ANTISCHISTOSOMAL STRUCTURE-ACTIVITY RELATIONSHIPS OF NITRATED *N*-PHENYL BENZAMIDE DERIVATIVES

By

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A dissertation submitted to the University of Zambia in partial fulfillment of the requirements for the degree of

Master of Science in

Chemistry

THE UNIVERSITY OF ZAMBIA

LUSAKA

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Co-Supervisor: Dr. Evelyn Funjika Sign: …….. Date: ……

ABSTRACT

Schistosomiasis is a chronic parasitic disease caused by blood-dwelling trematodes of the genus *Schistosoma*. It ranks second on a list of neglected tropical parasitic diseases. Globally, about 280,000 deaths are recorded annually while more than 240 million people get infected and more than 700 million people risk being infected. The disease is associated with approximately 3.3 million disability-adjusted life years. It also causes a total estimated annual global economic loss amounting to US$641,790,130. Africa alone accounts for more than 90% of schistosomiasis cases with *Schistosoma mansoni* and *Schistosoma haematobium* species being the most prevalent. For over 40 years, praziquantel has stood out as the principal drug of choice in the treatment and control of schistosomiasis. It is efficacious and safe. It has also been very instrumental in preventive chemotherapy. However, it faces emerging drug resistance, lacks antischistosomal activity against juvenile worms, does not provide a 100% cure, and does not prevent re-infection. *N*-phenylbenzamides have shown potential as antischistosomal agents although they have not been extensively studied. This study was inspired by one compound, **MMV687807,** which was active against *Schistosoma mansoni* worms and was 100% lethal at 10 µM against *Schistosoma haematobium* adult worms whose further medicinal chemistry exploration, identified **MK1-11,** from the recent study that reported its exhibited potency against *Schistosoma mansoni* adult worms attributed to trifluoromethyl group (CF_3), an electron-withdrawing group. This study, therefore, sought to further enhance activity by nitrating the core-scaffold of the front-runner compound, **MK1-11**. The analogs of this compound were thus synthesized and tested *in vitro* on *Schistosoma mansoni* juvenile worms. The successfully synthesized analogs were confirmed by HPLC-MS, ^{1}H-NMR, and ^{13}C-NMR spectroscopic techniques. The ^{1}H-NMR and ^{13}C-NMR results were analyzed using a spectral data analyzing software called *MestReNova*. The %-death-of-worms effect produced by the tested compounds were 62%, 28%, 56%, and 42% at 50 µM with reduced effects of 41.67%, 25%, 54.17%, and 25% at 10 µM respectively. Structure-activity relationships (SARs) appeared to confirm earlier findings being electron-withdrawing groups are essential for potency. Generally, analogs containing nitro and trifluoromethyl groups on the phenyl rings as depicted in **JS-03 and JS-05** exhibited enhanced activity. However, regioisomerism (**ortho** or **meta** substitution) in these two analogs did not seem to affect activity. Due to low activity on juvenile worms, the analogs could not be tested on adult worms. Consequently, enhancement or decrease in activity with respect to the front-runner compound, **MK1-11** which was only tested on adult worms and also using a different *in vitro* biological assay method could not be deduced. There is need, therefore, to consider resynthesizing the front-runner compound by further research, test it on both juvenile and adult worms using the same *in vitro* assay method as the newly synthesized analogs in order to assess to what extent activity is improved upon or compromised.

Keywords: *Synthesis, N-phenylbenzamides antischistosomal, schistosomiasis*

DEDICATION

In memory of my parents and my sister Bana Lewis. Bana Lewis, you were so instrumental during my early grades at school. Mum and Dad, your love for me knew no bounds and taught me the value of hard work. Although you are no longer of this world, your memories continue to regulate my life. I wish you could be here to witness this amazing day. Rest easy on the wings of eternity Sister, Mum, and Dad.

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CONFERENCES AND SEMINARS

Conference Presentations

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δ	Chemical shift
J	Coupling constant
^{o}C	Degrees celsius
g	Gram
σ	Hammett substituent constant
Hz	Hertz
h	Hour
MHz	Megahertz
µM	Micromolar
mg/kg	Milligram per kilogram
nm	Nanometre
ppm	Parts per million
%	Percent or percentage
π	Substituent hydrophobicity constant
$	United States Dollar
λ	Wavelength

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionization
AR	Analytical reagent
clog*P*	Calculated log in base 10 of lipophilicity
COSY	Correlation Spectroscopy
COVID-19	Coronavirus disease 2019
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
DALYs	Disability-adjusted life years
DCC	*N*, *N*′-Dicyclohexylcarbodiimide
DCM	Dichloromethane
1D	One-dimensional
2D	Two-dimensional
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublets of doublets
DHODH	Dihydroorotate dehydrogenase
DMAP	4-Dimethylaminopyridine
DMSO	Dimethylsulfoxide
DMSO-d_6	Deuterated dimethylsulfoxide
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
eq	Equivalent
EtOAc	Ethyl acetate
FGS	Female genital schistosomiasis
GPR	General purpose reagent
H3D	Drug discovery and development center
HBA	Hydrogen bond acceptors
HBD	Hydrogen bond donors
HDAC	Histone deacetylase
HPLC	High Performance Liquid Chromatography
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HSD	Honestly significant difference
HSQC	Heteronuclear Single Quantum Coherence
IC_{50}	Concentration of a drug required for 50% inhibition *in vitro*
iFCS	Inactivated foetal calf serum
JS	John Sichinga
m	Multiplet
m.p	Melting point
m/z	Mass to charge ratio
mAU	Milliabsorbance units
Me	Methyl

MGS	Male genital schistosomiasis
MHSRIP	May his soul rest in peace
MK	Masebe Kanyanta
MMV	Medicines for Malaria Venture
MoE	Ministry of Education
MW	Molecular weight
Na_2SO_4	Sodium sulphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
NDVI	Normalized difference vegetation index
NF54	Drug-sensitive strain of *Plasmodium falciparum*
NH_4Cl	Ammonium chloride
NPBAs	*N*-phenylbenzamides
NRIS	National Research and Innovation Symposium
NTDs	Neglected tropical diseases
NTS	Newly transformed schistosomula
OXA	Oxamniquine
***Pf*DHODH**	*Plasmodium falciparum* dihydroorotate dehydrogenase
PZQ	Praziquantel
q	Quartet
R_f	Retardation factor
s	Singlet
SARs	Structure-activity relationships
SD	Standard deviation
SDF	Staff development fellow
SHEPIZ	Strengthening Health Professional Workforce Education Programs for Improved Quality Health care in Zambia
S_N2	Nucleophilic substitution bimolecular
t	Triplet
td	Triplet of doublets
TLC	Thin Layer Chromatography
TPH	Tropical and Public Health Institute
t_r	Retention time
UCT	University of Cape Town
UNZA	University of Zambia
US	United States
UV	Ultraviolet
WBR	Worm burden reduction
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Chapter Overview

This chapter provides a brief overview of schistosomiasis as a disease. Schistosomiasis is described in the context of its brief history, etiology, global distribution of *Schistosoma* species, current schistosomiasis situation, and types of schistosomiasis. The *Schistosoma* parasite life cycle and its feeding characteristics are covered. The chapter further discusses the epidemiology, economic, and disease burden. Symptoms for schistosomiasis are equally presented. Antischistosomal drug development pipeline, drug repurposing, and the need to discover novel antischistosomal candidates are outlined. Schistosomiasis control and treatment, praziquantel activity, and its mode of action are highlighted. Lastly, the chapter lays out the statement of the problem, the significance of the study, the objectives, and the hypothesis.

1.2 Schistosomiasis: History and Background

Schistosomiasis is a neglected tropical debilitating disease.[1–8] It ranks second on the list of neglected tropical parasitic diseases.[9–11] It was formally called Bilharzia or Bilharziasis named after its discoverer, a German physician, and pathologist, Theodor Maximilian Bilharz.[12,13] He described the disease for the first time as a tropical parasitic disease in 1852.[14,15]

Theodor noticed a worm in white bumps on the mucous membranes of the bladder, ureters, seminal glands, and intestines in the year 1851 while working in one of the hospitals in Cairo, Egypt in the middle of the 19th century.[16,17] Therefore, this discovery marked the beginning of a new era for both tropical and sub-tropical parasitology. Henceforth, preventive and curative measures to schistosomiasis were instituted.[18–20] In the second half of the 19th and early 20th century hereafter, other discoveries began.[13]

1.3 Etiology of Schistosomiasis

Human schistosomiasis is caused by blood-dwelling flukes or trematode worms (Figure 1.1) of the genus *Schistosoma*.[21–25] Adult worms are paired, male and female.[26–28] The male worm is shorter, thicker and flatter with a groove wherein it grips a slender and longer female.[26–28]

Figure 1. 1: Paired adult Schistosoma worms.[27]

There are six *Schistosoma* species that exist, namely: *S. japonicum*, *S. mansoni*, *S. haematobium*, *S. mekongi*, *S. guineensis* and *S. intercalatum*.[15,29] However, *S. japonicum*, *S. mansoni* and *S. haematobium* are the most infective clinically.[30–36]

1.4 Global Distribution of Schistosoma Species

The *Schistosoma* species (Figure 1.2) are globally distributed as follows: *S. japonicum* (China, Indonesia and the Philippines), *S. mansoni* (Africa, the Middle East, South America and the Caribbean), *S. haematobium* (Africa and the Middle East), *S. guineensis* and *S. intercalatum* (rainforest areas of Central Africa) and *S. mekongi* (Cambodia and Lao People's Democratic Republic).[15,22]

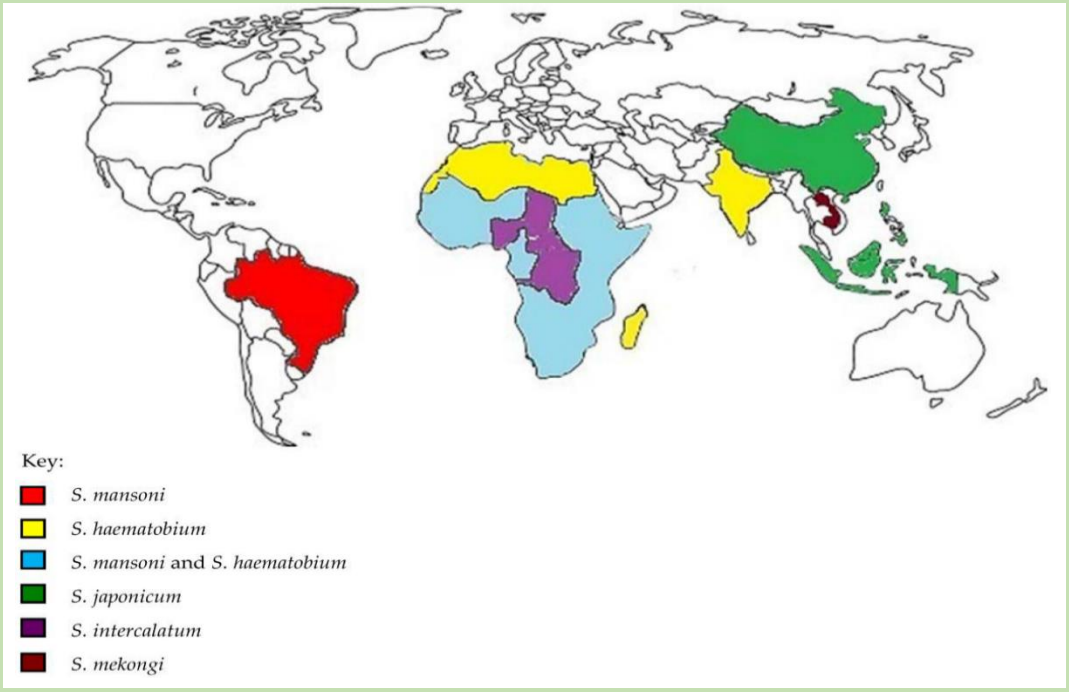

Figure 1. 2: Global distribution of Schistosoma species.[37]

1.5 Current Global Schistosomiasis Situation

The global distribution of schistosomiasis (Figure 1.3) has changed to a large extent in the past 50 years. Control successes have been achieved in Asia, the Americas, North Africa, and the Middle East.[38,39] In spite of this progress, the disease remains endemic in developing parts of the world, especially sub-Saharan Africa.[40]

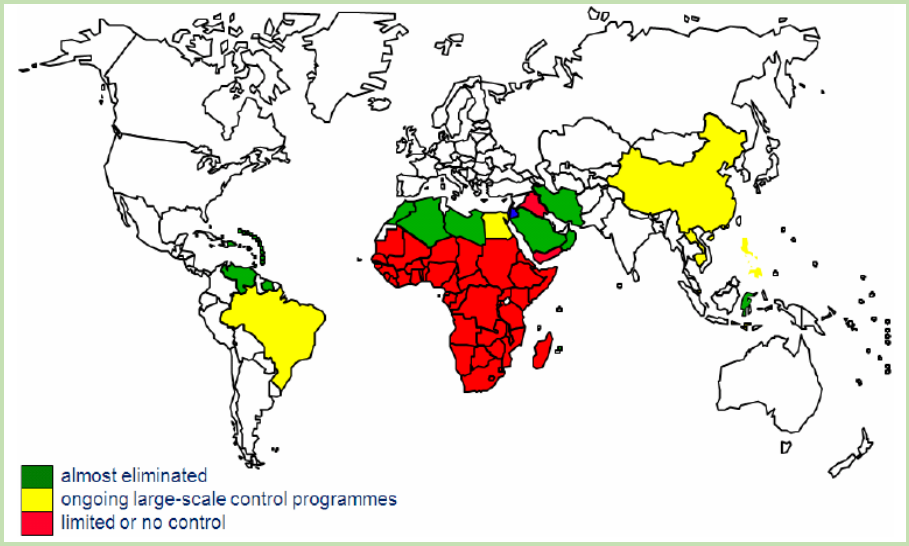

Figure 1. 3: Current global schistosomiasis situation.[38]

1.6 Types of Schistosomiasis

Table 1.1 shows two prominent types of human schistosomiasis, namely: Intestinal and urogenital schistosomiasis.[41,42] Intestinal schistosomiasis is associated with symptoms such as diarrhea, bloody stools and abdominal pain,[43] while urogenital schistosomiasis is characterized by hematuria (blood in urine).[44] Among the most infective Schistosoma species, *S. mansoni* and *S. japonicum* are responsible for intestinal schistosomiasis,[41,45,46] while *S. haematobium* is associated with urogenital schistosomiasis.[41,47–52]

Urogenital schistosomiasis affects both gender.[53] However, It is more apparent in women than in men.[54,55] If eggs of *S. haematobium* are trapped in the genital tract, they cause genital schistosomiasis.[54] In women, it is called female genital schistosomiasis (FGS) while in men it is called male genital schistosomiasis (MGS).[54,56] Apart from causing infertility in women, genital schistosomiasis also causes infertility in men but seldom reported.[54,57]

Table 1. 1: Types of schistosomiasis and their respective Schistosoma species.[a]

Type of schistosomiasis	Species	Geographical distribution
Intestinal Schistosomiasis	*S. mansoni*	Africa, Middle East, Caribbean, Brazil, Venezuela and Suriname.
	S. japonicum	China, Indonesia, and Philippines
	S. mekongi	Several districts of Cambodia and Lao People's Democratic Republic
	S. guineensis and related *S. intercalatum*	Rain forest areas of Central Africa
Urogenital Schistosomiasis	*S. haematobium*	Africa and Middle East

[a]Adapted from Drudge-Coates and Turner, 2013.

1.7 Schistosoma Parasite Life Cycle

Figure 1.4 depicts the *Schistosoma* parasite life cycle. It is very complex because it occurs in two alternate hosts.[58–60] Snails are the intermediate host,[58–64] while humans are the definitive host.[59,65] The parasite reproduces both sexually and asexually depending on the host.[60] Asexually in snails while sexually in mammals such as humans.[60,66] In humans, the parasites grow and pair up male and female.[67,68] After mating, the female lays eggs some of which are released into fresh water through faeces or urine while others get retained in host tissues thereby causing inflammation.[64] Eggs that reach fresh water eventually hatch releasing miracidia which then infect specific snails.[22,64] The miracidia of *S. mansoni*, *S. haematobium*, and *S. japonicum* infect *Biomphalaria*, *Bulinus* and *Oncomelania* snails respectively.[22,69] In the snail host, miracidia remove ciliated plates and grow into mother sporocysts which give birth asexually to daughter sporocysts.[70] Daughter sporocysts then produce cercariae which are released into fresh water.[71–75] At the infective stage, cercariae attach to humans when they get into contact with infested fresh water.[76–79] Penetration of the human skin then occurs aided by the secretion of enzymes that break down the natural protein of the skin.[41,78] During penetration, only the cercarial head enters the human skin leaving behind the forked tail which is shed off forming newly transformed schistosomula (NTS).[80–84]

Schistosomula then migrate to various body tissues via blood circulation. They later grow into adult worms and migrate to specific host organs where they reside.[84,85] *S. haematobium* migrates and settles in the perivesicular venule (surrounding the urinary bladder) where it causes urogenital schistosomiasis while *S. mansoni* and *S. japonicum* migrate and settle in the mesenteric venules (surrounding the small intestines) where they cause intestinal schistosomiasis.[22,86]

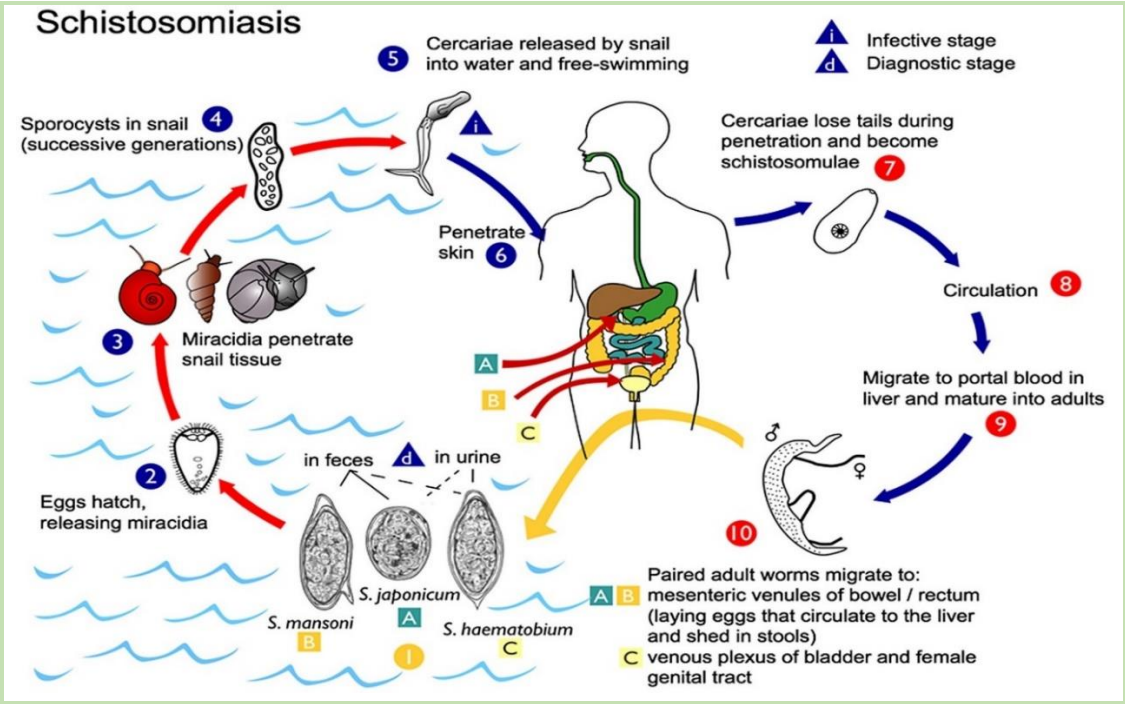

Figure 1. 4: Schistosoma parasite life cycle.[87]

1.8 Schistosoma Parasite Feeding Characteristics

Aside from sharing the same blood-feeding characteristics with Plasmodium parasites, *Schistosoma* parasites also share the same heme degradation mechanism with the former.[88–91] In order to supplement nutritional requirements as well as complete their sexual development, *Schistosoma* parasites need amino acids which they obtain by digesting host hemoglobin while releasing toxic heme (ferriprotoporphyrin) in the process. Heme may produce oxygen radicals or worse still damage cell membranes as well as parasite proteins. *Schistosoma* parasites, like Plasmodium parasites, avoid the toxic effects of heme by polymerization of heme molecules into a non-toxic, inert and insoluble hemozoin.[88–91]

1.9 Epidemiology, Economic and Disease Burden

Schistosomiasis is among the most prevalent parasitic diseases and presents serious economic and public health consequences.[92] In developing countries, health policy making demands estimates of the economic loss and global disease burden.[93]

The disease is endemic in 76 countries and poses serious health concerns.[40,94] It affects about 240 million people while more than 700 million people risk being infected globally.[95–98] It is associated with approximately 3.3 million disability-adjusted life years (DALYs).[99,100] It causes more than 280, 000 global annual deaths.[101,102] The total estimated annual global economic loss towards schistosomiasis control and treatment amounts to US$641,790,130.[103]

Africa alone accounts for more than 90% of schistosomiasis cases with *S. mansoni* and *S. haematobium* species being the most prevalent. In 2003, for instance, sub-Saharan Africa recorded 90% of schistosomiasis cases.[104]

A study that explored the relationship between the presence of *Schistosoma* infection and clinical morbidity reported a total of 70 million individuals out of 682 million in sub-Saharan Africa that experienced haematuria while 32 million experienced dysuria in a fortnight period resulting from *S. haematobium* infection. About 18 million people had bladder wall pathology while 10 million people had hydronephrosis. Additionally, *S. haematobium*-related renal failure and bladder cancer contributed to 150 000 estimated mortality rate per year in sub-Saharan Africa.[93]

It is estimated that 20 million people adversely suffer from serious manifestation of the disease.[105–107] It mostly affects young boys and girls.[108] Multiple risk factors, for instance, increased water activities, anatomical developmental changes that occur in the vasculature which supplies genitourinary structures harbouring the parasite and its eggs as well as development of immunological responses highly affect school-aged children.[109]

Schistosomiasis infection is highly prevalent in young adolescents. 60–80% of school going children in any population are vulnerable while only 20–40% of the old age group are vulnerable to infection.[22]

Zambia experiences a very high burden of schistosomiasis in spite of a number of efforts to mitigate it. *S. haematobium* and *S. mansoni* species are both common in all the 10 provinces, but *S. haematobium* is more prevalent causing urogenital or urinary schistosomiasis.[110] A study of 260 pupils aged 8–14 years in Ng'ombe Township, Lusaka showed *S. haematobium* infection prevalence rate of 13.1% (34/260).[111] In another study, it was noted that, at least one out of five school-age children was infected with *S. haematobium* according to a Ng'ombe Clinic document for a five-month period from January to May 2007.[112]

An observational study of 5–17-year-old school children in peri-urban compounds of Lusaka, Zambia reported schistosomiasis population prevalence of 20.72%.[112] The study revealed that peri-urban populations were prone to infection.

