

Synthesis and anti-infective activities of novel nitrofurantoin derivatives

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DEDICATION

To my parents:

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Babayi (Mr Landela O Zuma), thank you for always pushing me to be more.

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To my son

Akhe U A Zuma, you are the best part of life. My shining star, my heart; and a blessing in my life.

To my Sistra

Ntombikayise N Zuma: for lending an ear and a shoulder

PREFACE

This thesis is submitted in accordance with the General Academic Rules (A.13.7.3) of the North-West University. A published article and a submitted article manuscript are included in this thesis.

The thesis contains the following chapters:

Chapter 1: Thesis Introduction

Chapter 2: Literature Review

- Tuberculosis
- Leishmaniasis
- Malaria
- Nitrofurantoin and related drugs

Chapter 3: Review Article

The article entitled “**An update on derivatisation and repurposing of clinical nitrofuran drugs**” was published in the *European Journal of Pharmaceutical Sciences*.

Chapter 4: Submitted Article

The article entitled “**Single-step synthesis and anti-mycobacterial activity of novel nitrofurantoin analogues**” was submitted to the *Bioorganic Chemistry*.

The journals grant the author the right to include the article in a thesis. Permission from Elsevier: https://www.elsevier.com/___data/assets/pdf_file/0007/55654/AuthorUserRights.pdf

Chapter 5: Chemistry: synthesis and characterisation of hybrids

Chapter 6: Biological Evaluation: *in vitro* anti-infective activity and cytotoxicity evaluation

Chapter 7: Summary and Conclusion

Appendices: include supplementary information (IR, NMR and MS), various ethical and scientific approval certificates including permissions to reprint figures from other sources as well as the declaration from the language editor. Also included are the guides for authors for the scientific journal the article was submitted to.

ABSTRACT

Tuberculosis (TB) continues to be in the top ten lethal diseases. In 2018, it was responsible for 1.5 million deaths. *Mycobacterium tuberculosis* (*Mtb*) is the bacterium responsible for TB infection in humans. It is estimated that a third of the world's population is infected with latent TB (dormant infection), which serves as a reservoir for new active TB infections to occur. In latent TB, the mycobacteria survive under anaerobic and nutrient deficient conditions. Current anti-mycobacterial treatments are lengthy (a minimum of six to nine months depending on drug susceptibility) and involve a cocktail of drugs. The opportunistic development of resistant *Mtb* strains, the toxicity and adverse effects of current regimens, as well as latent TB, put importunate urgency on the need for drugs with activity against all active and latent infections. Furthermore, effective and low cost oral drugs that can reduce treatment duration and achieve mycobacterial clearance would have a positive impact on patient compliance which would reduce the spread of the disease as well as progression of drug susceptible to drug resistant *Mtb* strains.

Leishmaniasis disease burden tallies up to 350 million people at risk of infection, between 700 000 - 1 million new infection cases and up to 30 000 fatalities, annually. Of the many leishmanial species that can infect humans, *L. major*, and *Leishmania* (*L*) *donovani* responsible for cutaneous and visceral leishmaniasis, respectively, present the biggest concerns. Current anti-leishmanial therapies are unsatisfactory as they: (i) provide variable curative results, (ii) are too expensive, (iii) are impractical and inaccessible (since they are administered by intravenous and intramuscular injections), (iv) are not effective for all *Leishmania* species, and (v) are toxic. Therefore, there is a need for affordable oral drugs that would be effective and curative against both promastigote (infective) and amastigote (clinical and symptomatic) forms of leishmaniasis.

In 2017, malaria burden accounted for 219 million new cases and 435 000 deaths. *Plasmodium* (*P.*) *falciparum* which is responsible for the largest percentage of malaria morbidity and mortality has the ability to quickly develop drug resistance. Artemisinins are the cornerstone of malaria treatment. Indeed, the treatment of both uncomplicated and complicated malaria infections relies on artemisinin drugs. Artemisinins modulate parasite oxidative stress through the generation of reactive oxygen species (ROS) and reduce the levels of antioxidants and glutathione in the parasite which lead to its death. However, partial resistance to artemisinins has been reported in South-East Asia. The threat of widespread artemisinin resistance and the lack of alternative antimalarial drugs, amplify the urgency for the discovery and development of new chemotherapeutic agents that can (i) quickly and efficiently clear parasitaemia, (ii) block transmission, and (iii) deter the emergence of drug resistant strains, either by novel mode of action or by disrupting multiple crucial metabolic processes in the parasite.

In this study, nitrofurantoin (NFT) was investigated as a potential anti-infective nitroaromatic agent against mycobacterial, leishmanial and plasmodial infections. NFT is a staple urinary tract infection (UTI) drug that has multi-target activity. It overwhelms pathogens by attacking multiple critical metabolic pathways, including DNA replication, translation, transcription, as well as the Krebs cycle. Furthermore, it is active under both aerobic and anaerobic conditions. The anti-pathogenic effect occurs following a step-wise process involving activation by azoreduction, followed by nitroreduction. Azoreduction yields stable metabolites that have the ability to covalently bind to cellular proteins. Nitroreduction, on the other hand, occurs either by type I (anaerobic) or II (aerobic) reduction of the nitro group in the presence of pathogenic NADPH-cytochrome P450 reductases, producing toxic anti-pathogenic hydroxylamines and oxidative stress, respectively.

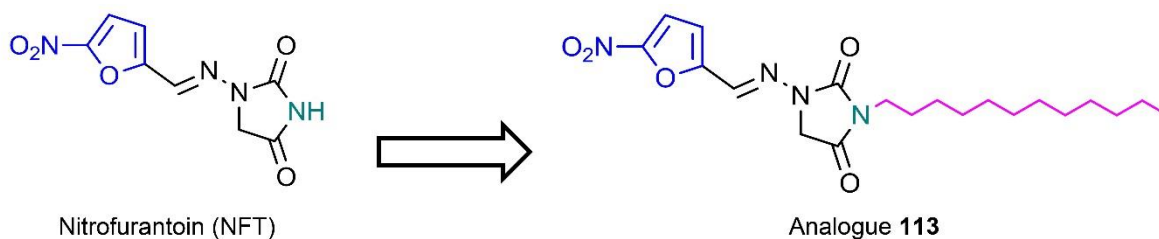
Akin to rifampicin, a first-line TB drug, NFT is active against both replicating and non-replicating mycobacteria. Despite the success of combination therapy involving nifurtimox (an NFT sister drug) in the treatment of human African trypanosomiasis (HAT) and its use in the treatment of Chagas' disease, nitroaromatic compounds, in general, have not been used for the treatment of leishmaniasis. Similar to HAT and Chagas, leishmaniasis is also a kinetoplastid disease. NFT has similar anti-pathogenic effects as artemisinins in that it generates ROS. However, nitroaromatics, including NFT, have rarely been investigated for malaria treatment. Furthermore, cases of NFT resistance in pathogens are rare, likely due to its multi-activity, as well as effectiveness under both aerobic and anaerobic conditions.

Triazoles are the building blocks for different anti-infective drugs; hence they are often used in molecular hybridisation drug design. Chemical properties such as strong dipole moments, bioisosteric effects, hydrogen bonding and their high affinity to bind with biological targets amplify their importance in medicinal chemistry.

In this study, aryl and *n*-alkyl NFT analogues (Series 1) as well as NFT-triazole hybrids (Series 2 to 4) were investigated as potential anti-infective agents. The NFT analogues were synthesised using single step nucleophilic substitution reactions. The NFT-triazole hybrids were synthesised by molecular hybridisation with aliphatic (Series 2 and 3) and aryl (series 4) linkers between NFT and 1,2,3-triazole, using click chemistry. All compounds were isolated by recrystallisation. The analogues had improved solubility and safety profiles as well as potent anti-infective activity, as evident from improved lipophilicity, low cytotoxicity, enhanced anti-mycobacterial, anti-leishmanial and anti-plasmodial potency. Analogue **113**, a twelve carbon aliphatic chain was the most active compound across all the tested pathogens. The NFT-triazole hybrids had pronounced cytotoxicity, with aryl-linked hybrids being the most toxic to mammalian cells. Furthermore, the hybrids showed varying and poor anti-mycobacterial and anti-plasmodial activity. The hybrids, particularly series 2,

performed better as potential anti-leishmanial agents, predominantly against *L. major*, the causal agent for cutaneous leishmaniasis.

In summary, molecular derivatisation of NFT was an effective strategy for improving its drug-like properties such as solubility and lipophilicity. Furthermore, NFT analogues performed best as potential anti-infective agents, as evident from good safety profiles, and potent anti-infective activity against all three tested pathogens, compared to the parent drug (NFT), and the NFT-triazole hybrids. Analogue **113** was identified as an anti-infective hit for further investigation in the urgent search for new, safe and affordable drugs. By contrast molecular hybridisation did not yield a favourable outcome. The introduction of the triazole produced hybrids with pronounced toxicity towards mammalian cells. Thus, 1,2,3-triazole did not stand as a viable bioactive companion for enhancement of the potency and safety profiles of nitrofurantoin.



• **Activity (μM)**

Mtb H37Rv: MIC₉₀ 15

L. major: IC₅₀ 56

L. donovani: IC₅₀ 34 (1S) & 26 (9515)

• **Cytotoxicity (μM)**

HEK-293: IC₅₀ 44

• **Selectivity Index SI:**

Mtb H37Rv 100

L. major 1

L. donovani 1 (1S) & 2 (9515)

• **Activity (μM)**

Mtb H37Rv: MIC₉₀ 0.99

L. major: IC₅₀ 0.034

L. donovani: IC₅₀ 0.012 (1S) & 0.099 (9515)

P. falciparum IC₅₀ 0.29 (3D7) & 0.25 (Dd2)

• **Cytotoxicity (μM)**

HEK-293: IC₅₀ > 100

• **Selectivity Index SI:**

Mtb H37Rv > 100

L. major > 29411

L. donovani > 8333 (1S) & > 1010 (9515)

P. falciparum > 345 (3D7) & > 400 (Dd2)

Keywords: molecular derivatisation; molecular hybridisation; nitrofurantoin; TB; malaria; leishmaniasis; toxicity; mycobacterial; leishmanial; plasmodial; nitroreductase

OPSOMMING

Tuberkulose (TB) is steeds onder die top tien dodelike siektes en was verantwoordelik vir 1.5 miljoen sterftes in 2018. TB in die mens word veroorsaak deur die bakterie *Mycobacterium tuberculosis* (*Mtb*). Daar word bereken dat 'n derde van die wêreld se populasie geïnfekteer is met latente TB, wat kan dien as 'n reservoir vir die ontwikkeling van aktiewe TB. In latente TB kan die mikobakterie oorleef in anaerobiese en voedingstof-arme toestande. Die huidige behandeling van TB is lank (‘n minimum van ses tot nege maande afhangende van geneesmiddel ontvanklikheid) en behels 'n kombinasie van verskeie geneesmiddels. Die waarskynlike ontwikkeling van weerstandige TB, die toksisiteit en nuwe-effekte van huidige behandelings, sowel latente TB, beklemtoon die groot behoefte om geneesmiddels teen die aktiewe en latente infeksies te verken en te ontwikkel. Verder sal effektiewe, verkorte en lae-koste behandeling om die mikobakterië uit te wis 'n positiewe invloed op pasiëntmeewerkendheid hê om sodoende die verspreiding van TB te bekamp en ook geneesmiddel ontvanklikheid te verbeter teen geneesmiddel weerstandige *Mtb* bakterië.

Leishmaniale infeksie stel ongeveer 350 miljoen mense vir infeksie gevaar, met tussen 700 000 - 1 miljoen nuwe gevalle van infeksie en tot 30 000 sterftes per jaar. Van die vele leishmaniasis spesies wat infeksie in die mens veroorsaak, is dit die *Leishmania major* en *Leishmania donovani*, onderskeidelik vir kutaneuse en viserale leishmaniasis verantwoordelik, waarvan viserale leishmaniasis die grootste bekommernis is. Huidige anti-leishmaniale behandeling is nie voldoende nie, weens (i) veranderde terapeutiese resultate, (ii) hoë kostes, (iii) onbekombaarheid en onpraktiese toediening (intraveneuse- en intramuskulêre inspuitings) (iv) oneffektiwiteit teen alle *Leishmania* spesies en (v) toksisiteit. Daarom is daar 'n noodsaaklikheid vir koste-effektiewe behandeling wat effektief en genesend teen beide die promastigoot (infektiewe) en amastigoot (kliniese en simptomatiese) vorms van leishmaniasis sal wees.

In 2017 was daar 219 miljoen nuwe gevalle van malaria en 435 000 sterftes. *Plasmodium* (*P.*) *falciparum* is verantwoordelik vir die grootste persentasie van siektes en sterftes en het ook die vermoë om geneesmiddelweerstandigheid te ontwikkel. Artemisiniene is die basis vir die behandeling van malaria, waar beide ongekompliseerde en gekompliseerde malaria infeksies afhanklik is van artemisiniene. Artemisiniene moduleer oksidatiewe stres in die parasiet deur die vorming van reaktiewe suurstof spesies wat lei tot vermindering van antioksidant en glutatioon vlakke en uiteindelik tot die dood van die parasiet. Daar is egter al in Suidoos-Asië weerstandigheid teen artemisiniene aangemeld. Die bedreiging van verspreide artemisinien weerstandigheid en die tekort aan alternatiewe antimalaria geneesmiddels beklemtoon die belangrikheid vir die ontwikkeling van nuwe chemoterapeutiese geneesmiddels wat: (i) vinnig en

effektief malaria kan stuit, (ii) oordrag kan blokkeer, en (iii) die verskyning van geneesmiddel weerstandige variante te verhoed deur 'n nuwe werkingsmeganisme of deur verskeie essensiële metabolisme prosesse in die parasiet te ontwrig.

In die huidige studie is nitrofurantoïen (NFT) as 'n potensiële anti-infektiewe nitroaromatiese middel teen mikobakteriële, leishmaniale en plasmodiese infeksies ondersoek. NFT is 'n primêre urienweginfeksie geneesmiddel met veelvuldige teikens vir aktiwiteit. NFT teiken verskeie metabolisme weë wat DNA replikasie, translasie, transkripsie en die Krebs-siklus insluit. Verder is NFT aktief in beide aërobiese en anaërobiese toestande. Die antipatogeniese effek is 'n stapsgewyse proses wat aktivering deur azoreduksie, gevolg deur nitroreduksie insluit. Azoreduksie lewer stabiele metaboliete wat kovalent bind aan sellulêre proteïene, waar nitroreduksie plaasvind deur tipe I (anaerobiese) of tipe II (aerobiese) reduksie van die nitrogroep in die teenwoordigheid van NADPH-sitochroom P450 reductase, om sodoende toksiese antipatogeniese hidroksielamien te lewer en oksidatiewe stres te weeg te bring.

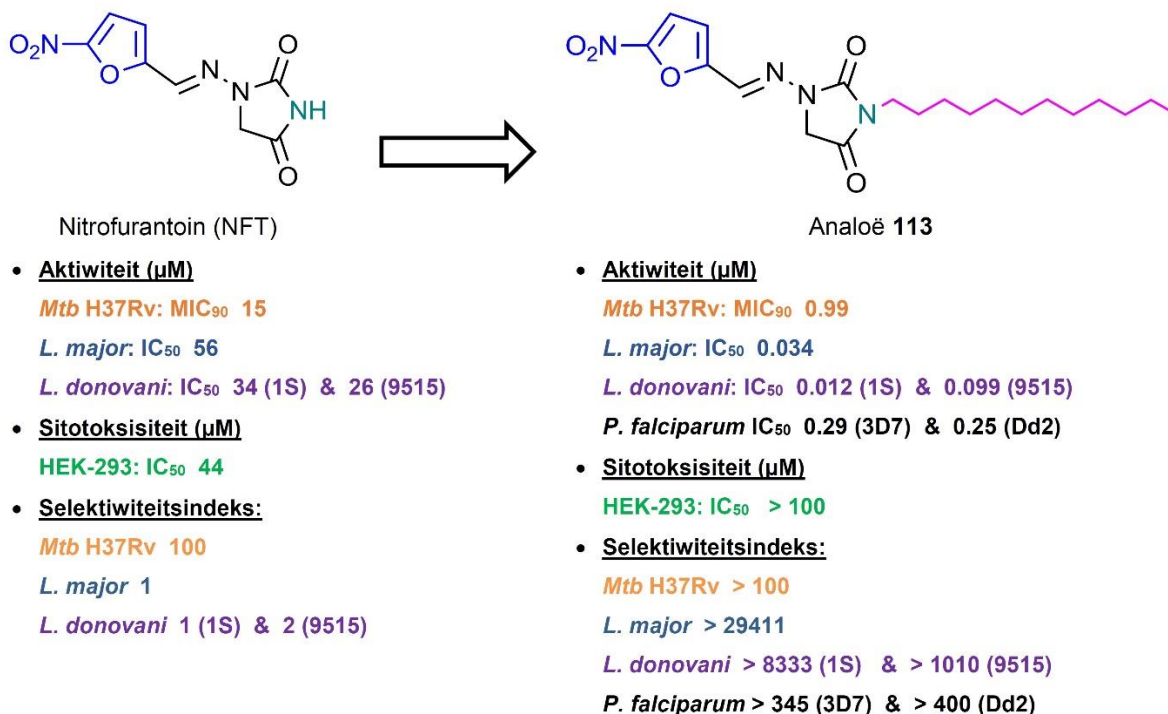
Soortgelyk aan rifampisien, 'n eersteliniebehandeling vir TB, is NFT aktief teen beide repliserende en nie-repliserende mikobakteriële. Nieteenstaande kombinasie terapie se sukses met nifurtimox ('n NFT derivaat) vir die behandeling van menslike Afrika tripanosomiasis (MAT) en Chaga se siekte, is nitroaromatiese verbindings in die algemeen nog nie gebruik vir die behandeling van leishmaniasis nie. Soortgelyk aan MAT en Chaga se siekte, is Leishmaniasis ook 'n kinetoplastiesiekte. NFT het anti-patogeniese effekte soortgelyk aan artemisiniene deurdat reaktiewe suurstof spesies geproduseer word. Nitroaromate, insluitend NFT, is egter baie min ondersoek vir die behandeling van malaria en gevalle van NFT weerstandigheid in patogene is baie raar, wat toegeskryf kan word aan NFT se veelvuldige teiken aktiwiteite sowel as sy effektiwiteit onder beide aërobiese en anaërobiese toestande.

Triasool is die boublokke vir verskei anti-infektiewe geneesmiddels en daarom word hulle meesal in molekulêre hibridiseringsgeneesmiddelontwerp gebruik. Chemiese eienskappe soos 'n sterk dipoolmoment, biosteriese effekte, waterstofbindings en 'n hoë affiniteit vir biologiese teikens, beklemtoon triasool se belangrikheid in medisinale chemie.

Verskei ariel en n-alkiel analoë (Reeks 1) sowel as NFT-triasool hibriede (Reeks 2 tot 4) is in dié studie ondersoek as moontlike anti-infektiewe geneesmiddels. Die NFT analoë is gesintetiseer deur 'n eenstap nukleofiliese substitusie reaksie. Die NFT-triasool hibriede is gesintetiseer deur molekulêre hibridisasie met alifatiese (Reeks 2 en 3) en ariel (Reeks 4) skakels tussen NFT en 1,2,3-triasool met die gebruik van kliek chemie. Alle verbindings is geïsoleer deur rekristallasie. Die analoë het verbeterde oplosbaarheid en veiligheidsprofile getoon so wel as potente anti-infektiewe aktiwiteit, soos duidelik gesien kan word deur verbeterde lipofilisiteit, verlaagde

toksiseit, verhoogde anti-mikobakteriële, anti-leishmaniale en anti-plasmodiese aktiwiteit. Analoo 113, wat 'n alifatiese ketting van twaalf koolstowwe bevat, was die mees aktiewe verbinding teen al die getoetsde patogene. Die NFT-triasool hibriede het merkbare sitotoksiseit getoon, met die ariel geskakelde hibriede wat die hoogste toksiseit getoon het teenoor soogdierselle. Verder het die hibriede varierende en swak anti mikobakteriële en anti-plasmodiese aktiwiteit getoon. Die hibriede, veral reeks 2, het beter gevaar as potensiële anti-leishmaniale verbindings, hoofsaaklik teen *L. Major*, verantwoordelik vir kutaneuse leishmaniasis, en gevolglik belowende potensiaal as voorkomende terapie toon.

Ten slotte, molekulêre derivatisering van NFT was 'n effektiewe strategie om die geneesmiddelsoortige eienskappe soos oplosbaarheid en lipofilisiteit te verbeter. Verder het die NFT analoë as die mees potensiële anti-infektiewe geneesmiddels geïdentifiseer soos gesien kon word uit goeie veiligheidsprofile en potente anti-infektiewe aktiwiteit teen al drie getoetsde patogene teenoor die moederverbinding (NFT) en die NFT-triasool hibriede. Analoo 113 is geïdentifiseer as 'n leidraadverbinding vir verdere ontwikkeling in die dringende soek na nuwe, veilige en bekostigbare geneesmiddels. Molekulêre hibridisasie was nie voordelig nie, en die insluiting van die triasool het hibriede gelever wat merkwaardige sitotoksiseit teenoor soogdierselle getoon het en daarom is 1,2,3-triasool nie 'n geskikte kandidaat om die aktiwiteit en veiligheidsprofiel van nitrofurantoin te verbeter nie.



Sleutelwoorde: molekulêre hibridisering; nitrofurantoin; TB; malaria; leishmaniasis; toksiseit; mikobakterieël; leishmaniale; plasmodiese; nitroreduksie

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LIST OF ABBREVIATIONS

5-NFs/NFs	5-nitrofurans
ACT	artemisinin-based combination therapy
ADEPT	antibody- directed enzyme prodrug therapy
AFB	Acid- Fast Bacilli
AHD	1-aminohydantoin
AhpC	alkyl hydroperoxide reductase
ALDH2	aldehyde dehydrogenase
AMOZ	3-amino-5- methylmorpholino-2-oxazolidinone
AOZ	3-amino-2-oxazolidinone
ARV	antiretroviral
BCG	Bacille Calmette-Giérin
BDQ	bedaquiline (
BSL-III	biosafety level III
CDC	Centers for Disease Control and Prevention
CHO	Chinese hamster ovarian
CL	cutaneous leishmaniasis
CMI	cell-mediated immunity
CPX	ciprofloxacin
CYP	cytochrome P
DCL	diffuse cutaneous leishmaniasis
DHA	dihydroartemisinin
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DN	diabetic nephropathy
DR-TB	drug-resistant TB
DR-TB	drug-resistant tuberculosis
DST	drug susceptibility testing
ECG	electrochemical gradient
EDG	electron donating groups
EFSA	European Food Safety Authority
EM	emetine
EMB	ethambutol
ESRD	end-stage renal disease

ETO	ethionamide
EWG	electron withdrawing groups
FDA	Food and Drug Administration
FQs	fluoroquinolones
FTD	furaltadone
FZ	furazolidone
GDEPT	gene-directed enzyme-prodrug therapy
GFR	glomerular filtration rate
GFX	gatifloxacin
GMS	Greater Mekong Sub-region
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulphide
GSSG	glutathione disulphide
HAT	human African trypanosomiasis
HBHA	Heparin-binding hemagglutinin adhesion
HEK-293	human embryonic kidney
HIV	Human Immunodeficiency virus
HOMO	highest occupied molecular orbital
IC ₅₀	concentration of compound inhibiting 50%
IFAT	Indirect Fluorescent Antibody Test
IGRAs	interferon-gamma release assays
IM	intramuscular
INH	isoniazid
IPT	intermittent preventive treatment
IPT	isoniazid preventive therapy
IRS	indoor residual spraying
ITNs	insecticide-treated mosquito nets
JAVMA	Journal of the American Veterinary Medical Association
KatG	catalase-peroxidase
LFX	levofloxacin
LNTB	lymph node tuberculosis
LTBI	latent TB infection
LUMO	lowest unoccupied molecular orbital
LZD	linezolid
MAO	monoamine-oxidase
MCL	muco-cutaneous leishmaniasis
MDR-TB	multi-drug resistant TB

MFX	moxifloxacin
MIC	minimum inhibitory concentration
MIC ₉₀	minimum concentration of compound inhibiting 90%
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
NECT	nifurtimox-eflornithine combination therapy
NFOH	hydroxymethylnitrofurazone
NFT	nitrofurantoin
NFT	nitrofurantoin
NFX	nifurtimox
NFZ	nitrofurazone
NO ₂	nitro group
NTDs	neglected tropical diseases
NTRs	nitroreductases
NX	nifuroxazide
OFX	ofloxacin
PABA	<i>p</i> -aminobenzoic acid
PAS	<i>p</i> -aminosalicylic acid
PfHRP2	<i>Plasmodium falciparum</i> histidine-rich protein 2
PI3K	phosphoinositide 3-kinase
PKDL	post-kala azar dermal leishmaniasis
pLDH	<i>Plasmodium</i> lactate dehydrogenase
PPD	purified protein derivative
PTO	prothionamide
PvHRP2	<i>Plasmodium vivax</i> histidine-rich protein 2
PZA	pyrazinamide
RDTs	Rapid Diagnostic Tests
RFB	rifabutin
RIF	rifampicin
RIF	rifampicin
RNS	reactive nitrogen species
ROS	reactive oxygen species
ROS	reactive oxygen species
RPT	rifapentine
RR-TB	rifampicin-resistant TB
Sb	antimony
SEM	semicarbazide
SI	selectivity index

SMC	Seasonal Malaria Chemoprevention
SOD	superoxide dismutase
SSM	sputum smear microscopy
STAT3	signal transducer and activator of transcription
TB	tuberculosis
TDR-TB	totally drug resistant TB
TRD	terizidone
TST	tuberculin skin
UTIs	urinary tract infections
VDEPT	virus-directed enzyme-prodrug therapy
VL	visceral leishmaniasis
WHO	World Health Organization
WRD	WHO-recommended rapid diagnostic
XDR-TB	extensively drug resistant TB

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1. Background

Together with the human immunodeficiency virus (HIV) and malaria, tuberculosis (TB) is one of the major communicable diseases, worldwide. TB is a bacterial infection caused by *Mycobacterium tuberculosis* (*Mtb*), which most often affects the lungs. When a person with lung TB coughs, sneezes or spits, the bacterium becomes airborne and causes person to person infection (WHO, 2017). In 2018, 10 million new *Mtb* incidents were reported, of which 1.1 million were children. People living with HIV accounted for 9% of new infections. 1.5 million TB related fatalities were reported during this period, of which 251 000 were HIV co-infected individuals and 250 000 were children. Furthermore, globally, multi-drug resistant TB (MDR-TB) accounted for 490 000 new cases with 10 000 new cases reported for South Africa (WHO, 2017; WHO, 2019). Moreover, South Africa is one of the highest TB burden countries, with 301 000 TB incidents, 177 000 of which are HIV co-infected and 11 000 infected with MDR-TB (WHO, 2019).

Current TB treatment regimens are toxic and their lengthy duration provides ample opportunity for the *Mycobacterium* to develop drug resistance (Smith *et al.*, 2013). Furthermore, lengthy treatment discourages patient compliance, which contributes to drug resistance and the development of MDR-TB and extensive drug resistant (XDR-TB). MDR-TB is the strain of TB that is resistant to at least one of the current first-line drugs, namely isoniazid and rifampicin. On the other hand, XDR-TB, in addition to first-line drugs, is resistant to any one of the fluoroquinolone drugs and an injectable anti-TB agent (Alexander & De, 2007).

Coinfection with other diseases, such as malaria and HIV, further contribute to the progression of TB. Consequently; control, prevention and treatment of both TB and the accompanying disease, become more complex. The complications may range from immune response (Enwere *et al.*, 1999) to drug-drug interactions which ultimately, result in reduced drug efficacy and toxicity (McIlleron *et al.*, 2007). Over and above the drawbacks associated with current treatment regimens and accompanying diseases, the inefficiency of current drugs against resistant TB emphasises the need for new and better chemotherapies through the identification of new agents or the improvement of existing ones.

Poverty, poor living conditions (poor ventilation and overcrowding), lack of sanitation, and pitiable access to health care services not only contribute to the spread of TB but other diseases such as neglected tropical diseases (NTDs) and malaria. NTDs account for one sixth of the world's population disease burden. Access to NTD medication is limited by affordability, poor and lack of access to health care systems. Additionally, drug administration is primarily intravenously (IV) or intramuscularly (IM), which often requires the presence of a healthcare practitioner. Furthermore, cost implications (in addition to the medicine itself, cost of transport for individuals residing far from medical facilities may be required) often dissuade infected persons from seeking medical attention (WHO, 2015).

Leishmaniasis is a high morbidity NTD which is also known as the disease of poverty, owing to its strong association with malnutrition and poor living conditions (Mitra & Mawson, 2017; WHO, 2015). The disease may contribute to social isolation, because of scarring that results as the lesions heal. Of the many leishmanial species that can infect humans, *Leishmania* (*L. donovani*) and *L. major*, are the biggest concerns. The former is responsible for visceral leishmaniasis (VL, kala azar, black fever) whereas the latter, causes cutaneous leishmaniasis (CL). VL predominantly occurs in East Africa and CL mainly afflicts Afghanistan, Peru, Saudi Arabia, Brazil, and the Syrian Arab Republic (McGwire & Satoskar, 2014; WHO, 2015).

On the other hand, malaria is caused by the *Plasmodium* parasite which is transmitted to humans through the bite of an infected female *Anopheles* mosquito. There are five species of the genus *Plasmodium* (*P.*) that can infect humans, namely *P. ovale*, *P. malariae*, *P. knowlesi*, *P. vivax* and *P. falciparum*, with the latter two being the most virulent (Pawluk *et al.*, 2013). In 2017, malaria accounted for 219 million new cases with 435 000 deaths of which 266 000 were children under the age of five (WHO, 2019a). Currently, uncomplicated malaria treatment is achieved by artemisinin combination therapy (ACTs), which is aimed at improving efficacy and delaying the emergence of drug resistance. In ACTs, artemisinins are administered together with a longer acting antimalarial drug based on the reasoning that the artemisinin will quickly reduce the parasite burden and when its concentration falls below therapeutic levels, the longer acting antimalarial will ensure continued treatment. However, even with the use of ACTs artemisinin, resistance has been reported (WHO, 2016). The threat of artemisinin resistance and the lack of alternative antimalarial drugs are further amplified by the neurotoxicity of artemisinins at high doses (Fairhurst & Dondorp, 2016; Starzengruber *et al.*, 2012; Van Neck *et al.*, 2007). Granted reports on artemisinin-induced neurotoxicity stem from studies on laboratory animals, exposed to the drugs for extended periods of time and at high dosages (Efferth & Kaina, 2010). However, in the wake of

resistance, the issue of neurotoxicity in humans becomes a concern, since strategies to maintain artemisinin efficacy may involve increased artemisinin concentrations, or increased dosages (Das et al., 2013) thus assigning urgency to the need for identifying and developing new antimalarial drugs.

Nitrofurans are a class of nitrocyclopentyl aromatic compounds that possess weak activity against both active and latent *Mtb* (Murugasu-Oei & Dick, 2000). Their activity is attributed to the metabolic reduction of the pharmacophore nitro group ($-\text{NO}_2$) by nitroreductase enzymes (Sutherland et al., 2010). Much like the first line TB drugs, isoniazid (Girling, 1977; LoBue & Moser, 2003), and rifampicin (RIF), nitrofurans kill latent forms of the *Mycobacterium* which is an invaluable advantage, since latent TB serves as a reservoir for new infections to occur (Lin & Flynn, 2010; Murugasu-Oei & Dick, 2000).

Redox homeostatic systems are crucial to the survival of *Mtb* in host macrophages and to its pathogenicity (Wolff et al., 2015). Therefore, compounds that target redox systems of the *Mycobacterium* play a crucial role in combating TB. In nitroaromatic compounds, the strong electron-withdrawing nitro group acts as a pharmacophore. This small group creates electron deficient sites which interact with biological nucleophiles such as proteins, amino acids, enzymes and nucleic acids. The biological nucleophilic interaction, which may occur as oxidation or reduction reactions, interrupts the normal electron flow (Strauss, 1979). The interruption in electron flow may result in microbial DNA damage and the disruption of mycobacterial homeostatic systems (de A. Farias et al., 2006; Herrlich & Schweiger, 1976).

The role of nitroaromatic compounds as antiprotozoal agents has been investigated, in fact benznidazole (BZ), nifurtimox (NFX) are currently used for the treatment of Chagas disease and human trypanosomiasis (HAT), respectively (de Andrade et al., 2015; Li et al., 2017). However, nitroaromatics have not been used for the treatment of leishmaniasis. In this study, nitrofurantoin derivatives were investigated as potential anti-mycobacterial and anti-protozoal agents. Nitrofurantoin (NFT, Figure 1.1) is a nitroaromatic drug that is widely used as an antibiotic for urinary tract infections (UTIs). Although it has been clinically used for decades, resistance against NFT remains scanty. Unfortunately, NFT has poor solubility in both water and oil. Therefore, it has poor tissue penetration, a short elimination half-life ($t_{1/2} \approx 20 - 60$ min) and a poor ability to reach therapeutic concentrations (40% is excreted unchanged through urine, hence its use in UTI treatment, which ultimately limit its effectiveness (Munoz-Davila, 2014).

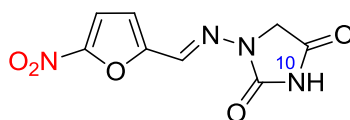
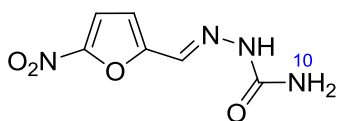
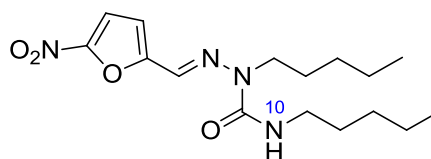


Figure 1.1: Structure of nitrofurantoin (NFT)

NFT shortfalls may be addressed by derivatisation by attaching *n*-alkyl and/or aryl substituents at the N-10 position of NFT (Figure 1.1) to yield analogues. Derivatisation may solve the problem of poor solubility (in both water and oil) by producing compounds with a better hydrophilicity/ lipophilicity balance, to enable passage through biological membranes in order to reach the site of action. This improvement may result in derivatives with (i) enhanced therapeutic concentration, (ii) the ability to cross cell membranes, (iii) improved tissue penetration and (iv) ultimately improved anti-infective (anti-mycobacterial, anti-leishmanial and anti-plasmodial) activity. This approach was successful in the investigation of lipophilic derivatives of nitrofurazone (NFZ, Figure 1.2); its lipophilic derivative (Figure 1.2) was found to be potent with bacteriostatic and bactericidal MIC values of 10 µg/mL and 23 mg/mL, respectively (de A. Farias *et al.*, 2006).



Nitrofurazone (NFZ, clogP = 0.204)



Lipophilic derivative of NFZ (clogP = 5.345)

Figure 1.2: Structures of nitrofurazone and its anti-*Mtb* active lipophilic derivative

1,2,3-Triazole (triazole, Figure 1.3) is a heterocyclic aromatic compound with anti-mycobacterial (Boechat *et al.*, 2011; Kharb *et al.*, 2011), anti-leishmanial (Ferreira *et al.*, 2007) and antiplasmodial (Boechat *et al.*, 2014) activity. This moiety is small and can create stable linkages (Haider *et al.*, 2014) between various molecular fragments. Additionally, its stability under reduction, oxidation as well as to hydrolysis at acidic and basic conditions is an advantage in biological systems which enables the triazole to bind to biological targets with ease (Haider *et al.*, 2014).

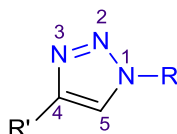


Figure 1.3: Structure of 1,2,3-Triazole

A well-known strategy to improve drug efficacy is molecular hybridisation. It consists of assembling through chemical transformations two or more different drug pharmacophores into a single chemical entity with multiple modes of action (Hulsman *et al.*, 2007). Advantageously, the hybrid molecule may have reduced side effects, offer less toxicity, improved bioavailability, the ability to interact with more than one pathogen target, and delay the development of drug resistance (Hulsman *et al.*, 2007; Muregi & Ishih, 2010). Therefore, in this study, nitrofurantoin derivatives were investigated as potential anti-infective agents.

Nitrofurantoin activity against *Mtb* (MIC = 50 μ M) has been reported (Murugasu-Oei & Dick, 2000) but not against *Plasmodium* nor *Leishmania* parasites. In this project, four series of nitrofurantoin derivatives were synthesised. The first series comprised of NFT analogues from direct substitution of *n*-alkyl and aryl substituents at N-10. The subsequent (second to fourth) series were triazole- hybrid derivatives. The first series provided a comparison basis with NFZ (*Mtb* MIC 75-150 μ M) (Kana *et al.*, 2010) and NFT (*Mtb* MIC 50 μ M) (Murugasu-Oei & Dick, 2000). This allowed for comparison between non-triazole derivatives and NFT-triazole (Figure 1.3) hybrids in order to determine the impact of the triazole pharmacophore on NFT as a potential anti-infective agent against mycobacterial, leishmanial and plasmodial infections.

1.2. Aim and Objectives of the study

The aim of this study was to investigate novel nitrofurantoin derivatives, as new effective, safe and affordable potential anti-infective agents.

In order to achieve this aim, the following objectives were set:

- Synthesis of novel, *n*-alkyl/ aryl nitrofurantoin analogues
- Synthesis of novel, nitrofurantoin-1,2,3-triazole hybrids
- Determination of *in vitro* anti-mycobacterial activity
- Determination of *in vitro* anti- leishmanial activity
- Determination of *in vitro* anti-plasmodial activity
- Cytotoxicity assessment of the synthesised compounds using human cell lines.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Globalisation, which drives the modern world through global trade and interaction, accentuates the importance of disease control. Migration and urbanisation render the era of segregation obsolete, consequently disease burden (a hindrance to socio-economic development) in third world countries becomes a global mission. However, this mission requires collaborative effort in terms of political will, scientific research and funding in order to accommodate access to facilities and basic health education in support of globalisation, particularly in an effort to manage, control and reduce the risk of the spread of infectious diseases.

For decades, diseases such as tuberculosis (TB), malaria and leishmaniasis continue to plague society with high morbidity and mortality rates. These diseases thrive and affect mainly resource-limited communities. The plight of these diseases is further complicated by the consistent development of resistance against effective drugs. In addition, the complex biochemistry and life cycles of the pathogens further complicate drug design and discovery, which ultimately negatively impacts efforts to eliminate and eradicate these diseases.

2.2 Tuberculosis

Despite being preventable and curable, TB continues to be responsible for large morbidity and mortality rates in the world. TB is caused by *Mycobacterium* (*M*) bacilli, namely *M. tuberculosis* (*Mtb*) and *M. africanum*. The former is the most prevalent and deadly tubercle bacillus in humans and the latter is responsible for TB infections only in certain West African regions (Delogu *et al.*, 2013). Additionally, other species that affect animals also exist, these include *M. bovis*, *M. caprae* and *M. pinnipedii* (Delogu *et al.*, 2013).

2.2.1. Transmission

An individual with active TB expels aerosol droplets infected with *Mtb* bacteria when coughing or sneezing. The infectious droplets, when inhaled by another person, can lead to: (i) immediate clearance of the organism by an immuno-competent host; (ii) latent infection of an immuno-competent yet susceptible host; (iii) in immuno-compromised individuals, such as patients with human immunodeficiency virus (HIV), the onset of active (primary) disease or reactivation of disease (Schluger & Burzynski; 2010, Wani, 2013). In addition to the

susceptibility and immuno-competency of the exposed potential host, successful TB infection can be governed by other factors, such as the environment, exposure and infectiousness. Environmental circumstances, such as poor ventilation, small enclosed spaces and a high probability of recirculation of infectious *Mtb* containing droplets, determine and increase the probability of infection (Srivastava *et al.*, 2015). Furthermore, exposure and infectiousness increase the risk of transmission, since the longer and the more frequently an individual is exposed, the higher the risk of developing active disease (Lönnroth *et al.*, 2009). In addition to infection through the inhalation of *Mtb* infested droplets, the pathogen can progress to a drug resistant strain (acquired), discussed in section 2.2.8. Larger *Mtb* infested droplets limit the penetration into the lungs, thus quickly activating innate macrophages to eliminate the pathogen. Smaller droplets, on the other hand, have the ability to travel deep into the lower lung and, as a result, may evade phagocytosis (Tang *et al.*, 2016).

2.2.2. Pathogenesis of tuberculosis infection and granuloma

Mtb thrives by using the host's immune defences to its own advantage (Figure 2.1). The bacilli reach the alveoli of the lungs by passage through the nasal passages, upper respiratory tract and bronchi (Wani, 2013). In the alveoli, phagocytosis of the tubercle bacilli by the alveolar macrophages occurs, thus eliminating the bacilli (Fogel, 2015). However, some mycobacteria may survive and multiply intracellularly, in which case, upon death of the macrophages, the surviving mycobacteria are released. The mycobacteria recruit other phagocytic cells (such as other alveolar macrophages, dendritic cells, monocytes and neutrophils) by manipulating infected macrophages to produce cytokines and chemokines (Guirado *et al.*, 2013; Wani, 2013). This recruitment process eventually leads to the formation of the tubercle, a nodular lesion (Russell, 2007). In the absence of intervention against bacterial replication, the tubercle continues to grow and enter the lymph, resulting in the formation of the Ghon complex (Smith, 2003). Bacterial cell proliferation continues until the host mediates an immune response by recruiting immune cells to surround the tubercle bacilli, ultimately forming a granuloma (Martin *et al.*, 2016; Russell, 2007). The granuloma is a structural aggregate of infected macrophages, foamy macrophages, mononuclear phagocytes, lymphocytes, collagen and other extracellular matrix components (Russell, 2007). In immuno-competent individuals, the granuloma can keep the bacilli contained by encasing the infected macrophages, thus terminating bacterial replication and giving rise to latent TB infection (LTBI), discussed in section 2.2.3 (Wani, 2013). Notably, LTBI is characterised by the presence of dormant bacilli that remain in the lungs for long periods of time without causing illness or clinical disease (Blumberg & Ernst, 2016). In this state, the bacilli survive in nutrient and oxygen scarce environments (Blumberg & Ernst, 2016; Moon *et al.*, 2015).

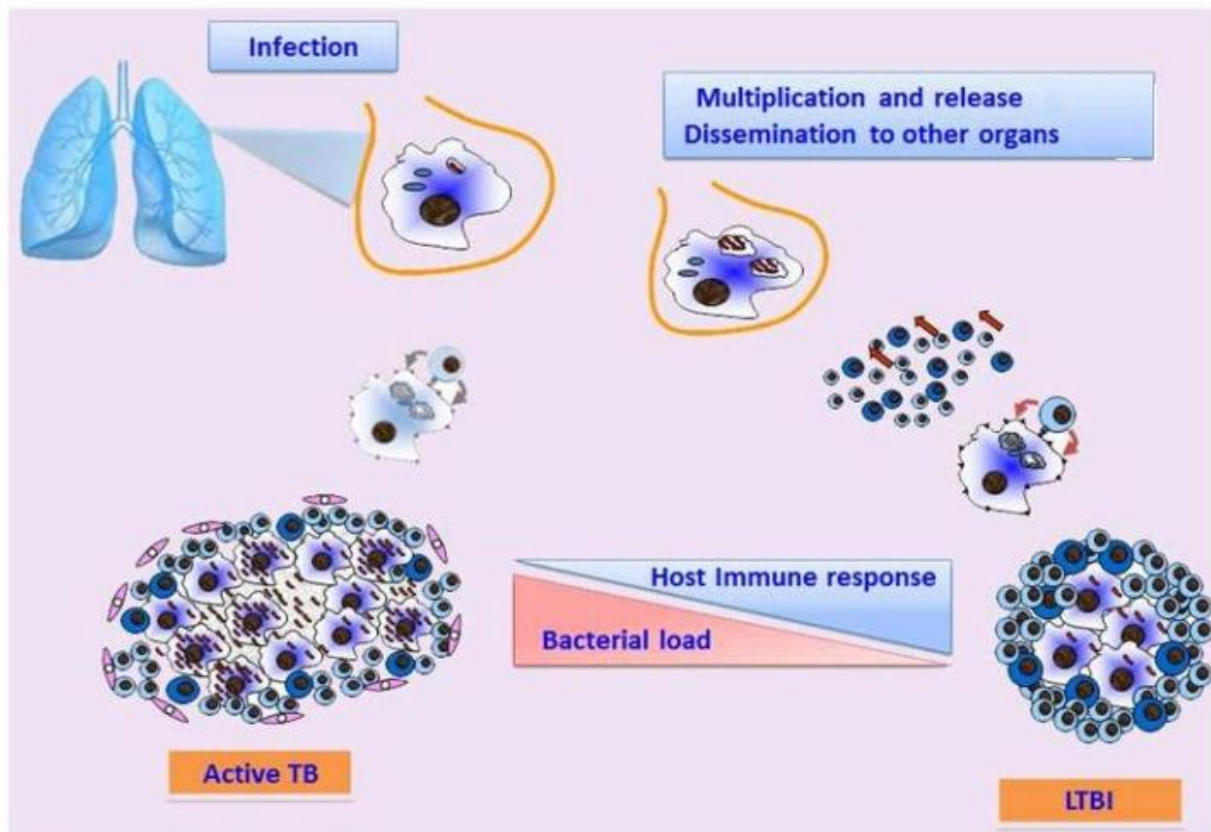


Figure 2.1: TB infection and its progression (Delogu *et al.*, 2013).

Mtb pathogenesis motivates and heightens both innate and adaptive host immune responses. The innate immune response is responsible for the recruitment of macrophages and neutrophils (Tang *et al.*, 2016). The former uses phagocytic receptors to identify the *Mtb*, promoting phagocytosis under harsh conditions of low pH and the presence of reactive oxygen and nitrogen species (ROS and RNS, respectively) in a bid to kill the mycobacteria (Bhatt & Salgame, 2007; Cambier *et al.*, 2014). The mycobacteria avoid these lethal efforts by employing several defence mechanisms including the: (i) disruption of phagosome-lysosome fusion (Armstrong & Hart, 1975; Tang *et al.*, 2016); (ii) employment of proteins such as catalase-peroxidase (KatG) and alkyl hydroperoxide reductase (AhpC) for protection against the toxicity of ROS and RNS (Tang *et al.*, 2016); and (iii) promotion of necrosis to enable infection of neighbouring cells by inhibiting apoptosis of infected macrophages (Guirado *et al.*, 2013). Humoral and cell-mediated immunity form adaptive host immune responses against TB. Of particular importance are CD4⁺ T cells, since HIV infected individuals are at a higher risk of TB infection (Tang *et al.*, 2016). Depletion of CD4⁺ T cells, which is synonymous with HIV infection, impairs cellular immunity and paves a way for opportunistic pathogens such as TB (Okoye & Picker, 2013).

2.2.3. Latent tuberculosis infection

LTBI occurs post exposure to *Mtb*, when the mycobacteria lives in deep tissues, where they survive for extended periods of time without activation and manifestation of active TB (Schluger & Burzynski, 2010). The bacilli are dormant and engage in a commensal symbiotic relationship with the host to ensure survival (Tang *et al.*, 2016). Unlike clinical infection, LTBI is not accurately detectable by tuberculin skin test (intradermal purified protein derivative, PPD, discussed in section 2.2.5.1), thus its detection for management and control is challenging (Parrish 1998). Disturbingly, LTBI accounts for 10 % of active TB infections and is catalysed by socioeconomic issues, poor disease control and coinfection with another disease (HIV and other diseases, such as diabetes and illnesses that require immunosuppressing medication may cause complications) (Fogel, 2015; Tang *et al.*, 2016). In fact, TB is the leading cause of death for people living with HIV/AIDS. All these factors depress efforts to control the scourge of TB, which are already strained by issues relating to diagnosis and treatment failure.

2.2.4. Extra-pulmonary tuberculosis

Although the focus is mainly on pulmonary TB (responsible for scores of deaths annually), this section briefly summarises extra-pulmonary TB, which is *Mtb* infection affecting any other organ of the body, except the lungs (Sharma & Mohan, 2004). The challenge with extra-pulmonary TB is the non-specificity of symptoms, features and manifestations, which may lead to a delay in diagnosis or misdiagnosis, thus promoting disease related complications.

TB of the urogenital tract (genitourinary TB), which affects the scrotum in men and the pelvis in women, occurs as a complication of pulmonary tuberculosis (Cruz-Knight & Blake-Gumbs, 2013; Sharma & Mohan, 2004). In fact, genitourinary TB may develop 5 to 25 years after pulmonary TB (Sharma & Mohan, 2004). The tubercle manifests in the kidneys, then enlarges and ruptures into the renal and urinary tracts (Sharma & Mohan, 2004; Zajackowski, 2012). Although, symptoms may be non-specific and, absent in some cases, scrotum/pelvic inflammation may occur. Other symptoms may include recurrent urinary tract infections (UTIs), the presence of blood or pus in the urine, difficulty urinating and flank pain (Zajackowski, 2012).

Abdominal TB, also known as TB of the digestive system, affects the ileocecal valve, ileum, cecum and ascending colon (Cruz-Knight & Blake-Gumbs, 2013). The mycobacteria may be inoculated into the neighbouring organs of the gastrointestinal system and/or spread via (i)

the lymph channels from the infected nodes; (ii) the ingestion of infected sputum from the pulmonary system; and (iii) haematogenous means (Sharma & Bhatia, 2004). Symptoms such as abdominal swelling, chronic abdominal pain, fever, irregularities in bowel movements and haem-positive faecal matter commonly occur (Debi *et al.*, 2014). Furthermore, intra-abdominal organs, such as the liver, pancreas and spleen, may also be affected, particularly in immuno-compromised individuals or in association with military TB (Ibrarullah *et al.*, 2002). Haematogenous disseminated TB or military TB is a severe form of TB that results in the haematogenous spread of mycobacteria characterised by lesions on the chest visible on chest radiography (Van Crevel & Hill, 2017).

Neurological TB affects the central nervous system and can manifest as TB meningitis or intracranial tuberculoma. The disease is prevalent in children, particularly in the first three (3) years. Signs and symptoms include ill-health, anorexia, behavioural irregularities, headache, neck stiffness, altered mental status and cranial nerve abnormalities (Sharma & Mohan, 2004). Long-term anomalies such as hydrocephalus, epilepsy, cognitive impairment, as well as sight and hearing loss may occur, which can negatively affect the individual's quality of life (Anderson *et al.*, 2010).

Lymph node TB (LNTB) also known as TB lymphadenitis or scrofula is the most common form of extra-pulmonary TB (Benjelloun *et al.*, 2015). The disease mainly affects children and young adults and occurs in the lymph nodes, particularly in the cervical and supraclavicular (Cruz-Knight & Blake-Gumbs, 2013). Mycobacterial infection of the lymph nodes may occur by lymphatic dissemination after *Mtb* is introduced into the respiratory tract or through the tonsils (Sharma & Mohan, 2004). Clinical features of LNTB may be confused with those of lymphoma, perpetuating misdiagnosis which promotes and prolongs chronic adverse effects from either incorrect treatment or the persevering undiagnosed disease (Sellar *et al.*, 2010). Signs and symptoms include slowly enlarging lymph nodes, fever, weight loss and lymph node abscesses that may rupture, causing ulcers and non-healing sinuses (Sharma & Mohan, 2004).

Skeletal TB also known as Pott's disease is a haematogenous infection that affects the vertebrae, mainly the spine and hip joint. TB of the spine is the most common form of skeletal TB and mainly affects the lower thoracic and lumbar vertebrae (Rasouli *et al.*, 2012). Other spinal areas that are often affected include the middle thoracic and cervical vertebrae. Infection often begins in the epiphyseal location or the anterior area of the vertebrae (Rasouli *et al.*, 2012; Sharma & Mohan, 2004). In addition to customary TB signs and symptoms, skeletal TB clinical features of skeletal TB may include severe pain in the afflicted area, local

swelling, suboccipital pain and neck stiffness (Cruz-Knight & Blake-Gumbs, 2013). Hip and knee joint TB is also accompanied by limited movement at the joints (Rasouli *et al.*, 2012). Spinal TB results in bone and spinal deformities leading to paraplegia. Paraplegia can sometimes set in years after the disease has become quiescent, possibly due to pressure to the spinal cord (Sharma & Mohan, 2004).

2.2.5. Diagnosis

Diagnosis is the first step of ensuring adequate and proper TB treatment. This goes beyond merely confirming mycobacterial infection but also determining proper course of treatment that includes avoiding drugs to which the mycobacteria is already resistant. However, in the neediest and vulnerable of populations, diagnosis remains inadequate. Complications presented by HIV coinfection and LTBI only serve to perpetrate disease prevalence, delay diagnosis and extend treatment, thus promoting transmission. Other diagnosis-related challenges include (i) over-diagnosis, which ultimately deplete the already sparse resources, and (ii) determining the afflicted area of the body. Currently available and used diagnostic tools include testing for latent infection, sputum smear microscopy (SSM), chest X-ray, TB culture, molecular testing and drug susceptibility testing.

2.2.5.1. Tuberculin skin test

The tuberculin skin test (TST or Mantoux tuberculin skin test) is the standard diagnostic tool for determining whether an individual has TB. The test involves the intradermal injection of purified protein derivative (PPD) that is derived from *Mtb* cultures (Fogel, 2015; Nayak & Acharjya, 2012). TST identifies prior exposure to TB via an inflammatory response as a result of memory T cells binding to the injected antigens. If the individual was previously exposed to *Mtb*, the result is a type IV delayed hypersensitivity skin reaction characterised by the formation of swollen, firm skin nodules 24 – 48 hours after injection (Fogel, 2015). A positive result is ascertained two to three days after injection, by the size of the raised, hard area or swelling of the injected area (CDC, 2011). The swelling indicates delayed hypersensitivity in response to tuberculin stimulated lymphocytes (Huebner *et al.*, 1993). TST diagnosis can be performed on any individual including infants, pregnant women and HIV-positive individuals. Additional tests may be necessary for Bacille Calmette-Guérin (BCG, section 2.2.6.1) vaccinated individuals as the test may give a false-positive result (CDC, 2011). Limitations of TST can include: (i) false-negative results which may occur in immunosuppressed individuals, including HIV positive people; (ii) improper administration of the intradermal injection; and (iii) the necessity for multiple visits to the healthcare facility which may lead to erratic results being obtained (Fogel, 2015; Schluger & Burzynski, 2010).

Other limitations, such as the age of the individual, the immunological status and coinfection with another disease, also negatively impact the specificity and sensitivity of TST (Nayak & Acharjya, 2012). Moreover, results are only be available 48 - 72 hours post-injection (Nayak & Acharjya, 2012; Sullivan & Kendig Jr, 1999). Reliability of the test, therefore, depends heavily on the standardisation of procedures, staff training and practice (Sullivan & Kendig Jr, 1999).

2.2.5.2. Rapid testing

Rapid testing for TB saves lives as it enables early detection, reducing the duration of infectiousness. Rapid tests detect antigens and antibodies in a sample and the sensitivity and specificity may vary from assay to assay (Singh & Grover, 2011). Of note, is the Xpert MTB/RIF assay which is a WHO-recommended rapid diagnostic (WRD) test progressively adopted as a diagnostic tool for individuals suspected of having pulmonary TB. The assay is used to test for TB and rifampicin resistance (Schito *et al.*, 2017; WHO, 2017a).

2.2.5.3. Sputum smear microscopy

Resource-limited and high TB burden countries rely on sputum smear microscopy (SSM) as a primary diagnostic tool for TB. This technique is inexpensive and can identify the most infectious patients (Desikan, 2013). In this technique, a sputum sample is smeared onto a glass slide and then stained with acid-fast stains, such as Ziehl-Neelsen stain, fluorescent acid-fast stain or fluorescein diacetate (Desikan, 2013; Kanade *et al.*, 2016). A limitation of this technique is compromised sensitivity in individuals with HIV coinfection and paediatric TB (Desikan, 2013). Furthermore, patient cooperation may lead to diagnostic default since the technique requires multiple sputum examinations.

2.2.5.4. Chest radiograph

Since pulmonary TB is the most predominant, chest radiograph (X-ray) is a useful diagnostic tool. The tool identifies abnormalities in the chest and lesions in the lungs that may be indicative of TB infection. Misdiagnosis is a major drawback with this method therefore, an accompanying diagnostic tool is often necessary to ensure prompt treatment (CDC, 2012; Ryu, 2015).

2.2.5.5. Tuberculosis culture

A TB culture test is used for TB diagnosis, drug-susceptibility testing and genotyping. A culture is grown in either solid (agar based) or liquid (culture broths) media. Detection of mycobacterial growth in culture broth is four – fourteen days compared to three – six weeks

in culture plates (CDC, 2012). The test is expensive, time consuming, requires laboratory equipment (e.g. incubators and safety cabinets) and relies on the competency of the health practitioner in order to avoid contamination and provide accurate results (CDC, 2012).

2.2.5.6. Extra-pulmonary tuberculosis diagnosis

Organ-related biopsies or tests (Table 2.1) are used in combination with other diagnostic tools (section 2.2.5) for the diagnosis of extra-pulmonary TB.

Table 2.1: Summary of extra-pulmonary TB diagnostic methods

Type	Afflicted region	Diagnosis
TB of the urogenital tract ^a	Men – scrotum Women – pelvis	• Urine culture for TB from morning urine samples • Tissue biopsy • Tuberculin skin test (TST)
TB of the digestive system (abdominal TB) ^b	Ileocecum, ileum, cecum, and ascending colon.	• TST • Ascetic fluid culture • Biopsy • Colonoscopy
TB of the central nervous system (meningitis/ intracranial tuberculoma) ^c	Meninges	• Cerebrospinal fluid examination using Acid- Fast Bacilli (AFB) assay
TB lymphadenitis (scrofula) ^d	Lymph nodes, particularly cervical and supraclavicular	• Fine-needle aspiration • Excisional biopsy
Spinal/Skeletal TB (Pott's disease) ^e	Vertebrae and results in bone and spinal deformities	• Positive TST • Tissue biopsy
Pericardial TB ^f	Heart	• Pericardial fluid • Biopsy

^a & ^b (Anderson *et al.*, 2010; Cruz-Knight & Blake-Gumbs, 2013; Debi *et al.*, 2014; Hegde *et al.*, 2014; Mayosi, 2009), ^c (Anderson *et al.*, 2010; Chin, 2014; Cruz-Knight & Blake-Gumbs, 2013), ^d(Cruz-Knight & Blake-Gumbs; 2013; Hegde *et al.*, 2014), ^e (Cruz-Knight & Blake-Gumbs, 2013), ^f (Cruz-Knight & Blake-Gumbs, 2013; Mayosi, 2009).

2.2.5.7. Testing for latent infection

Individuals with LTBI are at risk of developing active TB, making it imperative that a measure exists to identify the affliction in order to contain it. In the United States, LTBI diagnosis has led to an incredible decline in TB prevalence (Schluger & Burzynski, 2010). LTBI can be diagnosed by interferon-gamma release assays (IGRAs) which measure T cell responses to TB antigens (Cruz-Knight & Blake-Gumbs, 2013). IGRAs have better sensitivity and specificity compared to TST. Furthermore, there is no interference with BCG vaccine (section 2.2.6.1) antigens since the assay measures the release of cytokine IFN- γ from T cells that are absent in the vaccine (Diel *et al.*, 2012).

2.2.5.8. Drug susceptibility testing

This diagnostic tool is invaluable, although its aim is not to confirm TB infection, but to identify the strain's drug resistance in order to customise treatment cocktails. Drug susceptibility testing (DST) is used to ascertain and design customised regimens in order to ensure effective treatment, discourage the development of resistance, and create coherence with other drugs in patients who are undergoing additional treatments as a result of coinfection. This method is mainly aimed at reducing adverse effects, which is the motivating factor for patient non-compliance (D'Ambrosio *et al.*, 2015; Dheda *et al.*, 2016). DST is not meant to undercut standardised treatment regimens, merely improve overall TB treatment. Standardised treatment regimens cannot be understated nor undervalued, because DST is dependent on the availability of testing resources. Standardised treatment regimens ensure that treatment is commenced as soon as possible post diagnosis, thus reducing chances of transmission of the disease. Optimised strides for rapid and comprehensive DST readouts need to be improved in order to make the process of customised treatment efficient. Otherwise, empiric regimens continue to dominate treatment schedules and, unfortunately, this may amplify resistance (Dheda *et al.*, 2016; Schito *et al.*, 2017).

2.2.6. Prevention and control

The aim of preventive therapy is to minimise the risk of infection as much as possible. This form of therapy is targeted towards the most immune-vulnerable individuals (such as people living with HIV and infants) and those who are in contact with TB patients (such as caregivers). Furthermore, preventive therapy, such as a vaccine, should offer immunity from new infection as well as LTBI reactivation. Unfortunately, this form of therapy, accompanied by safety and effectiveness, remains elusive despite vigilant research, and is further limited by the complexity of *Mtb* biology and limited knowledge of basic immune responses (Schito *et al.*, 2017). However, there are some therapies that offer measurable preventive measures,

including isoniazid preventive therapy (IPT) and Bacille Calmette-Giérin (BCG) (WHO, 2014).

2.2.6.1. Bacille Calmette-Giérin

BCG is an attenuated live *Mycobacterium bovis* strain used in the vaccination of infants and children against *Mtb* infection. The vaccine offers a good safety profile and acceptable efficacy in infants and children, but not in adults (Luca & Mihaescu, 2013). It is speculated that the vaccine does not offer immunity against adult pulmonary TB as it cannot induce memory cell-mediated immunity (CMI) in the long-term. Furthermore, BCG efficacy is limited by prior exposure to the pathogen. Unfavourably, in children infected with HIV, the vaccine promotes disseminated BCG disease (Hesseling *et al.*, 2007).

Progress aimed at the improvement of BCG lead to the development of a recombinant BCG (rBCG) vaccine, specifically the live mycobacterium vaccine, rBCG30. It uses BCG to overexpress Ag85B (major *Mtb* secretory protein), an immune-dominant target of the host immune system. Unfortunately, this vaccine did not make it past Phase I clinical trials (da Costa *et al.*, 2014). Other vaccines, that utilise the live attenuation of *Mtb*, which may provide lasting immunity, are hindered by the risk of reverse mutation to virulent *Mtb*, especially in immunocompromised individuals. Fortunately, a live attenuated *Mtb* vaccine MTBVAC that is based on two independent, stable genetic deletions of genes coding for PhoP and fadD26 is under Phase IIa clinical trials (NCT03536117) in South African Neonates Living in a High-Burden Tuberculosis-Endemic Region, until December 2020 (Biofabri, 2019). PhoP is the transcription factor and fadD26 is essential for the synthesis of phthiocerol dimycocerosate (PDIM), a cell wall lipid, and both play a major role in virulence (Arbues *et al.*, 2013; Gonzalo-Asensio *et al.*, 2017). Furthermore, unlike in BCG, MTBVAC immunodominant antigens ESAT6 and CFP10 of the RD1 are conserved (Arbues *et al.*, 2013; Gonzalo-Asensio *et al.*, 2017). MTBVAC was at least as immunogenic as BCG and at the same dose level as BCG in healthy, BCG-naïve, HIV-negative adults (Gonzalo-Asensio *et al.*, 2017).

2.2.7. Chemotherapy

Chemotherapy is currently the best tool available to curb TB, considering the absence of a vaccine, natural and acquired immunity. In susceptible infection, although standard treatment regimens can be effective; cost, treatment duration and tolerability remain concerns. Drug-resistant TB (DR-TB) further presents a complication which necessitates (i) increased treatment duration; (ii) the use of expensive and toxic drugs; and (iii) additional clinical monitoring.

2.2.7.1. First-line drugs

First-line anti-TB drugs, also known as Group 1 TB drugs, are the cornerstone in the treatment of susceptible and first infection TB (Shakya *et al.*, 2012). The main mode of action for the first-line drugs is the disruption of cell wall synthesis (Table 2.2). Group 1 TB drugs are isoniazid, pyrazinamide, ethambutol, and the rifamycins. These drugs are orally administered and show potent activity as well as good tolerability.

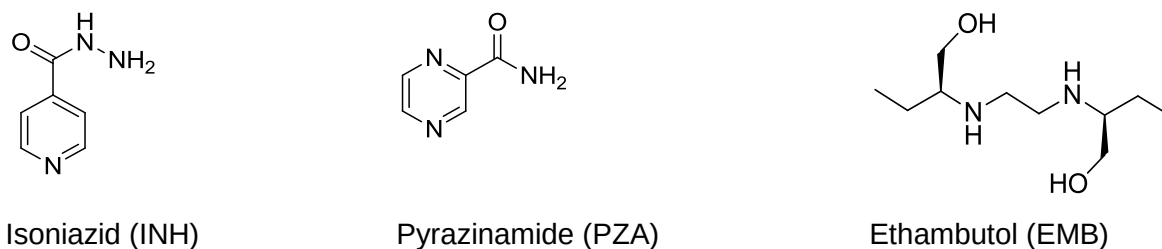


Figure 2.2: Non-rifamycin type of first-line TB drugs

Isoniazid

Isoniazid (INH, Figure 2.2) is an isonicotinic hydrazide with potent bactericidal activity against *Mtb* (WHO, 2017b). The drug is bactericidal against rapidly dividing cells and interferes with the bacterial cell wall by disrupting mycolic acid synthesis (Jena *et al.*, 2015; Jena & Harinath, 2015; WHO, 2014). In addition to use as a first-line treatment drug, it is also used for the prevention of TB in persons with HIV (Ayele *et al.*, 2015). The drug is commonly administered orally, but intramuscular (IM) and intravenous (IV) formulations are also available. The WHO dosage recommendations for INH-sensitive TB are 4 – 6 mg/kg/day (oral or IV, maximum 300 mg) for adults and 10 – 15 mg/kg/day (oral or IV, maximum 300 mg) for children. For preventive therapy, the WHO recommends, a daily dose of 5 mg/kg (maximum 300 mg) of INH for at least six months, ideally nine months. Additionally, 900 mg of isoniazid can be used in combination with 900 mg of rifapentine (section 2.2.7.1) once weekly for 12 doses (WHO, 2014).

INH is quickly absorbed and distributed into tissues and body fluids (WHO, 2017b). The drug is absorbed best on an empty stomach, while fatty meals lead to up to 50 % reduction in peak concentration (WHO, 2014). Since its metabolism occurs primarily by acetylation, this factor determines the plasma half-life that can vary from one hour in the presence of fast acetylators to three hours for slow acetylators. Its main excretion route is through the urine (WHO, 2014).

INH is well tolerated, but hypersensitivity may occur during the first initial weeks of treatment. In patients with epilepsy, INH interacts with anticonvulsant drugs that may necessitate dosage reduction of these drugs during isoniazid treatment (WHO, 2017b). INH should be used with caution in patients with hepatic disease as it may aggravate liver failure. Other adverse reactions include lethargy, diarrhoea, drug-induced lupus, and optic neuritis (WHO, 2014). Additionally, INH may interact with other drugs, interfere with their metabolism and increase their toxicity (e.g. serotonergic antidepressants and warfarin) (WHO, 2017b). Resistance occurs as a result of mutations in genes such as *katG* and *inhA* that play a role in the metabolism of the drug. KatG (*Mtb* catalase-peroxidase) is the enzyme that catalyses the synthesis of the isonicotinic acyl-NADH complex. The complex binds to InhA (an enoyl-acyl carrier protein reductase) that blocks fatty acid synthase and enoyl-AcpM, thus disrupting mycolic acid synthesis. Mutations in the aforementioned genes, therefore inhibit the formation of the isonicotinic acyl-NADH complex and disrupt the covalent bond with the carrier InhA (Shakya *et al.*, 2012).

Pyrazinamide

Pyrazinamide (PZA, Figure 2.2) is a nicotinamide analogue with potent sterilising activity and bactericidal activity against semi-dormant *Mtb* (WHO, 2017b). PZA is metabolised, by the enzyme pyrazinamidase, into its active metabolite, pyrazinoic acid. The active metabolite is particularly active at the low pH environment in the macrophages, where it disrupts the elongation of fatty acids by interfering with fatty acid synthase, ultimately disrupting mycolic acid synthesis (Shakya *et al.*, 2012). Moreover, PZA is potently efficacious in areas of acute inflammation and is, therefore, strategically used in the first three months of susceptible *Mtb* treatment, reducing the risk of relapse. The main advantage of PZA is that it accelerates the bactericidal effects of isoniazid and rifampicin, thus reducing treatment duration from nine to six months. This encourages patient compliance and reduces the risk of developing resistance.

The WHO dosage recommendations for adults are 25 mg/kg/day up to a maximum dose of 2 g and an additional intermittent dose of twice or thrice weekly of up to 50 mg/kg can be given and a 30 – 40 mg/kg/dose for children. In addition, excess weight considerations are made for this drug (Shakya *et al.*, 2012). PZA is administered orally, is well absorbed from the gastrointestinal tract, and is readily absorbed and distributed into tissues and body fluids. Plasma half-life is ten hours and peak hours of the drug are achieved in two hours (Prasanthi *et al.*, 2015). Metabolism of PZA occurs mainly in the liver and is excreted mainly in the urine. Unfortunately, PZA may cause gastrointestinal discomfort in addition to gout and

arthralgia (joint pains), hepatotoxicity, rash and photosensitivity. Much like other first-line drugs, PZA resistance occurs via mutations on the enzyme pyrazinamidase encoding gene *pncA* (Shakya *et al.*, 2012).

Ethambutol

Ethambutol (EMB, Figure 2.2) is a derivative of 1,2-ethanediamine that has bacteriostatic activity against actively growing mycobacteria. EMB disrupts *Mtb* cell wall formation by inhibiting arabinogalactan synthesis by interfering with the activity of the enzyme arabinosyltransferase. Arabinogalactan is a building block in the mycolyl-arabinogalactan-peptidoglycan complex of the *Mtb* cell wall (Telenti *et al.*, 1997). EMB is administered orally and absorbed from the gastrointestinal tract. EMB peak oral absorption occurs two – four hours, peak serum concentration two – three hours after the dose. EMB has a half-life of three – four hours and 80 % bioavailability, independent of food. It is mainly excreted in the urine with 20 % excreted unchanged in the faeces (WHO, 2014).

The WHO dosage recommendations for adults and children are 15 – 25 mg/kg/day, with higher doses recommended only during the initial months of therapy. Caution should be exercised in the calculation of weight-based dosages in order to avoid toxicity. Furthermore, dosage intervals and concentration should take into account renal function impairment. Side effects of ethambutol include red-green colour-blindness, joint pains and optic and peripheral neuritis. Resistance to ethambutol is associated with mutation in the gene *embB* that encodes for the enzyme arabinosyltransferase (Shakya *et al.*, 2012; Telenti *et al.*, 1997). However, long-term use of ethambutol may protect from the development of drug resistance (WHO, 2014)

Rifamycins

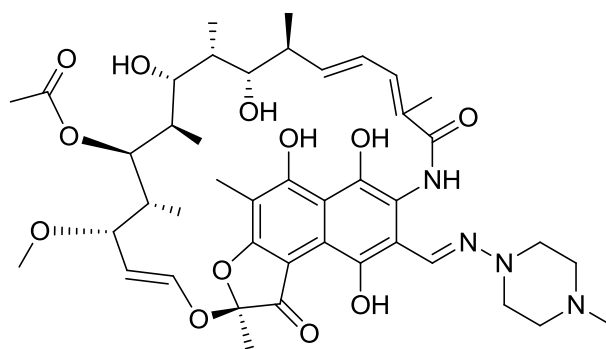
Rifamycins (Figure 2.3) bind to the β -subunit of the bacterial DNA-dependent RNA polymerase that is encoded by the *rpoB* gene, thus inhibiting protein translation and transcription. Furthermore, this method of binding uniquely equips rifamycins with activity against latent TB infection since inhibition occurs even during brief enzyme exposure. This subclass of TB drugs includes rifampicin, rifabutin and rifapentine. Inherently, cross-resistance between this class of drugs occurs and is mainly associated with mutation encoded by the *rpoB* gene (Craboli *et al.*, 2016; Munsiff *et al.*, 2006).

Rifampicin

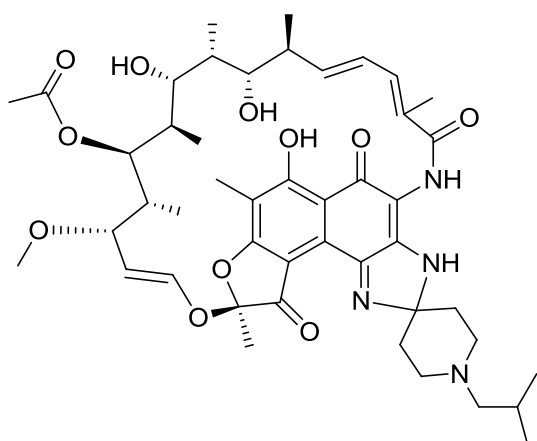
Rifampicin (RIF, Figure 2.3) is a potent anti-mycobacterial and a semisynthetic derivative of rifamycin. RIF is a broad-spectrum antibiotic, with potent efficacy against cellular and

extracellular mycobacteria that inhibits RNA synthesis and protein synthesis (WHO, 2014). This lipid soluble antibiotic, which can be administered orally or intravenously, is readily absorbed and distributed into tissues and body fluids, including cerebrospinal fluid (especially in the case of inflamed meninges) (Shakya *et al.*, 2012). The WHO dosage recommendations are 10 mg/kg/dose up to 600 mg for adults and 10 – 20 mg/kg/dose up to 600 mg for children. RIF has 80 % protein binding affinity and a rapid absorption which may be delayed or decreased by high-fat meals. The drug reaches peak serum concentration in two – four hours and has a half-life of two – three hours. Furthermore, RIF undergoes enterohepatic circulation and metabolism by deacetylation in the liver and its main excretion route is through the faeces (WHO, 2014).

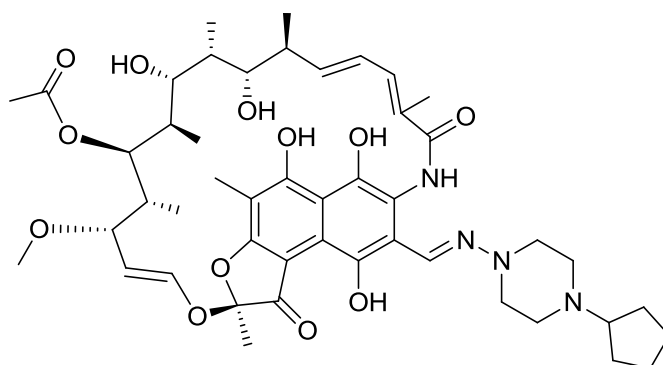
Although generally well tolerated by most patients, RIF may cause gastrointestinal reactions and potentially life-threatening dose-related hepatitis. Other adverse effects associated with this drug include fever, flu-like symptoms and thrombocytopenia. Additionally, HIV coinfecting individuals may suffer from exfoliative dermatitis. RIF induces hepatic enzymes, therefore, dose adjustments are necessary in co-administration with drugs metabolised in the liver such as warfarin, some antiretrovirals (ARVs) and anticonvulsants (WHO, 2017a). Precautions are also necessary when rifampicin is used by women on oral contraceptives (WHO, 2017b). Unfortunately, *Mtb* resistance to rifampicin develops speedily and cross resistance with other rifamycin antibiotics occurs (Shakya *et al.*, 2012; WHO, 2014). Rifampicin resistance is a consequence of conformational changes on the RNA polymerase as a result of mutations on the *rpoA* and *rpoC* genes, which encode for alpha- and beta-subunits of RNA polymerase (Telenti *et al.*, 1993). Furthermore, mutations on the *rpoB* gene, also known as a hot-spot region, play a role in rifampicin resistance (Qazi *et al.*, 2014).



Rifampicin (RIF)



Rifabutin (RFB)



Rifapentine (RPT)

Figure 2.3: Rifamycins

Rifabutin

Rifabutin (RFB, Figure 2.3) is a spiro-piperidyl-rifamycin derivative previously known as ansamycin (Crabol *et al.*, 2016). It is an antibiotic that is used for the treatment of drug-susceptible TB, mainly in HIV positive individuals on ARVs who show signs of intolerance to rifampicin (Narita *et al.*, 2000). RFB is an inhibitor of RNA polymerase that is administered orally. The WHO recommends a maximum dose of 300 mg (rarely 450 mg) for adults and 5-10 mg for children accompanied by extreme caution and monitoring (WHO, 2014). The drug is well absorbed from the gastrointestinal tract and can penetrate inflamed meninges. RFB has a low bioavailability (20 %); however, it has a higher intracellular tissue penetration and better tissue distribution in comparison with its sister drug, RIF (Crabol *et al.*, 2016).

Additionally, RFB has a half-life of 45 hours as a result of its large volume of distribution (9.3 L/kg at steady state) (Skinner & Blaschke, 1995).

Side effects of RFB include hepatotoxicity (similar to the sister drug, rifampicin), rash, skin discolouration and eye toxicity (WHO, 2014). Furthermore, RFB may decrease the effectiveness of oral hormone-based birth control agents as well as HIV drugs. Unfortunately, resistance to RIF also confers resistance to RFB and, as a result, about 80 % RIF-resistant strains are consequently resistant to RFB (WHO, 2014).

Rifapentine

Rifapentine (RPT, Figure 2.3) is a cyclopentyl-substituted semi-synthetic derivative of rifampicin that was discovered in 1965 (Munsiff *et al.*, 2006). The antibiotic acts by inhibiting RNA polymerase (WHO, 2014). RPT reaches peak concentration five – six hours post administration. The drug has 70 % oral bioavailability and a half-life of 13 hours. It is mainly metabolised in the liver by hydrolysis and deacetylation into an anti-mycobacterial active metabolite, desacetyl rifapentine. Furthermore, the drug is well tolerated, regardless of hepatic dysfunction, although monitoring of these patients is highly recommended (WHO, 2014).

The WHO recommends a dose of 600 mg once weekly during the continuation phase of treatment for adults and $450 \text{ mg} \leq 45 \text{ kg} \leq 600 \text{ mg}$ once weekly for children for the treatment of active TB. Moreover, the drug is recommended for LTBI (section 2.2.4). Adversely, RPT interacts with other drugs, such as oral contraceptives, and may cause hepatotoxicity, haematological disorders and hypersensitivity reactions. It is a bactericidal antibiotic of the same class as RIF and RFB hence it shares 100 % cross resistance with its sister drugs (WHO, 2014).

Table 2.2: Summary of current first-line TB drugs

Drug		MIC (µg/mL)	Half-life (hrs)	Activity	Mechanism of action
Isoniazid ^a		0.01-0.2	1-3	Bactericidal to rapidly dividing cells and bacteriostatic to non-dividing <i>Mtb</i>	Post <i>Mtb</i> catalase peroxidase activation - it disrupts cellular membrane by inhibiting mycolic acid synthesis
Pyrazinamide ^b		20-100	10	Bactericidal for semi-dormant <i>Mtb</i>	Requires activation by <i>Mtb</i> pyrazinamidase to disrupt mycobacterial growth
Ethambutol ^c		8.0	3-4	Bacteriostatic; bactericidal (high doses)	Inhibits the synthesis of arabinogalactan, which disrupts <i>Mtb</i> replication
Rifamycins ^d	Rifampicin	0.05-0.5	2-3	Bactericidal	Bind to DNA-dependent RNA polymerase, thus inhibiting RNA synthesis
	Rifabutin	2-4	45		
	Rifapentine	0.02	13-14		

^a (Jena et al., 2015; Shakya et al., 2012; WHO, 2017b), ^b (Shakya et al., 2012; WHO, 2017b), ^c (Shakya et al., 2012; Telenti et al., 1997; WHO, 2014), ^d (Craboli et al., 2016; Munsiff et al., 2006)

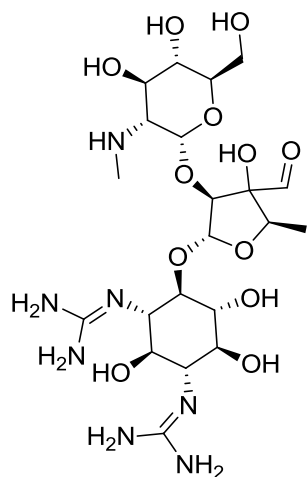
2.2.7.2. Second-line TB drugs

Injectable drugs

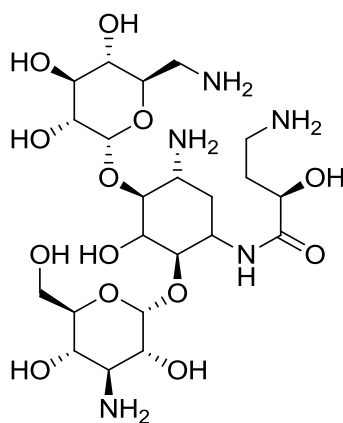
Injectable aminoglycosides (Figure 2.4) are second-line injectable drugs that include streptomycin, kanamycin, amikacin and capreomycin. Aminoglycosides are inhibitors of protein synthesis (Lee & Suh, 2016; Zhang, 2005). They are administered intravenously (IV) or intramuscularly (IM) mainly for the treatment of multi-drug resistant TB (MDR-TB) (Lee & Suh, 2016). They have poor activity compared to first-line drugs, which necessitates a longer MDR and XDR treatment duration and, consequently, increases toxicity exposure (Lee & Suh, 2016).

Aminoglycosides have variable penetration in the cerebrospinal fluid, but meninges inflammation improves drug penetration (Nau et al., 2010). These drugs disrupt translation by reversibly binding to the bacterial 30S ribosomal subunit (Leggett, 2017). The WHO recommends a maximum dose of 1 g at 15 mg/kg/day in a single daily dose for children and adults (except > 59 years of age in which case the maximum dose is 750 mg). Cross-resistance within this class often occurs; particularly kanamycin and amikacin which are preferred as a result of wide spread streptomycin-resistance. Adversely, kanamycin is more nephrotoxic than streptomycin. Furthermore, aminoglycosides cause ototoxicity (including

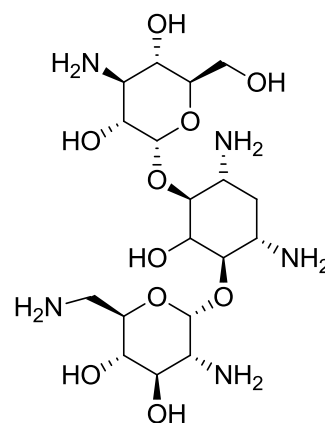
congenital deafness when used during pregnancy) and vestibular toxicity, whose severity increases with advanced age and long-term use. Electrolyte abnormalities and local pain are additional side effects associated with aminoglycosides. Reduced drug uptake and reduced ribosomal binding confer pathogen resistance to aminoglycosides. Furthermore, resistance is perpetuated by enzymatic deactivation as a result of acetylation, adenylation or phosphorylation (Leggett, 2017).



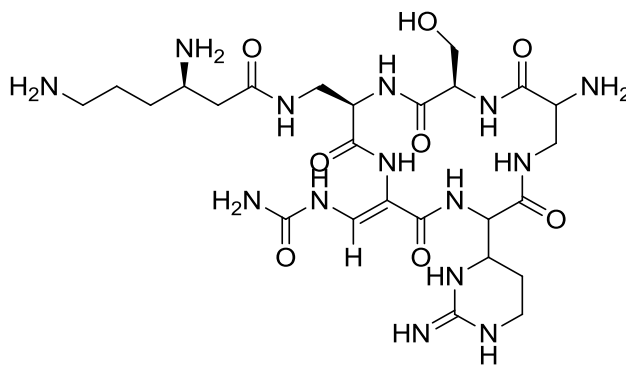
Streptomycin
MIC = 2–8 µg/mL



Amikacin
MIC = 4–8 µg/mL



Kanamycin
MIC = 1–8 µg/mL



Capreomycin
MIC = 1.25–2.5 µg/mL

Figure 2.4: Second-line TB drugs, also known as injectable protein synthesis inhibitors. (Shakya *et al.*, 2012)

Fluoroquinolones

Fluoroquinolones (FQs, Figure 2.5) are a class of antimicrobial compounds derived from nalidixic acid (Van Bambeke *et al.*, 2005). Despite shortcomings such as poor bioavailability, limited antimicrobial activity, and quick development of drug resistance, manipulation and derivatisation of nalidixic acid has led to the discovery of drugs such as levofloxacin, moxifloxacin, gatifloxacin, ofloxacin, and ciprofloxacin (Mandell & Tillotson, 2002). In addition to antibacterial activity, it has been reported that FQs also function as immunomodulators, antioxidants and nitric oxide (NO) regulators (Blower *et al.*, 2016). FQs act by inhibiting DNA gyrase, an enzyme that catalyses the process of negative supercoiling of DNA during replication (Blower *et al.*, 2016; Lee & Suh, 2016). This action leads to the disruption of DNA replication and transcription of the bacilli (Shakya *et al.*, 2012). FQs are generally well tolerated, however side effects such as nausea, vomiting, diarrhoea and abdominal pain commonly occur. In rare cases renal disturbances, skin hypersensitivity and blood disorders can occur (Schluger, 2013).

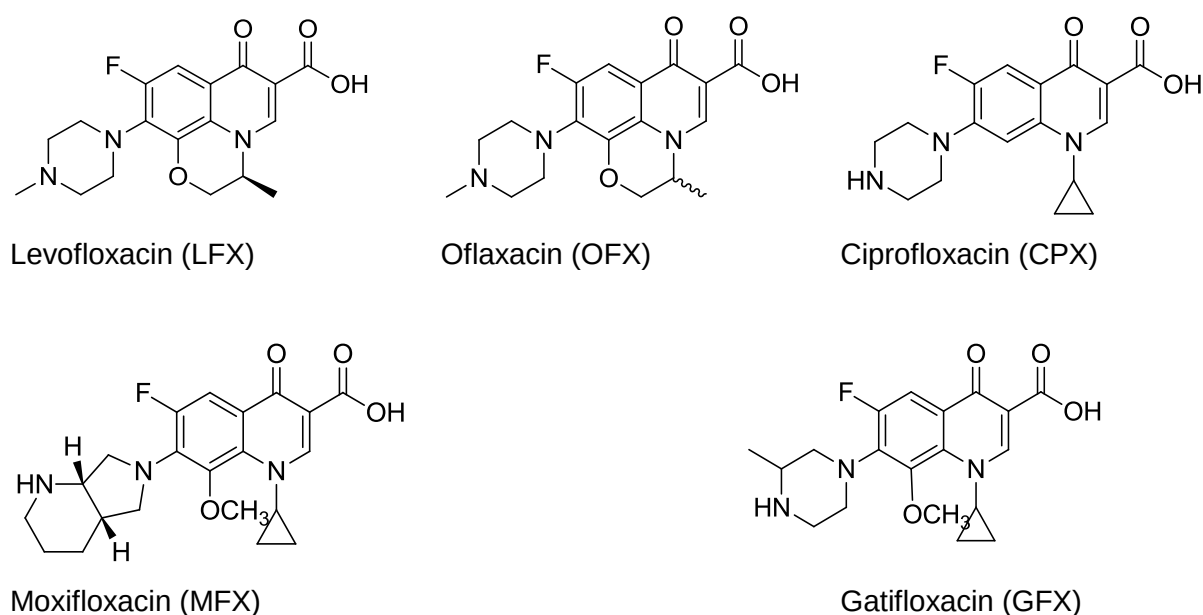


Figure 2.5: Fluoroquinolones (FQs), which form Group 3 of TB drugs

Levofloxacin (LFX, Figure 2.5) is bactericidal with potent anti-mycobacterial activity. For the treatment of TB, the WHO recommends a daily dose of 10 – 15 mg/kg of LFX once daily for adults, 15 – 20 mg/kg split into two doses (morning and evening) for children under the age five years and 10 – 15 mg/kg once daily for children over the age of five years. The drug is well absorbed, but antacids, milk-based products and divalent cation medication may reduce its absorption. Moreover, side effects such as headaches, dizziness, insomnia or tremulousness and hypoglycaemia may occur (WHO, 2014).

Ofloxacin (OFX) and ciprofloxacin (CPX) in Figure 2.5; are early generation FQs. These drugs demonstrate excellent activity against Gram negative bacteria. They were initially used for urological infections, because of their ability to reach high concentrations in the urinary tract (Mandell & Tillotson, 2002). Moxifloxacin (MFX, Figure 2.5) is well absorbed, evidently from 90 % availability. Gatifloxacin (GFX, Figure 2.5) is efficiently absorbed from the gastrointestinal tract, resulting in 96 % bioavailability (WHO, 2014). Similar to LFX, antacids, milk-based products and divalent cation medication may reduce GFX absorption; hence administration is advised two hours before or four hours after ingestion of these products (WHO, 2014). Additional undesirable effects of GFX include pain, swelling or tearing of a tendon, muscle or joint pain, and yellowing of the skin or eyes. Furthermore, GFX may worsen diabetes and glycaemic control. Patients may experience anxiety, confusion or dizziness, frequent urination and increased thirst, which could be symptomatic of hypoglycaemia or hyperglycaemia. The presence of the methoxy (OCH₃) at carbon-8 has an impact on toxicity in that FQs with this substitution (MFX, GFX) show reduced mammalian phototoxicity and cytotoxicity (Dong *et al.*, 1998). FQs inhibit DNA gyrase and mutations in this pathway confer both resistance and cross resistance with other FQs (Dong *et al.*, 1998).

Oral bacteriostatic drugs

Oral bacteriostatic drugs (Figure 2.6), such as ethionamide (ETO), prothionamide (PTO), *p*-aminosalicylic acid (PAS), *p*-aminosalicylate sodium, cycloserine (CS) and terizidone (TRD), are also second-line anti-TB drugs (D'Ambrosio *et al.*, 2015). ETO and PTO are pyridine-thioamide hybrids with weak bactericidal activity (Lee & Suh, 2016). These drugs act by inhibiting mycolic acid synthesis, thus disrupting cell wall synthesis (Lee & Suh, 2016). The WHO recommends an oral daily dose of 15 – 20 mg/kg/day up to a maximum of 500 – 750 mg for adults, and 15 – 20 mg/kg/day usually divided into two – three doses up to 1 g per day for children. The dose should be accompanied by vitamin B6 (WHO, 2014). The drugs are available for oral administration, although absorption data suggests inconsistencies as a result of gastrointestinal disturbances. Adverse reactions as a result of these derivatives of isonicotinic acid include gastrointestinal upset, anorexia, bruising, bleeding, vomiting, yellowing of skin or eyes, swollen breasts in men and hepatotoxicity. Possible drug-drug interaction with CS, may result in neurotoxicity (Shakya *et al.*, 2012, WHO, 2014).

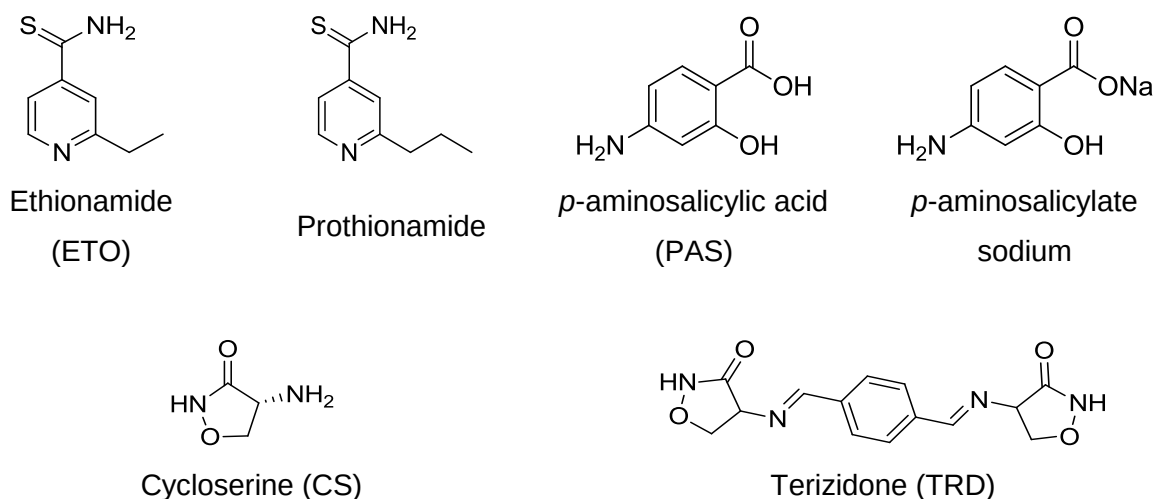


Figure 2.6: Oral bacteriostatic second-line anti-TB drugs

CS and TRD, in Figure 2.6, are bacteriostatic cyclic analogues of D-alanine. Dimerization of CS using *p*-xylene as a linker resulted in the discovery of TRD. TRD is better tolerated than CS, however efficacy may be compromised (Hwang *et al.*, 2013). These drugs inhibit cell wall synthesis by inhibiting peptidoglycan synthesis (Lee & Suh, 2016). CS and TRD are recommended by the WHO as second-line drugs for the treatment of MDR-TB (Hwang *et al.*, 2013). Advantageously, CS does not share cross-resistance with other anti-TB drugs, but drug toxicity is a concern. Since drug toxicity impacts patient compliance with the treatment regimen, it may perpetuate *Mtb* resistance. Side effects, such as headaches and depression, have precluded the use of CS by individuals with a history of seizures or epilepsy and psychiatric disorders (Hwang *et al.*, 2013).

PAS (Figure 2.6) and the sodium salt derivative *p*-aminosalicylate sodium are salicylic acid derivatives and oral bacteriostatic anti-TB drugs (D'Ambrosio *et al.*, 2015). PAS was a first-line drug, before the advent of better potency TB drugs, and it continues to be used in the treatment of drug-resistant TB infection (Minato *et al.*, 2015). It is thought to act via the disruption of iron acquisition. TB resistance to PAS was described shortly after it was introduced for clinical use and is mainly prevalent in monotherapy. However, the spread of DR-TB has seen the reintroduction of PAS into TB treatment regimens, despite gastrointestinal disturbances commonly associated with the drug (Zumla *et al.*, 2013).

Miscellaneous

Previously classified as Group 5 (WHO, 2012) or Group D add-on agents (WHO, 2016), this group consists of anti-TB drugs with limited data regarding efficacy and long-term safety in the treatment of drug-resistant TB. This group includes new anti-TB agents and repurposed

agents such as bedaquiline, delamanid, pretomanid, linezolid, sutezolid, clofazimine, amoxicillin/clavulanate, imipenem/cilastatin, meropenem, high-dose isoniazid, thioacetazone, and clarithromycin (D'Ambrosio *et al.*, 2015, Tiberi *et al.*, 2017).

Bedaquiline (BDQ, Figure 2.7) is a new bactericidal diarylquinoline drug, complete with a novel mode of action against *Mtb*. It is primarily metabolised by the CYP isoenzyme CYP3A4 into *N*-monodesmethyl metabolite (M2). BDQ inhibits mycobacterial ATP synthase by interacting with the subunit *c* of mycobacterial ATP synthase and depleting intracellular ATP (Dheda *et al.*, 2016). There are concerns about the selectivity of this process as it may also inhibit mitochondrial ATP synthase, causing mitochondrial toxicity, thus leading to conditions such as pancreatitis, cardiac and skeletal myopathies. However, mitochondrial ATP synthase displayed negligible sensitivity compared to mycobacterial ATP synthase (Haagsma *et al.*, 2009; Riccardi & Pasca, 2014). In consideration of the yet unexplained high mortality rate observed in the group treated with BDQ during clinical trials, an investigation into the safety and efficacy of this drug focusing on a nine month oral-only and a six month regimens was conducted (Gualano *et al.*, 2016). Although the outcome of the study led to the conclusion that the drug is safe to use in 6 month TB treatment regimens, concerns of its role in regimens that extend beyond 6 months, such as in cases of severe DR-TB, remain unaddressed (Mbuagbaw *et al.*, 2019).

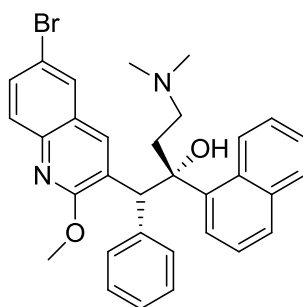


Figure 2.7: Bedaquiline (BDQ)

The WHO recommends a daily 400 mg dose for two weeks, followed by a 200 mg dose three times a week for 22 weeks, for adults (WHO, 2014). The drug is absorbed better when taken with food. BDQ is a long-lasting antibiotic with a half-life of 5.5 months. Gastrointestinal disorders, joint pain, headaches, QT (a measurement of some electrical properties of the heart) elongation (mainly associated with the *N*-monodesmethyl metabolite) and an increased risk of pancreatitis are some adverse effects associated with the use of this drug (Mbuagbaw, 2017). Adversely, inducers of CYP3A4 such as RIF (section 2.2.7.1) and efavirenz (HIV/AIDS treatment drug) reduce the efficacy of BDQ (WHO, 2014). Risk

assessment should, therefore, be conducted for use of BDQ in the presence of CYP3A4 inhibitors such as protease inhibitors, and azole anti-fungal drugs (WHO, 2014). Studies to determine the effectiveness and safety profile of BDQ in the treatment of paediatric MDR-TB are in Phase II (May 2016 - July 2025) clinical trials (Janssen, 2019).

Delamanid is a nitroimidazole (Figure 2.8) anti-TB agent that inhibits mycolic acid synthesis and produces reactive nitrogen species (Riccardi & Pasca, 2014). Clinical trials have shown that delamanid had good tolerability, but QT prolongation occurred frequently in patients receiving delamanid compared to those receiving placebo (D'Ambrosio *et al.*, 2015). The WHO recommends the use of delamanid at the dose of 100 mg twice daily for six months for adults (D'Ambrosio *et al.*, 2015).

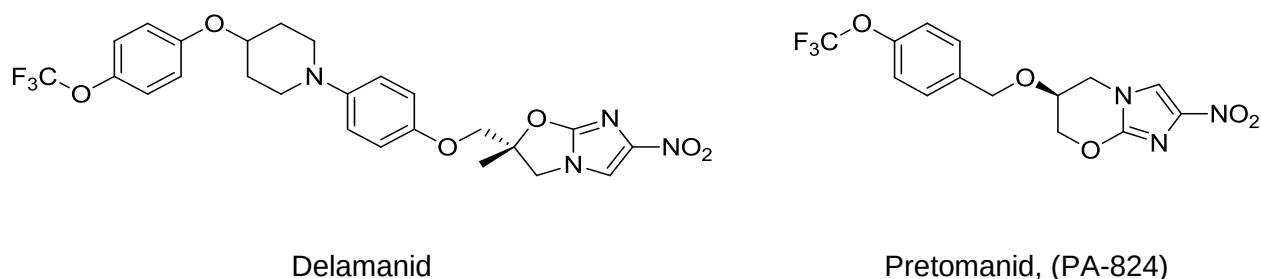


Figure 2.8: Nitroimidazole TB drugs

Pretomanid (previously known as PA-824) is a nitroimidazole anti-TB antibiotic of the same class (Figure 2.8) as delamanid. It disrupts the formation of the *Mtb* cell envelope by inhibiting the synthesis of mycolic acids (Singh *et al.*, 2008) and its activity is comparable to that of isoniazid (0.01-0.2 µg/mL). Although the drug showed good curative rates in both susceptible and DR-TB, as well as good interactions when combined with other drugs, clinical trials have been suspended amid hepatotoxicity concerns in HIV-positive individuals (Baptista *et al.*, 2018; D'Ambrosio *et al.*, 2015).

Linezolid (LZD) is a bactericidal oxazolidinone (Figure 2.9) that inhibits protein synthesis (McNeil *et al.*, 2017). The WHO recommends an oral daily dose of 600 mg for adults, and maximum dose of 600 mg at 10 mg/kg three times daily for children. LZD is well absorbed post oral administration. Furthermore, it can penetrate the cerebrospinal fluid and, as a result, it has been used for the treatment of meningitis in humans. Adversely, LZD diminishes platelet and white blood cell count (WHO, 2014) and other side effects include lactic acidosis accompanied by diarrhoea and nausea. It should be noted that these adverse effects could be related to mitochondrial toxicity as a result of the ability of LZD to interfere

with mitochondrial protein synthesis (Song *et al.*, 2015; Soriano *et al.*, 2005). The correlation between LZD and mitochondrial toxicity is also demonstrated by the cessation of adverse effects when LZD use is terminated. Mitochondrial toxicity thus limits its long-term use despite positive efficacy at a reduced dosage (300–600 mg/day). Fortunately, administration with clarithromycin (Figure 2.12) can boost LZD bioavailability, thus allowing for lower doses and reduced adverse effects. The drug is, therefore, recommended for use with standard regimen drugs at 300 and 600 mg for the treatment of MDR or extensively-drug resistant TB (XDR-TB), respectively (D'Ambrosio *et al.*, 2015).

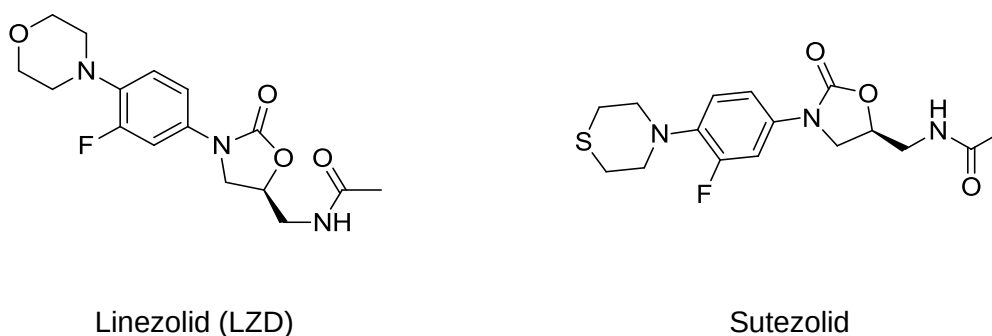


Figure 2.9: Oxazolidinone TB drugs

Owing to neuropathy and anaemia associated with LZD, sutezolid (PNU-100480), is a better oxazolidinone (Figure 2.9) with improved tolerability and efficacy (McNeil *et al.*, 2017). Sutezolid acts by inhibiting the initiation of protein synthesis by binding to 23S RNA in the 50S ribosomal subunit of the mycobacteria (McNeil *et al.*, 2017).

Clofazimine (CFZ, Figure 2.10) is an aminophenazine anti-TB agent reserved for use in cases where drug options are limited. Advantageously, the drug improved the treatment success rate, fast sputum culture conversion and cavity closure. Disadvantageously, CFZ may cause hyperpigmentation, gastrointestinal disorder, bleeding and bowel obstruction and QT prolongation (WHO, 2014). Additionally, CFZ may promote BDQ resistance (Dheda *et al.*, 2016).

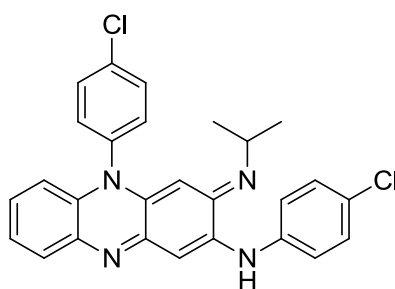


Figure 2.10: Clofazimine (CFZ)

Carbapenems (Figure 2.11), such as imipenem (Imp) and meropenem (Mpm), are dual action anti-*Mtb* drugs that target the mycobacterial cell wall as well as β -lactamase (Jaganath *et al.*, 2016). Imipenem is a β -lactam carbapenem used in combination with cilastatin (Figure 2.11) to elicit potent *in vitro* anti-*Mtb* activity (WHO, 2014). There is limited clinical data available for this combination; however, imipenem/ cilastatin co-administration is aimed at countering rapid degradation of imipenem by renal proximal tubule dipeptidases, since cilastatin inhibits dipeptidases (Keynan *et al.*, 1995). Diarrhoea, nausea and vomiting are the most common side effects associated with carbapenem drugs (WHO, 2014). Imipenem is contraindicated in children with meningitis since it is associated with high rates of seizures, in which case mpm is preferred. Mpm is a β -lactam carbapenem used in combination with clavulanate (Figure 2.11) to illicit potent *in vitro* anti-*Mtb* activity (WHO, 2014). On account of drug-resistance, clavunate is also administered in combination with amoxicillin (Amx, Figure 2.11) a broad spectrum penicillin antibiotic listed in Group 5 for the treatment of MDR-TB (WHO, 2014).

Although clinical TB-resistance to carbapenems has yet to be reported, Kumar and co-workers indicated that a mutation on an unannotated protein confers *Mtb* resistance to carbapenems (Kumar *et al.*, 2017).

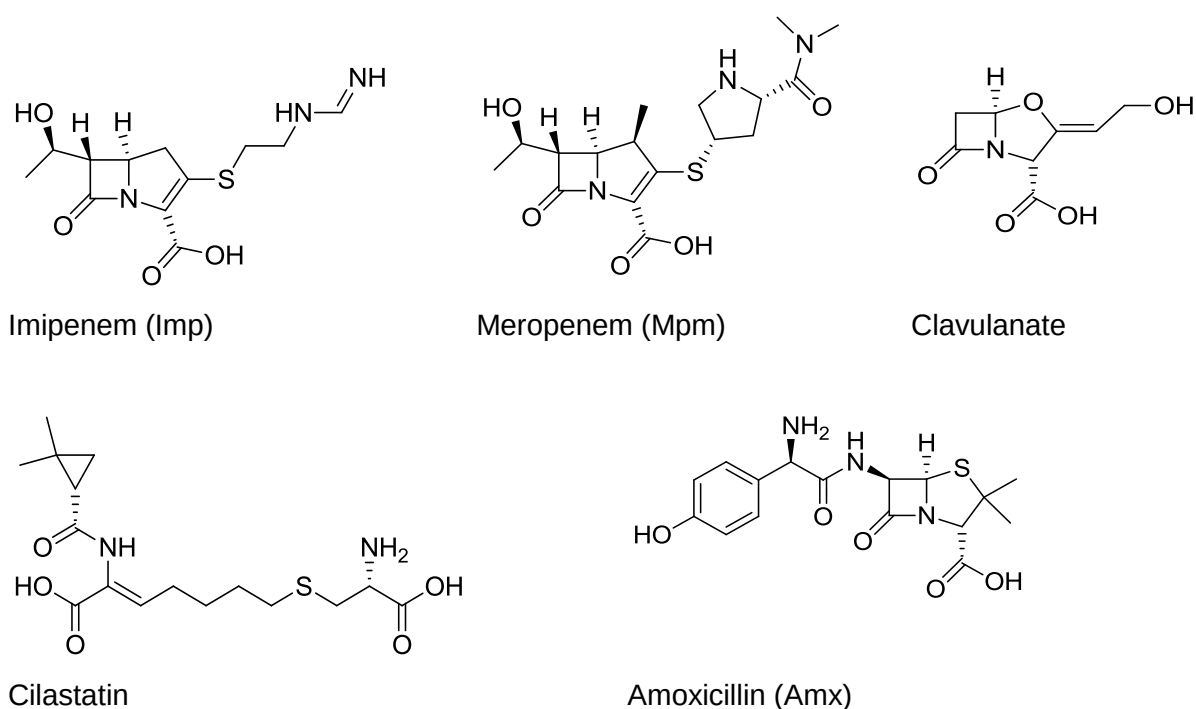
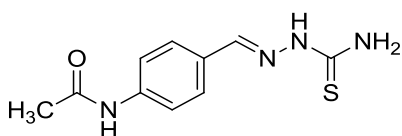
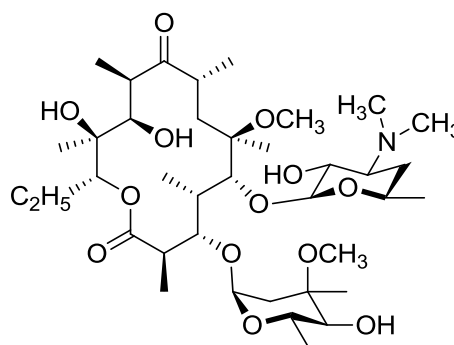


Figure 2.11: Structures of carbapenems and related drugs

Thioacetazone and clarithromycin (Figure 2.12) are no longer part of Group 5 anti-TB drugs, as recommended by the WHO (D'Ambrosio *et al.*, 2015). Thioacetazone is an oral antibiotic whose use has been stopped as a result of toxicity and severe side effects, such as neurological effects and exfoliative dermatitis. Toxicity was more pronounced in HIV positive individuals (Donald & McIlleron, 2009). It was one of the first anti-TB drugs that was soon replaced by drugs such as INH (Figure 2.2), but remained to serve as a reserve drug. Clarithromycin is a macrolide that used to be a Group 5 drug, but it is not recommended for use because of its limited bacteriostatic efficacy as well as inconsistent accuracy and reproducibility during laboratory testing (WHO, 2014). Furthermore, adverse effects headaches, nausea, diarrhoea and abdominal pain are frequently reported with this drug (Van der Paardt *et al.*, 2017).



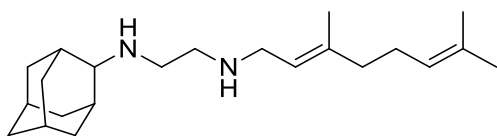
Thioacetazone



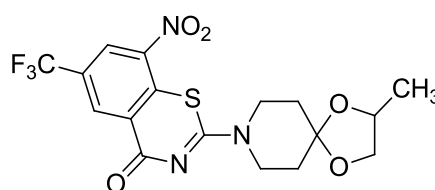
Clarithromycin

Figure 2.12: Structures of thioacetazone and clarithromycin

SQ109 in Figure 2.13, is a 1,2-ethylenediamine analogue of ethambutol with activity against both drug-susceptible and drug-resistant TB. The drug targets *MmpL3* in *Mtb* and inhibits protein synthesis. SQ109 interacts synergistically with BDQ and sutezolid (D'Ambrosio *et al.*, 2015).



SQ109



BTZ043

Figure 2.13: Newer TB drugs currently in clinical trials

BTZ043 in Figure 2.13, is a benzothiazine that impedes the synthesis of mycobacterial cell wall by inhibiting the synthesis of the arabinans precursor decaprenylphosphoarabinose (D'Ambrosio *et al.*, 2015; Hans-Knöll, 2018). BTZ043 is currently in Phase I clinical trials and, to date, the data suggests high selectivity and potency in drug-susceptible TB, MDR-TB and XDR-TB (Hans-Knöll, 2018). Furthermore, synergistic effects are observed when the drug is used with BDQ or delamanid (D'Ambrosio *et al.*, 2015). Synergistic effects observed with either new drug or drugs in clinical trials create an opportunity for the development of new anti-TB regimens. Together with the novel modes of action offered by these drugs, strides toward the containment of DR-TB may yield fruitful results.

2.2.8. Susceptible pulmonary tuberculosis treatment

The six-month rifampicin-based regimen (2HRZE/4HR) remains the recommended standard regimen for the treatment of drug-susceptible TB. The four drug regimen consists of INH, RIF, EMB and PZA also known as first-line TB drugs (section 2.2.7.1) (Tiberi *et al.*, 2017; WHO, 2017b). For HIV co-infected individuals, the WHO recommends antiretroviral treatment (ART) should be started or continued regardless of CD4 cell count (WHO, 2017b). In situations where both treatments are needed, the WHO recommends starting TB treatment first, followed by HIV treatment within the first eight weeks of TB treatment. However, for HIV patients with severe immunosuppression (CD4 cell counts less than 50 cells/mm³), HIV treatment should start within two weeks of initiating TB treatment (WHO, 2017b).

2.2.9. Drug Resistance in *Mycobacterium tuberculosis*

Proper treatment of TB involves strict adherence to the regimen of a cocktail of drugs over a minimum period of six months. However this is often accompanied by side effects and patient non-compliance is common during this time, particularly at cessation of TB symptoms (WHO, 2017b). The patient may feel there is no need for treatment or bearing with unfavourable side effects and becomes less vigilant with the treatment course, thus causing treatment failure. This undermining of treatment integrity creates an opportunity for the mycobacteria to develop drug resistance (WHO, 2017a).

The predicament of resistant *Mtb* has been persistent since the 1980s and is perpetuated by the TB treatment set-up which involves exposure to the same drugs for lengthy periods. This promotes patient non-compliance and an opportunity for resistant strains to emerge, which are described as rifampicin-resistant TB (RR-TB), MDR-TB, XDR-TB, and totally drug resistant (TDR-TB) *Mtb* strains (WHO, 2017a). MDR-TB is resistant to at least isoniazid and rifampicin. In addition to MDR-TB resistance, XDR-TB has resistance to any of the fluoroquinolones and to at least one of the injectable drugs such as amikacin, capreomycin, kanamycin (Tiberi *et al.*, 2017). Devastatingly, TDR-TB strains are resistant to all clinical anti-mycobacterial drugs (Zumla *et al.*, 2014). The pressing issue with resistant strains is the lack of chemotherapeutic agents particularly, since resistant strains are incurable by available TB drugs, which only serves to put pressure on the need for containment of transmission of resistant strains and the urgency of the development of new and effective drugs or, in the very least, the development of efficacious cocktails from available drugs (Smith *et al.*, 2013).

The treatment of resistant TB is governed by specific conditions in service of ensuring effectiveness, reduced toxicity and improved probability of cure. The most strict and standard rule is that drug-susceptibility testing should be conducted. An additional consideration is the patient's eligibility for a shorter treatment regimen; however, the standard recommended treatment duration for patients (excluding pregnant women) with RR-TB and MDR-TB is 9 - 12 months. RR-TB and MDR-TB patients who have never been treated with second-line drugs should be treated with a cocktail of at least five active drugs. The cocktail should include PZA and four core second-line drugs and at least two drugs from Group C (Tiberi *et al.*, 2017). An example of the cocktail would be PZA, core second-line drugs (ethionamide, cycloserine, clofazimine) and an injectable moxifloxacin (Tiberi *et al.*, 2017). Furthermore, newer drugs can be incorporated into the regimen, for example LZD, BDQ and delamanid may form part of the core second-line drugs.

2.2.10. Resistance to newer drugs

Resistance to BDQ (a diarylquinoline, Figure 2.7) and delamanid (a nitroimidazole, Figure 2.8) was described in section 2.2.7.5. In a case study involving a patient who had not received TB treatment before, an *Mtb* isolate displayed nine mutations in genes associated with resistance to seven drugs (namely INH, RIF, PZA, ETO, LZD, MFX and streptomycin). The mutation in *mmpR* is associated with BDQ resistance, whereas two mutations in *fbtA* and *fgd1* have been associated with phenotypic resistance to delamanid. This case highlights the need for the use of appropriate companion drugs in MDR-TB and XDR-TB treatment and emphasizes the importance of carefully designed TB cocktails, since it occurred in a well-resourced healthcare setting (Bloemberg *et al.*, 2015).

2.3 Leishmaniasis

Neglected tropical diseases (NTDs) are tropical and sub-tropical prevalent diseases that affect developing countries, mostly where poverty and resource limitations are rampant. NTDs affect more than 1 billion of the world's population (WHO, 2019). NTDs contribute to a large social and economic burden and have consequences, such as discrimination, malnutrition, social stigma, physical disabilities and disfigurement. In children, consequences include growth failure and impaired cognitive development. Economic divide, remoteness of endemic villages, poverty, and subpar access to healthcare contribute to delayed diagnosis and treatment. Consequently, transmission and clinical disease manifestation accentuates morbidity and mortality, thus limiting individuals from leading productive lives. NTDs diversely include bacterial, parasitic, viral, and fungal infections (WHO, 2019).

Leishmaniasis, is an NTD that is widely distributed across 89 countries, particularly across Asia, Africa, the Americas, and the Mediterranean region (Alvar *et al.*, 2006; Torres-Guerrero *et al.*, 2017). The disease burden tallies up to 350 million people at risk of infection, between 700 000 - 1 million new infection cases and up to 30 000 fatalities annually (WHO, 2018a). The prevalence of leishmaniasis can be attributed to socio economic conditions, such as development of new settlements, including deforestation, rural to urban migration, poor housing and sanitation as well as fast and unplanned urbanization, malnutrition, and climate change (WHO, 2018a).

2.3.1. Classification and clinical manifestations

Leishmaniasis is a parasitic disease caused by intracellular protozoan flagellates of the genus *Leishmania*, which invades and replicates in the macrophages (Cobo, 2014). There are twenty species of *Leishmania* (*L*) that can infect humans and thirty sand-fly species (pathogen vector, particularly *Phlebotomus* and *Lutzomyia*) that can transmit the disease (Torres-Guerrero *et al.*, 2017). There are different clinical forms of the disease, namely muco-cutaneous (MCL), diffuse cutaneous (DCL), cutaneous (CL, Aleppo boil) and visceral leishmaniasis (VL, kala azar, black fever) resulting in mutilation, aesthetic/social stigma (as a result of multiple lesions) and if left untreated, death (Berman, 1997).

2.3.1.1. Muco-cutaneous leishmaniasis

MCL, which is caused by *L. braziliensis*, *L. panamensis* and *L. amazonensis*, is predominant in Peru, Bolivia and Brazil with approximately 35 000 cases reported annually (Guerra *et al.*, 2011; Zulfiqar *et al.*, 2017). MCL manifests slowly and can proliferate over an average of 3

years, affecting mainly the mouth and nose, although it may occur on the oropharynx and larynx (Handler *et al.*, 2015).

Mucosal metastasis, a consequence of *L. braziliensis* infection, is caused by the dissemination of amastigotes through the blood and lymphatic system from the skin to the naso-oropharyngeal mucosa. The metastatic lesions cause facial disfigurement as a result of the progressive disintegration of nasal mucosa, soft and hard palate and ultimately the nasal septum (Kassi *et al.*, 2008; Zulfiqar *et al.*, 2017). Unfortunately, facial disfigurement can lead to social discrimination and poor quality of life (Handler *et al.*, 2015; Kassi *et al.*, 2008).

2.3.1.2. Cutaneous leishmaniasis

CL (Aleppo boil) is the most prevalent form of leishmaniasis (de Vries *et al.*, 2015). Globally, 600 000 to 1 million cases of CL are reported each year, 95 % of which occur in the Americas, the Mediterranean Basin, the Middle East and Central Asia, collectively (WHO, 2018a). *L. aethiopica*, *L. major* and *L. tropica*, and in rare cases *L. infantum*, are responsible for CL infection in countries such as India, Ethiopia, Sudan and Iran (Kroidl *et al.*, 2014; Zulfiqar *et al.*, 2017). In Central and South American countries (from Mexico to Argentina) the disease is commonly caused by members of the *Viannia* subgenus, namely *L. peruviana* (may progress into MCL), *L. braziliensis*, *L. amazonensis*, *L. guyanensis*, *L. venezuelensis* and *L. panamensis* (Showler & Boggild, 2015; Zulfiqar *et al.*, 2017).

CL infection is initiated when a lesion develops at the site of the sand fly bite. The initial size of the lesion may range from millimetres to centimetres in diameter, after which it enlarges and is accompanied by redness of the skin. Typically, plaques, papules and nodule formation, as well as skin thickening occur, which can cause scarring (Cobo, 2014; de Vries *et al.*, 2015).

2.3.1.3. Visceral leishmaniasis

VL (kala azar or black fever) is caused by *L. donovani* in the Indian subcontinent and Africa, whereas *L. infantum* (also known as *L. chagasi*) is responsible for infection in the Mediterranean regions and the New World (Chappuis *et al.*, 2007; Zulfiqar *et al.*, 2017). VL mainly affects bone marrow, liver and spleen (to a larger extent), thus presenting symptoms such as fever, hepatosplenomegaly, pancytopenia and weight loss. Clinical manifestations, such as the symptoms, the duration and presentation of the disease, differ from patient to patient. In its latent form, VL may survive and remain undiagnosed. Severity of the disease is diverse, ranging from mild VL (does not progress to classic kala azar) to Sudanese disease

(killing disease) and VL as a component of AIDS (Zijlstra, 2014). Mild VL is accompanied by symptoms such as fever, chronic fatigue, malaise, cough, irregular diarrhoea, abdominal pain and poor tolerance for work or play (Chappuis *et al.*, 2007; Gallina *et al.*, 2014).

Individuals with a compromised immune system, such as those living with HIV, are at high risk of VL infection. Third world countries such as Brazil, Ethiopia and India (Bihar state) have the highest leishmaniasis - HIV coinfection burden (WHO, 2018a). Furthermore, VL, as an opportunistic component of HIV/AIDS, can affect individuals from economically thriving countries such as southern Europe (Berman, 1997). HIV-induced immunosuppression may reactivate pre-existing latent leishmaniasis (Zijlstra, 2014). However, ARV treatment in co-infected patients may ward off the development of leishmaniasis (WHO, 2018a).

Sudan carries a 4 % VL burden with the Sudan endemic Sudanese disease, which results in post-kala azar dermal leishmaniasis (PKDL) and congenital leishmaniasis (Berman, 1997). In fact, 50 % of VL treated in Sudan develops into PKDL (Zulfiqar *et al.*, 2017). Symptoms of Sudanese disease include neurological symptoms, leishmanial cholecystitis (inflammation of the gall bladder) and burning feet sensation, (Handler *et al.*, 2015). Other adverse reactions include mouth macules and papules that can subsequently spread to the rest of the face and ultimately to the entire body (Berman, 1997; Srivastava *et al.*, 2011).

2.3.2. Life cycle of the *Leishmania* parasite

There are two developmental forms of the *Leishmania* parasite, namely the promastigote (infective, Figure 2.14 B) and amastigote (clinical, Figure 2.14 C). Both forms are morphologically similar (Figure 2.14), each with a kinetoplast, the DNA hub contained in the mitochondrion (Zulfiqar *et al.*, 2017). The promastigote is long and flagellated, whereas the amastigote is tear-drop shaped and lacks a flagellum. The life cycle of the *Leishmania* parasite occurs in vertebrates (including humans, dogs, rodents and wild animals, such as leopards and baboons) and the sand fly (the disease vector) (Handler *et al.*, 2015).

In the sand fly, the promastigotes multiply and differentiate by binary fission (Handler *et al.*, 2015). The process begins when the sand fly bites an infected vertebrate host, ingesting a macrophage containing *Leishmania* amastigotes with its blood meal. This initial process creates a peritrophic matrix (made from chitin and glycoproteins) that envelopes the meal and separates it from the midgut epithelium (Teixeira *et al.*, 2013). In the midgut, the amastigote is released from the macrophage and begins to differentiate into procyclic promastigotes (Figure 2.14). This step is characterised by the change in shape from spherical (Figure 2.14 C) to elongated (Figure 2.14 B), and the development of the motile

flagellum (Teixeira *et al.*, 2013). Procyclic promastigotes develop into nectomonad promastigotes which escape from the peritrophic matrix and attach themselves to the microvilli in the gut. Subsequently, nectomonad promastigotes travel into the thoracic midgut and stomodeal valve where they differentiate into leptomonad promastigotes that can develop into either haptomonad promastigotes that attach to the stomodeal valve or the vertebrate-infective metacyclic promastigotes (Rogers *et al.*, 2002). As the promastigote differentiates from one form to the next, other changes such as the opening of the neck region of the flagellar pocket, and the improvement of cell motility occur. These changes promote macromolecular uptake as well as the flexibility of movement (Sunter & Gull, 2017; Wheeler *et al.*, 2016).

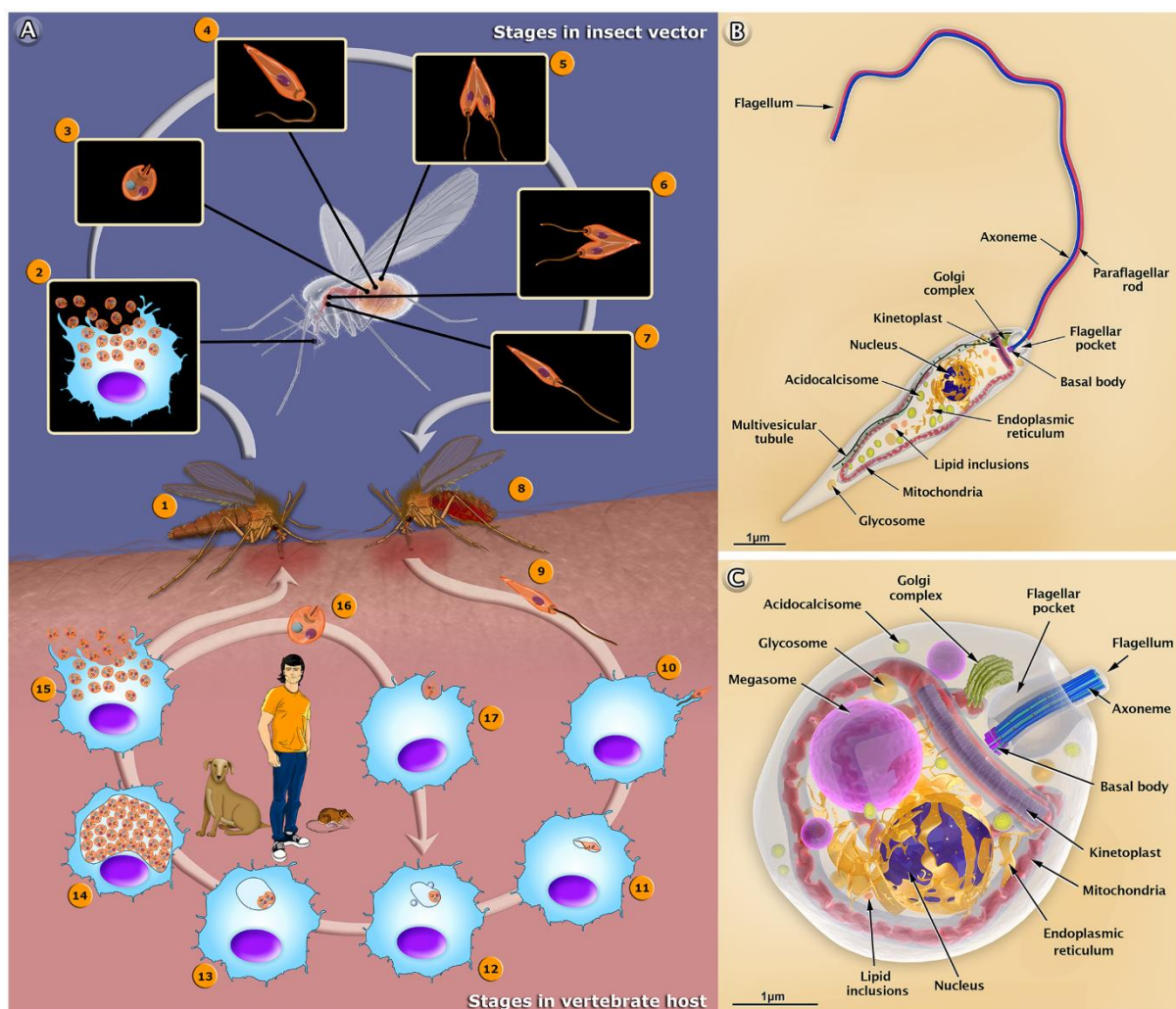


Figure 2.14: The life cycle of the *Leishmania* parasite (A). The structure of the flagellated promastigote (B) and amastigote (C) (Teixeira *et al.*, 2013)

In vertebrates, metacyclic promastigotes are deposited into the bite site when the sand fly has its blood meal. In response, the vertebrate immune system recruits macrophages to the

site. However, the parasite responds by invading the macrophages. Once inside, the promastigote (Figure 2.14 B) start differentiating into the amastigote (Figure 2.14 C). This process is characterised by the change in shape from an elongated cell with a flagellum to an immotile spherical structure with a small bulbous tip and the loss of the gap at the neck region (Teixeira *et al.*, 2013).

2.3.3. Preventive measures

There is no vaccine or preventive therapy available for leishmaniasis, therefore vector control is the cornerstone of prevention and control of the disease. This is achieved by the use of insecticides and insect repellent as well as removing stagnant water which is the breeding ground for the sand-flies. Individuals can reduce exposure by wearing protective clothing such as thick long-sleeved clothes and long pants.

2.3.4. Diagnosis

Leishmaniasis diagnosis involves the detection of parasite presence and the identification of the infecting species. *Leishmania* parasites or parasite DNA are detected from tissue specimens taken from skin lesions (CL) and aspirates from bone marrow, lymph nodes or the blood/buffy coat (VL) (Srivastava *et al.*, 2011). For optimal results and increased sensitivity, specimens are collected from different lesions, and different areas of the same lesion as well as dermal scrapings (Sharifi *et al.*, 2015). Parasite presence is detected using light-microscopic examination of stained slides, culture techniques and molecular methods (Srivastava *et al.*, 2011).

The leishmanin skin test (the Montenegro test) is used to determine the degree of exposure and immunity to the parasite, since some individuals may be long-term asymptomatic carriers of viable parasites (Cobo, 2014). The test involves the inoculation of *Leishmania* extracts in the forearm. A positive test is an indurated nodule that can be seen and measured after 48 hours in response to exposure to specific antigens (Krolewiecki *et al.*, 2017). The test is unreliable for VL infection, since a positive result may be seen 2 – 24 months post clinical recovery (Cobo, 2014).

Species identification can be achieved by using molecular methods and biochemical techniques, including isoenzyme analysis of cultured parasites. Molecular diagnostics, including polymerase chain reaction (PCR) based assays, have enabled a shift towards species-directed treatment (Showler & Boggild, 2015).

2.3.4.1. Parasitological diagnosis

Microscopic diagnosis of VL offers high specificity since the amastigote forms can be seen in tissue smears from the lymph nodes, bone marrow or spleen aspirates. Unfortunately, while splenic aspiration provides reliable diagnosis (sensitivity of 93.1 – 98.7 %), it is invasive and may cause haemorrhaging (Maurya *et al.*, 2010; Srivastava *et al.*, 2011). Bone marrow aspiration (BMA) and the lymph node aspirate smears have lower sensitivity ranging from 52 – 85 % and 52 – 58 %, respectively. BMA and splenic aspiration are painful and risky techniques. Variability in detection sensitivity, the requirement for a microscopy expert, time constraints and high cost limit the use of this technique (Srivastava *et al.*, 2011).

Diagnostic alternatives that offer sensitive and reproducible detection of parasites can be used. These include the buffy coat (white blood cell-rich layer) and peripheral blood mononuclear cells (PBMC) isolated from patient blood

2.3.4.2. Serological diagnosis

Serological testing, is the standard diagnostic tool which relies on the presence of specific humoral responses, as a result, sensitivity and specificity may vary. As a standard diagnostic tool for VL, it can be used to validate VL diagnosis, however in HIV co-infected individuals, sensitivity may be compromised (Lindoso *et al.*, 2016). Non-specific humoral response tests provide poor specificity and sensitivity, therefore they are unreliable and, as a result, have been abandoned. Specific tests, such as the Indirect Fluorescent Antibody Test (IFAT) which detect antibodies in the early stages of infection, offer sensitivity (96 %) and specificity (98 %); however, the need for sophisticated laboratory conditions, limits the use of this test (Srivastava *et al.*, 2011).

2.3.5. Chemotherapy

The complexity and diversity of the disease complicate and limit treatment. There are a number of available chemotherapeutic interventions albeit, inefficient and limited by poor efficiency (including efficacy limited to certain species and or species of certain geographical locations), extensive toxicity, limited drug administration routes, the high cost of treatment (particularly in the poorest communities), limited access to health facilities (in poverty stricken communities), and the emergence of drug resistance (Alvar *et al.*, 2006; WHO, 2018a). Furthermore, complications relating to the severity of the disease and the risk - benefit ratio mean that clinical drugs on the market are of limited use and produce unsatisfactory and varying results, depending on the form of the disease. Additionally, non-

lucrative commercial return and the prevalence of the disease in third world countries hinder anti-leishmanial drug research and development due to limited funding (WHO, 2018a).

Leishmaniasis treatment is individualised and takes into consideration the host and the infecting species, as treatment effectiveness may be host, parasite and/or geographically dependent. Furthermore, immuno-competency facilitates healing from leishmaniasis, for example, lesions of CL can heal without treatment, although this may take months. Vulnerable individuals, such as children, HIV patients, the elderly, pregnant and lactating women, may require adjusted regimens and dosages. Hindrances to treatment, such as the need for parenteral administration, the cost of treatment, and side effects, propagate morbidity. Consequently, cost implications cause individuals to use home 'cures' such as battery acid, petroleum or cauterization with fire. Unfortunately, these therapies may worsen the ailment or cause undetermined side effects (Alrajhi *et al.*, 2002; Alvar *et al.*, 2006).

2.3.5.1. Antimonial drugs

The first antimony-based anti-leishmanial drug was trivalent antimony (III) potassium tartrate also known as tartar emetic in Figure 2.15, (Boelaert & Sundar, 2014; Frezard *et al.*, 2009). Although it paved a way for the development of efficacious pentavalent drugs, its use was soon terminated due to severe side effects, such as severe nausea, vomiting, diarrhoea, hepatitis, renal failure, erythrocyte toxicity and cardiovascular collapse (Boelaert & Sundar, 2014; Duffin & René, 1991; Frezard *et al.*, 2009).

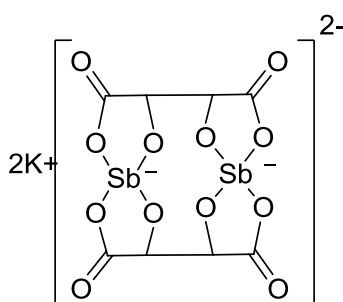


Figure 2.15: Antimony (III) potassium tartrate

In the 1940s, reduced toxicity antimonial drugs such as sodium stibogluconate (SSG, Pentostam®) and meglumine antimoniate (Glucantime) in Figure 2.16 were introduced (Boelaert & Sundar, 2014; Frezard *et al.*, 2009). These agents are the first-line drugs against all forms of leishmaniasis. They are administered intramuscularly for a minimum of three weeks, consequently causing localised pain. Furthermore, side effects such as nausea,

vomiting, weakness and myalgia, abdominal colic, diarrhoea and skin rashes may occur. Severe side effects such as hepatotoxicity and cardiotoxicity can also occur (Torres-Guerrero *et al.*; 2017). In endemic regions with no resistance, the WHO recommends intramuscular (IM) or intravenous (IV) administration of 20 mg/kg/day for 30 days for the treatment of VL and PKDL (Ansari *et al.*, 2017).

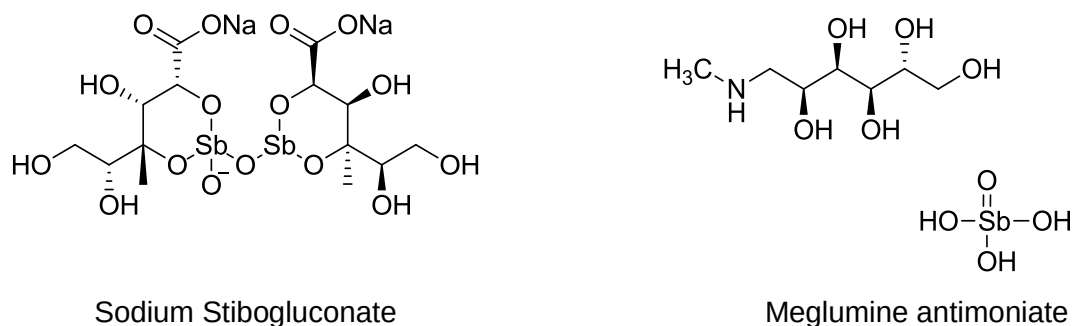


Figure 2.16: Pentavalent antimonial drugs

Although liposome- and cyclodextrin-based formulations of pentavalent antimonial (Sb (V)) drugs have better bioavailability, Sb (V) formulations act as prodrugs which are subsequently reduced to the active, albeit toxic trivalent antimonial (Sb (III)) metabolite (Boelaert & Sundar, 2014). The mechanism of action of pentavalent Sb is not clearly known, however the reduction of Sb (V) to Sb (III) is stage-specific. Amastigotes (clinical, Figure 2.14 C), but not promastigotes (infective, Figure 2.14 B), have the ability to reduce Sb (V) to Sb (III), hence amastigotes are more susceptible to Sb (V) compared to promastigotes (Haldar *et al.*, 2011). Furthermore, Sb (V) reduction may also take place within macrophages to a smaller extent. This reduction reaction involves mammalian thiols such as glutathione (GSH), cysteine and cysteinyl-glycine (lysosomal thiols) and the parasite glutathione-spermine conjugate thiol, trypanothione (Haldar *et al.*, 2011).

For Indian VL, resistance to antimonial drugs is widespread, mainly as a result of their misuse. CL resistance, however, is difficult to ascertain because of variation in sensitivity to pentavalent antimonials (Boelaert & Sundar, 2014, Haldar *et al.*, 2011).

2.3.5.2. Paromomycin

Paromomycin (formerly known as aminosidine, Figure 2.17) is an anti-leishmanial aminoglycoside, originally developed for use as an antibacterial (Boelaert & Sundar, 2014, Chawla *et al.*, 2011). In addition to being affordable, the drug is efficacious with 94 % (India) cure rates (Ansari *et al.*, 2017, Haldar *et al.*, 2011). It is administered daily by IM injections of

11 mg/kg/day for 21 days for the treatment of VL and PKDL in endemic areas. Adverse effects, such as localised pain at the site of injection, hepatotoxicity commonly occur (Ansari *et al.*, 2017). Paromomycin topical formulation for CL treatment provides inconsistent results, however efficacy is improved when a combination of paromomycin with methylbenzethonium chloride is applied twice a day for 20 days (Boelaert & Sundar, 2014).

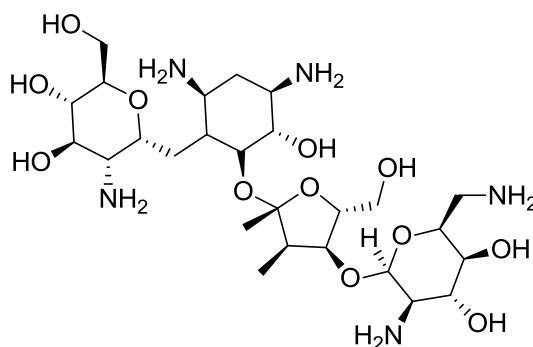


Figure 2.17: Paromomycin

In bacterial infections, paromomycin acts as inhibitors of protein synthesis by interacting with ribosomal RNA subunits, however in leishmanial infections its mechanism of action is not known, although some studies suggest that it targets the mitochondria (Chawla *et al.*, 2011; Halder *et al.*, 2011). The drug is not recommended for monotherapy in African VL because of its poor and varying (46 – 85 %) efficacy (Ansari *et al.*, 2017).

2.3.5.3. Miltefosine

Miltefosine (Figure 2.18) is an alkyl phospholipid originally developed for cancer chemotherapy. It was found to be a highly effective anti-leishmanial agent (94 – 97 %), particularly in India, Nepal and Bangladesh (Ansari *et al.*, 2017, Boelaert & Sundar, 2014). It was the first effective oral drug for the treatment of VL and CL (Sundar *et al.*, 2002). The treatment regimen entails 100 mg/day orally for 28 days. Unfortunately, the drug is expensive, particularly for leishmaniasis endemic and poor countries. Adverse effects, such as gastrointestinal complications which discourage patient compliance as well as kidney and liver toxicity, can occur. Furthermore, the drug is not recommended for use during pregnancy because of teratogenicity (Ansari *et al.*, 2017, Boelaert & Sundar, 2014).

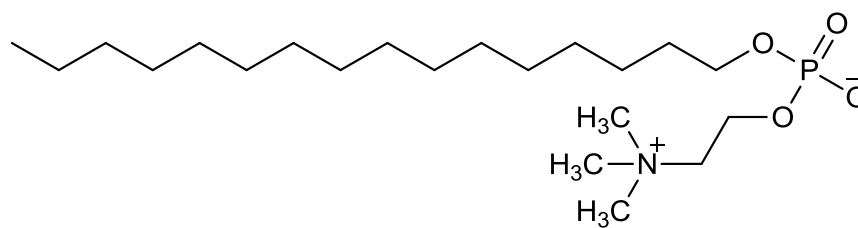


Figure 2.18: Miltefosine

2.3.5.4. Amphotericin B

Amphotericin B is a polyene antibiotic with anti-leishmanial activity. It causes cell death by irreversibly binding to ergosterols in the membrane of the *Leishmania* parasite (Boelaert & Sundar, 2014). It is a second-line VL treatment with high (90 % – 95 %) cure rates in Indian VL (Haldar *et al.*, 2011). Amphotericin B efficacy may be compromised in HIV-infected patients, which may also cause relapse (Lachaud *et al.*, 2009).

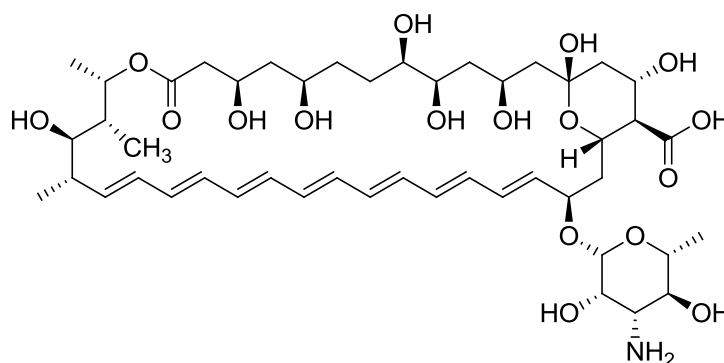


Figure 2.19: Amphotericin B

Amphotericin B (Figure 2.19) is clinically available as amphotericin B deoxycholate and in lipid formulations. Amphotericin B deoxycholate is a colloidal suspension administered as a slow IV infusion at 0.5 – 1 mg/kg for 15 – 20 infusions. Adversely, this formulation is associated with high fever and chills (Boelaert & Sundar, 2014). Lipid amphotericin B is formulated by incorporating amphotericin B into cholesterol esters and liposomes (phospholipid vesicles) as a means to improve drug uptake into macrophages and improve its therapeutic index (Boelaert & Sundar, 2014). Despite improved efficacy and reduced toxicity, amphotericin B formulations are expensive and require IV administration (Tiuman *et al.*, 2011).

2.3.5.5. Pentamidine

Pentamidine (Figure 2.20) is a water soluble aromatic diamidine with anti-leishmanial activity. It was used as a second-line anti-leishmanial of Sb-resistant VL in India, but it is no longer used due to reduced efficacy and treatment failure (Boelaert & Sundar, 2014). Nevertheless, the drug is used under strict observations at 4 mg/kg intramuscularly or slow IV injections for the treatment of CL caused by *L. guyanensis* and *L. panamensis* in the Northern Amazon (Boelaert & Sundar, 2014).

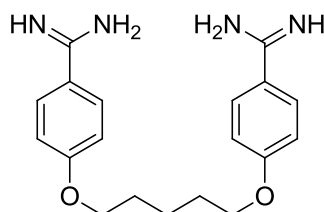


Figure 2.20: Pentamidine

Pentamidine inhibits the parasite's critical pathways by interfering with purine metabolism which ultimately disrupts the synthesis of the parasites macromolecules, such as DNA, RNA, phospholipids and proteins (Ansari *et al.*, 2017; Yang *et al.*, 2016). Unfortunately, pentamidine is associated with serious side effects, such as renal toxicity, inflammatory cardiomyopathy, insulin-dependent diabetes mellitus, hypoglycaemia, hypotension and unexplained shock (Ansari *et al.*, 2017).

2.3.5.6. Devices and non-chemotherapeutic treatments

In addition to available clinical drugs, CL treatment can be supplemented with non-chemotherapeutic modalities, such as thermotherapy and cryotherapy. Thermotherapy is the superficial or deep application of heat with the intention of increasing tissue temperature (Nadler *et al.*, 2004) since replication of CL amastigotes is inhibited at higher temperatures (Boelaert & Sundar, 2014). CL species such as *L. major*, *L. tropica*, and *L. mexicana* are thermosensitive and can be treated with one or two applications of localised heat (50 °C for 30 seconds) applied to the lesion under local anaesthesia. Unfortunately, the treatment may cause second degree burns and the heat therapy device is expensive (Boelaert & Sundar, 2014).

Cryotherapy is the therapeutic application of coolants to remove heat from the body to reduce tissue temperature. Liquid nitrogen is sprayed two millimetres outside the lesion margin to reduce tissue metabolism, oxygen utilisation, and inflammation (Boelaert &

Sundar, 2014, Nadler *et al.*, 2004). The treatment is repeated for up to six weeks. Local treatment of CL has variable efficacy (similar to Sb (V), in some cases) and better safety than conventional treatment (López-Carvajal *et al.*, 2016).

2.3.5.7. Combination therapy for visceral leishmaniasis

All clinical anti-leishmanial drugs are from different chemical classes, which greatly reduces the chance of cross resistance. Monotherapy encourages the emergence of drug resistant parasites, while combination therapy may provide shorter treatment periods, reduce toxicity, encourage patient compliance, reduce treatment (and treatment-associated) costs, varying cure rates and inconvenient administration methods, however combinations depend on the endemic region's response to therapy.

Current clinical combinations for the treatment of VL caused by *L. donovani* are (i) India, Bangladesh, and Nepal: a single dose of liposomal amphotericin B with miltefosine (for 7 days) or paromomycin (for 10 days) and miltefosine with paromomycin, both daily for 10 days; and (ii) East Africa: Sb (V) drugs in combination with paromomycin (Boelaert & Sundar, 2014).

2.4 Malaria

Malaria is a curable, but life-threatening disease caused by the *Plasmodium* parasite that is transmitted to humans by the female *Anopheles* mosquito. Five *Plasmodium* species, namely *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, can infect humans. Of global morbidity concern are *P. falciparum* and *P. vivax*. The latter can survive at low temperature and lengthy periods of time in a dormant stage. Furthermore, the dormant stage, known as the hypnozoite, can result in clinical relapse (Hulden & Hulden, 2011). *P. falciparum* is the deadliest of the *Plasmodium* species, accounting for the largest malaria burden and mortality (WHO, 2018b).

2.4.1. Life cycle and pathogenesis

The complex life cycle of the *Plasmodium* parasite occurs in two (the asexual and sexual) stages. The asexual stage occurs in the vertebrate (human) and the sexual stage takes place in a female *Anopheles* mosquito (Figure 2.21). The pre-erythrocytic or exoerythrocytic stage of the life cycle is initiated during the mosquito's blood meal. The parasite enters the human's bloodstream when an infected mosquito bites the human, thereby injecting sporozoites that are present in its saliva. The sporozoites enter the bloodstream and settle in the liver, where they invade the hepatocytes. In the hepatocytes, the sporozoites undergo mitosis to rapidly grow and differentiate into merozoites that can exit the hepatocytes, enter the bloodstream and invade the erythrocytes, initiating the intraerythrocytic stage (Hanssen *et al.*, 2010). The merozoites then develop into ring stages, where they are enclosed in the parasitophorous vacuoles until they develop into trophozoites. Upon maturation, the trophozoites differentiate into schizonts in which merozoite differentiation continues until it ruptures, releasing merozoites into the bloodstream. The released merozoites then infect and recruit more erythrocytes. This period of the asexual stage is responsible for the manifestation of symptoms and the disease in humans. The parasite uses the host's resources for nutrient supply and its development during this period. The host's erythrocyte cytoplasm, which contains about 95 % haemoglobin, is catabolised in order to generate amino acids that are used to nurture the parasite's growth and enable it to thrive (Francis *et al.*, 1997; Goldberg *et al.*, 1990; Soulard *et al.*, 2015).

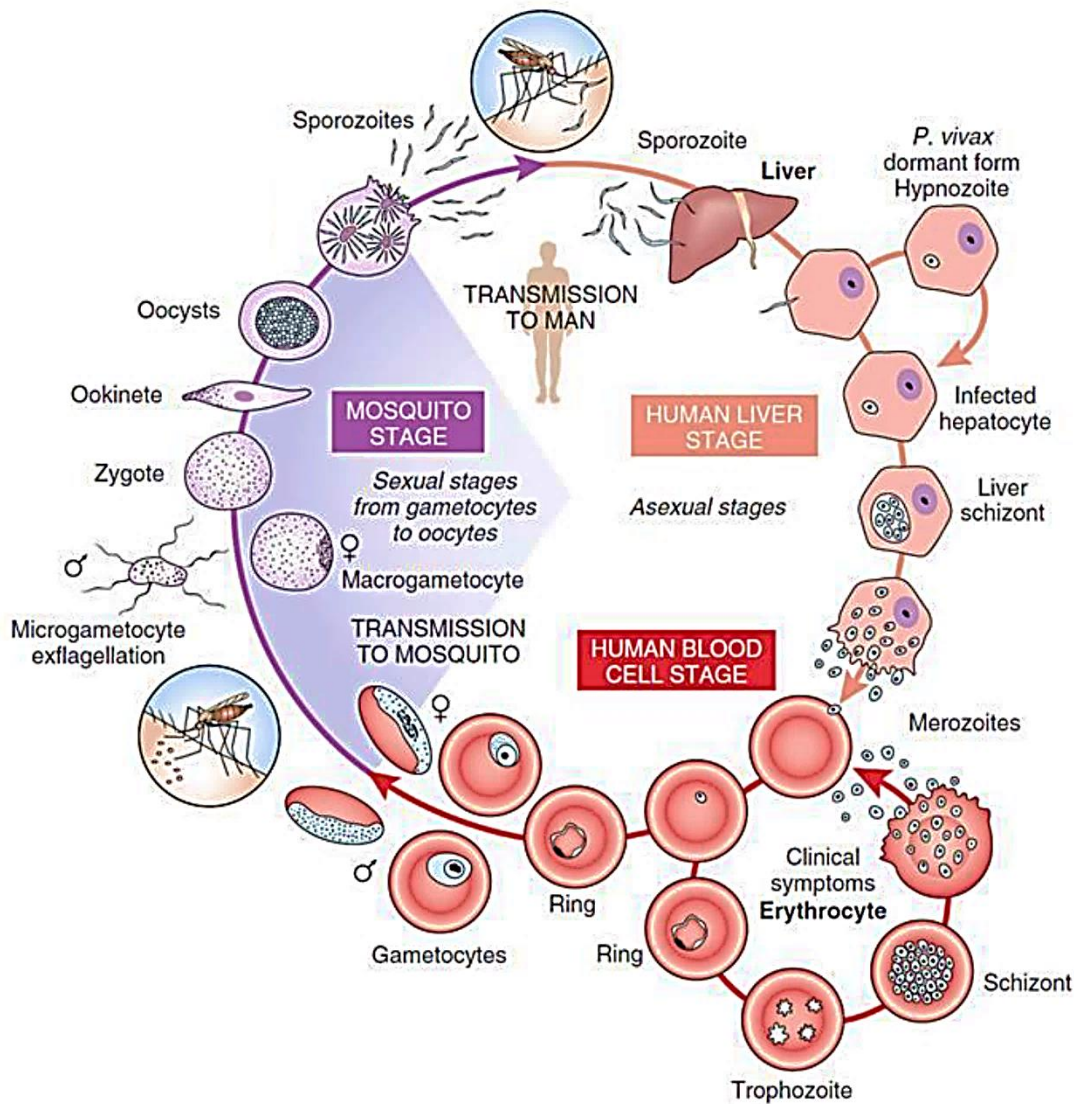


Figure 2.21: Life cycle of the *Plasmodium* parasite (McCarthy & Price, 2015)

Some merozoites develop into gametocytes (male and female) during replication and differentiation (Klein, 2013). The gametocytes are ingested by a female mosquito during its blood meal, thus beginning the sexual stage of the *Plasmodium* life cycle. Humidity and ambient temperature provide an ideal environment for the parasite to successfully develop in the mosquito. In the gut of the mosquito, the gametocytes mate and undergo meiosis to develop into ookinetes and then into oocysts in the midgut (Modrzynska *et al.*, 2017). Here, sporozoites develop and are transported across the mosquito's body, including the salivary glands where they can be transmitted to a human host and continue to perpetuate the transmission cycle (Klein, 2013; Modrzynska *et al.*, 2017).

2.4.2. Malaria signs and symptoms

The onset of malaria generally presents non-specific symptoms and may vary from species to species of the infecting *Plasmodium* parasite. The most common symptoms include fever, diarrhoea, headaches and chills (White *et al.*, 2014). Other symptoms may include impaired consciousness, respiratory distress and convulsions. The five *Plasmodium* species that infect humans each present their own manifestation time frames and complications, but focus is on *P. falciparum* infection which poses the biggest global concern (WHO, 2018b). Depending on the symptoms, malaria infection can be categorised as uncomplicated and complicated/ severe malaria (WHO, 2014). Uncomplicated malaria is characterised by a positive parasitological test with no clinical features of severe malaria (WHO, 2015b). Symptoms of uncomplicated malaria may resemble flu symptoms and a variety of other diseases and may, consequently, be misdiagnosed or undiagnosed. Although not life-threatening, uncomplicated malaria requires prompt attention and treatment because neglect may foster progression into complicated malaria. This is especially true for immunocompromised individuals such as children under the age of five years, pregnant woman and HIV co-infected individuals (WHO, 2018b).

2.4.2.1. Clinical features of complicated or severe malaria

Severe malaria can lead to vital organ dysfunction and can be fatal when left untreated (WHO, 2015b). This is mainly due to extensive parasitised erythrocyte sequestration (White *et al.*, 2014). Haemoglobin is catabolised to amino acids and by-products, such as oxygen radicals and haem during parasite growth and differentiation. This process consumes nitric oxide, consequently causing endothelial dysfunction and ultimately cytoadherence, which is the adherence of infected erythrocytes to endothelium cells (Ho & White, 1999; Magowan *et al.*, 1988; White *et al.*, 2014). In *P. falciparum* malaria, adherence is mediated by the strain-specific adhesive protein, PfEMP1 (Ghumra *et al.*, 2012). This is one of the *Plasmodium* parasite's strategies to evade the host's immune defences, since the parasite camouflages itself, thus avoiding splenic immune function and filtrative clearance (Carvalho *et al.*, 2013; Ho & White, 1999; White *et al.*, 2014). Furthermore, the camouflage allows the infected erythrocytes containing mature *P. falciparum* parasites into vital organs, such as the brain, causing cerebral malaria (Medana & Turner, 2006).

Cerebral malaria

Cerebral malaria is characterised by persistent coma and seizures as a result of erythrocyte sequestration in the cerebral capillaries and venules (Carvalho *et al.*, 2013; Ho & White, 1999; White *et al.*, 2014). Both children and adults may experience malarial retinopathy with

haemorrhages, as well as retinal and vessel whitening (White *et al.*, 2014). In African children, the disruption of endothelial intercellular tight junctions may cause blood–brain barrier permeability. Cerebral oedema is, therefore, observed in African children and absent in adults (Klein, 2013; White *et al.*, 2014). Consequently, 3 – 15 % of children who survive cerebral malaria, especially those who had hypoglycaemia, anaemia, repeated protracted seizures, and deep coma, may suffer neurological sequelae. Neurological deficits may include cerebral palsy, cortical blindness, deafness, and impaired cognition, which may affect learning and speech abilities (White *et al.*, 2014).

Severe anaemia

In areas of high malaria transmission young children commonly develop severe anaemia, generally as a consequence of repeated infection (Menendez *et al.*, 2000). Malaria infection triggers host splenic immune function and filtrative clearance (White *et al.*, 2014). During these processes, the spleen actively removes infected erythrocyte by-products from the rupture of the schizonts and those opsonised by immunoglobulin (del Portillo *et al.*, 2012, White *et al.*, 2014). In the erythrocyte stage, haemoglobin catabolism produces by-products, such as oxygen radicals and haem (Francis *et al.*, 1997). Since haem is toxic to the parasite, it is converted to haemozoin and released into the bloodstream. The spleen phagocytic and cellular immune functions actively remove infected erythrocyte by-products resulting from the rupture of schizonts (Urban *et al.*, 2005; White *et al.*, 2014). The intensified and accelerated splenic activity also removes unparasitised erythrocytes and compromises erythropoiesis causing severe anaemia (White *et al.*, 2014).

Acidosis and hypoglycaemia

Severe malaria patients can develop lactic acidosis as a consequence of the accumulation of organic acids. The condition is accelerated by ketoacidosis in children and acute kidney injury in adults (White *et al.*, 2014). Lactic acidosis is facilitated by the sequestered parasites obstruction of the host's microcirculatory flow, anaerobic glycolysis in tissue, and *Plasmodium* lactate production. Since severe malaria causes vital organ dysfunction, hepatic and renal lactate clearance mechanisms may be subpar, thus promoting lactic acidosis (Krishna *et al.*, 1994). Children and pregnant women can develop hypoglycaemia as a consequence of lactic acidosis (Day *et al.*, 2000, Krishna *et al.*, 1994). It should be noted that hypoglycaemia may also be the result of malnutrition in poor communities. Additionally, in adults hypoglycaemia may be a consequence of quinine that stimulates pancreatic insulin secretion (White *et al.*, 1987).

Pulmonary oedema and acute kidney injury

In severe malaria, careful fluid management is crucial, because rapid liquid infusion can be deadly, particularly for adults (especially pregnant women) with *P. falciparum* malaria (Taylor *et al.*, 2012). This is caused by the increased permeability of the pulmonary capillaries, particularly at the start of treatment. This complication can also occur in *P. vivax* and *P. knowlesi* infections (White *et al.*, 2014). Similarly, acute kidney injury may occur and may be deadly, since it can lead to the dysfunction of other vital organs. Although pathogenesis of this fatal condition is not clearly understood, it behaves similarly to acute tubular necrosis, a condition that involves the death of renal tubular epithelial cells of the kidneys (Nguansangiam *et al.*, 2007, White *et al.*, 2014).

Miscellaneous complications

Renal impairment, haemolysis and hepatocyte injury may result in jaundice. This condition is more common in adults than in children (Nguansangiam *et al.*, 2007; Taylor *et al.*, 2012). Children in endemic areas may also suffer from opportunistic pathogens, such as those causing severe sepsis, pneumonia and meningitis, which may contribute to misdiagnosis or delayed diagnosis (White *et al.*, 2014). Furthermore, coinfection with HIV/AIDS and TB may accelerate the severity and mortality of severe *P. falciparum* malaria (Sanyaolu *et al.*, 2013; Ter Kuile *et al.*, 2004).

Pregnant women are immunocompromised, as result, they are at risk of severe *P. falciparum* malaria and malaria-related death. Consequently, complications, such as high parasite burden, severe anaemia, hypoglycaemia, and acute pulmonary oedema, are more common (White *et al.*, 2014). Adversely, maternal anaemia and transmitting the *Plasmodium* parasite to the foetus are possible risks, which may result in congenital malaria, foetal distress, premature labour, low birth weight and stillbirth (Desai *et al.*, 2007). Fortunately, congenital malaria, which occurs in 5 % of neonates, clears out spontaneously in 62 % of the cases. Unfortunately, low birth weight is associated with infant mortality rates (Desai *et al.*, 2007; White *et al.*, 2014).

2.4.3. Diagnosis

The presentation of symptoms such as fever, chills and headaches can be a basis to suspect malaria infection, particularly in malaria endemic countries. However, these symptoms are not specific to malaria, in fact they are common to many diseases. Therefore, prompt and accurate diagnosis of malaria is a lifesaving exercise, with the following benefits: (i) it reduces malaria burden, effectively reducing the spread of infection; (ii) it ensures timeous diagnosis for prompt treatment; (iii) it ensures proper treatment to avoid

overtreatment and mistreatment that are precursors to drug resistance; (iv) it provides definite nature of the disease (whether it's complicated or uncomplicated malaria) and its associated complications (e.g, severe anaemia); (v) since malaria mostly affects the poorest communities, prompt and effective diagnosis can be a cost saving tool. Diagnosis is achieved by microscopic diagnosis, antigen detection and molecular diagnosis.

2.4.3.1. Microscopic diagnosis

Sensitivity and specificity make microscopic diagnosis an invaluable tool, mainly because it can be used to identify the infecting *Plasmodium* species and quantify parasitaemia (WHO, 2015b). The test involves smearing a drop of blood on a slide, staining it with Giemsa and examining it (Tangpukdee *et al.*, 2009). Additional advantages of this tool include (i) the detection of gametocytaemia and (ii) sensitivity, even at low parasite concentrations (WHO, 2015b). The biggest disadvantages of microscopic testing are: (i) cost implications, particularly since the disease is prevalent in poor communities, as well as the initial laboratory infrastructure setup; (ii) technical training and supervision required; (iii) heavy dependence of accuracy to the competency of the microscope technologist; (iv) dependence on infrastructure such as electricity; and (v) good quality and high performance microscopes as well as their maintenance (WHO, 2015b).

2.4.3.2. Antigen detection

Antigen detection, popularly known as Rapid Diagnostic Tests (RDTs), can provide results in 15 minutes, providing an alternative testing method to microscopy. The tests are designed to detect antigens such as *Plasmodium* lactate dehydrogenase (pLDH) and *Plasmodium* aldolase, which are characteristic of all *Plasmodium* species (Iqbal *et al.*, 2004). Additionally, *P. falciparum* and *P. vivax* specific antigen *Plasmodium* histidine-rich protein 2 (HRP2) can be detected and used to confirm diagnosis, specifically PfHRP2 and PvHRP2, respectively (Abba *et al.*, 2011; Tjitra *et al.*, 2001). Unfortunately, the *pfhrp2* gene may be absent in some *P. falciparum* strains, such as those isolated in the Brazilian Amazon Basin, that may lead to misdiagnosis as a result of a false-negative RDT (Rachid Viana *et al.*, 2017).

2.4.4. Prevention and control

Prevention and control play a vital role in transmission containment, particularly in the absence of a clinically approved effective vaccine, albeit not without effort. In fact RTS,S/AS01, an injectable malaria vaccine, is in the pipelines (Bejon *et al.*, 2008) and the WHO has deemed the vaccine safe and efficacious for use (RTS, 2015). Unfortunately, the vaccine is not a one size fits all, since it only provides partial protection against malaria,

particularly *P. falciparum*, in young children. The vaccine is currently in Phase III clinical trials in anticipation of implementation in 2020. The vaccine will be integrated into currently available and used prevention and control efforts (RTS, 2015).

Prevention aims is to prevent malaria infection by taking drugs that inhibit pre-erythrocytic development or drugs that kill the asexual stages of the parasite. Pre-erythrocytic development inhibition, also known as causal prophylaxis, can be achieved by drugs, such as atovaquone-proguanil or primaquine. Causal prophylaxis is started before an individual enters an endemic area. The intention is to assess drug tolerability and, in the case of slow elimination drugs, build up therapeutic concentrations (WHO, 2015b). Furthermore, preventive treatment maintains therapeutic drug levels in the blood throughout the high transmission period. This is achieved by programmes, such as intermittent preventive treatment (IPT) with sulfadoxine-pyrimethamine (SP) and seasonal malaria chemoprevention (SMC) in endemic areas. IPT is provided to all pregnant women (IPTp) in the first and second pregnancy during the second trimester (WHO, 2015b) in endemic areas. Similar treatment is recommended for infants (younger than 12 months) in areas of moderate to high malaria transmission (IPTi), such as in Africa where SP is still effective (WHO, 2015b). SMC involves monthly administration of amodiaquine and SP to all children under the age of six years during transmission season (WHO, 2015b). Suppressive prophylaxis which is aimed at killing asexual stages of the parasite is accomplished with IPT (White, 2005). It is important that suppressive prophylaxis is started at least four weeks after leaving the endemic country. In addition, by using a longer acting antimalarial drug in conjunction with a rapid killing drug, artemisinin-based combination therapy (ACT) (section 2.4.6.1) is designed to provide suppressive prophylaxis (White, 2005; WHO, 2015b).

Vector control is a means of reducing and disrupting mosquito breeding and is achieved by the use of mosquito repellent directly on the skin and insecticide-treated mosquito nets (ITNs), which provide barriers to transmission, as well as indoor residual spraying (IRS). In IRS, residual pesticides are sprayed to coat the walls and other surfaces of the house in order to kill mosquitoes that rest on the pesticide coated surface. This method does not prevent mosquito bites and infection; however, it is a good method to prevent transmission as it reduces the vector burden (White *et al.*, 2014).

2.4.5. Chemotherapy

Malaria chemotherapy remains the most effective intervention available against the disease, despite the constant development of drug resistance and the availability of drugs that target different stages of the parasite (Figure 2.22). Parasite resistance reduces drug effectiveness

(leading to treatment failure) or renders drugs ineffective, in which case the drug is obsolete as the parasite continues to thrive despite chemotherapeutic intervention. Quinolines (including related drugs), antifolates, certain antibiotics and artemisinins are groups of drugs that are currently available for the treatment of malaria.

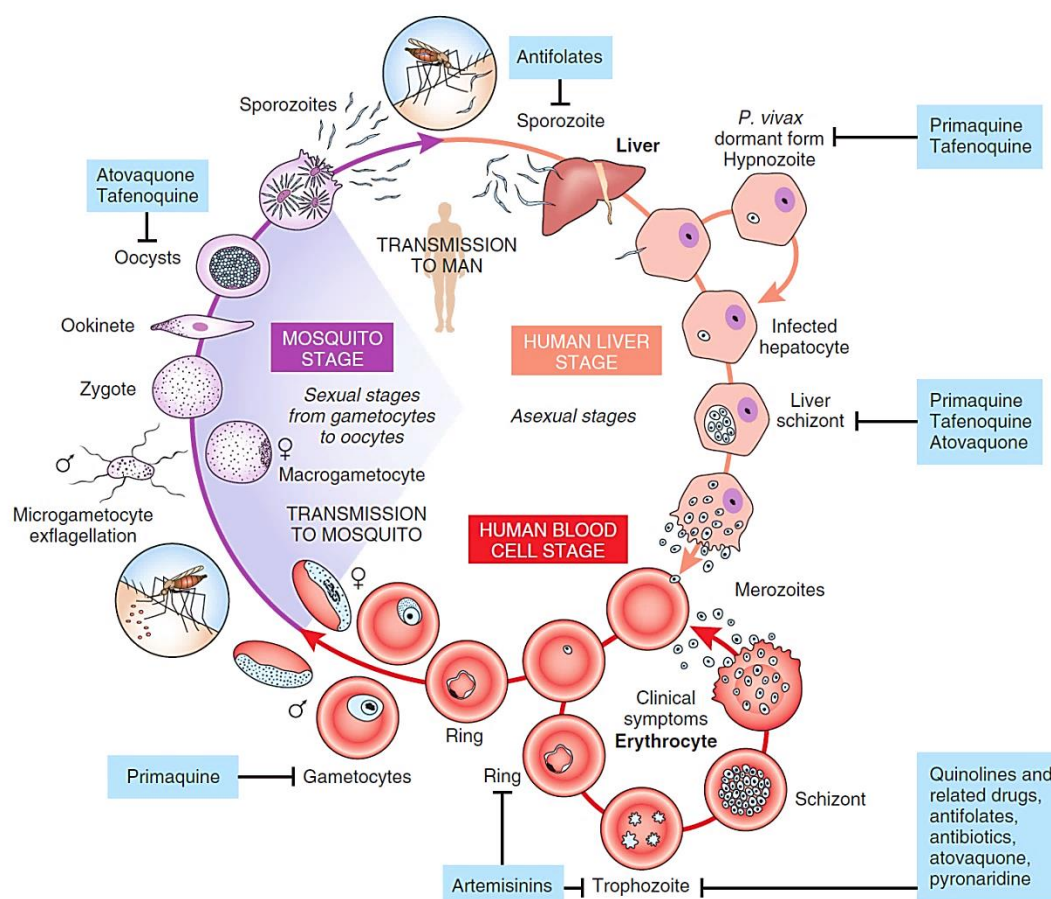


Figure 2.22: Summary of drug interventions with respect to the targeted stage in the life cycle of the *Plasmodium* parasite (McCarthy & Price, 2015).

2.4.5.1. Quinolines and related drugs

Quinolines are amongst the oldest antimalarials to date. These natural and synthetic compounds, which share a scaffold, can be sub-grouped into 4-aminoquinolines, 8-aminoquinolines and aryl aminoalcohols, also known as cinchona alkaloids or quinolines.

4-Aminoquinolines

The most important drugs in this group (Figure 2.23) include quinine, chloroquine, amodiaquine and piperazine. These drugs accumulate in the parasite's digestive vacuole to interfere with haemoglobin metabolism, inhibiting the parasite from detoxifying haem (Achan *et al.*, 2011).

Quinine

Quinine (Figure 2.23) is the naturally occurring cinchona alkaloid, primarily formulated as a salt with hydrochlorides, dihydrochlorides (the most commonly used), sulphates, bisulphates and gluconates (Achan *et al.*, 2011; Ibrahim *et al.*, 2007). Quinine kills large ring and trophozoite stages of the parasite. Furthermore, the drug kills gametocytes of *P. vivax* and *P. malariae* (WHO, 2015b). Its mechanism of action remains unknown; however, it is thought to be similar to that of CQ (Achan *et al.*, 2011; Petersen *et al.*, 2011; WHO, 2015b). Quinine is believed to accumulate in the parasite's digestive vacuole and inhibit hemozoin formation. It is available for oral and parenteral administration and becomes widely distributed throughout the body with detectable concentrations in the cerebrospinal fluid, breast milk and placenta (WHO, 2015b). It is primarily metabolised in the liver by CYP3A4, CYP2C9, CYP1A2 and CYP2D6 enzymes (WHO, 2015b).

Cinchonism, a collective term for the side effects associated with cinchona alkaloids that include tinnitus, slight hearing impairment, headaches and nausea, is concomitant with quinine usage. Other side effects include diarrhoea, vomiting, abdominal pain, vertigo, loss of vision and auditory loss (Achan *et al.*, 2011). Although uncommon, other side effects such as asthma and hepatic injury may occur (Achan *et al.*, 2011). Quinine resistance occurs, but is mainly associated with reduced or delayed activity and substandard quinine (Achan *et al.*, 2011).

Chloroquine

Since its discovery in 1934, chloroquine (CQ, Figure 2.23) plays an integral part in malaria chemotherapy. This synthetic quinoline kills blood stages of the parasite by interfering with intraparasitic haem detoxification (section 2.4.2.3) in the digestive vacuole of the parasite (Slater, 1993). CQ has 70 – 80 % bioavailability when taken orally and is metabolised in the gastrointestinal tract (WHO, 2015b). Although CQ is extensively distributed in body tissues (including the placenta) and fluids (including breast milk), 55 % of the drug is plasma protein-bound (WHO, 2015b). It is metabolised in the liver by CYP2C8 and CYP3A4 into the antimalarial active metabolite monodesethylchloroquine. Approximately 55 % of CQ is excreted via the kidneys and elimination is slow, with an elimination half-life of 175 – 290 hours for CQ and 175 – 290 hours for the metabolite (WHO, 2015b). CQ is well absorbed at therapeutic doses, but produces side effects that include vomiting, rashes, itching and pruritus (Foley & Tilley, 1997; Foley & Tilley, 1998). Since CQ has a high affinity for melanin-containing cells, pruritus is common and more severe in dark-skinned individuals (WHO, 2015b).

CQ is used for the treatment of uncomplicated malaria caused by all human infecting *Plasmodium* species, excluding *P. falciparum*. However, it is used by pregnant women in *P. falciparum* infections and in *P. falciparum* prophylaxis (WHO, 2015b). The cessation of CQ chemotherapy is by virtue of resistance which is associated with genetic mutations of PfCRT and PfMDR of the parasite's food vacuole (Bray *et al.*, 1998; WHO, 2015b).

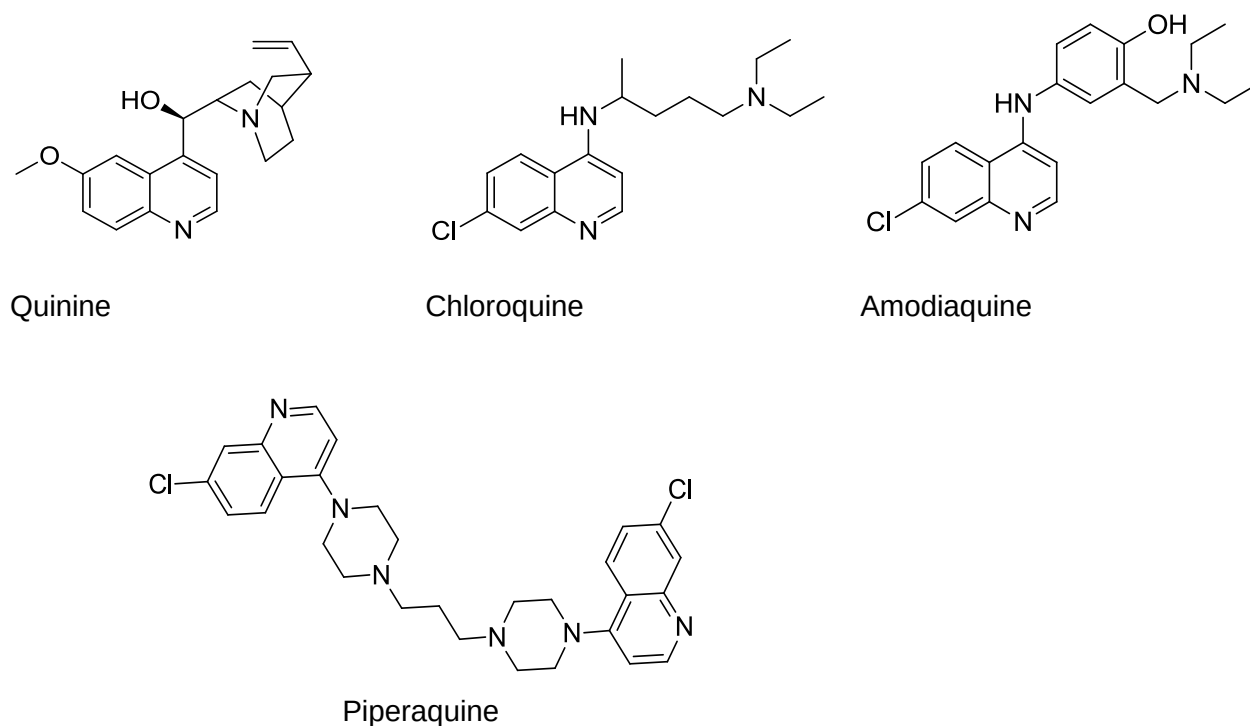


Figure 2.23: Clinical 4-aminoquinolines

Amodiaquine

Amodiaquine (Figure 2.23) was initially intended for CQ replacement, considering CQ resistance and itching associated with CQ use. It should be noted that amodiaquine may share structural similarities with and may be more palatable than CQ, but it is less active. Much like CQ, it is absorbed in the gut and well distributed throughout the body. Amodiaquine is metabolised in the liver by CYP2C8 into its antiparasmodial metabolite, monodesethylamodiaquine (desethylamodiaquine) which boosts the overall terminal elimination half-life of amodiaquine from ten hours to ten days (Tarning *et al.*, 2012). Unfortunately, this also contributes to the development of resistance to the drug (Petersen *et al.*, 2011). Amodiaquine and desethylamodiaquine are extensively bound (90 – 95 %) to plasma proteins. Desethylamodiaquine, but not amodiaquine, is accumulated in the erythrocytes, hence its activity as a blood schizonticide (Tarning *et al.*, 2012). Currently amodiaquine is used in ACT (section 2.4.6.1) in combination with artesunate (Figure 2.29)

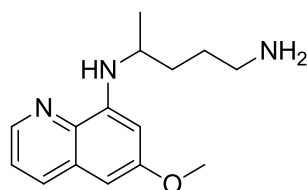
for the treatment of uncomplicated *P. falciparum* and *P. vivax* malaria. Furthermore, amodiaquine is considered to be effective against *P. ovale*, *P. knowlesi* and *P. malariae* (WHO, 2015b, Yakasai *et al.*, 2015). Moreover, it is used in prophylaxis in the SMC programme (section 2.2.4) in combination with SP (section 2.2.5.2).

