Synthesis and anti-leishmanial activity of novel 2,3-disubstituted-4(3*H*)-quinazolinone derivatives

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

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Examination:	August 2020
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The financial assistance of the National Research Foundation (NRF) towards this study is hereby acknowledged. The opinions expressed herein, and the conclusions arrived at, are solely those of the author and are not necessarily attributable to the NRF.

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PREFACE

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

Chapter 3: Article for submission

Synthesis and anti-leishmanial activity of novel 2,3-disubstituted-4(3*H*)-quinazolinone derivatives.

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ACKNOWLEDGEMENTS

I hereby wish to express my sincerest gratitude to the following individuals and institutions for their guidance and/or support during my MSc degree at the NWU (Potchefstroom Campus):

- My supervisor Prof. DD N'Da, for his continued support and for the opportunity to study a master's degree under his tutelage; and my co-supervisors Dr. J Aucamp and Ms. NH Zuma.
- Ms. NH Zuma, especially, for helping me with the practical aspects of carrying out routine laboratory techniques as well as for giving me advice on the synthetic portion of this thesis.
- Dr. J Aucamp for performing the *in vitro* anti-leishmanial assays of synthesised compounds as well as instructing me on how to interpret the results obtained in these assays.
- Dr. D Otto and Dr. JHL Jordaan for NMR and HRMS spectroscopic analyses.
- Prof. F Van Der Kooy for HPLC analyses.
- The North-West University (NWU) and National Research Foundation (NRF) for their financial assistance.
- My parents, Jeff and Carien, for their continued love, guidance and emotional support. Also, for their earnest sacrifice and hard work that allowed me the opportunity to study at university.

ABSTRACT

Leishmaniasis is a disease resulting from the ingress of protozoan protists of the genus *Leishmania* (*L*.) into the mononuclear phagocytes of vertebrate hosts. These parasites are mainly transmitted by the haematophagous activities of female phlebotomine sand flies in the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World). Leishmaniasis is traditionally classified into three clinical forms, namely: visceral leishmaniasis (a febrile chronic infection associated with hepatosplenomegaly and cytopenias), cutaneous leishmaniasis (associated with chronic, slow-healing skin lesions and ulcers) and mucosal/mucocutaneous leishmaniasis (an oligoparasitic form of the disease characterised by ulceration and destruction of the mucosa of the nose, pharynx, larynx and mouth). The life cycle of *Leishmania* parasites alternates between two main morphological forms. These are the promastigote (an elongated, motile and flagellated form of the parasite found in the digestive tract and proboscis of sand flies) and amastigote forms (a rounded, non-motile and non-flagellated form of the parasite that usually infects the mononuclear phagocytes of vertebrate hosts).

Leishmaniasis is endemic to over 98 countries, where an estimated 1 billion people are at risk of contracting the disease. Approximately 12 million individuals are estimated to be infected at any single point in time. The exact number of new cases per year is not known with certainty. The World Health Organisation (2020) estimates that around 600,000 - 1 million new cases of cutaneous leishmaniasis, and some 50,000 - 90,000 new cases of visceral leishmaniasis, occur annually. Visceral leishmaniasis is also estimated to be directly responsible for causing over 20,000 - 40,000 deaths every year. Unfortunately, there is no single preventative, commercial vaccine or chemoprophylaxis available for use in humans. Presently, there are only a limited number of drugs available for the treatment of leishmaniasis. These include the pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), amphotericin B, miltefosine, pentamidine and paromomycin - all of whom have limitations in terms of their efficacy, toxicity, cost, route of administration, frequency of administration and duration of treatment. The treatment of leishmaniasis is also complicated by the occurrence of treatment failure and the development of drug resistance in parasites. All of the above serves to underscore the urgent need to develop novel anti-leishmanial agents. In view of this need, a series of fourteen 4(3H)-quinazolinone derivatives, comprising ten 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids and their synthetic precursors, were synthesised in low to excellent yields (30 - 89%) using cyclisation, condensation, nucleophilic (S_N2) substitution and copper-catalysed alkyne-azide cycloaddition (CuAAC) reactions. The anti-leishmanial activity and cytotoxicity of these hybrids, and their associated synthetic intermediates, were duly investigated in the present study.

The anti-leishmanial activities of the derivatives (expressed as percentage growth inhibition ± standard deviation (SD)) against the promastigotes of three strains of *Leishmania* parasite (*L. donovani* 1S and 9515, and *L. major* IR-173) were determined using a resazurin-based assay. Amphotericin B (AMB) served as the reference drug. The cytotoxicity (IC_{50}, or half-maximal concentration) of the derivatives against Vero cells (expressed as the mean (μM) ± standard deviation (SD)) was also determined using a resazurin-based assay, with emetine (EM) as the reference drug. All 4(3*H*)-quinazolinone derivatives were established to be sparingly soluble in the screening medium and were tested as suspensions. All synthesised derivatives and reference drugs were screened at single-point concentrations of 100 μM.

All derivatives in this study were found to be non-toxic to Vero cells (IC_{50} > 100 μM). Two derivatives were found to possess moderate anti-leishmanial activity. These were compounds **2a** (58% and 43% growth inhibition of *L. donovani* 1S and 9515 promastigotes, respectively) and **4b** (48% growth inhibition of *L. major* IR-173 promastigotes). Compound **2a** is characterised by the inclusion of a propargyl group substituted at position 3 of its 4(3*H*)-quinazolinone scaffold, while compound **4b** has a brominated 1*H*-1,2,3-triazole-containing moiety substituted in this position. Both compounds contain a styryl moiety substituted at position 2 of their respective 4(3*H*)-quinazolinone scaffolds. The lack of significant anti-leishmanial activity of the synthesised derivatives may be attributed to their poor solubility in the aqueous screening medium, the remediation of which may be relegated to future studies.

Keywords: leishmaniasis; promastigote; molecular hybridisation; quinazolinone; triazole.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

ABBREVIATIONS

Abs	Absorbance.
Ace	Acetone.
AMB	Amphotericin B.
BCG	Bacillus Calmette-Guérin (vaccine).
CDC	Centers for Disease Control and Prevention.
$CDCl_3$	Deuterated chloroform.
$CH_3C(OEt)_3$	Triethyl orthoacetate.
cLogP	Calculated logarithm of the partition coefficient (LogP).
Cpd	Compound.
CuAAC	Copper(I)-catalysed alkyne-azide cycloaddition (reaction(s)).
$CuSO_4.5H_2O$	Copper(II) sulphate pentahydrate.
DEET	N,N-Diethyl-meta-toluamide.
DMF	N,N-dimethylformamide.
DMSO	Dimethyl sulfoxide.
DMSO-d_6	Deuterated dimethyl sulfoxide.
DNA	Deoxyribonucleic acid.
EC_{50}	Half-maximal effective concentration.
EM	Emetine dihydrochloride hydrate.
Et_2O	Diethyl ether.
EtAc	Ethyl acetate.
EtOH	Ethanol.

HBA	Hydrogen bond acceptor.
HBD	Hydrogen bond donor.
HIV	Human immunodeficiency virus.
HPLC	High-performance liquid chromatography.
HRMS	High-resolution mass spectrometry.
Hz	Hertz.
IC_{50}	Half-maximal inhibitory concentration.
IR	Infra-red spectroscopy.
K_2CO_3	Potassium carbonate.
LogS	Logarithm of [a compound's aqueous] solubility.
$MgSO_4$	Magnesium sulphate.
MW	Molecular weight.
Na L-ascorbate	Sodium L-ascorbate.
$NaHCO_3$	Sodium bicarbonate.
NaN_3	Sodium azide.
NCE	Novel chemical entity.
NH_4OAc	Ammonium acetate.
NMR	Nuclear magnetic resonance spectroscopy.
NTD	Neglected tropical disease(s).
PARP	Poly-(ADP-ribose) polymerase.
PB	Propargyl bromide.
PCR assay	Polymerase chain reaction (assay).
PKDL	Post-kala-azar dermal leishmaniasis.

ppm	Parts per million.
qPCR assay	Quantitative polymerase chain reaction (assay).
rt	Room (ambient) temperature (~25 °C).
SAR	Structure-activity relationship.
SD	Standard deviation.
S_N2	(Bi-molecular) nucleophilic substitution reaction.
TLC	Thin-layer chromatography.
V_d	Volume of distribution.
WHO	World Health Organization.
β–CD	Beta-cyclodextrin.

CHAPTER 1

INTRODUCTION AND RATIONALE OF STUDY

1.1. Introduction

Leishmaniasis is a spectrum of vector-borne diseases caused by an obligate intracellular parasite of the genus *Leishmania* (Georgiadou *et al*, 2015:43). It is mainly transmitted by way of the bite of infected female phlebotomine sand flies, primarily those in the genera *Phlebotomus* (in the Old World, or Eastern Hemisphere) and *Lutzomyia* (in the New World, or Western Hemisphere). Leishmaniasis is caused by over twenty species of *Leishmania* parasites. Traditionally, leishmaniasis has been divided into three major clinical syndromes, namely, visceral, cutaneous and mucosal/mucocutaneous leishmaniasis (Pearson & De Queiroz Sousa, 1996:1).

Leishmaniasis constitutes one of several infectious diseases of the tropics and sub-tropics, termed neglected tropical diseases (NTDs), which remain understudied as a result of limited research funding (Fenwick, 2012:233-234). These diseases mainly affect the poorest of the poor, primarily in developing countries. Leishmaniasis is endemic to 98 countries where an estimated 1 billion people are at risk of contracting the disease (WHO, 2016:292). It has been estimated that approximately 600,000 - 1 million new cases of cutaneous leishmaniasis, and some 50,000 - 90,000 new cases of visceral leishmaniasis, occur every year (WHO, 2020). The number of annual deaths attributable to visceral leishmaniasis is also estimated to be around 20,000 - 40,000 (Bi *et al*, 2018). The burden of disease of leishmaniasis is concentrated mainly in a few major foci throughout the world (Torres-Guerrero *et al*, 2017:750). In 2018, more than 95% of new cases of visceral leishmaniasis reported to the World Health Organization (WHO) occurred in only ten countries: Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, Sudan and South Sudan (WHO, 2020). Similarly, over 85% of new cases of cutaneous leishmaniasis occurred in only ten countries: Afghanistan, Algeria, the Islamic Republic of Iran, Iraq, the Syrian Arab Republic, Pakistan, Bolivia, Brazil, Colombia and Tunisia. Global distribution patterns of leishmaniasis are graphically illustrated in detail in Figure 1.1 and Figure 1.2 (Pigott *et al,* 2014).

Estimates of the burden that leishmaniasis poses are likely to be underestimations due to severe under-reporting of the disease (Tabbabi, 2019:1330). In addition, estimates also fail to account for secondary effects such as the social stigma attached to externally-presenting forms of the disease (as in cutaneous leishmaniasis and post-kala-azar dermal leishmaniasis) as well as the economic impact the disease and its treatment has on individuals and their communities (WHO, 2010:105).

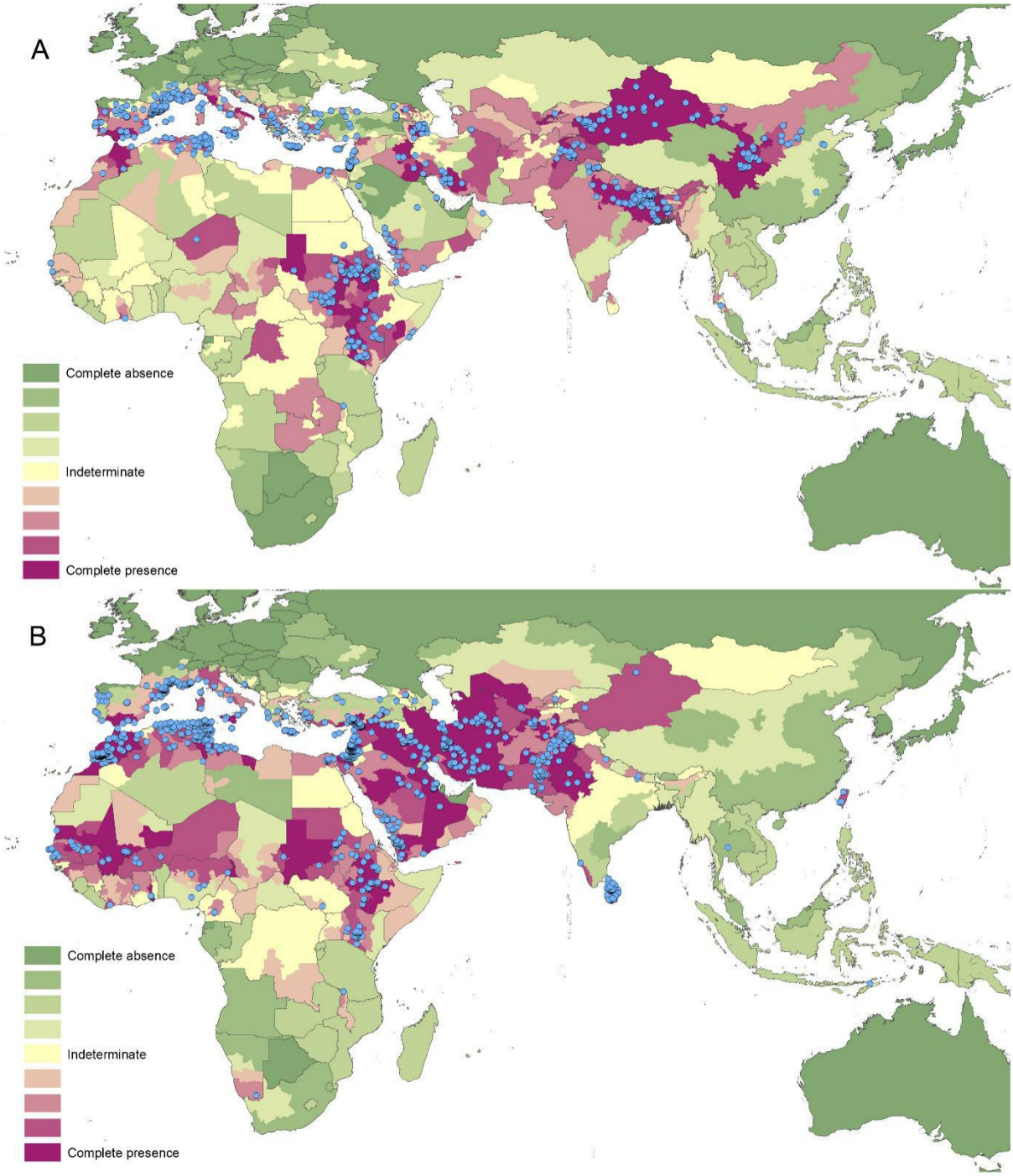

Figure 1.1. Reported geographical distribution of visceral and cutaneous leishmaniasis in the Old World based on empirical consensus (Pigott *et al,* 2014). A represents the geographical distribution of visceral leishmaniasis in the Old World (Eastern Hemisphere) and B represents the geographical distribution of cutaneous leishmaniasis in the Old World (Eastern Hemisphere). The blue spots indicate occurrence points or centroids of occurrences within small polygons.

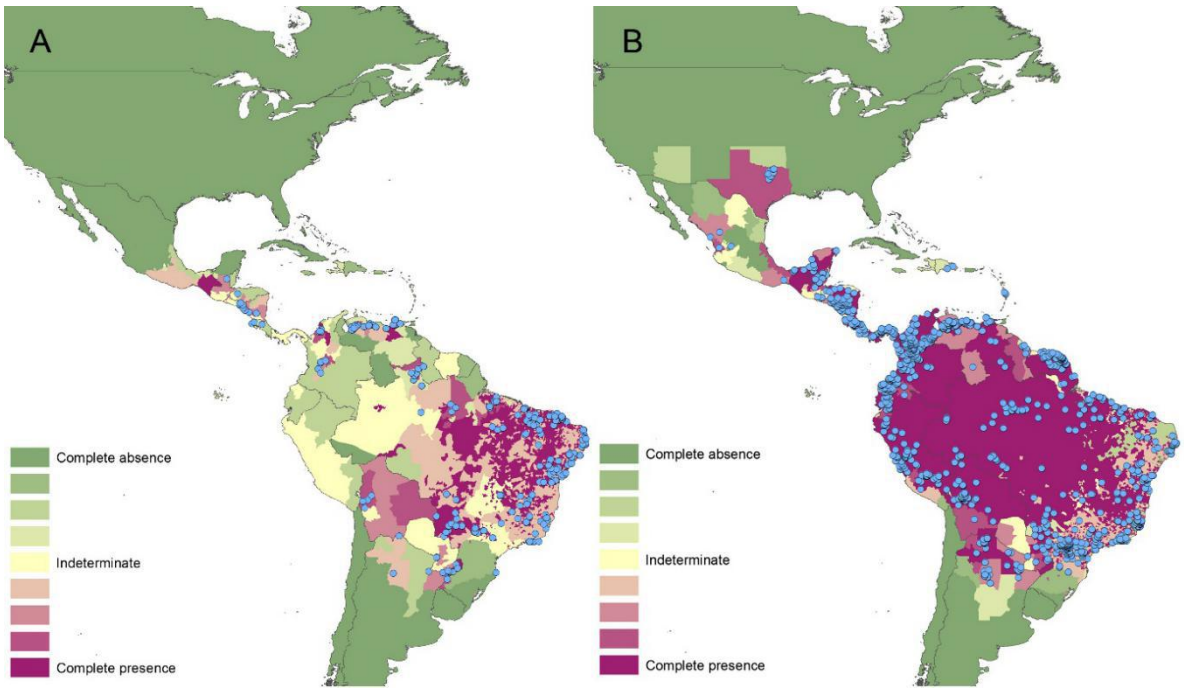

Figure 1.2. Reported geographical distribution of visceral and cutaneous leishmaniasis in the New World based on empirical consensus (Pigott *et al,* 2014). A represents the geographical distribution of visceral leishmaniasis in the New World (Western Hemisphere) and B represents the geographical distribution of cutaneous leishmaniasis in the New World (Western Hemisphere). The blue spots indicate occurrence points or centroids of occurrences within small polygons.

The problem that leishmaniasis poses is compounded by (i) the persistence of parasites in the tissues of the host's body, even after a successful clinical cure was fully affected (Conceição-Silva *et al*, 2018:1317-1318) and (ii) the occurrence of recrudescence and relapse of the disease, which is quite common, especially in those whose immune systems are compromised (Darcis *et al*, 2017:479-480; Vardy *et al*, 1999:914; Gradoni *et al*, 1996:234).

Currently, the co-infection of leishmaniasis with human immunodeficiency virus (HIV) represents an especially significant problem, as it is seen to intensify the burden of leishmaniasis by bringing about more severe forms of the disease that are difficult to manage (WHO, 2010:54). To date, leishmaniasis-HIV co-infection has been reported to occur in as many as 35 countries (Lindoso *et al*, 2016:148). The co-infection of HIV and leishmaniasis increases the likelihood of developing severe and atypical forms of leishmaniasis (WHO, 2010:54). Furthermore, leishmaniasis and HIV share an immunopathological mechanism wherein both are able to compromise the integrity of human dendritic cells and T helper (T_h or CD4) cells, which allows for the progressive worsening of both leishmaniasis and HIV, when the two diseases occur together in the same host (Garg *et al*, 2009; Bernier *et al*, 1995:7285).

The control, management and treatment of leishmaniasis are mainly effected by way of chemotherapy (Ponte-Sucre *et al*, 2017). Pentavalent antimonials, such as sodium stibogluconate and meglumine antimoniate, remain the primary chemotherapeutic agents with which leishmaniasis (regardless of causative species) is treated. The advent of resistance to pentavalent antimonial drugs necessitated the use of alternative agents such as miltefosine, amphotericin B (as both amphotericin deoxycholate and lipid formulations of amphotericin B), pentamidine and paromomycin. Even though treatment failure has been noted to occur with the use of most anti-leishmanial agents, its occurrence may be felt to be especially important with the use of miltefosine, as it is the only orally active anti-leishmanial agent currently available. The resistance of *Leishmania* parasites to miltefosine in rare cases, as well as in experimental laboratory settings, has been confirmed in the scientific literature (Deep *et* al, 2017; Ponte-Sucre *et al*, 2017; Srivastava *et al*, 2017:49).

Amphotericin B is highly efficacious as an anti-leishmanial agent, although its use is associated with the precipitous occurrence of toxicity and/or adverse events (Sundar *et al*, 2019:795 - 798). Toxicity and adverse events are more likely to occur with the use of the free deoxycholate form of amphotericin B. Lipid formulations of amphotericin B are similar to amphotericin B deoxycholate in terms of their efficacy but are significantly less toxic. The resistance of *Leishmania* parasites to amphotericin B (in rare cases and experimental laboratory settings), as well as several incidences of treatment failure associated with the use of amphotericin B, have been reported in the literature (Eichenberger *et al*, 2017; Morizot *et al*, 2016; Purkait *et al*, 2015:1031; Brotherton *et al*, 2014:126; Al-Mohammed *et al*, 2005:3274; Mbongo *et al*, 1998:357).

In addition to the above concerns, the disease presents those affected with a host of socio-economic problems. Leishmaniasis is observed to be related to poverty in a complex manner (Alvar *et al*, 2006:552). Even though poverty increases the risk for leishmaniasis, and aggravates the progression of the disease, leishmaniasis itself leads to further impoverishment of individuals and their families/communities (WHO, 2010:86). This may result from factors such as (i) increased expenditure on health care, (ii) the loss of income due to inability to gain employment or the inability to work and (iii) the deaths of those who are the prime breadwinners for their respective families and communities. The cost of effective anti-leishmanial therapeutic modalities in poverty-stricken countries (which are incidentally the countries that tend to have the highest burden of leishmanial disease) continues to be high and relatively unaffordable, despite price negotiations and price reductions instituted by the World Health Organization (WHO, 2010:89). All of the above, taken together, highlights the importance of investing in research to develop promising drugs/agents and therapeutic modalities against *Leishmania* parasites that are safe, cost-effective and clinically efficacious.

1.2. Rationale for this study

1.2.1. Molecular hybridisation

Molecular hybridisation is an approach employed in rational drug design that is based on the combination of pharmacophoric sub-units of different biologically active molecules to produce a novel hybrid compound with improved binding affinity and efficacy, compared to the parent molecules (Viegas-Junior et al, 2007:1829). The new hybrid thusly produced maintains/augments the pre-selected characteristics of the parent compounds. Hybridised drugs have the added advantage of being able to provide combination therapies in the form of single multi-functional agents that may provide more potent and targeted disease treatment than their non-hybridised counterparts (Bérubé, 2015:281). The molecular hybridisation approach may be used to improve the selectivity and side effect profile(s) of a compound and to produce compounds with altered or dual modes of action (Viegas-Junior *et al*, 2007:1829). The pharmacokinetics and pharmacodynamics of a given compound may also be improved via molecular hybridisation (Pawełczyk et al, 2018). Molecular hybridization has also been extensively used to produce agents with pronounced anti-leishmanial and anti-trypanosomal activities (Cardona-G *et al*, 2015).

1.2.2. Quinazolinones

Quinazolinones are a curious class of fused nitrogen-containing heterocyclic compounds that are known in the scientific literature for displaying a vast array of biological activities. These biological activities include activities as diverse as anti-cancer (Hu *et al*, 2012; Abouzid & Shouman, 2008), anti-bacterial (Nasab *et al*, 2017; El-Badry *et al*, 2012), anti-fungal (Ghorab *et al*, 2013; Ryu *et al*, 2012), anti-diabetic (Saeedi *et al*, 2019), hypnotic/sedative (Hammer *et al*, 2015), analgesic (Abdel-Aziz *et al*, 2016), anti-convulsant (Rajasekaran *et al*, 2013; Georgey *et al*, 2008), anti-tubercular (Khosropour *et al*, 2006), anti-viral (Krishnan *et al*, 2011), anti-malarial (Zhu *et al*, 2009) and anti-inflammatory (Zayed & Hassan, 2014) activities, amongst others (Kshirsagar *et al*, 2015). A small number of quinazolinone-containing drugs are currently in use as therapeutic modalities and are available for purchase in medical/healthcare markets (Hameed *et al*, 2018:281, 283). This includes drugs such as afloqualone (Ochiai, T. & Ishida, 1982), diproqualone (Audeval *et al*, 1988), halofuginone (Sundrud *et al*, 2009), methaqualone (Smyth *et al*, 1973:391), nolatrexed (Jodrell *et al*, 1999), quinethazone (Cohen & Vaughan, 1960), raltitrexed (Widemann *et al*, 1999), tiacrilast (Welton *et al*, 1986) and proquazone (Clissold & Beresford, 1987). A number of naturally occurring quinazolinones have also been isolated, identified and tested for biological activity in the last two decades (Kshirsagar *et al*, 2015:9336-9341). Amongst the myriad of these are alkaloids such as vasicinone and tryptanthrin with potent anti-leishmanial activities (Michael,

2003:485-486).

Quinazolinones are considered a privileged structure, that is, a molecular scaffold that is capable of producing a multitude of ligands that are able to potently and selectively bind to one or several biological targets through alteration of the functional groups in its molecular structure (Jafari *et al*, 2016:12, DeSimone *et al*, 2004). Privileged structure-based drug discovery has emerged as an approach in medicinal chemistry that yields very fruitful results due to its ability to find promising drug candidates in a very short time frame (Costantino & Barlocco, 2006). Privileged structures may be used to engender agents with a wide range of biological activities or, barring the ability to produce agents that elicit full activity in biological target(s), may also be used to produce compounds that exhibit drug-like properties. Thus, these kinds of structures are considered to be potentially important building blocks in medicinal chemistry and are invaluable in the ongoing effort to discover and develop viable drug candidates.

1.2.3. 1,2,3-Triazoles

In a manner similar to the quinazolinones, the 1,2,3-triazole-based heterocycles have been used to generate a multitude of medically useful compounds that exhibit a range of biological activities (Dheer *et al*, 2017:34; Agalave *et al*, 2011:2699; Tron *et al*, 2008). These biological activities include anti-microbial (Genin *et al*, 2000; Willner *et al,* 1972), anti-bacterial (Gregory *et al*, 1989), anti-fungal (Holla *et* al, 2005; Dong *et al*, 2001), anti-epileptic (Pålhagen *et al*, 2001), anti-inflammatory (Dong *et al*, 2001), analgesic (Dong *et al*, 2001), anti-retroviral (De Clercq, 2002; Alvarez *et al*, 1994), anti-obesity (Brockunier *et al*, 2000), anti-coccidial (Bochis *et al*, 1991), anti-allergic (Buckle, 1985), anti-neoplastic (Al-Masoudi & Al-Soud, 2002), anti-plasmodial (Raj *et al*, 2013), anti-anxiety (Martini *et al*, 1988) and anti-cancer (Norris *et al*, 1996) activities. The broad range of activities that may be elicited in biological targets by the 1,2,3-triazoles, coupled with the ease of their synthesis, have established the 1,2,3-triazole moiety as a promising and pharmacologically significant scaffold in medicinal chemistry (Totobenazara & Burke, 2015:2853; Kharb *et al*, 2011:1). A testament to this fact is the existence of a number of 1,2,3-triazole-containing compounds that are currently marketed for use as established drugs for a variety of diseases (Dheer *et al*, 2017:32). Drugs containing the 1,2,3-triazole moiety that are currently available for purchase on health care markets include the β-lactam anti-biotic tazobactam (Aziz Ali *et al*, 2017:3698; Zhang *et al*, 2017:501), the anti-fungal agent ravuconazole (Teixeira de Macedo Silva *et al*, 2018:2362) and the cephalosporin anti-biotic cefatrizine (Aziz Ali *et al*, 2017:3698; Zhang *et al*, 2017:501). Additionally, the development of 1,2,3-triazoles hybridised with other biologically active moieties/pharmacophores that possess anti-leishmanial activities has been thoroughly documented in the scientific literature (Dheer *et al*, 2017:39-41; Haider *et al*, 2014). Triazoles

(of which 1,2,3-triazoles are one sub-class) are a category of compounds that are also known to be active against other protozoal parasites, such as those in the genera *Plasmodium* and *Trypanosoma* (Uliassi *et al*, 2018). The usefulness of 1,2,3-triazoles even extends beyond the domain of medicinal chemistry to areas of study such as organic chemistry, materials science, food science as well as the biological sciences (Liu *et al*, 2018:650). This unanimously establishes the 1,2,3-triazoles as a class of compounds whose potential for use/research has not been exhausted.

1.2.4. Concluding rationale

Taking into account the rational drug design approaches and the promising biological activities mentioned so far, this study endeavours to investigate whether or not the derivatisation of 4(*3H*)-quinazolinones, by way of hybridization with another biologically active pharmacophore - the *1H*-1,2,3-triazole moiety - will yield anti-leishmanial drug-like candidates. This study will focus on the hybridisation of the 4(*3H*)-quinazolinone structural isomer and will take as its point of departure the work done by others such as Birhan *et al* (2014). Indeed, Birhan and colleagues (2014) were able to synthesise a series of 2,3-disubstituted-4(3H)-quinazolinones that compared favourably with existing anti-leishmanial agents such as miltefosine and amphotericin B deoxycholate. By deviating slightly from the scaffold proposed by Birhan and others (2014) (Figure 1.3 A), we instead propose an approach where a similar 2,3-disubstituted-4(3*H*)-quinazolinone molecular framework is synthesised, albeit with a 1*H*-1,2,3-triazole-containing moiety at the 3-position of the quinazolinone scaffold (Figure 1.3 B). This would permit us to hybridise *1H*-1,2,3-triazoles with 4(3*H*)-quinazolinones so as to produce molecules with the potentially promising biological activities constitutive of both scaffolds. Intermediates in the synthetic route will similarly be investigated for their potential anti-leishmanial activity.

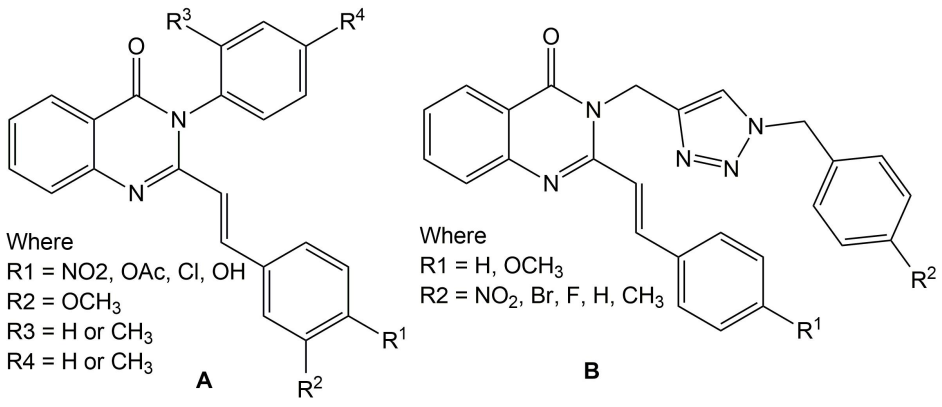

Figure 1.3. Molecular scaffold structures. A represents the scaffold structure of Birhan *et al* (2014) and B represents the scaffold structure being investigated (for its anti-leishmanial activity) in the current study.

1.3. Aims and Objectives

The aim of this study is to investigate *1H*-1,2,3-triazole-4(*3H*)-quinazolinone hybrids, with the ultimate goal of developing an entirely novel class of anti-leishmanial compounds that are safe, clinically efficacious and cost-effective to produce and disseminate.

Objectives of this study:

- Multi-step synthesis of novel 4(*3H*)-quinazolinone and *1H*-1,2,3-triazole hybrids.
- Assessment of the *in-vitro* anti-leishmanial activity of the synthesised compounds.
- Assessment of the safety profiles of the synthesised compounds using mammalian cell lines.

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CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Leishmaniasis is a diverse collection of diseases characterised by an infection with an obligate intracellular parasite of the genus *Leishmania,* in the order Kinetoplastida, class Trypanosomatida (Akhoundi *et al*, 2016). The clinical syndromes and presentation of leishmaniasis are considerably varied but may be classified into three distinct groupings, namely visceral (the most severe form of leishmaniasis), cutaneous (the most common form of leishmaniasis) and mucocutaneous leishmaniasis (WHO, 2020). Cutaneous leishmaniasis, in turn, is divided into Old World cutaneous leishmaniasis (caused by species that occur in the Eastern Hemisphere) and New World cutaneous leishmaniasis (caused by species found in the Western Hemisphere) (Shin *et al*, 2013:80). Each of the aforementioned clinical syndromes can be caused by more than one species of *Leishmania*, and a singular species of *Leishmania* is able to produce more than one clinical syndrome (see Table 2.1).

Leishmaniasis is still a disease of major concern in public health and the burden of disease thereof is steadily increasing (Hotez, 2018:421; Bailey *et al,* 2017; Desjeux, 2004:308; Desjeux, 2001:239-242). Of further concern is the fact that the reported number of *Leishmania* infections is currently inordinately underestimated (Tabbabi, 2019:1330; Alvar *et al*, 2012). Similarly, the full scope of the severity of leishmanial infections as well as the socio-economic impact are unknown (Bailey *et al,* 2017; Desjeux, 2004:310-311). The disease is endemic to 98 countries, where an estimated 1 billion people are at risk of being infected with the illness (WHO, 2016:292). Around 12 million people suffer as a result of leishmanial infection (Ribeiro *et al*, 2018), with 50,000 - 90,000 new cases of visceral leishmaniasis and some 600,000 - 1 million new cases of the cutaneous forms of the disease, estimated to occur every year (WHO, 2020). Visceral leishmaniasis also causes a large number of deaths every year (around 20,000 - 40,000) (Bi *et al*, 2018). Leishmaniasis constitutes one of several neglected tropical diseases (NTD), a group of infectious diseases that remain understudied and underfunded due to a multitude of reasons, ranging from social stigma to a lack of proper economic incentives (Feasey *et al*, 2009:180). In addition, access to anti-leishmanial drugs and therapeutic modalities are limited and these treatments are also fairly expensive (WHO, 2010:89). The problem that leishmaniasis represents is further compounded by the emergence of increasing resistance to existing therapeutic modalities as well as the occurrence of treatment failure (Ponte-Sucre *et al*, 2017).

2.2. Life Cycle

Leishmania is a genus of protozoal parasite that is largely homozygous and diploid, with 34 - 36 chromosomes that vary in size from 300 - 3000 kilobases (kb), depending on the species (Uliana *et al*, 2007). The genus is primarily dimorphic, occurring as the extracellular promastigote form in the insect vector (phlebotomine sand fly) and as the intracellular amastigote form in the vertebrate host (Pearson & De Queiroz Sousa, 1996:1). The invertebrate hosts (vectors) of *Leishmania* are insects of the order Diptera, in the family Psychodidae (subfamily: Phlebotominae) (Akhoundi *et al*, 2016). These small flying insects are colloquially known as sand flies. The subfamily consists of six genera: *Phlebotomus, Sergentomyia* and *Chinius* (in the Old World); as well as *Lutzomyia*, *Brumptomyia* and *Warileya* (in the New World). Of these genera, only *Phlebotomus* and *Lutzomyia* are of interest to the medical sciences, as all known vectors of *Leishmania* are of these two genera. The phlebotomine sand fly species responsible for the transmission of *Leishmania* varies by geographical location and the species of *Leishmania* being transmitted (Killick-Kendrick, 1999:282-283; Killick-Kendrick, 1990:3-12). Two types of phlebotomine vectors of *Leishmania* may be identified, namely, permissive vectors (able to support the development of several *Leishmania* species) and restrictive vectors, whose ability to support development of parasites is limited only to certain species of *Leishmania* (Sádlová *et al*, 2003:248; Kamhawi *et* al, 2000:25-26,31; Pimenta *et al*, 1994:9155,9159).

The life cycle of a *Leishmania* parasite starts in the phlebotomine insect vector's intestinal tract and proboscis, where the *Leishmania* parasite is found to occur as a metacyclic promastigote (a narrowed highly motile form of the parasite, measuring 5-8 µm). It is this form of the parasite that is transmitted to the vertebrate host when the female phlebotomine sand fly takes a blood meal (Bates *et al*, 2004:601-606). After inoculation into the vertebrate host, the metacyclic promastigote is phagocytised by the macrophages and other mononuclear phagocytic cells of the host. Once phagocytised, the metacyclic promastigote will transform into the intracellular form of the parasite, namely, the amastigote form (an ovoid or round, non-motile form of the parasite that lacks an exteriorised flagellum). The amastigote form is typically found in parasitophorous vacuoles, a structure produced by the parasite that allows them to evade the host's immune defences (Courret *et al*, 2002:2303). Amastigotes will multiply via binary fission and eventually rupture the host mononuclear phagocytic cells, releasing a multitude of amastigotes to once again infect other mononuclear phagocytic cells (Liu & Uzonna, 2012). More mononuclear phagocytic cells will subsequently make their way to the original infective site and become infected. The amastigotes can also spread to other regions of the host's body by way of the lymphatic and circulatory system, where they will then be able to infect other mononuclear phagocytic cells (Steverding, 2017).

Table 2.1. *Leishmania* species and their associated clinical syndromes and geographical distribution.

***Leishmania* species**	**Clinical syndrome**	**Geographic distribution**
Leishmania (L.) donovani.	Visceral leishmaniasis (Kala-azar).	Indian subcontinent, northern and eastern China, Pakistan, Nepal.
	Post-kala-azar dermal leishmaniasis.	Indian subcontinent.
Leishmania (L.) infantum.	Visceral leishmaniasis (Kala-azar).	Middle East, Mediterranean littoral, Balkans, central and south-western Asia, northern and north-western China, northern and sub-Saharan Africa.
	Old World cutaneous leishmaniasis.	Mediterranean basin.
Leishmania (L.) donovani.	Visceral leishmaniasis (Kala-azar).	Sudan, Kenya, Ethiopia.
(archibaldi).	Old World cutaneous leishmaniasis.	Sudan, East Africa.
Leishmania (L.) chagasi.	Visceral leishmaniasis (Kala-azar).	Latin America.
	New World cutaneous leishmaniasis.	Central and South America.
Leishmania (L.) amazonensis.	Visceral leishmaniasis (Kala-azar).	Brazil (Bahia State).
	New World cutaneous leishmaniasis.	Amazon basin, neighbouring areas, Bahia and other states in Brazil.
	Diffuse cutaneous leishmaniasis.	Amazon basin, neighbouring areas, Bahia and other states in Brazil.
Leishmania (L.) tropica.	Visceral leishmaniasis (Kala-azar).	Israel, India, and viscerotropic disease in Saudi Arabia (U.S. troops).
	Old World cutaneous leishmaniasis.	Mediterranean littoral, Middle East, western Asiatic area, Indian subcontinent.
Leishmania (L.) major.	Old World cutaneous leishmaniasis.	Middle East, north-western China, north-western India, Pakistan, Africa.
Leishmania (L.) aethiopica.	Old World cutaneous leishmaniasis.	Ethiopian highlands, Kenya, Yemen.
	Diffuse cutaneous leishmaniasis.	Ethiopian highlands, Kenya, Yemen.
Leishmania (L.) mexicana.	New World cutaneous leishmaniasis.	Central America, Mexico, Texas.
	Diffuse cutaneous leishmaniasis.	Mexico and Central America.
Leishmania (V.) braziliensis.	New World cutaneous leishmaniasis.	Multiple areas of Central and South America.
	Mucosal leishmaniasis.	Multiple areas of Latin America.
Leishmania (V.) guyanensis.	New World cutaneous leishmaniasis.	Guyana, Surinam, northern Amazon basin.
Leishmania (V.) peruviana.	New World cutaneous leishmaniasis.	Peru (western Andes), Argentinean highlands.
Leishmania (V.) panamensis.	New World cutaneous leishmaniasis.	Panama, Costa Rice, Colombia.
Leishmania (V.) pifanoi.	New World cutaneous leishmaniasis.	Venezuela.
	Diffuse cutaneous leishmaniasis.	Venezuela.
Leishmania (V.) garnhami.	New World cutaneous leishmaniasis.	Venezuela.
Leishmania (V.) venezuelensis.	New World cutaneous leishmaniasis.	Venezuela.

Where (*L.*) and (*V.*) refer to subgenus *Leishmania* and subgenus *Viannia*, respectively. Pearson & Sousa (1996:2).

The life cycle of a *Leishmania* parasite will be complete when a female phlebotomine sand fly takes a blood meal and ingests the parasitised cells of the vertebrate host (Liu & Uzonna, 2012). Once in the intestinal tract of the sand fly, the *Leishmania* parasite will develop through a series of flagellated (promastigote) stages (procyclic promastigote, nectomonad promastigote and leptomonad promastigote) to the final infective form of the parasite capable of infecting vertebrate hosts, namely, the metacyclic promastigote (Bates *et al*, 2004:601).

2.3. Taxonomy

The taxonomy of *Leishmania* parasites is tentative, complex, and subject to constant revision based on scientific understanding of the polymorphic patterns in the DNA markers, proteins and antigens of these parasites (Akhoundi *et al*, 2016; Bañuls *et al*, 2007:6-8). In the past, the taxonomical classification of *Leishmania* was based on a variety of miscellaneous criteria, including but not limited to: geographical distribution, epidemiological considerations, clinical manifestations, and biological characteristics such as tropism and antigenicity.

The genus of *Leishmania* is divided into two subgenera (*Leishmania* and *Viannia)* based on where the promastigote form of the parasite develops in the intestinal tract of the phlebotomine sand fly. Species of the subgenus *Viannia* develop in the hindgut before migrating to the foregut (peripylaria – intestinal tract anterior and inferior to the pylorus); whereas those of the subgenus *Leishmania* develop in the midgut and foregut (suprapylaria - intestinal tract anterior to the pylorus) (Rioux *et al,* 1990:111). *S*pecies in the subgenus *Viannia* are primarily endemic to Latin America, while species from the subgenus *Leishmania* occur throughout the world (see Table 2.1.).

Isozyme analyses (using electrophoresis) were used to define species complexes within the aforementioned two subgenera (Rioux *et al*, 1990). Initially, past taxonomical classifications of *Leishmania* species were based on miscellaneous criteria (as mentioned above), but since the 1980s intrinsic criteria such as biomolecular markers have been used to develop cladistic classification systems (Tibayrenc & Ayala, 1999:470). An example of a taxonomic scheme based on biomolecular markers is depicted in Figure 2.1.

2.4. Epidemiology

The large majority of cases of visceral leishmaniasis occur in three major foci distributed throughout disparate and unconnected parts of the world. The first of these foci (which accounts for around 67% of all reported cases of visceral leishmaniasis) consists of three Asiatic countries. These are the lowland regions of northern India and southern Nepal (termed the “Tarai or Terai”), as well as areas of Bangladesh with a similar biome (Saha *et al*, 2017). The second focus is limited to countries in the eastern portion of Africa, particularly in and around the Ethiopian-Sudan border (Zijlstra & El-Hassan, 2001), while the third and final focus is confined to the rural and peri-urban areas of eastern and north-eastern Brazil (Dupnik *et al*, 2011).

It has been well established that visceral leishmaniasis occurs with increased frequency in immunocompromised individuals such as, for example, the malnourished and those who have received organ transplants (Akuffo *et al*, 2018). The most notable contributing cause of developing visceral leishmaniasis in the immunocompromised individual is the human immunodeficiency virus (HIV). The majority of reported cases of leishmaniasis and HIV co-infection in the scientific literature are primarily from countries in southern European such as Spain, France, Portugal and Italy (Alvar *et al*, 2008:335).

Cutaneous leishmaniasis is found to be a major problem in large populations of susceptible individuals, especially when these populations are exposed as whole groups to the causative *Leishmania* or its vectors. The large-scale exposure of whole populations has been observed to occur with military operations and soldiers dispatched to serve in Iraq and Afghanistan (CDC, 2004; CDC, 2003), those who settle in an exposed area with the intent of setting up permanent residence (Du *et al*, 2016), as well as in travellers who travel to and from endemic areas (Magill, 2005). The majority (95%) of the world’s cutaneous leishmaniasis cases occur in Middle Eastern countries such as Iran (Islamic Republic of), the Syrian Arab Republic and Saudi Arabia; Central Asian countries such as Afghanistan; the Mediterranean basin and Latin America (Brazil, Bolivia and Colombia) (WHO, 2020, Reithinger *et al*, 2007:581). Finally, the majority (90%) of mucocutaneous leishmaniasis cases are limited to the four countries of Bolivia, Brazil, Peru, and Ethiopia (WHO, 2020).

The vertebrate reservoirs of *Leishmania* parasites are mainly limited to murid rodents, canids, edentates, procyanids, marsupials, primitive ungulates and primates (Akhoundi *et al*, 2016; Lainson & Shaw, 1992:95). Occasionally, vertebrate hosts may be infected by means other than the bite of the phlebotomine vector. These means include infection by way of blood transfusion (Dey & Singh, 2006), congenital transmission (Meinecke *et al*, 1999), sexual contact (St. C. Symmers, 1960), usage and sharing of contaminated needles amongst intravenous drugs users (Cruz *et al*, 2002), and occupational exposure (Herwaldt, 2001).

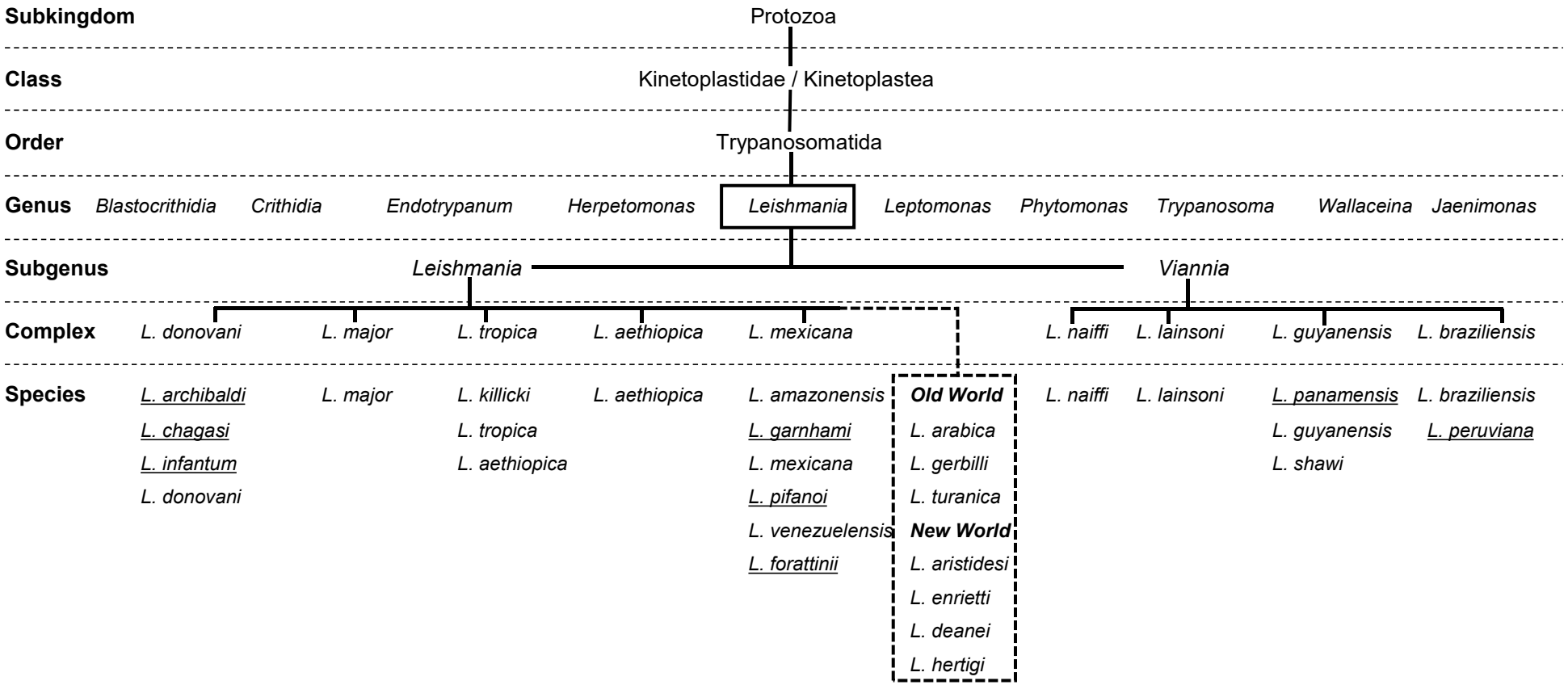

Figure 2.1. Taxonomy of the genus Leishmania. The taxonomical validity of underlined species is or has been disputed. The species in the dashed box represent species from the subgenus Leishmania who are considered non-pathogenic in human beings. Adapted from the taxonomy scheme published by the World Health Organization (WHO, 2010:6).

2.5. Signs and Symptoms

2.5.1. Visceral leishmaniasis

Symptoms of visceral leishmaniasis (synonyms: kala-azar, Dum-dum fever) include an irregular fever, enlargement of the spleen and liver (hepatomegaly and splenomegaly), deficiency of all three cellular components (white blood cells, red blood cells and platelets) of the blood (pancytopenia), and polyclonal gammopathy (Varma & Naseem, 2010:78). Emaciation and eventual death are common in those with progressed infections that have subsisted for extended periods of time (Coura-Vital *et al*, 2014). It is common for resistance to develop against *Leishmania* parasites (provided that there is no impairment in the body's cell-mediated immunity) in those who have survived a bout of visceral leishmaniasis (following successful treatment) and those who were asymptomatic but whose infection spontaneously resolved without any intervention (Nylén & Gautum, 2010:142). A form of dermal leishmaniasis, termed post-kala azar dermal leishmaniasis, may result in those who have been treated for visceral leishmaniasis (Zijlstra *et al*, 2003:87). Post-kala azar dermal leishmaniasis is characterized by the development of flat macular, maculopapular or nodular cutaneous lesions that contain a concentrated multitude of infective parasites. It usually occurs in East Africa and India in those who have been treated for visceral leishmaniasis. The lesions that characterize post-kala azar dermal leishmaniasis (PKDL) usually develop immediately but can also develop within six months to several years after the treatment of visceral leishmaniasis. The lesions of PKDL contribute to the spread of infection, as they act as reservoirs of parasites in interepidemic periods of visceral leishmaniasis (Zijlstra *et al*, 2003:87).

2.5.2. Cutaneous leishmaniasis

Cutaneous leishmaniasis (synonyms: oriental sore, tropical sore, Delhi boil, Aleppo boil, uta ulcer, chiclero ulcer or forest yaws) is characterized by the development of a skin lesion at the site of the sand fly bite (Bilgic-Temel, 2019:159-160). The borders of the lesion are usually erythematous, raised and well-demarcated, with an initial presentation as a small papule that slowly enlarges and ulcerates (usually from the centre outwards). These ulcers are typically painless and do not usually cause systemic symptoms unless the ulcer becomes secondarily infected. Ulcers may heal to form a scar that superficially resembles that of a healed burn-wound with a shallow central depression. The lesions exhibit a concentrated number of intracellular *Leishmania* parasites, even when the lesion has sufficiently healed (Mendonça *et al*, 2004:1018). Persons infected with *Leishmania* parasites may present with multiple lesions as a result of metastasising infection or due to a large number of individual sand fly bites (Hartley *et al*, 2014:412). A very rare form of cutaneous leishmaniasis, termed diffuse

cutaneous leishmaniasis, can result in those with an abnormal/absent immune response to invading *Leishmania* parasites (Convit *et al*, 1972:603). Symptoms specific to diffuse cutaneous leishmaniasis include: (i) a multitude of diffusely spread cutaneous lesions on several areas of the body, (ii) a high number of parasites inside the cutaneous lesions, (iii) anergy to skin tests (Montenegro test/Leishmanin skin test), (iv) slow and chronic progression of the disease, and (v) resistance to treatment or frequent relapse of the disease. Leishmaniasis recidivans, another form of cutaneous leishmaniasis, is a term given to a recurrent form of cutaneous leishmaniasis in which cutaneous lesions reoccur at, or close to, the original site of a previous (healed) cutaneous lesion (Torres-Guerrero *et al*, 2017).

2.5.3. Mucocutaneous/mucosal leishmaniasis

Mucocutaneous/mucosal leishmaniasis (synonyms: espundia) occurs as a result of the spread of infective parasites from the site of a cutaneous lesion(s), via the circulatory and lymphatic systems of the body, to the mucous membranes of the nose, oral cavity, pharynx or larynx where the parasites will be deposited and subsequently accumulate (Marra *et al*, 2014:3). Mucosal involvement typically occurs after the primary skin lesions have subsided, although mucosal involvement may occur concurrently with cutaneous manifestations of leishmaniasis (Cannella *et al*, 2011:847). Initially, those with mucosal involvement may experience symptoms such as discomfort, nasal discharge, nasal congestion, pain, inflammation, bleeding of the nose (epistaxis) or progressive structural deformity (Marsden, 1986:861). Small lesions eventually begin to develop on the mucous membranes of the nose, buccal cavity, pharynx and larynx. These lesions might go on to progressively ulcerate and cause gross mutilations of the nose, palate and face. The term “espundia” is generally used to refer to a kind of mucosal/mucocutaneous leishmaniasis where the sufferer presents with hypertrophy of the nose and lips (Larson & Marsden, 1987:880).

2.6. Diagnosis

Leishmaniasis is mainly diagnosed by identifying the *Leishmania* parasite, that is to say, both the amastigote and promastigote forms, in tissue cultures or smears (Sundar & Rai, 2002:951). The parasites are usually found in the mononuclear phagocytic cells of the vertebrate host (especially macrophages) and can be made to appear more pronounced through the use of Wright- and Giemsa-stains (Sundar & Rai, 2002:952). In Wright- and Giemsa-stained preparations the amastigote’s cytoplasm appears blue while the nucleus appears red and is seen to be situated eccentrically (off-centre). The amastigotes have a very distinct structure termed a kinetoplast, a specialised rod-like mitochondrial structure that is comprised of a large mass of extra-nuclear DNA. Visual observation of the kinetoplast confirms the diagnosis of *Leishmania* infection. As new technology emerges, it is very likely

that clinicians will be able to diagnose leishmaniasis by making use of polymerase chain reaction (PCR) assays. Unfortunately, a standardised rapid PCR assay for use in clinical settings is currently lacking (Galluzzi *et al*, 2018). Visceral leishmaniasis may be diagnosed with great accuracy by means of fine-needle aspiration of the spleen or bone marrow. The biological sample so obtained may then be grown as a tissue culture or it may be used to produce a touch preparation. Other tissues that can be used include those obtained from enlarged lymph nodes, the liver and the buffy coat of peripheral blood (Sundar & Rai, 2002:951-952). In the case of cutaneous leishmaniasis, a punch biopsy or aspirate of the margins of a dutifully cleaned skin lesion may be obtained and prepared for tissue culture, touch preparation (a technique in which a clinical specimen is dabbed onto a microscope slide to distribute a thin layer of cells for microscopic examination) and other modes of histopathological examination (Pearson & De Queiroz Sousa 1996:7-8). Immunologically orientated methods of diagnosis may be used as an adjunct in suspected cases of leishmaniasis. These methods include tests and assays such as antibody tests, cell-mediated assays and skin tests (Pearson & De Queiroz Sousa 1996:7-8). Ideally, in those forms of leishmaniasis in which the parasitic burden is low or not easily detectable by the usual means of diagnosis (such as in the case of mucocutaneous leishmaniasis and leishmaniasis recidivans), the more appropriate method of parasitological detection is a PCR assay. Quantitative PCR (qPCR) techniques may, in future, prove more specific in diagnosing acute forms of leishmaniasis. However, the proper standardization of PCR techniques, as well as the evaluation of their accuracy in being able to diagnose leishmaniasis in clinical settings, is still needed. (Galluzzi *et al*, 2018).

2.7. Prevention

There are a multitude of preventative measures that provide protection to individuals and their communities against sand fly bites. These protective measures will, by implication, also act to prevent the spread and contraction of leishmaniasis, provided that these measures are implemented and used correctly. One such a measure, considered to be a standard amongst personal protective measures, is the impregnation of clothes and fine-mesh bed nets with insecticides and/or insect-repellents such as N,N-diethyl-meta-toluamide (DEET or diethyltoluamide) and permethrins (Soto *et al*, 1995:599). However, the use of personal protective measures to prevent leishmaniasis, especially the use of impregnated bed nets, is not without controversy. The demonstrable efficacy of personal protective measures is variable, with some studies showing that these measures offer some degree of protective effect, while others have found no such protective effects (Dinesh *et al*, 2008; Courtenay *et al*, 2007). Treatment and prevention of leishmaniasis in reservoirs that play host to *Leishmania* parasites is another option in areas with both human and animal reservoirs. One approach,

the impregnation of dog collars with insecticides, has been proven to be a particularly effective preventative measure. This is of great importance as canids (both wild and domesticated) are major reservoirs of *Leishmania,* which means that the reduction of infection in these animals might contribute significantly to the reduction of human leishmanial infection (Mazloumi *et al,* 2002; Ashford *et al*, 1998). The identification and treatment of active or asymptomatic cases of leishmanial infections in human hosts is important as well, as greater proximity to infected persons increases the risk of transmission (Fakhar *et al*, 2008; Sakru *et al*, 2006).

Currently, there are no commercially available vaccines, although occurrences such as the spontaneous resolution of leishmanial infection in human beings suggest that there is no reason not to expect vaccines in the future (Khamesipour *et al*, 2005:423). The first efforts to develop vaccines have resulted in the first-generation vaccines which were generally intended to protect against infection with *Leishmania.* These vaccines were typically comprised of whole attenuated parasites that were administered with/without the Bacillus Calmette-Guérin (BCG) vaccine as an adjuvant (Noazin *et al*, 2008:6760). Despite their demonstrable safety and slight immunogenic effects, the overall failure of the first-generation vaccines paved the way for the development of the second- and third-generation vaccines. The efforts currently being put towards producing mass-marketable second-generation and third-generation vaccines include identifying and isolating anti-leishmanial immunogens and adjuvants, as well as the probable future use of live genetically engineered (non-pathogenic) strains of *Leishmania* parasites (Palatnik-de Sousa, 2008:1712-1718; Coler *et al*, 2007:245-247; Coler & Reed, 2005; Skeiky *et al*, 2002).

2.8. Treatment

It is currently not possible to standardise treatments or treatment recommendations for leishmaniasis worldwide due to the region-specific variability of leishmaniasis and its causative agent. Indeed, every geographic region throughout the world has its own combinations of vectors, hosts, and *Leishmania* species and/or species complexes, each with more or less genetic heterogeneity (Akhoundi *et al,* 2016). Optimal lines of leishmaniasis treatment are, therefore, specific to each geographic region and are best determined regionally by considering factors such as the observed efficacy of a drug in a given region and the resources/drugs available to health care professionals. Due consideration ought to be given to the risk-benefit ratio of a given therapeutic modality (WHO, 2010:54-55).

Although it is currently possible to affect a clinical cure of leishmaniasis (that is to say, the complete cessation of symptoms and signs within a set period of time), it is not altogether possible to affect a parasitological cure, that is, the complete elimination of parasites from

host tissues (Aebischer, 1994:25-26). In fact, viable (infective) *Leishmania* parasites can be isolated and cultured from host tissues such as old cutaneous leishmaniasis lesion scars (Mendonça *et al*, 2004:1018) and lymph nodes (Dereure *et al*, 2003:77), even after a successful clinical cure. There are currently only a handful of drugs available in clinics for the treatment of leishmaniasis. These include the pentavalent antimonials, miltefosine, amphotericin B, paromomycin and pentamidine (WHO, 2010:55-56).

2.8.1. Pentavalent antimonial drugs

The mainstay of anti-leishmanial therapy is the pentavalent antimony-containing drugs, namely, sodium stibogluconate (Figure 2.2A) and meglumine antimoniate (Figure 2.2B). The pentavalent (Sb^{5+}) antimonials are pro-drugs that undergo reduction in the body to the toxic trivalent (Sb^{3+}) species. It is this trivalent species that is able to bring about the death of the amastigotes in the phagolysosomes of the host's mononuclear phagocytic cells (macrophages). The trivalent antimony species is believed to interfere with the trypanothione redox system of the *Leishmania* parasite by causing an efflux of trypanothione/glutathione, as well as inhibiting trypanothione reductase. This interference causes a loss of thiol reduction potential in the parasite cells, leading to apoptotic cell death of the parasite (Frézard *et al*, 2009:2319-2323). The antimonials are either given intravenously or intramuscularly as they are not orally active. The compounds are administered in such a way so as to provide a dosage of 20 mg of antimony (Sb)/kg daily for at least three weeks, or 20 – 30 days (Frézard *et al*, 2009:2318, 2327). For cutaneous leishmaniasis, intra-lesional administration of antimonials is advocated as a safe, alternative method of administration with little to no toxicity (Monge-Maillo & López-Vélez, 2013:1906). The use of pentavalent antimonials is associated with a number of cardiological adverse events such as electrocardiographic changes, as well as cardiac toxicity (Lawn *et al*, 2006:266-267). Additionally, common adverse effects include drug-induced pancreatitis (Gasser *et al*, 1994:83), elevation of serum hepatic aminotransferase levels (Hepburn *et al*, 1993), bone marrow suppression, muscle and joint pain (myalgia and arthralgia), weakness, malaise, headache, nausea, vomiting, abdominal pain and skin rashes (Frézard *et al*, 2009:2318). Furthermore, peripheral neuropathy can occur with the use of the pentavalent antimonial drugs, however, it is reversible upon withdrawal/cessation of therapy (Brummitt *et al*, 1996:878-879). Increased refractoriness (unresponsiveness of a disease to treatment) to pentavalent antimonials in the state of Bihar in India, and to some degree in adjoining regions of Nepal, led to the adoption of alternative treatment strategies for these regions. However, in the rest of the world, the pentavalent antimony-containing compounds continue to be a particularly effective treatment for leishmaniasis. (Sundar & Chakravarty *et al*, 2015:54).

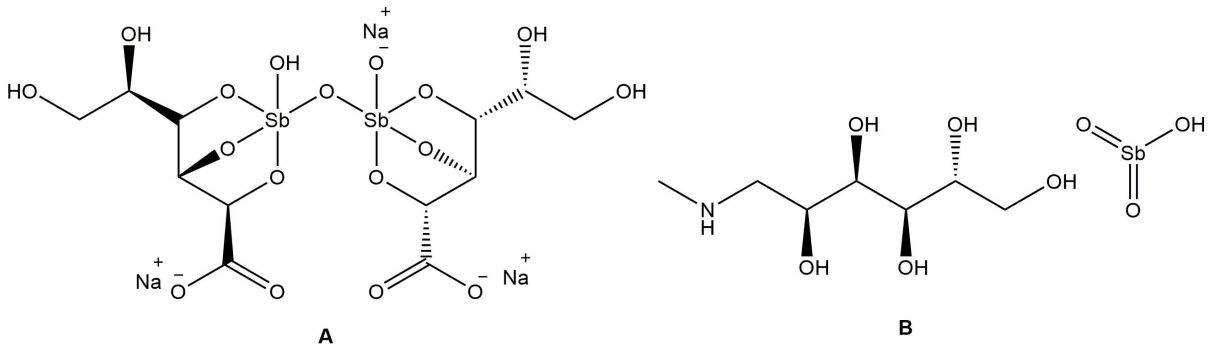

Figure 2.2. The chemical structures of the pentavalent (Sb^{5+}) antimonials sodium stibogluconate (A) and meglumine antimoniate (B).

2.8.2. Miltefosine

Miltefosine (Figure 2.3), an alkyl phospholipid or hexadecylphosphocholine, is the first orally active anti-leishmanial agent. It was approved as recently as 2014 by the United States Food and Drug Administration (FDA) for the treatment of visceral, cutaneous and mucocutaneous leishmaniasis (Sunyoto *et al*, 2018). The mechanism of action of miltefosine is currently poorly understood, although a plausible number have been proposed. Chief among these are that the drug causes disruptions or alterations in the ether-lipid metabolism, signal transduction or glycosylphosphatidylinositol anchor biosynthesis of the parasite (Dorlo *et al*, 2012:2581-2583). Nausea, vomiting and diarrhoea are the most commonly reported side effects in patients who undergo therapy with miltefosine. These side effects are usually self-limiting and subside as therapy is continued. Other observed side effects of miltefosine include renal insufficiency (rarely), elevated hepatic transaminases and serum creatine, and skin allergy (WHO, 2010:56). The use of miltefosine is limited due to its teratogenicity, and its use by women who are breastfeeding is strongly discouraged. Furthermore, the drug is contraindicated in women with child-bearing potential, particularly in circumstances where adequate contraception cannot be assured for the duration of treatment and for 3 months afterwards (WHO, 2010:56). Miltefosine has been found/proposed to be of use in treating canid leishmanial infections, an especially important discovery considering that dogs are an important reservoir of *Leishmania* parasites (Alvar *et al*, 2006:230-231).