A study on urinary schistosomiasis conducted in Zambia in 20 primary schools of Lusaka province to further understand the epidemiology of the infection demonstrated a serious health concern. The collected data from 1,912 (94%) of the total (2040) school children aged 6–15 years old from Kafue and Luangwa revealed a mean prevalence rate of 9.6%. Kafue district recorded a slightly higher prevalence rate than Luangwa, albeit the difference not significant. Kafue recorded 10.9% while Luangwa recorded 8.4%. The risk factors identified in that study included but not limited to geographical location, altitude, normalized difference vegetation index (NDVI), maximum temperature, age, sex of the child and intermediate host snail abundance among others.[18]

Studies conducted earlier in Siavonga in the Southern part of Zambia showed a high prevalence rate of *S. mansoni* and *S. haematobium*.[110] Another recent study involving 421 primary school children aimed at determining the prevalence and risk factors of urinary schistosomiasis and identifying the strain of *S. haematobium* among children in Siavonga and Lusaka districts in Zambia reported *S. haematobium* overall prevalence rate of 9.7%. Male participants made up 6.2% while female participants made up 3.5%. The observed higher prevalence rate in male participants could be attributed to their daily activities which include but not limited to playing, swimming and fishing in freshwater bodies. In that study, the highest overall prevalence rate (8.3%) was in the age group between 11–15 years.[113] A survey conducted in Livingstone in the year 2013 involving school-aged children reported prevalence ranges of between 3.3% and 73.3% for *S. haematobium* infections.[57]

1.10 Symptoms of Schistosomiasis

Acute schistosomiasis seldom shows symptoms in most people.[114,115] Chronic schistosomiasis, on the other hand, presents symptoms after a month or a year depending on the *Schistosoma* species.[114] The trapping of eggs in the host tissues is the major cause of schistosomiasis pathology.[116–119] Immune responses are induced by the trapped eggs in various body organs and tissues thereby forming granulomas.[120–123] Individuals with prior infection develop Katayama fever,[124] which is characterized by high temperatures.[114,125] In certain instances, a maculopapular rash appears at the site of entry while in others schistosomal dermatitis develops.[114,126] *S. mansoni* and *S. japonicum* cause abdominal pain, bloody diarrhea, colonic polyposis, portal hypertension, hematemesis, ascites, splenomegaly, esophageal variceal bleeding, chronic coughs, palpitations, atypical chest pain and pulmonary hypertension leading to death. Eggs from *S. haematobium* get deposited in the urinary tract system causing dysuria, hematuria, bladder polyps, ulcers, obstructive uropathies and squamous cell bladder cancer.[127–129]

1.11 Antischistosomal Drug Pipeline and Justification for New Drugs

Schistosomiasis is one of the most burdensome neglected tropical infectious diseases, yet the current armamentarium of antischistosomal drugs remains frighteningly small.[130–134] Massive research needs to be done to discover new drugs to serve as alternatives to praziquantel (PZQ), the only available drug, should resistance strike.[135–142]

Due to the costly nature of drug discovery and development, its high risk, laborious nature and the long duration to develop novel drugs *de novo*, drug repurposing is considered an efficient approach to circumvent these obstacles.[143–146] Drug repurposing is the discovery of a new therapeutic application of an already existing drug duly approved by a regulatory body for use in a specified medical indication.[145,147,148] Anticancer drugs, for instance, have been repurposed for use as antischistosomal agents against all forms of *Schistosoma* species.[32] In spite of their effectiveness, they have, unfortunately, presented severe adverse effects.[142]

Some of the lead compounds (Figure 1.5) that have undergone drug repurposing for use in the fight against schistosomiasis include but are not limited to artemether **(1**), artesunate (**2**), moxidectin (**3**), mefloquine (**4**), etc.[105,149] Although most of these were initially pursued as antimalarials, they were also discovered to exhibit good antischistosomal activity.[150]

A combination of mefloquine and PZQ demonstrated good activity against *S. mansoni.*[151–155] Artemisinins such as artemether and artesunate were lethal against immature forms of the parasite thereby presenting an opportunity for combination with PZQ.[131,156] Mefloquine, artesunate, mefloquine-artesunate and PZQ have exhibited lethal antischistosomal activity against *S. haematobium.*[157,158]A combination of artemether and PZQ or artemether administered alone was lethal on both juvenile and adult worms, albeit drug resistance in areas of high malaria endemicity exacerbated the situation.[159,160]

Artemisinin derivatives have suffered a very short *in vivo* half-life,[1] while moxidectin had suffered low efficacy against *S. haematobium.*[149] Notwithstanding all these efforts, there are currently no antischistosomal drug candidates under clinical trials and the pipeline is literally empty.[96,161]

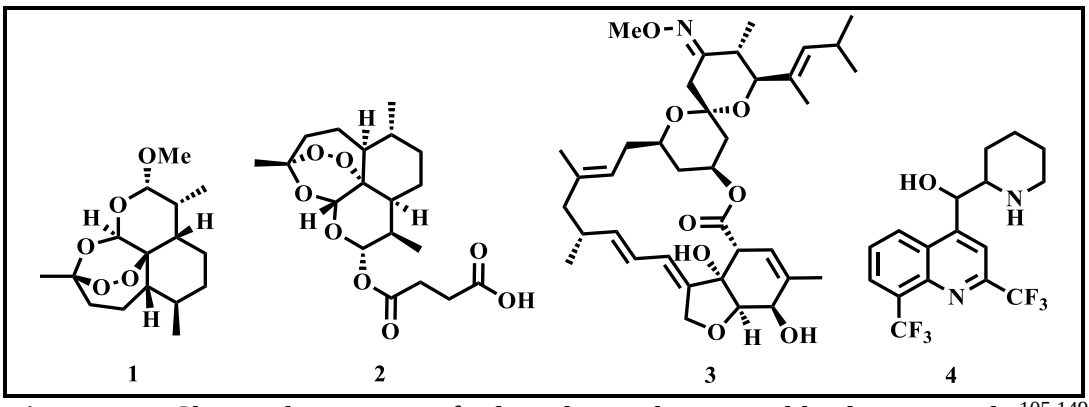

Figure 1. 5: Chemical structures of selected antischistosomal lead compounds.[105,149]

1.12 Schistosomiasis Control and Treatment

The World Health Organization (WHO) had set ambitious goals for the control and treatment of schistosomiasis with the vision, "a world free of schistosomiasis," aimed at eliminating it as a public health problem by 2020 and 2025.[162] In January 2021, a new road map was launched by WHO to set the targets attainable for all neglected tropical diseases (NTDs) by 2030 with a shift from use of a process indicator (coverage) to the use of impact indicator (elimination of morbidity).[163]

PZQ (**5**), oxamniquine (OXA, **6**) and metrifonate (**7**) (Figure 1.6) have been instrumental in the control and treatment of schistosomiasis.[164,165] PZQ is a pyrazinoisoquinoline derivative originally synthesized by E. Merck, Germany in 1970 as a prospective tranquilizer.[166,167] Later in 1972, its anthelmintic properties were detected by both Bayer A.G and E. Merck from a

screen of over 400 compounds.[168–170] It was found to exhibit antischistosomal activity against all the important *Schistosoma* adult species but had poor activity against juvenile worms.[171] The exhibited cure rates were 75–85, 63–85, 80–90 and 60–80% in patients with *S. haematobium*, *S. mansoni*, *S. japonicum* and co-infections with *S. mansoni* and *S. haematobium* respectively.[172]

OXA (a nitroquinoline) and metrifonate (an organophosphorus) had been used as alternative antischistosomal drugs.[96,173] OXA exhibited lethal activity on both juvenile and adult *S. mansoni* worms.[174,175] However, its efficacy was lower than that for praziquantel.[145,176] The low efficacy and narrow parasite specificity rendered it unreliable in the fight against schistosomiasis. It had also faced serious drug resistance.[177] Metrifonate, on the other hand, demonstrated lethal activity against *S. haematobium* making it instrumental in the treatment of urogenital schistosomiasis.[96,178,179] Nonetheless, it is no longer preferred for clinical use like OXA due to its narrow parasite specificity.[164,165,180] Having tried out the aforementioned antischistosomal drugs, none has come close to the efficacy of PZQ resulting in their withdrawal from the market. It is against this background that PZQ continues to be superior to any other antischistosomal drug for over 40 years.[181] It has so far been the only drug of choice in schistosomiasis control and treatment.[182–187] In view of the foregoing, all forms of schistosomiasis control and treatment are currently based on PZQ at a single dose of 40 mg/kg.[188–192]

Treatment with PZQ at 40 mg/kg affords cure rates of 70–90% in *S. mansoni* infections with worm egg reduction rates > 90%.[193] Cure rates are significantly associated with intensity of infection. The lighter the infection, the higher the cure rates.[194] Treatment of *S. haematobium* and *S. mansoni* infections requires a dose range of 40–60 mg/kg of body weight while *S. japonicum* treatment requires a much higher dose range.[166]

In the year 2012, about 27.5 million people were treated globally.[195] In 2015, out of 218.7 million people that needed preventive chemotherapy, 65.2 million people were treated with PZQ representing 29.8% global coverage.[105] In spite of pandemic-related disruptions, coronavirus disease 2019 (COVID–19) in particular, a total of 76.9 million (31.9%) people were treated globally in 2020 while 105 million (44.8%) people were treated in 2019. A drop in schistosomiasis treatment by 28.6 million in 2020 can be attributed to implementation of COVID–19 measures coupled with closure of schools.[196]

Interestingly for this period, more than 90% of treated cases were in the WHO's African Region that experiences the greatest global burden of schistosomiasis. Treatment of school-aged children decreased from 66.8% in 2019 to 44.9% in 2020. Furthermore, some countries such as the Democratic Republic of Congo, Ethiopia, Ghana and Yemen that are highly affected with schistosomiasis and requiring massive treatment, however, did not implement large-scale treatment.[196]

Deworming of school-aged children dropped by 25% to 342 million in 2020 compared to 455.7 million in 2019. A global coverage decrease by 42.9% from 165.8 million in 2019 to 94.7 million in 2020 for preschool-aged children was recorded. The number of preschool-aged children treated in 2020 decreased to 7.4 million from 53.3 million in 2019 in the WHO's African Region while recording a decrease to 0.9 million in 2020 from 10 million in 2019 in the Eastern Mediterranean Region.[196]

In other regions, the decrease was not so much, for instance, a drop from 90.2 million to 75.4 million in the South-East Asia Region while Western Pacific Region recorded a drop from 10.7 million to 10 million.[196]

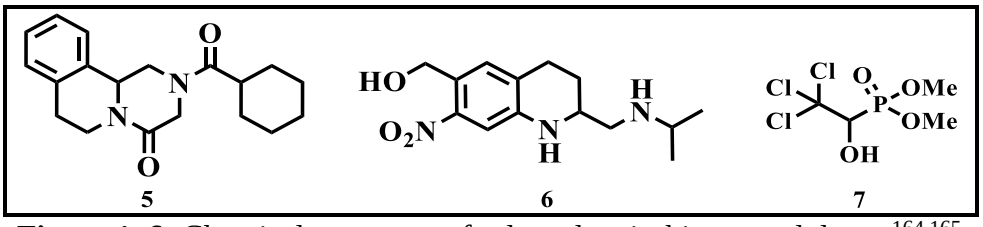

Figure 1. 6: Chemical structures of selected antischistosomal drugs.[164,165]

1.12.1 PZQ Activity

PZQ exhibits a broad-spectrum of antischistosomal activity and is efficacious against all forms of *Schistosoma* spp.[167,168,197] It is safe, cheap, well tolerated and effective against mature worms,[198] with little to no activity against sexually immature juvenile schistosomes.[199–201] PZQ is administered as a racemic mixture of (*R*) and (*S*) enantiomers (Figure 1.7).[202] The (*R*) enantiomer is the active component of the racemic mixture while the (*S*) enantiomer does not contribute to antischistosomal activity of the drug.[99]

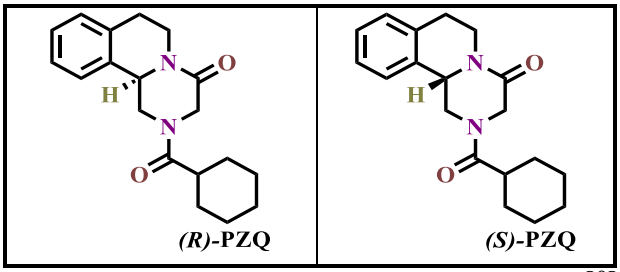

Figure 1. 7: (*R*) and (*S*) enantiomers of PZQ.[202]

1.12.2 PZQ Mode of Action

Despite being the mainstay treatment for schistosomiasis for over 40 years, the precise mode of action for PZQ is not well known.[166,182,203] However, one proposed mode of action suggests that the voltage-gated Ca^{2+} channels are the molecular targets for PZQ whose action causes a rapid influx of Ca^{2+} ions into the cells of the schistosomes.[89,203] This leads to muscle contraction and paralysis of the worm musculature.[30] PZQ is also thought to trigger vacuolation and blebbing in adult worms disrupting tegumental and subtegumental structures thereby exposing the worm surface antigens. The parasite then gets cleared by the host immune system due to easy recognition.[30]

1.13 Statement of the Problem

PZQ, the only drug of choice for over 40 years, faces emerging drug resistance.[204–207] It lacks efficacy against immature worms,[133,199] does not provide 100% cure,[208,209] and does not prevent re-infection.[210,211] Furthermore, its inactive enantiomer, (*S*)-PZQ is responsible for the bitter taste which is undesirable to patients' especially school-aged children who are more vulnerable to the disease.[30,212] *N*-phenylbenzamides have not been extensively explored as alternative antischistosomal agents in terms of structure-activity relationships in literature. There have been tests that have been reported from phenotypic screening but no medicinal chemistry optimization of the hit compound has been done. Therefore, no optimal compounds have been discovered yet. In an effort to address this problem, this study embarked on assessing the potential for efficacy enhancement through nitration of the *N*-phenylbenzamides core-scaffold.

1.14 Significance of the Study

The project envisaged to contribute to the search for novel, broad-spectrum and multi-stage antischistosomal agents with better efficacy than PZQ. It was further anticipated that the simple one-step synthetic protocol employed in this project would be cheaper than that for PZQ.

1.15 Objectives

1.15.1 Main Objective

To further enhance the antischistosomal activity of *N*-phenylbenzamides by nitrating the core-scaffold of the front-runner compound MK1–11.

1.15.2 Specific Objectives:

1. To synthesize and characterize target compounds using physical and spectroscopic techniques.
2. To evaluate antischistosomal structure-activity relationships (SARs) of various nitrated *N*-phenylbenzamide analogs.

1.16 Hypothesis

The *N*-phenylbenzamide core-scaffold can be utilized to generate small molecule drug leads having good *in vitro* and *in vivo* antischistosomal activities with favourable efficacy, safety, physico-chemical, drug metabolism and pharmacokinetic properties.

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CHAPTER 2

LITERATURE REVIEW

2.1 Chapter Overview

This chapter discusses *N*-phenylbenzamides (NPBAs) as a class of important biologically-active compounds. Their general pharmacological and antischistosomal activities are presented herein.

2.2 *N*-phenylbenzamides

2.2.1 General Pharmacological Activity

NPBAs (Figure 2.1) have exhibited a diverse range of pharmacological activities.[1–3] These include but are not limited to antimalarial,[4,5] antidiabetic,[6] antimicrobial,[7–14] antibacterial,[12,13] antiviral,[15,16] anticonvulsant,[17,18] antiallergic,[19] analgesics,[20,21] antifungal,[22–24] anticancer,[25–28] antitumor,[29,30], etc.

Ponatinib (**8**), for example, has been used as an anticancer drug.[31] A broadly active chidamide (**9**) is an oral histone deacetylase (HDAC) inhibitor used as an antitumor drug.[30] Mocetinostat (**10**) an antitumor drug, has been used in patients with hematologic malignancies.[29] In other studies, 4-amino-*N*-phenylbenzamides have been shown to possess anticonvulsant activity.[17] The front-runner compound (**11**), 4-amino-*N*-(2, 6-dimethylphenyl)benzamide (4-ADMPB), exhibited the most anticonvulsant activity in rats when dosed orally. However, the compound was observed to undergo *N*-acetylation upon oral administration having a short half-life of 15 min. Due to the consequence of metabolic disposition of 4-amino-*N*-phenylbenzamides, the intermediates, 4-nitro-*N*-phenylbenzamides, though initially overlooked, were then explored for possible anticonvulsant activity. One notable molecule (**12**), *N*-(2-chloro-6-methylphenyl)-4-nitrobenzamide, demonstrated the most anticonvulsant activity among the four compounds tested. It was more active than the parent compound 4-ADMPB and by far more active than phenytoin.[17]

In a recent study,[26] Imidazole-based *N*-phenylbenzamide derivatives (e.g., **13**) have been shown to exhibit some activity against some cancer cell lines (IC_{50} = 7.5–11.1 μM). Diimidazoline *N*-phenylbenzamide (**14**) has been reported to exhibit antimalarial potency against the NF54 strain of *Plasmodium falciparum* with an IC_{50} value of 1.9 nM.[4]

NPBAs have the potential to inhibit the mitochondrial permeability transition pore.[32] Studies have shown that they are a possible polymorphophore. Polymorphism is the crystallization of the molecule in a solid state via various packing arrangements.[33]

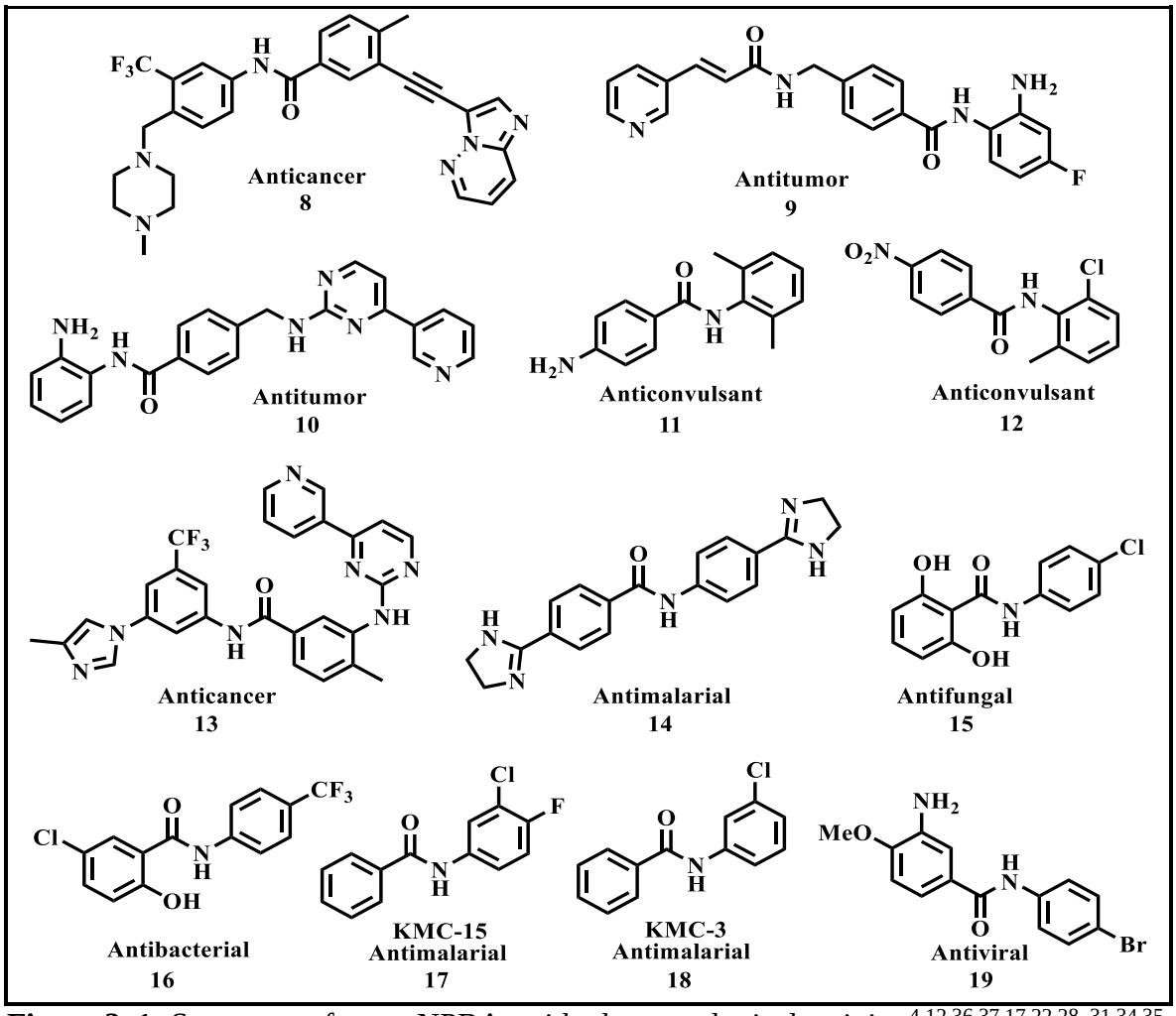

Figure 2. 1: Structures of some NPBAs with pharmacological activity.[4,12,36,37,17,22,28–31,34,35]

2.2.2 Antischistosomal Activity

NPBAs have demonstrated promising antischistosomal activity though they have not been extensively studied. Figure 2.2 exemplifies some NPBAs such as oxyclozanide (**24**),[38] niclosamide (**25**),[39] closantel (**26**),[40] rafoxanide (**27**),[41] among others with antischistosomal activity.

