Synthesis and *in vitro* antileishmanial efficacy of novel O-substituted derivatives of Nifuroxazide

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Preface

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

Chapter 1: Introduction and Problem Statement

Chapter 2: Literature Review

Chapter 3: Article for submission

Synthesis and in vitro antileishmanial evaluation of novel Nifuroxazide-based analogues

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

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Chapter 4: Summary of the study

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Abstract

Leishmaniasis is a neglected tropical disease (NTD) caused by the *Leishmania* parasite and this devastating vector-borne disease affects millions of people worldwide. There are up to 1 million new infections reportedly occurring on a yearly basis, in more than three clinical manifestation forms. The currently available treatments are limited to a handful of drugs that show significant toxicity, are delivered through invasive administration methods, are expensive and are threatened by emergence of parasitic resistance. Hence it is necessary to develop new, affordable and effective antileishmanial drugs.

5-Nitrofurans (NFs) are redox-active anti-infective drugs used to treat various infectious diseases. Research has shown that drugs containing nitro groups like NFs exhibit a wide spectrum of anti-infective activity against various diseases such as bacterial, mycobacterial, parasites and cancer. These drugs are very active owing to the presence of the nitro group that produces free radical species. These free radicals react with the pathogen cell wall enzymes and become lethal to the microorganisms.

Nifuroxazide (NFX) is one of the clinical nitrofurans (cNFs) in use as a treatment for gastro-intestinal infections. NFX contains the nitro moiety as well as a second hydrazone moiety that increases the activity of the drug. Studies have shown that NFX also possesses a variety of anti-infective activity including antiparasitic activity. However, the absorption of the drug from the intestinal tract is limited and the use of the drug may lead to various toxic effects associated with the generation of free radicals. These shortcomings may be overcome through hybridization, thus the possibility of NFX to act as a parent drug for the development of a new antiparasitic drug is promising.

This study evaluated nifuroxazide-based sulfonyl and benzyl analogues for their antileishmanial activity. The analogues contained three biologically active pharmacophores *i.e*., nitrofuran, hydrazone and sulfonyl or benzyl moiety and were synthesised in a single-step reaction. These analogues were screened for their toxicity on Vero cells and their antileishmanial activity against the promastigote forms of *Leishmania donovani* (1S and 9515) and *L. major* IR-173.

The activities of the synthesised analogues ranged from excellent, IC_{50} = 0.08 μM to moderate, IC_{50} = 9.74 μM. The *tert*-butyl substituted derivatives **1h**, **2d** were found to be the most potent of both series, possessing nanomolar activity against all three strains of *Leishmania*. The cytotoxicity

of the analogues ranged from moderately toxic to non-toxic. Overall, eight sulfonyl (**1d**, **1e**, **1f**, **1g**, **1h**, **1j, 1k** and **1l**) and six benzyl (**2b**, **2c**, **2d**, **2e**, **2f** and **2g**) analogues showed promise as anti-promastigote hit/lead compounds due to their high selectivity and activity as well as their low cytotoxicity and may serve as possible building blocks for future antileishmanial drug development.

Keywords: *Leishmania*, promastigote, nitrofuran, nifuroxazide, sulfonyl, benzyl.

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µM	Micromolar
ABC	ATP-dependent binding cassette
ALDH	Aldehyde dehydrogenase
AMB	Amphotericin B
CO_2	Carbon dioxide
CDC	Centers for Disease Control and Prevention
cNFs	Clinical Nitrofurans
CL	Cutaneous leishmaniasis
DNA	Deoxyribonucleic acid
DCL	Diffuse cutaneous leishmaniasis
DMF	Dimethylformamide
EDG	Electron donating group
EWG	Electron withdrawing group
EM	Emetine
ELISA	Enzyme-linked immunosorbent assay
Equiv	Equivalent
FBS	Fetal bovine serum
FZD	Furazolidone
GI	Gastrointestinal
HRMS	High resolution mass spectrometry

ICT	Immunochromatographic test
IR	Infrared
IM	Intramuscular
IV	Intravenous
K_2CO_3	Anhydrous potassium carbonate
L.	*Leishmania*
MCL	Mucocutaneous leishmaniasis
NFs	5-Nitrofurans
NFA	5-Nitro-2-furaldehyde
NTD	Neglected tropical disease
NADH	Nicotinamide adenine dinucleotide + hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate + hydrogen
NFX	Nifuroxazide
NFT	Nitrofurantoin.
NFZ	Nitrofurazone
N.D.	Not determined
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKDL	Post-kala-azar dermal leishmaniasis
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
Spp	Species
SI	Selectivity index
SD	Standard deviation
SAR	Structure activity relationship
SSG	Sodium stibogluconate
TLC	Thin layer chromatography
TEA	Triethylamine
VL	Visceral leishmaniasis
WB	Western blot assay
WHO	World Health Organization

CHAPTER 1

Introduction and Problem Statement

1.1 Introduction

Leishmaniasis is a widespread parasitic disease caused by the genus *Leishmania (L.)* (Hailu *et al.*, 2016). This disease is classified as a neglected tropical disease (Sangenito *et al.*, 2019), due to limited financing for its management in poor and developing countries (Oliveira *et al.*, 2021). As a result of this neglected status, at least 700 000 to 1 million new leishmaniasis cases are reported yearly worldwide (WHO, 2021a) with the majority occurring in the poverty-stricken developing countries. The transmission of this parasite occurs *via* the bite of an infected female sandfly (*Phlebotomus* and *Lutzomyia*) (Torres-Guerrero *et al.*, 2017), which carries the promastigote form of the parasites in its gut (Giraud *et al.*, 2019). After inoculation through the skin, the parasite undergoes phagocytosis by the host macrophages, causing it to transform into the non-flagellated amastigote form (Alemayehu & Alemayehu, 2017). This amastigote form multiplies inside the macrophage until it lyses, releasing the parasite and causing the multiplication process to be repeated (Alemayehu & Alemayehu, 2017; Liévin-Le Moal & Loiseau, 2016). These stages can be observed in Figure **1.1**.

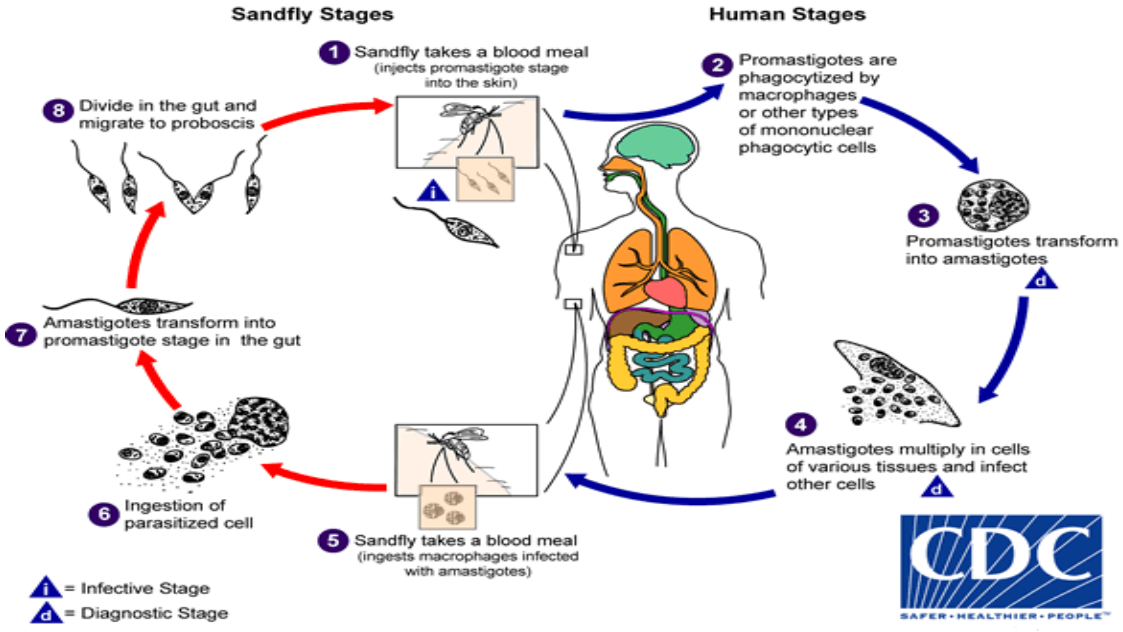


Figure 1.1: The life cycle of the *Leishmania* parasite (CDC, 2020a)

There are more than fifty species of *Leishmania* (divided into Old and New World species), twenty of which are pathogenic towards humans (Akhoundi *et al.*, 2016). *Leishmania donovani*, *L. major* and *L. braziliensis* are the highest priority species (CDC, 2020a; de Oliveira Guerra *et al.*, 2011) that contribute to the five clinical forms of leishmaniasis, namely visceral (VL) also known as kala-azar, post-kala-azar dermal (PKDL), cutaneous (CL), diffuse cutaneous (DCL), and mucocutaneous (MCL) leishmaniasis (Ehab Kotb *et al.*, 2014). The clinical form depends on the position of the parasite in the host's tissue (Akhoundi *et al.*, 2017).

Visceral leishmaniasis affects internal organ tissue and is the deadliest form of the disease, if left untreated (Braga, 2019), and is caused by *L. donovani* and *L. infantum* (Ready, 2014). Tens of thousands of VL cases have been reported in the last year (WHO, 2021a). VL presents with a variety of symptoms including severe symptoms like hepatosplenomegaly and bone marrow suppression (Ready, 2014; WHO, 2021b). Existing treatments for VL are limited to a handful of existing drugs namely amphotericin B, miltefosine, paromomycin and pentavalent antimonials (Freitas-Junior *et al.*, 2012). However, these treatments do not only come with efficacy but are costly and are associated with several adverse effects (No, 2016; Rauf *et al.*, 2016).

PKDL, which is also caused by *L. donovani* (Mukhopadhyay et al., 2014), is a sequela of VL (WHO, 2021a). This form is present in dermal tissue and can be identified by the unique clinical presentation in patients that have recovered from VL (Zijlstra, 2016). It mainly occurs in the Indian subcontinent and East Africa (WHO, 2021a). The current treatment consists of sodium stibogluconate (SSG) or miltefosine (Mondal *et al.*, 2019).

CL is the most common form of leishmaniasis (CDC, 2020b), and it is caused by a variety of Old (*L. major and L. infantum*) and New World (*L. amazonensis* and *L. mexicana*) species (de Vries *et al.*, 2015). CL manifests as ulcerating skin condition (Pagheh *et al.*, 2014). Treatment for CL consists of local and systemic treatments. Local treatments consist of wound care, paromomycin-containing ointments, intralesional antimonials combined with cryotherapy and thermotherapy while the systemic treatment consists of pentavalent antimony, liposomal amphotericin B, pentamidine, fluconazole and ketoconazole and miltefosine (Showler & Boggild, 2015).

DCL is a rare form of leishmaniasis that results in uncontrolled parasite growth in cutaneous lesions on the skin (Christensen *et al.*, 2019) and is mainly caused by *L. amazonensis* (Soares *et al.*, 2020). DCL manifests itself as non-ulcerating nodular lesions (Hashiguchi *et al.*, 2016). At present, there is no effective treatment for DCL (França-Costa *et al.*, 2014).

MCL is one of the least common manifestations of *Leishmania* infection (CDC, 2020b) and it is mainly caused by *L. braziliensis* (de Oliveira Guerra *et al*., 2011). MCL is a disfiguring form of leishmaniasis found in the nasopharyngeal mucosa (Braga, 2019). The treatment used for MCL consists of pentavalent antimony-containing compounds, such as *N*-metil-glucamine antimoniate, amphotericin B, pentamidine, miltefosine, ketoconazole and fluconazole (Amato *et al.*, 2008; Blum *et al.*, 2014).

There are, however, significant limitations to the above-mentioned treatments. All are toxic and must be administrated intravenously, with the exception of miltefosine that is administered orally (de Souza *et al.*, 2020). Amphotericin B has been shown to have acute toxicity, which includes fever, hypertension or hypotension, hypoxia, nausea, vomiting, rigors, and chronic nephrotoxicity (Hamill, 2013; Laniado-Laborín & Cabrales-Vargas, 2009). Due to these factors and high costs, its use is limited (Lanza *et al.*, 2019). Miltefosine has been reported to show embryo- and fetotoxicity, as well as teratogenic effects (Tiwari *et al.*, 2017) and should, be used with care in women of child-bearing age (Dorlo *et al.*, 2012). Pentavalent antimonials have shown cardiotoxicity (Cardona-Arias *et al.*, 2017) and hepatotoxicity (Kato *et al.*, 2014). Pentamidine has shown hypo- and hyperglycaemia as its main adverse effects (Hafiz & Kyriakopoulos, 2020). Further details on the current antileishmanial drugs in clinical use and their adverse effects will be provided in the subsequent Chapter 2.

Apart from the known toxicities, *Leishmania* spp*.* have developed resistance to the currently available drugs (Natera *et al.*, 2007; Ponte-Sucre *et al.*, 2013). Therefore, it is important to develop new, more cost-effective, safe and efficacious drugs for the effective treatment of leishmaniasis (Kato *et al.*, 2014).

Nifuroxazide (NFX) (Figure **1.2**) is a phenol-containing 5-nitrofuran derivative that was first synthesised in 1944 (B. Fernandes *et al.*, 2015; Dodd *et al.*, 1944). It has been used as an antibacterial drug since 1966 (Ernest, 1966) and as an antiprotozoal treatment since the 1970s (Cedillo-Rivera & Muñoz, 1992). NFX has also been shown to have anticancer effects (Bailly, 2019).

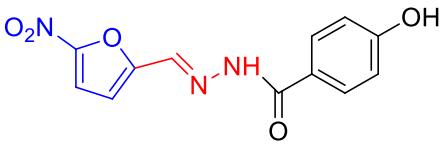


Figure 1.2: Structure of Nifuroxazide (NFX)

NFX's antibacterial and antiprotozoal activities are derived from the reduction of the nitro group, the main pharmacophore (blue in Figure **1.2**), by parasitic reductase enzymes (Krasavin *et al.*, 2019). The reduction of the nitro group produces toxic radical anions that form reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Pal & Bandyopadhyay, 2011; Zhou *et al.*, 2012). These species are responsible for both the antipathogenic effects and toxicity associated with NFX, due to oxidative stress causing protein carbonylation, membrane lipid peroxidation, DNA breakage, enzyme inactivation and inflammatory reactions, ultimately resulting in cell death (Liu *et al.*, 2017; Patel *et al.*, 2018).

In addition to the main mechanism of action, NFX also depletes the free thiol in parasites, which is an important part in the parasite's defence against ROS (Comini *et al.*, 2007; Pal & Bandyopadhyay, 2011). Furthermore, NFX also contains a second pharmacophore, the hydrazone moiety (red in Figure **1.2**), that contains intrinsic biological and pharmacological activity (Ryan, 2017). Its inclusion in NFX promotes chemical stability (Zuma *et al.*, 2019) and results in a compound with unique characteristics (Fernandes *et al.*, 2018; Verma *et al.*, 2014).

Currently, a variety of 5-nitrofuran drugs, including NFX, are in clinical use as antibacterial and antiparasitic treatments (Figure **1.3**), including Human African Trypanosomiasis (Zuma *et al.*, 2019) that is closely related to *Leishmania spp.* as both belong to the *Trypanosomatid* family, order Kinetoplastida (de Morais *et al.*, 2015). Studies have shown that there is very little risk of pathogenic resistance developing against 5-nitrofuran treatments (Le *et al.*, 2019; Zuma *et al.*, 2019), but the possibility for cross-resistance with other nitro-based treatments does exist (Bruhn *et al.*, 2016; Sokolova *et al.*, 2010). Thus, by exploring the use of NFX as a scaffold, novel antileishmanial compounds may be synthesised with reduced risks of resistance development and improved treatment efficacy, cost, and safety.

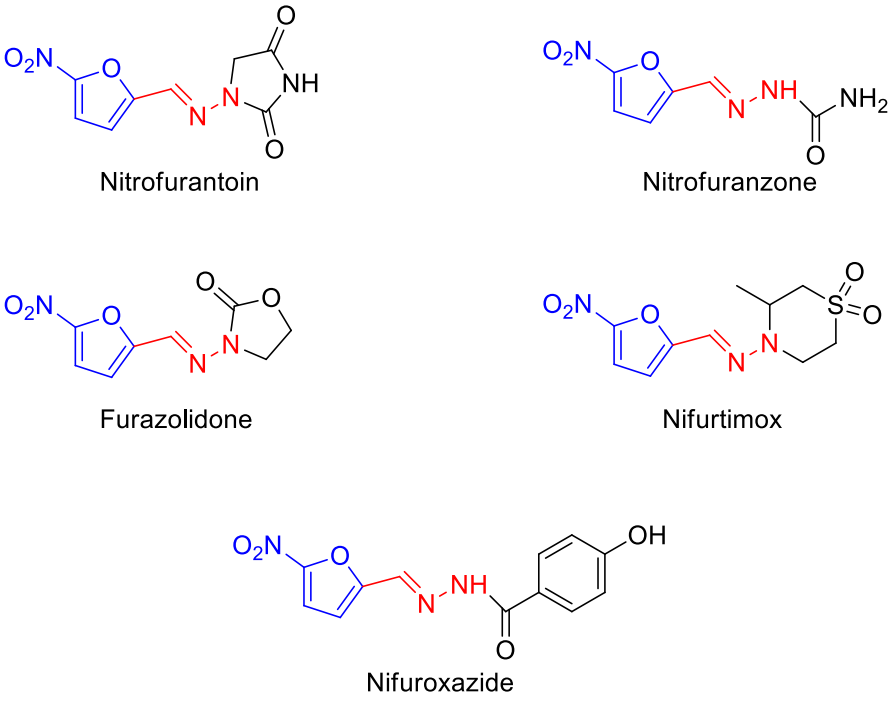


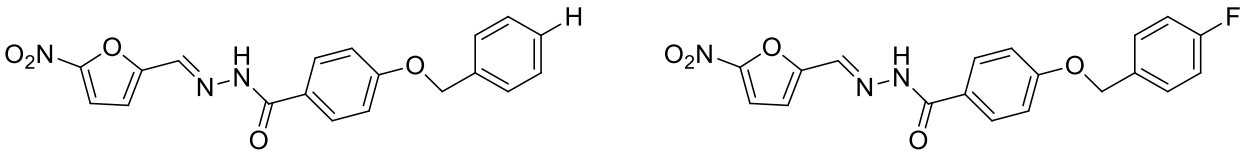
Figure 1.3: 5-Nitrofuran drugs currently in clinical use

The anti-infective efficacy of nifuroxazide (NFX) may be enhanced by substituting its hydroxy group with an additional functional moiety to introduce a third pharmacophore in its structure, with the nitrofuran and hydrazone acting as the first two pharmacophores (Kannigadu *et al.*, 2021; Sapkota *et al.*, 2011; Yousefi *et al.*, 2013). This may be achieved through the attachment of sulfonyl and benzyl groups to the already existing structure of NFX through the oxygen atom.

Previous research has indicated that sulfonyl and benzyl groups have good antiprotozoal activities and enhance the biological effectiveness of compounds (Sapkota *et al.*, 2011; Yousefi *et al.*, 2013) by activating enzymes in the host body that prevent tissue damage, thus decreasing adverse effects (Bölcskei *et al.*, 2017; Chang *et al.*, 2001). The sulfonyl substitution causes cysteine protease inhibition (Banerjee & Abagyan, 2019), which can result in an interruption in the life cycle of both protozoan and helminth parasites (Lehmann *et al.*, 2014).

The current research study is based on the further investigation of benzyl analogues of NFX, **1** and **2** (Figure **1.4**) from a previous study done by our research group (Kannigadu *et al.*, 2021). Intermediates **1** and **2** possessed nanomolar activity up to 10-fold higher than the parent compound NFX against *L. donovani* strains 1S and 9515 promastigotes, and *L. major*

promastigotes thus making it a potential early lead for further investigation into the search of drugs for the treatment of VL (Kannigadu *et al.*, 2021).



1

L. donovani 1S IC_{50}: 0.52 µM
L. donovani 9515 IC_{50}: 1.22 µM
L. major IR173 IC_{50}: 0.25 µM
Cytotoxicity: > 100 µM

2

L. donovani 1S IC_{50}: 0.52 µM
L. donovani 9515 IC_{50}: 1.22 µM
L. major IR173 IC_{50}: 0.25 µM
Cytotoxicity: > 100 µM

Figure 1.4: Benzyl analogues **1** and **2** of NFX (Kannigadu *et al.*, 2021)

That study also indicated that *O*-benzyl alkylation of NFX resulted in enhanced activity while the free NH group is essential for activity, which is demonstrated by the loss of activity observed with *O*, *N*- dialkylated NFX derivatives (Kannigadu *et al.*, 2021). Hence, in the current study, *O*-benzyl and -sulfonyl mono-substituted NFX derivatives were synthesized.

Thus, NFX derivatives might provide a suitable alternative antileishmanial drug with less toxicity and adverse effects. Accordingly, during this study, a series of novel NFX derivatives containing sulfonyl or benzyl moieties as depicted in Figure **1.5**, were synthesised and assessed for antileishmanial activity.

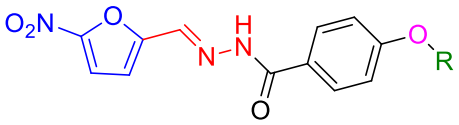


Figure 1.5: Proposed novel NFX derivatives where R is sulfonyl or benzyl substituent.

1.2 Aims and objectives

The aim of this study was to investigate novel nifuroxazide derivatives (Figure **1.5**) as potential in vitro effective antileishmanial agents.

The study's objectives are as follows:

- To synthesise a series of novel sulfonyl and benzyl nifuroxazide derivatives and characterize them using nuclear magnetic resonance (NMR), high resolution mass spectrometry (HRMS), melting point and infrared (IR) spectroscopy.

- Determination of *in vitro* antileishmanial activity against various *Leishmania* parasite strains and cytotoxicity assessment of the synthesised compounds using the Vero cell line.

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CHAPTER 2
Literature review

2.1 Introduction

Parasites have plagued humanity for many centuries (Steverding, 2017) causing chronic and debilitating diseases that are, to date, commonly confined to resource-poor populations (Hotez *et al.*, 2020). Consequently, there are up to twelve significant parasitic diseases that have become neglected over the years due to their limited global impact and their lower death rates in comparison to more serious global diseases (Hotez *et al.*, 2020). An example of this is leishmaniasis which is caused by infection with species (spp.) of the protozoan parasite *Leishmania* (*L*) (Sangenito *et al.*, 2019).

Leishmaniasis is a disfiguring parasitic neglected tropical disease (Sangenito *et al.*, 2019) that is found mostly in the tropical and sub-tropical parts of the world (Maia *et al.*, 2015; Nweze *et al.*, 2021). This vector-borne disease spreads through the bite of an infected female sandfly (*Phlebotomus* and *Lutzomyia* species) (Torres-Guerrero *et al.*, 2017) in both humans and a wide variety of mammalian reservoir hosts, including marsupials, hyraxes, humans, canine and rodent spp. (Steverding, 2017). The *Leishmania* parasite has a two-stage life cycle (Figure **2.1**). The first stage is the motile promastigote form of the parasite, which serves as the infective stage in the gut of the sandfly. The second stage is the tissue stage in the host's body after infection, where the promastigote form transforms into the non-motile amastigote form within the host's macrophages (CDC, 2020a; Damianou *et al.*, 2020).

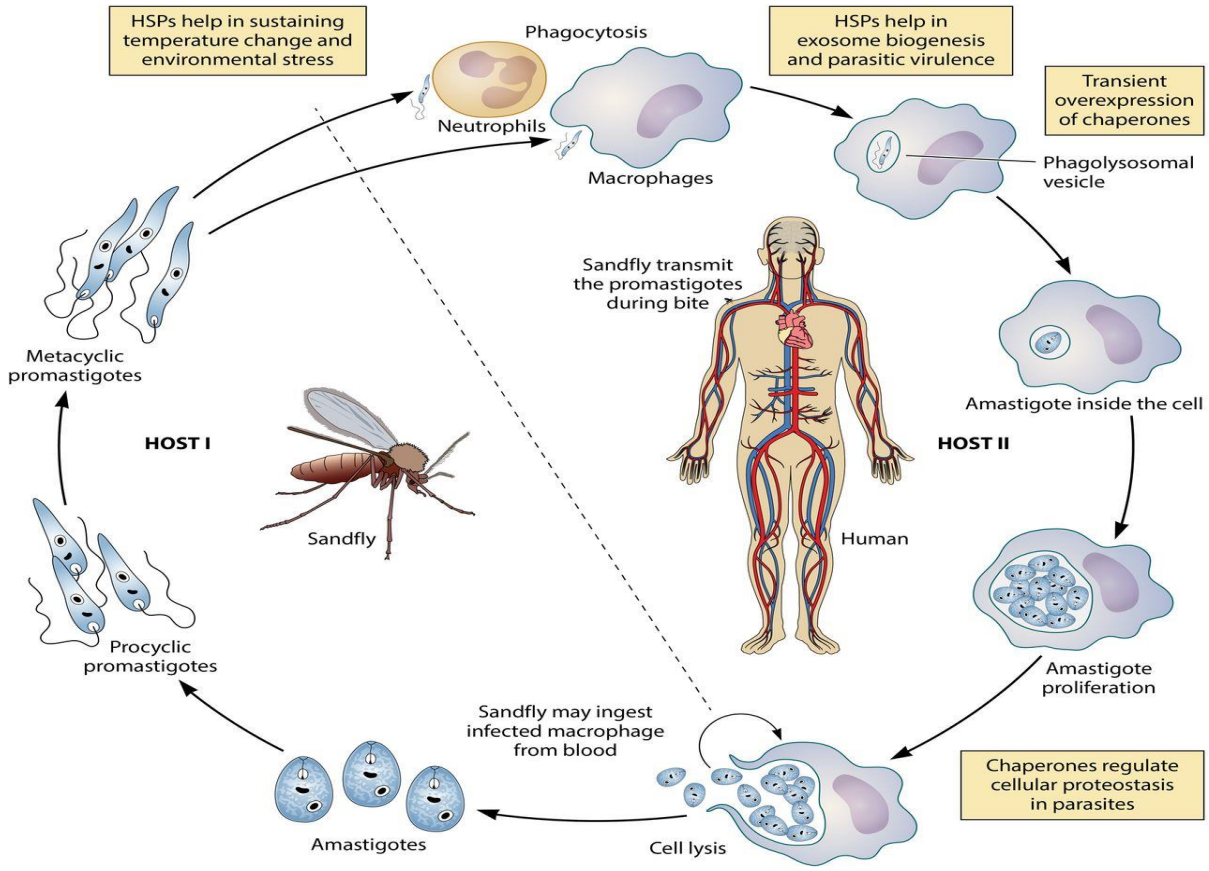


Figure 2.1: The life cycle of the *Leishmania* spp. parasite (Prasanna *et al.*, 2021)

2.2 Epidemiology

Leishmaniasis is a widespread disease that affects the poorest communities around the world that lack nutrition, resources, proper housing and have weakened immune systems (WHO, 2021a). It currently affects 90 countries worldwide (CDC, 2020b), including South America (Brazil, Colombia, Peru), Indian subcontinent (Bangladesh, India, Nepal), Central Asia (China), Middle East (Afghanistan, Iraq, Iran, Syria), Eastern Africa (Ethiopia, Eritrea, Kenya, Somalia, South Sudan, Sudan) and the Mediterranean basin (Algeria, Tunisia, Libya) (Figure **2.2**) (Rijal *et al.*, 2019; WHO, 2021a) . It is estimated that between 700 000 to 1 million new infections occur worldwide on a yearly basis and this disease is the third main cause of zoonotic infections (Pisarski, 2019; WHO, 2021a).

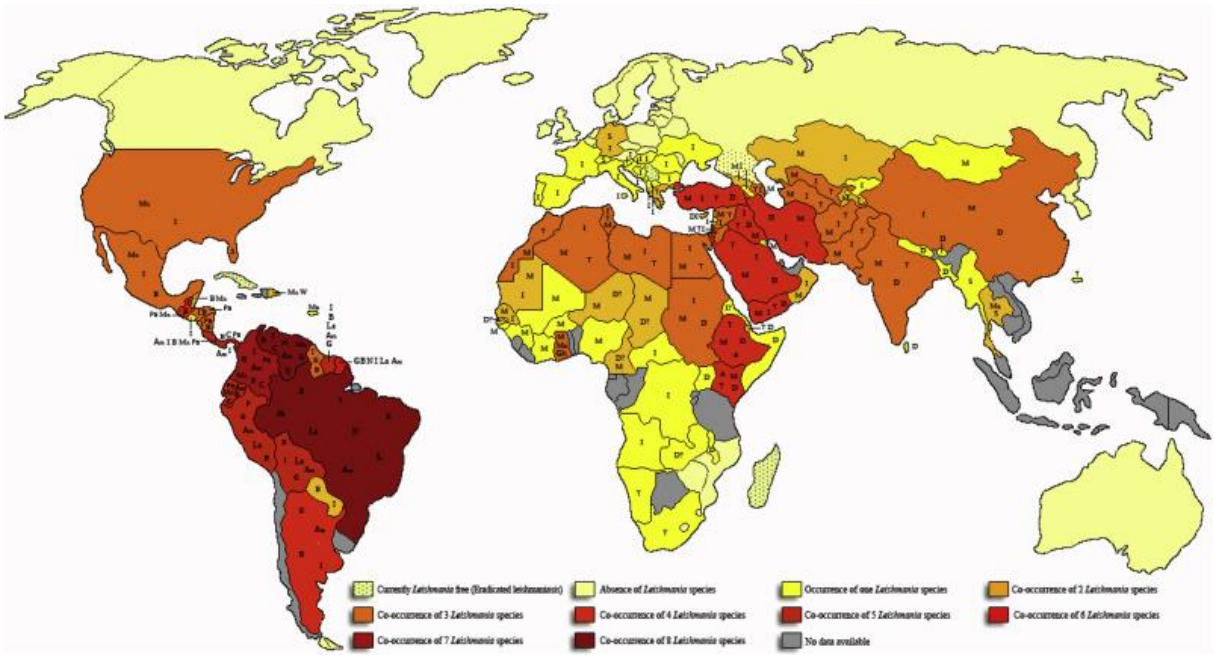


Figure 2.2: Global spread of *Leishmania* spp. (Akhoundi *et al.*, 2017)

Migration and urbanization pose an increased risk for the spread of the disease to endemic and non-endemic areas (Stamm, 2016; WHO, 2002). The development of drug resistance to currently available treatments also contributes to the proliferation of the parasite (Ponte-Sucre *et al.*, 2017). Furthermore, environmental changes play an important part in the life cycle and distribution of the disease. For example, temperature significantly affects the incubation time of the parasite (Bates, 2018), with warmer environments leading to a faster development and colder climates leading to a slower development (Torres-Guerrero *et al.*, 2017). Moreover, changes in the climate causes areas that were previously not affected to become endemic (Tidman *et al.*, 2021). Famine resulting in malnutrition and poor hygienic conditions caused by droughts and floods can also exacerbate the spread of the disease (WHO, 2021a). All of these factors show that leishmaniasis is still a very prevalent and neglected disease (Thakur *et al.*, 2018).

There are currently twenty *Leishmania* spp. that cause clinical manifestations in humans (Sasidharan & Saudagar, 2021). These species can be divided into two main groups, namely Old and New World *Leishmania* (Figure **2.3**) (Kevric *et al.*, 2015; Vojtkova *et al.*, 2020). Old World species are found in Africa, the Mediterranean basin and Middle East, whereas New World species are found throughout Central and South America and Mexico (Akhoundi *et al.*, 2016).

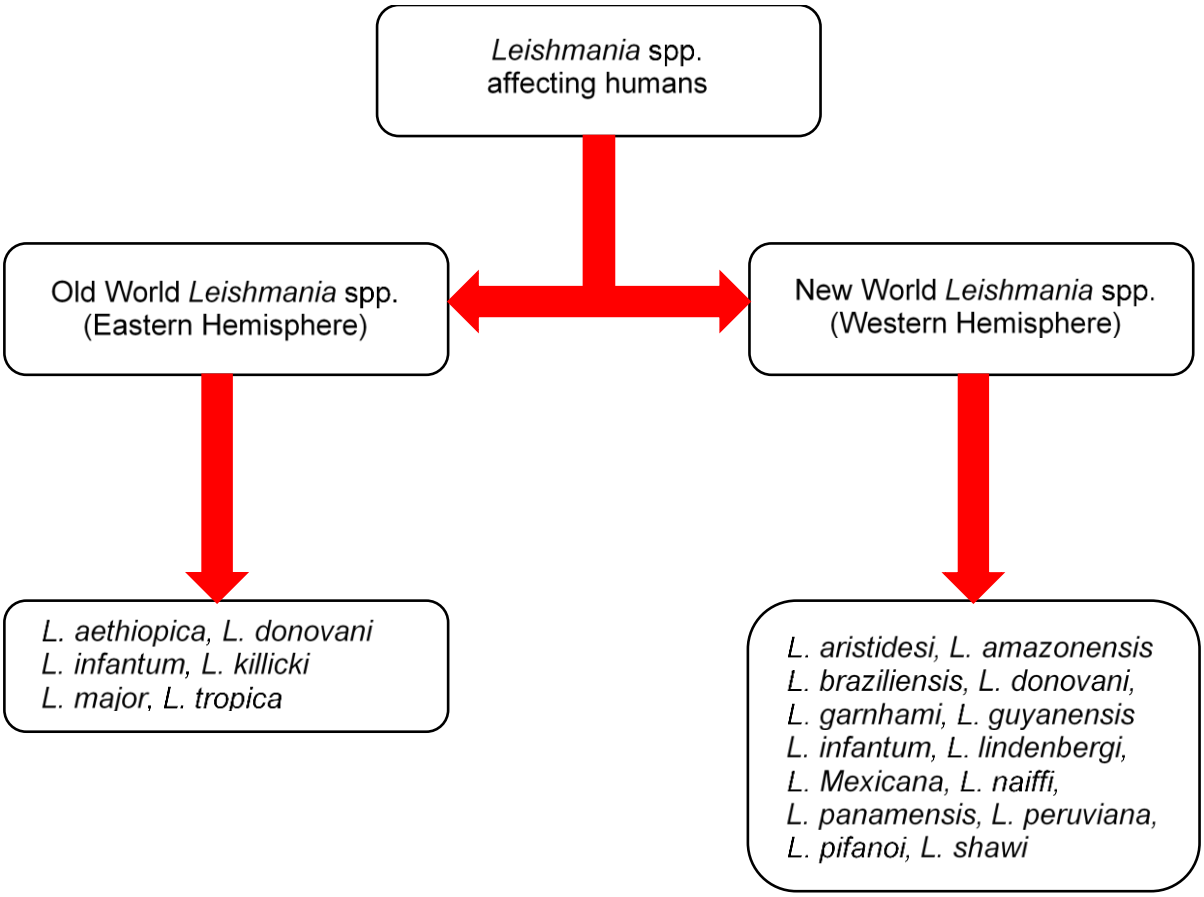


Figure 2.3: Division of *leishmania* spp. affecting humans (de Almeida *et al.*, 2021)

There are five clinical forms of leishmaniasis, namely: cutaneous (CL; most common form), diffuse cutaneous (DCL; rare form), mucocutaneous (MCL; least common form), visceral leishmaniasis, also known as kala-azar (VL; deadliest form), and post-kala-azar dermal leishmaniasis (sequel form of visceral leishmaniasis) (Akhoundi *et al.*, 2017; WHO, 2021b). The following section describes the symptoms and diagnoses of these clinical forms.

2.3 Diagnosis and clinical manifestations

Leishmania infection can be diagnosed based on its clinical symptoms and identified by using laboratory tests such as parasitological, and serological testing. These tests can be used individually or as a combination of diagnostic aids (WHO, 2021a). Parasitological diagnosis relies on the identification of leishmaniasis by firstly conducting a microscopic examination of tissue for the presence of parasites (Thakur *et al.*, 2020). Thereafter, a polymerase chain reaction (PCR) test can be performed to detect the presence of parasitic DNA in samples (Mugasa *et al.*, 2010).

Serological tests, on the other hand, use an enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay, and/or direct agglutination assay to obtain quantitative antibody results, which shows a higher antibody count in infected people. Alternatively, immunochromatographic test (ICT) and Western blot (WB) assays can be used to provide more detailed information on antibody responses to the various *Leishmania* antigens (Ehab Kotb *et al.*, 2014; Lévêque *et al.*, 2020).

The characteristics of leishmaniasis are unique to the clinical form in which it manifests and this can, accordingly, be used to identify the type of leishmaniasis (Figure **2.4**) and, to a degree, the *Leishmania* species involved (CDC, 2020c). As mentioned, the clinical forms of leishmaniasis consists of VL, post-kala-azar dermal leishmaniasis, CL, MCL and DCL (Inceboz, 2019).

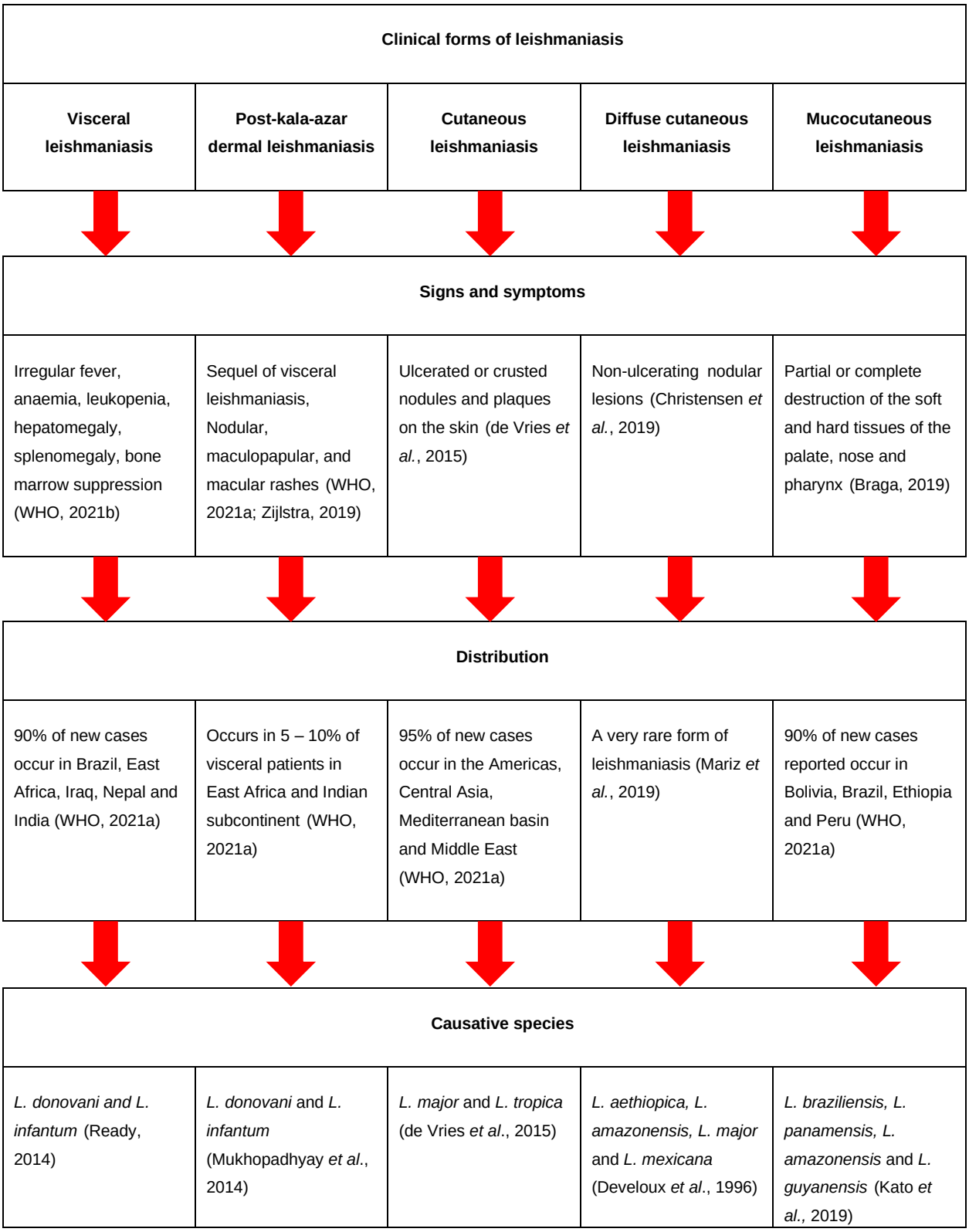


Figure 2.4: Clinical forms of Leishmaniasis

2.4 Disease control

The control of *Leishmania* infections consists of four main components, namely vector control, personal prevention, reservoir host control and disease treatment (WHO, 2021a). Vector control is done by spraying and using insecticides and insect repellents to either kill the vector or prevent a bite from the sandfly on a large scale.

This is, however, a time consuming and costly method of disease control (Reithinger *et al.*, 2007). Personal prevention methods entail the use of bed nets, wearing thick long-sleeved clothes and trousers and the use of insect repellent and insecticide spray (CDC, 2020d). Reservoir host control can be done using pesticide-impregnated collars or the culling of animal reservoir hosts to reduce the spread of the parasite (Boelaert *et al.*, 2018).

Vector control measures, together with prophylaxis and reservoir host control, are mainly used on the preventative side of disease control. Disease treatment with a variety of antileishmanial drugs forms part of the management side of disease control and remains the best way to control the disease in endemic and nonendemic areas (Alvar *et al.*, 2006).

2.5 Current treatments

2.5.1 Physical therapy

Physical therapy refers to localised non-pharmacological treatments for leishmaniasis and consists of carbon dioxide (CO_2) laser, thermo-, cryo- and electrotherapy (Roatt *et al.*, 2020). CO_2 laser treatments and thermotherapy both utilise thermolysis to treat leishmaniasis. *Leishmania* is temperature-sensitive, and temperatures above 39°C have been shown to negatively affect the parasite's ability to multiply (Wolf Nassif *et al.*, 2017). CO_2 laser treatment uses a carbon dioxide laser to incapacitate *Leishmania* parasites (Chakravarty & Sundar, 2019), whereas thermotherapy uses radio-frequency waves to increase the temperature of the infected area (Gonçalves & Costa, 2018). Both these forms of thermolysis actions are used to treat Old and New World CL and have been shown to be highly effective with minimal side effects and reduced treatment time (Chakravarty & Sundar, 2019).

Conversely, cryotherapy utilises liquid nitrogen (-195°C) to kill parasites through the formation of intracellular ice crystals, causing the disruption of the infected cells' functions and leading to localised ischemic necrosis. However, there are secondary effects associated with this treatment

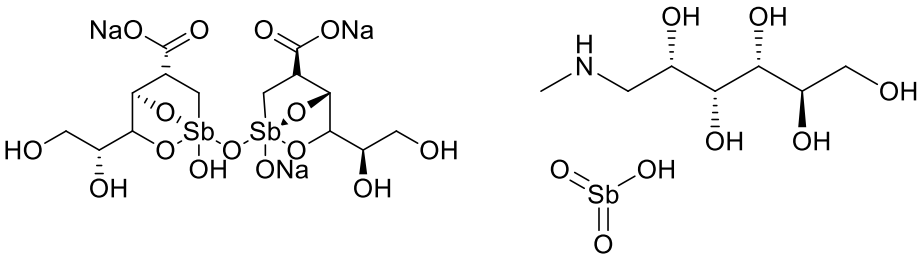
including oedema, erythema and hyper- or hypopigmentation (Roatt *et al.*, 2020). This modality is mainly used to treat Old World CL (Parvizi *et al.*, 2017). Alternatively, the use of electrotherapy to treat *L. major* CL has shown to be very effective with a 92.5% improvement after treatment. Electrotherapy uses localised direct electrical stimulation with an intensity between five and fifteen milliamperes and voltage below forty volts to kill the parasite (Hejazi *et al.*, 2004; Masmoudi *et al.*, 2013).

2.5.2 Chemotherapy

Despite the options of physical therapy, treatment of leishmaniasis still predominantly relies on chemotherapy. The current therapeutics for leishmaniasis consists of pentavalent antimonials, amphotericin B, azoles, paromomycin, pentamidine and miltefosine, which can be used as a single or combination therapy (Sundar *et al.*, 2019). These drugs are limited in their use due to them being toxic, expensive and requiring intravenous administrations. Another problem associated with the use of these drugs, is the development of parasitic resistance against them; hence, the extreme importance for the discovery of new antileishmanial drugs (Balaña-Fouce *et al.*, 2019).

2.5.2.1 Pentavalent antimonials

Pentavalent antimonials are a first-line antileishmanial monotherapy drug treatment used to treat VL, CL and MCL. The drugs that fall into the pentavalent antimonials class are sodium stibogluconate and meglumine antimoniate (Figure **2.5**). The emergence of drug resistance has resulted in these drugs being used in combinational therapy and being demoted to second-line treatment (Frézard *et al.*, 2009; Uliana *et al.*, 2018). Pentavalent antimonials are seen as prodrugs that have to be metabolised within the human and parasite *via* thiol-dependent reductase (Frézard *et al.*, 2009) into trivalent antimonials, which are considered the more effective forms (Carter *et al.*, 2006). Although both forms of antimonials have antileishmanial effects, their mechanism of action is not well understood (Mushtaq *et al.*, 2017). One of the proposed mechanisms is the destruction of parasitic DNA through degradation, which suggests that apoptosis, adenosine diphosphate phosphorylation and oxidation of fatty acid play a role (Kumar *et al.*, 2018).



Sodium stibogluconate (1) Meglumine antimoniate (2)

Figure 2.5: Structure of Sodium stibogluconate **(1)** and Meglumine antimoniate **(2)**

It is recommended that pentavalent antimonials form part of a combination therapy with amphotericin B (section 2.6.2.2) due to resistance development, as well as the severe adverse effects associated with them (Figure **2.11**) (Husein-ElAhmed *et al.*, 2020; Kevric *et al.*, 2015). These factors, together with the parenteral administration route, limit the use of pentavalent antimonials as treatment (Uliana *et al.*, 2018).