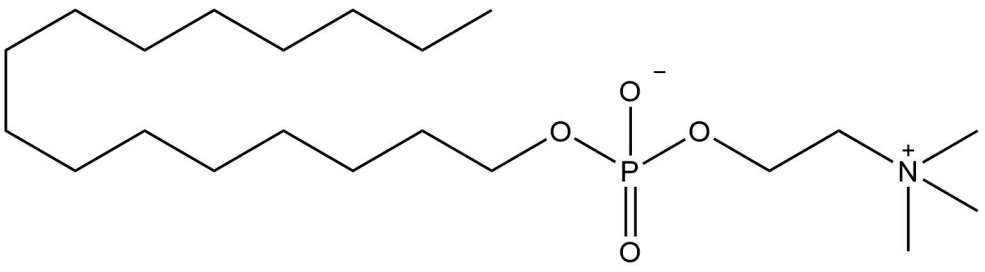

Figure 2.3. The chemical structure of miltefosine.

2.8.3. Amphotericin B

Amphotericin B (Figure 2.4) may be used as a highly effective agent in the treatment of visceral leishmaniasis, where it is able to affect a clinical cure in 90% of cases. It is also a drug that is preferentially used to treat cases of leishmaniasis that are resistant to therapy with pentavalent antimonial drugs (Mohamed-Ahmed *et al*, 2012:695,697). Amphotericin B is also used to treat both the cutaneous and mucocutaneous manifestations of leishmaniasis and has been successfully used to treat immunocompromised patients (Van Griensven *et al*, 2014:286). Lipid formulations (liposomal amphotericin B) and colloidal dispersion formulations of amphotericin B are less toxic than amphotericin B deoxycholate, although their use in endemic areas is limited by the difficulty of their administration and the fact that they are relatively expensive when compared to other anti-leishmanial drugs (Bern *et al*, 2006:917,923). The mechanism of action of amphotericin B, when used to treat leishmaniasis, is similar to its anti-fungal mechanism of action i.e. it complexes with ergosterol precursors in the cell membrane of the *Leishmania* parasite which causes the formation of transmembrane pores. These pores increase the permeability of the leishmanial membranes to a variety of ions (K^+, Ca^{2+} and Mg^{2+}), which promotes the leakage of these ions and other small molecules from the cell, thereby causing cell death (Mesa-Arango *et al*, 2012). *Leishmania* parasites have a sterol composition similar to that of fungi, and amphotericin B binds leishmanial sterols preferentially over host cholesterol (Moen *et al*, 2009:365). The literature has also shown that leishmanial infection of host macrophages may also be entirely circumvented upon sequestration of host membrane cholesterol as a result of using amphotericin B (Chattopadhyay & Jafurulla, 2011:7). Side effects that may occur as a result of using amphotericin B deoxycholate/lipid formulations of amphotericin B include fever, rigor, chills, thrombophlebitis (when administered intravenously), thrombocytopenia, back pain, nephrotoxicity, hypokalaemia and myocarditis (WHO, 2010:55-56).

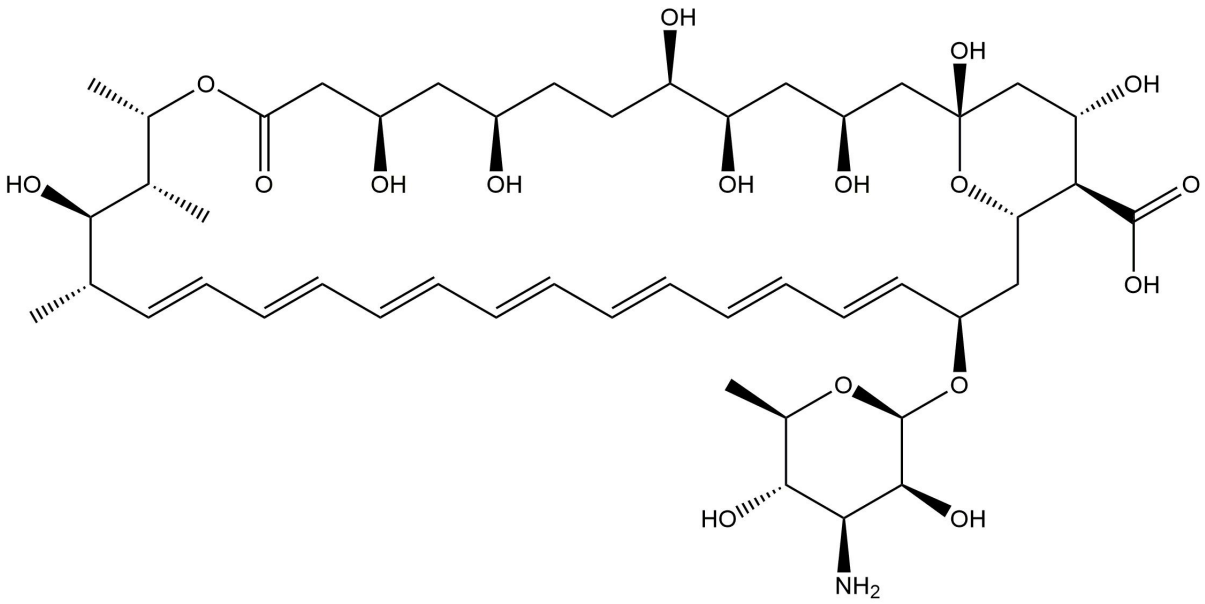

Figure 2.4. The chemical structure of amphotericin B.

2.8.4. Paromomycin

Paromomycin (aminosidine) is an aminoglycoside anti-bacterial agent that is sometimes used in the treatment of leishmaniasis. Paromomycin (Figure 2.5) has been used with limited success when administered parentally for visceral leishmaniasis, or as a topical agent for cutaneous leishmaniasis (El-on *et al*, 1985:704-705), either alone or in combination with pentavalent antimonial drugs (Sundar & Chakravarty, 2015:56). The mechanism of action of paromomycin in *Leishmania* parasites is poorly understood, but there is evidence to suggest that it may act by interfering with the nuclear transcription and vesicle-mediated trafficking processes of the parasite (Chawla *et al*, 2011). Paromomycin is usually administered intramuscularly as the sulphate, although doses are typically seen to be expressed in terms of the base (WHO, 2010:56). The most commonly experienced side effect is usually pain at the site of the injection. Other common side effects that may follow the intramuscular injection of paromomycin include: fever, hepatotoxicity (with elevated liver enzymes) and reversible ototoxicity (Sundar *et al*, 2007:2571). Tetany has been reported to occur following the parenteral administration of paromomycin. Complications associated with paromomycin-induced tetany may also include reversible renal tubular damage and the development of hypocalcaemia (Thakur, 2008:489). Development of pancreatitis has also been reported to occur with the use of paromomycin in the literature (Tan *et al*, 1995:22-23).

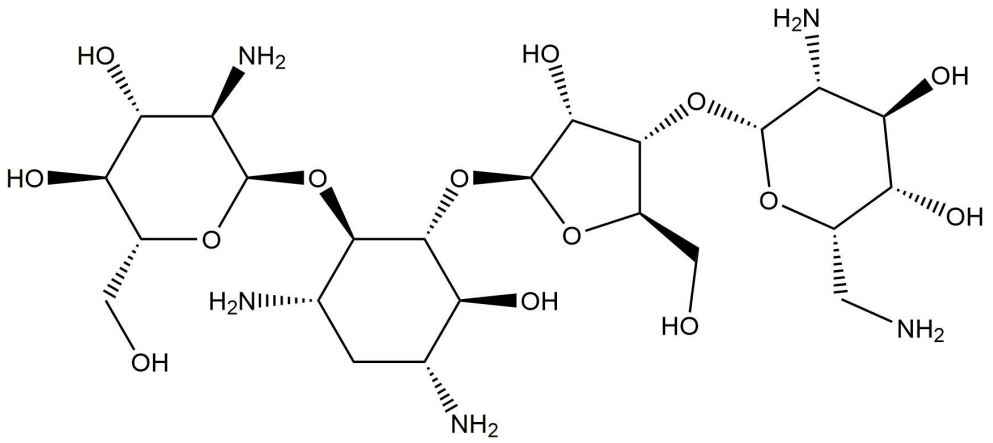

Figure 2.5. The chemical structure of paromomycin.

2.8.5. Pentamidine

Pentamidine (Figure 2.6) is a positively charged aromatic diamine broad-spectrum anti-microbial agent. It is usually found as the di-isethionate salt in a variety of dosage forms and formulations (Rex & Stevens, 2014:492; De *et al*, 1986:1486). Pentamidine is typically reserved for use as an alternative agent in the treatment of cutaneous leishmaniasis (Monge-Maillo & López-Vélez, 2013:1890). Out of all the anti-leishmanial agents, pentamidine is considered to be the least well-tolerated (Kuhlmann & Fleckenstein, 2017:1369). Approximately half of all patients who receive pentamidine at the recommended doses show some form of adverse event. Side effects, such as low blood pressure (hypotension), increased heart rate (tachycardia) and headache, may occur as a result of the intravenous administration of pentamidine. The occurrence of these side effects may be reduced by way of a slower infusion rate. It is advised that a patient's blood sugar levels be monitored during pentamidine treatment, as side effects such as pancreatitis, hyperglycaemia, hypoglycaemia and insulin-dependent diabetes may occur in some patients during the course of their treatment. Other side effects of pentamidine include dermal eruptions (skin rashes), anaemia, neutropenia, thrombophlebitis and the elevation of hepatic enzymes. Intramuscular injection of pentamidine is discouraged due to the increased likelihood of sterile abscesses (which may become secondarily infected) occurring at the site of injection. (Cheung *et al*, 1993:22).

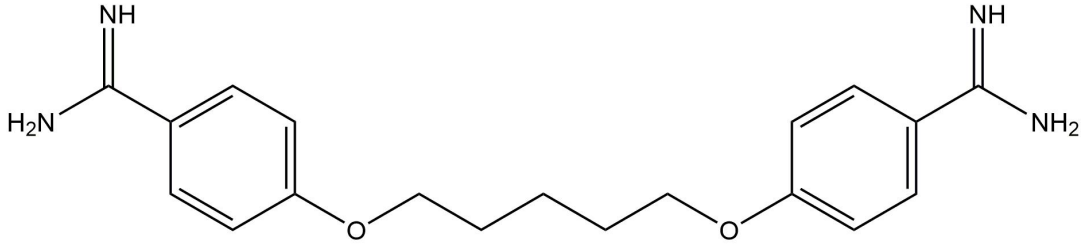

Figure 2.6. The chemical structure of pentamidine.

2.9. Future perspectives and potential anti-leishmanial agents

Despite many efforts on the part of governmental organisations and the scientific community, there is at present no available vaccine or single preventative measure that can fully protect individuals from contracting leishmaniasis. This fact renders health professionals totally reliant on the use of drugs (chemotherapy) when seeking to treat leishmaniasis (Didwania *et al*, 2017). Due to the limitations of the existing treatments (emerging resistance, treatment failure and toxicity), better drugs are urgently needed. In order to reinvigorate and strengthen the drug pipeline, a number of approaches in medicinal chemistry have recently attracted great interest in their ability to identify drug-like compounds that hold out the promise of being potential anti-leishmanial agents in the future. Chief among these strategies are (i) research into naturally-occurring scaffolds (*e.g.* quinazolinones) that might serve as the inspiration on which to base novel anti-leishmanial agents and (ii) the broad optimization and derivatisation (*e.g.* via hybridisation) of molecular pharmacophoric sub-units (e.g. 1,2,3-triazoles) that are known to have anti-leishmanial activity (Nagle *et al*, 2014:11316-11322).

2.9.1. Quinazolinones

Quinazolinones (Figure 2.7) are a class of fused, nitrogen-containing heterocyclic compounds, well known for possessing a varied number of biological properties (Jafari *et al*, 2016:1), including anti-bacterial (Al-Amiery *et al*, 2014; Deep *et al*, 2013; Pandey *et al*, 2009), anti-fungal (Ryu *et al*, 2012), anti-malaria (Jiang *et al*, 2005), anti-HIV (Deetz *et al*, 2001), anti-inflammatory (Giri *et al*, 2009; Laddha *et al*, 2006), anti-viral (Krishnan *et al*, 2011) and anti-mycobacterial/anti-tubercular (Khosropour *et al*, 2006) properties. Quinazolinones also have inhibitory effects on enzymes such as thymidylate synthase (Al-Rashood *et al*, 2006), poly-(ADP-ribose) polymerase (PARP) (Hattori *et al*, 2007) and tyrosine kinase (Khalil *et al*, 2003). Quinazolinones are considered a very important pharmacophore as they are considered a privileged structure, that is, they can provide useful ligands for more than one type of receptor or enzyme target by way of structural modification (Jafari *et al,* 2016:12; Duarte *et al*, 2007:1108). The basic quinazolinone scaffold occurs as two different structural isomers, namely, 2(*1H*)-quinazolinones and 4(3*H*)-quinazolinones. A dicarbonyl form, 2,4(1*H*,3*H*)-quinazolinedione is also known to exist as well (Hameed *et al*, 2018:281) (Figure 2.8).

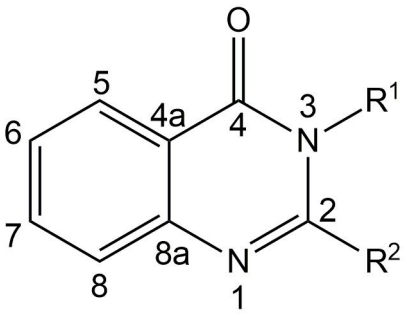

Figure 2.7. Basic quinazolinone scaffold (Jafari et al, 2016:3).

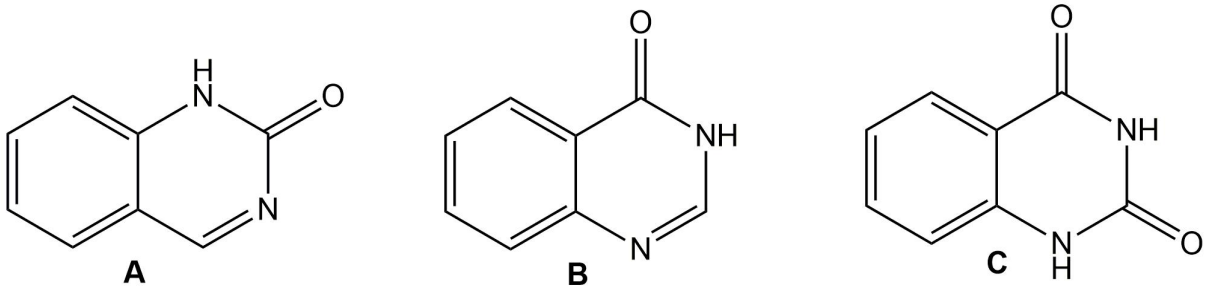

Figure 2.8. Isomeric forms of the quinazolinone basic scaffold. A represents the 2(*1H*)-quinazolinone structural isomer, B represents the 4(*3H*)-quinazolinone structural isomer and C represents the 4(*1H,3H*)-quinazolinedione structural isomer (Hameed *et al*, 2018:282).

A solvent-dependant tautomeric equilibrium between the lactam and lactim form of the quinazolinone scaffold has been observed to occur. Quinazolinones are usually found in the more polar conformer (the lactam form), as this form is the more stable of the two. The lactam conformer normally occurs in polar solvents, aqueous solvents or aqueous solvent/polar solvent mixtures. In contrast, the lactim conformer only occurs in non-polar solvents or in gas phase (Jafari *et al*, 2016:6). The resonance structures associated with the lactam and lactim conformer indicate that O-alkylation of the quinazolinone scaffold ring likely occurs in solvents of a more polar nature due to the inherent stability of the resonance structure of the lactam conformer compared to that of the lactim conformer (El-Badry *et al*, 2012:1361). As a result of the effects of the aforementioned tautomeric interaction, the quinazolinone scaffold shows a high degree of reactivity, hence why it is regarded as a privileged structure (Akbari *et al,* 2013:1462). The dynamic equilibrium between the two tautomeric conformers of the basic quinazolinone scaffold can be seen in Figure 2.9.

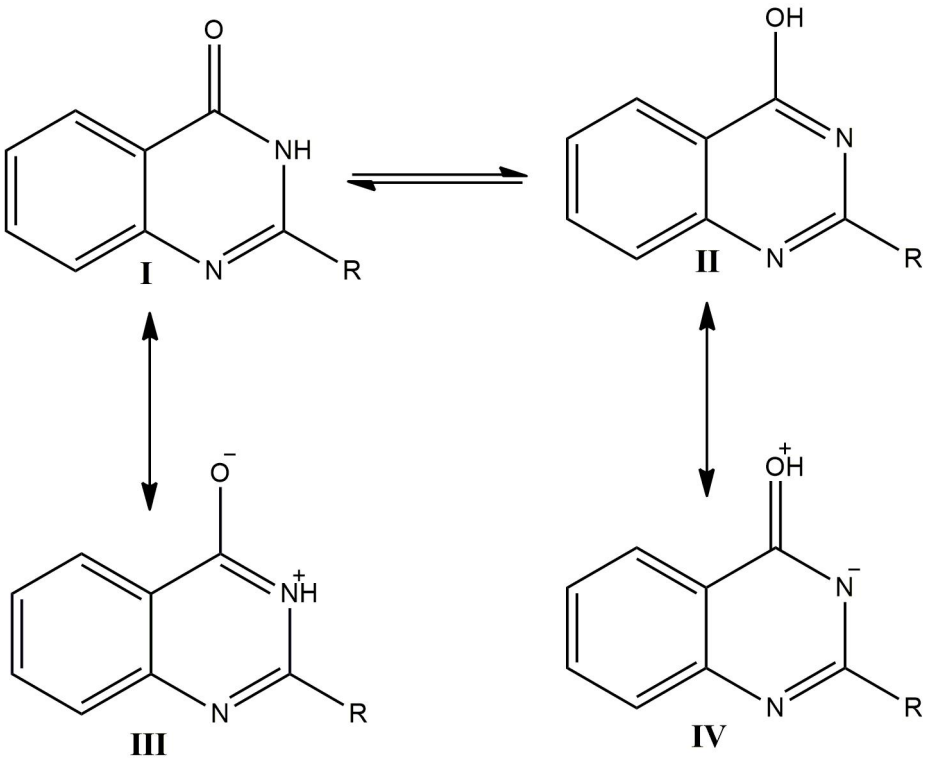

Figure 2.9. The tautomeric forms of the basic quinazolinone scaffold. I and II represent the lactam and lactim tautomers of the quinazolinone scaffold, respectively. The canonical forms (resonance structures) of the lactam and lactim tautomers are represented by III and IV, respectively. Adapted from Jafari *et al*, 2016, p. 7.

A few studies have shown that mono- or di-substituted quinazolinones are promising/possible anti-leishmanial compounds, as some of these compounds displayed some degree of marked anti-protozoal (anti-trypanosomal or anti-leishmanial) activity *in vitro*. For example, a study by Romero (2019) has identified a number of novel mono-substituted quinazolinones (specifically 2-aryl-substituted-4(*3H*)-quinazolinones) that showed significant anti-leishmanial activity (EC_{50}: 7.48 – 29.34 μM) *in vitro* against the promastigotes and amastigotes of three species of *Leishmania* - namely *L. braziliensis*, *L. mexicana* and *L. amazonensis* - when compared to an existing reference drug (Glucantime, EC_{50}: 12.30 – 22.71 μM) (Romero *et al*, 2019). Another study conducted by Birhan and colleagues (2014) supports the promising leishmanicidal activity of di-substituted quinazolinones. In that study, a number of 3-aryl-2-(substituted styryl)-4(*3H*)-quinazolinone derivatives were found to possess greater *in vitro* activity against promastigotes of *Leishmania donovani* (IC_{50}: 0.0128 to 3.1085 μg/ml) when compared to either miltefosine (IC_{50}: 3.1911 μg/ml) or amphotericin B (IC_{50}: 0.0460 μg/ml) (Birhan *et al*, 2014).

The development and identification of novel hits, and subsequently lead compounds, is often a very risky, challenging, labour-intensive and costly endeavour (Decker, 2017:1). This fact,

coupled with the limited understanding of the biology of *Leishmania* parasites, necessitates the development of alternative approaches in the rational design of anti-leishmanial drugs. One such an approach is the fusion of two or more anti-infective scaffolds, already known to possess biological activity (e.g. leishmanicidal activity), into a single molecular structure (Nagle *et al*, 2014:11318-11322). This form of derivatisation is termed molecular hybridisation, and is usually defined as the fusion of two or more pharmacophores/moieties with known biological activity into a singular structure to produce a novel chemical entity (NCE, hybrid) with better biological activity compared to the individual parent scaffolds (Cavalli & Bolognesi, 2009:7345; Meunier, 2008:69).

A growing body of literature exists wherein quinazolinones have been hybridised with other moieties that produced compounds with greater anti-leishmanial activity than any of the constituent parent structures. Ramu and colleagues (2017) effectively synthesised novel ß-carboline-quinazolinone hybrids that demonstrated potential use as anti-leishmanial agents against both promastigotes and intracellular amastigotes of *Leishmania donovani* parasites *in vitro* (Ramu *et al*, 2017). In a similar vein, Sharma (2013) hybridised quinazolinones with a variety of naturally occurring molecules such as pyrimidines, triazines and tetrazoles. Most of these hybrids exhibited excellent anti-leishmanial activity against intracellular amastigotes of *Leishmania donovani* (IC_{50}: 0.65 ± 0.2 - 7.76 ± 2.1 µM) when compared to a reference drug, miltefosine (IC_{50}: 8.4 ± 2.1 µM). At least two compounds were shown to exhibit significant *in vivo* inhibition (percentage parasite growth inhibition: 73.15 ± 12.69%, 80.93 ± 10.50%) of *Leishmania donovani* parasites in a golden hamster model (Sharma *et al*, 2013).

2.9.2. 1,2,3-Triazoles

The 1,2,3-triazoles are a group of aromatic five-membered heterocyclic compounds that consist of two carbon atoms and three nitrogen atoms. Triazoles have two isomeric varieties, namely, the 1,2,3-triazoles and 1,2,4-triazoles (Mignani *et al*, 2012:186). The general structures of these isomers are illustrated in Figure 2.10. The 1,2,3-Triazoles can be divided into three distinct groups (tautomers), based on the position of a substituent or moiety attached to one of the nitrogen atoms of the five-membered ring. The possible tautomeric forms of 1,2,3-triazoles are *1H*-1,2,3-triazole and *2H*-1,2,3-triazoles, respectively. A third tautomer exists, formally expressed as 3(*1H*)-1,2,3-triazole, that may be obtained only in rare instances (Belskaya *et al*, 2015:53). All three of the aforementioned tautomers can be seen in Figure 2.11. The 1,2,3-triazoles are anti-microbials (Genin *et al*, 2000; Willner *et al,* 1972), anti-bacterials (Gregory *et al*, 1989), anti-fungals (Holla *et* al, 2005; Dong *et al*, 2001), anti-epileptics (Pålhagen *et al*, 2001), anti-inflammatory agents (Dong *et al*, 2001), analgesic agents (Dong *et al*, 2001), anti-HIV agents (De Clercq, 2002; Alvarez *et al*, 1994), agents that regulate obesity (Brockunier *et al*, 2000), derivatives of anti-coccidial agents (Bochis *et al*,

1991), agents used to treat allergies (Buckle, 1985), potassium channel activators (Biagi *et al*, 2000), anti-neoplastic agents (Al-Masoudi & Al-Soud, 2002), anti-plasmodial agents (Raj *et al*, 2013), anti-anxiety agents (Martini *et al*, 1988), and tumour proliferation inhibitors (Norris *et al*, 1996) (de Carvalho da Silva *et al*, 2015:118-119; Mignani *et al*, 2012:186). Of note is the potential use of 1,2,3-triazoles to treat diseases caused by *Leishmania* (Tahghighi *et al*, 2012; Ferreira *et al*, 2007), as well as other kinetoplastid diseases, such as trypanosomiasis (Bakunov *et* al, 2010; da Silva Júnior *et al*, 2009).

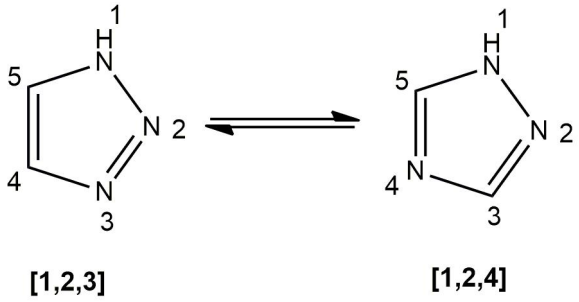

Figure 2.10. Molecular structure(s) of the isomeric forms of the triazole scaffold. Adapted from Kashyap & Silakari (2018:324).

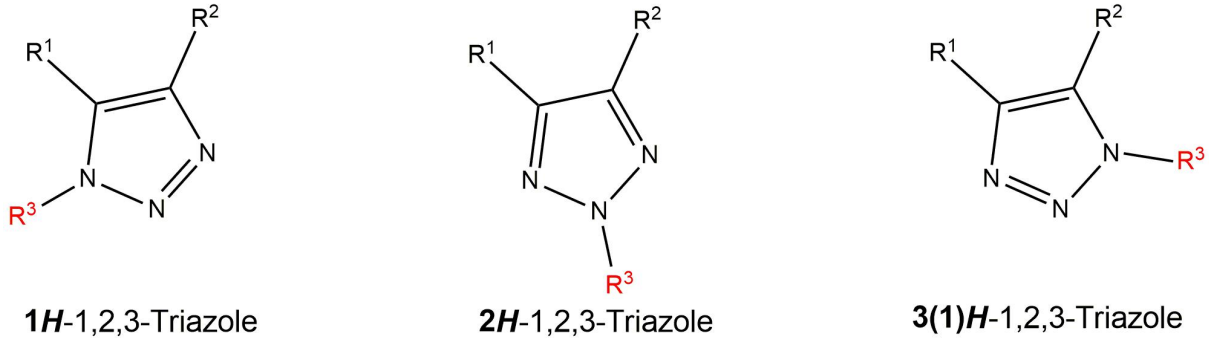

Figure 2.11. The tautomeric forms of 1,2,3-triazole(s). Adapted from Belskaya *et al*, 2015, p. 53.

The 1,2,3-triazoles may be prepared with ease using Huisgen cycloaddition reactions (Mignani *et al*, 2012:186-187) between azides and terminal alkynes (Huisgen, 1963). Alternatively, 1,2,3-triazoles may also be prepared by way of a copper-catalysed variant of the Huisgen cycloaddition, termed the CuAAC or copper(I)-catalysed alkyne-azide cycloaddition reaction (Tornøe *et al*, 2002). A mechanism for the copper (I)-catalysed alkyne-azide cycloaddition reaction (CuAAC) has been proposed by Worrell *et al* (2013). The mechanism is started by way of the generation of a sigma (σ)-bound copper-acetylide that bears another pi(π)-bound (in the form of a pi-complex) copper-atom (Figure 2.12a-b). The aforementioned is formed from copper (I) ion species (generated in situ) and a terminal alkyne. The σ-bound copper-acetylide bearing the π-bound copper atom (Figure 2.12b) will reversibly coordinate an organic azide and form a copper acetylide-azide-copper complex

(Figure 2.12c). After formation of this complex, a nucleophilic attack will occur at the third nitrogen (N3) of the azide by the ß-carbon of the aforementioned complex. This has the effect of forming a triazolyl-copper (I) intermediate (six-membered metallacycle) with one of the copper atoms serving as a stabilising ligand (Figure 2.12d). This intermediate ultimately undergoes ring-closure (Figure 2.12e) and subsequent protonation (Figure 2.12f) to yield the final triazole product. The catalyst mentioned in the beginning of the cycle is able to regenerate and continue the catalytic cycle until the limiting reagent is exhausted (Worrell *et al*, 2003:459). A diagrammatic representation of the mechanism for the CuAAC reaction, as proposed by Worrell *et al* (2003), can be seen in full in Figure 2.12.

The scientific literature abounds with examples of hybrid 1,2,3-triazoles molecules that exhibit moderate to excellent leishmanicidal activities. Temraz and colleagues (2018), for instance, reported 1,2,3-triazole-thiosemicarbazone hybrids that showed nanomolar activity (IC50: 140.3 - 227.4 nM) against *Leishmania major* promastigotes when compared to miltefosine (IC_{50}: 7.8 µM). These hybrids also displayed an eight-fold higher activity (IC_{50}: 1 – 1.4 µM) against axenic amastigotes when compared to the same reference drug, miltefosine, IC_{50}: 8.09 µM (Temraz *et al*, 2018).

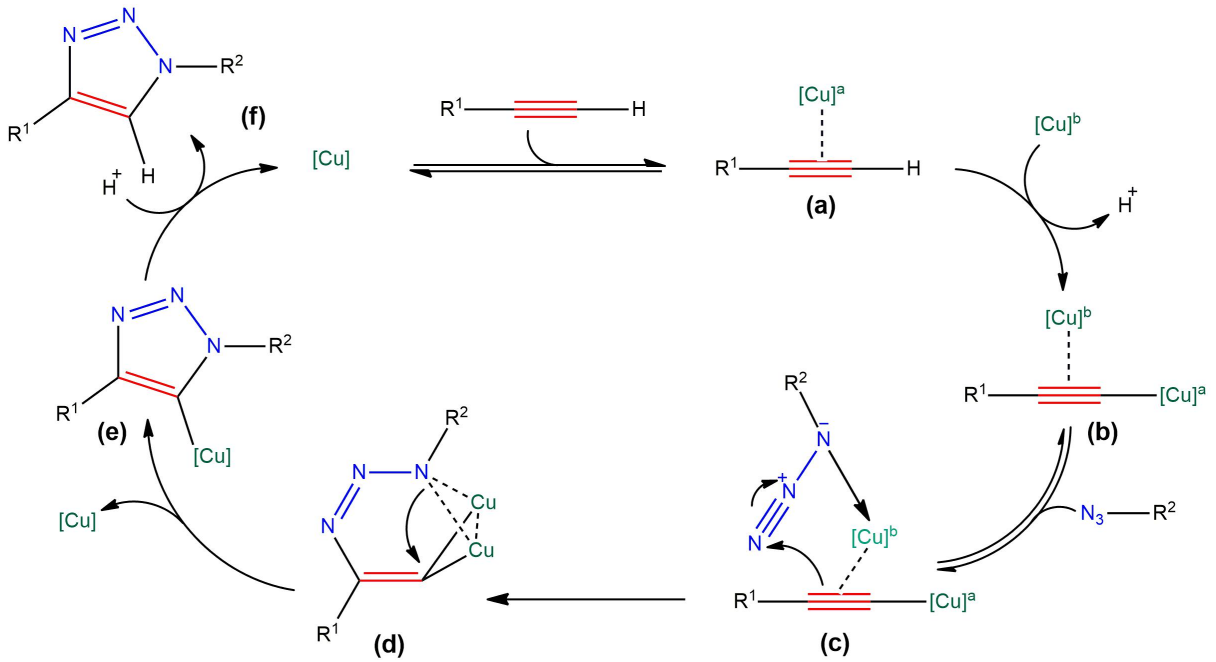

Figure 2.12. Diagrammatic representation of the catalytic model for CuAAC reactions as proposed by Worrell et al (2003:459).

Teixeira *et al* (2018) investigated the anti-leishmanial activity of 1,2,3-triazole-eugenol-based compounds. The study found that the most active compound in their series of synthesised compounds was active against promastigotes (IC_{50}: 7.4 ± 0.8 µmol L^{-1}) and amastigotes (IC_{50}: 1.6 µmol L^{-1}) of *Leishmania amazonensis*, when compared to reference drugs such as

pentamidine (promastigote IC_{50}: 4.2 ± 0.4 μmol L^{-1}, amastigote IC_{50}: 1.8 ± 0.2 μmol L^{-1}) and glucantime (amastigote IC_{50}: 45.5 ± 1.6 μmol L^{-1}). The compound was also impressively able to target intracellular parasites without interfering with the viability of the host macrophage (Teixeira *et al*, 2018). Upadhyay and colleagues (2018) have established 1,2,3-triazole-quinoline hybrids as a promising scaffold on which to base future anti-leishmanial drugs. Out of their series of synthesised compounds, it was found that most of the screened derivatives exhibited similar/greater activity against promastigotes (IC_{50}: 2.43 - 45.75 μM) and intracellular amastigotes (IC_{50}: 7.06 - 34.9 μM) of luciferase-expressing *Leishmania donovani* (with less cytotoxic effects) than the control, miltefosine (IC_{50}: 8.4 μM) (Upadhyay *et al*, 2018). In the work done by Sahu and colleagues (2019), a series of hybrids of quinine and 1,2,3-triazoles, were screened for anti-leishmanial activity against extracellular promastigotes of *Leishmania donovani* with amphotericin B (IC_{50}: 5.55 μM) as a reference drug. Of these compounds only four, ranging in activity (IC_{50}) from 1.78 – 4.40 μM, have shown promise as a future scaffold on which to base anti-leishmanial agents. Dwivedi *et al* (2015) were similarly able to synthesize and assay a series of 1,2,3-triazole-containing compounds (triazolyl O-benzylquercetin glycoconjugates) for their associated anti-leishmanial activity in *L. donovani* promastigotes and amastigotes *in vitro*. A small number of these compounds have shown comparable activity (promastigote IC_{50}: 7.76 ± 2.44 - 9.92 ± 2.16; amastigote IC_{50}: 6.08 ± 0.03 - 7.65±0.93) to miltefosine (promastigote IC_{50}: 5.95+0.95; amastigote IC_{50}: 4.16 + 0.20).

All of the above serves to establish the fact that 4(3*H*)-quinazolinone and 1,2,3-triazole scaffolds hold out the promise of being hybridised into potential anti-leishmanial agents.

The following chapter (Chapter 3) will include a manuscript intended for publication in the journal Bioorganic Chemistry, that details and discusses the syntheses and *in vitro* biological activities (anti-leishmanial activity and cytotoxicity) of a series of *1H*-1,2,3-triazole-4(*3H*)-quinazolinone hybrids.

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CHAPTER 3

ARTICLE FOR SUBMISSION

This chapter (Chapter 3) includes an article intended for submission to the journal Bioorganic Chemistry. This article is comprised of an abstract, graphical abstract, introduction, results and discussion, conclusion, materials and methods, syntheses of compounds and *in vitro* biological evaluations. This article was written in accordance with the journal's guidelines for authors (see Annexure C), which is available for download at:

https://www.elsevier.com/journals/bioorganic-chemistry/0045-2068?generatepdf=true.

Synthesis and in vitro anti-leishmanial activity of novel 2,3- disubstituted-4(3H)-quinazolinone derivatives

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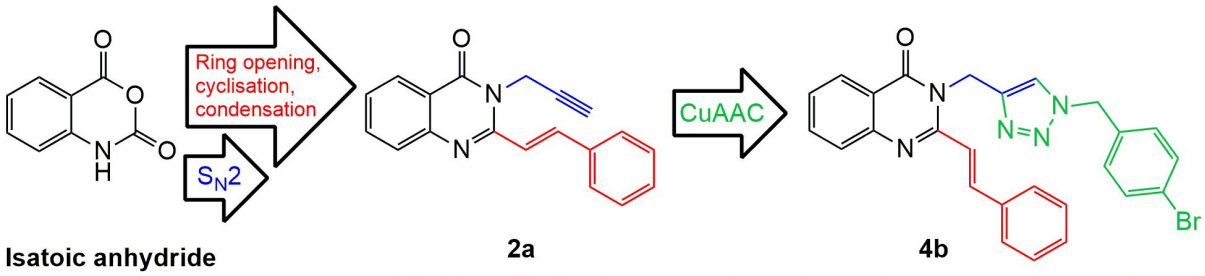
The article intended for publication was drafted and finalised by GL Ralph with input from NH Zuma, J Aucamp and DD N'Da. All syntheses were conducted by GL Ralph under the guidance of NH Zuma and DD N'Da. The anti-leishmanial activity and cytotoxicity assays were performed by J Aucamp.

ABSTRACT

Leishmaniasis is a sub-tropical and tropical disease typified by infection with protozoan parasites of the genus *Leishmania.* The disease is mainly spread by the bite of anautogenous female phlebotomine sand flies. Leishmaniasis is endemic to over 98 countries where approximately 1 billion people are at risk of infection. Over 12 million individuals are estimated to be afflicted by the disease at any given point in time. The World Health Organization (2020) estimates that approximately 600,000 - 1 million new cases of cutaneous leishmaniasis, and some 50,000 - 90,000 new cases of visceral leishmaniasis, occur annually. Approximately 20,000 - 40,000 deaths are also estimated to be caused by visceral leishmaniasis every year. The aforementioned serves to stress the renewed need for the development of novel anti-leishmanial agents. In light of this need, we investigated the *in vitro* cytotoxicity and anti-leishmanial activity of a series of fourteen 4(3*H*)-quinazolinone derivatives, comprising ten novel 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids and their associated synthetic precursors. Two derivatives, in particular, were observed to moderately inhibit the growth of *Leishmania* promastigotes. These were compound **2a**, which displayed 58% and 43% growth inhibition of *L. donovani* 1S and 9515 promastigotes, and compound **4b**, which exhibited a modest 47% growth inhibition of *L. major* strain IR-173 promastigotes. All synthesised derivatives were found to be non-toxic to mammalian (Vero) cells (IC_{50} > 100 μM) but exhibited poor parasite growth inhibition. The overall lack of significant promastigote growth inhibition may be attributed to the poor aqueous solubility of the derivatives, the remediation of which may be relegated to future endeavours.

Keywords: leishmaniasis; promastigote; quinazolinone; molecular hybridisation; triazoles.

GRAPHICAL ABSTRACT



Isatoic anhydride

2a

Activity (% growth inhib.) - 100 μM
L. donovani 1S: 58.4
L. donovani 9515: 43.5
Cytotoxicity, Vero IC_{50} >100 μM
cLogP: 3.44

4b

Activity (% growth inhib.) - 100 μM
L. major IR-173: 47.5
Cytotoxicity, Vero IC_{50} >100 μM
cLogP: 4.53

HIGHLIGHTS

- Synthesis of a series of novel 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids.
- Evaluation of the anti-leishmanial activity and cytotoxicity of the hybrids and their synthetic precursors.
- The hybrids and their synthetic precursors were non-toxic to Vero cells.
- Compound **2a** was the most active compound against *Leishmania donovani* 1S and 9515 promastigotes with 58% and 43% growth inhibition.
- Compound **4b** was the most active compound against *Leishmania major* IR-173 promastigotes with 47% growth inhibition.

3.1. Introduction

Leishmaniasis is a parasitic disease characterised by infection with an obligate intracellular parasite of the genus *Leishmania* [1]. The disease is primarily transmitted by the bite of infected female phlebotomine sand flies of two genera: *Phlebotomus* (in the Old World or Eastern Hemisphere) and *Lutzomyia* (in the New World or Western Hemisphere) [2]. Caused by over 20 species of *Leishmania* [3], leishmaniasis is one of several neglected infectious diseases of the sub-tropics and tropics that constitutes a public health problem whose burden is steadily increasing [4]. This increase is the result of a variety of factors such as the co-infection of leishmaniasis with human immunodeficiency virus (HIV) [5], increased international travel [6] as well as the migration of immigrants and refugees from areas of known endemicity [7]. Leishmaniasis is classified as a neglected tropical disease (NTD) due to low visibility of the disease amongst the public of high-income countries and a paucity of research investment on the part of the pharmaceutical sector and policymakers [8].

Leishmaniasis is endemic to 98 countries and territories, where an estimated 1 billion people are at risk of acquiring the disease [9]. The number of infected individuals is estimated to be around 12 million at any point in time [10]. Annually, around 600,000 - 1 million new cases of cutaneous leishmaniasis, and some 50,000 - 90,000 new cases of the visceral form of the disease, are estimated to occur [11], with some estimates attributing 20,000 - 40,000 deaths to visceral leishmaniasis [12].

Treatment of leishmaniasis in humans is mainly limited to the chemotherapeutic use of only a small number of drugs, such as the pentavalent (Sb^V) antimonials (sodium stibogluconate and meglumine antimoniate), amphotericin B, miltefosine, pentamidine, and paromomycin/aminosidine/monomycin [13]. The use of these drugs is negatively associated with the occurrence of treatment failure, prohibitively high costs, the emergence of drug-resistant forms of the leishmanial parasite (in both experimental and clinical settings), as well as the precipitous occurrence of toxicity and adverse events [14]. Altogether, these shortcomings serve to stress the need for new, safe and effective anti-leishmanial chemotherapeutic agents.

The development of novel chemical entities with entirely new mechanism(s) of action is expensive [15], time-consuming and fraught with risk [16]. It is, therefore, desirable instead to make use of drug development strategies that exploit structures with known biological activities. One such strategy is that of molecular hybridisation.

Molecular hybridisation is a strategy in rational drug design and development wherein two or more pharmacophoric subunits, derived from structures with known biological activity, are

joined into one single structure to produce a hybrid with greater potency, efficacy and affinity compared to their respective parent molecules [17]. A number of advantages are associated with this strategy, namely: (i) a greatly modified selectivity profile [17]; (ii) improvement of biopharmaceutical parameters (pharmacodynamics and pharmacokinetics) [18]; (iii) alterations in the mechanism of action or the ability to elicit biological activities via dual/multiple mechanisms of action; (iv) reduction of undesirable side effects and/or drug-induced adverse events; (v) decreased incidence of drug-drug interactions; (vi) increased patient compliance and therapy adherence; (vii) reduction in the emergence or spread of drug-resistant micro-organisms and protozoan parasites [19] and (viii) lower overall cost. Molecular hybrids may be produced either by linking two or more existing biologically active structures with bridging links (also termed linkers) or by integrating fragments of existing biologically active structures into a singular structure (a fusing/merging strategy) [20]. The approach has been utilised with great success by researchers to produce promising chemotherapeutic agents that may be used to prevent or treat tropical parasitic diseases such as malaria [21], trypanosomiasis and leishmaniasis [22].

Quinazolinones are a class of heterocycles comprised of fused nitrogen-containing rings that occur naturally in the form of some 200 alkaloids in plants [23] as well as metabolites derived from micro-organisms such as *Bacillus cereus* [24]. Synthetic, as well as semi-synthetic, quinazolinones are also extant in the scientific literature [25]. Quinazolinones have attracted considerable attention in medicinal chemistry owing to the relative ease of their synthesis, as well as the range of biological activities that may be elicited by their use. These biological activities include, *inter alia*, anti-malarial [26], anti-mycobacterial/anti-tubercular [27] and anti-leishmanial [28] activities.

Triazoles are another well-known class of heterocyclic organic compounds that hold great importance in the field of medicinal chemistry [29]. This class of compounds mainly refers to heterocycles that are comprised of a five-membered di-unsaturated ring that consists of two carbon and three nitrogen atoms [30]. The positions of the carbon and nitrogen atoms relative to one another give rise to two possible isomers, namely, 1,2,3-triazoles and 1,2,4 triazoles. Similar to quinazolinones, the 1,2,3-triazoles are also well known for their ability to evoke a myriad of biological activities, chief among these being their anti-protozoal activities against diseases such as malaria [31], leishmaniasis [32] and trypanosomiasis [33]. The 1,2,3-triazoles are also chemically stable and fairly resistant to reductive/oxidative chemical degradation, as well as degradation by hydrolysis, in both basic as well as acidic conditions [34]. This stability is attributed to the sp^2-hybridisation of the atoms of the ring as well as the delocalisation of six of the ring's electrons in π-molecular orbitals [30]. The 1,2,3-triazoles are also synthetically very accessible [30], as they are readily synthesised by Huisgen

cycloaddition [35] and/or copper-catalysed alkyne-azide cycloaddition (CuAAC) reactions [36] between terminal alkynes and azides.

In this study, a number of 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids were synthesised using a variety of synthetic pathways. The ability of these hybrids and their synthetic precursors to inhibit the growth of *Leishmania* promastigotes, as well as their cytotoxicity, was assessed *in vitro.* We herein report the syntheses, *in vitro* cytotoxicity and *in vitro* anti-leishmanial growth inhibitory properties of these hybrids and their associated synthetic intermediates.

3.2. Results and discussion

3.2.1. Chemistry

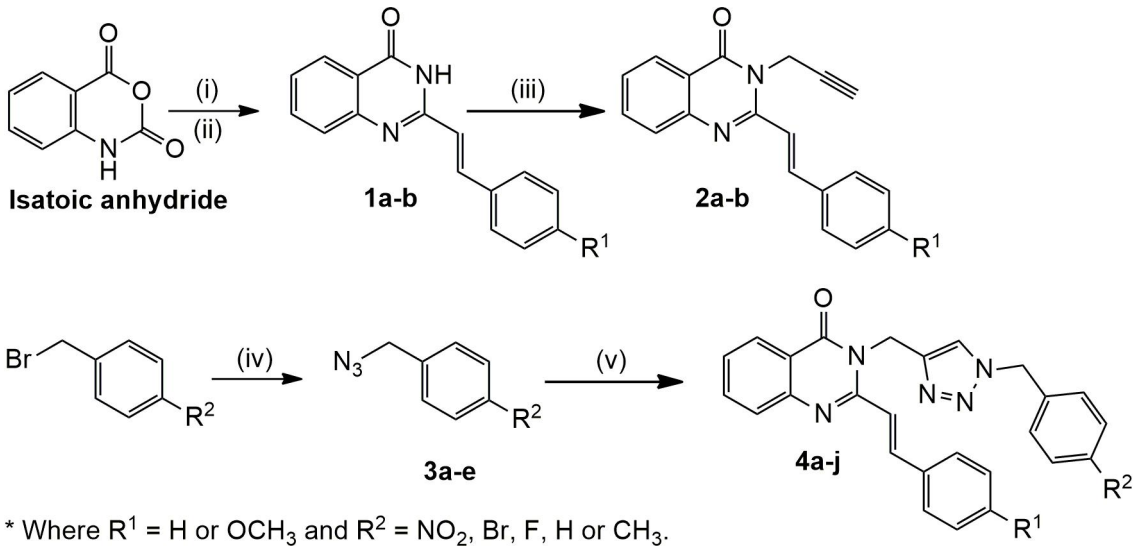
A series of fourteen 4(3*H*)-quinazolinone derivatives were synthesised using a variety of synthetic methods. These derivatives were divided into three distinct sub-series, namely: 2-(4-substituted-styryl)-4(3*H*)-quinazolinones (**1a-b**), 3-propyl-2-(4-substituted-styryl)-4(3*H*)-quinazolinones (**2a-b**) and 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids (**4a-j**) - as shown in Table 3.1. The entirety of the synthesis of the quinazolinone derivatives is illustrated in Scheme 1.

First, the 2-(4-substituted-styryl)-4(3*H*)-quinazolinones **1a-b** were synthesised in a two-step tandem one-pot process using an established literature method [37]. The first step of the one-pot synthesis involved the ring-opening of isatoic anhydride with ammonium acetate (NH_4OAc) to produce anthranilamide with loss of carbon dioxide, directly followed by cyclisation with triethyl orthoacetate ($CH_3C(OEt)_3$) at 120 °C for 6-8 hours to produce 2-methyl-4(3*H*)-quinazolinone. The second step involved the condensation of 2-methyl-4(3*H*)-quinazolinone with the appropriate *para*-substituted benzaldehyde, again at 120 °C for 6-8 hours, to yield the corresponding intermediate compounds **1a-b**, in moderate yields (45 - 60%), after purification by precipitation.

Next, the 3-propyl-2-(4-substituted styryl)-4(3*H*)-quinazolinones **2a-b** were synthesised by employing a literature method [38]. Compounds **2a-b** were produced by way of a nucleophilic (S_N2) aliphatic substitution (amine alkylation) reaction between the NH-3 of compounds **1a-b** and propargyl bromide (PB). The procedure was carried out at room temperature for a duration of 15 minutes (**2b**) to 48 hours (**2a**) in the presence of potassium carbonate. The target compounds were produced in moderate yields (40 - 44%) after purification by recrystallisation in ethanol or acetone.

Finally, the 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids **4a-j** were synthesised in two steps using a method outlined in the literature [39]. The first step consisted of the synthesis of *para*-substituted benzyl azides **3a-e** via nucleophilic substitution reaction (S_N2) between commercially available *para*-substituted benzyl bromides and sodium azide (NaN_3). The benzyl azides obtained in this manner were oil-like liquids that were suitable for use as reagents without any further purification. In the second step, the 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids **4a-j** were synthesised by copper-catalysed azide-alkyne cycloaddition (CuAAC) reactions wherein the 3-propyl-2-(4-substituted styryl)-4-(3*H*)-quinazolinones **2a-b** were subjected to benzyl azides **3a-e**, in the presence of copper (I)-species, at ambient temperatures (~25 °C) for 48 - 72 hours. This produced

compounds **4a-j** in low to excellent yields (30 - 89%) after purification by trituration with both ethanol and distilled water.



Scheme 1. Multi-step synthesis of 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids **4a-j**.

Reagents and conditions: (i): NH_4OAc (3.78 g, 1 eq.), $CH_3C(OEt)_3$ (1 eq.), 120 °C, 6 - 8 h; (ii): Benzaldehyde/*p*-methoxy benzaldehyde (1 eq.), 120 °C, 6 - 8 h; (iii): K_2CO_3 (12 eq.), DMF, PB (1.2 eq.), rt, 15 min - 48 h; (iv): DMSO, NaN_3 (1.1 eq.), rt, 10 - 12 h; (v): 3-propyl-2-(4-substituted-styryl)-4(3*H*)-quinazolinone **2a-b**, *para*(R^2)-substituted benzyl azide **3a-e** (1.1 eq.), DMSO/dH_2O (4:1), β–CD (0.03 - 0.07 eq.), Na L-ascorbate (0.3 eq.), $CuSO_4{\cdot}5H_2O$ (0.1 eq.), rt, 48 – 72 h.

Successful syntheses of all compounds were confirmed by routine molecular characterisation techniques such as infra-red spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR) and high-resolution mass spectrometry (HRMS). The ^1H and ^{13}C NMR spectra were inspected in order to identify peaks that are characteristic of 4(3*H*)-quinazolinone, styryl and 1,2,3-triazole moieties. The IR spectra were similarly investigated for the presence of characteristic absorptions that would allow for the identification of functional groups and substituents.

The ^1H NMR spectra of compounds **1a-b** bear a characteristic singlet (s) peak in the region of 12.06 - 11.98 ppm which is indicative of proton H-3 of the amide of the quinazolinone scaffold. The disappearance of this peak in the spectra of compounds **2a-b** indicated that the propargylation of compounds **1a-b** occurred at this site. The appearance of peaks that were indicative of the protons of a propargyl moiety in the ^1H NMR spectra of compounds **2a-b** were also observed. These peaks were the doublet (d) and triplet (t) of propargylic protons H-1'' (5.20 - 5.18 ppm) and H-3'' (3.24 - 3.22 ppm). Long-range coupling ($^4J_{H\text{-}1''\text{-}H\text{-}3''}$ 2.3 - 2.4 Hz)

was also seen to occur between H-3'' and H-1'' in the spectra of compounds **2a-b** [40]. The formation of a propargyl moiety was also confirmed by the appearance of three additional peaks in the ^{13}C spectra of compounds **2a-b**. These peaks were representative of the propargyl carbons C-1'' (31.75 - 31.71 ppm), C-2'' (78.67 - 78.61 ppm) and C-3'' (74.46 - 74.35 ppm).

The appearance of two doublets (d) in the ^{1}H NMR spectra of all quinazolinone derivatives provided evidence of the vinylic protons H-2' (8.00 - 7.85 ppm) and H-1' (7.63 - 6.86 ppm) of the styryl moiety attached to the quinazolinone scaffold. The literature reports the coupling constants of vicinal hydrogens of di-substituted alkenes ($^{3}J_{H\text{-}H}$), in one-dimensional ^{1}H-NMR, to be found in the range of 12 - 18 Hz for *trans*-isomers, while those of *cis*-isomers are found to be in the range of 0 - 12 Hz [40]. The coupling constants of the alkenyl protons H-1' and H-2' ($^{3}J_{H\text{-}1'\text{-}H\text{-}2'}$ 15.2 - 16.2 Hz) are therefore suggestive of the fact that the styryl moiety occurs as the trans-isomer for all the quinazolinone derivatives in this study. The coupling constants of proton H-5 indicate that this proton undergoes *ortho*-coupling with proton H-6 ($^{3}J_{H\text{-}5\text{-}H\text{-}6}$ 6.7 - 7.9 Hz) and *meta*-coupling with proton H-7 ($^{4}J_{H\text{-}5\text{-}H\text{-}7}$ 0.8 - 1.3 Hz) [40].

In the spectra of compounds **4a-j** the successful formation of the 1,2,3-triazole moiety was confirmed by the disappearance of the aforementioned propargyl peaks, coupled with the appearance of singlet (s) peaks representative of triazolyl proton H-5'' (8.28 - 8.05 ppm) and carbon C-5'' (124.25 - 123.11 ppm). Another signal in the ^{13}C spectra of compounds **4a-j**, occurring in the region of 134.81 - 132.37 ppm, was indicative of carbon C-4'' of the 1,2,3-triazole ring. This ring was also observed to be flanked on either side by methylene bridges (protons H-6''/H-7'' and carbons C-6''/C-7''). The chemical shifts associated with these bridge linkages occur in the regions of 5.73 - 5.57 ppm (H-7'') and 5.63 - 5.48 ppm (H-6'') on ^{1}H spectra; as well as in the regions of 55.32 - 51.84 ppm (C-7'') and 38.16 - 37.87 ppm (C-6'') on ^{13}C spectra.

The IR-spectra of all compounds were inspected for the presence of distinct absorptions that would allow for the identification of functional groups and substituents. Characteristic absorptions for carbonyl functional groups (C=O) were seen (1672 - 1654 cm^{-1}, C=O stretching) for all quinazolinone derivatives, along with absorptions belonging to the NH portion of the amide at position 3 of compounds **1a-b** (3186 - 3182 cm^{-1}, N-H stretching). Additional absorptions were also observed for the alkyne (propargyl) functional groups of compounds **2a-b** (3235 - 3209 cm^{-1}, ≡C-H stretching and 2115 - 2115 cm^{-1}, mono-substituted alkyne C≡C stretching). Other absorptions, specifically those pertaining to the C=N functionality at positions 1 and 2 of the quinazolinone scaffold in most of the quinazolinone derivatives, can be seen in the region of 1642 - 1631 cm^{-1} (C=N stretching). The C=N stretching absorptions of compounds **4a**, **4h** and **4i**, however, were not visible due to

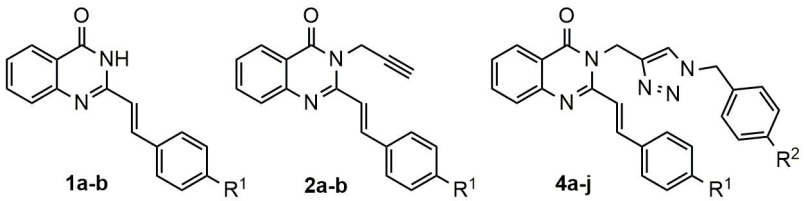
overlapping with the absorptions of their respective carbonyl (C=O) functional groups. The C-H bending absorptions of the methylene bridges in compounds **2a-b** and **4a-j** occur between 1473 - 1469 cm^{-1}. Nitro (NO_2) and alkyl aryl ether functional groups were observed to absorb in the regions of ~1519 cm^{-1} (N-O stretching) and 1256 - 1245 cm^{-1} (C-O stretching), respectively.

Absorptions specific to the 1*H*-1,2,3-triazole moiety were also observed. These included alkene =C-H stretching in the region of 3140 - 3108 cm^{-1} and C-N stretching in the region of 1348 - 1331 cm^{-1}. The successful formation of 1*H*-1,2,3-triazole (1,4-regioisomers) rings was confirmed by the disappearance of certain absorptions in the spectra of compounds **4a-j**. These were the absorptions specific to both the propargyl moiety of compounds **2a-b** and the azide (N_3) moiety of compounds **3a-e** (2090 - 2102 cm^{-1}, N=N=N stretching).

3.2.2. Physicochemical properties

In this section, we elaborate upon and discuss the physicochemical properties (lipophilicity and aqueous solubility) of the 1*H*-1,2,3-triazole-4(3*H*)-quinazolinone hybrids and their associated synthetic intermediates. The drug-likeness of the derivatives was determined with the use of Lipinski's rule of five, an index that is often used to also predict whether a compound may be orally-active in humans [41]. Due consideration was given to the oral activity of the compounds as only a limited number of orally active anti-leishmanial agents are currently available for use in the clinical setting, and it would be advantageous to facilitate research into potentially orally active agents [42]. All derivatives were predicted to be drug-like and/or orally active by conforming to the aforementioned index.

Table 3.1. Predicted physicochemical properties of all synthesised quinazolinone derivatives.



Cpd	Substituents		MW [a] (g/mol)	cLogP [b]	LogS [c]		HBD [f]	HBA [g]	Drug-like/orally active [h]
	R^1	R^2			ESOL [d]	Ali [e]			
1a	H	-	248.28	3.16	-3.92	-3.89	1	2	Yes
1b	OCH_3	-	278.31	3.13	-3.97	-4.06	1	3	Yes
2a	H	-	286.33	3.44	-3.89	-3.47	0	2	Yes
2b	OCH_3	-	316.35	3.43	-3.95	-3.63	0	3	Yes
4a	H	NO_2	464.48	3.29	-5.15	-5.71	0	6	Yes
4b		Br	498.37	4.53	-6.00	-5.64	0	4	Yes
4c		F	437.47	4.24	-5.25	-5.03	0	5	Yes
4d		H	419.48	3.89	-5.09	-4.92	0	4	Yes
4e		CH_3	433.50	4.28	-5.40	-5.31	0	4	Yes
4f	OCH_3	NO_2	494.50	3.23	-5.22	-5.87	0	7	Yes
4g		Br	528.40	4.63	-6.07	-5.80	0	5	Yes
4h		F	467.49	4.31	-5.32	-5.19	0	6	Yes
4i		H	449.50	3.91	-5.16	-5.09	0	5	Yes
4j		CH_3	463.53	4.35	-5.46	-5.47	0	5	Yes

[a] Molecular weight (units in grams per mole). [b] Calculated logP (consensus logP). [c] Predicted aqueous solubility, where log S is the logarithm of the amount of compound (in moles) able to dissolve a litre of water. [d] ESOL = Estimated Solubility. Calculated using a topological method [43]. [e] Calculated using a topological method [44]. [f] Number of hydrogen bond donors (NH and OH groups). [g] Number of hydrogen bond acceptors (nitrogen and oxygen atoms). [h] Determined with reference to Lipinski's rule of five (MW ≤ 500 g/mol; cLogP ≤ 5; HBD ≤ 5; HBA ≤ 10; no more than one violation allowed) [41]. All values in this table were calculated using the SwissADME web tool, http://www.swissadme.ch/ [45].

3.2.2.1. Lipophilicity

Lipophilicity is defined as the tendency of a compound to preferentially partition into either a non-polar/lipid or polar/aqueous matrix [46]. One of the traditional empirical techniques used to determine lipophilicity is to suspend and partition a compound between two immiscible non-polar (e.g. *n*-octanol) and polar (e.g. aqueous buffer) liquid phases. The partitioning value measured in this way is termed logP. More precisely, the logP value is the logarithm of the partition coefficient of the compound between an organic and an aqueous liquid phase at a pH where all of the compound molecules are in a neutral (non-ionised) state [46]. Compounds with a higher logP tend to be more lipophilic/non-polar and thus have poorer aqueous solubility, whilst those with a lower logP value tend be more hydrophilic/polar and thus have poorer lipid bilayer permeability [46].

Lipophilicity is an important physicochemical property as it is known to affect other drug properties such as permeability through bio-membranes, absorption, distribution, plasma protein binding, metabolism, elimination, volume of distribution (V_d) and toxicity [46]. The targeted logP value range for drugs intended for oral administration is usually between 0 and 5 [41]. It is within these ranges that a good balance between the lipophilicity and the aqueous solubility of a compound is struck. Calculated partition coefficient (cLogP) values are partition coefficients that are calculated using non-experimental methods [47]. These values are extensively used in drug discovery to predict drug-likeness and to optimise compound libraries for screening [48].

The cLogP values (range 3.13 - 4.63) for the quinazolinone-containing compounds in this study were well within the target range of cLogP < 5 set by Lipinski and others (Table 3.1). This indicates that all the synthesised derivatives are predicted to be drug-like in nature, suitable for oral administration, and similarly predicted to be well absorbed in the gastro-intestinal tract by way of passive diffusion [41].

3.2.2.2. Solubility

Solubility is the maximum amount of solute (by weight) that may dissolve in a given solvent (by volume) at a given set of solution conditions (pH, temperature). Compounds that are poorly soluble are deleterious to drug discovery and many negative effects may occur as a result of poor solubility [49]. These effects include: (i) poor absorption and bioavailability after oral administration, (ii) unsuitability for intravenous administration, (iii) artificially low activity or erratic results when screened in biological assays, (iv) drug discovery and formulation development challenges (e.g. increased drug development time and expensive formulations) and (v) inconvenience to patients (e.g. a patient may need to take the drug more frequently and in higher doses, which increases the likelihood that a patient may experience side effects/adverse events during the course of therapy). These are major challenges that deserve serious consideration in that the solubility of a compound may prove to be a much larger problem for drug discovery than the parameter of lipophilicity [41]. Solubility is influenced mainly by the structural properties inherent in the molecular structure of a compound [49]. Chief among these are the lipophilicity of a molecule (influenced by intra- and inter-molecular forces), the size (or molecular weight/shape) of a molecule as well as the properties of the crystal lattice of a solid compound (melting point, crystal stacking, crystal lattice energy etc) [49].

Two topological models were used to predict the aqueous solubility of the quinazolinone derivatives in this study (Table 3.1.). The first of these was an implementation of the ESOL model [43] and the second was derived from the work of Ali and colleagues [44]. These

models were chosen for use as they demonstrate a strong linear correlation between predicted and experimentally derived aqueous solubility values [45]. The logS values of the synthesised derivatives (calculated with the use of the aforementioned models) fell within the acceptable range for aqueous solubility, that is to say between -6 < logS (ESOL) < 0 [45]. However, aqueous solubility still presented a problem experimentally, as all the synthesised quinazolinone derivatives were unable to adequately solubilise in the aqueous screening medium at the intended concentration (100 μM) and were, instead, observed to form finely suspended precipitates. All synthesised derivatives were thus screened as suspensions.

3.2.3. Biological activity

Single-point screening experiments were performed to assess the anti-leishmanial activity (expressed as percentage growth inhibition ± standard deviation) of all synthesised quinazolinone derivatives against three strains of *Leishmania* promastigotes, with amphotericin B (AMB) serving as the reference drug. These strains were *L. donovani* strains 1S (MHOM/SD/62/1S) and 9515 (MHOM/IN/95/9515), and *L. major* strain IR-173 (MHOM/IR/-173). All strains were obtained from BEI resources. The cytotoxicity (half-maximal inhibitory concentration, IC_{50}) of all quinazolinone derivatives against Vero cells (expressed as the mean (μM) ± standard deviation) was also determined. Emetine (EM) served as the reference drug. The *Leishmania* species above were selected in order to determine the specificity of the derivatives against species that cause cutaneous forms of leishmaniasis (*L. major*) as well as the more serious and debilitating forms of the disease such as visceral leishmaniasis and post-kala azar dermal leishmaniasis (*L. donovani*) [50]. All quinazolinone derivatives and reference drugs were screened at concentrations of 100 μM in both the anti-leishmanial and cytotoxicity assays.

Table 3.2. *In vitro* anti-leishmanial activity (growth inhibition percentage ± standard deviation (SD)) and cytotoxicity (IC_{50}, mean (μM) ± standard deviation (SD)) of all synthesised quinazolinone derivatives.

