Synthesis and *in vitro* anti-protozoan activities of nitrofuran-based azine derivatives

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Dissertation accepted in fulfilment of the requirements for the degree Master of Science in Pharmaceutical Chemistry at the North-West University

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Graduation: December 2021

Student number: 27352935

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.

Chapter 1: Introduction and Problem Statement

Chapter 2: Literature Review

Chapter 3: Article for submission

Synthesis and *in vitro* antileishmanial activities of nitrofuran-based azine derivatives

This article will be submitted to the European Journal of Pharmaceutical sciences and was prepared according to author's guidelines, accessible in Annexure C and available on the Journal's homepage in the author information pack:

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

Chapter 4: Summary of the study

ACKNOWLEDGEMENTS

I hereby wish to express my sincere gratitude to the following individuals and institutions:

- My supervisor Prof. D.D. N'Da and my co-supervisors Dr. C Kannigadu and Dr. J Aucamp for their guidance and support throughout my MSc degree.
- Dr. D. Otto and Dr. J. Jordaan for NMR and HRMS spectroscopy.
- The NWU for financial support.
- To my family and friends for their love, kindness, and emotional support.
- To my fiancé, Shaun, for supporting and encouraging me throughout everything.
- To our Heavenly Father for providing me with the strength and endurance needed to complete my degree

.

ABSTRACT

Neglected tropical diseases (NTDs) are a group of diverse infectious conditions that are closely connected to poverty and affects more than a billion people worldwide. Leishmaniasis is a vector-borne NTD that is caused by the *Leishmania* parasite. Up to one million new cases of leishmaniasis are reported annually and current treatments are limited to a few chemotherapeutic drugs with significant toxicity, administration, cost, and parasite resistance issues. Therefore, there is an urgent need to search for new, safe, and affordable antileishmanial drugs.

In this study, the antileishmanial activity of several nitrofuran-based azine derivatives was investigated. The synthesised derivatives consist of three active pharmacophores, namely the nitrofuran, hydrazone and aromatic ring. Nitrofurans possess a broad range of biological activity against various diseases, including leishmaniasis. Therefore, in this study two sub-series of compounds (nitrofuran and nitrothiophene derivatives) were synthesised using the two-step process of hydrazone formation and Schiff base reactions. The derivatives were screened for activity against *L. donovani* and *L. major* promastigotes.

The antileishmanial activity ranged from good (IC_{50} = 0.42 µM) to no activity (IC_{50} >100 µM), with the nitrofuran sub-series exhibiting better activity compared to the nitrothiophene sub-series. The cytotoxicity of the compounds ranged from moderately toxic to non-toxic (IC_{50} 11.56- >100 µM). Compounds **3a**, **5a** and **7a** had good activity against all three *Leishmania* strains and were non-toxic, thus are potential anti-promastigote hits for further investigation. These derivatives could serve as building blocks for the development of future antileishmanial agents.

Keywords: *Leishmania*, promastigote, cytotoxicity, nitrofuran, nitrothiophene

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LIST OF ABBREVIATIONS

µM	Micromolar
AmB	Amphotericin B
APCI	Atmospheric pressure chemical ionisation
ATP	Adenosine triphosphate
CDC	Centers for Disease Control and Prevention
CL	Cutaneous leishmaniasis
cNFs	Clinical Nitrofurans
DAT	Direct agglutination test
DMEM	Dalbecco's modified Eagle's medium
DMF	*N*,*N*-dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDG	Electron donating group
ELISA	Enzyme-linked immunosorbent assays
EM	Emetine
EtOAc	Ethyl acetate
EtOH	Ethanol
EWG	Electron withdrawing group
FBS	Fetal bovine serum
FZD	Furazolidone
HAT	Human African trypanosomiasis
HIV	Human immunodeficiency virus
HRMS	High resolution mass spectrometry
IC_{50}	50% inhibitory concentration
IFA	Indirect fluorescent antibody test
IM	Intramuscular

IR	Infrared
IV	Intravenous
L-AmB	Liposomal amphotericin B
LFT	Liver function test
M199	Media 199
MCL	Mucocutaneous leishmaniasis
MHz	Megahertz
m.p.	Melting point
NADH	Nicotinamide adenine dinucleotide + hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate + hydrogen
NEAA	Non-essential amino acid
NFA	5-Nitro-2-furaldehyde
NFT	Nitrofurantoin
NFX	Nifuroxazide
NFZ	Nitrofurazone
NMR	Nuclear magnetic resonance
NNN	Novy-McNeal-Nicolle
NTA	5-Nitrothiophene-2-carboxaldehyde
NTD	Neglected tropical disease
PAHO	Pan American Health Organization
Pen/Strep	Penicillin-streptomycin
ppm	Parts per million
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
rt	Room temperature
SAR	Structure activity relationships
SD	Standard deviation

SI	Selectivity index
spp	Species
$t_{1/2}$	Terminal half-life
TB	Tuberculosis
TEA	Triethylamine
TLC	Thin layer chromatography
TPSA	Topological polar surface area
UK	United Kingdom
VL	Visceral leishmaniasis
WHO	World Health Organization

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 Introduction

Leishmaniasis is a group of vector-borne protozoan parasitic diseases that is caused by more than twenty *Leishmania* species (Georgiadou *et al*., 2015:44). It is transmitted to humans through the bite of an infected female phlebotomine sandfly and it can manifest as three clinical forms, namely visceral (VL), cutaneous (CL) and mucocutaneous (MCL), depending on the infecting *Leishmania* species (Salam *et al*., 2014:1; Braga, 2019:1; WHO, 2021*a*).

VL is the most severe form of the disease, and it can be contributed to the *L. donovani* and/or *L. infantum* species (CDC, 2021). VL affects the internal organs with symptoms such as fever, anaemia, weight loss, enlarged organs, and pancytopenia (Braga, 2019:2; CDC, 2021; WHO, 2021*a*) and this disease is usually fatal unless treated (WHO, 2021*a*). CL on the other hand, is predominantly caused by the species *L. tropica*, *L. major*, *L. aethiopica*, *L. infantum*, *L. donovani*, and *L. mexicana* (CDC, 2021) and these infections results in the development of skin lesions that heal very slowly, leaving behind atrophic scars (Braga, 2019:1). MCL is caused by the species *L. braziliensis*, *L. panamensis* or *L. guyanesis* and this form of the disease results in partial or complete destruction of soft and hard tissues of the palate, pharynx, and nose (Braga, 2019:2; CDC, 2021).

In general, diseases become neglected for three reasons: (1) when they are isolated to certain areas and are not a global threat, (2) when there is no financial benefit in treating them, and (3) when the disease is not considered deadly. Leishmaniasis is a NTD that is prevalent in tropical and sub-tropical developing countries and mostly affects poor individuals in rural areas (Wilson *et al*., 2020:2; WHO, 2021*b*;). There are no financial incentives to properly address leishmaniasis (Erber *et al*., 2020:2); although, the annual death toll is currently about 30 000 deaths per year (PAHO, 2020). Drugs currently available on their market to treat leishmaniasis have certain shortcomings, i.e., impractical intravenous administration (apart from the oral drug, miltefosine), toxicity, high cost, low efficacy and drug resistance (Georgiadou *et al*., 2015:46; Valle *et al*., 2019:2; Karamysheva *et al*., 2020:7). Drug efficacy is a major problem as it varies according to the infecting *Leishmania* species and the infection region. Another issue that is commonly experienced is infection relapse and this occurs especially in human immunodeficiency virus (HIV)-coinfected patients (Boelaert & Sundar, 2014:645).

Treatment of NTDs such as leishmaniasis are mostly in the form of global or organisational aid or donations to these poverty-stricken areas (WHO, 2021*c*). Furthermore, the cutaneous (skin

lesion) form is the most common form of leishmaniasis (CDC, 2021); hence, the disease is commonly regarded as debilitating, but not necessarily life threatening. This results in a perceived reduced need for global intervention as focus is rather placed on more deadly diseases (Erber *et al*., 2020:2).

In the past few years, there have been various efforts, such as vector control, disease surveillance, animal reservoir host control, early diagnosis and treatment, and social mobilisation, to control the spread of leishmaniasis (WHO, 2021*a*). However, the currently available statistics indicate that these methods have not yet been successful. Although there was a decrease in new VL cases in 2019 (13 809 cases) compared to 2018 (17 093 cases) (WHO, 2021*d*), there was an increase in new CL cases (277 058 cases reported in 2019 compared to 261 285 cases in 2018) (WHO, 2021*e*).

The treatment of leishmaniasis relies on a handful of diverse chemotherapeutic drugs, such as the pentavalent antimonies (sodium stibogluconate and meglumine antimonate), amphotericin B, miltefosine, pentamidine, paromomycin and the azoles (ketoconazole, itraconazole and fluconazole) (Figure **1-1**) (Georgiadou *et al*., 2015:46; Braga, 2019:2). Leishmaniasis is a difficult disease to diagnose, since it can present itself with symptoms that are similar to those of other infectious diseases such as malaria, Chagas' disease, tuberculosis (TB), schistosomiasis, and typhoid fever (Camargo & Langoni, 2006:534).

The reduction in efficacy and drug resistance of the parasites against antileishmanial treatment have led to the development and clinical implementation of combinational therapies. Combination therapy has the potential to shorten treatment duration, increase patient compliance and reduce medication dosage, thereby reducing toxicity, cost, and the risk of drug resistance development (WHO, 2010). Combination therapies that are currently in use include sodium stibogluconate with paromomycin, and miltefosine with amphotericin B (Uliana *et al*., 2018:471).

Although rare, there has been a report of resistance to combination therapy by *L. donovani* promastigotes. It was found that these parasites can develop resistance to miltefosine/paromomycin and sodium stibogluconate/paromomycin combinations (Ponte-Sucre *et al*., 2017:14). Hence, there is an urgent need for the discovery of new, safe and effective drugs to treat this disease (Capela *et al*., 2019:1).

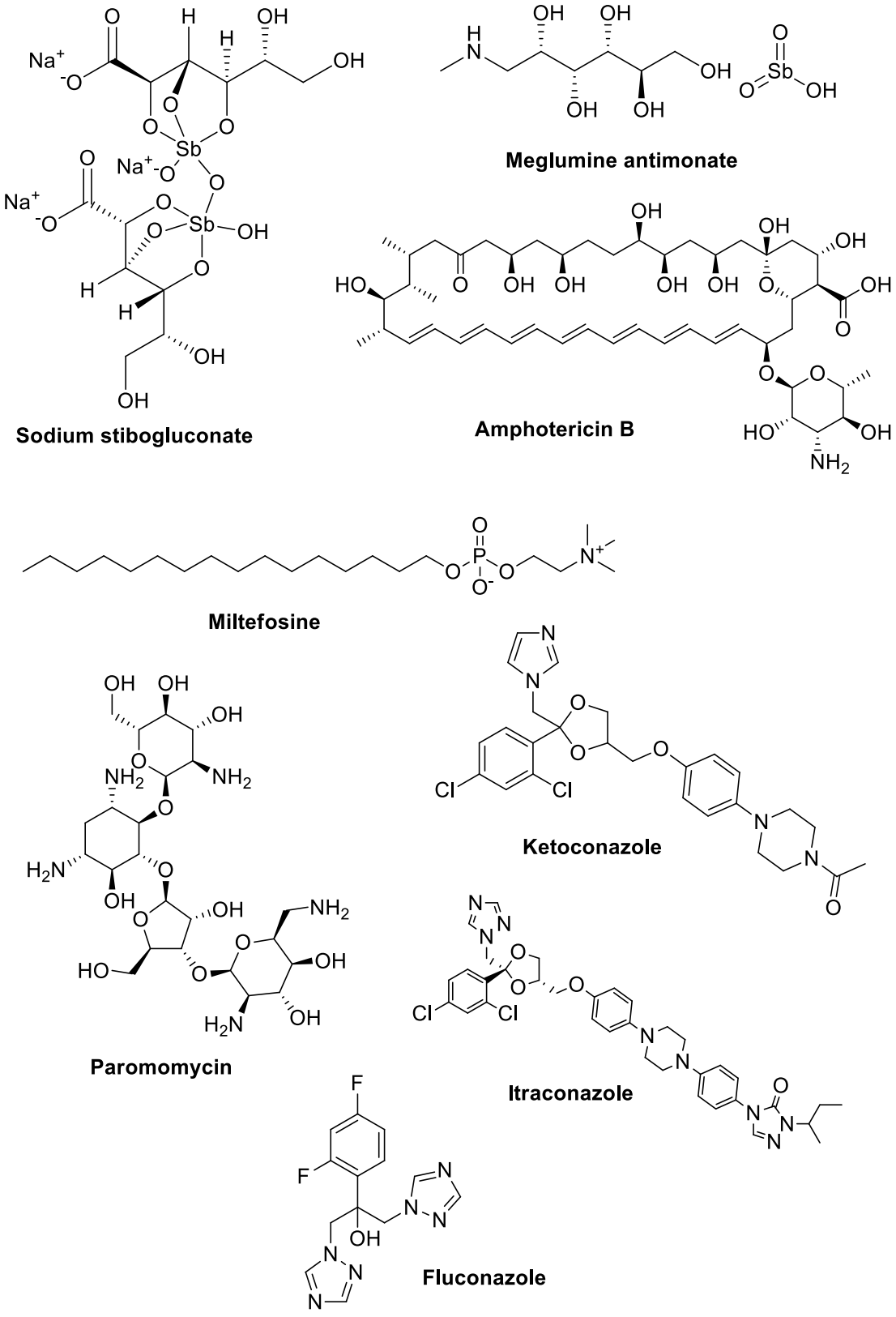


Figure **1-1**: Current clinical antileishmanial chemotherapeutic agents

1.2 Rationale for this project

Research has indicated that the nitrofuran class of drugs are biologically active against a broad spectrum of diseases, such as tuberculosis (Krasavin *et al*., 2019:128), cancer (Peterson, 2018:1439), urinary tract infections (Huttner *et al*., 2015:2456), and giardiasis (Ryan, 2018:1567). It has also been successfully used as pharmacophores in the treatment of Chagas disease (Sales Junior *et al*., 2017:1289), caused by flagellated protozoan parasites of the same family (*Trypanosomatidae*) and order (Kinetoplastida) as *Leishmania*.

Previous studies have also indicated that the nitrofuran moiety has good antileishmanial activity. In a study conducted by Sifontes-Rodríguez *et al*. (2015), they investigated the antileishmanial activity of nitrofuran-derivatives and found that all their compounds exhibited activity against the promastigote forms of *L. amazonensis* (MHOM/77/LTB0016 strain), *L. braziliensis* (MHOM/BR/75/M2 903 strain), and *L. infantum* (MHOM/FR/78/LEM75 strain). The activity IC_{50} values ranged between 0.8 µM to 4.7 µM against all three strains (Sifontes-Rodríguez *et al*., 2015:169).

Another study reported four antileishmanial promising nitrofuran-derivatives against the promastigote and amastigote forms of *L. major* with IC_{50} values in the 0.08 – 2.00 µM range (Tahghighi *et al*., 2011:2605).

Thus, the nitrofuran scaffold seems to be a good starting block to use in the discovery of new antileishmanial drugs. In this study, the antileishmanial activity of nitrofuran-based azine derivatives was investigated in an effort to discover potentially new anti-infective compounds. This study introduced three pharmacophores in the targeted compounds (Figure **1-2**). The nitrofuran moiety (green) was used as a starting block to enhance the drugs' anti-parasitic activity. The second pharmacophore, the azine bond (blue), is the linker between the nitrofuran and the aromatic ring and this group also contributes to the activity of the compound as it also possess' a broad spectrum of activity. The aromatic ring (red), pharmacophore three, not only brings stability to the compound but also contributes to its overall activity.

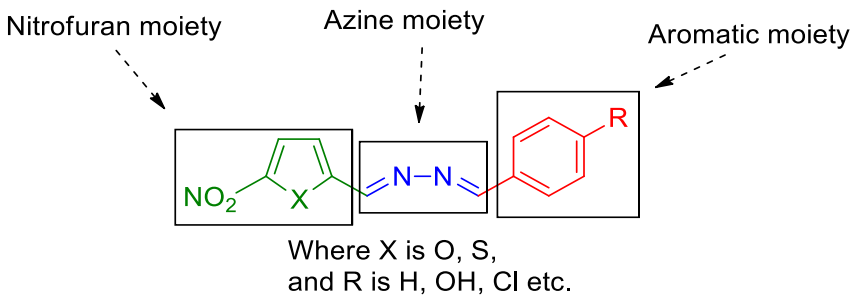


Figure **1-2**: General structure of target nitrofuran-based azine derivatives

1.3 Aim and objectives

The aim of this study is to investigate the antileishmanial activity of nitrofuran-based azine derivatives.

Objectives of this study are:

- To synthesise a series of 18 nitrofuran-based azine derivatives with the general structure as depicted in Figure **1-2**.
- To characterise the synthesised derivatives using routine techniques such as nuclear magnetic resonance (NMR), high resolution mass spectrometry (HRMS), and infrared (IR) spectroscopy.
- To assess *in vitro* antileishmanial activity of the synthesised compounds.
- To assess *in vitro* cytotoxicity of the active compounds using mammalian cell lines.

REFERENCE LIST

Boelaert, M. & Sundar, S. 2014. Leishmaniasis. In: Farrar, J., Hotez, P.J., Junghanss, T., Kang, G., Lalloo, D. & White, N.J., eds. *Manson's Tropical Infectious Diseases (Twenty-third Edition)*. London: W.B. Saunders. pp. 631-651.e4.

Bot, C., Hall, B.S., Álvarez, G., Di Maio, R., González, M., Cerecetto, H. & Wilkinson, S.R. 2013. Evaluating 5-nitrofurans as trypanocidal agents. *Antimicrobial Agents and Chemotherapy,* 57(4):1638-1647.

Braga, S.S. 2019. Multi-target drugs active against leishmaniasis: A paradigm of drug repurposing. *European Journal of Medicinal Chemistry,* 183, art. #111660. doi:10.1016/j.ejmech.2019.111660

Camargo, L.B. & Langoni, H. 2006. Impact of leishmaniasis on public health. *Journal of Venomous Animals and Toxins including Tropical Diseases,* 12(4):527-548.

Capela, R., Moreira, R. & Lopes, F. 2019. An overview of drug resistance in protozoal diseases. *International Journal of Molecular Sciences*, 20(22), art. #5748. doi:10.3390/ijms20225748

Centers for Disease Control and Prevention (CDC). 2021. *Parasites- Leishmaniasis: Resources for health professionals*. https://www.cdc.gov/parasites/leishmaniasis/health_professionals/index.html#tx Date of access: 16 Jun. 2021.

Erber, A.C., Arana, B., Ben Salah, A., Bennis, I., Boukthir, A., Castro Noriega, M.D.M., … Olliaro, P. 2020. Patients' preferences of cutaneous leishmaniasis treatment outcomes: Findings from an international qualitative study. *PLoS Neglected Tropical Diseases*, 14(2), e0007996. https://doi.org/10.1371/journal.pntd.0007996

Georgiadou, S.P., Makaritsis, K.P. & Dalekos, G.N. 2015. Leishmaniasis revisited: Current aspects on epidemiology, diagnosis and treatment. *Journal of translational internal medicine*, 3(2):43-50.

Huttner, A., Verhaegh, E.M., Harbarth, S., Muller, A.E., Theuretzbacher, U. & Mouton, J.W. 2015. Nitrofurantoin revisited: a systematic review and meta-analysis of controlled trials. *Journal of Antimicrobial Chemotherapy*, 70(9):2456-2464.

Karamysheva, Z.N., Guarnizo, S.A.G. & Karamyshev, A.L. 2020. Regulation of translation in the protozoan parasite *Leishmania*. *International Journal of Molecular Sciences*, 21(8), art. #2981. doi:10.3390/ijms21082981

Krasavin, M., Lukin, A., Vedekhina, T., Manicheva, O., Dogonadze, M., Vinogradova, T., … Sokolovich, E. 2019. Attachment of a 5-nitrofuroyl moiety to spirocyclic piperidines produces non-toxic nitrofurans that are efficacious *in vitro* against multidrug-resistant *Mycobacterium tuberculosis*. *European Journal of Medicinal Chemistry,* 166:125-135.

PAHO (Pan American Health Organization). 2020. *Leishmaniasis*. https://www3.paho.org/en/topics/leishmaniasis Date of access: 19 Jun. 2021.

Peterson, R.T. 2018. 5-Nitrofurans and cancer: teaching an old drug new tricks. *Cell Chemical Biology,* 25(12):1439-1440.

Ponte-Sucre, A., Gamarro, F., Dujardin, J.-C., Barrett, M.P., López-Vélez, R., García-Hernández, R., … Papadopoulou, B. 2017. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLoS Neglected Tropical Diseases*, 11(12), e0006052. https://doi.org/10.1371/journal.pntd.0006052

Ryan, E.T. 2018. Antiparasitic agents. In: Long, S.S., Prober, C.G. & Fischer, M., eds. *Principles and Practice of Pediatric Infectious Diseases (Fifth edition)*. Philadelphia, PA: Elsevier. pp. 1567-1587.e2.

Salam, N., Al-Shaqha, W.M. & Azzi, A. 2014. Leishmaniasis in the middle East: incidence and epidemiology. *PLoS neglected tropical diseases*, 8(10), e3208. https://doi.org/10.1371/journal.pntd.0003208

Sales Junior, P.A., Molina, I., Fonseca Murta, S.M., Sánchez-Montalvá, A., Salvador, F., Corrêa-Oliveira, R. & Carneiro, C.M. 2017. Experimental and clinical treatment of chagas disease: a review. *The American journal of tropical medicine and hygiene*, 97(5):1289-1303.

Sifontes-Rodríguez, S., Monzote-Fidalgo, L., Castañedo-Cancio, N., Montalvo-Álvarez, A.M., López-Hernández, Y., Diogo, N.M., … Cabrera-Pérez, M.Á. 2015. The efficacy of 2-nitrovinylfuran derivatives against *Leishmania* in vitro and in vivo. *Memorias do Instituto Oswaldo Cruz*, 110(2):166-173.

Tahghighi, A., Marznaki, F.R., Kobarfard, F., Dastmalchi, S., Mojarrad, J.S., Razmi, S., … Foroumadi, A. 2011. Synthesis and antileishmanial activity of novel 5-(5-nitrofuran-2-y1)-1,3,4-

thiadiazoles with piperazinyl-linked benzamidine substituents. *European Journal of Medicinal Chemistry*, 46(6):2602-2608.

Uliana, S.R.B., Trinconi, C.T. & Coelho, A.C. 2018. Chemotherapy of leishmaniasis: present challenges. *Parasitology*, 145(4):464-480.

Valle, I.V., Machado, M.E., Araújo, C.D.C.B., Da Cunha-Junior, E.F., Da Silva Pacheco, J., Torres-Santos, E.C., … Sathler, P.C. 2019. Oral pentamidine-loaded poly(d,l-lactic-co-glycolic) acid nanoparticles: an alternative approach for leishmaniasis treatment. *Nanotechnology,* 30(45), art. #455102. doi:10.1088/1361-6528/ab373e

WHO (World Health Organization) Expert Committee on the Control of the Leishmaniases & World Health Organization. 2010. *Control of the leishmaniases: report of a meeting of the WHO Expert Commitee on the Control of Leishmaniases*, Geneva: World Health Organization. https://apps.who.int/iris/handle/10665/44412 Date of access: 21 Mar. 2021.

WHO. 2021*a*. *Leishmaniasis: Key facts*. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis Date of access: 16 Jun. 2021.

WHO. 2021*b*. *Control of Neglected Tropical Diseases*. https://www.who.int/teams/control-of-neglected-tropical-diseases Date of access: 7 Feb. 2021.

WHO. 2021*c*. *Leishmaniasis- Access to essential antileishmanial medicines and treatment.* https://www.who.int/leishmaniasis/research/en/ Date of access: 4 May 2021.

WHO. 2021*d*. *Number of cases of visceral leishmaniasis reported. Data by country*. http://apps.who.int/gho/data/node.main.NTDLEISHVNUM?lang=en Date of access: 11 Feb. 2021.

WHO. 2021*e*. *Number of cases of cutaneous leishmaniasis reported. Data by country*. http://apps.who.int/gho/data/node.main.NTDLEISHCNUM?lang=en Date of access: 11 Feb. 2021.

Wilson, A.L., Courtenay, O., Kelly-Hope, L.A., Scott, T.W., Takken, W., Torr, S.J. & Lindsay, S.W. 2020. The importance of vector control for the control and elimination of vector-borne diseases. *PLoS Neglected Tropical Diseases*, 14(1), e0007831. https://doi.org/10.1371/journal.pntd.0007831

CHAPTER 2:

LITERATURE REVIEW

2. Introduction

NTDs are a group of diverse infectious conditions that differ in their epidemiology, distribution, impact and control (Kuper, 2019:838). NTDs are closely connected to poverty and affects more than a billion people worldwide (CDC, 2018; Engels & Zhou, 2020:2). There are more than a hundred and forty-nine countries and territories affected by at least one NTD. However, low-income countries are generally affected the most and can be affected by at least five NTDs simultaneously (CDC, 2018). These diseases are mainly caused by a variety of pathogens such as bacteria, viruses and parasites, which are common in tropical and sub-tropical climates (Engels & Zhou, 2020:1; Hotez *et al*., 2020:2; Nweze *et al*., 2021:2).

Parasites are organisms that live in or on another organism and depend on their host for survival. Parasites that cause diseases in humans can be divided into three main categories: protozoa, helminths, and ectoparasites (CDC, 2020*a*). *Leishmania* is a vector-borne neglected tropical protozoan (single-celled eukaryotic) parasite that possess a flagellum for motility (Paget, 2011:86). They are also referred to as *haemoflagellates* because they are parasitic in the host's blood and require haematin obtained from it. *Leishmania* is part of the *Trypanosomatidae* family (order *Kinetoplastida*) and possess a unique organelle called the kinetoplastid, which appears to be a DNA rich part of the mitochondrion (Paget, 2011:86).

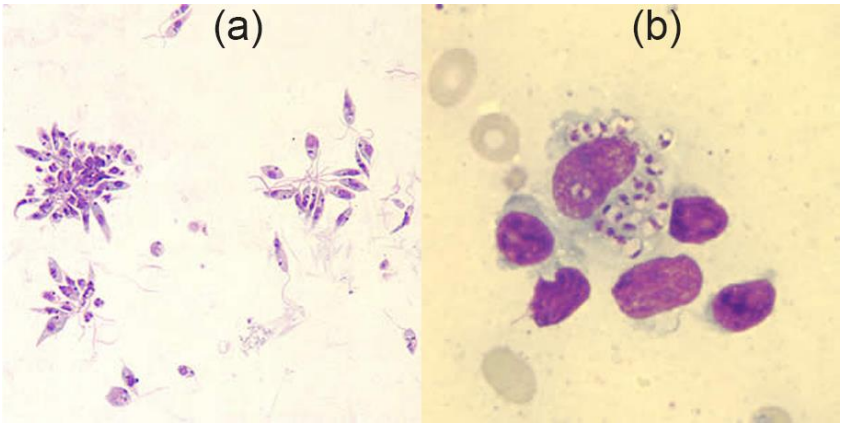


Figure **2-1**: Promastigote (a) and amastigote (b) forms of *Leishmania* spp (CDC, 2017).

There are more than twenty species belonging to the *Leishmania* genus that can infect humans, resulting in the development of the disease called leishmaniasis (Braga, 2019:1; Valle *et al*., 2019:2). *Leishmania* is transmitted to humans during hematophagy of an infected female sandfly of the *Phlebotomus* genus (WHO, 2021*a*). It can manifest itself in three different clinical forms

namely visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) (Salam *et al*., 2014:1). VL is the most dangerous form, causing excessive internal damage with a mortality rate of 95% if left untreated (WHO, 2021*b*). Of all of the species, *L. donovani* is responsible for the majority VL and *L. major* is responsible for the majority of CL (Güran, 2018:2). Leishmaniasis is ranked as one of the top ten most neglected diseases in the world (WHO, 2017). This disease is known to mostly affect individuals in developing countries and of low socioeconomic level (Georgiadou *et al*., 2015:44).

There are more than a billion individuals living in *Leishmania* endemic areas. It is estimated that about 90 000 new cases of VL and a million new cases of CL are reported annually worldwide (WHO, 2021*a*). Although leishmaniasis can cause significant mortality, there are very few efforts from the global community and pharmaceutical industry to invest in the research and development of novel therapeutics because of insufficient incentives (Salam *et al*., 2014:1).

The current drugs available for the treatment of leishmaniasis have several limitations including toxicity and intravenous administration, with the latter being a limiting factor in eradicating the disease in poverty stricken endemic areas (Freitas-Junior *et al.,* 2012:12). The eradication of leishmaniasis is further complicated by the incompatibility of the currently available drugs for pregnant women or individuals with hepatic or renal failure (Camargo & Langoni, 2006:536). Treatment recommendations varies in different regions due to the variation in response to drug therapy and the parasite species involved (Chakravarty & Sundar, 2010:167; WHO, 2021*b*). Therefore, there is an urgent need for new and effective antileishmanial drugs.

This chapter will focus on discussing current statistics, treatment and parasite resistance, as well as the challenges faced by leishmaniasis chemotherapy.

2.1 History of leishmaniasis

A study by Zink *et al*. (2006:1616) confirmed the presence of *L. donovani* found in DNA samples of Egyptian mummies of the Middle Kingdom in Thebes West dating back 4000 years ago. This disease is also mentioned in the Ebers Papyrus (a collection of ancient Egyptian medical documents dating back to 1500 BCE) as a skin condition named “Nile Pimple”, which was later identified as CL (Maspero, 1910; Steverding, 2016:4).

Scottish doctor, David Douglas Cunningham first observed *Leishmania* amastigotes in skin lesions of patients from India in 1885, but suggested that they were members of the fungi group *Mycetozoa* (Cunningham, 1885; Kobets *et al*., 2012:1443; Steverding, 2016:5).

In 1897, a Scottish pathologist, Lieutenant General Sir William Boog Leishman worked as an assistant to Ronald Ross at Netley, where he studied patients that was sent home from India for

suspected cases of malaria. At that time, he noticed a number of soldiers with remittent fever, muscular atrophy, anaemia and spleen enlargement. He considered the possibility that the symptoms indicated a specific disease that he named 'Dum-Dum fever' (Leishman, 1903:1252; Vincent, 2017:1584). In 1900, Leishman examined samples of a deceased patient who exhibited these symptoms before his death. He stained spleen samples to highlight the chromatin, where he found two chromatin masses in the bodies from the blood cells but he could not identify them (Leishman, 1903:1253; Vincent, 2017:1584). Three years later he found similar bodies in a rat that died of trypanosomiasis that led him to suggest that 'Dum-Dum fever' was caused by trypanosomes (Leishman, 1903:1253; Vincent, 2017:1584). Later in that same year, Charles Donovan reported that he found the same bodies as previously reported by Leishman. Ross examined Donovan's preparations and agreed that they matched Leishman's; hence, they concluded that Leishman had discovered a novel organism. This newly discovered species was then named *Leishmania donovani* (Ross, 1903:1262; Vincent, 2017:1584).

2.2 Epidemiology

Leishmaniasis is endemic to more than 98 countries and is among the most neglected and devastating tropical diseases in the world (Güran, 2018:2). This disease is the third biggest cause of parasite-related deaths in the world, with malaria being the first and schistosomiasis the second (WHO, 2017).

Leishmaniasis is prevalent in Africa, Latin America and Asia, and is usually correlated with malnutrition, poor living conditions, weakened immune systems, and shortage of resources (Georgiadou *et al*., 2015:44). There are about 12 to 15 million individuals in the world infected and about 1 billion people at risk of contracting the disease (WHO, 2021*a*). Leishmaniasis can spread to non-endemic areas through tourism, migration, and military movement (Inceboz, 2019:2). Environmental changes such as climate change, has the potential to extend the geographic range of the sandflies and therefore the areas in which leishmaniasis occurs (CDC, 2020*b*).

In 2019, the WHO reported 13 809 new cases of VL (20% of which occurred in India), a decrease compared to 2018 where 16 970 new cases were reported (WHO, 2021*d*). The total number of new CL cases reported in 2019 was 277 058 (26% of which occurred in the Syrian Arab Republic), an increase compared to the 244 005 cases reported the preceding year (WHO, 2021*e*). Since leishmaniasis is a NTD, there are very limited statistics available on MCL. Leishmaniasis causes about 30 000 deaths annually (PAHO, 2020).

VL, also known as 'kala-azar', is caused by *L. donovani* and *L. infantum* (also known as *L. chagasi*) (McGwire & Satoskar, 2014:9). VL is endemic to Brazil, South-East Asia, and East

Africa (Figure **2-2**). Approximately 90% of these reported cases occurs in Brazil, India, Ethiopia, Kenya, Sudan, South Sudan and Somalia (Braga, 2019:2).

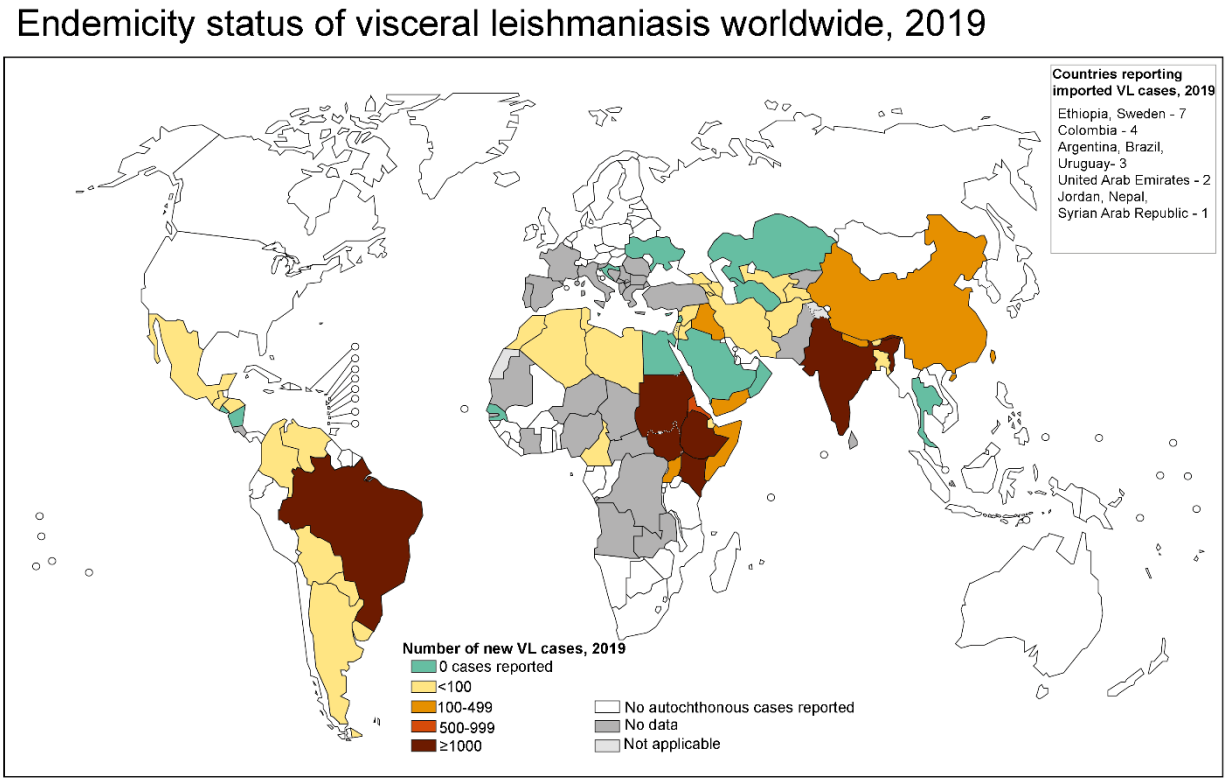


Figure **2-2**: Distribution of visceral leishmaniasis (VL) around the world. Adapted from WHO (2019*a*).

CL is caused by *L. major*, *L. infantum*, *L. tropica*, *L. Mexicana*, *L. amazonensis*, *L. guyanensis*, *L. panamensis*, *L. peruviana*, and *L. braziliensis* (Eiras *et al*., 2015:53). CL is endemic to regions such as the Mediterranean basin, Latin America, and western Asia from the Middle East to central Asia (Aoun & Bouratbine, 2014:2) (Figure **2-3**).

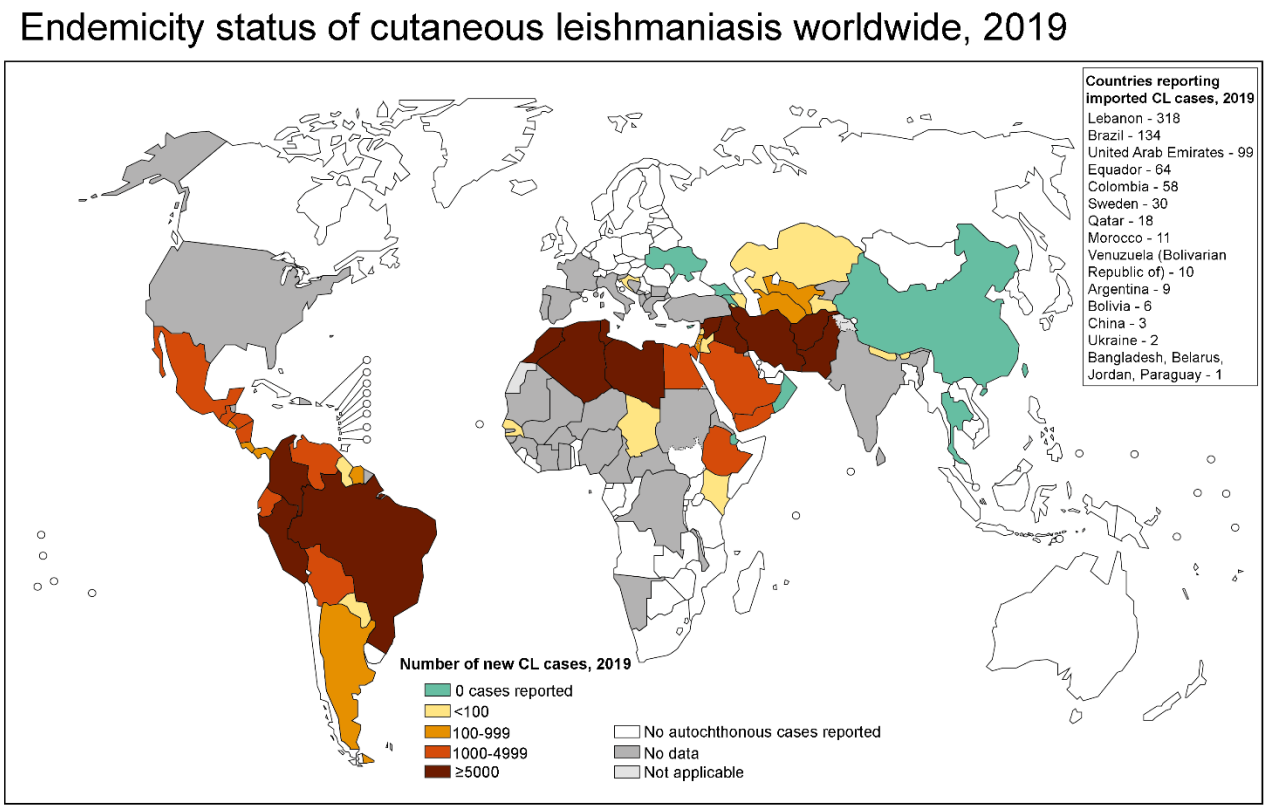


Figure **2-3**: Distribution of cutaneous leishmaniasis (CL) around the world. Adapted from WHO (2019*b*).

MCL is caused by *L. braziliensis* and it is found to occur mainly in South American countries. The majority of cases occurs in Peru, Brazil and Bolivia, but the disease is also found in lesser degrees in Ecuador, Colombia, Venezuela and Paraguay (McGwire & Satoskar, 2014:9).

2.3 Transmission and life cycle of *Leishmania*

Leishmania parasites are transmitted through vectors from host to host. Transmission can be anthroponotic (human to vector to human) or zoonotic (human to vector to animal or *vice versa*) and occurs in urban and rural environments, respectively (Avila-García *et al*., 2014:27). Transmission of the parasite from the vector to the host depends on how infective the host is, how infectious the sandfly's single bite is, the biting rate on average, and the number of sandflies that are present (Torres-Guerrero *et al*., 2017:6). The saliva of the vector contains several components that play an important role in the establishment of a *Leishmania* infection such as vasodilatory and anticlotting factors to aid blood flow, and immunomodulatory factors to inhibit healing of the wound and to stop the immune response (Torres-Guerrero *et al*., 2017:6).

Although not common, in 1960 in the United Kingdom (UK), there was a report of sexual transmission of leishmaniasis in which a woman developed a genital papule containing *Leishmania donovani* bodies, despite never visiting a *Leishmania*-endemic region. Her husband

was diagnosed with VL several years before, and although at the time of his wife's lesion he had no symptoms, it was assumed that he had transmitted the disease to his wife (Turchetti *et al*., 2014:403; Guedes *et al*., 2020:950).

The life cycle of *Leishmania* (Figure **2-4**) is divided into two stages; the motile flagellated promastigote stage (present in the vector), and the intracellular non-flagellated amastigote stage (present in mammalian host cells) (McGwire & Satoskar, 2014:7). When a sandfly bites an infected host (e.g. animal), it becomes infected and ingests the amastigote form from its host. The parasites in the fly's gut then transform from the amastigote form to the proliferative procyclic promastigote form, followed by the infectious metacyclic promastigote form. Thereafter, when the infected sandfly bites a new healthy host, the vector inoculates it *via* regurgitation of the metacyclic promastigotes (Torres-Guerrero *et al*., 2017:6). The infection is initiated through receptor-mediated binding of the infective promastigotes to phagocytising host cells, resulting in the encapsulation of the parasites in parasitophorous vacuoles within the host cells (McGwire & Satoskar, 2014:7). Parasitophorous vacuoles containing parasites fuse with lysosomes to form phagolysosomes. In these phagolysosomes, promastigotes are transformed into and replicate as amastigotes (McGwire & Satoskar, 2014:8).

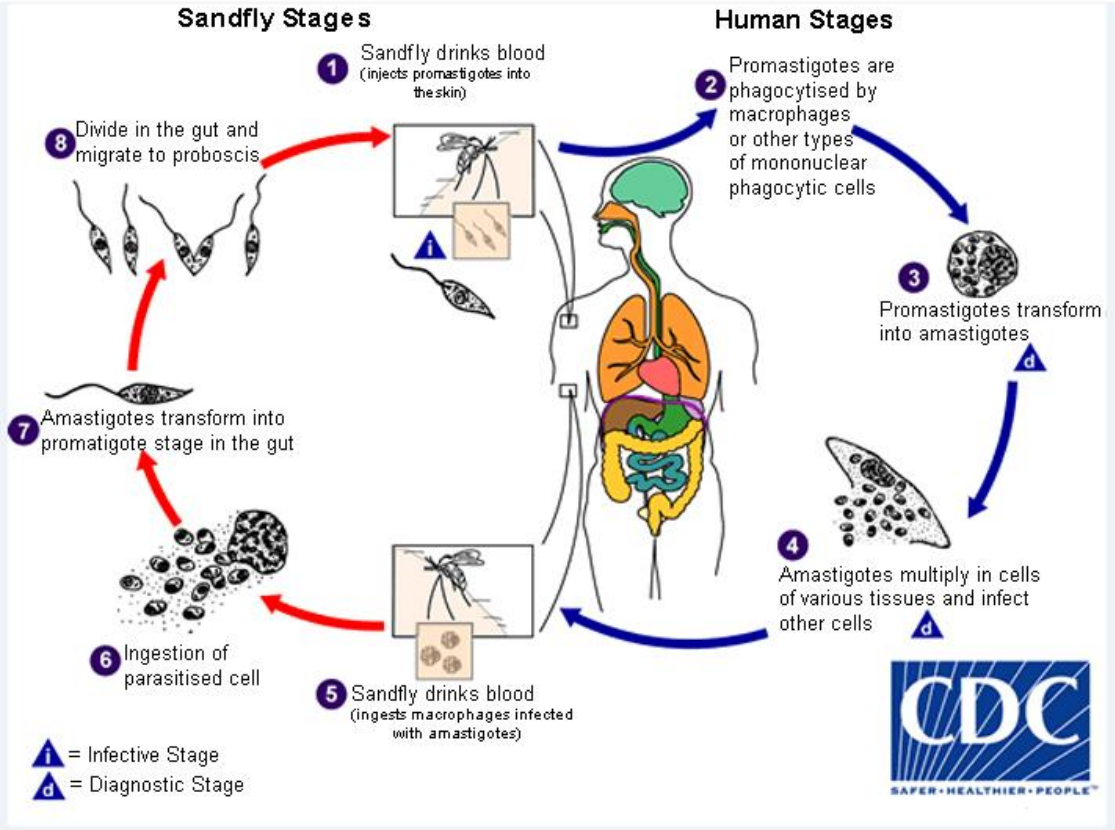


Figure **2-4**: Life cycle of leishmaniasis depicting the different stages that occurs. Adapted from CDC (2020*c*).

When the parasites enter the host, the hosts immune system activates the complement system (a large number of distinct plasma proteins that helps fight infection by reacting with one another to opsonise pathogens and induce inflammatory responses (Janeway *et al*., 2001)). However, in humans, the parasite can evade this immune response *via* inhibition of complement-mediated lysis, which takes place in the phagolysosomes of macrophages. This effect is brought on by the membrane protease gp63, or leishmanolysin, which is responsible for the inhibition of immune attacks against the parasite cell membrane by attaching to complement components (Avila-García *et al*., 2014:28). The parasite burden increases until the infected macrophages eventually gets disrupted and spreads extracellular amastigotes to surrounding tissue, infecting previously uninfected macrophages (McGwire & Satoskar, 2014:8).

2.4 Clinical features

The clinical features of leishmaniasis are determined by the three main human forms, namely VL, CL and MCL. The outcomes of each form are determined by the infecting parasite species and the host's genetic susceptibility (McGwire & Satoskar, 2014:8). However, most people infected with the *Leishmania* parasite, do not develop any symptoms at all. Thus, leishmaniasis refers to showing symptoms of *Leishmania* infection, rather than just being infected with the parasite (WHO, 2021*a*).

2.4.1 Visceral leishmaniasis (VL)

VL is a fatal form of leishmaniasis and has a mortality rate of 75-95% (Güran, 2018:7). VL has an incubation period of 3-8 months with preschool children, with the undernourished and immunocompromised individuals being the most at risk (Torres-Guerrero *et al*., 2017:9).

VL is most commonly characterised by irregular fever, lymphadenopathy, progressive pallor, splenomegaly, hepatomegaly, anorexia, weight loss, anaemia, thrombocytopenia, leukopenia, hypoalbuminemia, increased globulin, night sweats, oedema, asthenia, and cutaneous pigmentation (Pedrosa, 2017:17; Torres-Guerrero *et al*., 2017:9). Children infected with this disease can also experience chronic diarrhoea and growth retardation (Torres-Guerrero *et al*., 2017:9). Treatment varies for VL caused by *L. donovani*, but pentavalent antimonials and amphotericin B are the two preferred therapies in use (discussed in section 2.7).

2.4.2 Cutaneous leishmaniasis (CL)

CL is the most common form of leishmaniasis. It is a milder form of the disease and a sandfly bite causes skin lesions, ulcers or nodules on the host leaving behind lifelong scars and/or a serious disability (Güran, 2018:7). The incubation period of CL is 1-4 weeks but can proceed for several years (Torres-Guerrero *et al*., 2017:7).

CL can either be local (commonly presented with painless ulcerated lesions with raised edges), disseminated (presenting with less ulcerated lesions that can occur on all parts of the body), or diffused (a chronic and rare form that can compromise the skin of the patient, with frequent recurrence) (Camargo & Langoni, 2006:533). Diffused CL can cause lymphedema, lymphadenopathy, fever and poor health (Torres-Guerrero *et al*., 2017:8). CL is self-curing and nonlife-threatening, but accumulation of CL can often lead to permanent, disfiguring formations on the skin (Güran, 2018:7). The healing of cutaneous lesions can be sped up by treatment (McGwire & Satoskar, 2014:9). Treatment for CL consists of pentavalent antimonials, liposomal amphotericin B, miltefosine, fluconazole and ketoconazole (Eiras *et al*., 2015:52) (discussed in section 2.7).

2.4.3 Mucocutaneous leishmaniasis (MCL)

MCL, in contrast to CL, is a potentially life-threatening form of leishmaniasis that requires treatment, however it is a less common form (David & Craft, 2009:497). MCL can occur when there is dissemination of CL lesions *via* the lymphatic or hematogenic route, or the CL lesions were not properly treated. This is due to the formation of secondary cutaneous lesions and it can occur months to years after the primary lesions have healed (Camargo & Langoni, 2006:533; McGwire & Satoskar, 2014:9).

Generally, lesions begin in the nasal mucosa and spread to the pharynx and oral mucosa, the larynx, and skin of the nose and lip (Torres-Guerrero *et al*., 2017:9). In the early stages of MCL infection, symptoms of erythema and ulceration of the nostrils, as well as anterior and posterior nasal granulomas, lymphadenopathy, fever, and hepatomegaly can occur (David & Craft, 2009:498). During the mid-to-late stages of MCL infection, patients can present with oedema and erythema of the nostrils, ulceration of the hard or soft palate, perforation of the nasal septum, gingival oedema, and periodontitis (David & Craft, 2009:498). This disease is terribly disfiguring and causes chronic local destruction of tissue in the nose, oro- and naso-pharynx, mouth, and eyelids and it can progress to the point where respiratory functions is affected and nutrition is hindered (McGwire & Satoskar, 2014:9). MCL can usually be treated with pentavalent antimonials, pentamidine, miltefosine, amphotericin B, paromomycin or the azoles (fluconazole, ketoconazole and itraconazole) (discussed in section 2.7).

2.5 Diagnoses

Leishmaniasis is diagnosed based on its clinical features, endemicity and laboratory testing such as parasitological, immunological and serological testing (Salam *et al*., 2014:2; Georgiadou *et al*., 2015:44).

2.5.1 Clinical diagnosis

The infecting *Leishmania* species can be identified and confirmed by firstly examining patients for symptoms (previously discussed in section 2.4) and thereafter, further testing can be done in the laboratory by screening samples for either a parasitological or serological diagnosis (Torres-Guerrero *et al*., 2017:10). This is explained in detail below.

2.5.2 Parasitological diagnosis

Parasitological diagnosis is used to detect the parasites' amastigote forms. A sample is taken by performing a biopsy of lesions or by exploratory puncture of the liver, spleen, bone marrow and lymph nodes. The collected samples are then stained using Giemsa and cytologically examined by impressing them into the slides (Camargo & Langoni, 2006:534). In some cases, when it is possible, the samples are cultured in Novy-McNeal-Nicolle (NNN) or another appropriate medium so that the tissue amastigotes are converted to motile promastigotes and can multiply *in vitro* (Georgiadou *et al*., 2015:45). The cultures are checked weekly for about 4 weeks, with growth taking 2 weeks on average. The culture sensitivity depends on the parasite load but is estimated to be between 60-85% (Georgiadou *et al*., 2015:45).

2.5.3 Serological diagnosis

Several different techniques can be used for serological diagnosis such as indirect fluorescent antibody tests (IFA), enzyme-linked immunosorbent assays (ELISAs) and direct agglutination test (DAT). However, all of them differ in specificity, sensitivity, field conditions, practical application and availability of reagents (Camargo & Langoni, 2006:535).

IFA consists of an antigen-antibody reaction that is visualised through the use of a fluorescent conjugate. The results of the test are expressed in dilutions (Rodrigues *et al*., 2021:112). A study conducted on immunodiagnosis of CL showed that IFA has a sensitivity rating of 91.6% and a specificity rating of 81.0% (Sarkari *et al*., 2014:3).

ELISA tests allow for the detection of low antibody titers (Rodrigues *et al*., 2021:113). ELISAs are based on the bond between the antibody and the antigen. It is identified through the use of a conjugate that is labelled with an enzyme. The enzyme acts on a substrate and this reaction causes a colour change of the chromogen reagent (Rodrigues *et al*., 2021:113). The sensitivity of ELISA for VL was found to be 83.6%, while the specificity was found to be 90.5% (Mikaeili *et al*., 2007:116).

DAT is a semi-quantitative test that is usually used to diagnose VL and can be done in either serum, blood or urine. It involves a reaction between the antibodies and the antigens (dead promastigotes), where a visible agglutination reaction is generated after an incubation period

(Rodrigues *et al*., 2021:112). This test is easily readable and requires less equipment than ELISA, and can therefore be applied in low-resource settings (Georgiadou *et al*., 2015:45). The sensitivity of DAT for VL is 70.5%, while the specificity is 100.0% (Mikaeili *et al*., 2007:116).

These tests are not definitive proof of active disease as patients can test positive for serum antibodies against *Leishmania* spp. for months or years after recovery. These results must, therefore, be interpreted with caution and both clinical and epidemiological information must also be taken into account (Georgiadou *et al*., 2015:45).

2.6 Prevention

The most economical strategy for the prevention of infectious diseases is vaccination. Vaccines with induction immune responses can move toward the immunity pathway to control infections by producing memory lymphocytes, while also stimulating cellular and humoral immunity (Ghorbani & Farhoudi, 2018:30). To date, there are no existing vaccines for leishmaniasis; however, there are several undergoing studies in an effort to find one (U.S. National Library of Medicine, 2021).

In the meantime, vector control remains a highly effective preventative measure when thoroughly applied and sustained (Wilson *et al*., 2020:3). Eradicating the vector as a means of prevention can be done by using insecticide-impregnated thick clothes with long sleeves, curtains and/or bed nets, eliminating stagnant water, and using insect repellents (Eiras *et al*., 2015:53; Torres-Guerrero *et al*., 2017:11). Another preventative measure is to avoid outdoor activities in endemic areas, especially at night (Eiras *et al*., 2015:53; Torres-Guerrero *et al*., 2017:11).

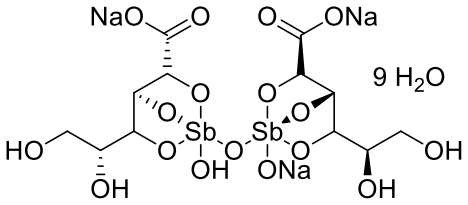
2.7 Treatment of leishmaniasis

The currently available treatment for leishmaniasis includes pentavalent antimonies (sodium stibogluconate and meglumine antimonate), amphotericin B, miltefosine, pentamidine, paromomycin and the azoles (ketoconazole, itraconazole and fluconazole) (Georgiadou *et al*., 2015:46; Braga, 2019:2). None of these drugs are ideal treatments for leishmaniasis as they require intravenous administration, are highly expensive, toxic, and are experiencing parasitic resistance (Freitas-Junior *et al.,* 2012:12). These drugs are discussed more in detail below.

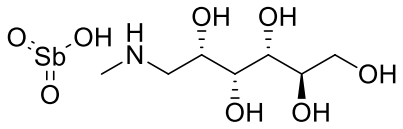
2.7.1 Pentavalent antimonies

Pentavalent antimonies are antimony-carbohydrate complexes (Figure **2-5**) that have been used for more than half a century to treat leishmaniasis (Pund & Joshi, 2017:449). There is evidence to suggest that pentavalent antimonies are prodrugs, and that they are converted within the organism to trivalent antimonies and become biologically active once in the bloodstream. However, the exact reduction site and the underlying reduction mechanisms is unknown (Domingues Passero *et al*., 2013:96). Pentavalent antimonies can be used to treat all forms of

leishmaniasis and are administered at 20 mg/kg/day for 28-30 days IV/IM for the VL form; 10-20 days for the CL form and 30 days for the MCL form (McGwire & Satoskar, 2014:11; Eiras *et al*., 2015:55).



Sodium stibogluconate



Meglumine antimoniate

Figure **2-5**: Sodium stibogluconate and meglumine antimonate for the treatment of leishmaniasis

The antimonies have a short half-life of 1-2 hours and a terminal elimination phase of 1-3 days where they are excreted in the urine (Aronson, 2016:619; De Aguiar *et al*., 2018:1114). General side effects of the pentavalent antimonies include nausea, vomiting, anorexia, abdominal pain, and headaches (WHO, 2010; Aronson, 2016:619). In the case of sodium stibogluconate, additional side effects such as serum transaminase elevations, pancreatitis, T-wave inversion or flattening, and rarely, myocardial, hepatic, and renal damage, may also be observed (Ryan, 2018:1568).

2.7.2 Amphotericin B

Amphotericin B (AmB) is a polyene macrolide antibiotic isolated from the *Streptomyces nodosus* strain (Pund & Joshi, 2017:449) (Figure **2-6**). This drug is used to treat patients with HIV-leishmaniasis coinfection or when parasites are resistant to pentavalent antimonies (Braga, 2019:3). AmB forms complexes with 24-substituted sterols, such as ergosterol, on the biological membrane. The formed complexes cause pores to open, which then alter the ion balance and kill the cell (Roberts *et al*., 2003:137; Freitas-Junior *et al.,* 2012:12). AmB deoxycholate is used to treat all forms of leishmaniasis and is administered intravenously at doses of 0.75-1.00 mg/kg/day for 15-20 alternate days for the treatment of VL caused by *L. donovani*, and for 30 days for VL caused by *L. infantum* (McGwire & Satoskar, 2014:11). For CL caused by *L. braziliensis*, the therapy length is 25-30 days, and 45 days for MCL (McGwire & Satoskar, 2014:11).

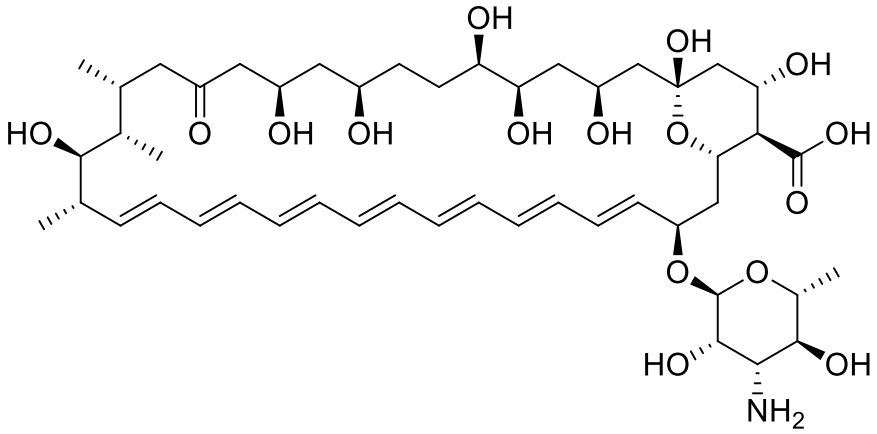


Figure **2-6**: Amphotericin B for the treatment of leishmaniasis

The terminal half-life of AmB is 127 hours ($t_{1/2}$ ~5 days) in healthy subjects, with the drugs being extensively excreted unchanged in the urine and faeces (Bekersky *et al*., 2002:833). AmB is generally well tolerated, although adverse effects may include infusion-related chills, high fever, and rigor, and in some cases, it can also cause thrombophlebitis of the injected vein, nephrotoxicity, and electrolyte abnormalities. Hypokalaemia and myocarditis are also uncommon, but can occur in extreme circumstances (WHO, 2010; McGwire & Satoskar, 2014:11).

In order to improve the bioavailability and pharmacokinetic properties of AmB, lipid formulations were developed. Liposomal AmB (L-AmB) has reduced side effects (mild fever, rigor, chills, and back pain, as well as transient nephrotoxicity and thrombocytopenia (WHO, 2010)) and the smaller liposomes stay in the bloodstream longer than free AmB. Therefore, it has a better half-life of 152 hours ($t_{1/2}$ ~6 days) and a high efficacy (Bekersky *et al*., 2002:828; Freitas-Junior *et al.,* 2012:12). L-AmB is administered at 3 mg/kg/day IV on days 1-5, 14 and 21 (Georgiadou *et al*., 2015:46).

2.7.3 Miltefosine

Miltefosine is the only oral drug currently available and is used to treat all forms of leishmaniasis. It is a phosphocholine derivative (Figure **2-7**) that has been shown to act as an inhibitor of lipid biosynthesis in parasites of the *Kinetoplastida* order (Pinto-Martinez *et al*., 2017:2). Indirectly, miltefosine causes cell death *via* a mechanism similar to apoptosis (Braga, 2019:4). It reduces the function of the overall replication mechanism of parasite cells and targets the homeostasis of Ca^{2+} in *Leishmania* parasites. This contributes to parasitic cell death through the eventual over accumulation of intracellular Ca^{2+} (Braga, 2019:4). Miltefosine is able to inhibit cytochrome-c oxidase in the mitochondria, causing an apoptosis-like cell death. Miltefosine has been reported to possess immunomodulatory properties that is believed to be another contributing factor to its antileishmanial activity (Dorlo *et al*., 2012:2583). For VL, miltefosine is given orally at

2.5 mg/kg/day for children ≤12 years old, and at 50.0 mg/kg/day for individuals >12 years and <25kg. For a weight of 25-50 kg, 100.0 mg/kg/day is substantial, and when the weight is >50 kg, 150.0 mg/kg/day is given, all for a total of 28 days (McGwire & Satoskar, 2014:12). To treat CL caused by *L. panamensis*, miltefosine is given at 2.0 mg/kg/day for 28 days, and for MCL caused by *L. braziliensis*, it is administered at 2.5-3.3 mg/kg/day for 4 weeks (WHO, 2010).

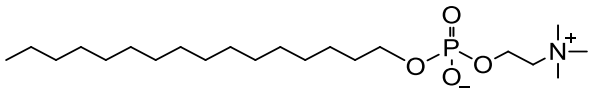


Figure **2-7**: Miltefosine for the treatment of leishmaniasis

Preclinical *in vitro* studies have shown no oxidative metabolisms by isoenzyme cytochrome P450, thus the main metabolic pathway of miltefosine appears to be exertion through phospholipases (Ghorbani & Farhoudi, 2018:30). Miltefosine has a long terminal elimination phase and a half-life of 31 days (Kip *et al*., 2018:166). Miltefosine is well tolerated but is teratogenic (Georgiadou *et al*., 2015:47) and causes symptoms such as headaches, nausea, vomiting, and/or diarrhoea during the first week of treatment (Eiras *et al*., 2015:56).

2.7.4 Pentamidine

Pentamidine is an aromatic diamidine (Figure **2-8**) that was previously used as a second-line treatment for VL in India, but due to a reduction in efficacy caused by parasitic resistance, it is no longer used to treat VL. It is, however, still an effective treatment for CL caused by *L. guyanensis* and *L. panamensis* in the Northern Amazon basin and it is administered as short courses, with four doses of 4 mg/kg IM or slow IV injections being given on alternate days (Boelaert & Sundar, 2014:647). The mechanism of action of pentamidine is poorly understood, but it is believed to undergo rapid active uptake into the cell of the protozoan parasite, where it binds to the transfer RNA. This binding inhibits the ribosomal protein synthesis process and the synthesis of nucleic acids and phospholipids (Rex & Stevens, 2015:492; Waller & Sampson, 2018:615). The accumulation of the drug in high concentrations *via* several transporter systems may contribute to the selectivity of pentamidine to the protozoan parasites. However, the loss of uptake of pentamidine into parasite cells can also contribute to resistance (Barrett & Croft, 2012:187).

