UNIVERSITY OF KWAZULU-NATAL

SYNTHESIS AND BIOLOGICAL EVALUATION OF DIHYDROPYRIMIDINONEDERIVATIVES AS A PROMISING ANTIMICROBIAL AGENTS

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Wadzanai Janet Muzenda

SYNTHESIS AND BIOLOGICAL EVALUATIONOF DIHYDROPYRIMIDINONEDERIVATIVES AS APROMISING ANTIMICROBIAL AGENTS

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By

WadzanaiJanet Muzenda

(B.Pharm)

Supervisor

Dr. RajshekharKarpoormathB.Pharm, M.Pharm, Ph.D

Preface

This study represents original work by the author and has not been submitted in any other form to another university. Where the use of work pertaining to others has been duly acknowledged in the text.

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Signed: **Name:**Wadzanai J.Muzenda **Date:** 1st of Dec. 2014

As the Candidate's supervisor, I have approved this thesis for submission.

Supervisor:

Signed:Name: Dr. R. Karpoormath **Date:** 1st of Dec. 2014

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I, **WADZANAI J. MUZENDA,** declare that the experimental work described in this dissertation was carried out at the School of Pharmacy, College of Health Sciences, University of KwaZulu-Natal, Westville campus under the supervision of Dr. Rajshekhar Karpoormath, and that:

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My first and sincere appreciation goes to my Saviour Jesus Christ and above all to my almighty living God. I would have never been able to finish without His strength, wisdom and perseverance. Philippians 4:13 “I can do all things through Christ who strengthened me”.

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May the Almighty living God bless you all.

List of Abbreviations

^{0}C	Degrees celsius
^{13}C NMR	Carbon-13 nuclear magnetic resonance spectroscopy
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
CDCl3	Deuterated chloroform
DCM	Dichloromethane
DMSO	Dimethyl sulfoxide
d	Doublet
EtOAc	Ethyl actetate
EtOH	Ethanol
FT-IR	Fourier transform infrared spectroscopy
Hz	Hertz
m.p.	Melting point
MIC	Minimum inhibitory concentration
min	Minutes
m	Multiplet
s	Singlet
TLC	Thin layer chromatography
t	Triplet
UV-VIS	Ultraviolet-visible spectroscopy

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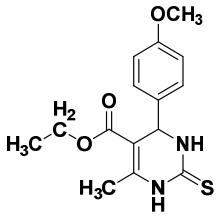
Abstract

The deterioration of human population due to an enhanced prevalence of infectious disease is becoming a global problem. The rising dominance of multi-drug resistant microorganism is driving research efforts towards the discovery, design and development of newer antimicrobial agents. As a contribution to these efforts, we synthesized compounds with same mechanism of action with available antimicrobial agents. A series of eleven dihydropyrimidinoneswere synthesized byBiginelli condensation reaction with various substituted aldehydes. The synthesized compounds were confirmed by melting point, TLC, IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy. Discovery CEM microwave reactor was employed for the synthesis of some compounds to increase the yield.The compounds obtained were in high yields of 80 – 98%. The synthesized compounds displayed low to moderate antifungal and good antibacterial activity. The compound below exhibited significant activity against *Bacillus subtilis.*



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CHAPTER I

INTRODUCTION

1.1 Medicinal Chemistry

Medicinal and pharmaceutical chemistry deals with the design, development and synthesis of drugs having therapeutic use and evaluation of their properties. The discipline encompasses pharmacology, chemistry, more specifically organic chemistry and several other biological specialties. Medicinal chemistry aims at developing novel chemical entities that can be put to therapeutic use after systematic and thorough synthetic alteration.[1] It involves identification of novel compounds and their chemical modifications so that the pharmacokinetic/pharmacodynamics properties and toxicological profiles are improved for human/animal administration.[2]Pharmaceutical chemistry primarily focuses on discovery of novel chemical entities of therapeutic value and quality control of medicinal formulations.[3]

1.2 Drug design and discovery

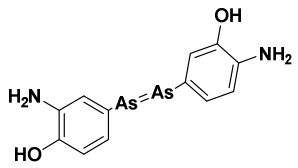
The primary objective of medicinal chemistry is the design and discovery of new compounds suitable for use as drugs. The discovery of a new drug requires not only its design and synthesis but also to understand its mechanism of action against a desired target in the body and its suitability for use as a drug.[2]Drug discovery also requires fundamental research as well as knowledge of the diseased state at a molecular level. This and other aspects of drug design and discovery require input from specialists in other fields such as biologists, biochemists, pharmacologist, biophysicist, computation and medical scientists amongst others.[4] A medicinal chemist should have an outline knowledge and understanding of these fields to design potent drugs.[3]

Since ancient times a wide range of natural products have been used for medicinal purposes. These products, obtained from animal, vegetable and mineral sources, were sometimes very effective. However, many of the products were toxic and had severe side effects.[1] Information about these ancient remedies was not readily available to users until the invention of the printing press in the 15th century.[1] This invention led to the widespread publication and circulation of natural products and pharmacopoeias, resulting in a rapid increase in the use as well as misuse of drugs of natural product origin and other remedies. However, improved communications between practitioners in the 18th and 19th centuries resulted in the progressive removal of preparations that were either ineffective or too toxic from herbals and pharmacopoeias. It also led to a more rational development of new drugs.

Initially this development was centered on the natural products isolated from plant and animal material, but as knowledge increased a wider range of pharmaceutically active

compounds were used as the starting point for the development of drugs. The compounds onwhich a development is based are now known as lead compounds, while the synthetic compounds developed from a lead are referred to as its analogues.[5]

The work of the medicinal chemist mainly focuses on the discovery of new lead compounds which has specific medical properties. This involves the development of more effective and safer analogues from both these new and existing lead compounds.This usually involves synthesizing and testing many hundreds of compounds before a suitable compound is produced.[3]The first rational development of synthetic drugs was carried out by Paul Ehrlich, who produced the antiprotozoal Arsphemamine**1**(also known as Salvarsan) in 1910, as shown in **Figure 1**.He combined synthesis with reliable biological screening and evaluation procedures. Ehrlich, at the beginning of the 20th century, had recognized that both the beneficial and toxic properties of a drug were important to its evaluation. He realized that the more effective drugs showed a greater selectivity for the target microorganism than its host. Consequently, to compare the effectiveness of different compounds, he statedthat a drug's selectivity was essential to improve the treatment of existing as well asnewly identified diseases and the production of safer drugs, with little or no adverse side effects.[5]



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Salvarsan

Figure 1. Structure of Salvarsan

1.3 Microbial infections

Healthy human beings live normally with microbial flora. There are approximately ten times bacterial cells in the human flora than the normal human cells in the body, with large number of bacteria's on the skin and as gut flora.[6]Normal flora can be pathogenic or nonpathogenic. Nonpathogenic microbes protect its host from invasion of pathogens into the human body.[7] Pathogenic normal flora have pathogenic effects if they are found at a site where they are not normally found e.g. bacteriods, normally found in intestines, can lead to production of abscess if they penetrate into a traumatic wound or surgical wound. *E. coli* have positive effects in gastrointestinal tract,but they also show negative effect, causing urinary tract

infections. Normal flora can prevent colonization of pathogens causing diseases through bacterial interference.[8]

1.3.1 Bacterial Infection

Bacteria are single-celled organisms which populate almost every niche on earth, from the hottest springs and chilliest waters to many spaces on and within the human body. As a group they are extremely diverse in size, shape, motility, nutrient requirements and pathogenicity (ability to cause disease), but share the common trait of lacking a true nucleus and hence are referred to as "prokaryotic".[9]

Bacteria are classified as Gram-negative and Gram-positive.The bacterial cell wall of Gram-positive consists of peptidoglycan and techoic acid. Gram negative bacterial cell wall consists of peptidoglycan, lipoprotein, lipopolysaccharides, phospholipids and proteins.[10]The major site of action of antimicrobials is on peptidoglycan which is one of the components of the cell wall.This layer is essential for the bacteria to survive in hypotonic environments. Loss or damage of this layer leads to destruction of the rigidity of the bacterial cell wall resulting in death. Antimicrobial agents easily pass through the outer cell wall of gram positive bacteria (as shown in **Figure 2)** while some penetrate through the narrow channels of Gram-negative species.[7]

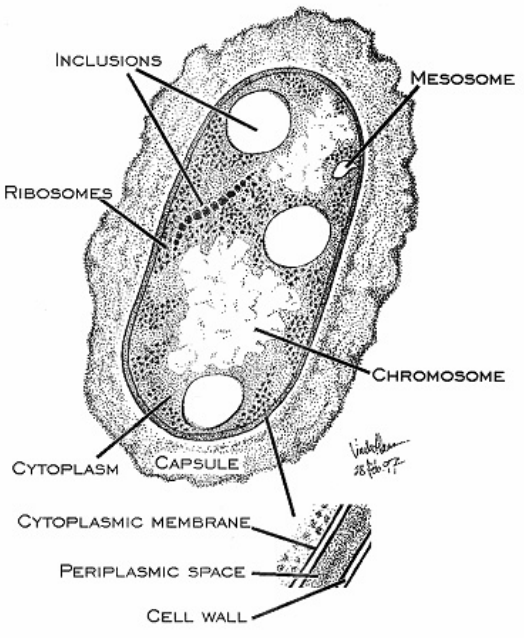

Figure 2. Structure of bacteria[9]

Though most of the earliest bacteria discovered were pathogens (disease-causing organisms) however a vast majority of bacteria on earth are harmless or even beneficial to humans. Many animals rely on gut bacteria to provide nutrients from food, which we are unable to synthesize ourselves (for example, ***E. coli*** in the gut produce Vitamin K).[11]Additionally, commensal (non-pathogenic) bacteria fill niches in our body and use resources that would otherwise be available to other pathogenic microorganisms. For instance, our skin and oral cavity are covered with bacteria, most of which will never harm us, as they live in a delicate balance with our immune system.[12]However pathogenic bacteria can cause disease in humans and animals as shown in **Table 1**. Some examples of pathogenic bacteria are: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*.

Table 1. An overview of bacterial infections[13]

Name of microorganism species	Site of body affected	Microbial infections caused
Streptococcus pneumoniae *Haemophilus influenza*	Brain	Bacterial meningitis
Streptococcus pneumoniae	Ears	Ear, nose and throat infections
Mycobacterium tuberculosis *Chlamydia pneumoniae*	Lungs	Respiratory tract infections
Staphylococcus aureus *Pseudomonusaeruginosa*	Skin	Skin infections
Neisseria gonorrhea *Treponemapallidum*	Reproductive system	Sexually transmitted infections
Escherichia coli *Pseudomonusaeruginosa*	Urinary System	Urinary tract infections
Streptococcus pyogenes *Haemophilus influenza*	Respiratory system	Respiratory tract infections
Staphylococcus aureus *Neiserriagonorrhoea*	Eyes	Eye infections

Fungi can also cause superficial, systemic and opportunistic infections such as candida infection, fungal meningitis and ***Pneumocystis pneumonia.***[14]Majority of the harmful microorganisms that a human body comes across are rendered harmless by the protective

effects of the body's immune system[7].However, some resistant species are pathogenic and can cause infectious diseases such as tuberculosis and typhoid,which are difficult to treat and need attention by medicinal chemists.

1.3.2 Fungal Infections

Fungi are eukaryotic (as shown in **Figure 3**)and include organisms such as molds and yeasts. Approximately 100 fungal species (out of ~100,000 known) are pathogenic.[15]Yeasts are unicellular and generally divide by simple binary fission (one cell divides into two).Molds, on the other hand, generally have complex life cycles.[16]

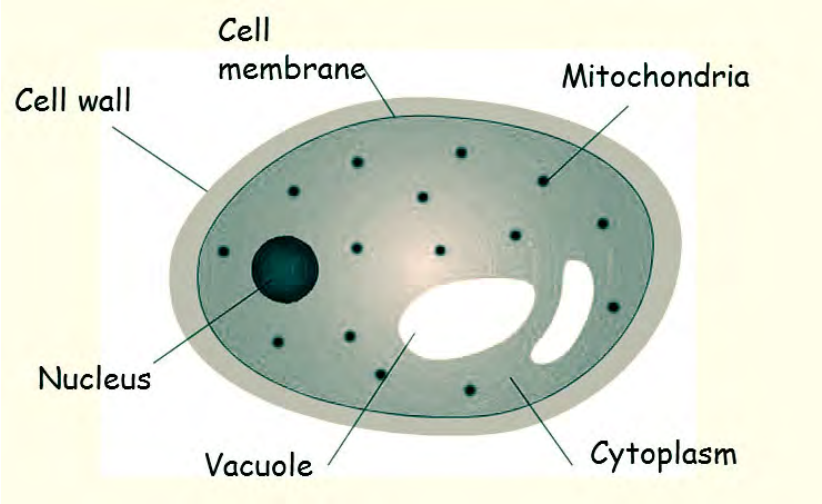

Figure 3. Structure of fungi[17]

Fungi life cycle pass through both an asexual and a sexual stage. They exist as multicellular organisms during much of this period and at this point are far from being "micro" organisms.[18]Nevertheless, because it is often their (microscopic) spores which are responsible for diseases, they remain classed as general "microbes". Diseases caused by fungi can be classified according to the parts of the body affected by the fungi, as depicted in the **Table 2**. Diseases due to fungi can also be caused by contact with or ingestion of toxins produced by certain species. For example, aflatoxin is a poison produced by the mold *Aspergillusflavus*, when ingested; symptoms may induce vomiting, convulsions or death.[20] In 1928, Alexander Fleming discovered *Penicilliumrubens*, which was the first antimicrobial fungus.[21]

Table 2. Some examples of superficial fungal infection[18]

Classification	Affected Site	Notes
Tineapedis (Athletes Foot)	Feet	The most common fungal infection. Initial infection can be dry and scaly, however secondary bacterial infection and accumulation of soggy debris can commonly occur.[19]
Tineacapitis	Scalp	More common in Children. Infected hair can break leaving a bald area.[19]
Tineabarbae	Beard	Common among men that work with animals e.g. agricultural workers due to animal human transmission. May be accompanied by bacterial folliculitis, secondary to ingrown hair.[19]
Tineacorporis (Ring worm)	Skin other than bearded area, scalp, groin, hands or feet	Tends to present as irregular expanding rings with a raised border.[18]
Tineacruris (Jock itch)	Groin, perineum and perianal areas	Lesion may be on the inner thighs, pubic, inguinal region or scrotum. Yeast infections (usually *Candida albicans*) are also commonly found in these areas.[18]

1.4 Antimicrobial agents

Antimicrobial came from Greek words which are anti, mikros and bios. Here Anti means against, mikrosindicated little, and bios means life.[22]Antimicrobial is the term used for all the agents that have an activity against microbial organisms: viruses (antiviral)[23], protozoa (antiprotozoal)[24], fungi (antifungal)[24]and bacteria (antibacterial).[25]They have been used for treatment in humans and animals. Antimicrobials have also been used prophylactically to avoid diseases and in food production.The term antimicrobial agent can also be used to designate synthetic as well as naturally obtained drugs that attenuate microorganisms.[13]

The chemicals used to treat infectious diseases fall into two main categories, namely natural products and chemotherapeutic agents.[26]Natural products are secondary metabolites that are only generated by certain microorganisms and are usually large, elaborated organic molecules that require complex enzymatic synthesis.[27]Alternately chemotherapeutic agents comprise of compounds that have been synthesized chemically. A hybrid of these two categories exists, in

which natural products that have been chemically modified to alter particular characteristics and is referred to as semi-synthetic antimicrobials.Antimicrobials are also classified according to spectrum activity effect on bacteria and also their mode of action as mentioned below.[28]

1.4.1 Classification of antimicrobials

1.4.1.1 Classification according to spectrum of activity:

Depending on the range of bacterial species susceptible to these agents, antibacterials are classified as broad-spectrum, intermediate-spectrum or narrow- spectrum.[28] Some examples are shown in**Figure 4**.