Cpd	Growth inhibition (% ± SD) [c]			Cytotoxicity, IC_{50} (μM ± SD) [d]
	L. donovani 1S	*L. donovani* 9515	*L. major IR*-173	Vero [e]
1a	27.46 ± 0.24	1.72 ± 0.32	13.34 ± 4.90	>100
1b	0.00 ± 0.01	21.41 ± 3.19	14.16 ± 1.85	>100
2a	58.40 ± 5.62	43.46 ± 1.90	24.12 ± 0.70	>100
2b	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	>100
4a	0.00 ± 0.00	0.00 ± 0.00	8.68 ± 1.36	>100
4b	21.97 ± 1.55	33.83 ± 1.04	47.47 ± 1.56	>100
4c	0.00 ± 0.00	0.00 ± 0.38	0.81 ± 0.025	>100
4d	0.00 ± 0.00	13.31 ± 4.01	0.00 ± 0.00	>100
4e	0.00 ± 0.77	15.26 ± 1.85	25.55 ± 1.46	>100
4f	0.00 ± 0.00	0.00 ± 0.00	2.29 ± 0.73	>100
4g	0.00 ± 0.43	0.00 ± 0.00	2.94 ± 0.19	>100
4h	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	>100
4i	0.00 ± 0.00	0.00 ± 0.00	20.05 ± 2.50	>100
4j	0.00 ± 0.00	0.00 ± 0.00	8.22 ± 1.25	>100
AMB [a]	100 ± 0.00	100 ± 0.00	100 ± 0.00	57.77 ± 5.58
EM [b]	-	-	-	0.05 ± 0.007

[a] Amphotericin B deoxycholate. [b] Emetine dihydrochloride hydrate. [c] Growth inhibition values are expressed as the mean of triplicate measurements ± standard deviation (SD). [d] IC_{50} values are expressed as the mean of triplicate measurements ± standard deviation (SD). [e] African green monkey kidney epithelial cells.

Only three compounds (**1a**, **2a** and **4b**) were observed to have activity against *L. donovani* strain 1S. Substitution of a propargyl moiety at position 3 of the quinazolinone scaffold of compound **1a** (27% inhibition of promastigote growth) effectively doubles the anti-leishmanial activity against strain 1S, as evidenced by the activity of compound **2a** (58% inhibition of promastigote growth). The anti-leishmanial activity of compound **4b** (22% inhibition of promastigote growth) against *L. donovani* 1S was structure-specific as no other hybrid had activity against this strain. None of the compounds with a methoxy functional group (OCH_3) substituted at position R^1 were seen to have activity against this strain.

The substitution of an electron-donating methoxy group (OCH_3) at position R^1 of compound **1a** (2% inhibition of promastigote growth) was seen to have the effect of improving its activity against *L. donovani* strain 9515, as demonstrated by the markedly increased promastigote growth inhibitory activity of compound **1b** (21% inhibition of promastigote growth). The same, however, could not be said for compounds **2a, 4b**, **4d** and **4e**, as a methoxy group substituted at position R^1 in these compounds negated activity altogether (*cf.* the biological activities of

these compounds and their corresponding counterparts that contain a methoxy at position R^1 i.e. **2b**, **4g**, **4i** and **4j**). The activities of compounds **4b**, **4d** and **4e** appear to be structure-specific, as no observable correlation could be discerned between the net electronic effect (electron-withdrawing or electron-donating) of their substituents at position R^2 and the compounds' ability to inhibit the growth of *L. donovani* strain 9515 promastigotes. In general, the substitution of a methoxy group at position R^1 had the effect of significantly diminishing/negating the promastigote growth inhibition activity of a compound against *L. donovani* strain 9515, however, compound **1b** constituted the exception. Amongst compounds **4a-j**, the greatest inhibition of *L. donovani* strain 9515 promastigote growth was seen to be elicited by compound **4b** which possessed no substituent at position R^1 and a bromine substituent at position R^2 (34% inhibition of promastigote growth). Overall, compound **2a** was observed to be the most active compound against *L. donovani* strain 9515, with a moderate promastigote growth inhibitory percentage of 43%.

L. major (strain IR-173) promastigotes were observed to be more susceptible to the synthesised compounds than were the promastigotes of both strains of *L. donovani*. Most of the compounds (11 out of 14 compounds) were observed to exhibit some degree of growth inhibitory activity against *L. major* promastigotes, with the exception of compounds **2b**, **4d** and **4h**. Incremental increases in the growth inhibition activity of compounds **1a** and **4d** were effected by substituting a methoxy group at position R^1 of these compounds, as demonstrated by the growth inhibitory activities of compounds **1b** and **4i**. This, however, constituted the exception, as the presence of a methoxy group at position R^1 in other compounds was seen to lead to an marked decline in promastigote growth inhibitory activity. Once again, the biological activities of all assayed compounds were structure-specific, as no observable trend could be discerned between the electron-withdrawing or electron-donating capacities of substituents at position R^1 and R^2 and the ability of these compounds to inhibit the growth of *L. major* (strain IR-173) promastigotes. Overall, the greatest inhibitor of *Leishmania major* strain IR-173 promastigote growth was observed to be compound **4b** (47% inhibition of promastigote growth).

During the assays, all synthesised derivatives were found to form finely dispersed suspensions in the aqueous growth medium, hence, the concentration of compound solubilised throughout the aqueous growth medium phase may be less than 100 μM in both the single-point anti-leishmanial assay as well as the cytotoxicity assay. The poor aqueous solubility may have, in part, contributed to the lack of anti-leishmanial activity in some instances, as well as the low cytotoxicity of the compounds, as seen in Table 3.2. All synthesised derivatives were determined to possess low cytotoxicity (IC_{50} > 100 μM).

3.3. Conclusion

A series of 4(3*H*)-quinazolinone derivatives (14 in total) were synthesised in low to excellent yields (30 - 89%) using a variety of synthetic methods including cyclisation, condensation, nucleophilic (S_N2) substitution and copper-catalysed alkyne-azide cycloaddition (CuAAC) reactions. The *in vitro* anti-leishmanial activity of these compounds against three strains of *Leishmania* promastigotes, as well as their *in vitro* cytotoxicity against mammalian (Vero) cells, were determined using resazurin-based assays.

In general, the synthesised compounds were determined to be non-toxic (IC_{50} > 100 µM) to Vero cells but displayed only moderate to no anti-leishmanial activity despite meeting the requisite criteria (optimal physicochemical parameters) for drug-likeness. The complete lack of cytotoxicity and the inability to adequately inhibit promastigote growth may be attributed to the poor aqueous solubility of the compounds. Two derivatives were observed to possess moderate anti-leishmanial activities. These were compound **2a** [*(E)*-3-(prop-2-yn-1-yl)-2-styryl-quinazolin-4(3*H*)-one] with 43% and 58% growth inhibition of *L. donovani* 9515 and 1S promastigotes, and compound **4b** [(*E*)-2-(4-methoxystyryl)-3-(prop-2-yn-1-yl)-quinazolin-4(3*H*)-one] with 47% growth inhibition of *L. major* IR-173 promastigotes. No conclusive structure-activity relationship (SAR) could be deduced from this study. The amelioration of the problematic aqueous solubility of the quinazolinone derivatives encountered in this study will be the topic of future research.

3.4. Materials and methods

3.4.1. Materials

Isatoic anhydride, triethyl orthoacetate, benzaldehyde, 4-methoxy benzaldehyde, propargyl bromide, potassium carbonate, sodium azide, 4-nitrobenzyl bromide, 4-bromobenzyl bromide, 4-fluorobenzyl bromide, 4-methylbenzyl bromide, 4-benzyl bromide, dimethyl sulfoxide (DMSO), β-cyclodextrin, sodium L-ascorbate and copper sulphate pentahydrate were purchased from Sigma–Aldrich (South Africa). Ammonium acetate and N,N-dimethylformamide (DMF) were purchased from Merck (South Africa). All other solvents listed in this particular study (excepting DMSO and DMF), and magnesium sulphate ($MgSO_4$), were purchased from Associated Chemical Enterprises; ACE (Johannesburg, South Africa). All chemicals and reagents were reagent/analytical grade and were used without further purification.

3.4.2. General procedures

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of all compounds (14 compounds) were recorded on a Bruker Avance III 600 spectrometer at frequencies of 600 MHz (^{1}H NMR) and 150.913 (^{13}C NMR), respectively, in deuterated dimethyl sulfoxide DMSO-d_6 or deuterated chloroform $CDCl_3$ at ambient temperature (~25 °C), or alternatively, 80 degrees Celsius (80 °C) where indicated. Chemical shifts in spectra so obtained are expressed and reported in parts per million δ (ppm), with the residual protons of the deuterated solvents serving as the reference. *J* (coupling constant) values are expressed in Hertz (Hz). Abbreviations used to indicate splitting patterns are as follows: singlet (s), doublet (d), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of triplets (dt), doublet of quartets (dq), triplet (t), triplet of doublets (td), triplet of triplets (tt), quartet of doublets (qd) and multiplet (m).

High-resolution mass spectrometry (HRMS) spectra were recorded on a micrOTOF-Q II mass spectrometer, using an APCI source type heated at 200 °C. The software used to record the mass spectra was the Bruker Compass DataAnalaysis 4.0 software package. Scans were performed from 50 – 1600 m/z at a capillary voltage of 4500 volts (V), an end plate offset voltage of -500 V, with the nebulizer set at 1.6 Bar, and a collision cell radio frequency (RF) voltage of 100 volts peak to peak (Vpp).

High-performance liquid chromatography (HPLC) was carried out on all compounds to determine their purity. The system used was an Agilent 1100 HPLC system outfitted with a quaternary pump and an Agilent 1100 series diode array detector. HPLC-grade solvents such as acetonitrile and deionised or Milli-Q (Millipore) water were used for chromatographic purity analysis. A Venusil XBP C18 column (measurements: 4.6 mm x 150mm; particle size: 5 µm) with an initial mobile phase of 70% acetonitrile and 30% deionised water was employed at a flow rate of 1 millilitre per minute (1 ml/min). A linear increase in the concentration of the acetonitrile portion of the mobile phase was affected over a period of five minutes to a final concentration of 85%. Equilibration time between runs of the system was five minutes and each run took approximately fifteen minutes to complete (total time: twenty minutes per compound analysed). Injection volumes varied from 5-10 microliters (µl). The eluent was monitored at wavelengths of 210, 230, 254, 280, 300 nanometres (nm).

Infra-red (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument system. Thin-layer chromatography (TLC) was performed using aluminium-backed silica gel plates from Merck (Johannesburg, South Africa) with a mean pore size of 60 ångströms (Å) with added fluorescent indicator (F254). TLC plates were visualised at 254 nm. Melting points of all compounds were performed on a Büchi melting point B-545 apparatus and are uncorrected.

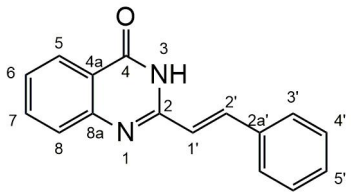
*3.4.3. General procedures for the synthesis of the 2-(4-substituted styryl)-4(3H)-quinazolinones (**1a-b**).*

Compounds **1a-b** were synthesised by using a literature method outlined in the work of Kumar and colleagues [37] as illustrated in Scheme 1 (i - ii). The method was carried out as follows:

To a mixture of isatoic anhydride (0.05 mol, 1 eq.) and ammonium acetate/NH_4OAc (1 eq.), in a flask outfitted with a magnetic stirrer, was added an equimolar amount of triethyl orthoacetate/$CH_3C(OEt)_3$ (1 eq.). The mixture was stirred neat and heated for 6-8 hours. Afterwards, the appropriate *para*-substituted benzaldehyde (benzaldehyde or 4-methoxybenzaldehyde) (1 eq.) was added and heating was continued for another 6-8 hours until TLC monitoring showed completion of the reaction. The contents of the flask were then suspended in a suitable amount of EtOH (25 ml), allowed to boil, and filtered. The residue was further purified by means of precipitation: the residue was heated (<153 °C) in a suitable amount of DMF and solubilised until a clear solution was obtained. The clear solution was then slowly decanted into a beaker containing an equivalent volume of ambient temperature distilled water. This resulted in the precipitation of compounds **1a-b** as suspended solid(s). The precipitate was filtered in a sintered glass funnel, washed three times with distilled water, and allowed to dry at room temperature.

*3.4.3.1. (E)-2-styrylquinazolin-4(3H)-one (**1a**)*

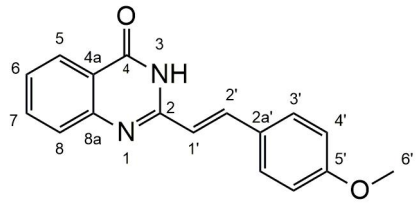
The reaction of intermediate 2-methyl-4(3*H*)-quinazolinone with benzaldehyde resulted in **1a**.



White powder; yield: 7.316 g (60%). m.p. 254.5-254.9 °C. IR ν_{max} (cm^{-1}): 3186.23 (N-H), 1664.30 (C=O), 1646.81 (C=N). ^1H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 12.06 (s, 1H, H-3), 8.13 (d, *J* = 7.9 Hz, 1H, H-5), 7.96 (d, *J* = 16.2 Hz, 1H, H-2'), 7.80 (t, *J* = 8.2 Hz, 1H, H-7), 7.68 (d, *J* = 8.2 Hz, 1H, H-8), 7.66 (d, *J* = 7.5 Hz, 2H, H-3'), 7.49 – 7.45 (m, 3H, H-6, H-4'), 7.42 (t, *J* = 7.2 Hz, 1H, H-5'), 7.01 (d, *J* = 16.2 Hz, 1H, H-1'). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 161.33 (C-4), 151.14 (C-2), 148.64 (C-8a), 137.92 (C-2'), 134.85 (C-2a'), 133.89 (C-7), 129.20 (C-5'), 128.58 (C-4'), 127.15 (C-3'), 126.65 (C-6), 125.68 (C-8), 125.44 (C-5), 121.03 (C-4a), 120.86 (C-1'). HRMS (APCI) *m/z*: $[M+H]^+$ 249.1018 (Calc. for $C_{16}H_{12}N_2O$: 249.1028). Purity (HPLC): 98.7%.

*3.4.3.2. (E)-2-(4-methoxy-styryl)-quinazolin-4(3H)-one (**1b**)*

The reaction of intermediate 2-methyl-4(3*H*)-quinazolinone with 4-methoxy benzaldehyde resulted in **1b**.



Light-yellow powder; yield: 6.813 g (49.9%). m.p. 285.0-285.5 °C. IR ν_{max} (cm^{-1}): 3182.22 (N-H), 1671.54 (C=O), 1642.27 (C=N), 1255.82 (Ar-OCH_3). ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 11.98 (s, 1H, H-3), 8.12 (dd, *J* = 7.9, 1.3 Hz, 1H, H-5), 7.91 (d, *J* = 16.1 Hz, 1H, H-2'), 7.78 (td, *J* = 8.3, 1.3 Hz, 1H, H-7), 7.65 (d, *J* = 8.1 Hz, 1H, H-8), 7.61 (d, *J* = 8.7 Hz, 2H, H-3'), 7.45 (t, *J* = 7.5 Hz, 1H, H-6), 7.03 (d, *J* = 8.7 Hz, 2H, H-4'), 6.86 (d, *J* = 16.1 Hz, 1H, H-1'), 3.84 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 161.37 (C-4), 160.39 (C-5'), 151.47 (C-2), 148.76 (C-8a), 137.68 (C-2'), 133.82 (C-7), 128.78 (C-3'), 127.51 (C-2a'), 126.50 (C-6), 125.42 (C-8), 125.37 (C-5), 120.71 (C-4a), 118.48 (C-1'), 114.28 (C-4'), 55.00 (C-6'). HRMS (APCI) *m/z*: $[M+H]^+$ 279.1117 (Calc. for $C_{17}H_{14}N_2O_2$: 279.1134). Purity (HPLC): 95.8%.

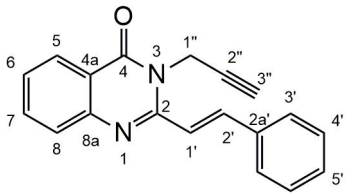
*3.4.4. General procedure for the synthesis of the 3-propyl-2-(4-substituted styryl)-4(3H)-quinazolinones (**2a-b**)*

Compounds **2a-b** were synthesised using the method described by Usifoh and Scriba [38], as illustrated in Scheme 1 (iii). The method is described as follows:

Propargyl bromide/PB (1.2 eq.) was added to a suspension of the appropriate 2-(4-substituted styryl)-4(3*H*)-quinazolinone **1a-b** (0.02 mol, 1 eq.) and potassium carbonate (12 eq.) in DMF (40 - 50 ml). The reaction mixture was allowed to stir at room temperature for 15 minutes - 48 hours until TLC monitoring indicated complete disappearance of the 2-(4-substituted styryl)-4(3*H*)-quinazolinone **1a-b**. The contents of the flask were then poured into distilled water and stirred until all of the potassium carbonate was sufficiently dissolved. This resulted in the precipitation of compounds **2a-b** as suspended solid(s). The precipitate was filtered off and washed with distilled water (3 x 50 ml). The resulting residue was then thoroughly dried and recrystallized using either Ace or EtOH.

*3.4.4.1. (E)-3-(prop-2-yn-1-yl)-2-styrylquinazolin-4(3H)-one (**2a**)*

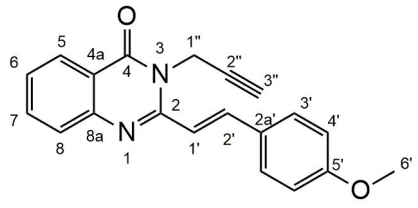
The reaction of (*E*)-2-styrylquinazolin-4(3*H*)-one (**1a**) with propargyl bromide resulted in **2a**.



Light-yellow needle-shaped crystals; yield: 3.075 g (44.4%). m.p. 181.3 - 182.3 °C. IR v_{max} (cm^{-1}): 3209.17 (≡CH), 2115.42 (C≡C), 1665.11 (C=O), 1633.33 (C=N), 1470.27 (CH_2 C-H). ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 8.17 (dd, *J* = 7.9, 0.8 Hz, 1H, H-5), 8.00 (d, *J* = 15.3 Hz, 1H, H-2'), 7.84 (td, *J* = 8.3, 0.8 Hz, 1H, H-7), 7.79 (d, *J* = 7.4 Hz, 2H, H-3'), 7.72 (d, *J* = 8.1 Hz, 1H, H-8), 7.52 (t, *J* = 7.5 Hz, 1H, H-6), 7.50 – 7.43 (m, 4H, H-1', H-4', H-5'), 5.20 (d, *J* = 2.4 Hz, 2H, H-1''), 3.24 (t, *J* = 2.4 Hz, 1H, H-3''). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 160.10 (C-4), 151.07 (C-2), 146.70 (C-8a), 139.98 (C-2'), 134.98 (C-2a'), 134.25 (C-7), 129.34 (C-5'), 128.52 (C-4'), 127.59 (C-3'), 126.75 (C-6), 126.19 (C-8), 126.06 (C-5), 119.47 (C-4a), 119.28 (C-1'), 78.61 (C-2''), 74.46 (C-3''), 31.75 (C-1''). HRMS (APCI) *m/z*: $[M+H]^+$ 287.1175 (Calc. for $C_{19}H_{14}N_2O$: 287.1184). Purity (HPLC): 97.8%.

*3.4.4.2. (E)-2-(4-methoxy-styryl)-3-(prop-2-yn-1-yl)-quinazolin-4(3H)-one (**2b**)*

The reaction of *(E)*-2-(4-methoxy-styryl)-quinazolin-4(3*H*)-one (**1b**) with propargyl bromide resulted in **2b**.



Yellow needle-shaped crystals; yield: 2.615 g (40.2%). m.p. 190.6 – 191.2 °C. IR v_{max} (cm^{-1}): 3235.38 (≡CH), 2114.67 (C≡C), 1659.77 (C=O), 1630.89 (C=N), 1468.15 (CH_2 C-H), 1245.52 (Ar-OCH_3). ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 8.15 (d, *J* = 7.8 Hz, 1H, H-5), 7.98 (d, *J* = 15.2 Hz, 1H, H-2'), 7.82 (t, *J* = 8.0 Hz, 1H, H-7), 7.74 (d, *J* = 8.6 Hz, 2H, H-3'), 7.69 (d, *J* = 8.1 Hz, 1H, H-8), 7.50 (t, *J* = 7.5 Hz, 1H, H-6), 7.30 (d, *J* = 15.2 Hz, 1H, H-1'), 7.05 (d, *J* = 8.6 Hz, 2H, H-4'), 5.18 (d, *J* = 2.3 Hz, 2H, H-1''), 3.85 (s, 3H, H-6'), 3.22 (t, *J* = 2.3 Hz, 1H, H-3''). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 160.55 (C-4), 160.16 (C-5'), 151.35 (C-2), 146.83 (C-8a), 139.86 (C-2'), 134.18 (C-7), 129.29 (C-3'), 127.70 (C-2a'), 126.61 (C-6), 126.04 (C-8), 125.87 (C-5), 119.31 (C-4a), 116.62 (C-1'), 114.23 (C-4'), 78.67 (C-2''), 74.35 (C-3''), 55.03 (C-6'), 31.71 (C-1''). HRMS (APCI) *m/z*: $[M+H]^+$ 317.1267 (Calc. for $C_{20}H_{16}N_2O_2$: 317.1290). Purity (HPLC): 98.2%.

*3.4.5. General procedures for the synthesis of the para-substituted benzyl azides (**3a-e**) and the 1H-1,2,3-triazole-4(3H)-quinazolinone hybrids (**4a-j**).*

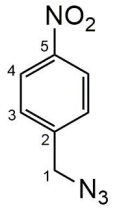
Compounds **3a-e** and **4a-j** were synthesised by employing a method derived from the work of Cilliers and colleagues [39], as illustrated in Scheme 1 (iv - v). The method is described as follows:

The appropriate *para*-substituted benzyl bromide (0.01 mol, 1 eq.) was dissolved in 20 ml of DMSO in a flask outfitted with a magnetic stirrer. Sodium azide/NaN_3 (1.1 eq.) was slowly added to the flask and the reaction mixture was allowed to stir for 10 - 12 hours at room temperature. After the elapsed time, the contents of the flask were slowly quenched by way of addition to a beaker containing 50 ml of room temperature distilled water. The aqueous mixture in the beaker was then extracted three times with 50 ml of Et_2O or EtAc. The extracted organic phase was then washed three times with 50 ml of a saturated brine solution, dried overnight with $MgSO_4$, and filtered. Lastly, the filtrate was evaporated using a rotary vacuum evaporator to produce compounds **3a-e** as oil-like liquids. These compounds were used in subsequent syntheses without any further purification.

Then, to a flask containing 10 ml of a mixture of DMSO/distilled water (4:1), was added the appropriate 3-propyl-2-(4-substituted styryl)-4(3*H*)-quinazolinone **2a-b** (0.0007 mol, 1 eq.), the appropriate *para*-substituted benzyl azide **3a-e** (1.1 eq.), β-cyclodextrin/β-CD (30-60 mg), sodium L-ascorbate/Na L-ascorbate (0.3 eq.) and copper sulphate pentahydrate (0.1 eq.). The mixture was stirred at room temperature for 48-72 hours until TLC monitoring showed the complete consumption of the 3-propyl-2-(4-substituted styryl)-4(3*H*)-quinazolinone **2a-b**. The reaction was then quenched by decanting the reaction mixture into a beaker filled with room temperature distilled water. Compounds **4a-j** were seen to be suspended as solid throughout the aqueous phase. The suspension was filtered and the resulting residue was thoroughly rinsed three times each with both room temperature distilled water and EtOH. Residues were dried in an oven at 70 °C for 2-3 hours to yield compounds **4a-j**.

3.4.5.1. 1-(azidomethyl)-4-nitrobenzene (**3a**)

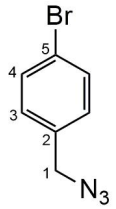
The reaction of 1-(bromomethyl)-4-nitrobenzene with sodium azide resulted in **3a**.



Reddish-orange oil (liquid); yield: 2.473 g (100%). m.p. oil. IR v_{max} (cm^{-1}): 2102 (N=N=N). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.24 (d, *J* = 8.6 Hz, 2H, H-4), 7.50 (d, *J* = 8.5 Hz, 2H, H-3), 4.50 (s, 2H, H-1). ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 147.88 (C-5), 142.83 (C-2), 128.72 (C-3), 124.18 (C-4), 53.86 (C-1).

*3.4.5.2. 1-(azidomethyl)-4-bromobenzene (**3b**)*

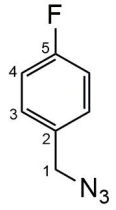
The reaction of 1-(bromomethyl)-4-bromobenzene with sodium azide resulted in **3b**.



Clear oil (liquid); yield: 2.545 g (100%). m.p. oil. IR v_{max} (cm^{-1}): 2093 (N=N=N). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.51 (d, *J* = 8.4 Hz, 2H, H-4), 7.19 (d, *J* = 8.4 Hz, 2H, H-3), 4.30 (s, 2H, H-1). ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 134.52 (C-2), 132.13 (C-3), 129.94 (C-4), 122.48 (C-5), 54.23 (C-1).

*3.4.5.3. 1-(azidomethyl)-4-fluorobenzene (**3c**)*

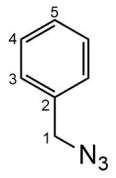
The reaction of 1-(bromomethyl)-4-fluorobenzene with sodium azide resulted in **3c**.



Yellow oil (liquid); yield: 1.680 g (70%). m.p. oil. IR v_{max} (cm^{-1}): 2093 (N=N=N). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.30 (dd, *J* = 8.5, 5.3 Hz, 2H, H-3), 7.08 (t, *J* = 8.6 Hz, 2H, H-4), 4.32 (s, 2H, H-1). ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 162.78 (C-5), 131.32 (C-2), 130.15 (C-3), 115.93 (C-4), 54.20 (C-1).

*3.4.5.4. (Azidomethyl)-benzene (**3d**)*

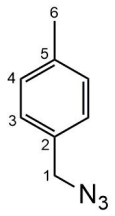
The reaction of (bromomethyl)-benzene with sodium azide resulted in **3d**.



Yellow oil (liquid); yield: 1.633 g (70%). m.p. oil. IR v_{max} (cm^{-1}): 2090 (N=N=N). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.40 (t, *J* = 7.4 Hz, 2H, H-4), 7.35 (t, *J* = 6.9 Hz, 1H, H-5), 7.33 (d, *J* = 7.4 Hz, 2H, H-3), 4.35 (s, 2H, H-1). ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 135.49 (C-2), 128.96 (C-4), 128.43 (C-5), 128.34 (C-3), 54.93 (C-1).

*3.4.5.5. 1-(azidomethyl)-4-methylbenzene (**3e**)*

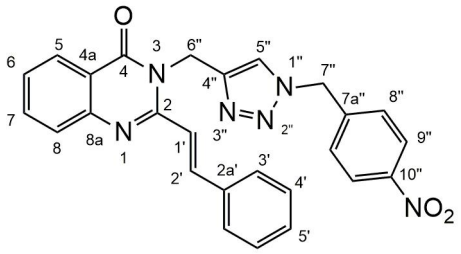
The reaction of 1-(bromomethyl)-4-methylbenzene with sodium azide resulted in **3e**.



Pale yellow oil (liquid); yield: 2.270 g (95.1%). m.p. oil. IR v_{max} (cm^{-1}): 2094 (N=N=N). ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.22 (d, *J* = 8.3 Hz, 2H, H-3), 7.20 (d, *J* = 8.3 Hz, 2H, H-4), 4.30 (s, 2H, H-1), 2.37 (s, 3H, H-6). ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 138.28 (C-2), 132.41 (C-5), 129.63 (C-4), 128.40 (C-3), 54.75 (C-1), 21.31 (C-6).

*3.4.5.6. (E)-3-{[1-(4-nitrobenzyl)-1H-1,2,3-triazol-4-yl]methyl}-2-styrylquinazolin-4(3H)-one (**4a**)*

The reaction of (*E*)-3-(prop-2-yn-1-yl)-2-styrylquinazolin-4(3*H*)-one (**2a**) with 1-(azidomethyl) -4-nitrobenzene (**3a**) resulted in **4a**.

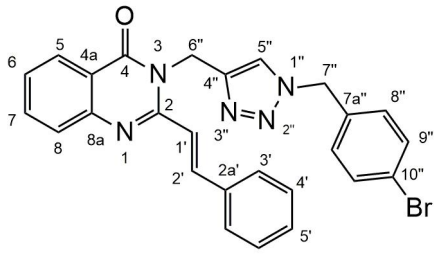


Pale yellow powder; yield: 0.347 g (52.4%). m.p. 248.9 – 251.6 °C. IR v_{max} (cm^{-1}): 3137.11 (=CH), 1661.45 (C=O), 1519.12 (NO_2), 1469.70 (CH_2), 1342.07 (C-N). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.28 (s, 1H, H-5''), 8.15 (d, *J* = 7.8 Hz, 1H, H-5), 8.06 (d, *J* = 8.6 Hz, 2H, H-9''), 7.88 (d, *J* = 15.3 Hz, 1H, H-2'), 7.83 (t, *J* = 7.6 Hz, 1H, H-7), 7.71 (d, *J* = 7.1 Hz, 2H, H-3'), 7.71 (d, *J* = 7.4 Hz, 1H, H-8), 7.62 (d, *J* = 15.3 Hz, 1H, H-1'), 7.51 (t, *J* = 7.5 Hz, 1H, H-6), 7.44 – 7.38 (m, 5H, H-4', H-5', H-8''), 5.73 (s, 2H, H-7''), 5.63 (s, 2H, H-6''). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 161.10 (C-4), 151.96 (C-2), 147.12 (C-8a), 147.09 (C-10''), 143.38 (C-7a''), 143.29 (C-2'), 139.85 (C-2a'), 135.16 (C-7), 134.65 (C-4''), 129.73 (C-5'),

128.91 (C-8''), 128.81 (C-4'), 127.92 (C-3'), 127.08 (C-6), 126.52 (C-8), 126.43 (C-5), 124.25 (C-5''), 123.75 (C-9''), 120.07 (C-4a), 119.90 (C-1'), 51.89 (C-7''), 38.07 (C-6''). HRMS (APCI) *m/z*: $[M+H]^+$ 465.1641 (Calc. for $C_{26}H_{20}N_6O_3$: 465.1675). Purity (HPLC): ~100%.

*3.4.5.7. (E)-3-{[1-(4-bromobenzyl)-1H-1,2,3-triazol-4-yl]methyl}-2-styrylquinazolin-4(3H)-one (**4b**)*

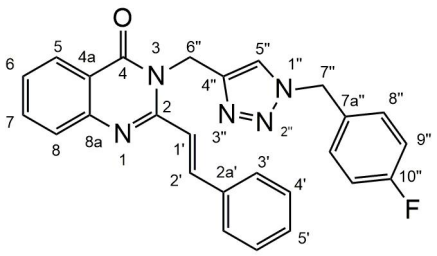
The reaction of (*E*)-3-(prop-2-yn-1-yl)-2-styrylquinazolin-4(3*H*)-one (**2a**) with 1-(azidomethyl) -4-bromobenzene (**3b**) resulted in **4b**.



Light yellow-orange powder; yield: 0.267 g (37.7%). m.p. 269.0 - 272.5 °C. IR ν_{max} (cm^{-1}): 3136.88 (=C-H), 1665.13 (C=O), 1634.74 (C=N), 1470.80 (CH_2), 1330.56 (C-N). ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 8.17 (dd, *J* = 7.9, 1.1 Hz, 1H, H-5), 8.12 (s, 1H, H-5''), 7.89 (d, *J* = 15.3 Hz, 1H, H-2'), 7.83 (td, *J* = 8.3, 1.1 Hz, 1H, H-7), 7.71 (d, *J* = 8.8 Hz, 1H, H-8), 7.69 (d, *J* = 7.5 Hz, 2H, H-3'), 7.58 (d, *J* = 15.3 Hz, 1H, H-1'), 7.51 (t, *J* = 7.9 Hz, 1H, H-6), 7.47 – 7.41 (m, 5H, H-4', H-5', H-9''), 7.19 (d, *J* = 8.3 Hz, 2H, H-8''), 5.59 (s, 2H, H-7''), 5.53 (s, 2H, H-6''). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 160.73 (C-4), 151.68 (C-2), 146.86 (C-8a), 142.83 (C-2'), 139.51 (C-2a'), 139.51 (C-7a''), 135.00 (C-7), 134.81 (C-4''), 134.05 (C-5'), 131.15 (C-9''), 129.58 (C-8''), 129.21 (C-6), 128.47 (C-4'), 127.43 (C-3'), 126.70 (C-8), 125.99 (C-5), 123.37 (C-5''), 120.93 (C-4a), 119.90 (C-10''), 119.68 (C-1'), 51.84 (C-7''), 37.97 (C-6''). HRMS (APCI) *m/z*: $[M+H]^+$ 498.0889, $[M+3H]^{3+}$ 500.0873 (Calc. for $C_{26}H_{20}BrN_5O$: 498.0929, 500.0909). Purity (HPLC): ~100%.

*3.4.5.8. (E)-3-{[1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl]methyl}-2-styrylquinazolin-4(3H)-one (**4c**)*

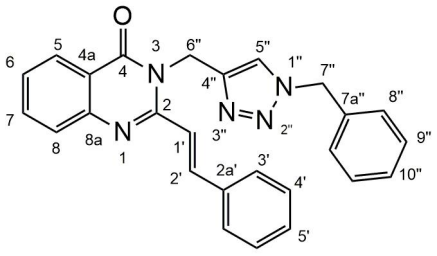
The reaction of (*E*)-3-(prop-2-yn-1-yl)-2-styrylquinazolin-4(3*H*)-one (**2a**) with 1-(azidomethyl) -4-fluorobenzene (**3c**) resulted in **4c**.



Light-yellow powder; yield: 0.182 g (29.8%). m.p. 236.5 – 238.6 °C. IR v_{max}: (cm^{-1}): 3135.04 (=CH), 1657.14 (C=O), 1632.59 (C=N), 1469.42 (CH_2), 1344.07 (C-N). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.20 (s, 1H, H-5''), 8.15 (d, *J* = 7.8 Hz, 1H, H-5), 7.89 (d, *J* = 15.3 Hz, 1H, H-2'), 7.83 (t, *J* = 7.6 Hz, 1H, H-7), 7.72 (d, *J* = 7.3 Hz, 2H, H-3'), 7.71 (d, *J* = 8.4 Hz, 1H, H-8), 7.63 (d, *J* = 15.3 Hz, 1H, H-1'), 7.51 (t, *J* = 7.5 Hz, 1H, H-6), 7.45 (t, *J* = 7.2 Hz, 2H, H-4'), 7.42 (t, *J* = 7.2 Hz, 1H, H-5'), 7.30 (dd, *J* = 8.4, 5.6 Hz, 2H, H-8''), 7.07 (t, *J* = 8.8 Hz, 2H, H-9''), 5.59 (s, 2H, H-7''), 5.53 (s, 2H, H-6''). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 162.58 (C-4), 161.08 (C-10''), 160.96 (C-10''), 151.97 (C-2), 147.11 (C-8a), 143.15 (C-2'), 139.88 (C-2a'), 135.21 (C-7), 134.63 (C-4''), 132.15 (C-7a''), 132.13 (C-7a''), 130.15 (C-8''), 130.09 (C-8''), 129.76 (C-5'), 128.94 (C-4'), 127.94 (C-3'), 127.08 (C-6), 126.50 (C-8), 126.41 (C-5), 123.76 (C-5''), 120.09 (C-4a), 119.91 (C-1'), 115.54 (C-9''), 115.39 (C-9''), 51.99 (C-7''), 38.16 (C-6''). HRMS (APCI) *m/z*: $[M+H]^+$ 438.1712 (Calc. for $C_{26}H_{21}FN_5O$: 438.1730). Purity (HPLC): ~100%.

*3.4.5.9. (E)-3-{[1-benzyl-1H-1,2,3-triazol-4-yl]methyl}-2-styrylquinazolin-4(3H)-one (**4d**)*

The reaction of (*E*)-3-(prop-2-yn-1-yl)-2-styrylquinazolin-4(3*H*)-one (**2a**) with (Azidomethyl)-benzene (**3d**) resulted in **4d**.

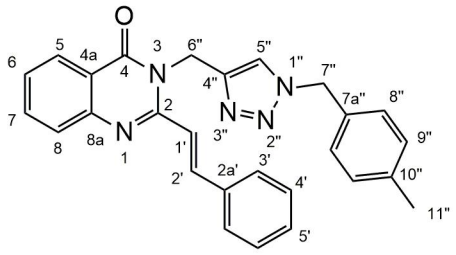


Light grey powder; yield: 0.523 g (88.8%). m.p. 259.2 - 260.8 °C. IR v_{max}: (cm^{-1}): 3135.77 (=CH), 1666.33 (C=O), 1635.61 (C=N), 1470.49 (CH_2), 1332.39 (C-N). ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 8.16 (dd, *J* = 7.9, 1.2 Hz, 1H, H-5), 8.09 (s, 1H, H-5''), 7.89 (d, *J* = 15.3 Hz, 1H, H-2'), 7.82 (td, *J* = 8.4, 1.2 Hz, 1H, H-7), 7.70 (dd, *J* = 7.6, 4.0 Hz, 3H, H-8, H-3'), 7.59 (d, *J* = 15.3 Hz, 1H, H-1'), 7.50 (t, *J* = 7.9 Hz, 1H, H-6), 7.45 (t, *J* = 7.2 Hz, 2H, H-4'), 7.41 (t, *J* = 7.2 Hz, 1H, H-5'), 7.29 - 7.26 (m, 3H, H-9'', H-10''), 7.23 (dd, *J* = 6.6, 2.9 Hz, 2H, H-8''), 5.58 (s, 2H, H-7''), 5.54 (s, 2H, H-6''). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 160.73

(C-4), 151.69 (C-2), 146.85 (C-8a), 142.73 (C-2'), 139.53 (C-2a'), 135.40 (C-7a''), 135.02 (C-7), 134.04 (C-4''), 129.20 (C-5'), 128.48 (C-4'), 128.19 (C-9''), 127.60 (C-6), 127.43 (C-3'), 127.36 (C-8''), 126.70 (C-10''), 125.98 (C-8), 125.98 (C-5), 123.29 (C-5''), 119.91 (C-4a), 119.68 (C-1'), 52.59 (C-7''), 38.01 (C-6''). HRMS (APCI) *m/z*: $[M+H]^+$ 420.1796 (Calc. for $C_{26}H_{22}N_5O$: 420.1824). Purity (HPLC): ~100%.

*3.4.5.10. (E)-3-{[1-(4-methylbenzyl)-1H-1,2,3-triazol-4-yl]methyl}-2-styrylquinazolin-4(3H)-one (**4e**)*

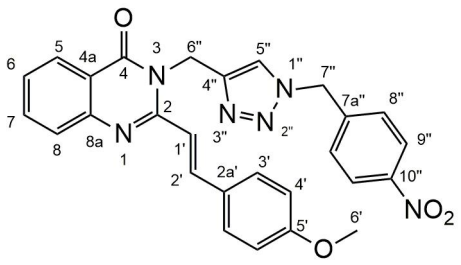
The reaction of (E)-3-(prop-2-yn-1-yl)-2-styrylquinazolin-4(3H)-one (2a) with 1-(azidomethyl)-4-methylbenzene (**3e**) resulted in **4e**.



Light yellow powder; yield: 0.407 g (67.2%). m.p. 238.5 – 240.7 °C. IR ν_{max}: (cm^{-1}): 3133.00 (=CH), 1664.55 (C=O), 1633.23 (C=N), 1472.55 (CH_2), 1346.60 (C-N). ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 8.16 (dd, *J* = 7.9, 1.1 Hz, 1H, H-5), 8.05 (s, 1H, H-5''), 7.89 (d, *J* = 15.3 Hz, 1H, H-2'), 7.82 (td, *J* = 8.4, 1.1 Hz, 1H, H-7), 7.70 (d, *J* = 7.0 Hz, 1H, H-8), 7.69 (d, *J* = 6.7 Hz, 2H, H-3'), 7.58 (d, *J* = 15.3 Hz, 1H, H-1'), 7.50 (t, *J* = 7.9 Hz, 1H, H-6), 7.45 (t, *J* = 7.2 Hz, 2H, H-4'), 7.41 (t, *J* = 7.2 Hz, 1H, H-5'), 7.13 (d, *J* = 8.0 Hz, 2H, H-8''), 7.07 (d, *J* = 7.9 Hz, 2H, H-9''), 5.57 (s, 2H, H-7''), 5.48 (s, 2H, H-6''), 2.25 (s, 3H, H-11''). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 160.72 (C-4), 151.69 (C-2), 146.85 (C-8a), 142.70 (C-2'), 139.52 (C-10''), 137.02 (C-2a'), 135.02 (C-7), 134.03 (C-7a''), 132.37 (C-4''), 129.19 (C-5'), 128.76 (C-9''), 128.46 (C-4'), 127.43 (C-3'), 127.40 (C-8''), 126.69 (C-6), 125.98 (C-8), 125.97 (C-5), 123.11 (C-5''), 119.90 (C-4a), 119.68 (C-1'), 52.40 (C-7''), 38.00 (C-6''), 20.14 (C-11''). HRMS (APCI) *m/z*: $[M+H]^+$ 434.1942 (Calc. for $C_{27}H_{24}N_5O$: 434.1981). Purity (HPLC): ~100%.

*3.4.5.11. (E)-2-(4-methoxy-styryl)-3-{[1-(4-nitrobenzyl)-1H-1,2,3-triazol-4-yl]-methyl}-quinazolin-4(3H)-one (**4f**)*

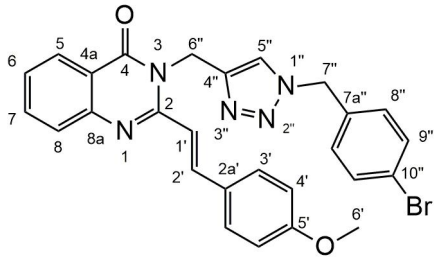
The reaction of (E)-2-(4-methoxy-styryl)-3-(prop-2-yn-1-yl)-quinazolin-4(3H)-one (2b) with 1-(azidomethyl)-4-nitrobenzene (**3a**) resulted in **4f**.



Pale orange powder; yield: 0.347 g (54.4%). m.p. 343.5 – 343.6 °C. IR v_{max}: (cm^{-1}): 3125.95 (=CH), 1666.39 (C=O), 1635.04 (C=N), 1519.48 (NO_2), 1472.66 (CH_2), 1347.82 (C-N), 1252.33 (Ar-OCH_3). ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 8.17 (s, 1H, H-5''), 8.15 (d, *J* = 7.9 Hz, 1H, H-5), 8.07 (d, *J* = 8.6 Hz, 2H, H-9''), 7.85 (d, *J* = 15.2 Hz, 1H, H-2'), 7.81 (t, *J* = 7.1 Hz, 1H, H-7), 7.67 (d, *J* = 8.1 Hz, 1H, H-8), 7.63 (d, *J* = 8.6 Hz, 2H, H-3'), 7.48 (t, *J* = 7.5 Hz, 1H, H-6), 7.44 (d, *J* = 8.6 Hz, 2H, H-8''), 7.40 (d, *J* = 15.3 Hz, 1H, H-1'), 6.99 (d, *J* = 8.6 Hz, 2H, H-4'), 5.71 (s, 2H, H-7''), 5.60 (s, 2H, H-6''), 3.83 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 160.81 (C-4), 160.43 (C-5'), 151.94 (C-2), 147.01 (C-8a), 146.98 (C-10''), 143.07 (C-7a''), 142.73 (C-2'), 139.36 (C-7), 134.01 (C-4''), 129.09 (C-3'), 128.47 (C-8''), 127.68 (C-2a'), 126.57 (C-6), 125.99 (C-8), 125.70 (C-5), 123.69 (C-5''), 123.22 (C-9''), 119.52 (C-4a), 117.27 (C-1'), 114.15 (C-4'), 54.98 (C-7''), 51.68 (C-6'), 37.87 (C-6''). HRMS (APCI) *m/z*: $[M+H]^+$ 495.1768 (Calc. for $C_{27}H_{23}N_6O_4$: 495.1781). Purity (HPLC): ~100%.

*3.4.5.12. (E)-3-{[1-(4-bromobenzyl)-1H-1,2,3-triazol-4-yl]methyl}-2-(4-methoxy styryl)-quinazolin-4(3H)-one (**4g**)*

The reaction of (*E*)-2-(4-methoxy-styryl)-3-(prop-2-yn-1-yl)-quinazolin-4(3*H*)-one (**2b**) with 1-(azidomethyl)-4-bromobenzene (**3b**) resulted in **4g**.

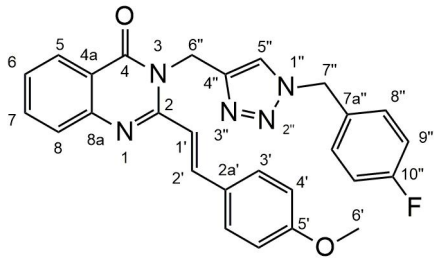


Light green powder; yield: 0.614 g (82.4%). m.p. 246.4 – 250.7 °C. IR v_{max}: (cm^{-1}): 3140.33 (=CH), 1661.21 (C=O), 1632.31 (C=N), 1470.85 (CH_2), 1335.56 (C-N), 1252.83 (Ar-OCH_3). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.19 (s, 1H, H-5''), 8.14 (d, *J* = 7.5 Hz, 1H, H-5), 7.87 (d, *J* = 15.2 Hz, 1H, H-2'), 7.82 (t, *J* = 7.6 Hz, 1H, H-7), 7.68 (d, *J* = 8.4 Hz, 3H, H-8, H-3'), 7.49 (t, *J* = 7.4 Hz, 1H, H-6), 7.46 – 7.41 (m, 3H, H-1', H-9''), 7.15 (d, *J* = 8.1 Hz, 2H, H-8''), 7.01 (d, *J* = 8.5 Hz, 2H, H-4'), 5.59 (s, 2H, H-7''), 5.53 (s, 2H, H-6''), 3.82 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 160.77 (C-4), 160.42 (C-5'), 151.93 (C-2), 146.96 (C-8a),

142.86 (C-2'), 139.35 (C-7a''), 134.80 (C-7), 133.94 (C-4''), 131.11 (C-9''), 129.52 (C-8''), 129.06 (C-3'), 127.72 (C-2a'), 126.54 (C-6), 125.94 (C-8), 125.64 (C-5), 123.30 (C-5''), 120.88 (C-4a), 119.51 (C-10''), 117.28 (C-1'), 114.17 (C-4'), 55.00 (C-7''), 51.81 (C-6'), 37.91 (C-6''). HRMS (APCI) *m/z*: $[M+H]^+$ 528.1022, $[M+3H]^{3+}$ 530.1005 (Calc. for $C_{27}H_{23}BrN_5O_2$: 528.1035, 530.1015). Purity (HPLC): ~100%.

*3.4.5.13. (E)-3-{[1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl]methyl}-2-(4-methoxystyryl)-quinazolin-4(3H)-one (**4h**)*

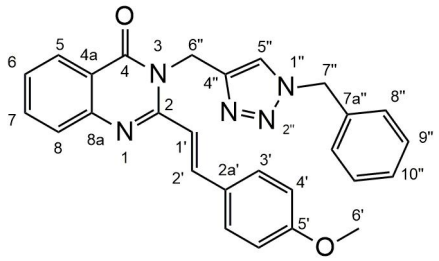
The reaction of (*E*)-2-(4-methoxy-styryl)-3-(prop-2-yn-1-yl)-quinazolin-4(3*H*)-one (**2b**) with 1-(azidomethyl)-4-fluorobenzene (**3c**) resulted in **4h**.



Light green powder; yield: 0.494 g (74.7%). m.p. 229.3 – 230.7 °C. IR ν_{max}: (cm^{-1}): 3111.97 (=CH), 1657.47 (C=O), 1472.79 (CH_2), 1343.42 (C-N), 1249.83 (Ar-OCH_3). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.18 (s, 1H, H-5''), 8.13 (d, *J* = 7.8 Hz, 1H, H-5), 7.87 (d, *J* = 15.2 Hz, 1H, H-2'), 7.82 (t, *J* = 8.1 Hz, 1H, H-7), 7.68 (d, *J* = 8.4 Hz, 3H, H-8, H-3'), 7.48 (t, *J* = 7.4 Hz, 1H, H-6), 7.46 (d, *J* = 15.2 Hz, 1H, H-1'), 7.30 (dd, *J* = 8.3, 5.6 Hz, 2H, H-8''), 7.08 (t, *J* = 8.8 Hz, 2H, H-9''), 7.01 (d, *J* = 8.6 Hz, 2H, H-4'), 5.57 (s, 2H, H-7''), 5.53 (s, 2H, H-6''), 3.82 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 162.30 (C-4), 160.76 (C-10''), 160.68 (C-10''), 160.41 (C-5'), 151.93 (C-2), 146.95 (C-8a), 142.81 (C-2'), 139.35 (C-7), 133.93 (C-4''), 131.61 (C-7a''), 131.59 (C-7a''), 129.66 (C-8''), 129.60 (C-8''), 129.05 (C-3'), 127.73 (C-2a'), 126.53 (C-6), 125.93 (C-8), 125.64 (C-5), 123.16 (C-5''), 119.51 (C-4a), 117.29 (C-1'), 115.03 (C-9''), 114.88 (C-9''), 114.16 (C-4'), 54.99 (C-7''), 51.77 (C-6'), 37.93 (C-6''). HRMS (APCI) *m/z*: $[M+H]^+$ 468.1819 (Calc. for $C_{27}H_{23}FN_5O_2$: 468.1836). Purity (HPLC): ~100%.

*3.4.5.14. (E)-3-{[1-benzyl-1H-1,2,3-triazol-4-yl]methyl}-2-(4-methoxystyryl)quinazolin-4(3H)-one (**4i**)*

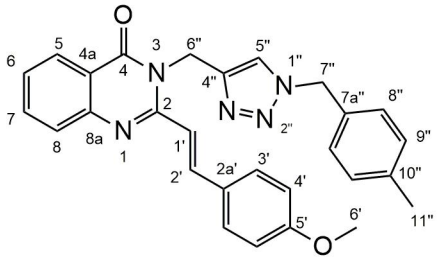
The reaction of (*E*)-2-(4-methoxy-styryl)-3-(prop-2-yn-1-yl)-quinazolin-4(3*H*)-one (**2b**) with (Azidomethyl)-benzene (**3d**) resulted in **4i**.



Yellow powder; yield: 0.235 g (36.8%). m.p. 224.7 – 226.9 °C. IR v_{max}: (cm^{-1}): 3107.92 (=CH), 1654.44 (C=O), 1469.56 (CH_2), 1346.12 (C-N), 1254.82 (Ar-OCH_3). ^{1}H NMR (600 MHz, DMSO-d_6, 80 °C) δ (ppm): 8.14 (dd, *J* = 7.9, 1.2 Hz, 1H, H-5), 8.08 (s, 1H, H-5''), 7.87 (d, *J* = 15.3 Hz, 1H, H-2'), 7.80 (td, *J* = 8.4, 1.2 Hz, 1H, H-7), 7.67 (d, *J* = 8.1 Hz, 1H, H-8), 7.64 (d, *J* = 8.7 Hz, 2H, H-3'), 7.47 (t, *J* = 7.5 Hz, 1H, H-6), 7.43 (d, *J* = 15.3 Hz, 1H, H-1'), 7.30 – 7.28 (m, 3H, H-9'', H-10''), 7.24 (dd, *J* = 6.7, 2.7 Hz, 2H, H-8''), 7.01 (d, *J* = 8.7 Hz, 2H, H-4'), 5.57 (s, 2H, H-7''), 5.54 (s, 2H, H-6''), 3.84 (s, 3H, H-6'). ^{13}C NMR (151 MHz, DMSO-d_6, 80 °C) δ (ppm): 161.02 (C-4), 160.58 (C-5'), 152.15 (C-2), 147.15 (C-8a), 143.03 (C-2'), 139.65 (C-7a''), 135.73 (C-7), 134.36 (C-4''), 129.45 (C-3'), 128.48 (C-9''), 127.87 (C-2a'), 127.84 (C-6), 127.63 (C-8''), 126.80 (C-8), 126.24 (C-5), 126.00 (C-10''), 123.62 (C-5''), 119.67 (C-4a), 117.35 (C-1'), 114.34 (C-4'), 55.21 (C-7''), 52.71 (C-6'), 38.09 (C-6''). HRMS (APCI) *m/z*: $[M+H]^+$ 450.1910 (Calc. for $C_{27}H_{24}N_5O_2$: 450.1930). Purity (HPLC): ~100%.

*3.4.5.15. (E)-2-(4-methoxystyryl)-3-{[1-(4-methylbenzyl)-1H-1,2,3-triazol-4-yl]-methyl}-quinazolin-4(3H)-one (**4j**)*

The reaction of (*E*)-2-(4-methoxy-styryl)-3-(prop-2-yn-1-yl)-quinazolin-4(3*H*)-one (**2b**) with 1-(azidomethyl)-4-methylbenzene (**3e**) resulted in **4j**.



Light green powder; yield: 0.361 g (55.8%). m.p. 217.5 – 221.1 °C. IR v_{max}: (cm^{-1}): 3125.76 (=CH), 1668.81 (C=O), 1631.55 (C=N), 1470.74 (CH_2), 1339.12 (C-N), 1254.10 (Ar-OCH_3). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.13 (s, 1H, H-5''), 8.13 (d, *J* = 6.7 Hz, 1H, H-5), 7.87 (d, *J* = 15.2 Hz, 1H, H-2'), 7.81 (t, *J* = 8.1 Hz, 1H, H-7), 7.67 (d, *J* = 8.6 Hz, 3H, H-8, H-3'), 7.48 (t, *J* = 7.6 Hz, 1H, H-6), 7.45 (d, *J* = 15.1 Hz, 1H, H-1'), 7.11 (d, *J* = 7.9 Hz, 2H, H-8''), 7.05 (d, *J* = 7.8 Hz, 2H, H-9''), 7.01 (d, *J* = 8.6 Hz, 2H, H-4'), 5.57 (s, 2H, H-7''), 5.48 (s, 2H, H-6''), 3.82 (s, 3H, H-6'), 2.22 (s, 3H, H-11''). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 161.14

(C-4), 160.66 (C-5'), 152.24 (C-2), 147.24 (C-8a), 143.15 (C-2'), 139.79 (C-10''), 137.35 (C-7), 134.56 (C-7a''), 132.89 (C-4''), 129.65 (C-3'), 129.17 (C-9''), 127.90 (C-2a'), 127.81 (C-8''), 126.93 (C-6), 126.40 (C-8), 126.18 (C-5), 123.62 (C-5''), 119.75 (C-4a), 117.37 (C-1'), 114.42 (C-4'), 55.32 (C-7''), 52.59 (C-6'), 38.14 (C-6''), 20.63 (C-11''). HRMS (APCI) *m/z*: $[M+H]^+$ 464.2077 (Calc. for $C_{28}H_{26}N_5O_2$: 464.2087). Purity (HPLC): ~100%.

3.4.6. In vitro biological evaluation

3.4.6.1. Anti-leishmanial activity assessment

The anti-promastigote activity of all synthesised compounds in this study was evaluated by using the following resazurin-based assay [51]:

Three strains of *Leishmania* - *Leishmania donovani* (strains 1S MHOM/SD/62/1S and 9515 MHOM/IN/95/9515 and *L.* major (strain IR-173 (MHOM/IR/-173)) - promastigotes were cultured in M199 with Hank's salts and 0.68 mM L-glutamine (purchased from Sigma Aldrich) supplemented with 4.2 mM sodium bicarbonate ($NaHCO_3$), 25 mM Hepes, 10 % fetal bovine serum and 50 units/mL Penicillin/Streptomycin solution with the pH adjusted to physiological pH of 7.3 – 7.4. The promastigotes were maintained at 26 °C. In the resazurin assay, promastigotes were harvested in the logarithmic phase of growth (1.25×10^5 cells/well or 1.25×10^6 cells/mL, final volume 100 µL/well) and seeded into 96 well plates (Nunc, Thermofisher Scientific) with the addition of 100 µM of compound for activity screening. The number of parasites was determined by counting the parasites using a haemocytometer. Amphotericin B served as the standard reference drug. Promastigotes cultured without any drugs served as the negative control while growth medium without any added parasites or drugs served as the blank. The prepared plates were incubated for 72 hours at a temperature of 26 ˚C in a humidified atmosphere. After the elapsed time in incubation, there was added 50 µL of resazurin solution (0.01 % resazurin sodium in phosphate-buffered saline/PBS) to each well and the plates were incubated again at 26 ˚C in the dark for 2 hours (1S promastigotes) to 4 hours (9515 and IR-173 promastigotes). Absorbance was measured at wavelengths of 570 nm and 600 nm using the Thermofisher Scientific GO Multiscan plate reader system. Data analyses were performed for each biological replicate using the SkanIt 4.0 Research Edition software package. Background absorbance of resazurin (600 nm) was subtracted from the absorbance values of resorufin (570 nm). The mean absorbance was also calculated along with the percentage growth inhibition. The following equation was used [52]:

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

3.4.6.2. Cytotoxicity assays

The cytotoxicity of all synthesised compounds in this study was also evaluated using the following resazurin-based assay [53]:

Vero cells (Cellonex, South Africa) were cultured in Hyclone Dulbecco's modified Eagle's medium with high glucose (Separations) supplemented with 10 % fetal bovine serum (purchased from Thermofisher Scientific), 1% L-glutamine, penicillin-streptomycin, amphotericin B and non-essential amino acids (Lonza). The cells were maintained in an appropriately humidified atmosphere at a constant temperature of 37 °C with 5% carbon dioxide (CO_2). For the resazurin assay, 96 well plates were prepared with 200 microliters (µL) of cell suspension (30 000 cells/mL) and incubated for a period of 24 hours. The cells were then treated with: (i) 100 µL of an emetine dihydrochloride solution (Sigma Aldrich) diluted with growth medium to the necessary concentrations (this served as the positive control); (ii) 80 µL of growth medium and 20 µL of solvent (this would serve as the negative control to compensate for any possible solvent effects); (iii) 80 µL of growth medium and 20 µL of experimental compound solutions. Blanks were made to only contain growth medium without any cells. The duly prepared plates were then incubated for a period of 48 hours. The resazurin assay was started by adding 50 µL of a sterile-filtered resazurin sodium salt (Sigma Aldrich) solution (0.01% in PBS) to the prepared plates and then incubating the plates for 2 hours. Absorbances were measured at wavelengths of 570 and 600 nm using the Thermofisher Scientific GO Multiscan plate reader system. Data analyses were performed for each biological replicate using the SkanIt 4.0 Research Edition software package. The background absorbance of resazurin (600 nm) was subtracted from the absorbance values of resorufin (570 nm). The mean absorbance was also calculated along with the percentage cell viability. The following equation was used:

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

The IC_{50} values were determined for each compound's three biological replicates using the GraphPad Prism 5 software package. For the final IC_{50} value of each compound, the mean IC_{50} of the biological replicates was calculated with standard deviation (SD) - as shown in Table 3.2.

3.4.6.3 Statistical analysis

In vitro cytotoxicity (IC_{50}, half-maximal inhibitory concentration) was determined by non-linear regression analysis. Results were expressed as the mean (µM) ± standard deviation (SD) from triplicate biological experiments. Statistical analysis was performed using SkanIt 4.0

Research Edition software (Thermofisher Scientific) and Prism V5 software (GraphPad). All reported data were determined to be statistically significant ($p < 0.05$).

Acknowledgements

This work was based on research that was financially supported by the National Research Foundation (NRF) (UID 98937 and IFRR 115349) and the North-West University. The authors extend their thanks to Dr. D Otto for NMR analysis, Dr. JHL Jordaan for NMR and HRMS analysis, and Prof. F Van Der Kooy for HPLC 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.

Disclaimer

All of the opinions, findings, conclusions, or recommendations, expressed herein are solely those of the authors and therefore the National Research Foundation (NRF) and the North-West university do not accept any liability in regard thereto.

Declaration of Competing Interests

The authors declare that they have no competing interests.

Ethics

Ethics approval for this study was obtained from the Human Research Ethics Committee of the North-West University (NWU-00943-19-A1).

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CHAPTER 4

SUMMARY AND CONCLUSION

Leishmaniasis is a neglected, infectious protozoan disease of the tropics and sub-tropics (Akhoundi *et al.*, 2016) that is spread through the bite of autogenous female phlebotomine sand flies (Killick-Kendrick, 1990). The disease still constitutes a major concern in public health, as the global burden of leishmaniasis (especially as regards the cutaneous forms of the disease) is gradually increasing (Hotez, 2018:421-422). Some 53 species of *Leishmania (L.)* parasites have been described in taxonomic literature, with 31 of these species known to infect mammals, and more than 20 species capable of causing disease in human beings (Akhoundi *et al.*, 2016).

Leishmaniasis is endemic to 98 countries, where an estimated 1 billion people are at risk of being affected by the disease (WHO, 2016:292). Over 12 million individuals are estimated to be infected with leishmaniasis at any given point in time (Ribeiro *et al.*, 2018). According to the World Health Organization (WHO, 2020), around 600,00 - 1 million new cases of cutaneous leishmaniasis, and some 50,000 - 90,000 new cases of visceral leishmaniasis, are estimated to occur every year. It is also estimated that around 20,000 - 40,000 annual deaths occur due to visceral leishmaniasis (Bi *et al.*, 2018). Current estimations of the burden of disease of leishmaniasis have been widely criticised as representing gross underestimates of the true burden of disease. This is mainly the result of severe under-reporting of the disease (Tabbabi, 2019:1330).

Presently, only a limited number of drugs are available for the treatment of leishmaniasis in the clinical setting (Uliana *et al.*, 2018:465-467). These drugs include the pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), miltefosine, amphotericin B deoxycholate, paromomycin and pentamidine. Unfortunately, the chemotherapeutic treatment of leishmaniasis is often complicated by the development of drug resistance in parasites (both in clinical and experimental settings), the occurrence of treatment failure, toxicity, side effects/adverse events as well as prohibitively exorbitant costs (Uliana *et al.*, 2018:473). These hindrances underline the urgent need for new anti-leishmanial drugs, as well as the need to make use of new or alternative approaches in rational drug design to engender treatment solutions. This study, in particular, made use of the drug design approach of molecular hybridisation.

Molecular hybridisation is an approach in rational drug design wherein new drug ligands and drug prototypes are engendered by fusing/linking a variety of common pharmacophores with

desirable biological activities into a single chemical entity (Viegas-Junior *et al.*, 2007:1829). This is done with the hope that the resulting hybrid(s) maintain, or perhaps even amplify, the sought-after biological activities of the molecules they were derived from. Molecular hybridisation is a drug development strategy that has also attracted considerable attention in medicinal chemistry due to the innumerable advantages that molecular hybrids possess over their non-hybridised counterparts. These advantages include the ability to elicit a greater number of biological activities with only a single chemical structure (Bérubé, 2016:281), the targeted and more potent treatment of disease(s), improvement of the selectivity profile of a compound (Viegas-Junior *et al.*, 2007:1829), the increased capacity to reduce the occurrence of undesired side effects as well as the ability to elicit biological activities by way of altered or dual modes of action. The biopharmaceutical parameters (pharmacokinetics and pharmacodynamics) of a given compound may also be markedly improved via molecular hybridisation (Pawełczyk *et al.*, 2018). Of particular interest is the use of the molecular hybridisation approach to produce agents that possess activity against the causative agents of diseases such as malaria (Muregi & Ishih, 2010), leishmaniasis and trypanosomiasis (Cardona-G *et al.*, 2018). This study has focused on the hybridisation of 4(*3H*)-quinazolinone and *1H*-1,2,3-triazole pharmacophores.

Quinazolinones are a class of fused nitrogen-containing bicyclical heterocycles that have received the renewed attention of medicinal chemists in recent decades due to the ease of their synthesis, as well as the wide range of biological activities that these compounds may elicit in biological targets (Jafari *et al.*, 2016). These biological activities include, amongst others, anti-cancer (Hu *et al.*, 2012), anti-bacterial (Nasab *et al.*, 2017), anti-diabetic (Saeedi *et al.*, 2019), sedative-hypnotic (Hammer *et al.*, 2015), anti-convulsive (Rajasekaran *et al.*, 2013), anti-tussive (Liu *et al.*, 2015) and anti-inflammatory (Zayed & Hassan, 2014) activities. Particularly noteworthy is the potential use of quinazolinones to treat infectious diseases such as tuberculosis (Khosropour *et al.*, 2006), malaria (Zhu *et al.*, 2009), trypanosomiasis (Patterson *et al.*, 2011) and leishmaniasis (Birhan *et al.*, 2014). All of the aforementioned serves to establish the promising prospect of producing future therapeutic agents that contain any number of quinazolinone moieties.