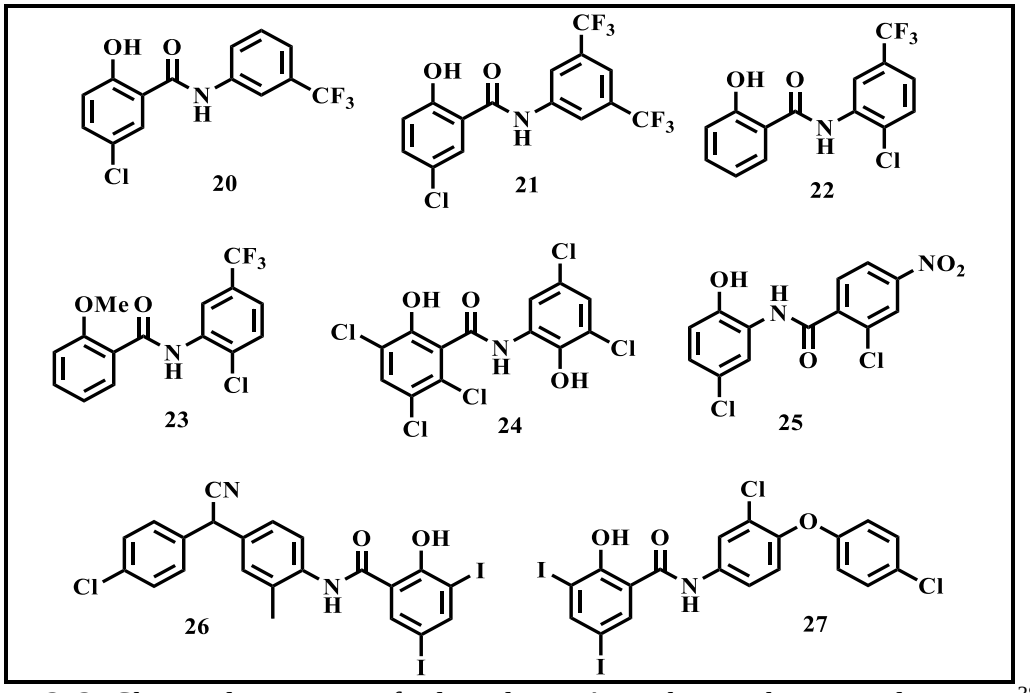

Figure 2. 2: Chemical structures of selected NPBAs with antischistosomal activity.[38–42]

MMV687807 (compound **28,** figure 2.3**),** an *N*-phenylbenzamide derivative was obtained from an *in vitro* antischistosomal screening of a library of 400 compounds. It was identified as a hit compound having exhibited lethal antischistosomal activity.[43]

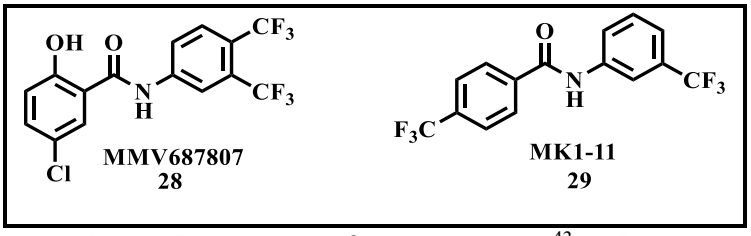

Figure 2. 3: Chemical structures of **MMV687807**,[43] and **MK1-11.**[44]

Further medicinal chemistry exploration of **MMV687807,** identified **MK1-11**(compound **29,** figure 2.3) which exhibited lethal *in vitro* activity at 5 µM on adult *Schistosoma mansoni* worms 48 h post-incubation. Nevertheless, it was not tested on juvenile worms. Table 2.1 illustrates *in vitro* antischistosomal results of **MMV687807** having exhibited submicromolar activity (IC_{50} = 0.3, 0.3 and 0.2 µM at 24, 48 and 72 h respectively) against juvenile *S. mansoni* worms while possessing micromolar activity on adult *S. mansoni* worms (IC_{50} = 9.7, 2.6, 2.2, 2.3 and 2.1 µM at 1, 16, 24, 48 and 72 h respectively). On *S. haematobium* adult worms, the compound was lethal at 10 µM. However, no structure-activity relationships and *in vivo* studies were conducted on this molecule.[43]

Table 2. 1*: In vitro* activity of MMV687807 *on S. mansoni* and *S. haematobium*.[b]

NTS[c] IC_{50} (µM)			adults IC_{50} (µM)					adults + albumin IC_{50} (µM)			effect (%) 10 µM *S. haematobium* adults		
24h	48h	72h	1h	16h	24h	48h	72h	24h	48h	72h	24h	48h	72h
0.3	0.3	0.2	9.7	2.6	2.2	2.3	2.1	5.7	5.5	5.6	100.0	100.0	100.0

[b]Adapted from Pasche et al., 2018.

[c]NTS, newly transformed schistosomula.

Figure 2.4 shows **MMV665852** (compound **30),** an *N,N'*-diarylurea that was also obtained from the screen of Medicines for Malaria Venture (MMV) Malaria box of 400 antimalarial compounds for antischistosomal activity.[45] Compound **30** emerged as the most active *in vitro* (IC_{50} = 0.8 µM) with 53% worm burden reduction (WBR) in *S. mansoni* infected mice.[46] It was active against both schistosomula and adult worms.[46]

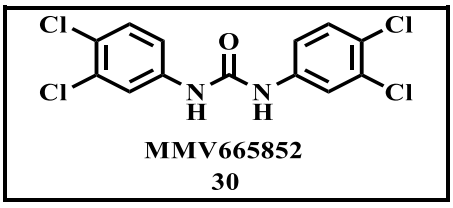

Figure 2. 4: Chemical structure of **MMV665852**.[42]

In a follow-up study, SARs of compound **30** against *S. mansoni* was investigated.[42] A total of 46 compounds with structures similar to compound **30** were identified using a Tanimoto-Rodgers similarity coefficient of 0.85 cut off from the screen of an MMV Open Access Malaria Box. These include 13 *N, N'*-diarylureas, 12 *N*-aryl, *N*'-alkylureas, 15 *N*-phenylbenzamides, 2 *N*-cyclohexylbenzamides, and 4 *N*-arylphenylcarbamates. The *in vitro* results showed that 13 compounds were lethal on NTS at 33.3 µM while 14 exhibited lethal activity on adult *S. mansoni* at the same concentration when incubated for 72 h. All the active compounds (IC_{50} ≤ 10 µM) were further evaluated for physicochemical properties such as solubility and passive intestinal permeability prior to *in vivo* studies.[42] The molecular weight (MW), hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) of the successful candidates were in the acceptable ranges of drug-likeness with a desirable clogP of ≤ 5. Aqueous solubilities were generally low (< 0.3 µM). Passive permeability was predicted to be good ($-logP_e$ < 5.7).[42]

Among the nine compounds that were progressed to *in vivo* studies ($IC_{90} \leq 10$ μM) in mice harboring adult *S. mansoni*, compound **22** (Figure 2.2) was the most active exhibiting WBR of 66% ($P < 0.05$) after a single oral dose of 400 mg/kg,[42] somewhat higher than 53% achieved with the same dose of **MMV665852**.[46] Compounds **20** and **21** (Figure 2.2) had decreased WBR of 12% and 36% respectively with observed toxicity in compound **21** causing death of two mice. Compound **23** (Figure 2.2) had reduced antischistosomal activity due to methylation of the phenol functional group, demonstrating the significance of salicylanilide.[42] Eight of the nine compounds were characterized as fast acting while seven had higher antischistosomal activity than compound **30** against both adult *S. mansoni* and NTS.[42]

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CHAPTER 3

METHODOLOGY

3.1 Chapter Overview

In this chapter, the rationale for the design of new target compounds is outlined. The chemistry employed, reagents, and solvents used are presented. The chapter further lays out the general procedure for the synthesis of new target compounds and the mechanism of DCC-DMAP-mediated amide coupling. The chapter concludes with the synthesis of nitrated *N*-phenyl benzamide target compounds.

3.2 Rationale for the Design of Target Compounds

In our laboratory, a series of *N*-phenylbenzamide derivatives have been generated. Figure 3.1 shows one notable compound, **MK1-11** demonstrating initial SARs suggesting that electron-withdrawing groups on the phenyl rings are superior to electron-donating groups activity-wise.

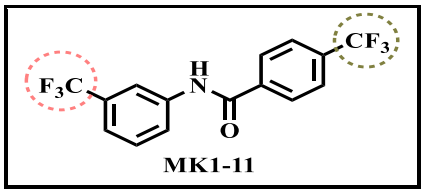


Figure 3. 1: Chemical structure of front-runner, **MK1-11**.[1]

In an attempt to enhance activity, the nitro group (NO_2), one of the strongest electron-withdrawing groups was adopted for bioisosteric replacement of trifluoromethyl group (CF_3) on the aniline portion of the molecule (Figure 3.2) for incorporation in the next generation of compounds. A bioisosteric replacement involves substitution of a part of a bioactive molecule with another substructure whose sizes and physicochemical properties are similar.[2,3]

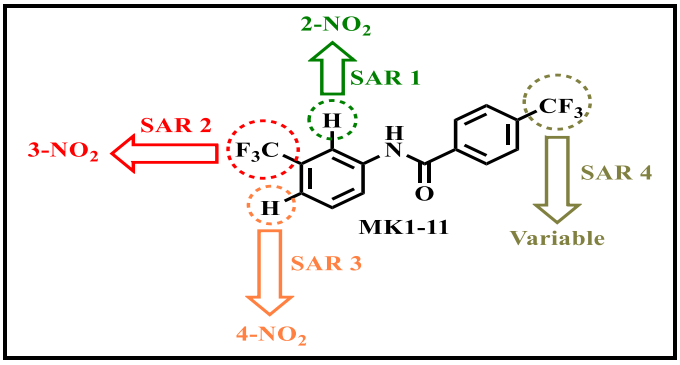


Figure 3. 2: Summary of the design of target compounds.

Figure 3.3 depicts a Craig plot, a very significant tool employed to predict the correlation that may exist between physicochemical properties of a molecule and its biological activity.[2]

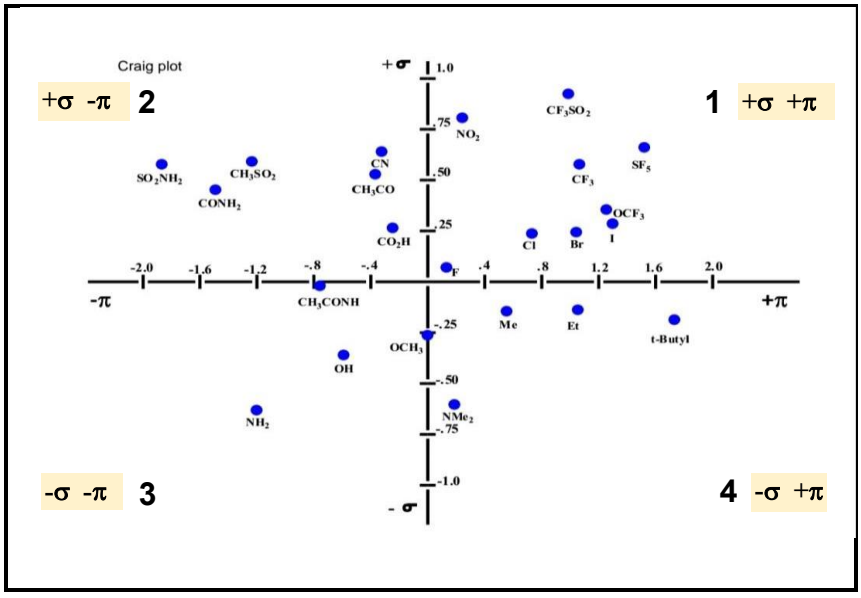


Figure 3. 3: Craig plot substituents in structure-activity relationships exploration.[4,5]

Analogs with the nitro on the *ortho*, *meta* and *para* positions were designed to understand whether or not positional changes are important for antischistosomal activity (Figure 3.4).

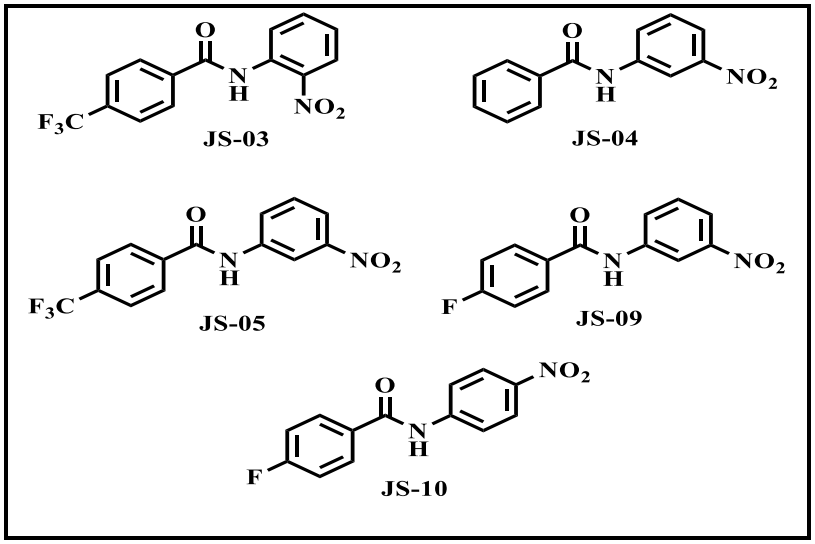


Figure 3. 4: Proposed target compounds.

3.3 Chemistry

3.3.1 Chemicals and Materials

Chemicals used in this MSc project were of general purpose reagent (GPR) and analytical reagent (AR) grade supplied by Sigma Aldrich. They were used as received without any purification. Materials used included but not limited to Whatman (125 mm) filter papers No.1, Merck KGaA TLC silica gel 60 F_{254} sheet (20 cm × 20 cm), aluminum foil and Aldrich silica gel (60–120 mesh).

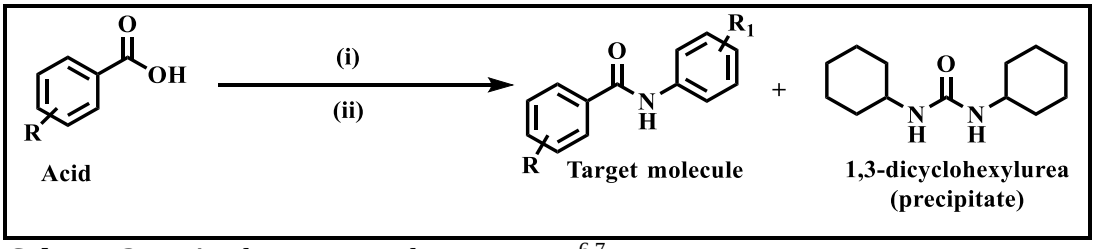
3.3.2 Equipment

Equipment used included a 254 nm handheld UV lamp, magnetic stirrer, laboratory thermometer (-10–110 ^{o}C), Analytical balance, BUCHI Rotavapor® R-100, GallenKamp Melting Point Apparatus, HPLC-MS, ^{1}H-NMR and ^{13}C-NMR Instruments.

3.3.3 Synthetic Protocol for Amide bond Formation

3.3.3.1 General Synthetic Scheme

The target compounds were synthesized via a simple one-step reaction involving acid-amine coupling of different anilines and different benzoic acid derivatives facilitated by *N*, *N*′-Dicyclohexylcarbodiimide (DCC) and 4-Dimethylaminopyridine (DMAP) (Scheme 3.1).



Scheme 3. 1: Acid-amine coupling reaction.[6,7]

Reagents and conditions: (i) DCM, DMAP, DCC, 0 ºC, 20 min; (ii) Nitroaniline, 25–33 °C, 24–72 h.

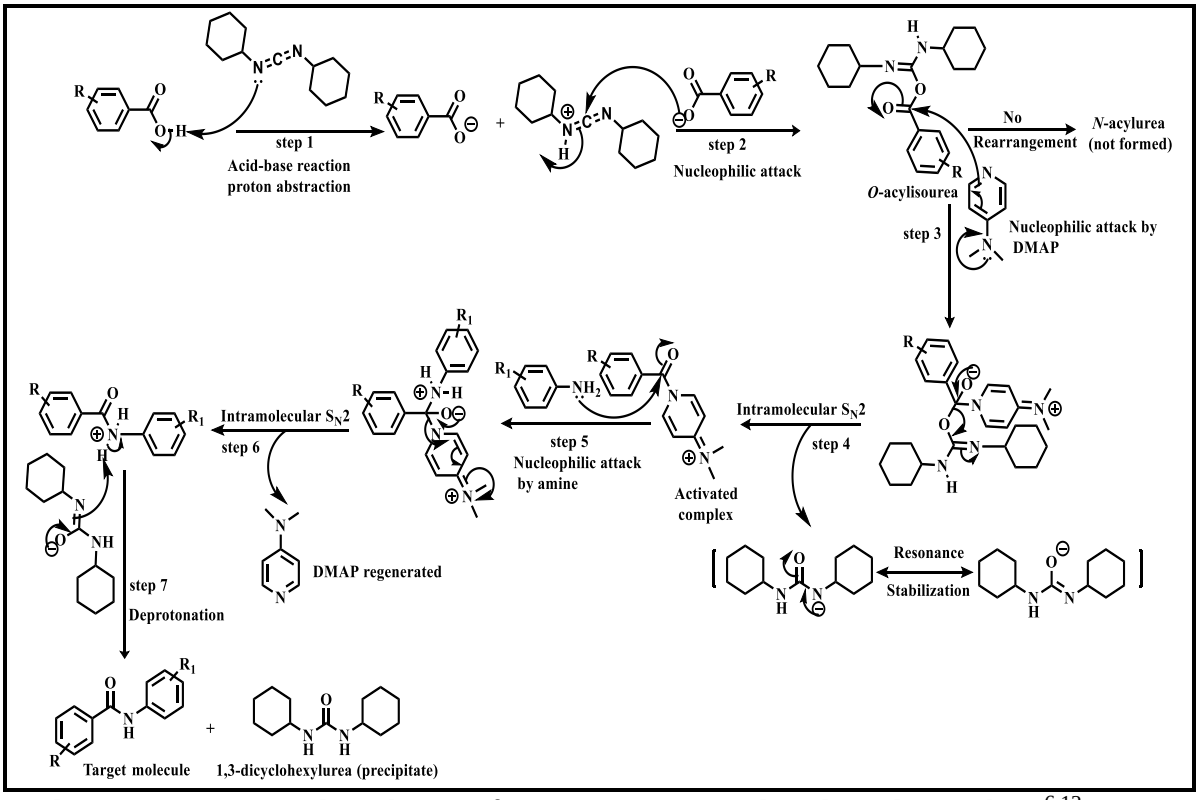
3.3.3.2 General Procedure for Synthesis of Target Compounds

The target compounds were synthesized by employing combined protocols from literature with minor modifications.[6–11]

1.2 eq of a suitable acid, 1.0 eq of DCC and 1.0 eq of DMAP were placed in a 50 mL round bottomed flask with 20 mL dichloromethane (DCM) sitting in a beaker containing crushed ice at 0 °C. The mixture was stirred to complete homogenization for 20 minutes with the aid of a magnetic stirrer. The reaction mixture was allowed to warm to above room temperature followed by addition of 1.0 eq of a particular amine. The resulting mixture was then kept above room temperature with continuous stirring while monitoring reaction progress with the aid of Thin Layer Chromatography (TLC). Upon completion of the reaction, the mixture was filtered to remove the precipitate (dicyclohexylurea). The collected filtrate was then washed with distilled water (30 mL × 5), saturated aqueous solutions of $NaHCO_3$ (30 mL × 3), NH_4Cl (30 mL × 3) and NaCl (30 mL × 3). The resulting organic layer was then dried using anhydrous sodium sulphate (Na_2SO_4) followed by filtration and *in vacuo* concentration (removal of solvents in the vacuum). To purify the resulting crude mixture, column chromatography was then employed using silica gel (60–120 mesh) and EtOAc in *n*-hexane as a mobile phase followed by trituration in diethylether to remove trace impurities. The compound was then dried *in vacuo* to furnish the desired product.

3.3.3.3 Mechanistic Details for DCC/DMAP-Mediated Coupling

The Formation of the amide bond in the mechanism (Scheme 3.2) involves deprotonation of the carboxylic acid by DCC.[6,12,13] A nucleophilic addition between the carboxylate ion and the protonated DCC (Step 2) affords an *O*-acylisourea intermediate.[6,14] The formed *O*-acylisourea intermediate is prone to rearrangement because of poor stability. It may rearrange to form the unreactive *N*-acylurea by acyl transfer in an intramolecular reaction.[6,7,15] If this rearrangement occurs, the amount of *O*-acylisourea available for further reaction may get compromised and in turn affect the yield of the product.[6,16] In view of the aforementioned, the acid is added to the coupling agent at 0 °C in the presence of DMAP, a catalytic nucleophile whose competitive nucleophilic attack on *O*-acylisourea (Step 3) is faster than the rearrangement side reaction thereby preventing it from taking place.[6,14,15] The *O*-acylisourea is, therefore, unavailable for rearrangement. An intramolecular S_N2 reaction (Step 4) produces an activated complex that reacts with the amine (Step 5) leading to the formation of a tetrahedral intermediate which then via an intramolecular S_N2 reaction (Step 6) leads to the formation of a protonated amide linkage while regenerating DMAP.[8] Deprotonation of the protonated amide linkage by the resonance stabilized molecule (Step 7) gives a neutral target molecule and a stable urea by-product as a precipitate.[6,12]

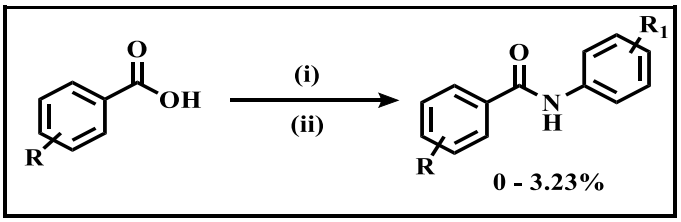


Scheme 3. 2: Proposed mechanism for DCC/DMAP-mediated amide coupling.[6,12]

3.3.4 Synthesis of Nitrated *N*-phenylbenzamide Target Compounds

3.3.4.1 Synthetic Scheme

The synthesis of target compounds was done by adopting the synthetic protocol reported in section 3.3.3.2. The yields generated at room temperature were very low despite relentless efforts to scale-up (Scheme 3.3).