Piperaquine

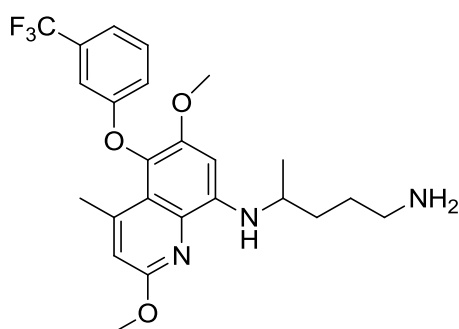
Piperaquine (Figure 2.23) is a bis-4-aminoquinoline with a similar mode of action as CQ, including its ability to accumulate in the parasite's digestive vacuole and its inhibition of haem detoxification (WHO, 2015b). Piperaquine is effective against chloroquine-resistant (CQR) *Plasmodium* parasites, particularly because of steric hindrance that hinders the parasite food vacuole proteins from being able to efflux the bulky bisquinoline structure (Tärning, 2007). Furthermore, the drug is less toxic and has better activity against blood stages of the parasite compared to CQ (Davis *et al.*, 2005). Piperaquine is readily absorbed in the gut and distributed throughout the body, and it is heavily protein bound (< 99 %), and reaches plasma peak concentrations within three – six hours. (WHO, 2015b). Currently, piperaquine (because of its long elimination half-life of two – four weeks) is used in ACT in combination with dihydroartemisinin (DHA; section 2.2.5.4), for the treatment of uncomplicated malaria (Kakuru *et al.*, 2016; WHO, 2015b).

2.4.5.2. 8-Aminoquinolines

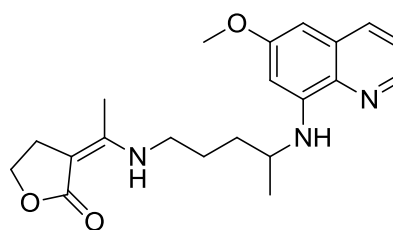
8-Aminoquinolines (Figure 2.24) are a hypnozoitocidal class of quinolines, hence their use in the treatment of *P. vivax* and *P. ovale* (Foley & Tilley, 1998). This class is comprised of primaquine, tafenoquine and bulaquine.



Primaquine



Tafenoquine



Bulaquine

Figure 2.24: Clinical 8-aminoquinoline antimalarial drugs

Primaquine

Primaquine (PQ, Figure 2.24), is an 8-aminoquinoline, with potent activity against the exoerythrocytic forms (hypnozoites) and the sexual (gametocytes) stages of malaria parasites (WHO, 2015b). As a result, the drug is mainly used for presumptive anti-relapse therapy. PQ has a short half-life (maximum five hours) (Potter *et al.*, 2015). It is well absorbed with about 96% bioavailability, and metabolised in the liver by CYP2C19, CYP2D6 and CYP3A4 into toxic intracellular oxidative species (WHO, 2015b). There is limited knowledge about its mechanism of action, but since PQ is relatively inactive compared to its metabolites, it is thought that the reactive metabolites interfere with electron transport in the parasite (Ganesan *et al.*, 2009; Pybus *et al.*, 2013). Fortunately, there are no reports of acquired resistance to its hypnozoitocidal or gametocytocidal potency; however, treatment failure is common in individuals with genetic polymorphisms associated with a decrease in CYP2D6 enzyme (WHO, 2015b).

Tafenoquine

Tafenoquine (TQ, WR238605, Figure 2.24), is structurally similar to PQ and is active against all human infecting *Plasmodium* parasites (McCarthy & Price, 2015). The drug is currently

registered for the radical cure of *P. vivax* with contraindication for pregnant women and children under the age of eight years (Lacerda *et al.*, 2019). Similar to 4-aminoquinolines, TQ inhibits haem polymerization and is potent against the asexual intraerythrocytic stages of *P. falciparum* and *P. vivax*. Additionally, TQ can play a major role in prevention, since it prevents sporozoite formation in the mosquito (McCarthy & Price, 2015).

TQ absorption is rapid and is further improved when the drug is taken with food. It is metabolised in the liver by CYP2D6, has a large volume of distribution (with an elimination half-life of 16 days) and accumulates in the erythrocytes (McCarthy & Price, 2015). In addition to minor side effects, such as gastrointestinal upset, headaches and myalgia, other potential side effects may include methemoglobinemia and the risk of haemolysis in 6GPD-deficient patients (McCarthy & Price, 2015; Vale *et al.*, 2009).

Bulaquine

Bulaquine, (BQ, Figure 2.24), a 2,4-dihydrofuran analogue of PQ, which was explored as a replacement for PQ, particularly because it is metabolised into PQ. BQ is a gametocytocidal agent that is active against all human- infecting *Plasmodium* parasites, including *P. falciparum* (Gogtay *et al.*, 2006). It has a better safety profile (Vale *et al.*, 2009) and an improved half-life (up to four days) compared to PQ. BQ is only licenced for use in India for the radical cure of *P. vivax* malaria (Gogtay *et al.*, 2006).

2.4.5.3. Aryl aminoalcohols

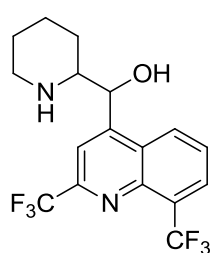
This class of antimalarials is comprised of synthetic alkaloids (Figure 2.25), such as mefloquine, lumefantrine and halofantrine.

Mefloquine

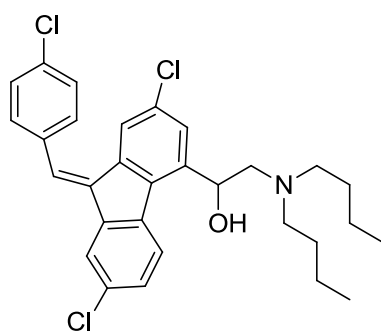
Mefloquine (Figure 2.25) was initially developed in response to CQ resistant (CQR) *P. falciparum* malaria (Foley & Tilley, 1997). This aminoalcohol is better tolerated and possesses better antimalarial activity compared to quinine. It is a potent blood schizonticide that exerts its antimalarial activity by interfering with haem detoxification. Furthermore, mefloquine inhibits solute import into the digestive vacuole of the parasite by interfering with PfMDR1 transport that, unfortunately, is associated with resistance (Price *et al.*, 2004). The drug persists in the body for extended periods of time (half-life of 20-30 days) and at sub-therapeutic levels which may also contribute to parasite resistance. The drug is contraindicated for epileptic individuals, as well as persons with psychotic disorders (González *et al.*, 2014).

Lumefantrine

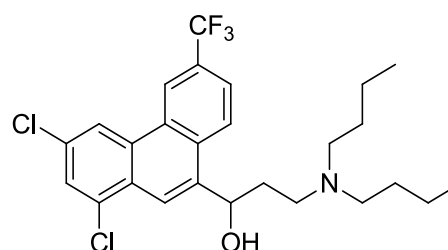
Lumefantrine (Figure 2.25) is a lipophilic aryl aminoalcohol with gametocytocidal (Makanga, 2014) and blood schizontocidal (Warhurst *et al.*, 2001) activity. It exerts antiplasmodial activity by binding to ferriprotoporphyrin IX to disrupt the synthesis of haemozoin (Warhurst *et al.*, 2001). Lumefantrine is heavily protein-bound and metabolised in the liver by CYP3A4 into its antiplasmodial metabolite, desbutyl-lumefantrine (Wong *et al.*, 2011). Although bioavailability is variable between individuals, it may be improved with fatty food (WHO, 2015b). Currently lumefantrine is used as a longer acting drug (elimination half-life of 32.7 – 275 hours) in ACT, in combination with artemether (Figure 2.29), for the treatment of uncomplicated *P. falciparum* malaria (WHO, 2015b).



Mefloquine



Lumefantrine



Halofantrine

Figure 2.25: Clinical aryl aminoalcohol antimalarial drugs

Halofantrine

Halofantrine, (Figure 2.25) is a blood schizonticide that is metabolised into an antiplasmodial metabolite, *N*-desbutylhalofantrine. Halofantrine and its metabolite have an elimination half-life of one – five days and three – seven days, respectively (Siriez *et al.*, 2012). The activity of halofantrine is thought to be similar to other members of its group and quinine (WHO, 2015b). Despite, good efficacy and rapid action, cardiotoxicity limits the use of halofantrine (Bouchaud *et al.*, 2009). Furthermore, drug resistance in both CQR and chloroquine sensitive (CQS) *P. falciparum* strains has been reported. Resistance is thought to be through

transporter mutations that alter the transport and accumulation of the drug into the parasite's digestive vacuole (Cui *et al.*, 2015; Duraisingh & Cowman, 2005).

2.4.5.4. Antifolates

There are two sub-classes of antifolates. Sub-class I (Figure 2.26) competes with *p*-aminobenzoic acid (PABA) for binding to dihydropteroate synthase (DHPS) enzyme, thus disrupting *de novo* folate synthesis. Sub-class I includes sulfadoxine, dapsone, sulfafurazole and sulfamethoxazole (Nzila, 2006). Only sulfadoxine will be briefly discussed as it is the only one still recommended for use by the WHO. Sub-class II (Figure 2.27) disrupts DNA replication and protein synthesis by inhibiting the activity of dihydrofolate reductase (DHFR). Sub-class II popularly includes pyrimethamine, proguanil and chlorproguanil. Only pyrimethamine, proguanil and the naphthoquinone analogue, atovaquone, will be discussed.

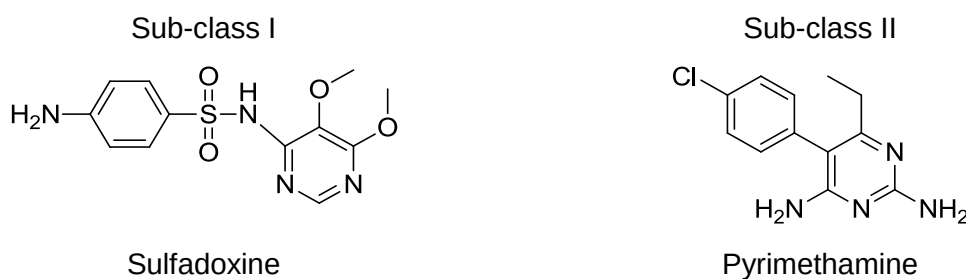


Figure 2.26: Clinical antifolate combination, used for prophylaxis and in ACT

Sulfadoxine

Sulfadoxine (Figure 2.26) has a long elimination half-life of 200 hours which, in addition to intermittent prophylaxis, may play a role in the development of resistance against the drug (Björkman & Phillips-Howard, 1991). The sulphonamide, sulfadoxine is an antifolate with an ability to inhibit DHPS (Nzila, 2006).

Pyrimethamine

Pyrimethamine (Figure 2.26) is a 2,4-diaminopyrimidine with schizontocidal activity (Nzila, 2006). It was initially developed to combat CQ resistance, but the development of parasite resistance was reported soon after its introduction as monotherapy. This prompted its synergistic combination with sulfadoxine (Watkins *et al.*, 1997).

Sulfadoxine-pyrimethamine combination therapy

Sulfadoxine – pyrimethamine (SP) is a combination of prophylactic drugs indicated for use in areas of moderate and high malaria transmission. SP is used specifically as an intermittent

preventive measure for pregnant women (in their first and second pregnancy) and infants. Moreover, SP can be used in treatment of acute uncomplicated malaria in combination with artesunate (WHO, 2015b).

Proguanil

Proguanil (Figure 2.27) is a highly protein-bound (75%) synthetic, biguanide, antifolate agent. It is metabolised in the liver by CYP2C19 (and possibly CYP3A4) into its antiparasmodial metabolite, cycloguanil that inhibits dihydrofolate reductase (Baggish & Hill, 2002, WHO, 2015b). It is readily absorbed with an elimination half-life of 8.0 – 17.6 hours and 15.6 – 22.6 hours for the metabolite (WHO, 2015b).

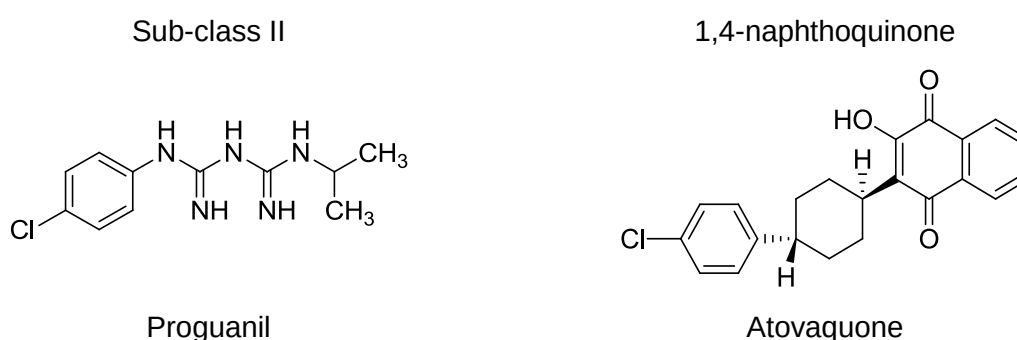


Figure 2.27: Clinical antifolate combination

Atovaquone

Atovaquone (Figure 2.27) is a broad-spectrum antiprotozoal hydroxy-1,4-naphthoquinone and an analogue of ubiquinone, a mitochondrial protein involved in electron transport (Baggish & Hill, 2002). Atovaquone has anti-plasmodial activity throughout all stages of all *Plasmodium* parasites (WHO, 2015b). It is highly lipophilic and protein bound (> 99 %). When used concurrently with rifampicin (an antimycobacterial discussed in section 2.3.6.1), there is a 40 – 50 % reduction in atovaquone levels (Baggish & Hill, 2002). In fact, it is almost exclusively excreted unchanged (WHO, 2015b). Atovaquone has a plasma half-life of two – three days, as a result of enterohepatic circulation (WHO, 2015b).

Atovaquone-proguanil combination therapy

Currently, atovaquone–proguanil combination is used (i) in malaria prophylaxis, (ii) sometimes for the treatment of uncomplicated malaria in travellers outside malaria endemic countries, and (iii) as an alternative treatment for uncomplicated malaria in combination with artesunate and primaquine, particularly in areas where the WHO recommended combinations are not effective (WHO, 2015b). The atovaquone–proguanil combination is

generally well tolerated in both children and adults; however side effects such as headaches, coughs, abdominal pain, nausea, vomiting and diarrhoea have been reported. Additionally, albeit rare, adverse events such as oral ulcerations and anaemia may occur. Furthermore, hepatic disorders, such as raised liver enzyme activity and hepatitis, have also been reported (WHO, 2015b). The atovaquone–proguanil combination should be taken with food in order to aid poor atovaquone absorption. The use of atovaquone–proguanil is exercised with caution in endemic areas because of the emergence of atovaquone resistance as a result of single mutations in the *cytb* gene (Khositnithikul *et al.*, 2008; White *et al.*, 2014).

2.4.5.5. Antibiotics

In addition to broad spectrum antibiotic activity, doxycycline and clindamycin (Figure 2.28) are used as antimalarial drugs (WHO, 2015b).

Doxycycline

Doxycycline (Figure 2.28) is a tetracycline antibiotic that is used in the treatment of uncomplicated *P. falciparum*, particularly as prophylactic treatment for individuals who travel to endemic areas (Gaillard *et al.*, 2015). The antibiotic has a terminal half-life of 14 – 24 hours (Newton *et al.*, 2005). It is used in combination with quinine and artesunate in augmentation of its slow activity (Gaillard *et al.*, 2015). It is efficacious against erythrocyte stages of the parasite. The drug is generally well tolerated; however, adverse reactions such as nausea, vomiting, abdominal pain, candidal vaginitis and skin reactions may occur. It may also reduce the efficacy of oral contraceptives (Back *et al.*, 1988). In TB coinfecting individuals, the drug should not be used with rifampicin (Garraffo *et al.*, 1987). It is also not recommended for use during pregnancy as it may cause fatal maternal liver necrosis and can cross the placenta (WHO, 2015b).

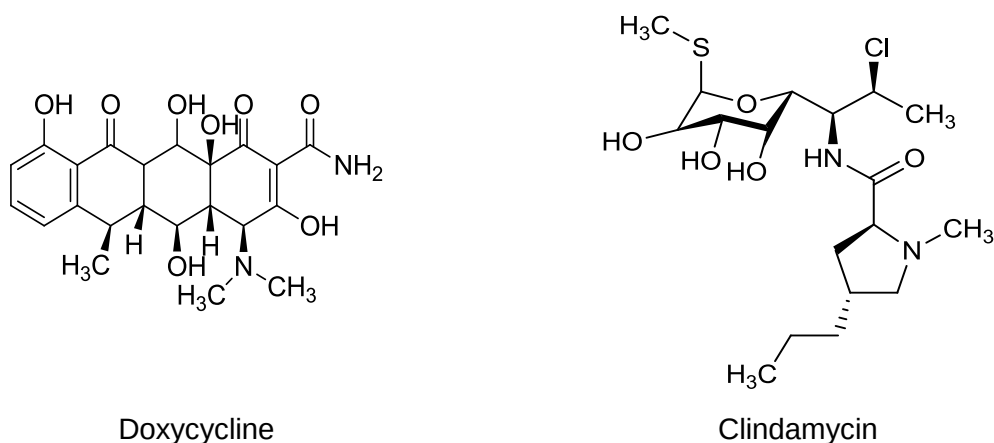


Figure 2.28: Antibiotics used in malaria chemotherapy

Clindamycin

Clindamycin (Figure 2.28) is a lincosamide derivative of lincomycin that exerts its antimalarial activity by inhibiting microbial protein synthesis by binding to the 50S ribosomal subunit and interfering with peptide chain initiation (Tenson *et al.*, 2003, WHO, 2015b). The antibiotic is heavily protein bound (90 %) and accumulates in the leukocytes, macrophages and the bile. Clindamycin is metabolised in the liver by CYP3A4 enzymes into its antiparasitodal *N*-demethyl and sulfoxide metabolites as well as some inactive metabolites (Carrasco-Portugal *et al.*, 2008). Clindamycin is detectable in breast milk, much like doxycycline (WHO, 2015b). The drug is generally well tolerated; however, caution should be exercised in patients with gastrointestinal diseases as the drug may cause diarrhoea and promote over growth of *Clostridium difficile* and pseudomembranous colitis (WHO, 2015b).

2.4.5.6. Artemisinin

Artemisinins (Figure 2.29) are a collective of synthetic analogues of artemisinin, a sesquiterpene lactone peroxide isolated from the plant *Artemisia annua* L. or *A. annua* of the genus *Artemisia* (Petersen *et al.*, 2011). These antimalarial drugs (Figure 2.29) are the backbone of both uncomplicated and severe malaria treatment. Although artemisinin is a potent antimalarial agent, its limited solubility is a source of problems associated with absorption and, ultimately, its bioavailability (White *et al.*, 2014). To address the solubility problem, lipophilic (DHA, artemether, and arteether) and hydrophilic (artesunate) semi-synthetic artemisinin derivatives were made and are currently used in ACT regimen, except for arteether (WHO, 2015b). Artemether and artesunate are rapidly metabolised in the liver into DHA, by cytochrome CYP3A4 and CYP2A6, respectively (Sangkuhl, 2012).

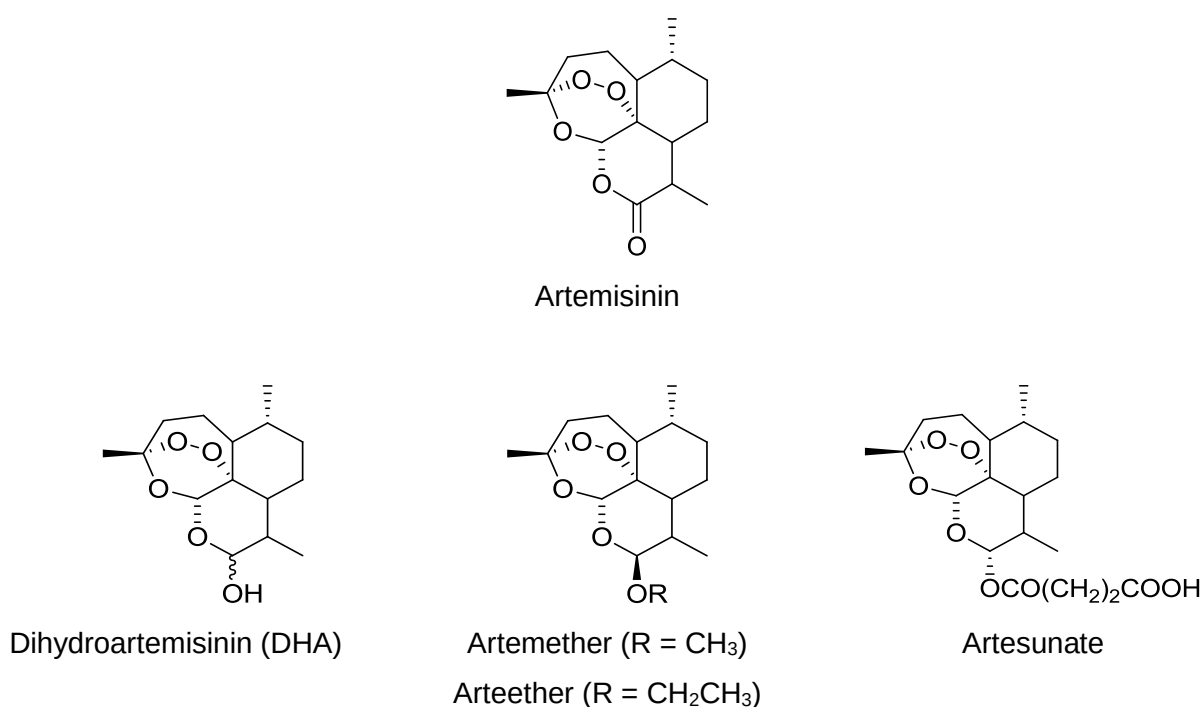


Figure 2.29: Artemisinin and its clinical derivatives

Artemisinins are fast acting antimalarials that rapidly clear parasite burden of all *Plasmodium* parasites that infect humans. They possess schizonticidal and gametocytocidal activity, as well as anti-trophozoite activity. The drugs are well tolerated; however, side effects such as mild gastrointestinal, disturbance, dizziness, neutropenia and elevated liver enzyme activity have been reported (WHO, 2015b). Furthermore, artemisinin monotherapy is contraindicated as it consequently leads to recrudescence and treatment failure which ultimately contributes to the parasite developing artemisinin resistance (Cheng *et al.*, 2012).

Dihydroartemisinin

DHA (Figure 2.29) is an artemisinin derivative with a short half-life (30 min) and it is the active metabolite of all clinical artemisinins (WHO, 2015b). As an ACT drug, it is used in combination with piperazine for the treatment of uncomplicated *P. falciparum* or *P. vivax* malaria, as well as in follow-up treatment for patients with severe malaria, when the patient can take oral medication (WHO, 2015b).

Artemether

Artemether (Figure 2.29) is a water-insoluble, lipophilic artemisinin derivative, usually administered intramuscularly or orally as an oil-based preparation. Artemether, is an artemisinin derivative with the longest half-life (four hours) (Croft & Seifert, 2010), although

some studies suggest a half-life of two to three hours (Makanga & Krudsood, 2009). It is 95% protein-bound and primarily metabolised by CYP3A4 and CYP2B6 (CYP2C9 and CYP2C19 may also play a small catabolic role) (WHO, 2015b). Its anti-plasmodial metabolite, DHA is responsible for the majority of activity post oral administration (WHO, 2015b).

Artesunate

Artesunate (Figure 2.29) is a hydrophilic, hemisuccinate artemisinin derivative used for the treatment of both uncomplicated and severe malaria (Morris *et al.*, 2011). It can be administered orally, rectally, intravenously and intramuscularly. Similar to other artemisinin derivatives, it is potent against all erythrocytic stages of malaria parasites, including the ring stages, early schizonts and gametocytes (WHO, 2015b; Woodrow *et al.*, 2005). However, it is poorly active against extra-erythrocytic forms, sporozoites, liver schizonts and merozoites (WHO, 2015b).

Mechanism of action of artemisinins

It is not clear what the mechanism of action of artemisinins is, however, the consensus is that the endoperoxide bridge is responsible for antiplasmodial activity, as artemisinin analogues that lack the endoperoxide bridge lack antimalarial activity (Wang *et al.*, 2010). Furthermore, it is widely accepted that the endoperoxide bridge exerts oxidative stress as a result of the formation of ROS (Cui & Su, 2009).

The subject of the mechanism of action of this class of antimalarials continues to be an ongoing debate, with the role of haem iron, *Plasmodium* mitochondria, PfATP6, and ROS homeostasis being the most common targets. In the haem involvement hypothesis, artemisinins react with haem ferrous ion (Fe^{2+}) to produce reactive free radicals which are subsequently rearranged to carbon centred radicals by intramolecular hydrogen-atom abstraction. The carbon centred radicals then react with biomolecules thus interfering with parasite's metabolism (Cui & Su, 2009; Olliaro *et al.*, 2001). Artemisinins disrupt normal *Plasmodium* mitochondrial function by interfering with the electron transport chain and generating ROS (Antoine *et al.*, 2013; Schmuck *et al.*, 2002).

2.4.6. WHO recommendations for the treatment of malaria

2.4.6.1. Uncomplicated malaria

ACT has the advantages of (i) simplicity of dosing (promoting adherence to therapy); (ii) better tolerance; and (iii) decreased risks of serious toxicity (Achan *et al.*, 2011). Treatment

of uncomplicated *P. falciparum* malaria is accomplished by ACT accompanied by a single PQ dose. Currently, the WHO recommends artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, piperaquine-dihydroartemisinin and artesunate-sulfadoxine-pyrimethamine three-day treatment regimens for both children and adults, except pregnant women who are in their first trimester (in which case, a seven-day course of quinine plus clindamycin is recommended). The three-day treatment is designed to cover two asexual cycles of the parasite which ensures optimal curative treatment (WHO, 2015b).

Special considerations are required for immunocompromised individuals, such as infants (infant body weight) pregnant women and HIV positive individuals. HIV treatment regimen and drug-drug interactions should be considered. For example, artesunate/amodiaquine ACT is not recommended when the patient is also receiving efavirenz or zidovudine. In children, the combination can cause neutropenia (low count of neutrophils) (Scarsi *et al.*, 2014). ACTs containing mefloquine should also be avoided because of increased incidences of neuropsychiatric complications (Nevin & Byrd, 2016).

2.4.6.2. Severe malaria

Effective treatment of severe malaria saves lives, therefore prompt and proper treatment in addition to supportive care are paramount. The standard treatment of severe malaria is IV or IM artesunate for at least 24 hours, followed by a three day ACT regimen and a single dose of primaquine, particularly in low transmission areas (WHO, 2015b).

2.4.6.3. Resistance to antimalarial drugs

Resistance to antimalarial drugs occurs in all malaria endemic regions and affects mostly the mainland South-East Asia. In fact, resistance of *P. falciparum* to chloroquine, sulfadoxine–pyrimethamine, mefloquine, atovaquone and artemisinins all originated from South-East Asia (WHO, 2018b).

Antimalarial drug resistance refers to the ability of the *Plasmodium* parasite to survive despite proper antimalarial treatment and absorption of the drug at the recommended dose, which results in either recrudescence or treatment failure. At the low end of the spectrum, at low levels of resistance, recrudescence may occur frequently. In contrast, treatment failures can be a result of poor parasitaemia clearance, and in dire situations, drug resistance may result in an increase in parasitaemia load, despite chemotherapeutic intervention. Currently, the threat is on artemisinin resistance, where (i) slow parasite clearance, (ii) reduction of ring

stage parasite susceptibility, and (iii) increasing rates of gametocytaemia are clear manifestations of resistance (WHO, 2015a; WHO, 2015b).

Despite recommendations and guidelines provided by the WHO, factors such as incorrect dosing, poor drug quality, drug-drug interactions and patient response, also play a role in drug resistance development. Patient response, in particular, may be a result of non-compliance or drug intolerance, which may prompt reactions such as poor drug absorption, nausea and vomiting (WHO, 2015b).

2.4.6.4. Treatment of artemisinin resistant malaria

Artemisinins kill ring stage parasites, which accelerates therapeutic response and ceases symptoms, a benefit that would be lost with artemisinin resistance. Slow parasite clearance, an increase in ACT treatment failure rates and high gametocytaemia count are signs of reduced *Plasmodium* susceptibility to artemisinin-based treatment (WHO, 2015a). The use of poor quality regimens only perpetuates the cycle and promotes the spread of drug resistance. *P. falciparum* has consistently developed resistance to antimalarial drugs and now the same fate has started to befall artemisinins. It should be noted that artemisinin resistant strains are isolated in the Greater Mekong Sub-region (GMS) including Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Vietnam. A longer course of artesunate-pyronaridine is the standard WHO recommendation for artemisinin resistant malaria (WHO, 2015b). Additionally, a single dose of primaquine should be added to all *P. falciparum* malaria treatment for effective gametocidal activity. In cases of severe malaria in areas where there's artemisinin resistance, the WHO recommends parenteral administration of artesunate and quinine (WHO, 2015b).

2.5 Identification of pathogenic targets for new anti-infective agents

The relationship between the mitochondria and TB infection is fundamental in that mitochondria are targeted by the mycobacteria at the point of entry as a way to promote their survival and ensure dissemination into other cells within the host (Sohn *et al.*, 2011). The mitochondrion is a double membraned organelle and a cellular powerhouse responsible for important metabolic processes, such as iron-sulphur biogenesis, ATP synthesis, ion homeostasis, calcium storage, biosynthesis of fatty acids and regulation of cell pathways, i.e. necrosis and apoptosis (Dubey, 2016; Lobet *et al.*, 2015). Processes such as ATP synthesis and regulation of cell pathways (i.e. necrosis and apoptosis), play an important role in the functioning of cells. They are required by the cell for regeneration, maintenance and death. Respiration consists of a chemical chain reaction that involves the transfer of electrons to oxygen and the transport of protons from the matrix across the inner membrane into the intermembrane, creating an electrochemical gradient (ECG). ECG provides energy to ATP synthase, thus catalysing the bulk of cellular ATP. The mitochondrial transmembrane potential signals the activation of pathways that repair or remove damaged mitochondria (Friedman & Nunnari, 2014). The diversity of mitochondrial functions makes it an ideal organelle for pathogens, either for purposes of survival and thriving in the host or evading the host's immune defences (Dubey, 2016; Lobet *et al.*, 2015).

Apoptosis is vital in ensuring that the body can get rid of damaged, superfluous, unnecessary and/or abnormal cells without compromising the integrity of host physiological processes (as a result the plasma membrane is preserved) (Elmore, 2007). Necrosis is the autolysis of cells, resulting in cell death and the disruption of plasma membranes. Virulent *Mtb* uses this process to infect neighbouring cells. Mitochondria play a crucial role in *Mtb* pathogenesis, especially pulmonary TB (Sohn *et al.*, 2011), through apoptosis and necrosis. In the macrophages, during *Mtb* infection, the bacterium causes the destruction of mitochondrial transmembrane potential, thus signalling the upregulation of anti-apoptotic factors and downregulation of pro-apoptotic factors (Abarca-Rojano *et al.*, 2003; Dubey, 2016; Sohn *et al.*, 2011). This phenomenon is observed in virulent mycobacteria and the inverse is observed for non-pathogenic mycobacteria. Furthermore, the bacterium induces necrosis as a means to escape the host's immune defences, allowing it to exit the macrophages and infect neighbouring cells (Chen *et al.*, 2006; Dubey, 2016).

Mycobacterial and host macrophage interaction (during phagocytosis, which is mediated in the alveolar macrophages) determine the success of TB infection. *Mtb* simultaneously inhibits host cell apoptosis while inducing pro-apoptotic signals. Heparin-binding hemagglutinin adhesin (HBHA) is a multifunctional mycobacterial antigen that interacts

specifically with non-phagocytic cells essential for the infection of lung epithelial cells and extra-pulmonary propagation of *Mtb*. This antigen releases cytochrome c (a haem protein that plays a role in the electron transport chain) from the mitochondria thus reducing mitochondrial transmembrane potential of macrophages (Sohn *et al.*, 2011).

Mitochondria play a role in both pathogenesis of diseases and ROS generation. Furthermore, ROS is essential in the host's immune response to pathogens (Turrens, 2003). Together these factors open an opportunity to explore chemotherapeutic agents that can complement the host's immune response and fight pathogens. Nitroaromatics with potent anti-mycobacterial activity have been described in literature (Tangallapally *et al.*, 2007; Tangallapally *et al.*, 2005). Nitroaromatics, such as 5-nitrofurans, make use of pathogenic enzymes for activation (Zhou *et al.*, 2012). This class of compounds has the ability to change mitochondrial membrane permeability, resulting in mitochondrial damage by oxidative stress and, ultimately, cell damage (Carbonera *et al.*, 1988).

Despite the success of combination therapy involving nifurtimox (a 5-nitrofuran, Figure 2.30) in the treatment of human African trypanosomiasis (HAT) and its use in the treatment of Chagas disease, nitroaromatics have not been used for the treatment of leishmaniasis (de Mecca *et al.*, 2002; Wyllie *et al.*, 2013). Much like, HAT and Chagas, leishmaniasis is a kinetoplastid disease (Barrett & Croft, 2012). In order to elicit anti-pathogenic activity, nitroaromatic compounds require activation by pathogen-specific nitroreductases (NTRs) (Zhou *et al.*, 2012). A homologous nitroreductase gene identified in the leishmania parasite genome suggests leishmanicidal activity via a type I NADH-dependent NTR, similar to anti-HAT activity of nifurtimox (Wyllie *et al.*, 2013, Wyllie *et al.*, 2012). VL *L. donovani* and CL *L. major* were both found to be sensitive to both, nifurtimox and fexinidazole (a nitroimidazole, Figure 2.30), suggesting potential chemotherapeutic activity of nitroaromatics, across different clinical forms of leishmaniasis (Wyllie *et al.*, 2013; Wyllie *et al.*, 2016).



Figure 2.30: Clinical nitroaromatics with anti-kinetoplastid activity

Nitrofurantoin (NFT, Figure 2.31) is a nitroaromatic and therapeutic agent whose structure consists of a 5-nitrofuran and a cyclopentyl amide ring joined together by a hydrazone moiety. This inexpensive drug has been extensively used in the treatment of urinary tract infections (UTIs) for over 60 years (Toole *et al.*, 1968). The drug has both bacteriostatic and bactericidal activity against a variety of Gram-negative (e.g. *Escherichia coli*) and many Gram-positive pathogens (Blass, 2015). NFT accumulates in the medullary tubular lumens and renal interstitium and is distributed to the renal medulla, with one-third of the drug appearing in the urine in its active form (Bains *et al.*, 2009). It is distributed in the kidneys at high concentrations; therefore, it is contraindicated for patients with renal impairment, since it may result in sensorimotor neuropathy (Bains *et al.*, 2009; Toole *et al.*, 1968). The cause is unclear; however it could be the result of the drug itself or the result of impaired renal clearance of metabolic products (Toole *et al.*, 1968). Although serious adverse events, such as pulmonary, neurotoxic, hepatic and haemolytic reactions occur with the use of NFT, the most commonly reported are nausea, headaches and rash (Bains *et al.*, 2009; Conklin, 1978). NFT elicits its biological efficacy through activation via nitroreduction which results in the formation of ROS, which then cause effects such as damage in DNA, lipids and proteins (Blass, 2015). NFT presents a single potential anti-infective agent with multiple sites of attack opportunity, thus reducing the chances of the mycobacteria or *Plasmodium* to develop drug resistance to the 5-nitrofuran.

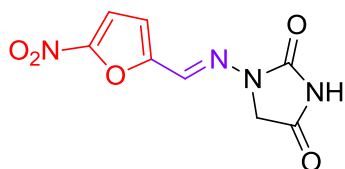


Figure 2.31: Nitrofurantoin (NFT)

Artemisinin are ROS-generating compounds with a clearly demonstrated role of oxidative stress in malaria treatment. Artemisinin modulate parasite oxidative stress through the generation of ROS and reduce the levels of antioxidants and glutathione (GSH) in the parasite (Cui & Su, 2009). NFT has similar anti-pathogenic effects, thus presenting an opportunity for new antimalarial agents (Carbonera *et al.*, 1988). Additionally, erythrocytes are the highway for oxygen transport with haemoglobin as courier (Francis *et al.*, 1997). This infers type II nitroreduction of NFT in malaria which, in the presence of ferrous iron, may proceed to form hydroxyl radicals from superoxide anion and hydrogen peroxide (Mason, 1990; Wang *et al.*, 2008). Anti-plasmodial activity by ROS-generating compounds presented

in a different structure, might present an opportunity for better and effective drugs. Pathogen resistance to nitrofurantoin is very rare, as is the use of nitroaromatics in malaria treatment, thus cross resistance is unlikely (Blass, 2015).

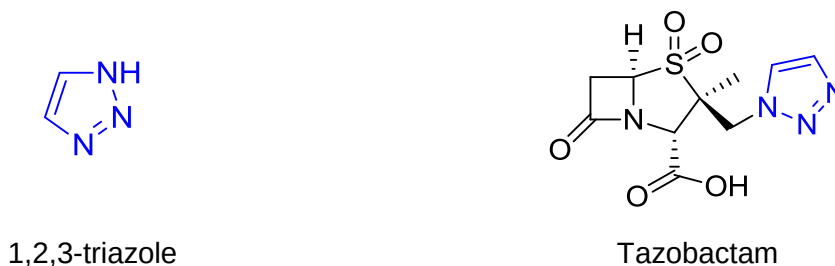


Figure 2.32: 1,2,3-triazole and related drug

1,2,3-Triazole (Figure 2.32), is an aromatic tri-nitrogen five-membered heterocyclic compound with significant biological and pharmaceutical importance. This small moiety can create stable linkages (Kantheti *et al.*, 2015). Additionally, its stability towards reduction, oxidation and hydrolysis under acidic and basic conditions is an advantage in biological systems, since it enables binding to biological targets with ease (Haider *et al.*, 2014). Moreover, triazole chemical properties such as strong dipole moments, bioisosteric (particularly as amide surrogates) effects, hydrogen bonding and their high affinity to bind with the biological targets amplifies their importance in medicinal chemistry (Dheer *et al.*, 2017).

Triazoles are metabolised in the liver and have long half-lives, ranging from 6 to 35 hours (Waller & Sampson, 2018). They interfere with the integrity of the cell wall by inhibiting lanosterol 14 α -demethylase. This consequently, reduces the synthesis of ergosterol, which is a cell membrane sterol found in fungi and protozoa (Waller & Sampson, 2018). Ergosterol has similar functions as cholesterol in animals, such as (i) maintain integrity and stability of cell membranes, cell wall permeability and membrane fluidity (ii) building block for biological molecules, and (iii) participation in metabolic pathways (Goluszko & Nowicki, 2005; Rodrigues, 2018).

The biological significance of the triazole moiety is well documented for anti-bacterial (Kant *et al.*, 2016), anti-cancer (Lakkakula *et al.*, 2019), anti-fungal (Dai *et al.*, 2015), anti-plasmodial (Mabasa *et al.*, 2019), anti-viral (El-Sayed *et al.*, 2017), anti-inflammatory (Assis *et al.*, 2012), anti-mycobacterial (Sajja *et al.*, 2017), and anti-leishmanial activity (Dheer *et*

al., 2017; Kamal *et al.*, 2013). Triazoles are the building blocks of different anti-infective therapies; hence they are often used in molecular hybridisation drug design. In fact, there are several clinical triazole drugs on the market; such as the penicillin derivative, tazobactam (Figure 2.32). Triazole presents a second pharmacophore and linker in the synthesis of potential anti-mycobacterial, anti-leishmanial and anti-plasmodial agents.

In this study, NFT derivatives will be synthesised and discussed. Chapter 4 will focus on the synthesis and anti-mycobacterial activity of the analogues, whereas, in Chapter 5 NFT-triazole hybrids incorporating linkers of varying chain length will be synthesised. Subsequently, anti-mycobacterial, anti-leishmanial and anti-plasmodial activity of the synthesised derivatives as well as cytotoxicity will be evaluated and discussed in detail in Chapter 6.

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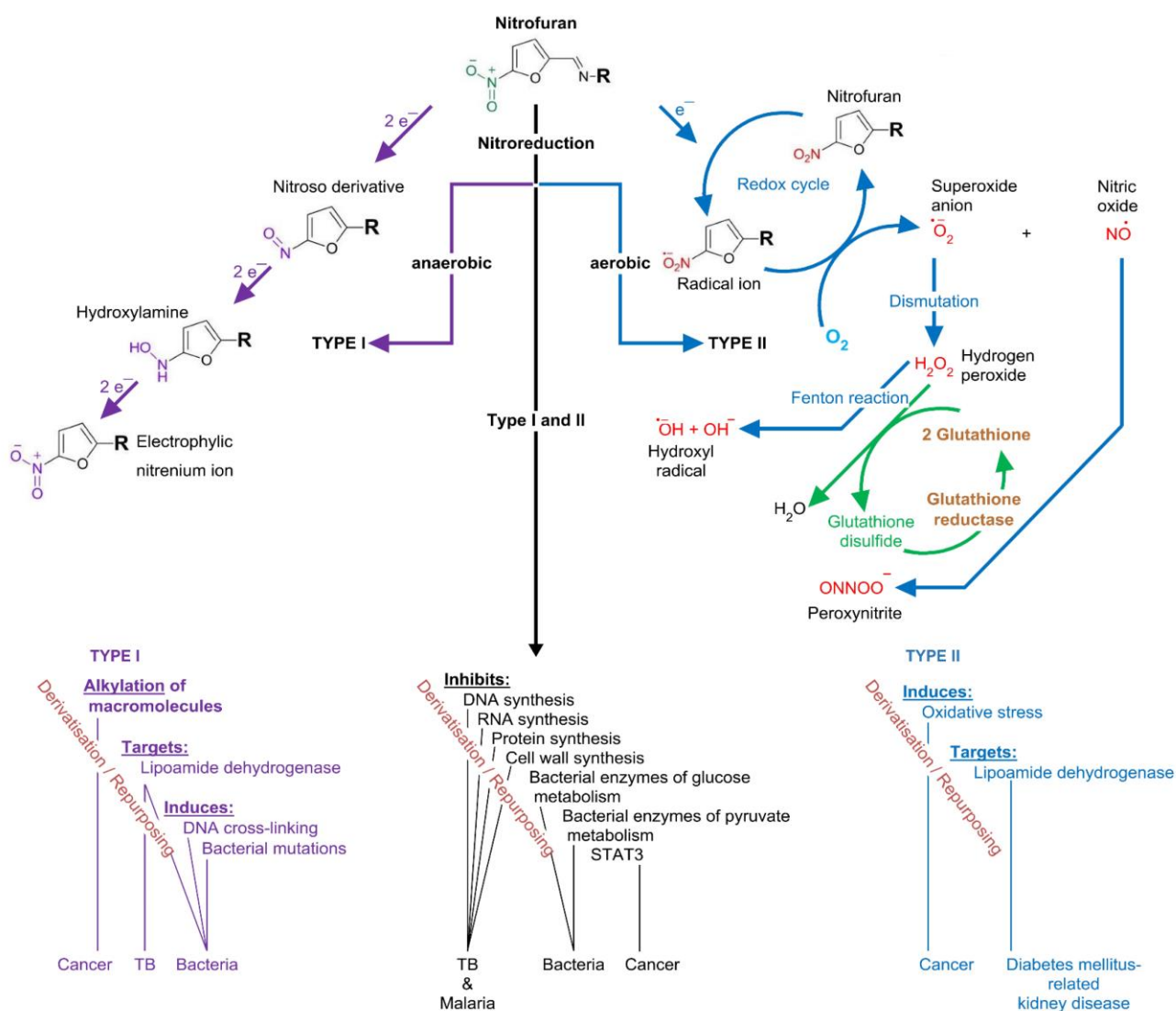
CHAPTER 3

PUBLISHED ARTICLE

Chapter 3 contains the article entitled “**An update on derivatisation and repurposing of clinical nitrofurans drugs**” which has been published in the European Journal of Pharmaceutical Sciences.

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Graphical Abstract





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An update on derivatisation and repurposing of clinical nitrofurans drugs

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ABSTRACT

5-nitrofurans (NFs) have been in clinical use for over 60 years. These affordable drugs are used for the treatment of a broad spectrum of diseases ranging from urinary tract infections to cancer. The anti-pathogenic effect of clinical NFs occurs following a step-wise process involving activation by azoreduction, followed by nitroreduction catalysed by azoreductases and nitroreductases (NTRs), respectively. Azoreduction yields stable metabolites that have the ability to covalently bind to cellular proteins. Nitroreduction, on the other hand, occurs by type I or II reduction of the nitro group in the presence of parasitic NADPH-cytochrome P450 reductases. Type I NTRs catalyse, under anaerobic conditions, the reduction of NFs to produce anti-pathogenic hydroxylamine. Under aerobic conditions, nitroreduction catalysed by type II NTRs produces reactive oxygen and nitrogen species (ROS/RNS), causing oxidative stress to pathogens and ultimate death. This multi-activity nature of NFs thus allows the repurposing of these drugs from agricultural chemicals and basic antibiotics to efficient therapies against human life-threatening diseases. Cases of NF resistance in pathogens are also limited likely due to this multi-activity, as well as effectiveness under both aerobic and anaerobic conditions. However, multi-activity of these drugs can also infer toxicity. Molecular derivatisation is an effective strategy to improve efficacy, lower toxicity, diversify activity and address pathogen resistance associated with the use of these drugs.

1. Introduction

The plight of drug resistance, patient non-compliance, multiple disease coinfection, drug-drug interactions and drug toxicity are factors that put immense pressure on the process of drug discovery. Economic issues, such as high burden of disease among the poorest of the poor, availability and access to medicine, as well as cost of drugs, further strain the process. As a result, drug repurposing is becoming a progressively used strategy in an effort to streamline treatment regimens, as well as to hasten efficiency in the lengthy and cutthroat process of drug discovery. Despite their existence spanning over six decades and their broad-spectrum activity, limited advances have been made in the development of new clinical 5-nitrofurans (NFs) in recent years. This review focuses on six clinical nitrofurans drugs which will be discussed in terms of their therapeutic benefits and shortcomings. Additionally, the activation, metabolism and toxicity of NFs will be discussed. Finally, their current use, repurposing and derivatisation efforts in current and modern chemotherapy will be explored.

NFs have been used in animal feed, and pharmaceutical applications (Conklin, 1978; Roldan et al., 2008). Until 1995, NFs were widely used in feed additives to promote growth in livestock, such as poultry, pigs and cattle, as well as in aquaculture and bee colonies (Vass et al., 2008).

Clinical NFs (Fig. 2) are broad spectrum redox-active antibiotics with bacteriostatic or bactericidal activity against Gram-positive and Gram-negative bacteria (Hall et al., 2010; Wang et al., 2008). These Schiff base derivatives of 5-nitrofurfuraldehyde are also effective against a variety of other pathogens (Hu et al., 2007), ranging from TB to malaria, leishmaniasis and trypanosomiasis and from urinary tract infections (UTIs) to cancer (Kaiser et al., 2015; Wyllie et al., 2016).

These yellow, and crystalline drugs are aromatic xenobiotic compounds whose main pharmacophore is a nitro group ($-\text{NO}_2$) attached to a furan ring (red in Fig. 1) (Spain, 1995). Interestingly, NFs have a second pharmacophore, a hydrazone moiety (blue in Fig. 1) whose carbon has zwitterionic (electrophilic and nucleophilic) properties, which aids in the overall chemical stability of the nitrofurans ring (Trukhacheva et al., 2005). The hydrazone pharmacophore has anti-pathogenic, particularly anti-TB (Bonnett et al., 2018; Jordão et al., 2011), antibacterial (Popielek and Biernasiuk, 2017), anti-trypanosomal (Katherine et al., 2017) and anticancer (Hristova-Avakumova et al., 2017) activities. In fact, the anticancer activity of hydrazones against breast carcinoma occurs via the inhibition of the phosphoinositide 3-kinase (PI3K) pathway, which is responsible for cancer survival by the phosphorylation of lipids in cell membranes (Kendall et al., 2012).

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Fig. 1. Illustration of pharmacophores present in the 5-nitrofuran drugs.

2. The clinical 5-nitrofurans

2.1. Nitrofurantoin

Nitrofurantoin (NFT, Fig. 2), is a cyclic amide containing 5-nitrofuran, whose staple use is the treatment of urinary tract infections (UTIs) (Horton, 2015). Its anti-UTI activity is enhanced by acidic pH (Horton, 2015). NFT is available as a suspension in a hydrophilic methylcellulose vehicle for use by children and elderly patients (Ensom and Decarie, 2006). It is a hypoxic agent and may have potential in the treatment of anaerobic strains of pathogens, such as latent TB (Miller et al., 2002). In fact, activity against *Mycobacterium tuberculosis* (*Mtb*, MIC₅₀ μM) has been reported (Murugasu-Oei and Dick, 2000). Moreover, no significant resistance has developed against this drug despite its use and existence spanning over five decades, probably because NFT has multiple biological targets (Blass, 2015). NFT overwhelms pathogens by attacking multiple critical metabolic pathways, such as replication, translation, transcription as well as the Krebs cycle (Horton, 2015; Kwon et al., 2001; McOsker and Fitzpatrick, 1994).

Under aerobic conditions, for example, in the liver and lungs, NFT metabolism occurs by redox cycling via one electron nitroreduction (Fig. 5) initiated by cytochrome P450 reductase at the expense of NADPH (Miller et al., 2002; Wang et al., 2008). Prolonged use of NFT is associated with pulmonary fibrosis, peripheral neuropathy, hepatocellular injury and haematological disorders (Wang et al., 2008). Pulmonary toxicity, such as progressive pulmonary fibrosis as well as hepatic toxicity in humans, has been associated with reactive oxygen species (ROS) generated by NFT (Adam et al., 1990). *In vitro* studies suggest that oxidant mechanisms are responsible for pulmonary toxicity, inferring type II nitroreduction (discussed in 3.2) (Horton, 2015; Martin, 1983). Remarkably, there is no observed correlation between NFT-induced ROS and cytotoxicity, suggesting independence between cytotoxicity and NFT redox cycling or ROS production; therefore, mechanisms underlying NFT remain unknown (Wang et al., 2008). Nevertheless, renal and glucose-6-phosphate dehydrogenase impairments are contraindicated as a precaution against haemolytic reactions (Gerk et al., 2001; Wang et al., 2008).

Under anaerobic conditions, nitroso, hydronitroso and amine

derivatives are formed (Miller et al., 2002; Voak et al., 2014). In UTI treatment, the nitro group is reduced to a nitroso (Fig. 4) that disrupts the Krebs cycle and inhibits DNA, RNA and protein synthesis, consequently killing the pathogen (Blass, 2015). NFs, therefore, possess anti-pathogenic activity in both hypoxic and oxygen-sensitive environments (Roldan et al., 2008). They can also interfere with the inner mitochondrial membrane, causing modifications in permeability and the disruption of potassium ion (K^+) efflux and respiratory rate. For example, NFT participates in redox cycling in the mitochondria by affecting the GSH/GSSG ratio in the mitochondria and ultimately disrupting the synchronised process of SH group reduction. This disrupts the regulated permeability of inner mitochondrial membrane, since critical SH groups regulate the activity of SH-dependent lysophospholipid acyltransferase which affords the mitochondria impermeability to Ca^{2+} post intramitochondrial phospholipase A2 (Carbonera & Azzone, 1988).

2.2. Nitrofurazone

Nitrofurazone, (NFZ, Fig. 2) is a hydrazinecarboxamide containing 5-nitrofuran. It is the only compound in its class that lacks the second cyclic substituent (Fig. 2). It is used as a topical cream for the treatment of superficial wounds, second- and third-degree burns, ulcers and skin infections (Boyce et al., 1995; Ryan et al., 2011). Additionally, the drug possesses trypanocidal activity, particularly against the amastigote and trypomastigote forms of the parasite (Trossini et al., 2010). NFZ interferes with the antioxidant system of trypanosomes by inhibiting trypanothione reductase. NFZ targets cruzain, a *T. cruzi* cysteine protease enzyme that is essential in the infection of mammalian cells, replication and pathogen metabolism (Trossini et al., 2010). Moreover, NFZ is effective as an antibacterial agent for the treatment or prevention of a wide variety of infections of the genito-urinary tract and is used as a food preservative (Mason, 1990). Its main significance is in cases where there is a risk of resistance in skin grafts, second- and third-degree burns (Boyce et al., 1995). The topical cream provides moisture and antibacterial efficacy which reduces the risk of bacterial colonisation (Abbas et al., 2015).

NFZ can bio-accumulate in avian eyes and pig retina. The azoreduction (discussed in 3.2) metabolite of NFZ, semicarbazide (Table 1), is associated with serious adverse effects, such as foetal deformation, underdeveloped testes, bone marrow formation abnormalities, haemorrhaging in the brain and, in some cases, death (Vass et al., 2008). The use of nitrofurantoin antibiotics as livestock antibacterial treatments, was banned by the European Union in 1995 and the Food and Drug Administration (FDA) in 2002 due to carcinogenicity risks of semicarbazide in edible tissues (Kaiser et al., 2015; McCracken and Kennedy, 2007). Despite this ban, nitrofurans are continuously used due to their effectiveness and availability. Semicarbazide, a compound

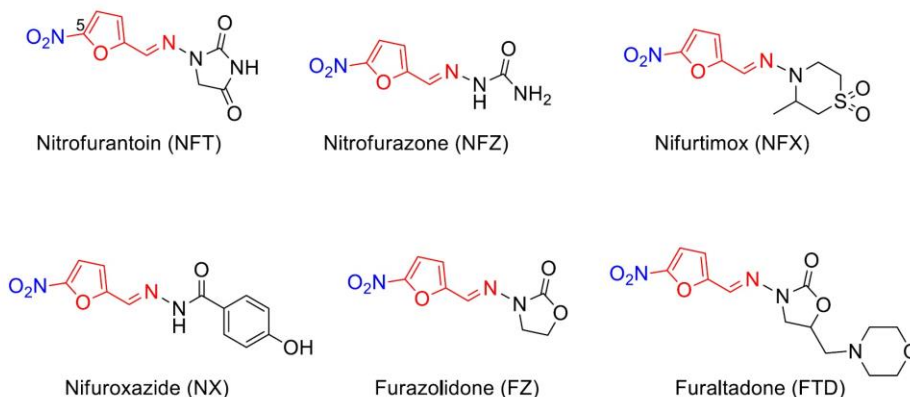


Fig. 2. Clinical 5-nitrofuran drugs.

Table 1

Azoreduction metabolites of the clinical NFs.

Drug	^a t _{1/2} (hr)	^b Peak conc. (hr)	^c Elim t _{1/2} (hr)	^d Azoreduction Metabolite & Structure	^e Protein binding (%)	^f oral Bioavail-ability (%)
Nitrofurantoin	0.3	cannot be maintained	0.3	 1-aminohydantoin (AHD)	60 - 77	60
Nitrofurazone	5	n/a	n/a	 Semicarbazide (SEM)	n/a	87 - 94
Nifurtimox	<1	3–4	3	 Methylthiomorpholine dioxide	39	n/a
Nifuroxazide	3.5	n/a	n/a	 4-hydroxybenzohydrazide	n/a	50
Furazolidone	4.9	3	5	 3-amino-2-oxazolidinone (AOZ)	75 –80	n/a
Furaltadone	n/a	n/a	n/a	 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ)	n/a	n/a

^a Half-life of NF drug (Gerk et al., 2001; Griffin, 2010; Jeganathan et al., 2011; Maddison et al., 2008; Page, 2008);.^b Peak concentration.(Grayson and Whitby, 2010; Page, 2008);.^c Elim t_{1/2} (hr) (Maddison et al., 2008; Page, 2008);.^d Metabolite (Vass et al., 2008; Verdon et al., 2007);.^e Protein binding percentage of azoreduction metabolite (Conklin, 1978; Jeganathan et al., 2011; Timperio et al., 2003);.^f Bioavailability: NFT (Nogueira Filho et al., 2013), NFZ (Bains et al., 2009) and NX (Fernandes et al., 2015).

associated with NFZ metabolism, (i) has also been detected in flour, salt and spices from azodicarbonamide additives (Becalski et al., 2004; Pereira et al., 2004), (ii) can form in products (such as egg white powder, thickening agents made of seaweed extracts and starches) disinfected with hypochlorite (Hoenicke et al., 2004) and (iii) has been found to occur naturally in certain edible crustaceans (e.g. shrimp, prawns and crab) and possibly honey (Crews, 2014). For humans, however, the concentration of this metabolite in food is limited enough to not elicit a significant threat of carcinogenicity (Vass et al., 2008). Nevertheless, care should be taken that storage containers, such as plastic gaskets and bottle lid seals that contain SEM, do not contaminate food products (Cooper et al., 2005) and thus pose health issues.

As with NFT, NFZ activation of the 5-nitrofur ring occurs by type I and II nitroreduction in target pathogens. Ryan and colleagues proposed that 5-nitrofur ring activation in humans occurs via

azoreductase-like enzymes to form a hydroxylamine that is associated with contact dermatitis and NFZ carcinogenicity (Ryan et al., 2011).

2.3. Nifurtimox

Nifurtimox, (NFX, Fig. 2), is a sulfone-containing 5-nitrofur that has been in use for over five decades, mainly in the treatment of Chagas disease (Montalto de Mecca et al., 2002). In addition, the drug is used in combination with eflornithine (NECT) for the treatment of late-stage human African trypanosomiasis (HAT) infection caused *Trypanosoma brucei*. (*T. b.*) *gambiense* parasite (Jeganathan et al., 2011).

Limitations of this drug, particularly in monotherapy, include varying unsatisfactory efficacy, toxicity, carcinogenicity, mutagenicity and lengthy treatment regimens (Hall et al., 2011). In fact, the efficacy of NFX is reduced in individuals with a long Chagas disease infection

history (Hall et al., 2011). This drug has a half-life of 50–60 min and a hepatic clearance of 3.5 mL/min that may result in low plasma concentrations (Gonzalez-Martin et al., 1994; Jeganathan et al., 2011). Adverse effects associated with NFX therapy include weight loss, skin allergies, hepatic intolerance, insomnia and polyneuropathy (Jeganathan et al., 2011; Montalto de Mecca et al., 2002). NFX is contraindicated for individuals with neurological disorders, kidney and liver failure conditions and pregnant women (WHO, 2017a).

NFX activation can occur by either type I (Fig. 4) or II nitroreduction (Figs. 5). NFX possesses antitrypanosomal activity conferred by the type II nitroreduction activation that ultimately generates the related oxidative stress (Docampo et al., 1981). However, the identification of prostaglandin F₂ α synthase (*in vitro* NFX reduction) and trypanosomal type I NTR (shares characteristics with many oxygen-insensitive enzymes) support type I nitroreduction (Hall et al., 2011). NFX resistance associated with NTR dysregulation e.g. via the inactivation of *Trypanosoma cruzi* NTR (TcNTR) gene, has been reported (Bruhn et al., 2016; Wilkinson et al., 2008).

2.4. Nifuroxazide

Nifuroxazide (NX, Fig. 2), is a phenol-containing 5-nitrofurant that is used as an antibiotic for the treatment of urogenital tract infections (Yang et al., 2015). The drug has antibiotic efficacy against Gram-positive such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Fernandes et al., 2012) and Gram-negative such as *Klebsiella pneumoniae* and *E. coli* bacteria (Squella et al., 1996). The activity of NX is bactericidal at high doses and bacteriostatic at low doses (Trukhacheva et al., 2005). It disrupts pathogenic metabolism by interfering with nucleic acids of pathogens (Tritilanunt and Vanprapar, 1991). Literature on activation and mechanism of action of this drug is limited, however, NX pharmacological (bactericidal and bacteriostatic) effects are thought to be similar to those of NFT (Dowling, 2013).

The broad-spectrum intestinal anti-infectious activity of NX, makes it suitable for colitis and diarrhoea treatment (Santiago et al., 1985; Tritilanunt and Vanprapar, 1991). Advantageously, even at high dosage, NX does not affect the integrity of intestinal flora (Buisson and Larribaud, 1989; Yang et al., 2015). However, the drug is poorly absorbed from the digestive tract, (Santiago et al., 1985; Tritilanunt and Vanprapar, 1991), resulting in rapid removal from the body without being metabolised (Buchberger et al., 1998). Nevertheless, NX is contraindicated for pregnant (despite lack of evidence of its teratogenicity) and breastfeeding women as a precautionary measure, (Timperio, 2003; Yang et al., 2015). The use of NX in combination with metronidazole, cephalosporins, chloramphenicol, NFT or griseofulvin and sedatives is not recommended as interaction may cause a disulfiram-like reaction. This reaction (inhibition of hepatic aldehyde dehydrogenases (ALDH) results in the accumulation of toxic amounts of acetaldehyde in the blood) is characterised by severe nausea and vomiting, headache and rapid heartbeat (Karamanakos et al., 2007; Timperio, 2003).

2.5. Furazolidone

Furazolidone (FZ, Fig. 2) is a 2-oxazolidone-containing 5-nitrofurant that is active against Gram-positive and Gram-negative bacteria. It is especially effective against pathogens of the gastrointestinal tract, such as *E. coli* and *Salmonella*, as well as protozoa, such as *Giardia lamblia* and *Eimeria* species (Wilson, 2012). Anti-leishmanial (Passos et al., 2014), antitrichomonal (Kuhlmann and Fleckenstein, 2017; Wendel and Workowski, 2007) and anti-trypanosomal (Sharma and Anand, 1997; Walzer et al., 1991) activities of FZ have also been reported, albeit clinical trials were not fruitful. After absorption, the drug levels are detectable in blood and other body fluids (Walzer et al., 1991). FZ may cause haemolysis in patients who have glucose-6-phosphate dehydrogenase (G6PD) deficiency. The metabolite of FZ, 3-amino-2-

oxazolidinone (AOZ), is an inhibitor of monoamine-oxidase (MAO) in rats (Hoogenboom et al., 2002). Furthermore, FZ hydrolysis produces the mutagenic and carcinogenic β -hydroxyethylhydrazine (Barbosa et al., 2007; Hoogenboom et al., 2002).

In veterinary medicine, FZ has been a staple antibiotic for over forty years (Nouws et al., 1987), either as prophylactic or therapeutic treatment against diseases caused by *E. coli* and *Salmonella* in calves, pigs and poultry. Disadvantageously, neurotoxicity, growth depression and haemorrhagic diathesis adverse effects of the drug have been reported (Kuhlmann and Fleckenstein, 2017; Nouws et al., 1987). Prevalently in veterinary use, FZ is activated by type I nitroreduction (Fig. 4) to form open chain nitriles (Timperio et al., 2003) that subsequently bind to and inhibit mitochondrial monoamine oxidase (Timperio et al., 2003).

2.6. Furaltadone

Since its introduction in October 1958, little effort has gone to the exploration of furaltadone (FTD, Fig. 2), a derivative of FZ, as an anti-pathogenic agent. *In vivo*, FTD is quickly metabolised to 3-amino-5-methylmorpholino-2-oxazolidinone (AMOX, Table 1), making it a short-lived drug with no detectable traces two hours post oral (200 mg) administration (McCarthy and Finland, 1961). The metabolite, on the other hand, complexes with proteins and is protein bound for extended periods of time. Under slight acidic (e.g. gastric) conditions, the AMOX-protein complex disintegrates, and AMOX is released and absorbed into the human body (Jin-Bo et al., 2015). FTD has been shown to have activity against Gram-positive and Gram-negative bacteria (Jin-Bo et al., 2015). However, numerous studies on efficacy have concluded that the drug is not effective as an antibiotic for *Salmonella* infection in chickens (Rampling et al., 1993) nor for both susceptible and resistant *Staphylococcus* infections in humans (Annotations, 1961). In fact, shortly after its introduction, there were reports of treatment failure (McCarthy and Finland, 1961). Both FTD and AMOX are mutagenic, teratogenic and carcinogenic (Barbosa et al., 2007; Jin-Bo et al., 2015). Toxicity induces neurological symptoms, including personality changes following oral treatment with FTD (Annotations, 1961).

3. Metabolism and activation

In order to exert their biological activities, NFs have to undergo at least one of two bio-activation processes, namely (i) hydrazone azoreduction and (ii) nitroreduction of the 5-nitrofurant ring.

3.1. Azoreduction

Azoreduction is a quick metabolic process; catalysed by azoreductase enzymes present in prokaryotic and eukaryotic organisms (Ryan, 2017). These enzymes are responsible for the reduction of azo compounds, including NFs, other nitroaromatics (such as methyl red) and quinones (Bin et al., 2004; Ryan et al., 2011). Azoreductases are NAD(P)H-dependent flavoenzymes present in a variety of bacteria found in the gut that can activate azo prodrugs into active metabolites (Ryan et al., 2011, 2010). Upon activation, azo pro-drugs elicit anti-inflammatory properties, hence they are used in the treatment of inflammatory bowel diseases, such as those affecting the colon and the small intestine (Ryan, 2017; Ryan et al., 2010). In humans, azoreductase homologues, NAD(P)H quinone oxidoreductases, confer cytotoxicity through the reduction of NFs, which occurs in a similar fashion as azoreduction by azoreductases (Ryan et al., 2011).

Food, veterinary and *in vivo* studies indicate that NFs undergo hydrazone cleavage (Fig. 3A) which results in active metabolites listed in Table 1 (Ryan et al., 2011; Vass et al., 2008; Verdon et al., 2007). Once cleaved off the parent drug, the metabolites (Table 1) covalently bind to cellular protein and persist *in vivo* for long periods of time (Chu and Lopez, 2005; Vass et al., 2008). Although precise half-lives of the

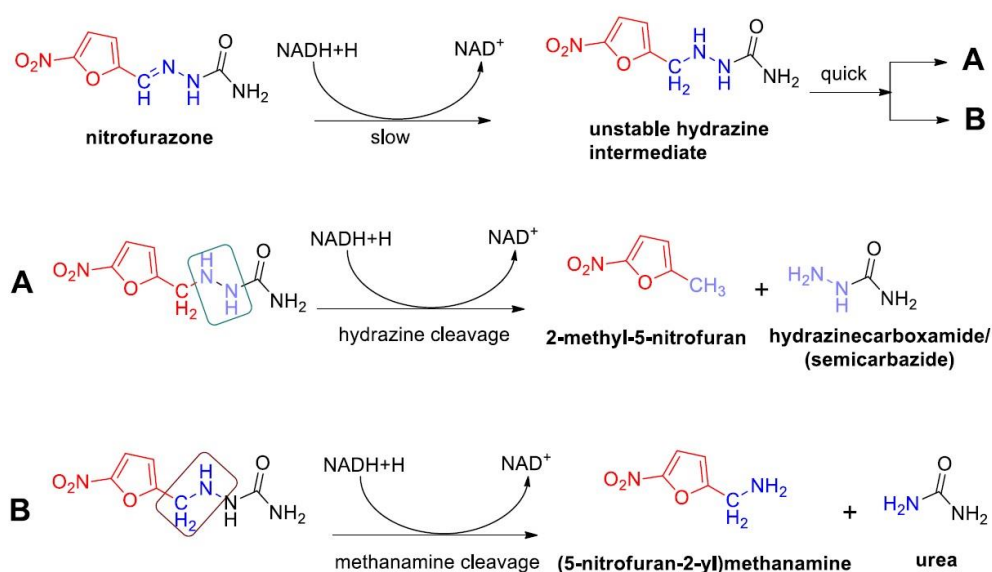


Fig. 3. Azoreduction reaction of NFs illustrated with NFZ. The two-step reaction occurs at the expense of two NADH+H. The first step is slow and results in the saturation of the diazene moiety (blue), leading to an unstable hydrazine intermediate. In the second step the hydrazine quickly undergoes an enzymatic reductive cleavage, resulting in either the active semicarbazide metabolite (SEM) as a product of hydrazine cleavage (route A) or urea as by-product of methanamine cleavage (route B) (Bin et al., 2004; Ryan et al., 2011).

metabolites remain unknown, NFZ metabolite SEM (discussed in 2.2) persists the longest and the NFT metabolite, 1-aminohydantoin (AHD), the shortest (Cooper et al., 2005). Since, some NFs have short half-lives (Cooper et al., 2005), the metabolites are used as markers in the monitoring and control of NFs for the detection in food production and animal growth enhancement (Verdon et al., 2007). In a study involving pigs, tissue-bound metabolites were still detectable six weeks after cessation of treatment (Cooper et al., 2005). Additionally, in animal studies where livestock was fed on 5-nitrofuran-containing feed, metabolites showed stability at both low (cold storage) and high (cooking) temperatures (Vass et al., 2008).

Azoreduction is a two-electron reduction process that occurs by cleavage of the azo bond facilitated by NADH or NADPH as electron donors (Ryan, 2017; Ryan et al., 2010). Activation is preceded by tautomerization of the hydrazone moiety to an azo tautomer (Bin et al., 2004; Ryan et al., 2011). In addition to the reduction of the hydrazone moiety, azoreductases may reduce the nitro group, resulting in the formation of a hydroxylamine (Ryan et al., 2011).

3.2. Nitroreduction

The pharmacological effect of NFs requires activation on the nitrofuran ring which is initiated at the nitro (NO_2) group substituent by pathogen-specific nitroreductases (NTRs) (Trukhacheva et al., 2005; Zhou et al., 2012). Indeed, the absence of NO_2 leads to either reduced or no therapeutic activity. The delocalisation of electrons in the aromatic nitrofuran ring is responsible for chemical stability. However, the strong electron-withdrawing nature of NO_2 results in electron deficiency in the aromatic ring, thus promoting the reduction of the nitroaromatic ring (Turrens, 2004). NTRs, which are abundantly present in bacteria, archaea, and eukaryotic organisms (but not in humans), catalyse the NADPH-dependent reduction of NFs (Roldan et al., 2008). Bacterial reductases, in particular, have gained interest in cancer chemotherapy involving enzyme-prodrug therapy, such as antibody-directed enzyme prodrug therapy (ADEPT), gene-directed enzyme-prodrug therapy (GDEPT), and virus-directed enzyme-prodrug therapy (VDEPT). These therapies are based on *E. coli* NTRs that are used to encourage tumour cell sensitivity to nitroaromatics (Roldan et al., 2008).

Nitroreduction occurs by either type I or II reduction and is catalysed by NADPH-cytochrome P450 reductases (Roldan et al., 2008). NADPH-cytochrome P450 reductase mediates the transfer of electrons to other enzymes such as heme oxygenase and other cytochrome P450 enzymes (Boyd et al., 1979; Wang et al., 2008). Enzymatic reduction of NFs produces highly reactive products that covalently bind to macromolecules in tissue by direct attachment to nucleophilic enzymes (Boyd et al., 1979). The majority of the resulting metabolites are responsible for the chemotherapeutic effects of NFs; however, some metabolites play a role in the carcinogenicity of NFs. In fact, carcinogenicity of NFs is attributed to the type I (Fig. 4) nitroreduction metabolite, hydroxylamine, that covalently binds to protein and DNA, disrupting the normal function of these macromolecules (Ryan, 2017).

Type I nitroreduction (i) occurs under anaerobic conditions, (ii) forms derivatives with a higher protein binding affinity compared to type II intermediates (McCalla et al., 1975; Peterson et al., 1979), (iii) is primarily responsible for the formation of mutagenic derivatives (McCalla et al., 1975), and (iv) is responsible for both the mechanism of action and selectivity of NFs. In the treatment of trypanosomiasis with NFX (discussed in 3.4), for example, type I trypanosomal NTRs are responsible for the mechanism of action, as well as the selectivity towards the trypanosomal parasite (Hall et al., 2011). In turn, type I NTR gene mutations are responsible for parasite resistance to NFs (Hall et al., 2011). Furthermore, the unsaturated open nitrile (Fig. 4h), a product of type I nitroreduction, is responsible for equipotent and indiscriminate toxicity to both mammalian and parasitic cells (Hall et al., 2011).

Type II nitroreduction activation of NFs occurs by futile redox cycling, a one electron reduction of the nitro group at the expense of NAD (P)H (Fig. 5). The process yields a superoxide ion radical ($\bullet\text{O}_2$) that then undergoes spontaneous or superoxide dismutase (SOD)-facilitated dismutation to produce hydrogen peroxide (Wardman, 1985). In the presence of an iron-complex, Fenton reactions take place, yielding an active and potent hydroxyl radical ($\bullet\text{OH}$) (Gutteridge, 1985). In summary, type II nitroreduction proceeds by the formation of a nitro-anion radical, redox cycling, generation of reductive oxygen species and lipid peroxidation, ultimately resulting in the inhibition of bacterial enzymes that disrupt crucial pathogenic metabolic pathways. The formation of the hydroxyl radical ($\bullet\text{OH}$) may further enhance the activity of NFs or

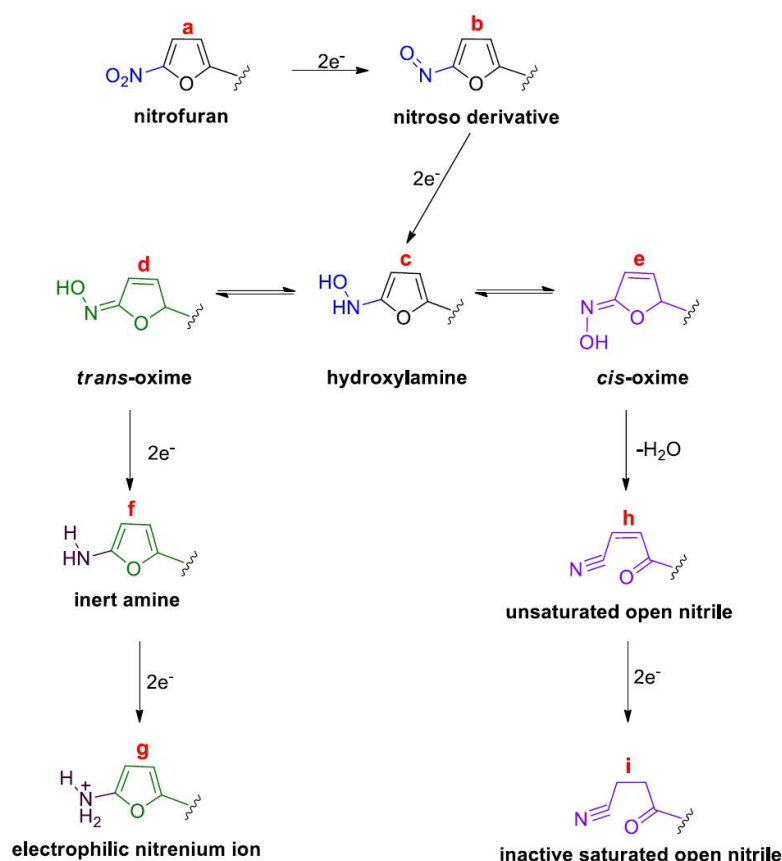


Fig. 4. In the absence of oxygen NFs, such as NFX (a), can undergo activation by type I nitroreduction via trypanosomal NTRs, at the expense of NAD(P)H. The reaction yields a nitroso derivative (b) that undergoes reduction into the hydroxylamine (c) (Hall et al., 2011). The hydroxylamine may undergo tautomerisation to form *trans*- and *cis*- NF oxime derivatives d and e, respectively (Gavin et al., 1966). d is reduced to an inert amine (f) and a reactive and electrophilic nitrenium ion (g) that disrupts DNA, causing its damage (Ford and Griffin, 1992). Dehydration of e results in ring opening, yielding a stable unsaturated open-chain nitrile (h). This process has antibacterial activity and anti-trypanosomal activity (Hall et al., 2011). To a smaller extent h undergoes reduction of the aliphatic double bond, forming an inactive saturated open nitrile derivative (i).

introduce toxicity such as carcinogenicity (Montalto de Mecca et al., 2002; Voak et al., 2014).

All NFs can undergo either type I or II nitroreduction, because the energy requirements for the reduction is similar (Hall et al., 2011; Squella et al., 1996). However, reduction potential, stability of the nitro radical, and the effect of the substituent attached to N-7 (Fig. 1), play a role in the type of nitroreduction that occurs (Miller et al., 2002; Squella et al., 1996). Consequently, the type of nitroreduction depends on the target NTR-containing pathogen, as well as the type of environment of the pathogen. This view supports the general broad-spectrum activity of these clinical compounds. Each type of

nitroreduction is accompanied by toxicity as result of the generation of the reactive hydroxylamine during type I (Fig. 4) nitroreduction and pharmacological activity due to the generation of ROS via redox cycling during type II (Fig. 5) nitroreduction (Ryan, 2017).

4. Toxicity

The chemotherapeutic effects of NF drugs are linked to the 5-nitrofur moiety in their structures, whereas toxic properties (such as carcinogenicity and mutagenicity) are attributed to the side chain to the moiety and by extension to their metabolites (Table 1) (Walzer et al.,

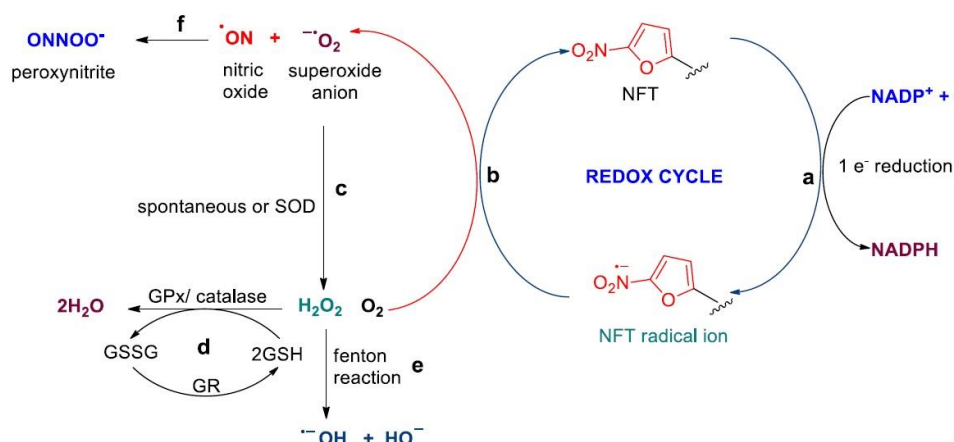


Fig. 5. Type II NTR, also known as a futile redox cycling, occurs under aerobic conditions mediated by NADPH-cytochrome P450 reductase (Wang et al., 2008). (a) In the presence of an electron donor NFT undergoes cyclic reduction to form the NFT radical anion. (b) Subsequent oxidation of the NFT radical returns the NFT molecule and reduces O_2 to $\bullet O_2^-$ (Mason, 1990). (c) Spontaneous or SOD-catalysed $\bullet O_2^-$ dismutation occurs and results in the formation of hydrogen peroxide. (d) Hydrogen peroxide can be safely degraded to water by glutathione peroxidase (GPx) in the presence of glutathione (GSH). The resulting oxidised GSH, glutathione disulphide (GSSG), can be reduced back to

GSH via glutathione reductase (GR). However, hydrogen peroxide and $\bullet O_2^-$ can also produce more potent free radicals via the Fenton reaction, in the presence of iron molecules (e), and/or peroxynitrite production, in the presence of nitric oxide radicals ($\bullet ON$) (f).

1991). In addition, toxicity may also result from activation of NFs by aldehyde dehydrogenase (ALDH2) or NTR mediated activation (Zhou et al., 2012). ALDH2 is a mitochondrial enzyme that is located on chromosome 12 in humans (Yu et al., 2016). This enzyme has reducing potential as well as dehydrogenase activity. Thus, it may reduce -NO_2 to a hydroxylamine that subsequently infers toxicity in humans, similarly to azoreductases reducing -NO_2 to a hydroxylamine with subsequent toxicity. Additional NF toxicity concerns involve the central nervous system as adverse effects, such as excitement, tremors, convulsions, and peripheral neuritis, gastrointestinal irregularities, poor weight gain, depression and alcohol intolerance, may occur (Zhou et al., 2012).

Overall, the toxicity of NF is dictated by several factors including chemical structure, exposure time, dosage and the type of the target cell. Studies based on chemical structures revealed NFT to be less cytotoxic than NFZ (Zuma et al., 2019) and NX (Fernandes et al., 2012). Furthermore, structural modifications (discussed in Section 7) on NFZ and NFT resulted in derivatives that are less toxic than parent NF (de A. Farias et al., 2006). Similarly, NX derivatisation led to analogues with reduced toxicity profile (Fernandes et al., 2012).

5. Resistance

NFs have been in clinical use for six decades and development of resistant pathogens against them are rare, although *H. pylori* and *E. coli* resistance to NFZ have been reported in literature (Ryan et al., 2011). In *E. coli*, nitrofurantoin sensitivity genes A and B (*NfsA* and *NfsB*) are the culprits for NFZ resistance (Ryan et al., 2011). Although the development of NF-resistant pathogens is slow, cross resistance with other nitro drugs is possible and may condemn other nitroaromatic agents to the same fate (Bruhn et al., 2016; Sokolova et al., 2010). Disconcertingly, the mechanism by which activation occurs may ultimately play a role in the development and growth of pathogen resistance against this entire class of agents (Sokolova et al., 2010). Beneficially, NFs have a broad spectrum of activity and adaptability in aerobic and anaerobic environments (Gavin et al., 1966; Kaiser et al., 2015; Wang et al., 2008). Advantageously, NF drug-resistant aerobic bacteria are susceptible when tested under anaerobic conditions (Maddison et al., 2008).

6. Repurposing 5-nitrofurans

There is a gap in what is currently known about NFs and their use in human chemotherapy, despite these drugs dating back more than six decades. Furthermore, nitroaromatics have generally been avoided by the pharmaceutical industry due to the toxic effect of the nitro group. However, positive contribution by NFX (one component of NECT) and fexinidazole (first introduced in the 1980s and now repurposed as the first oral treatment) as anti-HAT drugs, as well as pretomanid and delamanid as anti-TB agents, have ignited interest in nitroaromatics (Table 2) as viable drugs for human therapy. Success of these nitroaromatics is likely to spark an interest in the understanding of other nitroaromatics, including NFs from a medicinal chemistry perspective. This understanding may usher in an era of drug research concerned with effective, safe and affordable multi-activity nitroaromatic chemotherapeutic agents.

Prior to this renewed interest, typically veterinary and food industries invested in the monitoring and investigation of NFs. Consequently, studies in veterinary and food industries have clearly determined the residual effects (mutagenic, carcinogenic and teratogenic) of these drugs in humans (Vass et al., 2008). As a consequence of azoreduction, NFs are metabolised producing azoreduction (non-nitro) metabolites (Table 1) that covalently bind to tissue to form complexes (EFSA, 2015). These studies are based on the effect of NF metabolites in contaminated food introduced into the human body (Cooper et al., 2005; Pereira et al., 2004; Vass et al., 2008). Lamentably, studies driven by these industries don't take into account the effect of the infectious

target pathogen as well as its role and effect on the metabolism of the NFs. Furthermore, these studies are not concerned with the introduction of an intact NF in a human body, rather the resulting metabolites.

True to their multi-activity nature, NFs have potential for repurposing and development. Table 3 summarises the diversity in the clinical use of NFs, the broad spectrum of anti-pathogenic targets, as well as activation mechanisms eliciting their activities. Additionally, Table 3 outlines the repurposing/repositioning of clinical NFs, as they are or modified, for potential use in the treatment of other diseases.

6.1. Anti-plasmodial activity

The eukaryotic *Plasmodium* parasite has a very complex life cycle, whose asexual phase occurs in the vertebrate (human) and sexual phase in the female *Anopheles* mosquito. Although, there are five species that cause malaria in humans, *P. falciparum* presents the greatest threat. Blood stages of the malaria parasites present an opportunity of anti-plasmodial action for NFs via type II NTR (Fig. 5) activation. Indeed, in the presence of ferrous iron, hydrogen peroxide generated from NFs will undergo reduction to form the hydroxyl radical, a strong oxidant that may inhibit metabolic pathways, facilitating the synthesis of macromolecules, e.g. parasite proteins involved in the metabolic processes of uptake or digestion of haemoglobin. The targeting of haemoglobin-related processes may have deleterious effects on the *Plasmodium* cycle, considering that it is the parasite's primary source of nutrients. Advantageously, this may disrupt the sequestration of iron and heme into hemozoin, thus making iron available for reactions that are poisonous to the parasite (Sullivan, 2002). Indeed, hemozoin disruption is the hallmark mechanism of numerous successful antimalarial agents, including quinolines such as chloroquine, which binds to heme thus freeing iron (Meshnick, 1996). Furthermore, artemisinins, the gold standard antimalarials, use their pharmacophore (the endoperoxide bridge) to generate ROS that inhibits hemozoin formation, creating a toxic environment and subsequently causing the death of the *plasmodium* parasite (Meshnick, 1996; Meshnick et al., 1996).

Under aerobic conditions and in the presence of iron, NFs form highly reactive hydroxyl radicals from hydrogen peroxide (Turens, 2004). This may present an opportunity to repurpose NFs as antimalarial drugs. In fact, the role of nitroaromatic compounds as antimalarial agents has been investigated, particularly as glutathione (GSH) inhibitors and indeed, NFT is a glutathione reductase (GR) inhibitor (Dershwitz and Novak, 1980). GSH has a wide range of functions, including regulation of enzymes, degradation of proteins and the protection of cells against ROS. Furthermore, in *P. falciparum* high concentration of GSH increases resistance to antimalarials. GSH is a product of reduction of glutathione disulphide (GSSG) by GR during type II (Fig. 5) nitroreduction (Cakmak et al., 2011).

6.2. Anti-mycobacterial activity

Tuberculosis (TB), which is caused by *Mtb*, is responsible for high mortality. *Mtb* drug resistance and the synergistic relationship between HIV and TB only serves to exacerbate mortality and morbidity, which delays TB containment (WHO, 2017b). TB remains in the top ten lethal diseases with 1.7 million fatalities and 490 000 multidrug-resistant TB cases reported in 2016 (WHO, 2018). Furthermore, increased disease burden necessitates extended treatment periods on already lengthy treatment durations. NFs have anti-mycobacterial activity against both active and latent TB, as well as activity against resistant TB through the formation of ROS and subsequent generation of oxidative stress (Hurdle et al., 2008; Sutherland et al., 2009). These reactive species disrupt the *Mtb* redox homeostatic system. Wolff and colleagues observed that *Mtb* survival in human macrophages depends on eukaryotic-type protein kinase G (PknG), an important component of the redox homeostatic system RHOCS. PknG expression is uniquely induced by NADH, which also plays a key role in redox homeostasis. The disruption

Table 2
Efficacious anti-pathogenic nitroaromatics.

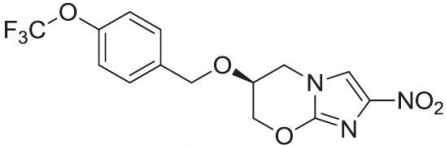
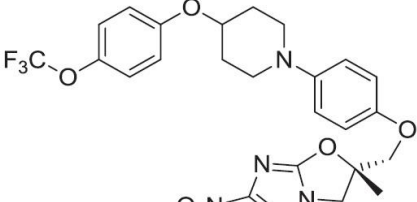
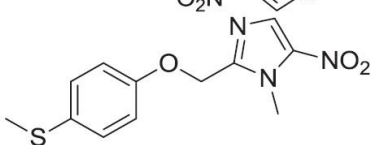
Drug	Structure	Disease	Challenges	Advantages	MOA
Pretomanid		TB	Cross resistance with: • other nitroimidazoles • other nitro aromatics	• Good interactions with other anti-TB drugs (Baptista et al., 2018)	• Inhibiting the synthesis of mycolic acid (Singh et al., 2008)
Delamanid		TB	Cross resistance with: • other nitroimidazoles • other nitro aromatics	• Safe use for children: 8 - 17 years (Dheda et al., 2016)	• Inhibits mycolic acid synthesis • Produces reactive nitrogen species (Riccardi and Pasca, 2014)
Fexinidazole		HAT Chagas treatment (Burrell-Saward et al., 2017)	Monotherapy use (Pollastri, 2018) Development of drug resistant strains	• Oral administration • Better efficacy and safety profile compared to NECT (Mesu et al., 2018). • Active metabolites (Burrell-Saward et al., 2017)	• DNA synthesis inhibitor (Deeks, 2019)

Table 3
The diversity in the clinical use of NFs, their broad spectrum of anti-pathogenic targets, and activation mechanisms eliciting their activities.

Drug	Clinical use	Target	Nitroreduction	Derivatisation & Repurposing	References
Nitrofurantoin	• Urinary tract infections	Inhibit the synthesis of DNA, RNA, protein and cell wall synthesis	Type I & II	TB Malaria	(Dershwitz and Novak, 1980; Zuma et al., 2019)
Nitrofurazone	Topical application for: • Infected burns • Skin infections	Inhibits bacterial enzymes involved in aerobic and anaerobic metabolism of glucose and pyruvate	Type I & II	TB	(de A. Farias et al., 2006)
Furazolidone	• Bacterial • Diarrhoea • Cholera • Giardiasis	Bind DNA and induce cross-linking, leading to bacterial mutations	Type I	Antibacterial furaltadone	(Annotations, 1961; Hussain and Koilpillai, 1960; McCarthy and Finland, 1961)
Nifurtimox	• Chagas disease • HAT	Disrupts structure and function through alkylation of macromolecules, such as proteins and nucleic acids. Induces oxidative stress in target cells	Type I	Hypoxic cancer cells	(Li et al., 2017)
Nifuroxazide	• Colitis • Diarrhoea	Lipoamide dehydrogenase	Type II Type I	MRSA TB	(Masunari and Tavares, 2007) (Rando et al., 2002)
		STAT3 inhibition	Type II Type I & II	Diabetes mellitus-related kidney disease Cancer	(Said et al., 2018) (Said et al., 2018; Zhu et al., 2016)

of PknG's pathway results in the accumulation of NADH and FAD, leading to increased susceptibility to oxidative stress. This causes impaired mycobacterial biofilm growth, a prohibition parameter to the growth and persistence of *Mtb* in host macrophages (Wolff et al., 2015).

Although the role of macrophages is phagocytosis and killing pathogens, they are also critical in the control and progression of infection (Cole et al., 2014; Flynn et al., 2011). In TB infection, the mycobacteria manipulate the host's immune system to its advantage, thus building suitable conditions to survive and proliferate (Queval et al., 2017). In phagocytosis, *Mtb*-macrophage complexes produce peroxynitrite (ONNO⁻), an oxidizing and nitrating agent that ultimately creates a bactericidal oxidative stress environment (Guirado et al., 2013; Turrens, 2003, 2004). Peroxynitrite is also a product of type II NTR of NFs (Fig. 5) that leads to oxidative stress. Thus, NFs have the opportunity of playing a synergistic role with the host's immune defences through the bombardment of the bacilli with ROS which may ultimately lead to faster bacteria death.

6.3. Anticancer activity

As a signal transducer and activator of transcription 3 (STAT3) inhibitor, NX is notably potent against malignant cells (Said et al., 2018; Zhu et al., 2016). STAT3 is a constitutively activated protein found in cancer cells. It is required by malignant cells for biological processes, such as cell proliferation, apoptosis, invasion and metastasis, but not necessary in normal cells. Much like other NFs, NX is involved in the mitochondrial – ROS pathway, which involves ROS and oxidative stress, through which the drug exerts its anticancer activity (Zhu et al., 2016). In addition to anticancer potential, the ability to inhibit STAT3 has a role in the treatment of diabetes mellitus and related complications, specifically diabetic nephropathy (DN) that can lead to irreversible end-stage renal disease (ESRD). DN is a five-stage chronic kidney disease characterised by deterioration in kidney function (Said et al., 2018). Clinical manifestations of DN include microalbuminuria, diabetic glomerular lesions and loss of glomerular filtration rate (GFR)

(Lim, 2014; Marshall, 2004; Said et al., 2018). NX inhibits the STAT3 cascade in diabetic-induced renal injury and reduces inflammation and fibrosis in diabetic rat kidneys (Said et al., 2018). Both cancer and diabetes have lifelong consequences on health and cost.

Furthermore, as a cancer chemotherapy against hypoxic tumour cells, NFX induces DNA damage (Li et al., 2017). Hypoxic tumour cells are responsible for malignant cell metastasis, altered cancer cells and metabolism. Additionally, hypoxia induces cell quiescence that promotes drug resistance, posing a threat to current clinical cancer interventions (Muz et al., 2015). Upon activation by human azoreductase homologues, NAD(P)H oxidoreductases, NFX subsequently inhibits the growth of hypoxic (including severely hypoxic) cells by inducing DNA damage (Li et al., 2017).

6.4. Anti-trypanosomal activity

NFX, (Fig. 2) is used for the treatment of Chagas disease and late stage HAT or sleeping sickness (Boiani et al., 2010). Chagas disease and HAT are trypanosomal diseases caused by *Trypanosoma cruzi* and *T. b.* species, respectively (Kirchhoff, 2011). Two sub-species of *T. b.* exist in humans, namely the slow-progressing and most prevalent form (98% of cases) caused by *T. b. gambiense*, and the fast-progressing form caused by *T. b. rhodesiense* (Büscher et al., 2017; WHO, 2018). The disease is most prevalent in Africa with approximately 70 million people estimated to be at different levels of risk of contracting HAT (WHO, 2018). To complicate matters, (i) treatment success is heavily dependent on the drug's ability to cross the blood-brain barrier, (ii) current treatment regimens are toxic and complicated to administer, and (iii) treatment outcomes require 24-month long follow up that include examination of body fluids, such as cerebrospinal fluid (WHO, 2018). Therefore, there is a dire need for drugs with a better overall profile, taking into consideration efficacy, toxicity and cost.

In line with the theme of preserving efficacious drugs from the plight of resistance as well as improving overall drug potency, NFX-eflornithine combination therapy (NECT) is used in the treatment of *T. b. gambiense* (WHO, 2019). NECT, which is currently recommended as first-line treatment for second stage *T. b. gambiense* HAT (Yun et al., 2010), is a better tolerated treatment combination used in replacement of the neurotoxic trivalent, arsenical melarsoprol. The combination has high cure rates (95 – 98%), lower fatality rates (< 1%), offers less severe adverse events as well as simpler administration and, as a result, it is included in the WHO Essential Medicines List (Priotto et al., 2007; Yun et al., 2010). NECT simplifies the treatment period, thus resulting in minimal hospital resources and reducing costs (Yun et al., 2010).

7. Molecular derivatisation of 5-nitrofurans

Structural modifications of clinical NFs have led to the considerable improvement of their properties, such as efficacy, solubility, bioavailability, protein binding affinity and toxicity. Derivatisation of NFZ at α -N (Table 4) resulted in compounds endowed with superior anti-mycobacterial (de A. Farias et al., 2006) and trypanomicidal activity (Trossini et al., 2010), respectively (Table 4) in comparison to the parent drug. For instance, the hydroxymethyl derivative of NFZ, NFOH (Table 4), possesses two-fold the trypanomicidal activity compared to NFZ and is superior to both NFZ and clinically used benznidazole (Table 4). In addition to total parasite (trypomastigotes and amastigotes) inhibition, NFOH is less mutagenic and is associated with low recrudescence (Chung et al., 2003; Trossini et al., 2010).

The derivatisation of NFT at α -N (Table 4) has resulted in *n*-alkyl or aryl synthesised analogues with better antimycobacterial activity when compared to the parent drug, with the lead derivative being equipotent to isoniazid with MIC₉₀ 0.5 μ M (Table 4). Furthermore, the derivatives have reduced protein binding affinity, cytotoxicity, as well as selectivity towards mammalian cells (Zuma et al., 2019).

NX derivatisation (Table 4), involving the substitution of oxygen

with sulphur at position 1 of the 5-nitrofurans ring, resulted in derivatives with improved potency against methicillin-resistant *Staphylococcus aureus* (MRSA), a multidrug-resistant Gram-positive bacteria responsible for nosocomial infections (Masunari and Tavares, 2007). The thiophenic derivatives have potential to provide efficacious therapy to immunocompromised and hospitalised patients suffering from opportunistic infections. Tavares and colleagues further explored thiophenic derivatives of NX as anti-tubercular agents (Masunari and Tavares, 2007). In this work, the thiophenic ring-containing derivative was identified as the most active with 10-fold less potency than isoniazid (Rando et al., 2002).

8. Conclusion

There is ample incentive to develop NF agents in an effort to reduce mortality and morbidity of diseases. Activity and toxicity of NFs are dependent on multiple factors, including chemical structure, exposure time, dosage and the type of cell, which together determine the type of activation and subsequent mode of action. The wide scope of prospects presented by this class of drugs provides a pool for the development of therapeutic agents for high burden diseases, as well as diseases with high potential for drug resistance development. Specifically, NFs present three significant advantages in chemotherapy. Firstly, to the disadvantage of the pathogen, NFs make use of the pathogen's readily available NTRs. These drugs are, therefore, activated by the action of pathogenic enzymes that are lacking in humans. In the treatment of HAT and Chagas disease, for example, trypanosomal NTRs are associated with NFX's mechanism of action as well as its selectivity towards the trypanosomal parasite. Secondly, the nitro (NO₂) group acts as an electron acceptor. In biological systems, this small group creates electron deficient sites that interact with biological nucleophiles, such as proteins, amino acids, enzymes and nucleic acids. The biological nucleophilic interaction, which may occur as oxidation or reduction reactions, interrupts the normal electron flow. The interruption in electron flow may result in pathogenic DNA damage and disruption of pathogenic homeostatic systems. Thirdly, NFs target multiple sites that likely contribute to the slow development of resistant pathogens.

In summary, mitochondrial inadequacies in the pathogen, the presence of crucial enzymes and/or metabolic pathways in the pathogen and lack thereof in humans, as well as the broad-spectrum activity against a wide variety of pathogens emphasises that NF drugs have a significant and important role in the development of new chemotherapeutic agents. Furthermore, derivatisation provides a library of lead compounds and a rapid, efficient and reliable tool for the discovery of new agents. Thus, derivatisation of current clinical NFs to address toxicity, improve efficacy, allow for cost effectiveness, diversify activity and address pathogen resistance, as well as discourage scepticism in the pursuit of these compounds as anti-pathogenic agents is a worthwhile strategy.

Disclaimer

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

CRediT authorship contribution statement

Nonkululeko H. Zuma: Data curation, Conceptualization, Investigation, Writing - original draft. **Janine Aucamp:** Writing - review & editing. **David D. N'Da:** Supervision, Conceptualization, Funding acquisition.

Declaration of Competing Interest

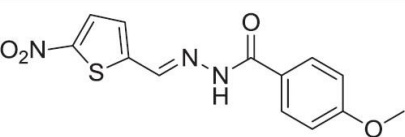
The authors have no relevant affiliations or financial involvement

Table 4
Potent anti-pathogenic molecular derivatives of NFs.

PARENT DRUG	DERIVATIVES	STANDARD CLINICAL DRUGS
NFZ Trypanomicidal activity: IC₅₀ = 23 μM (Trossini et al., 2010) NFZ Trypanomicidal activity: IC₅₀ = 23 μM (Trossini et al., 2010)	Hydroxymethylnitrofurazone (NFOH) Trypanomicidal activity: IC₅₀ = 11 μM (Trossini et al., 2010) Hydroxymethylnitrofurazone (NFOH) Trypanomicidal activity: IC₅₀ = 11 μM (Trossini et al., 2010)	Benznidazole Trypanomicidal activity: IC₅₀ = 34 μM (de Andrade et al., 2015) Benznidazole Trypanomicidal activity: IC₅₀ = 34 μM (de Andrade et al., 2015)
NFZ Anti-<i>Mtb</i> activity: MIC = 32 μM (Zuma et al., 2019) NFZ Anti-<i>Mtb</i> activity: MIC = 32 μM (Zuma et al., 2019)	de Farias 2a Anti-<i>Mtb</i> activity: MIC = 10 μM (de A. Farias et al., 2006) de Farias 2a Anti-<i>Mtb</i> activity: MIC = 10 μM (de A. Farias et al., 2006)	Isoniazid (INH) Anti-<i>Mtb</i> activity: MIC = 0.01–0.2 μM (Shakya et al., 2012) Isoniazid (INH) Anti-<i>Mtb</i> activity: MIC = 0.01–0.2 μM (Shakya et al., 2012)
NFT Anti-<i>Mtb</i> activity: MIC = 15 μM NFT Anti-<i>Mtb</i> activity: MIC = 15 μM (Zuma et al., 2019)	NFT-dodecyl derivative 13 Anti-<i>Mtb</i> activity: MIC = 1 μM (Zuma et al., 2019) NFT-dodecyl derivative 13 Anti-<i>Mtb</i> activity: MIC = 1 μM (Zuma et al., 2019)	
	NFT-octyl derivative 9 Anti-<i>Mtb</i> activity: MIC = 0.5 μM (Zuma et al., 2019) NFT-octyl derivative 9 Anti-<i>Mtb</i> activity: MIC = 0.5 μM (Zuma et al., 2019)	
NX Anti-MRSA MIC = 3.5 μM (Masunari and Tavares, 2007) NX Anti-MRSA MIC = 3.5 μM (Masunari and Tavares, 2007)	Thiophenic, 14 Anti-MRSA MIC = 0.44 μM (Masunari and Tavares, 2007) Thiophenic, 14 Anti-MRSA MIC = 0.44 μM (Masunari and Tavares, 2007)	

(continued on next page)

Table 4 (continued)

PARENT DRUG	DERIVATIVES	STANDARD CLINICAL DRUGS
	 Thiophenic, 3 Anti-<i>Mtb</i> activity: MIC = 2.0 μM (Rando et al., 2002) Thiophenic, 3 Anti-<i>Mtb</i> activity: MIC = 2.0 μM (Rando et al., 2002)	

with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript.

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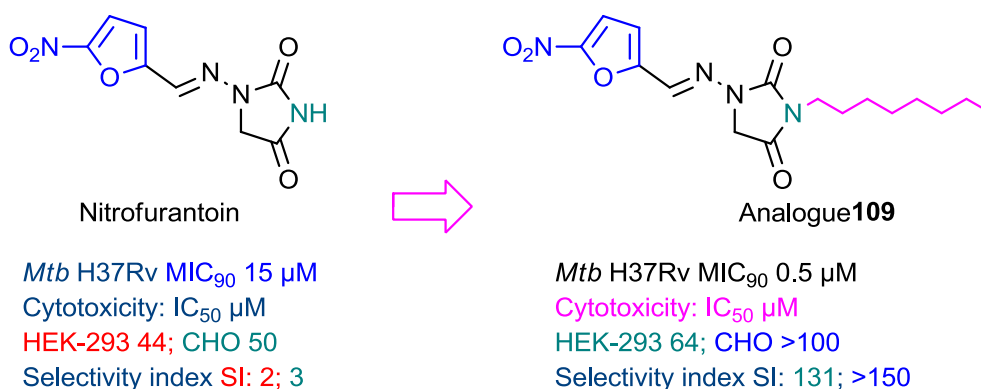
CHAPTER 4

SUBMITTED ARTICLE

Chapter 3 contains the article entitled “**Single-step synthesis and anti-mycobacterial activity of novel nitrofurantoin analogues**” which has been submitted to Bioorganic Chemistry. The article presents the Introduction, Results, Discussion, Conclusion and Materials and methods of the synthesised anti-mycobacterial compounds of this study.

The article was prepared according to the Author Guidelines available in the <https://www.elsevier.com/journals/bioorganic-chemistry/0045-2068/guide-for-authors>

Graphical abstract



Highlights

- Synthesis of a series of n-alkyl and aryl **nitrofurantoin analogues**.
- Evaluation of antimycobacterial H37Rv activity and cytotoxicity.
- *n*-Octyl analogue **109**, MIC₉₀ = 0.5 μ M, was 30-fold more potency than nitrofurantoin.
- Analogue **109**, 100-Fold more selective towards *Mtb*, stood as **anti-TB validated hit**.

Single-step synthesis and anti-mycobacterial activity of novel nitrofurantoin analogues

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Abstract

The emergence of drug-resistant tuberculosis (DR-TB) as well as the requirement for long, expensive and toxic drug regimens impede efforts to control and eliminate TB. Therefore, there's a need for effective and affordable anti-mycobacterial agents which can shorten the duration of therapy and are active against *Mycobacterium tuberculosis* (*Mtb*) in both active and latent phases. Nitrofurantoin (NFT) is a hypoxic agent with activity against a myriad of anaerobic pathogens and, like the first-line TB drug, rifampicin (RIF), kills non-replicating bacilli. However, the poor ability of NFT to cross host cell membranes and penetrate tissue means that it does not reach therapeutic concentrations. To improve TB efficacy of NFT, a series of NFT analogues was synthesized and evaluated *in vitro* for anti-mycobacterial activity against the laboratory strain, *Mtb* H37Rv, and for potential cytotoxicity using human embryonic kidney (HEK-293) and Chinese hamster ovarian (CHO) cells. The NFT analogues showed good safety profiles, enhanced anti-mycobacterial potency, improved lipophilicity, as well as reduced protein binding affinity. Analogue **109** which contains an eight carbon aliphatic chain was the most active, equipotent to isoniazid (INH), a major front-line agent, with MIC₉₀ = 0.5 µM, 30-fold more potency than the parent drug, nitrofurantoin (MIC₉₀ = 15 µM), and 100-fold more selective towards mycobacteria. Therefore, **109** was identified as a validated hit for further investigation in the urgent search for new, safe and affordable TB drugs.

Keywords: nitrofurans; nitrofurantoin; tuberculosis; analogues; nitroreductase; drug resistance

1. Introduction

Despite tuberculosis (TB) being a preventable and curable disease, it continues to be in the top ten lethal diseases, worldwide. In 2017, TB was responsible for 1.6 million deaths. This toll is in addition to the 10.0 million new TB cases that developed in the same year [1]. Hindrances in the efforts to eradicate this worldwide pandemic include absence of an effective vaccine, long, expensive and toxic drug regimens. Furthermore, social and economic issues complicate the problem since the most affected communities are poor and from sub-developed countries. Devastatingly, lengthy treatment regimens promote patient non-adherence which, in turn, promotes and exacerbates drug resistance [2].

In fact, the standard treatment regimen for drug susceptible TB involves numerous drugs, each with relatively serious side-effects. If the regimen shows poor curability or there are signs of resistance against one of the drugs, that drug is removed and two more are added. Each time resistance to a given drug is detected the same “rule” applies: the failed drug is removed and two take its place, each more toxic than the last. At a certain point, the regimen will start to include extremely painful injections given daily to weekly. The fear of needles and pain as well as the need to go to the clinic for each injection severely affects compliance. Together these problems add frustration and urgency to the already slow drug development process [3].

Mycobacterium tuberculosis (Mtb) is the bacterium responsible for TB infection in humans. While there are other forms of the disease, pulmonary TB is the most common and the form largely associated with high morbidity and mortality [4]. Infection occurs when an individual with active TB expels aerosol droplets containing *Mtb* bacteria which are subsequently inhaled by a naïve recipient. Inhalation of these infectious droplets can lead to immediate clearance of the organism by an immuno-competent host, latent infection of an immuno-competent yet susceptible host, or the onset of active (primary) disease or reactivation of disease in an immuno-compromised (such as patients with HIV) host [5, 6]. In latent TB, bacilli are thought to survive under anaerobic and nutrient deficient conditions. This sub-clinical infection is predicted to affect about one quarter of the world's population, providing a reservoir from which active disease occurs [7].

In an effort to address the need to provide drugs that have efficacy against both active and latent forms of the disease; the strategy of drug repurposing has been employed. Here, we considered 5-nitrofurans (NFs), compounds which have been used in animal feed, pharmaceutical and other applications [8, 9]. NFs are Schiff base derivatives of 5-nitrofuraldehyde, known to be effective against bacteria and variety of pathogens including trypanosomes [10, 11]. These broad-spectrum antibiotics are redox-active with dose

dependent bacteriostatic or bactericidal activity against gram-positive and gram-negative bacteria [12, 13].

NFs including nitrofurantoin (NFT, **101**) constitute a class of nitro-cyclopentyl aromatic compounds with weak activity against both active and latent *Mtb* [14]. This activity is attributed to the metabolic reduction of the pharmacophore nitro group (NO_2) by bacterial nitroreductase enzymes [15-17]. Indeed, in the presence of oxygen, NFT undergoes futile redox cycling, which is a single electron nitroreduction initiated by cytochrome P450 reductase at the expense of NADPH [12, 18, 19]. The formation of nitro-anion radical from redox cycling generates reactive oxygen species (ROS) and lipid peroxidation, which ultimately interfere with bacterial metabolic pathways leading to bacterial death [20]. Interestingly, there is no observed correlation between NFT-induced ROS and cytotoxicity, suggesting independence between cytotoxicity and NFT redox cycling or ROS production [12, 18]. Under anaerobic conditions however, nitroso, hydronitroso and amine derivatives are formed which have antibacterial activity [15, 18, 21].

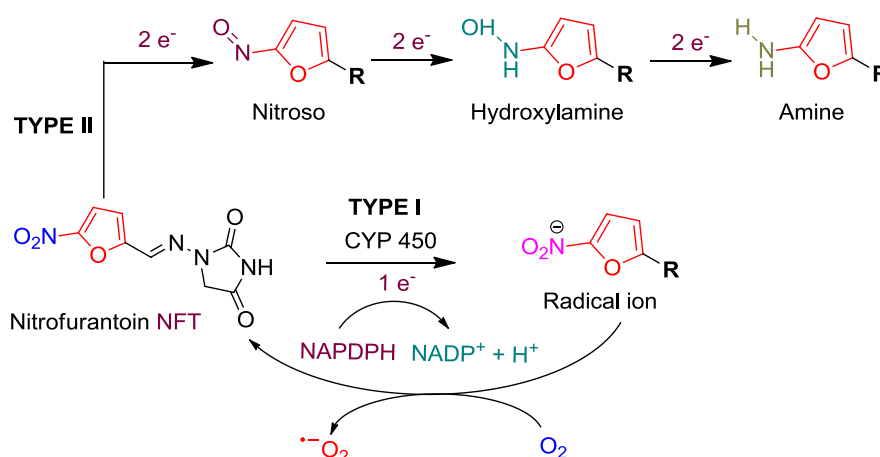


Figure 1: Schematic representation of NFT activation. Type I activation is initiated by cytochrome P450 (CYP 450) enzyme under aerobic conditions at the expense of NADPH. The process involves the alternating formation of oxygen radical and an NFT radical ion and results in bacterial death by oxidative stress. Type II NRT is an anaerobic process that results in the formation of NFT amine derivative (Spain, 1995)

Nitrofurantoin is highly protein-bound (60 - 77 %), seldom reaches plasma concentration and a large percentage is excreted unchanged [9, 23]. Furthermore, NFT poorly crosses cell membranes and therefore does not penetrate tissue well, resulting in a diminished ability to reach therapeutic concentrations [24, 25]. Nevertheless, it is effective at both low (50 μM) and high (300 μM) concentrations as well as over extended periods of exposure [18]. In addition to targeting the mitochondrion, NFT disturbs redox metabolism in the liver, which promotes glutathione disulphide (GSSG) efflux into the bile [26]. Moreover,

its prolonged use is associated with pulmonary fibrosis, peripheral neuropathy, hepatocellular injury and haematological disorders [12]. Pulmonary toxicity such as progressive pulmonary fibrosis and hepatic toxicity in humans have been associated with NFT generated reactive oxygen species. *In vitro* studies suggest that oxidant mechanisms are responsible for the pulmonary toxicity of NFT [27].

Upon activation by nitroreduction, NFT produces ROS which participate in futile redox (Type II, Figure 1) cycling in the mitochondria disrupting the GSH/GSSG ratio. Glutathione (GSH) is a product of reduction of GSSG by glutathione disulphide reductase (GR). GSH has a wide range of functions including regulation of enzymes, degradation of proteins, the protection of cells against reactive oxygen species as well as its role in the control of the permeability of the inner mitochondrial membrane [28].

Nitrofurantoin is a hypoxic agent with activity against many anaerobic pathogens including urinary tract infections [21, 29]. Indeed, its efficacy against urinary tract infections (UTIs) can be attributed to the large percentage (approximately one-third) excreted unchanged drug in the urine [9, 23]. Nitrofurantoin kills non-replicating forms of *Mtb* at MIC 50 μ M [14], similarly to the first-line TB drugs, isoniazid (INH) (MIC₉₀ ~0.01–0.2 μ g/mL) [30, 31] and rifampicin (RIF) (MIC₉₀ ~0.05–0.5 μ g/mL) [32] which are efficacious against non-replicating mycobacteria. The ability to kill non-replicating mycobacteria suggests the potential to preventively destroy the pool of *Mtb* infection, thus avoiding development of new infections [14, 33].

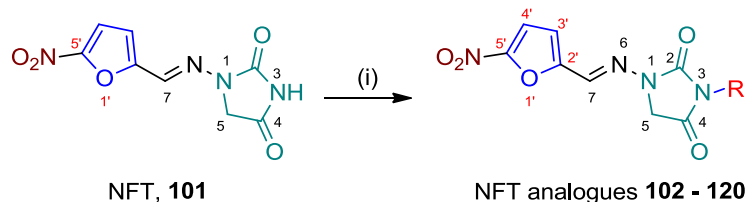
Additionally, the emergence of drug resistant *Mtb* amplifies the urgent need for a drug with a new mechanism of action. In this context, the non-specificity of nitrofurantoin in damaging DNA, lipids and proteins within the target pathogen, represents a potential advantage [34]. In fact, the seldom reported pathogen resistance to nitrofurantoin may be attributed to its ability to interfere with multiple targets, thus bombarding the pathogen by attacking multiple critical metabolic pathways such as replication, translation, transcription as well as the Krebs cycle synthesis [35, 36]. Thus, nitrofurantoin analogues, with improved solubility and lipophilicity, were synthesised and screened for anti-mycobacterial activity. Herein, we report the single step synthesis and anti-mycobacterial activity of these analogues.

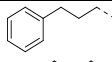
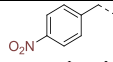
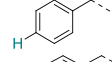
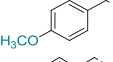
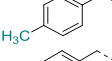
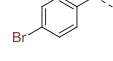
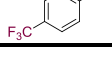
2. Results and discussion

2.1. Chemistry

Nitrofurantoin derivatives were successfully synthesised in average yields (55 -66%) using a modified single-step synthetic method described in the literature [37]. It involves the nucleophilic substitution of nitrofurantoin on the nitrogen N-3 using *n*-alkyl or (*para*-substituted) benzyl bromide. The latter bore electron donating groups, EDG and (H, OCH₃,

or CH₃) or electron withdrawing groups, EWG (Br, -NO₂, and CF₃). The analogues were isolated as crystals, powder or needles, following recrystallization in ethanol and water. They were grouped into *n*-alkyl and benzyl sub-series, where *n* ranged from 1 to 12.



Cpd	R	Cpd	R	Cpd	R	Cpd	R	Cpd	R
102	<i>n</i> -CH ₃ --	106	<i>n</i> -C ₅ H ₁₁ --	110	<i>n</i> -C ₉ H ₁₉ --	114		118	
103	<i>n</i> -C ₂ H ₅ --	107	<i>n</i> -C ₆ H ₁₃ --	111	<i>n</i> -C ₁₀ H ₂₁ --	115		119	
104	<i>n</i> -C ₃ H ₇ --	108	<i>n</i> -C ₇ H ₁₅ --	112	<i>n</i> -C ₁₁ H ₂₃ --	116		120	
105	<i>n</i> -C ₄ H ₉ --	109	<i>n</i> -C ₈ H ₁₇ --	113	<i>n</i> -C ₁₂ H ₂₅ --	117			

Scheme 1: Single step synthesis of target nitrofurantoin analogues.

Reagents and conditions: (i) **102 - 113**: *n*-alkyl bromide (1.5 eq), **114 - 120**: benzyl bromide (1.5 eq), TEA (1.5 eq), anhydrous DMF, rt, 24 hr

Nitrofurantoin is a weak acid *pK*_a 7.1 [38]. The synthesis of analogues *via* S_N2 nucleophilic substitution on N-3 with alkyl/benzyl bromide, was successfully accomplished in the presence of the moderate organic base, TEA (*pK*_a 10-11) as deprotonation agent. Brondani *et al.* previously reported the use of NaOH and heat for deprotonation [37]. However, in this study, applying heat led to decomposition and charring of the reaction mixture. Furthermore, other bases such as mineral K₂CO₃, and organic non-ionic DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene) were explored for deprotonation, however, TEA was found to achieve the best result. Subsequently, it was adopted for deprotonation of nitrofurantoin and the reaction was run at room temperature at the expense of time.

The structures of the analogues were confirmed by IR NMR and HRMS. The disappearance of H-3 singlet ca.11 ppm and the appearance of the singlet/triplet indicative of H-1'' protons ca. 4.65 ppm in ¹H spectra of the analogues (Supporting information) support the successful deprotonation and substitution on the nitrogen N-3. Signals pertaining to the resonance of furan ring aromatic protons H-4' and H-3' are present as doublets ca. 7.8 and 7.1 ppm, respectively. Hydantoin moiety H-5 proton resonates as a singlet in the 4.5-4.0 ppm region. The methine proton H-7 is also visible in the spectra as a singlet around 7.9 ppm.

Nitrofurantoin has low lipophilicity (clogP -0.22), poor solubility in oils and is slightly soluble in water (0.19 mg/mL) which is further affected by pH variations [9]. At pH 5, 7, and 9, NFT has aqueous solubility of 0.1, 0.3 and 50 g/L, respectively [9].

In general, drug development is largely hampered by toxicity and poor pharmacokinetics which also govern efficacy to a large extent [39]. Consequently, the lack of efficacy and high toxicity are considered the principal reasons for drug failure [40]. For all indications, 95% of all candidate drugs that enter clinical trials fail [41]. Compounds with favourable pharmacokinetics are more likely to be efficacious and safer in fact, toxicology and safety pharmacology are responsible for 20-40% of drug failures [41]. It is therefore essential to already determine drug-like properties during the early stages of developing any drug in an attempt to avoid future drug failure occurrence. The aqueous solubility of any compound largely influences its ability to be absorbed when taken orally. Poor water solubility is generally associated with high lipophilicity, while hydrophilic compounds generally show poor permeability and hence low absorption, which significantly influences the drug's bioavailability [39]. These two properties are therefore of high importance when designing new drugs. The *n*-octanol/water partition coefficient (log*P*) offers a reliable measure of the balance between lipophilicity and aqueous solubility, with values between 1 - 5 being targeted, and values between 1 - 3 being ideal [42, 43]. In this study, log*P* coefficients were estimated by calculating values using MarvinSketch software.

The analogues possessed enhanced lipophilicity in comparison to the parent drug as indicated by higher clogP values. A simple deprotonation of N-3 and subsequent substitution with a methyl group significantly raises the lipophilicity of NFT by 80% (**102** clogP = -0.04 vs. NFT **101** clogP -0.22). The lipophilicity of alkyl analogues linearly increases as the chain length (Figure 2). In the benzyl sub-series, however, no correlation could be found between lipophilicity and the electronic effect (EDG or EWG) of the substituent in *para* position on the phenyl ring.

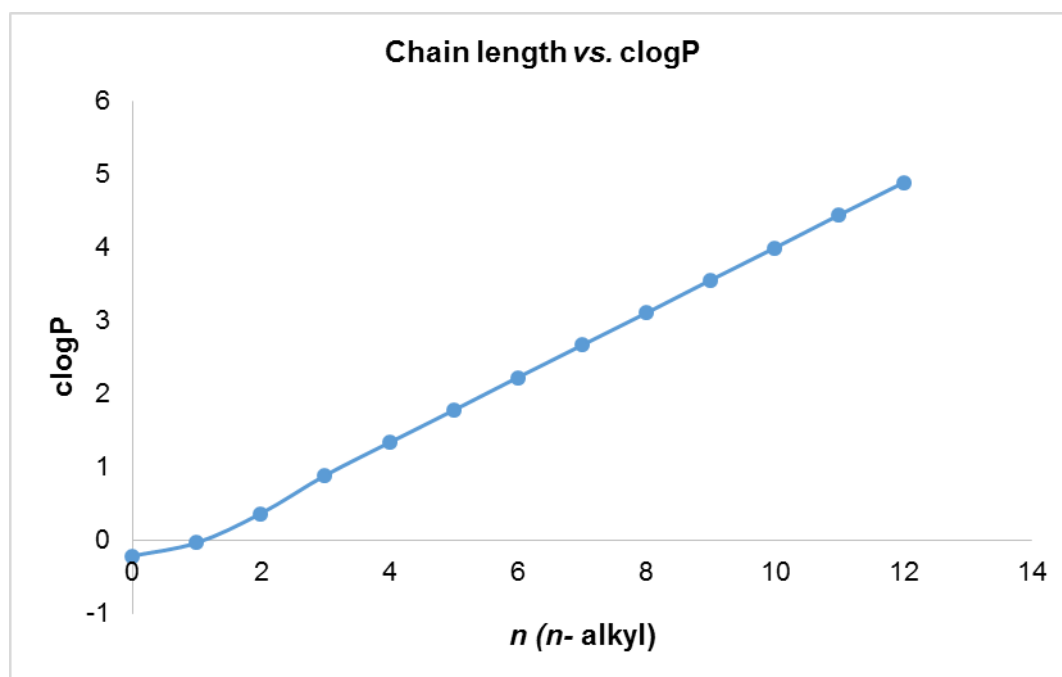


Figure 2: Graphical representation of linear variation of lipophilicity of alkyl analogues with chain length

2.2. *In vitro* biological activity evaluation

Mtb is a slow-growing mycobacterium. Furthermore, the bacilli can become air-borne and spread through an aerosol infection [44]. H37Rv, a human strain of *Mtb* was used to assess anti-mycobacterial activity of the synthesised nitrofurantoin analogues. In order to reduce the risk of exposure and infection, all the work with *Mtb* H37Rv was done under biosafety level III (BSL-III) conditions.

The synthesised nitrofurantoin analogues, **102** – **120** were screened *in vitro* for anti-mycobacterial activity, alongside standard drugs, rifampicin (RIF) and isoniazid (INH). The minimum concentration of compound inhibiting 90% (MIC₉₀) of mycobacterial growth was determined in two different media: ADC-containing medium with protein binding potential, and CAS-supplemented medium devoid of albumin. ADC medium mimics the biological environment and is therefore clinically relevant, whereas CAS provides a comparison parameter. On average, the analogues were more active than the parent drug (NFT) in both media. In the alkyl sub-series, short chain analogues ($n = 2 - 4$) showed poor activities (MIC₉₀ = 22.6 - 34.7 μ M) while the long-chain ($n = 6 - 12$) derivatives possessed better activities with MIC₉₀ values ranging from 0.5 to 3.4 μ M. In these assays, compounds **107** and **113** were equipotent to INH with micromolar activities while **109** exhibited sub-micromolar activity in the ADC-containing medium (Table 1). Furthermore, as the chain length increased, it appeared that protein binding diminished as from $n = 6$ to 12, the activities of analogues **107** – **113** were more pronounced than that of NFT in ADC and lower than in

CAS medium. In the benzyl sub-series, analogues **114**, **115**, **117** and **120** were found to be inactive while **116**, **118** and **119** possessed activities below 10 μM with MIC₉₀ values of 9.27, 5.03 and 9.49 μM , respectively.

Table 1: *In vitro* anti-mycobacterial activities and cytotoxicity data of analogues and standard drugs

Compd	n	clogP ^a	Antimycobacterial activity MIC ₉₀ (μM) ^b		Cytotoxicity IC ₅₀ (μM)		Selectivity index	
			ADC	CAS	HEK-293	CHO	^c SI ₁	^d SI ₂
NFZ	0	-0.14	31.5	3.55	55.1	97.8	2	3
NFT	0	-0.22	14.5	2.22	43.9	50.2	3	4
102	1	-0.04	22.6	2.25	77.8	>100	3	>4
103	2	0.36	34.7	3.42	38.3	60.4	1	2
104	3	0.88	33.3	3.79	13.5	40.2		1
105	4	1.33	18.3	8.53	53.0	77.8	3	4
106	5	1.77	2.1	0.24	29.1	>100	14	48
107	6	2.22	1	5.14	>100	>100	103	>100
108	7	2.66	2.0	2.34	>100	>100	50	>50
109	8	3.10	0.5	0.97	63.8	>100	131	>200
110	9	3.55	3.41	7.81	>100	>100	29	29
111	10	3.99	2.02	31.3	>100	>100	50	>50
112	11	4.44	1.88	3.91	>100	>100	53	53
113	12	4.88	0.99	62	>100	>100	100.	>101
114		2.46	>125	2.12	20.9	18.2		
115		1.73	>125	4.23	>100	40.0		
116		2.24	9.27	2.76	90.5	>100	10	>11
117		2.60	>125	1.66	>100	87.9	1	
118		1.67	5.03	0.97	17.6	>100	4	>20
119		1.57	9.49	2.14	15.8	91.3	2	10
120		2.50	nd	1.35	>100	38.2	nd	nd
RIF	nc		0.003	0.49				
INH	nc		0.96	0.01				
EM	nc				0.06	0.064		

^aCalculated using Chem Axon Marvin Beans Software (Version 5.2.3_1, May 26, 2009); ^bMIC₉₀ = determined *in vitro* against *Mtb* H37Rv; ^cSI₁ = Selectivity Index = IC₅₀ HEK-293/MIC₉₀ *Mtb* H37Rv (ADC); ^dSI₂ = Selectivity Index = IC₅₀ CHO/MIC₉₀ *Mtb* H37Rv (ADC); nd (not determined); nc (not calculated); NFZ = nitrofurazone; NFT = nitrofurantoin, 101; RIF = rifampicin; INH = isoniazid; EM = Emetine

Protein binding affinity of drug is a crucial factor affecting its efficacy, since this parameter influences the drug's ability to traverse through cell membranes [45]. Nitrofurantoin is highly plasma protein bound, ranges of 53-73% in rat, 12-45% in dog and 60–77% in human have been reported [9]. Additionally, nitrofurantoin binding primarily occurs to the albumin protein of the serum [9, 23]. Consequently, on the basis that NFT is highly protein bound [9], CAS medium was used as an alternative to ADC in order to determine the effect of the presence of albumin on the activity of the analogues. Notably, MIC₉₀ values were generally higher in ADC-supplemented medium than in CAS. This suggests that in ADC, NFT is largely protein-bound which results in limited quantity of drug

available to exert the anti-mycobacterial activity, whereas the opposite is observed in the CAS medium. However, this interpretation is speculative, and there could be additional effects including altered mycobacterial metabolism in the different growth media.

Interestingly, there was a clear correlation between lipophilicity/chain length (n) and anti-mycobacterial activity in the alkyl sub-series (Figure 3). As the chain length increases between $n = 1$ and 3, the activity decreases steadily, which portends an inverse proportionality between lipophilicity and activity. For these analogues, characterised by $\log P$ values outside the ideal range, higher protein-binding may be inferred with increasing chain length, in comparison to NFT. Possibly, the derivatisation was detrimental to NFT as it resulted in higher protein-binding and ultimate reduced activity. However, the trend (lipophilicity vs. activity) changes from $n = 4$ to 12, where the anti-mycobacterial activity (lower MIC_{90} values) increases significantly with the increasing lipophilicity reaching the optimum value of MIC_{90} 0.5 μM with **109** ($n = 8$, $clogP$ 3.1).

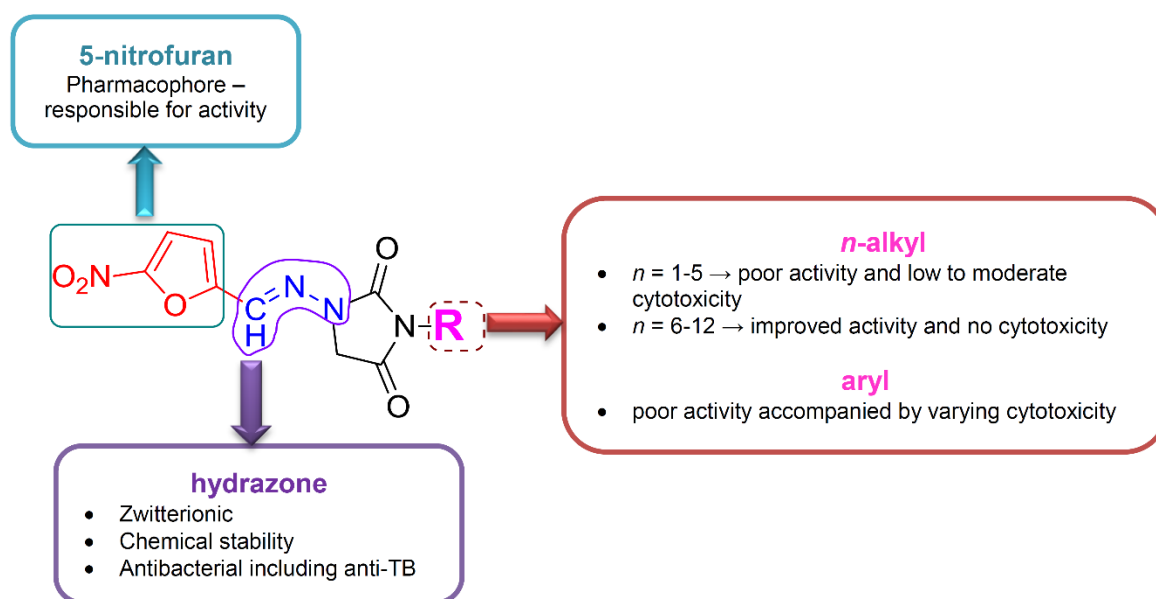


Figure 3: Schematic representation of structure-activity relationship.

Evidently, for the long chain analogues with $\log P$ values in the target range, there is a reduced protein binding which indicates an inverse relationship between n and MIC_{90} (Figure 4) and infers a proportional relationship between lipophilicity and anti-mycobacterial activity. It can be concluded in this case that the derivatization was beneficial to NFT as it led to reduced protein binding and ultimately significant antimycobacterial activity enhancement. This also confirmed the previously reported positive influence of long alkyl chains [46, 47] on antimycobacterial activity. Besides increased lipophilicity, this effect might also be mediated by facilitated transport through mycobacterial cell wall, via interaction of long carbon chains

of mycolic acids with alkyl chains of discussed compounds. Additionally, these findings deduce that position N-3 of the hydantoin ring is not the protein binding site of NFT since it is hindered through the derivatization, and yet the analogues were found to be more or less protein-bound. Overall, the activity of octyl analogue **109** ($MIC_{90} = 0.5 \mu M \sim 0.17 \mu g/mL$) against *M. tuberculosis* H37Rv was comparable to that of the clinically used first-line TB drug, INH ($MIC_{90} = 0.01-0.2 \mu g/mL$) [31, 32, 48].

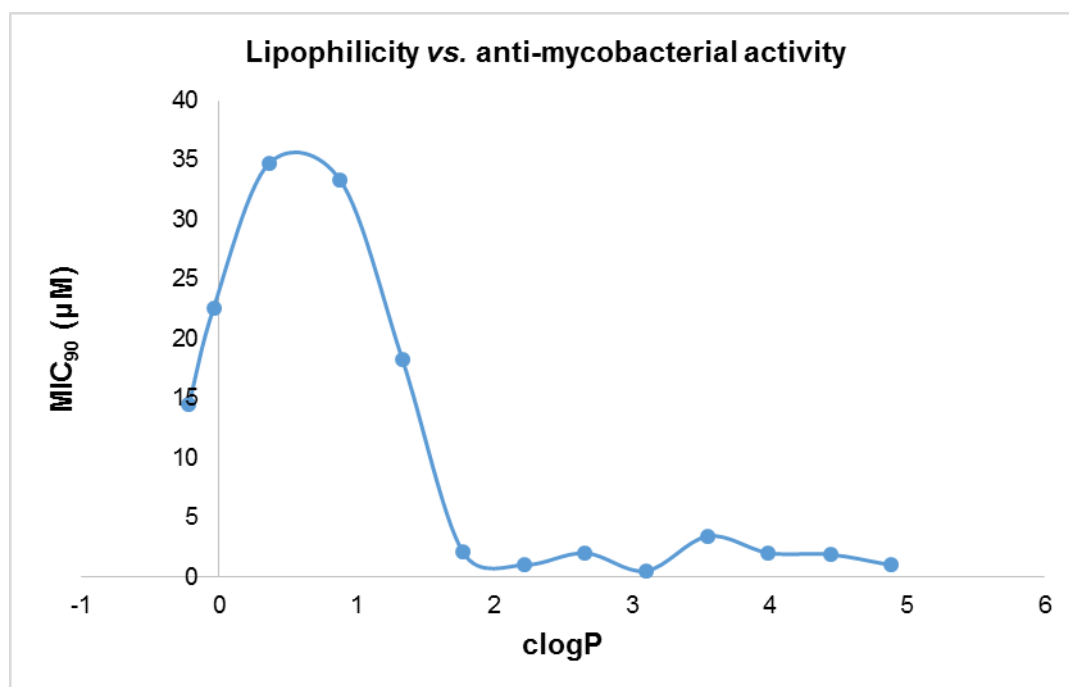


Figure 4: Illustration of the effect of lipophilicity on the anti-mycobacterial activity. An increase in lipophilicity results in decreased activity with the short chain ($n \leq 4$) analogues. With the long chain ($n \geq 5$), however, an increase in lipophilicity leads to increased activity.

Introduction of an aromatic moiety into the methyl side-chain of **102** resulted in N-benzyl analogues **115** – **120**, which had ideal lipophilicities but were found with either marginal increase or complete loss of activity in comparison to NFT. In fact, analogues **116**, **118** and **119** were the only actives in N-benzyl sub-series with MIC_{90} below $10 \mu M$. The presence of a second nitro (NO_2) conferred slightly higher anti-mycobacterial activity as seen with **118**. Inversely, no anti-mycobacterial activity improvement resulted from **114** despite this analogue containing a 3C chain in addition to the aromatic ring. It is unclear whether the electronic effect of the *para*-substituent played a role in anti-mycobacterial activity of the aryl analogues. For these structures, rigidity might be another parameter detrimental to their passage through *Mtb* cell wall. Overall, lipophilicity, molecule flexibility alongside protein-binding ability were identified as putatively important determinants governing the antimycobacterial activity of these analogues.

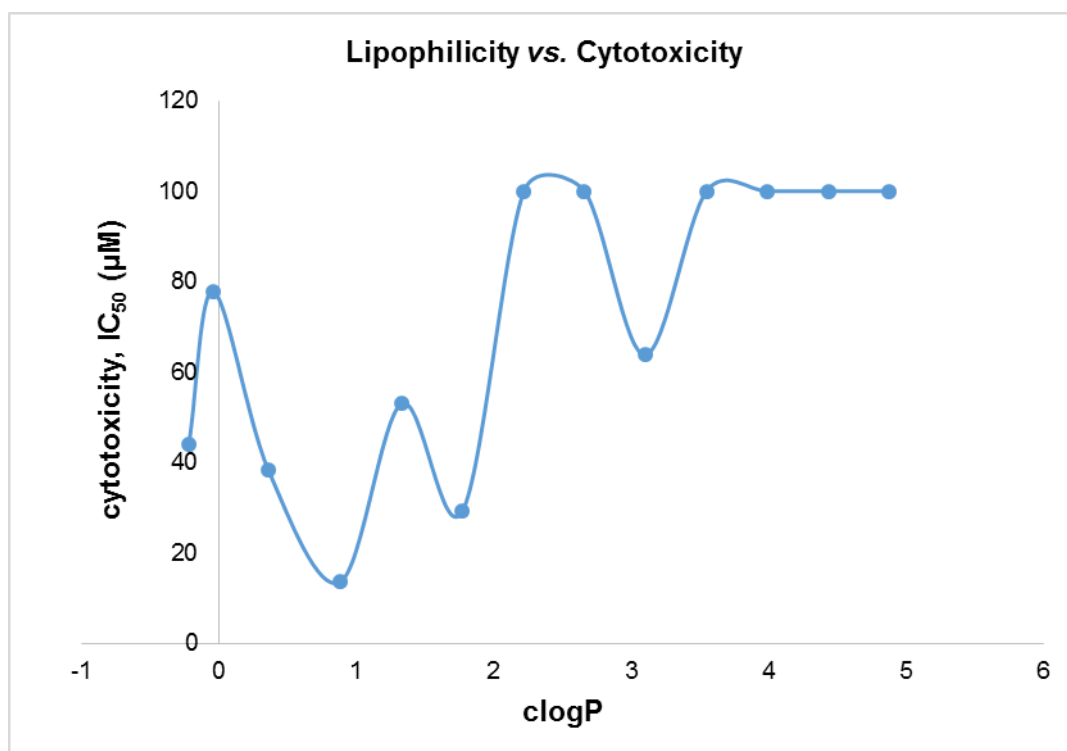


Figure 5: Graphical representation of the influence of lipophilicity on cytotoxicity. Inconsistent, zigzag pattern. As lipophilicity increases enhanced cytotoxicity is observed with short chain alkyl ($n \leq 5$). However, no induction of cytotoxicity in mammalian accompanied increasing lipophilicity in long chain alkyl analogues ($n \geq 6$, $\text{clogP} \geq 2$).

Emetine, an antiprotozoal drug known for its high cytotoxicity, was used as a reference drug in the cytotoxicity evaluation. An *in vitro* nephrotoxicity assay was performed in human kidney embryonic (HEK-293) cells alongside Chinese hamster ovarian (CHO) cells to assess the cytotoxic profile of the analogues with emetine as reference.

The decrease of viability of mammalian cells was measured using standard colorimetric assay based on the reduction of tetrazolium. Results were expressed as IC_{50} in μM . All synthesised nitrofurantoin analogues were less cytotoxic than emetine. Moreover, on average, the parent compound (NFT) was more toxic ($\text{IC}_{50} = 43.93 \mu\text{M}$) to mammalian cells (HEK-293) compared to the analogues. An increase in aliphatic chain length ($n = 6-12$) resulted in reduced cytotoxicity ($\text{IC}_{50} > 100 \mu\text{M}$), with the exception of $n = 8$ (**109** $\text{IC}_{50} = 63.80 \mu\text{M}$).

The course of *in vitro* nephrotoxicity of the analogues with varying the chain length/lipophilicity is illustrated in Figure 5. It followed an inconsistent “zigzag” pattern. However, the investigated derivatives can be divided into groups. The first group which includes short chain alkyl ($n \leq 5$) and benzyl/aryl (**114**, **118** and **119**) analogues, was moderately toxic than the parent drug in cells. The second group which comprised mostly long chain alkyl analogues ($n \geq 6$) was found to be non-toxic to the mammalian cells with

IC₅₀>100 µM. This finding suggests that the increase in lipophilicity did not induce toxicity in either human nephrotic or ovarian cells.

Furthermore, in the HEK-293 cell line, analogues **103**, **104**, **106**, **118** and **119** showed moderate toxicity with IC₅₀ values of 38.3, 13.5, 29.1, 17.6 and 15.8 µM, respectively while **102**, **105**, **109** and **116** had weak cytotoxicity. The N-alkyl analogues were nontoxic to CHO cells. Among active N-benzyl analogues, **116** and **118** were nontoxic while **114** (IC₅₀ = 18.16 µM) was the most cytotoxic, regardless of the mammalian cell line considered.

Selectivity index (SI), defined as IC₅₀ (cell)/MIC₉₀, was calculated using antimycobacterial activity against *Mtb* in ADC assay. Anti-mycobacterial agents are considered bacteria selective when the calculated SI is >10 [49, 50]. Short chain *n*-alkyl ($n \leq 5$) and all benzyl analogues showed poor bacterial selectivity as evidenced by their low selectivity indices <10 (Table 1), indicating that these analogues kill cells indiscriminately. Long chain *n*-alkyl ($n \geq 6$) analogues, however, were selective towards mycobacteria with the most active being **107** (n 6), **109** (n 8) and **113** (n 12) (MIC₉₀ <1 µM), all possessing indices ≥ 100 . These three analogues stand as validated hits [49] for further investigation in the search for novel anti-mycobacterial drugs.

One of the biggest issues with TB is the length of treatment, which can have a negative impact on gastrointestinal microbiota, particularly in oral therapy. Gastrointestinal microbiota is the microbial ecosystem inhabiting the gastrointestinal tract. These organisms have a large impact on the body including general human health, drug metabolism, as well as affecting how organs such as the liver (and its enzymes) metabolise drugs [51]. Since NFT is a clinical antibiotic [23], the identified validated hits may have an impact on the gastrointestinal microbiota.

3. Conclusion

There is worldwide need for novel effective anti-mycobacterial agents active against replicating organisms (including resistant strains) and non-replicating bacilli. The search for such agents led to the investigation of several nitro-aromatic compounds, including the discovery of Pretomanid (PA-824) which is currently undergoing Phase III clinical trials [52]. Subsequently, nitrofurantoin, a multi-target nitrofuran antibiotic was investigated as potential anti-mycobacterial agent taking into consideration both its low lipophilicity and poor solubility. Thus, *n*-alkyl and benzyl analogues of nitrofurantoin were synthesized in moderate yields in a single-step S_N2 process from this parent drug and appropriate *n*-alkyl/benzyl bromide reagents, and their activities were assessed against human *mycobacterium tuberculosis* H37Rv strain. The analogues (summarised in Figure 3) possessed improved lipophilicity in

reference to the parent drug. The short chain *n*-alkyl ($n \leq 5$) and benzyl analogues were poorly active while the long chain ($n \geq 6$) displayed good activities against the mycobacteria. In particular, analogues **107**, **109** and **113**, which featured *n*-hexyl, -octyl and -dodecyl chains, respectively, were equipotent to isoniazid (first line anti-TB drug) with macromolecular activity as a result of reduced protein-binding affinity. In addition, these analogues with 14 to 30-fold higher potency than nitrofurantoin were also non-toxic to mammalian cells and demonstrated highest selectivity against the mycobacteria.

Overall, the easy accessibility, equipotency to INH, improved lipophilicity, and good cytotoxicity profiles, all beneficial features displayed by analogues **107**, **109** and **113**, provide a good starting point in the optimization of nitrofurantoin as an anti-mycobacterial agent. These analogues were identified as validated antimycobacterial hits with **109** ($\text{MIC}_{90} = 0.5 \mu\text{M}$), 30 times more potent than nitrofurantoin as the logical choice for further development focusing on identification of both the protein-binding site in order to further improve the efficacy of nitrofurantoin, and the cellular target within the *Mtb*.

TB treatment habitually runs into several weeks, therefore in future work validated hits will be subjected to antibacterial (Gram -positive and -negative) assessment in order to ascertain their effect on the gastrointestinal microbiota. This evaluation is particularly important for prospective oral drugs since microbial integrity plays a role in general human health and in some cases drug metabolism.

4. Experimental section

4.1. Materials

Nitrofurantoin was purchased from Changzhou Kaixuan Chemical Co (Chunjiang, China). Anhydrous dimethyl formamide (DMF), trimethylamine (TEA), benzyl bromide, ethyl bromide, 4-bromobenzyl bromide, were purchased from Sigma–Aldrich (Johannesburg, South Africa). Dichloromethane (DCM), methanol (MeOH), ethanol (EtOH), ethyl acetate (EtOAc) were purchased from ACE chemicals (Johannesburg, South Africa). CHO cells (ATCC® CRL-9618™) were received as a gift from Prof. P.J. Smith from the Division of pharmacology, University of Cape Town, South Africa. HEK-293 cells (ATCC® CRL-1573™) were purchased from ATCC (Manassas, USA). All the chemicals and reagents were of analytical grade. Chemicals and reagents were used without further purification.

4.2. General procedures

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance™ III 600 spectrometer at a frequency of 600 MHz and 150.913 MHz, respectively, in $\text{DMSO}-d_6$. 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.

Mass spectra (MS) were recorded in positive mode on a Thermo Electron LXQ™ ion trap mass spectrometer, equipped with Xcalibur 2.2 data acquisition and analysis software. The MS had an APCI source set at 300°C, and was direct infusion with a Harvard syringe pump utilized at a flow rate of 10 µL/min. A full scan from 100 - 1200 amu was achieved in 1 s, with a capillary voltage of 7 V, while the corona discharge was 10 µA.

Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument. Thin layer chromatography (TLC) was performed, using silica gel plates (60F₂₅₄), obtained from Merck (Johannesburg, South Africa).

HPLC analyses was conducted using an Agilent 1100 series instrument equipped with a gradient pump, autosampler, diode Array UV detector and OpenLab CDS Chemstation Rev.C.01.07 SR3 data acquisition and analysis software was used (Agilent Technologies, Palo Alto, CA, USA). The column was a Venusil XBP C18 (2) -column, 150 x 4.6 mm, 5 µm spherical particles, 100 Å pore size (Agela Technologies, Newark, DE, USA), The mobile phase consisted of acetonitrile and 0.1% orthophosphoric acid in water with a linear gradient from 30% acetonitrile to 85% after five minutes and holding until 15 minutes before equilibrating with 30% acetonitrile to 20 minutes. The flow rate was set at 1 mL/min and the injection volume was 10 µL. The UV signal was monitored at 210 nm.

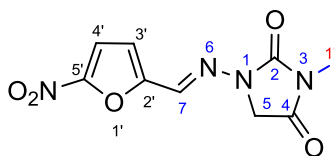
4.3. Syntheses

Synthesis of analogues 102 - 120

Nitrofurantoin, **101**, (2.94 mmol, 0.7 g) was dissolved in 10 mL of anhydrous dimethyl formamide (DMF), and trimethylamine (TEA, 4.41 mmol, 1.5 eq.) was added. The solution was stirred at room temperature for 15 min. To resulting yellow solution, the appropriate *n*-alkyl or benzyl bromide (4.41 mmol, 1.5 eq) was added. The reaction was stirred at room temperature and monitored by TLC eluting with DCM:MeOH (19:1, v/v). Upon completion, the reaction was quenched with 20 mL water and extracted with ethyl acetate (3 X 20 mL). The organic layer was washed once with saturated NH₄Cl and once with water. The solution

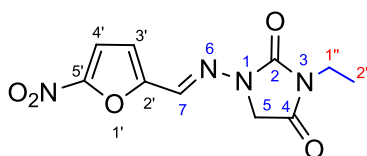
was concentrated *in vacuo*. The resulting paste was purified by recrystallisation in ethanol/water) to afford the desired analogue (Scheme 1).

4.3.1. (E)-3-Methyl-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **102**



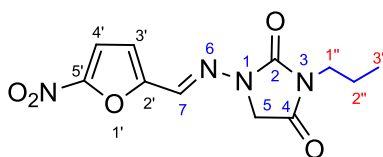
The reaction of NFT with bromomethane yielded **102** as yellow needles 740 mg (67%), m.p.: 136.1-136.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3138 (C-H), 1779 (C=O), 1719 (C=O), 1523 (NO₂), 1250 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.17 (d, J = 3.9 Hz, 1H, H-3'), 4.37 (s, 2H, H-5), 2.96 (s, 3H, H-1''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 169.15 (C-4), 152.37 (C-2), 152.37 (C-2'), 131.58 (C-7), 115.24 (C-4'), 115.21 (C-3'), 49.58 (C-5), 25.14 (C-5'). Purity 97%. HRMS m/z [M+H]⁺: 253.0516 (Calcd for C₉H₈N₄O₅⁺ 253.0494).

4.3.2. (E)-3-Ethyl-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **103**



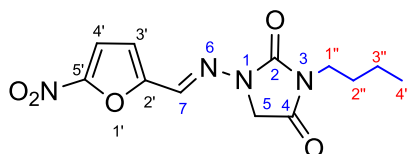
The reaction of NFT with bromoethane yielded **103** as fluffy yellow needles 658 mg (56%), m.p.: 211.3-212.5 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3150 (C-H), 1775 (C=O), 1713 (C=O), 1507 (NO₂), 1248 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.17 (d, J = 3.9 Hz, 1H, H-3'), 4.40 (s, 2H, H-5), 3.51 (q, J = 7.2 Hz, 2H, H-1''), 1.14 (t, J = 7.2 Hz, 3H, H-2''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.63 (C-4), 153.21 (C-2), 152.27 (C-2'), 152.21 (C-5'), 132.02 (C-7), 115.21 (C-3'), 115.21 (C-4'), 48.71 (C-5), 40.40 (C-1''), 13.52 (C-2''). Purity 91%. HRMS m/z [M+H]⁺: 267.0736 (Calcd for C₁₀H₁₀N₄O₅⁺ 267.0651).

4.3.3. (E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-3-propylimidazolidine-2,4-dione, **104**



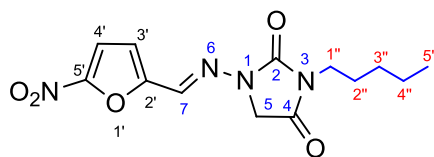
The reaction of NFT with 1-bromopropane yielded **104** as a fine yellow powder 754 mg (60%), m.p.: 192.7-196.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1776 (C=O), 1723 (C=O), 1508 (NO₂), 1254 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-7), 7.80 (d, J = 3.8 Hz, 1H, H-4'), 7.17 (d, J = 3.8 Hz, 1H, H-3'), 4.42 (s, 2H, H-5), 3.44 (dt, J = 15.6, 5.4 Hz, 2H, H-1''), 1.63 – 1.53 (m, 2H, H-2''), 0.88 (t, J = 6.8 Hz, 3H, H-3''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.90 (C-4), 153.42 (C-2), 152.27 (C-2'), 152.22 (C-5'), 132.01 (C-7), 115.21 (C-4'), 115.18 (C-3'), 48.64 (C-5), 40.41 (C-1''), 21.29 (C-2''), 11.55 (C-3''). Purity 94%. HRMS m/z [M+H]⁺: 281.0897 (Calcd for C₁₁H₁₂N₄O₅⁺ 281.0808).

4.3.4. (E)-3-Butyl-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **105**



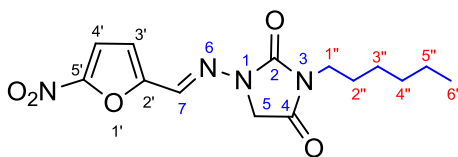
The reaction of NFT with 1-bromobutane yielded **105** as shiny yellow flakes 680 mg (52%), m.p.: 135.9-136.0 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1771 (C=O), 1718 (C=O), 1514 (NO₂), 1254 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, J = 3.6 Hz, 1H, H-4'), 7.16 (d, J = 3.6 Hz, 1H, H-3'), 4.40 (s, 2H, H-5), 4.05 – 3.99 (m, 2H, H-1''), 1.94 – 1.62 (m, 2H, H-2''), 1.36 – 1.34 (m, 2H, H-3''), 0.84 (t, J = 6.8 Hz, 3H, H-4''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-4), 153.17 (C-2), 152.39 (C-2'), 152.26 (C-5'), 132.02 (C-7), 115.22 (C-3'), 115.13 (C-4'), 49.80 (C-5), 48.28 (C-1''), 26.33 (C-2''), 18.03 (C-3''), 11.44 (C-4''). Purity 89%. HRMS m/z [M+H]⁺: 295.1013 (Calcd for C₁₂H₁₄N₄O₅⁺ 295.0964).

4.3.5. (E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-3-pentylimidazolidine-2,4-dione, **106**



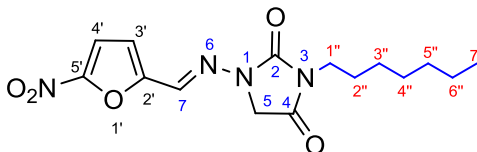
The reaction of NFT with 1-bromopentane yielded **106** as a dark yellow fine powder 891 mg (64%), m.p.: 146.5-147.9 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1775 (C=O), 1703 (C=O), 1529 (NO₂), 1250 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.17 (d, J = 3.9 Hz, 1H, H-3'), 4.41 (s, 2H, H-5), 3.64 – 3.48 (m, 2H, H-1''), 1.62 – 1.50 (m, 2H, H-2''), 1.33 – 1.23 (m, 4H, H-3'', -4''), 0.87 (t, J = 6.6 Hz, 3H, H-5''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-4), 153.40 (C-2), 152.27 (C-2'), 152.22 (C-5'), 132.01 (C-7), 115.20 (C-4'), 115.18 (C-3'), 48.64 (C-5), 40.41 (C-1''), 28.69 (C-2''), 27.54 (C-3''), 22.16 (C-4''), 14.29 (C-5''). Purity 97%. HRMS m/z [M+H]⁺: 309.1173 (Calcd for C₁₃H₁₆N₄O₅⁺ 309.1121).

4.3.6. (E)-3-Hexyl-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, 107



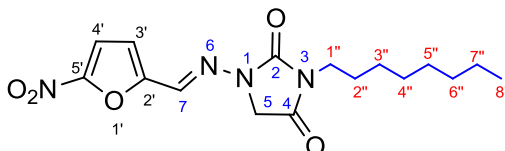
The reaction of NFT with 1-bromohexane yielded **107** as a pale yellow powder 855 mg (61%), m.p.: 152.9-163.5 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1769 (C=O), 1711 (C=O), 1520 (NO₂), 1250 (C-N). ¹H NMR (600 MHz, DMSO) δ (p pm): 7.86 (s, 1H, H-7), 7.80 (d, J = 3.8 Hz, 1H, H-4'), 7.17 (d, J = 3.8 Hz, 1H, H-3'), 4.41 (s, 2H, H-5), 3.67 – 3.45 (m, 2H, H-1''), 1.57 – 1.49 (m, 2H, H-2''), 1.31 – 1.22 (m, 6H, H-3'', -4'', -5''), 0.87 (t, J = 6.8 Hz, 3H, H-6''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-4), 153.39 (C-2), 152.27 (C-2'), 152.22 (C-5'), 132.01 (C-7), 115.20 (C-4'), 115.17 (C-3'), 48.64 (C-5), 40.14 (C-1''), 31.27 (C-4''), 27.83 (C-2''), 26.21 (C-3''), 22.46 (C-5''), 14.38 (C-6''). Purity 95%. HRMS m/z [M+H]⁺: 323.1373 (Calcd for C₁₄H₁₈N₄O₅⁺ 323.1278).

4.3.7. (E)-3-Heptyl-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, 108



The reaction of NFT with 1-bromoheptane yielded **108** a pale yellow powder 869 mg (58%), m.p.: 152.0-154.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1770 (C=O), 1708 (C=O), 1526 (NO₂), 1250 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.17 (d, J = 3.9 Hz, 1H, H-3'), 4.41 (s, 2H, H-5), 3.65 – 3.52 (m, 2H, H-1''), 1.63 – 1.50 (m, 2H, H-2''), 1.31 – 1.25 (m, 6H, H-3'', ..., -6''), 0.86 (t, J = 7.1 Hz, 3H, H-7''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-4), 153.39 (C-2), 152.27 (C-2'), 152.23 (C-5'), 132.01 (C-7), 115.20 (C-4'), 115.17 (C-3'), 48.64 (C-5), 40.28 (C-1''), 31.64 (C-5''), 28.72 (C-4''), 27.87 (C-2''), 26.51 (C-3''), 22.52 (C-6''), 14.43 (C-7''). Purity 97%. HRMS m/z [M+H]⁺: 337.1506 (Calcd for C₁₅H₂₀N₄O₅⁺ 337.1434).

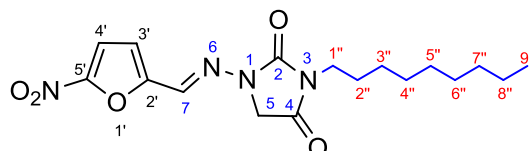
4.3.8. (E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-3-octylimidazolidine-2,4-dione, 109



The reaction of NFT with 1-bromooctane yielded **109** as a yellow powder 989 mg (66%), m.p.: 151.2-158.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1775 (C=O), 1702 (C=O), 1501 (NO₂), 1250 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.17 (d,

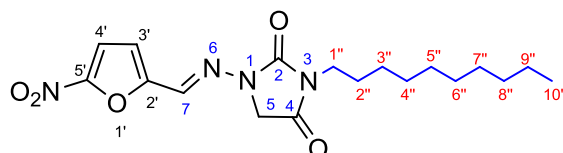
$J = 3.9$ Hz, 1H, H-3'), 4.41 (s, 2H, H-5), 3.67 – 3.45 (m, 2H, H-1''), 1.59 – 1.49 (m, 2H, H-2''), 1.30 – 1.21 (m, 10H, H-3'', ... -7''), 0.86 (t, $J = 6.8$ Hz, 3H, H-8''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-4), 153.40 (C-2), 152.27 (C-2'), 152.22 (C-5'), 132.00 (C-7), 115.20 (C-4'), 48.63 (C-5), 29.05 (C-2''), 31.70 (C-6''), 29.02 (C-5''), 27.85 (C-4''), 26.55 (C-3''), 22.57 (C-7''), 14.43 (C-8''). Purity 94%. HRMS m/z $[\text{M}+\text{H}]^+$: 351.1653 (Calcd for $\text{C}_{16}\text{H}_{22}\text{N}_4\text{O}_5^+$ 351.1590).

4.3.9. (E)-1-[(5-Nitrofuran-2-yl)methylene]amino}-3-nonylimidazolidine-2,4-dione, **110**



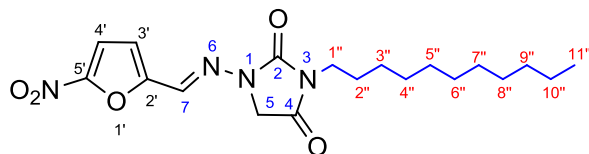
The reaction of NFT with 1-bromononane yielded **110** as a pale yellow powder 878 mg (55%), m.p.: 151.2-153.0 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1775 (C=O), 1704 (C=O), 1501 (NO_2), 1252 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, $J = 3.9$ Hz, 1H, H-4'), 7.17 (d, $J = 3.9$ Hz, 1H, H-3'), 4.41 (s, 2H, H-5), 3.67 – 3.45 (m, , 2H, H-1''), 1.61 – 1.49 (m, 2H, H-2''), 1.30 – 1.21 (m, 12H, H-3'', ...-8''), 0.86 (t, $J = 6.6$ Hz, 3H, H-9''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-4), 153.39 (C-2), 152.27 (C-2'), 152.23 (C-5'), 132.01 (C-7), 115.20 (C-4'), 115.16 (C-3'), 48.64 (C-5), 40.28 (C-1''), 31.76 (C-7''), 29.36 (C-2''), 29.13 (C-4''), 29.07 (C-6''), 26.54 (C-3''), 22.57 (C-8''), 14.44 (C-9''). Purity 89%. HRMS m/z $[\text{M}+\text{H}]^+$: 365.1848 (Calcd for $\text{C}_{17}\text{H}_{24}\text{N}_4\text{O}_5^+$ 365.1747).

4.3.10. (E)-3-Decyl-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-2,4-dione, **111**



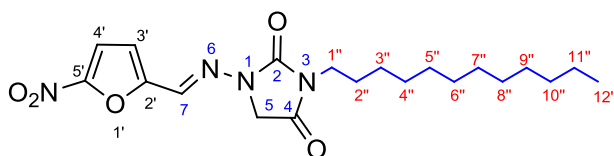
The reaction of NFT with 1-bromodecane yielded **111** as a fine dark yellow powder 992 mg (58%), m.p.: 149.6-150.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1776 (C=O), 1703 (C=O), 1502 (NO_2), 1252 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, $J = 3.9$ Hz, 1H, H-4'), 7.17 (d, $J = 3.9$ Hz, 1H, H-3'), 4.41 (s, 2H, H-5), 3.66 – 3.45 (m, 2H, H-1''), 1.59 – 1.49 (m, 2H, H-2''), 1.30 – 1.22 (m, 14H, H-3'',...H-9''), 0.86 (t, $J = 6.7$ Hz, 3H, H-10''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.83 (C-4), 153.39 (C-2), 152.27 (C-2'), 152.23 (C-5'), 132.01 (C-7), 115.20 (C-4'), 115.17 (C-3'), 48.64 (C-5), 39.86 (C-1''), 29.43 (C-2''), 26.54 (C-3''), 29.40 (C-5''), 29.19 (C-6''), 29.06 (C-4'', -7''), 31.77 (C-8''), 22.59 (C-9''), 14.44 (C-10''). Purity 88%. HRMS m/z $[\text{M}+\text{H}]^+$: 379.1946 (Calcd for $\text{C}_{18}\text{H}_{26}\text{N}_4\text{O}_5^+$ 379.1903).

4.3.11. (E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-3-undecylimidazolidine-2,4-dione, **112**



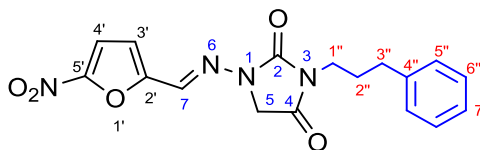
The reaction of NFT with 1-bromoundecane yielded **112** as a yellow powder 896 mg (53%). m.p.: 151.1-151.7 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1775 (C=O), 1703 (C=O), 1501 (NO₂), 1253 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.17 (d, J = 3.9 Hz, 1H, H-3'), 4.41 (s, 2H, H-5), 3.53 - 3.45 (m, 2H, 1''), 1.63 – 1.48 (m, 2H, H-2''), 1.28 – 1.19 (m, 16H, H-3'', ...H-10''), 0.85 (t, J = 6.8 Hz, 3H, H-11''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.83 (C-4), 153.39 (C-2), 152.27 (C-2'), 152.23 (C-5'), 132.00 (C-7), 115.20 (C-4'), 115.17 (C-3'), 48.64 (C-5), 39.85 (C-1''), 31.79 (C-9''), 29.49 (C-2''), 29.40 (C-5'', -6''), 29.20 (C-4'', -8''), 29.07 (C-7''), 26.55 (C-3''), 22.59 (C-10''), 14.44 (C-11''). Purity 92%. HRMS m/z [M+H]⁺: 393.2127 (Calcd for C₁₉H₂₈N₄O₅⁺ 393.2060).

4.3.12. (E)-3-Dodecyl-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **113**



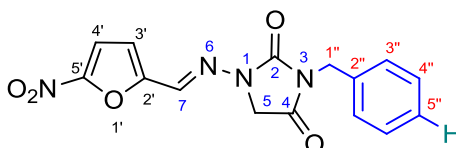
The reaction of NFT with 1-bromododecane yielded **113** as a fine yellow powder 992 mg (55%), m.p.: 209.5-216.9 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1776 (C=O), 1703 (C=O), 1503 (NO₂), 1254 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.17 (d, J = 3.9 Hz, 1H, H-3'), 4.41 (s, 2H, H-5), 3.53 - 3.45 (m, 2H, H-1''), 1.58 – 1.49 (m, 2H, H-2''), 1.29 – 1.19 (m, 18H, H-3'', ...H-11''), 0.85 (t, J = 6.7 Hz, 3H, H-12''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-4), 153.39 (C-2'), 152.23 (C-5'), 132.01 (C-7), 115.19 (C-4', -3'), 48.64 (C-5), C-1'' (39.71), C-10'' (31.79), 29.54 (C- 5'', -7''), 29.50 (C- 6''), 29.47 (C- 8''), 29.40 (C-4'', -9''), 29.21 (C-3''), 22.59 (C-11''), 14.44 (C-12''). Purity 91%. HRMS m/z [M+H]⁺: 407.2313 (Calcd for C₂₀H₃₀N₄O₅⁺ 407.2216).

4.3.13. (E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-3-(3-phenylpropyl)imidazolidine-2,4-dione, **114**



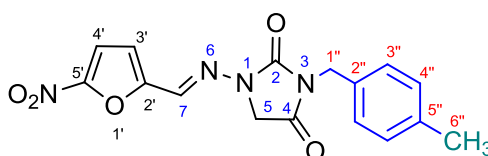
The reaction of NFT with (3-bromopropyl)benzene yielded **114** as shiny brownish yellow needles 993 mg (63%), m.p.: 194.7-195.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1770 (C=O), 1708 (C=O), 1518 (NO₂), 1247 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.29 (t, J = 7.5 Hz, 2H, H-6''), 7.23 (d, J = 7.0 Hz, 2H, H-5''), 7.19 (d, J = 7.3 Hz, 1H, H-7''), 4.37 (s, 2H, H-5), 3.64 - 3.36 (m, 2H, H-1''), 2.61 (t, J = 7.9 Hz, 2H, H-3''), 1.96 - 1.82 (m, 2H, H-2''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.88 (C-4), 153.39 (C-2), 152.28 (C-2'), 152.23 (C-5'), 141.68 (C-4'), 131.98 (C-7), 128.78 (C-6''), 128.73 (C-5''), 126.34 (C-7''), 115.21 (C-3'), 115.15 (C-4'), 48.68 (C-5), 40.41 (C-1''), 32.72 (C-3''), 29.48 (C-2''). Purity 96%. HRMS m/z [M+H]⁺: 356.1189 (Calcd for C₁₇H₁₆N₄O₅⁺ 356.1121).

4.3.14. (E)-3-Benzyl-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **115**



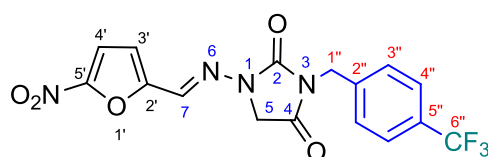
The reaction of NFT with benzyl bromide yielded ester **115** as a yellow powder 978 mg (67%), m.p.: 199.8-204.1°C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1776 (C=O), 1711 (C=O), 1510 (NO₂), 1247 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.89 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.37 - 7.33 (m, 4H, H-3'', -4''), 7.31 (t, J = 6.0, 2.6 Hz, 1H, H-5''), 7.18 (d, J = 3.9 Hz, 1H, H-3'), 4.68 (s, 2H, H-1''), 4.51 (s, 2H, H-5). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.79 (C-4), 153.24 (C-2), 152.31 (C-2'), 152.12 (C-5'), 136.43 (C-2''), 132.33 (C-7), 128.99 (C-4''), 128.04 (C-3''), 115.33 (C-3'), 115.18 (C-4'), 48.89 (C-5), 42.19 (C-1''). Purity 92%. HRMS m/z [M+H]⁺: 329.0887 (Calcd for C₁₅H₁₂N₄O₅⁺ 329.0808).

4.3.15. (E)-3-(p-Methylbenzyl)-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **116**



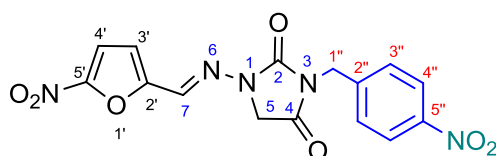
The reaction of NFT with 4-methyl benzyl bromide yielded **116** as fine yellow powder 977 mg (65%), m.p.: 240.8-241.4 °C. IR (ATR) $\nu_{\max}/\text{cm}^{-1}$: 1781 (C=O), 1710 (C=O), 1521 (NO₂), 1245 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.88 (s, 1H, H-7), 7.79 (d, J = 3.8 Hz, 1H, H-4'), 7.22 (d, J = 8.0 Hz, 2H, H-3''), 7.18 (d, J = 3.9 Hz, 1H, H-3'), 7.15 (d, J = 8.0 Hz, 2H, H-4''), 4.62 (s, 2H, H-1''), 4.35 (s, 2H, H-5), 2.28 (s, 3H, H-6''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.72 (C-4), 153.74 (C-2), 152.37 (C-2'), 152.21 (C-5'), 137.29 (C-5'), 132.29 (C-2''), 131.57 (C-7), 129.51 (C-3''), 128.08 (C-4''), 48.83 (C-5), 40.39 (C-1''), 21.15 (C-6''). Purity 97%. HRMS m/z [M+H]⁺: 343.1012 (Calcd for C₁₆H₁₄N₄O₅⁺ 343.0964).

4.3.16. (E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-3-[p-(trifluoromethyl)benzyl]imidazolidine-2,4-dione, **117**



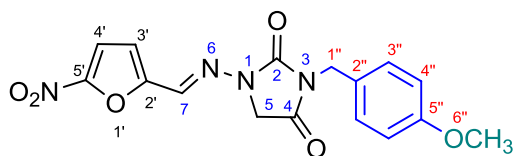
The reaction of NFT with 4-trifluoromethyl benzyl bromide yielded **117** as a fine yellow powder 988 mg (57%), m.p.: 211.6-213.6 °C. IR (ATR) $\nu_{\max}/\text{cm}^{-1}$: 1786 (C=O), 1718 (C=O), 1524 (NO₂), 1254 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.91 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.73 (d, J = 8.1 Hz, 2H, H-4''), 7.58 (d, J = 8.1 Hz, 2H, H-3''), 7.19 (d, J = 3.9 Hz, 1H, H-3'), 4.78 (s, 2H, H-1''), 4.51 (s, 2H, H-5). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.83 (C-4), 153.17 (C-2), 152.33 (C-2'), 152.08 (C-5'), 141.14 (C-2''), 132.44 (C-6''), 125.85 (C-4''), 125.88 (C-3''), 128.82 (C-5''), 115.34 (C-3'), 115.17 (C-4'), 49.03 (C-5), 41.79 (C-1''). Purity 89% HRMS m/z [M+H]⁺: 397.0712 (Calcd for C₁₆H₁₁F₃N₄O₅⁺ 397.0681).

4.3.17. (E)-3-(p-Nitrobenzyl)-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **118**



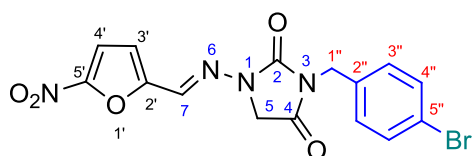
The reaction of NFT with 4-nitrobenzyl bromide yielded **118** as a fluffy yellow powder 857 mg (52%), m.p.: 216.9-218.2 °C. IR (ATR) $\nu_{\max}/\text{cm}^{-1}$: 1779 (C=O), 1721 (C=O), 1520 (NO₂), 1246 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.22 (d, J = 8.8 Hz, 2H, H-4''), 7.92 (s, 1H, H-7), 7.80 (d, J = 3.9 Hz, 1H, H-4'), 7.64 (d, J = 8.8 Hz, 2H, H-3''), 7.19 (d, J = 3.9 Hz, 1H, H-3'), 4.83 (s, 2H, H-1''), 4.52 (s, 2H, H-5). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-4), 153.13 (C-2), 152.33 (C-2'), 152.06 (C-5'), 147.41 (C-2''), 144.05 (C-5''), 132.50 (C-7), 129.11 (C-4''), 124.10 (C-3''), 115.39 (C-3'), 114.17 (C-4'), 49.08 (C-5), 41.68 (C-1''). Purity 84%. HRMS m/z [M+H]⁺: 374.0769 (Calcd for C₁₅H₁₁N₅O₇⁺ 374.0658)

4.3.18. (E)-3-(*p*-Methoxybenzyl)-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **119**



The reaction of NFT with 4-methoxy benzyl bromide yielded **119** as a yellow powder 871 mg (55%), m.p.: 197.2-219.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1782 (C=O), 1726 (C=O), 1604, 1512 (NO₂), 1243 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-7), 7.80 (d, *J* = 3.9 Hz, 1H, H-4'), 7.28 (d, *J* = 8.7 Hz, 2H, H-3''), 7.18 (d, *J* = 3.9 Hz, 1H, H-3'), 6.91 (d, *J* = 8.7 Hz, 2H, H-4''), 4.60 (s, 2H, H-5), 4.48 (s, 2H, H-1''), 3.73 (s, 3H, H-6''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.69 (C-4), 159.24 (C-2), 153.23 (C-5''), 152.30 (C-2'), 152.12 (C-5'), 132.27 (C-7), 129.72 (C-2''), 128.46 (C-1''), 115.31 (C-4'), 115.18 (C-3'), 114.35 (C-3''), 55.60 (C-6''), 48.82 (C-5), 41.73 (C-1'). Purity 89%. HRMS *m/z* [M+H]⁺: 359.0950 (Calcd for C₁₆H₁₄N₄O₆⁺ 359.0913).

4.3.19. (E)-3-(*p*-Bromobenzyl)-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, **120**



The reaction of NFT with 4-bromobenzyl bromide yielded **120** as a fine yellow powder, 981 mg (55%), m.p.: 269.4-269.5 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 1775 (C=O), 1703 (C=O), 1501 (NO₂), 1258 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.90 (s, 1H, H-7), 7.80 (d, *J* = 3.9 Hz, 1H, H-4'), 7.55 (d, *J* = 8.5 Hz, 2H, H-4''), 7.31 (d, *J* = 8.5 Hz, 2H, H-3''), 7.18 (d, *J* = 3.9 Hz, 1H, H-3'), 4.65 (s, 2H, H-1''), 4.49 (s, 2H, H-5). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.77 (C-4), 153.16 (C-2), 152.32 (C-2'), 152.09 (C-5'), 135.85 (C-2''), 132.38 (C-7), 131.86 (C-4''), 130.35 (C-3''), 121.22 (C-5''), 115.34 (C-3'), 115.17 (C-4'), 48.96 (C-5), C-1'' (41.61). Purity 87%. HRMS *m/z* [M+H]⁺: 406.9974 (Calcd for C₁₅H₁₁BrN₄O₅⁺ 406.9913)

4.4. In vitro biological activity evaluation

4.4.1. Anti-mycobacterial assays

Anti-mycobacterial assessment of compounds was carried out in two different media namely 7H9 ADC Glucose (Glu) Tween80 (Tw) and 7H9 Casitone Txloxapol (Tx) using previously described methods [53, 54]. 7H9 ADC Glu Tw medium comprises the standard 7H9 broth base supplemented with albumin-dextrose-catalase (ADC) and glucose, with Tween80 as

surfactant. 7H9 CAS Tx, on the other hand, contains the same 7H9 base, supplemented with casitone and tyloxapol as surfactant. ADC contains albumin, which can provide some indication of protein binding.

4.4.2. Cytotoxicity assays

Cytotoxicity of the synthesised compounds was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on two non-cancerous cell lines. The colorimetric MTT assay was developed by Mossman [55] to measure cytotoxicity and cell proliferation. Yellow MTT is reduced by viable cell mitochondria to form purple, insoluble formazan crystals. The cells and crystals are solubilised with organic solvents (e.g. isopropanol) and the solubilized formazan's absorbance measured spectrophotometrically in order to determine cell viability.

CHO cells (passage 10 – 23) were cultured in a 1:1 mixture of Hyclone Dulbecco's modified Eagle's medium (DMEM) with high glucose and Hyclone Ham's F12 growth medium (Kaighn's modification) and supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin mixture and amphotericin B, and 0.5% non-essential amino acids. HEK-293 cells (passage 24 – 32) were cultured in DMEM 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% CO₂. For the MTT assay, 96 well plates were prepared with 200 µL of cell suspension (25 000 cells/mL for both cell lines) and incubated for 24 hours. The growth media was aspirated and 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 48 hours.

To initiate the MTT assay, 20 µL of sterile-filtered MTT solution (1 mg/mL in PBS) was added to each well 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:

$$\text{Cell viability \%} = \frac{\Delta \text{ Abs sample} - \Delta \text{ Abs blank}}{\Delta \text{ Abs neg control} - \Delta \text{ Abs blank}} \times 100$$

The IC₅₀ and Z-score were determined for each compound's biological replicate using GraphPad Prism 5. For the final IC₅₀ of each compound, the mean IC₅₀ of the biological replicates were calculated and the mean dose-response curve, with standard error of the mean (SEM), prepared in GraphPad Prism 5.

Author contributions

DDN was responsible for the conceptualisation and design of the study. NHZ synthesised the compounds, analysed and interpreted the chemistry data and drafted the manuscript. JA performed the cytotoxicity experiments, analysed and interpreted cytotoxicity data. RS, AJ and DFW performed the antimycobacterial evaluation experiments. DFW, DDN, and NHZ analysed and interpreted the antimycobacterial data. DDN, and NHZ drafted the manuscript, developed the figures and tables. All authors were involved in revising and approved the final version of the manuscript.