Triazoles, another class of compounds that holds significant importance in medicinal chemistry, refers to any heterocyclic organic compound that consists of a five-membered heterocyclic ring with two carbon atoms and three nitrogen atoms, corresponding roughly to the molecular formula $C_2H_3N_3$ (Mignani *et al.*, 2012:186). The relative positions of the nitrogen atoms in the ring, with respect to one another and the carbon atoms, may differ. This has the effect of producing two sets of possible isomers *viz.* 1,2,3-triazoles and 1,2,4-triazoles. Each of these isomers may, in turn, be divided into a set of two tautomers that differ in regards to

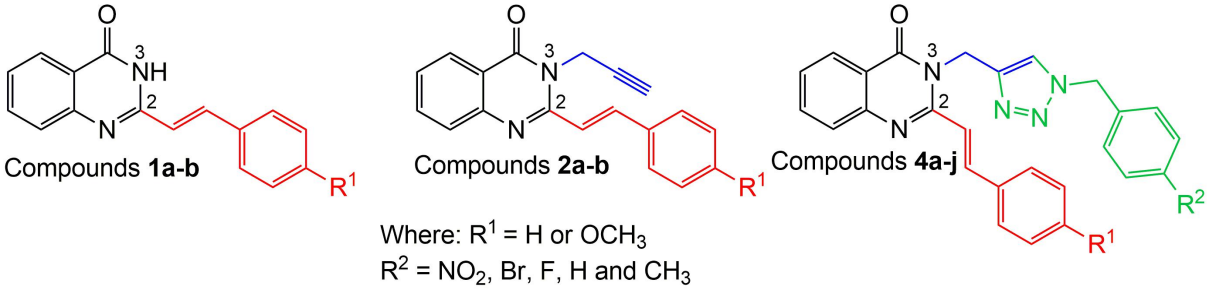
which nitrogen atom has a hydrogen atom bonded to it. In the case of the 1,2,3-triazole isomer (the isomer that pertains to this study in particular), these tautomers are the *1H*-1,2,3-triazoles and *2H*-1,2,3-triazoles. In rare instances, a third tautomeric form of the 1,2,3-triazoles - the 3(1*H*)-1,2,3-triazole tautomer - may be obtained (Belskaya *et al.*, 2015:53). The synthesis of the 1,2,3-triazoles may also be easily effected by way of Huisgen cycloaddition reactions (Huisgen, 1963), or their copper-catalysed variant (Tornøe *et al.*, 2002), between terminal alkynes and organic azides.

The 1,2,3-triazoles are stable and impervious to oxidative/reductive as well as hydrolytic degradation in both acidic and basic conditions (Pertino *et al.*, 2013:7662). The 1,2,3-triazoles have also been documented to exhibit a wide range of biological activities such as anti-microbial (Genin *et al.*, 2000), anti-bacterial (Gregory *et al.*, 1989), anti-fungal (Dong *et al.*, 2001), anti-epileptic (Pålhagen *et al.*, 2001), anti-inflammatory (Dong *et al.*, 2001), analgesic (Dong *et al.*, 2001), anti-HIV (De Clercq, 2002), anti-obesity (Brockunier *et al.*, 2000), anti-allergic (Buckle, 1985), potassium channel activating (Biagi *et al.*, 2000), anti-neoplastic (Al-Masoudi & Al-Soud, 2002), anti-anxiety (Martini *et al.*, 1988) and anti-proliferative (Norris *et al.*, 1996) activities. Furthermore, the 1,2,3-triazoles have shown promise in possibly being able to treat a number of infectious parasitic diseases such as trypanosomiasis (Bakunov *et al.*, 2010), leishmaniasis (Tahghighi *et al.*, 2012) and malaria (Raj *et al.*, 2013). All of the above, taken together, serve to establish the 1,2,3-triazoles as promising medicinal scaffold on which to base future therapeutic agents.

The aim of this study was to synthesise a series of 2,3-disubstituted-4(3*H*)-quinazolinone derivatives (hybrids of 4(3*H*)-quinazolinones and 1*H*-1,2,3-triazoles) with the express goal of producing a series of novel anti-leishmanial agents that are safe, clinically efficacious and cost-effective to produce and disseminate.

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

- Syntheses and characterisation of a series of novel 2,3-disubstituted-4(3*H*)-quinazolinone derivatives (4(3*H*)-quinazolinone and 1*H*-1,2,3-triazole hybrids) as depicted below.



- Determination of the *in vitro* cytotoxicity profiles of the hybrids and their synthetic intermediates against mammalian (Vero) cell lines using a resazurin-based assay.
- Assessment of the *in vitro* anti-leishmanial activity of the hybrids and their synthetic intermediates against three strains of *Leishmania* promastigotes (*L. donovani* strains 1S and 9515, and *L. major* strain IR-173) using a resazurin-based assay.

A series of fourteen 4(3*H*)-quinazolinone derivatives, comprising ten novel 1*H*-1,2,3-triazole-4(3*H*)-quinazolinones and their associated synthetic intermediates, were synthesised in a five-step process using a variety of synthetic methods including cyclisation, condensation, nucleophilic (S_N2) substitution and copper-catalysed alkyne-azide cycloaddition (CuAAC) reactions. The synthetic intermediates were produced with excellent purity (96 - 99%) in 40 - 60% yields, while the hybrids were produced with excellent purity (~100%) in 30 - 89% yields. The successful syntheses of all compounds were confirmed using routine molecular characterisation techniques such as infra-red spectroscopy (IR), proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance spectroscopy (NMR), and high-resolution mass spectrometry (HRMS). The physicochemical properties of the quinazolinone derivatives were also predicted using the SwissADME webtool (Daina *et al.*, 2017). This was done in order to predict their drug-likeness as well as their potential oral activity. Due consideration was given to the lipophilicity and aqueous solubility parameters of the synthesised derivatives as these properties are considered key parameters in drug discovery and design (Arnott & Planey, 2012:910-911). The cLogP (calculated logP) values (lipophilicity) of all synthesised derivatives were determined to be within the target range (logP < 5) as set by Lipinski and others (Lipinski *et al.*, 2001). All synthesised compounds were, therefore, predicted to be drug-like, suitable for oral administration and likely to be well-absorbed within the gastro-intestinal alimentary canal. Similarly, the logS (aqueous solubility) values of the synthesised derivatives fell within the acceptable range, that is to say between -6 < logS (ESOL) < 0. (Daina *et al.*, 2017). However, aqueous solubility still presented a challenge experimentally, as it was determined during the biological screening experiments that the synthesised derivatives were poorly soluble in the screening medium at the intended concentration (100 µM). The synthesised derivatives were observed, instead, to form finely suspended precipitates. All synthesised quinazolinone derivatives were, therefore, screened as suspensions.

Single-point resazurin-based assays were performed in order to determine the *in vitro* anti-leishmanial activity (expressed as percentage growth inhibition ± standard deviation (SD)) of all synthesised quinazolinone derivatives against three strains of *Leishmania* promastigotes. These strains were *L. donovani* strain 1S (MHOM/SD/62/1S), *L. donovani* strain 9515 (MHOM/IN/95/9515) and *L. major* strain IR-173 (MHOM/IR/-173) (obtained from

BEI resources). Amphotericin B (AMB) served as the reference drug. The *in vitro* cytotoxicity (IC_{50}, half-maximal inhibitory concentration, expressed as the mean (μM) ± standard deviation (SD)) of all synthesised derivatives was also determined against mammalian (Vero) cells using a resazurin-based assay. Emetine dihydrochloride hydrate (EM) served as the reference drug. All synthesised derivatives were determined to be non-toxic to Vero cells (IC_{50} > 100 μM). The synthesised derivatives and reference drugs were screened at concentrations of 100 μM in both the anti-leishmanial and cytotoxicity assays.

Only three compounds (**1a**, **2a** and **4b**) displayed activity against *L. donovani* strain 1S promastigotes, the most notable activity being that of compound **2a** (58% growth inhibition). Compound **2a** was also observed to be moderately active against *L. donovani* strain 9515 with 43% promastigote growth inhibitory activity. The remaining compounds either exhibited no promastigote growth inhibition, as was the case for eight compounds (compounds **2b**, **4a**, **4c**, **4f**, **4g**, **4h**, **4i** and **4j**), or low promastigote growth inhibition activity (2 - 34%) of *L. donovani* strain 9515. Out of a total of 14 compounds, only compound **4b** was observed to possess marked activity (47% parasite growth inhibition) against *L. major* strain IR-173. All other synthesised derivatives exhibited either no parasite growth inhibition (as was the case for compounds **2b**, **4d** and **4h**), or low activity (2.29 - 25.55% promastigote growth inhibition), against *L. major* strain IR-173. The poor aqueous solubility of the synthesised quinazolinone derivatives may have, in part, contributed to the general lack of pronounced anti-leishmanial activity. The remediation of the poor aqueous solubility of the synthesised derivatives will be the topic of future research. A number of structural modification strategies may be used to improve the poor aqueous solubility of a compound(s). These include: (i) the addition of ionizable groups, (ii) reducing the logP of a compound, (iii) the addition of polar groups or groups capable of hydrogen bonding, (iv) reducing the molecular weight of a compound, (v) the modification of planarity via out-of-plane substitution to reduce crystal packing and (vi) constructing a pro-drug (Kerns & Di, 2008:70-76).

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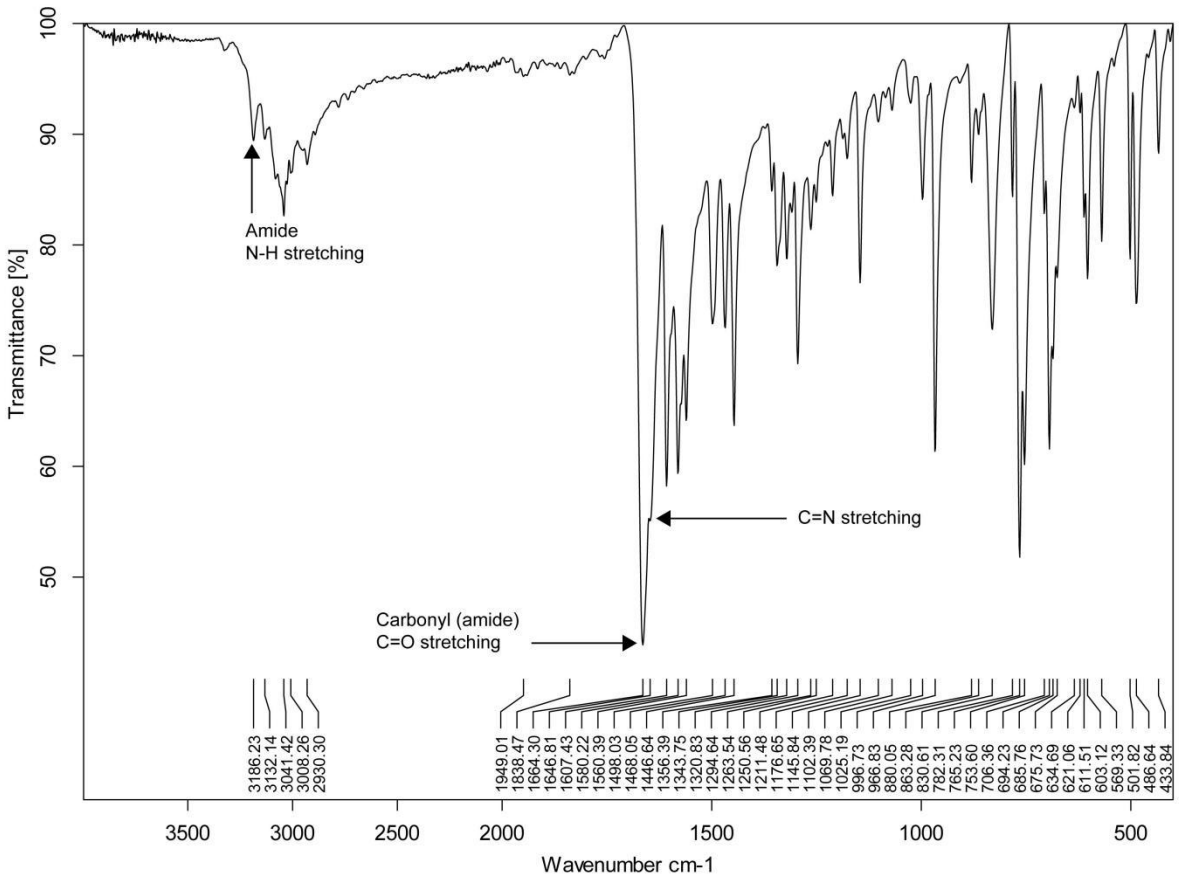
ANNEXURE A

SUPPLEMENTARY MATERIAL

Intermediate compounds

Compound 1a

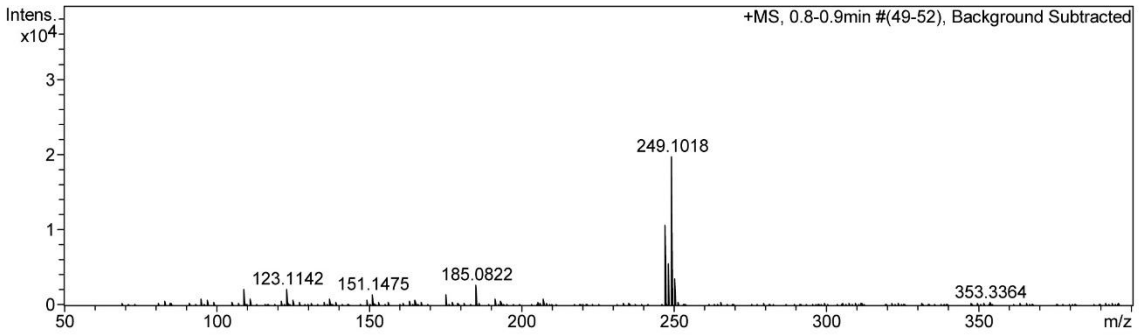
Infra-red spectroscopy (IR)



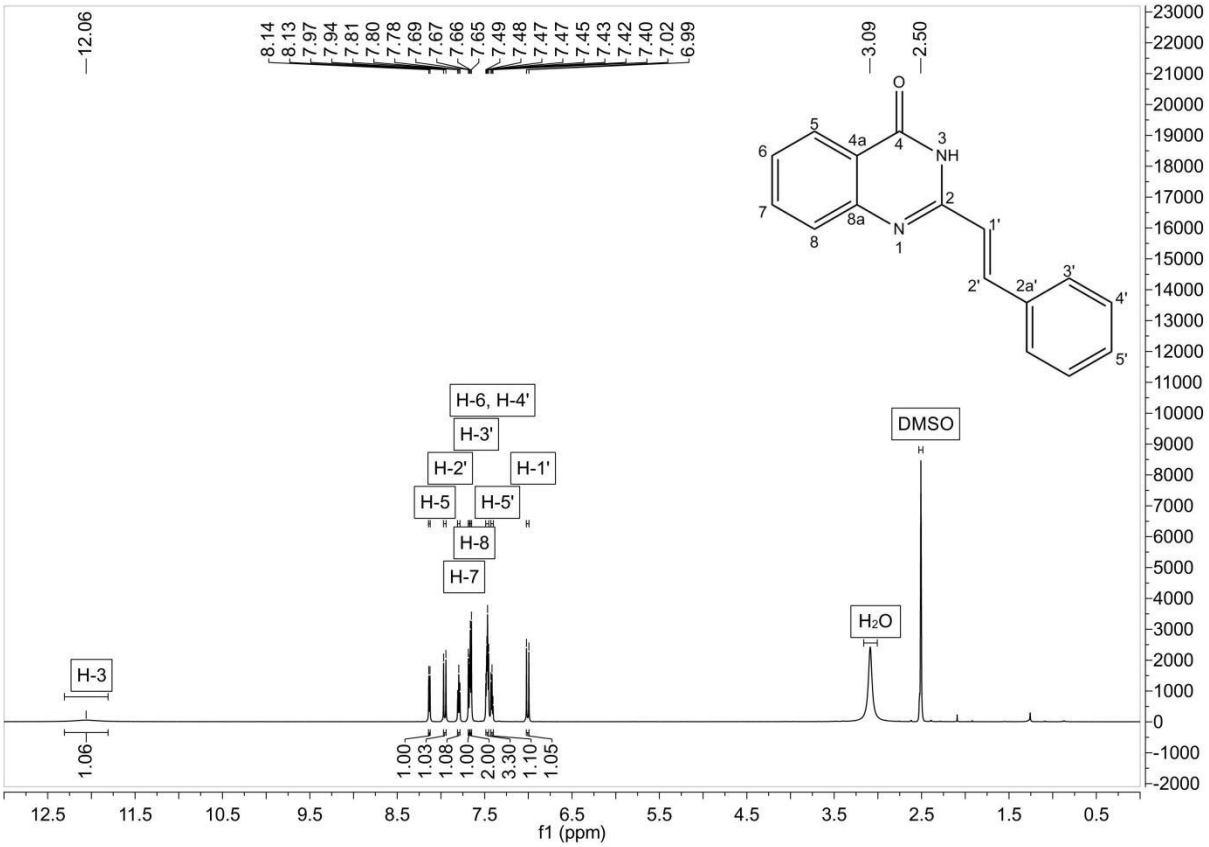
HRMS

Acquisition Parameter

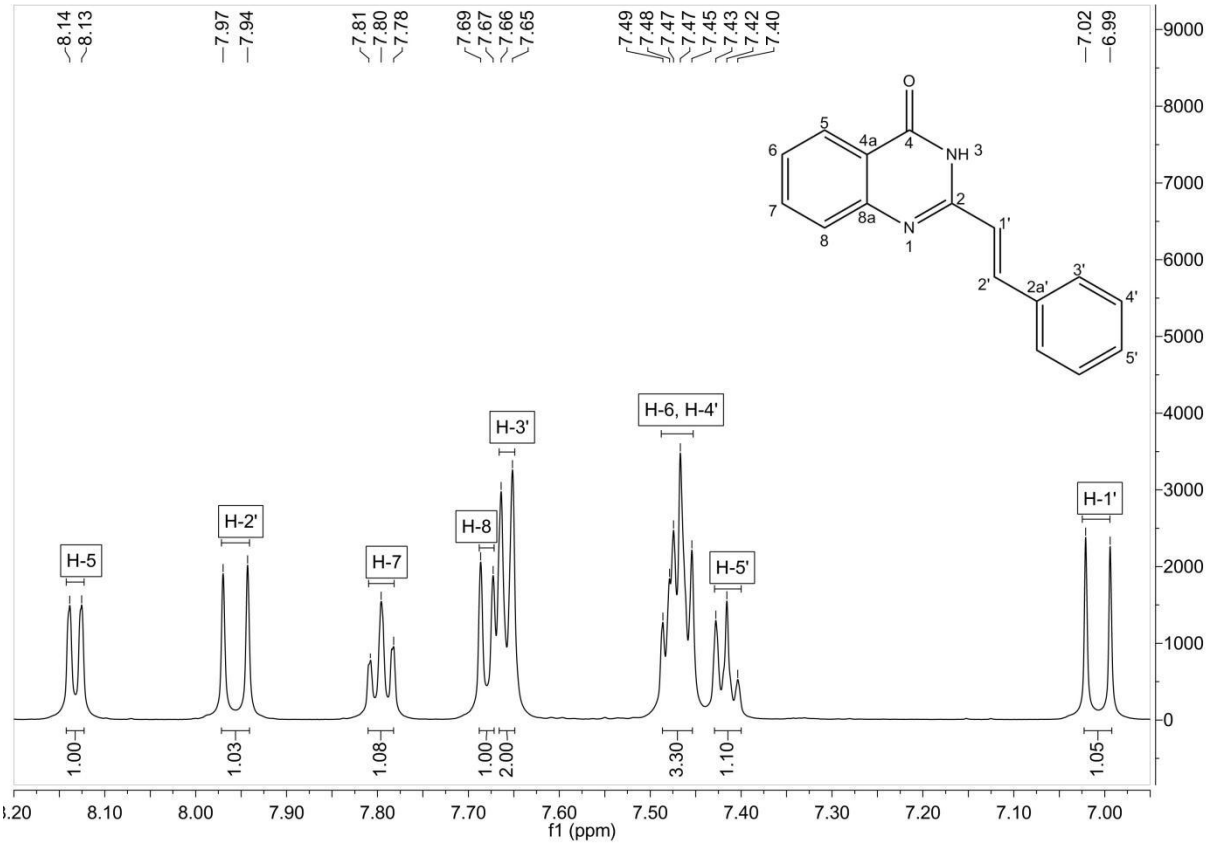
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



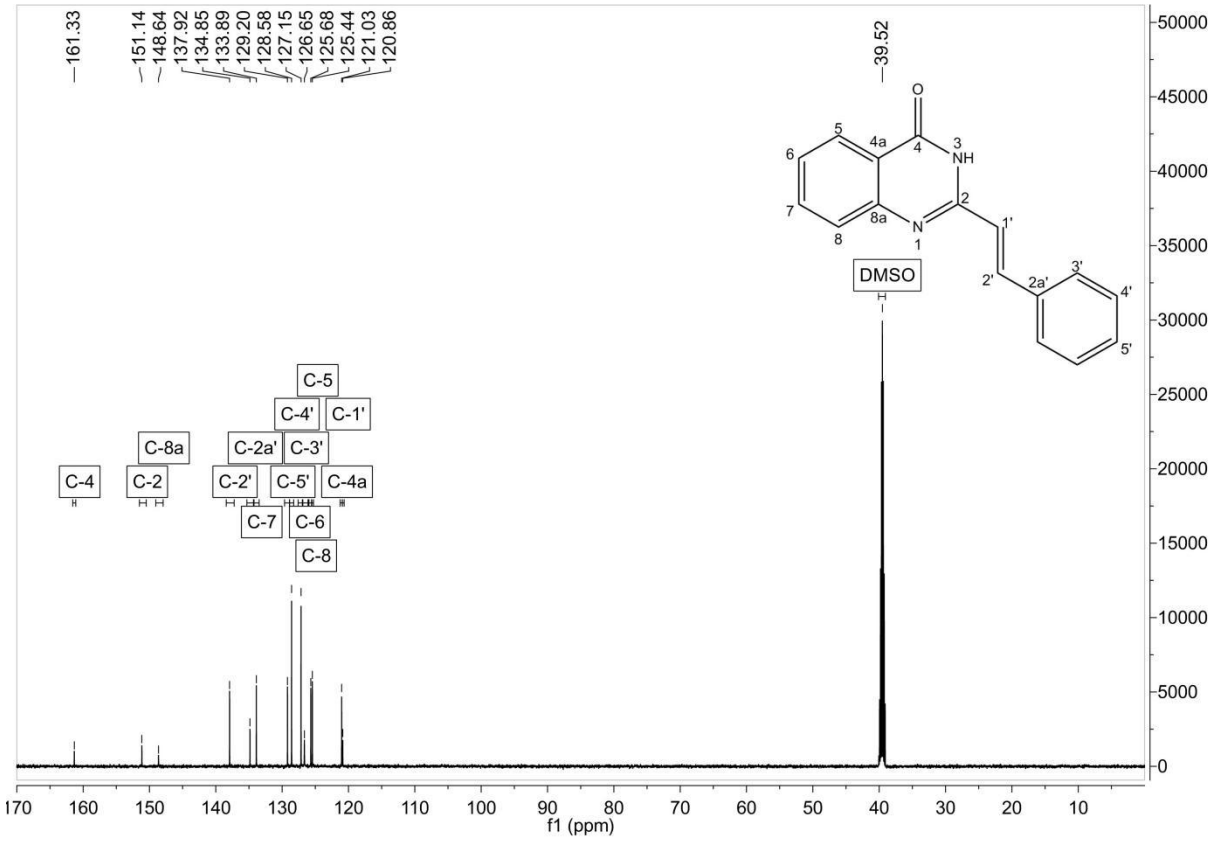
^{1}H NMR (DMSO at 80°C)



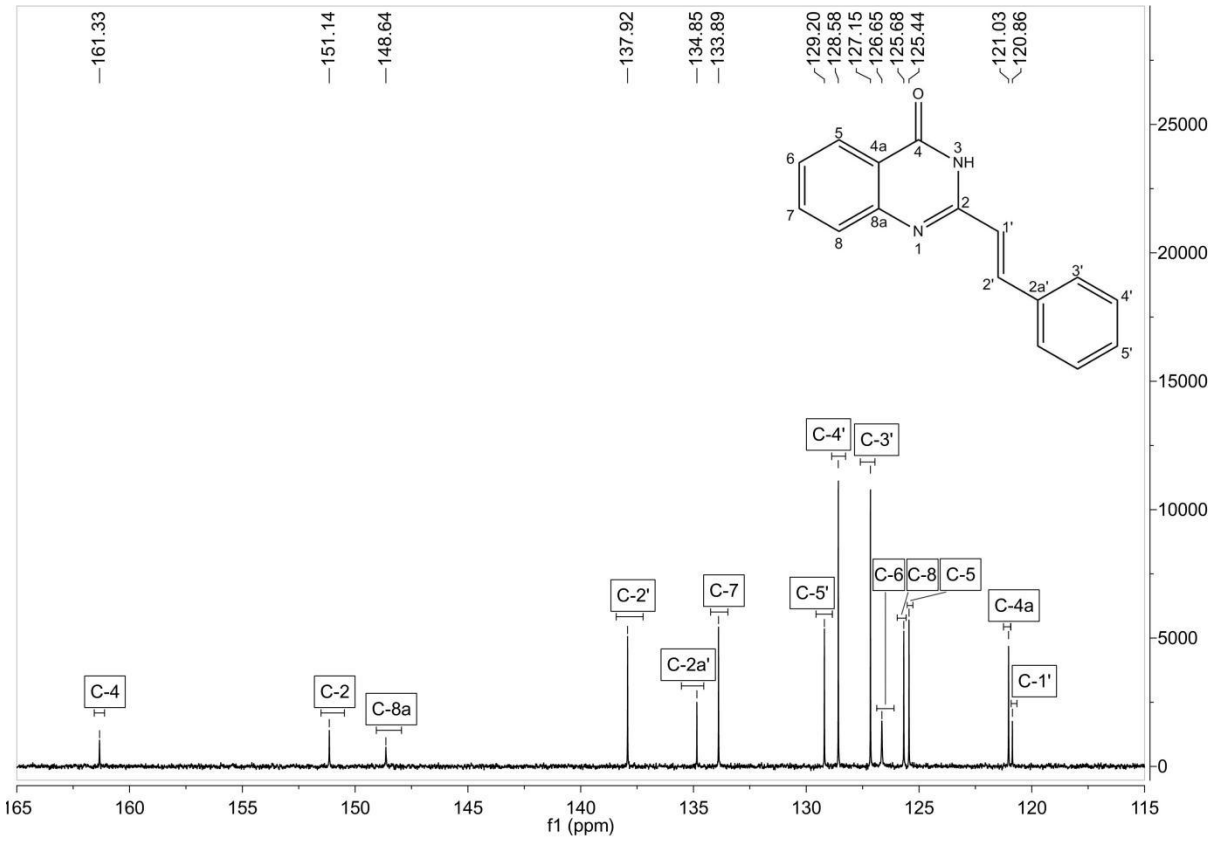
^{1}H NMR 8.20 - 6.95 ppm (DMSO at 80°C)



^{13}C NMR (DMSO at 80°C)

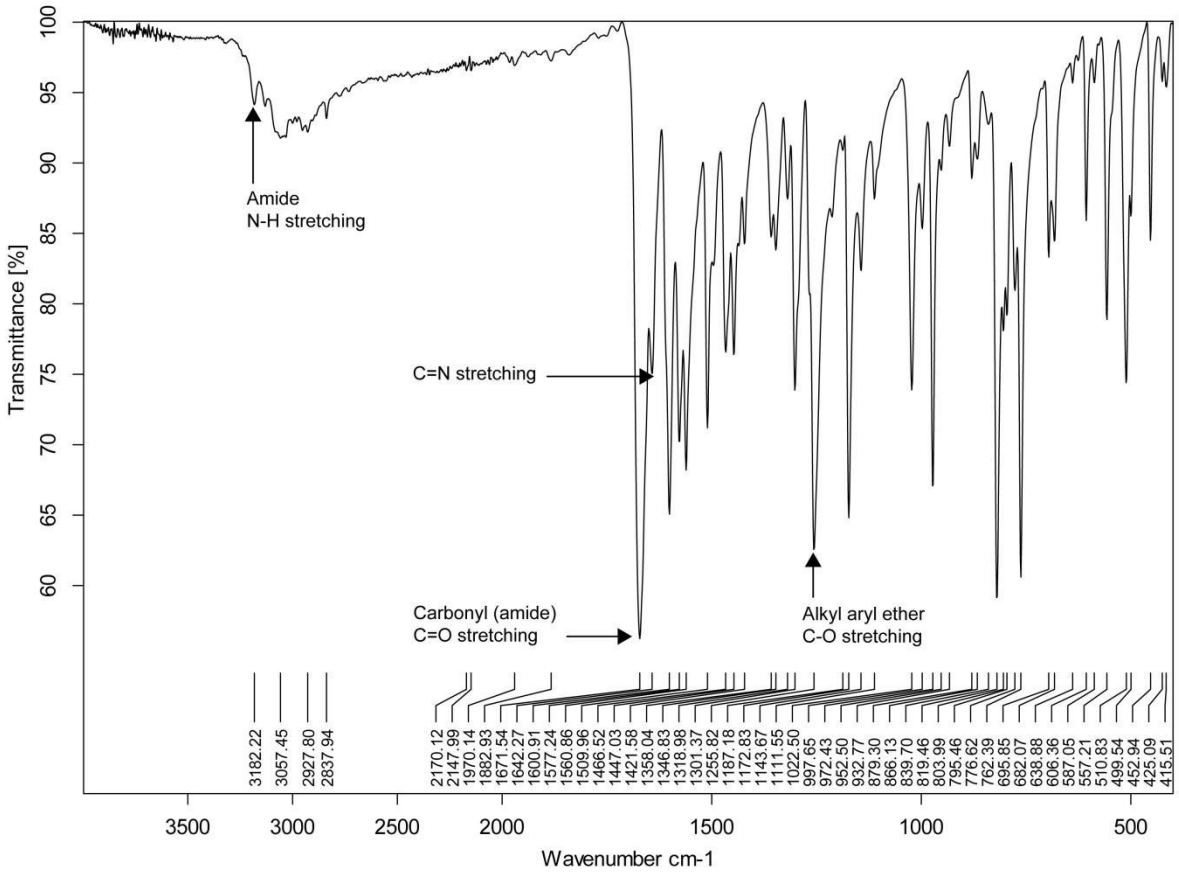


^{13}C NMR 165.00 - 115.00 ppm (DMSO at 80°C)



Compound 1b

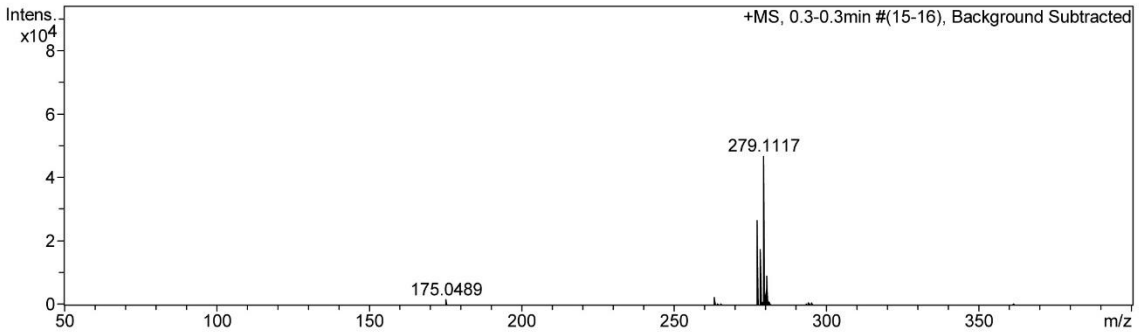
IR



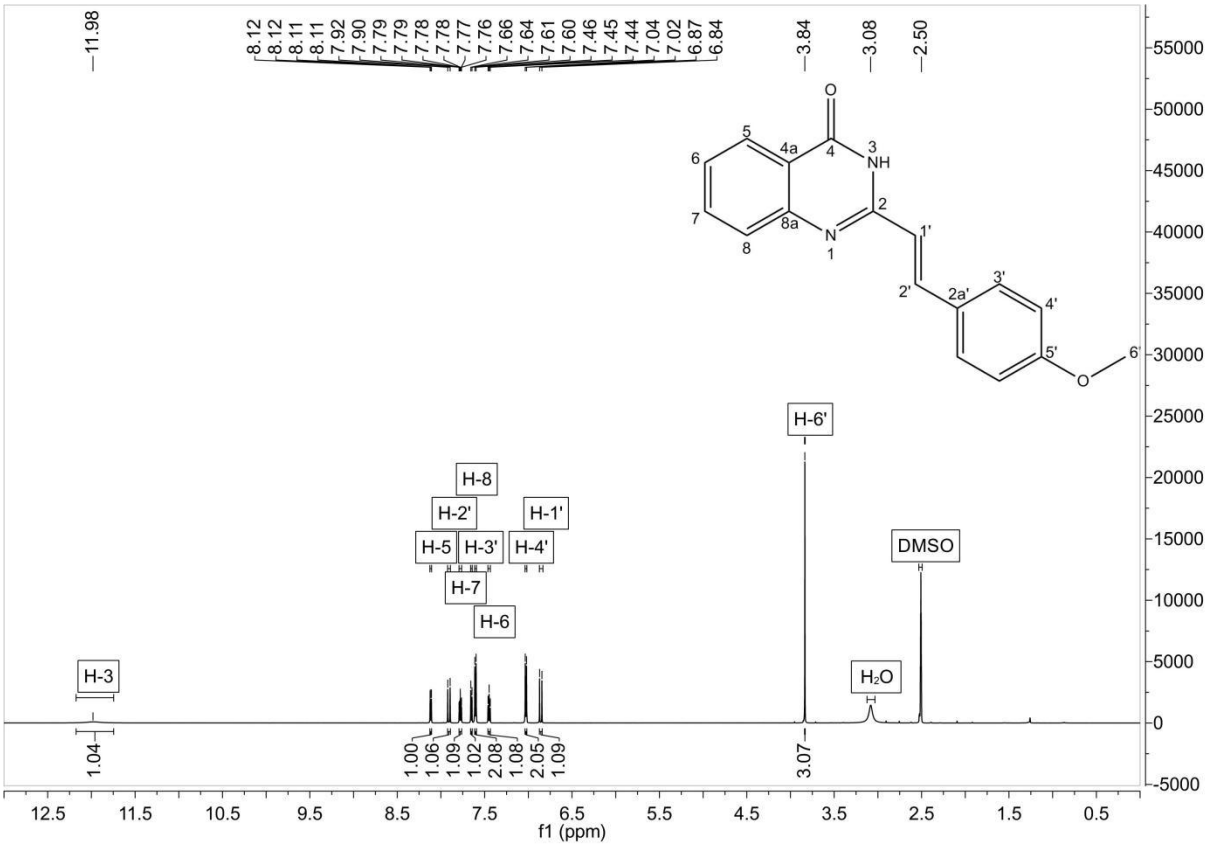
HRMS

Acquisition Parameter

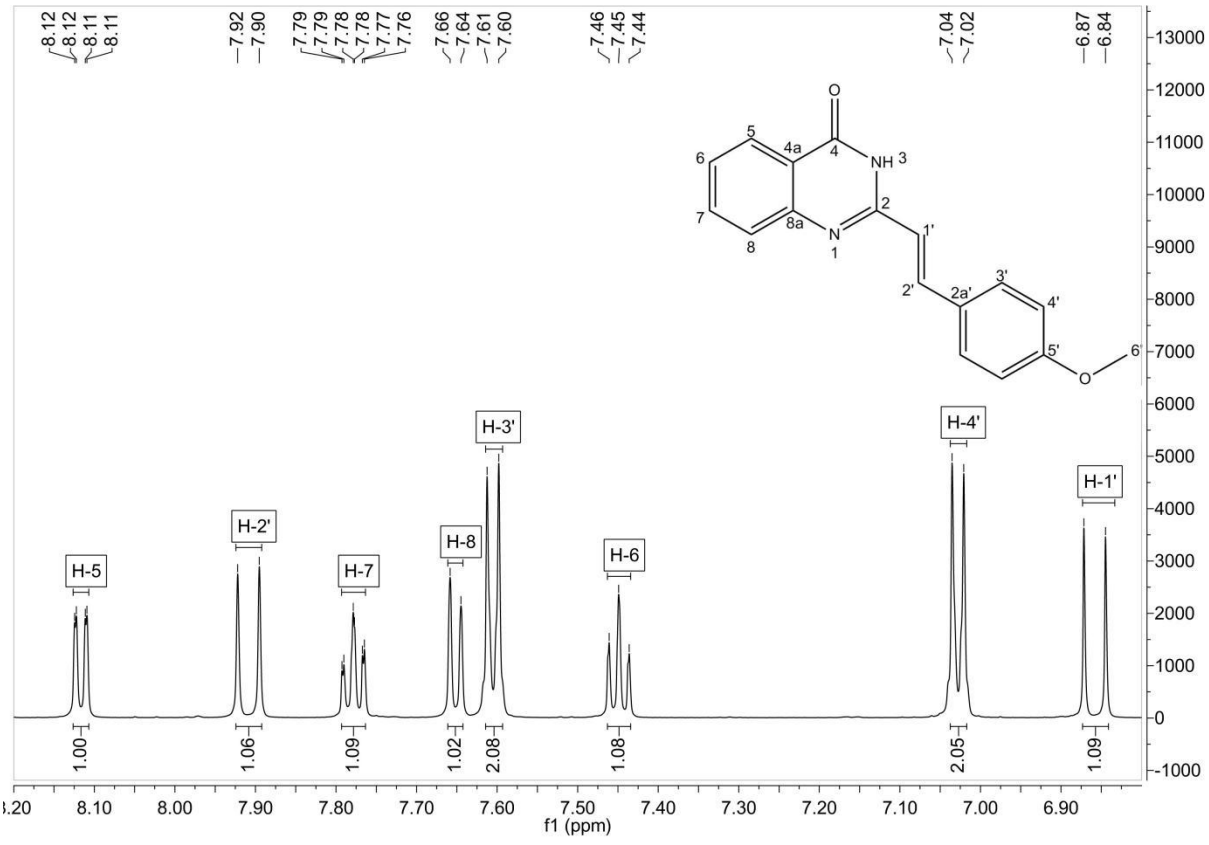
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



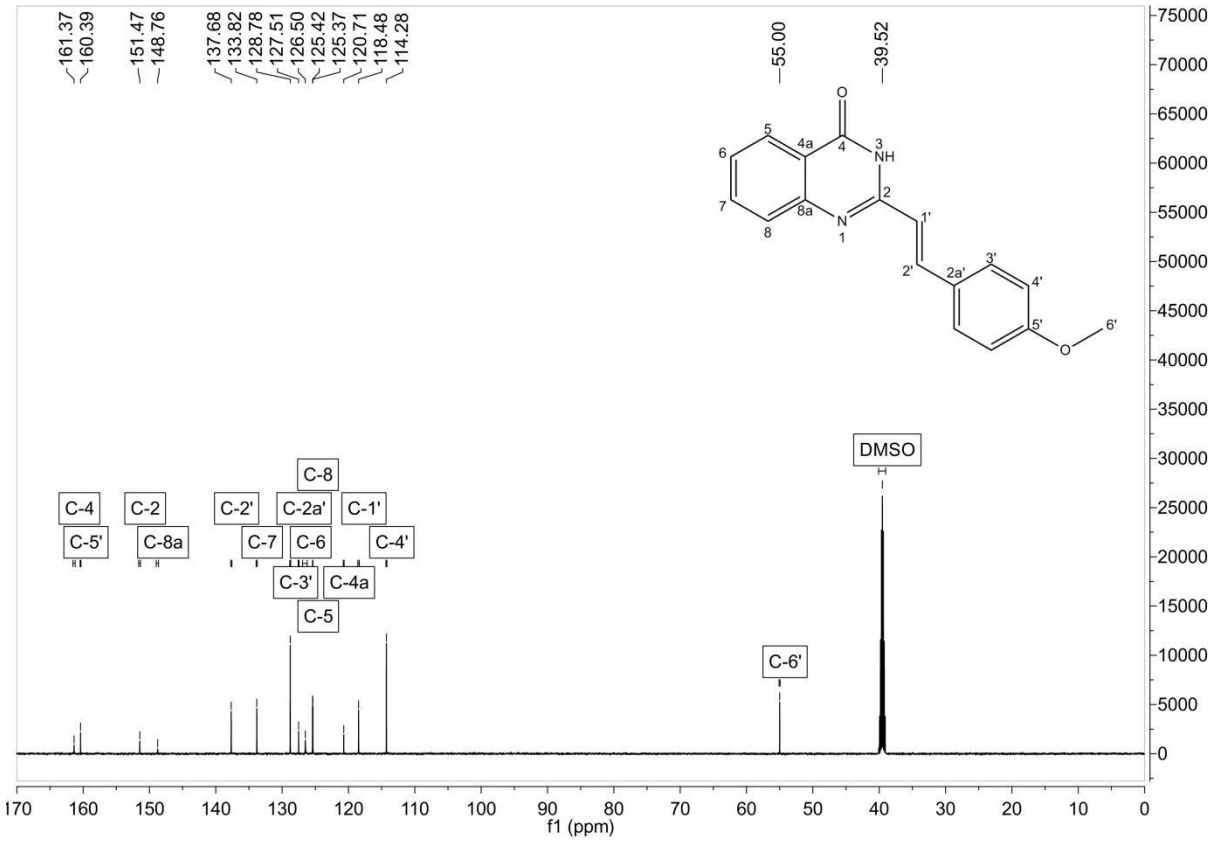
^{1}H NMR (DMSO at 80°C)



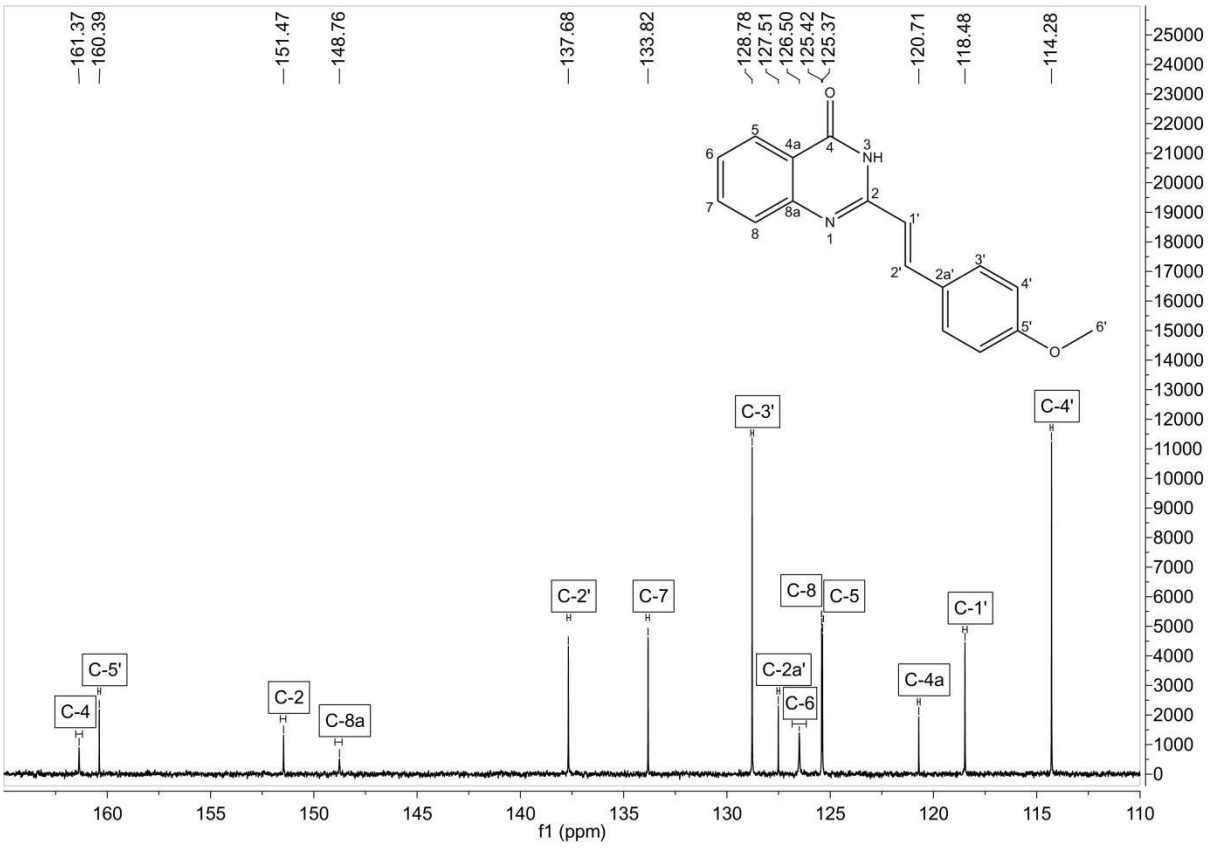
^{1}H NMR 8.20 - 6.80 ppm (DMSO at 80 °C)



^{13}C NMR (DMSO at 80°C)

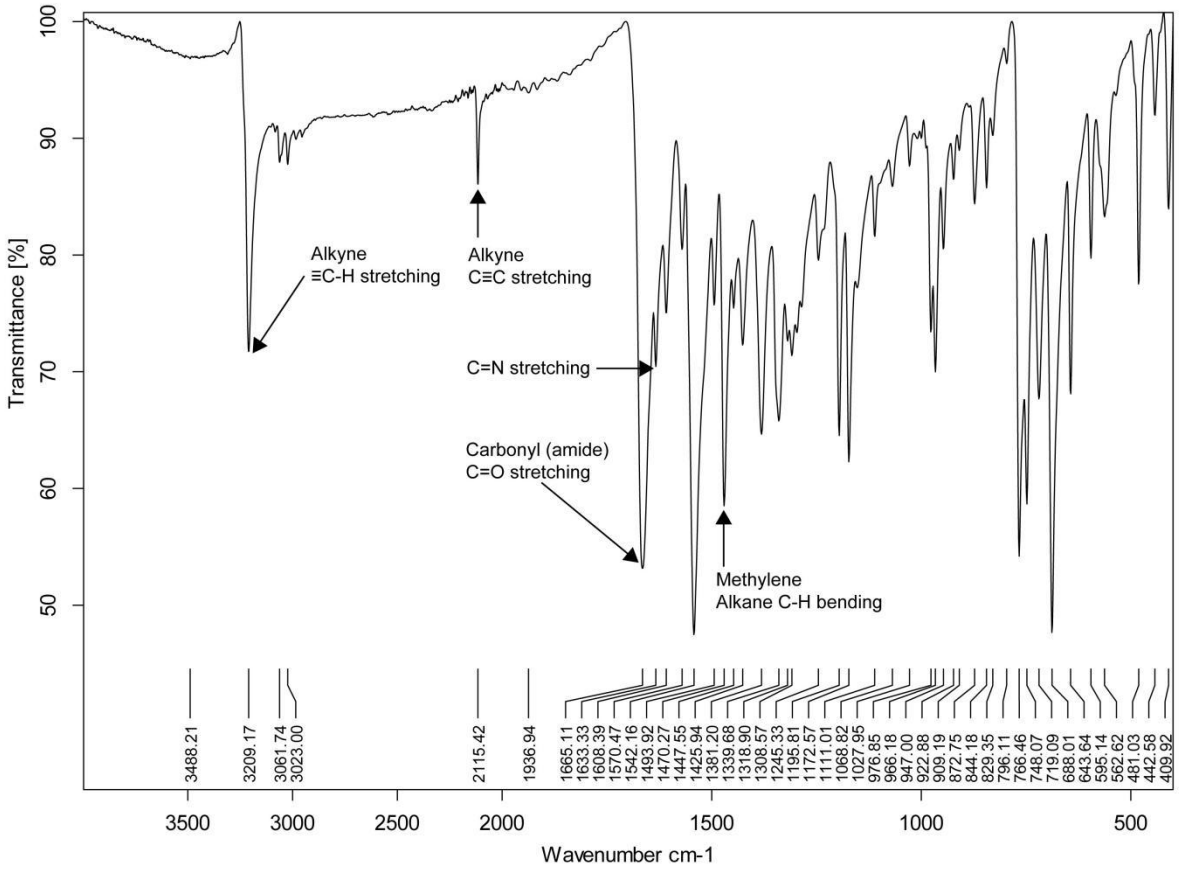


^{13}C NMR 165.00 - 110.00 ppm (DMSO at 80 °C)



Compound 2a

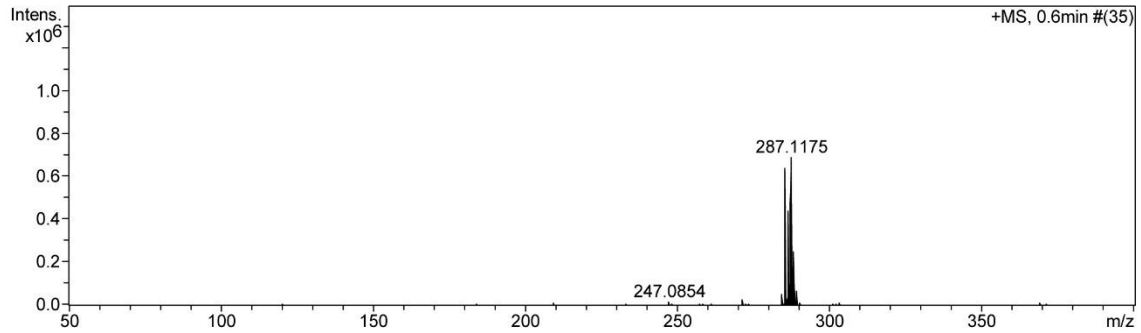
IR



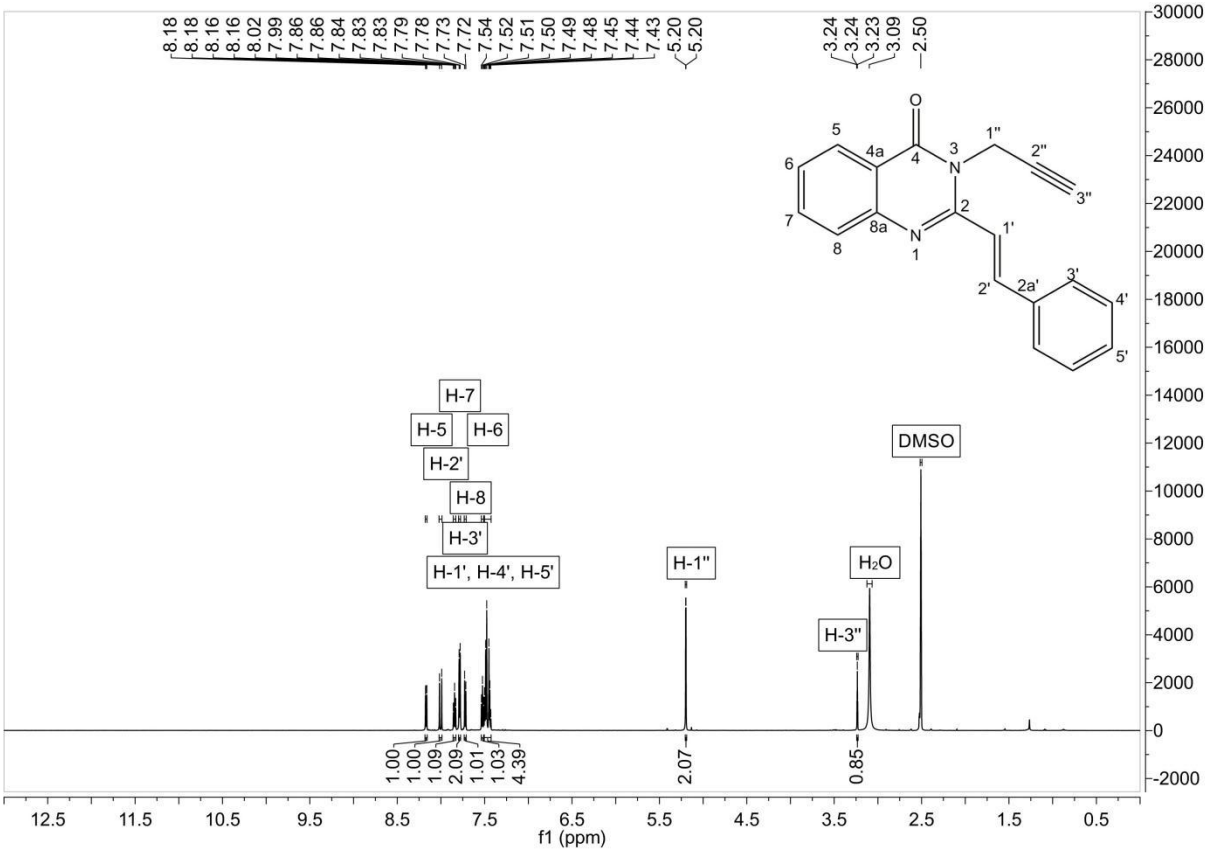
HRMS

Acquisition Parameter

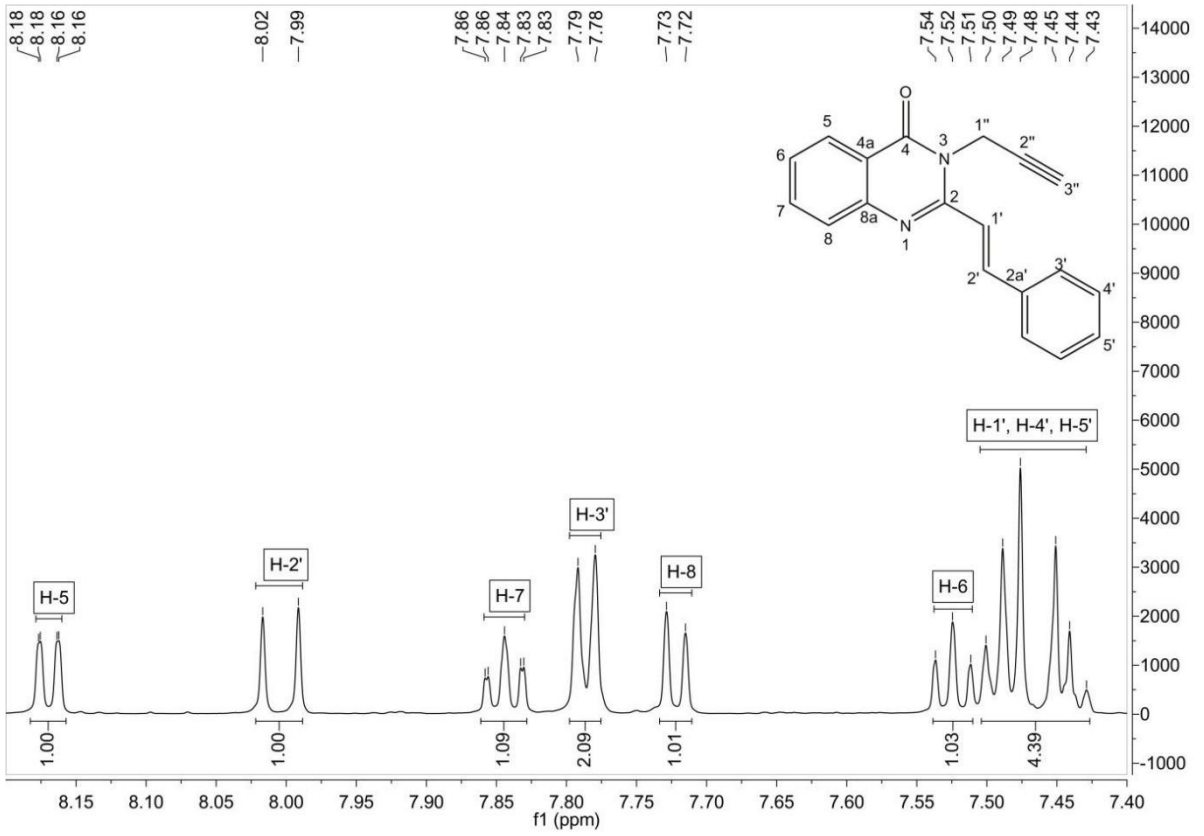
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



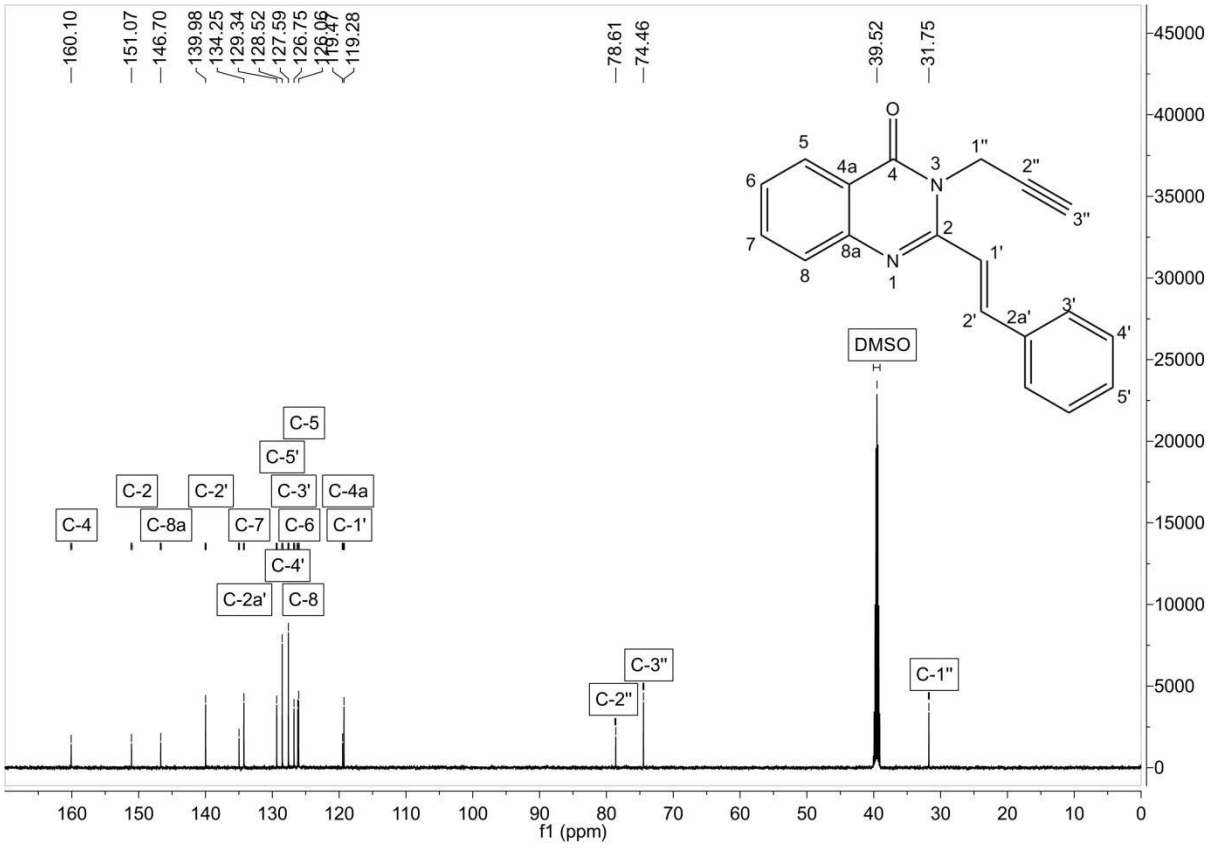
^{1}H NMR (DMSO at 80°C)



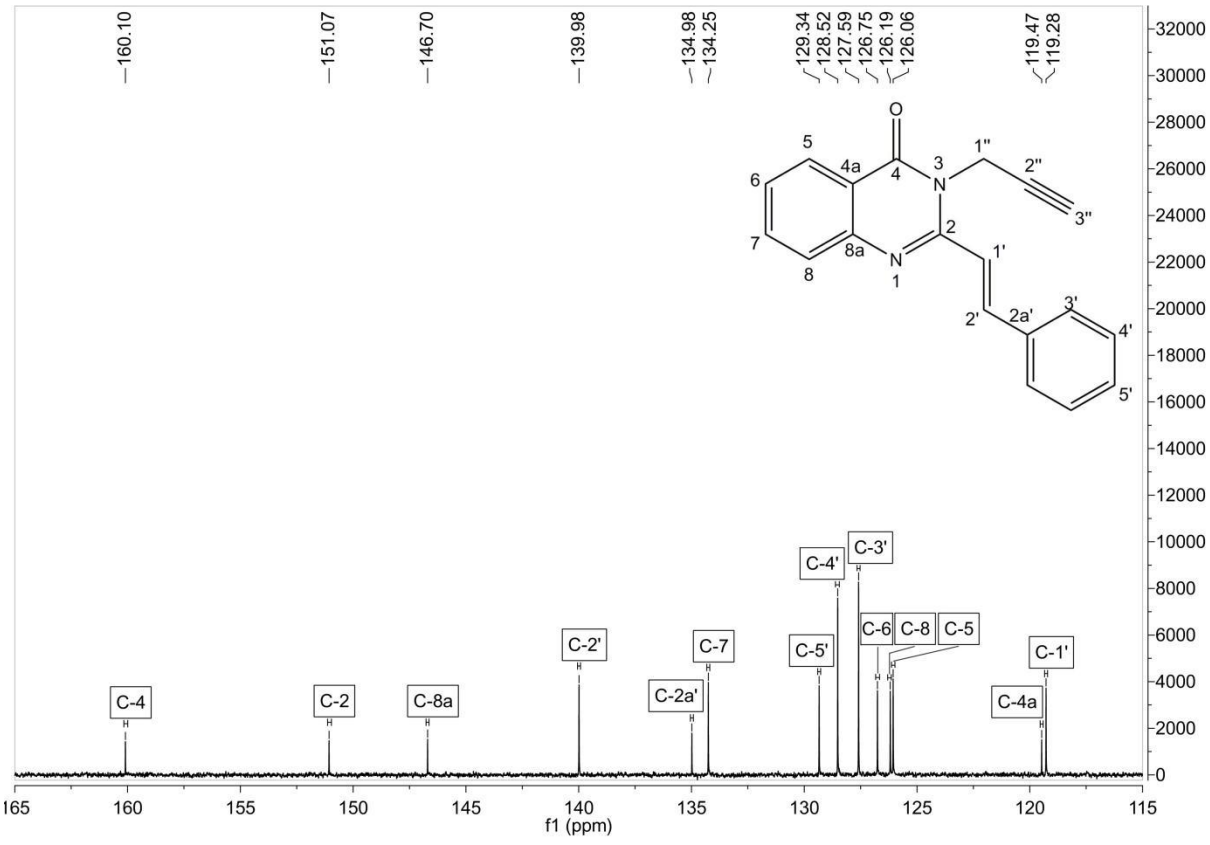
^{1}H NMR 8.20 - 7.40 ppm (DMSO at 80°C)



^{13}C NMR (DMSO at 80°C)