Antileishmanial drugs		
(1) Pentavalent antimonials	**(2) Amphotericin B**	**(3) Azoles**
Adverse effects		
Anorexia, arthralgia, cardiotoxicity, hepatotoxicity, headache, myalgia, nausea, nephrotoxicity, pancreatitis, renal failure and vomiting (Kip *et al.*, 2018)	Chills, hypokalaemia, high fever, myocarditis, nephrotoxicity, rigor and thrombocytopenia (Kip *et al.*, 2018)	Anaemia, anaphylaxis, diarrhoea, fever, headache, hepatitis, hypokalaemia hypotension, nausea, oedema, pruritus, seizure, thrombocytopenia and vomiting (El-Garhy, 2015)

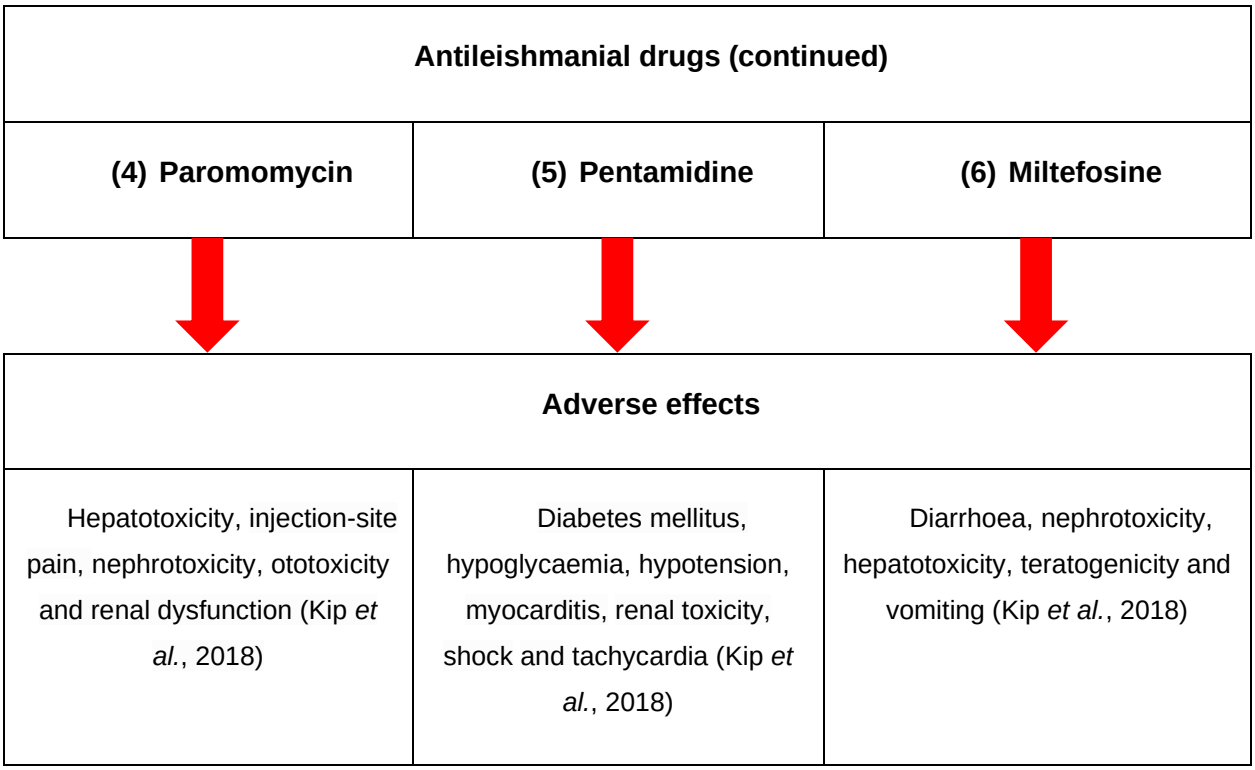
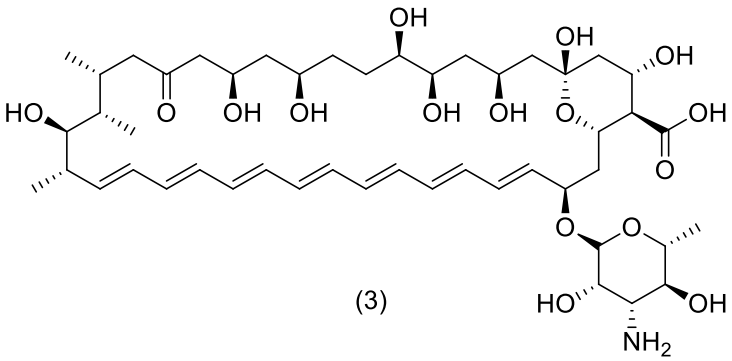


Figure 2.6: Adverse effects caused by antileishmanial compounds

2.5.2.2 Amphotericin B

Amphotericin B (Figure **2.7**) is considered to be an effective antileishmanial drug and is used as a first-line treatment for VL and can be administered alone or in combination with pentavalent antimonials (Chávez-Fumagalli *et al.*, 2015; Lanza *et al.*, 2019). Amphotericin B works by binding to the ergosterol in the cell membrane of the parasite, causing pores to form and ions to leak out of the cell, which then leads to metabolic shock and the death of the parasite (Shirzadi, 2019; Stone *et al.*, 2016). However, the use of this drug is limited due to its toxicity (Figure **2.11**), hence, the development of a lipid form that is less toxic (Rivnay *et al.*, 2019). Amphotericin B and its derivatives are administered by parenteral route, and together with their toxic effects, remain as areas for improvement (Hnik *et al.*, 2020).

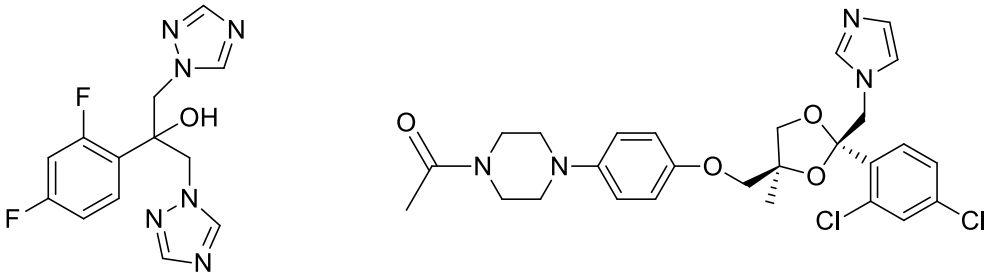


(3)

Figure 2.7: Structure of Amphotericin B **(3)**

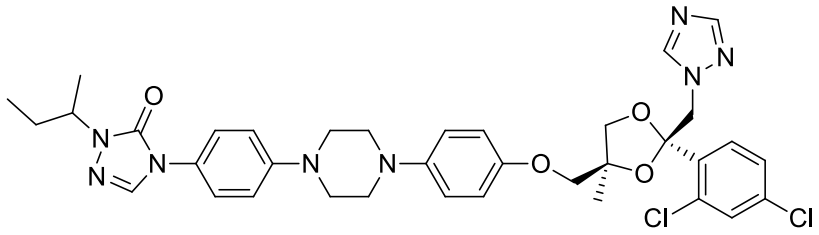
2.5.2.3 Azoles

Azoles are a class of five-membered heterocyclic compounds containing a nitrogen atom and at least one other non-carbon atom. Form this class of compounds, fluconazole, ketoconazole and itraconazole (Figure **2.8**) have shown moderate antileishmanial effects and have been used as first-line treatment for CL, MCL and DCL (Aburabie *et al.*, 2020; Galvão *et al.*, 2017). Azoles act by inhibiting the biosynthesis of ergosterol through the inhibition of the lanosterol 14-*α*-demethylase enzyme, leading to the formation of abnormalities in the cells of the parasite and causing death (Braga, 2019). With the oral fluconazole, ketoconazole and itraconazole formulation, the administration of the drug is very easy compared to the parenteral administered antileishmanial drugs (Eiras *et al.*, 2015). However, there are various adverse effects associated with the use of azoles (Figure **2.6**), which limits their therapeutic use (Benitez & Carver, 2019).



Fluconazole (4)

Ketoconazole (5)

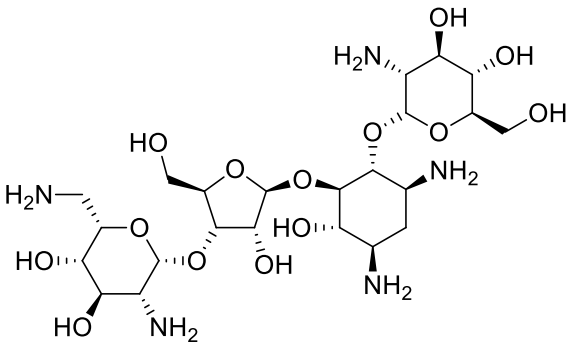


Itraconazole (6)

Figure 2.8: Structures of antileishmanial azoles: Fluconazole **(4)**, Ketoconazole **(5)** and Itraconazole **(6)**

2.5.2.4 Paromomycin

Paromomycin (Figure **2.9**) is part of the aminoglycoside antibiotic class, is used as second-line treatment to treat VL (parenteral formulation) and CL (topically/parenteral formulation) (Matos *et al.*, 2020). The mechanism of paromomycin remains under debate with a possibility being its binding to chain-elongating ribosomes to cause mistranslation of protein, resulting in protein synthesis blockade (Brugués *et al.*, 2015). Paromomycin is a low-cost treatment and with little parasitic resistance, making it an excellent candidate for combination therapy with various other antileishmanial drugs (Reguera *et al.*, 2019). However, this drug has unwanted effects (Figure **2.6**) which limits its clinical use (Lindoso *et al.*, 2012).

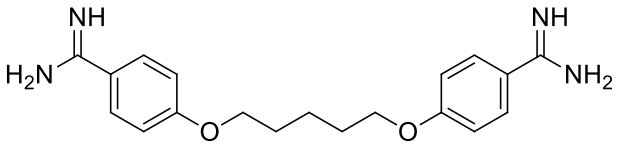


(7)

Figure 2.9: Structure of Paromomycin **(7)**

2.5.2.5 Pentamidine

A pentamidine (Figure **2.10**) is an aromatic diamidine that shows sufficient antiparasitic activity and is used as first-line treatment for VL and CL (Barioni *et al.*, 2015; Gadelha *et al.*, 2018). This drug works by causing mitochondrial membrane fragmentation through DNA synthesis interference, which leads to the parasite's death (Machado *et al.*, 2019). Pentamidine is a low-cost antileishmanial treatment which is mainly administered intravenously but can also be given intramuscularly (Kip *et al.*, 2018; Piccica *et al.*, 2021). However, the use of pentamidine is limited due to the toxic effects of the drug (Figure **2.6**) as well as the impractical routes of administration (WHO, 2010).



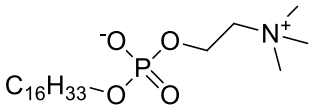
(8)

Figure 2.10: Structure of pentamidine **(8)**

2.5.2.6 Miltefosine

Miltefosine (Figure **2.11**), also known as hexadecylphosphocholine, is a first-line antileishmanial drug that is used to treat both VL and CL (Barioni *et al.*, 2015; CDC, 2020c). It acts by inhibiting protein kinase B (also known as Akt protein) which results in interruption of the intracellular signalling pathway necessary for cell survival (Dorlo *et al.*, 2012). The major advantages of miltefosine are that it is an orally administered drug and has mild adverse effects (Figure **2.6**)

(Vakil *et al.*, 2015). Besides, this drug has a high percentage of efficacy (>90%) in treating both VL and CL (Iranpour *et al.*, 2019). There are, however, adverse effects associated with miltefosine therapy, with the most dangerous being teratogenicity (Sundar *et al.*, 2012). Together with other disadvantages such as high cost of treatment and a long half-life of seven days, the use of the drug is also limited (Dorlo Thomas *et al.*, 2008; Sunyoto *et al.*, 2018).



(9)

Figure 2.11: Structure of Miltefosine **(9)**

2.6 Drug resistance

The emergence of drug resistance in *Leishmania* parasites is a cause for concern, due to the limited number of antileishmanial drugs approved for treatments and the slow drug development pace that prevents timely and sufficient solutions against the emergence of drug resistance (Balaña-Fouce *et al.*, 2019). The formation of drug resistance has been linked to the upregulation of two classes of the ATP-dependent binding cassette (ABC) transporter (Pérez-Victoria *et al.*, 2001; Van den Kerkhof *et al.*, 2020). The mutation is exacerbated by the overuse of the existing drugs, as well as poor patient compliance (Deep *et al.*, 2017; Sundar & Agarwal, 2016). Currently, pentavalent antimonials and pentamidine have been reported to show prevalent resistance, whereas amphotericin B, azoles, paromomycin and miltefosine have no significant resistance to date (Capela *et al.*, 2019). However, potential development of resistance similar to that of pentavalent antimonials is feared for miltefosine and amphotericin B (Chakravarty & Sundar, 2010; Torres-Guerrero et al., 2017). Thus, there is an urgent need for the development of new, effective and affordable antileishmanial drugs (Zulfiqar *et al.*, 2017).

2.7 Combination therapy

Recent reports have indicated antileishmanial treatments by monotherapy as is the case with pentavalent antimonials, to experience failures due to the development of parasitic drug resistance (Capela *et al.*, 2019; Torres-Guerrero *et al.*, 2017). This has led to the emergence of combination therapy (Sundar *et al.*, 2014). Combination therapy is used to delay or prevent the development of drug resistance, as well as lessen the toxic effects of the drugs used. This is

achieved by using drugs with different pharmacological effects that have a synergistic relationship to enhance the individual drugs effects, shorten the treatment time and prevent the survival of any *Leishmania* parasites that are partially resistant to one of the drugs used (Sundar *et al.*, 2019).

The available combination therapies for VL consist of amphotericin B with miltefosine, paromomycin with miltefosine, and pentavalent antimonials with paromomycin (Hendrickx *et al.*, 2017; van Griensven *et al.*, 2010). These treatments have shown a decrease in toxicity, dosage regimen and cost, while showing high efficacy in treating leishmaniasis in East Africa, Yemen and the Indian subcontinent (Uliana *et al.*, 2018). Currently, there is limited data on combination treatment for CL, although clinical trials have shown the combination of pentavalent antimonial with pentoxifylline as potential treatment for CL and severe MCL with success (Machado *et al.*, 2007; Sadeghian & Nilforoushzadeh, 2006). Thus, it is clear that the current combination therapies are an important component in the fight against the spread of leishmaniasis.

However, combination therapies do not eliminate the toxicity of the chosen drugs, thus adverse effects associated with toxicity may still appear. Alongside the toxicity, combination therapy does not address the issue of the impractical intravenous route of drug administration which is invasive and painful (Hnik *et al.*, 2020; WHO, 2010). Additionally, the emergence of multidrug-resistant strains of *Leishmania* further hinder the use of current combination therapies (Messaritakis *et al.*, 2013). Thus, the need for a new clinical antileishmanial drug is present (Zulfiqar *et al.*, 2017).

2.8 Novel antileishmanial drug development

The development of novel antileishmanial drugs for clinical use is a tedious and time-consuming endeavour that does not guarantee success. The development of drug-resistant *Leishmania* parasites against current therapies motivates the need for new clinically viable antileishmanial agents (Hendrickx *et al.*, 2019). A variety of antileishmanial scaffolds have been tested in the search for novel drugs, including 5-nitrofuran (Trefzger *et al.*, 2020), chroman (Ortiz *et al.*, 2020), thiazolidinone (Schadich *et al.*, 2020), and quinazolinone (Prinsloo *et al.*, 2021). The 5-nitrofuran scaffold, in particular, has shown promise in the development of new antileishmanial drugs and warrants further experimentation and modification, hence its derivatives are the focus of the subsequent sections.

2.8.1 The 5-nitrofuran scaffold

The 5-nitrofurans (NF, Figure **2.12**) are a class of redox-active anti-infective drugs that are used for the treatment of various infectious diseases (Pires *et al.*, 2001). The defining structural component is a furan ring linked to a nitro group. Research has indicated that nitro-containing drugs such as the 5-nitrofurans have a broad spectrum of activity against a variety of diseases caused by microbial infections such as bacterial (Gram-positive and -negative) (Kamal *et al.*, 2015), mycobacterial (tuberculosis) (Elsaman *et al.*, 2019) parasitic (leishmaniasis, malaria and trypanosomiasis) (Zuma *et al.*, 2019), and cancer (Bailly, 2019). These drugs are very active owing to the nitro group that produces free radical species, ROS and RNS which react with pathogen cell wall enzymes and become lethal to these microorganisms (Zuma *et al.*, 2019). This makes the nitro moiety a suitable primary building block in the discovery and development of new drugs for the targeting of infectious pathogens (Kannigadu & N'Da, 2020).

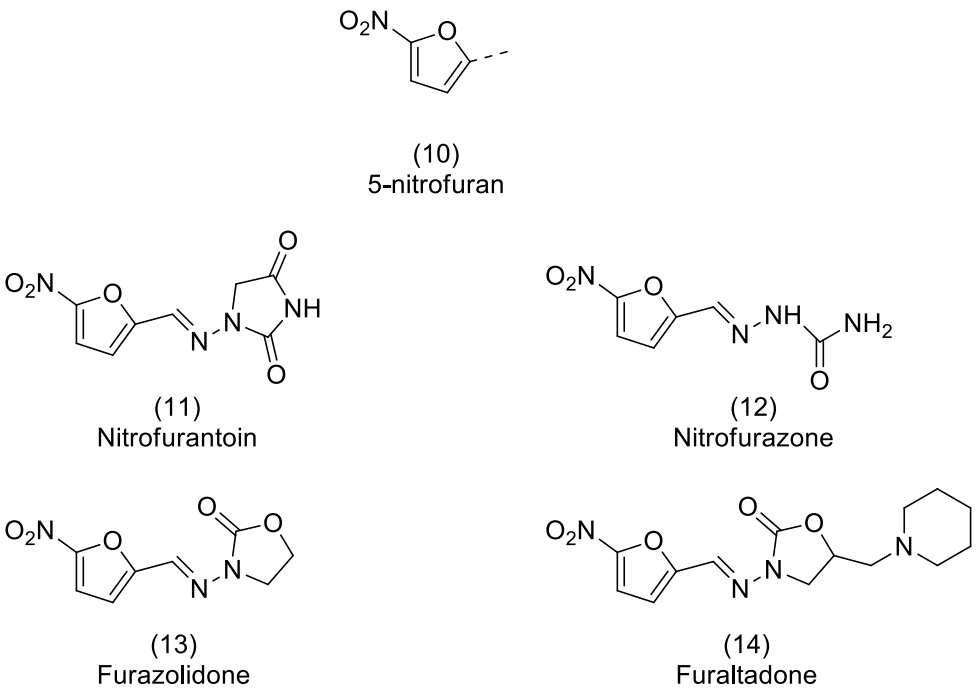
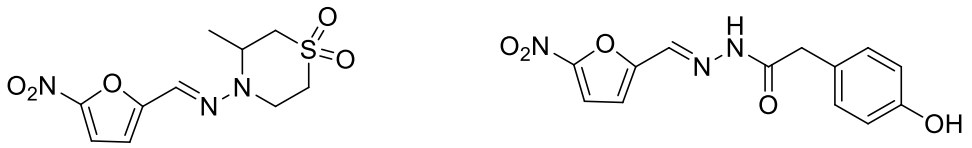


Figure 2.12: Basic structure of 5-nitrofuran **(10)**, and early 5-nitrofuran derivative **(11-14)**

The emergence of parasitic resistance to the currently available antileishmanial drugs (Figure **2.6**) has made the need for new drugs imperative (Zulfiqar *et al.*, 2017).

An increasingly common strategy for the development of new drugs is by fast-tracking (Jain & Sharma, 2017). This strategy entails the use of existing drugs or drugs' scaffold, refining and re-purposing them to form analogues with new applications. The advantage of this process is that the development time is shortened due to the existing drug having passed several checkpoints including pharmacokinetic and safety profiles (Kannigadu *et al.*, 2021). 5-Nitrofuran is such an existing scaffold that has led to the development of new clinical nitrofurans (cNFs) (Figure **2.13**)(Le *et al.*, 2019; Ponte-Sucre *et al.*, 2017).



Nifurtimox (15)

Nifuroxazide (16)

Figure 2.13: New clinical 5-Nitrofuran drugs **(15-16)**

Research on the anti-infective activities of the cNFs are also well documented (Hu *et al.*, 2007; Kannigadu & N'Da, 2020). cNFs have two important pharmacophores. The first is the redox active nitro group (blue in Figure **2.14**) whilst the second is the hydrazone linker (red in Figure **2.14**). As discussed above, the nitro group promotes activity through ROS and RNS (Zuma *et al.*, 2019) whilst the hydrazone pharmacophore improves the stability of the nitrofuran ring through zwitterionic properties (Trukhacheva *et al.*, 2005)

In order for the nitro group to form the reactive species discussed, the compound must undergo at least one of two activation mechanisms, hydrazone azoreduction and nitroreduction (Le & Rakonjac, 2021; Ryan *et al.*, 2011).

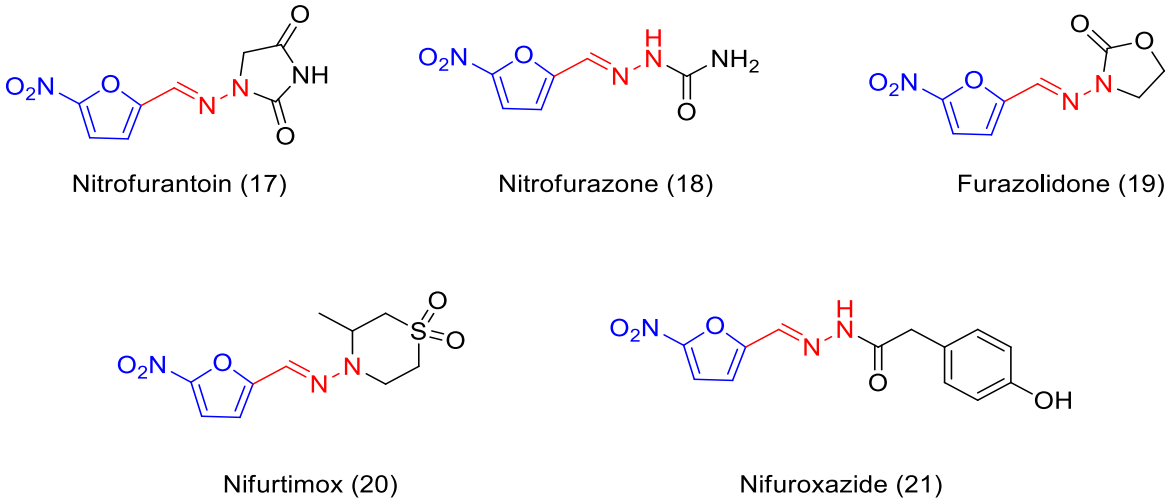
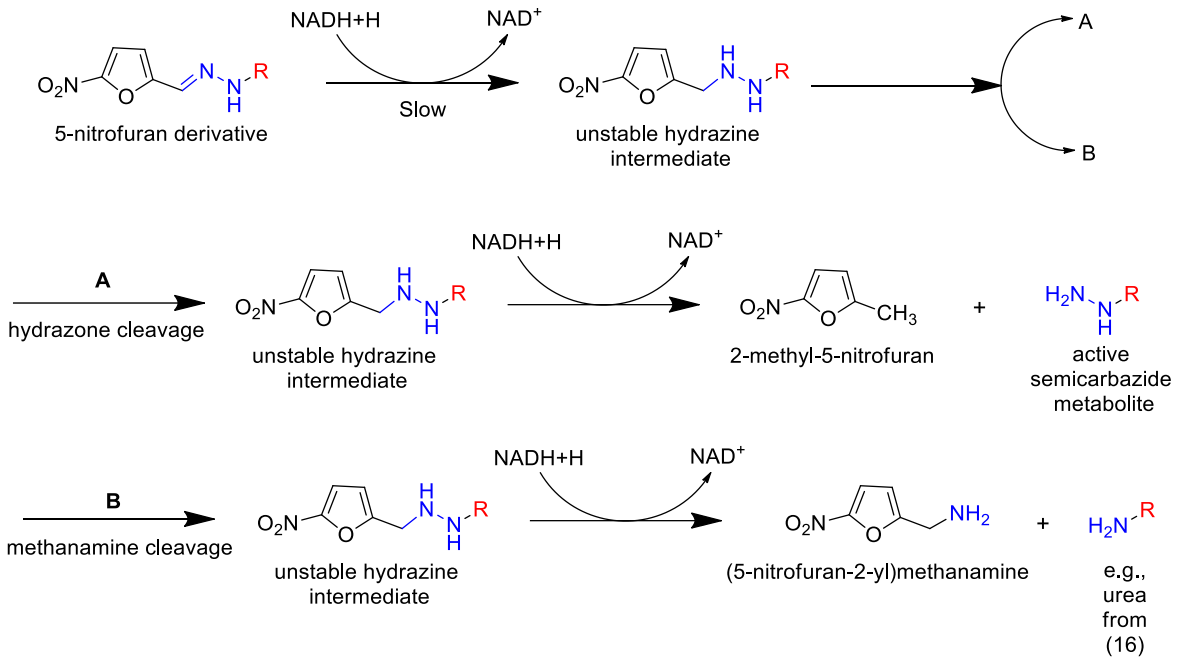


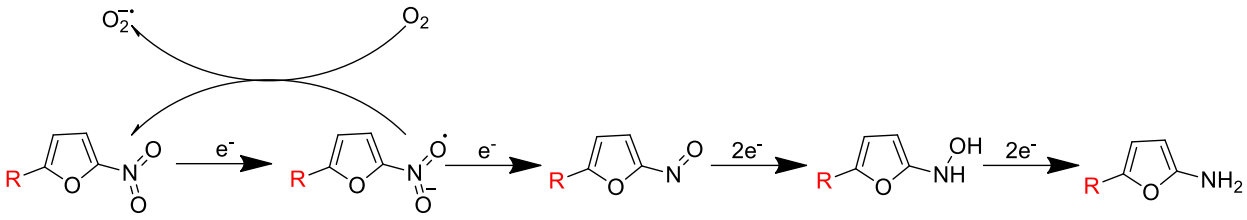
Figure 2.14: 5-Nitrofuran drugs currently in clinical use **(17-21)**

Hydrazone azoreduction is caused by a group of NADH/NADPH-dependent flavoenzymes that are present in many gut flora, including human gut flora (Ryan *et al.*, 2011). The flavoenzymes responsible for azoreduction in the human gut are known as NADH/NADPH quinone oxidoreductases (Cui *et al.*, 1995). The process of azoreduction (Scheme **2.1**) (Zuma *et al.*, 2019) is preceded by the tautomerisation of the hydrazone pharmacophore to an azo tautomer, followed by the two-electron reduction mechanism facilitated by NADH/NADPH as primary electron donors. This is achieved through the cleavage of the hydrazone bond, resulting in an active semicarbazide metabolite (**A**) due to hydrazone cleavage or a urea by-product through mathanamine cleavage (**B**) (Holland, 2017; Ryan, 2017). A secondary effect resulting from azoreduction is the reduction of the nitro moiety that results in the formation of toxic hydroxylamine, thus activating 5-nitrofuran compounds (Ryan *et al.*, 2011).

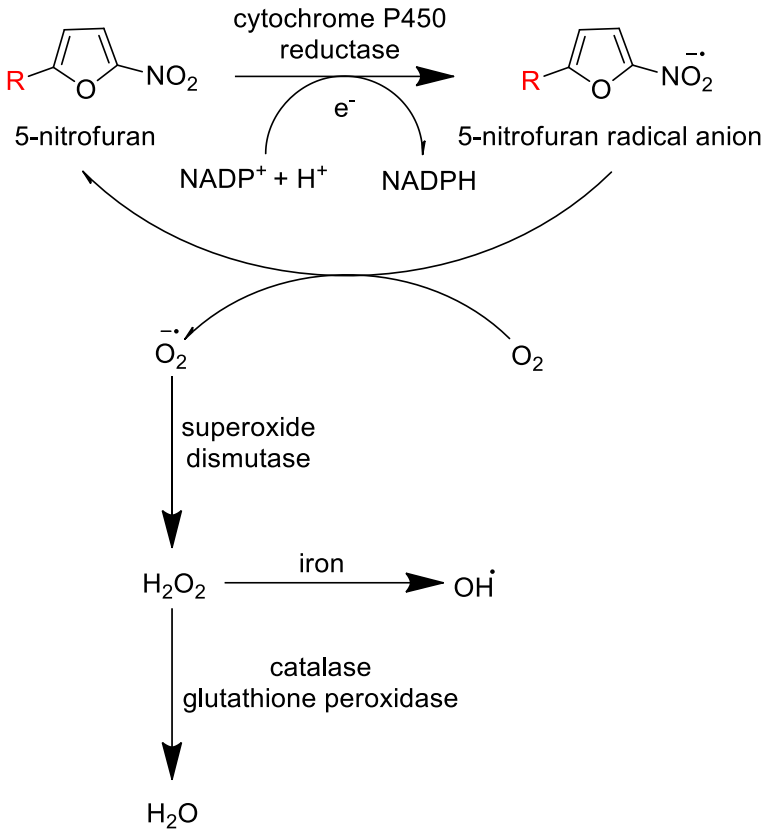


Scheme 2.1: Hydrazone azoreduction-induced activation of nitrofurans (adapted from Zuma *et al.* (2019))

Nitroreduction on the other hand, can occur in two forms (I and II) catalysed by two types of reductase enzymes i.e., type I (oxygen insensitive) and type II (oxygen sensitive) reductases (Bot *et al.*, 2013). Type I reductase (Scheme **2.2**) (Chen *et al.*, 2007) consists of flavin mononucleotide (FMN) enzymes that are NADH/NADPH-dependent and mainly found in prokaryotes and protozoan parasites. They induce a two-electron reduction of the nitro-group, forming a reactive and toxic hydroxylamine (Peterson *et al.*, 1979; Wilkinson *et al.*, 2008). Type II (Scheme **2.3**) (Wang *et al.*, 2008) contains NADH/HADPH-dependent FMN of flavin adenine dinucleotide (FAD) enzymes and reacts in oxygen-rich environments, which result in a one-electron reduction of the nitro-group through redox cycling to form ROS (Mason & Holtzman, 1975; Ryan, 2017). Both type I and II reduction reactions are mediated by a variety of enzymes including ferredoxin-$NADP^+$ reductase, NADPH-cytochrome P450 reductase and NADH-ubiquinone reductase (Roldán *et al.*, 2008).



Scheme 2.2: Type I nitroreductase activation of nitrofurans (adapted from Chen *et al.* (2007))

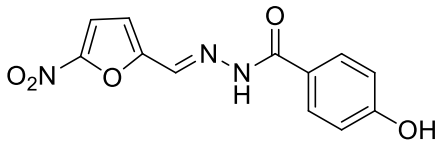


Scheme 2.3: Type II nitroreductase activation of 5-nitrofurans (adapted from Wang *et al.* (2008))

2.8.2 Nifuroxazide

Nifuroxazide (NFX, Figure **2.15**), is a cNF, a 5-nitrofuran derivative that is currently used as an oral antibiotic for the treatment of diarrhoea and colitis (Luo *et al.*, 2019). This drug shows notable activity against both gram-positive and negative bacteria without affecting the normal intestinal flora (Luo *et al.*, 2019; Zuma *et al.*, 2019). NFX can either be bacteriocidal (high dosage) or bacteriostatic (low dosage), depending on the dose (Trukhacheva *et al.*, 2005). Studies have also

shown that NFX has antiparasitic effects against numerous invasive species, including *Leishmania*, as well as possible anticancer effects (Kaiser *et al*., 2015; Zhao *et al*., 2020). Although the exact mechanism of action of NFX remains unclear, the accepted theory is through the occurrence of ROS and RNS as previously discussed in section 2.8.1 (Bailly, 2019) and the generation of oxidative stress. However, the bioactivation of the compound occurs *via* aldehyde dehydrogenase (ALDH) (Karlowicz-Bodalska *et al.*, 2019).



(22)

Figure 2.15: Structure of nifuroxazide **(22)**

The absorption of NFX in the intestinal tract is limited with a bioavailability of 50% that results in NFX acting primarily in the intestinal lumen (Kalia & Raines, 2008; Santiago *et al.*, 1985). The limited absorption and bioavailability are due to NFX being metabolised in the intestinal track (B. Fernandes *et al.*, 2015; Labaune *et al.*, 1986). The use of NFX may also result in toxic effects associated with the formation of the ROS and RNS, and include protein carbonylation, DNA breakage, enzyme inactivation and inflammatory reactions (Liu *et al.*, 2017; Patel *et al.*, 2018). These shortcomings may be overcome through structural modification and pharmacophore hybridisation that may improve the activity, bioavailability and toxicity of the compound (Agarwal *et al.*, 2017; Zhang *et al.*, 2019). Taking the anti-infective effects and shortcomings of NFX into consideration, the viability of the compound to act as a parent drug for the development of a new antiparasitic drug is promising (Petri e Silva *et al.*, 2016).

2.8.3 Pharmacophore hybridisation

The addition of another pharmacophore to an existing drug or scaffold is an important strategy in the development of new effective drugs (Claudio *et al.*, 2007). This addition of a second or third pharmacophore can increase the efficacy, stability, bioavailability and decrease the toxicity and adverse effects of a compound (Nepali *et al.*, 2014), and the effect of the hybridisation depends on the characteristics of the pharmacophore added (Zhang *et al.*, 2019). The chemical addition of sulfonyl and benzylated moieties have shown to enhance biological activity, chemical and

enzymatic stabilities of an existing structure/compound thus warranting further hybridisation studies (Shakhatreh *et al.*, 2016; Zhao *et al.*, 2019).

2.8.4 Sulfonyl derivatives

Sulfonyl derivatives form an important part of drug development due to the various pharmacological properties that they possess, including anti-Alzheimer (Mutahir *et al.*, 2016), antibacterial (Kumar Verma *et al.*, 2020), anticancer (Rakesh *et al.*, 2018), antidiabetic (Sharma & Soman, 2015), antifungal (Lal *et al.*, 2013), anti-inflammatory (Ahmad *et al.*, 2021), antileishmanial (Dar *et al.*, 2015), antimalaria (Nicoleti *et al.*, 2016), antioxidant (Badgujar *et al.*, 2018), antitubercular (Quintana *et al.*, 2017) and antiviral (Dash *et al.*, 2020). The functional groups that form part of the sulfonyl derivatives are mainly sulfonyl chlorides, sulfonic esters, sulfonamides and sulfones (Figure **2.16**) (Hofman *et al.*, 2018). The addition of a sulfonyl group to a drug-scaffold forms an electron withdrawing compound with increased stability against hydrolysis and resistance to the reduction of its sulphur (Zhao *et al.*, 2019).

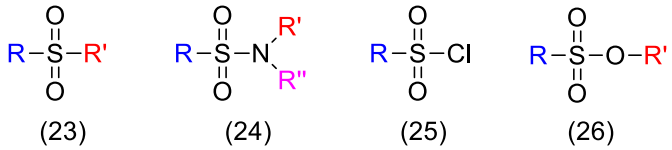
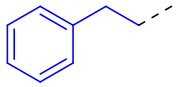


Figure 2.16: Sulfonyl functional groups: sulfones **(23)**, sulfonamides **(24)**, sulfonyl chlorides **(25)** and sulfonate ester **(26)**

2.8.5 Benzyl derivatives

The addition of a benzyl moiety (Blue in Figure **2.17**) to compounds have been used to enhance biological activity and chemical stability (Gaunt *et al.*, 1998). Benzyl substituted drugs have shown to have antibacterial and antifungal properties as well as enhanced other biological activities, and this effect has attributed to tissue damage through enzyme activation associated with the benzyl moiety (Chang *et al.*, 2001; Shakhatreh *et al.*, 2016). Through the addition of a benzyl moiety to NFX, the formation of hydrazone will enhance due to the electrophilic carbonyl group that may enhance the activation resulting in increased biological activity of NFX (Fernandes *et al.*, 2018).



(27)

Figure 2.17: Benzyl moiety **(27)**

2.9 Rationale for current study

In the current, study 2 series of NFX-based analogues were synthesised containing an additional sulfonyl or benzyl moiety, respectively. The new analogues contained three pharmacophores i.e., 5-nitrofuran, hydrazone and either sulfonyl or benzyl pharmacophore. As discussed, (section 2.8.1), cNFs are redox-active drugs with various biological and pharmacological properties and contains a secondary hydrazone pharmacophore for additional stability and redox activity. The addition of the third sulfonyl or benzyl pharmacophore is based on previous studies that indicated that *O*-benzyl alkylation of NFX resulted in enhanced activity (Kannigadu *et al.*, 2021). Thus, by mono-substituting NFX with a *O*-benzyl or -sulfonyl substituent it may provide an antileishmanial drug with less toxicity, adverse effects and higher activity.

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Chapter 3
Article for submission

Chapter **3** consists of the article entitled “Synthesis and *in vitro* antileishmanial evaluation of novel Nifuroxazide-based analogues”, which will be submitted to the Journal of Bioorganic & Medicinal Chemistry. This article contains the chemical synthesis and biological findings of the synthesised antileishmanial compounds of the study.

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Synthesis and *in vitro* antileishmanial activity of novel Nifuroxazide-based analogues

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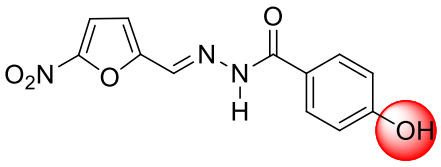
Highlights

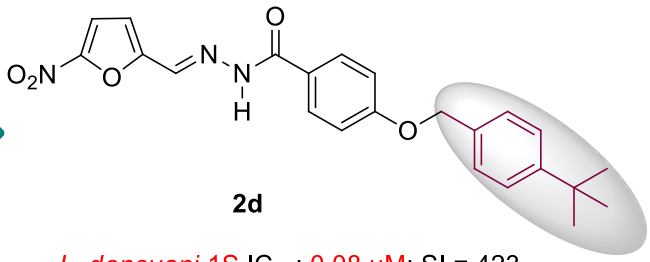
- Novel *O*-substituted nifuroxazide derivatives were synthesised
- Their antileishmanial activity was evaluated *in vitro* against L. promastigotes
- Overall good activity found against *Leishmania donovani* and *L. major* promastigotes
- Derivatives showed moderate-to-no cytotoxicity towards Vero cells
- Derivative **2d** was the most active, with IC_{50} values ranging between 0.08 – 0.24 μM

Graphical Abstract



Nifuroxazide - NFX

L. donovani 1S IC_{50}: 4.53 µM; SI = 22
L. donovani 9515 IC_{50}: 4.48 µM; SI = 22
L. major IR173 IC_{50}: 38.48 µM; SI = 3
Cytotoxicity, Vero IC_{50}: > 100 µM



2d

L. donovani 1S IC_{50}: 0.08 µM; SI = 423
L. donovani 9515 IC_{50}: 0.24 µM; SI = 141
L. major IR173 IC_{50}: 0.09 µM; SI = 376
Cytotoxicity, Vero IC_{50}: 33.85 µM

Abstract

Leishmaniasis is a parasitic disease affecting millions of people worldwide and it is endemic to 98 countries. It is considered a neglected tropical disease and can be fatal if left untreated. Between 650 000 and 1.1 million new infections are annually reported worldwide by the WHO. The current treatments for leishmaniasis are unsatisfactory due to the development of parasitic resistance and toxicity, and this highlights the need for the development of new antileishmanial drugs. In the process of searching for new treatments, a series of twenty nifuroxazide analogues were synthesised in moderate to excellent yields (25-81%) and investigated for its antileishmanial potential. It was found that analogue **2d**, which contains a 4 -(*tert*-butyl) benzyl moiety was the most potent of the series, possessing nanomolar activities up to 427-fold higher than the parent drug nifuroxazide against all three tested *Leishmania* strains. This analogue is considered an anti-promastigote hit compound and future investigation will focus on the anti-amastigote activity determination required to assess its potential to act as new antileishmanial agent.

Keywords: *Leishmania*, nitrofuran, nifuroxazide, sulfonyl, benzyl

1. Introduction

Leishmaniasis is an infection caused by protozoan parasites belonging to the genus *Leishmania (L.)* and is transmitted by the bite of an infected female *Phlebotomus* sandfly[1, 2]. There are approximately twenty species of *Leishmania* that can affect humans, with *L. donovani* and *L. major* being the most prevalent of the species[3]. In addition to this, leishmaniasis has three main clinical forms of manifestation; visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL), with CL being the most common form and VL being the most dangerous form[4].

This disease is still one of the world's most neglected tropical diseases (NTD) due to limited financing for its management. Leishmaniasis is found in 98 countries worldwide, mostly affecting developing countries in the tropical and sub-tropical regions of Africa, Asia, the Americas and the Mediterranean basin[5]. In 2021, the WHO estimated that 50 000 to 90 000 new cases of VL and 600 000 to 1 million new cases of CL occur worldwide annually, however, only 25% to 45% cases were reported to WHO[5].

To date, there is no effective vaccine available for the prevention of leishmaniasis[6], and the currently available treatments rely solely on chemotherapy. The pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) are first line drugs that have been used clinically for decades, and are the drugs of choice for treating all forms of leishmaniasis[7].

The development of antimonial resistance poses a major concern for the effective treatment of leishmaniasis[7]. In severe cases where the patient is unresponsive to the antimonials, second line drugs such as amphotericin B, paromomycin, pentamidine and miltefosine can be used either individually or in combination to treat leishmaniasis[8, 9]. However, all of these drugs are toxic, require long term treatment and, with the exception of miltefosine, have poor bioavailability thus requiring intravenous (IV) and intramuscular (IM) administration routes[10].

Furthermore, the currently available therapies have become inadequate for the treatment of leishmaniasis due to their overuse, resulting in the development of pathogenic resistance that is aggravating the public health risk[11]. Thus, urgent attention is required for the development of new agents due to the lack of alternative chemotherapeutic approach for the treatment of leishmaniasis. Over recent years, a great number of synthetic compounds have been evaluated for their antileishmanial potential[12-14].

In particular, the use of nitroaromatic scaffolds such as the 5-nitrofuran, 5-nitrothiophene, 5-nitroimidazoles and the clinical nitrofurans drugs (cNFs) (Figure **1**) in the development of treatments for infectious diseases has been well established over the years[7, 15]. These drugs (cNFs) and scaffolds have also shown potential as anticancer agents[16, 17].

The diverse biological activities of nitrofurans (NFs) result from the mechanisms of action of their two pharmacophores. The first pharmacophore is the nitrofuran (red, in Figure **1**) and the second is the hydrazone moiety (blue, in Figure **1**). The nitro group on the nitrofuran is able to form reactive oxygen species (ROS) and reactive nitrogen species (RNS) once activated by parasitic nitroreductase enzymes, hence causing oxidative stress which ultimately results in pathogen cell death[18]. The hydrazone moiety improves the stability of the compound, and has intrinsic biological activities[19], such as antibacterial[20], antiparasitic[21], antifungal[22], antiviral[23] and anti-inflammatory activities[24]. The hydrazone moiety is activated by hydrazone azoreductase enzymes, resulting in the formation of either an active semicarbazide metabolite or an urea by-product[17]. The resulting multi-active nature of the cNFs contributes to the reduced development of pathogenic resistance, thus making them good candidates for drug repurposing for the treatment of infectious diseases.

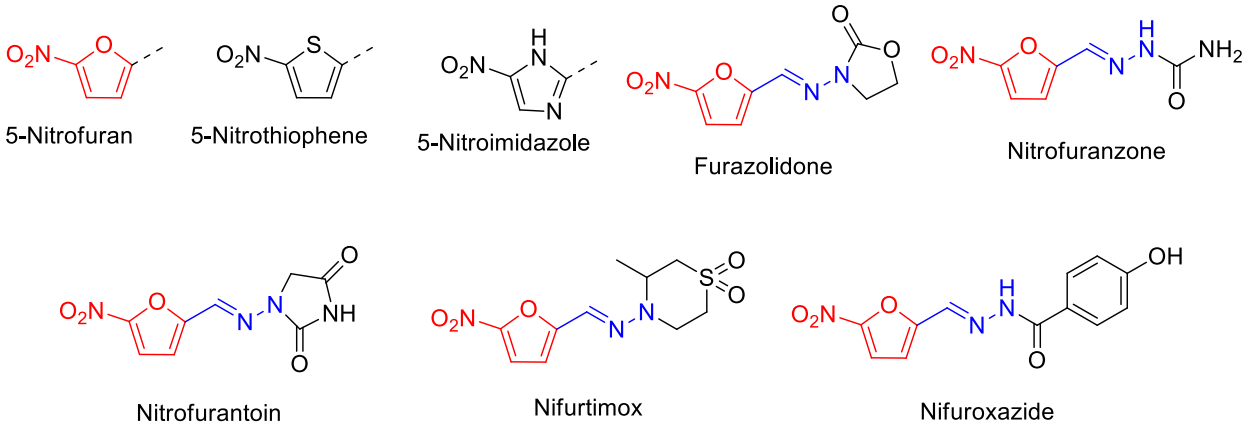


Figure 1: Nitroaromatic scaffolds and current cNFs

Nifuroxazide (NFX) is used as oral antibiotic for the treatment of various gastric infections[25]. NFX shows notable antibacterial activity without affecting the normal intestinal flora[17, 25]. It is dose dependent and can either be bacteriocidal at high dosages or bacteriostatic at low dosage[26]. NFX also contains antiparasitic effects, which include antileishmanial effects[27, 28]. NFX's mechanism of

action is unclear, but the widely accepted theory is the formation of ROS and RNS that generate oxidative stress resulting in cell death[18].

However, NFX shows limited gastrointestinal absorption as result of limited intestinal metabolism[29, 30] hence it primarily acts in the intestinal lumen[31, 32]. The use of NFX may cause toxic effects including DNA breakage, inflammatory reactions, and enzyme inactivation[33, 34] due to the formation of ROS and RNS. By making use of structural modification and hybridisation, these shortcomings may be overcome, and the efficacy of the drug may be improved[35, 36]. Taking the shortcomings and wide-spread anti-infective of NFX into consideration, the possibility of NFX to act as a building block for the development of a new antiparasitic drugs is promising[37].

Furthermore, the addition of a benzyloxy or sulfonyl group to existing drugs/scaffolds have been shown to increase the efficacy, stability, bioavailability and decrease the toxicity and adverse effects of the drugs[38, 39]. Studies have indicated benzyloxy drugs have antibacterial and antifungal properties and that the benzyloxy group can enhance the biological activity of compounds by preventing tissue damage through enzyme activation[40, 41]. Benzyloxy groups are also electrophilic groups that enhances the formation of the hydrazone when in contact with a strong nucleophilic group like hydrazine[42]. Alternatively, the addition of a sulfonyl group to a drug-scaffold promotes chemical stability forming an electron withdrawing compound with increased stability against hydrolysis and resistance to the reduction of its sulphur[43].

Based on this evidence, *O*-benzylated and *O*-sulfonated analogues of nitrofuran antibiotic nifuroxazide (NFX) were synthesized and their antileishmanial activities were examined *in vitro*. We herein report the synthesis and the biological activities of these analogues.

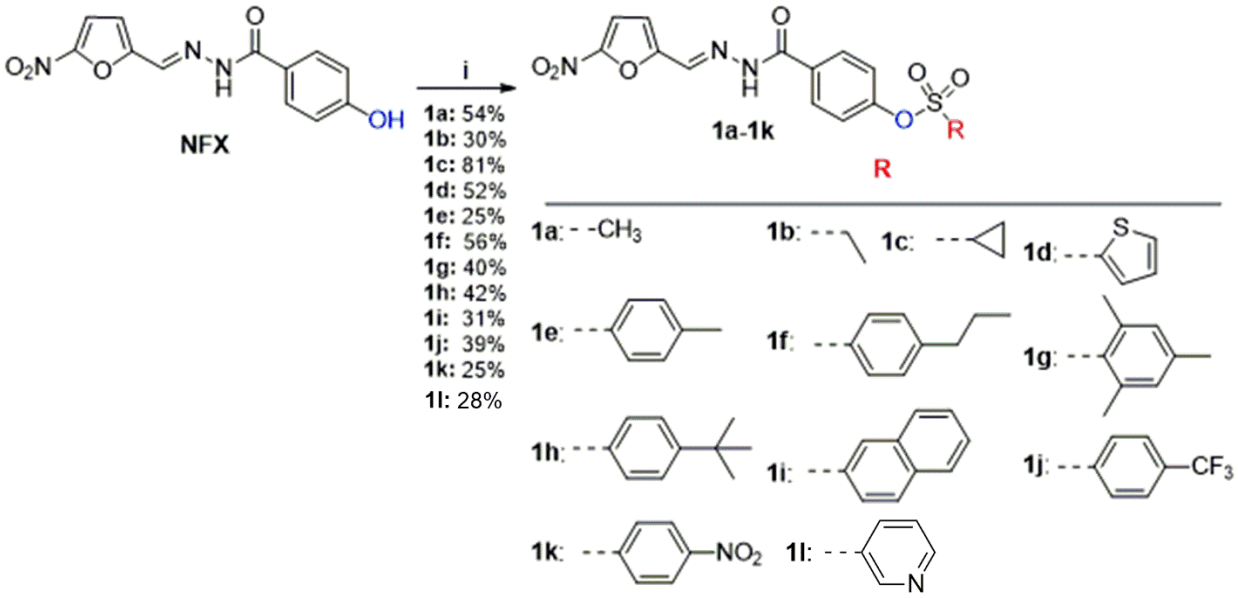
2. Results and discussion

2.1 Chemistry

Previously, *O*- and *N*- alkylated analogues of NFX were investigated for their antileishmanial activity[44]. The study, determined that *O*-benzylated derivatives performed better than *O, N*-di-benzylated analogues as they were found to possess nanomolar activity up to 10-fold higher than the parent compound NFX against *L. donovani* and *L. major* promastigotes, thus making them potential early hits for further investigation into the search of drugs for the treatment of VL[44]. It was also noticed that the NH group is essential for activity as all di-substituted NFX compounds lost activity[44].