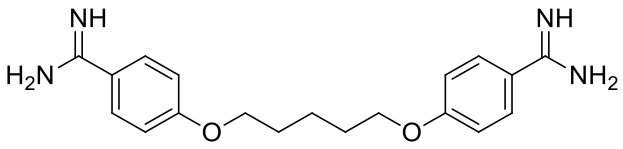


Figure **2-8**: Pentamidine used for the treatment of *T.b. gambiense*

Pentamidine is distributed mainly to the liver and kidneys, where it is excreted slowly without undergoing biotransformation and remains unchanged in the faeces and urine (Rex & Stevens, 2015:492). The half-life of pentamidine is between 10 and 14 days (Waller & Sampson, 2018:615). Pentamidine is relatively well tolerated in the body; however, it still has several adverse effects that include hypotension, hypoglycaemia or hyperglycaemia, nephrotoxicity, prolongation of the QT interval, and gastrointestinal effects. In rare occasions, more severe side effects such as highly abnormal hepatic and pancreatic functions, and Stevens-Johnson syndrome, may also be observed (Babokhov *et al*., 2013:244; Kennedy, 2013:191).

2.7.5 Paromomycin

Paromomycin (previously known as aminosidine) is a broad-spectrum antibiotic belonging to the aminoglycosides class (Boelaert & Sundar, 2014:647) (Figure **2-9**). Its antileishmanial activity is believed to be mediated by inhibiting parasite metabolism and mitochondrial respiration (McCarthy *et al*., 2015:512). It is also suggested that paromomycin inhibits the protein synthesis of protozoa by binding to the 30S ribosomal subunit, which causes accumulation of abnormal 30S-50S ribosomal complexes, eventually causing cell death (Kip *et al*., 2018:164). Paromomycin is administered at 15 mg/kg/day for 21 days as a monotherapy for Indian VL. It can also be administered as a combinational therapy with L-AmB (McGwire & Satoskar, 2014:11).

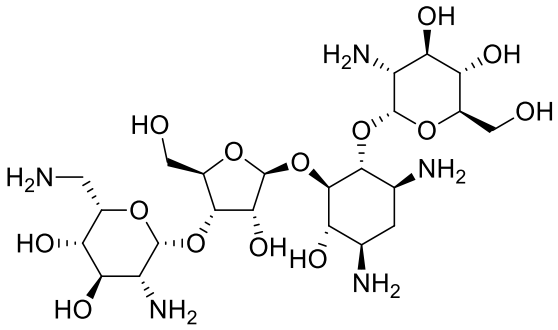


Figure **2-9**: Paromomycin for the treatment of VL and CL

Paromomycin is primarily excreted unchanged by the kidneys through glomerular filtration and is therefore not metabolised. The half-life of paromomycin is 2-3 hours (Kip *et al*., 2018:164). Side-effects of paromomycin include nephrotoxicity (renal injury), ototoxicity, and hepatotoxicity (LFTs increase) (Freitas-Junior *et al.,* 2012:12; Georgiadou *et al*., 2015:47).

2.7.6 Azoles

Ketoconazole, fluconazole and itraconazole are derived from the family of azoles and can be used to treat all forms of leishmaniasis (Figure **2-10**). These drugs all share the same molecular target, the enzyme lanosterol 14-*α*-demethylase, which is involved in the biosynthesis of the main cell membrane sterol, ergosterol, in yeasts (Braga, 2019:2). *Leishmania* spp. cells have ergosterol as

the main sterol component in their membranes, thus the depletion of ergosterol by these azoles causes the parasite's cells to undergo a number of structural changes that results in the inhibition of cell growth (Braga, 2019:3). An oral dose of 200 mg/day of fluconazole is recommended by the WHO for a total of 6 weeks for treating VL caused by *L. major* (WHO, 2020). WHO also recommends 600 mg/day of ketoconazole for 28 days for treating VL caused by *L. mexicana* (WHO, 2020). Itraconazole was found to be effective to treat CL at a dose of 200 mg/day for 3 months, after which the dose was reduced to 100 mg/day for another month (Consigli *et al*., 2006:46). A study was conducted by Amato *et al*. (2000) where they tested the efficacy of itraconazole in MCL. In this study, ten patients were given 4 mg/kg/day (up to 400 mg/day) for 6 weeks. Six out of the ten patients had healed lesions 3 months after treatment, thus concluding that itraconazole can be effective against MCL (Amato *et al*., 2000:153).

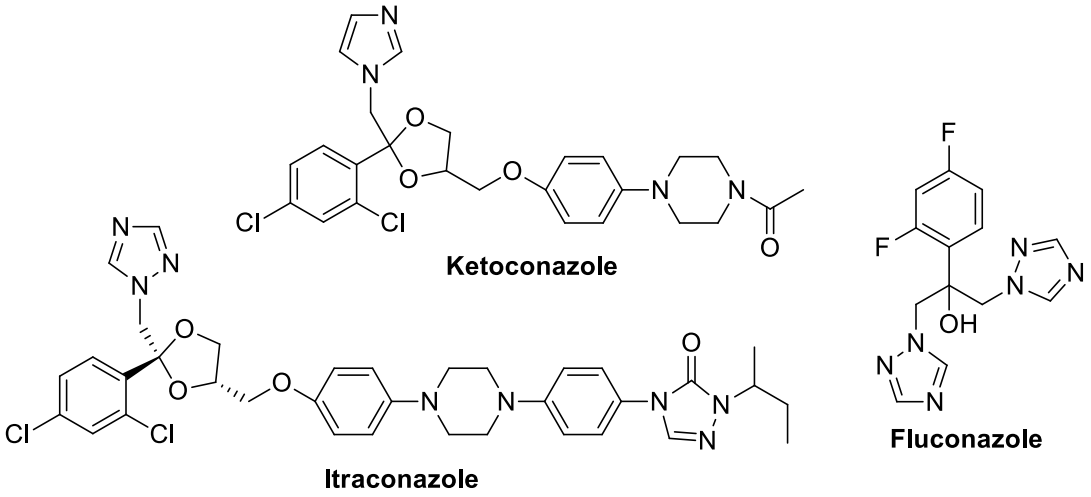


Figure **2-10**: Ketoconazole, itraconazole, and fluconazole for the treatment of leishmaniasis

The elimination half-life of fluconazole is 31.6 hours (Debruyne & Ryckelynck, 1993:10), 7.5-7.9 hours for ketoconazole (Huang *et al*., 1986:206), and 34.0 hours for itraconazole (Prentice & Glasmacher, 2005:i18). Common side-effects of the azoles include gastrointestinal effects and hepatotoxicity (Eiras *et al*., 2015:57).

2.8 Drug resistance

The interaction of drugs with one or more targets is the primary effect in cell destruction. Therefore, for a parasite's survival, the altering of intracellular drug levels or the drug's ability to affect targets are of great importance. A variety of mechanisms can be used to lower drug levels at the target action site, including increased export, decreased uptake, and inactivation by sequestration or metabolism. Inhibition of primary targets often trigger cell damage and death (Croft *et al*., 2006:114). Many anti-parasitic drugs undergo redox reactions, which produce reactive oxygen species (ROS), which in turn can result in peroxidative damage of membrane

proteins, lipids or DNA and ultimately parasite death. Therefore, overexpressed repair systems can contribute to drug resistance *via* multiple mechanisms (Croft *et al*., 2006:114). Discussed below are the most common resistance mechanisms of *Leishmania* parasites to the previously discussed antileishmanial drugs.

2.8.1 Pentavalent antimonies

Drug resistance to pentavalent antimonies was first indicated in the early 1980s in North Bihar, India, when 30% of patients were found to not respond to the administered drugs (Chakravarty & Sundar, 2010:168). This number has drastically increased over the years and today, up to 60% of patients do not respond to the pentavalent antimony drugs in North Bihar (India) (Maltezou, 2010:2). Despite several studies being done on *in vitro* parasite mutants, resistance mechanisms in field parasites are not yet fully understood. This is because of the differences between *in vitro* and field parasite antimonial resistance mechanisms, and also because *in vitro* unresponsiveness does not necessarily mean clinical resistance (Maltezou, 2010:2). Antimony is a heavy metal that shares characteristics with the heavy metal arsenic, and thus arsenic resistance renders cross-resistance with antimonies. This offers a possible reason for antimony resistance in North Eastern India, as high levels of arsenic in drinking water may have led to parasites becoming resistant to arsenic and therefore to antimonies (Ponte-Sucre *et al*., 2017:6). Another setback for antimony treatment is HIV-VL coinfected patients, as antimonies need an immune system that is intact to be effective and these patients tend to relapse. Thus, HIV-VL coinfected patients serve as a potential source to increase drug resistance (Chakravarty & Sundar, 2010:169). *Leishmania* strains that have shown resistance to antimonies include *L. donovani* (Mukhopadhyay *et al*., 2011:1311; Zheng *et al*., 2020:11), *L. infantum* (Brotherton *et al*., 2013:1; Seblova *et al*., 2014:6274), *L. braziliensis* (Rojas *et al*., 2006:1378), *L. panamensis* (Rojas *et al*., 2006:1378) and *L. tropica* (Hadighi *et al*., 2007:1319).

2.8.2 Amphotericin B

Clinical isolates of *L. donovani* have been found to be resistant to AmB. *Leishmania* strains that are AmB-resistant have shown significant reduction in ROS accumulation, leading to an increase in the tryparedoxin cascade that could play a key role in kinetoplastides' antioxidative defence against ROS (Ghorbani & Farhoudi, 2018:29). The AmB-R strain of *L. infantum* can upregulate members of the tryparedoxin cascade, and their resistance to AmB is connected to ATP depletion, followed by increased ion leakage. Thus, by preventing ion leakage, ATP depletion could be inhibited, and AmB-R strains could possibly be controlled (Ghorbani & Farhoudi, 2018:29).

Previously, it was assumed that clinical resistance of AmB was rare, but the overuse of this drug has now caused it to experience parasitic resistance. This was confirmed in a recent study by Kariyawasam *et al*. (2019) were they found resistance of AmB at 0.12 µg/mL by *L. donovani*,

L. infantum, *L. tropica*, *L. major*, *L. amazonensis*, *L. chagasi*, *L. mexicana*, *L. braziliensis* and *L. guyanensis.*

2.8.3 Miltefosine

Promastigotes that are resistant to miltefosine can be created in a laboratory by chemically mutating the parasites' proteins (Coelho *et al*., 2012:1). Mutations in the MT gene causes imperfections in drug internalisation, which in turn is considered to be the mechanism of resistance (Ghorbani & Farhoudi, 2018:30). The reduction in internalisation is mainly due to reduced uptake or increased efflux of miltefosine. The addition of inactivating mutations or deletions in the translocation apparatus LMT and/or LRos3 in miltefosine showed drastic increase in miltefosine resistance in both *in vitro* and *in vivo L. donovani* assays (Ponte-Sucre *et al*., 2017:12).

Resistance to miltefosine has been reported in *L. donovani* (Srivastava *et al*., 2017:9) and *L. infantum* (Van Bockstal *et al*., 2020:16).

2.9 Rationale for new antileishmanial drug development

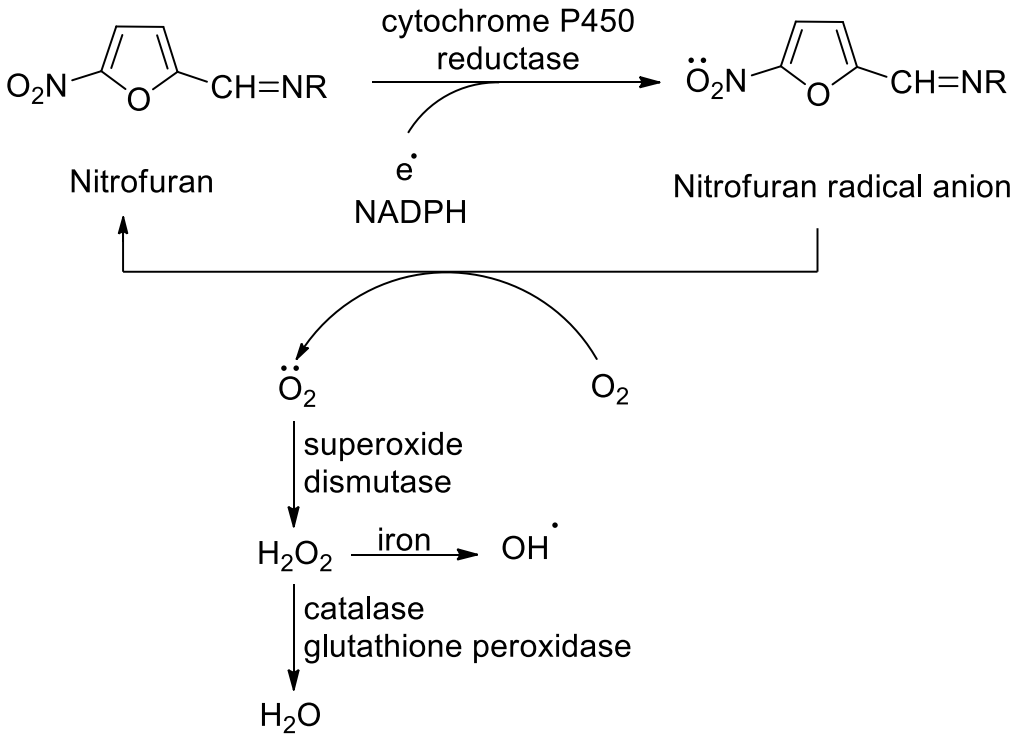
The pathogens of infectious diseases are able to survive in human hosts by multiplying and adapting. This leads to the generation of new strains of parasites, thus causing existing drugs to become less effective for the treatment of these diseases.

In the search for new, safe and effective antileishmanial drugs, extensive research on several scaffolds such as azoles (Mathur *et al*., 2020:2), triazoles (Teixeira de Macedo Silva *et al*., 2018:2360), nitrofurans (Sifontes-Rodríguez *et al*., 2015:166), flavonoids (Boniface & Ferreira, 2019:2473), and various others have been explored. The nitrofuran moiety is of particular interest of all these scaffolds, as it is redox-active, and there are several reports indicating its broad spectrum of activity (Wyllie *et al*., 2016:1; Foroumadi *et al*., 2017:904). Also, nifurtimox (a nitrofuran drug) and fexinidazole (a nitroimidazole drug) are currently used for treating human African trypanosomiasis (HAT) (a disease caused by a taxonomically related protozoan parasite) and both these drugs are orally available, hence it may be a suitable backbone to use in the search for new drugs.

2.9.1 Nitrofuran

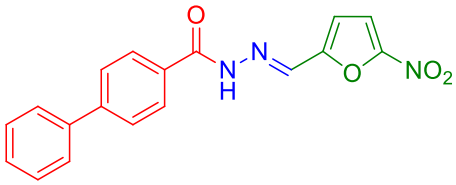
Nitrofurans are a class of redox-active anti-infective drugs that are used for the treatment of various infectious diseases. Structurally, nitrofurans contain a furan ring with a nitro group. These drugs have a broad range of biological activity against various diseases such as cancer (Peterson, 2018:1439), tuberculosis (Krasavin *et al*., 2019:130), giardiasis (Ryan, 2018:1567) and urinary tract infections (Huttner *et al*., 2015:2456). Drugs that contain the nitro group are very active

because the nitro group itself produces free radical species, reactive oxygen species (ROS) and reactive nitrogen species (RNS), which react with pathogen cell wall enzymes, thereby becoming lethal to these microorganisms (Pal & Bandyopadhyay, 2012:556; Kim *et al*., 2019:203). The generation of ROS (such as O_2^-, H_2O_2 and OH) are induced by nitrofurans, and the reduction of the nitro group is catalysed by a flavin containing nitroreductase, such as NADPH or NADH (Sharma & Anand, 1997:432; Le *et al*., 2019:2). Furthermore, nitrofurans possess several other mechanisms of antimicrobial action, which may contribute to the lack of pathogenic resistance against such therapeutic agents.

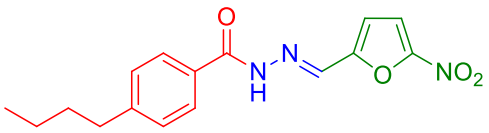


Scheme **2-1**: Mechanism of action of nitrofuran drugs. Adapted from Wang *et al*. (2008)

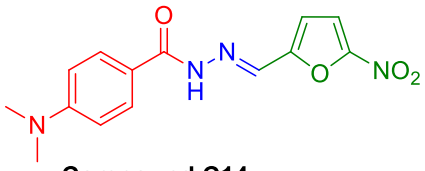
There have been extensive studies reported on nitrofuran drugs as anti-infective drugs. In a recent study by Palace-Berl *et al*. (2015), a series of 5-nitro-2-furfuriliden derivatives were synthesised and tested for its anti-*T. cruzi* activity. Their results have indicated that all synthesised compounds, with the exception of compound C14, showed similar or better activity than the reference drug benznidazole. Compounds C20 and C15 (Figure **2-11**) were the most potent against the epimastigote form of three *T. cruzi* strains. Compound C20 showed activity of 1.17 µM (Y- strain), 3.17 µM (Silvio X10 cl1 strain), and 1.81 µM (Bug 2149 cl10 strain). Compound C15 showed activity of 3.10 µM (Y- strain), 4.75 µM (Silvio X10 cl1 strain), and 4.41 µM (Bug 2149 cl10 strain).



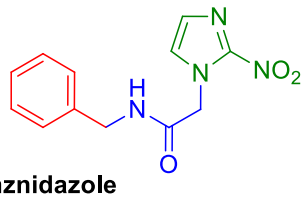
Compound C20
Y- IC_{50} 1.17 µM
Silvio X10 cl1- IC_{50} 3.17 µM
Bug 2149 cl10- IC_{50} 1.81 µM



Compound C15
Y- IC_{50} 3.10 µM
Silvio X10 cl1- IC_{50} 4.75 µM
Bug 2149 cl10- IC_{50} 4.41 µM



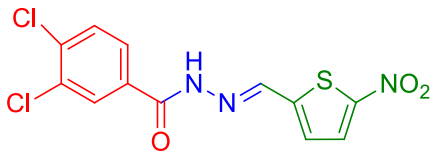
Compound C14
Y- IC_{50} 100 µM
Silvio X10 cl1- IC_{50} 100 µM
Bug 2149 cl10- IC_{50} 47.29 µM



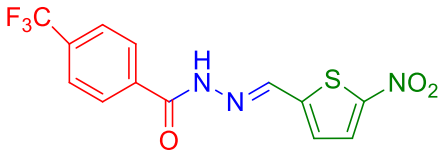
Benznidazole
Y- IC_{50} 40.40 µM
Silvio X10 cl1- IC_{50} 29.16 µM
Bug 2149 cl10- IC_{50} 30.63 µM

Figure **2-11**: The two most active compounds, compound C20 and C15, as well as the least active compound, compound C14, and reference drug, benznidazole (Palace-Berl *et al*., 2015)

In another study, the antileishmanial activity of 5-nitro-2-heterocyclic benzylidene hydrazides was investigated. It has been found that compounds 2/S, 6/S, 8/S, 18/S, 18/O, and 28/O showed better activity than the drugs pentamidine (IC_{50} 1.06 µM) and AmB (IC_{50} 1.19 µM). The most potent compounds of this series were compounds 18/S and 8/S (Figure **2-12**) showing IC_{50} values of 0.41 µM and 0.82 µM, respectively, against *L. donovani* promastigotes (Rando *et al*., 2008).



Compound 18/S
L. donovani promastigote- IC_{50} 0.41 µM



Compound 8/S
L. donovani promastigote- IC_{50} 0.82 µM

Figure **2-12**: Two analogues, 18/S and 8/S, with promising anti-promastigote activity (Rando *et al*., 2008)

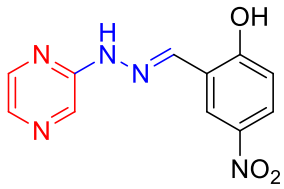
2.9.2 Hydrazone and azine

In addition to the nitrofuran moiety, clinical nitrofurans have a hydrazone moiety (=C-NH-NH_2) that possesses diverse biological and pharmacological properties, such as anti-cancer (Iliev *et al*., 2019:756), anti-inflammatory (Kumar *et al*., 2015:2580), anti-protozoal (Rollas & Küçükgüzel,

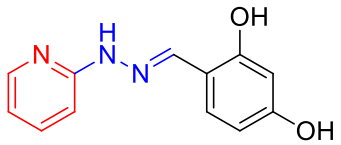
2007:1919), antibacterial (Popiołek *et al*., 2020:260), anti-trypanosomal (Glinma *et al*., 2015:001), and antileishmanial (Vargas *et al*., 2018:1) activity. Research has indicated that the combination of hydrazones with other biologically active functional groups results in compounds with unique chemical and physical character (Verma *et al*., 2014:69).

The exact mechanism of action of hydrazones are not yet fully understood. However, it is theorised that hydrazones are inhibitors of parasite cysteine proteases (Vargas *et al*., 2018:2), which is hydrolase enzymes that degrade proteins. In parasitic diseases, cysteine proteases are involved in hemoglobin degradation, surface protein processing, and parasite egress (Verma *et al*., 2016:1). Therefore, this makes promising targets for drug development.

A study conducted by Coimbra *et al*. (2018) investigated the antileishmanial activity of hydrazone-containing compounds against the promastigote and amastigote forms of several *Leishmania* strains. Their study found a few active compounds, with their best two compounds (figure **2-13**) showing better activity against the amastigote form of *L. amazonensis* (IFLA/BR/1967/PH8 strain) than their reference drug, miltefosine (IC_{50} 12.74 µM). Compounds 2s and 2c showed IC_{50} values of 11.09 µM and 11.98 µM, respectively (Coimbra *et al*., 2018).



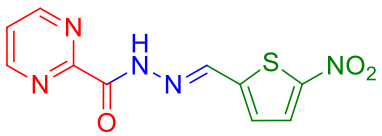
Compound 2s
L. amazonensis amastigote- IC_{50} 11.09 µM



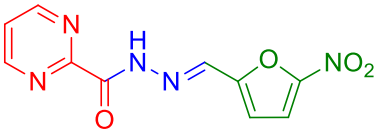
Compound 2c
L. amazonensis amastigote- IC_{50} 11.98 µM

Figure **2-13**: Compounds 2s and 2c, with the best antileishmanial activity (Coimbra *et al*., 2018)

Another study conducted by Coimbra *et al*. (2019) also investigated the antileishmanial activity of hydrazone-containing compounds against the promastigote and amastigote forms of *L. amazonensis* (IFLA/BR/67/PH8 strain). Their study showed two compounds (figure **2-14**) with fairly good anti-amastigote activity. Compounds 6i and 6h showed IC_{50} values of 8.41 µM and 10.8 µM, respectively (Coimbra *et al*., 2019).



Compound 6i
L. amazonensis amastigote- IC_{50} 8.41 µM



Compound 6h
L. amazonensis amastigote- IC_{50} 10.8 µM

Figure **2-14**: Compounds 6i and 6h, with the best anti-amastigote activity (Coimbra *et al*., 2019)

Azines are organic molecules which bear the C=N–N=C functional unit. The azine moiety consists of two imine bonds groups joined by a N–N single bond, that are polar acceptor groups orientated in opposite directions. Azine compounds have been receiving increasing attention because of their chemical, physical, and biological properties (Singh & Pandey, 2009:57). They are thermally and chemically more stable than their hydrazone tautomers. Some azines are also redox-active (Chourasiya *et al*., 2019:8487). The attachment of the azine moiety to the nitrofuran pharmacophore may lead to derivatives with enhanced hydrophilicity, chemical and thermally stability, and possibly increased biological activity.

2.9.3 Benzene ring

Aromatic rings are extensively used in drug design due to their well-known synthetic and modification paths (Aldeghi *et al*., 2014:450). A benzene ring introduces chemical stability, diverse reactivity paths and possible enhancement of biological activity. In this study, the aromatic moiety (a benzene ring) will be incorporated into nitrofuranylazine through the use of aromatic aldehydes. Aldehydes contain a carbonyl group which is a mild electrophile with increased reaction rate that is of more interest to use in biological systems upon interaction with strong nucleophiles such as hydrazine. This causes an equilibrium shift that favours the formation of hydrazone (Fernandes *et al*., 2018:905).

In this study, nitrofuran-based azine derivatives will be synthesised, characterised and evaluated for its antileishmanial activity. The synthesised derivatives will consist of three active pharmacophores, namely the nitrofuran, the hydrazone and the aromatic ring (figure **2-15**). As already discussed (section 2.9.1), nitrofuran possesses diverse biological and pharmacological properties and it is redox-active. The addition of the azine may improve solubility, thermal and chemical stability, as well as redox activity, and the addition of a benzene ring may further enhance biological activity. Thus, the combination of these three pharmacophores may form biologically active anti-parasitic agent with good stability, efficacy and long half-life.

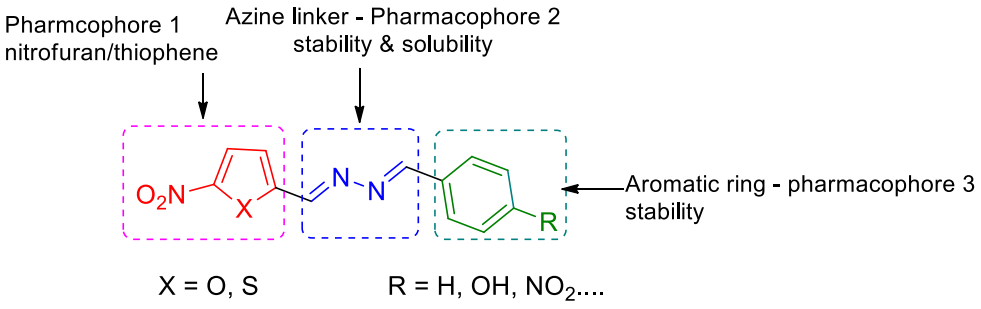


Figure **2-15**: Compound design model of the study

REFERENCE LIST

Aldeghi, M., Malhotra, S., Selwood, D.L., and Chan, A.W. 2014. Two-and three-dimensional rings in drugs. *Chemical Biology & Drug Design*, 83(4):450-461.

Amato, V.S., Padilha, A.R.S., Nicodemo, A.C., Duarte, M.I.S., Valentini, M., Uip, D.E., … Neto, V.A. 2000. Use of itraconazole in the treatment of mucocutaneous leishmaniasis: A pilot study. *International Journal of Infectious Diseases*, 4(3):153-157.

Aoun, K. & Bouratbine, A. 2014. Cutaneous leishmaniasis in North Africa: a review. *Parasite,* 21(14), art. #14. doi:10.1051/parasite/2014014

Aronson, J.K. 2016. Antimony and antimonials. In: Aronson, J.K., ed. *Meyler's Side Effects of Drugs (Sixteenth Edition)*. Oxford: Elsevier. pp. 619-624.

Avila-García, M., Mancilla, J., Segura-Cervantes, E. & Galindo-Sevilla, N. 2014. Transmission to humans. In: Claborn, D.M., ed*. Leishmaniasis- Trends in Epidemiology, Diagnosis and Treatment*. London, UK: IntechOpen. pp. 27-44.

Babokhov, P., Sanyaolu, A.O., Oyibo, W.A., Fagbenro-Beyioku, A.F. & Iriemenam, N.C. 2013. A current analysis of chemotherapy strategies for the treatment of human African trypanosomiasis. *Pathogens and global health,* 107(5):242-252.

Barrett, M.P. & Croft, S.L. 2012. Management of trypanosomiasis and leishmaniasis. *British medical bulletin,* 104(1):175-196.

Bekersky, I., Fielding, R.M., Dressler, D.E., Lee, J.W., Buell, D.N. & Walsh, T.J. 2002. Pharmacokinetics, excretion, and mass balance of liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate in humans. *Antimicrobial agents and chemotherapy*, 46(3):828-833.

Boelaert, M. & Sundar, S. 2014. Leishmaniasis. In: Farrar, J., Hotez, P.J., Junghanss, T., Kang, G., Lalloo, D. & White, N.J., eds. *Manson's Tropical Infectious Diseases (Twenty-third Edition)*. London, UK: W.B. Saunders. pp. 631-651.e4.

Boniface, P.K. & Ferreira, E.I. 2019. Flavonoids as efficient scaffolds: Recent trends for malaria, leishmaniasis, Chagas disease, and dengue. *Phytotherapy Research*, 33(10):2473-2517.

Braga, S.S. 2019. Multi-target drugs active against leishmaniasis: A paradigm of drug repurposing. *European Journal of Medicinal Chemistry,* 183, art. #111660. doi:10.1016/j.ejmech.2019.111660

Brotherton, M.-C., Bourassa, S., Leprohon, P., Légaré, D., Poirier, G.G., Droit, A. & Ouellette, M. 2013. Proteomic and genomic analyses of antimony resistant *Leishmania infantum* mutant. *PLOS ONE*, 8(11), e81899. https://doi.org/10.1371/journal.pone.0081899

Camargo, L.B. & Langoni, H. 2006. Impact of leishmaniasis on public health. *Journal of Venomous Animals and Toxins including Tropical Diseases,* 12(4):527-548.

CDC (Centers for Disease Control and Prevention). 2017. *Leishmaniasis [Leishmania spp.] Image gallery*. https://www.cdc.gov/dpdx/leishmaniasis/index.html Date of access: 16 May 2021.

CDC. 2018. *Neglected tropical diseases (NTDs)*. https://www.cdc.gov/globalhealth/newsroom/topics/ntds/index.html Date of access: 29 Apr. 2021.

CDC. 2020*a*. *Parasites – About parasites*. https://www.cdc.gov/parasites/about.html#:~:text=There%20are%20three%20main%20classes,protozoa%2C%20helminths%2C%20and%20ectoparasites Date of access: 19 Mar. 2021.

CDC. 2020*b*. *Parasites - Leishmaniasis: Epidemiology & Risk Factors*. https://www.cdc.gov/parasites/leishmaniasis/epi.html Date of access: 18 Feb. 2021.

CDC. 2020*c*. *Parasites - Leishmaniasis: Biology.* https://www.cdc.gov/parasites/leishmaniasis/biology.html Date of access: 22 Nov. 2020.

Chakravarty, J. & Sundar, S. 2010. Drug resistance in leishmaniasis. *Journal of Global Infectious Diseases*, 2(2):167-176.

Chourasiya, S.S., Kathuria, D., Wani, A.A. & Bharatam, P.V. 2019. Azines: synthesis, structure, electronic structure and their applications*. Organic & Biomolecular Chemistry*, 17(37):8486-8521.

Coelho, A.C., Boisvert, S., Mukherjee, A., Leprohon, P., Corbeil, J. & Ouellette, M. 2012. Multiple mutations in heterogeneous miltefosine-resistant *Leishmania major* population as determined by whole genome sequencing. *PLoS Neglected Tropical Diseases*, 6(2), e1512. https://doi.org/10.1371/journal.pntd.0001512

Coimbra, E.S., Antinarelli, L.M.R., De A. Crispi, M., Nogueira, T.C.M., Pinheiro, A.C. & De Souza, M.V.N. 2018. Synthesis, biological activity, and mechanism of action of 2-pyrazyl and pyridylhydrazone derivatives, new classes of antileishmanial agents. *ChemMedChem*, 13(14):1387-1394.

Coimbra, E.S., Nora De Souza, M.V., Terror, M.S., Pinheiro, A.C. & Da Trindade Granato, J. 2019. Synthesis, biological activity, and mechanism of action of new 2-pyrimidinyl hydrazone and

N-acylhydrazone derivatives, a potent and new classes of antileishmanial agents. *European Journal of Medicinal Chemistry*, 184, art. #111742. doi:10.1016/j.ejmech.2019.111742

Consigli, J., Danielo, C., Gallerano, V., Papa, M. & Guidi, A. 2006. Cutaneous leishmaniasis: successful treatment with itraconazole. *International Journal of Dermatology*, 45(1):46-49.

Croft, S.L., Sundar, S. & Fairlamb, A.H. 2006. Drug resistance in leishmaniasis. *Clinical microbiology reviews*, 19(1):111-126.

Cunningham, D.D. 1885. *On the presence of peculiar parasitic organisms in the tissue of a specimen of Delhi boil*. 1st ed. Calcutta, India: Government printing.

David, C.V. & Craft, N. 2009. Cutaneous and mucocutaneous leishmaniasis. *Dermatologic Therapy*, 22(6):491-502.

De Aguiar, M.G., Gonçalves, J.E., Souza, M.d'A., De Silva, R.E., Silveira, J.N. & Cota, G. 2018. Plasma antimony determination during cutaneous leishmaniasis treatment with intralesional infiltration of meglumine antimoniate. *Tropical Medicine & International Health*, 23(10):1110-1117.

Debruyne, D. & Ryckelynck, J.P. 1993. Clinical pharmacokinetics of fluconazole. *Clinical Pharmacokinetics,* 24(1):10-27.

Domingues Passero, L.F., Laurenti, M.D., Santos-Gomes, G., Soares Campos, B.L., Sartorelli, P. & Lago, J.H.G. 2013. *In vivo* antileishmanial activity of plant-based secondary metabolites. In: Rai, M.K. & Kon, K.V., eds. *Fighting Multidrug Resistance with Herbal Extracts, Essential Oils and Their Components*. San Diego: Academic Press. pp. 95-107.

Dorlo, T.P.C., Balasegaram, M., Beijnen, J.H. & De Vries, P.J. 2012. Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *Journal of Antimicrobial Chemotherapy*, 67(11):2576-2597.

Eiras, D.P., Kirkman, L.A. & Murray, H.W. 2015. Cutaneous leishmaniasis: current treatment practices in the USA for returning travelers. *Current treatment options in infectious diseases*, 7(1):52-62.

Engels, D. & Zhou, X.-N. 2020. Neglected tropical diseases: an effective global response to local poverty-related disease priorities. *Infectious Diseases of Poverty*, 9(1), art. #10. doi:10.1186/s40249-020-0630-9

Fernandes, C.S.M., Teixeira, G.D.G., Iranzo, O. & Roque, A.C.A. 2018. Engineered protein variants for bioconjugation. In: Sarmento, B. & Das Neves, J., eds. *Biomedical Applications of Functionalized Nanomaterials*. Amsterdam: Elsevier. pp. 105-138.

Foroumadi, A., Adibi, H., Kabudanian Ardestani, S., Shirooie, S., Bozorgomid, A. & Jafari, A. 2017. Synthesis and leishmanicidal activity of 1-[5-(5-nitrofuran-2-yl)-1,3,4-thiadiazole-2-yl]-4-benzoylepiperazines. *Iranian journal of pharmaceutical research,* 16(3):904-909.

Freitas-Junior, L.H., Chatelain, E., Kim, H.A. & Siqueira-Neto, J.L. 2012. Visceral leishmaniasis treatment: what do we have, what do we need and how to deliver it? *International journal for parasitology. Drugs and drug resistance*, 2:11-19.

Georgiadou, S.P., Makaritsis, K.P. & Dalekos, G.N. 2015. Leishmaniasis revisited: Current aspects on epidemiology, diagnosis and treatment. *Journal of translational internal medicine*, 3(2):43-50.

Ghorbani, M. & Farhoudi, R. 2018. Leishmaniasis in humans: drug or vaccine therapy? *Drug design, development and therapy*, 12:25-40.

Glinma, B., Gbaguidi, F.A., Kassehin, U.C., Kpoviessi, S.D.S., Houngbeme, A., Houngue, H.D., … Poupaert, J.H. 2015. Synthesis and trypanocidal activity of salicylhydrazones and p-tosylhydrazones of S-(+)-carvone and arylketones on African trypanosomiasis. *Journal of Applied Pharmaceutical Science*, 5(06):001-007.

Guedes, D.L., Van Henten, S., Cnops, L., Adriaensen, W. & Van Griensven, J. 2020. Sexual transmission of visceral leishmaniasis: A neglected story. *Trends in Parasitology*, 36(12):950-952.

Güran, M. 2018. An overview of leishmaniasis: historic to future perspectives. In: Savić, S., ed. *Vectors and Vector-Borne Zoonotic Diseases*. London, UK: IntechOpen. pp. 1-14.

Hadighi, R., Boucher, P., Khamesipour, A., Meamar, A.R., Roy, G., Ouellette, M. & Mohebali, M. 2007. Glucantime-resistant *Leishmania tropica* isolated from Iranian patients with cutaneous leishmaniasis are sensitive to alternative antileishmania drugs. *Parasitology Research*, 101(5):1319-1322.

Hotez, P.J., Aksoy, S., Brindley, P.J. & Kamhawi, S. 2020. What constitutes a neglected tropical disease? *PLoS Neglected Tropical Diseases*, 14(1), e0008001. https://doi.org/10.1371/journal.pntd.0008001

Huang, Y.C., Colaizzi, J.L., Bierman, R.H., Woestenborghs, R. & Heykants, J. 1986. Pharmacokinetics and dose proportionality of ketoconazole in normal volunteers. *Antimicrobial agents and chemotherapy*, 30(2):206-210.

Huttner, A., Verhaegh, E.M., Harbarth, S., Muller, A.E., Theuretzbacher, U. & Mouton, J.W. 2015. Nitrofurantoin revisited: a systematic review and meta-analysis of controlled trials. *Journal of Antimicrobial Chemotherapy*, 70(9):2456-2464.

Iliev, I., Kontrec, D., Detcheva, R., Georgieva, M., Balacheva, A., Galić, N. & Pajpanova, T. 2019. Cancer cell growth inhibition by aroylhydrazone derivatives. *Biotechnology & Biotechnological Equipment*, 33(1):756-763.

Inceboz, T. 2019. Epidemiology and Ecology of Leishmaniasis. In: Rodriguez-Morales, A.J., ed. *Current Topics in Neglected Tropical Diseases*. London, UK: IntechOpen. pp. 1-15.

Janeway, C.A. Jr., Travers, P., Walport, M. & Shlomchik, M.J. 2001. The complement system and innate immunity. In: Janeway, C.A. Jr., Travers, P., Walport, M. & Shlomchik, M.J., eds. *Immunobiology: The Immune System in Health and Disease (Fifth edition)*. New York, NY: Garland Science.

Kariyawasam, R., Challa, P., Lau, R. & Boggild, A.K. 2019. Susceptibility testing of *Leishmania* spp. against amphotericin B and fluconazole using the Sensititre™ YeastOne™ YO9 platform. *BMC Infectious Diseases*, 19(1), art. #593. doi:10.1186/s12879-019-4237-3

Kennedy, P.G.E. 2013. Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). *The Lancet Neurology*, 12(2):186-194.

Kim, S.Y., Park, C., Jang, H.-J., Kim, B.-O., Bae, H.-W., Chung, I.-Y., … Cho, Y.-H. 2019. Antibacterial strategies inspired by the oxidative stress and response networks. *Journal of Microbiology*, 57(3):203-212.

Kip, A.E., Schellens, J.H.M., Beijnen, J.H. & Dorlo, T.P.C. 2018. Clinical pharmacokinetics of systemically administered antileishmanial drugs. *Clinical Pharmacokinetics*, 57(2):151-176.

Kobets, T., Grekov, I. & Lipoldova, M. 2012. Leishmaniasis: prevention, parasite detection and treatment. *Current Medicinal Chemistry*, 19(10):1443-1474.

Krasavin, M., Lukin, A., Vedekhina, T., Manicheva, O., Dogonadze, M., Vinogradova, T., … Sokolovich, E. 2019. Attachment of a 5-nitrofuroyl moiety to spirocyclic piperidines produces

non-toxic nitrofurans that are efficacious *in vitro* against multidrug-resistant *Mycobacterium tuberculosis*. *European Journal of Medicinal Chemistry,* 166:125-135.

Kumar, N., Chauhan, L.S., Sharma, C.S., Dashora, N. & Bera, R. 2015. Synthesis, analgesic and anti-inflammatory activities of chalconyl-incorporated hydrazone derivatives of mefenamic acid. *Medicinal Chemistry Research*, 24(6):2580-2590.

Kuper, H. 2019. Neglected tropical diseases and disability—what is the link? *Transactions of The Royal Society of Tropical Medicine and Hygiene*, 113(12):838-844.

Le, V.V.H., Davies, I.G., Moon, C.D., Wheeler, D., Biggs, P.J. & Rakonjac, J. 2019. Novel 5-nitrofuran-activating reductase in *Escherichia coli*. *Antimicrobial agents and chemotherapy*, 63(11), e00868-19. https://doi.org/10.1128/AAC.00868-19

Leishman, W.B. 1903. On the possibility of the occurrence of trypanosomiasis in India. *British Medical Journal*, 1(2213):1252-1254.

Maltezou, H.C. 2010. Drug resistance in visceral leishmaniasis. *Journal of Biomedicine and Biotechnology*, 2010, art. #617521. doi:10.1155/2010/617521

Maspero, G. 1910. *The dawn of civilization - Egypt and Chaldaea*. 5th ed. London, UK: Society for the Promotion of Christian Knowledge.

Mathur, T., Kumar, M., Barman, T.K., Raj, V.S., Upadhyay, D.J. & Verma, A.K. 2020. A novel class of azoles with potent anti-leishmanial activity. *bioRxiv*, doi:10.1101/2020.10.16.342329

McCarthy, J.S., Wortmann, G.W. & Kirchhoff, L.V. 2015. Drugs for protozoal infections other than malaria. In: Bennett, J.E., Dolin, R. & Blaser, M.J., eds. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*. Philadelphia, PA: Elsevier. pp. 510-518.e3.

McGwire, B.S. & Satoskar, A.R. 2014. Leishmaniasis: clinical syndromes and treatment. *QJM: An International Journal of Medicine,* 107(1):7-14.

Mikaeili, F., Fakhar, M., Sarkari, B., Motazedian, M.H. & Hatam, G. 2007. Comparison of serological methods (ELISA, DAT and IFA) for diagnosis of visceral leishmaniasis utilizing an endemic strain. *Iranian Journal of Immunology*, 4(2):116-121.

Mukhopadhyay, R., Mukherjee, S., Mukherjee, B., Naskar, K., Mondal, D., Decuypere, S., … Roy, S. 2011. Characterisation of antimony-resistant *Leishmania donovani* isolates: Biochemical and

biophysical studies and interaction with host cells. *International journal for parasitology*, 41(13):1311-1321.

Nweze, J.A., Mbaoji, F.N., Li, Y.-M., Yang, L.-Y., Huang, S.-S., Chigor, V.N., … Yang, D.-F. 2021. Potentials of marine natural products against malaria, leishmaniasis, and trypanosomiasis parasites: a review of recent articles. *Infectious Diseases of Poverty*, 10(1), art. #9. doi:10.1186/s40249-021-00796-6

Paget, T. 2011. Protozoa. In: Denyer, S.P., Hodges, N., Gorman, S.P. & Gilmore, B.F., eds. *Hugo and Russell's Pharmaceutical Microbiology (Eighth edition)*. Chichester, UK: Blackwell Publishing. pp. 84-105.

PAHO (Pan American Health Organization). 2020. *Leishmaniasis*. https://www3.paho.org/en/topics/leishmaniasis Date of access: 19 Jun. 2021.

Pal, C. & Bandyopadhyay, U. 2012. Redox-active antiparasitic drugs. *Antioxidants & redox signaling,* 17(4):555-82.

Palace-Berl, F., Pasqualoto, K., Jorge, S., Zingales, B., Zorzi, R., Nunes, M., … Tavares, L. 2015. Designing and exploring active N'-[(5-nitrofuran-2-yl) methylene] substituted hydrazides against three Trypanosoma cruzi strains more prevalent in Chagas disease patients. *European Journal of Medicinal Chemistry*, 96(96):330-9.

Pedrosa, C.M.S. 2017. Clinical manifestations of visceral leishmaniasis (American visceral leishmaniasis). In: Claborn, D., ed. *The Epidemiology and Ecology of Leishmaniasis*. London, UK: IntechOpen. pp. 17-30.

Peterson, R.T. 2018. 5-Nitrofurans and cancer: teaching an old drug new tricks. *Cell Chemical Biology,* 25(12):1439-1440.

Pinto-Martinez, A.K., Rodriguez-Durán, J., Serrano-Martin, X., Hernandez-Rodriguez, V. & Benaim, G. 2017. Mechanism of action of miltefosine on *Leishmania donovani* involves the impairment of acidocalcisome function and the activation of the sphingosine-dependent plasma membrane Ca^{2+} channel. *Antimicrobial agents and chemotherapy*, 62(1), e01614-17. https://doi.org/10.1128/AAC.01614-17

Ponte-Sucre, A., Gamarro, F., Dujardin, J.-C., Barrett, M.P., López-Vélez, R., García-Hernández, R., … Papadopoulou, B. 2017. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLoS Neglected Tropical Diseases*, 11(12), e0006052. https://doi.org/10.1371/journal.pntd.0006052

Popiołek, Ł., Rysz, B., Biernasiuk, A. & Wujec, M. 2020. Synthesis of promising antimicrobial agents: hydrazide-hydrazones of 5-nitrofuran-2-carboxylic acid. *Chemical Biology & Drug Design*, 95(2):260-269.

Prentice, A.G. & Glasmacher, A. 2005. Making sense of itraconazole pharmacokinetics. *Journal of Antimicrobial Chemotherapy*, 56:i17-i22.

Pund, S. & Joshi, A. 2017. Nanoarchitectures for neglected tropical protozoal diseases: challenges and state of the art. In: Grumezescu, A.M., ed. *Nano- and Microscale Drug Delivery Systems*. Amsterdam: Elsevier. pp. 439-480.

Rando, D.G., Avery, M.A., Tekwani, B.L., Khan, S.I. & Ferreira, E.I. 2008. Antileishmanial activity screening of 5-nitro-2-heterocyclic benzylidene hydrazides. *Bioorganic & Medicinal Chemistry*, 16(14):6724-6731.

Rex, J.H. & Stevens, D.A. 2015. Drugs Active against fungi, pneumocystis, and microsporidia. In: Bennett, J.E., Dolin, R. & Blaser, M.J., eds. *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition).* Philadelphia, PA: Elsevier. pp. 479-494.e4.

Roberts, C.W., McLeod, R., Rice, D.W., Ginger, M., Chance, M.L. & Goad, L.J. 2003. Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. *Molecular and Biochemical Parasitology*, 126(2):129-142.

Rodrigues, W.F., Mendes, N.S., De Carvalho Ribeiro, P., Mendes Filho, D., Parreira, R.C., Chaves, K.C.B., … Miguel, C.B. 2021. A critical review of the applicability of serological screening for leishmaniasis in blood banks in Brazil. *Journal of Parasitic Diseases*, 45(1):109-117.

Rojas, R., Valderrama, L., Valderrama, M., Varona, M.X., Ouellette, M. & Saravia, N.G. 2006. Resistance to antimony and treatment failure in human *Leishmania (viannia)* infection. *The Journal of Infectious Diseases*, 193(10):1375-1383.

Rollas, S. & Küçükgüzel, S.G. 2007. Biological activities of hydrazone derivatives. *Molecules (Basel, Switzerland)*, 12(8):1910-1939.

Ross, R. 1903. Note on the bodies recently described by Leishman and Donovan. *British Medical Journal*, 2(2237):1261-1262.

Ryan, E.T. 2018. Antiparasitic agents. In: Long, S.S., Prober, C.G. & Fischer, M., eds. *Principles and Practice of Pediatric Infectious Diseases (Fifth edition)*. Philadelphia, PA: Elsevier. pp. 1567-1587.e2.

Salam, N., Al-Shaqha, W.M. & Azzi, A. 2014. Leishmaniasis in the middle East: incidence and epidemiology. *PLoS neglected tropical diseases*, 8(10), e3208. https://doi.org/10.1371/journal.pntd.0003208

Sarkari, B., Ashrafmansouri, M., Hatam, G., Habibi, P. & Abdolahi Khabisi, S. 2014. Performance of an ELISA and indirect immunofluorescence assay in serological diagnosis of zoonotic cutaneous leishmaniasis in Iran. *Interdisciplinary Perspectives on Infectious Diseases*, 2014, art. #505134. doi:10.1155/2014/505134

Seblova, V., Oury, B., Eddaikra, N., Aït-Oudhia, K., Pratlong, F., Gazanion, E., … Sereno, D. 2014. Transmission potential of antimony-resistant leishmania field isolates. *Antimicrobial agents and chemotherapy*, 58(10):6273-6276.

Sharma, S. & Anand, N. 1997. Nitroheterocycles. In: Sharma, S. & Anand, N., eds. *Pharmacochemistry Library.* Amsterdam: Elsevier Science B.V. pp. 421-438.

Sifontes-Rodríguez, S., Monzote-Fidalgo, L., Castañedo-Cancio, N., Montalvo-Álvarez, A.M., López-Hernández, Y., Diogo, N.M., … Cabrera-Pérez, M.Á. 2015. The efficacy of 2-nitrovinylfuran derivatives against *Leishmania* in vitro and in vivo. *Memorias do Instituto Oswaldo Cruz*, 110(2):166-173.

Singh, B. & Pandey, A. 2009. Synthesis, characterisation and mesomorphic properties of unsymmetrical N-(o-hydroxybenzylidene)-N'-(4-n-alkoxybenzylidene)azines and their copper (II) complexes. *Liquid Crystals,* 37(1):57-67.

Srivastava, S., Mishra, J., Gupta, A.K., Singh, A., Shankar, P. & Singh, S. 2017. Laboratory confirmed miltefosine resistant cases of visceral leishmaniasis from India. *Parasites & Vectors,* 10(1), art. #49. doi:10.1186/s13071-017-1969-z

Steverding, D. 2016. The history of leishmaniasis. *Parasites & Vectors*, 10(1), art. #82. doi: 10.1186/s13071-017-2028-5

Teixeira de Macedo Silva, S., Visbal, G., Lima Prado Godinho, J., Urbina, J.A., De Souza, W. & Cola Fernandes Rodrigues, J. 2018. In vitro antileishmanial activity of ravuconazole, a triazole antifungal drug, as a potential treatment for leishmaniasis. *Journal of Antimicrobial Chemotherapy*, 73(9):2360-2373.

Torres-Guerrero, E., Quintanilla-Cedillo, M.R., Ruiz-Esmenjaud, J. & Arenas, R. 2017. Leishmaniasis: a review. *F1000Research,* 6(750):1-15.

Turchetti, A.P., Souza, T.D., Paixão, T.A. & Santos, R.L. 2014. Sexual and vertical transmission of visceral leishmaniasis. *The Journal of Infection in Developing Countries*, 8(4):403-407.

U.S. National Library of Medicine. 2021. *Clinical trials- Leishmaniasis*. https://clinicaltrials.gov/ct2/results?cond=leishmaniasis&term=vaccine&cntry=&state=&city=&dist=#wrapper Date of access: 24 Apr. 2021.

Valle, I.V., Machado, M.E., Araújo, C.D.C.B., Da Cunha-Junior, E.F., Da Silva Pacheco, J., Torres-Santos, E.C., … Sathler, P.C. 2019. Oral pentamidine-loaded poly(d,l-lactic-co-glycolic) acid nanoparticles: an alternative approach for leishmaniasis treatment. *Nanotechnology,* 30(45), art. #455102. doi:10.1088/1361-6528/ab373e

Van Bockstal, L., Bulté, D., Hendrickx, S., Sadlova, J., Volf, P., Maes, L. & Caljon, G. 2020. Impact of clinically acquired miltefosine resistance by *Leishmania infantum* on mouse and sand fly infection. *International Journal for Parasitology: Drugs and Drug Resistance*, 13:16-21.

Vargas, E., Echeverri, F., Upegui, Y.A., Robledo, S.M. & Quiñones, W. 2018. Hydrazone derivatives enhance antileishmanial activity of thiochroman-4-ones. *Molecules*, 23(1), art. #70. doi:10.3390/molecules23010070

Verma, G., Marella, A., Shaquiquzzaman, M., Akhtar, M., Ali, M.R. & Alam, M.M. 2014. A review exploring biological activities of hydrazones. *Journal of pharmacy & bioallied sciences,* 6(2):69-80.

Verma, S., Dixit, R. & Pandey, K.C. 2016. Cysteine proteases: modes of activation and future prospects as pharmacological targets. *Frontiers in Pharmacology*, 7, art. #107. doi:10.3389/fphar.2016.00107

Vincent, H.M. 2017. William Boog Leishman: parasitologist and politician. *Parasitology*, 144(12):1582-1589.

Waller, D.G. & Sampson, A.P. 2018. Chemotherapy of infections. In: Waller, D.G. & Sampson, A.P., eds. *Medical Pharmacology and Therapeutics (Fifth Edition)*. Elsevier. pp. 581-629.

Wang, Y., Gray, J., Mishin, V., Heck, D., Laskin, D. & Laskin, J. 2008. Role of cytochrome P450 reductase in nitrofurantoin-induced redox cycling and cytotoxicity. *Free radical biology & medicine*, 44:1169-79.

Wilson, A.L., Courtenay, O., Kelly-Hope, L.A., Scott, T.W., Takken, W., Torr, S.J. & Lindsay, S.W. 2020. The importance of vector control for the control and elimination of vector-borne diseases. *PLoS Neglected Tropical Diseases*, 14(1), e0007831. https://doi.org/10.1371/journal.pntd.0007831

WHO (World Health Organization) Expert Committee on the Control of the Leishmaniases & World Health Organization. 2010. *Control of the leishmaniases: report of a meeting of the WHO Expert Commitee on the Control of Leishmaniases*, Geneva: World Health Organization. https://apps.who.int/iris/handle/10665/44412 Date of access: 21 Mar. 2021.

WHO. 2017. *Global vector control response 2017–2030: A strategic approach to tackle vector-borne diseases.* https://www.who.int/publications/i/item/WHO-HTM-GVCR-2017.01 Date of access: 16 Jun. 2021.

WHO. 2019*a*. *Status of endemicity of visceral leishmaniasis worldwide*. https://www.who.int/images/default-source/maps/leishmaniasis_vl_2019.png?sfvrsn=49da7180_1 Date of access: 21 Mar. 2021.

WHO. 2019*b*. *Status of endemicity of cutaneous leishmaniasis worldwide*. https://www.who.int/images/default-source/maps/leishmaniasis_cl_2019.png?sfvrsn=23653e5f_1 Date of access: 21 Mar. 2021.

WHO. 2020. *Box 1. Recommended treatment regimens for visceral leishmaniasis, ranked by preference*. https://www.who.int/leishmaniasis/research/978924129496_pp67_71.pdf Date of access: 20 Des. 2020.

WHO. 2021*a*. *Leishmaniasis: Overview*. https://www.who.int/health-topics/leishmaniasis#tab=tab_1 Date of access: 16 Jun. 2021.

WHO. 2021*b*. *Leishmaniasis: Key facts*. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis Date of access: 16 Jun. 2021.

WHO. 2021*d*. *Number of cases of visceral leishmaniasis reported. Data by country*. http://apps.who.int/gho/data/node.main.NTDLEISHVNUM?lang=en Date of access: 11 Feb. 2021.

WHO. 2021*e*. *Number of cases of cutaneous leishmaniasis reported. Data by country*. http://apps.who.int/gho/data/node.main.NTDLEISHCNUM?lang=en Date of access: 11 Feb. 2021.

Wyllie, S., Roberts, A.J., Norval, S., Patterson, S., Foth, B.J., Berriman, M., … Fairlamb, A.H. 2016. Activation of bicyclic nitro-drugs by a novel nitroreductase (NTR2) in *Leishmania*. *PLoS Pathogens*, 12(11), e1005971. https://doi.org/10.1371/journal.ppat.1005971

Zheng, Z., Chen, J., Ma, G., Satoskar, A.R. & Li, J. 2020. Integrative genomic, proteomic and phenotypic studies of *Leishmania donovani* strains revealed genetic features associated with virulence and antimony-resistance. *Parasites & Vectors*, 13(1), art. #510. doi:10.1186/s13071-020-04397-4

Zink, A.R., Spigelman, M., Schraut, B., Greenblatt, C.L., Nerlich, A.G. & Donoghue, H.D. 2006. Leishmaniasis in ancient Egypt and Upper nubia. *Emerging infectious diseases*, 12(10):1616-1617.

CHAPTER 3

ARTICLE FOR SUBMISSION

Chapter 3 contains the article entitled “Synthesis and *in vitro* antileishmanial activities of nitrofuran-based azine derivatives” which will be submitted to European Journal of Pharmaceutical Sciences. This article contains the biological findings of the synthesised antileishmanial compounds of the study.

The manuscript was prepared according to the Author Guidelines of the journal (see Annexure C).

Synthesis and *in vitro* antileishmanial activities of nitrofuran-based azine derivatives

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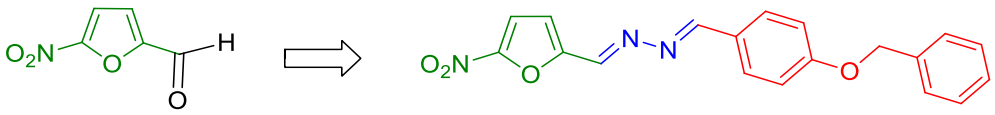
The synthesis was conducted by Ms M Viljoen under the guidance of Dr C Kannigadu and Prof DD N'Da. The cytotoxicity and antileishmanial activity properties were determined by Ms M Viljoen and Dr J Aucamp.

HIGHLIGHTS

- Synthesis of 2 sub-series of nitrofuran-based azine derivatives
- *In vitro* evaluation of antileishmanial activity and cytotoxicity
- Overall good activity of derivatives against various *Leishmania* promastigotes
- Moderate to non-cytotoxic derivatives against Vero cells
- Activity with IC_{50} values 0.34 µM - 0.70 µM were reported for the most active compound (**7a**) against all parasite strains

GRAPHICAL ABSTRACT



5-Nitro-2-furaldehyde, NFA

L. donovani 1S IC_{50} 11.06 µM
L. donovani 9515 IC_{50} 13.71 µM
L. major IR-173 IC_{50} 7.94 µM
$LogP_{o/w}$ 0.36
Vero IC_{50} 17.82 µM

Compound **7a**

L. donovani 1S IC_{50} 0.49 µM
L. donovani 9515 IC_{50} 1.61 µM
L. major IR-173 IC_{50} 0.45 µM
$LogP_{o/w}$ 3.43
Vero IC_{50} >100 µM

ABSTRACT

Leishmaniasis is a vector-borne infectious disease affecting both humans and animals. This neglected tropical disease can be fatal if not treated. Hundreds of thousands of new leishmaniasis cases are being reported by the WHO every year, and currently available treatments are unsatisfactory. Severe adverse effects, short half-lives and increased pathogen resistance against current clinical treatments emphasise the urgent need for the development of new antileishmanial drugs. In search for such drugs, we investigate a series of nitrofuran-based azine derivatives. Herein, we report the design, synthesis and biological activity of the derivatives against promastigotes of *Leishmania donovani* (1S and 9515), and *L. major* IR-173 strains. Compounds **3a**, **5a** and **7a** featuring chloro, methyl and benzyl substituents, respectively, were the most active against the tested *Leishmania* strains, possessing nanomolar activities up to 18-fold higher than the parent compound 5-nitro-2-furaldehyde. These three compounds were identified as anti-promastigote hits for further investigation into novel antileishmanial agents.

Keywords: *Leishmania*, promastigote, nitrofuran, thiophene, hydrazone, azine

1. Introduction

Leishmaniasis is a neglected tropical disease (NTD) caused by the *Leishmania* parasite and it is transmitted to humans during hematophagy of an infected female *Phlebotomus* sandfly (WHO, 2021a). There are over twenty species that can affect humans and three clinical forms in which the disease can manifest itself in (Salam et al., 2014). These forms are cutaneous (CL), which can be identified by lesions on the skin; mucocutaneous (MCL), a differentiated form with lesions on the mouth and skin; and visceral leishmaniasis (VL), identified by skin lesions and a systemic infection that results in an immune deficiency (Torres-Guerrero et al., 2017). VL is the most dangerous form, causing excessive internal damage with a mortality rate of 95% if left untreated (WHO, 2021b).

Leishmaniasis is closely associated with poor living conditions, malnutrition, weak immune systems and the lack of finances (WHO, 2021b). In 2019, the World Health Organisation (WHO) estimated 50,000 to 90,000 new cases of VL (mostly in Brazil, East Africa, and India) and 600,000 to 1 million new cases of CL (mostly in the Americas, the Mediterranean basin, the Middle East, and Central Asia) to have occurred worldwide (WHO, 2021b).

There is no vaccine for protection against this infection; however, various chemotherapeutic drugs, such as the pentavalent antimonies (sodium stibogluconate and meglumine antimonate), amphotericin B, miltefosine, pentamidine, paromomycin and the azoles (ketoconazole, itraconazole and fluconazole) (**Fig. 1**), are available for the treatment of leishmaniasis (Georgiadou et al., 2015; Ghorbani and Farhoudi, 2018; Braga, 2019). These drugs have several limitations, such as impractical administration, toxicity, cost, and parasite resistance (Freitas-Junior et al., 2012). Fight against the latter is an ongoing battle due to the misuse of drugs, financial constraints, and the unavailability of new drugs (Maltezou, 2010).

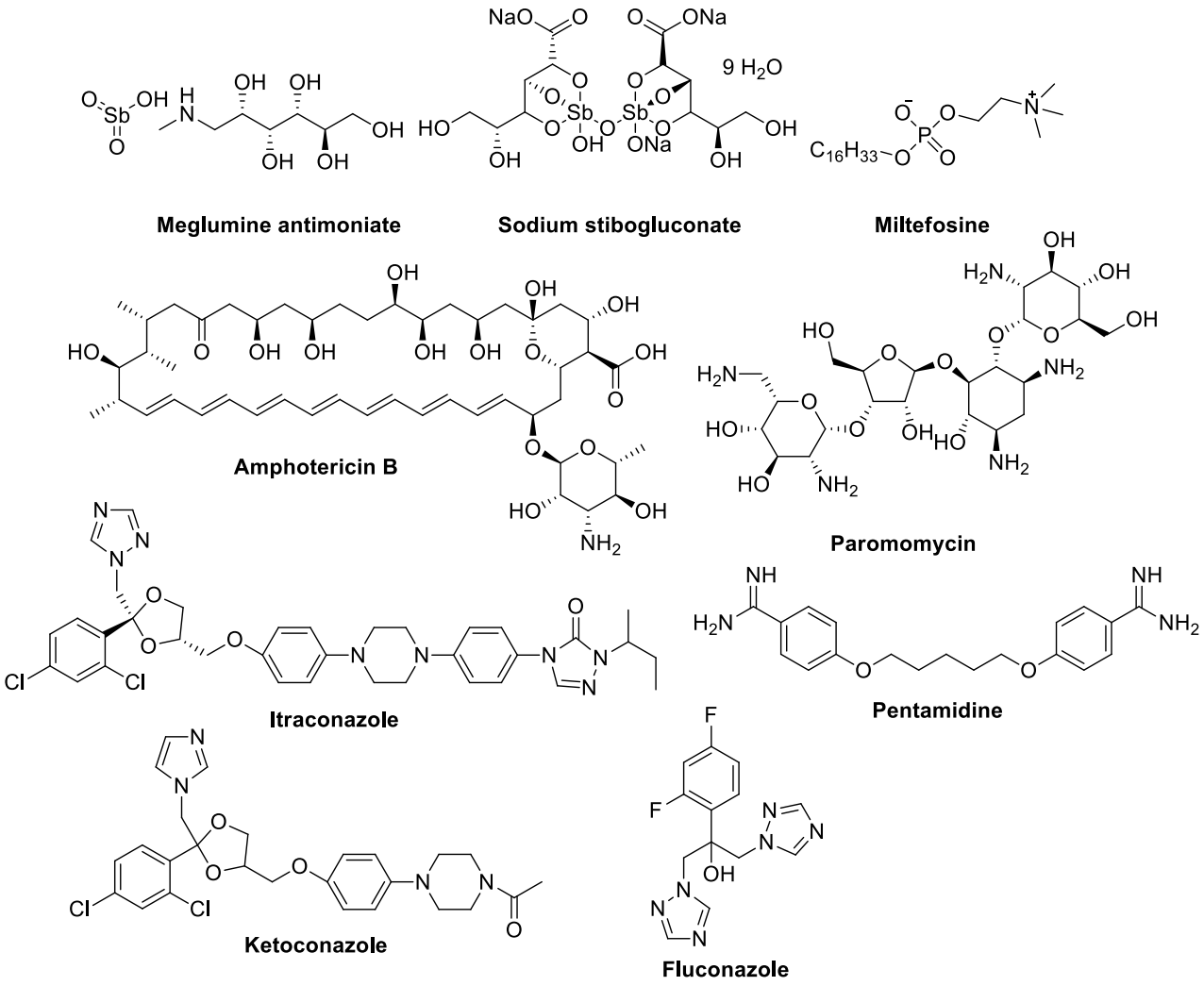


Fig. 1: Clinical drugs used for the treatment of leishmaniasis

The use of clinical nitrofurans (cNFs) to treat various infectious diseases has been well established over the recent years (Patterson and Wyllie, 2014; Zuma et al., 2019). The diverse biological activities of nitrofurans have been attributed to its nitro group and its ability to form reactive oxygen species (ROS) upon Fenton's reactions with enzymes (Pal and Bandyopadhyay, 2012; Patel et al., 2018). Additionally, cNFs (**Fig. 2**) also have a hydrazone moiety (blue in **Fig. 2**) that possess anti-infective activity therefore making them good candidates for drug repurposing for the treatment of infectious diseases such as leishmaniasis.