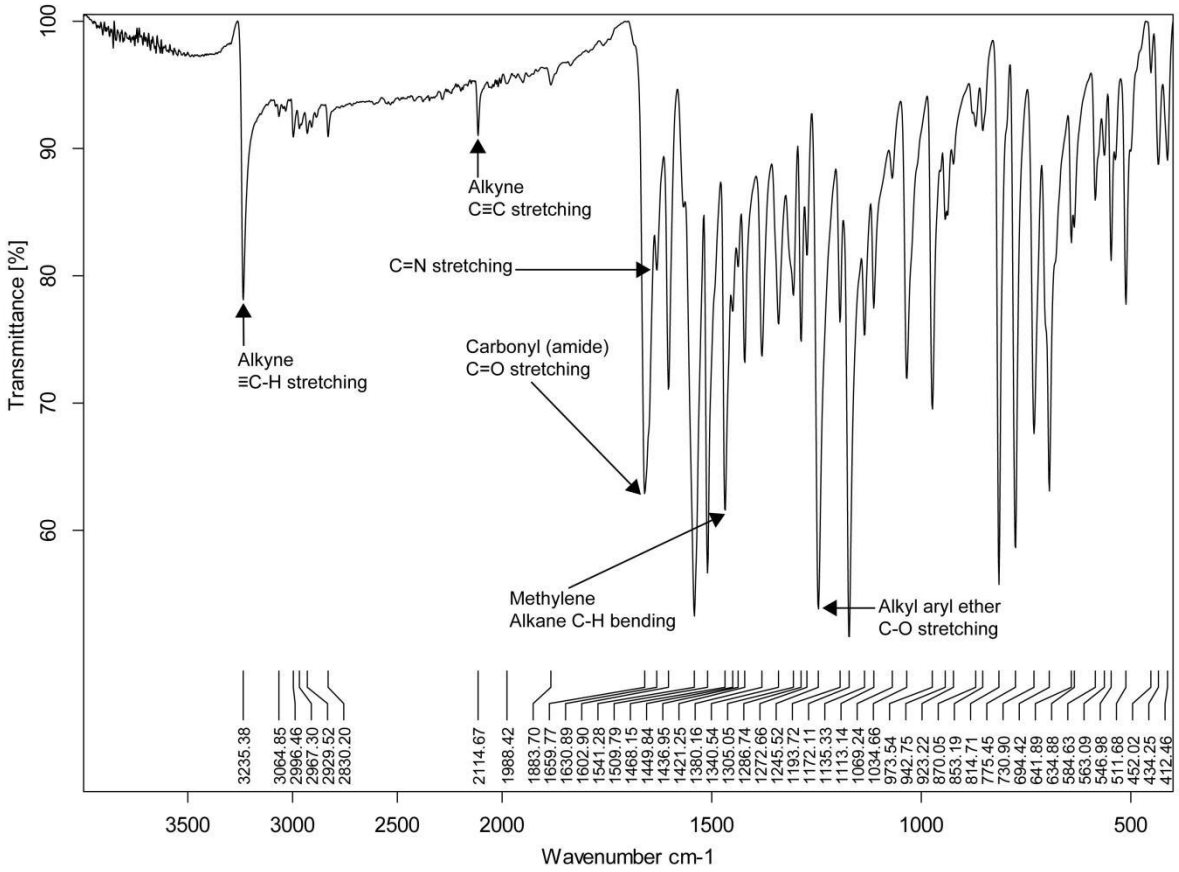


^{13}C NMR 165.00 - 115.00 ppm (DMSO at 80°C)



Compound 2b

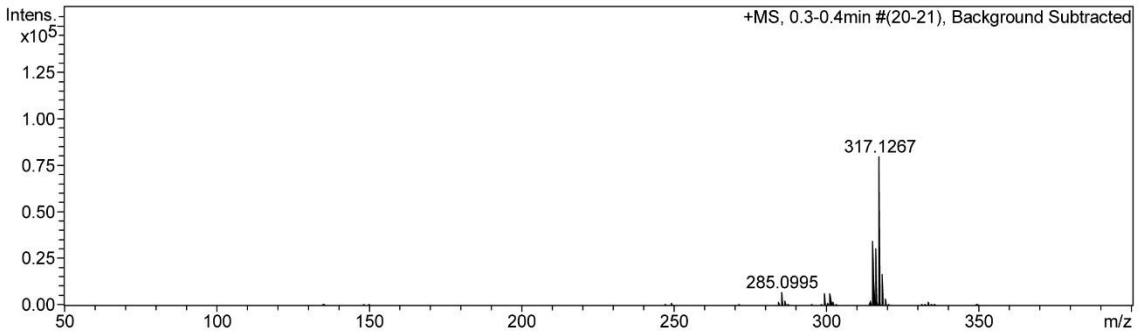
IR



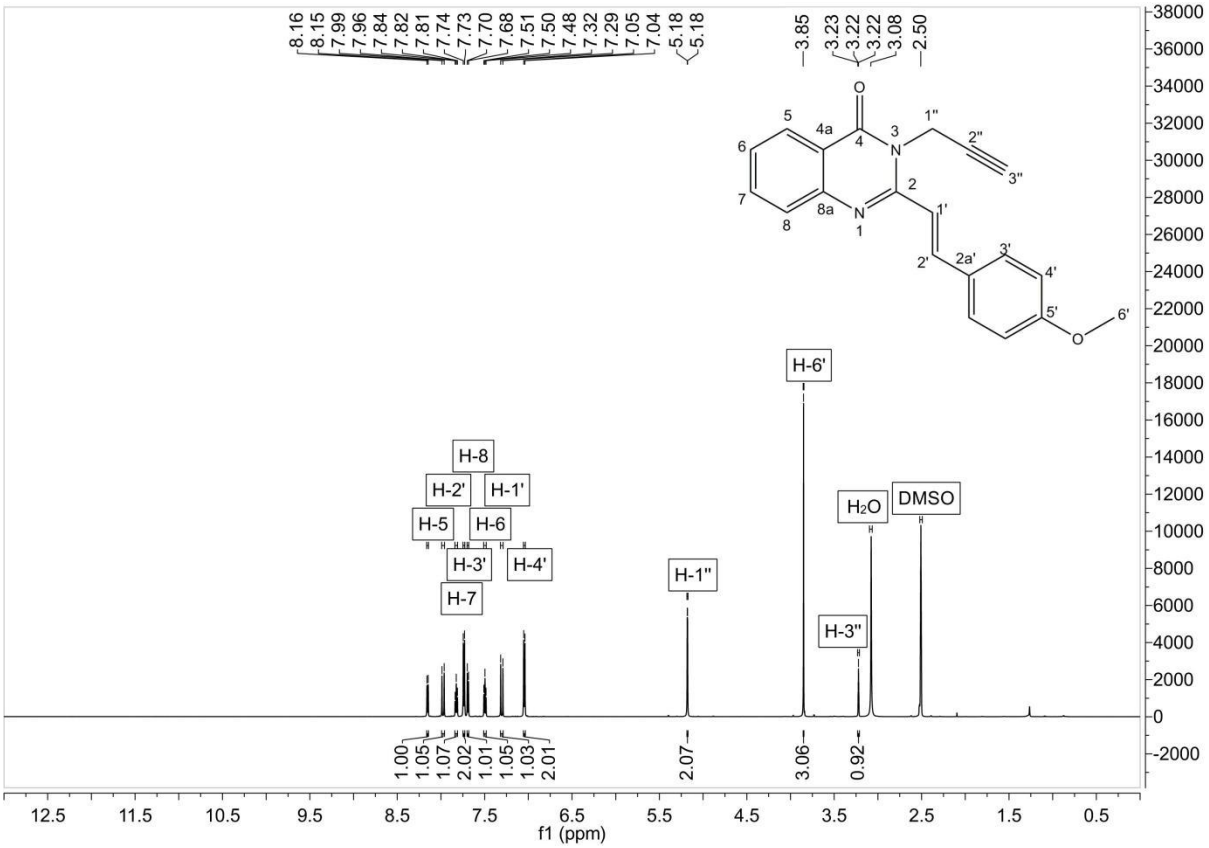
HRMS

Acquisition Parameter

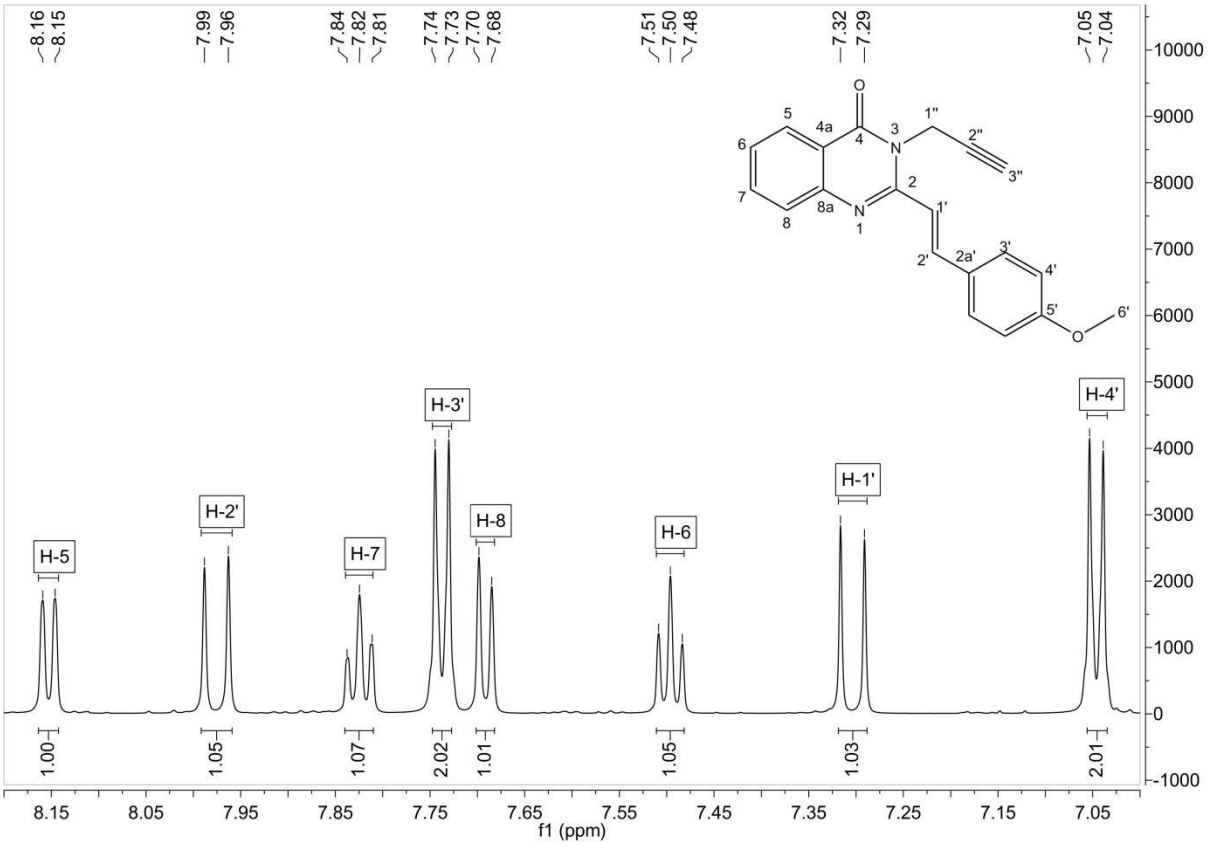
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



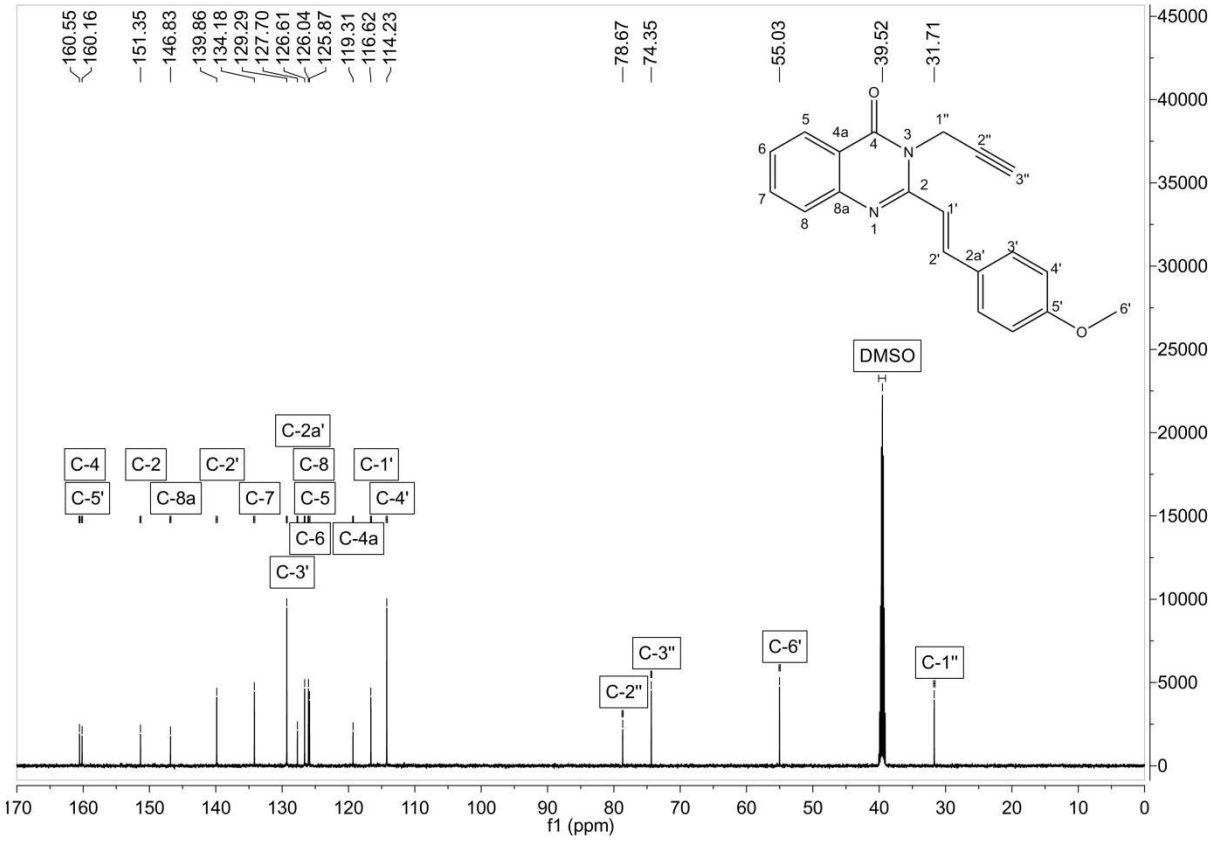
^{1}H NMR (DMSO at 80°C)



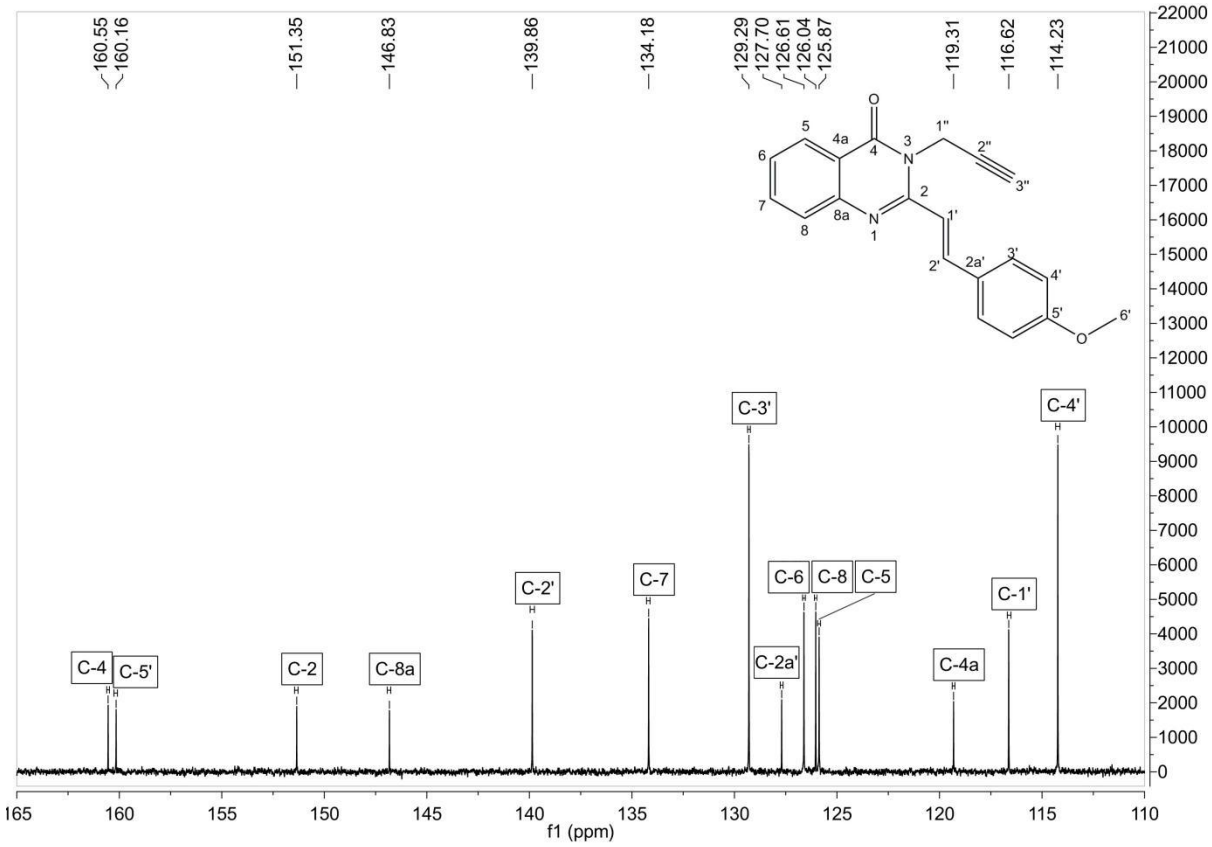
^{1}H NMR 8.20 - 7.00 ppm (DMSO at 80°C)



^{13}C NMR (DMSO at 80°C)



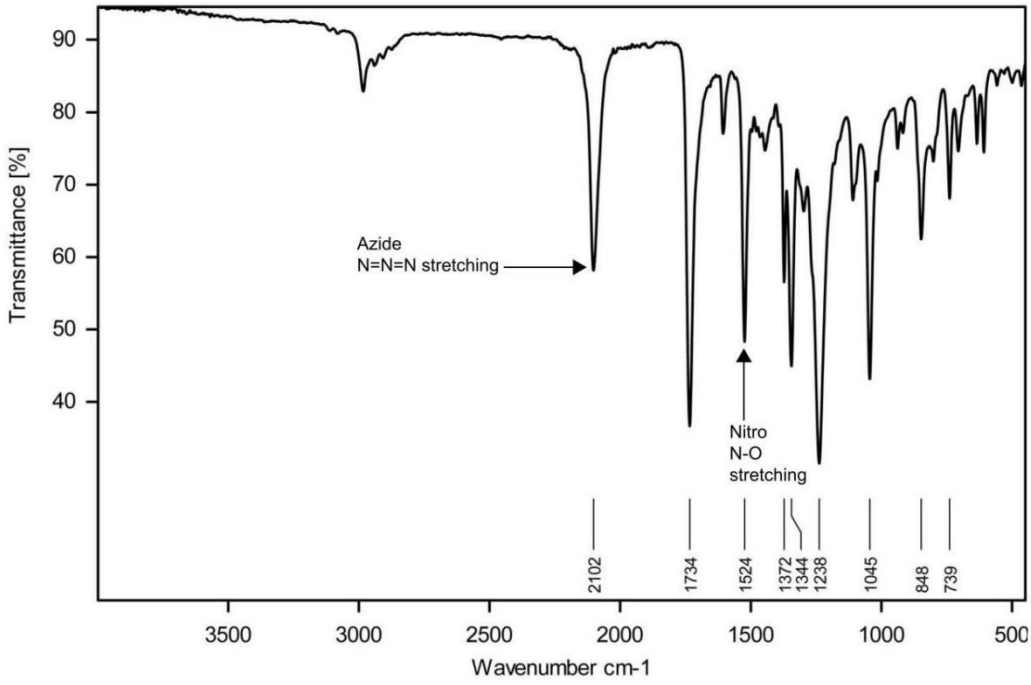
^{13}C NMR 165.00 - 110 ppm (DMSO at 80°C)



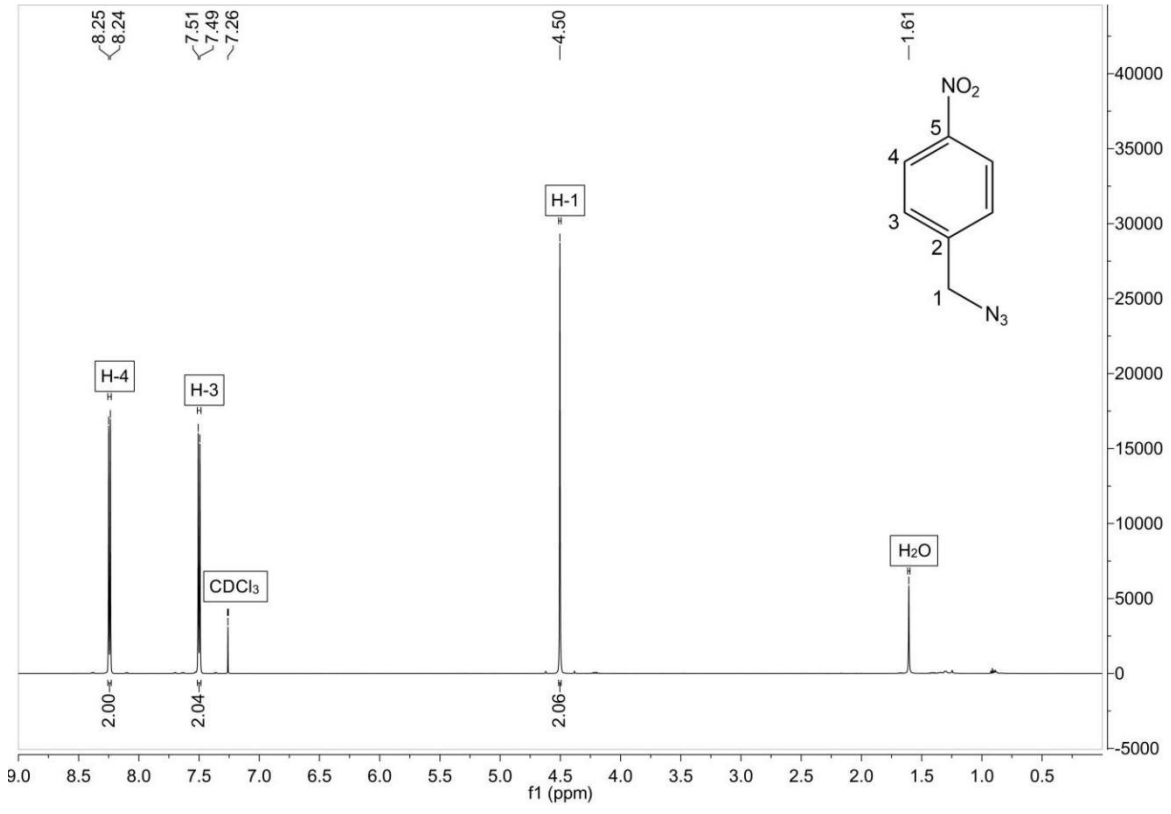
Para-substituted benzyl azide (reagents)

1-(azidomethyl)-4-nitrobenzene (Compound 3a)

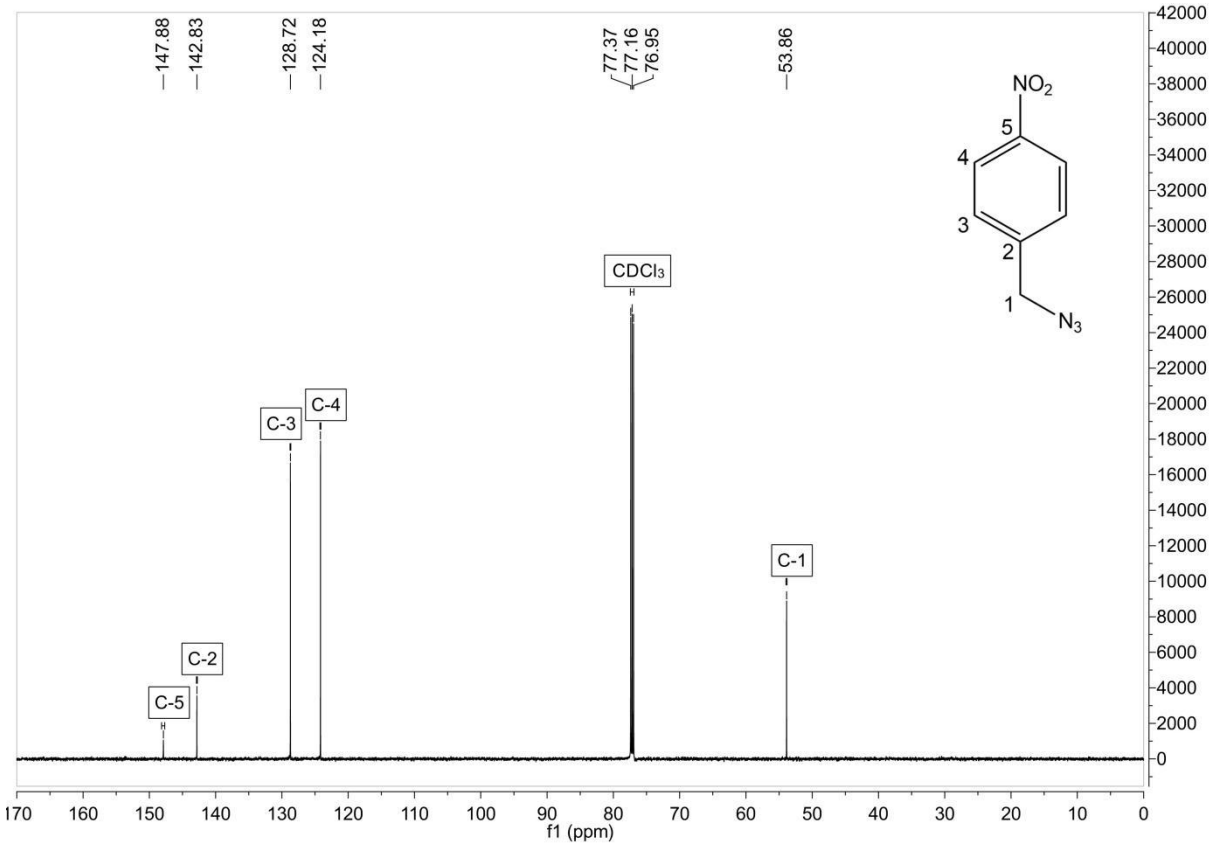
IR



^{1}H NMR (Chloroform at ambient temperature, ~25 °C)

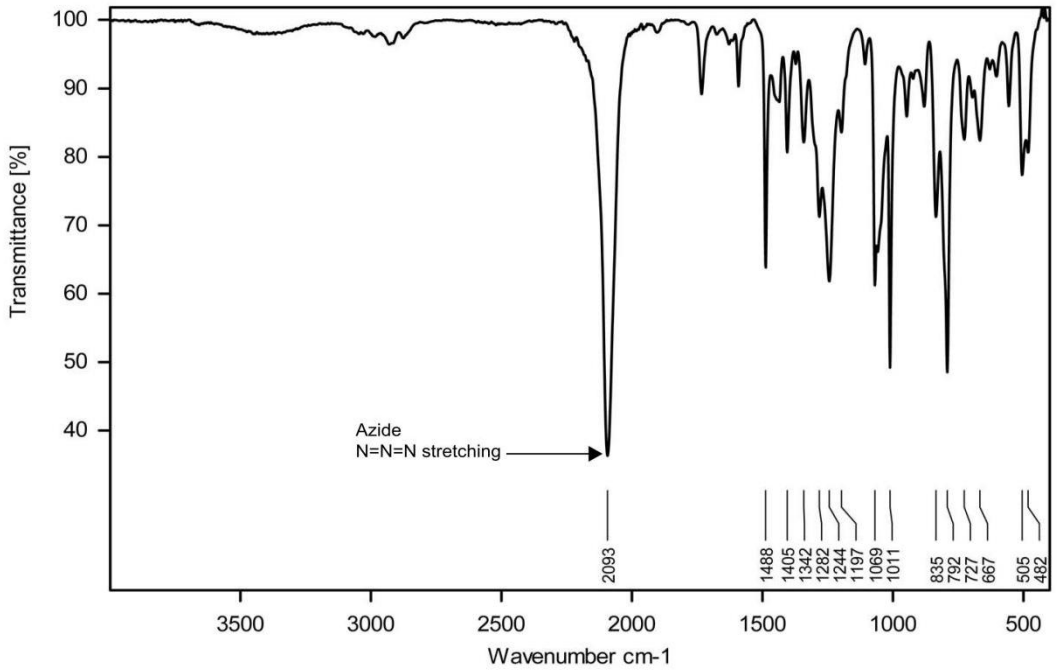


^{13}C NMR (Chloroform at ambient temperature, ~25 °C)

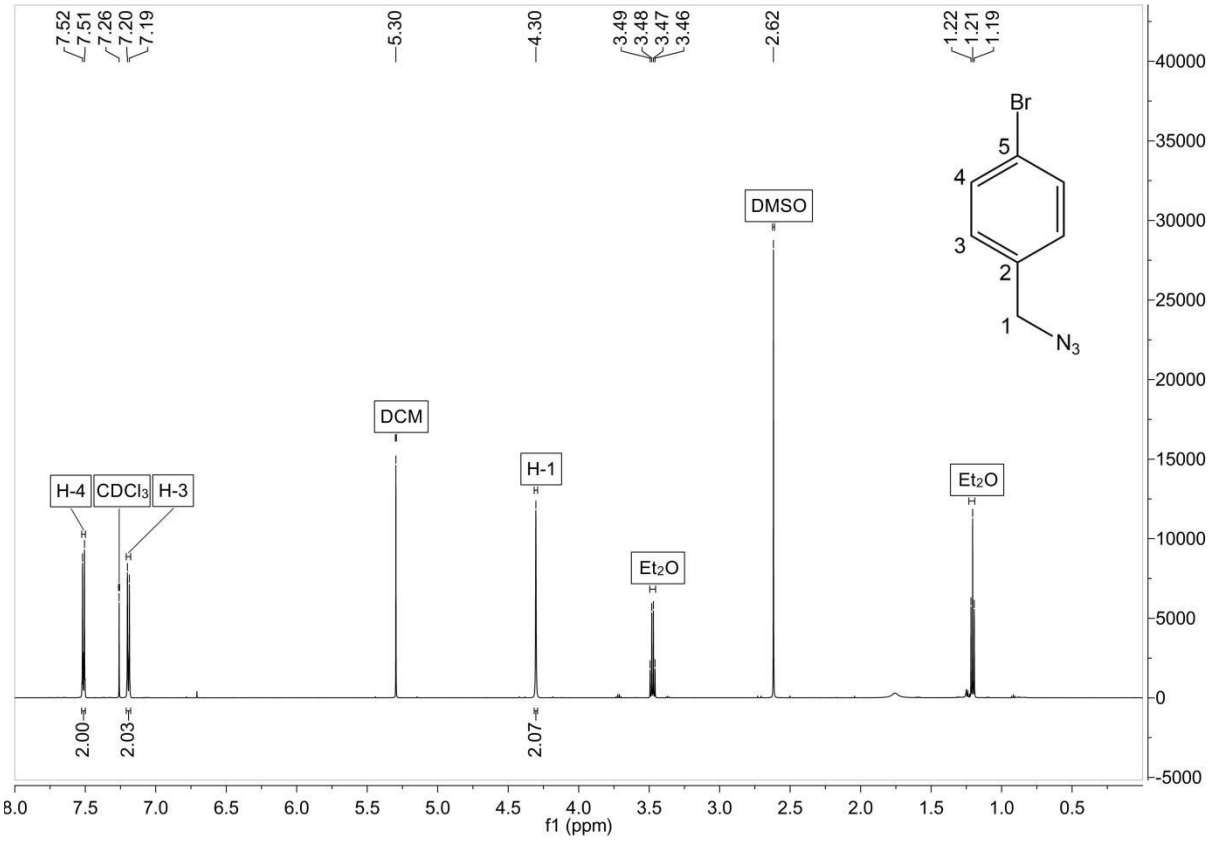


1-(azidomethyl)-4-bromobenzene (Compound 3b)

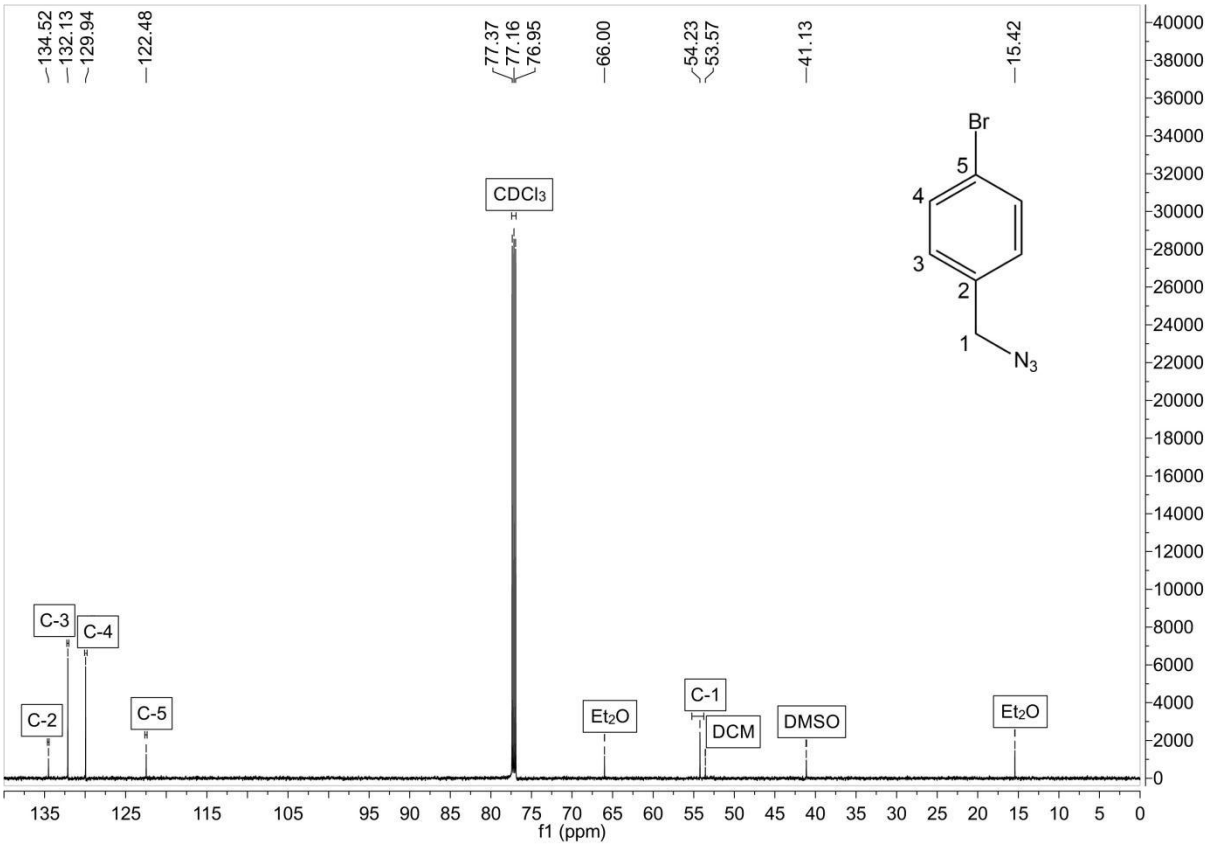
IR



^{1}H NMR (Chloroform at ambient temperature, ~25 °C)

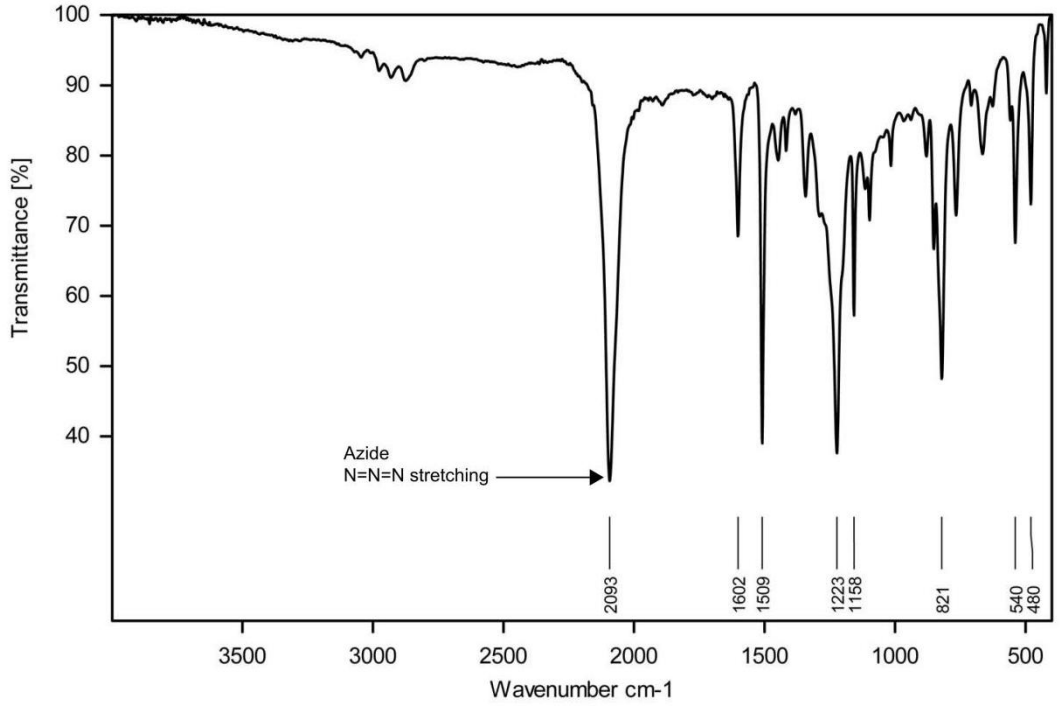


^{13}C NMR (Chloroform at ambient temperature, ~25 °C)

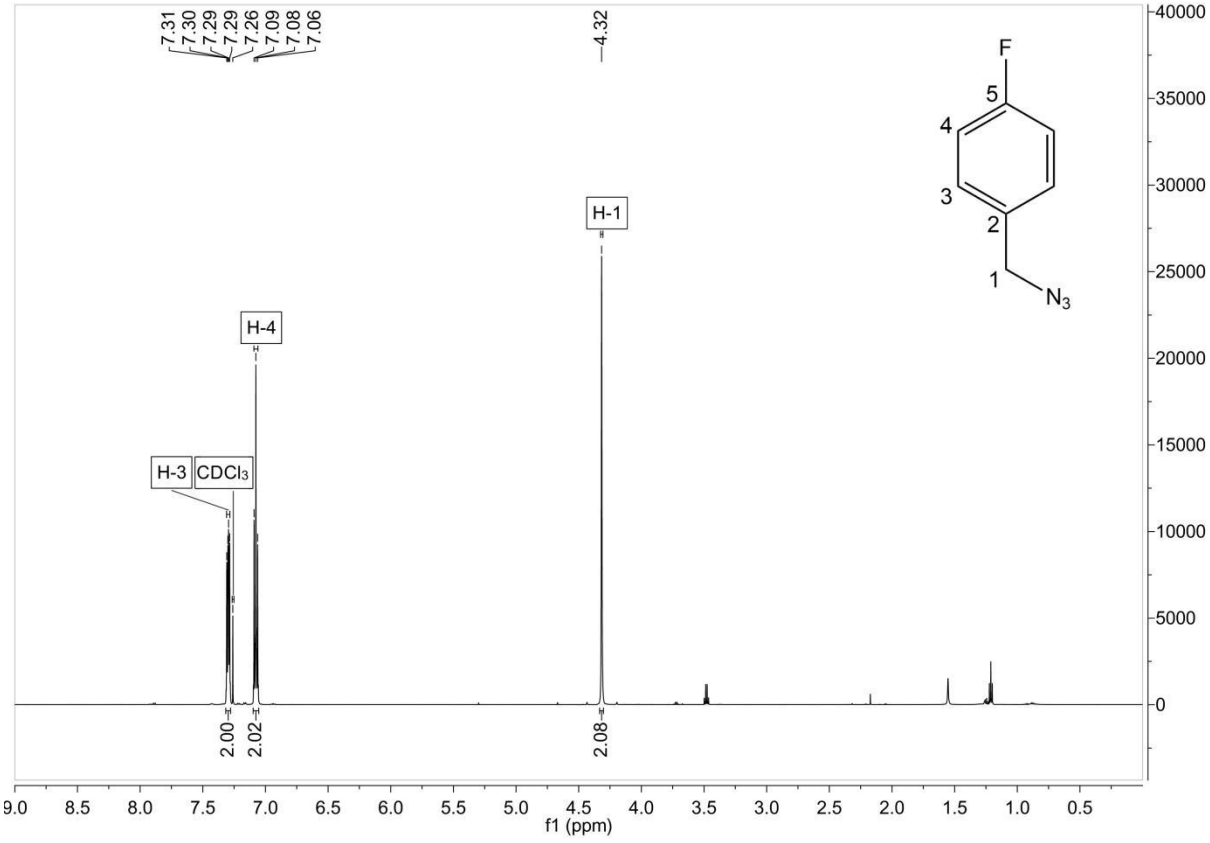


1-(azidomethyl)-4-fluorobenzene (Compound 3c)

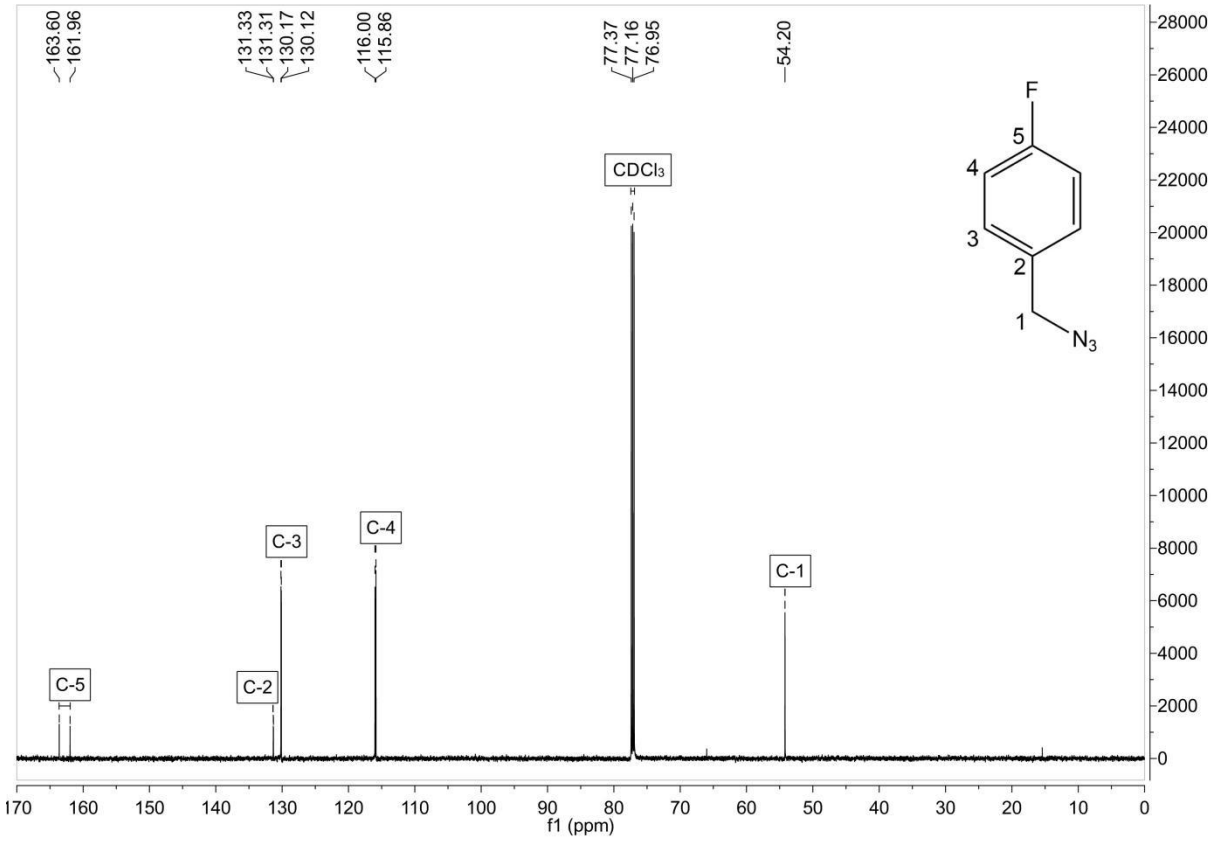
IR



^{1}H NMR (Chloroform at ambient temperature, ~25 °C)

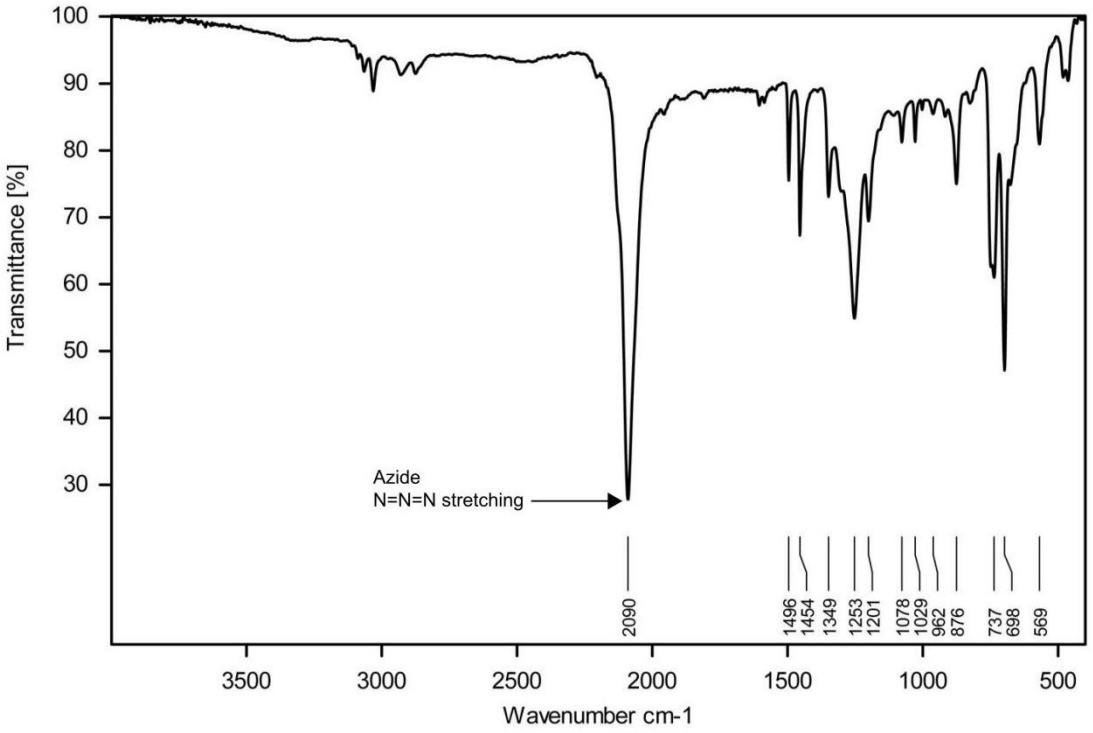


^{13}C NMR (Chloroform at ambient temperature, ~25 °C)

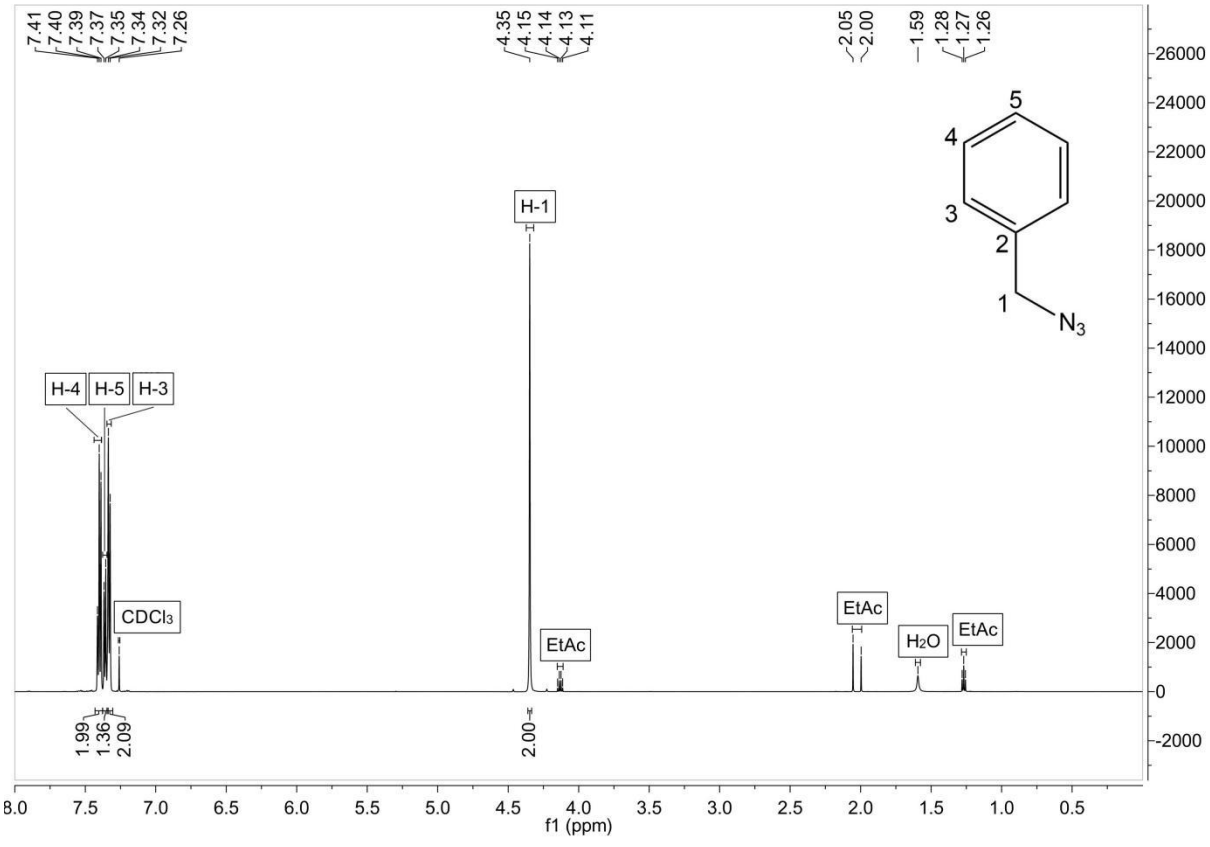


(Azidomethyl)-benzene (Compound 3d)

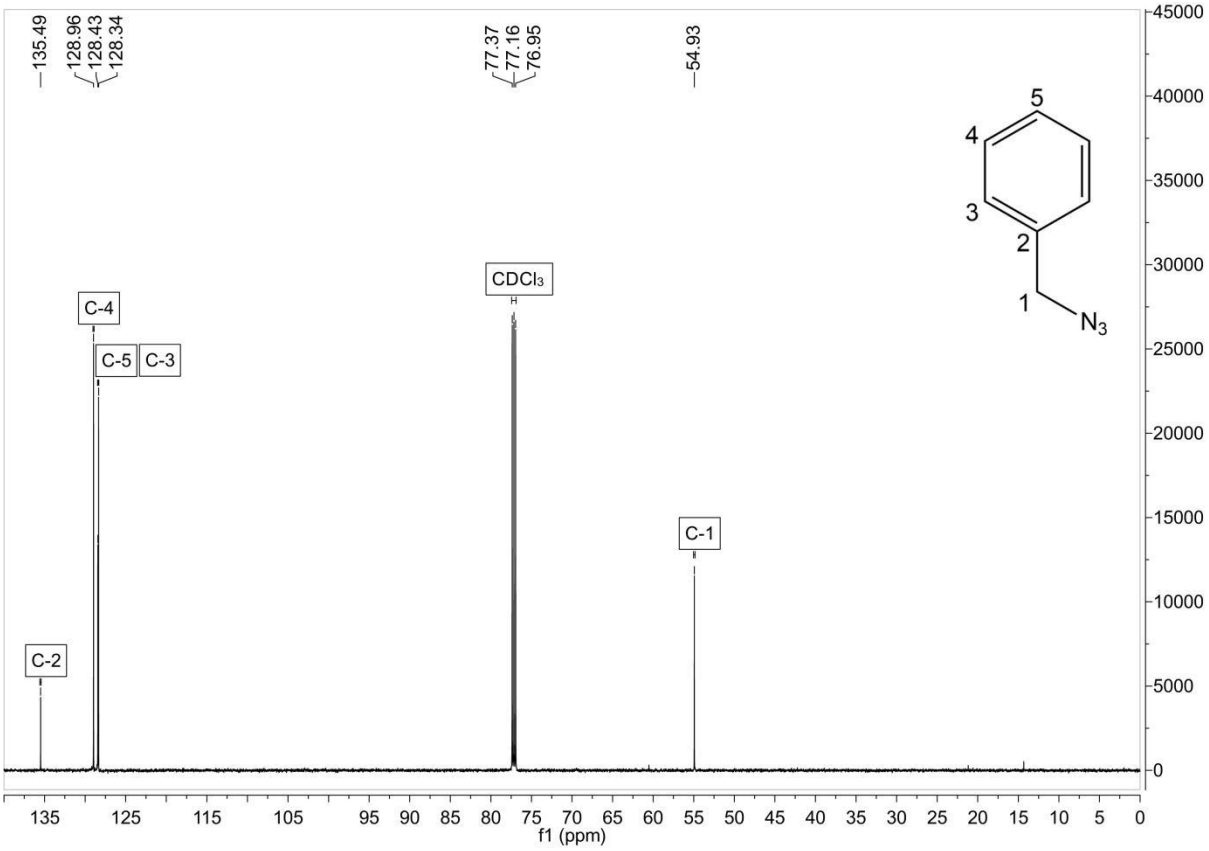
IR



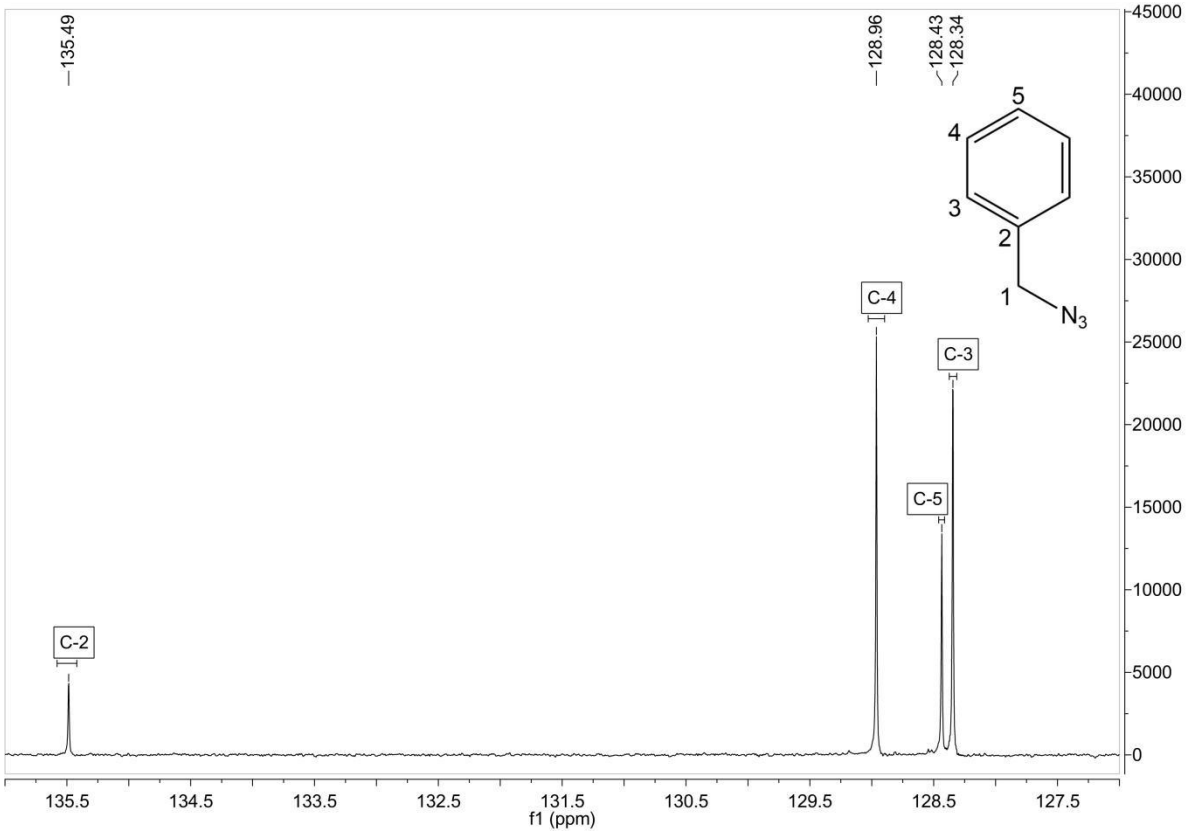
^{1}H NMR (Chloroform at ambient temperature, ~25 °C)



^{13}C NMR (Chloroform at ambient temperature, ~25 °C)

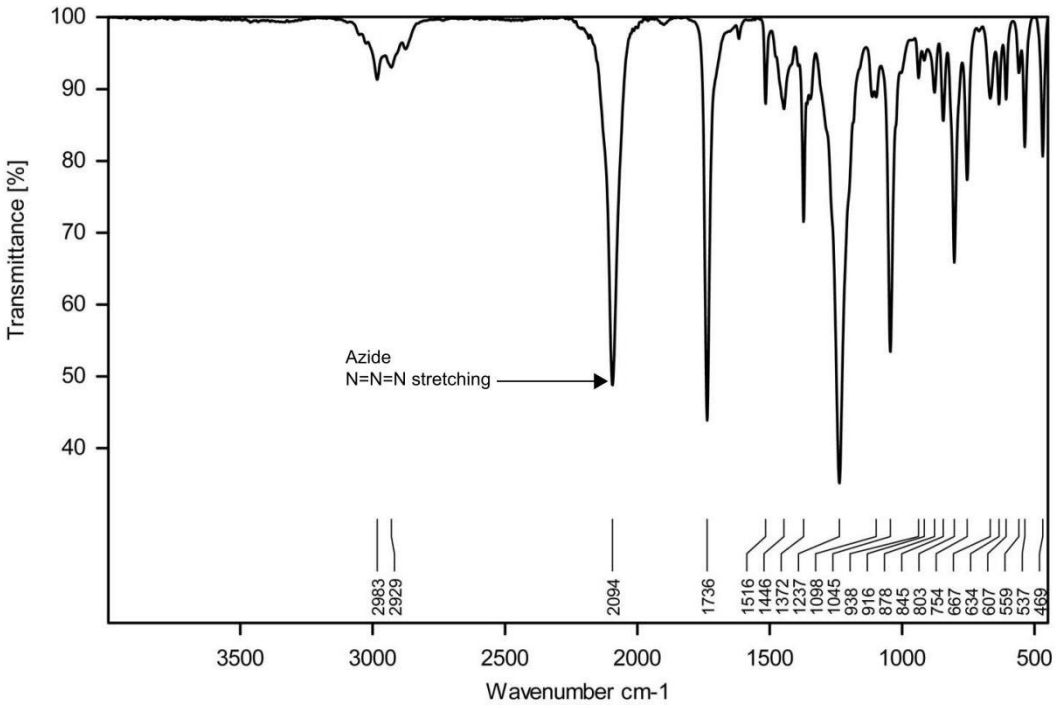


^{13}C NMR 136.00 - 127.00 ppm (Chloroform at ambient temperature, ~25 °C)

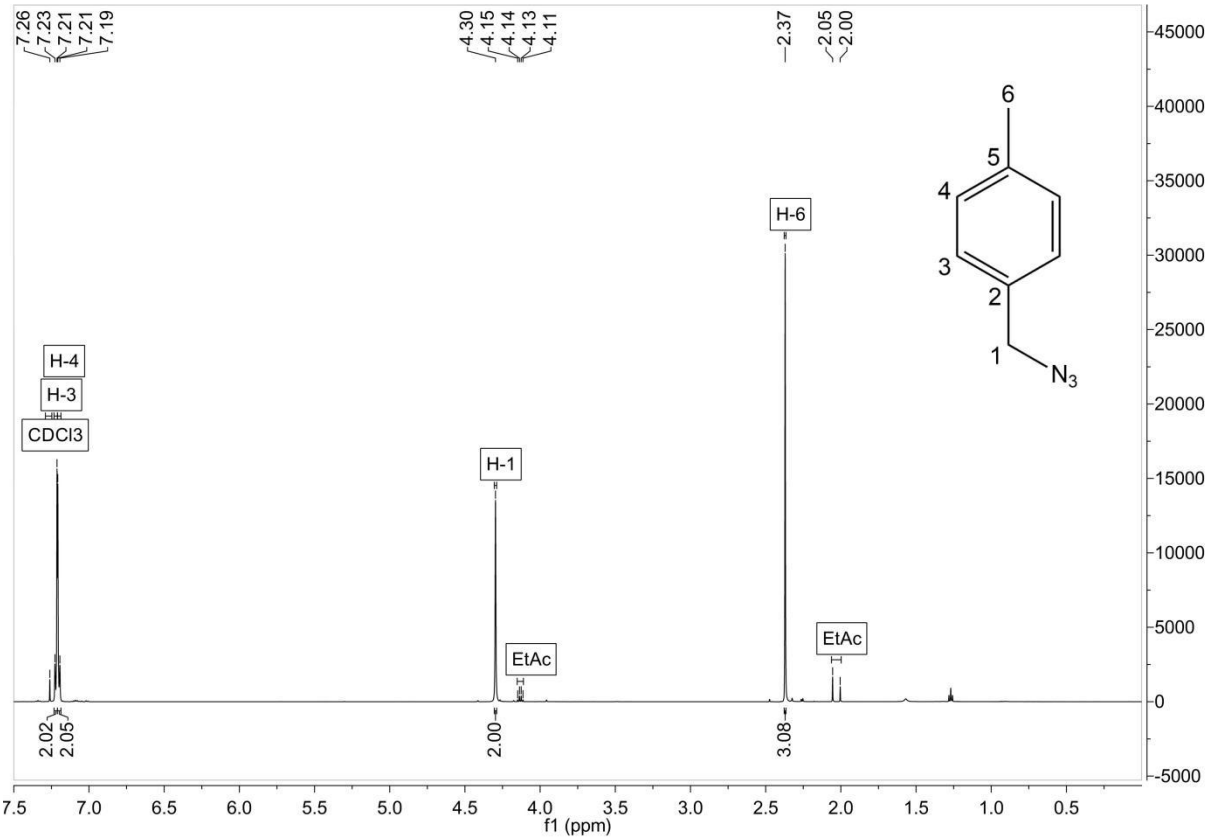


1-(azidomethyl)-4-methylbenzene (Compound 3e)