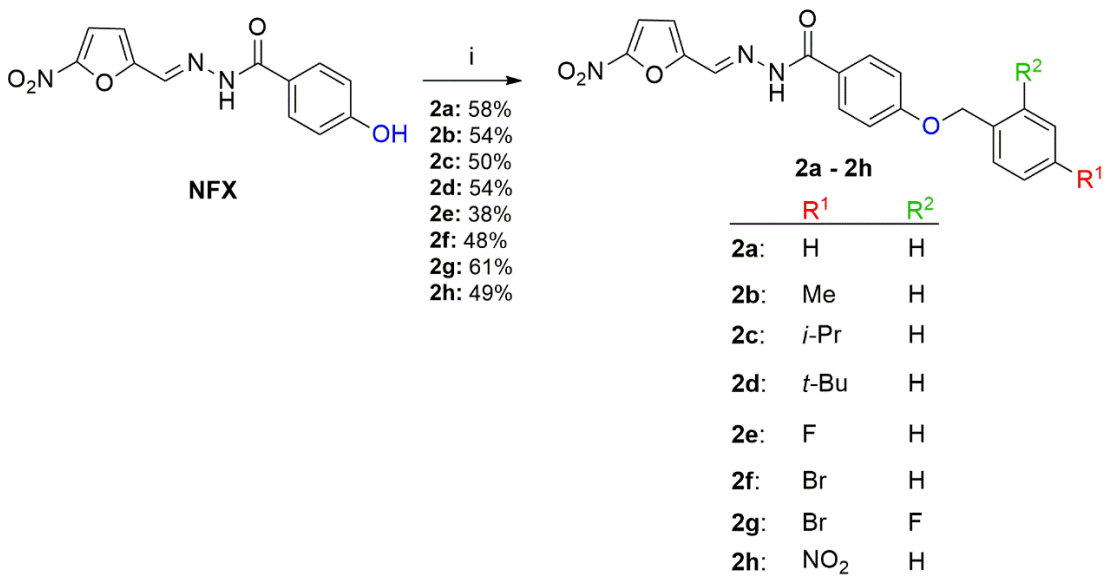
Based on these previous findings, *O*-benzyl and -sulfonyl analogues of nifuroxazide (NFX) (**1a-1k**; **2a-2h**) were synthesized in a one-step process as depicted in Schemes **1** and **2**, respectively, starting from commercially available NFX. NFX was deprotonated *in situ* by reacting it in a basic medium provided by either triethylamine or potassium carbonate, followed by the S_N2 nucleophilic substitution with various substituted benzyl bromides or sulfonyl chlorides to afford the benzyl or sulfonyl NFX analogues **1a-1l** and **2a-2h** in moderate to good yields (25 - 81%) after recrystallization with ethyl acetate: *n*-hexane (1:4, v/v).

It is noteworthy to indicate that compounds **2a** and **2e** had previously been reported[44], but were not assessed for antileishmanial activity against host intracellular amastigotes hence their inclusion in this study.



Scheme 1: One-step synthesis of *O*-sulfonyl NFX analogues (**1a-1l**)

Reagents and conditions: i) Sulfonyl chloride (1 equiv), TEA (2 equiv), DMF (10 ml), 80-90 °C, 12 h.



Scheme 2: One-step synthesis of *O*-benzyl NFX analogues (**2a-2h**)
Reagents and conditions: i) Benzyl bromide (1 equiv), K_2CO_3 (0.5 equiv), DMF (10 mL), 12 h, rt.

The structures of all the synthesized compounds were confirmed using routine molecular analysis techniques, such as NMR (^{1}H and ^{13}C), HRMS and IR. A successful SN_2 reaction was confirmed by the disappearance of the OH peak of the starting material nifuroxazide at *ca* δ5.35 ppm and the appearance of aromatic peaks in the region 6-8 ppm. A characteristic singlet was observed in the ^{1}H NMR spectrum around *ca* δ12.08 ppm for all analogues in series **1a-1l** and **2a-2h** and this indicated the presence of the acidic proton H-1 of the hydrazone moiety.

All analogues also possessed a singlet *ca* δ 8.39 ppm and this signal was assigned to the hydrazone bond of the vinylic proton H-3. The aromatic protons H-6 and H-7 of the furan ring gave a pair of doublets (d) with a *J* value of 3.9 Hz at δ7.68 and δ6.87 ppm, respectively. The deshielding of H-6 was due to the electronic effect of the nitro group that withdraws electron density away from H-6, while the unsaturated hydrazone bond pushes electron density towards H-7.

NFX phenyl ring showed two aromatic resonances that appeared as two pairs of coupled doublets at δ7.83 and δ7.13 ppm (*J* = 8.9 Hz) respectively. These signals were attributed to the aromatic protons H-3' and H-4'. The ^{1}H and ^{13}C NMR of the *O*-benzyl and -sulfonyl substituents varied depending on the type of group attached. However, in both series, where aromatic substituents

were attached, it was usual to observe two pairs of coupled doublets in the δ 7.16 – 6.89 ppm region *J* value found in the 8-9 Hz range.

The **2e** and **2g** derivative presented with an aromatic resonance consisting of a doublet of doublets (dd) in δ 7.53-7.24 ppm the region with $J_{H\text{-}H}$ = 8.6 and $^{3}J_{H\text{-}F}$ = 5.6 Hz assigned to resonance of proton 8' with the latter doublet as a result of the coupling with the adjacent fluorine atom. In summary, all the protons of each analogue were accounted for.

In the ^{13}C NMR, the carbonyl carbon (C-1') was observed at δ161.37 whereas the four aromatic carbons of the furan ring appeared as singlets at δ152.03 (C-4), 151.91 (C-5), 115.12 (C-7), 114.66 (C-6). The vinylic carbon (C-3) was seen as a singlet at δ136.53. The phenyl ring aromatic carbons of NFX appeared at δ161.07 (C-5'), 127.96 (C-3'), 124.96 (C-2'),114.68 (C-4') whilst the *O*-benzylated or *O*-sulfonated phenyl ring carbons appeared as singlets at δ136.53 (C-7'),128.46 (C-9'), 127.76 (C-8'), 124.96 (C-10').

For the derivatives **2e** and **2g**, the carbon C-10' resonated as a doublet at δ161.82 ppm resulting from the coupling with adjacent fluorine with $^{1}J_{C\text{-}F}$ = 244.0 Hz. The carbon C-9 expressed a weaker coupling with the fluorine (2 bonds away) showing a doublet at δ115.29 ppm with $^{2}J_{C\text{-}F}$ = 21.4 Hz and C-8' demonstrated an even weaker coupling the fluorine (3 bonds further) evidenced by the doublet at δ 130.08 ppm with $^{3}J_{C\text{-}F}$ = 8.3 Hz. A quartet was observed at δ 123.12 ppm with $^{1}J_{CF3}$ = 273.2 Hz for compound **1j** and this was attributed to the trifluoro coupling occurring at C-10. A doublet was also seen for C-9' at δ134.47 ppm with $^{2}J_{CF3}$ = 32.6 Hz and a quartet for C-8' at δ127.14 ppm with $^{3}J_{CF3}$ = 3.9 Hz. IR analysis further confirmed the success of the S_N2 reaction (**1a-l**; **2a-h**) by the disappearance of the broad OH peak 3200-3600 cm^{-1} and the appearance of characteristic absorption of S=O (1410-1380 cm^{-1} and 1204-1177 cm^{-1}) and C-O (1225-1200 cm^{-1} and 1075-1020 cm^{-1}).

2.2 Predicted physicochemical and pharmacokinetic properties

Oral administration is the preferred form of drug delivery by virtue of it being the least invasive route[45]. In order to achieve effective drug delivery *via* oral administration, a compound's physicochemical properties must adhere to Lipinski's rule of five[46]. Hence, the physicochemical properties of all synthesised analogues were predicted using SwissADME web tool. All data was summarized in Table **1** and used to determine the drug-likeness of the synthesised compounds.

Table 1: ADME and physicochemical data of synthesized NFX derivatives and reference NF drugs as predicted by SwissADME web tool, http://www.swissadme.ch.

Compd	MW (g/mol)	Log $P_{o/w}$ [a]	RA [b]	Log *S* [c]		HBD[f]	HBA[g]	Lipinski's violation	GI absorption	Leadlike-ness [h]	Drug-likeness [i]
				ESOL[d]	Ali[e]						
NFX	275.22	0.90	5	-2.95	-4.27	2	6	0	High	Yes	Yes
1a	353.31	1.08	7	-3.17	-4.82	1	8	0	Low	No	Yes
1b	367.33	1.32	8	-3.41	-5.21	1	8	0	Low	No	Yes
1c	379.34	1.74	8	-3.59	-5.40	1	8	0	Low	No	Yes
1d	421.40	2.53	8	-4.56	-6.98	1	8	0	Low	No	Yes
1e	429.40	2.49	8	-4.83	-6.75	1	8	0	Low	No	Yes
1f	457.46	3.18	10	-5.45	-7.76	1	8	0	Low	No	Yes
1g	457.46	3.15	8	-5.43	-7.51	1	8	0	Low	No	Yes
1h	471.48	3.51	9	-5.80	-8.10	1	8	0	Low	No	Yes
1i	465.44	3.27	8	-5.65	-7.67	1	8	0	Low	No	Yes
1j	483.37	3.47	9	-5.38	-7.29	1	11	1	Low	No	Yes
1k	460.37	1.81	9	-4.59	-7.15	1	10	1	Low	No	Yes
1l	450.81	2.33	8	-4.66	-6.52	1	9	0	Low	No	Yes
2a	365.34	2.83	8	-4.52	-5.93	1	6	0	High	No	Yes
2b	379.37	2.90	8	-4.82	-6.31	1	6	0	High	No	Yes
2c	407.42	3.62	9	-5.37	-7.09	1	6	0	High	No	Yes
2d	421.45	3.88	9	-5.79	-7.66	1	6	0	High	No	Yes
2e	383.33	3.02	8	-4.68	-6.04	1	7	0	High	No	Yes
2f	444.24	3.36	8	-5.43	-6.65	1	6	0	High	No	Yes
2g	462.23	3.67	8	-5.54	-6.68	1	7	0	High	No	Yes
2h	410.34	2.13	9	-4.53	-6.65	1	8	0	Low	No	Yes
FZD	225.16	0.32	3	-1.24	-1.62	0	6	0	High	No	Yes
NFZ	198.14	-0.59	4	-1.21	-2.45	2	5	0	High	No	Yes
NFT	238.16	-0.50	3	-1.04	-1.60	1	6	0	High	No	Yes

[a] Calculated log $P_{o/w}$ (consensus log $P_{o/w}$). [b] Number of rotatable bonds. [c] Predicted aqueous solubility, where log *S* is the logarithm of the amount of compound (in moles) that can dissolve in a litre of water. [d] ESOL = estimated aqueous solubility, derived from using a topological method[47]. [e] Derived from using a topological method[48] with log *S* scale: insoluble < -10 < poorly soluble < -6 < moderately soluble < -4 < soluble < -2 very soluble < 0 highly soluble. [f] Number of hydrogen bond donors. [g] Number of hydrogen bond acceptors. [h] Calculated from Teague *et al.* (1999) , 250 ≤ MW ≤ 350; XLOGP ≤ 3.5; Number of rotatable bonds ≤ 7 – good oral bioavailability. [i] Determined by using Lipinski's rule of five: MW ≤ 500 g/mol; LogP ≤ 5; HBD ≤ 5; HBA ≤ 10; no more than one violation allowed[46]. All values in this table were calculated using SwissADME web tool, http://www.swissadme.ch[50]. Abbreviations: NFX: nifuroxazide; FZD: furazolidone; NFZ: nitrofurazone; NFT: nitrofurantoin.

All synthesised analogues except compounds **1j** and **1k** complied with Lipinski's rules and showed predicted physicochemical properties well within the target ranges as set out by Lipinski *et al.*[46]. Compounds **1j** and **1k** violated one of Lipinski's rules. All benzyl analogues, with the exception of **2h,** showed a high GI absorption while all sulfonyl analogues showed low GI

absorption. All synthesised analogues were predicted to be drug-like in nature, but none was predicted to be a lead compound.

2.3 Pharmacology

The synthesised NFX analogues were assessed for their *in vitro* antileishmanial properties against the promastigote form of three strains of *Leishmania*, namely *L. donovani* strains 1S and 9515 and *L. major* strain IR-173. These strains and species of *Leishmania* were chosen to determine the specificity of the synthesised analogues against *L. major,* which causes CL and, *L. donovani* which causes the deadly and debilitating VL[51, 52]. Amphotericin B (AMB) was used as reference antileishmanial drug while cNFs, NFX, furazolidone, nitrofurazone and nitrofurantoin were tested as reference drugs for comparison against the synthesised analogues. Cytotoxicity profiles of the compounds were established using Vero cells with emetine (EM) as a positive control. All of the results are reported in Table **2**.

The cytotoxicity data indicated that the majority of the sulfonyl analogues (**1d-1k**) were non-toxic to Vero cells (IC_{50} > 100 µM), while **1a**, **1b** and **1c** showing moderate toxicity to mammalian cells (10 µM < IC_{50} < 50 µM). In contrast, all of the benzyl analogues (**2a**-**2h**), (except **2a** and **2d** which showed moderate toxicity), were weak to non-toxic to Vero cells (50 µM < IC_{50} > 100 µM). All of the synthesised analogues, with the exception of **2h**, were active against the promastigotes of all three strains of *Leishmania*, with IC_{50} values comparable or better than the parent NFX and reference cNFs. The most active analogues of the series, **1f**, **1h, 1j, 2c** and **2d**, demonstrated improved anti-promastigote activity of up to 56-fold against *L. donovani* and 427-fold against *L. major* in comparison to NFX.

Table 2: Biological results of synthesised NFX analogues and reference nitrofuran antibiotics

Compound	Cytotoxicity Vero[b] IC_{50} (µM)	*L. donovani* strain 1S		*L. donovani* strain 9515		*L. major* strain IR-173	
		IC_{50} (µM)[a]	SI_1[c]	IC_{50} (µM)[a]	SI_2[d]	IC_{50} (µM)[a]	SI_3[e]
NFX	>100	4.53 ± 0.19	22	4.48 ± 0.10	22	38.48 ± 2.72	3
1a	31.86 ± 3.81	5.55 ± 0.17	6	2.15 ± 0.08	15	9.74 ± 0.36	3
1b	26.99 ± 6.12	3.28 ± 0.02	8	1.51 ± 0.18	18	5.56 ± 0.20	5
1c	23.92 ± 0.36	2.98 ± 0.01	8	1.88 ± 0.02	13	5.21 ± 0.16	5
1d	>100	4.33 ± 0.46	23	0.23 ± 0.01	435	4.57 ± 0.03	22
1e	>100	7.06 ± 0.05	14	0.38 ± 0.09	263	0.83 ± 0.04	120
1f	>100	0.30 ± 0.05	333	0.64 ± 0.04	156	0.82 ± 0.07	122
1g	>100	0.49 ± 0.01	204	1.38 ± 0.19	72	0.21 ± 0.06	476
1h	>100	0.80 ± 0.12	125	0.60 ± 0.01	167	0.32 ± 0.02	313
1i	>100	2.62 ± 0.05	38	1.86 ± 0.13	54	2.80 ± 0.06	36
1j	>100	0.84 ± 0.15	119	0.69 ± 0.09	145	0.26 ± 0.05	385
1k	>100	0.98 ± 0.12	102	1.13 ± 0.11	88	0.51 ± 0.02	196
1l	84.10 ± 4.53	5.02 ± 0.54	17	>10		>10	
2a	46.97 ± 3.93	3.20 ± 0.38	15	1.92 ± 0.06	24	2.87 ± 0.01	16
2b	92.03 ± 8.55	0.96 ± 0.02	96	1.27 ± 0.13	72	0.72 ± 0.02	128
2c	62.37 ± 1.29	0.12 ± 0.01	520	0.25 ± 0.01	249	0.14 ± 0.01	446
2d	33.85 ± 2.28	0.08 ± 0.00	423	0.24 ± 0.01	141	0.09 ± 0.00	376
2e	96.32 ± 5.21	1.63 ± 0.13	59	0.90 ± 0.03	107	0.62 ± 0.05	155
2f	80.97 ± 6.08	0.28 ± 0.00	289	1.05 ± 0.03	77	0.35 ± 0.03	231
2g	>100	0.41 ± 0.02	244	1.98 ± 0.09	51	0.28 ± 0.04	357
2h	78.74 ± 9.94	N.D.	N.D.	N.D.	N.D.	3.71 ± 0.06	21
AMB	57.77 ± 3.22	0.02 ± 0.00	2 889	0.02 ± 0.003	2 889	0.03 ± 0.006	1 926
FZD	>100	0.32 ± 0.00	313	0.28 ± 0.04	357	0.34 ± 0.03	294
NFZ	>100	6.54 ± 0.93	15	1.85 ± 0.14	54	1.85 ± 0.06	54
NFT	>100	33.95 ± 1.82	3	27.55 ± 0.00	4	86.04 ± 2.35	1
EM	0.08 ± 0.01	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a] Represented as the mean ± SD from the triplicate biological experiments.
[b] African green monkey kidney epithelial cells.
[c] Selectivity index: SI_1 = IC_{50} Vero/IC_{50} *L. donovani* 1S.
[d] Selectivity index: SI_2 = IC_{50} Vero/IC_{50} *L. donovani* 9515.
[e] Selectivity index: SI_3 = IC_{50} Vero/IC_{50} *L. major* IR-173.
Abbreviations: AMB: Amphotericin B; NFX: nifuroxazide; FZD: furazolidone; NFZ: nitrofurazone; NFT: nitrofurantoin; EM: emetine; N.D.: not determined.

The majority of the sulfonyl analogues showed high selectivity (SI>10) towards all three strains of *Leishmania* tested with the exception of **1a**, **1b** and **1c**, which indicates that their anti-promastigote activity was intrinsic. Half of the sulfonyls showed nanomolar activity against at least two strains with the best performing analogues, **1f**, **1h** and **1j** showing nanomolar antileishmanial activity against all three strains. Based on the criteria set out in Katsuno *et al* (2015), with a hit compound being considered a compound with antipromastigote IC_{50} of below 10 uM and SI above 10 or a compound with antipromastigote IC_{50} of below 1 µM and SI between 10 and 100. Compared to a

lead compound that is considered a compound with antipromastigote IC_{50} below 1 µM and SI above 100[53]. All of the sulfonyl analogues were identified as hit compounds against the strains tested. The analogues **1d**, **1e**, **1f**, **1g**, **1h**, **1j, 1k** and **1l** all show promise as possible lead compounds with IC_{50} values below 1 µM and SI values above 100[53].

With the exception of **2h,** SI>10 was shown by all of the benzyl analogues towards all three strains of *Leishmania* tested**,** which was indicative of the intrinsic anti-promastigote activity of these compounds. The benzyl analogues were more potent against all three strains, and this may be attributed to the benzyl group's electrophilic nature that causes the formation of hydrazone resulting in the activation of the nitro-group and formation of more ROS and RNS[42, 54]. Many of the benzyl analogues showed nanomolar activity against the *Leishmania* strains tested with the best performers being **2c** and **2d.**

Based on the criteria set out in Katsuno *et al* (2015) as seen above, all of the benzyl analogues with the exception of **2h** were hit compounds against all three strains tested. Six analogues namely **2b**, **2c**, **2d**, **2e**, **2f** and **2g** shows characteristics of possible lead compounds. Both series showed similar cell viability effects, with only three sulfonyl (**1a, 1b** and **1c**) and two benzyl (**2a** and **2d**) analogues showing moderate toxicity to Vero cells.

When considering the electronic effect, the substituents (R) were be divided into three categories namely electron withdrawing groups (EWGs), neutral group (H), and electron donating groups (EDGs).

In the sulfonyl series, the R were: methyl (Me), ethyl (Et), cyclopropyl (*c*Pr), thiophene (Th), 4-methyl phenyl (4-Me Phe), 4-*n*propyl phenyl (4-*n*Pr Phe), 4-*tert*-butyl Phenyl (4-*t*Bu Phe), 2,4,6-trimethyl phenyl (TMP), naphtyl (Naph), 4-trifluoromethyl phenyl (4-CF_3 Phe) and nitro (NO_2).
The increasing order of EWGs was CF_3< NO_2 and Me<Et<*c*Pr< Th <4-Me Phe <4-*n*Pr Phe<4-*t*Bu Phe<TMP<Naph for EDGs. The weaker EWG bearing analogue **1j** was the most active against all three parasite strains as compared to **1k** carrying the stronger EWG. Among EDG bearing analogues, the SAR profiles were disparate. However, of notice, analogues **1a**, **1b** and **1c** which bore the least EDGs in the increasing strength order Me, Et and *c*Pr were distinctively the least active compounds in the same order regardless of the strain considered.

The benzyl series presented with R on the benzylic phenyl ring, 4-Me, 4-isopropyl (4-*i*Pr), 4-*t*Bu, 4-F, 4-Br, 2-F 4-Br and 4-NO_2. The increasing EDG strength order was, Me<*i*-Pr<*t*-Bu and

Br<F<2-F 4-Br< NO_2 for EWGs. The neutral analogue **2a** bearing unsubstituted benzyl moiety and the stronger EWG bearing derivative **2g** were distinctively the least active compounds in this sub-series. Moreover, an increase in activity was observed also with an increase in EDG strength irrespective of the strain considered. A similar pattern was noticed among the remainder of EWG analogues against *L.* major parasites. However, an increase in EWG strength caused a decrease in activity against *L. donovani* 9515 while no discernible SAR pattern could be observed against *L. donovani* 1S among EWG bearing analogues. Hence, a correlation could be found between structure and activity against *1S* and L. *major* parasites.

Furthermore, bioisosteres are substituents or groups with comparable/similar chemical or physical properties that produce similar biological features[55]. Chief benefits of bioisosterism include the rational modification of a lead compound into a safer and more clinically effective agent through attenuation of toxicity, modification of activity, and/or alteration of pharmacokinetics[55]. The ability of bioisosteres to elicit similar biological activities has been attributed to common physicochemical properties, such as electronegativity, steric size, and lipophilicity[56]. The substitution of hydrogen by fluorine is one of the most commonly employed monovalent isosteric replacements. Hydrogen and fluorine atoms have similar steric parameters with their van der Waal's radii being 1.2 and 1.35 Å, respectively[57]. Analogue **2a** and its bioisostere **2e** had comparable lipophilicity with Log $P_{o/w}$ values of 2.83 and 3.02, respectively. However, the bioisoteric substitution of H with F resulted in an enhancement of biological activity as analogue **2e** was found to be 2- to 5-fold more active, and safer (SI 59-155) than 2a across all parasites. Hence, bioisoterism was beneficial in this research.

3. Conclusion

A series of nineteen novel NFX analogues were synthesised by an SN_2 reaction with moderate to good yields starting from the parent. The majority of the synthesised analogues were found to be weakly to non-toxic. The benzyl analogues showed more potent activity against the three *Leishmania* strains. The electronic effect analysis indicated a direct correlation between anti-promastigote activities and the strength of EWGs in the sulfonyl series. A direct correlation could also be identified between anti-promastigote activities and the strength of EDGs in the benzyl series. Furthermore, the *tert*-butyl analogues in both sulfonyl and benzyl sub-series were very potent against all three tested strains. Eight sulfonyl (**1d**, **1e**, **1f**, **1g**, **1h**, **1j**, **1k** and **1l**) and six benzyl (**2b**, **2c**, **2d**, **2e**, **2f** and **2g**) analogues showed promise as anti-promastigote lead

compounds. However, the amastigote form of *Leishmania* is the clinically relevant form, invading the host macrophages, multiplying and infecting cells, causing dissemination of the infection to secondary sites. Thus, future work regarding the potential activity of these compounds against the amastigote form of the parasite will be investigated to possibly identify new potential antileishmanial hits/leads.

4. Experimental section

4.1 General procedures

All chemicals were purchased from Sigma-Aldrich (Johannesburg, South Africa) and solvents from Associated Chemical Enterprises, ACE (South Africa). All chemicals and reagents were of analytical grade and no further purification was needed. The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra was recorded on a Bruker Avance^{TM} III 600 spectrometer at a frequency of 600 and 151 MHz, respectively, in deuterated dimethyl sulfoxide (DMSO-d_6). The chemical shifts were reported in parts per million (ppm) with the residual protons of the solvent used as reference. The abbreviations of the splitting patterns were as follows: singlet (s), doublet (d), doublet of doublet (dd), doublet of doublets of doublets (ddd), doublet of triplets (dt), 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. It was equipped with an atmospheric pressure chemical ionisation (APCI) source set at 200 °C or 180 °C and analysed using a Bruker Compass Data Analysis 4.0 software. A full scan that ranged from 50 - 1500 m/z at a capillary voltage of 4500 V, an end plate offset voltage of -500 V, with the nebuliser set at 1.6 and 0.4 Bar, respectively, and a collision cell RF voltage of 100 V. Infrared (FTIR) spectra were recorded on a Bruker Alpha-P FTIR instrument. A BÜCHI melting point B-545 instrument was used to determine melting points (mp) and were uncorrected. Thin layer chromatography (TLC) was performed on silica gel plates ($^{60}F_{254}$) that was acquired from Merck (South Africa).

4.2 Syntheses

4.2.1 Synthesis of *O*-sulfonated nifuroxazide derivatives (1a-1l)

Nifuroxazide (NFX, 1.81 mmol, 0.5 g, 1 equiv) was dissolved in anhydrous dimethylformamide (DMF) (10 mL). Triethylamine (TEA, 10.76 mmol 1.5 mL, 6 equiv) was added dropwise to the reaction followed by substituted sulfonyl chloride (1 equiv). The reaction was then heated to

between 80-90 °C for 12 h and monitored by thin layer chromatography (TLC). Upon completion, DMF was removed *in vacuo*, the residue suspended in water (40 mL) and extracted with ethyl acetate (3 x 40 mL). The organic phase was then dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to result in a solid crude compound. The targeted compound was then recrystallised with ethyl acetate:*n*-hexane (1:4, v/v) to afford the desired compounds.

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl methanesulfonate* (1a)**

Yellow powder; yield: 54%; mp: 184-187 °C (EtOAc); IR υ_{max} (cm^{-1}): 3137 (N-H), 1677 (C=O), 1565 (C=N), 1479 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.37 (s, 1H, H-1), 8.30 (s, 1H, H-3), 7.97 (d, *J* = 8.3 Hz, 2H, H-3'), 7.68 (d, *J* = 3.8 Hz, 1H, H-6), 7.47 (d, *J* = 8.3 Hz, 2H, H-4'), 7.20 (d, *J* = 3.8 Hz, 1H, H-7), 3.37 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 162.41 (C-1'), 151.96 (C-5'), 151.60 (C-4), 151.60 (C-5), 135.85 (C-3), 131.75 (C-2'), 129.94 (C-3'), 122.43 (C-4'), 115.54 (C-7), 114.60 (C-6), 37.74 (C-6'). HRMS-APCI (*pos*) *m/z* 354.0378 $[M + H]^+$ (calcd for $C_{13}H_{12}N_3O_7S^+$, 354.0396).

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl ethanesulfonate* (1b)**

Yellow powder; yield: 30%; mp: 192-195 °C (EtOAc); IR υ_{max} (cm^{-1}): 3346 (N-H), 1665 (C=O), 1564 (C=N), 1476 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.30 (s, 1H, H-1), 8.40 (s, 1H, H-3), 8.02 (d, *J* = 8.0 Hz, 2H, H-3'), 7.80 (d, *J* = 3.7 Hz, 1H, H-6), 7.51 (d, *J* = 8.0 Hz, 2H, H-4'), 7.29 (*d, *J* = 3.7 Hz, 1H, H-7), 3.60 (d, *J* = 7.3 Hz, 2H, H-6'), 1.40 (s, 3H, H-7'). ^{13}C NMR (151 MHz, DMSO) δ 162.40 (C-1'), 151.96 (C-5'), 151.60 (C-5), 151.47 (C-4), 135.85 (C-3), 131.64 (C-2'), 129.94 (C-3'), 122.32 (C-4'), 115.55 (C-7), 114.61 (C-6), 45.01 (C-6'), 8.07 (C-7'). HRMS-APCI (*pos*) *m/z* 368.0529 $[M + H]^+$ (calcd for $C_{14}H_{14}N_3O_7S^+$, 368.0552).

*Coalesced doublet

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl cyclopropanesulfonate* (1c)**

Yellow powder; yield: 81%; mp: 174-177 °C (EtOAc); IR υ_{max} (cm^{-1}): 3209 (N-H), 1655 (C=O), 1562 (C=N), 1476 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.31 (s, 1H, H-1), 8.40 (s, 1H, H-3), 8.03 (d, *J* = 8.3 Hz, 2H, H-3'), 7.80 (d, *J* = 3.8 Hz, 1H, H-6), 7.54 (d, *J* = 8.3 Hz, 2H, H-4'), 7.30 (*d, *J* = 3.8 Hz, 1H, H-7), 3.14 (tt, *J* = 7.9, 4.7 Hz, 1H, H-6'), 1.21 (qd, *J* = 5.6, 1.0 Hz, 2H, H-7'a/8'a), 1.05 (qd, *J* = 5.6, 1.0 Hz, 2H, H-7'b/8'b). ^{13}C NMR (151 MHz, DMSO) δ 162.43 (C-1'), 151.98 (C-5'), 151.61 (C-5), 135.87 (C-3), 131.73 (C-2), 129.88 (C-3'), 122.59 (C-4'), 115.55 (C-7), 114.62 (C-6), 27.65

(C-6'), 6.08 (C-7'), 6.08 (C-8'). HRMS-APCI (*pos*) *m/z* 380.0559 $[M + H]^+$ (calcd for $C_{15}H_{14}N_3O_7S^+$, 380.0552).

*Coalesced doublet

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl thiophene-2-sulfonate* (1d)**

Yellow powder; yield: 52%; mp: 198-201 °C (EtOAc); IR υ_{max} (cm^{-1}): 3095 (N-H), 1664 (C=O), 1560 (C=N), 1476 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.29 (s, 1H, H-1), 8.37 (s, 1H, H-3), 8.14 (dd, *J* = 5.0, 1.3 Hz, 1H, H-7'), 7.95 (d, *J* = 8.0 Hz, 2H, H-3'), 7.85 (d, *J* = 3.8 Hz, 1H, H-6), 7.80 (dd, *J* = 5.0, 1.3 Hz, 1H, H-9'), 7.27 (dd, *J* = 5.0, 3.9 Hz, 1H, H-8'), 7.28 (d, *J* = 8.0 Hz, 2H, H-4'), 7.26 (*d, *J* = 3.8 Hz, 1H, H-7). ^{13}C NMR (151 MHz, DMSO) δ 162.27 (C-1'), 151.95 (C-5'), 151.55 (C-5), 137.57 (C-4), 136.78 (C-3), 135.89 (C-2'), 129.90 (C-8'), 129.90 (C-9'), 128.62 (C-3'), 122.21 (C-7'), 115.55 (C-7), 114.59 (C-6). HRMS-APCI (*pos*) *m/z* 422.0107 $[M + H]^+$ (calcd for $C_{16}H_{12}N_3O_7S_2^+$, 422.0117).

*Coalesced doublet

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 4-methylbenzene sulfonate* (1e)**

Yellow powder; yield: 25%; mp: 204-207 °C (EtOAc); IR υ_{max} (cm^{-1}): 3147 (N-H), 1662 (C=O), 1567 (C=N), 1469 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.26 (s, 1H, H-1), 8.37 (s, 1H, H-3), 7.91 (d, *J* = 7.9 Hz, 2H, H-3'), 7.80 (d, *J* = 3.9 Hz, 1H, H-6), 7.78 (d, *J* = 8.2 Hz, 2H, H-7'), 7.49 (d, *J* = 8.2 Hz, 2H, H-8'), 7.29 (*d, *J* = 3.9 Hz, 1H, H-7), 7.22 (d, *J* = 7.9 Hz, 2H, H-4'), 2.43 (s, 3H, H-10'). ^{13}C NMR (151 MHz, DMSO) δ 162.26 (C-1'), 151.92 (C-5'), 151.54 (C-5), 146.05 (C-4), 135.81 (C-3), 131.82 (C-2'), 131.16 (C-9'), 130.32 (C-8'), 129.83 (C-3'), 128.23 (C-7'), 122.24 (C-4'), 115.51 (C-7), 114.57 (C-6), 21.17 (C-10'). HRMS-APCI (*pos*) *m/z* 430.0687 $[M + H]^+$ (calcd for $C_{19}H_{16}N_3O_7S^+$, 430.0687).

*Coalesced doublet

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl4-propylbenzene sulfonate* (1f)**

Yellow powder; yield: 56%; mp: 172-175 °C (EtOAc); IR υ_{max} (cm^{-1}): 3102 (N-H), 1666 (C=O), 1562 (C=N), 1478 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.27 (s, 1H, H-1), 8.37 (s, 1H, H-3), 7.92 (d, *J* = 8.1 Hz, 2H, H-3'), 7.81 (d, *J* = 3.9 Hz, 1H, H-6), 7.79 (d, *J* = 8.4 Hz, 2H, H-7'), 7.50 (d, *J* = 8.4 Hz, 2H, H-8'), 7.28 (*d, *J* = 3.9 Hz, 1H, H-7), 7.22 (d, *J* = 8.1 Hz, 2H, H-4'), 2.69 (t, *J* = 7.5 Hz, 2H,

H-10'), 1.61 (ddd, *J* = 22.0, 15.0, 7.5 Hz, 2H, H-11'), 0.88 (t, *J* = 7.5 Hz, 3H, H-12'). ^{13}C NMR (151 MHz, DMSO) δ162.29 (C-1'), 151.94 (C-5'), 151.56 (C-5), 150.36 (C-4), 135.84 (C-3), 131.81 (C-3'), 131.44 (C-9'), 129.83 (C-2'), 129.75 (C-8'), 128.28 (C-7'), 122.25 (C-4'), 115.53 (C-7), 114.58 (C-6), 36.93 (C-10'), 23.50 (C-11'), 13.43 (C-12'). HRMS-APCI (*pos*) *m/z* 458.1012 $[M + H]^+$ (calcd for $C_{21}H_{20}N_3O_7S^+$, 458.1022).

*Coalesced doublet

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 2,4,6-trimethylbenzene sulfonate* (1g)**

Brown powder; yield: 40%; mp: 213-216 °C (EtOAc); IR υ_{max} (cm^{-1}): 3232 (N-H), 1655 (C=O), 1558 (C=N), 1476 (S=O); ^{1}H NMR (600 MHz, DMSO) δ12.26 (s, 1H, H-1), 8.36 (s, 1H, H-3), 7.91 (d, *J* = 8.0 Hz, 2H, H-3'), 7.80 (d, *J* = 3.9 Hz, 1H, H-6), 7.29 (*d, *J* = 3.9 Hz, 1H, H-7), 7.19 (d, *J* = 8.0 Hz, 2H, H-4'), 7.17 (s, 2H, H-8'), 2.51 (s, 6H, H-11'/12'), 2.31 (s, 3H, H-10'). ^{13}C NMR (151 MHz, DMSO) δ162.28 (C-1'), 151.94 (C-5'), 151.57 (C-5), 144.51 (C-4), 139.77 (C-9'), 135.83 (C-3), 131.94 (C-8'), 131.70 (C-3'), 129.85 (C-7'), 129.38 (C-2'), 121.99 (C-4'), 115.52 (C-7), 114.58 (C-6), 22.12 (C-11'/12'), 20.59 (C-10'). HRMS-APCI (*pos*) *m/z* 458.0998 $[M + H]^+$ (calcd for $C_{21}H_{20}N_3O_7S^+$, 458.1022).

*Coalesced doublet

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 4-(tert-butyl)benzene sulfonate* (1h)**

Light yellow powder; yield: 42%; mp: 192-195 °C (EtOAc); IR υ_{max} (cm^{-1}): 3216 (N-H), 1660 (C=O), 1561 (C=N), 1476 (S=O); ^{1}H NMR (600 MHz, DMSO) δ12.27 (s, 1H, H-1), 8.37 (s, 1H, H-3), 7.93 (d, *J* = 8.0 Hz, 2H, H-3'), 7.84 (d, *J* = 8.5 Hz, 2H, H-7'), 7.80 (d, *J* = 3.8 Hz, 1H, H-6), 7.72 (d, *J* = 8.5 Hz, 2H, H-8'), 7.29 (*d, *J* = 3.8 Hz, 1H, H-7), 7.25 (d, *J* = 8.0 Hz, 2H, H-4'), 1.32 (s, 9H, H-11'/12'/13'). ^{13}C NMR (151 MHz, DMSO) δ162.30 (C-1'), 158.51 (C-9'), 151.95 (C-5'), 151.57 (C-5), 135.85 (C-3), 131.81 (C-2'), 131.44 (C-6'), 129.87 (C-3'), 128.11 (C-8'), 126.78 (C-7'), 122.20 (C-4'), 115.54 (C-7), 114.58 (C-6), 35.17 (C-10'), 30.62 (C-11'/12'/13'). HRMS-APCI (*pos*) *m/z* 472.1156 $[M + H]^+$ (Calculated for $C_{22}H_{22}N_3O_7S^+$, 472.1178).

*Coalesced doublet

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl naphthalene-2-sulfonate* (1i)**

Yellow powder; yield: 31%; mp: 223-226 °C (EtOAc); IR υ_{max} (cm^{-1}): 3245 (N-H), 1662 (C=O), 1560 (C=N), 1477 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.22 (s, 1H, H-1), 8.62 (s, 1H, H-3), 8.33 (s, 1H, H-15'), 8.25 (t, *J* = 8.2 Hz, 2H, H-10'/13'), 8.14 (d, *J* = 8.2 Hz, 1H, H-7'), 7.93 (dd, *J* = 8.2, 1.9 Hz, 1H, H-8'), 7.88 (d, *J* = 8.2 Hz, 2H, H-3'), 7.82-7.77 (m, 3H, H-6/11'/12'), 7.25 (d, *J* = 8.2 Hz, 2H, H-4'), 7.24 (d, *J* = 3.9 Hz, 1H, H-7). ^{13}C NMR (151 MHz, DMSO) δ 162.27 (C-1'), 151.93 (C-5'), 151.54 (C-4'), 151.49 (C-5), 143.80 (C-6'), 135.82 (C-3), 135.15 (C-9'), 131.46 (C-14'), 130.37 (C-10'), 130.16 (C-11'), 130.13 (C-12'), 129.89 (C-8'), 129.69 (C-3'/13'), 128.17 (C-15'), 128.03 (C-2'), 122.43 (C-7'), 115.52 (C-4'), 115.14 (C-6), 114.57 (C-7). HRMS-APCI (*pos*) *m/z* 466.0698 $[M + H]^+$ (calcd for $C_{22}H_{16}N_3O_7S^+$, 466.0709).

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 4-(trifluoromethyl)benzenesulfonate* (1j)**

Light yellow powder; yield: 39%; mp: 191-194 °C (EtOAc); IR υ_{max} (cm^{-1}): 3258 (N-H), 1666 (C=O), 1565 (C=N), 1480 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.27 (s, 1H, H-1), 8.37 (s, 1H, H-3), 8.14 (d, *J* = 8.3 Hz, 2H, H-8'), 8.09 (d, *J* = 8.5 Hz, 2H, H-3'), 7.95 (d, *J* = 8.3 Hz, 2H, H-7'), 7.79 (d, *J* = 3.9 Hz, 1H, H-6), 7.29 (d, *J* = 8.5 Hz, 2H, H-4'), 7.24 (d, *J* = 3.9 Hz, 1H, H-7). ^{13}C NMR (151 MHz, DMSO) δ 162.23 (C-1), 152.06 (C-5'), 151.96 (C-4), 151.54 (C-5), 138.02 (C-6'), 135.91 (C-3), 134.47 (d, *J* = 32.6 Hz, C-9'), 130.03 (C-7'), 129.38 (C-3'), 127.14 (q, *J* = 3.9 Hz, C-8'), 123.12 (q, *J* = 273.2 Hz, $\underline{C}F_3$), 122.34 (C-2'), 115.59 (C-4'), 115.14 (C-6), 114.58 (C-7). HRMS-APCI (*pos*) *m/z* 484.0421 $[M + H]^+$ (calcd for $C_{19}H_{13}F_3N_3O_7S^+$, 484.0426).

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 4-nitrobenzenesulfonate* (1k)**

Yellow powder; yield: 25%; mp: 159-162 °C (EtOAc); IR υ_{max} (cm^{-1}): 3249 (N-H), 1672 (C=O), 1539 (NO_2), 1472 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.28 (s, 1H, H-1), 8.47 (d, *J* = 8.8 Hz, 2H, H-8'), 8.37 (s, 1H, H-3), 8.19 (d, *J* = 8.8 Hz, 2H, H-7'), 7.94 (d, *J* = 8.2 Hz, 2H, H-3'), 7.79 (d, *J* = 3.9 Hz, 1H, H-6), 7.24 (d, *J* = 3.9 Hz, 1H, H-7), 6.88 (d, *J* = 8.2 Hz, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ 162.21 (C-1'), 152.05 (C-5'), 151.53 (C-9'), 151.17 (C-5), 139.24 (C-6'), 135.94 (C-3), 130.08 (C-8'), 125.13 (C-7'), 123.18 (C-2'), 122.39 (C-3'), 115.14 (C-4'), 114.73 (C-6), 114.58 (C-7). HRMS-APCI (*pos*) *m/z* 461.0393 $[M + H]^+$ (calcd for $C_{18}H_{13}N_4O_9S^+$, 461.0403).

***(E)-4-{2-[(5-nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl pyridine-3-sulfonate* (1l)**

Brown powder; yield: 27.6%; mp: 198-201 °C (EtOAc); IR υ_{max} (cm-1): 3066 (N-H), 1664 (C=O), 1569 (C=N), 1476 (S=O); ^{1}H NMR (600 MHz, DMSO) δ 12.28 (s, 1H, H-1), 9.03 (s, 1H, H-3), 9.00 (dd, J = 4.8, 1.4 Hz, 1H, H-7'), 8.37 (s, H-10'), 8.35 (dt, J = 8.0, 4.0, 1.4 Hz, 1H, H-9'), 7.94 (d, J = 8.0 Hz, 2H, H-3'), 7.80 (d, J = 3.9 Hz, 1H, H-6), 7.74 (ddd, J = 8.2, 4.9, 0.6 Hz, 1H. H-8'), 7.31 (d, J = 8.0 Hz, 2H, H-4'), 7.29 (d, J = 3.9 Hz, 1H, H-7). 13C NMR (151 MHz, DMSO) δ 162.70 (C-1'), 152.53 (C-5'), 152.43 (C-9'), 152.29 (C-4), 152.02 (C-5), 151.63 (C-10'), 138.50 (C-6), 134.84 (C-3), 130.51 (C-10'), 129.86 (C-3'), 127.64 (C-7'), 127.62 (C-2'), 122.81 (C-8'), 116.06 (C-4'), 115.62 (C-6), 115.05 (C-7). HRMS-APCI (*pos*) *m/z* 417.0488 $[M + H]^+$ (Calcd for C17H13N4O7S+, 417.0460).

4.2.2 Synthesis of *O*-benzylated nifuroxazide derivatives (2a-2h)

Nifuroxazide (NFX, 1.81 mmol, 0.5 g, 1 equiv) was dissolved in 10 mL of DMF. Thereafter, anhydrous potassium carbonate (K_2CO_3, 0.91 mmol, 0.19 g, 0.5 equiv) was added to the flask, followed by the addition of substituted benzyl bromide (1 equiv). The reaction was left to stir at room temperature for 12h and monitored by TLC. Upon completion, DMF was removed *in vacuo* and water (10 mL) added to initiate precipitation. The precipitate was filtered and recrystallised with ethyl acetate:hexane (1:4, v/v) to yield the desired product.

***(E)-4-(benzyloxy)-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide* (2a)[44]**

Light yellow powder; yield: 58%; mp: 227-229 °C (EtOAc) ; IR υ_{max} (cm^{-1}): 3281 (N-H), 1663 (C=O), 1556 (C=N), 1347 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 12.08 (s, 1H, H-1), 8.39 (s, 1H, H-3), 7.91 (d, *J* = 8.8 Hz, 2H, H-3'), 7.81 (d, *J* = 3.9 Hz, 1H, H-6), 7.47 (d, *J* = 7.5 Hz, 2H, H-8'), 7.41 (t, *J* = 7.5 Hz, 2H, H-9'), 7.35 (t, *J* = 7.5 Hz, 1H, H-10'), 7.16 (d, *J* = 8.8 Hz, 2H, H-4'), 6.88 (d, *J* = 3.9 Hz, 1H, H-7), 5.21 (s, 2H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 161.37 (C-1'), 161.07 (C-5'), 152.03 (C-4), 151.91 (C-5), 136.53 (C-3/7'), 128.46 (C-9'), 127.96 (C-3'), 127.76 (C-8'), 124.96 (C-2'/10'), 115.12 (C-7), 114.66 (C-6), 114.68 (C-4'), 69.44 (C-6'). HRMS-APCI (*pos*) *m/z* 366.1092 $[M + H]^+$ (calcd for $C_{19}H_{16}N_3O_5^+$, 366.1090).

***(E)-4-[(4-methylbenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide* (2b)**

Yellow powder; yield: 54%; mp: 226-229 °C (EtOAc); IR υ_{max} (cm^{-1}): 3247 (N-H), 1666 (C=O), 1580 (C=N), 1347 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 12.06 (s, 1H, H-1), 8.39 (s, 1H, H-3), 7.90 (d, *J* = 8.0 Hz, 2H, H-3'), 7.79 (d, *J* = 3.9 Hz, 1H, H-6), 7.35 (d, *J* = 8.0 Hz, 2H, H-4'), 7.25 (d, *J* = 7.8

Hz, 2H, H-9'), 7.20 (d, *J* = 7.8 Hz, 2H, H-8'), 7.14 (d, *J* = 3.9 Hz, 1H, H-7), 5.15 (s, 2H, H-6'), 2.31 (s, 3H, H-11'). ^{13}C NMR (151 MHz, DMSO) δ 161.43 (C-1'), 161.12 (C-5'), 152.06 (C-4), 151.95 (C-5), 137.26 (C-3), 133.49 (C-3'), 129.02 (C-9'), 127.88 (C-8'), 124.90 (C-10'), 115.14 (C-7), 114.99 (C-6), 114.70 (C-4'), 69.37 (C-6'), 20.76 (C-11'). HRMS-APCI (*pos*) *m/z* 380.1234 $[M + H]^+$ (calcd for $C_{20}H_{18}N_3O_5S^+$, 380.1246).