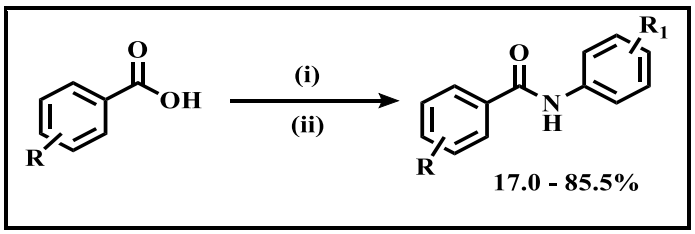


Scheme 3. 3: Acid-amine coupling reaction.

Reagents and conditions: (i) DCM, DMAP, DCC, 0 ºC, 20 min; (ii) Nitroaniline, 25–26 ºC, 24–72 h.

3.3.4.2 Synthetic Scheme at Optimized Temperature

Following poor percentage yields recorded on initial attempts to synthesize the target compounds (section 3.3.4.1), it was noted that a slight increase in temperature (≥ 0.5 °C) enhanced the % yields of the products. Further improvement of the % yields of the products was achieved by subjecting the reaction mixture to temperature optimization (≥ 5 °C) as depicted by scheme 3.4.



Scheme 3. 4: Acid-amine coupling reaction.

Reagents and conditions: (i) DCM, DMAP, DCC, 0 °C, 20 min; (ii) Nitroaniline, 30–33 °C, 24–26 h.

3.3.4.3 General Optimized Procedure for Synthesis of Target Compounds

4-(Trifluoromethyl)benzoic acid (0.100 g, 0.526 mmol, 1.2 eq), DCC (0.090 g, 0.438 mmol, 1.0 eq) and DMAP (0.054 g, 0.438 mmol, 1.0 eq) (**JS-03 & JS-05**), Benzoic acid (0.100 g, 0.819 mmol, 1.2 eq), DCC (0.141 g, 0.682 mmol, 1.0 eq) and DMAP (0.083 g, 0.682 mmol, 1.0 eq) (**JS-04**), 4-Fluorobenzoic acid (0.100 g, 0.713 mmol, 1.2 eq), DCC (0.123 g, 0.594 mmol, 1.0 eq) and DMAP (0.073 g, 0.594 mmol, 1.0 eq) (**JS-09 & JS-10**) were dissolved in 20 mL DCM using a 50 mL round bottomed flask sitting in a beaker containing crushed ice at 0 °C. The mixture was stirred for 20 minutes with the aid of a magnetic stirrer. The mixture was then allowed to warm to above room temperature in readiness for addition of the amine. The amine, 2-nitroaniline (0.061 g, 0.438 mmol, 1.0 eq, **JS-03**), 3-nitroaniline (0.094 g, 0.682 mmol, 1.0 eq, **JS-04**), 3-nitroaniline (0.061 g, 0.438 mmol, 1.0 eq, **JS-05**), 3-nitroaniline (0.082 g, 0.594 mmol, 1.0 eq, **JS-09**), 4-nitroaniline (0.082 g, 0.594 mmol, 1.0 eq, **JS-10**) was then added when the reaction mixture had reached a temperature of 30 °C (**JS-04**), 30.5 °C (**JS-03**), 31 °C (**JS-09**), 33 °C (**JS-05 & JS-10**). The resulting mixture was kept at this temperature while monitoring reaction progress with the aid of TLC. Upon completion of the reaction after 24 h (**JS-04, JS-05, JS-09, JS-10**), 26 h (**JS-03**), the reaction mixture was then filtered to remove the precipitate (dicyclohexylurea) while the filtrate was collected. The obtained filtrate was then washed with distilled water (30 mL × 5), saturated aqueous solutions of $NaHCO_3$

(30 mL × 3), NH_4Cl (30 mL × 3) and NaCl (30 mL × 3). The resulting organic layer was dried over anhydrous Na_2SO_4 for 48 hours followed by filtration and *in vacuo* concentration. Purification of the resulting crude mixture was performed using column chromatography with silica gel (60–120 mesh) as stationary phase and 20% EtOAc (**JS-03 & JS-04**), 40% EtOAc (**JS-09 & JS-10**), 60% EtOAc (**JS-05**) in *n*-hexane as mobile phase. To remove trace impurities and enhance crystallization, the purified compound was subjected to trituration in *n*-pentane/diethylether followed by drying *in vacuo* resulting in a desired product as greenish-yellow solid (0.0231 g, **JS-03**), yellow solid (0.0284 g, **JS-04**), ivory silk solid (0.0422 g, **JS-05**), white solid (0.1321 g, **JS-09**), pearl ivory solid (0.04286 g, **JS-10**).

The temperature for each reaction (Figure 3.5) was well noted and recorded using a laboratory thermometer (-10–110 ^oC).

Figure 3. 5: Reaction progress in the fume hood.

Figure 3.6 depicts an acid-amine reaction mixture for a complete trial reaction followed by filtration to remove the precipitate (dicyclohexylurea).

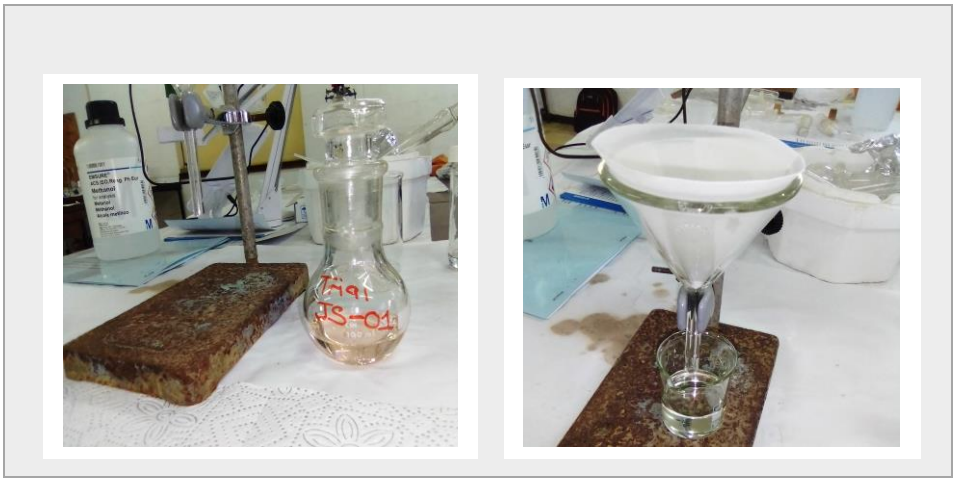
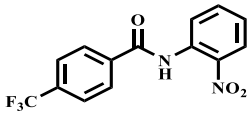
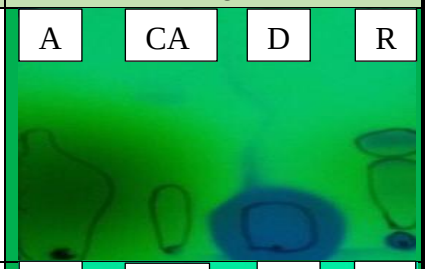
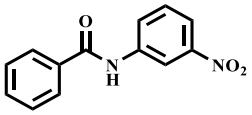
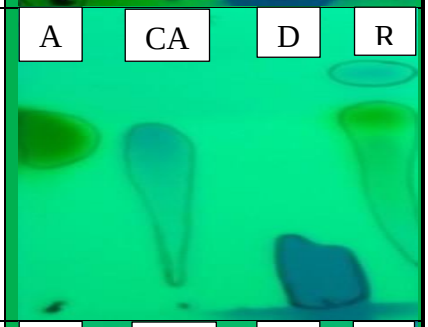
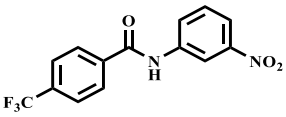
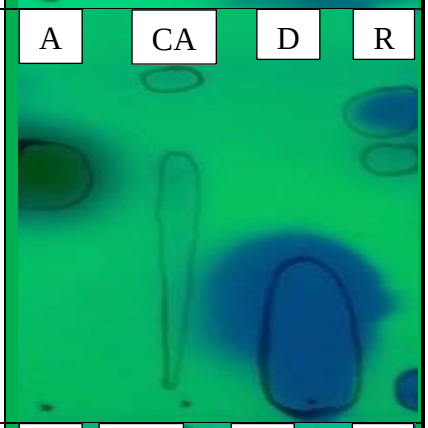
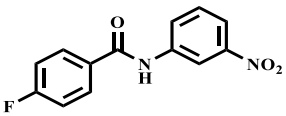
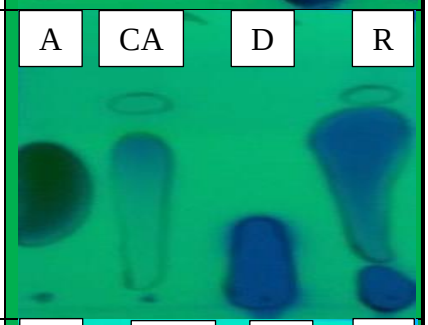
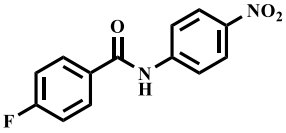
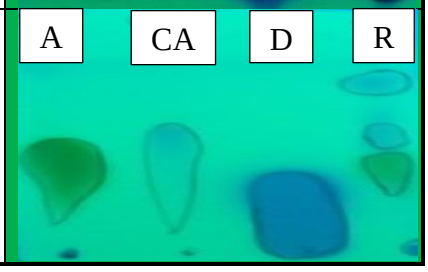


Figure 3. 6: Filtration of the trial acid-amine reaction mixture.

The progress of acid-amine coupling reactions was monitored by TLC (Table 3.1) using 254 nm pre-coated TLC plates and a 254 nm UV handheld lamp.

Table 3. 1: Monitoring acid-amine coupling reactions by thin layer chromatography.

Entry	Code	Chemical structure	TLC
1	**JS-03**		
2	**JS-04**		
3	**JS-05**		
4	**JS-09**		
5	**JS-10**		

Key: A = Amine, CA = Carboxylic acid, D = DMAP, R = Reaction mixture

The reaction mixture (R) spots when compared to reactants spots: A, CA and D in all the reactions showed one distinct spot that was attributed to the formation of the product.

Figure 3.7 demonstrates the purification of the crude mixture by column chromatography.

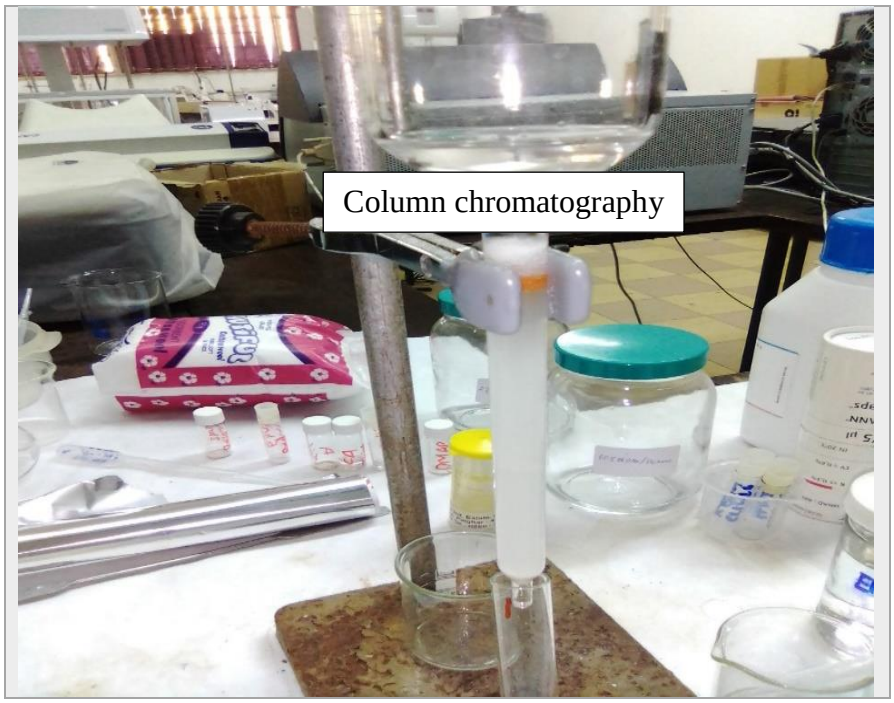


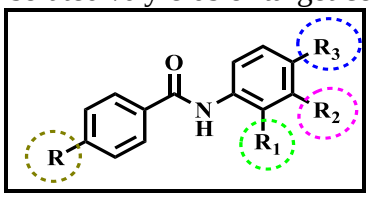
Figure 3. 7: Purification of crude mixture by column chromatography.

The obtained organic sample was then dried *in vacuo* aided by a BUCHI Rotavapor® R-100 to furnish the desired product (Figure 3.8).

Figure 3. 8: Drying of the final product *in vacuo*.

Table 3.2 summarizes corresponding isolated percentage yields for the synthesized final compounds obtained in low to good yield (17.0–85.5%). Their successful synthesis was then carefully scrutinized by HPLC-MS, ^{1}H-NMR and ^{13}C-NMR spectroscopic techniques for confirmation as outlined in the next chapter (Chapter four).

Table 3. 2: Isolated % yields of target compounds.



Code	R	R_1	R_2	R_3	% Yield
JS-03	CF_3	NO_2	H	H	17.0
JS-04	H	H	NO_2	H	17.2
JS-05	CF_3	H	NO_2	H	31.1
JS-09	F	H	NO_2	H	85.5
JS-10	F	H	H	NO_2	27.73

3.4 Media, Compound Dilution and Assay Plates

The antischistosomal assay experiment was carried out at the Swiss TPH (Basel, Switzerland), in accordance with Swiss National animal welfare regulations.

S. mansoni cercariae were harvested from *S. mansoni*-infected *B. glabrata*, and mechanically transformed to NTS using standard procedures.[17–29] The resulting NTS suspension was then kept in TSS 199 culture medium enriched with 5% heat-inactivated foetal calf serum (iFCS) and 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, USA) incubated at 37 °C in 5% CO_2 atmosphere for a minimum period of 3-12 h before use.[30] Wells containing 100 NTS each were then incubated in a 96-well plate (BD Falcon) with 10 µM and 50 µM compound diluted in supplemented medium 199.[17] Incubation of NTS in a compound-free DMSO in the assay at a volume equivalent to the highest compound concentration served as control (negative control).[17,31,32] The assay was performed twice in triplicate (three wells).[17] A viability scale was employed to evaluate the effects of the compounds via microscopical readout (Carl Zeiss, Germany, magnification 80×) with respect to death, changes in motility, viability, and morphological alterations 72 h post compound exposure.[24]

3.5 References

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chapter Overview

In this chapter, the synthesis of five target compounds is discussed followed by the presentation of their characterization encompassing HPLC-MS, ^{1}H-NMR, ^{13}C-NMR, melting point (m.p), and retardation factor, R_f. Furthermore, *in vitro* antischistosomal results of target compounds are covered. The chapter concludes with preliminary structure-activity relationships drawn from *in vitro* antischistosomal results and comments on statistical analysis.

4.2 Synthesis of target compounds

The carboxy activating agent, DCC is one of the most powerful and common coupling reagents used in the synthesis of amides, anhydrides, esters, etc.[1] It facilitates the coupling of free primary amines and carboxylic acids to produce amide bonds aided by a nucleophilic catalyst, DMAP at room temperature in high yield.[2]

The first attempts to synthesize the target compounds were met with challenges and did not work well in our hands at room temperature (25–26 °C) based on the synthetic protocols from literature sources as reported in the methodology (section 3.3.3.2). Yields of the products were drastically compromised ranging from low to moderate.

The difficulties encountered in this coupling strategy were thought to arise from poor nucleophilicity of the amino nitrogen of aniline due to its attachment to a phenyl group with a highly electron-withdrawing nitro group. According to Due-Hansen and co-workers, coupling at elevated temperatures improved the yields for such electron-deficient amines.[3]

In a quest to enhance the yields of the products, the synthetic protocol was then modified by optimizing the temperature (30–33 °C) leading to the synthesis of five target compounds in low to good yield (17.0–85.5%).

Ghosh et al (2021) have reported that 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI)/DMAP-mediated coupling was more convenient for electron deficient amines because of the formation of a water-soluble urea by-product leading to production of better yields than DCC-based coupling reactions.[4] They further postulated that chromatographic purification and removal of *N,N'*-Dicyclohexylurea a by-product constituted the major bottlenecks of DCC-based coupling reactions thereby compromising the yields of the products.[4]

4.3 Characterization of target compounds

All the synthesized target compounds were characterized by HPLC-MS, ^{1}H-NMR, ^{13}C-NMR, melting point (m.p), and retardation factor, R_f. ^{1}H-NMR and ^{13}C-NMR results were analyzed using a spectral data analyzing software called *MestReNova.*

4.3.1 Characterization of *N*-(2-nitrophenyl)-4-(trifluoromethyl)benzamide (JS-03)

4.3.1.1 HPLC-MS Analysis

The HPLC-MS (Figure 4.1) shows an HPLC chromatogram with a peak at 1.152 min retention time having observed purity (100%) based on peak area and total area of peaks under interest. However, due to the likelihood of inorganic impurities (residual on ignition), the percentage purity was considered as **99.90%**. The APCI^{+} mass spectrum revealed a pseudo-molecular ion $[M + H]^{+}$ (*m/z* = 311.1) that was consistent with the calculated exact mass (310.06) for the target compound.

HPLC-MS analysis is, however, not exhaustive to fully confirm the successful synthesis of the target compound. The more reliable and superior ^{1}H-NMR and ^{13}C-NMR analysis were then employed. This approach was extended to the characterization of the other target compounds.

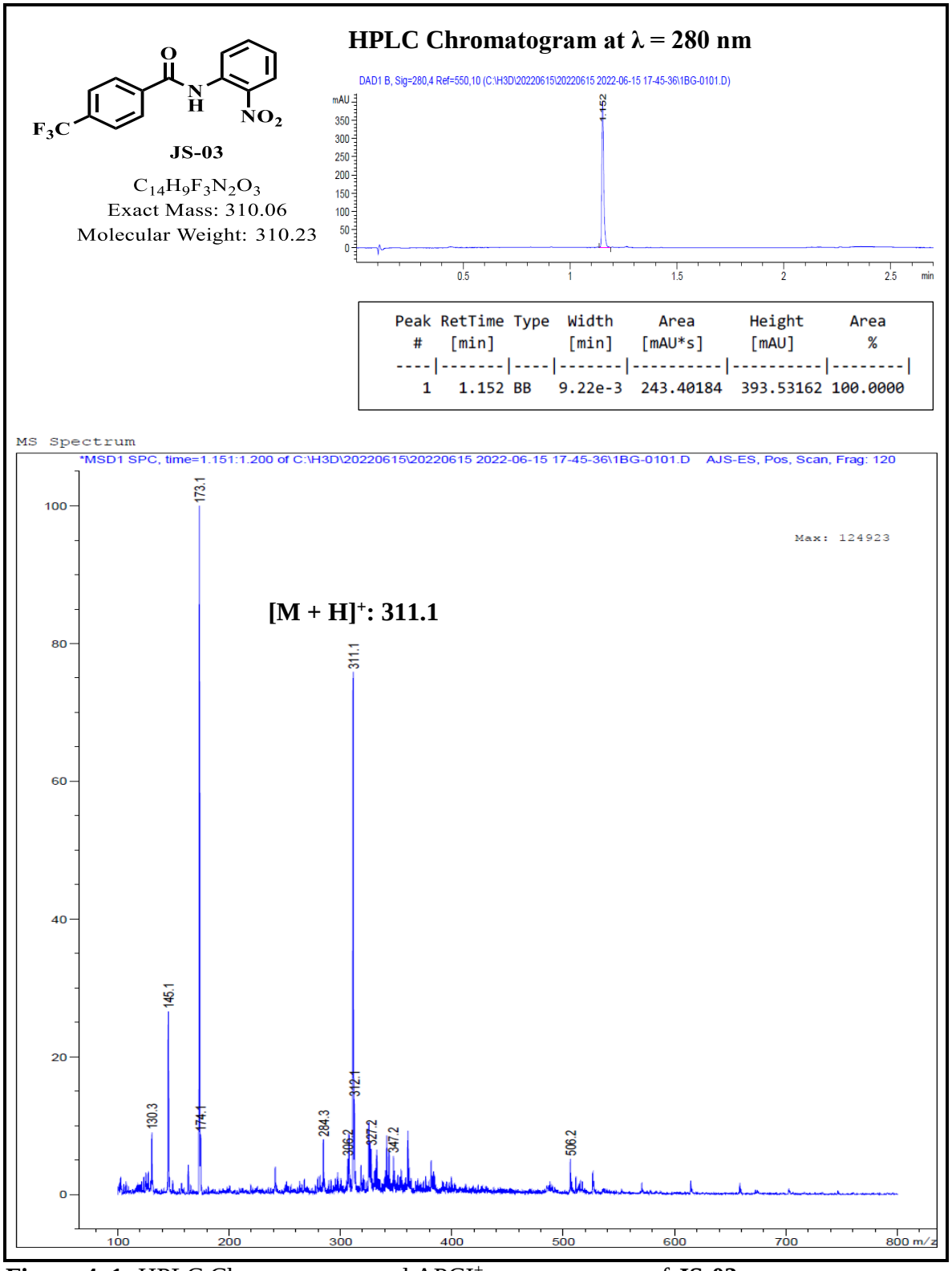


Figure 4. 1: HPLC Chromatogram and APCI$^+$ mass spectrum of **JS-03.**

4.3.1.2 ^{1}H-NMR Analysis

The ^{1}H-NMR spectrum (Figure 4.2) shows six proton signals representative of seven chemically distinct proton environments (numbered), and nine protons present in the chemical structure of the target compound.