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Disclaimer

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

Appendix A. Supplementary data

Supplementary data to this article can be found online <https://doi.org/????>

Abbreviations

BSL-III	biosafety level III
CHO	Chinese hamster ovarian
DR-TB	drug-resistant tuberculosis
EDG	electron donating groups

EM	emetine
EWG	electron withdrawing groups
GR	glutathione disulfide reductase
GSH	glutathione
GSSG	glutathione disulphide
HEK-293	human embryonic kidney
IC ₅₀	concentration of compound inhibiting 50%
INH	isoniazid
MIC ₉₀	minimum concentration of compound inhibiting 90%
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
NFT	Nitrofurantoin
NFs	5-nitrofurans
NO ₂	nitro group
RIF	rifampicin
ROS	reactive oxygen species
SI	selectivity index
TB	tuberculosis
UTIs	urinary tract infections

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CHAPTER 5

CHEMISTRY AND SYNTHESIS

5.1. Introduction

Traditional drug development is an expensive and laborious task. The process from drug design to clinical drug can take an estimated 13.5 years (Guha, 2015). Furthermore, the process involves building a library from a variety of new chemical entities. An alternative to this costly and lengthy process is drug repurposing, which is the repositioning of a clinically approved drug for the treatment of other diseases, besides the drug's originally intended disease (Shim & Liu, 2014). The drug may be repurposed as is or it can be modified either by molecular derivatisation or hybridisation. The former involves exploitation of structure-activity relationships (SAR) of clinical drugs to deliver analogues with improved therapeutic profiles (Guha, 2013; Hu *et al.*, 2017). The latter is the assemblage of two or more different drugs into a single chemical entity by chemical reactions, (Hulsman *et al.*, 2007). The advantage of drug repurposing is that properties such as pharmacokinetic and safety profiles of the drug of interest are already known, consequently, shortening the drug development process (Charlton *et al.*, 2018).

This chapter describes and discusses the synthesis of hybrids by molecular hybridisation of nitrofurantoin (NFT, Figure 5.1) and triazole (Tz, Figure 5.2) using click chemistry. NFT (in depth discussion in Chapters 2 - section 2.5 and 3 - section 2.1) is an inexpensive drug that possesses activity against aerobic and anaerobic pathogens (Miller *et al.*, 2002; Wang *et al.*, 2008). Triazoles (Chapter 2 section 2.5) are the building blocks for different anti-infective drugs; hence they are often used in molecular hybridisation drug design. Poor solubility and the fact that at least a third of the drug is excreted unchanged (Bains *et al.*, 2009) motivated the synthesis of NFT analogues (Chapter 4) and NFT-Tz hybrids with the intention of improving NFT shortfalls and boosting overall anti-infective activity.

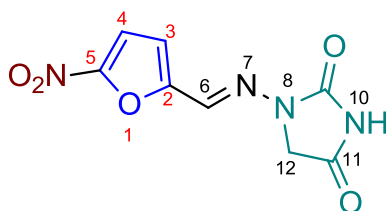


Figure 5.1: Chemical structure of nitrofurantoin (NFT)

The compounds synthesised in the study are for the potential use in the treatment of diseases that affect mainly poor and resource limited populations. Therefore, the main considerations were efficient synthesis, i.e. (i) simple synthesis (few steps, preferably less than five); (ii) minimal and simple purification (hence no chromatography, only recrystallisation - using green solvents, such as ethanol and water); and (iii) provide good yields (~70%). Consequently, molecular hybridisation was accomplished using click chemistry and Huisgen cycloaddition, in the synthesis of NFT-Tz hybrids (Series 2 to Series 4).

Click chemistry entails stereo-selective, regio- and stereo-specific reactions performed under operationally simple and mild reaction conditions (Heravi *et al.*, 2016; Kolb *et al.*, 2001). The success of copper (I)-catalysed (Cu(I)-catalysed) Huisgen type cycloaddition has led to its classification as click chemistry, since the reaction: (i) is simple to perform; (ii) is often stereo-specific; (iii) can be easily conducted in a variety of solvents, including water; (iv) generally, does not require chromatography purification (Das *et al.*, 2014). Furthermore, pure products can be isolated by simple filtration or extraction and simple recrystallisation (Das *et al.*, 2014; Heravi *et al.*, 2016). The 1,2,3-triazole cyclopentyl ring (Figure 5.2) was formed by the Cu(I)-catalysed exergonic fusion or the Cu-catalysed azide-alkyne cycloaddition (CuAAC) of a terminal azide with a terminal alkyne (Rostovtsev *et al.*, 2002). The process was born from the Huisgen 1,3-dipolar cycloaddition (Figure 5.2), owing to the contribution of Huisgen and his assistant in 1960 (Heravi *et al.*, 2016).

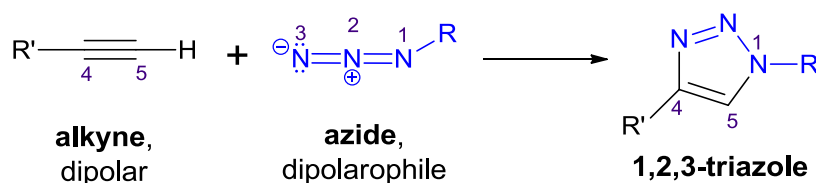
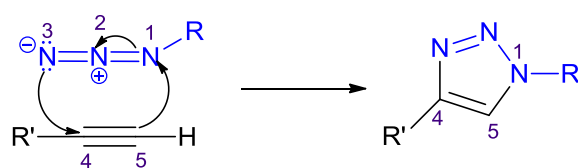
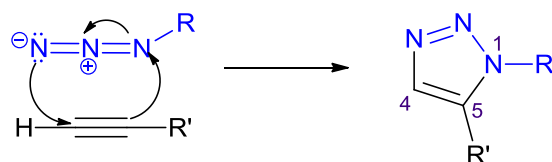


Figure 5.2: The 1,3 Huisgen 1,3-dipolar cycloaddition reaction

Essentially, two π -electrons of the dipolarophile (e.g. azide) and four electrons of the dipolar (e.g. alkyne) compound engage in a $[2_s+4_s]$ suprafacial cycloaddition (Figure 5.3) which requires compatibility of HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) of both participating compounds (Das *et al.*, 2014; Heravi *et al.*, 2016). Since, HOMO electrons are weakly held and require less activation energy, they are readily donated (Clayden *et al.*, 2012). Furthermore, electronic and steric effects play a major role in regio-selectivity of the reaction (Das *et al.*, 2014).



A: Formation of 1,4-regioisomer



B: Formation of 1,5-regioisomer

Figure 5.3: Mechanism of the Huisgen 1,3-dipolar cycloaddition reaction

The classic Huisgen 1,3-dipolar cycloaddition reaction often results in a mixture of regioisomers. The Cu-catalysed reaction allows for the synthesis of the 1,4-disubstituted regioisomers specifically. The regio-selectivity of the reaction depends on electronic and steric effects and is somewhat predictable hence the highly regio-selective Cu(I)-catalysed alkyne-azide, 1,3-dipolar cycloaddition (CuAAC) reaction has been broadly and widely studied in the recent past (Das *et al.*, 2014; Heravi *et al.*, 2016). The 1,3-dipolar Cu(I)-catalysed cycloaddition reaction of an azide source and the alkyne is a significant strategy for the regio- and stereo-selective synthesis of 1,2,3-triazoles (Heravi *et al.*, 2016; Kolb *et al.*, 2001). The active Cu(I) catalyst can be generated from Cu(I) salts or Cu(II) salts using sodium ascorbate as the reducing agent. Addition of a slight excess of sodium ascorbate prevents the formation of oxidative homo-coupling products (Das *et al.*, 2014).

The combination of NFT and Tz for hybrid synthesis should result in hybrids with favourable pharmacokinetic properties and ultimately synergistically amplify each other's potency against targeted pathogens. The synthesised hybrids were divided into three NFT-Tz series namely Series 2 (single carbon linker, 1C), Series 3 (extended *n*-alkyl linker) and Series 4 (aryl linker). Series 3 was further subdivided into 3.3 (three carbon linker, 3C), 3.5 (five carbon linker, 5C) and 3.6 (six carbon linker, 6C).

5.2. Materials and methods

5.2.1. Materials

Nitrofurantoin was purchased from Changzhou Kaixuan Chemical Co (Chunjiang, China). Anhydrous dimethyl formamide (DMF), trimethylamine (TEA), benzyl bromide, ethyl bromide, 4-bromobenzyl bromide, 1-decyne, 1-octyne, 1-heptyne, 1-hexyne, 1-Ethynyl-4-fluorobenzene, 1-ethynyl-4-methoxybenzene, 1-ethynyl-3,5-bis(trifluoromethyl)benzene, 1-ethynyl-4-pentylbenzene, phenyl propargyl sulphide, 1,3-dibromopropane, 1,5-dibromopentane, 1,6-dibromohexane, sodium azide, sodium ascorbate, and copper (II) sulphate were purchased from Sigma–Aldrich (Johannesburg, South Africa). Dichloromethane (DCM), methanol (MeOH), ethanol (EtOH) and ethyl acetate (EtOAc) were purchased from ACE chemicals (Johannesburg, South Africa). All the chemicals and reagents were of reagent grade and were used without further purification.

5.2.2. General procedures

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance™ III 600 spectrometer at a frequency of 600 MHz and 150.913 MHz, respectively, in $\text{DMSO}-d_6$. 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 nebuliser set at 1.6 Bar and 0.4 Bar, respectively, and a collision cell RF voltage of 100 Vpp.

Mass spectra (MS) were recorded in positive mode on a Thermo Electron LXQ™ ion trap mass spectrometer, equipped with Xcalibur 2.2 data acquisition and analysis software. The MS had an APCI source set at 300 °C, and was direct infusion with a Harvard syringe pump utilised at a flow rate of 10 $\mu\text{L}/\text{min}$. A full scan from 100 - 1200 amu was achieved in 1 s, with a capillary voltage of 7 V, while the corona discharge was 10 μA .

Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument. Thin layer chromatography (TLC) was performed, using silica gel plates (60F₂₅₄), obtained from Merck (Johannesburg, South Africa).

HPLC analyses was conducted using an Agilent 1100 series instrument equipped with a gradient pump, autosampler, diode Array UV detector. OpenLab CDS Chemstation Rev.C.01.07 SR3 data acquisition and analysis software were used (Agilent Technologies, Palo Alto, CA, USA). The column was a Venusil XBP C18 (2) -column, 150 x 4.6 mm, 5 µm spherical particles, 100 Å pore size (Agela Technologies, Newark, DE, USA), The mobile phase consisted of acetonitrile and 0.1% orthophosphoric acid in water with a linear gradient from 30% acetonitrile to 85% after five minutes and holding until 15 minutes before equilibrating with 30% acetonitrile to 20 minutes. The flow rate was set at 1 mL/min and the injection volume was 10 µL. The UV signal was monitored at 210 nm.

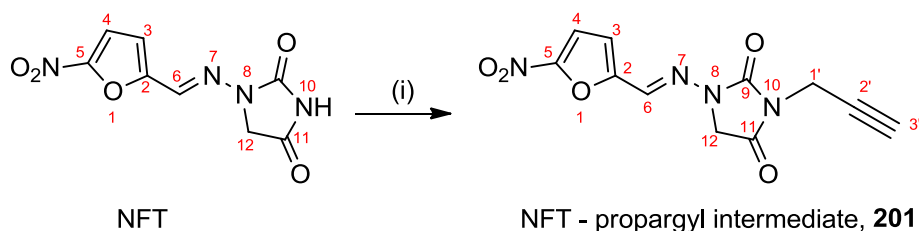
5.2.3. Syntheses

5.2.3.1. Synthesis of Series 2 hybrids

The synthesis of Series 2 occurred in two steps, namely (i) the propargylation of NFT to the propargylated intermediate **201** (Scheme 5.1), and (ii) the synthesis of the hybrids from **201** using click chemistry.

Synthesis of propargylated NFT intermediate, **201**

NFT (4.20 mmol, 1.0 g) was dissolved in anhydrous DMF (10 mL), after which TEA (4.41 mmol, 1.5 eq.) was added. The solution was stirred at room temperature for 15 min. To the resulting yellow solution, propargyl bromide (4.41 mmol, 1.5 eq) was added. The reaction was stirred at room temperature for 24 h and monitored by TLC, eluting with DCM:MeOH (19:1, v/v).



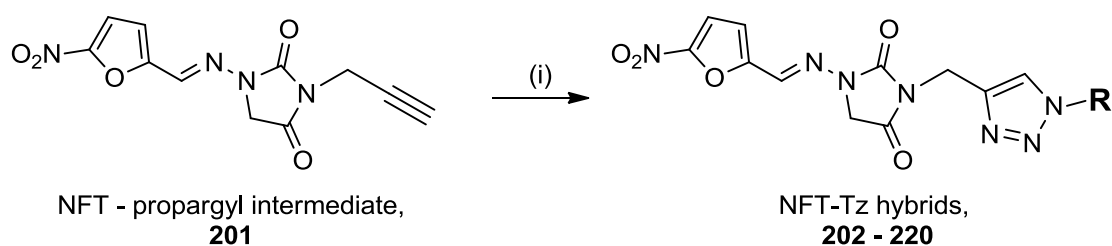
Scheme 5.1: Single step synthesis of NFT-propargyl intermediate, **201**.

Reagents and conditions: (i) propargyl bromide (1.5 eq), TEA (1.5 eq), anhydrous DMF, rt, 24 hr

Upon completion, the reaction was quenched with 20 mL water and extracted with ethyl acetate (3 X 20 mL). The organic layer was washed once with saturated NH_4Cl (20mL) and once with water. The solution was concentrated *in vacuo*. The resulting paste was purified by recrystallisation in ethanol/water to afford the NFT-propargyl intermediate, **201** as a yellow powder, 1.06 g (91%). m.p.: 136.1-136.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 2138 ($\text{C}\equiv\text{C}$), 1779 ($\text{C}=\text{O}$), 1719 ($\text{C}=\text{O}$), 1523 (NO_2). ^1H NMR (600 MHz, DMSO) δ (ppm): 7.89 (s, 1H, H-6), 7.80 (d, J = 3.8 Hz, 1H, H-4), 7.20 (d, J = 3.8 Hz, 1H, H-3), 4.49 (s, 2H, H-12), 4.29 (d, J = 2.5 Hz, 2H, H-1'), 3.31 (t, J = 2.5 Hz, 1H, H-3'). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 166.89 (C-11), 152.29 (C-9, 2), 151.97 (C-5), 132.64 (C-4), 115.51 (C-3), 115.14 (C-6), 78.12 (C-2'), 74.76 (C-3'), 48.87 (C-5), 28.20 (C-1'). HRMS m/z $[\text{M}+\text{H}]^+$: 277.0547 (Calc. for $\text{C}_{11}\text{H}_8\text{N}_4\text{O}_5^+$ 277.0528).

Synthesis of hybrids, 202 – 220

Intermediate **201** (0.5 g, 1.81 mmol) was dissolved in 10 mL of DMSO:water (4:1, v/v). To the resulting solution, *n*-alkyl or benzyl azide (1.5 eq) synthesised using a previously described method (Cilliers *et al.*, 2019), was added. To the reaction mixture, catalytic amounts of β -cyclodextrin (0.02 eq), sodium ascorbate (0.3 eq) and copper sulphate pentahydrate (0.1 eq) were added in sequence. After 12 h, an additional portion of sodium ascorbate (0.3 eq) was added. The reaction (Scheme 5.2) was stirred at room temperature for 48 hr and monitored by TLC, eluting with DCM:MeOH (19:1, v/v). Upon completion, the reaction was quenched with water (20 mL) and extracted with ethyl acetate (3 X 20 mL). The organic layer was washed once with saturated NH_4Cl (30 mL) and once with water (30 mL). The solution was concentrated *in vacuo* and the resulting paste was purified by recrystallisation in ethanol/water to afford the desired analogue.

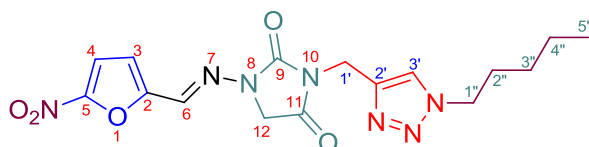


Cpd.	R	Cpd.	R	Cpd.	R	Cpd.	R	Cpd.	R
202	<i>n</i> -C ₅ H ₁₁ --	206	<i>n</i> -C ₉ H ₁₉ --	210		214		218	
203	<i>n</i> -C ₆ H ₁₃ --	207	<i>n</i> -C ₁₀ H ₂₁ --	211		215		219	
204	<i>n</i> -C ₇ H ₁₅ --	208	<i>n</i> -C ₁₁ H ₂₃ --	212		216		220	
205	<i>n</i> -C ₈ H ₁₇ --	209	<i>n</i> -C ₁₂ H ₂₅ --	213		217			

Scheme 5.2: Synthesis of hybrids, **202 - 220**.

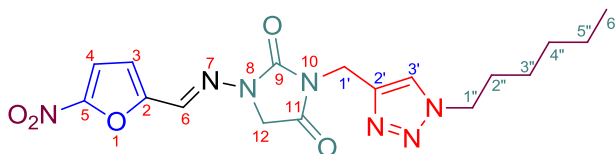
Reagents and conditions: (i) **202 - 209**: *n*-alkyl azide (1.5 eq), **210 - 220**: benzyl azide (1.5 eq), β -cyclodextrin (0.02 eq), sodium ascorbate (0.3 eq) and copper sulphate pentahydrate (0.1 eq), DMSO: water (4:1, v/v), rt, 48 h.

(E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino}-10-[(4-pentyl-1*H*-1,2,3-triazol-1-yl)methyl]imidazolidine-9,11-dione, **202**



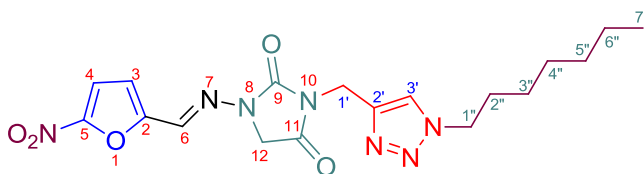
The reaction of **201** with 1-pentyl azide yielded **202** as a fine brownish yellow powder, 351 mg (50%), m.p.: 105-109-6 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3139 (C-H, alkene), 2925 (C-H, alkane), 1785 (C=O), 1712 (C=O), 1344 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.08 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.80 (d, *J* = 3.9 Hz, H-4), 7.18 (d, *J* = 3.9 Hz, 1H, H-3), 4.73 (s, 2H, H-1'), 4.48 (s, 2H, H-12), 4.32 (t, *J* = 7.2 Hz, 2H, H-1''), 1.84 – 1.73 (m, 2H, H-2''), 1.35 – 1.14 (m, 4H, H-3'', -4''), 0.89 – 0.80 (m, 3H, H-5''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.34 (C-11), 152.88 (C-9), 152.31 (C-5), 152.09 (C-2), 132.40 (C-6, 2'), 123.83 (C-3'), 115.31 (C-4), 115.17 (C-3), 49.81 (C-1''), 48.83 (C-1'), 39.72 (C-2''), 39.58 (C-3''), 21.99 (C-4''), 14.26 (C-5''). Purity 92%. HRMS *m/z* [M+H]⁺: 390.1496 (Calc. for C₁₆H₁₉N₇O₅⁺ 390.1481).

(E)-1-[[5-Nitrofuran-2-yl)methylene]amino}-10-[(4-hexyl-1*H*-1,2,3-triazol-1-yl)methyl]imidazolidine-9,11-dione, **203**



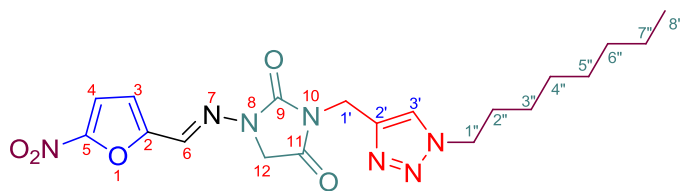
The reaction of **201** with 1-hexyl azide yielded **203** as a dark orange powder, 583 mg (80%), m.p.: 123.8-124.6 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 2918 (C-H, alkane), 2850 (C-H, C-12), 1785 (C=O), 1729 (C=O), 1340 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.08 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.19 (d, $J = 3.9$ Hz, 1H, H-3), 4.71 (t, $J = 6.8$ Hz, 2H, H-1''), 4.48 (s, 2H, H-12), 4.32 (s, 2H, H-1'), 1.77 (m, 2H, H-2''), 1.27 – 1.22 (m, 6H, H-3'...-5''), 0.85 – 0.80 (m, 3H, H-6''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.33 (C-11), 152.88 (C-9), 152.31 (C-5), 152.09 (C-2), 132.40 (C-6, 2'), 123.83 (C-3'), 115.31 (C-4), 115.17 (C-3), 49.81 (C-1''), 48.83 (C-1'), 39.72 (C-2''), 39.58 (C-3''), 25.96 (C-4''), 22.39 (C-5''), 14.32 (C-6''). Purity 91%. HRMS m/z $[\text{M}+\text{H}]^+$: 404.1637 (Calc. for $\text{C}_{16}\text{H}_{19}\text{N}_7\text{O}_5^+$ 404.1638).

(E)-1-[[5-Nitrofuran-2-yl)methylene]amino}-10-[(4-heptyl-1*H*-1,2,3-triazol-1-yl)methyl]imidazolidine-9,11-dione, **204**



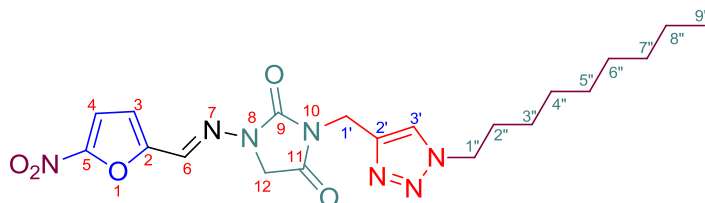
The reaction of **201** with 1-heptyl azide yielded **204** as a yellow powder, 558 mg (74%), m.p.: 130.3-132.7 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3138 (C-H, alkene), 2925 (C-H, alkane), 1784 (C=O), 1712 (C=O), 1344 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.08 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.18 (d, $J = 3.9$ Hz, 1H, H-3), 4.73 (s, 2H, H-1'), 4.48 (s, 2H, H-12), 4.31 (t, $J = 7.2$ Hz, 2H, H-1''), 1.79 – 1.75 (m, 2H, H-2''), 1.26 – 1.21 (m, 8H, H-3'...-6''), 0.98 - 0.85 (m, 3H, H-7''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.33 (C-11), 152.87 (C-9), 152.63 (C-5), 152.09 (C-2), 132.40 (C-6, 2'), 123.83 (C-3'), 115.31 (C-4), 115.17 (C-3), 49.83 (C-1''), 48.83 (C-1'), 39.72 (C-2''), 39.58 (C-3''), 28.50 (C-4''), 26.26 (C-5''), 22.47 (C-6''), 14.32 (C-7''). Purity 91%. HRMS m/z $[\text{M}+\text{H}]^+$: 418.1811 (Calc. for $\text{C}_{18}\text{H}_{23}\text{N}_7\text{O}_5^+$ 418.1794).

(E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-10-[(4-octyl-1*H*-1,2,3-triazol-1-yl)methyl]imidazolidine-9,11-dione, **205**



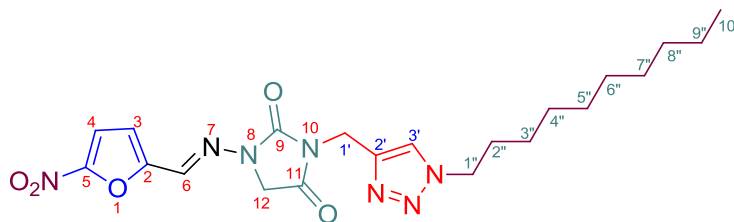
The reaction of **201** with 1-octyl azide yielded **205** as a yellow powder, 598 mg (77%), m.p.: 127.9-130.3 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3137 (C-H, alkene), 2924 (C-H, alkane), 1785 (C=O), 1718 (C=O), 1343 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.08 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.18 (d, $J = 3.9$ Hz, 1H, H-3), 4.73 (s, 2H, H-1'), 4.48 (s, 2H, H-12), 4.31 (t, $J = 7.2$ Hz, 2H, H-1''), 1.79 – 1.75 (m, 2H, H- 2''), 1.26 – 1.21 (m, 10H, H- 3''...-7''), 0.92 -0.84 (m, 3H, H-8''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.33 (C-11), 152.87 (C-9), 152.32 (C-5), 152.09 (C-2), 132.40 (C-6, 2'), 123.83 (C-3'), 115.31 (C-4), 115.17 (C-3), 49.83 (C-1''), 48.83 (C-1'), 39.72 (C-2''), 39.58 (C-3''), 28.50 (C-4''), 28.81 (C-5''), 22.53 (C-7''), 14.42 (C-8''). Purity 92%. HRMS m/z $[\text{M}+\text{H}]^+$: 432.1966 (Calc. for $\text{C}_{19}\text{H}_{25}\text{N}_7\text{O}_5^+$ 432.1951).

(E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-10-[(4-nonyl-1*H*-1,2,3-triazol-1-yl)methyl]imidazolidine-9,11-dione, **206**



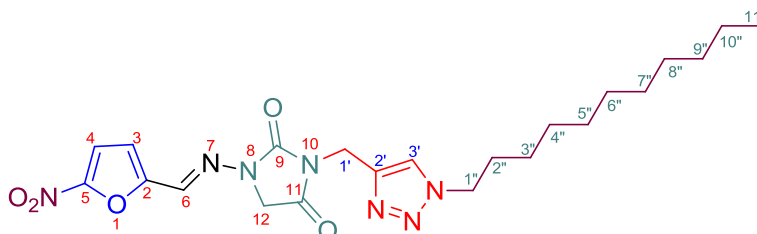
The reaction of **201** with 1-nonyl azide yielded **206** as a dark orange powder, 467 mg (58%), m.p.: 123.8-126.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3142 (C-H, alkene), 2920 (C-H, alkane), 1783 (C=O), 1713 (C=O), 1345 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.08 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.18 (d, $J = 3.9$ Hz, 1H, H-3), 4.73 (s, 2H, H-1'), 4.48 (s, 2H, H-12), 4.31 (t, $J = 7.2$ Hz, 2H, H-1''), 1.79 – 1.75 (m, 2H, H- 2''), 1.26 – 1.21 (m, 12H, H-3''...-8''), 0.91 - 0.85 (m, 3H, H-9''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.32 (C-11), 152.87 (C-9), 152.31 (C-5), 152.09 (C-2), 132.40 (C-6, 2'), 123.83 (C-3'), 115.41 (C-4), 115.17 (C-3), 49.76 (C-1''), 48.62 (C-1'), 31.72 (C-7''), 29.29 (C-5''), 29.07 (C-4''), 28.85 (C-6), 26.30 (C-3''), 22.57 (C-8''), 14.43 (C-9''). Purity 93%. HRMS m/z $[\text{M}+\text{H}]^+$: 445.2131 (Calc. for $\text{C}_{20}\text{H}_{27}\text{N}_7\text{O}_5^+$ 445.2074).

(E)-1-[(5-Nitrofuran-2-yl)methylene]amino}-10-[(4-decyl-1*H*-1,2,3-triazol-1-yl)methyl]imidazolidine-9,11-dione, **207**



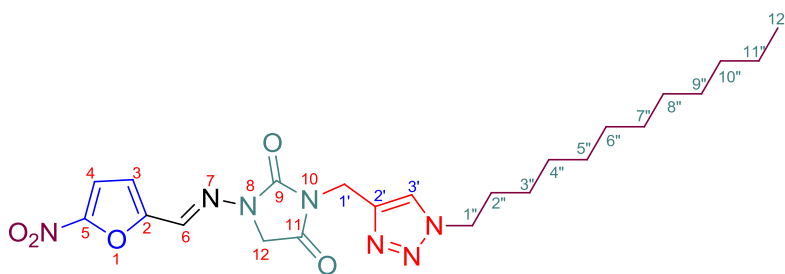
The reaction of **201** with 1-decyl azide yielded **207** as a fine orange powder, 638 mg (77%), m.p.: 148.0-148.8 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 2920 (C-H, alkane), 1785 (C=O), 1713 (C=O), 1344 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.08 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.80 (d, $J = 4.2$ Hz, 1H, H-4), 7.19 (d, $J = 4.2$ Hz, 1H, H-3), 4.73 (s, 2H, H-1'), 4.48 (s, 2H, H-12), 4.31 (t, $J = 7.1$ Hz, 2H, H-1''), 1.87 – 1.74 (m, 2H, H-2''), 1.38 – 1.24 (m, H-3''...9''), 0.98 – 0.84 (m, $J = 7.1$ Hz, 3H, H-10''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.32 (C-11), 152.87 (C-9), 152.09 (C-5), 151.93 (C-2), 132.39 (C-6), 132.12 (C-2'), 123.91 (C-3'), 115.30 (C-4), 115.16 (C-3), 49.52 (C-1''), 48.62 (C-1'), 31.45 (C-7''), 30.25 (C-5''), 26.26 (C-3''), 22.50 (C-9''), 14.51 (C-10''). Purity 91%. HRMS m/z $[\text{M}+\text{H}]^+$: 460.2295 (Calc. for $\text{C}_{21}\text{H}_{29}\text{N}_7\text{O}_5^+$ 460.2264).

(E)-1-[(5-Nitrofuran-2-yl)methylene]amino}-10-[(4-undecyl-1*H*-1,2,3-triazol-1-yl)methyl]imidazolidine-9,11-dione, **208**



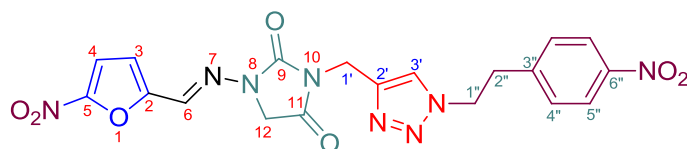
The reaction of **201** with 1-undecyl azide yielded **208** as a dark yellow powder, 604 mg (70%), m.p.: 163.3-163.9 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3145 (C-H, alkene), 2919 (C-H, alkane), 2849 (C-H), 1781 (C=O), 1715 (C=O), 1345 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.08 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.81 (d, $J = 4.1$ Hz, 1H, H-4), 7.19 (d, $J = 4.1$ Hz, 1H, H-3), 4.71 (s, 2H, H-1'), 4.48 (s, 2H, H-12), 4.31 (t, $J = 7.1$ Hz, 2H, H-1''), 1.78 (dt, $J = 14.4, 7.2$ Hz, 2H, H-2''), 1.36 - 1.02 (m, 16H, H-3''...10''), 0.99 - 0.84 (m, 3H, H-11''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.20 (C-11), 152.82 (C-9), 151.92 (C-5), 132.34 (C-6, 2'), 123.70 (C-3'), 115.17 (C-4), 114.73 (C-3), 49.74 (C-1''), 48.62 (C-1', 12), 29.45 (C-7''), 29.43 (C-2''), 29.19 (C-8''), 26.25 (C-3''), 22.58 (C-10''), 14.44 (C-11''). Purity 93%. HRMS m/z $[\text{M}+\text{H}]^+$: 474.2406 (Calc. for $\text{C}_{22}\text{H}_{31}\text{N}_7\text{O}_5^+$ 474.2420).

(E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-10-[(4-dodecyl-1*H*-1,2,3-triazol-1-yl)methyl]imidazolidine-9,11-dione, **209**



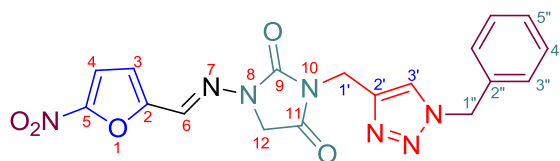
The reaction of **201** with 1-dodecyl azide yielded **209** as an orange yellow powder, 566 mg (64%), m.p.: 163.9-165.8 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3145 (C-H, alkene), 2919 (C-H, alkane), 2849 (C-H, alkane), 1782 (C=O), 1715 (C=O), 1345 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.08 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.18 (d, J = 3.9 Hz, 1H, H-3), 4.73 (s, 2H, H-1'), 4.48 (s, 2H, H-12), 4.31 (t, J = 7.1 Hz, 2H, H-1''), 1.77 (dd, J = 14.4, 7.2 Hz, 2H, H-2''), 1.34 – 1.01 (m, 18H, H-3'...-11''), 0.88 – 0.85 (m, 3H, H-12''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.44 (C-11), 152.83 (C-9), 152.16 (C-5), 151.40 (C-2), 132.57 (C-6), 132.35 (C-2'), 123.69 (C-3'), 115.31 (C-4), 115.17 (C-3), 49.97 (C-1''), 48.61 (C-1'), 40.42 (C-2''), 39.59 (C-3''), 31.78 (C-10''), 30.25 (C-5'...8''), 29.49 (C-4''), 29.43 (C-9''), 26.04 (C-3''), 22.58 (C-11''), 14.44 (C-12''). Purity 91%. HRMS m/z $[\text{M}+\text{H}]^+$: 488.2566 (Calc. for $\text{C}_{23}\text{H}_{33}\text{N}_7\text{O}_5^+$ 488.2577).

(E)-1-[[[(5-Nitrofuran-2-yl)methylene]amino]-10-[(1-(4-nitrophenethyl)-1*H*-1,2,3-triazol-4-yl)methyl]imidazolidine-9,11-dione, **210**



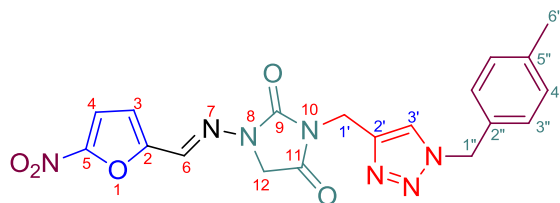
The reaction of **201** with 4-nitrophenethyl azide yielded **210** as a dark orange powder, 334 mg (39%), m.p.: 125.4-126.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3240 (C-H, alkene), 2921 (C-H, alkane), 1783 (C=O), 1726 (C=O), 1339 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.16 (d, J = 8.7 Hz, 2H, H-5''), 8.05 (s, 1H, H-6), 7.89 (s, 1H, H-3'), 7.80 (d, J = 3.8 Hz, 1H, H-4), 7.46 (d, J = 8.7 Hz, 2H, H-4''), 7.19 (d, J = 3.8 Hz, 1H, H-3), 4.49 (s, 2H, H-12), 4.29 (d, J = 7.4 Hz, 2H, H-1''), 3.30 (t, J = 7.4 Hz, 2H, H-2''), ^{13}C NMR (151 MHz, DMSO) δ (ppm): 166.88 (C-11), 152.34 (C-9), 152.28 (C-5), 151.97 (C-2), 143.92 (C-6''), 142.88 (C-3''), 132.64 (C-6), 130.59 (C-4''), 123.92 (C-3'), 115.49 (C-4), 115.14 (C-3), 48.88 (C-1''), 39.59 (C-2''). Purity 91%. HRMS m/z $[\text{M}+\text{H}]^+$: 469.1232 (Calc. for $\text{C}_{19}\text{H}_{16}\text{N}_8\text{O}_7^+$ 469.1175).

(E)-10-[(4-Benzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, **211**



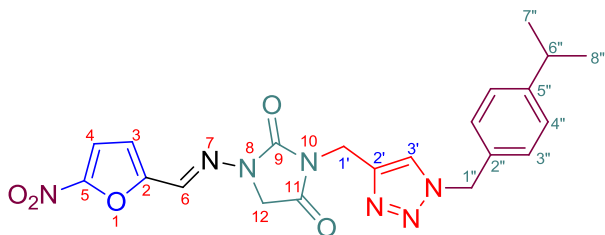
The reaction of **201** with 4-benzyl azide yielded **211** as an orange powder, 523 mg (71%), m.p.: 143.5-145.4 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3130 (C-H, alkene), 2921 (C-H, alkane), 2851 (C-H, alkane), 1781 (C=O), 1714 (C=O), 1343 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.14 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.41 – 7.30 (m, 5H, H-2''...5''), 7.18 (d, $J = 3.9$ Hz, 1H, H-3), 5.58 (s, 2H, H-1''), 4.73 (s, 2H, H-1'), 4.47 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.66 (C-11), 152.60 (C-9), 152.37 (C-5), 152.14 (C-2), 142.51 (C-2''), 136.11 (C-2'), 132.35 (C-4''), 128.65 (C-3''), 124.14 (C-3'), 115.31 (C-4), 114.95 (C-3), 78.36 (C-2'), 74.76 (C-3'), 53.30 (C-1''), 48.39 (C-12). Purity 94%. HRMS m/z $[\text{M}+\text{H}]^+$: 410.1170 (Calc. for $\text{C}_{18}\text{H}_{15}\text{N}_7\text{O}_5^+$ 410.1168).

(E)-10-[(5-Methylbenzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, **212**



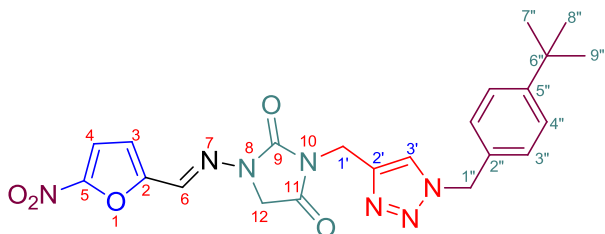
The reaction of **201** with 4-methylbenzyl azide yielded **212** as a fluffy, shiny and yellow crystals, 424 mg (55%), m.p.: 154.9-155.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3155 (C-H, alkene), 2918 (C-H, alkane), 1780 (C=O), 1719 (C=O), 1345 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.09 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.21-7.17 (m, 5H, H-3, 3'', 4''), 5.52 (s, 2H, H-1''), 4.72 (s, 2H, H-1'), 4.47 (s, 2H, H-12), 2.28 (s, 3H, H-6''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 166.98 (C-11), 152.82 (C-9), 152.38 (C-2), 151.62 (C-5), 137.99 (C-2''), 133.55 (C-6), 132.79 (C-2'), 129.77 (C-4''), 128.55 (C-3''), 123.69 (C-3'), 115.31 (C-4), 115.17 (C-3), 53.06 (C-1''), 48.84 (C-12), 40.42 (C-1'), 21.18 (C-6''). Purity 92%. HRMS m/z $[\text{M}+\text{H}]^+$: 424.1323 (Calc. for $\text{C}_{19}\text{H}_{17}\text{N}_7\text{O}_5^+$ 424.1325).

(E)-10-[(5-Isopropylbenzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, **213**



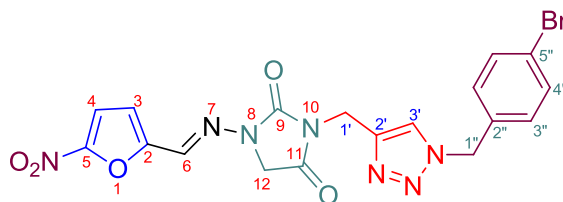
The reaction of **201** with 4-isopropylbenzyl azide yielded **213** as an orange powder, 627 mg (77%), m.p.: 154.9-155.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3135 (C-H, alkene), 2958 (C-H, alkane), 1783 (C=O), 1714 (C=O), 1344 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.13 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.26 – 7.22 (m, 4H, H-3'', 4''), 7.18 (d, J = 3.9 Hz, 1H, H-3), 5.52 (s, 2H, H-1''), 4.73 (s, 2H, H-1'), 4.47 (s, 2H, H-12), 2.92 – 2.80 (m, 1H, H-6''), 1.17 (d, J = 6.9 Hz, 6H, H-7'', 8''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.95 (C-11), 152.55 (C-9), 151.99 (C-5), 148.73 (C-2), 142.13 (C-2''), 133.87 (C-2'), 128.57 (C-3''), 127.15 (C-4''), 124.08 (C-3'), 115.32 (C-4), 115.02 (C-3), 52.62 (C-1''), 48.85 (C-12), 24.28 (C-7'', 8''). Purity 90%. HRMS m/z $[\text{M}+\text{H}]^+$: 452.1716 (Calc. for $\text{C}_{21}\text{H}_{21}\text{N}_7\text{O}_5^+$, 452.1638).

(E)-10-[[5-(*tert*-Butyl)benzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, **214**



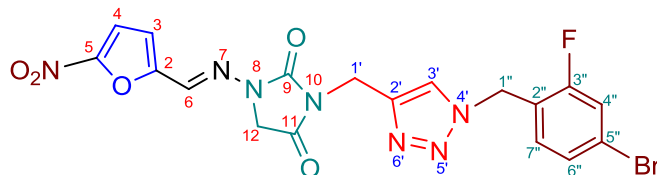
The reaction of **201** with 4-(*tert*-butyl)benzyl azide yielded **214** as an orange powder, 484 mg (57%), m.p.: 143.5-145.4 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3136 (C-H, alkene), 2960 (C-H, alkane), 1780 (C=O), 1720 (C=O), 1344 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.13 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.39 (d, J = 8.4 Hz, 2H, H-4''), 7.25 (d, J = 8.4 Hz, 2H, H-3''), 7.18 (d, J = 3.9 Hz, 1H, H-3), 5.53 (s, 2H, H-1''), 4.73 (s, 2H, H-1'), 4.47 (s, 2H, H-12), 1.25 (s, 9H, H-7''...-9''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.35 (C-11), 152.88 (C-9), 152.08 (C-5), 151.14 (C-2), 142.63 (C-2''), 133.47 (C-2'), 128.29 (C-3''), 126.01 (C-4''), 124.00 (C-3'), 115.31 (C-4), 115.17 (C-3), 53.01 (C-1''), 48.62 (C-12), 31.53 (C-7''...-9''). Purity 90%. HRMS m/z $[\text{M}+\text{H}]^+$: 466.1806 (Calc. for $\text{C}_{22}\text{H}_{23}\text{N}_7\text{O}_5^+$, 466.1794).

(E)-10-[(5-Bromobenzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **215**



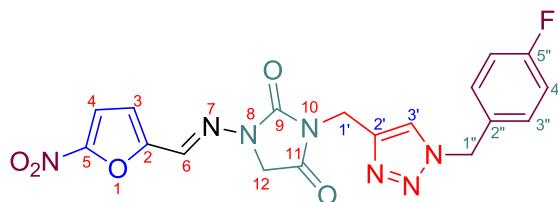
The reaction of **201** with 4-bromobenzyl azide yielded **215** as a dark orange powder, 431 mg (49%), m.p.: 171.7-172.3 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3129 (C-H, alkene), 2923 (C-H, alkane), 1785 (C=O), 1713 (C=O), 1344 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.19 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.58 (d, J = 8.4 Hz, 2H, H-4''), 7.28 (d, J = 8.4 Hz, 2H, H-3''), 7.18 (d, J = 3.9 Hz, 1H, H-3), 5.60 (s, 2H, H-1''), 4.74 (s, 2H, H-1'), 4.48 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.36 (C-11), 152.88 (C-9), 152.15 (C-2), 152.08 (C-5), 142.41 (C-2''), 131.55 (C-2'), 131.47 (C-3''), 127.61 (C-4''), 124.36 (C-3'), 122.31 (C-5''), 115.31 (C-4), 115.17 (C-3), 52.15 (C-1''), 49.06 (C-12). Purity 91%. HRMS m/z $[\text{M}+\text{H}]^+$: 488.0309 (Calc. for $\text{C}_{18}\text{H}_{14}\text{BrN}_7\text{O}_5^+$, 488.0240).

(E)-10-[(5-Bromo-7-fluorobenzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **216**



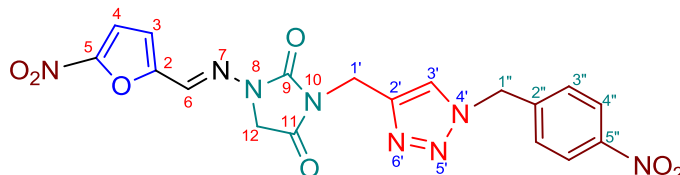
The reaction of **201** with 3-fluoro-4-bromobenzyl azide yielded **216** as a dark orange powder, 554 mg (60%), m.p.: 175.1-175.8 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3135 (C-H, alkene), 1778 (C=O), 1720 (C=O), 1343 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.13 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.63 (d, J = 8.2, 1H, H-6''), 7.47 (dd, J = 8.2, 1.8 Hz, 1H, H-4''), 7.32 (d, J = 8.2 Hz, 1H, H-7''), 7.18 (d, J = 3.9 Hz, 1H, H-3), 5.63 (s, 2H, H-1''), 4.73 (s, 2H, H-1'), 4.47 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.32 (C-11), 161.29 (C-3''), 159.65 (C-9), 152.87 (C-2), 152.29 (C-5), 142.32 (C-2''), 132.82 (C-7''), 132.41 (C-6), 128.55 (C-6''), 124.33 (C-3'), 122.76 (C-5''), 115.31 (C-4), 115.17 (C-3), 48.84 (C-1''), 46.74 (C-12). Purity 90%. HRMS m/z $[\text{M}+\text{H}]^+$: 506.0239 (Calc. for $\text{C}_{18}\text{H}_{13}\text{BrFN}_7\text{O}_5^+$, 506.0146).

(E)-10-[(5-Fluorobenzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, **217**



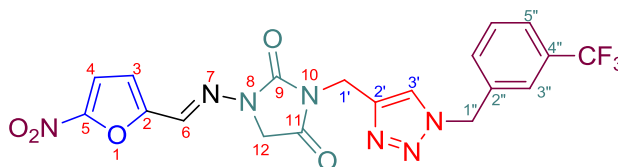
The reaction of **201** with 4-fluorobenzyl azide yielded **217** as a dark orange powder 374 mg (48%), m.p.: 184.4-185.4 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 2921 (C-H, alkane), 1783 (C=O), 1713 (C=O), 1343 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.15 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.40 (dd, J = 8.7, 5.5 Hz, 2H, H-3''), 7.21 (t, J = 8.9 Hz, 2H, H-4''), 7.18 (d, J = 3.9 Hz, 1H, H-3), 5.57 (s, 2H, H-1''), 4.73 (s, 2H, H-1'), 4.46 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.35 (C-11), 161.56 (C-5''), 152.88 (C-9), 152.29 (C-2), 152.07 (C-5), 142.39 (C-2''), 132.40 (C-2'), 130.85 (C-3''), 130.79 (C-4''), 124.09 (C-3'), 116.16 (C-5'), 115.32 (C-4), 115.16 (C-3), 52.48 (C-1''), 48.84 (C-12). Purity 90%. HRMS m/z $[\text{M}+\text{H}]^+$: 428.1113 (Calc. for $\text{C}_{18}\text{H}_{14}\text{FN}_7\text{O}_5^+$, 428.1074).

(E)-10-[(5-Nitrobenzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, **218**



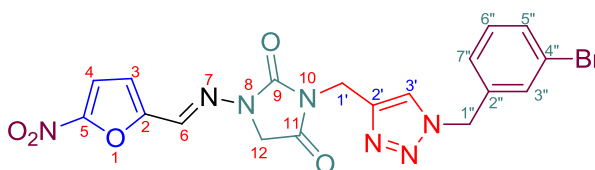
The reaction of **201** with 4-nitrobenzyl azide yielded **218** as a dark yellow powder, 249 mg (30%), m.p.: 181.2-181.8 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 2918 (C-H, alkane), 1785 (C=O), 1729 (C=O), 1340 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.39 (s, 1H, H-6), 8.37 (s, 1H, H-3'), 8.30 (d, J = 8.6 Hz, 2H, H-4''), 7.83 (d, J = 8.6 Hz, 2H, 3''), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.19 (d, J = 3.9 Hz, 1H, H-3), 5.57 (s, 2H, H-1''), 4.73 (s, 2H, H-1'), 4.29 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 166.88 (C-11), 152.28 (C-9), 151.98 (C-2), 144.76 (C-5''), 142.57 (C-2''), 132.64 (C-6), 126.80 (C-3''), 124.83 (C-4''), 124.56 (C-3'), 115.46 (C-4), 115.12 (C-3), 57.28 (C-1''), 48.62 (C-12). Purity 88%. HRMS m/z $[\text{M}+\text{H}]^+$: 455.1000 (Calc. for $\text{C}_{18}\text{H}_{14}\text{N}_8\text{O}_7^+$, 455.1019).

(E)-10-[(4-Trifluoromethylbenzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **219**



The reaction of **201** with 3-trifluoromethylbenzyl azide yielded **219** as a dark orange powder, 453 mg (52%), m.p.: 185.4-186.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3133 (C-H, alkene), 2924 (C-H, alkane), 1785 (C=O), 1721 (C=O), 1326 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.23 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.73 – 7.62 (m, 4H, H-3'', 6'', 5'', 7''), 7.19 (d, J = 3.9 Hz, 1H, H-3), 5.71 (s, 2H, H-1''), 4.75 (s, 2H, H-1'), 4.48 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.32 (C-11), 161.29 (C-3'), 159.65 (C-9), 152.87 (C-2), 152.29 (C-5), 142.32 (C-2''), 132.82 (C-7''), 132.41 (C-6), 128.55 (C-6''), 124.33 (C-3'), 122.76 (C-5''), 115.31 (C-4), 115.17 (C-3), 48.84 (C-1''), 46.74 (C-12). Purity 90%. HRMS m/z $[\text{M}+\text{H}]^+$: 478.1034 (Calc. for $\text{C}_{19}\text{H}_{14}\text{FN}_7\text{O}_5^+$, 478.1042).

(E)-10-[(4-Bromobenzyl-1*H*-1,2,3-triazolyl)methyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **220**



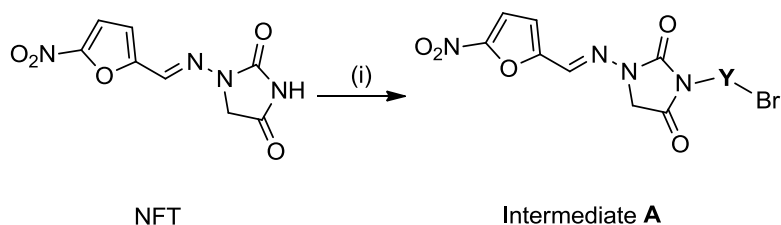
The reaction of **201** with 3-bromobenzyl azide yielded **220** as a fine dark orange powder, 546 mg (62%), m.p.: 188.0-188.7 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3134 (C-H, alkene), 2924 (C-H, alkane), 1783 (C=O), 1715 (C=O), 1343 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.19 (s, 1H, H-6), 7.88 (s, 1H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.58 – 7.53 (m, 2H, H-5'', 3''), 7.37 – 7.29 (m, 2H, H-6'', 7''), 7.18 (d, J = 3.9 Hz, 1H, H-3), 5.59 (s, 2H, H-1''), 4.74 (s, 2H, H-1'), 4.48 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.36 (C-11), 152.88 (C-9), 152.08 (C-2, 5), 142.44 (C-2''), 139.03 (C-3''), 131.55 (C-6), 131.47 (C-6''), 131.29 (C-5''), 127.61 (C-7''), 124.36 (C-3'), 122.31 (C-4''), 115.31 (C-4), 115.17 (C-3), 52.44 (C-1''), 48.85 (C-12), Purity 92%. HRMS m/z $[\text{M}+\text{H}]^{+2}$: 490.0334 (Calc. for $\text{C}_{19}\text{H}_{14}\text{BrN}_7\text{O}_5^{+2}$, 490.0340).

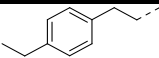
5.2.3.2. Synthesis of Series 3 and 4

The synthesis of Series 3 and 4 hybrids occurred in three steps, namely (i) the formation of intermediate **A** (Scheme 5.3) by attachment of the *n*-alkyl/ aryl linkers on NFT, respectively, (ii) the synthesis of azide intermediate **B** from intermediate **A** (Scheme 5.4), and (iii) the synthesis of the hybrids from azide intermediate **B** using click chemistry.

Synthesis of intermediates A1 – A4

NFT (2.94 mmol, 0.7 g) was dissolved in anhydrous DMF (10 mL), after which TEA (4.41 mmol, 1.5 eq.) was added. The solution was stirred at room temperature for 15 min. To the resulting yellow solution, the appropriate dibromide (8.82 mmol, 3 eq) was added (Scheme 5.3 (i)). The reaction was stirred at room temperature and monitored by TLC, eluting with DCM:MeOH (19:1, v/v). Upon completion, the reaction was quenched with 20 mL of water and extracted with ethyl acetate (3 X 20 mL). The organic layer was washed once with saturated NH₄Cl (30 mL) and once with water (30 mL). The solution was concentrated *in vacuo*. The resulting paste was purified by recrystallisation in ethanol/water to afford the NFT- intermediate, **A**. All intermediates were confirmed by ¹H and ¹³C NMR.

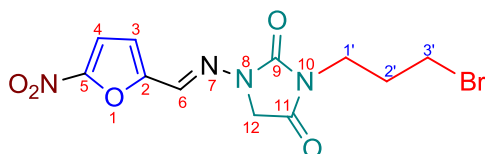


Cpd.	A1	A2	A3	A4
Y	<i>n</i> -C ₃ H ₇ --	<i>n</i> -C ₅ H ₁₁ --	<i>n</i> -C ₆ H ₁₃ --	

Scheme 5.3: Synthesis of intermediate, **A**

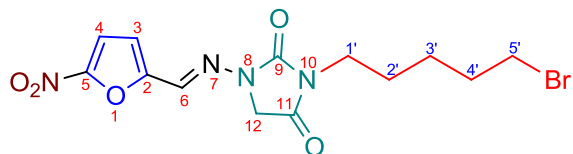
Reagents and conditions: (i) **A1 – A3**: *n*-alkyl dibromide (1.5 eq), **A4**: 1,4-bis(bromomethyl)benzene (3 eq), TEA (1.5 eq), anhydrous DMF, rt, 24 hr

(*E*)-10-(3-Bromopropyl)-1-[[5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, **A1**



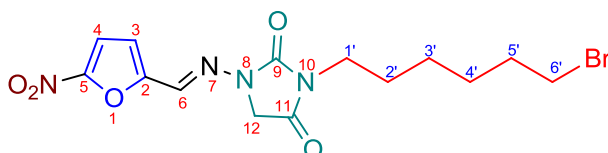
¹H NMR (600 MHz, DMSO) δ (ppm): 7.88 (s, 1H, H-6), 7.80 (d, *J* = 3.9 Hz, 1H, H-4), 7.18 (d, *J* = 3.9 Hz, 1H, H-3), 4.39 (s, 2H, H-12), 3.55 (t, *J* = 6.7 Hz, 2H, H-1'), 3.42 (t, *J* = 6.7 Hz, 2H, H-3'), 1.81 (p, *J* = 6.7 Hz, 2H, H-2'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.88 (C-11), 153.28 (C-9), 152.14 (C-2), 132.06 (C-6), 115.21 (C-4), 115.15 (C-3), 48.65 (C-12), 40.42 (C-1'), 36.35 (C-3'), 27.32 (C-2').

(E)-10-(3-Bromopentyl)-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, A2



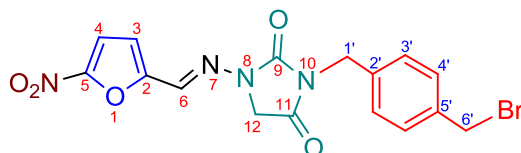
^1H NMR (600 MHz, DMSO) δ (ppm): 7.88 (s, 1H, H-6), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.17 (d, J = 3.9 Hz, 1H, H-3), 4.42 (s, 2H, H-12), 3.44 – 3.36 (m, 4H, H-1', 5'), 1.84 – 1.81 (m, 2H, H-2'), 1.62 – 1.36 (m, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-11), 153.38 (C-9), 152.21 (C-2), 132.08 (C-6), 115.19 (C-4), 48.68 (C-12), 40.42 (C-1'), 32.19 (C-5'), 31.78 (C-3'), 26.72 (C-4'), 25.15 (C-3').

(E)-10-(3-Bromohexyl)-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, A3



^1H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-6), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.17 (d, J = 3.9 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 3.53 (t, J = 6.7 Hz, 2H, H-1'), 3.47 (t, J = 7.0 Hz, 2H, H-6'), 1.84 – 1.77 (m, 2H, H-5'), 1.60 - 1.54 (m, 2H, H-2'), 1.44 – 1.37 (m, 2H, H-3'), 1.31 (m, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-11), 153.39 (C-9), 152.22 (C-2), 132.02 (C-6), 115.20 (C-3, 4), 48.68 (C-12), 40.43 (C-1'), 35.51 (C-6'), 32.61 (C-5'), 32.52 (C-2'), 27.12 (C-4'), 25.67 (C-3').

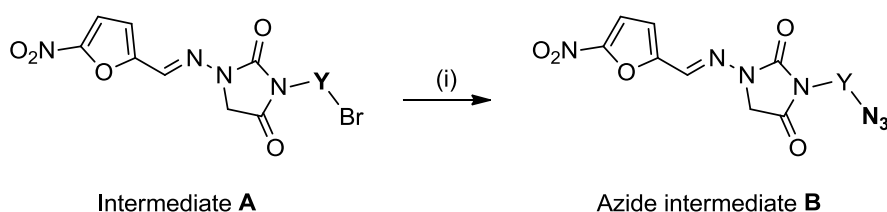
(E)-10-[3-Bromomethyl)benzyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, A4



^1H NMR (600 MHz, DMSO) δ (ppm): 7.90 (s, 1H, H-6), 7.80 (d, J = 3.7 Hz, 1H, H-4), 7.37 (d, J = 8.4 Hz, 2H, H-3'), 7.29 (d, J = 8.4 Hz, 2H, H-4'), 7.18 (d, J = 3.7 Hz, 1H, H-3), 5.15 (s, 2H, H-6'), 4.68 (s, 2H, H-1'), 4.43 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.79 (C-11), 153.23 (C-9), 152.31 (C-2), 152.11 (C-5), 135.46 (C-5'), 132.34 (C-6), 128.85 (C-3'), 128.39 (C-4'), 115.33 (C-4), 115.18 (C-3), 48.92 (C-12), 41.92 (C-1'), 39.57 (C-6').

Synthesis of intermediate B1 – B4

Intermediate **A** (0.5 g) was dissolved in DMSO (10 mL). To this solution NaN₃ (1.5 eq) was added. The reaction was stirred at room temperature for 36 hr. Thereafter, the reaction was quenched with 20 mL of water and then extracted with ethyl acetate (3 X 20 mL). The organic layer was washed with water (2 X 20 mL) and then brine (2 X 20 mL). The organic solution was concentrated *in vacuo*. The resulting muddy paste was purified by recrystallisation in ethanol/water to afford intermediate, **B**. All intermediates were confirmed by ¹H and ¹³C NMR.

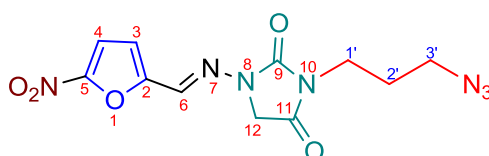


Cpd	B1	B2	B3	B4
Y	<i>n</i> -C ₃ H ₇ --	<i>n</i> -C ₅ H ₁₁ --	<i>n</i> -C ₆ H ₁₃ --	

Scheme 5.4: Synthesis of intermediate **B1-B4**

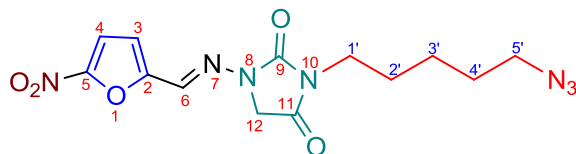
Reagents and conditions: (i) **A1-A4** (0.5 g), NaN₃, (1.5 eq), DMSO, rt, 36 hr

(*E*)-10-(Propyl-3-azide)-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **B1**



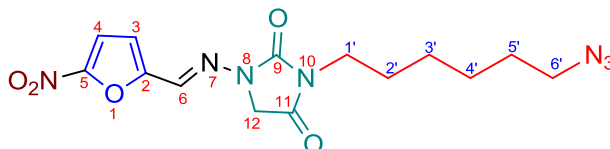
¹H NMR (600 MHz, DMSO) δ (ppm): 7.88 (s, 1H, H-6), 7.80 (d, *J* = 3.9 Hz, 1H, H-4), 7.18 (d, *J* = 3.9 Hz, 1H, H-3), 4.40 (s, 2H, H-12), 3.55 (t, *J* = 6.7 Hz, 2H, H-1'), 3.42 (t, *J* = 6.7 Hz, 2H, H-3'), 1.82 – 1.76 (m, 2H, H-2'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.88 (C-11), 153.28 (C-9), 152.14 (C-2), 132.06 (C-6), 115.21 (C-4), 115.15 (C-3), 48.65 (C-12), 40.42 (C-1'), 36.35 (C-3'), 27.32 (C-2').

(E)-10-(Pentyl-3-azide)-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, B2



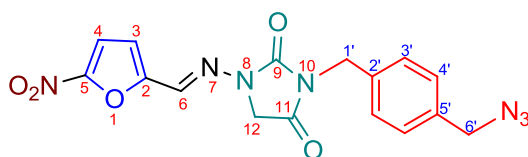
^1H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-6), 7.79 (d, J = 3.9 Hz, 1H, H-4), 7.17 (d, J = 3.8 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 3.55 – 3.48 (m, 2H, H-1'), 1.58 – 1.54 (m, 2H, H-2'), 1.39 – 1.33 (m, 4H, H-4'..5'). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.83 (C-11), 153.37 (C-9), 152.27 (C-2), 152.23 (C-5), 132.05 (C-6), 115.15 (C-3, 4), 50.98 (C-5'), 48.65 (C-12), 40.41 (C-1'), 28.27 (C-4'), 23.76 (C-3').

(E)-10-(Hexyl-3-azide)-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, B3



^1H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-6), 7.80 (d, J = 3.8 Hz, 1H, H-4), 7.17 (d, J = 3.8 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 3.47 (t, J = 7.0 Hz, 2H, H-1'), 1.57 – 1.51 (m, 4H, H-2', 6'), 1.34 (dd, J = 8.6, 5.4 Hz, 6H, H-3'...5'). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.83 (C-11), 153.38 (C-9), 152.24 (C-5), 132.01 (C-6), 115.17 (C-4), 115.14 (C-3), 51.02 (C-6'), 48.65 (C-12), 40.41 (C-1'), 28.59 (C-2'), 27.75 (C-5'), 26.16 (C-4'), 26.07 (C-3').

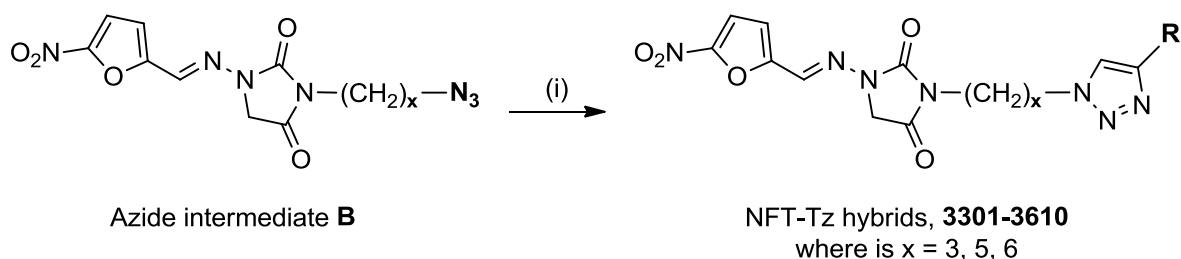
(E)-10-[3-(Azidomethyl)benzyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, B4



^1H NMR (600 MHz, DMSO) δ (ppm): 8.23 (s, 1H, H-6), 7.89 (d, J = 3.6 Hz, 1H, H-4), 7.41 – 7.32 (m, 4H, H-3',-4'), 7.18 (d, J = 3.6 Hz, 1H, H-3), 5.16 (s, 2H, H-6'), 4.67 (s, 2H, H-1'), 4.48 (s, 2H, H-12). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.79 (C-11), 153.23 (C-9), 152.31 (C-2), 152.11 (C-5), 136.36 (C-6'), 135.46 (C-5'), 132.34 (C-6), 128.85 (C-3'), 128.39 (C-4'), 115.33 (C-4), 115.18 (C-3), 48.92 (C-12), 41.92 (C-1'), 39.57 (C-6').

Synthesis of hybrids, 3301 - 3610

Intermediate **B**, (0.5 g) was dissolved in 10 mL of DMSO:water (4:1, v/v). To the resulting solution, the appropriate alkyne (1.5 eq.) was added, after which catalytic amounts of β -cyclodextrin (0.02 eq), sodium ascorbate (0.3 eq) and copper sulphate pentahydrate (0.1 eq) were added in sequence (Scheme 5.5). After 12 h an additional amount of sodium ascorbate (0.3 eq) was added. The reaction was stirred at room temperature for 48 h and monitored by TLC, eluting with DCM:MeOH (19:1, v/v). Upon completion, the reaction was quenched with 20 mL of water and extracted with ethyl acetate (3 X 20 mL). The organic layer was washed once with saturated NH_4Cl and once with water. The solution was concentrated *in vacuo*. The resulting paste was purified by recrystallisation in ethanol/water to afford the desired analogue.

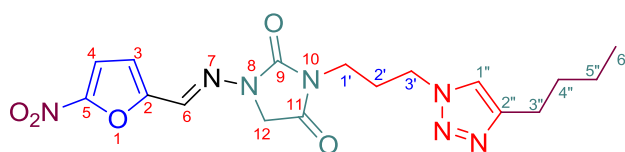


Cpd.	x	Cpd.	x	Cpd.	x	R	Cpd.	x	Cpd.	x	Cpd.	x	R
3301	3	3501	5	3601	6	$n\text{-C}_4\text{H}_9$ --	3306	3	3506	5	3606	6	
3302	3	3502	5	3602	6	$n\text{-C}_5\text{H}_{11}$ --	3307	3	3507	5	3607	6	
3303	3	3503	5	3603	6	$n\text{-C}_6\text{H}_{13}$ --	3308	3	3508	5	3608	6	
3304	3	3504	5	3604	6	$n\text{-C}_8\text{H}_{17}$ --	3309	3	3509	5	3609	6	
3305	3	3505	5	3605	6		3310	3	3510	5	3610	6	

Scheme 5.5: Synthesis of *n*-alkyl NFT-Tz hybrids, **3301-3610**.

Reagents and conditions: (i) *n*-alkyne or phenyl ethynyl (1.5 eq), β -cyclodextrin (0.02 eq), sodium ascorbate (0.3 eq) and copper sulphate pentahydrate (0.1 eq), 10 ml DMSO:water (8:2) rt, 48 h.

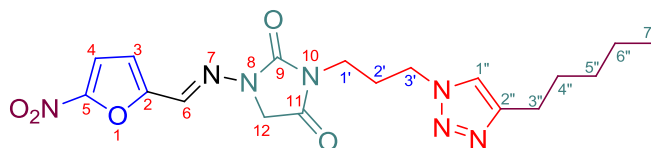
(*E*)-3-[3-(2-Butyl-1*H*-1,2,3-triazol-1-yl)propyl]-1-[[5-(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, **3301**



The reaction of **B1** (1.56 mmol) with 1-hexyne yielded **3301** as an orange powder, 331 mg (53%), m.p.: 154.7-156.3 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3135 (C-H, alkene), 2928 (C-H, alkane), 1776 (C=O), 1708 (C=O), 1496 (NO_2), 1388 (C-N), 1294 (C-N). ^1H NMR (600 MHz, DMSO)

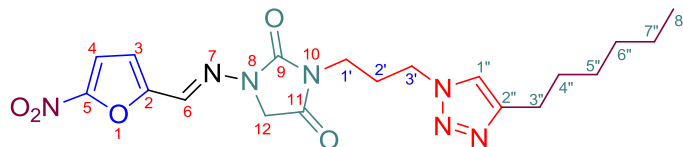
δ (ppm): 7.89 (s, 1H, H-6), 7.85 (s, 1H, H-1''), 7.81 (d, $J = 3.9$ Hz, 1H, H-4), 7.18 (d, $J = 3.9$ Hz, 1H, H-3), 4.36 (s, 2H, H-12), 3.82 (t, $J = 7.6$ Hz, 2H, H-1'), 2.60 (t, $J = 7.6$ Hz, 2H, H-3'), 2.18 – 2.10 (m, 4H, H-2', 3''), 1.60 – 1.51 (m, 2H, H-4''), 1.36 – 1.27 (m, 2H, H-5''), 1.01 – 0.97 (m, 3H, H-6''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.88 (C-11), 153.34 (C-9), 152.29 (C-6), 152.20 (C-5), 132.06 (C-2''), 122.35 (C-1''), 115.21 (C-4, 3), 48.84 (C-12), 47.42 (C-3'), 40.41 (C-1'), 28.58 (C-4'), 25.15 (C-2'), 22.14 (C-5''), 14.17 (C-6''). Purity 94%. HRMS m/z $[\text{M}+\text{H}]^+$: 404.1654 (Calc. for $\text{C}_{17}\text{H}_{21}\text{N}_7\text{O}_5^+$ 404.1604).

(E)-3-[3-(2-Pentyl-1H-1,2,3-triazol-1-yl)propyl]-1-[[5-nitrofur-2-yl)methylene]amino}imidazolidine-9,11-dione, 3302



The reaction of **B1** (1.56 mmol) with 1-heptyne yielded **3302** as a yellow powder, 366 mg (56%), m.p.: 161.1-161.8 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3153 (C-H, alkene), 2927 (C-H, alkane), 1770 (C=O), 1710 (C=O), 1495 (NO_2), 1370 (C-N), 1250 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 7.89 (s, 1H, H-6), 7.85 (s, 1H, H-1''), 7.81 (d, $J = 3.9$ Hz, 1H, H-4), 7.18 (d, $J = 3.9$ Hz, 1H, H-3), 4.41 (s, 2H, H-12), 4.34 (t, $J = 6.7$ Hz, 2H, H-1'), 3.52 (t, $J = 6.7$ Hz, 2H, H-3'), 2.59 - 2.45 (m, 2H, H-2'), 2.16 – 2.10 (m, 2H, H-3''), 1.61 – 1.55 (m, 2H, H-4''), 1.33 – 1.25 (m, 4H, H-6'', 5''), 1.01 - 0.96 - (m, $J = 6.7$ Hz, 3H, H-7''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.66 (C-11), 153.05 (C-9), 152.15 (C-6, 5), 131.89 (C-2''), 122.27 (C-1''), 115.17 (C-4, 3), 48.40 (C-12), 47.19 (C-3'), 40.41 (C-1'), 28.36 (C-4'), 25.45 (C-2'), 22.34 (C-6''), 14.38 (C-7''). Purity 96%. HRMS m/z $[\text{M}+\text{H}]^+$: 418.1828 (Calc. for $\text{C}_{18}\text{H}_{23}\text{N}_7\text{O}_5^+$ 418.1761).

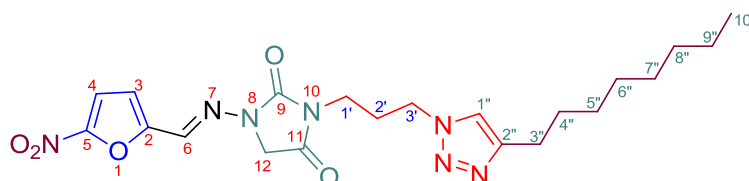
(E)-3-[3-(2-Hexyl-1H-1,2,3-triazol-1-yl)propyl]-1-[[5-nitrofur-2-yl)methylene]amino}imidazolidine-9,11-dione, 3303



The reaction of **B1** (1.56 mmol) with 1-octyne yielded **3303** as a yellow powder, 273 mg (41%), m.p.: 171.1-171.7 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3137 (C-H, alkene), 2924 (C-H, alkane), 1778 (C=O), 1707 (C=O), 1497 (NO_2), 1386 (C-N), 1250 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 7.89 (s, 1H, H-6), 7.85 (s, 1H, H-1''), 7.81 (d, $J = 3.3$ Hz, 1H, H-4), 7.18 (d, $J = 3.3$ Hz, 1H, H-3), 4.42 (s, 2H, H-12), 4.33 (t, $J = 6.5$ Hz, 2H, H-1'), 3.52 (t, $J = 6.5$ Hz, 2H, H-3'), 2.62 – 2.56 (m, 2H, H-2'), 2.17 – 2.09 (m, 2H, H-3''), 1.60 – 1.53 (m, 2H, H-4''), 1.37 – 1.21 (m, 6H, H-5''...7''), 0.97 – 0.82 (m, 3H, H-8''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.89

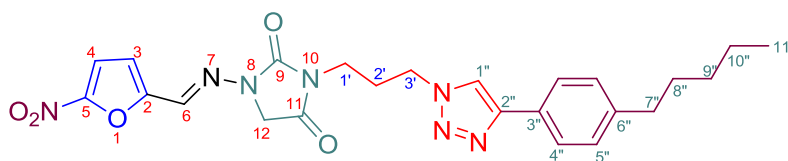
(C-11), 153.05 (C-9), 152.16 (C-6, 5), 132.11 (C-2''), 122.48 (C-1''), 115.16 (C-4,3), 48.62 (C-12), 47.38 (C-3'), 40.42 (C-2''), 31.47 (C-6''), 29.32 (C-2'), 28.80 (C-4''), 28.42 (C-5''), 22.52 (C-7''), 14.42 (C-8''). Purity 90 %. HRMS m/z $[M+H]^+$: 432.1985 (Calc. for $C_{19}H_{25}N_7O_5^+$ 432.1917).

(E)-3-[3-(2-Octyl-1H-1,2,3-triazol-1-yl)propyl]-1-[(5-nitrofuran-2-yl)methylene]imidazolidine-9,11-dione, 3304



The reaction of **B1** (1.56 mmol) with 1-decyne yielded **3304** as a yellow powder, 152 mg (21%), m.p.: 197.2-198.1 °C. IR (ATR) ν_{max}/cm^{-1} : 3119 (C-H, alkene), 2954 (C-H, alkane), 1770 (C=O), 1711 (C=O), 1490 (NO₂), 1370 (C-N), 1251 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.89 (s, 1H, H-6), 7.84 (s, 1H, H-1''), 7.81 (d, J = 3.9 Hz, 1H, H-4), 7.18 (d, J = 3.9 Hz, 1H, H-3), 4.40 (s, 2H, H-12), 4.35 t, J = 6.7 Hz, 2H, H-1'), 3.52 (t, J = 6.7 Hz, 2H, H-3'), 2.61 – 2.56 (m, 2H, H-2'), 2.15 – 2.10 (m, 2H, H-3''), 1.59 – 1.53 (m, 2H, H-4''), 1.30 – 1.21 (m, 10H, H-5''...H-9''), 1.02 - 0.97 (m, 3H, H-10''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.89 (C-11), 153.28 (C-9), 151.92 (C-6, 5), 132.11 (C-2''), 122.25 (C-1''), 115.16 (C-3,4), 48.63 (C-12), 47.19 (C-3'), 40.42 (C-1'), 31.76 (C-3'', 8''), 29.47 (C-5''), 29.24 (C-7''), 29.13 (C-6''), 28.36 (C-4''), 22.58 (C-9''), 14.44 (C-10''). Purity 90%. HRMS m/z $[M+H]^+$: 460.2293 (Calc. for $C_{21}H_{29}N_7O_5^+$ 460.2230).

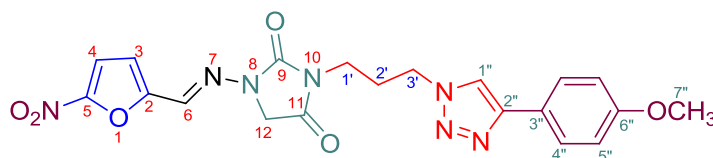
(E)-3-[3-[2-(6-pentylphenyl)-1H-1,2,3-triazol-1-yl]propyl]-1-[(5-nitrofuran-2-yl)methylene]imidazolidine-9,11-dione, 3306



The reaction of **B1** (1.56 mmol) with 1-ethynyl-4-pentylbenzene yielded **3306** as a yellow powder, 102 mg (13%), m.p.: 194.0-195.2 °C. IR (ATR) ν_{max}/cm^{-1} : 3130 (C-H, alkene), 2956 (C-H, alkane), 1772 (C=O), 1712 (C=O), 1494 (NO₂), 1369 (C-N), 1250 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.51 (s, 1H, H-6), 7.87 (s, 1H, H-1''), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.73 (d, J = 7.9 Hz, 2H, H-4''), 7.24 (d, J = 7.9 Hz, 2H, H-5''), 7.16 (d, J = 3.9 Hz, 1H, H-3), 4.48 (s, 2H, H-12), 4.35 (t, J = 6.6 Hz, 2H, H-1'), 3.58 (t, J = 6.6 Hz, 2H, H-3'), 2.59 – 2.55 (m, 2H, H-2'), 2.25 – 2.19 (m, 2H, H-7''), 1.59 – 1.55 (m, 2H, H-8''), 1.34 – 1.23 (m, 4H, H-9'', 10''), 0.87 (t, J = 6.4 Hz, 3H, H-11''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.89 (C-11),

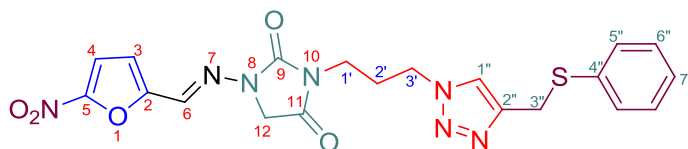
164.76 (C-9), 153.33 (C-2), 152.20 (C-5), 146.77 (C-2"), 142.50 (C-6"), 132.06 (C-6), 129.22 (C-1"), 128.70 (C-5"), 125.58 (C-3"), 121.59 (C-4"), 115.20 (C-4,3), 48.81 (C-12), 47.77 (C-3'), 40.98 (C-1'), 35.32 (C-7"), 31.36 (C-9"), 31.01 (C-8"), 28.32 (C-2'), 22.43 (C-10"), 14.41 (C-11"). Purity 89%. HRMS m/z $[M+H]^+$: 494.2133 (Calc. for $C_{24}H_{27}N_7O_5^+$ 494.2074).

(E)-3-{3-[2-(6-methoxyphenyl)-1H-1,2,3-triazol-1-yl]propyl}-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3307



The reaction of **B1** (1.56 mmol) with 1-ethynyl-4-methoxybenzene yielded **3307** as a yellow powder, 268 mg (38%), m.p.: 191.9-192.3 °C. IR (ATR) ν_{max}/cm^{-1} : 3117 (C-H, alkene), 2938 (C-H, alkane), 1778 (C=O), 1709 (C=O), 1498 (NO₂), 1368 (C-N), 1245 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.45 (s, 1H, H-6), 7.85 (s, 1H, H-1"), 7.80 (d, J = 3.8 Hz, 1H, H-4), 7.75 (d, J = 8.7 Hz, 2H, H-4"), 7.15 (d, J = 3.8 Hz, 1H, H-3), 6.99 (d, J = 8.7 Hz, 2H, H-5"), 4.46 (t, J = 6.7 Hz, 2H, H-1'), 4.34 (s, 2H, H-12), 3.77 (s, 3H, H-7"), 3.58 (t, J = 6.7 Hz, 2H, H-3'), 2.24 – 2.19 (m, 2H, H-2'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.87 (C-11), 165.56 (C-6"), 159.40 (C-9), 153.31 (C-2), 152.18 (C-5), 146.61 (C-2"), 132.04 (C-6), 126.94 (C-4"), 123.82 (C-), 121.03, 115.15 (C-5"), 114.73 (C-4,3), 55.59 (C-7"), 48.80 (C-12), 47.77 (C-3'), 40.41 (C-1'), 28.29 (C-2'). Purity 89%. HRMS m/z $[M+H]^+$: 454.1430 (Calc. for $C_{20}H_{19}N_7O_6^+$ 454.1397).

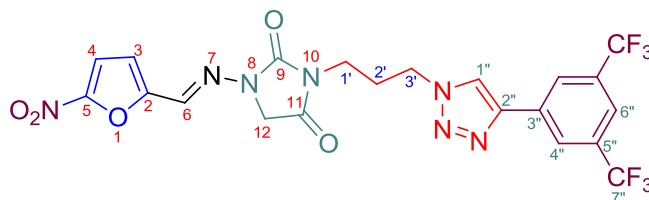
(E)-3-{3-[2-(6-(phenylthio)methyl)-1H-1,2,3-triazol-1-yl]propyl}-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3308



The reaction of **B1** (1.56 mmol) with phenylpropargyl sulfide yielded **3308** as a yellow powder, 237 mg (32%), m.p.: 172.1-178.1 °C. IR (ATR) ν_{max}/cm^{-1} : 3120 (C-H, alkene), 2927 (C-H, alkane), 1785 (C=O), 1708 (C=O), 1482 (NO₂), 1367 (C-N), 1249 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.97 (s, 1H, H-6), 7.88 (s, 1H, H-1'), 7.80 (d, J = 3.8 Hz, 1H, H-4), 7.36 (d, J = 7.6 Hz, 2H, H-5"), 7.31 (d, J = 7.6 Hz, 2H, H-6"), 7.19 (d, J = 7.3 Hz, 1H, H-7"), 7.18 (d, J = 3.8 Hz, 1H, H-3), 4.41 – 4.36 (m, 4H, H-1',3"), 4.28 (s, 2H, H-12), 3.50 (t, J = 6.7 Hz, 2H, H-3'), 2.13 – 2.07 (m, 2H, H-2'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.92 (C-11), 153.34 (C-9), 152.20 (C-5, 2), 136.31 (C-4"), 132.08 (C-6), 129.47 (C-5"), 128.69 (C-6"), 126.38 (C-7"), 123.84 (C-1"), 115.21 (C-4), 115.16 (C-3), 48.81 (C-12), 47.52 (C-3'), 40.46

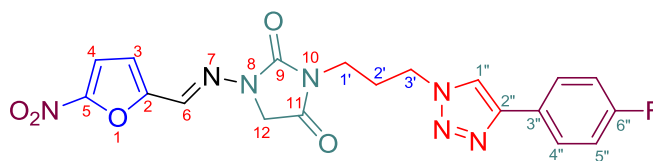
(C-1'), 36.33 (C-3''), 27.72 (C-2'). Purity 89%. HRMS m/z $[M+H]^+$: 470.1210 (Calc. for $C_{20}H_{19}N_7O_5S^+$ 470.1168).

(E)-3-{2-[6-(3,5-Bis(trifluoromethyl)phenyl)-1H-1,2,3-triazol-1-yl]propyl}-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, 3309



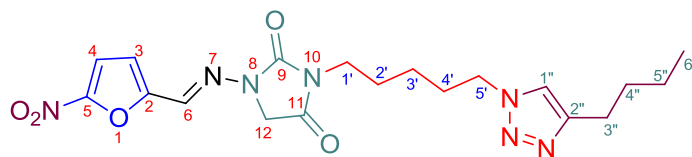
The reaction of **B1** (1.56 mmol) with 1-ethynyl-3,5-bis(trifluoromethyl)benzene yielded **3309** as a yellow powder, 340 mg (39%), m.p.: 198.1-199.1 °C. IR (ATR) ν_{max}/cm^{-1} : 3138 (C-H, alkene), 2957 (C-H, alkane), 1798 (C=O), 1725 (C=O), 1493 (NO₂), 1382 (C-N), 1274 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.99 (s, 1H, H-6''), 8.50 (s, 2H, H-4''), 8.04 (s, 1H, H-6), 7.85 (s, 1H, H-1''), 7.79 (d, J = 3.8 Hz, 1H, H-4), 7.12 (d, J = 3.8 Hz, 1H, H-3), 4.54 (t, J = 7.0 Hz, 2H, H-1'), 4.35 (s, 2H, H-12), 3.61 (t, J = 6.5 Hz, 2H, H-3'), 2.29 – 2.23 (m, 2H, H-2'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.87 (C-11), 158.02 (C-9), 153.28 (C-2), 152.12 (C-4), 145.05 (C-2''), 133.23 (C-3''), 132.03 (C-5''), 131.60 (C-6), 125.76 (C-7''), 124.08 (C-6''), 115.14 (C-4, 3), 48.81 (C-12), 48.19 (C-3'), 41.47 (C-1'), 28.04 (C-2'). Purity 88%. HRMS m/z $[M+H]^+$: 560.1098 (Calc. for $C_{21}H_{15}F_6N_7O_5^+$ 560.1039).

(E)-3-{3-{2-[6-(*p*-fluorophenyl)]-1H-1,2,3-triazol-1-yl}propyl}-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, 3310



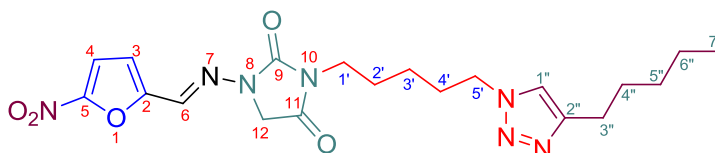
The reaction of **B1** (1.56 mmol) with 1-ethynyl-4-fluorobenzene yielded **3310** as a yellow powder 359 mg (52%), m.p.: 184.5-191.9 °C. IR (ATR) ν_{max}/cm^{-1} : 3130 (C-H, alkene), 2923 (C-H, alkane), 1780 (C=O), 1708 (C=O), 1496 (NO₂), 1369 (C-N), 1248 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.56 (s, 1H, H-6), 7.88 – 7.85 (m, 3H, H-4'', -1''), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.27 (t, J = 8.8 Hz, 2H, H-5''), 7.16 (t, J = 6.0 Hz, 1H, H-3), 4.49 (t, J = 6.6 Hz, 2H, H-3'), 4.35 (s, 2H, H-12), 3.58 (t, J = 6.6 Hz, 2H, H-1'), 2.25 – 2.20 (m, 2H, H-2'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.65 (C-11), 161.38 (C-6''), 153.32 (C-9), 152.27 (C-2), 152.16 (C-5), 145.81 (C-2''), 132.05 (C-6), 127.80 (C-1''), 127.63 (C-4''), 127.58 (C-3''), 121.92 (C-5''), 116.34 (C-4), 116.20 (C-3), 48.80 (C-12), 47.84 (C-3'), 40.40 (C-1'), 28.14 (C-2'). Purity 88%. HRMS m/z $[M+H]^+$: 442.1244 (Calc. for $C_{19}H_{16}FN_7O_5^+$ 442.1197).

(E)-3-[3-(2-Butyl-1*H*-1,2,3-triazol-1-yl)pentyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, 3501



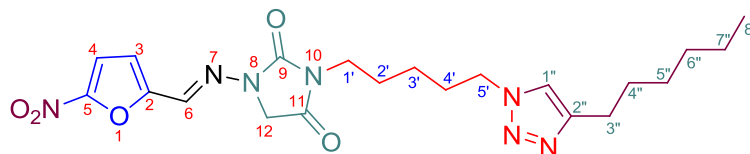
The reaction of **B2** (1.43 mmol) with 1-hexyne yielded **3501** as a yellow powder, 484 mg (78%), m.p.: 112.7-112.9 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3131 (C-H, alkene), 2953 (C-H, alkane), 1779 (C=O), 1708 (C=O), 1497 (NO₂), 1370 (C-N), 1251 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.88 (s, 1H, H-6), 7.83 (s, 1H, H-1''), 7.80 (d, J = 3.7 Hz, 1H, H-4), 7.18 (d, J = 3.7 Hz, 1H, H-3), 4.39 (t, J = 5.1 Hz, 2H, H-5'), 4.28 (s, 2H, H-12), 3.45 (t, J = 6.9 Hz, 2H, H-1'), 2.62 – 2.56 (m, 2H, H-3''), 1.81 – 1.66 (m, 2H, H-4'), 1.58 – 1.42 (m, 2H, H-4''), 1.35 – 1.28 (m, 4H, H-3', 2''), 0.90 (t, J = 6.5 Hz, 3H, H-6''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-11), 152.23 (C-9), 132.03 (C-2''), 122.05 (C-1''), 115.18 (C-4, 3), 49.34 (C-12), 40.41 (C-1'), 31.62 (C-3''), 29.51 (C-2', 4''), 25.18 (C-4'), 23.28 (C-3'), 22.17 (C-5''), 14.18 (C-6''). Purity 93%. HRMS m/z [M+H]⁺: 432.1999 (Calc. for C₁₉H₂₅N₇O₅⁺ 432.1917).

(E)-3-[3-(2-Pentyl-1*H*-1,2,3-triazol-1-yl)pentyl]-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, 3502



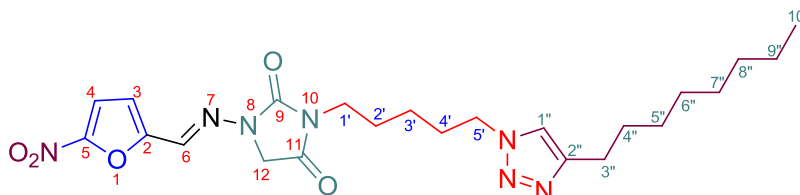
The reaction of **B2** (1.43 mmol) with 1-heptyne yielded **3502** as a yellow powder, 242 mg (40%), m.p.: 128.5-129.3 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3139 (C-H, alkene), 2925 (C-H, alkane), 1779 (C=O), 1708 (C=O), 1496 (NO₂), 1369 (C-N), 1251 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-6), 7.83 (s, 1H, H-1''), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.18 (d, J = 3.9 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 4.28 (t, J = 5.3 Hz, 2H, H-5'), 3.45 (t, J = 6.9 Hz, 2H, H-1'), 2.65 – 2.58 (m, 2H, H-3''), 1.84 – 1.78 (m, 2H, H-4'), 1.59 – 1.55 (m, 4H, H-2', 4''), 1.28 – 1.21 (m, 6H, H-3', 5'', 6''), 0.85 (t, J = 6.9 Hz, 3H, H-7''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-11), 153.37 (C-9), 152.23 (C-2, 5), 132.03 (C-6), 122.01 (C-1''), 115.19 (C-4, 3), 49.33 (C-5'), 48.66 (C-12), 40.41 (C-1'), 31.31 (C-2'), 29.63 (C-4'), 29.16 (C-2''), 27.19 (C-4''), 23.37 (C-3'), 22.34 (C-6''), 14.38 (C-7''). Purity 94%. HRMS m/z [M+H]⁺: 446.2159 (Calc. for C₂₀H₂₇N₇O₅⁺ 446.2074).

(E)-3-[3-(2-Hexyl-1*H*-1,2,3-triazol-1-yl)pentyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3503



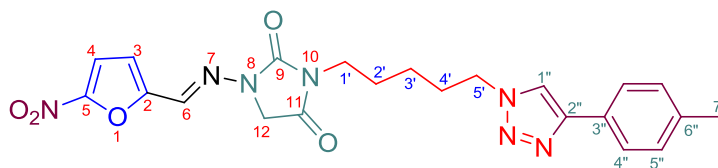
The reaction of **B2** (1.43 mmol) with 1-octyne yielded **3503** as a yellow powder, 438 mg (67%), m.p.: 134.7-134.9 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3126 (C-H, alkene), 2952 (C-H, alkane), 2923, (C-H, alkane), 1780 (C=O), 1710 (C=O), 1496 (NO₂), 1371 (C-N), 1255 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.88 (s, 1H, H-6), 7.83 (s, 1H, H-1'), 7.81 (d, *J* = 3.8 Hz, 1H, H-4), 7.18 (d, *J* = 3.8 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 4.26 (t, *J* = 6.6 Hz, 2H, H-5'), 3.44 (d, *J* = 6.6 Hz, 2H, H-1'), 2.57 (t, *J* = 7.4 Hz, 2H, H-3''), 1.83 - 1.77 (m, 2H, H-4'), 1.59 - 1.55 (m, 2H, H-4''), 1.42 - 1.22 (m, 6H, H-3', 5'', 6''), 0.89 - 0.83 (m, 3H, H-8''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.66 (C-11), 151.70 (C-9), 132.03 (C-2''), 122.07 (C-1''), 115.22 (C-4), 115.19 (C-3), 49.33 (C-12), 40.41 (C-5'), 40.27 (C-1'), 31.50 (C-3''), 29.50 (C-4''), 29.45 (C-5''), 28.76 (C-4'), 25.52 (C-3'), 22.52 (C-7''), 14.42 (C-8''). Purity 90%. HRMS *m/z* [M+H]⁺: 460.2304 (Calc. for C₂₁H₂₉N₇O₅⁺ 460.2230).

(E)-3-[3-(2-Octyl-1*H*-1,2,3-triazol-1-yl)pentyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3504



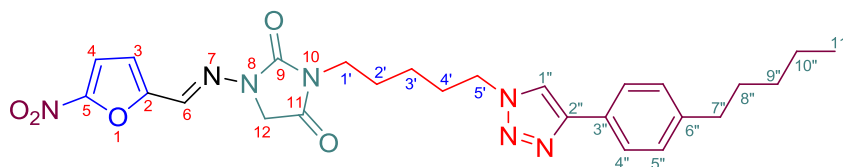
The reaction of **B2** (1.43 mmol) with 1-decyne yielded **3504** as a yellow powder, 426 mg (61%), m.p.: 135.4-135.6 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3125 (C-H, alkene), 2952 (C-H, alkane), 1780 (C=O), 1709 (C=O), 1496 (NO₂), 1370 (C-N), 1252 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-6), 7.83 (s, 1H, H-1''), 7.80 (d, *J* = 3.9 Hz, 1H, H-4), 7.18 (d, *J* = 3.9 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 4.27 (t, *J* = 5.6 Hz, 2H, H-5'), 3.44 (t, *J* = 6.6 Hz, 2H, H-1'), 2.69 - 2.57 (m, 2H, H-3''), 1.82 - 1.79 (m, 2H, H-4'), 1.58 - 1.53 (m, 2H, H-4''), 1.27 - 1.23 (m, 12H, H-3',-5''...-9''), 0.86 - 0.84 (m, 3H, H-10''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-11), 153.37 (C-9), 152.23 (C-2,5), 132.02 (C-2''), 115.19 (C-4,3), 49.32 (C-5') 48.66 (C-12), 31.75 (C-3'', 8''), 29.63 (C-5''), 29.21 (C-2'), 29.12 (C-7''), 27.17 (C-4'), 25.51 (C-3'), 22.58 (C-9''), 14.45 (C-10''). Purity 90%. HRMS *m/z* [M+H]⁺: 488.2621 (Calc. for C₂₃H₃₃N₇O₅⁺ 488.2543).

(E)-3-[3-(2-*p*-Tolyl-1*H*-1,2,3-triazol-1-yl)pentyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **3505**



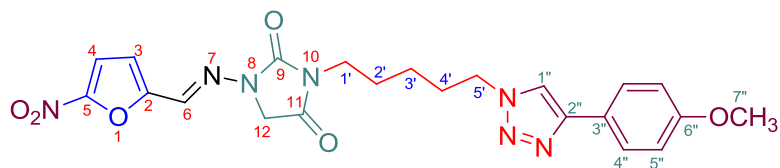
The reaction of **B2** (1.43 mmol) with 4-ethynyltoluene yielded **3505** as a yellow powder, 494 mg (74%), m.p.: 171.5-172.9 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3104 (C-H, alkene), 2919 (C-H, alkane), 1780 (C=O), 1709 (C=O), 1498 (NO₂), 1370 (C-N), 1249 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.49 (s, 1H, H-6), 7.87 (s, 1H, H-1''), 7.79 (d, *J* = 3.6 Hz, 1H, H-4), 7.71 (d, *J* = 7.8 Hz, 2H, H-4''), 7.24 (d, *J* = 7.8 Hz, 2H, H-5''), 7.17 (d, *J* = 3.6 Hz, 1H, H-3), 4.41 – 4.38 (m, 4H, H-12, -5'), 3.48 (t, *J* = 6.6 Hz, 2H, H-1'), 2.51 (s, 3H, H-7''), 1.62 – 1.57 (m, 2H, H-2'), 1.48 – 1.31 (m, 2H, H-3'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.96 (C-11), 153.19 (C-9), 152.25 (C-1, 5), 146.81 (C-2''), 132.19 (C-1'), 129.88 (C-5''), 128.56 (C-3''), 125.52 (C-4''), 115.14 (C-4, 3), 51.25 (C-5'), 49.69 (C-12), 40.44 (C-1'), 29.44 (C-2'), 27.47 (C-4'), 23.30 (C-3'), 21.31 (C-7''). Purity 90%. HRMS *m/z* [M+H]⁺: 466.1848 (Calc. for C₂₂H₂₃N₇O₅⁺ 466.1761).

(E)-3-{3-[2-(6-Pentylphenyl)-1*H*-1,2,3-triazol-1-yl]pentyl}-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **3506**



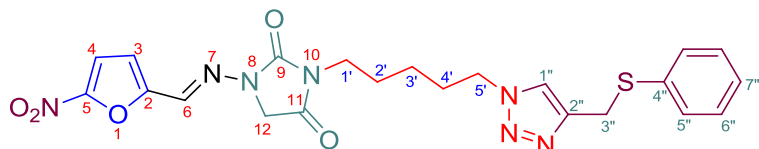
The reaction of **B2** (1.43 mmol) with 1-ethynyl-4-pentylbenzene yielded **3506** as a yellow powder, 320 mg (43%), m.p.: 182.5-182.6 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3016 (C-H, alkene), 2954 (C-H, alkane), 1782 (C=O), 1720 (C=O), 1498 (NO₂), 1375 (C-N), 1252 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.51 (s, 1H, H-6), 7.87 (s, 1H, H-1''), 7.80 (d, *J* = 3.9 Hz, 1H, H-4), 7.73 (d, *J* = 7.9 Hz, 2H, H-4''), 7.24 (d, *J* = 7.9 Hz, 2H, H-5''), 7.16 (d, *J* = 3.9 Hz, 1H, H-3), 4.48 (t, *J* = 6.7 Hz, 2H, H-5'), 4.35 (s, 2H, H-12), 3.58 (t, *J* = 6.7 Hz, 2H, H-1'), 2.57 (t, *J* = 7.6 Hz, 2H, H-7''), 2.28 - 2.22 (m, 2H, H-4'), 1.59 – 1.55 (m, 2H, H-8''), 1.42 – 1.29 (m, 4H, H-3', 9''), 0.87 (t, *J* = 6.4 Hz, 3H, H-11''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.89 (C-11), 153.33 (C-9), 152.20 (C-1, 5), 146.77 (C-2''), 142.50 (C-6''), 132.06 (C-6), 129.22 (C-1'', 5''), 128.70 (C-3''), 125.58 (C-4''), 121.59 (C-), 115.20 (C-4, 3), 48.81 (C-12), 40.98 (C-1'), 35.32 (C-7''), 31.01 (C-3', 9''), 28.32 (C-4'), 22.43 (C-10''), 14.41 (C-11''). Purity 89%. HRMS *m/z* [M+H]⁺: 522.2456 (Calc. for C₂₆H₃₁N₇O₅⁺ 522.2387)

(E)-3-{3-[2-(6-Methoxyphenyl)-1H-1,2,3-triazol-1-yl]pentyl}-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3507



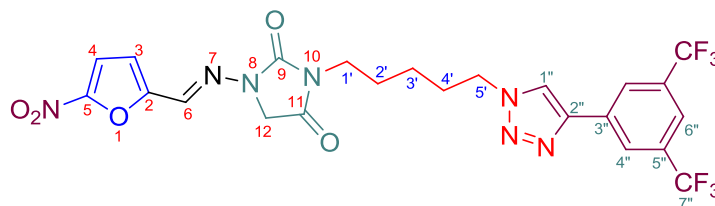
The reaction of **B2** (1.43 mmol) with 1-ethynyl-4-methoxybenzene yielded **3507** as a yellow powder, 139 mg (20 %), m.p.: 204.1-204.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3134 (C-H, alkene), 2938 (C-H, alkane), 1780 (C=O), 1716 (C=O), 1496 (NO₂), 1368 (C-N), 1246 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.45 (s, 1H, H-6), 7.87 (s, 1H, H-1''), 7.80 (d, *J* = 3.6 Hz, 1H, H-4), 7.74 (t, *J* = 7.2 Hz, 2H, H-4''), 7.17 (d, *J* = 3.6 Hz, 1H, H-3), 6.99 (t, *J* = 7.2 Hz, 2H, H-5''), 4.41 – 4.36 (m, 4H, H-5', 12), 3.78 (s, 3H, H-7''), 3.47 (d, *J* = 6.4 Hz, 2H, H-1'), 1.93 – 1.86 (m, 2H, H-4'), 1.62 – 1.59 (m, 2H, H-2'), 1.35 – 1.32 (m, 2H, H-3'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.88 (C-11), 160.59 (C-6''), 153.40 (C-9), 152.21 (C-2, 5), 147.99 (C-2''), 132.05 (C-6, 1''), 126.91 (C-4''), 120.69 (C-3''), 115.21 (C-4, 3), 114.71 (C-5''), 55.60 (C-7''), 50.97 (C-5'), 49.63 (C-12), 40.41 (C-1'), 28.26 (C-2'), 27.43 (C-4'), 23.75 (C-3'). Purity 91%. HRMS *m/z* [M+H]⁺: 482.1793 (Calc. for C₂₂H₂₃N₇O₆⁺ 482.1710)

(E)-3-{3-[2-[6-(Phenylthio)methyl]-1H-1,2,3-triazol-1-yl]pentyl}-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3508



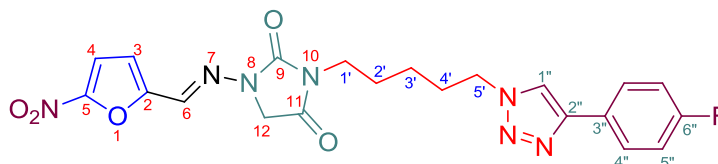
The reaction of **B2** (1.43 mmol) with phenylpropargyl sulfide yielded **3508** as a yellow powder, 408 mg (57%), m.p.: 129.6-130.5 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3125 (C-H, alkene), 2950 (C-H, alkane), 1780 (C=O), 1710 (C=O), 1480 (NO₂), 1345 (C-N), 1252 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.97 (s, 1H, H-6), 7.88 (s, 1H, H-1''), 7.80 (d, *J* = 3.9 Hz, 1H, H-4), 7.36 (d, *J* = 7.8 Hz, 2H, H-5''), 7.30 (d, *J* = 7.7 Hz, 2H, H-6''), 7.19 (d, *J* = 7.3 Hz, 1H, H-7''), 7.17 (d, *J* = 3.9 Hz, 1H, H-3), 4.41 – 4.36 (m, 4H, H-5', 3''), 4.28 (s, 2H, H-12), 3.50 (t, *J* = 6.7 Hz, 2H, H-1'), 1.81 – 1.51 (m, 6H, H-2', 4'), 1.20 – 1.01 (m, 2H, H-3'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.92 (C-11), 153.34 (C-9), 152.20 (C-2, 5), 136.31 (C-4''), 132.08, 129.47 (C-5''), 128.69 (C-6''), 123.84 (C-7''), 115.21 (C-4), 115.16 (C-3), 49.49 (C-5), 48.81 (C-12), 40.46 (C-1'), 36.33 (C-3''), 28.59 (C-4'), 27.72 (C-3'). Purity 91%. HRMS *m/z* [M+H]⁺: 498.1555 (Calc. for C₂₂H₂₃N₇O₅S⁺ 498.1481).

(E)-3-{3-[2-{6-[3,5-Bis(trifluoromethyl)phenyl]-1H-1,2,3-triazol-1-yl}pentyl]-1-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3509



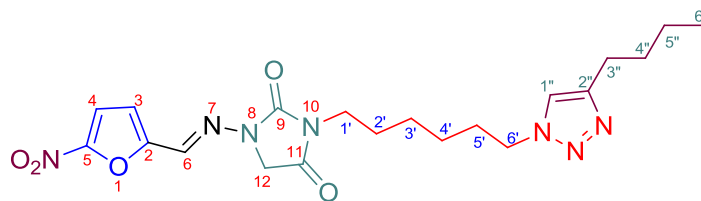
The reaction of **B2** (1.43 mmol) with 1-ethynyl-3,5-bis(trifluoromethyl)benzene yielded **3509** as a yellow powder, 377 mg (45%), m.p.: 174.6-178.8 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3157 (C-H, alkene), 2929 (C-H, alkane), 1788 (C=O), 1723 (C=O), 1531 (NO₂), 1351 (C-N), 1280 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.97 (s, 1H, H-6''), 8.50 (s, 2H, H-4''), 8.06 (s, 1H, H-6), 7.87 (s, 1H, H-1''), 7.80 (d, J = 3.8 Hz, 1H, H-4), 7.12 (d, J = 3.8 Hz, 1H, H-3), 4.54 (t, J = 6.8 Hz, 2H, H-5'), 4.35 (s, 2H, H-12), 3.61 (t, J = 6.8 Hz, 2H, H-1'), 1.95 – 1.87 (m, 2H, H-4'), 1.63 – 1.55 (m, 2H, H-2'), 1.35 – 1.22 (m, 2H, H-3'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.87 (C-11), 158.02 (C-9), 153.28 (C-2), 152.12 (C-5), 145.05 (C-2''), 144.03, 134.69, 133.82 (C-3''), 131.60 (C-5''), 131.38 (C-1''), 122.82 (C-4''), 121.85 (C-6''), 115.14 (C-4, 3), 48.81 (C-12), 40.41 (C-1'), 34.78 (C-2'), 28.04 (C-3'). Purity 89%. HRMS m/z [M+H]⁺: 588.1382 (Calc. for C₂₃H₁₉F₆N₇O₅⁺ 588.1352).

(E)-3-{3-[2-(6-fluorophenyl)]-1H-1,2,3-triazol-1-yl]pentyl]-1-[[5-Nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3510



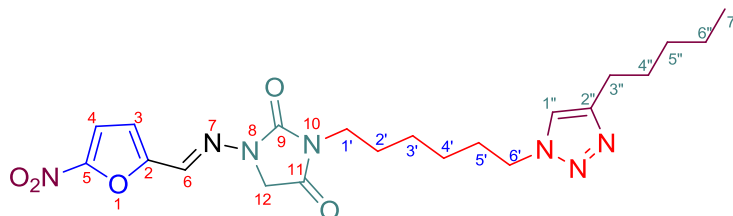
The reaction of **B2** (1.43 mmol) with 1-ethynyl-4-fluorobenzene yielded **3510** as a yellow powder, 311 mg (46%), m.p.: 143.7-145.7 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3128 (C-H, alkene), 2934 (C-H, alkane), 1780 (C=O), 1710 (C=O), 1497 (NO₂), 1343 (C-N), 1234 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.55 (s, 1H, H-6), 7.88 – 7.84 (m, 3H, H-4'', -1''), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.30 – 7.24 (m, 1H, H-5''), 7.17 (d, J = 3.9 Hz, 1H, H-3), 4.48 (t, J = 6.8 Hz, 2H, H-5'), 4.42 – 4.39 (m, 4H, H-12, 5'), 3.47 (t, J = 6.8 Hz, 2H, H-1'), 1.93 – 1.90 (m, 2H, H-4'), 1.57 – 1.46 (m, 2H, H-2'), 1.33 – 1.29 (m, 2H, H-3'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.87 (C-11), 162.91 (C-6''), 161.33 (C-9), 152.21 (C-5, 2), 145.88 (C-2''), 132.05 (C-6), 127.89 (C-1''), 127.59 (C-3''), 116.34 (C-5''), 116.20 (C-4), 115.17 (C-3), 50.96 (C-5'), 49.74 (C-12), 29.41 (C-2'), 27.43 (C-4'), 23.23 (C-3'). Purity 92%. HRMS m/z [M+H]⁺: 470.1597 (Calc. for C₂₁H₂₀FN₇O₅⁺ 470.1510).

(E)-3-[3-(2-Butyl-1*H*-1,2,3-triazol-1-yl)hexyl]-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **3601**



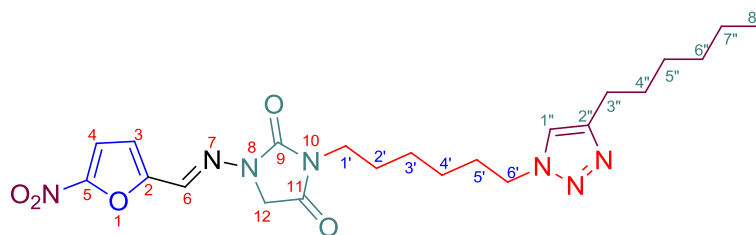
The reaction of **B3** (1.38 mmol) with 1-hexyne yielded **3601** as a yellow powder, 284 mg (46%), m.p.: 111.2-113.4 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3128 (C-H, alkene), 2954 (C-H, alkane), 1778 (C=O), 1709 (C=O), 1554 (NO₂), 1376 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-6), 7.83 (s, 1H, H-1''), 7.80 (d, *J* = 3.9 Hz, 1H, H-4), 7.17 (d, *J* = 3.9 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 4.26 (t, *J* = 5.2 Hz, 2H, H-6'), 3.45 (t, *J* = 7.0 Hz, 2H, H-1'), 2.59 (t, *J* = 7.6 Hz, 2H, H-3''), 1.79 – 1.73 (m, 4H, H-2', 5'), 1.59 – 1.52 (m, 2H, H-4''), 1.34 – 1.27 (m, 4H, H-4', 5''), 0.88 (t, *J* = 6.4 Hz, 3H, H-6''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-11), 152.23 (C-9), 147.27 (C-2), 132.01 (C-5, 2''), 122.02 (C-1''), 115.16 (C-4, 3), 49.46 (C-12), 40.42 (C-1'), 31.62 (C-3''), 29.97 (C-5'), 25.70 (C-4'), 25.17 (C-3'), 22.15 (C-5''), 14.17 (C-6''). Purity 91%. HRMS *m/z* [M+H]⁺: 446.2153 (Calc. for C₂₀H₂₇N₇O₅⁺ 446.2074).

(E)-3-[3-(2-Pentyl-1*H*-1,2,3-triazol-1-yl)hexyl]-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **3602**



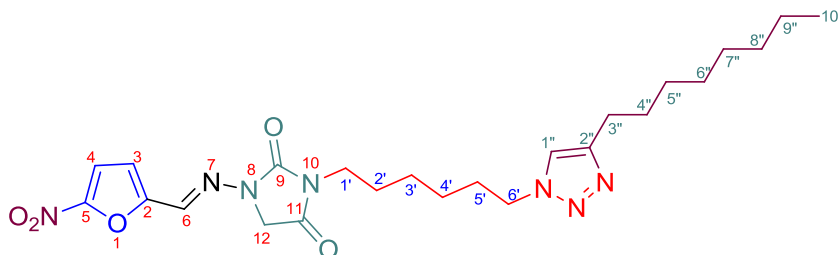
The reaction of **B3** (1.38 mmol) with 1-heptyne yielded **3602** as a yellow powder, 391 mg (62%), m.p.: 122.3-125.6 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3129 (C-H, alkene), 2954 (C-H, alkane), 1778 (C=O), 1708 (C=O), 1344 (C-N), 1249 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-6), 7.84 (s, 1H, H-1''), 7.80 (d, *J* = 3.9 Hz, 1H, H-4), 7.17 (d, *J* = 3.9 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 4.29 (d, *J* = 5.4 Hz, 2H, H-6'), 3.44 (d, *J* = 7.1 Hz, 2H, H-1'), 2.58 (t, *J* = 7.6 Hz, 2H, H-3''), 1.81 – 1.72 (m, 2H, H-5'), 1.58 – 1.52 (m, 4H, H-2', 4''), 1.31 – 1.23 (m, 8H, H-3', 4', 5'', 6''), 0.86 (t, *J* = 6.8 Hz, 3H, H-7'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.35 (C-11), 153.20 (C-9), 151.70 (C-5, 1), 122.03 (C-1''), 114.65 (C-4, 3), 49.45 (C-12), 40.42 (C-1'), 31.29 (C-3'', 5''), 29.97 (C-2'), 29.17 (C-2''), 25.70 (C-4'), 25.47 (C-3'), 22.34 (C-6''), 14.38 (C-7''). Purity 93%. HRMS *m/z* [M+H]⁺: 460.2306 (Calc. for C₂₁H₂₉N₇O₅⁺ 460.2230).

(E)-3-[3-(2-Hexyl-1*H*-1,2,3-triazol-1-yl)hexyl]-1-[[5-nitrofur-2-yl)methylene]amino}imidazolidine-9,11-dione, 3603



The reaction of **B3** (1.38 mmol) with 1-octyne yielded **3603** as a yellow powder, 307 mg (47 %), m.p.: 128.9-130.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3124 (C-H, alkene), 2954 (C-H, alkane), 2040, 1778 (C=O), 1709 (C=O), 1554 (NO₂), 1347 (C-N), 1254 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.86 (s, 1H, H-6), 7.83 (s, 1H, H-1''), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.17 (d, $J = 3.9$ Hz, 1H, H-3), 4.40 (s, 2H, H-12), 4.29 – 4.26 (m, 2H, H-6'), 3.45 (t, $J = 7.0$ Hz, 2H, H-1'), 2.58 (t, $J = 7.6$ Hz, 2H, H-3''), 1.79 – 1.75 (m, 2H, H-5'), 1.61 - 1.54 (m, 4H, H-2', 4''), 1.27 – 1.19 (m, 10H, H-3', 4', 5'', 6''), 0.91 – 0.87 (m, 3H, H-8''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-11), 152.22 (C-9, 5), 132.01 (C-2''), 122.05 (C-1''), 115.17 (C-4, 3), 49.51 (C-6'), 48.65 (C-12), 28.71 (C-5''), 27.69 (C-4''), 25.90 (C-4'), 25.50 (C-3'), 22.51 (C-7''), 14.41 (C-8''). Purity 93%. HRMS m/z [M+H]⁺: 474.2476 (Calc. for C₂₂H₃₁N₇O₅⁺ 474.2387).

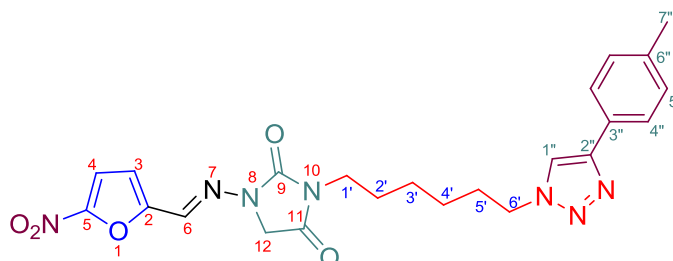
(E)-3-[3-(2-Octyl-1*H*-1,2,3-triazol-1-yl)hexyl]-1-[[5-nitrofur-2-yl)methylene]amino}imidazolidine-9,11-dione, 3604



The reaction of **B3** (1.38 mmol) with 1-decyne yielded **3604** as a yellow powder, 197 mg (28%), m.p.: 202.5-202.6 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3123 (C-H, alkene), 2954 (C-H, alkane), 2850, 2034, 1778 (C=O), 1708 (C=O), 1555 (NO₂), 1455, 1347 (C-N), 1255 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.87 (s, 1H, H-6), 7.84 (s, 1H, H-1''), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.17 (d, $J = 3.9$ Hz, 1H, H-3), 4.40 (s, 2H, H-12), 4.28 (t, $J = 5.7$ Hz, 2H, H-6'), 3.45 (t, $J = 6.8$ Hz, 2H, H-1'), 2.58 (t, $J = 7.6$ Hz, 2H, H-3''), 1.80 – 1.75 (m, 2H, H-5'), 1.58 – 1.52 (m, 4H, H-2', 4''), 1.28 – 1.23 (m, 14H, H-3', 4', 5''...9''), 0.95 - 0.84 (m, 3H, H-10''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-11), 152.22 (C-9, -5), 132.01 (C-6, -2''), 122.05 (C-1''), 115.21 (C-4, -3), 49.51 (C-6'), 48.65 (C-12), 31.75 (C-8''), 30.06 (C-2''), 29.48 (C-7''), 29.22

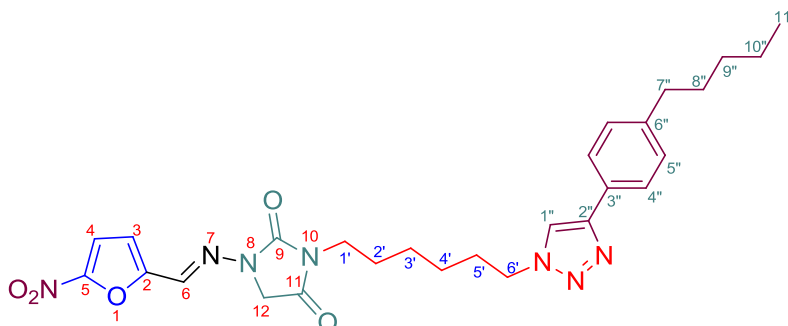
(C-6''), 29.12 (C-5''), 29.06 (C-4''), 27.69 (C-5'), 25.90 (C-4'), 25.50 (C-3'), 22.58 (C-9''), 14.45 (C-10''). Purity 93%. HRMS m/z $[M+H]^+$: 502.2759 (Calc. for $C_{24}H_{35}N_7O_5^+$ 502.2700).

(E)-3-{3-[2-(*p*-Tolyl)-1*H*-1,2,3-triazol-1-yl]hexyl}-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3605



The reaction of **B3** (1.38 mmol) with 4-ethynyltoluene yielded **3605** as a yellow powder, 264 mg (40%), m.p.: 197.3-197.8 °C. IR (ATR) $\nu_{\max}/\text{cm}^{-1}$: 3108 (C-H, alkene), 2930 (C-H, alkane), 1774 (C=O), 1708 (C=O), 1564 (NO₂), 1370 (C-N), 1253 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.51 (s, 1H, H-6), 7.86 (s, 1H, H-1''), 7.79 (d, J = 3.9 Hz, 1H, H-4), 7.72 (d, J = 7.9 Hz, 2H, H-4''), 7.25 (d, J = 7.9 Hz, 2H, H-5''), 7.17 (d, J = 3.9 Hz, 1H, H-3), 4.42 – 4.38 (m, 4H, H-12, 6'), 3.46 (t, J = 6.7 Hz, 2H, H-1'), 2.33 (s, 3H, H-7''), 1.59 – 1.50 (m, 2H, H-2'), 1.35 – 1.26 (m, 4H, H-3', 4'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-11), 153.39 (C-9), 152.23 (C-2, -5), 146.81 (C-2''), 132.02 (C-6), 129.88 (C-1''), 128.59 (C-3''), 125.52 (C-4''), 121.27 (C-4''), 115.19 (C-4), 115.14 (C-3), 49.88 (C-5'), 48.65 (C-12), 29.97 (C-5', -2'), 27.69 (C-4'), 25.94 (C-3'), 21.31 (C-7''). Purity 92%. HRMS m/z $[M+H]^+$: 480.1980 (Calc. for $C_{23}H_{25}N_7O_5^+$ 480.1917).

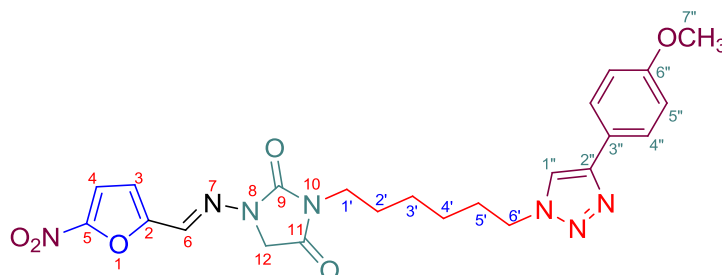
(E)-3-{3-[2-(6-Pentylphenyl)-1*H*-1,2,3-triazol-1-yl]hexyl}-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3606



The reaction of **B3** (1.38 mmol) with 1-ethynyl-4-pentylbenzene yielded **3606** as a yellow powder, 359 mg (49%), m.p.: 129.6-131.0 °C. IR (ATR) $\nu_{\max}/\text{cm}^{-1}$: 3128 (C-H, alkene), 2927 (C-H, alkane), 1778 (C=O), 1707 (C=O), 1496 (NO₂), 1373 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.52 (s, 1H, H-6), 7.87 (s, 1H, H-1''), 7.79 (d, J = 3.8 Hz, 1H, H-4), 7.73 (d, J = 7.8 Hz, 2H, H-4''), 7.25 (d, J = 7.8 Hz, 2H, H-5''), 7.17 (d, J = 3.8 Hz, 1H, H-3), 4.43 –

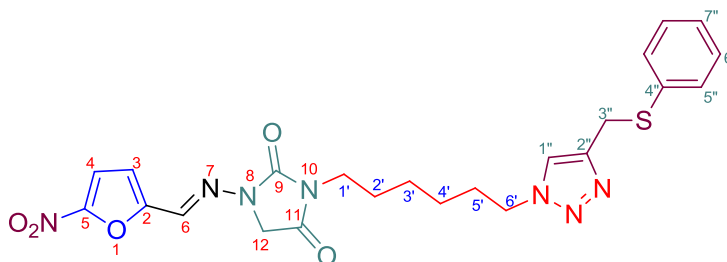
4.36 (m, 4H, H-12, 6'), 3.47 (t, $J = 6.9$ Hz, 2H, H-1'), 2.59 (t, $J = 7.5$ Hz, 2H, H-7''), 1.88 – 1.83 (m, 2H, H-5'), 1.60 – 1.50 (m, 4H, H-2', 8''), 1.40 – 1.23 (m, 6H, H-3', 4', 9''), 0.86 (t, $J = 6.9$ Hz, 3H, H-11''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.85 (C-11), 153.39 (C-9), 152.23 (C-2, 5), 132.02 (C-6''), 129.22 (C-5''), 125.55 (C-4''), 115.19 (C-4), 115.14 (C-3), 51.03 (C-6'), 48.66 (C-12), 40.44 (C-1'), 35.33 (C-7''), 31.35 (C-9''), 31.01 (C-8''), 28.58 (C-5'), 26.05 (C-4'), 25.93 (C-3'), 22.43 (C-10''), 14.40 (C-11''). Purity 89%. HRMS m/z $[\text{M}+\text{H}]^+$: 536.2563 (Calc. for $\text{C}_{27}\text{H}_{33}\text{N}_7\text{O}_5^+$ 536.2543).

(E)-3-{3-[2-(6-Methoxyphenyl)-1H-1,2,3-triazol-1-yl]hexyl}-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3607



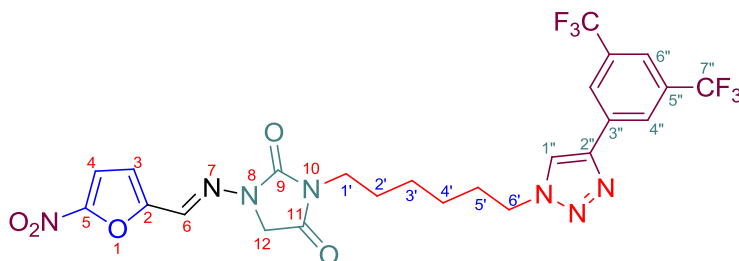
The reaction of **B3** (1.38 mmol) with 1-ethynyl-4-methoxybenzene yielded **3607** as a yellow powder, 95 mg (14%), m.p.: 171.7-173.0 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3103 (C-H, alkene), 2930 (C-H, alkane), 1771 (C=O), 1709 (C=O), 1561 (NO_2), 1370 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.47 (s, 1H, H-6), 7.86 (s, 1H, H-1''), 7.80 (d, $J = 3.9$ Hz, 1H, H-4), 7.76 (d, $J = 8.6$ Hz, 2H, H-4''), 7.17 (d, $J = 3.9$ Hz, 1H, H-3), 7.00 (d, $J = 8.6$ Hz, 2H, H-5''), 4.40 (s, 2H, H-12), 4.37 (t, $J = 6.9$ Hz, 2H, H-6'), 3.79 (s, 3H, H-7''), 3.46 (t, $J = 6.9$ Hz, 2H, H-1'), 1.88 – 1.83 (m, 2H, H-5'), 1.58 – 1.53 (m, 2H, H-2'), 1.34 – 1.28 (m, 4H, H-3', 4'). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.86 (C-11), 159.39 (C-6''), 153.39 (C-9), 152.22 (C-2, -5), 146.67 (C-2''), 132.01 (C-6, -1''), 126.91 (C-4''), 123.96 (C-3''), 115.20 (C-4, 3), 114.75 (C-5''), 55.62 (C-7''), 49.85 (C-6'), 48.65 (C-12), 40.42 (C-1'), 29.99 (C-2'), 27.70 (C-4'), 25.95 (C-3'). Purity 93%. HRMS m/z $[\text{M}+\text{H}]^+$: 496.1963 (Calc. for $\text{C}_{23}\text{H}_{25}\text{N}_7\text{O}_6^+$ 496.1866).

(E)-3-{3-{2-[6-(phenylthio)methyl]-1H-1,2,3-triazol-1-yl}hexyl}-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3608



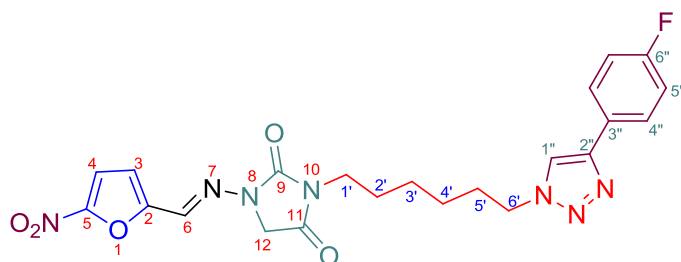
The reaction of **B3** (1.38 mmol) with phenylpropargyl sulfide yielded **3608** as a yellow powder, 435 mg (62 %), m.p.: 193.2-194.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3122 (C-H, alkene), 2928 (C-H, alkane), 1778 (C=O), 1707 (C=O), 1532 (NO₂), 1370 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.92 (s, 1H, H-6), 7.87 (s, 1H, H-1''), 7.79 (d, J = 3.9 Hz, 1H, H-4), 7.35 (d, J = 7.5 Hz, 2H, H-5''), 7.35 (d, J = 7.5 Hz, 2H, H-6''), 7.19 (d, J = 7.5 Hz, 1H, H-7''), 7.18 (d, J = 3.9 Hz, 1H, H-3), 4.41 (s, 2H, H-12), 4.30 – 4.24 (m, 4H, H-3'', 6'), 3.44 (t, J = 6.8 Hz, 2H, H-1'), 1.79 – 1.65 (m, 2H, H-5'), 1.56 – 1.48 (m, 2H, H-2'), 1.31 – 1.22 (m, 2H, H-3'), 1.20 – 1.08 (m, 2H, H-4'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.84 (C-11), 153.20 (C-9), 152.23 (C-5, -2), 136.24 (C-4''), 132.03 (C-6), 129.43 (C-5''), 128.75 (C-6''), 128.72 (C-2''), 126.38 (C-7''), 123.56 (C-1''), 115.19 (C-4), 115.14 (C-3), 49.68 (C-6'), 48.66 (C-12), 40.44 (C-1'), 38.73 (C-3''), 30.00 (C-2'), 27.68 (C-5'), 25.80 (C-4'), 25.56 (C-3'). Purity 88%. HRMS m/z [M+H]⁺: 512.1711 (Calc. for C₂₃H₂₅N₇O₅S⁺ 512.1638).

(E)-3-{2-[6-[3,5-Bis(trifluoromethyl)phenyl]-1H-1,2,3-triazol-1-yl]hexyl}-1-[(5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 3609



The reaction of **B3** (1.38 mmol) with 1-ethynyl-3,5-bis(trifluoromethyl)benzene yielded **3609** as a yellow powder, 88 mg (11%), m.p.: 182.5-183.2 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3152 (C-H, alkene), 2931 (C-H, alkane), 1778 (C=O), 1707 (C=O), 1530 (NO₂), 1345 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.98 (d, J = 2.5 Hz, 1H, H-6''), 8.50 (d, J = 2.5 Hz, 2H, H-4''), 8.06 (s, 1H, H-6), 7.87 (s, 1H, H-1''), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.17 (d, J = 3.9 Hz, 1H, H-3), 4.46 – 4.39 (m, 4H, H-12, 6'), 3.47 (t, J = 7.0 Hz, 2H, H-1'), 1.92 – 1.85 (m, 2H, H-5'), 1.58 – 1.50 (m, 2H, H-2'), 1.33 – 1.30 (m, 4H, H-3', 4'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.86 (C-11), 153.40 (C-9), 152.22 (C-2, 5), 144.11 (C-2''), 133.75 (C-3''), 132.01 (C-5''), 130.98 (C-1''), 125.74 (C-7''), 121.20 (C-6''), 115.20 (C-4, 3), 51.02 (C-6'), 48.66 (C-12), 40.41 (C-1'), 28.58 (C-5'), 26.18 (C-4'), 26.05 (C-3'). Purity 88%. HRMS m/z [M+H]⁺: 602.1567 (Calc. for C₂₄H₂₁F₆N₇O₅⁺ 602.1508).

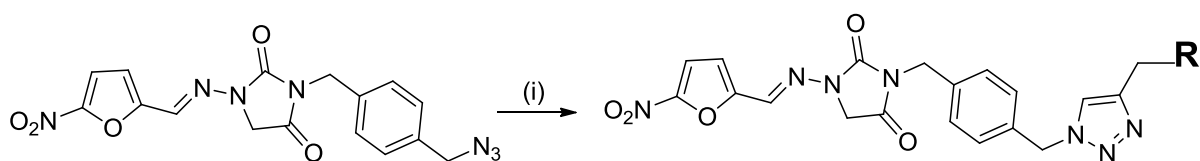
(E)-3-{3-[2-(6-Fluorophenyl)]-1H-1,2,3-triazol-1-yl]hexyl}-1-[[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-9,11-dione, 3610



The reaction of **B3** (1.38 mmol) with 1-ethynyl-4-fluorobenzene yielded **3610** as a yellow powder, 81 mg (12%), m.p.: 202.5-202.6 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3103 (C-H, alkene), 2930 (C-H, alkane), 1771 (C=O), 1713 (C=O), 1561 (NO₂), 1370 (C-N), 1246 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.58 (s, 1H, H-6), 7.89 – 7.84 (dd, J = 7.7, 2.5 Hz, 2H, H-4''), 7.86 (s, 1H, H-1''), 7.80 (d, J = 3.9 Hz, 1H, H-4), 7.28 (t, J = 7.7 Hz, 2H, H-5''), 7.17 (d, J = 3.9 Hz, 1H, H-3), 4.41 – 4.38 (m, 4H, H-12, 6'), 3.46 (t, J = 6.9 Hz, 2H, H-1'), 1.88 – 1.84 (m, 2H, H-5'), 1.57 – 1.54 (m, 2H, H-2'), 1.36 – 1.25 (m, 4H, H-3', 4'). ¹³C NMR (151 MHz, DMSO) δ (ppm): 169.54 (C-11), 161.11 (C-6''), 153.40 (C-9), 152.21 (C-2, 5), 145.89 (C-2''), 132.01 (C-1'', 4''), 127.93 (C-3''), 116.35 (C-5''), 116.21 (C-4), 115.20 (C-3), 49.94 (C-6'), 48.66 (C-12), 40.42 (C-1'), 29.97 (C-5'), 27.70 (C-4'), 25.94 (C-3'). Purity 90%. HRMS m/z [M+H]⁺: 484.1746 (Calc. for C₂₂H₂₂FN₇O₅⁺ 484.1666).

Synthesis of hybrids, 401-409

Intermediate **B4**, (0.4 g) was dissolved in 10 mL of DMSO:water (4:1, v/v). To the resulting solution, the appropriate alkyne (1.5 eq.) was added, after which catalytic amounts of β -cyclodextrin (0.02 eq), sodium ascorbate (0.3 eq) and copper sulphate pentahydrate (0.1 eq) were added in sequence (Scheme 5.6). After 12 h an additional amount of sodium ascorbate (0.3 eq) was added. The reaction was stirred at room temperature and monitored by TLC, eluting with DCM:MeOH (19:1, v/v). Upon completion, the reaction was quenched with 20 mL of water and extracted with ethyl acetate (3 X 20 mL). The organic layer was washed once with saturated NH₄Cl and once with water. The solution was concentrated *in vacuo*. The resulting paste was purified by recrystallisation in ethanol/water to afford the desired analogue.

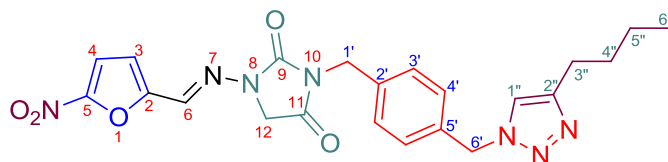


intermediate B4				hybrids, 401 - 409					
Cpd	R	Cpd	R	Cpd	R	Cpd	R	Cpd	R
401	<i>n</i> -C ₄ H ₉ --	403	<i>n</i> -C ₆ H ₁₃ --	405		407		409	
402	<i>n</i> -C ₅ H ₁₁ --	404		406		408			

Scheme 5.6: Synthesis of aryl NFT-Tz hybrids.

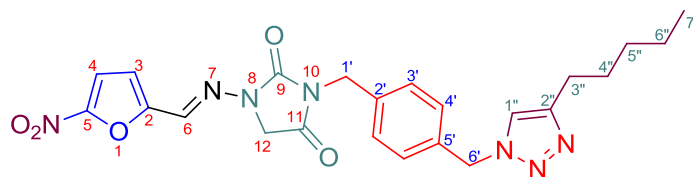
Reagents and conditions: (i) **401 - 403**: *n*-alkyne (1.5 eq), **404 - 409**: phenyl ethynyl (1.5 eq), β -cyclodextrin (0.02 eq), sodium ascorbate (0.3 eq) and copper sulphate pentahydrate (0.1 eq), 10 ml DMSO:water, rt, 48 h.

(E)-10-{8-[(2-Butyl-1*H*-1,2,3-triazol-1-yl)methyl]benzyl}-6-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **401**



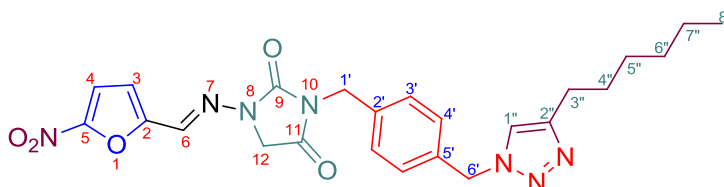
The reaction of **B4** (1.30 mmol) with 4-methoxy benzyl bromide yielded **401** as a yellow powder, 220 mg (45%), m.p.: 191.2-192.4 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3143 (C-H, alkene), 2916 (C-H, alkane), 1780 (C=O), 1721 (C=O), 1561 (NO₂), 1368 (C-N), 1250 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.88 (s, 1H, H-6), 7.79 (s, 1H, H-1''), 7.35 (d, *J* = 3.7 Hz, 1H, H-4), 7.30 (d, *J* = 7.6 Hz, 2H, H-3'), 7.25 (d, *J* = 7.6 Hz, 2H, H-4'), 7.17 (d, *J* = 3.7 Hz, 1H, H-3), 5.51 (s, 2H, H-1'), 4.65 (s, 2H, H-6'), 4.49 (s, 2H, H-12), 2.58 (t, *J* = 6.6 Hz, 2H, H-3''), 1.58 – 1.50 (m, 2H, H-5''), 1.33 – 1.24 (m, 2H, H-4''), 0.88 (t, *J* = 6.6 Hz 3H, H-6''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.74 (C-11), 153.18 (C-9), 152.31 (C-2), 152.10 (C-5), 136.26 (C-5'), 136.15 (C-2'), 132.32 (C-2''), 128.84 (C-6), 128.53 (C-3'), 122.34 (C-1''), 115.33 (C-4), 115.17 (C-3), 63.06 (C-6'), 52.78 (C-1'), 48.89 (C-12), 31.57 (C-3''), 22.16 (C-5''), 14.16 (C-6''). Purity 90%. HRMS *m/z* [M+H]⁺: 466.1816 (Calc. for C₂₂H₂₃N₇O₅⁺ 466.1761).

(E)-10-{8-[(2-Pentyl-1*H*-1,2,3-triazol-1-yl)methyl]benzyl}-6-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **402**



The reaction of **B4** (1.30 mmol) with 4-methoxy benzyl bromide yielded **402** as a yellow powder, 241 mg (48%), m.p.: 197.2-197.8 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3121 (C-H, alkene), 2920 (C-H, alkane), 1774 (C=O), 1708 (C=O), 1523 (NO₂), 1369 (C-N), 1247 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 7.88 (s, 1H, H-6), 7.79 (s, 1H, H-1''), 7.33 (d, J = 3.7 Hz, 2H, H-4), 7.28 (d, J = 7.6 Hz, 2H, H-3'), 7.23 (d, J = 7.6 Hz, 2H, H-4'), 7.17 (d, J = 3.7 Hz, 1H, H-3), 5.51 (s, 2H, H-1'), 4.67 (s, 2H, H-6'), 4.49 (s, 2H, H-12), 2.57 (t, J = 7.6 Hz, 2H, H-3''), 1.59 – 1.56 (m, 2H, H-4''), 1.32 – 1.26 (m, 4H, H-5'', -6''), 0.91 - 0.85 (m, 3H, H-7''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.73 (C-11), 153.18 (C-9), 152.30 (C-2), 152.10 (C-5), 147.77 (C-2''), 136.26 (C-5'), 136.16 (C-2'), 132.32 (C-2''), 128.53 (C-3'), 127.85 (C-4'), 122.35 (C-1''), 115.32 (C-4), 115.17 (C-3), 63.06 (C-6'), 52.78 (C-1'), 48.89 (C-12), 31.30 (C-3''), 29.11 (C-5''), 25.43 (C-4''), 22.31 (C-6''), 14.37 (C-7''). Purity 92%. HRMS m/z [M+H]⁺: 480.1976 (Calc. for C₂₃H₂₅N₇O₅⁺ 480.1917).

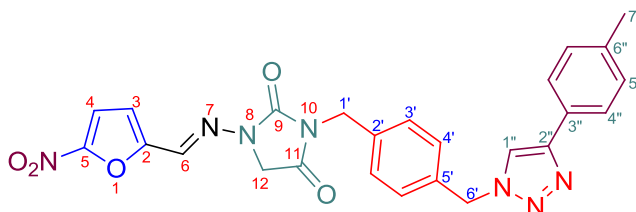
(E)-10-{8-[(2-Hexyl-1*H*-1,2,3-triazol-1-yl)methyl]benzyl}-6-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **403**



The reaction of **B4** (1.30 mmol) with 4-methoxy benzyl bromide yielded **403** as a yellow powder, 138 mg (27%), m.p.: 197.2-219.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3121 (C-H, alkene), 2920 (C-H, alkane), 1774 (C=O), 1708 (C=O), 1523 (NO₂), 1342 (C-N), 1247 (C-N). ¹H NMR (600 MHz, DMSO) δ 8.32 (s, 1H, H-6), 7.89 (s, 2H, H-1''), 7.78 (d, J = 3.8 Hz, 1H, H-4), 7.37 (d, J = 7.6 Hz, 2H, H-3'), 7.23 (d, J = 7.6 Hz, 2H, H-4'), 7.17 (d, J = 3.8 Hz, 1H, H-3), 5.51 (s, 2H, H-1'), 5.16 (s, 2H, H-6'), 4.49 (s, 2H, H-12), 2.57 (t, J = 7.5 Hz, 2H, H-3''), 1.58 – 1.54 (m, 2H, H-4''), 1.33 – 1.18 (m, 6H, H-7'', -5'', -6''), 0.96 - 0.85 (m, 3H, H-8''). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.73 (C-11), 153.18 (C-9), 152.31 (C-2), 152.11 (C-5), 136.25 (C-5'), 136.17 (C-2'), 135.56 (C-5'), 132.32 (C-6), 128.84 (C-2''), 128.52 (C-3), 127.85 (C-4), 122.38 (C-1''), 115.32 (C-4), 115.17 (C-3), 65.03 (C-6'), 52.78 (C-1'), 48.89 (C-12), 31.73 (C-3''),

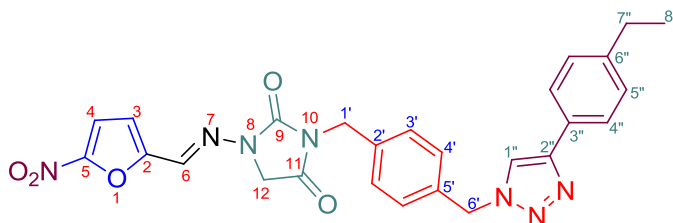
29.10 (C-5"), 29.08 (4"), 22.56 (C-7"), 14.43 (C-8"). Purity 89%. HRMS m/z $[M+H]^+$: 494.2133 (Calc. for $C_{24}H_{27}N_7O_5^+$ 494.2107).

(E)-10-{8-[(2-*p*-Tolyl-1*H*-1,2,3-triazol-1-yl)methyl]benzyl}-6-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **404**



The reaction of **B4** (1.30 mmol) with 4-methoxy benzyl bromide yielded **404** as a yellow powder, 287 mg (55 %), m.p.: 197.2-219.1 °C. IR (ATR) $\nu_{\max}/\text{cm}^{-1}$: 3135 (C-H, alkene), 1798 (C=O), 1720 (C=O), 1521 (NO₂), 1348 (C-N), 1253 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.32 (s, 1H, H-6), 7.89 (s, 1H, H-1"), 7.80 (d, J = 3.7 Hz, 1H, H-4), 7.72 (d, J = 7.3 Hz, 2H, H-4"), 7.41 (d, J = 7.3 Hz, 2H, H-5"), 7.28 (d, J = 7.5 Hz, 2H, H-4'), 7.26 (d, J = 7.5 Hz, 1H, H-3'), 7.16 (d, J = 3.7 Hz, 1H, H-3), 5.62 (s, 2H, H-1'), 5.17 (s, 2H, H-6'), 4.48 (s, 2H, H-12), 2.32 (s, 3H, H-7"). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.74 (C-11), 162.41 (C-9), 153.23 (C-2), 152.31 (C-5), 142.44 (C-2"), 134.74 (C-5"), 132.31 (C-6), 129.90 (C-3"), 128.85 (C-1"), 128.65 (C-3'), 128.21 (C-4"), 127.06 (C-4'), 125.57 (C-6"), 115.32 (C-4), 115.18 (C-3), 65.03 (C-6'), 63.06 (C-1'), 48.85 (C-12), 21.31 (C-7"). Purity 90%. HRMS m/z $[M+H]^+$: 500.1659 (Calc. for $C_{25}H_{21}N_7O_5^+$ 500.1604).

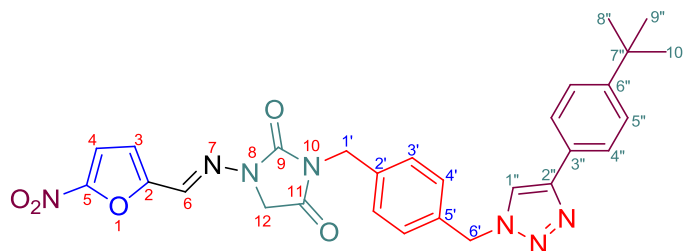
(E)-10-{8-[[2-(6-Ethylphenyl)-1*H*-1,2,3-triazol-1-yl]methyl]benzyl}-6-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **405**



The reaction of **B4** (1.30 mmol) with 1-ethyl-4-ethynylbenzene yielded **405** as a yellow powder 171 mg (32%), m.p.: 197.2-219.1 °C. IR (ATR) $\nu_{\max}/\text{cm}^{-1}$: 3147 (C-H, alkene), 2962 (C-H, alkane), 1774 (C=O), 1711 (C=O), 1523 (NO₂), 1344 (C-N), 1249 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.57 (s, 1H, H-6), 8.32 (s, 1H, H-1"), 7.89 (d, J = 3.9 Hz, 1H, H-4), 7.87 (d, J = 7.4 Hz, 2H, H-4"), 7.37 (d, J = 7.4 Hz, 2H, H-5"), 7.31 (d, J = 7.3 Hz, 2H, H-4'), 7.24 (d, J = 7.3 Hz, 2H, H-3'), 7.17 (d, J = 3.9 Hz, 1H, H-3), 5.62 (s, 2H, H-1'), 5.17 (s, 2H,

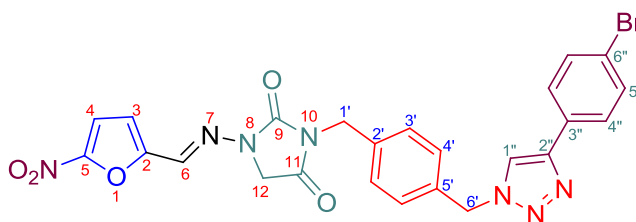
H-6'), 4.48 (s, 2H, H-12), 2.61 (q, $J = 7.6$ Hz, 2H, H-7''), 1.19 (t, $J = 7.6$ Hz, 3H, H-8''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.76 (C-11), 162.41 (C-9), 153.21 (C-2), 152.31 (C-5), 147.21 (C-2''), 143.98 (C-6''), 136.43 (C-5'), 135.56 (C-2'), 132.33 (C-1'), 128.85 (C-6), 128.71 (C-5''), 128.55 (C-3'), 127.86 (C-4'), 127.06 (C-3''), 125.66 (C-4''), 115.33 (C-4), 115.18 (C-5), 63.06 (C-6'), 53.15 (C-1'), 48.90 (C-12), 28.40 (C-7''), 15.97 (C-8''). Purity 90%. HRMS m/z $[\text{M}+\text{H}]^+$: 514.1842 (Calc. for $\text{C}_{26}\text{H}_{23}\text{N}_7\text{O}_5^+$ 514.1761).

(E)-10-{8-[[2-(*tert*-Butylphenyl)-1*H*-1,2,3-triazol-1-yl]methyl]benzyl}-6-[[5-nitrofur-2-yl)methylene]amino}imidazolidine-9,11-dione, **406**



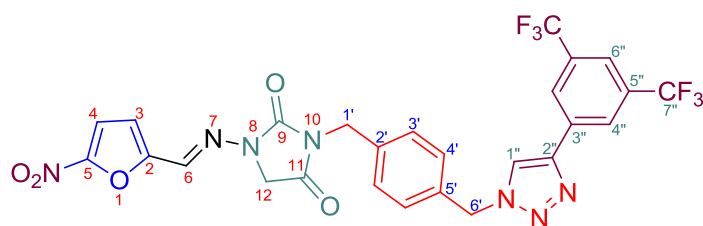
The reaction of **B4** (1.30 mmol) with 4-*tert*-butylphenylacetylene yielded **406** as a yellow powder, 107 mg (19%), m.p.: 197.2-219.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3156 (C-H, alkene), 2959 (C-H, alkane), 1773 (C=O), 1712 (C=O), 1527 (NO_2), 1346 (C-N), 1252 (C-N). ^1H NMR (600 MHz, DMSO) δ (ppm): 8.58 (s, 1H, H-6), 8.33 (s, 1H, H-1''), 7.88 (d, $J = 3.4$ Hz, 1H, H-4), 7.79 (d, $J = 7.8$ Hz, 2H, H-4''), 7.40 (d, $J = 7.8$ Hz, 2H, H-5''), 7.29 (d, $J = 6.8$ Hz, 2H, H-4'), 7.18 (d, $J = 6.8$ Hz, 2H, H-3'), 7.16 (d, $J = 3.4$ Hz, 1H, H-3), 5.62 (s, 2H, H-1'), 4.67 (s, 2H, H-6'), 4.48 (s, 2H, H-12), 1.26 (s, 9H, H-8''...10''). ^{13}C NMR (151 MHz, DMSO) δ (ppm): 167.73 (C-9), 153.18 (C-9), 152.30 (C-4), 152.10 (C-3), 150.83 (C-6''), 147.12 (C-2''), 136.41 (C-5'), 135.89 (C-2'), 132.32 (C-6), 131.92 (C-1''), 128.63 (C-3'), 128.54, 128.37, 127.86 (C-4'), 127.06 (C-3''), 126.08, 125.98 (C-4''), 125.43 (C-5''), 115.32 (C-4), 115.17 (C-3), 63.06 (C-6'), 53.13 (C-1'), 48.89 (C-12), 34.83 (C-7''), 31.34 (C-8''...9''). Purity 90%. HRMS m/z $[\text{M}+\text{H}]^+$: 542.2125 (Calc. for $\text{C}_{28}\text{H}_{27}\text{N}_7\text{O}_5^+$ 542.2074).

(E)-10-{8-[[2-(4-Bromophenyl)-1*H*-1,2,3-triazol-1-yl]methyl]benzyl}-6-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **407**



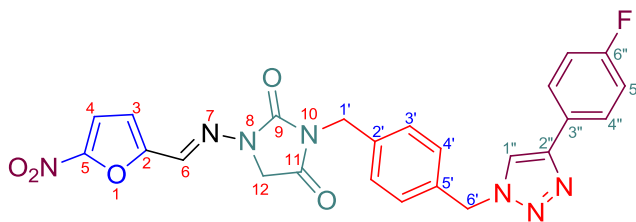
The reaction of **B4** (1.30 mmol) with 1-bromo-4-ethynylbenzene yielded **407** as a yellow powder 171 mg (29%), m.p.: 197.2-219.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3121 (C-H, alkene), 2920 (C-H, alkane), 1774 (C=O), 1708 (C=O), 1523 (NO₂), 1342 (C-N), 1247 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.55 (s, 1H, H-6), 8.32 (s, 1H, H-1''), 7.89 (d, J = 3.6 Hz, 1H, H-4), 7.80 (d, J = 7.7 Hz, 2H, H-4''), 7.67 (d, J = 7.7 Hz, 2H, H-5''), 7.37 (d, J = 7.4 Hz, 2H, H-4'), 7.29 (d, J = 7.4 Hz, 2H, H-3'), 7.18 (d, J = 3.6 Hz, 1H, H-3), 5.17 (s, 2H, H-1'), 4.68 (s, 2H, H-6'), 4.47 (s, 2H, H-12). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.79 (C-11), 162.41 (C-9), 153.23 (C-), 152.31, 152.11 (C-2''), 136.36 (C-5'), 135.46 (C-3'), 132.34 (C-6), 129.10 (C-1''), 128.85 (C-3''), 128.39 (C-4''), 128.21 (C-3'), 127.06 (C-4'), 115.33 (C-4), 115.18 (C-3), 63.06 (C-6'), 53.70 (C-1'), 48.92 (C-12). Purity 90 %. HRMS m/z [M+H]⁺: 564.0604 (Calc. for C₂₄H₁₈N₇O₅⁺ 564.0553).

(E)-10-{8-[[2-[3,5-Bis(Trifluoromethyl)phenyl]-1H-1,2,3-triazol-1-yl)methyl]benzyl}-6-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, **408**



The reaction of **B4** (1.30 mmol) with 1-octyne yielded **408** as a yellow powder 122 mg (19%), m.p.: 197.2-219.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3135 (C-H, alkene), 1798 (C=O), 1720 (C=O), 1564 (NO₂), 1348 (C-N), 1253 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 9.01 (d, J = 2.5 Hz, 1H, H-6''), 8.51 (d, J = 2.5 Hz, 2H, H-4''), 8.06 (s, 1H, H-6) 7.88 (s, 1H, H-1''), 7.79 (d, J = 3.7 Hz, 1H, H-4), 7.37 (d, J = 7.2 Hz, 2H, H-4'), 7.29 (d, J = 7.2 Hz, 2H, H-3'), 7.17 (d, J = 3.7 Hz, 1H, H-3), 5.63 (s, 2H, H-1'), 4.66 (s, 2H, H-1'), 4.47 (s, 2H, H-12). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.73 (C-11), 153.18 (C-9), 152.30 (C-2), 152.09 (C-5), 146.29 (C-2''), 136.45 (C-5'), 135.79 (C-2', 3''), 132.33 (C-6, 3'), 128.65 (C-5''), 128.55 (C-4'), 127.85 (C-4''), 116.35 (C-7''), 116.21 (C-6''), 115.32 (C-4), 115.17 (C-3), 63.06 (C-6'), 53.20 (C-1'), 48.85 (C-12). Purity 90%. HRMS m/z [M+H]⁺: 622.1247 (Calc. for C₂₆H₁₇F₆N₇O₅⁺ 622.1665).

(E)-10-{8-[[2-(*p*-Methoxyphenyl)-1*H*-1,2,3-triazol-1-yl]methyl]benzyl}-6-[[5-nitrofuran-2-yl)methylene]amino}imidazolidine-9,11-dione, 409



The reaction of **B4** (1.30 mmol) with 4-methoxy benzyl bromide yielded **409** as a yellow powder 87 mg (17 %), m.p.: 197.2-219.1 °C. IR (ATR) $\nu_{\text{max}}/\text{cm}^{-1}$: 3141 (C-H, alkene), 2918 (C-H, alkane), 1776 (C=O), 1716 (C=O), 1493 (NO₂), 1345 (C-N), 1248 (C-N). ¹H NMR (600 MHz, DMSO) δ (ppm): 8.51 (s, 1H, H-6), 8.06 (s, 1H, H-1''), 7.88 (d, *J* = 7.2 Hz, 2H, H-5''), 7.79 (d, *J* = 3.7 Hz, 1H, H-4), 7.37 (d, *J* = 7.2 Hz, 2H, H-5''), 7.30 (d, *J* = 7.3 Hz, 1H, H-4') 7.24 (d, *J* = 7.3 Hz, 1H, H-3') 7.14 (d, *J* = 3.7 Hz, 1H, H-3), 5.70 (s, 2H, H-1'), 4.66 (d, *J* = 2H, H-6'), 4.51 (s, 2H, H-12). ¹³C NMR (151 MHz, DMSO) δ (ppm): 167.74 (C-11), 153.18 (C-9), 152.30 (C-2), 152.08 (C-5), 144.53 (C-2''), 136.63 (C-5'), 135.39 (C-2'), 133.71 (C-1''), 132.36 (C-6), 115.33 (C-4), 115.16 (C-3), 53.49 (C-1'), 48.90 (C-12). Purity 90%. HRMS *m/z* [M+H]⁺: 504.1403 (Calc. for C₂₈H₂₇FN₇O₅⁺ 504.1353).

5.3. Results

NFT is poorly soluble in most organic solvents, as well as in water (0.19 mg/mL). Water solubility is improved under basic conditions (Conklin, 1978). DMSO (25 mg/mL) and DMF (50 mg/mL) were the only solvents in which the drug has moderate solubility. However, the reaction did not proceed in DMSO, hence DMF was the only solvent choice. No special conditions and precautions, such inert conditions or high/low temperature, were necessary for the synthesis. Molecular hybridisation resulted in hybrids (Series 2 - 4) with better solubility in organic solvents such as ethyl acetate and ethanol. The hybrids required extra effort to purify, hence the compounds were recrystallised at least twice.

In Series 2, NFT and Tz moieties were linked by a single, carbon (1C) chain. In Series 3, the linker was extended to three (3C), five (5C) and six (6C) carbon chains which allowed for structural diversity and improved flexibility of the hybrids. Moreover, for structural diversity, an aryl linker (1,4-dimethylbenzene) was used for Series 4. Additionally, *n*-alkyl (*n* = 5 - 12) or benzyl substituents (**R**) were used on the triazole ring. In order to establish if electronic effect influenced potency, electron donating groups (EDG), H, OCH₃, CH₃, or *t*-Bu and electron withdrawing groups (EWG), Br, F, NO₂, and CF₃, were added to further diversify benzyl substituents. By contrast, to allow for structural comparison, either similar or the same substituents were used across the hybrid series.

The single carbon linker hybrids (Series 2) were successfully synthesised in moderate (50%) to good (80%) yields with the exception of hybrid **219**, which had a poor yield (30%). The extended *n*-alkyl linker hybrids (Series 3) were synthesised in very low (12%) to moderate (62%) yields, with the exception of **3501** (78%), whereas aryl linker hybrids (Series 4) were prepared in poor yields (17- 45%). All the compounds were confirmed by ¹H and ¹³C NMR as well as HRMS and IR. HPLC purity determination indicated good purity margins (≥ 90) for the majority of the compounds.

5.4. Discussion

Simple nucleophilic substitutions are a useful tool in any synthetic chemist's arsenal. They provide an efficient method for synthesis aimed at targeting a particular functional group or reaction site in order to produce the desired target compound. In this study, the synthesis design flow included: (i) the maintenance of the original NFT scaffold, (ii) the synthesis of compounds from the scaffold (NFT) using simple and cost-effective reactions, and (iii) the creation of a library of compounds from which comparisons could be made. With this in mind N-10 containing the acidic α -H (flanked by C=O at C-9 and C-11 positions) was targeted as the reaction site for reason of easy deprotonation under mild basic conditions.

On average, Series 2 had the highest yields, possibly as a result of inductive effect from the two C=O (C-9 and C-11) in close proximity to the alkyne (C-2'≡C-3'). The uneven distribution of electrons created a greater electron density towards the more electronegative carbonyl groups (C=O, C-9 and C-11) which contributed to the electrophilic reactivity of C-3' (Clayden *et al.*, 2012). By contrast, in Series 3 and 4, as the linker chain length increased, the electronegative carbonyl groups got further away from the alkyne and the effect became less significant. Furthermore, for the aryl linker (Series 4), conjugation reduced electrophilic reactivity (Clayden *et al.*, 2012), thus contributing to the poor yields.

The molecular integrity of the NFT scaffold was confirmed by markers such as: (i) resonance of hydantoin ring H-12 protons which were consistently found in the region of ca. ~4.50 ppm regardless of the linker or substituent, (ii) the peaks associated with H-6 (the hydrazone proton) resonance appearing in the ca. 8.65 to 7.99 ppm range, and finally (iii) the preservation of the 5-Nitrofuran ring (blue in the hybrids) confirmed by the H-3 and H-4 peaks resonance appearing in the ca. 7.17 - 7.03 ppm and ca. 7.91 – 7.70 ppm ranges, respectively. The Nitrofuran ring is the NFT pharmacophore (responsible for anti-infective activity, Chapter 3). Furthermore, IR confirmed the presence of the carbonyl groups (C=O)

C-9 and C-11 on the hydantoin ring, which occurred in the regions of ca.1790 - 1768 and 1730-1703.

The synthesis of NFT intermediates, **201** and **A1-A4** by nucleophilic substitution (S_N2) at N-10 (α -H) was successful. The N-10 α -H with a pKa of 9.23 (Marvin sketch) was satisfactorily deprotonated by TEA (pKa = 10.21). Successful hybridisation was evident from the disappearance of ca.11.47 ppm singlet peak associated with α -H (H-10) on ^1H NMR, in accompaniment to the appearance of the methylene protons H-1' whose resonance peak appeared in the ca. 3.5 - 4.5 ppm (Series 2) region, depending on the substituent (alkyl or aryl). For the extended *n*-alkyl linker hybrids (Series 3), the peaks associated with H-1' resonance appearing in the ca. 3.50 to 4.70 ppm range, and ca. 4.70 ppm for the aryl linker hybrids (Series 4) was an indication of successful reaction. Additionally, in Series 3 and 4, the aryl H-1" proton associated with the triazole ring, appeared in the aromatic region of ca. 7.95 to 7.40 ppm. Furthermore, the methylene protons (H-1') appeared as a singlet in the region of 5.60 - 5.52 for aryl substituents and 4.29 – 4.73 for alkyl substituents. IR confirmed the presence of the triazole (C-N) as indicated by the peaks in the region of ca.1340 - 1388.

Good solubility improves drug permeability, which in turn enhances drug absorption and consequently drug bioavailability (Waring, 2010). Therefore, the lipophilicity and hydrophilicity balance (by inference $\log P$) is crucial. High lipophilicity is generally associated with drug toxicity, whereas high hydrophilicity indicates poor permeability and hence low absorption (Van De Waterbeemd & Gifford, 2003). The Lipinski's rule of five for drug-like properties indicates that $\log P$, which is defined as the octanol/water partition coefficient, should be ≤ 5 (Goodwin *et al.*, 2017). The $\log P$ offers a reliable measure of the balance between lipophilicity and aqueous solubility. In this study, the $\log P$ values (Table 1) were calculated ($\text{clog}P$) using Chem Axon Marvin Beans Software. Although, it is not a true reflection of experimental values, $\text{clog}P$ provides a good estimate of the balance between lipophilicity and hydrophilicity. For validated hit and lead compounds for the treatment of infectious diseases, the requirement is $\log P$ values <5 , and ideally <3 (Goodwin *et al.*, 2017, Katsuno *et al.*, 2015).

Table 1: Calculated log*P* values of hybrids

Series 2				Series 3						Series 4	
Cpd.	clog <i>P</i>	Cpd.	clog <i>P</i>	Cpd.	clog <i>P</i>	Cpd.	clog <i>P</i>	Cpd.	clog <i>P</i>	Cpd.	clog <i>P</i>
NFT	-0.22	212	1.58	3301	1.19	3501	2.59	3601	3.04	401	4.11
203	1.62	213	2.09	3302	2.08	3502	3.04	3602	3.48	402	3.66
204	2.07	214	2.82	3303	2.52	3503	3.48	3603	3.93	403	3.22
205	2.51	215	3.12	3304	3.41	3504	4.37	3604	4.82	404	4.04
206	2.96	216	2.35	3305	2.46	3505	3.42	3605	3.86	405	4.49
207	3.40	217	2.49	3306	4.24	3506	4.66	3606	5.64	406	5.08
208	3.85	218	1.72	3307	1.79	3507	2.75	3607	3.19	407	4.30
209	4.29	219	1.52	3308	2.03	3508	3.00	3608	3.44	408	5.29
210	4.74	220	2.46	3309	3.70	3509	5.20	3609	5.11	409	3.67
211	1.81	221	2.35	3310	2.09	3510	3.05	3610	3.49		

log*P* calculated using Chem Axon Marvin Beans Software (Version 5.2.3_1, May 26, 2009)

One of the limitations of NFT is poor solubility, in fact this is one of the reasons of its use mainly in UTI treatment (Bains *et al.*, 2009). Consequently, compounds with an improved log*P* (NFT log*P* = -0,22) were targeted. All the synthesised compounds had better log*P* values than the parent drug. The chain length of both the linker and the alkyl substituent had an effect on the log*P* value, i.e, an increase in chain length led to an increase in log*P* (Table 1). For the alkyl substituted aryl linker hybrids, an increase in chain length led to a decrease in log*P*. In contrast, the aryl substituents had no discernible pattern on the log*P*, regardless of the EDG or EWG attached. Thus, the linker had more effect on log*P* compared to the substituent.

The extended *n*-alkyl linker hybrids, **3509**, **3606** and **3609** with log*P* values of 5.20, 5.64 and 5.11 respectively, as well as **408** (log*P*= 5.29) failed to meet Lipinski's rule of log*P* values <5. Incidentally, **3509**, **3609** and **408** were 3,5-bis(trifluoromethyl)benzene hybrids. Hybrids **3509** (5C linker) and **3609** (6C linker) differ by a single carbon on the linker, whereas **408** was an aryl linker derivative. Trifluoromethyl (-CF₃) does not naturally occur in biological systems, but plays an important role in drug stability (Purser *et al.*, 2008). It is polar and unreactive thus protects drug from rapid metabolism by metabolic enzymes, consequently improving metabolic stability, bioavailability and protein-ligand interactions (Clayden, 2019; Purser *et al.*, 2008). Additionally, the density of the positive and the negative electrical charge distribution on NFT (Chapter 3) carbon C-6 which has zwitterionic (electrophilic and nucleophilic) properties, aids in the overall chemical stability (Trukhacheva *et al.*, 2005).

Therefore, despite the $\log P$ parameter, (marginally off the 5 threshold) **3509**, **3609** and **408** possess sound drug-like properties from the physicochemical properties viewpoint.

5.5. Conclusion

The synthesis of NFT-Tz hybrids using efficient methods was successful i.e. simple synthesis was achieved in three steps, ((i) linker attachment, (ii) azide introduction, and (iii) cycloaddition by click chemistry); and simple purification (only recrystallisation in ethanol and water - no chromatography). Despite varying yields and the hybrids were isolated with good purity margins (average ~ 90%).

Furthermore, the synthesised hybrids had favourable calculated $\log P$ values (within the ≤ 5 range) as well as better solubility in organic solvents (such as ethanol or ethyl acetate) in comparison to the parent drug, NFT. This indicates improved lipophilicity, which may positively contribute to better bioavailability. This library of hybrids which was synthesised from known anti-infective moieties (NFT and Tz), has better solubility and improved lipophilicity compared to Nitrofurantoin, therefore it is likely to be efficacious and safer, as well as perform better as anti-infective agents. Thus, in Chapter 6, anti-mycobacterial, anti-leishmanial and anti-plasmodial activities as well as cytotoxicity of the synthesised compounds will be evaluated and discussed in detail.

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CHAPTER 6

IN VITRO BIOLOGICAL ACTIVITY AND CYTOTOXICITY EVALUATION

6.1. Introduction

The strategies of drug repurposing by molecular derivatisation and hybridisation of clinical drugs, in order to optimise and improve chemotherapeutic properties is instrumental to address and combat pathogenic infections and deter the emergence of drug resistant pathogens. Molecular derivatisation (Chapter 4) exploited structure-activity relationships (SAR) of Nitrofurantoin to deliver analogues with improved therapeutic profiles as previously reported on other clinical drugs (Hu *et al.*, 2017, Guha, 2013). Molecular hybridisation (Chapter 5), on the other hand, involved the combination of Nitrofurantoin (NFT) with triazole (Tz) to generate new hybrids, on the principle of combining two or more pharmacophores in the design and development of new bioactive agents (Ivasiv *et al.* 2019). The selection of both pharmacophores was based on characteristics (section 2.5) such as pharmacological features, physicochemical properties, toxicity profiles, and the mechanisms of action of the selected bioactive agents (Croft & Seifert, 2010).

This chapter details the anti-infective activity of NFT analogues (Series 1, Chapter 4) and NFT-Tz hybrids (Series 2 to 4, Chapter 5) as potential anti-mycobacterial, anti-leishmanial and anti-plasmodial agents as well as the cytotoxicity (on mammalian cells) of the compounds.

6.2. Materials and methods

6.2.1. Materials

CHO cells (ATCC® CRL-9618™) were received as a gift from Professor P.J. Smith from the Division of Pharmacology, University of Cape Town, South Africa. HEK-293 cells (ATCC® CRL-1573™) were purchased from ATCC (Manassas, USA). L-glutamine, sodium bicarbonate and HEPES, were purchased from Sigma-Aldrich (Johannesburg, South Africa). Foetal bovine serum, Penicillin/Streptomycin solution and Nunc 96 well plates were purchased from Thermofisher Scientific (Johannesburg, South Africa). All the chemicals and reagents were of analytical grade and suitable for cell cultures.

6.2.2. Cytotoxicity evaluation

Cytotoxicity of the synthesised compounds was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on two non-cancerous cell lines. The colorimetric MTT assay was developed by Mossmann to measure cytotoxicity and cell proliferation (Mossmann, 1983). Yellow MTT is reduced by viable cell mitochondria to form purple, insoluble formazan crystals. The cells and crystals are solubilised with organic solvents (e.g. isopropanol) and the solubilised formazan's absorbance measured spectrophotometrically in order to determine cell viability.

CHO cells (passage 10 – 23) were cultured in a 1:1 mixture of Hyclone Dulbecco's modified Eagle's medium (DMEM) with high glucose and Hyclone Ham's F12 growth medium (Kaighn's modification) and supplemented with 10% foetal bovine serum, 1 % penicillin-streptomycin mixture and amphotericin B, and 0.5% non-essential amino acids.

HEK-293 cells (passage 24 – 32) were cultured in DMEM 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 were maintained in a humidified atmosphere at 37 °C and 5% CO₂. For the MTT assay, 96 well plates were prepared with 200 µL of cell suspension (25 000 cells/mL for both cell lines) and incubated for 24 hours. The growth media were aspirated and cells were then treated with: (i) 100 µL of emetine dihydrochloride solution diluted with growth medium to the necessary concentrations (positive control); (ii) 80 µL of growth medium and 20 µL of solvent (negative control to compensate for possible solvent effects); and (iii) 80 µL of growth medium and 20 µL of experimental compound solutions. Blanks contained growth medium without cells. The treated plates were incubated for 48 hours.

To initiate the MTT assay, 20 µL of sterile-filtered MTT solution (1 mg/mL in PBS) was added to each well 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 the MTT reagent, the assay was performed in the dark. Thus, the plates were covered with aluminium foil and the contents gently mixed for 5 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:

$$\text{Cell viability \%} = \frac{\Delta \text{ Abs sample} - \Delta \text{ Abs blank}}{\Delta \text{ Abs neg control} - \Delta \text{ Abs blank}} \times 100$$

The IC₅₀ and Z-score were determined for each compound's biological replicate using GraphPad Prism 5. For the final IC₅₀ of each compound, the mean IC₅₀ of the biological replicates was calculated.

6.2.3. *In vitro* anti-infective activity evaluation

6.2.3.1. Anti-mycobacterial activity

Mtb H37Rv, a human strain of Mtb was used to assess anti-mycobacterial activity of the synthesised Nitrofurantoin analogues. In order to reduce the risk of exposure and infection, all the work with Mtb H37Rv was done under biosafety level III (BSL-III) conditions. Anti-mycobacterial assessment of the synthesised compounds (Chapters 3 and 5) was carried out in two different media namely 7H9 ADC Glucose (Glu) Tween80 (Tw) and 7H9 Casitone Tyloxapol (Tx) using previously described methods (Naran *et al.*, 2016; Wilson *et al.*, 2017). The 7H9 ADC Glu Tw medium comprised the standard 7H9 broth base supplemented with albumin-dextrose-catalase (ADC) and glucose, with Tween80 as surfactant. 7H9 CAS Tx medium, on the other hand, contained the same 7H9 base, supplemented with casitone and tyloxapol as surfactant.

6.2.3.2. Anti-leishmanial activity

The anti-leishmanial (promastigote) activity of the synthesised (Chapters 3 and 5) compounds was evaluated using the resazurin assay, also known as the AlamarBlue® assay on three *Leishmania* strains. The assay involved the irreversible enzymatic reduction of oxidised blue resazurin dye to pink, highly fluorescent resorufin by viable cells (Czekanska, 2011). This non-toxic reagent serves as an effective tool for assessing cell proliferation and drug toxicity. *L. donovani* (strains 1S (MHOM/SD/62/1S) and 9515 (MHOM/IN/95/9515)), and *L. major* (strain IR-173 (MHOM/IR/-173)) were used in this project. *L. donovani* causes visceral leishmaniasis (VL, section 2.3.1.3) and *L. major* is associated with cutaneous leishmaniasis (CL, section 2.3.1.2).

Promastigotes were cultured in M199 with Hank's salts and 0.68 mM L-glutamine supplemented with 4.2 mM sodium bicarbonate, 25 mM Hepes, 10% fetal bovine serum and 50 U/mL Penicillin/Streptomycin solution and the pH adjusted to 7.3 – 7.4. The promastigotes were maintained at 26 °C. For the resazurin assay, logarithmic phase promastigotes (1.25 x 10⁵ cells/mL, final volume 100 µL/well) were seeded in 96 well plates in the presence of twelve two-fold dilution concentrations of compounds for IC₅₀

determination. Amphotericin B (10 µM) served as the standard drug and growth medium without parasites served as the blank. The plates were incubated for 72 hours at 26 °C in humidified atmosphere. After incubation, 50 µL of resazurin solution (0.01 % in phosphor-buffered saline) was added to each well and the plates were further incubated at 26 °C in the dark for 2 hours (1S promastigotes) to 4 hours (9515 and IR-173 promastigotes). Absorbance was measured at 570 nm and 600 nm using the Thermofisher Scientific GO Multiscan plate reader. Data analysis was performed for each biological replicate using SkanIt 4.0 Research Edition software. Background absorbance of resazurin (600 nm) was subtracted from the absorbance values of resorufin (570 nm). The mean absorbance calculated and cell viability was determined by the following equation:

$$\text{Cell viability \%} = \frac{\Delta \text{Abs sample} - \Delta \text{Abs blank}}{\Delta \text{Abs neg control} - \Delta \text{Abs blank}} \times 100$$

The IC₅₀ and Z-score were determined for each compound's three biological replicates using GraphPad Prism 5 and the mean IC₅₀ of the biological replicates served as the final IC₅₀ of each compound.

6.2.3.3. Anti-plasmodial activity

The anti-plasmodial activity of the synthesised compounds (Chapter 3 and 5) was evaluated using the [³H]hypoxanthine incorporation assay on the *P. falciparum* strains, 3D7 (drug susceptible) and Dd2 (drug resistant). The assay method was adapted from Trager and Jensen and was used to measure parasite toxicity (Trager & Jensen, 1976). [³H]hypoxanthine was added to parasite cultures treated with novel compounds, taken up by the parasites as a precursor of purine deoxynucleotides for DNA synthesis (Desjardins *et al.*, 1979) and radioisotope incorporation was measured, which was proportional to the number of parasitised erythrocytes present.

Blood stage parasites of *P. falciparum* strains 3D7 and Dd2 were cultured in human A⁺ erythrocytes and RPMI 1640 medium supplemented with 0.5% Albumax II, 0.4% D-(+)-glucose, 0.025 M HEPES, 0.323 mM hypoxanthine and 48 µg/mL gentamicin. The cells were maintained at 37 °C and an atmosphere of 95% N₂, 5% O₂ and 5% CO₂. For the assay, 96 well plates were prepared with: (i) 100 µL of reference drug solution diluted with hypoxanthine-free medium to the necessary concentrations (positive control); (ii) 100 µL hypoxanthine-free medium without drug (negative control); and (iii) 100 µL of a two-fold dilution of experimental compound solutions in hypoxanthine-free medium. To the prepared plates, 100 µL of infected erythrocyte suspension (parasitemia 0.15 % and hematocrit 2.5 %) was added.

in hypoxanthine-free medium was added. The treated plates were incubated for 46 hours at 37 °C and an atmosphere of 95% N₂, 5% O₂ and 5% CO₂.

To initiate [³H]hypoxanthine incorporation, 0.5 µCi of [³H]hypoxanthine in 50 µL was added and the plates incubated for a further 24 hours. The parasites were then harvested onto glass fibre filters, washed with distilled water and dried. Radioactivity was recorded as counts per minute (cpm) per well at each drug concentration, and growth inhibition was expressed as the percentage of ³H incorporation compared with untreated controls. The IC₅₀ values of three biological replicates were individually calculated from sigmoidal dose-response curves and the mean IC₅₀ with standard deviation (SD) determined.

6.3. Results

6.3.1. Cytotoxicity

Emetine, an antiprotozoal drug known for its high cytotoxicity, was used as a reference drug in the cytotoxicity evaluation. Toxicity classification criteria are summarised in Table 6.1 and the cytotoxicity results are presented in Tables 6.2 to 6.5.

Table 6.1: Toxicity classification

Toxicity	IC ₅₀ (µM)	References
Low	> 100	(Adewusi <i>et al.</i> , 2013; Fu <i>et al.</i> , 2014)
Weak	50 < IC ₅₀ <100	(Liu <i>et al.</i> , 2017)
Mild to moderate	10 < IC ₅₀ <50	(Finiuk <i>et al.</i> , 2017; Liu <i>et al.</i> , 2017)
Significant to high	< 10	(Finiuk <i>et al.</i> , 2017; Kolf-Clauw <i>et al.</i> , 2013; Liu <i>et al.</i> , 2017)

Series 4 stood out as the most cytotoxic of all the series, whereas Series 2 and 3 had similar cytotoxicity profiles. In these series (2 and 3) the majority of the hybrids were mildly to moderately cytotoxic, and only hybrids **206** and **212** for Series 2, as well as **3307** and **3309** for Series 3 (Figure 6.1) were found to be nontoxic to mammalian cells.