***(E)-4-[(4-isopropylbenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide* (2c)**

Yellow powder; yield: 50%; mp: 213-216 °C (EtOAc); IR υ_{max} (cm^{-1}): 3243 (N-H), 1656 (C=O), 1556 (C=N), 1308 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 12.04 (s, 1H, H-1), 8.39 (s, 1H, H-3), 7.91 (d, *J* = 8.8 Hz, 2H, H-3'), 7.79 (d, *J* = 3.9 Hz, 1H, H-6), 7.38 (d, *J* = 8.8 Hz, 2H, H-9'), 7.27 (d, *J* = 8.8 Hz, 2H, H-4'), 7.25 (d, *J* = 3.9 Hz, 1H, H-7), 7.15 (d, *J* = 8.8 Hz, 2H, H-8'), 5.16 (s, 2H, H-6'), 2.98 (ddd, *J* = 20.7, 13.9, 6.8, 1H, H-11'), 1.21 (s, *3*H, H-12') 1.20 (s, 3H, H-13'). ^{13}C NMR (151 MHz, DMSO) δ 161.46 (C-1'), 161.17 (C-5'), 152.07 (C-4), 151.96 (C-5), 148.26 (C-10'), 133.90 (C-3), 127.98 (C-3'), 126.37 (C-8'), 124.90 (C-9'), 115.15 (C-4'), 114.80 (C-7), 114.63 (C-6), 69.37 (C-6'), 33.18 (C-11'), 23.84 (C-12'), 23.84 (C-13'). HRMS-APCI (*pos*) *m/z* 408.1568 $[M + H]^+$ (calcd for $C_{22}H_{22}N_3O_5^+$, 408.1559).

***(E)-4-[(4-{tert-butyl)benzyl]oxy}-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide* (2d)**

Yellow powder; yield: 54%; mp: 197-200 °C (EtOAc); IR υ_{max} (cm^{-1}): 3280 (N-H), 1655 (C=O), 1558 (C=N), 1308 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 12.02 (s, 1H, H-1), 8.38 (s, 1H, H-3), 7.91 (d, *J* = 8.7 Hz, 2H, H-3'), 7.79 (d, *J* = 3.8 Hz, 1H, H-6), 7.42 (d, *J* = 8.7 Hz, 2H, H-9'), 7.39 (d, *J* = 8.7 Hz, 2H, H-4'), 7.25 (d, *J* = 3.8 Hz, 1H, H-7), 7.15 (d, *J* = 8.7 Hz, 2H, H-8'), 5.16 (s, 2H, H-6'), 1.28 (s, 9H, H-12'/13'/14'). ^{13}C NMR (151 MHz, DMSO) δ 161.46 (C-1'), 161.20 (C-5'), 152.08 (C-4), 151.97 (C-5), 150.49 (C-10'), 133.53 (C-3), 132.87 (C-7'), 127.70 (C-3'), 125.21 (C-8'), 124.91 (C-9'), 123.10 (C-2'), 115.16 (C-4'), 114.70 (C-6), 114.63 (C-7), 69.27 (C-6'), 34.29 (C-11'), 31.10 (C-12'/13'/14'). HRMS-APCI (*pos*) *m/z* 422.1716 $[M + H]^+$ (calcd for $C_{23}H_{24}N_3O_5^+$, 422.1716).

***(E)-4-[(4-fluorobenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide* (2e)**[44]

Yellow powder; yield: 38%; mp: 198-201 °C (EtOAc); IR υ_{max} (cm^{-1}): 3209 (N-H), 1662 (C=O), 1557 (C=N), 1347 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 12.02 (s, 1H, H-1), 8.38 (s, 1H, H-3), 7.92 (d, *J* = 8.8 Hz, 2H, H-3'), 7.79 (d, *J* = 3.9 Hz, 1H, H-6), 7.53 (dd, J_{H-H} = 8.6, $^3J_{H-F}$ = 5.6 Hz, 2H, H-8'), 7.24 (*d, *J* = 8.6 Hz, 2H, H-9'), 7.16 (d, *J* = 8.8 Hz, 2H, H-4'), 6.87 (d, *J* = 3.9 Hz, 1H, H-7), 5.19 (s, 2H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 161.82 (d, $^1J_{C-F}$ = 244.0 Hz, C-10'), 161.27 (C-1'), 161.04 (C-5'), 152.07 (C-4), 151.94 (C-5), 132.78 (C-3), 130.12 (C-7'), 130.08 (d, $^3J_{C-F}$ = 8.3 Hz

C-8'), 125.05 (C-2'), 115.29 (d, $^{2}J_{C\text{-}F}$ = 21.4 Hz, C-9'), 115.23 (C-4'), 114.73 (C-6, C-7), 68.73 (C-6'). HRMS-APCI (*pos*) *m/z* 384.0993 $[M + H]^+$ (calcd for $C_{19}H_{15}FN_3O_5^+$, 384.0996).
*Coalesced doublet

***(E)-4-[(4-bromobenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide* (2f)**

Yellow powder; yield: 48%; mp: 215-218 °C (EtOAc); IR υ_{max} (cm^{-1}): 3276 (N-H), 1657 (C=O), 1558 (C=N), 1316 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 12.10 (s, 1H, H-1), 8.39 (s, 1H, H-3), 7.91 (d, *J* = 8.0 Hz, 2H, H-3'), 7.79 (d, *J* = 3.7 Hz, 1H, H-6), 7.60 (d, *J* = 8.3 Hz, 2H, H-9'), 7.43 (d, *J* = 8.3 Hz, 2H, H-8'), 7.25 (d, *J* = 8.0 Hz, 1H, H-4'), 7.15 (d, *J* = 3.7 Hz, 1H, H-7), 5.20 (s, 2H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 161.18 (C-1'), 161.12 (C-9'), 152.06 (C-4), 151.93 (C-5), 136.05 (C-3), 134.89 (C-7'), 131.41 (C-9'), 129.88 (C-8'), 125.12 (C-3'), 123.16 (C-10'), 121.10 (C-2'), 115.14 (C-7), 115.02 (C-6), 114.70 (C-4'), 68.63 (C-6'). HRMS-APCI (*pos*) *m/z* 444.0171 $[M + H]^+$ (calcd for $C_{19}H_{15}BrN_3O_5^+$, 444.0195).

***(E)-4-[(4-bromo-2-fluorobenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide* (2g)**

Yellow powder; yield: 61%; mp: 224-227 °C (EtOAc); IR υ_{max} (cm^{-1}): 3278 (N-H), 1657 (C=O), 1579 (C=N), 1318 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 12.07 (s, 1H, H-1), 8.39 (s, 1H, H-3), 7.93 (d, *J* = 8.8 Hz, 2H, H-3'), 7.81 (d, *J* = 3.8 Hz, 1H, H-6), 7.63 (dd, $J_{H\text{-}H}$ = 9.6 Hz, $^{4}J_{H\text{-}F}$ = 1.8 Hz, 1H, H-11'), 7.55 (*d, *J* = 8.1 Hz, 1H, H-9'), 7.48 (dd, $J_{H\text{-}H}$ = 8.2, $^{3}J_{H\text{-}F}$ = 1.8 Hz, 1H, H-12'), 7.25 (d, *J* = 3.8 Hz, 1H, H-7), 7.18 (d, *J* = 8.8 Hz, 2H, H-4'), 5.22 (s, 2H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 162.71 (d, $^{1}J_{C\text{-}F}$ = 245.4 Hz, C-8'), 161.11 (C-1'), 161.01 (C-5'), 159.45 (C-4), 151.95 (C-5), 132.18 (d, $^{3}J_{C\text{-}F}$ = 4.7 Hz, C-12'), 129.91 (C-3), 127.70 (d, $^{4}J_{C\text{-}F}$ = 3.5 Hz, C-11'),125.36 (C-3'), 123.06 (C-10'), 118.93 (d, $^{2}J_{C\text{-}F}$ = 24.6 Hz, C-9'),119.01 (C-6), 118.85 (C-7), 114.61 (C-4'), 63.37 (C-6').). HRMS-APCI (*pos*) *m/z* 462.0078 $[M + H]^+$ (calcd for $C_{19}H_{14}BrFN_3O_5^+$, 462.0101).
*Coalesced doublet

***(E)-4-[(4-nitrobenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide* (2h)**

Yellow powder; yield: 49%; mp: 205-208 °C (EtOAc); IR υ_{max} (cm^{-1}): 3280 (N-H), 1665 (C=O), 1559 (C=N), 1353 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 12.06 (s, 1H, H-1), 8.39 (s, 1H, H-3), 8.27 (d, *J* = 8.7 Hz, 2H, H-9'), 7.93 (d, *J* = 8.7 Hz, 2H, H-3'), 7.79 (d, *J* = 3.9 Hz, 1H, H-6), 7.74 (d, *J* = 8.7 Hz, 2H, H-8'), 7.24 (d, *J* = 3.9 Hz, 1H, H-7), 6.88 (d, *J* = 8.7 Hz, 2H, H-4'), 5.39 (s, 2H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 161.14 (C-1'), 160.95 (C-5'), 152.07 (C-4), 151.91 (C-5), 147.10 (C-10'), 144.47 (C-7'), 129.87 (C-3), 128.33 (C-3'), 125.39 (C-2'), 123.65 (C-8'), 123.15 (C-9'), 115.15

(C-4'), 114.82 (C-7), 114.73 (C-6), 68.23 (C-6'). HRMS-APCI (*pos*) *m/z* 411.0929 $[M + H]^+$ (calcd for $C_{19}H_{15}N_4O_7^+$, 411.0941).

5. Biological screening

5.1 Cytotoxicity assay

The cytotoxicity of the synthesised NFX derivatives was evaluated using the resazurin assay. The assay involves the irreversible enzymatic reduction of oxidised blue resazurin dye to pink, highly fluorescent resorufin by viable cells[58]. This non-toxic reagent serves as an effective tool for assessing cell proliferation and drug toxicity.

Vero cells were cultured using Hyclone Dulbecco's modified Eagle's medium with high glucose (Separations). The medium was supplemented with 10% fetal bovine serum (FBS) (Thermofisher Scientific, South Africa) and 1% L-glutamine, penicillin-streptomycin and non-essential amino acids (Lonza). The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. For the resazurin assay, 96 well plates were prepared using 100 µL of a 60 000 cells/mL suspension and incubated for 24 hours. After the incubation period, the cells were treated with 100 µL of emetine dihydrochloride solution (Sigma Aldrich) diluted with growth medium to the necessary concentrations (positive control), 80 µL of growth medium and 20 µL of solvent (negative control), or 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.

The resazurin assay was initiated by adding 50 µL of sterile-filtered resazurin sodium salt (Sigma Aldrich) solution (0.01% in phosphate-buffered saline (PBS) and incubating the plates for 2 hours. The Thermofisher Scientific GO Multiscan plate reader was used to measure absorbance at 570 and 600 nm. SkanIt 4.0 Research Edition software was used to analyse data for each biological replicate. Background absorbance (600 nm) was subtracted from absorbance values (570 nm), the mean absorbance calculated, and the percentage cell viability was determined by the following equation:

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

The IC_{50} and Z-score were determined for each compound's biological replicate using GraphPad Prism 5. For the final IC_{50} of each compound, the mean IC_{50} of the biological replicates were calculated with standard deviation (SD).

5.2 Antileishmanial assay

The anti-promastigote activity of the synthesised NFX derivatives were evaluated using the resazurin assay and three *Leishmania* strains. A modified method of Kulshrestha *et al.* (2013) and Siqueira-Neto *et al.* (2010) was used.

The promastigote form of *L. donovani* (strains 1S (MHOM/SD/62/1S) and 9515 (MHOM/IN/95/9515)) and *L. major* (strain IR-173 (MHOM/IR/-173)) were cultured in M199 with Hank's salts and 0.68 mM L-glutamine (Sigma Aldrich) supplemented with 4.2 mM sodium bicarbonate, 25 mM Hepes, 0.0005% hemin, 0.0001% biotin, 0.1 mM adenine (Sigma Aldrich), 10% FBS 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 (1.25×10^6 cells/mL, final volume 100 µL/well) were seeded in 96 well plates (Nunc, Thermofisher Scientific) in the presence of either 10 µM of compound for activity screening or seven two-fold dilution concentrations of compounds for IC_{50} determination, with a starting concentration of 10 µM. Amphothericin B (10 µM) was chosen as the standard drug and growth medium without parasites served as the blank. The plates were incubated for 48 hours at 26 ˚C in humidified atmosphere.

After incubation, 50 µL of resazurin solution (0.01% in PBS) was added to each well and the plates were further incubated at 26 ˚C in the dark for 24 hours. The Thermofisher Scientific GO Multiscan plate reader was used to measure absorbance at 570 nm and 600 nm. 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).

For the single-point activity screening, the following equation was used to determine growth inhibition percentage:

Growth inhibition % = 100 – [(Δ Abs sample – Δ Abs blank) / (Δ Abs neg control – Δ Abs blank) X 100]

Compounds that showed growth inhibition of >70% qualify for further IC_{50} evaluation[60]. To calculate the IC_{50}, cell viability was determined using the following equation:

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

The IC_{50} and Z-score were determined for each compound's three biological replicates using the cell viability % values and GraphPad Prism 5. The mean IC_{50}, with standard deviation, of the biological replicates served as the final IC_{50} of each compound.

Abbreviations

AMB: Amphotericin B; CL: Cutaneous leishmaniasis; cNFs: clinical nitrofurans; EDG: electron donating group; DMF: anhydrous dimethylformamide; EM: emetine; EWG: electron withdrawing groups; FBS: fetal bovine serum; FZD: furazolidone; IM: intramuscular; IV: intravenous; *L.*: *Leishmania*; MCL: mucocutaneous leishmaniasis; NFs: nitrofurans; NFA: 5-nitro-2-furaldehyde; NFT: nitrofurantoin; NFX: Nifuroxazide; NFZ: nitrofurazone; NMR: nuclear magnetic resonance; NTD: neglected tropical diseases; PBS: phosphate-buffered saline; RNS: reactive nitrogen species; ROS: reactive oxygen species; SD: standard deviation; TEA: triethylamine; TLC: thin layer chromatography; VL: visceral leishmaniasis; WHO: World Health Organization.

Author contributions

Conceptualization: [David D. N'Da]; Methodology: [Gideon Badenhorst, Christina Kannigadu, Janine Aucamp]; Formal analysis and investigation: [David D. N'Da, Gideon Badenhorst, Christina Kannigadu and Janine Aucamp]; Writing - original draft preparation: [Gideon Badenhorst]; Writing - review and editing: [David D. N'Da]; Funding acquisition: [David D. N'Da]; Resources: [David D. N'Da]; Supervision: [David D. N'Da].

Declaration of Competing Interests

The authors declare that they have no competing interest

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Leishmania donovani, Strain 1S (MHOM/SD/62/1S), NR-48821
Leishmania donovani, Strain 9515 (MHOM/IN/95/9515), NR-48822
Leishmania major, Strain IR173 (MHOM/IR/-173), NR-48816

Availability of data and materials

Supporting information can be found in the Supplementary Information

Ethics approval

Ethics approval for this study was obtained from the Human Research Ethics Committee of the North-West University (NWU-00445-20-A1).

Disclaimer

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

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CHAPTER 4

Summary and Conclusion

Leishmaniasis is a parasitic disease caused by infection with *Leishmania* parasites and is spread through the bite of infected *Phlebotomus* sandflies (Hailu *et al.*, 2016; WHO, 2021a). This neglected tropical disease (NTD) is endemic to tropical and sub-tropical regions of more than 90 countries (CDC, 2020; Sangenito *et al.*, 2019) and can manifest in five clinical forms consisting of: the most common cutaneous leishmaniasis (CL), the least common mucocutaneous leishmaniasis (MCL), the rare diffuse cutaneous leishmaniasis (DCL), the deadly visceral leishmaniasis (VL) (kala-azar) and post-kala-azar dermal leishmaniasis (PKDL) (Ehab Kotb *et al.*, 2014). The WHO estimates that approximately 700 000 to 1 million new cases of leishmaniasis occurs worldwide on an annual basis and it was also found that this disease accounts for the third highest zoonotic infections worldwide (Pisarski, 2019; WHO, 2021a).

Leishmania has two developmental forms, promastigote found in the vector (the female sandfly) and amastigote located in the human host. The latter is responsible for the clinical symptoms of the infection hence is the most relevant in the discovery of new antileishmanial drugs.

Leishmaniasis can be treated with a limited amount of chemotherapy drugs namely, pentavalent antimonials, amphotericin B, paromomycin, pentamidine and miltefosine (Roatt *et al.*, 2020; Sundar *et al.*, 2019). However, there are several limitations regarding the use of these drugs such as toxicity, high cost, and invasive parenteral administration with the exception of miltefosine that is delivered orally. Furthermore, the overuse of these drugs has led to the emergence of parasite resistance (Freitas-Junior *et al.*, 2012). Hence, there is an need for the development of new, affordable and effective antileishmanial agents (Zulfiqar *et al.*, 2017).

The use of clinical nitrofurans (cNFs) such as nifuroxazide (NFX), nitrofurantoin (NFT) and furazolidone (FZD), to treat numerous infectious diseases has been well established over the years (Pires *et al.*, 2001; Zuma *et al.*, 2019). These nitrofurans have shown activity against bacterial, mycobacterial and parasitic infections (Elsaman *et al.*, 2019; Kamal *et al.*, 2015; Zuma *et al.*, 2019). Their activities have been attributed to the generation of free radical species (reactive oxygen species ROS and reactive nitrogen species RNS) that are produced by the nitro group, which reacts with the pathogen cell wall enzymes and become lethal to these microorganisms (Pal & Bandyopadhyay, 2011). However, this group is also a source of toxicity (Gallardo-Garrido

et al., 2020). In addition to the reactive nitro group, molecules of cNFs also contain a hydrazone moiety with nitro activating effects (Trukhacheva *et al.*, 2005).

NFX is currently used as an antibiotic for the treatment of diarrhoea and colitis (Luo *et al.*, 2019). This drug shows noteworthy activity against both gram-positive and negative bacteria without affecting the normal intestinal flora (Luo *et al.*, 2019; Zuma *et al.*, 2019). Studies have shown that NFX has antiparasitic effects against a variety of invasive species, including *Leishmania*, (Kaiser *et al*., 2015; Zhao *et al*., 2020) and its biological action occurs through the generation of ROS and RNS (Bailly, 2019) which induces oxidative stress a and ultimately causes parasite death.

However, the intestinal absorption of NFX is limited with a bioavailability of 50% (Kalia & Raines, 2008; Santiago *et al.*, 1985). The limited absorption is due to the metabolism of NFX in the intestinal track (B. Fernandes *et al.*, 2015; Labaune *et al.*, 1986). Other shortcomings of NFX include toxic effects such as protein carbonylation, DNA breakage, enzyme inactivation and inflammatory reactions (Liu *et al.*, 2017; Patel *et al.*, 2018). Through structural modification and pharmacophore hybridisation, these shortcomings may be reduced upon (Agarwal *et al.*, 2017; Zhang *et al.*, 2019). Taking the anti-infective effects and shortcomings of NFX into consideration, the viability of the compound to act as a parent drug for the development of a new antiparasitic drug is promising (Petri e Silva *et al.*, 2016).

Research indicates that the incorporation of sulfonyl or benzyl groups into the molecular structures of compounds results in derivatives that have unique and strong interaction modes with target proteins, and these groups are able to introduce chemical stability, diverse reactivity paths and the possible enhancement of biological activities (Shakhatreh *et al.*, 2016; Zhao *et al.*, 2019). Thus, the main objective of this study was to investigate the in vitro antileishmanial activity and safety of synthesized NFX-based sulfonyl and benzylated analogues.

The analogues were synthesised in a single-step type-2 nucleophilic substitution reaction by reacting NFX with various substituted benzyl bromides or sulfonyl chlorides in anhydrous dimethylformamide (DMF) basified with the addition of Triethylamine (TEA) or anhydrous potassium carbonate (K_2CO_3). They were isolated in moderate to good yields (25-81%) after recrystallisation with ethyl acetate:*n*-hexane (1:4, v/v). The molecular structures of all the analogues were confirmed using NMR, HRMS and IR characterization techniques.

These analogues were thereafter evaluated for *in vitro* cytotoxicity on Vero cells and screened for *in vitro* antileishmanial activity against promastigotes of three *Leishmania* strains namely *L. donovani* (strains 1S and 9515) which causes the deadly and debilitating VL, and *L. major* (strain IR-173), which causes CL (Ready, 2014; Remadi *et al.*, 2016).

The most active analogues in the sulfonyl series were **1f**, **1h** and **1k**, showing no cytotoxicity (100 µM > IC_{50}), selectivity index of above 100 and sub-micromolar antileishmanial activity against all three strains with IC_{50} values ranging between 0.26 µM and 0.84 µM. In the benzyl series, the best performers were analogues **2c** and **2d**, showing moderate to low cytotoxicity (IC_{50} = 30-65 µM), selectivity index of above 100 and sub-micromolar antileishmanial activity against all three strains with IC_{50} values found in the 0.09 - 0.25 µM range. Based on the criteria set out by Katsuno *et al.* (Katsuno *et al.*, 2015), it was established that analogues **1d**, **1e**, **1f**, **1g**, **1h**, **1j**, **1k, 1l**, **2b**, **2c**, **2d**, **2e**, **2f** and **2g** all showed promise as possible anti-promastigote hit/lead compounds due to their high selective activity and low cytotoxicity.

Furthermore, in both series, the *tert*-butyl substituents (**1h**, **2d**) performed extremely well with sub-micromolar antileishmanial activity and high selectivity indexes against all three strains of *Leishmania*. This may be attributed to the strength of electron donating nature of the group which allows it to undergo activation of the nitro group *via* nitroreduction by either a single or double electron transfer.

In summary, the NFX-based analogues obtained in a simple one-step synthetic route in moderate to good yields from the commercial parent drug and showed good to excellent antileishmanial activity with minimal mammalian cytotoxicity. Both the sulfonyl and benzylated analogues proved to be potent with both series resulting in anti-promastigote hits. Further testing is required; thus, the current synthesised analogues will be screened against the amastigote form of *Leishmania* to confirm their potential as possible antileishmanial hit compounds.

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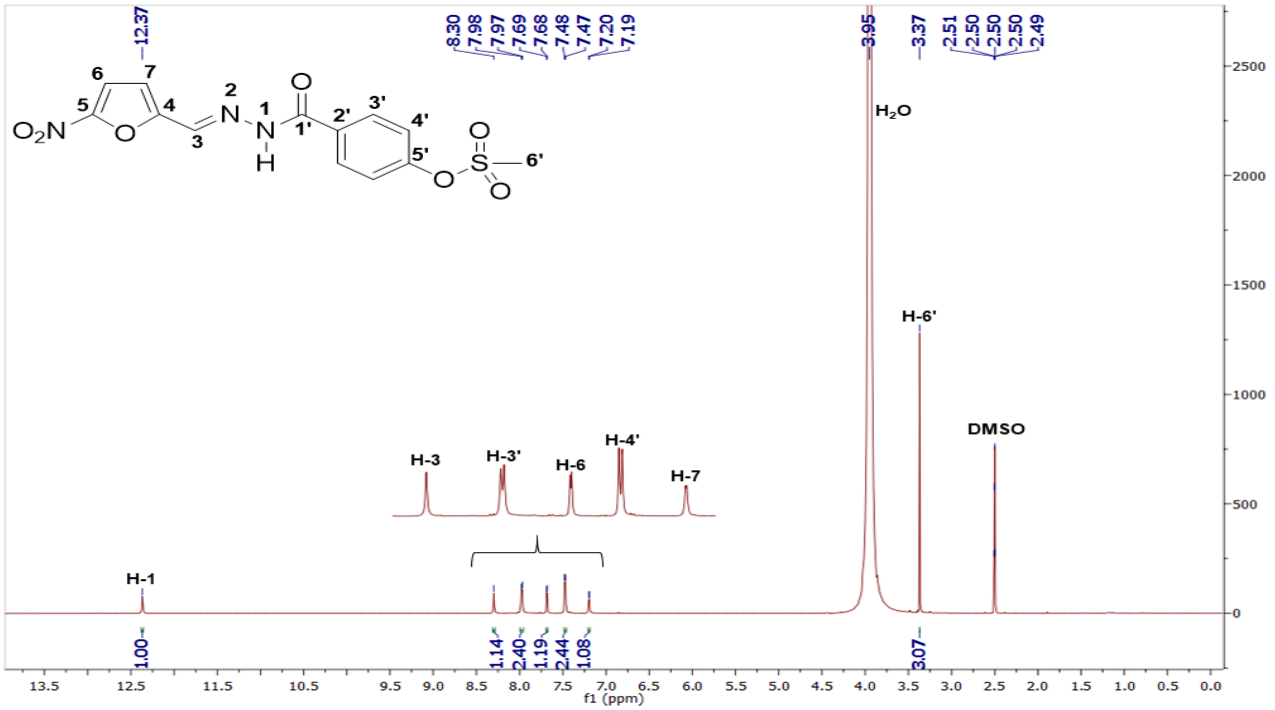
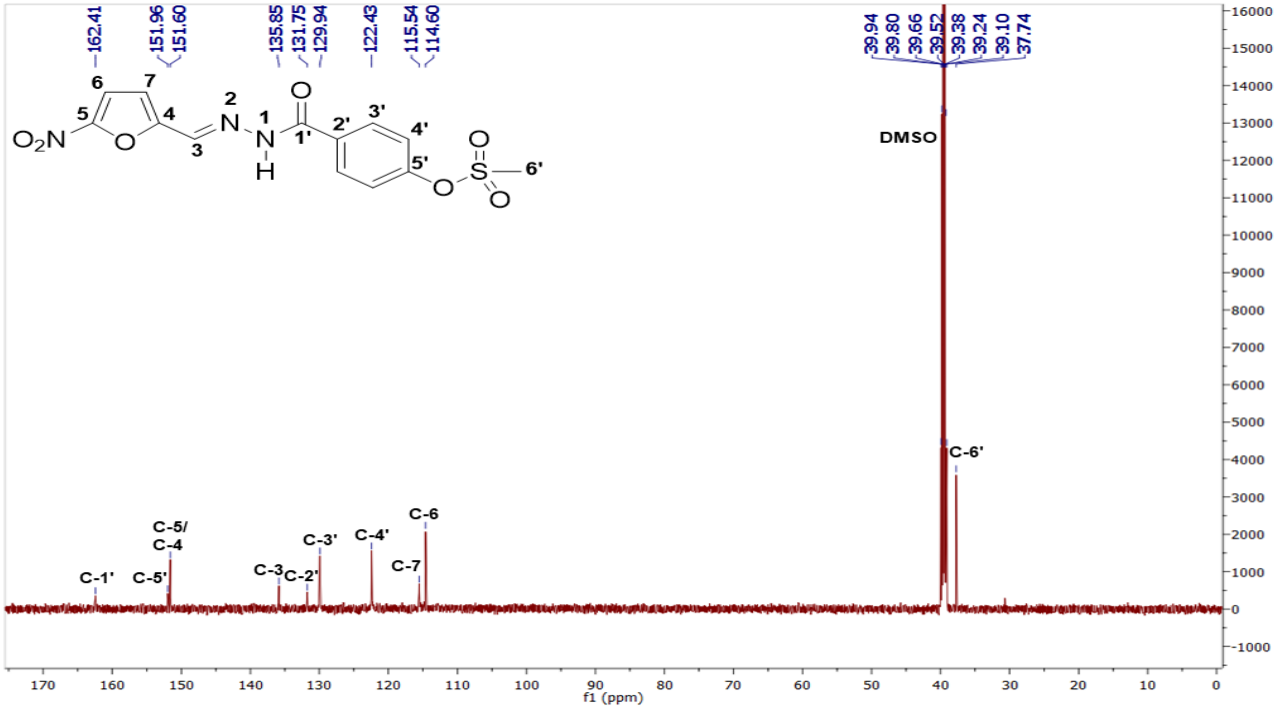
Annexure A: Analytical Data for Chapter 3

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl methanesulfonate (1a)**

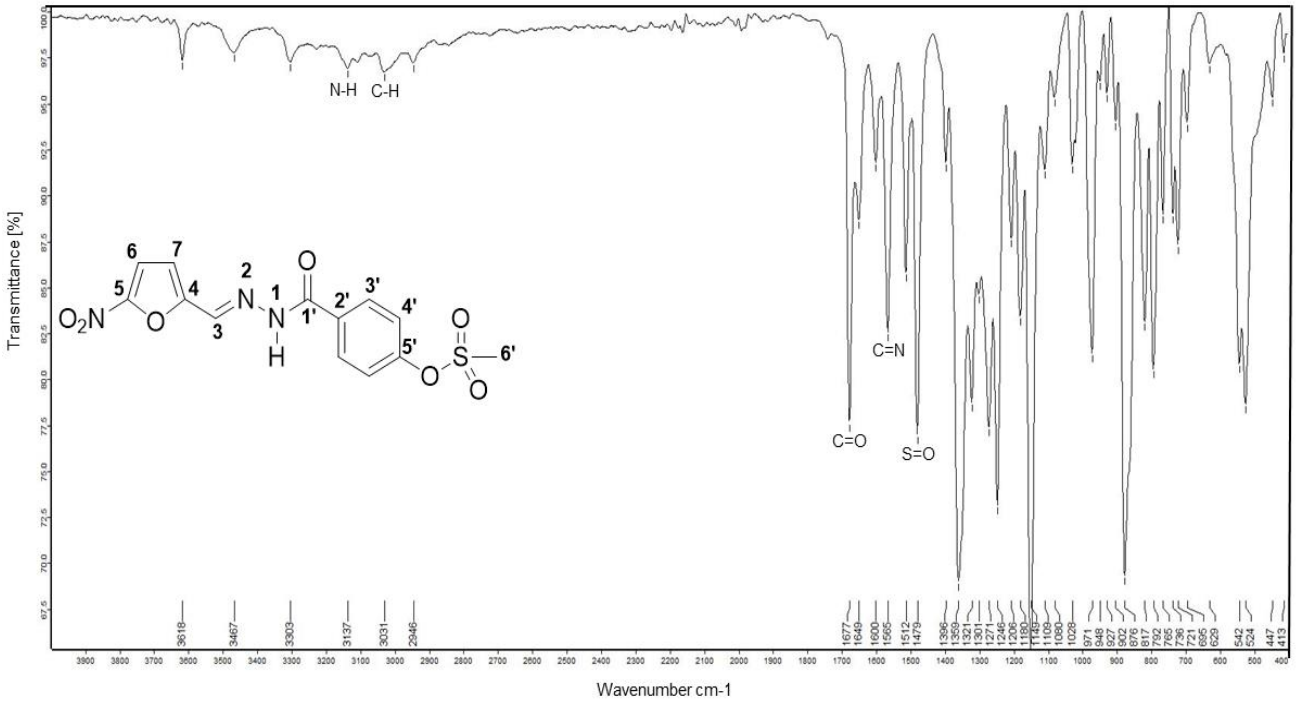
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

Acquisition Parameter

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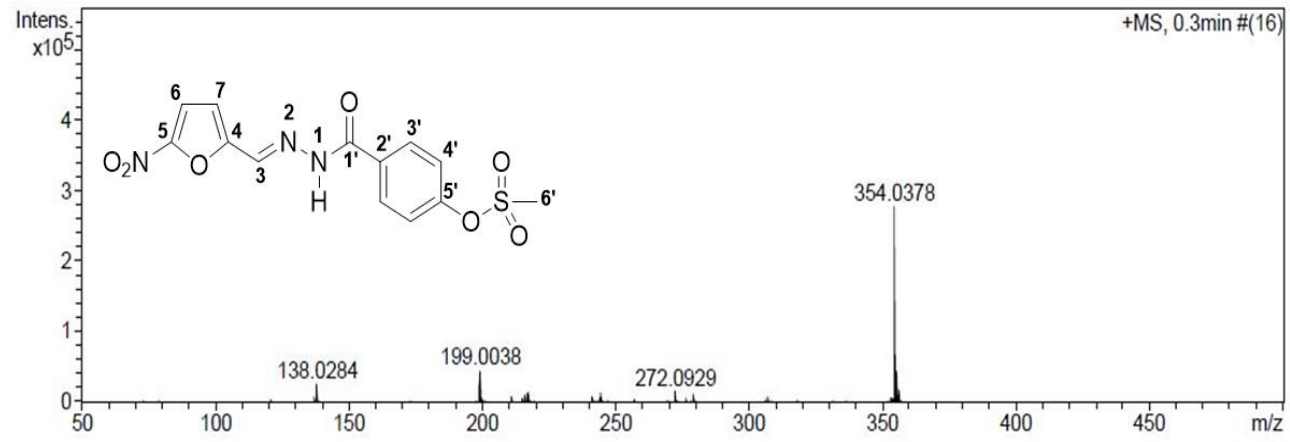
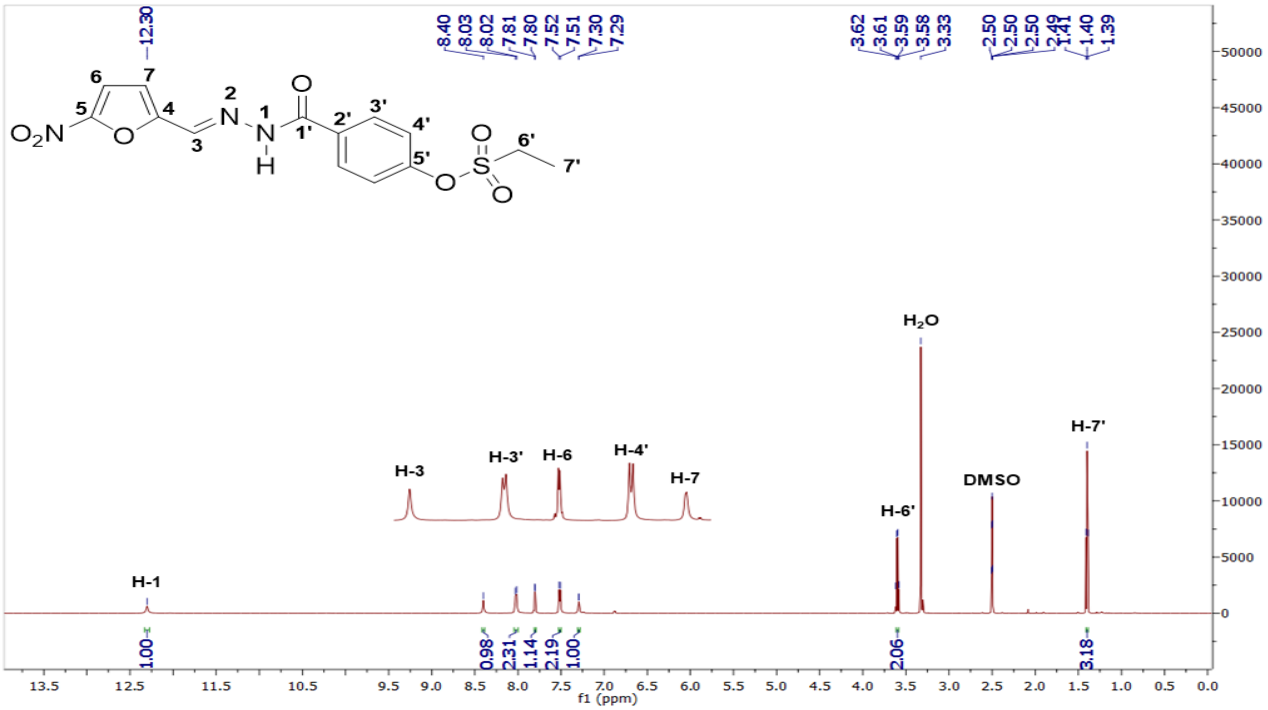
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



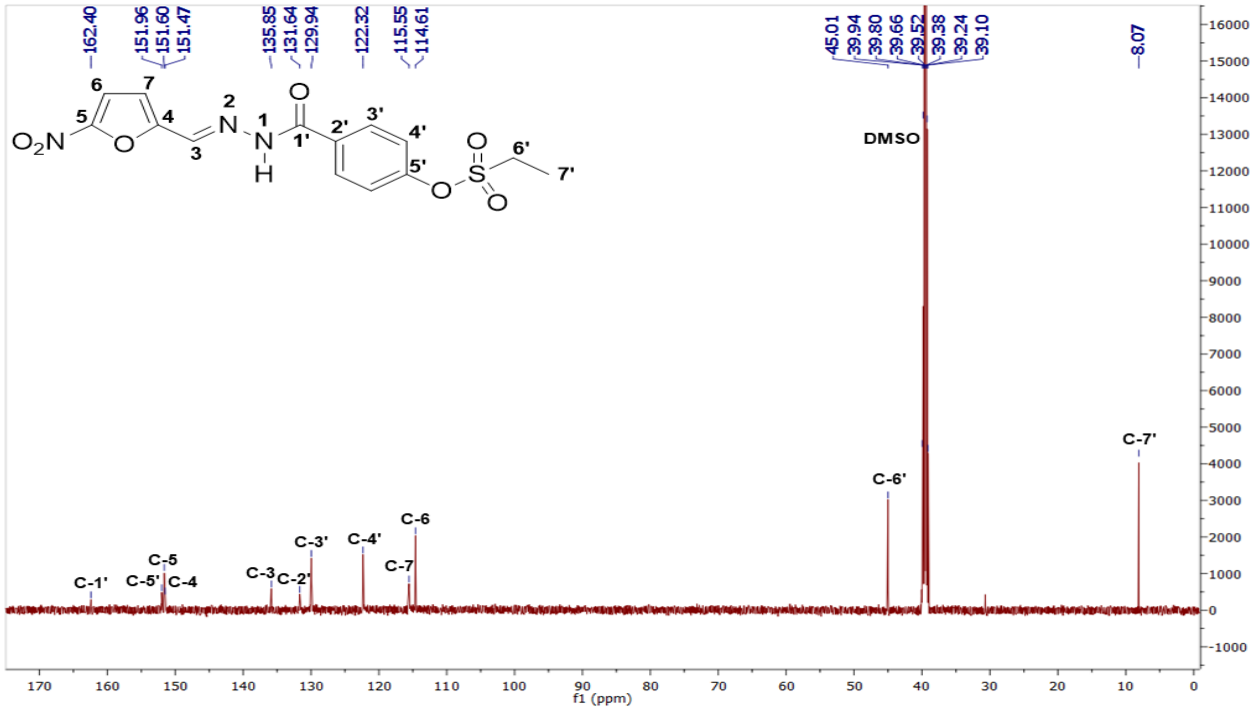
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
354.0378	1	C 13 H 12 N 3 O 7 S	100.00	354.0390	1.3	3.6	1.7	9.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl ethanesulfonate (1b)**

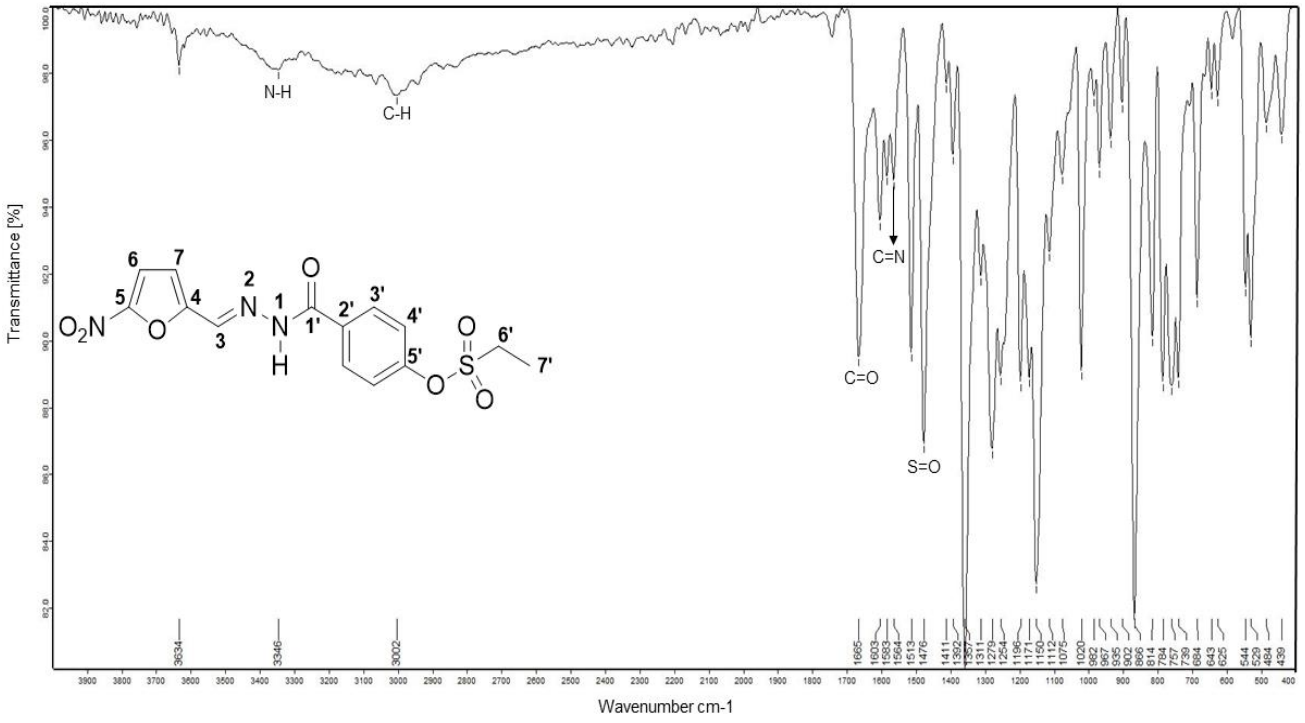
^{1}H NMR in DMSO



^{13}C NMR in DMSO



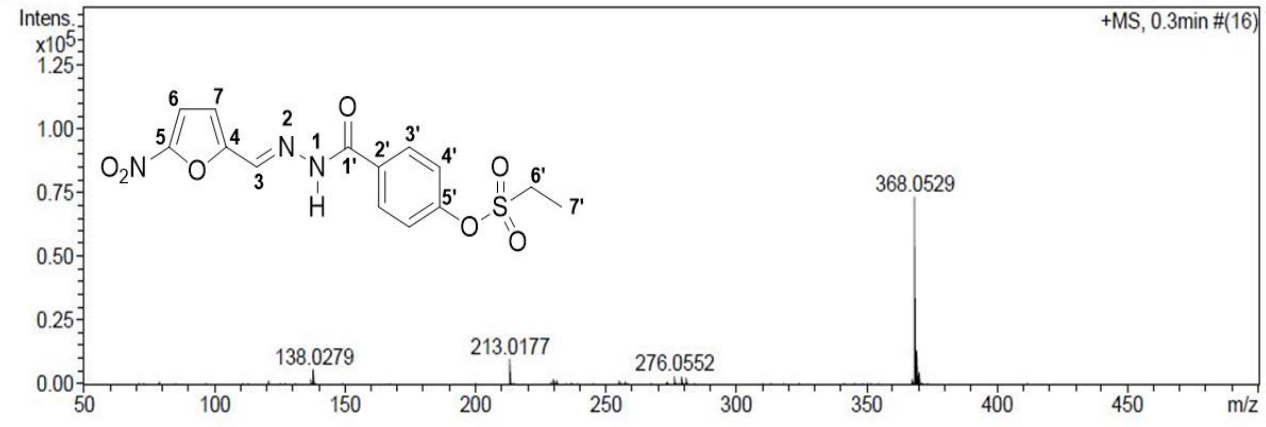
IR Spectrum



HRMS

Acquisition Parameter

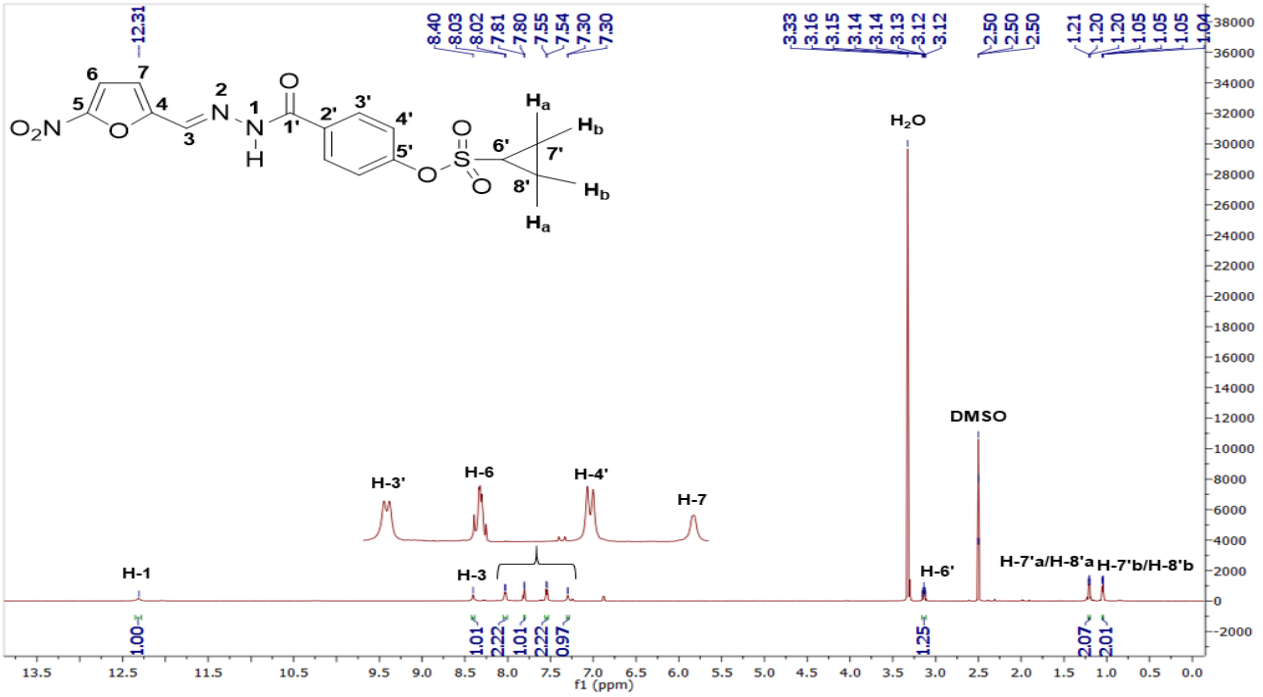
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