- Broad spectrum antibacterials are active against both Gram-positive and Gram-negative organisms. Examples include: tetracyclines, phenicols, fluoroquinolones, “third-generation” and “fourth-generation” cephalosporin.[29]
- Narrow spectrum antibacterials have limited activity and are primarily only useful against particular species of microorganisms. For example, glycopeptides and bacitracin are only effective against Gram-positive bacteria, whereas polymixins are usually only effective against Gram negative bacteria. Aminoglycosides and sulfonamides are only effective against aerobic organisms, while nitroimidazoles are generally only effective for anaerobes.[28]

1.4.1.2 Classification based on effect on microorganism:

- Bactericidal drugs are those that kill target organisms. Examples of bactericidal drugs include, penicillins**2**, fluoroquinolones**3**and cephalosporins**4**.[30]
- Bacteriostatic drugs inhibit or delay bacterial growth and replication. Examples of such include tetracyclines**5** and sulphonamides**9**.[31]
- Some antibiotics can be both bacteriostatic and bactericidal, depending on the dose, duration of exposure and the state of the invading bacteria. For example ciprofloxacin and ofloxacin.[32]

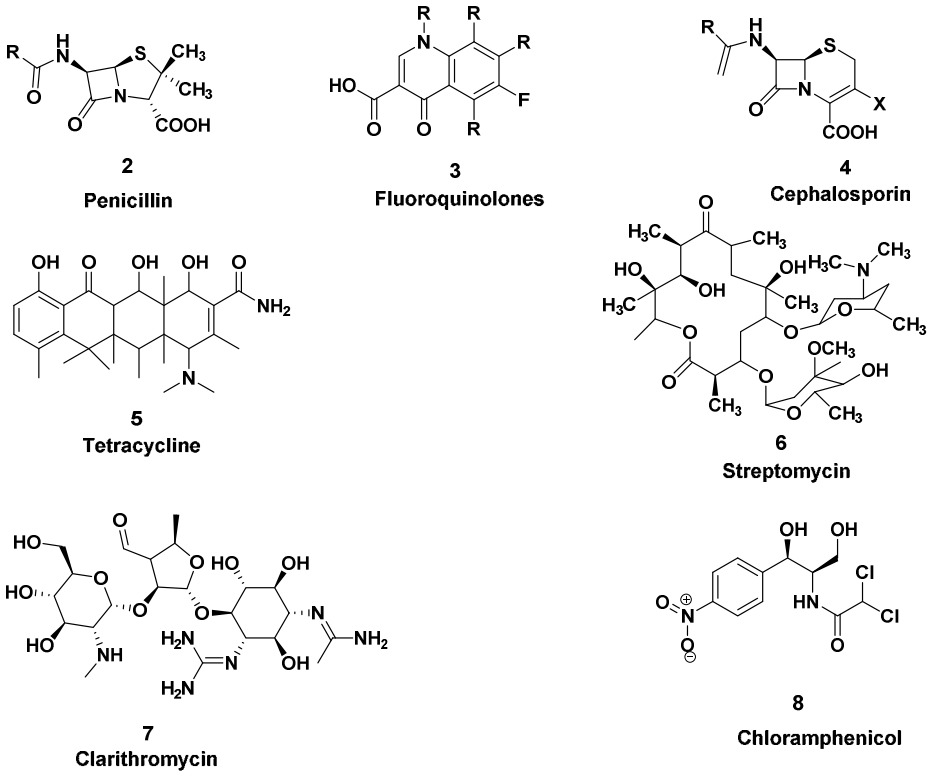

Figure 4. Examples of antimicrobial agents based on their classification

Numerous antimicrobial agents are known, however, not all of them can be used in vivoas in addition to their antimicrobial activity they can be toxic to human beings.[33]Antimicrobials have to be non-toxic, non-allergenic, effective and selective, chemically stable, active against possibly more than one bacterium and inexpensive. The ratio between the therapeutic effect and the toxic effect in the human body is described by the drugs therapeutic index (TI). Antimicrobial drugs can have several different modes of action.[18]Penicillins**2** and cephalosporin**4**act by interfering with the synthesis of cell wall, while tetracyclines**5** act by interrupting the synthesis of proteins and sulfonamides and fluoroquinolones**3** obstruct DNA functions.[18]

1.5 History and Development of Antimicrobial Agents

Looking back on the history of human diseases, infectious diseases have accounted for a very large proportion of diseases as a whole. It was not until the latter half of the 19thcentury that microorganisms were found to be responsible for a variety of infectious diseases that had been plaguing humanity from ancient days.Accordingly, chemotherapy aimed at the causative organisms was developed as the main therapeutic strategy.[34]

The first antimicrobial agent discovered was Salvarsan**1**, a remedy for syphilis that was synthesized by Ehrlichin 1910.[5]In 1935, sulfonamides **9**(**Figure 5**) were developed by Domagk and other researchers.[35]These drugs were synthetic compounds and had limitations in terms of safety and efficacy.[13]

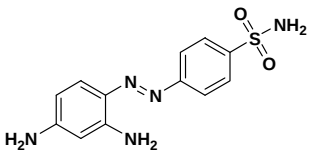

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Figure 5. Structure of Sulphonamide

In 1928, Fleming discovered penicillin. He observed that the growth of *Staphylococcus aureus* was inhibited in a zone surrounding a contaminated blue mold (a fungus from the *Penicillium* genus) in culture dishes, leading to the finding that a microorganism could produce substances that might inhibit the growth of other microorganisms. This antibiotic was named penicillin and it came into clinical use in the 1940s. Penicillin, which is an outstanding agent in terms of safety and efficacy, lead the era of antimicrobial chemotherapy by saving lives of many wounded soldiers during world war II.[21] In subsequent decades new classes of potent antimicrobial agents were developed, leading to a golden age of antimicrobial chemotherapy, as depicted in **Figure 6**.

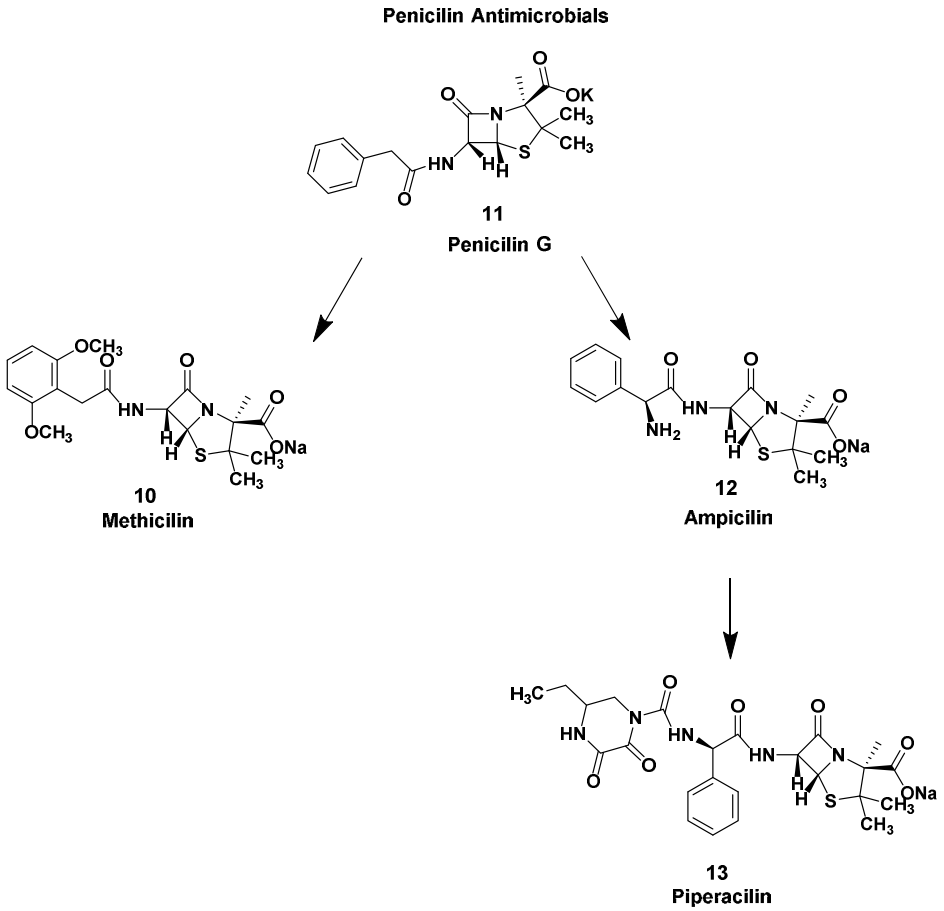

Figure 6. Development of penicillin

In 1944, streptomycin **6**, an aminoglycoside antibiotic, was obtained from the soil containing bacterium *Streptomyces griseus.*[36, 37]Thereafter, chloramphenicol **8**, tetracycline **5** and glycopeptide e.g. Vancomycin**14**(**Figure 7**) was discovered from soil bacteria. The synthesized antimicrobial agent which is quinolone antimicrobial drug, was discovered in 1962.[37]

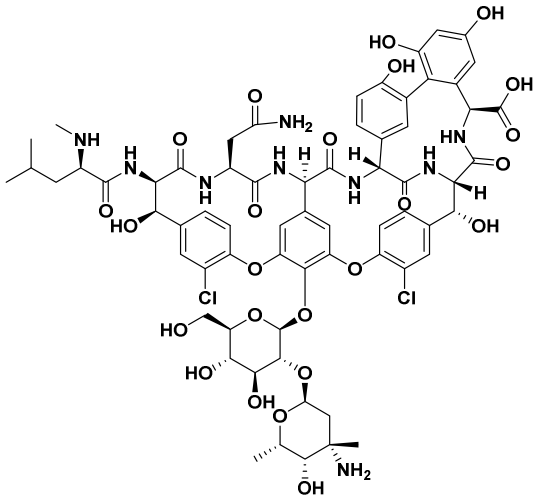

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Figure 7. Structure of Vancomycin

Improvements in each class of antimicrobial agents continued to achieve a broader antimicrobial spectrum and higher antimicrobial activity. For example, in case of β-lactam antibiotics (penicillins, cephems, carbapenems and monobactams); penicillins were originally effective for Gram-positive organisms such as *S. aureus*. Later, to address penicillin-resistant *S. aureus* which produces the penicillin hydrolyzing enzyme penicillinase, methicillin was developed.[38]On the other hand, attempts to expand the antimicrobial spectrum yielded ampicillin, which is also effective against Gram-negative *Enterobacteriaceae*and piperacillin as well as against *Pseudomonas aeruginosa.*

Cephems were developed in the 1960s, and came into widespread use.Cephems are classifiedinto several generations according to their antimicrobial spectra. First-generation e.g. cefazolin**15**(**Figure 8**),are effective only for Gram-positive organisms and *Escherichia coli*, although their antimicrobial activity against these organisms is potent.

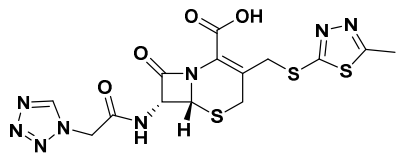

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Figure 8. Structure of Cefazolin

Second-generation cephems e.g. cefotiam**16**(**Figure 9**)have an extended antimicrobial spectrum that covers not only Gram-positive but also Gram negative organisms including other *Enterobacteriaceae*.[18]

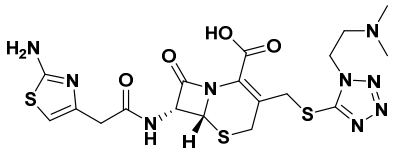

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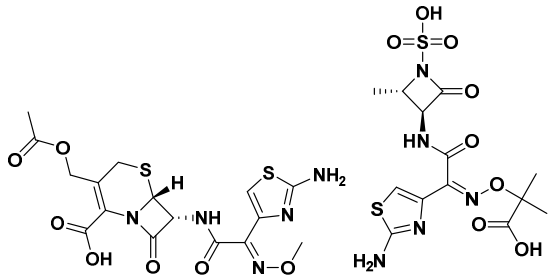
Figure 9. Structure of Cefotiam

Third-generation cephems e.g. Cefotaxime**17**have higher efficacy for Gram-negative organisms and some drugs of this generation are also effective for *P. aeruginosa*, although the antimicrobial activity against Gram-positive organisms is generally lower than that of the first generation. These drugs are effective not only for Gram-positive and Gram-negative bacteria but also anaerobes and their antimicrobial activity is strong. The monobactam antibiotic aztreonam**18** exerts an antimicrobial effect only on Gram-negative bacteria.[39] The structures of compounds **17** and **18** are depicted in **Figure 10**.