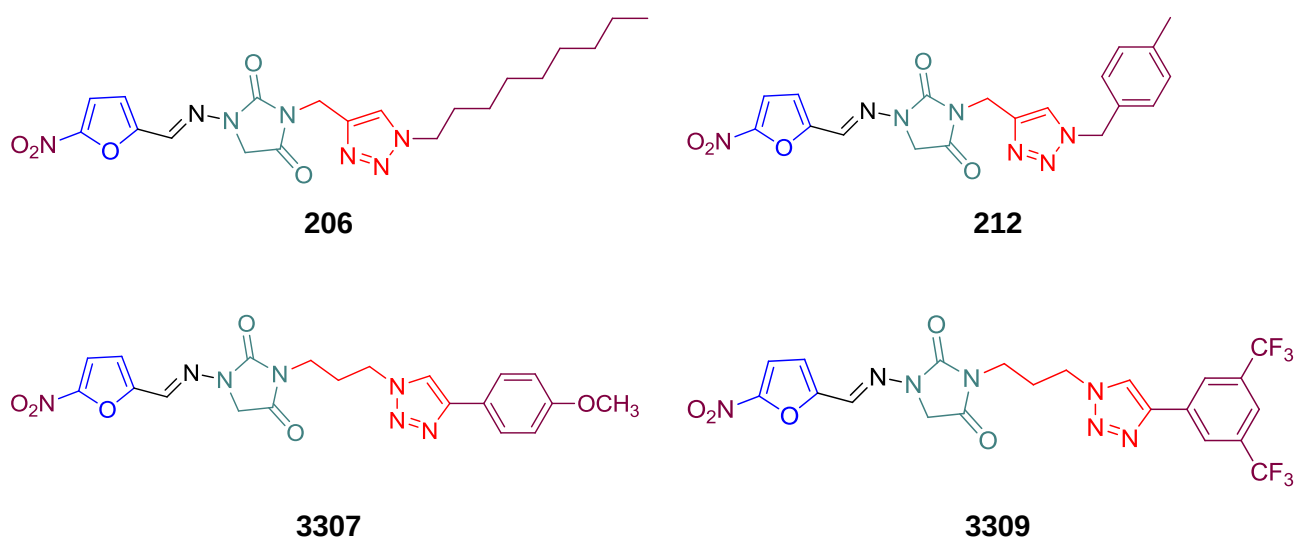


Figure 6.1: Hybrids with low toxicity to mammalian cells

6.3.2. Anti-mycobacterial activity

The synthesised compounds were screened *in vitro* for anti-mycobacterial activity, alongside standard TB drugs, rifampicin (RIF) and isoniazid (INH), against virulent laboratory strain, *Mtb* H37Rv. The minimum concentration of compound inhibiting 90% (MIC₉₀) of mycobacterial growth was determined in two different media: ADC-containing medium with protein binding potential, and CAS-supplemented medium devoid of albumin. The MIC₉₀ values are presented in Tables 6.2 to 6.5. On average, the hybrids had poor anti-mycobacterial activity. No hybrid was found to be more active than the parent drug, NFT. However, hybrids **3507**, **3510** and **3609** in Series 3 (Figure 6.2) were equipotent to the parent drug in CAS medium.

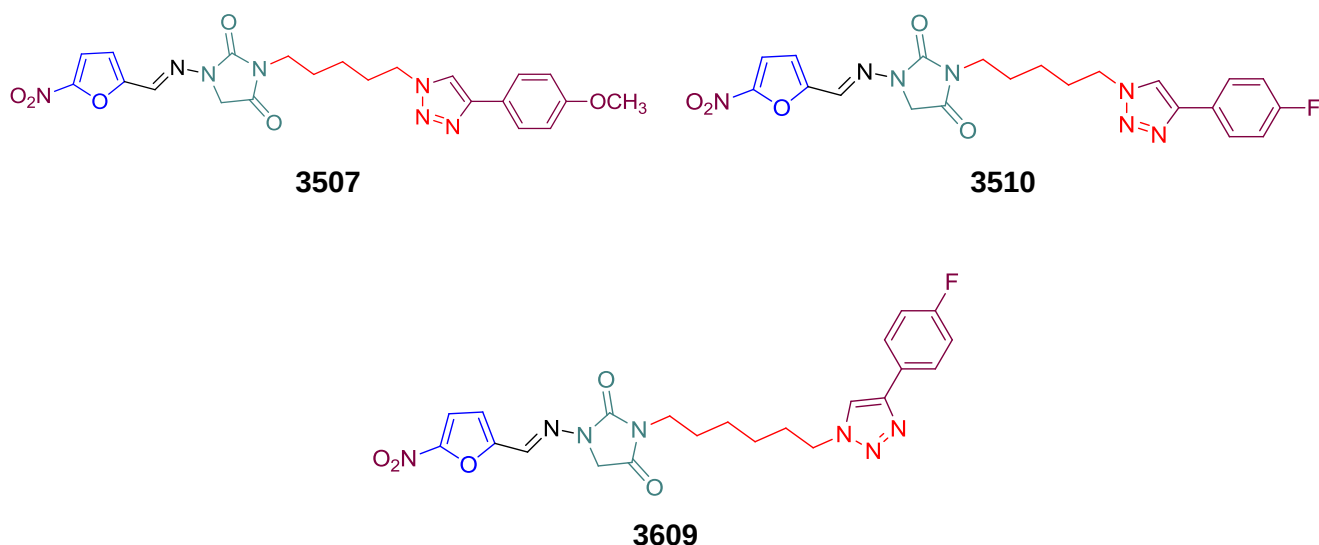


Figure 6.2: Series 3 hybrids equipotent to the parent drug

6.3.3. Anti-leishmanial activity

All the synthesised derivatives were screened *in vitro* for promastigote anti-leishmanial activity against *L. major* IR -173 (CL strain) as well as *L. donovani* 1S and 9515 (VL strains), alongside the standard anti-leishmanial drug, amphotericin B (AmB) and NFT sister drugs furazolidone (FZ), nifuroxazide (NX), and nitrofurazone (NFZ) as well as 5-nitro-2-furaldehyde (contains the Nitrofuran pharmacophore without the side chain). In literature, a 70% growth inhibition cut-off criterion was reported (Siqueira-Neto *et al.*, 2010), however in this study anti-leishmanial IC₅₀ determination was reserved for compounds with growth inhibition percentage $\geq 30\%$. As a result, poorly performing hybrids in Series 2 (Table 6.3) and Series 3 (Table 6.4) were excluded, particularly against *L. donovani* (both 1S and 9515) parasites. The anti-leishmanial activity IC₅₀ results are presented in Tables 6.2 to 6.5.

6.3.4. Anti-plasmodial activity

All the synthesised derivatives were first subjected to dual point screening according to following parameters: (i) compounds must show $> 70\%$ growth inhibition against 3D7 at 10 μM to be screened for growth inhibition at 1 μM , and (ii) exclude all compounds with moderate to high cytotoxicity (IC₅₀ $< 30 \mu\text{M}$) against HEK-293 cells (Tougan *et al.* 2019; Weisman *et al.*, 2006). The latter consideration resulted in the exclusion of all Series 4 (Table 6.5) hybrids from further screening. In Series 1 (analogues), only **113** (Figure 6.3) had $> 70\%$ growth inhibition at 1 μM . None of Series 2 hybrids had $> 50\%$ growth inhibition at 1 μM , whereas only hybrids **3302** and **3604** in Series 3 (Figure 6.3) had $\geq 50\%$ growth inhibition at 1 μM . All the % growth inhibition data is presented in Tables 6.2 to 6.5.

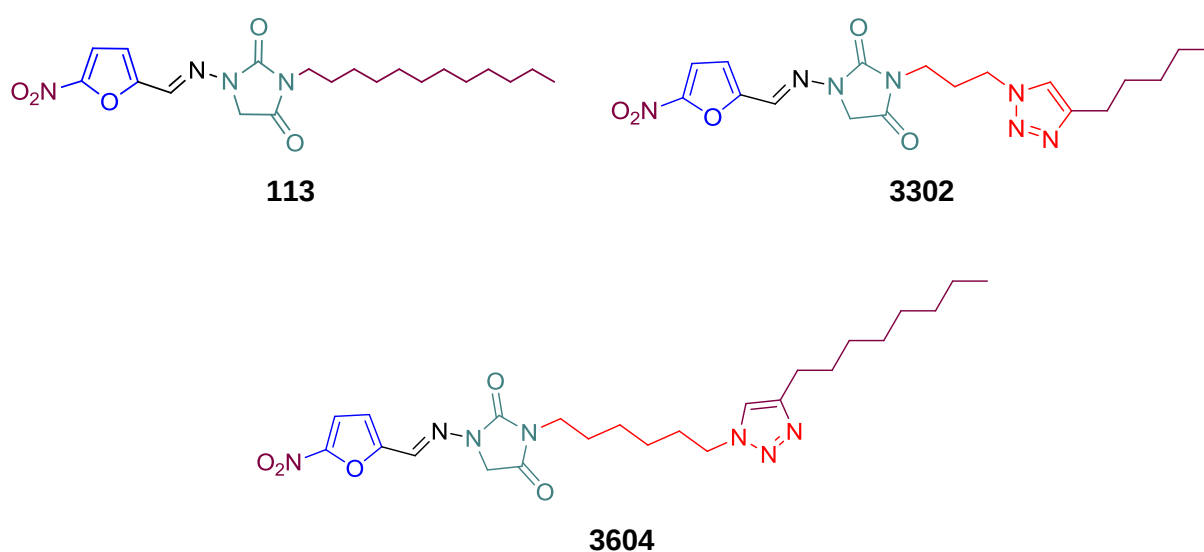


Figure 6.3: Hybrids with anti-plasmodial activity

Anti-plasmodial activity IC₅₀ was determined *in vitro* alongside chloroquine (CQ), and methylene blue against *P. falciparum* CQ-susceptible (3D7) and CQ-, pyrimethamine and mefloquine resistant (Dd2) strains. Antimalarial standard drugs such as dihydroartemisinin (DHA), artemether (ARM) and artesunate (ARS) were also used for comparison. The anti-plasmodial IC₅₀ results are presented in Table 6.8.

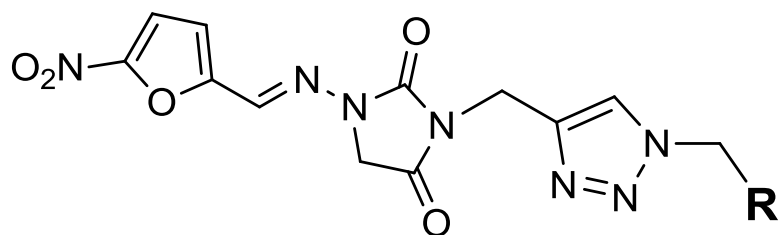
Cpd.	R	Cpd.	R
102	<i>n</i> -CH ₃ --	112	<i>n</i> -C ₁₁ H ₂₃ --
103	<i>n</i> -C ₂ H ₅ --	113	<i>n</i> -C ₁₂ H ₂₅ --
104	<i>n</i> -C ₃ H ₇ --	114	
105	<i>n</i> -C ₄ H ₉ --	115	
106	<i>n</i> -C ₅ H ₁₁ --	116	
107	<i>n</i> -C ₆ H ₁₃ --	117	
108	<i>n</i> -C ₇ H ₁₅ --	118	
109	<i>n</i> -C ₈ H ₁₇ --	119	
110	<i>n</i> -C ₉ H ₁₉ --	120	
111	<i>n</i> -C ₁₀ H ₂₁ --		

Figure: 6.4: Series 1 – NFT analogues

Table 6.2: Biological data of NFT analogues

Cpd	n	logP _a	Cytotoxicity IC ₅₀ (μM)		Anti-infective Activity										Anti-plasmodial (3D7)		
					Anti- <i>Mtb</i> MIC ₉₀ (μM) ^b		Selectivity Index		Anti-leishmanial, IC50 (μM)			Selectivity Index					
			HEK- 293	CHO	ADC	CAS	SI ₁	SI ₂	Promastigotes			SI ₃	SI ₄	SI ₅	% inhibition		
									<i>L. major</i>	<i>L. donovani</i> 1S					10 μM	1 μM	0.1 μM
NFZ	0	-0.14	55.1	97.8	31.5	3.55	2	3	1.22	1.45	1.82	45	38	30	-	-	-
NFT	0	-0.22	43.9	50.2	14.5	2.22	3	4	55.9	34.0	26.3	1	1	2	60	-	-
102	1	-0.04	77.8	>100	22.6	2.25	3	>4	1.32	-	0.438	59	-	178	56	-	-
103	2	0.36	38.3	60.4	34.7	3.42	1	2	0.436	0.780	0.141	88	49	271	52	-	-
104	3	0.88	13.5	40.2	33.3	3.79	-	1	0.821	-	0.245	16	-	55	55	-	-
105	4	1.33	53.0	77.8	18.3	8.53	3	4	0.961	-	-	55	-	-	74	3.1	0.0
106	5	1.77	29.1	>100	2.1	0.24	14	48	0.728	0.314	4.42	40	93	7	79		
107	6	2.22	>100	>100	1	5.14	103	>100	0.442	0.254	2.14	226	394	47	77	5.7	0.0
108	7	2.66	>100	>100	2.0	2.34	50	>50	0.562	0.119	2.43	178	840	41	79	17	0.2
109	8	3.10	63.8	>100	0.5	0.97	131	>200	0.0780	0.056	1.19	818	1139	54	82	4.5	0.0
110	9	3.55	>100	>100	3.41	7.81	29	29	0.0190	0.013	0.485	5263	7692	206	89	9.3	0.0
111	10	3.99	>100	>100	2.02	31.3	50	>50	0.0230	0.010	0.242	4348	10000	413	91	24	0.0
112	11	4.44	>100	>100	1.88	3.91	53	53	0.0200	0.0080	0.260	5000	12500	385	86	34	0.0
113	12	4.88	>100	>100	0.99	62	100.	>101	0.0340	0.012	0.099	2941	8333	1010	89	85	1.1
114		2.46	20.9	18.2	>125	2.12			1.33	0.212	0.111	16	98	188	67	-	-
115		1.73	>100	40.0	>125	4.23			0.275	1.25	0.222	364	80	450	86	-	-
116		2.24	90.5	2.76	9.27	2.76	10	>11	2.83	1.93	13.2	32	47	7	75	8.9	0.0
117		2.60	>100	87.9	>125	1.66	1		0.168	0.501	0.252	595	200	397	70		
118		1.67	17.6	>100	5.03	0.97	4	>20	0.133	0.479	10.0	132	37	2	79	1.2	0.0
119		1.57	15.8	91.3	9.49	2.14	2	10	0.449	0.979	5.49	35	16	3	64		
120		2.50	>100	38.2	nd	1.35	nd	nd	0.391	0.131	1.48	256	763	67	72		
RIF		nc	-	-	0.003	0.49	-	-	-	-	-	-	-	-	-	-	-
INH		nc	-	-	0.96	0.01	-	-	-	-	-	-	-	-	-	-	-
AmB		-	-	-	-	-	-	-	0.03	0.02	0.02	-	-	-	-	-	-
FZ		-	-	-	-	-	-	-	0.37	0.41	0.57	-	-	-	-	-	-
NX		-	-	-	-	-	-	-	-	4.53	4.48	-	-	-	-	-	-
EM		nc	0.06	0.064	-	-	-	-	-	-	-	-	-	-	-	-	-

^aCalculated using Chem Axon Marvin Beans Software (Version 5.2.3_1, May 26, 2009); ^bMIC₉₀ = determined *in vitro* against *Mtb* H37Rv; nc (not calculated); NFZ = nitrofurazone; NFT = Nitrofurantoin; AmB = amphotericin B; FZ = furazolidone; NX = nifuroxazide; RIF = rifampicin; INH = isoniazid; EM = Emetine; Selectivity Index = IC₅₀ HEK-293/MIC₉₀/IC₅₀; SI₁ = ADC, SI₂ = CAS; SI₃ = *L. major*; SI₄ = *L. donovani*, 1S, SI₅ = *L. donovani* 9515



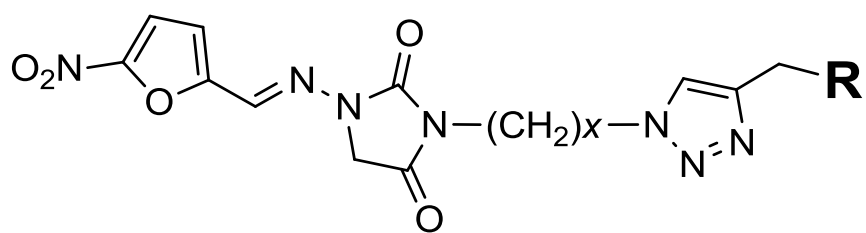
Cpd.	R	Cpd.	R
202	<i>n</i> -C ₅ H ₁₁ --	212	
203	<i>n</i> -C ₆ H ₁₃ --	213	
204	<i>n</i> -C ₇ H ₁₅ --	214	
205	<i>n</i> -C ₈ H ₁₇ --	215	
206	<i>n</i> -C ₉ H ₁₉ --	216	
207	<i>n</i> -C ₁₀ H ₂₁ --	217	
208	<i>n</i> -C ₁₁ H ₂₃ --	218	
209	<i>n</i> -C ₁₂ H ₂₅ --	219	
210		220	
211			

Figure: 6.5: Series 2 – Single carbon linker NFT-Tz hybrids

Table 6.3: Biological data of the single carbon linker NFT-Tz hybrids

Cpd	n	logP ^a	Cytotoxicity IC ₅₀ (μM)	Anti-infective Activity												
				Anti- <i>Mtb</i> MIC ₉₀ (μM) ^b		Selectivity index		Anti-leishmanial IC ₅₀ (μMm)			Selectivity index			Anti-plasmodial (3D7)		
								Promastigotes			% inhibition					
				HEK-293	ADC	CAS	SI ₁	SI ₂	<i>L. major</i>	<i>L. donovani</i> 1S	SI ₁	SI ₂	SI ₃	10 μM	1 μM	0.1 μM
202	5	1.62	67.1	>125	>125	0.5	0.5	0.343	-	-	196	-	-	52	-	-
203	6	2.07	41.8	>125	71.5	0.3	0.6	0.343	-	-	122	-	-	58	-	-
204	7	2.51	31.9	>125	59.0	0.3	0.5	0.272	-	-	117	-	-	67	-	-
205	8	2.96	43.8	>125	>125	0.4	0.4	6.51	0.051	4.68	7	860	139	65	-	-
206	9	3.40	>100	>125	>125	0.8	0.8	6.85	0.257	0.177	15	389	565	83	10	-
207	10	3.85	20.3	>125	>125	0.2	0.2	1.24	-	1.60	16	-	13	84	-	-
208	11	4.29	7.27	>125	>125	0.1	0.1	0.570	-	-	13	-	-	85	-	-
209	12	4.74	23.2	>125	>125	0.2	0.2	0.450	-	-	41	-	-	84	-	-
210		1.81	32.7	62.4	>125	0.5	0.3	0.496	0.057	7.59	66	573	4	85	10	-
211		1.58	88.5	>125	>125	0.7	0.7	0.505	-	3.03	175	-	29	72	-	-
212		2.09	>100	>125	>125	0.8	0.8	0.090	-	-	1111	-	-	75	11	0.0
213		2.82	15.8	>125	>125	0.1	0.1	0.133	0.305	-	118	5	-	86		
214		3.12	60.2	--	>125	-	0.5	--	-	-	-	-	-	86	35	7.2
215		2.35	43.7	--	>125	-	0.3	0.173	-	-	253	-	-	72	3.5	0.0
216		2.49	12.6	>125	>125	0.1	0.1	0.478	0.0240	1.75	26	523	7	76	-	-
217		1.72	21.0	61.3	>125	0.3	0.2	0.676	-	-	31	-	-	78	-	-
218		1.52	1.24	>125	34.8	0.01	0.04	0.378	-	-	3	-	-	88	-	-
219		2.46	48.4	>125	>125	0.4	0.4	1.05	-	-	46	-	-	72	13	0.0
220		2.35	14.4	29.9	>125	0.5	0.1	0.191	-	-	75	-	-	85	-	-
RIF		nc	-	0.003	0.49	-	-	-	-	-	-	-	-	-	-	-
INH		nc	-	0.96	0.01	-	-	-	-	-	-	-	-	-	-	-
AmB		nc	-	-	-	-	-	0.03	0.02	0.02	-	-	-	-	-	-
FZ		nc	-	-	-	-	-	0.37	0.41	0.57	-	-	-	-	-	-
NX		nc	-	-	-	-	-	-	4.53	4.48	-	-	-	-	-	-
EM		nc	0.06	-	-	-	-	-	-	-	-	-	-	-	-	-

^aCalculated using Chem Axon Marvin Beans Software (Version 5.2.3_1, May 26, 2009); ^bMIC₉₀ = determined *in vitro* against *Mtb* H37Rv; nc (not calculated); NFZ = nitrofurazone; NFT = Nitrofurantoin; RIF = rifampicin; INH = isoniazid; AmB = amphotericin B; FZ = furazolidone; NX = nifuroxazide; EM = Emetine; Selectivity Index = IC₅₀ HEK-293/MIC₉₀/IC₅₀; SI₁ = ADC, SI₂ = CAS; SI₃ = *L. major*; SI₄ = *L. donovani*, 1S, SI₅ = *L. donovani* 9515.



x= chain length

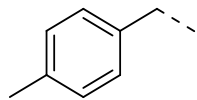
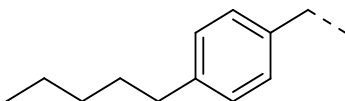
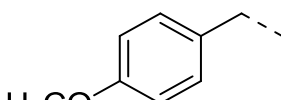
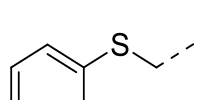
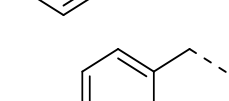
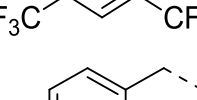
Cpd.	x	Cpd.	x	Cpd.	x	R
3301	3	3501	5	3601	6	<i>n</i> -C ₄ H ₉
3302	3	3502	5	3602	6	<i>n</i> -C ₅ H ₁₁
3303	3	3503	5	3603	6	<i>n</i> -C ₆ H ₁₃
3304	3	3504	5	3604	6	<i>n</i> -C ₈ H ₁₇
3305	3	3505	5	3604	6	
3306	3	3506	5	3606	6	
3307	3	3507	5	3607	6	
3308	3	3508	5	3608	6	
3309	3	3509	5	3609	6	
3310	3	3510	5	3610	6	

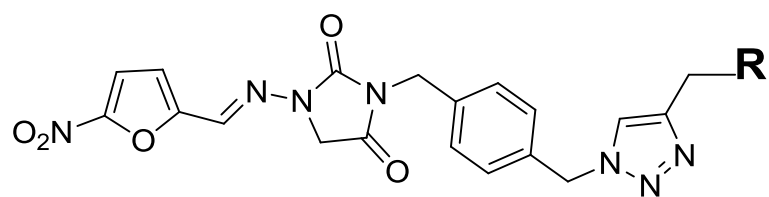
Figure: 6.6: Series 3 – The extended *n*-alkyl linker NFT-Tz hybrids

Table 6.4: Biological data of the extended *n*-alkyl linker NFT-Tz hybrids

Cpd	n	logP ^a	Cytotoxicity IC ₅₀ (μM)	Anti-infective Activity								Anti-plasmodial (3D7)		
				Anti- <i>Mtb</i> MIC ₉₀ (μM) ^b	Selectivity Index	Anti-leishmanial IC ₅₀ (μM)			Selectivity Index					
						Promastigotes			SI ₂	SI ₃	SI ₄			
						<i>L.</i> <i>major</i>	<i>L. donovani</i>							
			HEK-293	CAS	SI ₁		1S	9515	% inhibition	10 μM	1 μM	0.1 μM		
3301	4	1.19	35.6	>125	0.3	0.449	-	-	79	-	-	74		
3302	5	2.08	16.5	63.5	0.3	0.551	-	-	30	-	-	82		
3303	6	2.52	11.5	>125	0.1	0.475	-	-	24	-	-	83		
3304	8	3.41	37.4	>125	0.3	0.729	-	-	51	-	-	85	13.8	0.0
3305		2.46	79.8	>125	0.6	0.674	-	-	114	-	-	57		
3306		4.24	10.8	>125	0.1	12.9	-	-	1	-	-	88		
3307		1.79	>100	>125	0.8	0.804	-	-	124	-	-	78	9.8	1.8
3308		2.03	30.6	43.7	0.7	0.737	-	-	42	-	-	88	12.3	0.0
3309		3.70	>100	>125	0.8	0.672	0.424	1.20	149	236	83	76	3.1	0.0
3310		2.09	12.0	>125	0.1	0.435	-	-	28	-	-	78		
3501	4	2.59	65.9	74.0	0.9	1.07	-	-	62	-	-	0		
3502	5	3.04	28.6	34.0	0.8	1.50	0.071	0.610	19			88	53.2	0.0
3503	6	3.48	87.9	>125	0.7	-	-	-	-	-	-	82	12.6	0.0
3504	8	4.37	13.9	>125	0.1	1.70	0.152	0.012	8	91	1158	86	-	-
3505		3.42	9.73	32.2	0.3	1.86	-	-	5	-	-	76	-	-
3506		4.66	22.7	>125	0.2	-	-	-	-	-	-	85	-	-
3507		2.75	37.2	14.7	0.3	0.503	-	-	74	-	-	0	-	-
3508		3.00	17.7	31.1	0.6	0.979	-	-	18	-	-	87	-	-
3509		5.20	44.5	>125	0.4	-	-	-	-	-	-	0	-	-
3510		3.05	15.5	15.9	1	1.19	-	-	13	-	-	0	-	-
3601	4	3.04	83.4	33.3	3	0.270	-	-	309	-	-	82	42.9	4.8
3602	5	3.48	50.3	>125	0.4	5.30	0.038	0.481	9	1324	105	66		
3603	6	3.93	19.6	>125	0.2	2.62	0.091	0.109	7	215	179	84		
3604	8	4.82	31.1	>125	0.2	2.75	0.045	0.059	11	691	527	84	49.5	1.0
3605		3.86	-	>125	-	0.661	0.078	0.979	11	91	7	80	-	-
3606		5.64	5.39	23.0	0.23	2.19	-	0.048	2	-	112	84	-	-
3607		3.19	8.97	>125	0.07	0.593	0.084	0.229	15	107	39	-	-	-
3608		3.44	-	>125	-	0.218	--	--	32	-	-	88	-	-
3609		5.11	16.4	20.0	0.8	0.799	0.271	5.35	21	60	3	86	-	-

Cpd	n	logP ^a	Cytotoxicity IC ₅₀ (μM)	Anti-infective Activity										
				Anti- <i>Mtb</i> MIC ₉₀ (μM) ^b	Selectivity Index	Anti-leishmanial IC ₅₀ (μM)			Selectivity Index			Anti-plasmodial (3D7)		
						Promastigotes								
						<i>L.</i> <i>major</i>	<i>L. donovani</i>		SI ₂	SI ₃	SI ₄	% inhibition		
							1S	9515				10 μM	1 μM	0.1 μM
HEK-293	CAS	SI ₁												
3610	3.49	6.24	>125	0.05	1.73	0.376	0.296	4	21	17	81	-	-	
RIF	nc	0.003	-	-	-	-	-	-	-	-	-	-	-	
INH	nc	0.96	-	-	-	-	-	-	-	-	-	-	-	
AmB	nc	-	-	-	0.03	0.02	0.02	-	-	-	-	-	-	
FZ	nc	-	-	-	0.37	0.41	0.57	-	-	-	-	-	-	
NX	nc	-	-	-	-	4.53	4.48	-	-	-	-	-	-	
EM	nc	0.06	-	-	-	-	-	-	-	-	-	-	-	

^aCalculated using Chem Axon Marvin Beans Software (Version 5.2.3_1, May 26, 2009); ^bMIC₉₀ = determined *in vitro* against *Mtb* H37Rv; nc (not calculated); NFZ = nitrofurazone; NFT = Nitrofurantoin, **1**; RIF = rifampicin; INH = isoniazid; EM = Emetine; Selectivity Index = IC₅₀ HEK-293 / IC₅₀; Sl₁= *Mtb* , Sl₂ = *L. major*; Sl₃ = *L.donovani*, 1S, Sl₄ = *L.donovani* 9515



Cpd.	R
401	<i>n</i> -C ₄ H ₉ --
402	<i>n</i> -C ₅ H ₁₁ --
403	<i>n</i> -C ₆ H ₁₃ --
404	
405	
406	
407	
408	
409	

Figure 6.7: Series 4 - The aryl linker NFT-Tz hybrids

Table 6.5: Biological data of the aryl linker NFT-Tz hybrids

Cpd.	n	logP ^a	Cytotoxicity IC ₅₀ (μM)	Anti-infective activity									Anti-plasmodial (3D7)
				Anti- <i>Mtb</i> MIC ₉₀ (μM) ^b		Anti-leishmanial IC ₅₀ (μM)				Selectivity Index			
						Promastigotes				SI ₂	SI ₃	SI ₄	
				HEK-293	ADC	CAS	SI ₁	<i>L. major</i>	<i>L. donovani</i>				
							1S	9515					
A4	0	4.49	5.56	18.0	18.0	0.3	0.192	1.18	--	29	5	-	87
B4	0	-	15.2	>125	>125	0.1	2.40	--	--	6	-	-	85
401	6	4.11	7.18	>125	>125	0.1	0.788	0.348	0.819	9	21	9	89
402	5	3.66	16.9	>125	>125	0.1	0.174	1.99	--	97	9	-	90
403	4	3.22	16.5	>125	>125	0.1	1.23	2.97	--	13	6	-	88
404		4.04	13.8	>125	>125	0.1	0.491	0.014	0.911	28	984	15	84
405		4.49	6.12	>125	>125	0.05	0.741	--	2.67	8	-	2	88
406		5.08	5.06	>125	>125	0.05	2.32	--	2.55	2	-	2	88
407		4.30	12.9	>125	>125	0.1	0.361	0.656	0.228	36	20	56	84
408		5.29	5.03	>125	>125	0.05	0.195	0.066	0.670	26	76	8	91
409		3.67	12.0	>125	>125	0.1	0.361	0.656	0.228	5	-	3	84
RIF		nc	-	0.003	0.49	-	-	-	-	-	-	-	-
INH		nc	-	0.96	0.01	-	-	-	-	-	-	-	-
AmB		nc	-	-	-	-	0.03	0.02	0.02	-	-	-	-
FZ		nc	-	-	-	-	0.37	0.41	0.57	-	-	-	-
NX		nc	-	-	-	-	-	4.53	4.48	-	-	-	-
EM		nc	0.06	-	-	-	-	-	-	-	-	-	-

^aCalculated using Chem Axon Marvin Beans Software (Version 5.2.3_1, May 26, 2009); ^bMIC₉₀ = determined *in vitro* against *Mtb* H37Rv; *Mtb* H37Rv (ADC); IC₅₀ CHO/MIC₉₀ *Mtb* H37Rv (ADC); nd (not determined); nc (not calculated); NFZ = nitrofurazone; NFT = Nitrofurantoin; RIF = rifampicin; INH = isoniazid; EM = Emetine; Selectivity Index = IC₅₀ HEK-293/ IC₅₀; Sl₁ = *Mtb*, Sl₂ = *L. major*; Sl₃ = *L. donovani*, 1S, Sl₄ = *L. donovani* 9515

6.4. Discussion

6.4.1. Cytotoxicity

Toxicity accounts for more than a third of drug failure in clinical settings, as well as for a significant amount of drug development expenses such as clinical trials and post-marketing costs (Guengerich, 2010). Therefore, screening for toxicity early in the drug development process is paramount. Starting off from a clinical drug (NFT) established a benchmark for comparison. The single carbon linker NFT-Tz hybrids (Series 2, Figure 6.2) had similar cytotoxicity (Table 6.3) as the parent drug (NFT, $IC_{50} = 43.93 \mu M$). The majority of these compounds were mildly to moderately toxic ($10 < IC_{50} < 50$, classification, Table 6.1) to mammalian cells (HEK-293). In addition, there was no distinctive difference in cytotoxicity, between *n*-alkyl and aryl hybrids. Hybrids **206** (alkyl) and **212** (aryl) were the only two compounds (Figure 6.8) in this series with low cytotoxicity ($IC_{50} > 100 \mu M$).

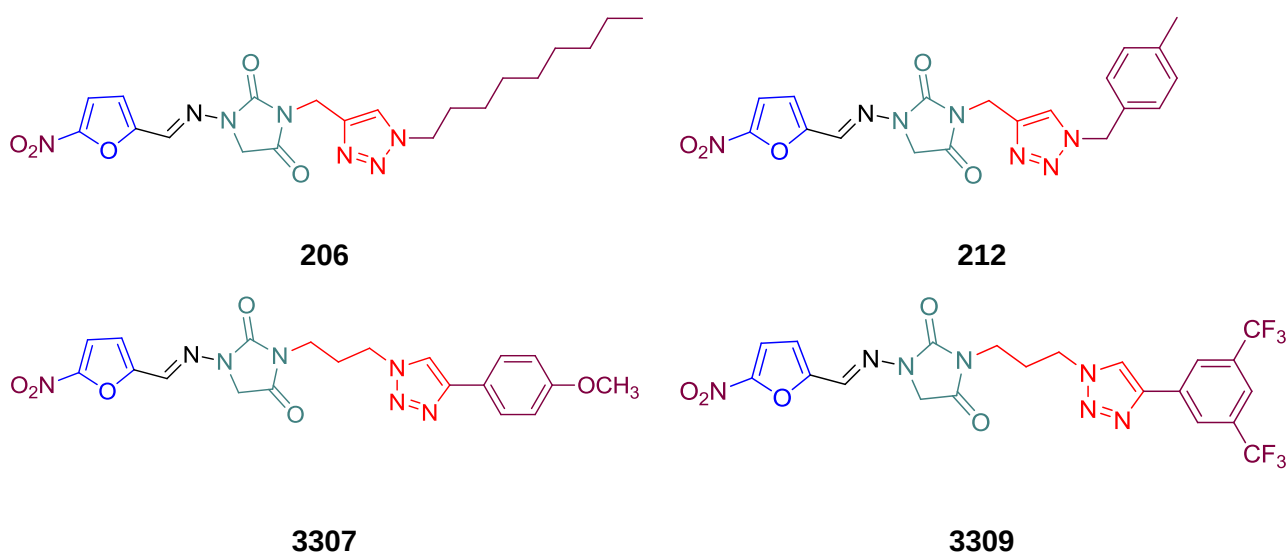


Figure 6.8: Hybrids that exhibited low cytotoxicity

The extended *n*-alkyl linker NFT-Tz hybrids (Series 3, Figure 6.3) and the aryl linker NFT-Tz hybrids (Series 4, Figure 6.5) ranged from mildly - moderately cytotoxic ($10 \mu M < IC_{50} < 50 \mu M$) to significantly toxic ($IC_{50} < 10 \mu M$), regardless of the substituent (**R**) attached. These findings were consistent with the observation from series 2 that introduction of the Tz moiety enhanced the cytotoxicity of the clinical drug. The exceptions were **3307** (**R**: -OCH₃) and **3309** (**R**: 2,4-CF₃), where cytotoxicity was low, $IC_{50} > 100 \mu M$. Extension of the linker did not improve the cytotoxicity as evident from comparable cytotoxicity between series 2 and 3. In fact, the majority of these hybrids was mildly to moderately cytotoxic and only two hybrids (Series 2: **206** & **212** (Figure 6.8) and Series 3: **3307** & **3309**) from each series were nontoxic to mammalian cells. The structural differences of the substituents on these four compounds, **206** (**R**: 9C *n*-alkyl), **212** (**R**: isopropylbenzyl), **3307** (**R**: methoxybenzyl) and **3309** (**R**: bis-trifluorobenzyl) did not provide a pattern from which to make comparisons and draw conclusions.

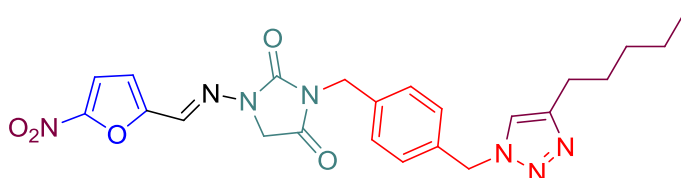
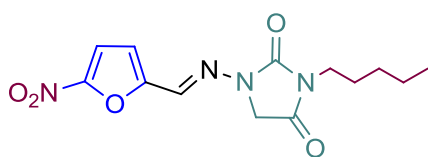
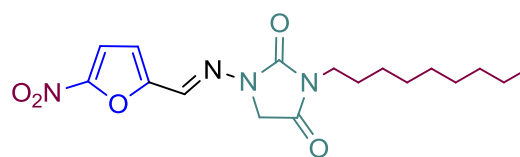


Figure 6.9: Hybrid **402**

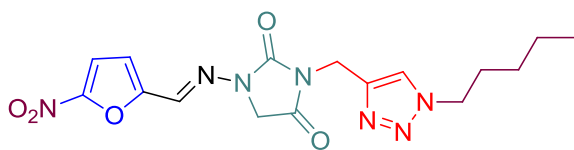
The use of an aryl linker (Series 4), however, conferred indiscriminate cytotoxicity regardless of the substituent (**R**) attached. In fact, Series 4 was the most toxic of all the series in this project, indeed the least cytotoxic hybrid in this series was **402** (IC_{50} 17 μ M, Figure 6.9), thus this series is not safe for human cells.



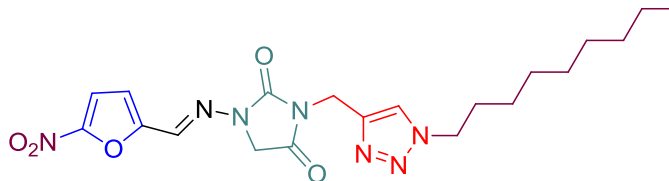
106



110



202

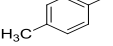


206

Figure 6.10: Cytotoxicity comparison between the analogues and hybrids

The majority of Series 1 and 2 derivatives had the same substituents (**R**); as such, comparison between these series was possible. The cytotoxicity data for the NFT analogues (Series 1) was discussed at length in Chapter 4, and was re-entered in Table 6.2 for ease of access as well as in Table 6.6 for comparison. 60% of the Series 1 and 2 derivatives differed only by the presence of the triazole. From these compounds, the observation that the introduction of Tz to NFT led to pronounced cytotoxicity was apparent (Table 6.6). The exceptions (Figure 6.10) were *n*-alkyl *n* = 5 (analogue **106** and the hybrid **202**) which had reduced cytotoxicity, where cytotoxicity IC_{50} were 29 and 67 μ M, respectively and *n*-alkyl *n* = 9 (analogue **110** and the hybrid **206**) remained non-cytotoxic (IC_{50} >100 μ M) in both series. Series 3 and 4 hybrids with the same substituents (**R**) as Series 1 and 2, did not form a pattern to allow for comparison.

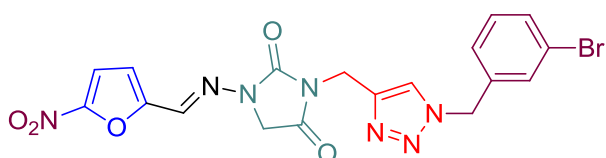
Table 6.6: Cytotoxicity comparison between the analogues and hybrids

Subst.		Cytotoxicity IC ₅₀ (μM) HEK-293					
		Series 1	Series 2	Series 3			Series 4
	n			3301-3310	3501-3510	3601-3610	
Chain length	5	29.1	67.1	16.5	28.6	50.3	16.9
	6	>100	41.8	11.5	87.9	19.6	4.11
	7	>100	31.9	-	-	-	-
	8	63.8	43.8	3.41	13.9	31.1	-
	9	>100	>100	-	-	-	-
	10	>100	20.3	-	-	-	-
	11	>100	7.27	-	-	-	-
	12	>100	23.2	-	-	-	-
		>100	32.7	-	-	-	-
		90.5	88.5	79.8	9.73	-	13.8
		>100	60.2	-	-	-	12.9
		15.8	21.0	-	-	-	-

NFT is weakly cytotoxic (IC₅₀ HEK-293 55.1 μM) while triazole moiety has no reported cytotoxicity. However, the hybrids were in general increasingly more cytotoxic than the drug. This could be attributed to the triazole moiety, as a consequence of its chemical properties such as strong dipole moments and hydrogen bonding which result in high affinity to bind to macromolecules, including possibly those in the cell membrane (Dheer *et al.*, 2017; Smit *et al.*, 2019).

6.4.2. Anti-mycobacterial activity

Anti-mycobacterial activity of the NFT analogues (Series 1) was discussed in depth in Chapter 4 and was re-entered in Table 6.2 for ease of access as well as in Table 6.7 for comparison. The single carbon linker (Series 2) hybrids had poor activity (61 μM < MIC₉₀ and >125 μM) against *Mtb* H37Rv, with the exception of **220** (MIC₉₀ 29.9 μM, Figure 6.11) which was in the same range (MIC₉₀ 18.3 – 34.7 μM) as short chain analogues (Series 1, *n*-alkyl *n* 1 - 4). The extended *n*-alkyl linker NFT-Tz hybrids (Series 3, Figure 6.3) and the aryl linker NFT-Tz hybrids (Series 4, Figure 6.5) were inactive (MIC₉₀ > 125) regardless of the linker or the attached substituent (**R**).

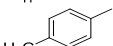
**Figure 6.11:** Hybrid **220**

This data indicates that the introduction of Tz led to the loss in anti-mycobacterial activity (Table 6.7). The only difference between compounds represented in Table 6.7 was the Tz moiety in the

1C linker NFT-Tz hybrids. Overall, the introduction of the Tz moiety to NFT led to a loss of anti-mycobacterial activity. The length or flexibility of the linker did not improve anti-mycobacterial activity of the NFT-Tz hybrids.

The majority of Series 1 and 2 derivatives had the same substituents (**R**); as such anti-mycobacterial activity comparison between these series was possible. 60% of the Series 1 and 2 derivatives differed only by the presence of the triazole. From these compounds, it was apparent that the introduction of Tz into the NFT structure led to the loss of anti-mycobacterial activity (Table 6.7) in addition to pronounced cytotoxicity.

Table 6.7: Anti-mycobacterial activity comparison between the analogues hybrids.

Subst.		Anti-TB <i>Mtb</i> H37Rv MIC ₉₀ (μM),					
Chain length	n	Series 1	Series 2	Series 3			Series 4
				3301-3310	3501-3510	3601-3610	
	5	2.1	>125	63.5	34.0	>125	>125
	6	1	>125	>125	>125	>125	>125
	7	2.0	>125	-	-	-	-
	8	0.5	>125	>125	>125	>125	-
	9	3.41	>125	-	-	-	-
	10	2.02	>125	-	-	-	-
	11	1.88	>125	-	-	-	-
	12	0.99	>125	-	-	-	-
		>125	62.4	-	-	-	-
		9.27	>125	>125	32.2	>125	
		-	60.2	-	-	-	>125
	5.03	61.3	-	-	-	-	

The parent drug (NFT, MIC₉₀ 14.5 μM) had better anti-mycobacterial activity than the hybrids. It is worth noting that some series 3 hybrids (Figure 6.12) namely **3507** (MIC₉₀ 14.7 μM) and **3510** (MIC₉₀ 15.9 μM) were as potent as the parent drug. However, taking into account cytotoxicity this observed potency was intrinsic as seen from the poor selectivity index values SI<1. A validated anti-TB hit should be >10-fold selective in killing mycobacteria as opposed to mammalian cells (Katsuno *et al.*, 2015). Inopportunately, none of the hybrids met this criteria, in fact, the hybrids with highest selectivity index were **3510** and **3601** (Figure 6.12) at 1 and 3, respectively. Further, selectivity index supports the observation of intrinsic activity as a result of cytotoxicity, suggesting that these hybrids may be toxic to human cells at concentrations below their mycobacterial efficacy.

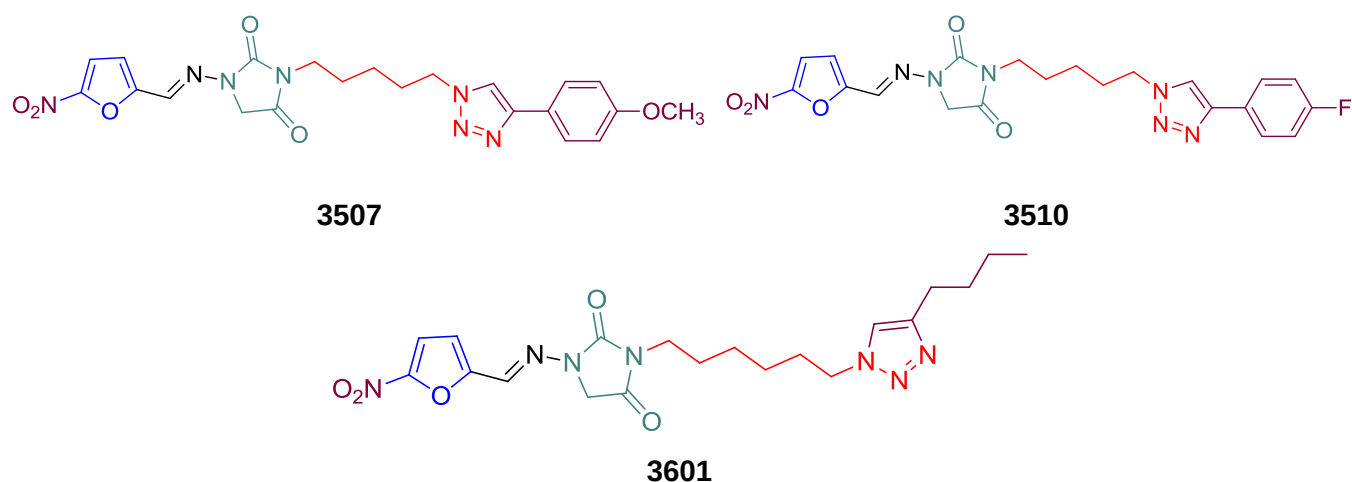


Figure 6.12: Hybrids with potential toxicity at concentrations below their mycobacterial efficacy

6.4.3. Anti-leishmanial activity

NFT had poor anti-leishmanial (promastigotes) activity against *L. major* (IC_{50} 56 μ M) and *L. donovani* (IC_{50} 26 μ M). However, the introduction of a methyl ($-CH_3$, **102** Figure 6.13) group at N-10 led to 42 times better activity against *L. major* (IC_{50} 1.32 μ M) and 60 times for *L. donovani* (IC_{50} 0.438 μ M). Overall, NFT analogues (Table 6.2) were potent against all tested leishmanial strains.

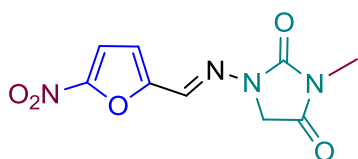


Figure 6.13: Analogue **102**

The least active analogue was **116** (IC_{50} >1.5 μ M, Figure 6.14) across all strains. The long chain analogues ($n \leq 12$) were at least 10-fold more potent than the short chain analogues (n 2 - 4). Furthermore, the aliphatic analogues had superior activity compared to the aryl analogues. Aryl analogues and short chain analogues (n 1 - 4) had equipotent anti-leishmanial activity. The $clogP$ value ranges: short chain analogues (n 1 - 4), -0.04 to 2.66 and aryl analogues 1.57 to 2.60 compared to the 2.66 to 4.88 of long chain analogues (n 7 - 12). Overall, the improvement in NFT lipophilicity improved anti-leishmanial activity against promastigotes.

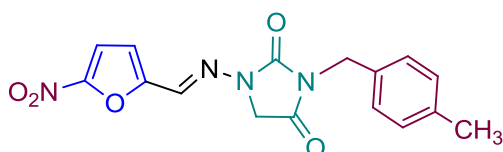


Figure 6.14: Analogue **116**

The addition of Tz on NFT did not negatively affect anti-leishmanial activity for *L. major* (CL). In fact, Series 2 hybrids were equipotent to shorter chain analogues ($n \leq 7$) and aryl analogues (Series 1) against *L. major*. However, only six Series 2 hybrids exhibited good anti-leishmanial activity (Table 6.3) against *L. donovani* (VL) and the rest had poor growth inhibition ($< 30\%$). Series 3 hybrids: **3301** - **3310** (3C linker) had poor growth inhibition (particularly, against *L. donovani*), therefore IC_{50} was not determined. Hybrid **3309** (Figure 6.15) was an exception among these hybrids as it had good activity (IC_{50} 1.2 μ M, *L. donovani*). Similarly, for **3501** – **3510** (5C linker hybrids), only **3502** and **3504** (Figure 6.15) exhibited good anti-leishmanial activity against *L. donovani* and *L. major*. On the other hand, the majority of 6C linker hybrids (**3601** - **3610**) also exhibited good anti-leishmanial activity against both *L. major* and *L. donovani*. Series 4 (aryl linker hybrids) were potent against all the tested strains; possibly indiscriminate activity presumably as a consequence of high cytotoxicity ($5 < IC_{50} < 17 \mu$ M). However, SI (majority between 13 and 97) was in line with the generic criteria hit selection ($SI > 10$) for infectious diseases (Katsuno *et al.*, 2015).

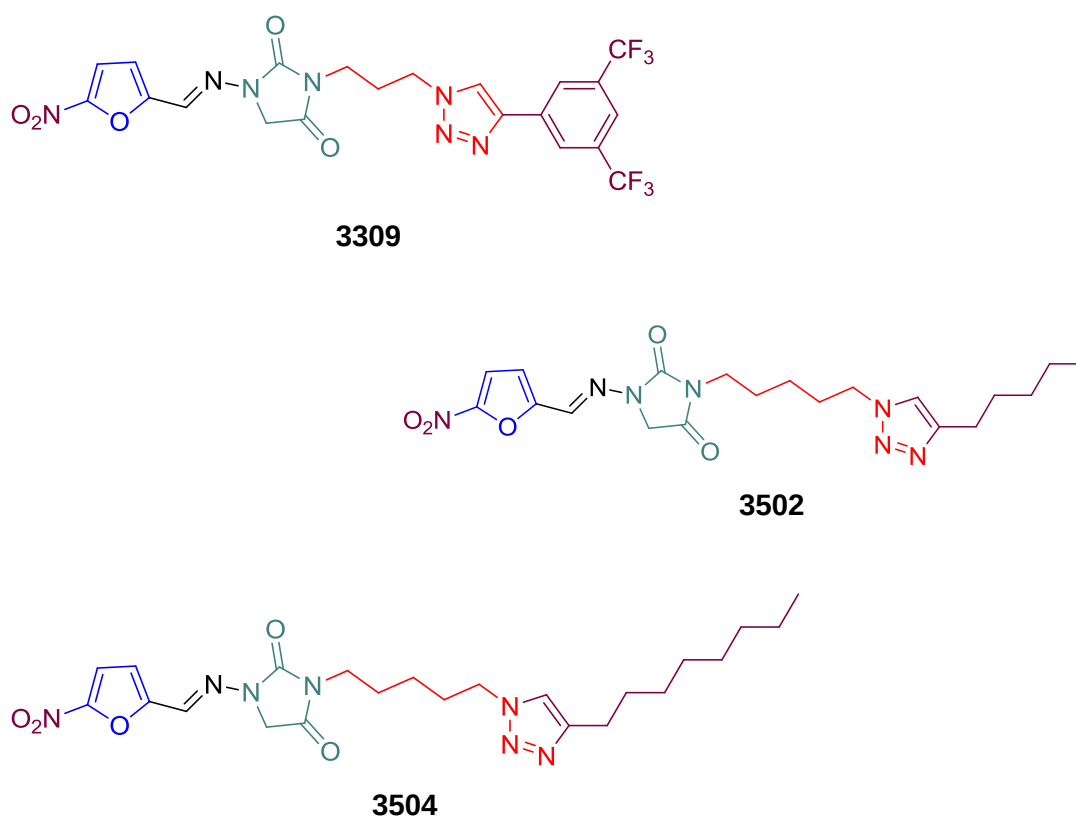


Figure 6.15: Series 3 hybrids with good anti-leishmanial activity against *L. donovani*

The focus of this study was to identify potentially affordable and effective anti-infective agents. Leishmaniasis is endemic in poverty stricken and resource limited areas. Therefore, the synthesised compounds were screened against promastigotes as the assays are simpler and cheaper (Siqueira-Neto *et al.*, 2010). In this study, it has been established that NFT has potential as an anti-leishmanial scaffold. Overall, the synthesised compounds were significantly potent

against *L. major* (CL) promastigotes with anti-leishmanial activity below 10 μ M. In fact, **113** (IC_{50} (μ M) 0.0340 (*L. major*), 0.012 (*L. donovani* 1S) and 0.099 (*L. donovani* 9515)) was equipotent to amphotericin B (an expensive, IV administered clinical anti-leishmanial drug) with an IC_{50} (μ M) of 0.03 (*L. major*), 0.02 (*L. donovani* 1S), 0.02 (*L. donovani* 9515). In addition, analogues **109** – **112** (Figure 6.16, highlighted green in Table 6.2), as well as hybrids **3309** and **3601** - **3610** (6C linker, Figure 6.17) exhibited good anti-leishmanial activity against both *L. major* and *L. donovani* promastigotes, therefore these hybrids may be candidates for further testing, i.e. amastigote anti-leishmanial activity to assess the ability to stand as anti-leishmanial hits (Katsuno *et al.*, 2015, Rao *et al.*, 2019). Therefore, future work, will include determination of anti-leishmanial activity against amastigotes since they are the clinically relevant forms of the parasite.

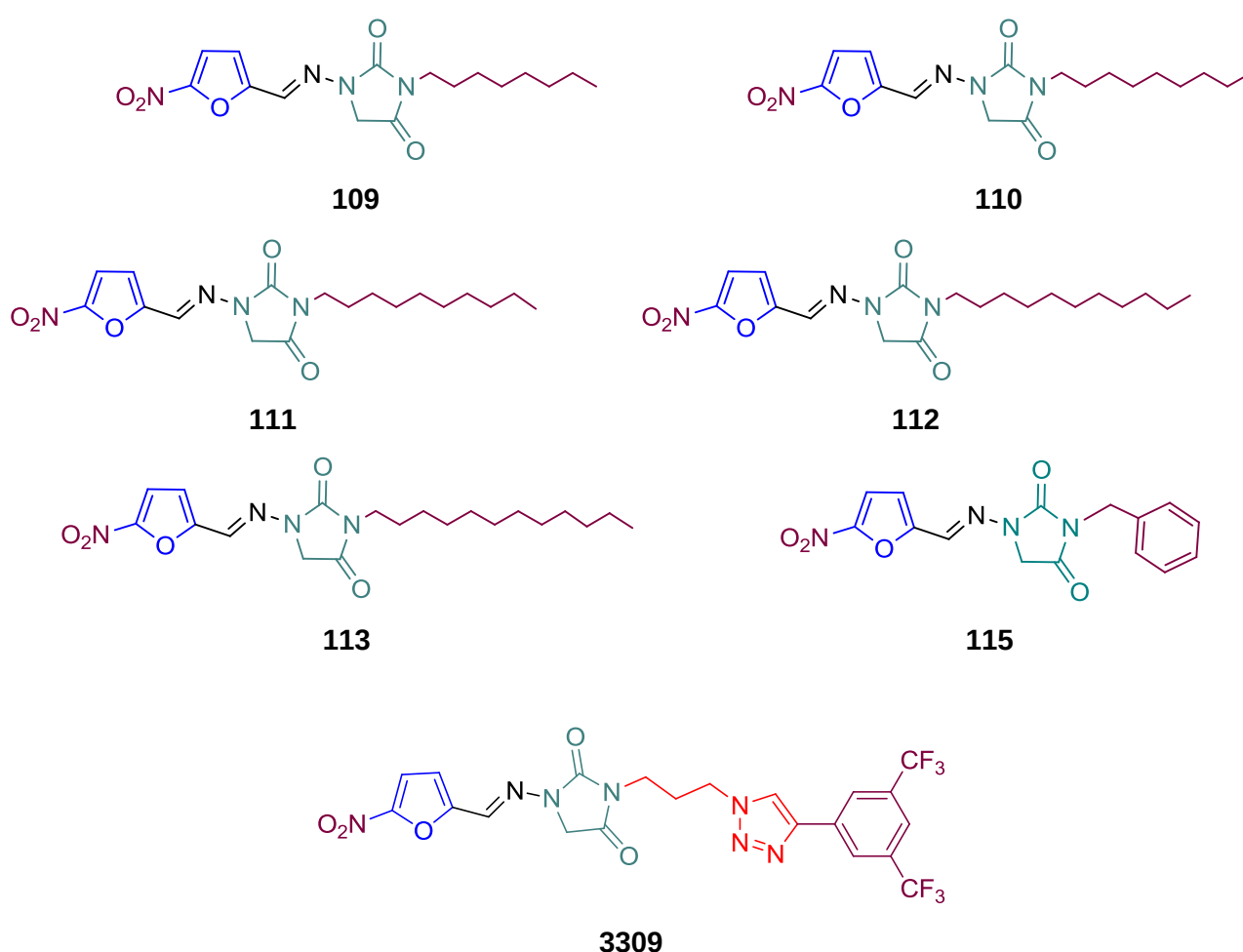
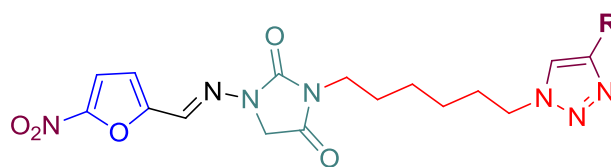


Figure 6.16: Derivatives with good anti-leishmanial activity against *L. major* and *L. donovani* promastigotes



Cpd.	R	Cpd.	R	Cpd.	R	Cpd.	R	Cpd.	R
3601	<i>n</i> -C ₄ H ₉	3603	<i>n</i> -C ₆ H ₁₃	3605		3607		3609	
3602	<i>n</i> -C ₅ H ₁₁	3604	<i>n</i> -C ₈ H ₁₇	3606		3608		3610	

Figure 6.17: Six carbon linker hybrids

6.4.4. Anti-plasmodial activity

NFT analogues (Series 1): at 10 μ M, only analogue **111** (an aliphatic 10-carbon analogue, Figure 6.16) had 91% growth inhibition while analogues (Figure 6.16) **109**, **111**, **112**, **113** and **115** had between 80- 89% growth inhibition. In this series, only **113** (an aliphatic 12-carbon analogue) had significant (85%) growth inhibition at 1 μ M. Consequently, after target based high-throughput screening, only **113** was advanced to anti-plasmodial IC₅₀ determination (Table 6.8) which was found to be 0.29 μ M and 0.25 μ M for 3D7 (drug sensitive) and Dd2 (drug resistant) strains of *P. falciparum*, respectively. Furthermore, the selectivity index (SI) for **113** was calculated to be 345 for 3D7 and 400 for Dd2. Despite a good toxicity profile, NFT analogues (Table 6.2) were devoid of significant activity against *P. falciparum*. The most active compound, **113** (IC₅₀ 0.25 against 3D7 and 0.29 against Dd2) was identified as a potential validated hit as it met the potency check-point criteria (IC₅₀ <1 μ M), for antimalarial validated hit (Katsuno *et al.*, 2015).

Table 6.8: Anti-plasmodial activity IC₅₀ (μ M) and selectivity index of the most active compounds

Cpd	Antiplasmodial IC ₅₀ (μ M)		Cytotoxicity (μ M)	Selectivity Index	
	3D7	Dd2		3D7	Dd2
113	0.29	0.25	>100	>345	>400.0
214	0.86	0.54	60.2	70.0	112
3502	0.56	0.18	28.6	51.1	159
3604	0.57	0.69	31.1	54.5	45.1
CQ	0.0116	0.1125*	nd	nd	nd
DHA	n/d	0.0012*	nd	nd	nd
ARM	n/d	0.0023*	nd	nd	nd
ARS	n/d	0.0075*	nd	nd	nd
MB	n/d	0.0045	nd	nd	nd

Selectivity Index = (IC₅₀ HEK-293 / IC₅₀ *P.falciparum* strain), CQ =Chloroquine, DHA = dihydroartemisinin, ARM = artemether, ARS = artesunate, *(Zuma *et al.*, 2016), MB =methylene blue , n/d = not determined

The synthesised hybrids had no significant anti-plasmodial activity. In fact, at 10 μM , all hybrids exhibited less than 90% growth inhibition. A total of nine hybrids (47%) of the single carbon linker (Series 2) had growth inhibition of >80%, and of those, six (67%) hybrids had growth inhibition of >85%. Although, none of the compounds in this series met the IC_{50} 0.1 μM (100 nM) of cellular potency check-point criteria for antimalarial early leads, the most potent anti-plasmodial hybrid, **214** (IC_{50} 3D7 0.86 μM ; IC_{50} Dd2 0.54 μM , Figure 6.18) was identified as a validated hit (Katsuno *et al.*, 2015).

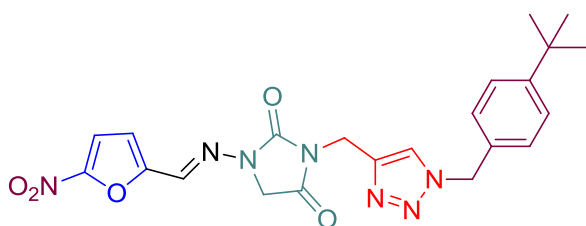


Figure 6.18: The most potent anti-plasmodial hybrid, **214**

For the extended *n*-alkyl linker hybrids, the IC_{50} (Table 6.8) was determined for **3502** (Figure 6.15) and **3604** (Figure 6.17) as they had the highest growth inhibition (50%) at 1 μM . The aryl linker hybrids were excluded on account of cytotoxicity ($\text{IC}_{50} < 30 \mu\text{M}$) in HEK-293 cells. Both selected hybrids showed sub-macromolar activities against the two strains of blood stage *P. falciparum* parasite, and good selectivity index ($\text{SI} > 10$). Thus, they stand as potential antimalarial validated hits (Katsuno *et al.*, 2015).

None of the identified potential hits possessed activity comparable to the artemisinins and other antimalarial standard drugs. However, in this study, it has been established that Nitrofurantoin does possess anti-plasmodial activity. Therefore, the data encourages further (i) structural modifications to improve efficacy; and (ii) *in vivo* studies and assessment across the malaria life cycle, particularly in the liver stage to ascertain the viability of Nitrofurantoin as a potential antimalarial drug.

6.5. Conclusion

NFT analogues had the best drug properties ranging from good safety profiles to good anti-infective activity. Analogue **113** stood out as the most active compound, with pronounced activity against all tested pathogens including *P. falciparum*. Analogue **109** was also a hit against mycobacterial and leishmanial pathogens. In contrast, NFT - Tz hybrids had high cytotoxicity as well as poor anti-mycobacterial and anti-plasmodial activity regardless of the linker or the attached substituent (**R**).

In summary molecular derivatisation was an effective strategy in the improvement of NFT as an anti-infective agent. NFT analogues provide a good starting point in the optimisation of Nitrofurantoin as an anti-infective agent. Overall, **113** (*n*-alkyl, *n* 12, in Table 6.2) stood out as a validated hit against two diseases, TB and malaria. In contrast, molecular hybridisation (NFT - Tz hybrids) was detrimental to anti-mycobacterial activity and produced hybrids with high cytotoxicity. However, future studies to determine of anti-leishmanial activity against amastigotes are envisaged to ascertain the overall anti-leishmanial activity of both the analogues and the hybrids.

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CHAPTER 7

SUMMARY AND CONCLUSION

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* (*Mtb*), is responsible for scores of deaths annually, despite the fact that it can be prevented and cured (with prompt treatment). TB was responsible for 1.5 million deaths in 2018 (WHO, 2019). The principal concerns with regards to TB include: (i) coinfection with other diseases such as HIV; (ii) the widespread of drug resistant *Mtb* strains (Smith *et al.*, 2013; WHO, 2018a), (iii) the length of treatment, which increases in the case of resistant TB; (iv) the toxicity of TB drug cocktails; and (v) latent TB infection, a reservoir for new active TB infections, which is estimated to reside in a third of the world's population (WHO, 2017).

Annually, 350 million people are at risk of infection by the *Leishmania* (*L.*) parasite, the causal agent for leishmaniasis (WHO, 2018c). Leishmaniasis is a high morbidity neglected tropical disease (NTD) also known as the disease of poverty, owing to its strong association with malnutrition and poor living conditions (Mitra & Mawson, 2017; WHO, 2015). The disease is particularly endemic in Asia, Africa, the Americas, and the Mediterranean region (Alvar *et al.*, 2006; Torres-Guerrero *et al.*, 2017). There are twenty species of *Leishmania* that can infect humans and thirty sand-fly species (pathogen vector) that can transmit the disease (Torres-Guerrero *et al.*, 2017). However, infections caused by *L. major*, and *L. donovani* responsible for cutaneous and visceral leishmaniasis, respectively, account for the largest burden. Despite being customised (takes into consideration the host and the infecting species), current leishmaniasis treatments are inadequate on account of: (i) the cost of treatment (anti-leishmanial drugs are expensive); (ii) impractical and inaccessible drug administration (intravenous and intramuscular injections); (iii) toxicity of clinical anti-leishmanial drugs; (iv) the varying cure rates (not effective for all *Leishmania* species, and in some cases against the same species from different geographic locations); (v) the absence of a vaccine; and (vi) coinfection with other diseases such as HIV (McGwire & Satoskar, 2014; WHO, 2018b).

Malaria, a vector-borne disease caused by the *Plasmodium* (*P*) parasite, transmitted to humans through the bite of an infected female *Anopheles* mosquito. Although, there are five species of the genus *Plasmodium* (*P*) that can infect humans, *P. vivax* and *P. falciparum*, are of large health concern (Pawluk *et al.*, 2013). The former can result in clinical relapse due to its ability to become dormant (hypnozoite), whereas the latter accounts for the largest malaria burden and mortality (Hulden & Hulden, 2011; WHO, 2018c). In 2017, malaria was responsible for 219 million new cases and 435 000 deaths. Additionally, *P. falciparum* has the ability to quickly develop drug resistance. Malaria chemotherapy heavily relies on artemisinins. In uncomplicated malaria, cure is

achieved by artemisinin combination therapy (ACT), which is aimed at improving efficacy and delaying the emergence of drug resistance. ACT is the administration of an artemisinin drug together with a longer acting antimalarial drug. This strategy is built on the principle that artemisinin, due its high potency and fast onset of action, will quickly reduce the parasite burden and when its concentration falls below therapeutic levels, the longer acting antimalarial will ensure continued treatment. However, artemisinin partially resistant *P. falciparum* strains have been reported in the Great Mekong Region (WHO, 2016). In response to this partial resistance and in the absence of new drugs; the approach may involve increased artemisinin concentrations, or increased dosages (Das et al., 2013). Adversely, this approach amplifies neurotoxicity concerns of artemisinins at high doses (Fairhurst & Dondorp, 2016, Starzengruber *et al.*, 2012, Van Neck *et al.*, 2007). It is noteworthy that reports on artemisinins induced neurotoxicity stem from studies on laboratory animals, exposed to the drugs for extended periods of time and at high dosages (Efferth & Kaina, 2010). However, in the wake of resistance, the issue of neurotoxicity in humans becomes a concern thus assigning urgency to the need for new antimalarial drugs.

Thus, there is a dire need for new effective, and low cost oral anti-infective agents against mycobacterial, leishmanial and plasmodial infections that can: (i) quickly and efficiently clear pathogen burden; (ii) block transmission; and (iii) deter the emergence of drug resistant strains, either by novel mechanism of action or by disrupting multiple crucial metabolic processes in the pathogen. Consequently in this study, Nitrofurantoin (NFT, Figure 7.1) was investigated on the following basis: (i) its activity against both replicating and non-replicating mycobacteria (Miller *et al.*, 2002; Wang *et al.*, 2008); (ii) the success of nitroaromatic compounds in the treatment of other kinetoplastid diseases (under which leishmaniasis is classified), such as the use of nifurtimox (an NFT sister drug) in combination therapy for human African trypanosomiasis (HAT) and in the treatment of Chagas' disease (Montalto de Mecca *et al.*, 2002; Jeganathan *et al.*, 2011); (iii) NFT has similar anti-pathogenic effects as artemisinins in that it generates ROS (Cui & Su, 2009); and (iv) cases of NFT resistance in pathogens are rare, likely due to its multi-activity, as well as effectiveness under both aerobic and anaerobic conditions (Blass, 2015).

NFT (Figure 7.1) is a common drug for the treatment of urinary tract infections (Horton, 2015). It is a redox active agent with activity under both aerobic and anaerobic conditions (Voak *et al.*, 2014; Wang *et al.*, 2008). NFT activation occurs on the Nitrofuran ring (red in Figure 7.1) by pathogen-specific nitroreductases (NTRs) (Zhou *et al.*, 2012). Aerobic conditions favour type II nitroreduction, which is the one electron reduction of the nitro (NO₂) group at the expense of NAD(P)H by futile redox cycling (Wardman, 1985). The process generates reductive oxygen species (ROS) and lipid peroxidation, consequently resulting in the inhibition of pathogenic enzymes that disrupt crucial pathogenic metabolic pathways (Mason, 1990; Wang *et al.*, 2008). Under anaerobic conditions, the

nitro group is reduced to a nitroso species that disrupts the Krebs cycle and inhibits DNA, RNA and protein synthesis, consequently killing the pathogen (Blass, 2015).

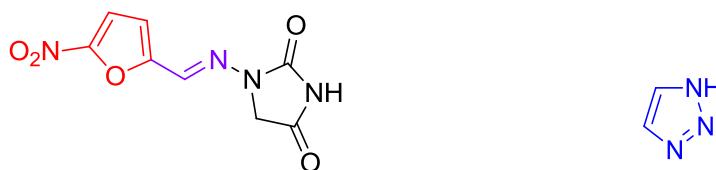


Figure 7.1: Nitrofurantoin (NFT)

Triazole (Tz)

Triazole (Tz, Figure 7.1) is an important biological and pharmaceutical agent with anti-mycobacterial (Sajja *et al.*, 2017), anti-leishmanial (Dheer *et al.*, 2017; Kamal *et al.*, 2013) and anti-plasmodial (Mabasa *et al.*, 2019) activities. This moiety possesses chemical properties such as strong dipole moments, and hydrogen bonding. As a result, it binds to biological targets with high affinity (Dheer *et al.*, 2017). Triazole inhibits lanosterol 14 α -demethylase which compromises the integrity of the cell wall by reducing the synthesis of ergosterol - a fungal and protozoal analogue of cholesterol (Goluszko & Nowicki, 2005; Rodrigues, 2018; Waller & Sampson, 2018).

Drug repurposing is increasingly becoming a common and successful alternative to the expensive traditional *de novo* drug discovery. The strategy of drug repurposing, explores clinically approved drugs for the treatment of other diseases, besides the drug's originally intended disease (Shim & Liu, 2014). The drug may be repurposed as is or it can be modified either by molecular derivatisation or hybridisation. The former is dedicated to producing analogues with improved therapeutic profiles (Hu *et al.*, 2017, Guha, 2013). The latter is a strategy of drug design, in which two or more different drug pharmacophores are assembled into a single chemical entity with multiple modes of action (Hulsman *et al.*, 2007). The premise for molecular hybridisation is that a hybrid molecule may have reduced side effects, reduce toxicity, improve bioavailability, interact with more than one pathogen target, and delay the development of drug resistance (Hulsman *et al.*, 2007, Muregi & Ishih, 2010). Therefore, in this study, Nitrofurantoin derivatives (Series 1) and Nitrofurantoin-triazole hybrids (Series 2 – 4) were investigated as potential anti-infective agents.

The aim of this study was to investigate novel Nitrofurantoin derivatives, as new effective, safe and affordable potential anti-infective agents.

In order to achieve this aim:

- A series of novel, *n*-alkyl/ aryl Nitrofurantoin analogues was synthesised
- Three series of novel, Nitrofurantoin-triazole hybrids with varying linkers were prepared

- H37Rv, a human strain of *Mtb* was used to assess *in vitro* anti-mycobacterial activity of all the derivatives
- *In vitro* anti-plasmodial activity of the derivatives was determined against, 3D7 (drug susceptible) and Dd2 (drug resistant), *P. falciparum* strains
- *L. donovani* (1S and 9515) and *L. major* (IR-173) strains which are responsible for visceral and cutaneous leishmaniasis; respectively, were used to assess *in vitro* anti-leishmanial (promastigote) activity of the derivatives
- An *in vitro* assay was performed in human kidney embryonic (HEK-293) cells alongside Chinese hamster ovarian (CHO) cells to assess the cytotoxicity profile of all the synthesised derivatives.

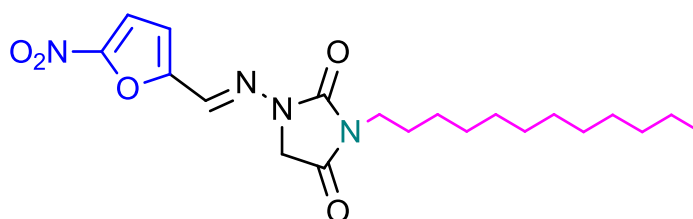
TB, leishmaniasis and malaria are diseases that affect mainly poor and resource limited populations. Therefore, the main consideration in bringing about these NFT derivatives was cost-effective and efficient synthesis, i.e. (i) simple synthesis (few steps, preferably less than five); (ii) minimal and simple purification (hence no chromatography, only recrystallisation - using green solvents, such as ethanol and water); and (iii) provide good yields (~70%). Consequently, the NFT analogues (Series 1) were obtained by single step synthesis and purified by recrystallisation in ethanol and water. In addition, the NFT-Tz hybrids (Series 2-4) were synthesised in three steps namely (i) the formation of first intermediate by propargylation (Series 2) or the attachment of the *n*-alkyl (Series 3) and aryl (Series 4) linkers on NFT; (ii) the synthesis of azide intermediate; and (iii) the synthesis of the hybrids using click chemistry (varying yields, majority 50 – 80%). When compared to the parent drug (NFT), all the derivatives (Series 1-4) had improved drug-like properties on the physicochemical point of view.

NFT analogues had the best drug-like properties, from good safety profile, low toxicity to good anti-infective activity. The analogues may be a good foundation for the development of anti-infective agents, particularly the *n*-alkyl derivatives against mycobacterial and leishmanial infections. Analogues **109 – 113** show promising activity against all pathogens tested in this study. Further studies including (i) *in vitro* evaluation of the analogues against the clinically relevant amastigote forms of leishmanial infections; (ii) evaluation in *in vivo* models, particularly, against mycobacterial and leishmanial infections; and (ii) structural modification, possibly replacing triazole with its bioisostere, the imidazole moiety (Bonandi *et al.*, 2017) might be a step in the right direction for the development of safe, effective, affordable and accessible agents with potential for oral administration that will be accessible to those who need them the most.

NFT-Tz hybrids resulted in a loss of anti-mycobacterial activity, and increased cytotoxicity. Thus, it became apparent that the introduction of the triazole moiety on the NFT scaffold confers cytotoxicity and leads to a loss of anti-mycobacterial activity, despite the improvement on the log*P*

(and by inference lipophilicity). Adversely, triazole is not an ideal companion bioactive agent for Nitrofurantoin in the synthesis of molecular hybrids with potent anti-mycobacterial or anti-plasmodial activity and a good safety profile. However, the hybrids (particularly Series 2 and Series 3) had good activity against *L. major* (promastigotes) the causal agent for cutaneous leishmaniasis. Although, the hybrids reported herein did not possess potent growth inhibition against *L. donovani* promastigotes (infective form), *L. major* data encourages future evaluation for anti-leishmanial activity against amastigotes (clinical form).

In conclusion, molecular derivatisation of NFT produced analogues (series 1) with improved anti-infective (particularly, anti-mycobacterial and anti-leishmanial) properties and good safety profiles. In contrast, molecular hybridisation (NFT - Tz hybrids, series 2 - 4) was detrimental to anti-mycobacterial activity and cytotoxicity. Neither strategy produced efficacious potential anti-plasmodial agents comparable to artemisinins, although both strategies produced derivatives identified as antimalarial validated hits. Analogue **113** (Figure 7.2) stood out as an anti-infective hit for further investigation in the urgent search for new, safe and affordable drugs.



Activity (μM)		Cytotoxicity (μM)	Selectivity Index
IC ₅₀			
HEK-293	-	-	> 100
<i>Mtb</i> H37Rv	MIC ₉₀	0.99	> 100
<i>L. major</i>	IR-173	0.034	> 29411
<i>L. donovani</i>	1S	0.012	> 8333
	9515	0.099	> 1010
<i>P. falciparum</i>	3D7	0.29	> 345
	Dd2	0.025	> 400

Figure 7.2: Summary of biological data for analogue **113**

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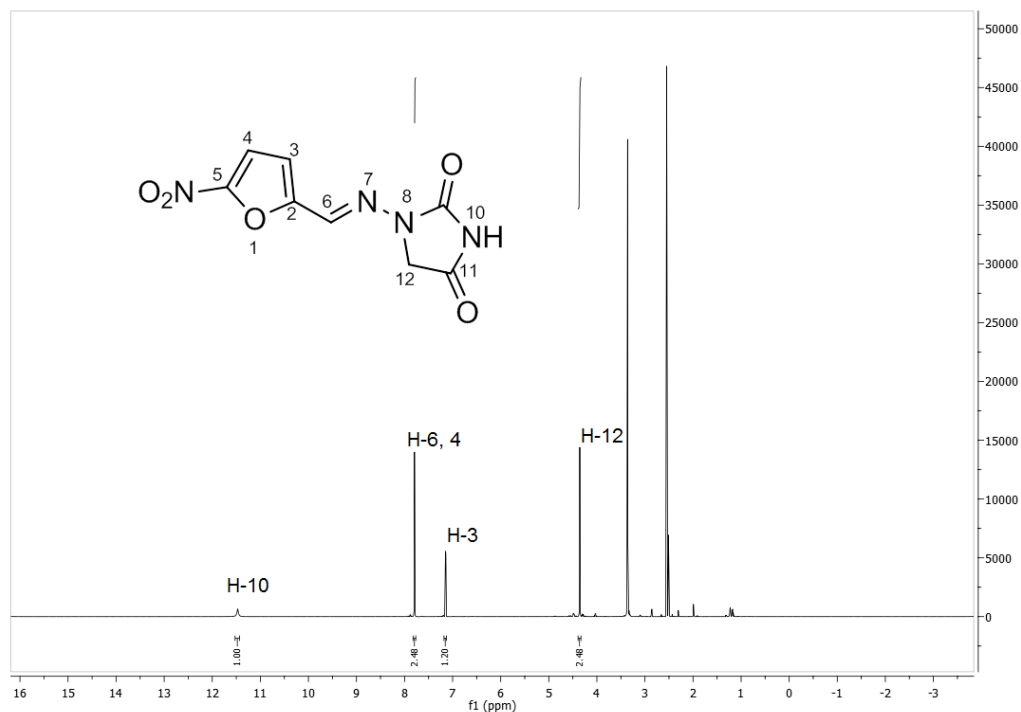
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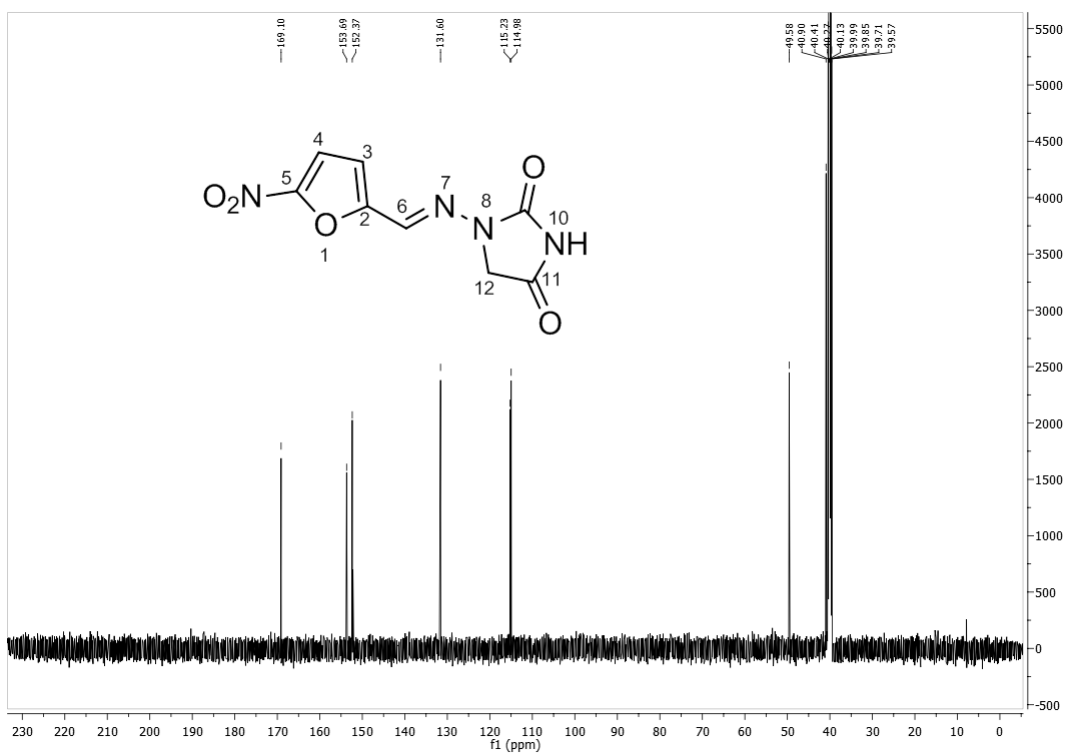
APPENDIX A: SPECTRA

STARTING SCAFFOLD: NITROFURANTOIN, 101

^1H NMR IN DMSO

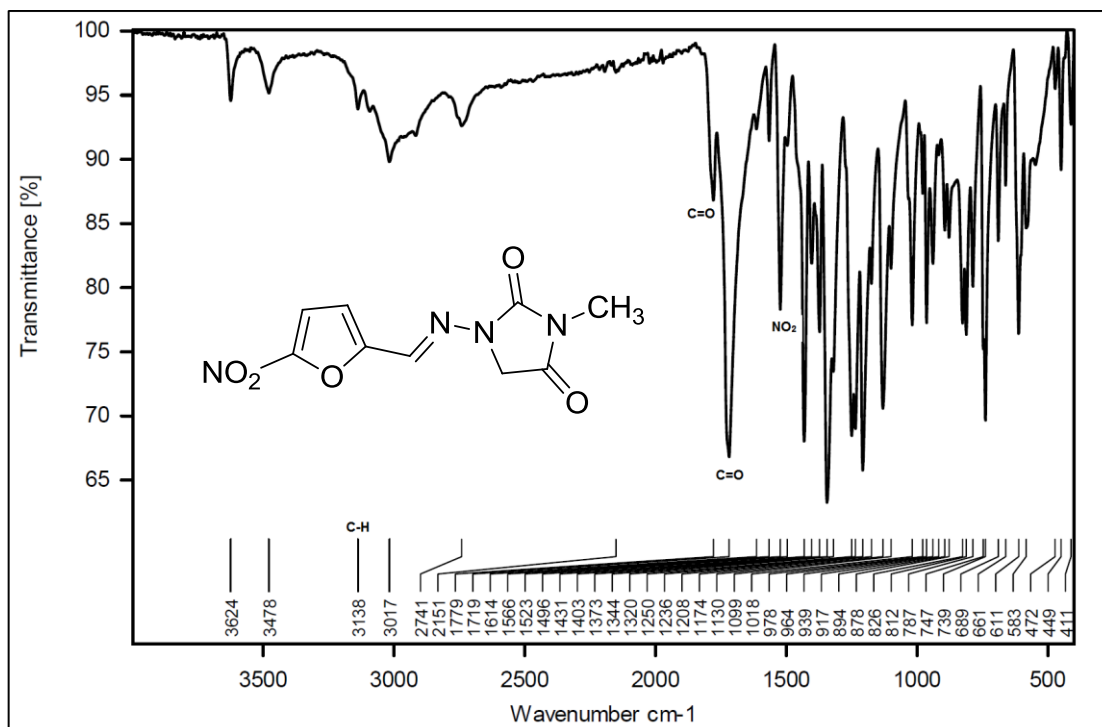


^{13}C NMR in DMSO

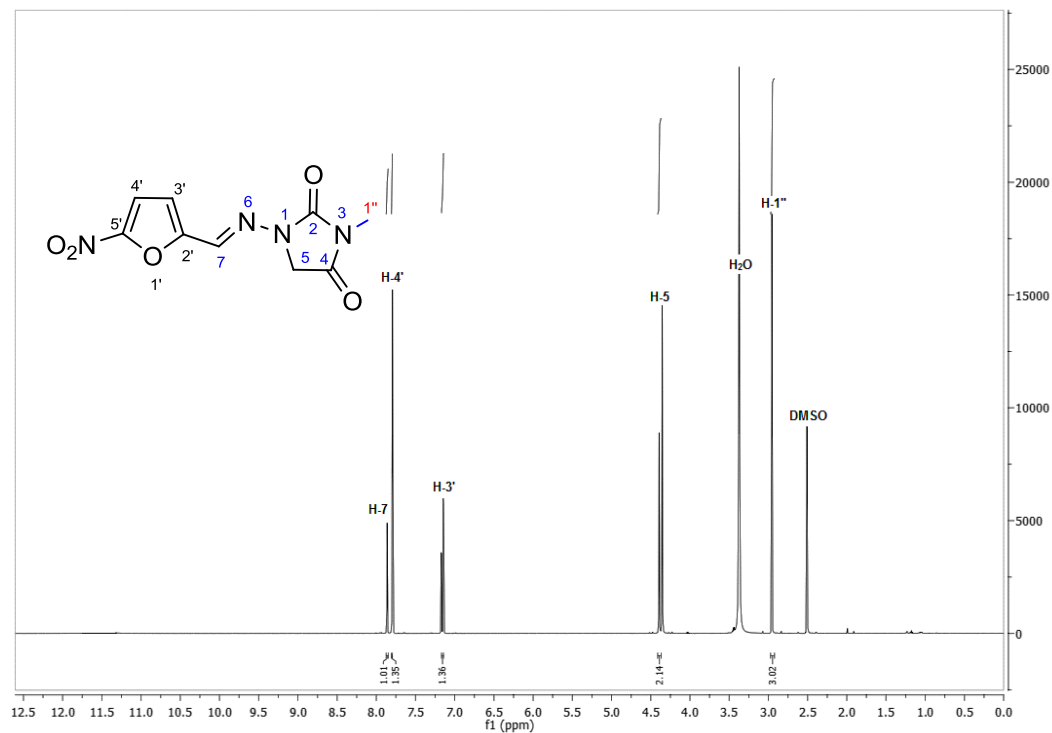


ANALOGUE 102

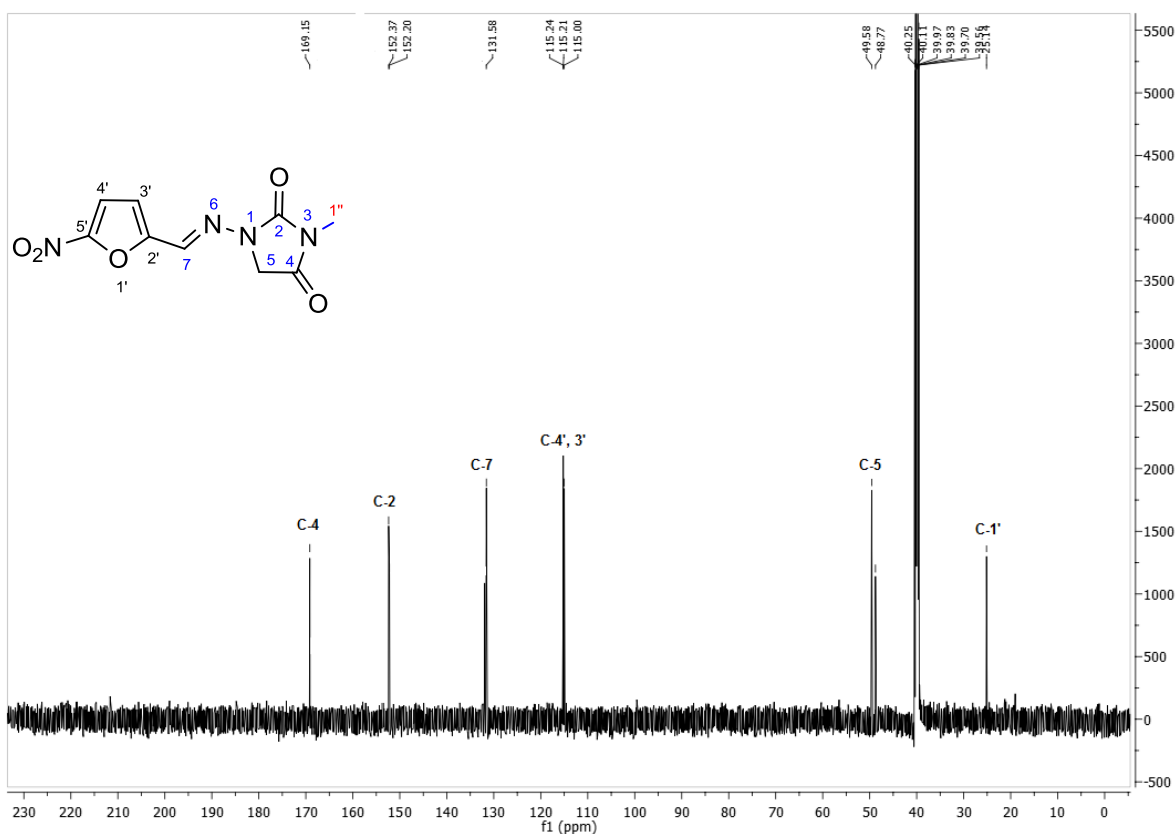
IR



¹H NMR in DMSO



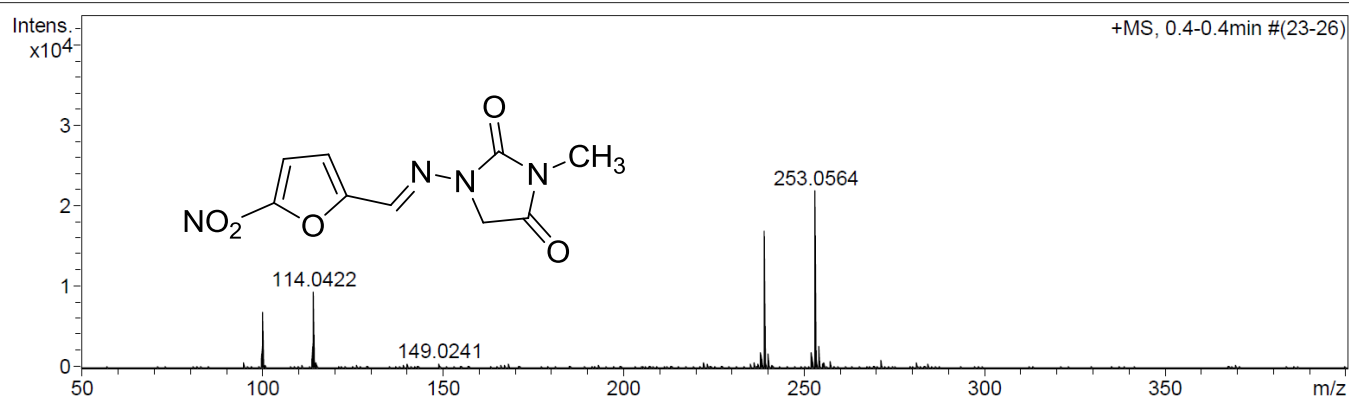
¹³CNMR in DMSO



HRMS

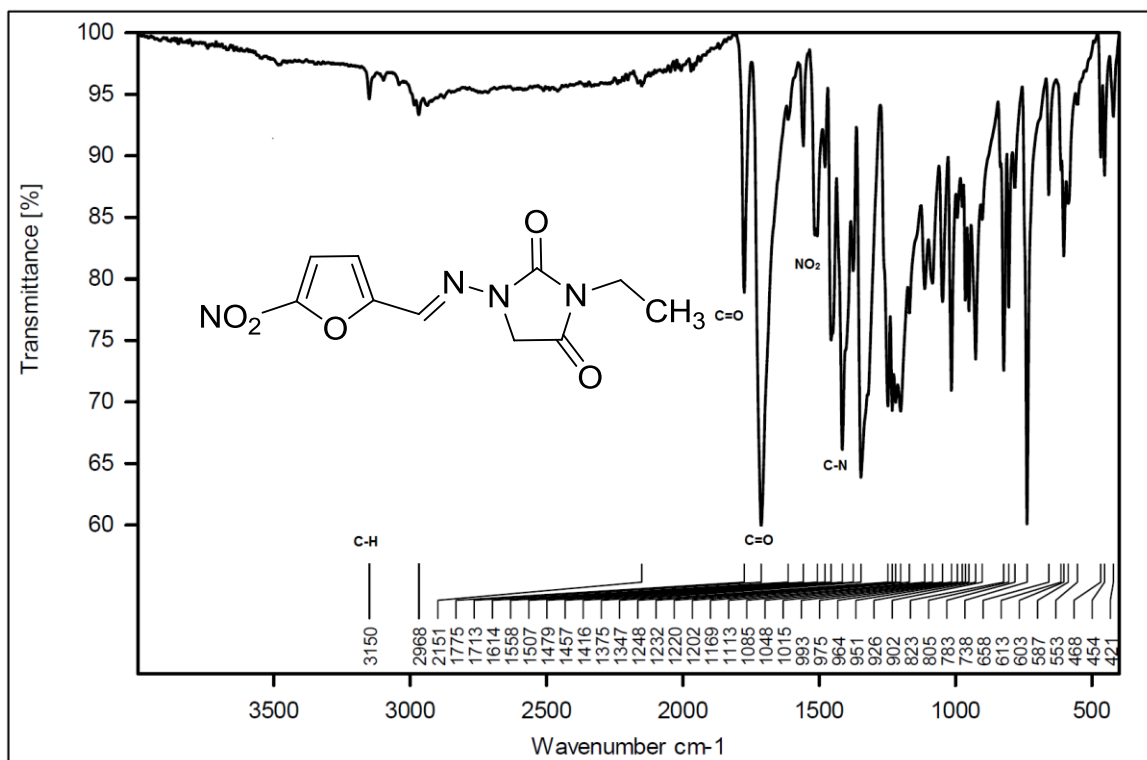
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	80.0 Vpp	Set Divert Valve	Waste

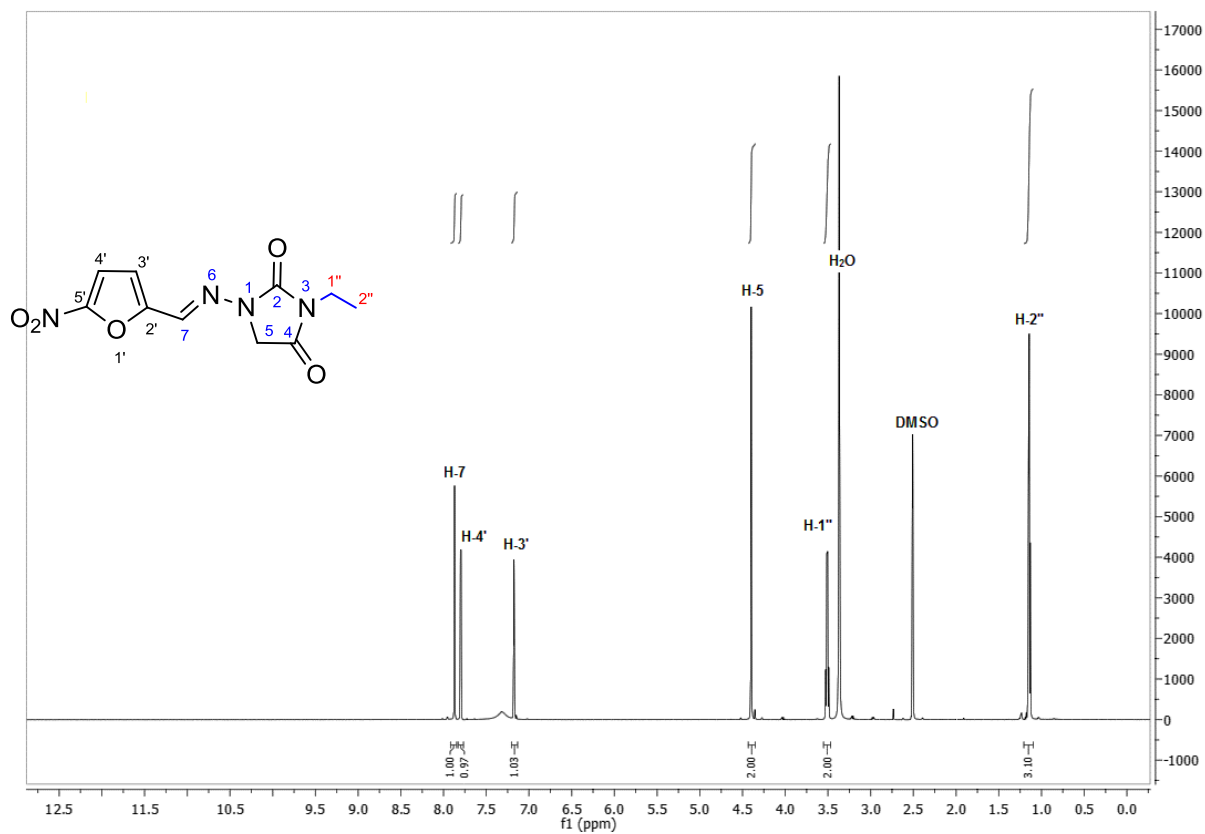


ANALOGUE 103

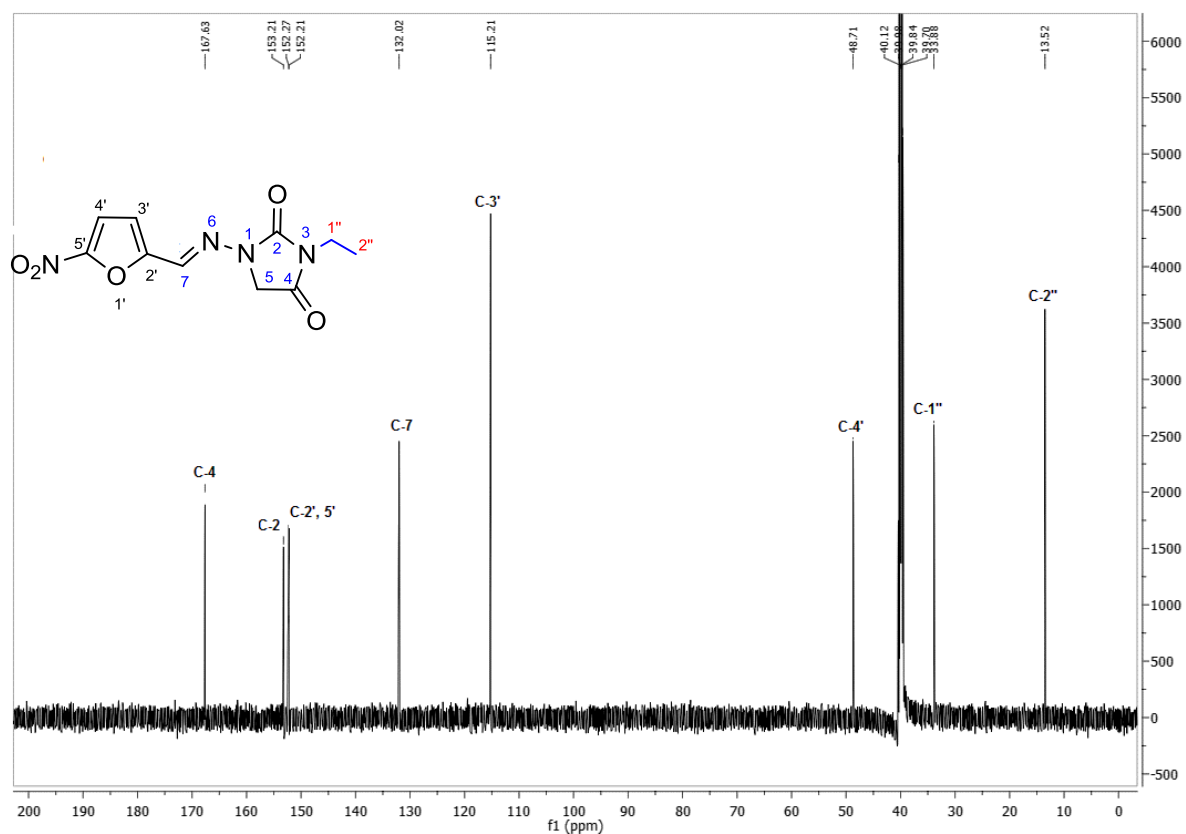
IR



^1H NMR in DMSO



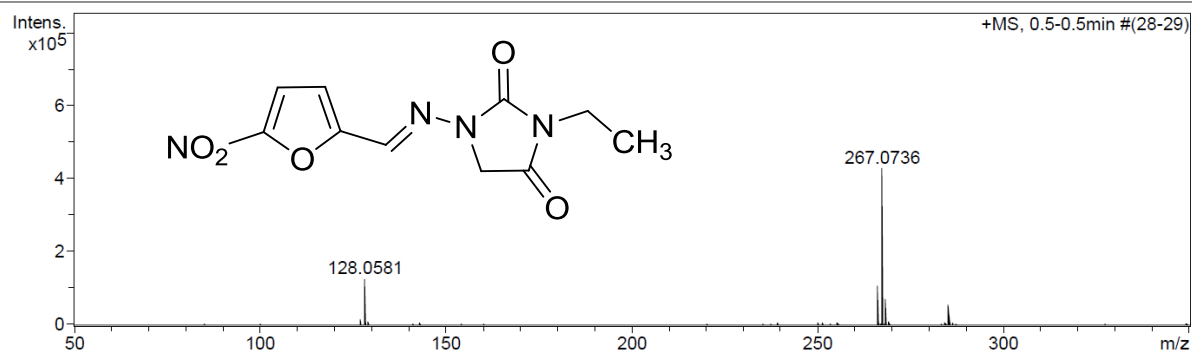
¹³CNMR in DMSO



HRMS

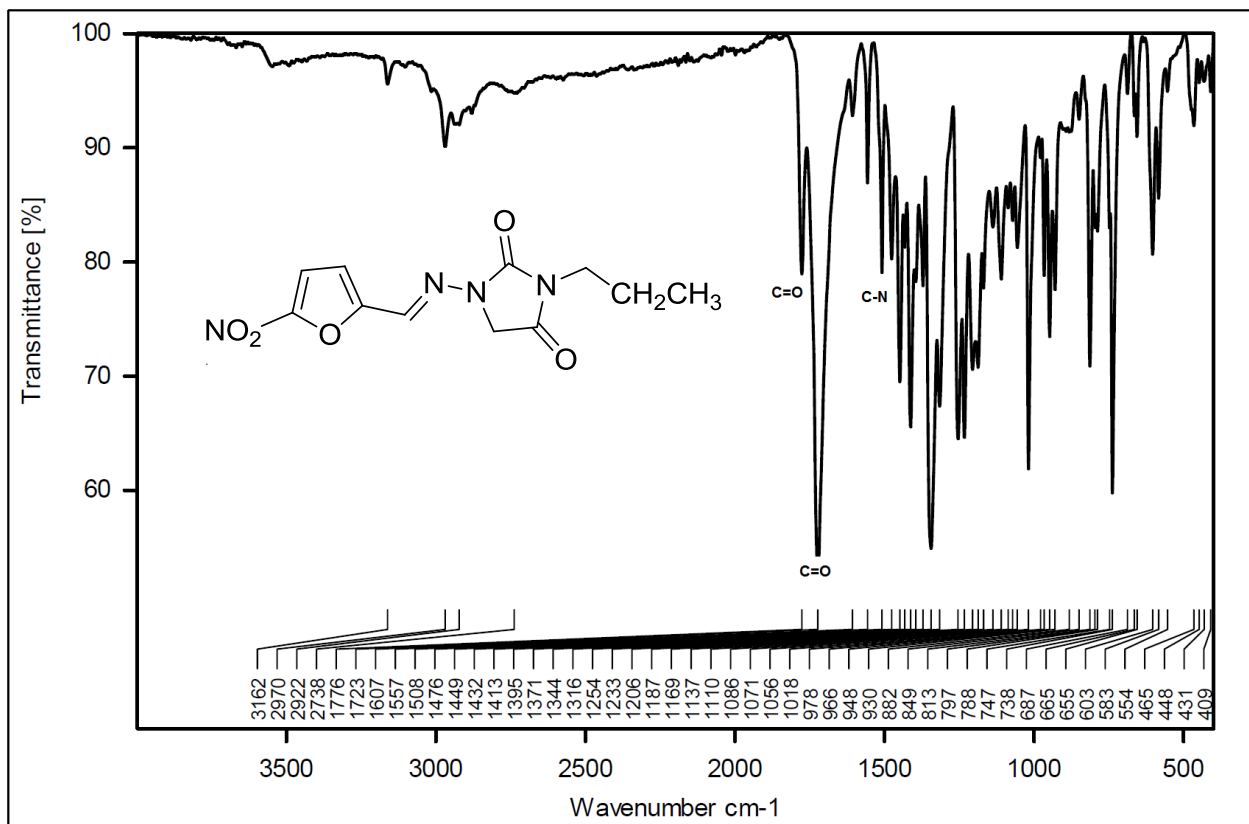
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

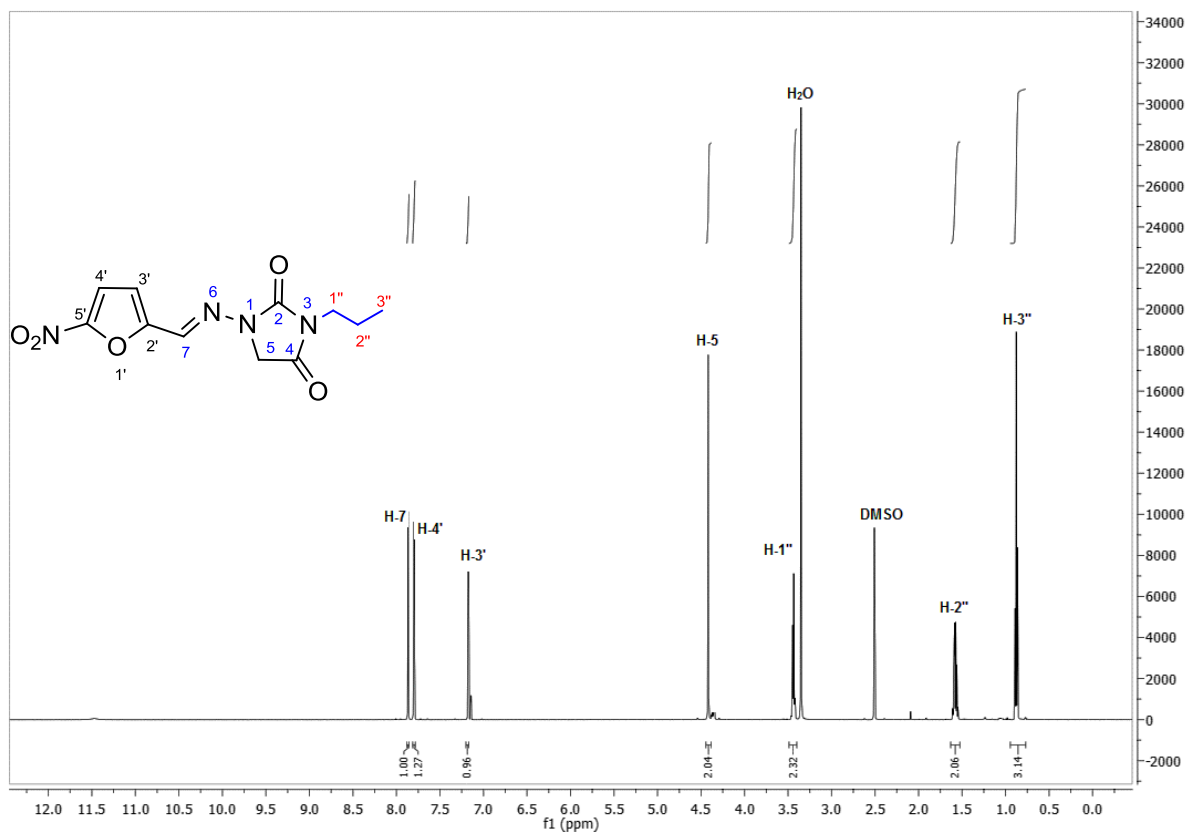


ANALOGUE 104

IR



¹H NMR in DMSO



Chemical structure of 1-(4-nitrophenyl)-2-((2-ethyl-1H-imidazol-1-yl)methyl)-1H-imidazole-5-carboxamide is shown. The structure includes a 4-nitrophenyl group, a 1H-imidazole-5-carboxamide group, and a 2-ethyl-1H-imidazole group. The carbon atoms are labeled with numbers 1' through 7 and 1'' through 3''.

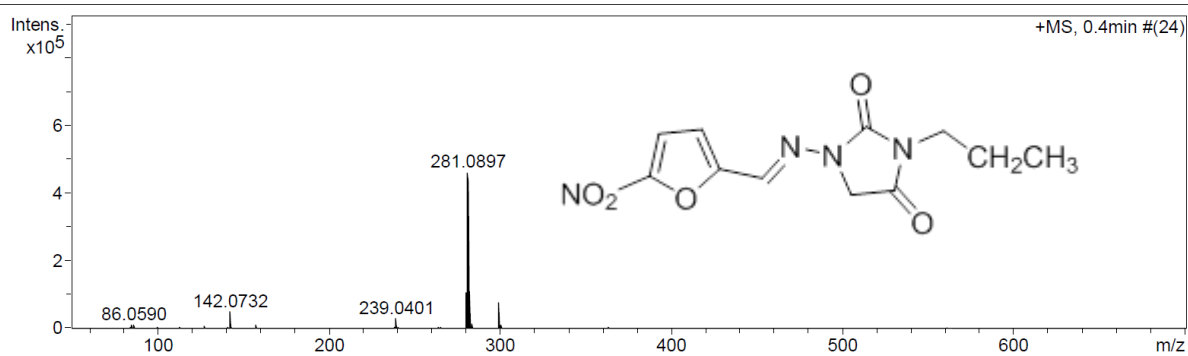
¹³C NMR spectrum (f1 (ppm)) showing peaks corresponding to the structure. The x-axis ranges from 0 to 200 ppm. The y-axis ranges from -500 to 6500. The spectrum shows several sharp peaks, with the following chemical shifts (ppm) labeled above the peaks:

- 167.90 (C-4)
- 153.42 (C-2', 5')
- 152.27 (C-2)
- 132.01 (C-7)
- 115.21 (C-4', 3')
- 48.64 (C-5)
- 21.29 (C-2'')
- 11.55 (C-3'')

Additional peaks are visible in the aliphatic region (0-40 ppm) corresponding to the ethyl group, with chemical shifts labeled as 48.64, 40.48, 40.41, 40.37, 39.89, 39.85, 39.71, 39.57, and 21.29 ppm.

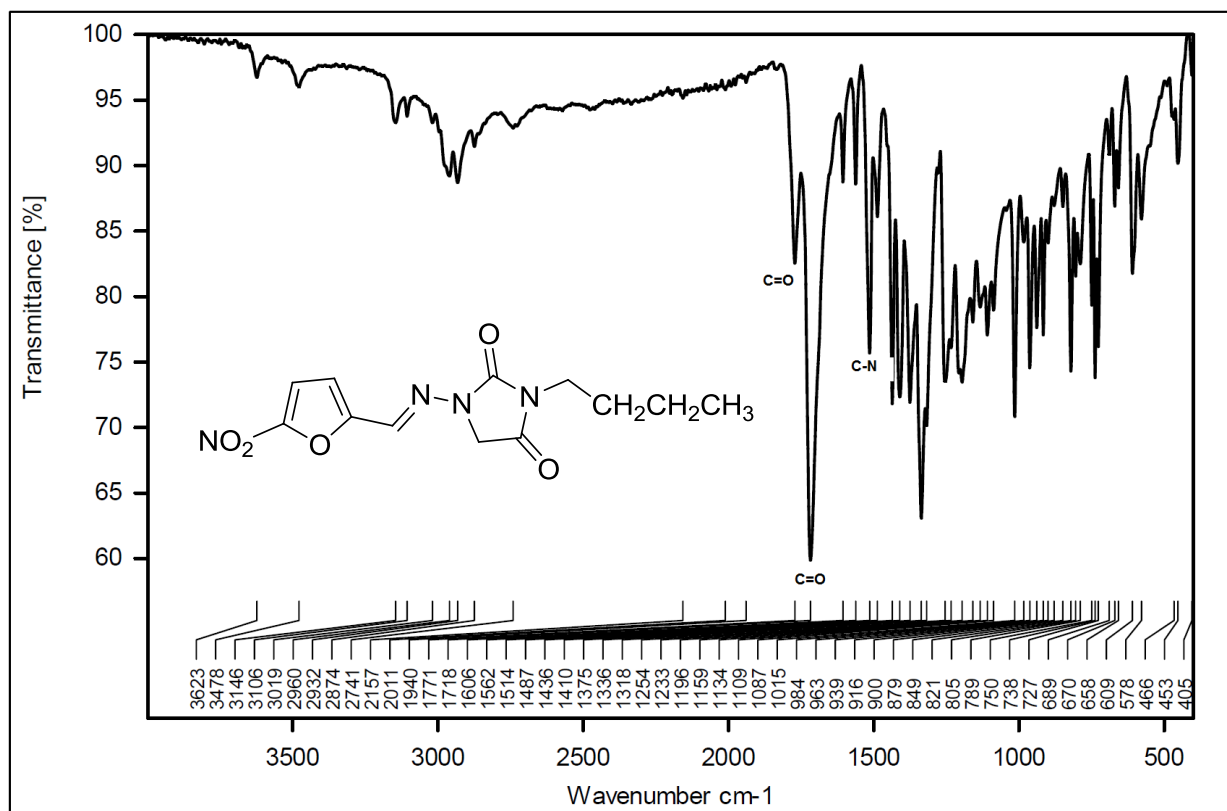
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	280 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

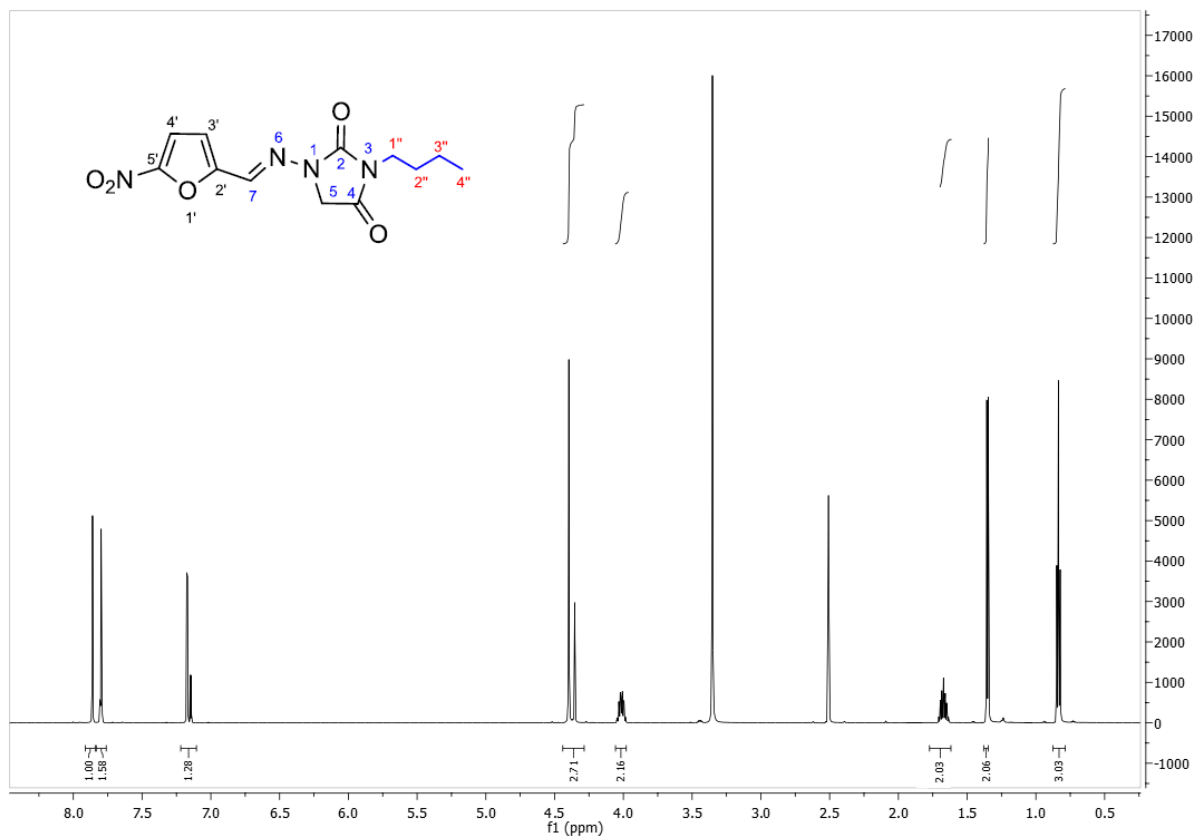


ANALOGUE 105

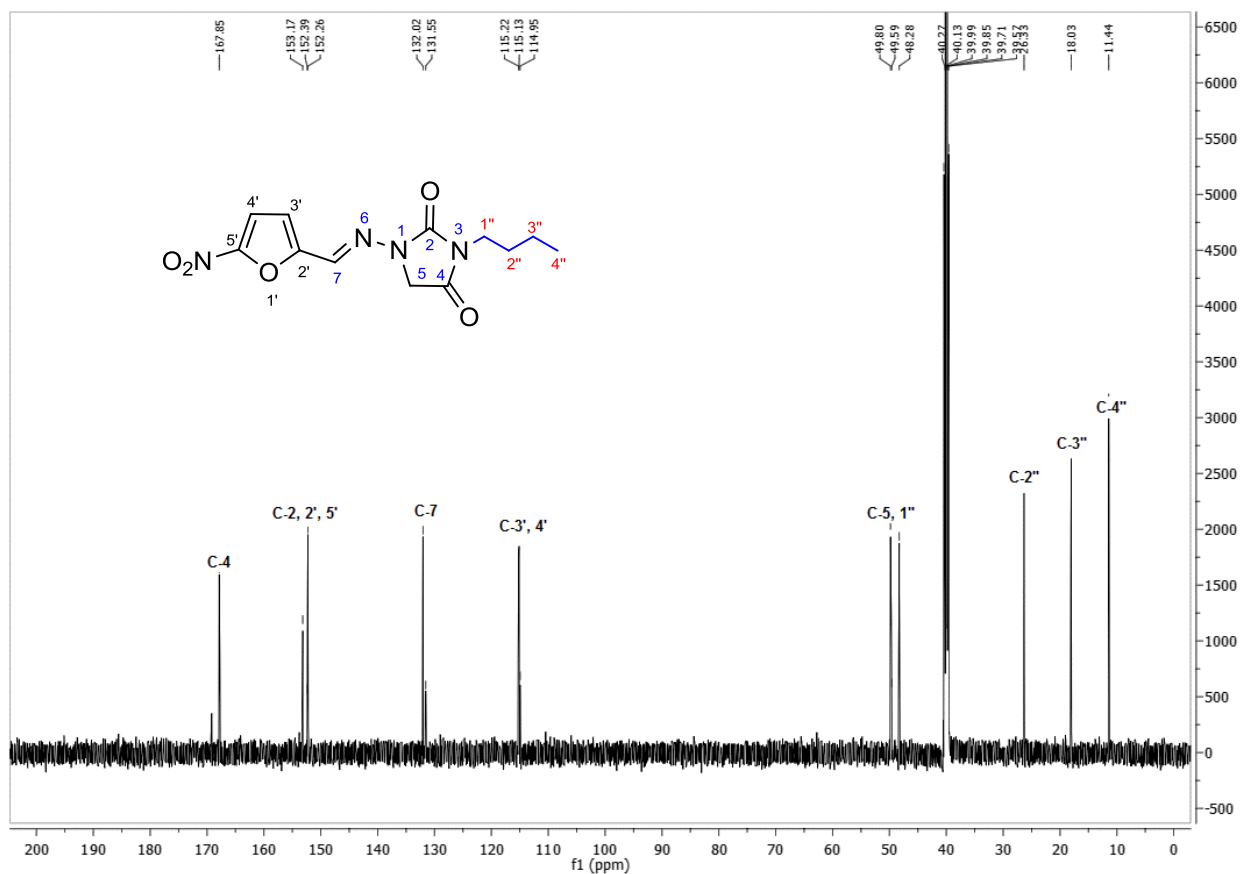
IR



^1H NMR in DMSO



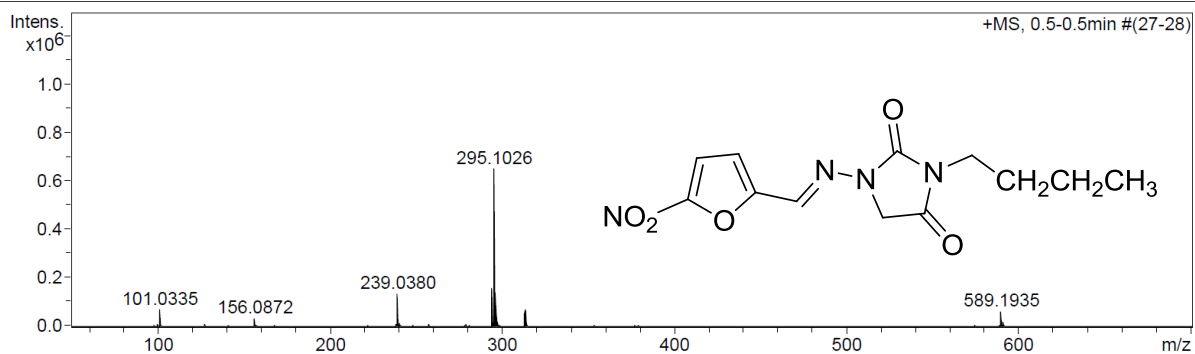
¹³CNMR in DMSO



HRMS

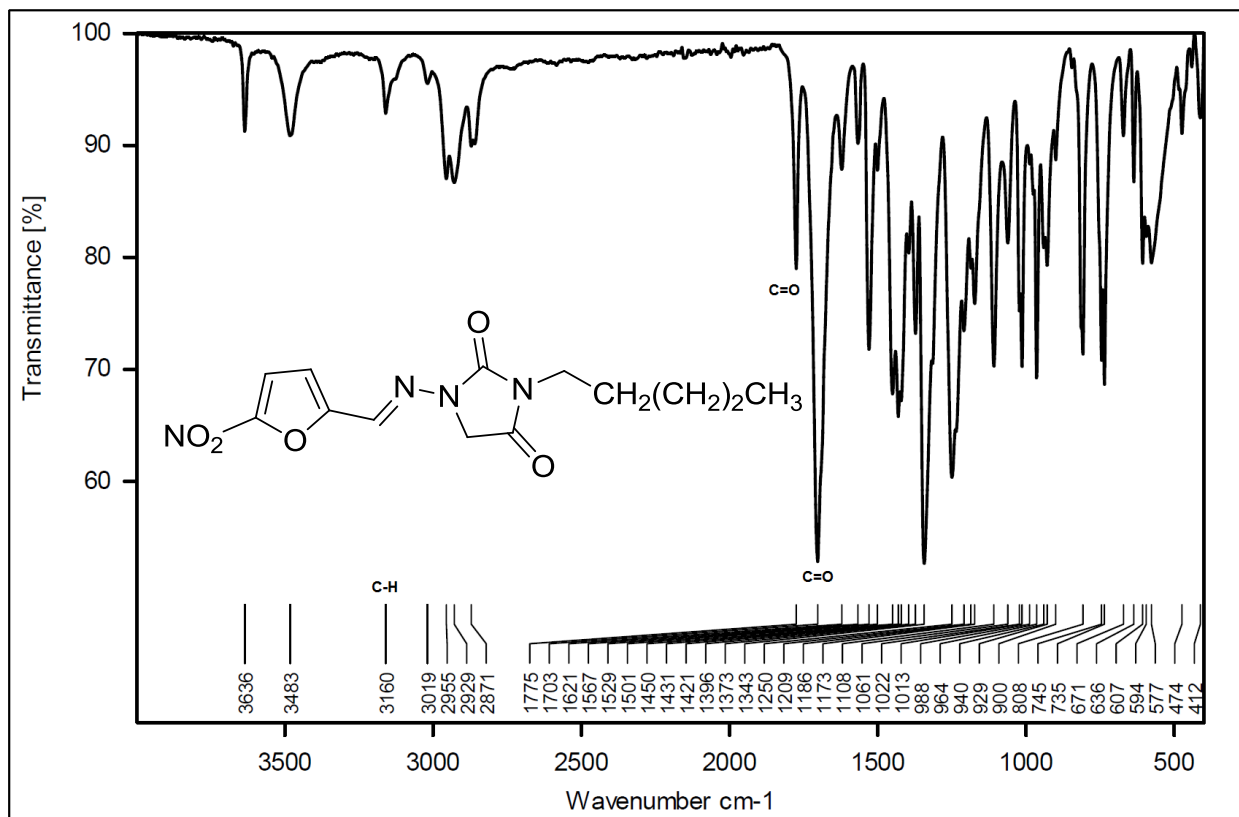
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

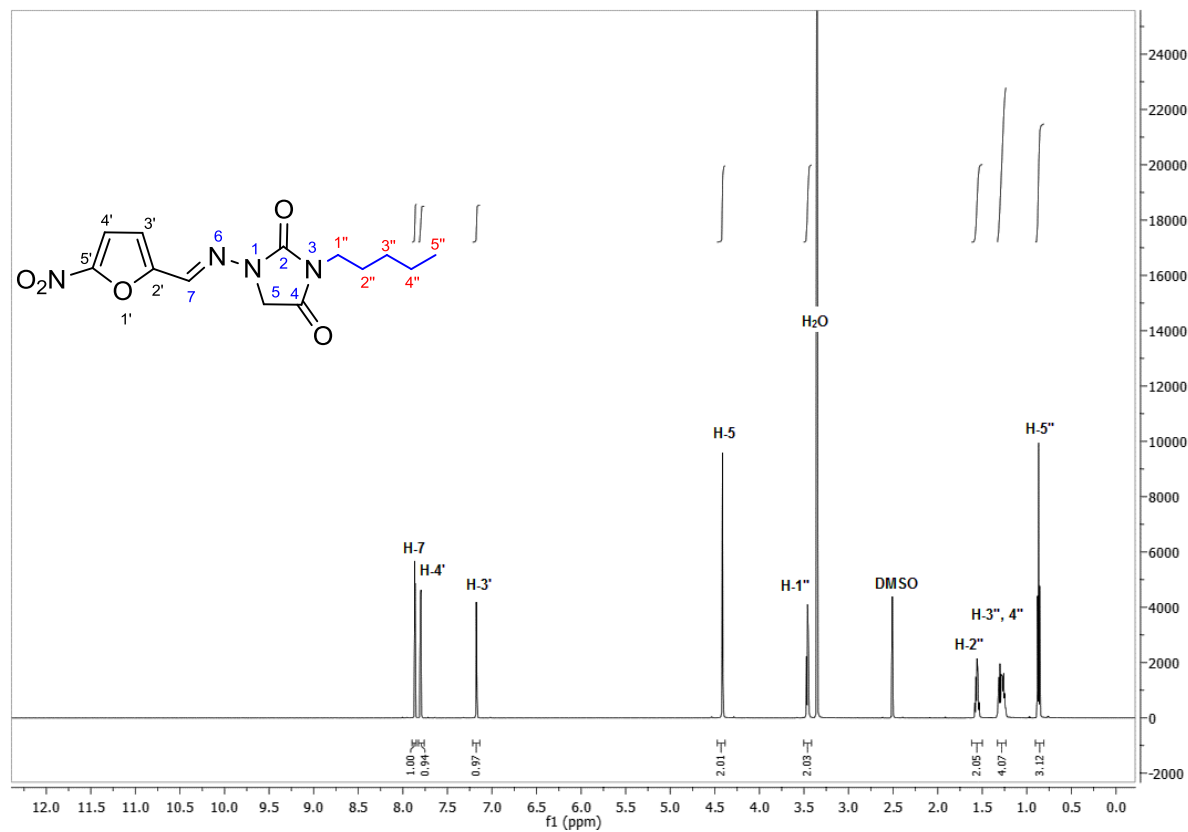


ANALOGUE 106

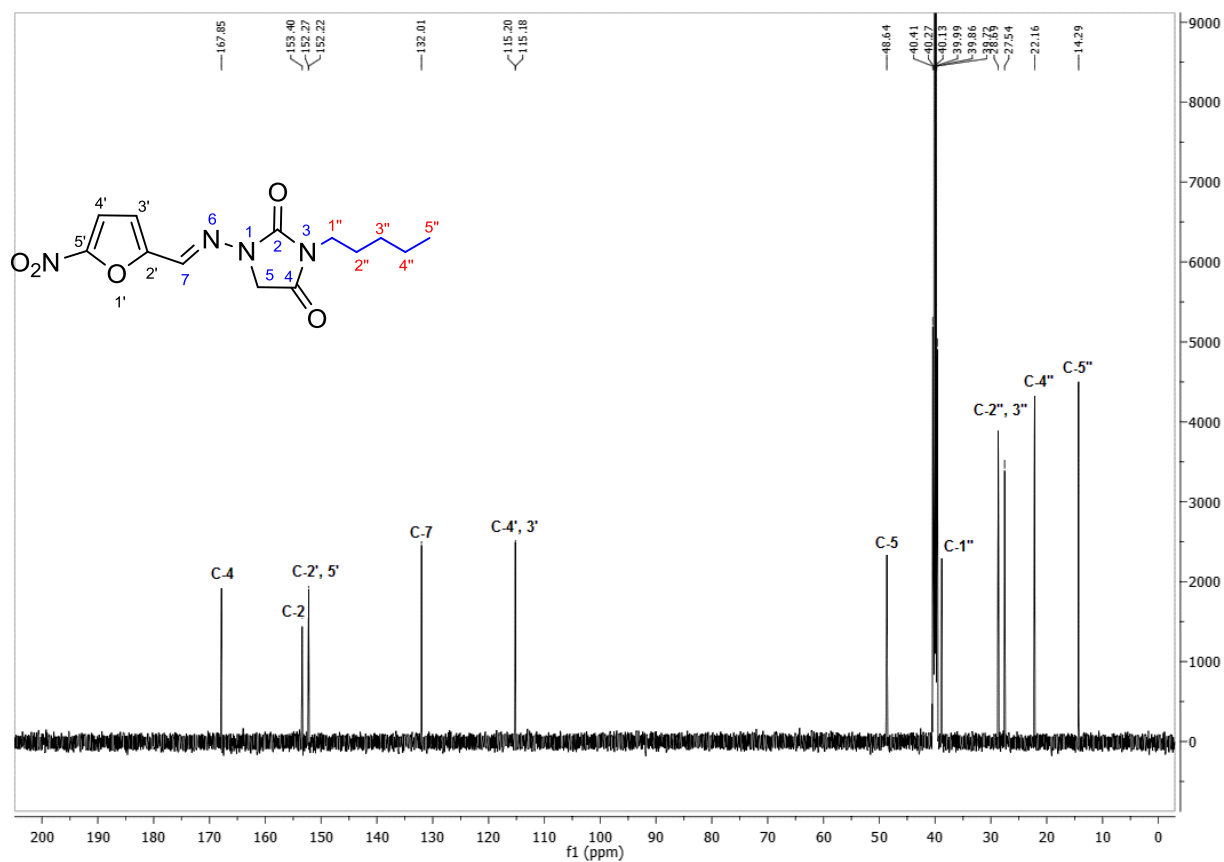
IR



^1H NMR in DMSO



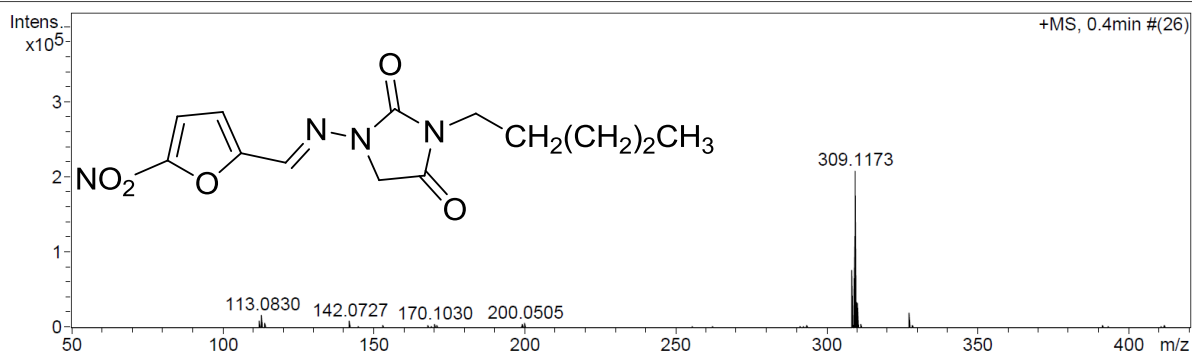
¹³CNMR in DMSO



HRMS

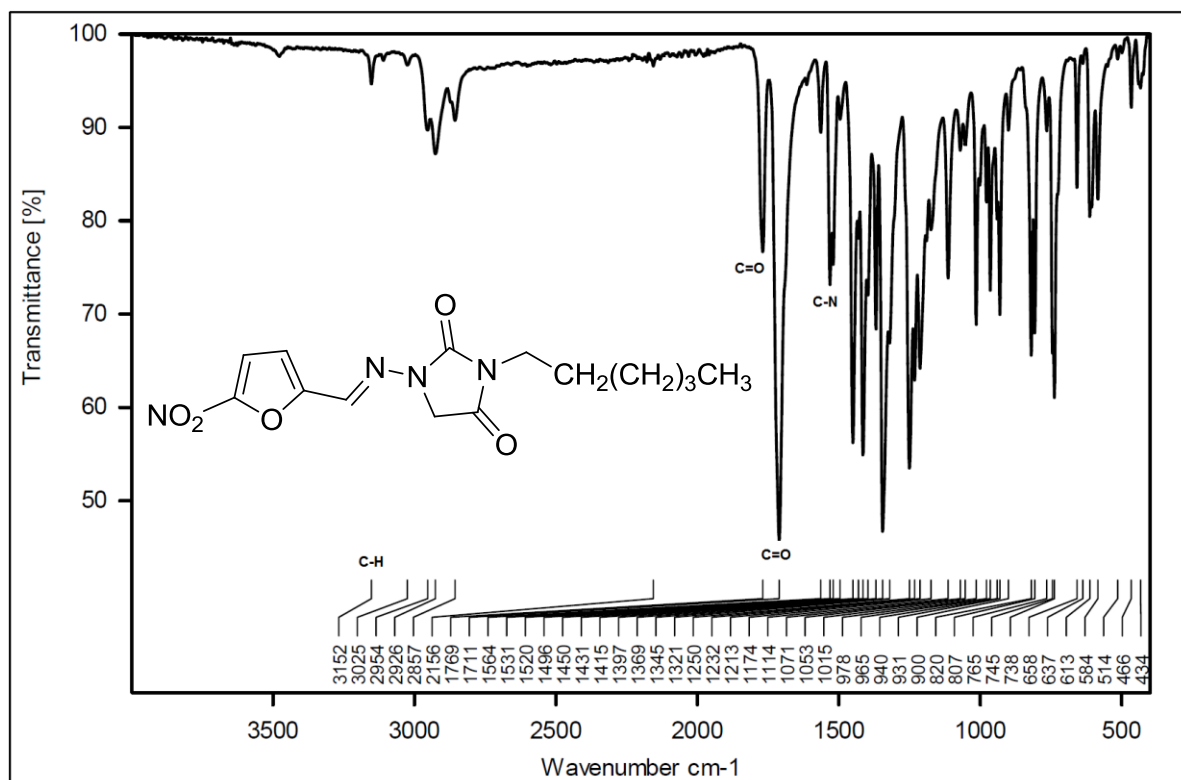
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

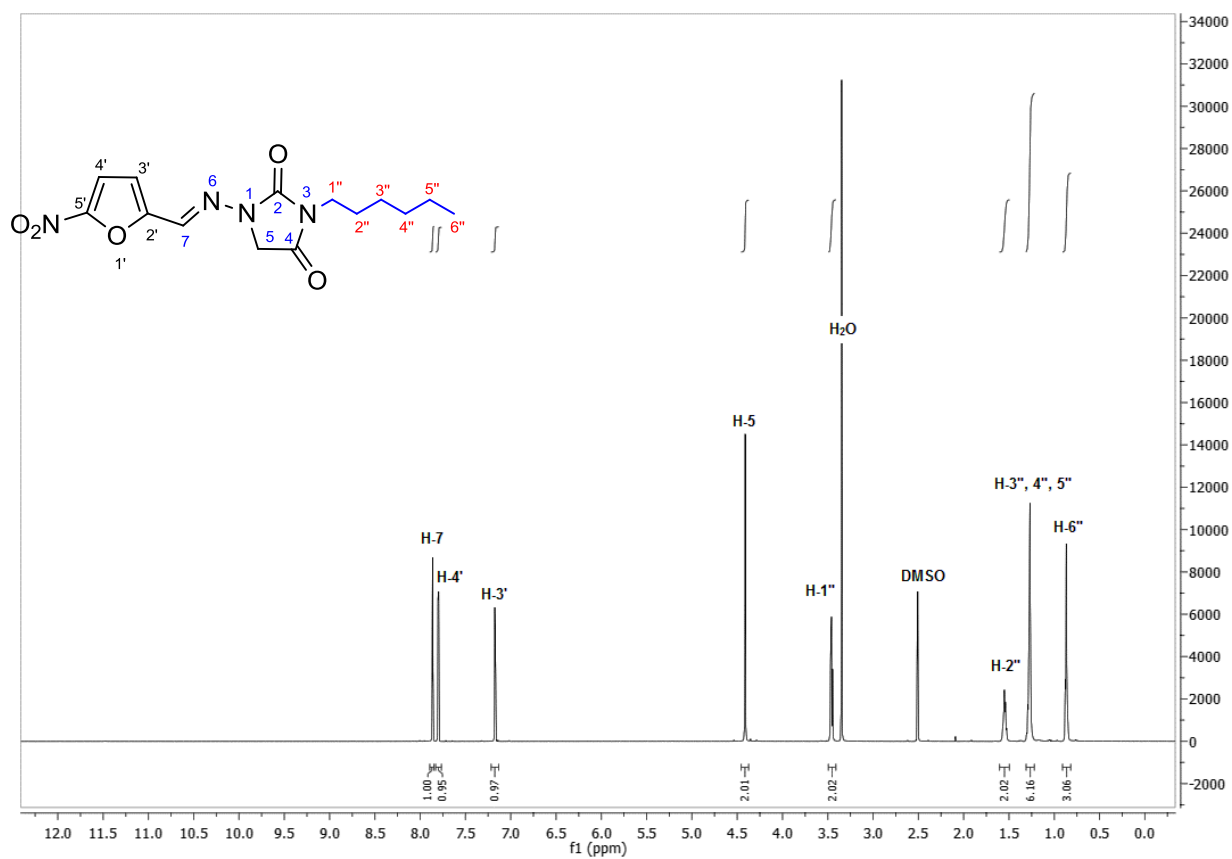


ANALOGUE 107

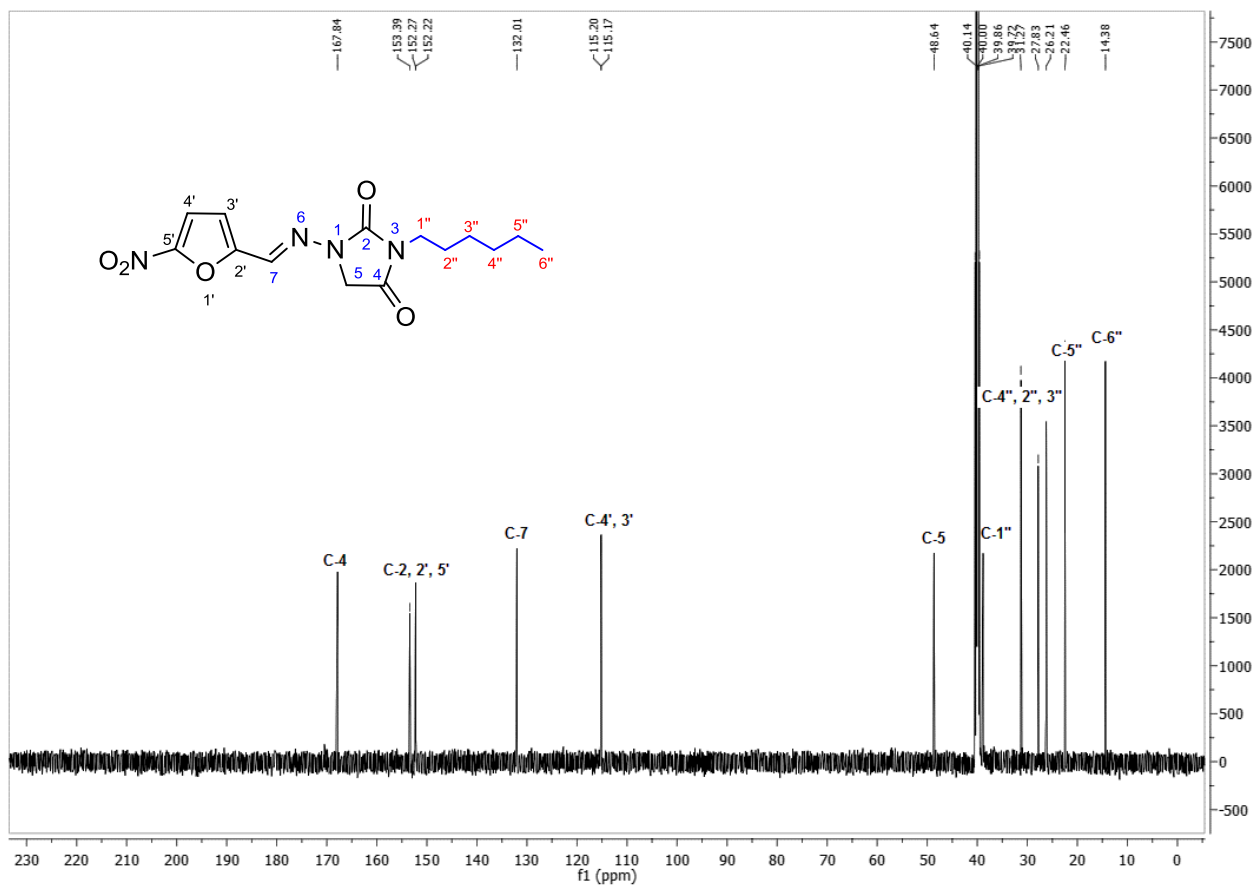
IR



^1H NMR in DMSO

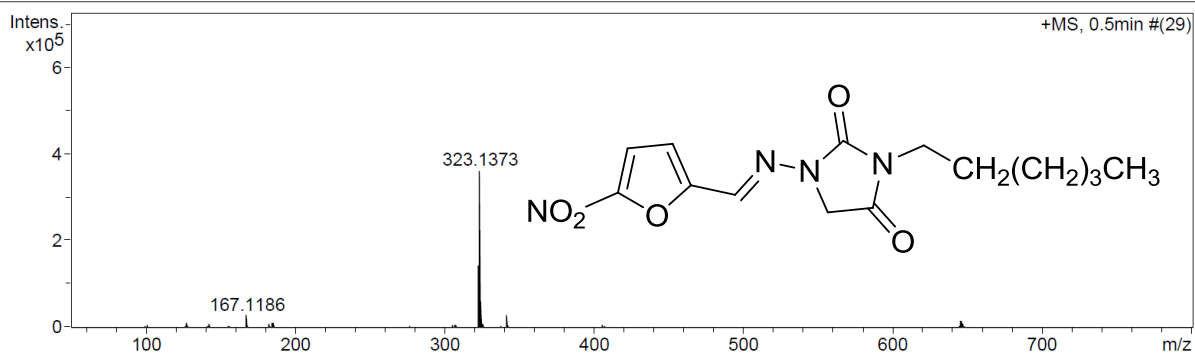


¹³CNMR in DMSO

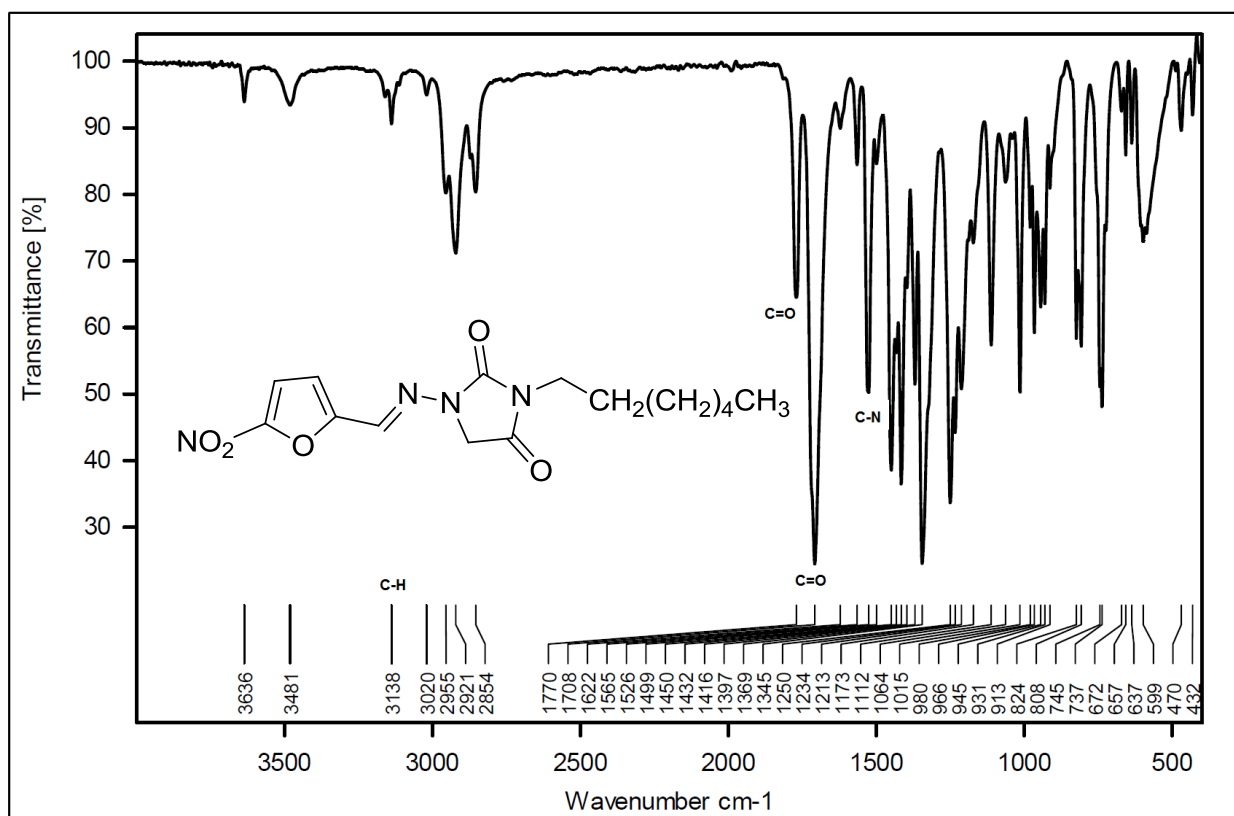
**HRMS**

Acquisition Parameter

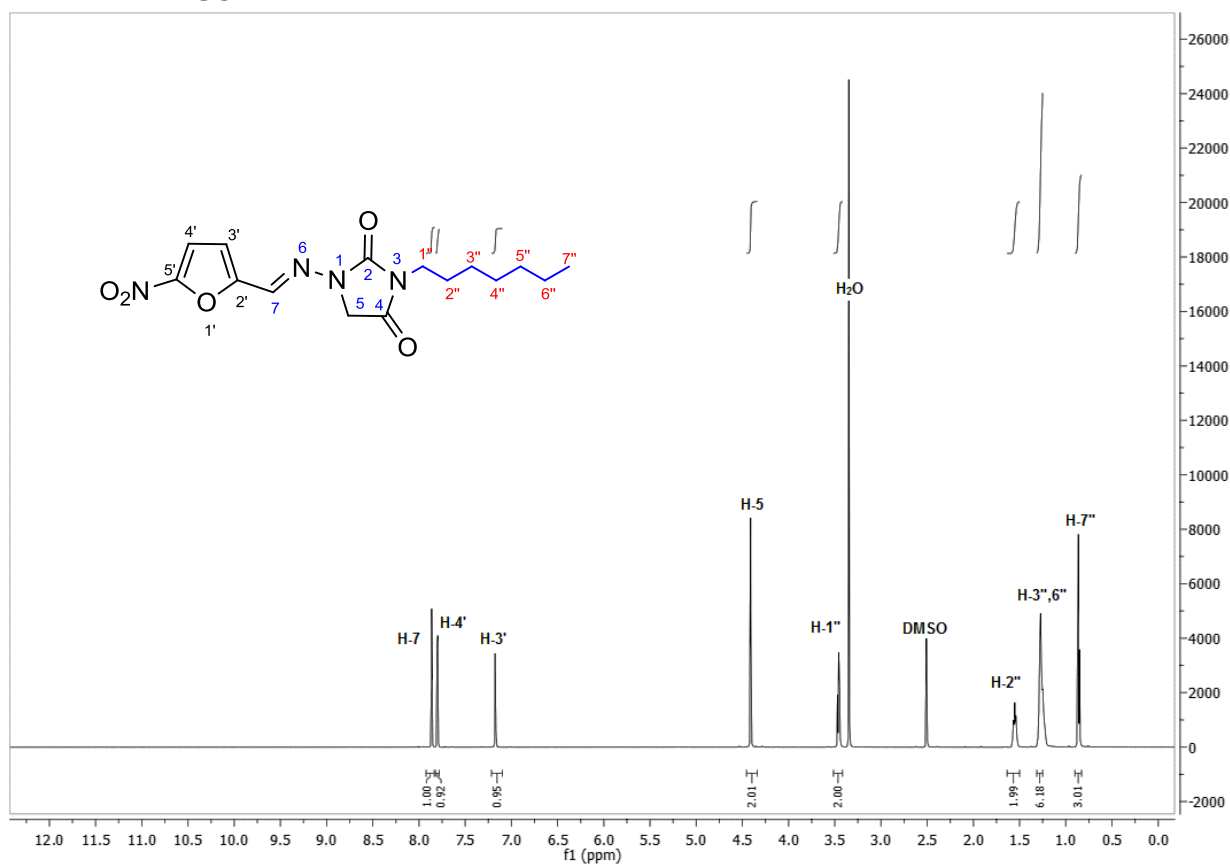
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Work



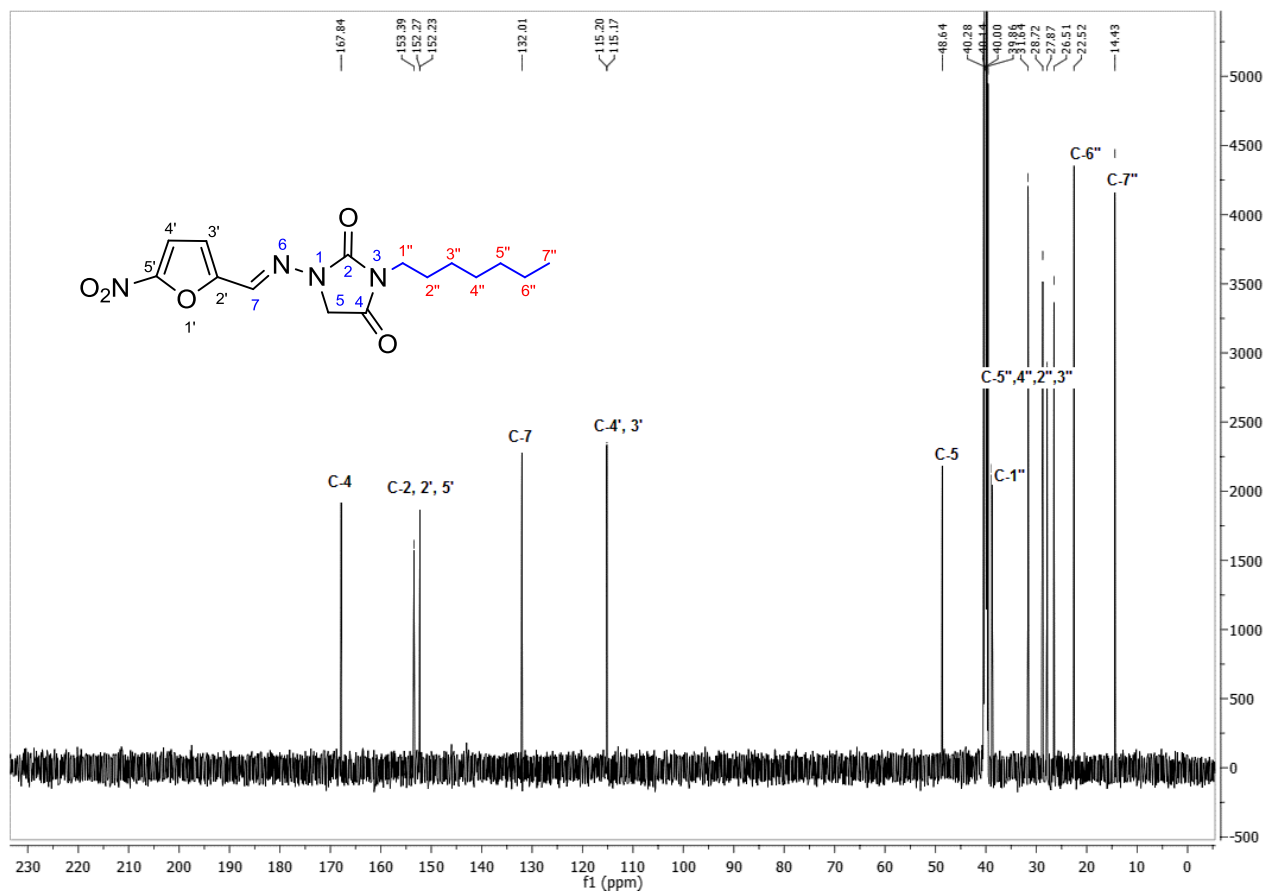
IR



¹H NMR in DMSO

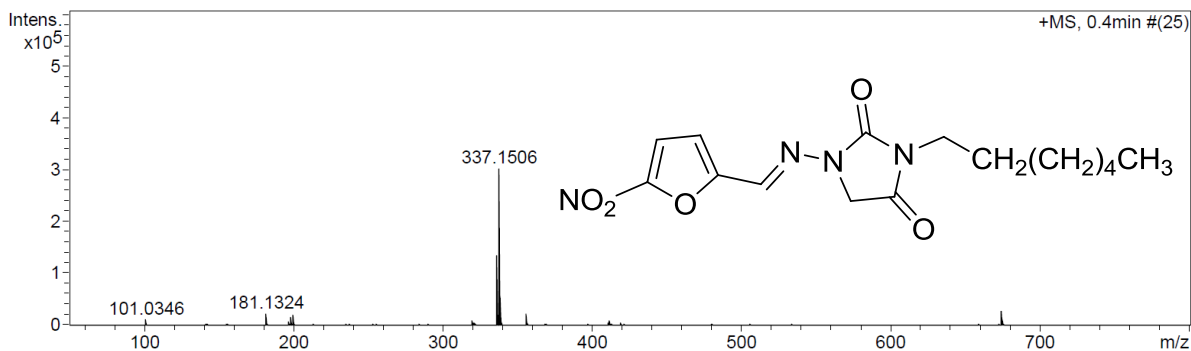


¹³CNMR in DMSO

**HRMS**

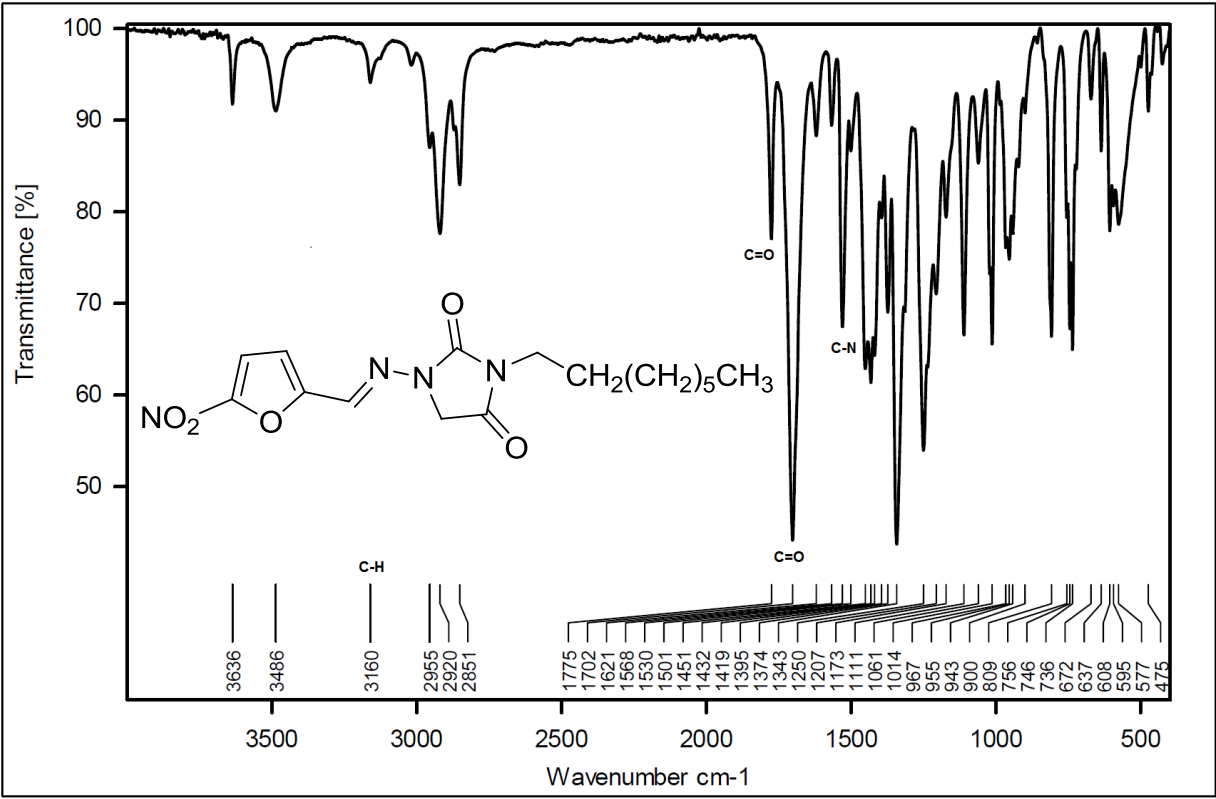
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Work

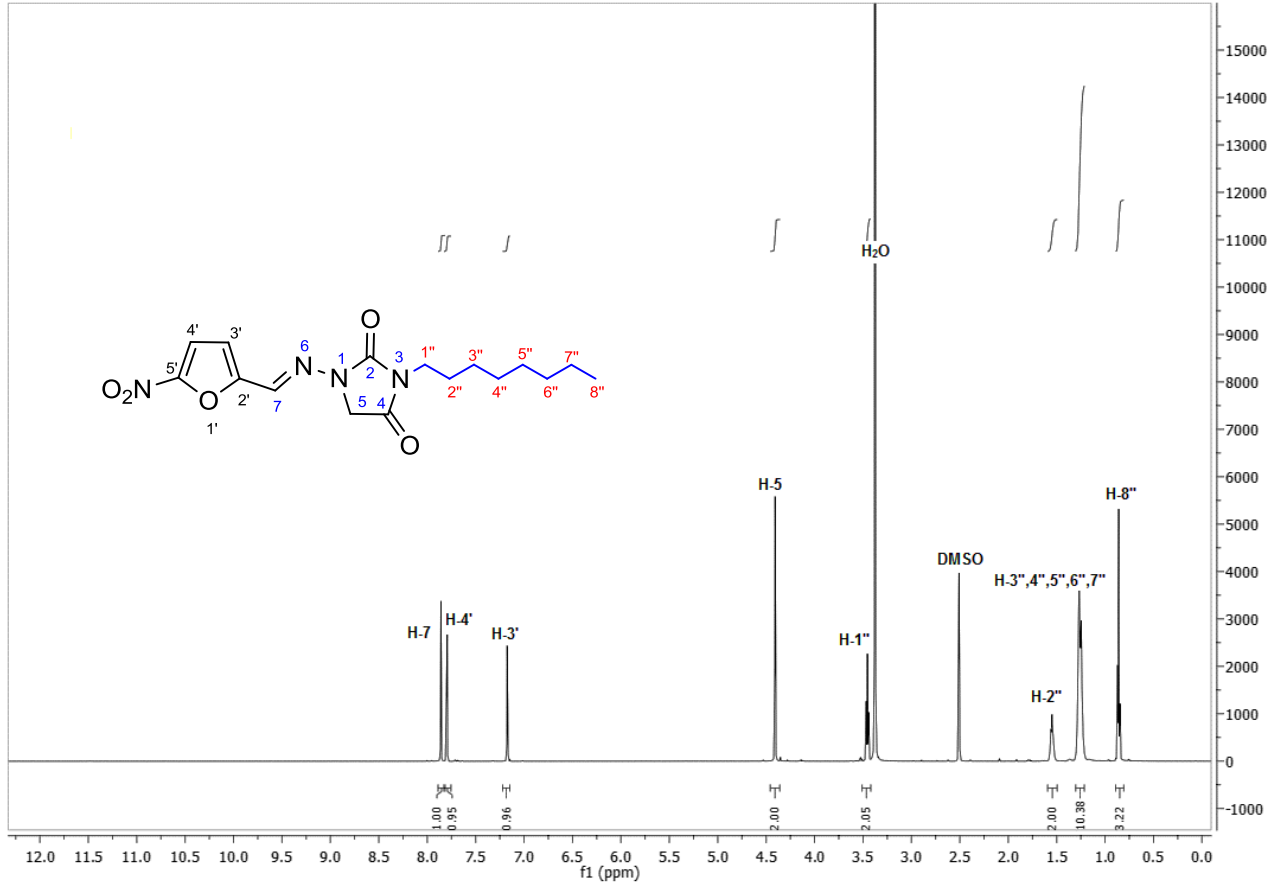


ANALOGUE 109

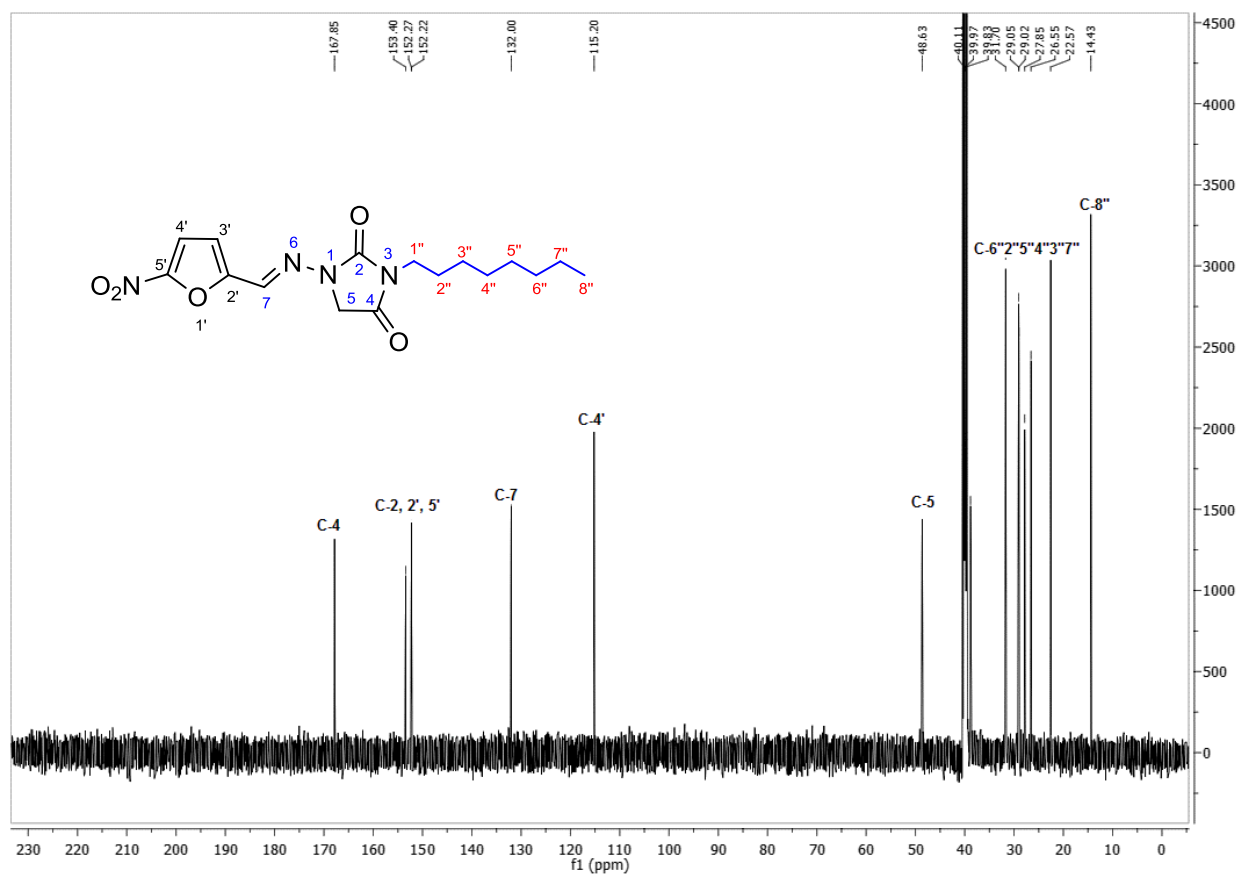
IR



¹H NMR in DMSO



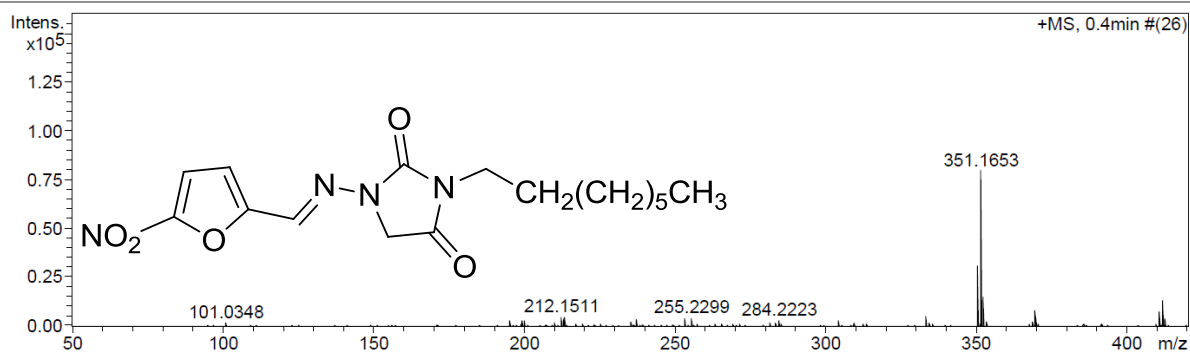
¹³CNMR in DMSO



HRMS

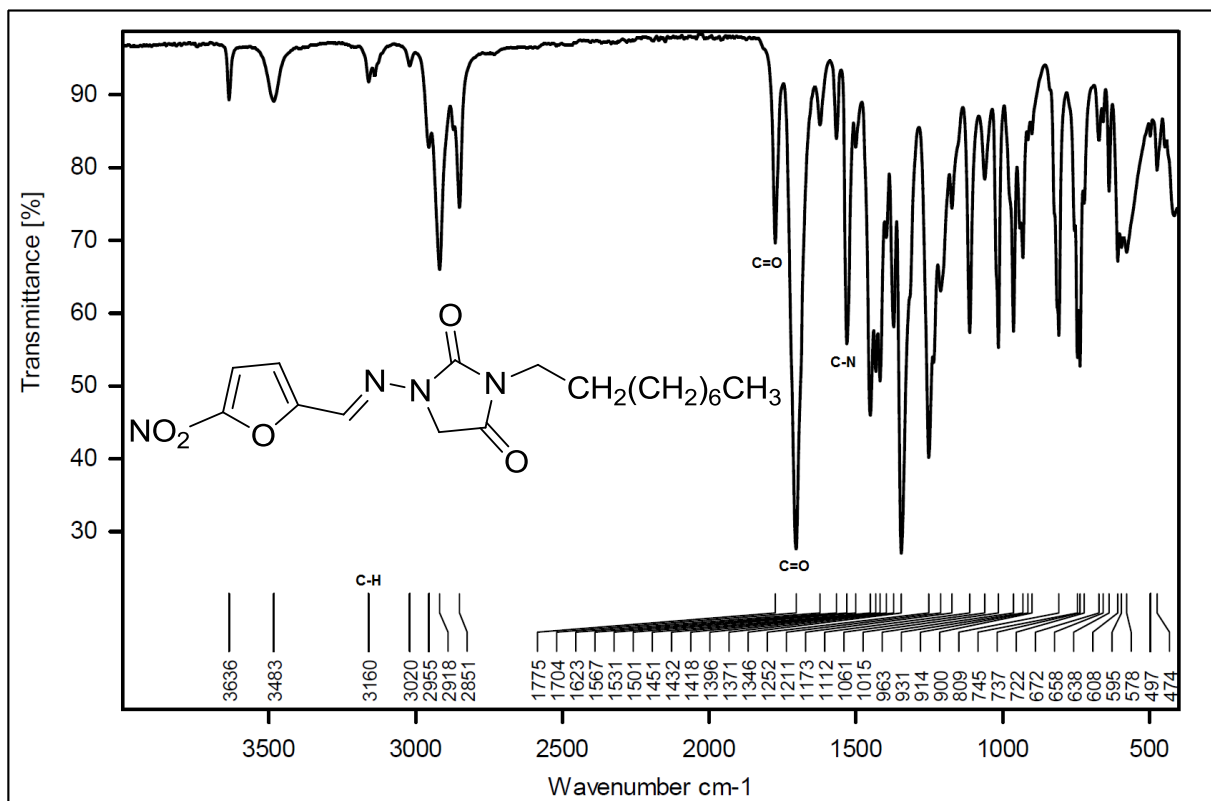
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

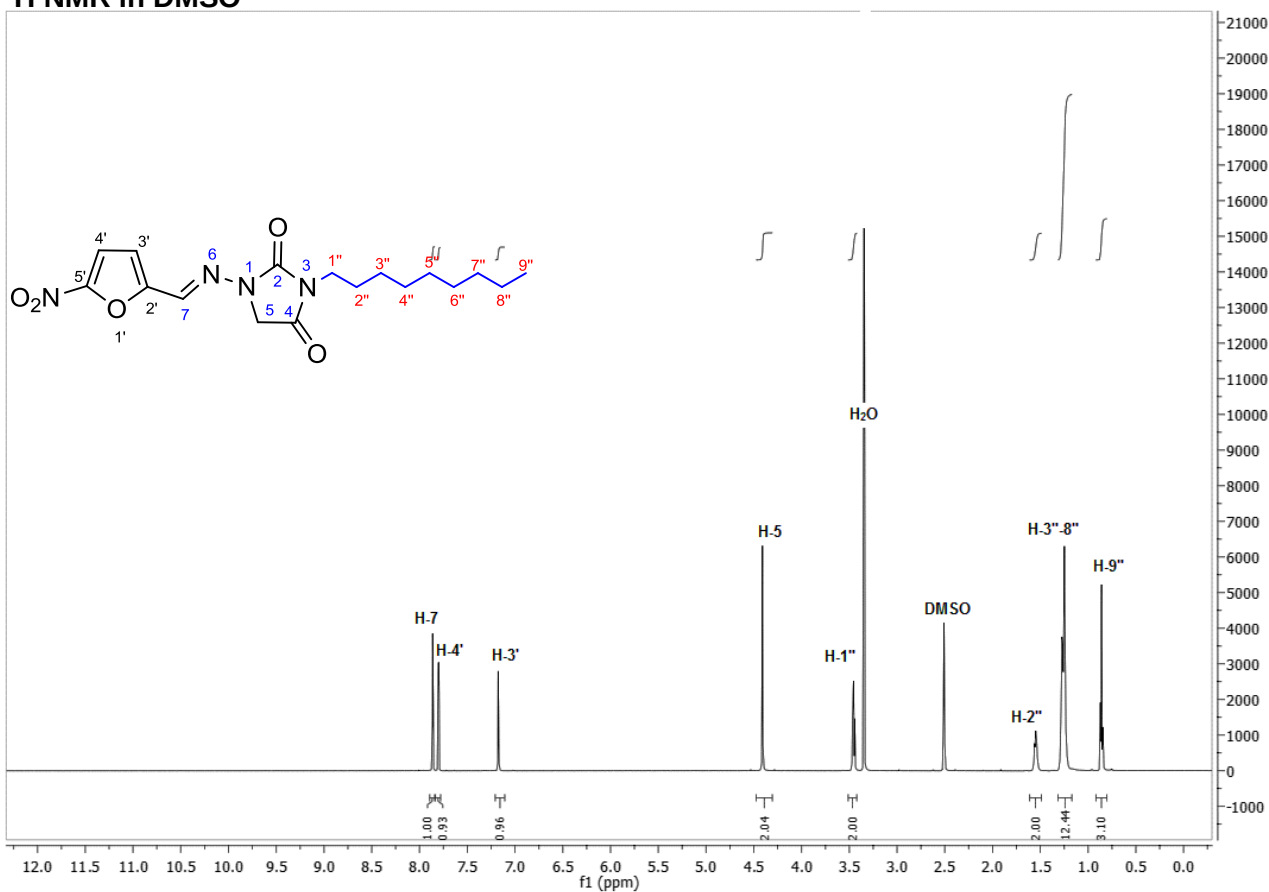


ANALOGUE 110

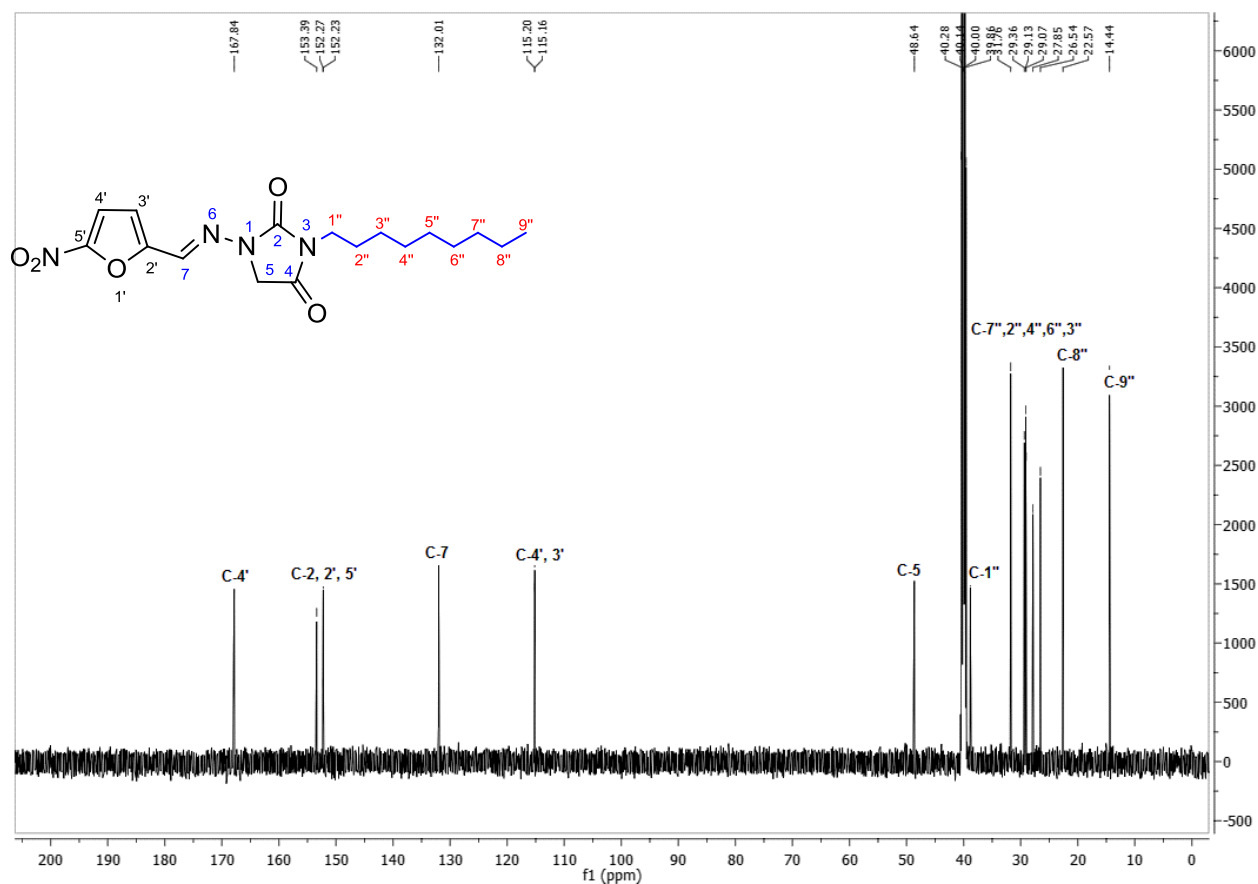
IR



¹H NMR in DMSO



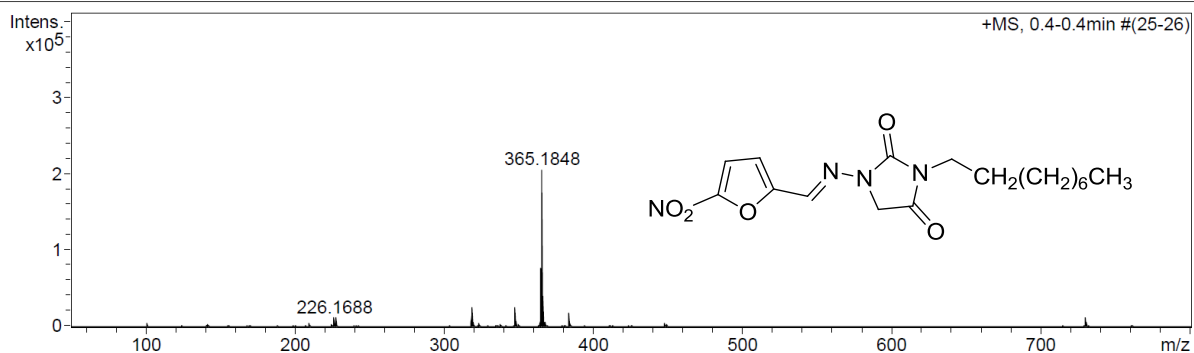
¹³CNMR in DMSO



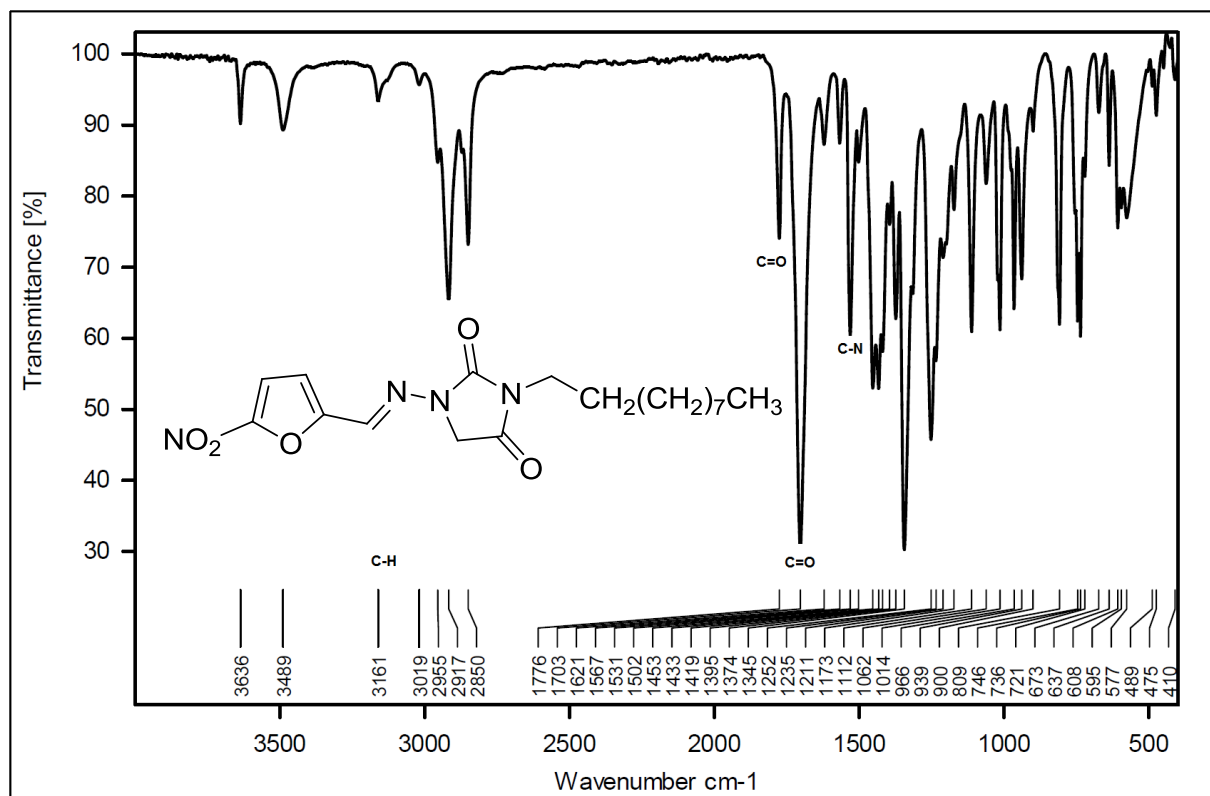
HRMS

Acquisition Parameter

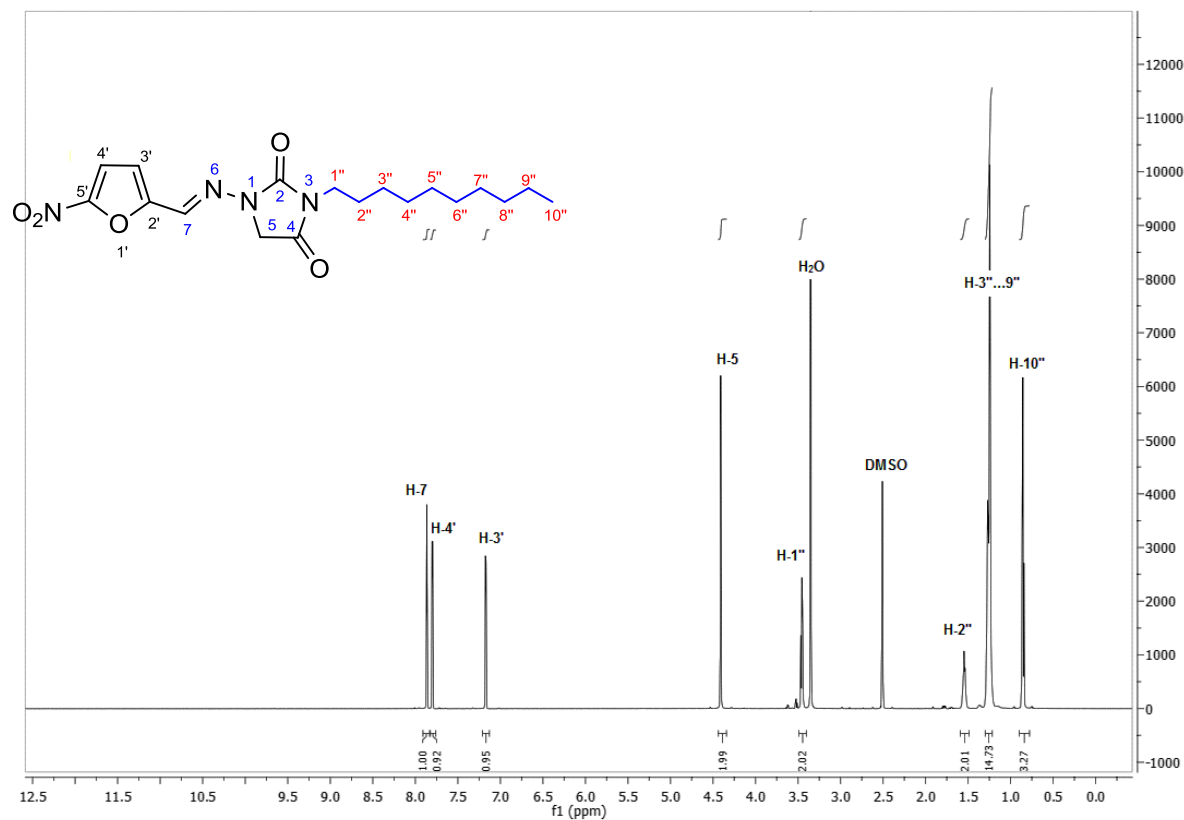
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



IR



¹H NMR in DMSO



Chemical structure of 1-(4-nitrophenyl)-4-(10-oxodecyl)-1H-imidazole-2-carboxamide is shown. The structure is labeled with carbon atoms 1' through 10'.

