MULTICOMPONENT SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL EVALUATION OF PYRAZOLE DERIVATIVES

Thesis submitted to the Durban University of Technology in fulfilment of the requirements for the award of the degree of

MASTER OF APPLIED SCIENCES

IN

CHEMISTRY

By

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2024

DECLARATION

This dissertation is submitted to the Durban University of Technology for the Master of Applied Sciences in Chemistry degree. I declare that this work is my own and has not been submitted before for any degree or examination to this or any other institution or university for this degree or any other degree or award.

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ACKNOWLEDGEMENT

Firstly, I would like to express my deepest gratitude to my **Lord and Saviour Jesus Christ**, son of the living **God** for keeping my life through His righteousness by believing in His Word which has strengthened and encouraged me in afflicting times and uncertainty during my studies. **Ephesians 3:20-21 -** "*20 Now to Him who is able to do exceedingly abundantly above all that we ask or think, according to the power that works in us, 21 to Him be glory in the church by Christ Jesus to all generations, forever and ever. Amen.*"

My sincere appreciation, deep sense of gratitude, and indebtedness to my supervisor and mentor **Dr. Talent Raymond Makhanya** *Department of Chemistry, Durban University of Technology, Durban,* for his guidance, constant encouragement, remarkable patience, perusal of the manuscript, and motivation throughout my course of study. You are a motivating factor, a great source of inspiration, and resilience given the fact that you endured and recovered from a life-compromising period, I thank God for you. You are a hero and a true academician par excellence. Further, I extend my extreme gratitude to **Prof. Robert M Gengan** for his continuous motivation, support, and critical editing and proof reading of my dissertation.

To Prof**. Sabiu** Saheed and **Jamiu Aribisala**, *Department of Biotechnology and Food Science, Durban University of Technology*, I am deeply grateful for your invaluable contributions to my work. Your insightful feedback and training in biological studies significantly improved the quality of my research, and I cannot thank you enough for your support.

My sincere appreciation goes to all the members of the Organic Research Group, Chemistry Department, Faculty of Applied Sciences, Durban University of Technology, especially to Ms Senzekile Majola and Mr Zamokuhle Miya for their constant support, and words of encouragement whenever I needed them.

Sincere thanks also go to Mrs. Caryl Janse Van Rensburg from UKZN, Pietermaritzburg Campus for TOF-MS sample analysis and UKZN, Westville Campus staff for providing NMR spectral data analysis. I want to also thank Dr. Avinash Ramsaroop *Department of Mechanical Engineering, Durban University of Technology* for I.R analysis.

I also would like to convey my utmost heartfelt appreciation to my mother and best friend, **Ms Nombuso Amanda Bridgette Mntambo** for being ever so loving, and tender and being my pillar. Thank you, Mom, for always being available for me, and bearing with me when I was

going through frustrations and gloomy days of ailing. You nurtured me back to life, you have played a profound role during this tenure of my research, thank you for being my safe place and tolerating my pleonastic tendencies when explaining chemistry concepts to you which frequently bore you, it felt like torture. Thank you for the laughs we had despite the circumstances, I LOVE YOU MOM.

To my uncle, **Sikhumbuzo Sibiya** who has become a father to me since the passing of my dad, has brought me up to be a responsible and educated man. I wouldn't have reached this far in life if it wasn't for his relentless support and fatherly love. You are awesome *Baba* and I appreciate you so much.

I would like to thank my spiritual parents, **Mr Micheal Yaw Owusu**, and his wife **Mrs. Patricia Owusu** for their unwavering support and guidance throughout my academic journey. They have been instrumental in keeping me focused and motivated, and their teachings have greatly influenced my conduct as a believer.

I dedicate my success to those who look up to me—my incredible siblings. **Babalwa**, my younger sister, embodies character, strength, and intelligence. **Ntokozo**, the quiet yet uniquely creative last born, is a bright human being who is destined for the highest stature. And **Lindokuhle**, my charismatic brother, inspires me to pave the way for his achievements because he always says that he looks up to me. I have unwavering faith in him. **Hlomla** and **Hleloluhle** whom I cherish and wish excellence in their education.

My sincere gratitude goes to the National Research Foundation (South Africa) and the Mining Qualification Authority (MQA) for their financial support, which funded my study and enabled me to pursue my academic goals.

ABSTRACT

People have been ailing regularly nowadays and conventional antibiotics have become less potent due to drug resistance. The molecular architecture of the antibiotics issued to patients has only marginally changed since their discovery, thus this has caused microorganisms to adapt or survive the repeated administration of the same drugs over time, causing medication to be less effective, and people becoming sicker because the lack of new, different and potent antibiotic structures has enabled bacteria to mutate. This presented the pressing need to develop pyrazole compounds because they suit the category of being structurally different and have been reported to exhibit excellent antibacterial activities. To obtain pyrazole derivatives, Microwave-Assisted Organic Synthesis (MAOS) was utilized to synthesize ten novel compounds of (4Z)-4-arylidene-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide derivatives (**4a-4j**) by a one-pot multicomponent reaction (MCR) methodology that is eco-friendly and adhering to green chemistry principles, occurring by catalyst-free in a water-ethanol solvent system. The methodology to access novel pyrazoles (**4a-4j**) in this study is superior to conventional pyrazole synthesis because it requires harsh reaction conditions and expensive catalysts, which deviates from green chemistry. All synthesized compounds (**4a-4j**) were fully characterized and confirmed by ^{1}H NMR, ^{13}C NMR, 2D NMR, FTIR and TOF-MS. Antimicrobial study was performed against two Gram-negative and two Gram-positive strains *viz. Escherichia Coli (E.Coli), Pseudomonas aeruginosa (P. aeruginosa), Staphylococcus Aureus (S. Aureus), and Streptococcus pneumoniae (S. pneumoniae),* respectively, and amoxicillin was used as the reference drug.

It was found that only **4c** and **4j** exhibited activity against *E. coli* and their Minimum Inhibitory Concentration (MIC) values were 1.38 and 2.50 mg/mL, respectively. In contrast, Amoxicillin displayed a lower MIC value of 0.0306 mg/mL, thereby suggesting that amoxicillin is more effective than the synthesized pyrazoles against *E. coli*. Pyrazoles **4a**, **4d**, and **4g** showed no antibacterial activity against *P. aeruginosa*. Compounds **4b**, **4c**, **4e**, **4f**, **4h**, **4i**, and **4j** displayed varying MIC values ranging from 0.212 to 0.625 mg/mL against *S. aureus*, 4i being 0.212 mg/mL the lowest MIC value. Amoxicillin displayed a lower MIC value than all the pyrazoles against *P. aeruginosa*. Against *S. pneumoniae*, **4j** demonstrated an excellent antibacterial potential with a lower MIC value of 0.0156 mg/mL compared to that of amoxicillin (0.0306 mg/mL). The other pyrazoles displayed marginally higher MIC values than amoxicillin; **4g** showed no activity for *S. aureus*. However, **4i** displayed the lowest MIC value, suggesting its

potential as an antibacterial agent. It was percieved that the unsubstituted phenyl ring in **4i** contributed to its enhanced potency against test bacteria.

Molecular docking studies were performed to predict the binding sites and affinities of the pyrazoles, revealing that they primarily target Penicillin Binding Proteins (PBPs) in the test bacteria. Docking scores for *E. coli* ranged from -6.7 to -7.9 kcal/mol and compound **4f** exclusively exhibited the best docking score of -8.1 kcal/mol against *E. coli* better than amoxicillin's docked score of -7.0 kcal/mol. Docking scores for *P. aeruginosa* ranged from -6.8 to -7.7 kcal/mol. Compounds **4g** and **4j** displayed the highest negative docking scores of -7.7 kcal/mol, outperforming amoxicillin thereby suggesting their potential as inhibitors, however **4b** and **4f** were comparable. Furthermore, in silico simulation, Molecular Dynamics (MD) studies were conducted for **4g** and **4j**. Docking scores for *S. pneumoniae* range from -7.2 to -8.1 kcal/mol. Compound **4j** displayed the highest negative docking score. Agreement between docking and in vitro results reinforces **4j** as a potential inhibitor. The pyrazoles exhibited docking scores between -5.5 and -6.6 kcal/mol for *S. aureus* with **4j** showing potential as an inhibitor. While lower than other PBPs, some pyrazoles are comparable to amoxicillin.

Furthermor, the pyrazoles exhibited LogP (Lipophilicity) values oscillating between 1 and 2.5, indicating better bioavailability. Amoxicillin, partitioned close to an aqueous phase, showed by a negative LogP value of -0.29. Amoxicillin's LD_{50} (lethal dose for killing 50% of the bacteria) is 15 g/kg, suggesting non-toxicity orally. Among pyrazoles, only compound **4c** shares this safety profile as amoxicillin, qualifying **4c** to be an orally administered antibacterial agent. Other pyrazoles fall into toxicity class 4, necessitating alternative administration routes, such as IV (Intravenous) or IM (Intramuscular). All studied compounds showed no affinity for BBB (Blood-Brain Barrier) permeability or P-glycoprotein binding, indicating they can traverse the system without hindrance from nonspecific enzymes or tissues. Compound **4d** does not inhibit any CYP (Cytochrome P450) isoenzymes, similar to amoxicillin. Compound **4e** selectively inhibits CYP 3A4, relevant for drug metabolism.

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ABBREVIATIONS

MCRs	- Multi-component reactions
MS	- Mass Spectrometry
MAOS	– Microwave Assisted Organic Synthesis
Calcd	- Calculated
m.p.	- melting point
mL	- milliliter
mmol	- millimole
µL	- microliter
h	- hour (s)
min	- minutes
mg	- milligram
µg	- microgram
µM	- micromolar
%	- percent
α	- alpha
β	- beta
g	- gram
^{o}C	- degree Celsius
m	- meter
cm	- centimeter
ppm	- parts per million
TLC	- thin-layer chromatography
LRo5	– Lipinski's Rule of 5
NaOH	- sodium hydroxide
KBr	- potassium bromide
$CDCl_3$	- deuterated-chloroform
DMSO-d_6	- deuterated-dimethyl sulfoxide
$POCl_3$	- phosphorus oxy trichloride

VHR	- Vilsmeier-Haack reaction
rt	- room temperature
PPh_3	- triphenyl phosphine
ZnO	- zinc oxide
CuO/ZrO_2	– Copper Oxide/Zirconium Oxide
NH_3	– ammonia
CAN	- cerium ammonium nitrate
N-atoms	- Nitrogen atoms
DABCO	- Diazobicyclo[2,2,2]octane
TSC	– Thiosemicarbazide
EAA	– Ethylaceto acetate
HCl	- hydrochloric acid
SA	- South Africa
TB	- mycobacterium tuberculosis
PBP	– Penicillin Bonding Protein
NSAID	– Non-Steroidal Anti-Inflammatory Drugs
S. aureus	– Staphylococcus
S. pneumonaie	– Streptococcus pneumonaie
E. coli	– Escherichia coli
P. aeruginosa	– Pseudomonas aeruginosa
IDSA	- Infectious Diseases Society of America
MDR	– Multidrug Resistant
QRSA	- Quinolone-resistant S. aureus
$CHCl_3$	- chloroform
CH_2Cl_2	– dichloromethane (DCM)
DMF	- dimethylformamide
MeOH	- methanol
EtOH	– ethanol
CH_3CN	- acetonitrile
H_2O	- water

H_2SO_4	- sulphuric acid
DMSO	- dimethyl sulfoxide
MIC	- minimium inhibitory concentration
FT-IR	- fourier transform infrared spectrometry
^{1}H NMR	- proton nuclear magnetic resonance
^{13}C NMR	- carbon 13 nuclear magnetic resonance
TOF-MS	- time of flight mass spectrometry
COSY	- homonuclear correlation spectrometry
HSQC	- heteronuclear single quantum correlation
HMBC	- heteronuclear multiple bond correlation
DEPT	- distortion-less enhancement polarisation transfer
2D-NMR	- two dimensional nuclear magnetic resonance
NMR	- Nuclear magnetic resonance
UV	– Ultra violet
FDA	– Food & Drug Administration
BBB	- Blood Brain Barrier
MD	- Molecular Docking
CYP 450	- Cytochrome P450 enzymes
PDB	- Protein Data Bank
HBD	- Hydrogen Bond Donator
HBA	- Hydrogen Bond Acceptor
MW	- Molecular Weight
LogP	- Octanol/Water partition co-efficient (Lipophilicity)
ADMET	- Absorption, Distribution, Metabolism, Excretion & Toxicity
RTB	- Rotatable Bonds
G.I	- Gastrointestinal
B.S	- Bioavailability Score
LD_{50}	- Lethal Dose able to kill 50% of Bacteria
T.C	- Toxicity Class
Pgp	- P-Glycoprotein

Chapter One: Introduction

1.1 Introduction

1.1.1 The Structure of Pyrazoles

Pyrazole, **Figure 1**, is an aromatic heterocyclic compound with the chemical formula $C_3H_4N_2$ (**1**) comprising six delocalized electrons. The reduced forms of pyrazole are pyrazoline **4**, **5**, **6** and pyrazolidine **3,** while the oxidized form is pyrazolone **2.** Pyrazoline and pyrazolidine are non-aromatic compounds (Faisal et al. 2019) since they neither have electron conjugation nor delocalized (Faisal *et al.* 2019) electrons. They are rarely found in nature, probably due to difficulty in the formation of *N-N* bonds by living organisms (Kumar *et al.* 2013). Owing to the diverse pharmacological activities of this pyrazole ring, several researchers across the globe are engaged in the development of pharmacologically active agents bearing it (Khan *et al.* 2016).

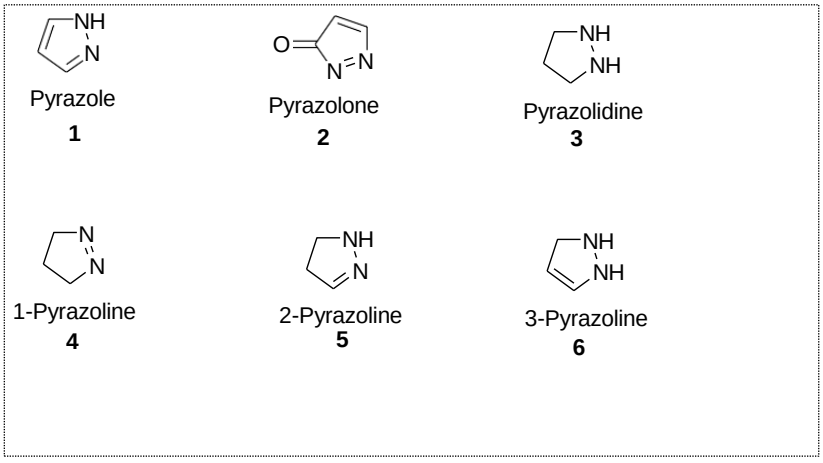

Figure 1: Simple structural forms of pyrazoles (Faisal *et al.* 2019)

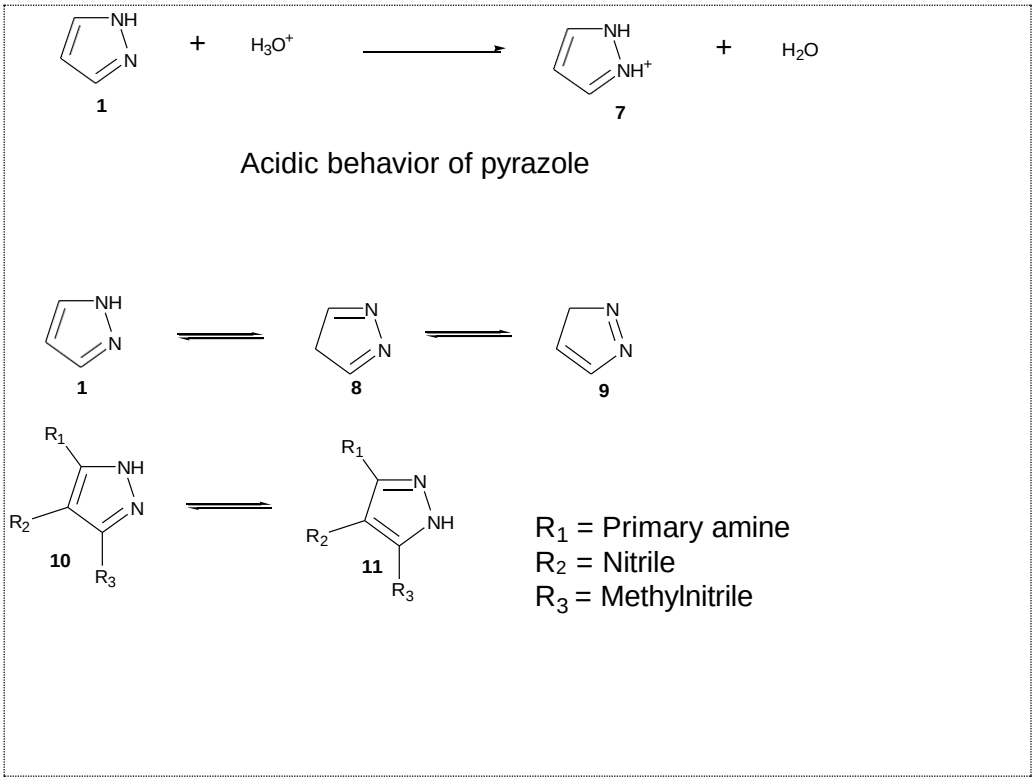

Figure 2: Acid-base and tautomeric behaviour of typical pyrazoles (Faisal *et al.* 2019)

Pyrazoles have served as synthetic intermediates for various biological, physical-chemical, material science, and industrial applications (Ríos and Portilla 2022). In addition, pyrazoles have been reported to possess a broad spectrum of biological profiles such as antitubercular (Modi, Patel and Chhabria 2023), anticancer (Alsayari *et al.* 2021), anti-HIV (Liu *et al.* 2016), antifungal (Sun and Zhou 2015), antimicrobial (Saleh *et al.* 2021) and antioxidant activities (Matta *et al.* 2023). This was also reported in docking studies on COVID-19 strains, proving good binding affinities and potentially the pyrazole scaffold becoming an anti-corona virus candidate (Matta *et al.* 2023).

1.1.2 Synthesis of Pyrazoles

The synthesis of pyrazole derivatives has a rich history, marked by evolved classical methods. Some strategies for synthesizing pyrazoles include Knorr synthesis, which is named after Ludwig Knorr. This method involves the reaction of α-diketones with hydrazine or hybrids (Quereshi, Guru and Jain 2024). It laid the foundation for subsequent advancements in pyrazole synthesis. Cyclo-condensation reactions (Ríos and Portilla 2022) are a traditional approach involving condensations of 1,3-bis-electrophilic compounds with hydrazine hybrids and [3+2]

cycloaddition reactions (Ríos and Portilla 2022). These reactions occur between diazo compounds and different alkynes, leading to the construction of a pyrazole ring. While these classical methods have been widely used to access pyrazoles, recent advances include multicomponent one-pot protocols, photo-redox reactions, and transition metal-catalyzed reactions, which enhance the synthesis of pyrazole derivatives (Ameziane El Hassani *et al.* 2023).

The future of pyrazole research lies in integrating green chemistry principles into synthesis methodologies. The traditional methods of pyrazole synthesis often involve harsh reaction conditions and the use of toxic reagents that are environmentally unfriendly, generating waste and consuming energy (Quereshi, Guru and Jain 2024). Modern innovative approaches, including microwave-assisted synthesis, flow chemistry, and biocatalysis, offer opportunities to reduce the environmental footprint of pyrazole synthesis (Quereshi *et al*. 2024).

1.1.3 Application of Pyrazoles

The usage of pyrazoles is not just limited to biological architectures for drug design. However, it has also been reported to have applications in the agrochemical industry as pesticides (Wu *et al.* 2012), herbicides (Fu *et al.* 2020) and plant growth regulators (Chaudhari and Rajput 2018) as **Figure 3** portrays. Pyrazole-based herbicides have proven effective in selectively targeting and eliminating unwanted plant species, preserving the integrity of cultivated crops. Pyrazoles also serve as plant growth regulators, interacting with crucial plant hormones and providing a tool for farmers to manipulate crop growth for improved yield and quality (Quereshi, Guru and Jain 2024). Pyrazoles has found many applications in various industries because of its versatility. As a result, this study investigates the biological activities of pyrazole pharmacophores and adds to the medicinal chemistry library that may be further modified to mitigate drug-resistant bacteria.

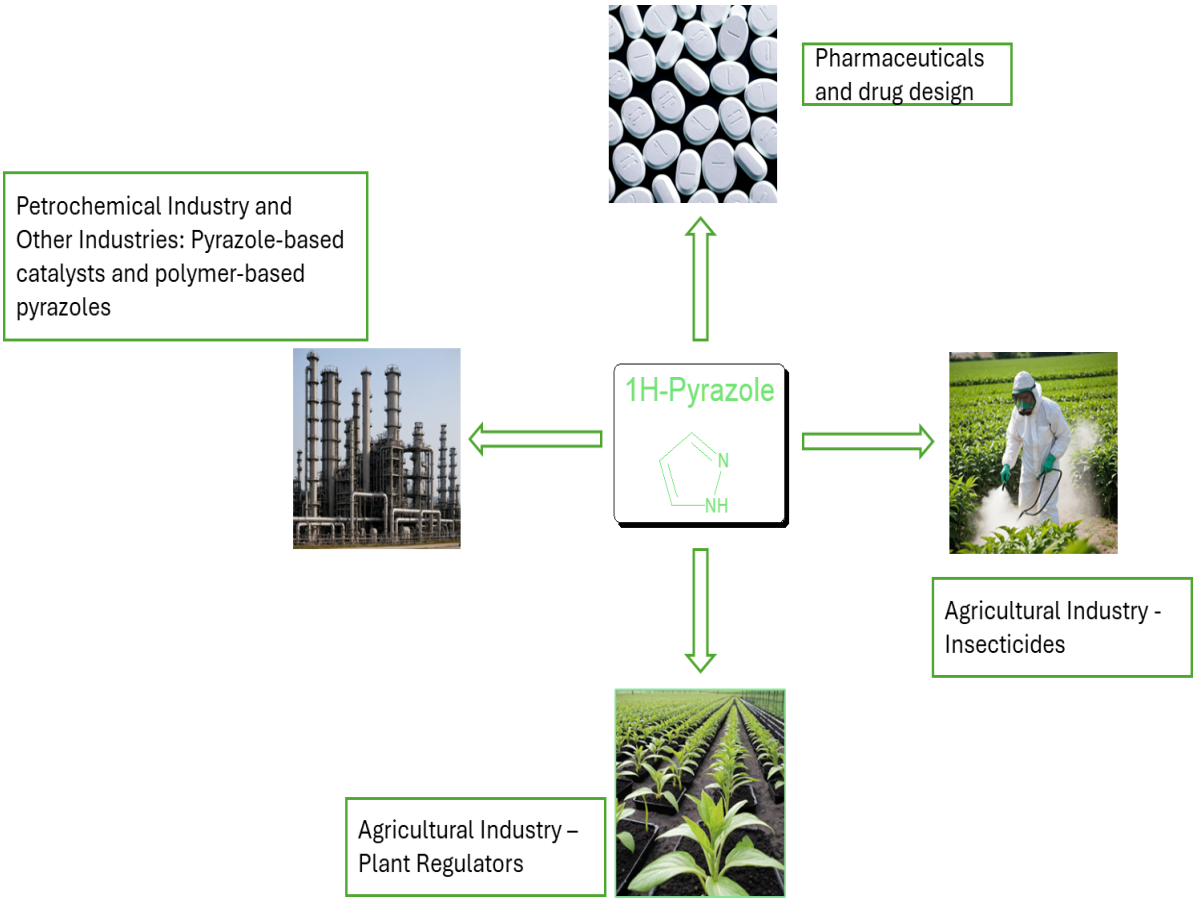

Figure 3: Application of pyrazole scaffold in various industries

The pyrazole moiety as a medicinal agent has proven to relieve certain infections and ailments that our society faces. However, illness-causing microorganisms have become unresponsive to conventional antibiotics. Therefore, medicinal chemists and other scientists have improved the existing antibiotics by incorporating a pyrazole core to enhance their potency against resistant microorganisms (Muniyasamy *et al.* 2024).

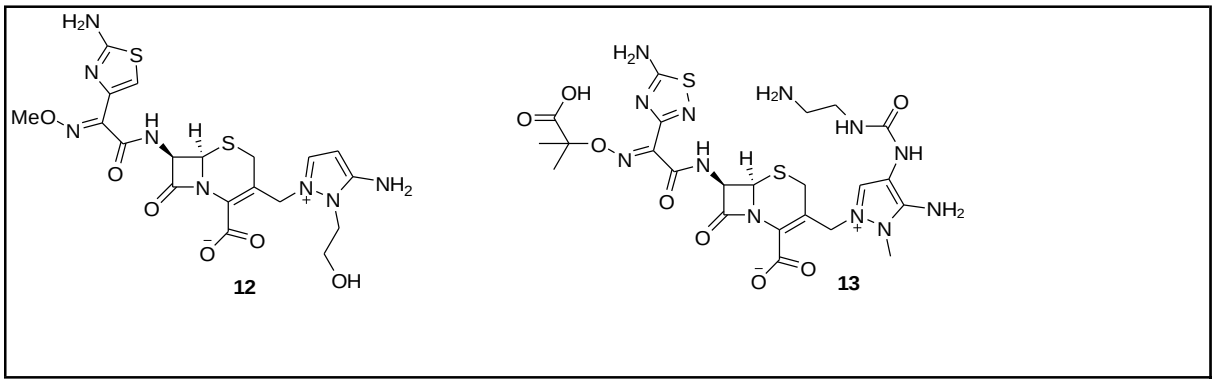

Figure 4: Approved pyrazole containing antibiotics (A Alam 2022)

In **Figures 4**, **12**, and **13**, pyrazole-based antibiotics are approved for treating bacterial infections. In this study, a feasible, efficient protocol will be utilized through a multicomponent reaction to synthesize novel pyrazole derivatives with potential improved biological efficacy. The research scope is presented in **Figure 5**.

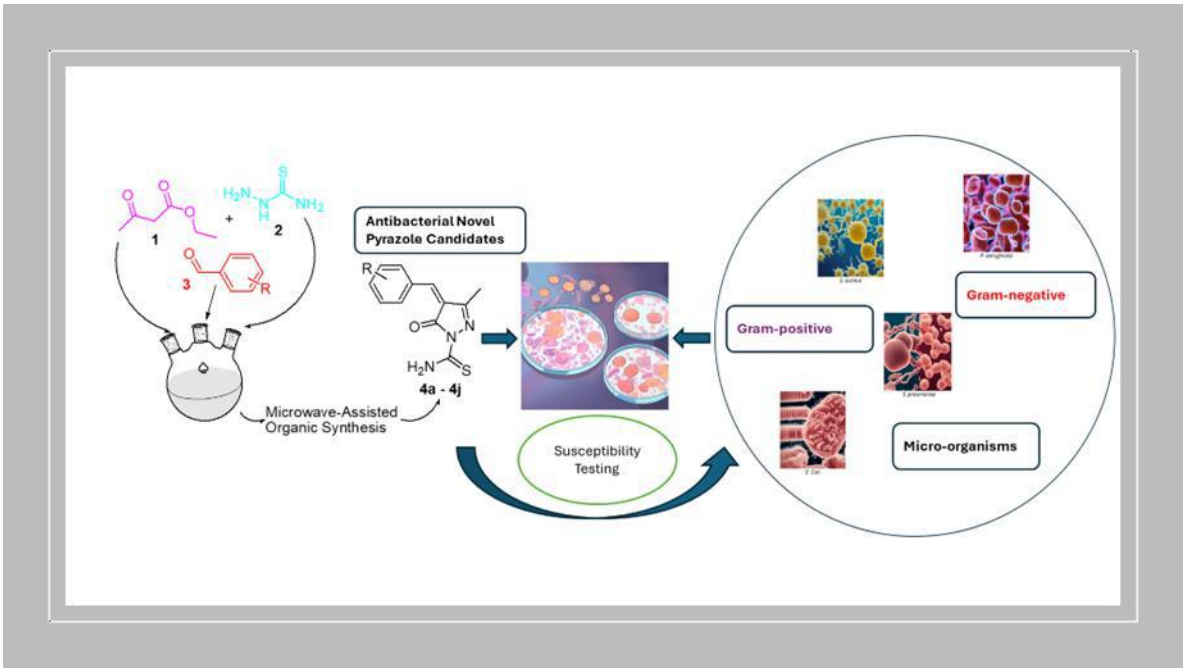

Figure 5: Graphical overview of the methodology of investigated pyrazoles

1.2 Aim and Objectives

1.2.1. Aim

The study aims to synthesize novel pyrazole derivatives using a multicomponent reaction, evaluate their antibacterial activities, and determine their molecular binding properties.

1.2.2 Objectives

- Design and optimize the multicomponent synthesis of novel pyrazoles using green chemistry
- Characterize the pyrazole using spectroscopic techniques
- Evaluate the antibacterial activities of pyrazole derivatives by agar-well diffusion

- Determine the Minimum Inhibitory Concentrations and Zone of inhibition of pyrazoles
- Determine the binding affinities of pyrazoles by molecular docking
- Evaluate the silico pharmacokinetics and toxicity prediction of pyrazoles

1.3 Structure of the Thesis

This thesis comprises five chapters, which are set out under the following headings:

Chapter 1: Introduction

Brief introduction to pyrazole compounds, their synthetic methodology, the aim and objective of the study.

Chapter 2: Literature Survey

Encompasses a background of pyrazoles, their methods of synthesis, green chemistry, recent approaches in synthesis, catalytic approaches of pyrazole synthesis, microwave synthesis of pyrazoles, and antimicrobial activity of pyrazoles.

Chapter 3: Methodology

This chapter elaborates on the general methodology and the general procedure for the preparation of pyrazoles, characterization techniques, ligand acquisition, protein preparation and molecular docking of penicillin binding protein of the bacteria, minimum inhibitory concentration and sensitivity assays, and in silico pharmacokinetic and toxicity predictions

Chapter 4: Results and Discussion

This section encapsulates the Synthesis of (4Z)-4-Arylidene-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide derivatives which are pyrazoles and chemistry involved, spectroscopic data analysis, optimization conditions, biological study results, molecular docking studies of pyrazoles, and pharmacokinetics and toxicology evaluation of pyrazoles

Chapter 5: Conclusion and Recommendation

The section highlights the key aspect of the study, mentioning the efficacy of the methodology, the yield obtained utilizing green techniques, the derivatives which proved potent against test bacteria, docking score results, and finally the pyrazole's oral bioavailability results.

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Chapter Two: Literature Review

2.1 Background of Pyrazole

Nitrogen-containing heterocyclic compounds are crucial in developing a new class of structural components for use in the pharmaceuticals and agrochemical industry (Keri *et al.* 2015). The pyrazole heterocyclic ring is an essential building block in organic, bioorganic, and pharmaceutical chemistry. It has attracted much attention for its simplicity in preparation and therapeutic potential as the foundation of cutting-edge medical applications (Verma *et al.* 2021). One of the most potent chemical groups is pyrazole and its derivatives because they exhibit various pharmacological effects, including antibacterial, anticonvulsant, anticancer, analgesic, anti-inflammatory, and antitumor properties (Khan *et al.* 2016).

Pyrazoles are planar, and their anions are less reactive to nucleophilic attack but susceptible to electrophilic attack. In the reactivity of adjacent *N*-atoms, as described by (Faisal *et al.* 2019), the nitrogen atom in position two has a lone pair of electrons and is mildly basic. The nitrogen atom in position one is not reactive but releases the H^+ in the presence of a base (Faisal *et al.* 2019). The combined impact of two nitrogen atoms lowers the charge density at carbon-3 and carbon-5, making them susceptible to electrophilic attack (Faisal *et al.* 2019).

Pyrazoles possess pivotal pharmacological activities such as antipyretic, sedative and antidiabetic (Faisal *et al.* 2019). In addition to their therapeutic applications, they're also employed in material chemistry and food industries (Baiju and Namboothiri 2017). The *N*-substituted pyrazole derivatives can inhibit and deactivate liver alcohol dehydrogenase (Bhaskaruni *et al.* 2020). Pyrazoles have attracted considerable attention in supramolecular and polymer chemistry, herbicides, cosmetic colourings and UV stabilizers. Furthermore, some pyrazoles have liquid crystal properties (Quang-Hung *et al.* 2023). Other pyrazole derivatives, such as Viagra **18**, celecoxib **19**, and fipronil **27**, are widely used as therapeutic agents on the market today (Doddaramappa *et al.* 2015) shown in **Figure 6**. Amongst FDA-approved medications that contain the pyrazole scaffold are Rimonabant **20**, Difenamizole **22**, Betazole **23**, Lonazolac, Mepirizole **24**, Fezolamine **25**, Tepoxalin **26**, and Deracoxib **28** and Pyrazomycin **29** (Becerra, Abonia and Castillo 2022b) revealed by **Figure 7**. Hence, pyrazoles' biological activity potency has made them ideal synthetic targets. Numerous methods for preparing substituted pyrazoles have been established in the literature (Kumar and Govindaraju 2015). For example, in **Scheme 6**, page 31, at 50-70°C, the reaction of Baylis-Hillman adduct

54 and phenyl hydrazine **55** yielded tetrasubstituted pyrazole derivatives **56** with very good regioselectivity of products in 89% yield (Kumar and Govindaraju 2015).

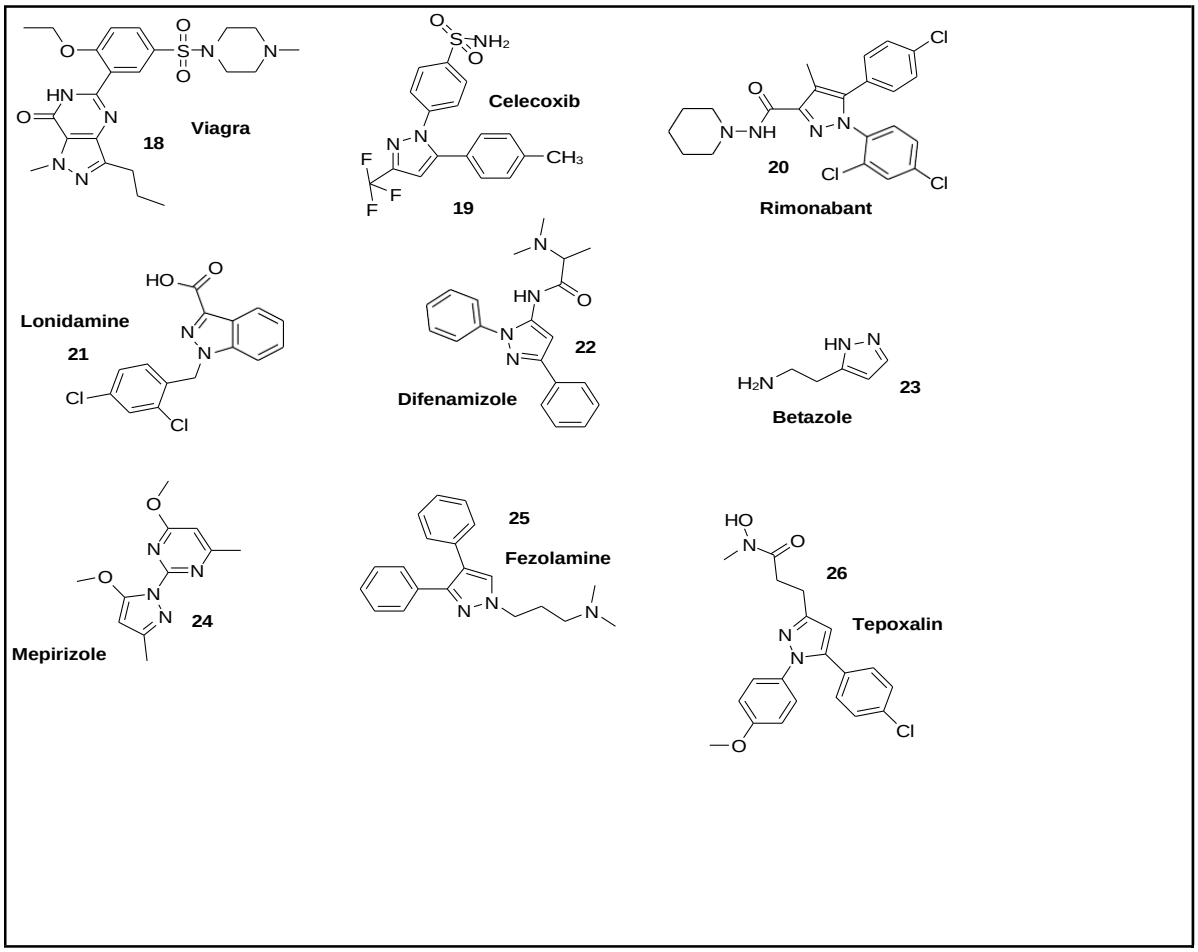


Figure 6: Therapeutic pyrazole core treatments on market

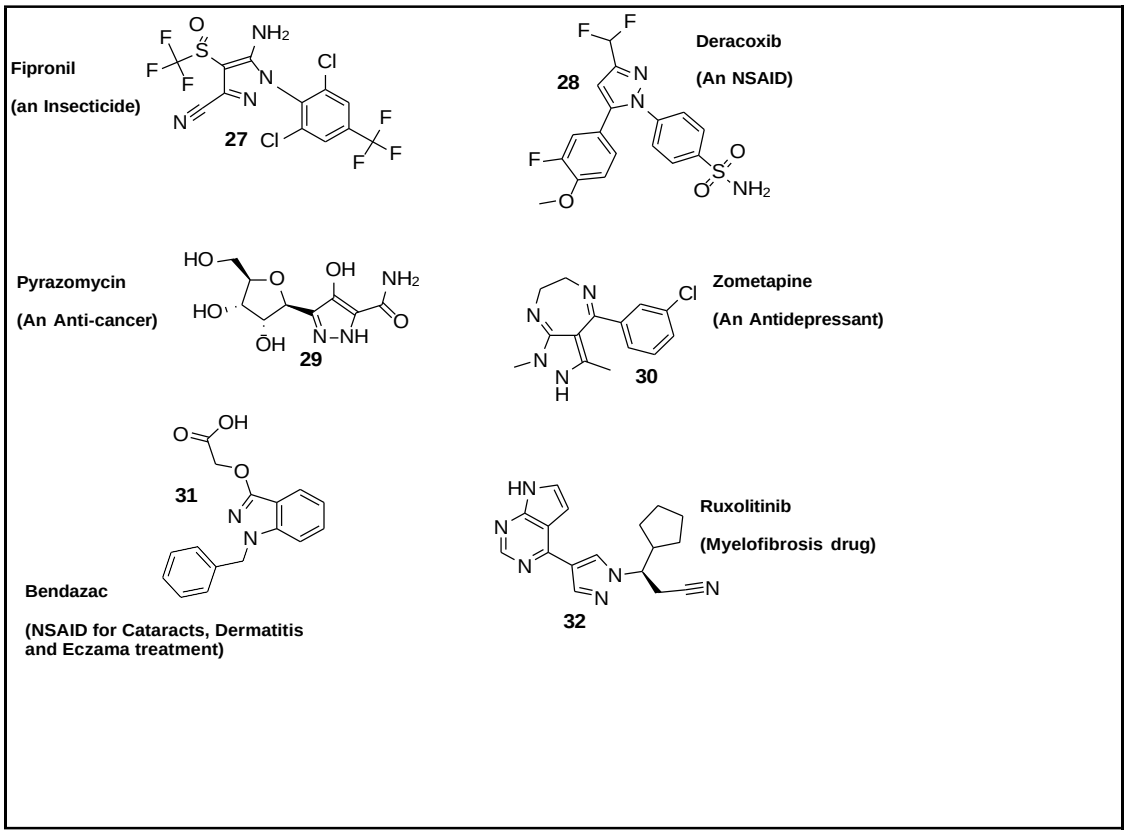
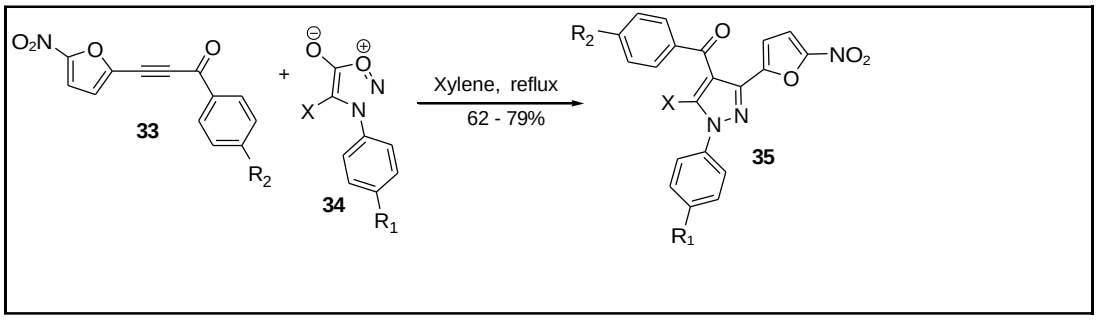


Figure 7: Pharmacological pyrazole marketed drugs

2.2 Conventional Methods of Synthesizing Pyrazole

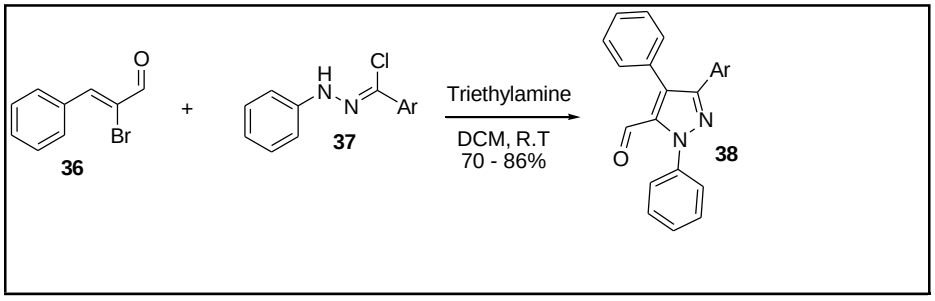
Traditional methods for the synthesis of substituted pyrazoles utilize condensations of hydrazines with 1,3-dicarbonyl compounds and their 1,3-electrophile equivalents or intermolecular [3+2] cycloadditions of 1,3-dipoles to alkynes (Fustero, Simón-Fuentes and Sanz-Cervera 2009). Thus, the annulation reactions of substituted hydrazines with α,β-unsaturated aldehydes, α,β -unsaturated ketones, and β-aminoacrylates are the focal point of classical procedures for the synthesis of functionalized pyrazoles (Singh *et al.* 2021). Other synthetic techniques, such as the [1,5]-sigmatropic rearrangement, selective rearrangement of polyazaheterocycles, the Pechmann pyrazole synthesis, and inter/intramolecular cyclization of conjugated azoalkenes with triphenylphosphine followed by thermolysis, have also been reported in the literature (Singh *et al.* 2021). 1,3-Dipolar cycloadditions are bimolecular and involve the addition of a 1,3-dipole to a multiple-bond system, leading to five-membered heterocycles, as mentioned by (Amblard, Cho and Schinazi 2009). As in the allyl anion system, a 1,3-dipole is a system of three atoms distributed with four pi electrons. The three atoms can be combinations of C, O, and N. The dipolarophile can be virtually any double or triple bond. 1,3-Dipolar cycloadditions of diazoalkanes to multiple bonds were first observed in 1888 by

Buchner (Breugst and Reissig 2020). Examples of 1,3-dipoles used to synthesize pyrazoles include nitrile, imines and sydnones. The usual dipolarophile for this process is an alkene, alkyne or an alkyne surrogate **33** (Dadiboyena and Nefzi 2011). Sydnones **34** are mesoionic heterocycles that exhibit potential biological properties and serve as valuable precursors in synthesising substituted pyrazoles **35**, **Scheme 1**.

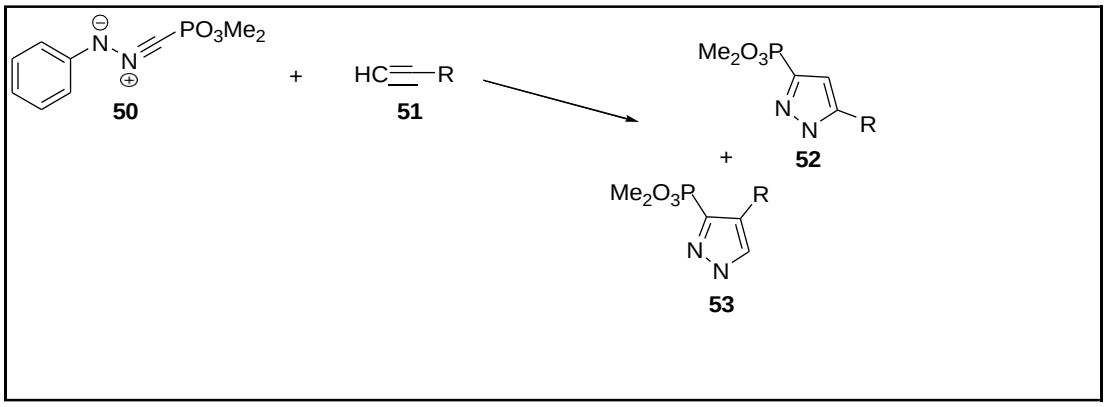


Scheme 1: A 1,3-Dipolar cycloaddition pyrazole synthesis from sydnones (Dadiboyena and Nefzi 2011)

Tetrasubstituted pyrazoles **38** are obtained in high yield through the regioselective 1,3-dipolar cycloaddition of α-bromocinnamaldehyde **36** to C-aryl-*N*-phenyl nitrile imines **37** at room temperature **Scheme 2**, reports (Fustero *et al.* 2011) and (Dadiboyena and Nefzi 2011). A 1,3-dipolar cycloaddition of nitrile imine **50** to monosubstituted alkynes **51** produced *N*-phenyl-5-substituted-3-dimethoxyphosphonopyrazoles **52** & **53** (**Scheme 3**) with strong regioselectivity but low yields as potential *N*-methyl D-aspartate (NMDA) antagonists. The cycloadditions of nitrile amine to alkenes produces regioselectively 5-substituted pyrazoline derivatives, which were oxidized to provide high yields of pyrazoles (Dadiboyena and Nefzi 2011). Highly functionalized unsymmetrical α,β-acetylenic phenones with a 5-nitrofurane unit were used as dipolarophiles in 1,3-dipolar cycloadditions with *N*-arylsydnones to create a series of 1-aryl-3-(5-nitro-2-furyl)-4-aroylpyrazoles. The antibacterial and antifungal properties of the resultant pyrazoles were investigated further (Fustero *et al.* 2011).