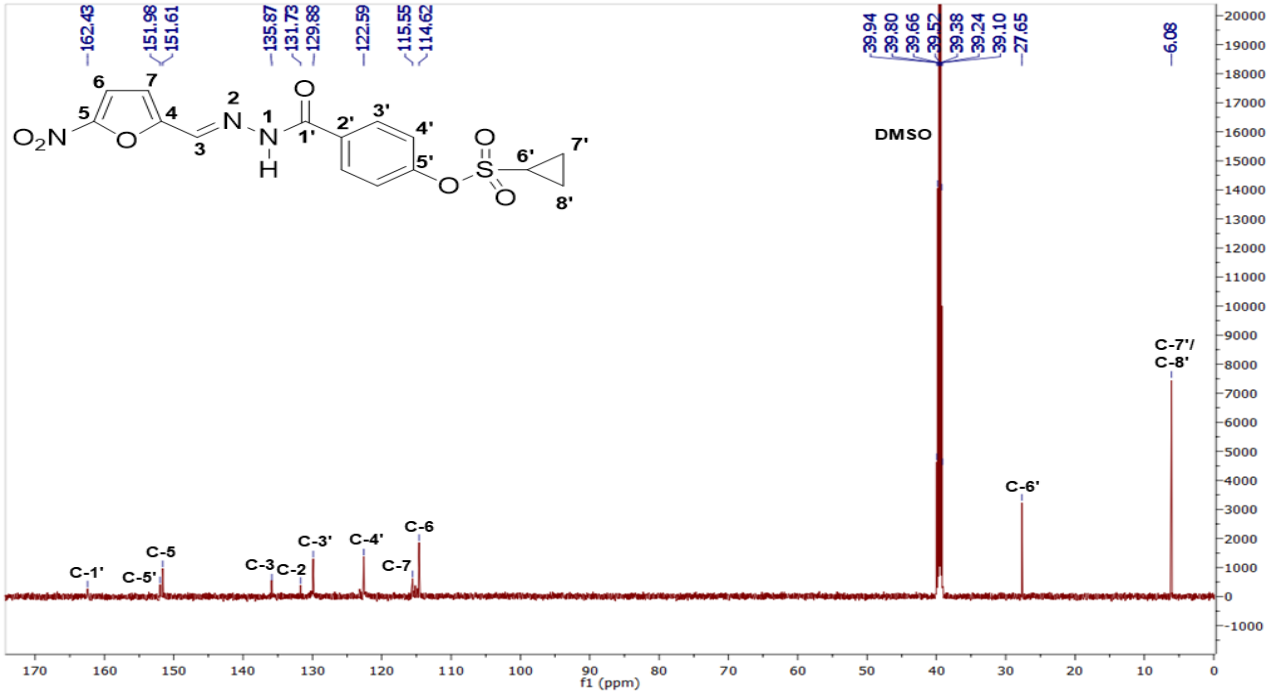
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
368.0529	1	C 14 H 14 N 3 O 7 S	100.00	368.0547	1.8	4.9	7.6	9.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl cyclopropanesulfonate (1c)**

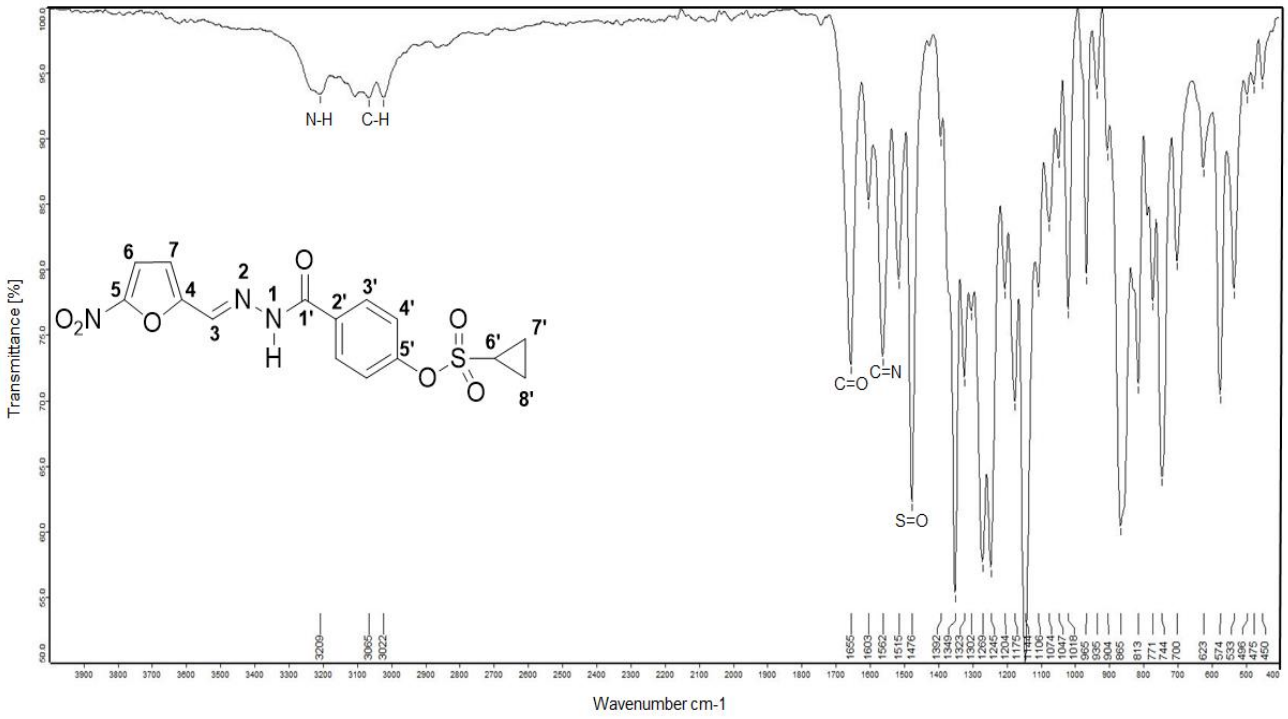
^{1}H NMR in DMSO



^{13}C NMR in DMSO



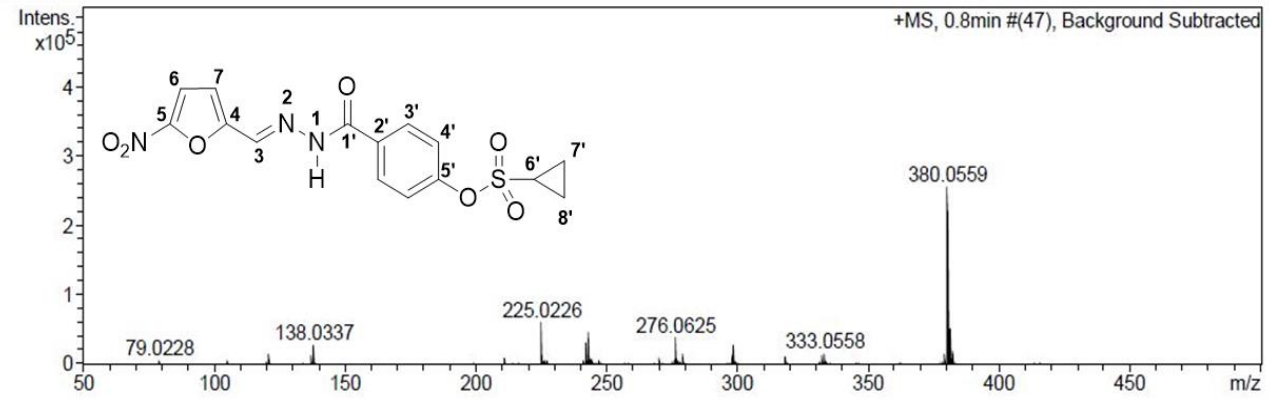
IR Spectrum



HRMS

Acquisition Parameter

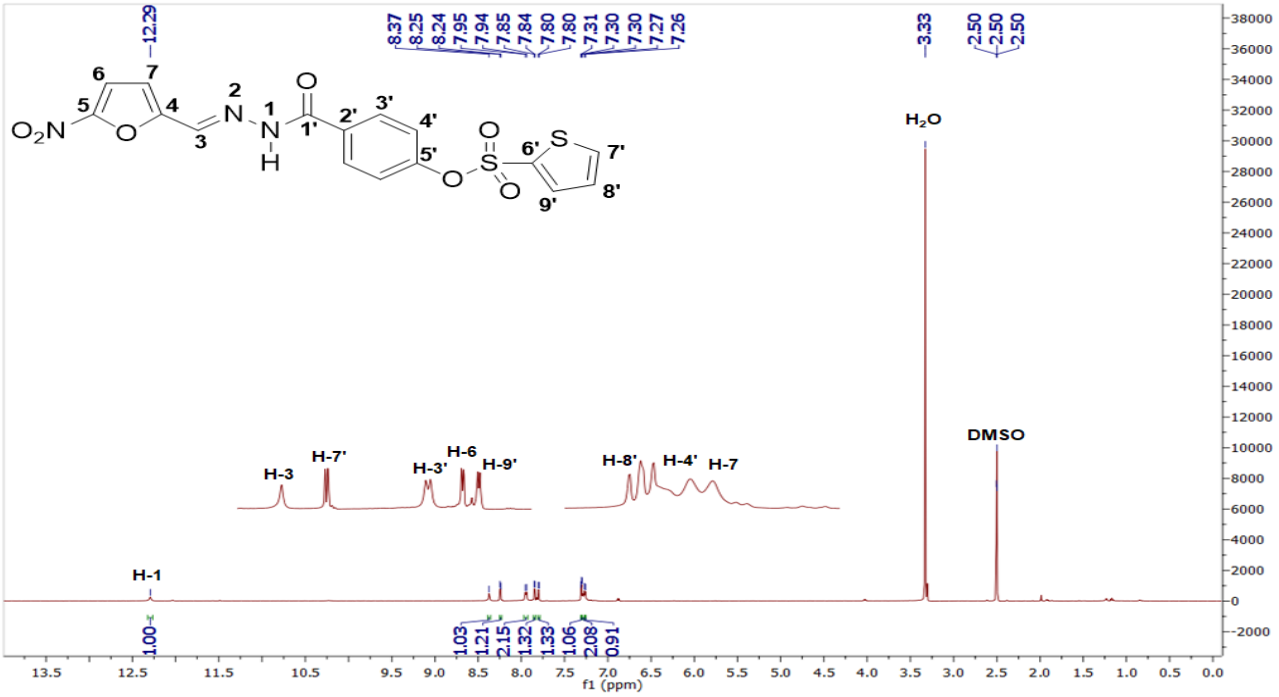
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



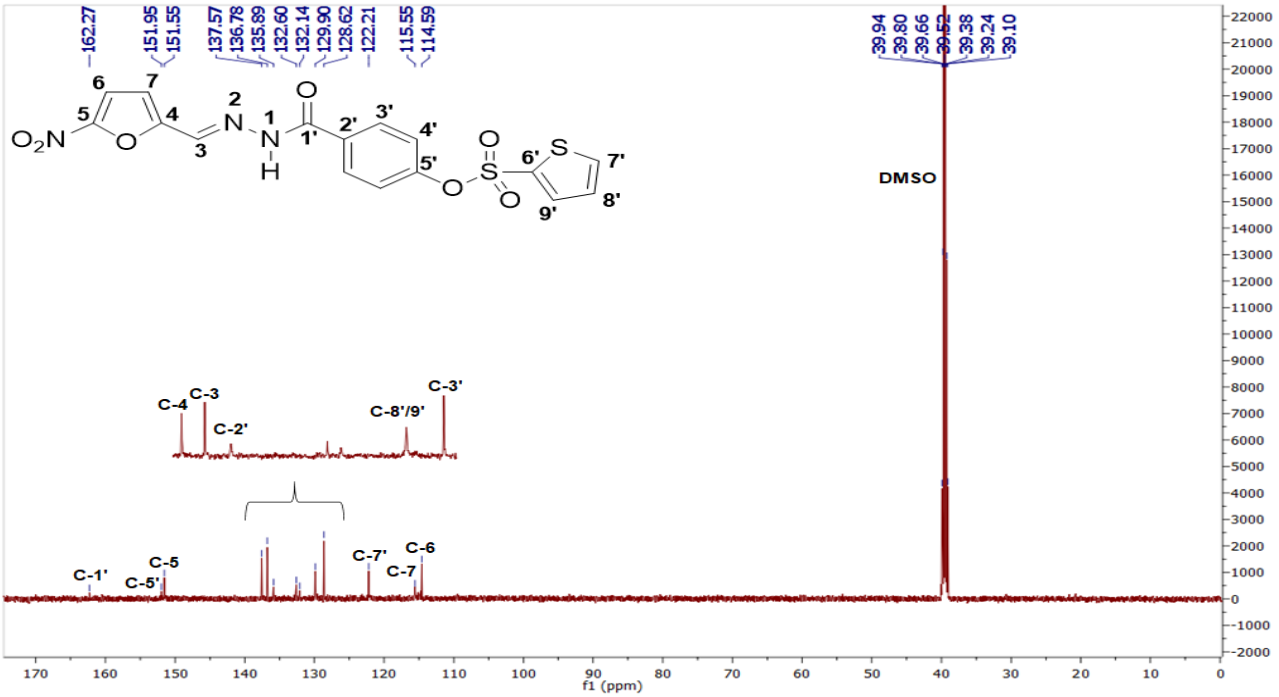
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
380.0559	1	C 15 H 14 N 3 O 7 S	100.00	380.0547	-1.2	-3.1	11.2	10.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl thiophene-2-sulfonate (1d)**

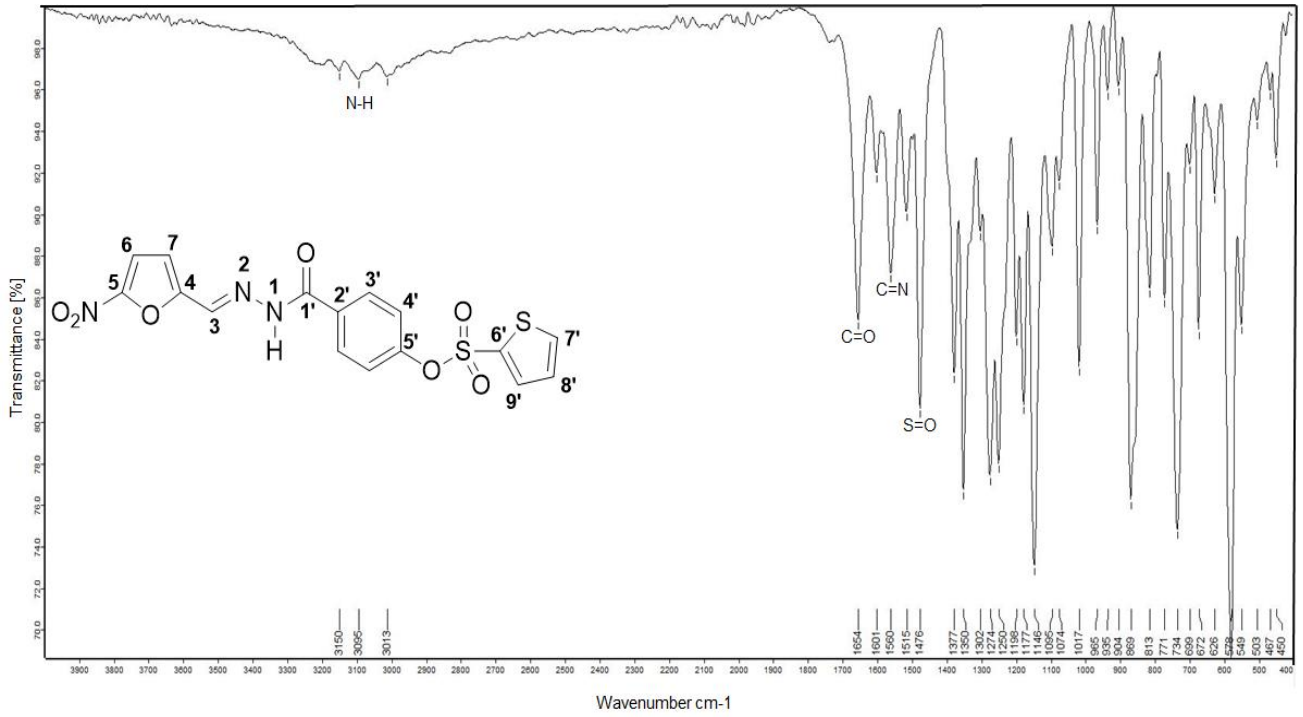
^{1}H NMR in DMSO



^{13}C NMR in DMSO



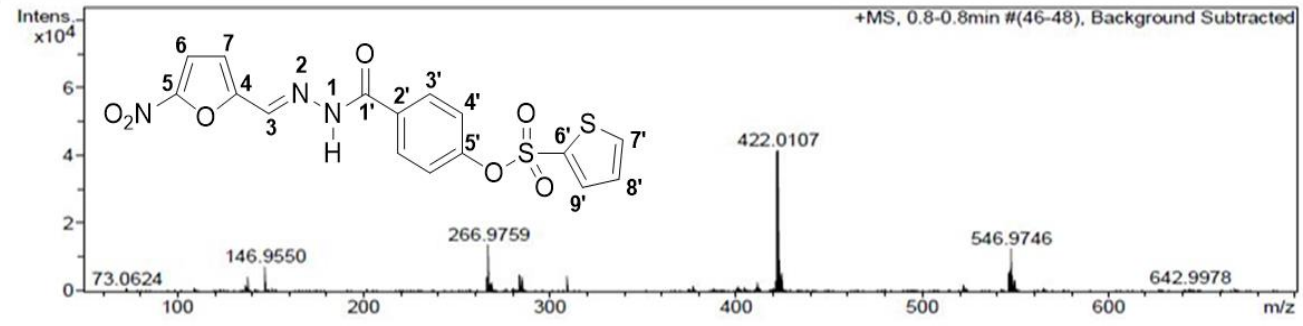
IR Spectrum



HRMS

Acquisition Parameter

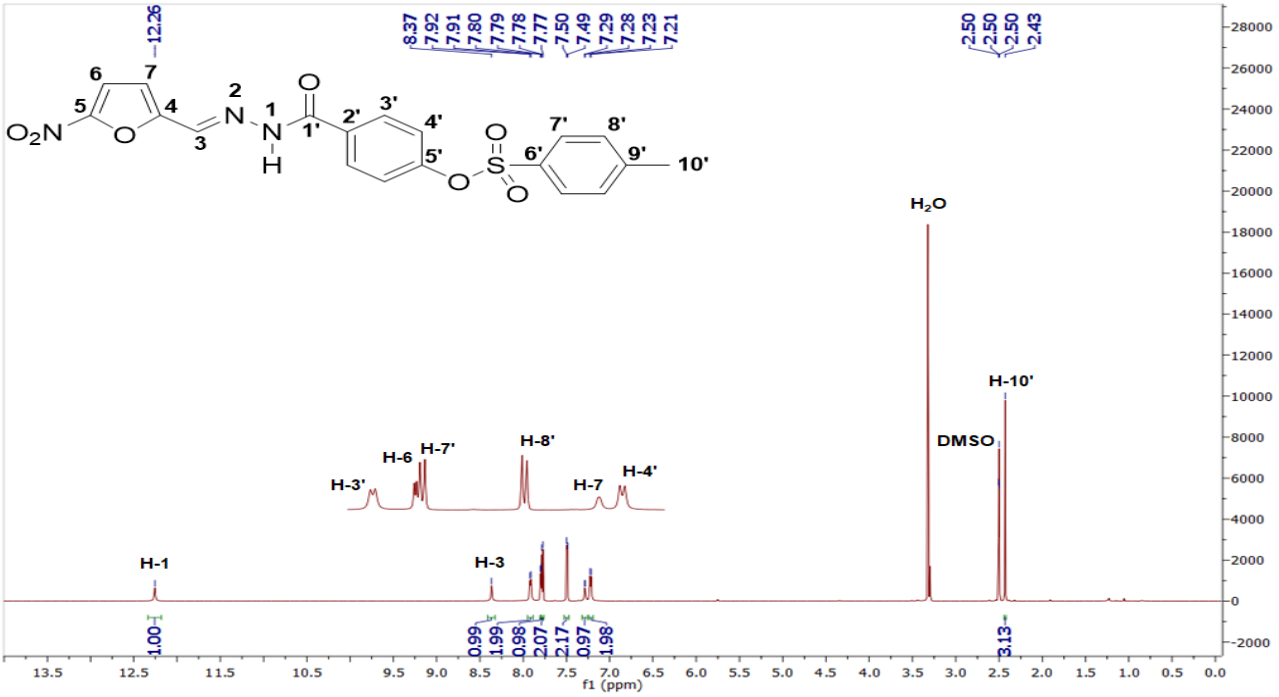
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



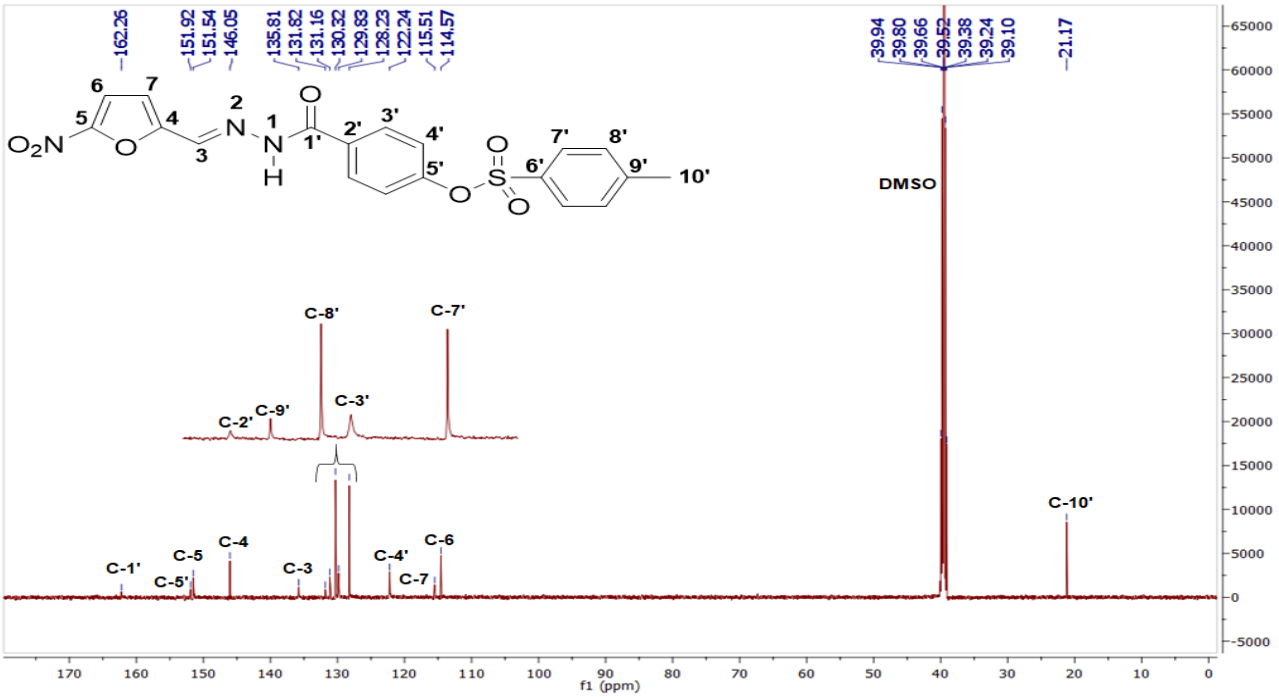
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
422.0107	1	C 16 H 12 N 3 O 7 S 2	100.00	422.0111	0.5	1.1	11.8	12.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 4-methylbenzene sulfonate (1e)**

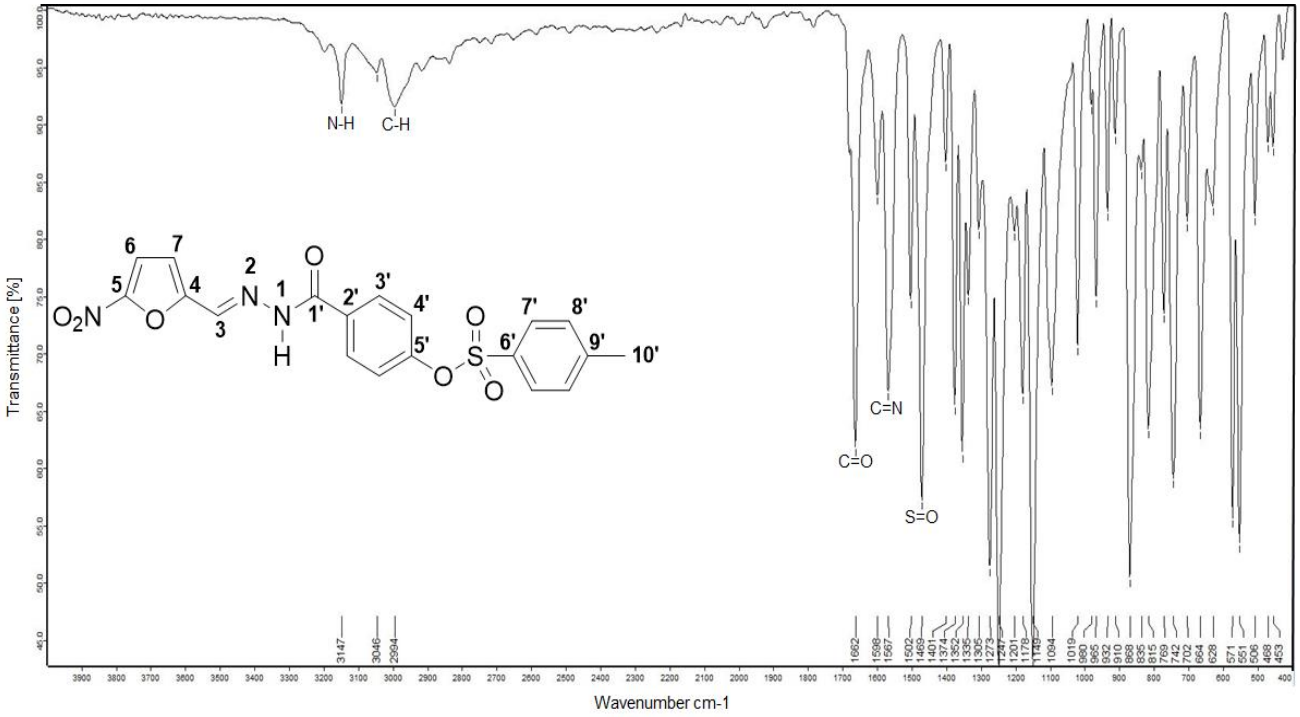
^{1}H NMR in DMSO



^{13}C NMR in DMSO



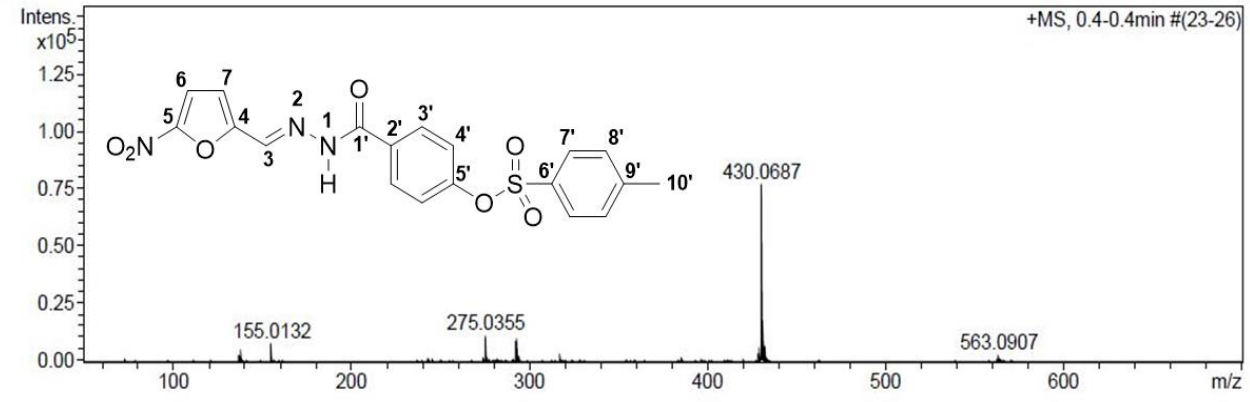
IR Spectrum



HRMS

Acquisition Parameter

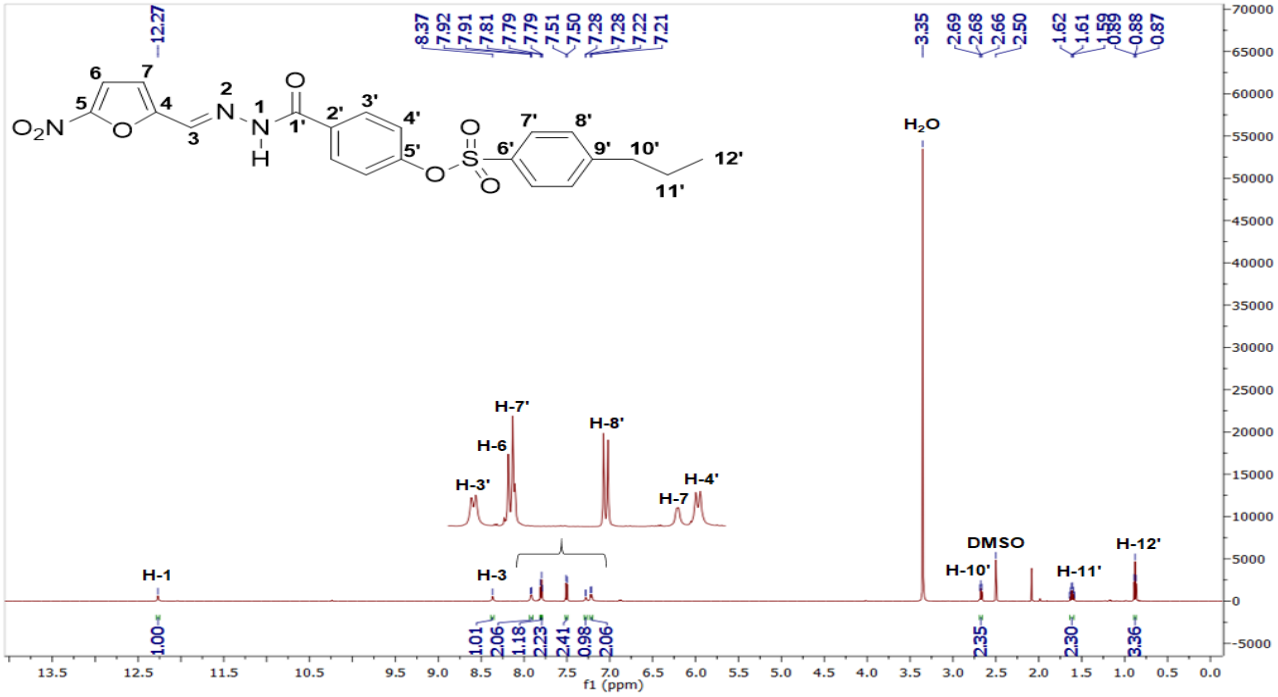
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



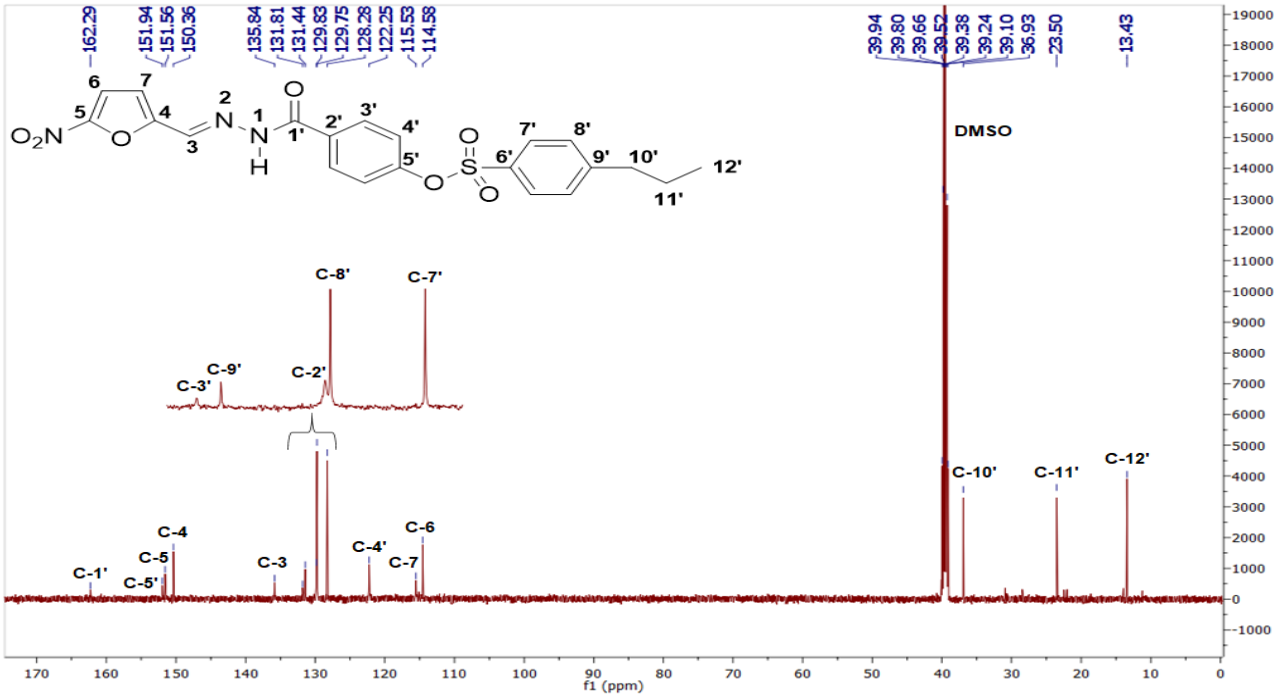
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
430.0687	1	C 19 H 16 N 3 O 7 S	100.00	430.0703	1.6	3.7	2.1	13.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl4-propylbenzene sulfonate (1f)**

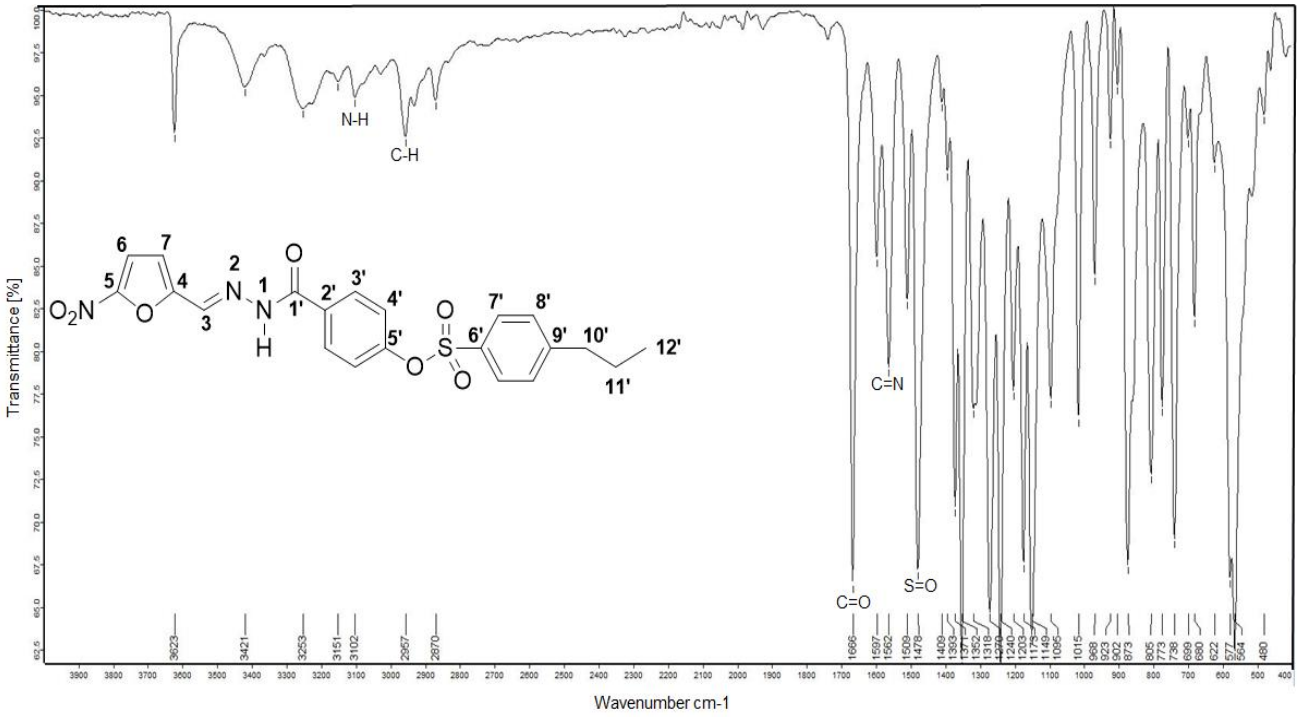
^{1}H NMR in DMSO



^{13}C NMR in DMSO



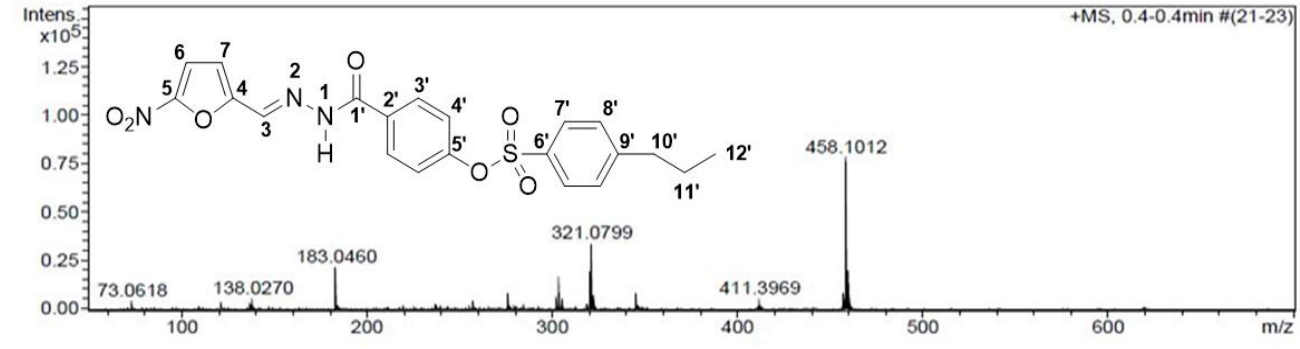
IR Spectrum



HRMS

Acquisition Parameter

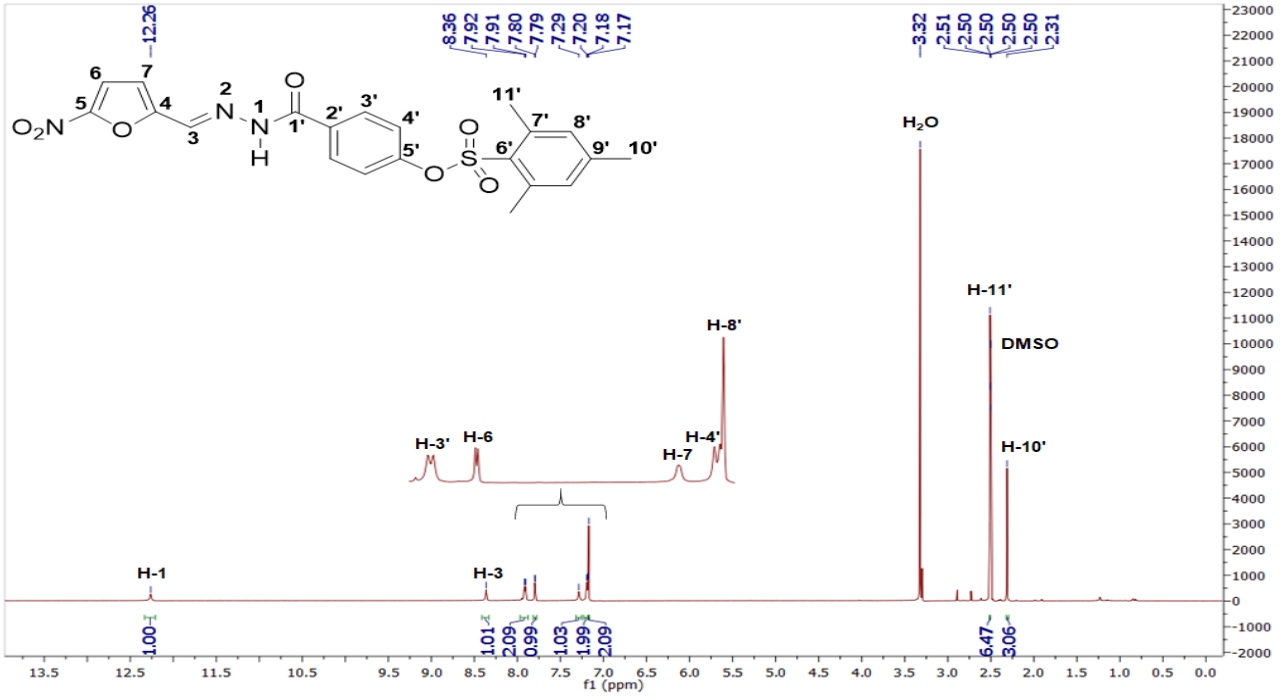
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



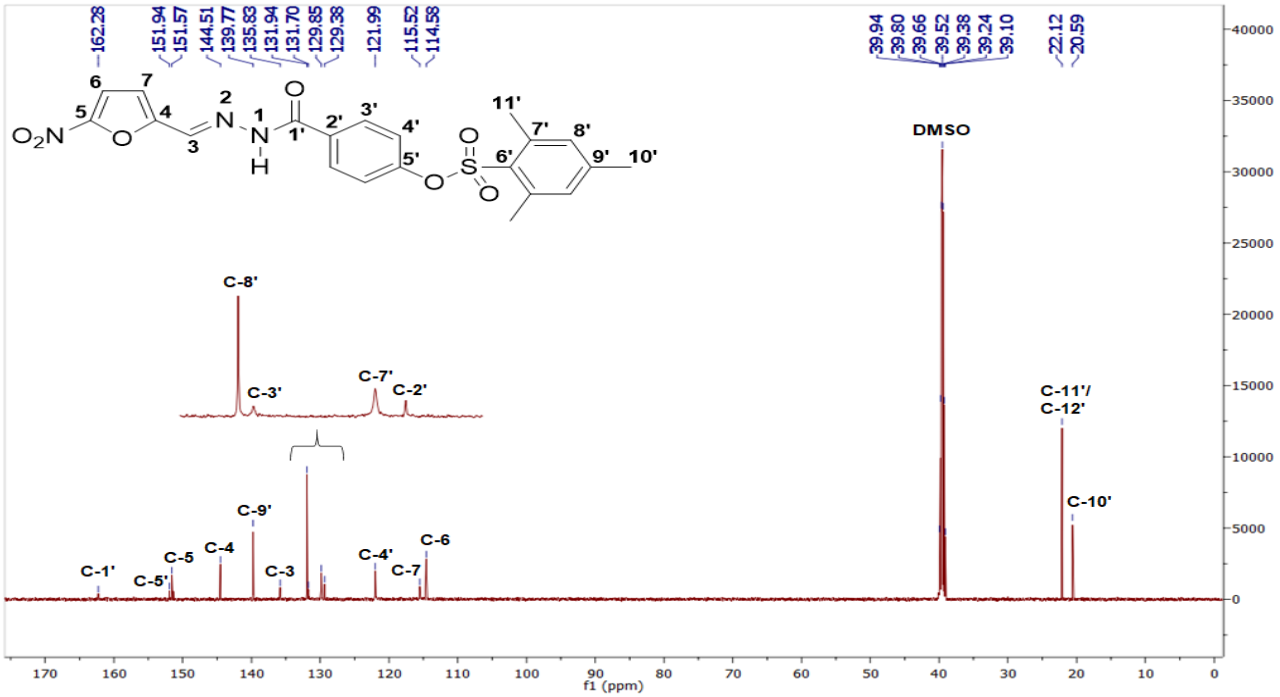
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
458.1012	1	C 21 H 20 N 3 O 7 S	100.00	458.1016	0.5	1.0	6.2	13.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 2,4,6-trimethylbenzene sulfonate (1g)**

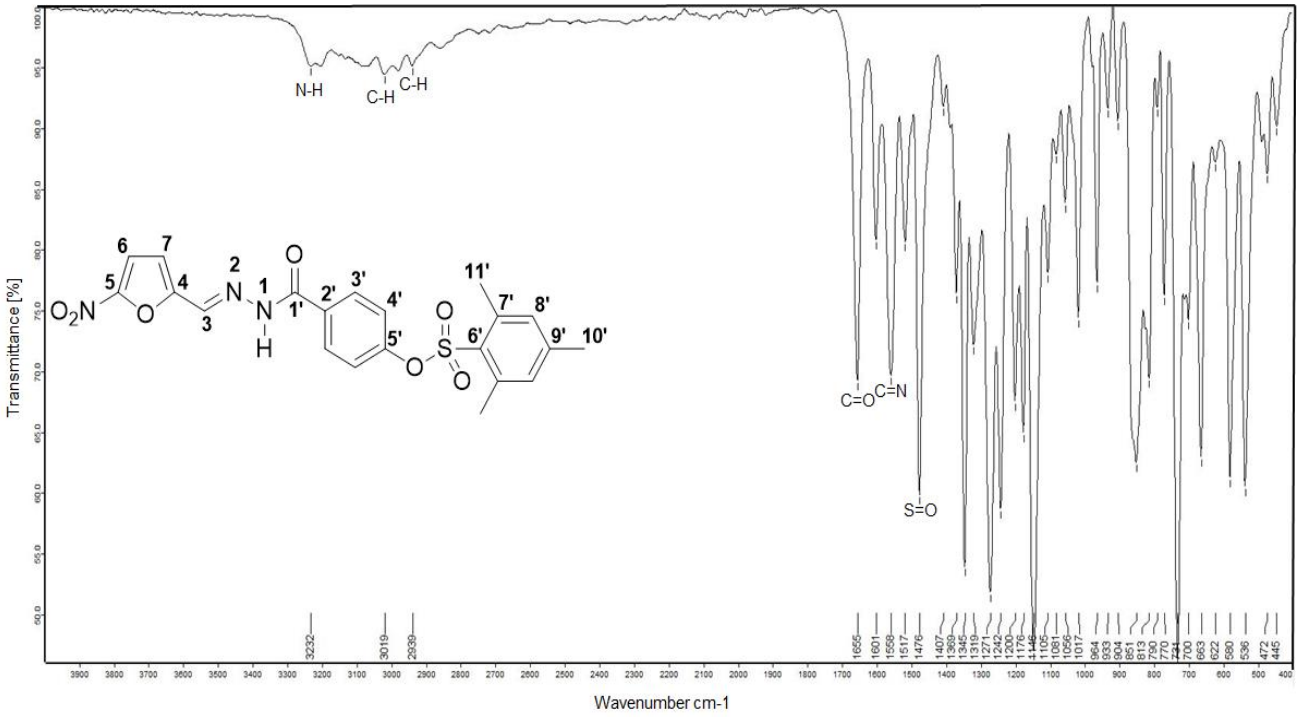
^{1}H NMR in DMSO



^{13}C NMR in DMSO



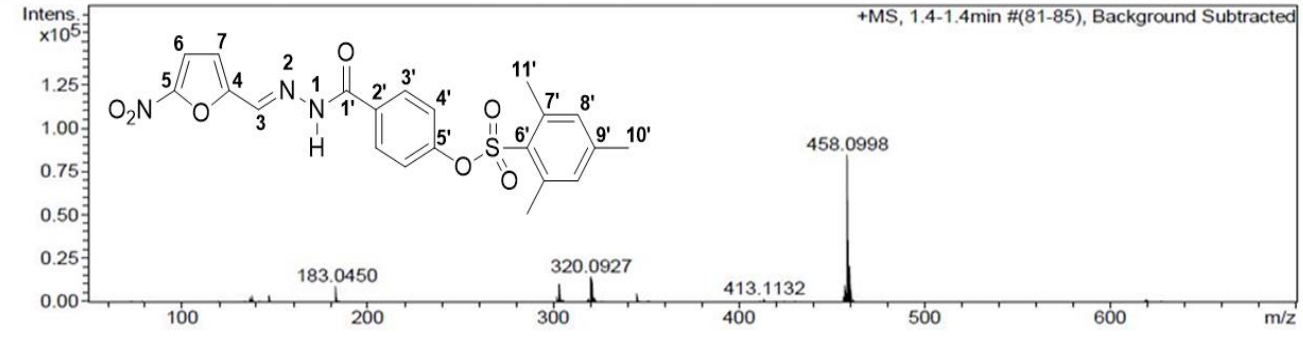
IR Spectrum



HRMS

Acquisition Parameter

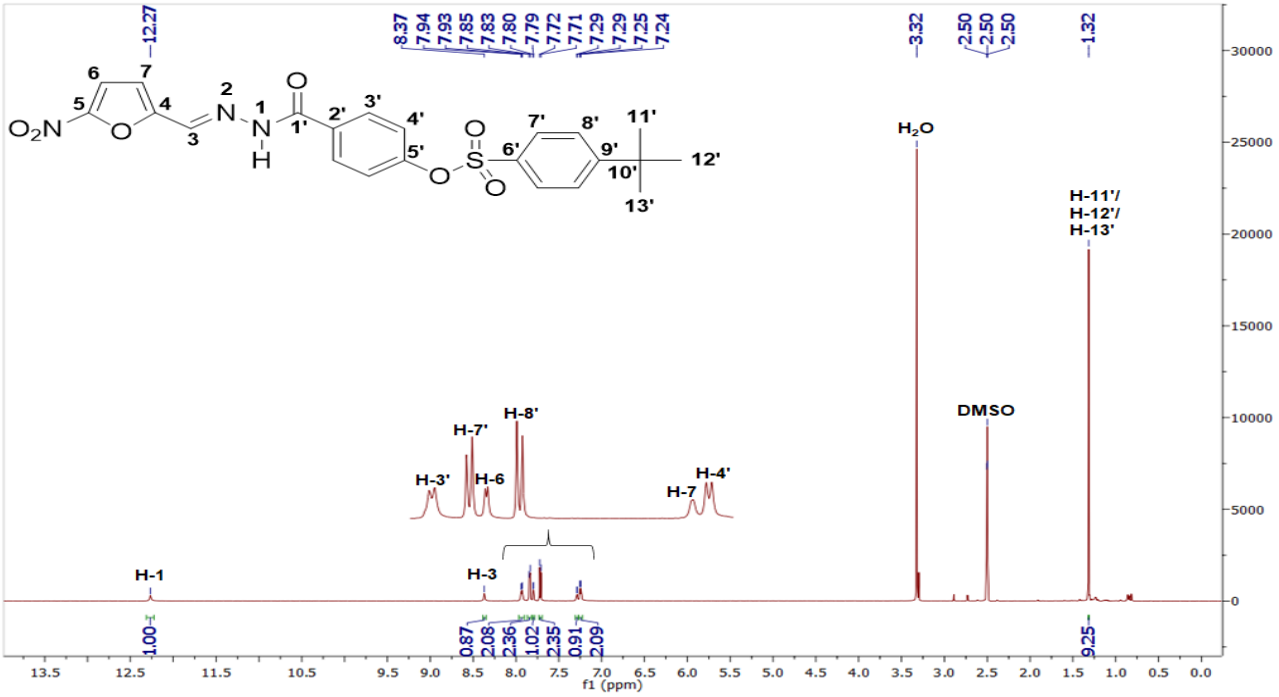
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