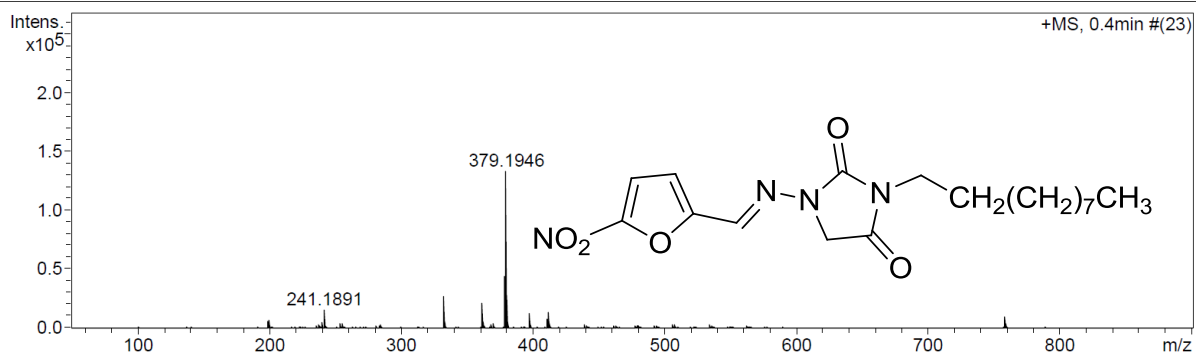
¹³C NMR spectrum (f1 (ppm)) showing peaks corresponding to the structure. The x-axis ranges from 0 to 200 ppm. The y-axis represents intensity.

Key peaks and their assignments (ppm):

- 167.83 (C-4)
- 153.39 (C-2, 2', 5')
- 132.01 (C-7)
- 115.20 (C-4', 3')
- 40.28 (C-5)
- 39.86 (C-1'')
- 31.77 (C-2''...8'')
- 29.43 (C-9'')
- 28.40 (C-10'')

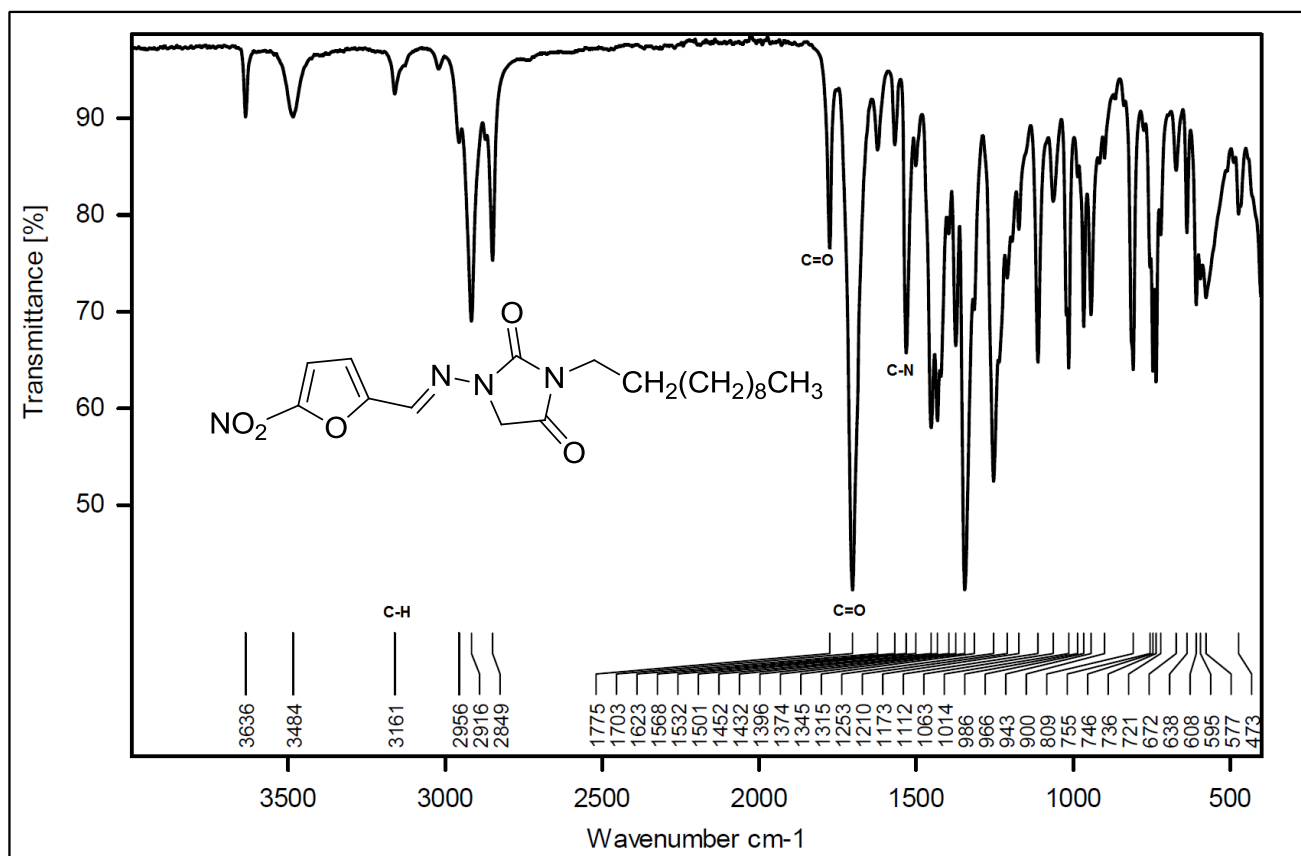
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Work

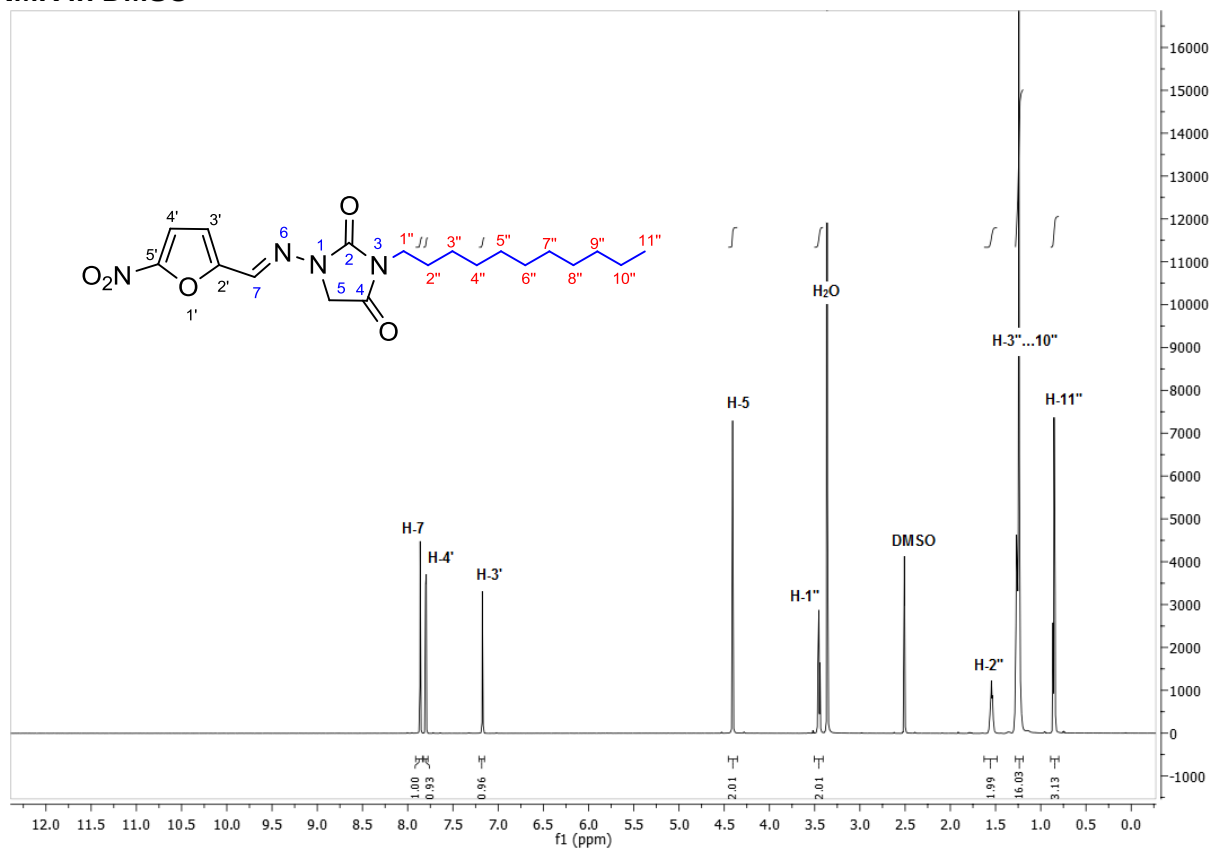


ANALOGUE 112

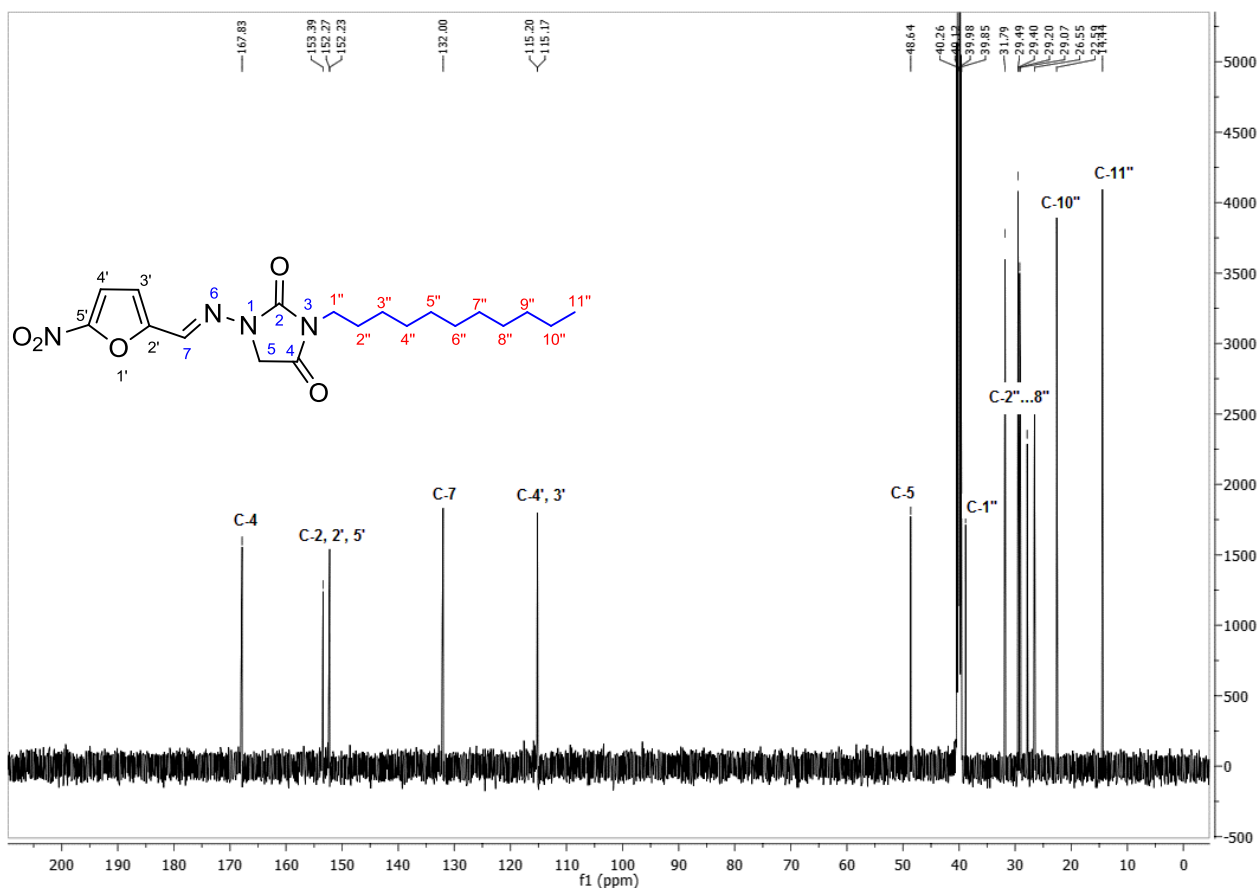
IR



^1H NMR in DMSO



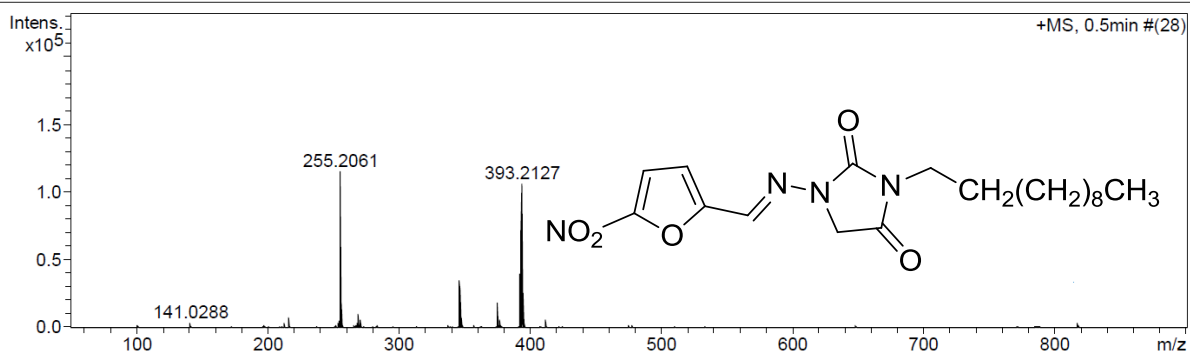
¹³CNMR in DMSO



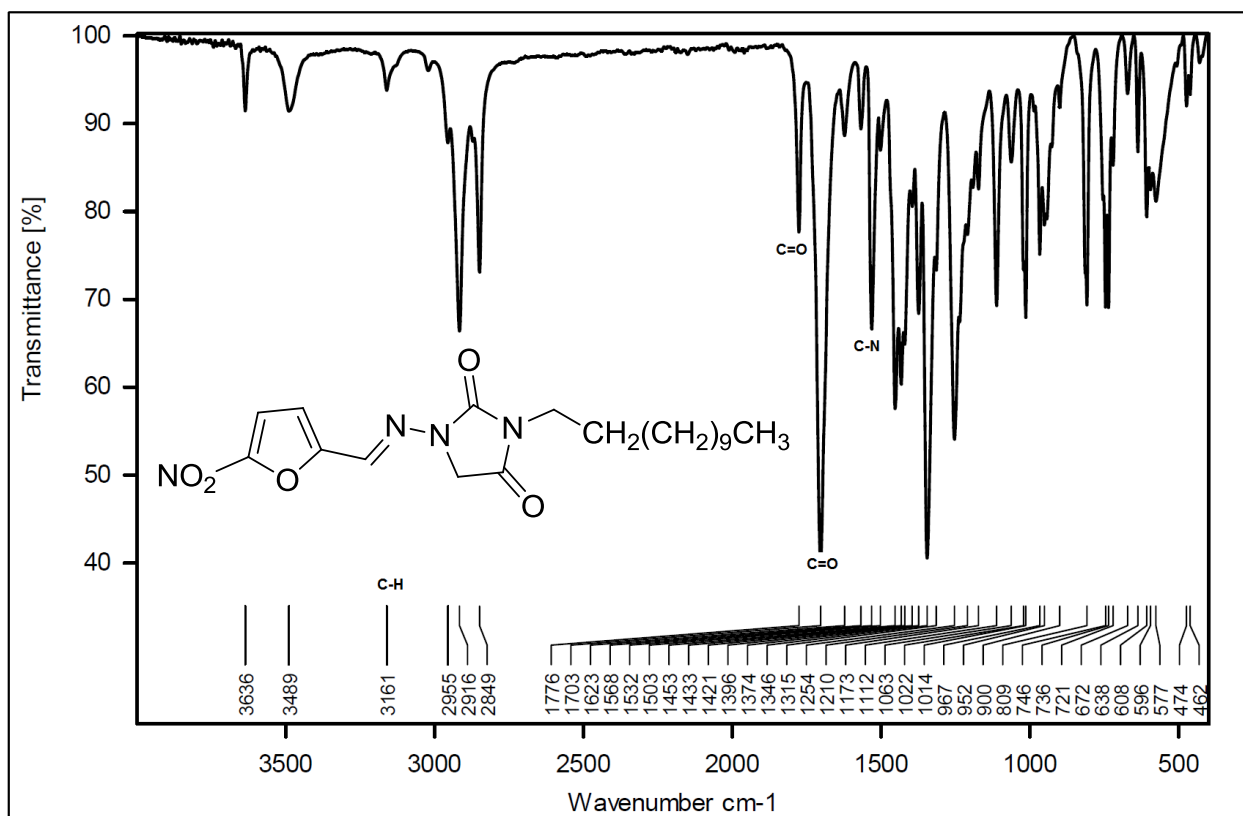
HRMS

Acquisition Parameter

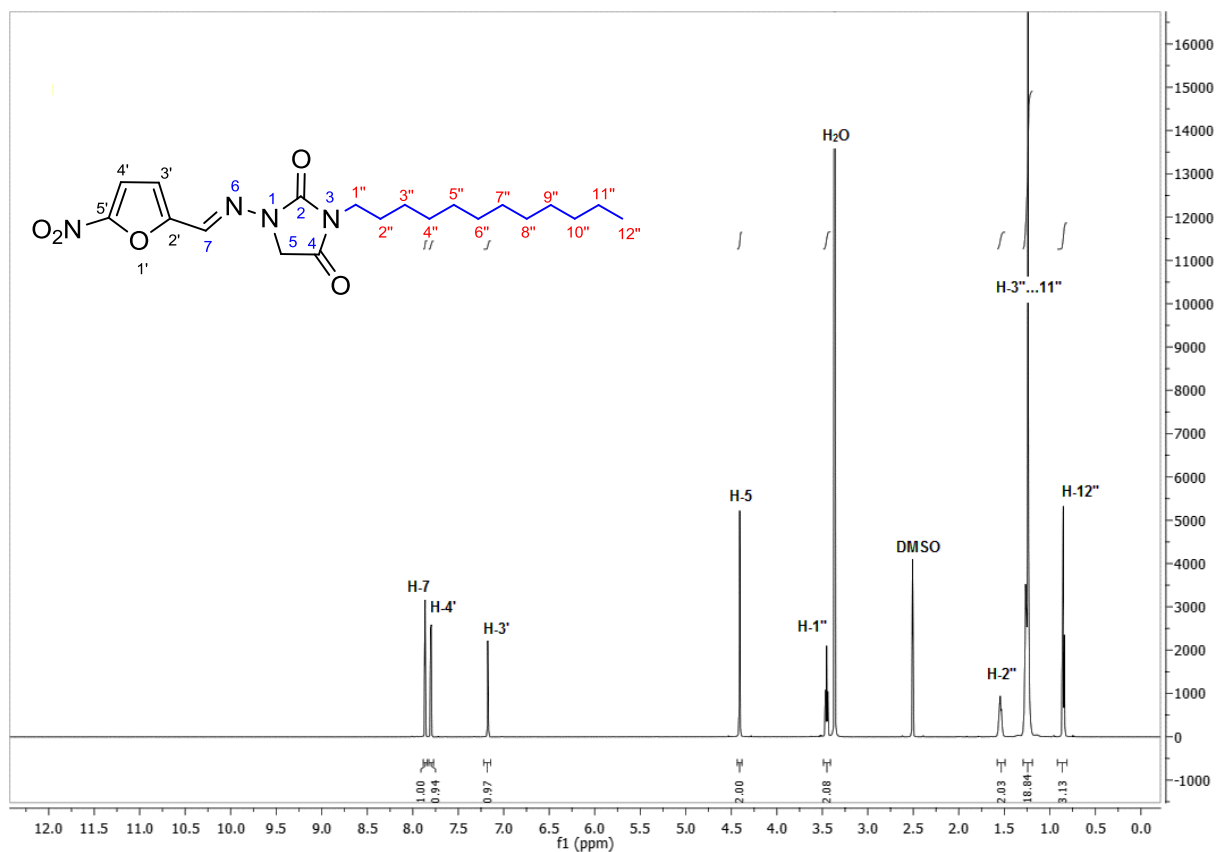
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



IR



¹H NMR in DMSO

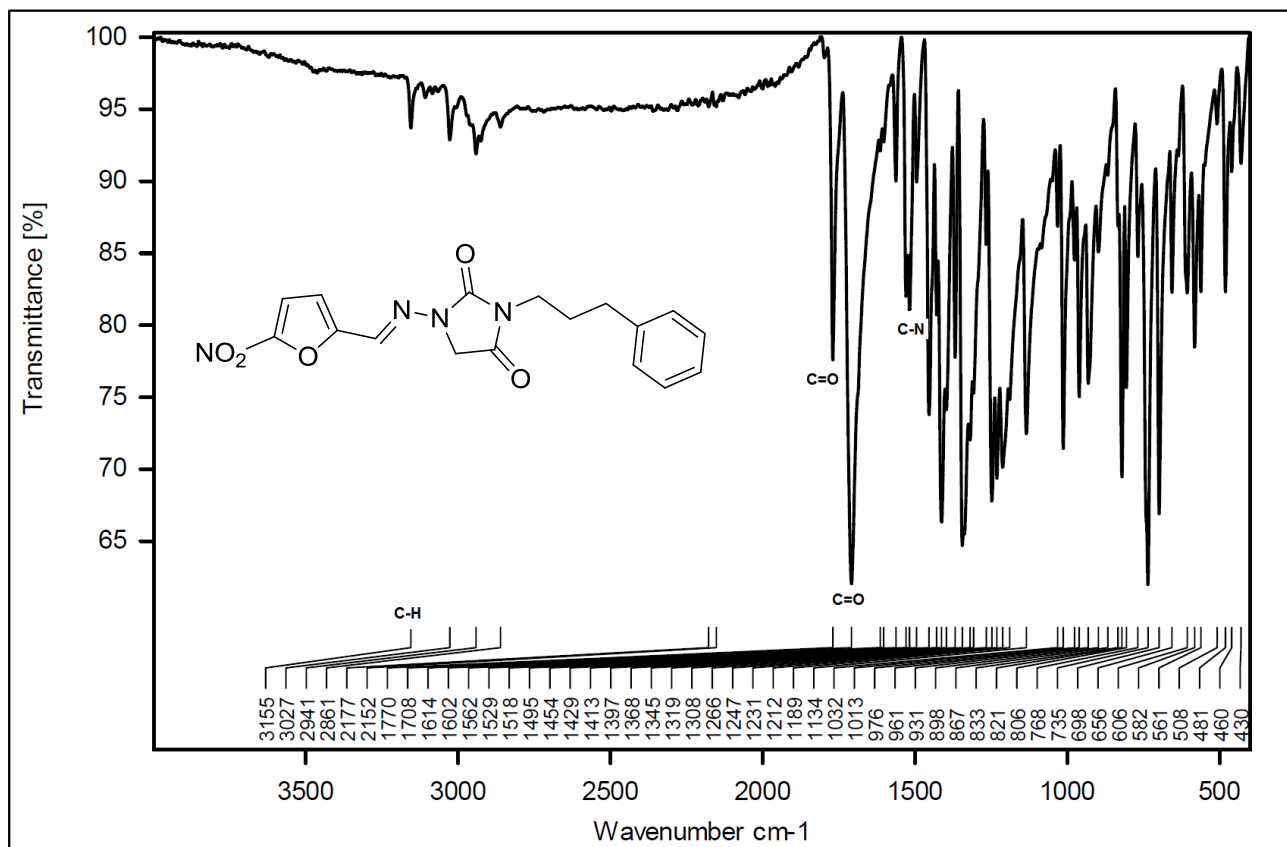


Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

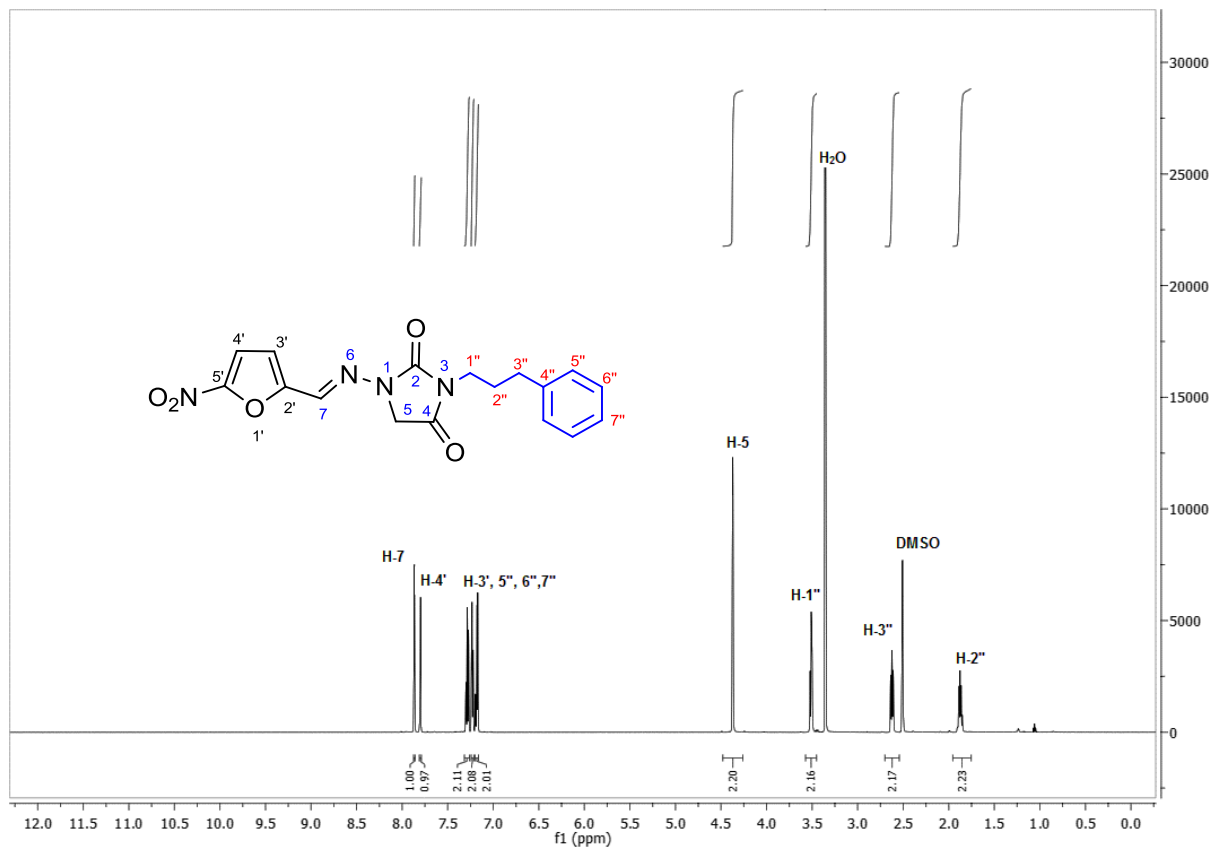


ANALOGUE 114

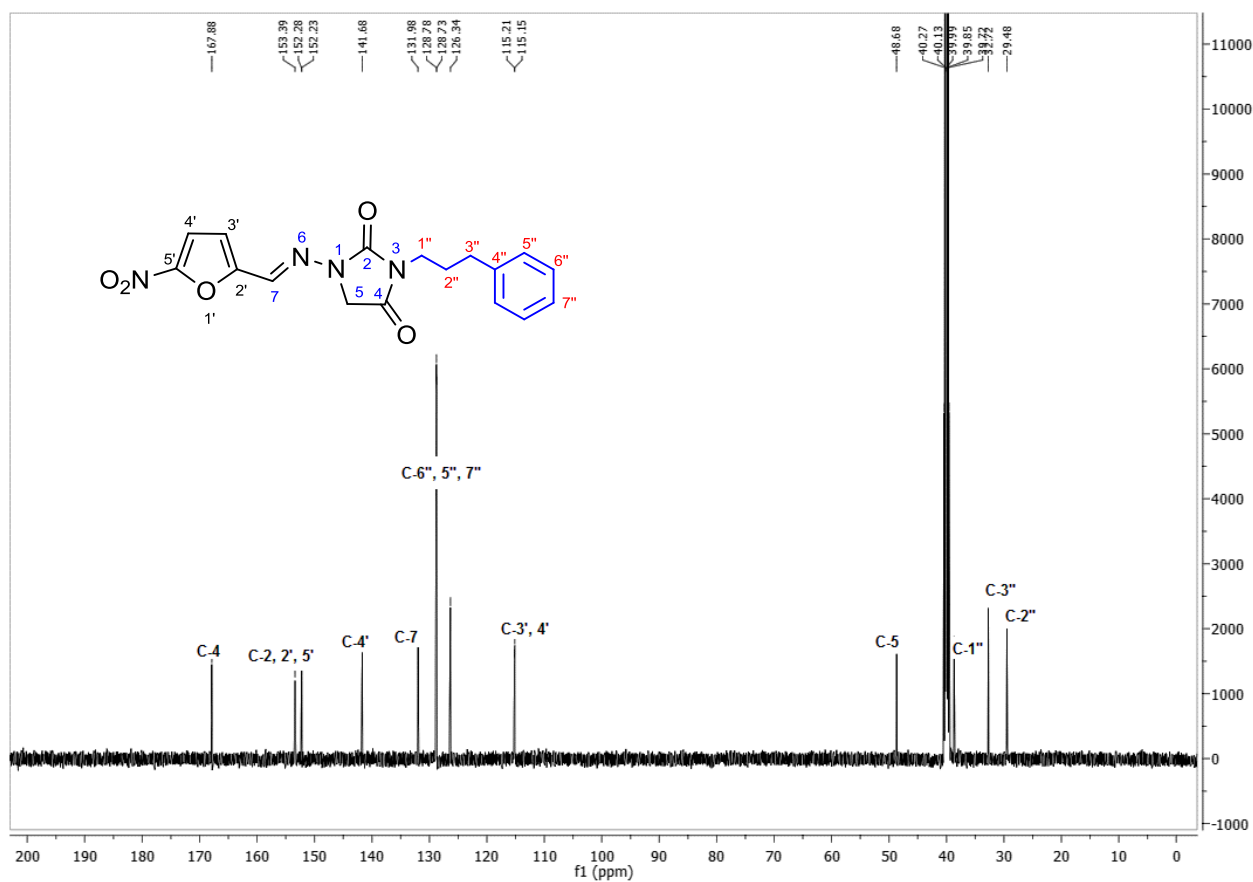
IR



¹H NMR in DMSO



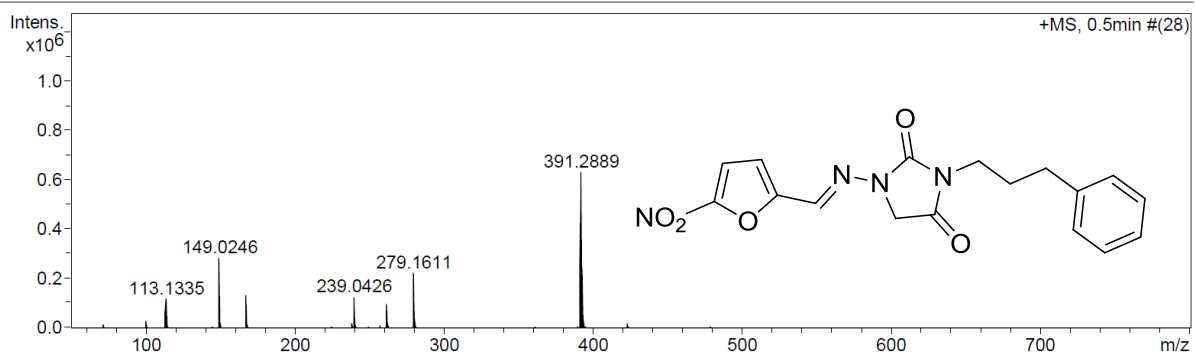
¹³CNMR in DMSO



HRMS

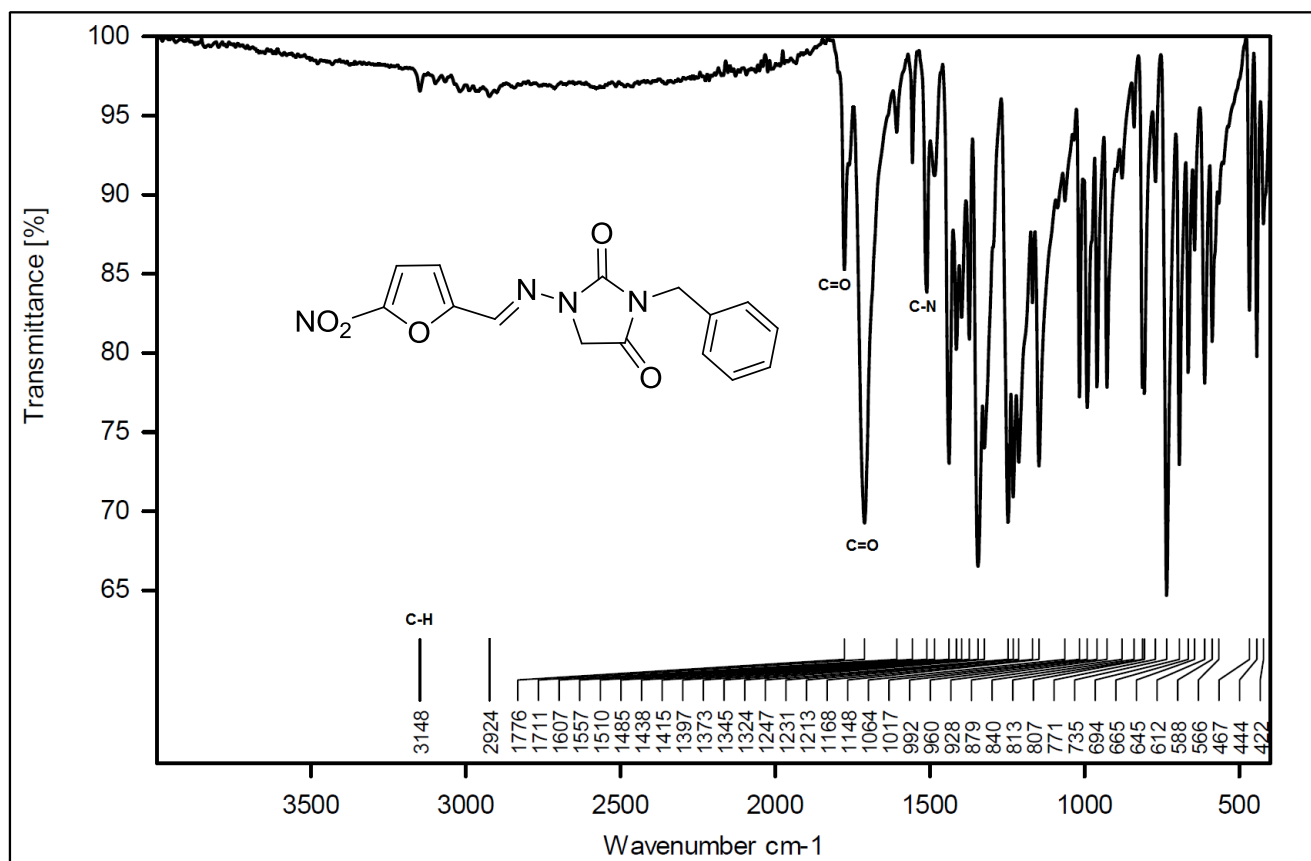
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

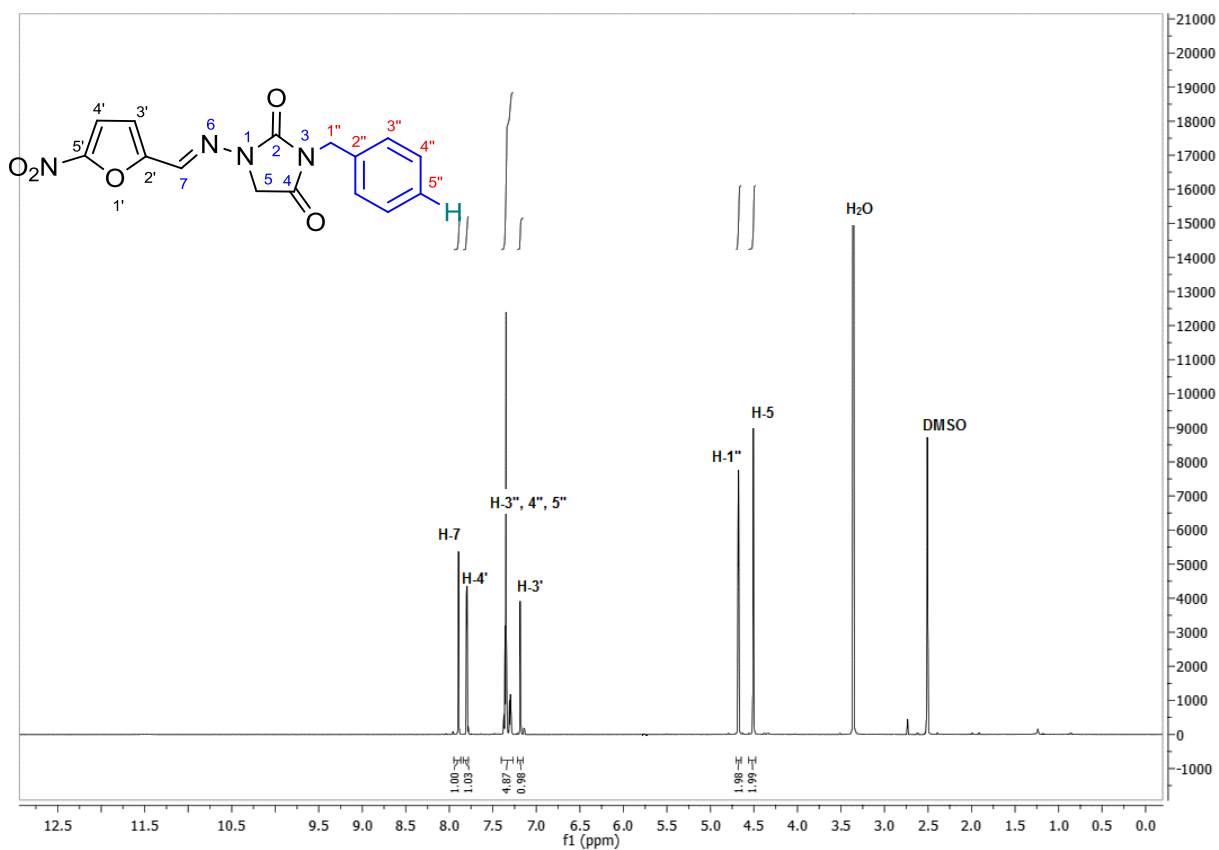


ANALOGUE 115

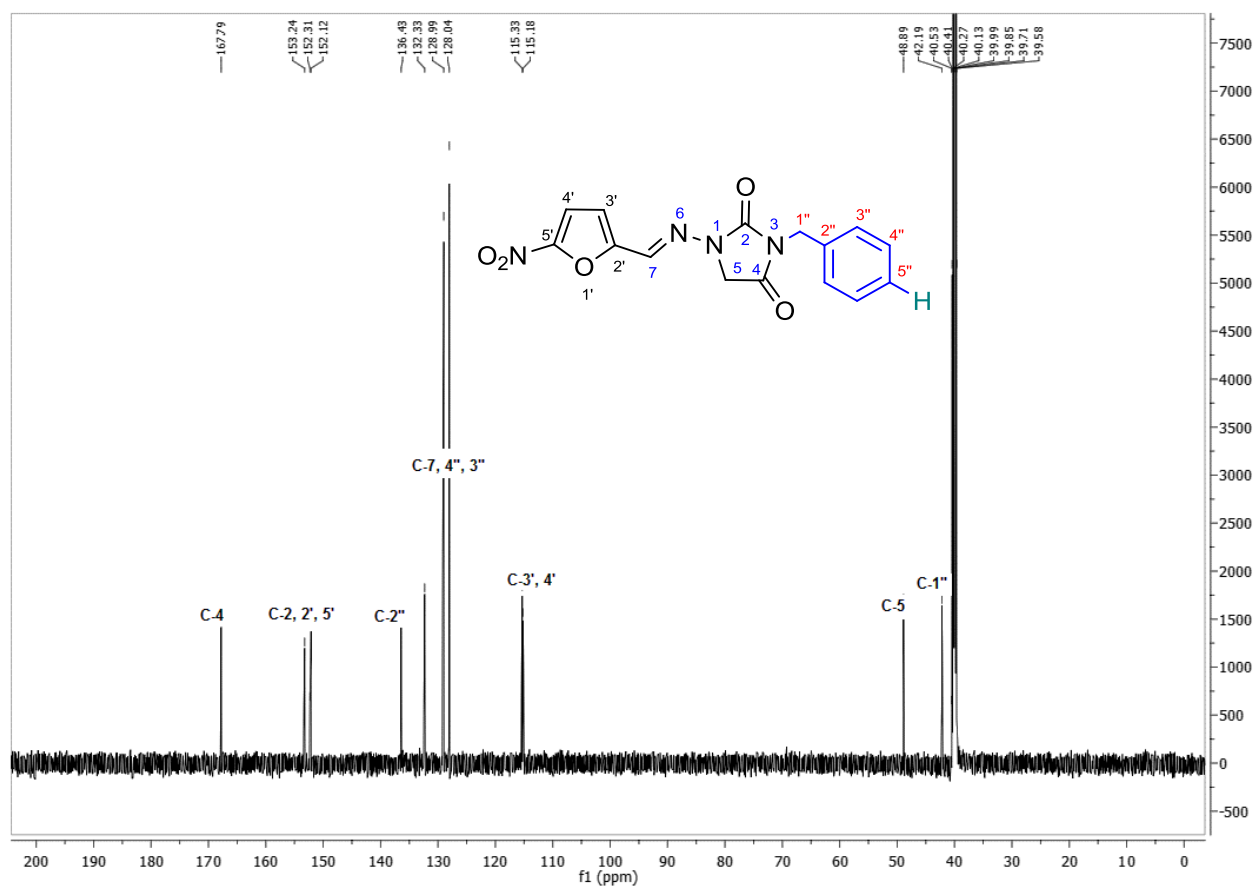
IR



¹H NMR in DMSO



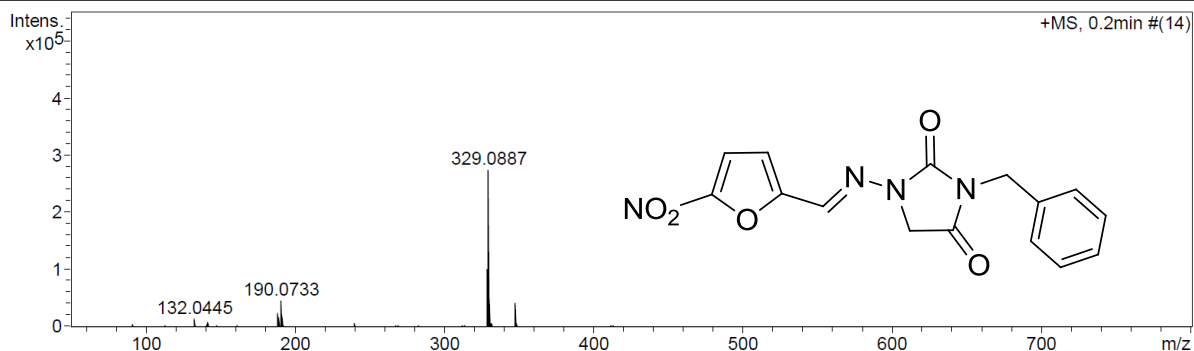
¹³CNMR in DMSO



HRMS

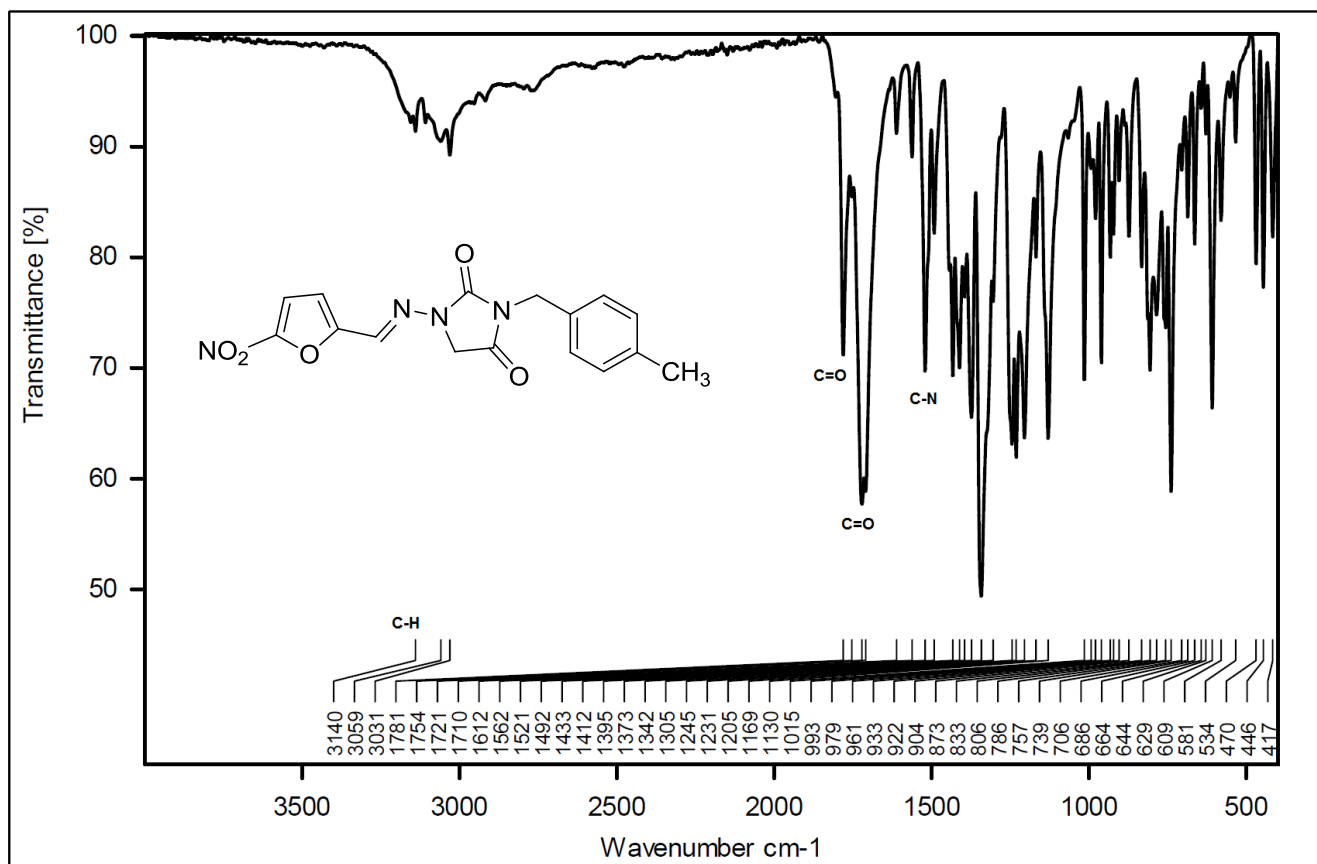
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

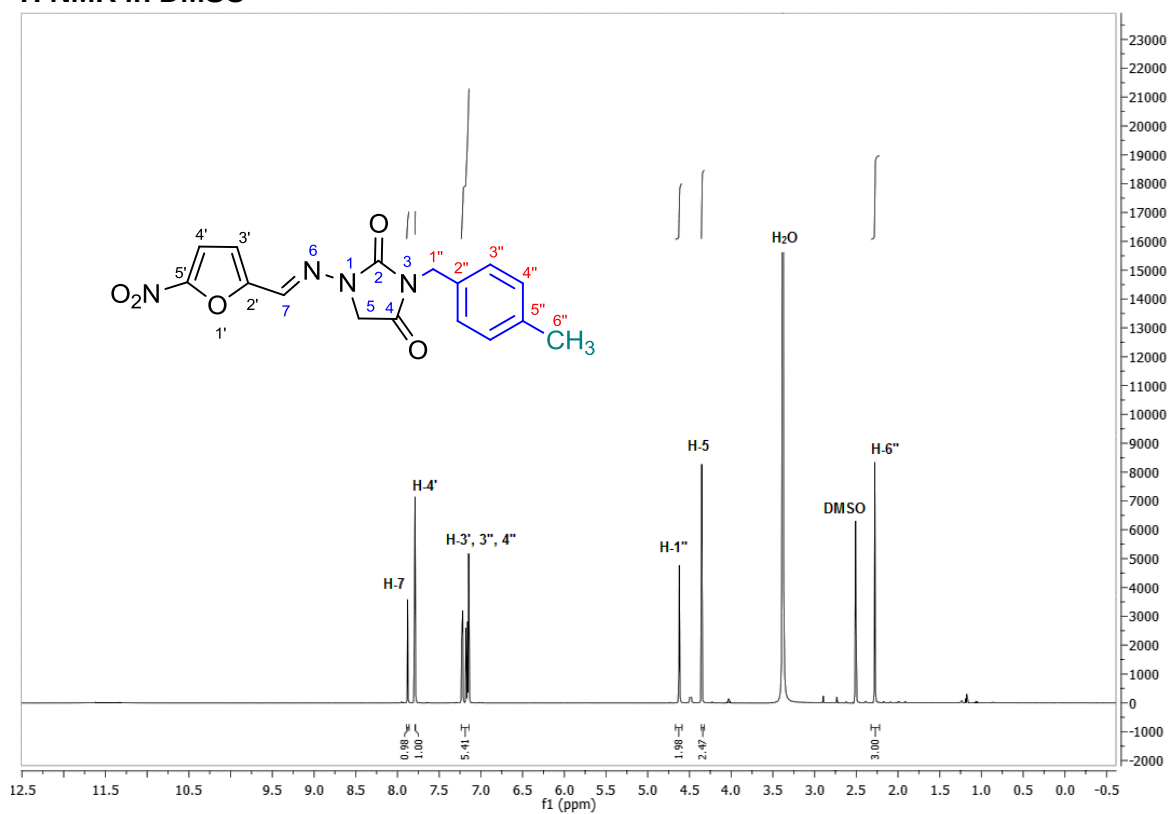


ANALOGUE 116

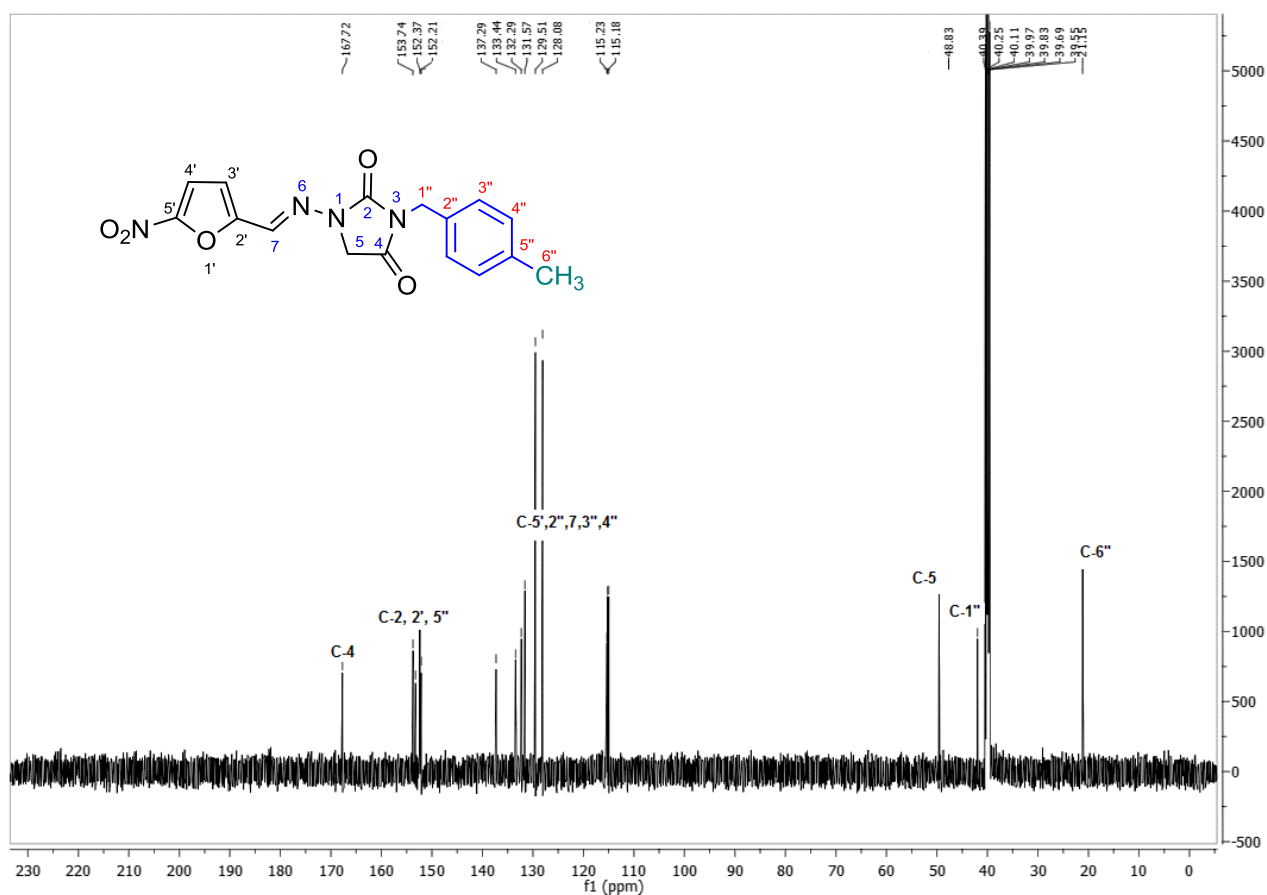
IR



^1H NMR in DMSO



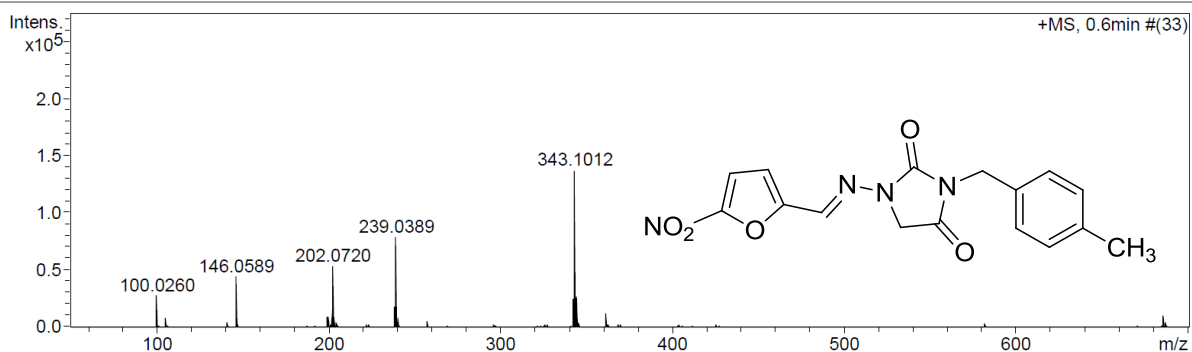
¹³CNMR in DMSO



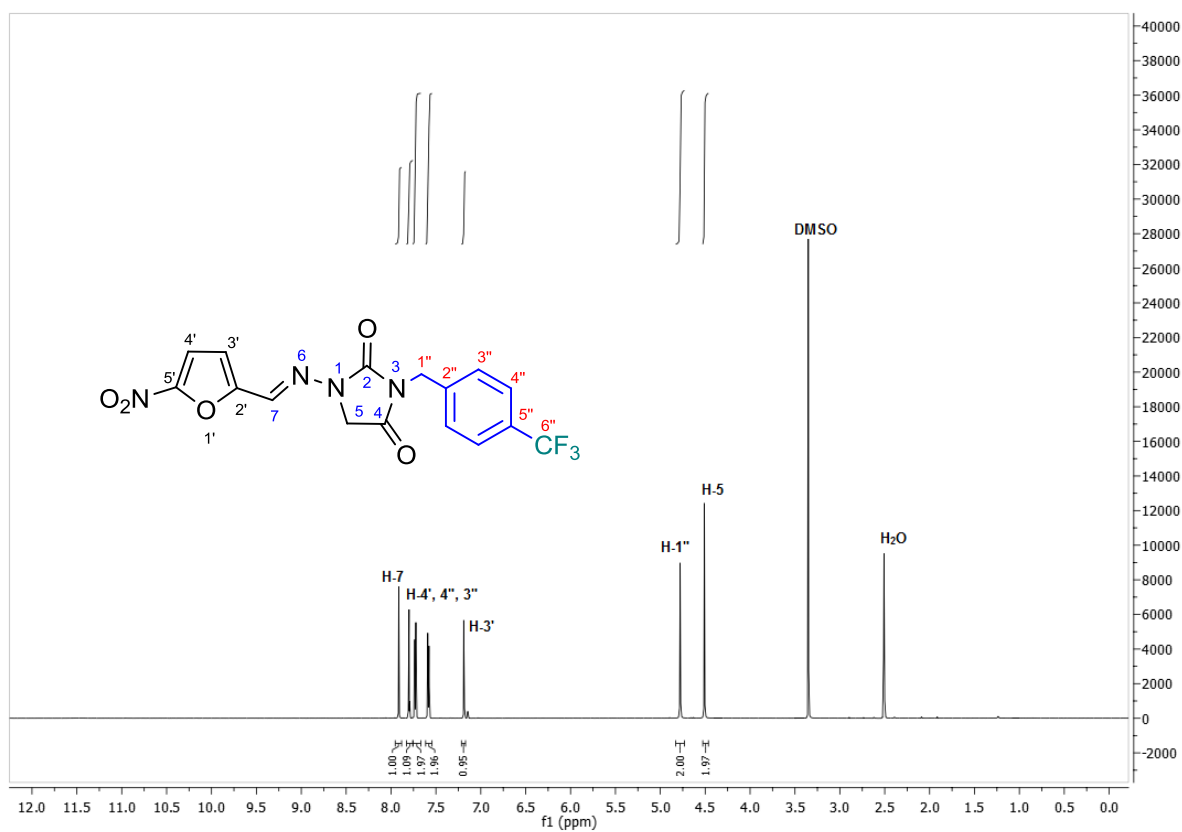
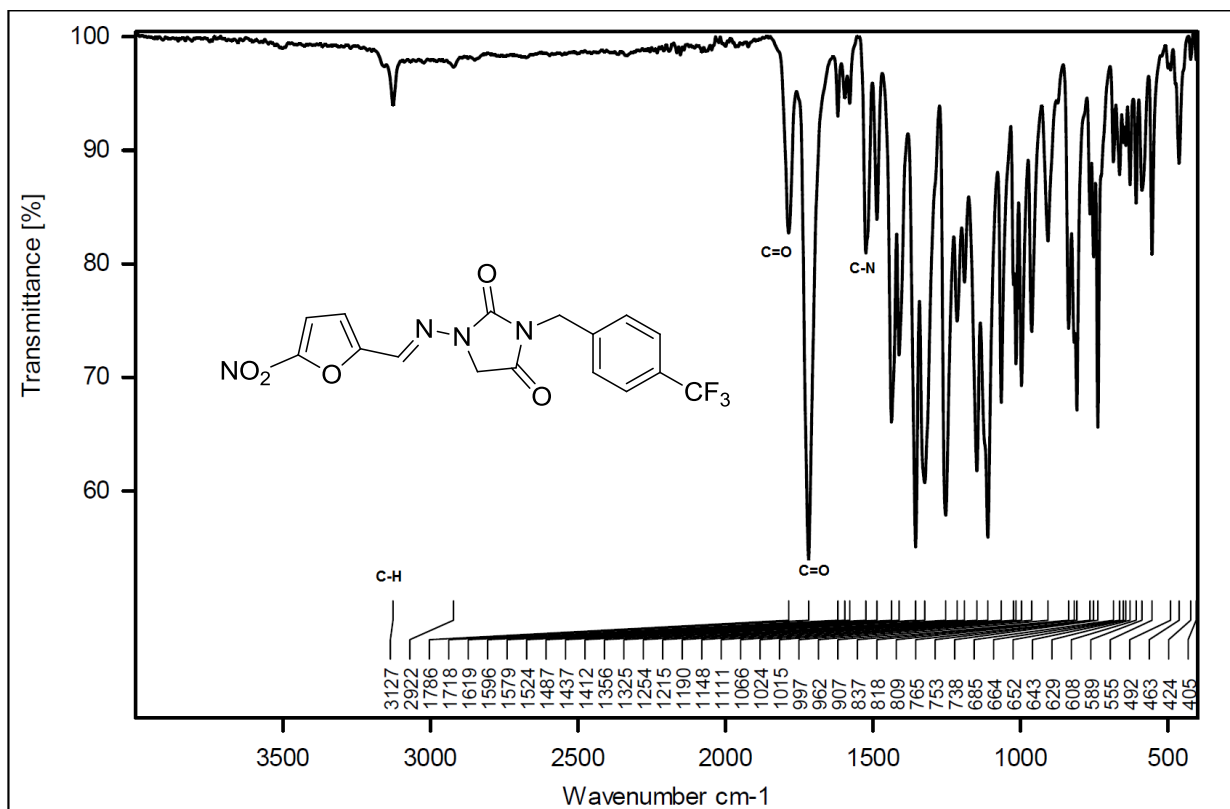
HRMS

Acquisition Parameter

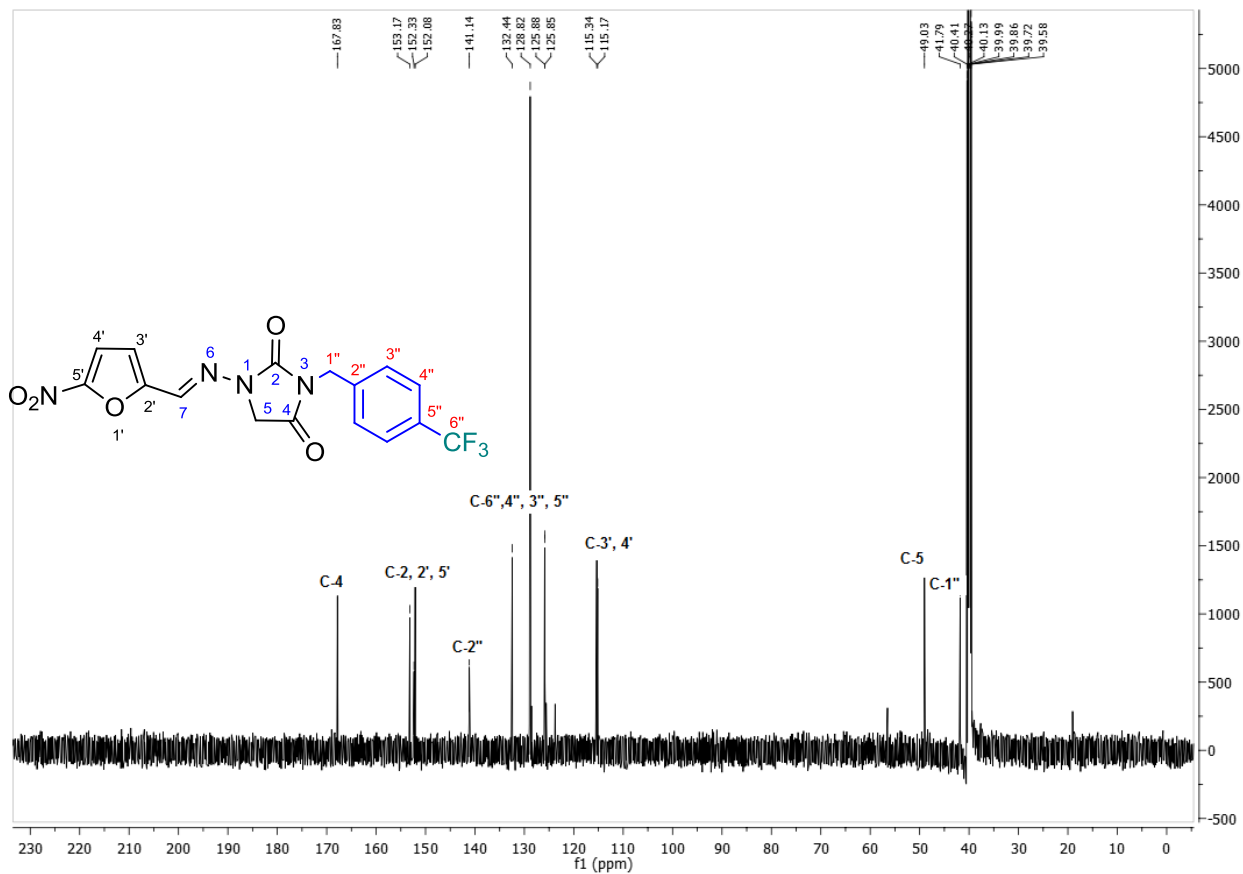
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



IR

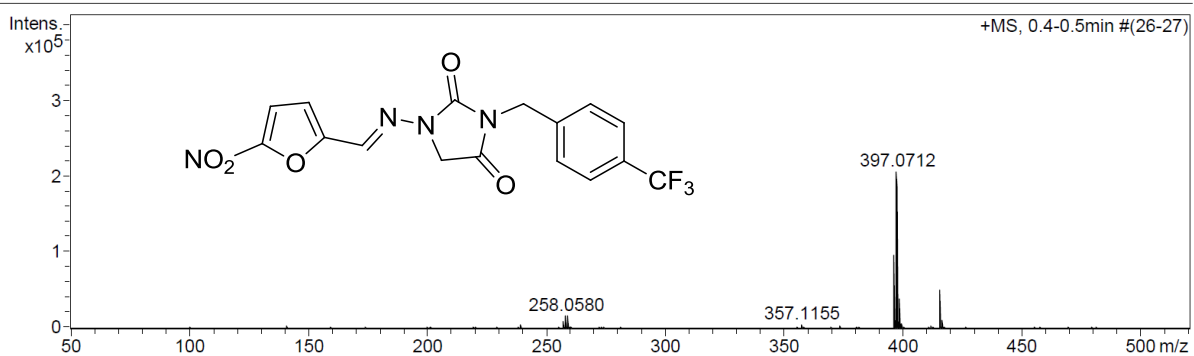


¹³CNMR in DMSO

**HRMS**

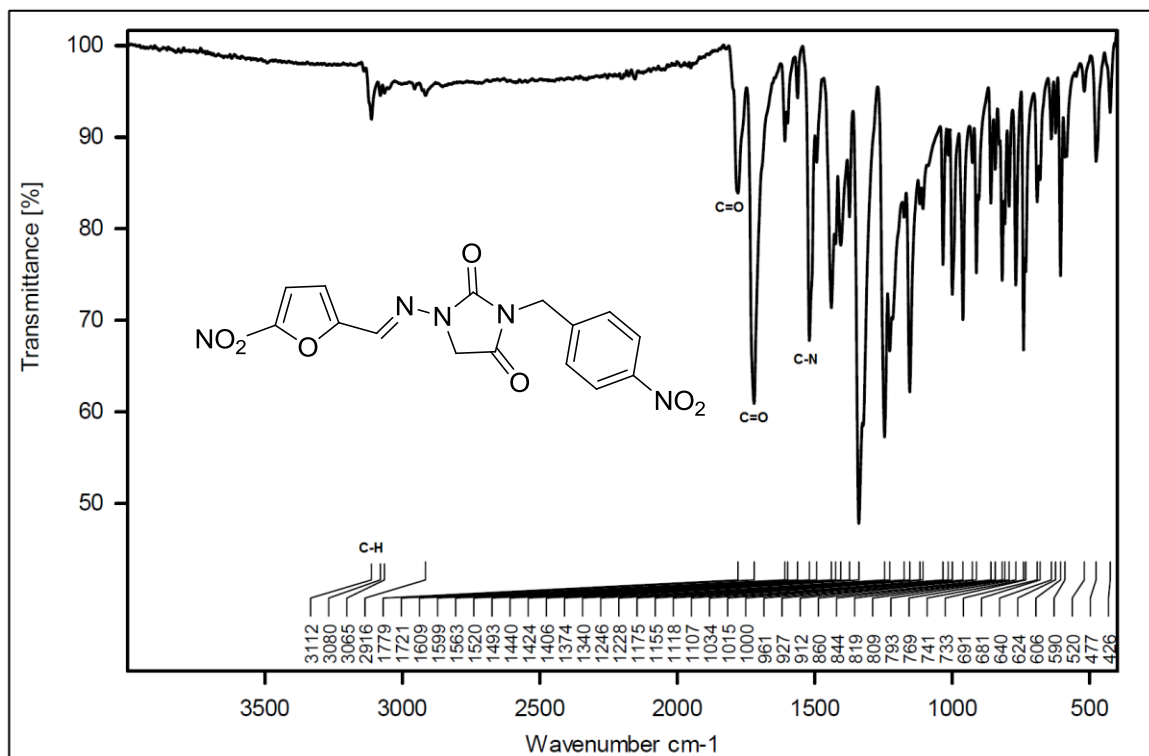
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Work

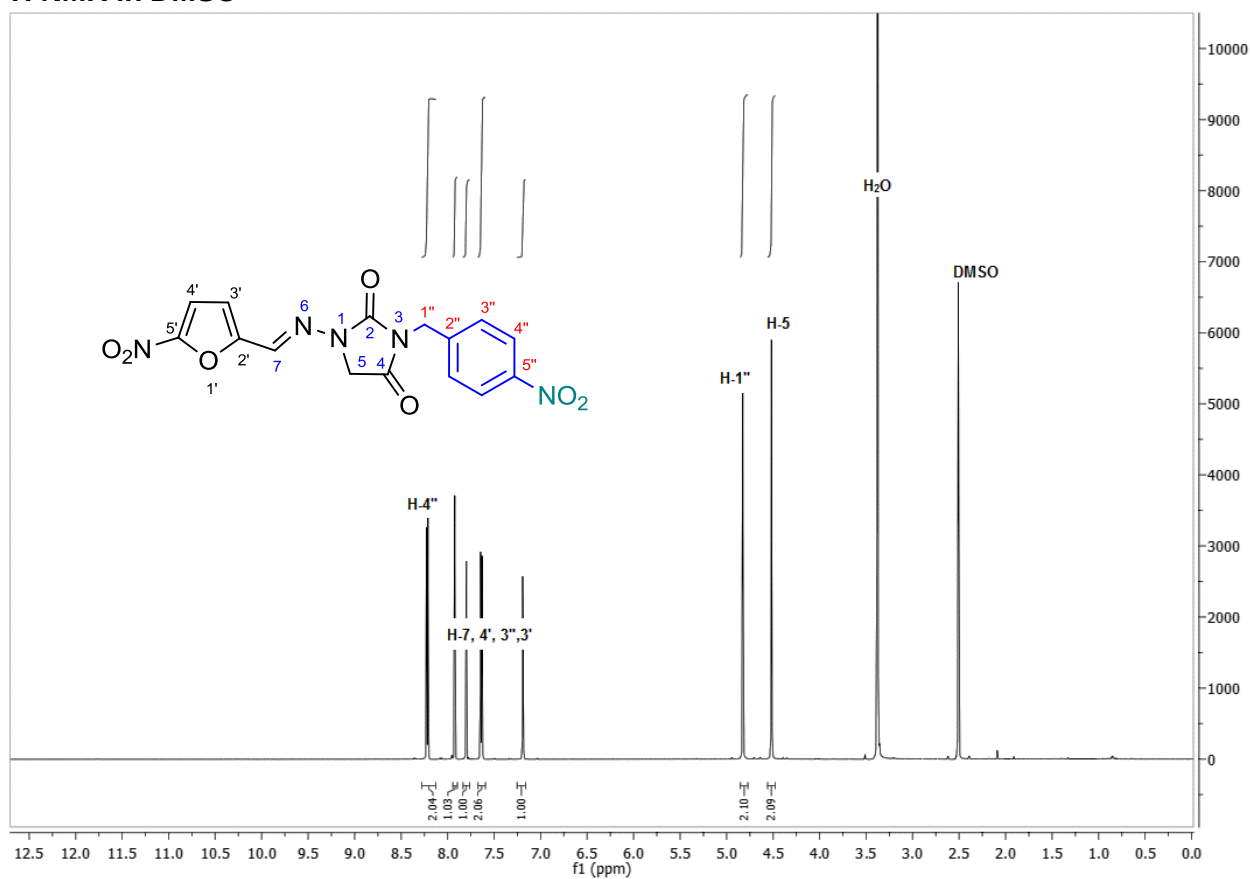


ANALOGUE 118

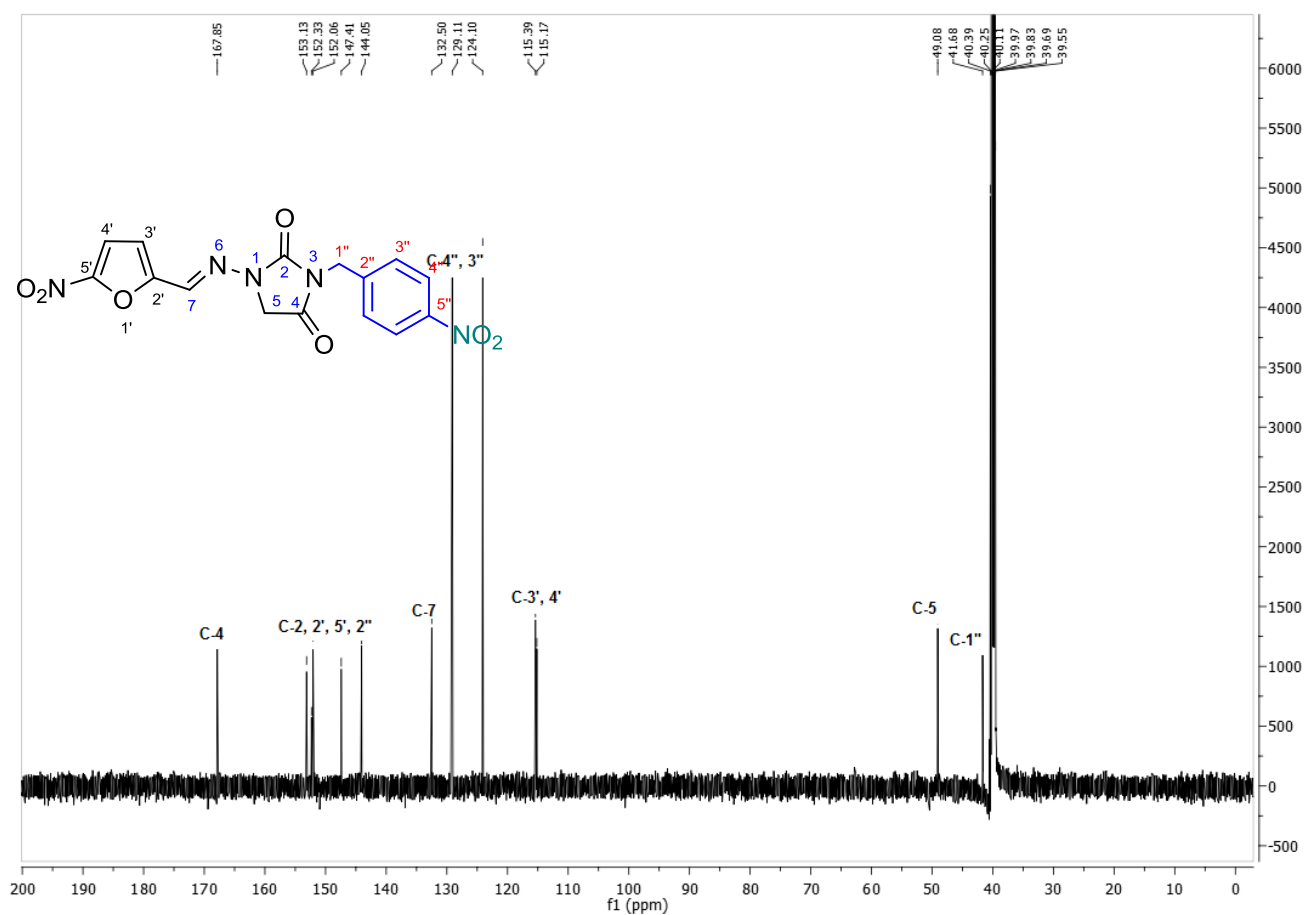
IR



¹H NMR in DMSO



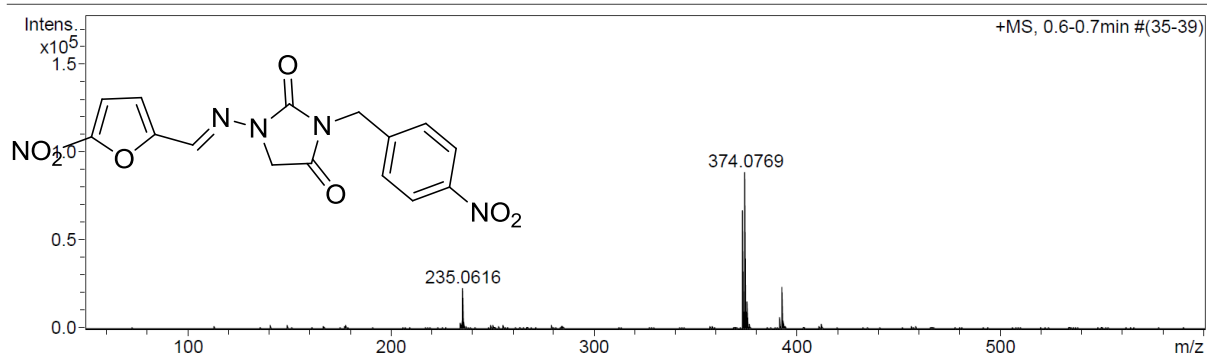
¹³CNMR in DMSO



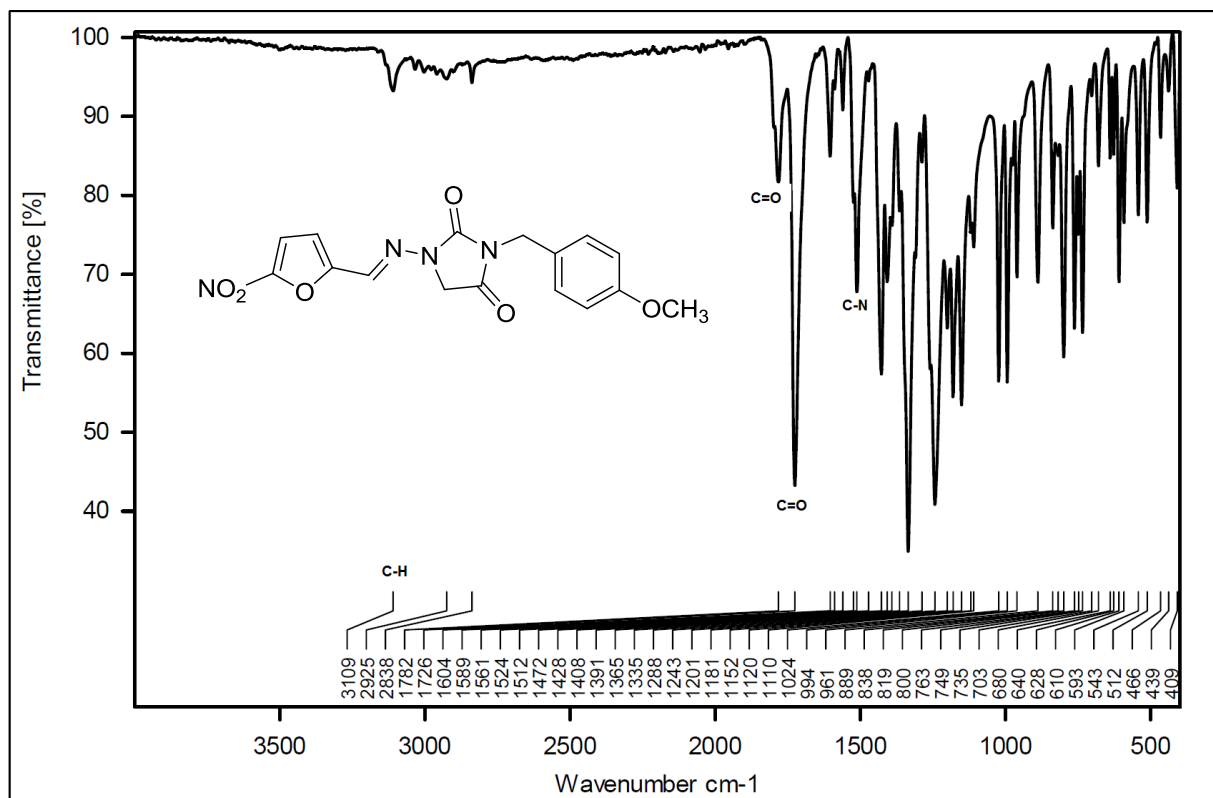
HRMS

Acquisition Parameter

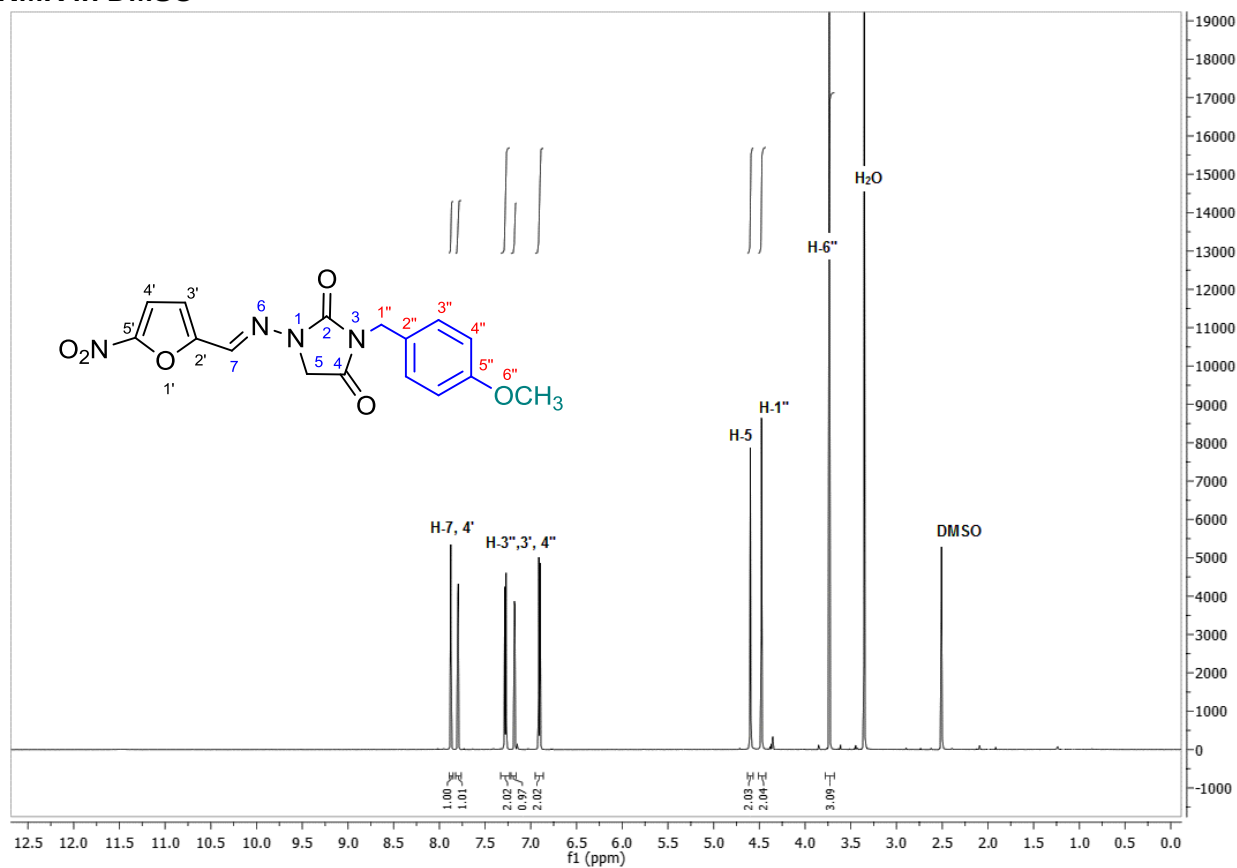
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



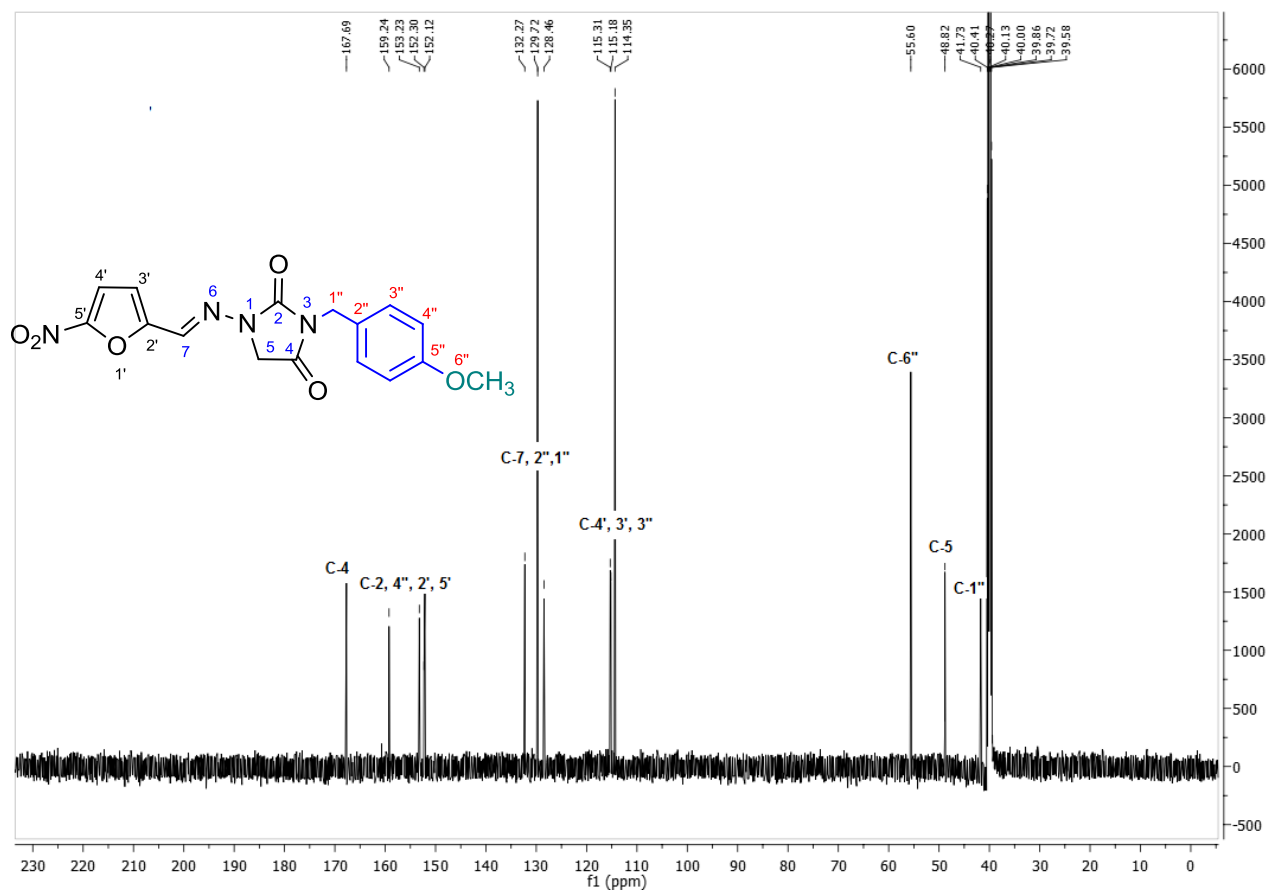
IR



¹H NMR in DMSO



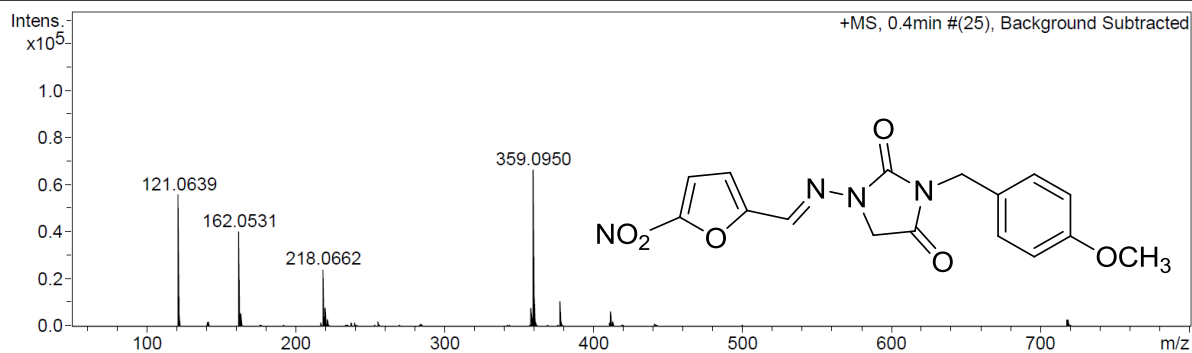
¹³CNMR in DMSO



HRMS

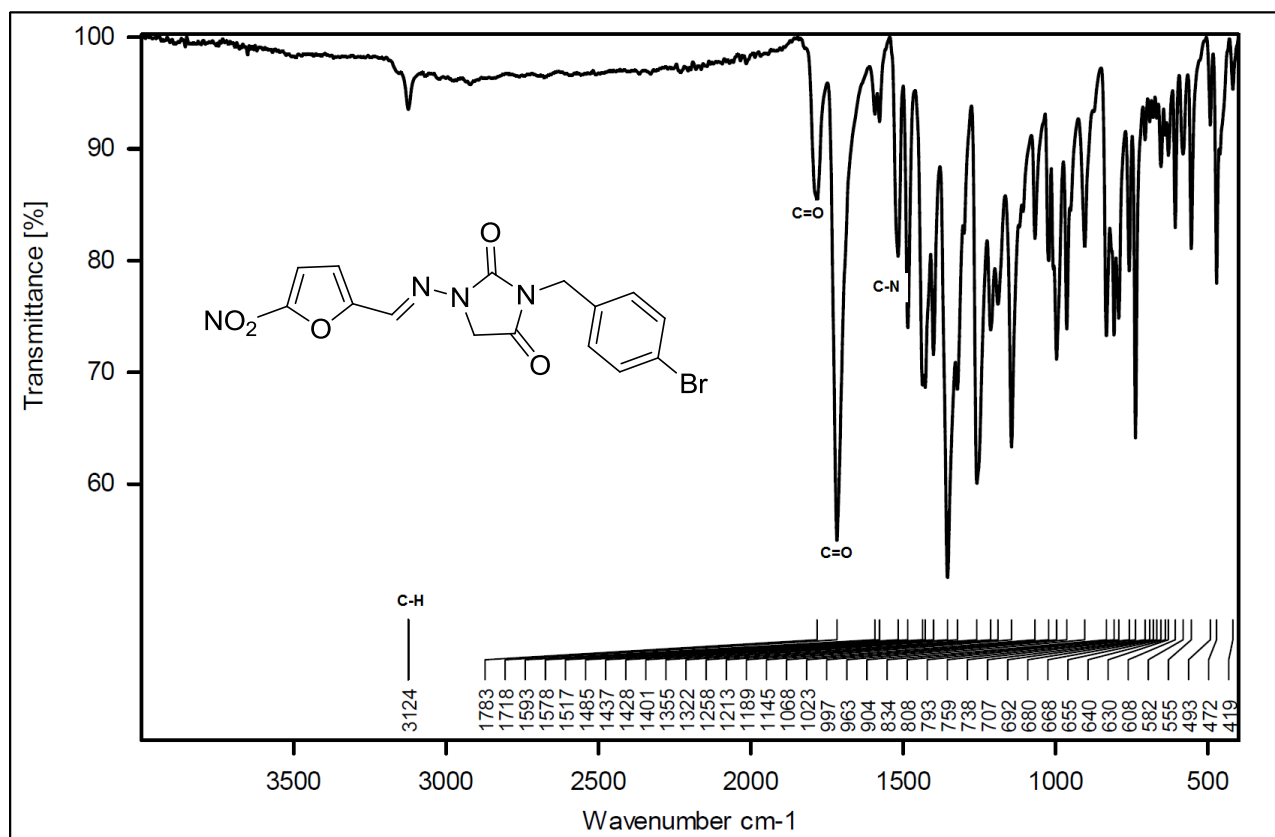
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

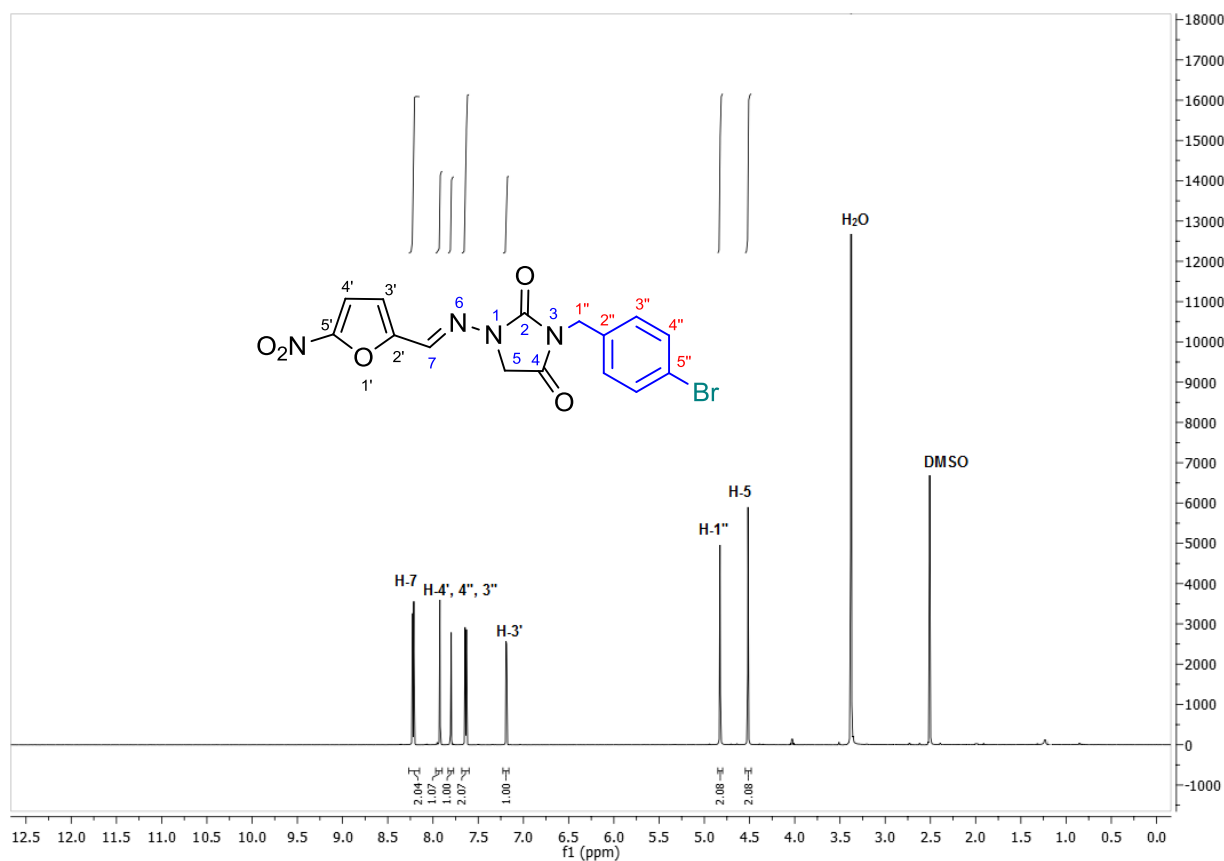


ANALOGUE 120

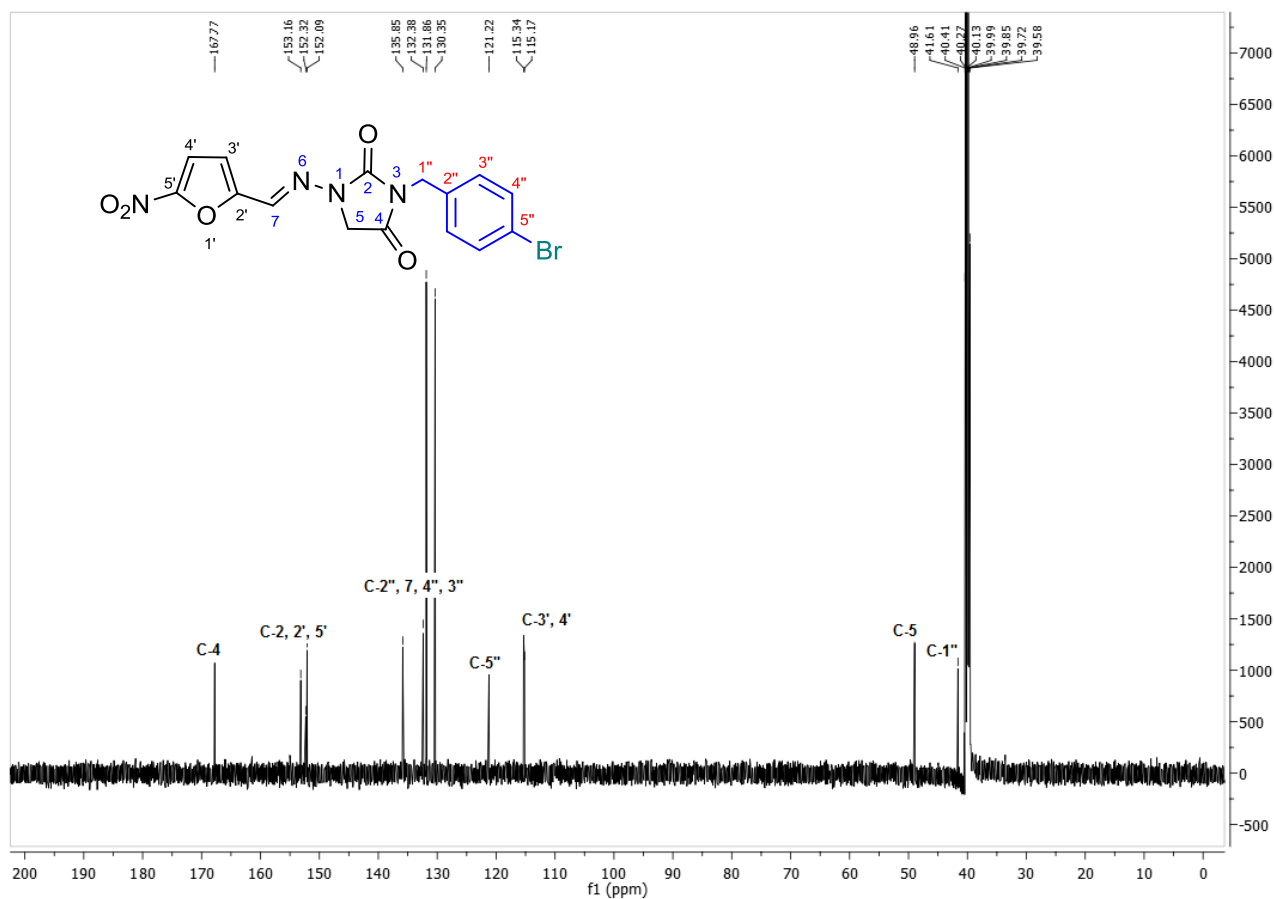
IR



^1H NMR in DMSO



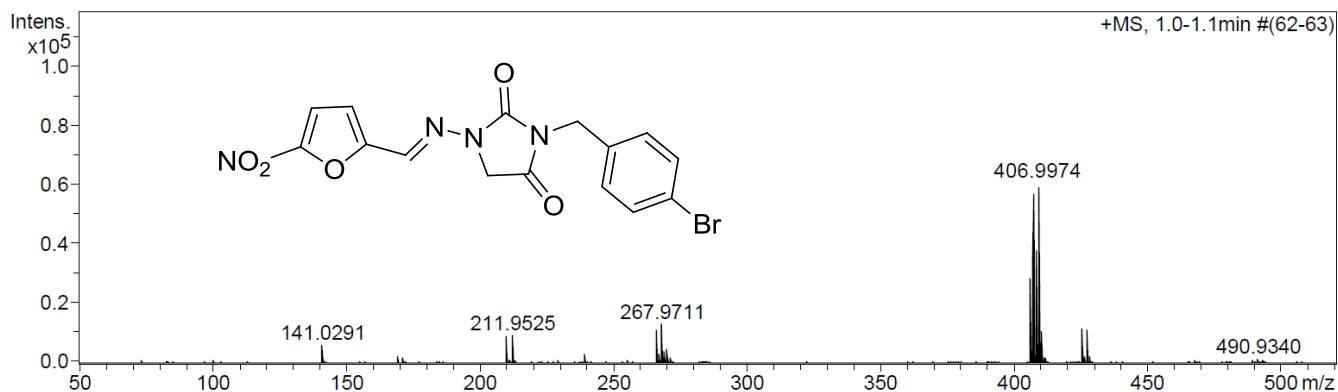
¹³CNMR in DMSO



HRMS

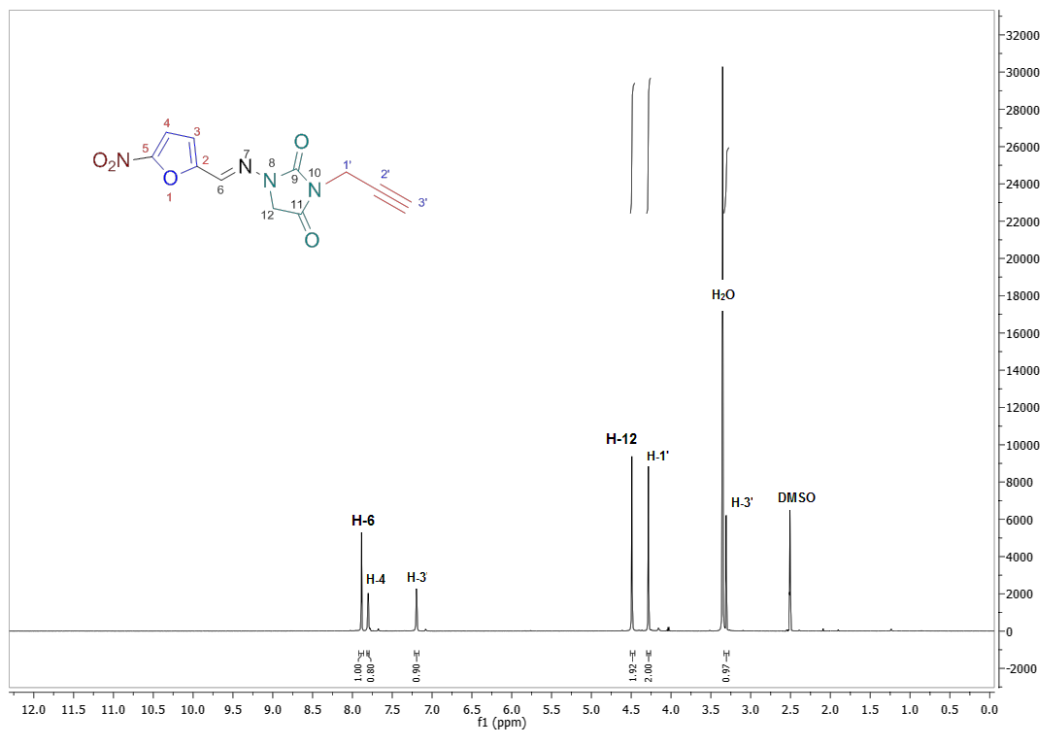
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

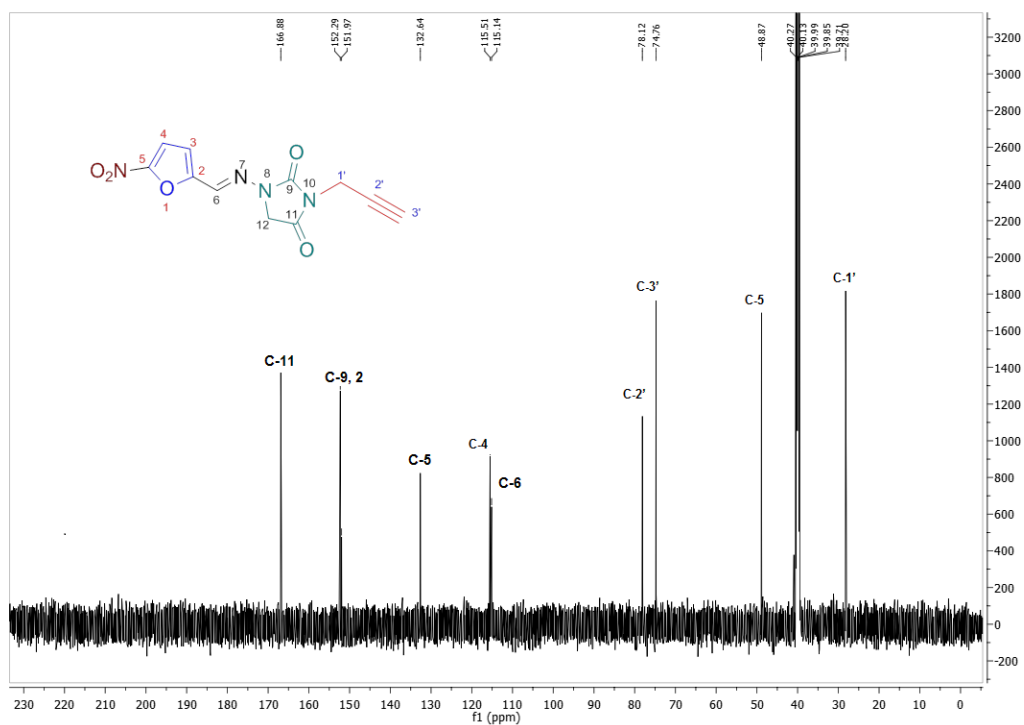


INTERMEDIATE 200

¹H NMR in DMSO



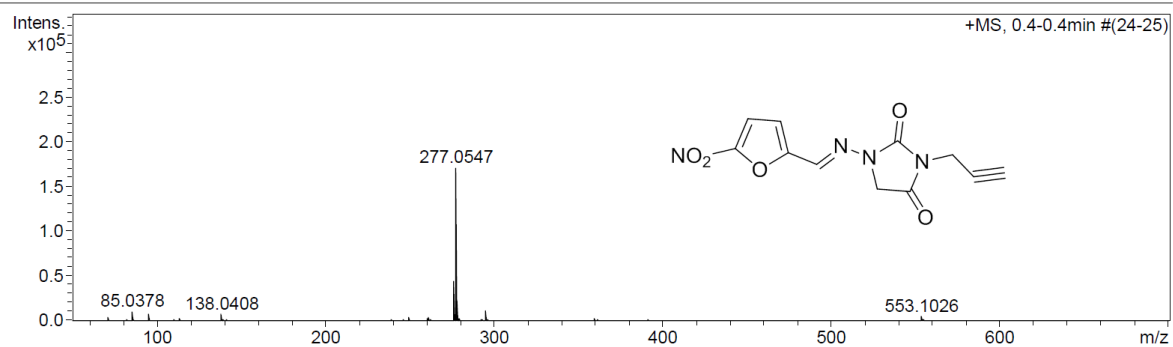
¹³C NMR in DMSO



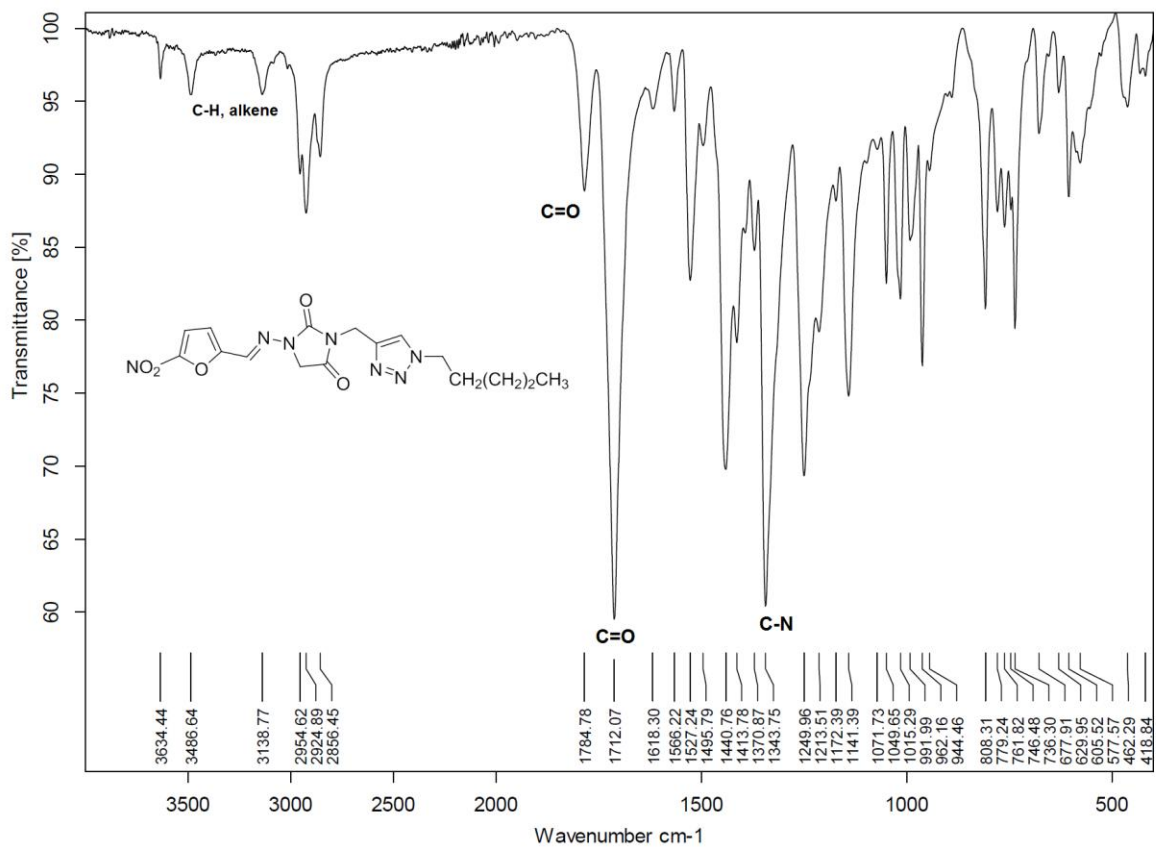
HR-MS

Acquisition Parameter

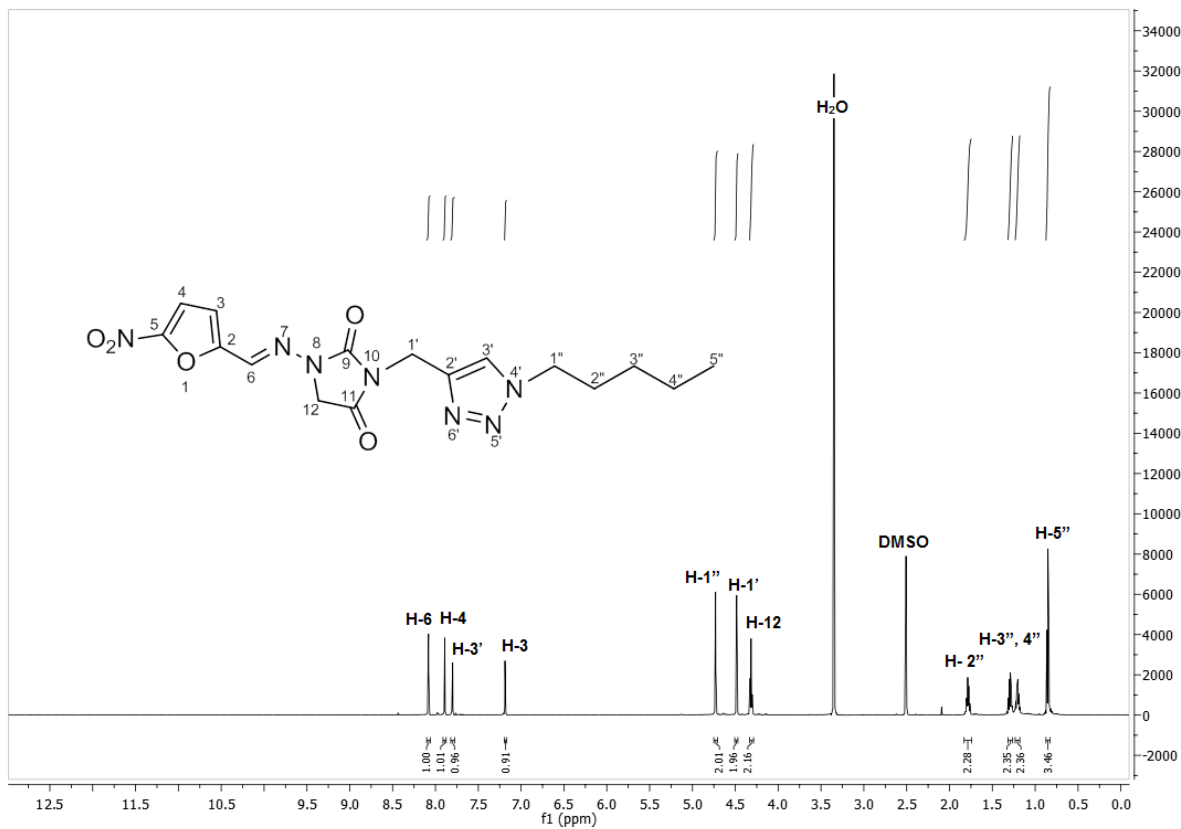
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



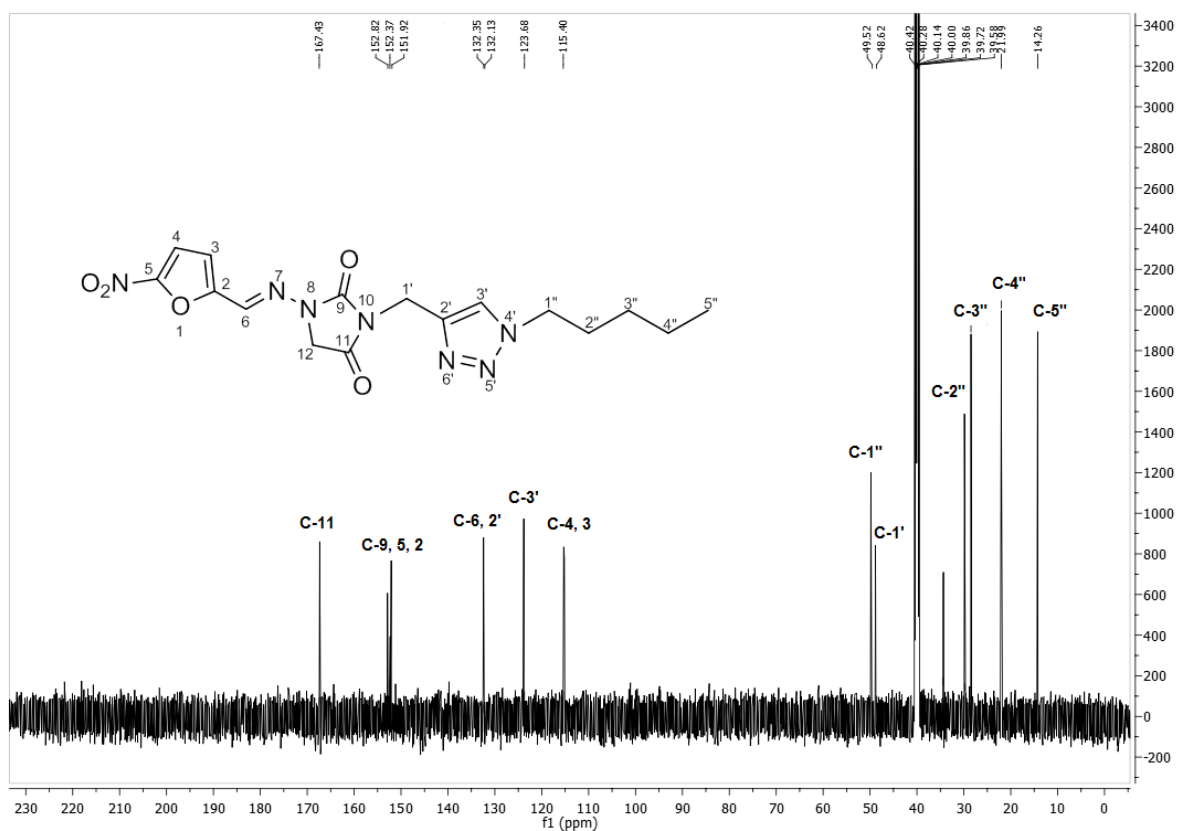
HYBRID 202
IR



¹H NMR in DMSO



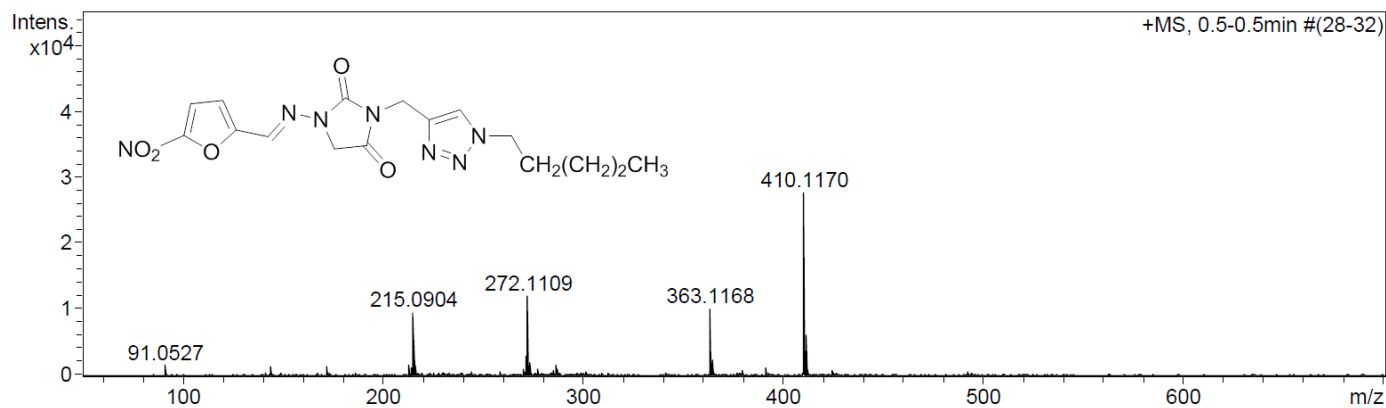
¹³C NMR in DMSO

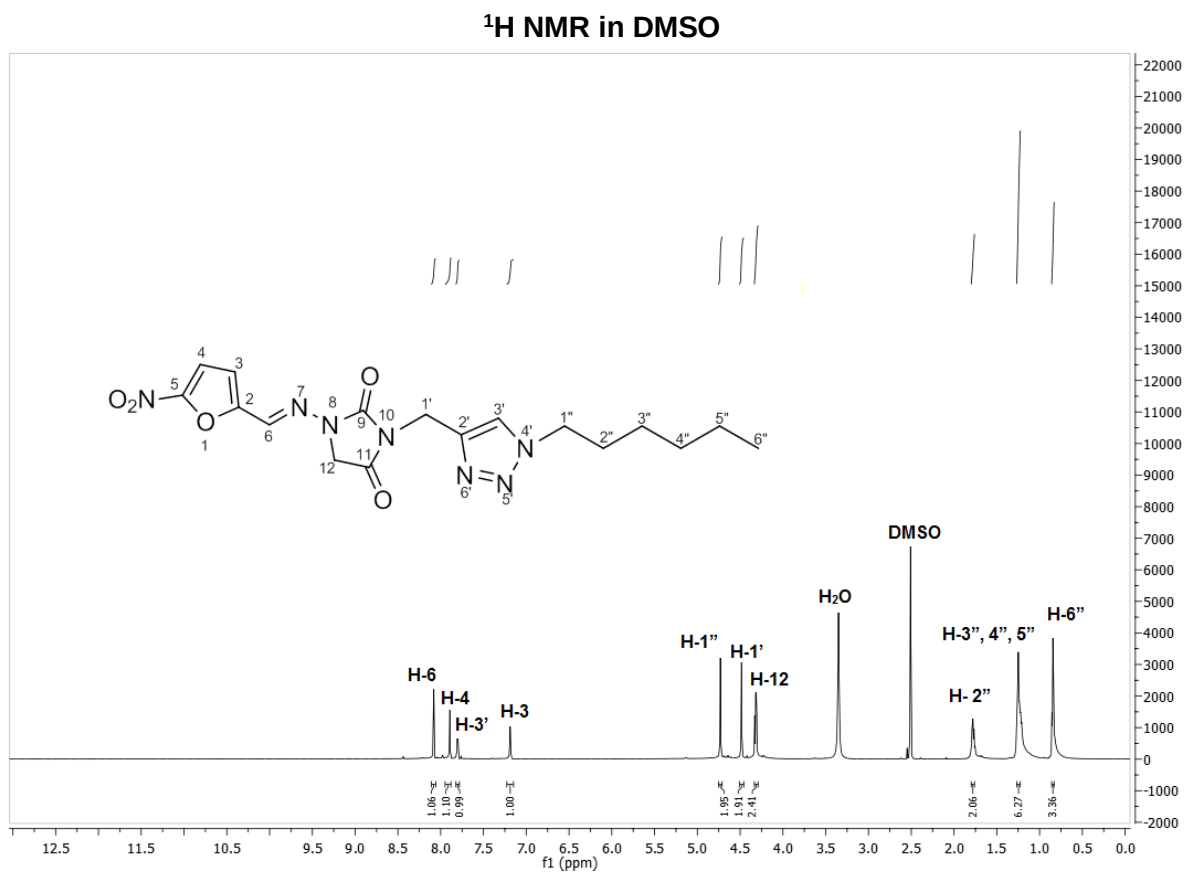
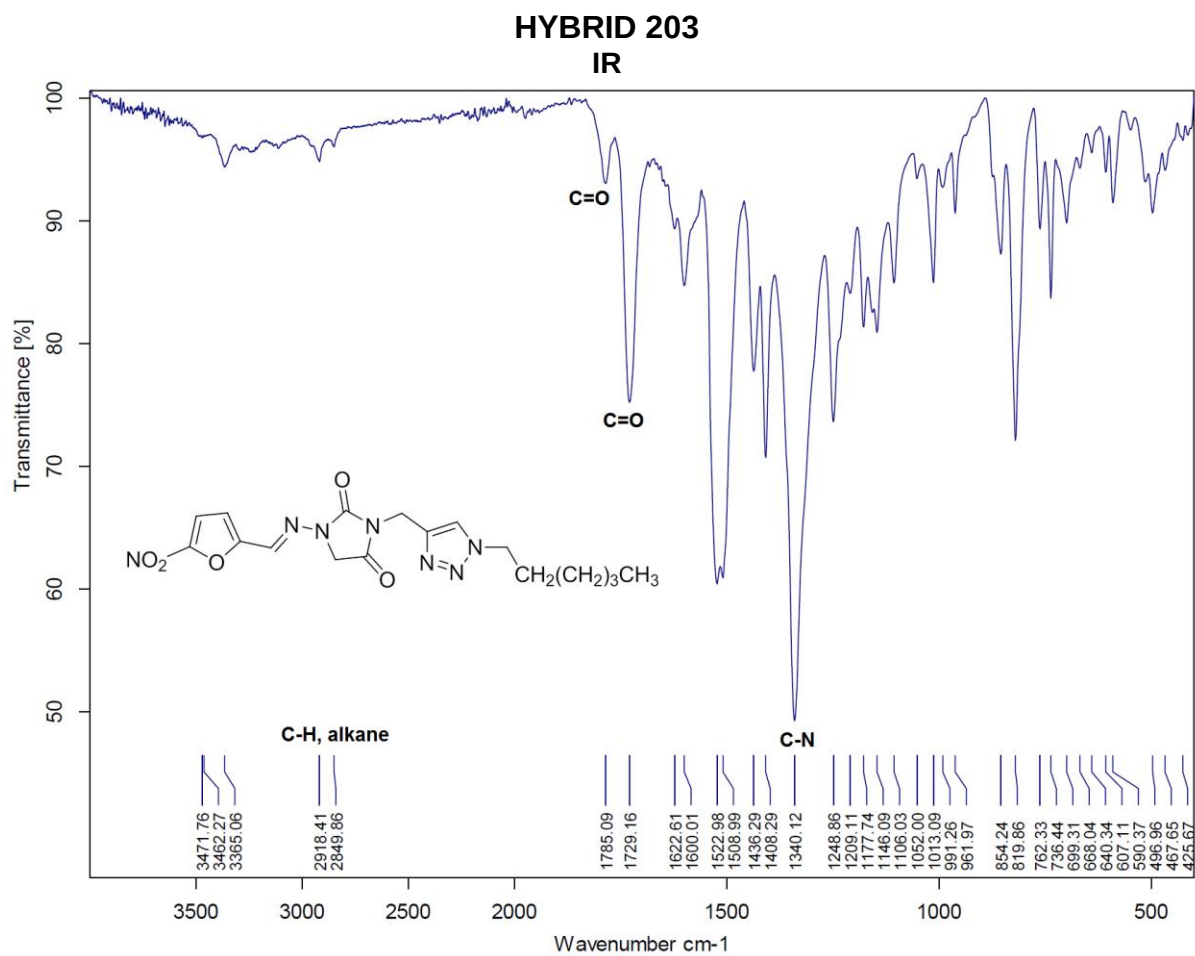


HR-MS

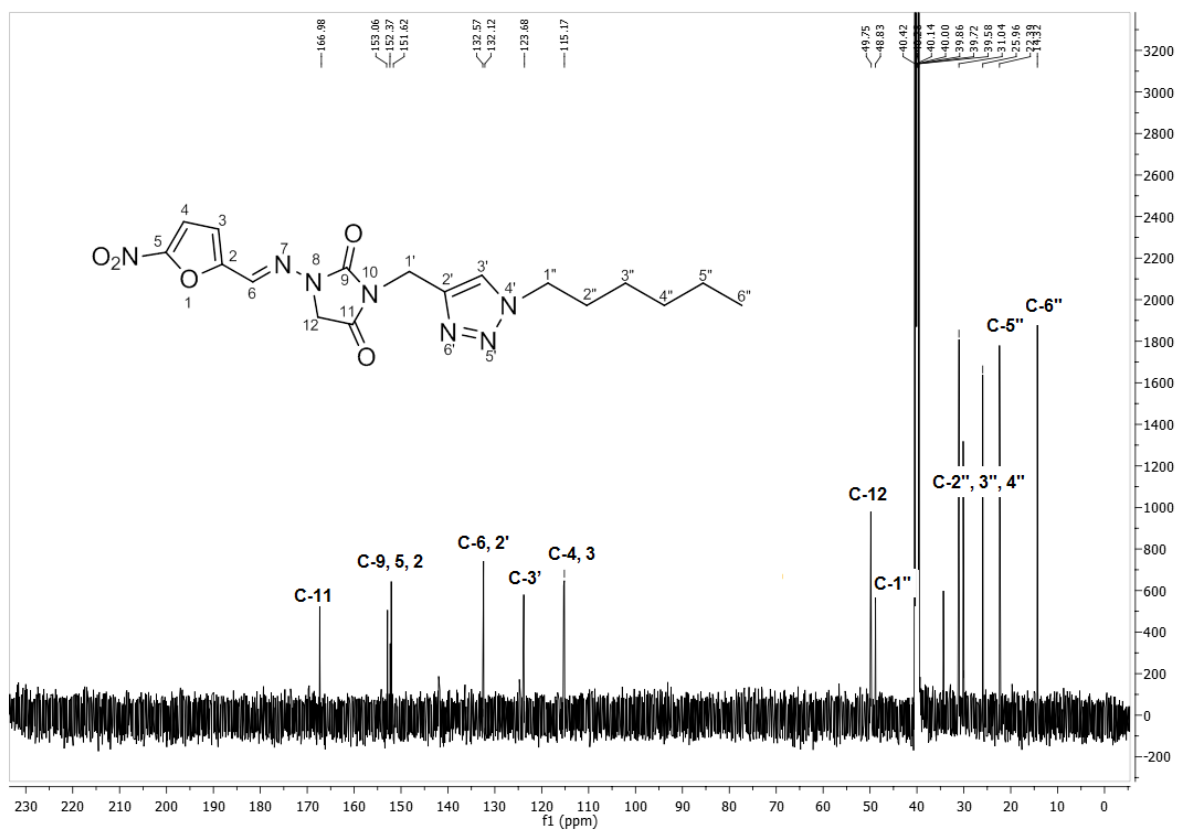
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste





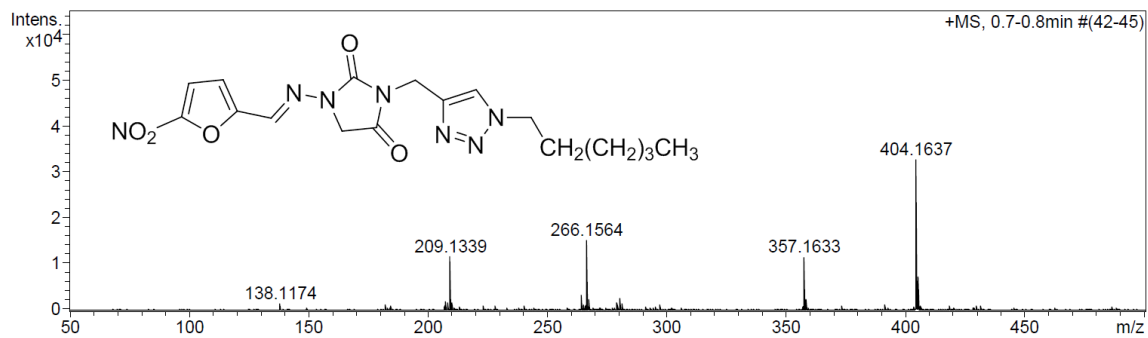
¹³C NMR in DMSO



HR-MS

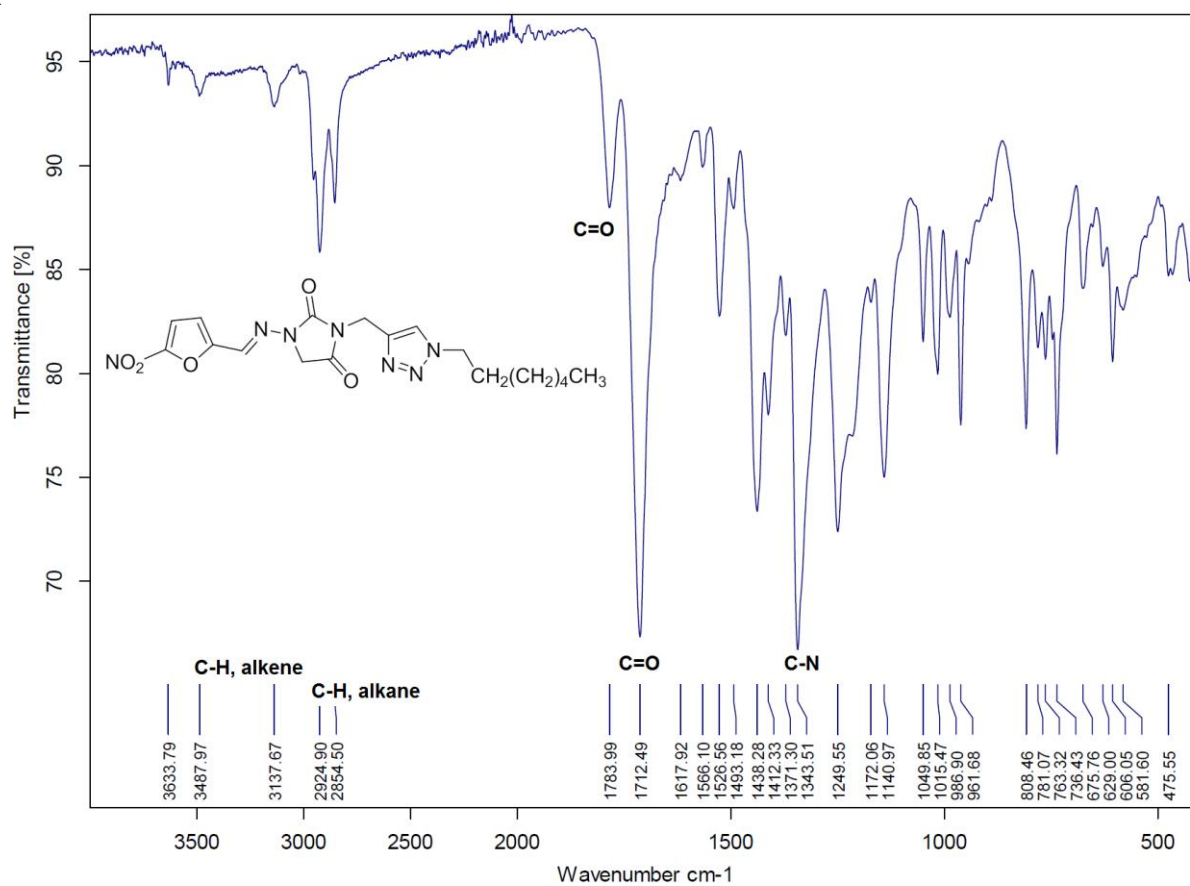
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

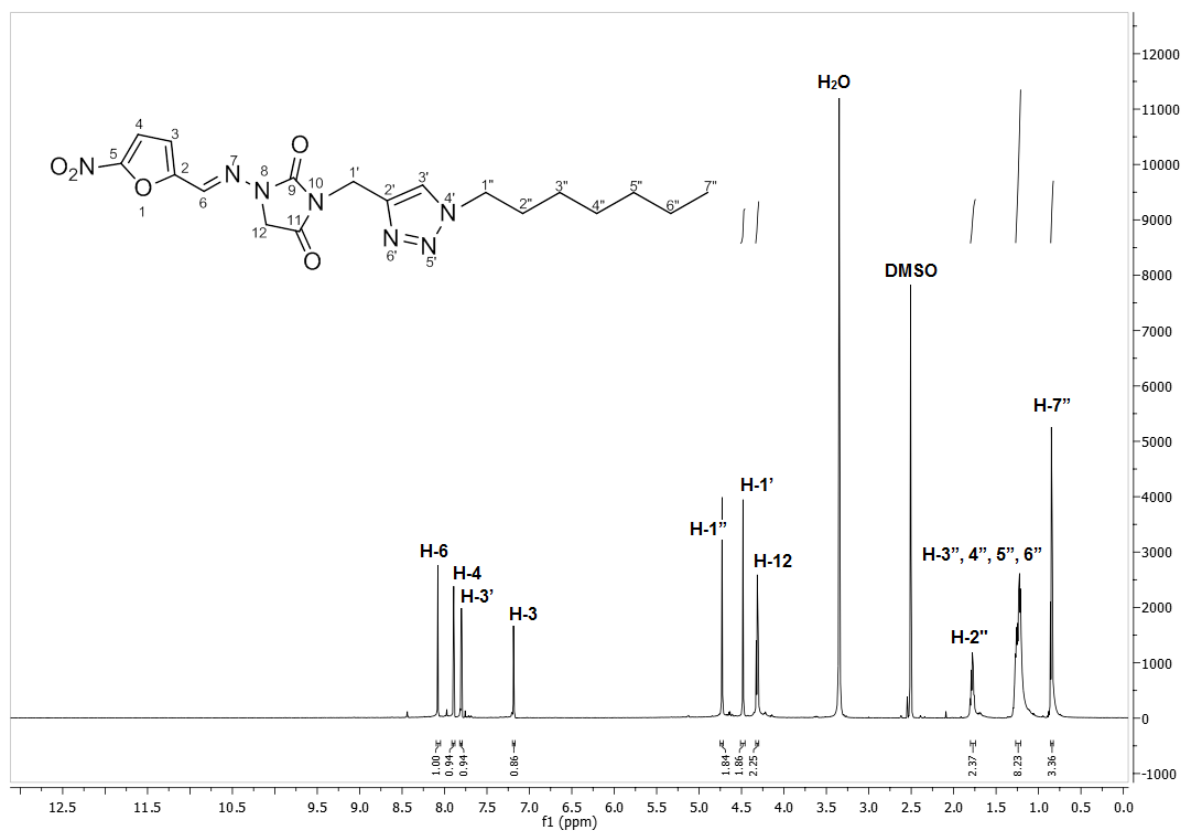


HYBRID 204

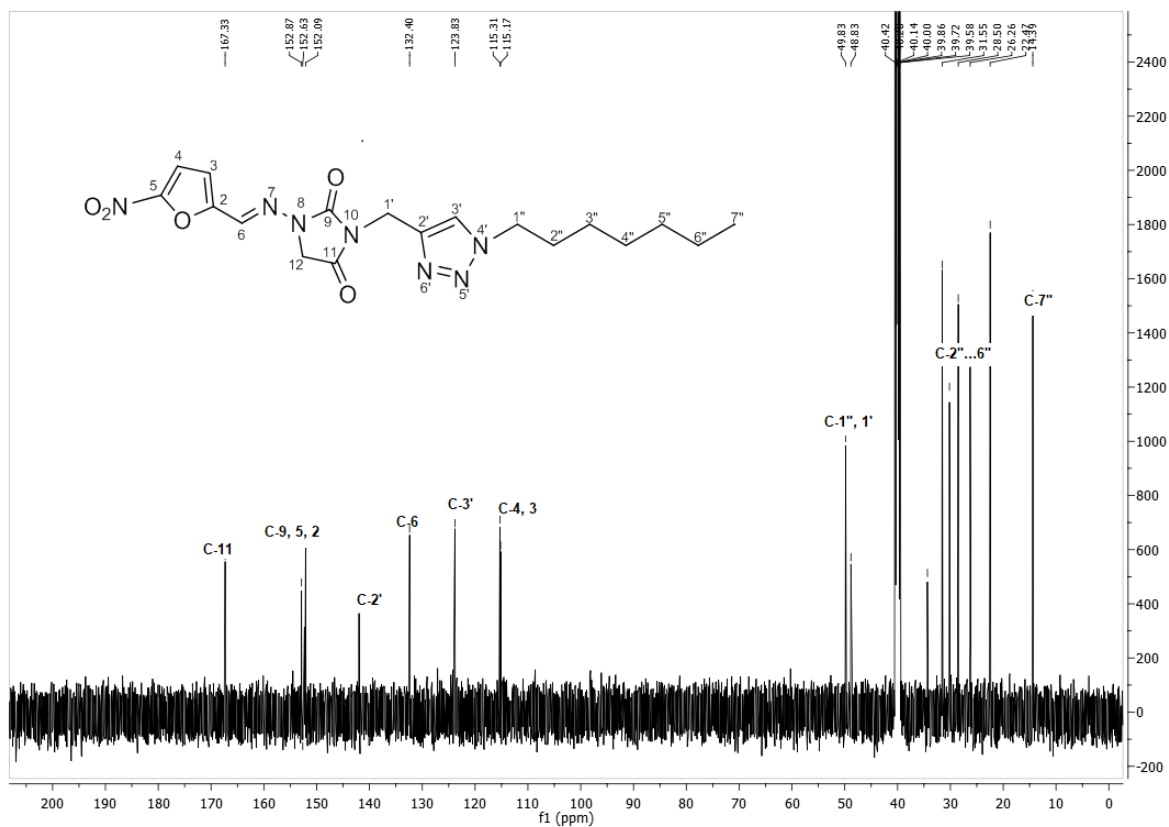
IR



¹H NMR in DMSO



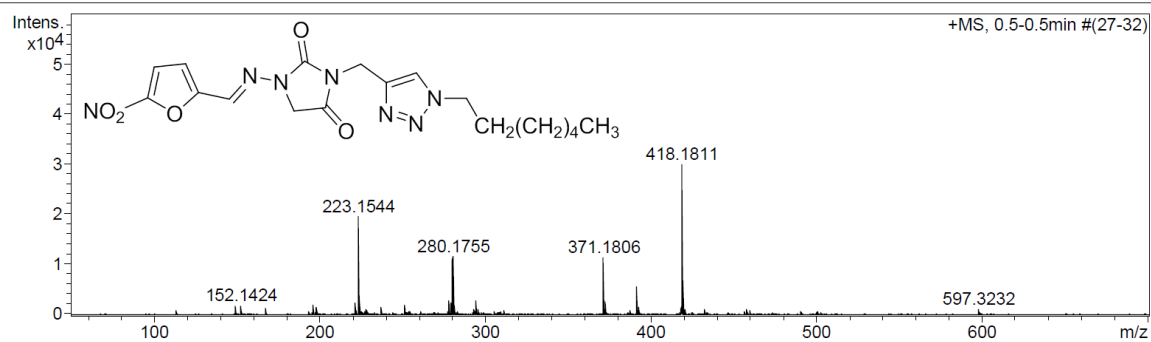
¹³C NMR in DMSO



HR-MS

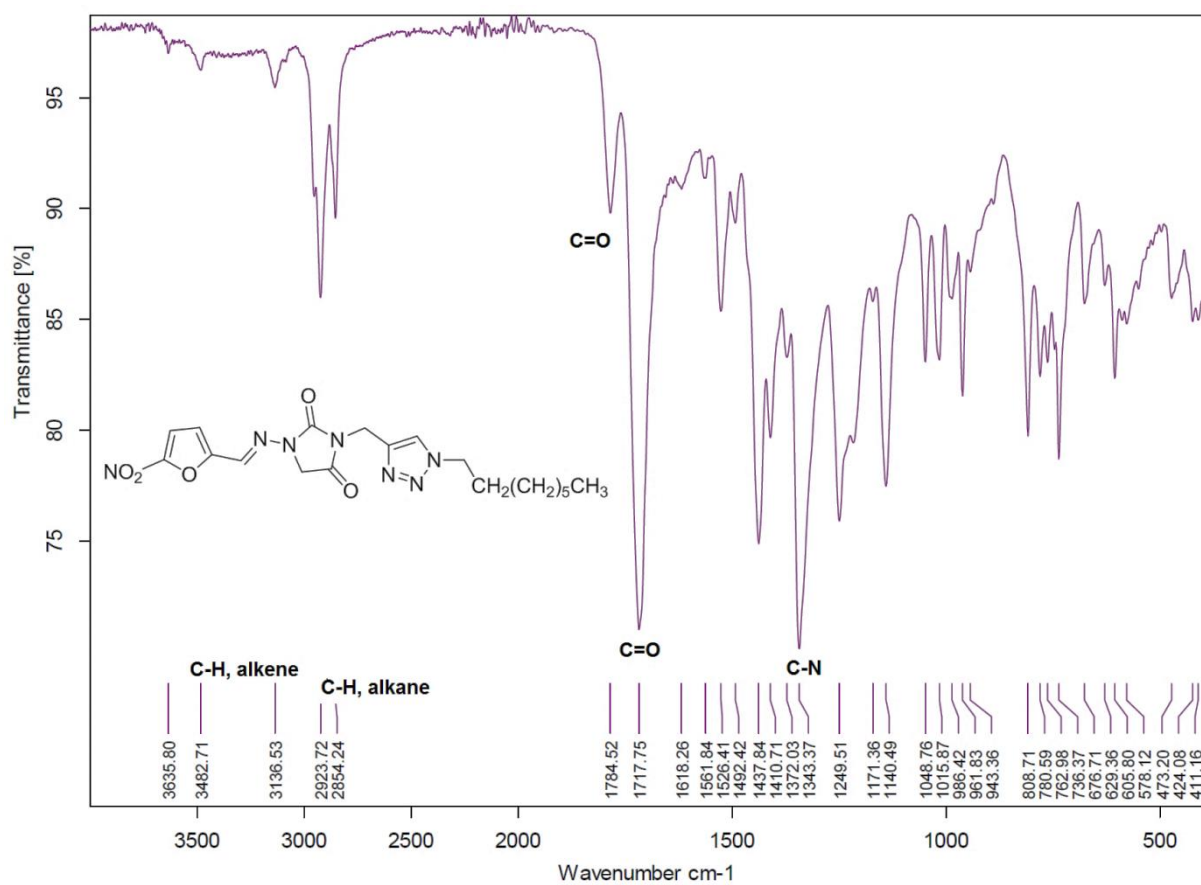
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

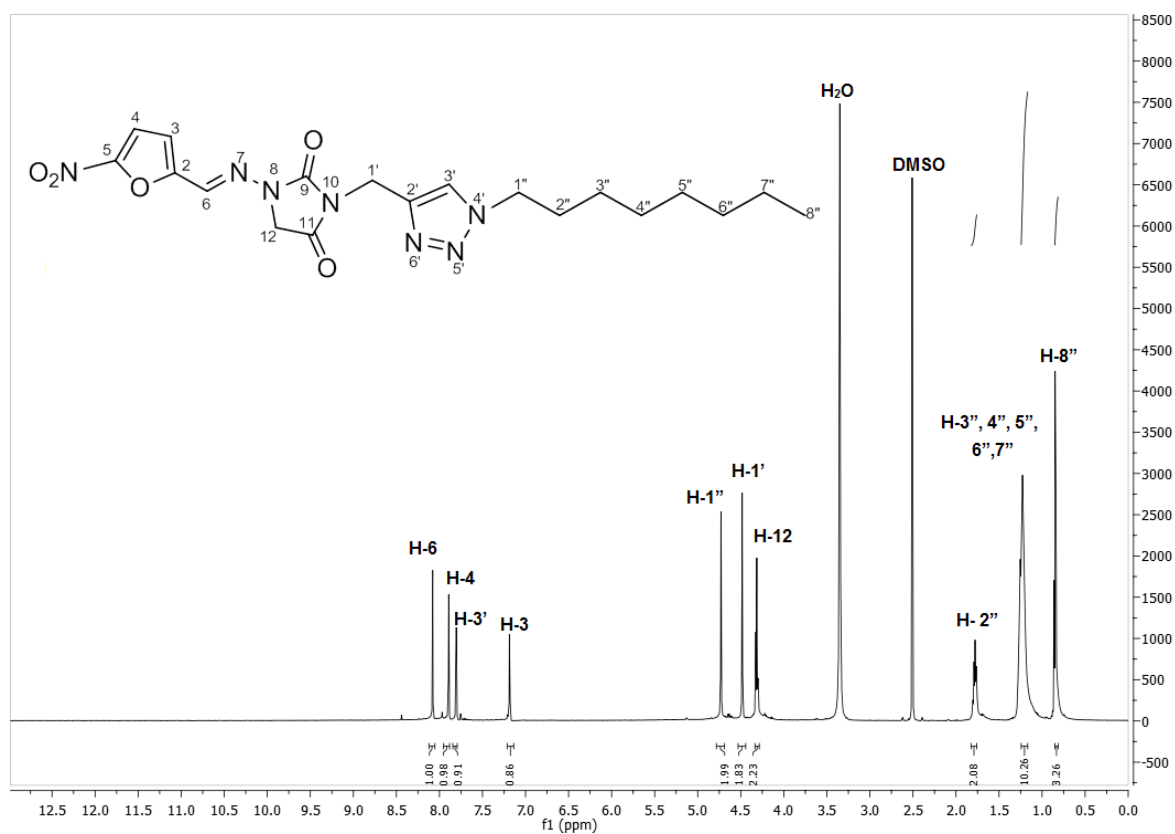


HYBRID 205

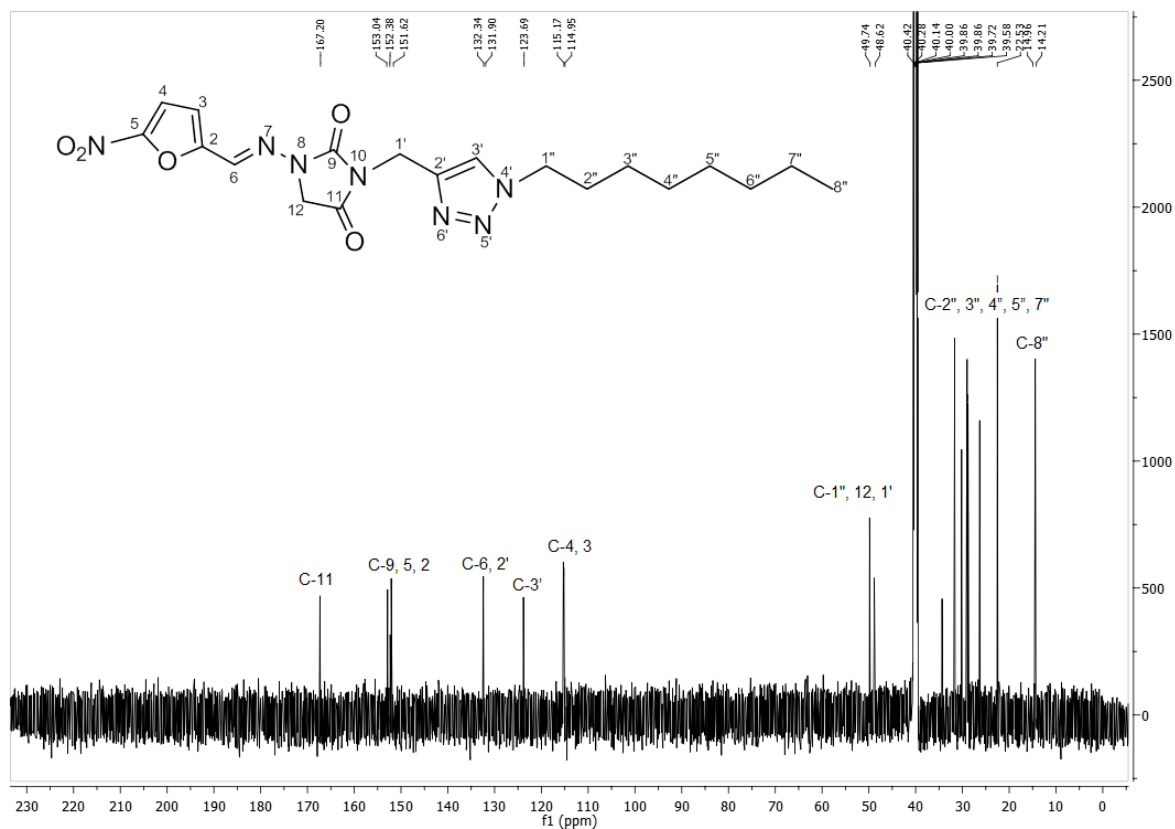
IR



¹H NMR in DMSO



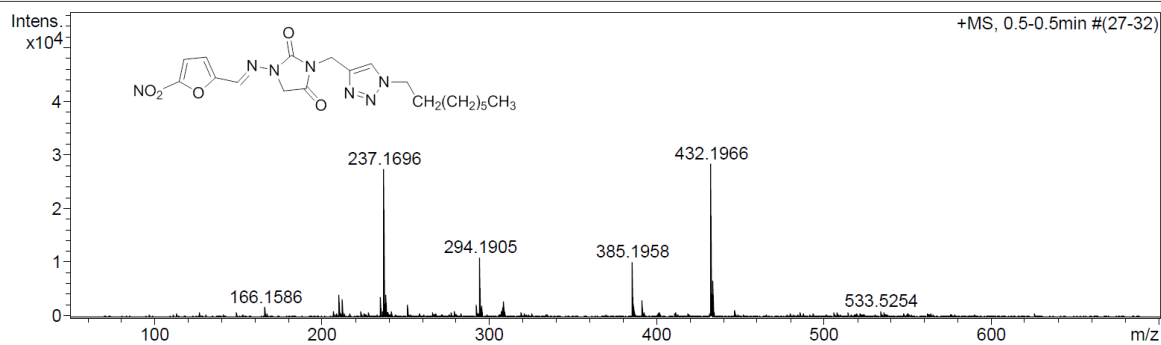
¹³C NMR in DMSO



HR-MS

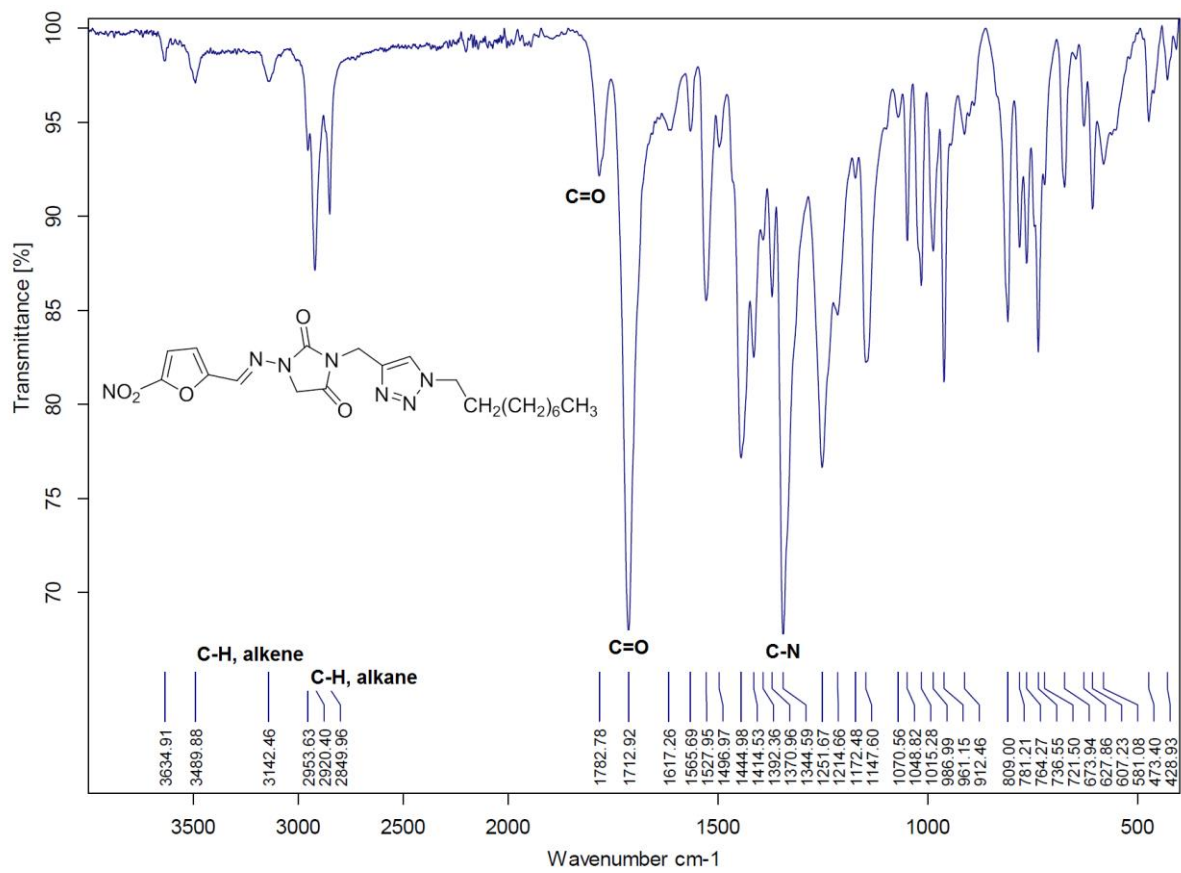
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

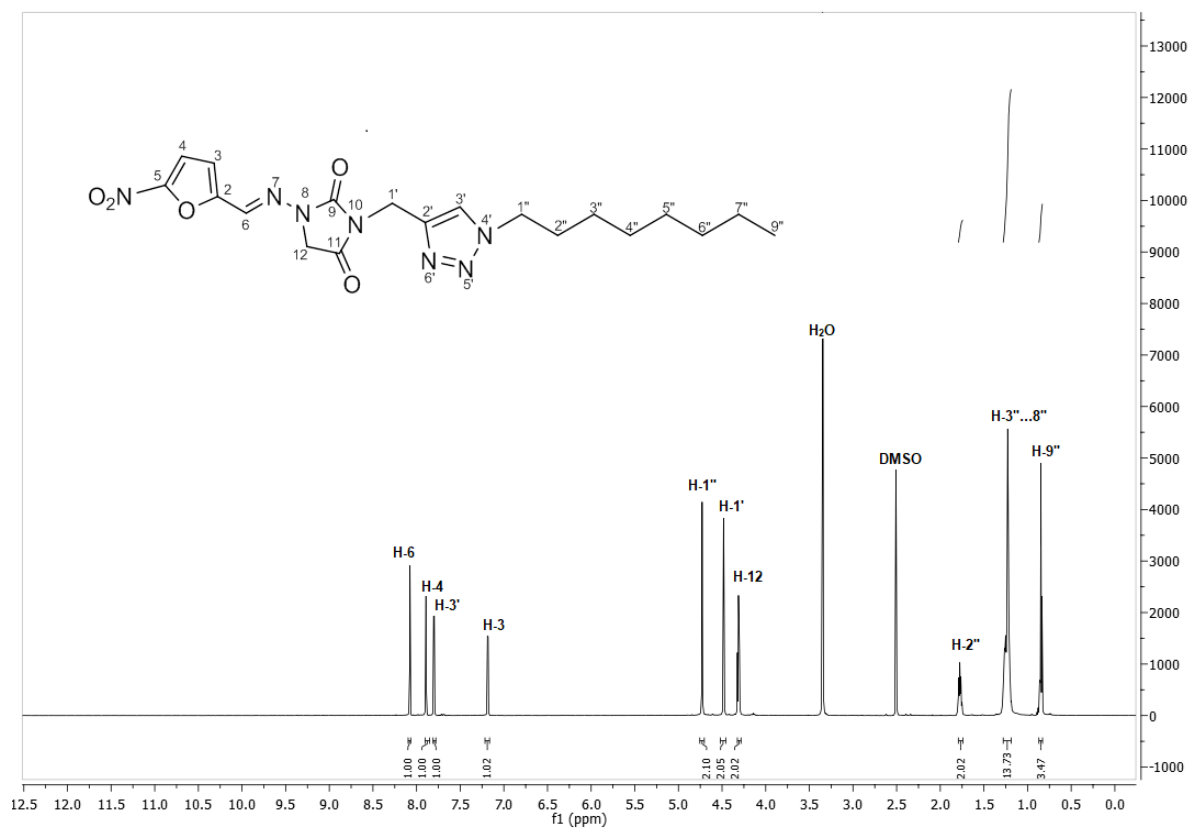


HYBRID 206

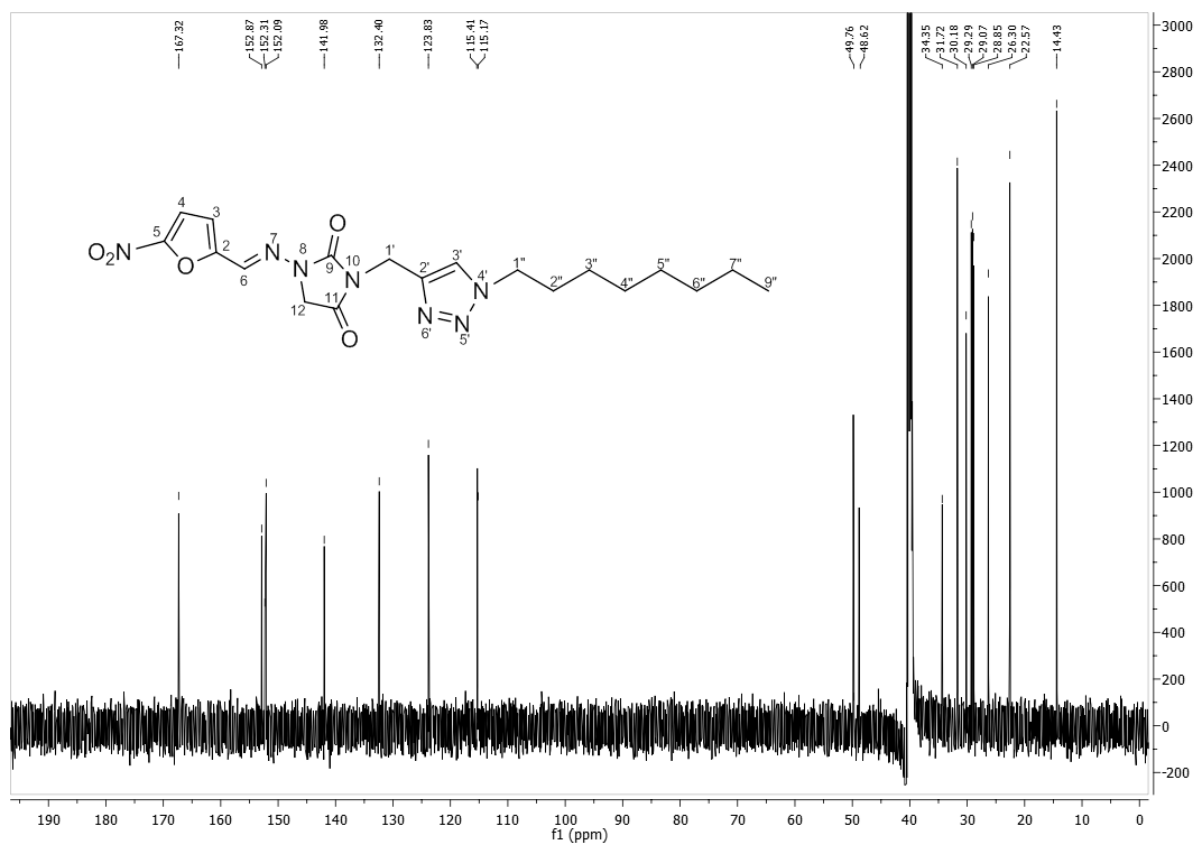
IR



¹H NMR in DMSO



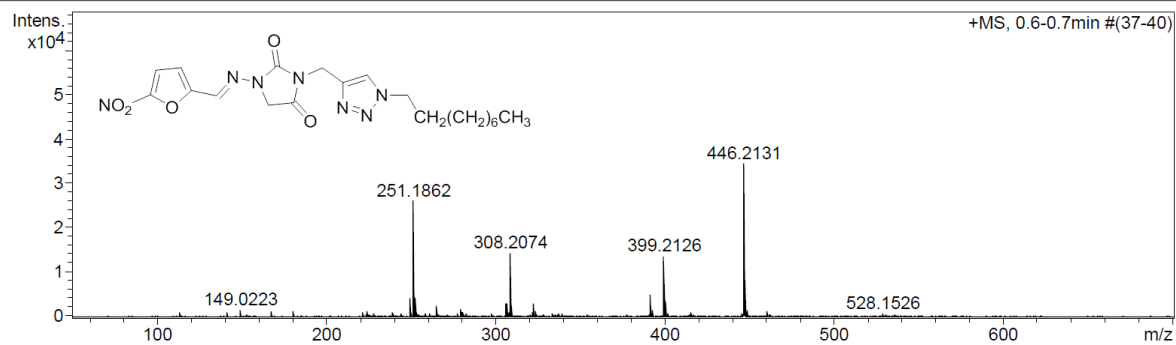
¹³C NMR in DMSO



HR-MS

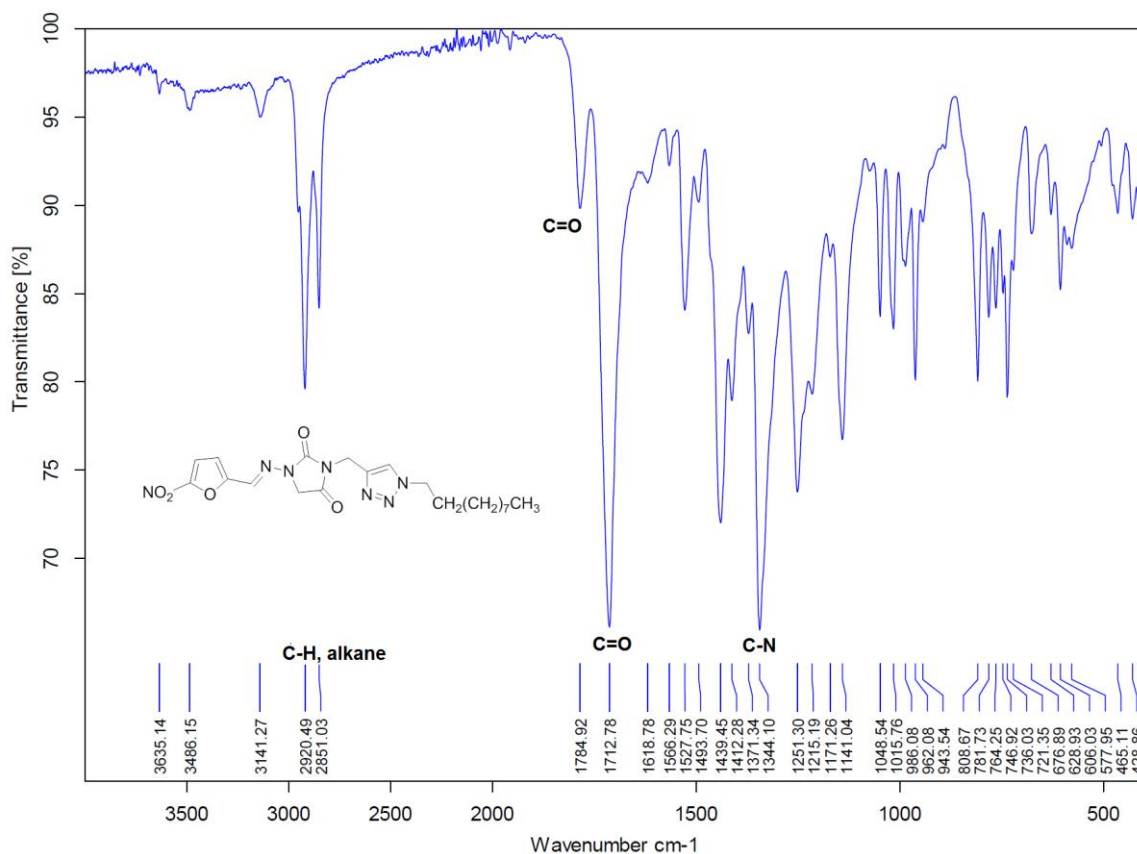
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