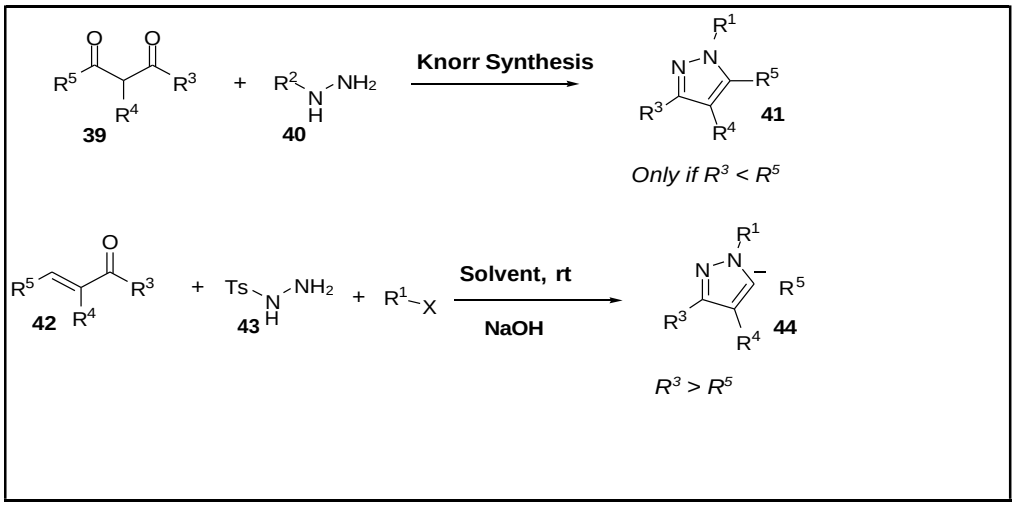


Scheme 2: Functionalized pyrazole from cyclocondensation of bromocinnamaldehyde (Fustero *et al.* 2011)



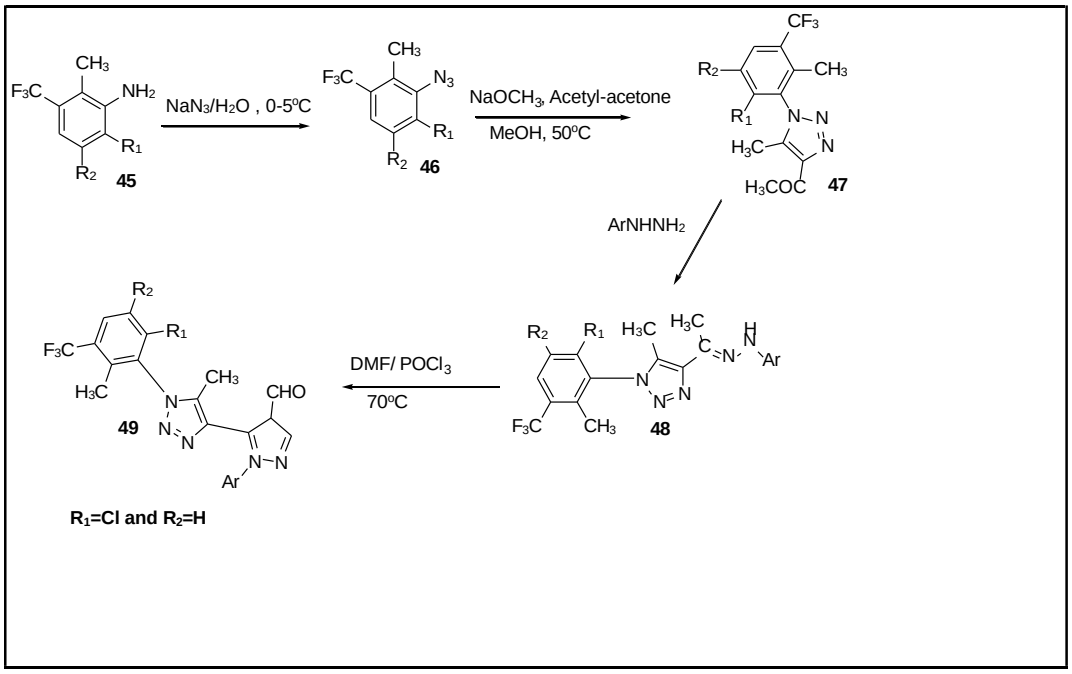
Scheme 3: Nitrile imine and alkyne generating 5-substituted-3-dimethoxyphosphonopyrazoles (Dadiboyena and Nefzi 2011)

With particular emphasis on its regioselective synthesis, the original Knorr condensation method was inevitably found to be associated with poor regioselectivity as in the reaction of a 1,3-dicarbonyl **39** compound with an unsymmetrical hydrazine **40** (Yoon, Lee and Shin 2011) reported and shown in **Scheme 4**. The limitation of this preparation method was the failure or difficulty in separating the two pyrazole regio-isomers by chromatography or repeated crystallization. They subsequently demonstrated a dramatically reduced yield of the desired pyrazole product (Yoon, Lee and Shin 2011). In their publication (Tang and Zhang 2013) devised a general and convenient Knorr method for synthesising pyrazoles with substituent at C-3 larger than that of C-5 in the pyrazole moiety. This was formerly deemed unsuitable, and only the vice versa was viable, C-5 larger than C-3. Regioselectivity in this study was established in one-pot and synthesized functionalized pyrazoles **41** and **44** in good yields (Tang and Zhang 2013) delineates (**Scheme 4**).

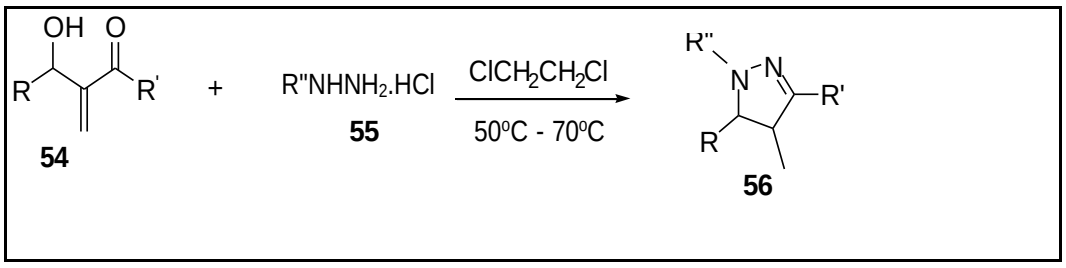


Scheme 4: Regioselective pyrazole synthesis (Tang and Zhang 2013)

According to the literature, the formylation of hydrazones produces formyl pyrazoles. On treatment with DMF and $POCl_3$, Schiff's bases of aldehydes and ketones undergo cyclization reactions, yielding pyrazole derivatives and formylation on the pyrazole ring (Rajput and Rajput 2011). A popular approach in pyrazole chemistry is the production of 4-formylpyrazole via the Vilsmeier-Haack reaction. There is still much interest in finding novel methods for preparing 4-formylpyrazoles because of their potential applications as precursors in synthesising various chemical and heterocyclic compounds (Kumari *et al.* 2020). In a recent study Manjunatha *et al*. 2016., explored a synthetic protocol for the title compounds, 3-{5-methyl-1-[2-methyl-3-(trifluoromethyl)-substituted-phenyl]-1H-1,2,3-triazol-4-yl}-1-(aryl)-1H-pyrazole-4-carbaldehyde **49** employing a Vilsmeier–Haack formylation (Manjunatha *et al.* 2016). In this study shown in **Scheme 5**, novel 1,2,3-triazole **47** bearing pyrazoles as a critical nucleus are evaluated for their anti-microbial and anti-oxidant potentials with the hope of improving the biological activities.



Scheme 5: Vilsmeier– Haack formylation of pyrazole by (Manjunatha *et al*. 2016)



Scheme 6: Pyrazoline Synthesis from Baylis-Hillman adduct by (Kumar and Govindaraju 2015)

2.3 Modern Synthesis of Pyrazole Derivatives

2.3.1 Green Chemistry

Since the release of Paul Anastas and John Warner's ground-breaking book Green Chemistry: Theory and Practice 21 years ago, the world has witnessed some amazingly inventive discoveries to improve the sustainability of industrial and manufacturing processes (Bastin and Dicks 2019) and encompassed in the book are principles of green chemistry (Long 2018). Modes to implement in synthesis methodology design may include green techniques like microwave, grinding and ultrasonication. In addition, solvent-free or green solvents like ethanol, water, and ionic liquids. Using heterogeneous catalysts is the operational tool to

achieve sustainability using green chemistry—also, a one-pot multicomponent reaction which improves the efficiency of chemical reactions (Singh *et al.* 2021).

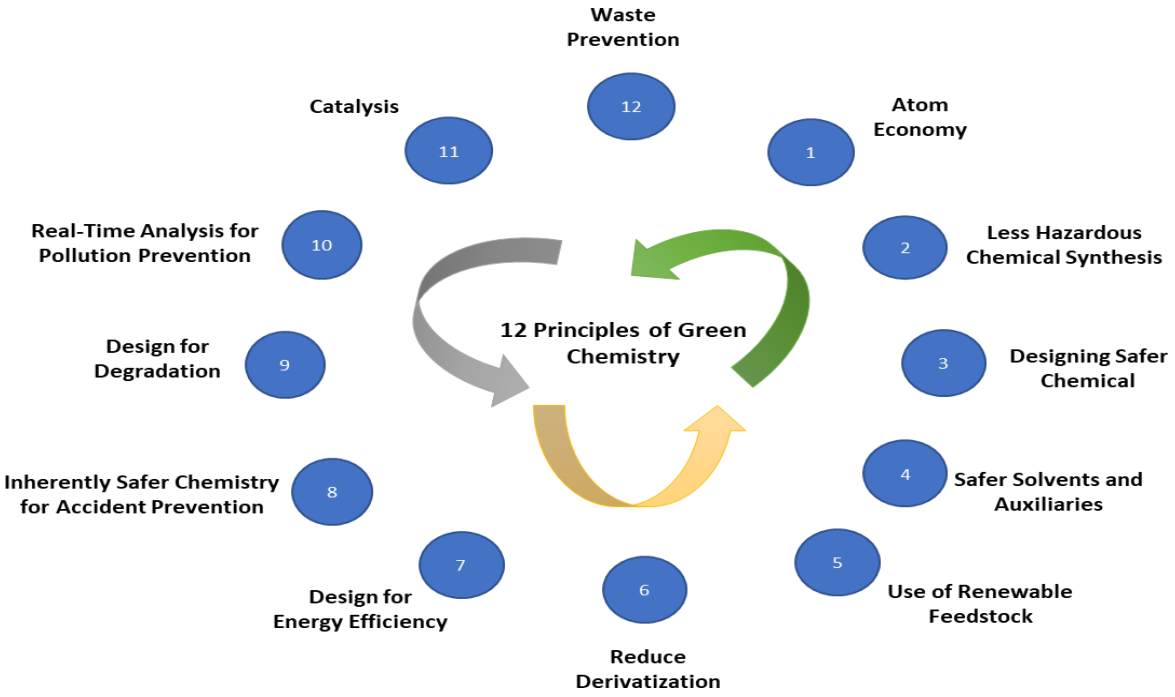


Figure 8: 12 Principles of Chemistry by Paul Anastas (Dicks 2016)

Nowadays, many complex molecules can be synthesized in the laboratory, and such structures are well-published. However, despite the achievements scientists and researchers made in constructing these molecular architectures, methods used to accomplish complex products are extensive. They could be more efficient in yield due to protection, defunctionalization, purification and side products (Li and Trost 2008). Trost proposed the atom economy, a set of cohesive guiding principles for evaluating the efficiency of specific chemical processes (Li and Trost 2008). The atomic economy attempts to maximize the incorporation of the starting components into the ultimate product of any given reaction. If the maximal incorporation cannot be accomplished, the quantities of side products produced should ideally be minute and ecologically harmless (Li and Trost 2008).

By utilizing one-pot synthesis, diversity and high atom economy is attained to produce the necessary products in a single step. Through the simultaneous production of two or more bonds without isolating or purifying intermediates (Fustero *et al.* 2011), multicomponent reactions (MCRs) are highly relevant, allowing the combination of more than two building blocks. Furthermore, by changing the reactive elements, great amounts of variation can be achieved

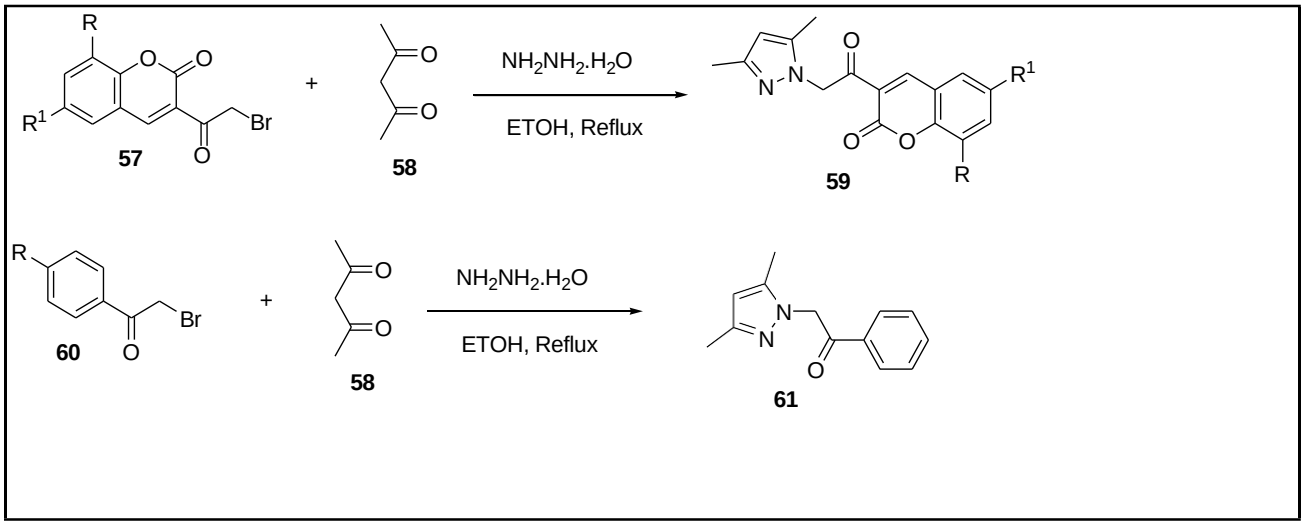
(Fustero *et al.* 2011). MCRs in the aqueous phase have received a lot of interest, mainly due to the great synthetic efficiency of these procedures (Srivastava *et al.* 2014b). Given the need for green chemistry. Using water as a solvent instead of organic solvent has resulted in remarkable advances. The development of organic synthesis is due to its low cost, safety, and environmental friendliness (Wen *et al.* 2011). Therefore, in this investigation, water and ethanol will be utilized in a one-pot MCR expedited by microwave irradiation.

2.3.2 Recent Approaches of Pyrazole Synthesis

Most uneconomical classical reactions in which intrinsic wastes are unavoidable are substitutions and eliminations. Simple additions, cycloadditions, and rearrangements are desirable reactivities (Li and Trost 2008). The new eco-friendly methods for synthesizing pyrazole are multicomponent synthesis, microwave irradiation and green heterogeneous catalysis (Chaudhari and Dhivare 2016). Multi-component reactions can create numerous bonds in a single step. Thus, MCRs are advantageous in synthetic, combinatorial, and medicinal chemistry (Ablajan *et al.* 2013). By decreasing the number of synthetic steps, waste output can be reduced, and energy consumption in the pharmaceutical sector can be saved (Tangeti *et al.* 2016). The traditional multi-step synthesis of molecules involves more than one process, including compound purification after each step. By-products are created, which affects the atom economy (Srivastava *et al.* 2014a). MCRs, on the other hand, are efficient at developing complexity, exhibit higher atom economy, and enhance product yield.

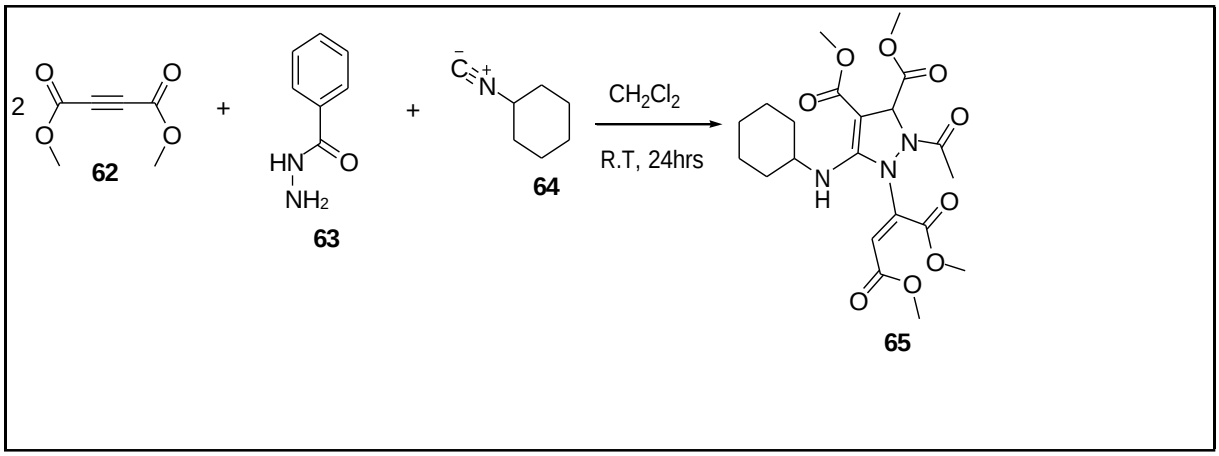
2.4. Multicopmonent Synthesis of Pyrazoles

The synthesis by multicomponent approach, (P. Santhosh, Chunduru and Rajeswar-Rao 2011) incorporated coumarin moieties to study their biological activities. By condensing various 3-(2-bromoacetyl)coumarins **57** and phenacyl bromides **60** with hydrazine hydrate and acetylacetone **58**, resulted in the formation of the cyclic 3-[2-(3,5-dimethyl-1H-pyrazol-1-yl)acetyl]-2H-chromen-2-one **59**, and 2-(3,5-dimethyl-1H-pyrazol-1-yl)-1-phenylethanone **61** derivatives displayed in **Scheme 7**.



Scheme 7: Novel pyrazole synthesis from 3-(2-bromoacetyl)coumarins and phenacyl bromides with hydrazine in water (P. Santhosh, Chunduru and Rajeswar-Rao 2011)

A one-pot, four-component reaction involving arylcarbohydrazides **63**, dialkyl acetylenedicarboxylates **62**, and cyclohexyl isocyanide **64** in **Scheme 8** produced the highly functionalized 1H-pyrazole derivatives **65**. Due to the straightforward availability of the synthetic technique and the neutral ring closure conditions, this method can potentially be used in synthesising numerous functionalized 1H-pyrazole derivatives (Kumar and Jayaroopa 2013).

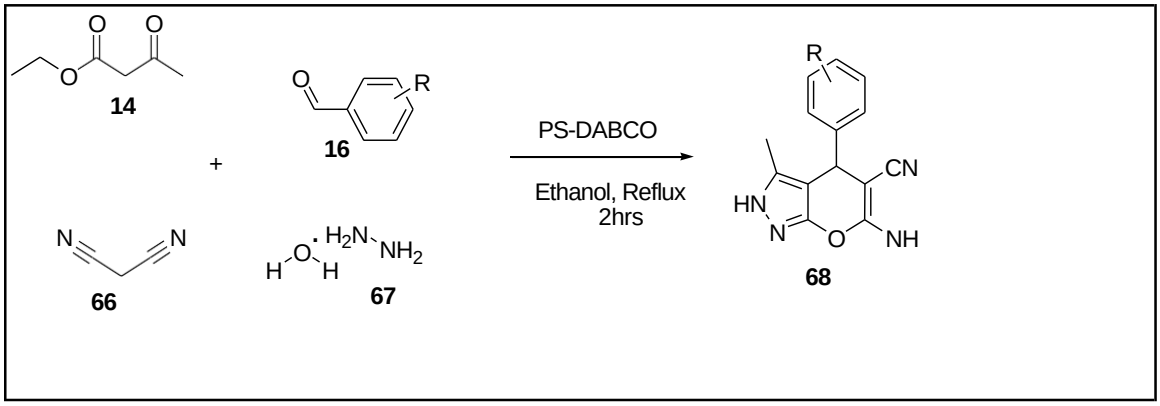


Scheme 8: A multicomponent and highly functionalized pyrazole synthesis reported by (Kumar and Jayaroopa *et al*. 2013)

2.5 Catalytic Synthesis of Pyrazoles

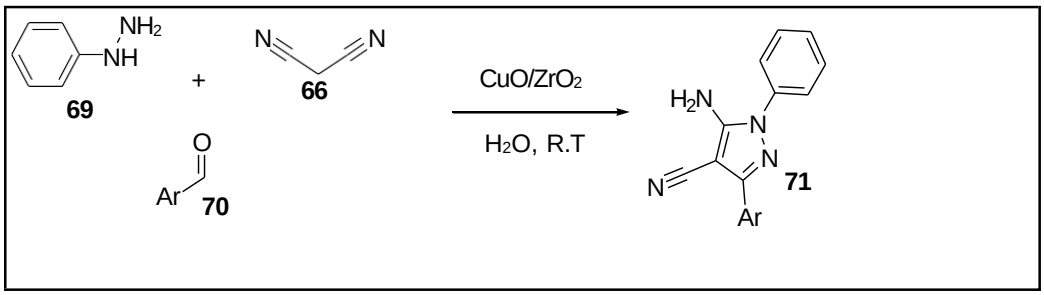
2.5.1 Heterogeneous Catalysis

Polymer-supported heterogeneous catalysts and heterogeneous catalysts have various advantages in organic synthesis, including ease of product separation, isolation and catalyst regeneration (Khairnar, Mane and Chaudhari 2019). However, most known approaches use homogeneous catalysts containing expensive, toxic chemicals and high temperatures without recycling or reusing the catalyst. As a result, there has been a lot of interest in developing novel reusable heterogeneous catalysts to solve these difficulties (Quang-Hung *et al*. 2022). The heterogeneous Diazobicyclo[2,2,2]octane (DABCO-Polymer Supported) catalyst in **Scheme 9** has good catalytic activity in a variety of C-C and C-N bond-forming processes, according to the literature by (Khairnar, Mane and Chaudhari 2019). Its significance in pyrazole formation is reported, as seen in **Scheme 9**, the PS-DABCO furnishes pyrazole scaffolds **68**, from ethyl acetoacetate **14**, substituted benzaldehydes **16**, malononitrile **66**, and hydrazine hydrate **67**.



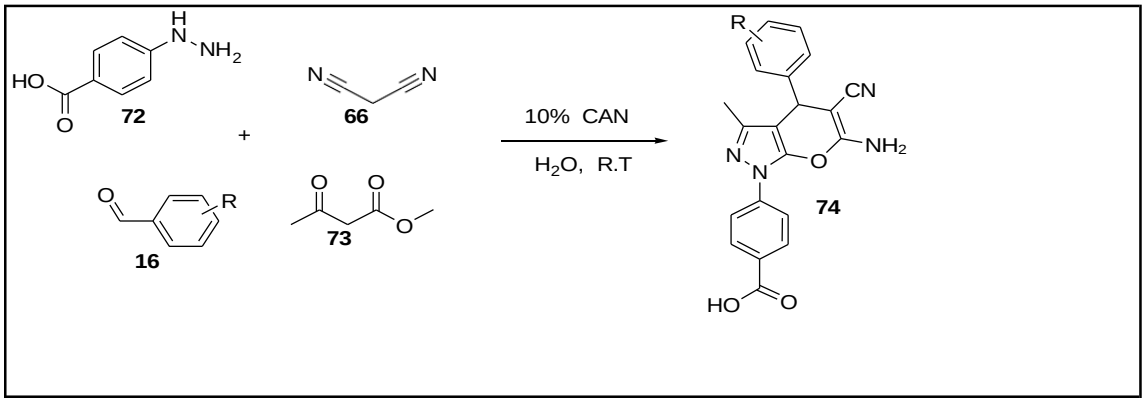
Scheme 9: Polymer Supported-DABCO catalyzed multicomponent synthesis of pyranopyrazole by (Khairnar, Mane and Chaudhari 2019)

With the aid of a CuO/ZrO_2 catalyst in a three-component Mannich-type reaction involving phenyl hydrazine **69**, malononitrile **66**, and substituted aromatic aldehydes **16**, novel pyrazole-4-carbonitrile **71** analogues in **Scheme 10** were created in good to outstanding yields in under two hours (Bhaskaruni *et al*. 2020). A straightforward wet-impregnation approach created a novel mixed oxide catalyst with increased activity and long-term stability. The heterogeneous catalyst can be used repeatedly for up to five cycles without losing its high activity (Bhaskaruni *et al*. 2020).



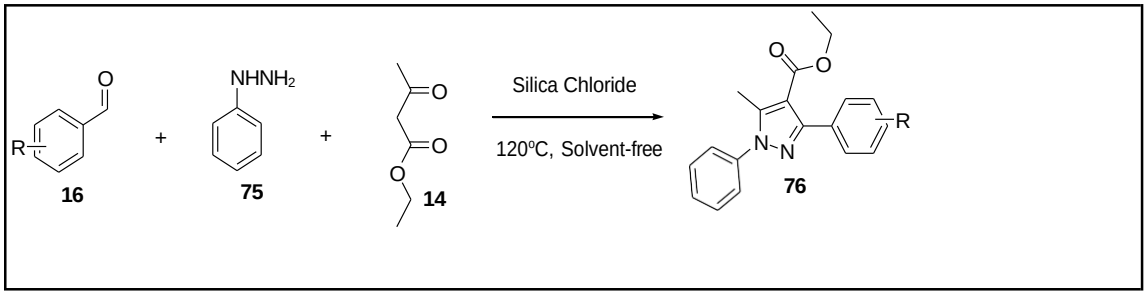
Scheme 10: Bhaskaruni *et al*.2020, reported the pyrazole-4-carbonitrile synthesis (Bhaskaruni *et al*. 2020).

Ablajan *et al*. (2013) investigation describes a simple, one-pot, four-component pyranopyrazole **74** synthesis catalyzed by cerium ammonium nitrate (CAN). Ablajan *et al*. 2013 **Scheme 11**, elucidates the positive outcomes of CAN in terms of its high-yielding capacity, low cost, environmental friendliness, and straightforward operation in pyrazole derivatives.



Scheme 11: Pyrazno(2.3-c)pyrazoles synthesis under ultrasound, cerium ammonium nitrate catalyzed (Ablajan *et al*. 2013)

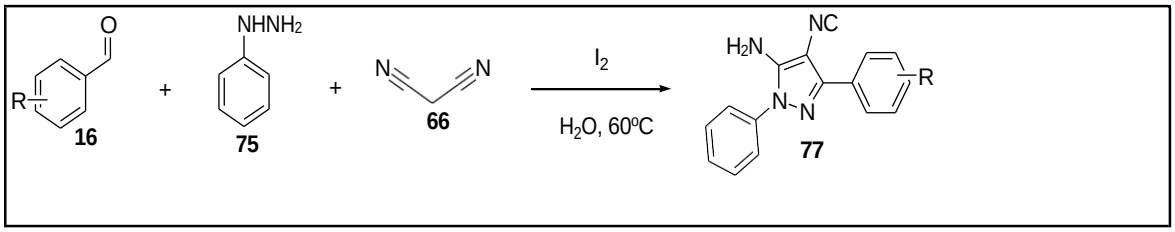
Progressing on their early work on the development of convenient synthetic protocols for bioactive pyrazole derivatives **76**, (Jawale *et al.* 2011), reported an efficient one-pot cyclocondensation of aromatic aldehydes **16**, phenylhydrazine **75** and ethylacetoacetate **14** allowed to reflux at solvent-free conditions with the influence of silica chloride, a heterogeneous catalyst in **Scheme 12**.



Scheme 12: Silica chloride catalyzed-solvent free one pot pyrazole synthesis by (Jawale *et al.* 2011)

2.5.2 Homogeneous Catalysis

The use of enzymes, organo-catalysts or transition metal catalysts, as opposed to the employment of stoichiometric quantities of other traditional promoters of different organic synthetic processes (like inorganic/organic bases, Brønsted acids, radicals). Allow the discovery of many new synthetic protocols within the toolbox of organic chemists (García-Álvarez 2020). Among all the facets of green chemistry, selecting a green homogeneous catalyst is crucial to the chemical process (Sangwan *et al.* 2021). A practical one-pot multicomponent synthesis in **Scheme 13** was reported by the Srivastava group in 2014, bringing together phenyl hydrazine **75**, malononitrile **66**, and a varied spectrum of aldehydes **16** utilizing water and iodine as catalysts to generate functionalized pyrazoles **77** (Singh *et al.* 2021).

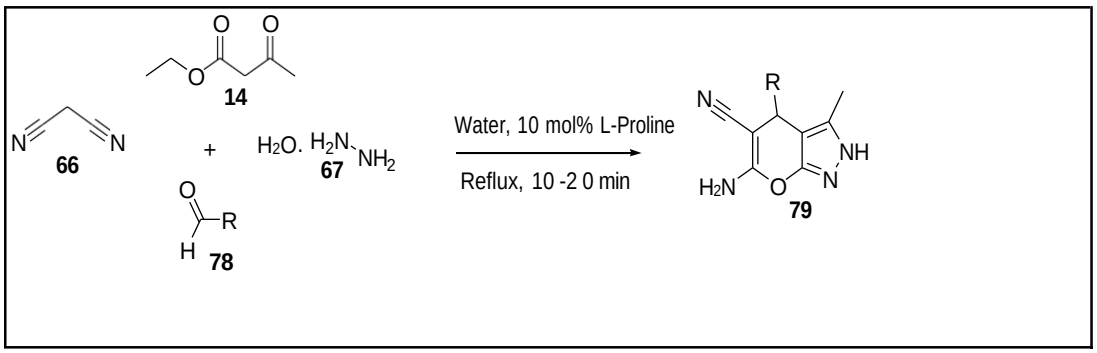


Scheme 13: A 3-multicomponent Iodine catalyzed one pot pyrazole synthesis reported in (Srivastava *et al*. 2014)

The use of organo-catalysts for organic synthesis has become safe and earned a valuable place in the literature (Sangwan *et al.* 2021). This branch of chemistry is a novel idea that involves the isolation, characterization, and synthesis of new chemicals with a primary focus on environmental safety. A recent study by (Sihtmäe *et al.* 2022) reveals a paucity of green

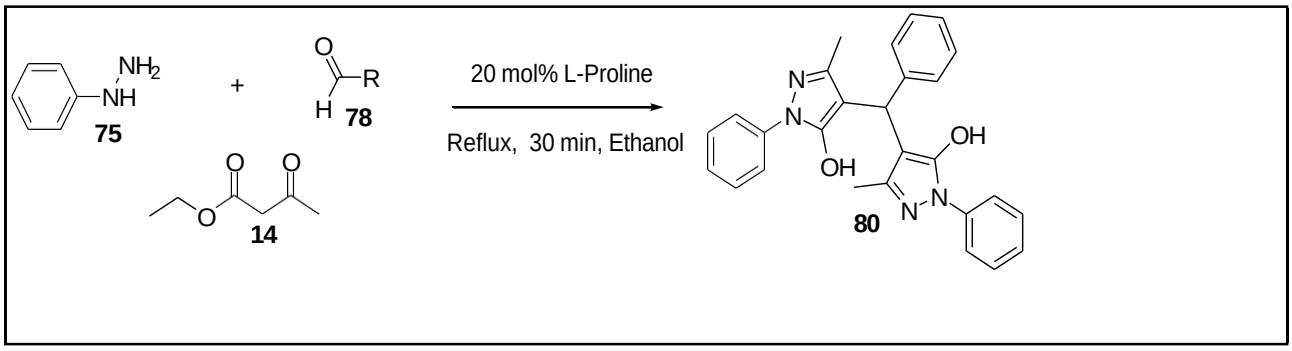
chemistry aspects of homogeneous catalysts. They have conducted a study analysing the ecotoxicity of a library of selected amino-catalysts and chiral hydrogen-bonding organo-catalysts. Amino-catalysts proved to be of low ecotoxicity and were deemed not harmful, but thioureas and squaramide were toxic, and some were shown to be harmful (Sihtmäe *et al.* 2022).

Mecadon *et al.* (2011) investigated the efficiency of L-proline against three other catalysts: ϒ-alumina, basic alumina, and KF-alumina. In a water-based multicomponent synthesis of 6-amino-4-aryl-3-methyl-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitriles **79** in **Scheme 14**, the amino acid was revealed to be a better catalyst in terms of yield and eco-friendliness (Mecadon *et al.* 2011).



Scheme 14: One-pot synthesis of 6-amino-4-alkyl/aryl-3-methyl-2,4-dihydropyrano[2,3-c]pyrazole-5-carbonitriles in water catalyzed by L-proline, (Mecadon *et al*. 2011)

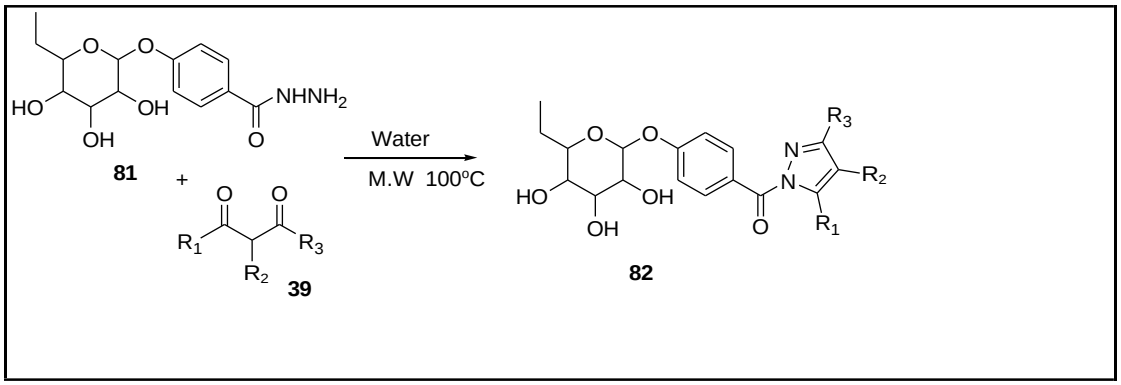
This facile, L-proline catalyzed method furnished high yields, is non-toxic and needs no column chromatography for purification. The ability of L-proline as an organo-catalyst to form iminium intermediates with carbonyls. Playing the role of both Lewis acid and base existing as a zwitterion in organic reactions has made it appealing, considering its benignity (Heravi, Danafar and Heravi 2019). The study by (Mahajan *et al.* 2017) indicates an eco-friendly synthesis of 4,4′-(arylmethylene)bis(3-methyl-1-phenyl-1H-pyrazol-5-ols) **80** in ethanol and catalytic amounts of L-proline. The advantages of the reported procedure highlighted in **Scheme 15**, were shorter reaction times, non-chromatographic purification, readily available starting materials and inexpensive non-toxic catalyst. (Mahajan *et al.* 2017)



Scheme 15: Organo-catalysed efficient one pot synthesis of 4,4′-(Arylmethylene)bis-(3-methyl-1-phenyl-1H-pyrazol-5-ols), (Mahajan *et al.* 2017)

2.6 Synthesis of Pyrazoles Through Microwave Irradiation

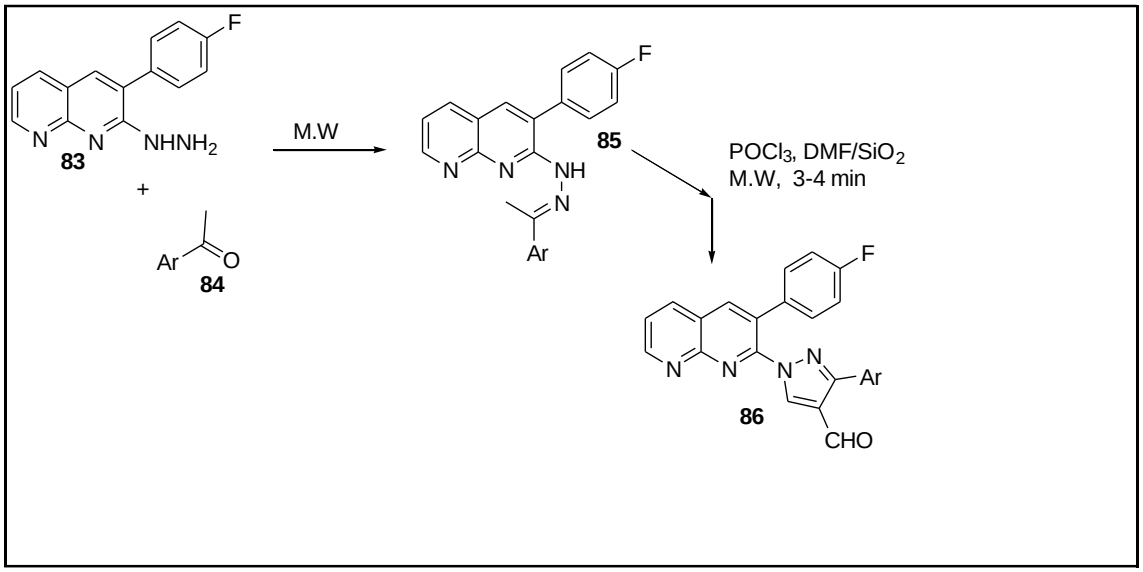
Microwave (MW) irradiation is beneficial in biologically important *N*-heterocycle preparation, leading to high yield and low environmental impact (Henary *et al.* 2020). Microwave-assisted organic synthesis (MAOS) exploits dielectric volumetric heating as an alternative heat source, which results in faster and more selective reactions. Due to the uniform heat distribution (Henary *et al.* 2020). In **Scheme 16**, compound **82** was synthesized through conventional and microwave methods described by Henry *et al.* 2020. In the case of conventional methods, the investigation yielded no yield, deeming the approach ineffective. However, microwave methods gave superior outcomes due to their low reaction times and relatively high yields. (Henary *et al.* 2020)



Scheme 16: Microwave synthesis of sugar-based pyrazole derivatives (Henary *et al.* 2020)

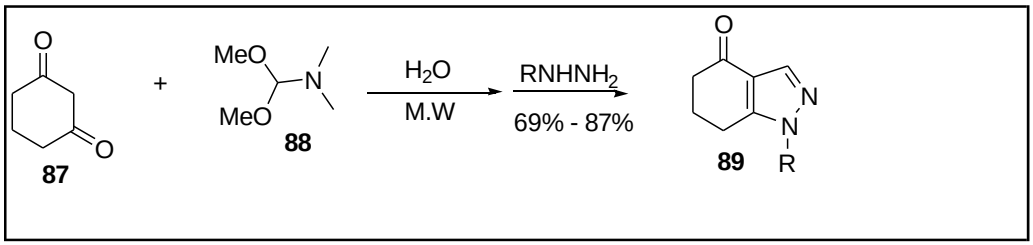
In their review (Singh *et al.* 2021) highlighted work done by (Mogilaiah and Reddy 2005) where they prepared 3-aryl-4-formyl-1-[3-(4-fluorophenyl)-1,8-naphthyridin-2-yl]-pyrazoles,

86 using acetophenone **84**, 3-(4-fluorophenyl)-1,8-naphthyridin-2-ylhydrazine **83** with $POCl_3$-DMF over silica gel under microwave irradiation which gave the product in 84-97% yield in 3-5 minutes depicted in **Scheme 17**, reported by (Singh *et al.* 2021).



Scheme 17: Synthesis of 3-aryl-4-formyl-1-[3-(4-fluorophenyl)-1,8-naphthyridin-2-yl]-pyrazoles under microwave irradiation (Singh *et al*. 202)1

An aqueous one-pot synthesis of 4,5-fused-1-substituted pyrazoles **89** from 1,3-cycloalkanediones **87** through enamino-ketones **88** in which microwave irradiation is used (**Scheme 18**) has been described in (Fustero, Simón-Fuentes and Sanz-Cervera 2009). The short reaction times (120 seconds at 200◦ C) and the simple purification through precipitation of the products in aqueous media make this a remarkably elegant procedure (Fustero, Simón-Fuentes and Sanz-Cervera 2009).



Scheme 18: One-pot synthesis of 4,5-fused-1-substituted pyrazoles using Microwave Irradiation (Fustero, Simón-Fuentes and Sanz-Cervera 2009)

2.7 Antimicrobial Activity of Pyrazoles

Since their discovery, pyrazoles have enchanted scientists with their vast range of biological and pharmacological impacts on diseases. As time progresses and our way of life evolving, the population increases, and so do other socioeconomic factors introduce uncharted medical conditions people suffer from and illnesses that former prescriptions no longer cure or mitigate. The Infectious Diseases Society of America (IDSA) has designated the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, and Pseudomonas aeruginosa Enterobacter*) family of pathogens as dangerous due to their propensity to evade the bactericidal effect of conventional antibiotics (Verma *et al.* 2021). When an ill-causing bacteria is no longer responsive to the prescription to bring about a remedial effect from the medical condition experienced, that bacteria is multi-drug resistant, also known as MDR. This terminology is well known to patients diagnosed with the bacterium *Mycobacterium tuberculosis*, and MDR bacteria or fungi can be fatal. To advance the study of functionalized pyrazoles moiety and their antimicrobial activities substantiated in several kinds of literature (Sahu *et al.* 2008), (Shivapura *et al.* 2015), (Barakat *et al.* 2016). It is imperative to delve further into this scaffold and its biological effects in alleviating MDRs and adding to the medicinal chemistry library. Research in medicinal chemistry is still looking for new analgesic and antibacterial drugs without adverse effects. Despite the development of new, more expensive pharmaceuticals, the average person cannot afford their price. These changes have, therefore, highlighted the urgent need for new, more potent, affordable, and secure antimicrobial agents (Vijesh *et al.* 2013).

The synthesized pyrazole entities, **Figure 9**, were tested against three Gram-positive and three Gram-negative bacteria and other pathogenic strains like *M.tubercolosis* and *P.fulciparam* strain (Bhatt *et al.* 2018) reports. In their study, they demonstrated that compound **90** exhibited a higher potency (62.5 µg/mL) against Gram-negative strain *E.coli* compared to the standard drugs ampicillin and chloramphenicol (100 µg/mL) and (50µg/mL), respectively. Also, concerning *V. cholera* strain, compound **91** revealed enhanced potency (MIC: 62.5 mg/mL). As compared to ampicillin both compounds **90** and **91** showed activity against *B. subtilis* and were found to be highly potent with MIC of 62.5 mg/mL as compared to ampicillin (MIC: 250 mg/mL) and norfloxacin (MIC: 100 mg/mL) standard drugs (Bhatt *et al.* 2018).

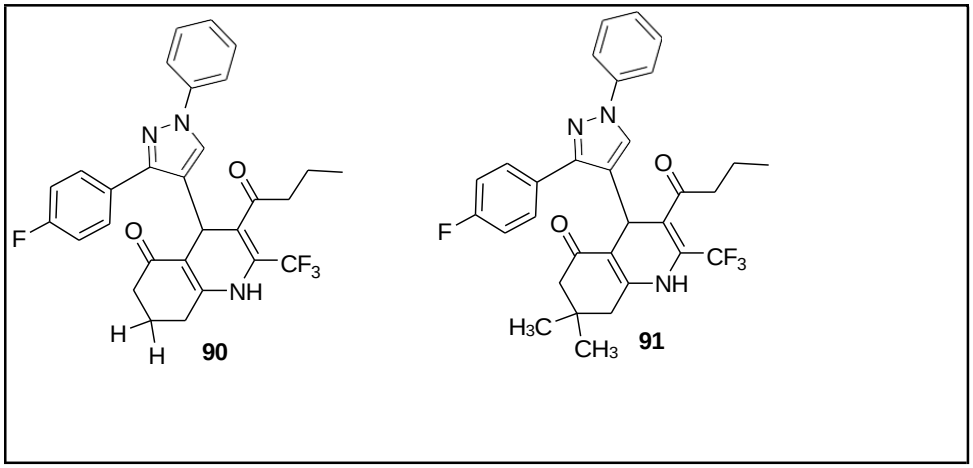


Figure 9: Biologically active pyrazole entities developed by (Bhatt *et al.* 2018)

A series of rhodanine-based 5-aryloxy pyrazoles in **Figure 10** have been constructed by (Song *et al.* 2013), and their antimicrobial action tested against *Methicillin-resistant S. aureus* (MRSA) and *Quinolone-resistant S. aureus* (QRSA) strains. It was found that compound **92** depicted the highest antimicrobial activity amongst the derivatives developed with a MIC of 1 and 2 µg/mL against the aforementioned strains. Bis- and Tris-heterocycles developed by (Bhanu Prakash *et al.* 2014) presented compounds **93** and **94** as effective antibacterial agents and compound **95** showing high potency as an antimicrobial agent better than the standard drug Ketoconazole. Furthermore, all the mentioned compounds exhibited greater antimicrobial activity than the standard drug Chloramphenicol, particularly against *Staphylococcus aureus*.