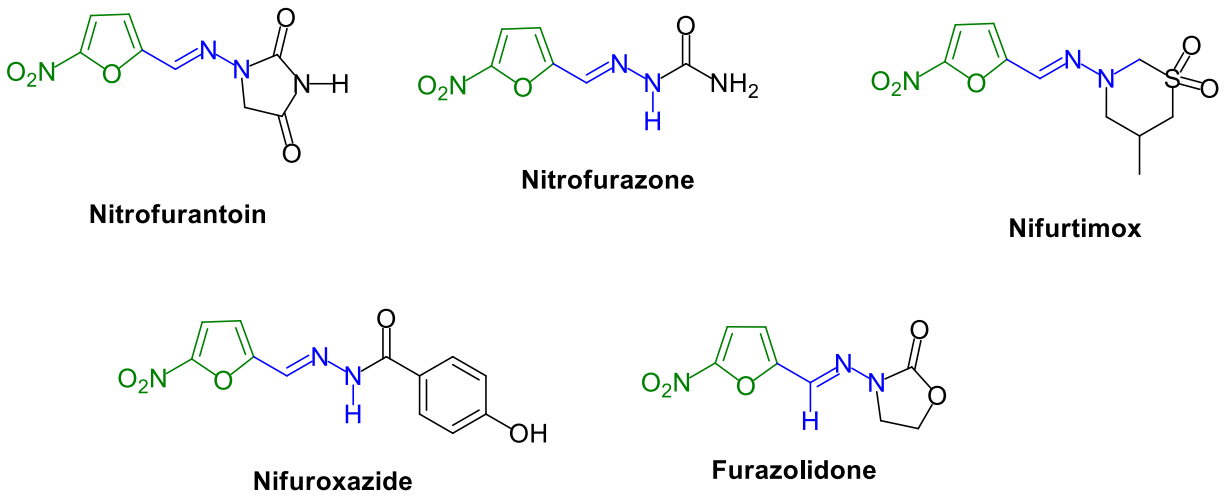


Fig. 2: Clinical nitrofuran drugs

The hydrazone moiety possesses diverse biological and pharmacological properties, such as anti-cancer (Sreenivasulu et al., 2019), anti-microbial (Manikandan et al., 2017), anti-trypanosomal (Glinma et al., 2015), anti-mycobacterial (Angelova et al., 2017), anti-convulsive (Dehestani et al., 2018), anti-inflammatory (Kajal et al., 2014) and analgesic (Navidpour et al., 2014) properties. Research has indicated that azines bearing the C=N–N=C functional unit are bactericidal and anti-parasitic (**Fig. 3**). These compounds are highly basic and form salts very easily, thus they are endowed with increased water solubility and become equivalent to hydrazones in the protonated state. They are also thermally and chemically more stable than their hydrazone tautomer's (Singh and Pandey, 2009; Chourasiya et al., 2019).

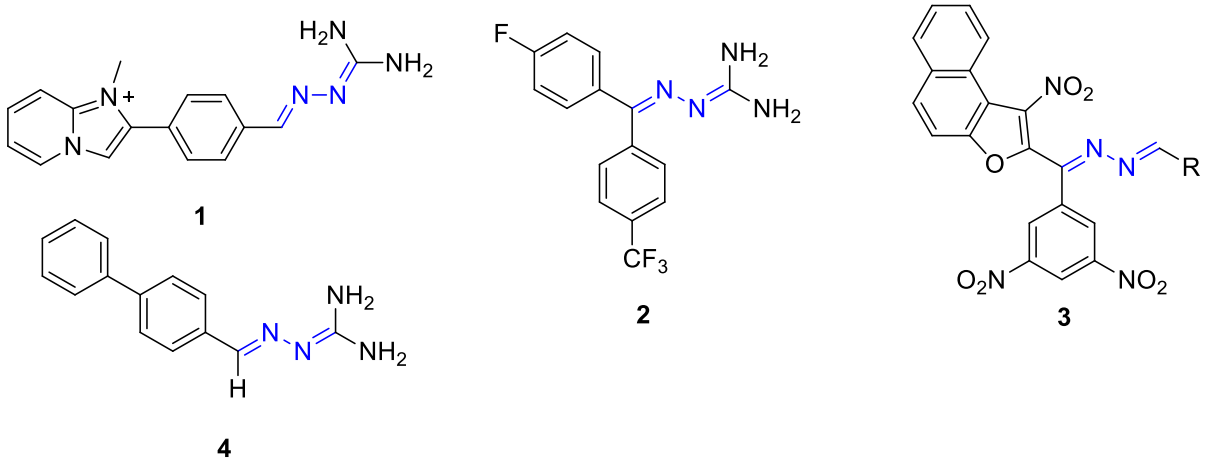


Fig. 3: Structures of biologically active azines where (**1**) trypanocidal agent (**2**) antimalarial agent (**3**) and (**4**) antibacterial agent (Chourasiya et al., 2019)

Furthermore, the use of aromatic rings in drug design is a well-known strategy as they offer numerous unique and strong interaction modes with target proteins, which include classical

arene-arene (π-stacking) interactions, as well as arene-H bonding (edge-to-face interactions), and other interactions, such as p-cation stabilisation and sulphur-arene interactions (Ward and Beswick, 2014). Thus, the linking of nitrofuran, azines and aromatic rings may result in compounds that are more active and stable.

Based on the evidence, nitrofuran-based azine derivatives and their antileishmanial activities were examined *in vitro*. We herein report the synthesis and the biological activities of these analogues.

2. Materials and methods

2.1. Materials

Reagents hydrazine monohydrate (N_2H_4), triethylamine ($C_6H_{15}N$), benzaldehyde (C_7H_6O), 4-nitrobenzaldehyde ($C_7H_5NO_3$), 4-chlorobenzaldehyde (C_7H_5ClO), 4-bromobenzaldehyde (C_7H_5BrO), 4-methylbenzaldehyde (C_8H_8O), 4-methoxybenzaldehyde ($C_8H_8O_2$), 4-fluorobenzaldehyde (C_7H_5FO), 4-hydroxybenzaldehyde ($C_7H_6O_2$), 4-(benzyloxy)benzaldehyde ($C_{14}H_{12}O_2$), 5-nitro-2-furaldehyde ($C_5H_3NO_4$), 5-nitro-2-thiophenecarboxaldehyde ($C_5H_3NO_3S$), p-Toluenesulfonic acid (PTSA), and sulphuric acid (H_2SO_4), were obtained from Sigma-Aldrich (Johannesburg, South Africa). *N*,*N*-dimethylformamide (DMF) were purchased from Merck (Johannesburg, South Africa). All other solvents; ethanol (EtOH), dichloromethane (DCM), ethyl acetate (EtOAc), and hexane, as well as potassium carbonate (K_2CO_3) and magnesium sulphate ($MgSO_4$), were purchased from Associated Chemical Enterprises, ACE (South Africa). All chemicals and reagents were of analytical grade and no further purification was needed.

2.2. General procedures

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra was recorded on a Bruker Avance™ 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 as a reference. The abbreviations of the splitting patterns were as follows: singlet (s), doublet (d), doublet of doublet (dd), 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 Vpp. 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 ($60F_{254}$) that was acquired from Merck (South Africa).

2.3. Syntheses

*2.3.1 Synthesis of phenylhydrazone intermediates (**1-8**)*

Substituted phenylaldehydes (10 mmol, 1 eq.) were dissolved in DCM (10 mL), triethylamine (1.5 eq.) was added, and the reaction was left to stir at room temperature for 10 minutes. Thereafter, excess hydrazine monohydrate (60 mmol, 4 eq.) was added, and the reaction was left to stir at room temperature for 12 hours while monitored by TLC. Upon completion, the reaction mixture was extracted with DCM (2 × 30 mL) and water (2 × 40 mL). The organic phase was dried over anhydrous magnesium sulphate, filtered and concentrated in vacuum to yield a crude product, which was then recrystallised with ethyl acetate to form the phenylhydrazone compounds **1-8**.

(E)-Benzylidenehydrazine **(1)**

Dark yellow powder; yield: 47%; m.p. 88-90 °C; IR $_{Vmax}$ (cm^{-1}): 2921 (N-H), 2851 (C-H), 1649 (C=C); ^{1}H NMR (600 MHz, DMSO) δ 8.72 (s, 1H, H-2), 8.89 (dd, *J* = 7.6, 1.8 Hz, 2H, H-4), 7.53 (dd, *J* = 13.1, 5.8 Hz, 3H, H-5/6), 6.62 (s, 2H, H-1). ^{13}C NMR (151 MHz, DMSO) δ 161.21 (C-2), 133.73 (C-3), 131.23 (C-6), 128.80 (C-4), 128.25 (C-5).

(E)-(4-Fluorobenzylidene)hydrazine **(2)**

Light yellow powder; yield: 62%; m.p. 181-183 °C; IR $_{Vmax}$ (cm^{-1}): 3324 (N-H), 2924 (C-H), 1596 (C=C), 1220 (C-F); ^{1}H NMR (600 MHz, DMSO) δ 7.70 (s, 1H, H-2), 7.50 (dd, J_{H-F} = 8.6, 5.8 Hz, 2H, H-4), 7.14 (t, J_{H-F} = 8.6 Hz, 2H, H-5), 6.70 (s, 2H, H-1). ^{13}C NMR (151 MHz, DMSO) δ 161.44 (d, $^{1}J_{C-F}$ = 244.0 Hz, C-6), 137.11 (C-2), 132.92 (d, $^{2}J_{C-F}$ = 3.0 Hz, C-3), 126.81 (d, $^{3}J_{C-F}$ = 8.0 Hz, C-4), 115.26 (d, $^{4}J_{C-F}$ = 21.7 Hz, C-5).

(E)-(4-Chlorobenzylidene)hydrazine **(3)**

Yellow powder; yield: 65%; m.p. 196-198 °C; IR $_{Vmax}$ (cm^{-1}): 3315 (N-H), 2933 (C-H), 1590 (C=C), 815 (C-Cl); ^{1}H NMR (600 MHz, DMSO) δ 8.72 (s, 1H, H-2), 7.90 (d, *J* = 8.5 Hz, 2H, H-4), 7.59 (d, *J* = 8.5 Hz, 2H, H-5), 6.62 (s, 2H, H-1). ^{13}C NMR (151 MHz, DMSO) δ 160.56 (C-2), 136.01 (C-6), 132.59 (C-3), 130.01 (C-4), 129.08 (C-5).

(E)-(4-Bromobenzylidene)hydrazine **(4)**

White crystal-like powder; yield: 61%; m.p. 72-74 °C; IR $_{Vmax}$ (cm^{-1}): 3353 (N-H), 2912 (C-H), 1588 (C=C), 507 (C-Br); ^{1}H NMR (600 MHz, DMSO) δ 7.65 (s, 1H, H-2), 7.50 (d, *J* = 8.5 Hz, 2H, H-4), 7.41 (d, *J* = 8.5 Hz, 2H, H-5), 6.91 (s, 2H, H-1). ^{13}C NMR (151 MHz, DMSO) δ 136.53 (C-2), 135.76 (C-3), 131.36 (C-5), 126.91 (C-4), 119.97 (C-6).

(E)-(4-Methylbenzylidene)hydrazine **(5)**

Yellow powder; yield: 85%; m.p. 150-152 °C; IR $_{Vmax}$ (cm^{-1}): 3316 (N-H), 2915 (C-H), 1612 (C=C); ^{1}H NMR (600 MHz, DMSO) δ 7.66 (s, 1H, H-2), 7.36 (d, *J* = 8.0 Hz, 2H, H-4), 7.13 (d, *J* = 8.0 Hz, 2H, H-5), 6.62 (s, 2H, H-1), 2.28 (s, 3H, H-7). ^{13}C NMR (151 MHz, DMSO) δ 138.56 (C-2), 136.61 (C-6), 133.59 (C-3), 128.96 (C-5), 125.06 (C-4), 20.72 (C-7).

(E)-(4-Methoxybenzylidene)hydrazine **(6)**

Light yellow powder; yield: 67%; m.p. 170-172 °C; IR $_{Vmax}$ (cm^{-1}): 2927 (N-H), 2836 (C-H), 1595 (C=C); ^{1}H NMR (600 MHz, DMSO) δ 8.63 (s, 1H, H-2), 7.81 (d, *J* = 8.7 Hz, 2H, H-4), 7.05 (d, *J* = 8.7 Hz, 2H, H-5), 6.62 (s, 2H, H-1), 3.83 (s, 3H, H-7). ^{13}C NMR (151 MHz, DMSO) δ 161.65 (C-6), 160.45 (C-2), 129.95 (C-4), 126.55 (C-3), 114.38 (C-5), 55.37 (C-7).

(E)-[4-(Benzyloxy)benzylidene]hydrazine **(7)**

White powder; yield: 84%; m.p. 125-127 °C; IR $_{Vmax}$ (cm^{-1}): 3314 (N-H), 3031 (C-H), 1597 (C=C), 1236 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 7.66 (s, 1H, H-2), 7.44 (d, *J* = 8.7 Hz, 2H, H-4), 7.41 (d, *J* = 8.5 Hz, 2H, H-9), 7.38 (d, *J* = 8.5 Hz, 2H, H-10), 7.32 (t, *J* = 7.3 Hz, 1H, H-11), 6.98 (d, *J* = 8.7 Hz, 2H, H-5), 6.48 (s, 2H, H-1), 5.10 (s, 2H, H-7). ^{13}C NMR (151 MHz, DMSO) δ 157.91 (C-6), 138.58 (C-2), 136.98 (C-8), 129.26 (C-4), 128.32 (C-10), 127.71 (C-3), 127.55 (C-9), 126.42 (C-11), 114.84 (C-5), 69.20 (C-7).

(E)-4-(Hydrazonomethyl)phenol **(8)**

Yellow powder; yield: 71%; m.p. 275-277 °C; IR $_{Vmax}$ (cm^{-1}): 3315 (O-H), 2939 (N-H), 2750 (C-H), 1585 (C=C); ^{1}H NMR (600 MHz, DMSO) δ 9.99 (s, 1H, H-7), 8.54 (s, 1H, H-2), 7.68 (d, *J* = 8.5 Hz, 2H, H-4), 6.86 (d, *J* = 8.5 Hz, 2H, H-5), 6.62 (s, 2H, H-1). ^{13}C NMR (151 MHz, DMSO) δ 160.16 (C-6), 129.96 (C-4), 126.56 (C-3), 125.05 (C-2), 115.66 (C-5).

*2.3.2 Synthesis of phenylhydrazone intermediate (**9**)*

4-Nitrobenzaldehyde (10 mmol, 1 eq.) was dissolved in ethanol (20 mL) and then hydrazine monohydrate (70 mmol, 5 eq.) and PTSA (1 mmol, 0.08 eq.) were added consecutively. The reaction was refluxed for 60 minutes and monitored by TLC. Upon completion, it was cooled down to 5-10 °C and the precipitate formed was filtered, washed with cold water and dried under vacuum to yield intermediate **9**. No further purification was done.

(E)-(4-nitrobenzylidene)hydrazine **(9)**

Bright yellow powder; yield: 91%; m.p. 134-136 °C; IR $_{Vmax}$ (cm^{-1}): 3419 (N-H), 3064 (C-H), 1572 (C=C), 1499 (N-O); ^{1}H NMR (600 MHz, DMSO) δ 8.16 (d, *J* = 8.9 Hz, 2H, H-5), 7.73 (s, 1H, H-2), 7.67 (d, *J* = 8.9 Hz, 2H, H-4), 7.55 (s, 2H, H-1). ^{13}C NMR (151 MHz, DMSO) δ 145.62 (C-6), 143.48 (C-2), 134.15 (C-3), 125.29 (C-4), 123.98 (C-5).

*2.3.3 Synthesis of azine derivatives (**1a-9a** and **1b-9b**)*

5-Nitro-2-furaldehyde or 5-nitro-2-thiophenecarboxaldehyde (3 mmol, 1 eq.) was dissolved in anhydrous ethanol (10 mL) and then substituted phenylhydrazone (3 mmol, 1 eq.) and 1 drop of catalytic sulphuric acid was added to the reaction. The reaction was left to stir for 12 hours and monitored by TLC. Upon completion, the reaction was quenched with water (50 mL) and the precipitate formed was filtered and recrystallised with ethyl acetate to yield the desired azines (**1a-9a**, **1b-9b**). Compounds **6a** and **7b** were further purified by column chromatography on silica gel eluting EtOAc/hexane (2:8, v/v) to yield the pure azine products.

In the case of compounds **5a**, **9a**, **5b**, **6b** and **9b**, the acid method was not suitable as the product could not be formed. For these specific derivatives, the previously used sulphuric acid was substituted for anhydrous potassium carbonate (6 mmol, 2 eq.).

(1E,2E)-1-Benzylidene-2-[(5-nitrofuran-2-yl)methylene]hydrazine **(1a)**

Brown powder; yield: 98%; m.p. 169-171 °C; IR $_{Vmax}$ (cm^{-1}): 1629 (C=N), 1565 (C=C), 1471 (N-O), 1246 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 8.78 (s, 1H, H-1'), 8.64 (s, 1H, H-6), 7.90 (d, *J* = 6.9 Hz, 2H, H-3'), 7.81 (d, *J* = 3.8 Hz, 1H, H-4), 7.56 (t, *J* = 7.1 Hz, 1H, H-5'), 7.53 (d, *J* = 6.9 Hz, 2H, H-4'), 7.40 (d, *J* = 3.8 Hz, 1H, H-3). ^{13}C NMR (151 MHz, DMSO) δ 163.77 (C-1'), 152.52 (C-5), 150.68 (C-2), 149.56 (C-6), 133.30 (C-5'), 132.02 (C-2'), 129.02 (C-3'), 128.77 (C-4'), 118.54 (C-3), 114.13 (C-4). HRMS-APCI *m/z*: 244.0717 $[M+H]^+$ (calculated for $C_{12}H_9N_3O_3^+$, 244.0722).

(1E,2E)-1-(4-Fluorobenzylidene)-2-[(5-nitrofuran-2-yl)methylene]hydrazine **(2a)**

Yellow powder; yield: 66%; m.p. 191-193 °C; IR $_{Vmax}$ (cm^{-1}): 3113 (C-H), 1631 (C=N), 1597 (C=C), 1516 (N-O), 1342 (C-F), 1247 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 8.81 (s, 1H, H-1'), 8.65 (s, 1H, H-6), 7.97 (dd, J_{H-F} = 8.8, 5.7 Hz, 2H, H-3'), 7.82 (d, *J* = 3.9 Hz, 1H, H-4), 7.41 (d, *J* = 3.9 Hz, 1H, H-3), 7.38 (t, J_{H-F} = 8.8 Hz, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ 164.24 (d, $^{1}J_{C-F}$ = 250.3 Hz, C-5'), 162.64 (C-1'), 151.39 (C-5), 150.64 (C-2), 149.64 (C-6), 131.19 (d, $^{2}J_{C-F}$ = 9.1 Hz, C-3'), 129.96 (d, $^{3}J_{C-F}$ = 2.9 Hz, C-2'), 118.55 (C-3), 116.21 (d, $^{4}J_{C-F}$ = 22.0 Hz, C-4'), 114.11 (C-4). HRMS-APCI *m/z*: 262.0635 $[M+H]^+$ (calculated for $C_{12}H_8FN_3O_3^+$, 262.0628).

(1E,2E)-1-(4-Chlorobenzylidene)-2-[(5-nitrofuran-2-yl)methylene]hydrazine **(3a)**

Yellow powder; yield: 60%; m.p. 175-177 °C; IR $_{Vmax}$ (cm^{-1}): 3092 (C-H), 1630 (C=N), 1562 (C=C), 1352 (N-O), 1246 (C-O), 820 (C-Cl); ^{1}H NMR (600 MHz, DMSO) δ 8.81 (s, 1H, H-1'), 8.65 (s, 1H, H-6), 7.92 (d, *J* = 8.5 Hz, 2H, H-3'), 7.82 (d, *J* = 3.9 Hz, 1H, H-4), 7.61 (d, *J* = 8.5 Hz, 2H, H-4'), 7.42 (d, *J* = 3.9 Hz, 1H, H-3). ^{13}C NMR (151 MHz, DMSO) δ 162.58 (C-1'), 152.53 (C-5), 150.56 (C-2), 149.79 (C-6), 136.56 (C-5'), 132.19 (C-2'), 130.34 (C-3'), 129.16 (C-4'), 118.68 (C-3), 114.08 (C-4). HRMS-APCI *m/z*: 278.0334 $[M+H]^+$ (calculated for $C_{12}H_8ClN_3O_3^+$, 278.0332).

(1E,2E)-1-(4-Bromobenzylidene)-2-[(5-nitrofuran-2-yl)methylene]hydrazine **(4a)**

Yellow powder; yield: 78%; m.p. 196-198 °C; IR $_{Vmax}$ (cm^{-1}): 3092 (C-H), 1629 (C=N), 1563 (C=C), 1475 (N-O), 1243 (C-O), 513 (C-Br); ^{1}H NMR (600 MHz, DMSO) δ 8.79 (s, 1H, H-1'), 8.65 (s, 1H, H-6), 7.84 (d, *J* = 8.5 Hz, 2H, H-3'), 7.82 (d, *J* = 3.9 Hz, 1H, H-4), 7.75 (d, *J* = 8.5 Hz, 2H, H-4'), 7.42 (d, *J* = 3.9 Hz, 1H, H-3). ^{13}C NMR (151 MHz, DMSO) δ 162.72 (C-1'), 152.55 (C-5), 150.56 (C-2), 149.81 (C-6), 132.52 (C-5'), 132.10 (C-3'), 130.51 (C-4'), 125.57 (C-2'), 118.73 (C-3), 114.10 (C-4). HRMS-APCI *m/z*: 321.9824 $[M+H]^+$ (calculated for $C_{12}H_8BrN_3O_3^+$, 321.9827).

(1E,2E)-1-(4-Methylbenzylidene)-2-[(5-nitrofuran-2-yl)methylene]hydrazine **(5a)**

Yellow powder; yield: 13%; m.p. 167-169 °C; IR $_{Vmax}$ (cm^{-1}): 3118 (C-H), 1626 (C=N), 1571 (C=C), 1336 (N-O), 1241 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 8.76 (s, 1H, H-1'), 8.64 (s, 1H, H-6), 7.82 (d, *J* = 3.9 Hz, 1H, H-4), 7.79 (d, *J* = 8.1 Hz, 2H, H-3'), 7.39 (d, *J* = 3.9 Hz, 1H, H-3), 7.34 (d, *J* = 8.1 Hz, 2H, H-4'), 2.38 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 163.90 (C-1'), 152.46 (C-5), 150.79 (C-2), 149.28 (C-6), 142.27 (C-5'), 130.63 (C-2'), 129.62 (C-3'), 128.80 (C-4'), 118.31 (C-3), 114.14 (C-4), 21.23 (C-6'). HRMS-APCI *m/z*: 258.0880 $[M+H]^+$ (calculated for $C_{13}H_{11}N_3O_3^+$, 258.0879).

(1E,2E)-1-(4-Methoxybenzylidene)-2-[(5-nitrofuran-2-yl)methylene]hydrazine **(6a)**

Orange powder; yield: 27%; m.p. 169-171 °C; IR $_{Vmax}$ (cm^{-1}): 2921 (C-H), 1706 (C=N), 1600 (C=C), 1347 (N-O), 1239 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 8.75 (s, 1H, H-1'), 8.62 (s, 1H, H-6), 7.86 (d, *J* = 8.8 Hz, 2H, H-3'), 7.82 (d, *J* = 3.9 Hz, 1H, H-4), 7.38 (d, *J* = 3.9 Hz, 1H, H-3), 7.09 (d, *J* = 8.8 Hz, 2H, H-4'), 3.84 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 163.78 (C-1'), 162.39 (C-5), 150.97 (C-2), 148.86 (C-6), 130.73 (C-3'), 125.86 (C-5'), 118.03 (C-2'), 114.56 (C-4'), 114.56 (C-4), 114.20 (C-3), 55.48 (C-6'). HRMS-APCI *m/z*: 274.0840 $[M+H]^+$ (calculated for $C_{13}H_{11}N_3O_4^+$, 274.0828).

(1E,2E)-1-[4-(Benzyloxy)benzylidene]-2-[(5-nitrofuran-2-yl)methylene]hydrazine **(7a)**

Yellow powder; yield: 75%; m.p. 175-177 °C; IR $_{Vmax}$ (cm^{-1}): 3102 (C-H), 1618 (C=N), 1573 (C=C), 1352 (N-O), 1246 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 8.74 (s, 1H, H-1'), 8.61 (s, 1H, H-6), 7.86 (d, *J* = 8.8 Hz, 2H, H-3'), 7.80 (d, *J* = 3.9 Hz, 1H, H-4), 7.47 (d, *J* = 7.5 Hz, 2H, H-8'), 7.41 (t, *J* = 7.5 Hz, 2H, H-9'), 7.37 (d, *J* = 3.9 Hz, 1H, H-3), 7.35 (t, *J* = 7.5 Hz, 1H, H-10'), 7.16 (d, *J* = 8.8 Hz, 2H, H-4'), 5.21 (s, 2H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 163.50 (C-1'), 161.41 (C-5'), 152.33 (C-5), 150.92 (C-2), 148.72 (C-6), 136.48 (C-7'), 130.63 (C-3'), 128.38 (C-9'), 127.89 (C-2'), 127.68 (C-8'), 126.02 (C-10'), 117.86 (C-3), 115.32 (C-4'), 114.09 (C-4), 69.46 (C-6'). HRMS-APCI *m/z*: 350.1151 $[M+H]^+$ (calculated for $C_{19}H_{15}N_3O_4^+$, 350.1141).

4-(E)-(E)-[(5-Nitrofuran-2-yl)methylene]hydrazonomethylphenol **(8a)**

Red powder; yield: 14%; m.p. 216-218 °C; IR $_{Vmax}$ (cm^{-1}): 3426 (O-H), 3112 (C-H), 1598 (C=N), 1565 (C=C), 1347 (N-O), 1211 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 10.27 (s, 1H, H-6'), 8.69 (s, 1H, H-1'), 8.59 (s, 1H, H-6), 7.81 (d, *J* = 3.9 Hz, 1H, H-4), 7.75 (d, *J* = 8.6 Hz, 2H, H-3'), 7.35 (d, *J* = 3.9 Hz, 1H, H-3), 6.89 (d, *J* = 8.6 Hz, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ 164.15 (C-1'), 161.26 (C-5'), 152.35 (C-5), 151.12 (C-2), 148.44 (C-6), 130.99 (C-3'), 124.31 (C-2'), 117.77 (C-4), 115.94 (C-4'), 114.23 (C-3). HRMS-APCI *m/z*: 260.0686 $[M+H]^+$ (calculated for $C_{12}H_9N_3O_4^+$, 260.0671).

(1E,2E)-1-(4-Nitrobenzylidene)-2-[(5-nitrofuran-2-yl)methylene]hydrazine **(9a)**

Light brown powder; yield: 62%; m.p. 170-172 °C; IR $_{Vmax}$ (cm^{-1}): 3111 (C-H), 1633 (C=N), 1572 (C=C), 1526 (N-O), 1342 (N-O), 1251 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 8.92 (s, 1H, H-1'), 8.69 (s, 1H, H-6), 8.37 (d, *J* = 8.7 Hz, 2H, H-4'), 8.15 (d, *J* = 8.7 Hz, 2H, H-3'), 7.83 (*d, *J* = 3.9Hz, 1H, H-4), 7.46 (*d, *J* = 3.9 Hz, 1H, H-3). ^{13}C NMR (151 MHz, DMSO) δ 161.43 (C-1'), 150.45 (C-5), 150.27 (C-2), 149.05 (C-6), 139.19 (C-5'), 137.53 (C-2'), 129.72 (C-4'), 124.13 (C-3'), 119.30 (C-3), 114.04 (C-4). HRMS-APCI *m/z*: 289.0577 $[M+H]^+$ (calculated for $C_{12}H_8N_4O_5^+$, 289.0573).

*****d is a coalesced doublet

(1E,2E)-1-Benzylidene-2-[(5-nitrothiophen-2-yl)methylene]hydrazine **(1b)**

Yellow powder; yield: 56%; m.p. 166-168 °C; IR $_{Vmax}$ (cm^{-1}): 3096 (C-H), 1610 (C=N), 1531 (C=C), 1495 (N-O); ^{1}H NMR (600 MHz, DMSO) δ 8.94 (s, 1H, H-1'), 8.75 (s, 1H, H-6), 8.19 (d, *J* = 4.3 Hz, 1H, H-4), 7.90 (d, *J* = 7.5 Hz, 2H, H-3'), 7.72 (d, *J* = 4.3 Hz, 1H, H-3), 7.56 (d, *J* = 7.5 Hz, 1H, H-5'), 7.52 (d, *J* = 7.5 Hz, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ 163.60 (C-1'), 155.28 (C-5), 152.25 (C-2), 144.95 (C-6), 133.31 (C-5'), 132.42 (C-3), 132.00 (C-2'), 130.36 (C-4), 129.01 (C-3'), 128.78 (C-4'). HRMS-APCI *m/z*: 260.0512 $[M+H]^+$ (calculated for $C_{12}H_9N_3O_2S^+$, 260.0494).

(1E,2E)-1-(4-Fluorobenzylidene)-2-[(5-nitrothiophen-2-yl)methylene]hydrazine **(2b)**

Yellow powder; yield: 55%; m.p. 187-189 °C; IR $_{Vmax}$ (cm^{-1}): 3082 (C-H), 1613 (C=N), 1505 (C=C), 1335 (N-O), 1221 (C-F); ^{1}H NMR (600 MHz, DMSO) δ 8.93 (s, 1H, H-1'), 8.76 (s, 1H, H-6), 8.19 (d, *J* = 4.3 Hz, 1H, H-4), 7.97 (dd, J_{H-F} = 8.8, 5.7 Hz, 2H, H-3'), 7.72 (d, *J* = 4.3 Hz, 1H, H-3), 7.37 (t, J_{H-F} = 8.8 Hz, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ 164.24 (d, $^{1}J_{C-F}$ = 250.4 Hz, C-5'), 162.48 (C-1'), 157.29 (C-5), 155.36 (C-2), 152.27 (C-6), 144.90 (C-2'), 132.44 (C-3), 131.17 (d, $^{2}J_{C-F}$ = 9.0 Hz, C-3'), 130.35 (C-4), 116.19 (d, $^{3}J_{C-F}$ = 22.0 Hz, C-4'). HRMS-APCI *m/z*: 278.0404 $[M+H]^+$ (calculated for $C_{12}H_8FN_3O_2S^+$, 278.0400).

(1E,2E)-1-(4-Chlorobenzylidene)-2-[(5-nitrothiophen-2-yl)methylene]hydrazine **(3b)**

Yellow powder; yield: 27%; m.p. 179-181 °C; IR $_{Vmax}$ (cm^{-1}): 3094 (C-H), 1604 (C=N), 1522 (C=C), 1321 (N-O), 815 (C-Cl); ^{1}H NMR (600 MHz, DMSO) δ 8.77 (s, 1H, H-1'), 8.57 (s, 1H, H-6), 8.17

(d, *J* = 4.4 Hz, 1H, H-4), 8.02 (d, *J* = 8.5 Hz, 2H, H-3'), 7.74 (d, *J* = 4.4 Hz, 1H, H-3), 7.70 (d, *J* = 8.5 Hz, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ 161.84 (C-1'), 155.96 (C-5), 150.88 (C-2), 137.13 (C-6), 136.35 (C-5'), 133.65 (C-3), 131.99 (C-2'), 130.88 (C-3'), 129.47 (C-4'), 129.12 (C-4). HRMS-APCI *m/z*: 294.0097 $[M+H]^+$ (calculated for $C_{12}H_8ClN_3O_2S^+$, 294.0104).

(1E,2E)-1-(4-Bromobenzylidene)-2-[(5-nitrothiophen-2-yl)methylene]hydrazine **(4b)**
Yellow powder; yield: 41%; m.p. 193-195 °C; IR $_{Vmax}$ (cm^{-1}): 3083 (C-H), 1610 (C=N), 1550 (C=C), 1332 (N-O), 536 (C-Br); ^{1}H NMR (600 MHz, DMSO) δ 8.77 (s, 1H, H-1'), 8.59 (s, 1H, H-6), 8.18 (d, *J* = 4.4 Hz, 1H, H-4), 7.95 (d, *J* = 8.4 Hz, 2H, H-3'), 7.85 (d, *J* = 8.4 Hz, 2H, H-4'), 7.75 (d, *J* = 4.4 Hz, 1H, H-3). ^{13}C NMR (151 MHz, DMSO) δ 161.93 (C-1'), 155.97 (C-5), 150.83 (C-2), 136.34 (C-6), 133.65 (C-3), 132.40 (C-3'), 132.31 (C-5'), 131.00 (C-4'), 129.12 (C-4), 126.18 (C-2'). HRMS-APCI *m/z*: 337.9592 $[M+H]^+$ (calculated for $C_{12}H_8BrN_3O_2S^+$, 337.9599).

(1E,2E)-1-(4-Methylbenzylidene)-2-[(5-nitrothiophen-2-yl)methylene]hydrazine **(5b)**
Yellow powder; yield: 24%; m.p. 166-168 °C; IR $_{Vmax}$ (cm^{-1}): 2920 (C-H), 1609 (C=N), 1532 (C=C), 1327 (N-O); ^{1}H NMR (600 MHz, DMSO) δ 8.92 (s, 1H, H-1'), 8.71 (s, 1H, H-6), 8.18 (d, *J* = 4.1 Hz, 1H, H-4), 7.80 (d, *J* = 8.0 Hz, 2H, H-3'), 7.70 (d, *J* = 4.1 Hz, 1H, H-3), 7.34 (d, *J* = 8.0 Hz, 2H, H-4'), 2.38 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 163.67 (C-1'), 154.96 (C-5), 152.15 (C-2), 145.10 (C-6), 142.26 (C-5'), 132.24 (C-3), 130.64 (C-2'), 130.36 (C-4), 129.62 (C-3'), 128.79 (C-4'), 21.23 (C-6'). HRMS-APCI *m/z*: 274.0644 $[M+H]^+$ (calculated for $C_{13}H_{11}N_3O_2S^+$, 274.0650).

(1E,2E)-1-(4-Methoxybenzylidene)-2-[(5-nitrothiophen-2-yl)methylene]hydrazine **(6b)**
Orange powder; yield: 46%; m.p. 171-173 °C; IR $_{Vmax}$ (cm^{-1}): 2924 (C-H), 1608 (C=N), 1529 (C=C), 1329 (N-O); ^{1}H NMR (600 MHz, DMSO) δ 8.90 (s, 1H, H-1'), 8.69 (s, 1H, H-6), 8.17 (d, *J* = 4.3 Hz, 1H, H-4), 7.86 (d, *J* = 8.7 Hz, 2H, H-3'), 7.68 (d, *J* = 4.3 Hz, 1H, H-3), 7.08 (d, *J* = 8.7 Hz, 2H, H-4'), 3.84 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 163.48 (C-1'), 162.40 (C-5), 154.46 (C-2), 151.97 (C-6), 145.37 (C-5'), 131.96 (C-2'), 130.72 (C-3'), 130.37 (C-3), 125.88 (C-4), 114.56 (C-4'), 55.47 (C-6'). HRMS-APCI *m/z*: 290.0595 $[M+H]^+$ (calculated for $C_{13}H_{11}N_3O_3S^+$, 290,0599).

(1E,2E)-1-[4-(Benzyloxy)benzylidene]-2-[(5-nitrothiophen-2-yl)methylene]hydrazine **(7b)**
Yellow powder; yield: 13%; m.p. 136-138 °C; IR $_{Vmax}$ (cm^{-1}): 2921 (C-H), 1599 (C=N), 1547 (C=C), 1328 (N-O), 1158 (C-O); ^{1}H NMR (600 MHz, DMSO) δ 8.72 (s, 1H, H-1'), 8.52 (s, 1H, H-6), 8.16 (d, *J* = 4.4 Hz, 1H, H-4), 7.98 (d, *J* = 8.8 Hz, 2H, H-3'), 7.71 (d, *J* = 4.4 Hz, 1H, H-3), 7.49 (d, *J* = 7.5 Hz, 2H, H-8'), 7.42 (t, *J* = 7.5 Hz, 2H, H-9'), 7.36 (t, *J* = 7.5 Hz, 1H, H-10'), 7.26 (d, *J* = 8.8 Hz, 2H, H-4'), 5.24 (s, 2H, H-6'). ^{13}C NMR (151 MHz, DMSO) δ 162.90 (C-1'), 161.87 (C-5'), 155.47 (C-5), 150.13 (C-2), 136.64 (C-6), 136.48 (C-7'), 132.99 (C-3), 131.39 (C-3'), 129.07 (C-4), 128.49 (C-9'), 128.02 (C-2'), 127.85 (C-8'), 125.80 (C-10'), 115.67 (C-4'), 69.58 (C-6'). HRMS-APCI *m/z*: 366.0916 $[M+H]^+$ (calculated for $C_{19}H_{15}N_3O_3S^+$, 366.0912).

4-(E)-(E)-[(5-Nitrothiophen-2-yl)methylene]hydrazonomethylphenol **(8b)**

Orange powder; yield: 42%; m.p. 243-245 °C; IR $_{Vmax}$ (cm^{-1}): 3453 (O-H), 3089 (C-H), 1602 (C=N), 1492 (C=C), 1332 (N-O); ^{1}H NMR (600 MHz, DMSO) δ 10.22 (s, 1H, H-6'), 8.88 (s, 1H, H-1'), 8.63 (s, 1H, H-6), 8.17 (d, *J* = 4.4 Hz, 1H, H-4), 7.75 (d, *J* = 8.6 Hz, 2H, H-3'), 7.67 (d, *J* = 4.4 Hz, 1H, H-3), 6.88 (d, *J* = 8.6 Hz, 2H, H-4'). ^{13}C NMR (151 MHz, DMSO) δ 163.80 (C-1'), 161.26 (C-5'), 154.01 (C-5), 151.83 (C-2), 145.55 (C-6), 131.75 (C-4), 130.97 (C-3'), 130.39 (C-3), 124.32 (C-2'), 115.94 (C-4'). HRMS-APCI *m/z*: 276.0434 $[M+H]^+$ (calculated for $C_{12}H_9N_3O_3S^+$, 276.0443).

(1E,2E)-1-(4-Nitrobenzylidene)-2-[(5-nitrothiophen-2-yl)methylene]hydrazine **(9b)**

Yellow powder; yield: 66%; m.p. 239-241 °C; IR $_{Vmax}$ (cm^{-1}): 3108 (C-H), 1624 (C=N), 1595 (C=C), 1505 (N-O), 1336 (N-O); ^{1}H NMR (600 MHz, DMSO) δ 8.98 (s, 1H, H-1'), 8.87 (s, 1H, H-6), 8.35 (d, *J* = 8.7 Hz, 2H, H-4'), 8.19 (d, *J* = 4.3 Hz, 1H, H-4), 8.14 (d, *J* = 8.7 Hz, 2H, H-3'), 7.76 (d, *J* = 4.3 Hz, 1H, H-3). ^{13}C NMR (151 MHz, DMSO) δ 161.46 (C-1'), 156.45 (C-5), 152.71 (C-2), 149.05 (C-6), 144.40 (C-5'), 139.19 (C-2'), 133.09 (C-3), 130.33 (C-4), 129.71 (C-4'), 124.10 (C-3'). HRMS-APCI *m/z*: 305.0335 $[M+H]^+$ (calculated for $C_{12}H_8N_4O_4S^+$, 305.0345).

3. Biological evaluation

3.1. Cytotoxicity assay

The cytotoxicity of synthesised compounds was evaluated using the resazurin assay. The assay involved the irreversible enzymatic reduction of oxidised blue resazurin dye to pink, highly fluorescent resorufin by viable cells (Czekanska, 2011). This non-toxic reagent serves as an effective tool for assessing cell proliferation and drug toxicity.

Vero cells were cultured in Hyclone Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Thermofisher, South Africa) and 1% L-glutamine, non-essential amino acids (NEAA) and penicillin-streptomycin (Pen/Strep) solution (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 with 100 µL of 60 000 cells/mL Vero cell suspension. The Vero cell plates were incubated for 24 hours, after which the growth medium was replaced with 100 µL medium containing: (1) emetine dihydrochloride (Sigma Aldrich) solution diluted with growth medium to the necessary concentrations (positive control); (2) growth medium and solvent (negative control to compensate for possible solvent effects); (3) growth medium and experimental compound solutions. Blanks contained growth medium without cells. The treated plates were incubated for 48 hours. After incubation, 50 µL of sterile-filtered resazurin sodium salt (Sigma Aldrich) solution (0.01% in PBS) was added, and the plates were incubated for 2 hours. The Thermofisher Scientific GO Multiscan plate reader was used at 570 and 600 nm to measure absorbance. SkanIt 4.0 Research Edition software was used to perform data analysis 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).

3.2. In vitro antileishmanial assay

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

Promastigotes 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 Media 199 (M199) growth medium with Hank's salts and 0.68 mM L-glutamine, supplemented with 25 mM Hepes, 4.2 mM sodium bicarbonate, 0.0005% hemin, 0.0001% biotin, 0.1 mM adenine (Sigma Aldrich), 10% FBS and 50 U/mL Pen/Strep solution, the pH adjusted to 7.3 – 7.4. The promastigotes were maintained at 25 ˚C. For the resazurin assay, logarithmic phase promastigotes (1.25×10^5 cells/well, final volume 100 µL/well) were seeded in 96 well plates (Nunc, Thermofisher Scientific) in the presence of 7 two-fold dilution concentrations of compounds for IC_{50} determination. The standard drug was amphothericin B (10 µM), while growth medium without parasites served as the blank. The plates were incubated for 48 hours at 25 ˚C in humidified atmosphere. After the incubation period, 50 µL of resazurin solution (0.01% in phosphor-buffered saline) was added to each well and the plates were further incubated in the dark at 25 ˚C for 24 hours. The Thermofisher Scientific GO Multiscan plate reader was used at 570 nm and 600 nm to measure absorbance. SkanIt 4.0 Research Edition software was used to perform data analysis for each biological replicate. Background absorbance of resazurin (600 nm) was subtracted from the absorbance values of resorufin (570 nm). The mean absorbance calculated, and the 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 three biological replicates using the cell viability % values and GraphPad Prism 5. The mean IC_{50} of the biological replicates served as the final IC_{50} of each compound.

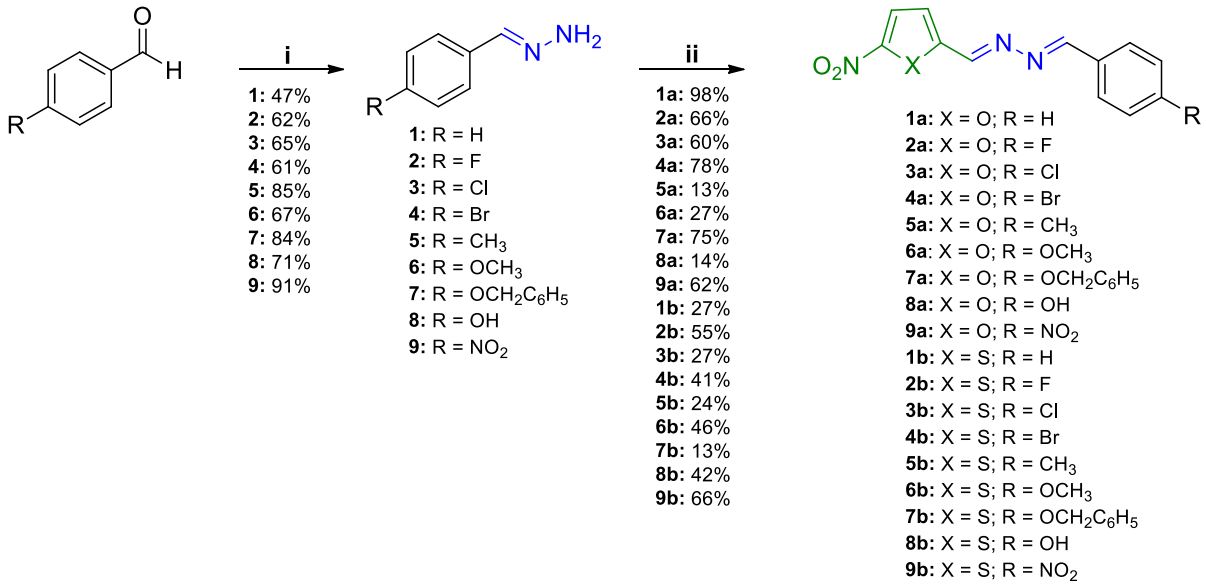
3.3. Statistical analysis

In vitro antileishmanial activities and cytotoxicity, indicated as IC_{50} values, were derived from non-linear regression analysis. Results were represented as the mean ± SD from the triplicate biological experiments. Statistical analysis was performed, using SkanIt 4.0 Research Edition software (Thermofisher Scientific) and Prism V5 software (GraphPad). All reported data was significant at p <0.05.

4. Results and discussion

4.1. Chemistry

A series of nitrofuran-based azine derivatives (**1a-9a**, **1b-9b**) was synthesised *via* a two-step process (Scheme **3-1**), starting from commercially available 5-nitro-2-furaldehyde (NFA) or 5-nitrothiophene-2-carboxaldehyde (NTA). Firstly, hydrazone formation occurs by the reaction of substituted aldehydes with excess hydrazine hydrate (**1-9**). This reaction is then followed by a Schiff base reaction of the hydrazones (**1-9**) with NFA or NTA, and upon elimination of water, the targeted nitrofuran-based azine derivatives (**1a-9a**, **1b-9b**) are formed in poor to excellent yields of 13-98% after recrystallisation with ethyl acetate or column chromatography.



Scheme **3-1**: Two-step reaction for the formation of nitrofuran-based azine derivatives
Reagents and conditions: i. hydrazine hydrate (4 eq.), TEA (1.5 eq.), EtOH (10 mL), rt, 12 h; ii. Phenylhydrazone (**1-9**) (1 eq.), EtOH (10 mL), rt, 12 h.

The structures of all compounds were confirmed using routine analysis techniques, such as NMR, HRMS and IR. The formation of the hydrazone compounds (**1-9**) was confirmed on ^{1}H NMR by the disappearance of the characteristic aldehyde proton at *ca δ* 10.0 ppm and the appearance of a distinct singlet at *ca δ* 7.65 ppm indicative of the C-H proton. This is further collaborated by the

appearance of the characteristic singlet at *ca* δ 6.60, indicating the presence of the NH_2 proton of the hydrazone moiety. The disappearance of this NH_2 peak from the spectra **1a-9a** and **1b-9b** confirmed the success of the Schiff based reaction. This was further corroborated by the appearance of two separate vinylic protons H-1' and H-6 of the hydrazone bond as characteristic singlets at *ca* δ 8.79 and 8.65 ppm. The aromatic protons H-4 and H-3 of the furan ring gave a pair of doublets (d) with a *J* value of 3.9 Hz at δ 7.82 and δ 7.41 ppm, respectively. The deshielding of H-4 was due to the electronic effect of the nitro group that withdraws electron density away from H-4, while the unsaturated hydrazone bond pushes electron density towards H-3. The phenyl ring showed two aromatic resonances that appeared as two pairs of coupled doublets at δ 7.84 and δ 7.75 ppm (^3J = 8.5 Hz), which were attributed to the aromatic protons H-3' and H-4', respectively. For the fluorine containing compounds (**2**, **2a**, **2b**), proton-fluorine coupling ($J_{H\text{-}F}$) was determined, and it was found that these atoms underwent mostly ortho couplings with $J_{H\text{-}F}$ ~8-9 Hz. In summary, all the protons of each analogue were accounted for.

The ^{13}C spectra of all analogues showed the two hydrazone moiety peaks of C-1' at δ 162.7 and C-6 at δ 149.8, whereas the four aromatic carbons of the furan ring appeared as singlets at δ 152.5 (C-5), δ 150.6 (C-2), δ 118.7 (C-3) and δ 114.1 (C-4). The aromatic protons of the phenyl ring appeared as singlets at δ 132.5 (C-5'), δ 132.1 (C-3'), δ 130.5 (C-4') and δ 125.6 (C-2'). Carbon-fluorine coupling ($J_{C\text{-}F}$) also occurred in compounds **2**, **2a** and **2b** with the ^1J, ^2J, ^3J and ^4J values within the expected ranges. IR further confirmed the success of the Schiff base reactions (**1a-9a**, **1b-9b**) by the appearance of its commonly showed characteristic absorption of C=N stretching (1600 – 1560 cm^{-1}) and N-O stretching (1550-1500 and 1350-1300 cm^{-1}).

4.2. Predicted physiochemical and pharmacokinetic properties

The most used method in drug discovery for quantifying the lipophilicity of compounds is $logP_{o/w}$. The $logP_{o/w}$ value of a molecule is the partition coefficient between octanol (lipophilic phase) and water (hydrophilic phase) (Lindsley, 2014). $LogP_{o/w}$ is used to examine important biological properties for drug action, which include lipid solubility, distribution in tissue, binding to receptors, cellular uptake, metabolism, and drug bioavailability (Ghose et al., 1998). According to Lipinski et al. (2001), $logP_{o/w}$ values are targeted to be between 1 and 5, with values between 1 and 3 being ideal.

Table 1: Physicochemical and ADME data of synthesised nitrofuran-based azine derivatives and standard nitrofuran drugs as predicted by SwissADME web tool, http://www.swissadme.ch

Cpd.	MW (g/mol)	Log $P_{o/w}$ [a]	RA [b]	Log S [c]		TPSA Å² [f]	HBD [g]	HBA [h]	Lipinski's violation	GI absorption	Leadlikeness [i]	Druglikeness [j]
				ESOL[d]	Ali[e]							
NFA	141.08	0.36	2	-1.59	-2.20	76.03	4	2	0	High	No	Yes
1a	243.22	2.01	4	-2.77	-3.34	83.68	0	5	0	High	No	Yes

2a	261.21	2.32	4	-2.92	-3.45	83.68	0	6	0	High	Yes	Yes
3a	277.66	2.52	4	-3.35	-3.99	83.68	0	5	0	High	Yes	Yes
4a	322.11	2.64	4	-3.67	-4.06	83.68	0	5	0	High	Yes	Yes
5a	257.24	2.15	4	-3.06	-3.72	83.68	0	5	0	High	Yes	Yes
6a	273.24	1.82	5	-2.83	-3.51	92.91	0	6	0	High	Yes	Yes
7a	349.34	3.43	7	-4.75	-5.99	92.91	0	6	0	High	No	Yes
8a	259.22	1.41	4	-2.62	-3.39	103.91	1	6	0	High	Yes	Yes
9a	288.22	1.26	5	-2.81	-4.12	129.50	0	7	0	High	Yes	Yes
NTA	157.15	0.70	2	-2.04	-3.08	91.13	0	3	0	High	No	Yes
1b	259.28	2.66	4	-3.82	-5.23	98.78	0	4	0	High	Yes	Yes
2b	277.27	2.88	4	-3.97	-5.33	98.78	0	5	0	High	No	Yes
3b	293.73	3.11	4	-4.41	-5.88	98.78	0	4	0	High	No	Yes
4b	338.18	3.28	4	-4.72	-5.94	98.78	0	4	0	High	No	Yes
5b	273.31	2.82	4	-4.12	-5.61	98.78	0	4	0	High	No	Yes
6b	289.31	2.51	5	-3.88	-5.39	108.01	0	5	0	High	Yes	Yes
7b	365.41	3.94	7	-5.24	-6.95	108.01	0	5	0	High	No	Yes
8b	275.28	2.08	4	-3.68	-5.29	119.01	1	5	0	High	Yes	Yes
9b	304.28	1.96	5	-3.86	-6.01	144.60	0	6	0	Low	Yes	Yes
NFX	275.22	0.90	5	-2.95	-4.27	120.65	2	6	0	High	Yes	Yes
FZD	225.16	0.32	3	-1.24	-1.62	100.86	0	6	0	High	No	Yes
NFZ	198.14	-0.59	4	-1.21	-2.45	126.44	2	5	0	High	No	Yes
NFT	238.16	-0.50	3	-1.04	-1.60	120.73	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, implemented from a topological method (Delaney, 2004). [e] Implemented from a topological method (Ali et al., 2012) with log S scale: insoluble < -10 < poorly soluble < -6 < moderately soluble < -4 < soluble < -2 very soluble < 0 highly soluble. [f] Topological polar surface area (TPSA): Calculated from (Ertl et al., 2000). [g] Number of hydrogen bond donors. [h] Number of hydrogen bond acceptors. [i] Calculated from (Teague et al., 1999), 250 ≤ MW ≤ 350; XLOGP ≤ 3.5; Number of rotatable bonds ≤ 7. [j] Determined by using Lipinski's rule of five: MW ≤ 500 g/mol; LogP ≤ 5; HBD ≤ 5; HBA ≤ 10; no more than one violation allowed (Lipinski et al., 2001). All values in this table were calculated using SwissADME web tool, http://www.swissadme.ch (Daina et al., 2017). NFA: 5-nitro-2-furaldehyde; NTA: 5-nitro-2-thiophenecarboxaldehyde; NFX: nifuroxazide; FZD: furazolidone; NFZ: nitrofurazone; NFT: nitrofurantoin.

All synthesised derivatives complied with Lipinski's rules and had physicochemical properties well within the target ranges as set by Lipinski et al. (2001). Lipophilicity, although a crucial physiochemical parameter, is not the only determining characteristic of good drug design, as evident by other successful drugs that do not fall within the target range (Arnott and Planey, 2012). Therefore, other drug parameters, such as topological polar surface area (PTSA), were predicted for these compounds. PTSA is the surface area of a molecule that emerges from nitrogen or oxygen atoms, as well as hydrogen atoms that are attached to oxygen or nitrogen atoms (Caron and Ermondi, 2016). This shows the correlation with passive molecular transport through membranes, which allows the prediction of GI absorption, Caco-2 monolayer permeability, and penetration of the blood-brain barrier. All the derivatives, with the exception of **9b**, were predicted

to be highly absorbed in the GI tract through passive diffusion and were, therefore, expected to be druglike by nature.

4.3. Pharmacology

The synthesised derivatives were assessed for its *in vitro* antileishmanial activity against the promastigotes of three *Leishmania* promastigote strains, *L. donovani* strains (1S and 9515) and *L. major* strain IR-173. These *Leishmania* spp. were selected to determine the specificity of the synthesised derivatives against *L. major* parasites that cause CL, and *L. donovani* parasites that cause the more serious and debilitating VL (Salam et al., 2014). Amphotericin B (AmB) was used as the reference antileishmanial drug alongside 5-nitro-2-furaldehyde, 5-nitrothiophene-2-carboxaldehyde and the cNFs, nifuroxazide, furazolidone, nitrofurazone and nitrofurantoin were tested as reference drugs for comparison. Cytotoxicity profiles of the derivatives were determined using Vero cells with emetine (EM) as a positive control. These results are reported in **Table 2**.

Table 2: Biological results of synthesised nitrofuran-based azine derivatives and nitrofuran antibiotics

	[a]Antileishmanial activity, IC_{50} ± SD (μM)[b] (n=3)						Cytotoxicity
Cpd.	***L. donovani* 1S**	**SI_2[d]**	***L. donovani* 9515**	**SI_3[e]**	***L. major* IR-173**	**SI_4[f]**	**Vero[c], IC_{50} (μM)**
NFA	11.06 ± 1.50	2	13.71 ± 2.37	1	7.94 ± 0.79	2	17.82 ± 0.25
1a	1.19 ± 0.03	14	1.45 ± 0.19	11	1.19 ± 0.29	14	16.29 ± 3.50
2a	0.73 ± 0.17	65	1.18 ± 0.14	40	0.73 ± 0.09	65	47.42 ± 1.12
3a	0.56 ± 0.04	179	0.67 ± 0.04	149	0.42 ± 0.07	238	> 100
4a	4.42 ± 0.00	23	3.56 ± 0.00	28	0.75 ± 0.18	133	> 100
5a	0.55 ± 0.20	182	0.76 ± 0.05	132	0.42 ± 0.08	238	> 100
6a	1.24 ± 0.10	41	1.16 ± 0.11	44	0.89 ± 0.12	57	51.01 ± 1.26
7a	0.49 ± 0.10	204	1.61 ± 0.22	62	0.45 ± 0.02	222	> 100
8a	1.20 ± 0.36	16	1.02 ± 0.08	18	0.64 ± 0.10	29	18.66 ± 1.31
9a	0.34 ± 0.20	34	0.70 ± 0.06	17	0.70 ± 0.10	17	11.56 ± 1.12
NTA	3.18 ± 0.59	4	5.07 ± 0.45	2	3.75 ± 0.39	3	12.42 ± 0.88
1b	1.94 ± 0.13	22	0.93 ± 0.06	45	1.43 ± 0.19	29	42.00 ± 8.79
2b	2.81 ± 0.00	36	2.00 ± 0.15	50	3.79 ± 0.70	26	> 100
3b	> 100	>1	> 100	>1	42.78 ± 0.00	2	> 100
4b	> 100	>1	81.1 ± 0.00	1	> 100	>1	> 100
5b	> 100	>1	> 100	>1	> 100	>1	> 100
6b	> 100	>1	> 100	>1	> 100	>1	> 100
7b	> 100	>1	> 100	>1	4.39 ± 0.24	23	> 100
8b	4.14 ± 0.41	8	1.66 ± 0.18	20	2.22 ± 0.00	15	33.12 ± 1.22
9b	> 100	>1	> 100	>1	> 100	>1	> 100
AMB	0.02 ± 0.00	2890	0.02 ± 0.009	2890	0.03 ± 0.01	1927	57.8 ± 3.2
NFX	10.73 ± 1.03	9	9.43 ± 0.85	11	31.10 ± 2.16	3	> 100

FZD	0.32 ± 0.00	313	0.28 ± 0.28	357	0.34 ± 0.03	294	> 100
NFZ	6.54 ± 0.93	15	1.85 ± 0.14	54	1.85 ± 0.06	54	> 100
NFT	> 100	>1	23.71 ± 5.44	4	87.00 ± 2.35	1	> 100
EM	n.d		n.d		n.d		0.08 ± 0.009

[a] Compounds **1a**, **4a**, **7a**, **8a**, **1b** and **7b**, were tested in suspension, therefore this may account for high SD values
[b] Represented as the mean ± SD from the triplicate biological experiments.
[c] African green monkey kidney epithelial cells.
[d] Selectivity index: $SI_2 = IC_{50}$ Vero/IC_{50} *L. donovani* 1S.
[e] Selectivity index: $SI_3 = IC_{50}$ Vero/IC_{50} *L. donovani* 9515.
[f] Selectivity index: $SI_4 = IC_{50}$ Vero/IC_{50} *L. major* IR-173
Abbreviations: AMB: Amphotericin B; NFA: 5-nitro-2-furaldehyde; NTA: 5-nitro-2-thiophenecarboxaldehyde; NFX: nifuroxazide; FZD: furazolidone; NFZ: nitrofurazone; NFT, nitrofurantoin; EM, emetine; n.d, not determined.

The synthesised intermediates **1-9** exhibited no activity thus were not reported in **Table 2**. The cytotoxicity data indicated that most of the nitrofuran derivatives (**1a-9a**) were moderately toxic to the mammalian cells, except compounds **3a**, **4a**, **5a** and **7a** which were non-toxic (IC_{50} > 100 µM). On contrary, all the nitrothiophene derivatives (**1b-9b**), with the exceptions of **1b** and **8b**, were not toxic to the mammalian cells, inferring that their observed antileishmanial activities were intrinsic.

The most active derivatives of the series were compounds **3a**, **5a** and **7a** that demonstrated significant nanomolar activities against promastigotes of the three tested *Leishmania* strains, with high selectivity indexes (SI 179-238). These analogues possessed up to 32-fold superior activity in comparison to its parent compound NFA and the reference nitrofuran drugs.

Compounds **3a** and **5a** were active against all three strains showing nanomolar activity of 0.56 ± 0.04 µM and 0.55 ± 0.20 µM for *L. donovani* (1S), 0.67 ± 0.04 µM, 0.76 ± 0.05 µM for *L. donovani* (9515) and 0.42 ± 0.07 µM, 0.42 ± 0.08 µM for *L. major* promastigotes, respectively. The most active of these three compounds was **7a** with nanomolar activity of 0.49 ± 0.01 µM for *L. donovani* (1S), 1.61 ± 0.22 µM for *L. donovani* (9515) and 0.45 ± 0.02 µM for *L. major* promastigotes. Compound **9a** showed excellent activity against all three tested strains with IC_{50} 0.34 ± 0.20 µM, 0.70 ± 0.06 µM and 0.70 ± 0.10 µM against *L. donovani* (1S and 9515) and *L. major* promastigotes. However, this compound was also moderately toxic to the mammalian cells, disqualifying it as a potential hit compound. Compound **7a** was not predicted to be a good lead compound. However, this compound turned out to be the most active compound of the series.

In this series, two moieties (furan and thiophene) were compared with the only difference between them being that one bares an oxygen atom whereas the other had a sulphur. The antileishmanial results indicate that the nitrofuran sub-series (**1a-9a**) exhibited a much more potent activity than the nitrothiophene sub-series (**1b-9b**). Thus, this may be explained by the presence of the nitrofuran extra hydrogen bond acceptor i.e., O atom in compounds **1a-9a**. Hydrogen bonds form non-covalent interactions that are significant in both medicinal and biochemistry. These bonds

are important because a single hydrogen bond interaction can enhance the potency of oxygen-containing drugs towards parasites in comparison to their sulphur containing counterparts (Bauer et al., 2019).

Because of its overall superior activity, the nitrofuran sub-series compounds were analysed for structure activity relationships (SARs). When considering the electronic effect and strength, the R groups of compounds can be divided into electron withdrawing groups (EWG) (NO_2> F> Cl> Br> OCH_3), neutral groups (H), and electron donating groups (EDG) (OH> $OCH_2C_6H_5$> CH_3). The general pattern of EWG compounds suggests that the activity of these compounds decrease as the strength of the EWG decreases. This is evident by the activities of **3a** bearing the strongest of these EWGs i.e., NO_2, that exhibits IC_{50} values of 0.34, 0.70, and 0.70 µM, against *L. donovani* 1S, *L. donovani* 9515 and *L. major*, respectively, compared to 1.24, 1.16, and 0.89 µM of the weakest EWG (the OCH_3 group).