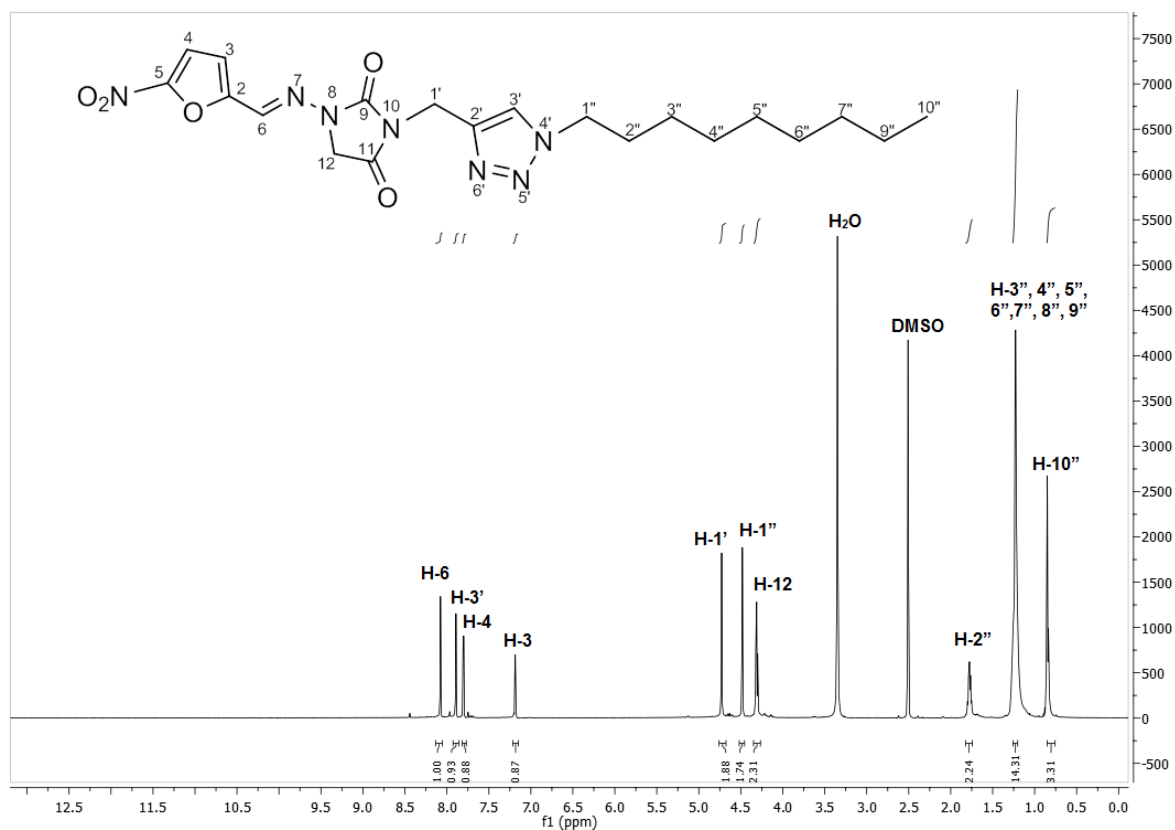


HYBRID 207

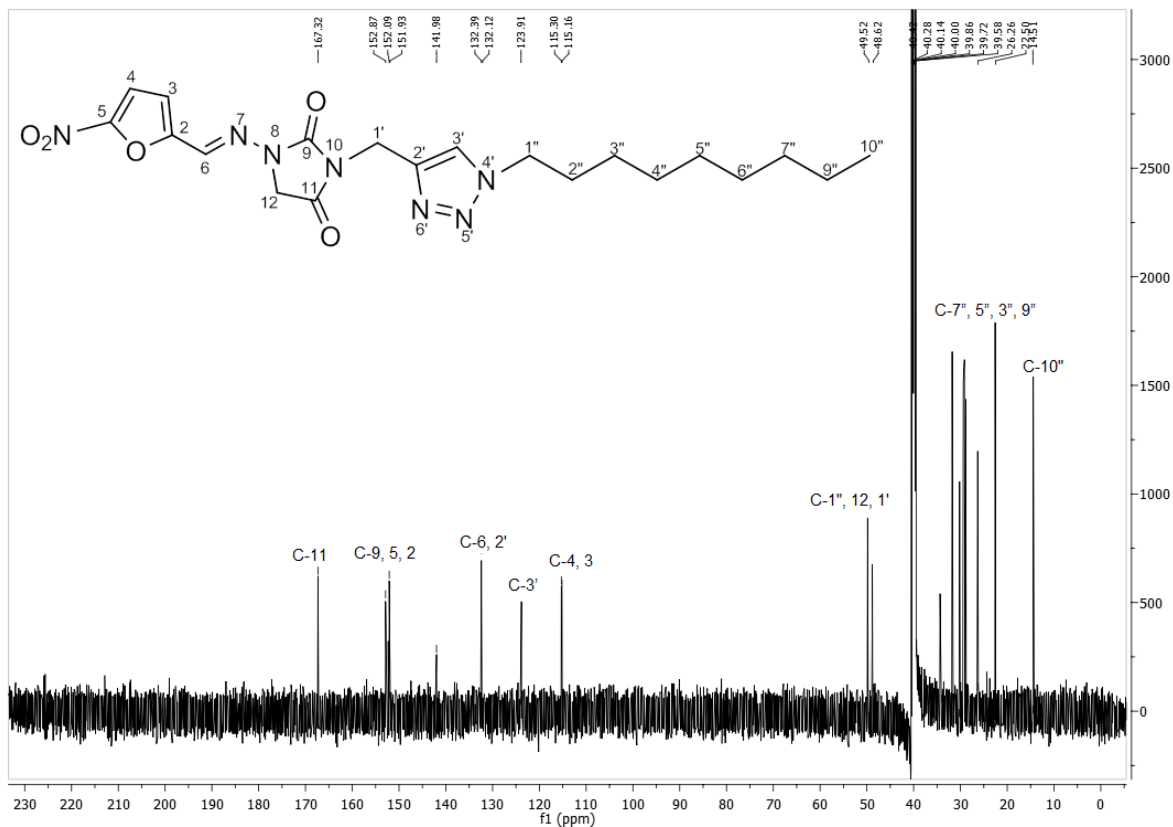
IR



¹H NMR in DMSO



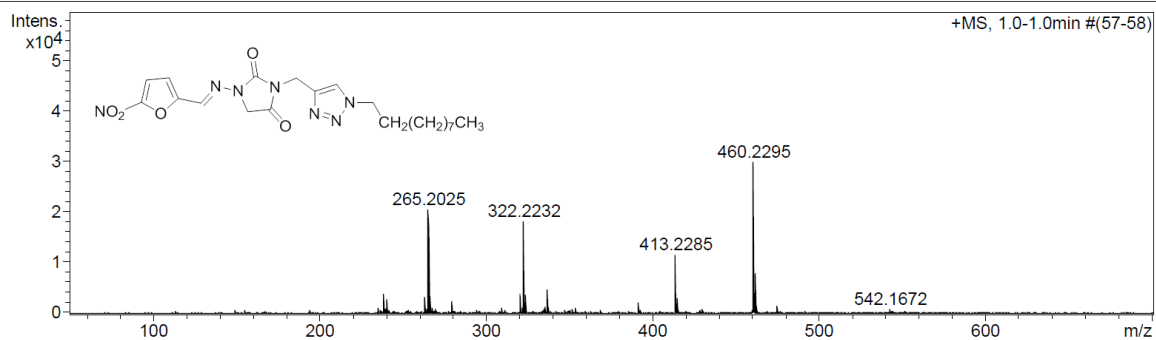
¹³C NMR in DMSO



HR-MS

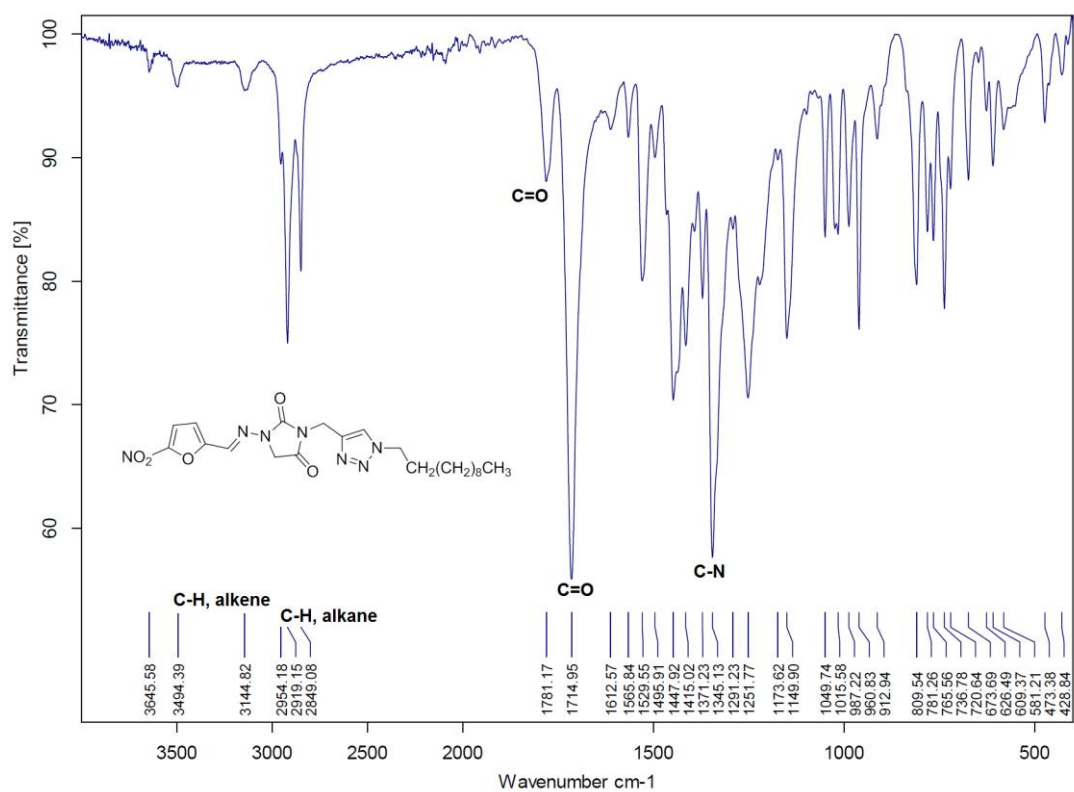
Acquisition Parameter

Acquisition Parameters					
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Work

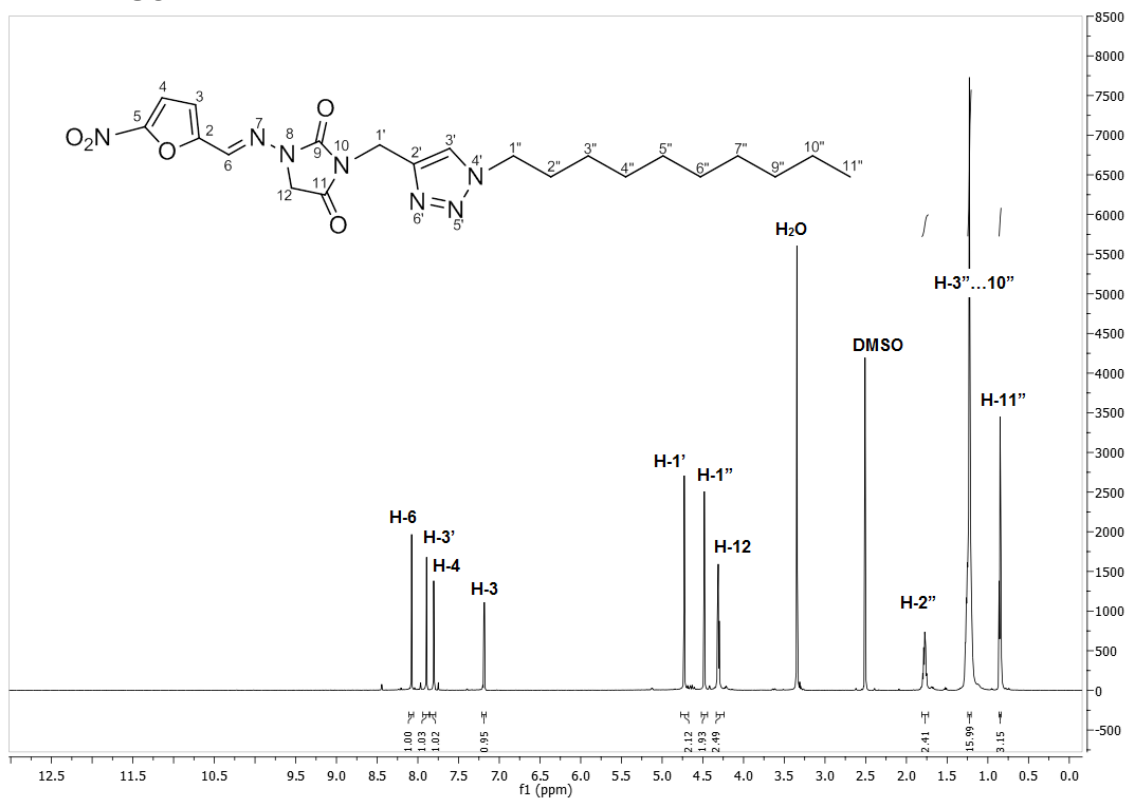


HYBRID 208

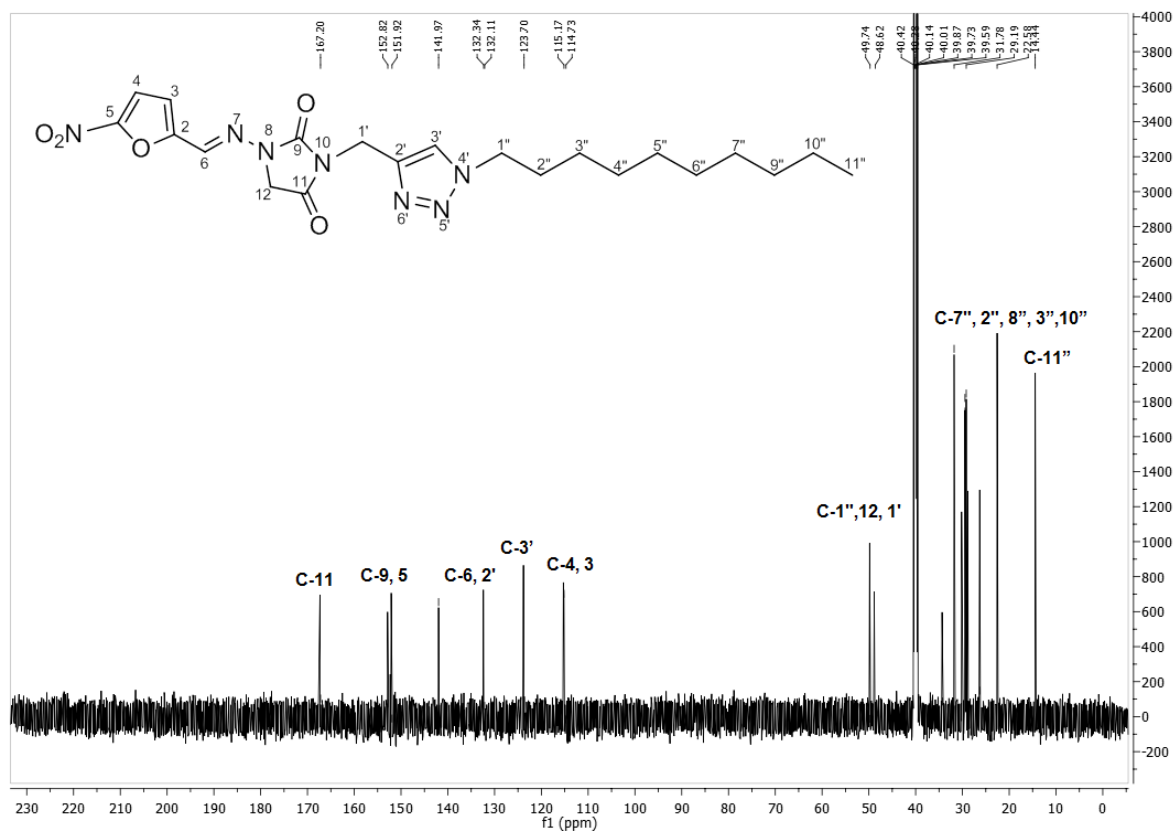
IR



¹H NMR in DMSO



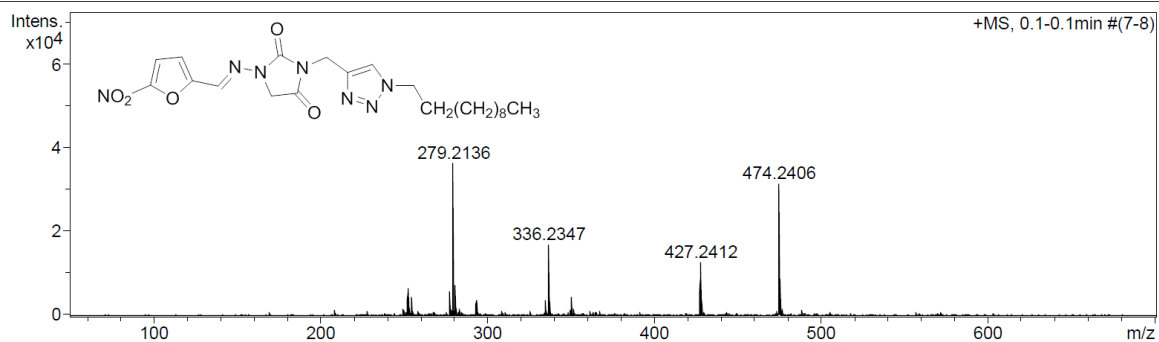
¹³C NMR in DMSO



HR-MS

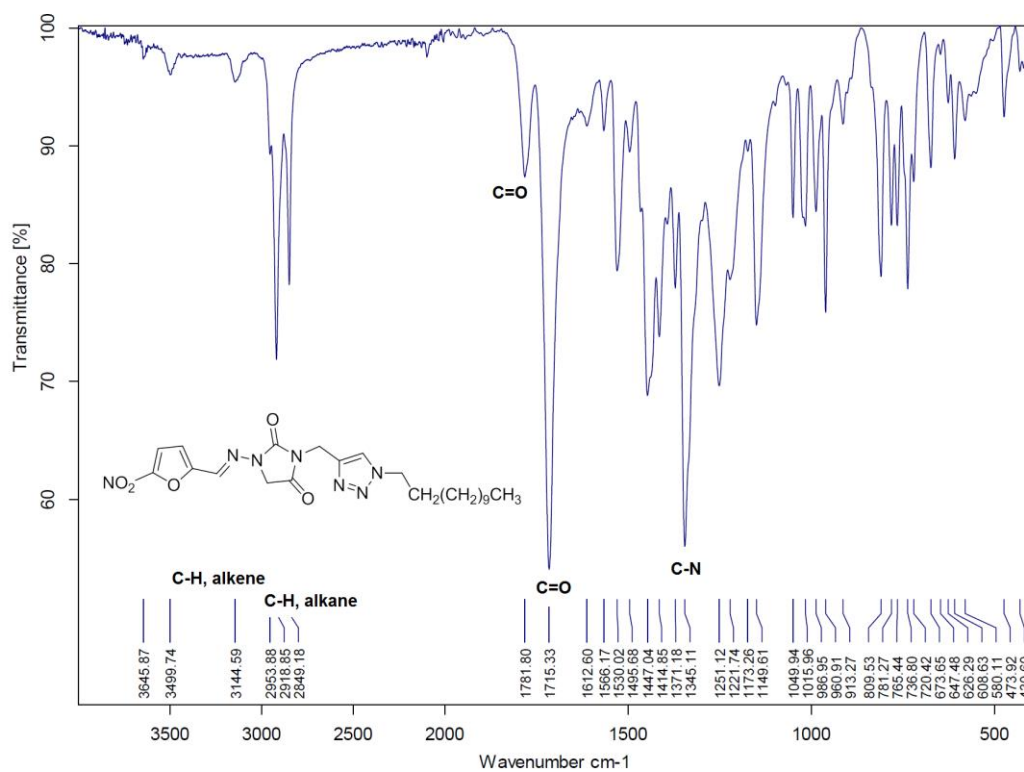
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

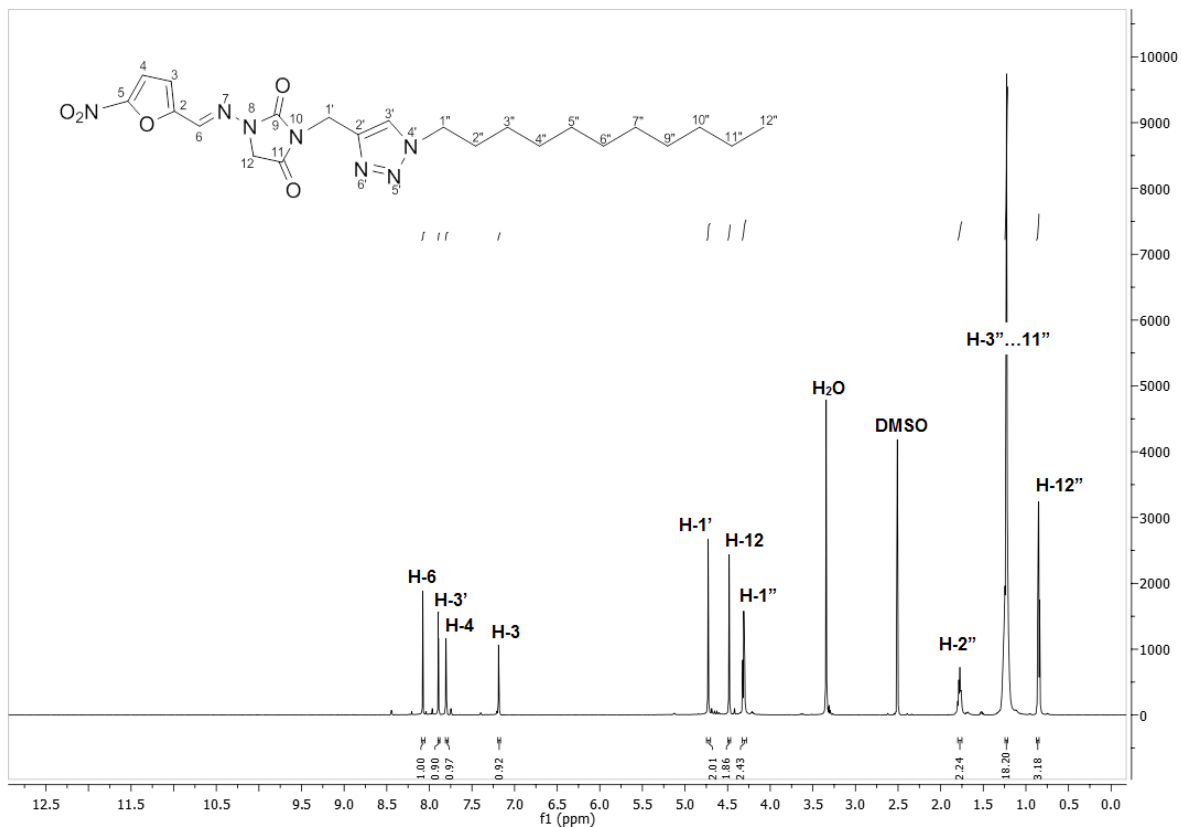


HYBRID 209

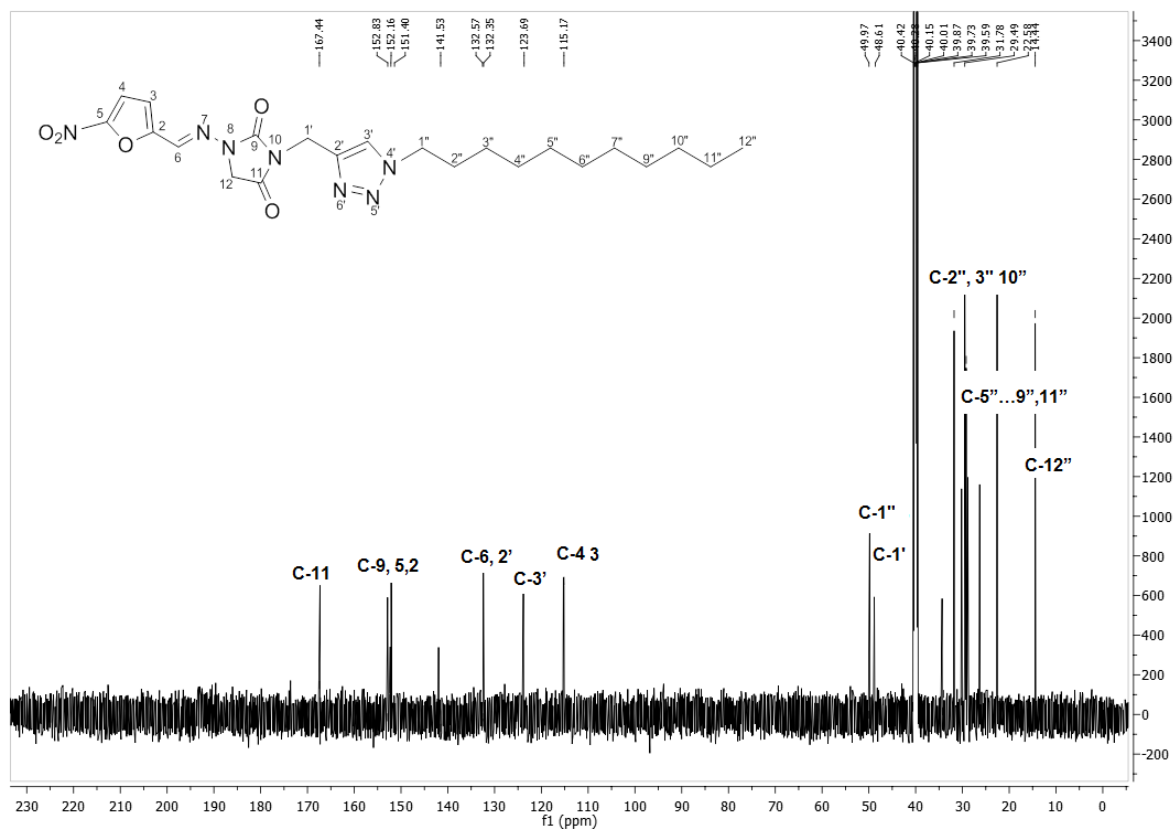
IR



¹H NMR in DMSO



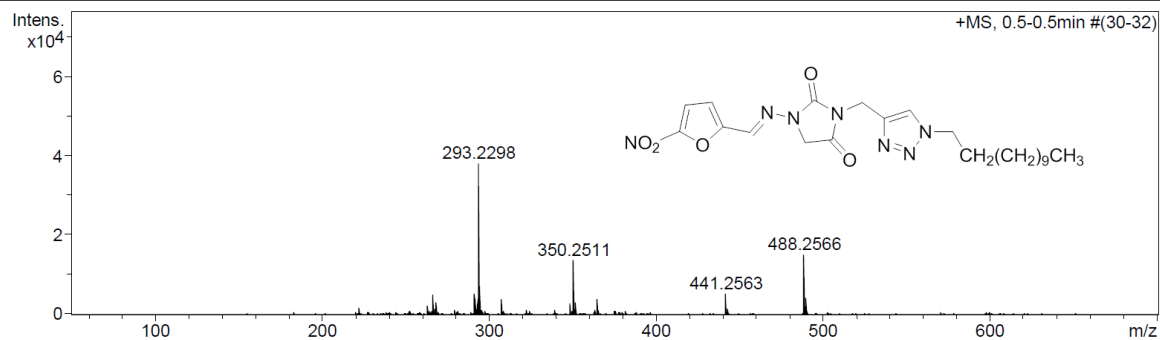
¹³C NMR in DMSO



HR-MS

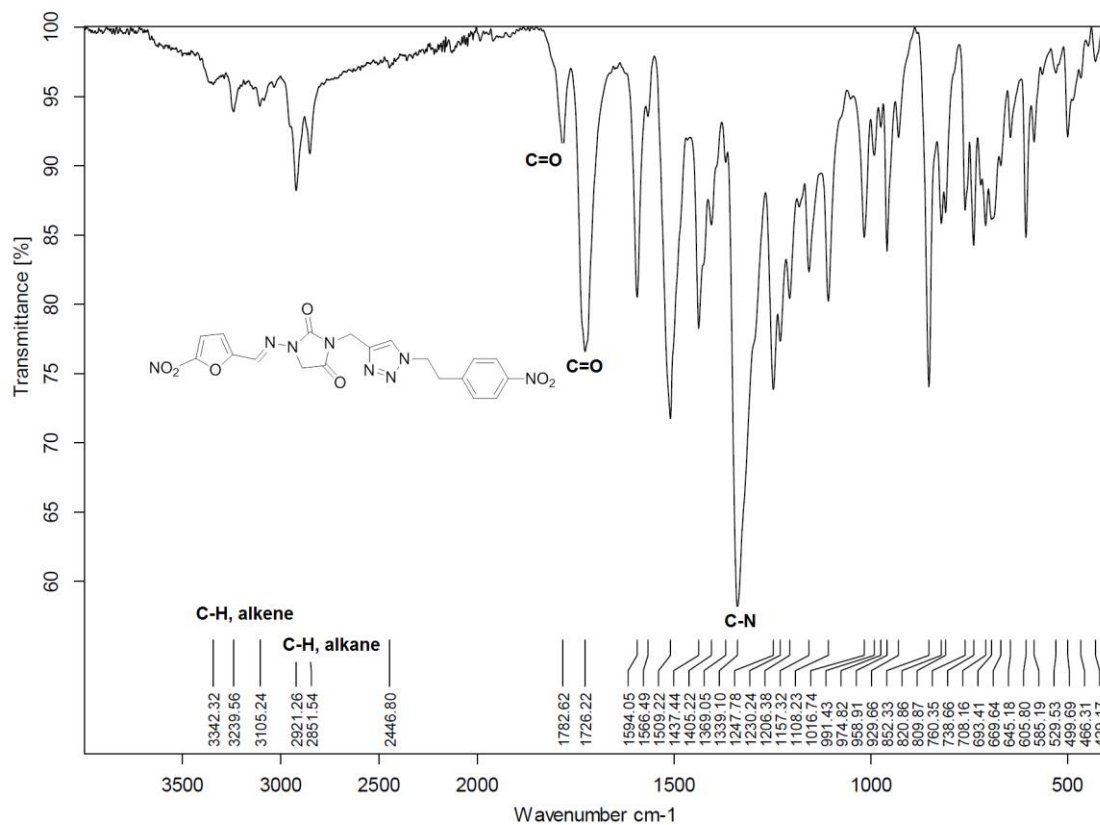
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

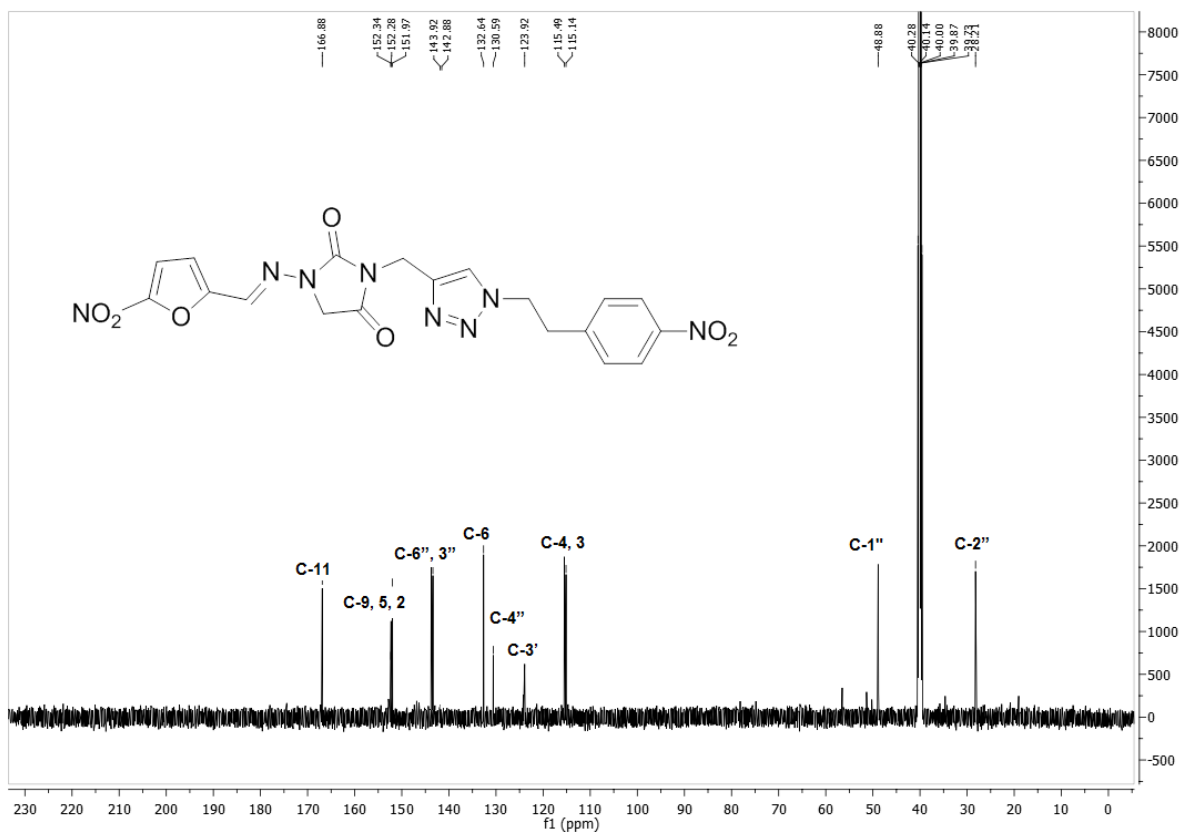


HYBRID 210

IR



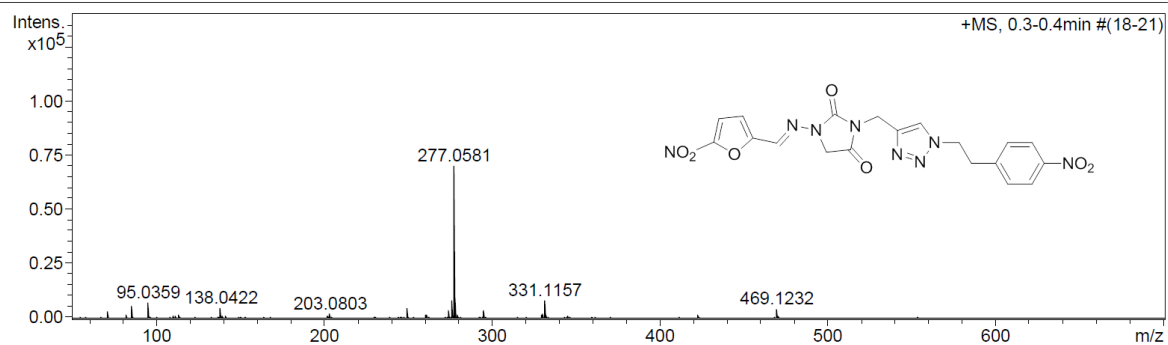
¹³C NMR in DMSO



HR-MS

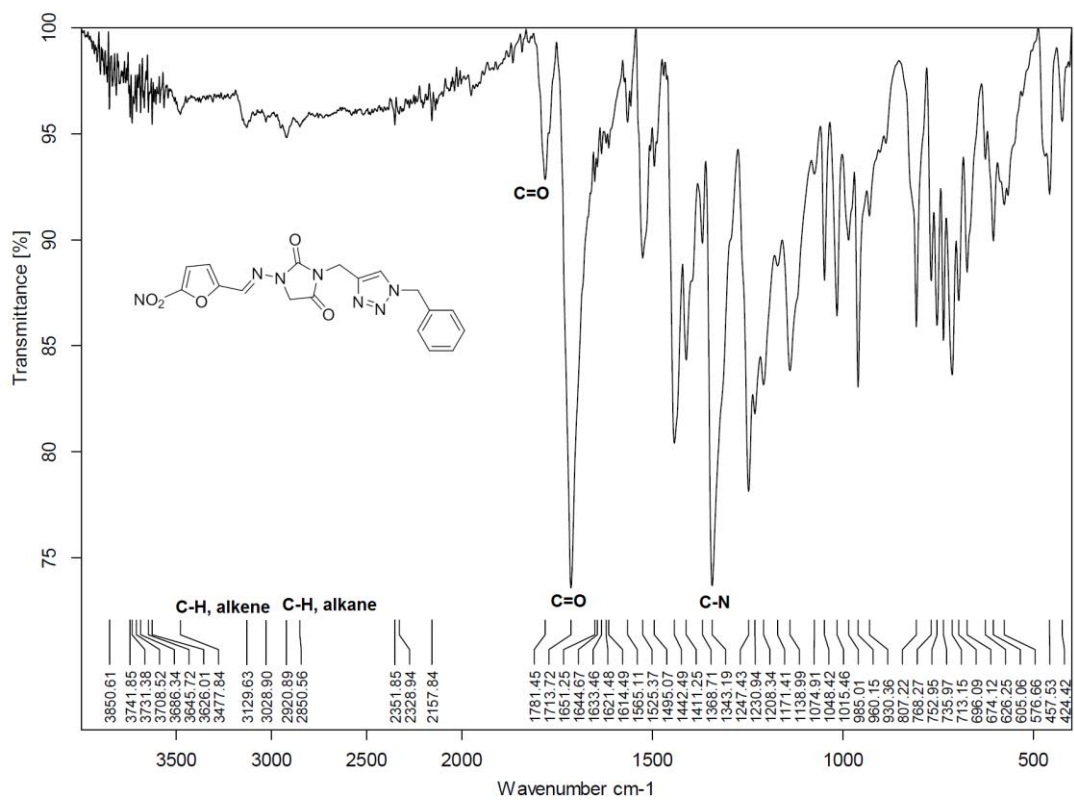
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
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Scan End	1600 m/z	Set Collision Cell RF	80.0 Vpp	Set Divert Valve	Waste

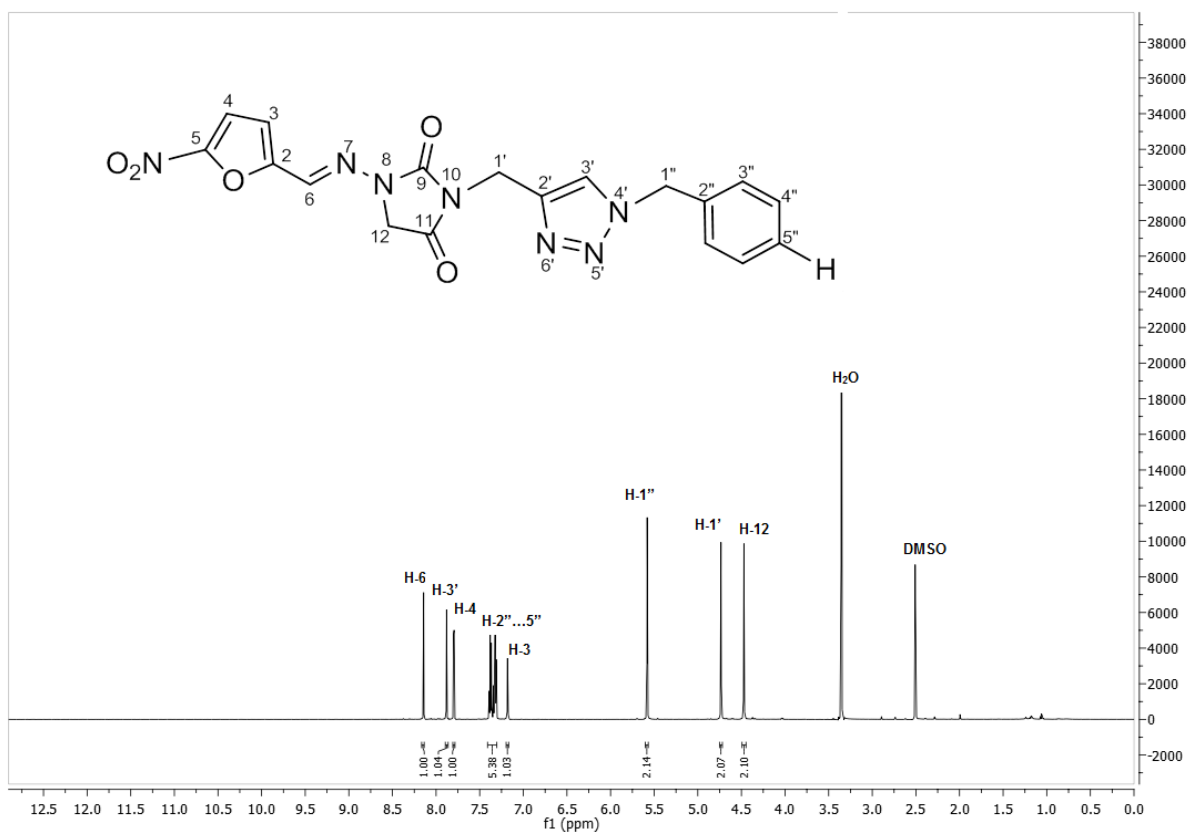


HYBRID 211

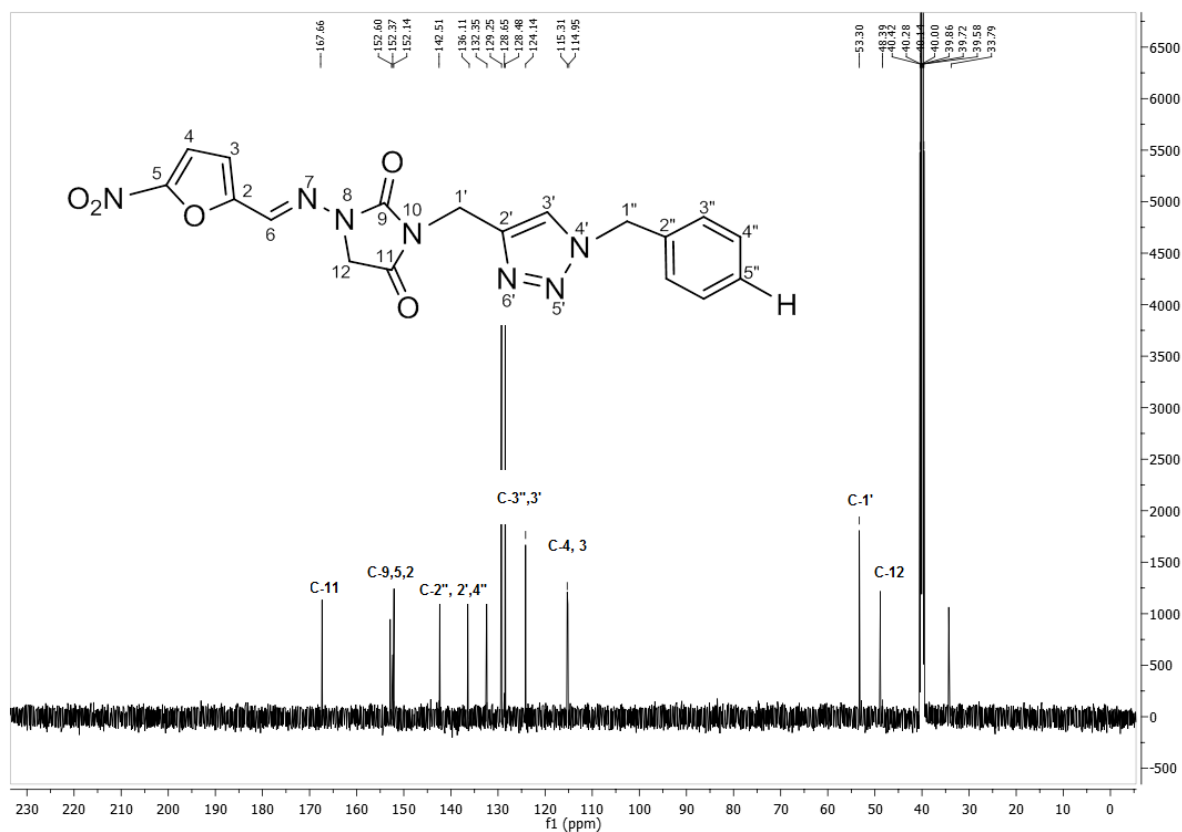
IR



¹H NMR in DMSO



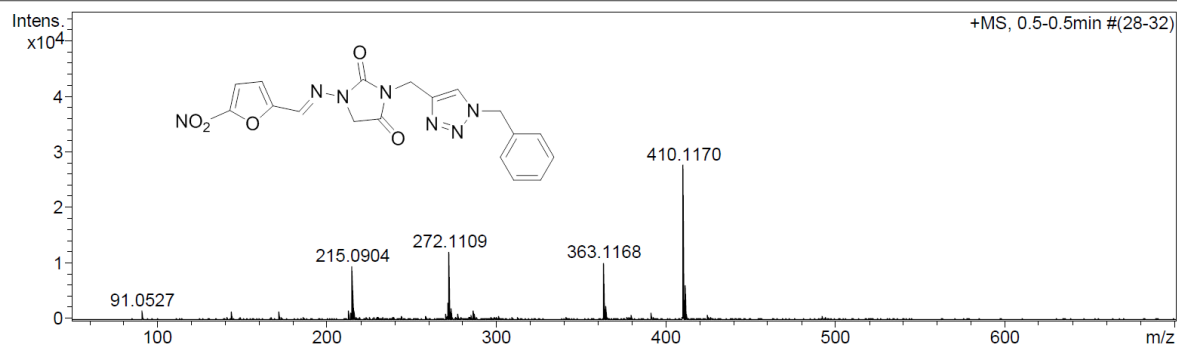
¹³C NMR in DMSO



HR-MS

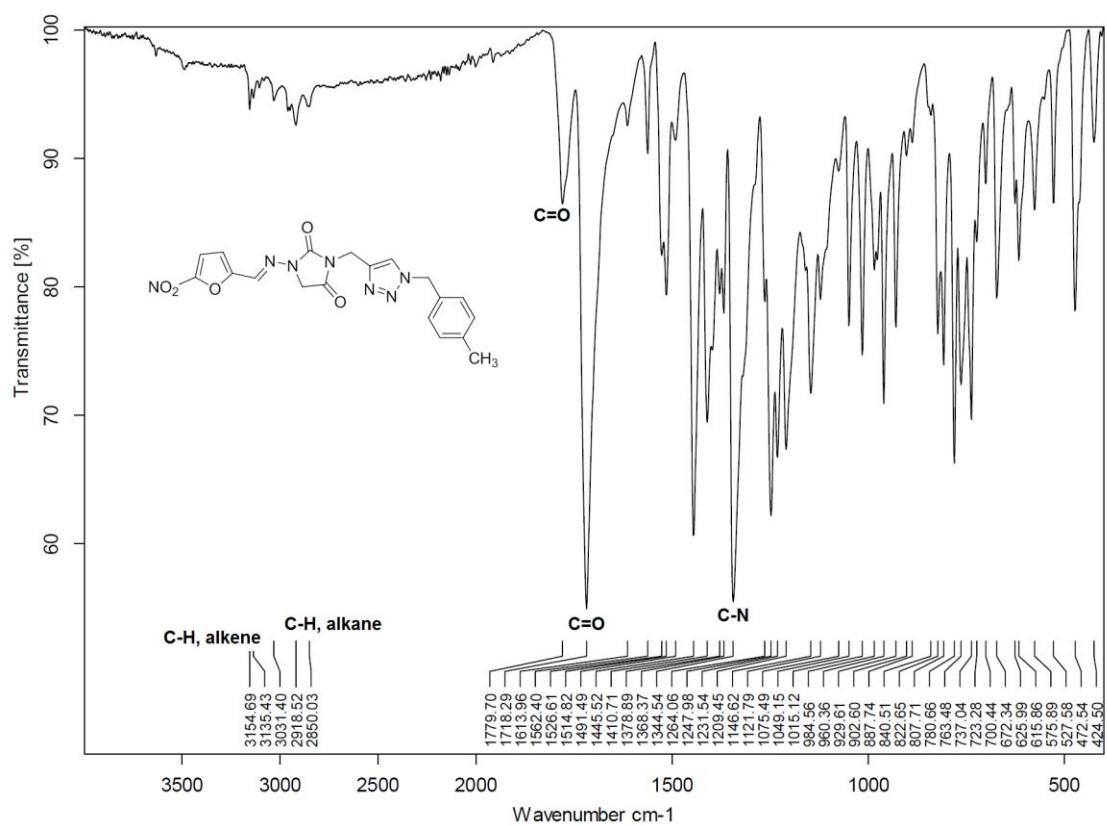
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

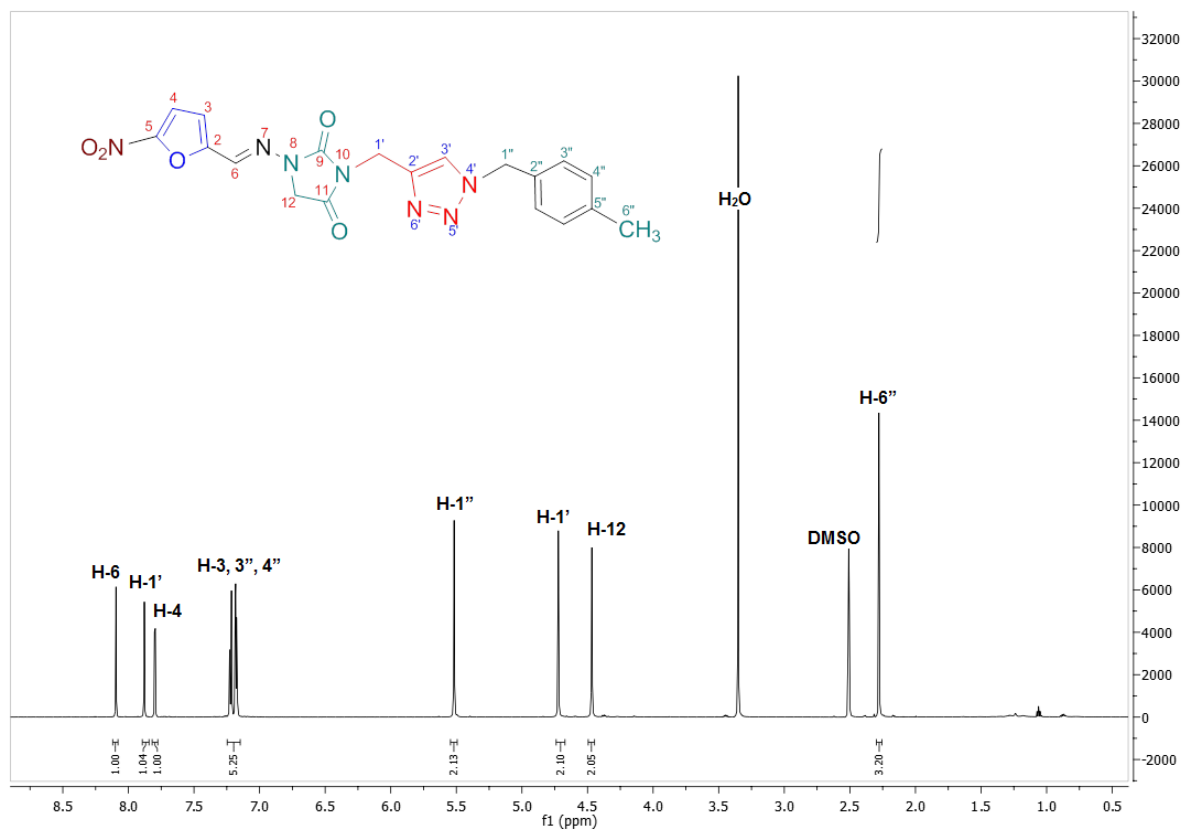


HYBRID 212

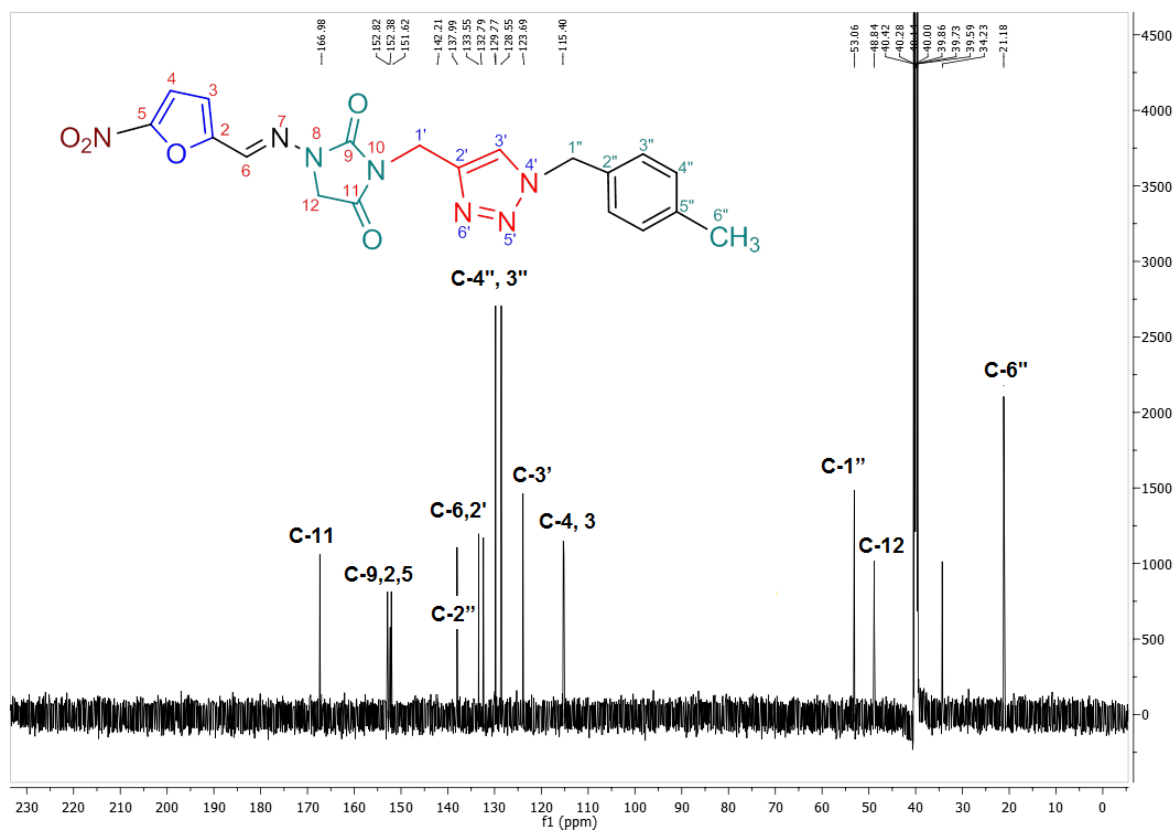
IR



¹H NMR in DMSO



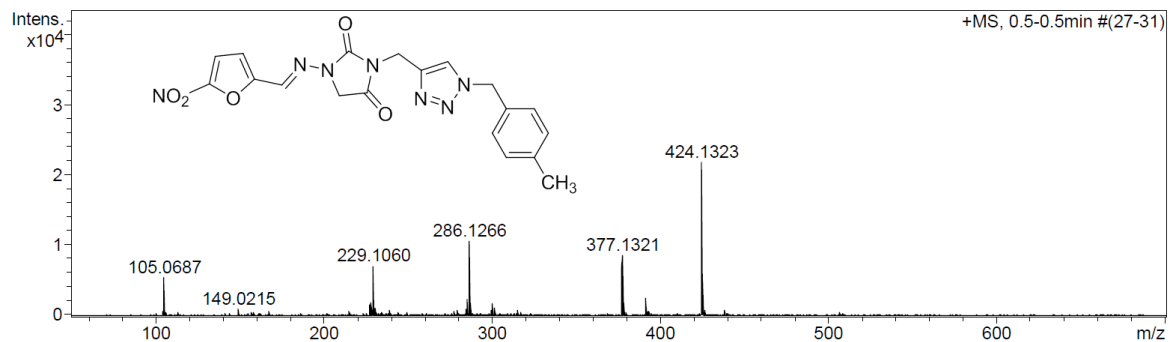
¹³C NMR in DMSO



HR-MS

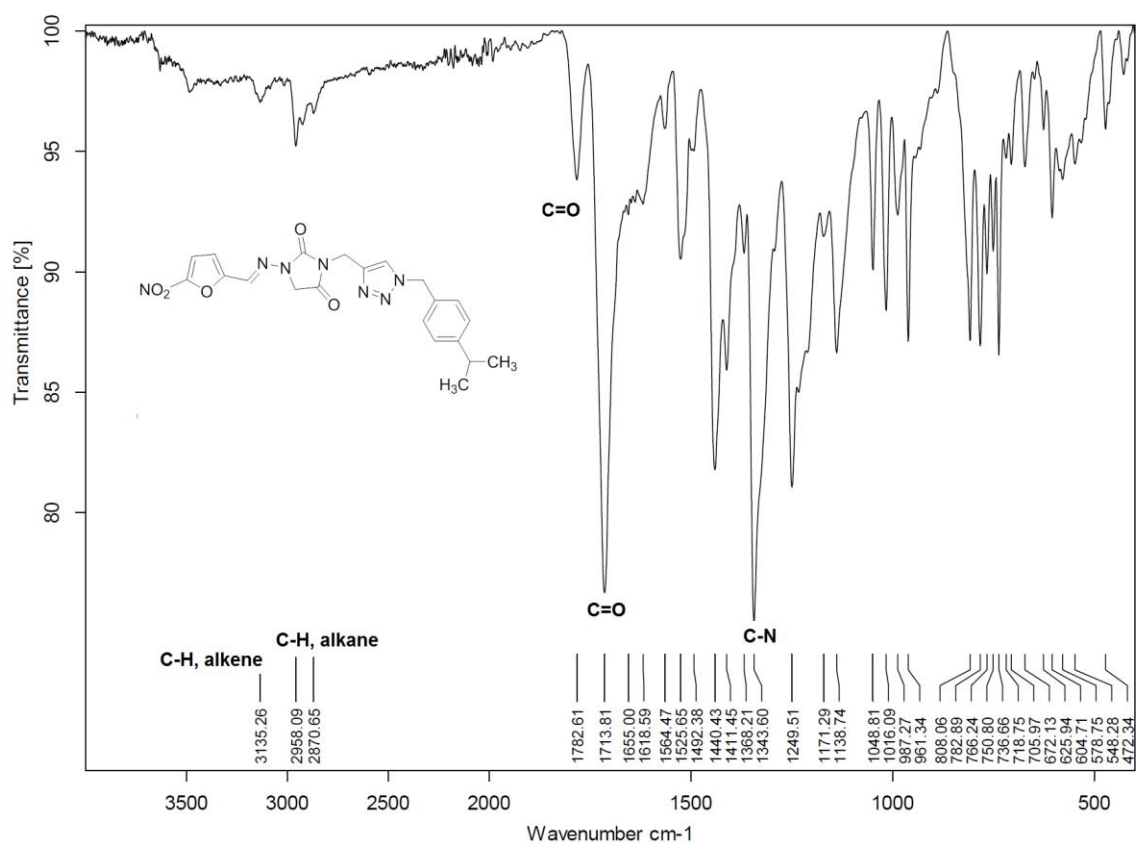
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

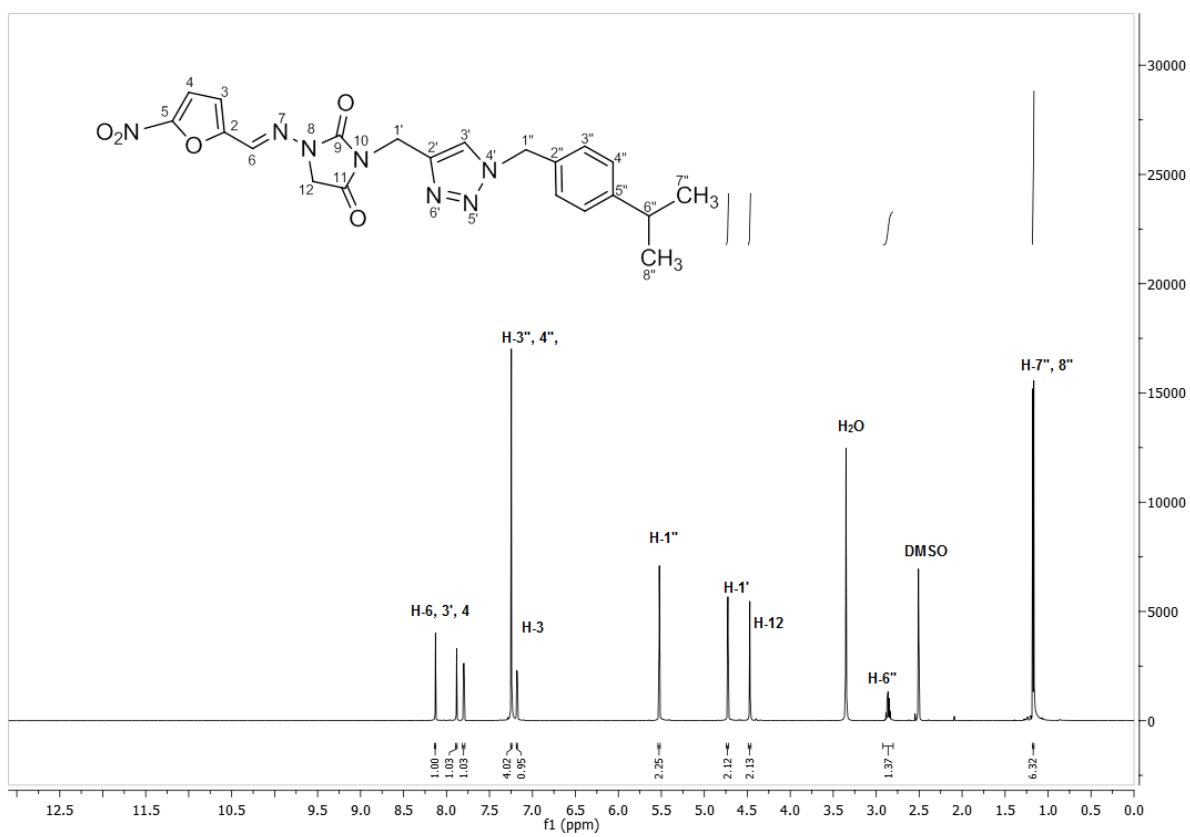


HYBRID 213

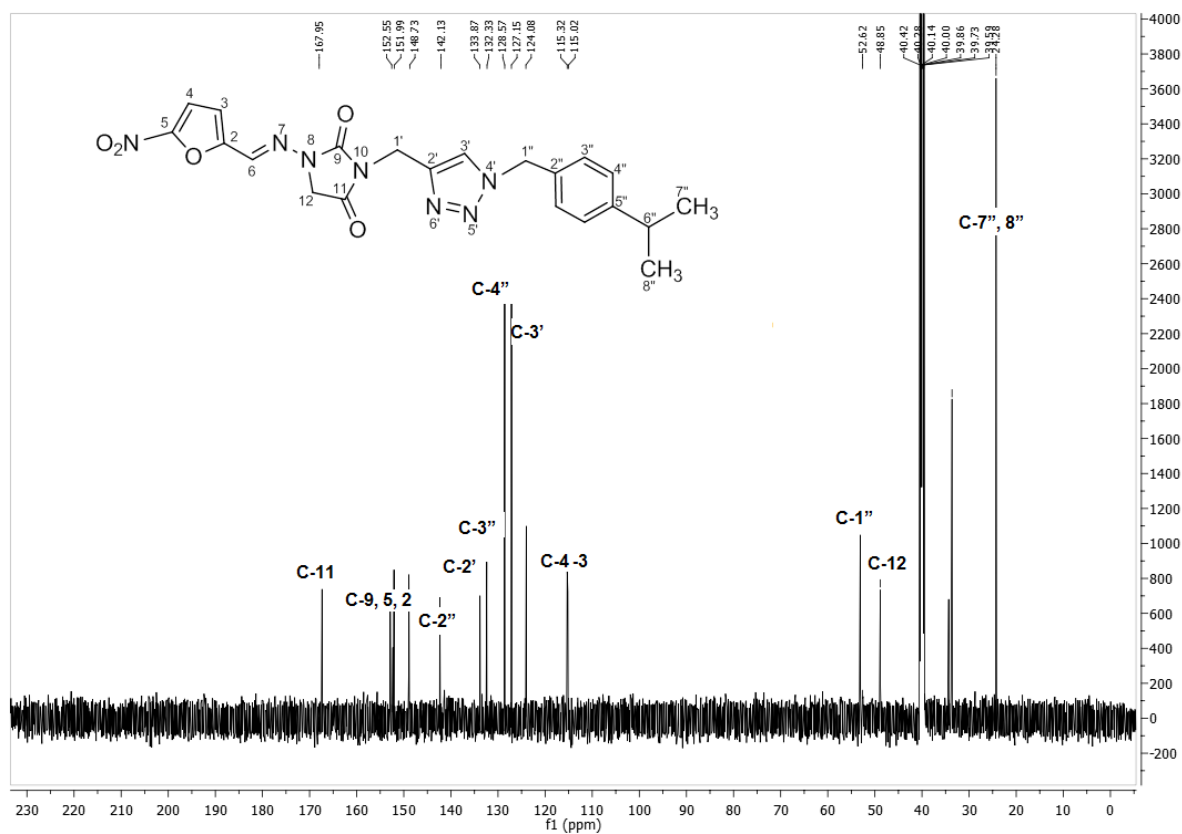
IR



¹H NMR in DMSO



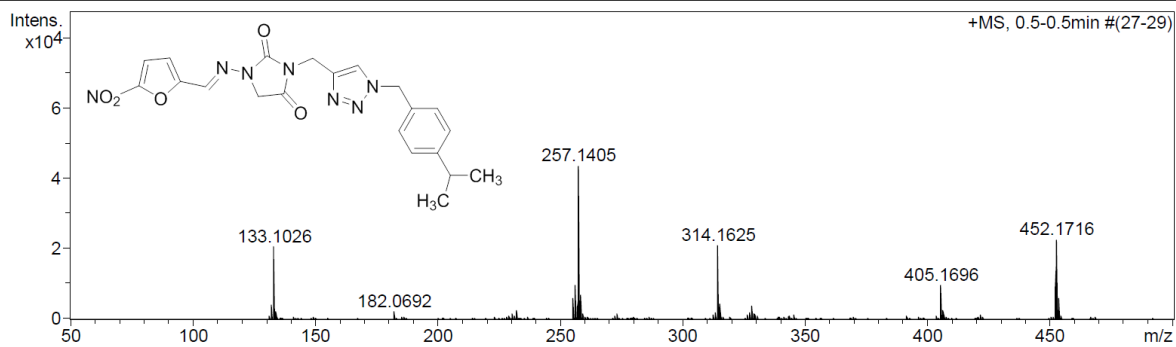
¹³C NMR in DMSO



HR-MS

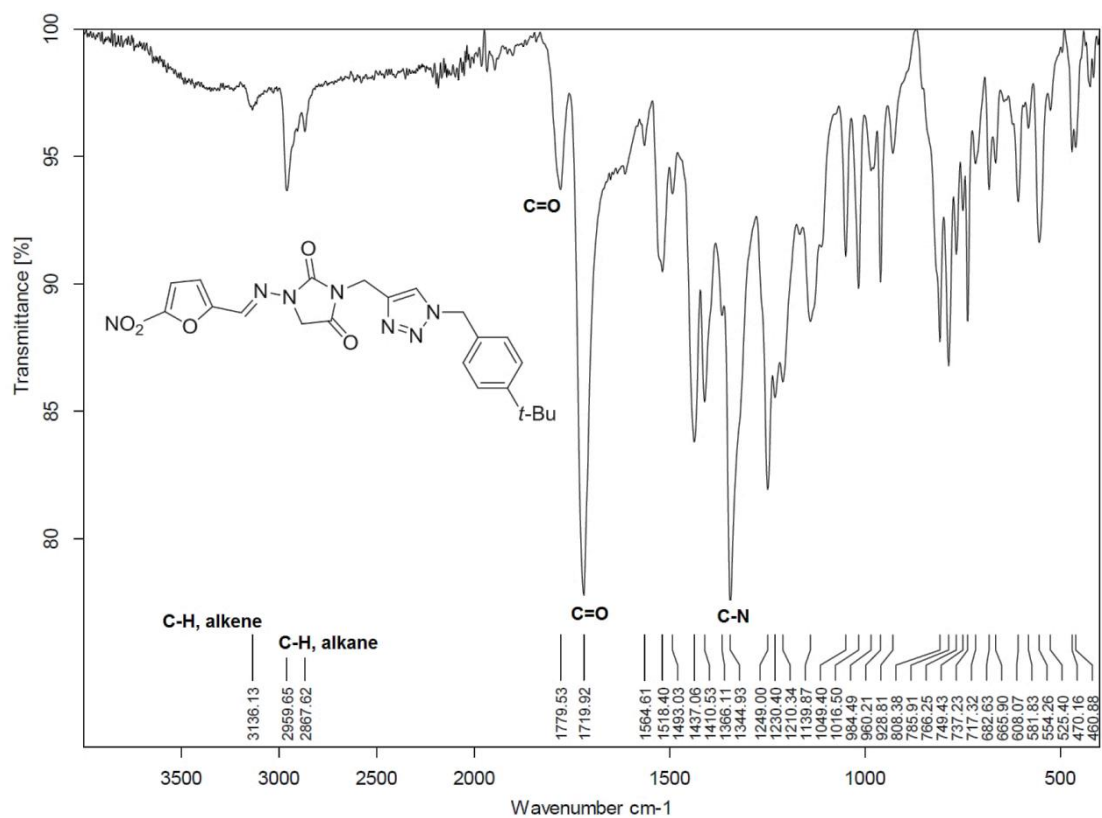
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

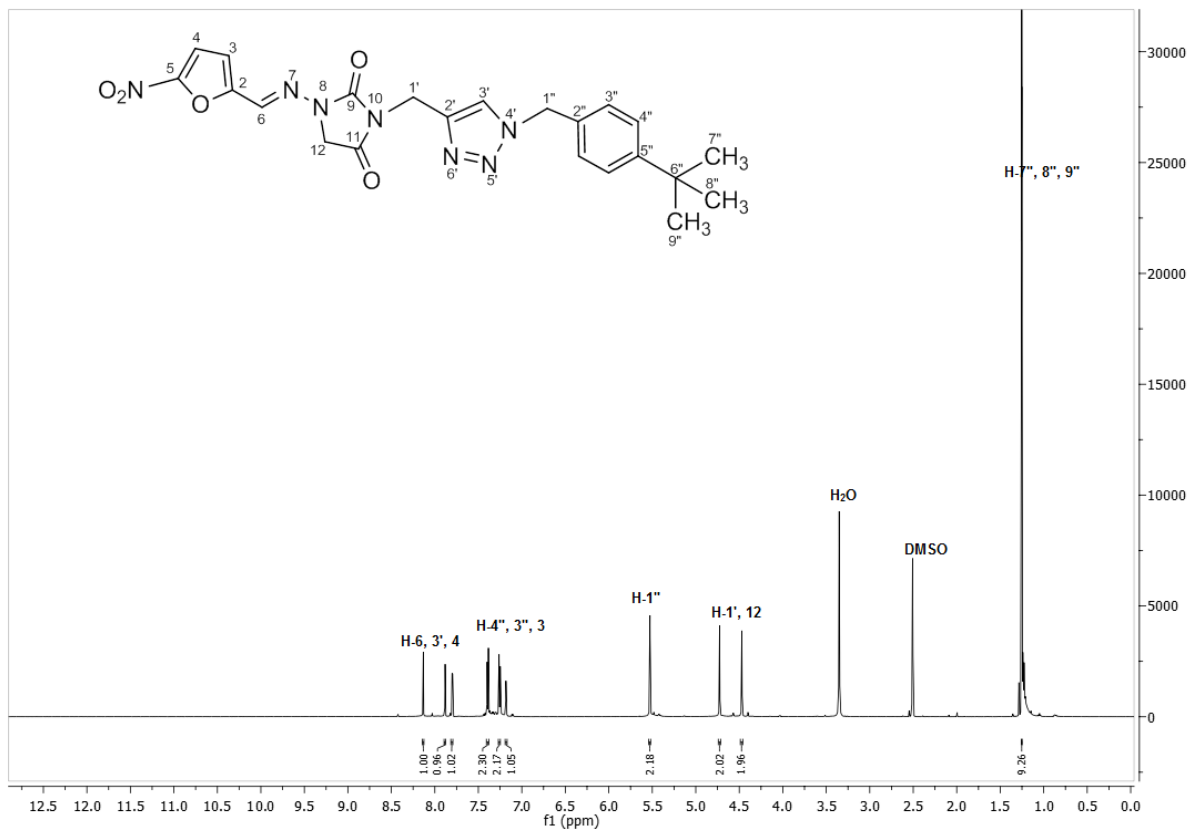


HYBRID 214

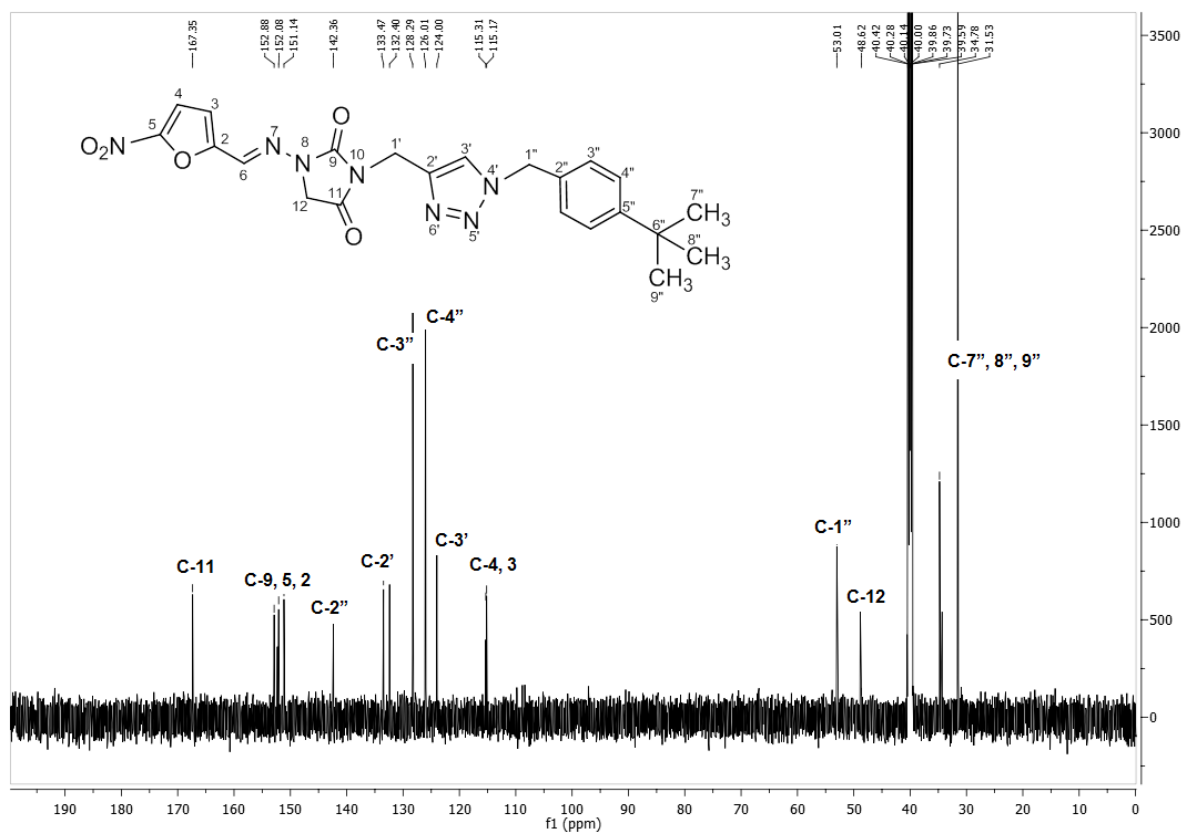
IR



¹H NMR in DMSO



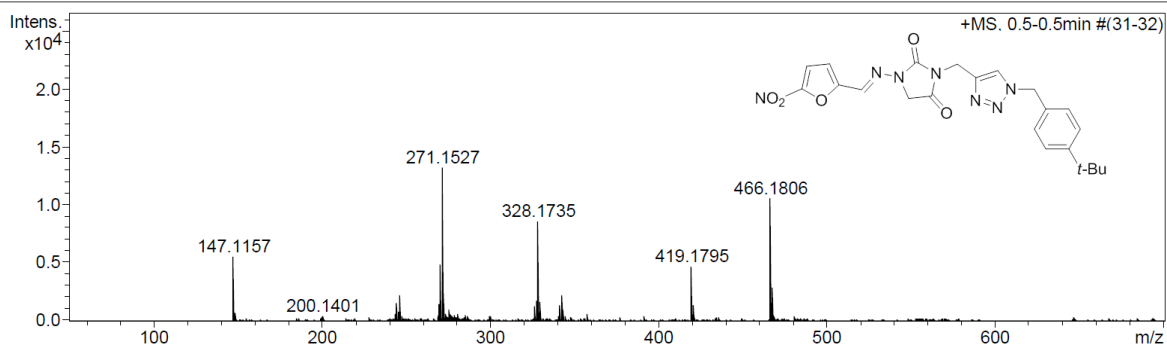
¹³C NMR in DMSO



HR-MS

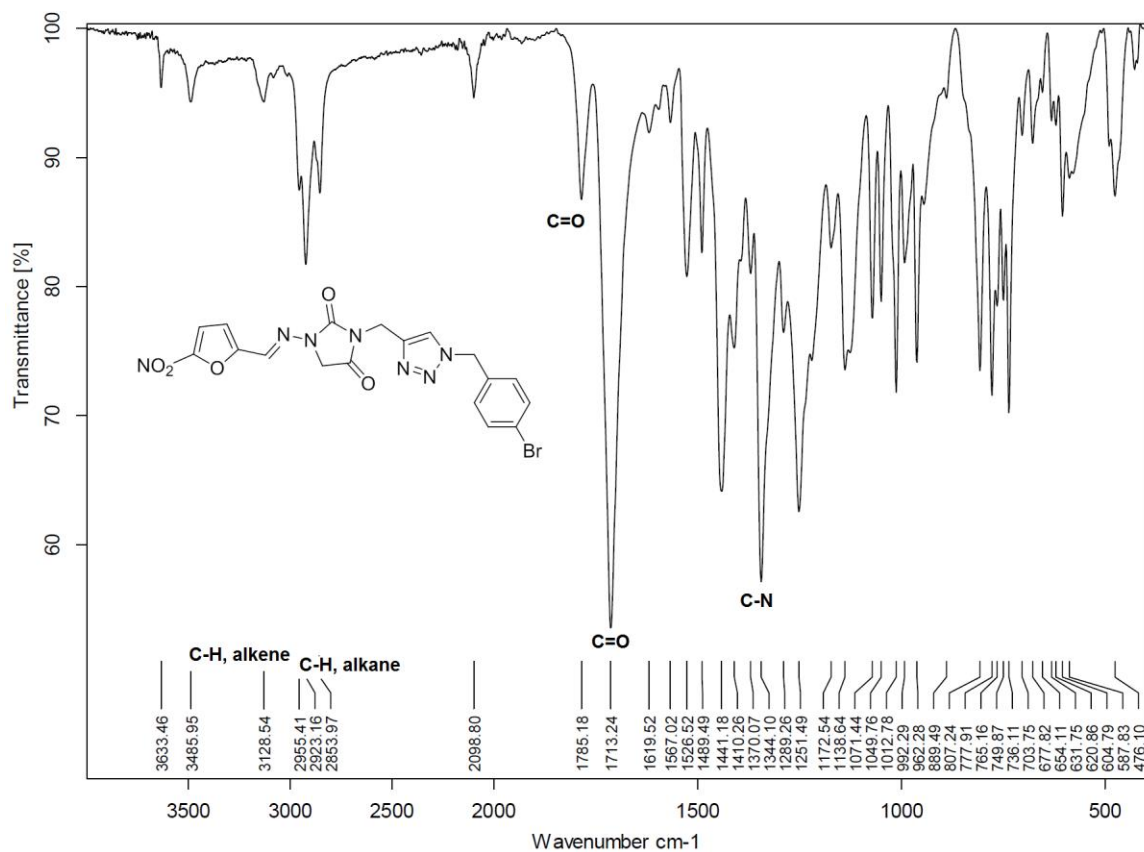
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

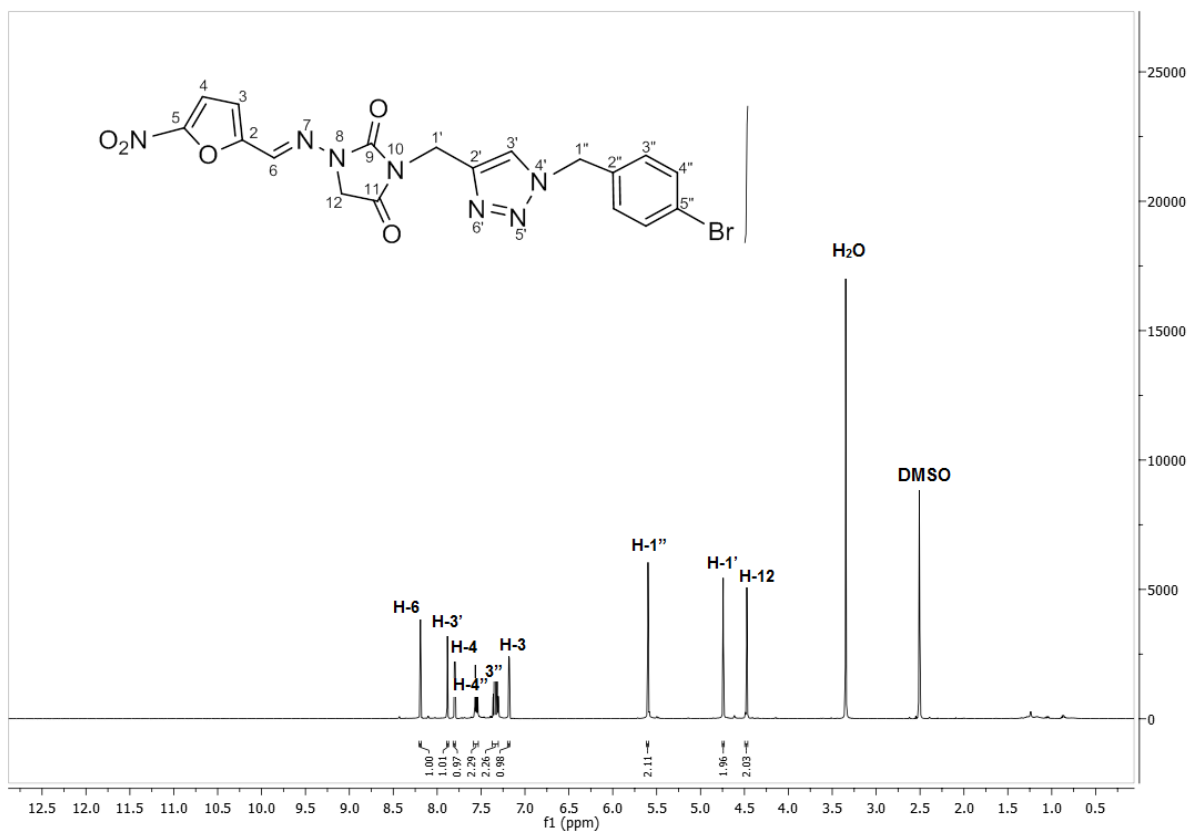


HYBRID 215

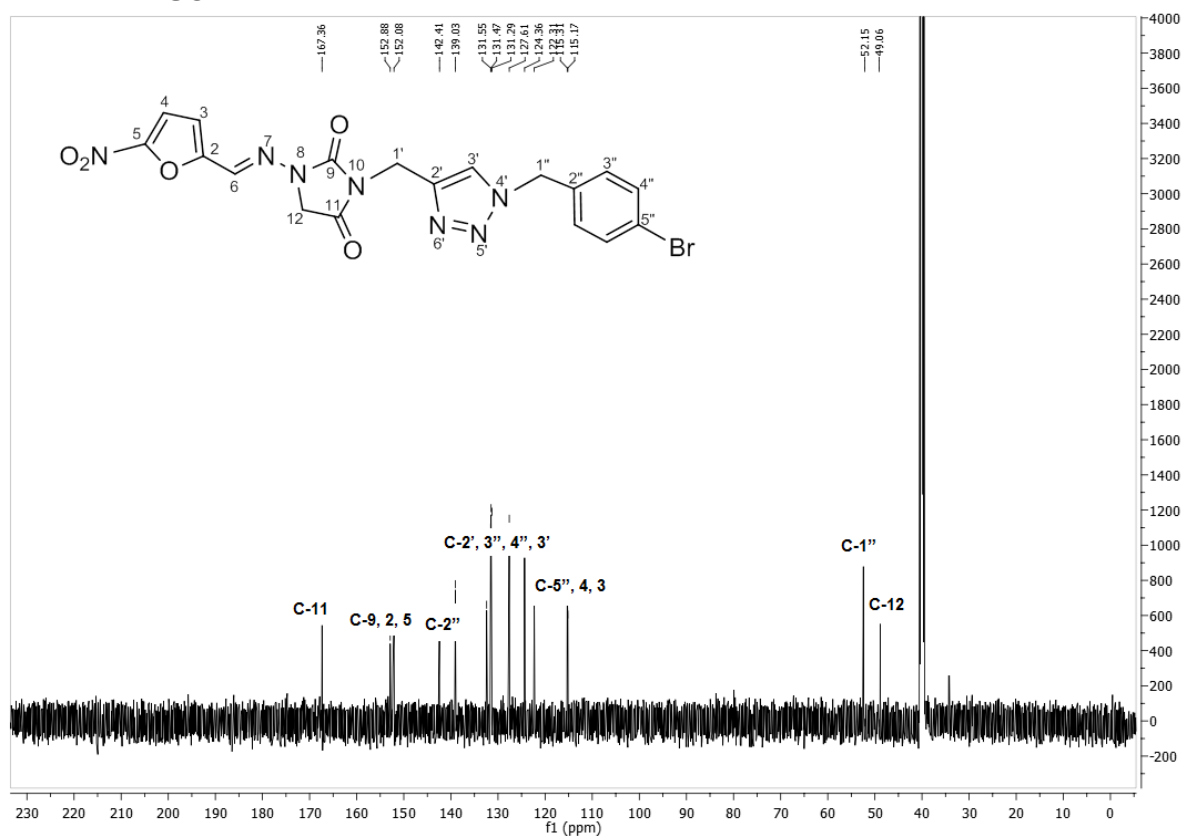
IR



¹H NMR in DMSO



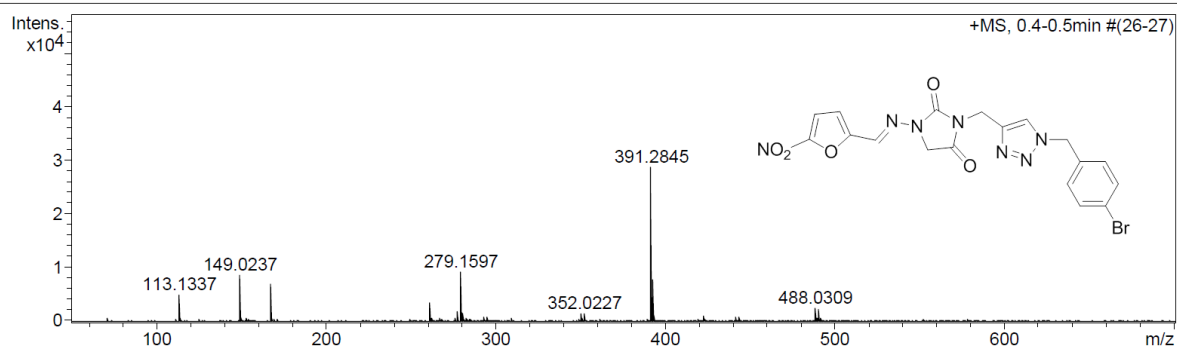
¹³C NMR in DMSO



HR-MS

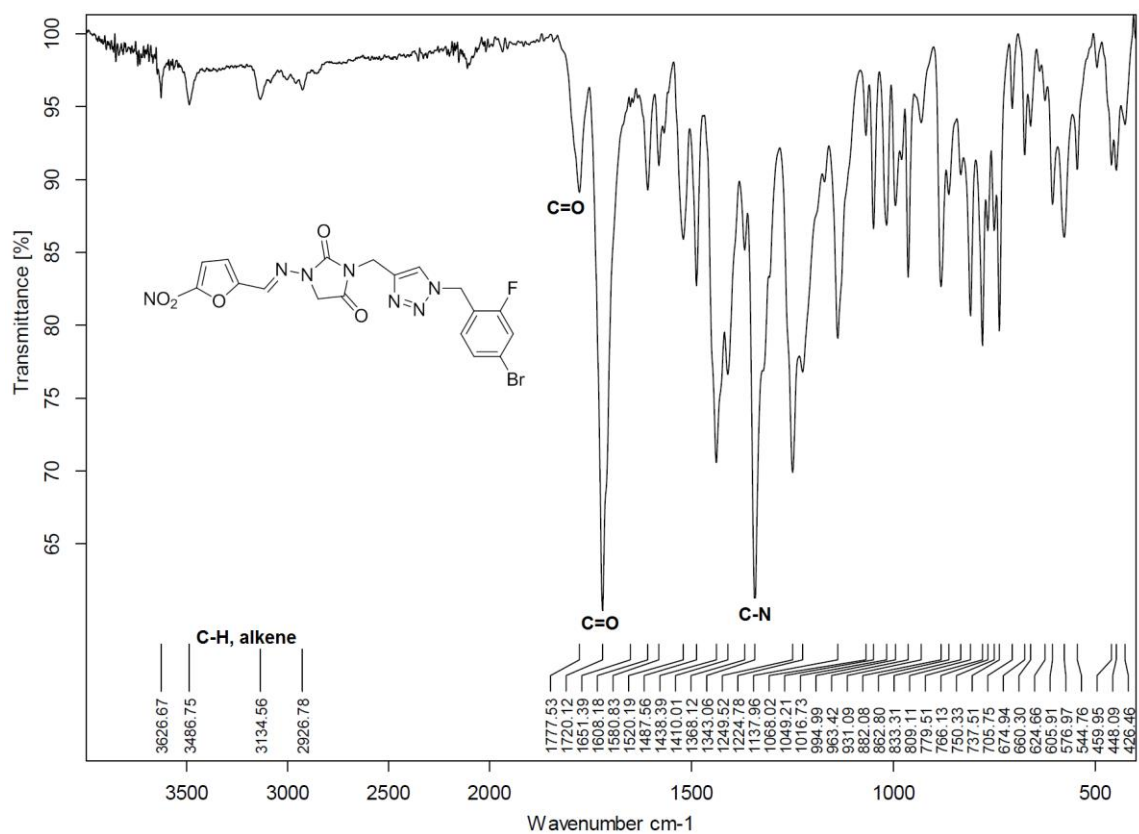
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

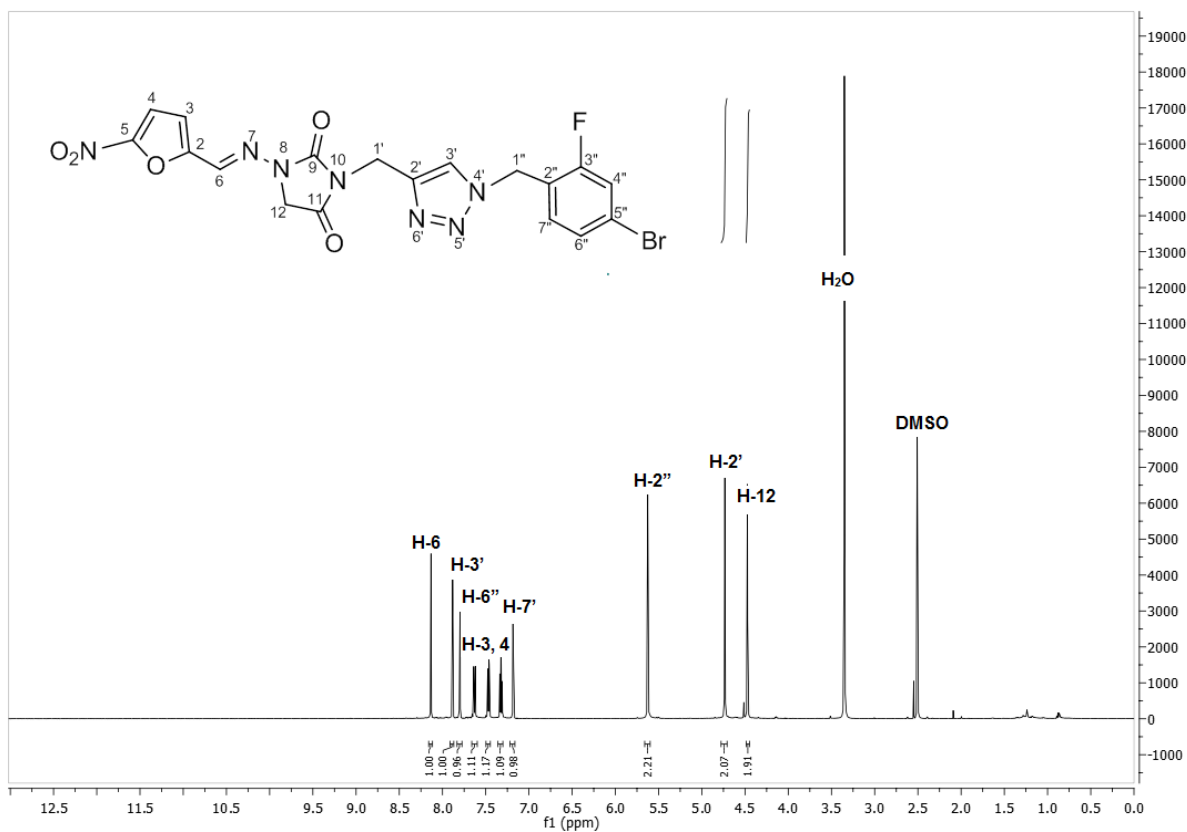


HYBRID 216

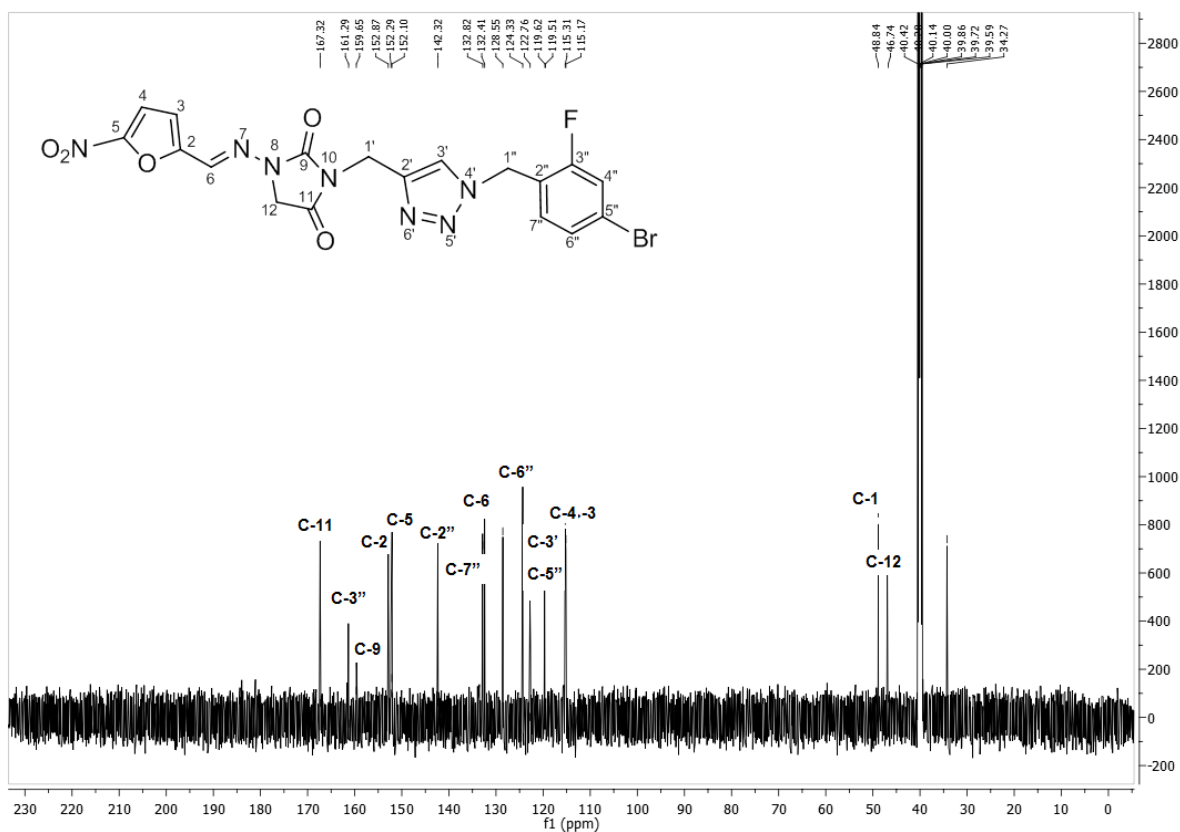
IR



¹H NMR in DMSO



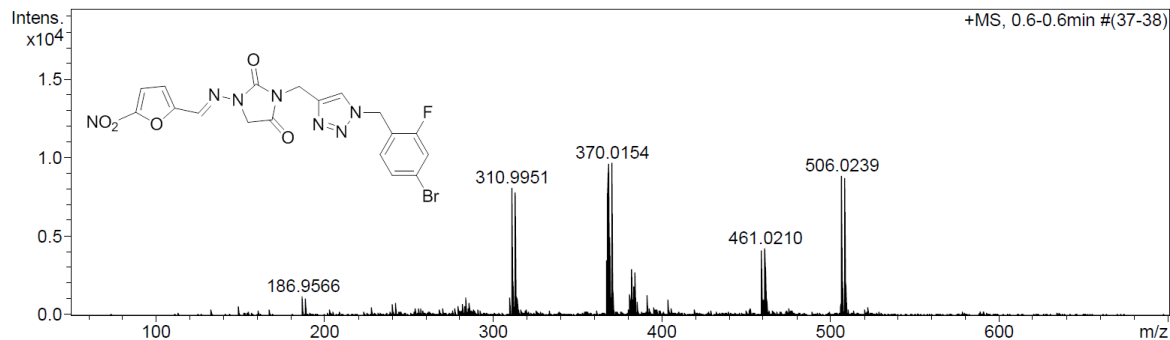
¹³C NMR in DMSO



HR-MS

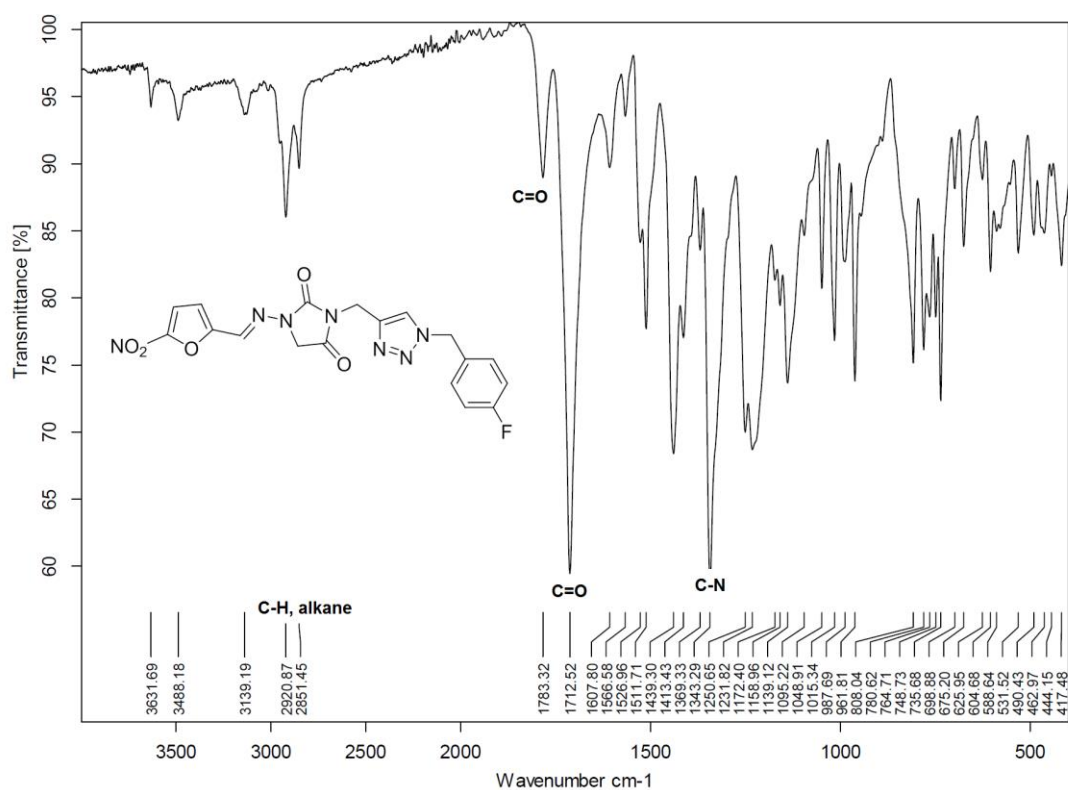
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

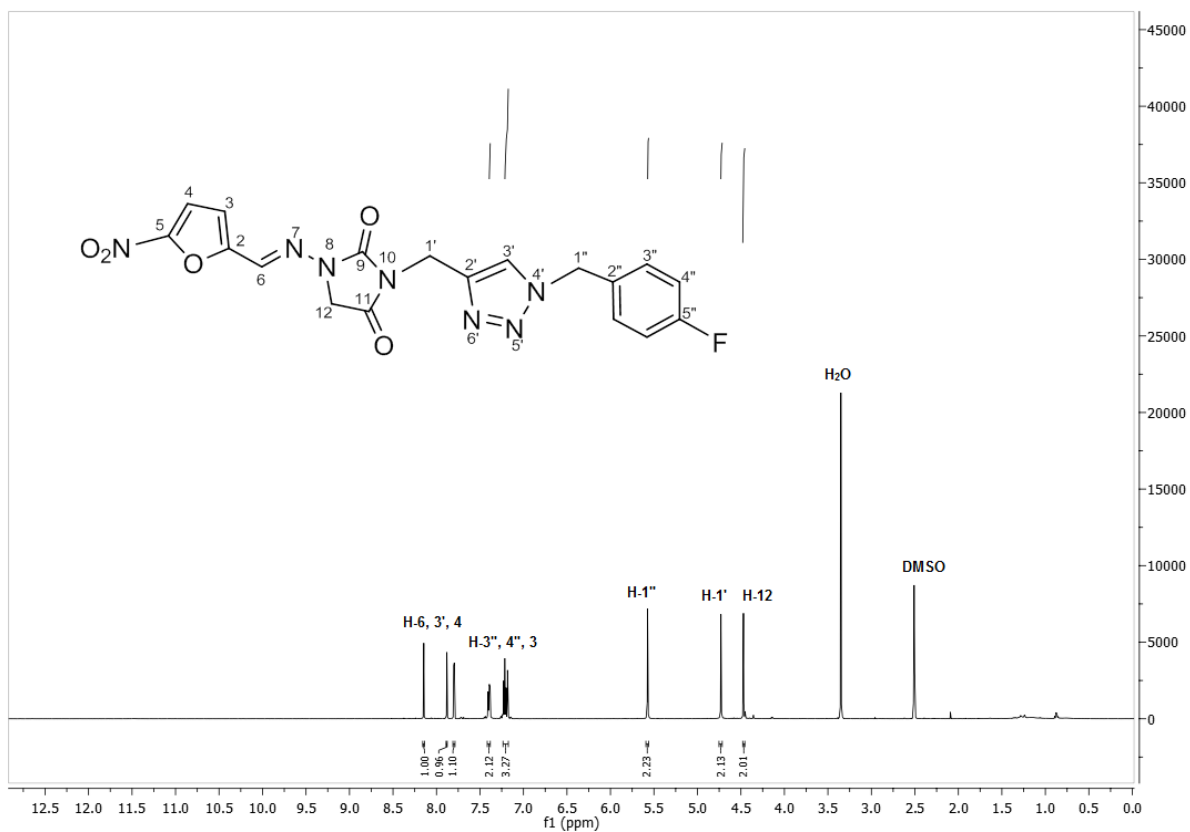


HYBRID 217

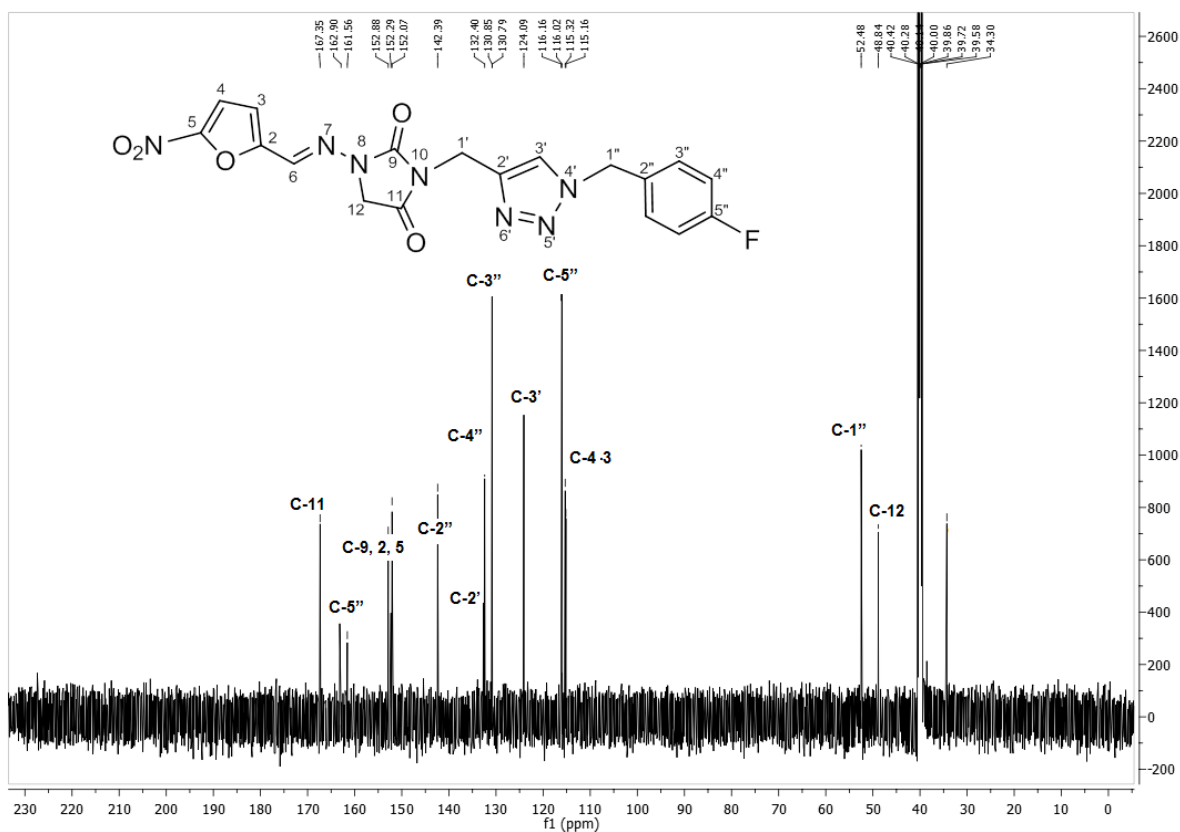
IR



¹H NMR in DMSO



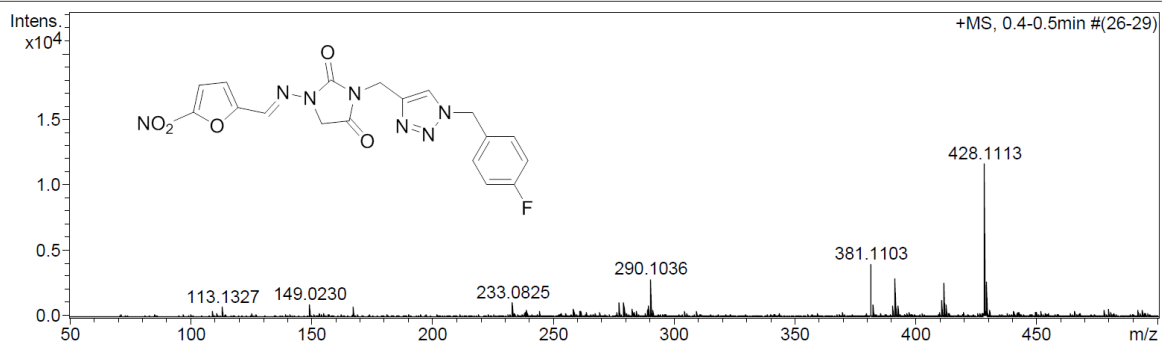
¹³C NMR in DMSO



HR-MS

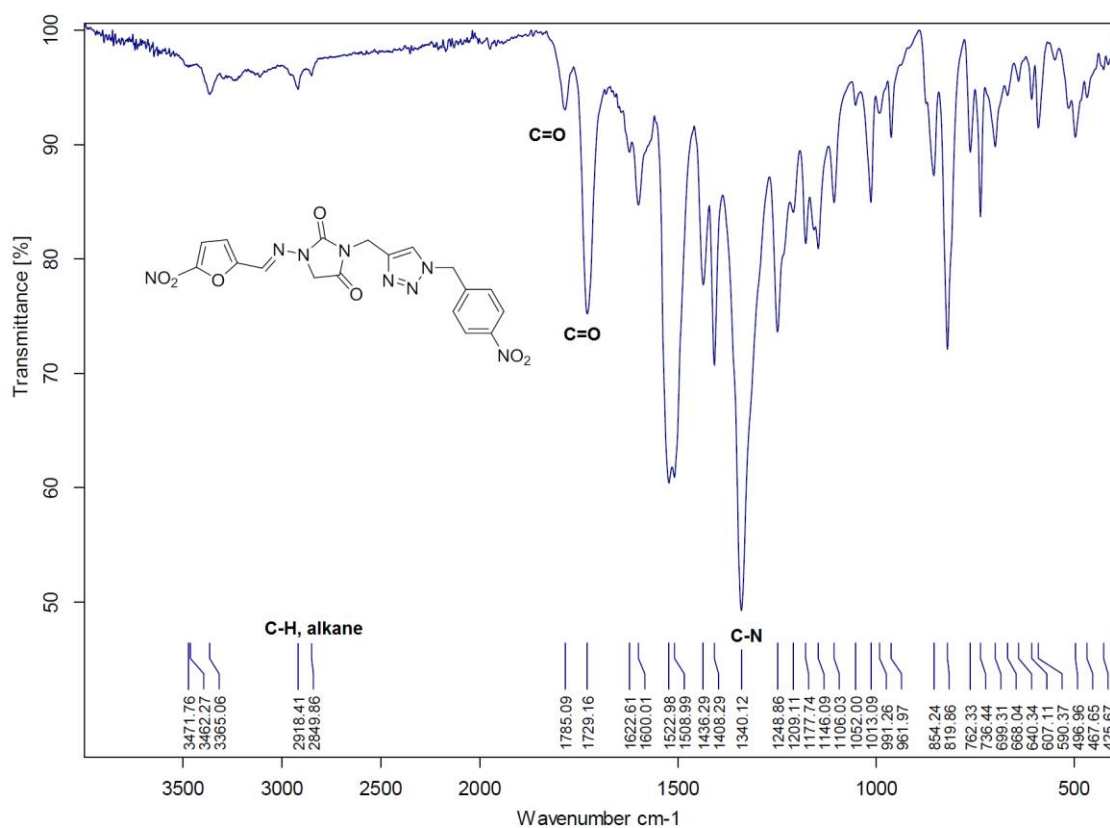
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

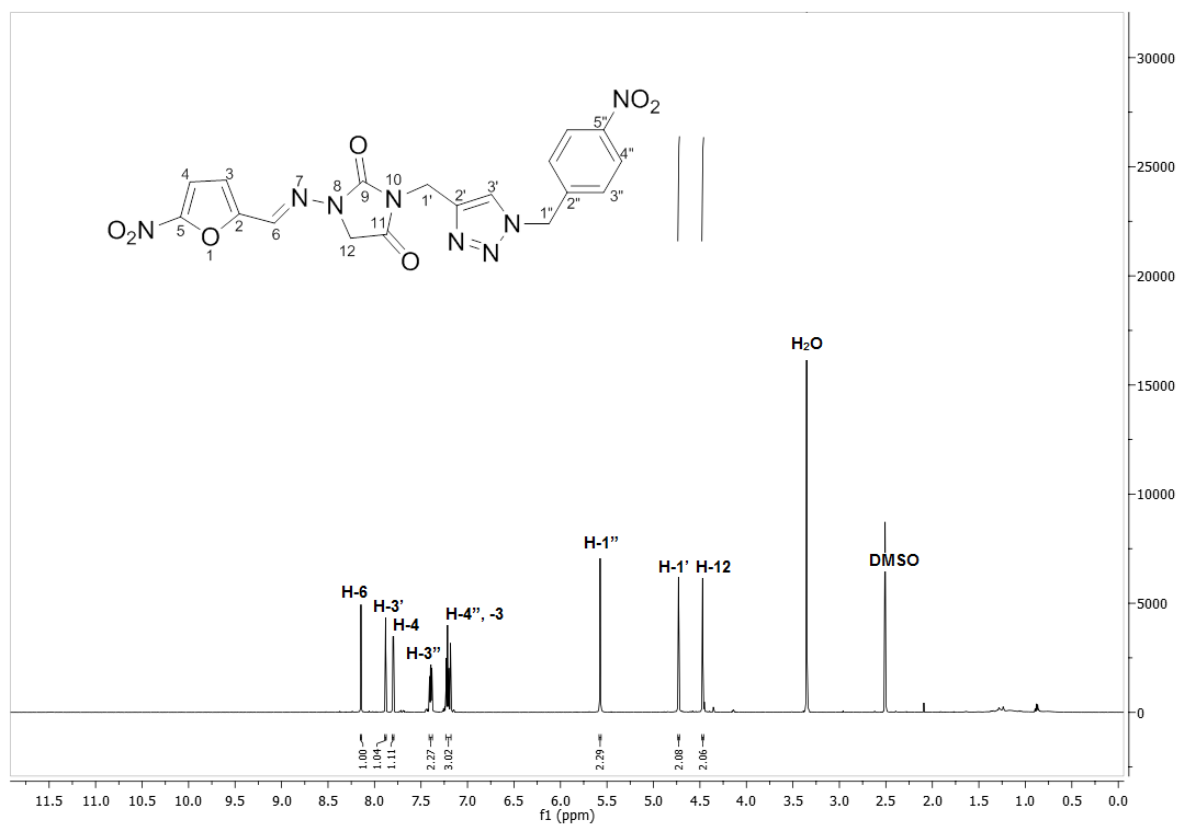


HYBRID 218

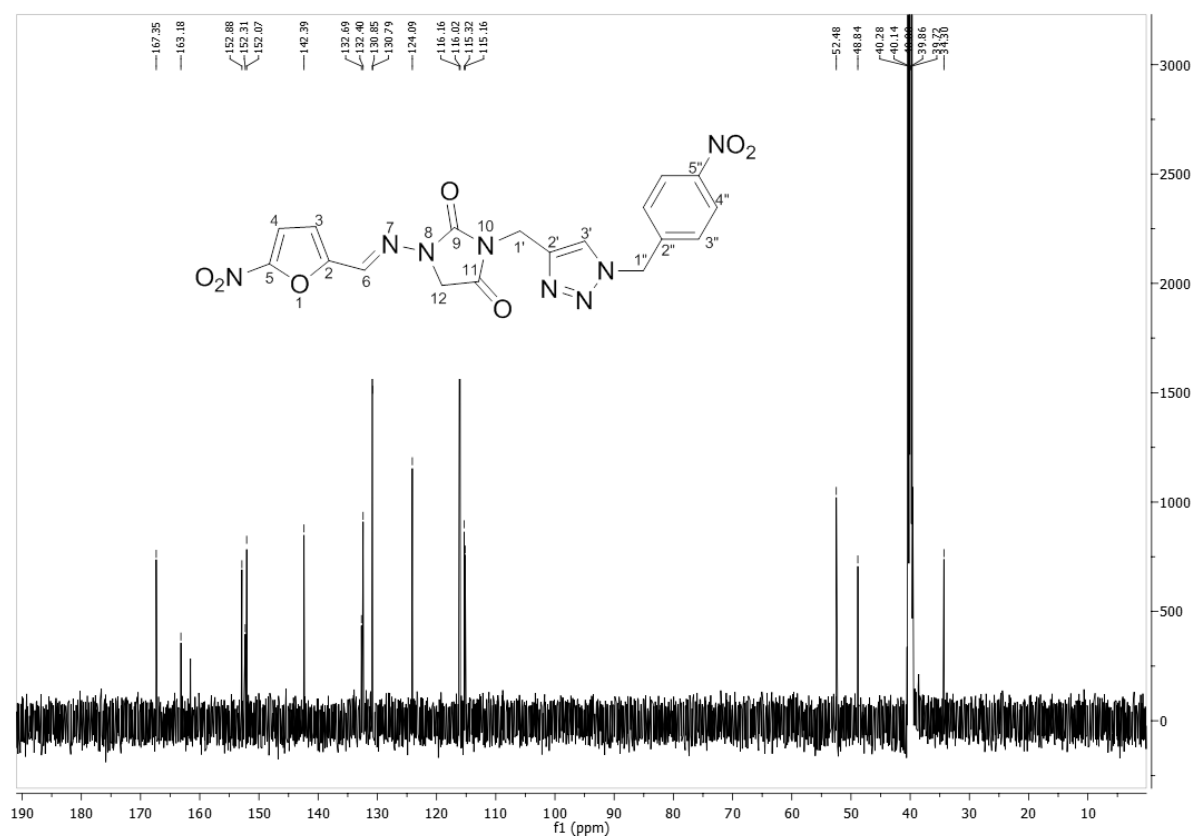
IR



¹H NMR in DMSO



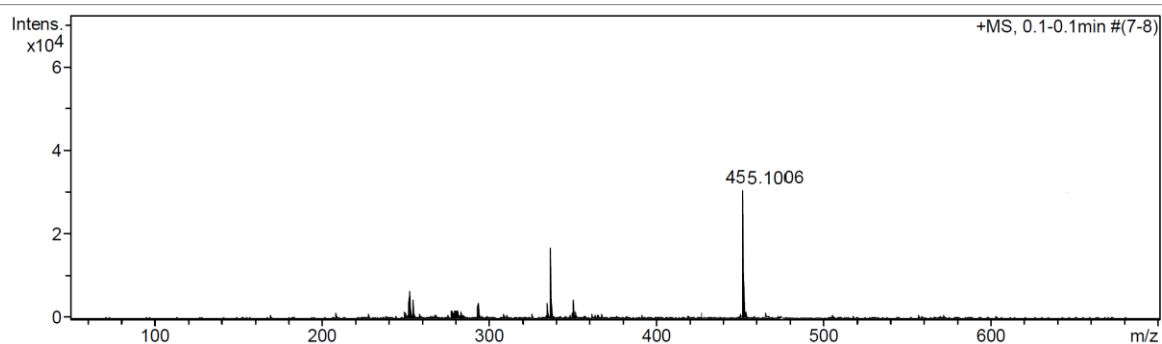
¹³C NMR in DMSO



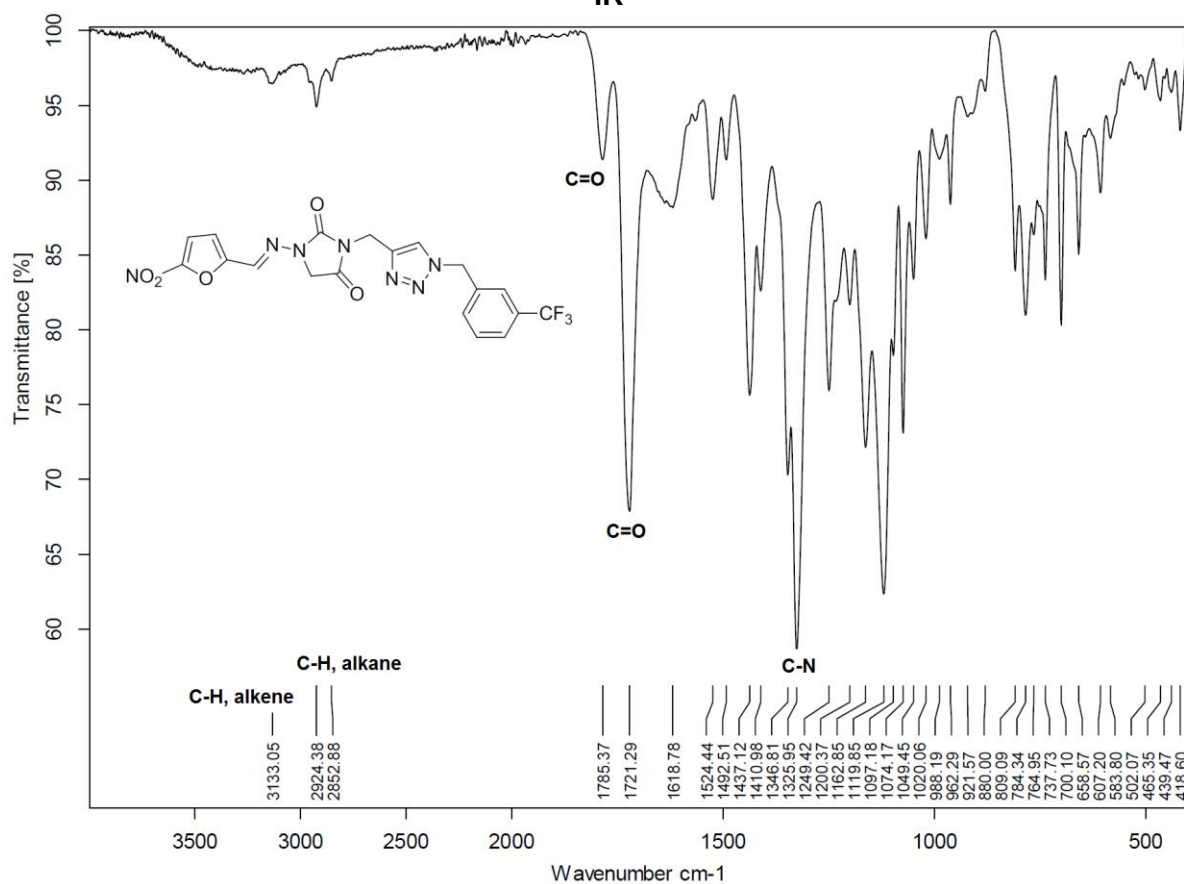
HR-MS

Acquisition Parameter

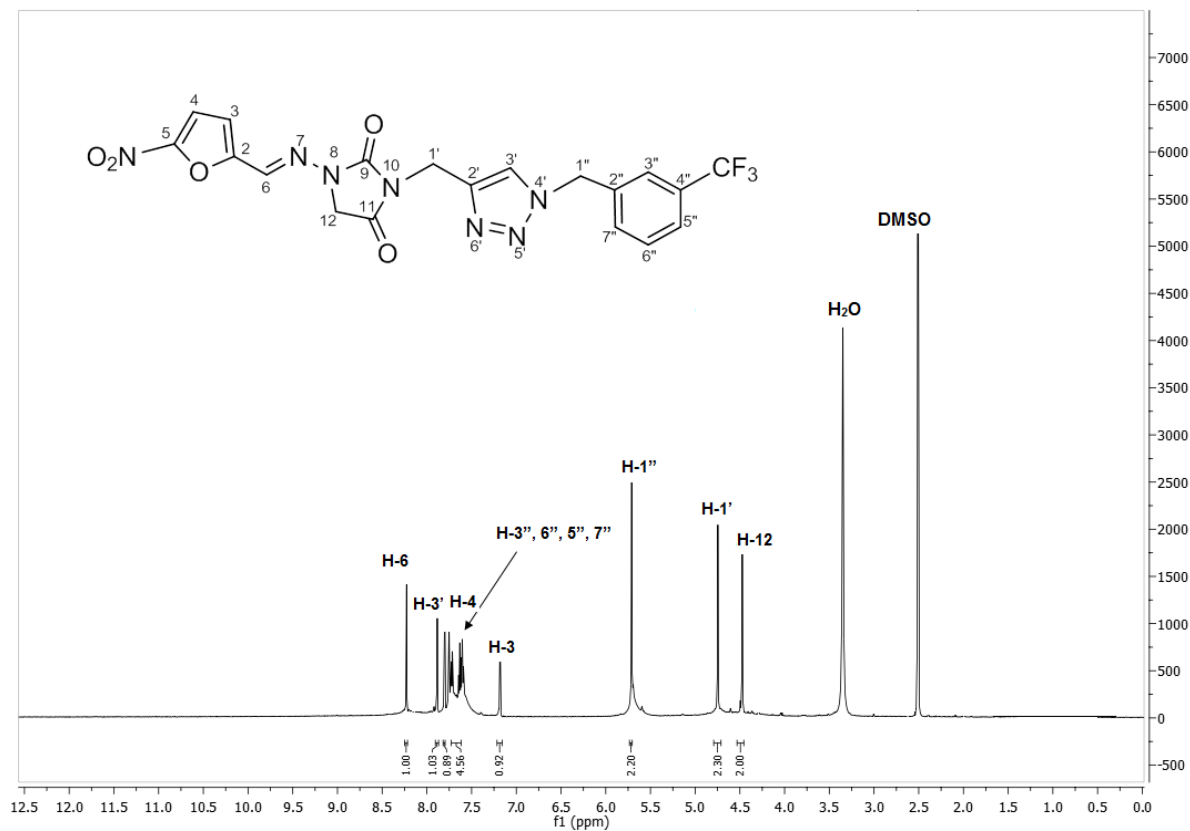
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



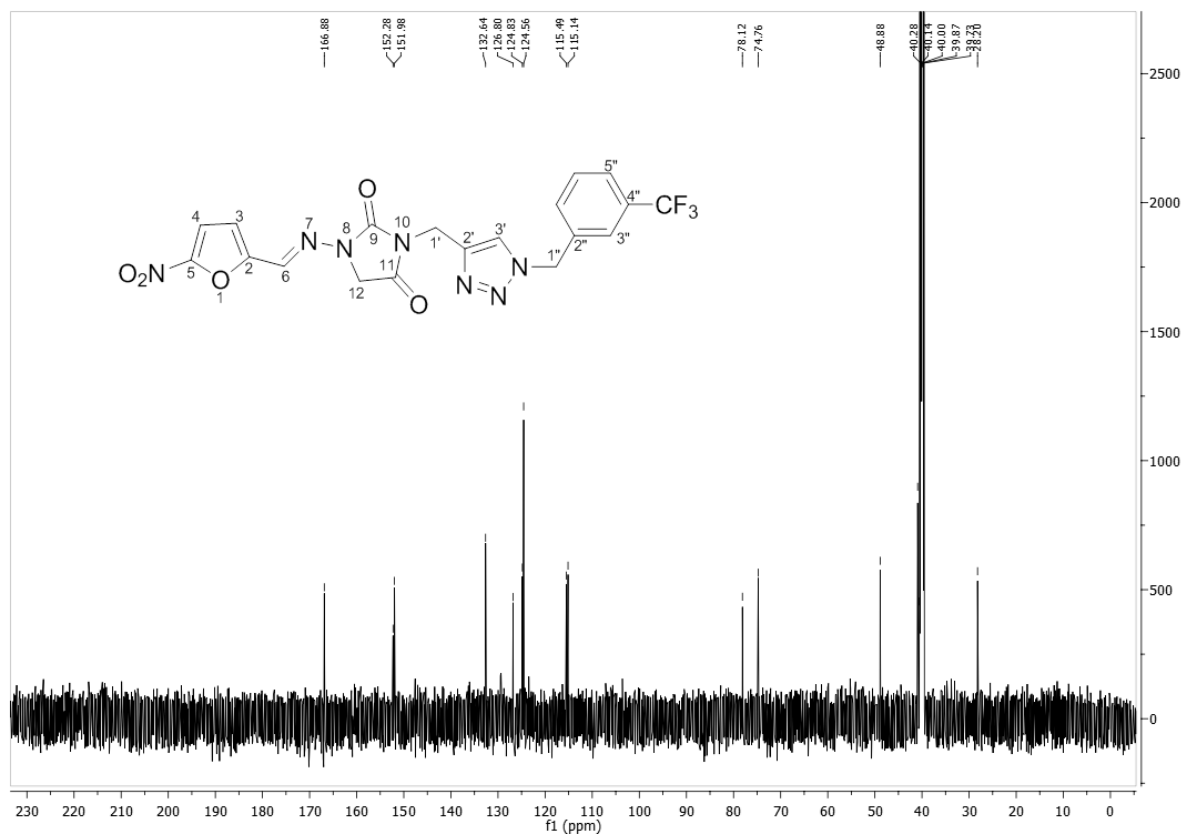
HYBRID 219 IR



¹H NMR in DMSO



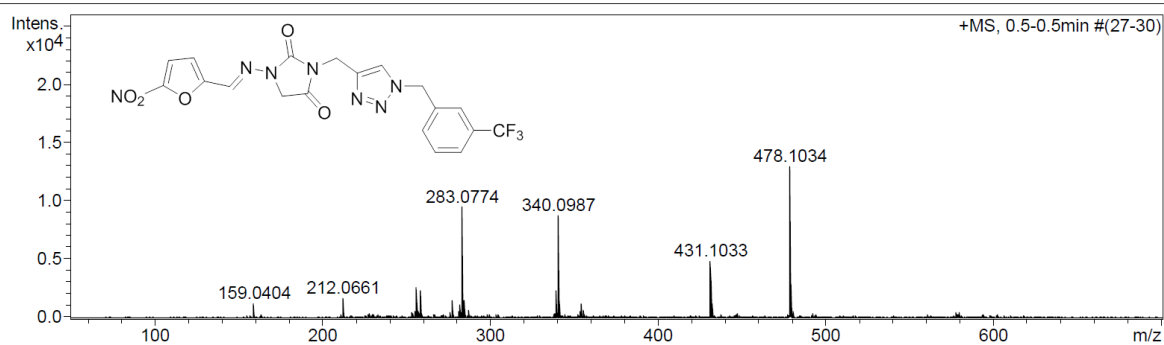
¹³C NMR in DMSO



HR-MS

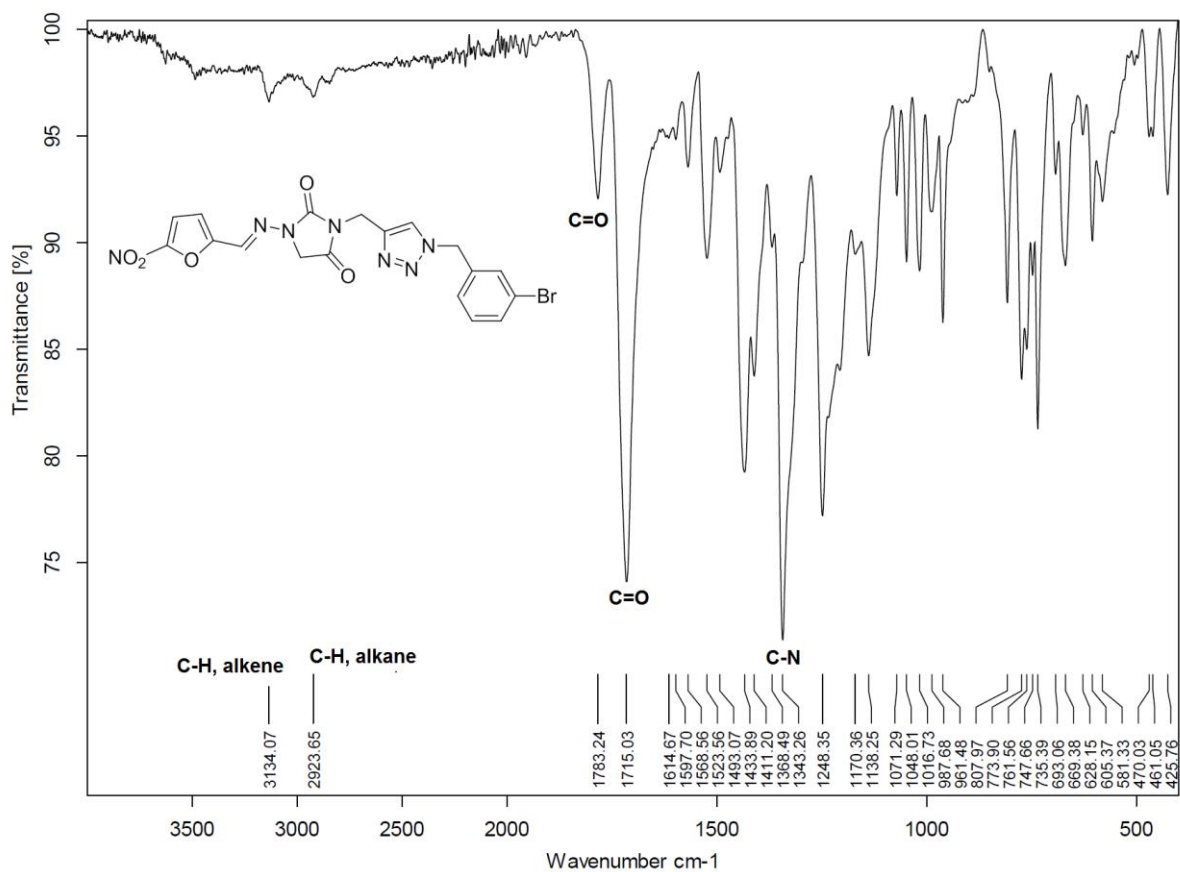
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

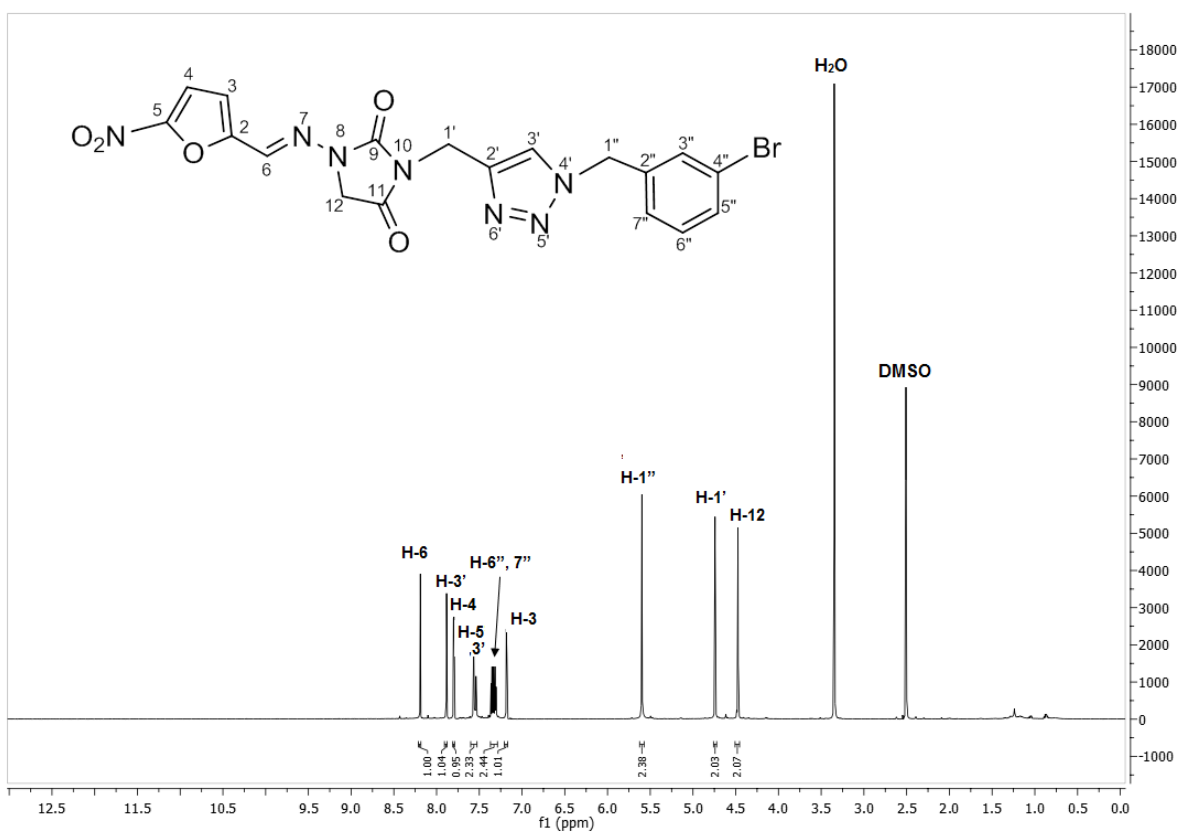


HYBRID 220

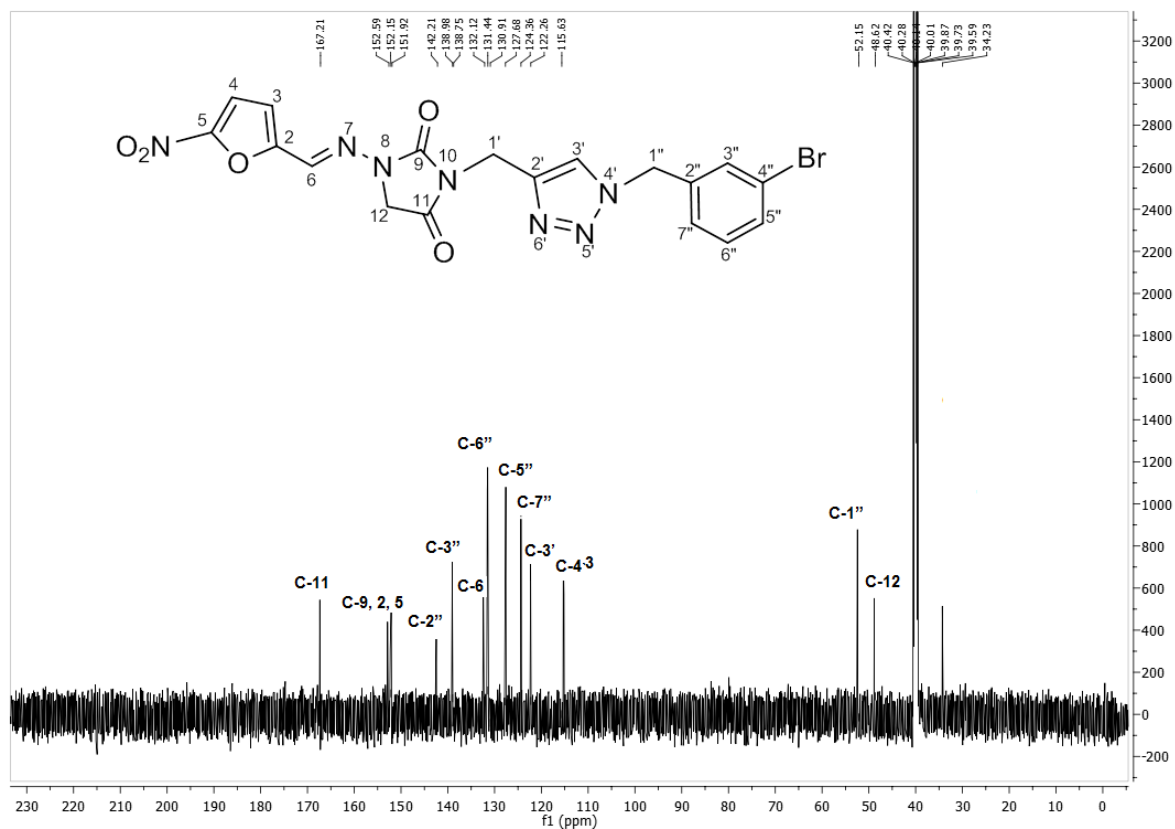
IR



¹H NMR in DMSO



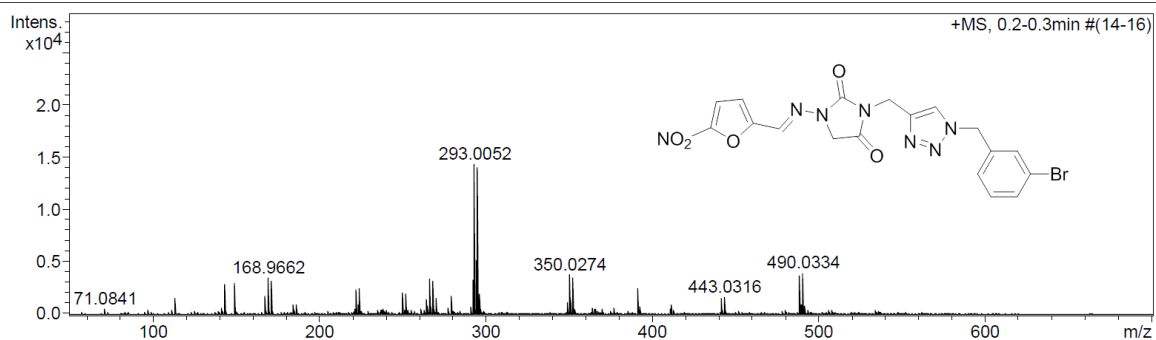
¹³C NMR in DMSO



HR-MS

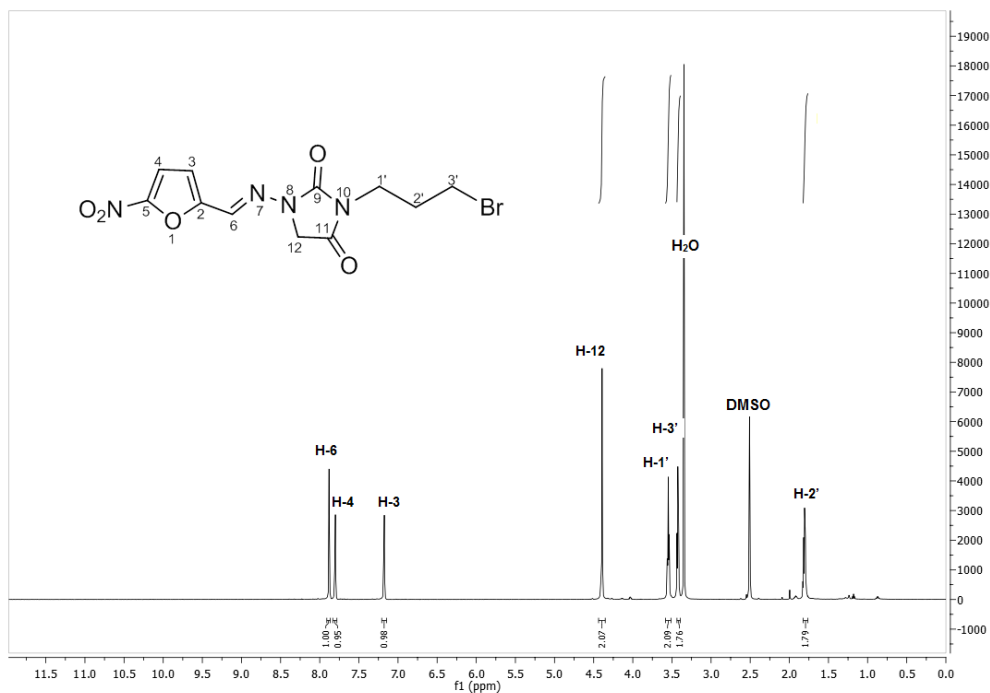
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	80.0 Vpp	Set Divert Valve	Waste

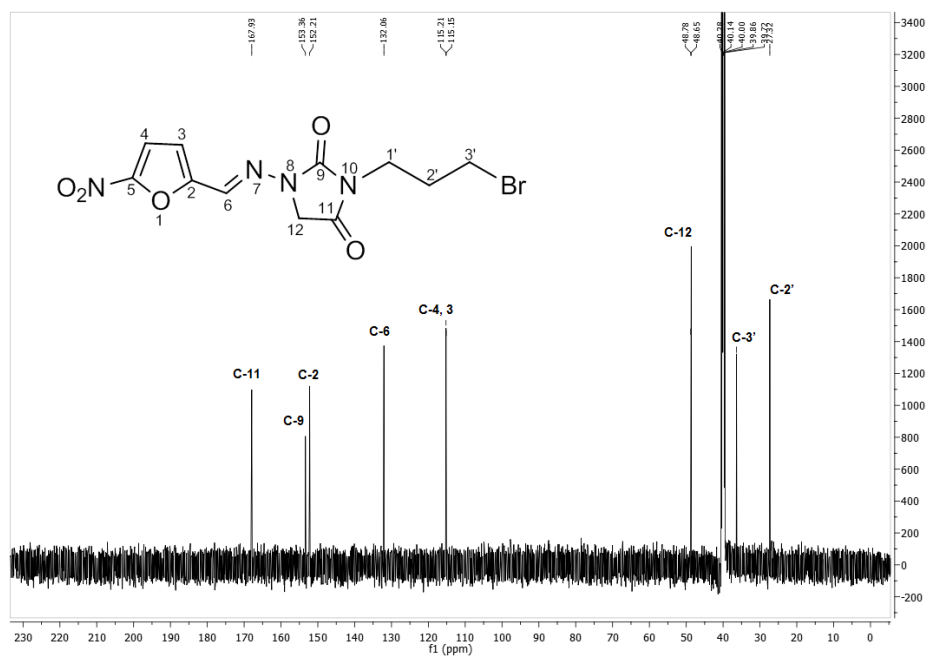


A1

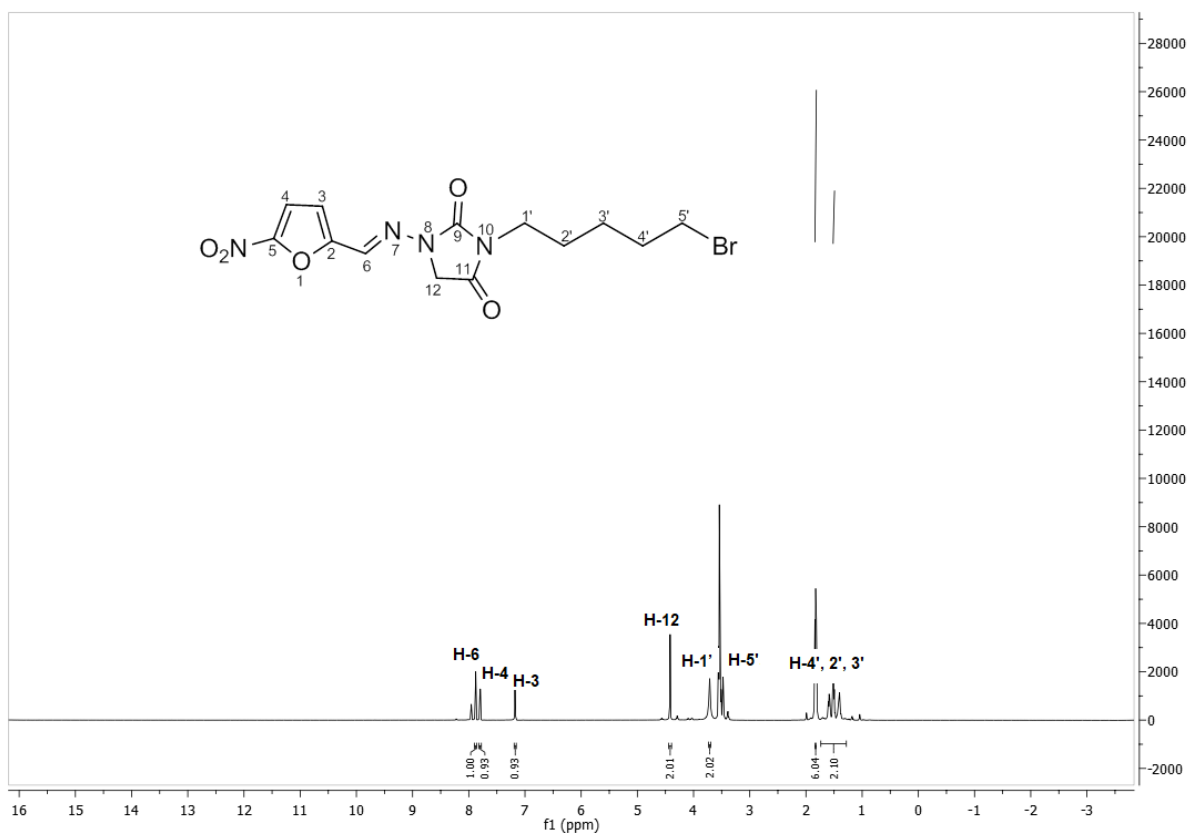
¹H NMR in DMSO



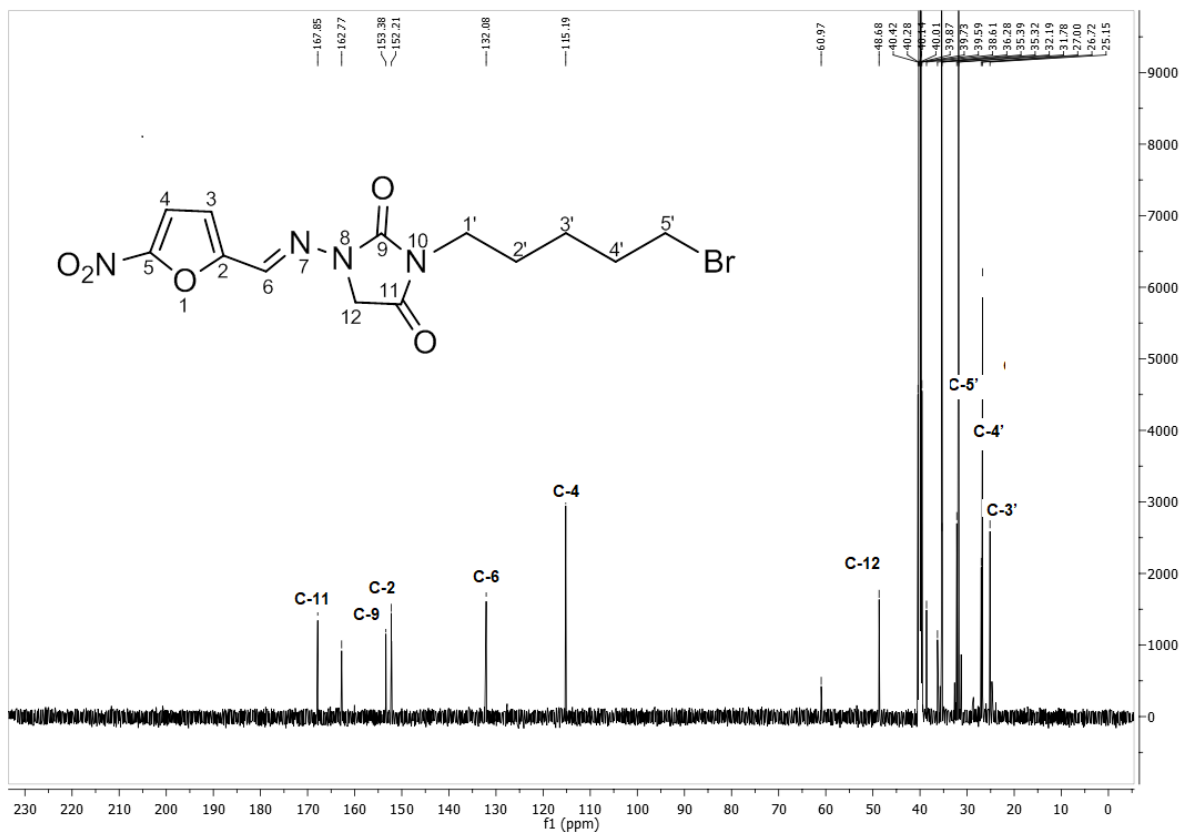
¹³C NMR in DMSO



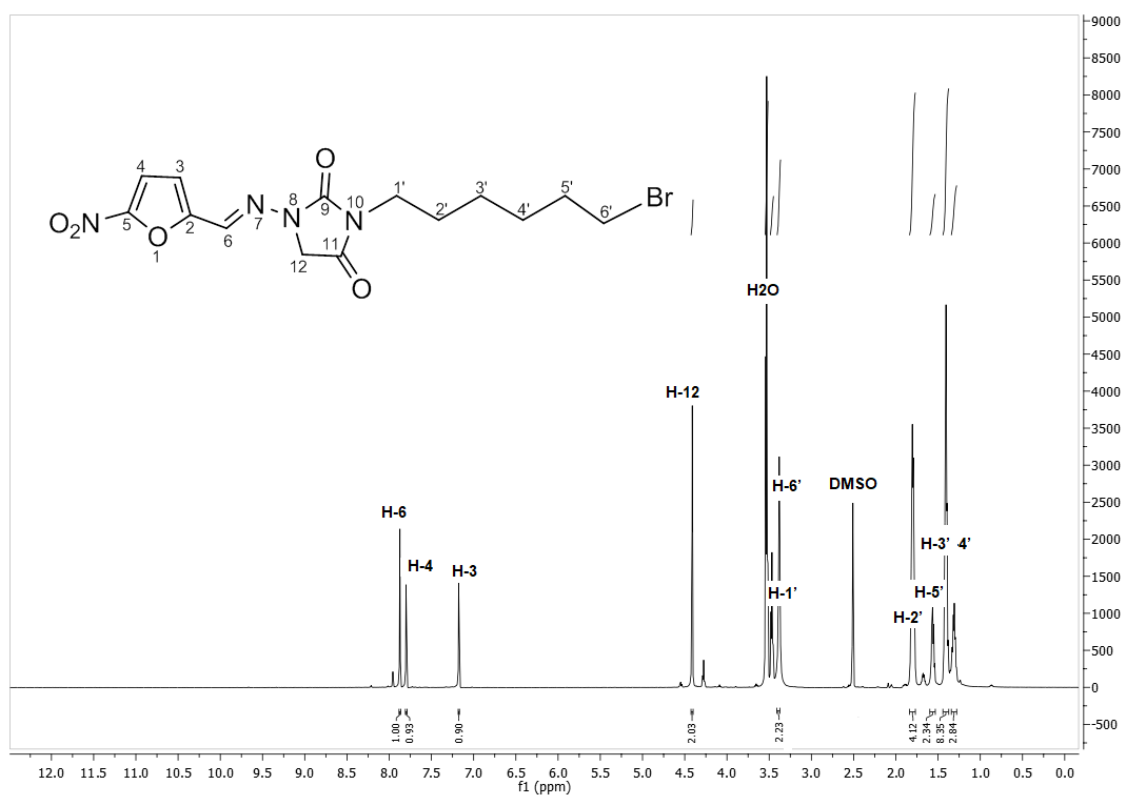
A2
¹H NMR in DMSO



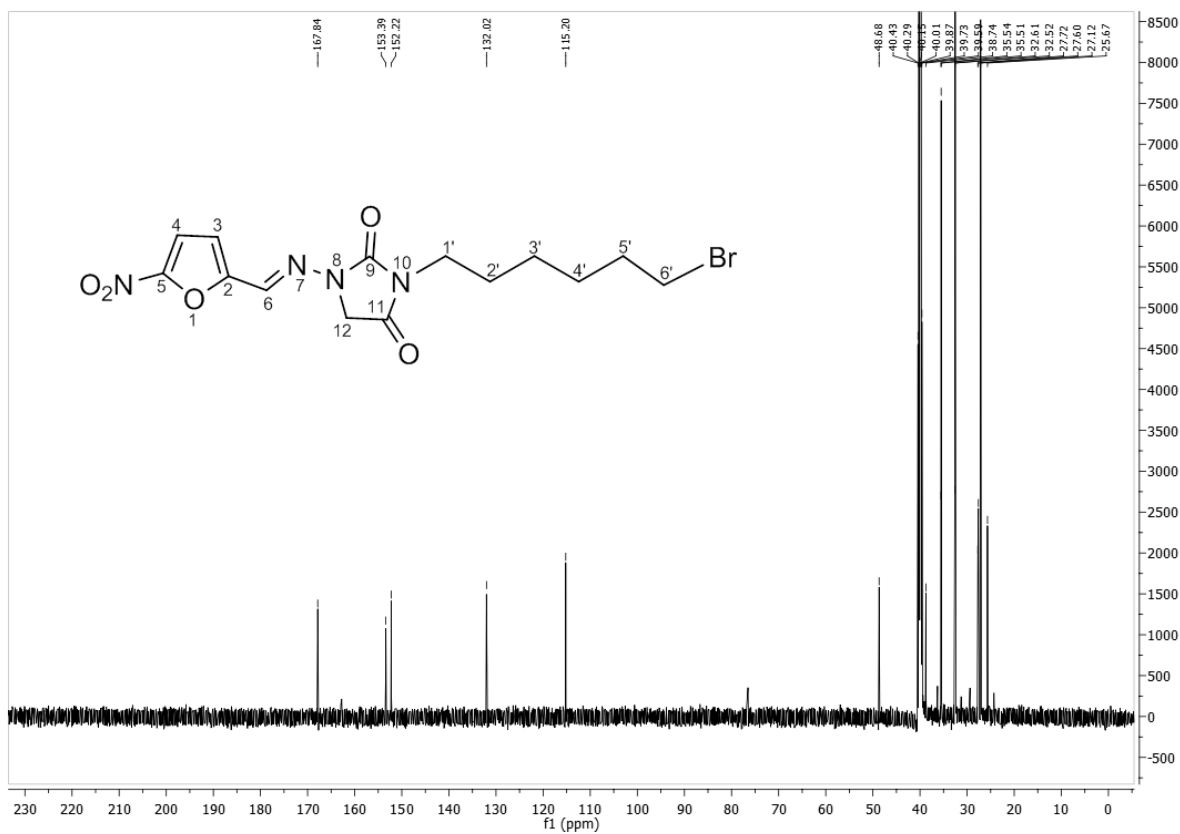
¹³C NMR in DMSO



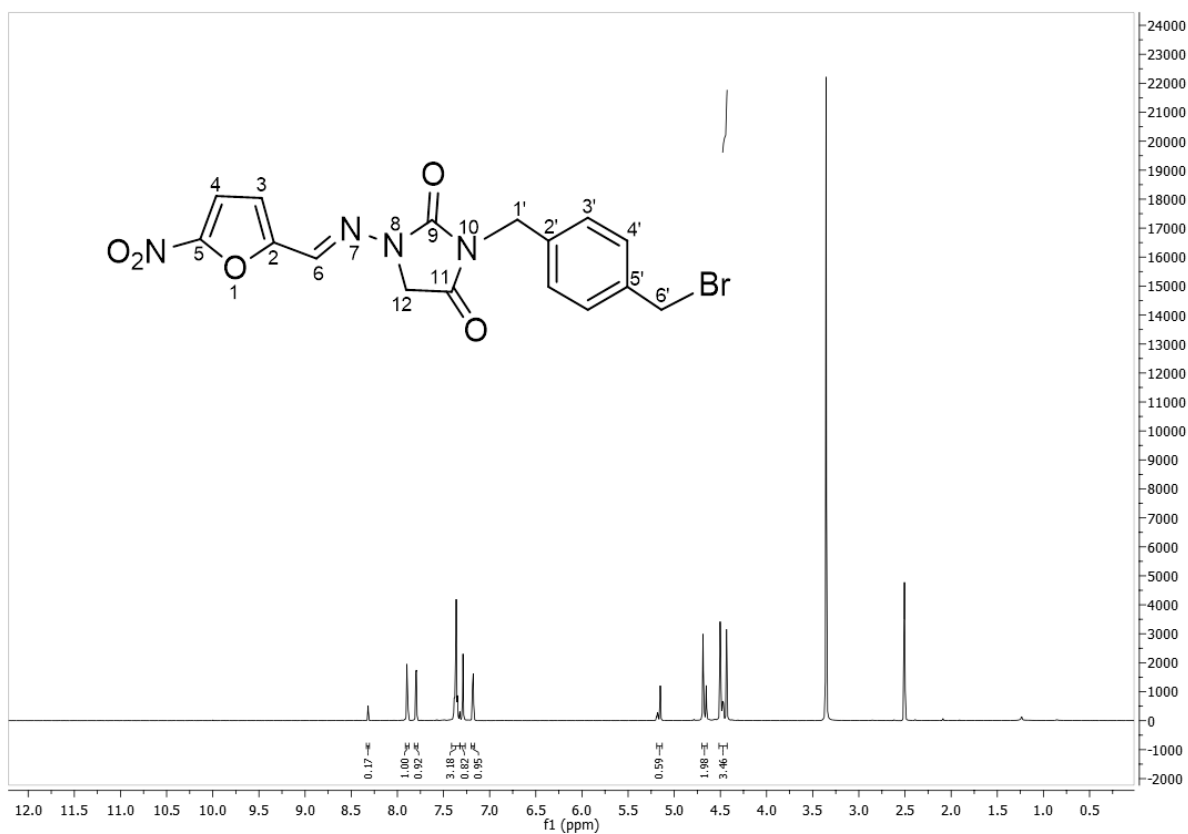
A3
¹H NMR in DMSO



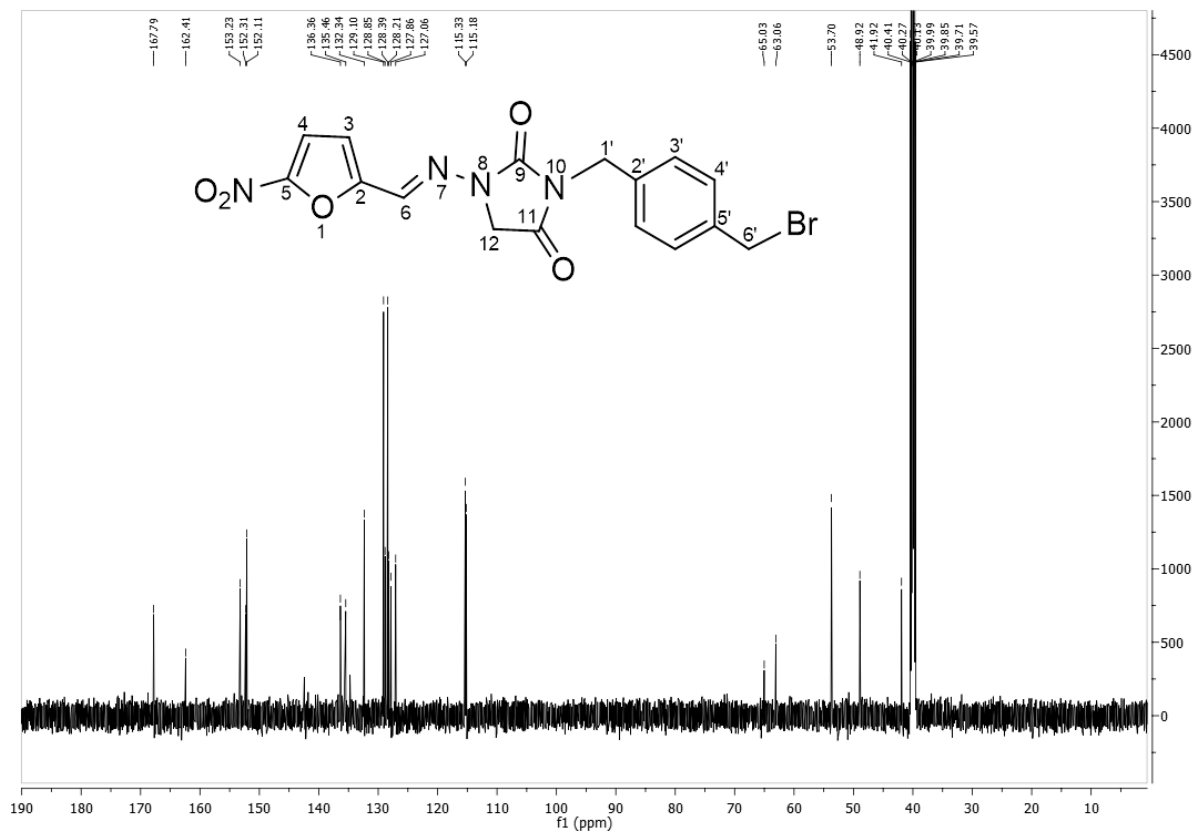
¹³C NMR in DMSO



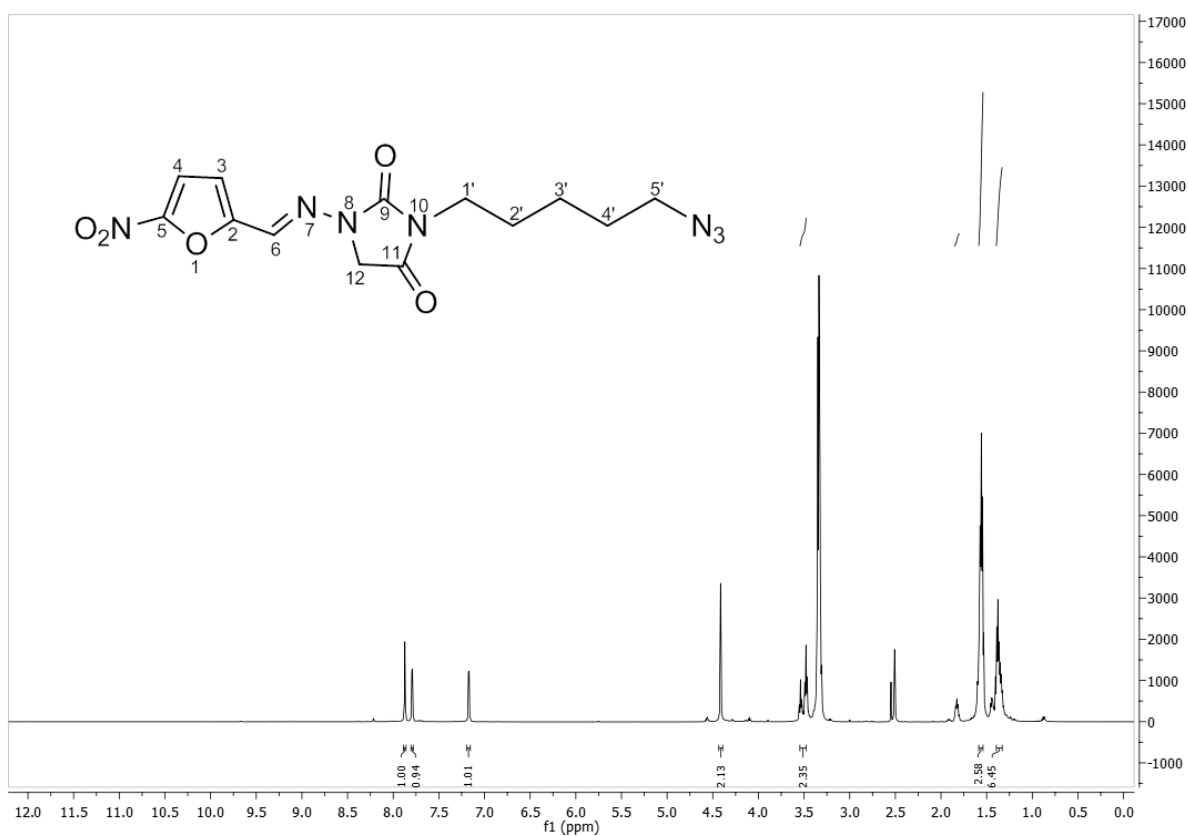
A4
¹H NMR in DMSO



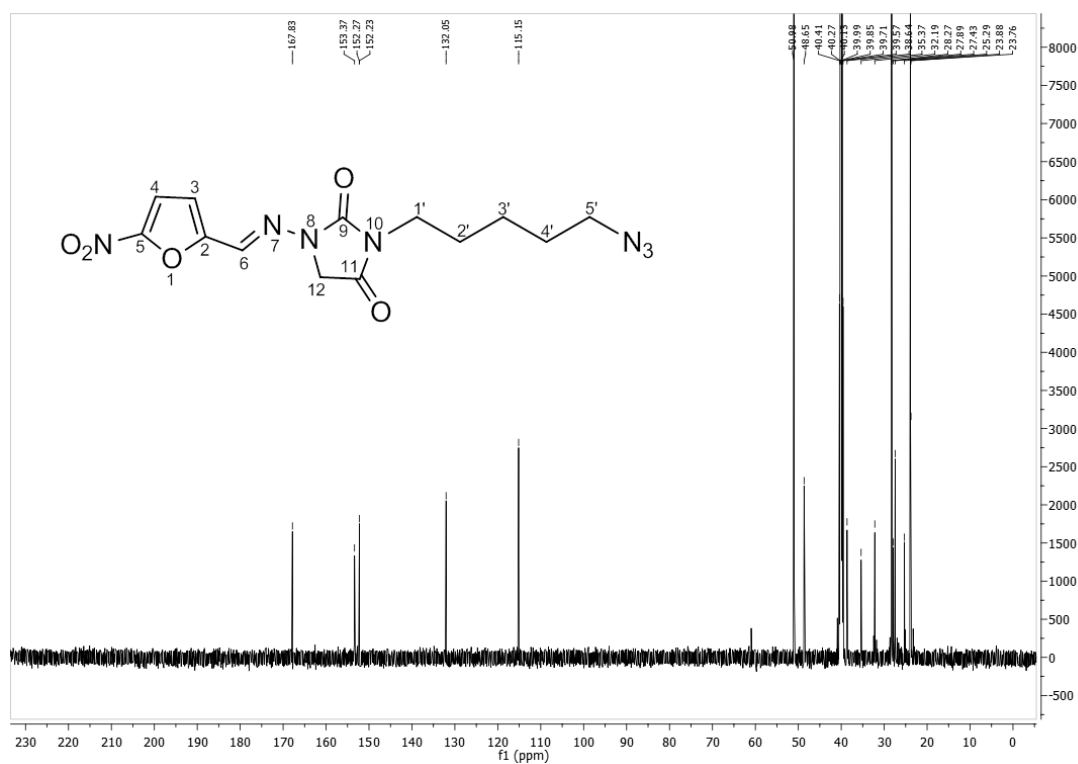
¹³C NMR in DMSO



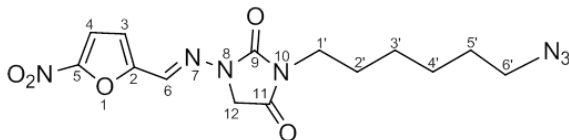
B2
¹H NMR in DMSO



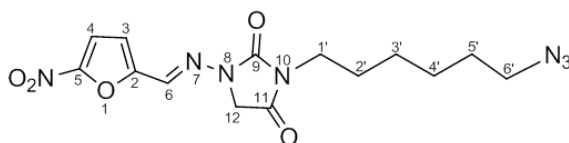
¹³C NMR in DMSO



¹H NMR in DMSO

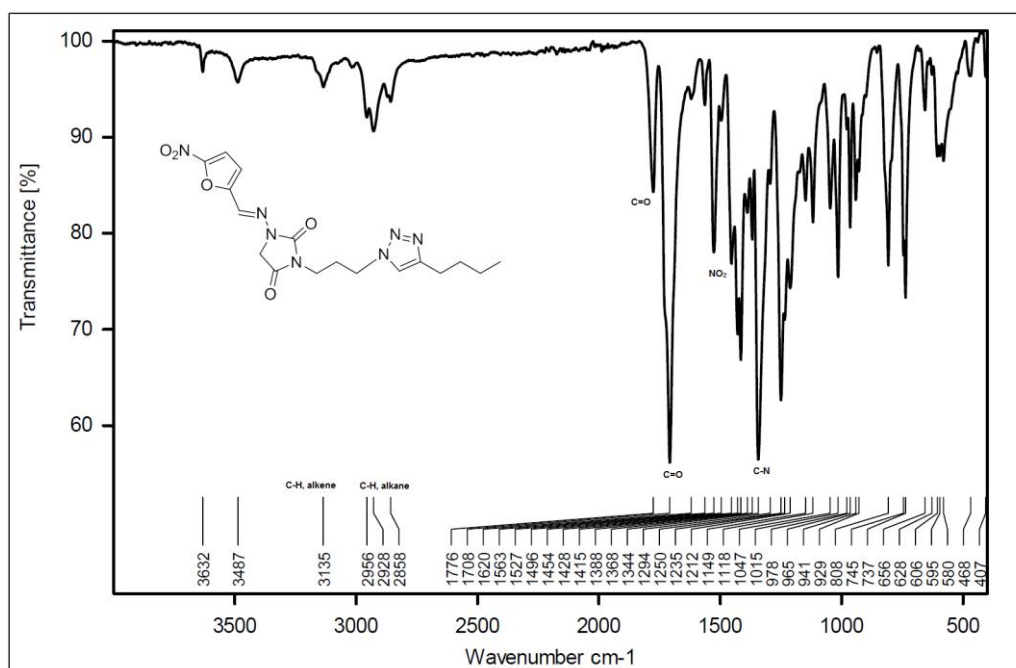


¹³C NMR in DMSO

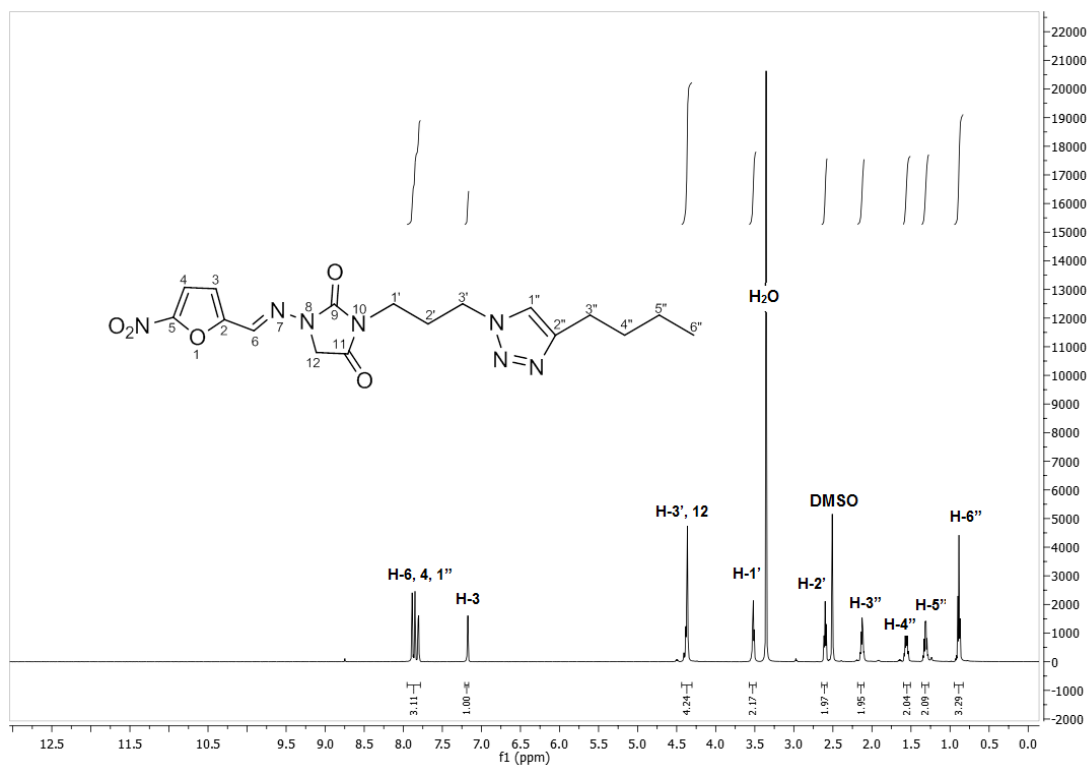


Hybrid 3301

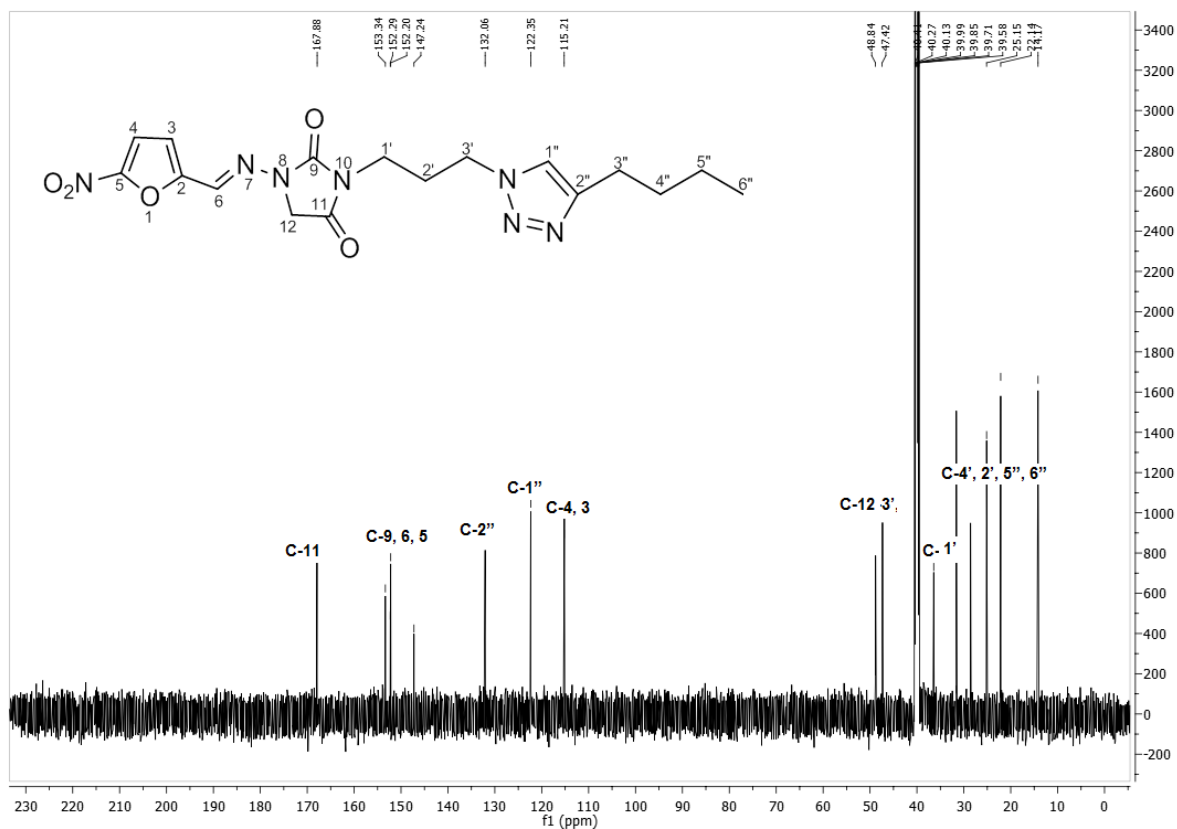
IR



¹H NMR in DMSO



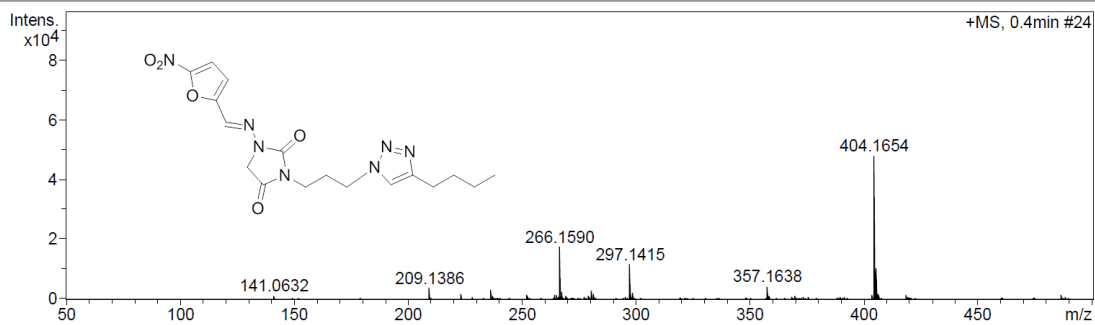
¹³C NMR in DMSO



HR-MS

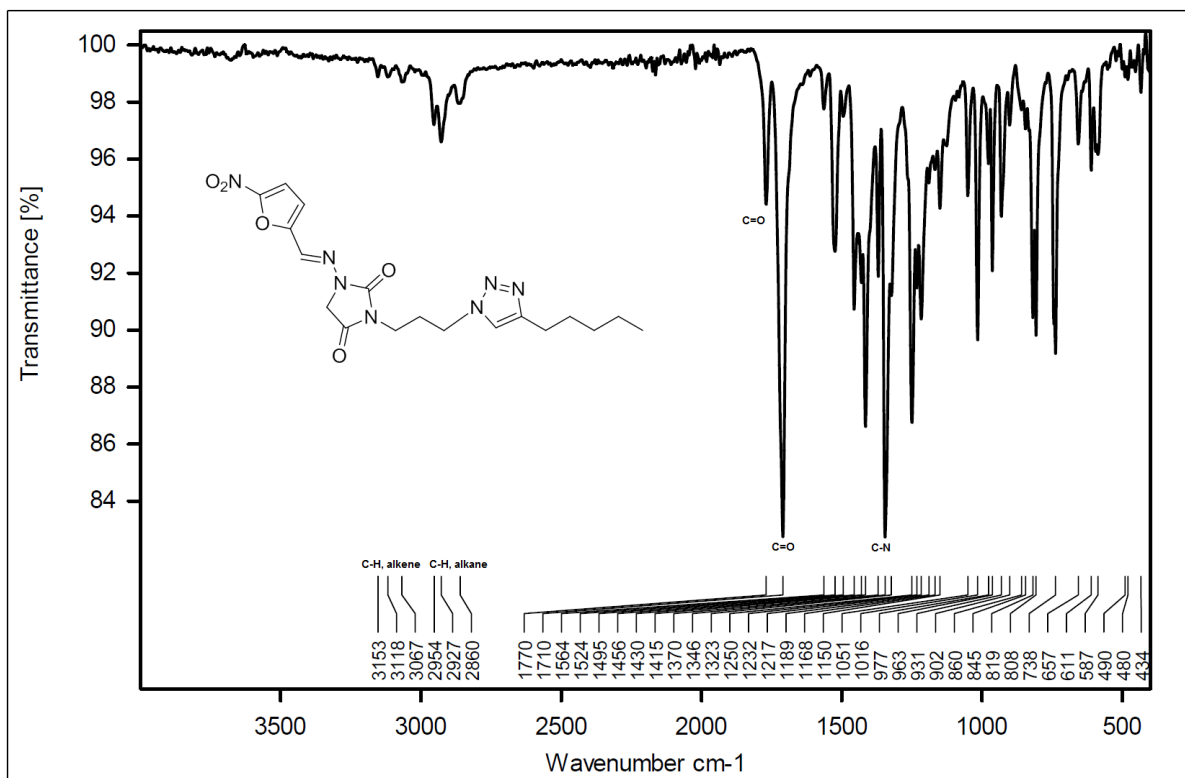
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