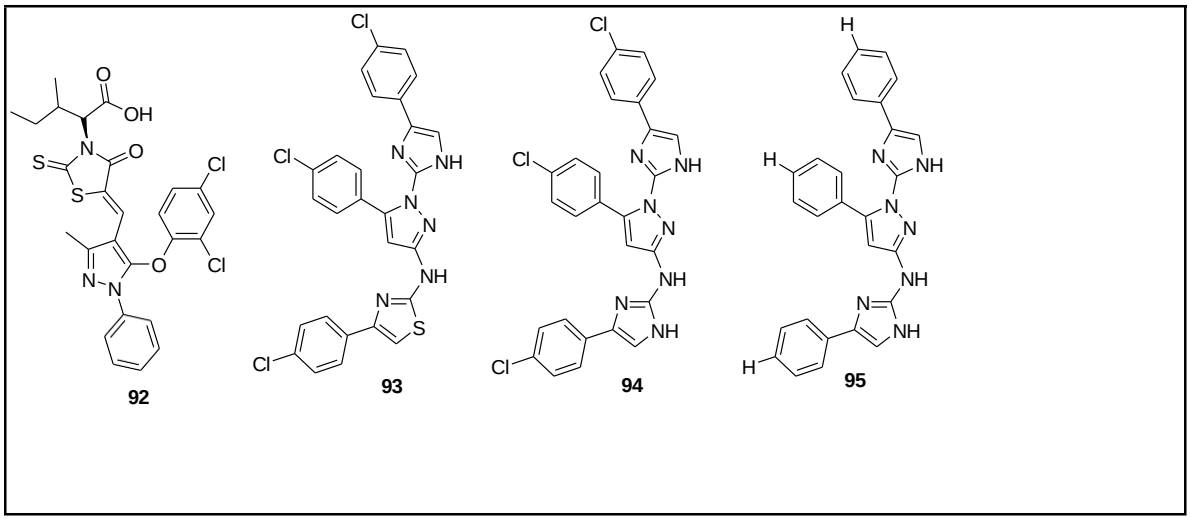


Figure 10: Novel pyrazole moieties presented by (Song *et al.* 2013) and (Bhanu Prakash *et al.* 2014) that show antimicrobial activity

The review published by (Khan *et al.* 2016) reports on studies presented by (Ningaiah *et al.* 2014) and (Nasr, Bondock and Eid 2014) on their antimicrobial evaluation. Ningaiah *et al*. 2014, synthesized a novel series of pyrazole 1,3,4- oxadiazole-based hybrids and evaluated their antimicrobial potential. Consequently, compound **96** emerged as the best antibacterial agent against different Gram-negative and Gram-positive strains of bacteria. Nasr *et al*. 2014 synthesized a series of pyrazole-based compounds and evaluated their antimicrobial activity. Compound **97** was reported to be the most active antimicrobial. Compound **98** was active against *Penicillium notatum* (P. notatum) and *B. subtilis* having a MIC 46 and 44 mg/mL, respectively. Compound **99** was found active against *P. notatum, B. subtilis and Escherichia coli* (E. coli) with MIC value of 57, 54 and 43 mg/mL, substantiated in (Khan *et al.* 2016). Their structures elucidated in **Figure 11** below.

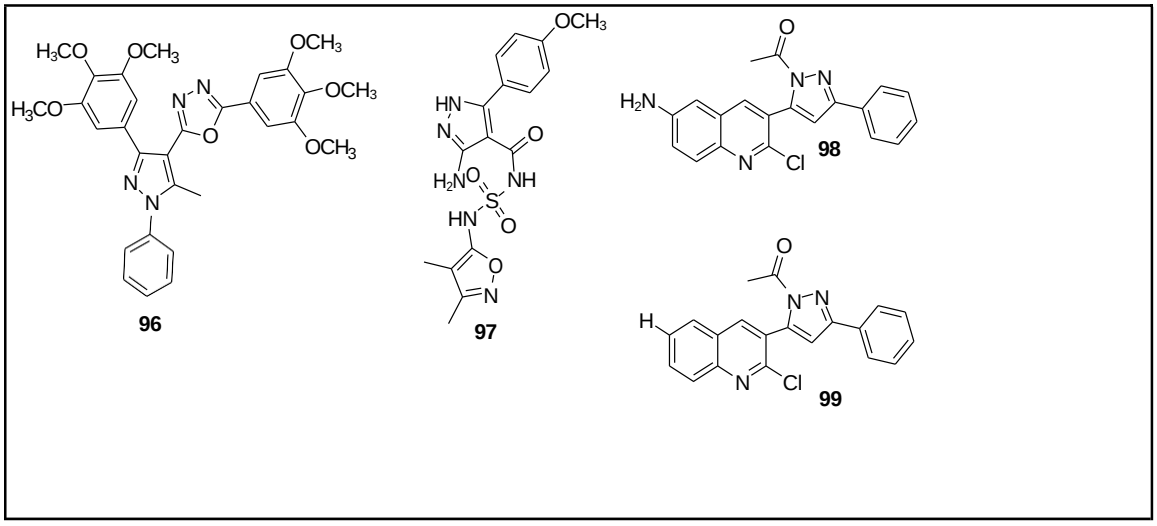


Figure 11: Pyrazole architectures demonstrating antifungal and antibacterial activity (Khan *et al.* 2016)

The outcome of functionalized pyrazoles in **Figure 12**, which showed moderate to good antibacterial activity, had been developed by (Govindaraju, Ningappa and Kumar 2013) and excellent action against all of the investigated species has been demonstrated, 3-(4-Methoxyphenyl)-1,4-diphenyl-1H-pyrazol-5-yl) (phenyl)methanone **101** and 3-(4-Chlorophenyl)-1,4-diphenyl-1H-pyrazol-5-yl) (phenyl)methanone **102** appeared to elucidate better antibacterial activity. These compounds can be studied as potential antimicrobial agents (Govindaraju, Ningappa and Kumar 2013). In contrast to *B. subtilis* and *S. aureus species*, 3-

(4-methylphenyl)-1,4-diphenyl-1H-pyrazol-5-yl) (phenyl) methanone was found to be only modestly potent against *E. coli* and *S. typhimurium* organisms.

Against Gram-positive and Gram-negative stains, this pyrazole moiety **100** displayed good to powerful in vitro antibacterial activity (Doddaramappa *et al.* 2015) compared to other freshly synthesized pyrazoles **Figure 12**. This unique molecule and its analogues also showed strong antidiabetic efficacy. The literature of (Becerra, Abonia and Castillo 2022a) reports using ampicillin as the reference medication. The pyrazole derivatives were tested against Gram-positive (Bacillus subtilis and Staphylococcus aureus) and Gram-negative (Escherichia coli and Pseudomonas aeruginosa) bacterial strains, a study reported initially by (Kendre, Landge and Bhusare 2019). In contrast to ampicillin, it was discovered that pyrazoles had an inhibitory zone in the range of (21–24 mm). Intriguingly, compound **103** outperformed ampicillin in antibacterial activity against *Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa*, with zones of inhibition of 17, 20, and 22 mm; respectively.

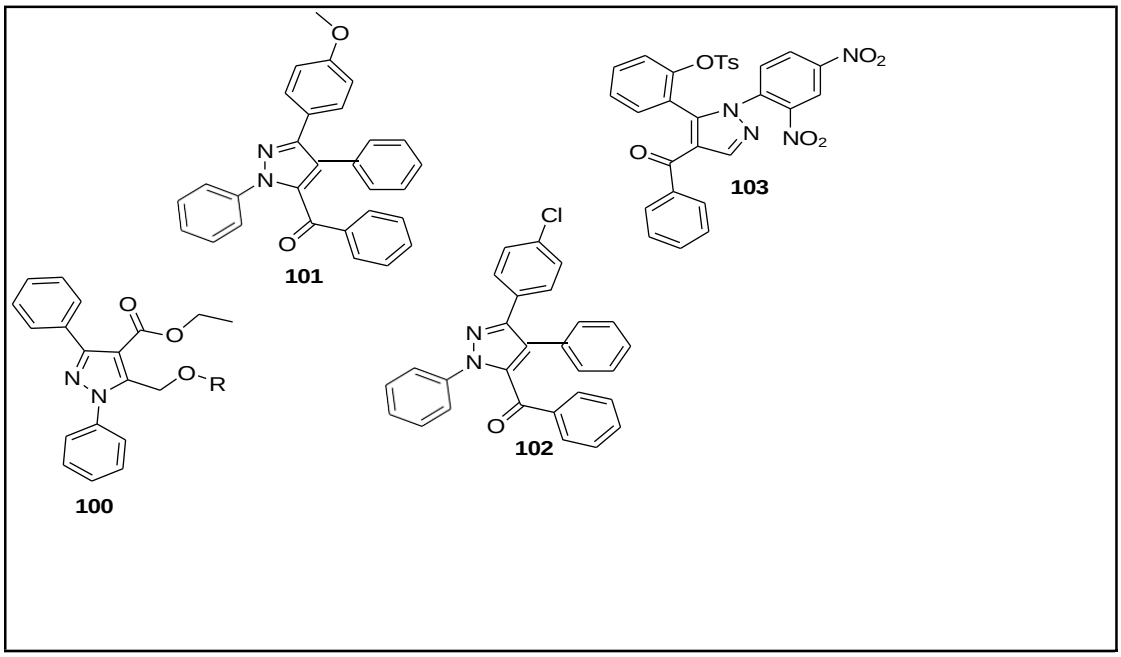


Figure 12: Novel pyrazole derivatives displaying good antimicrobial activity (Govindaraju, Ningappa and Kumar 2013), (Doddaramappa *et al.* 2015) and (Becerra, Abonia and Castillo 2022a)

It is clear from compound **104** that adding more electronegative atoms, such as halogens and oxygen on the benzene ring, boosts the pyrazole scaffold's effectiveness against MRSA as factually highlighted in (Verma *et al.* 2021) depicted in **Figure 13**. A 5-hydroxy-pyrazole

compound with a general structure similar to **105**, characterized by a MIC of 25 µg/mL, emerged as a hit candidate from virtual screening. Consequently, optimization of the above and synthesis of a library of derivatives led (Castagnolo *et al.* 2008) to obtain several pyrazoles as beguiling compounds with enhanced antimycobacterial activity. Pharmacophores such as halogens enhanced the activities as proven by pyrazole derivative **105** with the p-bromophenyl group at N1 position (MIC: 4 µg/mL) better than the lead compound it was derived from (Castagnolo *et al.* 2008).

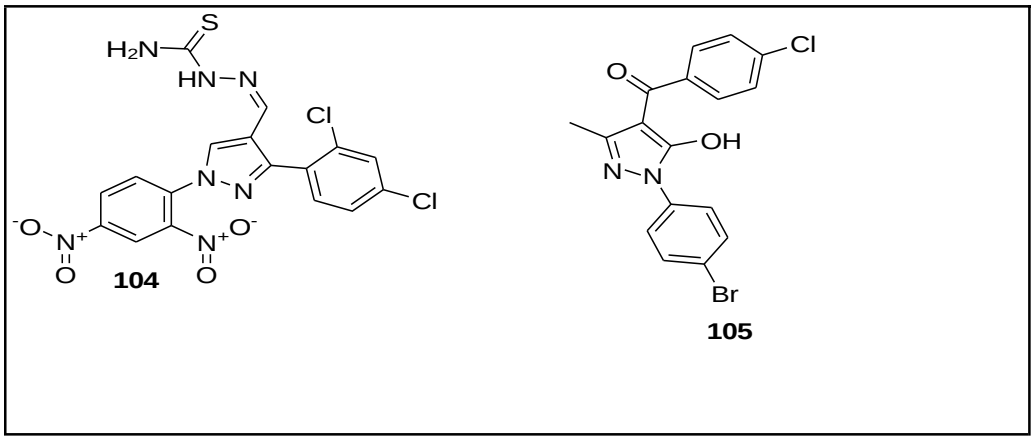


Figure 13: Novel pyrazole derivatives with halogen pharmacophores (Verma *et al.* 2021)

The research study by (El-Sabbagh *et al.* 2009), reports novel acetyl **106** and *N*-thiocarbamoyl 4,5-dihydropyrazole **107** compounds. The new pyrazolothiazol-4(5H)-ones **108** and pyrazolothiazoles **109** were produced by cyclizing N-thiocabamoylpyrazole derivatives shown by **Figure 14**. The described compounds demonstrated antiviral activity against several viruses, including the herpes simplex virus types 1 (KOS) and 2 (G), vaccinia virus, vesicular stomatitis virus, coxsackie virus B4, respiratory syncytial virus, and parainfluenza-3 virus (Ansari *et al.* 2017). According to (Zhang *et al.* 2010), most of the target pyrazole compounds display vigorous inhibition activity against *Corynespora cassiicola* in their study. Compound **110** was the most effective against *Pseudomonas syringae pv. Lachrymans*. When the benzene ring is substituted by halogen, the compounds generally show excellent antimicrobial action against *Corynespora cassiicola* reported in the review (Ansari *et al.* 2017).

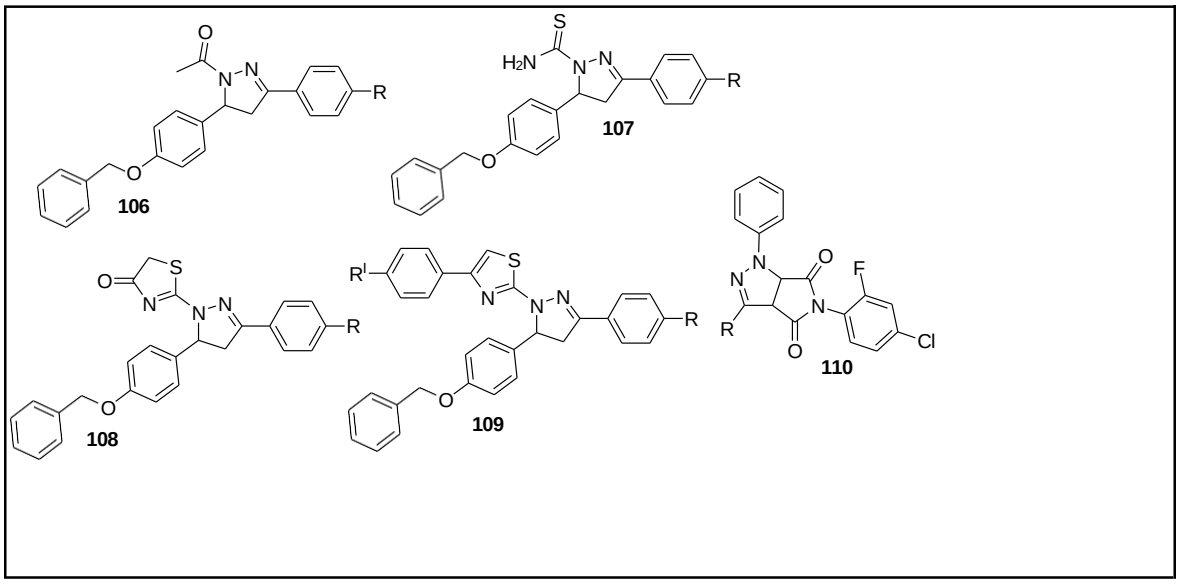


Figure 14: Novel pyrazole derivatives showing antiviral activities and antimicrobial activities (El-Sabbagh *et al.* 2009) and (Ansari *et al.* 2017).

Docking is a popular method for predicting how small molecules will align with their target proteins to determine the small molecule's affinity and activity (Morris and Lim-Wilby 2008). The goal of docking studies is to optimize the shape of both the ligand and protein and the relative orientation of the protein and ligand to reduce the total system's free energy (Raval and Ganatra 2022). The discovery of a new drug is a challenging task. Thus, docking plays a pivotal role in the drug design and discovery (Chaudhary and Mishra 2016) and the structural characterization of medications (Raval and Ganatra 2022). The critical steps involved in molecular docking are in silico filters which describe the compound's Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) and select the most promising candidate (Raval and Ganatra 2022). The proteins (receptors) are retrieved from the protein data bank (PDB), active site prediction of protein, and the preparation of ligands, which consider the Lipinski Rule of 5 (LRo5) (Raval and Ganatra 2022). Lipinki's rule aids in predicting the drug-likeness of a molecule by appraising it through the stipulated physicochemical parameters; hydrogen bond donors ≤ 5, hydrogen bond acceptors be ≤ 10, molecular mass be ≤ 500 Da, lipophilicity (expressed as LogP ≤ 5) and Polar Surface Area (TPSA) < 140 Å (Petit *et al.* 2012). In this study, molecular docking and LRo5 have been considered to assess the oral bioavailability of pyrazole derivatives.

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Chapter Three: Materials and Methods

3.1 General Methodology

All experiments under microwave irradiation were carried out in a MW Discover CEM 2.0 instrument using modes of operation with simultaneous cooling. The instrument has a maximum power output of 300 W, a maximum temperature of 300 ^{o}C, and a maximum pressure of 300 psi.

Thin-layer chromatography (TLC) was performed using Merck pre-coated silica gel, and the components were visualized under a UV or an iodine chamber. Isolated product solids were put in crushed ice (500 mL), filtered, washed with hexane and water, and recrystallized with ethanol. All the reagents were of analytical grade obtained from Sigma-Aldrich. Time of flight Low-resolution mass-spectroscopy with Electrospray ionization was used to determine masses of **4a – 4j**

3.1.1 General Procedure for the Preparation of Pyrazoles

In a 30 mL Pyrex glass tube, 130 µL ethylaceto acetate **14** (≈ 1 mmol) was added, followed by 0.1 g thiosemicarbazide **15** (≈ 1 mmol). To this mixture, 35 µL was added for all liquid benzaldehydes **16** (0.25 – 0.38 mmol) while 0.05g (0.5 mmol) was used for solid benzaldehydes. After that, a 10 mL aliquot of ethanol: water (1:1) was added. The Pyrex glass tube was placed into the microwave organic synthesizer set at 125℃and 195 W and run for 10 minutes, as shown in **Scheme 19.** In a preliminary experiment, small aliquots were removed and run on TLC to determine the time for the reaction to reach completion. After the solids had been formed, they were filtered and washed with water, followed by hexane and dried at room temperature to obtain final crystals. A similar protocol was used for the synthesis of all pyrazoles.

3.1.2 Characterization Techniques

FTIR spectra were recorded on a Perkin-Elmer FTIR spectrophotometer 65 as KBr pellets and the absorption expressed in cm^{-1}. ^{1}H-NMR spectra were recorded in DMSO on 400 MHz & 600 MHz, ^{13}C-NMR spectrometer at 25 ^{o}C with tetramethylsilane (TMS) as the internal standard, and resonances (δ) are given in ppm. Data are reported as follows: chemical shift (d),

multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constants (Hz), and integration.

3.1.3 Minimum Inhibitory Concentration and Sensitivity Assays

The agar well diffusion method was employed to assess the antibacterial properties of the synthesized compounds (Ramakrishnan *et al.* 2011) . Test organisms (*Staphylococcus aureus* ATCC 12600, *Streptococcus pneumonia* ATCC 49619, *Pseudomonas aeruginosa* ATCC BAA-1744 and *Escherichia coli* ATCC BAA-2452) were standardized to the 0.5 McFarland standard. An aliquot of 0.1 mL of the standardized test organisms was evenly inoculated on an already solidified Mueller Hinton agar plate. Thereafter, wells were made on the inoculated plate using a sterile cork borer (6 mm), and the synthesized compounds were introduced using 2 % DMSO as negative control and amoxicillin as positive control. After incubation at 37 °C for 24 hours, the antibacterial effect was gauged by the appearance of zones of inhibition. Additionally, the Minimum Inhibition Concentration was determined using the microdilution method. (Sharma *et al.* 2011) Briefly, 0.2 mL of the standardized test organisms were added to a 96-well plate containing 2-fold serial diluted antibacterials (**4a**, **4b**,**4c**, **4d**, **4e**, **4f**, **4g**, **4h**, **4i** and **4j**). The MIC was identified as the lowest concentration preventing bacterial growth after overnight incubation. Amoxicillin served as the positive control.

3.1.4 Ligand Acquisition, Protein Preparation and Molecular Docking of Penicillin Binding Protein of the Bacteria

The X-ray crystal structures of PBP2a (6H5O) of *S. aureus* (Janardhanan *et al*., 2019), PBP2x (1QMF) of *S. pneumonieae* (Gordon *et al*., 2000) and PBP3 of *E. coli* (6I1I) (Sainsbury *et al*., 2011) and *P. aeruginosa* (6VJE) (Kumar et al., 2020) were acquired from the Protein Data Bank (https://www.rcsb.orgs/) and optimised through the removal of non-standard amino acids and water molecules using UCSF chimera software v 1.14 (Aribisala *et al.* 2022). The cleaned structures were then saved in PDB format for molecular docking.

The x-y-z coordinates of the active sites of PBP2a (Center: x: 26.80 y: -4.50 z: 27.04 size: x: 26.41 y: 25.0 z: 28.58), PBP2x (Center: x: 110.48 y: 58.05 z: 79.69 size: x: 23.98 y: 26.01 z: 19.72) and PBP3 of *E. coli* (Center: x: 27.83 y: 17.07 z: -14.16 size: x: 20.92 y: 13.92 z: 18.94) and *P. auruginosa* (Center: x: 5.40 y: 35.27 z: 19.27 size: x: 25.0 y: 20.65 z: 19.22) were defined using Discovery Studio v 21.1.0 (MubarakAli *et al.* 2021) and afterward confirmed from literature (Janardhanan *et al*., 2019; Gordon *et al*., 2000; Sainsbury *et al*., 2011; Kumar

et al., 2020). The synthesised compounds (**4a**, **4b**, **4c**, **4d**, **4e**, **4f**, **4g**, **4h**, **4i** and **4j**) were construted and converted to 3D format using the ChemDraw Ultra 8.0 and afterward optimised using the open babel program present on Python Prescription v 0.9.5 (PyRx) (Dallakyan and Olson, 2015). Subsequent docking at the active site of the proteins was ensured by dragging the grid box to fit the established, well-defined x-y-z coordinates. Thereafter, the optimized 3D structures of the ligands (**4a**, **4b**, **4c**, **4d**, **4e**, **4f**, **4g**, **4h**, **4i** and **4j** reference amoxicillin) and cleaned proteins (PBP2a of *S. aureus*, PBP2x of *S. pneumoniae* and PBP3 of *E. coli* and *P. auruginosa*) were subjected to molecular docking using the Autodock vina package plug-in on PyRx (Dallakyan and Olson, 2015), which allows for multiple docking of ligands (Aribisala *et al.* 2022). Finally, ranking of the ligands was done based on their binding affinity, and the docked complexes of the best pose with the highest affinity for the compounds were saved in PDB format. Validation of the docking conformation was carried out using the superimposition techniques and appropriate measurement of the root mean square deviation (RMSD) of the best docked synthesised compound and amoxicillin from position of the native inhibitors in each case of the investigated PBPs (Fahad *et al*., 2013; Bajorath *et al.*, 2001)

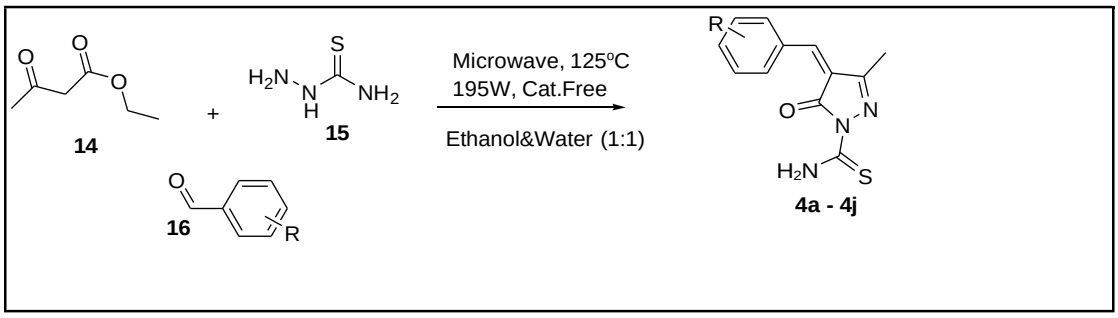
3.1.5 In Silico Pharmacokinetics and Toxicity Prediction

The *in silico* prediction of the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of the synthesised pyrazoles were studied using online software tools SwissADME (http://swissadme.ch/index.php) and Protox II (https://tox-new.charite.de/protox_II/).

4.1 Chemistry

4.1.1 Synthesis of 4Z)-4-Arylidene-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide derivatives

The three component one-pot synthesis of (4Z)-4-arylidene-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide derivatives, initially was investigated by conventional thermal reflux, reacting equimolar amounts of benzaldehyde, ethyl acetoacetate and thiosemicarbazide in ethanol solvent. This approach proved tedious as the reaction produced poor yield, required long reaction times and gave impurities that required purification by column chromatography. Therefore, the same reaction was conducted using microwave irradation (MW) by mixing equimolar amounts of ethyl acetoacetate, benzaldehyde and thiosemicarbazide to yield the lead compound **4i** (4Z)-4-benzylidene-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide in **Scheme 19**.



Scheme 19: Multicomponent synthesis of novel pyrazoles in microwave *catalyst-free*

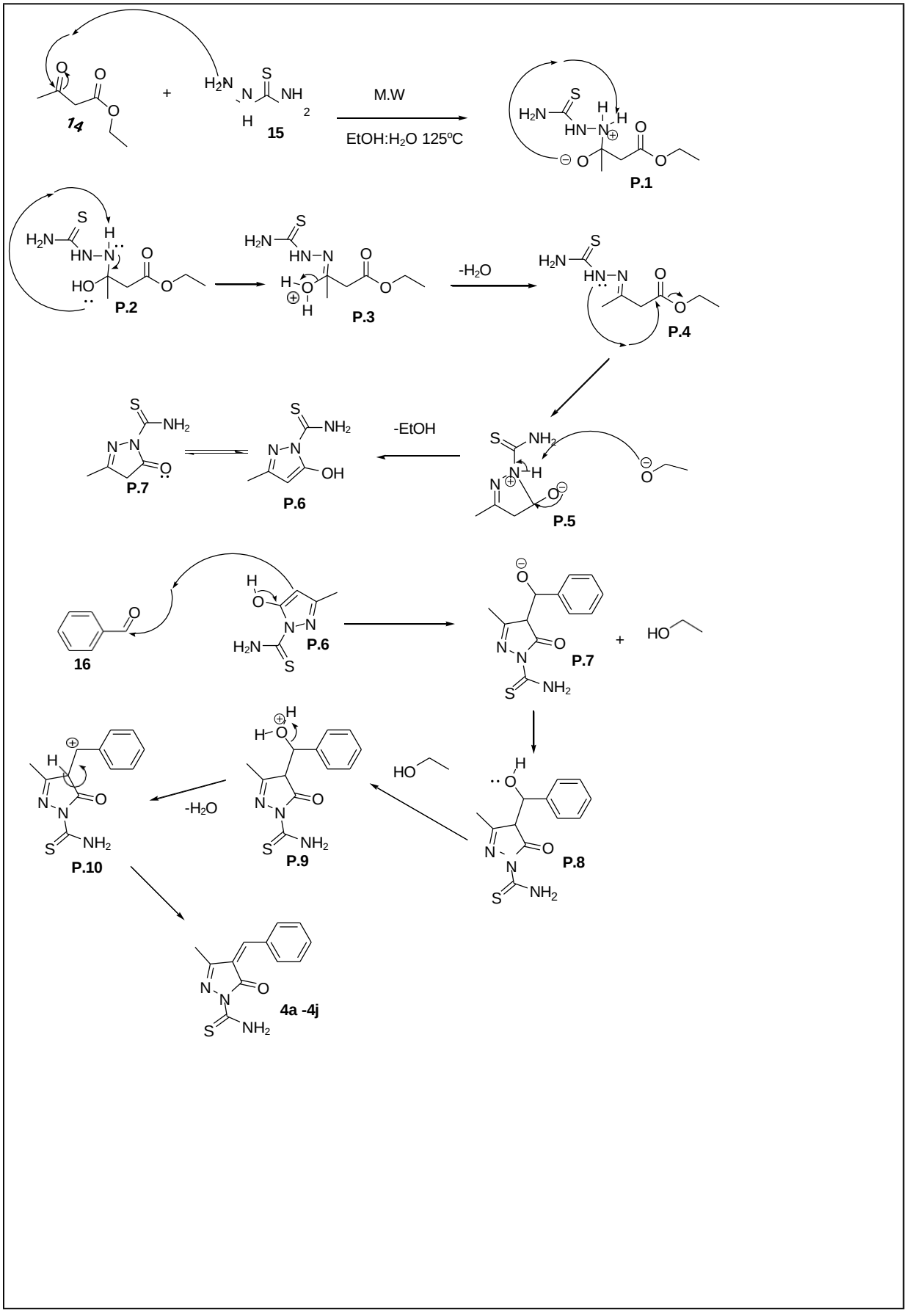
MW gave excellent yield in a short reaction time and the crude product was purified without using column chromatography. After the work-up process the crude collected by vacuum filtration was washed with hexane and thereafter recrystallized by dissolving the powdered product in ethanol. The latter was slowly evaporated in the fume hood to produce crystals. This approach enables a multicomponent reaction to occur by catalyst-free conditions in aqueous media, adhering to green principles. The reaction conditions were optimised by investigating parameters at 125 - 145 °C, 180 – 200 W and 300 psi. The reaction was conducted in a solvent

mixture of ethanol and deionized (1:1) for 10 minutes. The stoichiometry of the reagents used were 130 µL ethyl acetoacetate, 35 µL benzaldehyde derivatives and 0.125 g thiosemicarbazide catalyst-free, adjustments done on the premise of TLC monitoring. Regarding powdered phenylaldehydes 0.05 – 0.2 g were used under stipulated microwave conditions and yielded pyrazole analogs.

Table 1: Optimization of reaction conditions for synthesis of pyrazole derivatives

Entry	**Solvent**	**Temperature**	**Catalyst**	**Time**	**Yield (%)**
1	Ethanol	Reflux	Piperidine	12 h	32
2	Neat	Reflux	Piperidine	12 h	-
3	DMSO	Reflux	TEA	12 h	22
4	DMSO	Reflux	NaOH	12 h	-
5	Water	Reflux	NaOH	12 h	42
6	Water	Reflux	Glycine	12 h	63
7	Ethanol	Reflux	L-Proline	12 h	59
8	Ethanol: Water (3:1)	Reflux	L-Proline	12 h	66
9	Ethanol	Microwave	-	22 min	79
10	Ethanol: Water (2:1)	Microwave	-	22 min	83
11	Ethanol: Water (1:1)	Microwave	-	10 min	91

The reaction **Scheme 20** below depicts the plausible mechanism that furnished (4Z)-4-Arylidene-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide derivatives. Under thermodynamically controlled conditions and employment of polar protic solvents, ethylaceto acetate **14** has been envisaged to react with thiosemicarbazide **15** by a Knorr pyrazole synthesis. The lone pair of electrons on the terminal amine of thiosemicarbazide **15** facilitates a nucleophilic attack on the carbonyl carbon of ethylacetoacetate **14** and predominantly forms the first intermediate **P.4** azomithine structures (Schiff-Base) by condensation. Remarkably, this reaction is spontaneous and can be initiated solely by irradiation without a catalyst. The cyclocondensation forming the precursor tautomers **P.6** and **P.7** results from a lone pair on the interior secondary amine of **P.4** forming a C-N bond which re-configures its structure as seen in **P.4** by expelling the ethoxy group, which is a good leaving group. The instability of **P.5** arising from the positively charged nitrogen due to quadruple bonds releases a proton that is subsequently taken by ethoxy to form ethanol. Thus, both tautomers, **P.6** and **P.7**, have achieved a thermodynamically controlled product.



Scheme 20: Plausible synthetic pathway under MW of novel pyrazole compounds

While conventional thermal reflux reactions are slower due to gradual temperature increases, MW reactions exhibit higher collision frequencies, reaching maximum temperatures within seconds. Although substituents near reactive sites and their orientation play a role, their effect on reaction time is negligible in MW reactions. Precursor **P.6** was predicted to be favored for the production of (4Z)-4-Arylidene-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide derivatives when substituted benzaldehyde **16** derivatives were introduced. Monitoring the TLC revealed the disappearance of **P.6** and the formation of the product in situ. The enol aspect of **P.6** is reactive with electrophiles, so carbon 4 of **P.6** facilitates a nucleophilic attack to the oncoming benzaldehyde **16** to form **P.7**. From P.7 the scheme proceeds through Knoevenagel condensation up to the final product. The oxygen anion on **P.7** will accept a proton donated by the solvent to form **P.8**, which will continue to **P.9,** where water molecules are eliminated. The α-hydrogens in **P.10** are readily released (polarized bond) to form an sp^2-hydridized bond that neutralizes the carbocation to complete the final pyrazole product derivatives investigated in this study **4a – 4j**. The choice of solvent system in this regard has proved to play a crucial role in aiding product formation. All the synthesized compounds were confirmed by spectral data (^{1}H,^{13}C NMR, FTIR and TOF-MS), which correlates with the compound of the proposed structure in **Scheme 20**.

Various aromatic aldehydes furnished our target pyrazole derivatives, which resulted in the synthesis of ten novel compounds (**4a**-**4j**). The presence of electron-withdrawing/electron-donating substitutions did not seem to significantly limit the overall reactivity, giving the desired product in quantitative yields (Table 2 and **Scheme 19**).

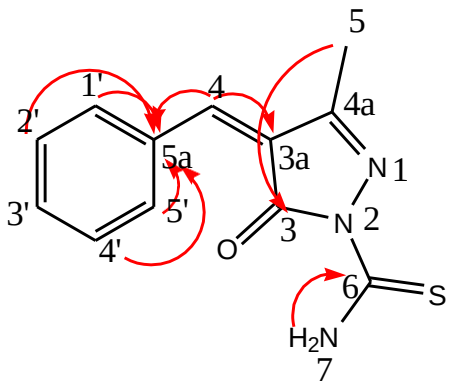
Table 2: Synthesis of target pyrazole derivatives under microwave

Entry	R^1	R^2	R^3	Product	Time (min)	Yield	Melting Points (°C)
3a	4-Cl	-	-	4a	10	90	194
3b	2-OH	-	-	4b	10	89	201
3c	4-$N(CH_3)_2$	-	-	4c	10	92	210
3d	2-NO_2	-	-	4d	10	87	241
3e	3-Br	4-OH	5-OCH_3	4e	10	96	211
3f	4-OCH_3	-	-	4f	10	94	160
3g	4-NO_2	-	-	4g	10	95	241
3h	4-SCH_3	-	-	4h	10	90	216
3i	4-H	-	-	4i	10	91	194
3j	4-CH_3			4j	10	91	170

After the work-up process (**Table 1 & 2**), product **4i** was collected as cream white solids by vacuum filtration with a 91% yield and a 192 -194°C melting point. **FT-IR** (KBr, *v*, cm^{-1}): 3297 – 3303 (N-H), 3048 (=C-H), 1643 (C=N), 995 – 1053 (C=S) and 489-554 (C – H aromatic bend) (Salum *et al.* 2020), (Boudjellal *et al.* 2020) and (Khatab *et al.* 2021). The **^{1}H-NMR** spectrum **(600 MHz, DMSO)** (Appendix, page 85) and also succinctly displayed in **Table 3** presented the following distinctive results, at signal δ11.44 ppm is a downfield thio-amide group H_2N-C=S, a singlet at δ2.08 ppm (3H, *s*) chemical shift is for a methyl group (H5), the phenyl ring revealed signals at δ7.39 ppm (1H, *d*, H1' & H5'), δ7.79 ppm (1H, *t*, H2' & H4') and H4 is a significant signal at δ8.05 ppm (1H, *s*) showing an sp^2 hybridized -CH verifying the formation of pyrazole compound **4i**. The **^{13}C NMR** spectrum and **APT** (Table 3 & Appendix, page 85) showed five -CH signals which were δ142.7 ppm (C4), δ127.7 ppm (C1' & C5'), δ129.1 ppm (C2' & C4'), δ130.3 ppm for (C3') and δ31.13 ppm (C5). Three quaternary carbon signals had chemical shifts at δ178.4 ppm for C6, δ206.9 ppm for (C3&6) because of electron cloud movement due to mesomeric and resonance effects, although this is plausible δ206.9 ppm is rendered to be C3 because the carbonyl has deshielding neighbor of nitrogen and δ134.6 ppm for (C3a, 5a), respectively. For further verification of compound **4i** correlation study by ^{1}H-^{1}H coupling COSY and ^{1}H quaternary carbon coupling **HMBC** was performed. A **COSY** study (Table 3 and Appendix, page 85) presented a strong coupling between protons of H1'& H5' (δ7.39 ppm, 1H, *d, J* = 7.74 *Hz*) correlating to those of H2' & H4' (δ7.79 ppm, 1H, *t, J* = 5.2 Hz) and vice versa as substantiated on **Table 3**. The HMBC spectrum (Appendix, page 85) correlated

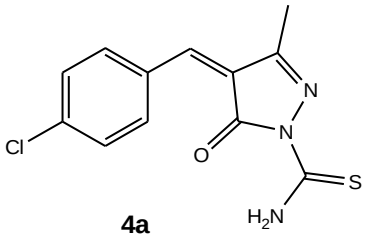
protons of H1' & H5' coupling with C5a, and that of H2' & H4' seeing C5a as well. Proton H4 is strongly correlated to C3a & C5a combination and other correlations are illustrated in **Table 3**. Looking into all other derivatives, they divulged similar chemical shifts. **TOF-MS ES** found [M^+], 243.0 m/z: **TOF-MS ES** calculated [M^+], 245 m/z (Appendix, page 85).

Table 3: ^{13}C NMR and ^{1}H NMR Spectra of Compound 4i and Structural Representation

Compound 4i - Lead	δ_C	δ_H	COSY	HMBC	Compound 4i Structure
C					
6	178.4	-	-	H7	
3	206.9	-	-	H5	
3a&5a	134.6	-	-	H4	
CH					
4	142.7	8.05 (1H, s)	-	C3a&C5a	
1'&5'	127.7	7.39 (1H, d)	H2', H4'	C5a	
2'&4'	129.1	7.79 (1H, t)	H1', H5'	C5a	
3'	130.3	-	-	-	
CH_3					
5	31.13	2.08 (3H, s)	-	C3	
NH					
7	-	11.44	-	C6	

4.2 **Spectroscopic data analysis of ten synthesized pyrazole derivatives 4a – 4j**

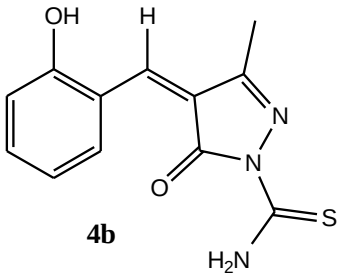
(4Z)-4-(4-chlorobenzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4a**



4a

Yield is 90%, pale grey solids, m.p 190 – 194 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3308 – 3322 (N-H), 3059 (=C-H), 1635 (C=N), 1033 – 1077 (C=S) and 791 (C - Cl) (Salum *et al.* 2020), (Boudjellal *et al.* 2020) and (Khatab *et al.* 2021). The **^{1}H NMR (400 MHz, DMSO)** of **4a**, **Table 4** revealed a signal at δ 3.45 ppm (6H, *s*) being that of DMSO. A downfield shift was observed for H_2N-C=S at δ 11.47 ppm (1H, *s*) due to amide and thioamide delocalization in the pyrazole ring by mesomeric effects. Benzene moiety protons were consistent, revealing doublets multiplicity in that H1' & H5' was δ 7.83 ppm (2H, *d*) and that of H2' & H4' was at δ 7.44 ppm (2H, *d*). The **^{13} C NMR** of **4a** confirmed functional groups, thioamide at δ 178.5 ppm (C6) as a single chemical shift. The chemical shift shown at C4 (δ 141.4 ppm) signifies the formation of our pyrazole derivative, an sp^2 carbon. Benzene carbons were also agreeable, with C1' & C5' being at δ129.4 ppm, C3' being δ134.7 ppm, C2' & C4' being δ128.0 ppm and C3a, 5a being δ133.6 ppm. The appendix substantiates the compound's characterization. **TOF-MS ES** found [M^+], 279.1 m/z; **TOF-MS ES** calculated [M^+], 279.0 m/z.

(4Z)-4-(2-hydroxybenzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4b**

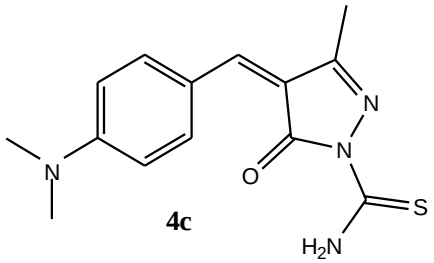


4b

Yield is 89%, pale lemon solids, m.p 198 – 201 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3302 – 3333 (N-H), 3045 (=C-H), 1593 (C=N), 1003 – 1083 (C=S), 3423 (C - OH) and 1208 (C - O) (Salum *et al.* 2020),

(Boudjellal *et al.* 2020) and (Khatab *et al.* 2021). **^{1}H NMR (400 MHz, DMSO)** of compound **4b**. Shifts observed for H2' (1H, *d*), H4' (1H, *t*), and H3' (1H, *t*), were δ 6.85, δ 7.22, and δ 6.81 ppm, respectively. An sp^2 -CH chemical shift happening at H4 revealed δ 8.37 ppm (1H, *s*), showcasing the formation of pyrazole. A hydroxyl shift was found to occur at δ 9.88 ppm (1H, *s*). The value at δ 3.42 ppm is consistent with DMSO-d_6, and δ 11.34 ppm revealed protons of thioamide H_2N-C=S (2H, *s*), which was consistent throughout pyrazole analogues. In the **^{13}C NMR** of **4b**, C2', C3', C4', and C5' chemical shifts corresponded to δ 120.7 ppm, δ 127.2 ppm, δ 119.7 ppm, δ 116.5 ppm respectively. For (C3a, 5a) δ 131.5 ppm, and δ 140.2 ppm for C4 signify a pyrazole formation. The thioamide C6 was seen at δ 178.1 ppm, and all chemical shifts of ^{13}C NMR and ^{1}H NMR of pyrazole analogues were consistent, proving our compound structures. **TOF-MS ES** found [M^+], 258.0 m/z; **TOF-MS ES** calculated [M^+], 260.3 m/z.

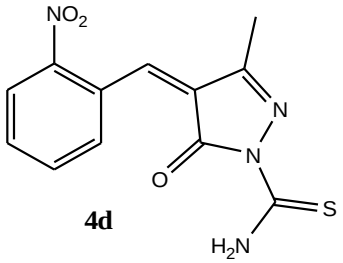
(4Z)-4-(4-(dimethylamino)benzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4c**



4c

Yield is 92%, reddish brown solids, m.p 208 – 210 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3301 – 3314 (N-H), 3046 (=C-H), 1590 (C=N), 995 – 1079 (C=S) and 1221 (C - N) (Salum *et al.* 2020), (Boudjellal *et al.* 2020) and (Khatab *et al.* 2021). The **^{1}H NMR (400 MHz, DMSO)** of **4c** revealed a signal indicative of a shielding methyl group (H6') to nitrogen at δ 2.96 ppm (6H, *s*), a more downfield shift is seen for H_2N-C=S (2H, *s*), at δ 11.18 ppm due also to amide and thioamide delocalization in pyrazole ring by mesomeric effects. Phenyl protons were consistent in that protons from H1' & H5' were δ 7.59 ppm (2H, *d*) and H2' & H4' were doublets at δ 6.69 ppm (2H, *d*). The **^{13} C NMR** of **4c** confirmed functional groups carbonyl and thioamide, C3 (δ 190.3 ppm) and C6 (δ 177.5 ppm) respectively, and also a shift of δ 45.8 ppm (C6') correlating to $N(CH_3)_2$ was seen. The chemical shift shown at C4 (δ 143.8 ppm) signifies the formation of our pyrazole derivative, an sp^2 carbon, -CH. Benzene carbons were also agreeable, with C1' & C5' being at δ 124.9 ppm, C2' & C4' being δ 121.9 ppm, C3' at δ151.9 ppm and C3a,5a being δ 129.1 ppm. **TOF-MS ES** found [Na + M^+], 311.2 m/z; **TOF-MS ES** calculated [Na + M^+], 311.4 m/z.

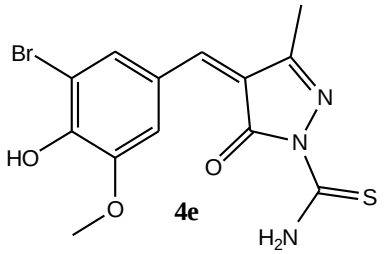
(4Z)-4-(2-nitrobenzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4d**



4d

Yield is 87%, bright yellow solids, m.p 239 – 241 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3314 – 3341 (N-H), 3055 (=C-H), 1630 (C=N), 1013 – 1086 (C=S), 1304 & 1485 (N - O) (Salum *et al.* 2020), (Boudjellal *et al.* 2020) and (Khatab *et al.* 2021). The **^{1}H-NMR (400 MHz, DMSO)** of **4d** gave distinctive results. At signal δ 11.74 ppm a downfield H_2N-C=S (2H, *s*), the phenyl ring revealed doublet signals H2' at δ 8.43 ppm (1H, *d*), H3' was recorded to be at δ 7.73 ppm (1H, *trip*) and H4' had a chemical shift of δ 7.63 (1H, *trip*) and lastly a signal corresponding to H5' being δ 8.02 ppm (1H, *d*). A significant signal at δ 8.12 ppm (1H, *s*) of *sp*^{2} hybridized -CH (H4) verified the formation of pyrazole compound 4d. In the **^{13}C NMR** of **4d**, C1', C2', C3', C4' & C5' chemical shifts corresponded to δ148.7 ppm (NO_2), δ128.7 ppm, δ 130.7 ppm & δ 124.9 ppm, δ128.8 respectively, and C3a,5a chemical shifts appeared to be δ 133.7. The shift value at δ137.7 ppm for C4 signifies a pyrazole formation. The thioamide group C6 was seen at δ 178.9 ppm, and all chemical shifts of ^{13}C NMR and ^{1}H NMR of pyrazole analogues were consistent proving our compound structures. **TOF-MS ES** found [M^{+}], 292.1 m/z; **TOF-MS ES** calculated [M^{+}], 290.3 m/z.

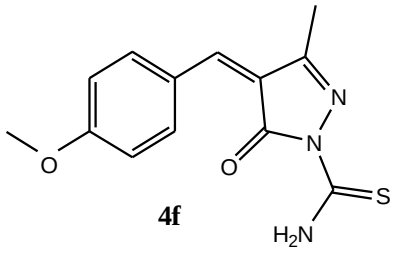
(4Z)-4-(3-bromo-4-hydroxy-5-methoxybenzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4e**



4e

Yield is 96%, pale brown solids, m.p 209 – 211 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3310 – 3333 (N-H), 3057 (=C-H), 1624 (C=N), 1013 – 1088 (C=S) and 3413 - 3499 (C - OH) (Salum *et al.* 2020), (Boudjellal *et al.* 2020) and (Khatab *et al.* 2021). **^{1}H NMR (400 MHz, DMSO)** of compound **4e**, shifts observed at H1' and H5' were δ 7.49 and δ 7.43 ppm both (1H, s), respectively. An *sp*^{2} -CH chemical shift happening at H4 revealed δ 7.91 ppm (1H, *s*) showcasing the formation of pyrazole. A proton (OH) at δ 9.92 ppm (1H, *s*) was for the hydroxyl group, and a shift value of δ 3.88 ppm (3H, *s*) being H8 was typical of a methoxy group. Furthermore, δ 11.36 ppm revealed protons of H_2N-C=S (2H, *s*), which this value was consistent throughout pyrazole analogues. In the **^{13}C NMR** of **4e**, C1', C2', C3', C4' and C5' chemical shifts corresponded to δ 124.8 ppm, δ 129.4 ppm, δ 150.3 ppm and 149.1 ppm and δ126.9 ppm respectively, C8 was δ56.8 (methoxy). The value at δ 141.8 ppm for C4 signifies a pyrazole formation. The thioamide C6 was seen at δ 178.1 ppm, and all chemical shifts of ^{13}C NMR and ^{1}H NMR of pyrazole analogues were consistent. **TOF-MS ES** found [M^{+}], 372.0 m/z; **TOF-MS ES** calculated [M^{+}], 370.2 m/z.