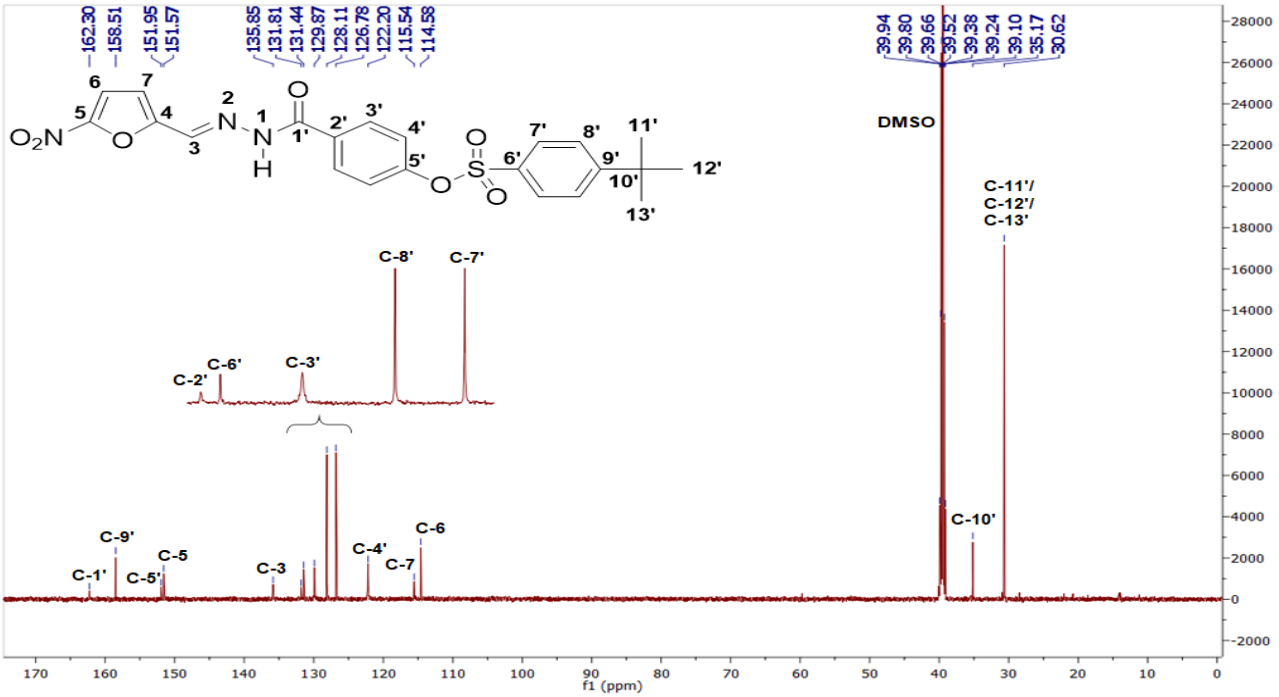
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
458.0998	1	C 21 H 20 N 3 O 7 S	100.00	458.1016	1.9	4.1	1.8	13.5	even	ok

***(E)-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 4-(tert-butyl)benzene sulfonate* (1h)**

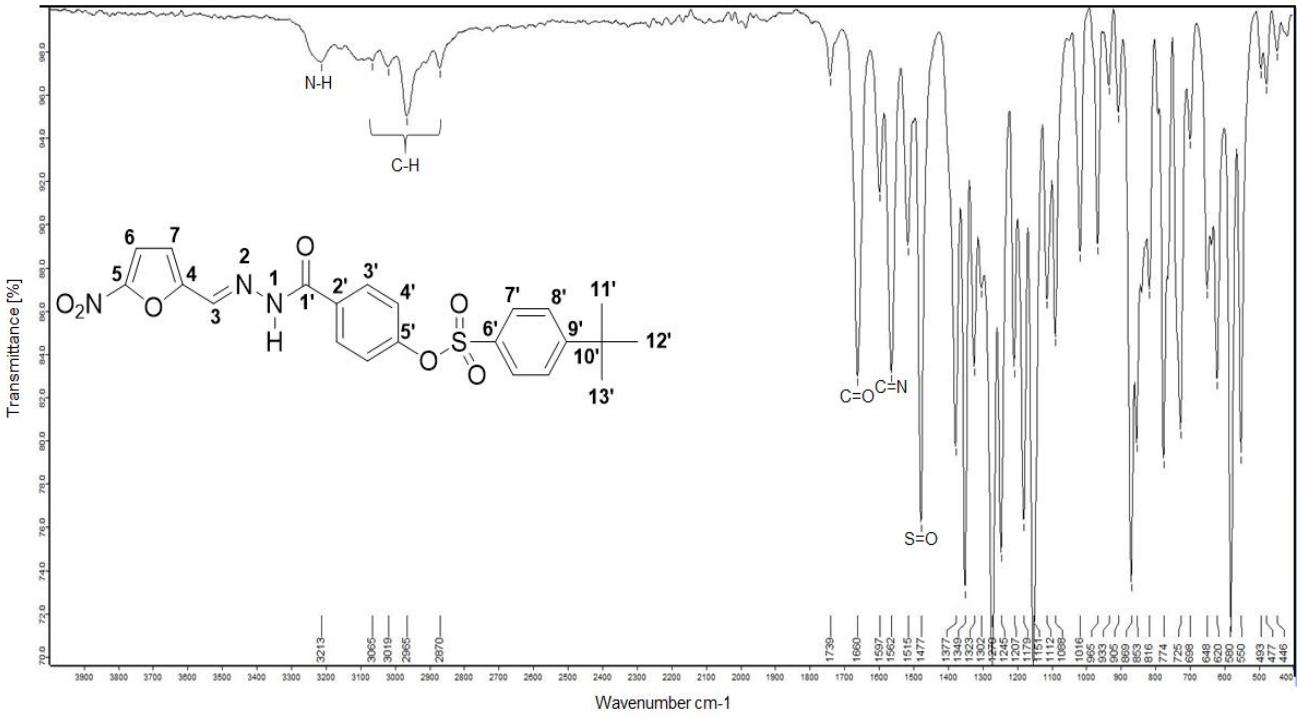
^{1}H NMR in DMSO



^{13}C NMR in DMSO



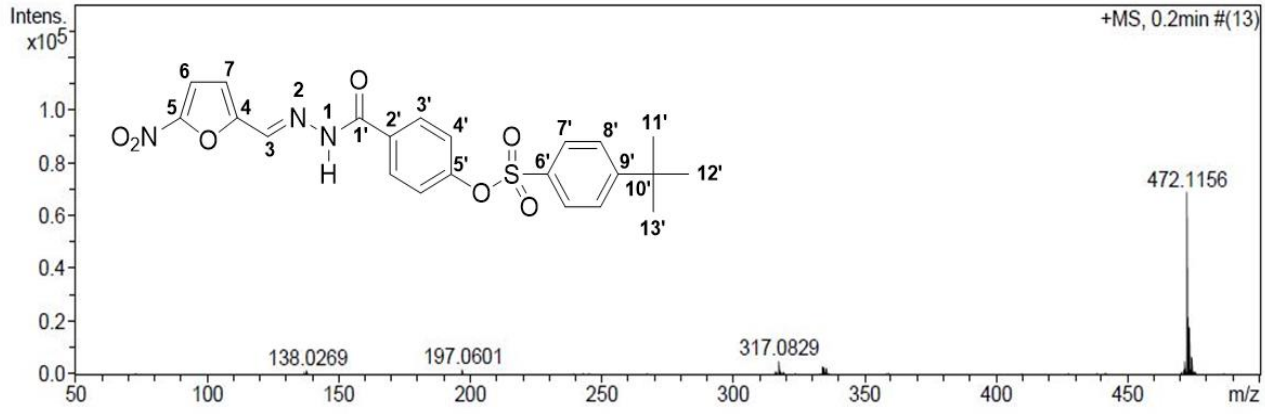
IR Spectrum



HRMS

Acquisition Parameter

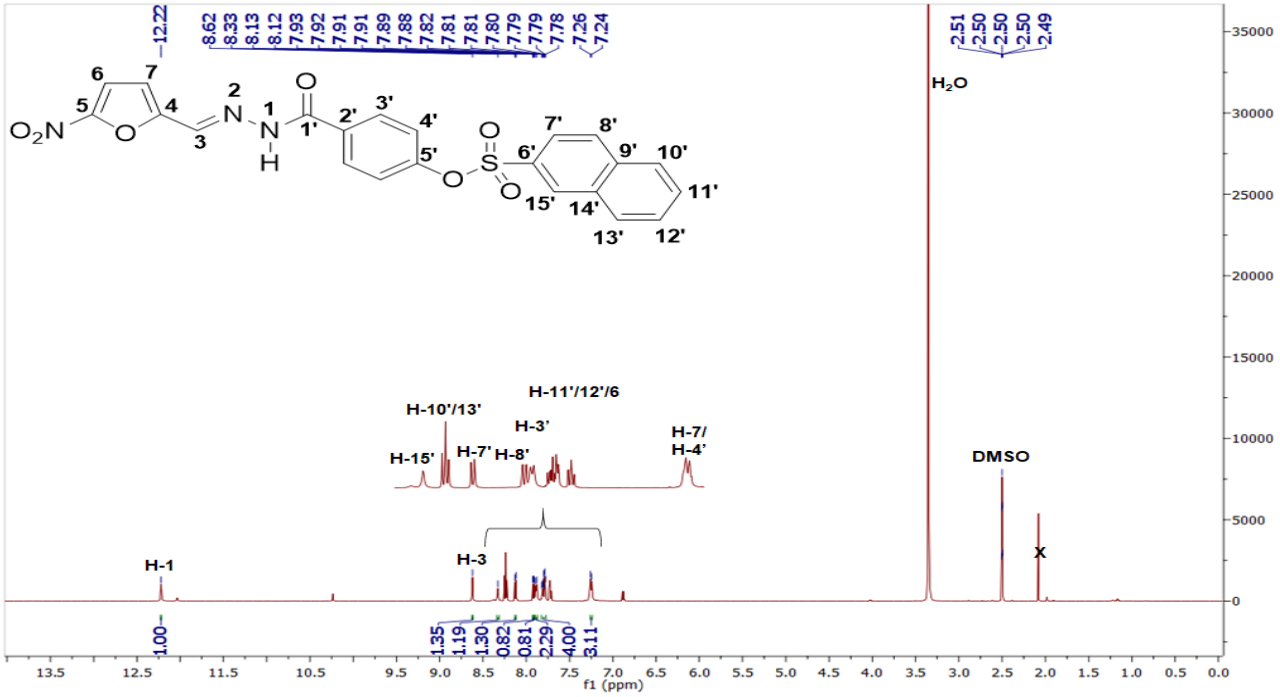
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



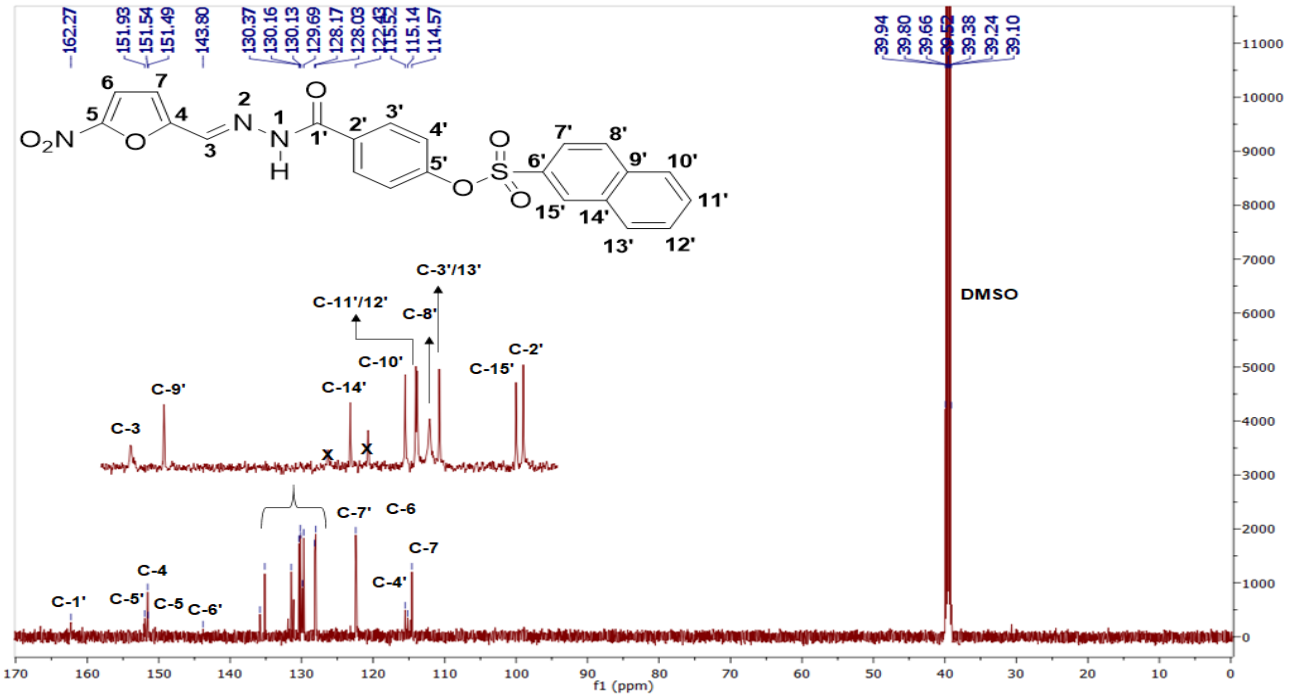
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
472.1156	1	C 22 H 22 N 3 O 7 S	100.00	472.1173	1.7	3.5	8.4	13.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl naphthalene-2-sulfonate (1i)**

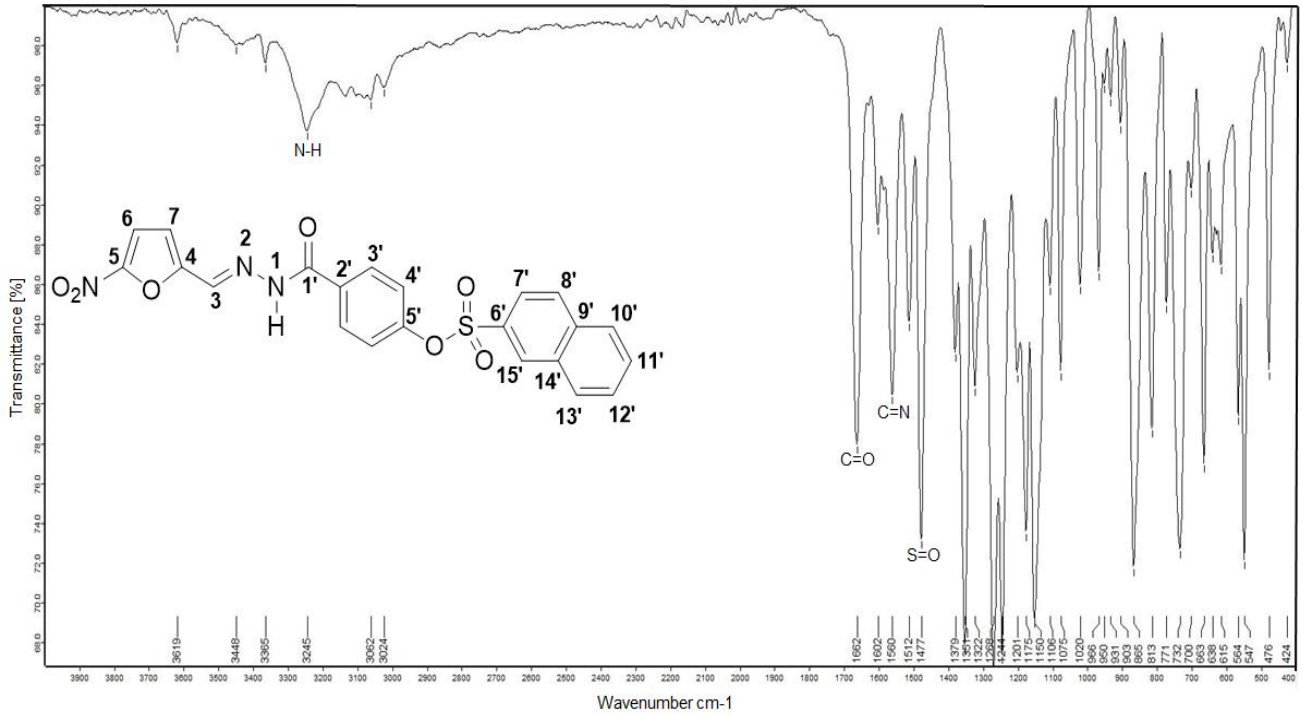
^{1}H NMR in DMSO



^{13}C NMR in DMSO



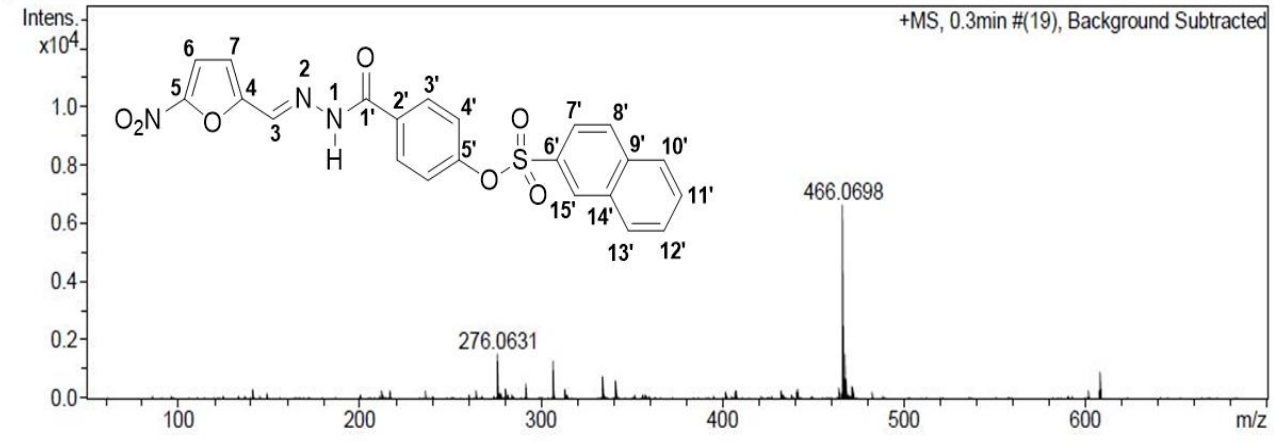
IR Spectrum



HRMS

Acquisition Parameter

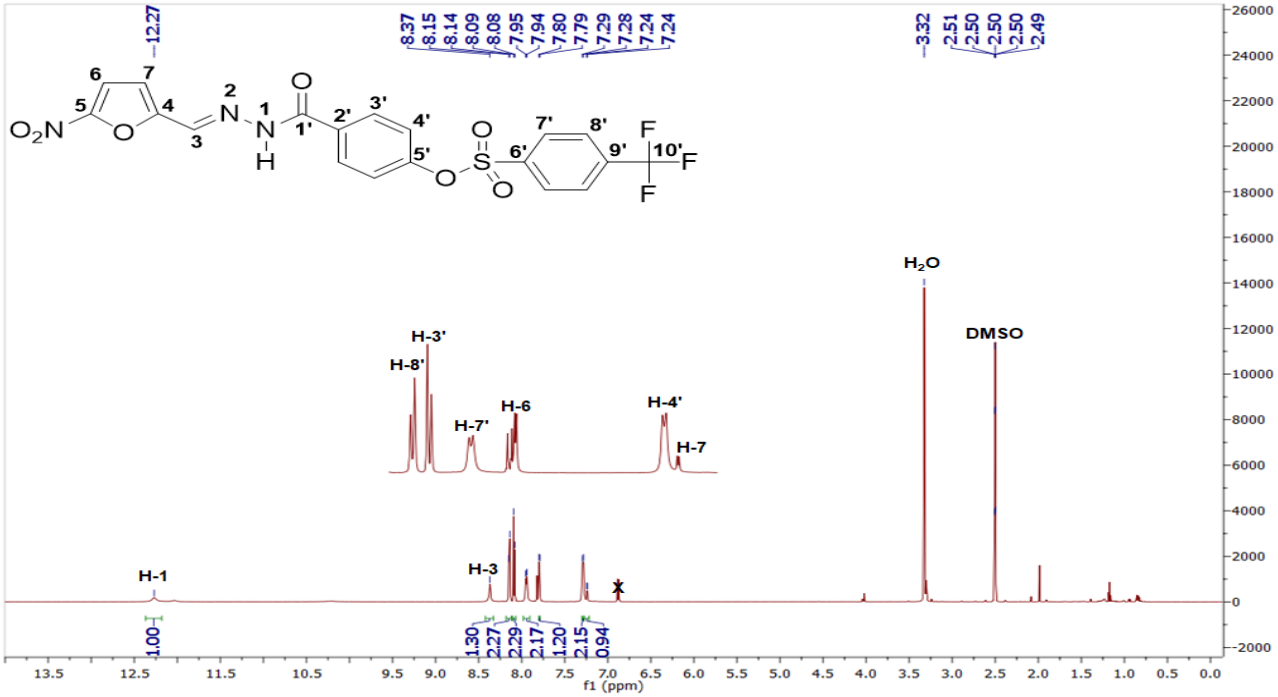
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



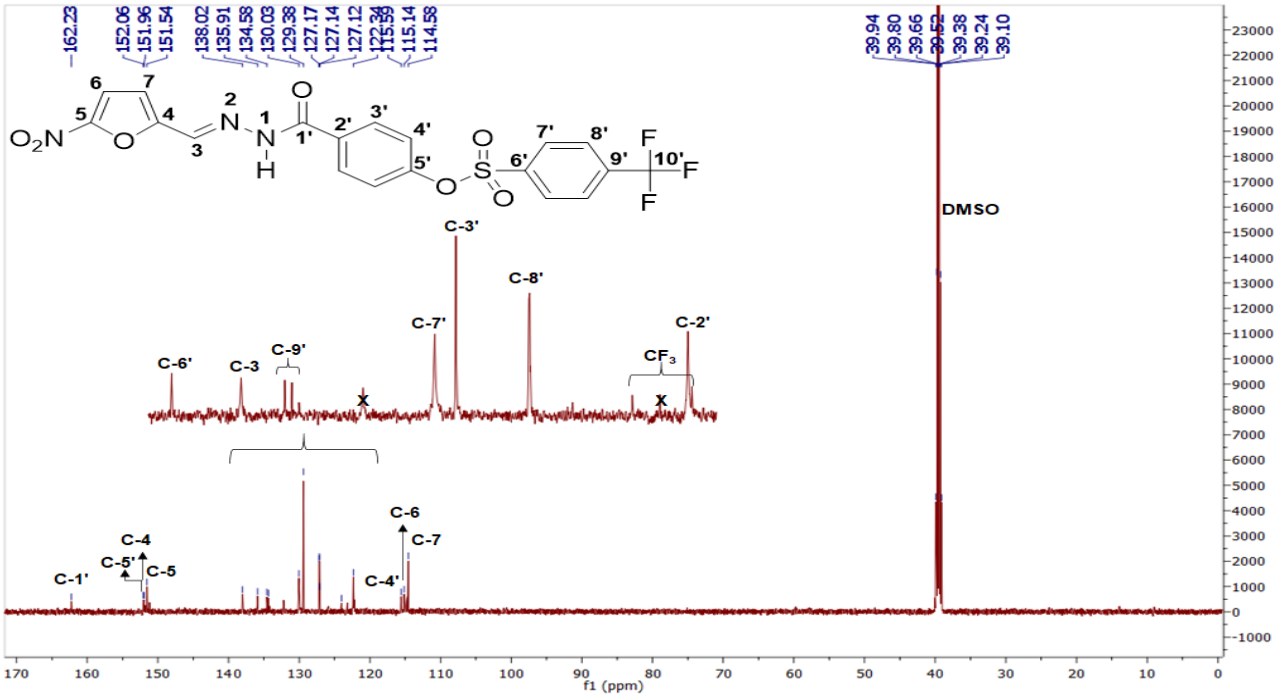
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
466.0698	1	C 22 H 16 N 3 O 7 S	100.00	466.0703	0.6	1.2	136.6	16.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 4-(trifluoromethyl)benzenesulfonate (1j)**

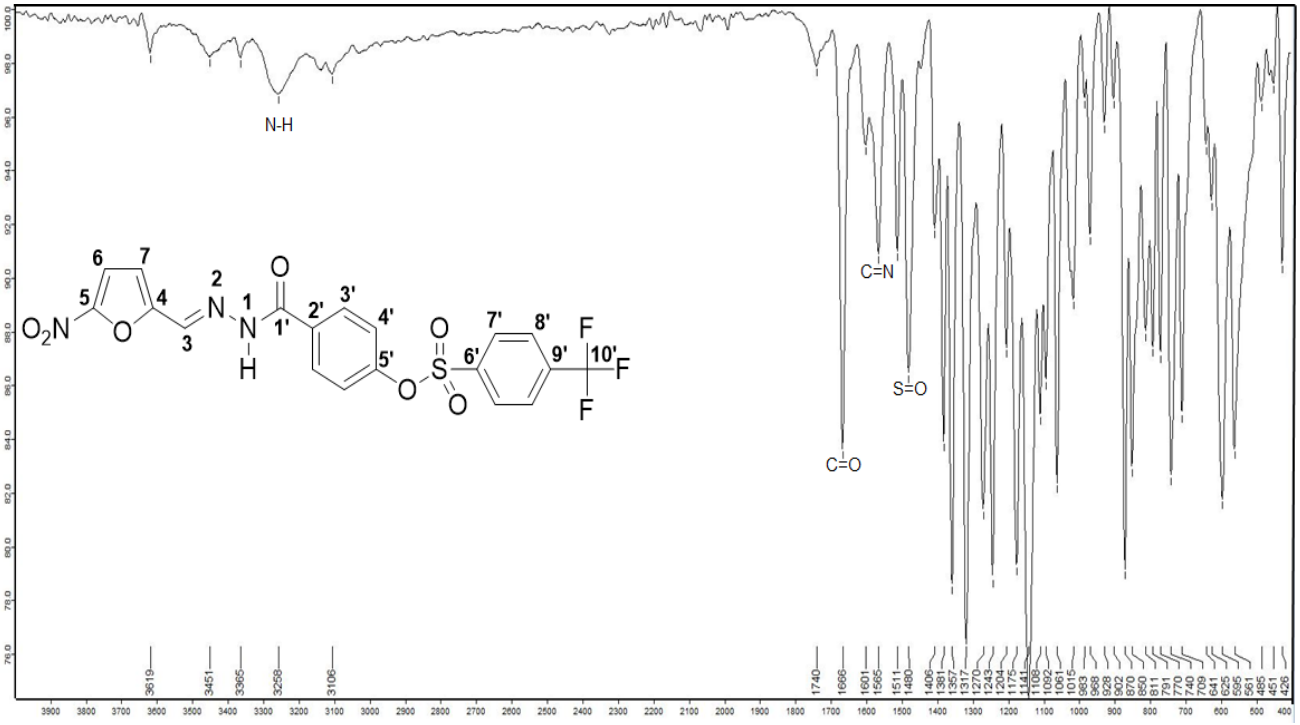
^{1}H NMR in DMSO



^{13}C NMR in DMSO

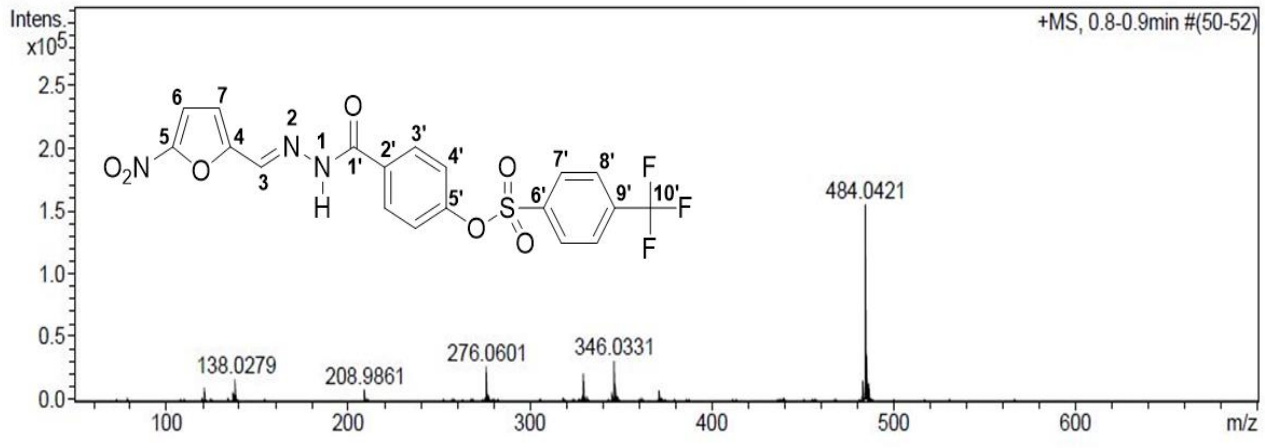


IR Spectrum



HRMS

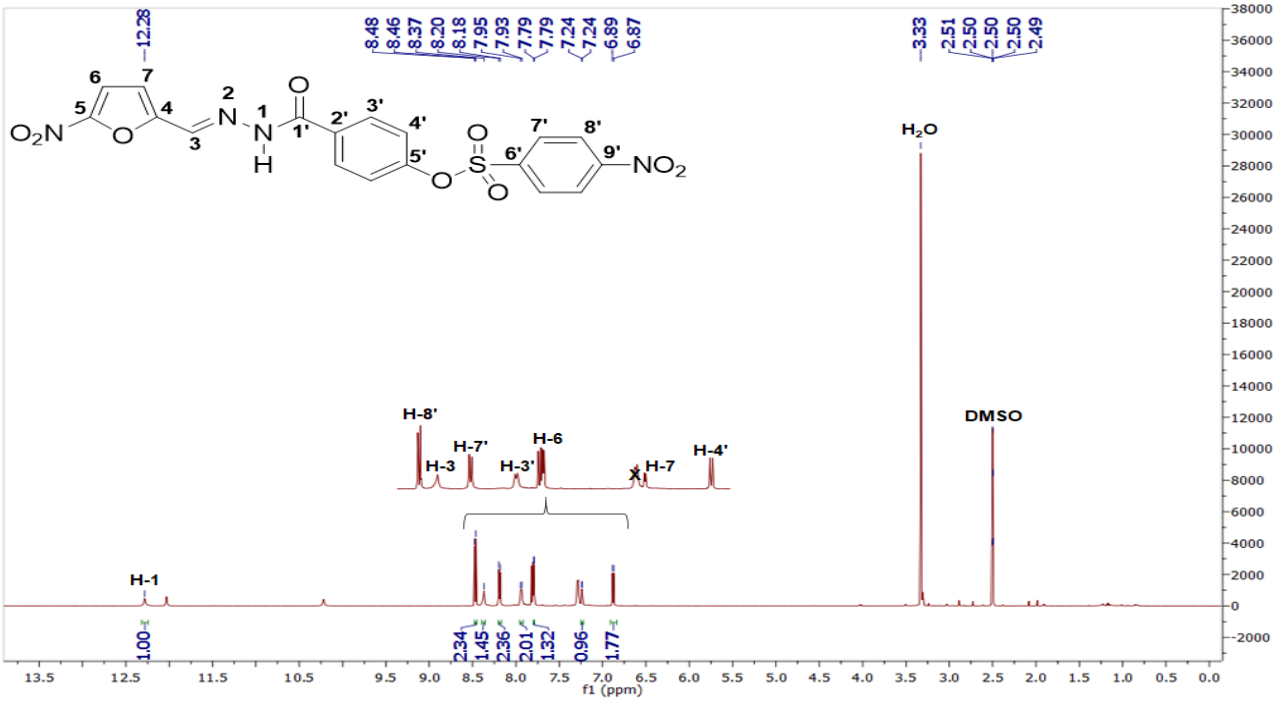
Acquisition Parameter					
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



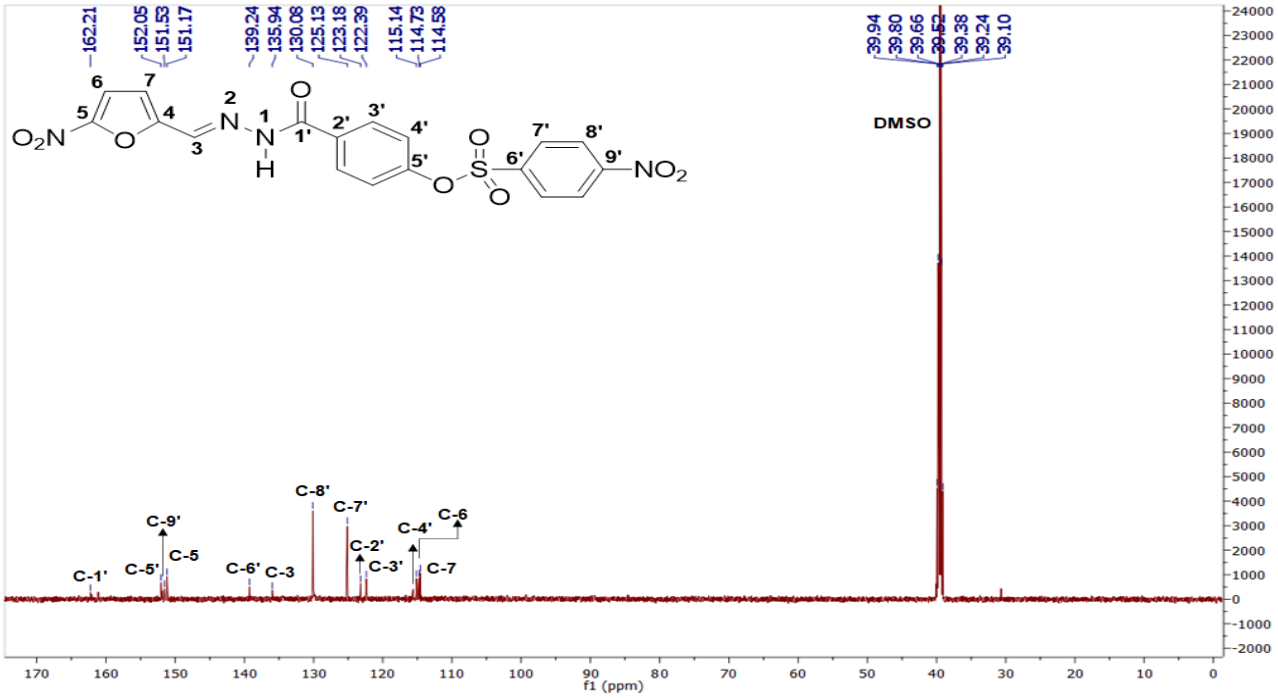
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
484.0421	1	C 19 H 13 F 3 N 3 O 7 S	100.00	484.0421	-0.1	-0.1	8.5	13.5	even	ok

***(E)*-4-{2-[(5-Nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl 4-nitrobenzenesulfonate (1k)**

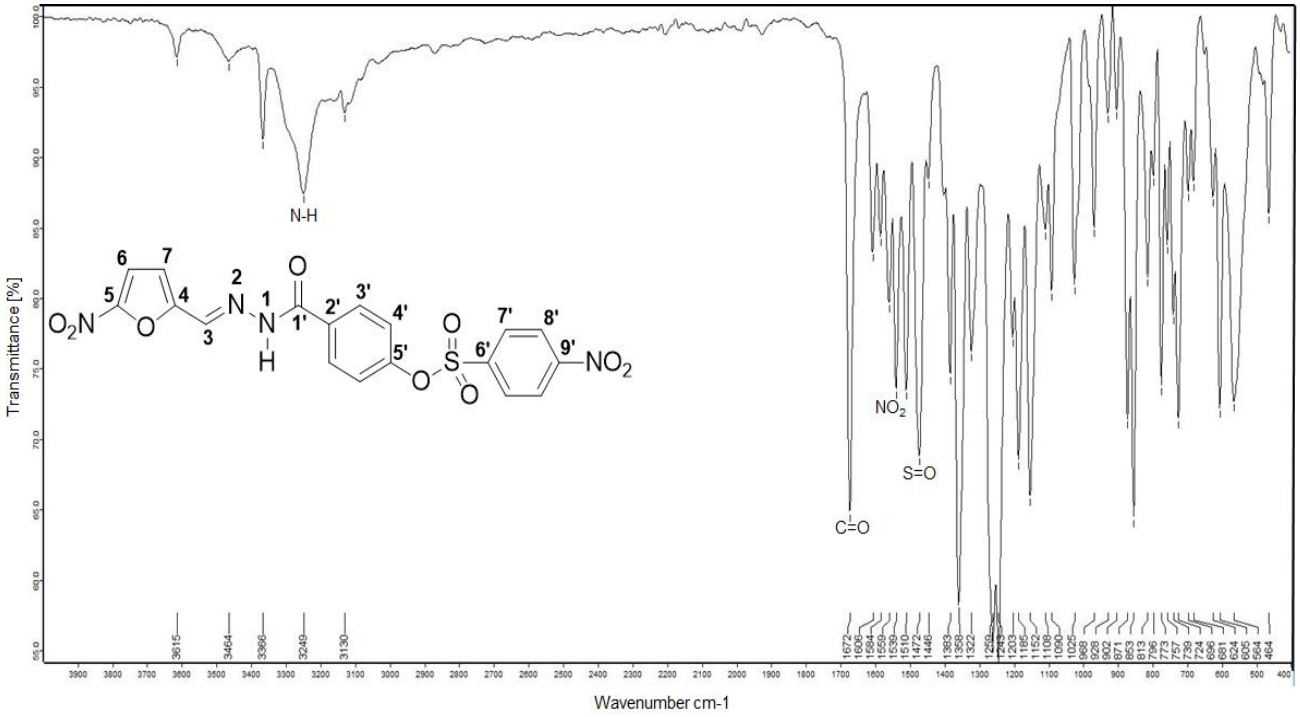
^{1}H NMR in DMSO



^{13}C NMR in DMSO



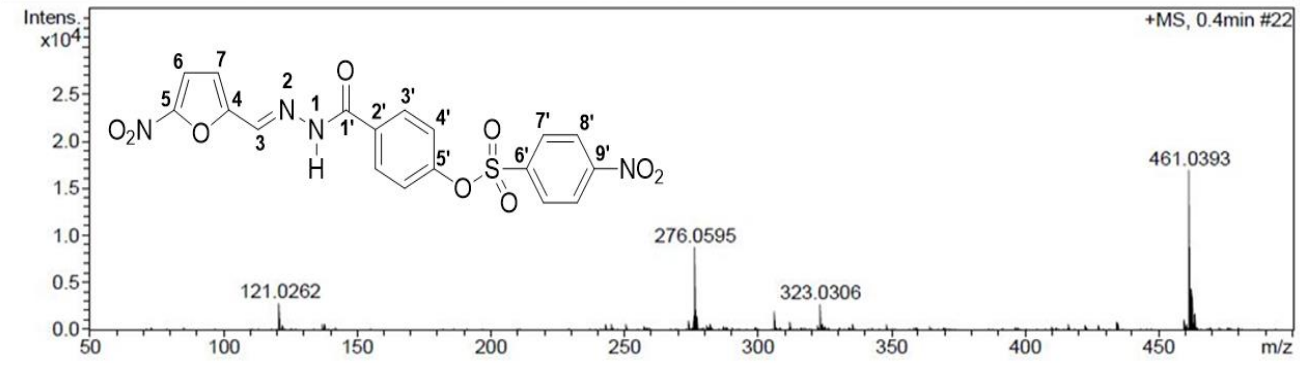
IR Spectrum



HRMS

Acquisition Parameter

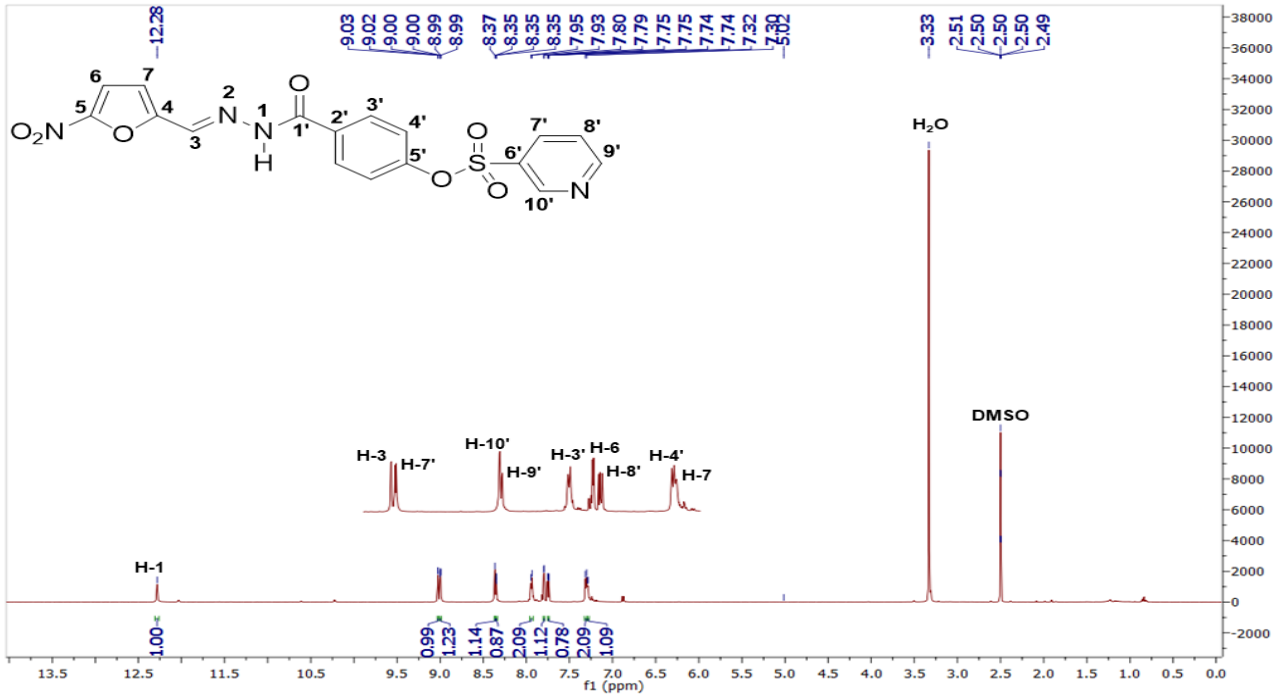
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



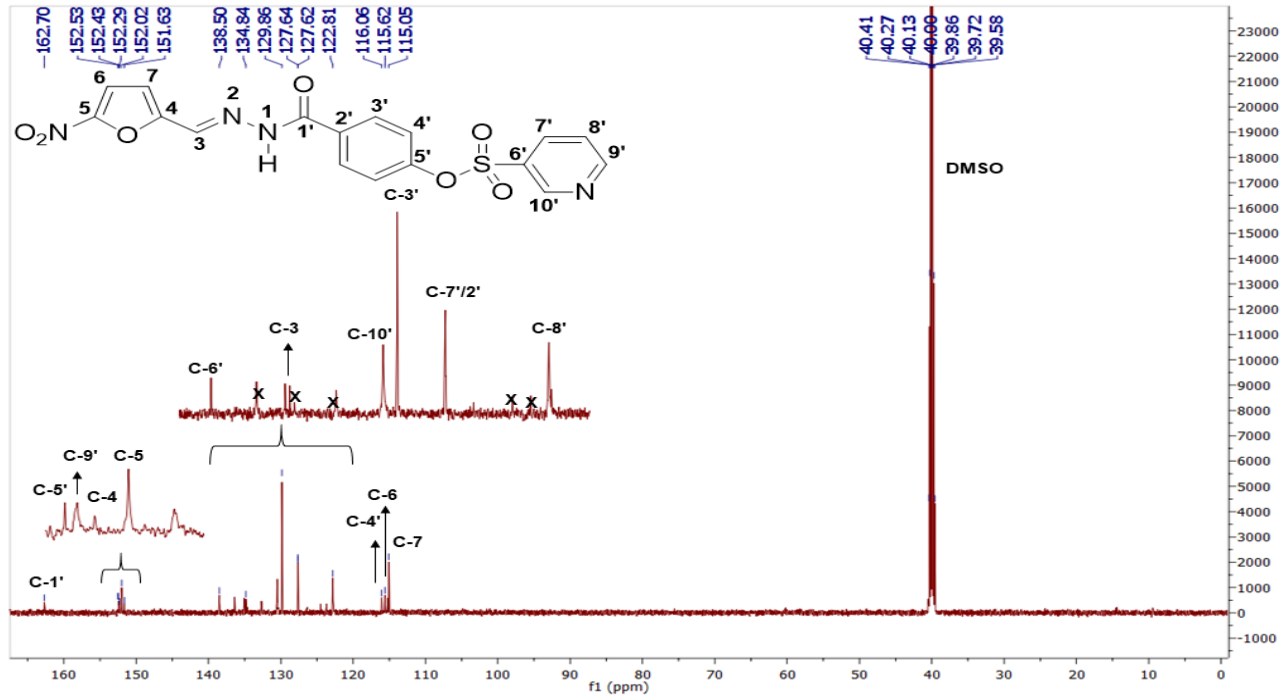
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
461.0393	1	C 18 H 13 N 4 O 9 S	100.00	461.0398	0.4	0.9	22.0	14.5	even	ok