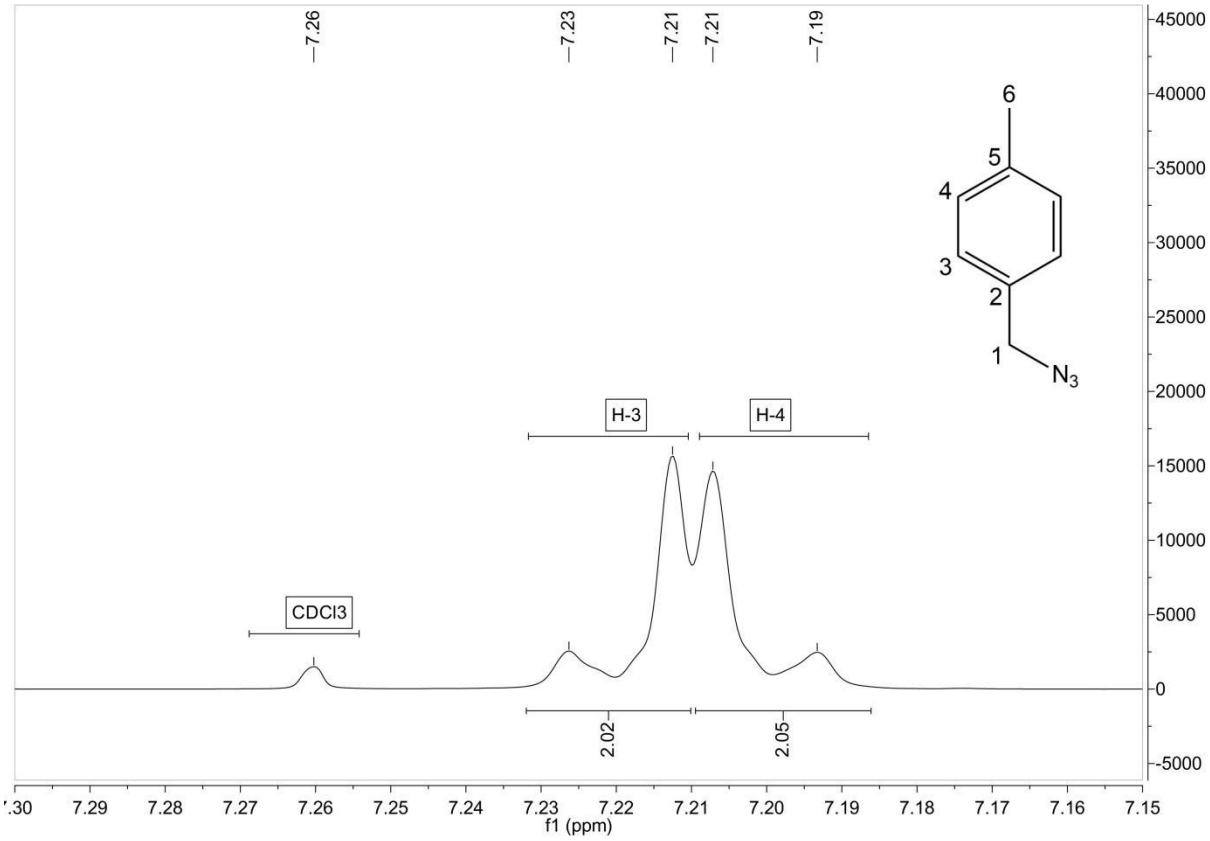
IR



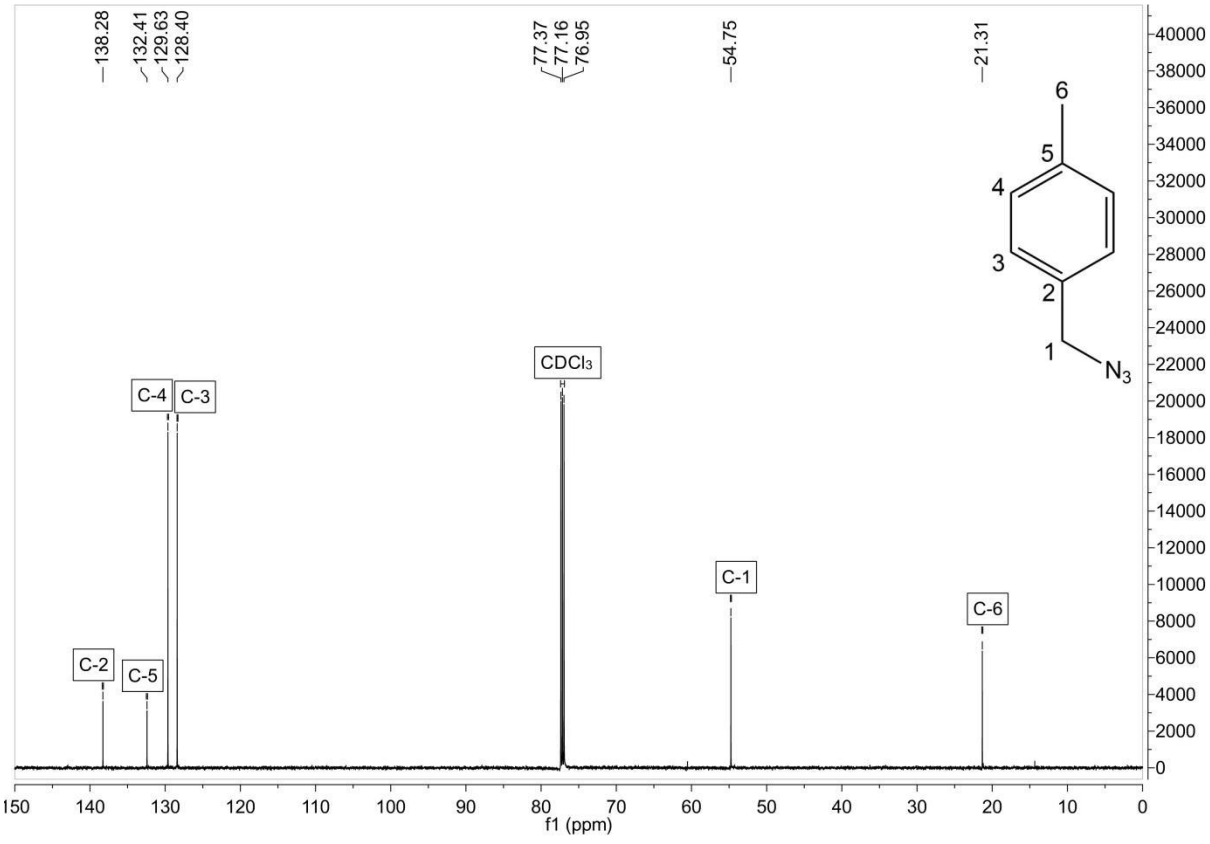
^{1}H NMR (Chloroform at ambient temperature, ~25 °C)



^{1}H NMR 7.30 - 7.15 ppm (Chloroform at ambient temperature, ~25 °C)



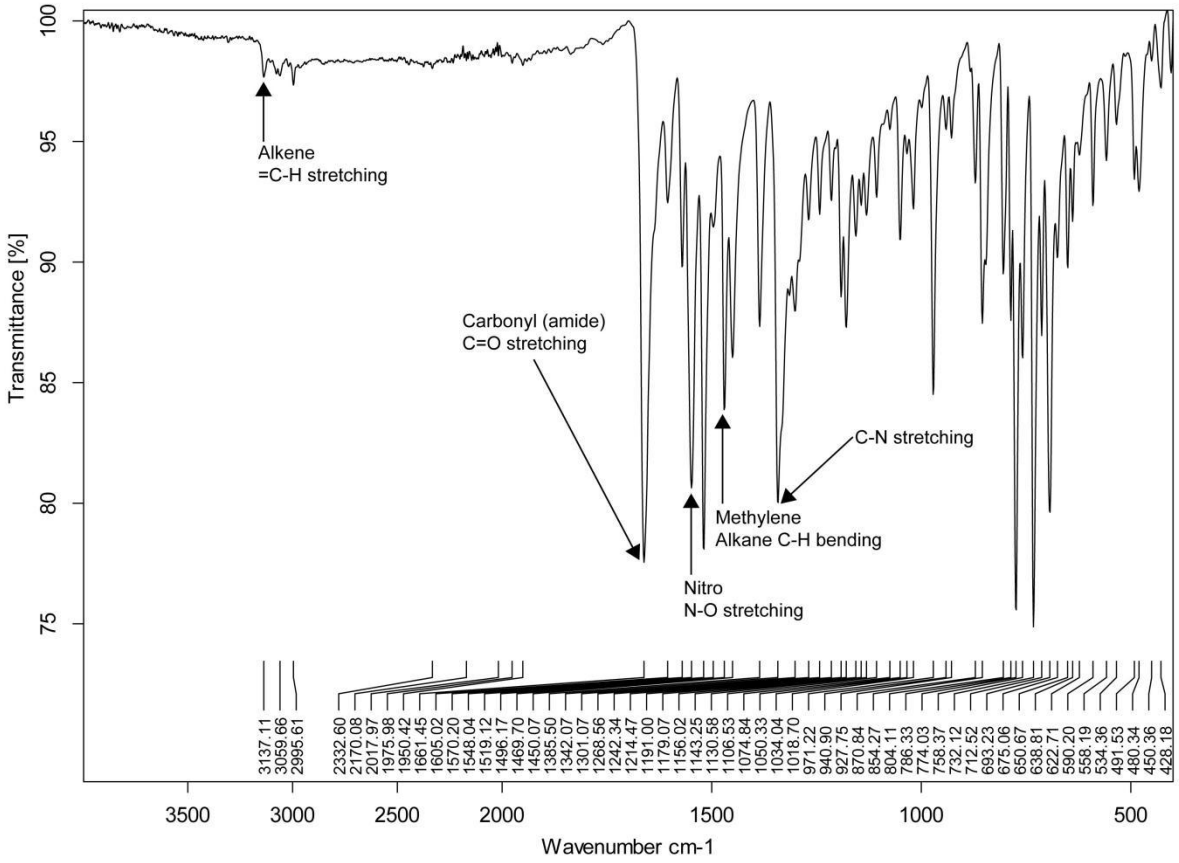
^{13}C NMR (Chloroform at ambient temperature, ~25 °C)



Final compounds

Compound 4a

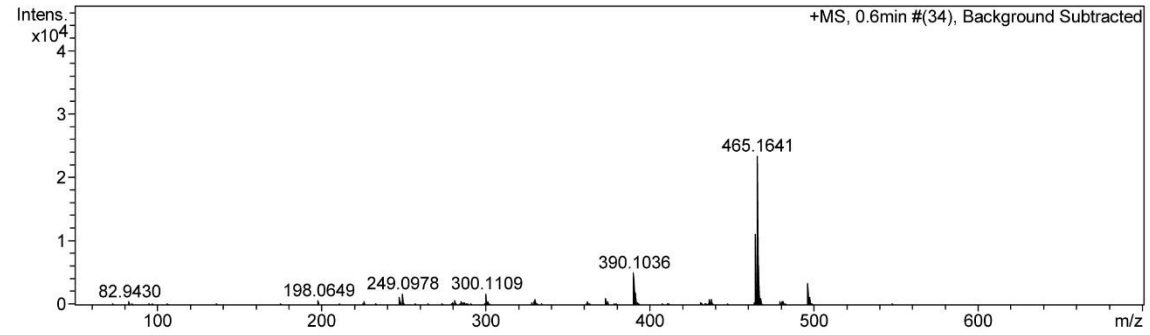
IR



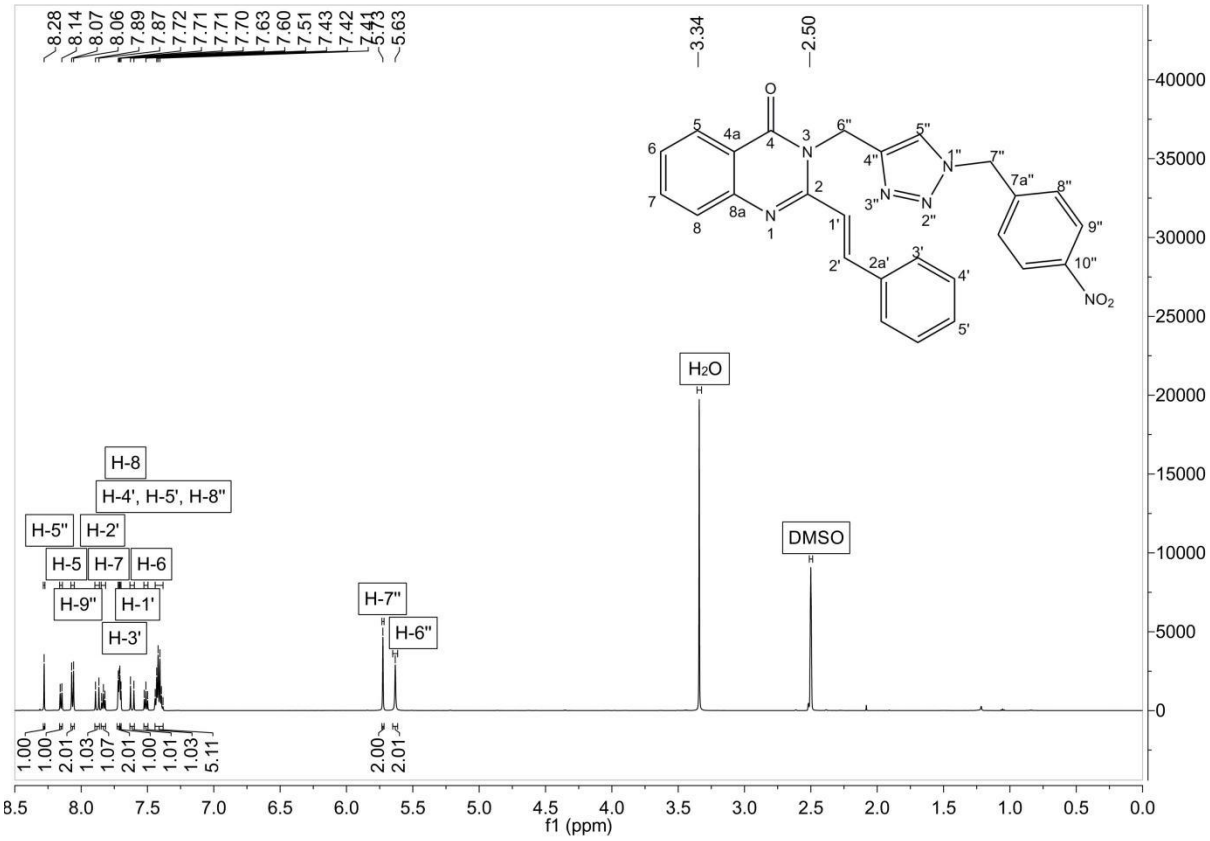
HRMS

Acquisition Parameter

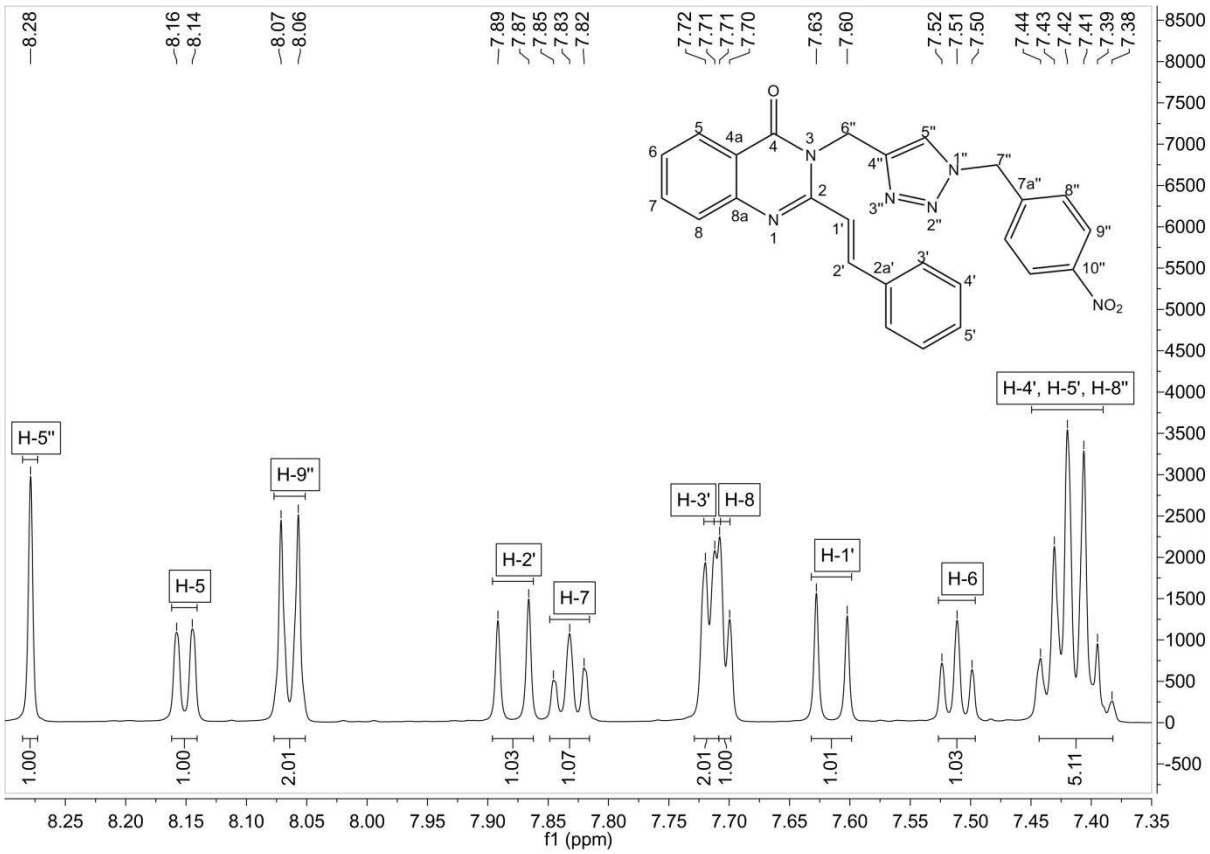
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



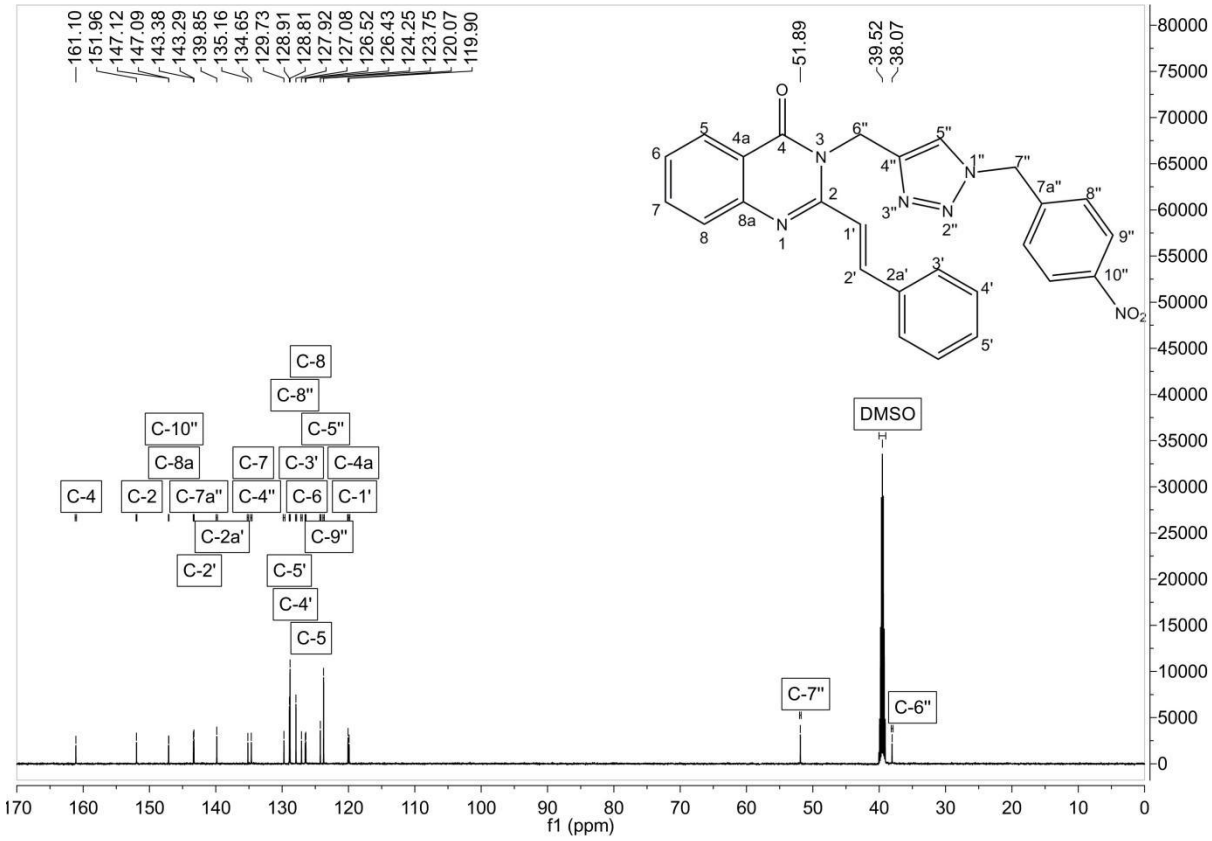
^{1}H NMR (DMSO at ambient temperature, ~25 °C)



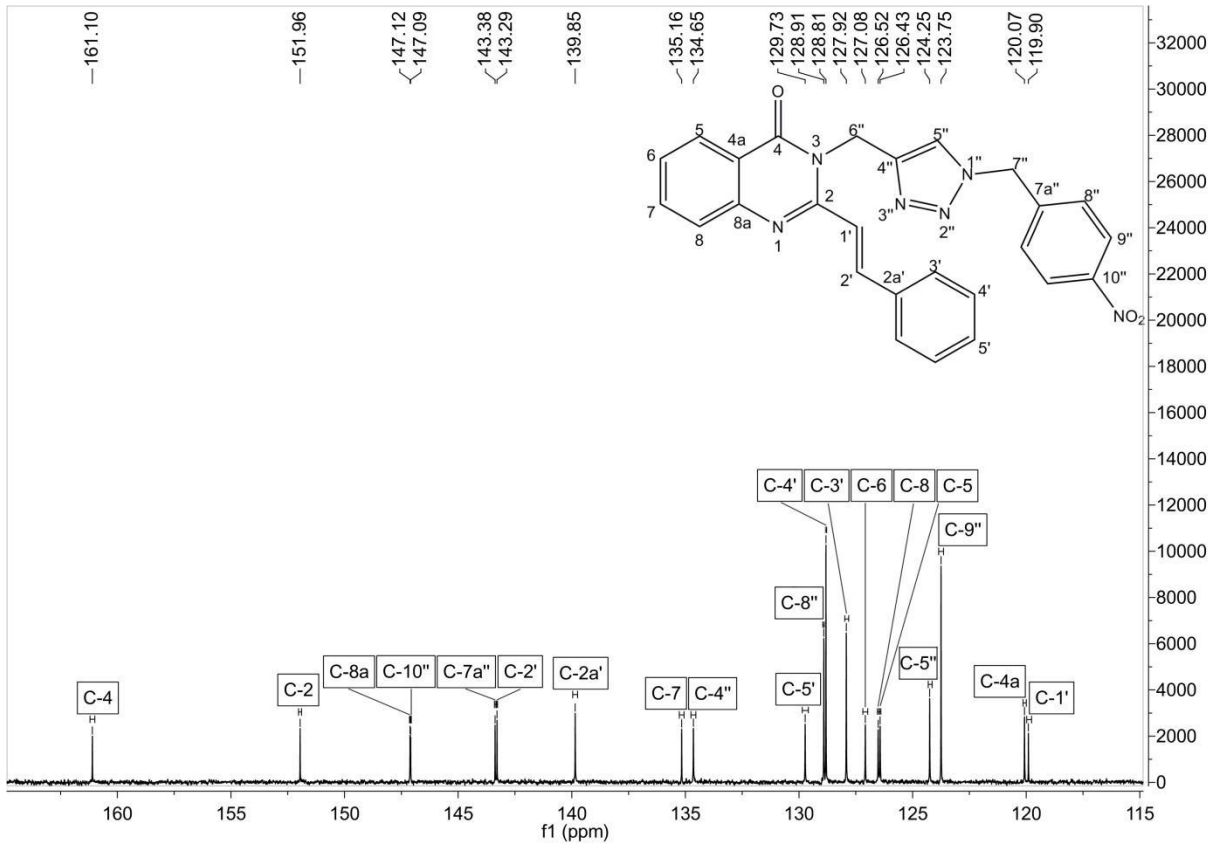
^{1}H NMR 8.30 - 7.35 ppm (DMSO at ambient temperature, ~25 °C)



^{13}C NMR (DMSO at ambient temperature, ~25 °C)

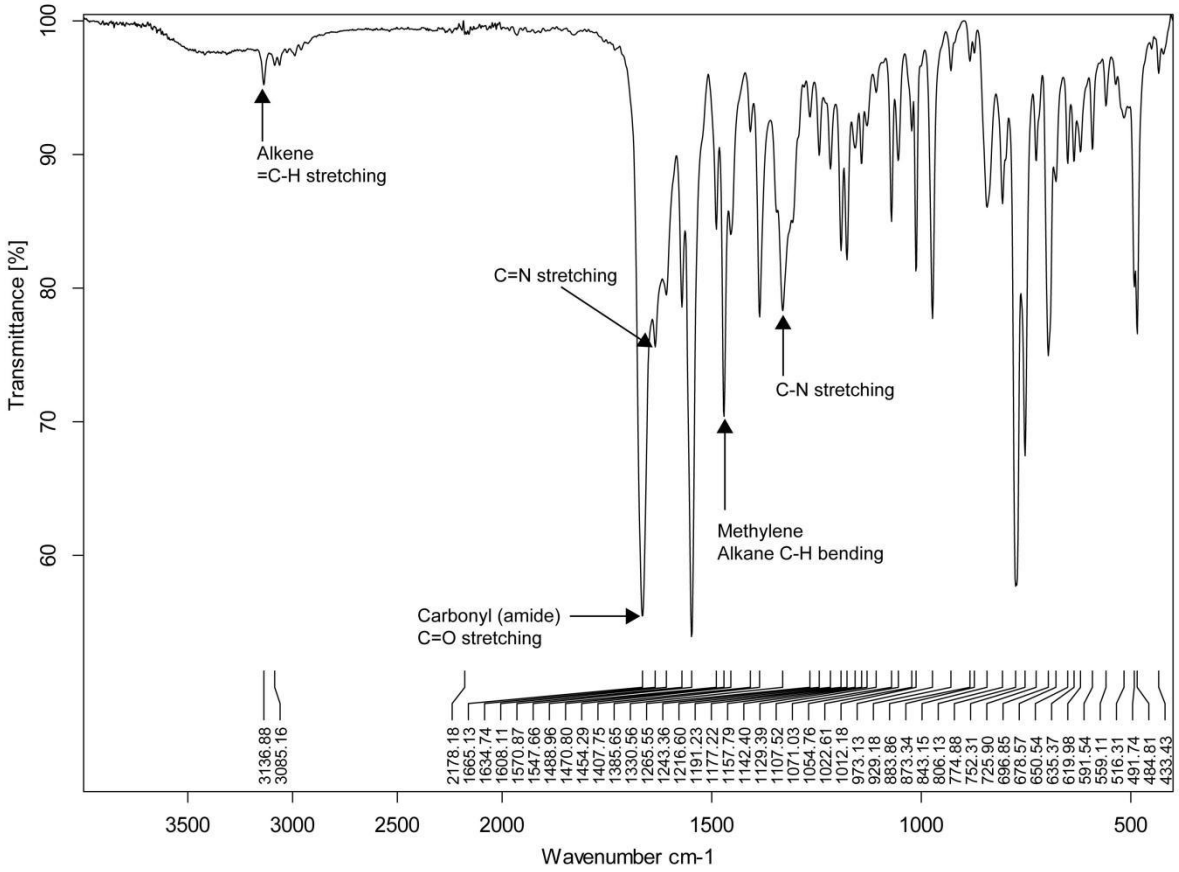


^{13}C NMR 165.00 - 115.00 ppm (DMSO at ambient temperature, ~25 °C)



Compound 4b

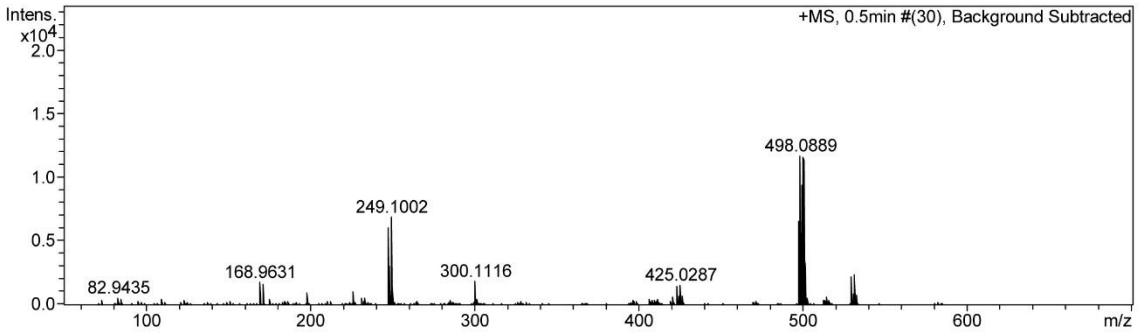
IR

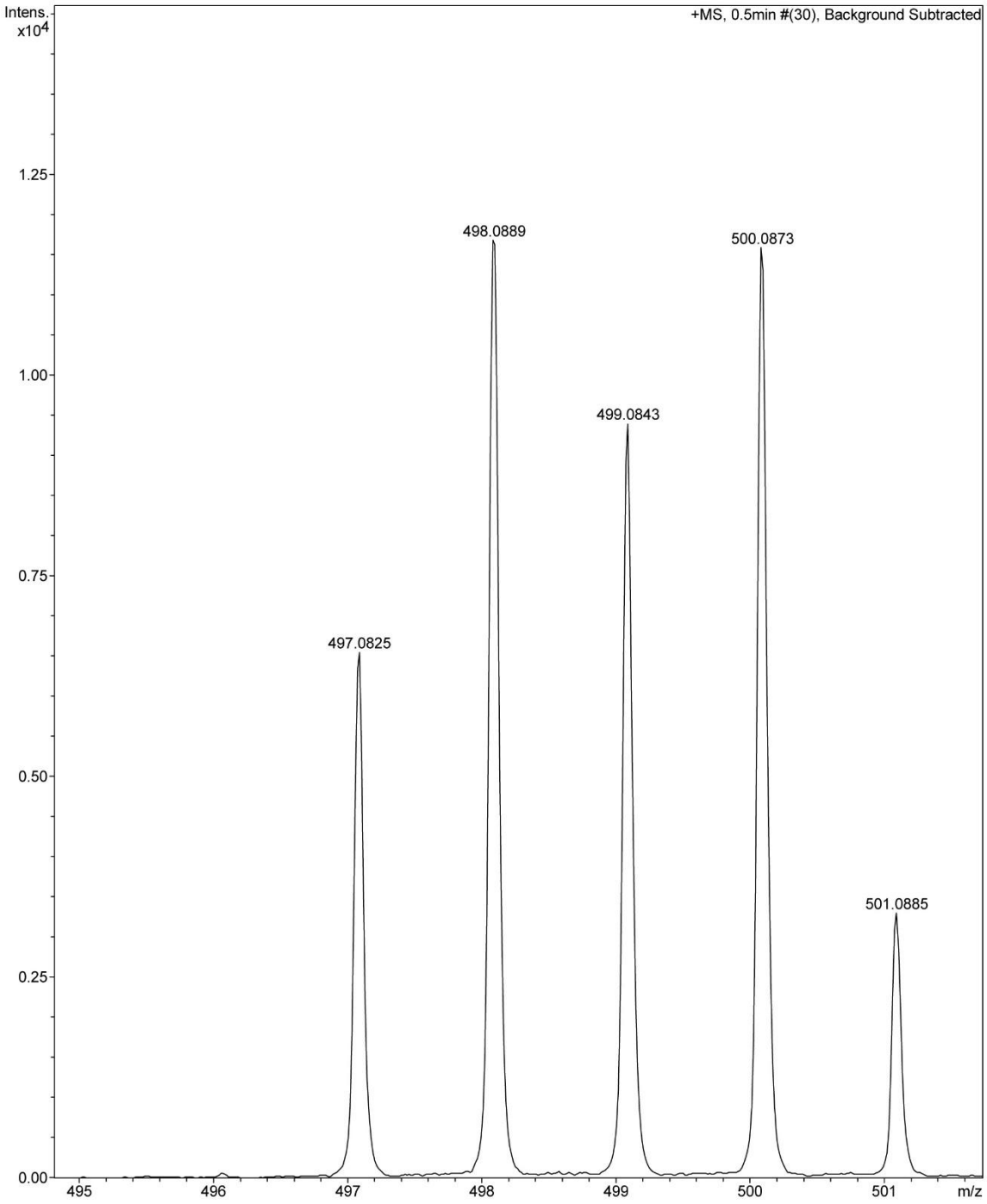


HRMS

Acquisition Parameter

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




Intens.
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0.50
0.25
0.00
498.0889
500.0873
499.0843
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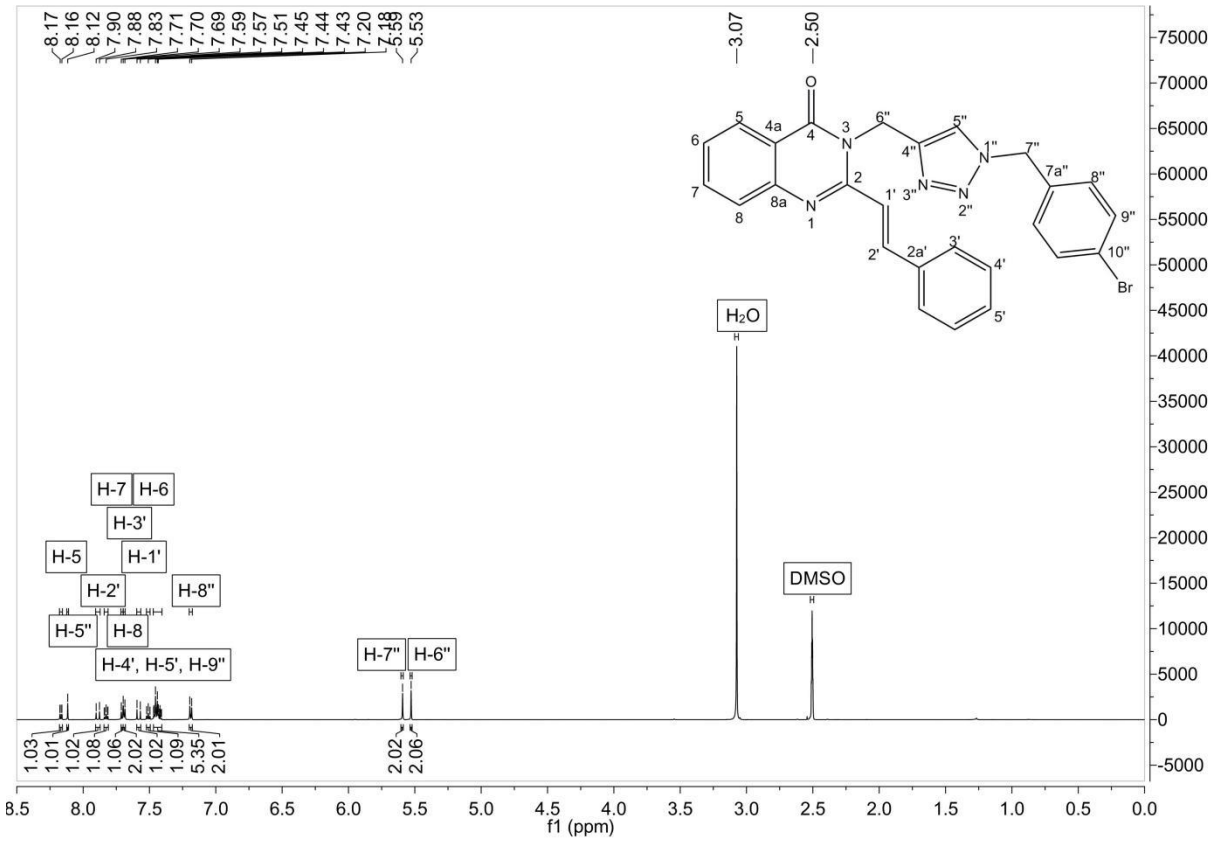
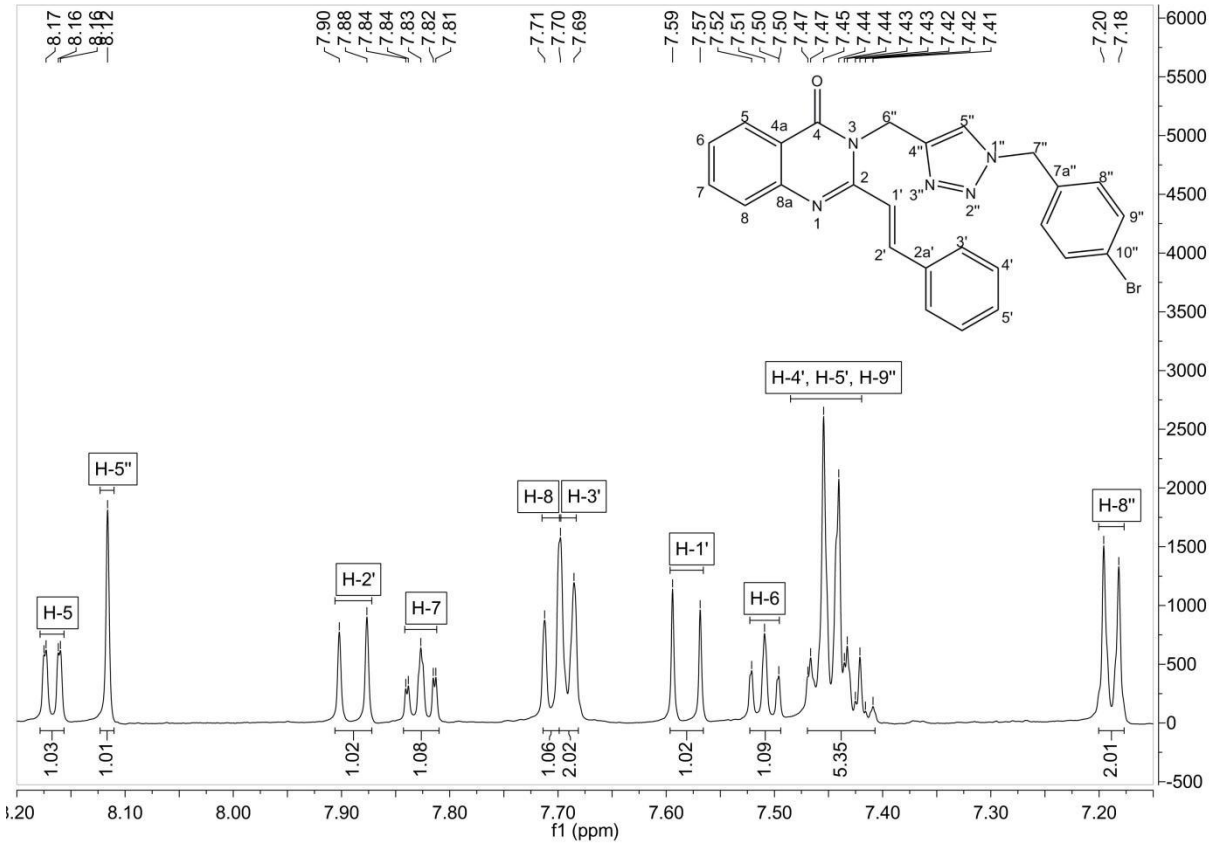
501.0885
495
496
497
498
499
500
501
m/z

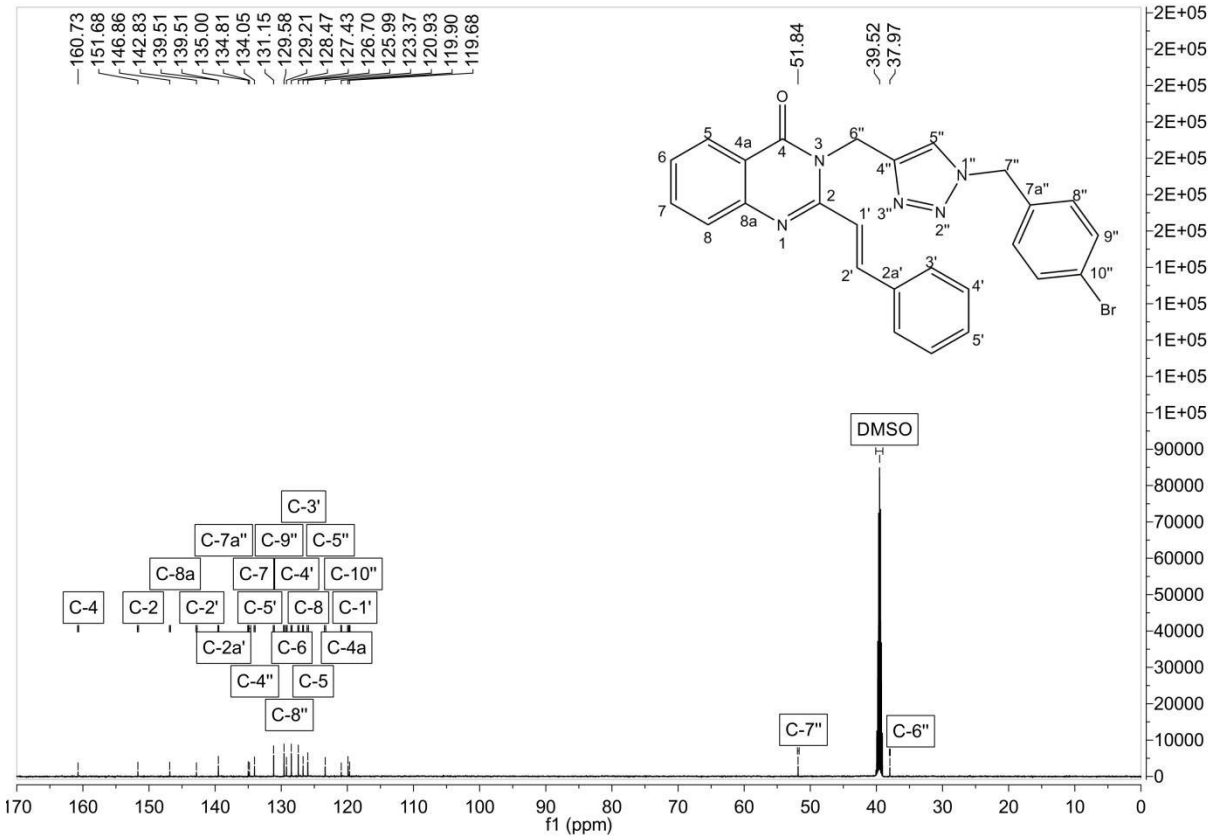
^{1}H NMR (DMSO at 80°C)



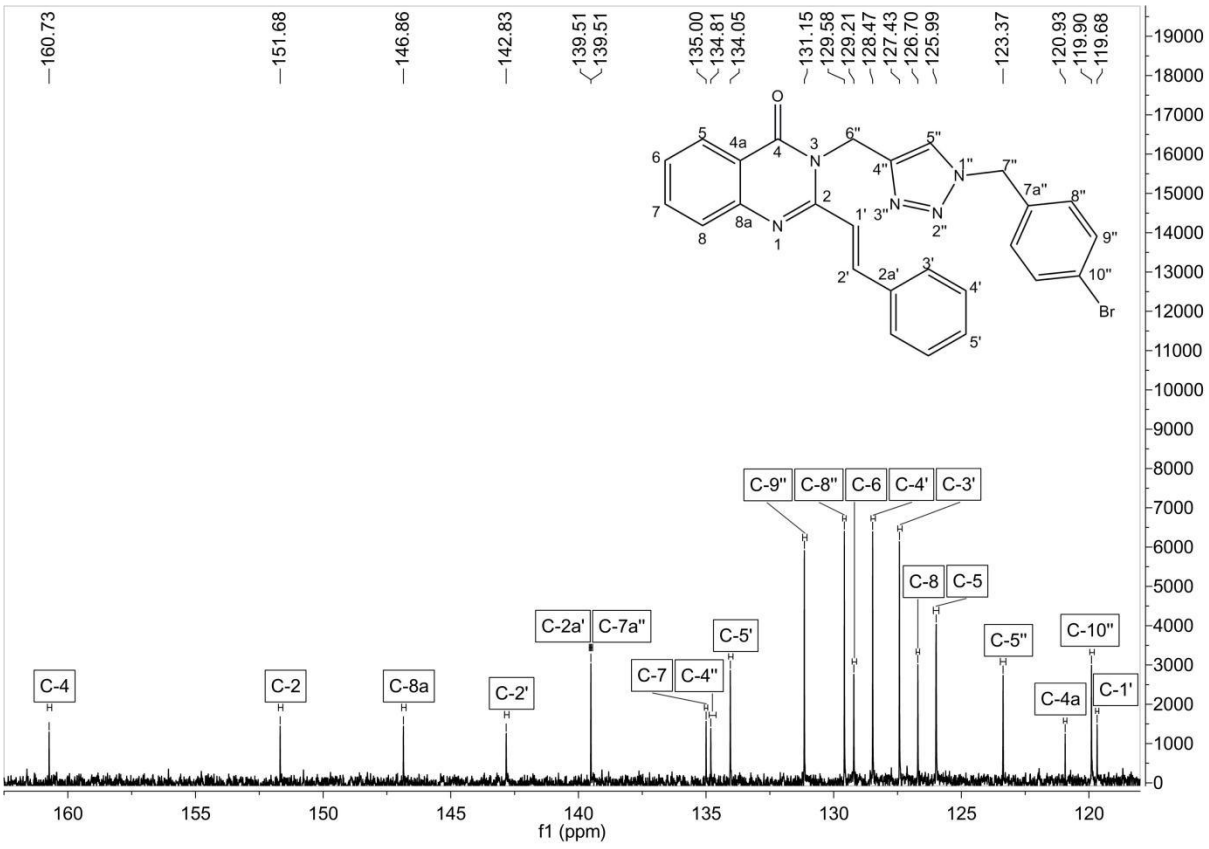
^{1}H NMR 8.20 - 7.15 ppm (DMSO at 80°C)



^{13}C NMR (DMSO at 80°C)

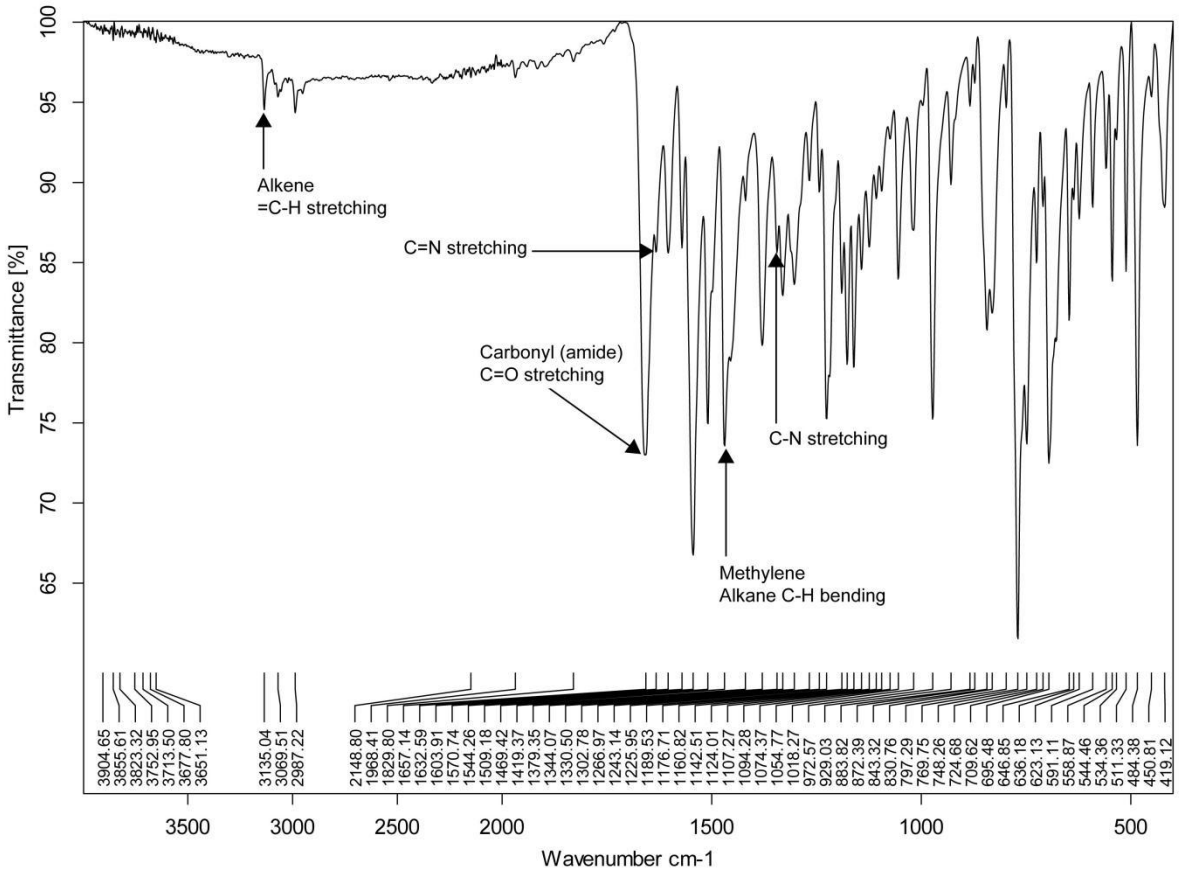


^{13}C NMR 162.50 - 118.00 ppm (DMSO at 80°C)



Compound 4c

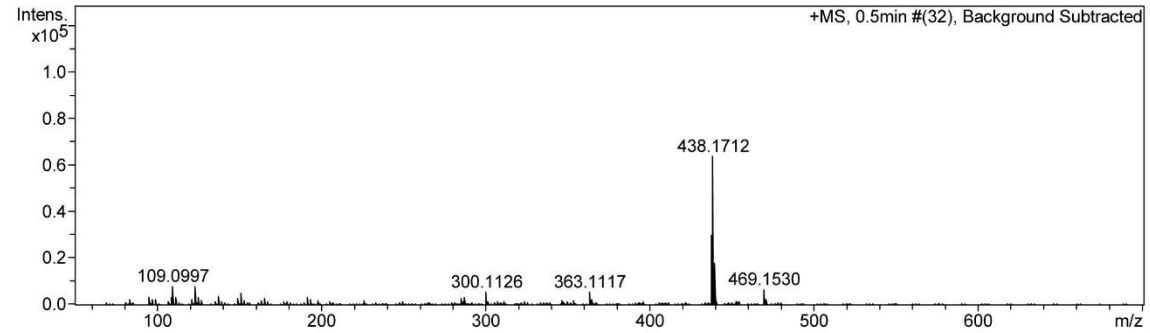
IR



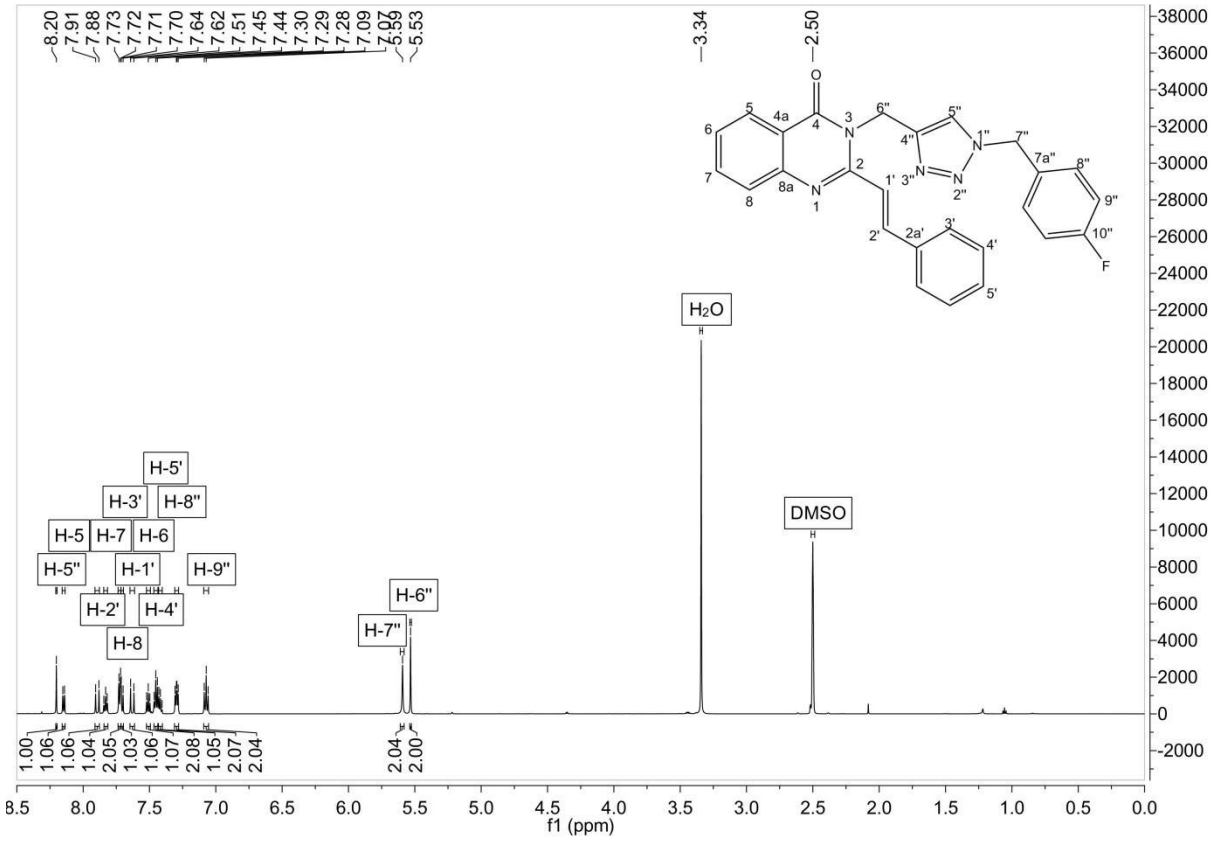
HRMS

Acquisition Parameter

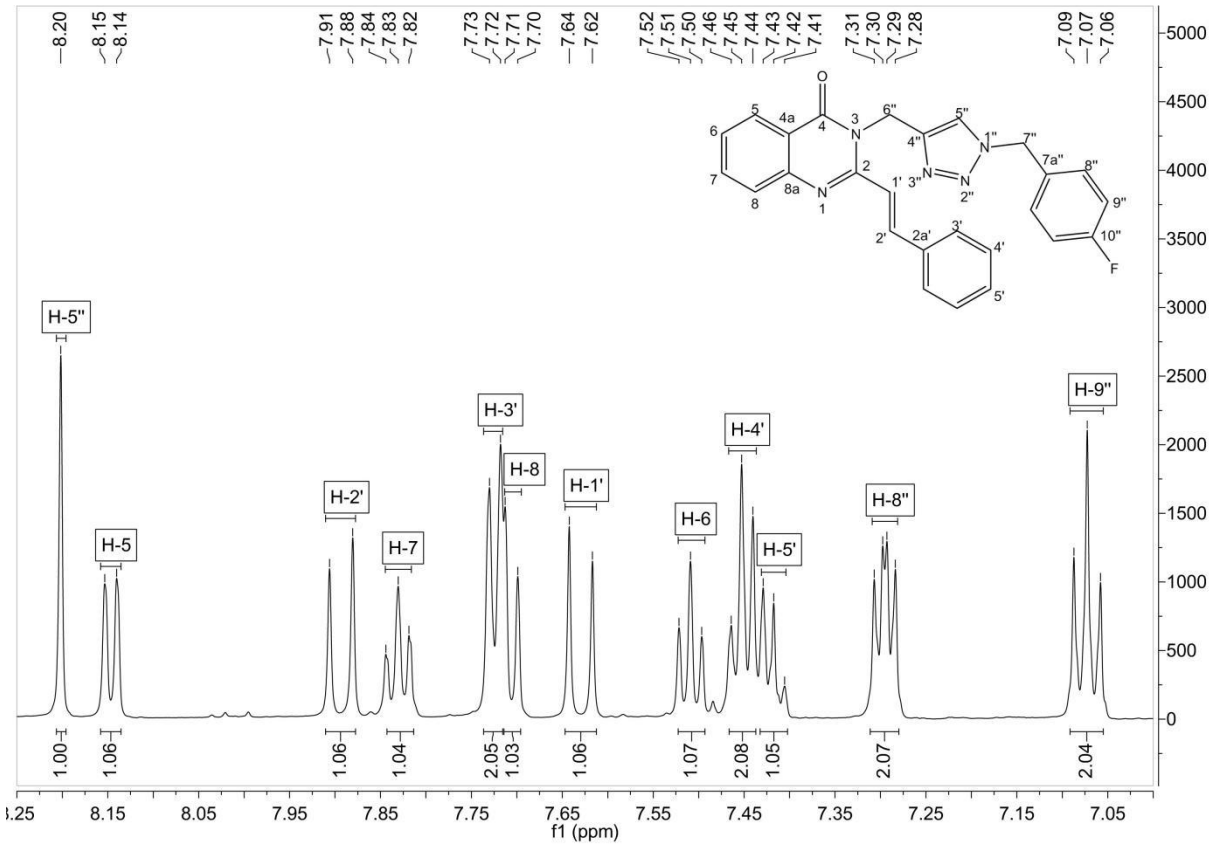
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



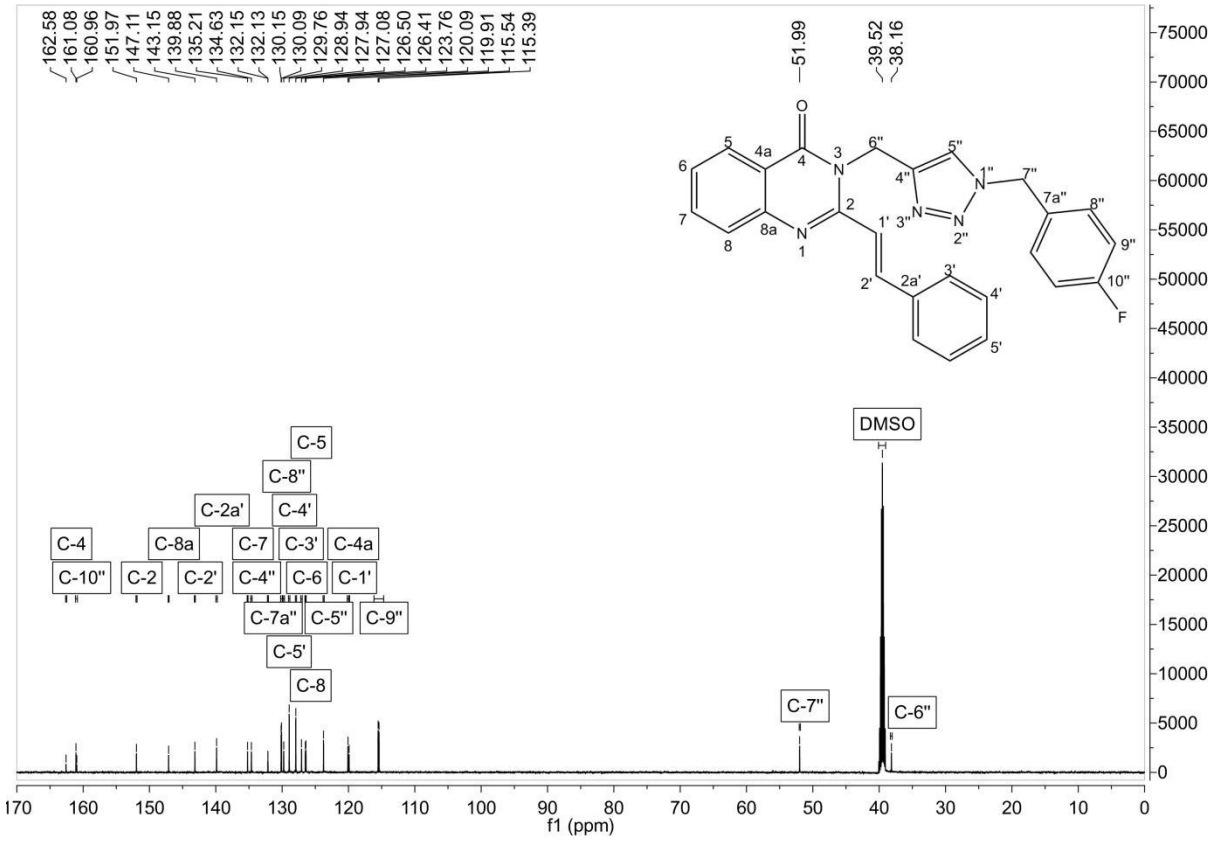
^{1}H NMR (DMSO at ambient temperature, ~25 °C)



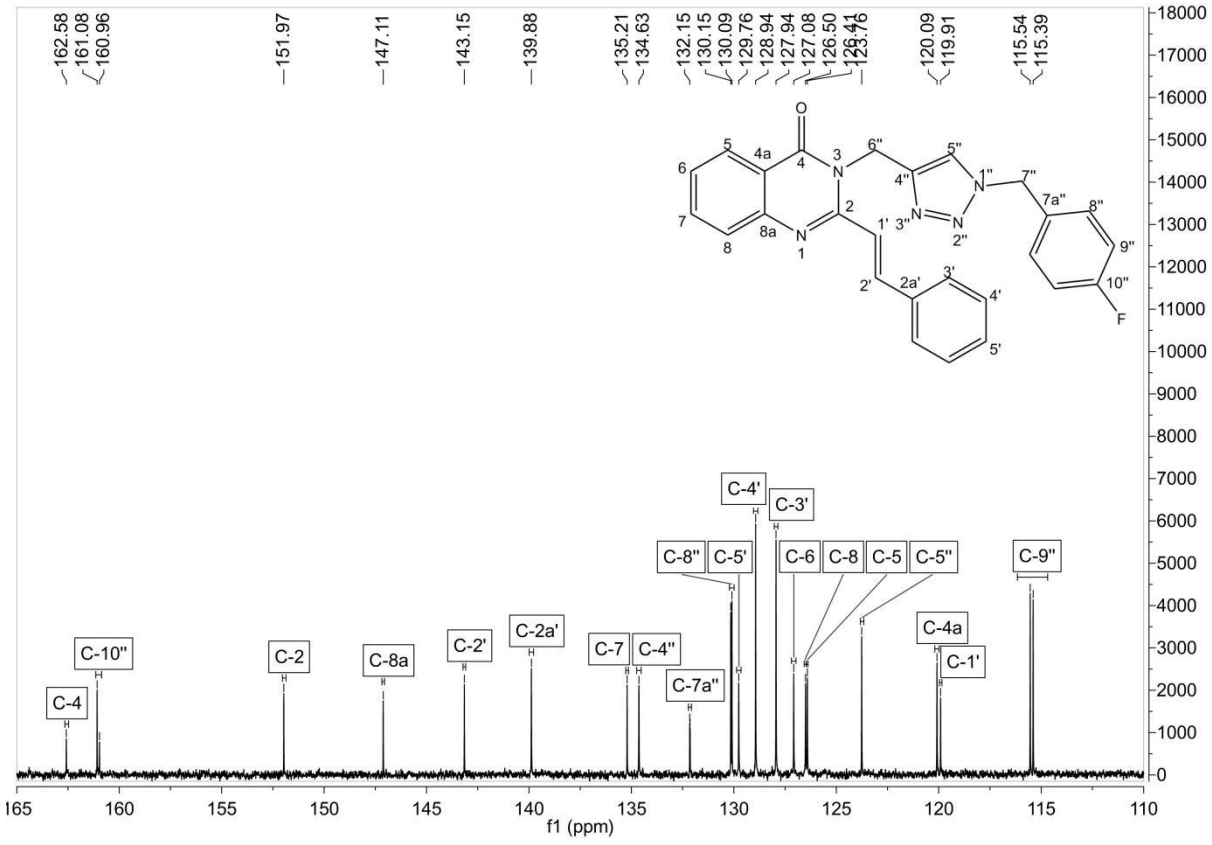
^{1}H NMR 8.25 - 7.00 ppm (DMSO at ambient temperature, ~25 °C)



^{13}C NMR (DMSO at ambient temperature, ~25 °C)

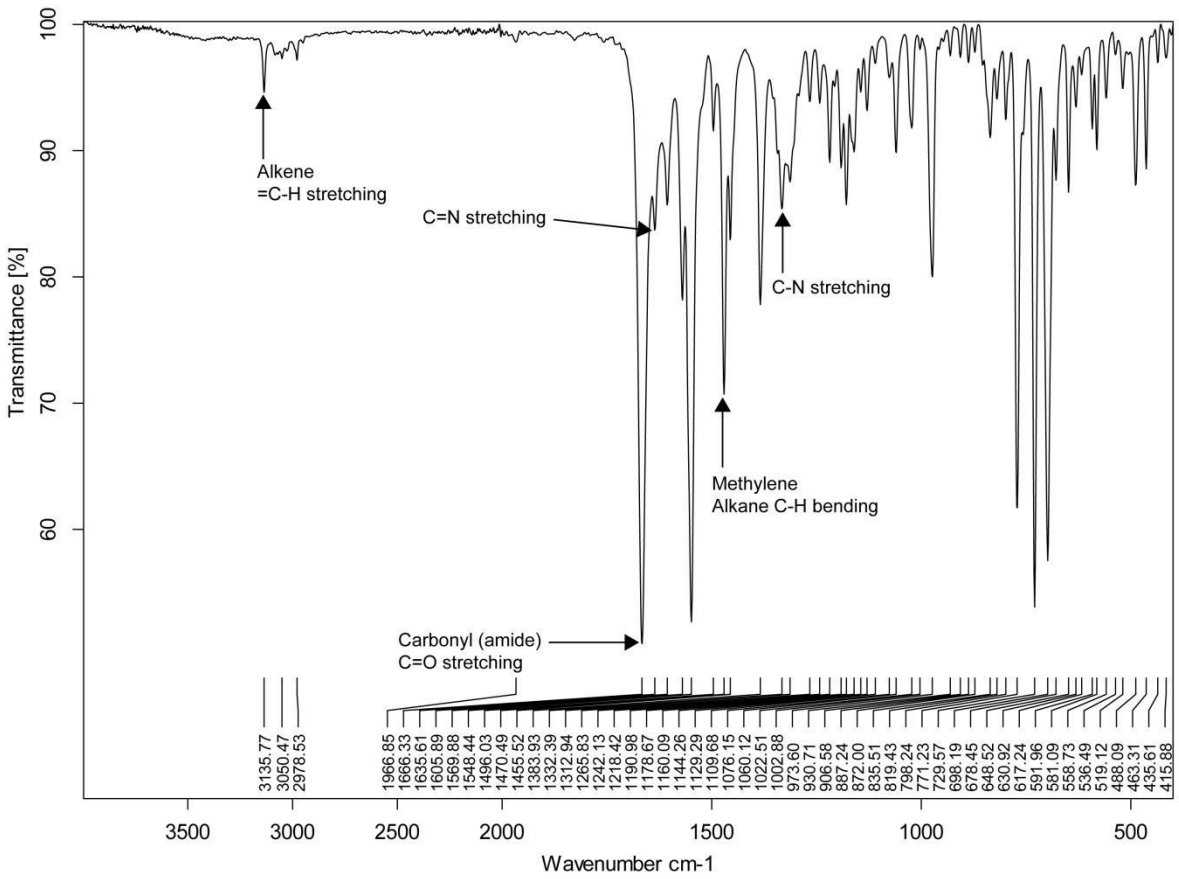


^{13}C NMR 165.00 - 110.00 ppm (DMSO at ambient temperature, ~25 °C)