On contrary, EDG compounds had increased activity as the strength of the EDG decreased. This is evident by the strongest of these EDGs (the OH group) exhibiting IC_{50} values of 1.20, 1.02, and 0.64 µM, respectively, compared to 0.55, 0.76, and 0.42 µM of the weakest EDG (the CH_3 group). The neutral group (H group) overall exhibited weaker activity, with IC_{50} values of 1.19, 1.45, and 1.19 µM, respectively against the same parasites.

5. Conclusion

A series of nitrofuran-based azine derivatives were synthesised in poor to excellent yields following a two-step process that included hydrazone formation and Schiff base reaction. Most of the compounds were found to be non-cytotoxic, apart from compounds **1a**, **2a**, **6a**, **8a**, **9a**, **1b** and **8b** which exhibited moderate toxicity. It was observed that the nitrofuran sub-series (**1a-9a**) was more potent than the nitrothiophene sub-series (**1b-9b**), and this might be related to the oxygen atom of the furan ring's ability to form hydrogen bonding. The electronic effect analysis showed strong EWGs has good activity, while weak EDG has the better activity. Derivatives **3a** and **5a** were the most potent against all three *Leishmania* tested strains and **7a** against two *Leishmania* strains (*L. donovani* 1S and *L. major*), and these compounds could be identified as anti-promastigote hits. Further work on the nitrofuran series will be done as a possible route to enhancing its antileishmanial activity. Furthermore, the activities of the current derivatives will also be tested against the intracellular amastigote form to confirm their potential as antileishmanial hits.

Acknowledgements

The work was based upon research financially supported by the North-West University, Potchefstroom Campus. The authors thank Dr. J Aucamp for biological activity screening, Dr. Johan Jordaan for HRMS analysis and Dr. Daniel Otto for NMR analysis.

The following reagents were obtained through BEI Resources, NIAID, NIH:

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

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REFERENCE LIST

Ali, J., Camilleri, P., Brown, M.B., Hutt, A.J., Kirton, S.B., 2012. Revisiting the general solubility equation: In silico prediction of aqueous solubility incorporating the effect of topographical polar surface area. J. Chem. Inf. Model. 52, 420-428.

Angelova, V.T., Valcheva, V., Vassilev, N.G., Buyukliev, R., Momekov, G., Dimitrov, I., Saso, L., Djukic, M., Shivachev, B., 2017. Antimycobacterial activity of novel hydrazide-hydrazone derivatives with 2H-chromene and coumarin scaffold. Bioorg. Med. Chem. Lett. 27, 223-227.

Arnott, J.A., Planey, S.L., 2012. The influence of lipophilicity in drug discovery and design. Expert Opin. Drug Discov. 7, 863-875.

Bauer, C.A., Schneider, G., Göller, A.H., 2019. Machine learning models for hydrogen bond donor and acceptor strengths using large and diverse training data generated by first-principles interaction free energies. J. Cheminform. 11, 59.

Braga, S.S., 2019. Multi-target drugs active against leishmaniasis: A paradigm of drug repurposing. Eur. J. Med. Chem. 183, 111660-111660.

Caron, G., Ermondi, G., 2016. Molecular descriptors for polarity: the need for going beyond polar surface area. Future Med. Chem. 8, 2013-2016.

Chourasiya, S.S., Kathuria, D., Wani, A.A., Bharatam, P.V., 2019. Azines: synthesis, structure, electronic structure and their applications. Org. Biomol. Chem. 17, 8486-8521.

Czekanska, E.M., 2011. Assessment of cell proliferation with resazurin-based fluorescent dye. Methods Mol. Biol. 740, 27-32.

Daina, A., Michielin, O., Zoete, V., 2017. SwissADME: A free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep. 7, 42717.

Dehestani, L., Ahangar, N., Hashemi, S.M., Irannejad, H., Honarchian Masihi, P., Shakiba, A., Emami, S., 2018. Design, synthesis, *in vivo* and *in silico* evaluation of phenacyl triazole hydrazones as new anticonvulsant agents. Bioorg. Chem. 78, 119-129.

Delaney, J.S., 2004. ESOL: Estimating aqueous solubility directly from molecular structure. J. Chem. Inf. and Comput. Sci. 44, 1000-1005.

Ertl, P., Rohde, B., Selzer, P., 2000. Fast calculation of molecular polar surface area as a sum of fragment-based contributions and its application to the prediction of drug transport properties. J. Med. Chem. 43, 3714-3717.

Freitas-Junior, L.H., Chatelain, E., Kim, H.A., Siqueira-Neto, J.L., 2012. Visceral leishmaniasis treatment: what do we have, what do we need and how to deliver it? Int. J. Parasitol. Drugs Drug Resist. 2, 11-19.

Georgiadou, S.P., Makaritsis, K.P., Dalekos, G.N., 2015. Leishmaniasis revisited: Current aspects on epidemiology, diagnosis and treatment. J. Transl. Int. Med. 3, 43-50.

Ghorbani, M., Farhoudi, R., 2018. Leishmaniasis in humans: drug or vaccine therapy? Drug Des Devel Ther. 12, 25-40.

Ghose, A.K., Viswanadhan, V.N., Wendoloski, J.J., 1998. Prediction of hydrophobic (lipophilic) properties of small organic molecules using fragmental methods: an analysis of alogP and clogP methods. The J. Phys. Chem. A 102, 3762-3772.

Glinma, B., Gbaguidi, F.A., Kassehin, U.C., Kpoviessi, S.D.S., Houngbeme, A., Houngue, H.D., Accrombessi, G.C., Poupaert, J.H., 2015. Synthesis and trypanocidal activity of salicylhydrazones and p-tosylhydrazones of S-(+)-carvone and arylketones on African trypanosomiasis. J. Appl. Pharm. Sci. 5, 001-007.

Kajal, A., Bala, S., Sharma, N., Kamboj, S., Saini, V., 2014. Therapeutic potential of hydrazones as anti-inflammatory agents. Int. J. Med. Chem. 2014, 761030-761030.

Kulshrestha, A., Bhandari, V., Mukhopadhyay, R., Ramesh, V., Sundar, S., Maes, L., Dujardin, J.C., Roy, S., Salotra, P., 2013. Validation of a simple resazurin-based promastigote assay for routine monitoring of miltefosine susceptibility in clinical isolates of *Leishmania donovani*. Parasitol. Res. 112, 825-828.

Lindsley, C.W., 2014. Lipophilicity, in: Stolerman, I., Price, L. (Eds.), Encyclopedia of Psychopharmacology, Springer Berlin Heidelberg., Berlin, pp. 1-6.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug Deliv. Rev. 46, 3-26.

Maltezou, H.C., 2010. Drug resistance in visceral leishmaniasis. J. Biomed. Biotechnol. 2010, 617521.

Manikandan, V., Balaji, S., Senbagam, R., Vijayakumar, R., Rajarajan, M., Vanangamudi, G., Arulkumaran, R., Sundararajan, R., Thirunarayanan, G., 2017. Synthesis and antimicrobial activities of some (E)-N'-1-(substituted benzylidene)benzohydrazides. Int. J. Adv. Chem. 5, 17-24.

Navidpour, L., Shafaroodi, H., Saeedi-Motahar, G., Shafiee, A., 2014. Synthesis, anti-inflammatory and analgesic activities of arylidene-2-(3-chloroanilino)nicotinic acid hydrazides. Med. Chem. Res. 23, 2793-2802.

Pal, C., Bandyopadhyay, U., 2012. Redox-active antiparasitic drugs. Antioxid. Redox Signal. 17, 555-82.

Patel, R., Rinker, L., Peng, J., Chilian, W.M., 2018. Reactive oxygen species: the good and the bad, in: Filip, C., Albu, E. (Eds.), Reactive Oxygen Species (ROS) in Living Cells, IntechOpen., London, pp. 7-20.

Patterson, S., Wyllie, S., 2014. Nitro drugs for the treatment of trypanosomatid diseases: past, present, and future prospects. Trends Parasitol. 30, 289-298.

Salam, N., Al-Shaqha, W.M., Azzi, A., 2014. Leishmaniasis in the middle East: incidence and epidemiology. PLoS Negl. Trop. Dis. 8, e3208-e3208.

Singh, B., Pandey, A., 2009. Synthesis, characterisation and mesomorphic properties of unsymmetrical N-(o-hydroxybenzylidene)-N’-(4-n-alkoxybenzylidene)azines and their copper (II) complexes. Liq. Cryst. 37, 57-67.

Siqueira-Neto, J.L., Song, O.R., Oh, H., Sohn, J.H., Yang, G., Nam, J., Jang, J., Cechetto, J., Lee, C.B., Moon, S., Genovesio, A., Chatelain, E., Christophe, T., Freitas-Junior, L.H., 2010. Antileishmanial high-throughput drug screening reveals drug candidates with new scaffolds. PLoS Negl. Trop. Dis. 4, e675.

Sreenivasulu, R., Reddy, K.T., Sujitha, P., Kumar, C.G., Raju, R.R., 2019. Synthesis, antiproliferative and apoptosis induction potential activities of novel bis(indolyl)hydrazide-hydrazone derivatives. Bioorg. Med. Chem. 27, 1043-1055.

Teague, S.J., Davis, A.M., Leeson, P.D., Oprea, T., 1999. The design of leadlike combinatorial libraries. Angew. Chem. Int. Ed. Engl. 38, 3743-3748.

Torres-Guerrero, E., Quintanilla-Cedillo, M.R., Ruiz-Esmenjaud, J., Arenas, R., 2017. Leishmaniasis: a review. F1000Res. 6, 1-15.

Ward, S.E., Beswick, P., 2014. What does the aromatic ring number mean for drug design? Expert Opin. Drug Discov. 9, 995-1003.

WHO (World Health Organization), 2021a. Leishmaniasis: Overview. https://www.who.int/health-topics/leishmaniasis#tab=tab_1 (accessed 25 May 2021).

WHO. 2021b. Leishmaniasis: Key facts. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis (accessed 25 May 2021).

Zuma, N.H., Aucamp, J., N'Da, D.D., 2019. An update on derivatisation and repurposing of clinical nitrofuran drugs. Eur. J. Pharm. Sci. 140, 105092.

CHAPTER 4

SUMMARY AND CONCLUSION

Leishmaniasis is a vector-borne protozoan parasitic disease that is transmitted to humans during hematophagy of an infected female *Phlebotomus* sandfly (WHO, 2021*a*). This tropical disease is endemic to more than 98 countries and is amongst the top ten most neglected and devastating diseases in the world (WHO, 2017; Güran, 2018:2). This disease can manifest in three clinical forms; the most common being the CL form, the rarer MCL form, and/or the most lethal VL form (Salam *et al*., 2014:1). There are more than a billion individuals living in *Leishmania* endemic areas, putting them at higher risk of contracting the disease. According to WHO, about 90 000 new cases of VL and a million new cases of CL are reported annually worldwide (WHO, 2021*b*). Leishmaniasis is estimated to cause 20 000 to 30 000 deaths every year (PAHO, 2020).

Leishmaniasis can be treated and cured with chemotherapeutic drugs, such as the pentavalent antimonies, amphotericin B, miltefosine, pentamidine, paromomycin and the azoles (Georgiadou *et al*., 2015:46; Braga, 2019:2). However, most of these available drugs are limited by impractical administration (except for miltefosine), overall toxicity, cost, and the occurrence of pathogenic resistance (Freitas-Junior *et al*., 2012:12). There have been several reports of parasitic resistance against the pentavalent antimonies, amphotericin B, and miltefosine. This has led to the discontinued use of some of these drugs against leishmaniasis in areas where resistance is prevalent, and patient cure rates have declined. Hence, the emphasis on the urgent need for the discovery of new, safe, and effective drugs to treat this disease (Capela *et al*., 2019:1).

It has been well established over recent years that clinical nitrofurans (cNFs) can be used to treat infectious diseases (Patterson & Wyllie, 2014:291; Zuma *et al*., 2019:1). The diverse biological activities of nitrofurans have been attributed to their nitro group and its ability to form ROS upon Fenton reactions with enzymes (Pal & Bandyopadhyay, 2012:556; Patel *et al*., 2018:8). These enzymes are crucial for the growth and development of parasites. When ROS are produced, the parasite membranes are damaged *via* oxidation of their enzymes, which eventually leads to cell death (Pal & Bandyopadhyay, 2012:556). These drugs are active against various infections, including Chagas disease (Sales Junior *et al*., 2017:1289) and *Leishmania* (Rando *et al*., 2008:6724; Sifontes-Rodríguez *et al*., 2015:166).

In addition to the nitrofuran moiety, cNFs also have a hydrazone moiety that possess anti-infective activity. The combination of hydrazones with other biologically active functional groups have been shown to result in compounds with unique chemical and physical characteristics (Verma *et al*., 2014:69). This includes compounds that are easily synthesised, highly active against a variety of

conditions, and has low toxicity (Asif & Husain, 2013:2). Therefore, this makes nitrofurans good candidates for drug development for the treatment of infectious diseases such as leishmaniasis.

Furthermore, the use of aromatic rings in drug design is common, as these rings offer numerous unique and strong interaction modes with target proteins and, therefore, introduce chemical stability, diverse reactivity paths and possible enhancement of biological activity (Aldeghi *et al*., 2014:50).

Thus, the aim of this study was to investigate the antileishmanial activity of nitrofuran-based azine derivatives, synthesised and characterised using routine techniques such as NMR, HRMS, and IR spectroscopy. These compounds were also evaluated for *in vitro* cytotoxicity on mammalian cell lines.

The nitrofuran-based azine derivatives were synthesised *via* a two-step process, starting from commercially available 5-nitro-2-furaldehyde (NFA) or 5-nitrothiophene-2-carboxaldehyde (NTA). Firstly, hydrazone formation occurred by the reaction of substituted aldehydes with excess hydrazine hydrate. This reaction was then followed by a Schiff base reaction of the hydrazones with NFA or NTA, and upon elimination of water, the targeted nitrofuran-based azine derivatives were formed in poor to excellent yields of 13-98% after recrystallisation with ethyl acetate or column chromatography. The structures of all the synthesised compounds were confirmed using routine analysis techniques, namely NMR, HRMS and IR.

The synthesised derivatives were screened for *in vitro* antileishmanial activity against the promastigotes of three *Leishmania* promastigote strains, *L. donovani* (strains 1S and 9515) and *L. major* (strain IR-173). These *Leishmania* spp. were selected to determine the specificity of the synthesised derivatives against *L. donovani* parasites that cause the serious and debilitating VL, and *L. major* parasites that cause CL (Salam *et al*., 2014:1). Compounds **1a-9a**, **1b-2b** and **8b** all exhibited good activity against the three *Leishmania* promastigote strains, with IC_{50} values ranging between 0.34 µM and 4.42 µM. The remaining compounds, **3b-7b** and **9b**, showed little to no activity against the promastigotes.

Most of the compounds were non-toxic to the mammalian cells (IC_{50} >100 µM), except compounds **1a**, **2a**, **8a**, **9a**, **1b** and **8b**, which were moderately toxic, and **6a** that had low toxicity. The most active compounds of the series were compounds **3a**, **5a** and **7a** as they demonstrated significant nanomolar activities against the promastigotes of the three tested *Leishmania* strains.

The antileishmanial results indicated that the nitrofuran sub-series (**1a-9a**) exhibited a much more potent activity than the thiophene sub-series (**1b-9b**). This might be related to the oxygen atom of the furan ring's ability to form hydrogen bonding. An electronic effect analysis in relation to

activity, was performed on the nitrofuran sub-series, and it revealed that strong electron withdrawing groups (EWGs) has good activity, while weak electron donating groups (EDGs) has the better activity.

During the course of this study a few challenges were faced. The original method to synthesise the phenylhydrazone compounds did not work. This method consisted of using ethanol as solvent, along with hydrazine hydrate (1 eq.) and the required aldehyde (1 eq.). To resolve this issue, the method was changed to the one described in chapter 3 (2.3.1). When performing the biological assays, several compounds exhibited solubility issues. This resulted in high SD values and can affect the overall accuracy of the results. In future studies, if the solubility of the compounds can be increased, it may result in more accurate assays.

In summary, the synthesised nitrofuran-based azine derivatives were brought about through simple synthetic routes in poor to excellent yields and additionally possessed overall good antileishmanial activity. The nitrofuran sub-series proved to be much more active against the three *Leishmania* promastigote strains in comparison to the nitrothiophene sub-series. Most of the compounds proved to be safe against mammalian cells. For further testing, the activities of the current derivatives will be screened against the intracellular amastigote form to confirm their potential as antileishmanial hits.

REFERENCE LIST

Aldeghi, M., Malhotra, S., Selwood, D.L., and Chan, A.W. 2014. Two-and three-dimensional rings in drugs. *Chemical Biology & Drug Design*, 83(4):450-461.

Asif, M. & Husain, A. 2013. Analgesic, anti-inflammatory, and antiplatelet profile of hydrazones containing synthetic molecules. *Journal of Applied Chemistry*, 2013, art. #247203. doi:10.1155/2013/247203

Braga, S.S. 2019. Multi-target drugs active against leishmaniasis: A paradigm of drug repurposing. *European Journal of Medicinal Chemistry,* 183, art. #111660. doi:10.1016/j.ejmech.2019.111660

Capela, R., Moreira, R. & Lopes, F. 2019. An overview of drug resistance in protozoal diseases. *International Journal of Molecular Sciences*, 20(22), art. #5748. doi:10.3390/ijms20225748

Freitas-Junior, L.H., Chatelain, E., Kim, H.A. & Siqueira-Neto, J.L. 2012. Visceral leishmaniasis treatment: what do we have, what do we need and how to deliver it? *International journal for parasitology. Drugs and drug resistance*, 2:11-19.

Georgiadou, S.P., Makaritsis, K.P. & Dalekos, G.N. 2015. Leishmaniasis revisited: Current aspects on epidemiology, diagnosis and treatment. *Journal of translational internal medicine*, 3(2):43-50.

Güran, M. 2018. An overview of leishmaniasis: historic to future perspectives. In: Savić, S., ed. *Vectors and Vector-Borne Zoonotic Diseases*. London, UK: IntechOpen. pp. 1-14.

PAHO (Pan American Health Organization). 2020. *Leishmaniasis*. https://www3.paho.org/en/topics/leishmaniasis Date of access: 19 Jun. 2021.

Pal, C. & Bandyopadhyay, U. 2012. Redox-active antiparasitic drugs. *Antioxidants & redox signaling,* 17(4):555-82.

Patel, R., Rinker, L., Peng, J. & Chilian, W.M. 2018. Reactive oxygen species: the good and the bad. In: Filip, C. & Albu, E., eds. *Reactive Oxygen Species (ROS) in Living Cells*. London: IntechOpen. pp. 7-20.

Patterson, S. & Wyllie, S. 2014. Nitro drugs for the treatment of trypanosomatid diseases: past, present, and future prospects. *Trends in Parasitology*, 30(6):289-298.

Rando, D.G., Avery, M.A., Tekwani, B.L., Khan, S.I. & Ferreira, E.I. 2008. Antileishmanial activity screening of 5-nitro-2-heterocyclic benzylidene hydrazides. *Bioorganic & Medicinal Chemistry*, 16(14):6724-6731.

Salam, N., Al-Shaqha, W.M. & Azzi, A. 2014. Leishmaniasis in the middle East: incidence and epidemiology. *PLoS neglected tropical diseases*, 8(10), e3208. https://doi.org/10.1371/journal.pntd.0003208

Sales Junior, P.A., Molina, I., Fonseca Murta, S.M., Sánchez-Montalvá, A., Salvador, F., Corrêa-Oliveira, R. & Carneiro, C.M. 2017. Experimental and clinical treatment of chagas disease: a review. *The American journal of tropical medicine and hygiene*, 97(5):1289-1303.

Sifontes-Rodríguez, S., Monzote-Fidalgo, L., Castañedo-Cancio, N., Montalvo-Álvarez, A.M., López-Hernández, Y., Diogo, N.M., … Cabrera-Pérez, M.Á. 2015. The efficacy of 2-nitrovinylfuran derivatives against *Leishmania* in vitro and in vivo. *Memorias do Instituto Oswaldo Cruz*, 110(2):166-173.

Verma, G., Marella, A., Shaquiquzzaman, M., Akhtar, M., Ali, M.R. & Alam, M.M. 2014. A review exploring biological activities of hydrazones. *Journal of pharmacy & bioallied sciences,* 6(2):69-80.

WHO (World Health Organization). 2017. *Global vector control response 2017–2030: A strategic approach to tackle vector-borne diseases.* https://www.who.int/publications/i/item/WHO-HTM-GVCR-2017.01 Date of access: 19 Jun. 2021.

WHO. 2021*a*. *Leishmaniasis: Overview*. https://www.who.int/health-topics/leishmaniasis#tab=tab_1 Date of access: 16 Jun. 2021.

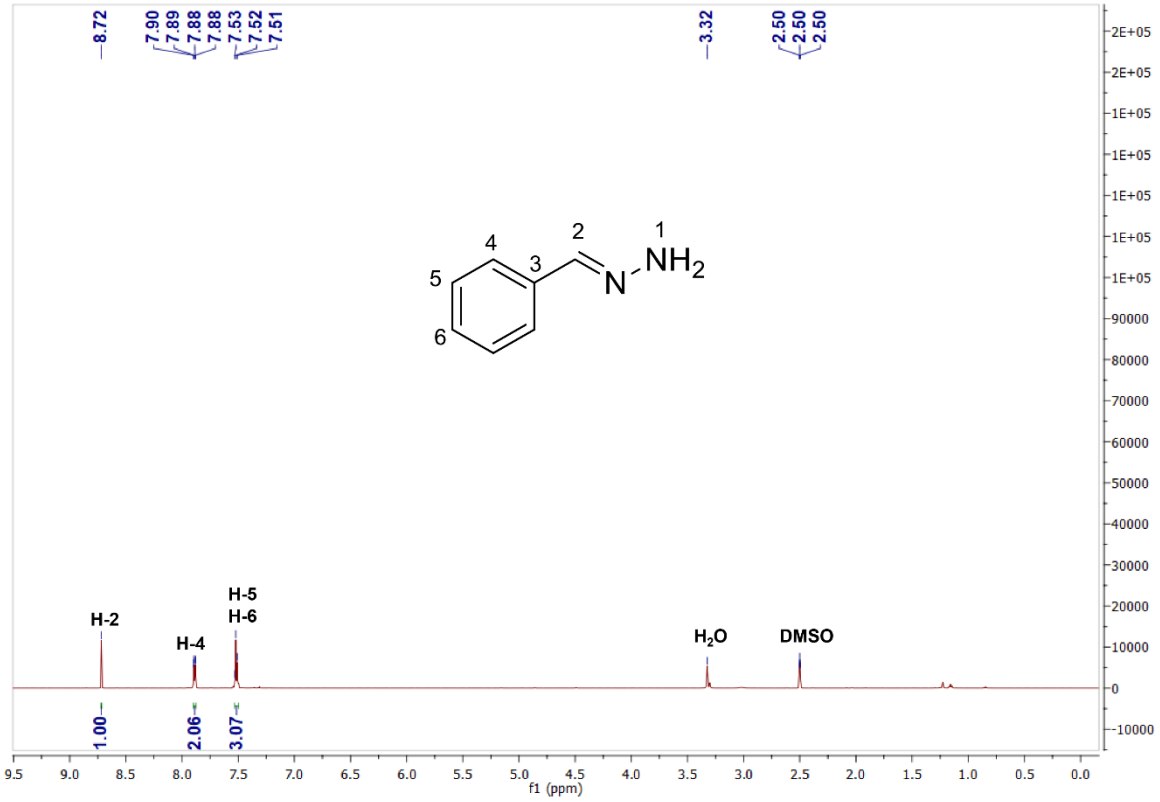
WHO. 2021*b*. *Leishmaniasis: Key facts*. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis Date of access: 16 Jun. 2021.

Zuma, N.H., Aucamp, J. & N'Da, D.D. 2019. An update on derivatisation and repurposing of clinical nitrofuran drugs. *European Journal of Pharmaceutical Sciences*, 140, art. #105092. doi:10.1016/j.ejps.2019.105092

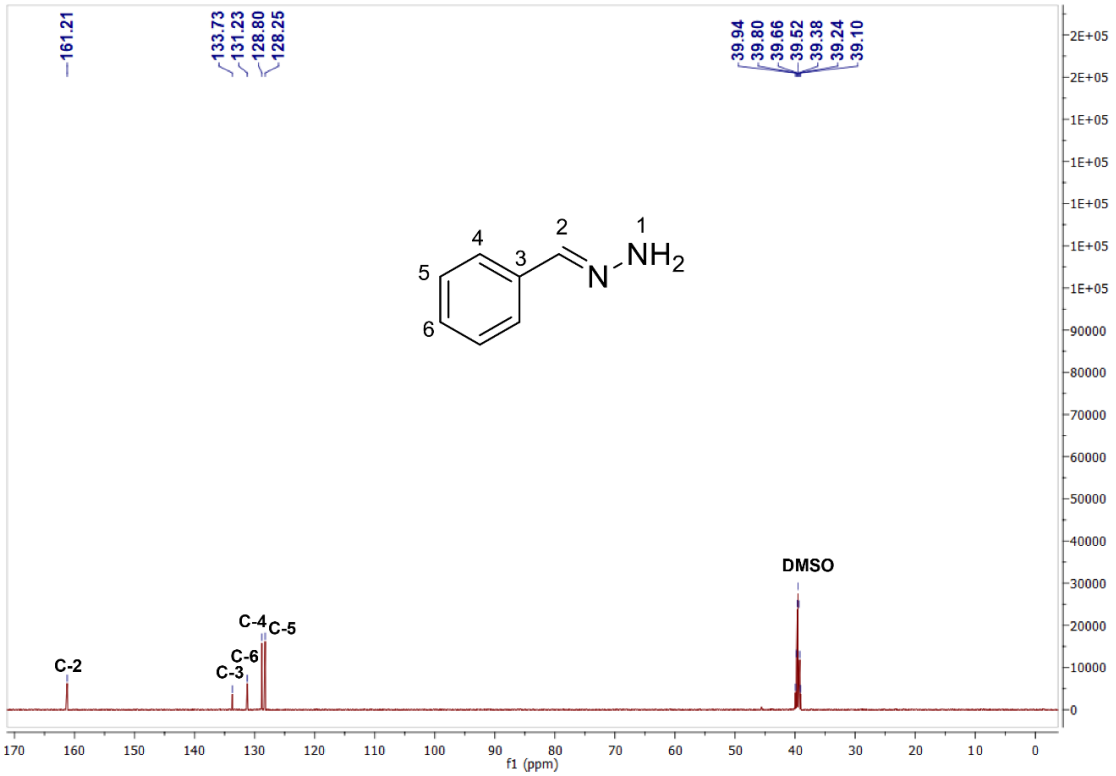
ANNEXURE A: ANALYTICAL DATA FOR CHAPTER 3

(E)-benzylidenehydrazine (1)

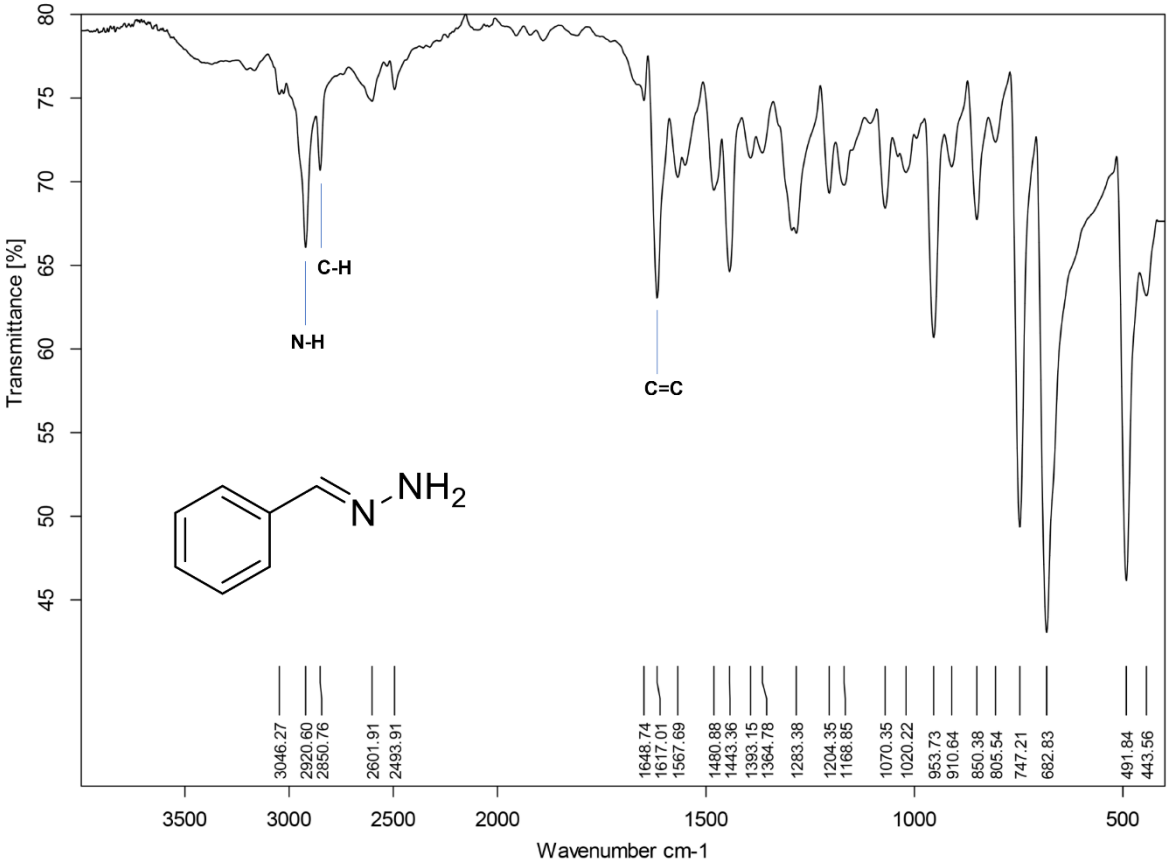
^{1}H NMR in DMSO



^{13}C NMR in DMSO

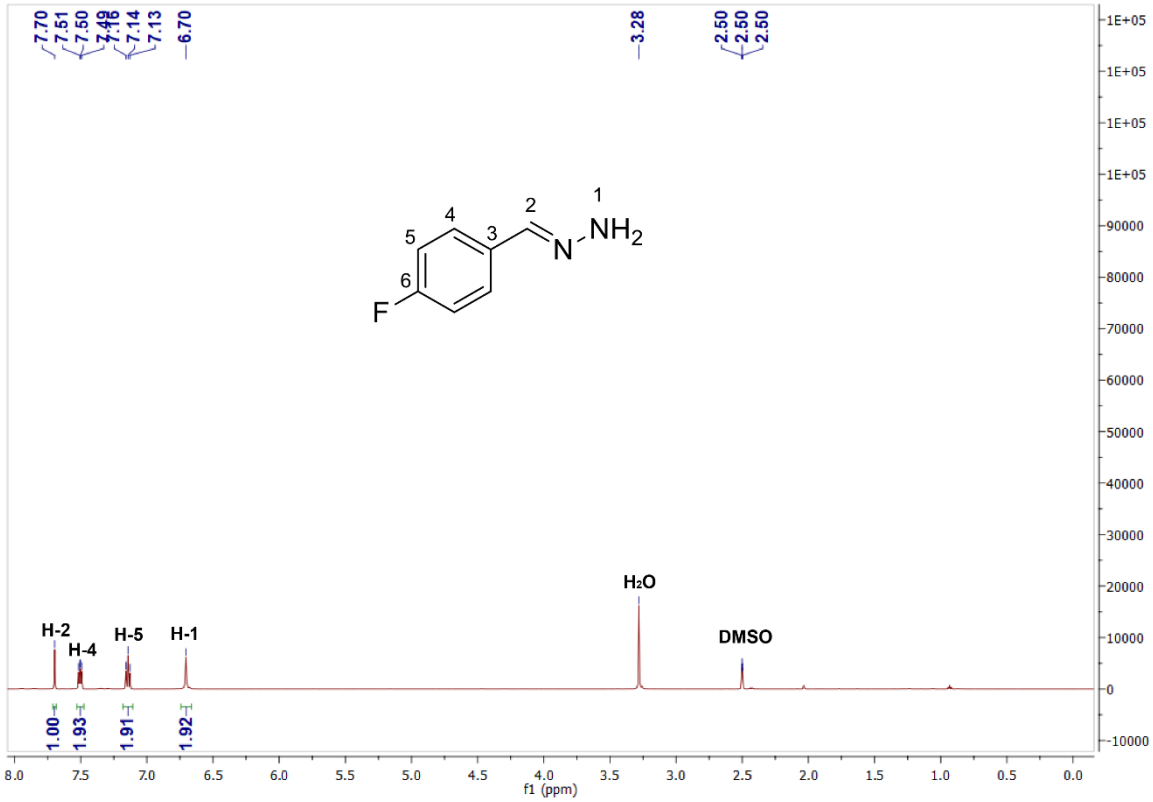


IR Spectrum

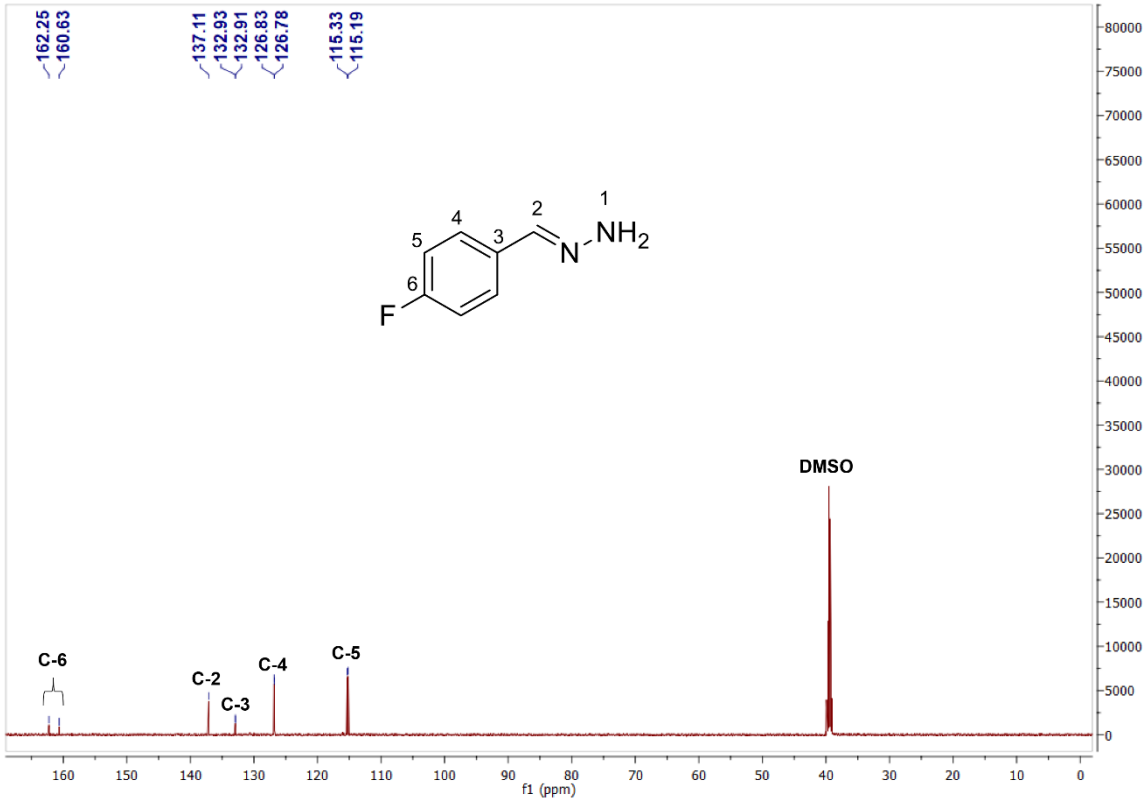

Transmittance [%]
80
75
70
65
60
55
50
45
C-H
N-H
C=C
NH2
N
3046.27
2920.60
2850.76
2601.91
2493.91
1648.74
1617.01
1567.69
1480.88
1443.36
1393.15
1364.78
1283.38
1204.35
1168.85
1070.35
1020.22
953.73
910.64
850.38
805.54
747.21
682.83
491.84
443.56
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(E)-(4-fluorobenzylidene)hydrazine (2)

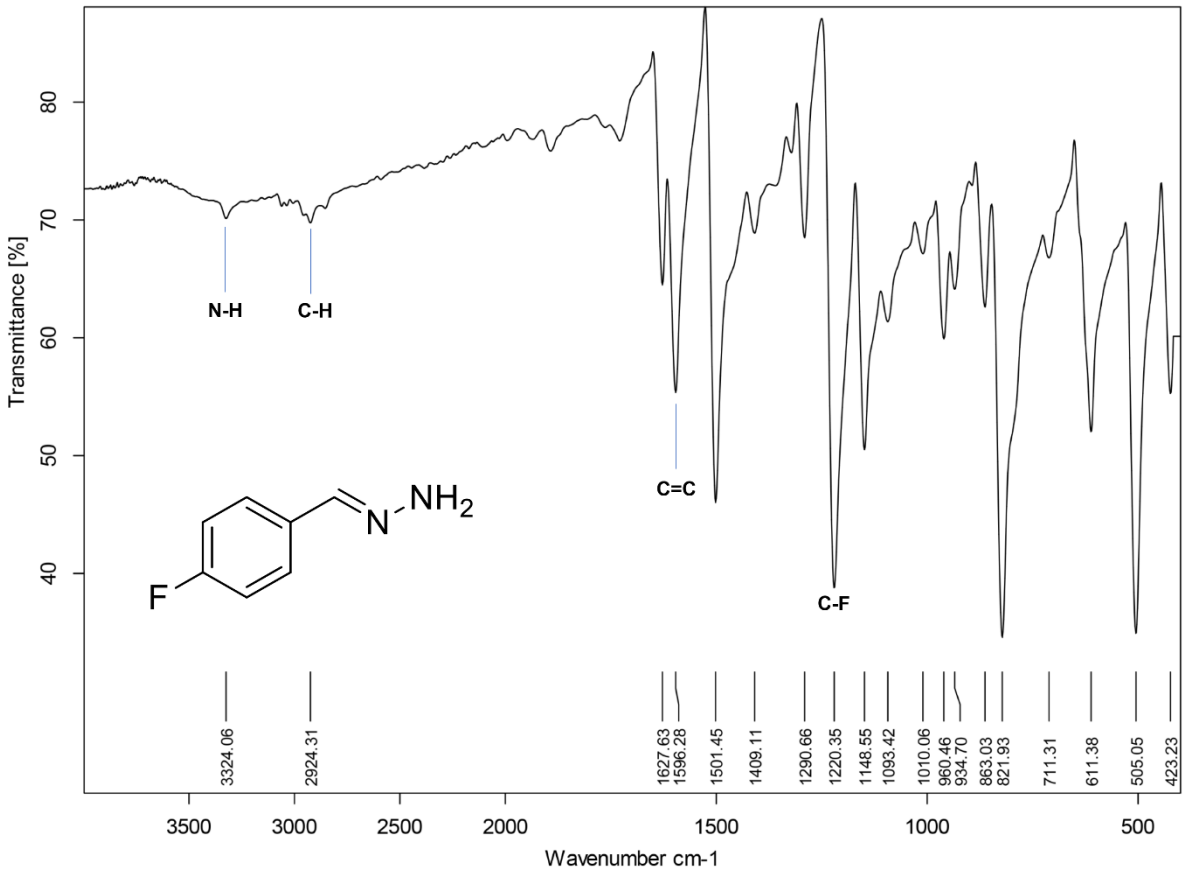
^{1}H NMR in DMSO



^{13}C NMR in DMSO

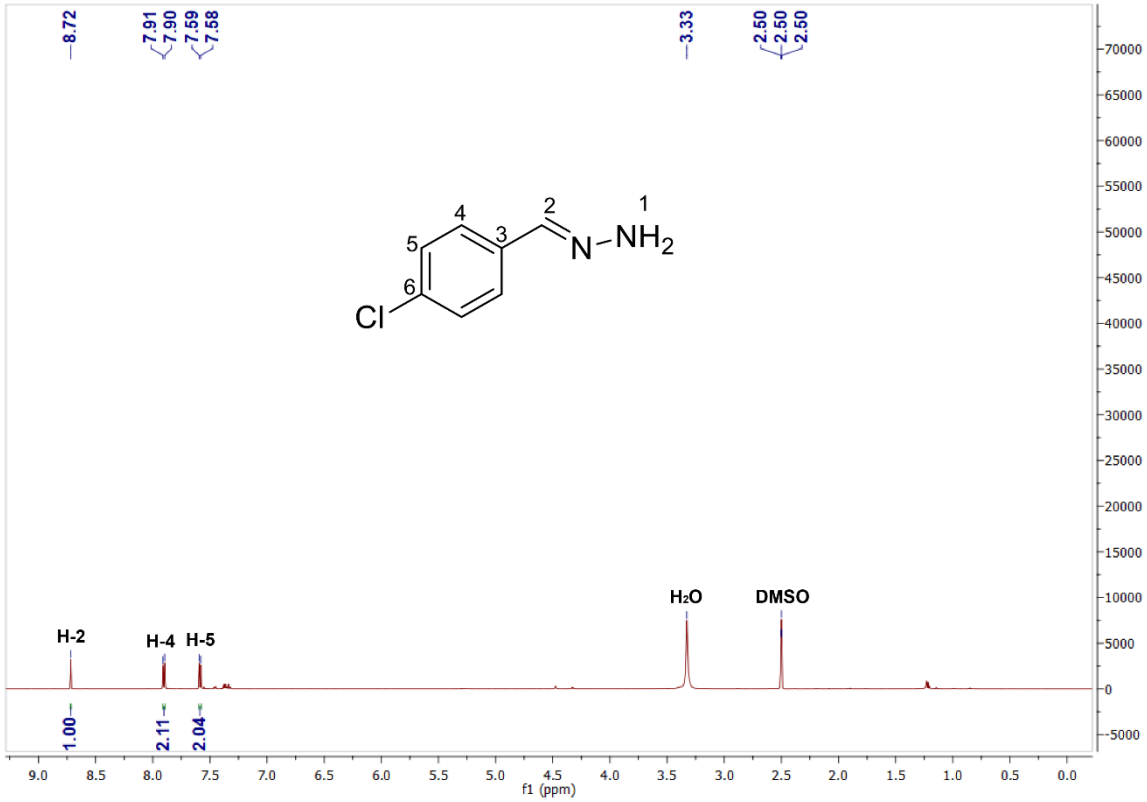


IR Spectrum

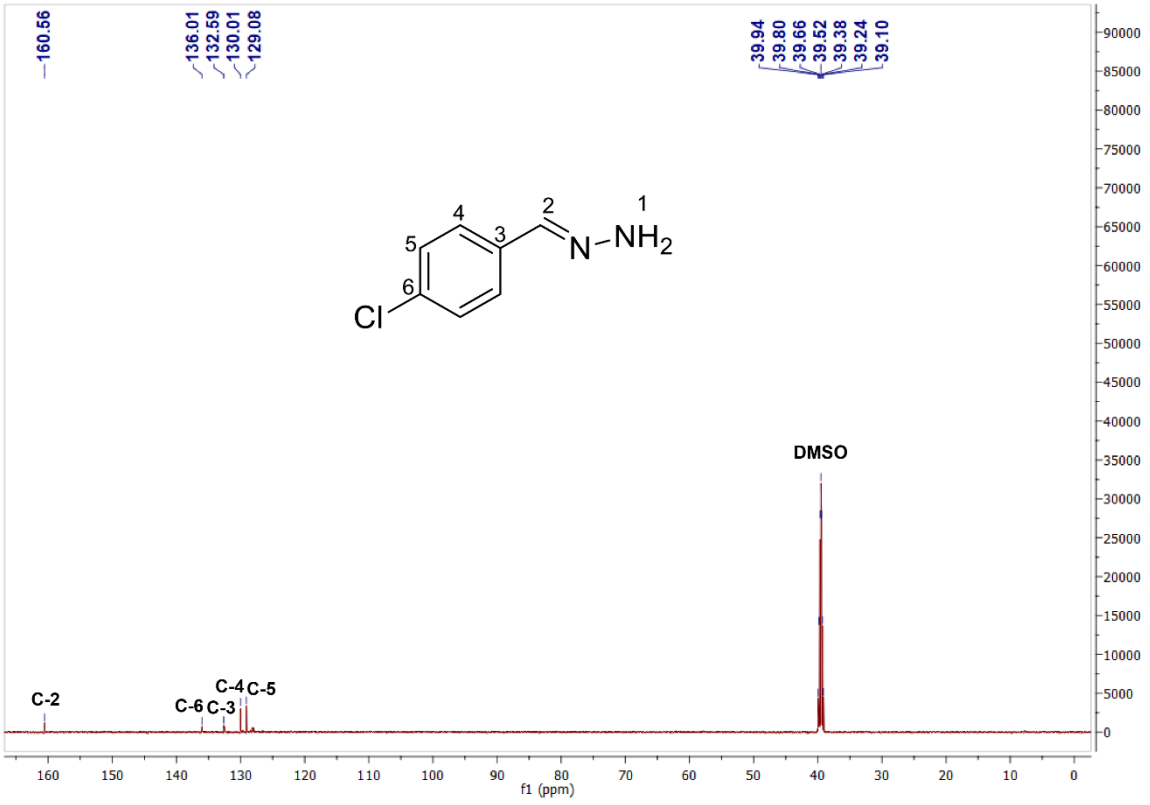

Transmittance [%]
80
70
60
50
40
N-H
C-H
C=C
C-F
NH2
N
F
3324.06
2924.31
1627.63
1596.28
1501.45
1409.11
1290.66
1220.35
1148.55
1093.42
1010.06
960.46
934.70
863.03
821.93
711.31
611.38
505.05
423.23
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(E)-(4-chlorobenzylidene)hydrazine (3)

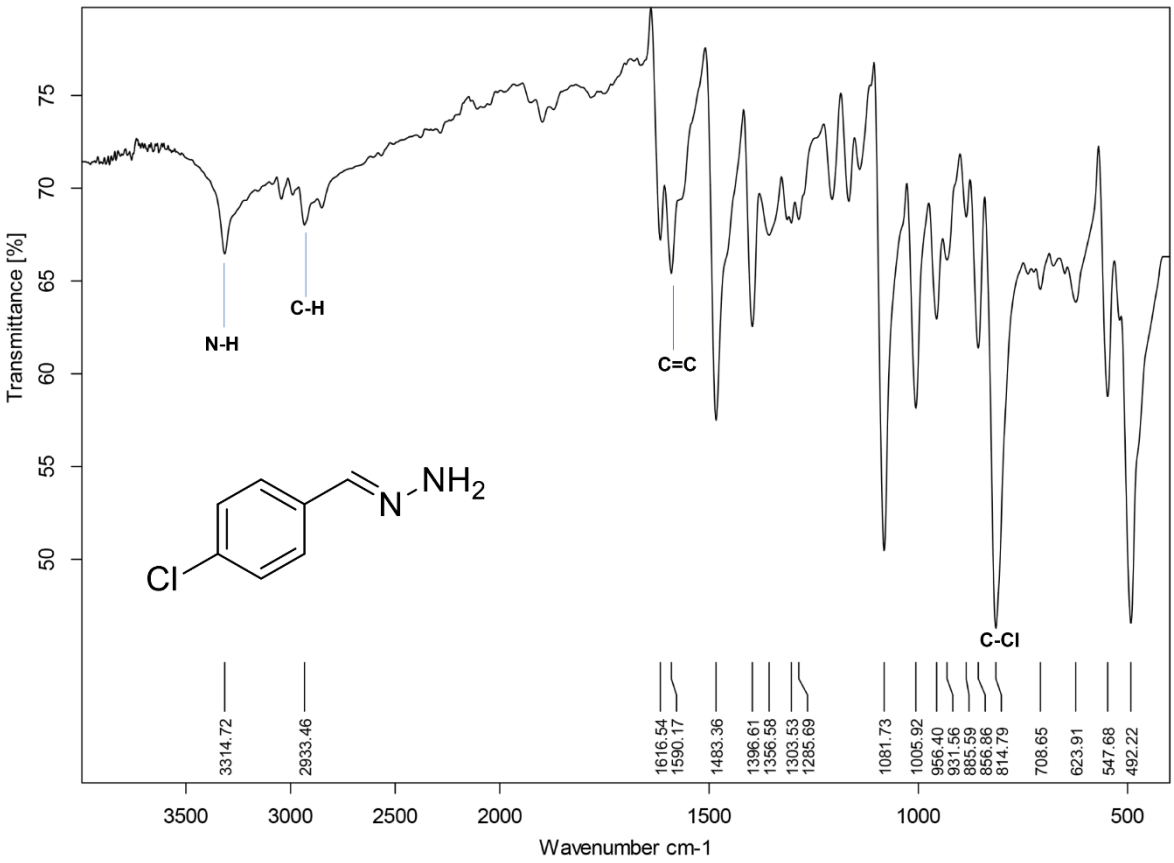
^{1}H NMR in DMSO



^{13}C NMR in DMSO

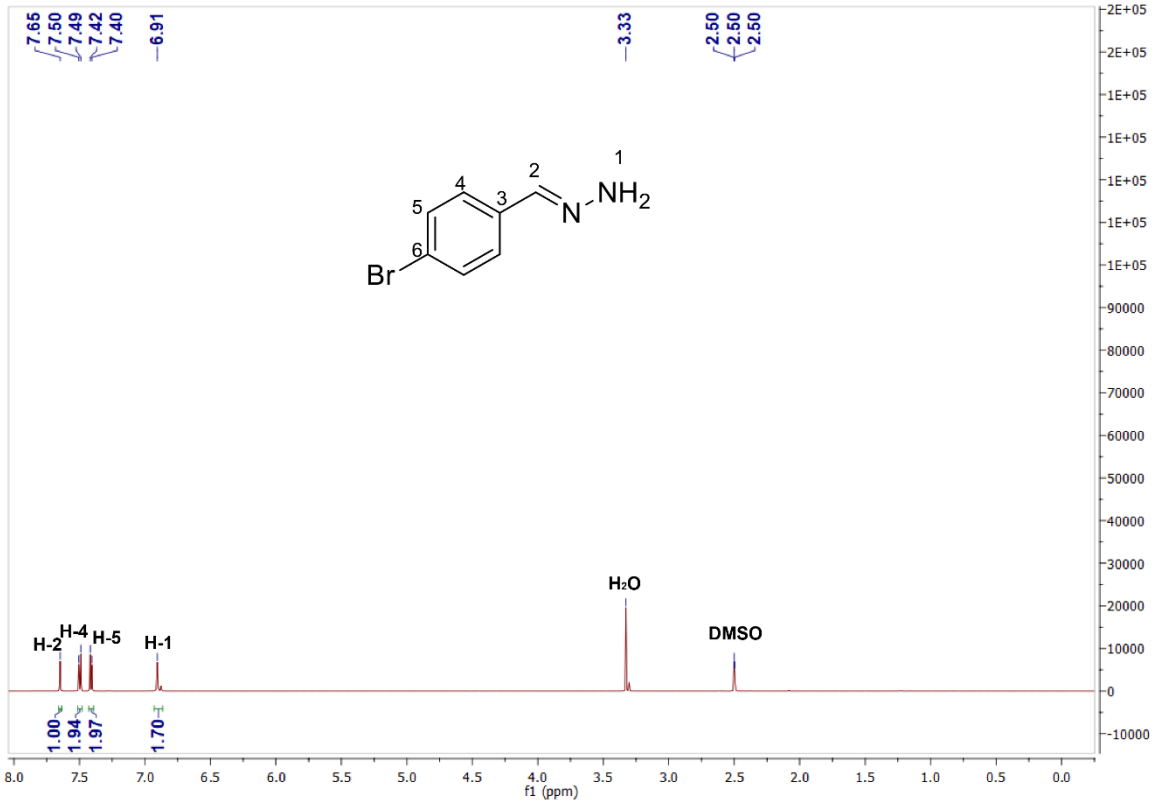


IR Spectrum

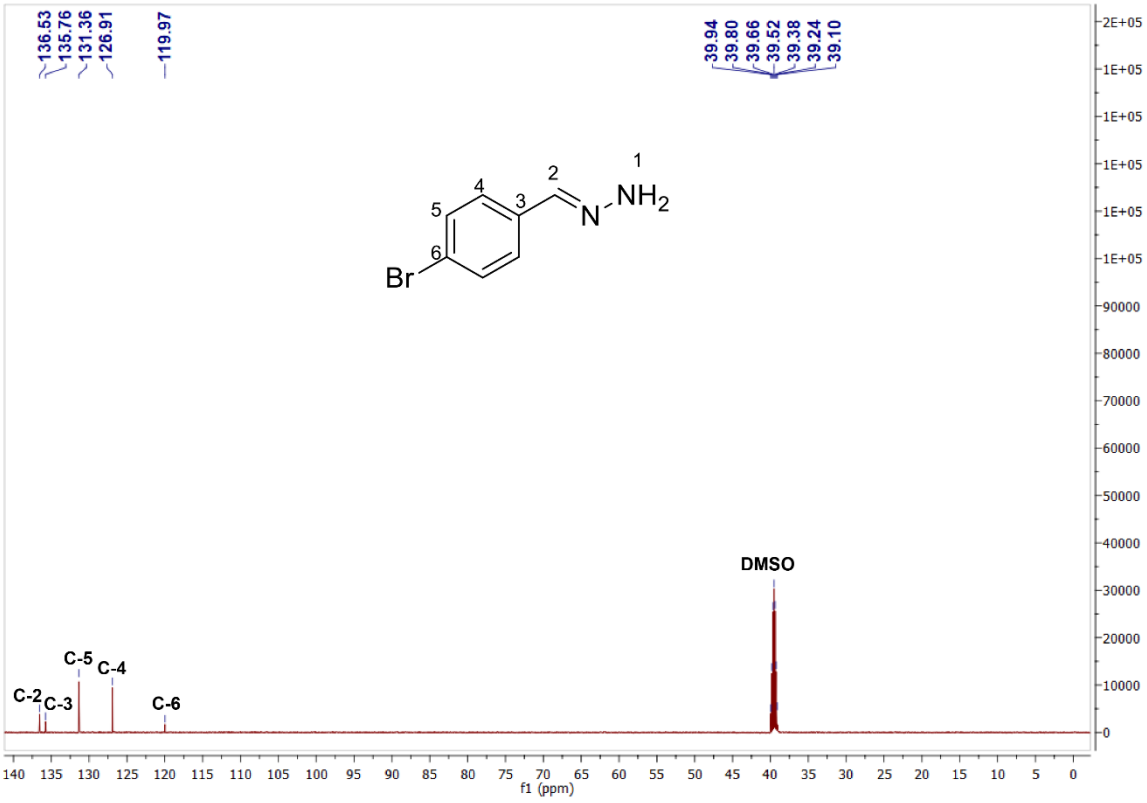

Transmittance [%]
75
70
65
60
55
50
N-H
C-H
C=C
C-Cl
NH2
N
Cl
3314.72
2933.46
1616.54
1590.17
1483.36
1396.61
1356.58
1303.53
1285.69
1081.73
1005.92
956.40
931.56
885.59
856.86
814.79
708.65
623.91
547.68
492.22
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(E)-(4-bromobenzylidene)hydrazine (4)

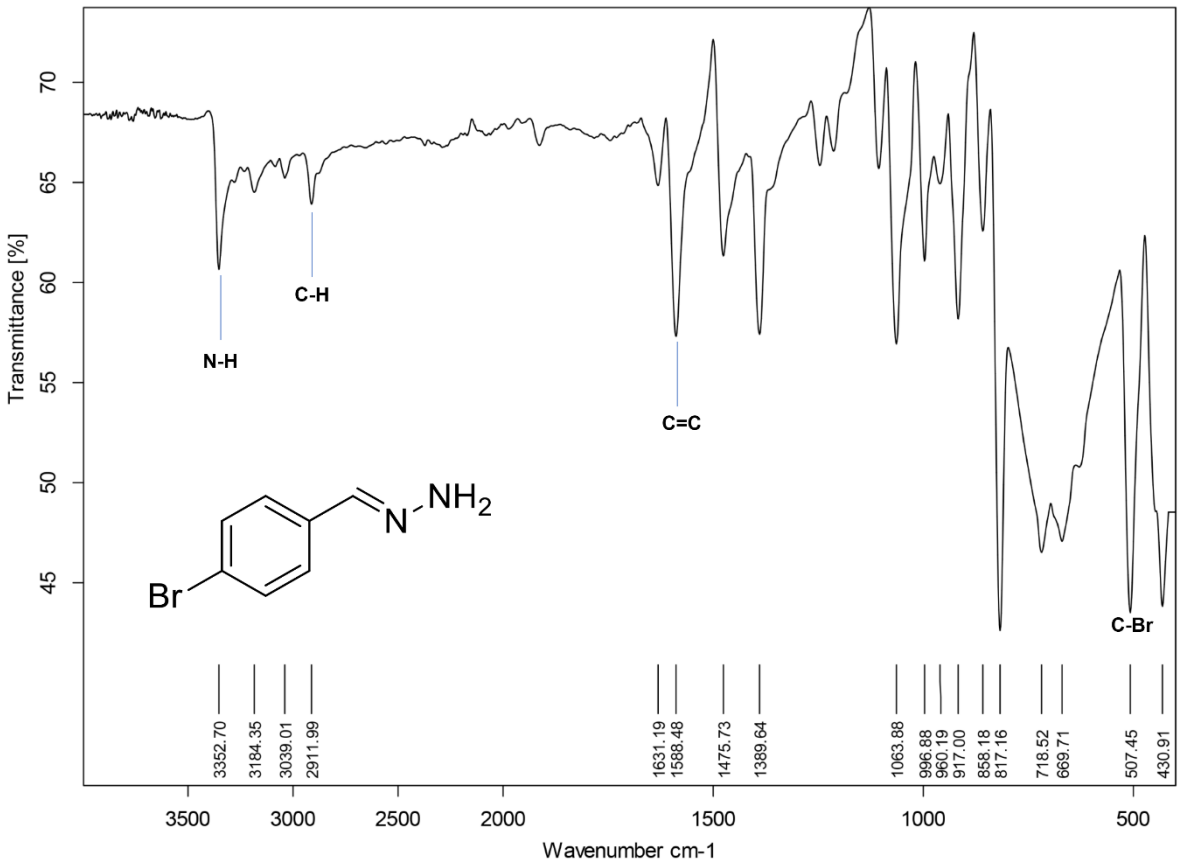
^{1}H NMR in DMSO



^{13}C NMR in DMSO

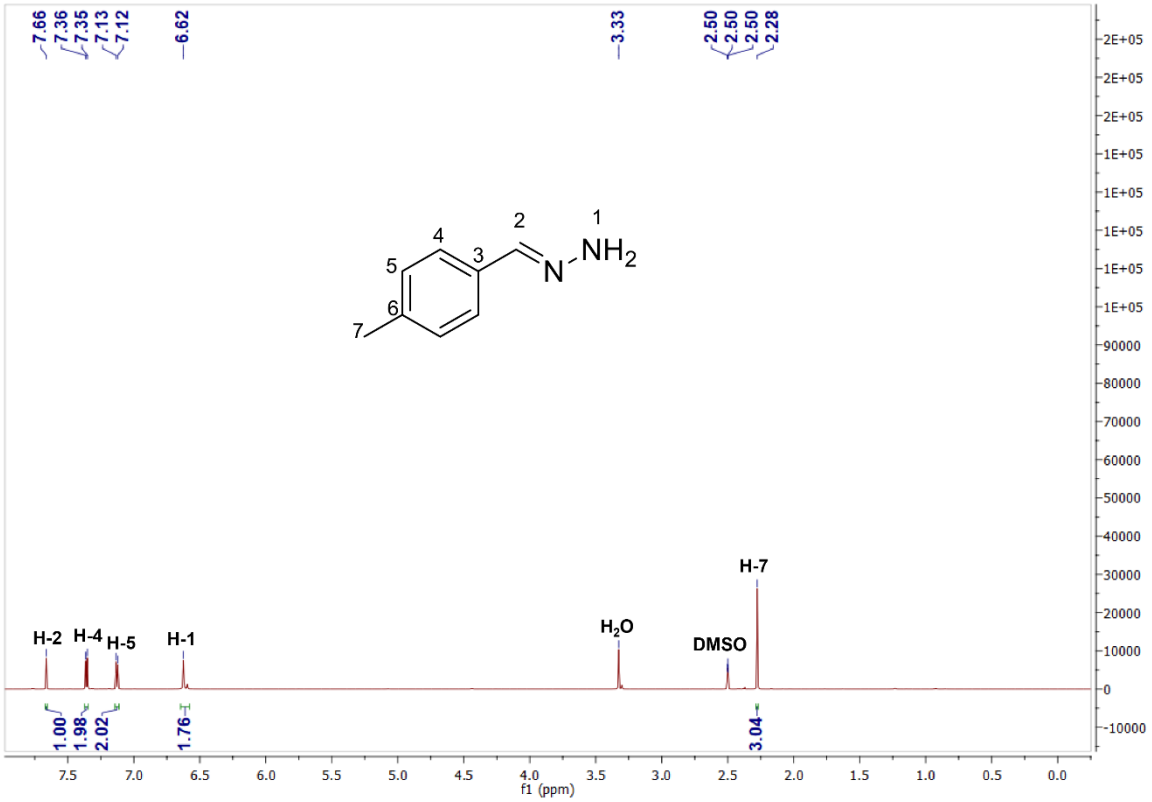


IR Spectrum

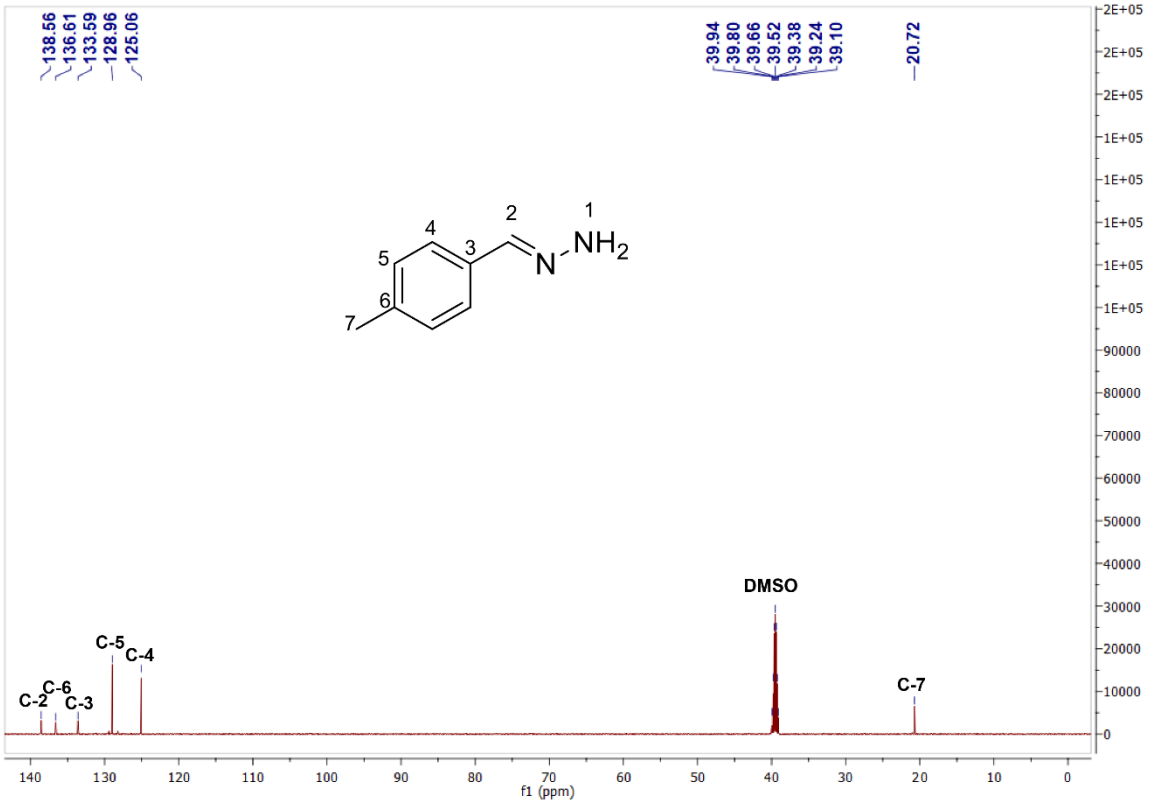

Transmittance [%]
70
65
60
55
50
45
N-H
C-H
C=C
C-Br
Br
N
NH2
3352.70
3184.35
3039.01
2911.99
1631.19
1588.48
1475.73
1389.64
1063.88
996.88
960.19
917.00
858.18
817.16
718.52
669.71
507.45
430.91
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(E)-(4-methylbenzylidene)hydrazine (5)