(4Z)-4-(4-methoxybenzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4f**

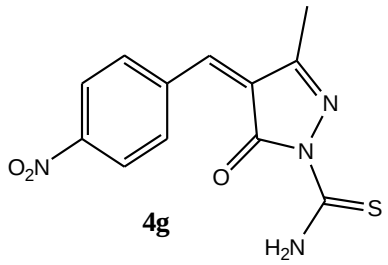


4f

Yield is 94%, cream white solids, m.p 158 – 160 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3298 – 3331 (N-H), 3049 (=C-H), 1605 (C=N), 1017 – 1085 (C=S) and 1150 (C-O-C) (Salum *et al.* 2020), (Boudjellal *et al.* 2020) and (Khatab *et al.* 2021). The **^{1}H NMR (400 MHz, DMSO)** of **4f** shifts observed for H1' & H5' (1H,

d), was δ 6.96 ppm, and H2' & H4' (1H, *d*) was δ 7.74 this is all due to symmetry. An sp^2 -CH chemical shift at H4 revealed δ 8.11 ppm (1H, *s*), showcasing pyrazole formation. A methoxy group (H8) chemical shift for (3H,s) was found to occur at δ 3.85 ppm and δ 11.32 ppm revealed protons of H_2N-C=S (2H, *s*), which was consistent throughout pyrazole analogues. In the **^{13}C NMR** of **4f**, C1', C2', C3', C4' and C5' chemical shifts corresponded to shifts at δ 132.1 ppm, δ 129.3 ppm, δ 161.2 ppm, δ 127.1 ppm and δ 115.1 ppm. A methoxy branch, C8 was δ 55.7 ppm and δ 142.8 ppm for C4 signifying a pyrazole formation. Shift values of δ 132.1 ppm for (C3a,5a) and C6 was seen at δ 178.1 ppm. **TOF-MS ES** found [Na + M^+], 297.1 m/z; **TOF-MS ES** calculated [Na + M^+], 298 m/z.

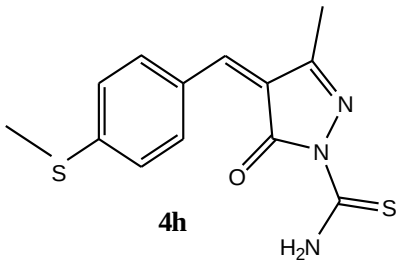
(4Z)-4-(4-nitrobenzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4g**



4g

Yield is 95%, bright yellow solids, m.p 239 – 241 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3302 – 3332 (N-H), 3046 (=C-H), 1540 (C=N), 1015 – 1086 (C=S) and 1485 (NO-stretch) (Salum *et al.* 2020), (Boudjellal *et al.* 2020) and (Khatab *et al.* 2021).The **^{1}H NMR (400 MHz, DMSO)** of **4g** shifts observed for H1' & H5' (1H, *d*), was δ 8.09 ppm, and H2' & H4' (1H, *d*) was δ 8.22 this is all due to symmetry. An sp^2 -CH chemical shift happening at H4 was revealed to be δ 8.12 ppm (1H, *s*), showcasing pyrazole formation. The value of δ 11.72 ppm revealed protons of H_2N-C=S (2H, *s*) consistent throughout pyrazole analogues. In the **^{13}C NMR** of **4g**, C1', C2' and C3' chemical shifts corresponded to shifts at δ 128.6 ppm, δ 124.2 ppm, δ 148.0 ppm. A chemical shift at δ 141.2 ppm for C4 signifies a pyrazole formation. Shift values of δ 140.0 ppm for (C3a,5a) and C6 was seen at δ 178.9 ppm. **TOF-MS ES** found [M^+], 295.0 m/z; **TOF-MS ES** calculated [M^+], 290.3 m/z.

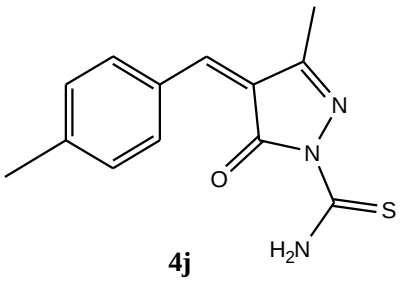
(4Z)-4-(4-(methylthio)benzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4h**



4h

Yield is 90%, pale grey solids, m.p 210 – 216 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3314 – 3300 (N-H), 3057 (=C-H), 1601 (C=N), 1012 – 1061 (C=S) and 1284 (C – H methyl bend) (Salum *et al*. 2020), (Boudjellal *et al*. 2020) and (Khatab *et al*. 2021). The **^{1}H NMR (600 MHz, DMSO)** of **4h** was as follows. A downfield shift was observed at δ 11.40 ppm (1H, *s*) due to amide and thioamide delocalization in the pyrazole ring by mesomeric effects, and the 2 methyl groups H5 & H8 appeared at δ2.08 ppm (6H, *s*). Benzene moiety protons were consistent, revealing doublets multiplicity of H1' & H5' being δ7.26 ppm (1H, *d*) and that of H2' & H4' being at δ 7.73 ppm (2H, *d*), also a chemical shift at δ8.00 ppm signifies H4 (=C-H) pyrazole vinyl link to benzene. The **^{13} C NMR** of **4h** confirmed the functional groups' carbonyl, thioamide, and methyl thio, having δ 192.4 ppm (C3), δ 206.9 ppm (C3&6), and δ14.6 ppm (C8) respectively. The chemical shifts shown at C4 being δ 142.3 ppm signifies the formation of our pyrazole derivative, an *sp*^{2} carbon. Benzene carbons C1', C2', C3', C4' and C5'were agreeable at δ 129.1 ppm, δ 125.9 ppm, δ 161.4 ppm, δ 128.1 ppm, and δ 131.1 ppm respectively, and lastly C3a,5a at δ 141.1 ppm and δ 178.3 ppm C6. **TOF-MS ES** found [M^{+}], 290.0 m/z; **TOF-MS ES** calculated [M^{+}], 291.4 m/z.

(4Z)-4-(4-methylbenzylidene)-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide **4j**



4j

Yield is 91%, cream-colored solids, m.p 166 – 170 ^{o}C. **FT-IR** (KBr, *v*, cm^{-1}): 3346 (N-H), 2916 (-C-H Aromatic), 1065 – 1148 (C=S) and 2328-2622 (methyl stretch) (Salum *et al.* 2020), (Boudjellal *et al.* 2020) and (Khatab *et al.* 2021).The **^{1}H NMR (600 MHz, DMSO)** of **4j** shifts observed were for H1' & H5' (1H, *d*) δ7.22 ppm, δ7.69 ppm for H2' & H4' (1H, *d*) respectively, δ8.01 ppm for H4 (1H, *s*) an *sp*^{2} carbon linking the pyrazole moiety with benzene. Are six protons on C5 and C3'' (2 methyl groups) at δ2.08 ppm (6H, *s*), methyl protons (H3'') at δ 2.50 ppm (3H, *s*), and thioamide group, H_2N-C=S (2H, *s*) at δ11.37 ppm were also observed. In the **^{13}C NMR** of **4j**, the chemical shifts for C1', C2', and C3' were δ129.7 ppm, δ127.9 ppm, δ 140.1 ppm. The value of C4 signifies a pyrazole formation with a shift value of δ 142.9 ppm, C5 was confirmed at δ 31.1 ppm, and C3'' (methyl) was δ 21.5 ppm. The thioamide C3&6 was seen at δ 206.9 ppm, and the thioamide was δ178.3 ppm. **TOF-MS ES** found [M^{+}], 261.1 m/z; **TOF-MS ES** calculated [M^{+}], 259.3 m/z.

4.2 Biological Studies

4.2.1 Antimicrobial Susceptibility Evaluation

The antimicrobial activities of the synthesized compounds (**4a**–**4j**) were assessed using two Gram-positive (*S. aureus* ATCC 12600, *S. pneumonia* ATCC 49619) and two Gram-negative (*P. aeruginosa* ATCC BAA-1744 and *E. coli* ATCC BAA-2452) bacteria strains, using amoxicillin as positive control and 2% DMSO as negative control. The antibacterial susceptibility was evaluated based on the zone of inhibition measured after incubation. The compounds observed with a zone of inhibition diameter greater than eight (> 8 mm) were regarded as active compounds based on the report of (Makhanya, Gengan and Kasumbwe 2020). Two synthesized compounds **4d** and **4h**, showed no antimicrobial activity against Gram-negative organisms suggesting that the organisms might be resistant to the two synthesized compounds relative to amoxicillin (10 mm). Compounds **4b**, **4e**, **4i**, and **4j** had a higher zone of inhibition of 15, 20, 25 and 14 mm, respectively against *P. aeruginosa*. This observation might suggest the superior antibacterial potential of **4b**, **4e**, **4i**, and **4j** against *Pseudomonas aeruginosa* relative to amoxicillin (**Table 4**). However, against *S. aureus*, *S. pneumoniae*, and *E. coli*, amoxicillin had higher inhibition zones at 50 mm, 51 mm, and 32 mm, respectively, compared to the synthesized pyrazoles. Generally, the synthesized pyrazoles had a better antibacterial effect against the Gram-positive organisms than the Gram-negative. Compounds **4a** and **4b** have the highest zone of inhibition against *S. pneumoniae* and *S. aureus*, with a zone of inhibition between 22 and 23 mm (**Table 4**). All synthesized compounds showed excellent activity against Gram-positive bacteria with moderate to good activity against *Pseudomonas aeriginosa* and inactivity for *E. coli.*

Table 4: Antibacterial susceptibility effect of compounds **4a** – **4j** against test organisms

	Zones of Inhibition (mm)				
Entry	Compounds	*S. aureus*	*S. pneumoniae*	*P. aeruginosa*	*E. coli*
1	4a	22±0.58	22±1.2	10±1.2	-
2	4b	30±1	28±0.58	15±0.58	-
3	4c	20±1	20±0	10±0.58	8±1
4	4d	14±0.58	14±1	-	-
5	4e	23±1.5	22±1	20±1.5	-
6	4f	20±1.2	15±0.58	10±1.7	-
7	4g	16±0	15±0.58	10±0	-
8	4h	20±1.7	20±1.2	-	-
9	4i	20±1	30±0	25±1.2	-
10	4j	25±1.5	25±1.1	14±1.2	8±0
11	Amoxicillin	50±0	51±0.58	10±0.58	32±1.2
12	2% DMSO	-	-	-	-

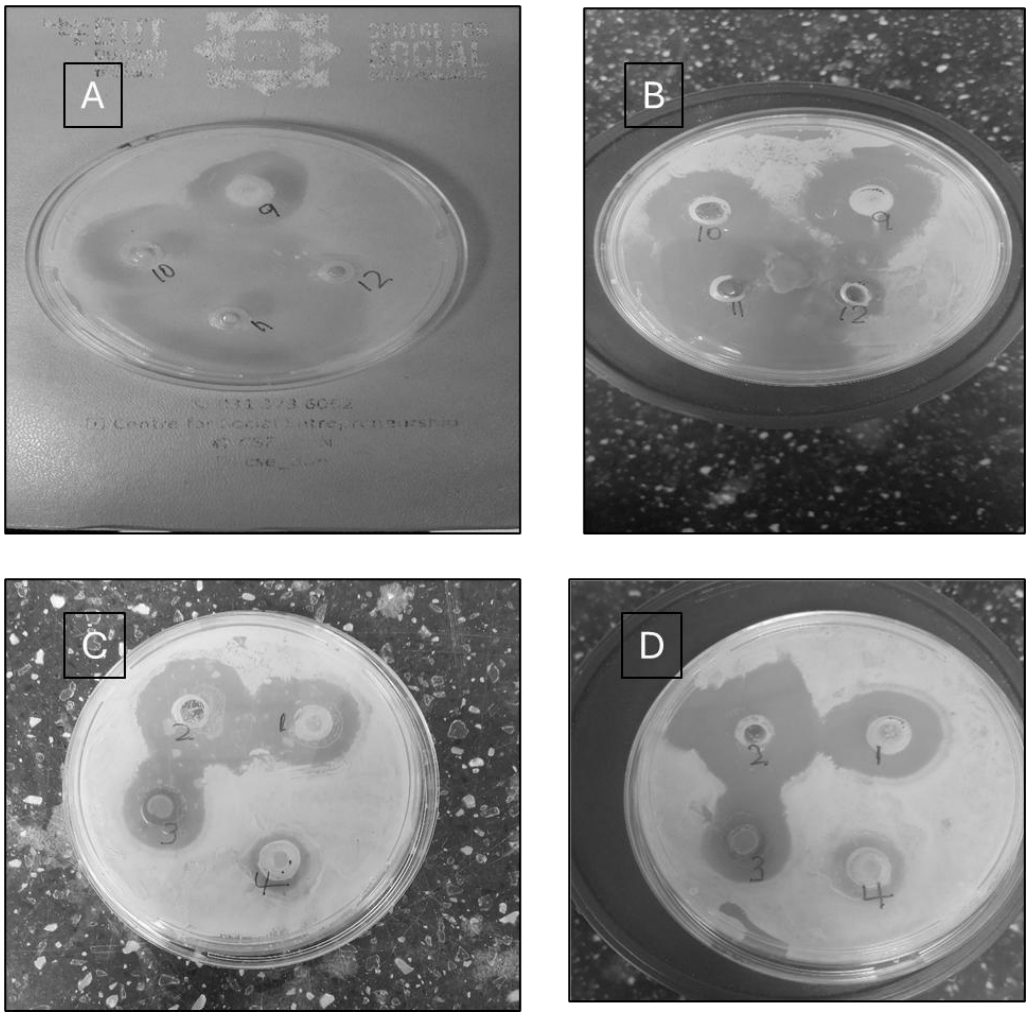


Figure 15: Zones of inhibition of selected pyrazoles [4a(1), 4b(2), 4c(3), 4d(4), 4i(9), 4j(10), Amoxicillin(11)] against (A and C) *S. pneumoniae* and (B and D) *S. aureus*

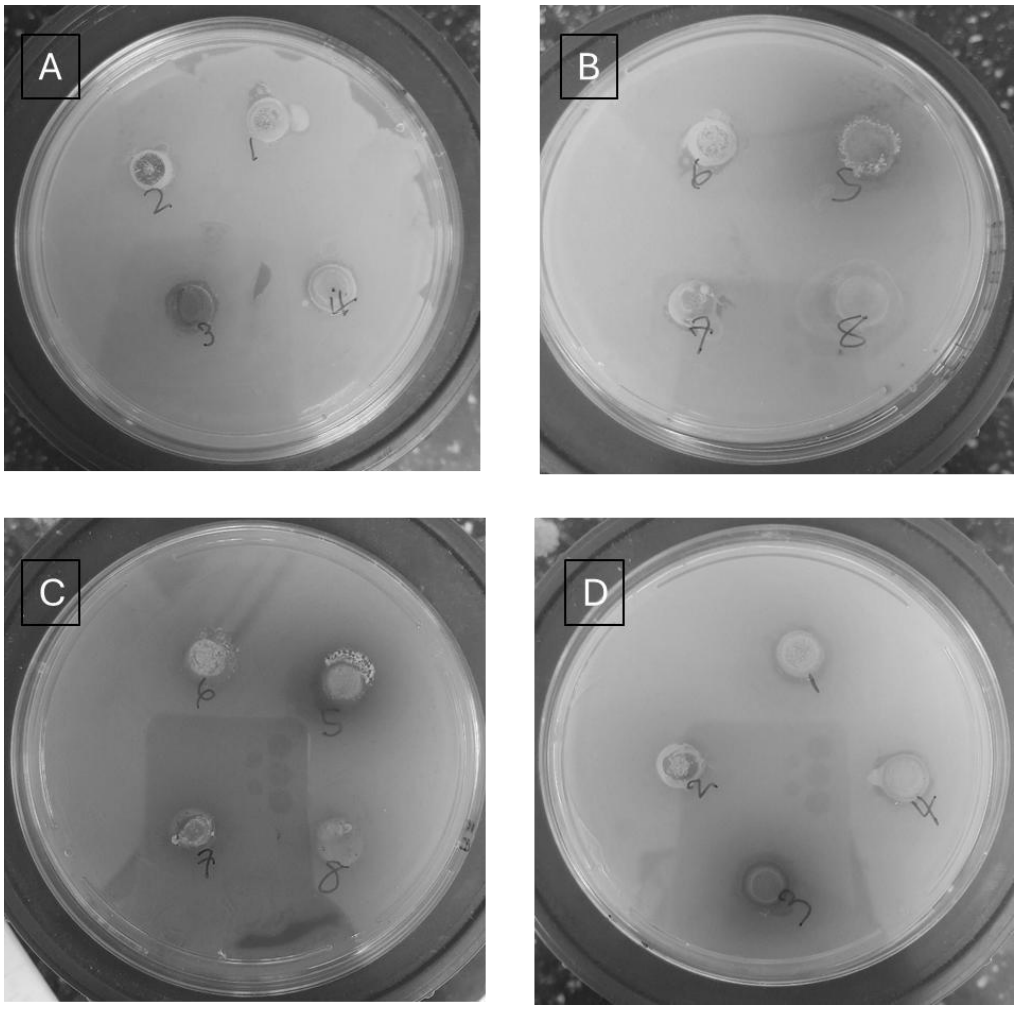


Figure 16: Zones of inhibition of selected pyrazoles [4a(1), 4b(2), 4c(3), 4d(4), 4e(5), 4f(6), 4g(7), 4h(8)] against (A and B) *Escherichia coli* and (C and D) *Pseudomonas aeruginosa*

4.2.2 Minimum Inhibitory Concentration of Pyrazoles

In this study, the antibacterial efficacy of the synthesized pyrazole derivatives (**4a - 4j**) was assessed using the microdilution susceptibility test as outlined by (Sharma *et al.* 2011). The data indicates that all compounds investigated exhibited a range of antibacterial activity, from moderate to strong, against Gram-positive and Gram-negative bacteria (**Table 5**). This suggests that these compounds have broad-spectrum antimicrobial effects. The difference in antibacterial activity between the pyrazole could be related to the type of substituent attached to the phenyl ring. A notable observation was observed in compounds **4d** and **4g**, which both contain a nitro group (-NO_2) as substituent on the phenyl ring. Despite the same substituent,

the compounds differ in placement around the phenyl ring: **4d** has an ortho configuration while **4g** has a para configuration, an isomer. Both compounds demonstrated moderate inhibition zones, ranging between 10 and 15 mm, against all the investigated organisms. However, their MIC values were markedly different. At the same time, compound **4d** had an MIC of 0.438 mg/mL against Gram-positive bacteria investigated (*S. aureus* and *S. pneumoniae*) and showed no inhibitory effect on Gram-negative bacteria. Compound **4g**, in contrast, did not exhibit any inhibitory effect against both Gram-positive and Gram-negative organisms. These findings highlight the complexity of the antibacterial activity within this group of pyrazole derivatives, thereby highlighting the need for further investigation to understand the influence of molecular structure on their efficacy. This observation was noted in a previous study by Sharma *et al.* (2011), who described the nitro group's behaviours as enigmatic.

Against *E. coli*, except for compounds **4c** and **4j** with MIC values of 1.38 mg/mL and 2.50 mg/mL, respectively (**Table 5**), all the synthesised pyrazoles had no activity. This observation is consistent with this study's antibacterial susceptibility pattern assay, where only **4c** and **4j** had inhibition zones, suggesting their potential as a prospective drug candidate for infections caused by *E. coli*. Comparatively, amoxicillin had a lower MIC value against *E. coli* at 0.0306 mg/mL relative to **4c** and **4j**. This observation substantiates a better activity of amoxicillin against *E. coli* than the synthesized pyrazole. This might suggest that further structural alteration of the **4c** and **4j** are required to further increase their potency against *E. coli*. Like *E. coli*, pyrazole compounds **4a**, **4d** and **4g** depicted no antibacterial activity against *P. aeruginosa.* While compounds **4b**, **4c**, **4e**, **4f**, **4h**, **4i**, and **4j** had MICs values 0.224 mg/mL, 1.38 mg/mL, 0.189 mg/mL, 0.438 mg/mL, 2.5 mg/mL, 0.170 mg/mL and 1.25 mg/mL respectively (**Table 5**) with **4i** having the lowest value. Amoxicillin, conversely, had a lower MIC value at 0.0765 mg/mL than all the pyrazoles, suggestive of its better antibacterial activity. The impotent or diminished antibacterial activity of the pyrazoles against both Gram-negative organisms might be due to the membrane structure of Gram-negative bacteria. It functions as a barrier that prevents the penetration of antimicrobials (Mert *et al.* 2014) coupled with the tendency of Gram-negative bacteria. To express genes that enable them to become resistant against antimicrobials.

Against *S. pneumoniae* while only three pyrazole compounds (**4a**, **4f** and **4g**) had no activity, compound **4j** had a lower MIC value at 0.0156 mg/mL than amoxicillin (0.0306 mg/mL). This observation is consistent with the antibacterial sensitivity assay. 4j is a potential antibacterial agent against *S. pneumoniae*. Other synthesized pyrazoles had a slightly higher MIC value relative to amoxicillin. Against *S. aureus*, compound **4g** showed no activity. The other synthesized pyrazoles had MIC values ranging between 0.212 mg/mL and 0.625 mg/mL, with **4i** having the lowest value, suggestive of its better potential as an antibacterial agent against *S. aureus*.

Generally, **4c**, **4e**, **4j**, and **4i** were the best-synthesized pyrazoles against *E. coli*, *P. aeruginosa*, *S. pneumoniae* and *S. aureus*, respectively. It is interesting to observe that **4g** had no MIC value against all the test organisms. The unsubstituted phenyl ring present in **4i** might have increased the potency of the compound. Against all the test bacteria relative to other synthesized pyrazoles, such as **4a**, **4b**, **4c**, **4d**, **4e**, **4f** and **4h**, that contain electron-withdrawing groups on their benzene ring. Similarly, the presence of an electron-donating group (-CH_3) on the benzene ring of **4j** might have potentiated the antibacterial effect against *S. pneumoniae*. Furthermore, the position of the substituents on the benzene ring seemed to affect the activity of the pyrazoles (**Table 5**). With compounds consisting of ortho-phenyl substituents (**4b**), meta and para-trisubstituted phenyl analogue (**4e**), and unsubstituted phenyl compound (**4i**), they consistently exhibited excellent inhibition zones and better MIC values. When compared to ortho-substituted counterparts, **4a**, **4f**, **4g** and **4h** against both Gram-positive and Gram-negative bacteria. These differences may be attributed to the variations in atom size, electronegativity, and polarity of the substituents (Turos *et al.* 2005). Influencing their interaction with bacterial targets and contributing to the pyrazole pharmacophore's activity.

Table 5: MIC activity of compounds **4a** – **4j**

	Antimicrobial Minimum Inhibitory Concentration (MIC) of Compounds 4a – 4j (mg/mL)				
Entry	Compounds	*S. aureus*	*S. pneumonia*	*P. aeruginosa*	*E. coli*
1	4a	0.453	-	-	-
2	4b	0.280	0.196	0.224	-
3	4c	0.344	1.38	1.38	1.38
4	4d	0.438	0.438	-	-
5	4e	0.236	0.165	0.189	-
6	4f	0.438	-	0.438	-
7	4g	-	-	-	-
8	4h	0.312	0.625	2.5	-
9	4i	0.212	0.149	0.170	-
10	4j	0.625	0.0156	1.25	2.50
11	Amoxicillin	0.0153	0.0306	0.0765	0.0306
12	2%DMSO	-	-	-	-

4.2.3 Molecular Docking Studies

4.2.3.1 Docking scores of the pyrazoles

Protein-to-ligand interaction was studied by molecular docking the synthesized pyrazoles at the active site of the essential PBPs of the test organisms [*E. coli* (PBP3), *P. aeruginosa* (PBP3), *S. aureus* (PBP2a), and *S. pneumoniae* (PBP2x)]. Subsequent docking score energies were recorded for each complex, signifying the ligand's possible orientation at the active site. The conformation with the lowest docking score (highest negative docking score) was considered the best-docked complex with a better potential of forming a stable complex. The docking score of all compounds against all the PBPs is shown in **Table 6**. Against the PBP3 enzyme of *E. coli*, the docking scores ranged between -6.7 kcal/mol to -7.9 kcal/mol, with **4f** having the highest negative docking score at -8.1 kcal/mol. Except for compounds **4a** and **4e,** with docking scores of -6.7 kcal/mol and -6.9 kcal/mol, all synthesized pyrazoles had better docked scores than amoxicillin (-7.0 kcal/mol) against the PBP3 enzyme of *E. coli*. Compound

4f had the best docked score among the synthesized pyrazoles against PBP3 enzyme of *E. coli* in this study, which contrasts with the *in vitro* findings where only **4c** and **4j** had activity. Deduction from this observation might mean that molecular docking is too preliminary in identifying active molecules, as reported by earlier studies (Cerón-Carrasco, 2022; Aribisala and Jamiu, 2022). Thus, further advances in silico studies, such as molecular dynamic (MD) simulation, might be required to understand the dynamics of the interaction of the pyrazoles against PBP3 of *E. coli*.

Against PBP3 enzyme of *P. aeruginosa*, the docking scores ranged between -6.8 kcal/mol and -7.7 kcal/mol with compounds **4g** and **4j** having the highest negative docking scores both at -7.7 kcal/mol. Comparatively, only **4g** and **4j** had higher negative docking scores than amoxicillin (-7.5 kcal/mol) with **4b** (-7.4 kcal/mol) and **4f** (-7.5) having comparable values. This observation points to the potential of **4g** and **4j** as potential inhibitors of PBP3 of *P. aeruginosa*. However, the docking result only predicts the best pose of a ligand on a protein target. Further advances *in silico* studies, such as MD simulation, are also recommended against the target for compounds **4j** and **4g**.

Against PBP2x of *S. pneumoniae,* the docking scores range between -7.2 kcal/mol and -8.1 kcal/mol (**Table 6**), with **4j** having the highest negative docking scores at -8.1 kcal/mol, followed closely by **4d** at -7.9 kcal/mol. The observation that compound **4j** had the highest negative docking score is consistent with the *in vitro* result of this study. **4j** had the lowest MIC value (0.0156 mg/mL) among the pyrazoles, with a value that was lesser than amoxicillin (0.0306 mg/mL) suggesting that there is a correlation between the molecular docking and the *in vitro* result against *S. pneumoniae*. This observation thus further reinforces the potential of **4j** as a potential inhibitor of PBP2x of *S. pneumoniae*. As earlier stated, further MD simulation of the complex (PBP2x-4j) might be necessary to understand the extent of the dynamics, stability and flexibility over a period.

Against PBP2a of *S. aureus*, the synthesized pyrazoles had docking scores ranging between -5.5 kcal/mol to -6.6 kcal/mol (**Table 6**), with **4j** having the highest negative docking scores. This observation indicates the better potential of **4j** as an inhibitor of PBP2a. The docking scores of the pyrazole were relatively lower against PBP2a relative to other investigated PBPs targets of test bacteria. Nonetheless, compounds **4a**, **4b**, **4e**, **4g**, and **4h** had comparable docking scores at -5.5 kcal/mol as amoxicillin. Compounds **4b** and **4f**, with a score of -6.0 kcal/mol, were better complex stabilized in the cavity of PBP2a than amoxicillin.

To investigate the validity of the docking protocol employed in the study. The superimposition techniques employed showed that relative partial binding position (RMSD: 2 Å) exists for the best docked synthesized pyrazole (**4f** against PBP3 of E. coli and **4j** against other investigated PBPS) and amoxicillin at the native ligand position of all the investigated PBPs [PBP3 of *E. coli*, PBP3 of *P. aeruginosa* PBP2x of *S. pneumoniae* and PBP2a of *S. aureus*] (**Figure 17**). Despite partial binding position, the best docked synthesised pyrazoles and amoxicillin were observed to interact with active site amino acids residues that have been reported in previous studies such as Lys310, Asn361, Ser359, Ser307, Thr308 in PBP3 of *E. coli* (Dom et al., 2019; Aribisala *et al*., 2023), Ser548, Ser337, Gln552, Ser396, Thr550, and Lys547 in PBP2x of *S. pneumoniae* (Gordon *et al*., 2000), Ser294, Tyr503, Arg489 and Ser349 in PBP3 of *P. aeruginosa* (Kumar et al., 2020) and Ser462, Thr404, Ser403, Gly402, and Asn464 in PBP2a *S. aureus* (Janardhanan *et al*., 2019) (**Figure 17**). These observations are suggestive of the reliability of the docking score observed in this study for the targets.

Table 6: Docking scores in kcal/mol of synthesized pyrazole derivative against essential PBPs of test bacteria

Synthesized Pyrazoles	**PBP3 of *E. coli***	**PBP3 of *P. aeruginosa***	**PBP2x of *S. pneumoniae***	**PBP2a of *S. aureus***
4a	-6.7	-7.3	-7.6	-5.5
4b	-7.5	-7.4	-7.6	-5.5
4c	-7.2	-7.1	-7.5	-5.6
4d	-7.6	-7.3	-7.9	-6.0
4e	-6.9	-6.8	-7.3	-5.5
4f	-8.1	-7.5	-7.6	-6.0
4g	-7.3	-7.7	-7.2	-5.5
4h	-7.2	-7.0	-7.6	-5.5
4i	-7.2	-7.1	-7.5	-5.7
4j	-7.9	-7.7	-8.1	-6.6
Amoxicillin	-7.0	-7.5	-7.5	-5.5

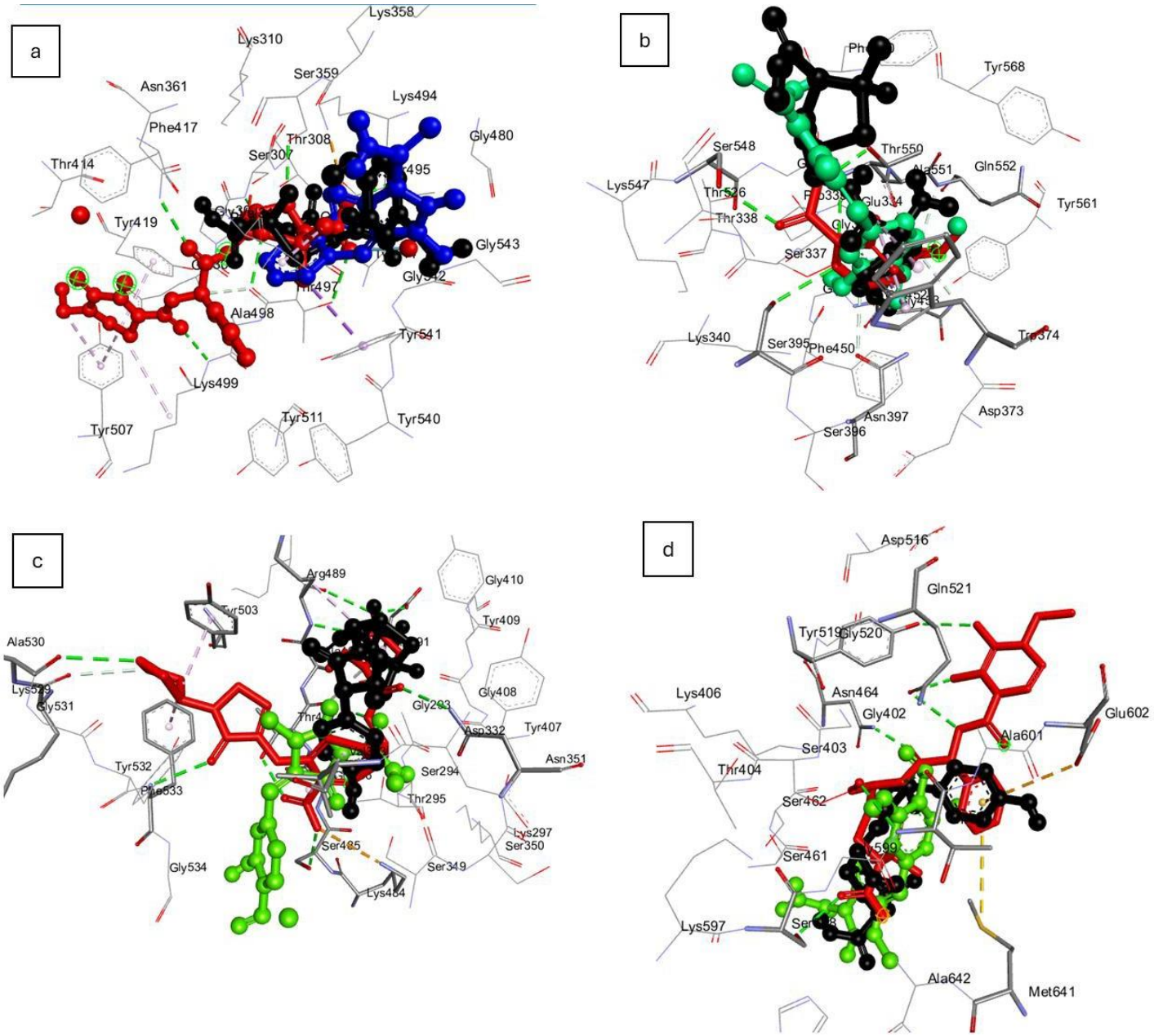


Figure 17: Docking validation via superimposition of best docked synthesized pyrazole derivative [(a) **4f** (blue) against PBP3 of *E. coli* (6I1I), and **4j** (green) against (b) PBP2x (1QMF) of *S. pneumonieae* (c) PBP3 of *P. aeruginosa* (6VJE) (d) PBP2a (6H5O) of *S. aureus*] and amoxicillin (blue) against the native ligand (red) in each case of the investigated target PBPs.

4.2.4 Pharmacokinetics and Toxicology evaluation of Pyrazoles

Nowadays, it has become essential to develop lead compounds that can be easily absorbed orally and transported to the desired site of action. Also, leads that are not easily converted into toxic metabolites before reaching the target site of action and can excrete from the body without any complications, before they accumulate in sufficient quantities that can cause adverse side effects (Ntie-Kang 2013). Therefore, the pharmacokinetics of a drug are known as ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) and are imperative to determine when developing drug-like molecules. The in-silico predictions of ADMET characteristics provides insight into the compound's oral bioavailability and relative toxicity when used as a drug (Segall and Barber 2014; Aribisala *et al.* 2022). Lipinski's Rule of 5 (LRo5) was considered for determination of drug-likeness of the studied pyrazoles because of its wide application and accuracy (Raval and Ganatra 2022). Lipinski Rule describes an orally bioavailable molecule as that having a molecular weight ≤ 500, hydrogen bond donors ≤ 5, hydrogen acceptors ≤ 10, octanol/water partition coefficient ≤ 5 and rotatable bonds ≤ 5 as a bioactive drug; represent as MW≤ 500, HBDs ≤ 5, HBAs ≤ 10, log P(o/w) ≤ 5, and RTBs ≤ 5 respectively.

In **Table 7**, all pyrazole derivatives adhered to the LRo5 parameters, showing good bioavailability as drug candidates. This indicates that after administration, the compounds may be able to actively diffuse from the gastro-intestinal (G.I) tract's lumen to the vasculature's lumen, where they can circulate the system and reach their target sites at adequately high concentrations (Aribisala *et al.* 2022) to induce therapeutic effects. This is supported by the bioavailability score (BS) of 55% seen in **Table 8** for pyrazoles being as good as amoxicillin. However, the high gastrointestinal (GI) tract absorptions of pyrazoles showed their advantage over amoxicillin having a low GI absorption (**Table 8**).

Table 7: Lipinki rule of 5 parameters for pyrazole derivatives

Ligands	M.W <500 (g/mol)	HB-A≤10	HB-D≤5	Log P_o/W ≤5	WS	LV
4a	279.7	2	1	2.25	S	No
4b	261.3	3	2	1.34	S	No
4c	288.4	2	3	1.73	S	No
4d	288.3	3	1	1.59	MS	No
4e	368.2	3	2	2.39	MS	No
4f	275.3	3	1	1.70	S	No
4g	290.3	1	4	1.17	MS	No
4h	291.4	2	1	2.26	MS	No
4i	245.3	2	1	1.73	S	No
4j	259.3	2	1	2.05	MS	No
Amoxicillin	365.4	6	4	-0.29	VS	No

MW: Molecular weight, HB-D: Hydrogen bond donor, HB-A: Hydrogen bond acceptor, Log P o/w: Partition coefficient, WS: Water solubility, LV: Lipinski Violation, S: Soluble, MS: Moderate soluble, VS: Very soluble

The Log P ≤ 5 parameter of LRo5 is a crucial factor in determining bioavailability of a compound as well. A LogP >5 suggests high lipophilicity, which produces undesirable drug features that promote nonspecific plasma protein binding, poor water solubility and potentially crosses blood brain barrier (BBB), reducing the chances of the drug reaching targeted sites (Morak-Młodawska *et al.* 2023). Studies have shown that compounds with a moderate lipophilicity oscillating around 2 have optimal abilities to reach molecular targets (Kathuria *et al.* 2021); (Morak-Młodawska *et al.* 2023). In this regard, the pyrazoles exhibited better bioavailability as their LogP oscillates between 1-2.5 compared to amoxicillin which is partitioning close to an aqueous phase, given its negative value (Rutkowska *et al.* 2012) seen in **Table 7**.

In terms of toxicity, amoxicillin has an LD_{50} (Lethal dose to kill 50% bacteria) of 15g/kg, indicating it is safe for oral consumption. It falls under toxicity class (TC) level 6, which categorizes it as non-toxic and orally friendly, with minimal adverse effects. This antibiotic is commonly administered orally (P.O. form) in hospitals. Among the pyrazoles, only compound **4c** shared the same toxicity profile (toxicity class 6) as amoxicillin with a LD_{50} of 5.4g/kg, indicating that it is safe to develop **4c** as a P.O drug because it's non-toxic like amoxicillin. Other pyrazole compounds were all predicted to be toxicity class 4 (TC4) drug candidates [harmful if swallowed ($300 < LD_{50} \leq 2000$)], with such variable LD_{50} values, the oral administration may not be viable in this regard. However, other routes of administration such as buccal, sublingual, inhalation, intramuscular, subtopical or even intravenous may be

considered to curb many unnecessary adverse effects. All studied compounds showed no affinity for BBB permeability and no P-glycoprotein binding, signifying that they can travel through the system without impediments by nonspecific enzymes and tissues, thus achieving their desired pharmacological effects.

Table 8: Predicted ADMET parameters for pyrazole derivatives

Ligands	G.I Absorption	B.S	LD_{50} (mg/kg)	T.C	BBB Permeability	Pgp
4a	High	0.55	1012	4	No	No
4b	High	0.55	960	4	No	No
4c	High	0.55	5400	6	No	No
4d	High	0.55	1040	4	No	No
4e	High	0.55	1000	4	No	No
4f	High	0.55	450	4	No	No
4g	High	0.55	711	4	No	No
4h	High	0.55	1000	4	No	No
4i	High	0.55	960	4	No	No
4j	High	0.55	960	4	No	No
Amoxicillin	High	0.55	15000	6	No	No

GI absorption: Gastrointestinal absorption, BS: Bioavailability score, LD: Lethal dose, TC: Toxicity class, BBB permeant: Blood-brain barrier permeation, Pgp substrate: Permeability glycoprotein substrate

Table 9: Pyrazole derivative inducing or inhibiting CYP 450 Isoenzymes prediction

Ligands	CYP -1A2	CYP-2C19	CYP-2C9	CYP-2D6	CYP-3A4
4a	Yes	Yes	Yes	No	No
4b	Yes	No	No	No	No
4c	Yes	Yes	No	No	No
4d	No	No	No	No	No
4e	No	No	Yes	No	Yes
4f	Yes	Yes	No	No	No
4g	Yes	No	No	No	No
4h	Yes	Yes	Yes	No	No
4i	Yes	Yes	No	No	No
4j	Yes	Yes	No	No	No
Amoxicillin	No	No	No	No	No

Compound **4d**, in **Table 9** above, was predicted to not inhibit any of the cytochrome (CYP) isoenzymes like amoxicillin, suggesting their advantage over other pyrazole compounds, which may result in drug toxicity (Aribisala *et al.* 2022). Only compound **4e** showed an affinity to inhibit CYP 3A4, an isoenzyme responsible for breaking down or metabolizing more than 60%

of drugs (Verma *et al.* 2022). Metabolism is imperative in pharmacokinetics because it involves the biotransformation of an active drug to an inactive form. Biotransformation is aided by cytochrome P450 (CYP 450) isoenzymes (Ogu and Maxa 2000). CYP 450 enzymes are primarily located in the liver and intestine. They metabolize most drugs through oxidation and reduction or hydrolysis (Durán-Iturbide, Díaz-Eufracio and Medina-Franco 2020). CYP 450 enzymes can either be induced or inhibited by various drugs and substances, which results in drug interactions that lead to toxicity or reduction in the therapeutic effect (Issa *et al.* 2017). None of the pyrazole compounds seemed to inhibit CYP 2D6. Only two compounds, which are **4d** and **4e** including amoxicillin were non-inhibitors of CYP1A2 (**Table 9**). Further investigations are needed to optimize pyrazole structures for therapeutic use.

Table 10: Predicted adverse effects of pyrazole derivatives

Ligands	Hepatotoxicity	Carcinogenicity	Mutagenicity	Immunotoxicity	Cytotoxicity
4a	-	-	-	I	I
4b	-	-	-	I	I
4c	-	-	-	I	I
4d	-	A	A	I	I
4e	-	-	-	A	-
4f	-	-	-	I	I
4g	-	A	-	I	I
4h	-	-	-	I	I
4i	-	-	-	I	I
4j	-	-	-	I	I
Amoxicillin	I	I	I	I	-

A : Active, I :Inactive, and "-" : Not defined

In drug discovery, mutagenicity is an issue that needs to be avoided (Hsu *et al.* 2016). Potentially mutagenic impurities will likely be formed in any drug substance since their synthesis requires reactive intermediates, which may also react with DNA (Waechter *et al.* 2024). Evidence of mutagenic activity may indicate that a chemical substance can potentially encourage carcinogenic effects (Hsu *et al.* 2016). In therapeutic agents, carcinogenicity is strongly correlated with mutagenicity. Mutagens are agents that cause an increase in the frequency of DNA modifications or mutations (Basak, Nair and Mittra 2016). When in silico studies were performed on pyrazole derivatives to assess probable adverse effects, carcinogenicity and mutagenicity (**Table 10**). Because **4d** and **4g** are isomers, they gave different probable adverse effects; **4d** having a nitro-group attached at the ortho position may

be both carcinogenic and mutagenic. Whereas **4g** has been revealed to be potentially carcinogenic but not mutagenic, its nitro-group attachment is at the para position.

The nitrogen dioxide substituent has been observed to potentially be an active carcinogen and mutagen. These effects in early drug design are detected by in silico studies. The recognized mutagenic groups of scaffolds can serve as a guide for medicinal chemists. To prevent the development of potentially mutagenic therapeutic agents in early drug design, the core structures of mutagenic compounds must be modified to form non-mutagenic compounds (Hsu *et al.* 2016) documents. The rest of the pyrazole derivatives were undetected as active or threats as carcinogens or mutagens. Immunotoxicity is the adverse effects of foreign substances or drugs on the immune system (Semwal *et al.* 2022). Only compound **4e**, **Table 10**, showed an active immunotoxicity outcome, and the rest of the pyrazole derivatives were inactive. The degree to which a substance can cause damage to a cell is known as cytotoxicity. This was also appraised by in silico, and none of the studied pyrazoles are cytotoxic. None of the pyrazoles in this study were shown to be hepatotoxic. Hence, they are safe and have no affinity for damaging the liver, which was substantiated by the CYP 450 enzymes outcome in **Table 9**.