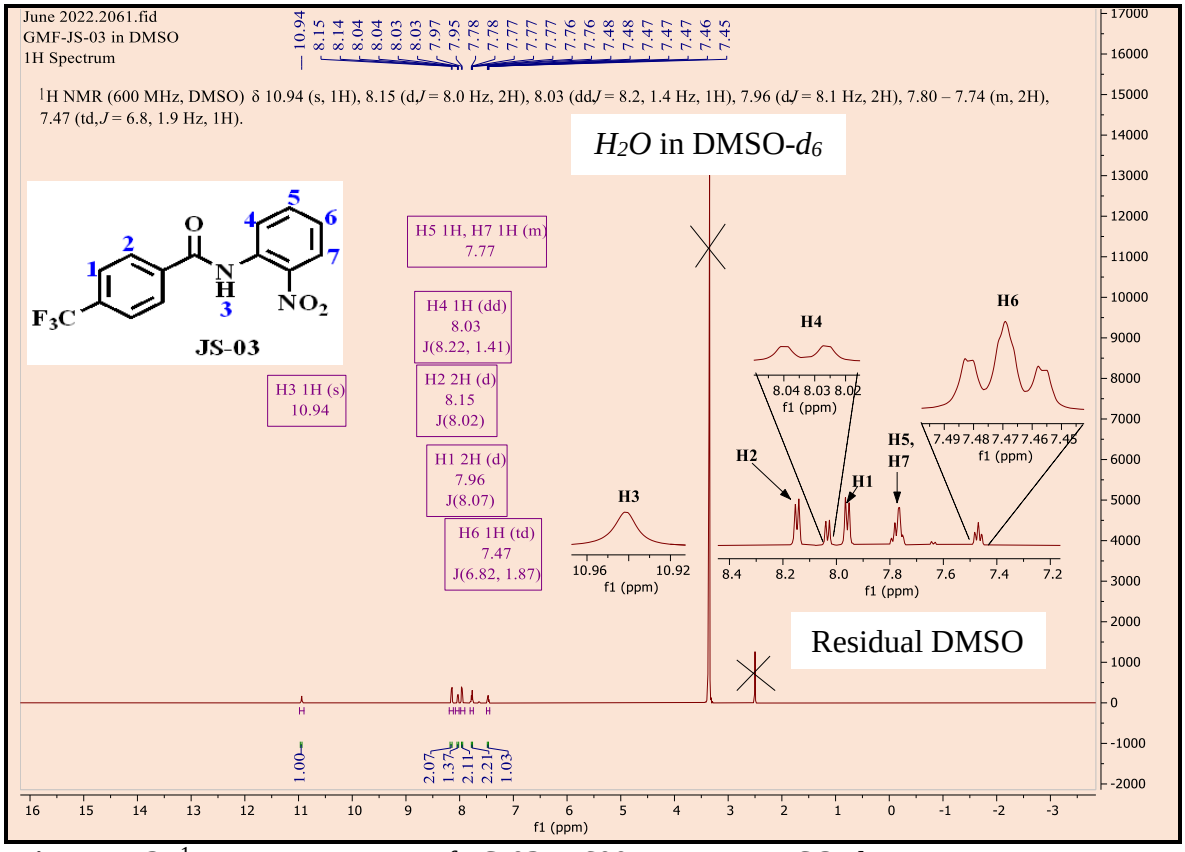


Figure 4. 2: ^{1}H-NMR spectrum of **JS-03** at 600 MHz in DMSO-d_6.

As anticipated, a sharp signal integrating for one proton and most downfield was observed at δ =10.94 ppm as a singlet (*s*) corresponding to the N*H* proton (H3). A doublet signal at δ = 8.15 ppm, integrating for two protons, was attributed to H2 with the multiplicity pattern arising from a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H1 justified by a large coupling constant (J = 8.02 Hz). The signal at δ = 8.03 ppm (H4) integrating for one proton appears as a doublet of doublets (*dd*) having undergone both a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H5 with a larger coupling constant (J = 8.22 Hz) and a long-range coupling ($^{4}J_{H\text{-}H}$) to H6 with a smaller coupling constant (J = 1.41 Hz). H1, resonating at δ = 7.96 ppm as a two-proton signal, experiences a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H2 affording a doublet (*d*) with a large coupling constant (J = 8.07 Hz). The multiplicity pattern observed at δ = 7.77 ppm, integrating for two protons, was attributed to H5 (1H) and H7 (1H) resonating as a multiplet (*m*). A well-defined triplet of

doublets (*td*) integrating for one proton appears at δ = 7.47 ppm. This signal corresponds to H6 that experiences a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to the magnetically equivalent protons H5 and H7 (J = 6.82 Hz) yielding a triplet (*t*) that further undergoes a long-range coupling ($^{4}J_{H\text{-}H}$) to H4 (J = 1.87 Hz) splitting into a triplet of doublets (*td*). The splitting of H6 into a triplet by H5 and H7 followed by its subsequent conversion to a triplet of doublets by H4 justifies that H5 and H7 are magnetically equivalent protons and resonate at the same frequency as evidenced by the multiplet observed at δ = 7.77 ppm .This multiplet justifies the observed six signals as representative of seven chemically distinct proton environments in the molecule. The total integral value of nine for the signals correlated with the total number of protons in the molecule demonstrating a well-resolved ^{1}H-NMR spectrum with all the protons assigned, mapped and accounted for.

4.3.1.3 ^{13}C-NMR Analysis

The ^{13}C-NMR spectrum (Figure 4.3) shows 12 carbon signals representative of 12 chemically distinct carbon environments (numbered), and 14 carbons present in the chemical structure of the target compound.

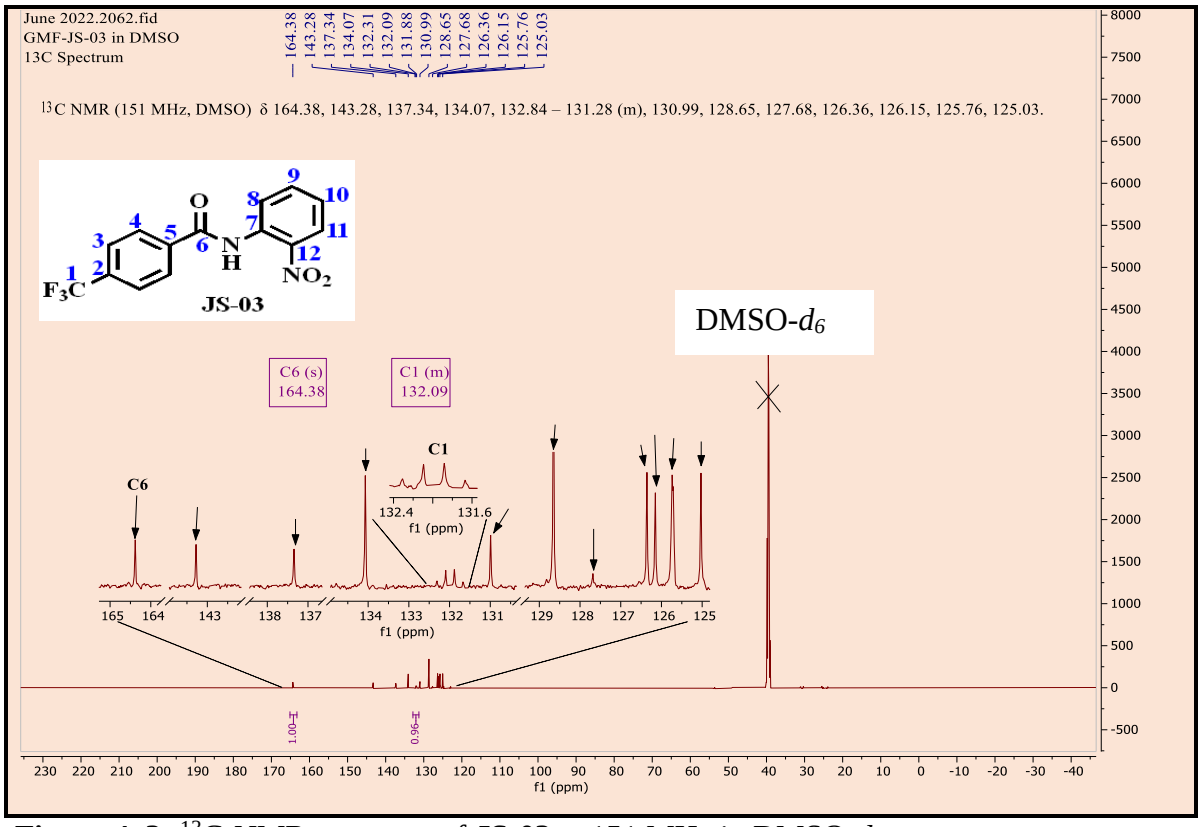


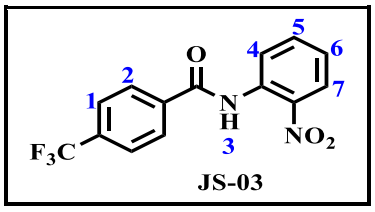
Figure 4. 3: ^{13}C-NMR spectrum of **JS-03** at 151 MHz in DMSO-d_6.

Expectedly, the most downfield diagnostic signal at δ = 164.38 ppm appears as a singlet (*s*) and is associated with the carbonyl carbon (C6). The other notable diagnostic signal observed at δ = 132.09 ppm and attributed to C1 is an expected quartet (*q*) from a very short-range geminal coupling (two-bond coupling, $^{2}J_{C\text{-}F}$). However, a multiplet (*m*) was resolved instead despite it having a typical quartet appearance. A clear quartet was obtained in its regioisomer, **JS-05** (see section 4.3.3.3).

The other carbons could not be assigned because further sophisticated NMR experiments such as the two-dimensional (2D) Correlation Spectroscopy (COSY) and Heteronuclear Single Quantum Coherence (HSQC) that could aid in assigning them were beyond the scope of this MSc project. However, the one-dimensional (1D) ^{13}C-NMR analysis employed in this project focused on a few carbons giving rise to key diagnostic signals which was sufficient to confirm the successful synthesis of the molecules. Therefore, all the synthesized target compounds were analyzed and confirmed in the same manner.

The 12 chemically distinct carbon environments in the structure of the target compound were accounted for. The 12 signals observed in the ^{13}C-NMR spectrum represent 12 chemically distinct carbon environments.

4.3.1.4 Comprehensive Characterization of JS-03



JS-03

^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.94 (s, 1H, H^{3}), 8.15 (d, *J* = 8.0 Hz, 2H, H^{2}), 8.03 (dd, *J* = 8.2, 1.4 Hz, 1H, H^{4}), 7.96 (d, *J* = 8.1 Hz, 2H, H^{1}), 7.80 – 7.74 (m, 2H, H^{5}, H^{7}), 7.47 (td, *J* = 6.8, 1.9 Hz, 1H, H^{6}); ^{13}C-NMR (151 MHz, DMSO-d_6) δ 164.38, 143.28, 137.34, 134.07, 132.84 – 131.28 (m), 130.99, 128.65, 127.68, 126.36, 126.15, 125.76, 125.03, m.p. 110.8–113.8 °C; R_f (EtOAc:Hex, 1:4) 0.56; APCI^{+}: *m/z* [M + H]$^{+}$ = 311.1, calculated exact mass = 310.06, purity = 99.90%, t_r = 1.152 min.

4.3.2 Characterization of *N*-(3-nitrophenyl)benzamide (JS-04)

4.3.2.1 HPLC-MS Analysis

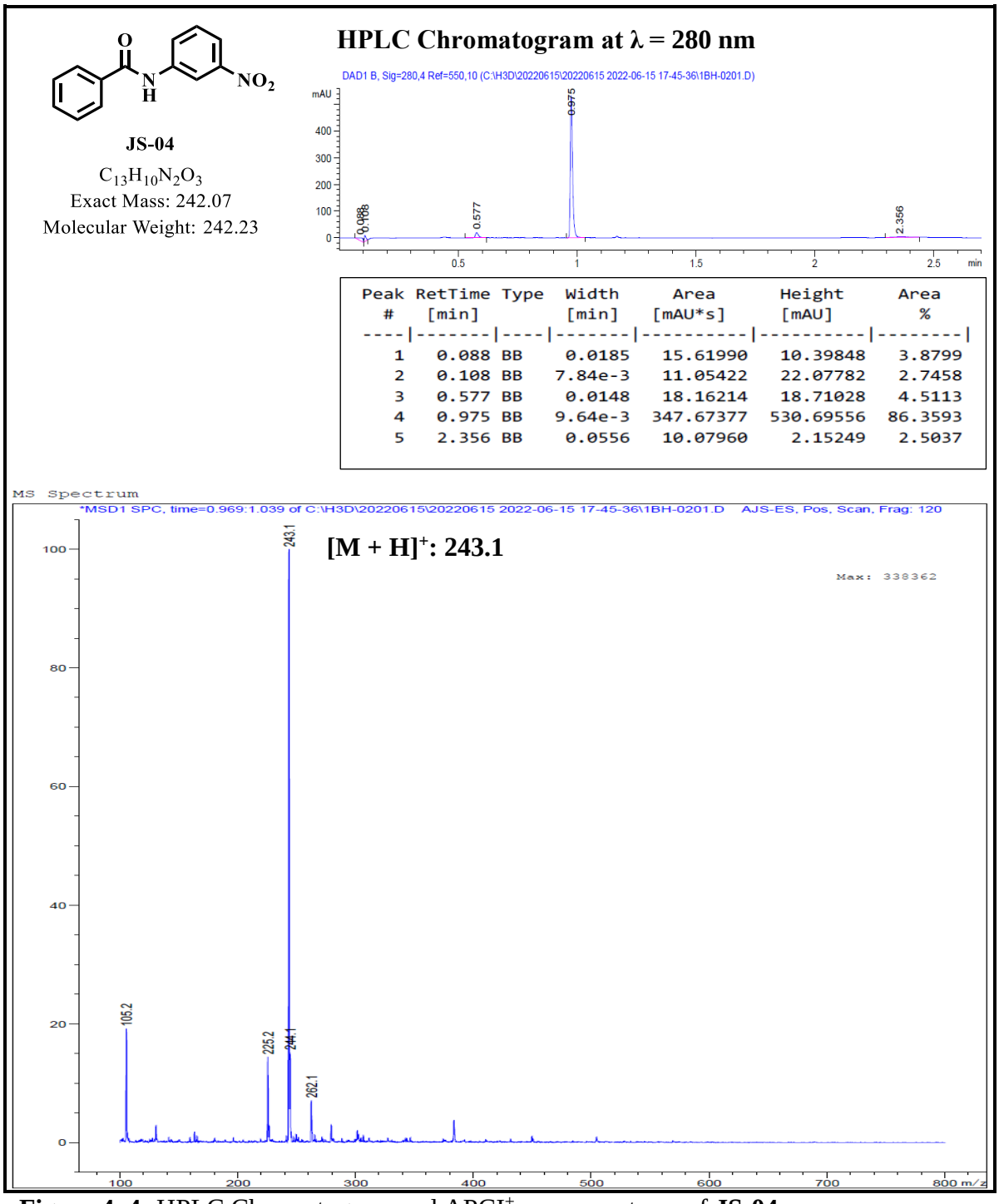


Figure 4. 4: HPLC Chromatogram and APCI$^+$ mass spectrum of **JS-04.**

Figure 4.4 depicts a peak at 0.975 min retention time on the HPLC chromatogram with observed purity (86.3593%) attributed to both the sample and solvent peaks having been integrated as well, hence, included in the calculation by the instrument.

The correct purity was, therefore, determined by recalculating it manually while ignoring solvent peaks. It was calculated as peak area percentage of total area of peaks under interest.[5] The recalculated purity obtained was **97.18%**. The APCI$^+$ mass spectrum shows a pseudo-molecular ion $[M + H]^+$ (m/z = 243.1) that was comparable to the calculated exact mass (242.07) for the target compound.

4.3.2.2 ^{1}H-NMR Analysis

The ^{1}H-NMR spectrum (Figure 4.5) consisting of seven proton signals accounted for eight chemically distinct proton environments (numbered), and 10 protons present in the chemical structure of the target compound.

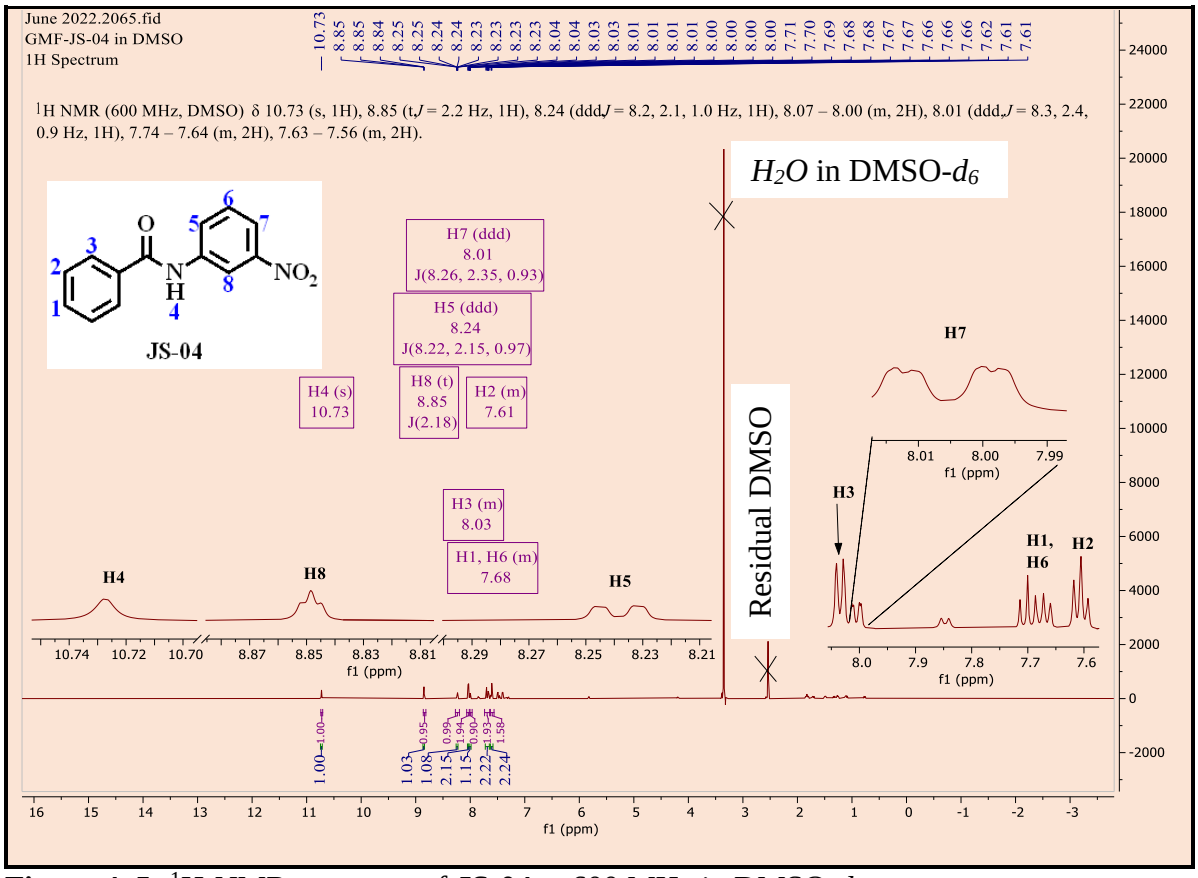


Figure 4. 5: ^{1}H-NMR spectrum of **JS-04** at 600 MHz in DMSO-d_6.

The most downfield signal at δ = 10.73 ppm integrating for one proton appears as a singlet (*s*) just as expected. This signal is attributed to H4. The observed signal slightly upfield at δ = 8.85 ppm integrating for one proton appears as a triplet (*t*) and is attributed to H8 that experiences equal long-range coupling ($^4J_{H\text{-}H}$) to H5 and H7 justified by a small coupling constant (J = 2.18 Hz). Further upfield, a signal integrating for one proton at δ = 8.24 ppm corresponds to H5 giving rise to a doublet of doublets of doublets (*ddd*). It undergoes a short-range vicinal coupling ($^3J_{H\text{-}H}$) to H6 with a larger coupling constant (J = 8.22 Hz), a long-range coupling ($^4J_{H\text{-}H}$) to H7 with a smaller coupling constant (J = 2.15 Hz) and a further long-range coupling ($^4J_{H\text{-}H}$) to H8 with the smallest coupling constant (J = 0.97 Hz). A signal at δ = 8.03 ppm integrating for two protons gives rise to a multiplet (*m*) attributed to H3. Another doublet of doublets of doublets (*ddd*) appears at δ = 8.01 ppm. It is attributed to H7 that undergoes a short-range vicinal coupling ($^3J_{H\text{-}H}$) to H6 with a larger coupling constant (J = 8.26 Hz), a long-range coupling ($^4J_{H\text{-}H}$) to H5 with a smaller coupling constant (J = 2.35 Hz) and a further long-range coupling ($^4J_{H\text{-}H}$) to H8 with the smallest coupling constant (J = 0.93 Hz). A multiplet (*m*) arising from a signal observed at δ = 7.68 ppm integrating for two protons is attributed to H1 and H6. The most upfield signal at δ = 7.61 ppm integrating for two protons corresponds to H2. It appears as a multiplet (*m*) contrary to the expected triplet (*t*) from a short-range vicinal coupling ($^3J_{H\text{-}H}$) to H1 and H3. The multiplet at δ = 7.68 ppm justifies the observed seven signals as representative of eight chemically distinct proton environments in the molecule. Relatedly, the total integral value of 10 confirms the presence of 10 protons in the molecule.