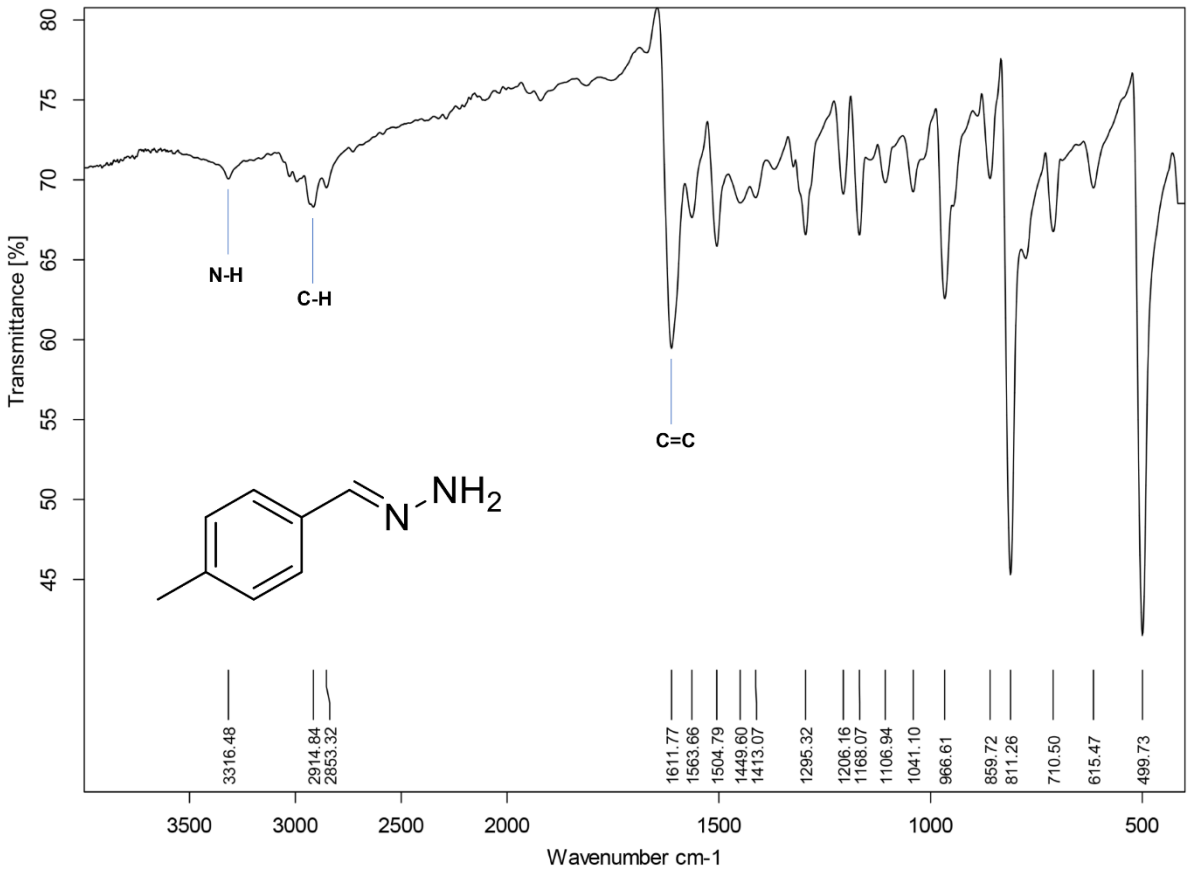
^{1}H NMR in DMSO



^{13}C NMR in DMSO

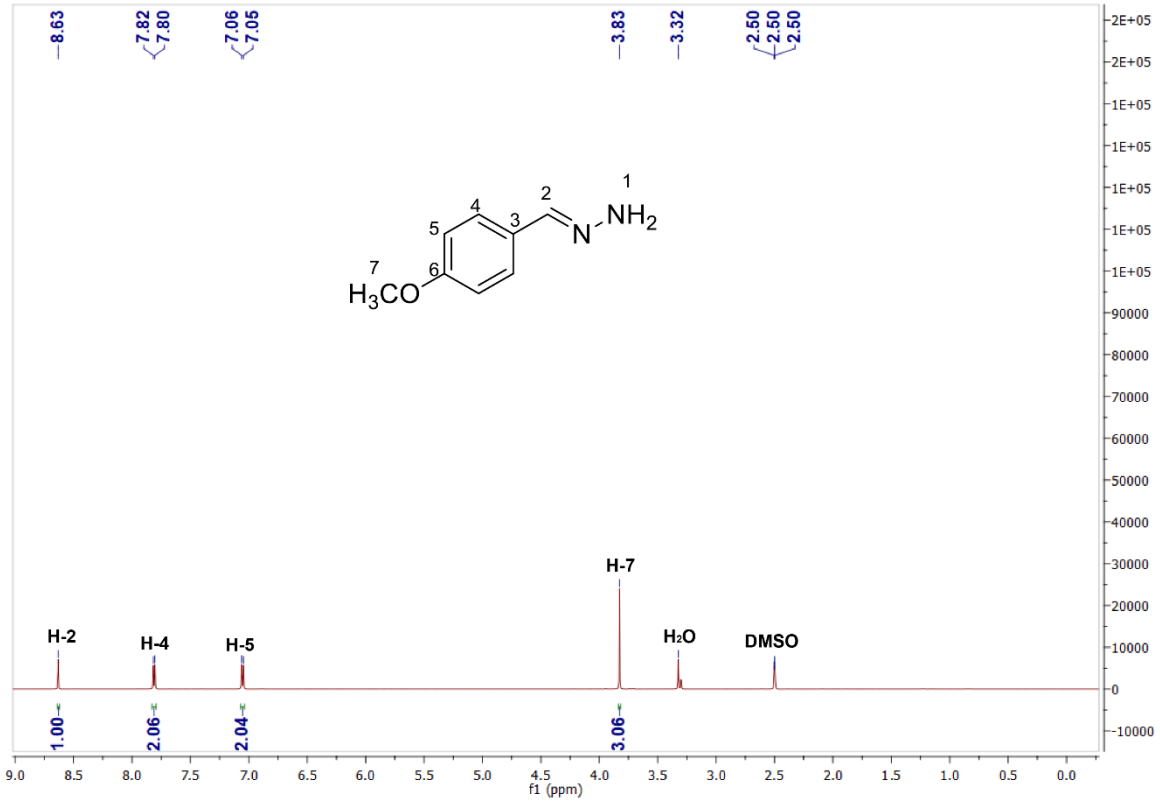


IR Spectrum

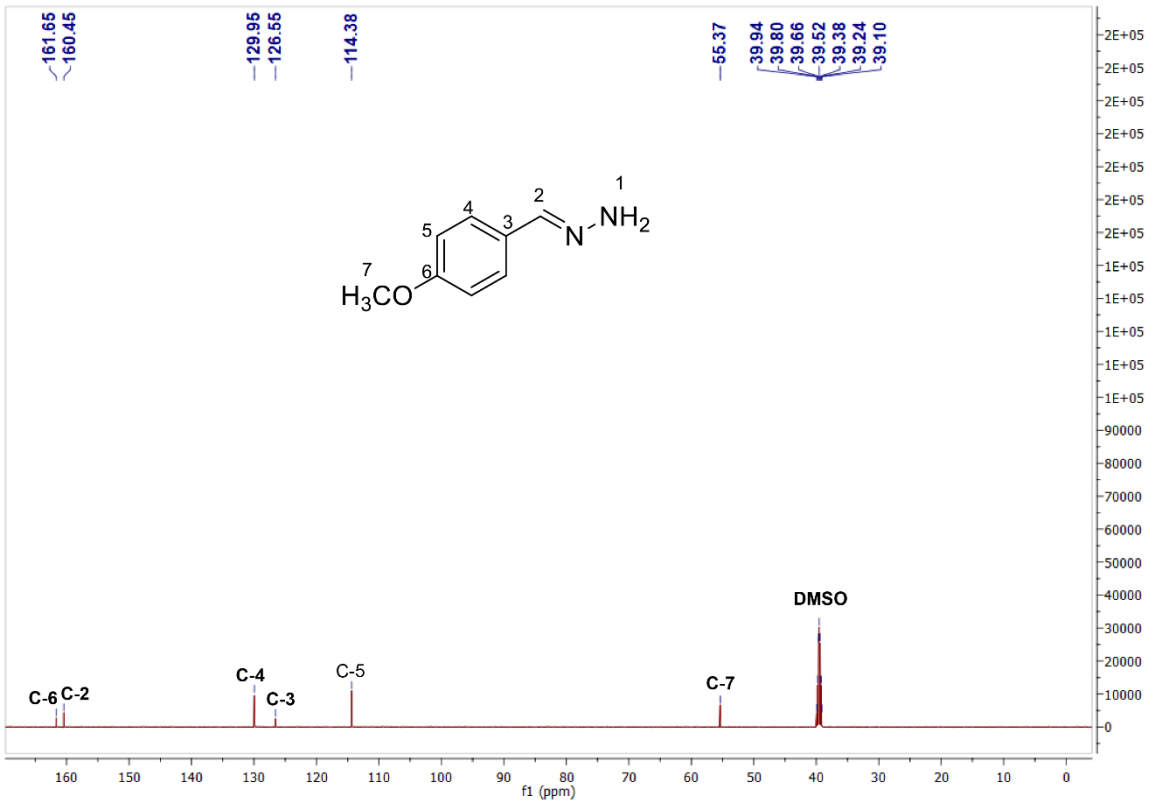

Transmittance [%]
80
75
70
65
60
55
50
45
N-H
C-H
C=C
NH2
N
3316.48
2914.84
2853.32
1611.77
1563.66
1504.79
1449.60
1413.07
1295.32
1206.16
1168.07
1106.94
1041.10
966.61
859.72
811.26
710.50
615.47
499.73
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(E)-(4-methoxybenzylidene)hydrazine (6)

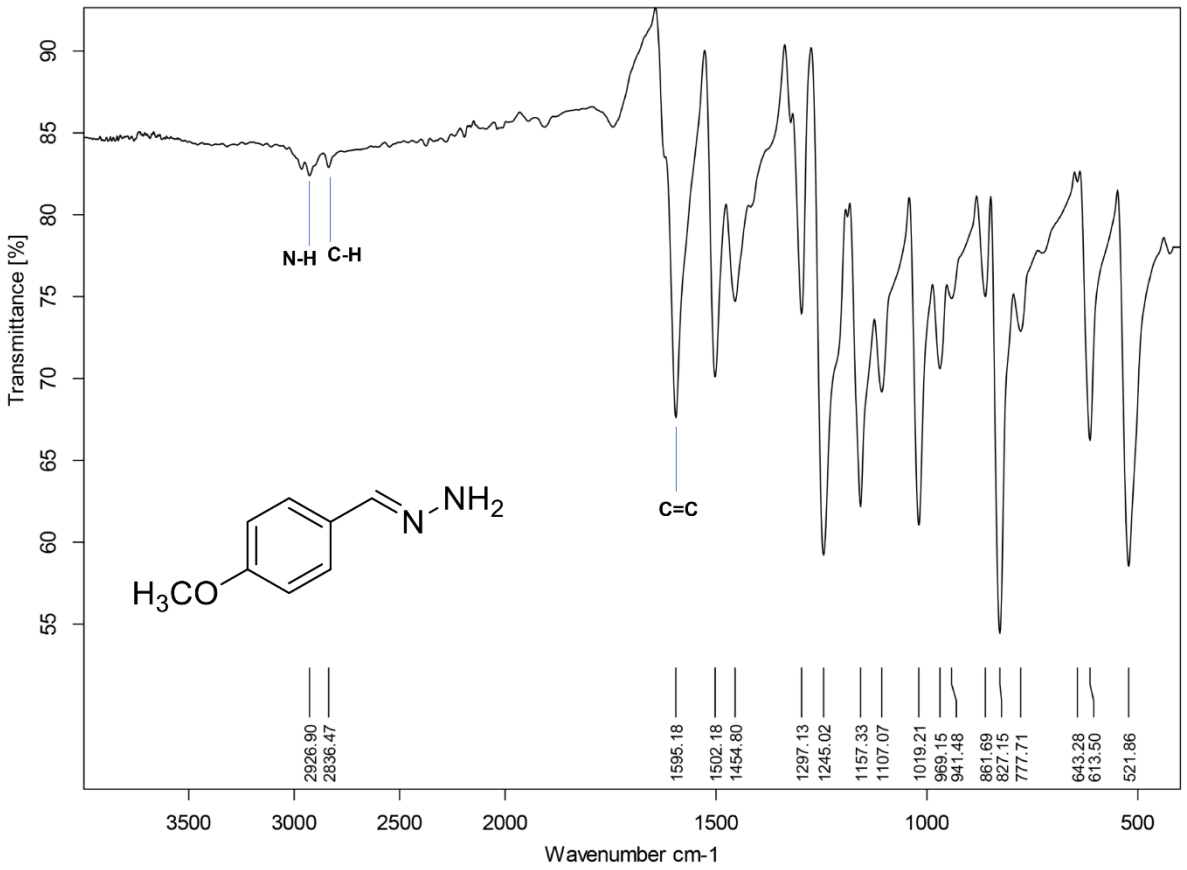
^{1}H NMR in DMSO



^{13}C NMR in DMSO

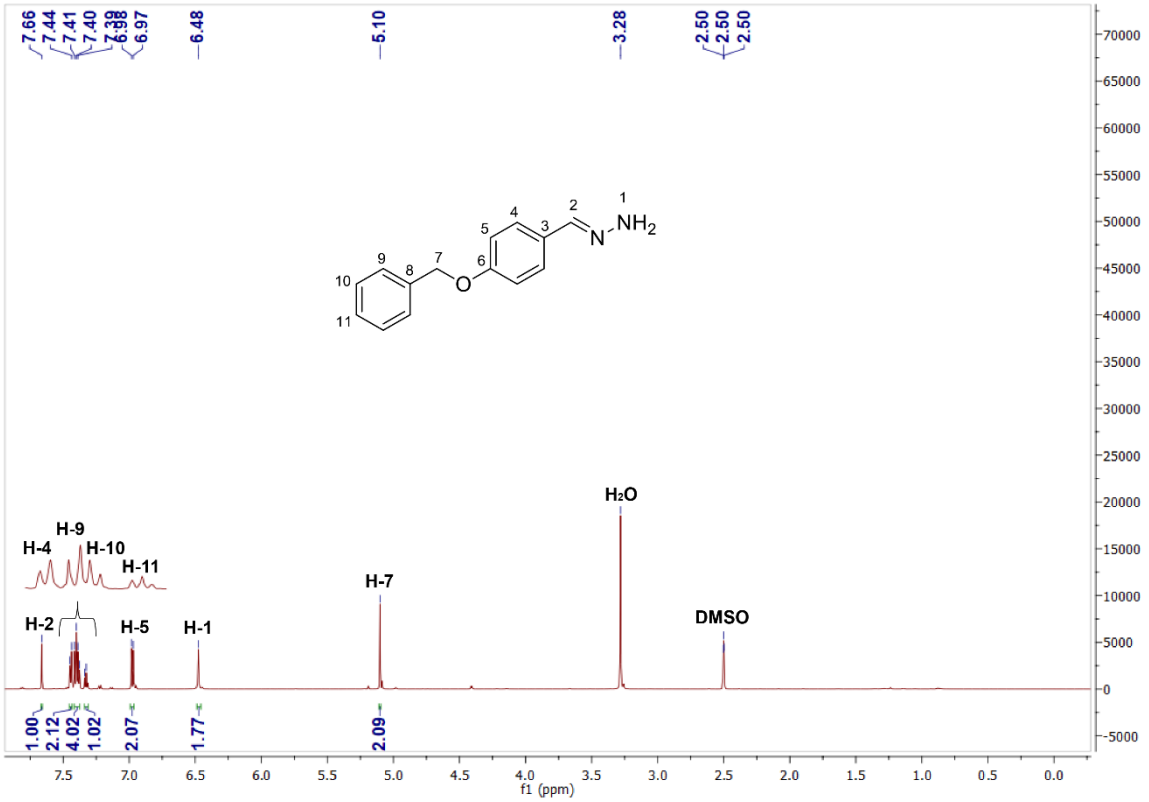


IR Spectrum

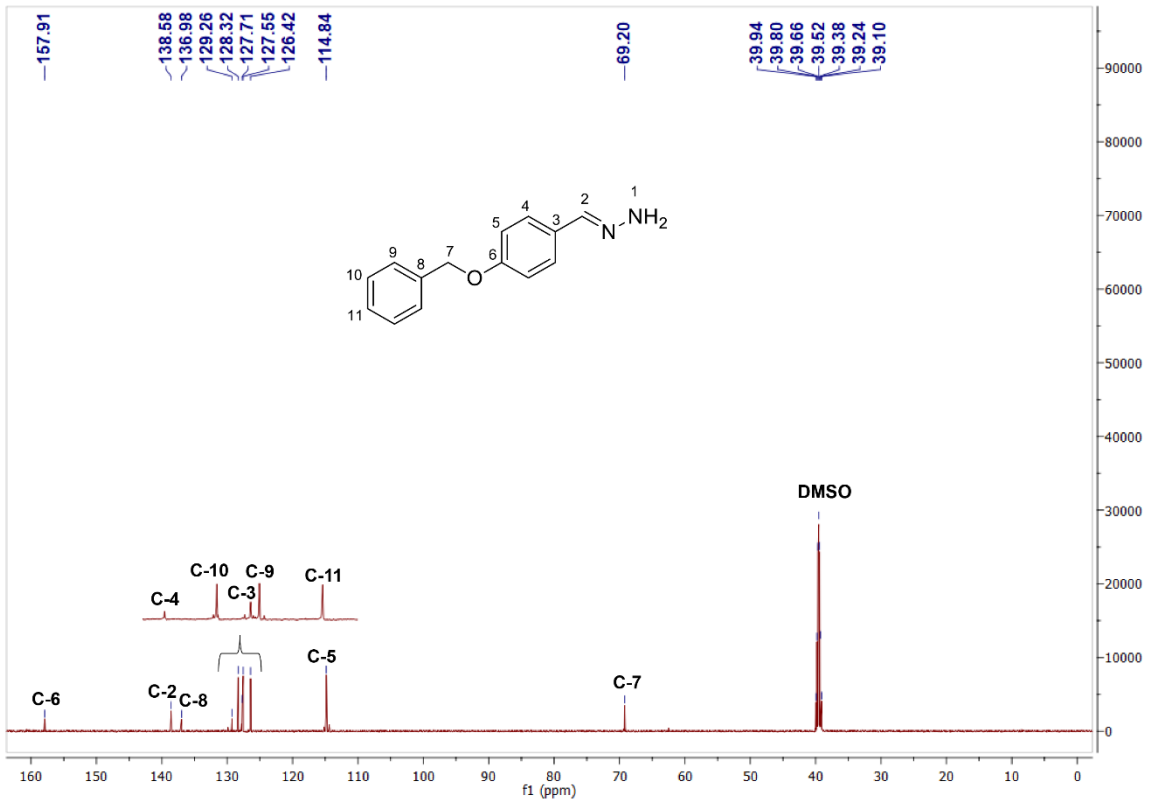

Transmittance [%]
90
85
80
75
70
65
60
55
N-H
C-H
C=C
H_3CO
N
NH_2
2926.90
2836.47
1595.18
1502.18
1454.80
1297.13
1245.02
1157.33
1107.07
1019.21
969.15
941.48
861.69
827.15
777.71
643.28
613.50
521.86
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(E)-(4-(benzyloxy)benzylidene)hydrazine (7)

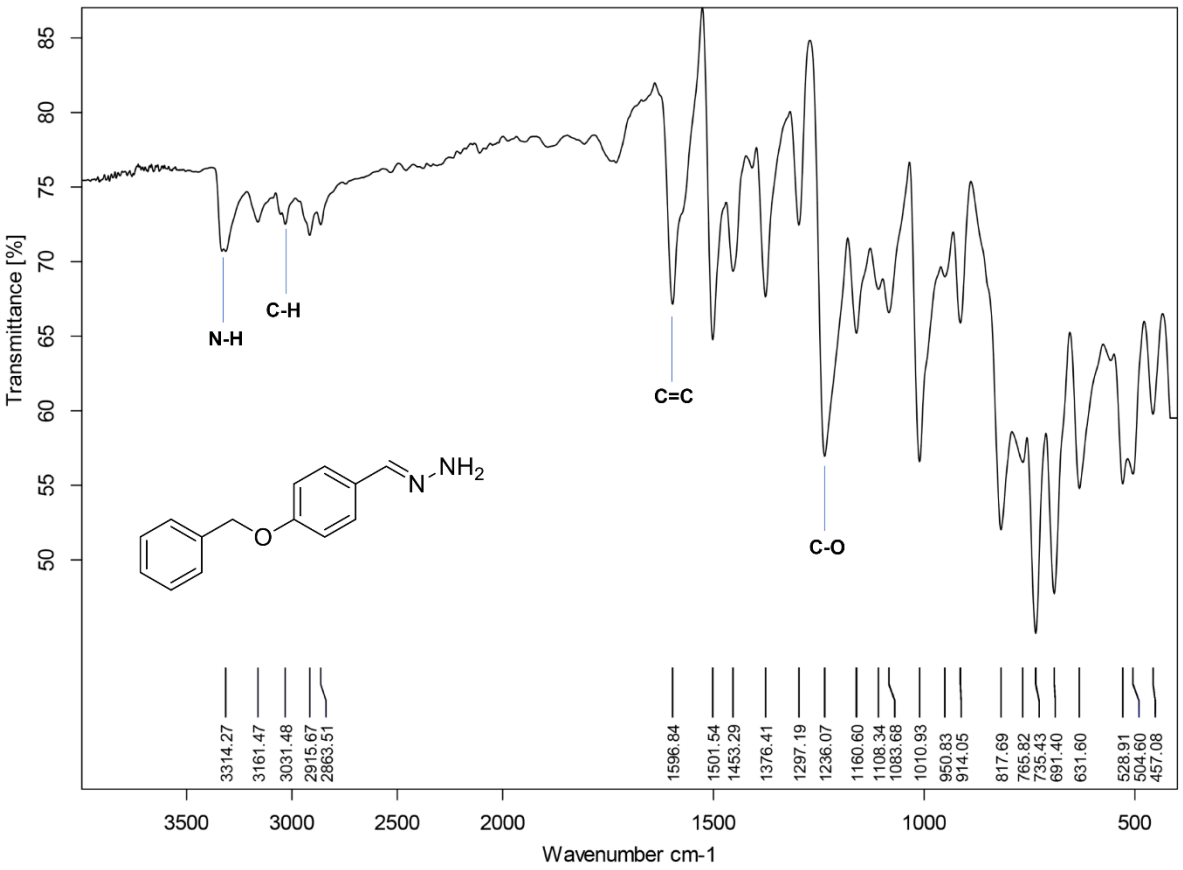
^{1}H NMR in DMSO



^{13}C NMR in DMSO

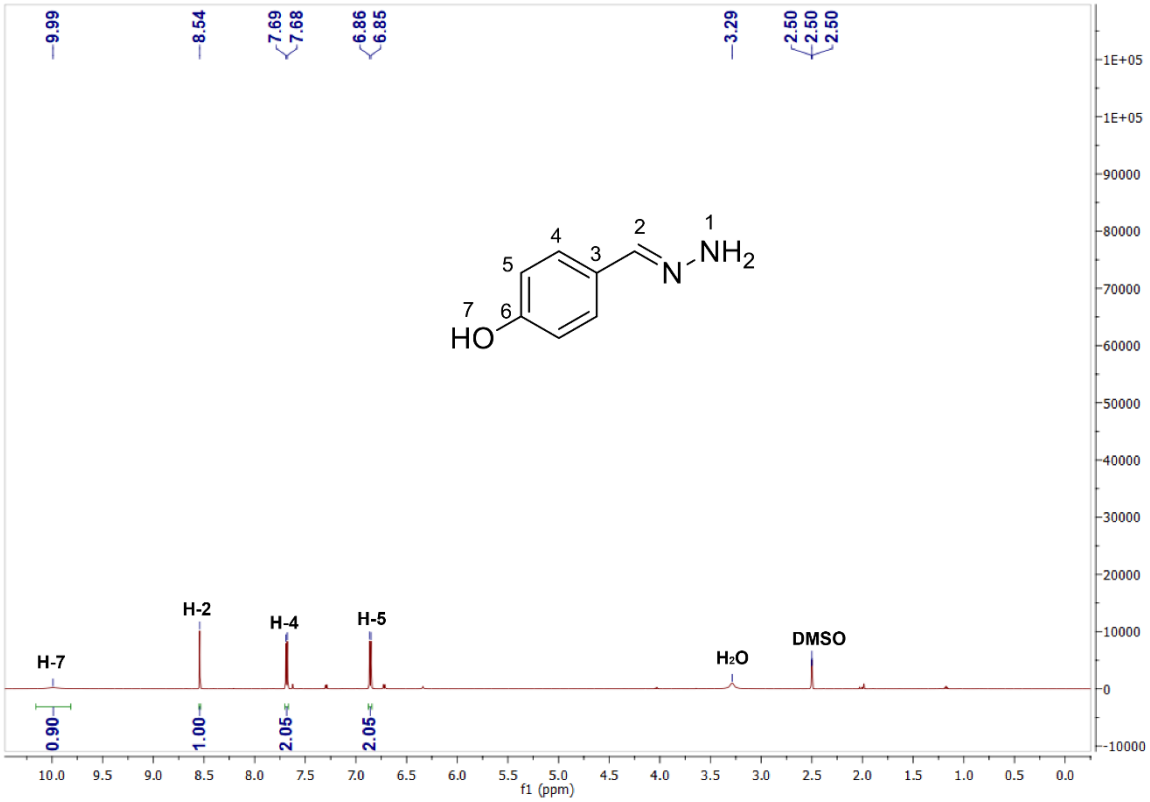


IR Spectrum

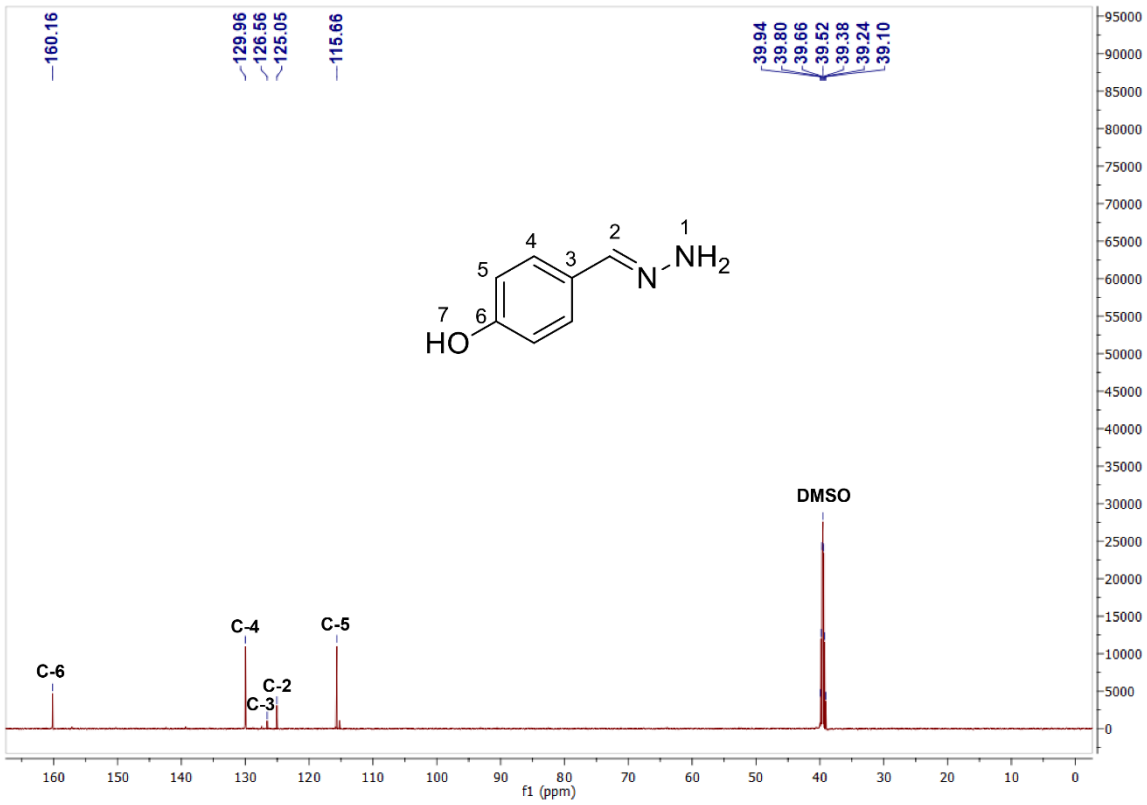

Transmittance [%]
85
80
75
70
65
60
55
50
N-H
C-H
C=C
C-O
NH2
N
O
3314.27
3161.47
3031.48
2915.67
2863.51
1596.84
1501.54
1453.29
1376.41
1297.19
1236.07
1160.60
1108.34
1083.68
1010.93
950.83
914.05
817.69
765.82
735.43
691.40
631.60
528.91
504.60
457.08
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(E)-4-(hydrazonomethyl)phenol (8)

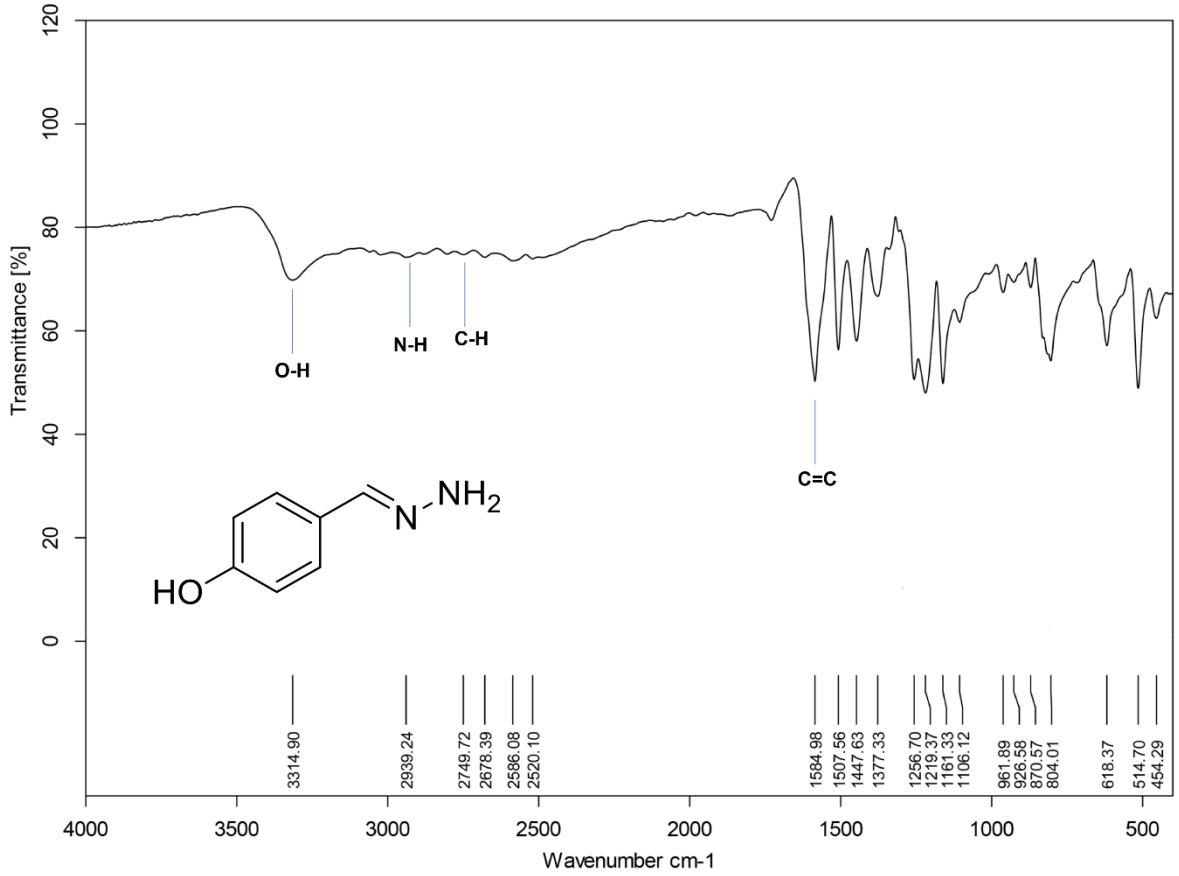
^{1}H NMR in DMSO



^{13}C NMR in DMSO

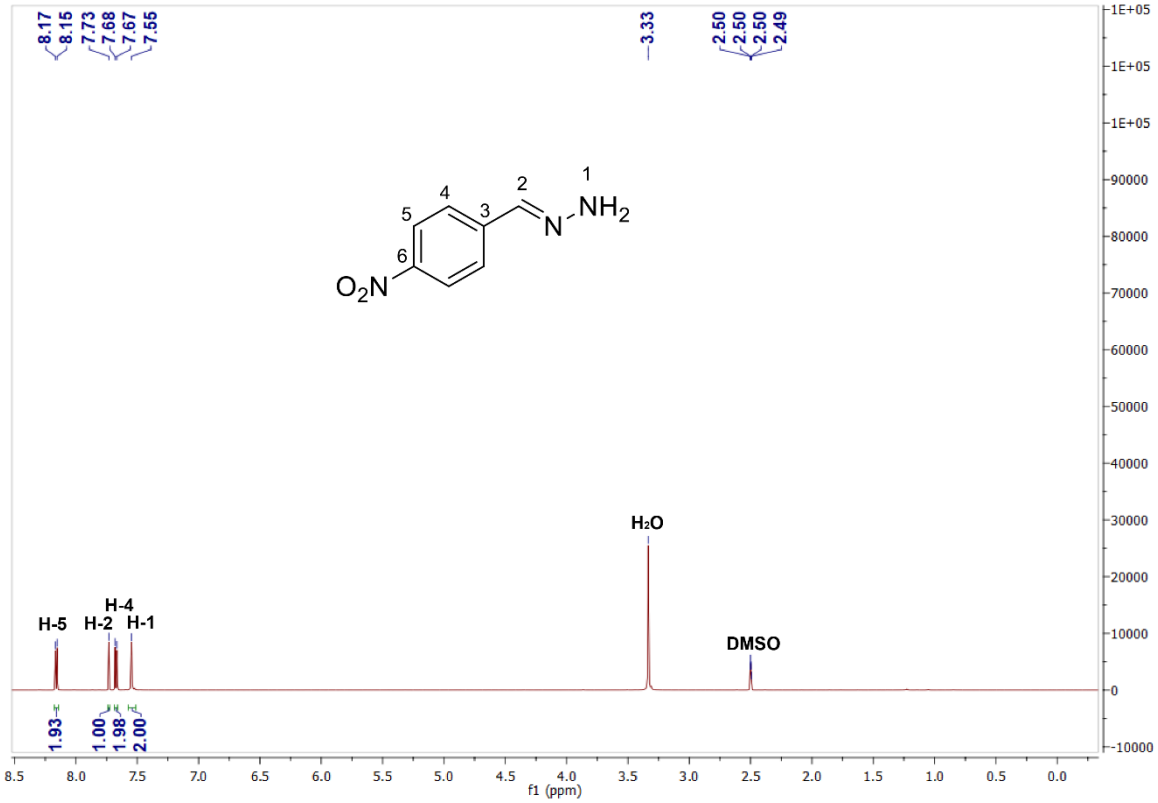


IR Spectrum

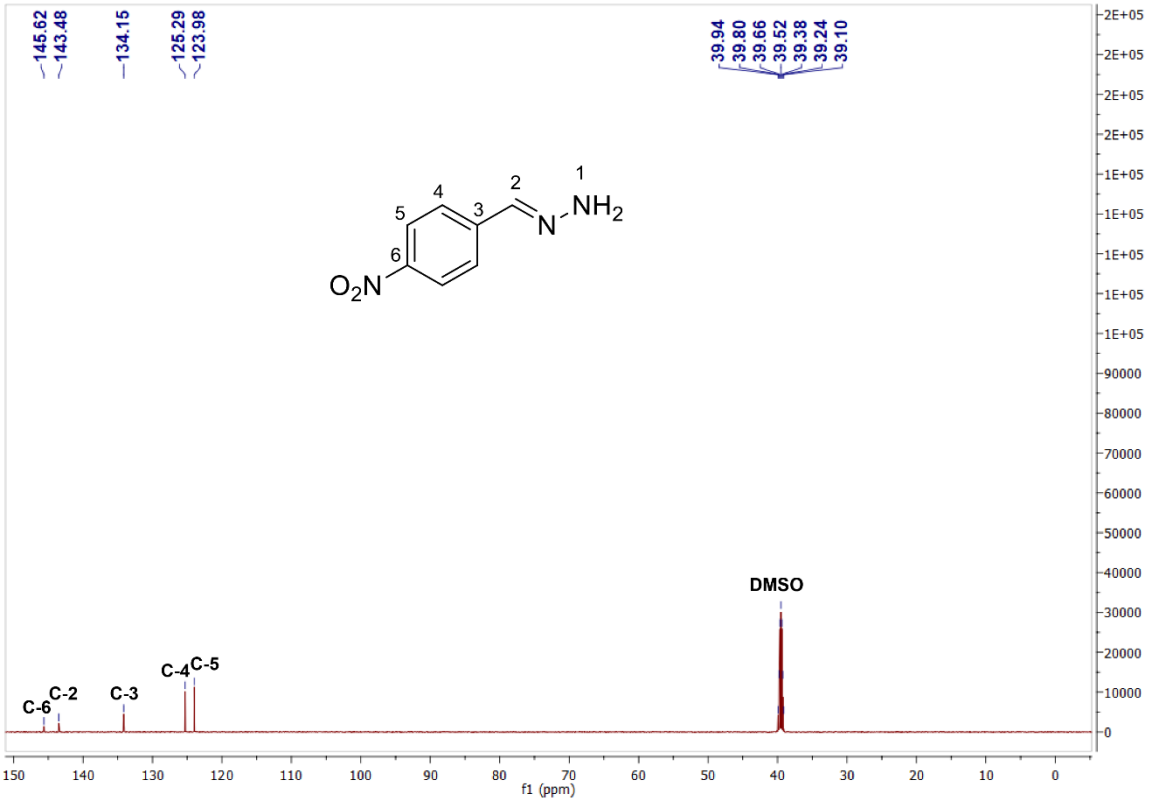

Transmittance [%]
120
100
80
60
40
20
0
O-H
N-H
C-H
C=C
NH2
N
HO
3314.90
2939.24
2749.72
2678.39
2586.08
2520.10
1584.98
1507.56
1447.63
1377.33
1256.70
1219.37
1161.33
1106.12
961.89
926.58
870.57
804.01
618.37
514.70
454.29
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(E)-(4-nitrobenzylidene)hydrazine (9)

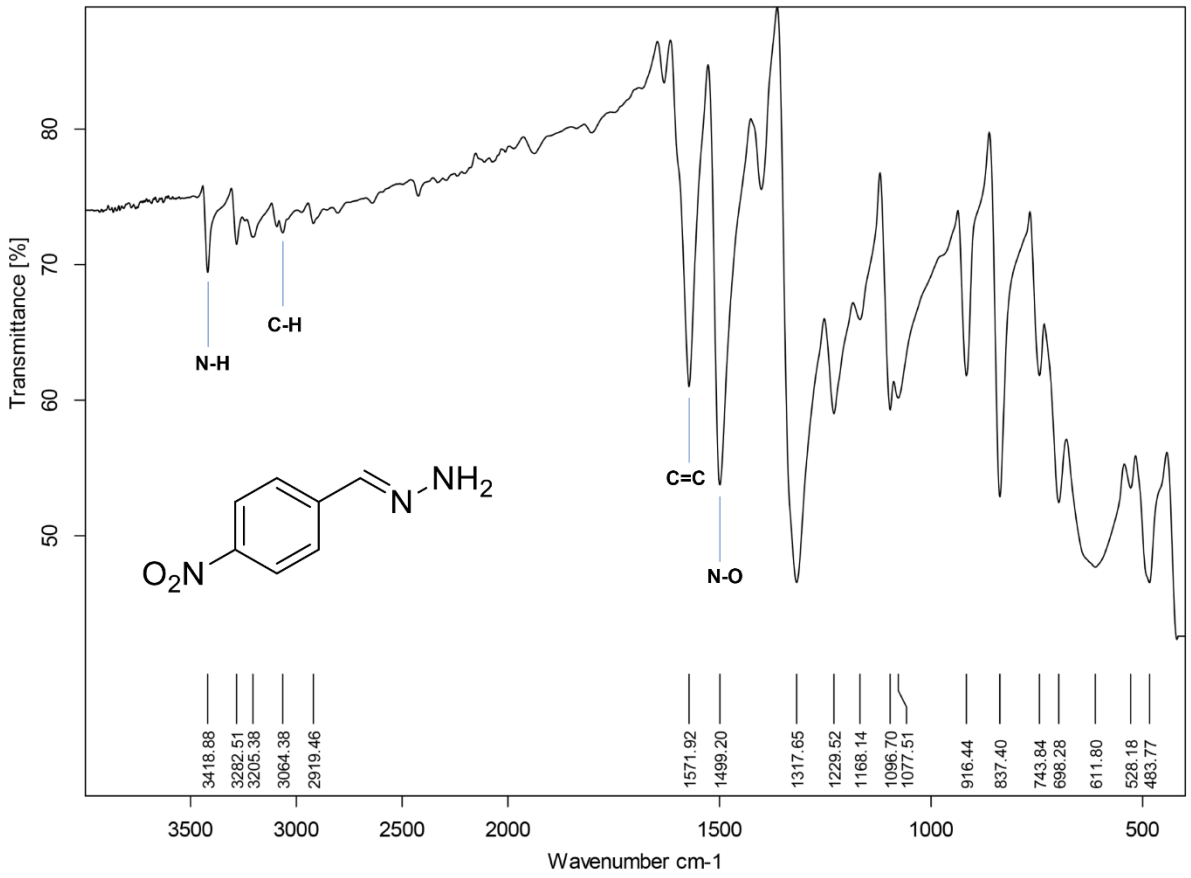
^{1}H NMR in DMSO



^{13}C NMR in DMSO

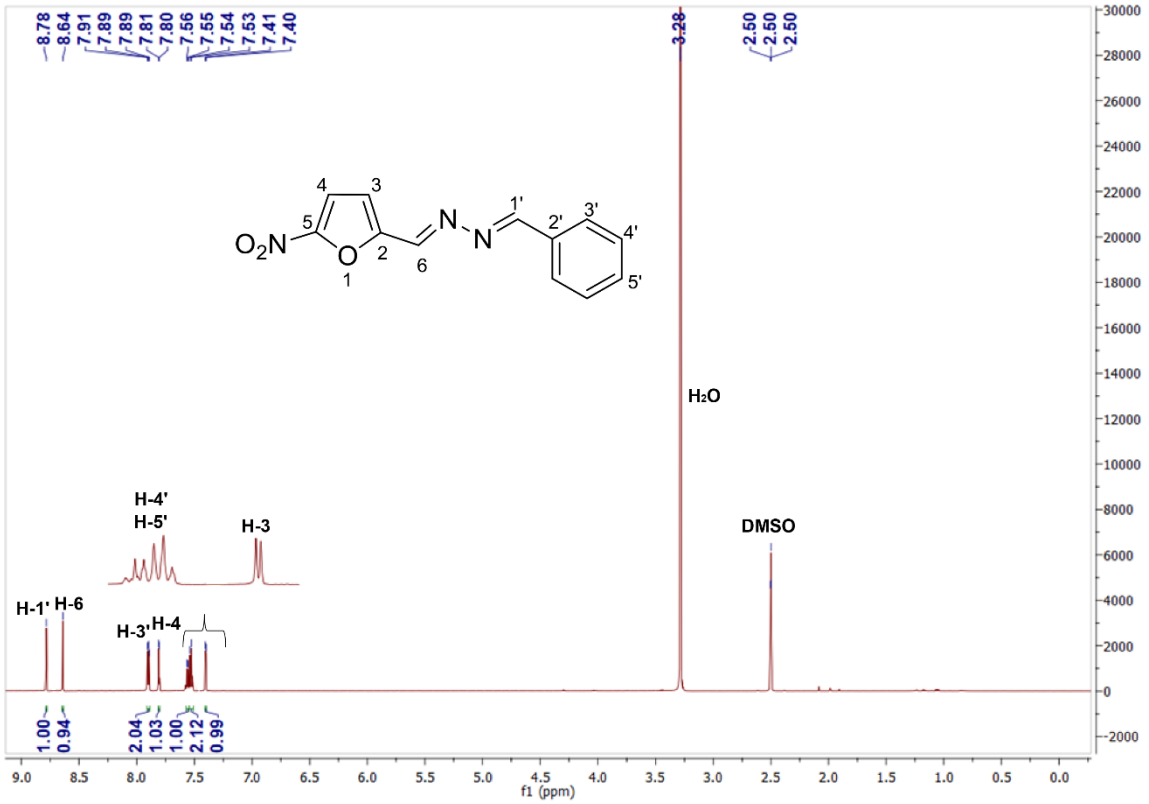


IR Spectrum

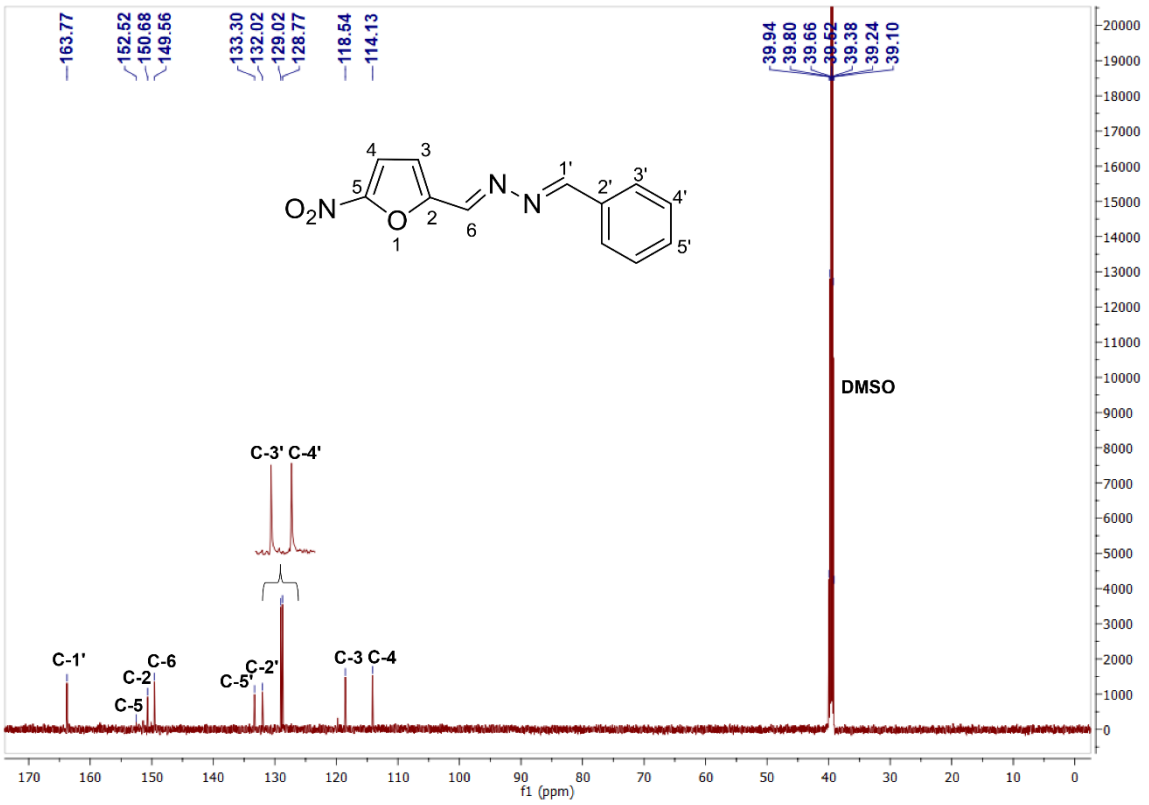

Transmittance [%]
80
70
60
50
N-H
C-H
C=C
N-O
O_2N
N
NH_2
3418.88
3282.51
3205.38
3064.38
2919.46
1571.92
1499.20
1317.65
1229.52
1168.14
1096.70
1077.51
916.44
837.40
743.84
698.28
611.80
528.18
483.77
3500
3000
2500
2000
1500
1000
500
Wavenumber cm-1

(1E,2E)-1-benzylidene-2-([5-nitrofuran-2-yl]methylene)hydrazine (1a)

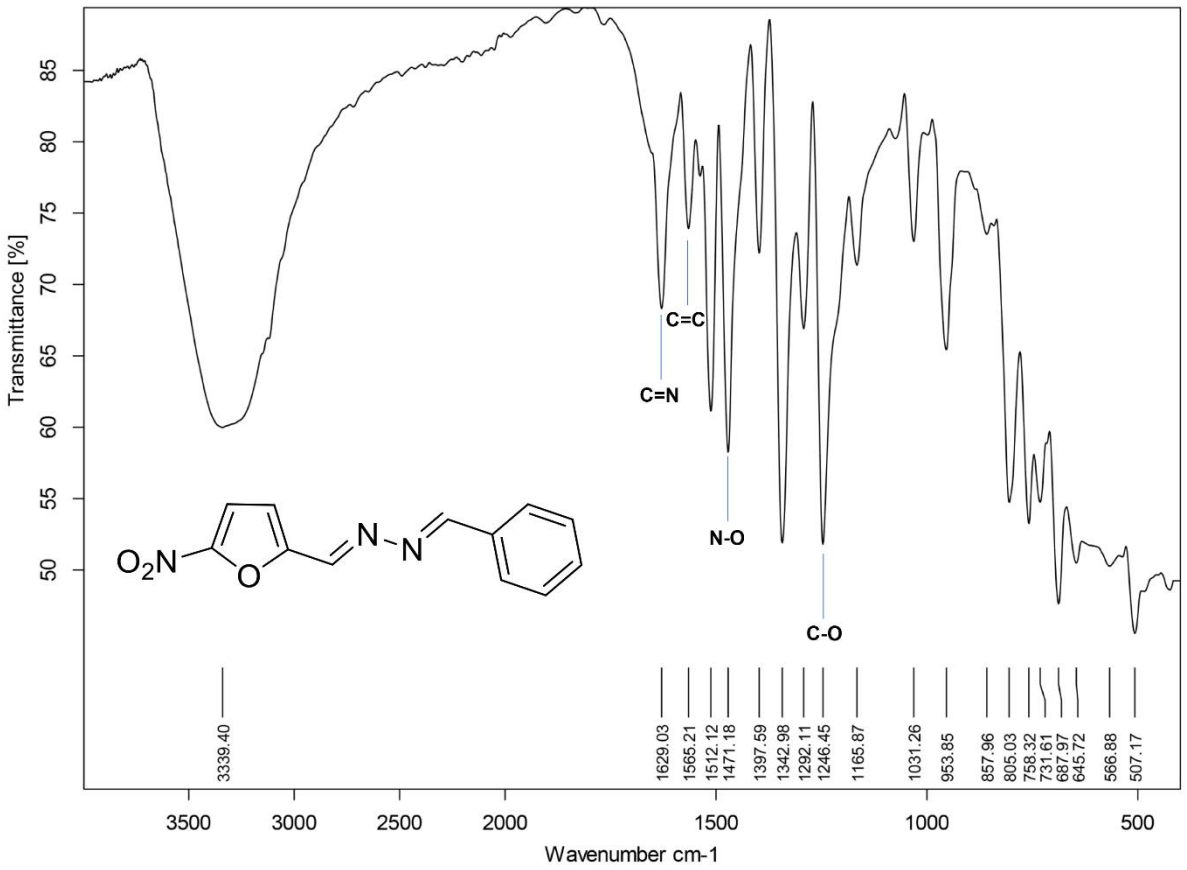
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

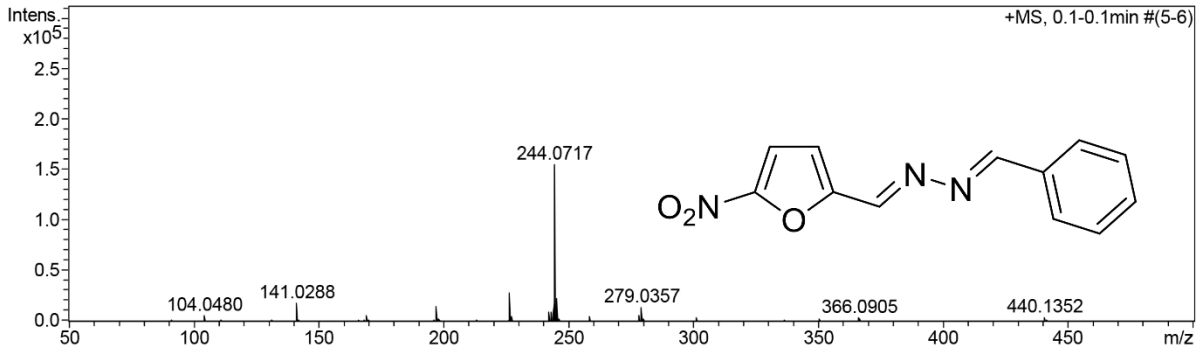
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000033.d	Acquisition Date	10/12/2020 3:33:00 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-1	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

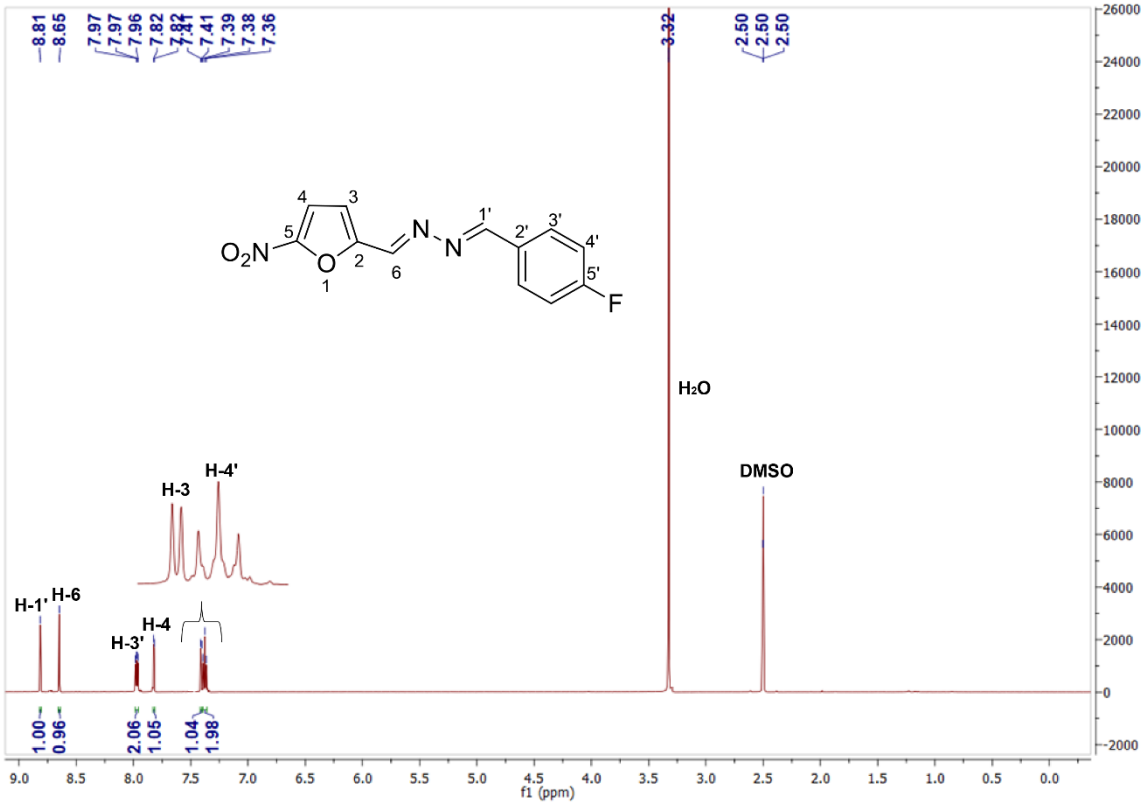
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



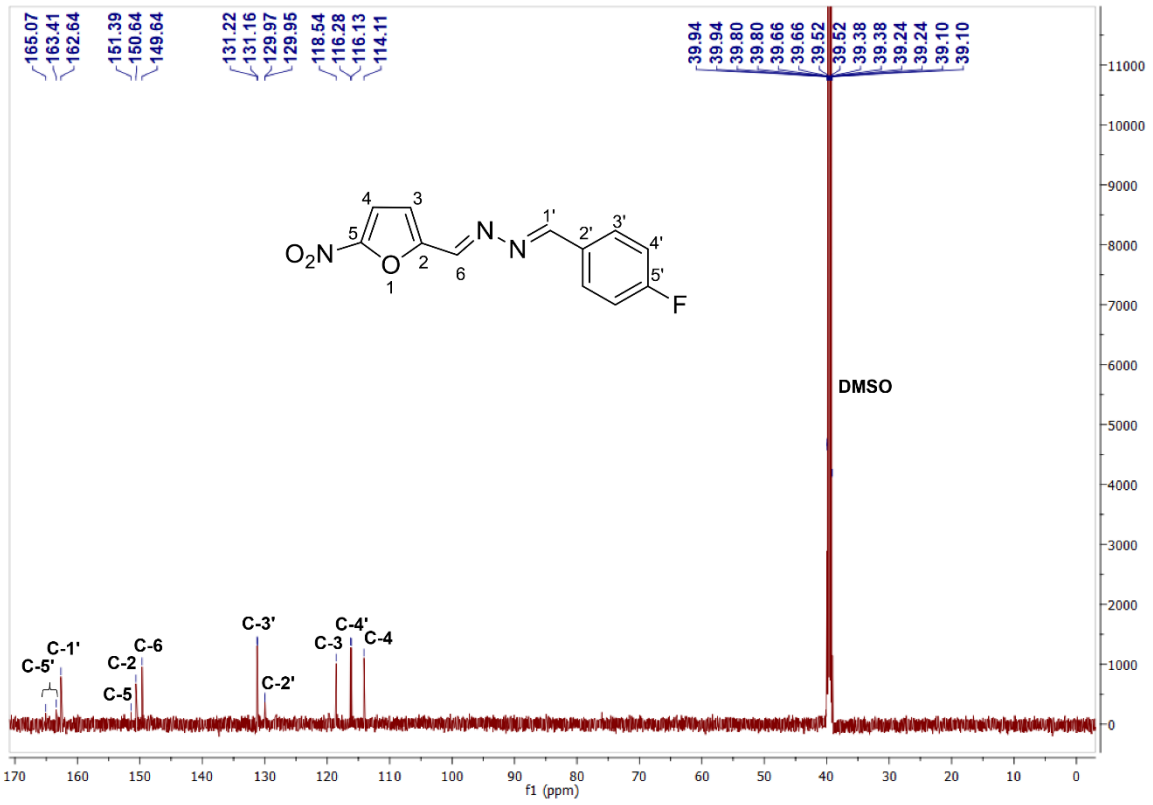
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
244.0717	1	C 12 H 10 N 3 O 3	100.00	244.0717	-0.1	-0.3	2.1	9.5	even	ok

(1E,2E)-1-(4-fluorobenzylidene)-2-([5-nitrofuran-2-yl]methylene)hydrazine (2a)

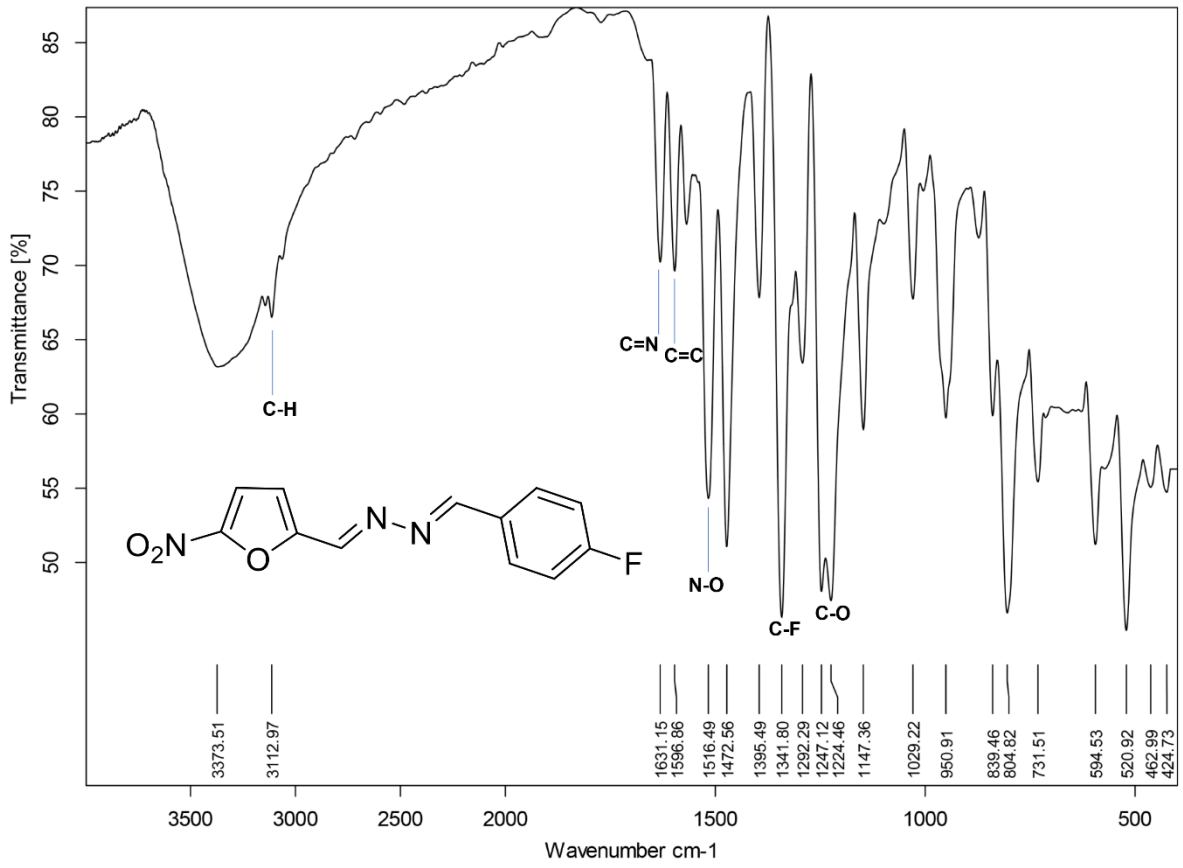
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