Chapter Five: Conclusion and Recommendation

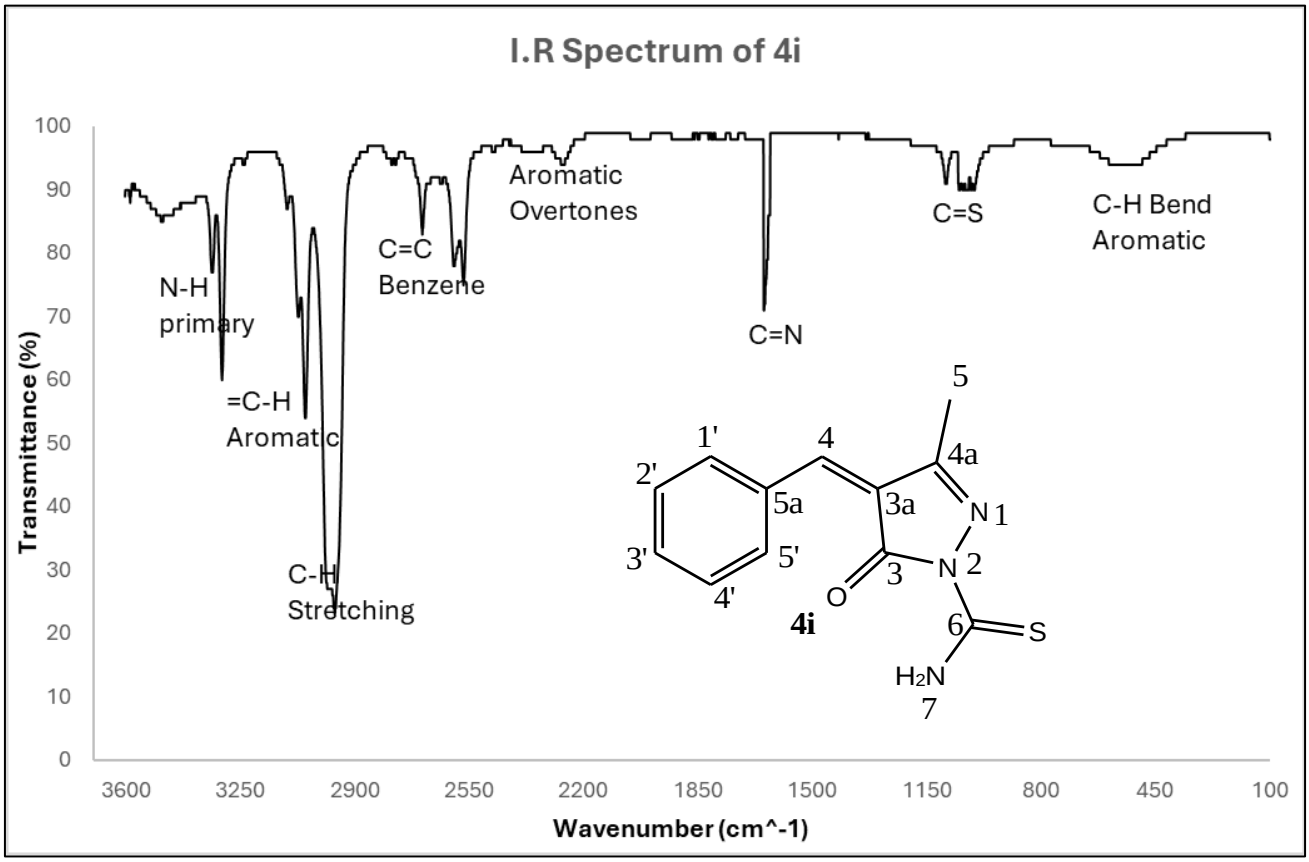
This study presents an efficient, eco-friendly method for synthesizing (4Z)-4-arylidene-4,5-dihydro-3-methyl-5-oxopyrazole-1-carbothioamide derivatives (**4a–4j**) using a one-pot, microwave-assisted approach. Compared to conventional thermal reflux, microwave irradiation drastically improved reaction times, increased product yields (87-96%), and minimized purification requirements. The method performed well in an ethanol-water (1:1) solvent system, without using a catalyst. This protocol was effective with various aromatic aldehydes, regardless of substituent type. The optimized approach offered a rapid, green pathway to high-purity pyrazole derivatives. Structural confirmation of pyrazoles was verified by ^{1}H-NMR, ^{13}C-NMR, TOF-MS, and IR spectrometry, with consistent results across derivatives.

The comprehensive study of pyrazole derivatives has revealed a nuanced landscape of antibacterial activity. Our studies demonstrate that these compounds possess a broad-spectrum efficacy, challenging the traditional understanding of substituent influence on antimicrobial action. Despite sharing a nitro group, the distinct behaviours of compounds **4d** and **4g** underscore the critical role of molecular positioning in determining antibacterial potency. Similarly, the contrasting activities of **4f** and **4h** highlight the impact of substituent characteristics on pharmacological interactions. Pyrazoles exhibited higher negative docking scores than amoxicillin. Despite this, in vitro activity was observed only for compounds **4c** and **4j** for *E. coli*. Molecular docking on PBP3 of *E. coli* alone may not fully predict active molecules. Compounds **4g** and **4j** showed potential as PBP3 inhibitors of *P. aeruginosa*. Further in silico studies, including molecular dynamics simulations, are recommended. Compound **4j** had the highest negative docking score, consistent with its low MIC value. Agreement between docking and in vitro results supports **4j** as a PBP2x inhibitor. Pyrazoles exhibited lower docking scores against PBP2a. **4j** emerged as a promising inhibitor, comparable to amoxicillin. **4b** and **4f** also stabilized well in the PBP2a cavity. These insights contribute to our understanding of pyrazole-PBP interactions and highlight the need for further investigations to optimize these compounds for potential therapeutic use.

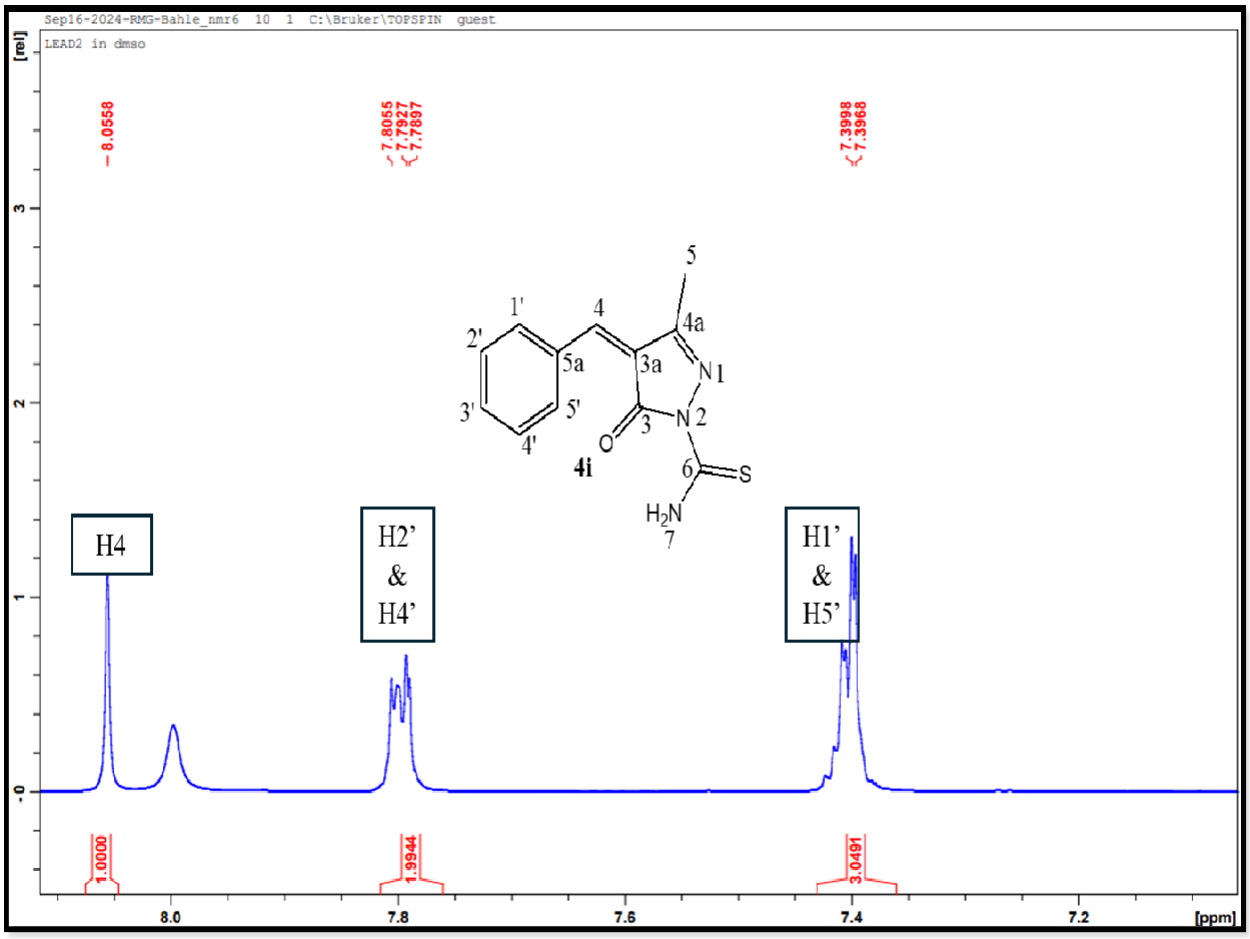
Our investigation of pyrazole compounds reveals their potential as oral bioavailable drug candidates. All evaluated pyrazoles adhere to Lipinski Rule of Five (LRo5) criteria. They are likely to be orally bioavailable, diffusing from the GI tract to the vasculature and reaching

therapeutic concentrations. Pyrazoles exhibit a BS (Bioavailability Score) of 55%, comparable to amoxicillin. Their high GI tract absorption advantage over amoxicillin suggests improved bioavailability. Pyrazoles' LogP oscillates between 1-2.5, favorable for bioavailability. Amoxicillin's partitioning close to an aqueous phase contrasts with pyrazoles studied. Regarding toxicity and safety, pyrazoles exhibit varying LD50 values, suggesting alternative administration routes. Compound **4c** shares safety characteristics with amoxicillin. Pyrazoles show no BBB affinity or P-glycoprotein binding. Compound **4d** is a non-inhibitor of CYP isoenzymes, minimizing drug toxicity. Thus, the series has demonstrated to be potential antibacterial drug candidates for Gram-positive bacteria especially and should be considered for further study. Notably, some compounds like **4c**, **4e**, **4j** and **4i** were the best-synthesised pyrazoles against *E. coli*, *P. aeruginosa*, *S. pneumoniae* and *S. aureus*, respectively, indicating potential as alternative antimicrobial agents. However, amoxicillin generally exhibited superior efficacy across all tested bacteria. These results underscore the promise of pyrazole derivatives in antibacterial treatment and the importance of continued research to optimize their effectiveness. Future studies are warranted to decipher the intricate mechanisms at play and to harness the full therapeutic potential of pyrazole derivatives. Hence, pyrazoles hold promise as potential drug candidates.

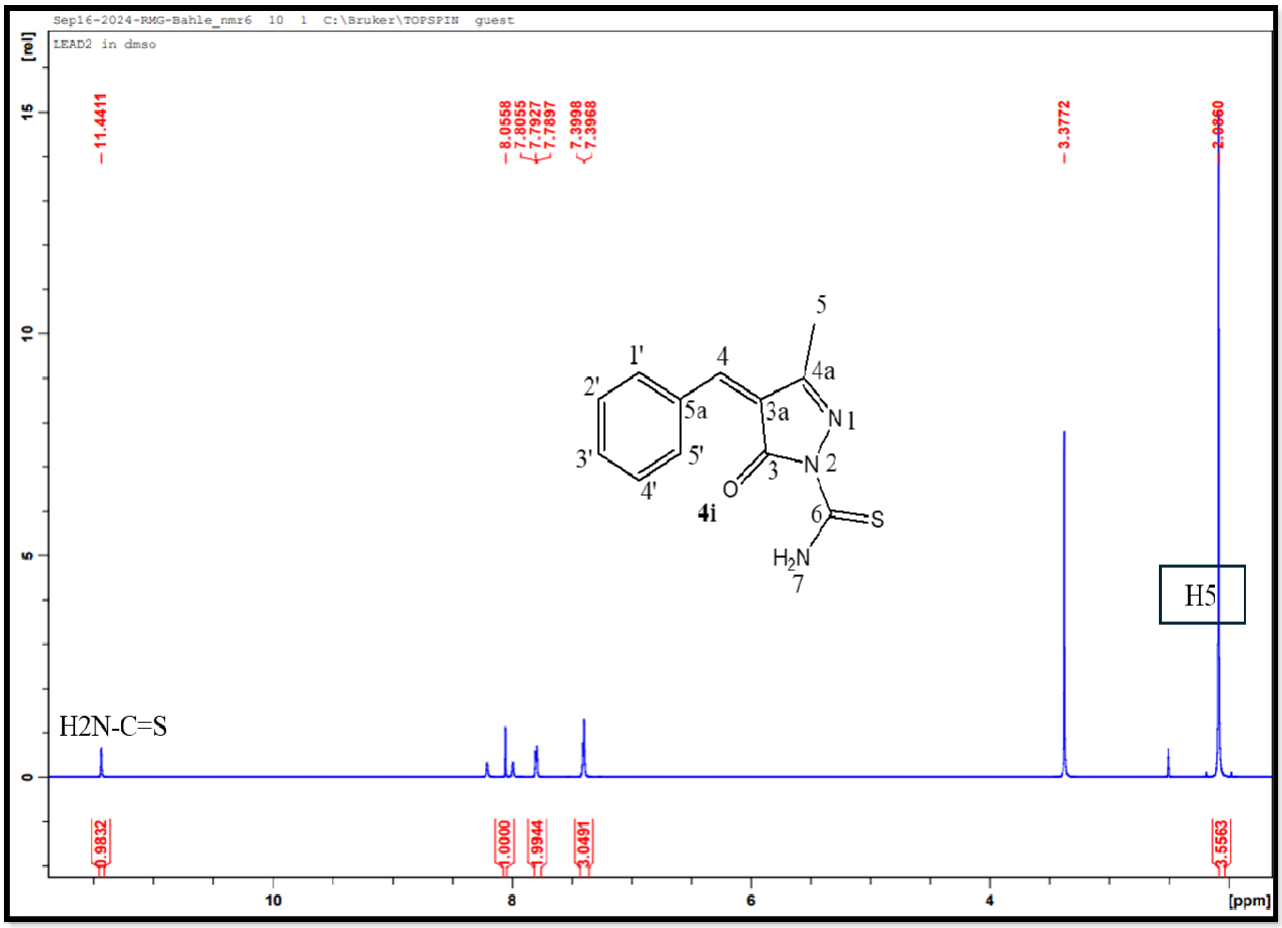
APPENDIX



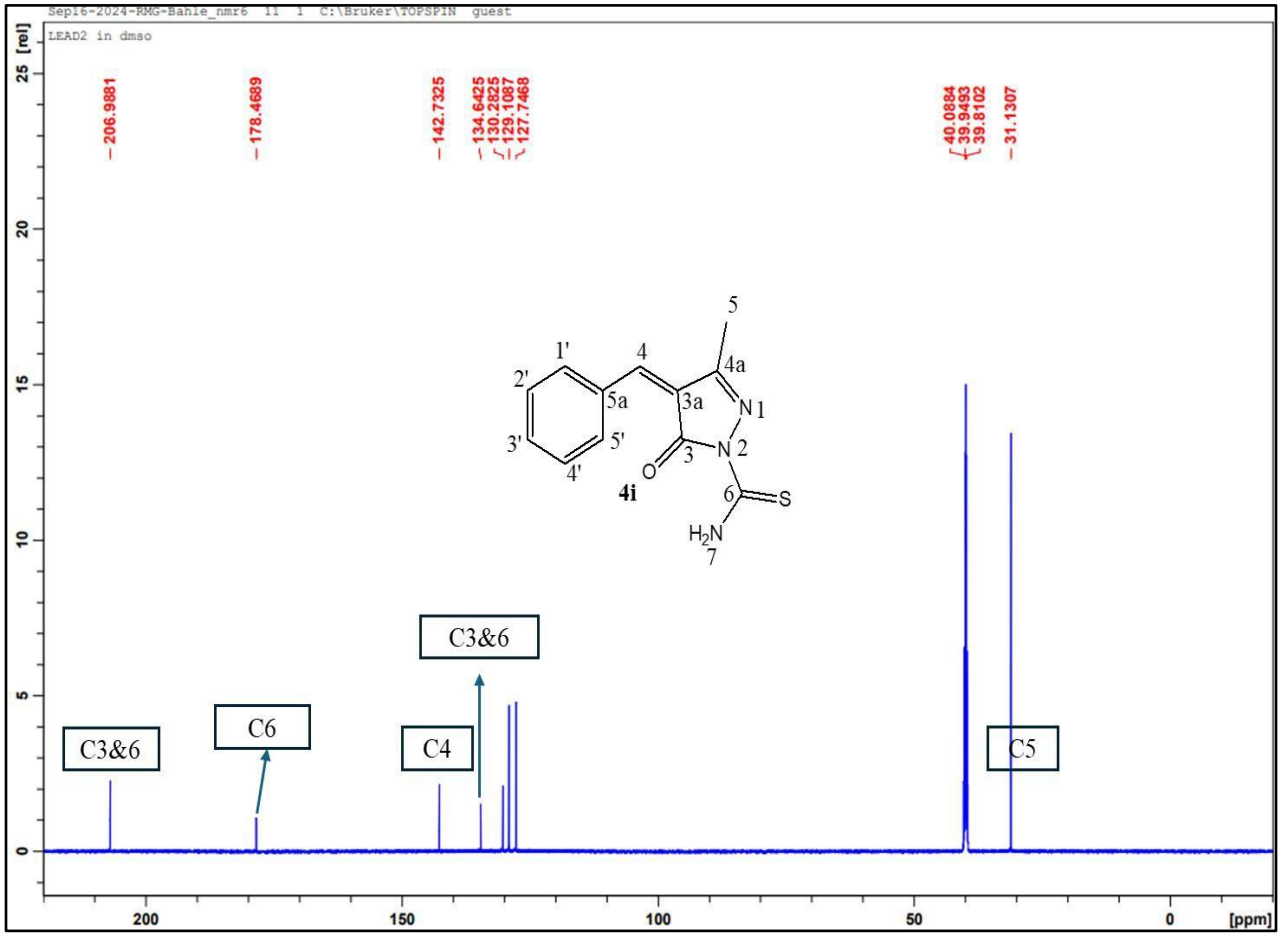
Appendix 1: I.R Spectrum of **4i**



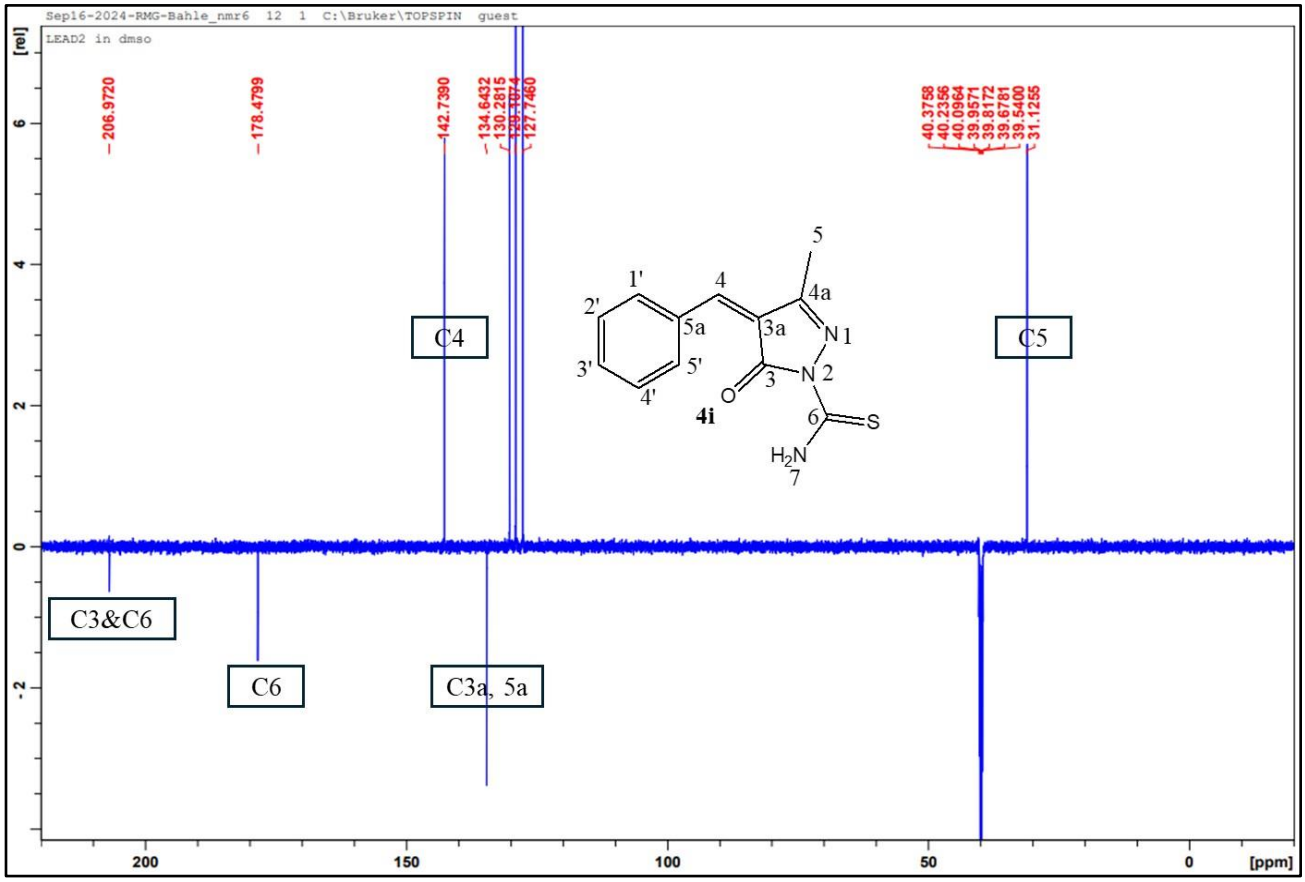
Appendix 2: Expanded ^{1}H NMR spectrum for compound **4i**



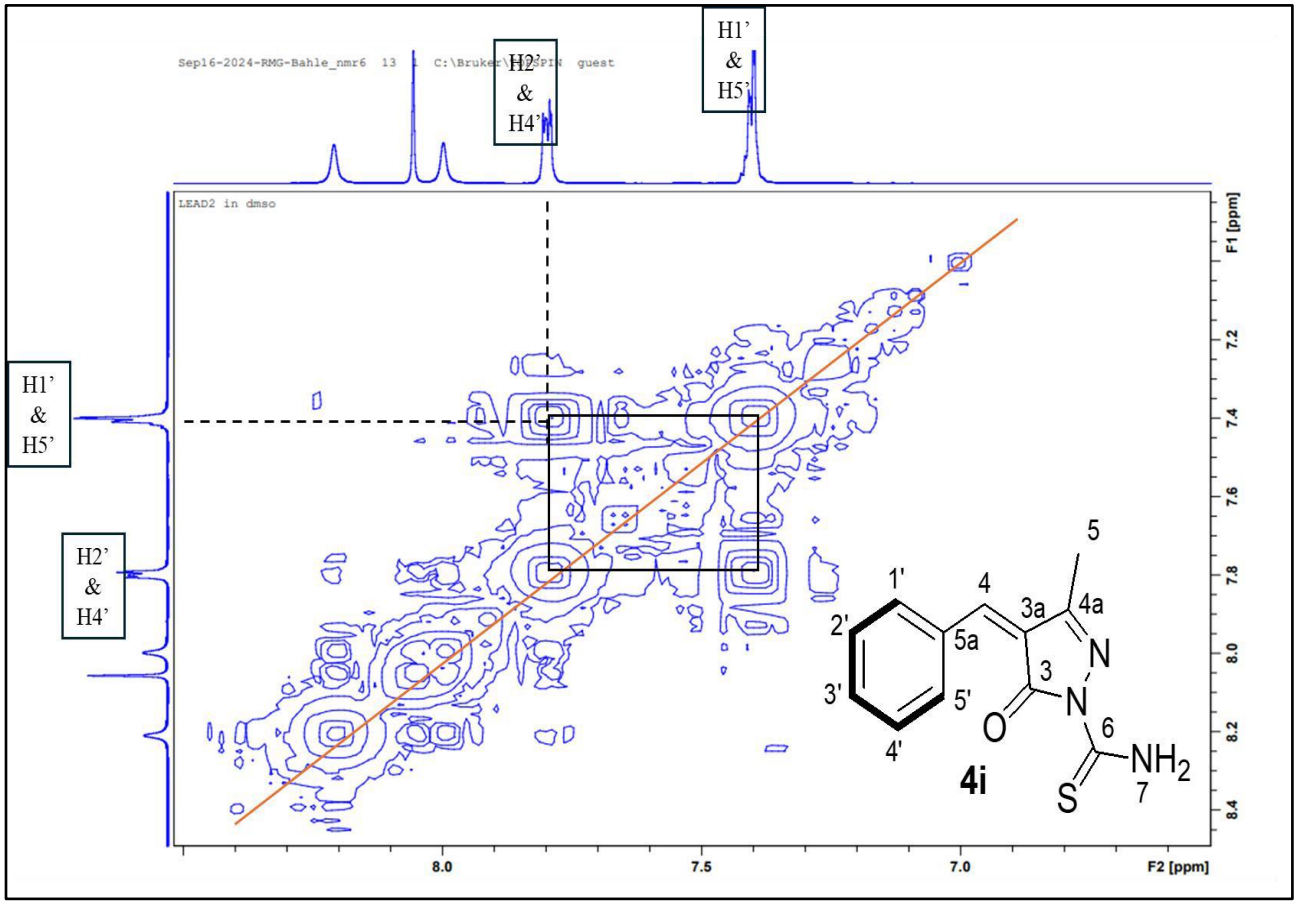
Appendix 3: ^{1}H NMR spectrum for compound **4i**



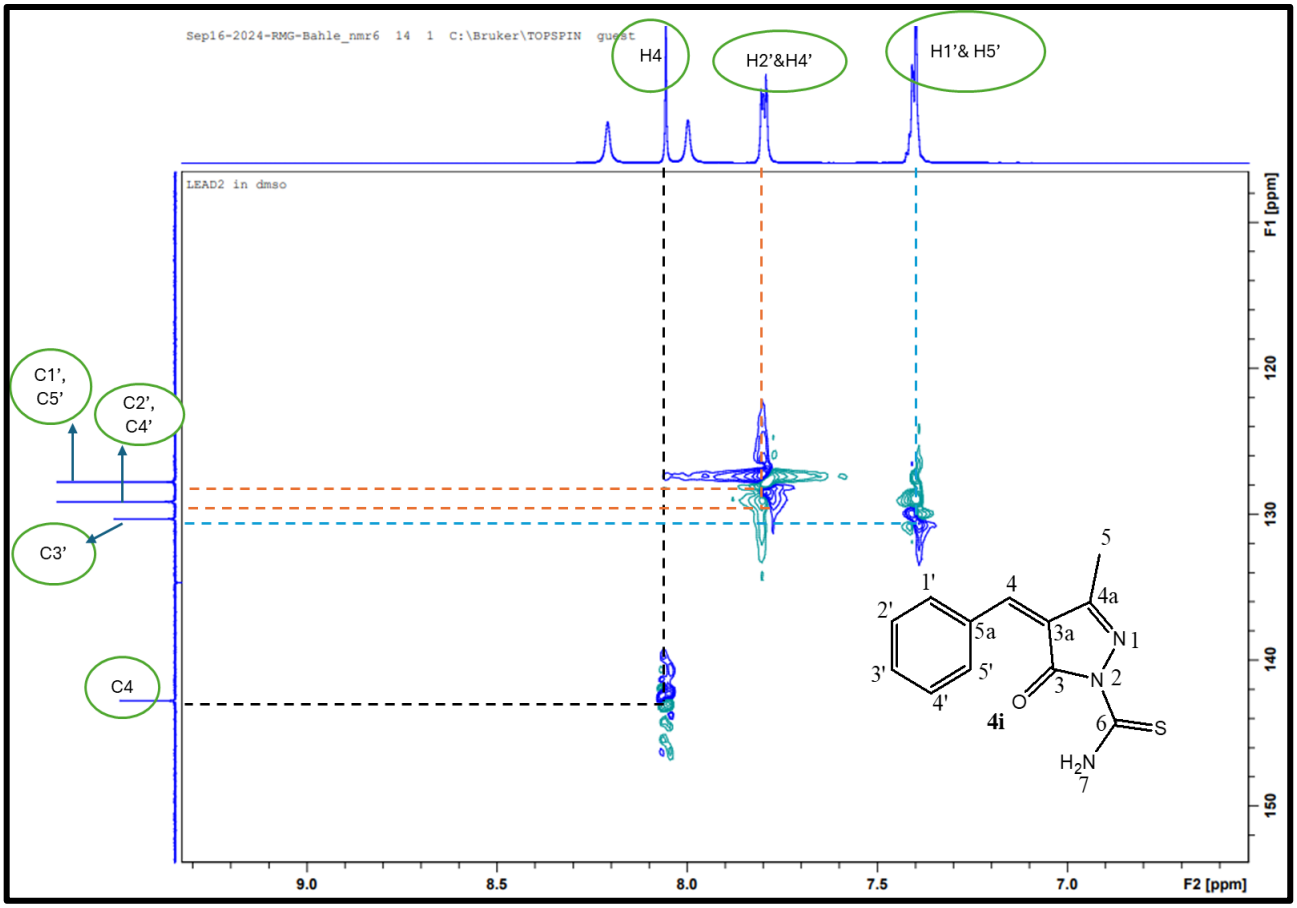
Appendix 4: ^{13}C NMR spectrum for compound **4i**



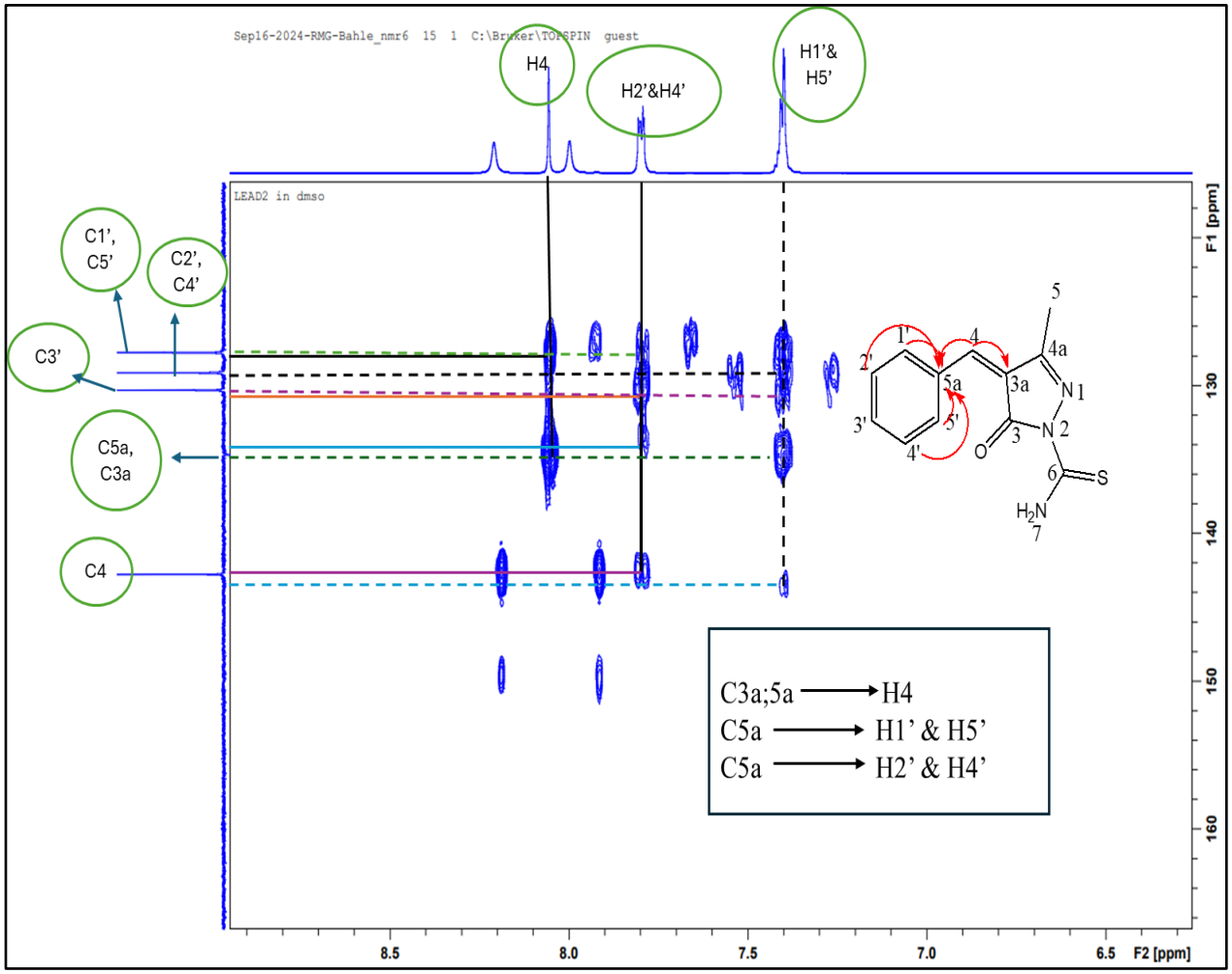
Appendix 5: APT of compound **4i**



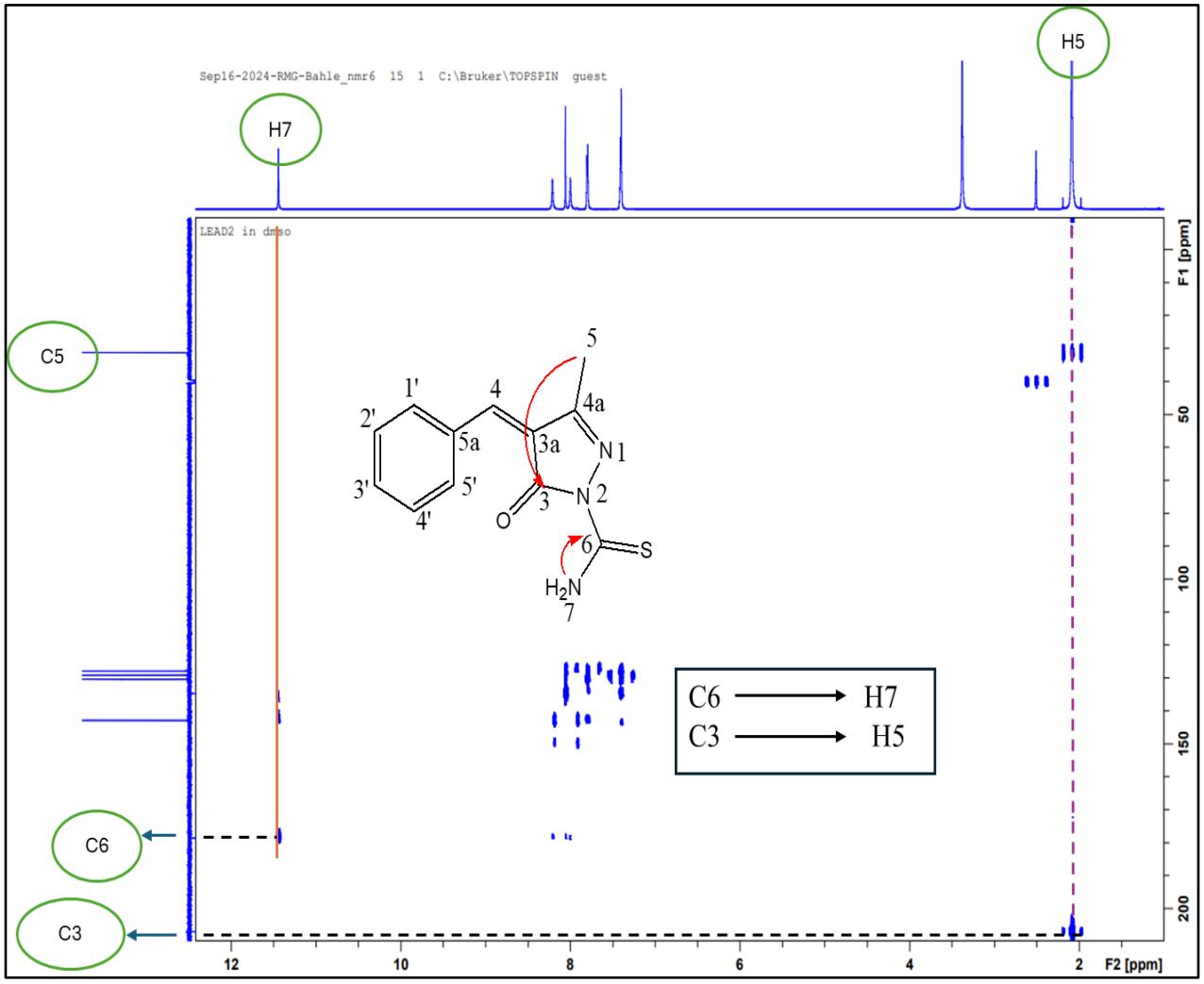
Appendix 6: Proton COSY of compound **4i**



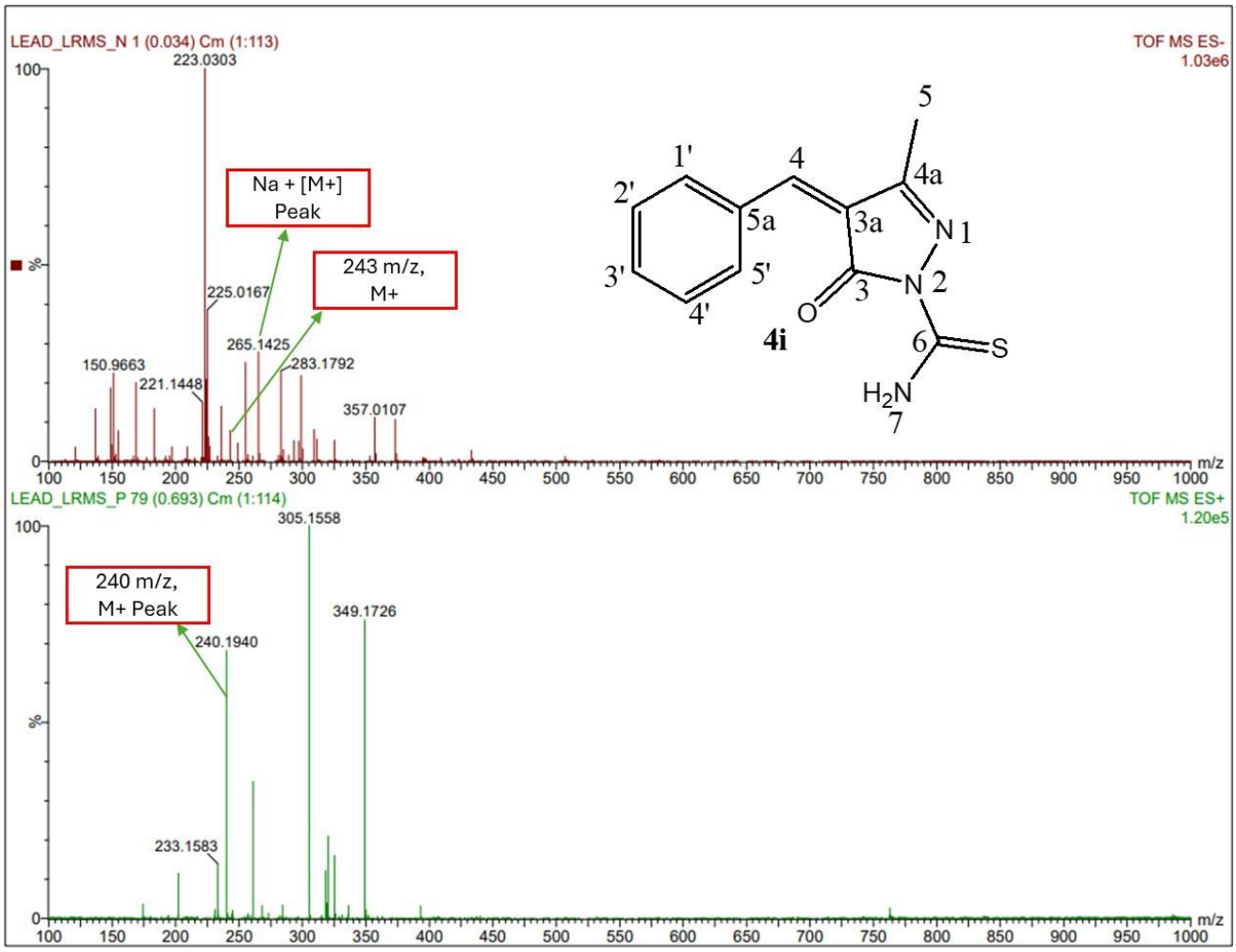
Appendix 7: Expanded HSQC diagram of compound **4i**



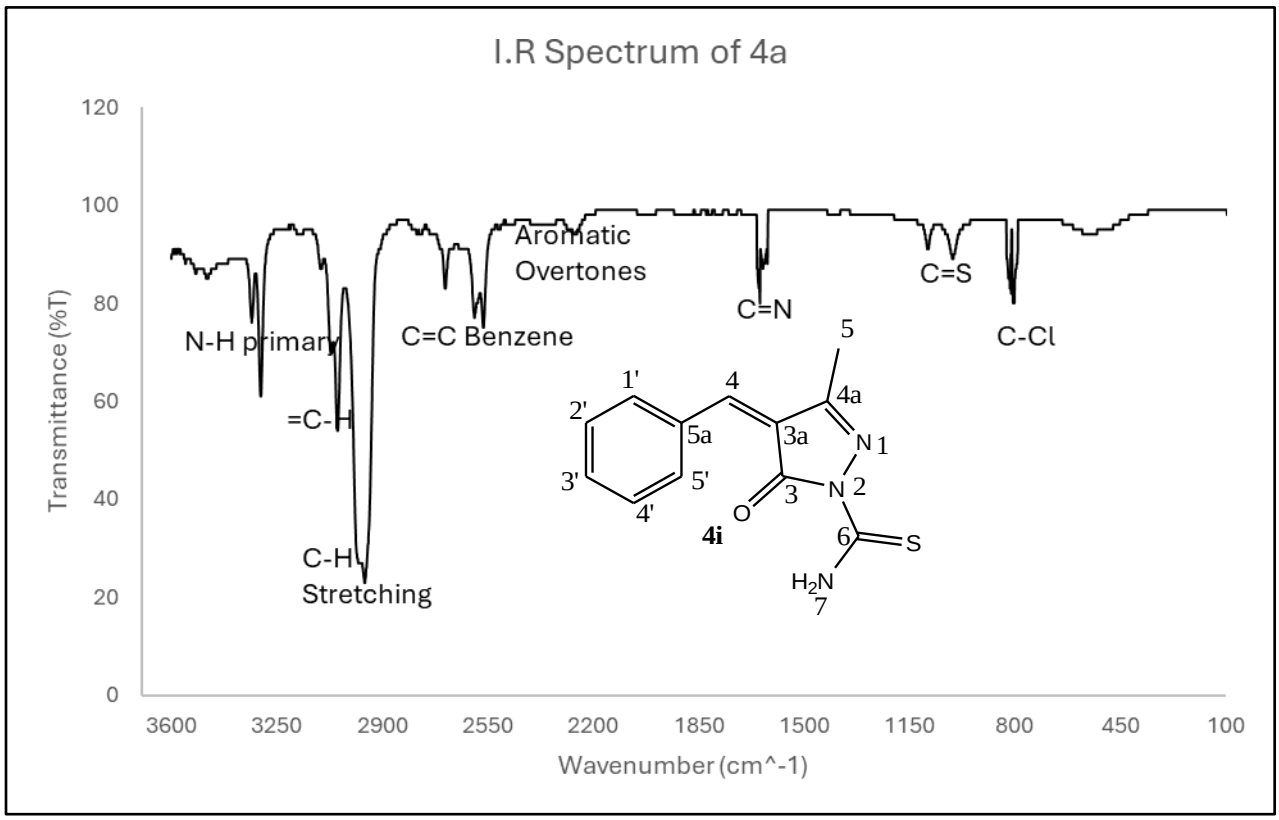
Appendix 8: Expanded HMBC diagram of compound **4i**



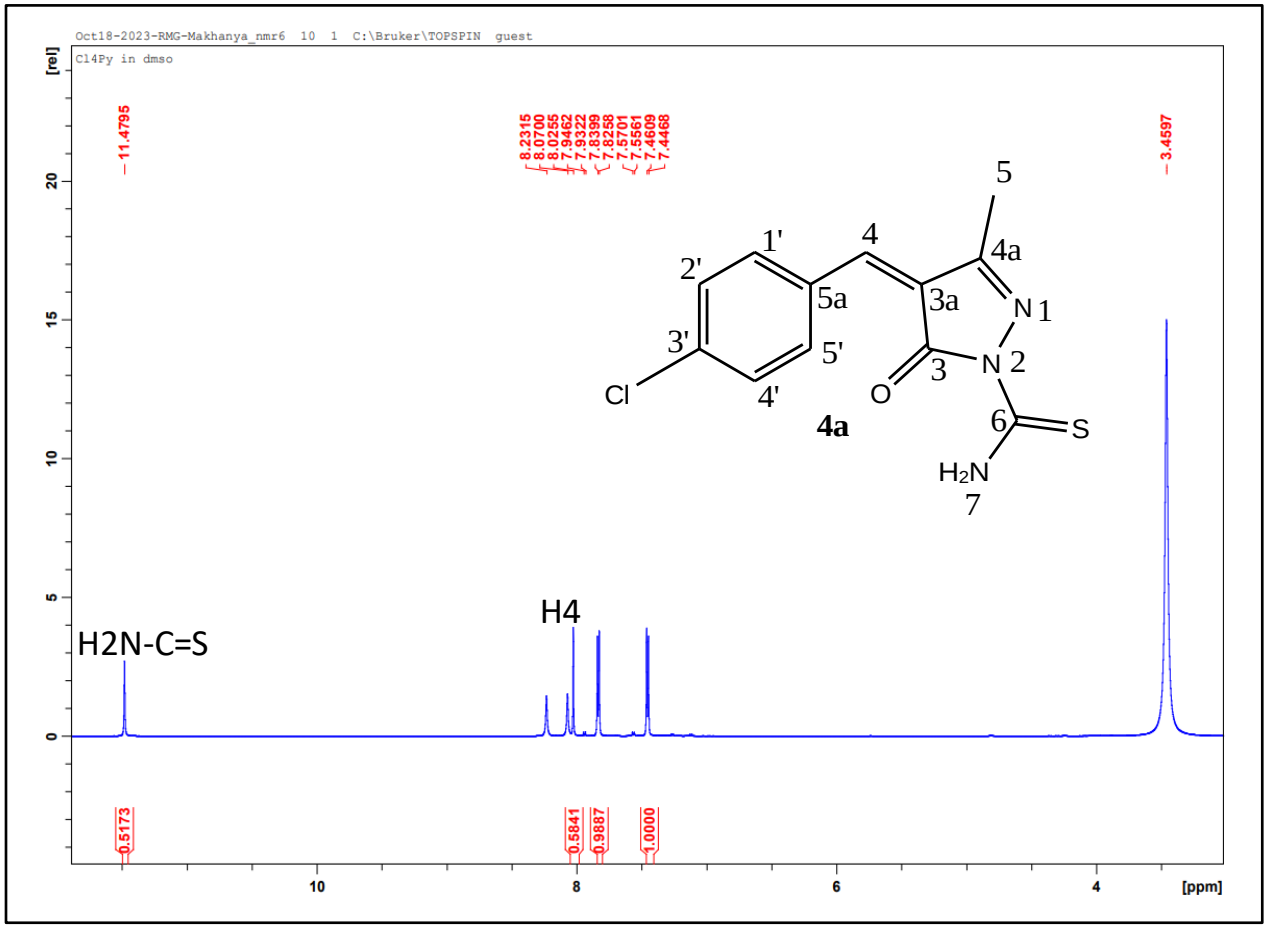
Appendix 9: HMBC diagram of compound **4i**