4.3.2.3 ^{13}C-NMR Analysis

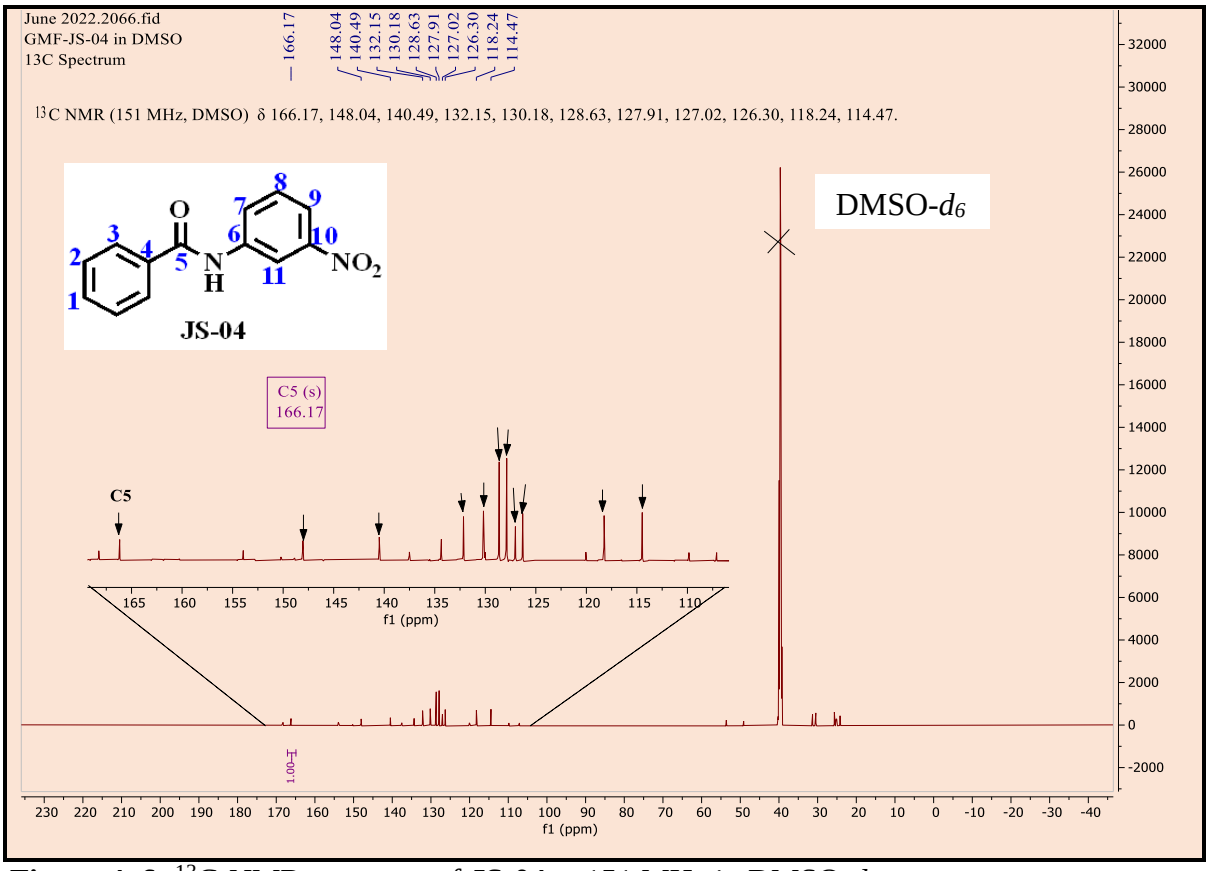


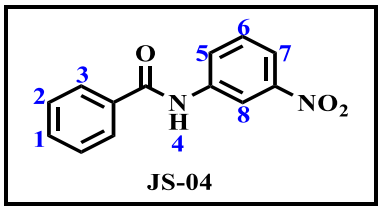
Figure 4. 6: ^{13}C-NMR spectrum of **JS-04** at 151 MHz in DMSO-d_6.

Figure 4.6 shows a ^{13}C-NMR spectrum comprising 11 carbon signals representative of 11 chemically distinct carbon environments (numbered), and 13 carbons present in the chemical structure of the target compound.

As expected, a very intense and most downfield diagnostic signal observed at δ = 166.17 ppm appears as a singlet (*s*) attributed to the carbonyl carbon (C5).

Admittedly, the sample for this compound appears to have some impurities as evidenced from the extra peaks in the ^{1}H-NMR and ^{13}C-NMR spectra. Current studies in our research group are focusing on obtaining such a compound in a more pure form.

4.3.2.4 Comprehensive Characterization of JS-04



JS-04

^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.73 (s, 1H, H^4), 8.85 (t, J = 2.2 Hz, 1H, H^8), 8.24 (ddd, J = 8.2, 2.1, 1.0 Hz, 1H, H^5), 8.07 – 8.00 (m, 2H, H^3), 8.01 (ddd, J = 8.3, 2.4, 0.9 Hz, 1H, H^7), 7.74 – 7.64 (m, 2H, H^1, H^6), 7.63 – 7.56 (m, 2H, H^2); ^{13}C-NMR (151 MHz, DMSO-d_6) δ 166.17, 148.04, 140.49, 132.15, 130.18, 128.63, 127.91, 127.02, 126.30, 118.24, 114.47, m.p. 124.9 – 126.8 °C; R_f (EtOAc:Hex, 1:4) 0.35; APCI$^+$: m/z $[M + H]^+$ = 243.1, calculated exact mass = 242.07, purity = 97.18%, t_r = 0.975 min.

4.3.3 Characterization of *N*-(3-nitrophenyl)-4-(trifluoromethyl)benzamide (JS-05)

4.3.3.1 HPLC-MS Analysis

Figure 4.7 shows an HPLC chromatogram with purity (95.1702%) at 1.113 min retention time. However, the observed purity is not a correct value as it includes solvent peaks that were equally integrated. The correct value (**97.73%**) was obtained by recalculating it manually as described in the case of **JS-04**.[5] The molecular ion $[M + H]^+$ (m/z = 311.1) observed on the APCI$^+$ mass spectrum was consistent with the calculated exact mass (310.06) for the target compound.

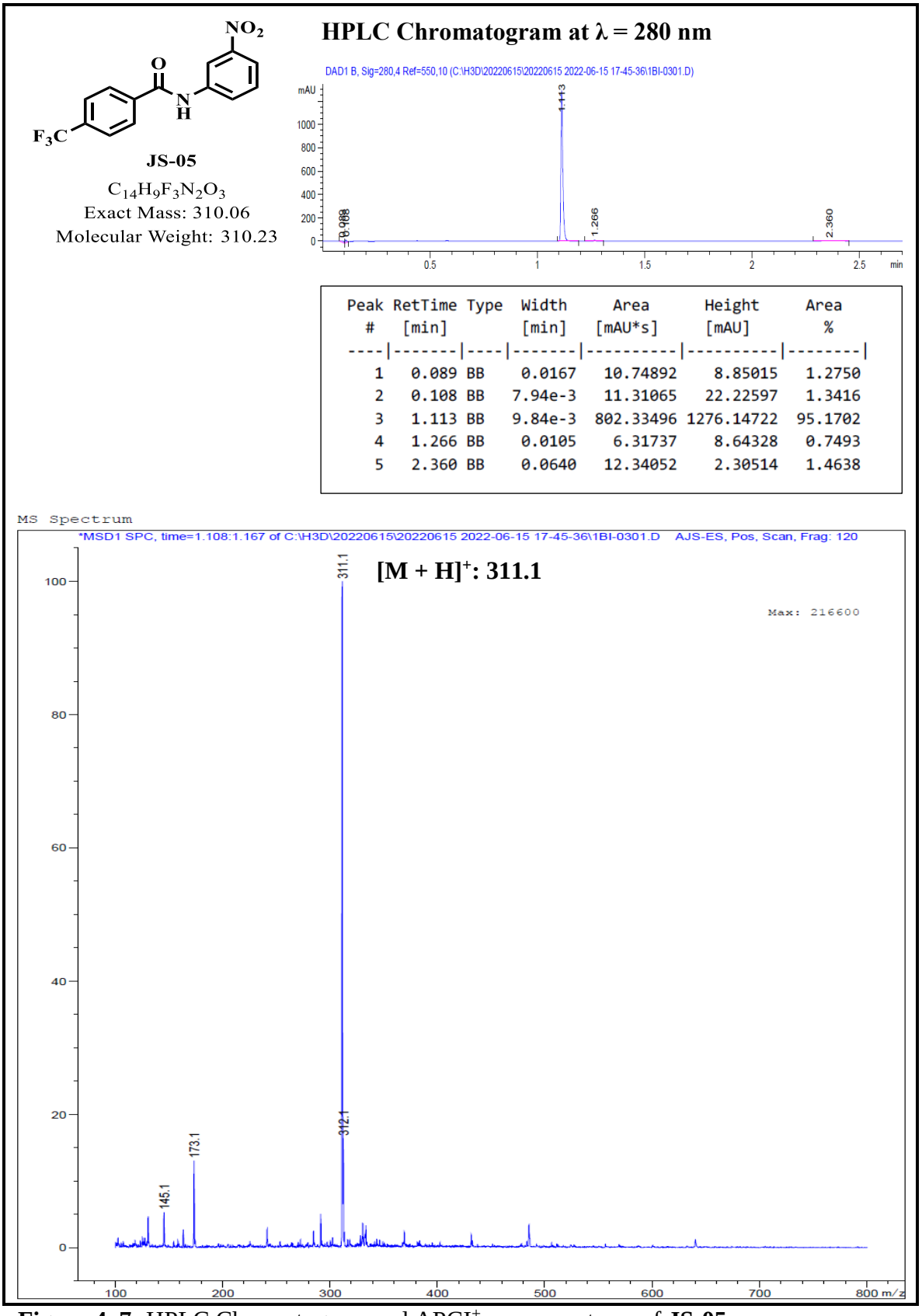


Figure 4. 7: HPLC Chromatogram and APCI$^+$ mass spectrum of **JS-05.**

4.3.3.2 ^{1}H-NMR Analysis

The ^{1}H-NMR spectrum (Figure 4.8) shows six proton signals representative of seven chemically distinct proton environments (numbered), and nine protons present in the chemical structure of the target compound.

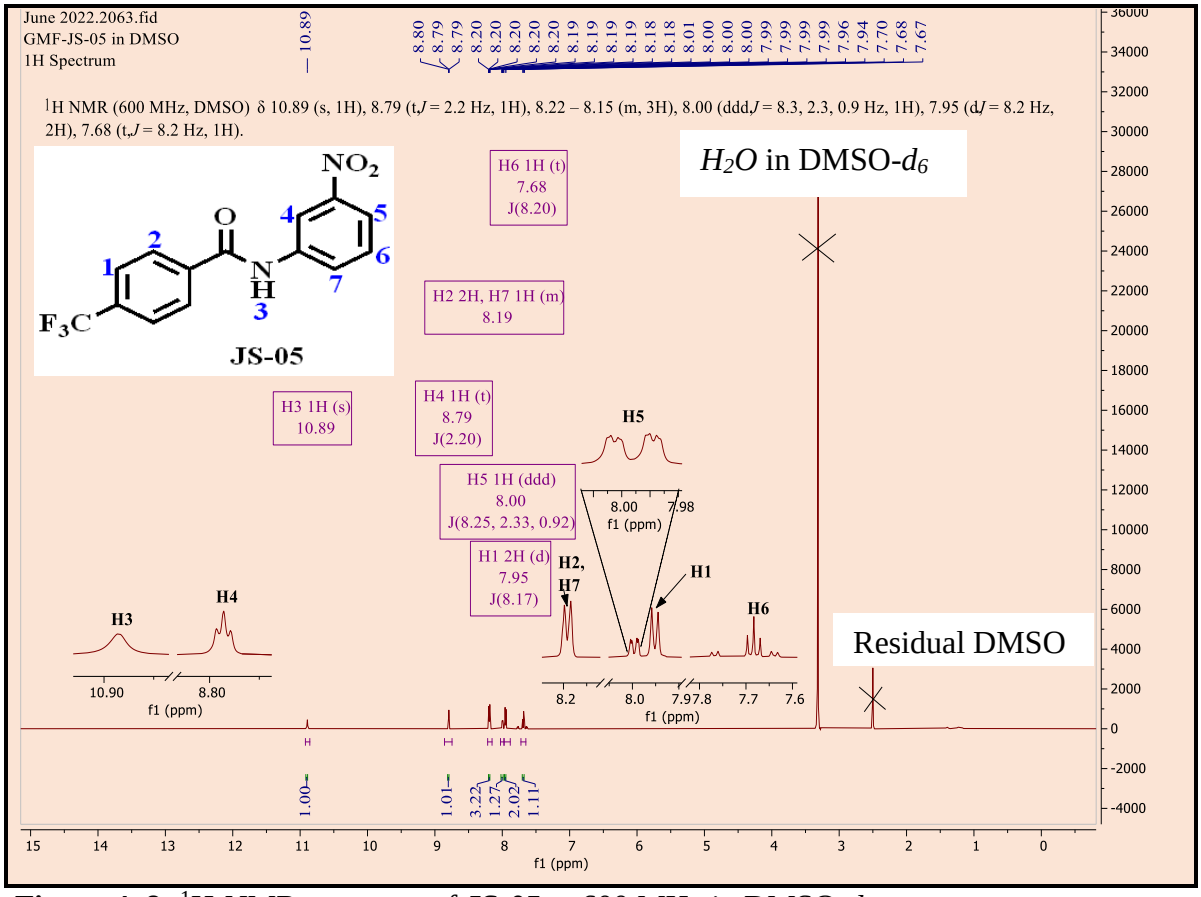


Figure 4. 8: ^{1}H-NMR spectrum of **JS-05** at 600 MHz in DMSO-d_6.

A sharp and most downfield diagnostic singlet signal observed at δ = 10.89 ppm, integrating for one proton, corresponds to H3. A relatively upfield triplet signal (δ = 8.79 ppm), integrating for one proton, is attributed to H4 that experiences equal long-range coupling ($^{4}J_{H\text{-}H}$) to H5 and H7 evidenced by a small coupling constant (J = 2.20 Hz). Protons H2 and H7 resonated as a multiplet (*m*) (δ = 8.19 ppm). A very vivid signal (δ = 8.00 ppm), integrating for one proton, was associated with H5. This proton experiences a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H6 (J = 8.25 Hz), a long-range coupling ($^{4}J_{H\text{-}H}$) to H7 (J = 2.33 Hz) and a further long-range coupling ($^{4}J_{H\text{-}H}$) to H4 (J = 0.92 Hz) giving rise to a doublet of doublets of doublets (*ddd*).

A signal at δ = 7.95 ppm integrating for two protons was associated with H1. This proton gives rise to a doublet (*d*) having undergone a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H2, evidenced by a large coupling constant (J = 8.17 Hz). The most upfield signal (δ = 7.68 ppm) integrating for one proton is attributed to H6 and appears as a triplet (*t*). It experiences equal short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H5 and H7 (J = 8.20 Hz). The multiplet observed at δ = 8.19 ppm justifies the six signals observed on the ^{1}H-NMR spectrum. The total integral value of nine for the signals was consistent with the nine protons in the molecule.

4.3.3.3 ^{13}C-NMR Analysis

The ^{13}C-NMR spectrum (Figure 4.9) shows 12 carbon signals representative of 12 chemically distinct carbon environments (numbered), and 14 carbons present in the chemical structure of the target compound.

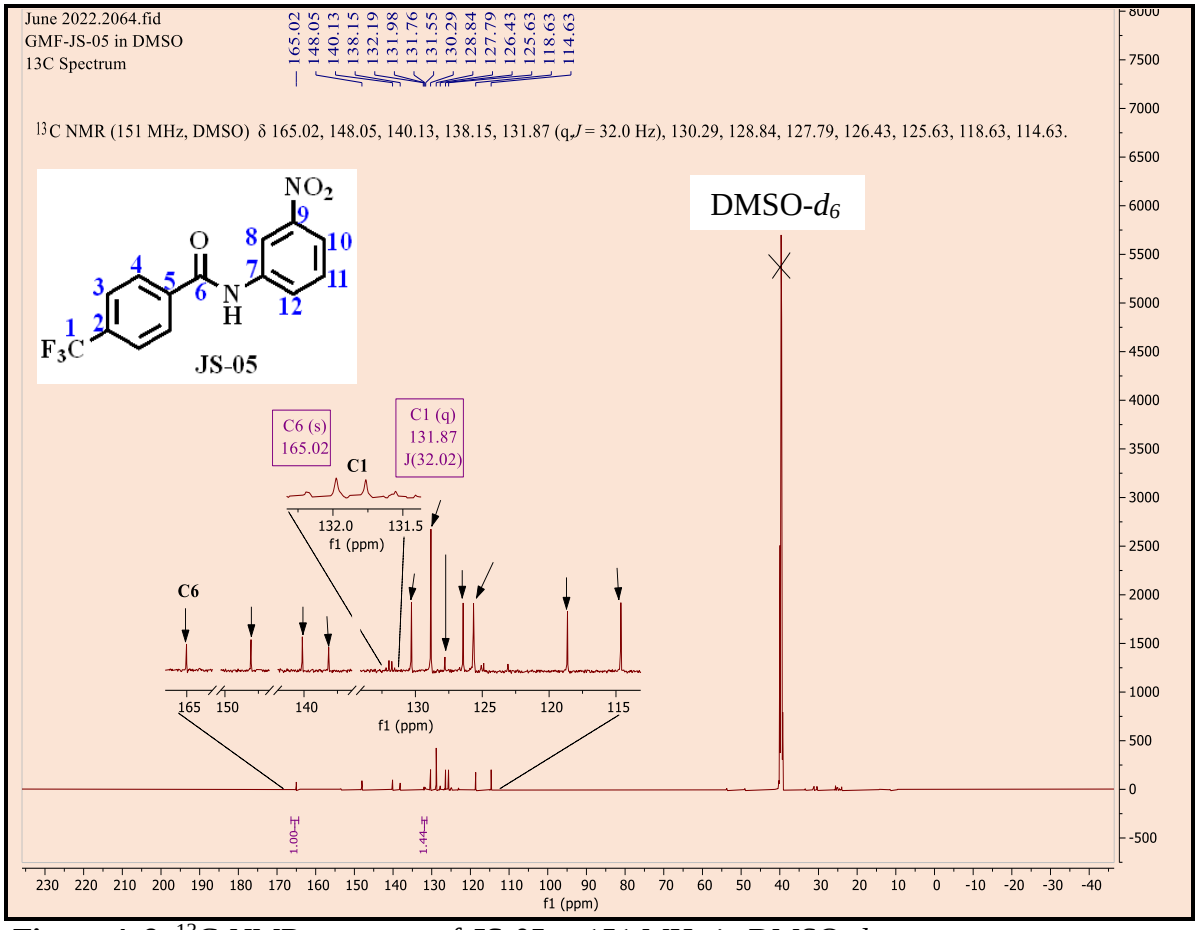
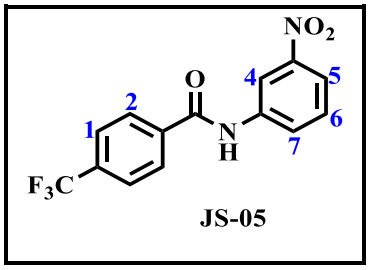


Figure 4. 9: ^{13}C-NMR spectrum of **JS-05** at 151 MHz in DMSO-d_6.

As anticipated, the most downfield diagnostic signal at δ = 165.02 ppm appears to correspond to the carbonyl carbon (C6). The other notable diagnostic signal, observed as a quartet (*q*) at δ = 131.87 ppm, was attributed to C1 and arose from a very short-range geminal coupling between C1 and its three fluorine neighbors ($^{2}J_{C\text{-}F}$) (short-range coupling evidenced by a large coupling constant, J = 32.02 Hz).

4.3.3.4 Comprehensive Characterization of JS-05



JS-05

^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.89 (s, 1H, H^3), 8.79 (t, J = 2.2 Hz, 1H, H^4), 8.22 – 8.15 (m, 3H, H^2, H^7), 8.00 (ddd, J = 8.3, 2.3, 0.9 Hz, 1H, H^5), 7.95 (d, J = 8.2 Hz, 2H, H^1), 7.68 (t, J = 8.2 Hz, 1H, H^6); ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.02, 148.05, 140.13, 138.15, 131.87 (q, J = 32.0 Hz), 130.29, 128.84, 127.79, 126.43, 125.63, 118.63, 114.63, m.p. 178.2 – 180.8 °C; R_f (EtOAc:Hex, 3:2) 0.81; APCI$^+$: *m/z* $[M + H]^+$ = 311.1, calculated exact mass = 310.06, purity = 97.73%, t_r = 1.113 min.

4.3.4 Characterization of 4-Fluoro-*N*-(3-nitrophenyl)benzamide (JS-09)

4.3.4.1 HPLC-MS Analysis

Figure 4.10 shows an HPLC chromatogram with percentage purity of 100% at 1.002 min retention time. Like **JS-03** (section 4.3.1.1), the percentage purity was considered as **99.90%**. The APCI$^+$ mass spectrum revealed a pseudo-molecular ion $[M + H]^+$ (*m/z* = 261.1) comparable to the calculated exact mass (260.06) for the target compound.