***(E)*-4-{2-[(5-nitrofuran-2-yl)methylene]hydrazinecarbonyl}phenyl pyridine-3-sulfonate (1l)**

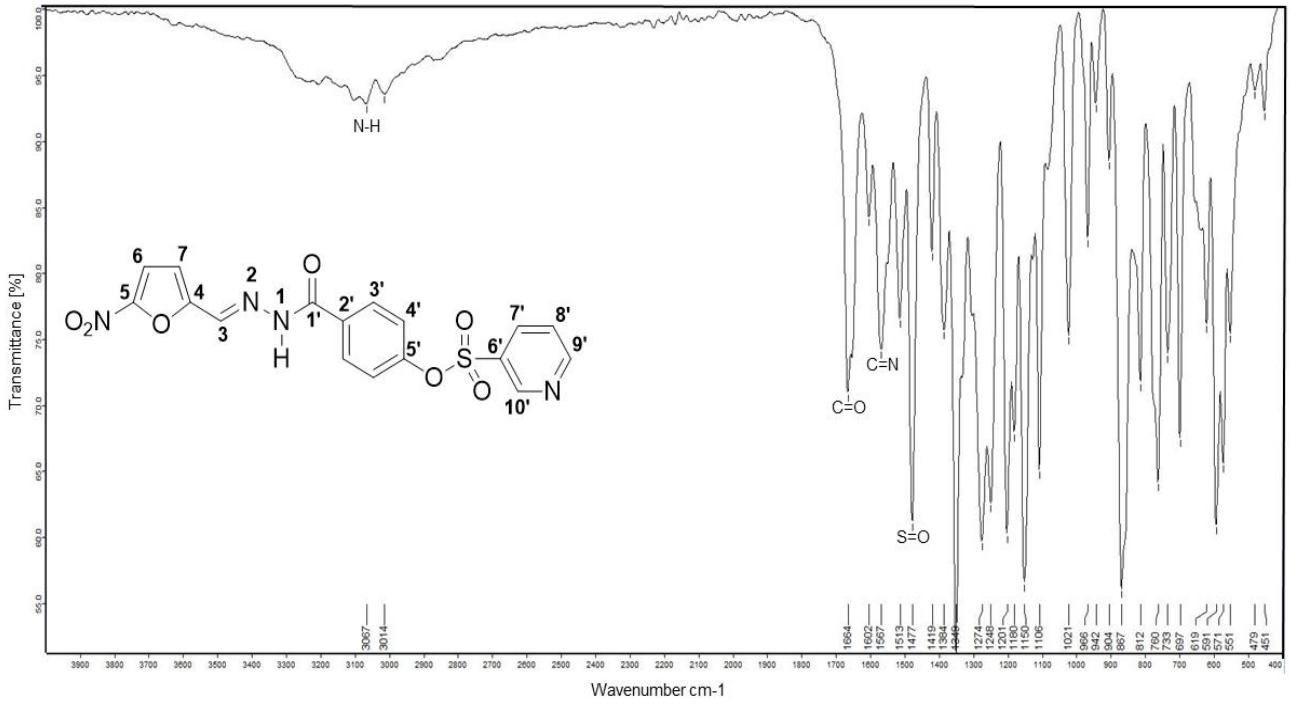
^{1}H NMR in DMSO



^{13}C NMR in DMSO



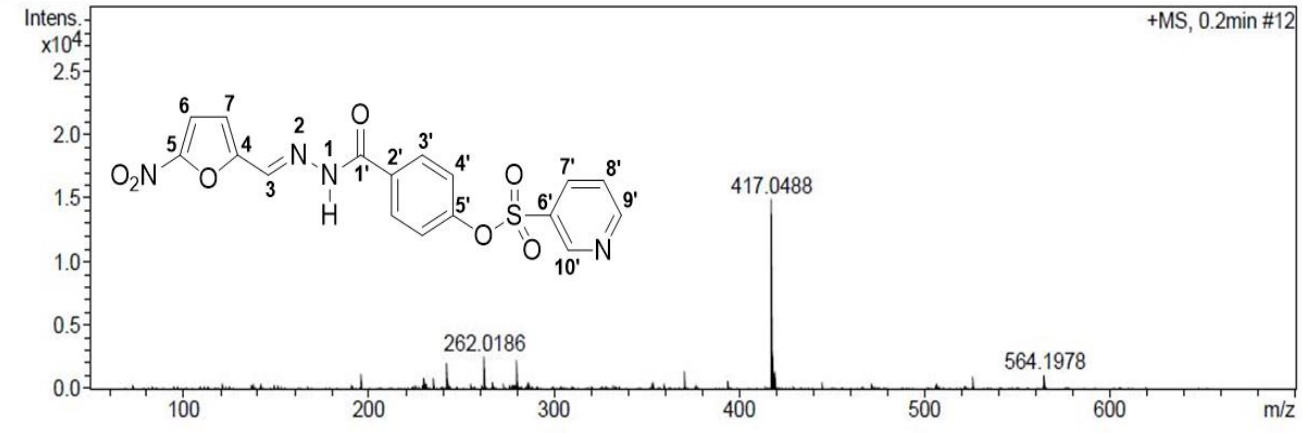
IR Spectrum



HRMS

Acquisition Parameter

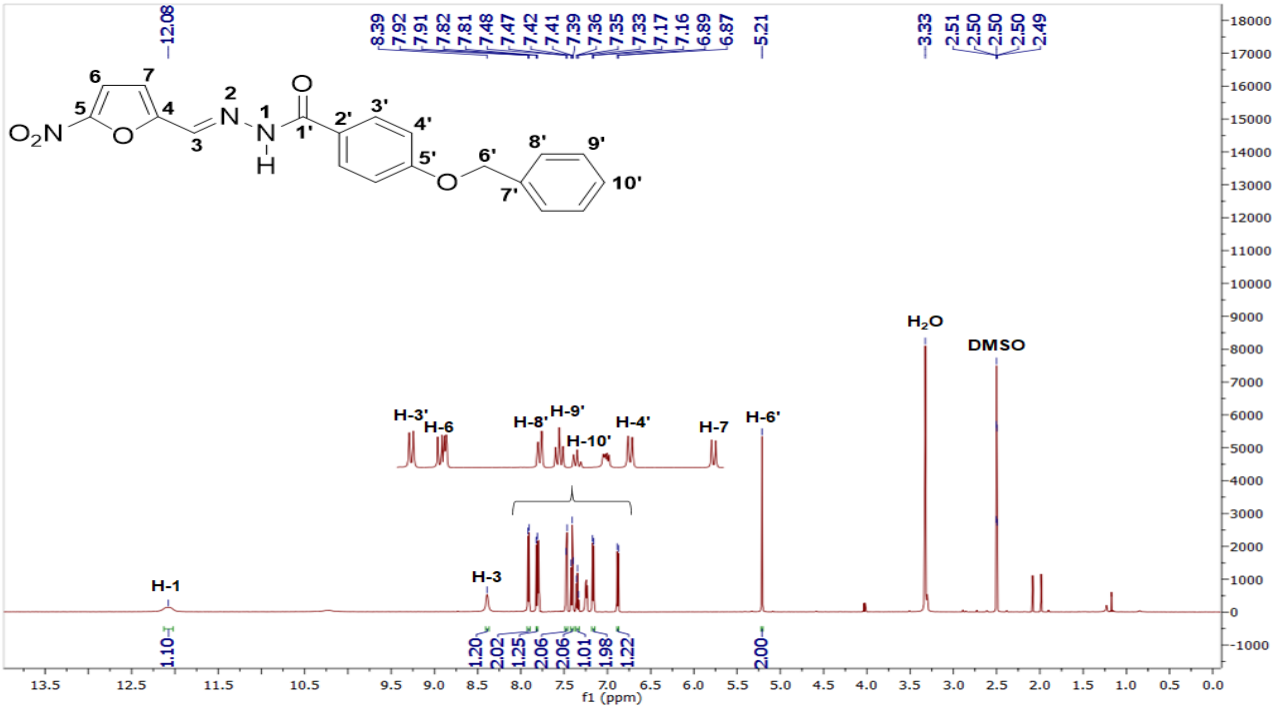
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



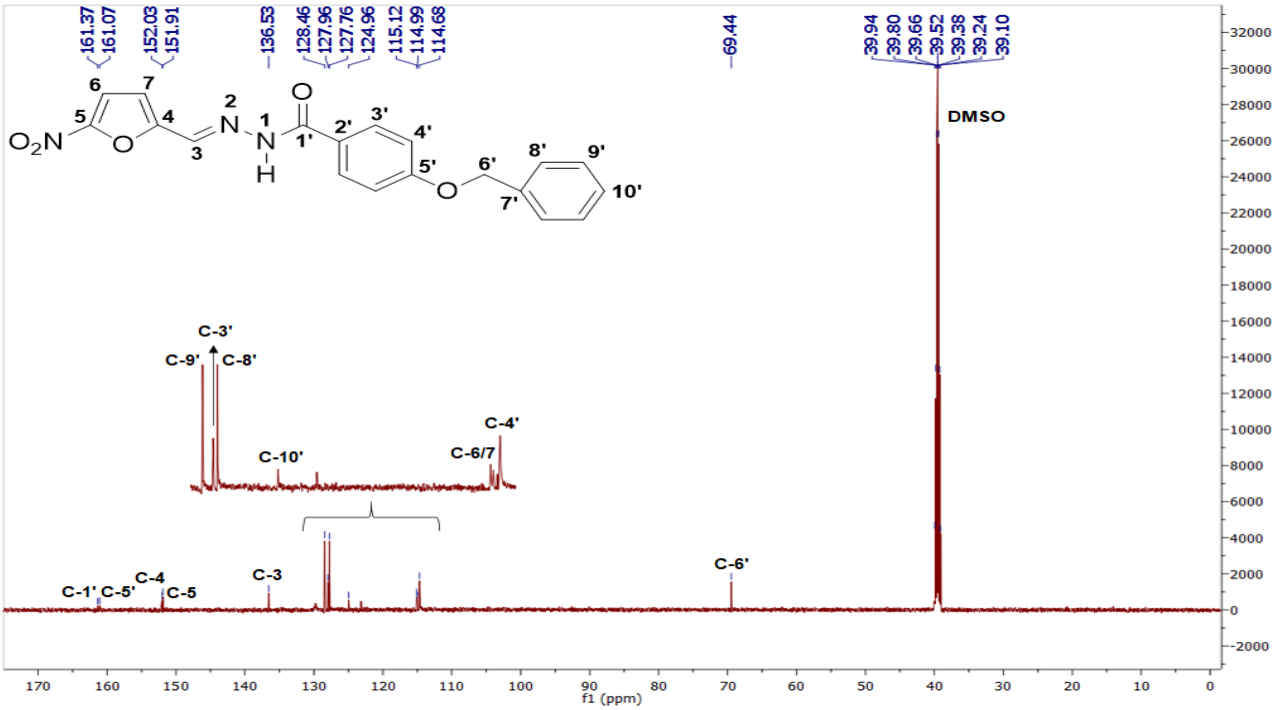
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
417.0488	1	C 17 H 13 N 4 O 7 S	100.00	417.0499	1.2	2.9	40.5	13.5	even	ok
	2	C 16 H 18 Cl N 2 O 7 S	0.02	417.0518	3.0	7.3	180.4	8.5	even	ok

(E)-4-(benzyloxy)-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide (2a)

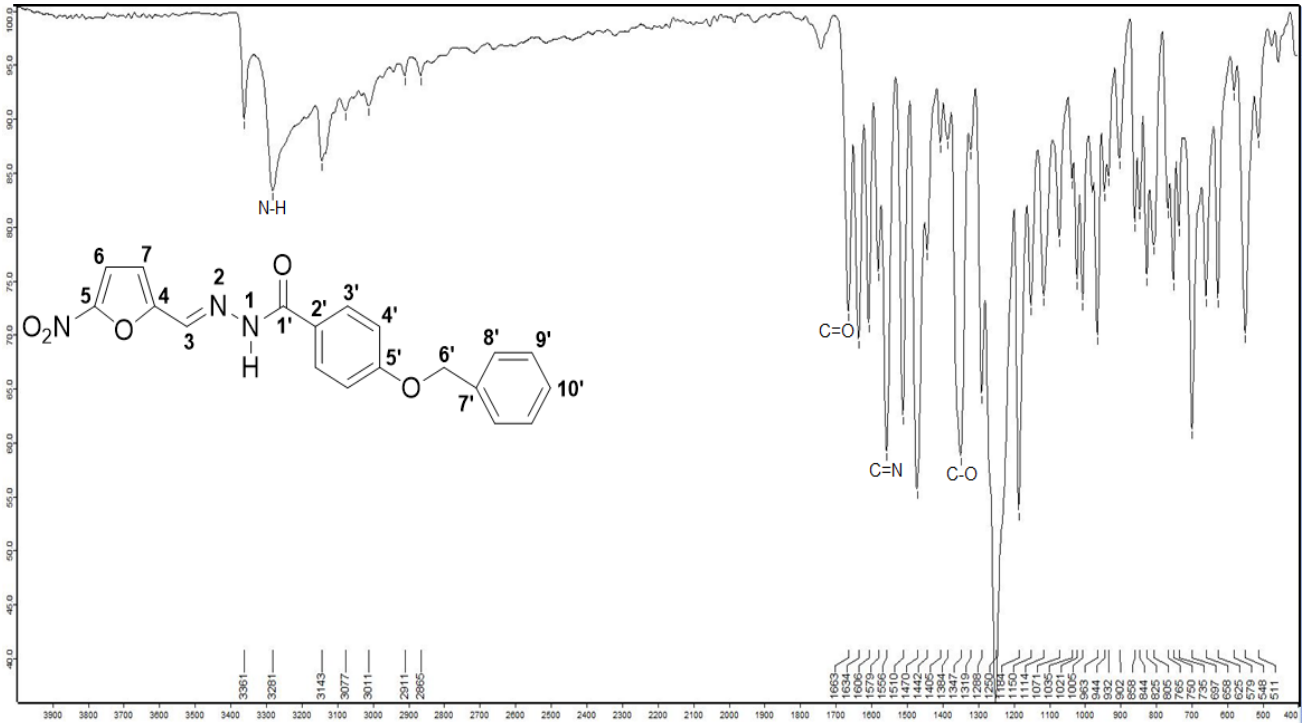
^{1}H NMR in DMSO



^{13}C NMR in DMSO

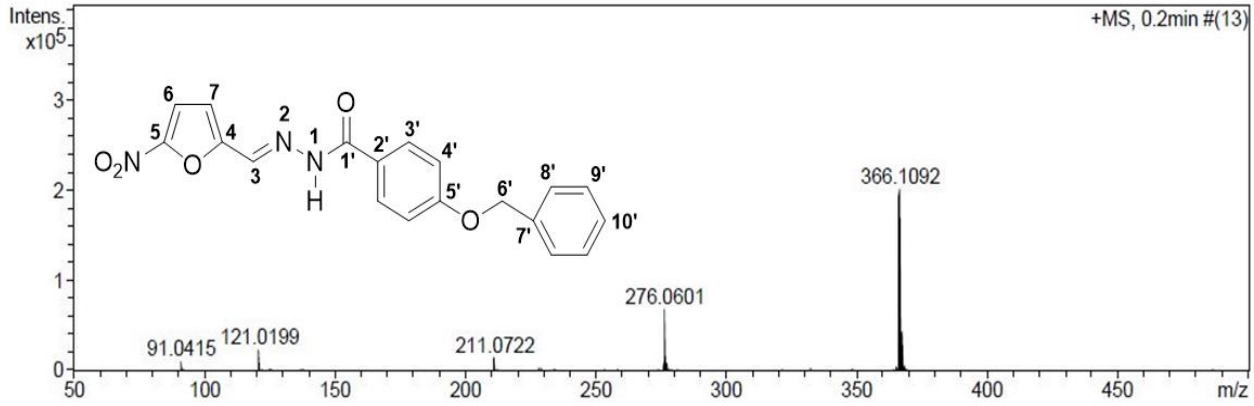


IR Spectrum



HRMS

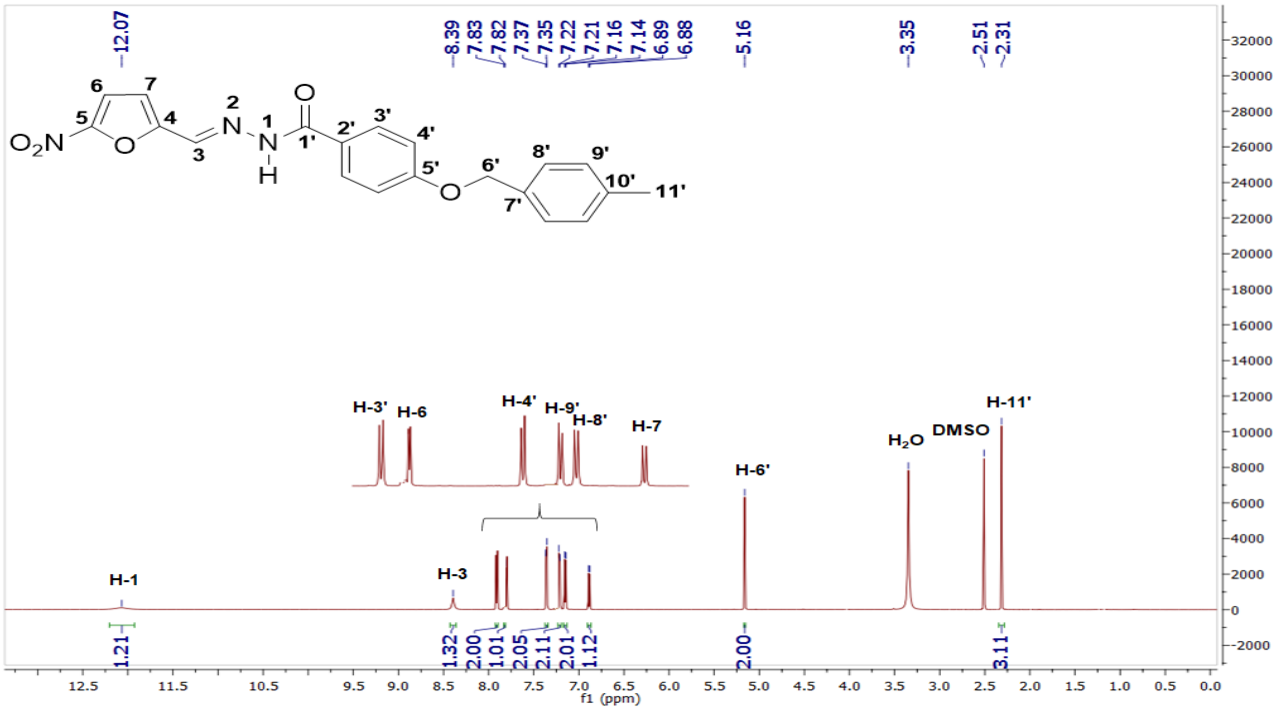
Acquisition Parameter					
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



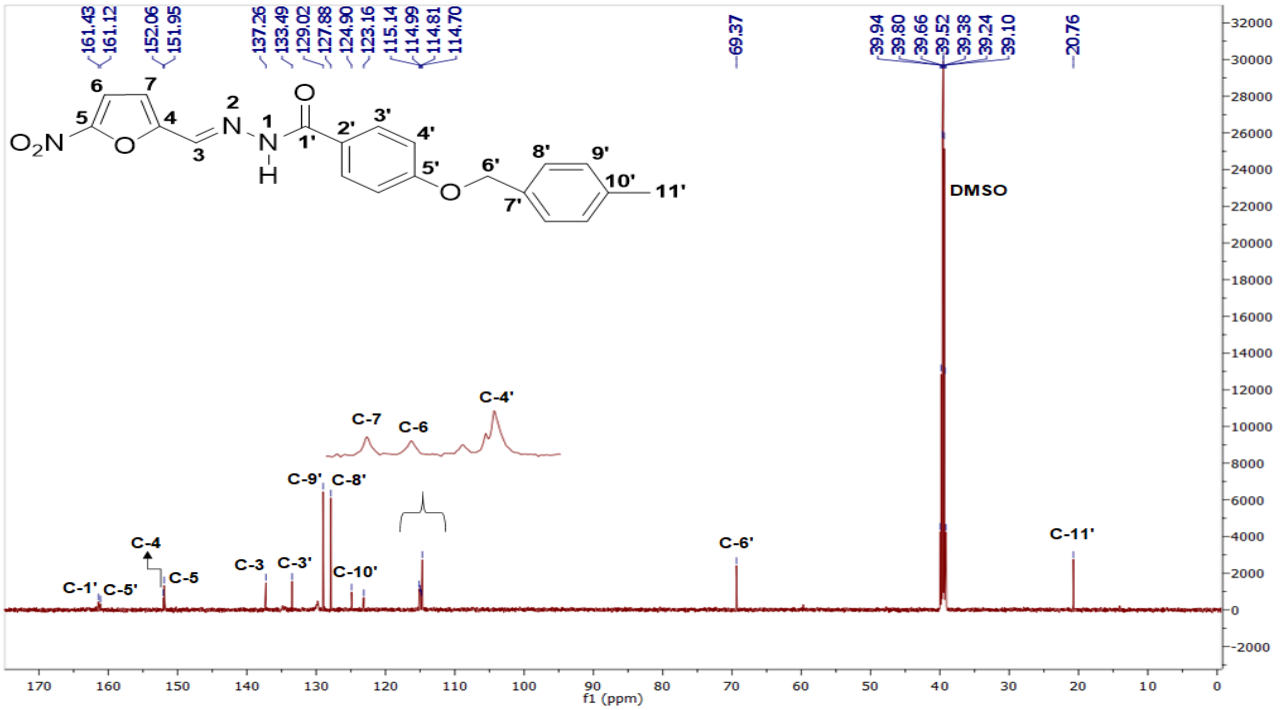
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
366.1092	1	C 19 H 16 N 3 O 5	100.00	366.1084	-0.8	-2.1	0.7	13.5	even	ok

***(E)*-4-[(4-methylbenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide (2b)**

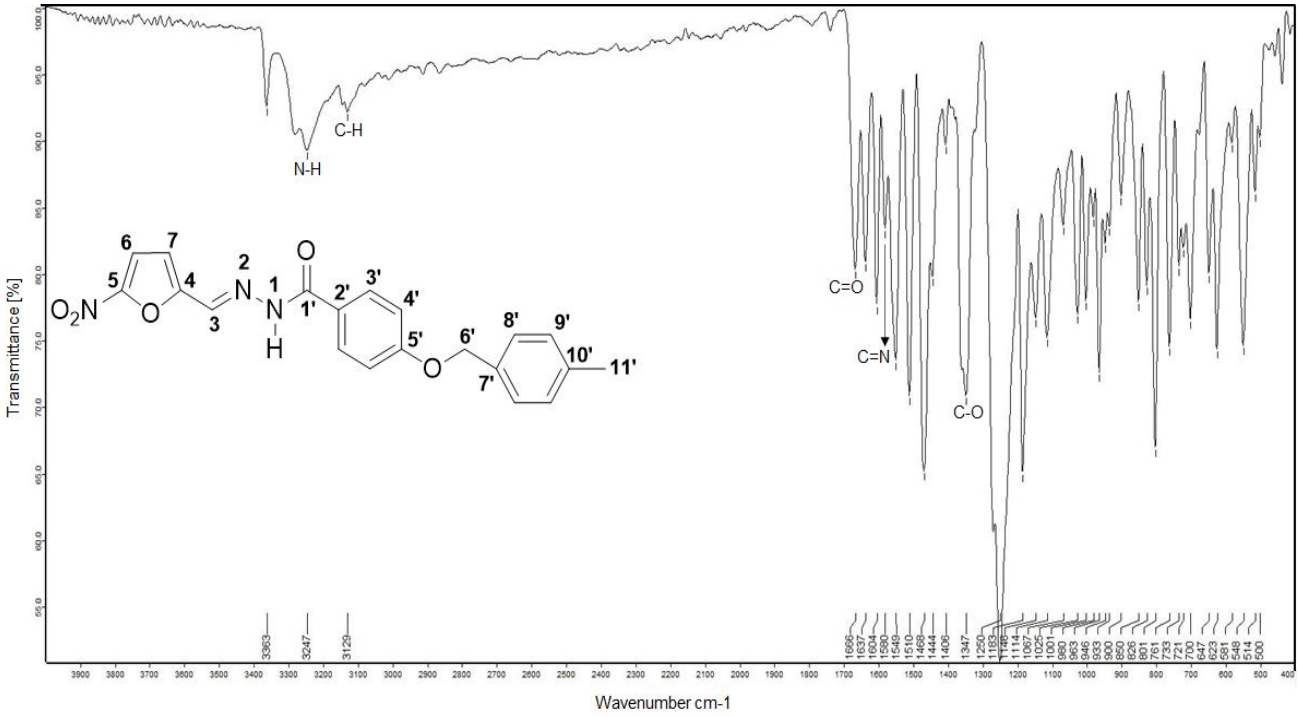
^{1}H NMR in DMSO



^{13}C NMR in DMSO



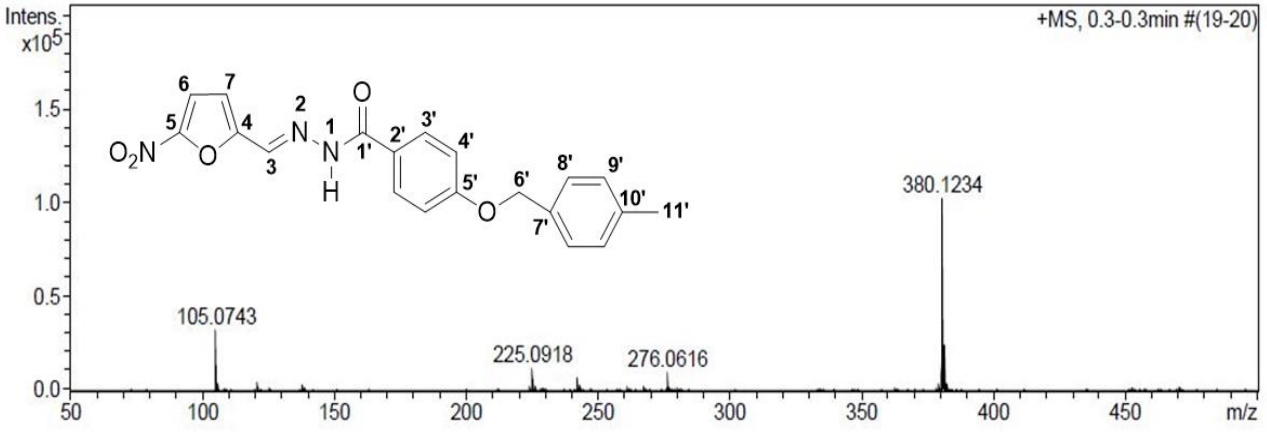
IR Spectrum



HRMS

Acquisition Parameter

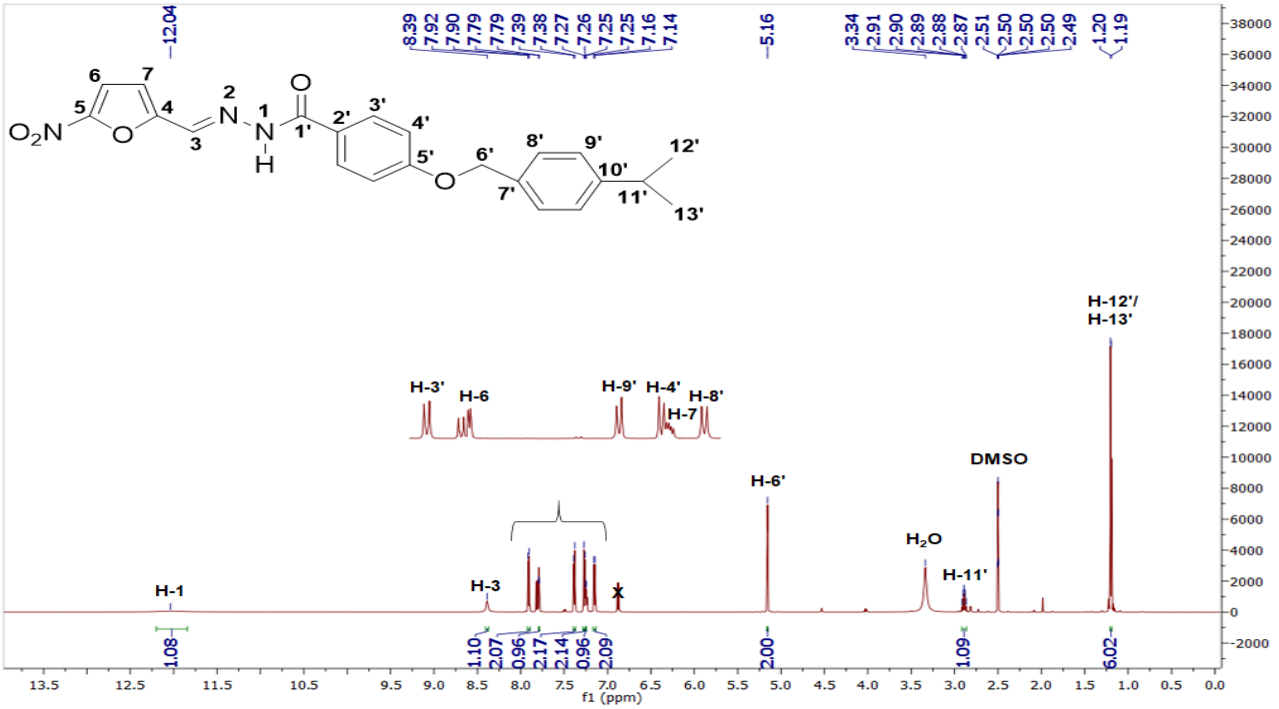
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



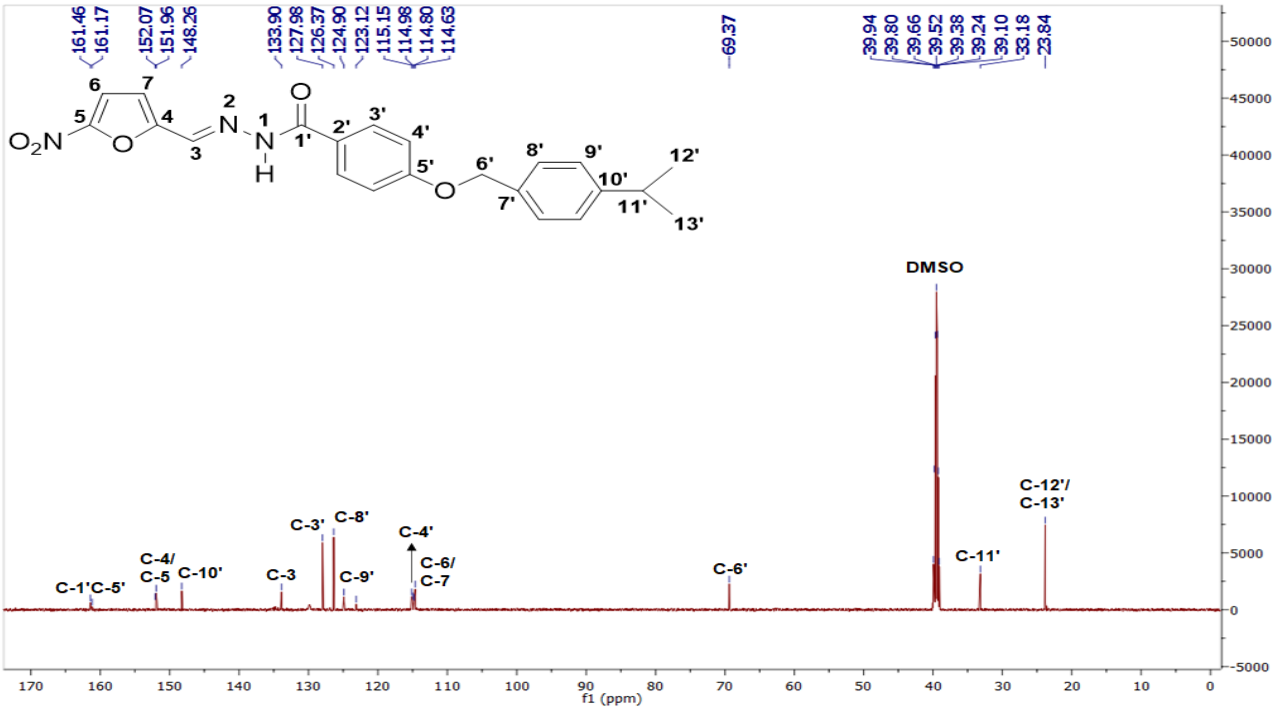
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
380.1234	1	C 20 H 18 N 3 O 5	100.00	380.1241	0.7	1.9	5.2	13.5	even	ok

(E)-4-[(4-isopropylbenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide (2c)

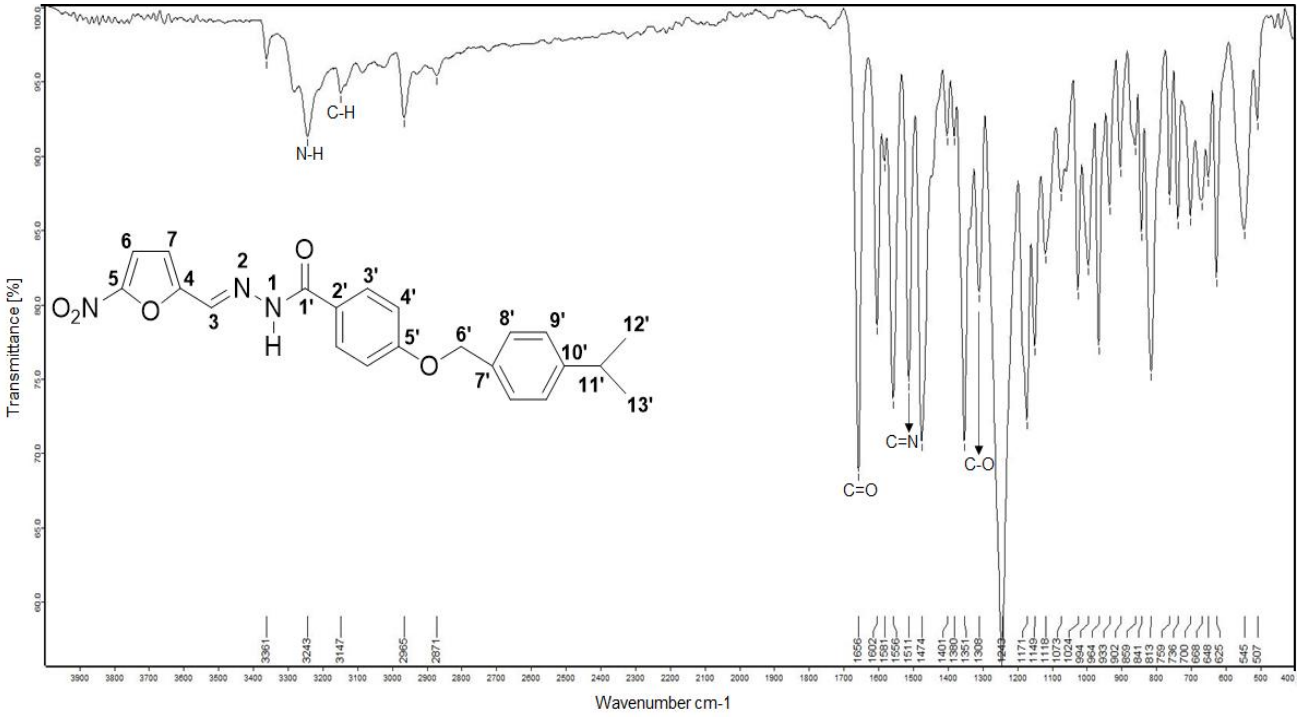
^{1}H NMR in DMSO



^{13}C NMR in DMSO



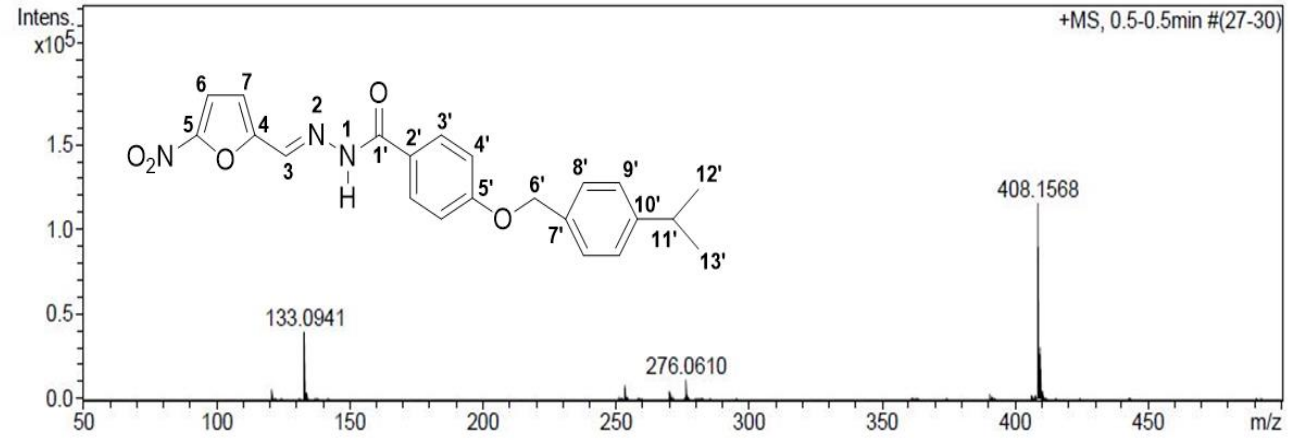
IR Spectrum



HRMS

Acquisition Parameter

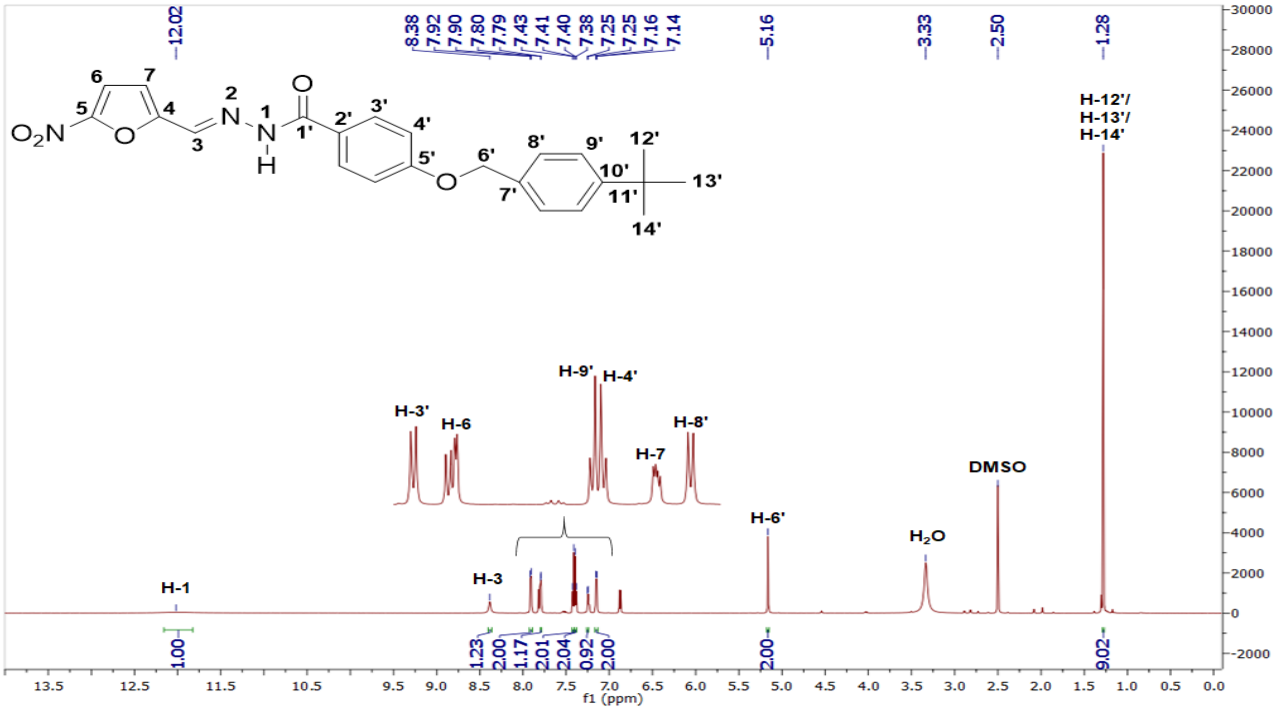
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



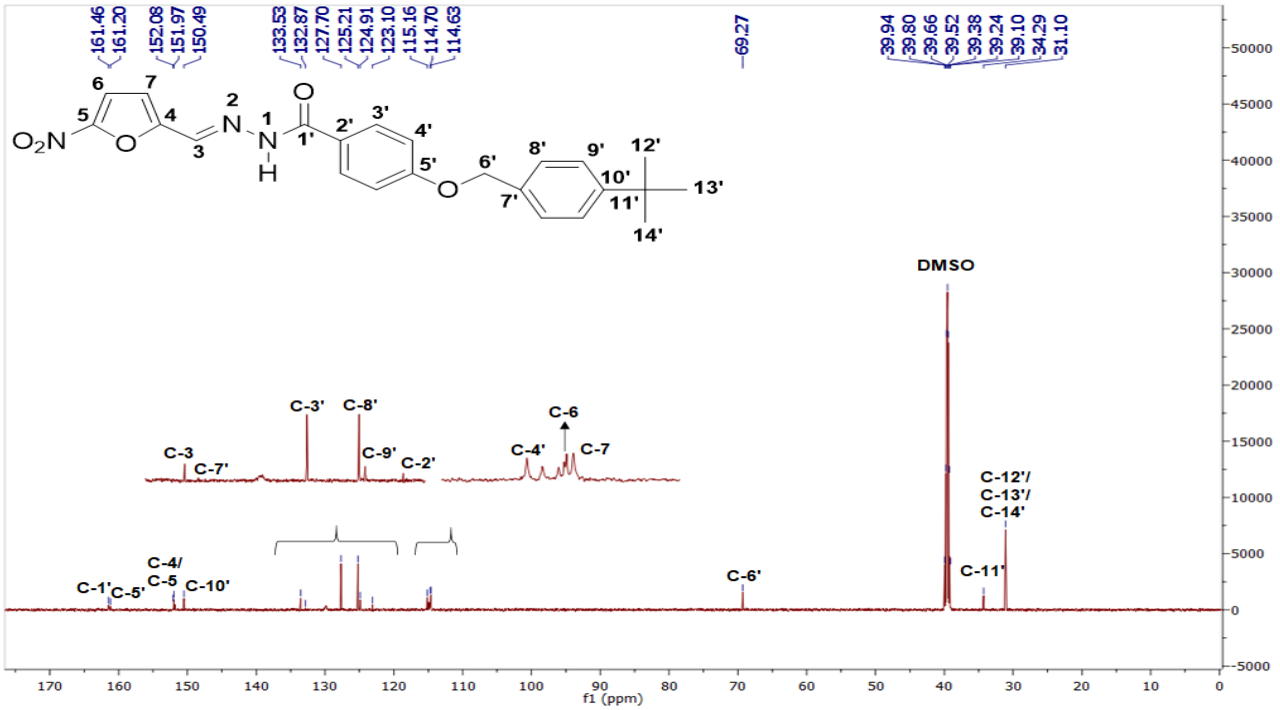
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
408.1568	1	C 22 H 22 N 3 O 5	100.00	408.1554	-1.4	-3.5	8.7	13.5	even	ok