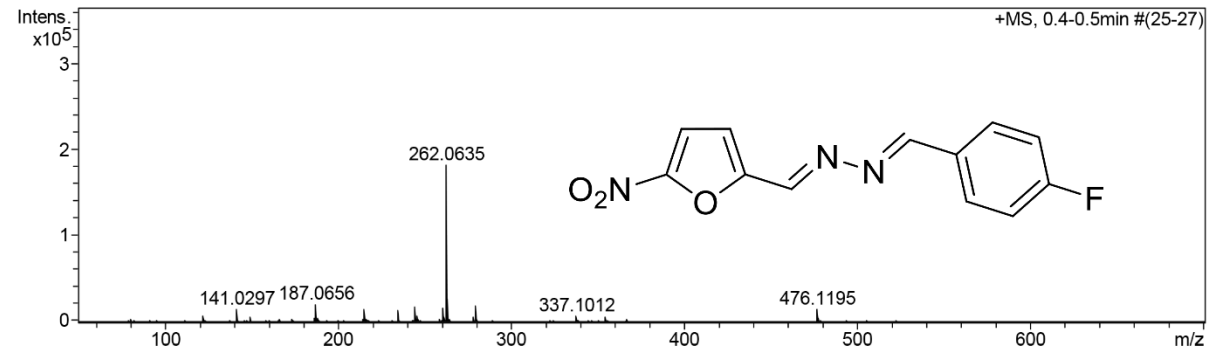
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000037.d	Acquisition Date	10/12/2020 3:37:28 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-7	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

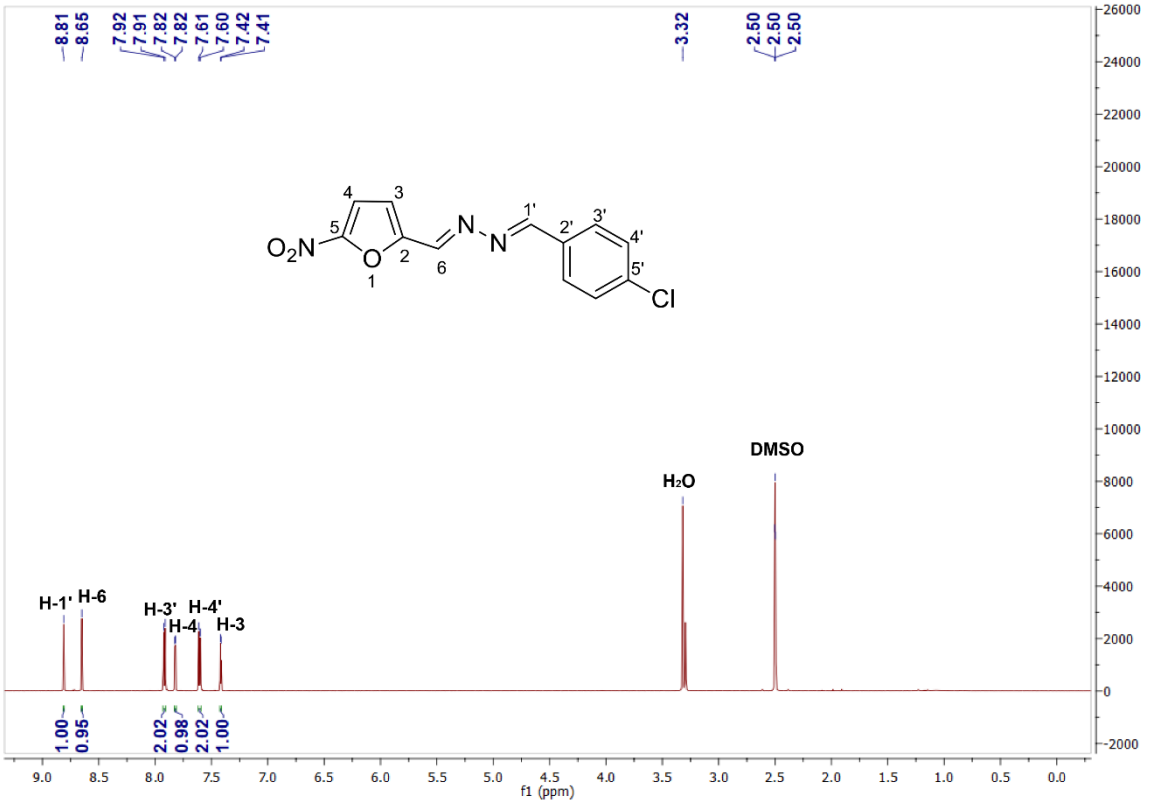
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



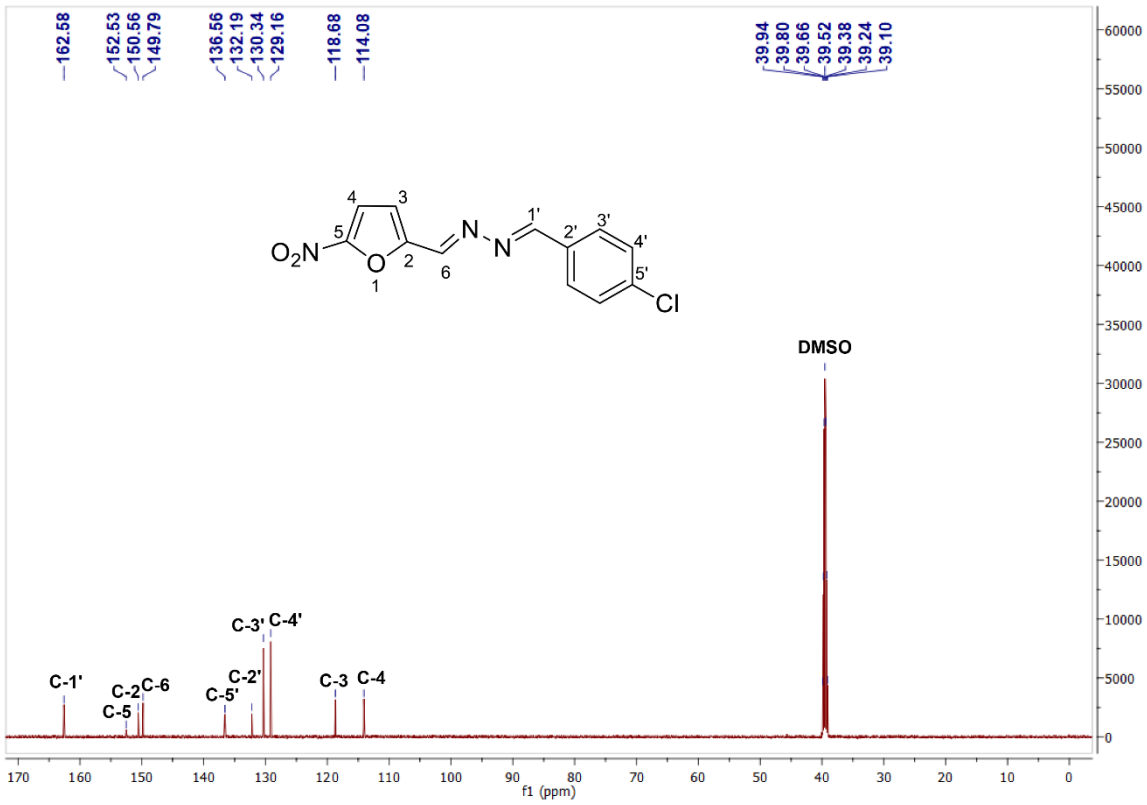
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
262.0635	1	C 12 H 9 F N 3 O 3	100.00	262.0622	-1.3	-5.0	1.2	9.5	even	ok

(1E,2E)-1-(4-chlorobenzylidene)-2-([5-nitrofuran-2-yl]methylene)hydrazine (3a)

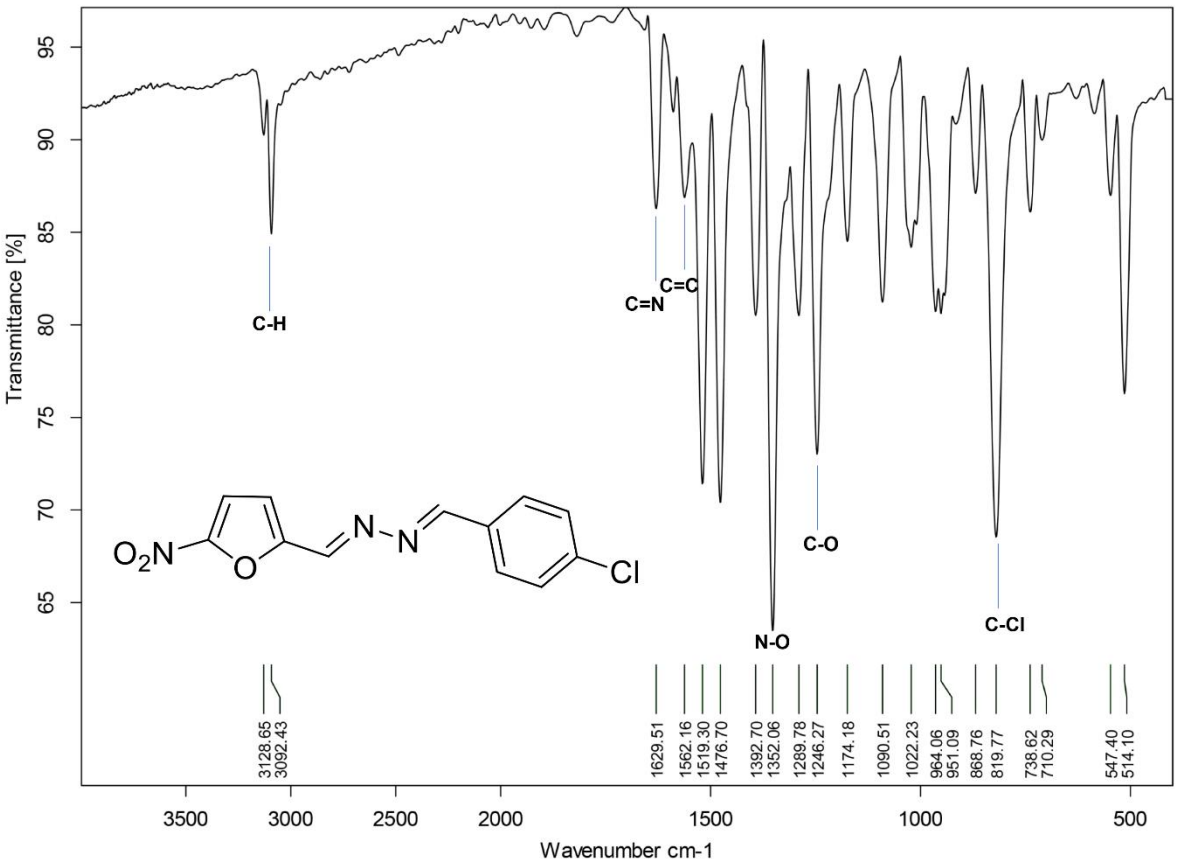
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR spectrum



HRMS

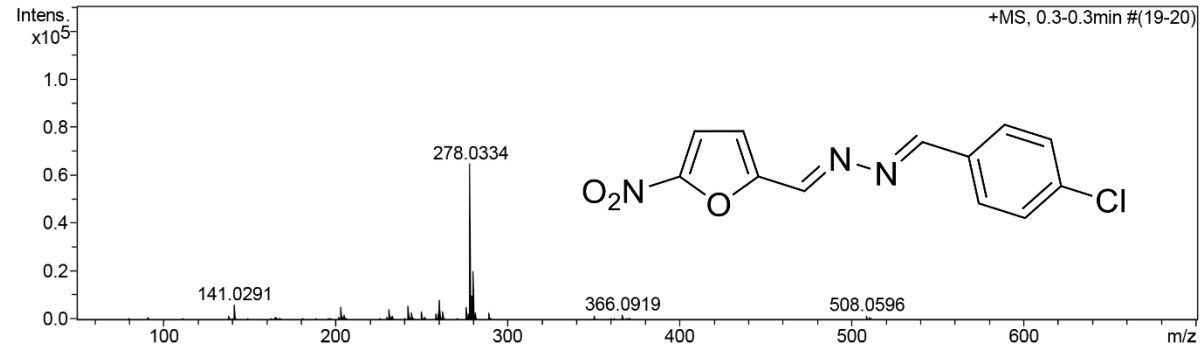
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000035.d	Acquisition Date	10/12/2020 3:35:23 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-3	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

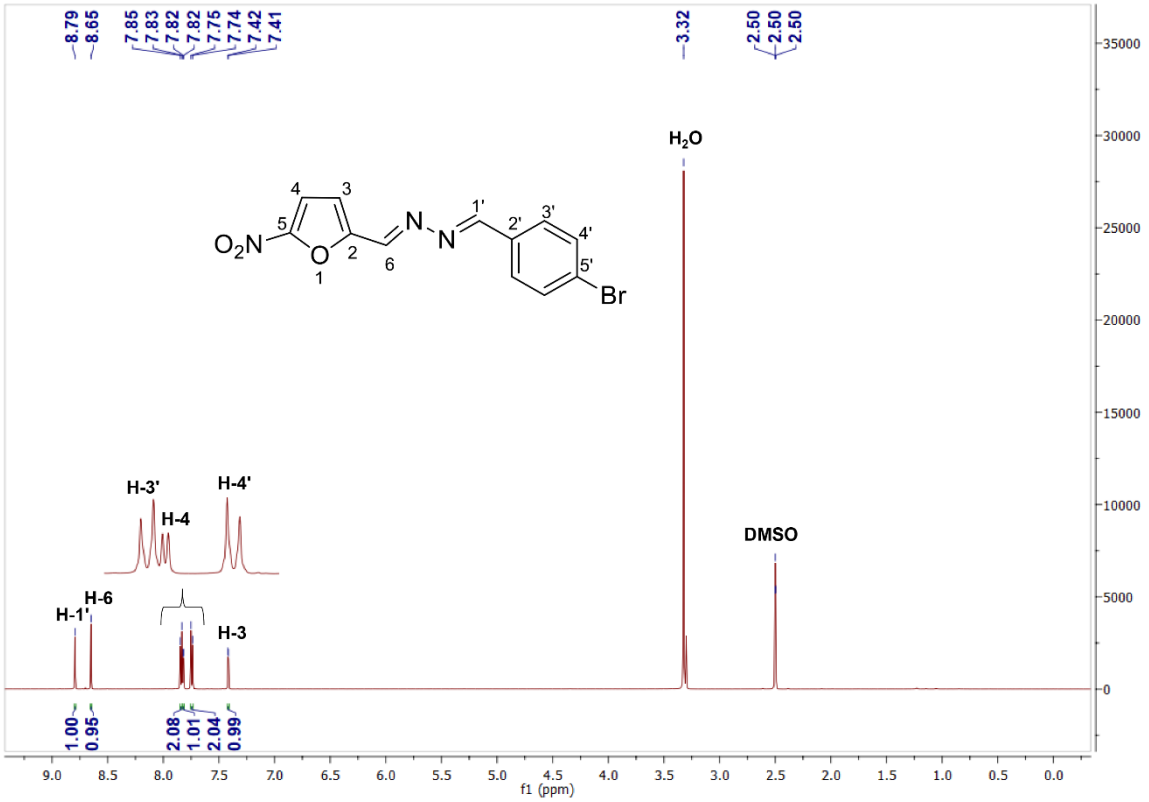
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



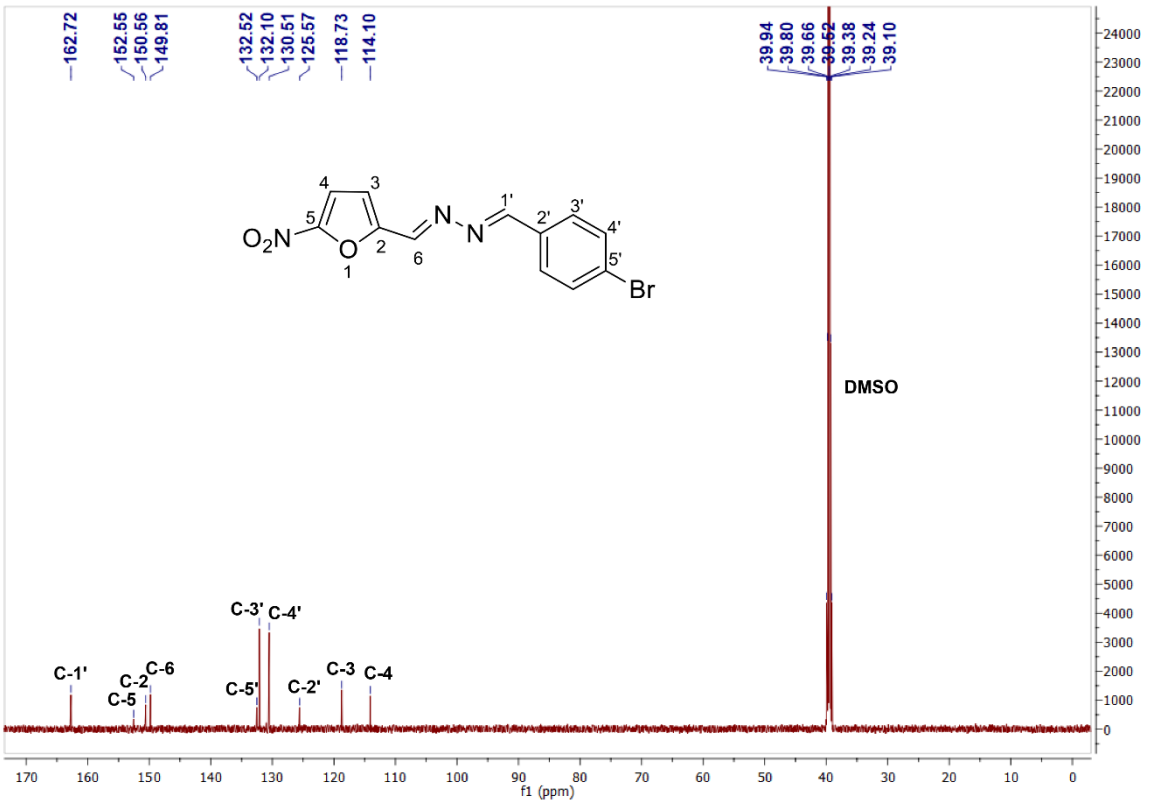
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
278.0334	1	C 12 H 9 Cl N 3 O 3	100.00	278.0327	-0.7	-2.5	12.6	9.5	even	ok

(1E,2E)-1-(4-bromobenzylidene)-2-([5-nitrofuran-2-yl]methylene)hydrazine (4a)

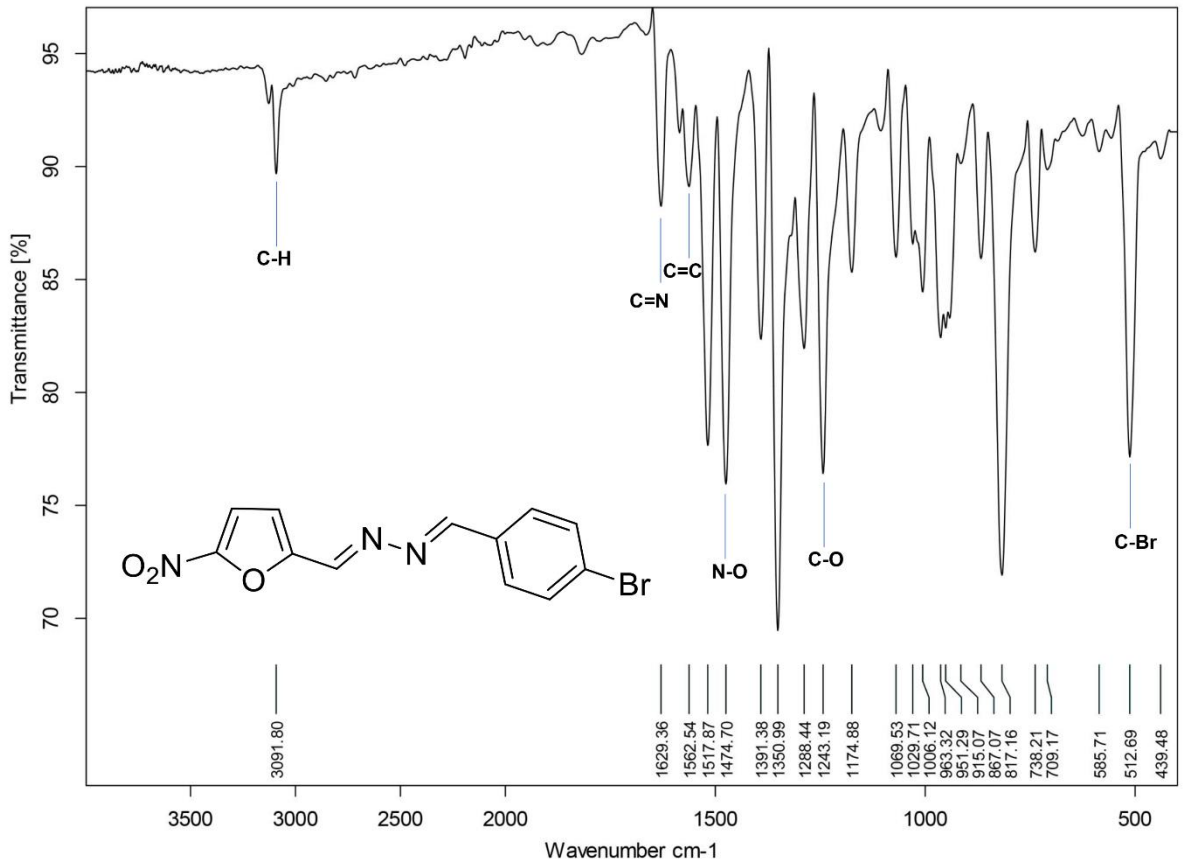
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

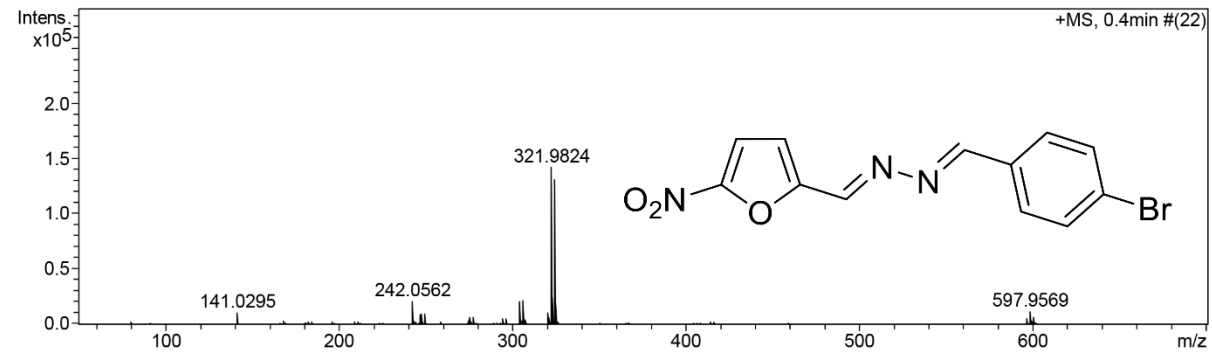
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000036.d	Acquisition Date	10/12/2020 3:36:35 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-4	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

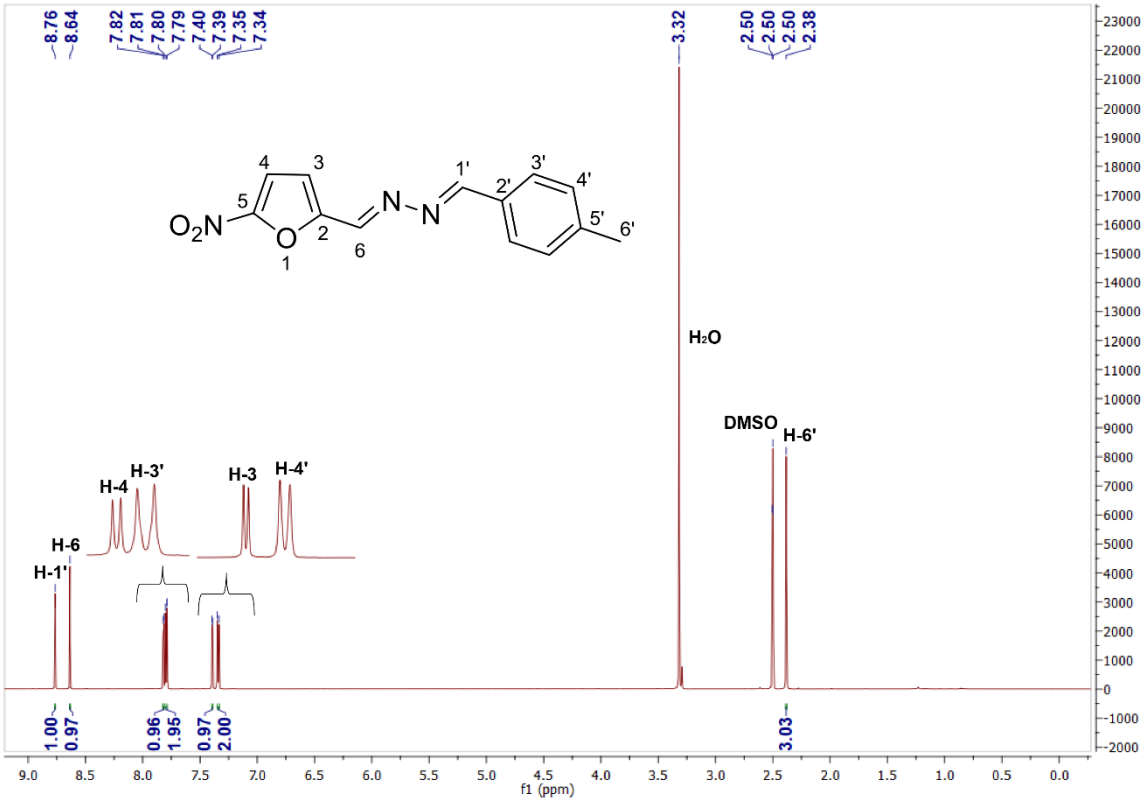
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



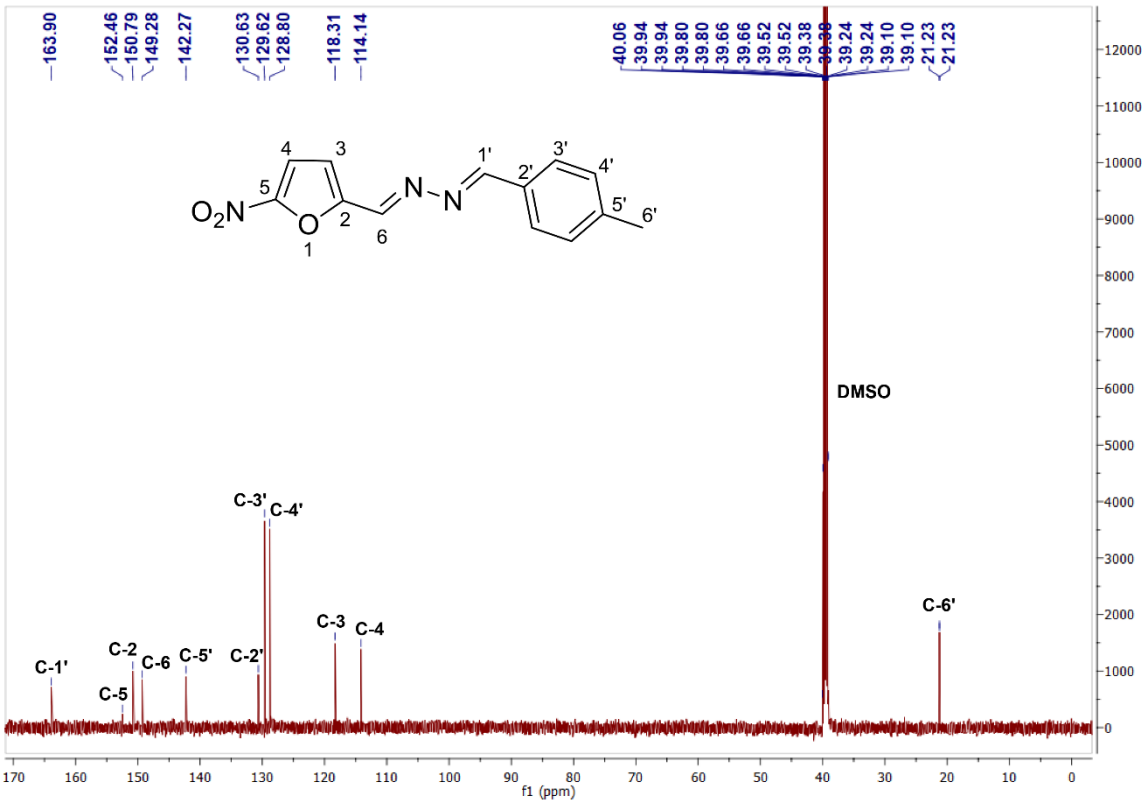
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
321.9824	1	C 12 H 9 Br N 3 O 3	100.00	321.9822	-0.3	-0.8	32.5	9.5	even	ok

(1E,2E)-1-(4-methylbenzylidene)-2-([5-nitrofuran-2-yl]methylene)hydrazine (5a)

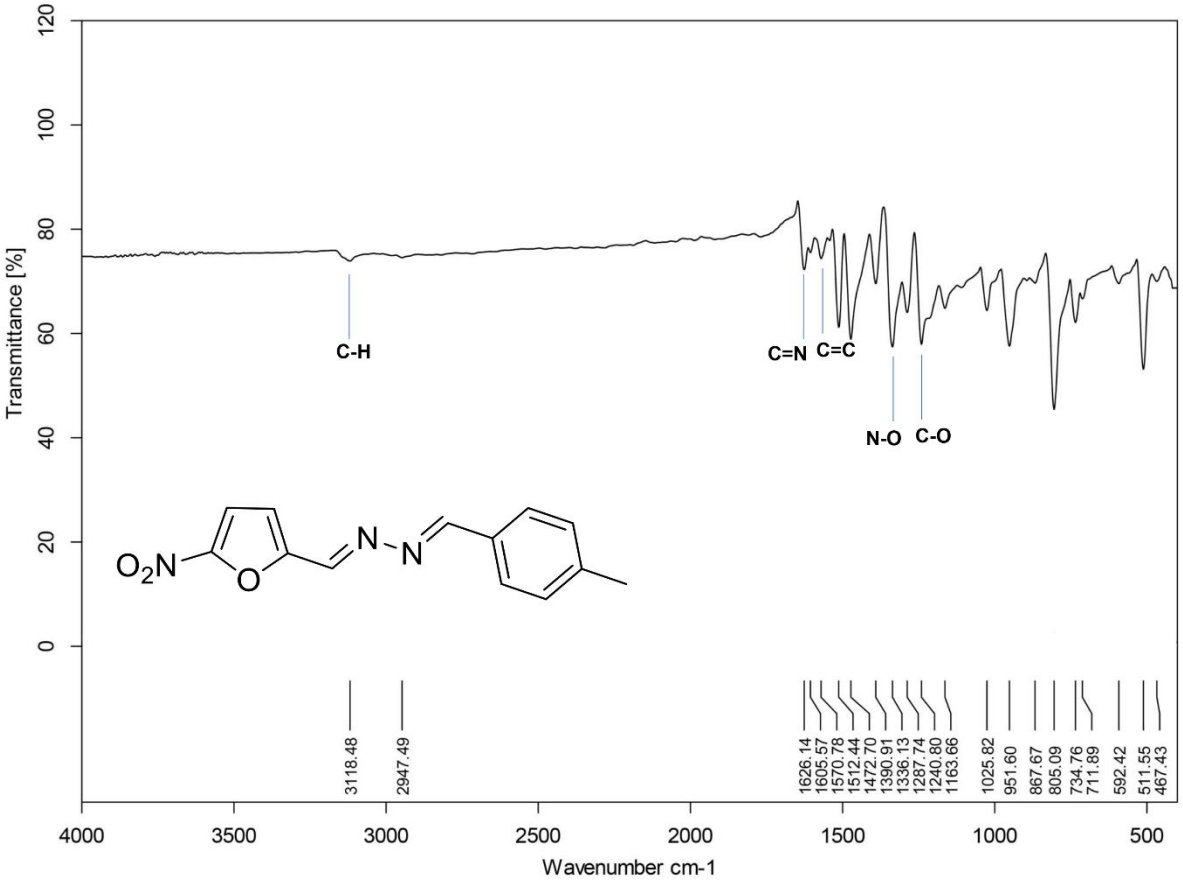
^{1}H NMR in DMSO



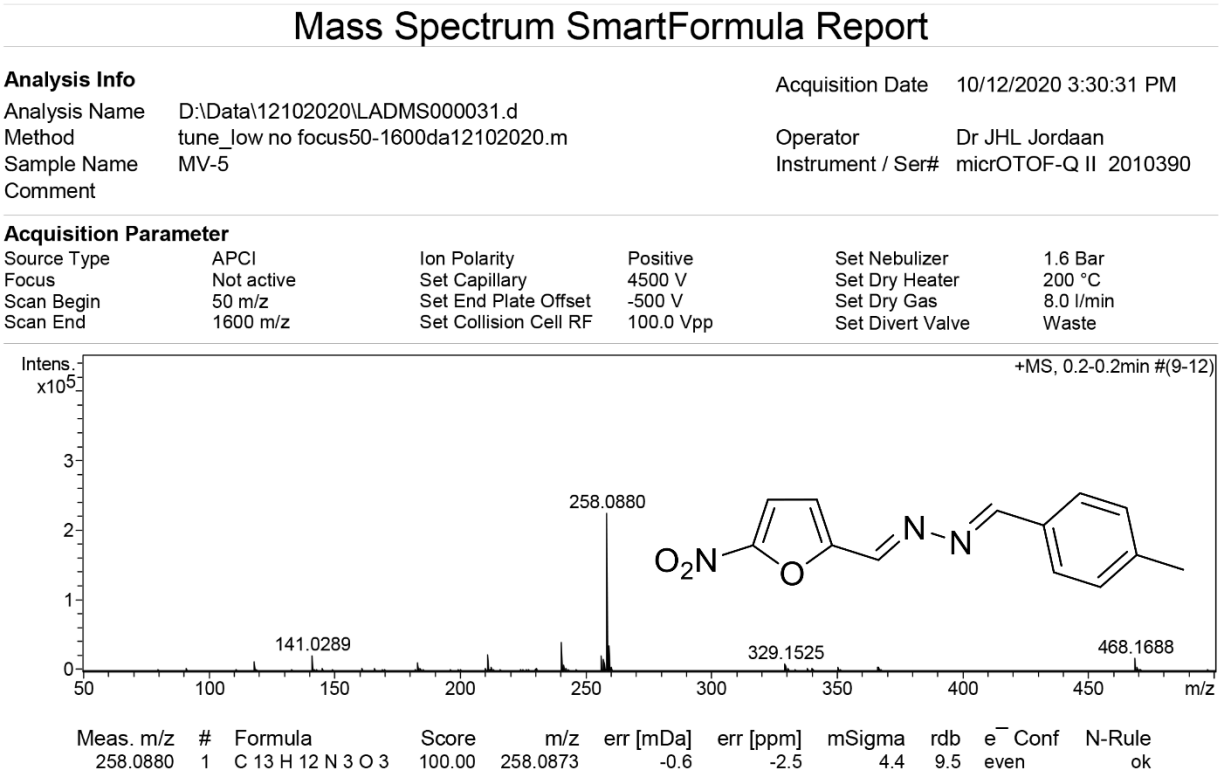
^{13}C NMR in DMSO



IR Spectrum

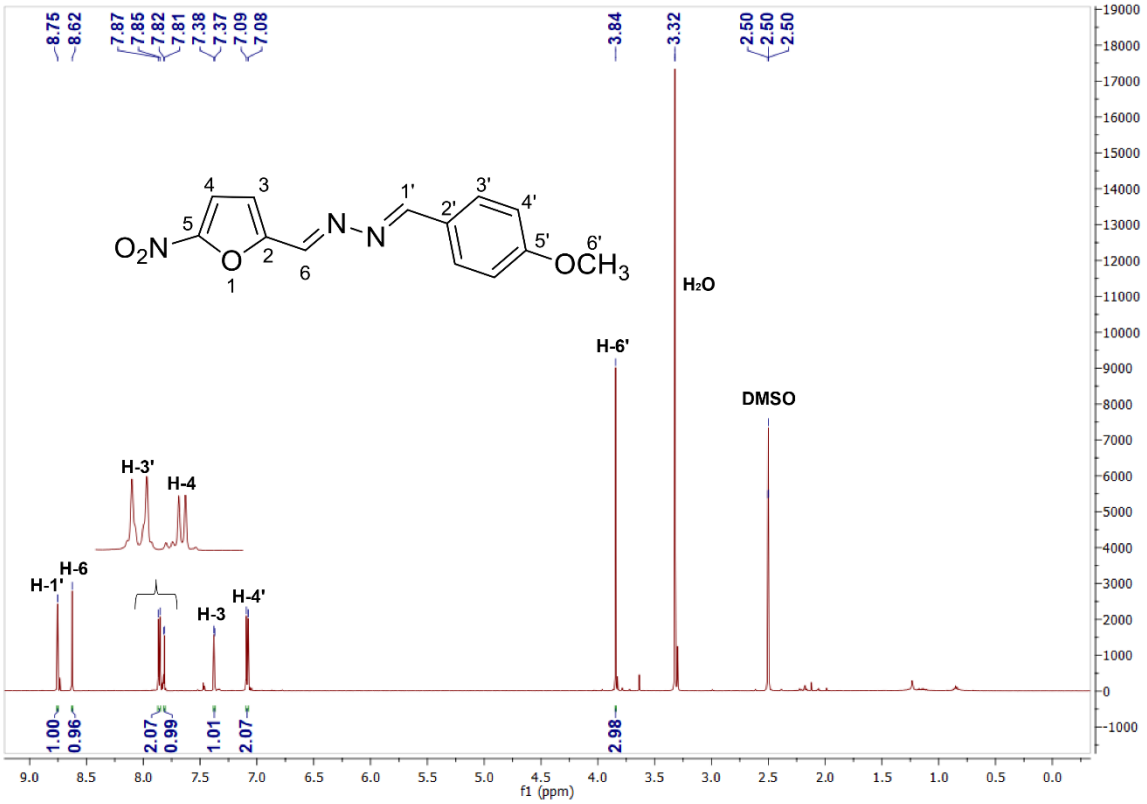


HRMS

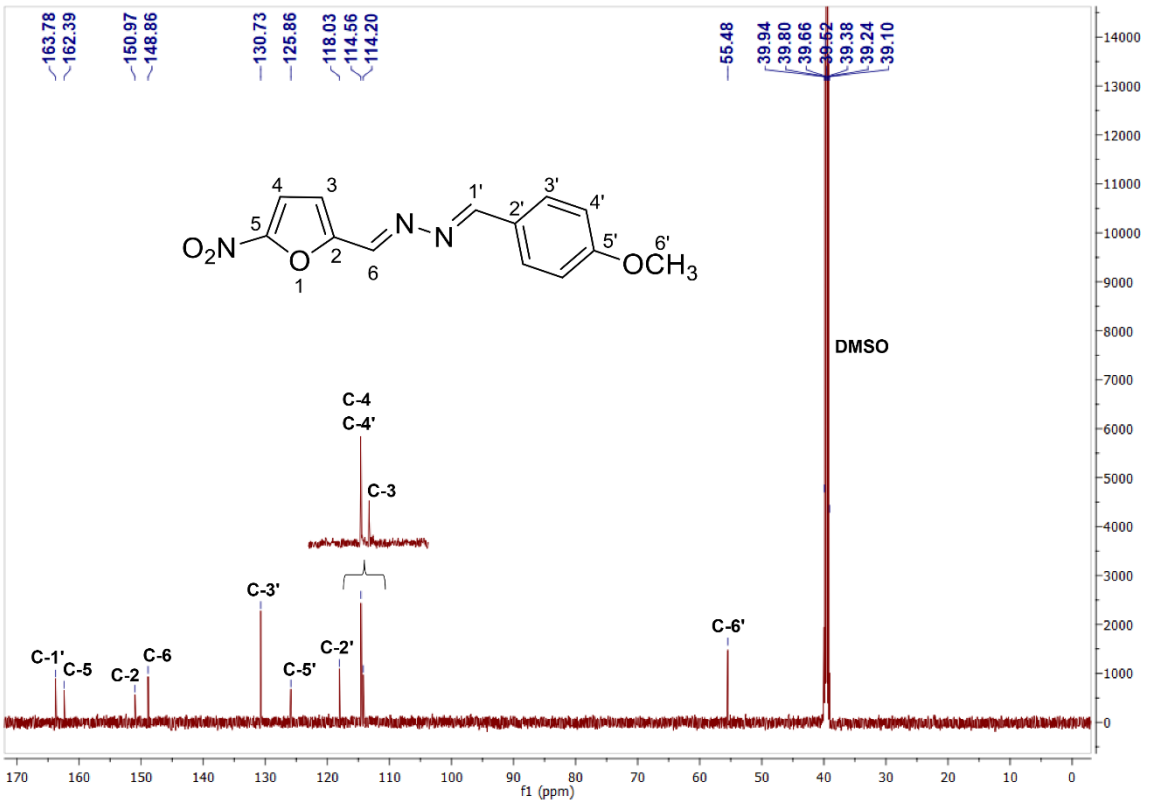


(1E,2E)-1-(4-methoxybenzylidene)-2-([5-nitrofuran-2-yl]methylene)hydrazine (6a)

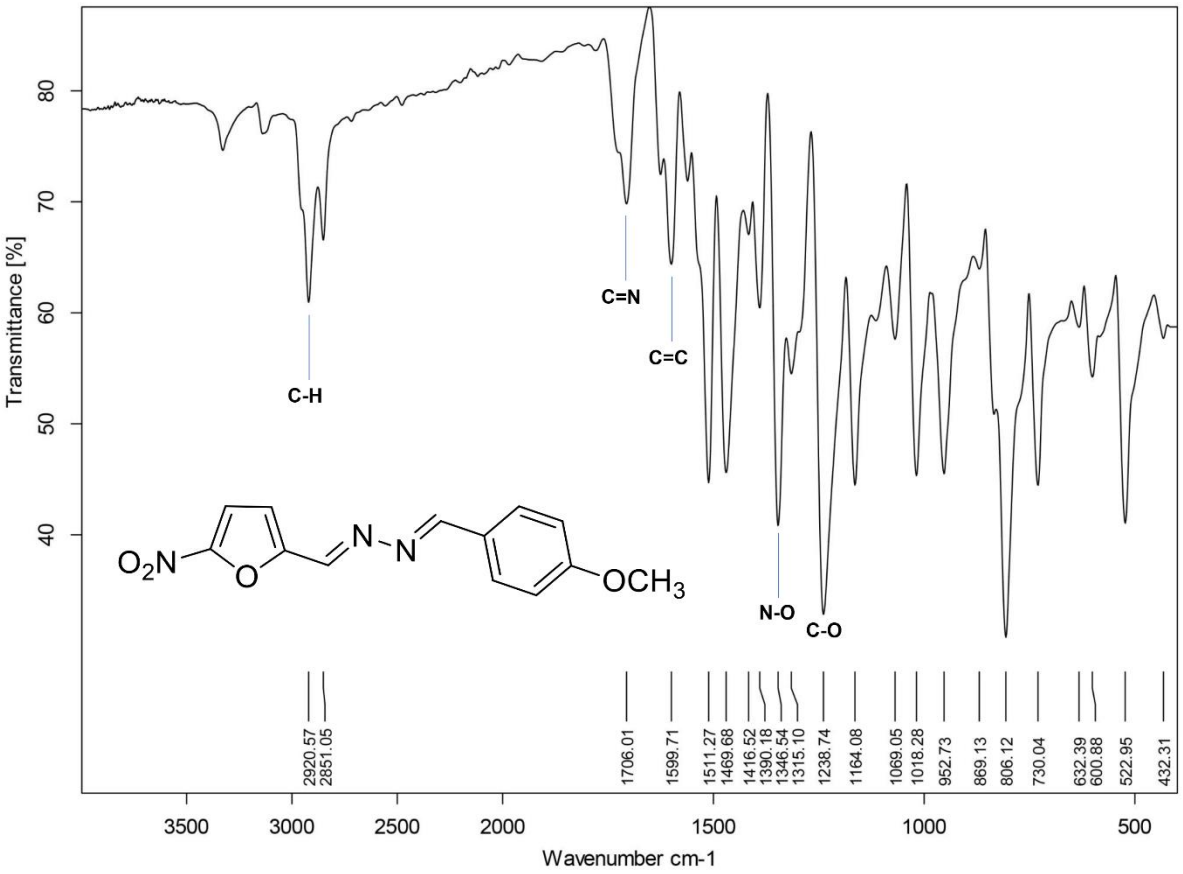
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

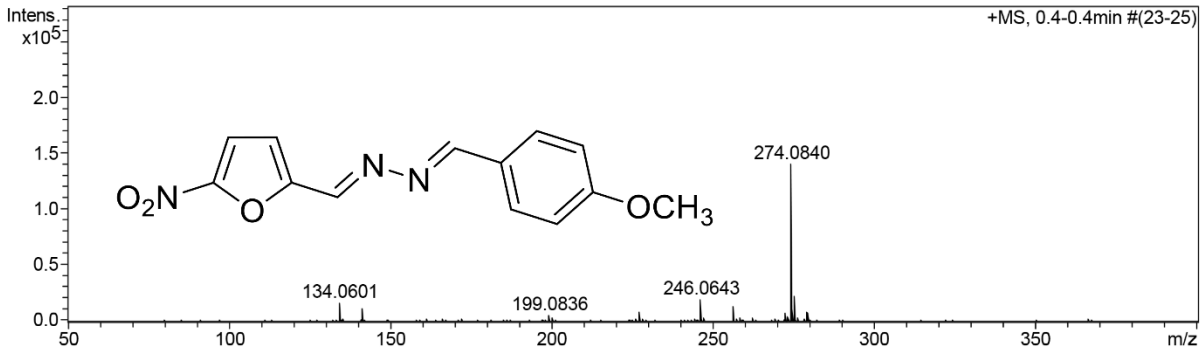
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000038.d	Acquisition Date	10/12/2020 3:38:40 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-6	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

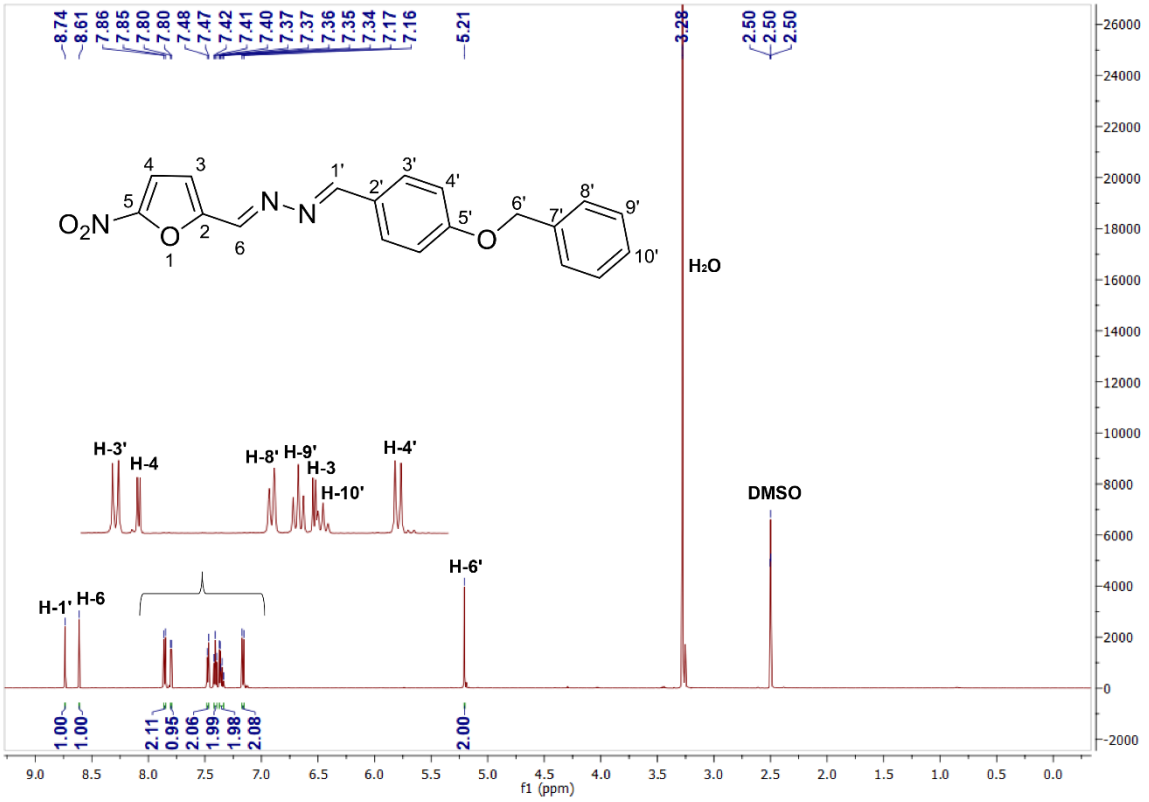
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



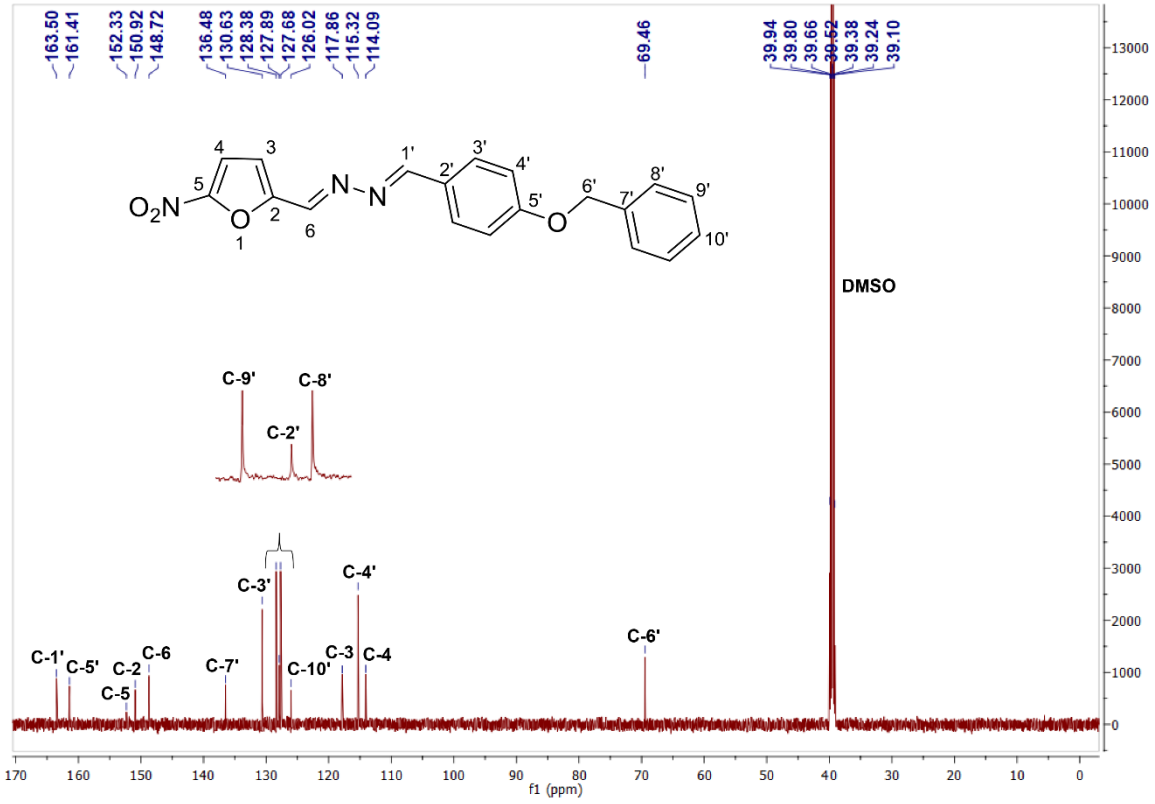
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
274.0840	1	C 13 H 12 N 3 O 4	100.00	274.0822	-1.8	-6.5	2.0	9.5	even	ok

(1E,2E)-1-(4-[benzyloxy]benzylidene)-2-([5-nitrofuran-2-yl]methylene)hydrazine (7a)

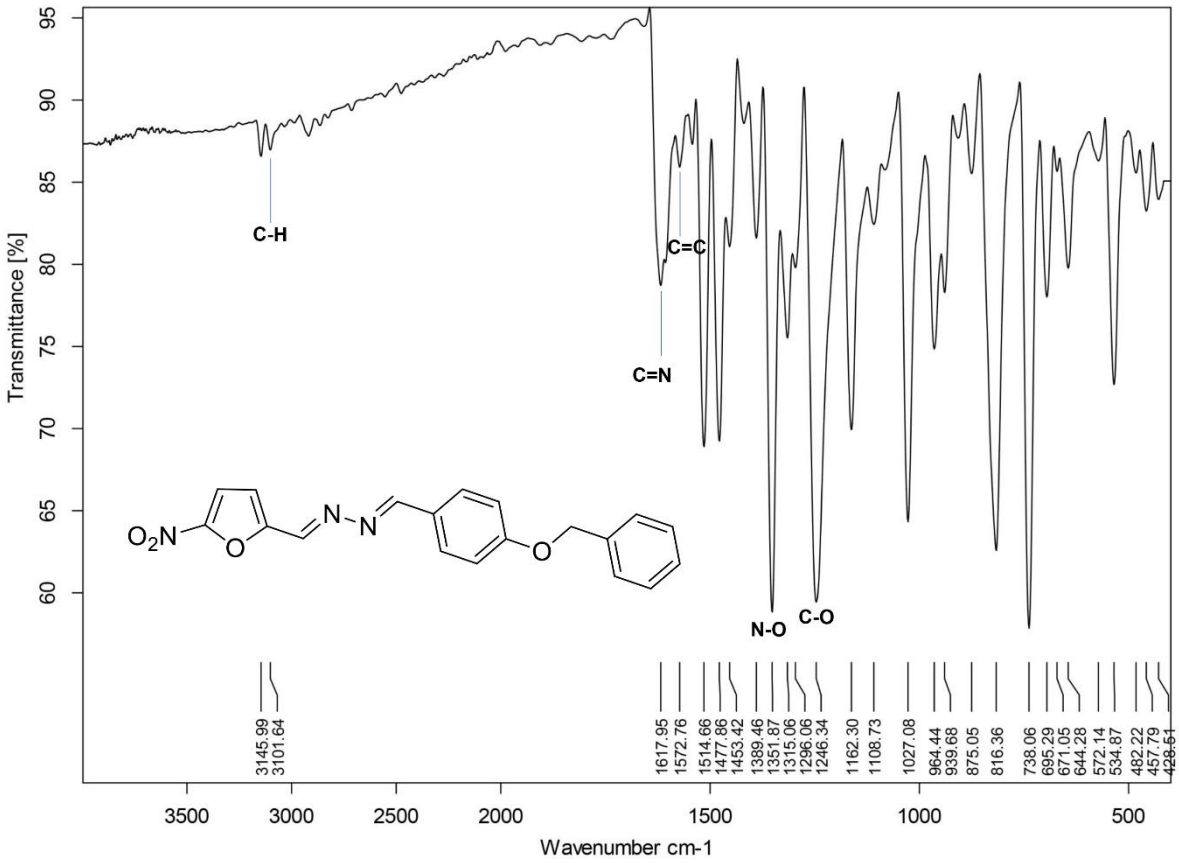
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

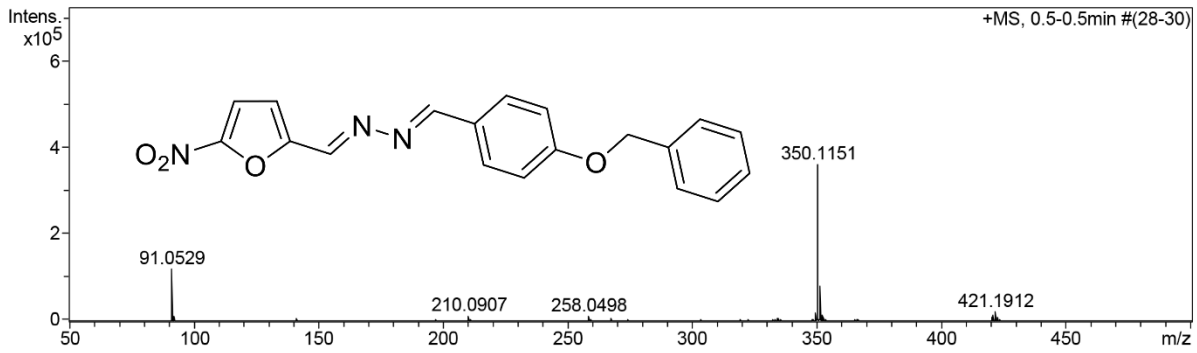
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000028.d	Acquisition Date	10/12/2020 3:26:16 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-09	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

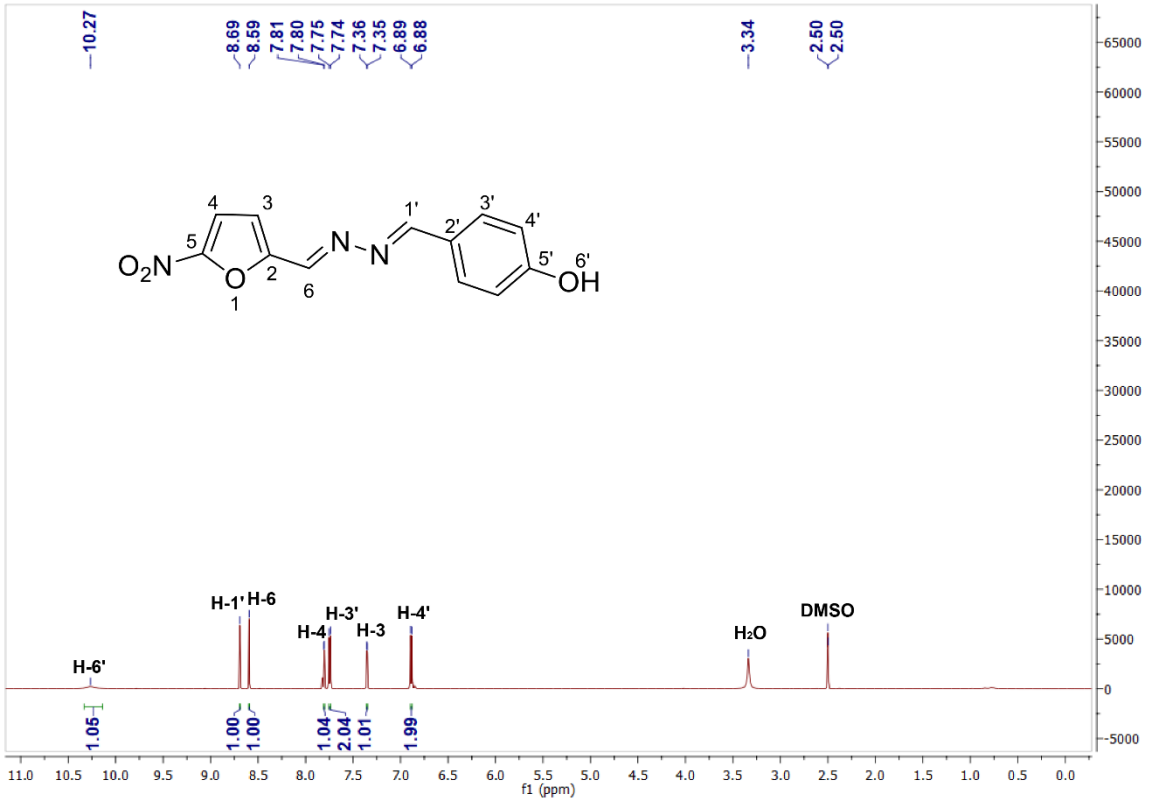
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



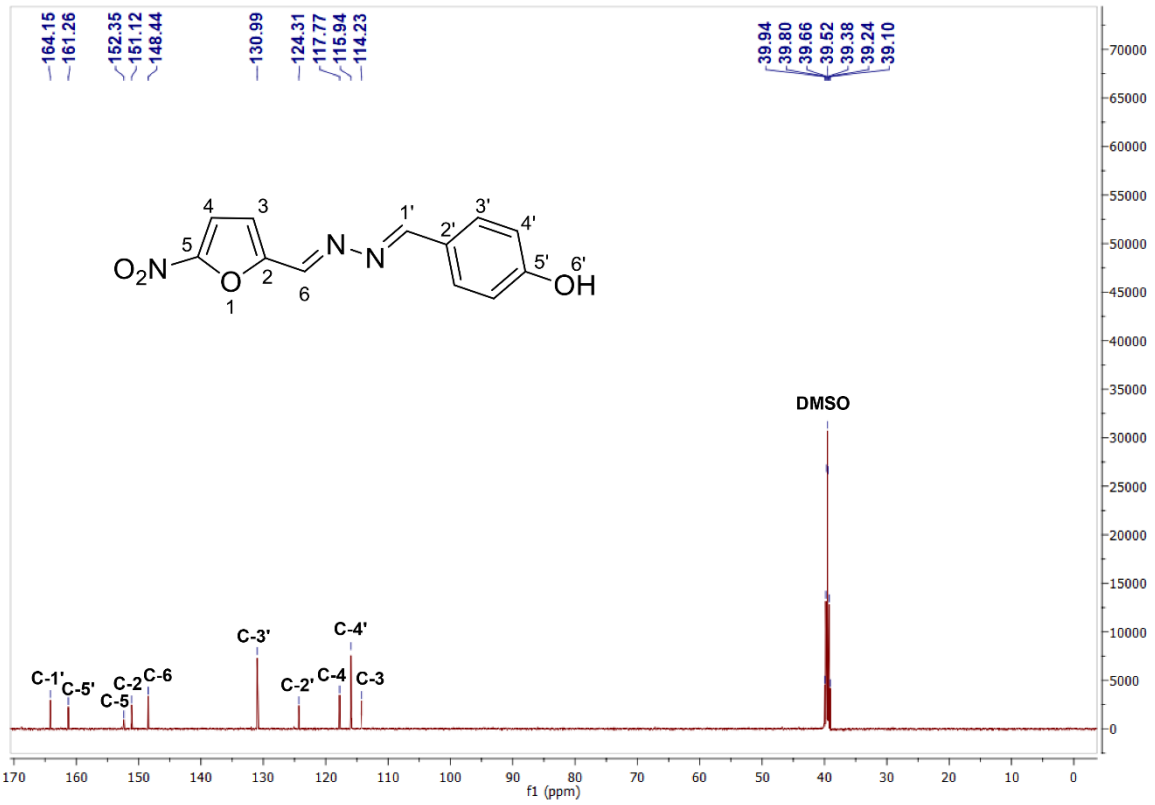
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
91.0529	1	C 7 H 7	100.00	91.0542	1.4	15.1	1.9	4.5	even	ok
350.1151	1	C 19 H 16 N 3 O 4	100.00	350.1135	-1.6	-4.5	2.3	13.5	even	ok

4-(E)-(E)-([5-nitrofuran-2-yl]methylene)hydrazonomethylphenol (8a)