17 18

Figure 10. Structure of some of the third generation cephalosporin

1.6 Mode of action of antimicrobials

Antimicrobial agents act by altering the cell wall synthesis, ribosomal function, folate synthesis, nucleic acid synthesis and plasma membrane integrity as indicated in **Table 3**.[40]

- **Inhibition of cell metabolism**

Antibacterial agents which inhibit cell metabolism are called antimetabolites. These compounds inhibit the metabolism of a microorganism, but not the metabolism of the host.

They do this by inhibiting an enzyme catalyzed reaction which is present in the bacterial cell, but not in animal cells. The best known examples of antibacterial agents acting in this way are the sulfonamides.

- **Inhibition of bacterial cell wall synthesis**

Inhibition of cell wall synthesis leads to bacterial cell lysis (bursting) and death. Agents operating in this way include penicillins and cephalosporins. Since animal cells do not have a cell wall, they are unaffected by such agents e.g. penicillin **2**.

- **Interactions with the plasma membrane**

Some antibacterial agents interact with the plasma membrane of bacterial cells to affect membrane permeability. This has fatal results for the cell e.g. Polymyxins B, depicted in **Figure 1**, operate in this way.

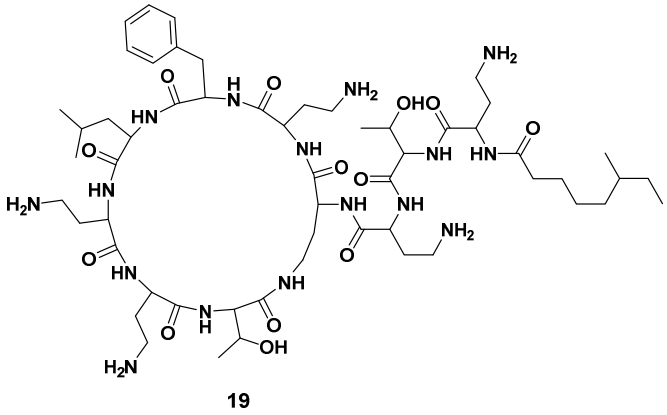


Figure 11. Structure of Polymixin B

- **Disruption of protein synthesis**

Disruption of protein synthesis means that essential enzymes required for the cell's survival can no longer be made. Agents which disrupt protein synthesis include the Tetracycline **5** and Chloramphenicol **8**.

- **Inhibition of nucleic acid transcription and replication**

Inhibition of nucleic acid function prevents cell division and/or the synthesis of essential enzymes. Agents acting in this way include Proflavin**20**, as depicted in **Figure 12.**

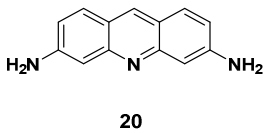


Figure 12. Structure of Proflavin

Table 3. Mode of antimicrobial actions[18]

Mode of action	Drugs
Cell membrane	• polymixin • bacitracin • colistin
Cell wall synthesis beta-lactams	• penicillins • carbapenems • cephalosporins • vancomycin
DNA replication	• quinolones
DNA –dependent RNA polymerase	• rifampicin
Folic Acid metabolism	• trimethoprim • ormetoprim • sulphonamides
Protein synthesis	30S Ribosome • tetracycline • aminoglycosides
Protein synthesis	50S Ribosome • chloramphenicols • clindamycin • erythromycin

When bacterial cells multiply and divide they make new molecules of DNA, RNA and protein. From their environment they obtain smaller units of amino acids and sugars which are found on their cell walls and cell membrane. Antimicrobial agents act on specific targets,which is the basis of their antimicrobial action.[13]

1.7 Mode of antimicrobial resistance

There are basic mechanisms by which microorganisms can be resistant to antimicrobial agents. These cases of acquired resistance are of greater concern, where initially a number of susceptible bacteria become unresponsive to an antibacterial agents and spreads under the selective pressure of use of that agent, as shown in **Figure 13**.

These mechanisms are:

- Development of altered receptors for a drug.
- Decrease in the amount of drugs that reach the receptor by altering bacterial cell wall that no longer contain the binding site of antimicrobial agent or increase in removal of the drug by efflux pumps.
- Inactivation of the drug.
- Synthesis of resistant metabolic pathways.
- Organism possess enzymes that destroy the antibacterial agent before it can have an effect e.g. β-lactamases.
- Bacteria can acquire mutations that limit entry of antimicrobial agents to the intracellular target site.[41]

Usually the bacteria can possess one or all of these mechanisms.While antibiotics are effective against majority of infections, there is also an increase in resistance by some pathogens. This threatens to undermine few remaining drugs on market that are still effective against them.

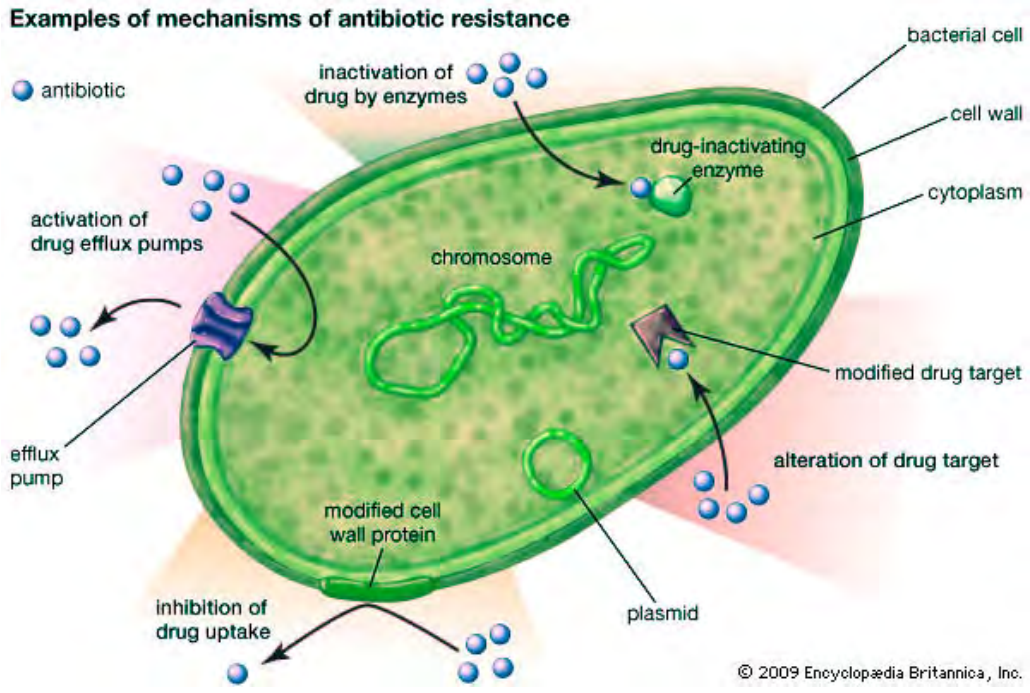


Figure 13. Mode of antimicrobial resistance.[42]

The ability of the bacteria to establish new mutant variants against man made antibiotics means antibiotic resistance will remain a threat for many coming decades. However there is

an urgent need to develop new drugs to combat resistance at different levels in pathogenic microorganism.[43]

1.8 Need for development of antimicrobial agents

The remarkable success of antimicrobial drugs generated a misconception in the late 1960s and early 1970s that infectious diseases had been conquered. However, 40 years later, infectious diseases remain the second-leading cause of death worldwide. Furthermore, the emergence of multidrug-resistant bacteria has created a situation in which there are few or no treatment options for infections with certain microorganisms.[44]The threat of bioterrorism, which gained widespread public attention after 11 September 2001, has expanded the problem because genetic changes in pathogens could render them resistant to currently available antimicrobials.[45] Pattern in rates of death from infectious disease in the 20th century (from 1900 to 1980) revealed that the rate has dropped from 797 per 100,000 people to 36 per 100,000 people, a reduction by a factor of more than 20 and a testament in part to the efficacy of antibiotics(**Figure 14**).However, from 1980 to 2000, that rate doubled, largely because of HIV but also due to the spread of drug-resistant bacterial pathogens, such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant E*nterococci*, multiple-drug-resistant gram-negative bacteria, and multiple-drug-resistant tuberculosis (MDR-TB).[45]

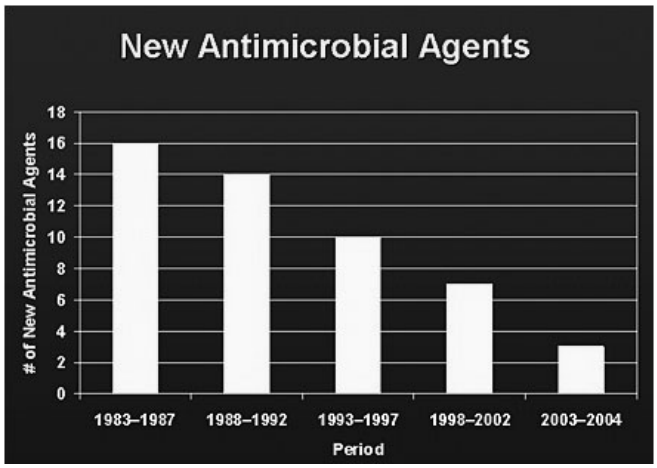


Figure 14. Trends in developments of antimicrobial agents

While the rise in mortality is due partly to infection in more seriously ill or immuno-compromised patients, there is no doubt for the need for new strategies and new molecules to treat pathogens that are resistant to nearly the full range of contemporary antibiotics. We are at a critical point at which infections caused by some bacterial pathogens are untreatable. The

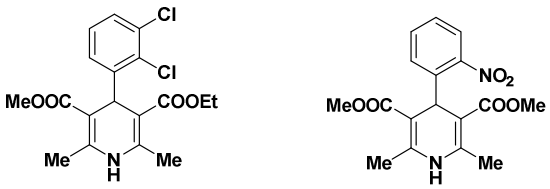
need for new antimicrobial agents is greater than ever because of the emergence of multidrug resistance in common pathogens, the rapid emergence of new infections, and the potential for use of multidrug-resistant agents. Unexpectedly, some pharmaceutical companies have indicated that they are limiting anti-infective research programs. Despite the critical need for new antimicrobial agents, the development of these agents is declining. Solutions encouraging and facilitating the development of new antimicrobial agents are needed.

1.9 Synthesis of Dihydropyrimidinones

1.9.1 Introduction dihydropyrimidine

Diverse Biginelli products by structural manipulation have shown various biological activities such as anti-tubercular[46], antiviral[47], antitumor[48], antifungal[24], anti-inflammatory[49] and antibacterial activity.[50]

Dihydropyrimidinones, aBiginelli productis an important class of medicinal scaffold and found as structural component of many biologically active drugs in market. Hence the development of efficient and general synthetic methods for construction of dihydropyrimidinones is an important research challenge with high applicability in pharmaceutical industry. **Figure 15**shows some of dihydropyrimidinones i.e. felodipine**21**and nifedipine**23.**



21
Felodipine

22
Nifedipine

Figure 15. Structure of Dihydropyrimidinones

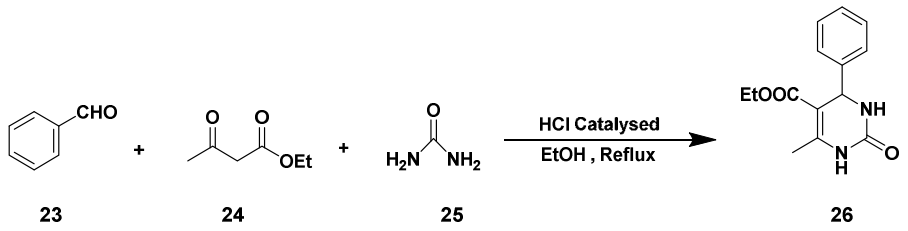
Monastrol was the first drug congener of dihydropyrimidines class synthesized by Biginelli reaction with significant anticancer activity. Monastrol has ester functional in its structural scaffold. During an attempt in anticancer research another dihydropyrimidine withfuryl substitution was synthesized and was found to be more potent than monastrol.

Chemistry of dihydropyrimidines is recorded long back in the history. Dihydropyrimidines are of great interest for Pharmaceutical Chemistry. Heterocycles that consists of dihydropyrimidinepharmacophore are reported to exhibit antihypertensive, anti-HIV, antitumor, antiepileptic, antimalarial, antimicrobial, anti-inflammatory, antitubercular and

antibacterial activity. Over the years, much attention was given for the synthesis of dihydropyrimidines. Nowadays the emphasis has been put on understanding the course of reaction with more emphasis on structural variants. Different methods for the synthesis of dihydropyrimidines are explained below in brief. Present work deals with exploration of Biginelli's reaction for development of heterocyclic motifs with antimicrobial potential activity.