Compound 4d

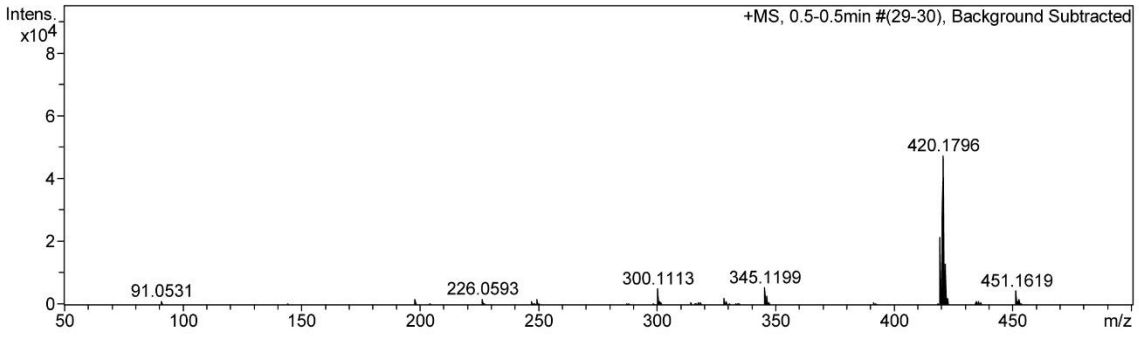
IR



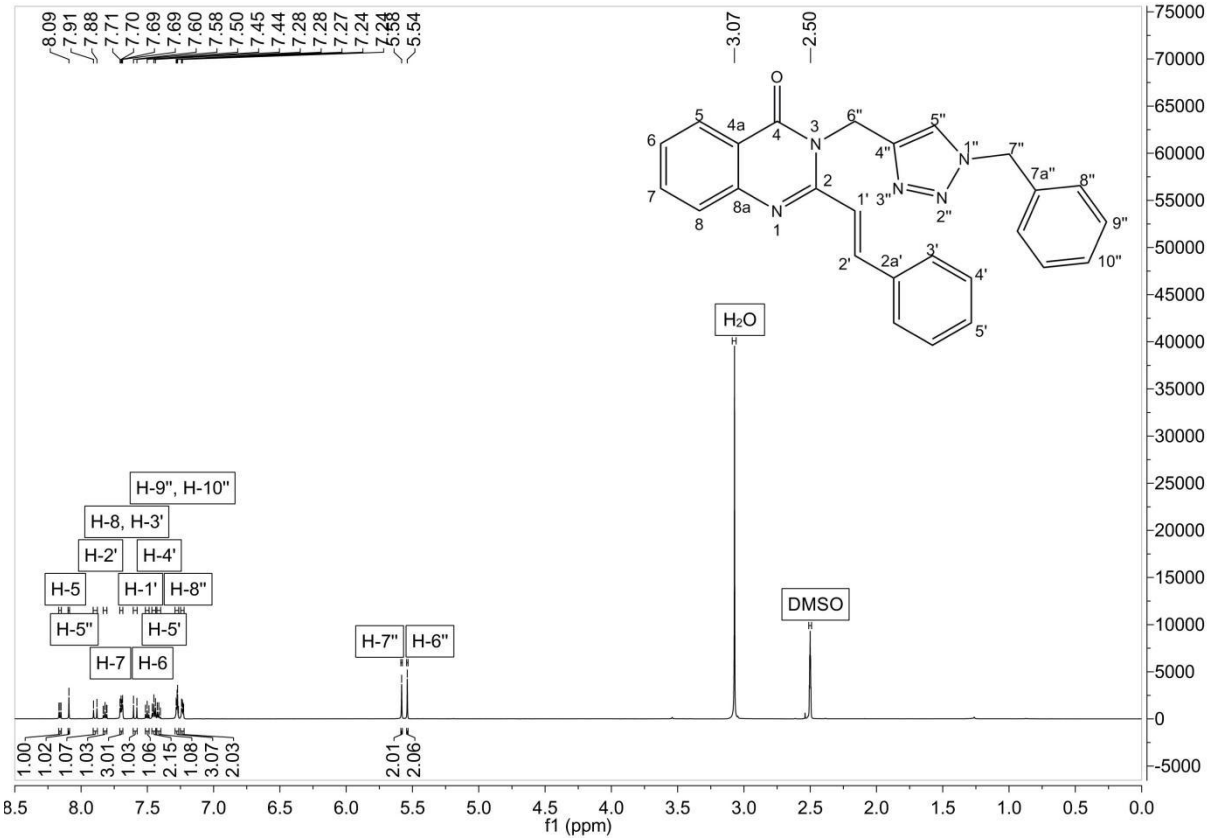
HRMS

Acquisition Parameter

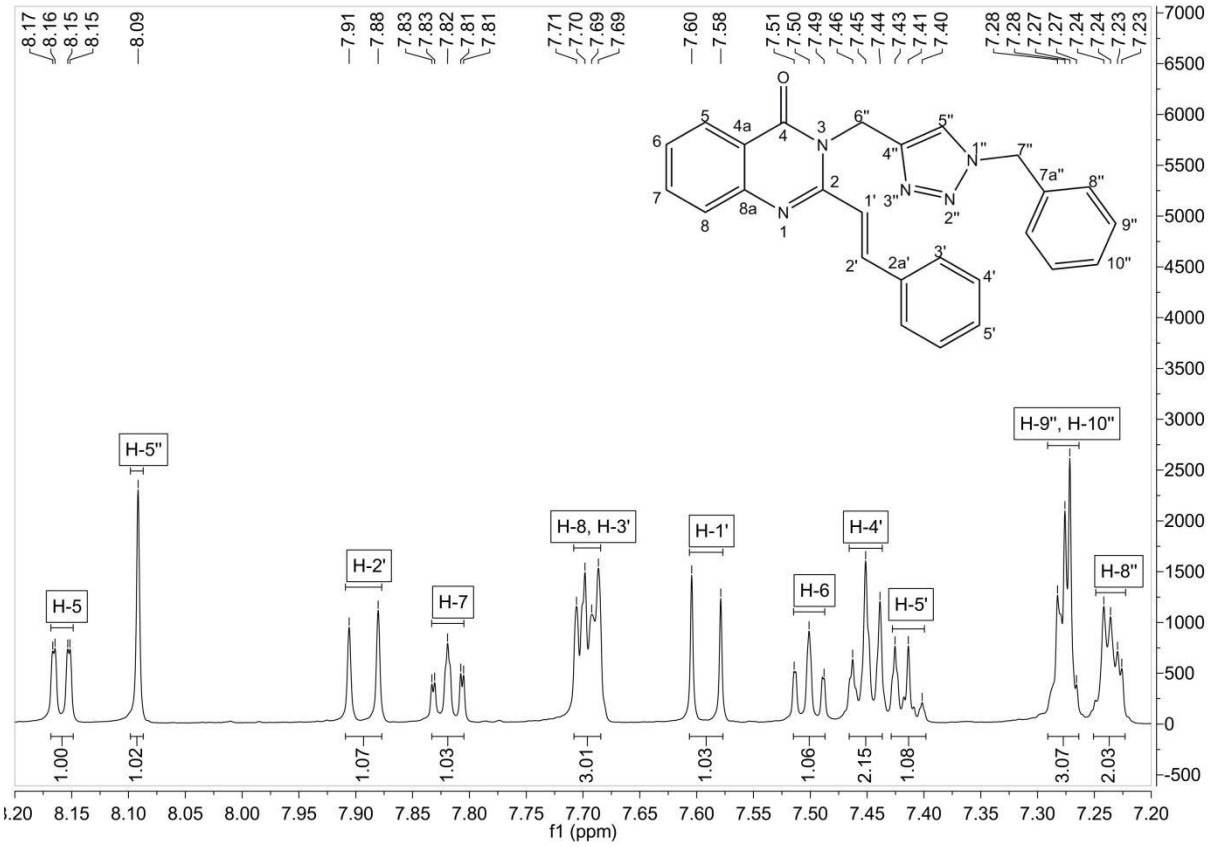
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



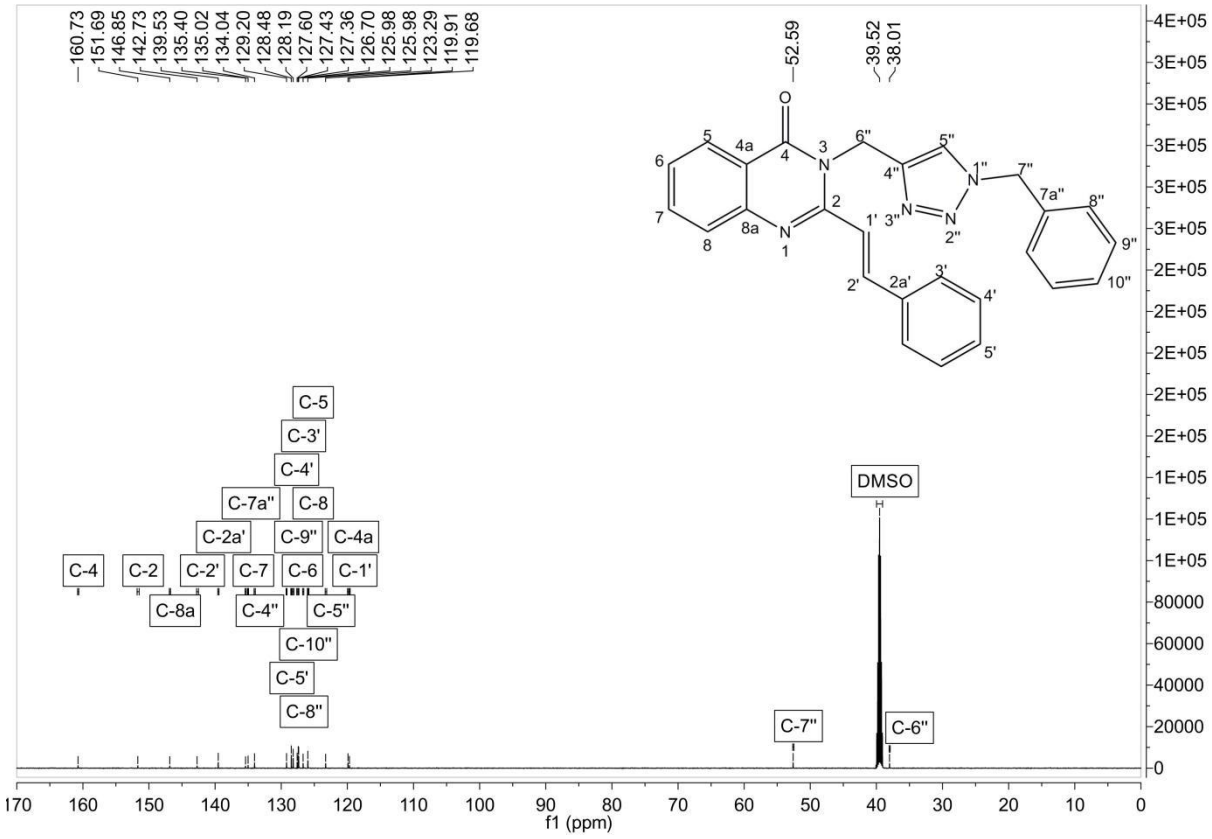
^{1}H NMR (DMSO at 80°C)



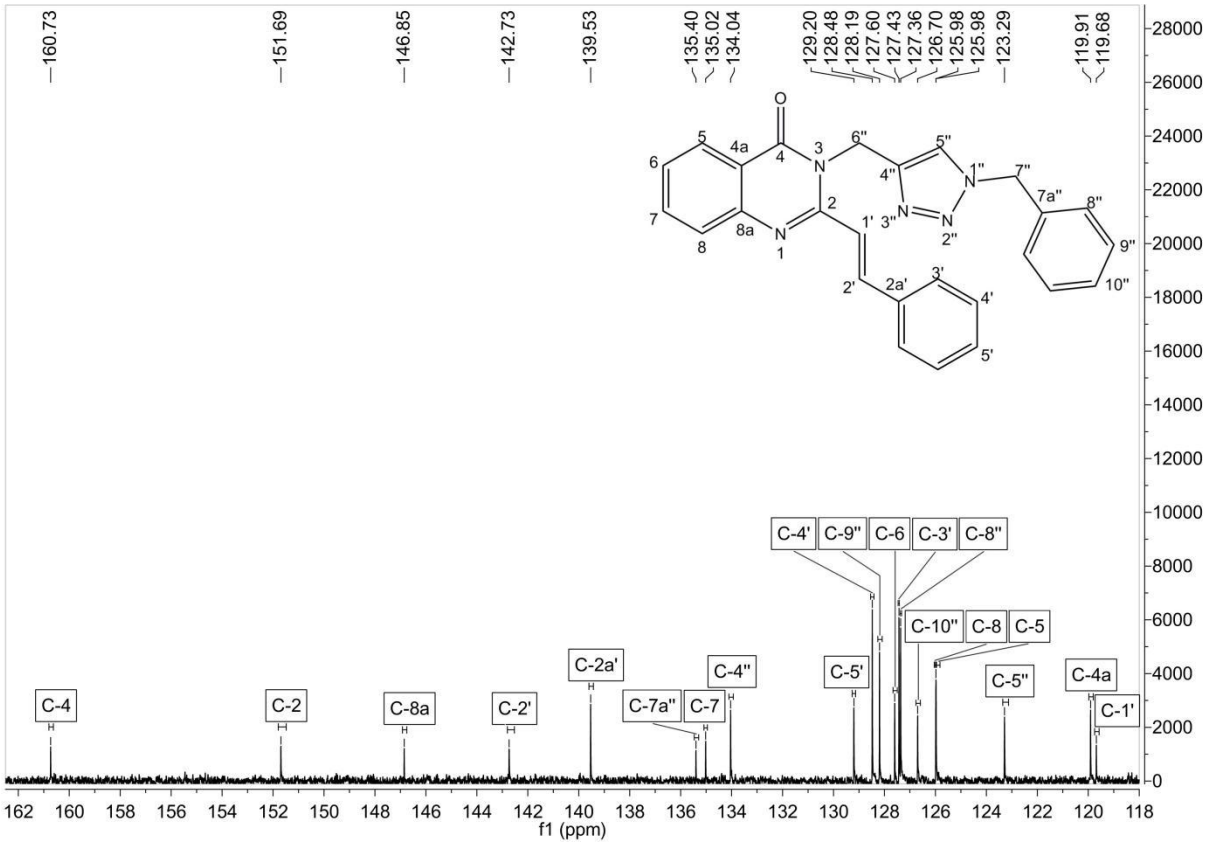
^{1}H NMR 8.20 - 7.20 ppm (DMSO at 80 °C)



^{13}C NMR (DMSO at 80°C)

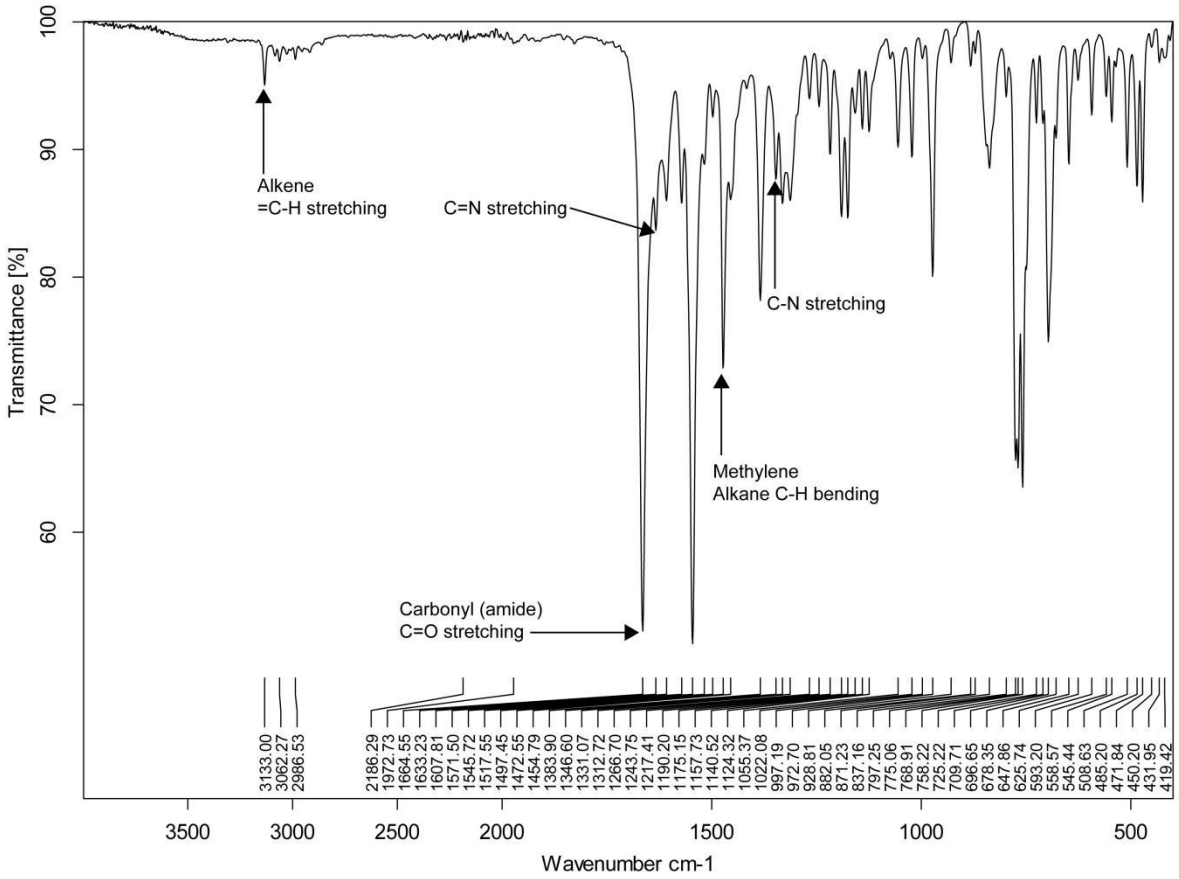


^{13}C NMR 162.50 - 118.00 ppm (DMSO at 80°C)



Compound 4e

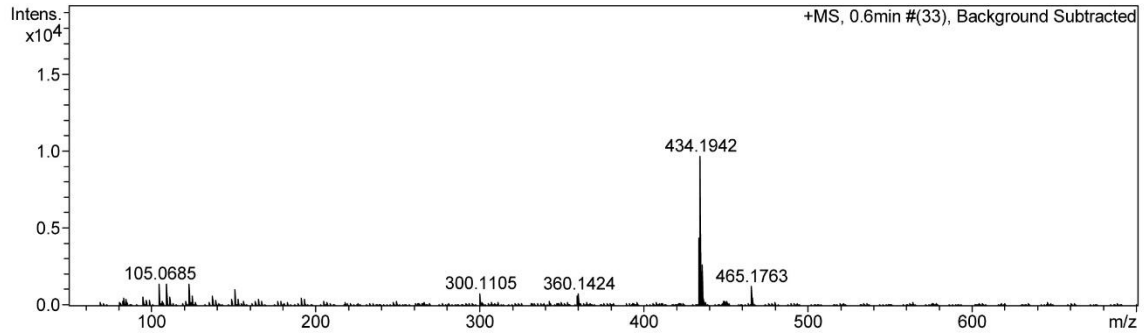
IR



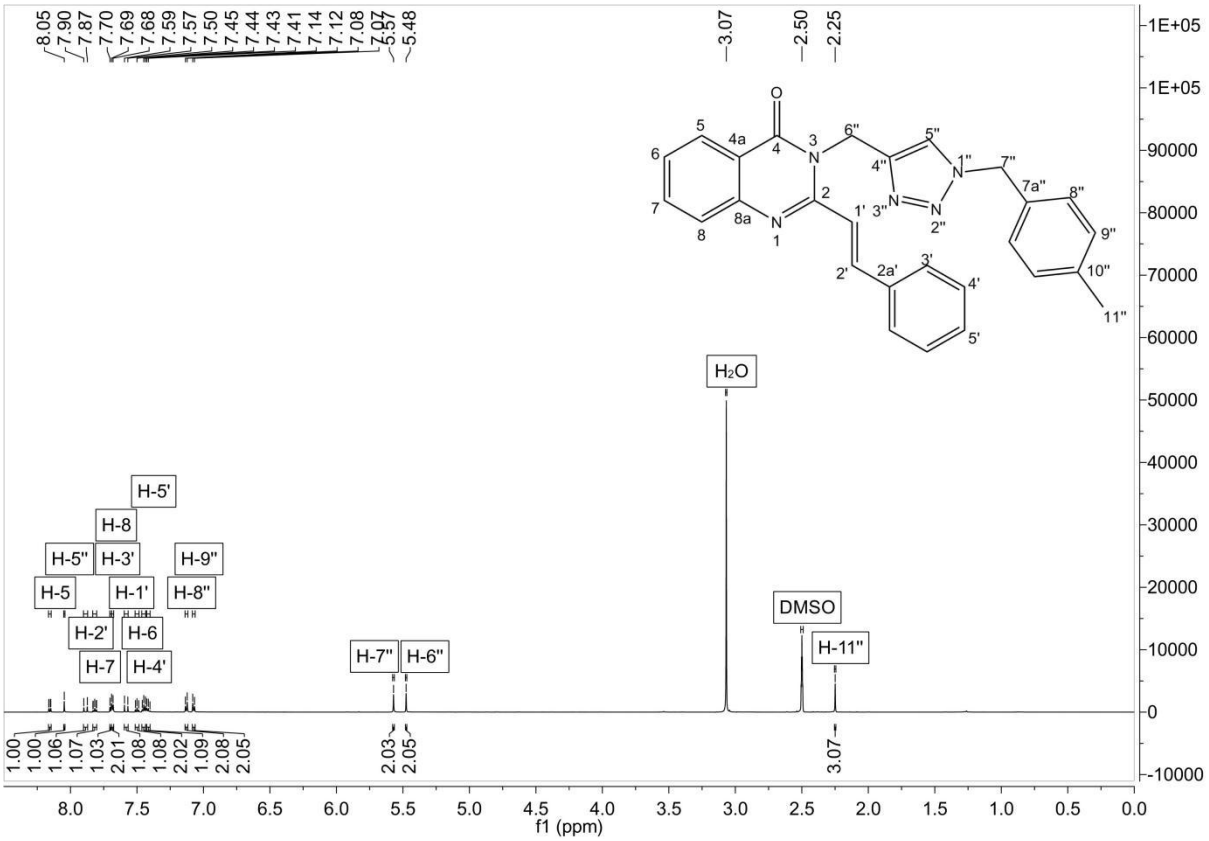
HRMS

Acquisition Parameter

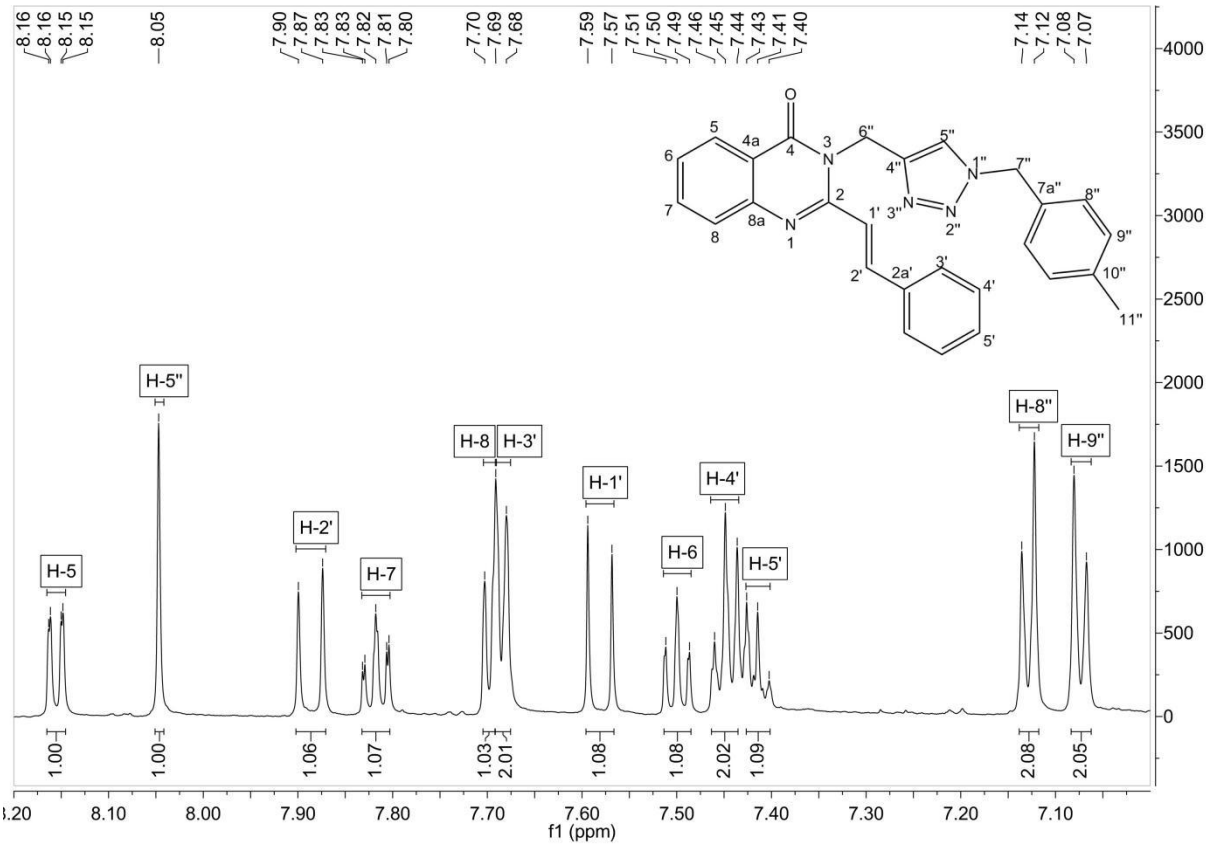
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



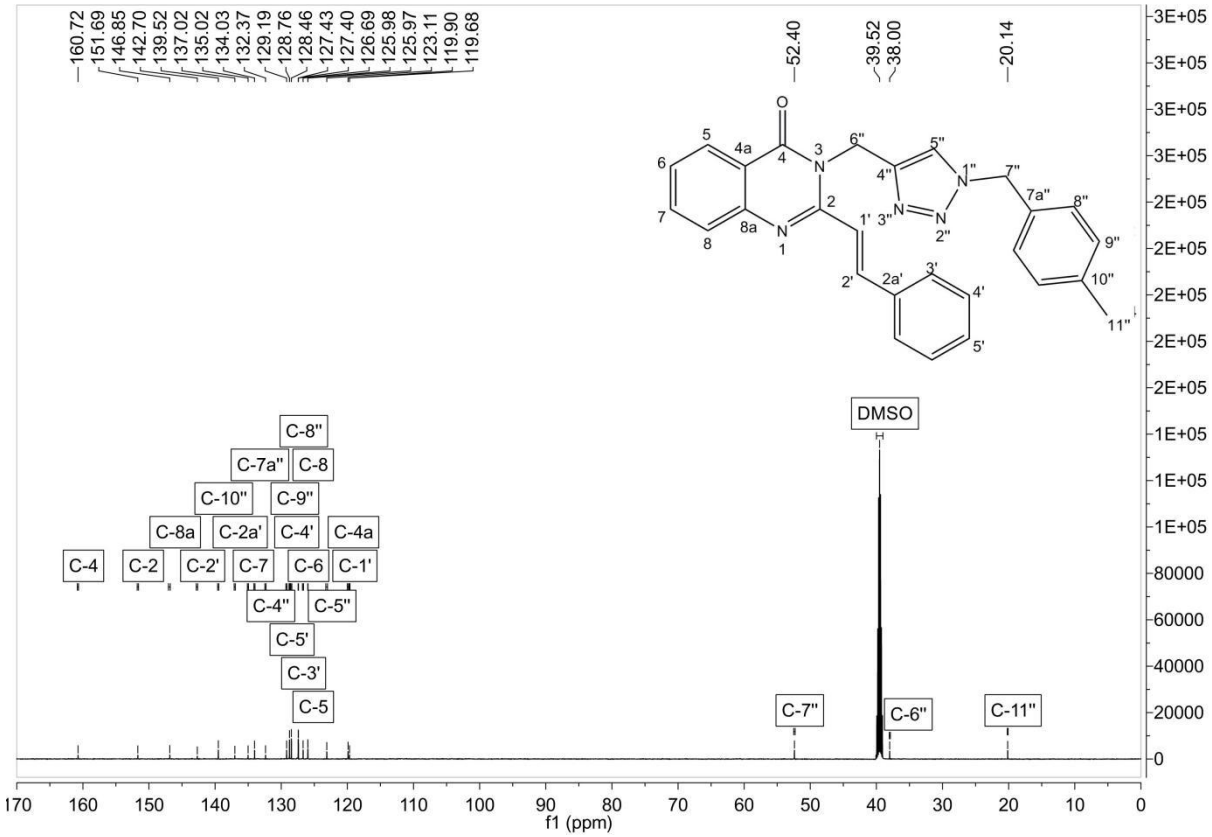
^{1}H NMR (DMSO at 80°C)



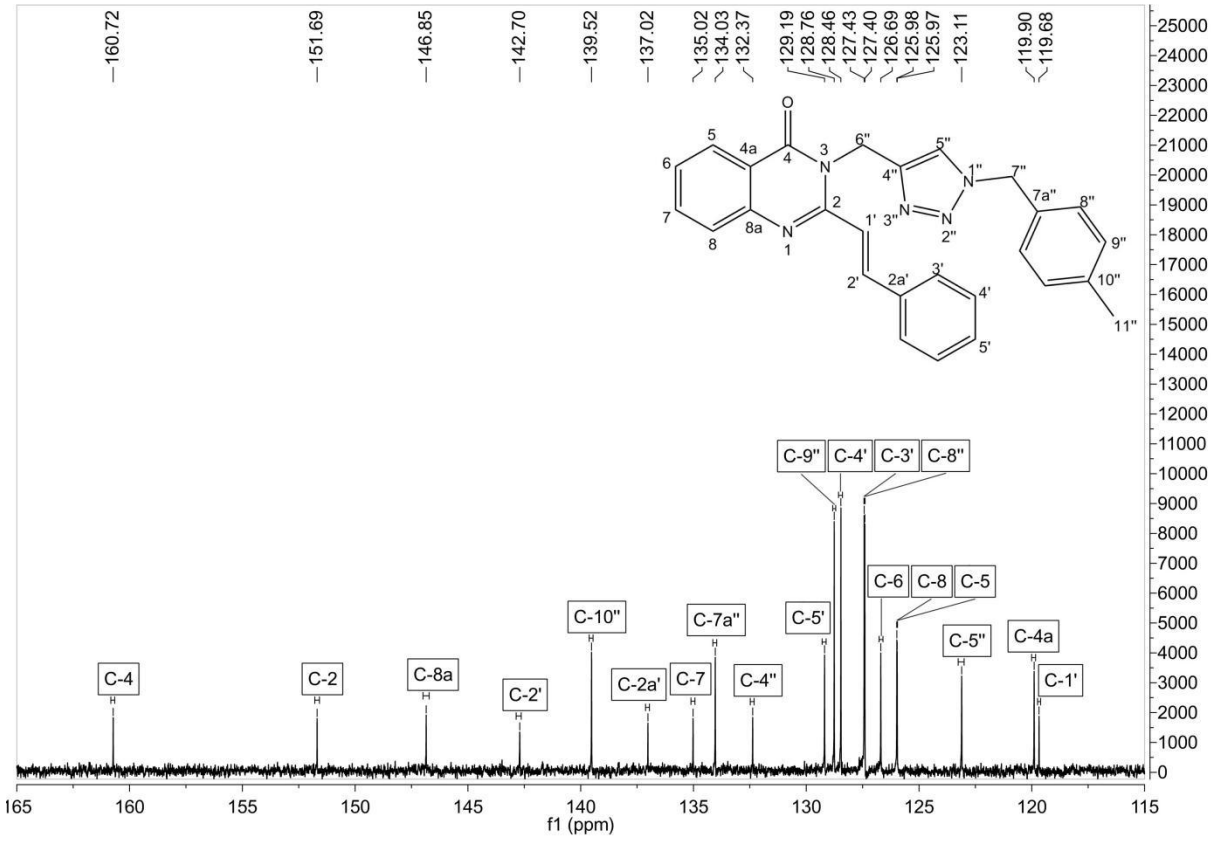
^{1}H NMR 8.20 - 7.00 ppm (DMSO at 80°C)



^{13}C NMR (DMSO at 80°C)

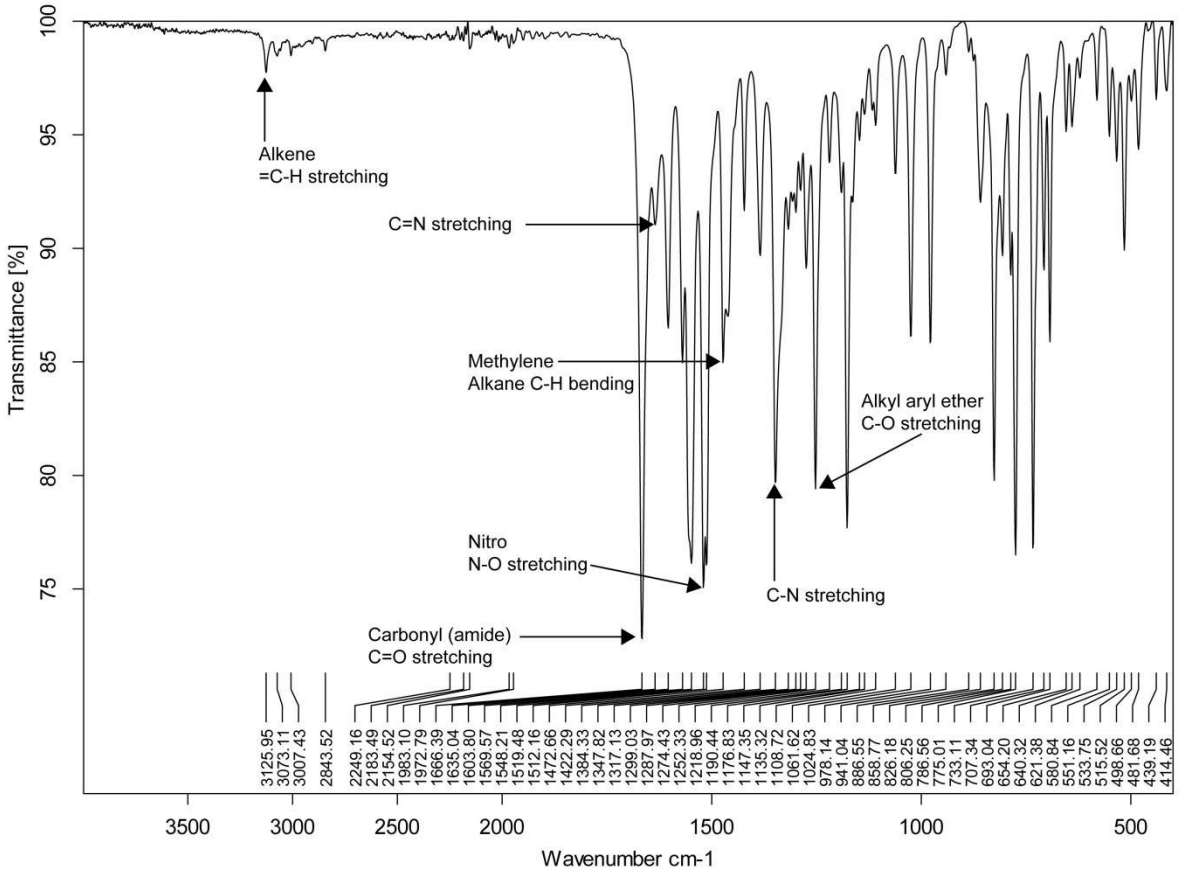


^{13}C NMR 165.00 - 115.00 ppm (DMSO at 80°C)



Compound 4f

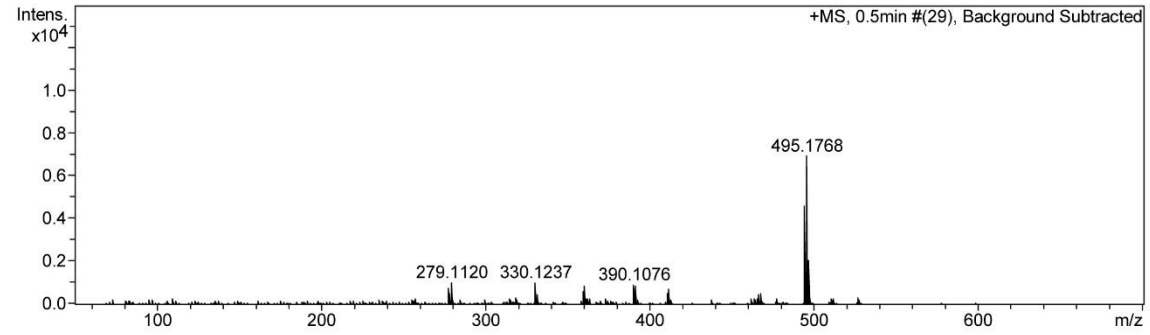
IR



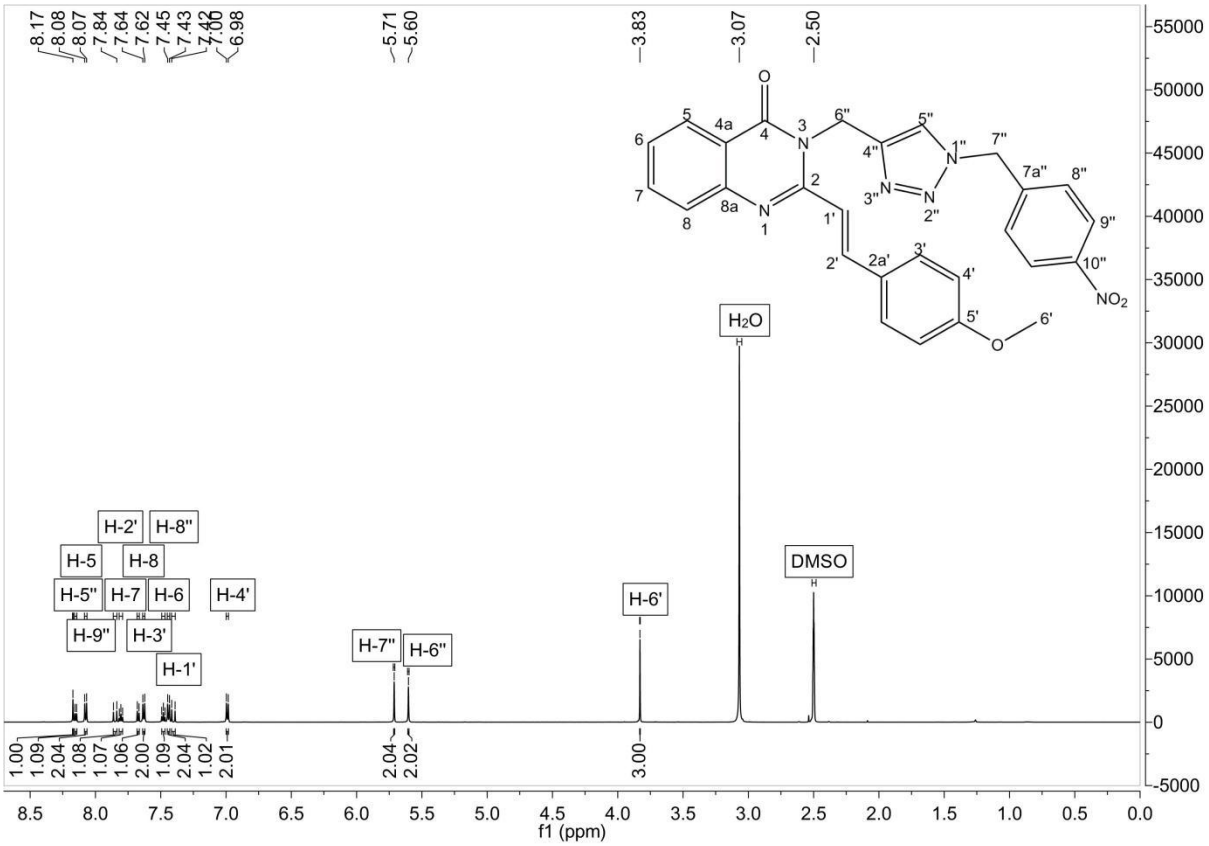
HRMS

Acquisition Parameter

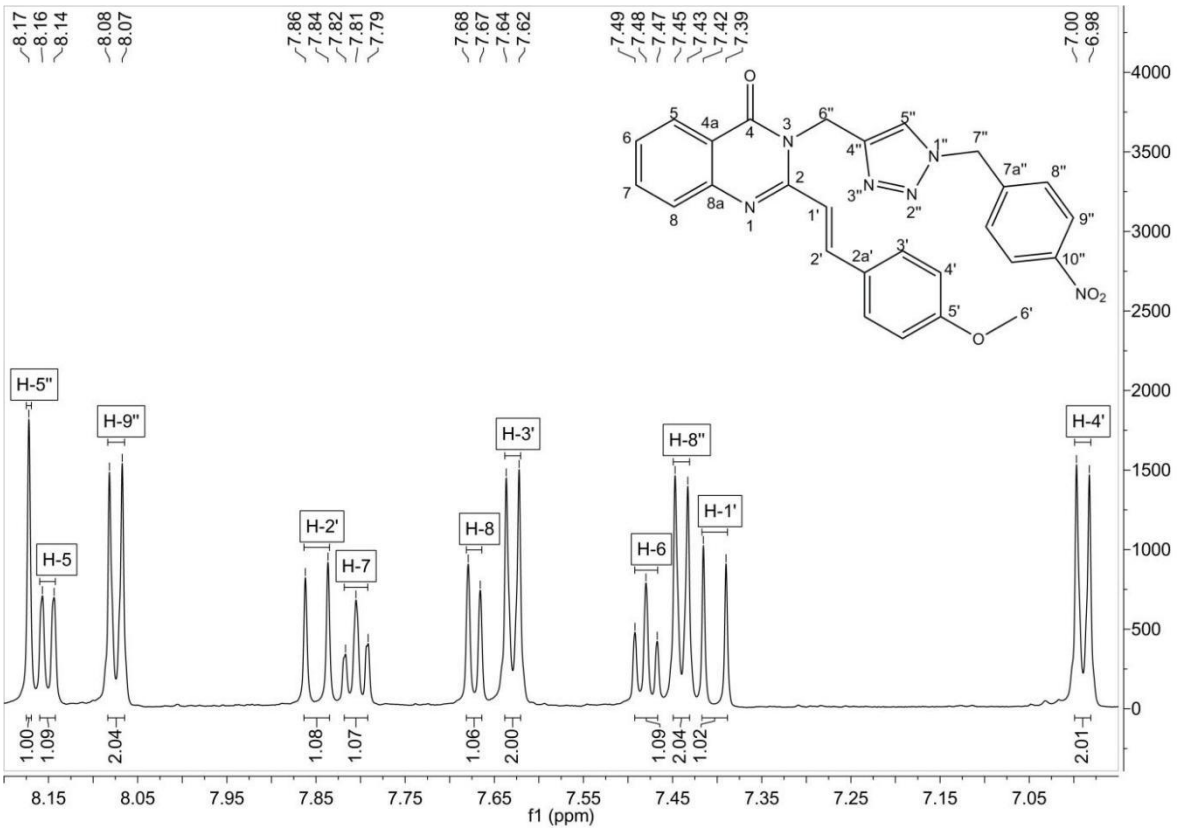
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



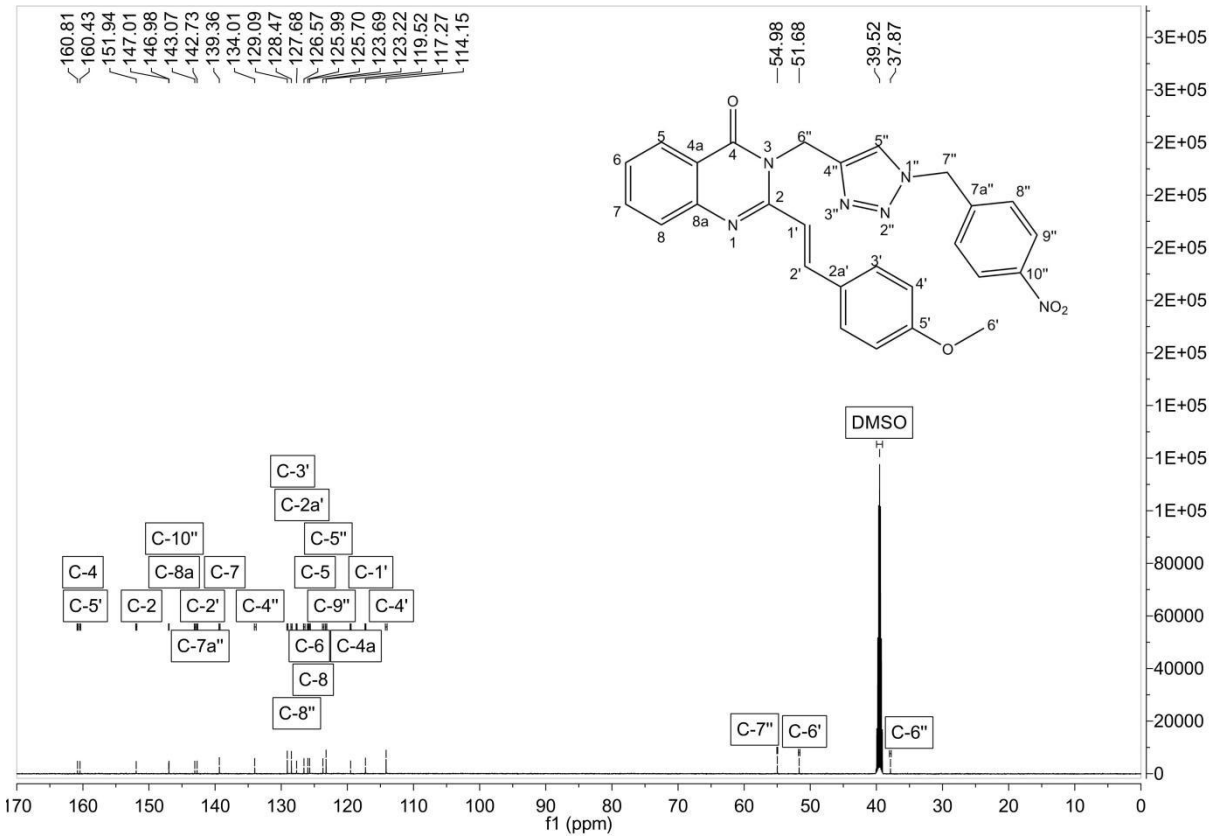
^{1}H NMR (DMSO at 80°C)



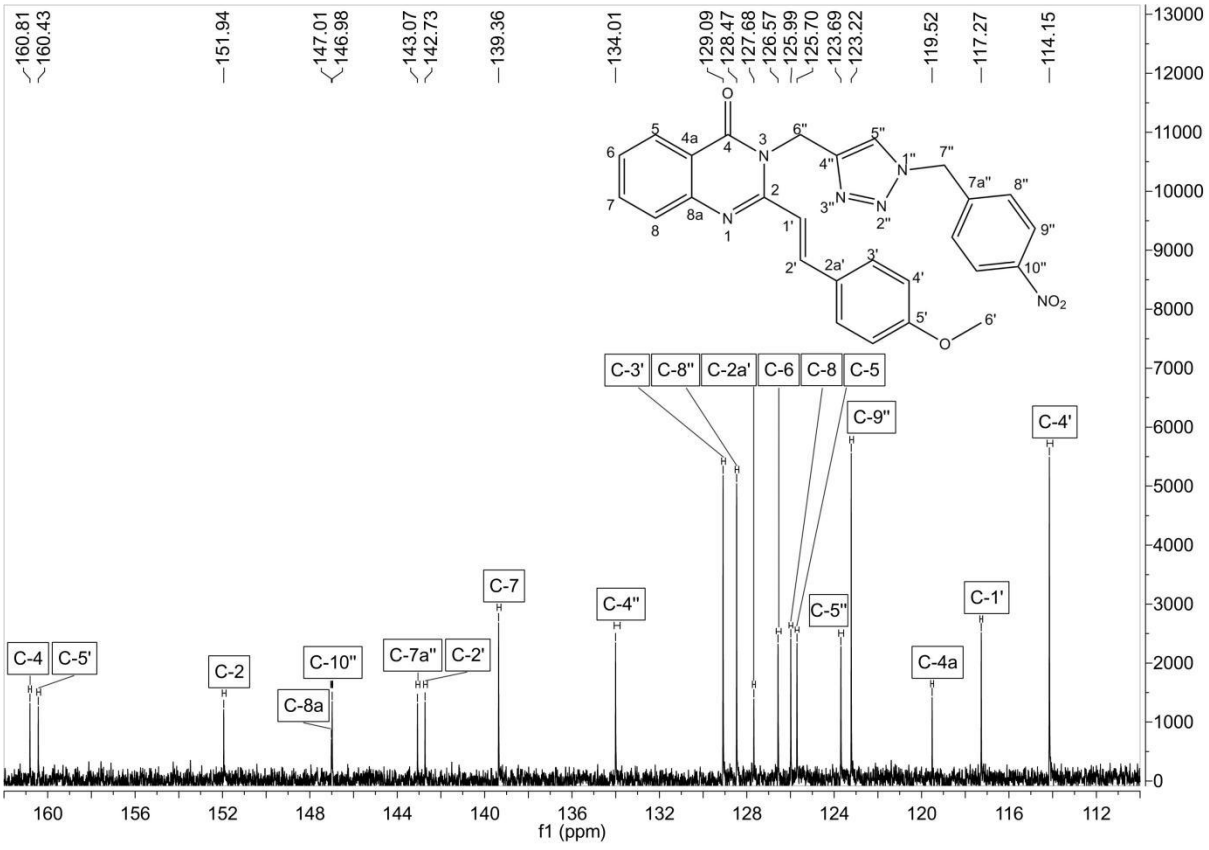
^{1}H NMR 8.20 - 6.95 ppm (DMSO at 80°C)



^{13}C NMR (DMSO at 80°C)

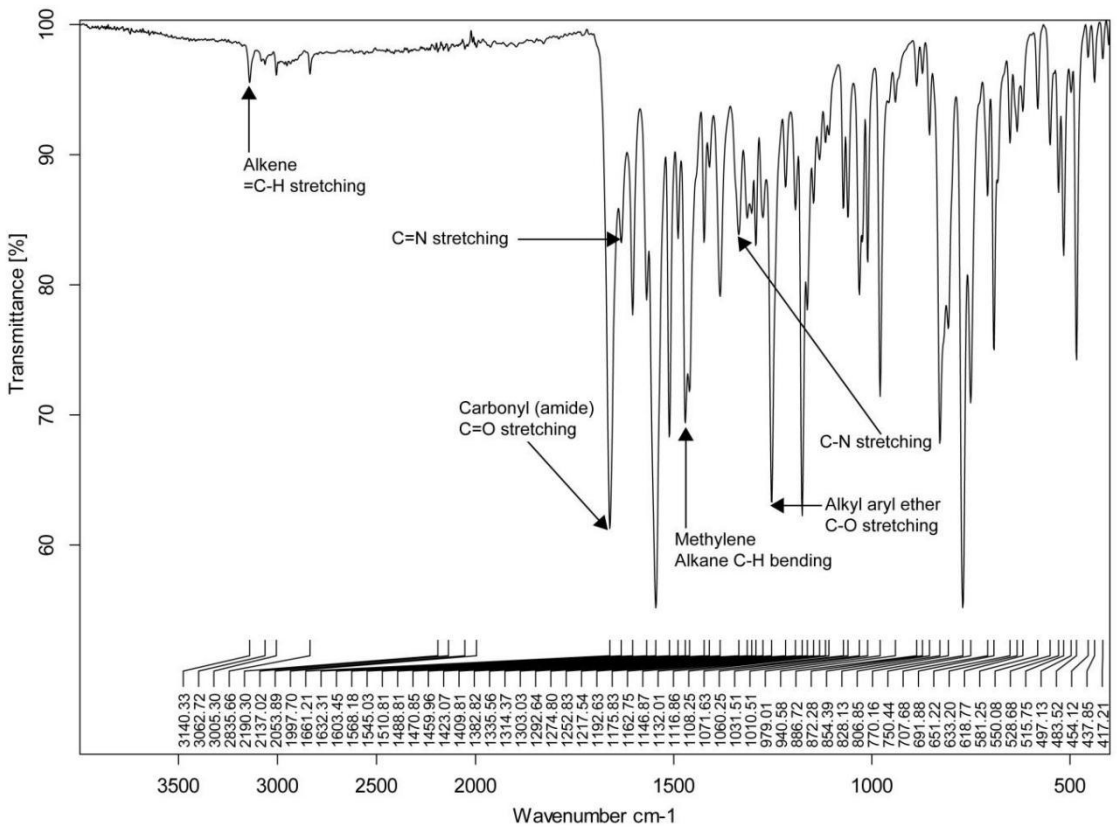


^{13}C NMR 162.00 - 110.00 ppm (DMSO at 80°C)



Compound 4g

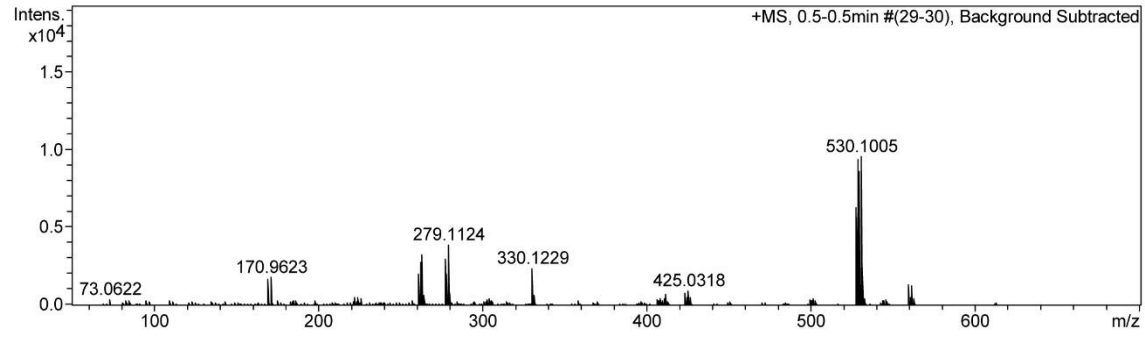
IR

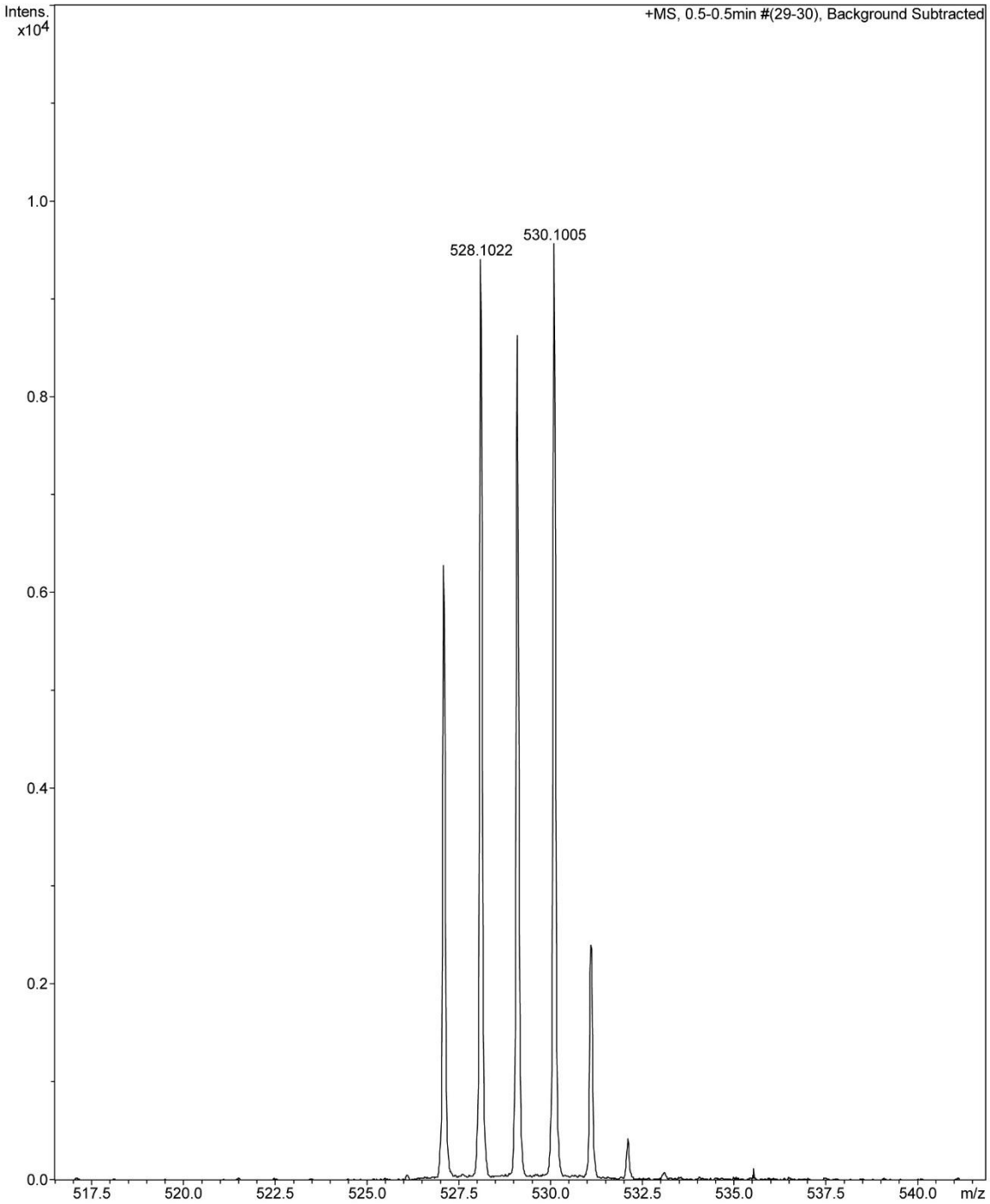


HRMS

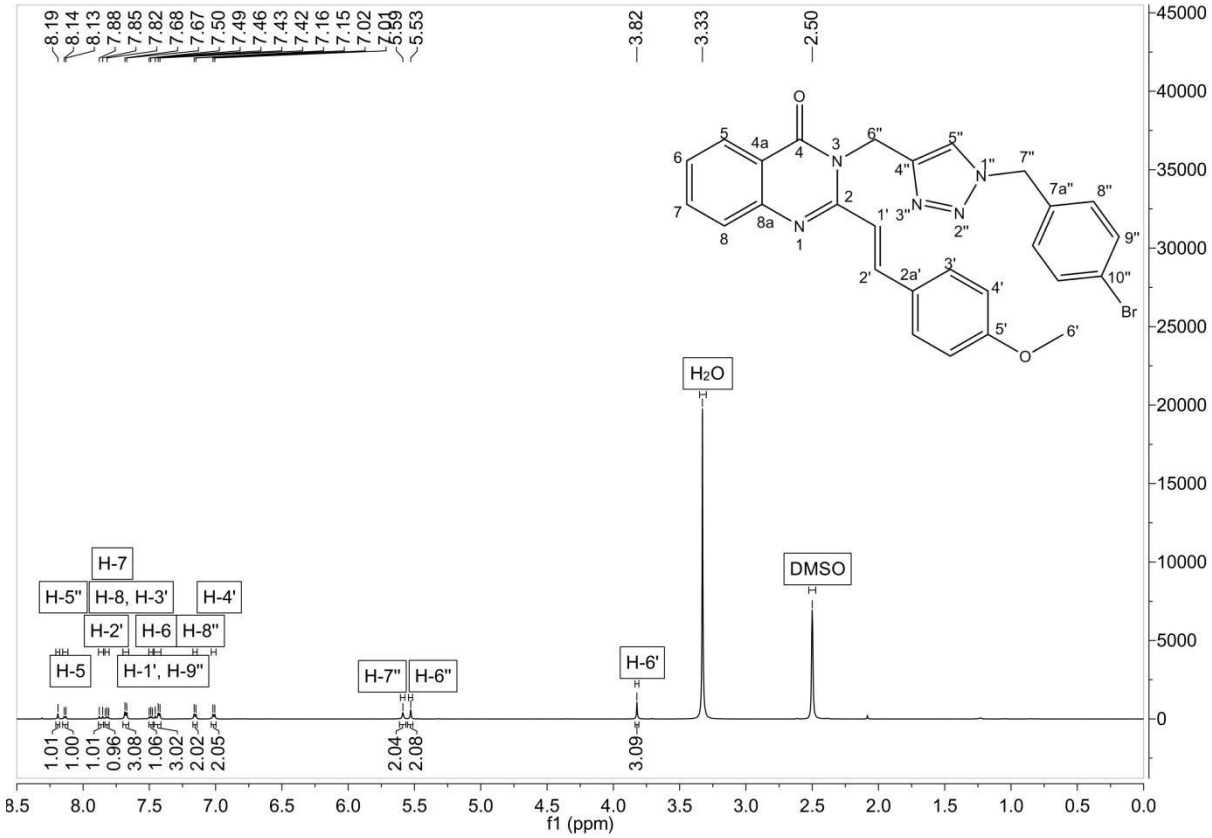
Acquisition Parameter

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

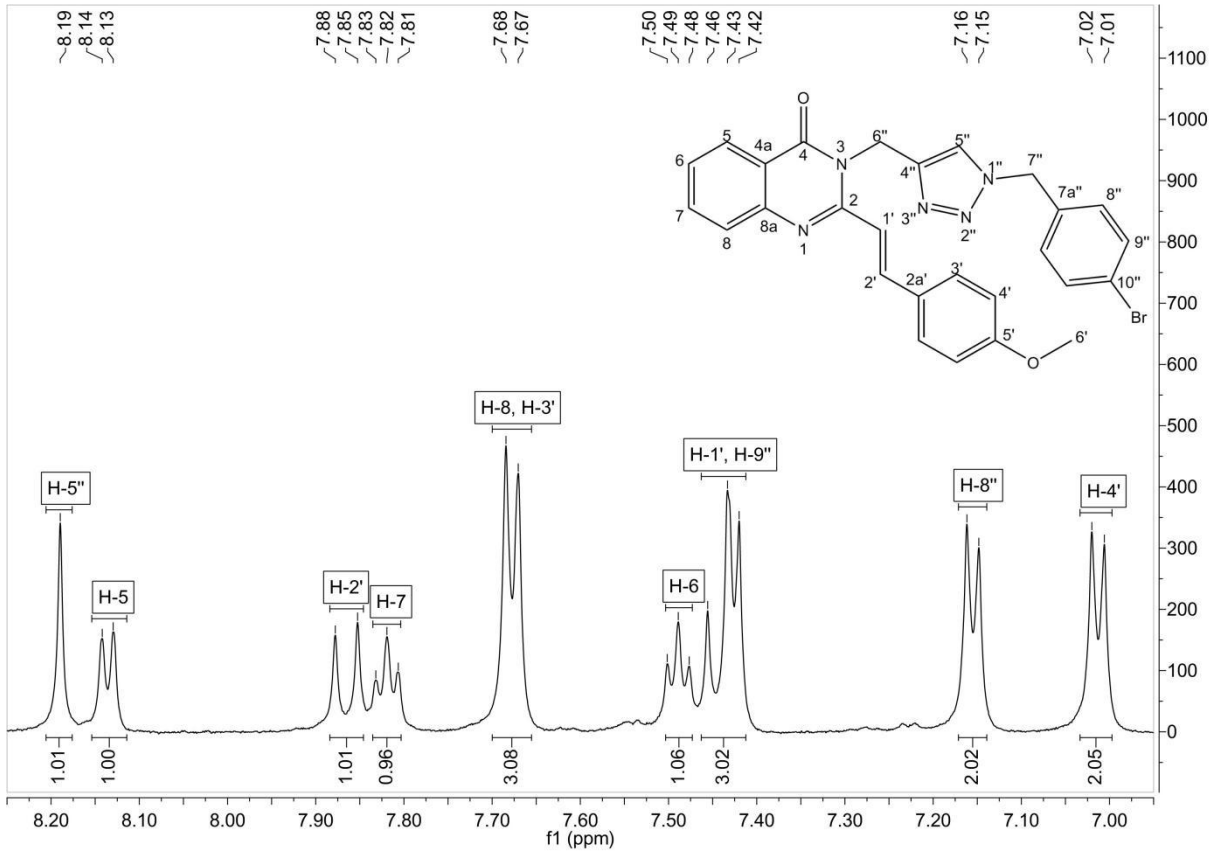



Intens.
x10^4
+MS, 0.5-0.5min #(29-30), Background Subtracted
1.0
0.8
0.6
0.4
0.2
0.0
528.1022
530.1005
517.5
520.0
522.5
525.0
527.5
530.0
532.5
535.0
537.5
540.0
m/z