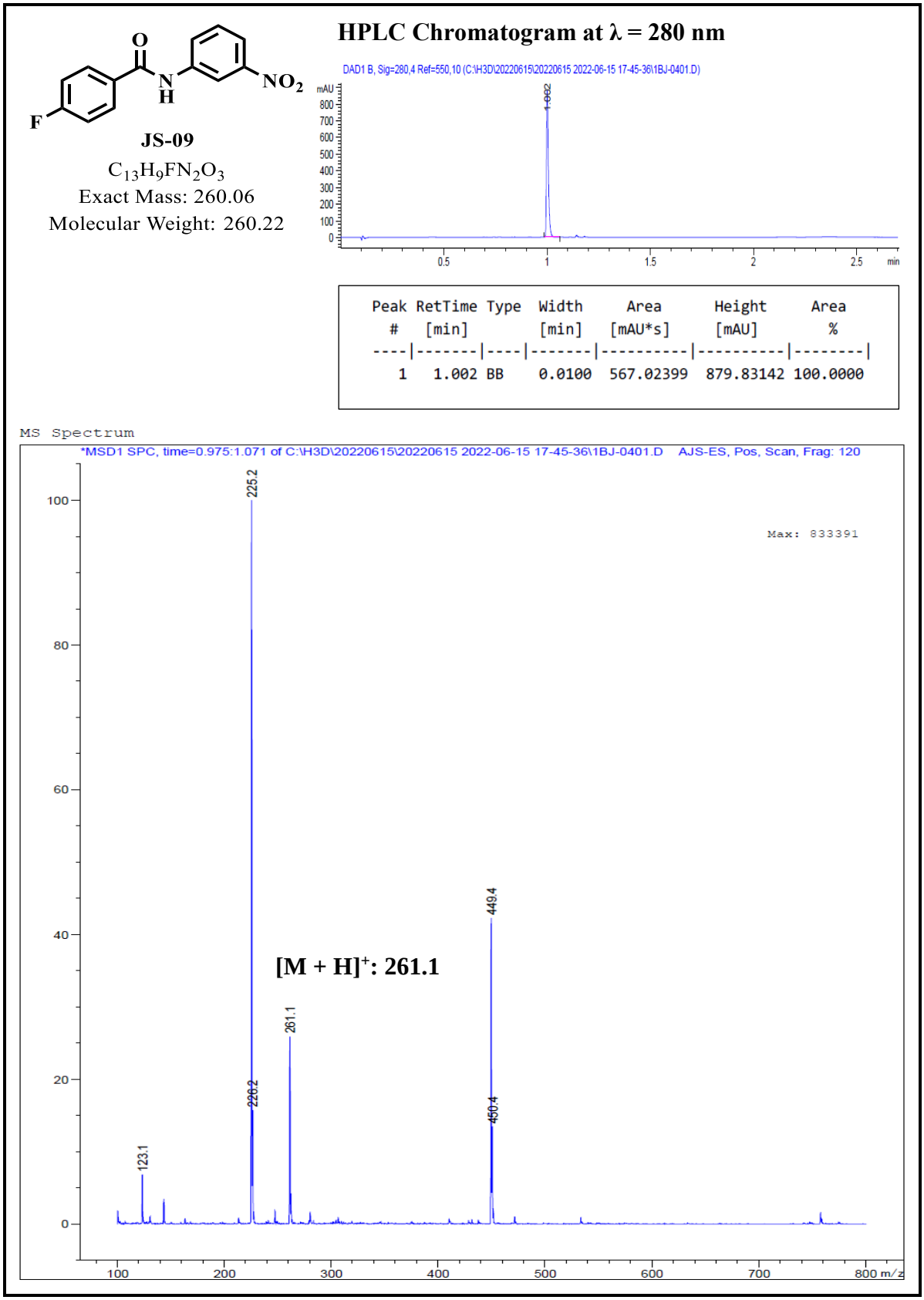


Figure 4. 10: HPLC Chromatogram and APCI$^+$ mass spectrum of **JS-09.**

4.3.4.2 ^{1}H-NMR Analysis

The ^{1}H-NMR spectrum (Figure 4.11) shows seven proton signals in accordance with seven chemically distinct proton environments (numbered), and nine protons present in the chemical structure of the target compound.

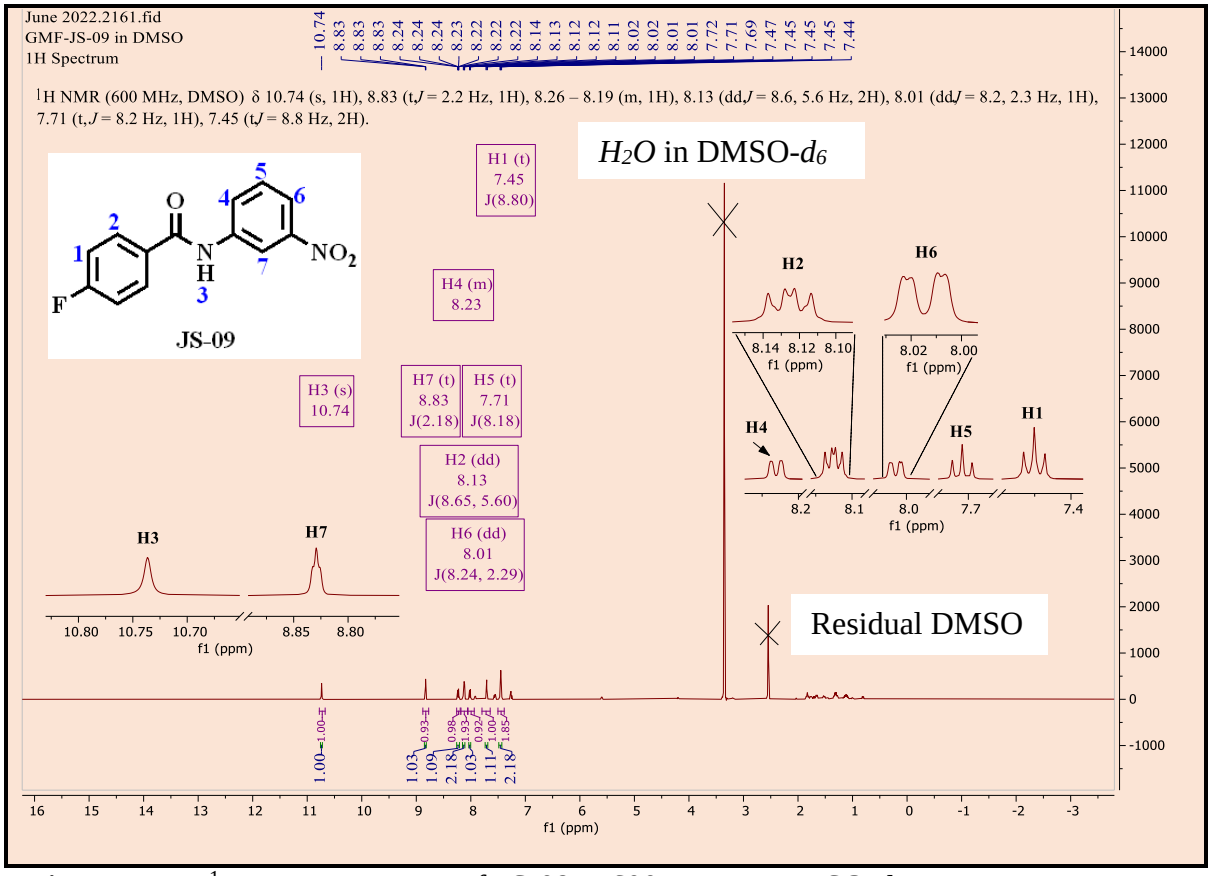


Figure 4. 11: ^{1}H-NMR spectrum of **JS-09** at 600 MHz in DMSO-d_6.

A well-resolved multiplicity pattern was observed for all the nine protons in the molecule. As expected, the most downfield signal (δ = 10.74 ppm) integrating for one proton was unambiguously associated with H3 and appears as a singlet (*s*). A triplet (*t*) peak integrating for one proton is evident at δ = 8.83 ppm corresponding to H7 having equal long-range coupling ($^{4}J_{H\text{-}H}$) to H4 and H6 justified by a small coupling constant (J = 2.18 Hz). A signal at δ = 8.23 ppm integrating for one proton appears as a multiplet (*m*) and corresponds to H4, albeit having a typical doublet appearance. A doublet of doublets (*dd*) attributed to H2 is observed at δ = 8.13 ppm integrating for two protons. It arises from a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H1 (J = 8.65 Hz) and a long-range coupling ($^{4}J_{H\text{-}F}$) evidenced by a smaller coupling constant (J = 5.60 Hz).

Another well-defined doublet of doublets (*dd*) appears at δ = 8.01 ppm from a signal integrating for one proton. It was unambiguously attributed to H6 having undergone both a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H5, justified by a larger coupling constant (J = 8.24 Hz) and a long-range coupling ($^{4}J_{H\text{-}H}$) to H4 justified by a smaller coupling constant (J = 2.29 Hz). Further upfield, a triplet (*t*) peak integrating for one proton appears at δ = 7.71 ppm, and is associated with H5 that experiences equal short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H4 and H6 evidenced by a large coupling constant (J = 8.18 Hz). The most upfield signal at δ = 7.45 ppm integrating for two protons corresponds to H1 and resonated as a triplet ($^{3}J_{H\text{-}F,}$ = 8.80 Hz) arising from equal short-range coupling to H2 and F.

4.3.4.3 ^{13}C-NMR Analysis

Figure 4.12 depicts a ^{13}C-NMR spectrum exhibiting 11 carbon signals representative of 11 chemically distinct carbon environments (numbered), and 13 carbons present in the chemical structure of the target compound.

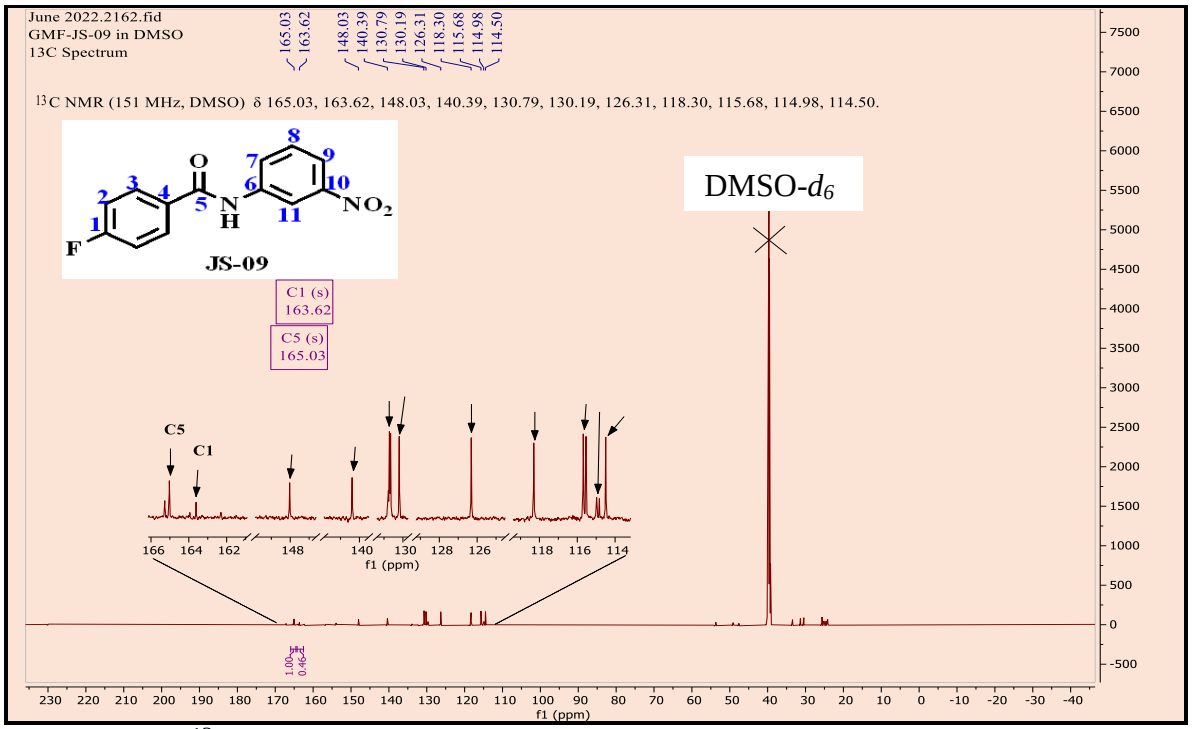
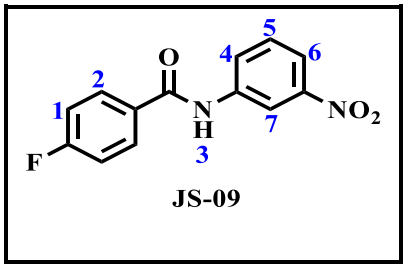


Figure 4. 12: ^{13}C-NMR spectrum of **JS-09** at 151 MHz in DMSO-d_6.

The most downfield and more intense diagnostic signal appeared at δ = 165.03 ppm as a singlet (*s*) and was unambiguously associated with the carbonyl carbon (C5). The other notable and less intense diagnostic signal relatively upfield was observed at δ = 163.62 ppm and was attributed to C1.

The 11 signals observed on the ^{13}C-NMR spectrum for the target compound were attributed to 11 chemically distinct carbon environments.

4.3.4.4 Comprehensive Characterization of JS-09



JS-09

^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.74 (s, 1H, H^{3}), 8.83 (t, *J* = 2.2 Hz, 1H, H^{7}), 8.26 – 8.19 (m, 1H, H^{4}), 8.13 (dd, *J* = 8.6, 5.6 Hz, 2H, H^{2}), 8.01 (dd, *J* = 8.2, 2.3 Hz, 1H, H^{6}), 7.71 (t, *J* = 8.2 Hz, 1H, H^{5}), 7.45 (t, *J* = 8.8 Hz, 2H, H^{1}); ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.03, 163.62, 148.03, 140.39, 130.79, 130.19, 126.31, 118.30, 115.68, 114.98, 114.50, m.p. 150.8 – 151.9 °C; R_f (EtOAc:Hex, 2:3) 0.62; APCI^{+}: *m/z* [M + H]$^{+}$ = 261.1, calculated exact mass = 260.06, purity = 99.90%, t_r = 1.002 min.

4.3.5 Characterization of 4-fluoro-*N*-(4-nitrophenyl)benzamide (JS-10)

4.3.5.1 HPLC-MS Analysis

Figure 4.13 depicts an HPLC chromatogram with observed purity (37.6497%) at 1.007 min retention time. However, this purity value is not correct because it includes solvent peaks that were equally integrated. The correct value (**79.16 ≈ 80%**) was obtained as described in the case of **JS-04**.[5] The molecular ion [M + H] $^{+}$ (*m/z* = 261.1) observed on the APCI^{+} mass spectrum was comparable to the calculated exact mass (260.06) for $C_{13}H_9FN_2O_3$ (**JS-10**).

Due to low purity (≈ 80%), which was less than the acceptable purity requirement (≥ 95%) for biological testing, compound **JS-10** could not progress to *in vitro* antischistosomal testing. The high impurity level in this compound is evident on the HPLC chromatogram, and the ^{1}H-NMR and ^{13}C-NMR spectra in section 4.3.5.2 and 4.3.5.3 respectively.

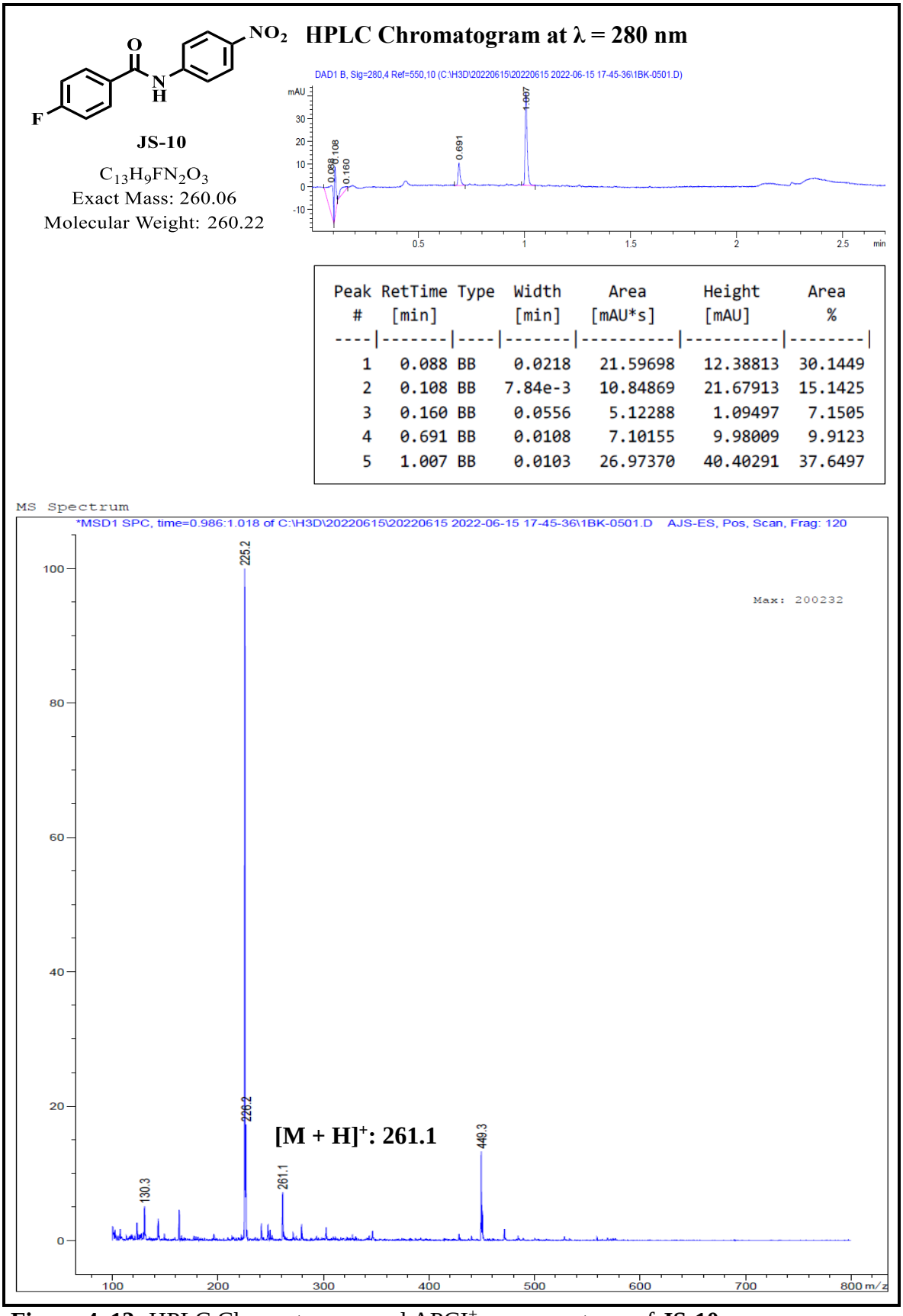


Figure 4. 13: HPLC Chromatogram and APCI$^+$ mass spectrum of **JS-10.**

4.3.5.2 ^{1}H-NMR Analysis

The ^{1}H-NMR spectrum (Figure 4.14) shows four proton signals representative of five chemically distinct proton environments (numbered), and nine protons present in the chemical structure of $C_{13}H_9FN_2O_3$ (JS-10) the target compound.

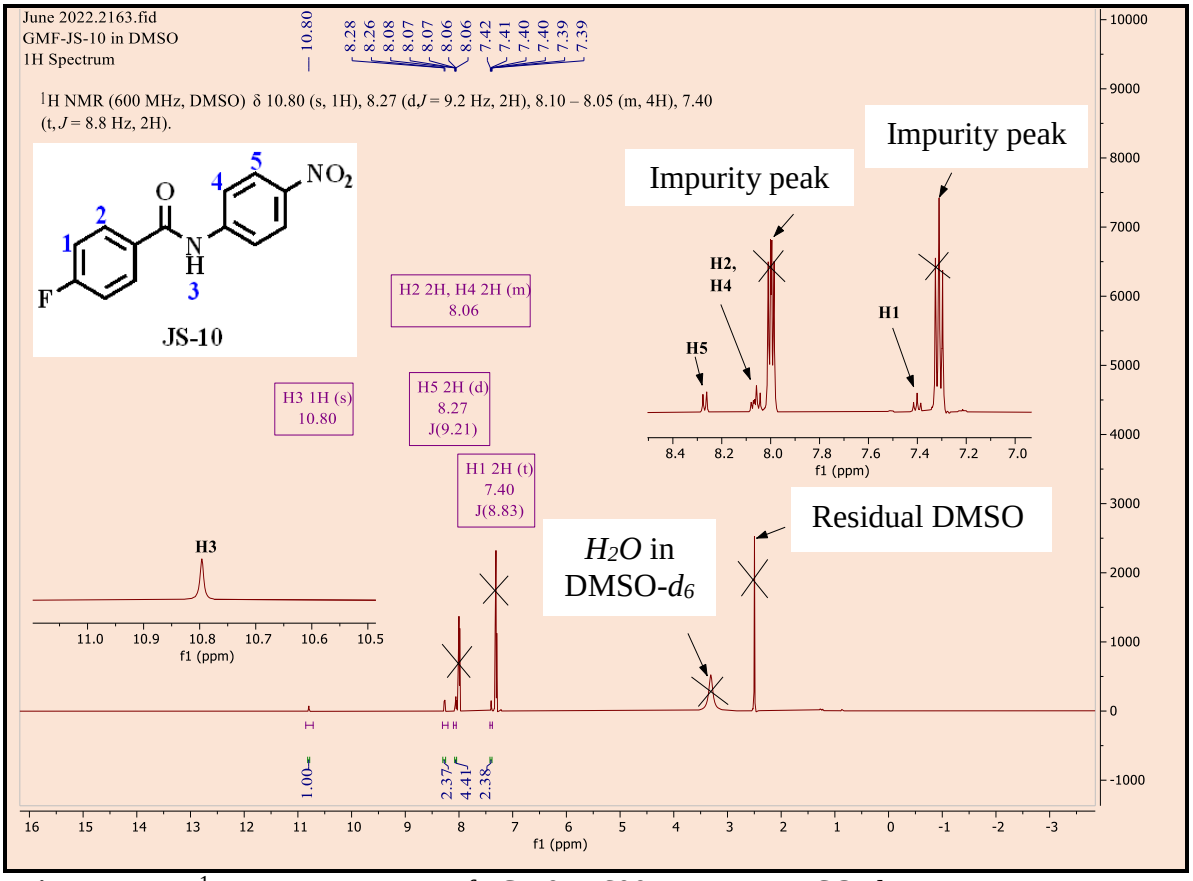


Figure 4. 14: ^{1}H-NMR spectrum of **JS-10** at 600 MHz in DMSO-d_6.