1.9.2 Synthesis: Biginelli condensation reaction

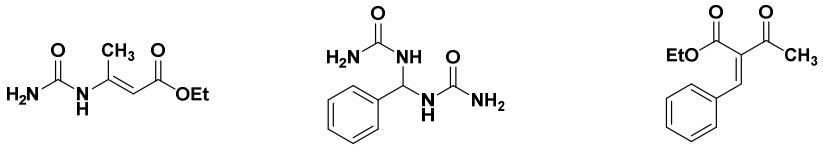
Historically the first reaction was reported by Pietro Biginelli from University of Florence[40](1893).Biginelli did the reaction as acid catalyzed condensation of ethyl acetoacetate, benzaldehyde and urea in ethanol. The mixture was refluxed and on cooling he obtained a product that was solid crystals and the product was 3,4-dihydropyrimine-2(1H)-one[43]. It was a three component reaction as depicted below.[50]



Scheme 1. The acid catalyzed formation of 3, 4- dihydropyrimine-2(1H)-one (**26**)

Hydrochloric acid was the acid used and the reaction was called Biginelli condensation reaction. In the early part of the 20th century Biginelli's work was ignored. The work remained unexplored for some time. Interest started increasing around 1970s and 1980s and it was eventually extended by alteration of all the three building blocks.

Folkers*et al.* made the first attempt to research and understand Biginelli's reaction in 1933. The reaction was done under acidic condition. They could prove intermediate 1,1-(phenylmethanediyl)-di-urea, as depicted in **Figure 16**,to transform to end product of Biginelli compound.[51]After few decades Sweet and Fissekis (1973) reinvestigated the same reaction.[51]They came up with a mechanism that opposed Folkers suggestion.

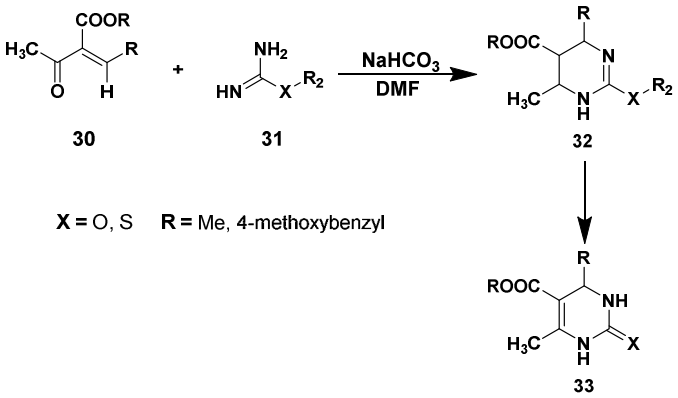


27 28 29

Figure 16. Structure of Intermediates compounds by Folkers and Johnson

1.9.3 Knoevenagel condensation and reaction with urea (2-step reaction)

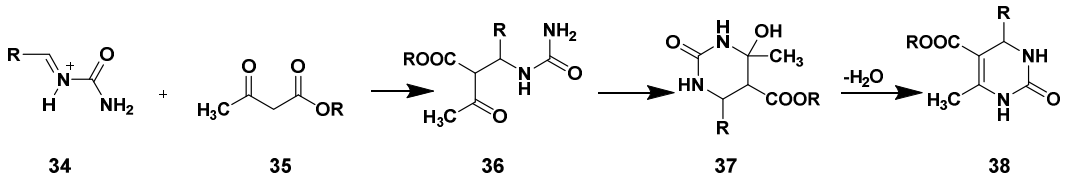
Atwal and O'Reilly *et al* (in 1987) did a two-step process and reported troubles associated with Biginelli compounds. There was a poor yield, with aliphatic aldehydes and aldehydes having hindered carbonyl function with*ortho* substituents. The first step was about synthesis of unsaturated carbonyl compound by Knoevenagel condensation.[50]The second step was base catalyzed addition of substituted ureas, as depicted below.



30 31 32 33

Scheme 2. The base catalyzed formation of dihydropyrimidines

The modified Biginelli reaction was not exploring much since it involves two steps. Kappe (1997)[52]further reexamined the mechanism of Biginelli reaction based on spectral techniques like $^{1}H/^{13}C$ NMR. According to his mechanism shown below the first step evidently involved nucleophilic attack of urea. The nucleophilic attack was on the electron deficient carbon of aldehyde function. It was an acid catalyzed reaction that resulted in the formation of N-acyliminium ion as the precursor from an aldehyde and urea compound. The second step was addition of a *pi* nucleophile ***i.e.*** methylene adding onto the intermediate. He concluded that dihydropyrimidines were formed in small quantities.[43, 53-61]

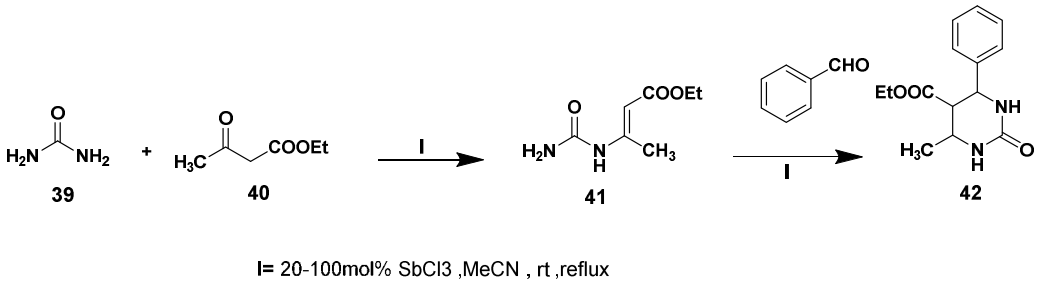


Scheme 3. Synthesis of 3,4-dihydropyrimidinone through N-acyliminium ion

Saloutina and coworkers replaced acetoacetic ester with $CF_3COCH_2CO_2Et$. They isolated intermediate on the scheme. The next step was dehydration using *p*-toluene sulphonic acid. The dehydration part in this mechanism was also investigated using $GaCl_3$ as Lewis Acid. They found out that anhydrous $GaCl_3$ gives high yields as compared to hydrated $GaCl_3$. Hydrated $GaCl_3$ does not perform well in the reaction.

1.9.4 Reaction with antimonytrichloride as a catalyst

In 2007 Cepenac*et al*[62] used a different catalyst antimonytrichloride, which was a Lewis acid catalyst. The reaction was found under that condition that proceeds *via* intermediate **41,** which was different as proposed by Kappe*et al.* with iminium formation, as depicted in **Scheme 4**.



Scheme 4. Synthesis of 3, 4-dihydropyrimidinone through 3-ureido-crotonates

Antimony (III) Chloride was found to be very efficient and the reaction proceeds with good to high yields. Although it's not an ideal catalyst, it promoted preparation to sterically hindered dihydropyrimidines.

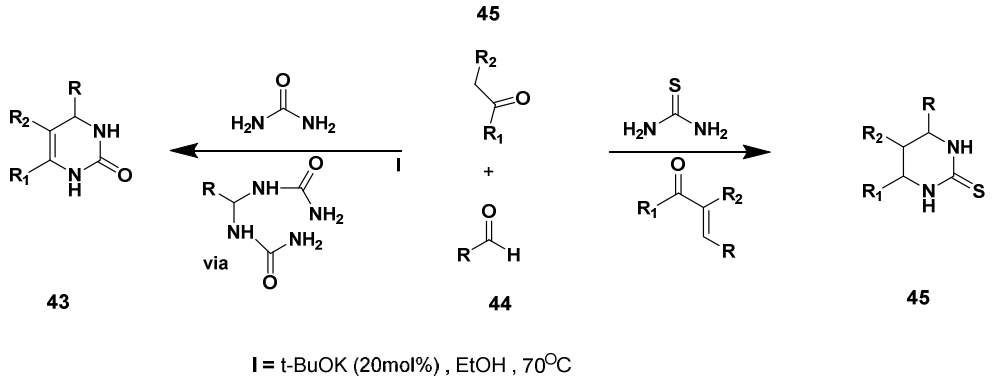
1.9.5 DFT Study

In 2008 Jian–Hua Zhou (DFT study) investigated the procedure described by Biginelli, which is condensation of urea, benzaldehyde and ethylacetoacetate under classical reaction conditions. According to Kappe's mechanism, five intermediate structures were optimized and four transition states found. Their calculated results proved that Kappe's proposed mechanism was right.[63]

In 2009 De Souz d *et al*did some mechanistic investigations of Biginelli reaction.[64]They supported Fokker's and Johnson's proposal and their conclusion was based on density functional theory calculations (DFT). Mass spectrometer with accessories for various ionizations was used whereas DFT calculations was used to investigate the Biginelli's proposed major competing mechanism.[64]

1.9.6 Reaction with Nickel (II) nitrate hexahydrate as a catalyst

Boumoud*et al* also reported their investigations using nickel (II) nitrate hexahydrate as catalyst. They supported Folker's mechanism. Most of the investigations were on the use of the Lewis acidand Lewis acid like catalysts and few papers which described the use of basic catalysts. Chinese researchers recently came up with the use of strong bases and also proposed a different pathway, as depicted in **Scheme 5**.The reaction proceeded well in the presence of Bronsted base and they obtained moderate to good yield. Enone and bisurea were suggested as reaction intermediates for reactions involving thiourea and urea as substrates.[65]



Scheme 5. The base catalyzed formation of 3,4-dihydropyrimidinone (11)

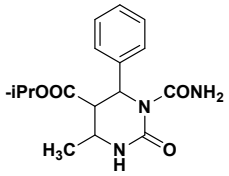
The above mentioned mechanisms and processes re-investigations with catalysts were found to be perspective trend in the course of Biginelli reaction as compared to what have been reported earlier on motto that reaction seemed to follow Kappe Mechanism. There is another mechanism report using hexaaqua-Al (III)BF4 [66] as a mild acid catalyst.

1.10 Biological activity of dihydropyrimidinones

1.10.1 Antihypertensive

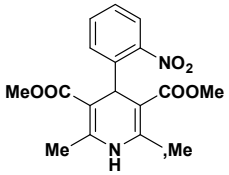
Some biologists found Biginelli products resembling with that of Hantzsh's 1,4-dihydropyridine. These were the side products of Biginelli reaction in the initial experiment such as the *aza-* analogues of Nifedipine**47** and other known compounds which are calcium

channel modulators. Biginelli compounds are promising structural targets for bringing them to medicinal use. **Figure 17** depicted below showing some resemblance between Biginelli product and an existing antihypertensive drug.



46

Biginelli product

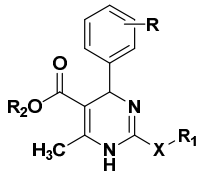


47

Nifedipine

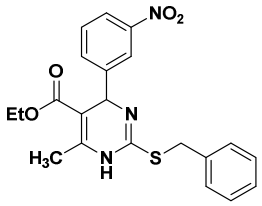
Figure 17. Biginelli's product **46** and antihypertensive compounds**47**

Figure 18 displayssomeorally effective antihypertensive agents. Hetero-substituted dihydroxypyrimidine (DHPM) shown below with branched ester and an ***alkyl-thio-*** group like S-Me, was found to have optimal antihypertensive activity.



48

R =2-NO_2, 3-NO_2, 2-CF_3 , 3-Cl
R_1 = Me , $CH_2CH{=}CH_2$
R_2 =ET , i-Pr , Me, SBu
X = O, S

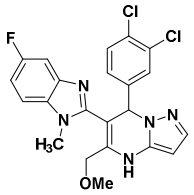


49

Figure 18.Antihypertensive compounds

1.10.2 Potassium channel antagonists

The compound shown in **Figure 19** was annulation of benzamidole ring with Biginelli displayed potassium channel antagonistic activity. These are under preclinical developments.[67-69]

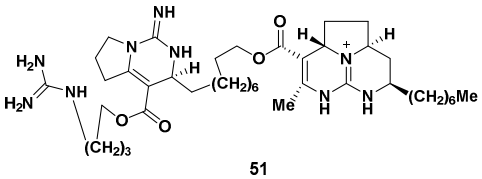


50

Figure 19. Potential Potassium antagonist

1.10.3 Anti-HIV

Batzelladine and its derivatives of DHPMs were obtained from marine natural source. They have promising anti-HIV activity. Batzelladine's low molecular weight derivatives were found to inhibit the HIV virus by binding to gp-120 to CD4 cells.[47]**Figure 20** displays the structure of BatzelladineA**51**.



51
Batzelladine A

Figure 20. Structure ofBatzelladine A

1.10.4 Antitumor

Monastrol is the first Biginelli compound which has very good anticancer activity. Some compounds have also been investigated for their action as kinesin Eg5 inhibitors. Furyl derivatives **53** were also discovered during this attempt and it was found to be more potent than Monastrol as depicted in **Figure 21**.[48]

52 53

(S)Monastrol

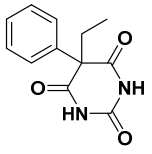
Figure 21. Monastrol**52** and potential anticancer compounds**53**

Some of the reported Eg5 inhibitors were Enastron, Dimethylenastron and Fluostrastrol.[48]Monastrol's**52.**Thepotency was compared to these new inhibitors and they

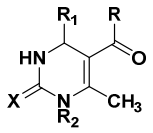
were found to have a better fit of the ligand at the allosteric binding site. Some pyrimidinone-peptide hybrid molecules were also identified as Hsp70 modulators that inhibit cell proliferation.

1.10.5 Antiepileptic

Phenobarbital is the drug of choice of epilepsy. Biginelli's compounds have a structural similarity with Phenobarbital. The compounds,shown in **Figure 22**, were investigated for epilepsy. Theyshowed promising antiepileptic activity.[70]



54



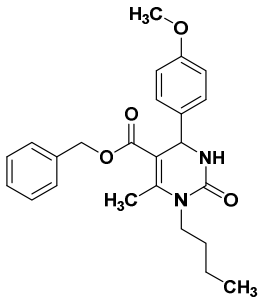
55

R = OMe, Me, NH_2 , OMe
R1= Ph, 4-Br-C_6H_4, 3-OMeC_6H_4
R2= H, Me
X = O, S

Figure 22. Structure of Phenobarbital**54**and potential antiepileptic compounds**55**

1.10.6 Antimalarial

Other pyrimidine-amides derivatives of DHPMs are new class. They belong to Hsp 70 modulators. Nine compounds are under clinical investigation against antimalarial activity because they inhibited the replication of pathogenic *P. falciparum.*[24] The structure of thepotential antimalarial compound is shown in **Figure 23**.