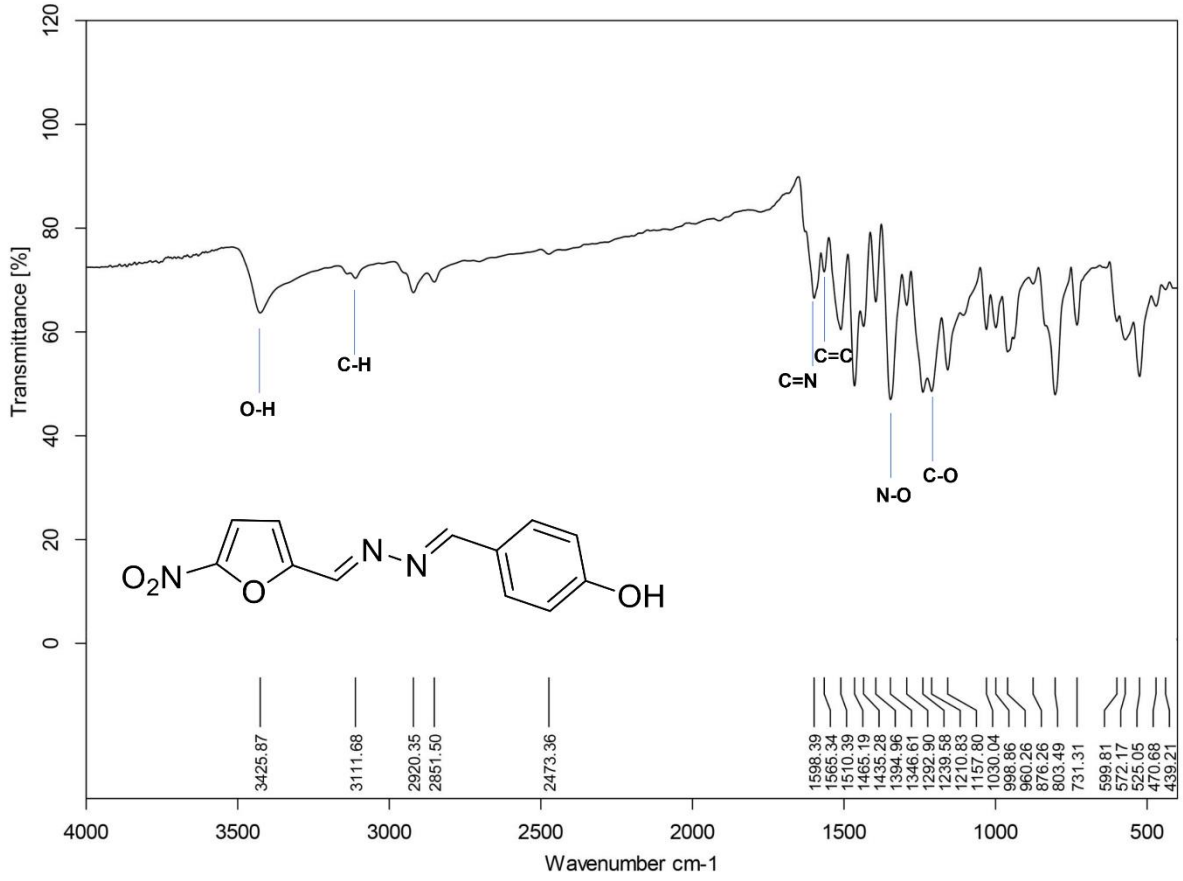
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

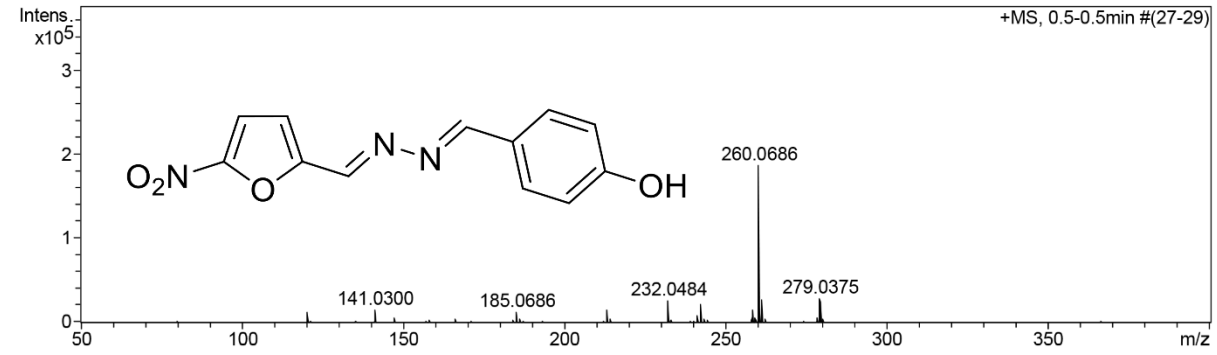
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000039.d	Acquisition Date	10/12/2020 3:39:46 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-8	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

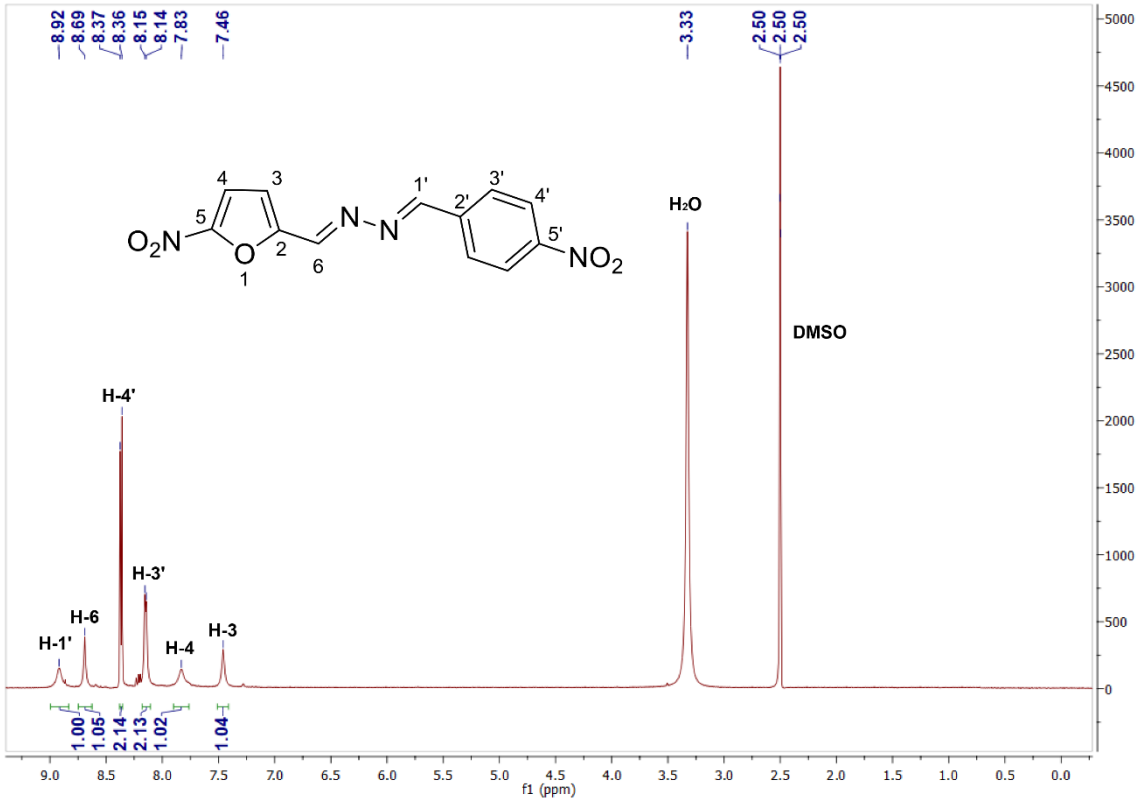
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



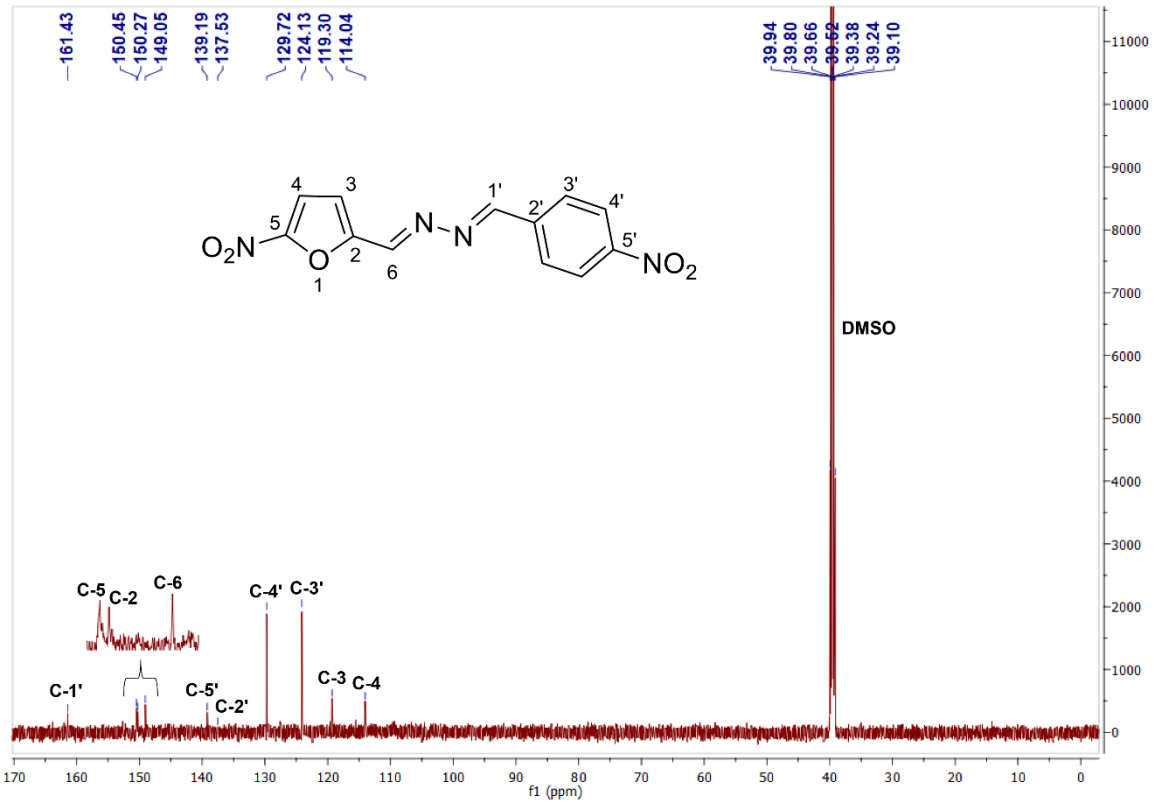
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
260.0686	1	C 12 H 10 N 3 O 4	100.00	260.0666	-2.0	-7.6	6.3	9.5	even	ok

(1E,2E)-1-(4-nitrobenzylidene)-2-([5-nitrofuran-2-yl]methylene)hydrazine (9a)

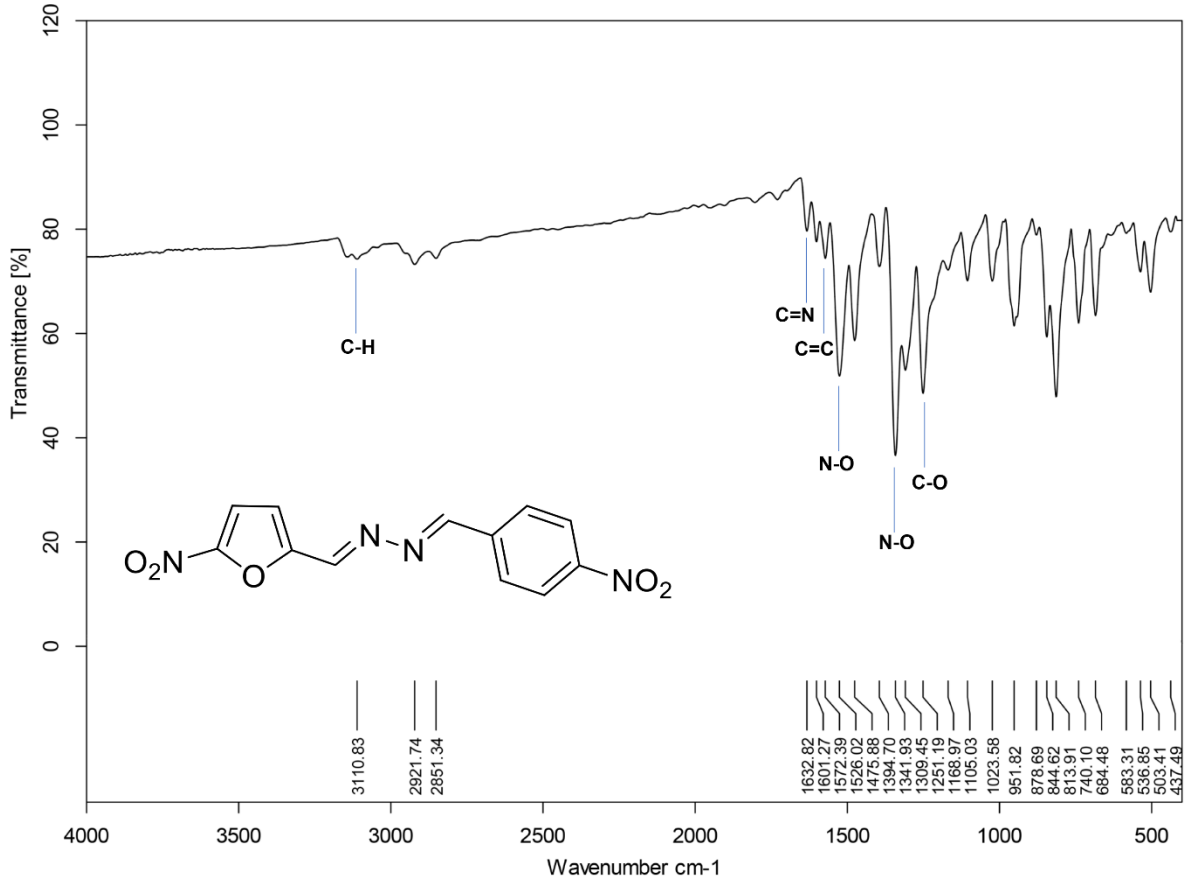
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

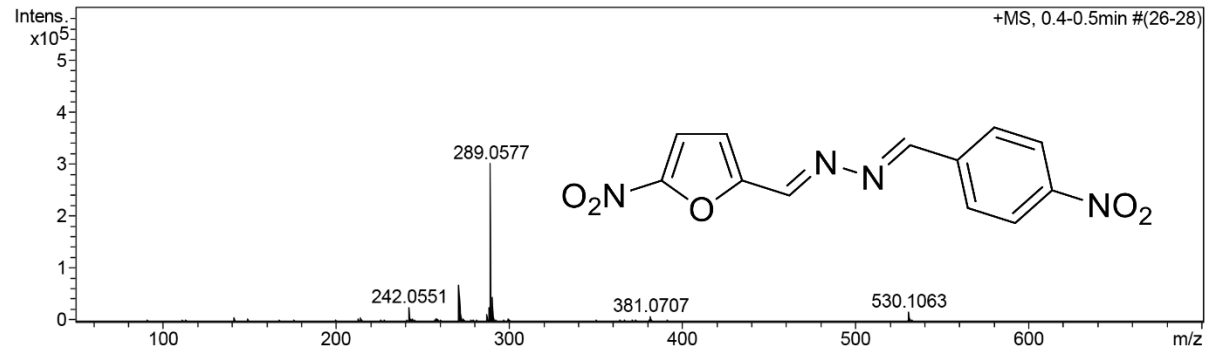
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000034.d	Acquisition Date	10/12/2020 3:34:00 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-2	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

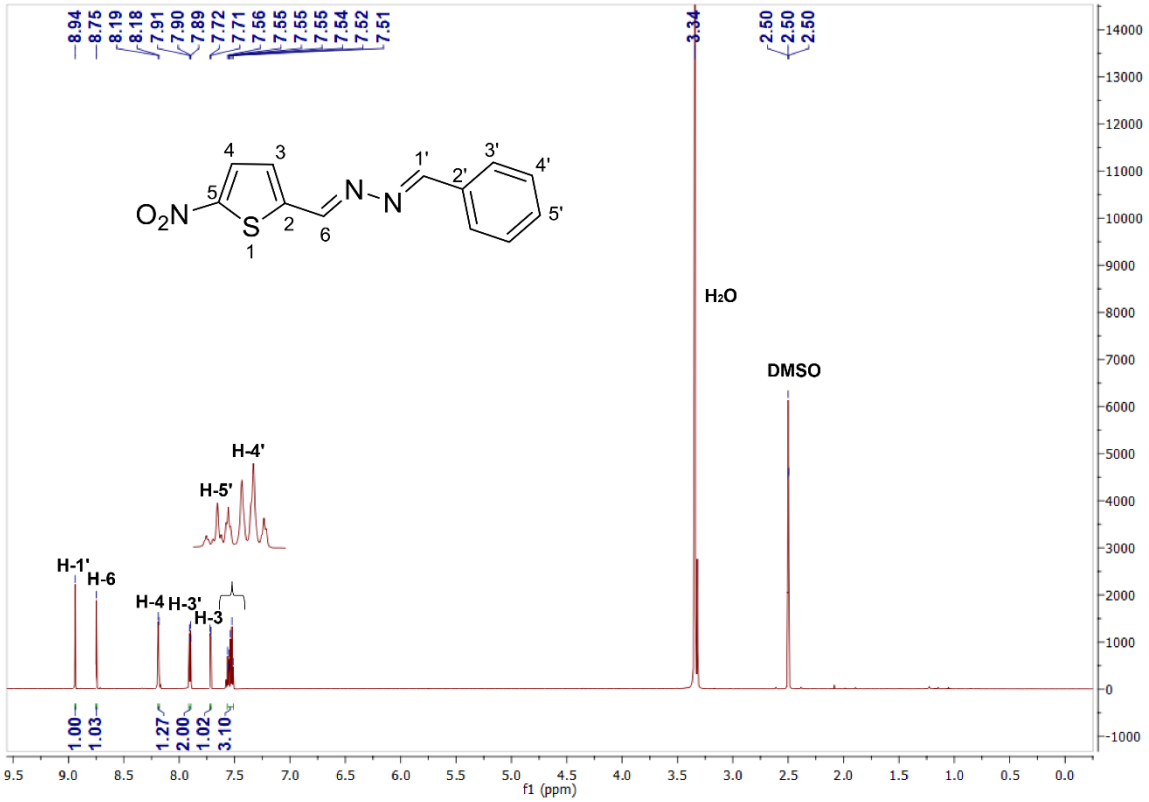
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



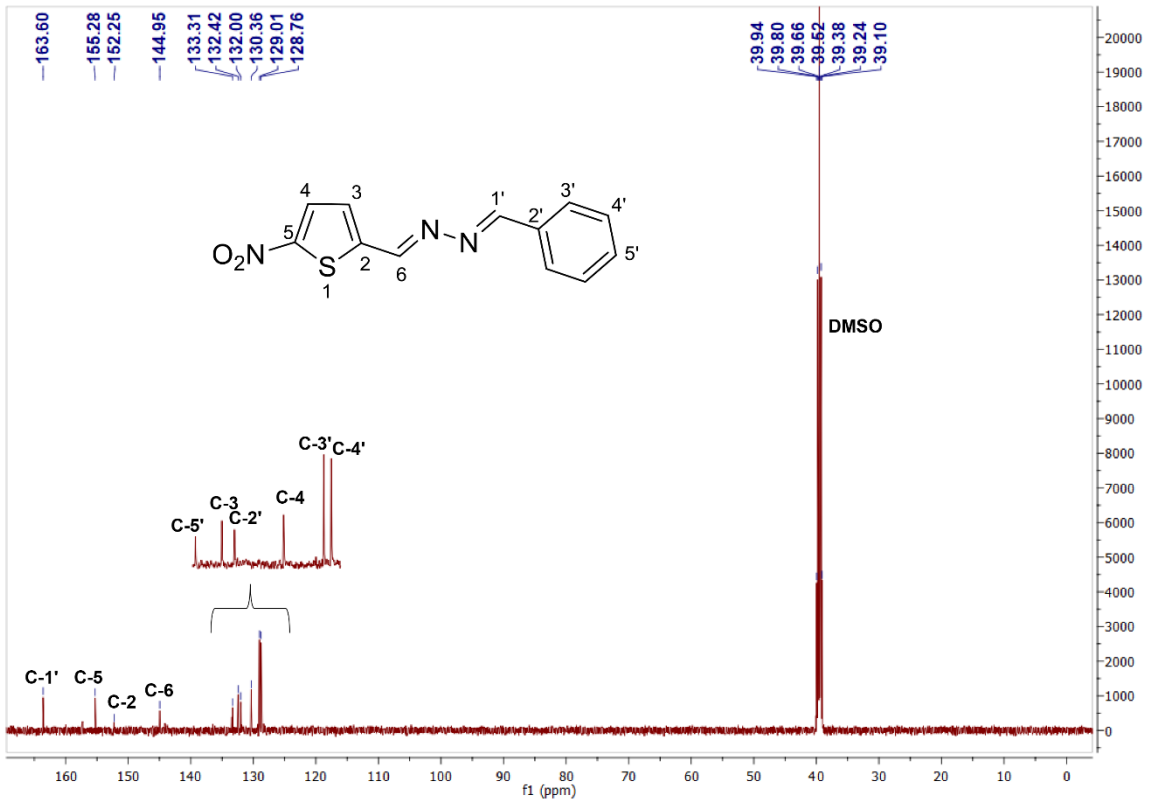
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
271.0462	1	C 12 H 7 N 4 O 4	100.00	271.0462	0.0	0.1	32.4	11.5	even	ok
289.0577	1	C 12 H 9 N 4 O 5	100.00	289.0567	-0.9	-3.2	2.1	10.5	even	ok

(1E,2E)-1-benzylidene-2-([5-nitrothiophen-2-yl]methylene)hydrazine (1b)

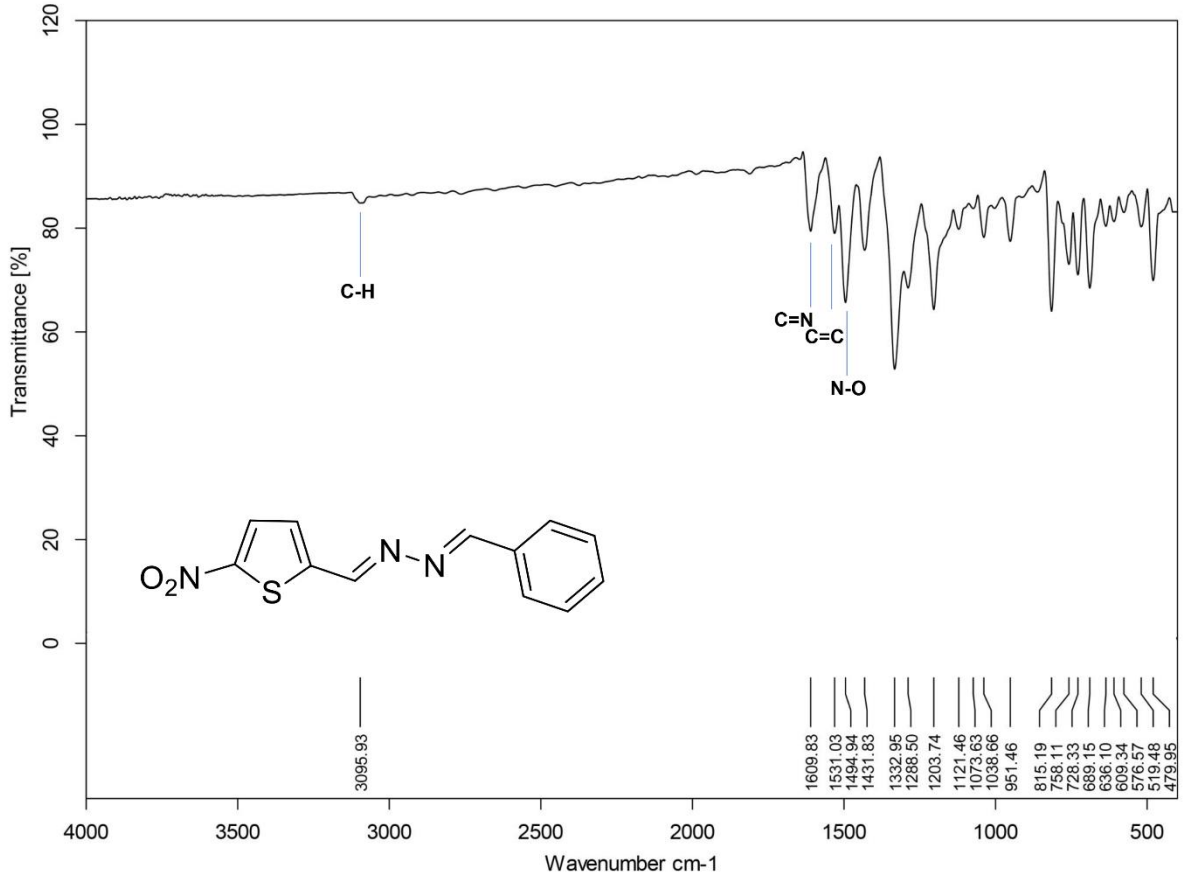
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

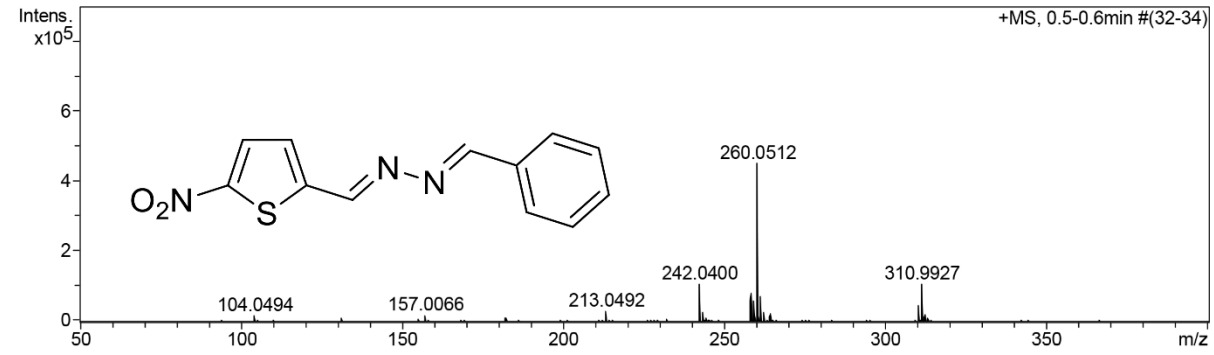
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000040.d	Acquisition Date	10/12/2020 3:41:12 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-10	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

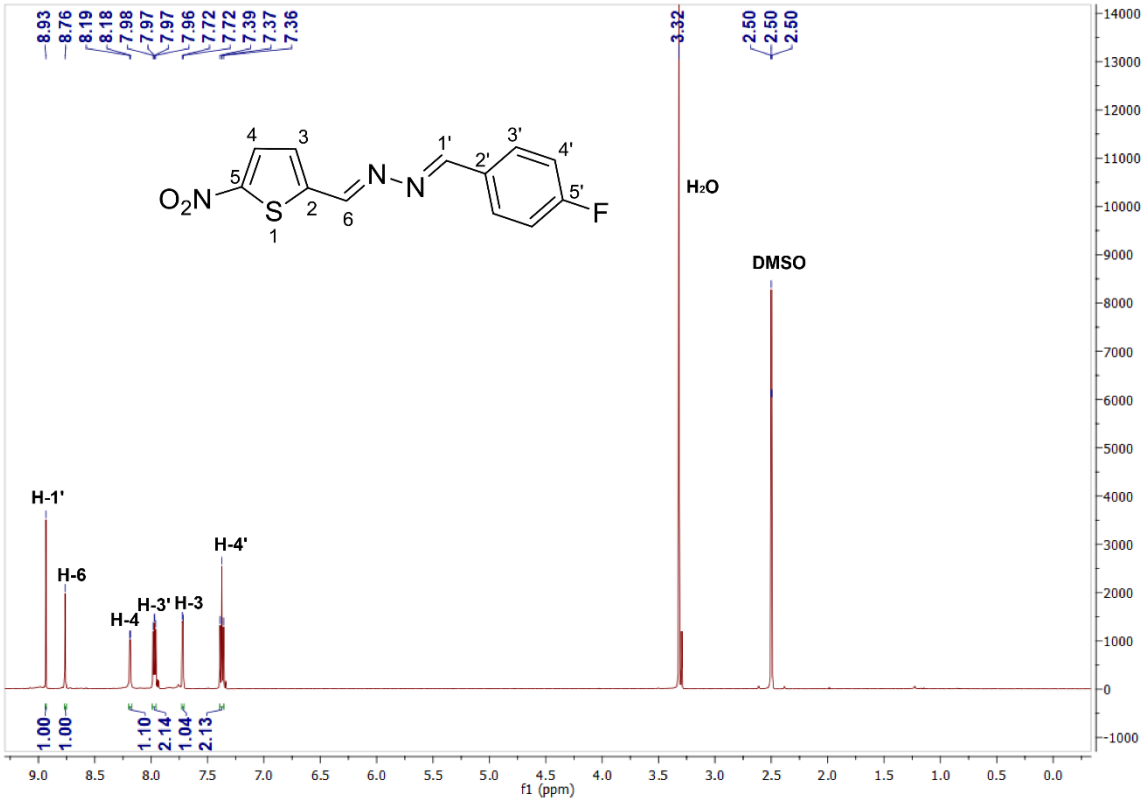
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



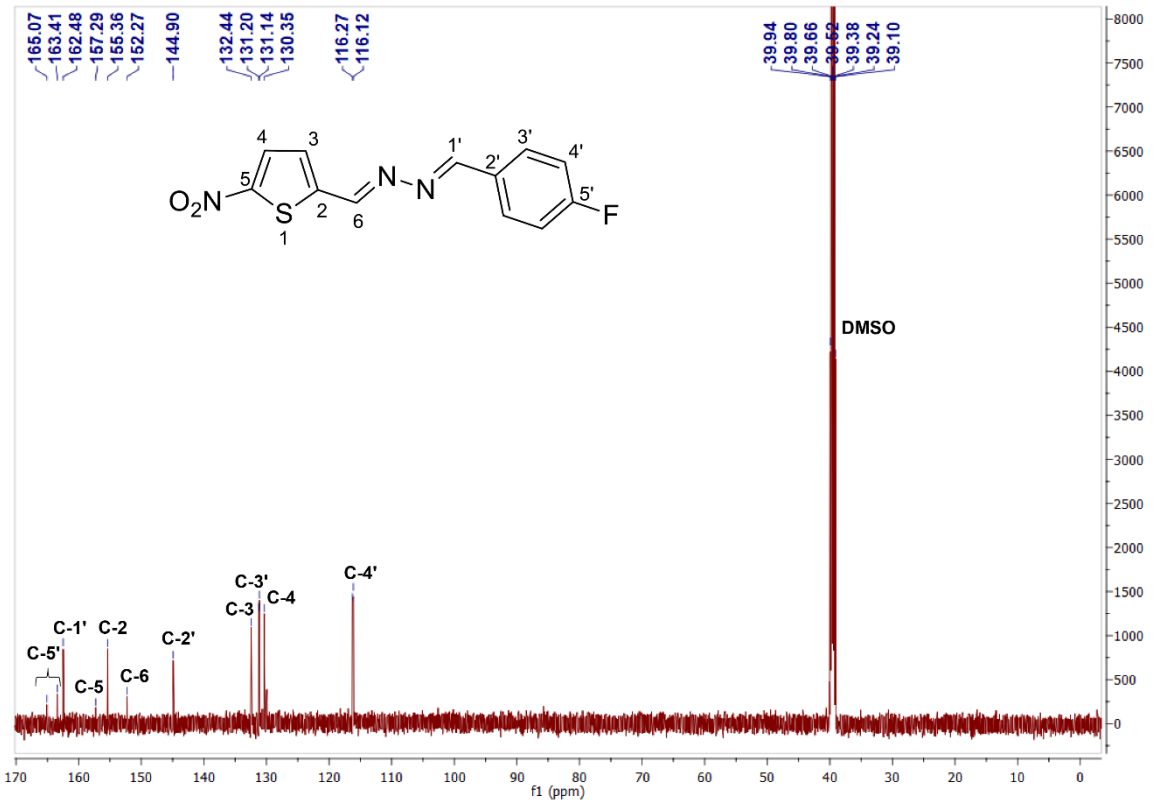
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
260.0512	1	C 12 H 10 N 3 O 2 S	100.00	260.0488	-2.4	-9.3	3.1	9.5	even	ok

(1E,2E)-1-(4-fluorobenzylidene)-2-([5-nitrothiophen-2-yl]methylene)hydrazine (2b)

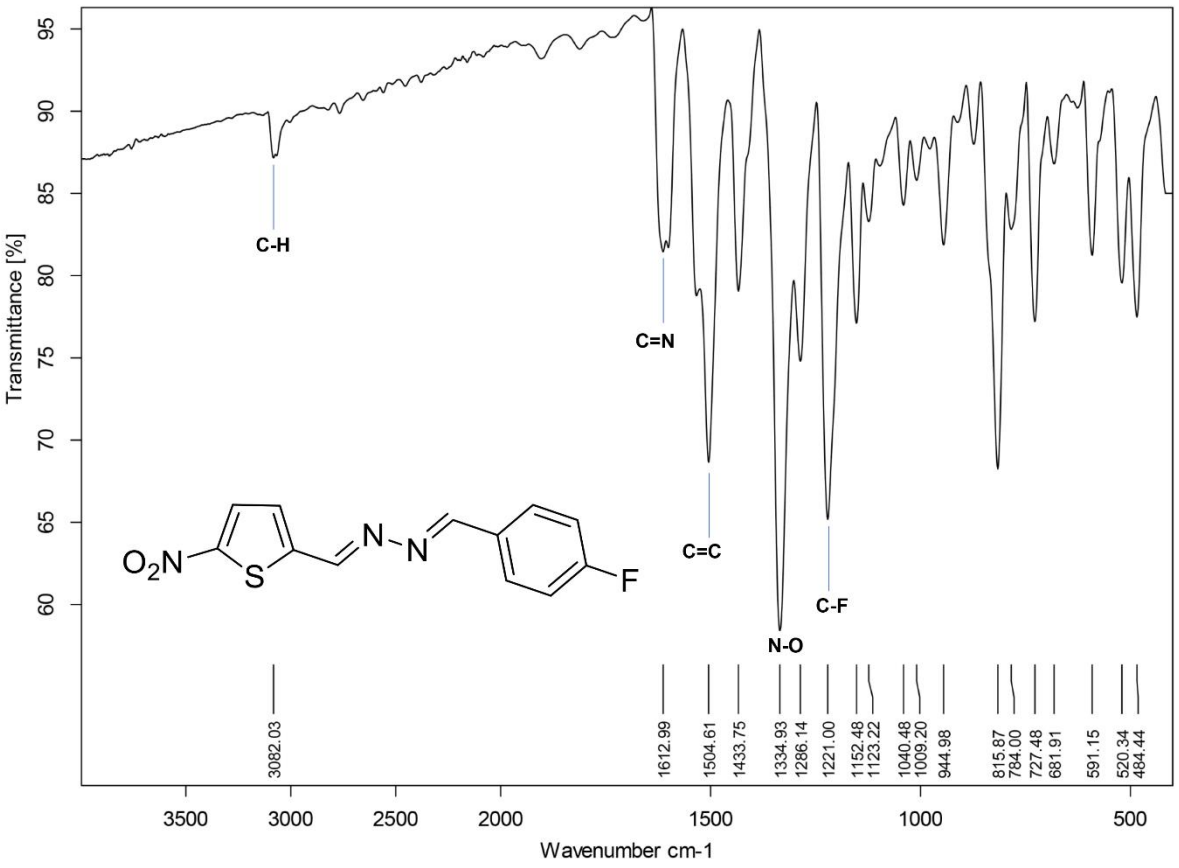
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

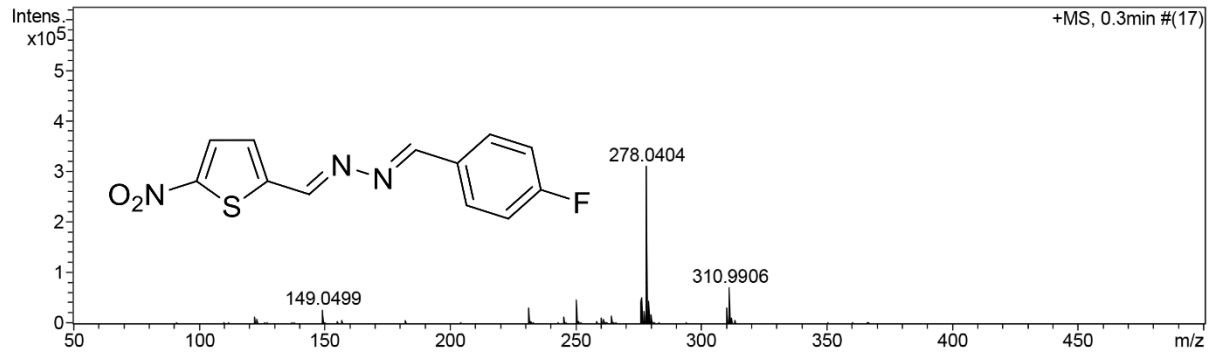
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000032.d	Acquisition Date	10/12/2020 3:31:41 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-16	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

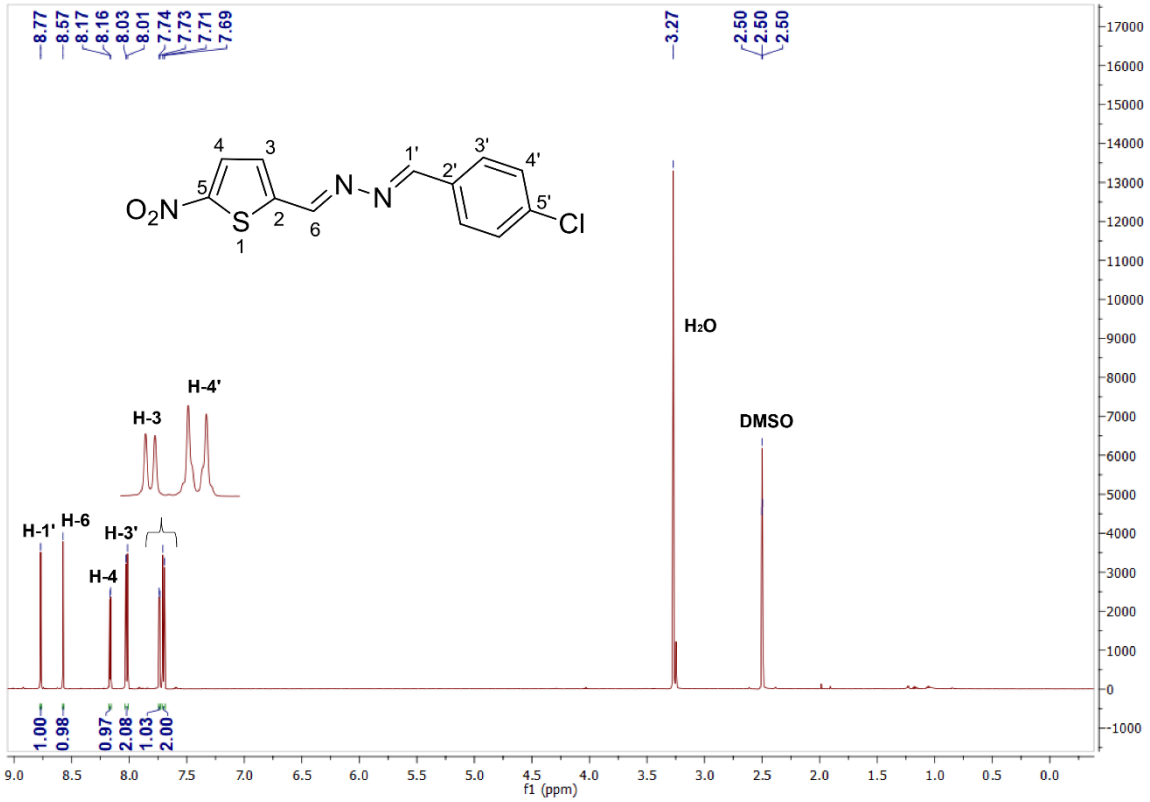
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



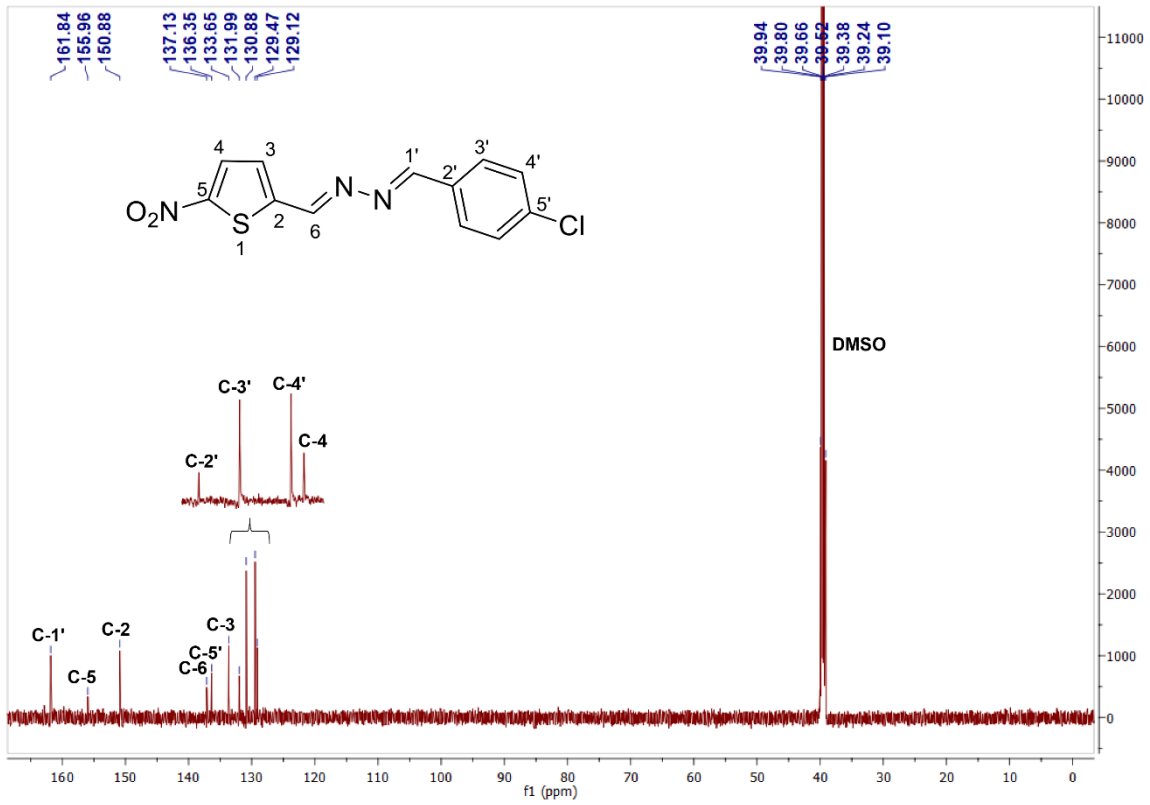
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
278.0404	1	C 12 H 9 F N 3 O 2 S	100.00	278.0394	-1.0	-3.5	0.9	9.5	even	ok

(1E,2E)-1-(4-chlorobenzylidene)-2-([5-nitrothiophen-2-yl]methylene)hydrazine (3b)

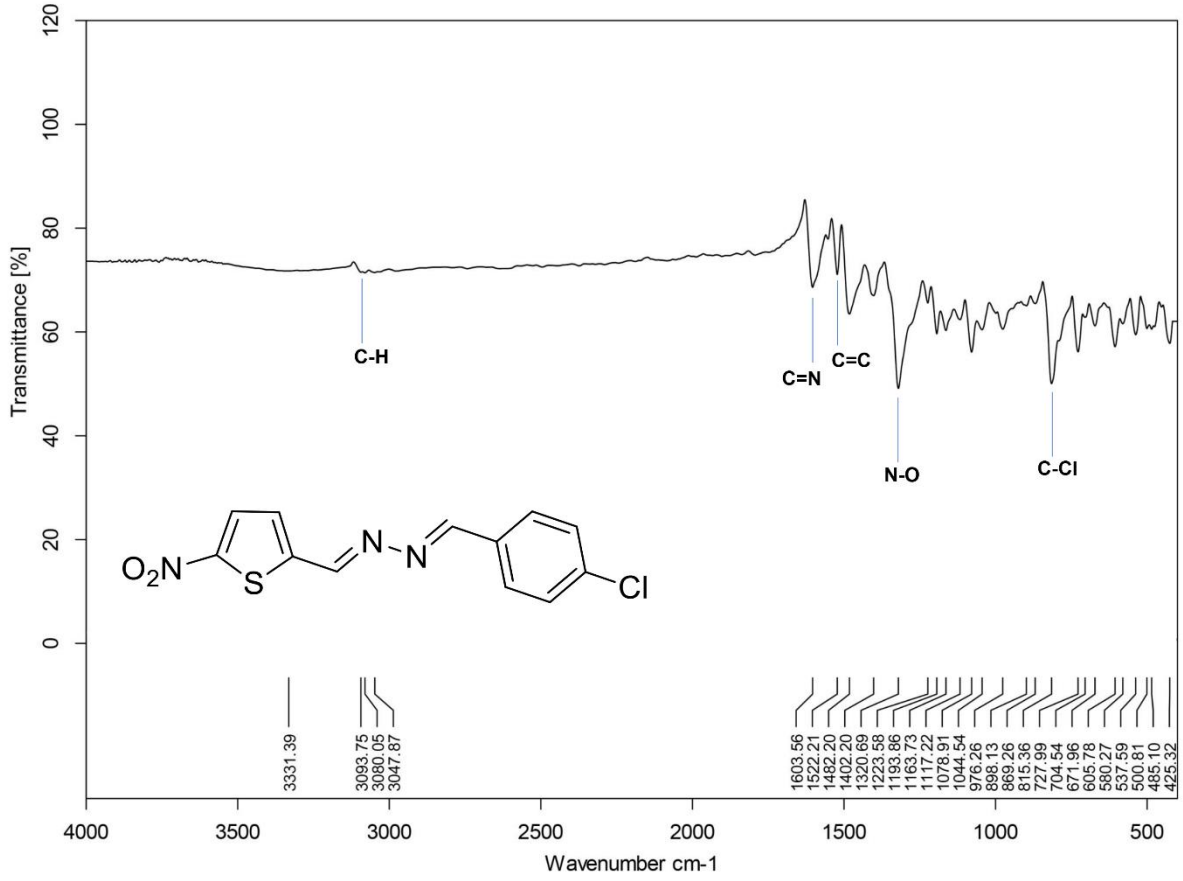
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

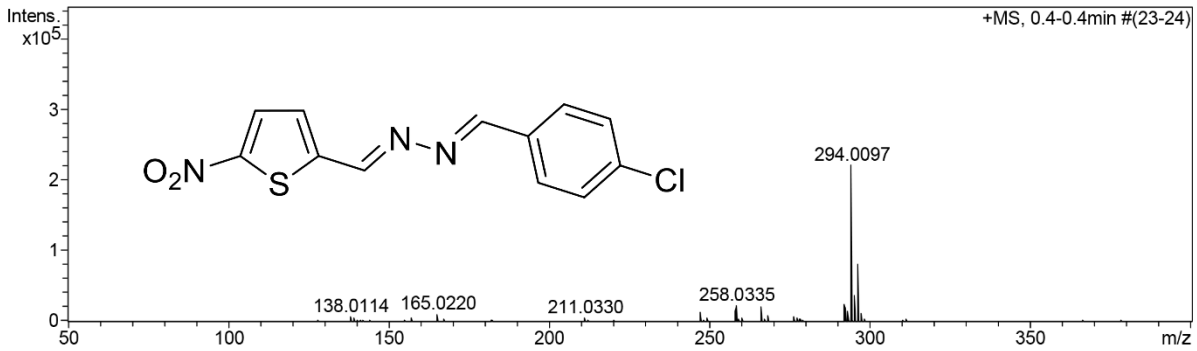
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000042.d	Acquisition Date	10/12/2020 3:44:39 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-12	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

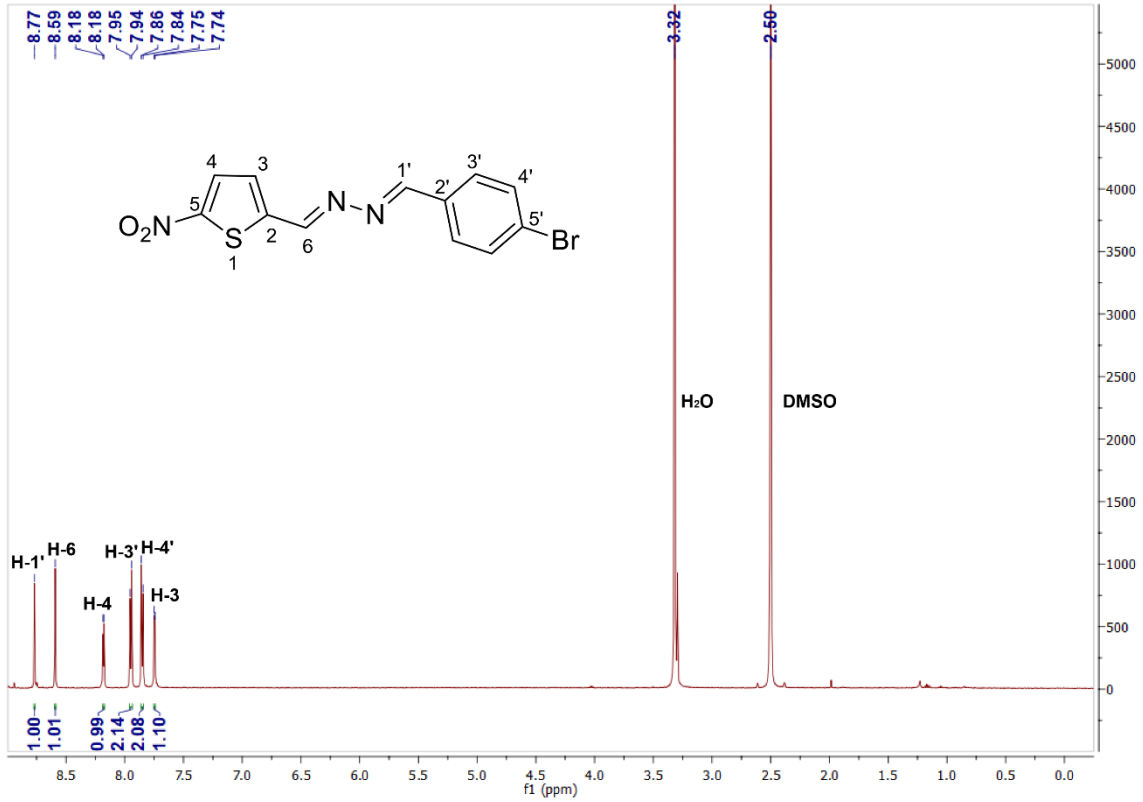
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



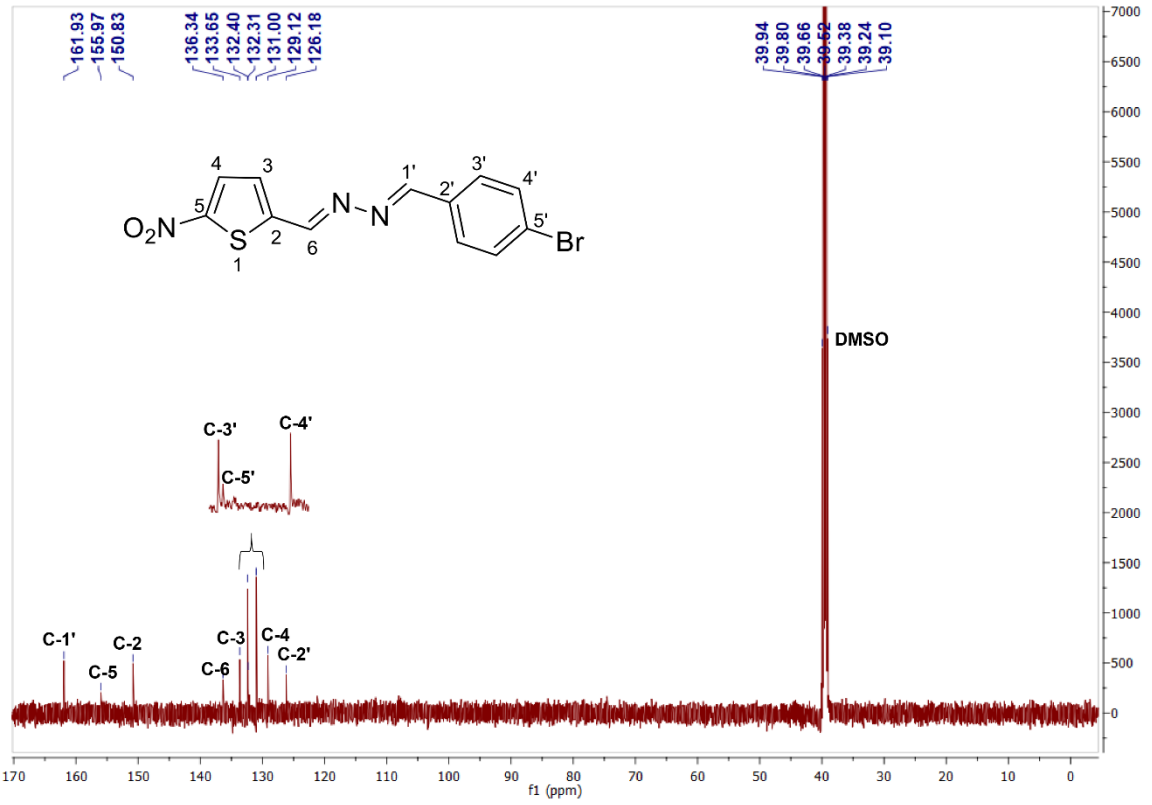
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
294.0097	1	C 12 H 9 Cl N 3 O 2 S	100.00	294.0099	0.2	0.6	8.0	9.5	even	ok

(1E,2E)-1-(4-bromobenzylidene)-2-([5-nitrothiophen-2-yl]methylene)hydrazine (4b)

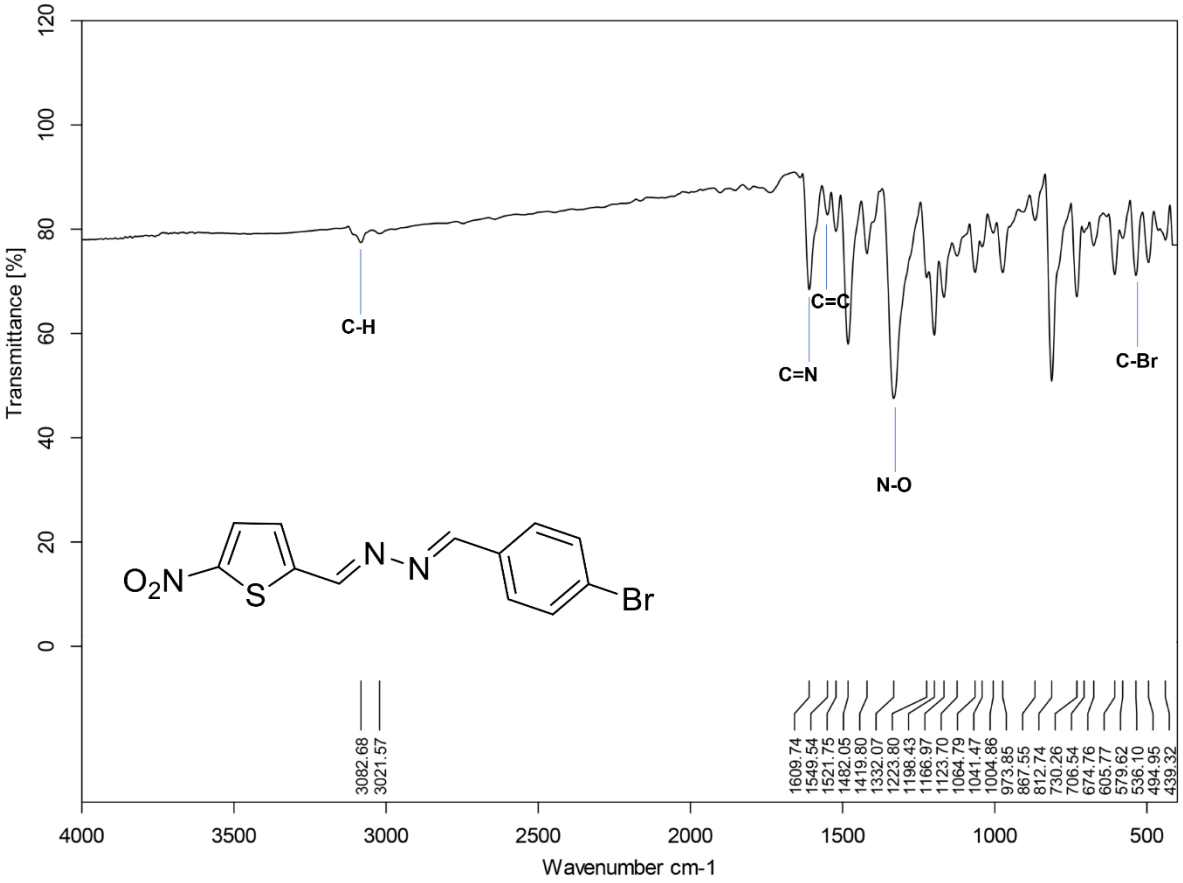
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

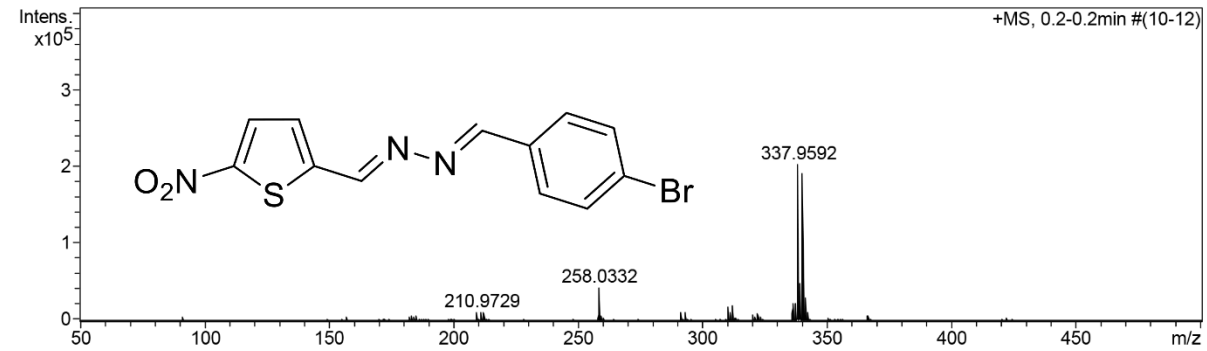
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000030.d	Acquisition Date	10/12/2020 3:29:29 PM
Method	tune_low no focus50-1600da12102020.m		
Sample Name	MV-13	Operator	Dr JHL Jordaan
Comment		Instrument / Ser#	micrOTOF-Q II 2010390

Acquisition Parameter

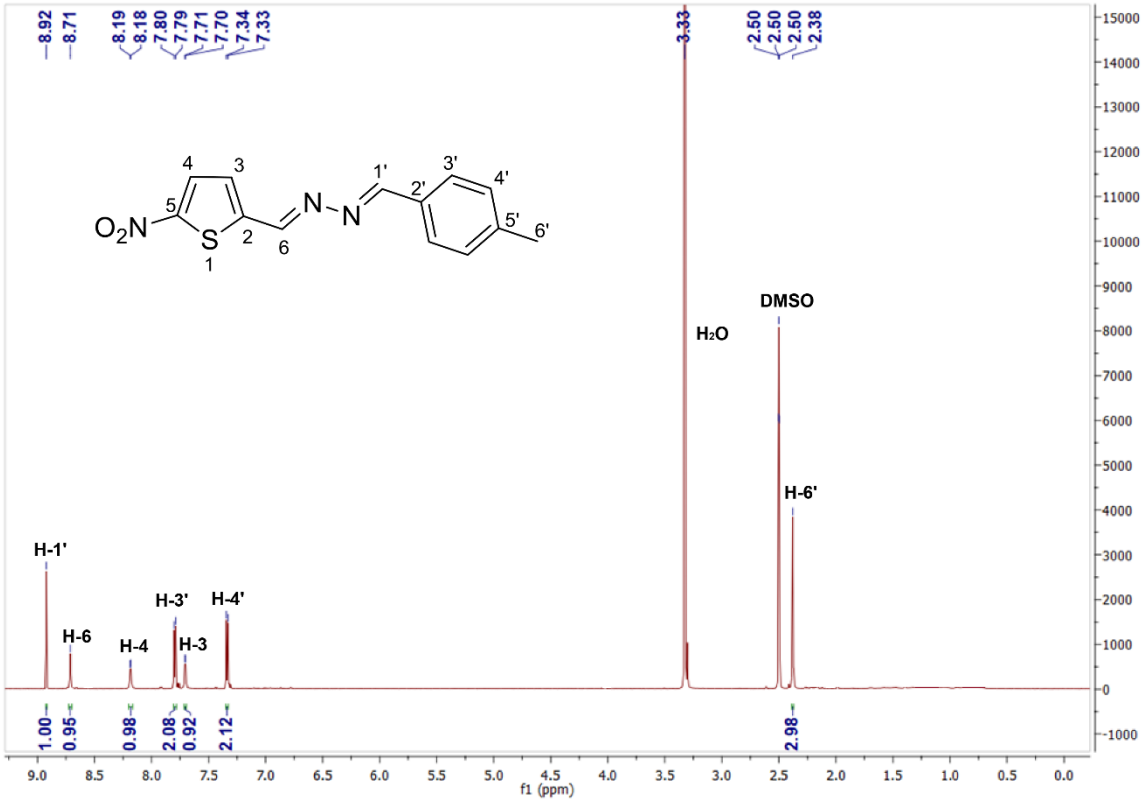
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