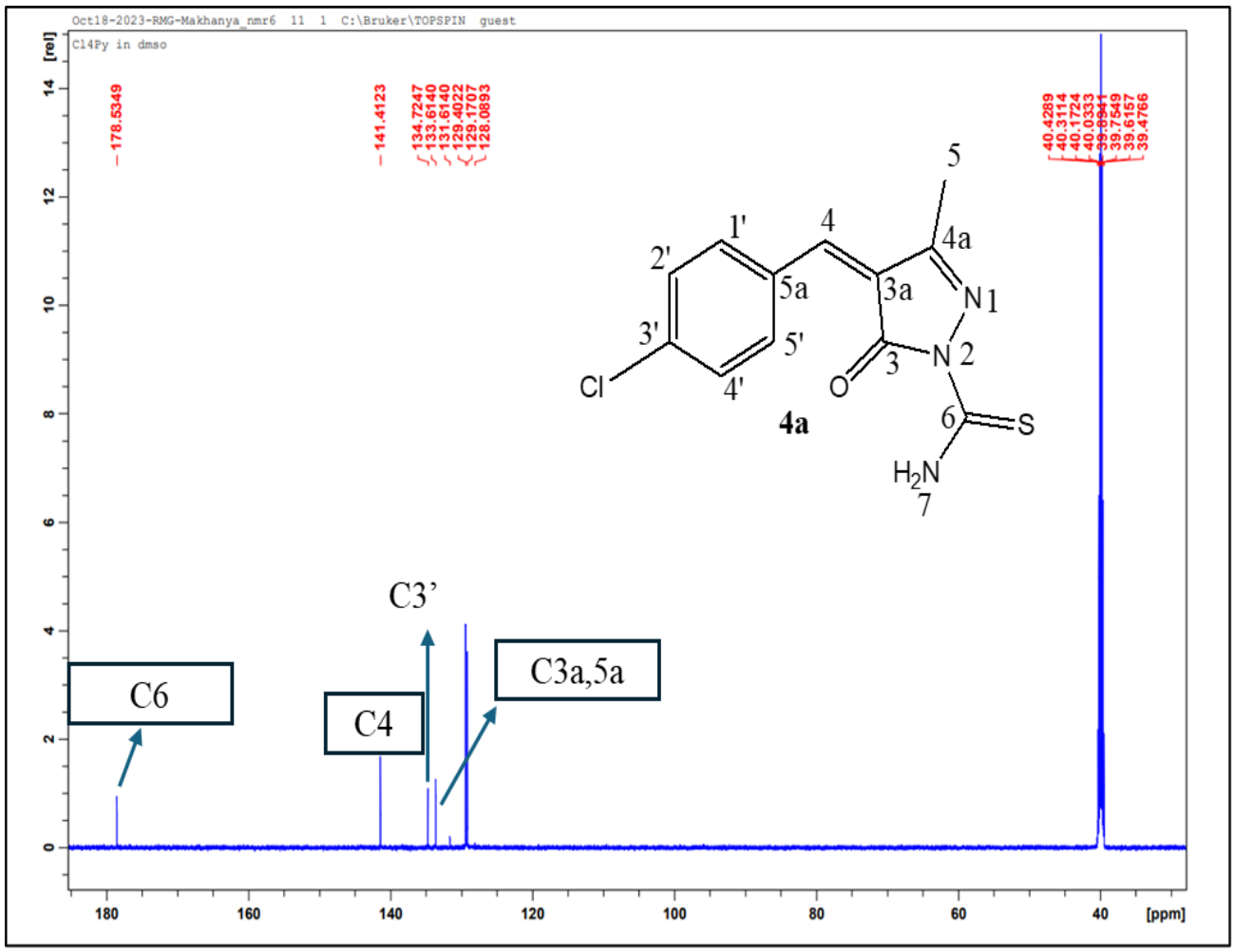
Appendix 10: Time of Flight-Mass Spectrometry for compound **4i**



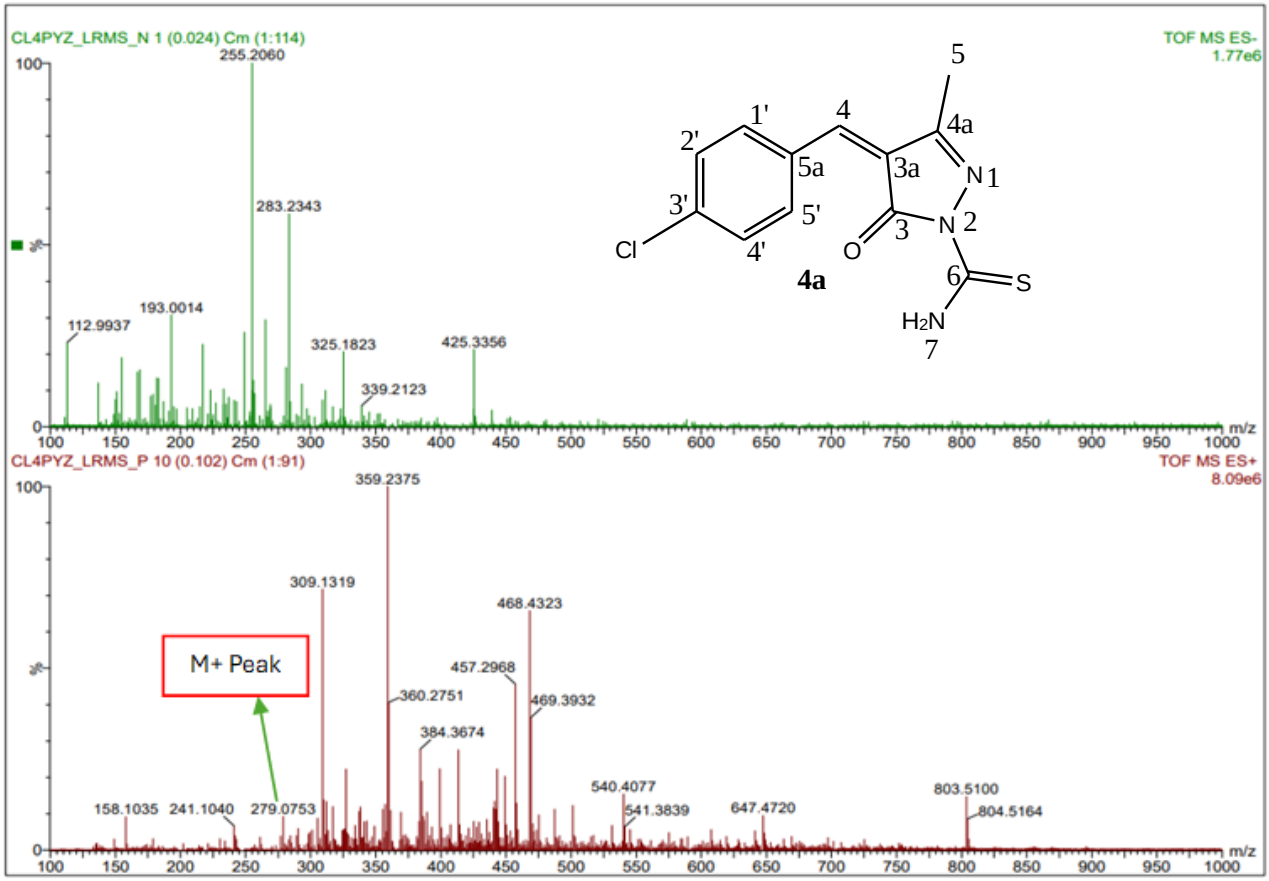
Appendix 11: I.R spectrum of **4a**



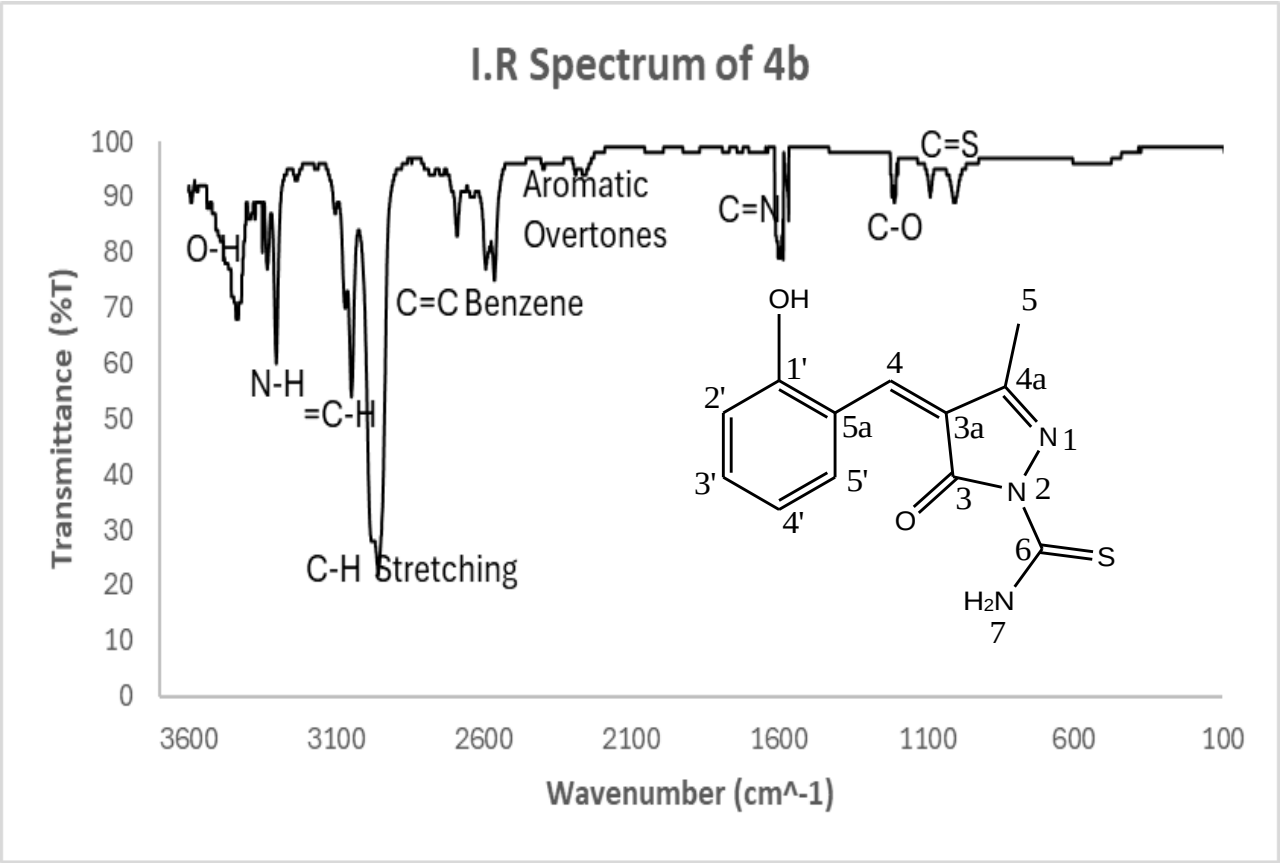
Appendix 12: ^{1}H NMR spectrum for compound **4a**



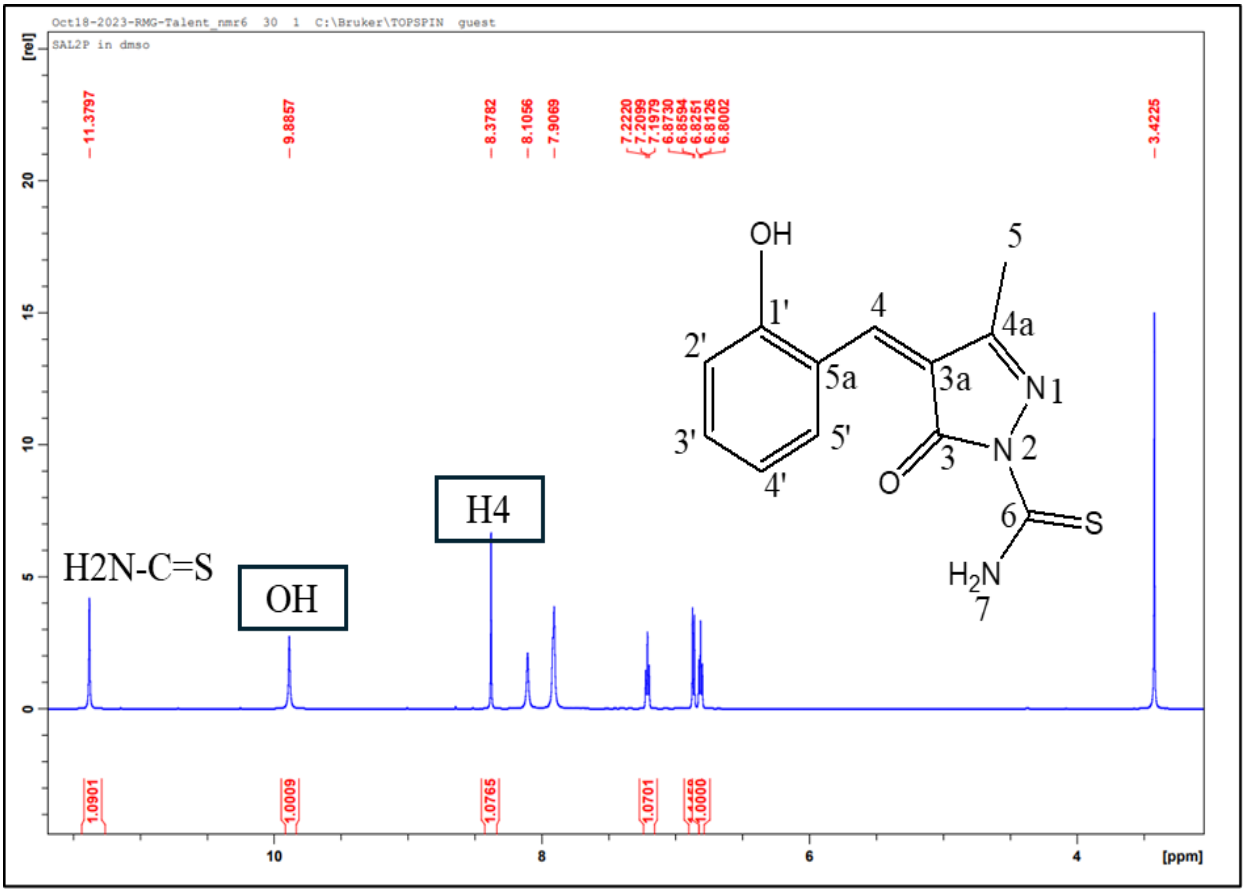
Appendix 13: ^{13}C NMR spectrum for compound **4a**



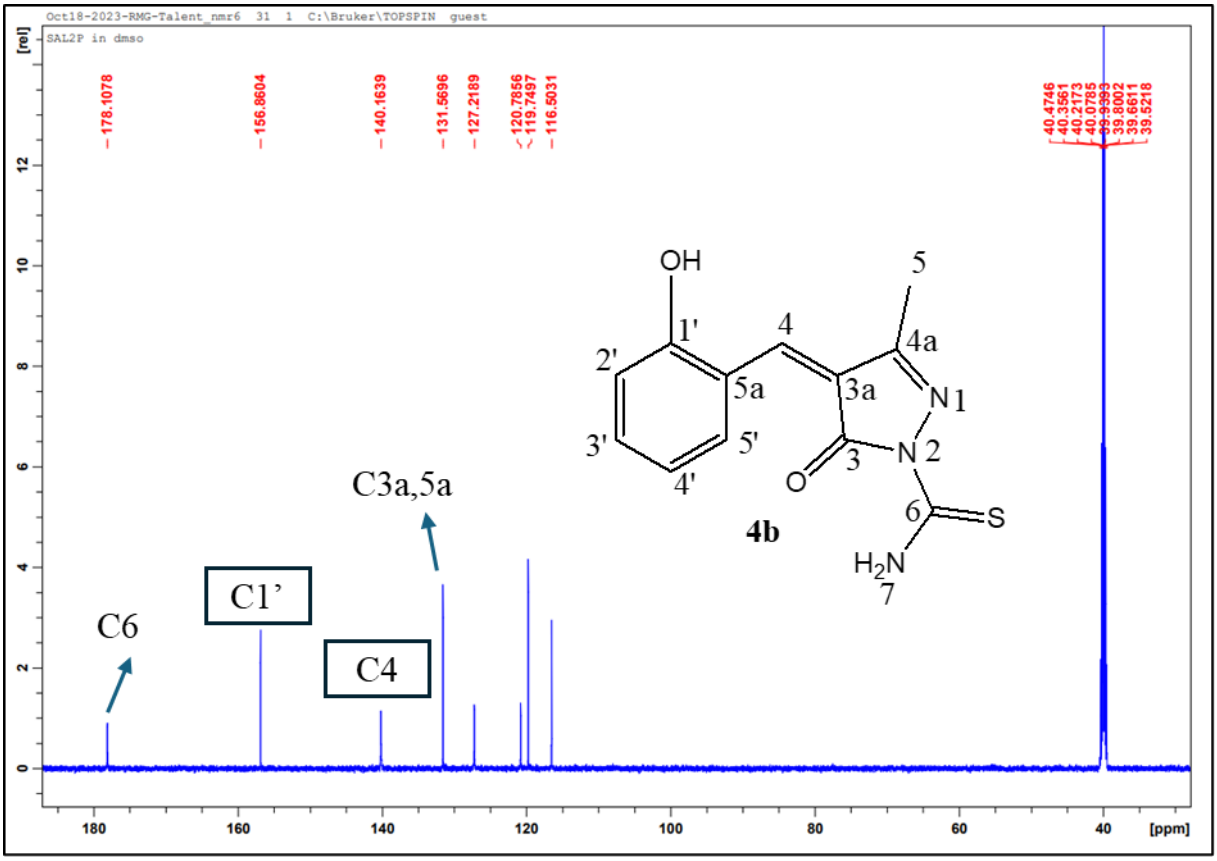
Appendix 14: Time of Flight-Mass Spectrometry of compound **4a**



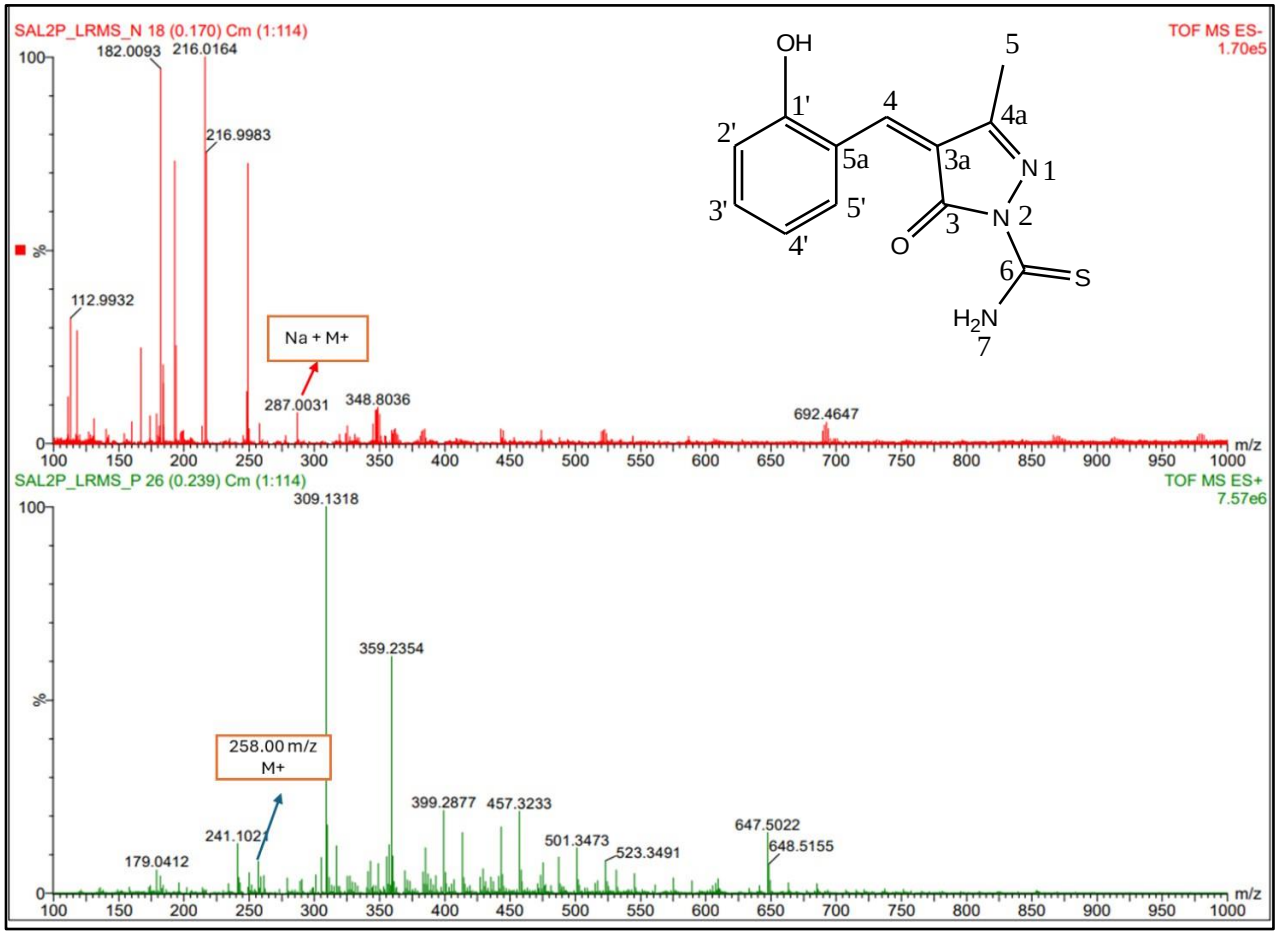
Appendix 15: I.R spectrum of **4b**



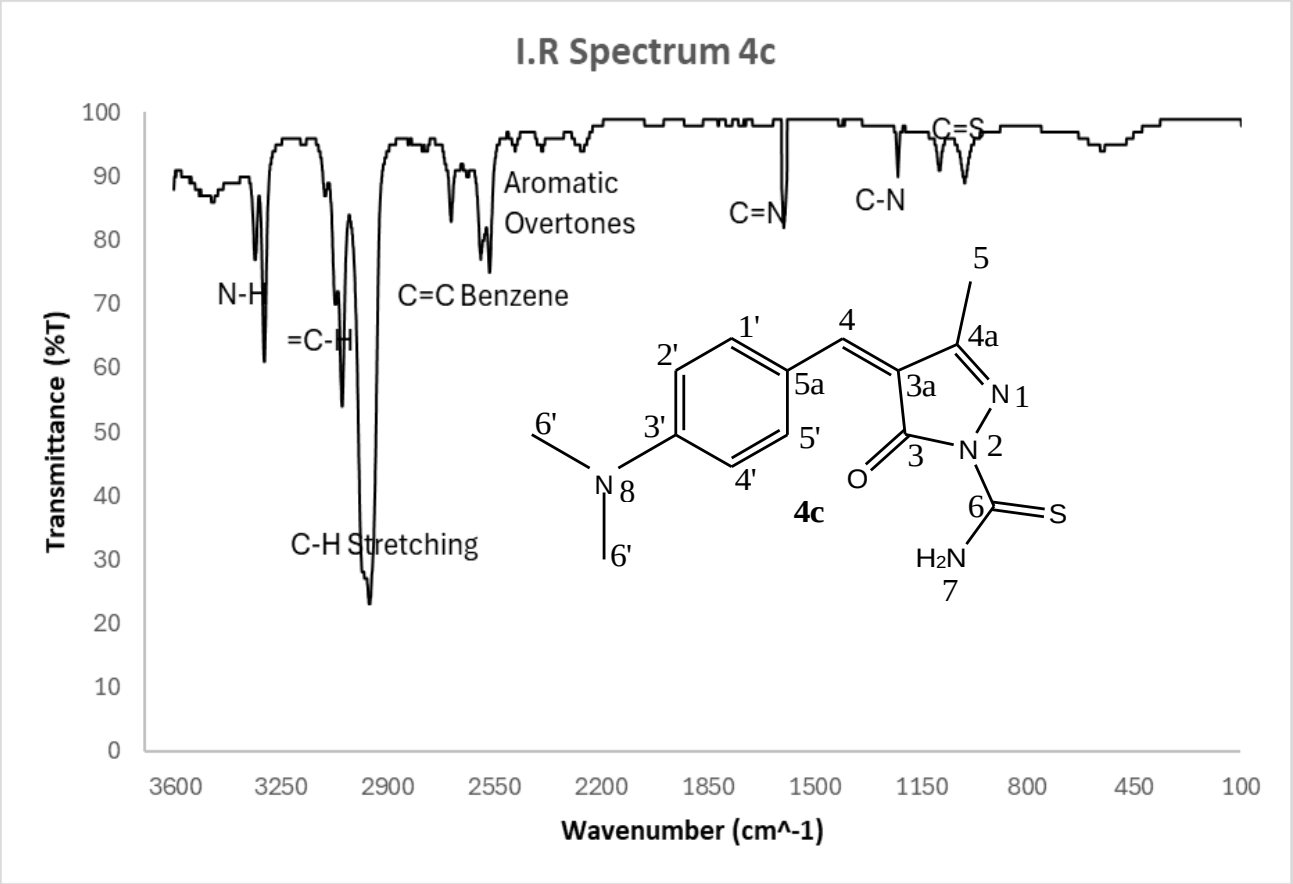
Appendix 16: ^{1}H NMR spectrum for compound **4b**



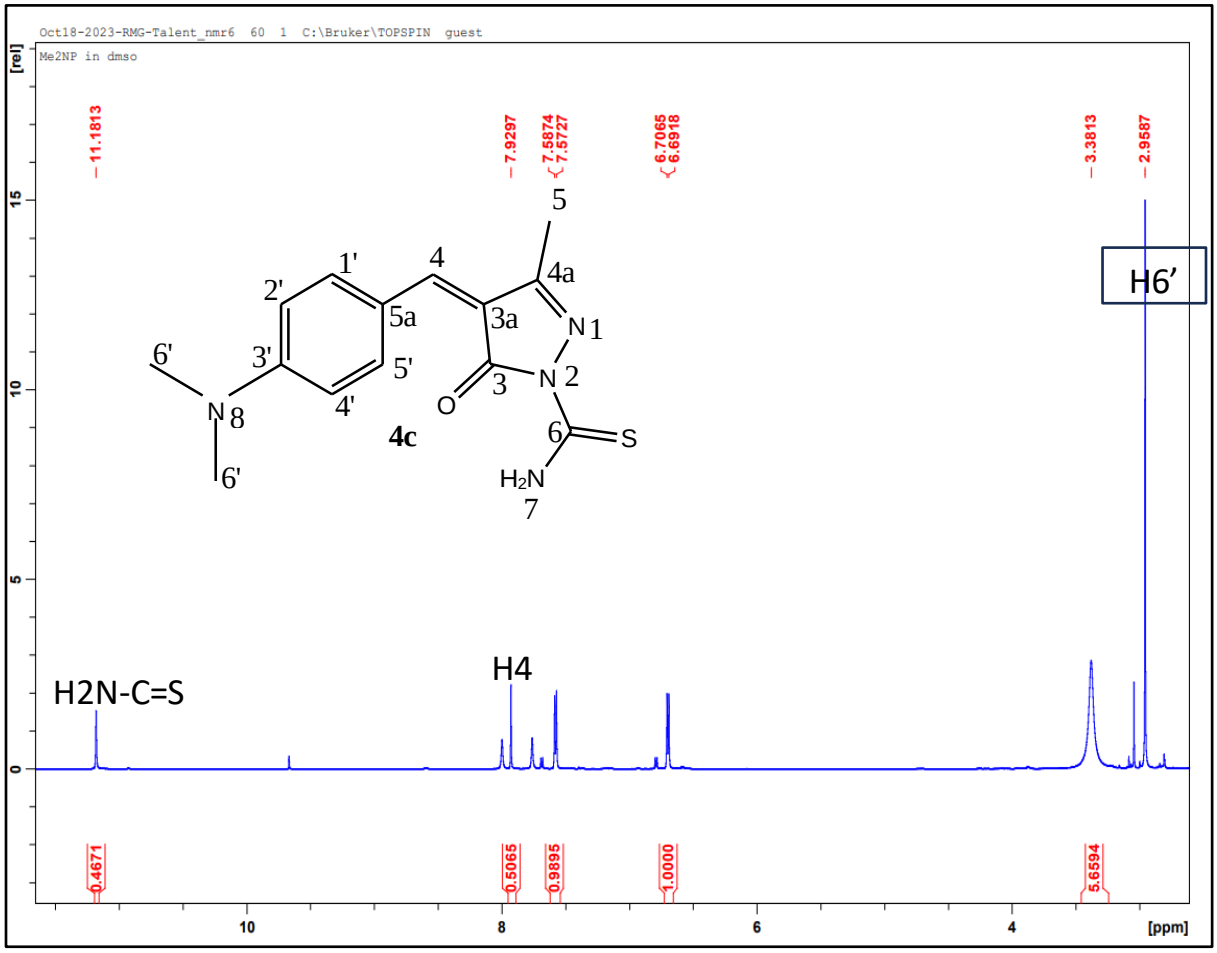
Appendix 17: ^{13}C NMR spectrum for compound **4b**



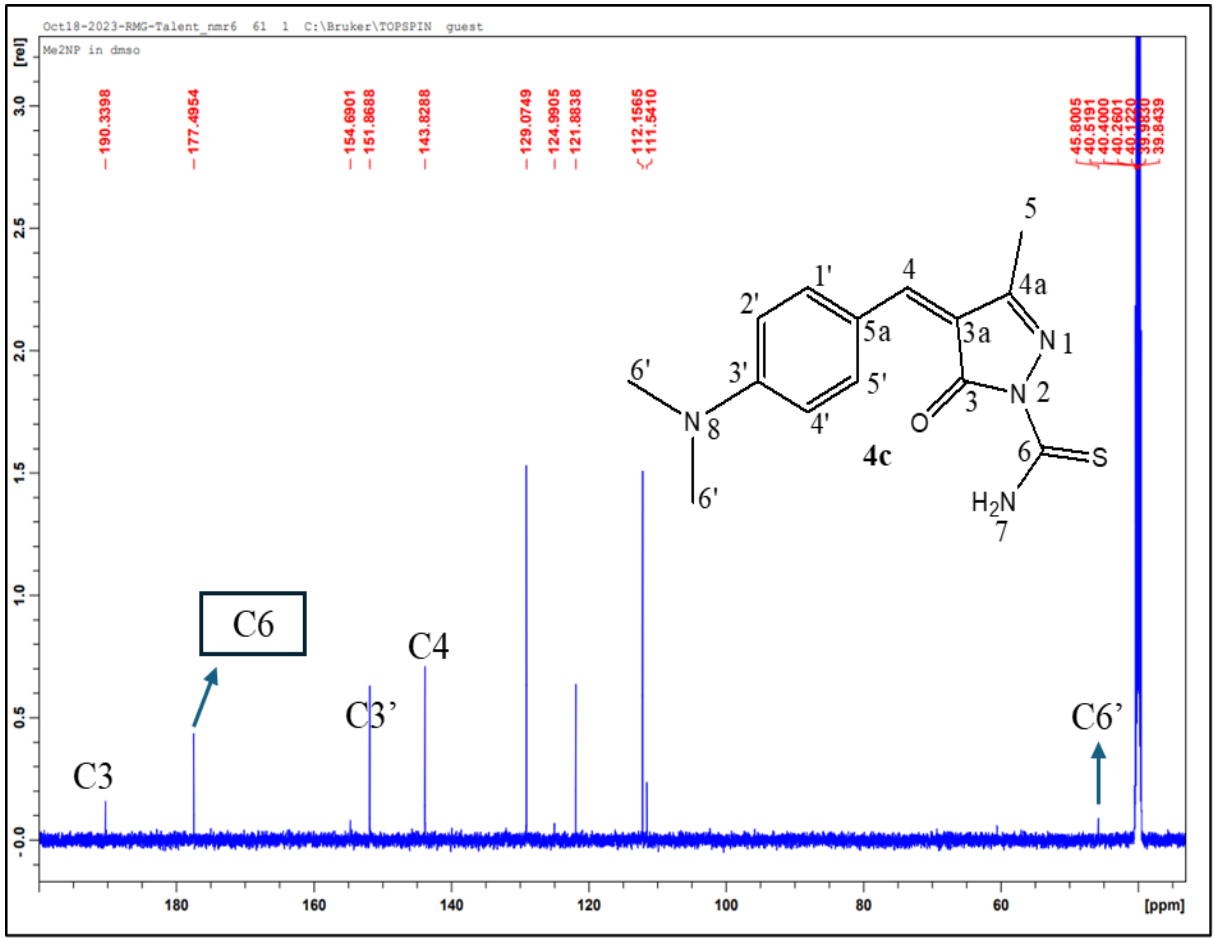
Appendix 18: Time of Flight-Mass Spectrometry of compound **4b**



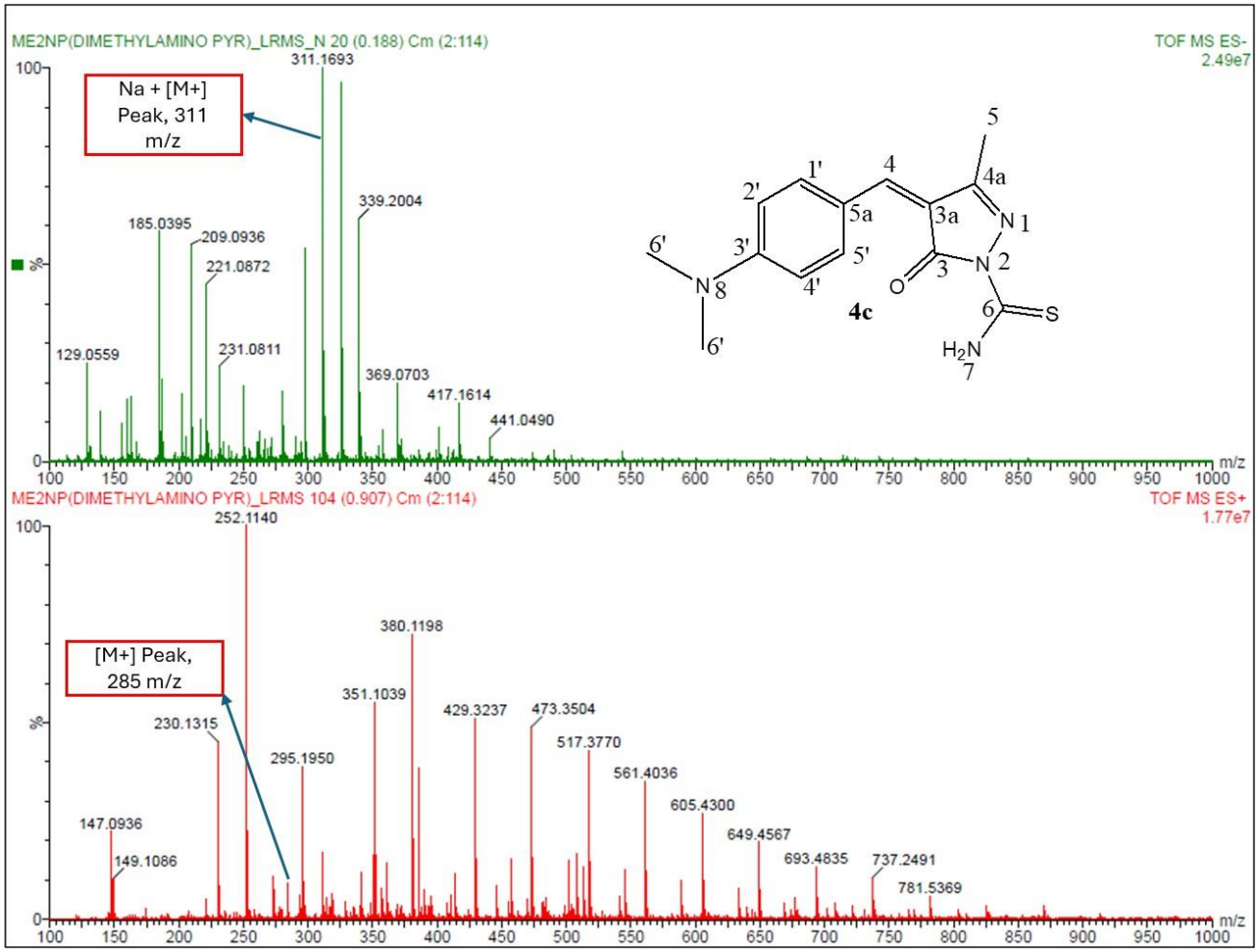
Appendix 19: I.R Spectrum of **4c**



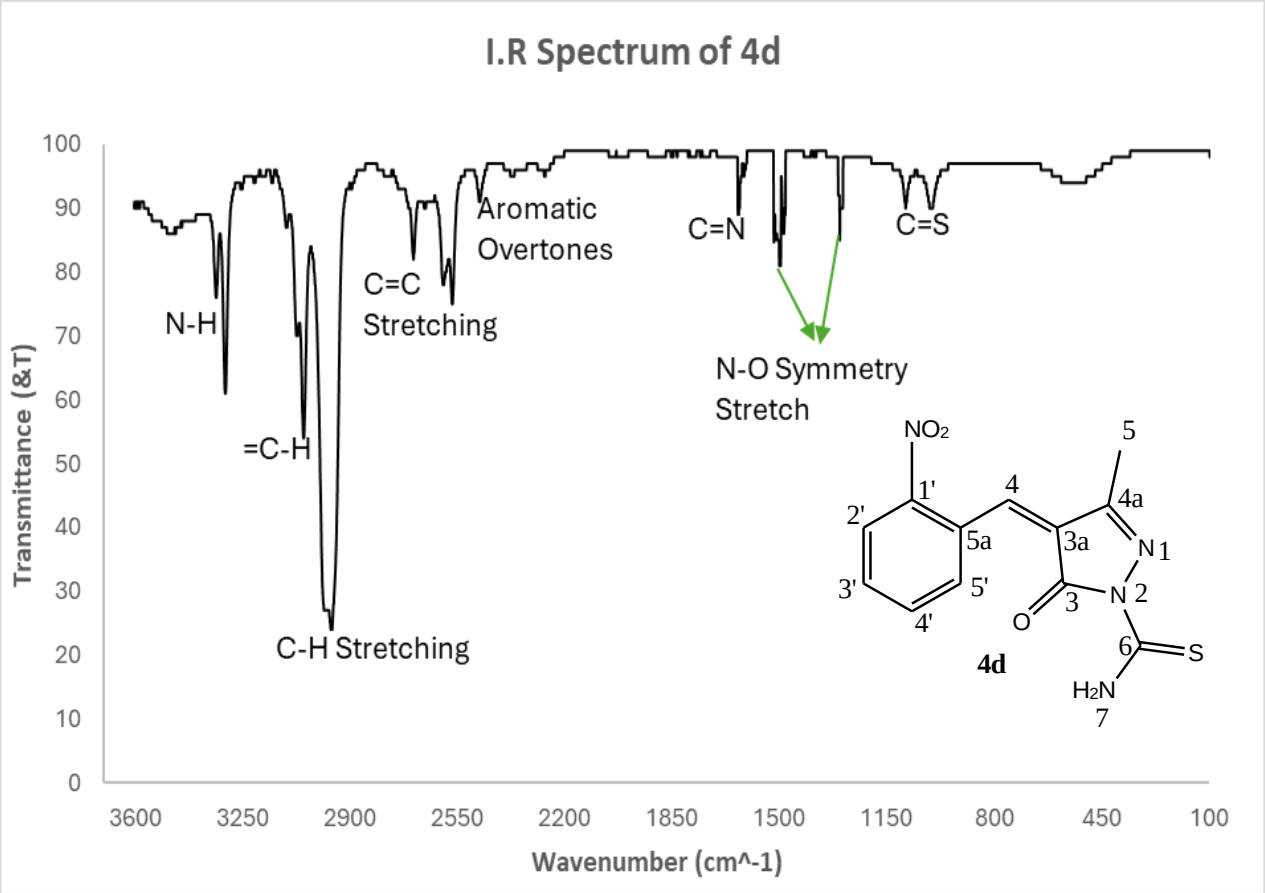
Appendix 20: ^{1}H NMR spectrum for compound **4c**



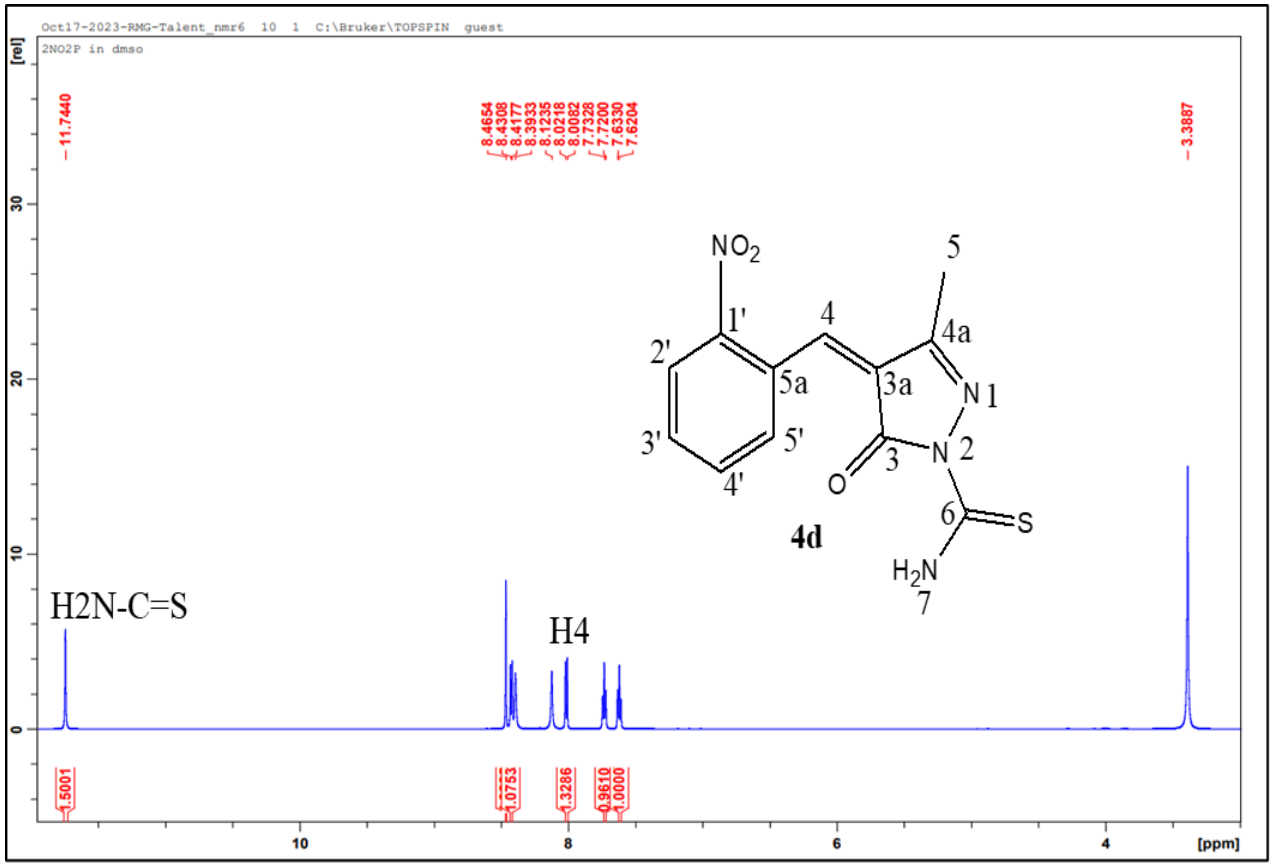
Appendix 21: ^{13}C NMR spectrum for compound **4c**



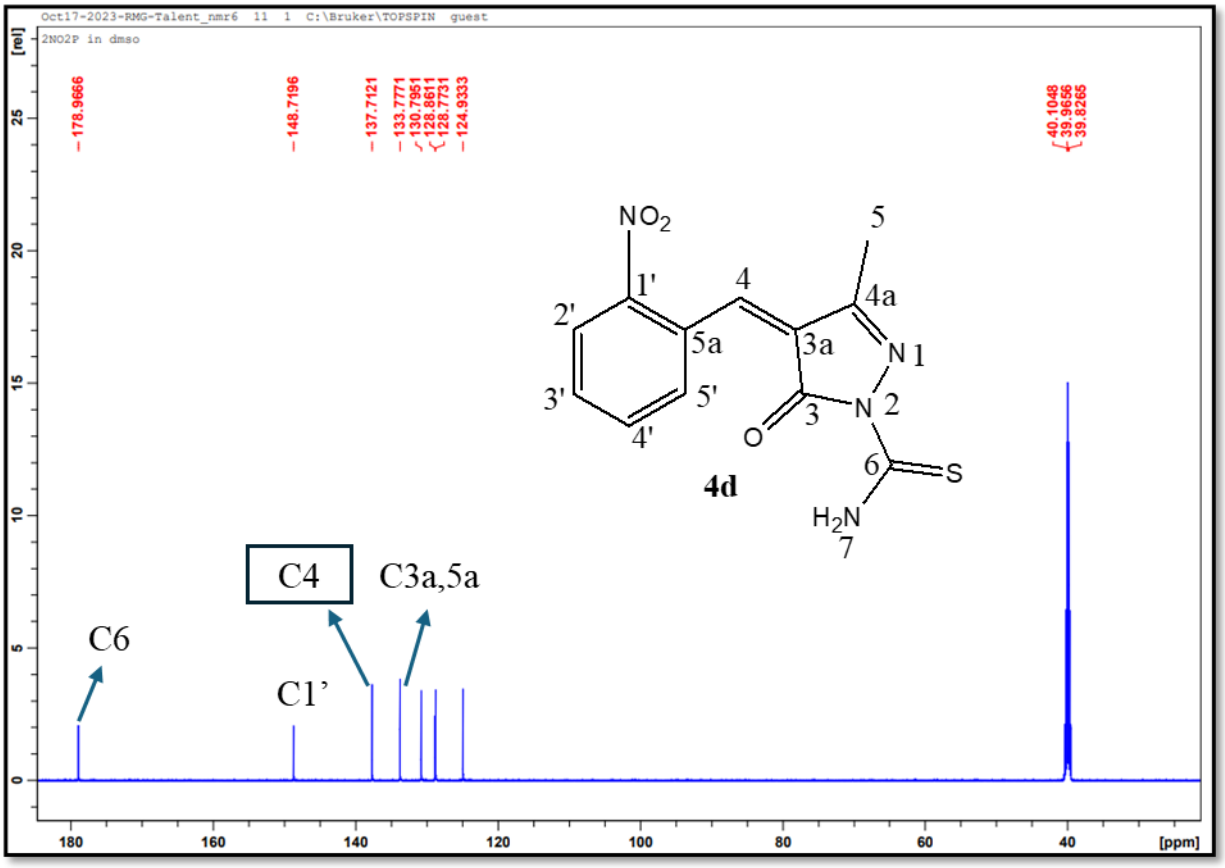
Appendix 22: Time of Flight-Mass Spectrometry of compound **4c**