The most downfield signal integrating for one proton confirms the amide-bond formation in the structure of the target compound and is unambiguously associated with H3 that appears as a singlet (*s*) at δ = 10.80 ppm. A doublet (*d*) (J = 9.21 Hz) was observed at δ = 8.27 ppm integrating for two protons corresponding to H5 that experiences a short-range vicinal coupling ($^{3}J_{H\text{-}H}$) to H4. Further upfield, a multiplet signal at δ = 8.06 ppm integrating for four protons was associated with H2 (2H) and H4 (2H). Protons H1 resonated as a triplet ($^{3}J_{H\text{-}F,} = 8.83$ Hz) at δ = 7.40 ppm with this multiplicity pattern arising from equal short-range coupling to H2 and F. The total integral value correlated well with the total number of protons in the structure of **JS-10**.

4.3.5.3 ^{13}C-NMR Analysis

Figure 4.15 shows a ^{13}C-NMR spectrum with seven carbon signals representative of nine chemically distinct carbon environments (numbered), and 13 carbons present in the chemical structure of the target compound.

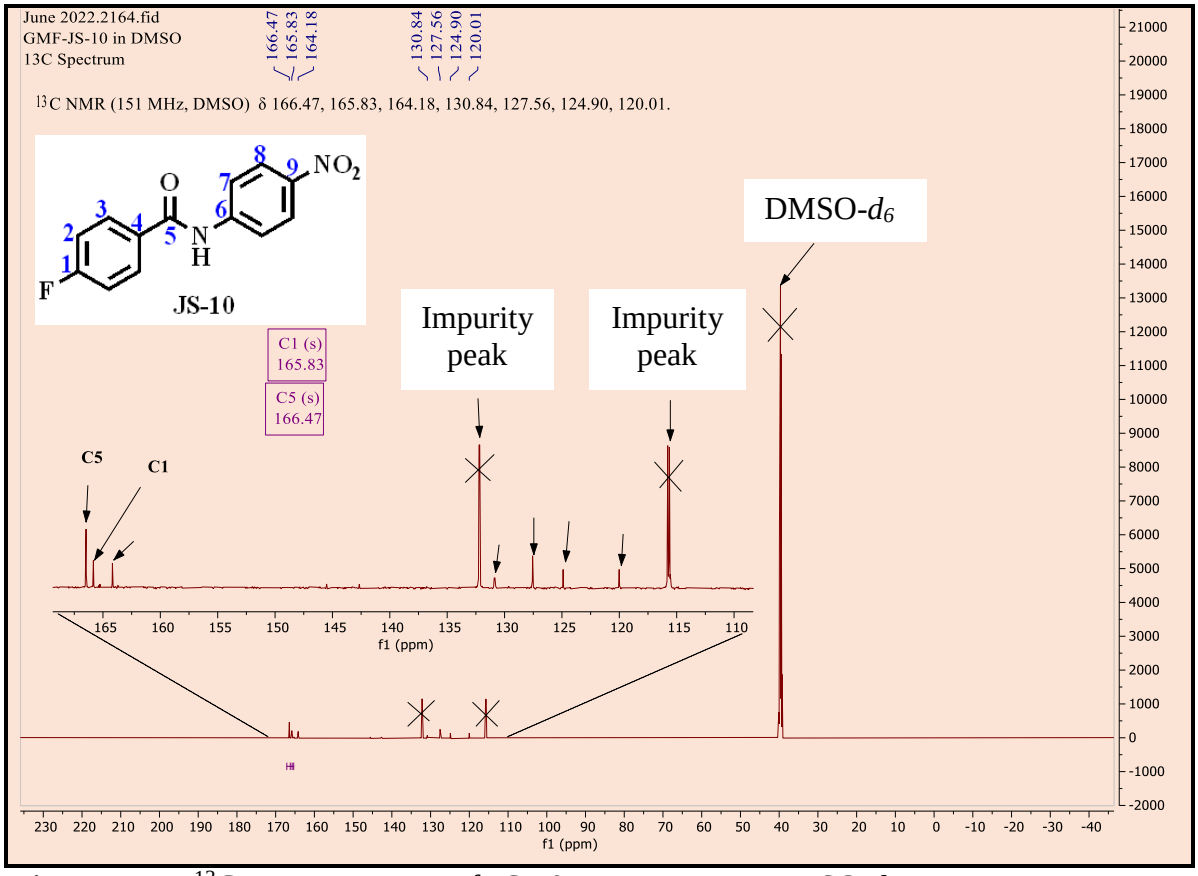
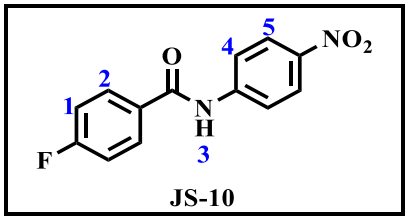


Figure 4. 15: ^{13}C-NMR spectrum of **JS-10** at 151 MHz in DMSO-d_6.

The Presence of two diagnostic signals one at δ = 166.47 ppm (most downfield) attributed to the carbonyl carbon (C5) and another one relatively upfield at δ = 165.83 ppm associated with the carbon-fluorine bond (C1) confirmed the formation of the target compound. The carbonyl carbon (C5) gave rise to a singlet (*s*) as anticipated while the expected doublet (*d*) associated with the carbon-fluorine bond (C1) was not observed.

The inconsistency between the observed seven signals and the nine carbon environments could be due to some carbons resonating at the same chemical shift or peaks from carbons in **JS-10** being obscured by impurity peaks.

4.3.5.4 Comprehensive Characterization of JS-10



JS-10

^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.80 (s, 1H, H^3), 8.27 (d, J = 9.2 Hz, 2H, H^5), 8.10 – 8.05 (m, 4H, H^2, H^4), 7.40 (t, J = 8.8 Hz, 2H, H^1); ^{13}C-NMR (151 MHz, DMSO-d_6) δ 166.47, 165.83, 164.18, 130.84, 127.56, 124.90, 120.01, m.p. 90.9 – 102.8^{o}C; R_f (EtOAc:Hex, 2:3) 0.76; APCI^{+}: *m/z* $[M + H]^+$ = 261.1, calculated exact mass = 260.06, purity ≈ 80%, t_r = 1.007 min.

4.4 *In vitro* Antischistosomal Evaluation

The four target compounds which met the acceptable purity requirement (≥ 95%) progressed to *in vitro* antischistosomal biological testing conducted in collaboration with Professor Jennifer Keiser, University of Basel, Switzerland at the Swiss TPH.

4.4.1 *In vitro* Antischistosomal Activity against *S. mansoni* NTS

The %-death-of-worms effect produced by the tested compounds (Table 4.1) were 62%, 28%, 56% and 42% at 50 µM with reduced effect of 41.67%, 25%, 54.17% and 25% at 10 µM. Results are presented as mean value (average) of triplicate measurements with standard deviation in brackets.

Table 4. 1: *In vitro* antischistosomal activity of target compounds on *S. mansoni* NTS.

Compound code	Effect in % (dead, 72h) and SD[d] Test conc. 50 µM	Effect in % (dead, 72h) and SD[d] Test conc. 10 µM
JS-03	62 (0)	41.67 (8.3)
JS-04	28 (0)	25 (0)
JS-05	56 (4)	54.17 (8.3)
JS-09	42 (2)	25 (0)

[d]SD, Standard deviation

The *in vitro* antischistosomal biological results (Figure 4.16) were plotted in bar graph format using Microsoft Excel 2013.

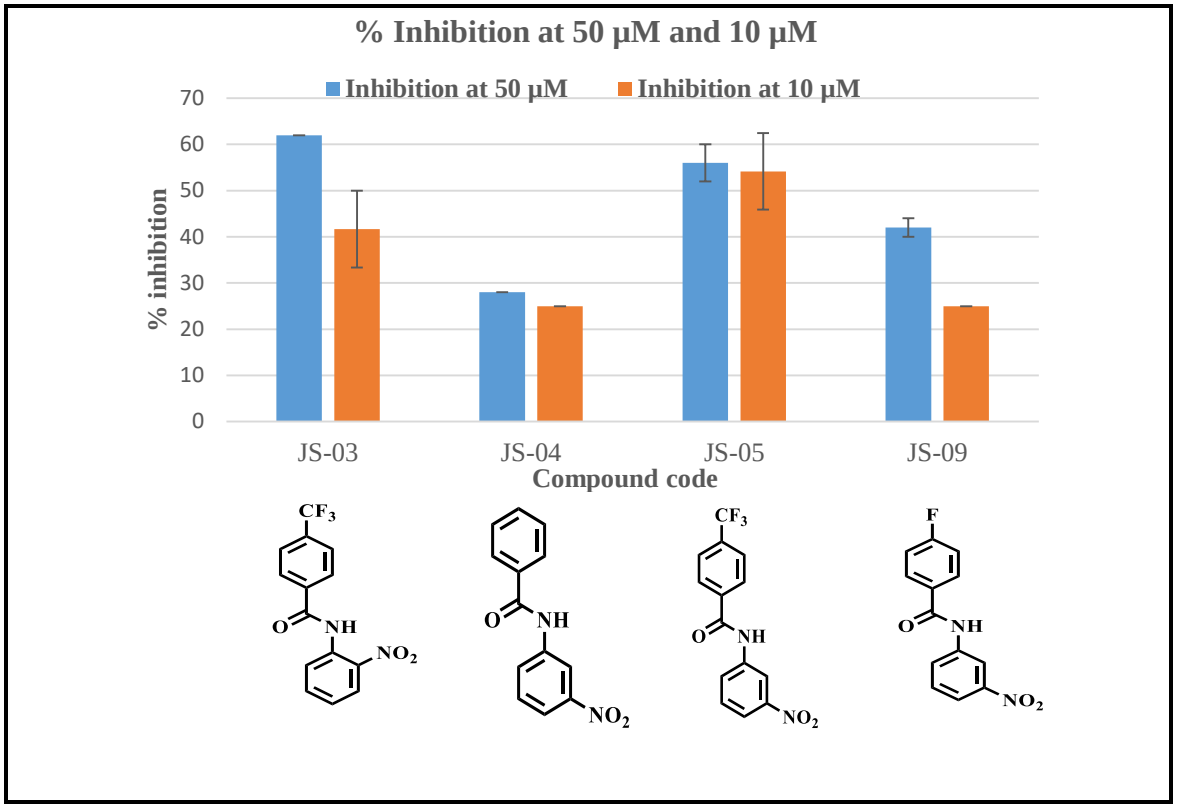


Figure 4. 16: In vitro antischistosomal activity of tested compounds on *S. mansoni* NTS.

4.5 Structure-activity Relationships

To start with, it should be acknowledged that the number of compounds reported in this study is too limited to derive conclusive SARs. The previous work conducted in our research group seems to suggest that the presence of electron-withdrawing groups on both phenyl rings enhances antischistosomal potency.[6] This pattern also seems to have emerged when **JS-03** and **JS-05** (both with strongly electron-withdrawing groups on both phenyls) are compared with **JS-04** and **JS-09**. In **JS-05**, the presence of the *para*-CF_3 on the top phenyl was associated with enhanced potency compared to the *para*-F (a much lesser electron-withdrawing substituent, see Craig plot in section 3.2) in **JS-09**. This pattern was even more striking when **JS-05** was compared to **JS-04** which is devoid of any electron-withdrawing substituent on the top phenyl group. Regioisomerism (**ortho** or **meta** substitution), as depicted in **JS-03** and **JS-05**, does not seem to affect activity.

4.6 Statistical Analysis

Statistical analysis to determine whether there was a significant difference between two mean values of tested compounds could not be done because statistical tools such as One-way Analysis of Variance (ANOVA) with post-hoc Tukey's Honestly Significant Difference Test (Tukey's HSD Test) require input of raw data (replicate measurements).[7] However, the results received from our collaborator as indicated on Table 4.1, had mean and standard deviation values already calculated.

4.7 References

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(3) Due-Hansen, M. E.; Pandey, S. K.; Christiansen, E.; Andersen, R.; Hansen, S. V. F.; Ulven, T. A Protocol for Amide Bond Formation with Electron Deficient Amines and Sterically Hindered Substrates. *Org. Biomol. Chem.* **2016**, *14* (2), 430–433.

(4) Ghosh, A. K.; Shahabi, D. Synthesis of Amide Derivatives for Electron Deficient Amines and Functionalized Carboxylic Acids Using EDC and DMAP and a Catalytic Amount of HOBt as the Coupling Reagents. *Tetrahedron Lett.* **2021**, *63*, 1–12.

(5) *How to calculate the percentage purity using the HPLC?* https://www.researchgate.net/post/How_to_calculate_the_percentage_purity_using_the_HPLC (accessed 2022-11-24).

(6) Kanyanta, M.; Lengwe, C.; Mambwe, D.; Francisco, K. R.; Liu, L. J.; Sun, Y. U.; Amarasinghe, D. K.; Caffrey, C. R.; Cheuka, P. M. Activity of N-Phenylbenzamide Analogs against the Neglected Disease Pathogen, Schistosoma Mansoni. *Bioorg. Med. Chem. Lett.* **2023**, *82*, 1–6.

(7) Williams, L. J.; Abdi, H. Tukey's Honestly Significant Difference (HSD) Test. *Encyclopedia of Research Design*; 2010; pp 1–5.

CHAPTER 5

CONCLUSIONS, LIMITATIONS & RECOMMENDATIONS

5.1 Conclusions

In this MSc project, synthesis of five nitrated *N*-phenylbenzamide derivatives was executed, and the percentage yields were obtained in low to good yield (17.0–85.5%). Spectroscopic characterization (HPLC-MS, ^{1}H-NMR, and ^{13}C-NMR) revealed that four target compounds exhibited purity (> 95%) thereby satisfying the acceptable purity requirement (≥ 95%) for progression to *in vitro* biological testing while the purity of one target compound, **JS-10** was compromised (≈ 80%) and was not progressed to *in vitro* antischistosomal biological testing. Physical characterization with regards to melting points (m.p) and retardation factors, R_f, of the target compounds revealed ranges from 90.9–180.8 °C and 0.35–0.81 respectively. The %-death-of-worms effect against *S. mansoni* NTS produced by the tested compounds were 62%, 28%, 56%, and 42% at 50 μM with reduced effects of 41.67%, 25%, 54.17% and 25% at 10 μM respectively. Whether activity was enhanced or decreased in relation to the front-runner compound, **MK1-11** could not be deduced because its *in vitro* assay method was different from the one used for **JS** compounds in this MSc project. Furthermore, the front-runner compound was only tested against adult worms. The tested compounds demonstrated activity against *S. mansoni* juvenile worms, a property that praziquantel lacks. However, the activity on juvenile worms was too low to progress the analogs and test them against adult worms. The work to identify analogs with better activity profile is ongoing in our research group.

Preliminary SARs appear to confirm earlier findings – electron-withdrawing substituents on the phenyl rings are essential for potency.

5.2 Limitations of the Study

The following were the limitations of the study:

1. DCC was the only coupling agent available at the time of this project while EDCI, the alternative and a more suitable coupling agent for electron-deficient amines was under procurement. Consequently, DCC was used instead, thereby jeopardizing the success of reactions.

2. Lack of own instrumentation for characterization: In this project, samples had to be shipped to UCT through collaboration to confirm their successful synthesis by HPLC-MS, ^{1}H-NMR, and ^{13}C-NMR. This was a costly process in terms of time constraints, and wastage of reagents in cases of unsuccessful synthesis. Having your own HPLC-MS, for example, would enable you to check the feasibility of the reaction at the initial stage and avoid wastage of reagents by changing the direction of the project.

3. *In vitro* antischistosomal biological testing of the samples could not be done at UNZA and the country at large. Hence, samples had to be shipped to Switzerland for *in vitro* testing through the established collaboration with Prof. Jennifer Keiser (University of Basel, Switzerland) at the Swiss TPH. The wait for biological results equally affected the progress of the project.

4. The analysis of biological results was restrictive. This was due to the form in which *in vitro* antischistosomal assay results were provided by the collaborator. As such, statistical analysis could not be done. Results were not provided in raw form (replicate measurements).

5. Further purification of the target compound, **JS-10** with compromised purity (≈ 80%) could not be done because it was costly to ship one sample back for recharacterization to South Africa at UCT, and also ship it to Switzerland for *in vitro* biological testing.

6. Assay validation with PZQ, the positive control was not done due to lack of resources.

5.3 Recommendations of the Study

1. The front-runner compounds, **MK1-09** and **MK1-11** as reported by Masebe Kanyanta and co-workers, should be resynthesized and tested under the same assay conditions to assess to what extent activity is improved upon or compromised.

2. Additional compounds with a diverse range of electron withdrawing groups need to be synthesized in order to elucidate a clear and more conclusive SARs.

3. Future studies should consider dinitro-substitution on the aniline portion of the scaffold, and use the same *in vitro* assay method as **JS** compounds in this MSc project to be able to deduce whether there would be enhancement or decrease in potency.

4. For any acid-amine coupling with electron-deficient amines, future studies should consider using EDCI as a coupling agent.

5. Future studies should consider repeating experiments to ascertain the cause of similarity in activity of an analog at two different concentrations that are far apart as observed in **JS-04** and **JS-05**.

Appendix A: Approval letter for Protocol

THE UNIVERSITY OF ZAMBIA

DIRECTORATE OF RESEARCH AND GRADUATE STUDIES

Great East Road Campus | P.O. Box 32379 | Lusaka 10101 | **Tel:** +260-290 258/291 777
Fax: (+260) 211 290 258/253 952 | **Email:** director.drgs@unza.zm | **Website:** www.unza.zm

APPROVAL OF STUDY

IORG No. 0005376
NASREC IRB No. 00006465

8th December, 2022

REF NO. NASREC-2022-OCT.-012

Mr.John Sichinga,
The University of Zambia
School of Natural Sciences,
P.O. Box 32379,
LUSAKA.

Dear Mr. J. Sichinga,

RE: "ANTISCHISTOSOMAL STRUCTURE - ACTIVITY RELATIONSHIPS OF NITRATED *N*- PHENYLBENZAMIDE DERIVATIVES"

Reference is made to your protocol dated as captioned above. NASREC resolved to approve this study and your participation as Principal Investigator for a period of one year.

REVIEW TYPE	ORDINARY REVIEW	APPROVAL NO. NASREC-2022-OCT.-012
Approval and Expiry Date	Approval Date: 8th December, 2022	Expiry Date: 8th December, 2023
Protocol Version and Date	Version - Nil.	28th November, 2023
Information Sheet, Consent Forms and Dates	• English.	To be provided
Consent form ID and Date	• Version - Nil	To be provided
Recruitment Materials	• Nil	Nil
Other Study Documents	• Questionnaire.	

Specific conditions will apply to this approval; As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be

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suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

CONDITIONS OF APPROVAL

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to NASREC within 5 days.
- All protocol modifications must be approved by NASREC prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to NASREC within 5 working days.
- All recruitment materials must be approved by NASREC prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. NASREC will only approve a study for a period of 12 months.
- It is the responsibility of the PI to renew his/her ethics approval through a renewal application to NASREC.
- Where the PI desires to extend the study after expiry of the study period, documents for study extension must be received by NASREC at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Documents received within 30 days after expiry will be labelled "late submissions" and will incur a penalty fee of K500.00. No study shall be renewed whose documents are submitted for renewal 30 days after expiry of the certificate.
- Every 6 (six) months a progress report form supplied by The University of Zambia Natural and Applied Sciences Research Ethics Committee as an IRB must be filled in and submitted to us. There is a penalty of K500.00 for failure to submit the report.
- When closing a project, the PI is responsible for notifying, in writing or using the Research Ethics and Management Online (REMO),both NASREC
- and the National Health Research Authority (NHRA) when ethics certification is no longer required for a project.
- In order to close an approved study, a Closing Report must be submitted in writing or through the REMO system. A Closing Report should be filed when data collection has ended and the study team will no longer be using human participants or animals or secondary data or have any direct or indirect contact with the research participants or animals for the study.
- Filing a closing report (rather than just letting your approval lapse) is important as it assists NASREC in efficiently tracking and reporting on projects. Note that some funding agencies and sponsors require a notice of closure from the IRB which had approved the study and can only be generated after the Closing Report has been filed.
- A reprint of this letter shall be done at a fee.
- All protocol modifications must be approved by NASREC by way of an application for an

amendment prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address or methodology and methods. Many modifications entail minimal risk adjustments to a protocol and/or consent form and can be made on an Expedited basis (via the IRB Chair). Some examples are: format changes, correcting spelling errors, adding key personnel, minor changes to questionnaires, recruiting and changes, and so forth. Other, more substantive changes, especially those that may alter the risk-benefit ratio, may require Full Board review. In all cases, except where noted above regarding subject safety, any changes to any protocol document or procedure must first be approved by NASREC before they can be implemented.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of NASREC, we would like to wish you all the success as you carry out your study.

Yours faithfully,

Dr. E. M. Mwanaumo

CHAIRPERSON
THE UNIVERSITY OF ZAMBIA NATURAL AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE - IRB

cc: Director, Directorate of Research and Graduate Studies
Assistant Director (Research), Directorate of Research and Graduate Studies
Assistant Registrar (Research), Directorate of Research and Graduate Studies