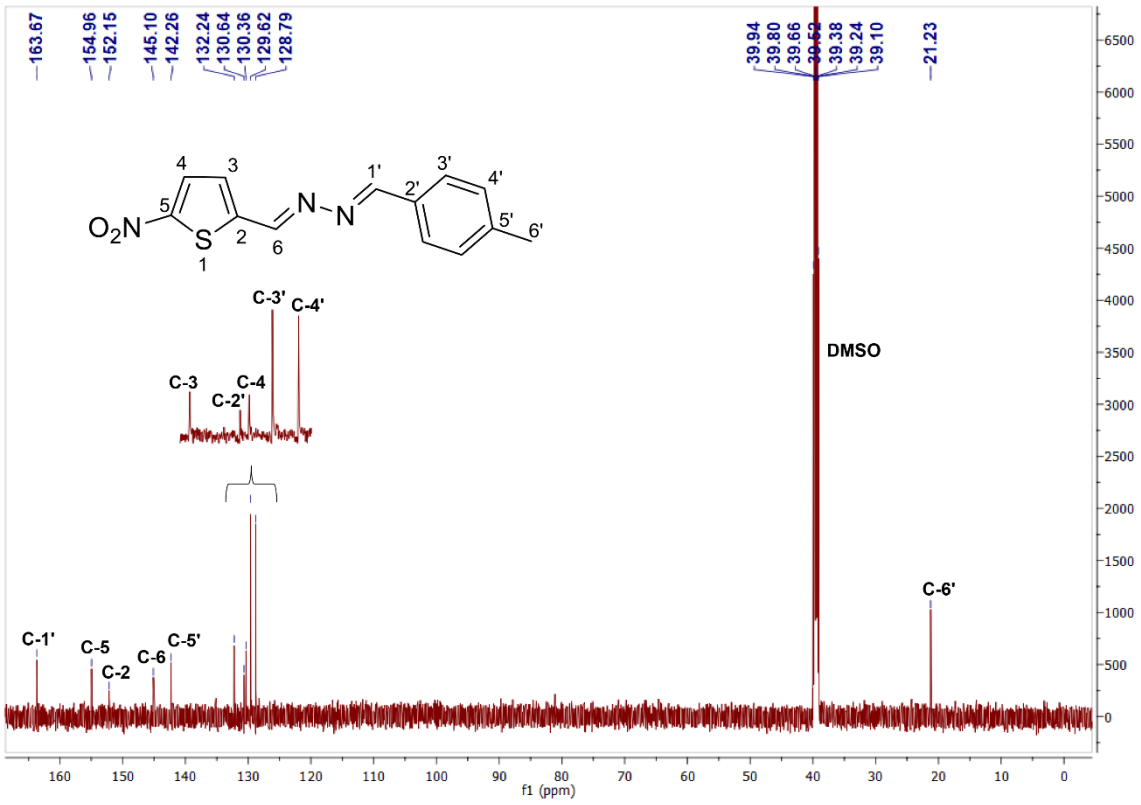
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
337.9592	1	C 14 H 11 Br O 3 S	51.79	337.9607	1.5	4.3	44.4	9.0	odd	ok
	2	C 12 H 9 Br N 3 O 2 S	100.00	337.9593	0.1	0.4	46.5	9.5	even	ok

(1E,2E)-1-(4-methylbenzylidene)-2-([5-nitrothiophen-2-yl]methylene)hydrazine (5b)

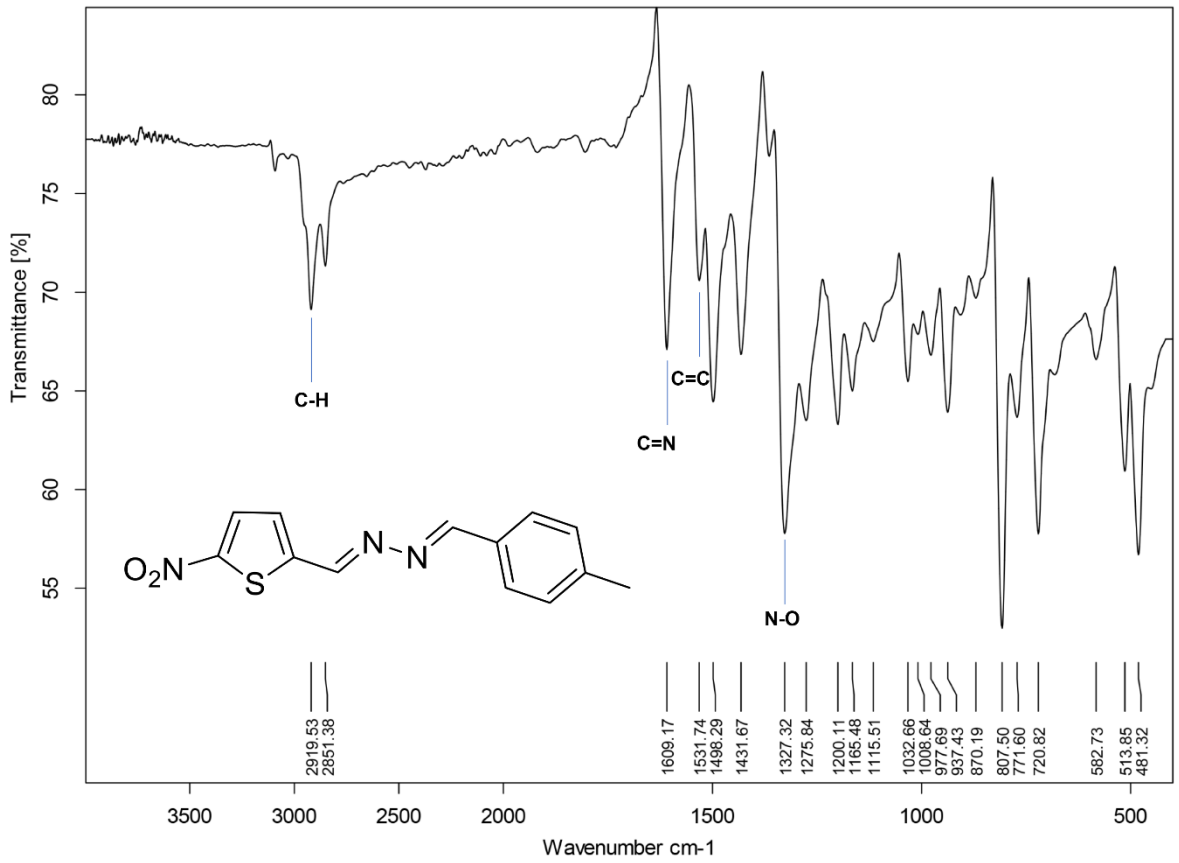
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

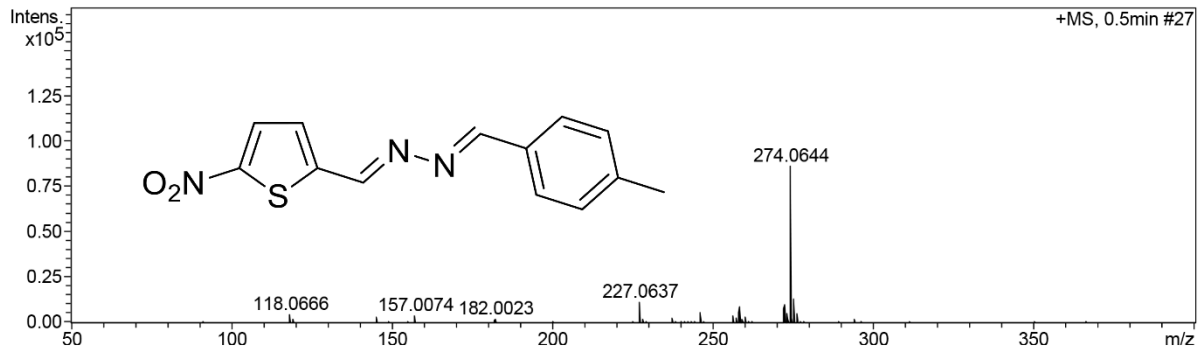
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000043.d	Acquisition Date	10/12/2020 3:45:37 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-14	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

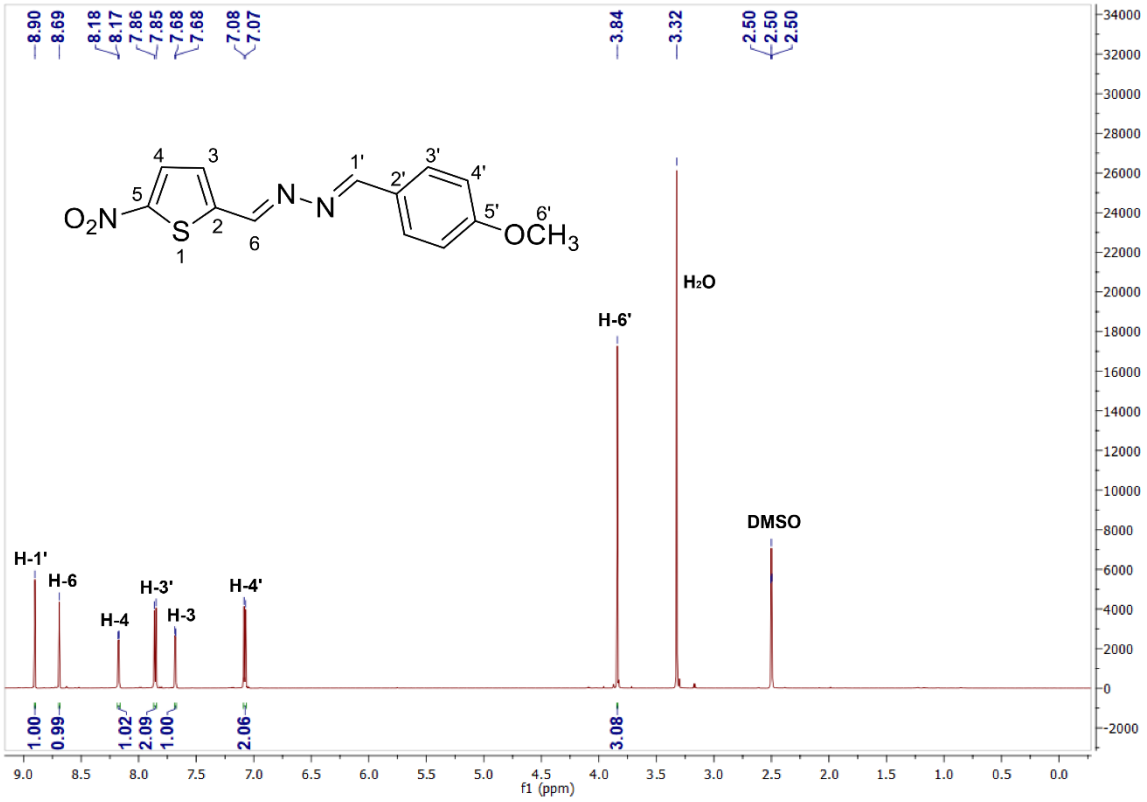
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



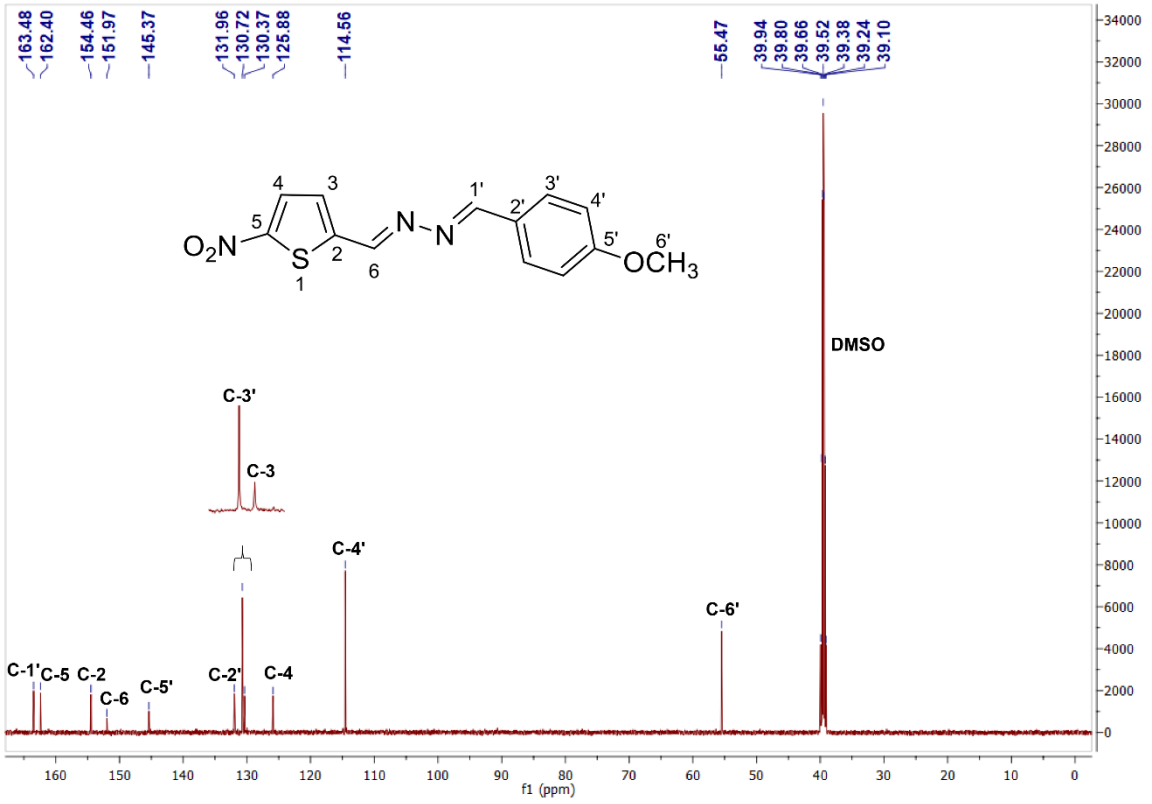
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
274.0644	1	C 13 H 12 N 3 O 2 S	100.00	274.0645	0.1	0.3	2.0	9.5	even	ok

(1E,2E)-1-(4-methoxybenzylidene)-2-([5-nitrothiophen-2-yl]methylene)hydrazine (6b)

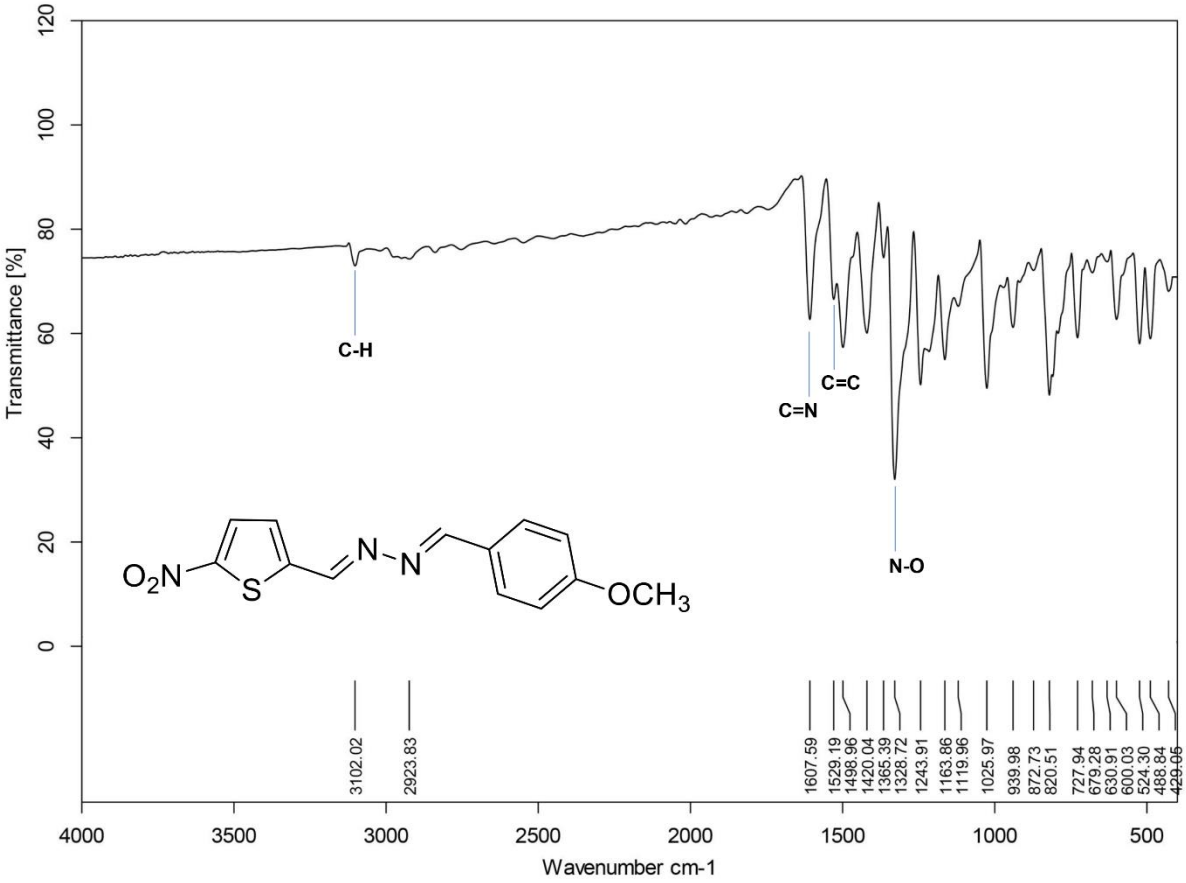
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

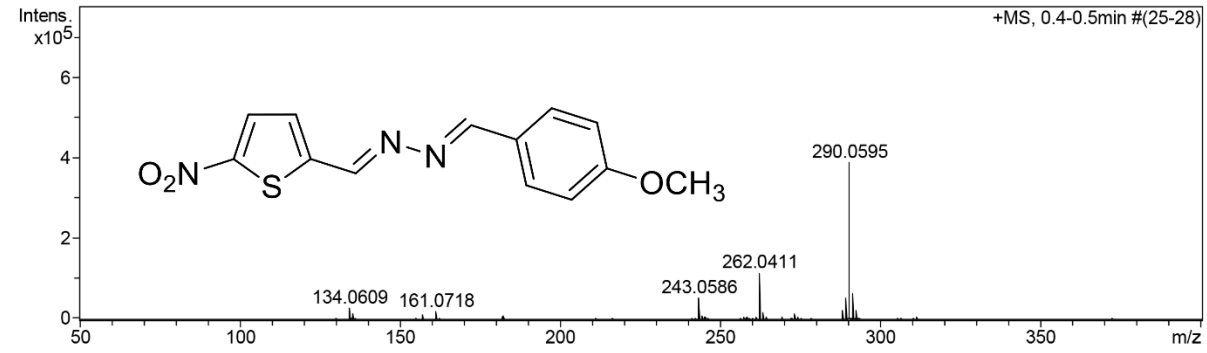
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000044.d	Acquisition Date	10/12/2020 3:46:50 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-15	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

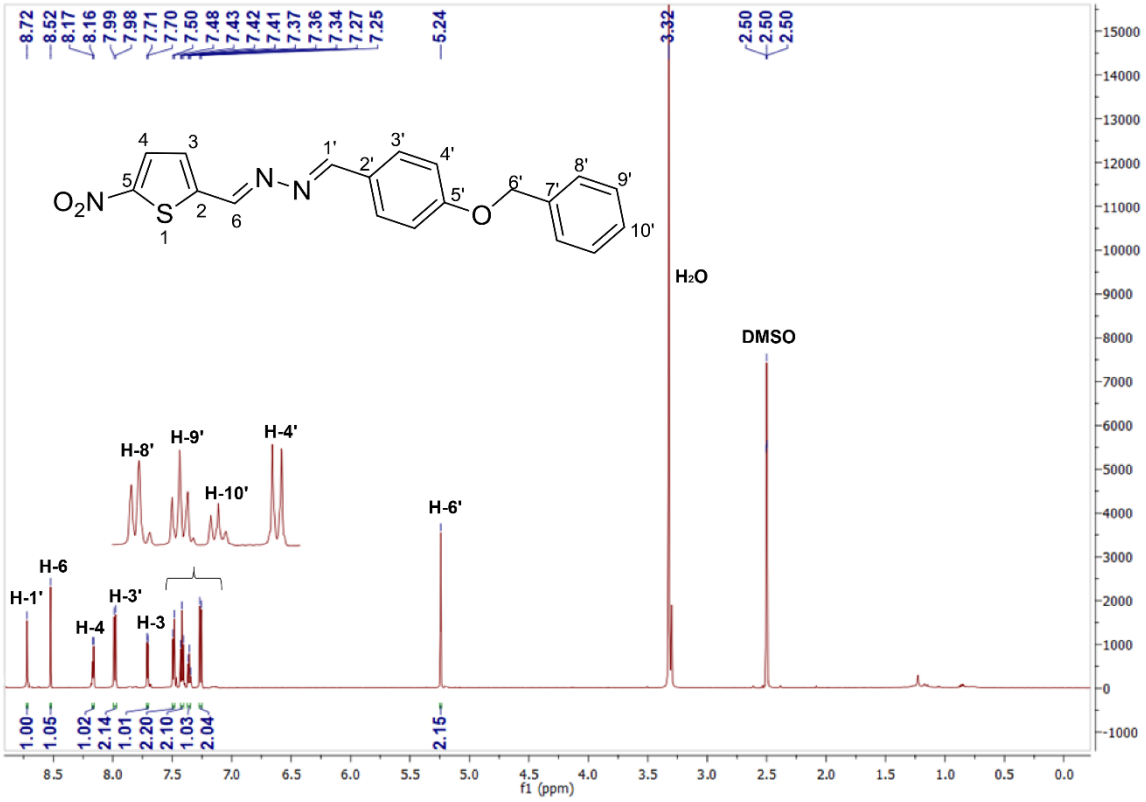
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



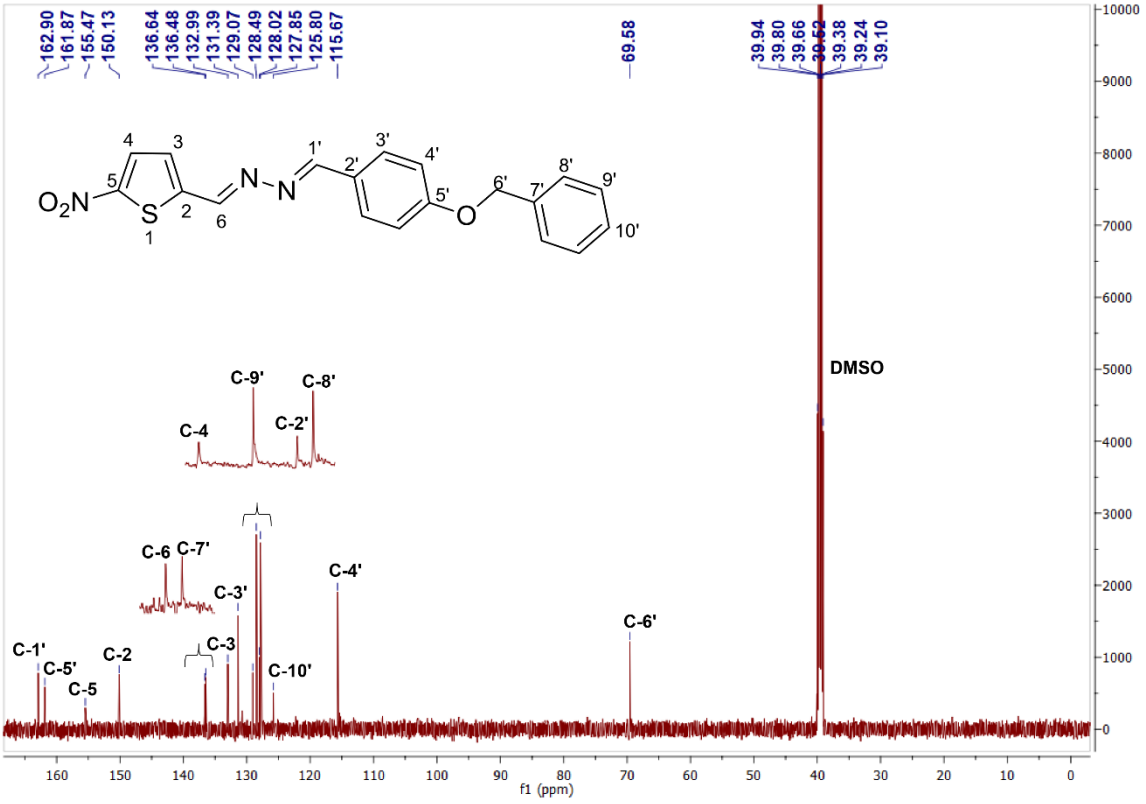
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
290.0595	1	C 13 H 12 N 3 O 3 S	100.00	290.0594	-0.1	-0.3	2.6	9.5	even	ok

(1E,2E)-1-(4-(benzyloxy)benzylidene)-2-((5-nitrothiophen-2-yl)methylene) hydrazine (7b)

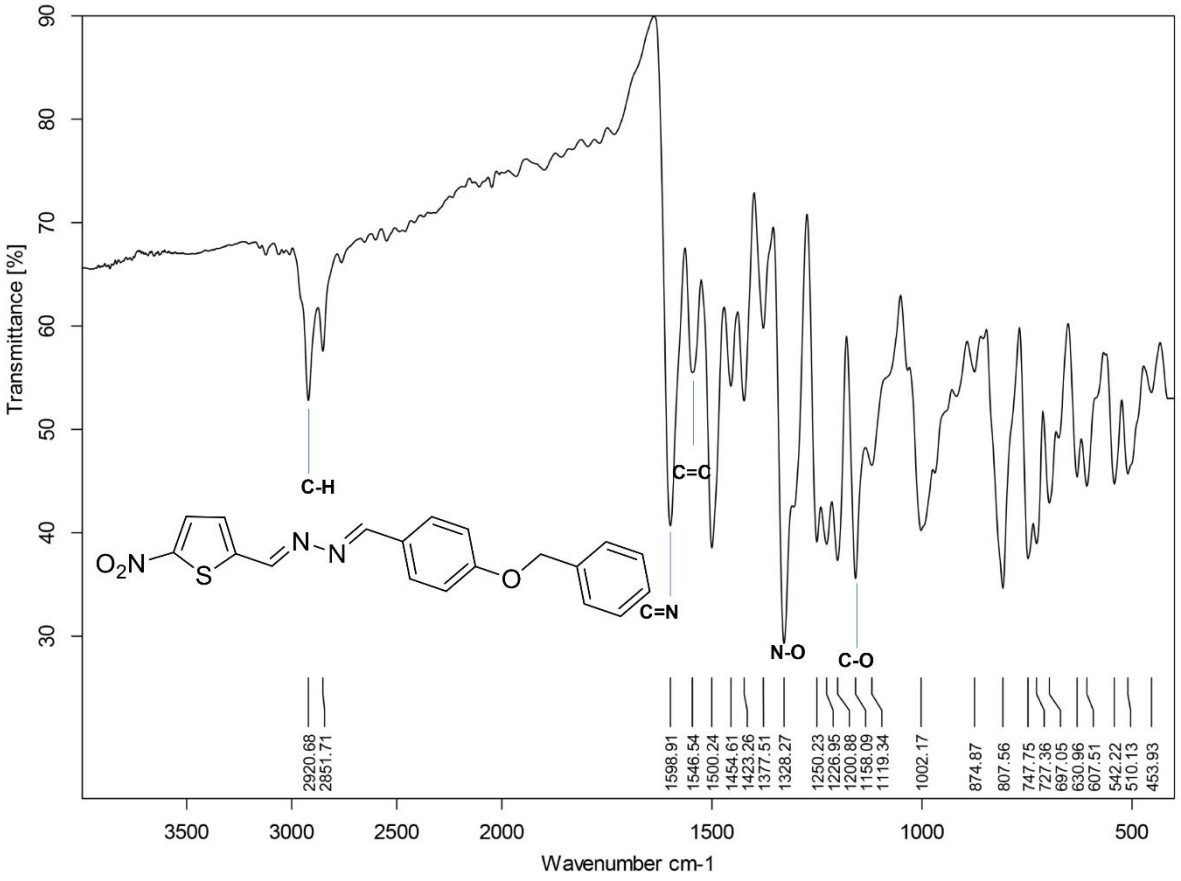
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

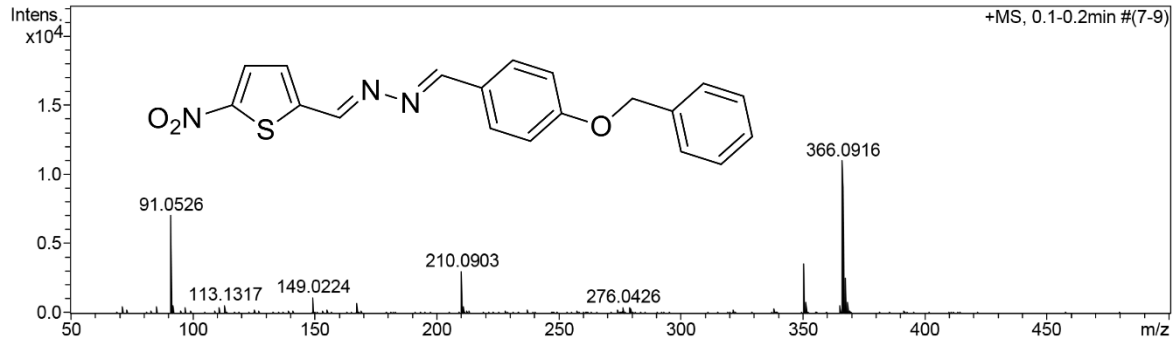
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000029.d	Acquisition Date	10/12/2020 3:28:30 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-18	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

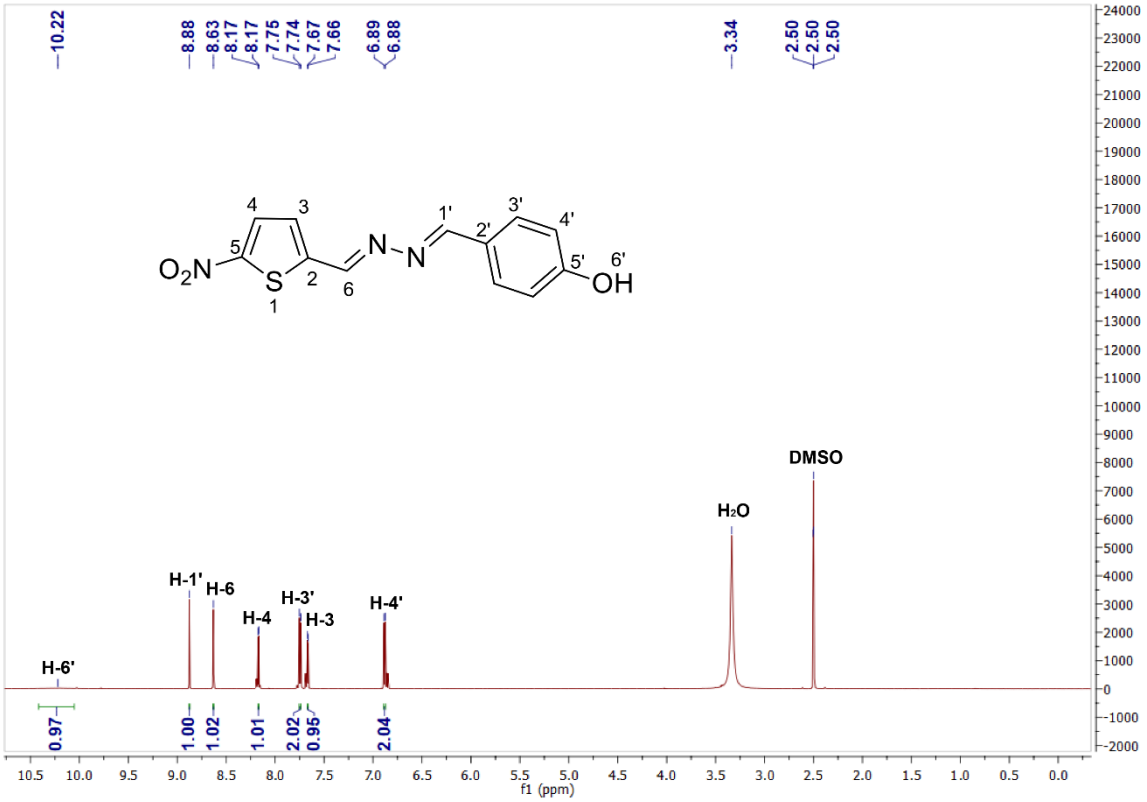
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



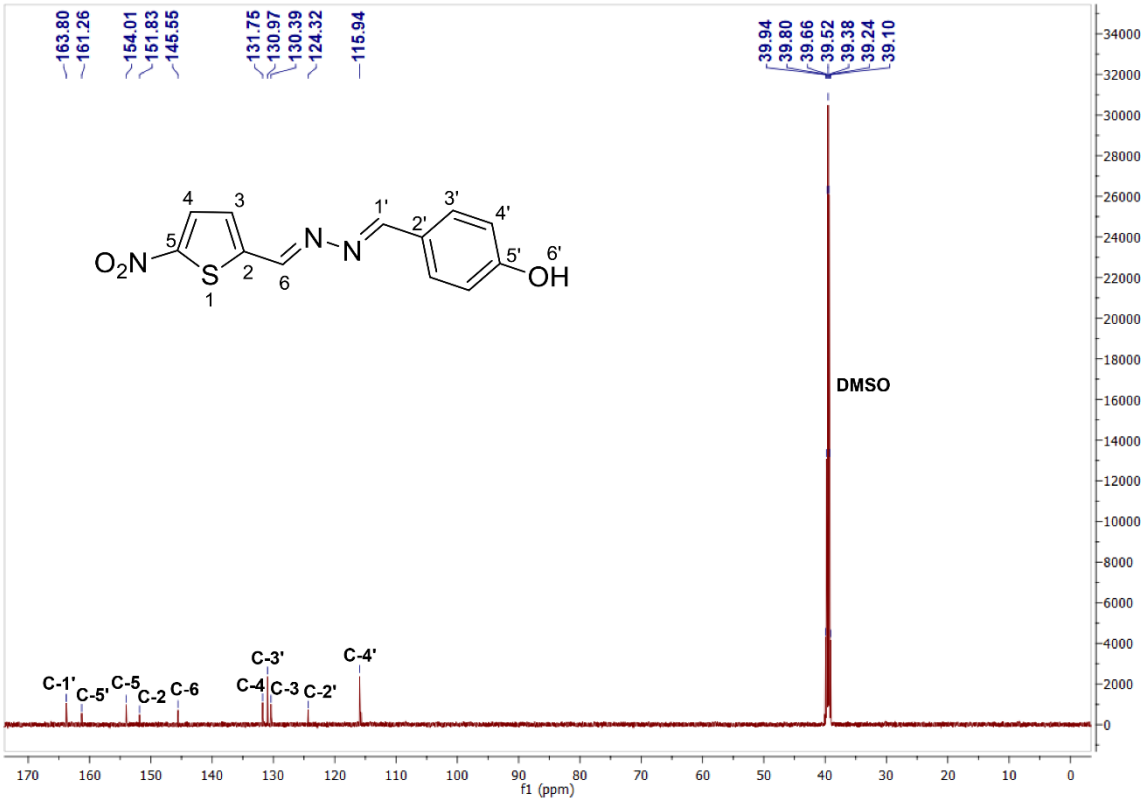
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
91.0526	1	C 7 H 7	100.00	91.0542	1.7	18.2	5.0	4.5	even	ok
210.0903	1	C 14 H 12 N O	100.00	210.0913	1.0	4.8	14.1	9.5	even	ok
	2	C 11 H 14 O 4	41.62	210.0887	-1.7	-7.9	34.4	5.0	odd	ok
350.1128	1	C 19 H 16 N 3 O 4	100.00	350.1135	0.7	2.1	17.7	13.5	even	ok
366.0916	1	C 19 H 16 N 3 O 3 S	100.00	366.0907	-0.9	-2.4	7.2	13.5	even	ok

4-(E)-(E)-([5-nitrothiophen-2-yl]methylene)hydrazonomethylphenol (8b)

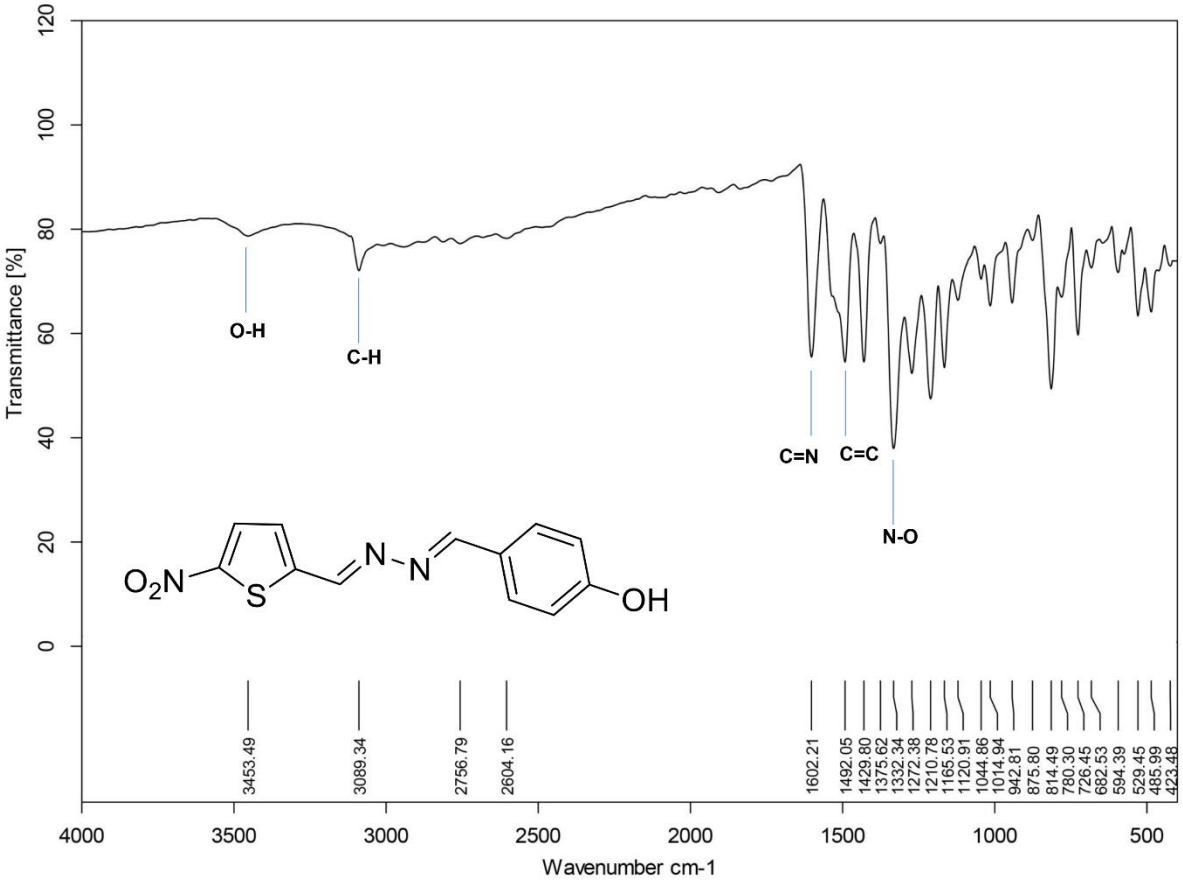
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

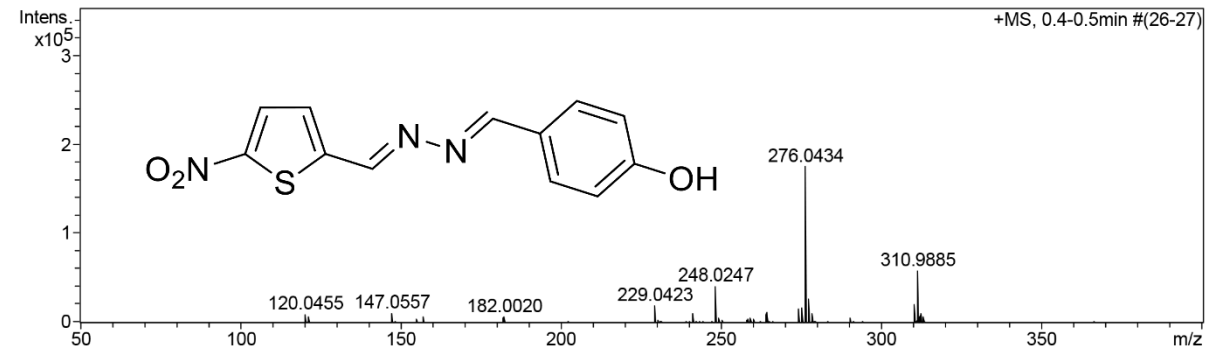
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000045.d	Acquisition Date	10/12/2020 3:48:48 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-17	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

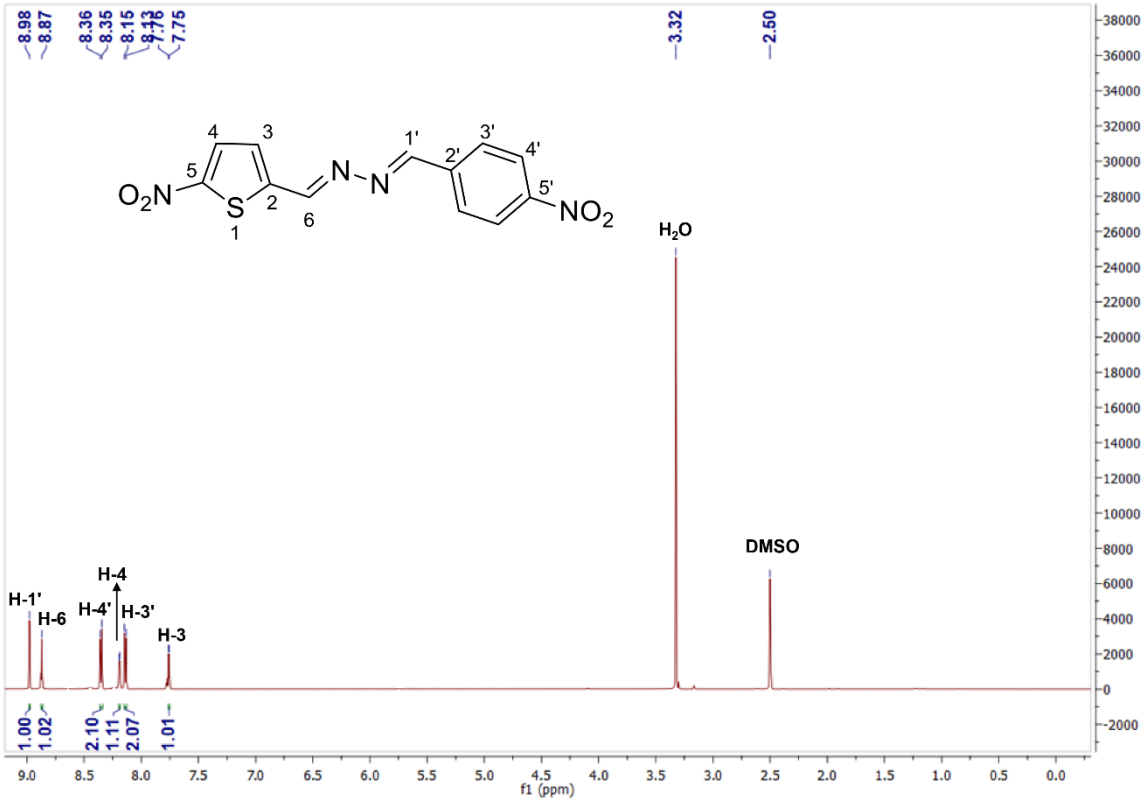
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



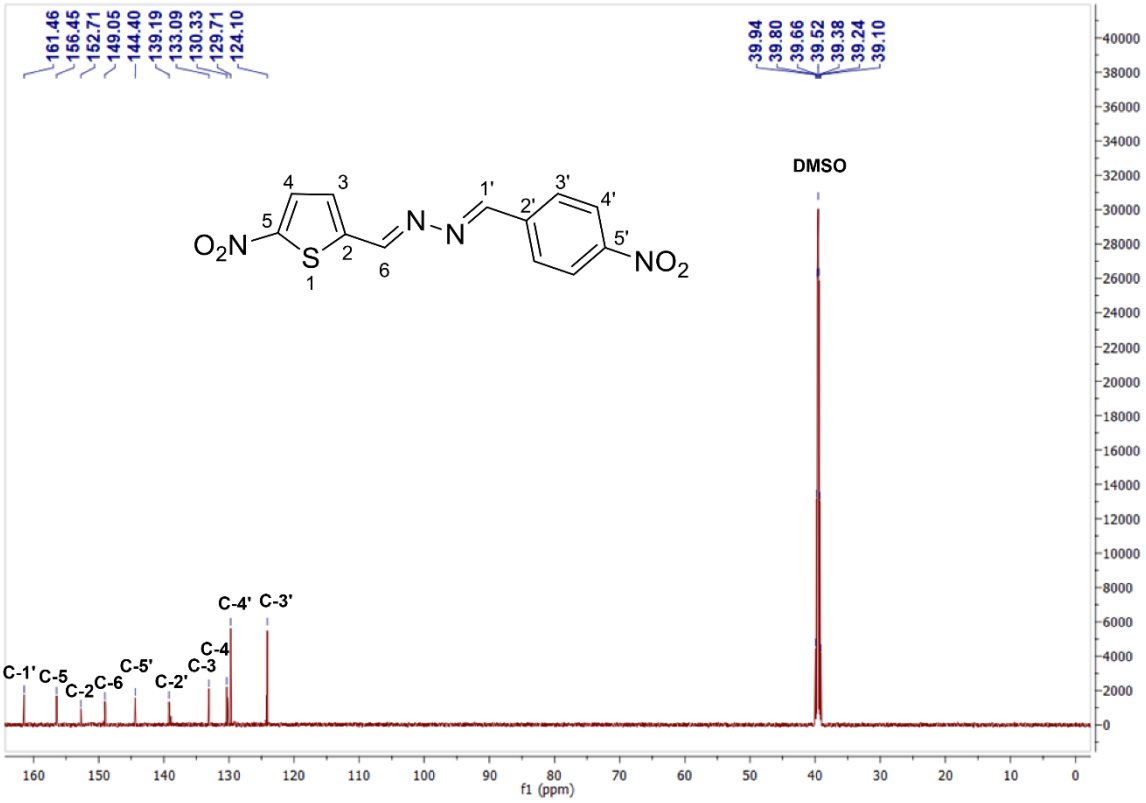
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
276.0434	1	C 12 H 10 N 3 O 3 S	100.00	276.0437	0.3	1.2	0.8	9.5	even	ok

(1E,2E)-1-(4-nitrobenzylidene)-2-([5-nitrothiophen-2-yl]methylene)hydrazine (9b)

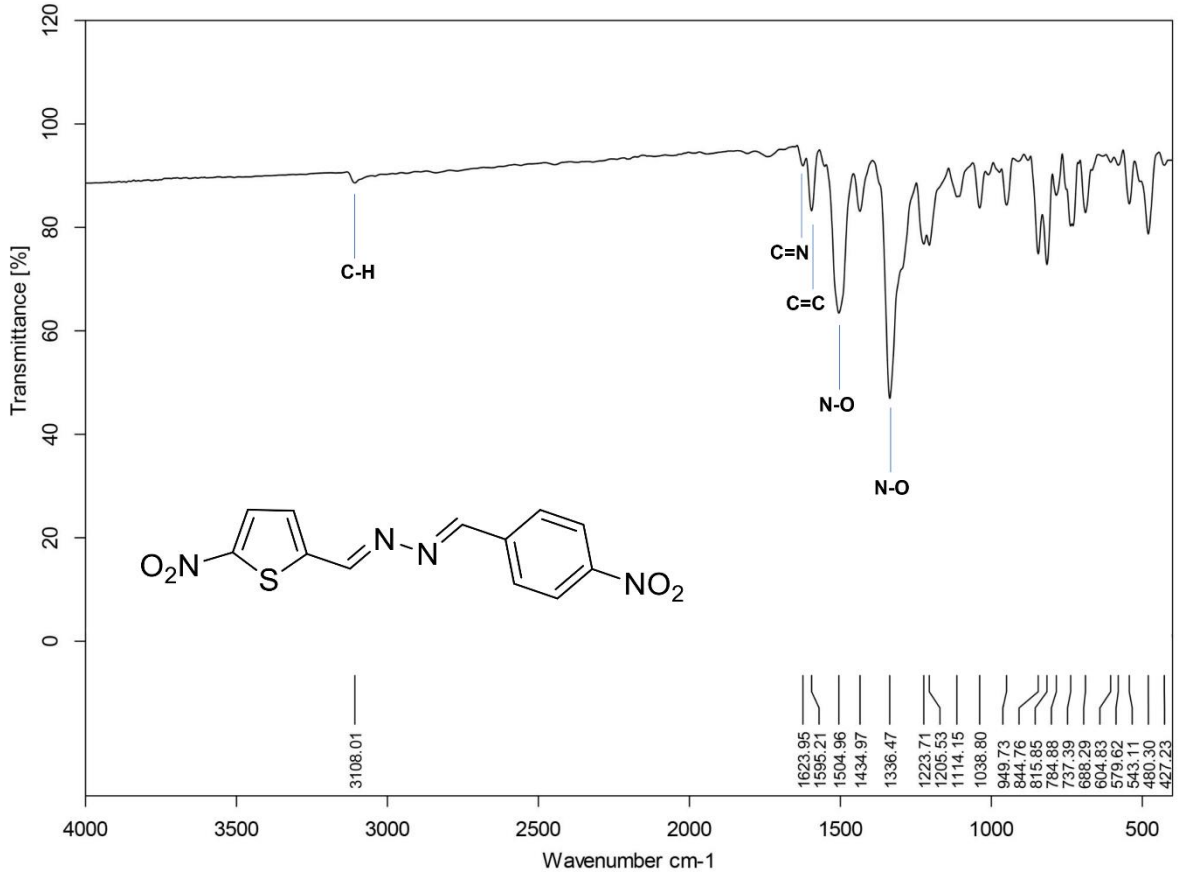
^{1}H NMR in DMSO



^{13}C NMR in DMSO



IR Spectrum



HRMS

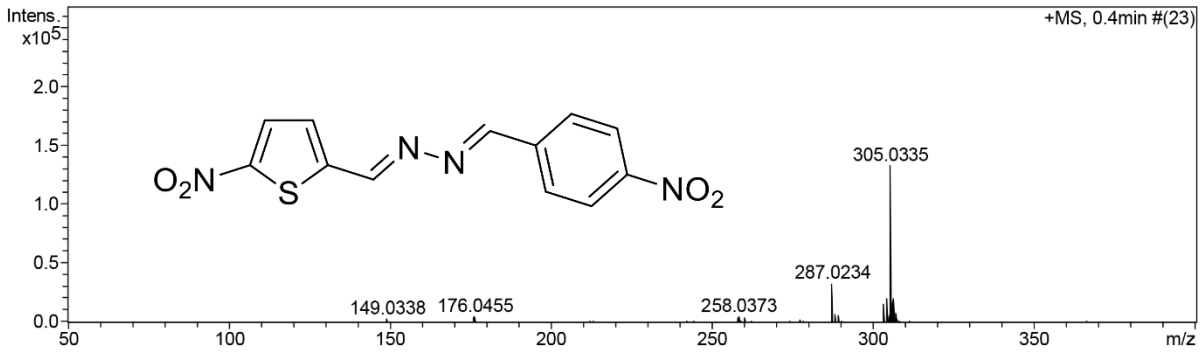
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\12102020\LADMS000041.d	Acquisition Date	10/12/2020 3:43:42 PM
Method	tune_low no focus50-1600da12102020.m	Operator	Dr JHL Jordaan
Sample Name	MV-11	Instrument / Ser#	micrOTOF-Q II 2010390
Comment			

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
305.0335	1	C 12 H 9 N 4 O 4 S	100.00	305.0339	0.4	1.4	2.3	10.5	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)

8 July 2020

RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Health Research Ethics Committee (NWU-HREC) on 08/07/2020, 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* anti-protozoan activities of nitrofuran-based azine derivatives
Principal Investigator/Study Supervisor/Researcher: Prof DD N'Da
Student: M Viljoen - 27352935

Ethics number:

N	W	U	-	0	0	3	8	5	-	2	0	-	A	1
Institution				Study Number						Year			Status	

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

Application Type: Single study
Commencement date: 08/07/2020

Risk: No Risk

General conditions:

The following general terms and conditions will apply:

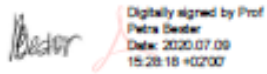
- *The commencement date indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, 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 ethical clearance due to the COVID-19 pandemic:

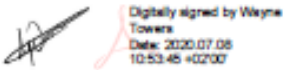
Please note: Due to the nature of the study i.e. laboratory work involving the synthesis of medicinal compounds and *in vitro* testing, this study will be able to proceed during the current alert level, following receipt of this decision letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure.

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,



NWU-HREC Chairperson



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

Current details: (13210572) G:\My Drive\My Documents 2019\0227\NWU-HREC\NWU-HREC_Approval Letters\9.1.5.4.3_LOD_NWU-00000-20-A1_2020mmdd.docm
13 February 2020

File reference: 9.1.5.4.3

ANNEXURE C:

The European Journal of Pharmaceutical Sciences Author Guidelines.

EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

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

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0928-0987

DESCRIPTION

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

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

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

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

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AUDIENCE

Pharmaceutical and Biopharmaceutical Scientists, Medicinal Chemists, Pharmacologists, Analytical Chemists, Clinical Pharmacologists, Pharmaceutical Engineers

IMPACT FACTOR

2019: 3.616 © Clarivate Analytics Journal Citation Reports 2020

ABSTRACTING AND INDEXING

BIOSIS Citation Index
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EDITORIAL BOARD

GUIDE FOR AUTHORS

INTRODUCTION

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

Types of Paper

Research articles

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

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

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

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

Review articles

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

Commentaries and Mini-reviews

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

Commentaries (Guidance)

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

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

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

Mini-review (Guidance)

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

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

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

Submission checklist

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

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- All tables (including titles, description, footnotes)
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- Indicate clearly if color should be used for any figures in print

Graphical Abstracts / Highlights files (where applicable)
Supplemental files (where applicable)

Further considerations
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- Journal policies detailed in this guide have been reviewed
- Referee suggestions and contact details provided, based on journal requirements

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BEFORE YOU BEGIN

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Informed consent and patient details

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Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. To verify originality, your article may be checked by the originality detection service Crossref Similarity Check.

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Author contributions

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

Changes to authorship

Authors are expected to consider carefully the list and order of authors **before** submitting their manuscript and provide the definitive list of authors at the time of the original submission. Any addition, deletion or rearrangement of author names in the authorship list should be made only **before** the manuscript has been accepted and only if approved by the journal Editor. To request such a change, the Editor must receive the following from the **corresponding author**: (a) the reason for the change in author list and (b) written confirmation (e-mail, letter) from all authors that they agree with the addition, removal or rearrangement. In the case of addition or removal of authors, this includes confirmation from the author being added or removed.

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Additional Information

Editorial review: All manuscripts are generally submitted to 2-3 reviewers who are selected for their ability to evaluate the work. Supplementary material may be included to facilitate the review process. Authors may request that certain reviewers should not be chosen, but should then also explain why. Members of the editorial board will usually be called upon for advice when there is disagreement among the reviewers or between reviewers and authors, or when the editors feel that the manuscript has not received adequate consideration by the reviewers.

Please submit, with the manuscript, the names, postal addresses and e-mail addresses of at least four potential reviewers. Good suggestions lead to faster processing of your paper. Please note: Reviewers who do not have an institutional e-mail address will only be considered if their affiliations are given and can be verified. Please ensure that the e-mail addresses are current. International reviewers who have recently published in the appropriate field should be nominated, and their areas of expertise must be stated clearly. Note that the editor retains the sole right to decide whether or not the suggested reviewers are contacted. To aid the editorial process when suggested reviewers are not chosen or decline to review, ensure that the classifications chosen are as detailed as possible. It is not sufficient to select e.g. 'analytical chemistry' or 'physical pharmacy and pharmaceutical technology'.

All reviewers' comments must be responded to, and suggested changes be made. The author should detail the changes made in response to the referees' comments and suggestions in an accompanying letter. If the author disagrees with some changes, the reason, supported by data, should be given. The editors may refuse to publish manuscripts from authors who persistently ignore referees' comments. A revised manuscript should be received by the editorial office no later than 2 months after the editorial decision was sent to the author(s); otherwise it will be processed as a new manuscript.

PREPARATION

Peer review

This journal operates a single anonymized review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. Editors are not involved in decisions about papers which they have written themselves or have been written by family members or colleagues or which relate to products or services in which the editor has an interest. Any such submission is subject to all of the journal's usual procedures, with peer review handled independently of the relevant editor and their research groups. More information on types of peer review.

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Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

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

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

Results
Results should be clear and concise.

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

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

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

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

Essential title page information
• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
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The manuscript must be submitted by the person who is in charge of correspondence at all stages of the editorial process, production, and post-publication. Ensure that phone numbers (with country and area codes) are provided, in addition to the e-mail address (preferably an institutional e-mail address) and the complete postal address. Contact details of the other authors must be kept up to date by the corresponding author.
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Highlights

Highlights are optional yet highly encouraged for this journal, as they increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract

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

Graphical abstract

A Graphical abstract is mandatory for this journal. It should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more, but should be readable on screen at a size of 200 × 500 pixels (at 96 dpi this corresponds to 5 × 13 cm). Bear in mind readability after reduction, especially if using one of the figures from the article itself. Preferred file types: TIFF, EPS, PDF or MS Office files. See https://www.elsevier.com/graphicalabstracts for examples.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

Abbreviations are a hindrance for the reader. Use as few abbreviations as possible and write out names of compounds, receptors, etc., in full throughout the text of the manuscript, with the exceptions given below. Unnecessary and nonsense abbreviations are not allowed. Generic names should not be abbreviated. As an example, AMP, HAL, HIST, RAMH, TAM, SST, for amphetamine, haloperidol, histamine, (R)-α -methylhistamine, tamoxifen, somatostatin, are not accepted. Abbreviations which have come to replace the full term (e.g., GABA, DOPA, PDGF, 5-HT, for Y -aminobutyric acid, 3,4-dihydroxyphenylalanine, PDGF, 5-hydroxytryptamine) may be used, provided the term is spelled out in the abstract and in the body of the manuscript the first time the abbreviation is used. Unwieldy chemical names may be abbreviated. As an example, 8-OH-DPAT, DOI, DTG, BAPTA, for 8-hydroxy-2-(di-*n*-propylamino)tetralin, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, 1,3-di(2-tolyl)-guanidine, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, are acceptable; however, the full chemical name should be given once in the body of the manuscript and in the abstract, followed in both cases by the abbreviation. Code names may be used, but the full chemical name should be given in the text and in the abstract. *Authors not conforming to these demands may have their manuscripts returned for correction with delayed publication as a result.*

Some abbreviations may be used without definition:

1 ADP,CDP, GDP, IDP5'-pyrophosphates of adenosine UDPcytidine, guanosine, inosine, uridine AMP etc.adenosine 5'-monophosphate etc. ADP etc.adenosine 5'-diphosphate etc. ATP etc.adenosine 5'-triphosphate etc. CM-cellulosecarboxymethylcellulose CoA and acetyl-CoAcoenzyme A and its acyl derivatives DEAE-cellulose*O*-(diethylaminoethyl)-cellulose DNAdeoxyribonucleic acid EGTAethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid FADflavin-adenine dinucleotide FMNflavin mononucleotide GSH, GSSGglutathione, reduced and oxidized Hepes4-(2-hydroxyethyl)-1-

piperazine-ethanesulphonic acid NADnicotinamide-adenine dinucleotide NADPnicotinamide-adenine dinucleotide phosphate NMNnicotinamide mononucleotide P_i, PP_iorthophosphate, pyrophosphate RNAribonucleic acid Tris2-amino-2-hydroxymethylpropane-1,3-diol

Two alternative conventions are currently in use in some cases. For example, for the phosphoinositides there are both the abbreviations recommended by the IUPAC-IUB and those of the Chilton Convention (e.g., PtdIns(4,5)P_2 vs. PIP $_2$for phosphatidylinositol 4,5-biphosphate). The journal will accept either of these forms but not their combination.

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

Units of mass

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

Units of time

1 hourh minutemin seconds millisecondms microsecondμs

Units of volume

1 litrel millilitreml microlitreμl

Units of length

1 metrem centimetrecm millimetremm micrometreμm nanometrenm

Units of concentration

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

Units of heat, energy, electricity

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

Units of radiation

1 curieCi counts per minutecpm disintegrations per minutedpm becquerelBq

Miscellaneous

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

The isotope mass number should appear before the atomic symbol, e.g., [^{3}H]noradrenaline, [^{14}C]choline. Ions should be written: Fe^{3+}, Ca^{2+}, Mg^{2+}. The term absorbance (A) is preferred to extinction or optical density. For abbreviations not included in this list consult: *Units, Symbols and Abbreviations, A Guide for Biological and Medical Authors and Editors*, 1994 (The Royal Society of Medicine, London), ISBN 0-905958-78-0, or *Scientific Style and Format. The CBE Manual for Authors, Editors, and Publishers*, 6th edn. (Cambridge University Press, Cambridge), ISBN 0-521-47154-0.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g. lab technicians, statisticians, colleagues providing help preparing the manuscript).

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List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

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Nomenclature and Units

Only generic and chemical names of drugs should be used, although a proprietary equivalent may be indicated once, in parentheses. *Pharmacological and Chemical Synonyms*, E.E.J. Marler, 9th edn. (Elsevier, Amsterdam, 1990) may be consulted.

The nomenclature of chemical substances should be consistent, clear and unambiguous, and should conform to the usage of the American Chemical Society and the convention recommended by the International Union of Pure and Applied Chemistry (IUPAC). When in doubt, writers should consult the indexes of *Chemical Abstracts*; the various reports and pamphlets of the American Chemical Society Committee on Nomenclature, Spelling and Pronunciation; and from the International Union of Biochemistry and Molecular Biology (IUBMB): *Biochemical Nomenclature and Related Documents* (Portland Press, London).

When drugs, which are mixtures of stereoisomers are used, the fact that they have a composite nature and the implication of this for interpretation of the data and drawing of conclusions should be made clear. The use of the appropriate prefix is essential. Use of the generic name alone without prefix would be taken to refer to agents with no stereoisomers. The nomenclature of the various isomers and isomeric mixtures can be found in: (i) *IUPAC, Nomenclature of Organic Chemistry*, eds. J. Rigaudy and S.P. Klesney (Pergamon Press, London), 1979, p. 481; (ii) *Signs of the times: the need for a stereochemically informative generic name system*, Simonyi, M., J. Gal and B. Testa, 1989, Trends Pharmacol. Sci. 10, 349. For nomenclature of peptides, see *Neuropeptides*, Vol. 1, 1981, p. 231.

The nomenclature of receptors and their subtypes should conform to the *TIPS 1995 Receptor & Ion Channel Nomenclature Supplement (Trends Pharmacol. Sci.* Receptor Nomenclature Supplement 1995). Copies of this supplement are available from the publisher (Elsevier Trends Journals, Oxford Fulfilment Centre, P.O. Box 800, Kidlington, Oxford OX5 1DX, UK. Tel.: (44-1865) 843-699; Fax: (44-1865) 843-911).

The trivial name of the enzyme may be used in the text, but the systematic name and classification number according to *Enzyme Nomenclature*, rev. edn. (Academic Press, New York, NY, 1984) should be quoted the first time the enzyme is mentioned.

GenBank accession numbers

Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should reference this information in the following manner:

For each and every accession number cited in an article, authors should type the accession number in **bold, underlined text** . Letters in the accession number should always be capitalised. (See Example 1 below.) This combination of letters and format will enable Elsevier's typesetters to recognize the relevant texts as accession numbers and add the required link to GenBank's sequences.

Example 1: "GenBank accession nos. **AI631510** , **AI631511** , **AI632198** , and **BF223228**), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. **BE675048**), and a T-cell lymphoma (GenBank accession no. **AA361117**)".

Authors are encouraged to check accession numbers used very carefully. **An error in a letter or number can result in a dead link.**

In the final version of the ***printed* article**, the accession number text will not appear bold or underlined (see Example 2 below).

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In the final version of the ***electronic* copy**, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article (see Example 3 below).

Example 3: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

Formulas and equations

Structural chemical formulas, process flow diagrams and complicated mathematical expressions should be very clearly presented. All subscripts, superscripts, Greek letters and unusual characters must be identified. Structural chemical formulas and process flow diagrams should be prepared in the same way as graphs.
Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

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M Viljoen

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Dissertation submitted in partial fulfilment of the requirements for the degree Master of Science in Pharmaceutical Chemistry at the North-West University

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