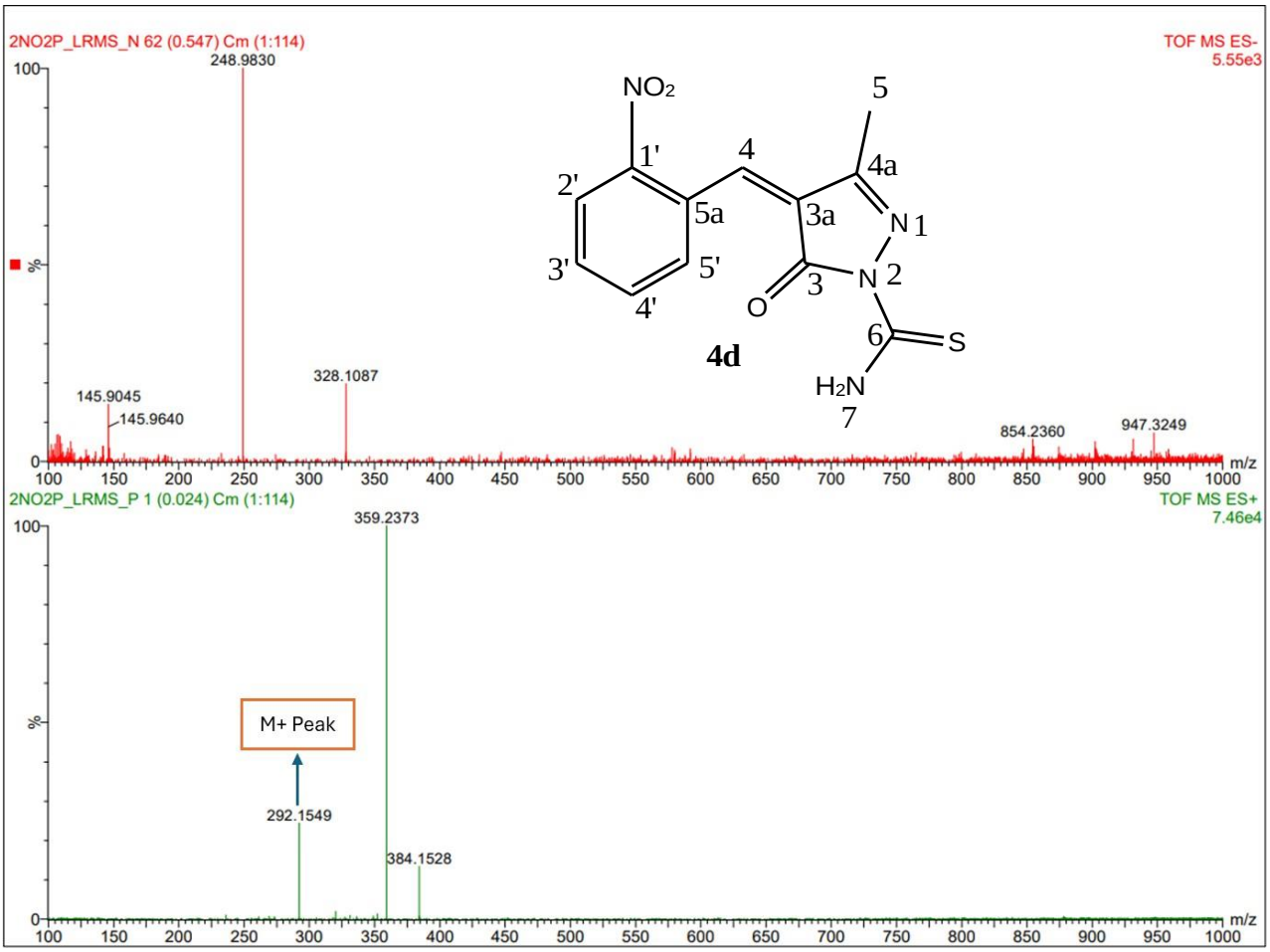
Appendix 23: I.R spectrum of **4d**



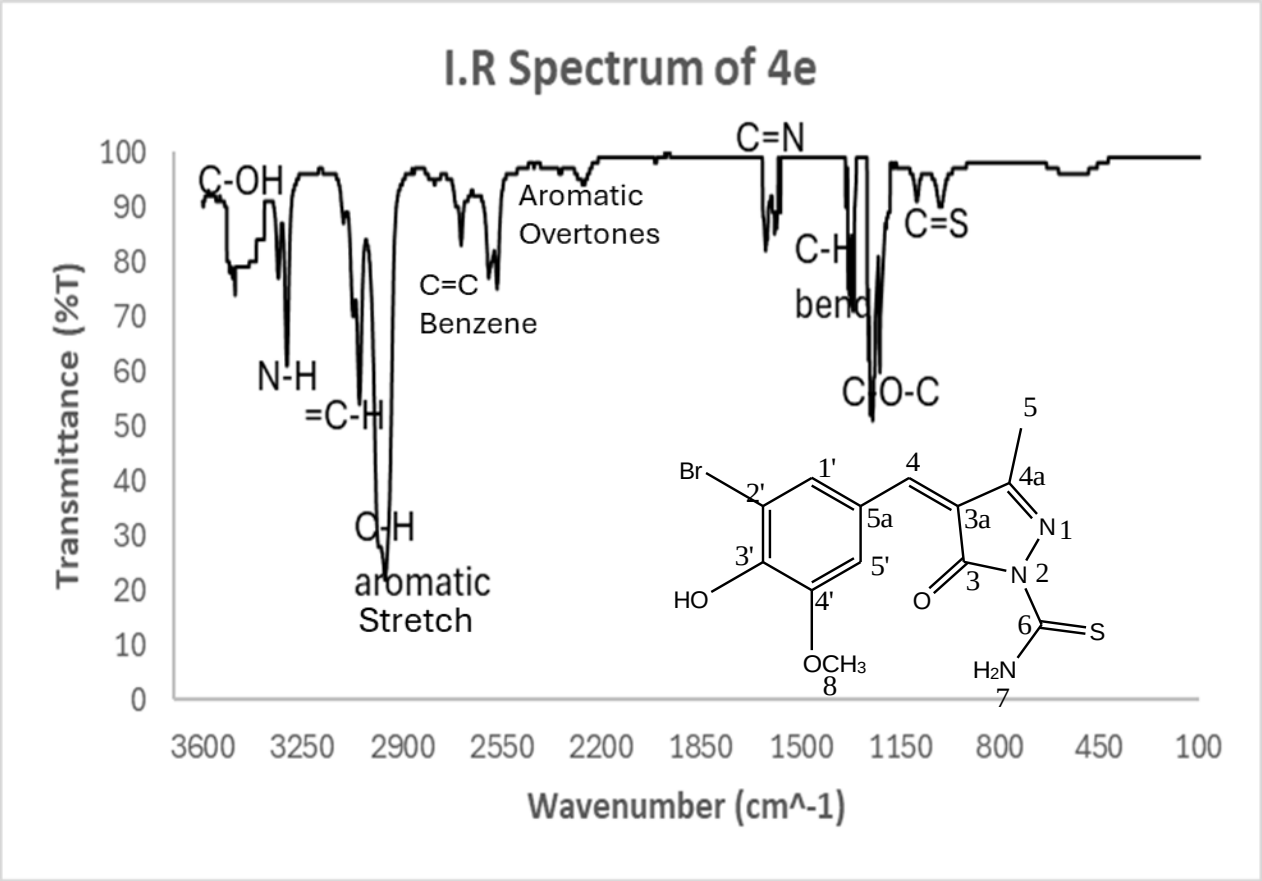
Appendix 24: ^{1}H NMR spectrum for compound **4d**



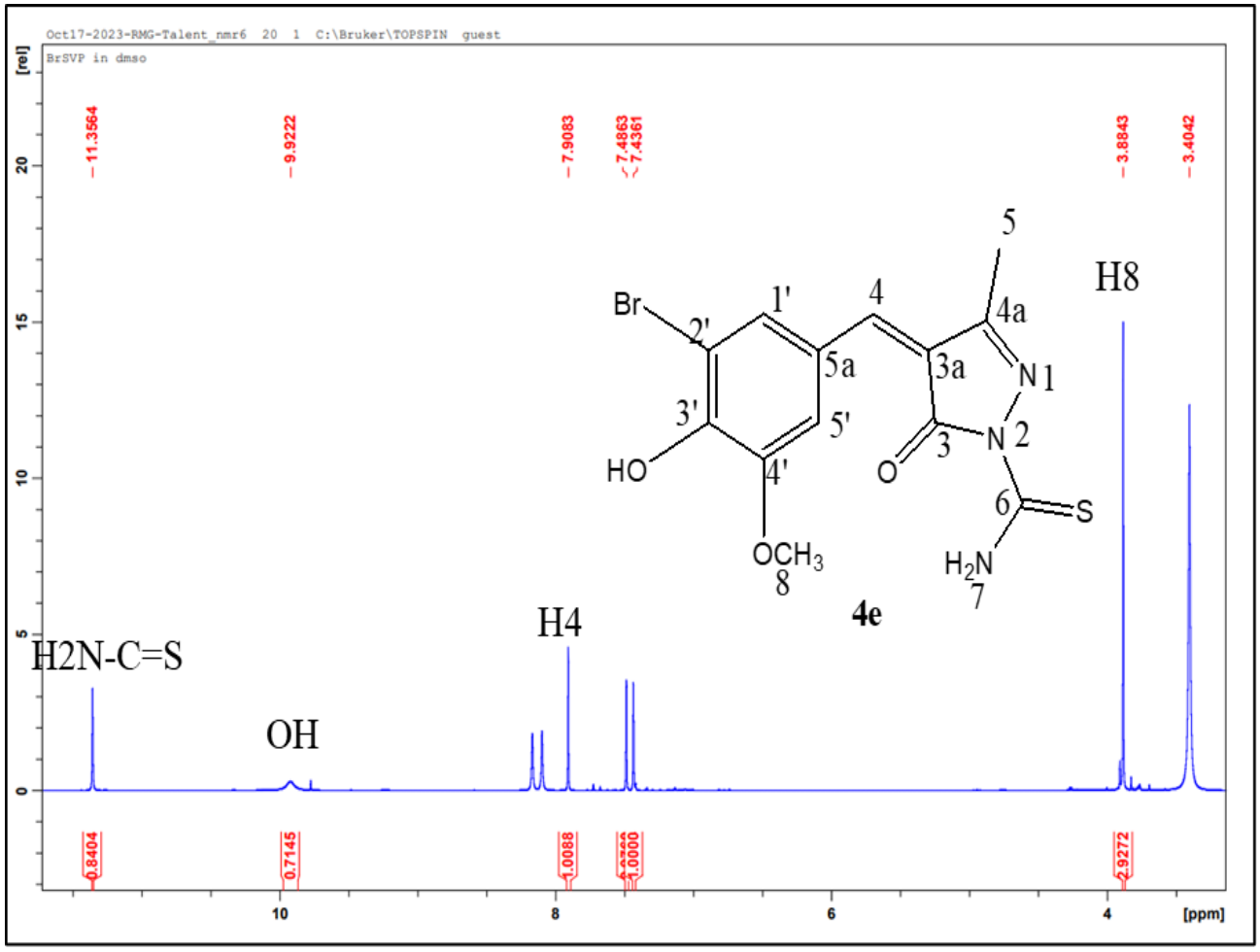
Appendix 25: ^{13}C NMR spectrum for compound **4d**



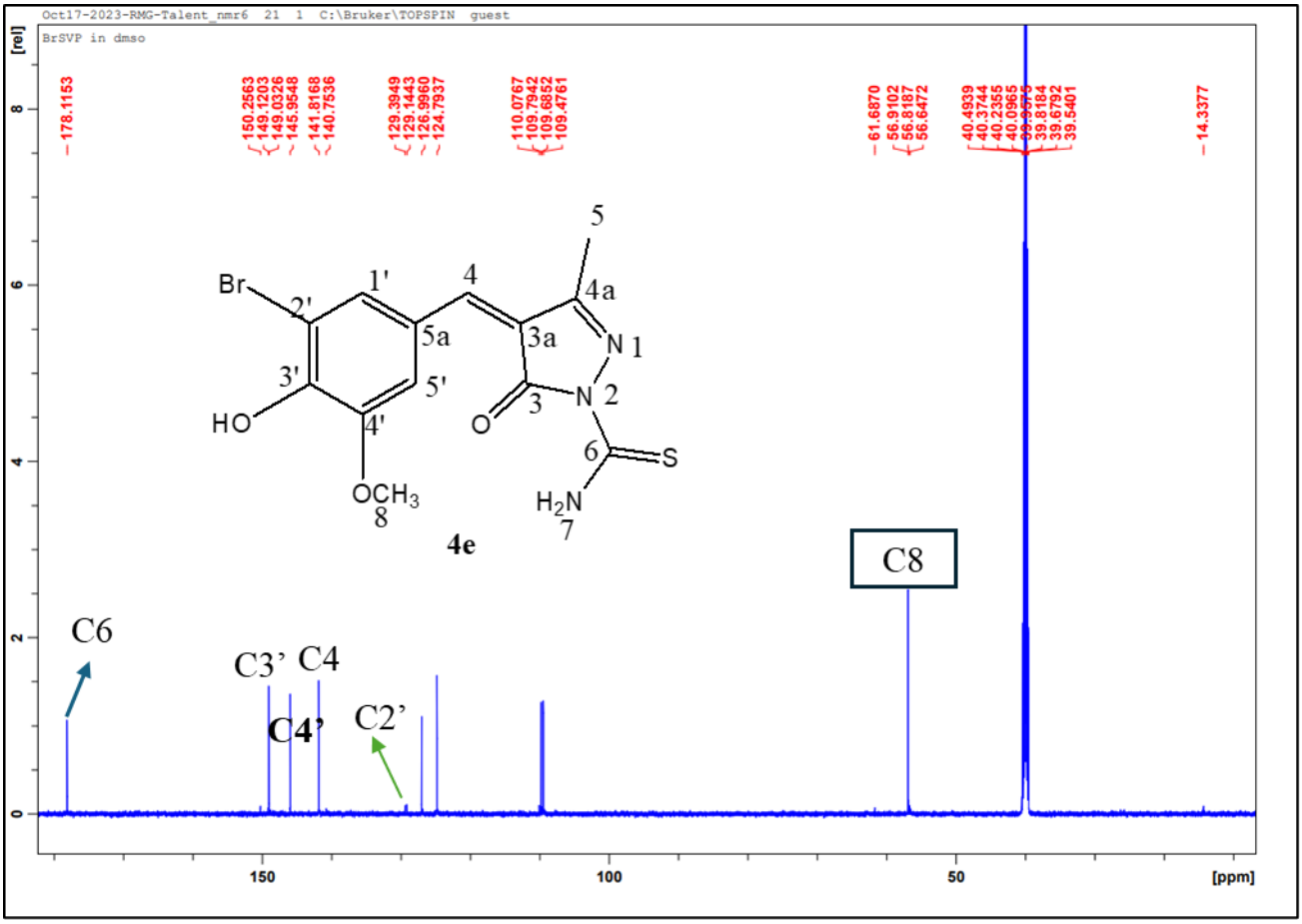
Appendix 26: Time of Flight-Mass Spectrometry for compound **4d**



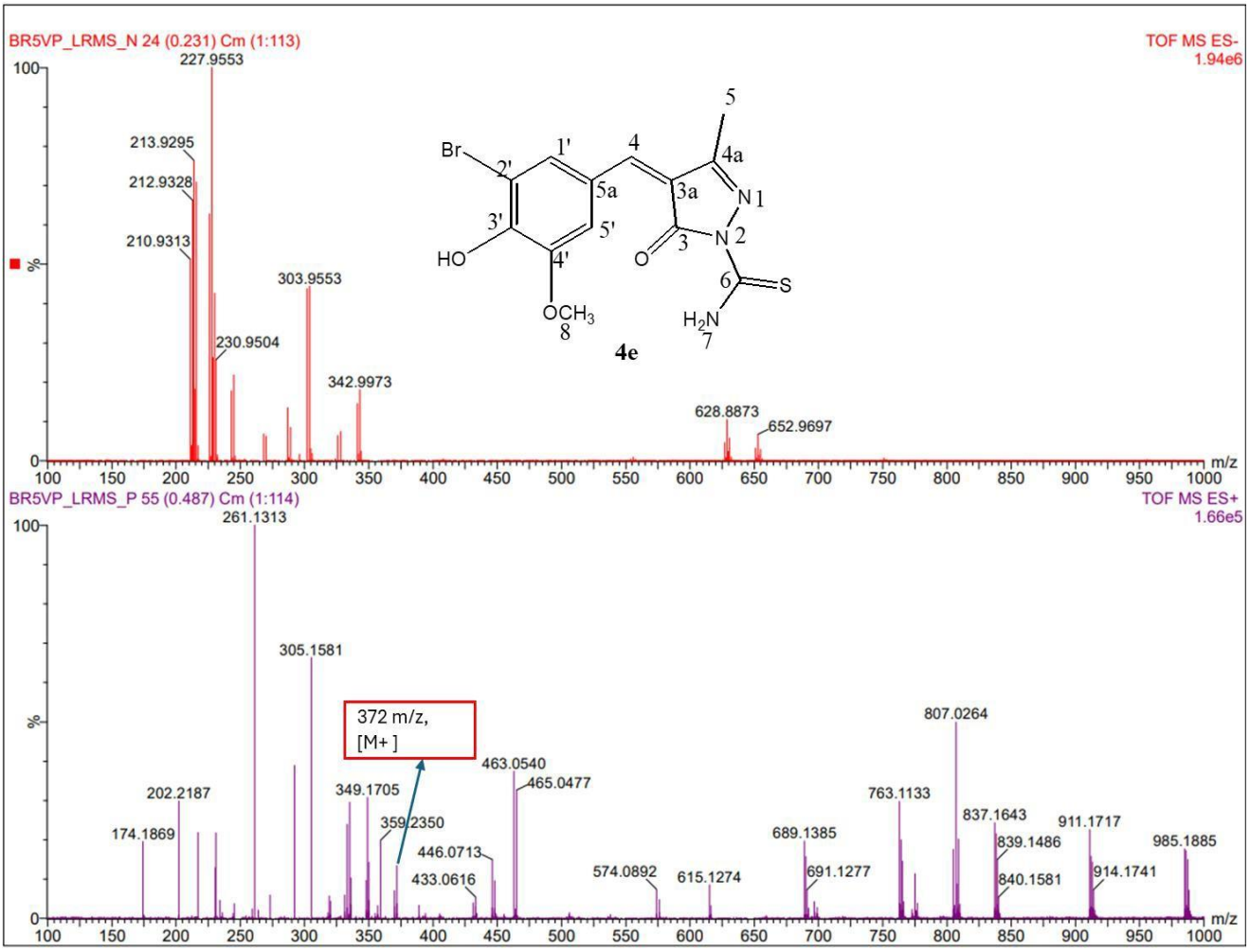
Appendix 27: I.R spectrum of **4e**



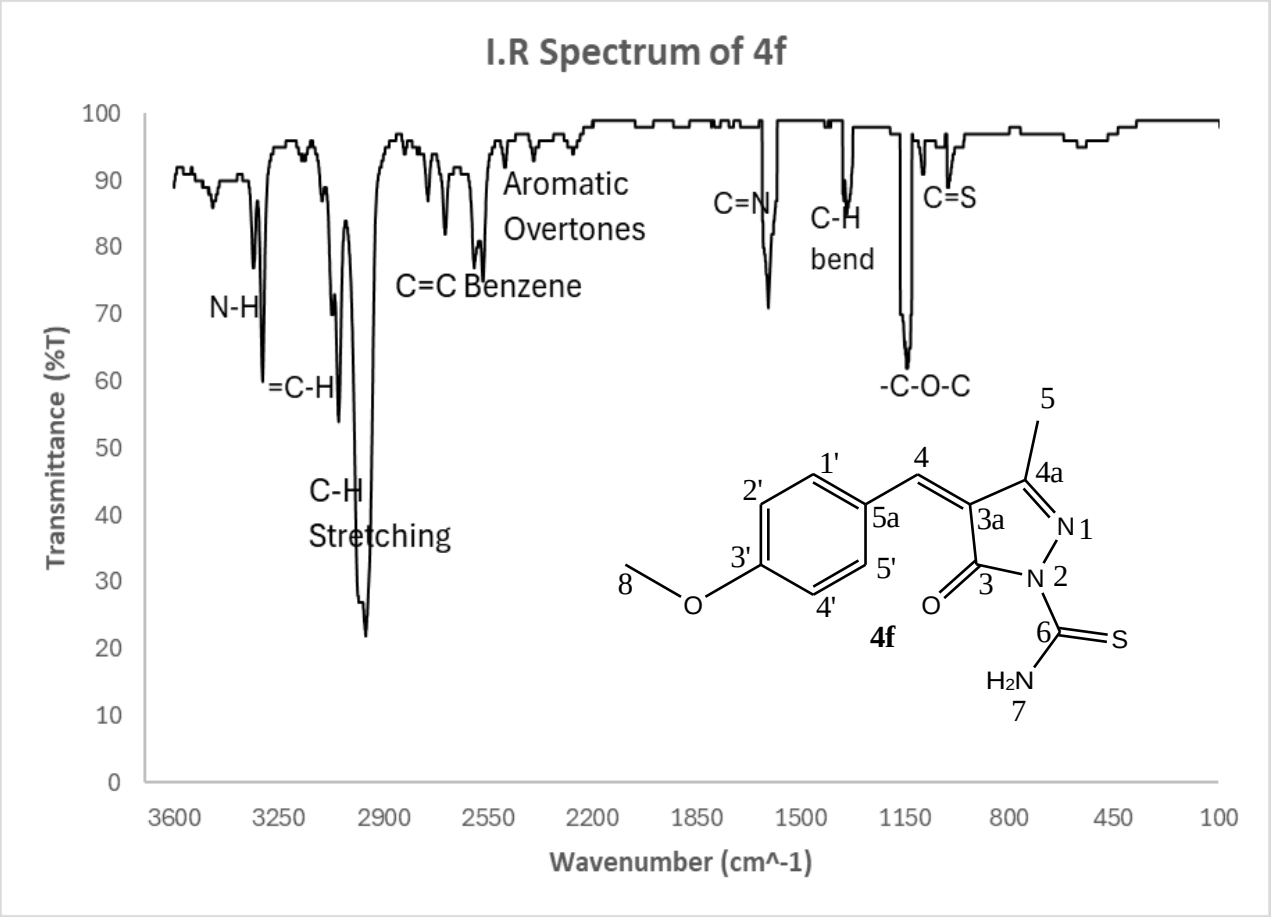
Appendix 28: ^{1}H NMR spectrum for compound **4e**



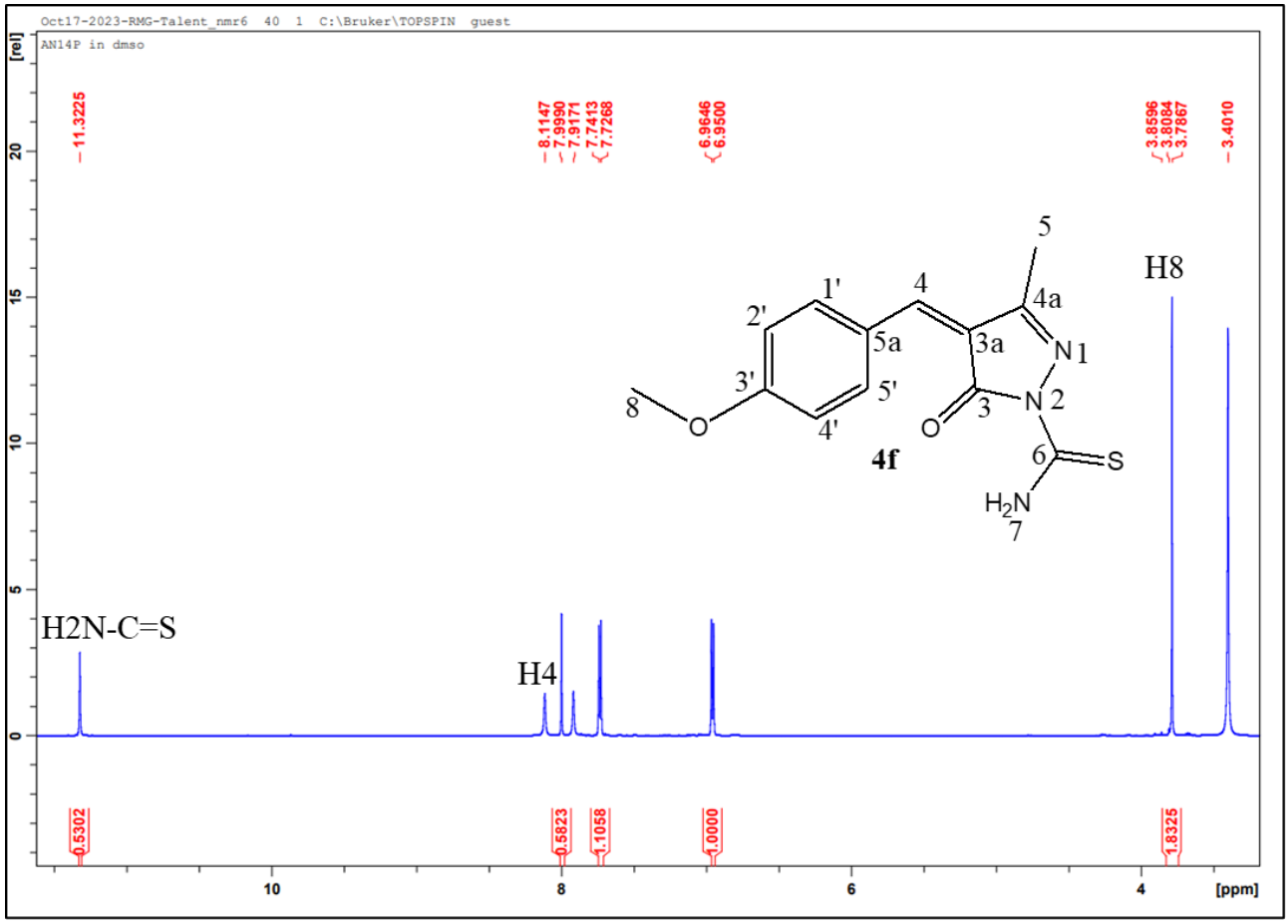
Appendix 29: ^{13}C NMR spectrum for compound **4e**



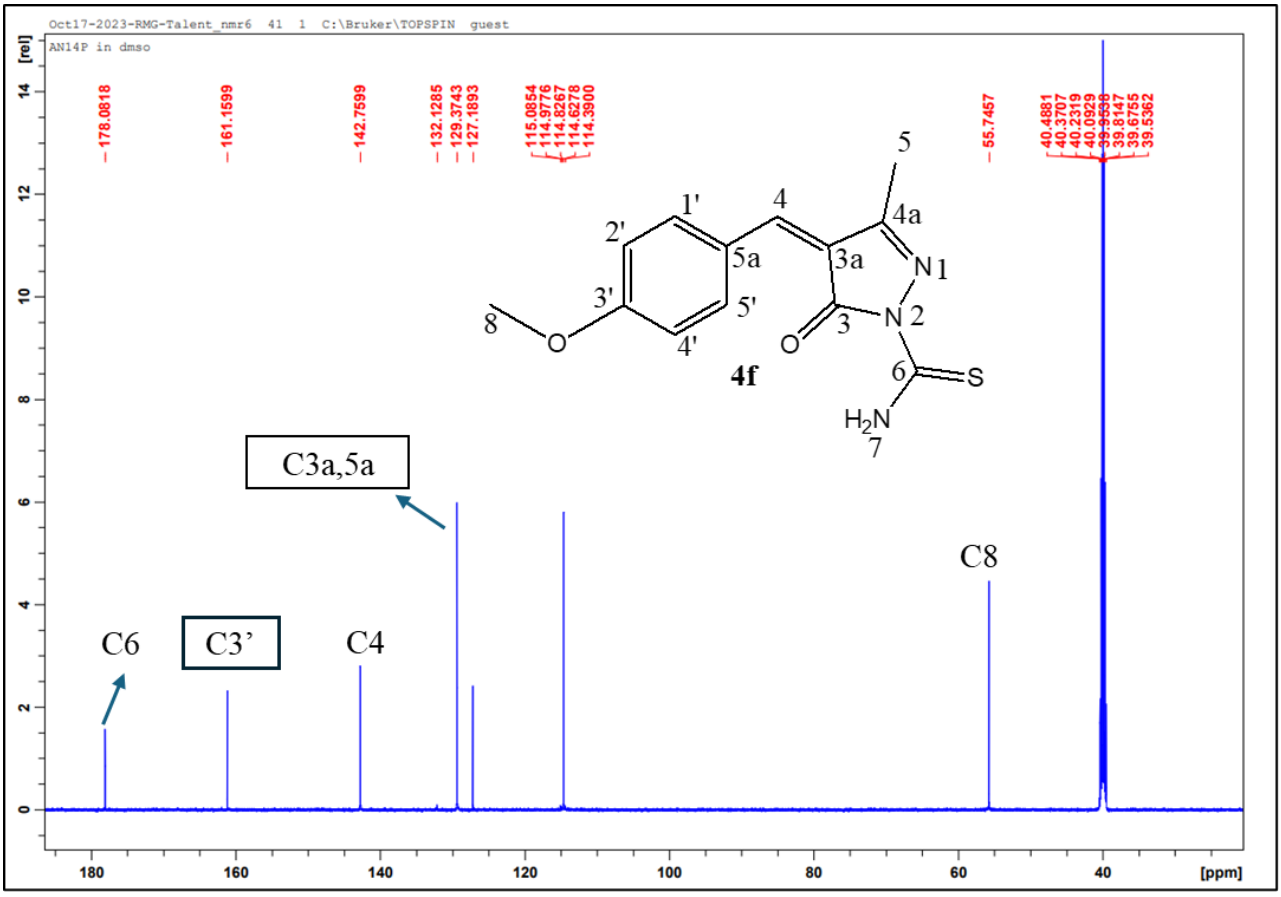
Appendix 30: Time of Flight-Mass Spectrometry for compound **4e**



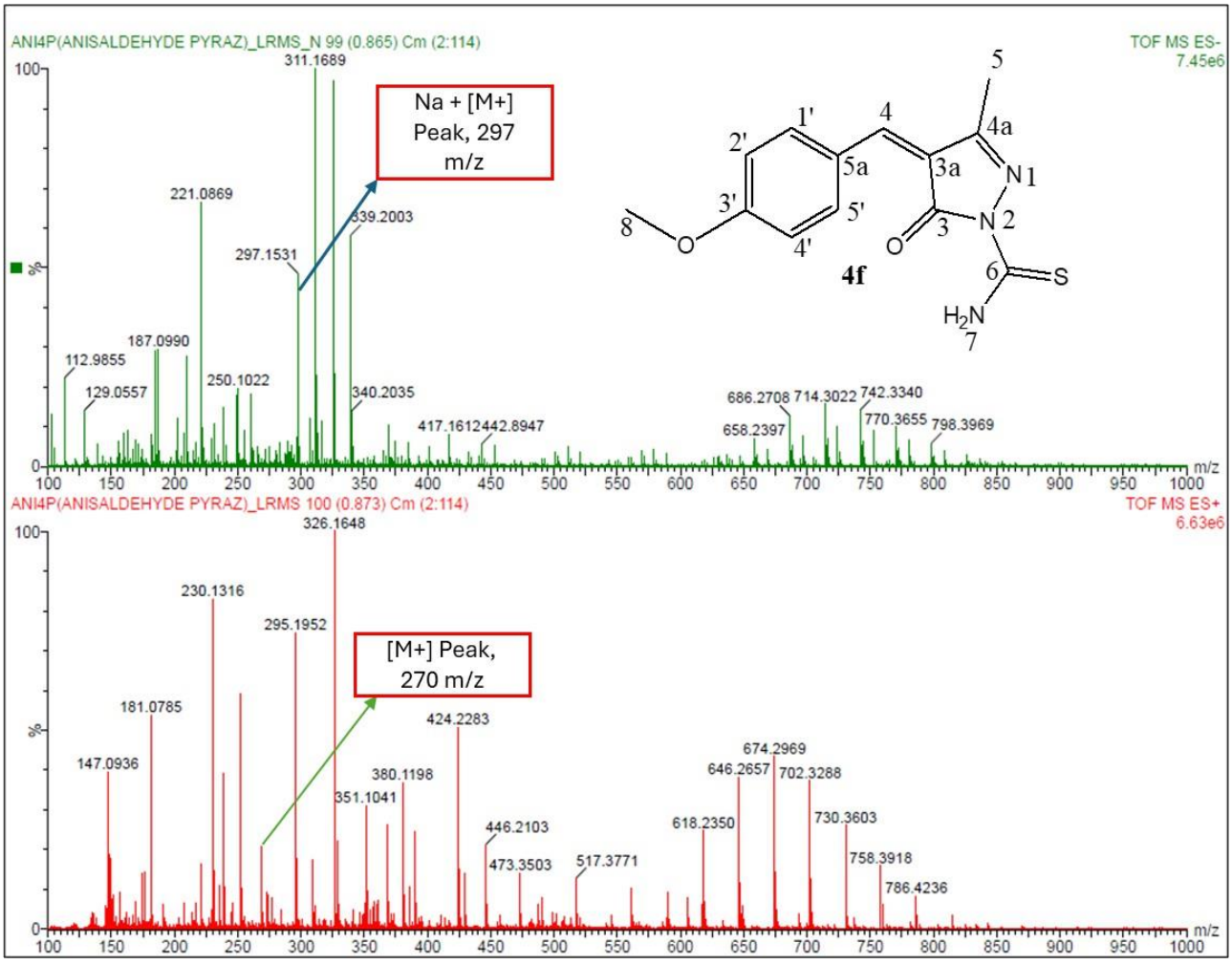
Appendix 31: I.R spectrum of **4f**



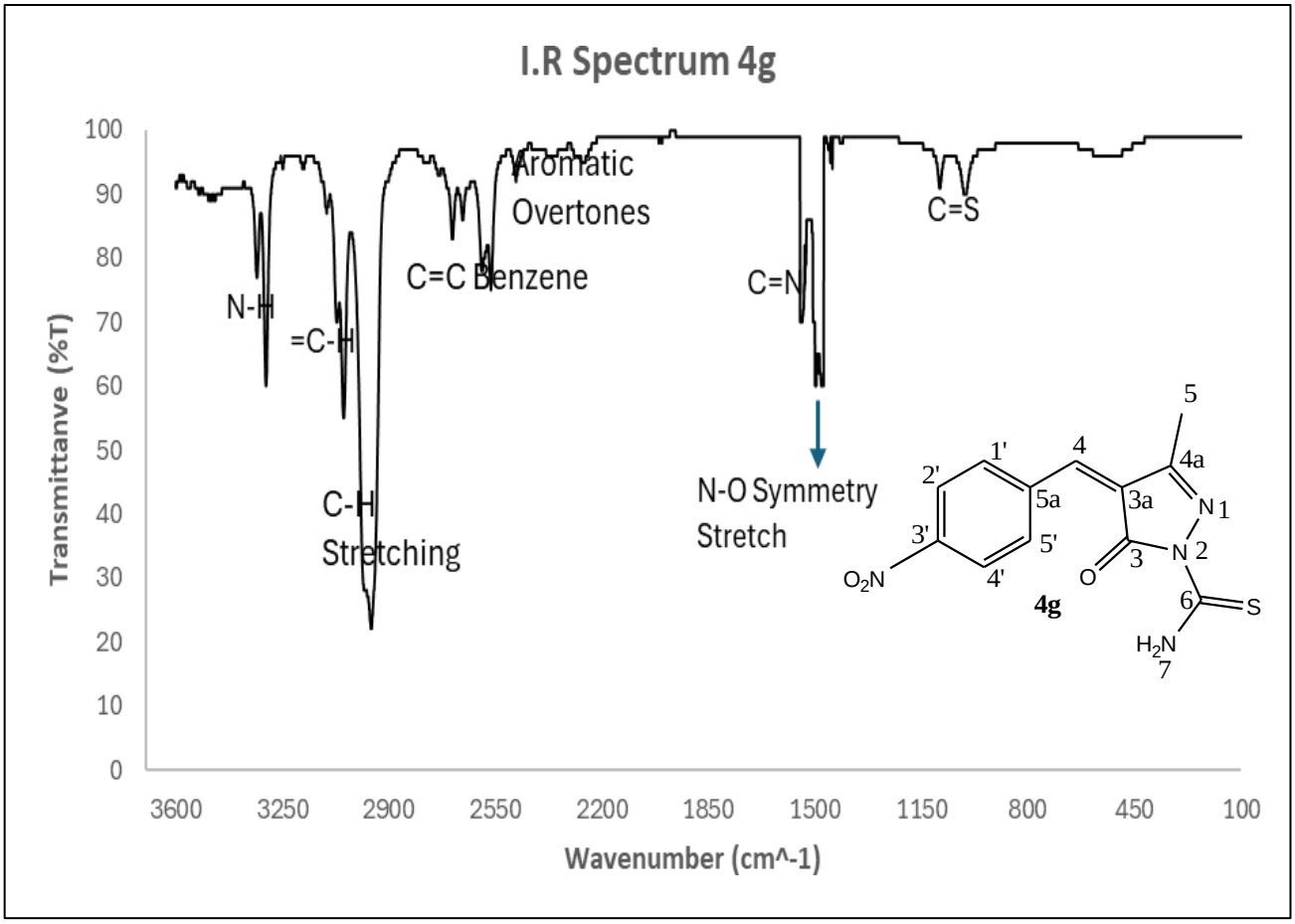
Appendix 32: ^{1}H NMR spectrum for compound **4f**



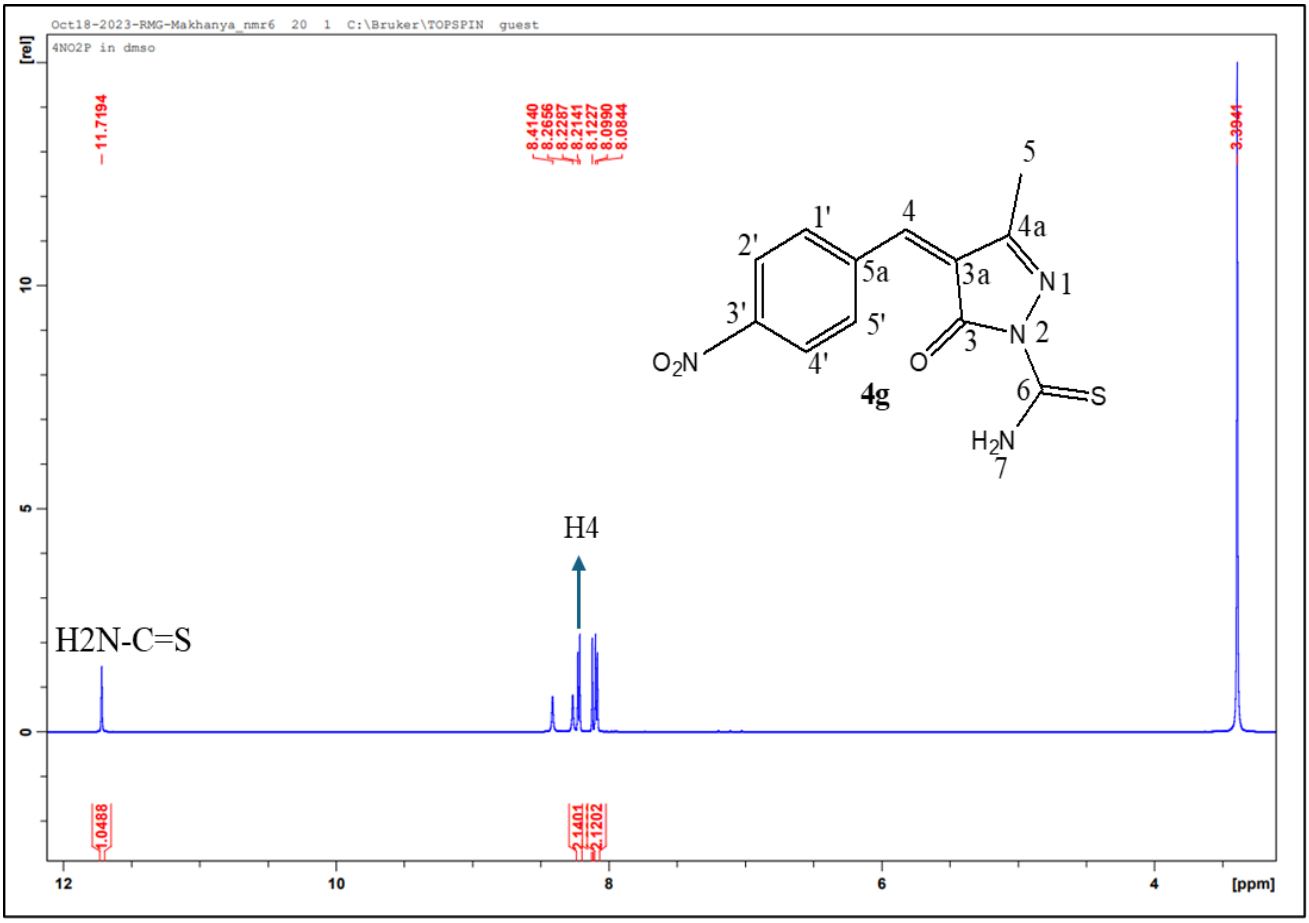
Appendix 33: ^{13}C NMR spectrum for compound **4f**



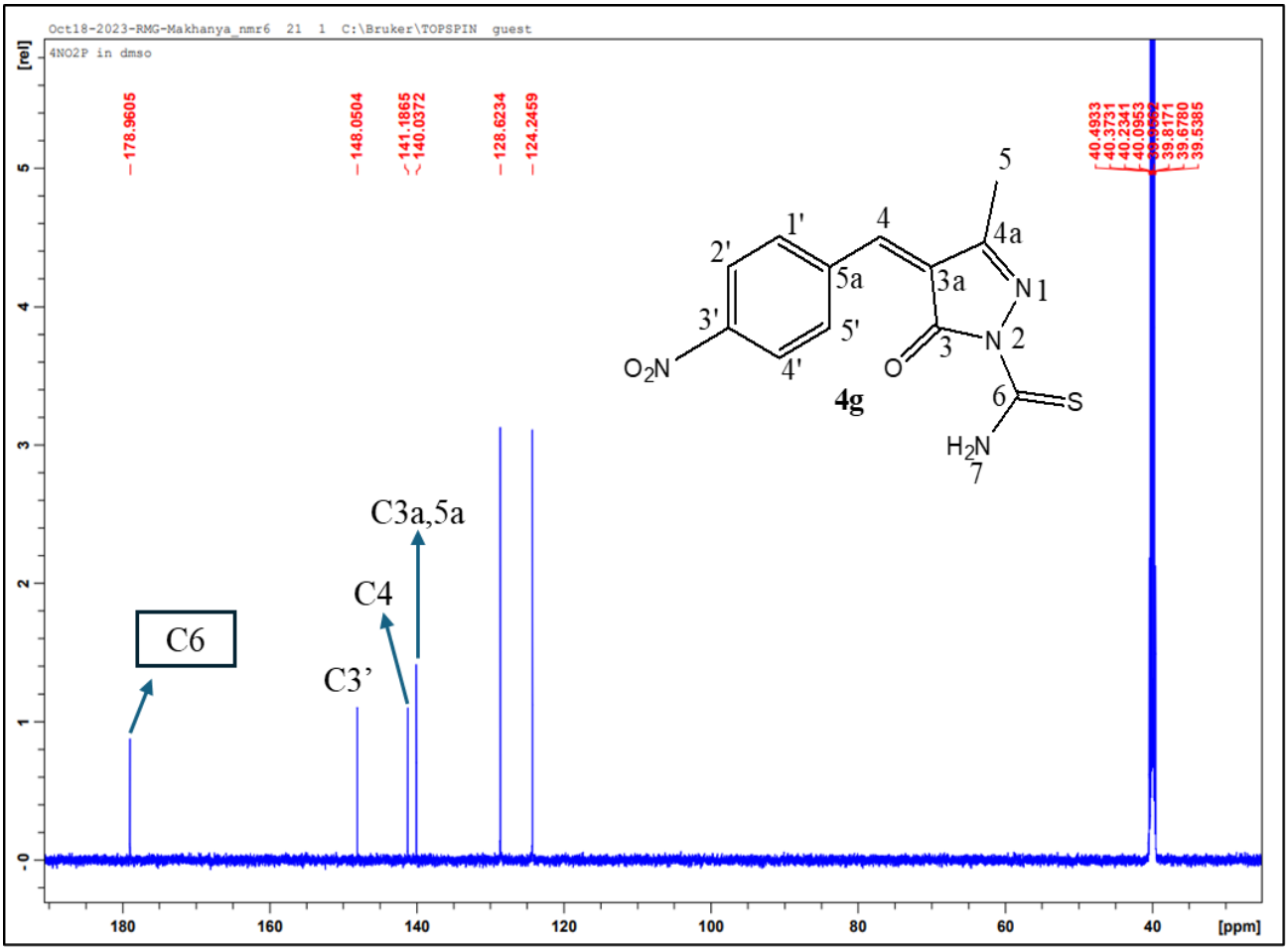
Appendix 34: Time of Flight-Mass Spectrometry for compound **4f**