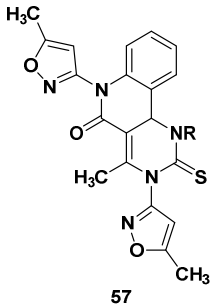


56

Figure 23. Structure of potential antimalarial compounds

1.10.7 Anti-microbial

1-aryl-4-methyl-3,6-bis(5-methylisoxazol-3-yl)-2-thioxo-2,3,6,*10b*-tetrahydro-1H pyrimido[5,4-c]quinolin-5-ones, which was a Biginelli product, has shown potent antimicrobial activity such as antibacterial, antifungal and antimalarial activities.[24]



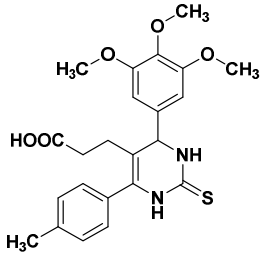
57

R= C_6H_5 , 4-$CH_3OC_6H_4$, 4-BrC_6H_4 , $C_6H_5CH_2$, 4-ClC_6H_4 , 3-$CH_3OC_6H_5$, 2-ClC_6H_4

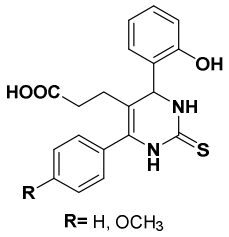
Figure 24. Structure of potential antimicrobial compounds

1.10.8 Anti-inflammatory

3-(4,6-disubstituted-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-propionic acid derivatives were screened and showed anti-inflammatory activity by rat paw edema method. Further some of the substituted Biginelli compounds in this series with cyanide and ester functionalities were found to have promising antibacterial agents.[49, 71]**Figure 25** depicts structures of two potential anti-inflammatory compounds.



58



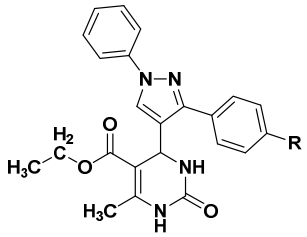
R= H, OCH_3

59

Figure 25. Structure of potential anti-inflammatory compounds

1.10.9 Antitubercular agents

Dihydropyrimidines were found to have antitubercular activity against *Mycobacterium tuberculosis* H37Rv. Significantly, two compounds ethyl 4-[3-(4-fluorophenyl)-1-phenyl-1H-pyrazol-4-yl]-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate and ethyl-4–[3-(4-nitrophenyl)-1-phenyl-1H-pyrazol-4-yl]-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[72, 73] (as shown in **Figure 26**)were found to be more potent and more active than isoniazid.



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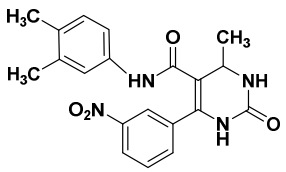


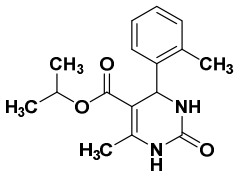
61

R= F, NO_2

Figure 26. Potential antitubercular compounds

1.10.10 Antibacterial

Substituted Biginelli compounds (**Figure 27**)were found to exhibit promising antibacterial activity. This includes some ester and cyanide as depicted below.[23, 25, 46, 74]



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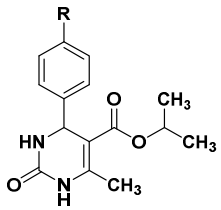


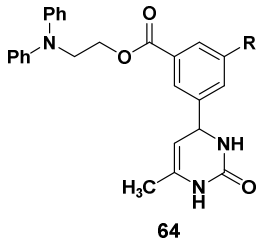
63

R= Cl, NO_2 , F

Figure 27. Potential antibacterial compounds

1.10.11 Antioxidants and antifilarial

Some Biginelli compounds (**Figure 28**)have been found to have antioxidantand antifilarialactivity.[75-77]



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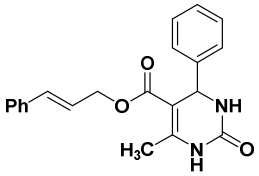


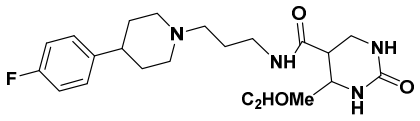
65

R= H, NO_2

Figure 28. Potential Antioxidants and antifilarial compounds

1.10.12α_{-1A} adrenergic receptor antagonists

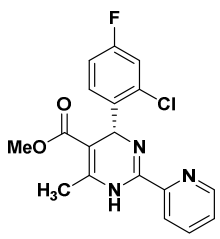
Biginelli class of compounds have been found to act as alpha 1A adrenergic receptor antagonists.[78]



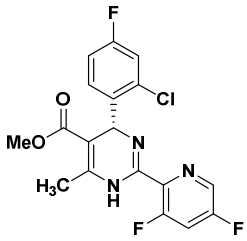
66

Figure 29. Potential antiadrenergic compounds

1.10.13Anti-HBV (hepatitis B virus) agents.[79]



67

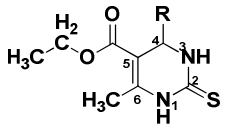


68

Figure 30. Potential anti HBV compounds

1.11 Characterization of dihydropyrimidinones

The principal tools used for the characterization of dihydropyrimidines were Nuclear Magnetic Resonance (NMR) Spectroscopy and Infra-red spectroscopy (IR).Confirmation of dihydropyrimidines structure was done by spectroscopic techniques such as ^{1}H NMR and ^{13}C NMR. Fromliterature, the presence of a characteristic singlet peak in ^{1}H NMR at 2.36 ppm corresponds to the CH_3 of the pyrimidine. A singlet peakresonating around 8.54 ppm was attributed to NH proton of the pyrimidine ring.[80]In the ^{13}CNMR spectra of dihydropyrimidine, the carbon signals (C2, C3, C5, and C6)appeared at δ166.3, 144.9, 159, and 17.9 respectively. Different functional groups of synthesised compounds were determined using Infra-red spectroscopy. The presence of the characteristic band around 3100-3300cm^{-1}show the presence of (N-H stretches) of primary amines in compounds 3a-3k.



R = Cl, Fl, OCH_3, C_6H_6, $C_5H_{10}O$, $C_8H_8O_2$

69

Figure 31. Dihydropyrimidine

1.12 Rationale and motivation

Currently due to various factors (resistance, misuse/abuse of drugs and other environmental factors) infections caused by some bacterial and fungal pathogens are untreatable. The need for development of new antimicrobial agents is more than ever because of the emergence of multidrug resistance in common pathogens, the rapid emergence of new infections, and the potential for use of multidrug-resistant agents. In spite the urgent need for new antimicrobial agents, the development of these agents is declining. Solutions encouraging and facilitating the development of new antimicrobial agents are needed. It is well documented as mentioned earlier thatBiginelli products have shown diverse biological activities such as anti-tubercular, antiviral, antitumor, antifungal, anti-proliferative, anti-inflammatory and antibacterial activity. Dihydropyrimidinones, aBiginelli productis an important class of privileged medicinal scaffold and found as structural component of many biologically active drugs in market including antibacterial and antifungals. These observations have motivated us to synthesize some analogs of dihydropyrimidinones by using Biginelli reaction conditions and evaluate them for potential antibacterial and antifungal activities.

1.13Research aims and objectives

- To design the scheme for synthesis of proposed compounds
- To synthesize the title compounds by an proposed method
- To carry out the required confirmatory test such as TLC for monitoring/completion of the reaction.
- To confirm the structures of the synthesized compounds by IR, ^{1}HNMR and Mass spectroscopy.
- To evaluate the proposed compounds for their antimicrobial activity.

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CHAPTER II

EXPERIMENTAL

2 Experimental

2.1 General Procedure

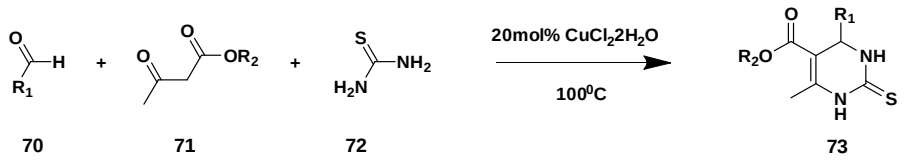
The synthesized compounds were identified and characterized using the following methods:

- Melting point determination
- Thin Layer Chromatography
- Nuclear Magnetic Resonance Spectroscopy (NMR).

This was done to show that all prepared compounds were of different nature than the starting material. Melting point determination was carried out in closed capillaries using electrothermal 9300 digital melting point apparatus. H^1 and ^{13}C were all recorded using a Bruker Advanced III 400MHz spectrometer. All the spectras were all recorded at room temperature. The chemical shifts were recorded in deuterated chloroform ($CDCl_3$) against the internal standard of trimethylsilane. The reactions were all monitored by using TLC Thin Layer Chromatography on MERCK 0.25 SILICA GEL ($60F_{254}$) and were visualized with UV light. Perkin Elmer spectrum 100 FT-IR spectrophotometer was used to record IR spectra.

2.2 Synthesis of dihydropyrimidinones

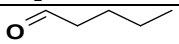
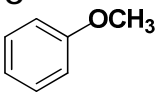
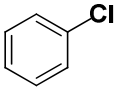
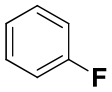
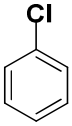
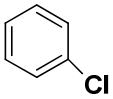
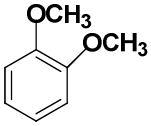
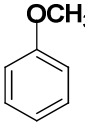
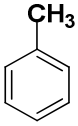
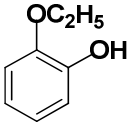
In a 100 ml round bottomed flask, benzaldehyde (10mmol, 1g) was added.Ethylacetoacetate(10 mmol) and urea (12mmol) was also added into the same 50ml round bottomed flask .$CuCl_2.2H_2O$ was used as a catalyst. The mixture was stirred rapidly and refluxed at 100^oC - 110^oC for 3 hrs in the absence of a solvent. Cupric chloride and urea dissolved after some few minutes and the solid mixture changed to oily after about 20mins. After 30mins of gradual stirring the oily mixture changed to solid. Stirring was continued for the remaining 2hrs 30mins. TLC was used to monitor the reaction. After 3hrs elapsed the reaction was cooled at room temperature. Ice was added to the solid mixture and crushed until separated solid particles were obtained. The separated solid particles were filtered and checked using TLC for purity. If not pure the solid particles were purified by crystallization whereby the particles were dissolved in a beaker of 20ml ethanol and heated until 5ml ethanol remained. The mixture was left to crystallize. The same process was successfully applied to structurally varied aldehydes to yield 3,4-dihydropyrimidine-2(1H)-ones as depicted in**Scheme 6**and **Table 4**below.[81]



R_1 = C_4H_9, C_7H_80, C_6H_5Cl, C_6H_5F. C_6H_5Cl, C_6H_5Cl, $C_8H_{10}O_2$, C_7H_8O, $C_8H_{10}O_2$, C_6H_6
R_2 = C_2H_5

Scheme 6. Overall Procedure of the synthesized dihydropyrimidinones compounds

Table 4. Dihydropyrimidines derivatives

Serial number	-R_1	-R_2
3a		C_2H_5
3b		C_2H_5
3c		C_2H_5
3d		C_2H_5
3e		C_2H_5
3f		C_2H_5
3g		C_2H_5
3h		C_2H_5
3i		C_2H_5
3j		C_2H_5
3k		C_2H_5

Some of the compounds were synthesized using microwave reactor. Absolute ethanol was used as a solvent in the microwave.The purifying method was the same as the one mentioned above.[80]

2.3 *In vitro* antimicrobial activity

The dihydropyrimidinones **(3a-3k)** were further assessed for antimicrobial activity against panel of bacterial and fungal strains by following earlier reported MIC assay methodusing resazurin dye.[82]

2.3.1 Microorganism used

Standard cultures of two gram +ve [***Staphylococcus aureus***ATCC25923**,** ***Bacillus subtilis***ATCC6051]**,** two gram -ve [***Escherichia coli*** ATCC35218**,** ***Pseudomonas aeruginosa*** ATCC27853], three fungal strains [***Candida albicans***ATCC90028, ***Cryptococcus neoformans***ATCC66031 and ***Aspergillusniger***ATCC16404]were used for the antibacterial and antifungal activity respectively. Culturing and subculturing (one day prior to testing) of these microorganisms was carried out at the department of microbiology, Inkosi Albert Luthuli hospital, Durban, South Africa. Subculturing of these microorganisms was used in this assay.

2.3.2 Preparation of medium

The nutrient medium was prepared by dissolving 22 g of Muller-Hinton Broth containing acid hydrolysate of casein, beef extract and starch in 1 L of double distilled water. The pH of this medium was adjusted to 7.4 ± 0.1 and sterilized by autoclave for 15 min at 121℃. The solution was allowed to cool and stored at a temp of 4℃. Sterility check was performed by incubating un-inoculated media in an aerobic incubator at 37 ℃ for 18-24 h. For antifungal activity, RPMI 1640 medium with L-glutamine and 0.165 M MOPS and without sodium bicarbonate (Lonza) was used.

2.3.3 Preparation of test compounds (stock solution and working standard)

An accurately weighed quantity (4.000 mg) of the synthesized compounds and standard drugs were dissolved in 1 ml of DMSO to give stock solution (4000 µg/mL). Further, 100µl of stock solution was diluted with 900 µl of double distilled water to afford working standard solution (400 µg/mL).

2.3.4 Preparation of inoculum

One day prior to testing one or more identical colonies of microorganisms were suspended in4.5 ml sterile double distilled water. The inoculates were adjusted to 0.5 McFarland standard (1.5 X 108 cfu/mL). A density check turbidmeter was used to ensure that the inoculum was a 0.5 McFarland standard.