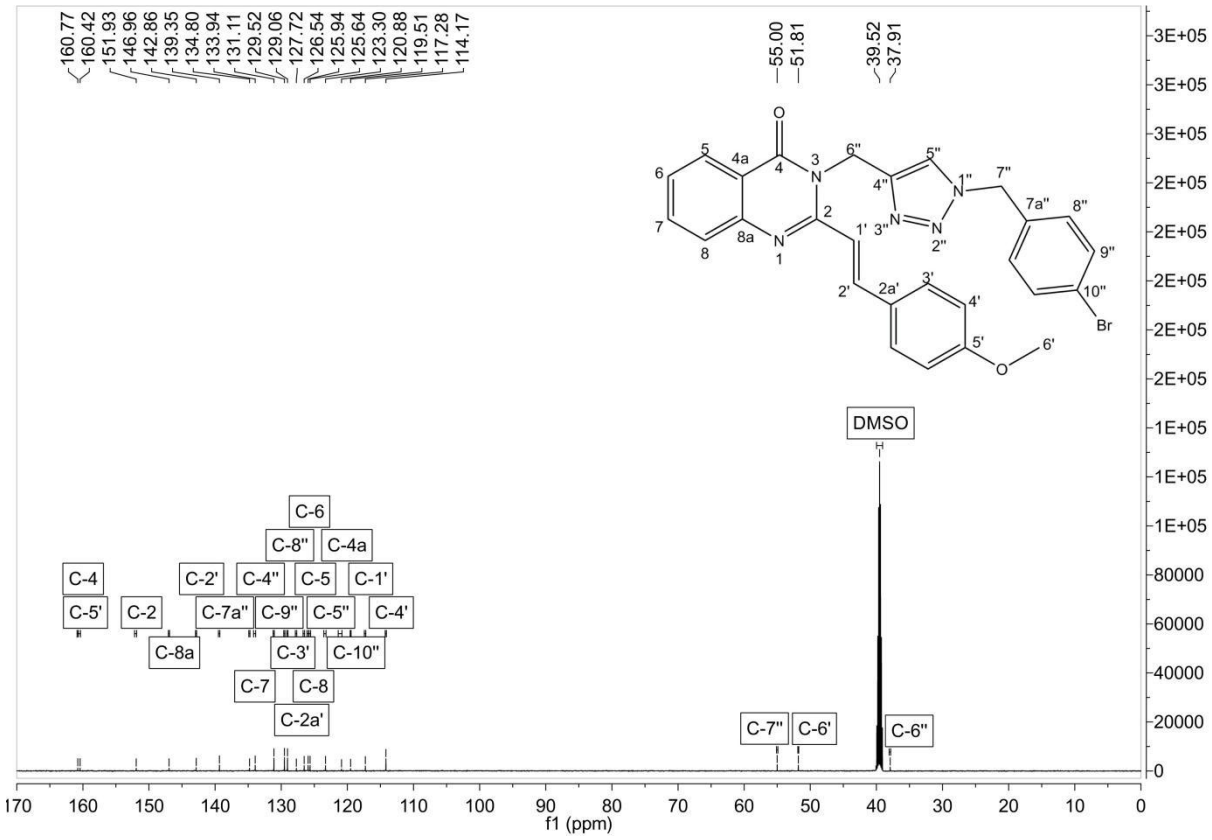
^{1}H NMR (DMSO at ambient temperature, ~25 °C)



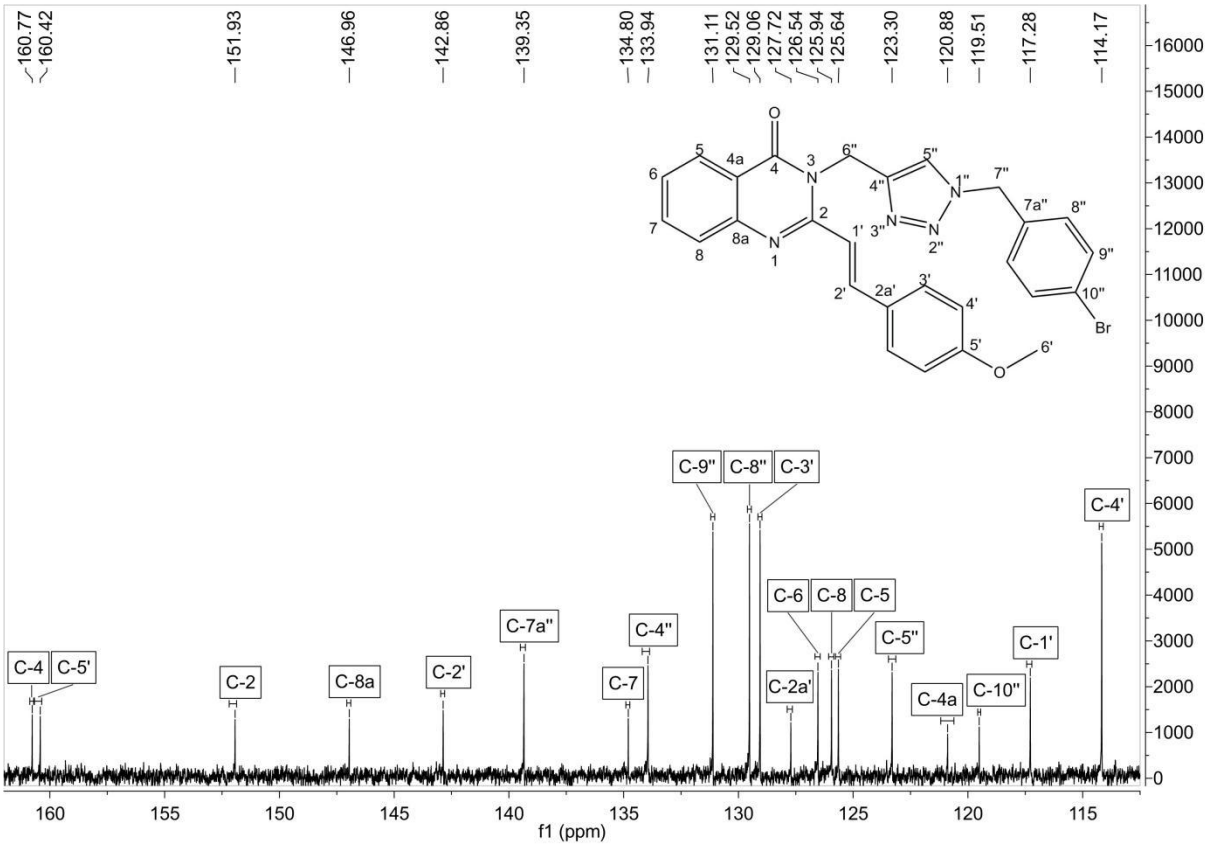
^{1}H NMR 8.25 - 6.95 ppm (DMSO at ambient temperature, ~25 °C)



^{13}C NMR (DMSO at ambient temperature, ~25 °C)

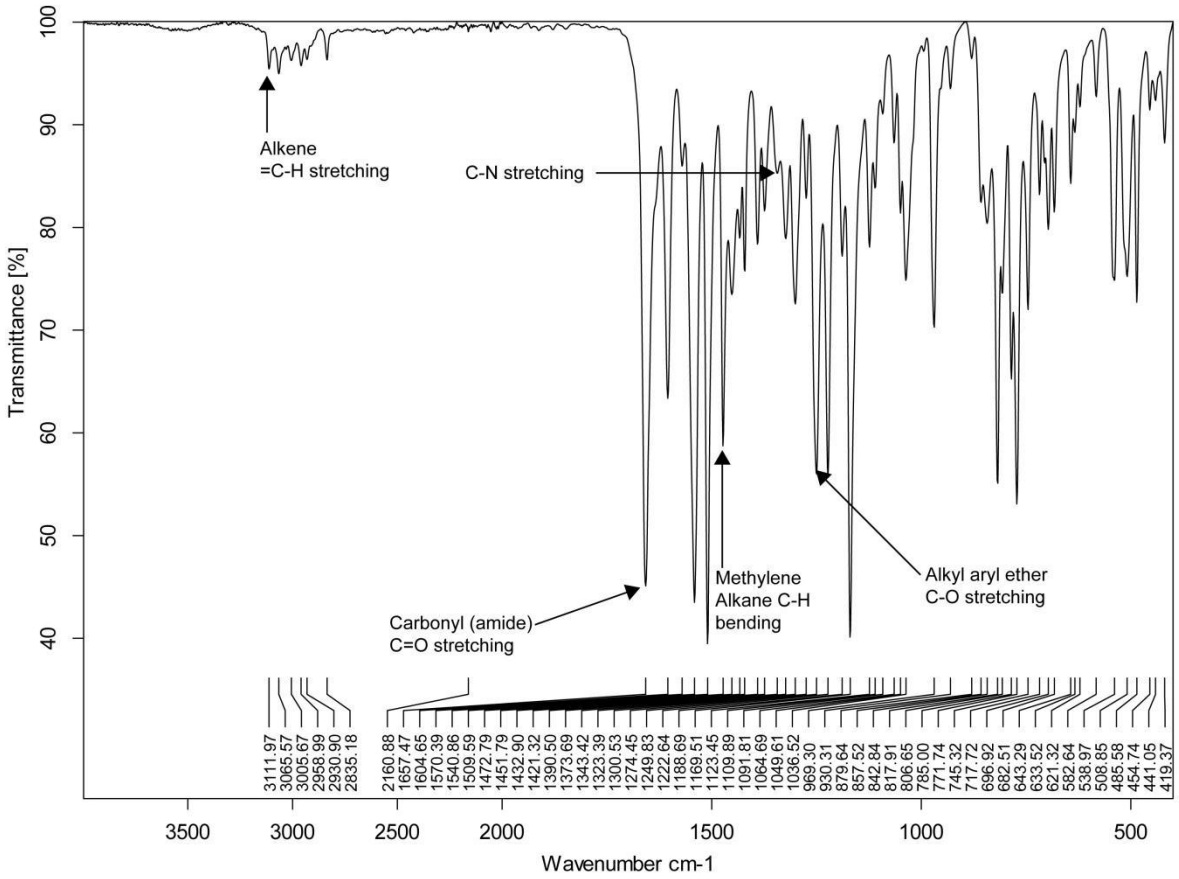


^{13}C NMR 162.00 - 112.50 ppm (DMSO at ambient temperature, ~25 °C)



Compound 4h

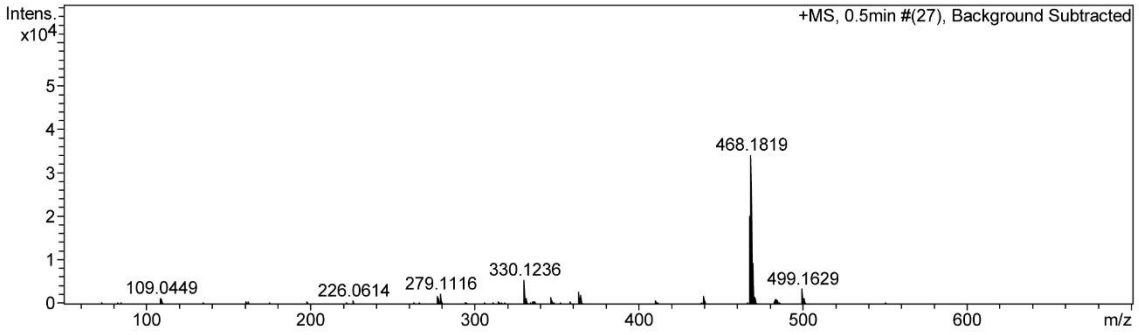
IR



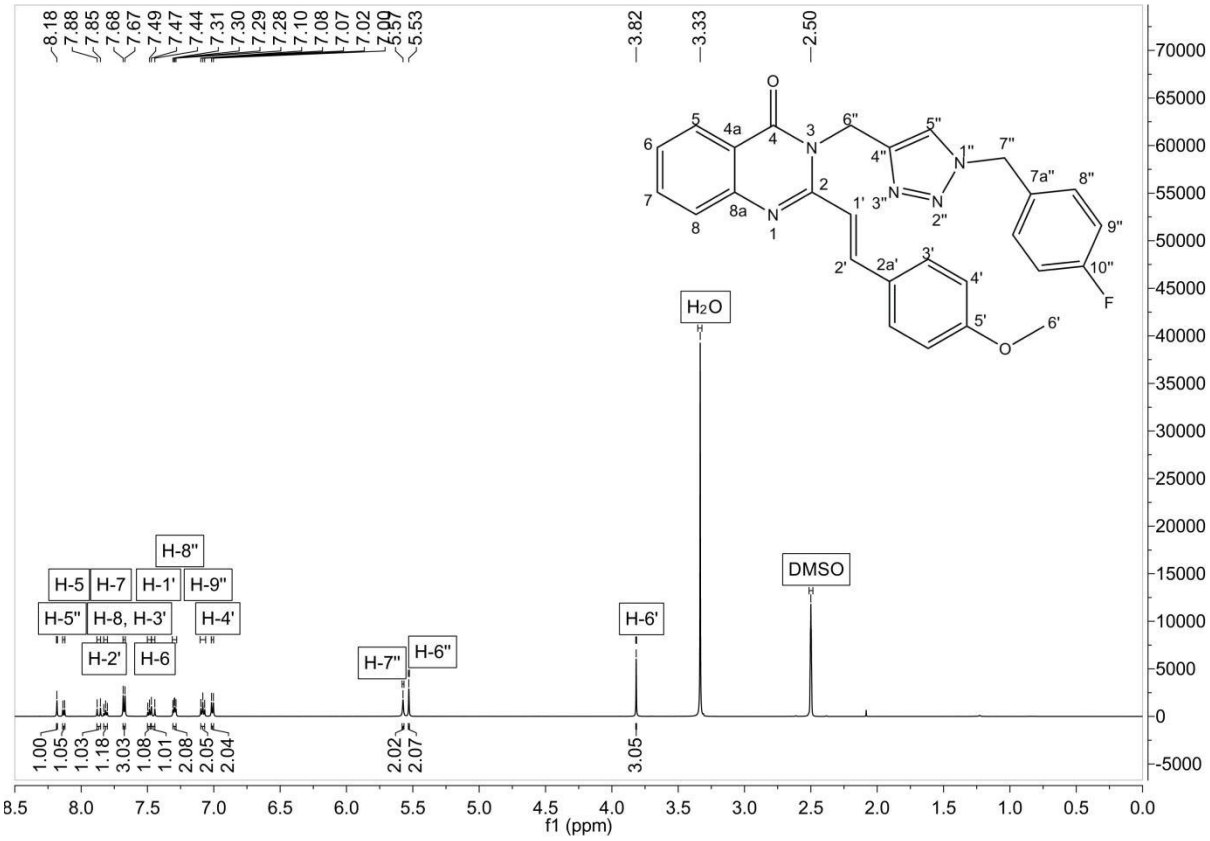
HRMS

Acquisition Parameter

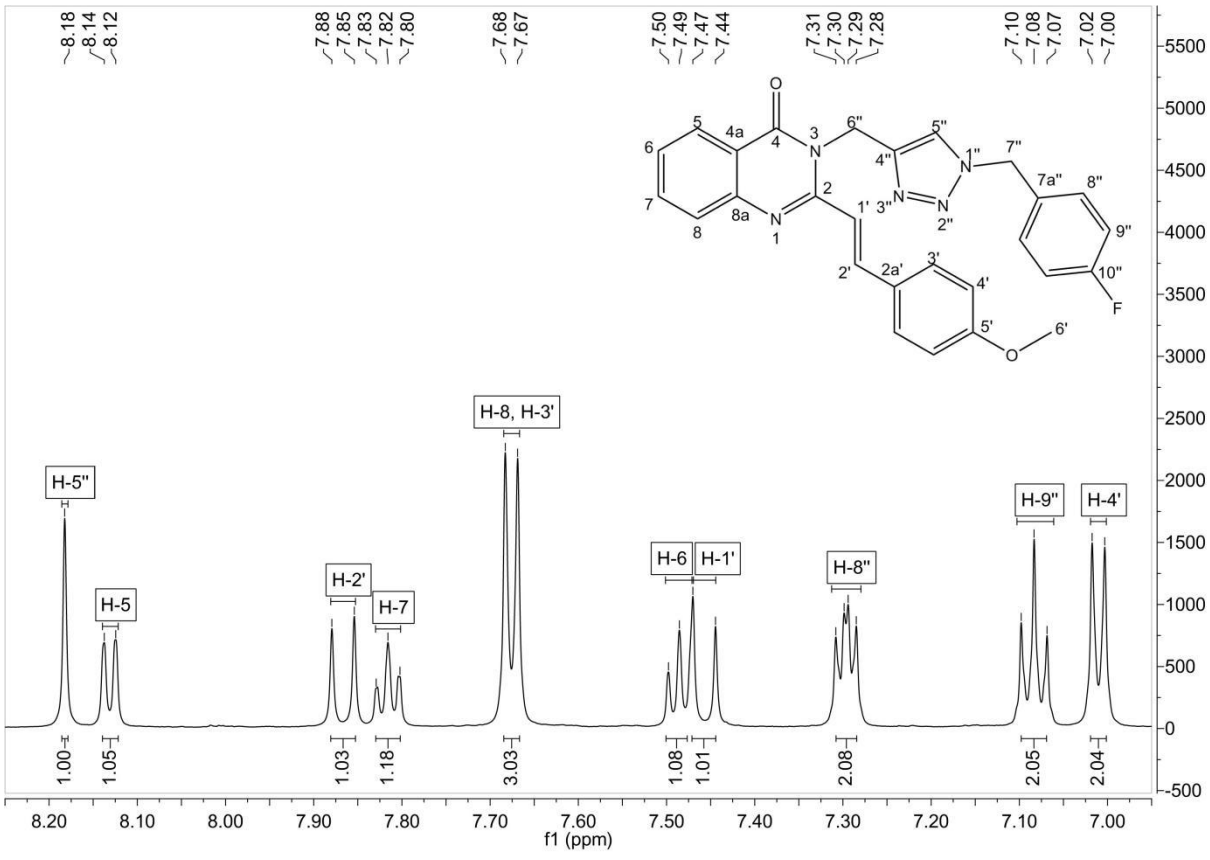
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



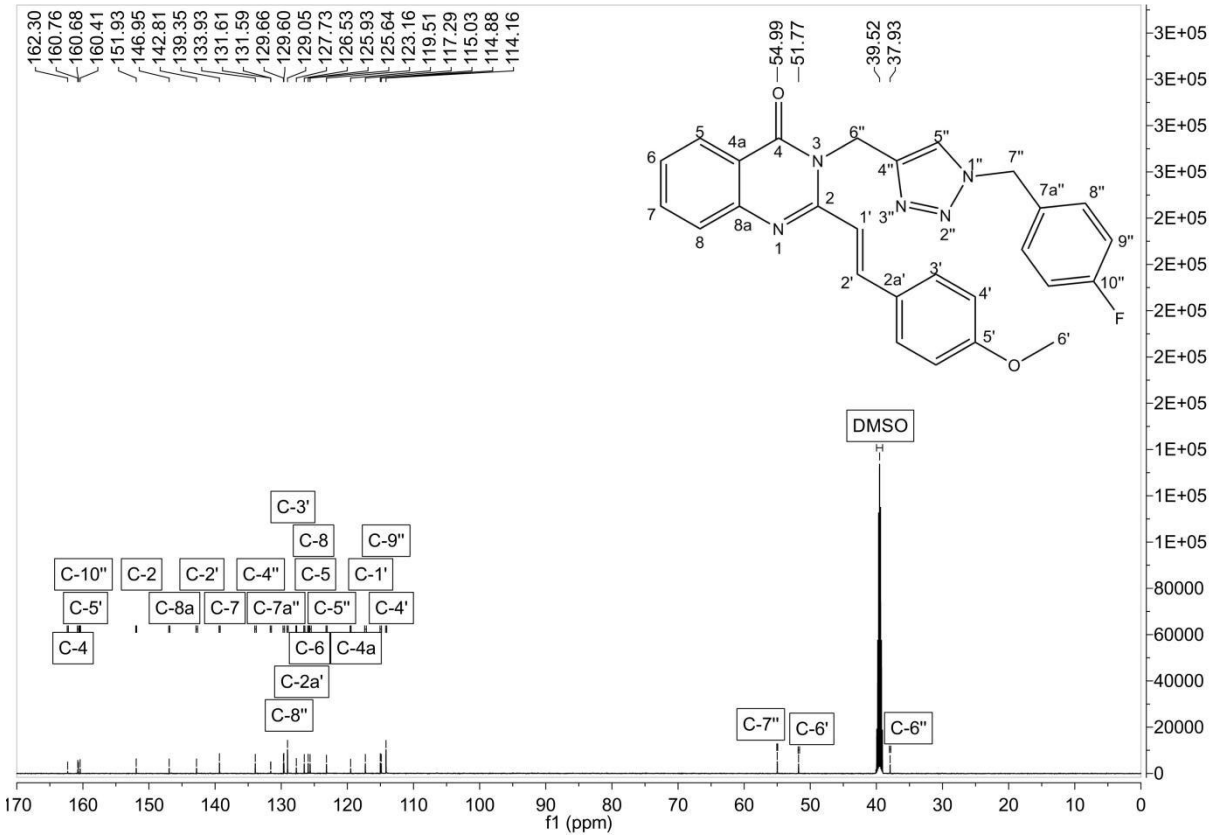
^{1}H NMR (DMSO at ambient temperature, ~25 °C)



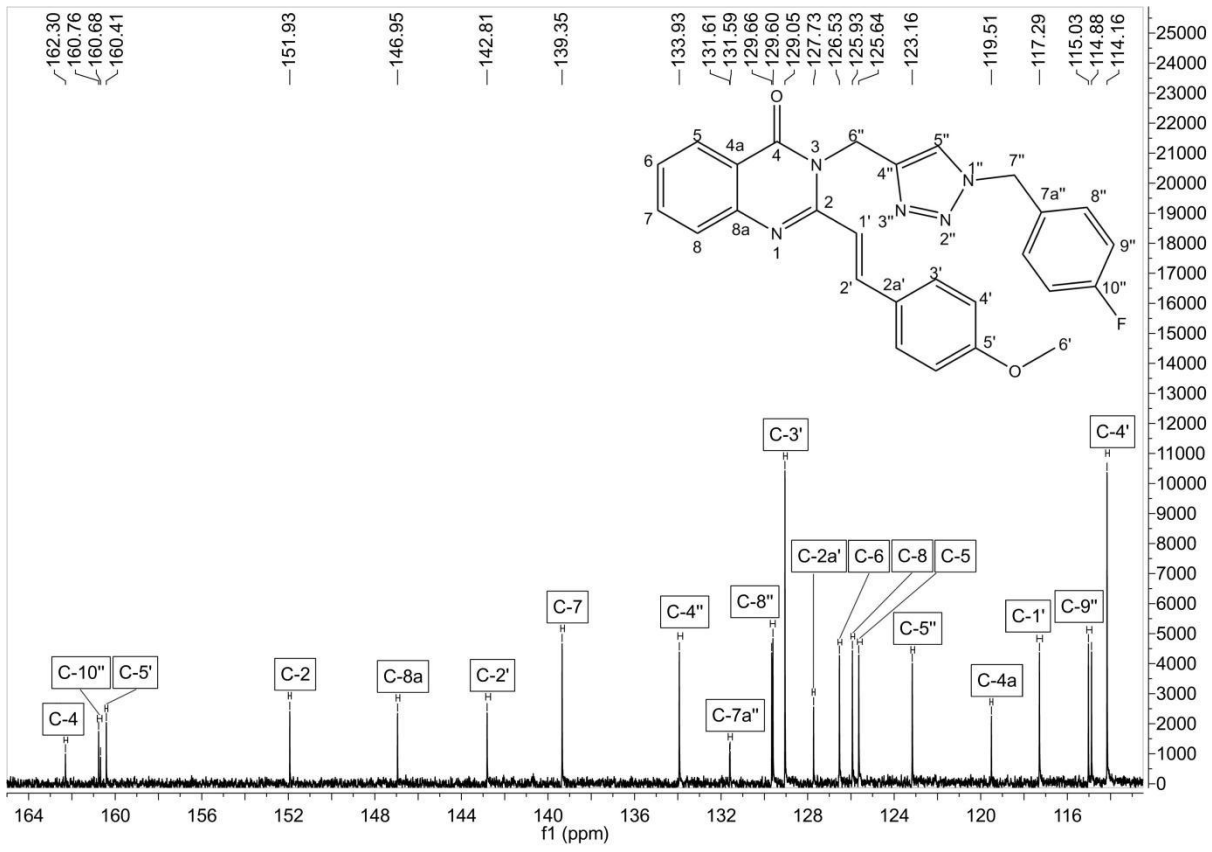
^{1}H NMR 8.25 - 6.95 ppm (DMSO at ambient temperature, ~25 °C)



^{13}C NMR (DMSO at ambient temperature, ~25 °C)

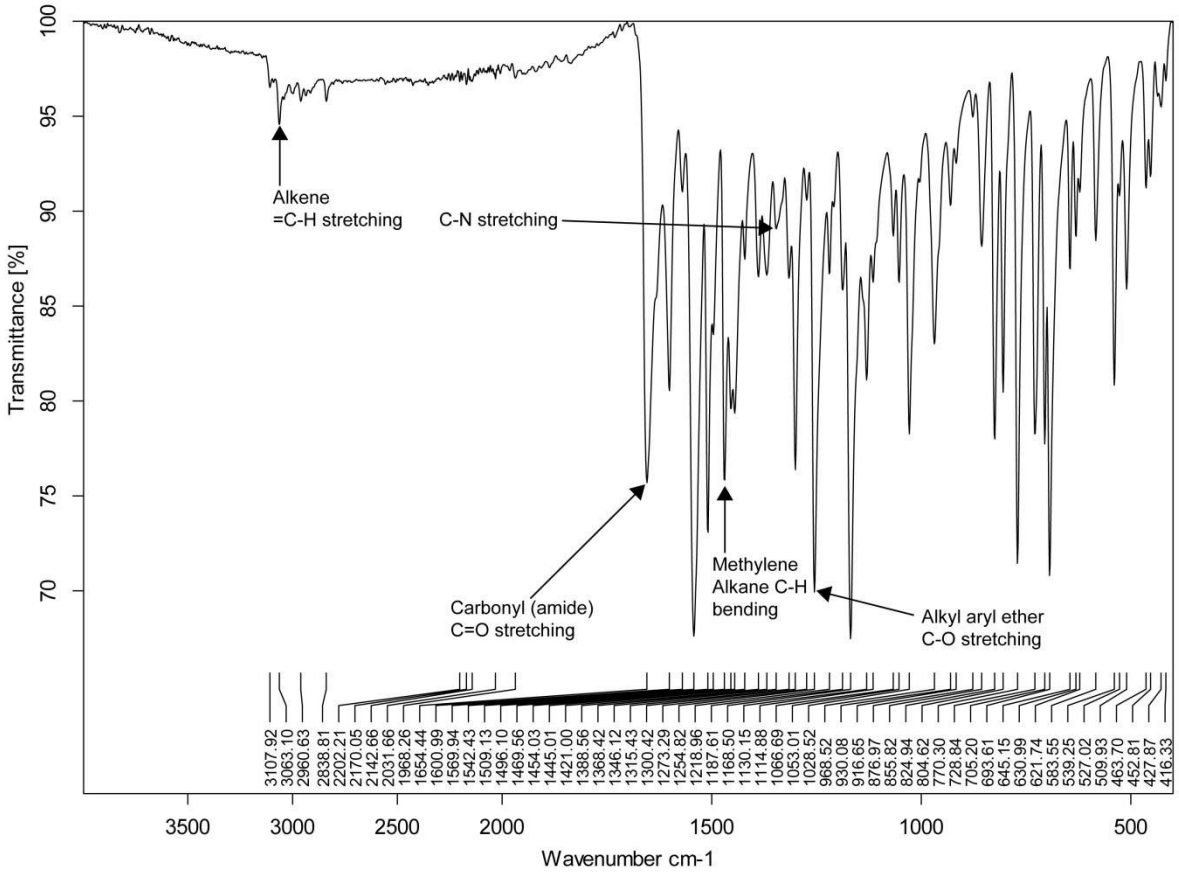


^{13}C NMR 165.00 - 112.50 ppm (DMSO at ambient temperature, ~25 °C)



Compound 4i

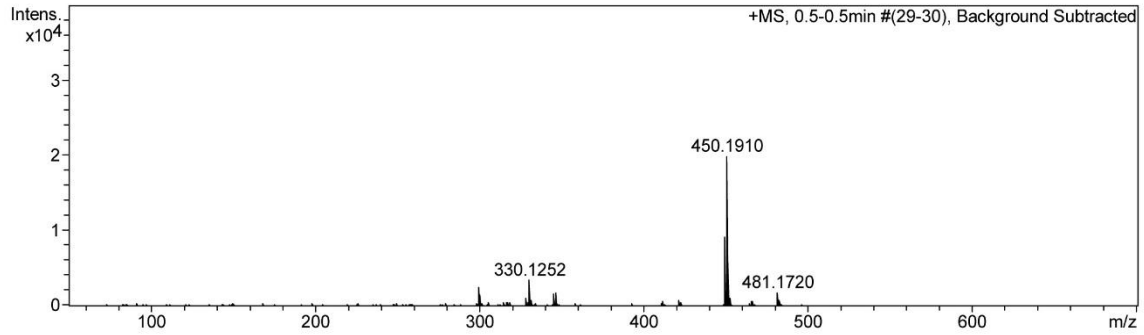
IR



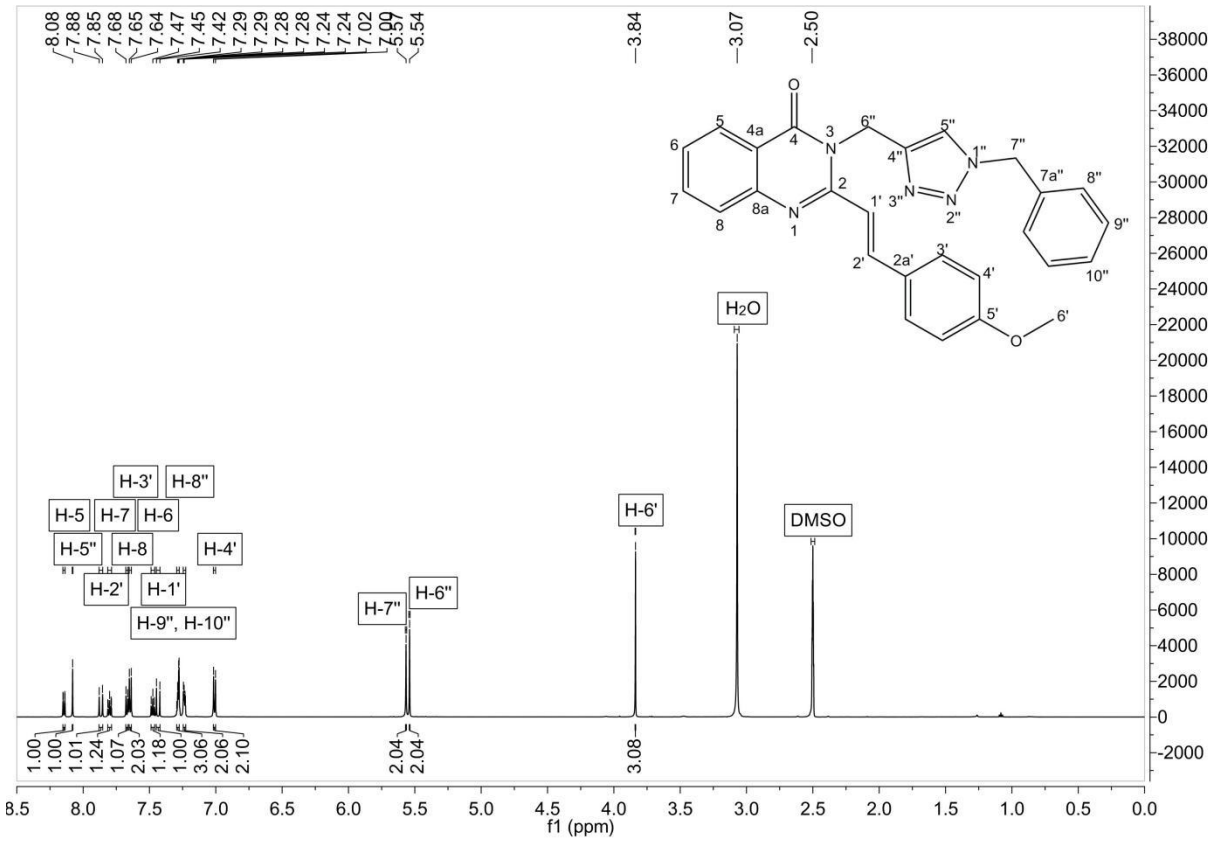
HRMS

Acquisition Parameter

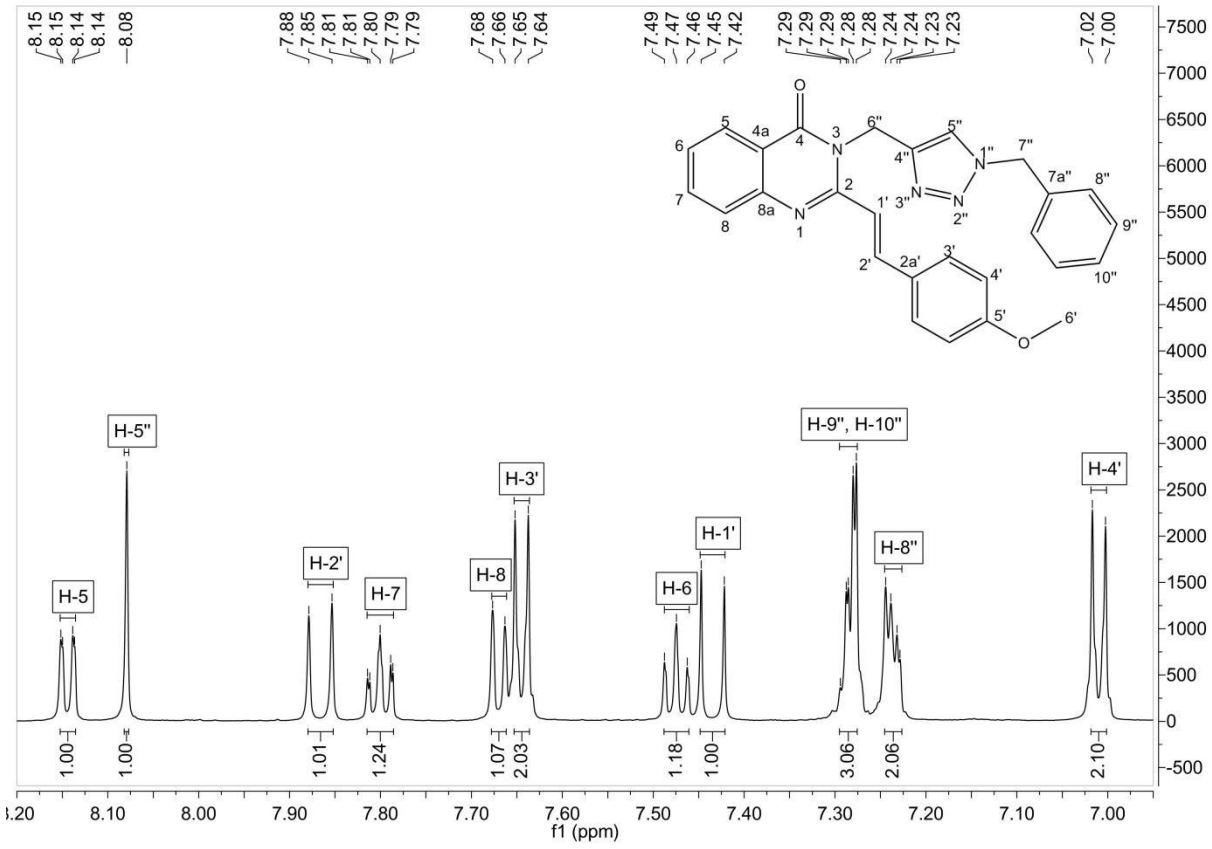
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



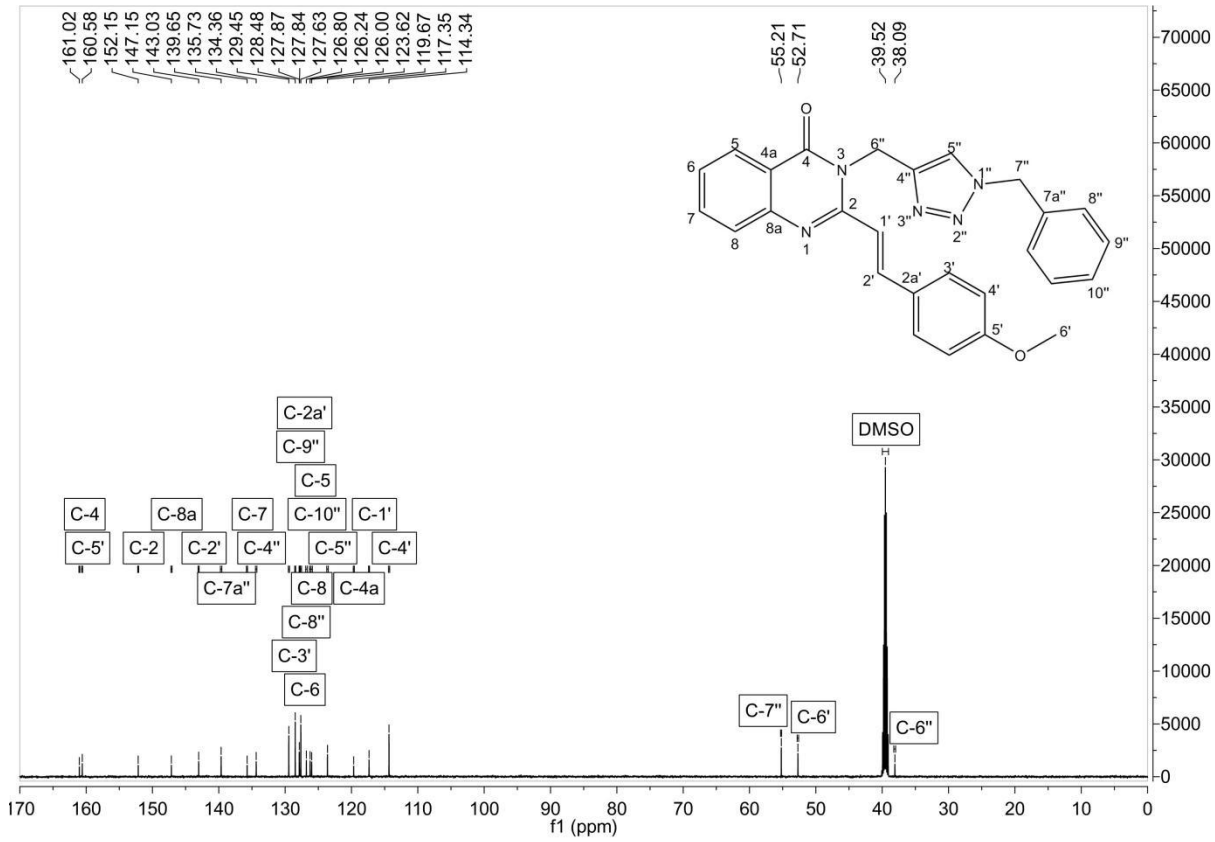
^{1}H NMR (DMSO at 80°C)



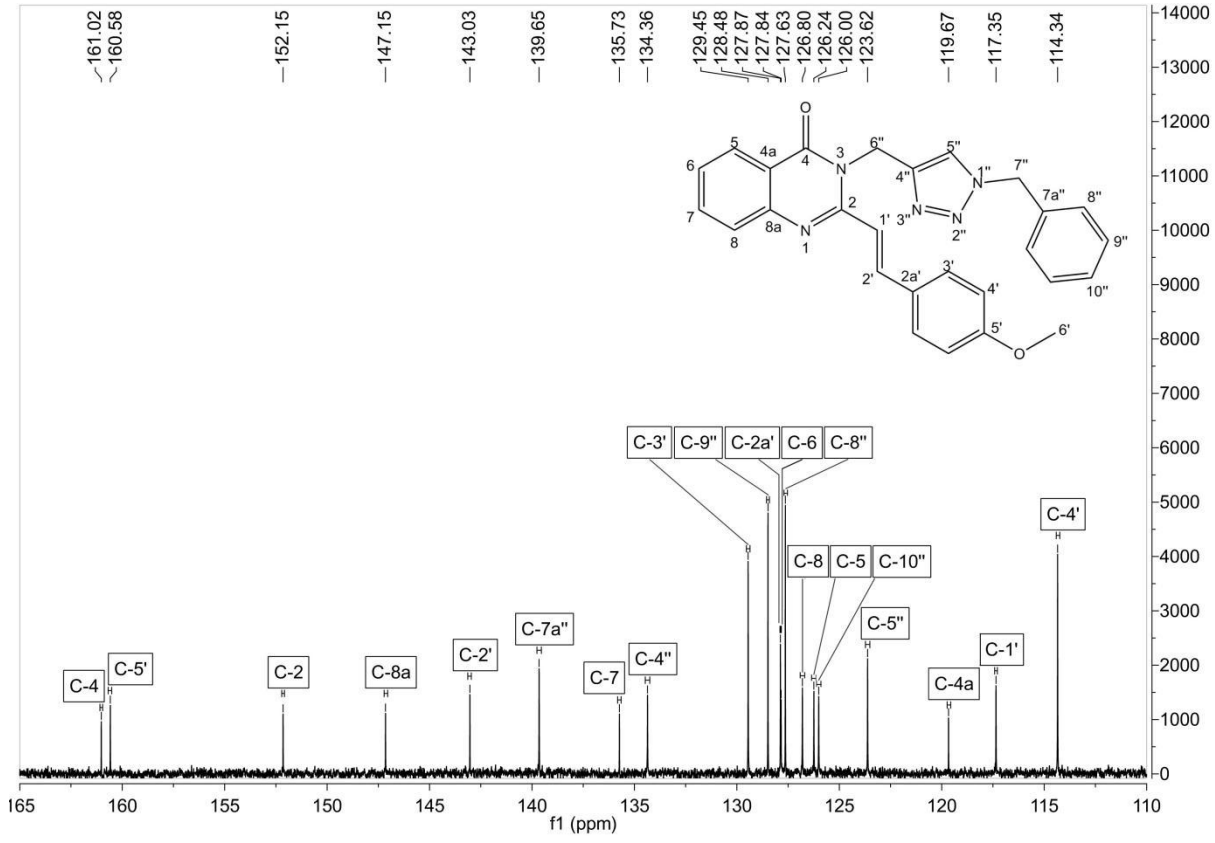
^{1}H NMR 8.20 - 6.95 ppm (DMSO at 80°C)



^{13}C NMR (DMSO at 80°C)

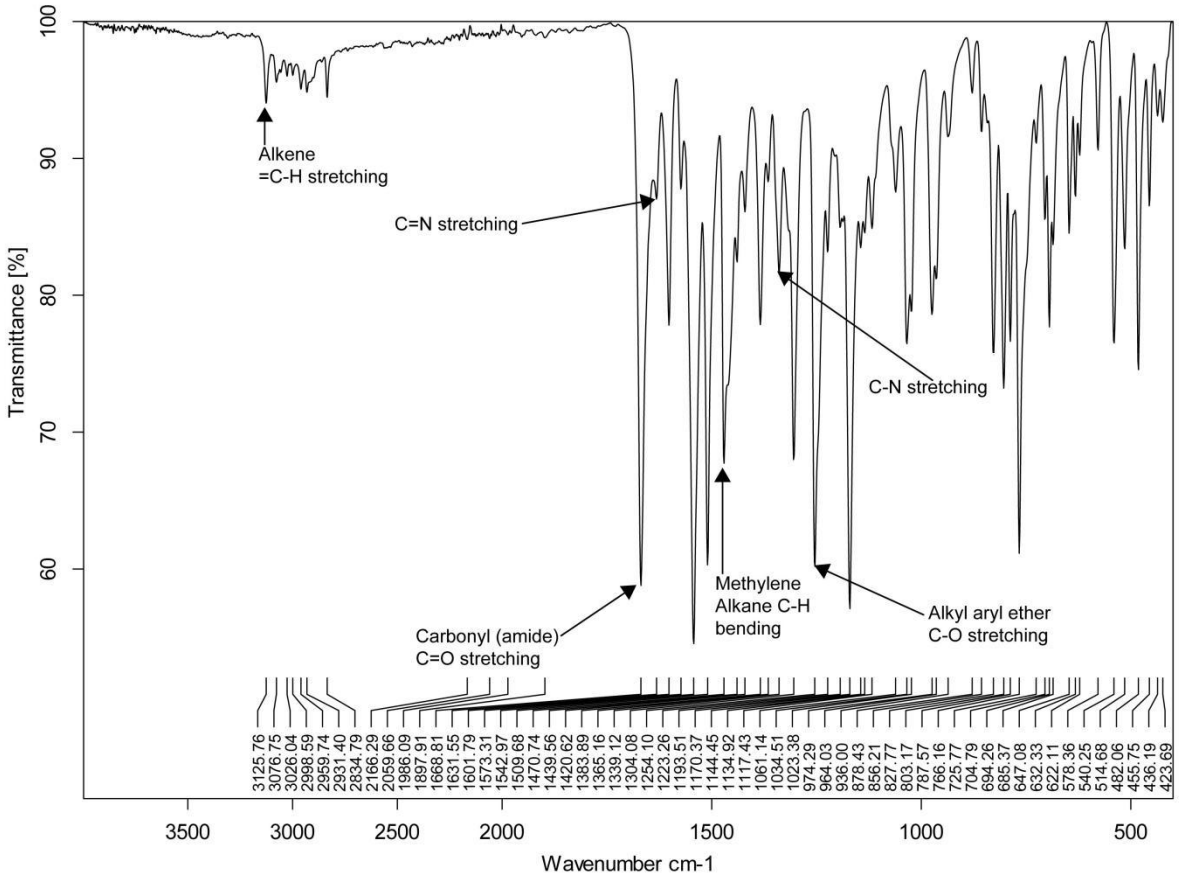


^{13}C NMR 165.00 - 110.00 ppm (DMSO at 80°C)



Compound 4j

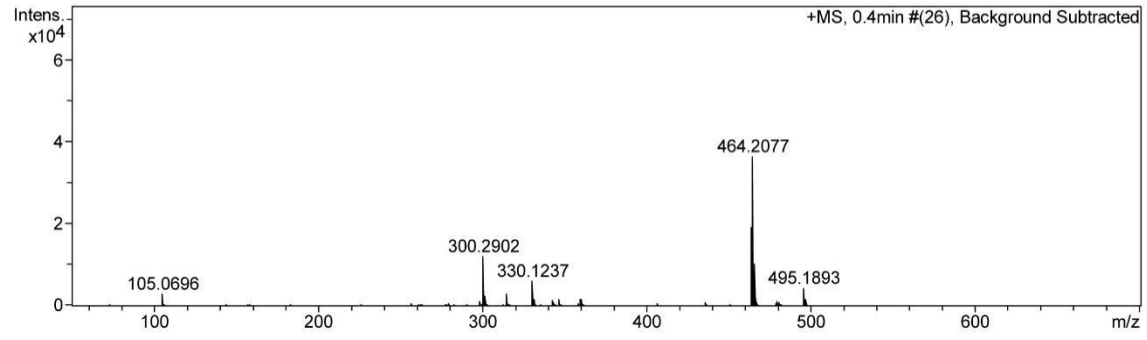
IR



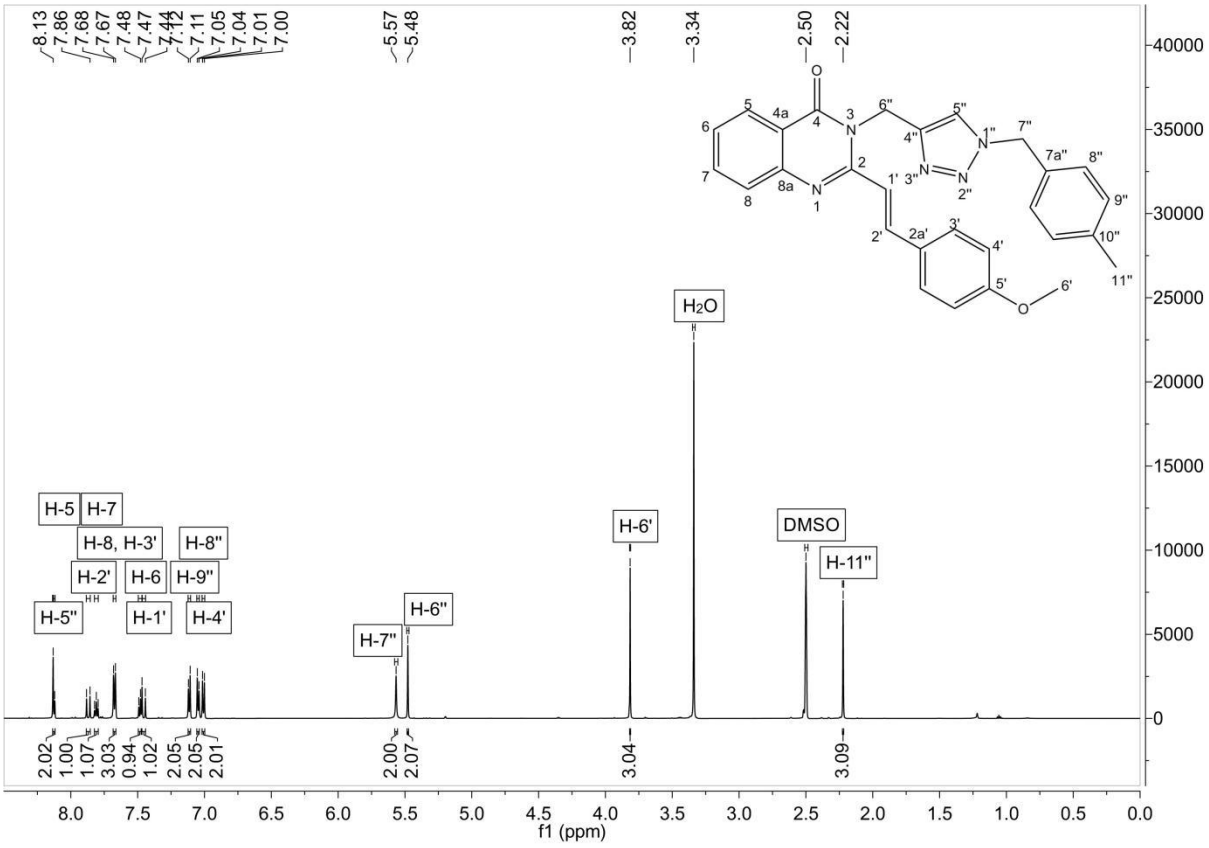
HRMS

Acquisition Parameter

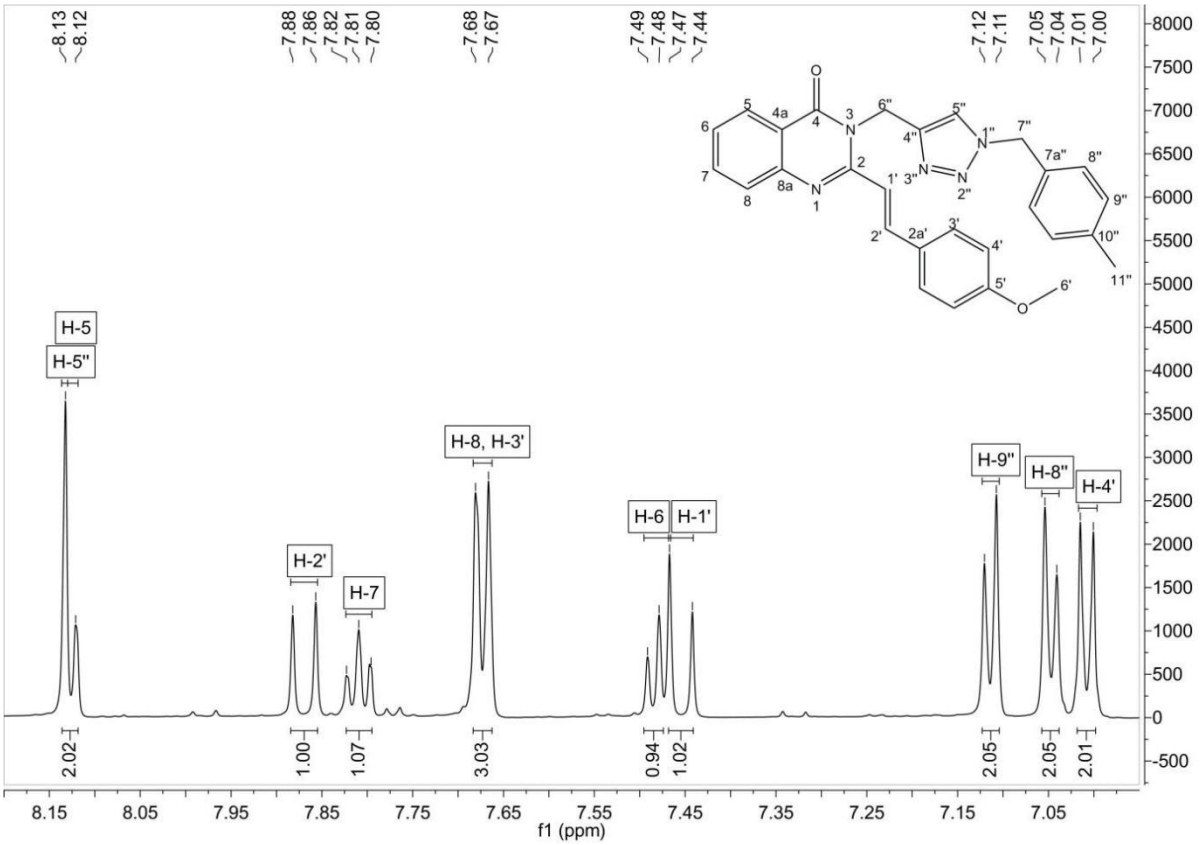
Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.6 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



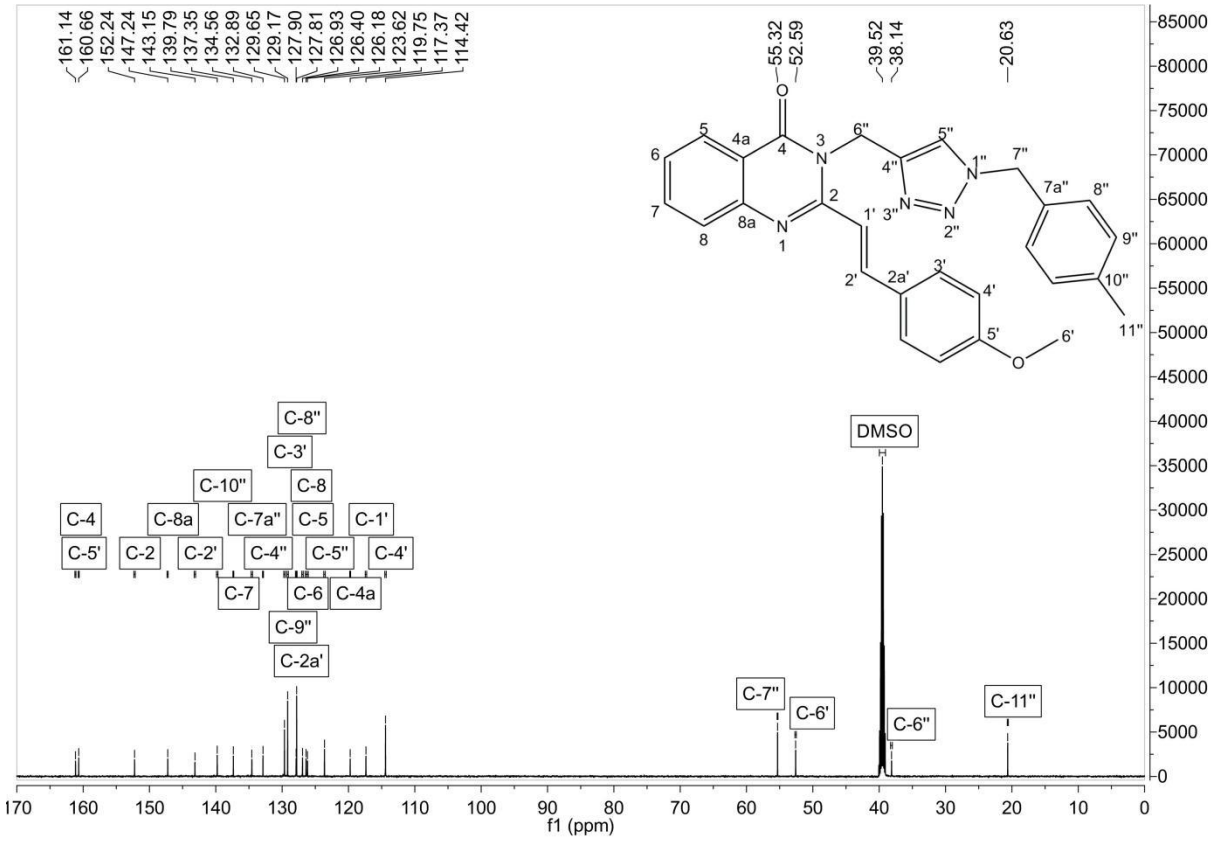
^{1}H NMR (DMSO at ambient temperature, ~25 °C)



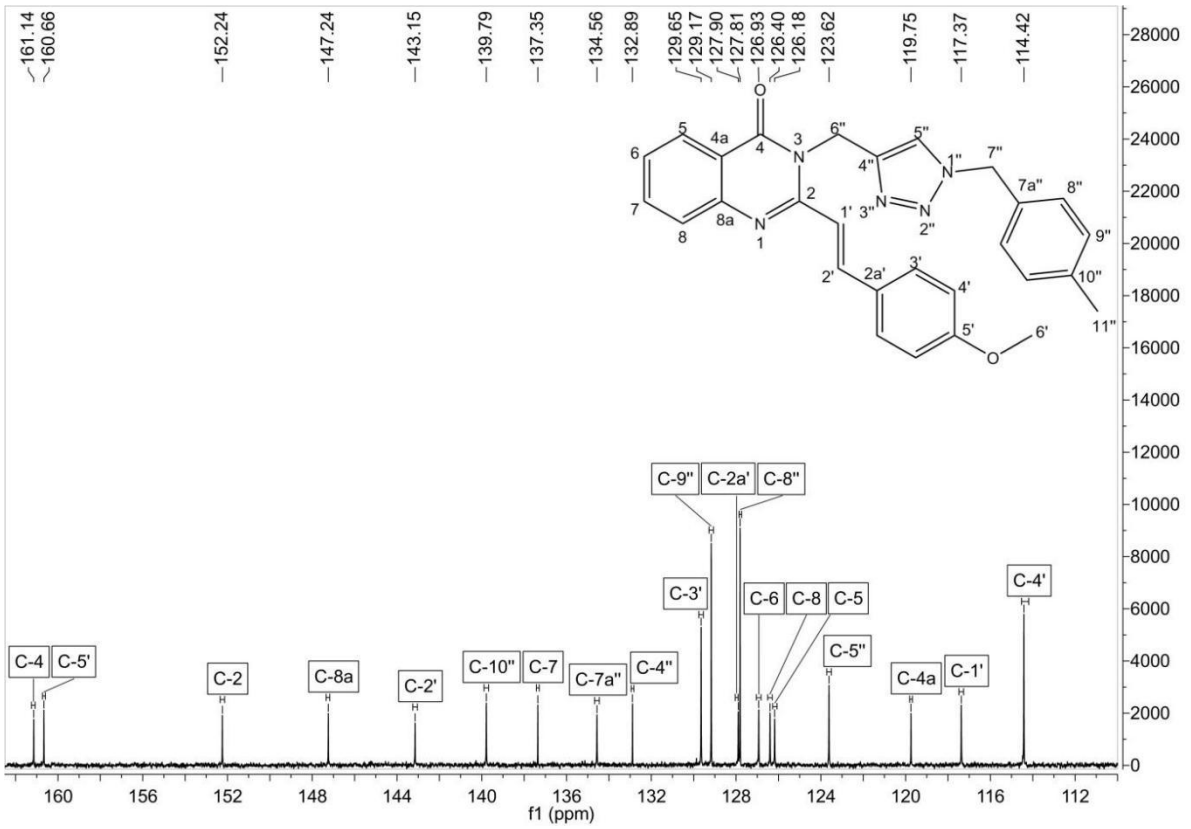
^{1}H NMR 8.20 - 6.95 ppm (DMSO at ambient temperature, ~25 °C)



^{13}C NMR (DMSO at ambient temperature, ~25 °C)



^{13}C NMR 162.50 - 110.00 ppm (DMSO at ambient temperature, ~25 °C)



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Quinazolinone and quinazoline derivatives: recent structures with potent antimicrobial and cytotoxic activities

Elham Jafari,[1,2] Marzieh Rahmani Khajouei,[2,3] Farshid Hassanzadeh,[1,2] Gholam Hossein Hakimelahi,[4] and Ghadam Ali Khodarahmi[1,2,*]

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Quinazoline and quinazolinone as important medicinal scaffolds: a comparative patent review (2011–2016)

Author: Abdul Hameed, , Mariya Al-Rashida, et al
Publication: Expert Opinion on Therapeutic Patents
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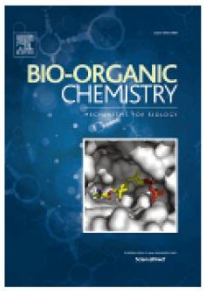
ANNEXURE C

GUIDE TO AUTHORS

BIOORGANIC CHEMISTRY

AUTHOR INFORMATION PACK

TABLE OF CONTENTS



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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
[2] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, 2018. The art of writing a scientific article. Heliyon. 19, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).
Reference to a dataset:
[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/ xwj98nb39r.1.

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25 October 2019

RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Health Research Ethics Committee (NWU-HREC) on 25/10/2019, the NWU-HREC hereby clears your study as a no risk study. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Synthesis and antileishmanial activity of novel 2, 3-disubstituted-4(3*H*) quinazolinone derivatives
Principal Investigator/Study Supervisor/Researcher: Prof DD N'Da
Student: GL Ralph - 24896160

Ethics number:

N	W	U	-	0	0	9	4	3	-	1	9	-	A	1
	Institution			Study Number						Year			Status	

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

Application Type: Single study
Commencement date: **25/10/2019**

Risk: **No Risk**

General conditions:

The following general terms and conditions will apply:

- *The commencement date indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research;*
  - *withdraw or postpone clearance if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
    - *submission of the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by
Prof Petra Bester
Date: 2019.10.25
09:32:57 +02'00'

NWU-HREC Chairperson

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