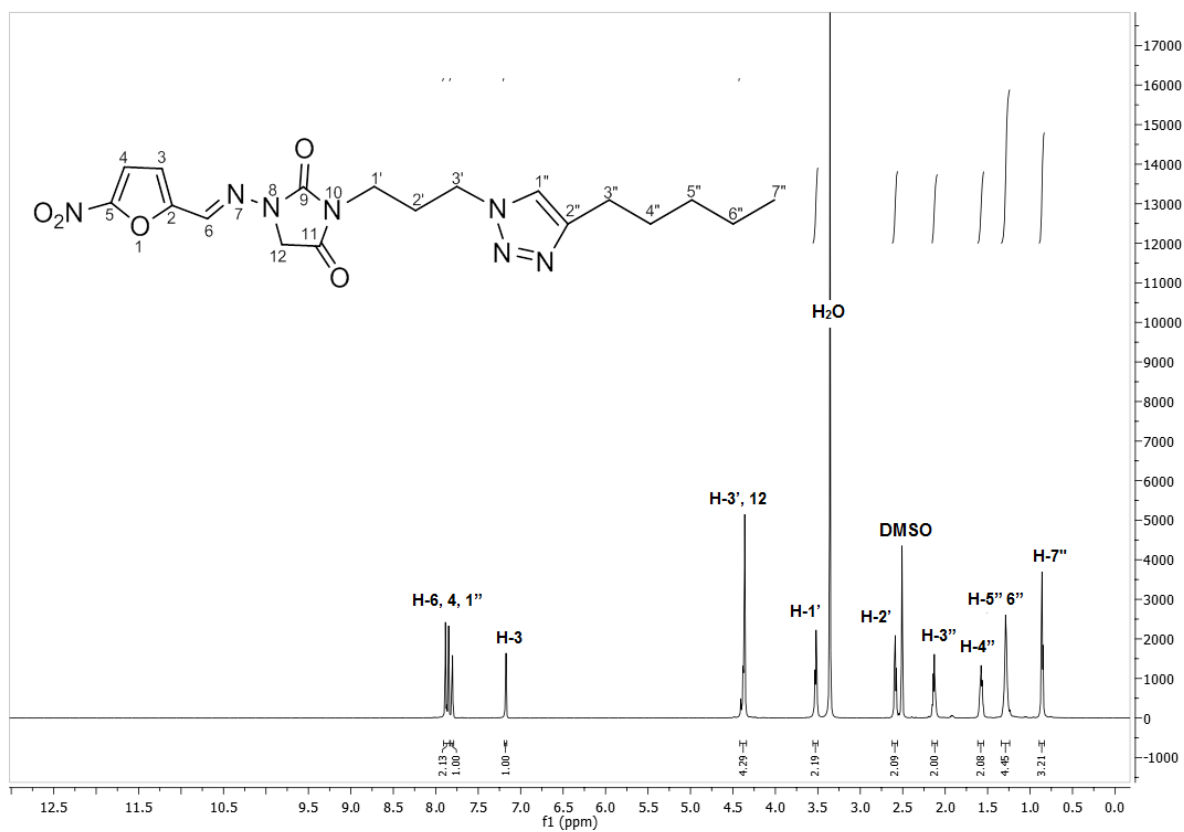


Hybrid 3302

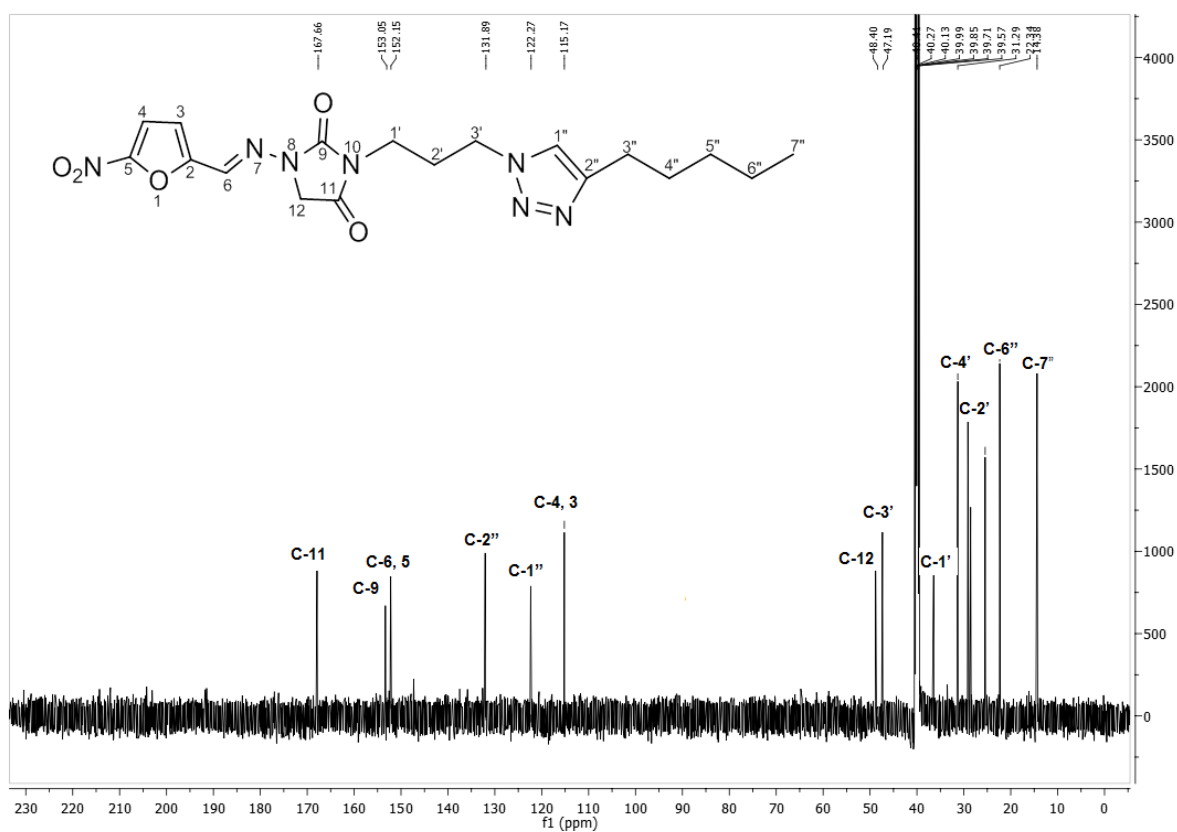
IR



¹H NMR in DMSO



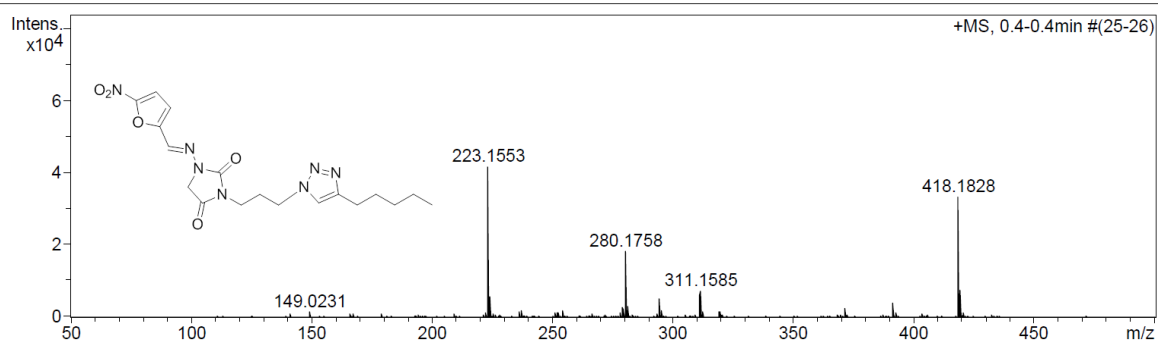
¹³C NMR in DMSO



HR-MS

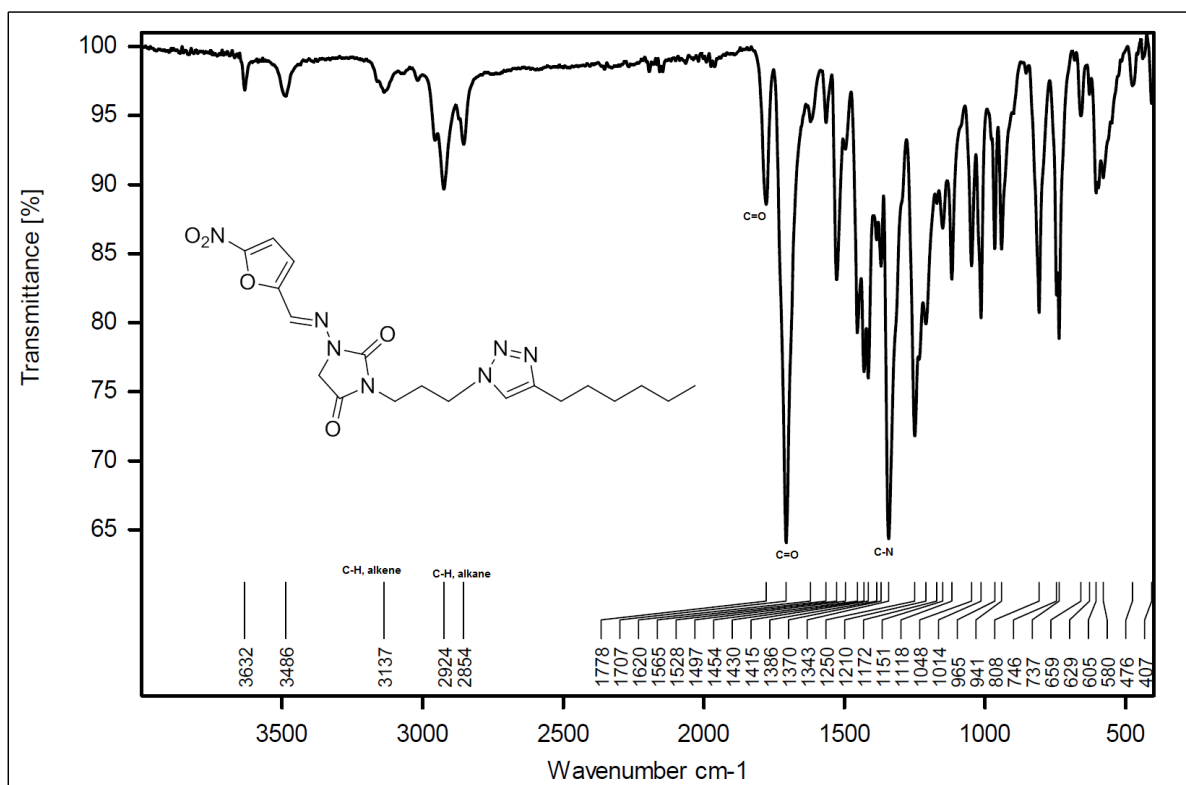
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

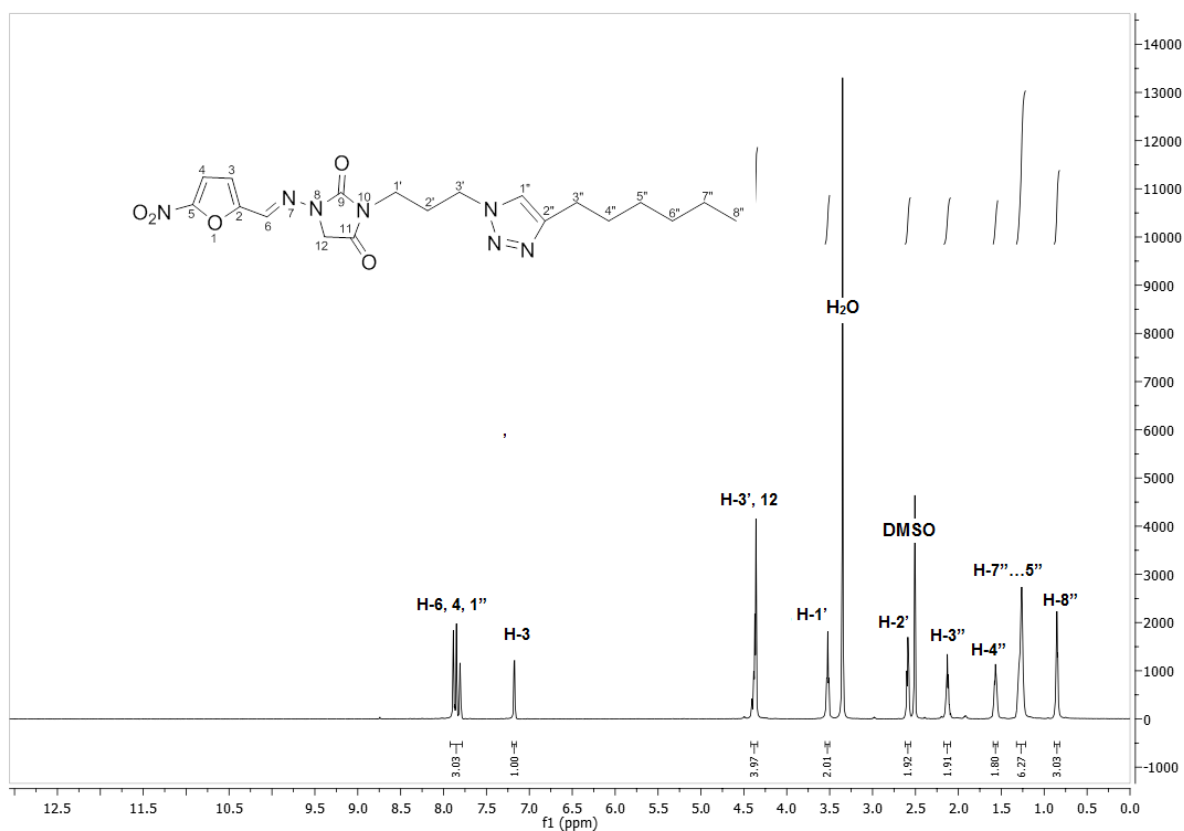


Hybrid 3303

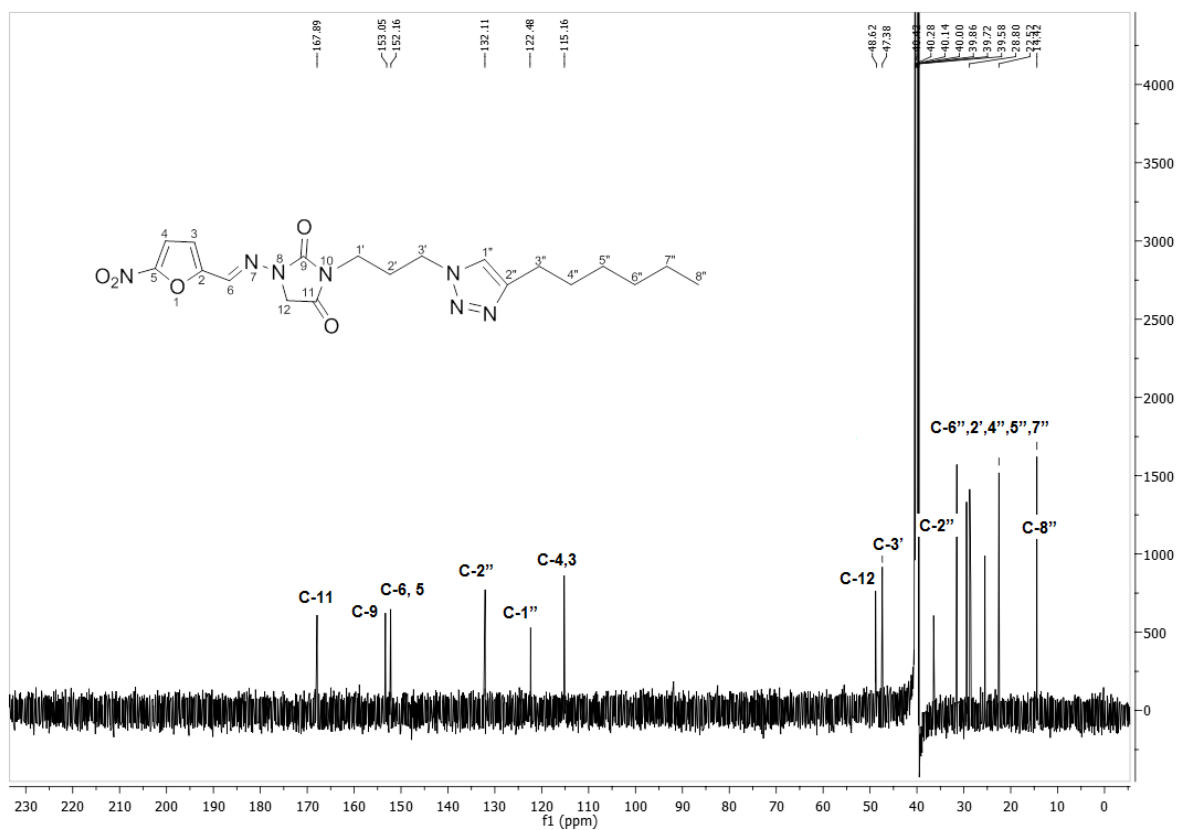
IR



¹H NMR in DMSO



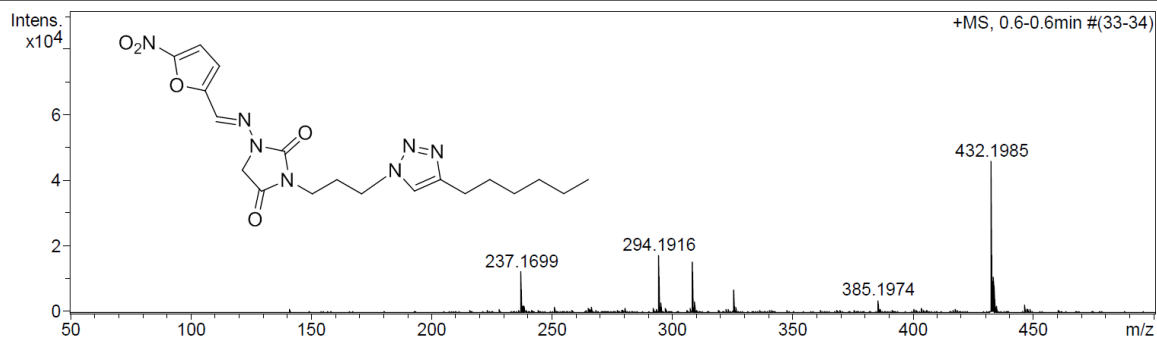
¹³C NMR in DMSO



HR-MS

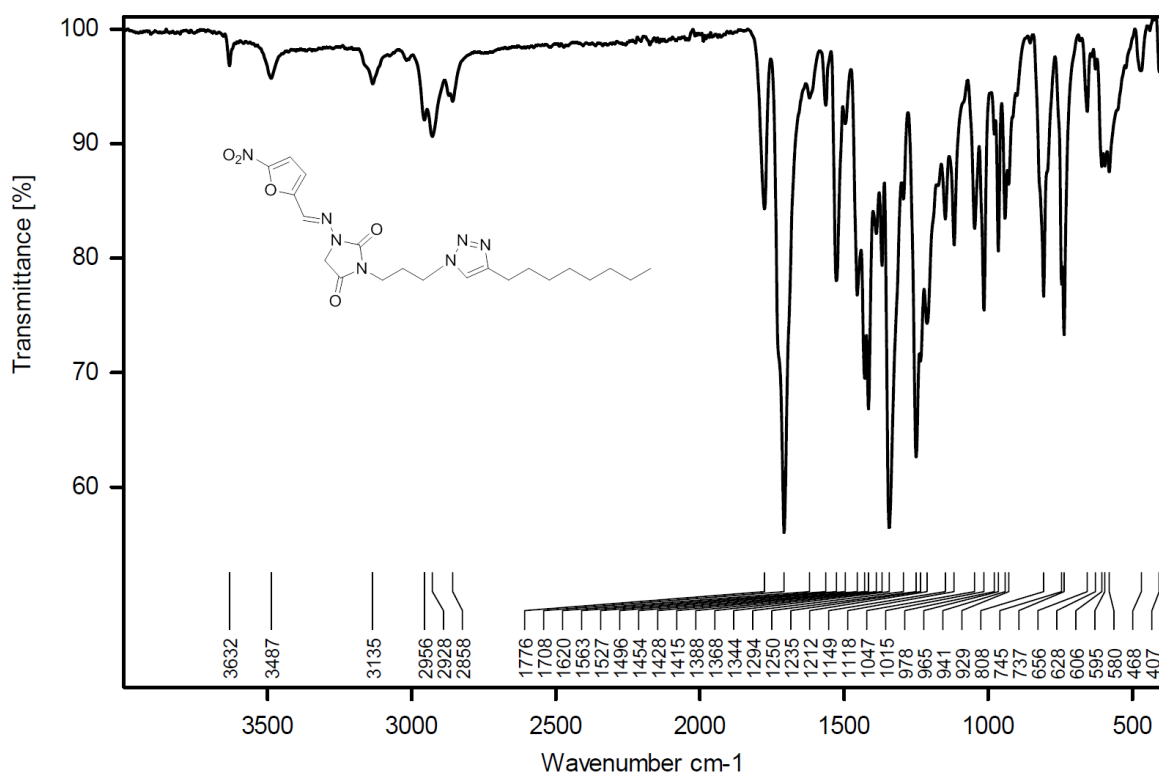
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

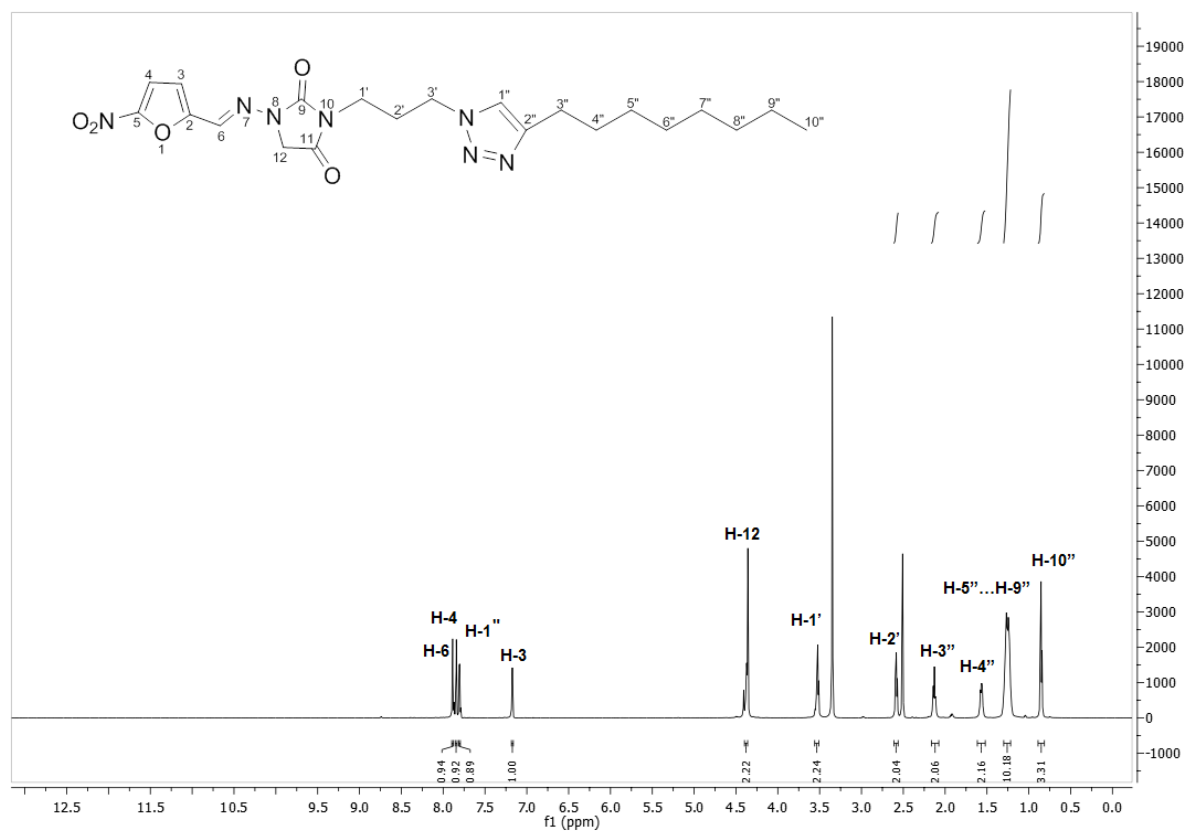


Hybrid 3304

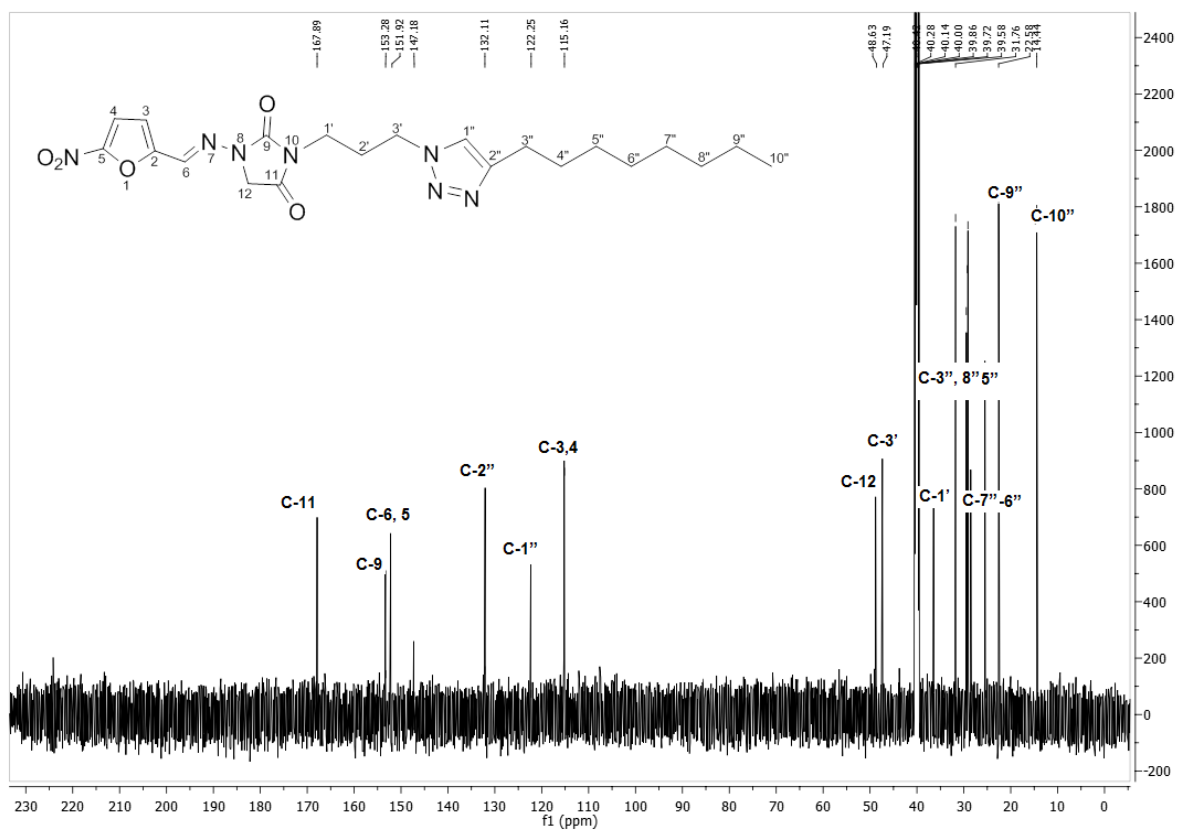
IR



¹H NMR in DMSO



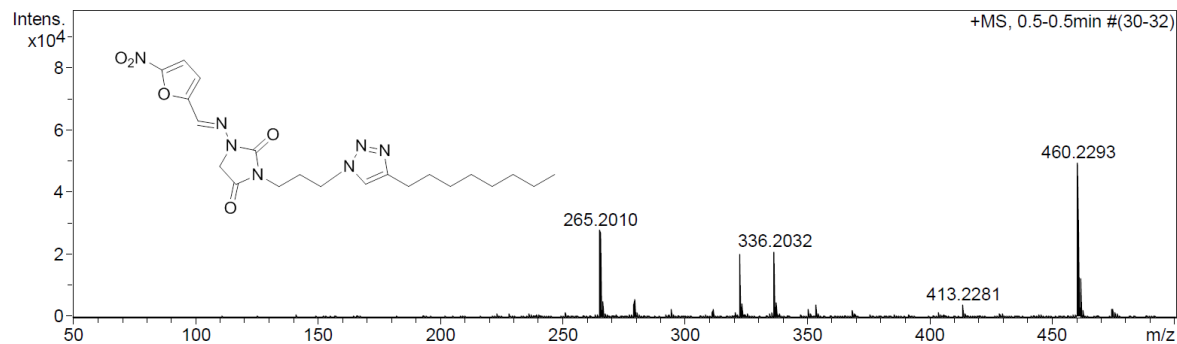
¹³C NMR in DMSO



HR-MS

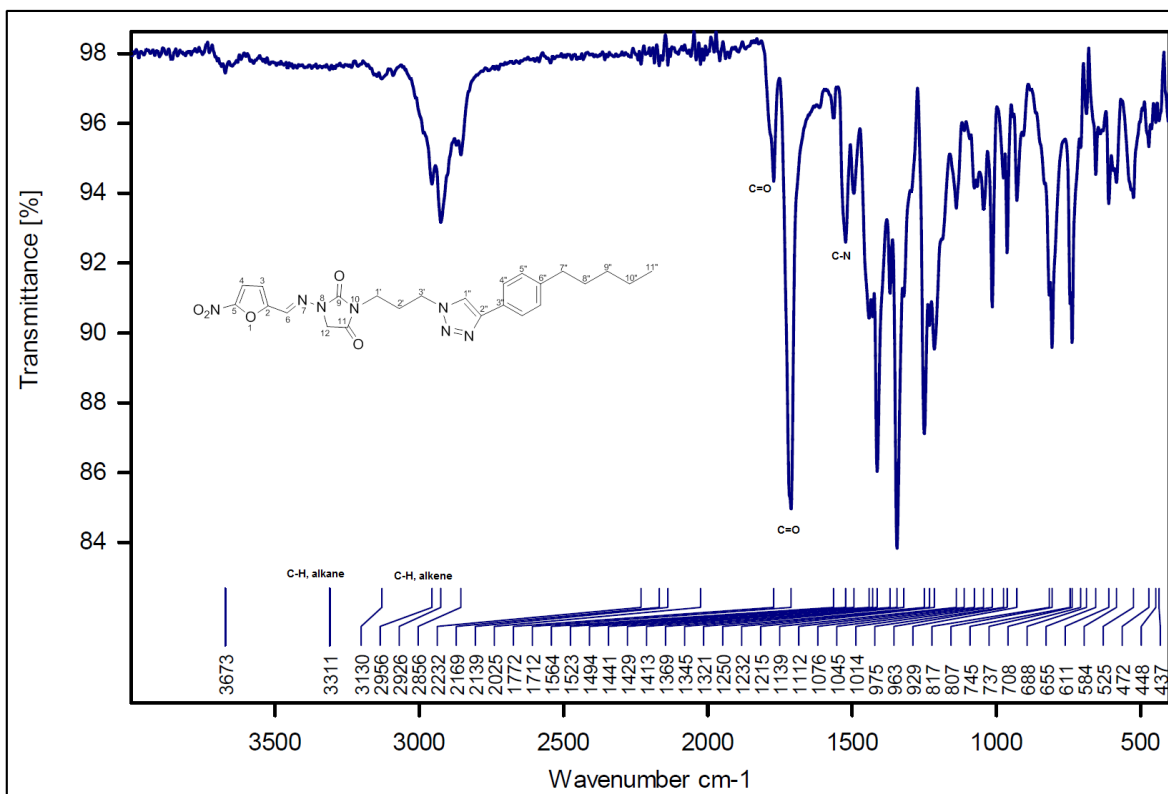
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

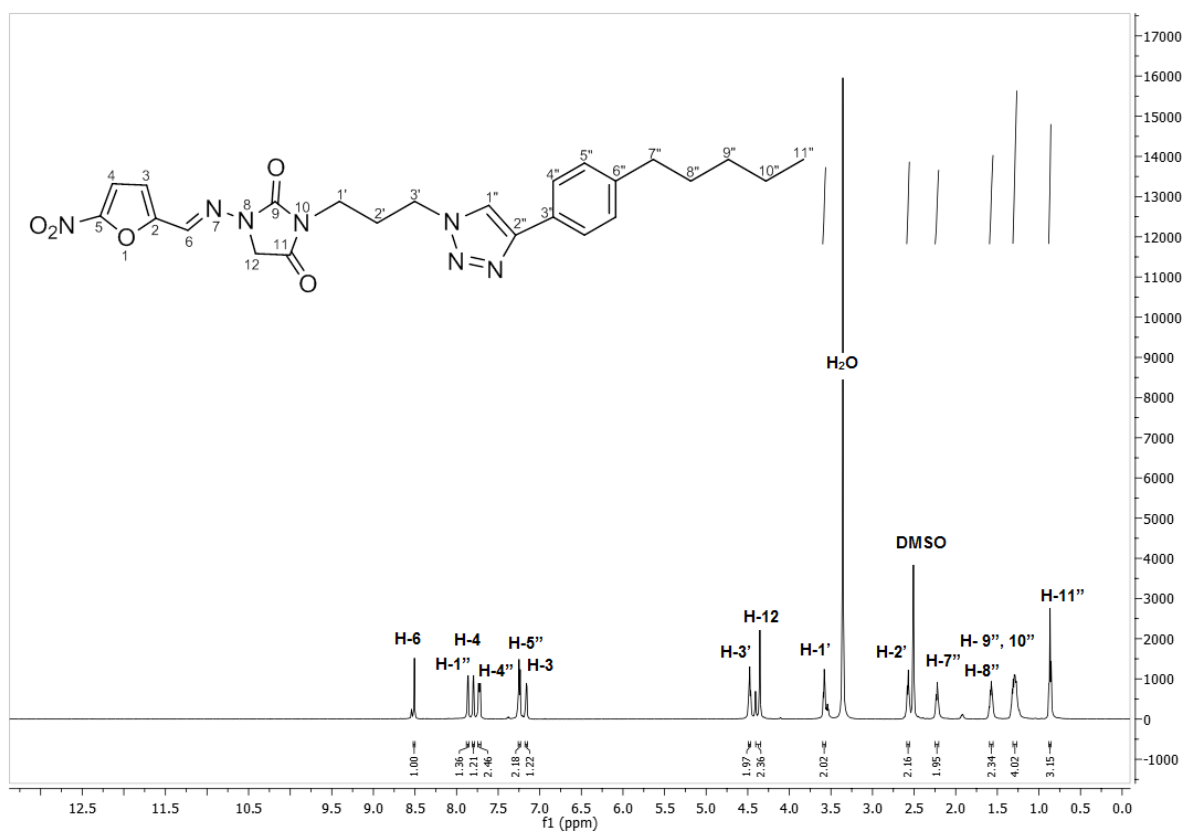


Hybrid 3306

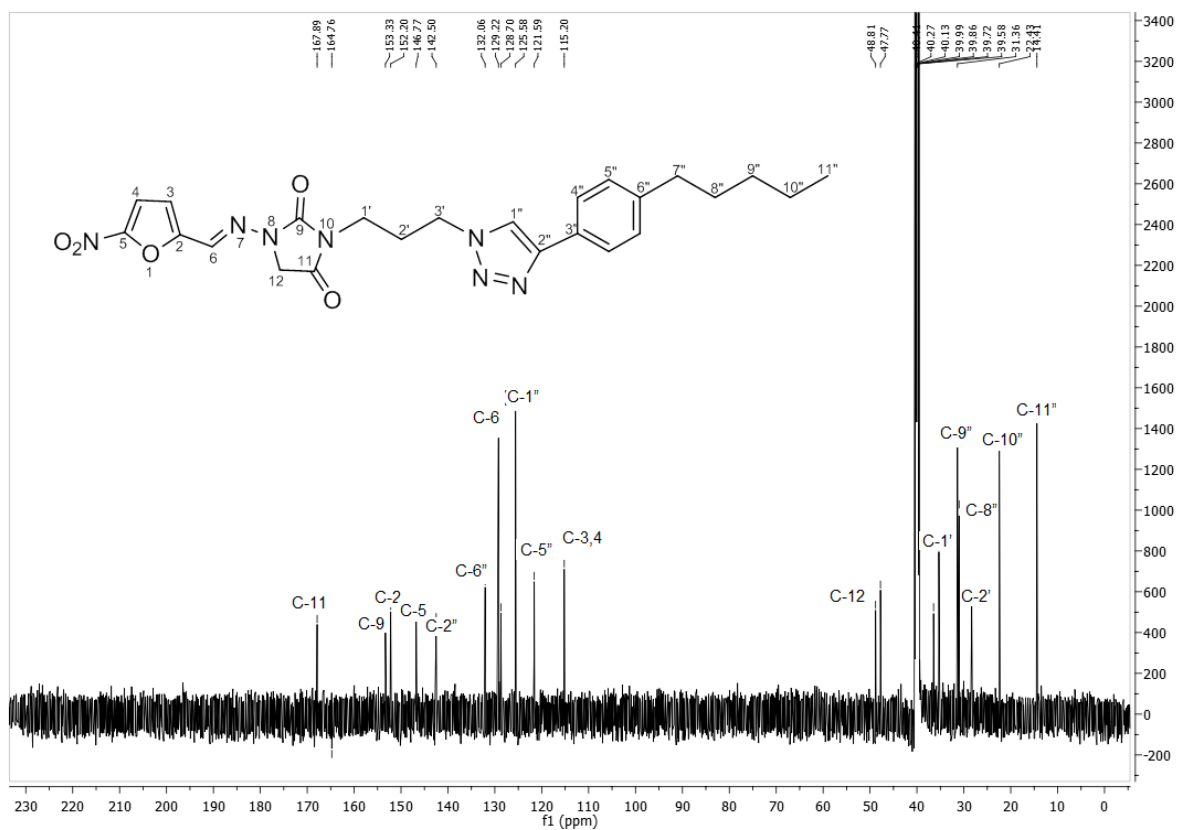
IR



¹H NMR in DMSO



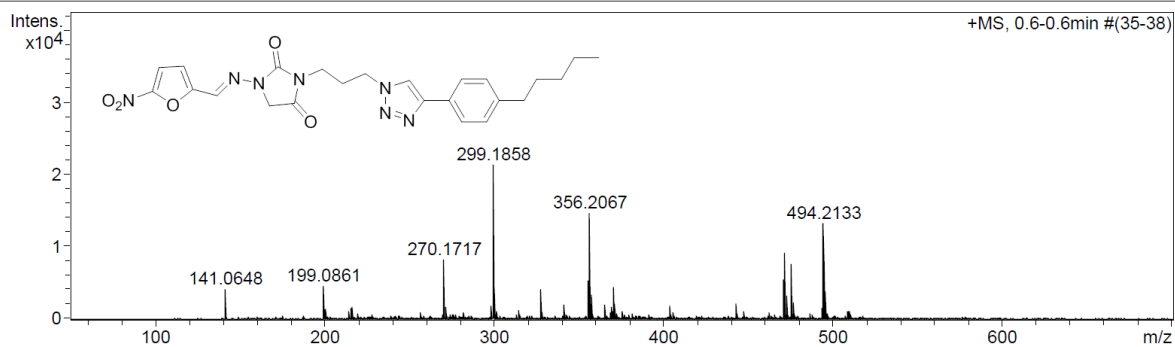
¹³C NMR in DMSO



HR-MS

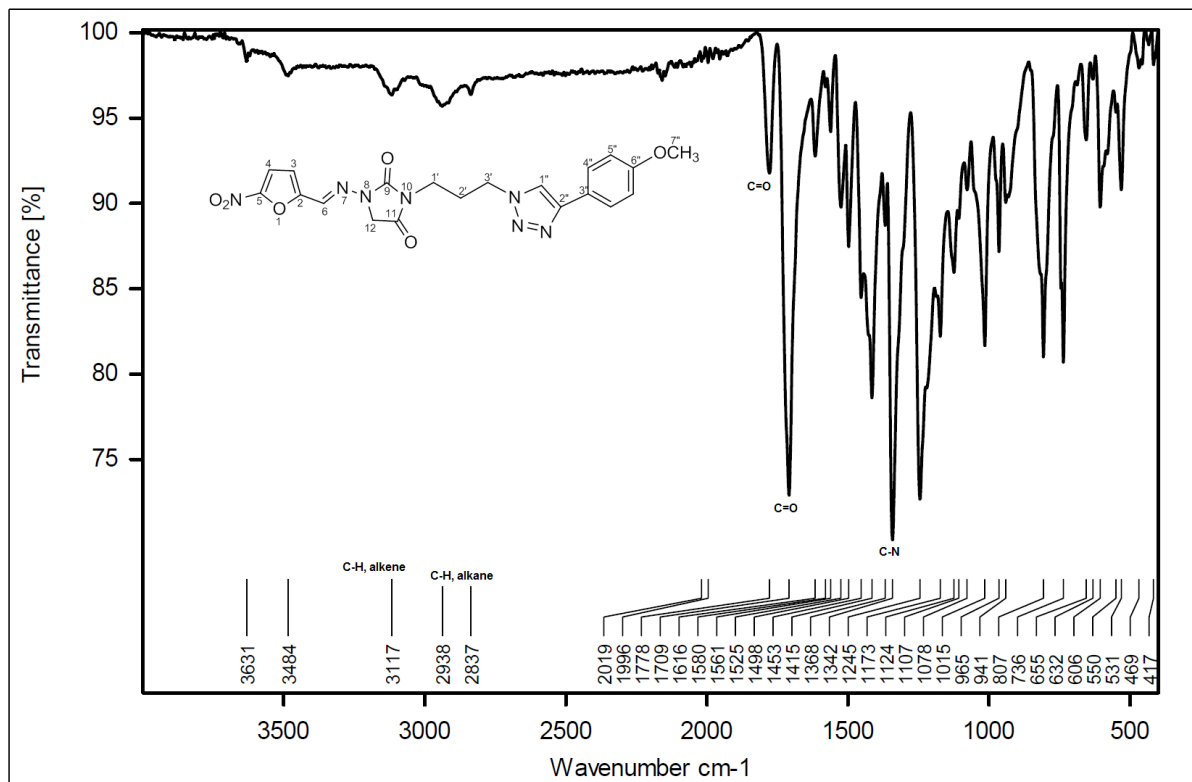
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

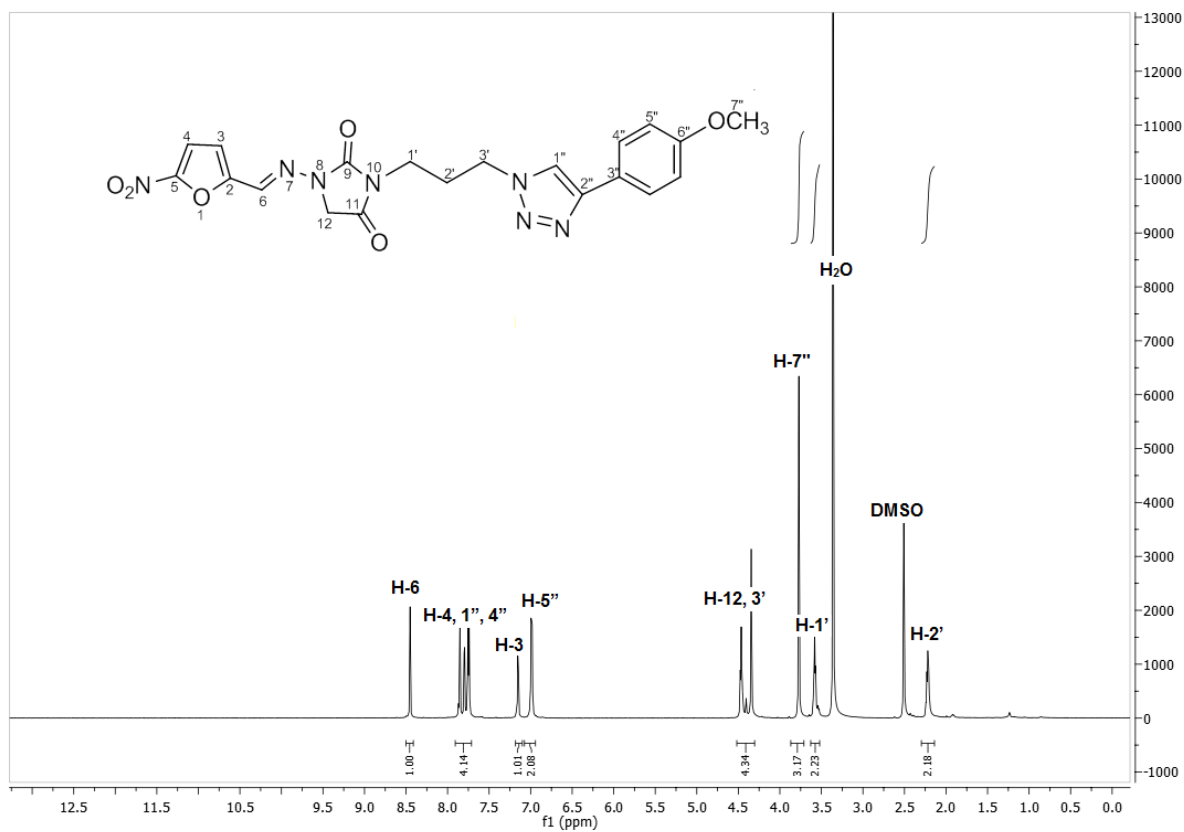


Hybrid 3307

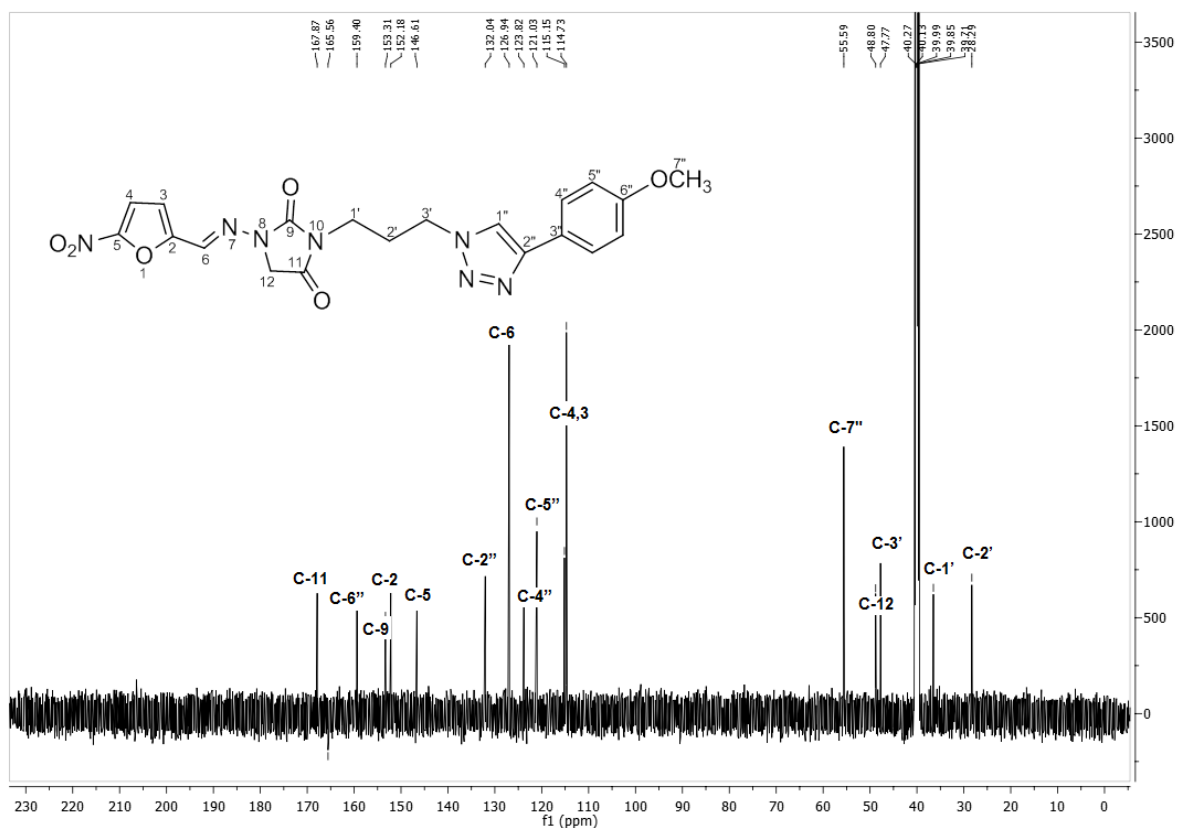
IR



¹H NMR in DMSO



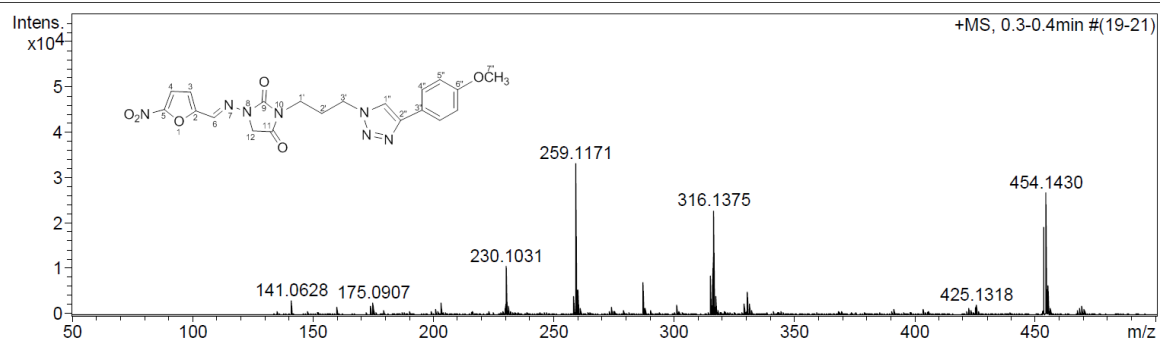
¹³C NMR in DMSO



HR-MS

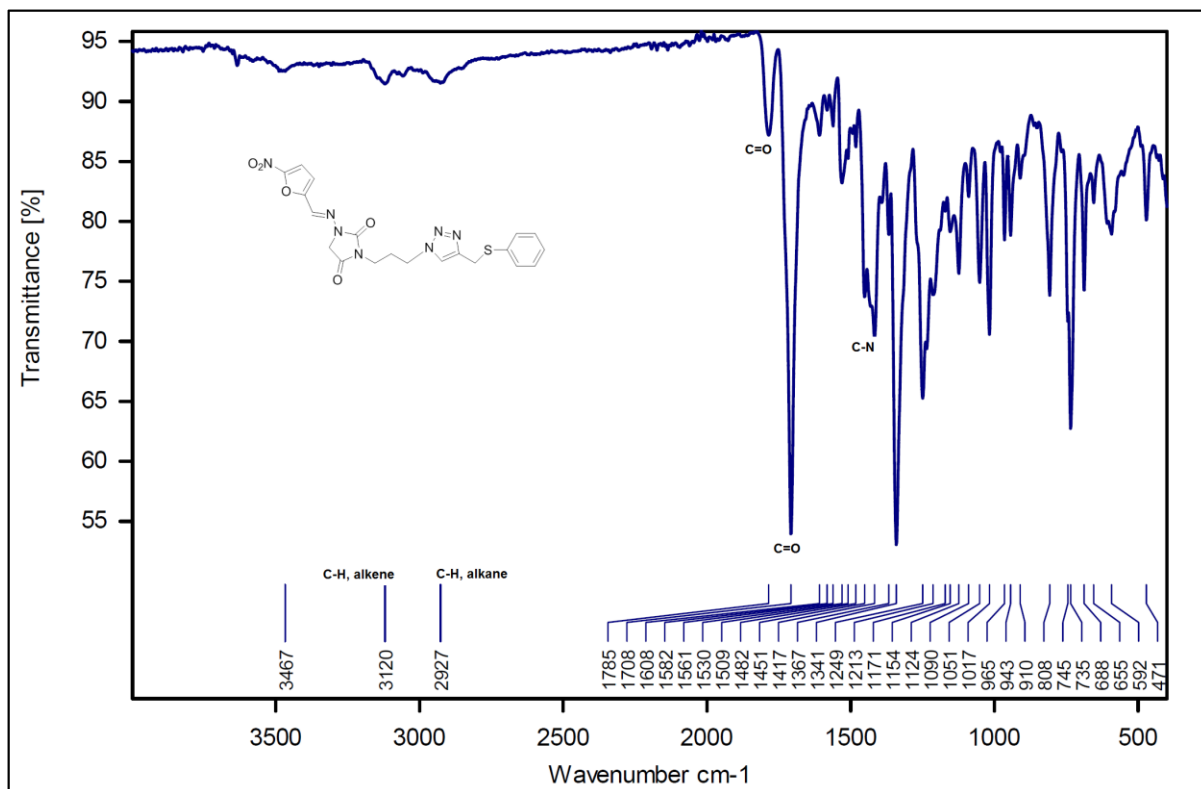
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

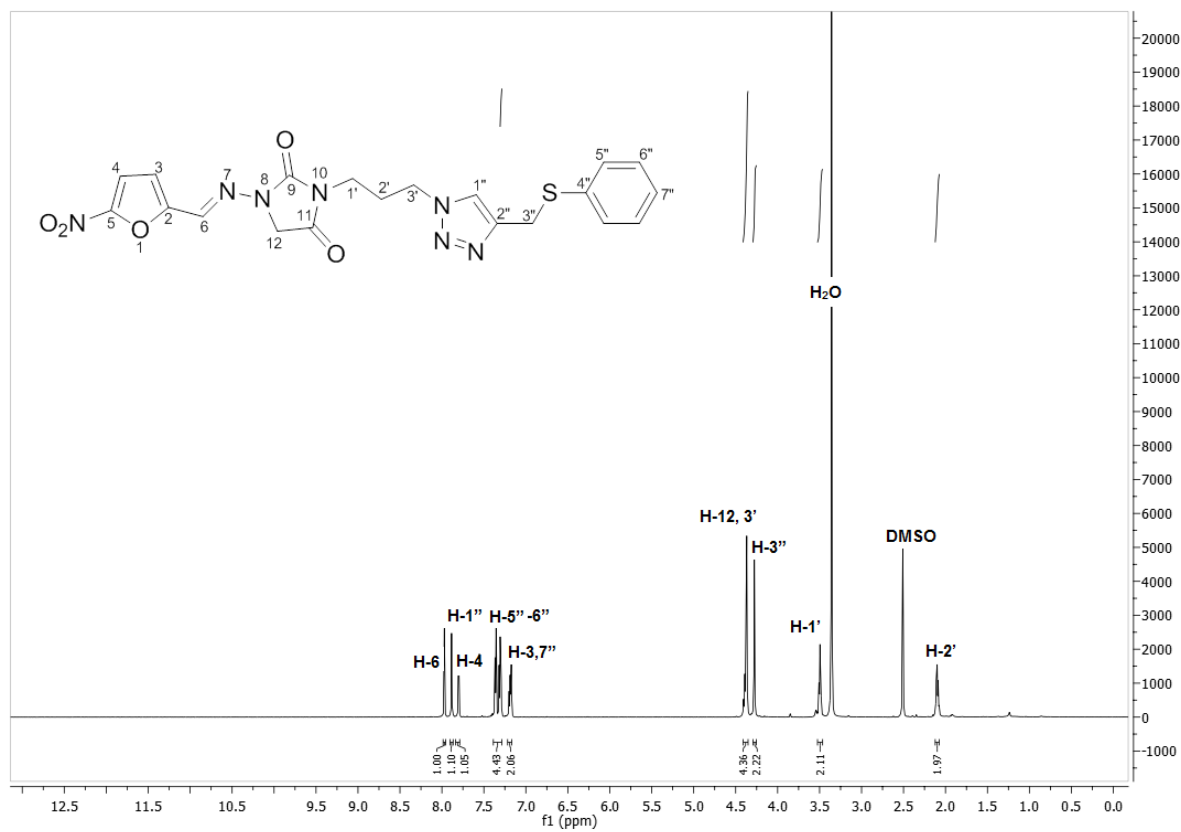


Hybrid 3308

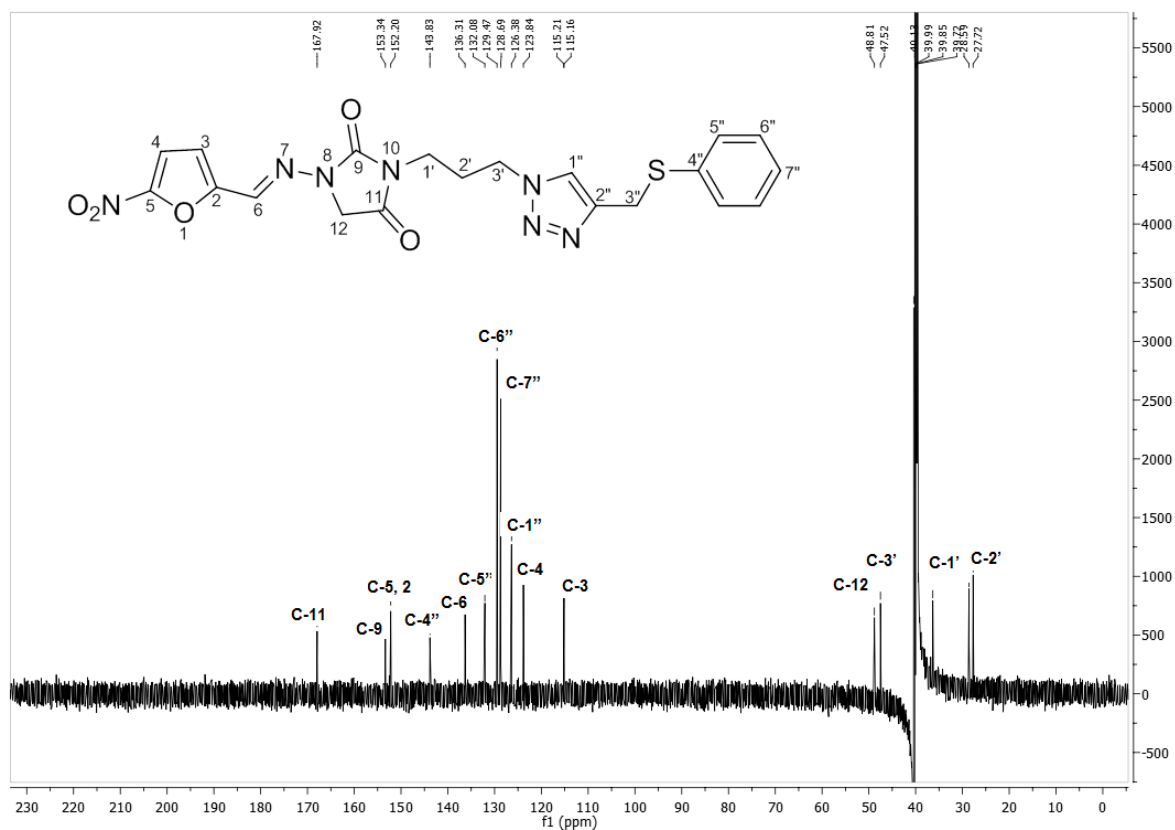
IR



¹H NMR in DMSO



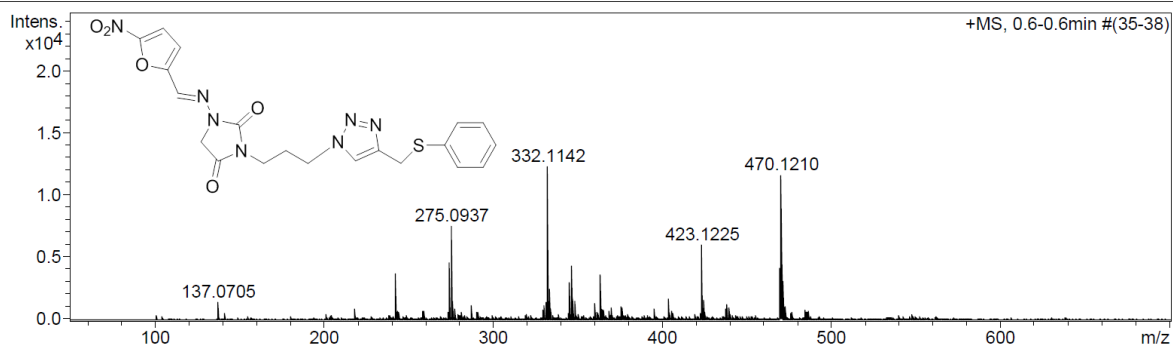
¹³C NMR in DMSO



HR-MS

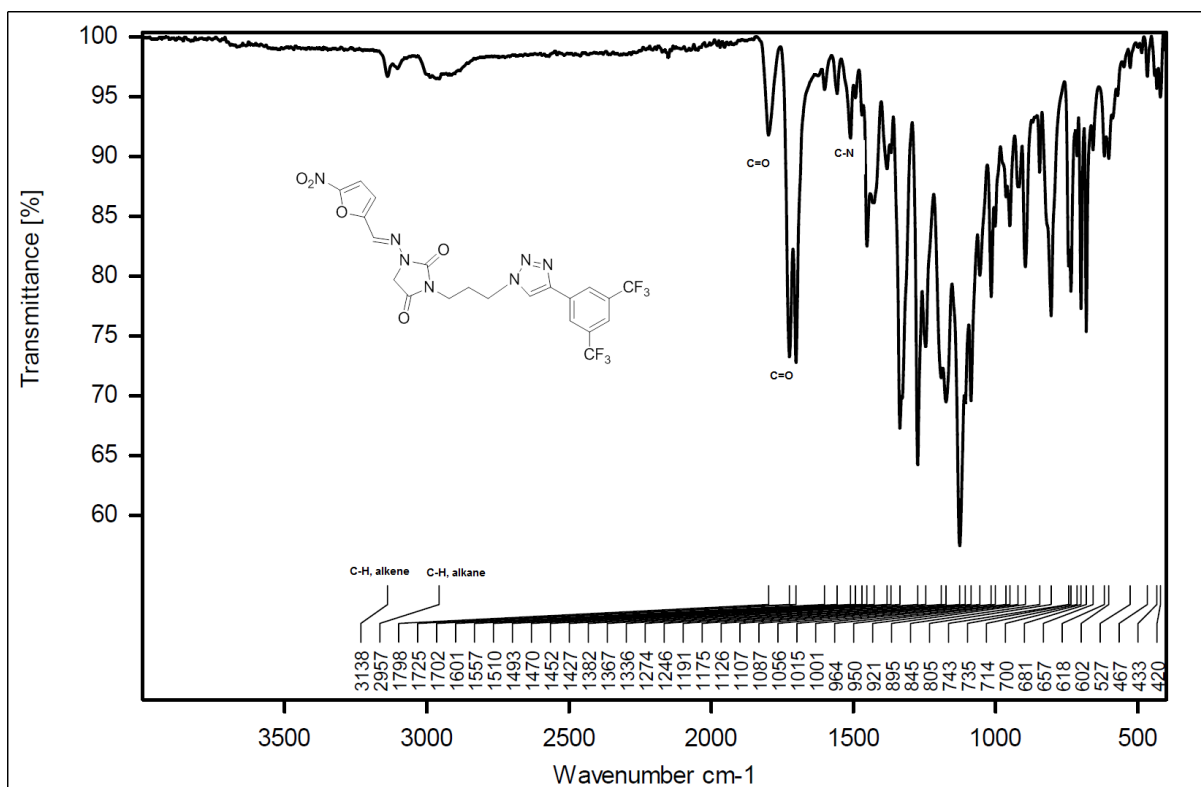
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

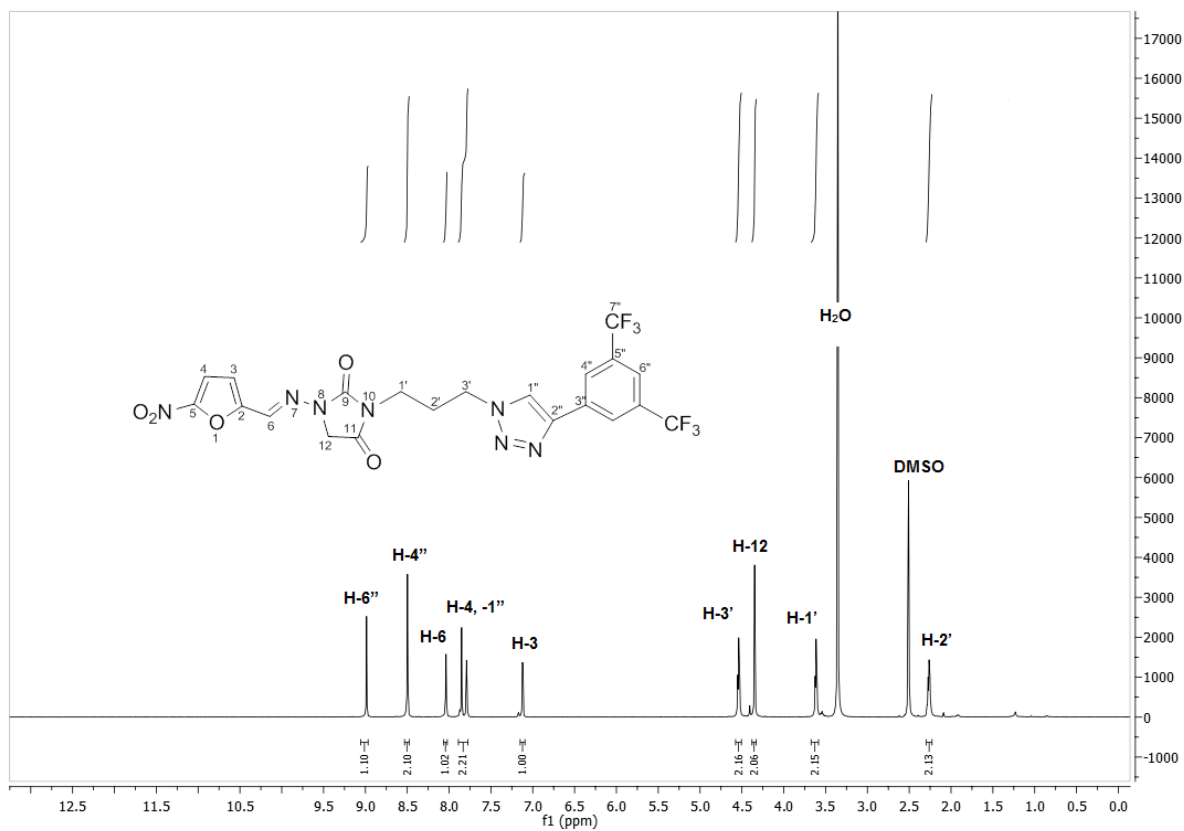


Hybrid 3309

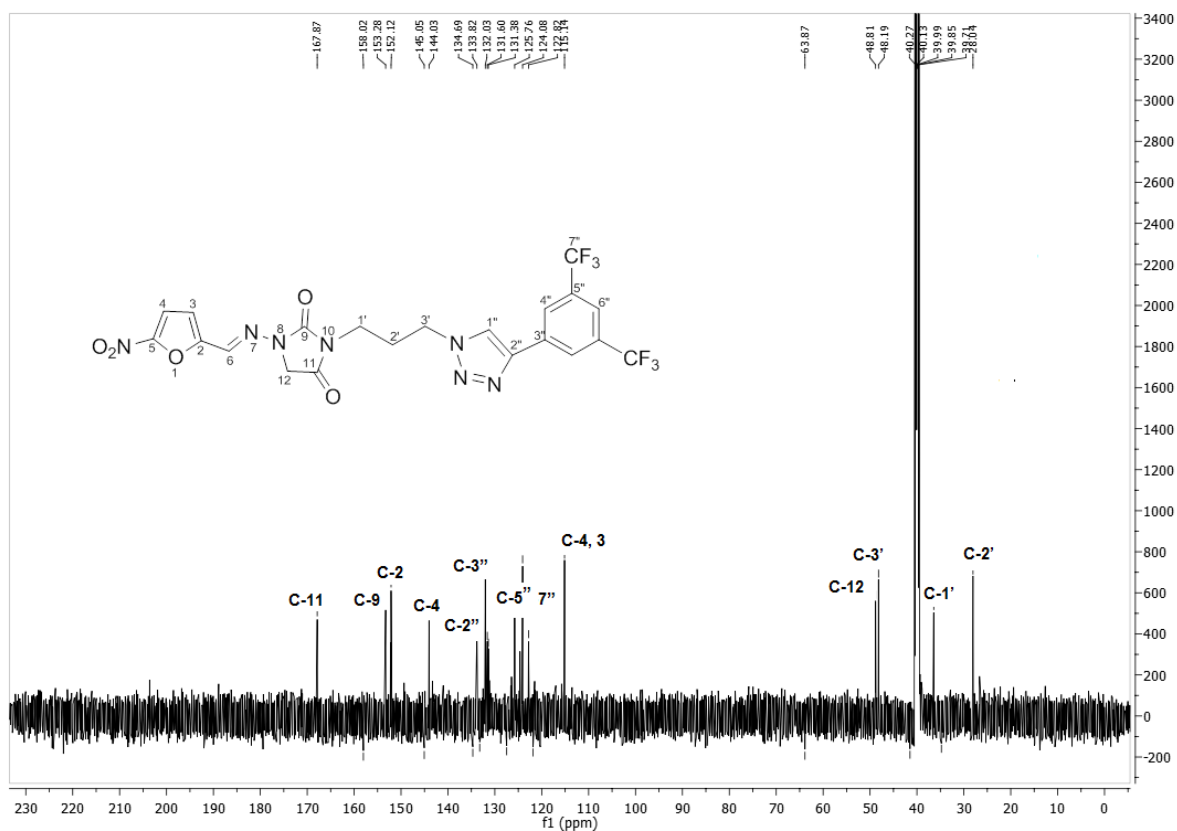
IR



¹H NMR in DMSO



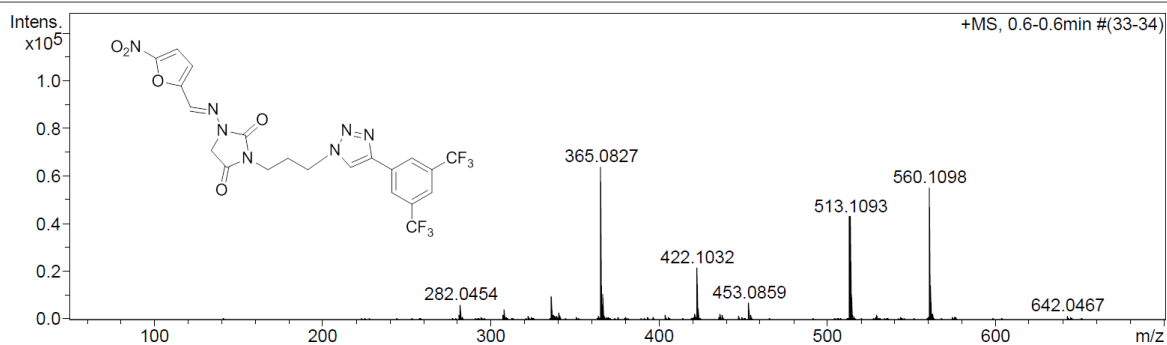
¹³C NMR in DMSO



HR-MS

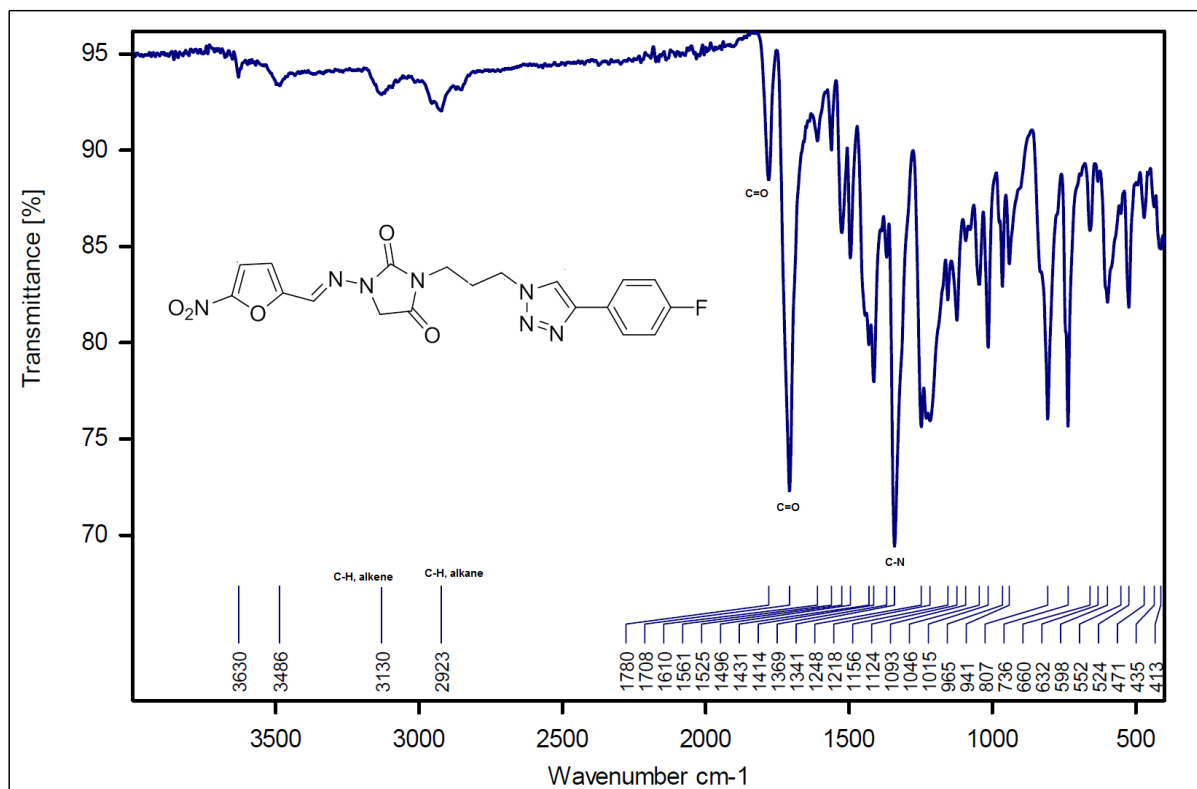
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

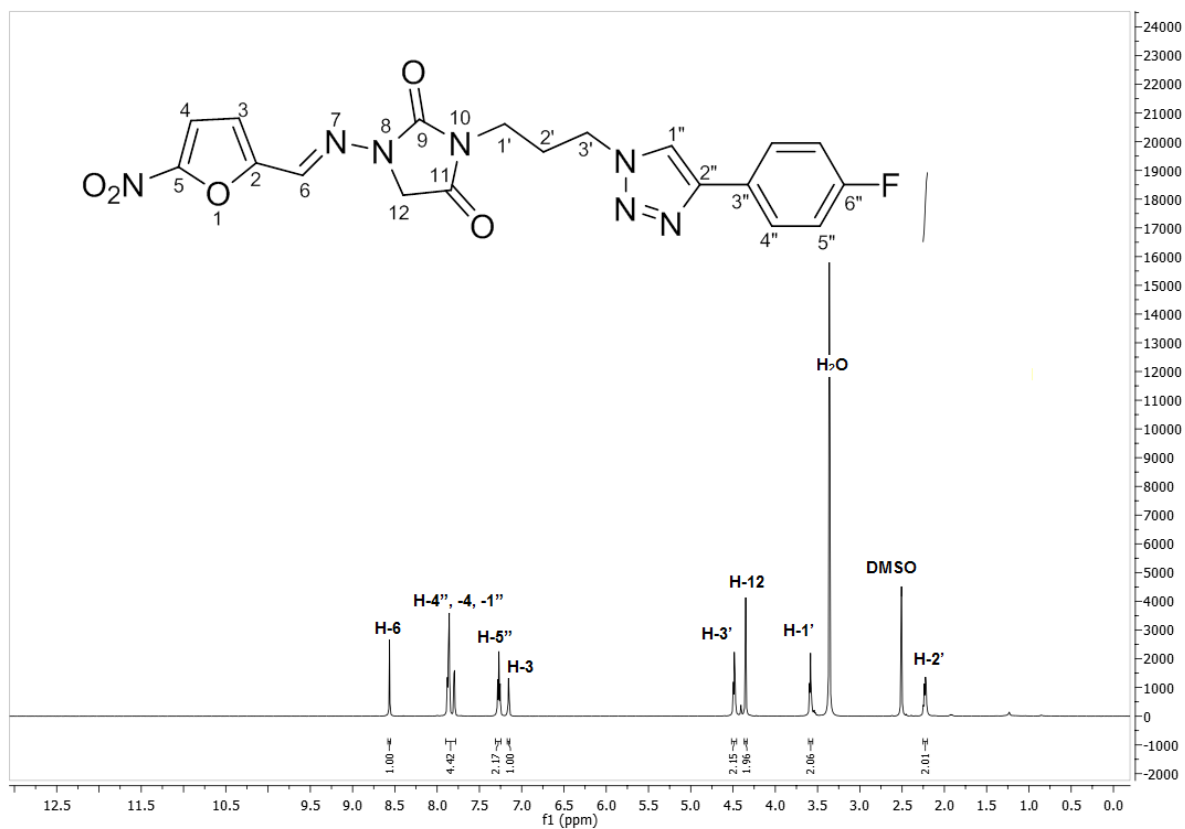


Hybrid 3310

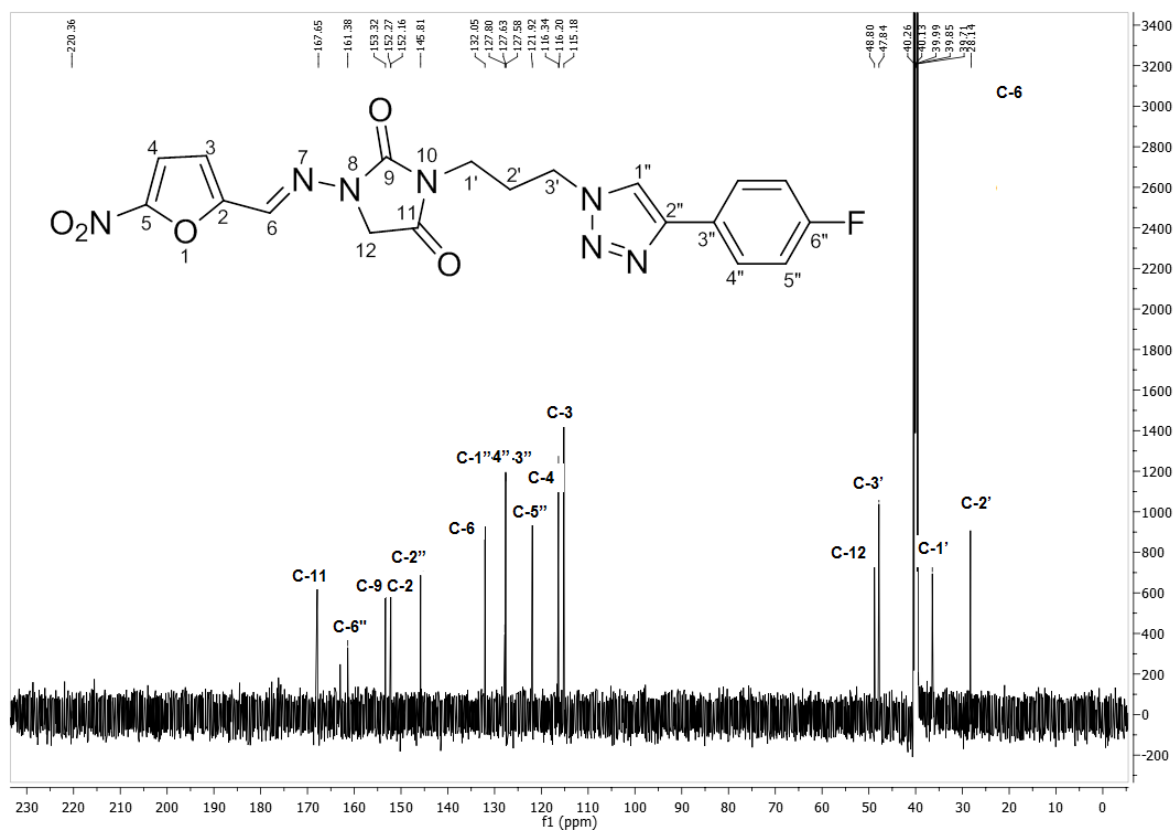
IR



¹H NMR in DMSO



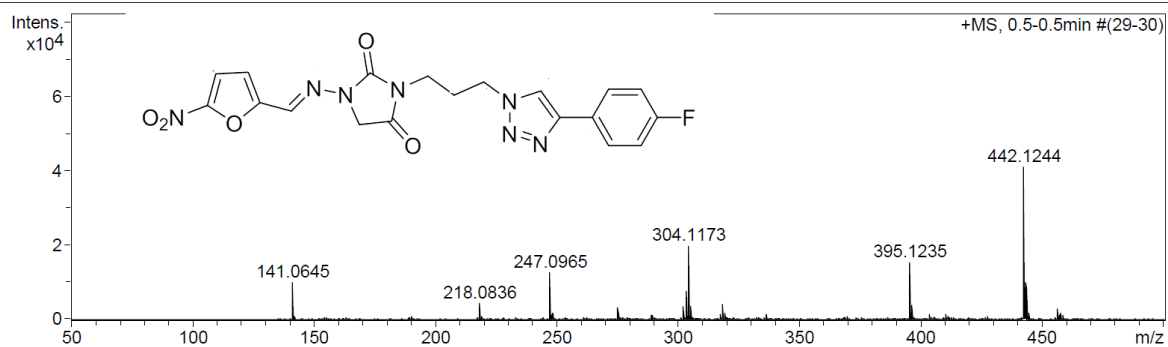
¹³C NMR in DMSO



HR-MS

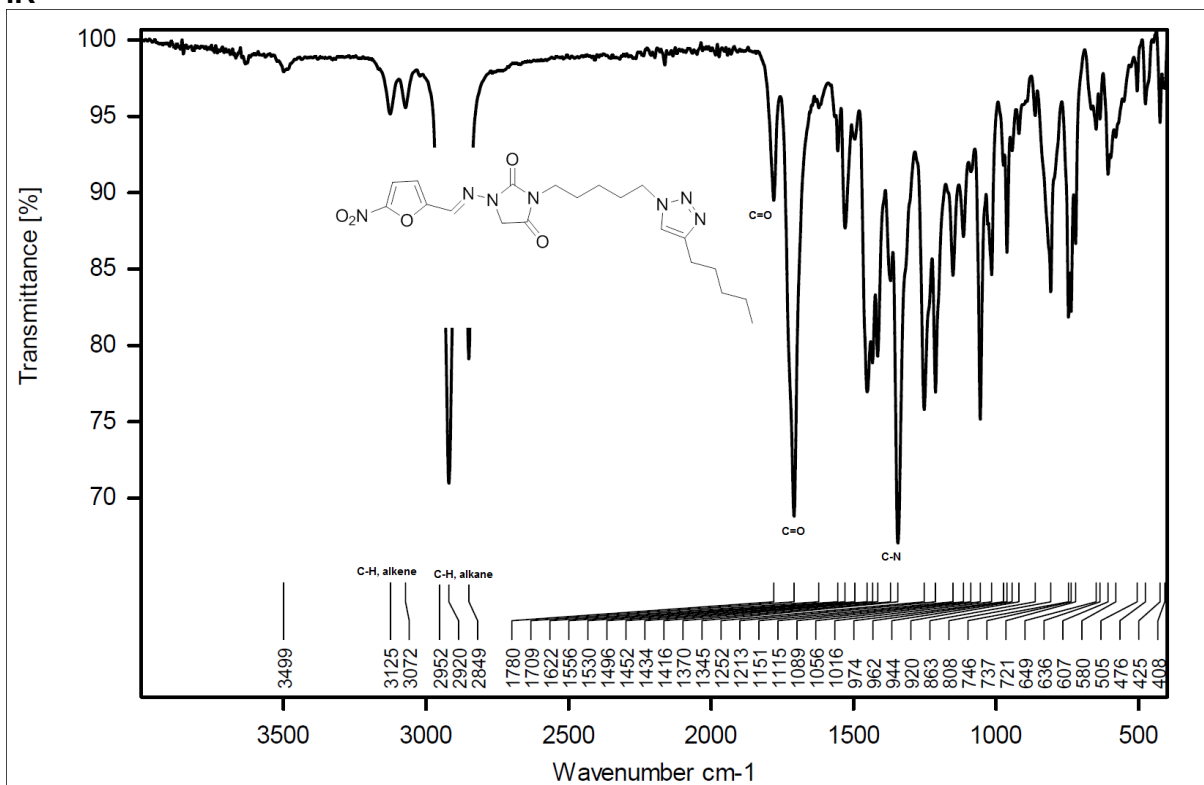
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

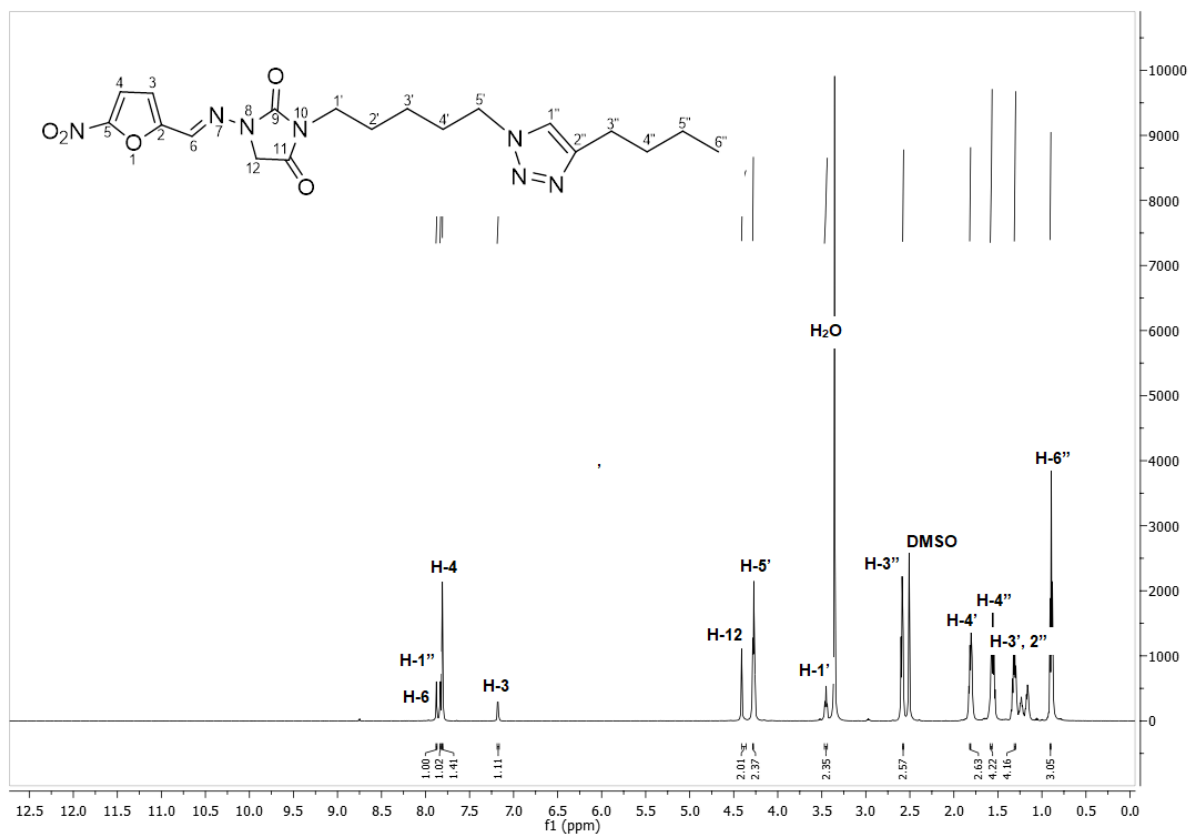


Hybrid 3501

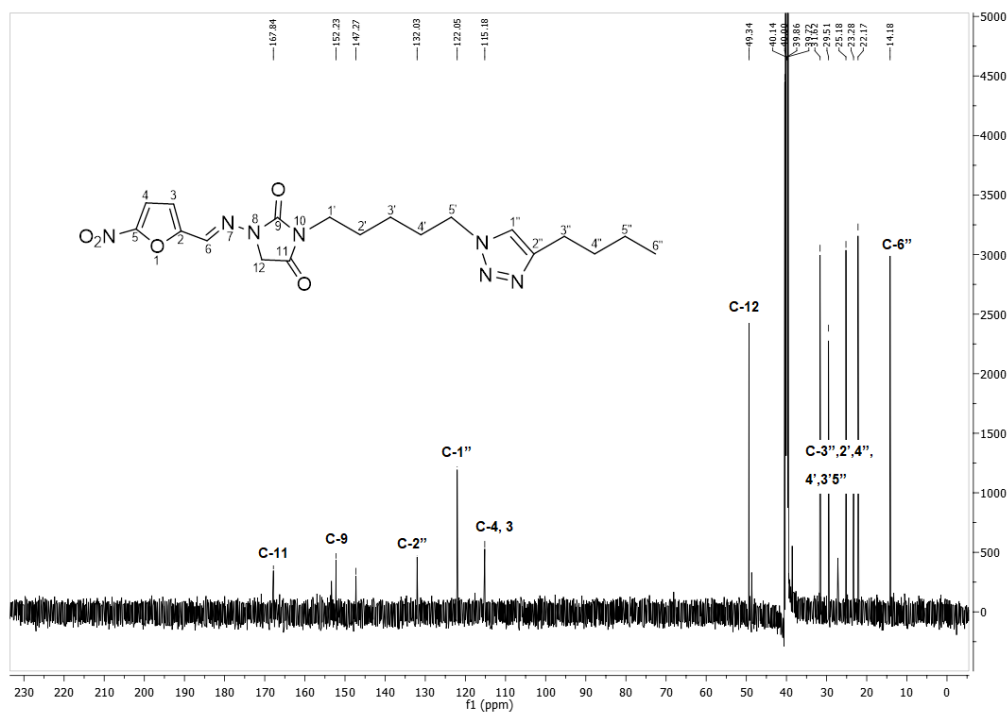
IR



¹H NMR in DMSO



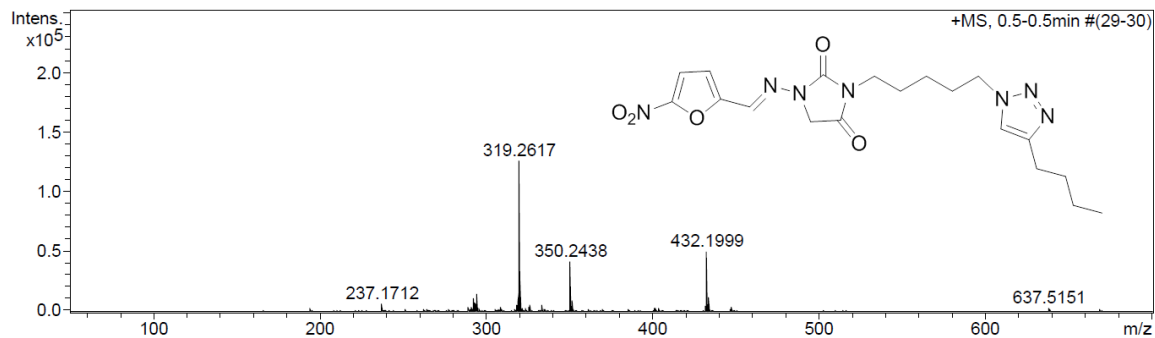
¹³C NMR in DMSO



HR-MS

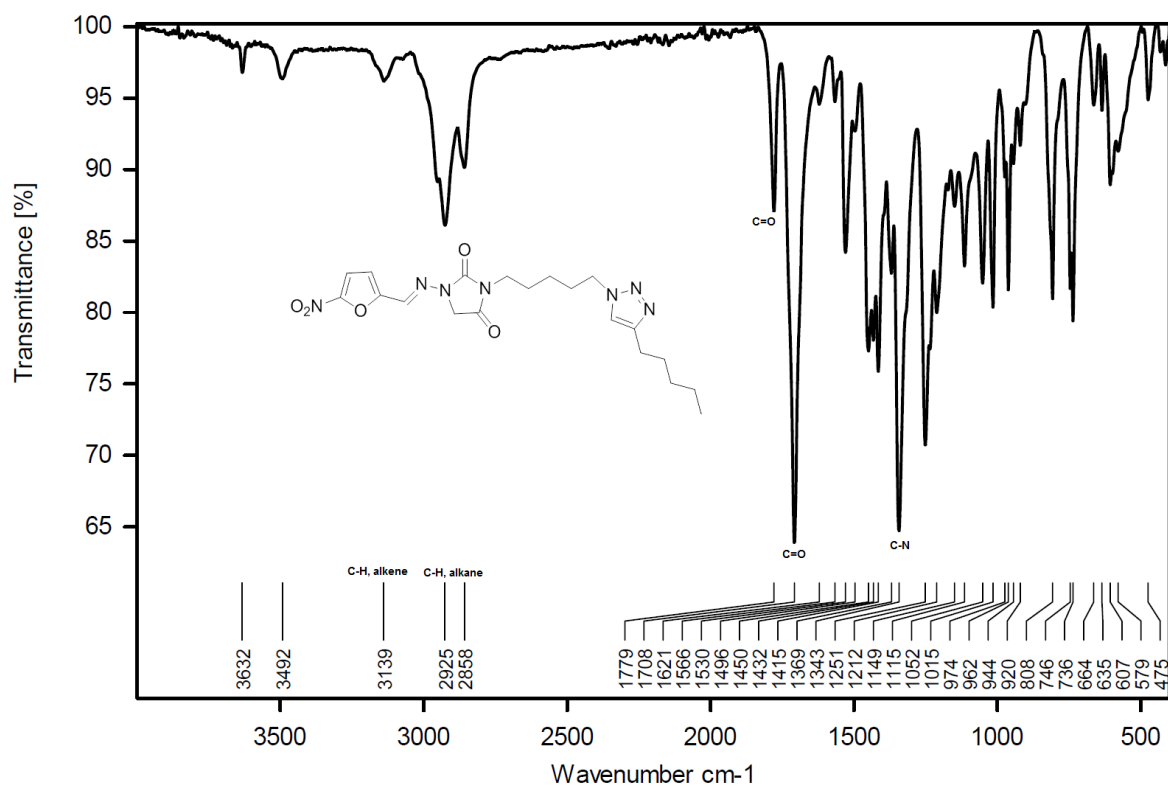
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
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Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

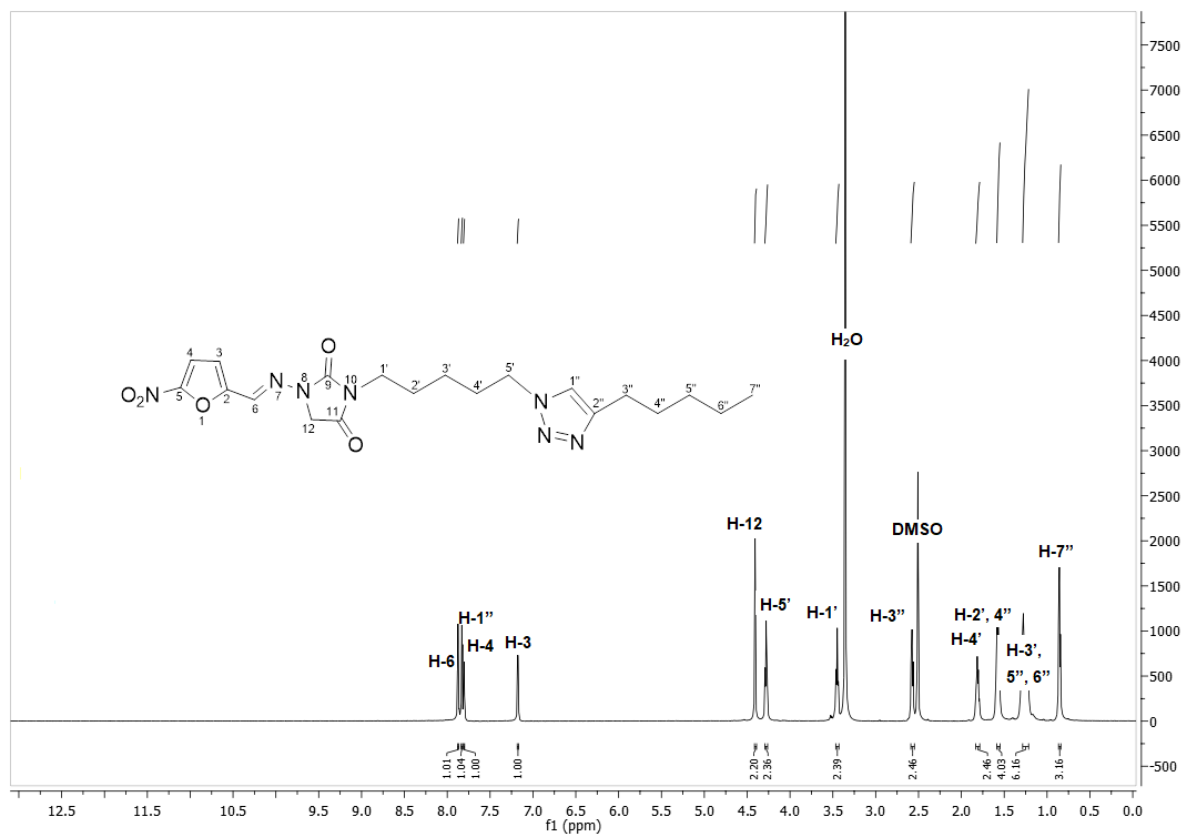


Hybrid 3502

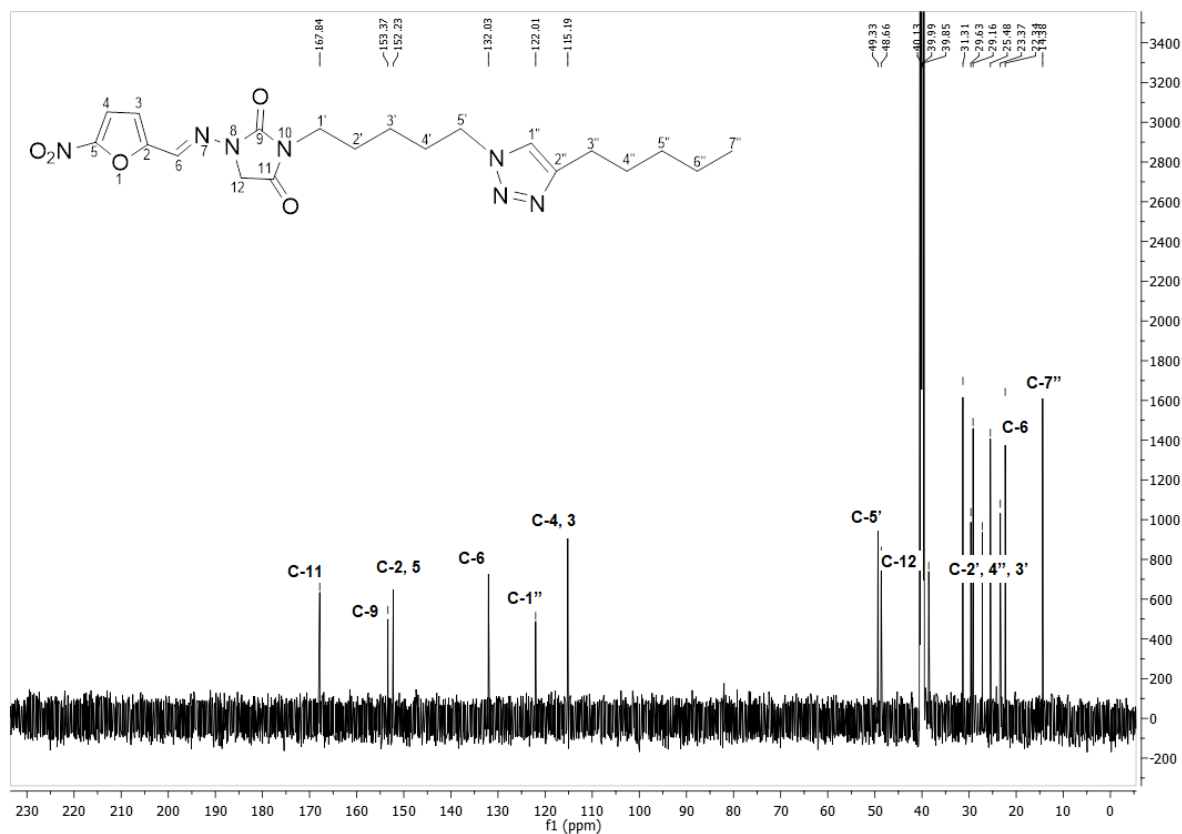
IR



¹H NMR in DMSO



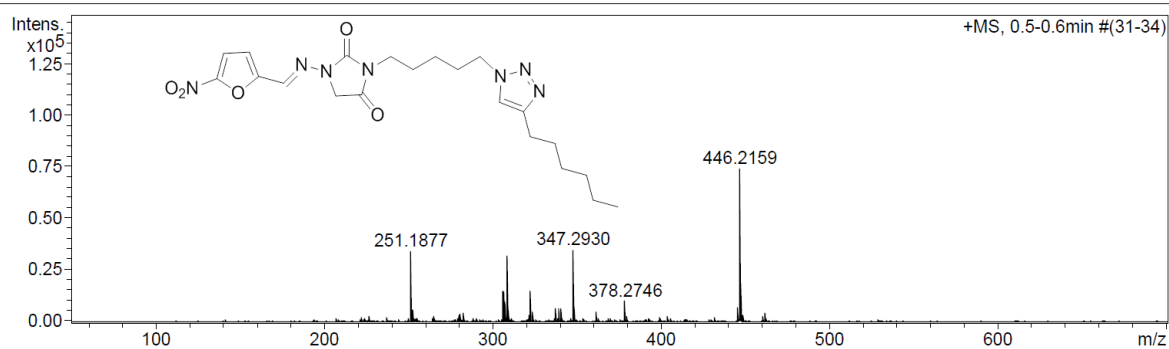
¹³C NMR in DMSO



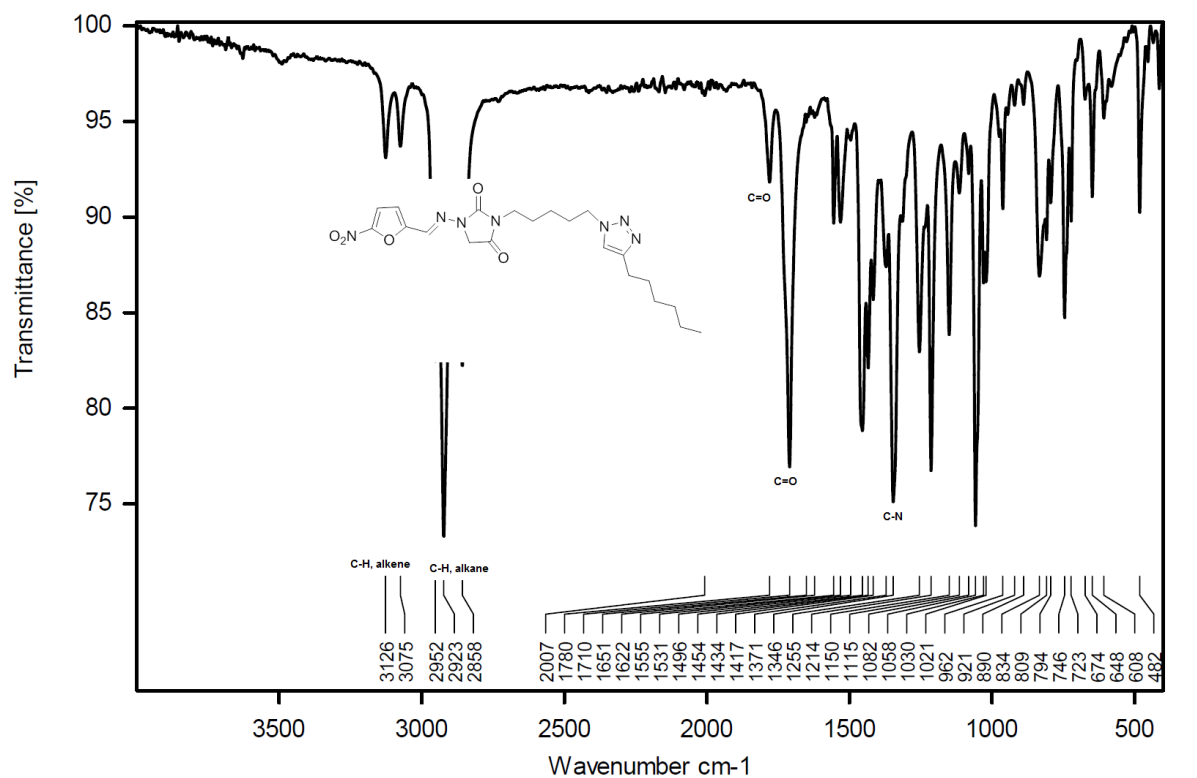
HR-MS

Acquisition Parameter

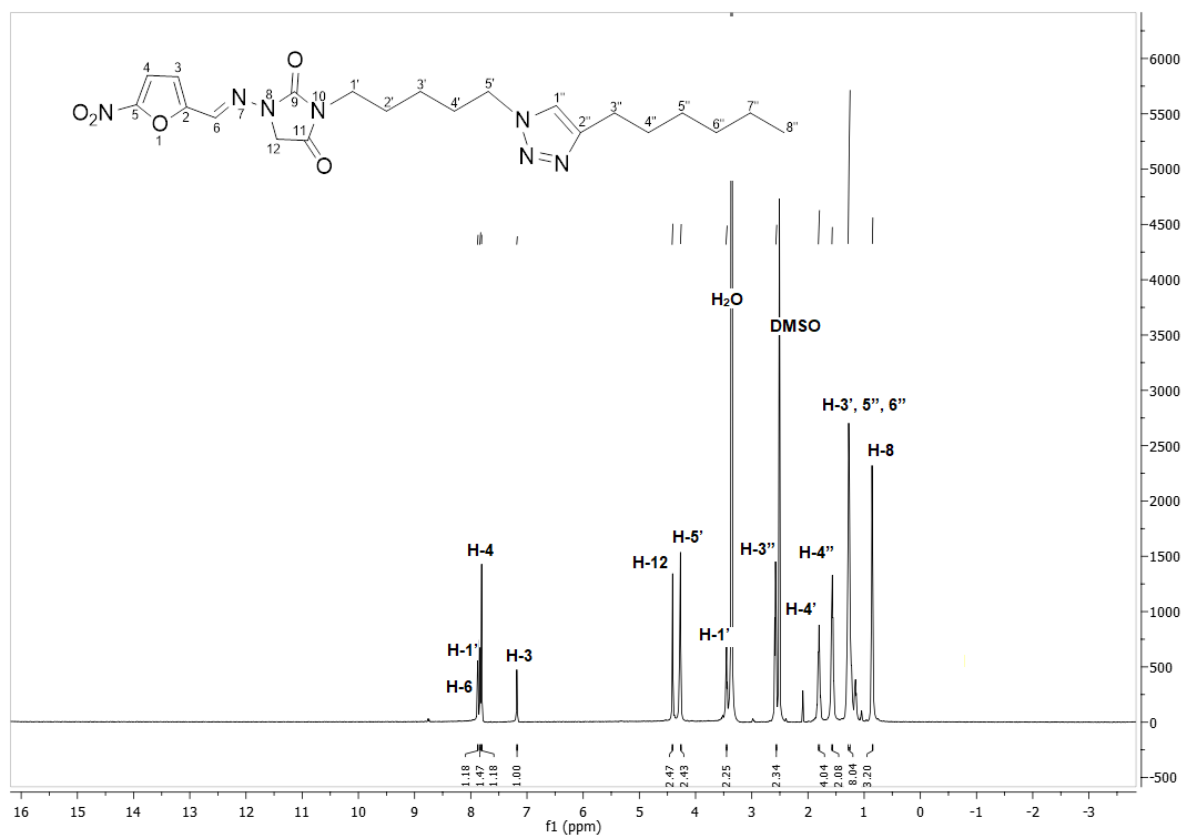
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Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



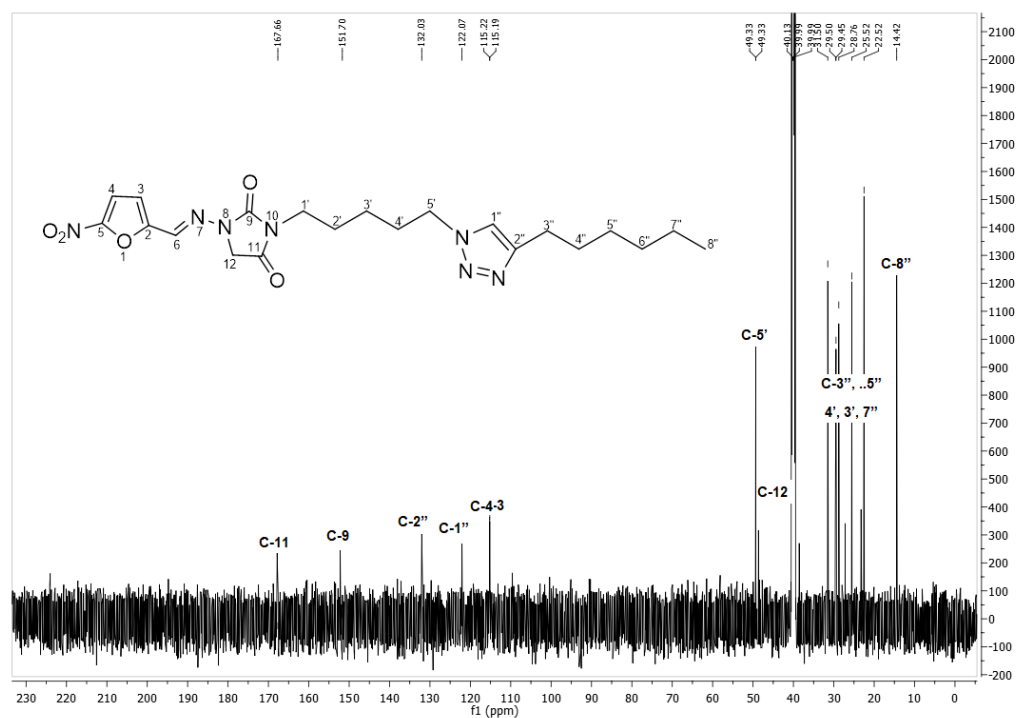
IR



¹H NMR in DMSO



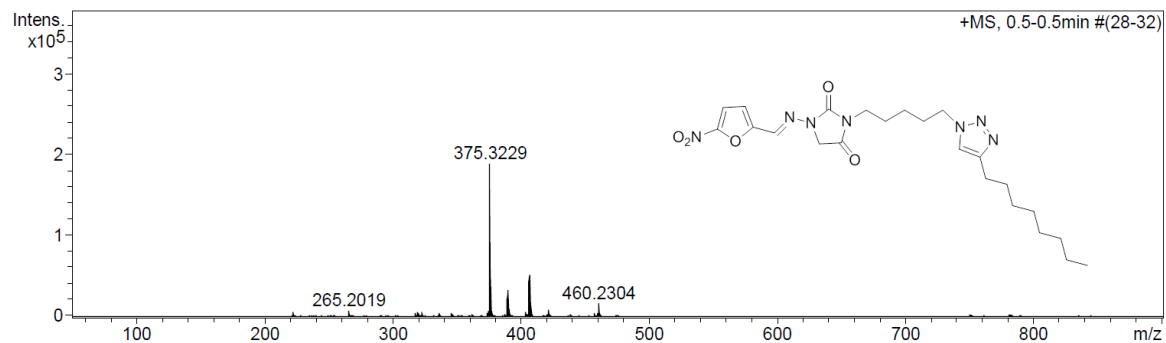
¹³C NMR in DMSO



HR-MS

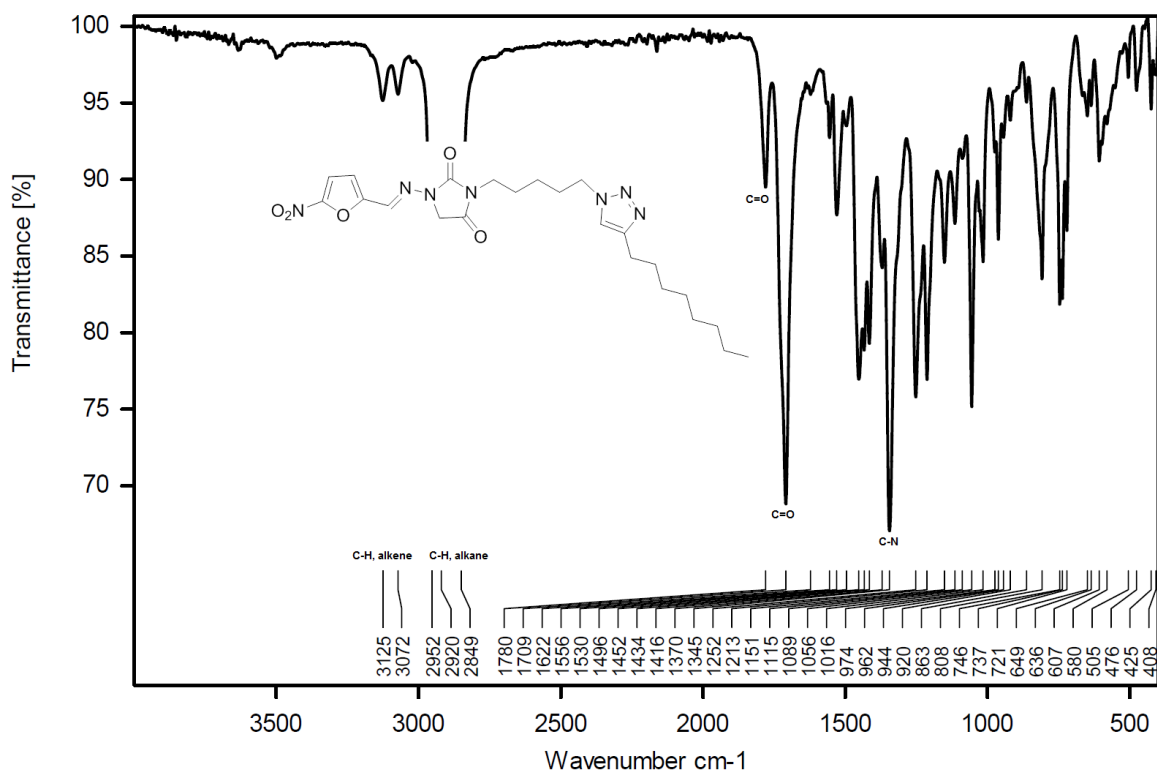
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

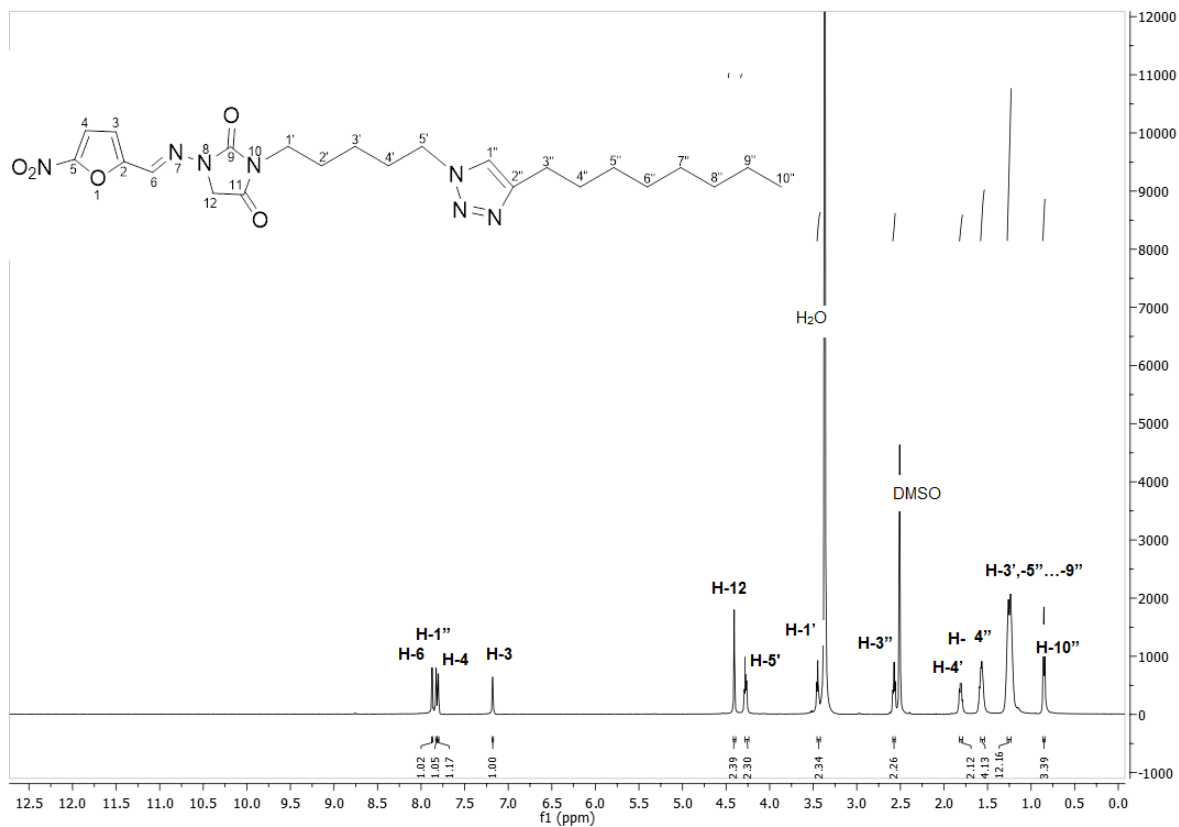


Hybrid 3504

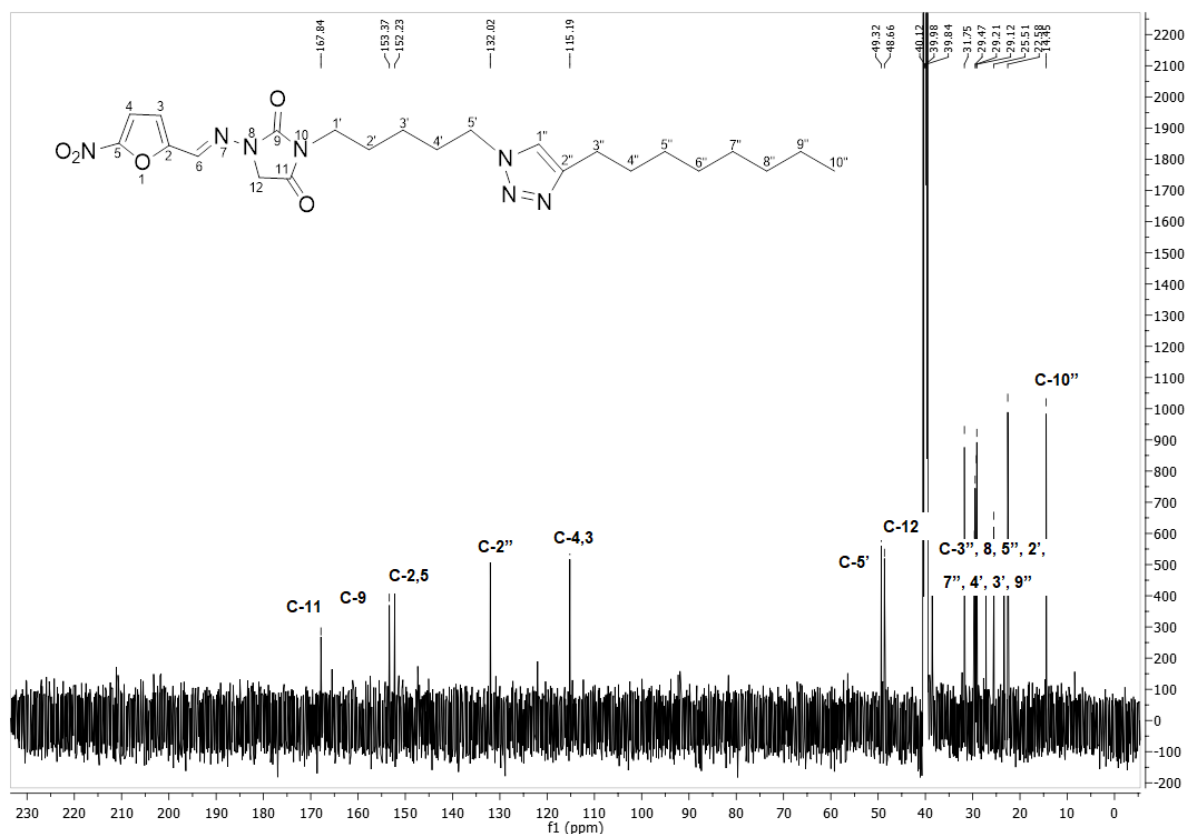
IR



¹H NMR in DMSO



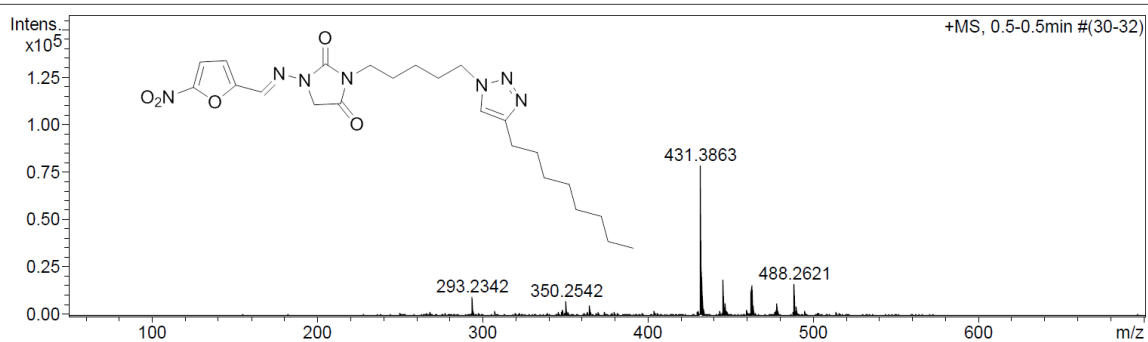
¹³C NMR in DMSO



HR-MS

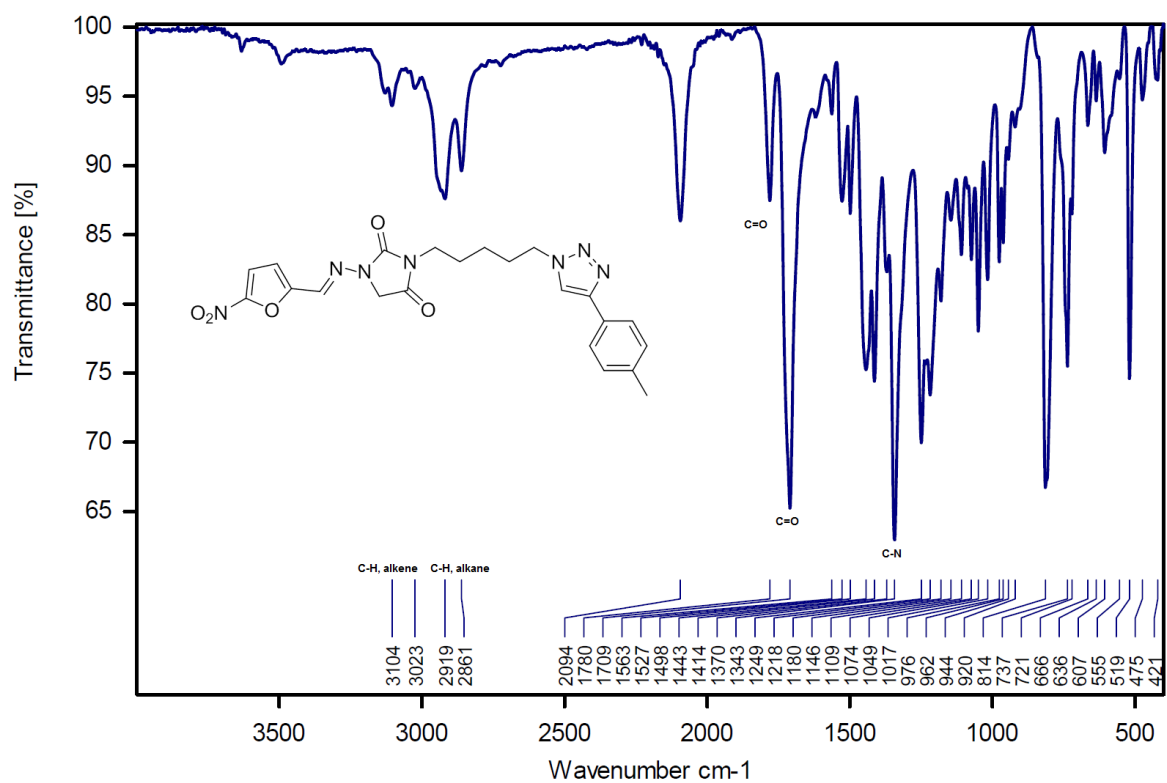
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Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

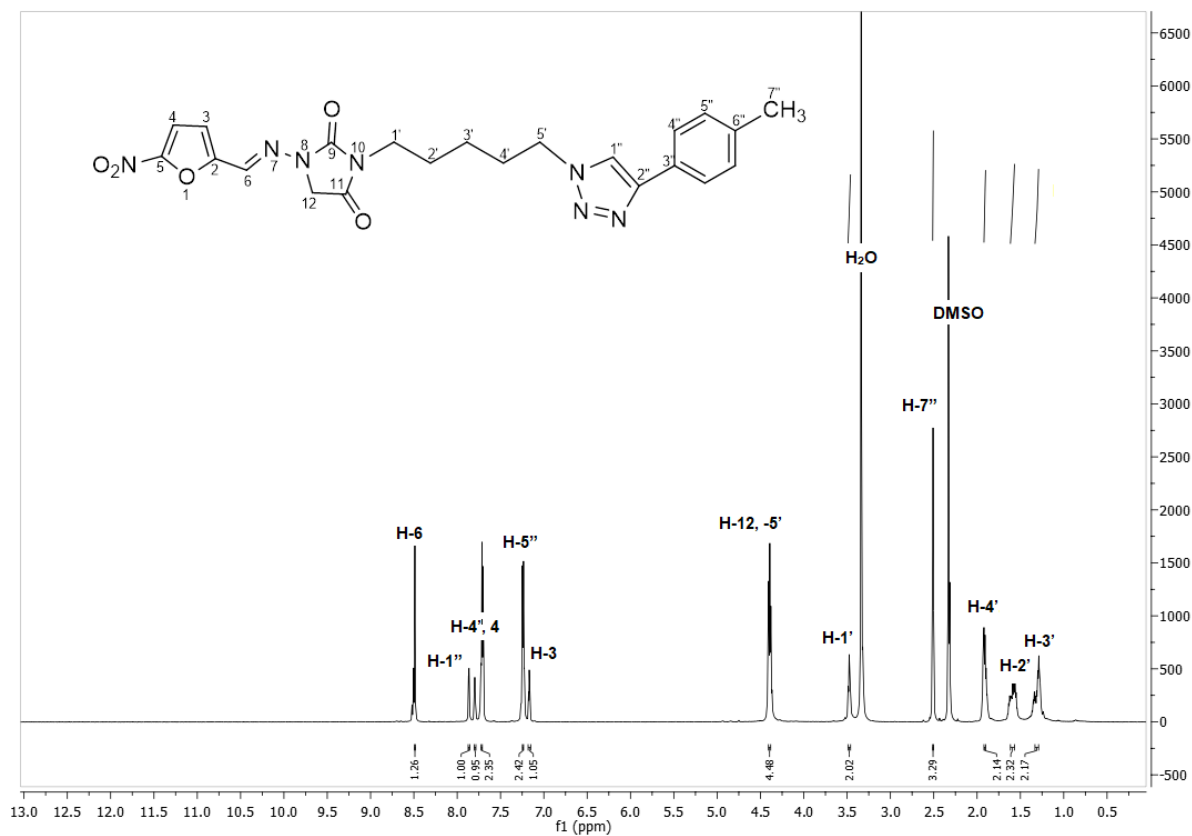


Hybrid 3505

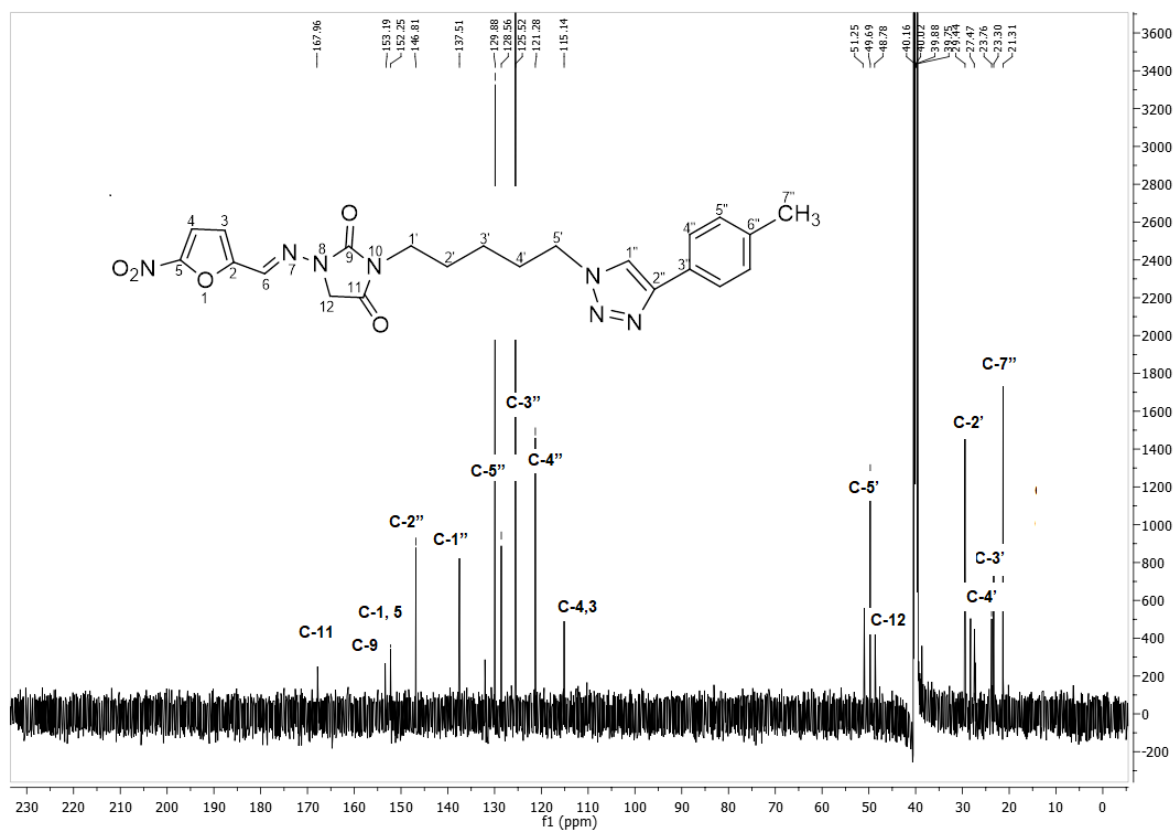
IR



¹H NMR in DMSO



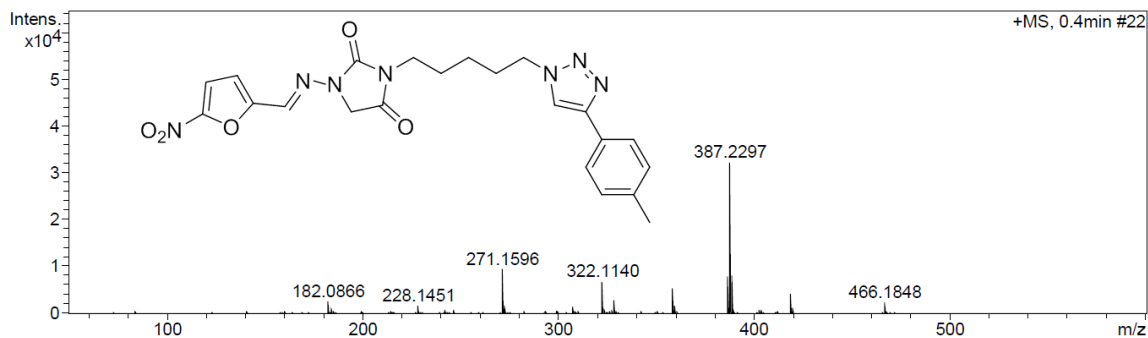
¹³C NMR in DMSO



HR-MS

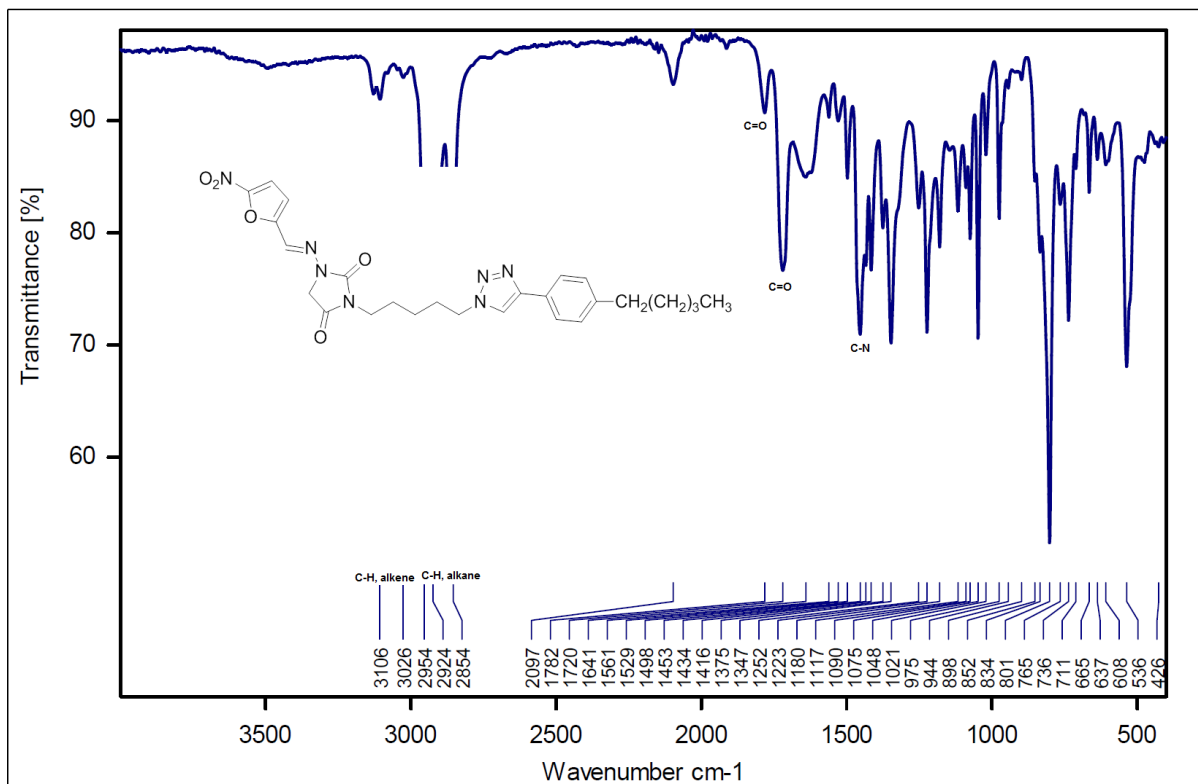
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

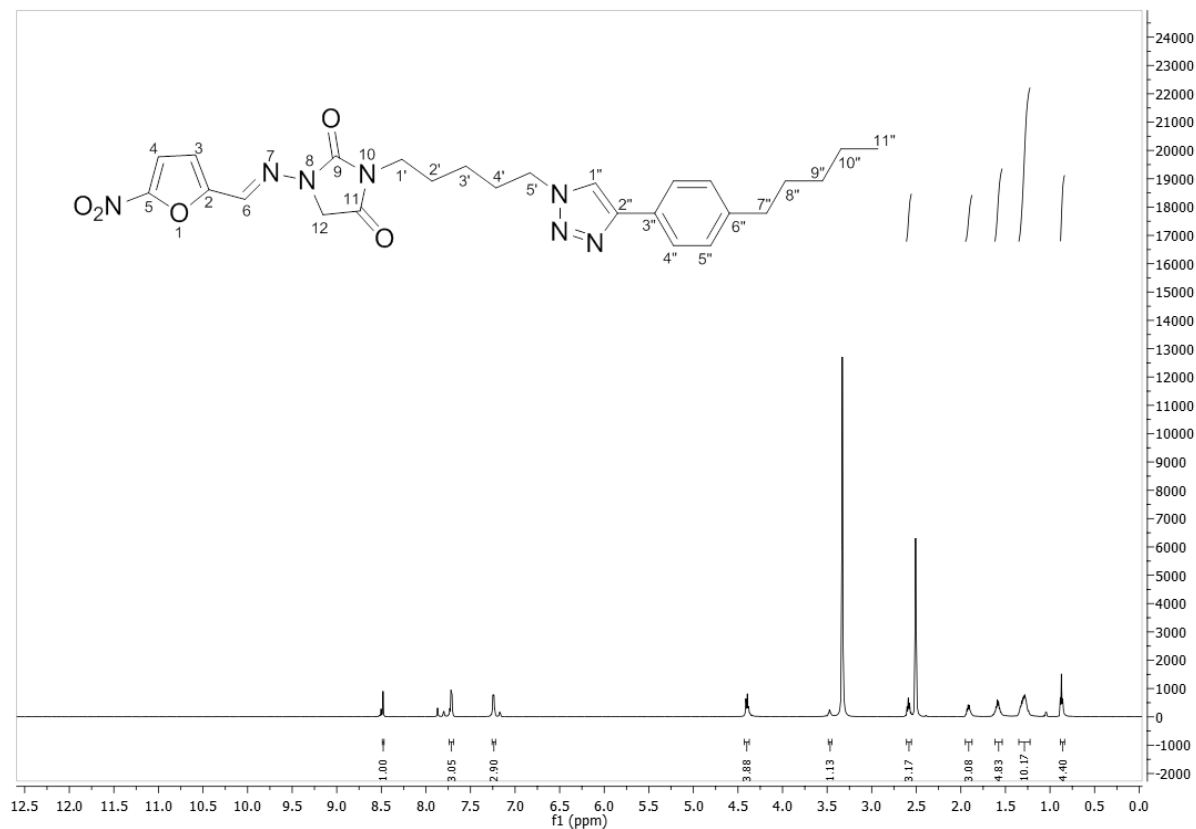


Hybrid 3506

IR



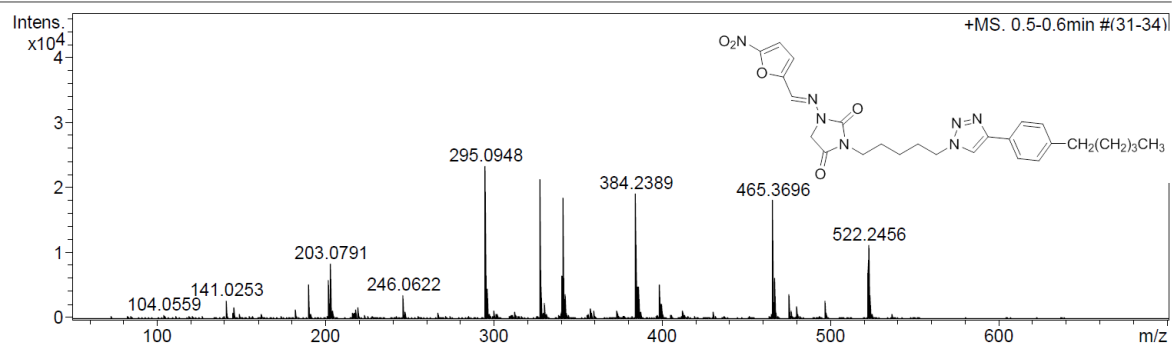
¹H NMR in DMSO



HR-MS

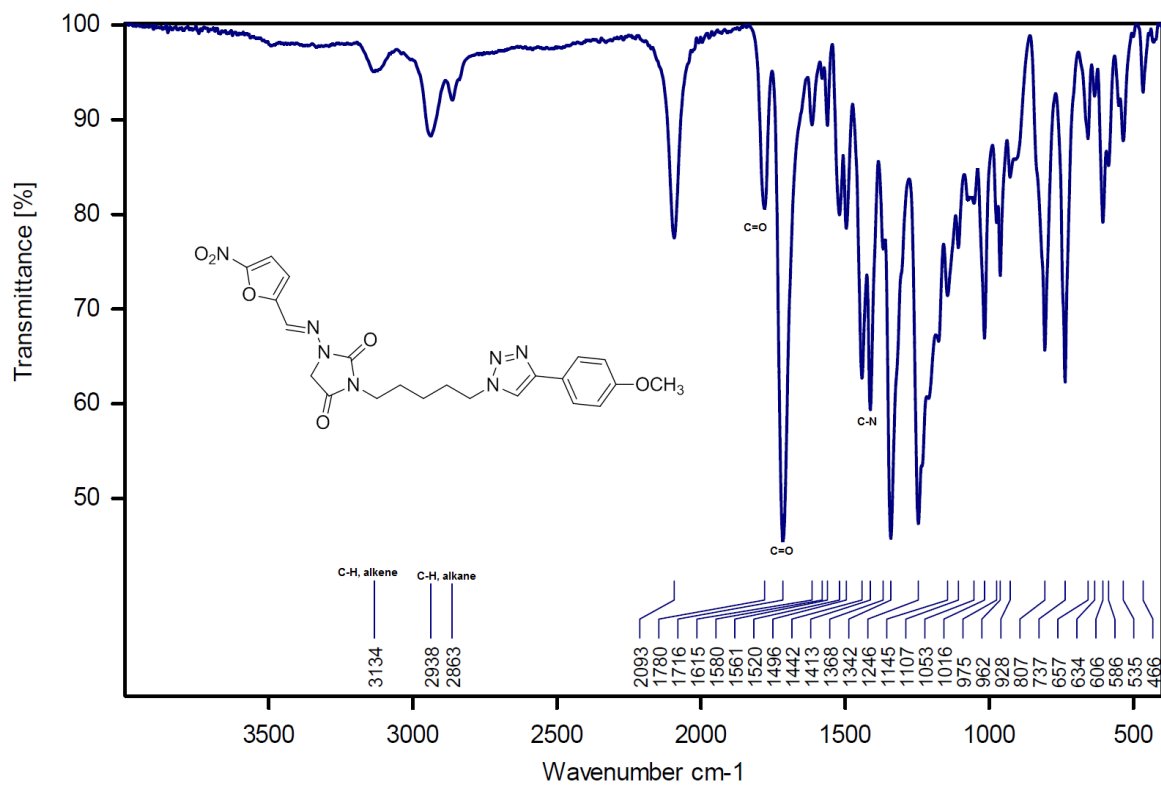
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
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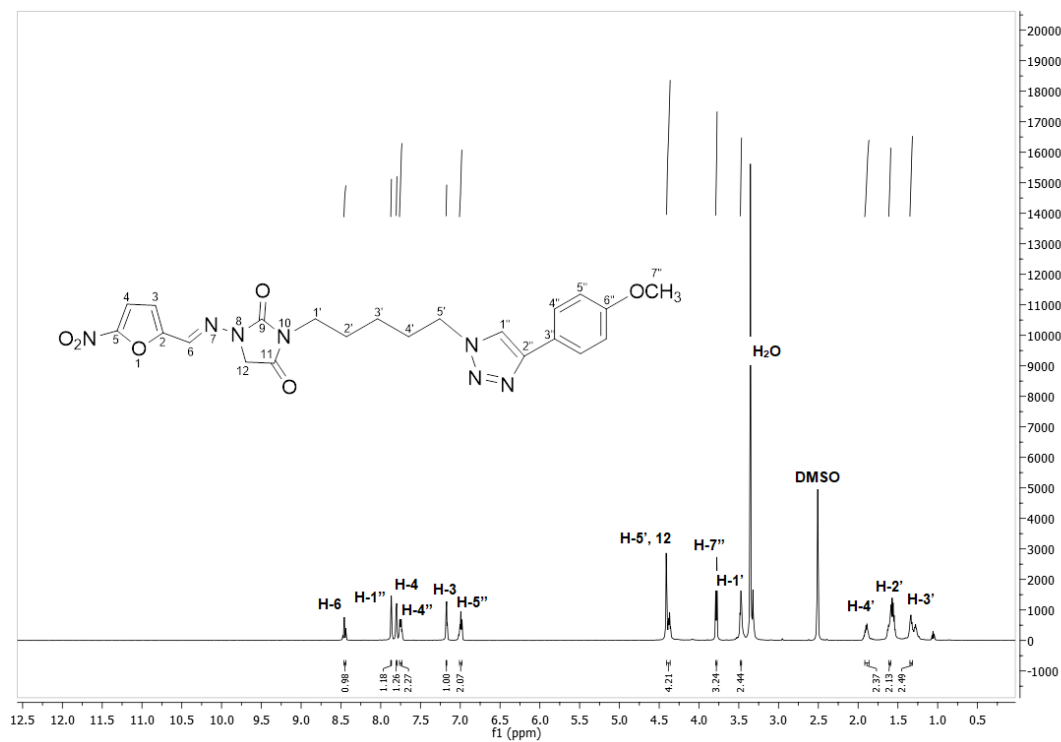


Hybrid 3507

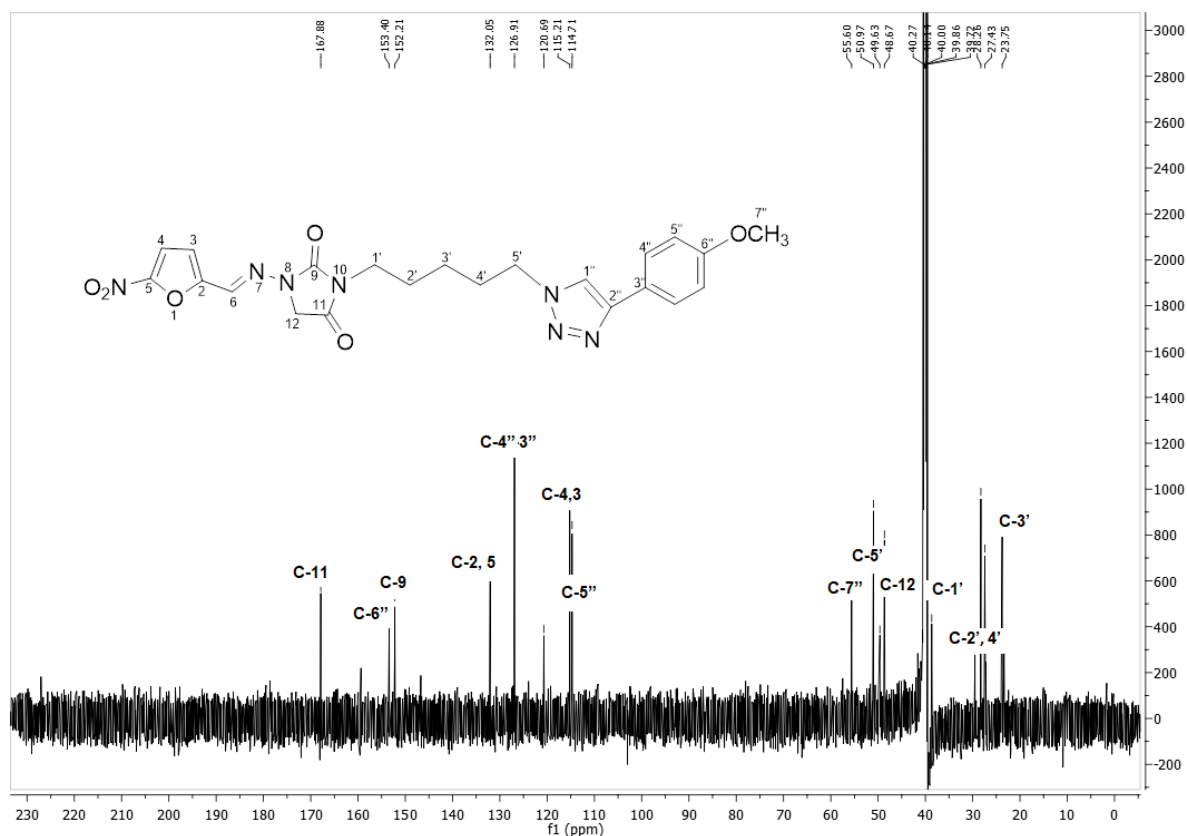
IR



¹H NMR in DMSO



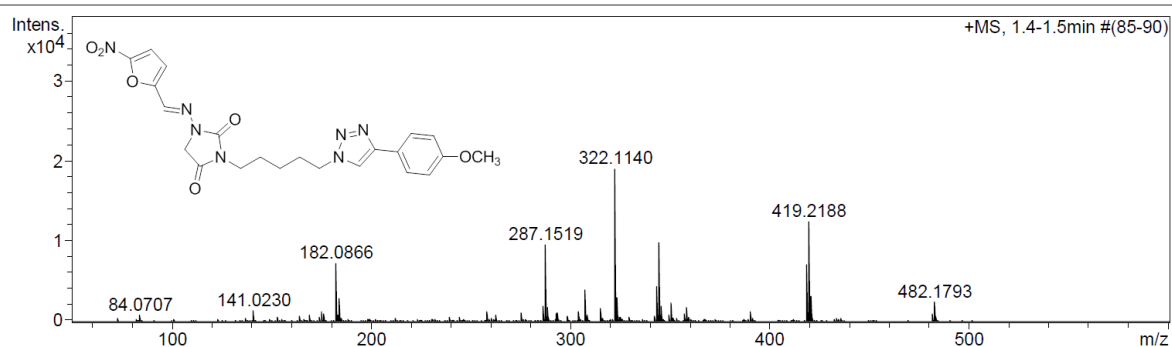
¹³C NMR in DMSO



HR-MS

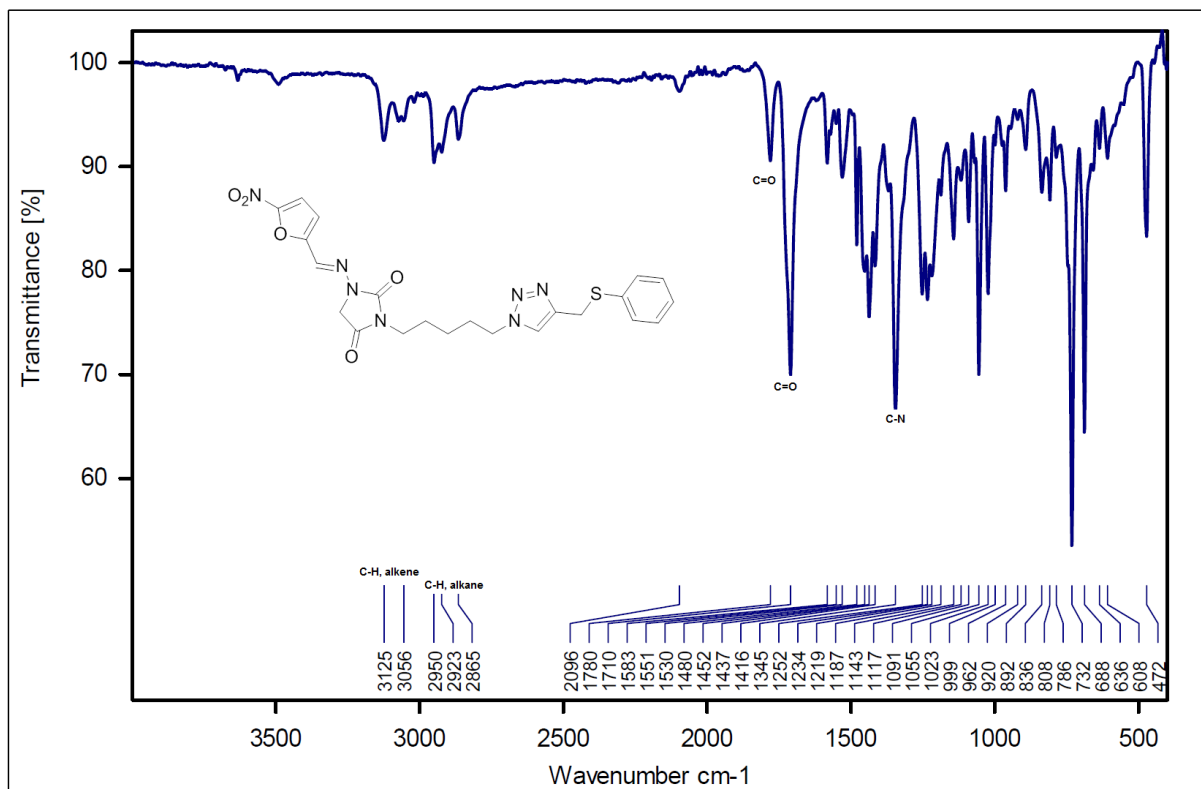
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

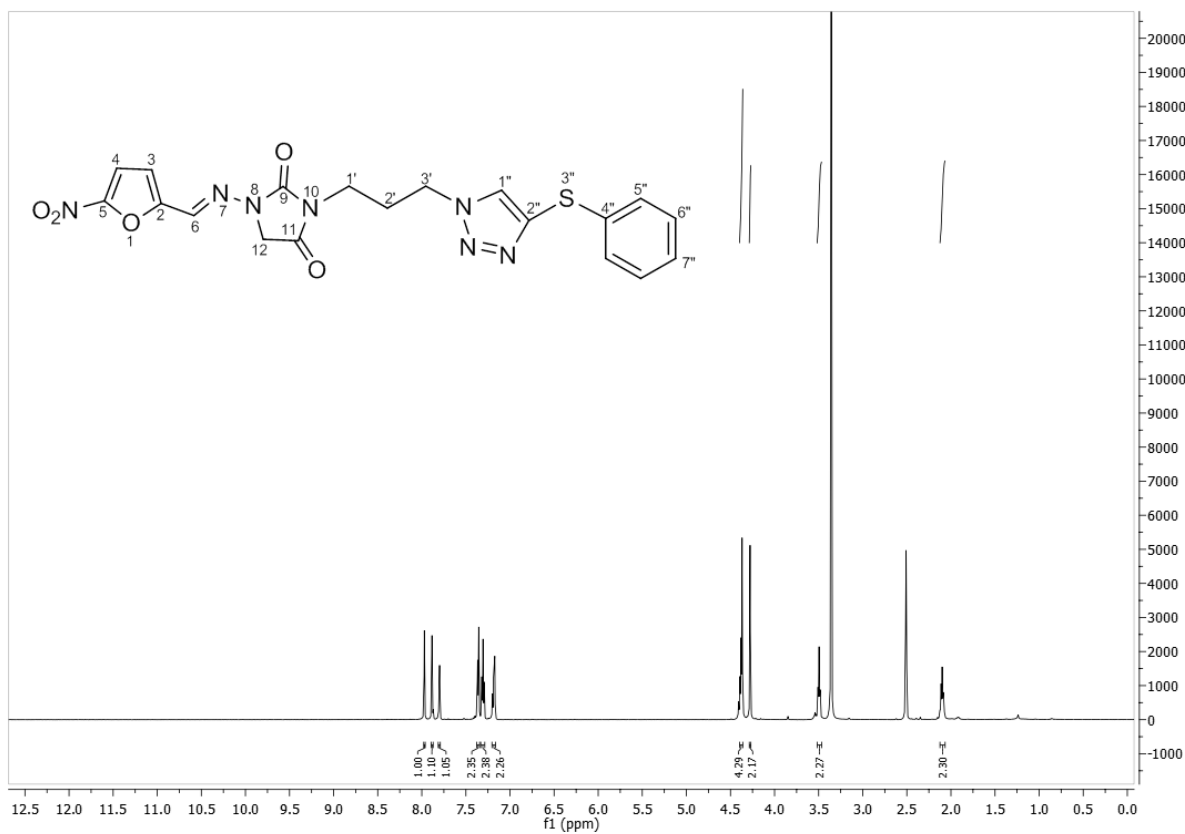


Hybrid 3208

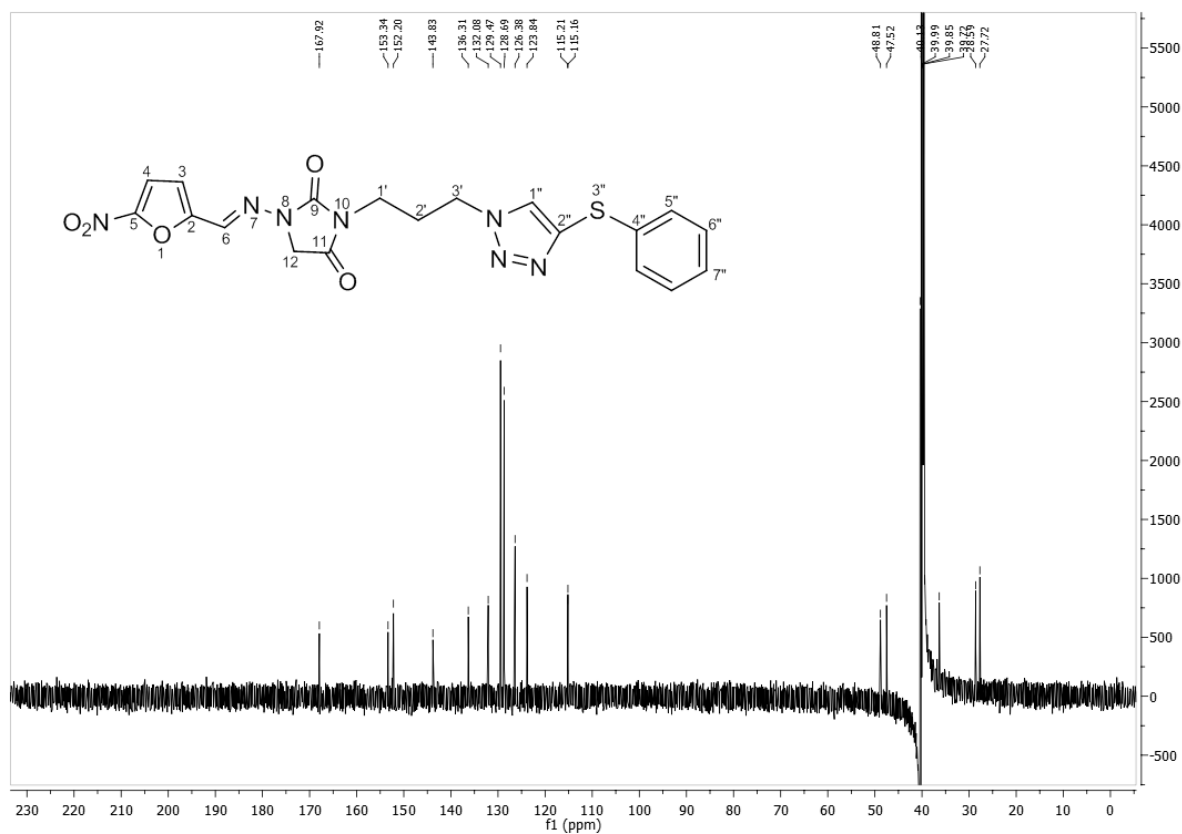
IR



¹H NMR in DMSO



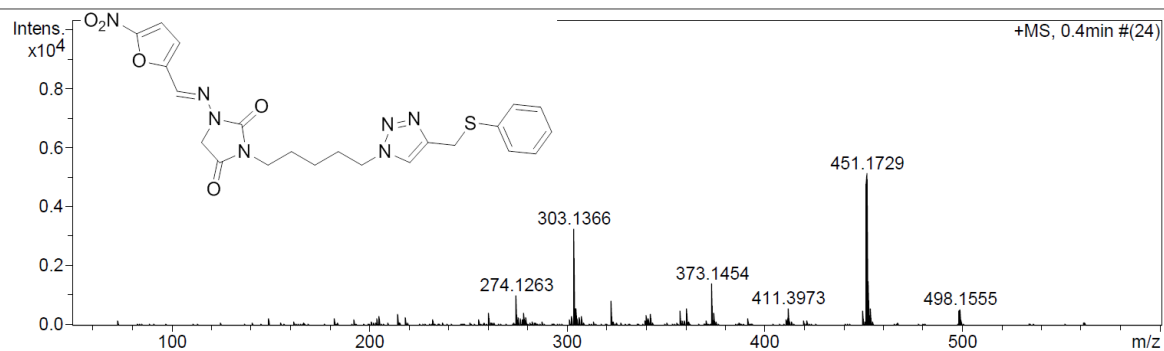
¹³C NMR in DMSO



HR-MS

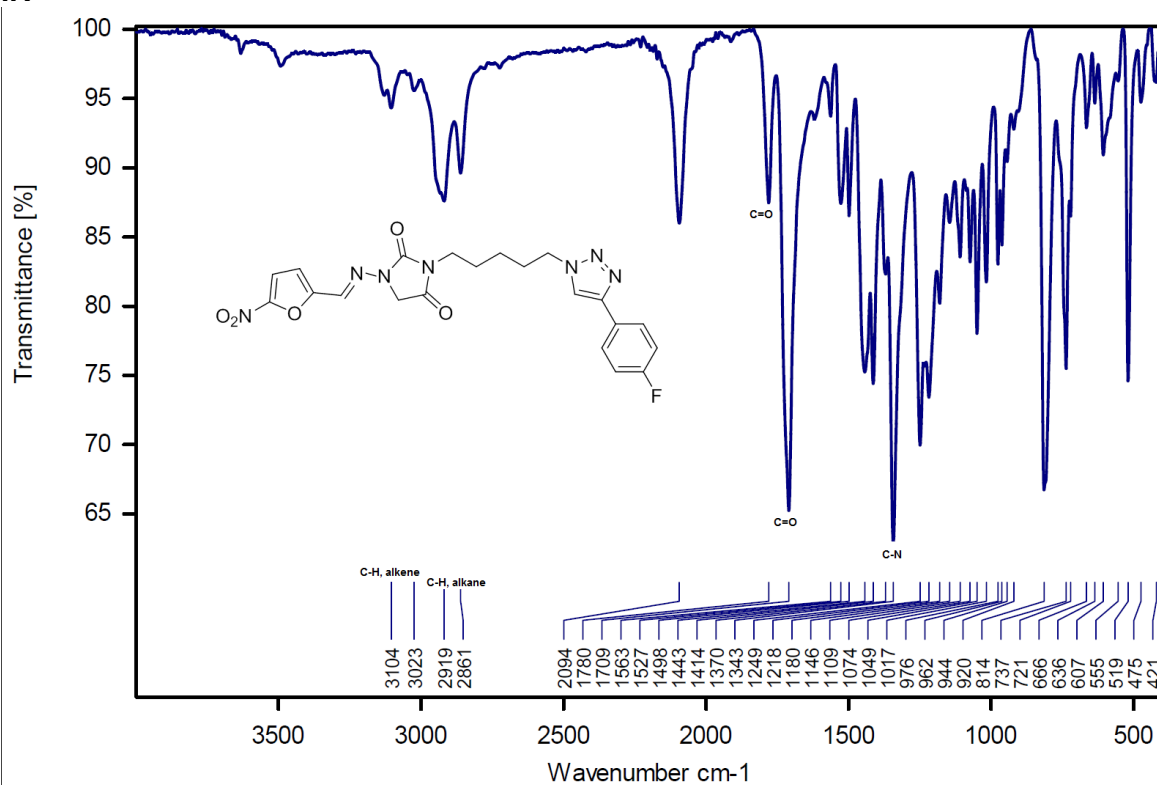
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

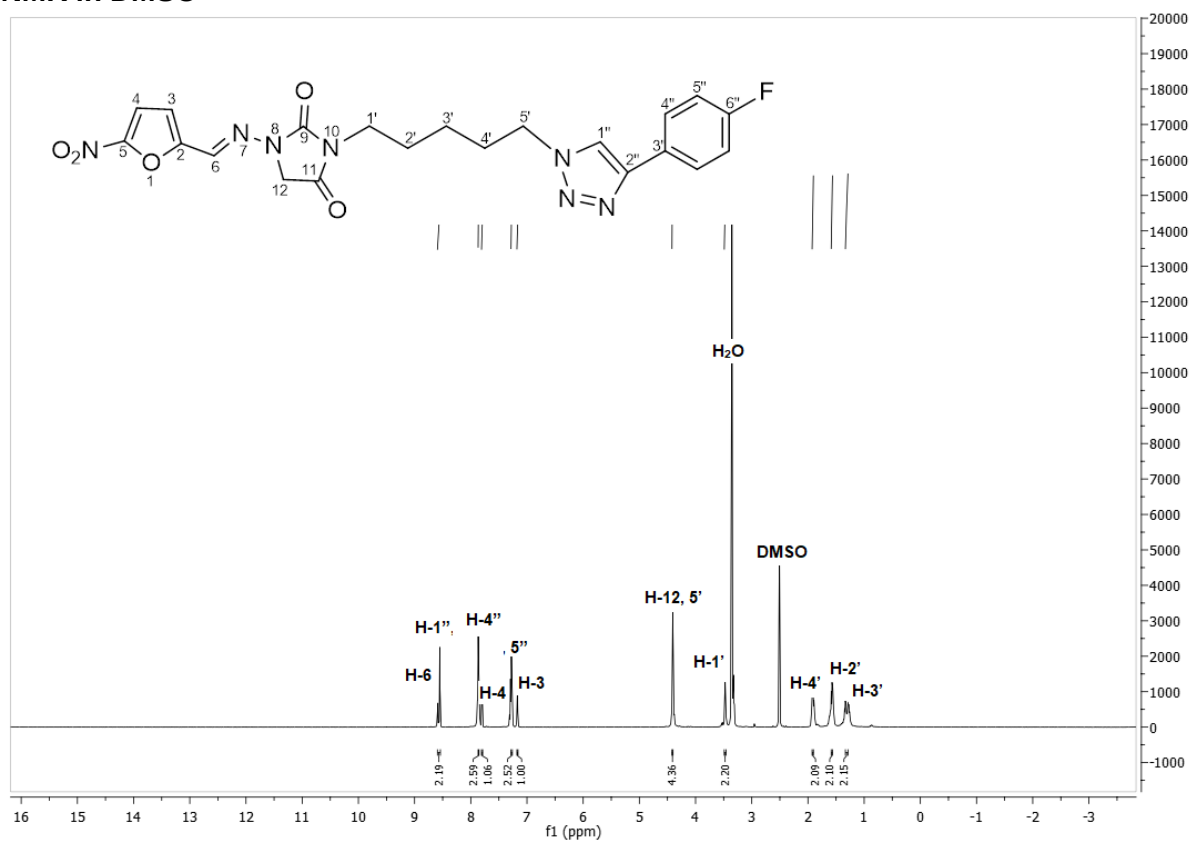


Hybrid 3510

IR

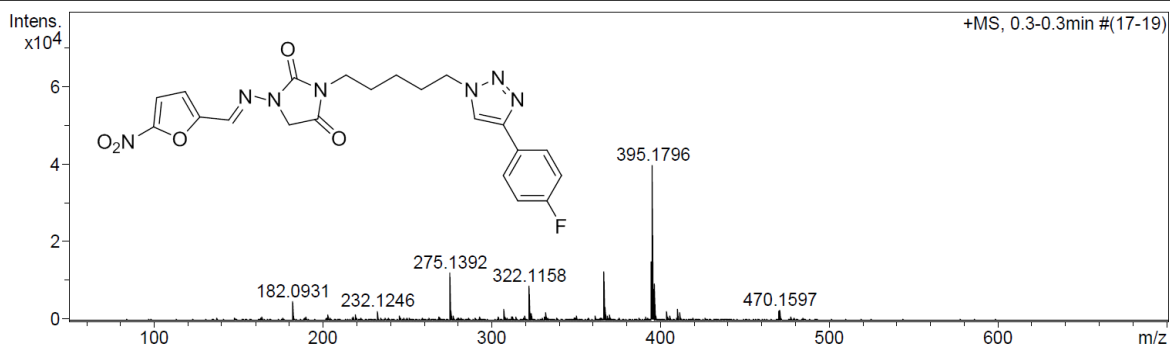


¹H NMR in DMSO



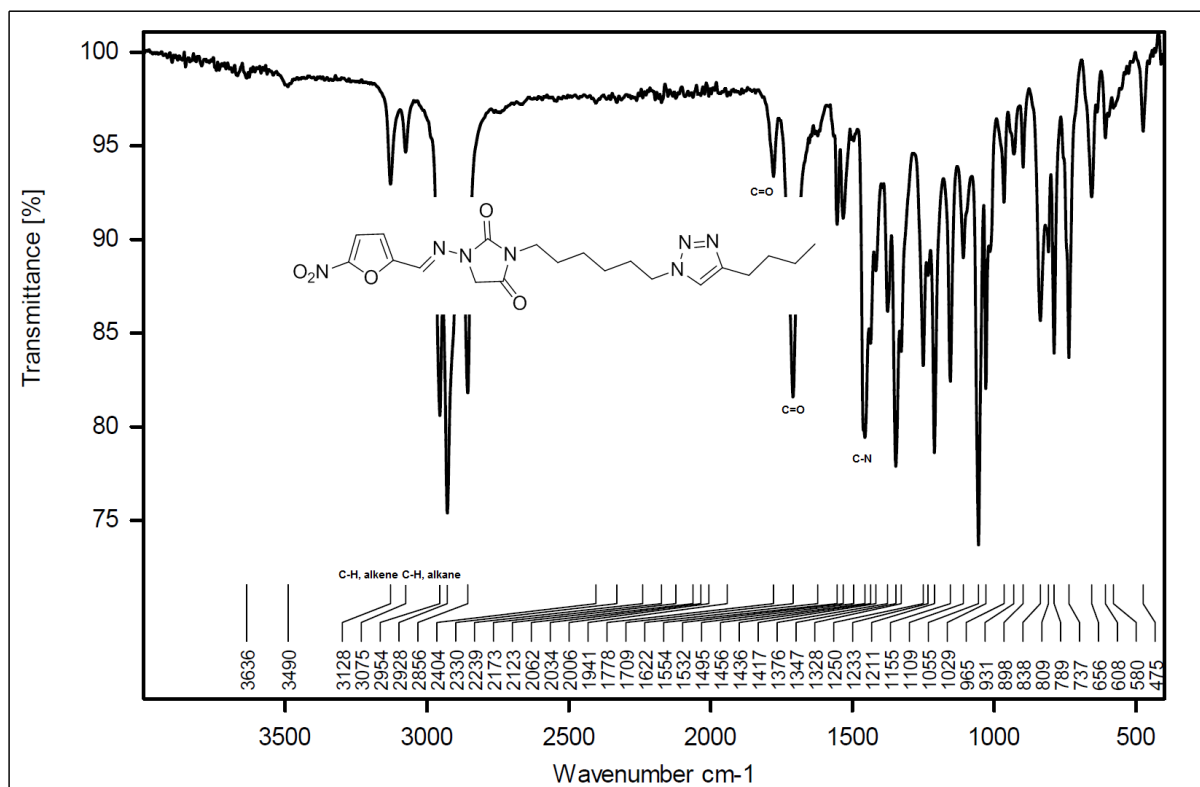
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Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
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Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