2.3.5 Broth micro-dilution method

The preliminary in vitro antimicrobial activity for the newly synthesized title compounds (**3a to 3k**) was evaluated using the broth micro-dilution method.[83, 84]100 µl of sterile

doubledistilled water was added to all outer-perimeter wells of a 96-well microliter plates tominimize evaporation of the medium in the test wells during incubation. To the remainingtest wells 100 µl of MHB was added. Two fold serial dilutions of the test compounds andstandard drugs (amoxicillin and Amphotericin B) were made directly on the microplate usingMHB. The compounds were tested at final concentration of (200, 100, 50, 25, 12.5, 6.25, 3.125,1.56, 0.78, 0.39 µg/ml). Finally, 10 µl of the freshly prepared bacterial or fungal inoculumwas added to the wells. The microliter plates were covered and sealed with parafilm andincubated at 37 ± 1 ^{0}C for 24 h. After this, 10 µl of freshly prepared resazurin (0.4 mg/ml)was added to the test wells and incubated further for 5h. MIC was determined as a bluecolour in the test well was interpreted as no bacterial growth and a pink colour was scored asgrowth. The MIC was thus defined at the lowest drug concentration that prevented a colourchange from blue to pink. This experiment was conducted in duplicate and the average MICvalues in µg/ml.

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CHAPTER III

RESULTS AND DISCUSSION

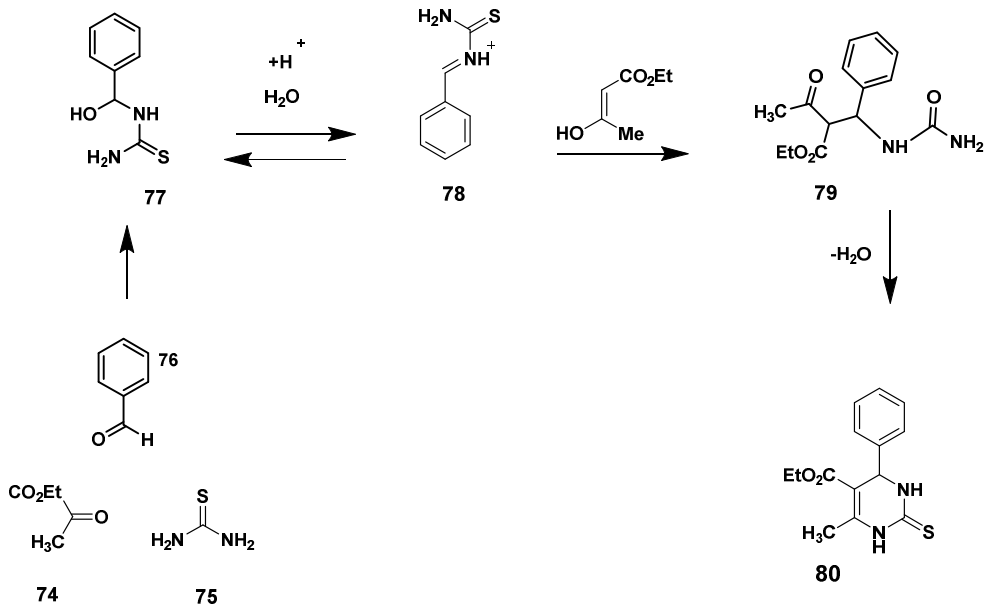
3 Results and discussion

Eleven compounds were successfully synthesized using Biginelli condensation reaction. It was a one-step reaction. All products were substituted at 1,2,3,4 positions of the heterocyclic ring with positions 1, 2, 3 being the same for all compounds with NH group on carbon 1 and 2 and S group on carbon 3. An ester was found at position 5 and a methyl group at position 6.

3.1 Chemistry

3.1.1 Synthetic data

During the reaction there is a formation of N-acyliminium**79** after thiourea **75** and benzaldehyde**76** have been mixed. This is through an unobserved (^{1}HNMR) hemiaminal**77**. Combination of an enol tautomer of ethylacetoacetate leads to the formation of dihydropyrimidine**80**as a precursor. The rate limiting step is the first step that leads to the formation of **79**, hence preventing **77** and **78** from being observed on NMR.



Scheme7. The mechanistic pathway for the formation of dihydropyrimidines

3.1.2 Physical properties

Generally all the compounds appeared orange, yellow and light yellow solids. The yields were between 85-95% for all compounds synthesized. The melting points were between 135 and 221^{0}C. The data is summarized in **Table 5.**

Table 5. Physical properties of the synthesized compounds

Serial No.	Nature of crystals	% Yield	Melting Point ^{0}C
3a	Light yellow crystals	85	137-138
3b	Yellow crystals	95	152-153
3c	Yellow crystals	96	201-202
3d	Yellow crystals	85	135-136
3e	Light yellow crystals	96	177-178
3f	Yellow crystals	80	142-143
3g	Orange crystals	95	160-161
3h	Light yellow crystals	96	150-151
3i	Light yellow crystals	98	175-176
3j	Yellow crystals	98	220-221
3k	Light yellow crystals	85	204-205

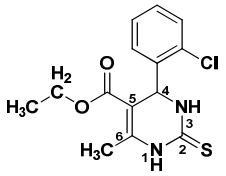
3.1.3 Spectroscopic data

3.1.3.1 Characterization by ^{13}CNMR and ^{1}H NMR

The dihydropyrimidine which is the pharmacophore of the structure was identified in all compounds with NH-proton resonating around δH 7.3–8.0 as a singlet. The aromatic hydrogen appeared around δH7.0-7.8, while the methine proton signalsobserved as multiplet in the ^{1}H NMR, appeared around δH 5.30-5.91. The multipletsignal around δH 4.00-4.15was attributed to CH_2protons. The CH_3 (methyl) protons of the pyramidine ring resonatedas a singlet around δH1.14-2.50.

In^{13}C NMR, the C=S aromatic carbon peak was observed around δ145.88-176.00 and heterocyclic carbons were found just down filed of the solvent peak around δ 100.00-160.00. The methine and methyl carbon signalsappeared slightly up filed of the solvent peak betweenδ 60.0 and 13.0.The structures of synthesized compounds were established on the basis of their spectral analytical data (FT-IR, ^{1}H NMR, and ^{13}C NMR) as shown below.

Ethyl-4-(2-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[85]



81

Molecular Formula: $C_{14}H_{15}ClN_2O_2S$ **Formula weight**:310.80gmol^{-1}

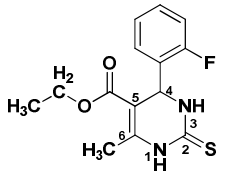
Physical description: Yellow crystals; **Yield**: 80% (0.8 g); **Melting point**:142^{0}C

IR (υ_{max}, cm^{-1}): 3173.73(N-H), 1706.51(C=O), 1651.31(C=C), 726.19 (C-H)

^{1}H NMR (400MHz, CDCl$_3$): δ8.29 (s,1H,NH), 7.40-7.38 (m,1H,Ar-H), 7.34 (s,1H,NH), 7.27-7.22 (m,3H,Ar-H), 5.91-5.90 (m,1H,CH), 4.05-4.00 (q,2H,CH_2), 2.46 (s,3H,CH_3 of pyrimidine ring), 1.08-1.05 (t,3H,CH_3) ppm.

^{13}C NMR(100MHz, CDCl$_3$):δ174.35 (C=S), 164.85 (C=O), 144.50-100.70 (Aromatic and Heterocyclic carbons), 60.37 (methine carbon), 52.68 (O-CH_2), 18.01 (C-4 of pyrimidine ring), 13.86 (CH_3).**Reported MP is 219^{0}C.**[85]

Ethyl-4-(2-fluorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[85]



82

Molecular Formula:$C_{14}H_{15}FN_2O_2S$ **Formula weight**:294.34gmol^{-1}

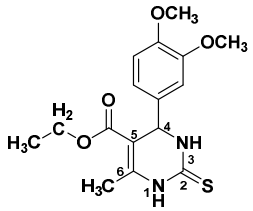
Physical description: Orange crystals;**Yield**:85% (0.8453g); **Melting point**:136^{0}C

IR (υ_{max}, cm^{-1}): 3178.05 (N-H), 1706.77 (C=O), 1650.29 (C=C), 755.44(C-H).

^{1}H NMR (400MHz, CDCl$_3$, δ):8.53 (s,1H,NH), 7.42-7.03 (m,4H,Ar-H), 7.51(s,1H,NH), 5.73 (s,1H,CH), 4.08-4.03 (q,2H,CH_2), 2.42 (s,3H,CH_3 of pyrimidine ring), 1.11 (t,3H,CH_3) ppm.

^{13}C NMR (100MHz, CDCl$_3$, δ):174.46 (C=S), 164.92 (C=O), 161.30-100.42 (Aromatic and heterocyclic carbons), 60.36 (C-4 of pyrimidine ring), 49.96 (O-CH_2), 18.06 (CH_3), 13.88 (CH_3). **Reported MP is 90^{0}C.**[85]

Ethyl-4-(3,4-dimethoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[85]



83

Molecular Formula:$C_{16}H_{20}N_2O_4S$ **Formula weight**: 336.41gmol^{-1}

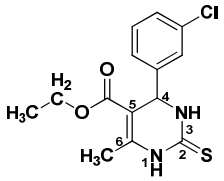
Physical description:Orange Crystals; **Yield**: 95 % (0.9653g); **Melting Point**: **161**^{0}C

IR(υ_{max}, cm^{-1}): 3305.40 (N-H), 1658.77 (C=O), 1573.76 (C=C), 762.30 (C-H)

^{1}H NMR (400MHz , $CDCl_3$, δ): 8.37(s,1H,NH), 7.80 (s, 1H, NH), 6.80-6.78 (m,3H,Ar-H), 5.33 (s,1H,CH), 4.10-4.05(q,2H,CH_2), 3.82 (s,6H,OCH_3), 2.33 (s,3H,CH_3 of pyrimidine ring), 1.17-1.14 (t,3H,CH_3) ppm.

^{13}C NMR (100MHz, $CDCl_3$, δ): 174.16 (C=S), 165.35(C=O), 148.99-102.95(Aromatic and heterocyclic carbons), 60.36(C-4 of pyrimidine ring), 55.87-55.65(O-CH_3), 18.15 (CH_3), 14.09 CH_3.**Reported MP is 214^{0}C.**[85]

Ethyl-4-(3-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[85]



84

Molecular Formula:$C_{14}H_{15}ClN_2O_2S$**Formula weight**:310.80gmol^{-1}

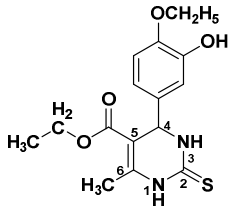
Physical description:Yellow crystal; **Melting point**: 202^{0}C;**Yield**: 96% (0.9763g)

IR (υ_{max}, cm^{-1}): 3309.86(N-H), 1661.07 (C=O), 1566.86 (C=C), 744.35(C-H)

^{1}H NMR (400MHz, $CDCl_3$, δ): 8.07(s,1H,NH), 7.55(s,1H,NH); 7.26-7.16(m,4H,Ar-H), 5.37 (s,1H,CH),4.12-4.07 (q,2H,CH_2), 2.36 (s, 3H, CH_3 of pyrimidine ring), 1.20-1.16 (t, 3H, CH_3) ppm.

^{13}C NMR(100MHz, $CDCl_3$, δ):174.84(C=S), 164.93(C=O), 144.17-102.42 (Aromatic and heterocyclic), 60.61 (C-4 of pyrimidine ring), 55.85(O-CH_2), 18.57 (CH_3), 14.08(CH_3).**Reported MPis 240 ^{0}C.**[85]

Ethyl 4-(4-ethoxy-3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[86]



85

Molecular Formula:$C_{16}H_{20}N_2O_4S$ **Formula weight**:336.41gmol^{-1}

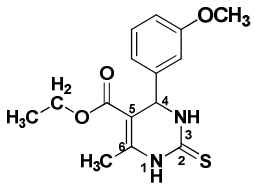
Physical description: Yellow crystals; **Melting point**:220^{0}C;**Yield**:98% (0.9876g)

IR (υ_{max}, cm^{-1}):3176.27(N-H), 1682.59(C=O), 1583.65(C=C), 740.47(C-H)

^{1}H NMR (400MHz, $CDCl_3$, δ): 7.62(s, 1H, NH), 7.09(s, 1H, NH), 6.86-6.76(m,3H,Ar-H), 5.68 (s,1H,OH), 5.32-5.31(m,1H,CH), 4.13-4.05(q,2H,CH_2), 2.34 (s,3H,CH_3 of pyrimidine ring), 2.16(s,2H,CH_2), 1.45-1.41 (t,3H,CH_3), 1.20-1.16 (t, 3H, CH_3) ppm.

^{13}C NMR (100MHz, $CDCl_3$, δ): 145.99-145.88(C=S), 141.92(C=O), 134.41(C=O), 119.69-103.27(Aromatic and heterocyclic carbons), 64.5(C-4 of pyrimidine ring), 60.45 (OCH_2), 56.15 (OCH_2), 18.51(CH_3), 1480-1414(CH_3).**Reported MP is 203^{0}C.**

Ethyl-4-(3-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate [47]



86

Molecular Formula:$C_{15}H_{18}N_2O_3S$ **Formula weight**:306.10g/mol^{-1}

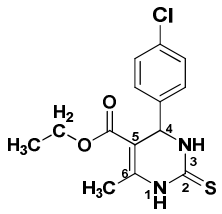
Physical description: Yellow Crystals; **Melting point**:152^{o}C;**Yield**:95% (0.9632g)

IR (υ_{max}, cm^{-1}):3294.9(N-H), 1657.8 (C=O), 1570.5(C=C), 699.6(C-H)

^{1}H NMR (400MHz , $CDCl_3$, δ):8.088(s,1H,NH), 7.41(s,1H,NH), 7.26-6.80(m,4H,Ar-H), 5.37(s,1H,CH),4.11-4.06(q,2H,CH_2),3.77(s,3H,OCH_3), 2.351(s,3H,CH_3 of pyrimidine), 1.19-1.15(t,3H,CH_3) ppm.