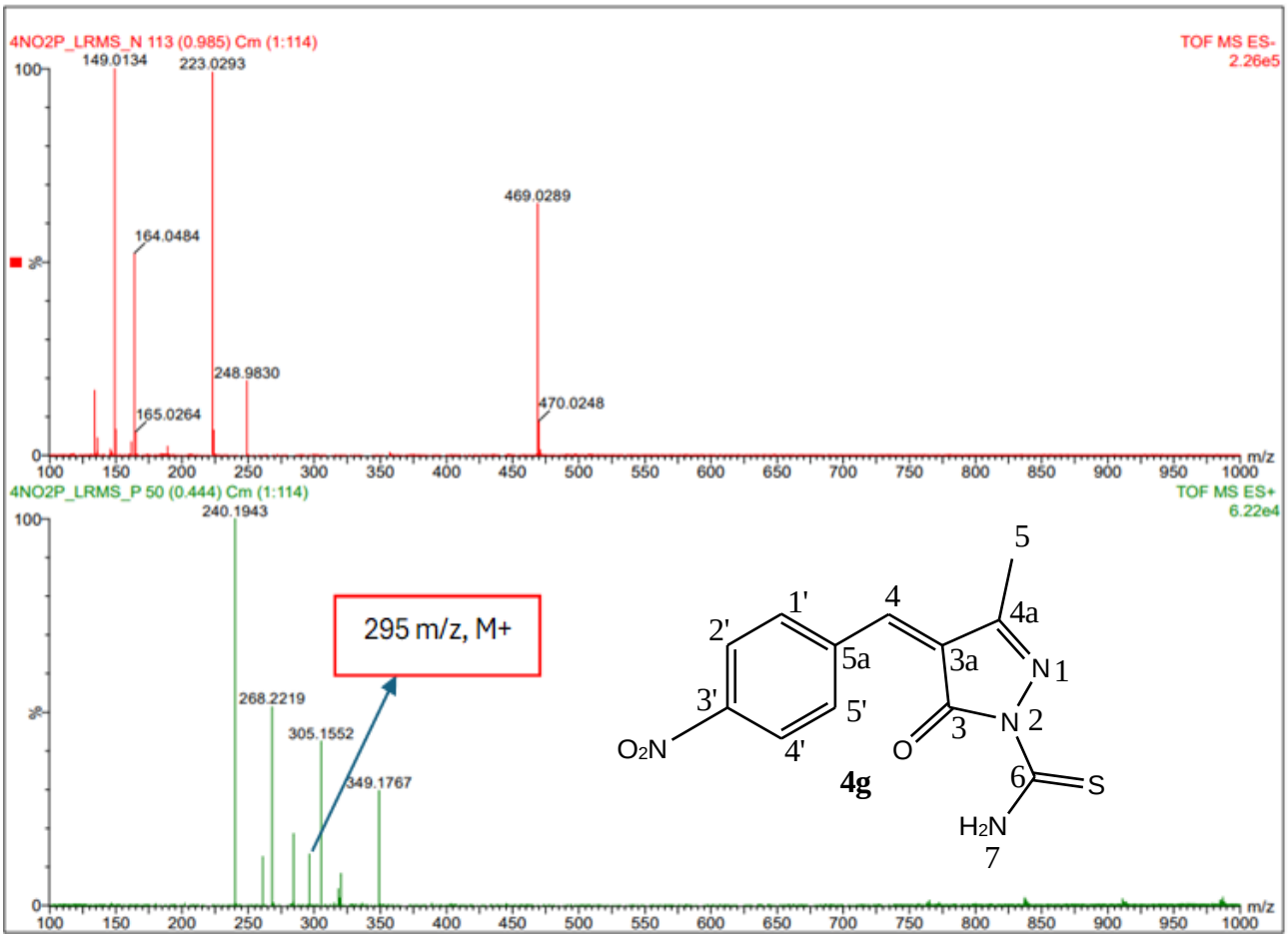
Appendix 35: I.R Spectrum of **4g**



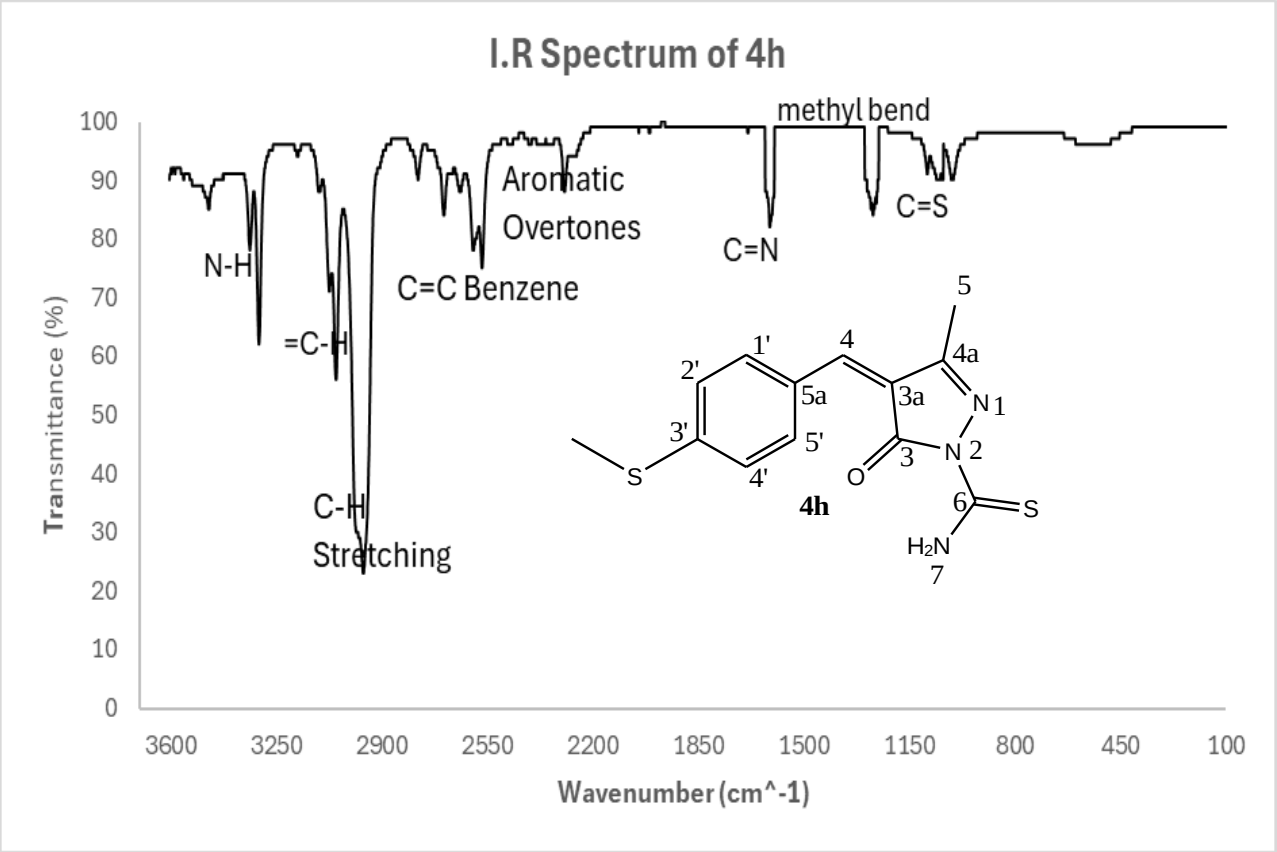
Appendix 36: ^{1}H NMR spectrum for compound **4g**



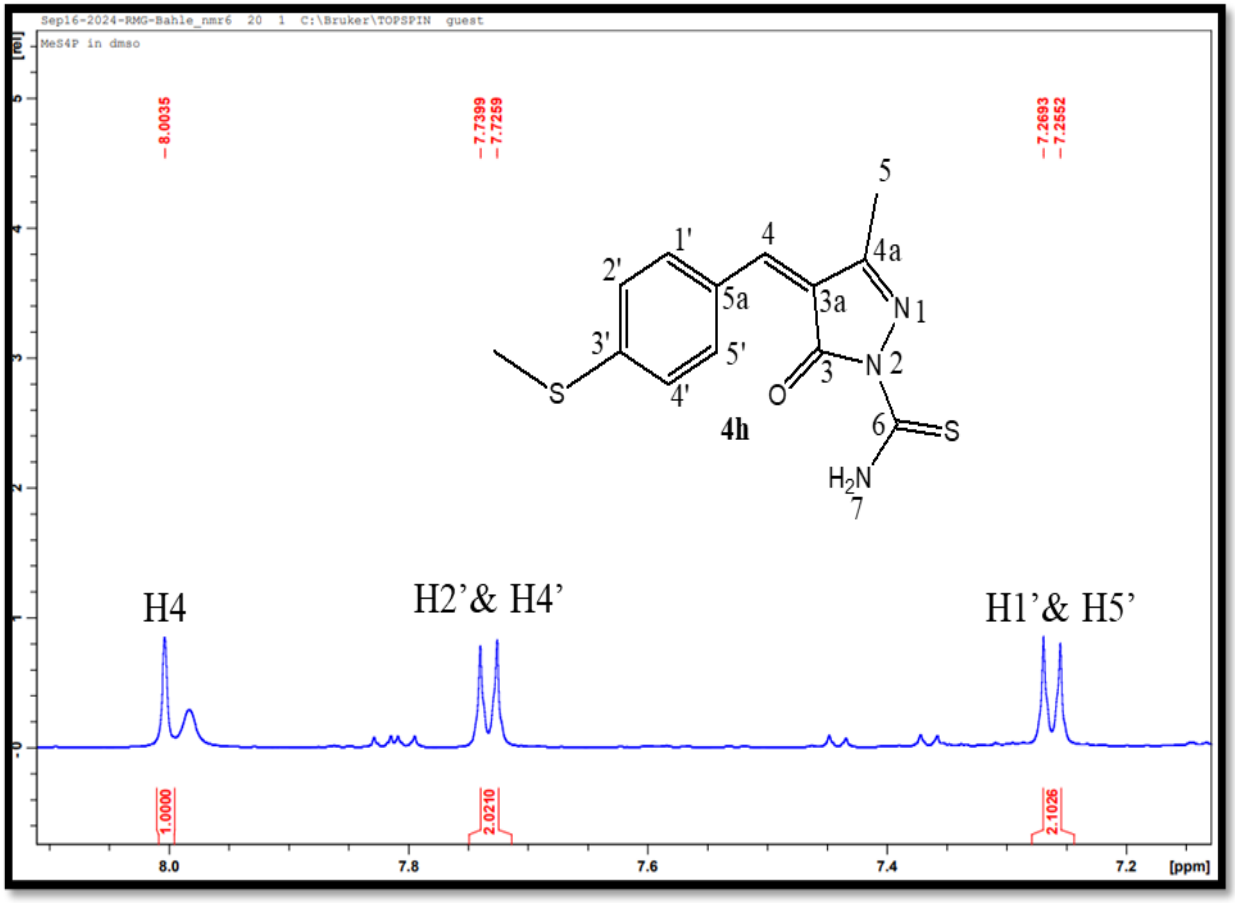
Appendix 37: ^{13}C NMR spectrum for compound **4g**



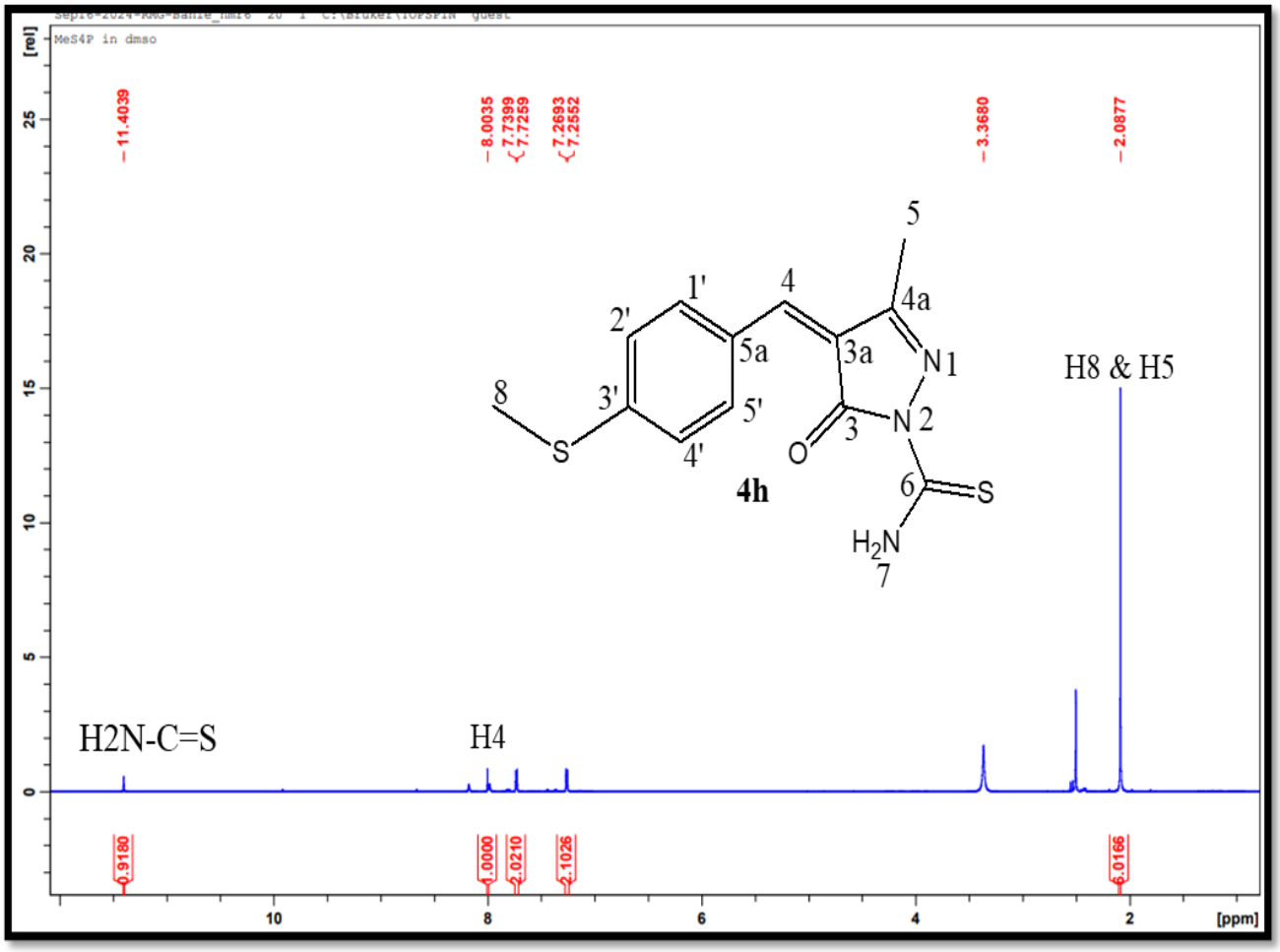
Appendix 38: Time of Flight-Mass Spectrometry for compound **4g**



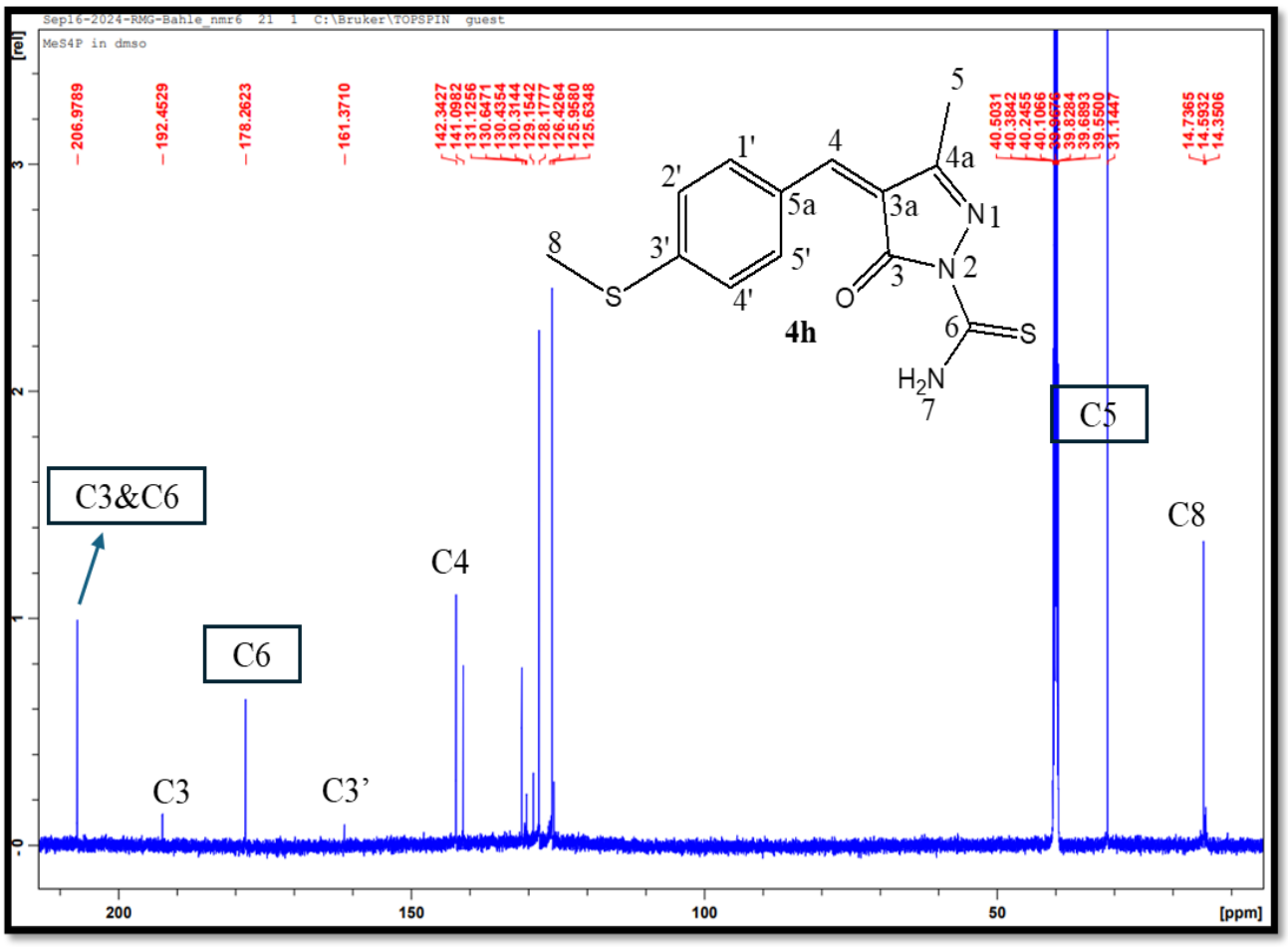
Appendix 39: I.R Spectrum of **4h**



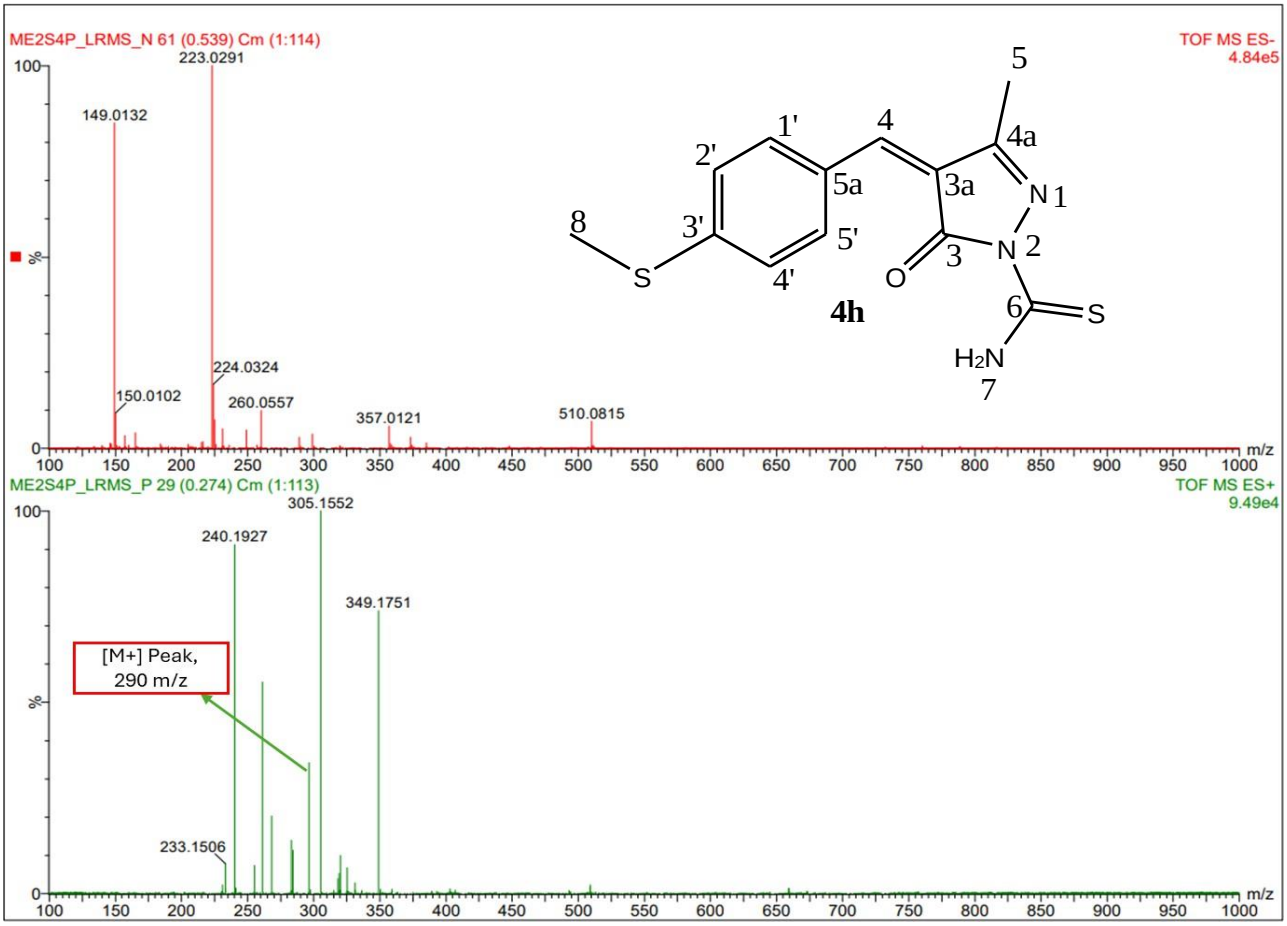
Appendix 40: Expanded ^{1}H NMR spectrum for compound **4h**



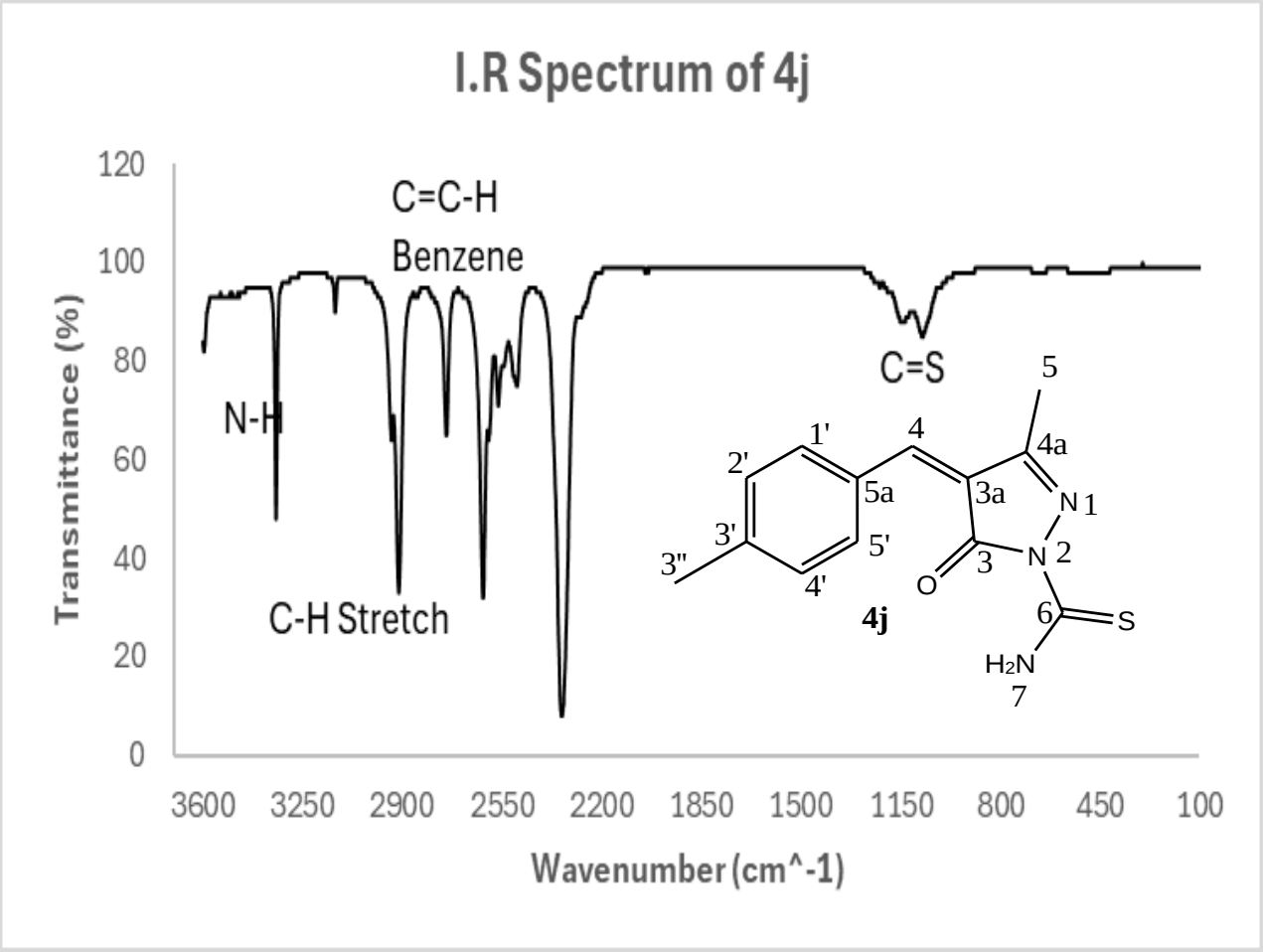
Appendix 41: ^{1}H NMR spectrum for compound **4h**



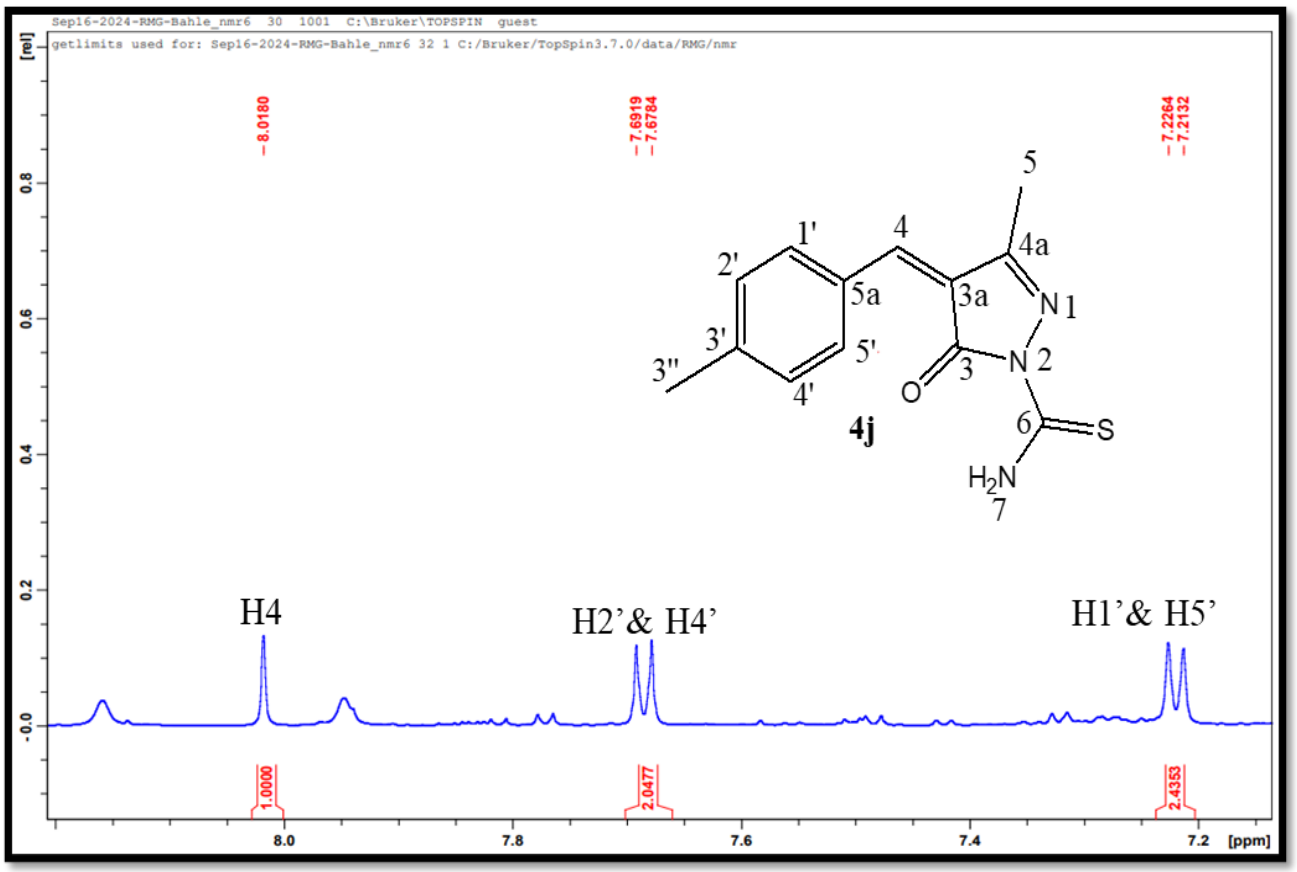
Appendix 42: ^{13}C NMR spectrum for compound **4h**



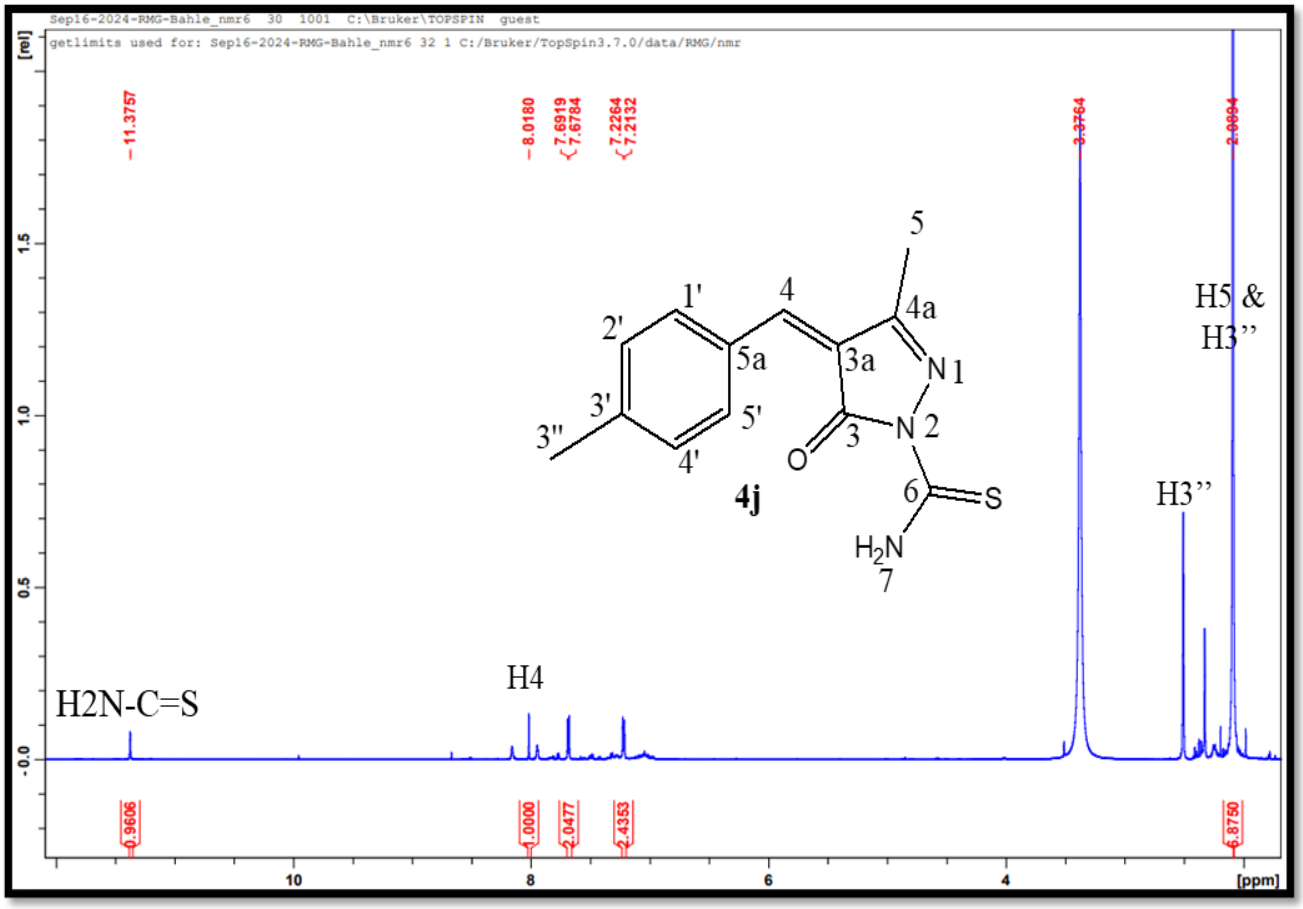
Appendix 43: Time of Flight-Mass Spectrometry for compound **4h**



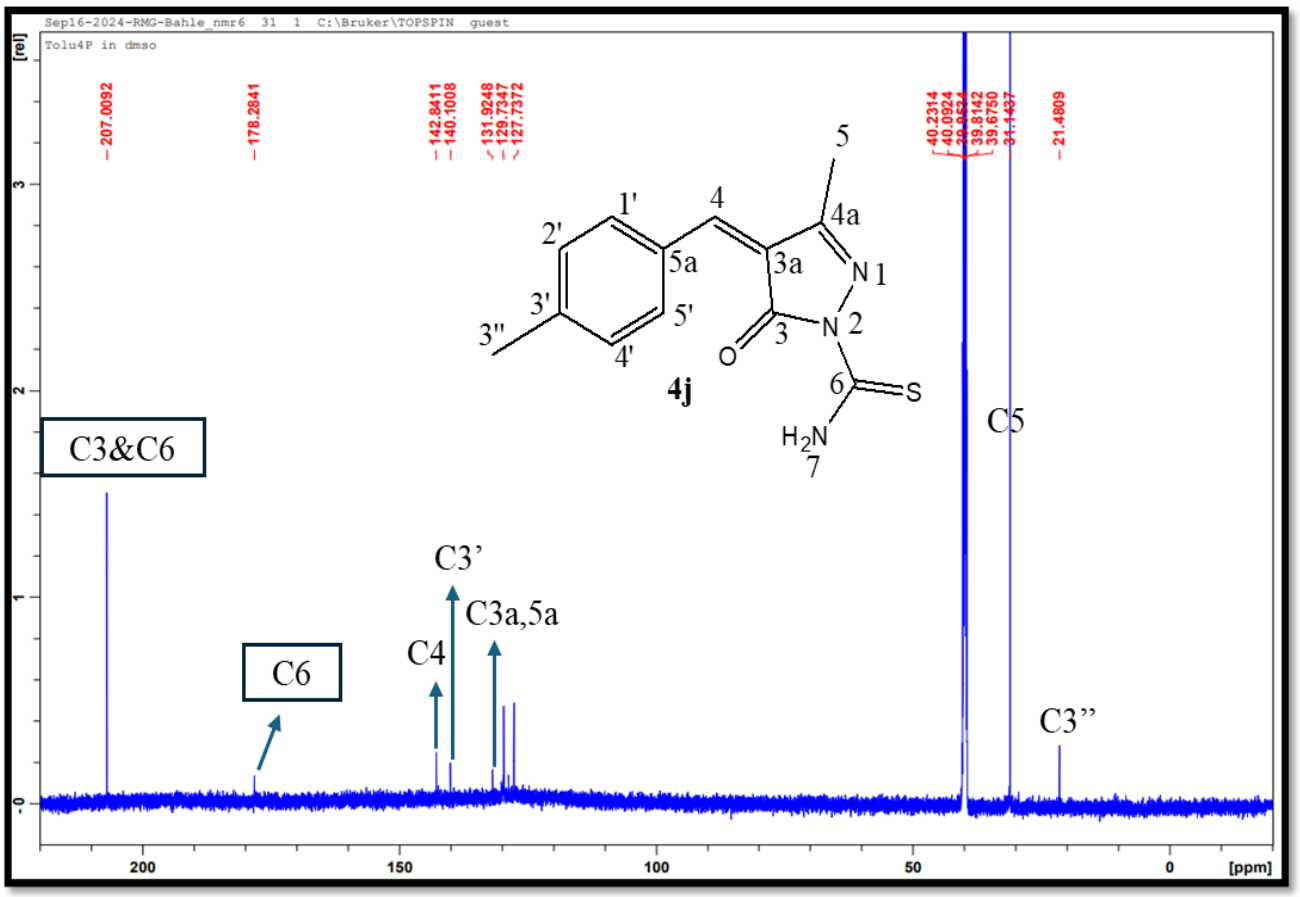
Appendix 44: I.R Spectrum of **4j**



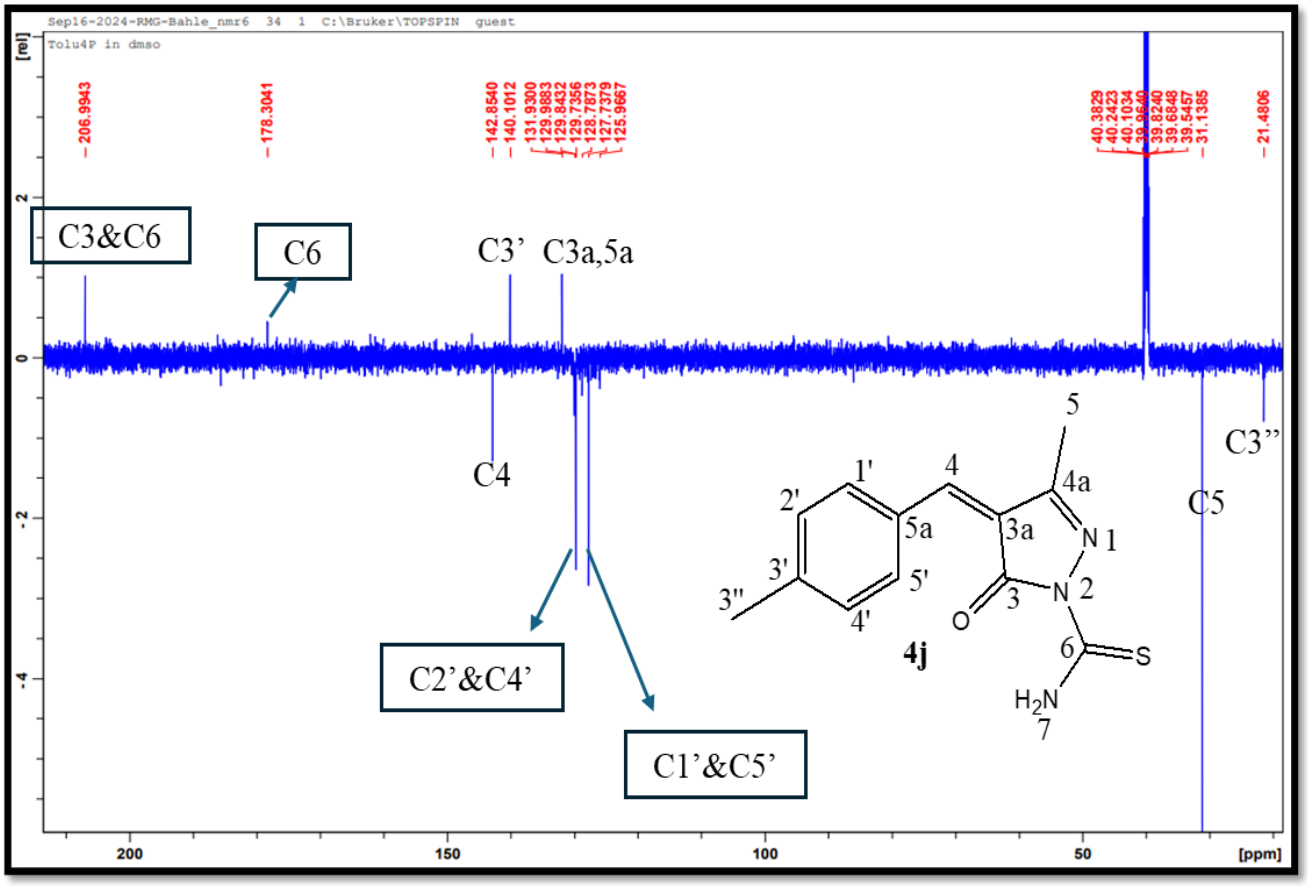
Appendix 45: Expanded ^{1}H NMR spectrum for compound **4j**



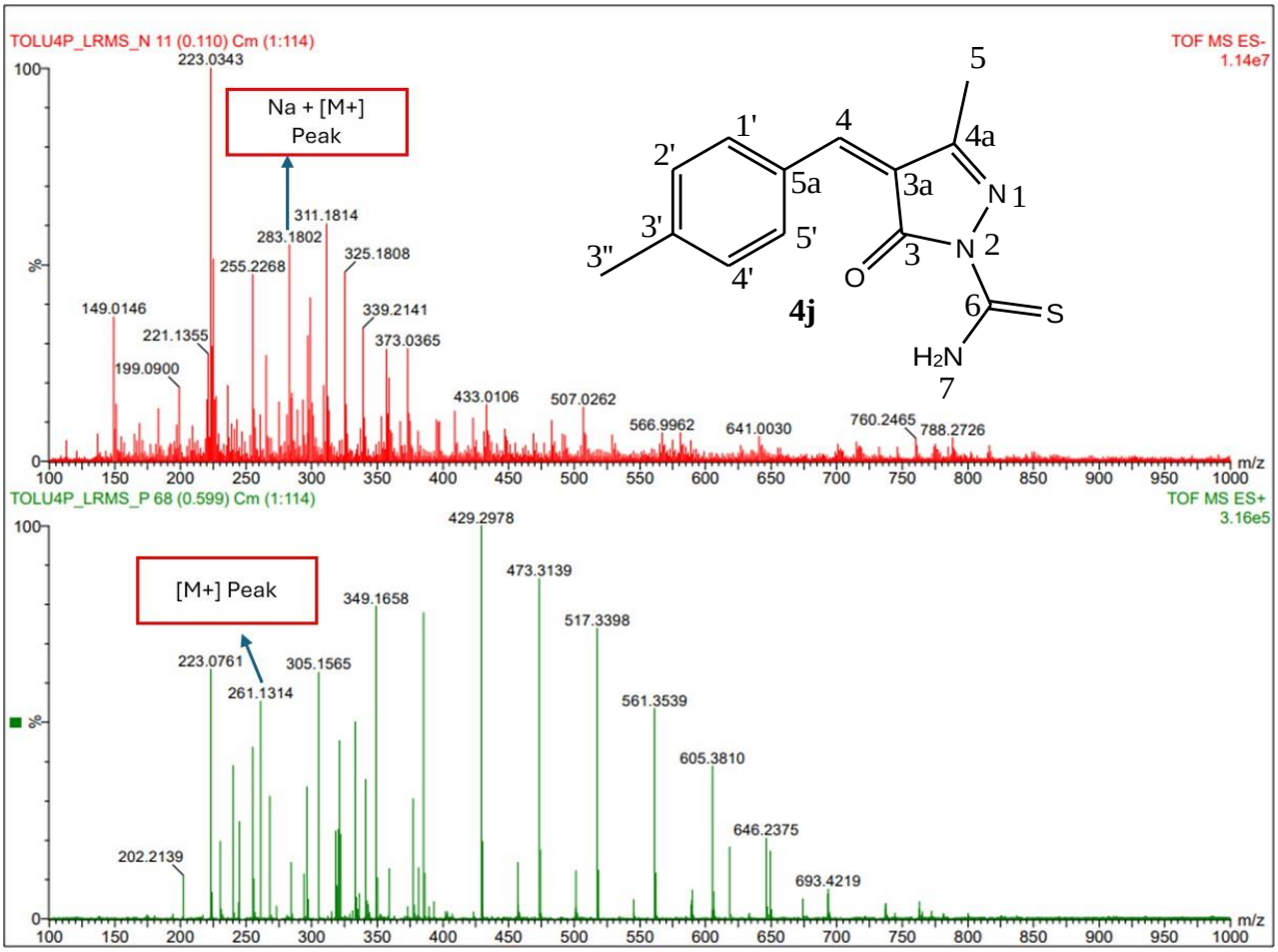
Appendix 46: ^{1}H NMR spectrum for compound **4j**



Appendix 47: ^{13}C NMR spectrum for compound **4j**



Appendix 48: APT of compound **4j**



Appendix 49: Time of Flight-Mass Spectrometry for compound **4j**

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Conference: *Oral Presentation*

Bahle L. Mntambo, T.R Makhanya and R.M Gengan, "Catalyst-free microwave-assisted multicomponent synthesis of pyrazole derivatives and evaluation of their antibacterial potential." **SACI-KZN Postgraduate Research Colloquium, 29 November 2024 - (University of Zululand)**.

Publication: *Manuscript*

Title: Microwave Irradiation Promote Eco-Friedly Multicomponent Synthesis of Substituted Pyrazole Derivatives and Study Their Antibacterial Activities.

Authors: Bahle Luyanda Mntambo, Jamiu Aribasala, Sabiu Saheed, Senzekile Majola, Robert Gengan, Talent Makhanya.

Journal Name: Molecules

Manuscript ID: molecules-3589479

Year: 31 March 2025

Status: Submitted