***(E)*-4-[(4-{tert-butyl)benzyl]oxy}-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide (2d)**

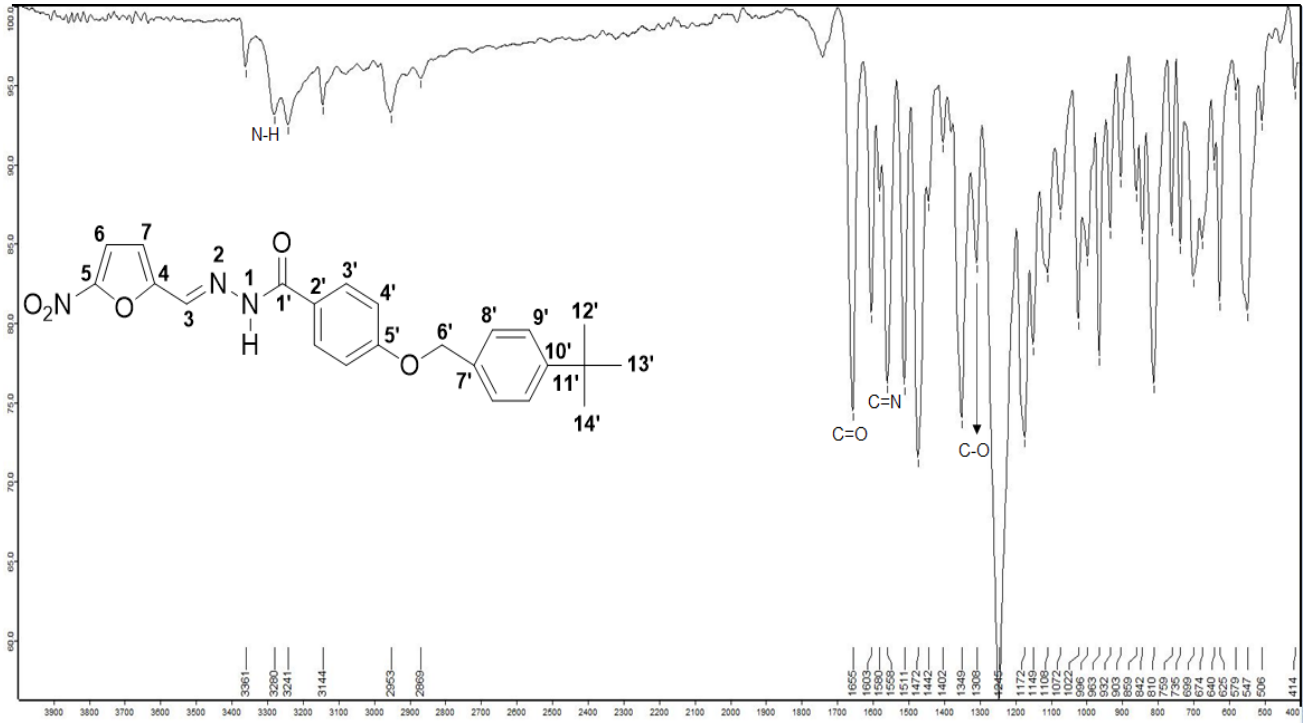
^{1}H NMR in DMSO



^{13}C NMR in DMSO

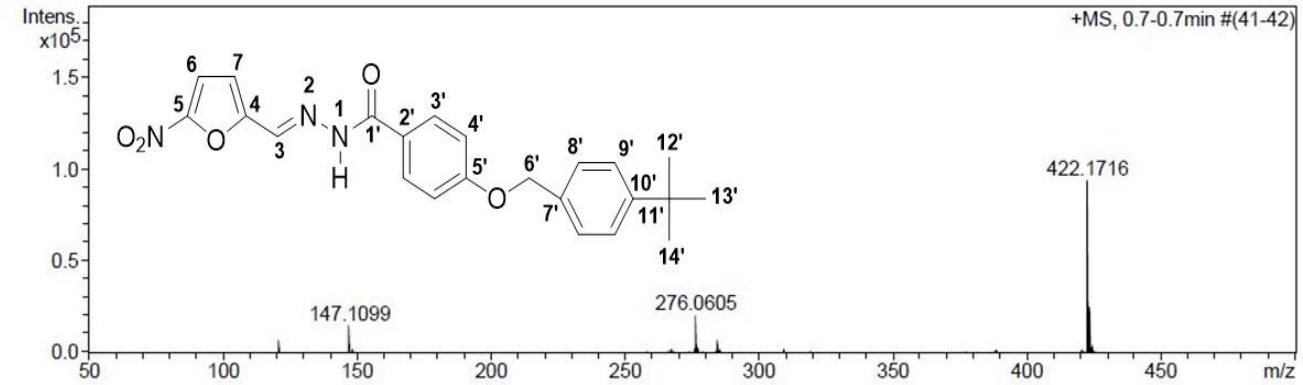


IR Spectrum



HRMS

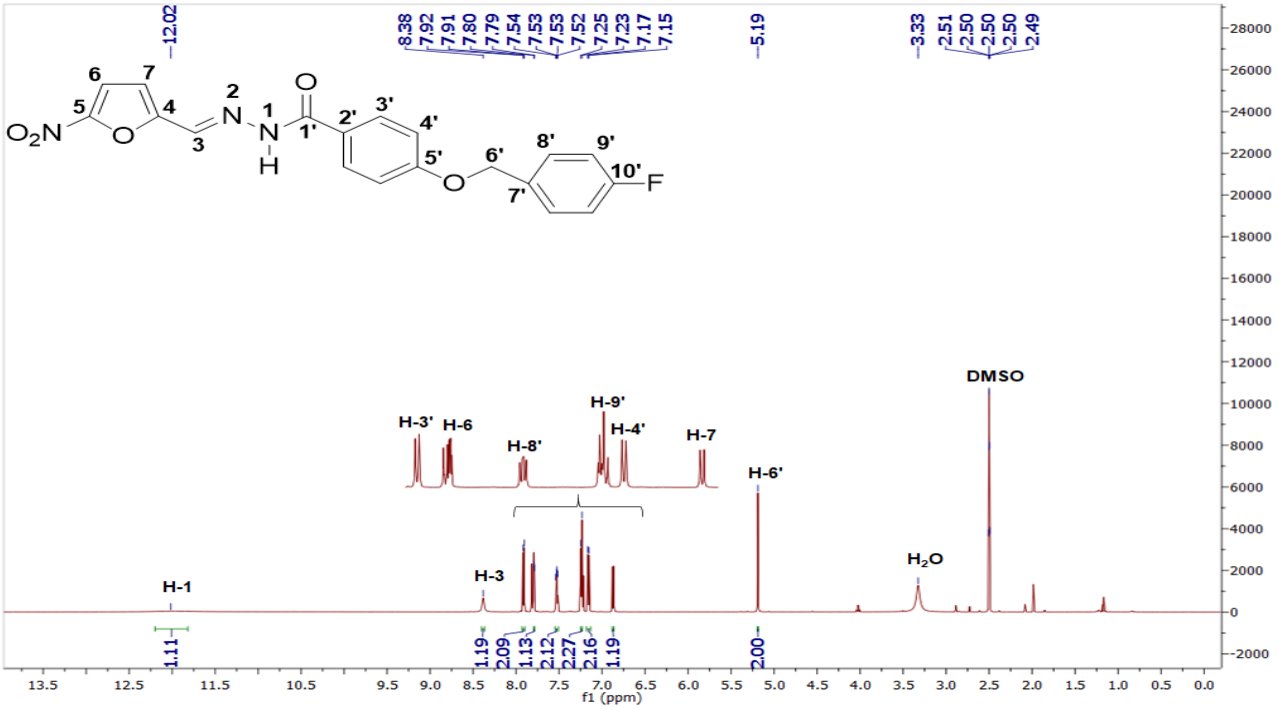
Acquisition Parameter					
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



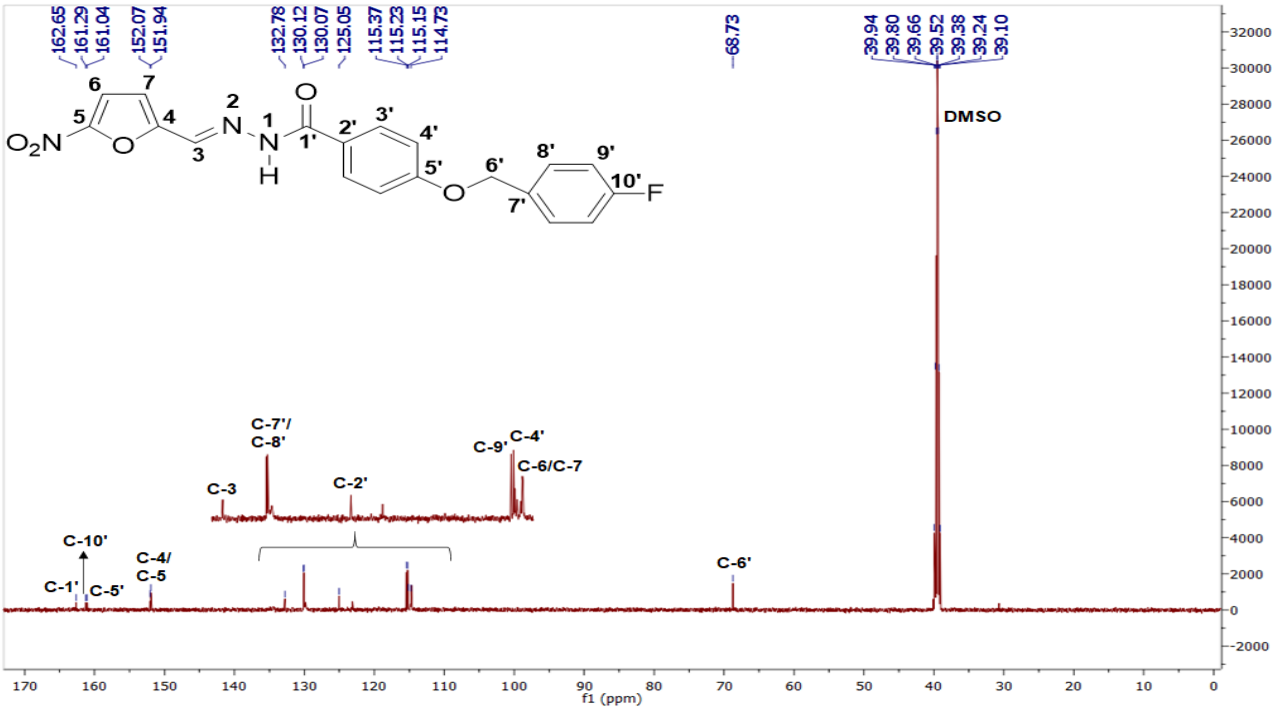
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
422.1716	1	C 23 H 24 N 3 O 5	100.00	422.1710	-0.6	-1.4	5.2	13.5	even	ok

(E)-4-[(4-fluorobenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide (2e)

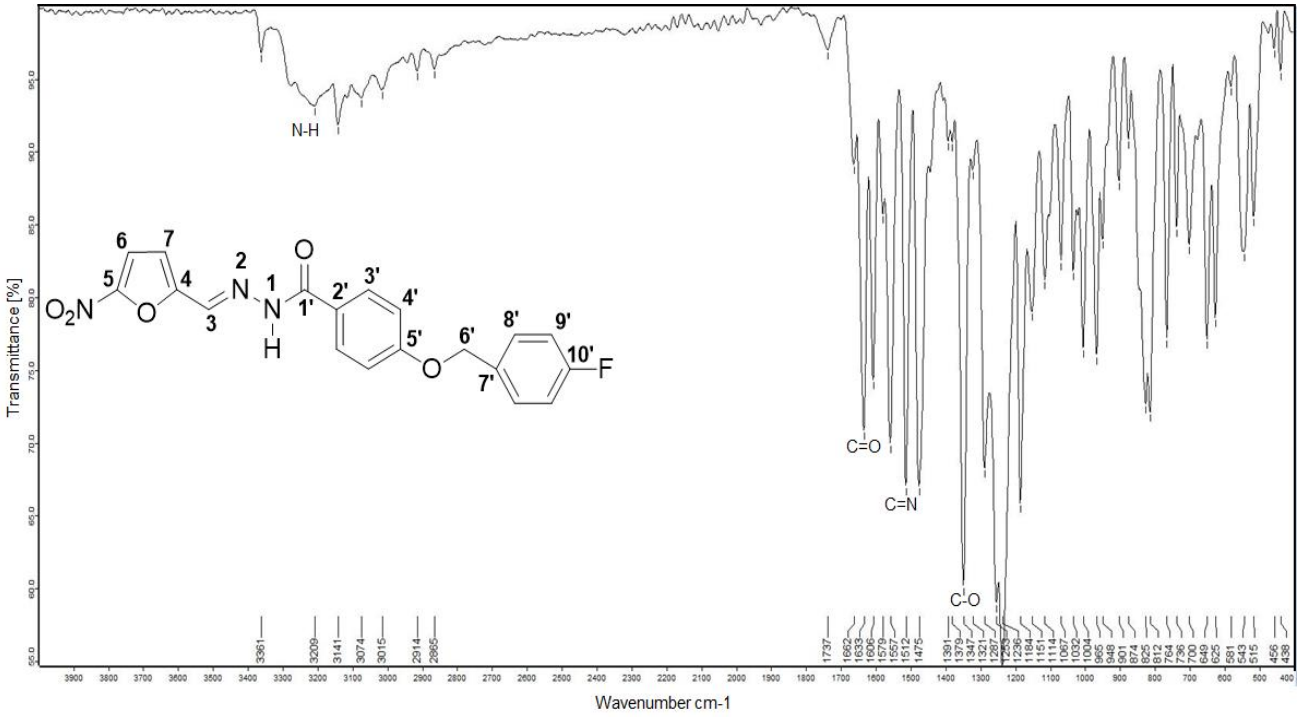
^{1}H NMR in DMSO



^{13}C NMR in DMSO



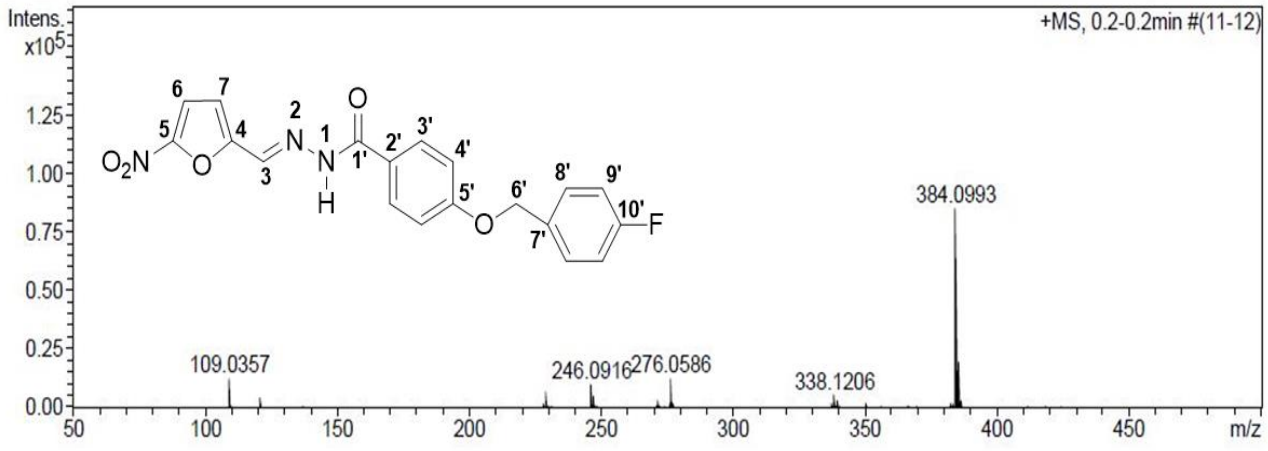
IR Spectrum



HRMS

Acquisition Parameter

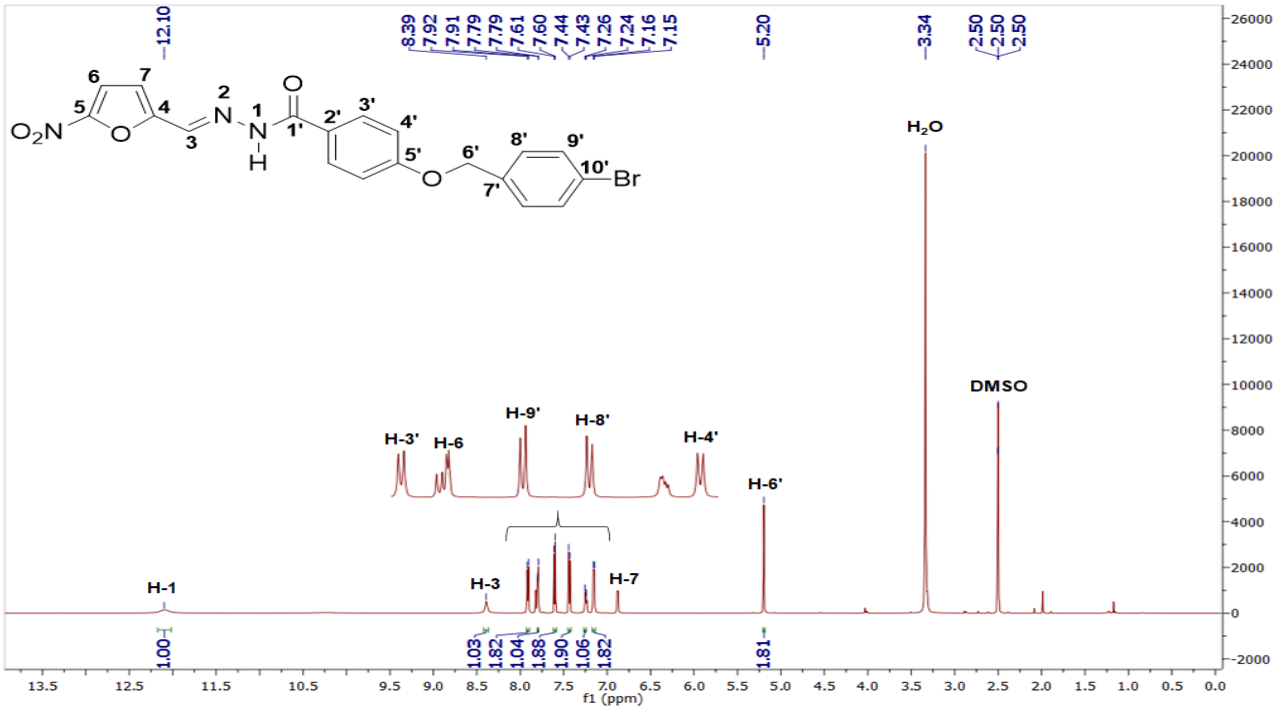
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



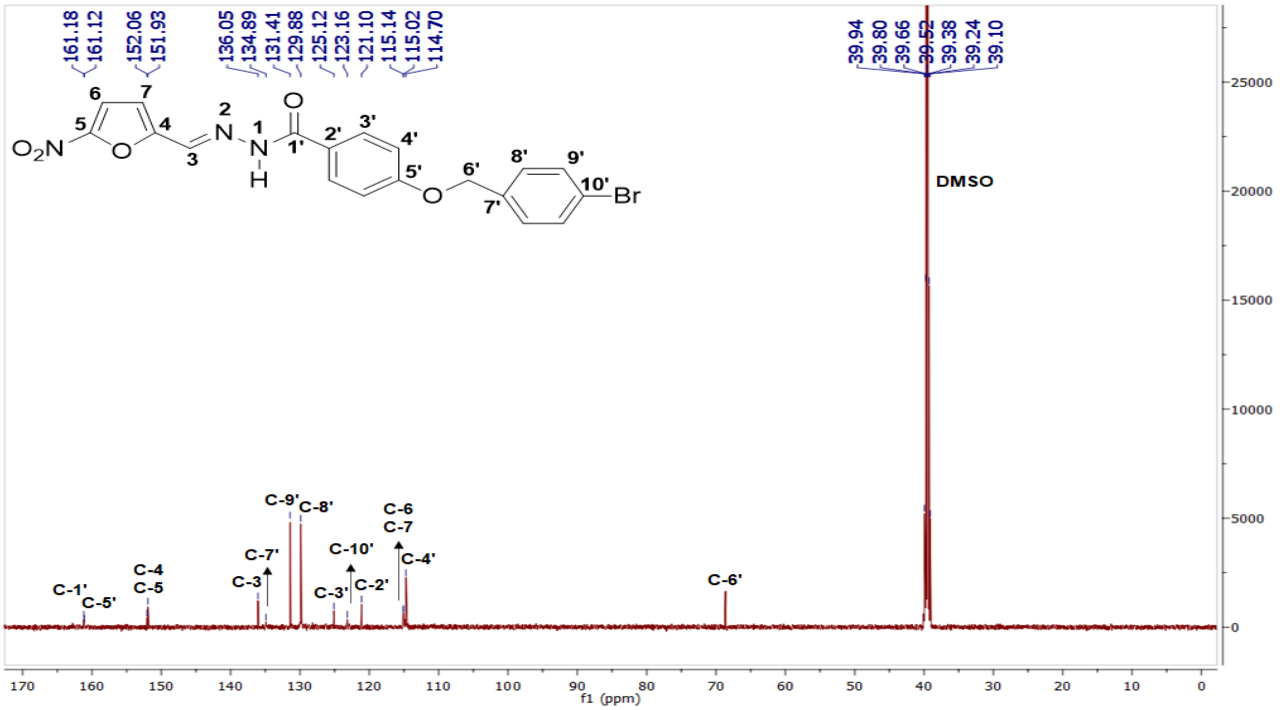
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
384.0993	1	C 19 H 15 F N 3 O 5	100.00	384.0990	-0.2	-0.6	10.1	13.5	even	ok

(E)-4-[(4-bromobenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide (2f)

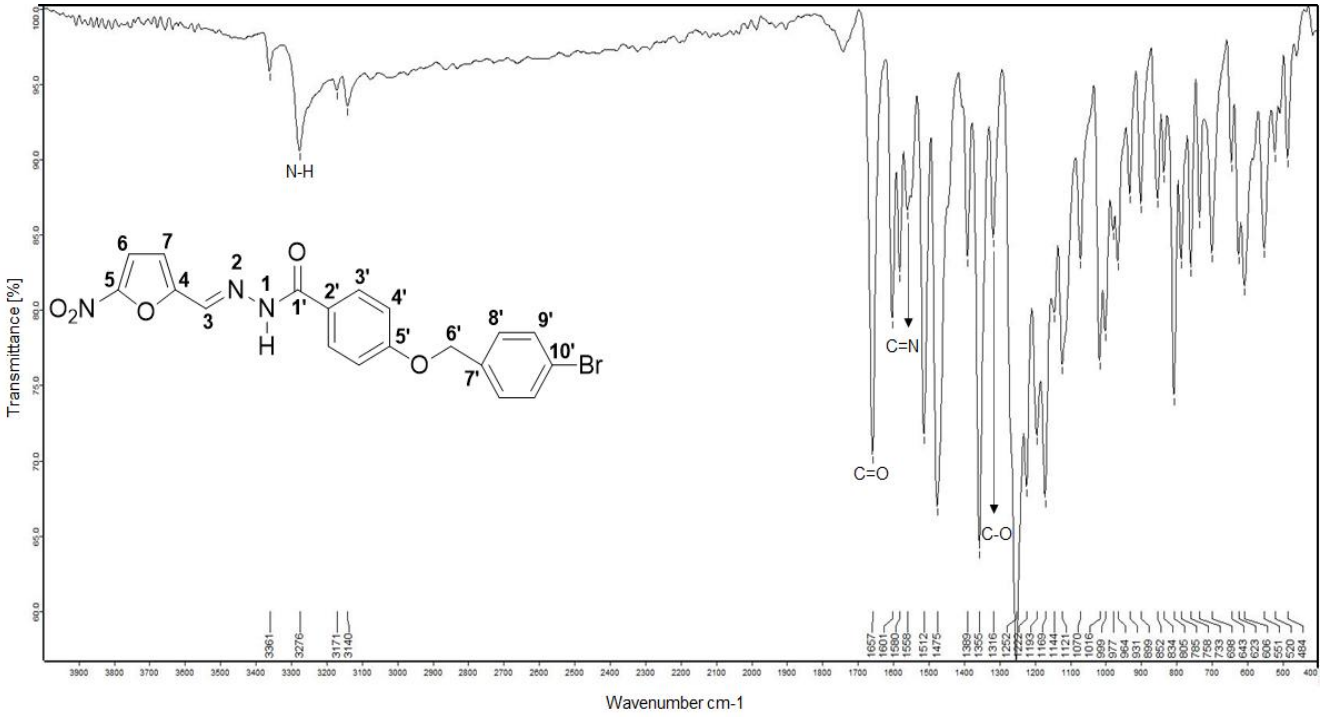
^{1}H NMR in DMSO



^{13}C NMR in DMSO



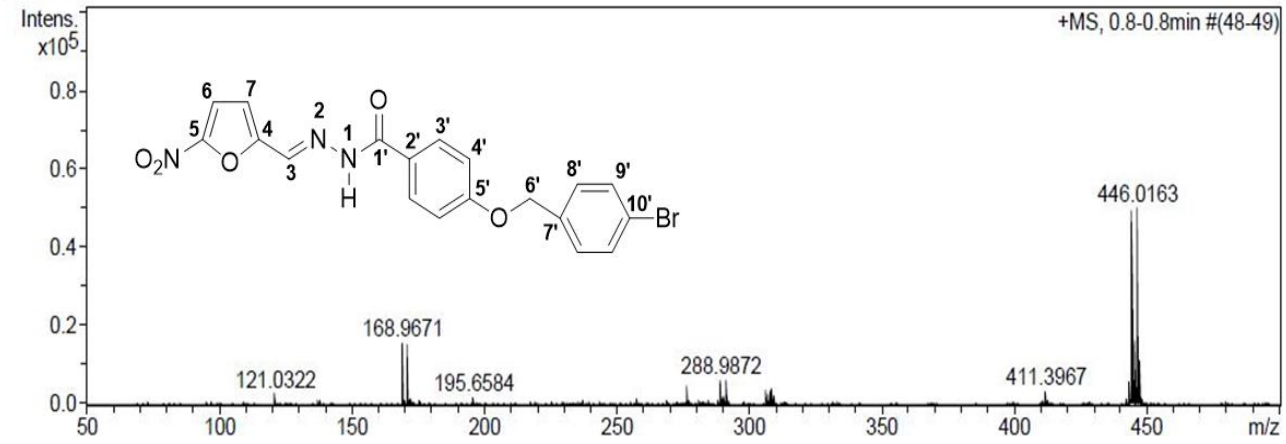
IR Spectrum



HRMS

Acquisition Parameter

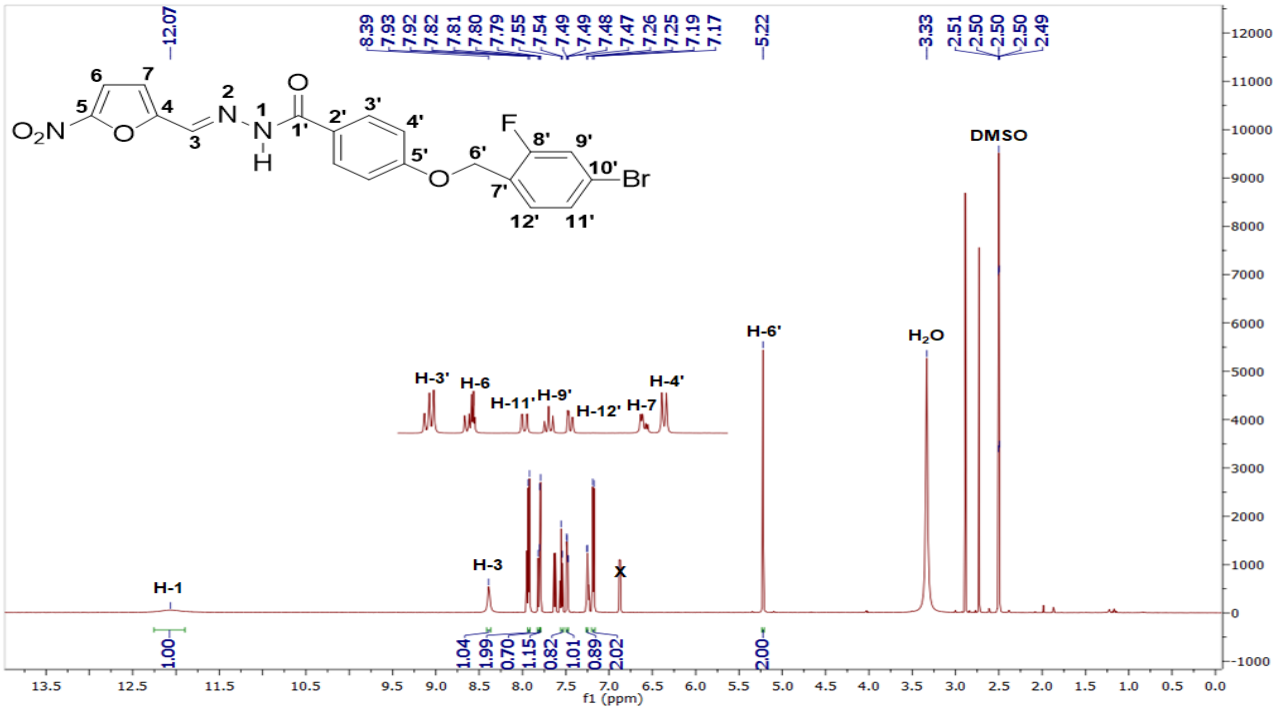
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



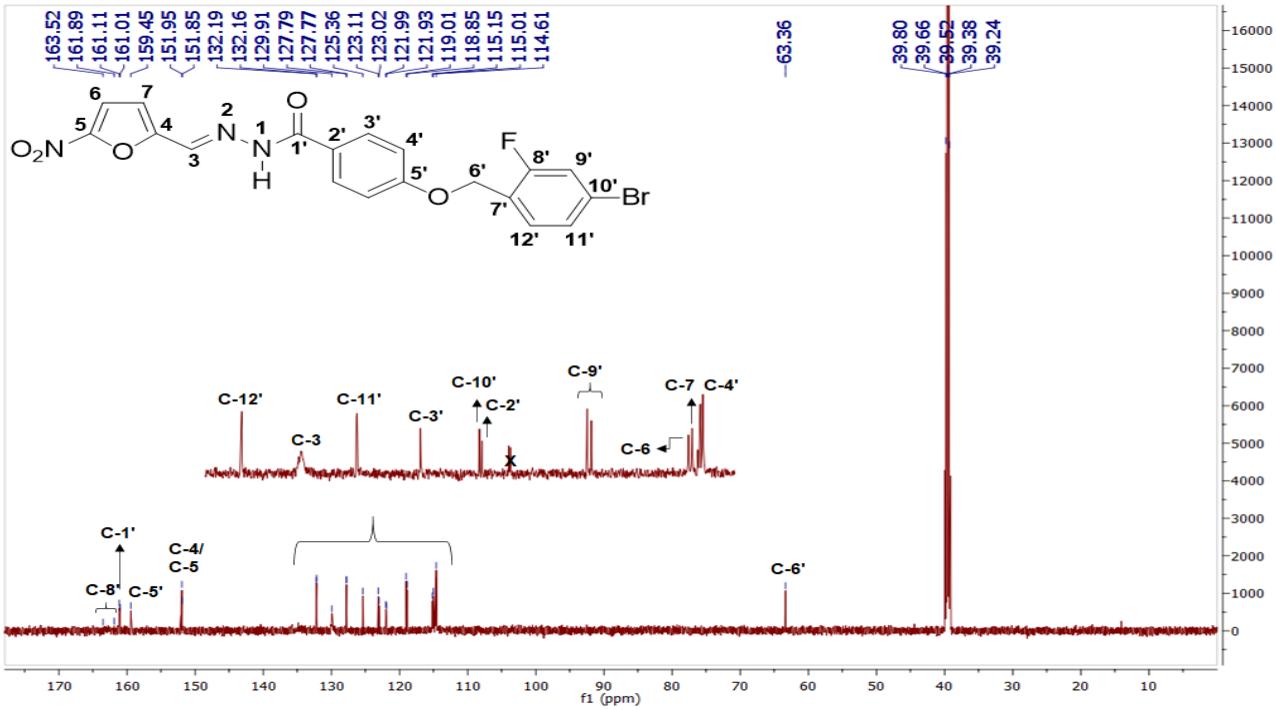
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
444.0171	1	C 19 H 15 Br N 3 O 5	100.00	444.0190	1.9	4.2	47.6	13.5	even	ok

***(E)*-4-[(4-bromo-2-fluorobenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide (2g)**

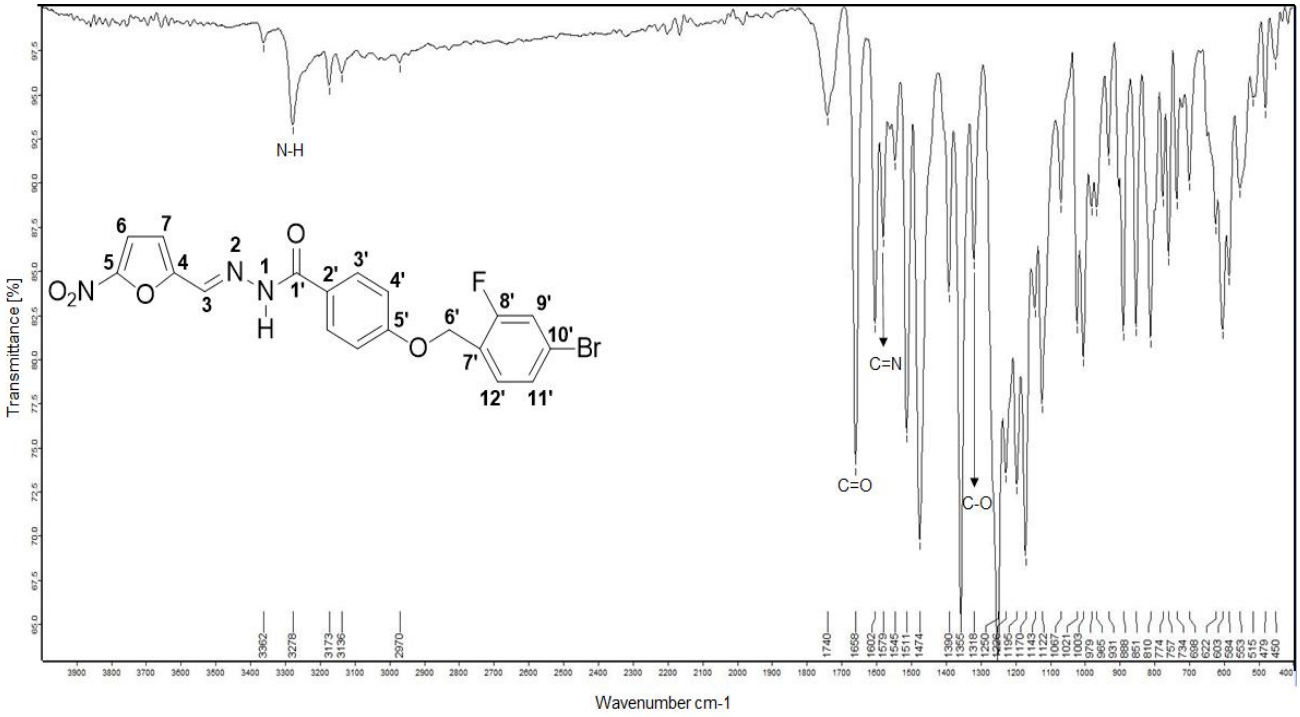
^{1}H NMR in DMSO



^{13}C NMR in DMSO



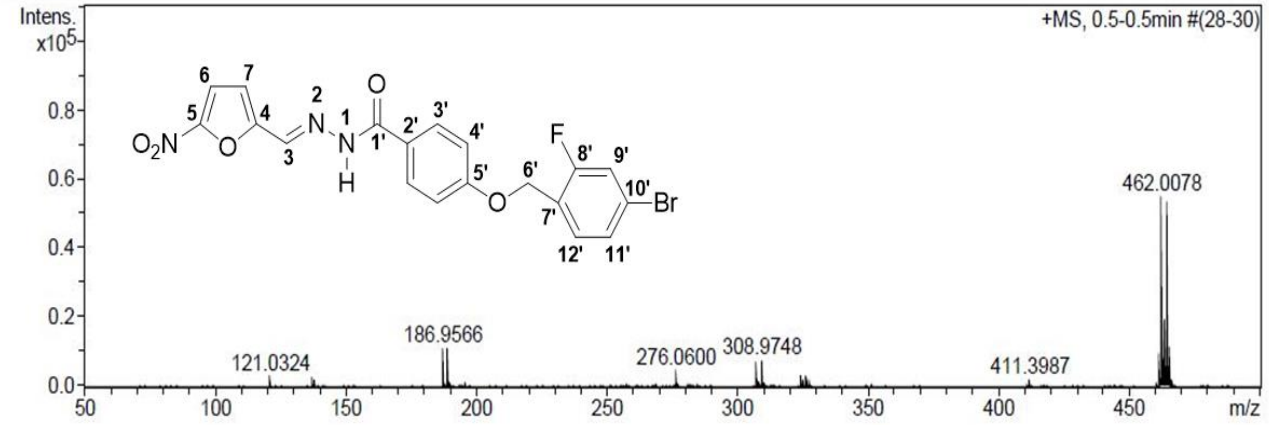
IR Spectrum



HRMS

Acquisition Parameter

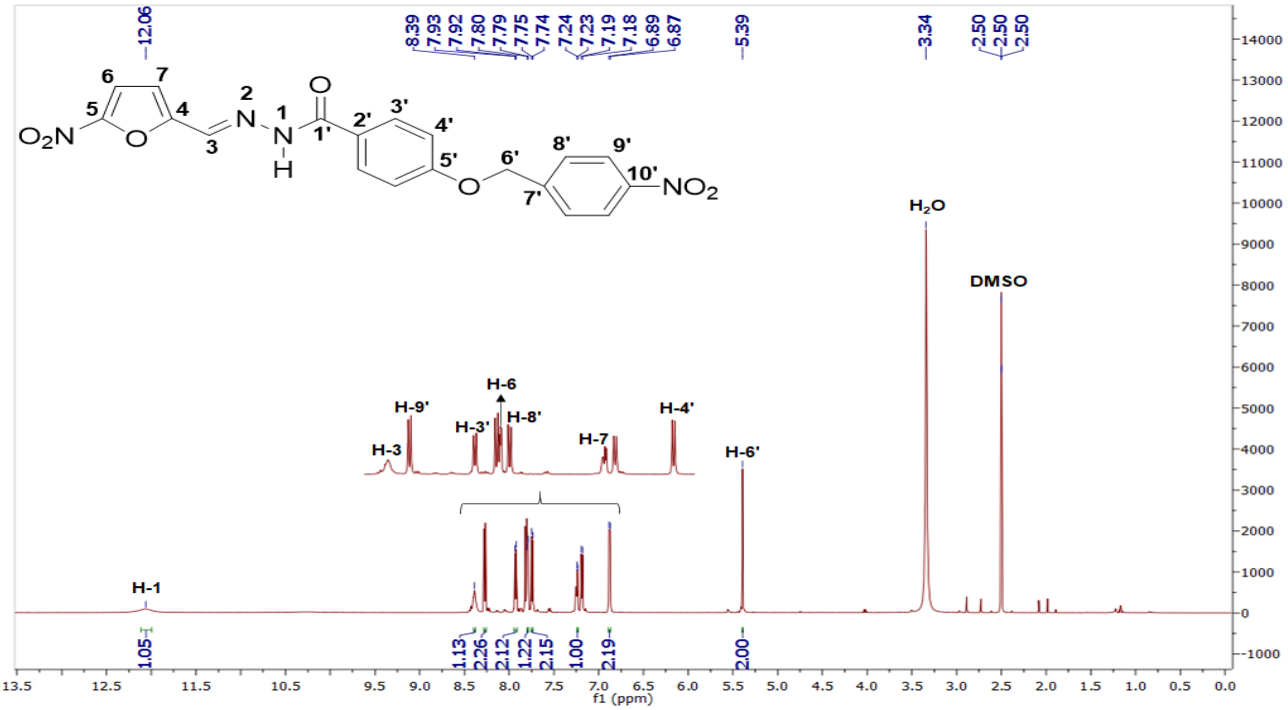
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



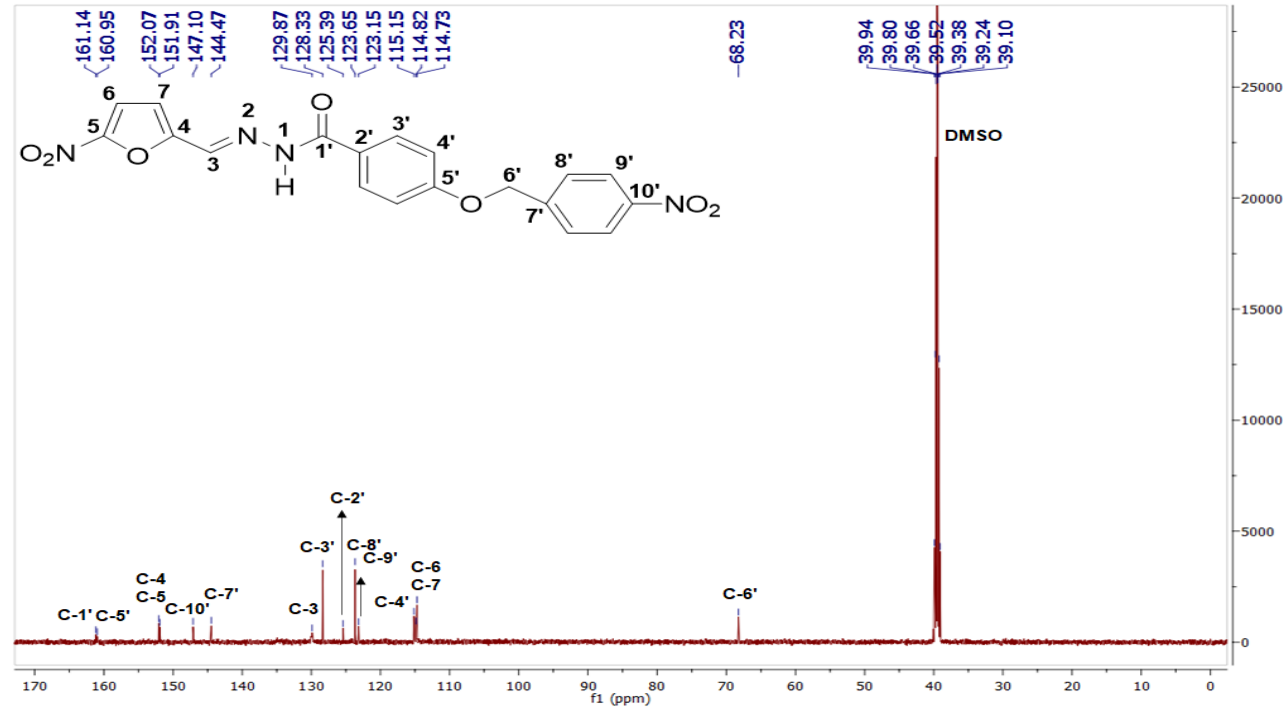
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
462.0078	1	C 19 H 14 Br F N 3 O 5	100.00	462.0095	1.8	3.8	63.4	13.5	even	ok

(E)-4-[(4-nitrobenzyl)oxy]-N'-[(5-Nitrofuran-2-yl)methylene]benzohydrazide (2h)

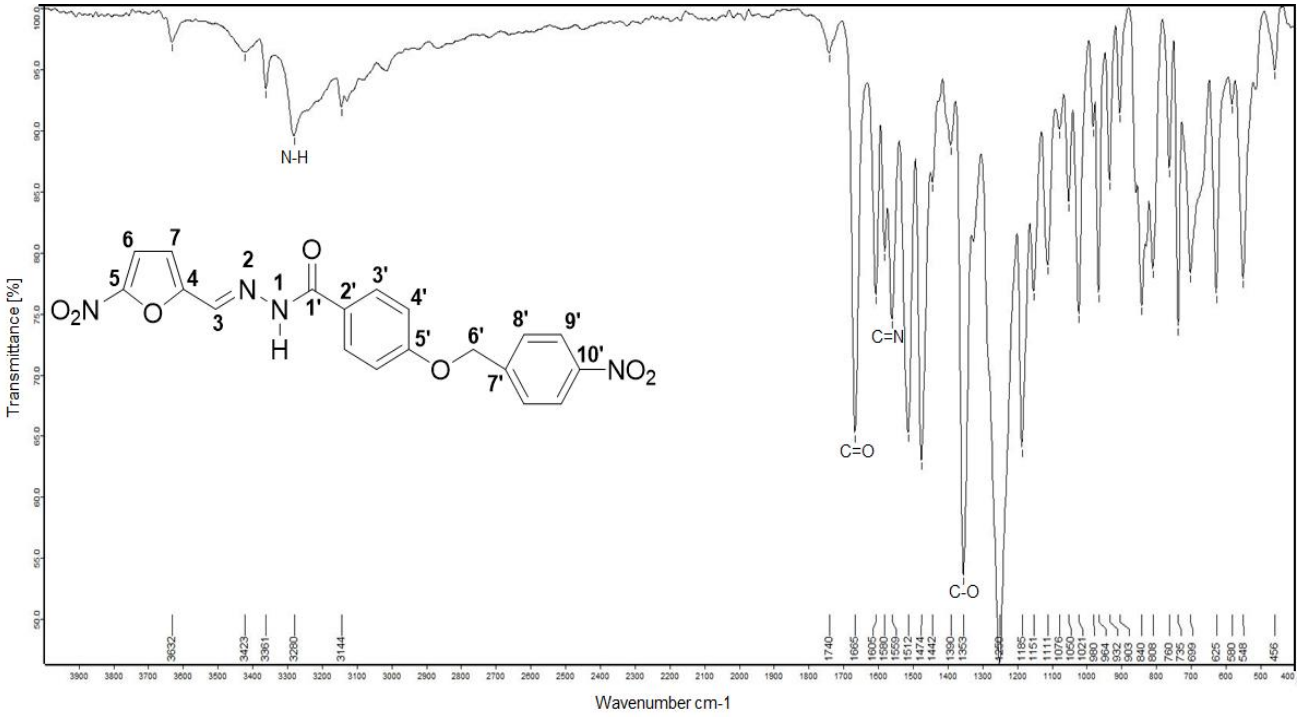
^{1}H NMR in DMSO



^{13}C NMR in DMSO



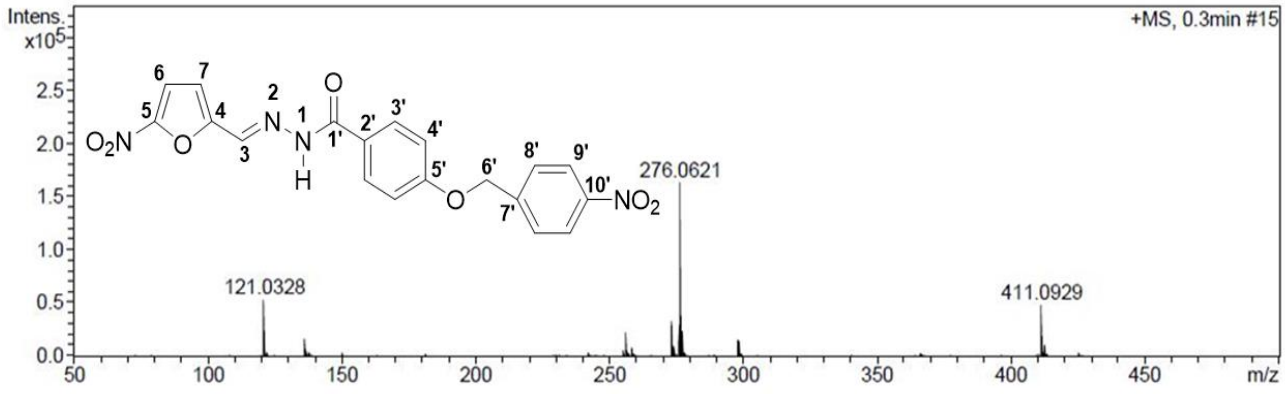
IR Spectrum



HRMS

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
411.0929	1	C 19 H 15 N 4 O 7	100.00	411.0935	0.6	1.5	21.8	14.5	even	ok

Annexure B: Ethics Approval Certificate (NWU-HREC).

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za

North-West University Health Research Ethics Committee (NWU-HREC)

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

16 August 2021

RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Health Research Ethics Committee (NWU-HREC) on 16/08/2021, 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 *in vitro* antileishmanial efficacy of novel O-substituted derivatives of Nifuroxazide
Principal Investigator/Study Supervisor/Researcher: Prof DD N'Da
Student: GD Badenhorst - 27129039

Ethics number:

N	W	U	-	0	0	2	6	2	-	2	1	-	A	1
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 16/08/2021

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-HRECApply@nwu.ac.za or 018 299 1206*

Special conditions of the research approval due to the COVID-19 pandemic:

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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: 2021.08.17 13:47:12 +02'00'

NWU-HREC Chairperson

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Head of the Faculty of Health Sciences Ethics Office for Research, Training and Support

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Annexure C: Author Guidelines of The Journal of Bioorganic & Medicinal Chemistry

BIOORGANIC & MEDICINAL CHEMISTRY

The Tetrahedron Journal for Research at the Interface of Chemistry and Biology

AUTHOR INFORMATION PACK

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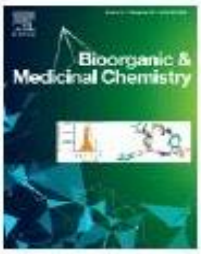

ISSN: 0968-0896

DESCRIPTION

Bioorganic & Medicinal Chemistry publishes complete accounts of research of outstanding significance and timeliness on all aspects of molecular interactions at the interface of chemistry and biology, together with critical review articles. The journal publishes reports of experimental results in medicinal chemistry, chemical biology and drug discovery and design, emphasizing new and emerging advances and concepts in these fields. The aim of the journal is to promote a better understanding at the molecular level of life processes, and living organisms, as well as the interaction of these with chemical agents.

The Journal welcomes papers on: the medicinal chemistry and associated biology (including target identification and validation) of established or new disease targets the reporting of the discovery, design or optimization of potent new compounds or biological agents the analysis and discussion of structure-activity relationships and pharmacological issues relevant to drug design and action using in vitro and in vivo models, including the use of computational techniques when closely linked to experimental data the reporting of "first-in-class" new therapeutic compounds the chemical biology or bioorganic/bioinorganic chemistry that significantly advances knowledge of a biological mechanism methodological advances that are chemistry-based and which significantly impact on medicine or biology the preparation and examination of biotherapeutics for the treatment of pathophysiological disease states the development of materials for specific therapeutic targeting

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AUDIENCE

Chemists, Medicinal Chemists, Pharmacologists, Biochemists, Molecular Biologists.

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5. Cancer Research UK. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/; 2003 Accessed 13 March 2003.
Reference to a dataset:
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