^{13}C NMR(100MHz, CDCl$_3$, δ):174.63(C=S), 165.21(C=O), 143.76-102.72 (Aromatic and heterocyclic carbons), 60.42 (C-4 of pyrimidine ring), 56.04(O-CH_2), 55.23(O-CH_3), 18.33 (CH_3), 13.06(CH_3). **Reported MP is 210^{0}C.**[47]

Ethyl-4-(4-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate [47]



87

Molecular Formula:$C_{14}H_{15}ClN_2O_2S$ **Formula weight**:310.80 gmol^{-1}

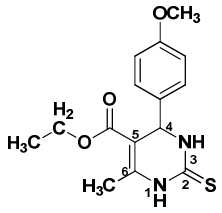
Physical description: Yellow Crystals; **Meltingpoint**:178^{0}C; **Yield**:96% (0.9735g)

IR (υ_{max}, cm^{-1}):3325.59(N-H), 1668.50(C=O), 1572.52(C=C),744.89(C-H)

^{1}H NMR (400MHz, CDCl$_3$, δ):8.31(s,1H,NH),7.82(s,1H,NH), 7.30- 7.21(m,4H,Ar-H), 5.38(s,1H,CH), 4.13-4.07(q,2H,CH_2), 2.36(s,3H,CH_3 of pyrimidine ring), 1.20-1.17 (t,3H,CH_3) ppm.

^{13}C NMR(100MHz, CDCl$_3$, δ): 174.39(C=S), 165.08(C=O), 143.05-102.61(Aromatic and heterocyclic carbons),60.55(C-4 of pyrimidine ring), 55.40(O-CH_2), 18.29(CH_3), 14.08(CH_3). **Reported MP is 215^{0}C.**[47]

Ethyl-4-(4-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[47]



88

Molecular Formula:$C_{15}H_{18}N_2O_3S$ **Formula weight**:306.10gmol^{-1}

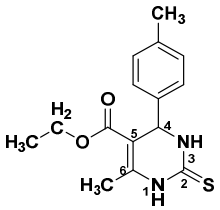
Physical description:Yellow Crystals; Melting **point:** 150^{0}C**;Yield**:96% (0.9712g)

IR (υ_{max}, cm^{-1}):3310.02(N-H), 1664.94(C=O), 1573.31(C=C), 764.60(C-H)

^{1}H NMR (400MHz, $CDCl_3$, δ): 8.20(s,1H,NH), 7.56(s,1H,NH), 7.26-6.81(m,4H,Ar-H), 5.33(s,1H,CH), 4.09-4.05(q,2H,CH_2),3.77(s,3H, OCH_3),2.34(s, 3H, $CH_3$of pyrimidine ring), 1.18-1.14(t, 3H, CH_3) ppm.

^{13}C NMR(100MHz, $CDCl_3$, δ):174.19(C=S), 165.28(C=O), 59.52-103.14(Aromatic and heterocyclic carbons), 60.36(C-4 of pyrimidine ring),55.59-55.25(OCH_3), 18.22(CH_3), 13.06(CH_3). **Reported MP is 214^{0}C.**[47]

Ethyl-6-methyl-2-thioxo-4-p-tolyl-1,2,3,4-tetrahydropyrimidine-5-carboxylate[87]



89

Molecular Formula:$C_{15}H_{18}N_2O_2S$ **Formula weight**:290.38gmol^{-1}

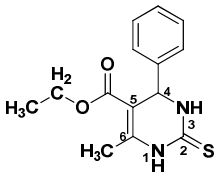
Physical description:Yellow Crystals; **Melting point:** 176^{0}C; **Yield**:98% (0.9912g)

IR(υ_{max}, cm^{-1}):3321.40(N-H), 1669.72(C=O), 1573.95(C=C), 758.80(C-H)

^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.33(s, 1H, NH), 7.67(s, 1H, NH), 7.26-7.09 (m, 4H, Ar-H), 5.34(s, 1H,CH), 4.10-4.04(q,2H,CH_2), 2.33-2.30(s,3H,CH_3 of pyrimidine ring), 2.163(s,1H,CH_3), 1.18-1.4 (t,3H,CH_3)ppm.

^{13}C NMR(100MHz, $CDCl_3$, δ):174.26(C=S), 165.29(C=O),142.79-102.99 (Aromatic and heterocyclic carbons), 60.35 (C-4 of pyrimidine ring), 55(OCH_2), 21.09(CH_3), 18.17(CH_3), 14.05(CH_3).**Reported MP is 214^{0}C.**[87]

Ethyl 6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[87]



90

Molecular Formula:$C_{14}H_{16}N_2O_2S$ **Formula weight**:276.35gmol^{-1}

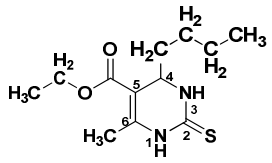
Physical description:Yellow Crystals;**Melting point:**204^{0}C; **Yield**:85% (0.8632g)

IR (υ_{max}, cm^{-1}):3324.36(N-H), 1666.27(C=O), 1573.06(C=C), 758.66(C-H)

^{1}H NMR (400MHz , $CDCl_3$, δ): 8.02(s,1H,NH), 7.401(s,1H,NH), 7.34-7.26(m,4H,Ar-H), 5.397(s,1H,CH), 4.11-4.05(q,2H,CH_2), 2.35(s,3H,CH_3 of pyrimidine ring), 1.17-1.14(t,3H,CH_3) ppm.

^{13}C NMR(100MHz, $CDCl_3$, δ): 174.57(C=S), 165.20(C=O), 142.64-102.93(Aromatic and heterocyclic carbon), 60.42(C-4 of pyrimidine ring), 56.22(OCH_2), 18.34(CH_3), 14.05(CH_3). **Reported MP is 202^{0}C.** [87]

Ethyl (4-butyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate[88]



91

Molecular Formula:$C_{12}H_{20}N_2O_2S$ **Formula weight**:256.36 $gmol^{-1}$

Physical description: Yellow Crystals;**Yield**:85% (0.8623g); **Melting Point**: 138^{0}C

IR (υ_{max}, cm^{-1}): 3175.66(N-H), 1708.75(C=O), 1593.79(C=C), 746.89(C-H)

^{1}H NMR (400 MHz, $CDCl_3$, δ):8.18(s,1H,NH), 7.68(s,1H,NH), 4.34(s,1H,CH), 4.14-4.11(q,2H,CH_2), 2.28(s,3H,CH_3 of pyrimidine ring), 1.60-1.54(m,7H), 1.521-1.46 (d,3H,CH_3) ppm.

^{13}C NMR (100MHz, $CDCl_3$, δ): 175.48(C=S), 165.48(C=O), 143.60(C-6 of pyrimidine ring), 103.02(C-5 of pyrimidine ring), 60.34 (C-4 of pyrimidine ring), 52.27(OCH_2), 36.29-22.30 (CH_2 of veraldehyde), 18.18(CH_3), 14.27(CH_3). **Reported MP is 230^{0}C.**[88]

3.1.4 Interpretation of spectral analysis

3.1.4.1 Infra-red spectroscopy

Different functional groups of synthesised compounds were determined using Infra-red spectroscopy. The presence of the characteristic band around 3100-3300cm^{-1}show the presence of (N-H stretches) of primary amines in compounds **3a-3k**as shown in **Figure 32.**

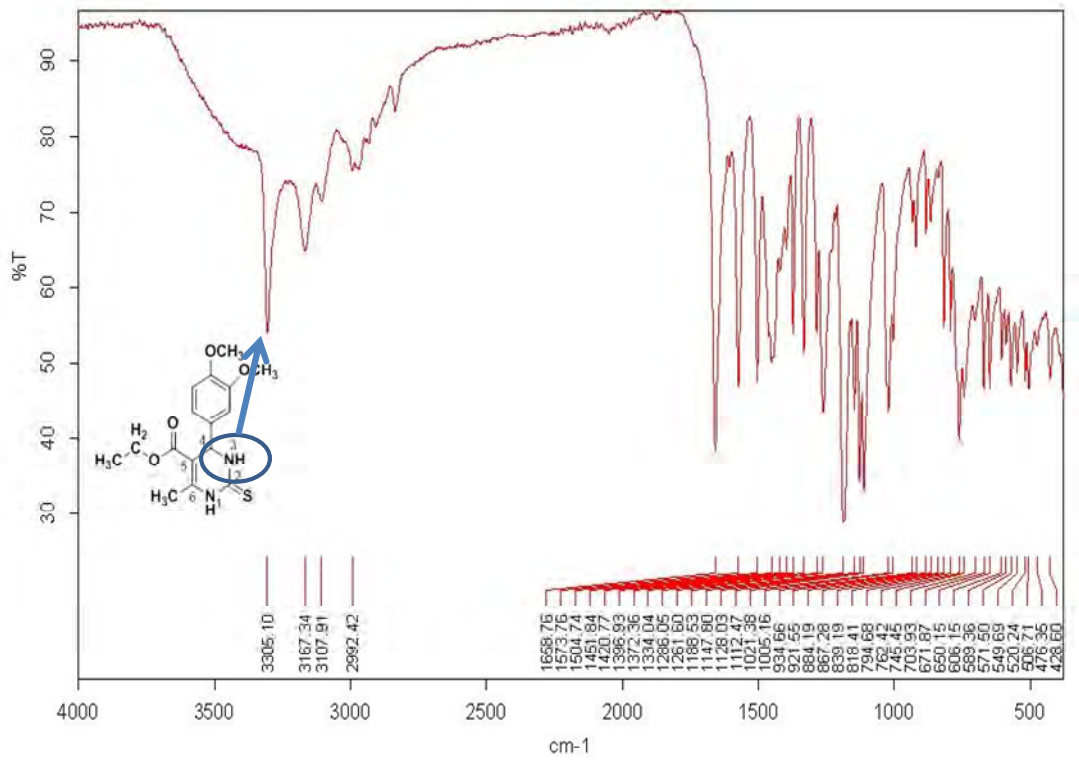


Figure 32.IR spectra of compound **3g**

3.1.4.2 ^{1}H NMR spectroscopy

From^{1}H NMRspectra of compounds **3a-3k**, it showed that the most prominent singlet signal appeared around δ 7.8 ppm and δ 8.37 ppm. It was attributed to two (NH) protons while one distinctive multiplet signal around δ 6.80-6.78ppm were assigned to aromatic protons as depicted on **Figure 33.**

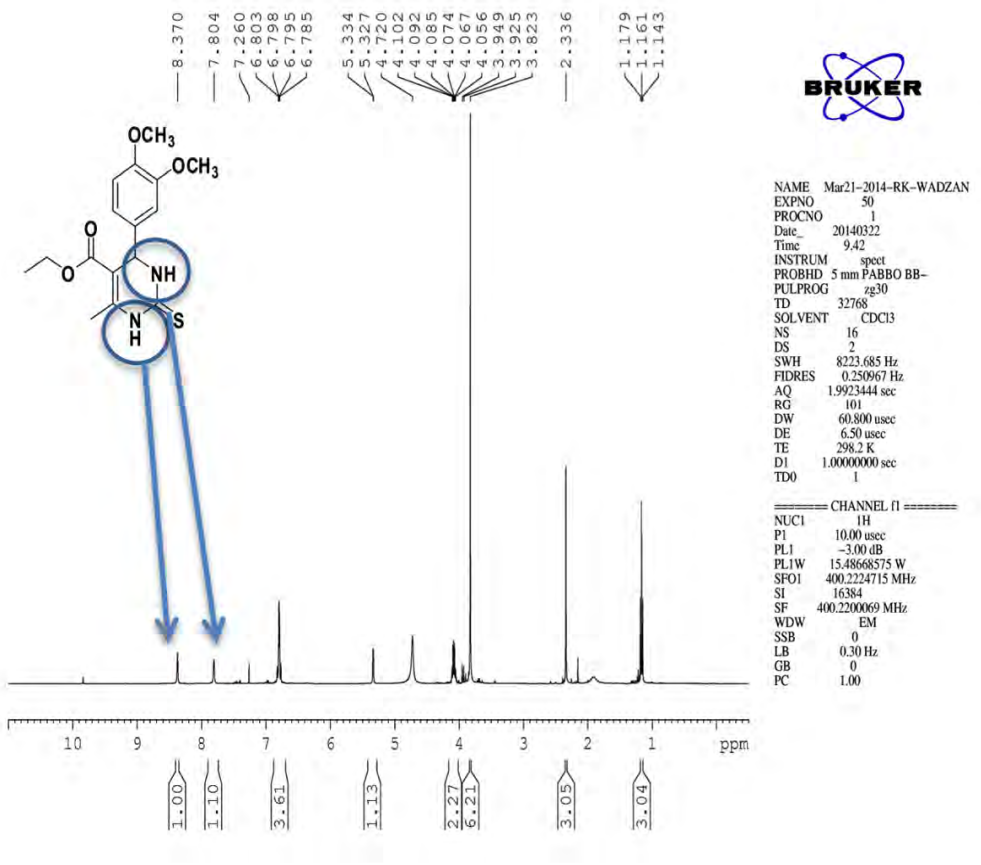


Figure 33. ^{1}H NMR spectroscopy of compound**3g**

3.1.4.3 ^{13}C NMR spectroscopy

In ^{13}C NMR spectrum of compounds **3a**-**3k**the appearance of a distinctive signal around δ165.3ppm confirmed the presence of carbonyl carbon (C=O). Methoxy carbons resonated around δ 55.87-55.65ppm as depicted on **Figure 34.**