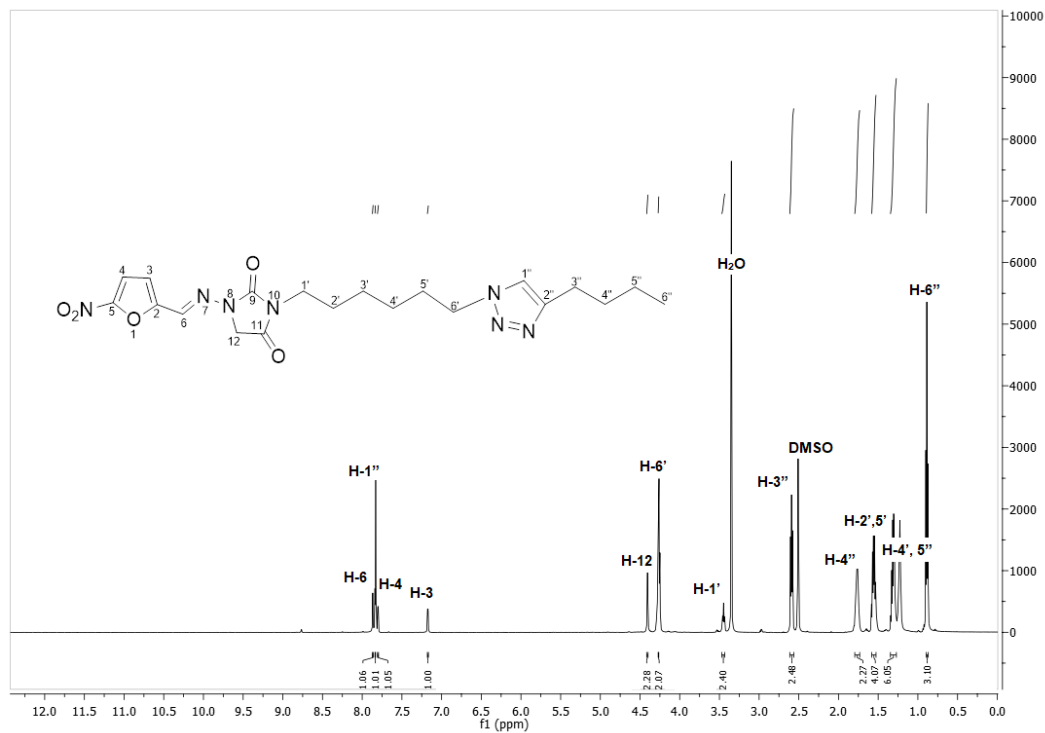


Hybrid 3601

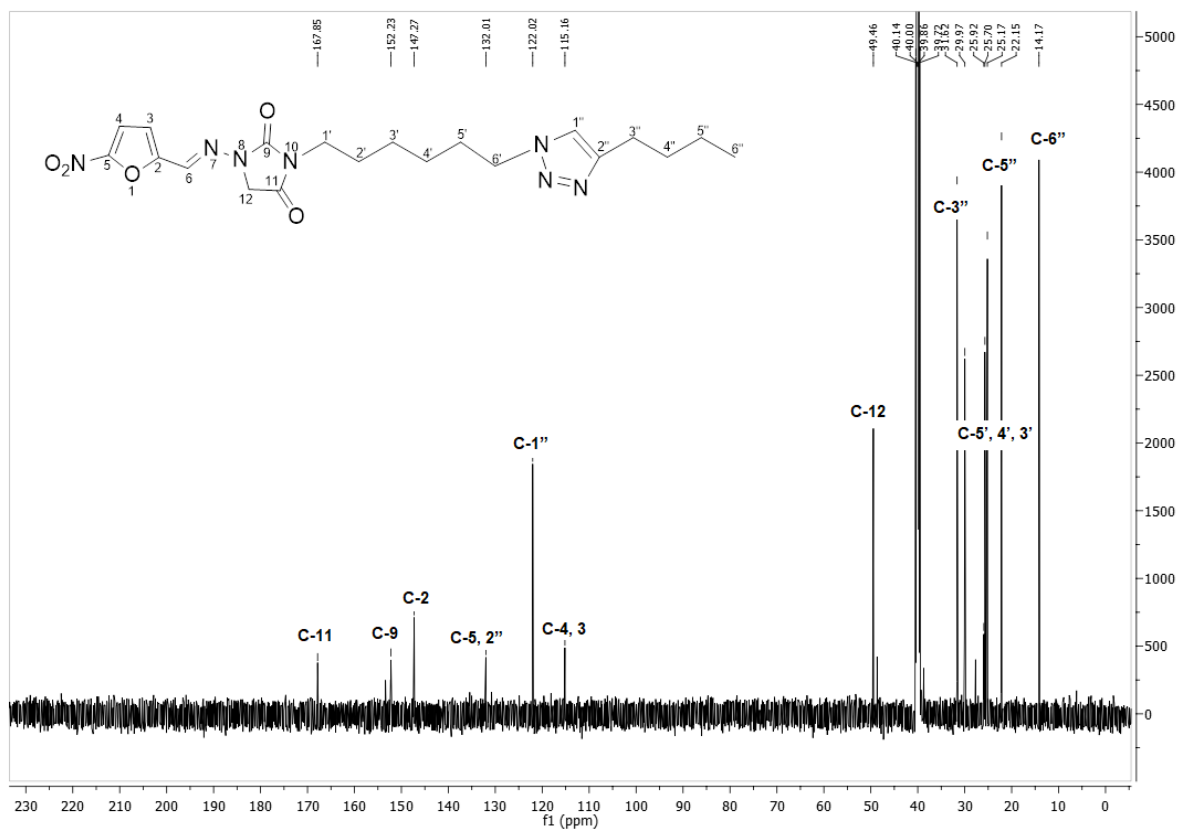
IR



¹H NMR in DMSO



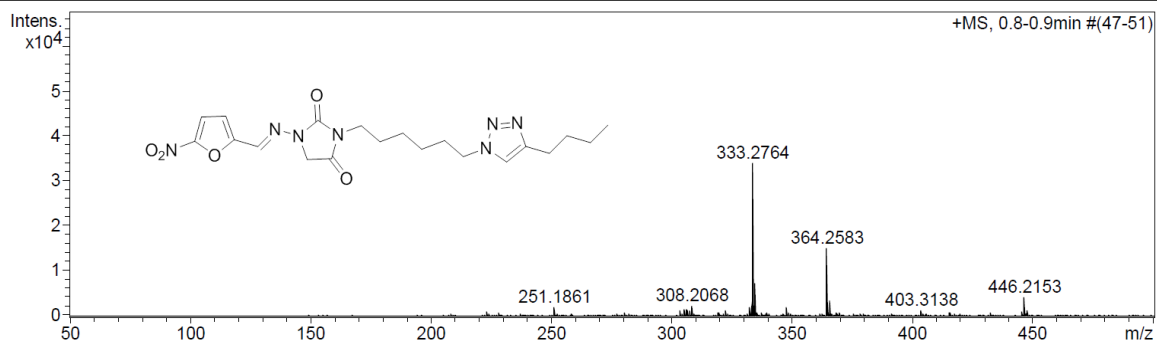
¹³C NMR in DMSO



HR-MS

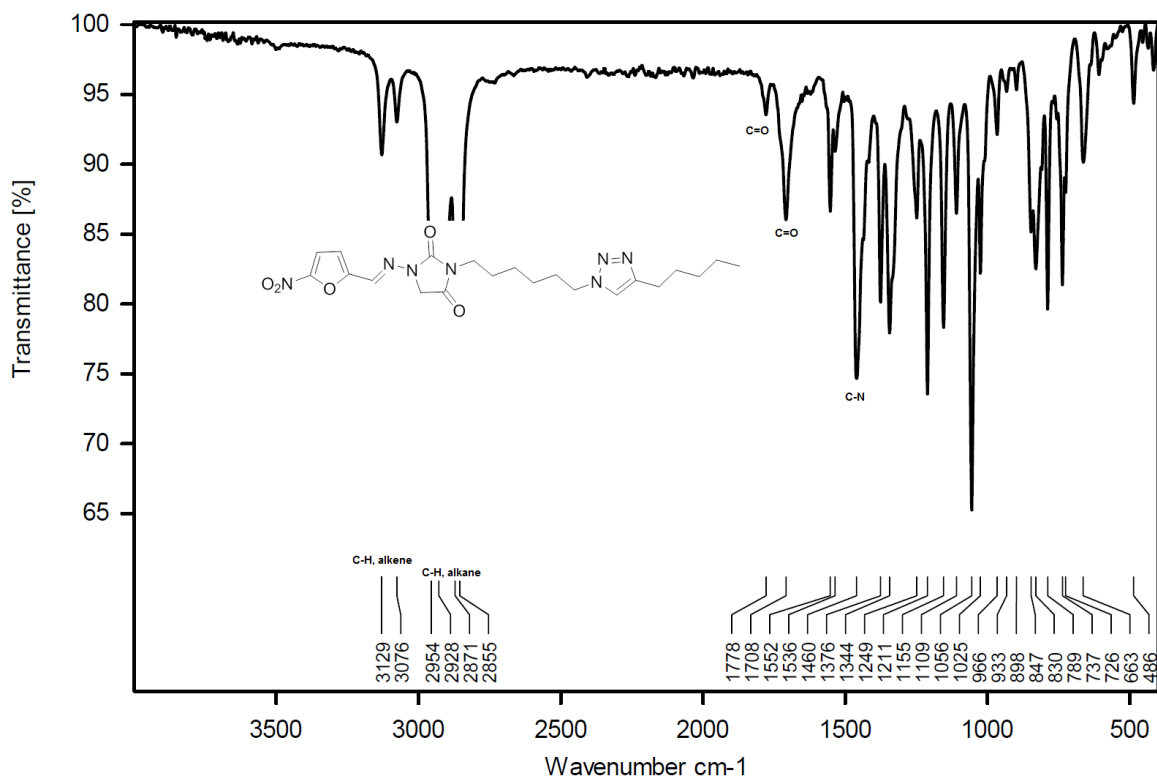
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Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
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Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

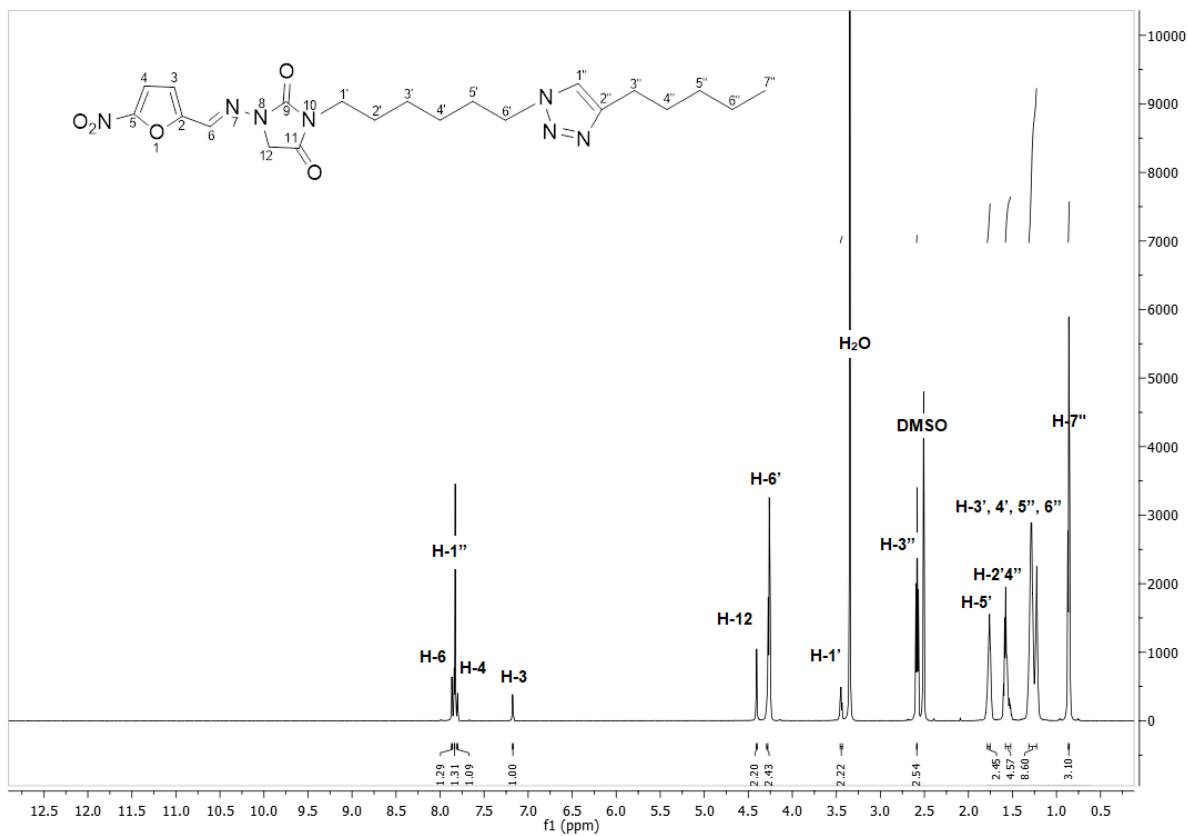


Hybrid 3602

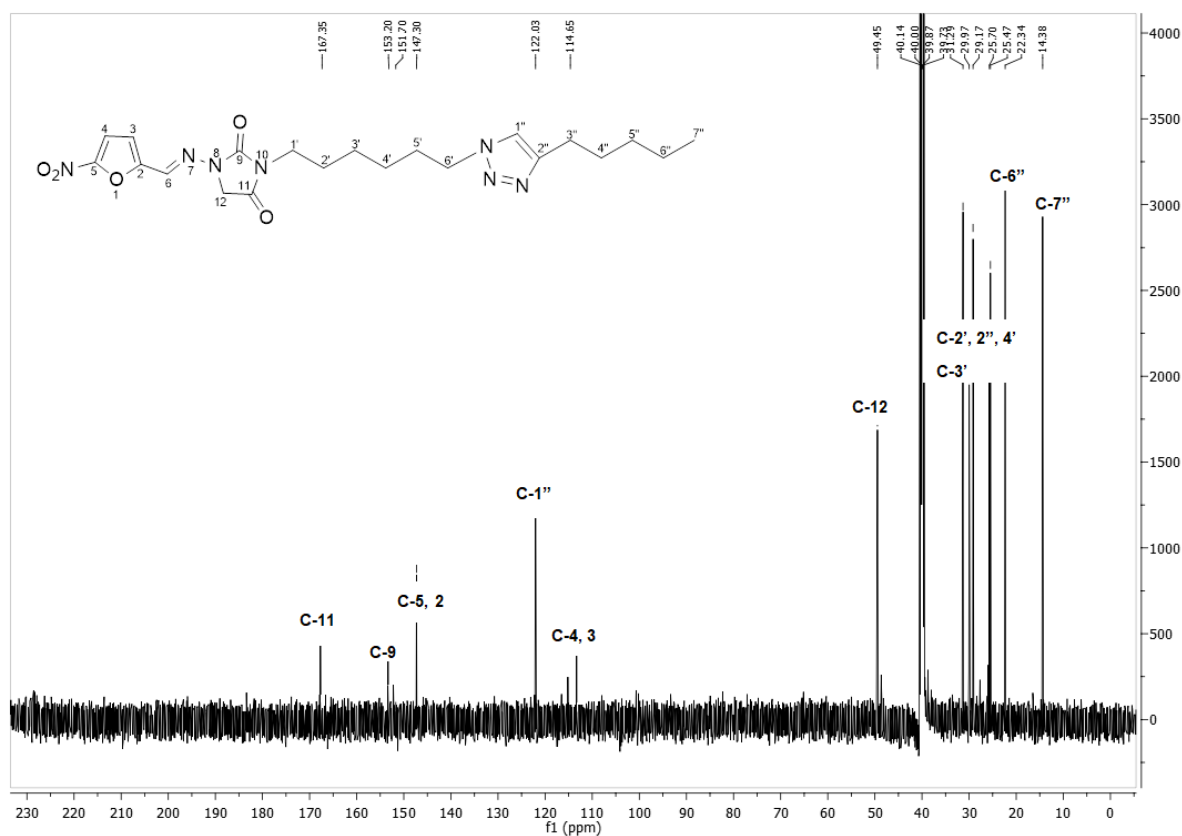
IR



¹H NMR in DMSO



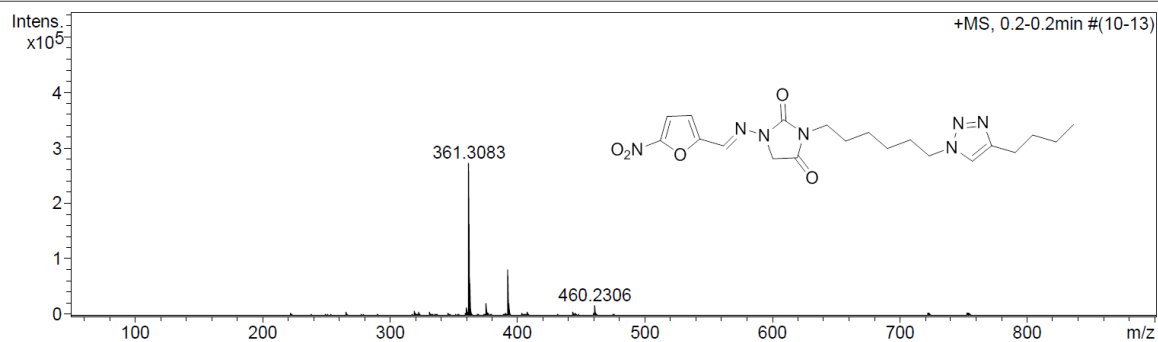
¹³C NMR in DMSO



HR-MS

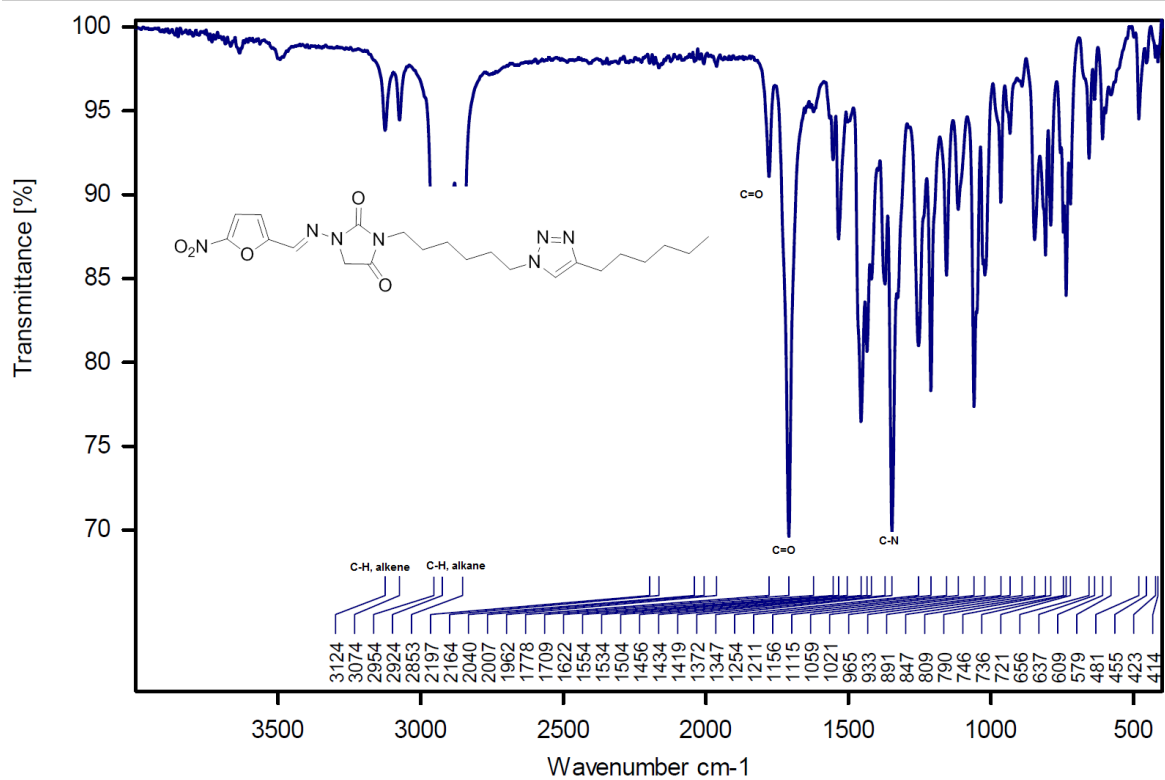
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

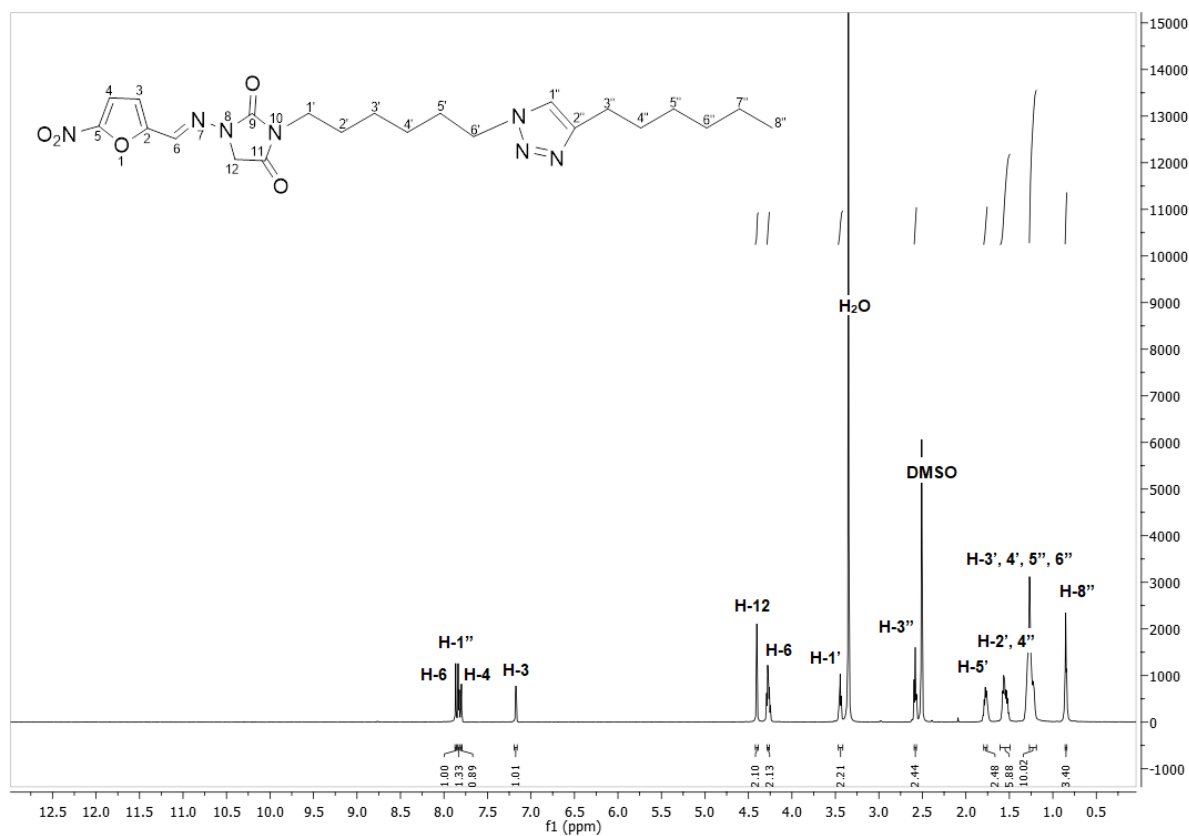


Hybrid 3603

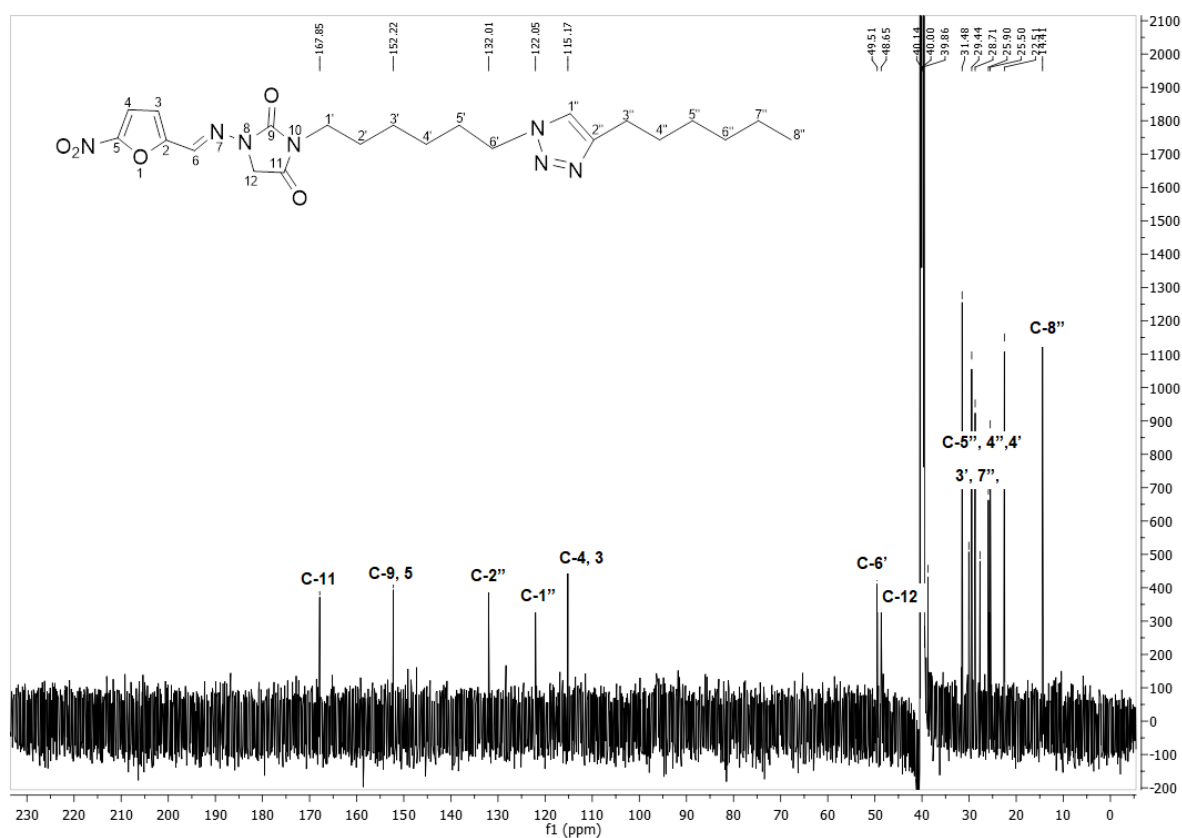
IR



¹H NMR in DMSO



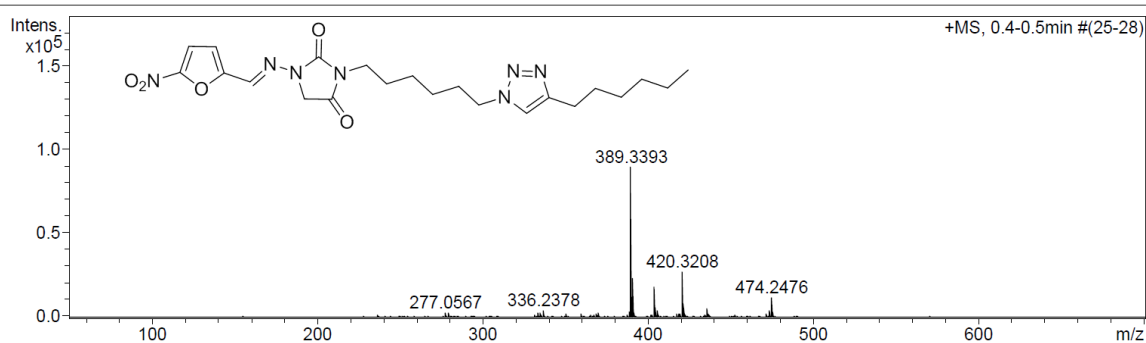
¹³C NMR in DMSO



HR-MS

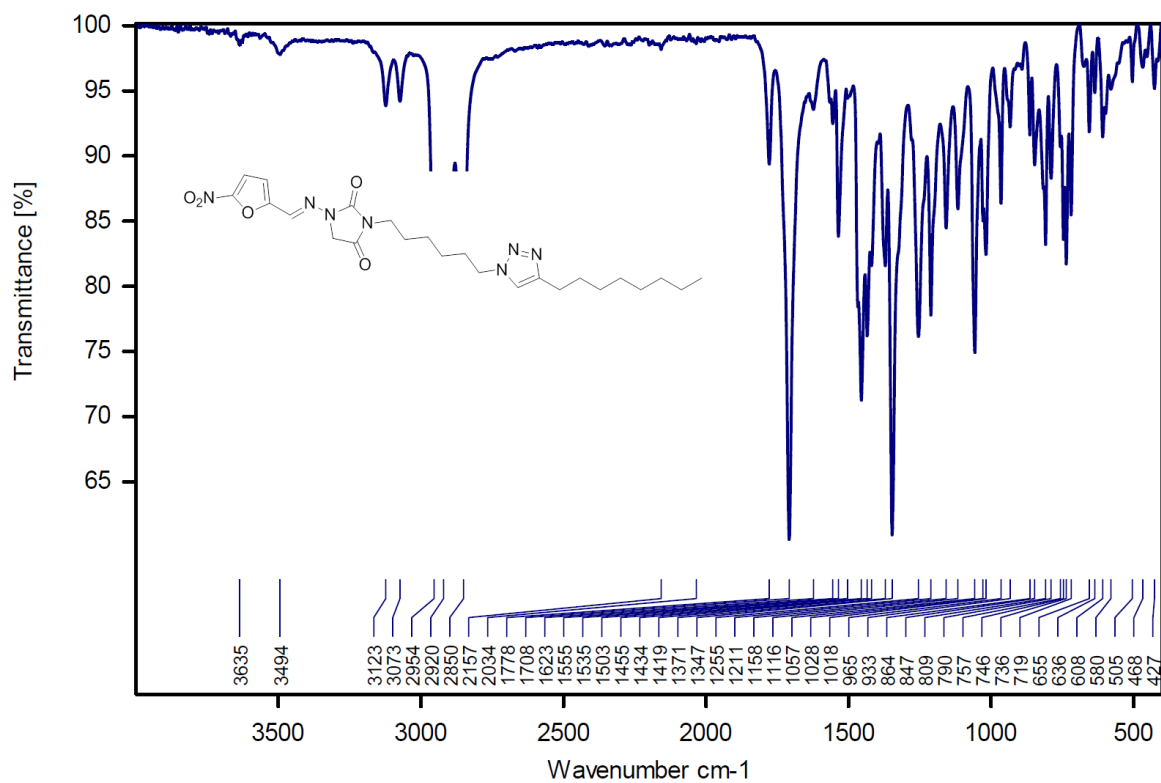
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Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
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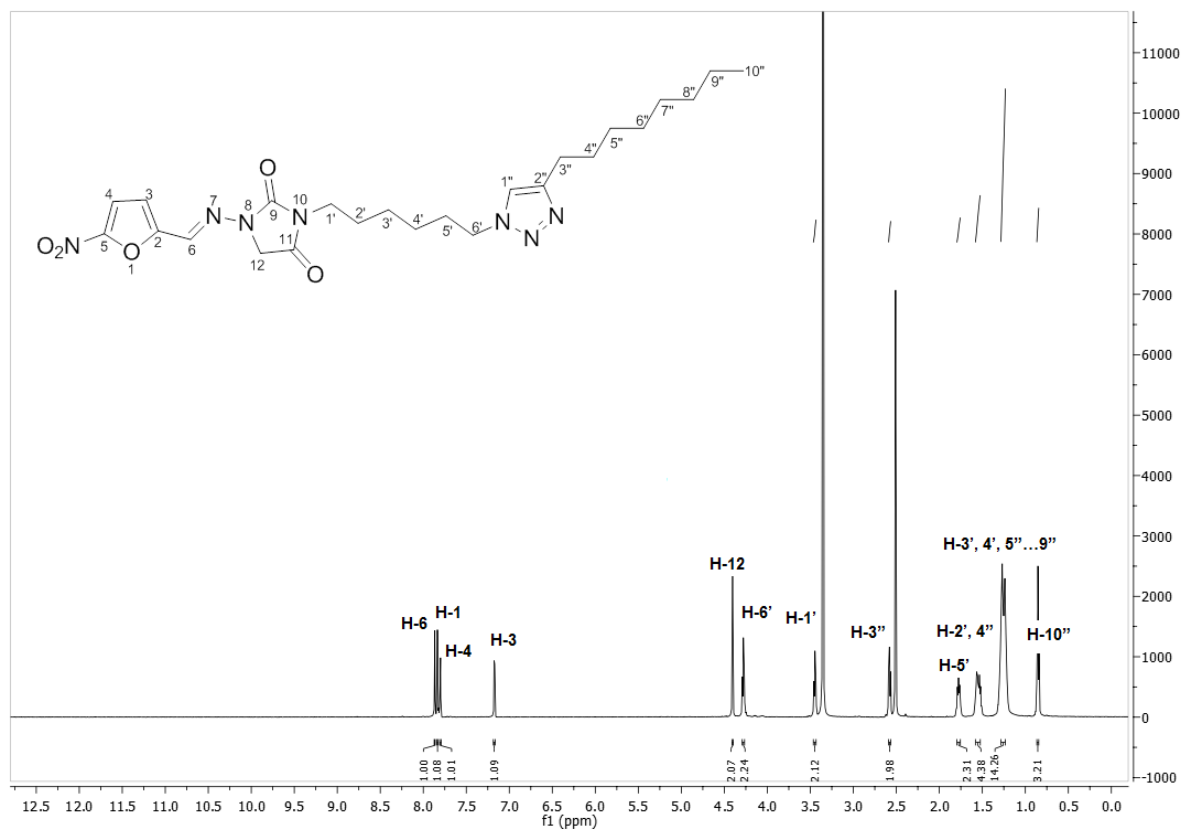


Hybrid 3604

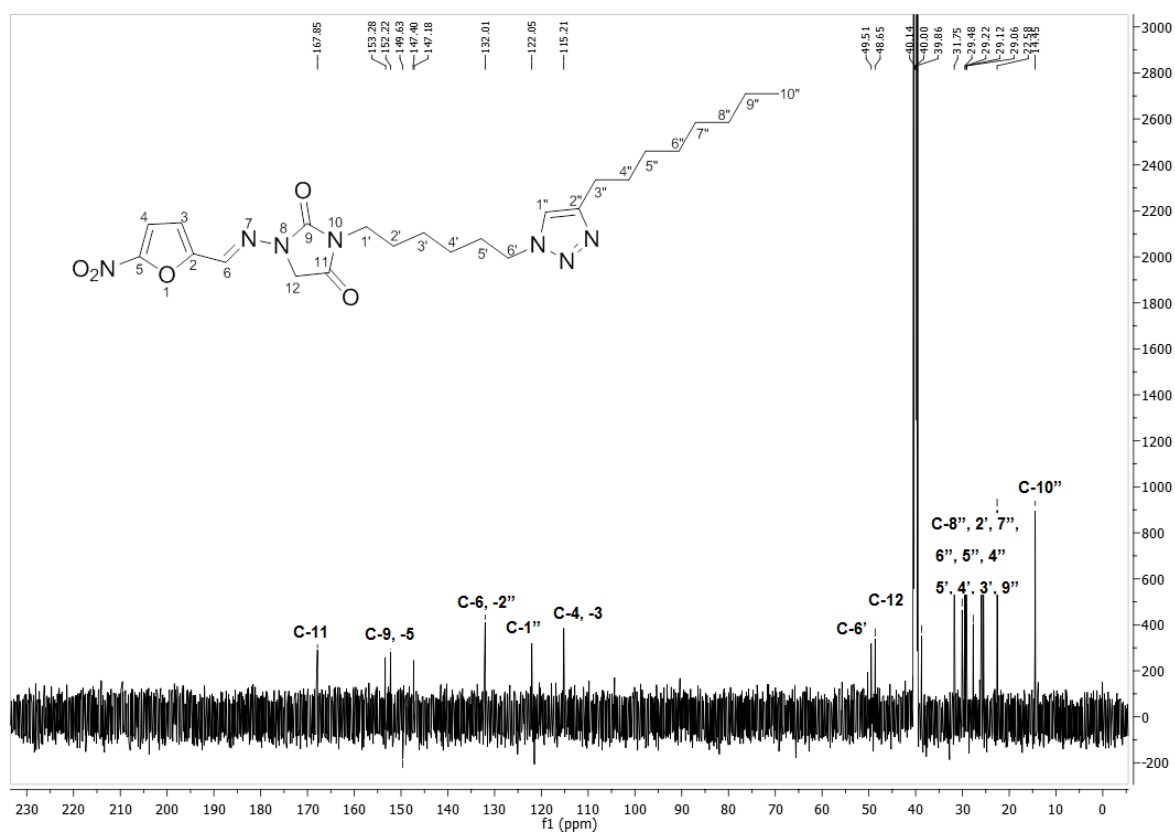
IR



¹H NMR in DMSO



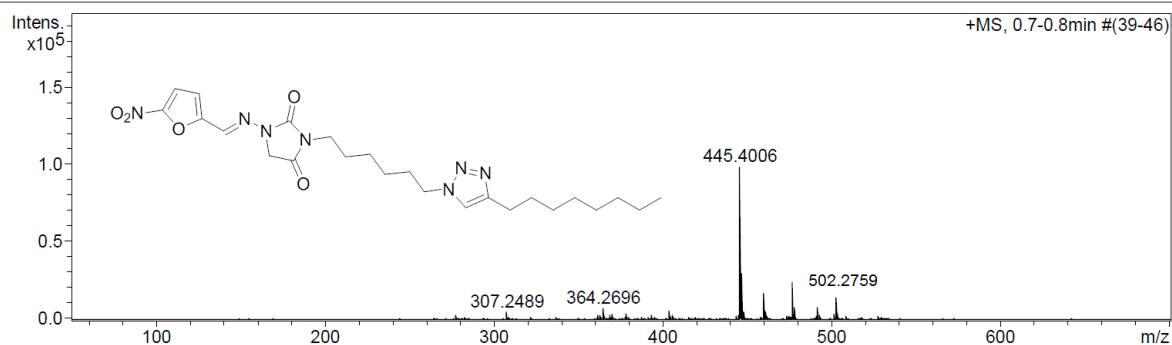
¹³C NMR in DMSO



HR-MS

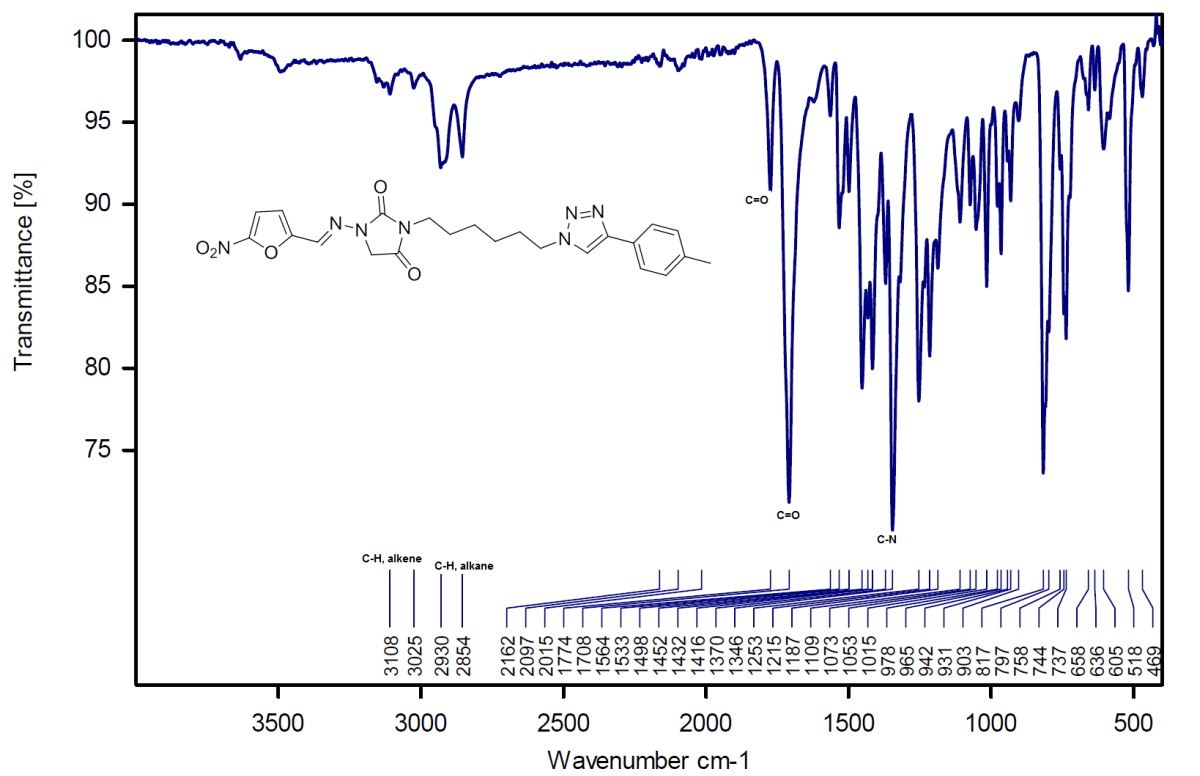
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
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Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

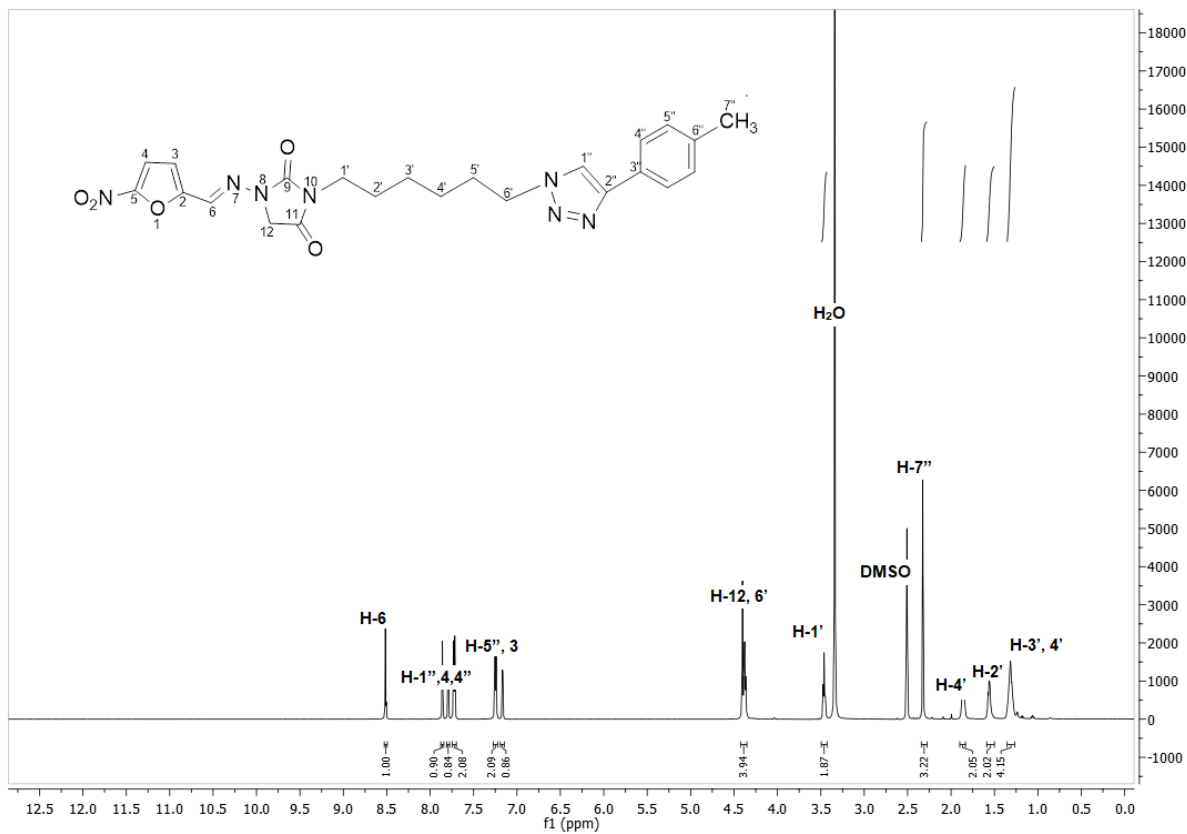


Hybrid 3605

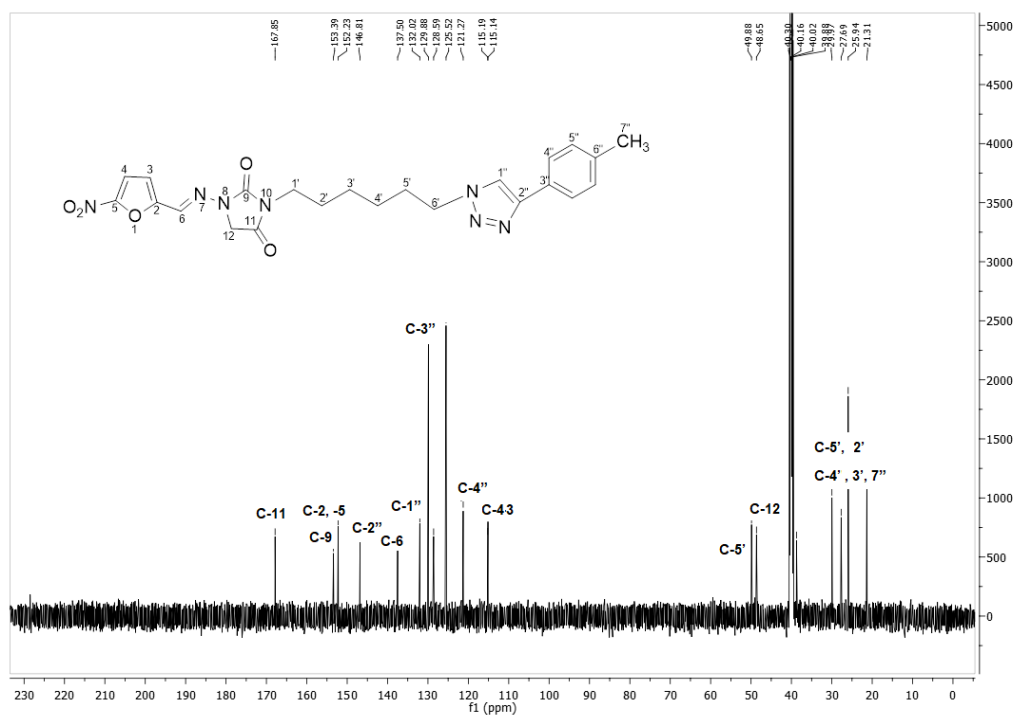
IR



¹H NMR in DMSO



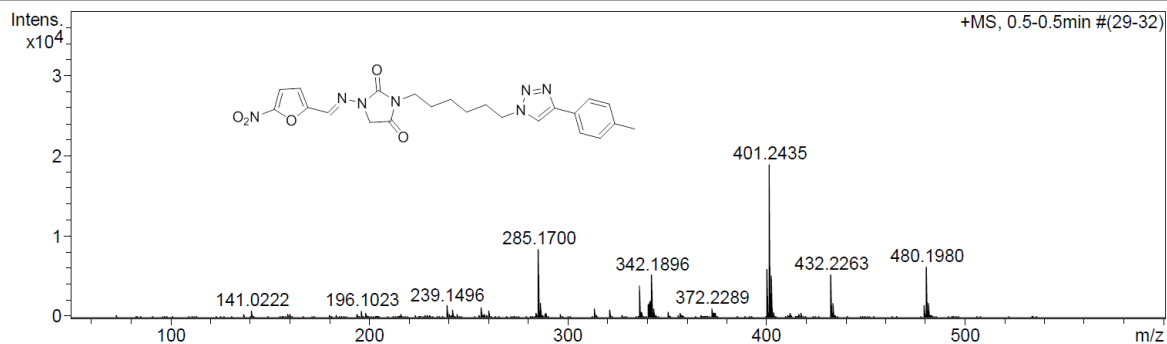
¹³C NMR in DMSO



HR-MS

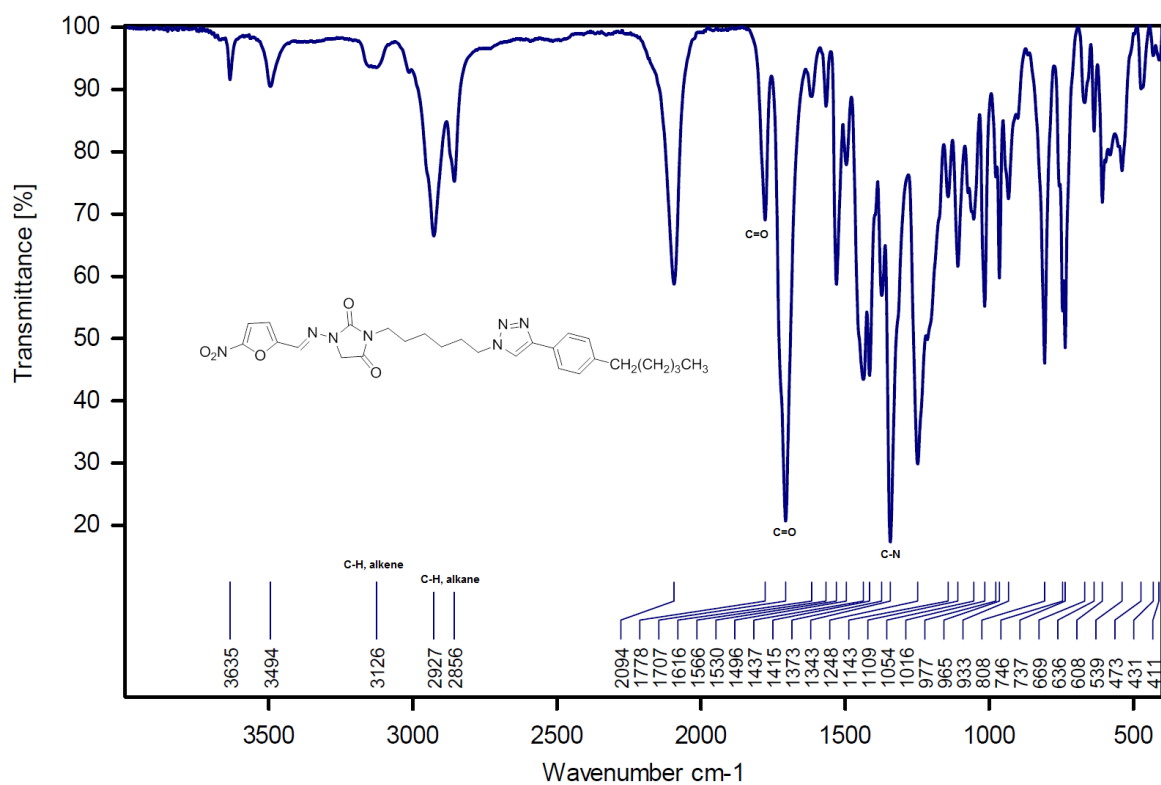
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Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
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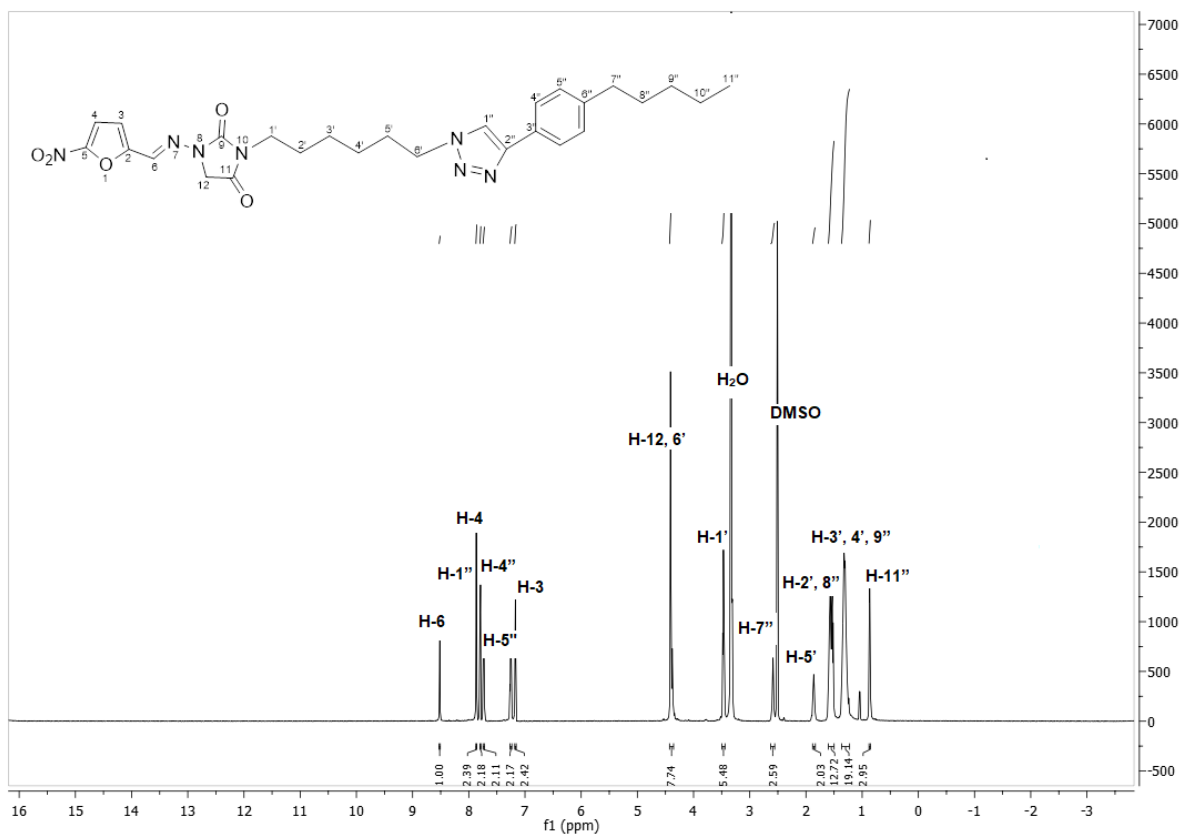


Hybrid 3606

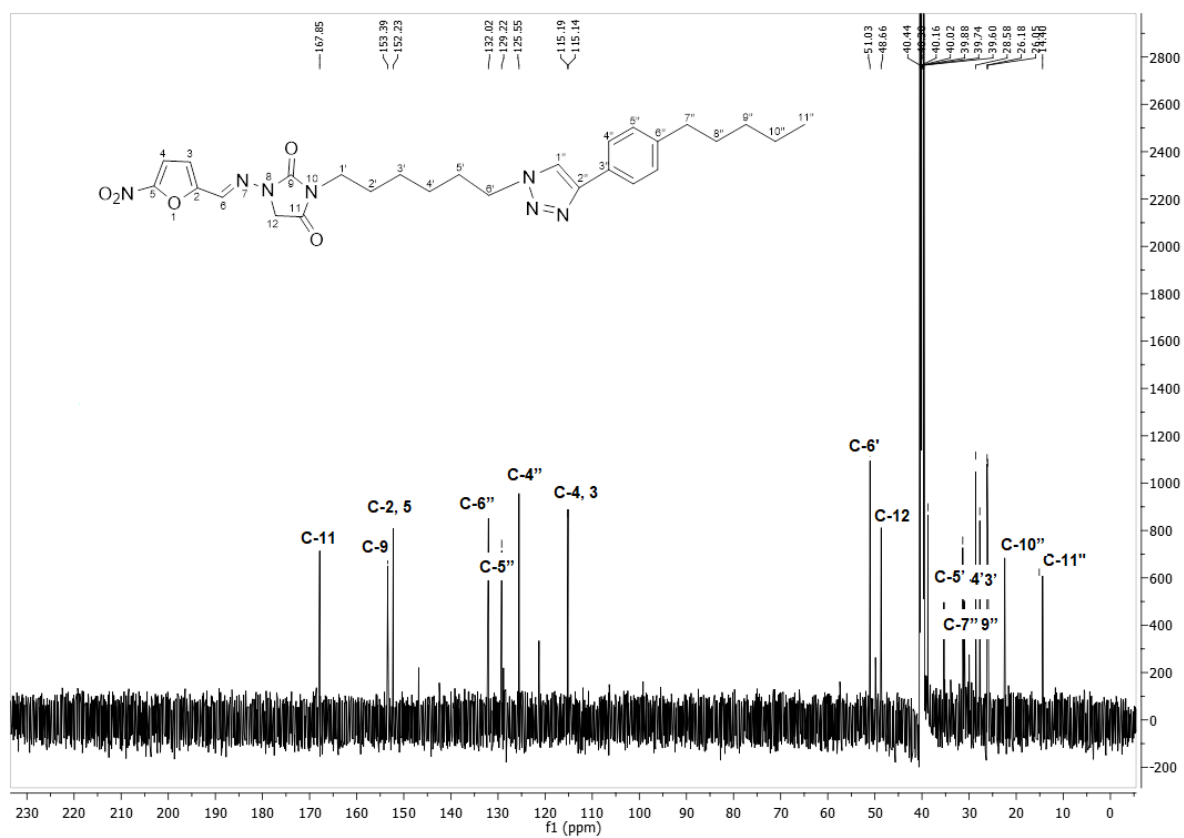
IR



¹H NMR in DMSO

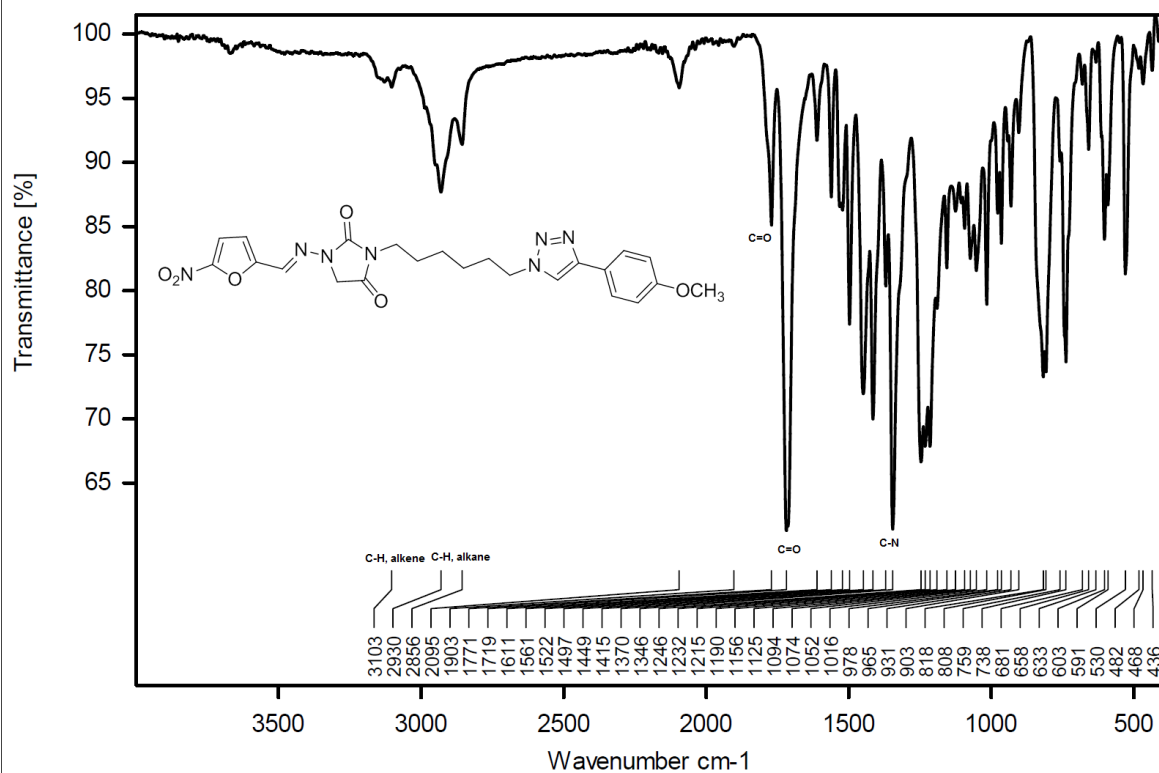


¹³C NMR in DMSO

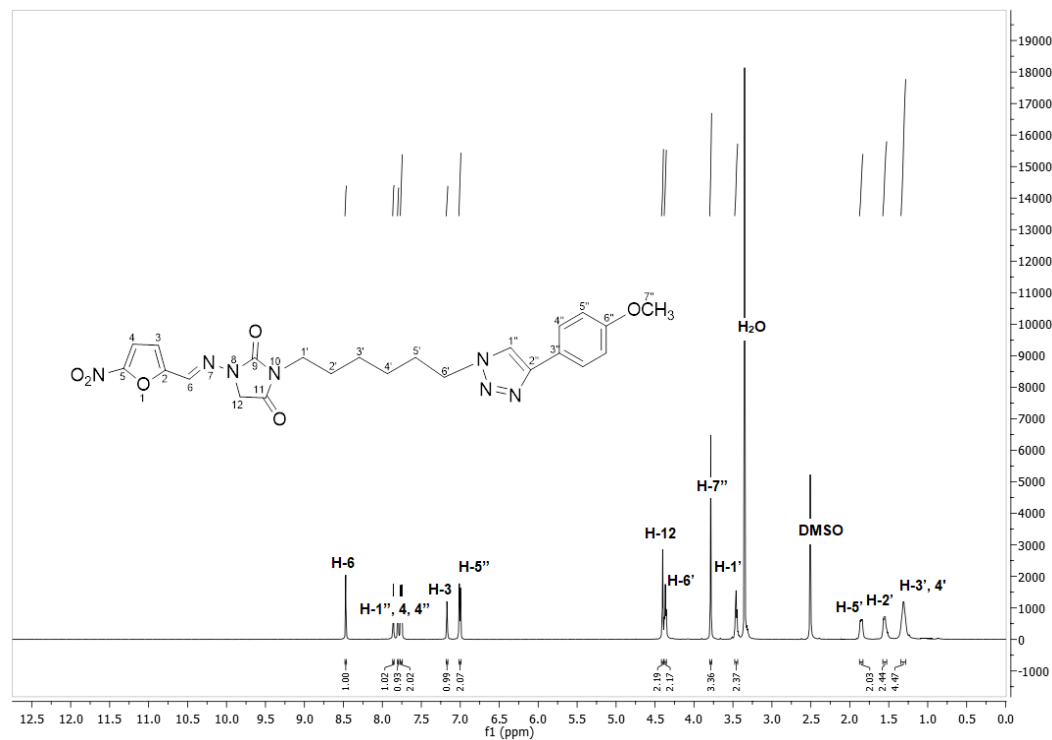


Hybrid 3607

IR

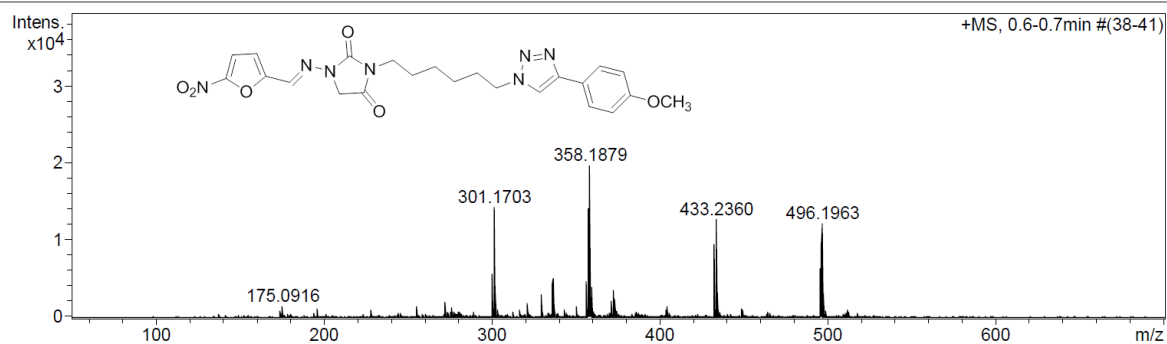


¹H NMR in DMSO



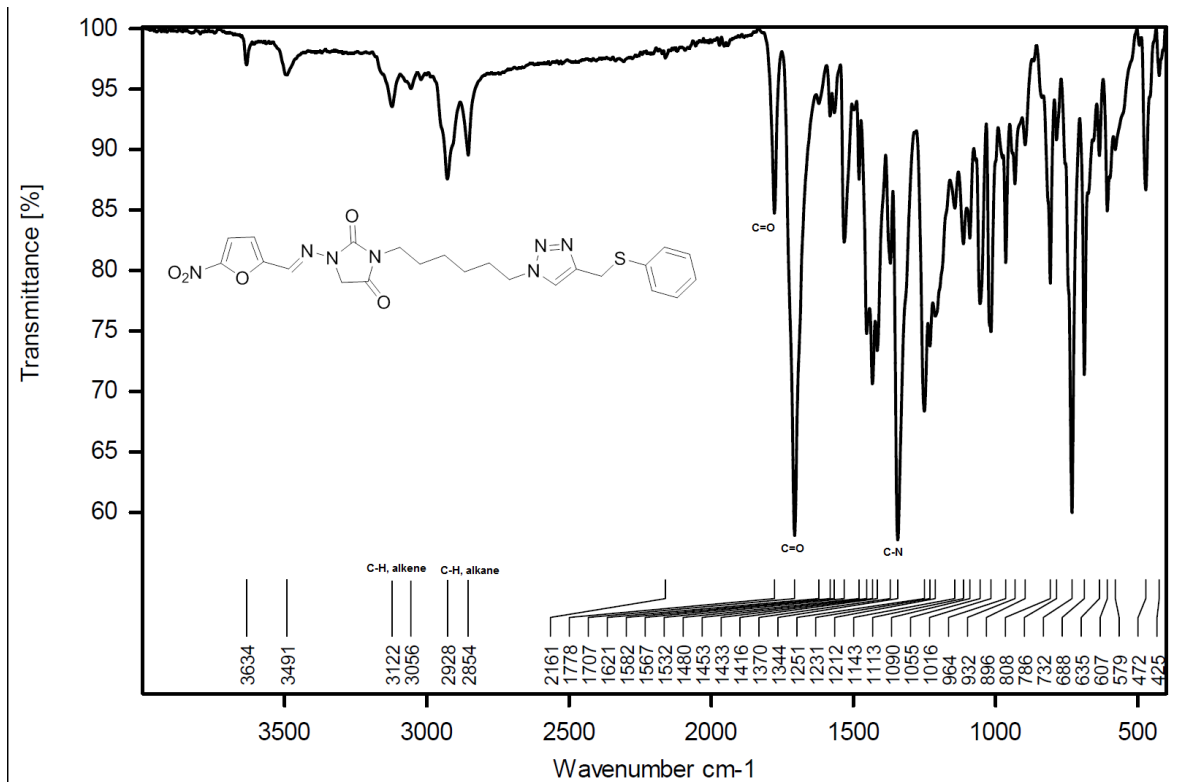
Acquisition Parameter

Acquisition Parameters					
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
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Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

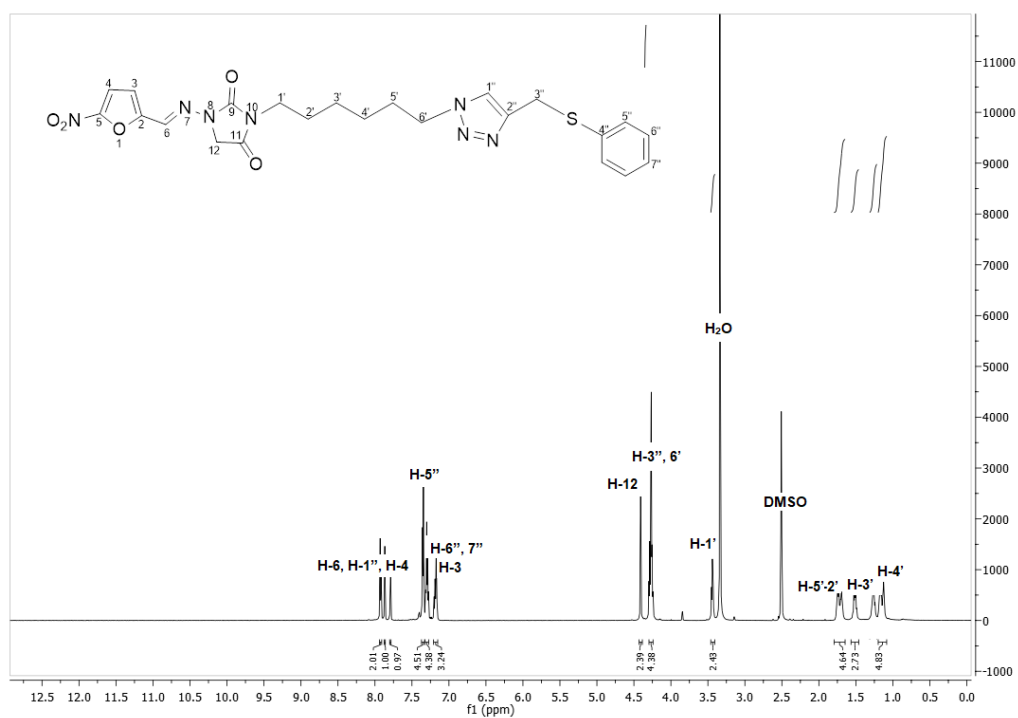


Hybrid 3608

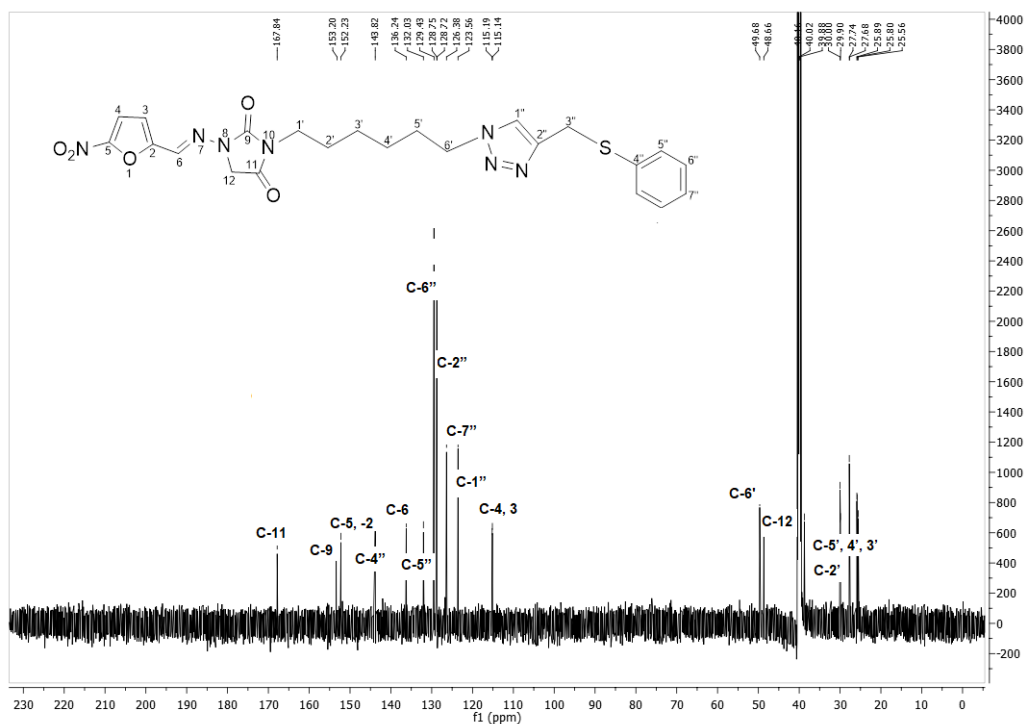
IR



¹H NMR in DMSO



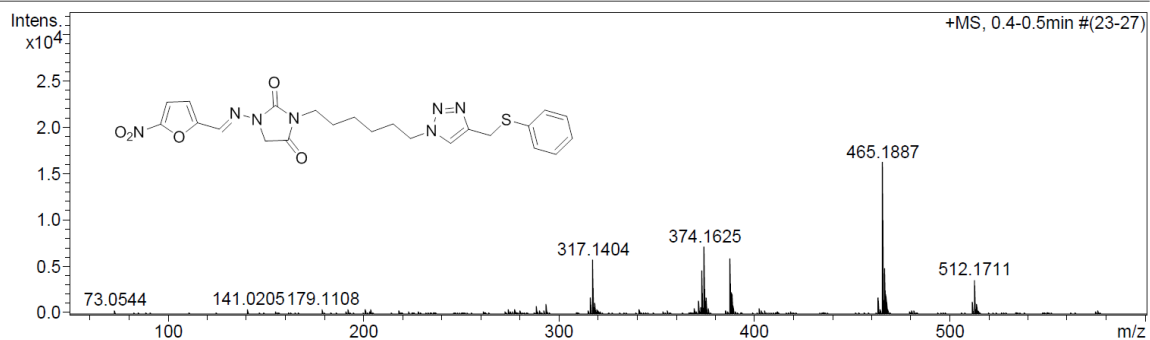
¹³C NMR in DMSO



HR-MS

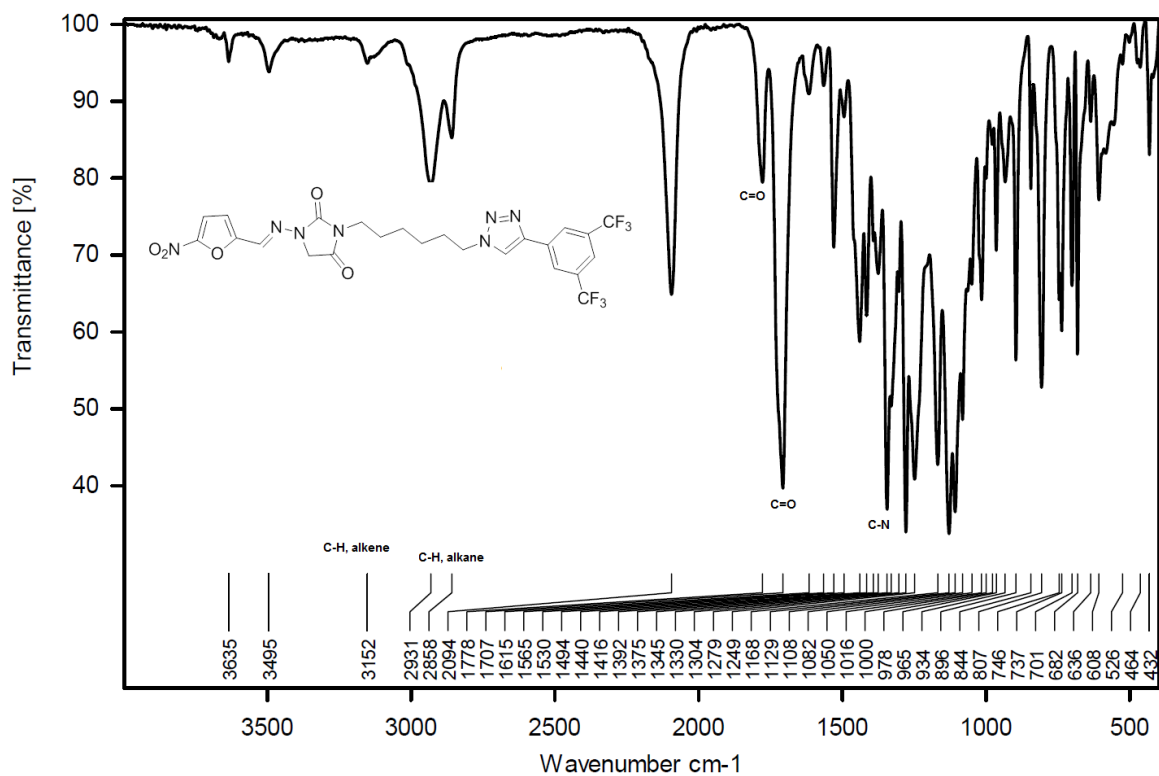
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

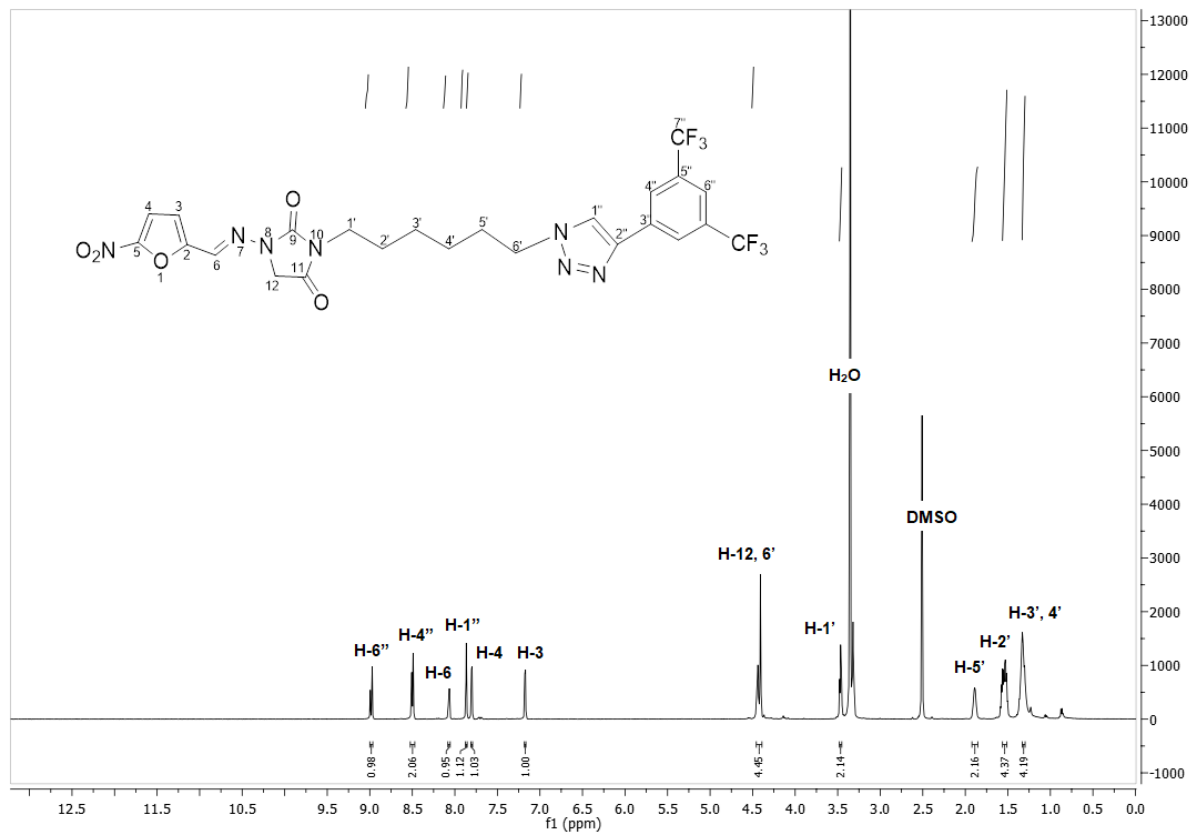


Hybrid 3609

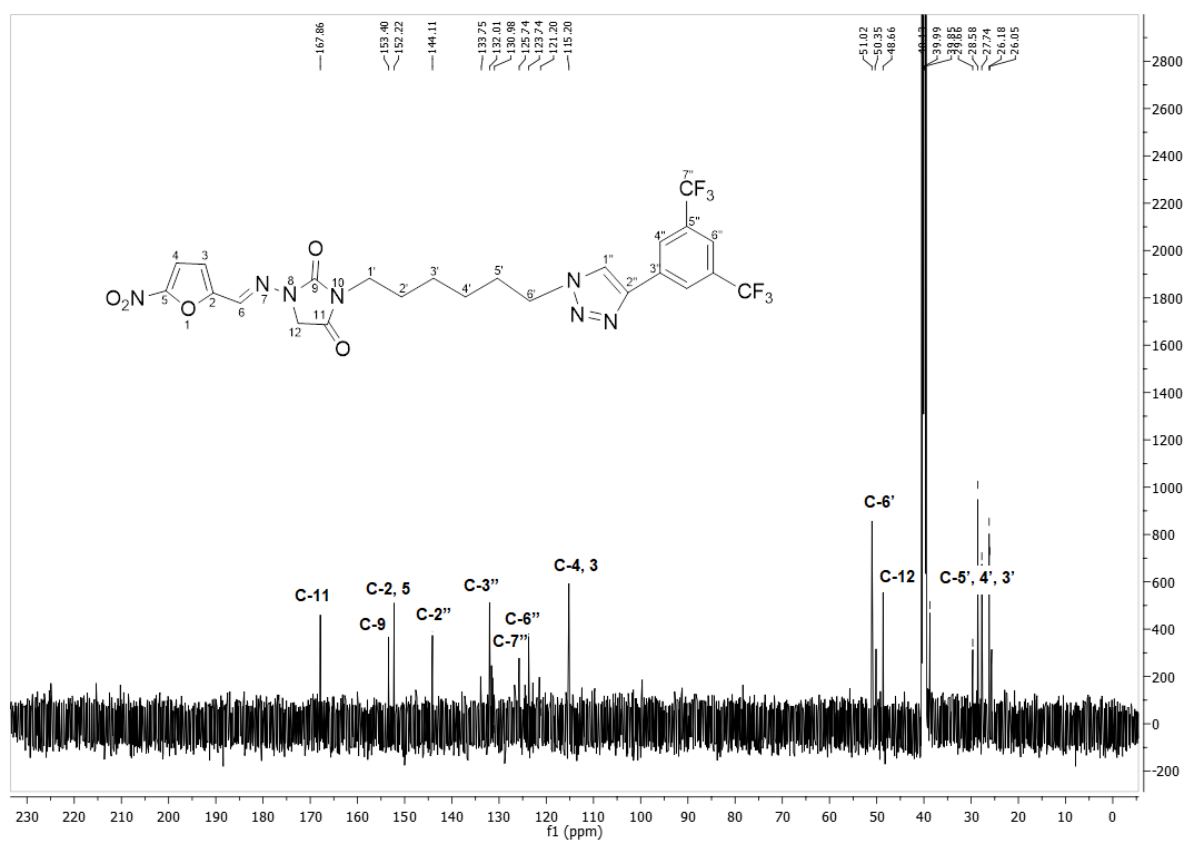
IR



¹H NMR in DMSO

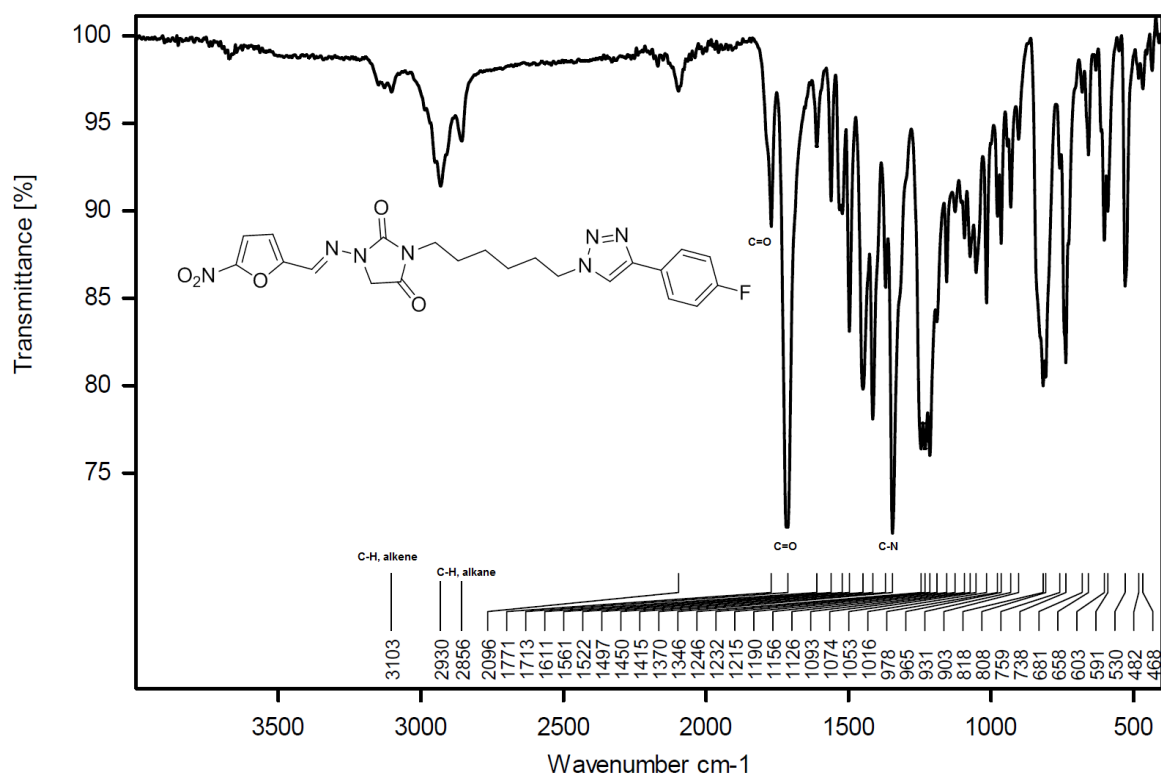


¹³C NMR in DMSO

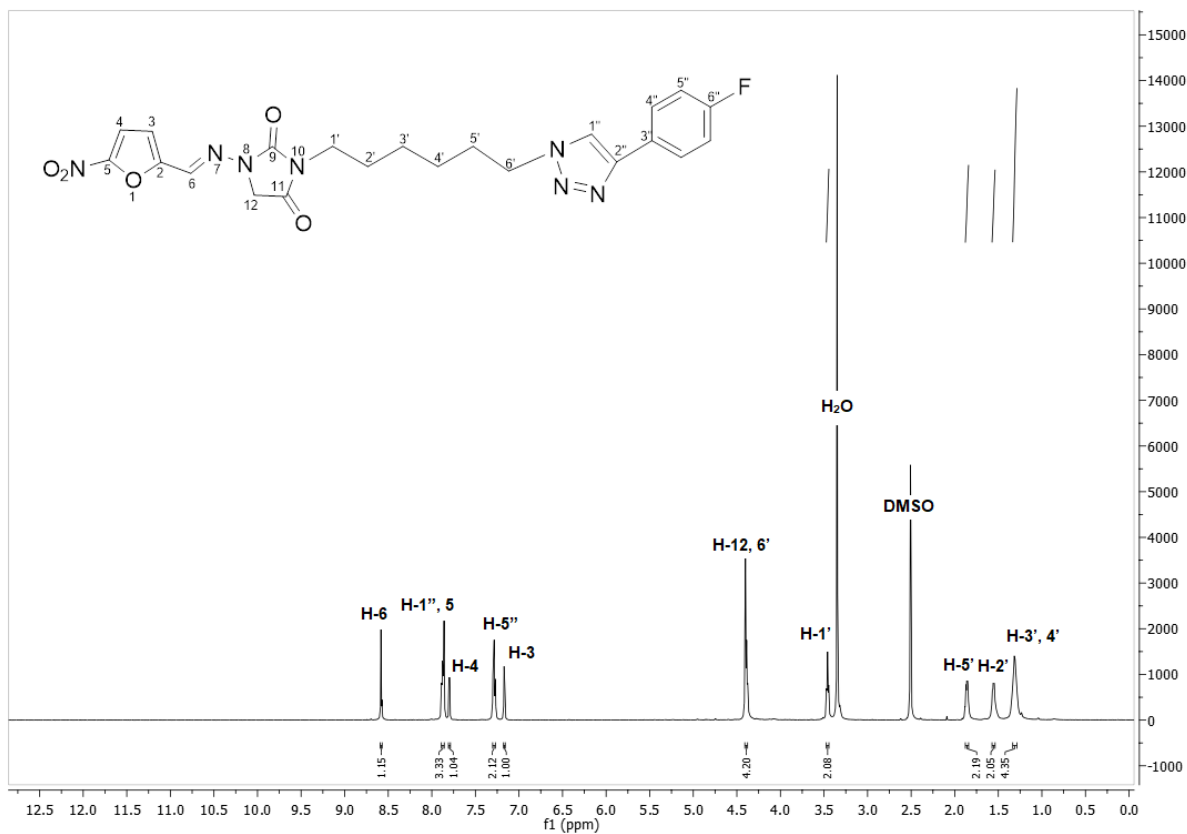


Hybrid 3610

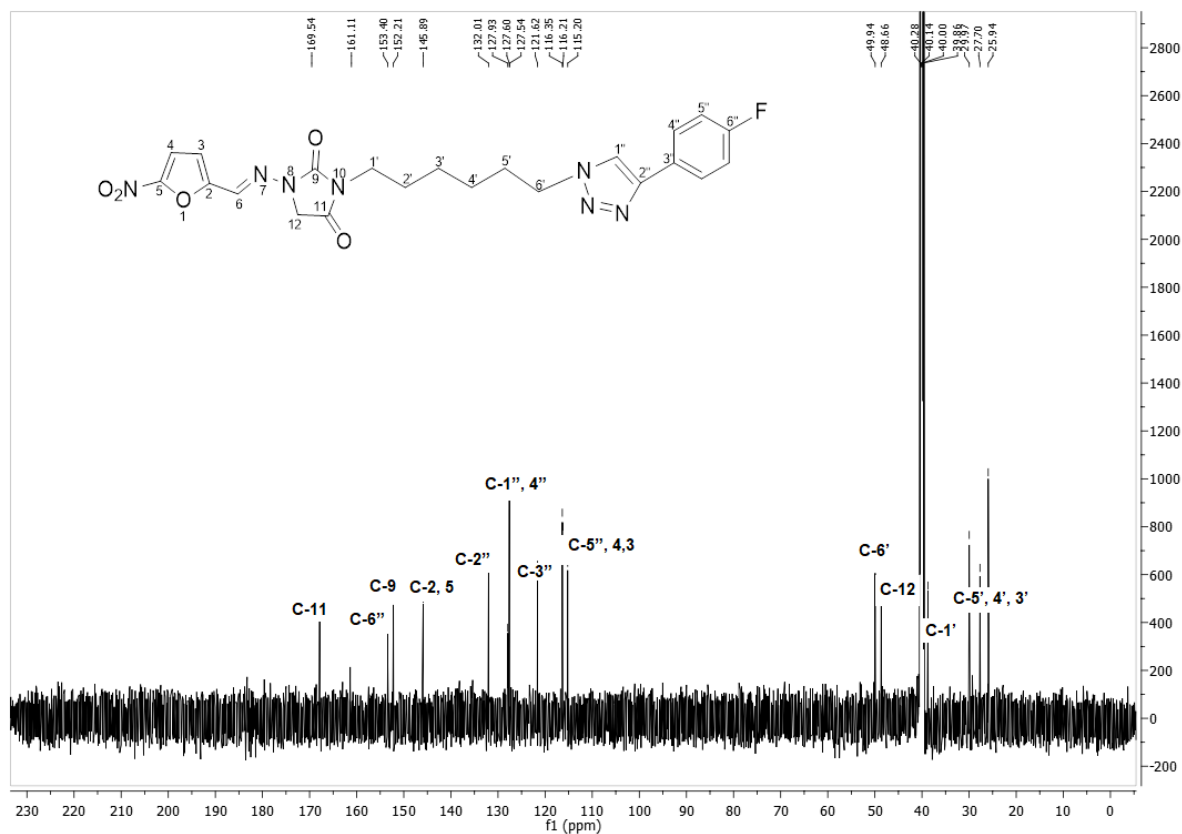
IR



¹H NMR in DMSO



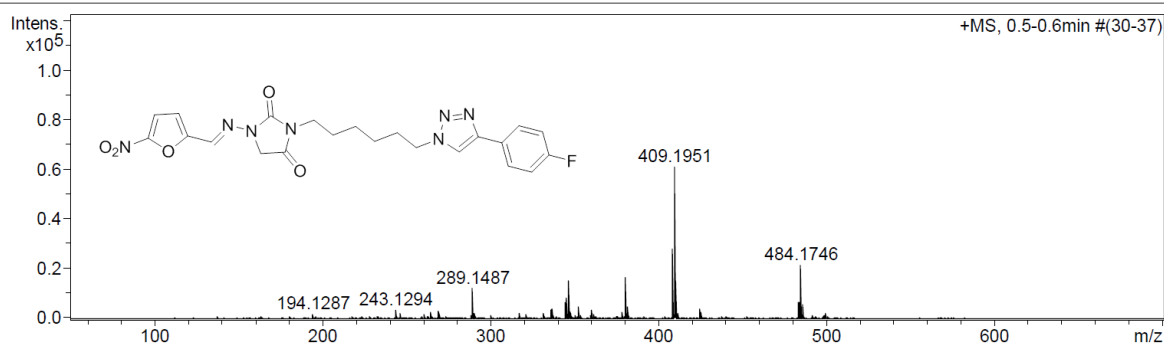
¹³C NMR in DMSO



HR-MS

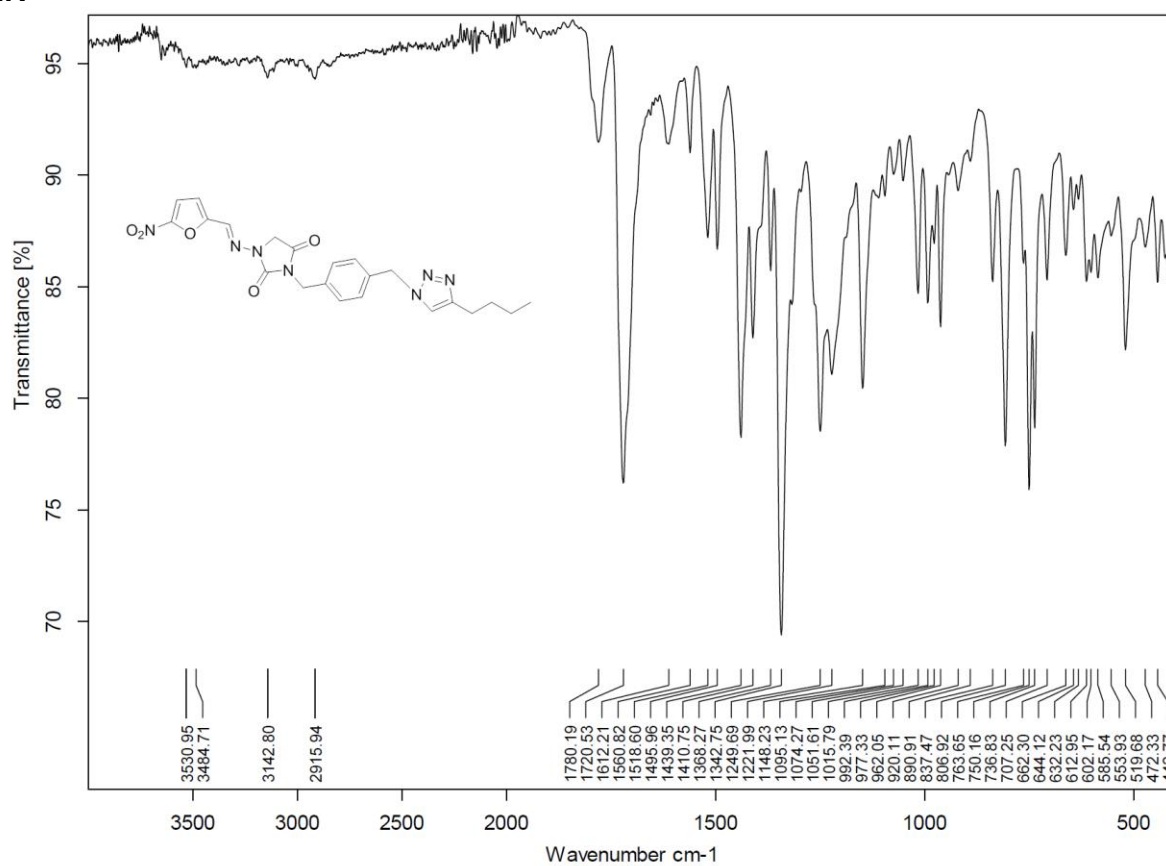
Acquisition Parameter

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

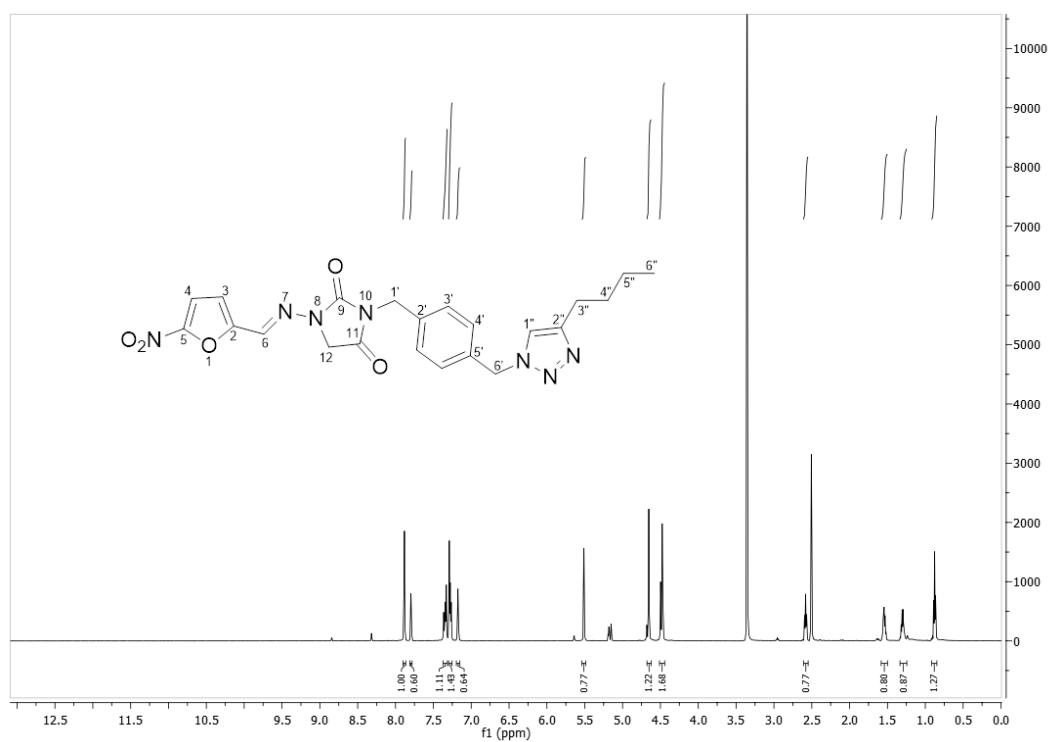


Hybrid 401

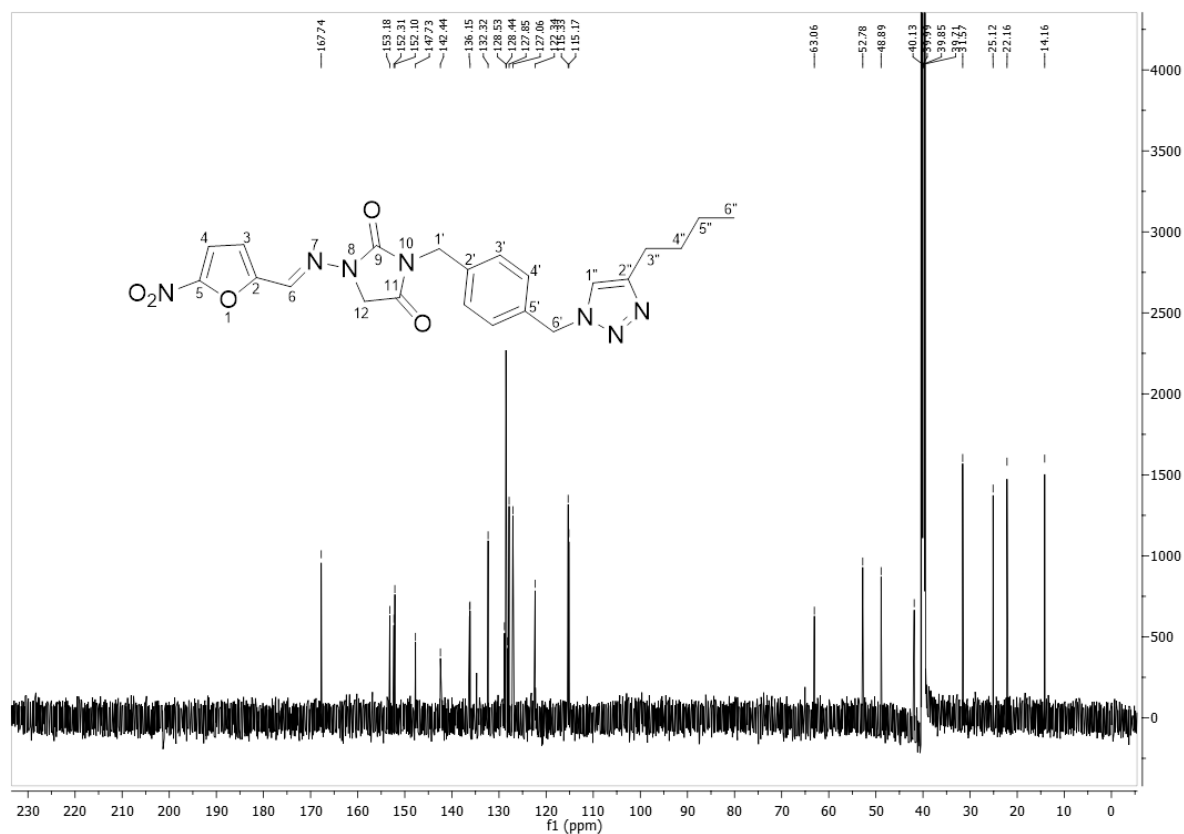
IR



¹H NMR in DMSO



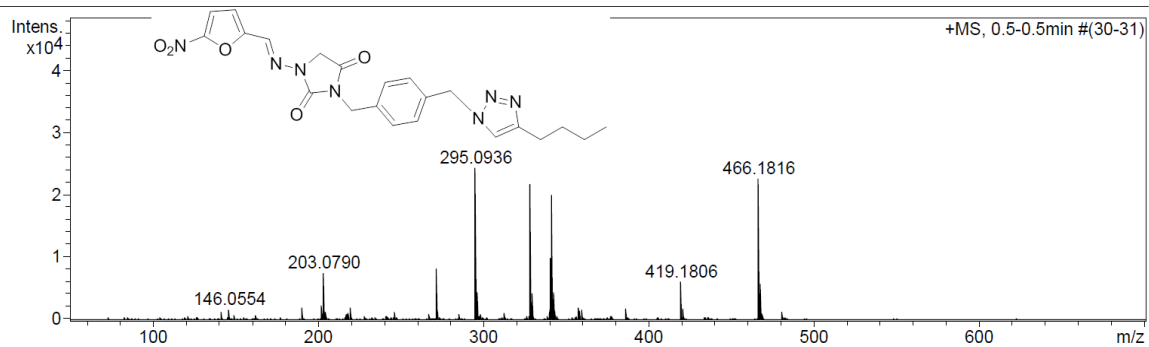
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HR-MS

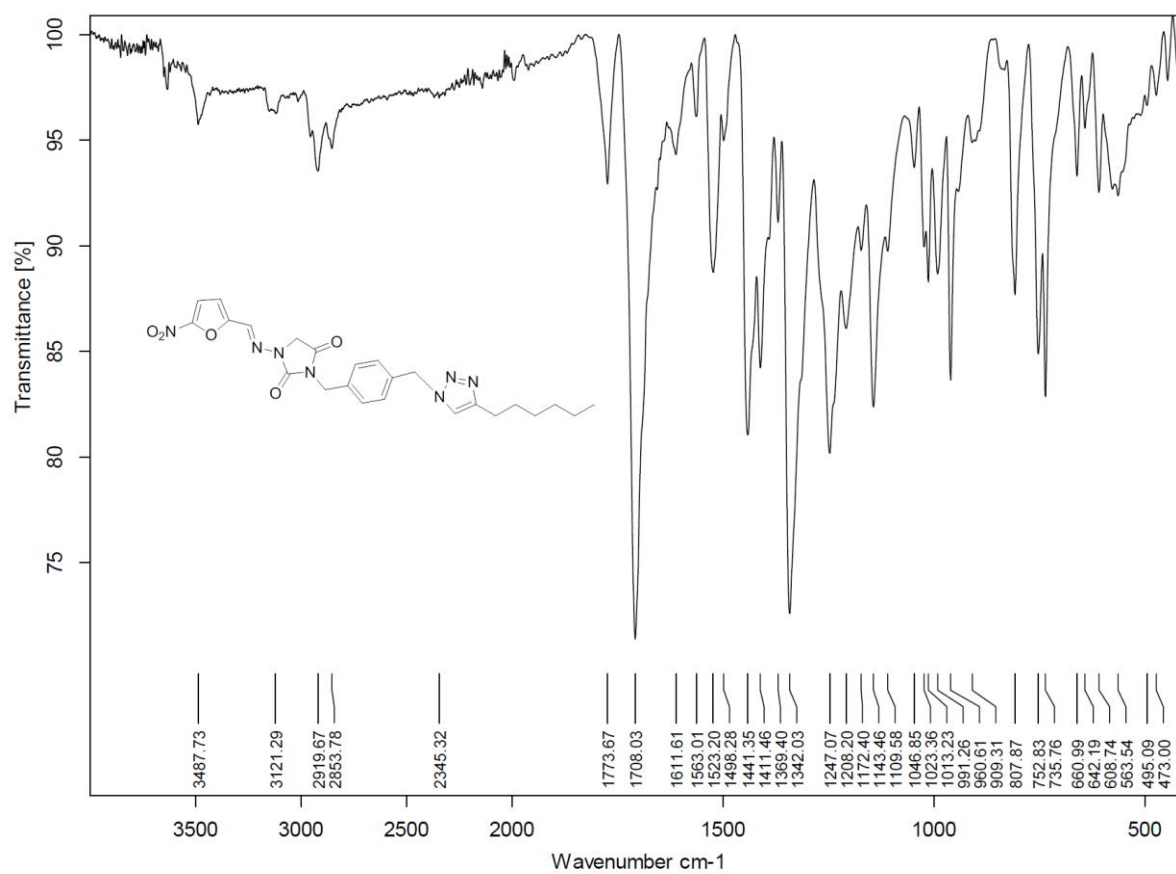
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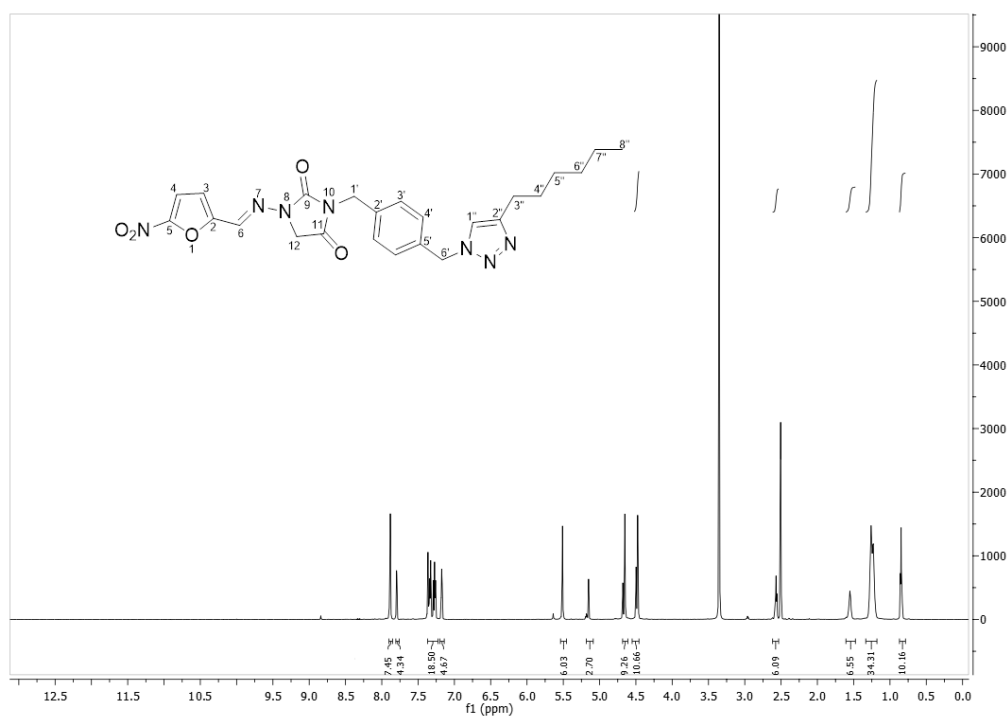


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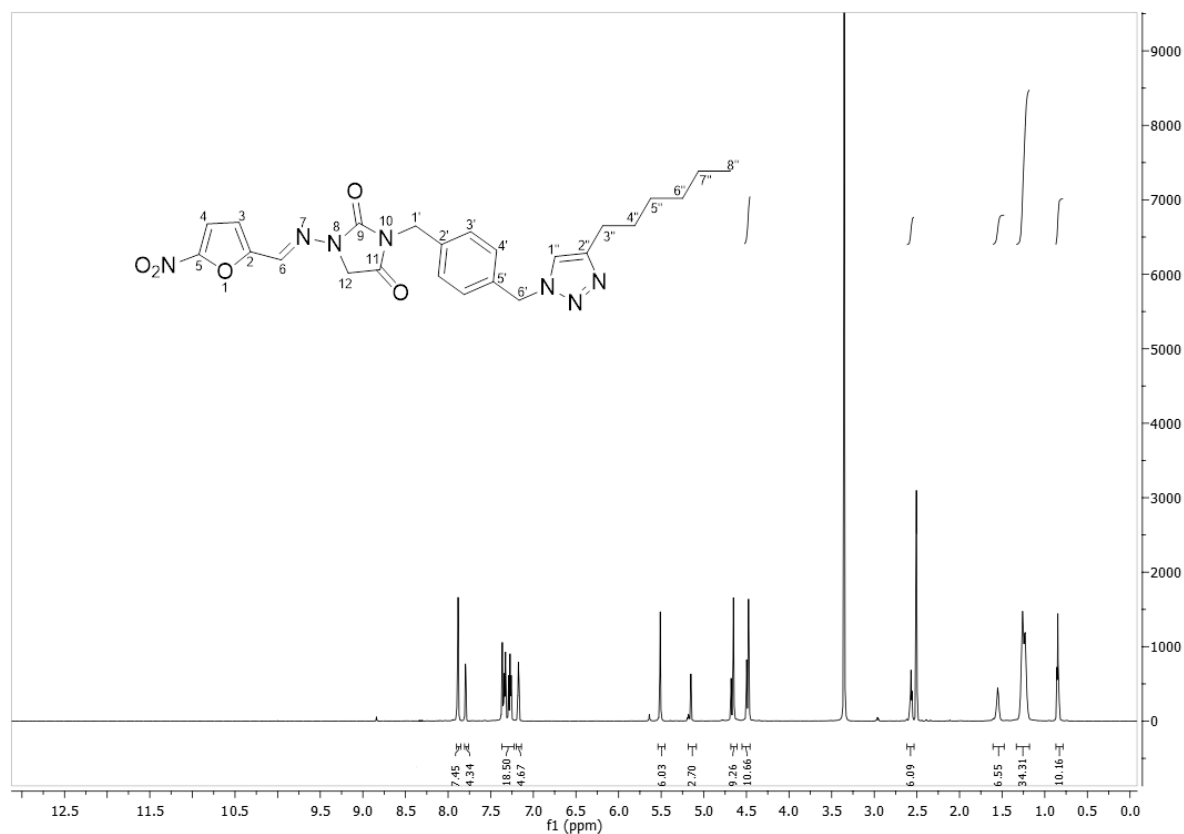
IR



¹H NMR in DMSO



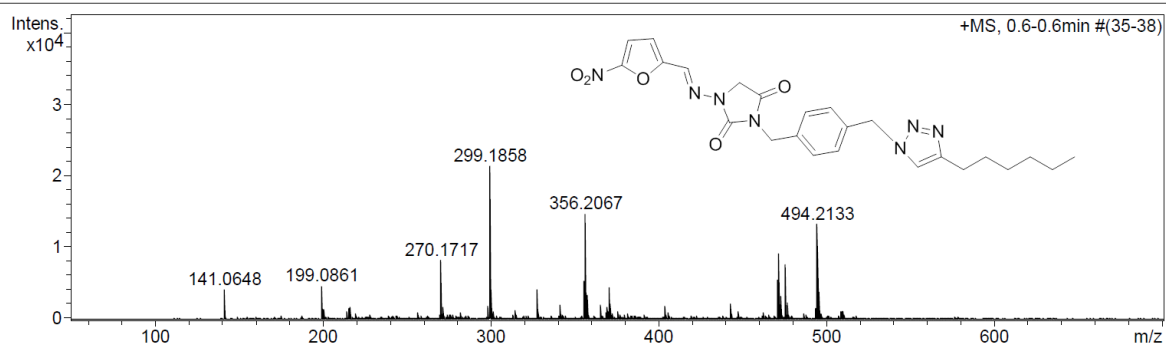
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HRMS

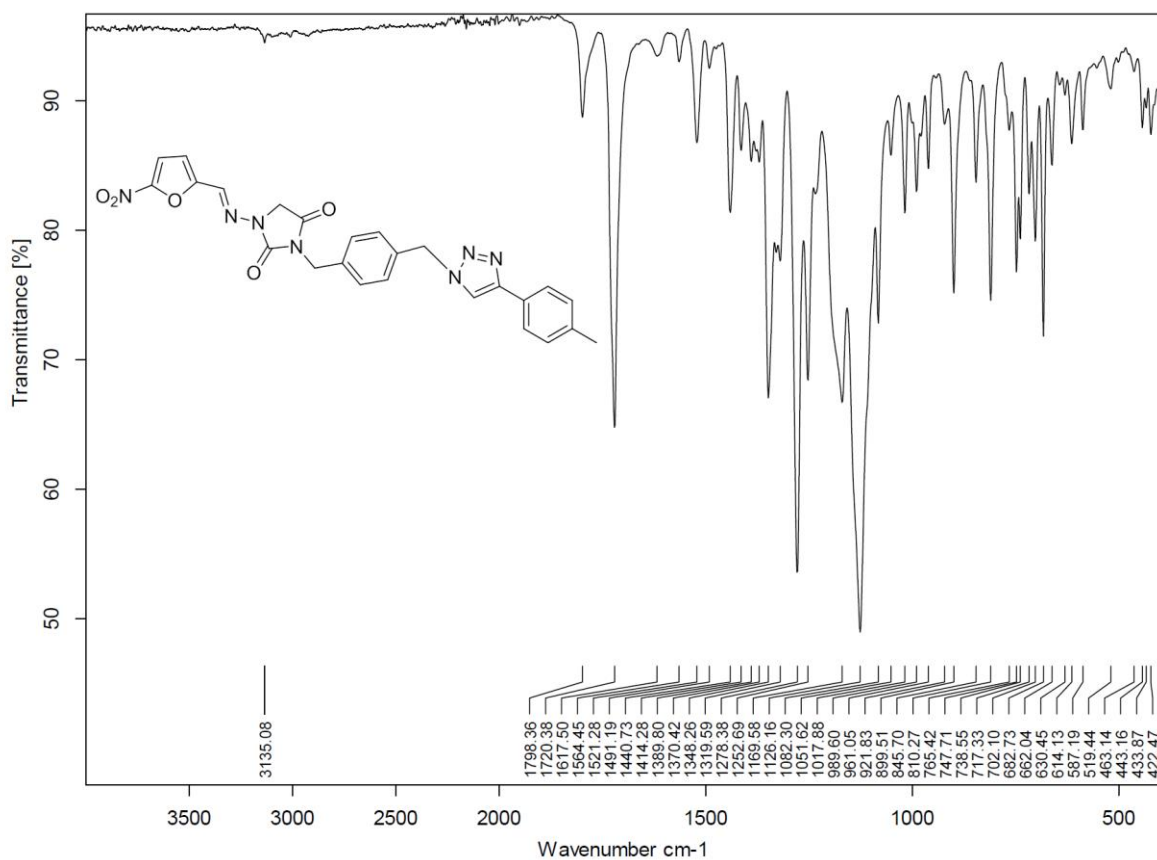
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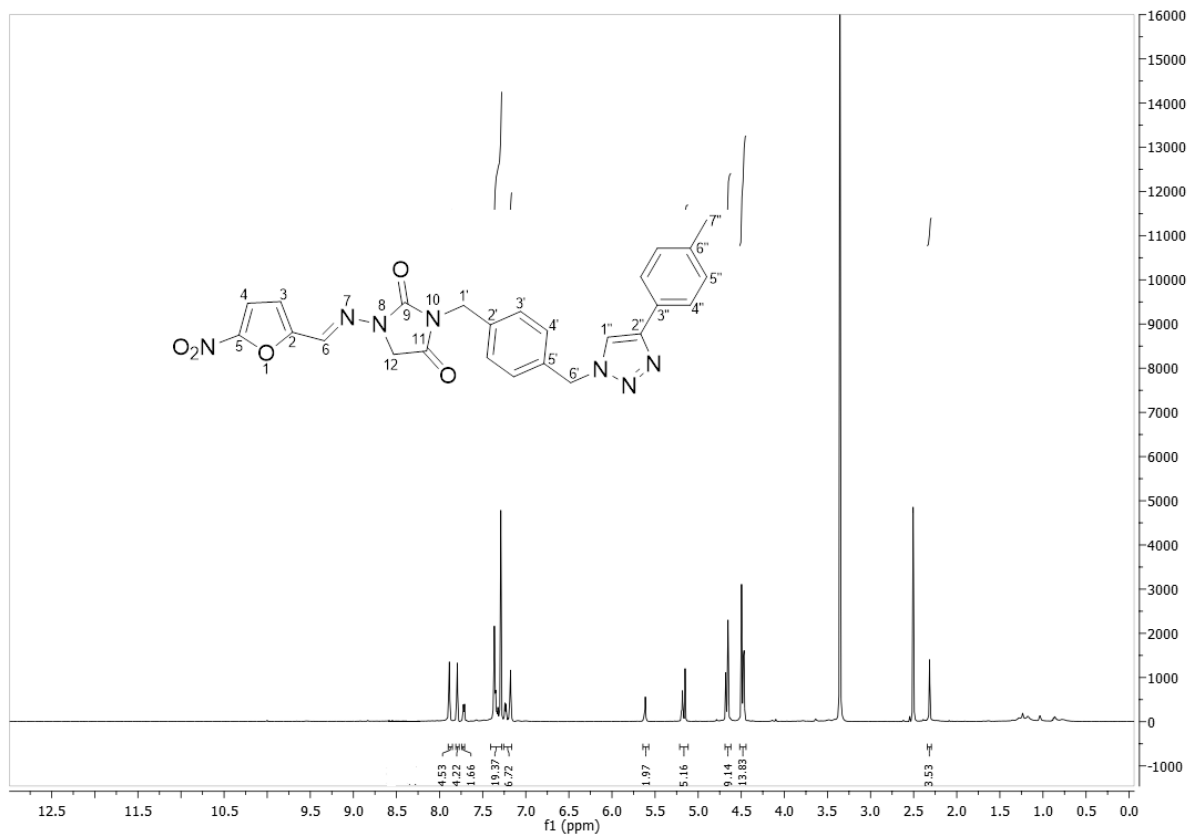


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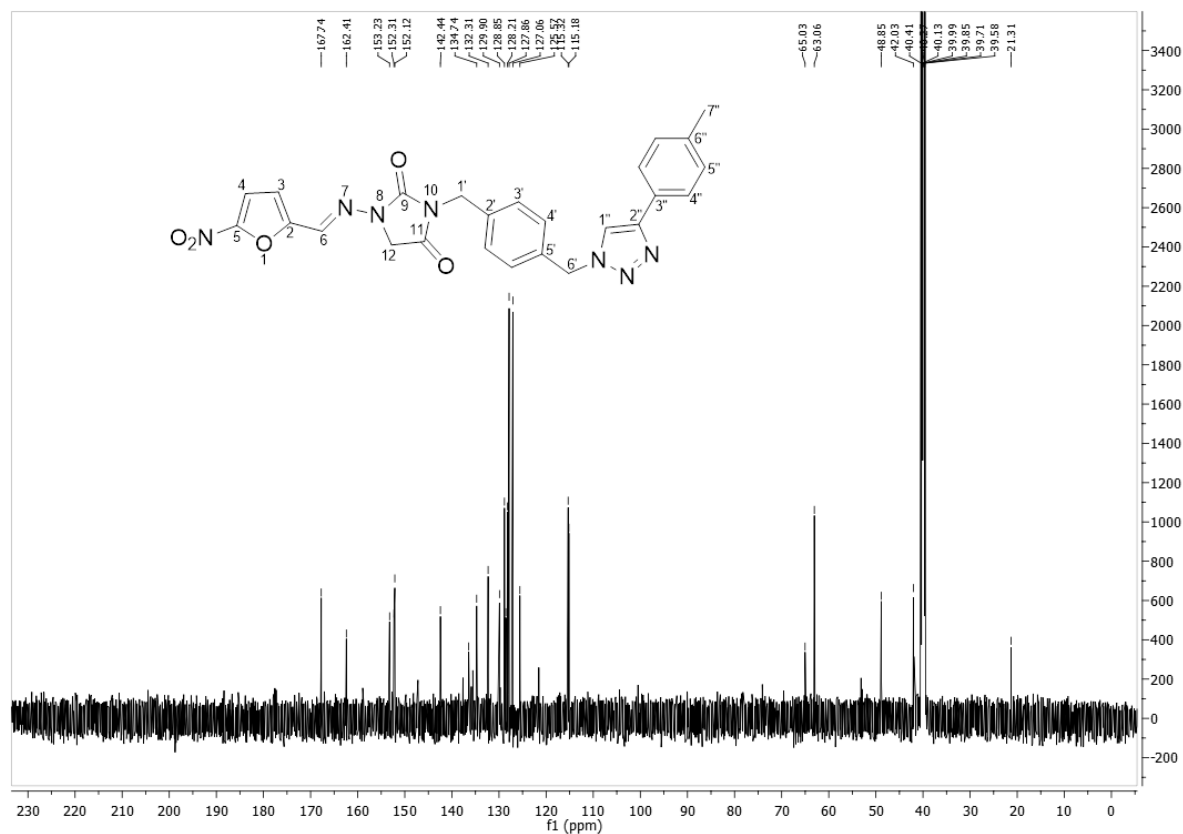
IR



¹H NMR in DMSO



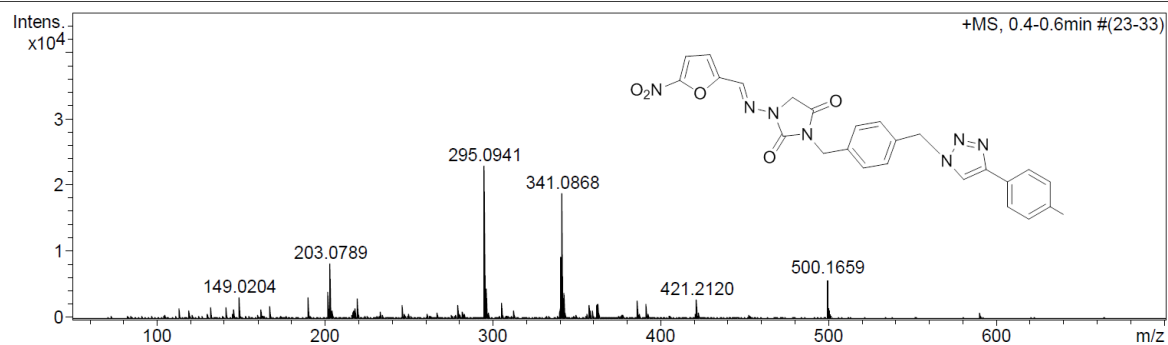
¹³C NMR in DMSO



HRMS

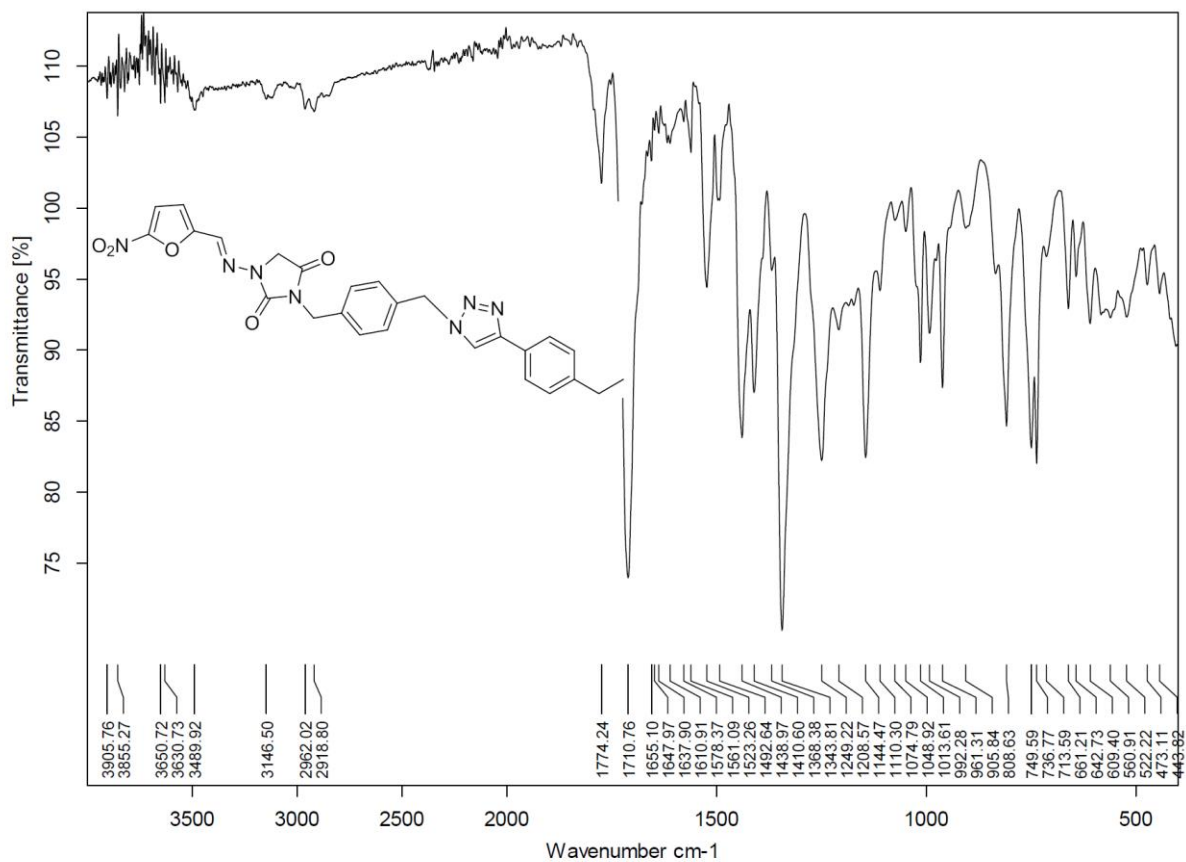
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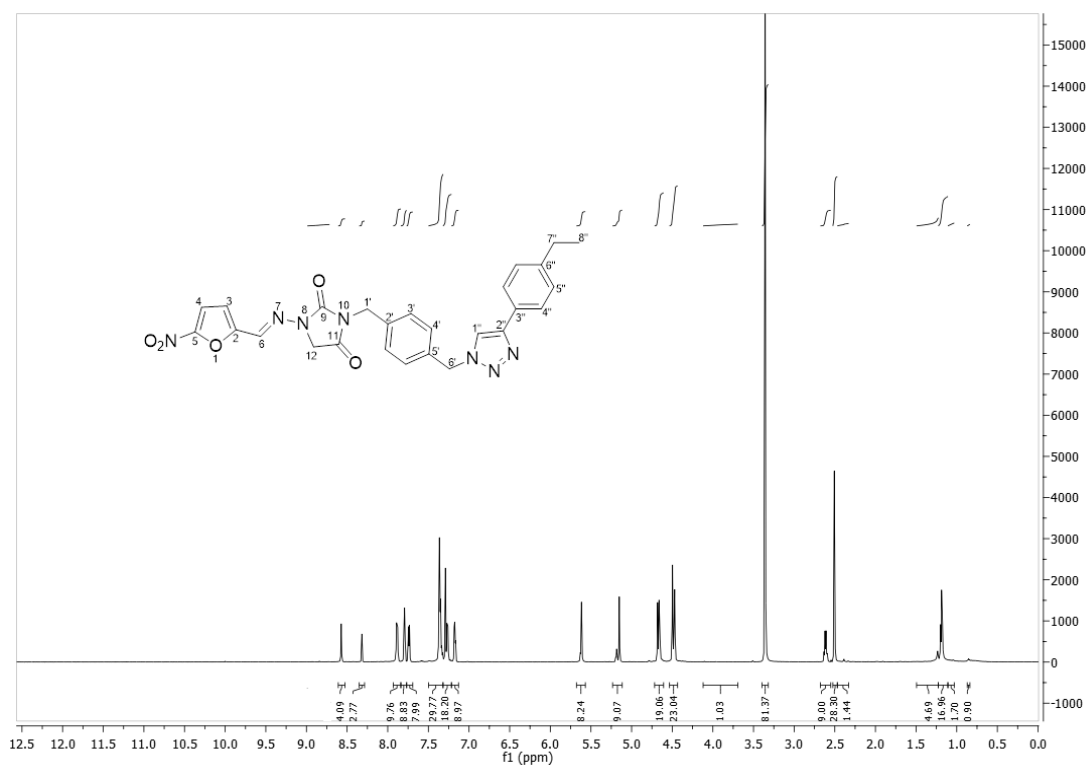


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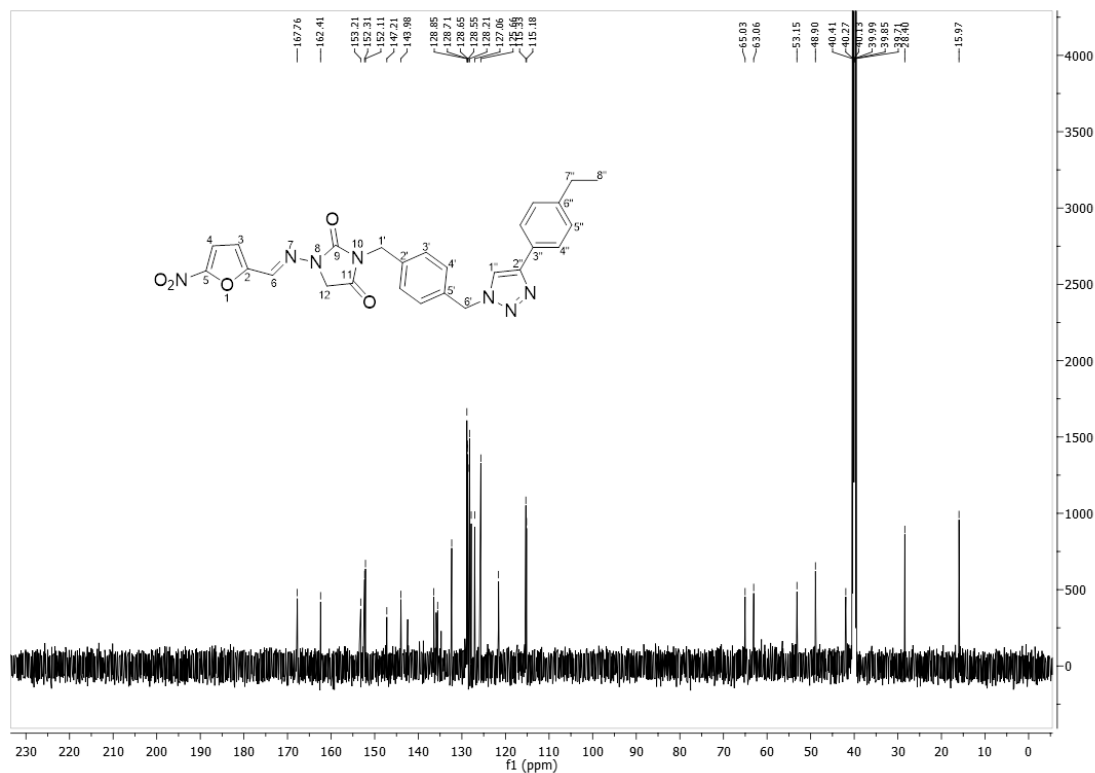
IR



¹H NMR in DMSO



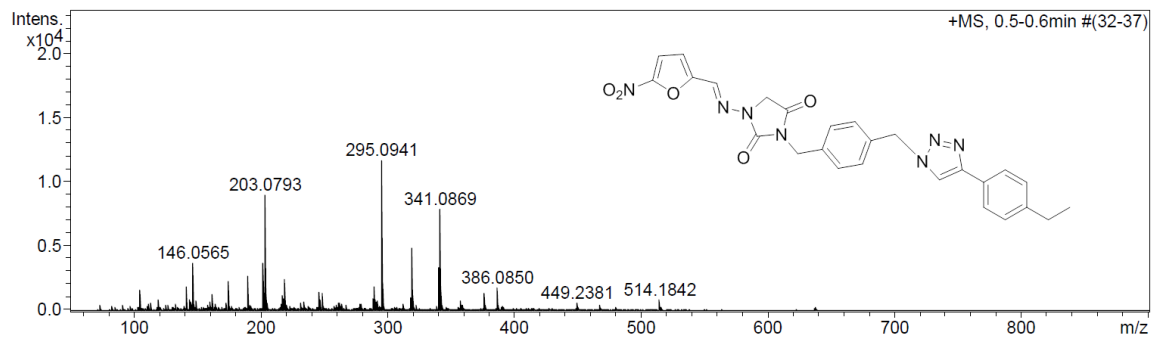
¹³C NMR in DMSO



HRMS

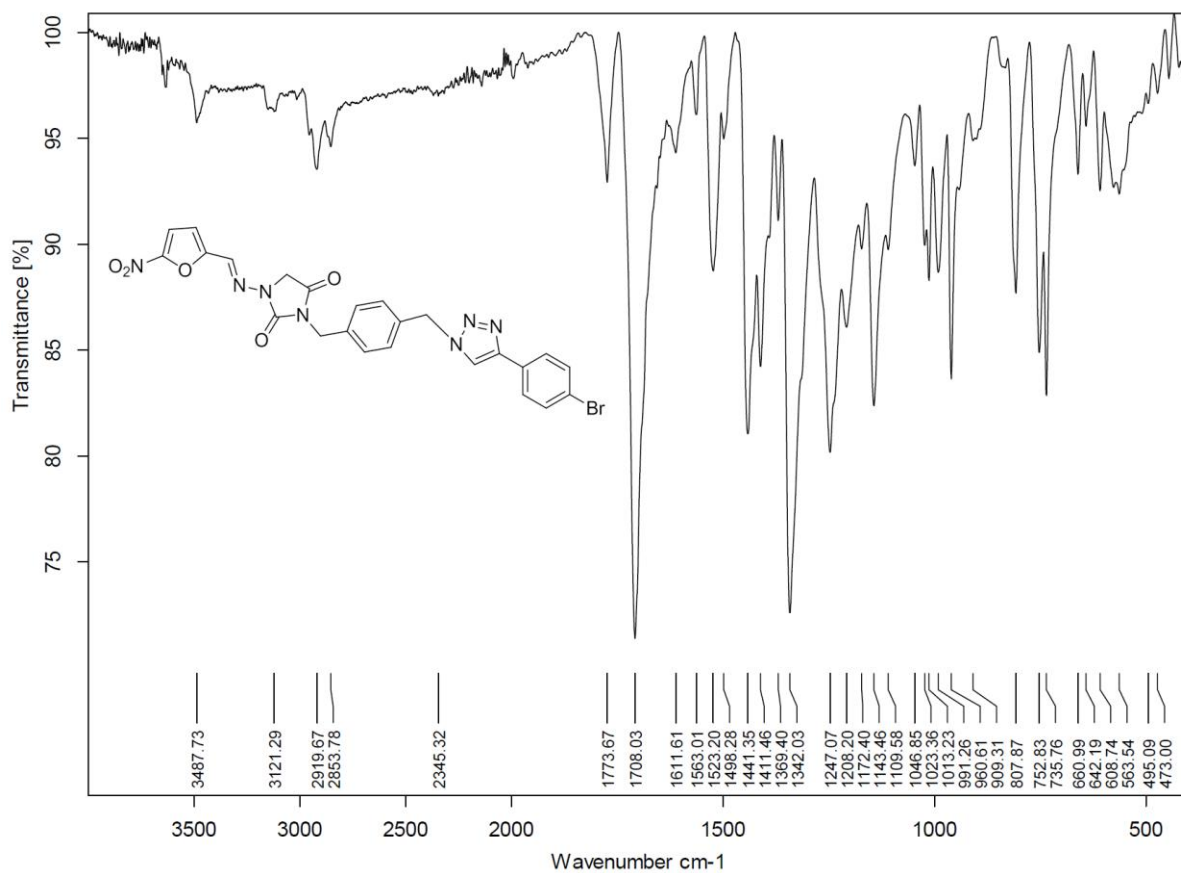
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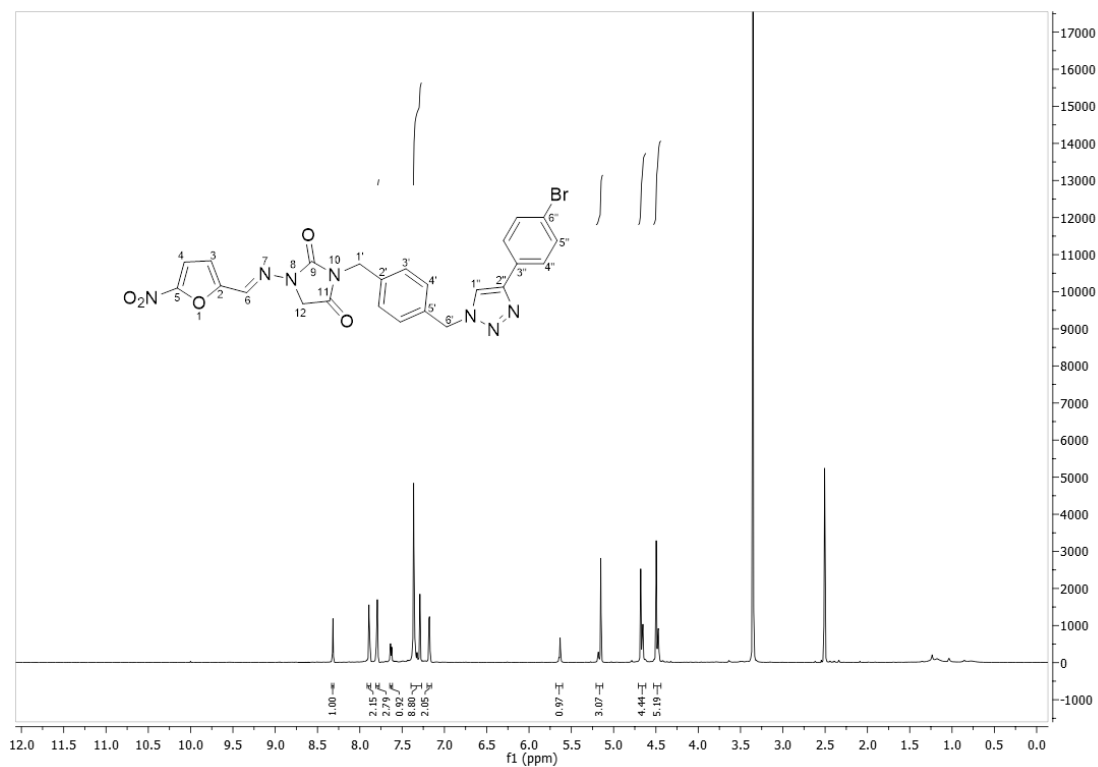


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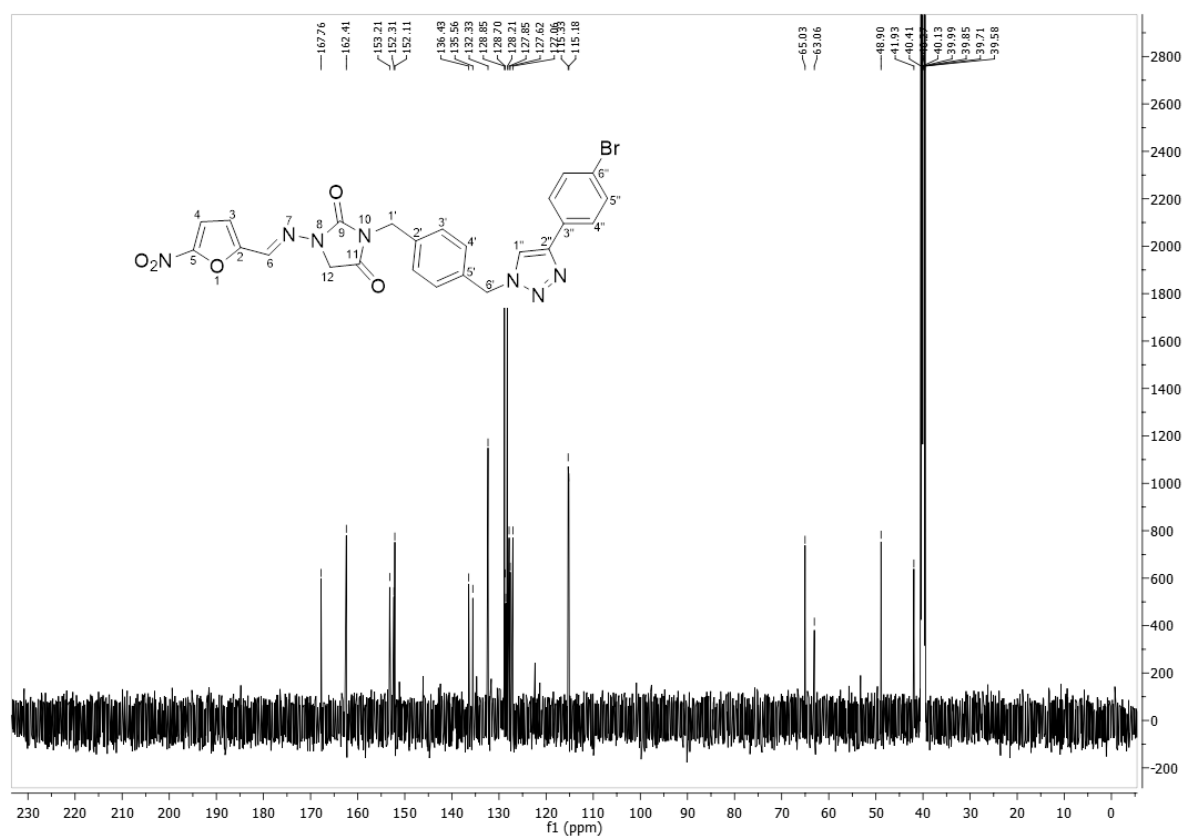
IR



¹H NMR in DMSO

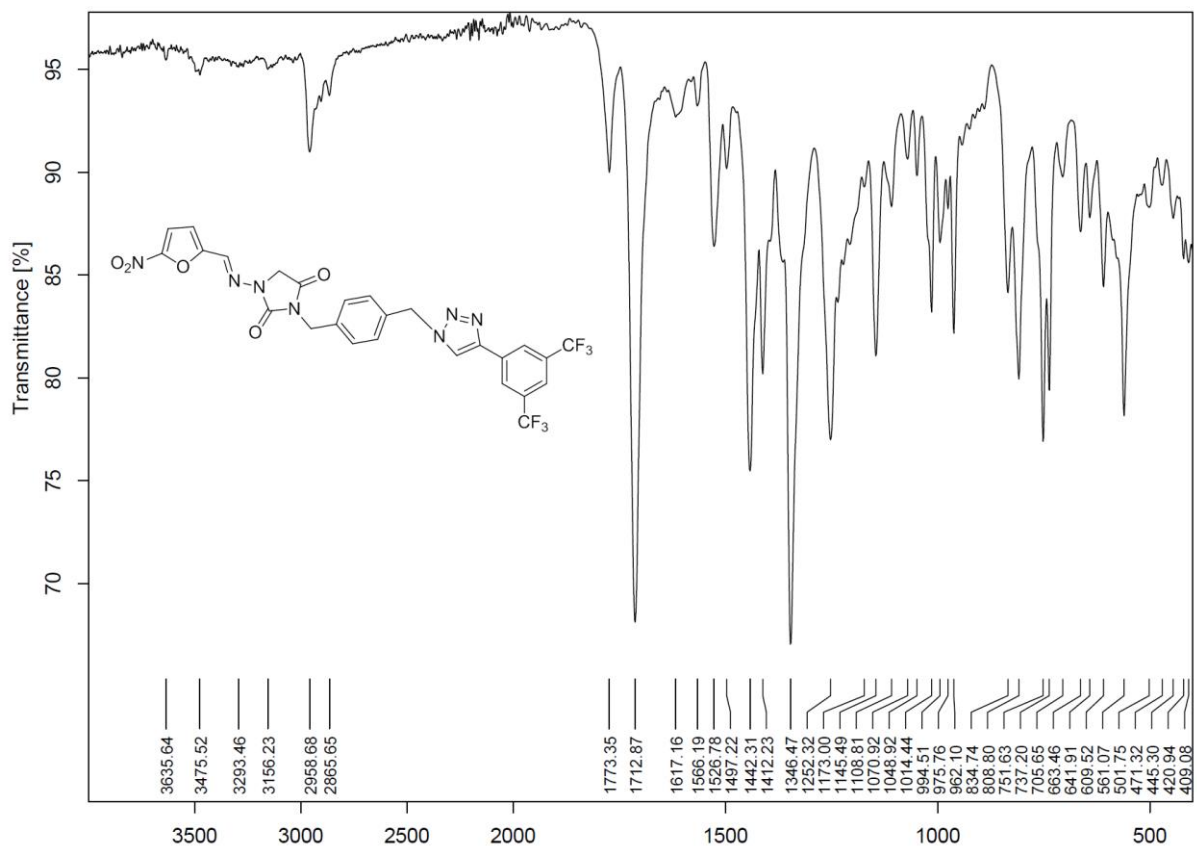


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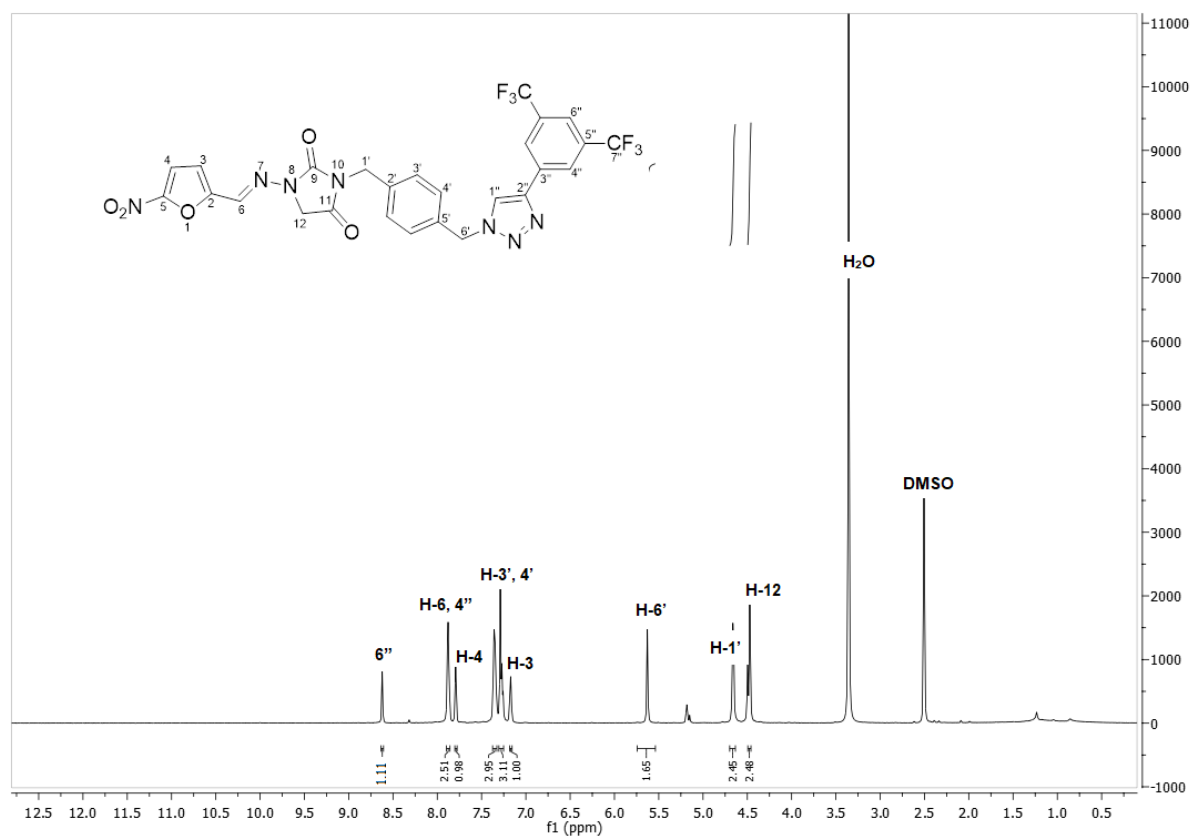


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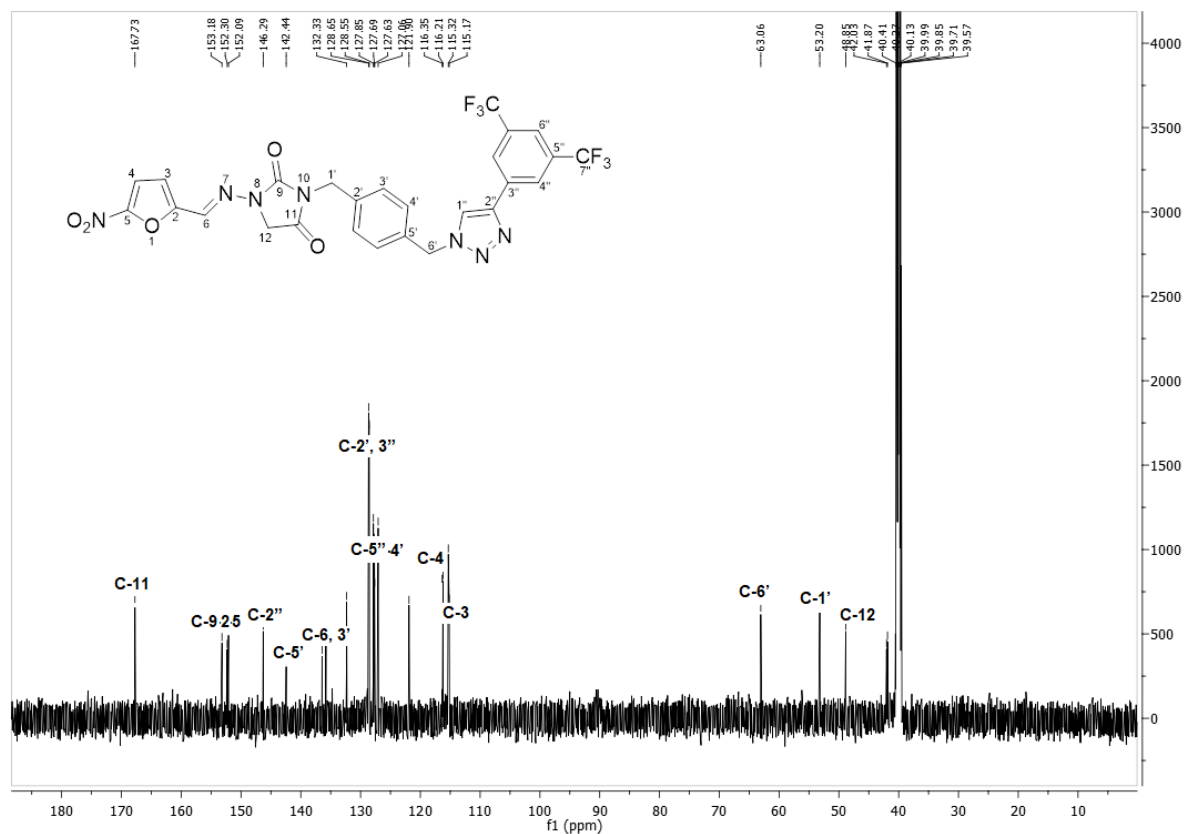
IR



¹H NMR in DMSO



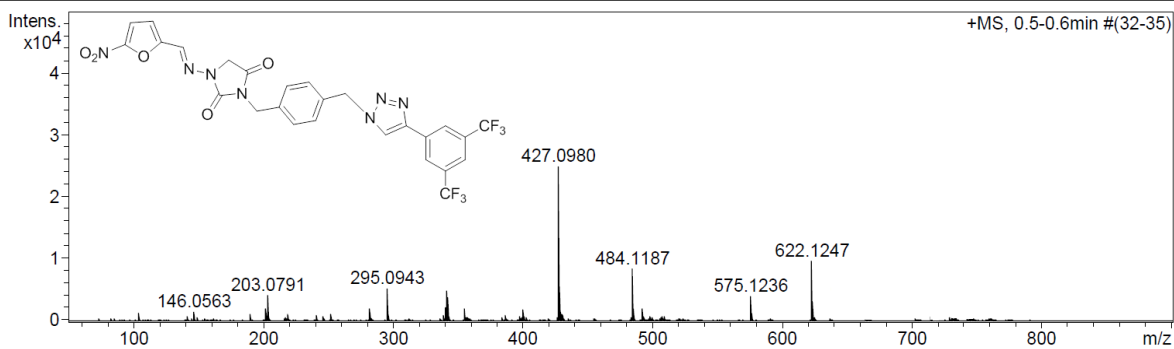
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HR-MS

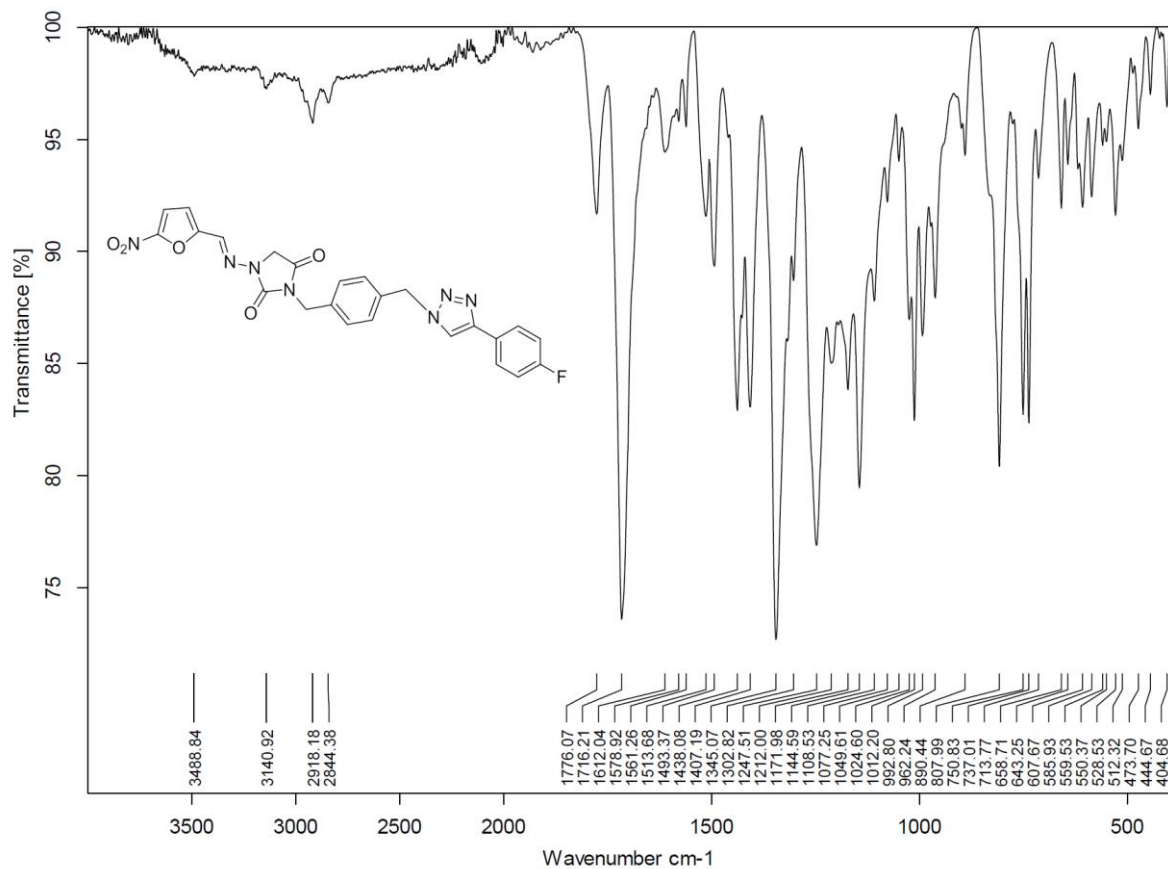
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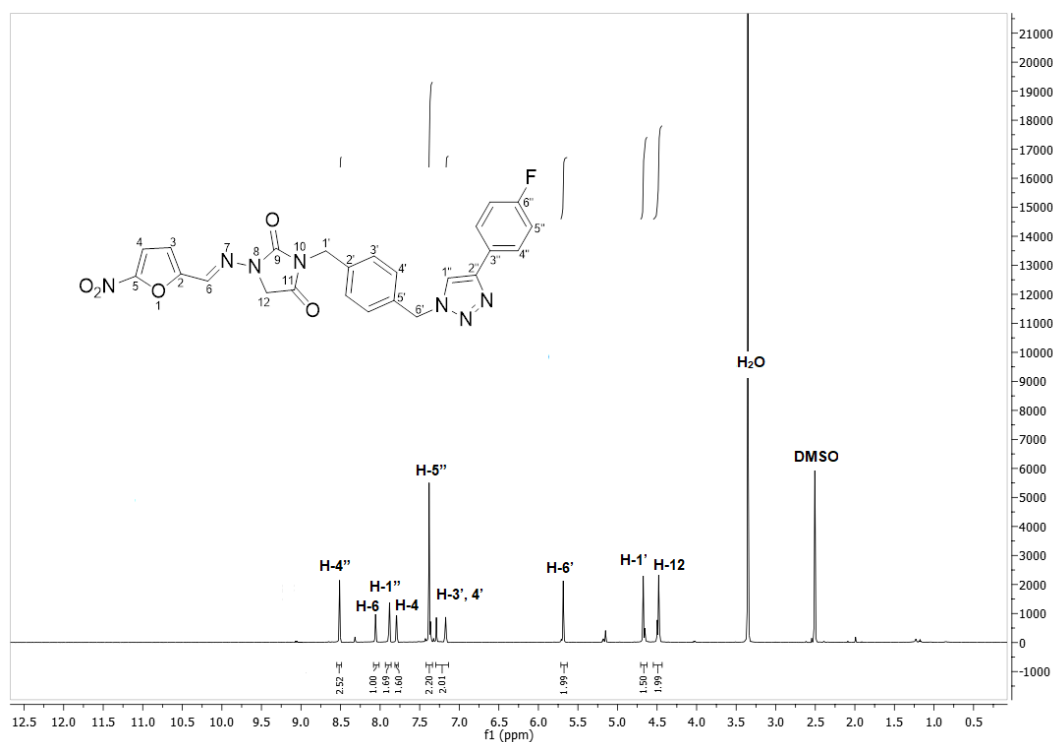


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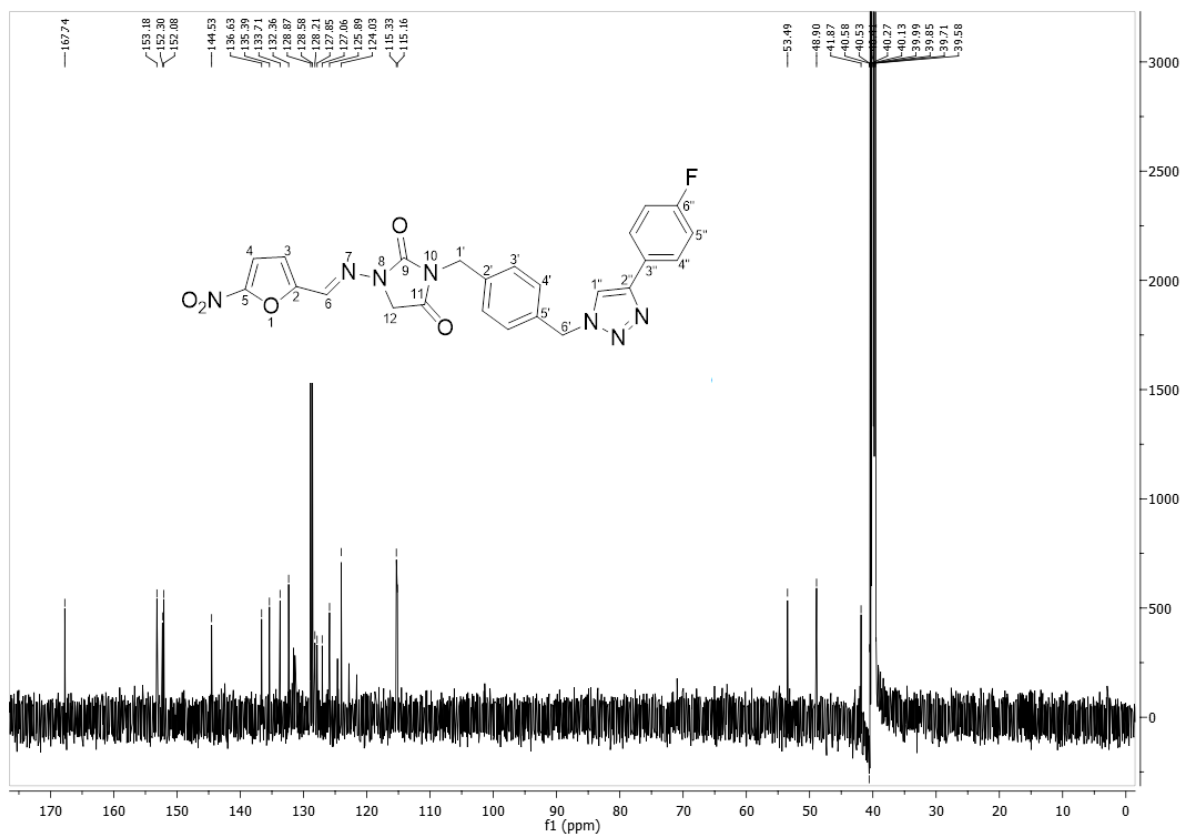
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¹H NMR in DMSO



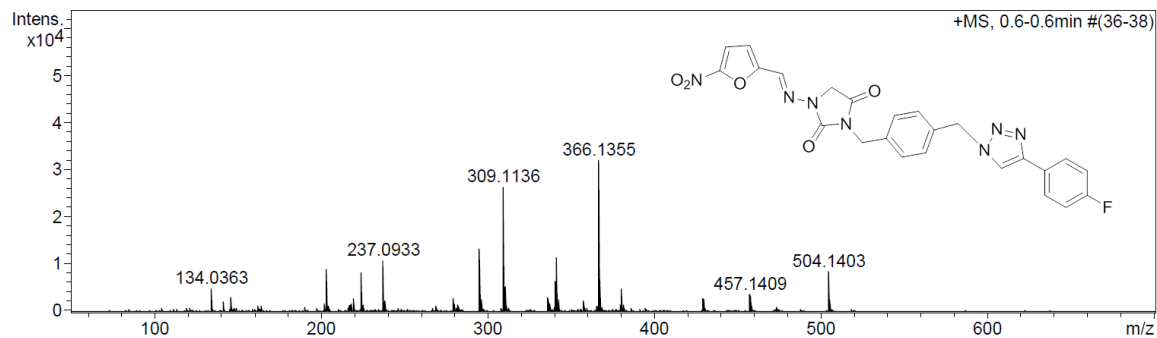
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HR-MS

Acquisition Parameter

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Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



APPENDIX B

Ethical and scientific approval certificates including permissions to reprint figures from other sources as well as the declaration from the language editor and the *Bioorganic Chemistry* Author Guidelines

25 October 2019

RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Health Research Ethics Committee (NWU-HREC) on 25/10/2019, the NWU-HREC hereby clears your study as a no risk study. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Synthesis and anti-infective activities of novel nitrofurantoin derivatives

Principal Investigator/Study Supervisor/Researcher: Prof DD N'Da

Student: NH Zuma - 23978538

Ethics number:

N	W	U	-	0	0	9	4	1	-	1	9	-	A	1
---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Institution Study Number Year Status

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation;
A = Authorisation

Application Type: Single study
Commencement date: 25/10/2019

Risk:

No Risk

General conditions:

The following general terms and conditions will apply:

- *The commencement date indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
 - *request access to any information or data at any time during the course or after completion of the study;*
 - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research;*
 - *withdraw or postpone clearance if:*
 - *any unethical principles or practices of the study are revealed or suspected;*
 - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
 - *submission of the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
 - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECAppl@nwu.ac.za or 018 299 1206*

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,



Digitally signed by
Prof Petra Bester
Date: 2019.10.25
09:35:00 +02'00'

NWU-HREC Chairperson



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25 October 2019

File reference: 9.1.5.4.3

PMC full text: [Mediterr J Hematol Infect Dis. 2013; 5\(1\): e2013070.](#)

<< Prev Figure 2

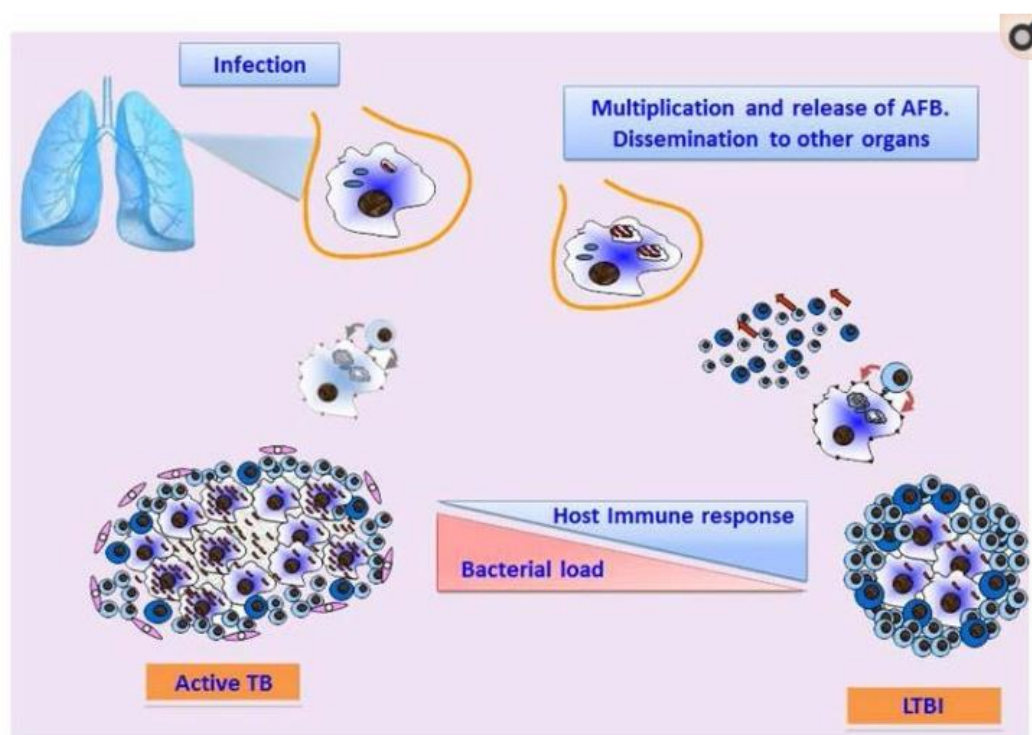
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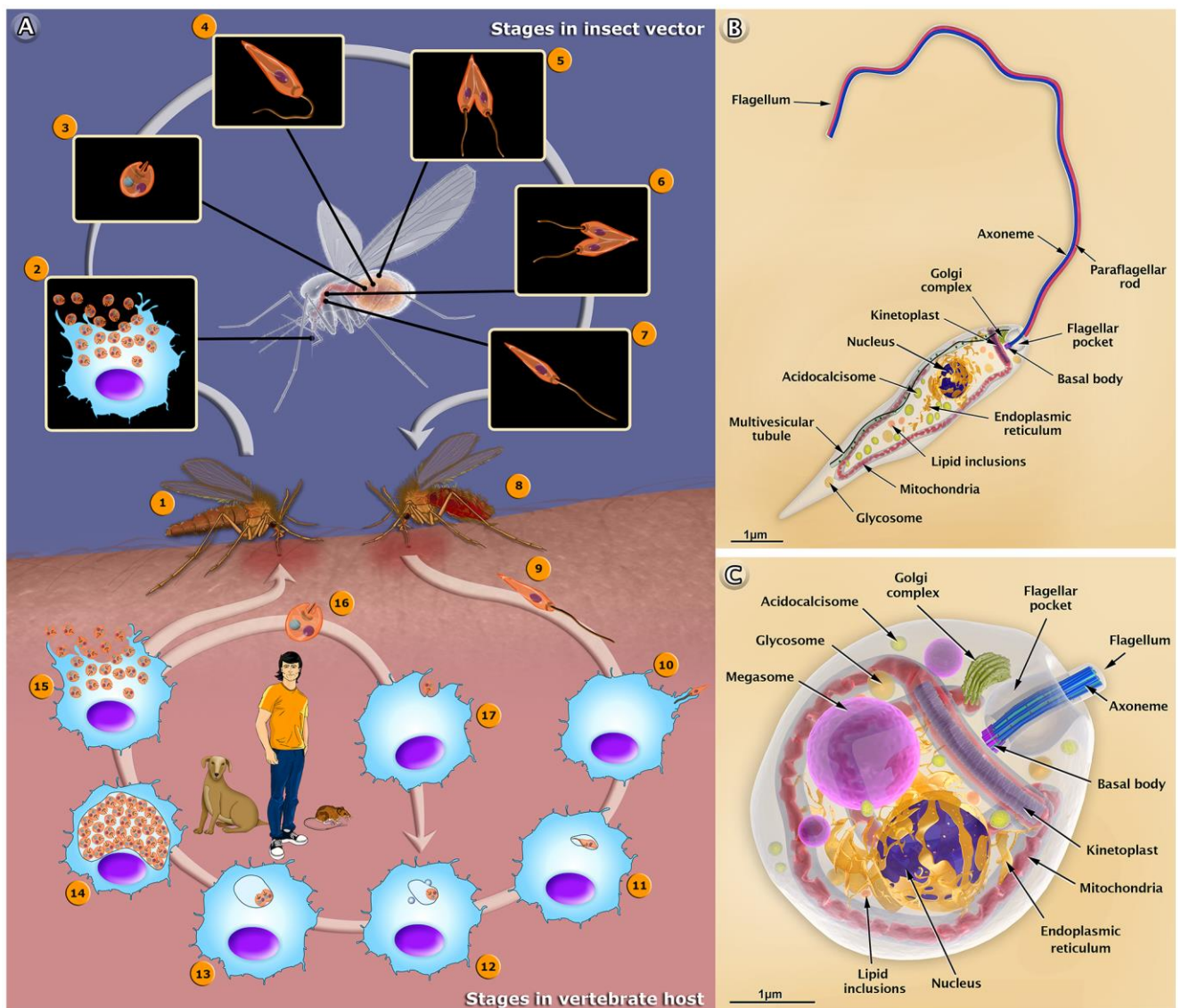
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Figure 2



TB pathogenesis



Citation: Teixeira DE, Benchimol M, Rodrigues JCF, Crepaldi PH, Pimenta PFP, de Souza W (2013) The Cell Biology of *Leishmania*: How to Teach Using Animations. PLoS Pathog 9(10): e1003594. <https://doi.org/10.1371/journal.ppat.1003594>

Editor: Laura J. Knoll, University of Wisconsin Medical School, United States of America

Published: October 10, 2013

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Competing interests: The authors have declared that no competing interests exist.



Chapter: 40 Antimalarial Drugs

Book: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases

Author: James S. McCarthy, Richard N. Price

Publisher: Elsevier

Date: Jan 1, 2015

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To whom it may concern,

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Yours truly,

A handwritten signature in black ink, appearing to read 'J. Handford', written over a horizontal line.

Signature and credentials of language editor

JULIA S HANDFORD [MBA, BCom (Acc), BSc (Hons), HED]



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