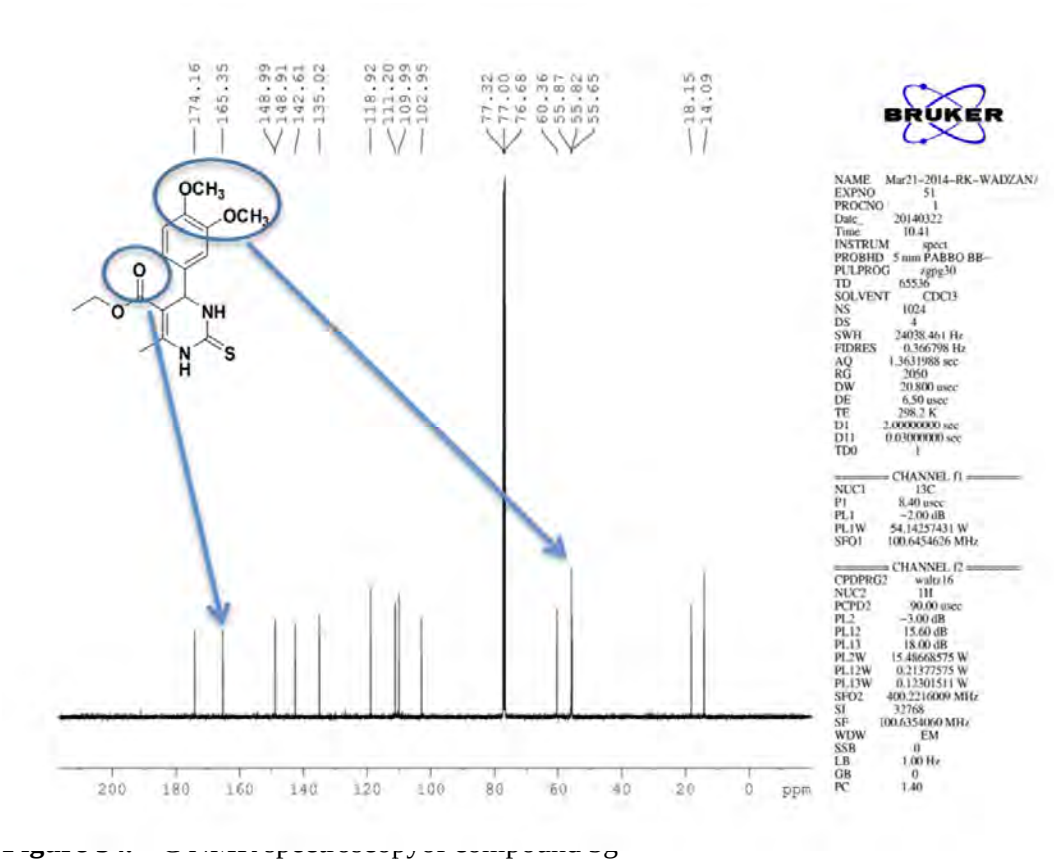


Figure 3.1: ^{13}C NMR spectroscopy of compound 2g

3.2 *Invitro* biological assay

The synthesized dihydropyrimidinones were evaluated for their minimum inhibitory concentration (MIC) by broth dilution method against bacterial *Staphyloccocusaureus* ATCC25923, *B. subtilis*ATCC6051, *E. coli* ATCC35218, *P. aeruginosa* ATCC27853 and fungal *C. albicans* ATCC90028, *C. neoformans* ATCC66031 at Albert Luthuli Hospital, Durban. In general these compounds were found to be less effective against tested bacterial strains. All the tested compounds were inactive towards Gram-Positive *Staphyloccocusaureus* ATCC25923 and Gram-negative *E. coli* ATCC35218 at the tested concentration (200 µg/mL). Compound **2** with *para*-methoxy substituent on phenyl ring was found to be most active compound against *B. subtilis*ATCC6051 with MIC value of 100 µg/mL. This indicated that substitution of electron donating methoxy- substitution at *para*-position of phenyl ring is favorable for activity toward *B. subtilis*ATCC6051. Compound **1** with para-methyl substituent at phenyl ring was found to be most active against Gram-negative *P. aeruginosa* ATCC27853. This revealed that substitution of electron donating methyl- group at *para*-position is more favored than other substations for activity towards *P. aeruginosa*

ATCC27853. Standard Moxicillin showed highly potent activity (MIC = <0.39 µg/mL)towards all tested bacterial strains.

Table 6. Antibacterial activity (MIC in µg/mL) of a series of dihydropyrimidinones (3a-3k)

Compound	*Staphylococcus aureus*	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosa*
1	>200	200	200	100
2	>200	100	200	200
3	>200	200	>200	>200
4	>200	>200	>200	>200
5	200	>200	200	>200
6	>200	200	200	>200
7	>200	200	200	>200
8	>200	>200	200	>200
9	>200	>200	200	>200
10	>200	>200	200	200
11	200	200	200	200
Moxycillin	**<0.39**	**<0.39**	**<0.39**	**<0.39**

The synthesized dihydropyrimidinones were also evaluated for their minimum inhibitory concentration (MIC) by broth dilution method against fungal *C. albicans* ATCC90028, *C. neoformans* ATCC66031 at Albert Luthuli Hospital, Durban. In general these compounds were found to be less effective against tested fungal strains. All the tested compounds were inactive towards tested fungal strains (MIC = >200 µg/mL). Standard Amphotericin B showed highly potent activity towards *C. albicans* ATCC90028and *C. neoformans* ATCC66031 at MIC of 0.25 and1. 2 µg/mL, respectively.

Table 7. Antifungal activity (MIC in µg/mL) of a series of dihydropyrimidinones (3a-3k)

Compound	***Candida albicans*** **ACTT**	***Cryptococcus neoformans*** **ACTT**
1	200	>200
2	200	>200
3	200	>200
4	200	>200
5	200	>200
6	200	>200
7	200	>200
8	200	>200
9	200	>200
10	200	>200
11	200	>200
Amphotericin B	**0.25**	**1-2**

References

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CHAPTER IV

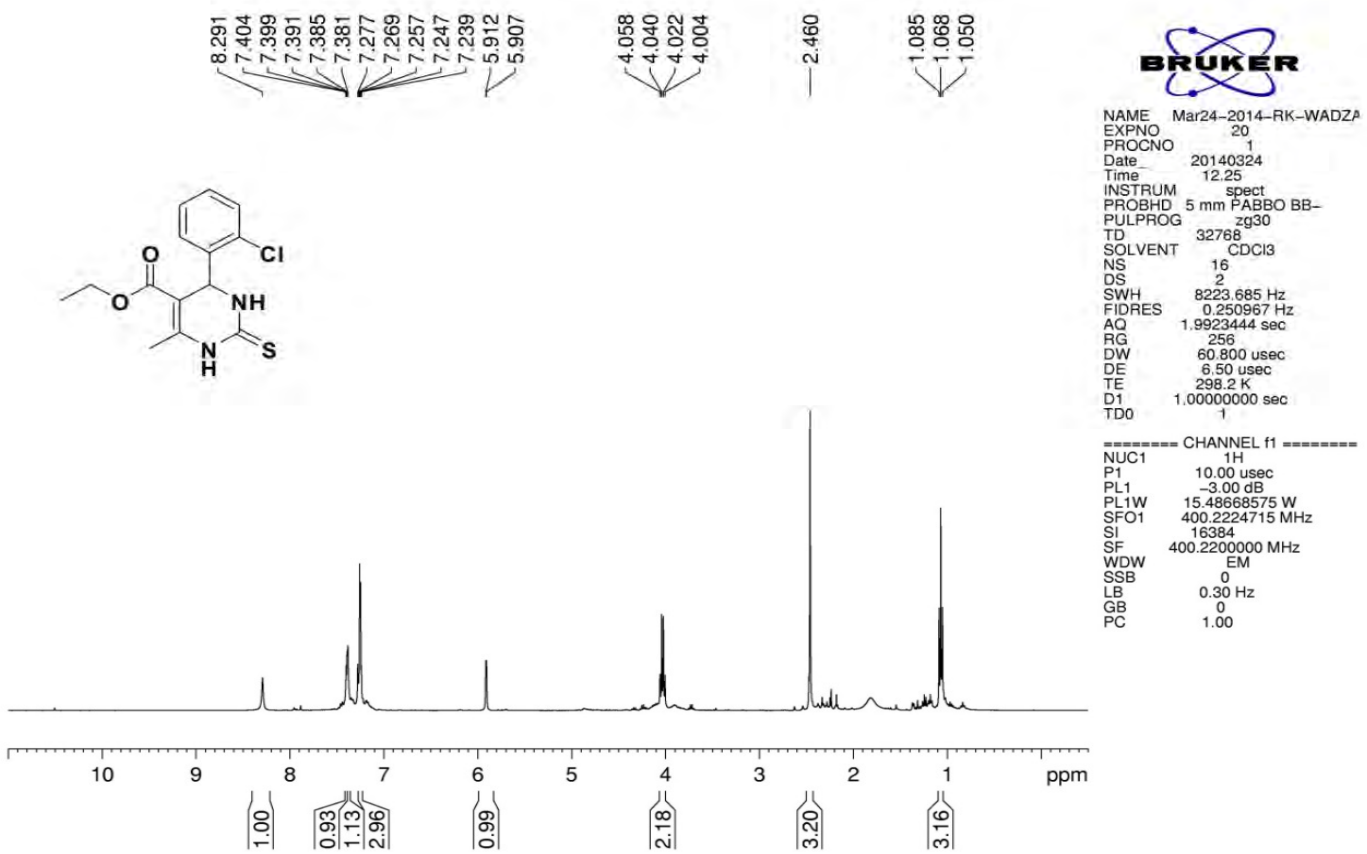
CONCLUSION AND FUTURE WORK

4 Conclusion and Future Work

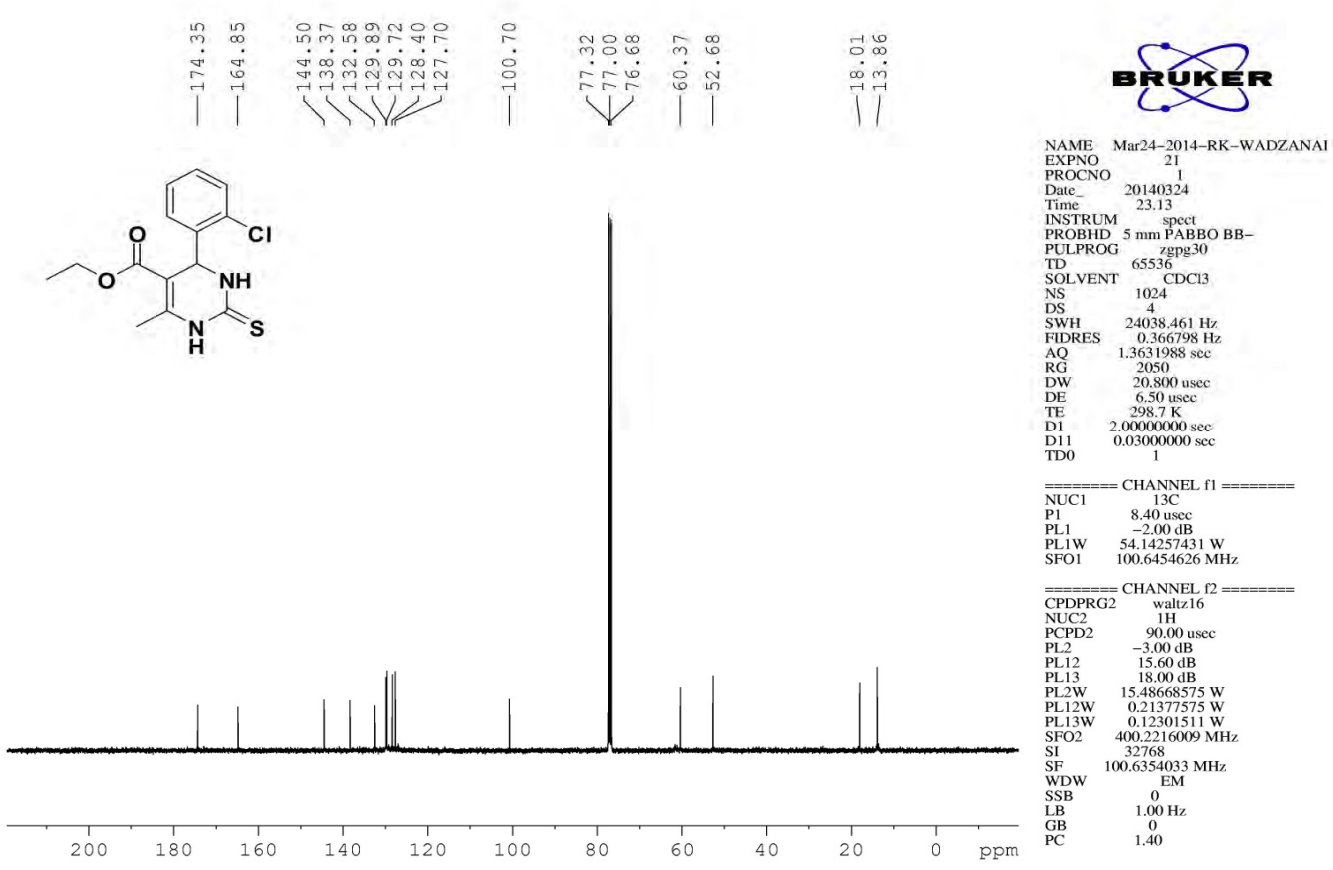
Conclusively, a series of 1,2,3,4-tetrahydropyrimidine **3(a-k)** have been successfully synthesized, characterized and evaluated for their antibacterial and antifungal activity. In general these compounds were found to be less effective against tested bacterial strains. All the tested compounds were inactive towards Gram-Positive ***Staphyloccocusaureus*** ATCC25923 and Gram-negative ***E. coli*** ATCC35218 at the tested concentration (200 µg/mL). Compound 2 with para-methoxy substituent on phenyl ring was found to be moderately active against ***B. subtilis*** ATCC6051 with MIC value of 100 µg/mL. This indicated that substitution of electron donating methoxy- substitution at para-position of phenyl ring is favorable for activity toward ***B. subtilis***ATCC6051 and no compound was found to be active against fungal strain.

Possible improvements in the antimicrobial activity can be further achieved by slight modifications in the substituent's and/or additional structural activity investigations to have good antimicrobial activity.

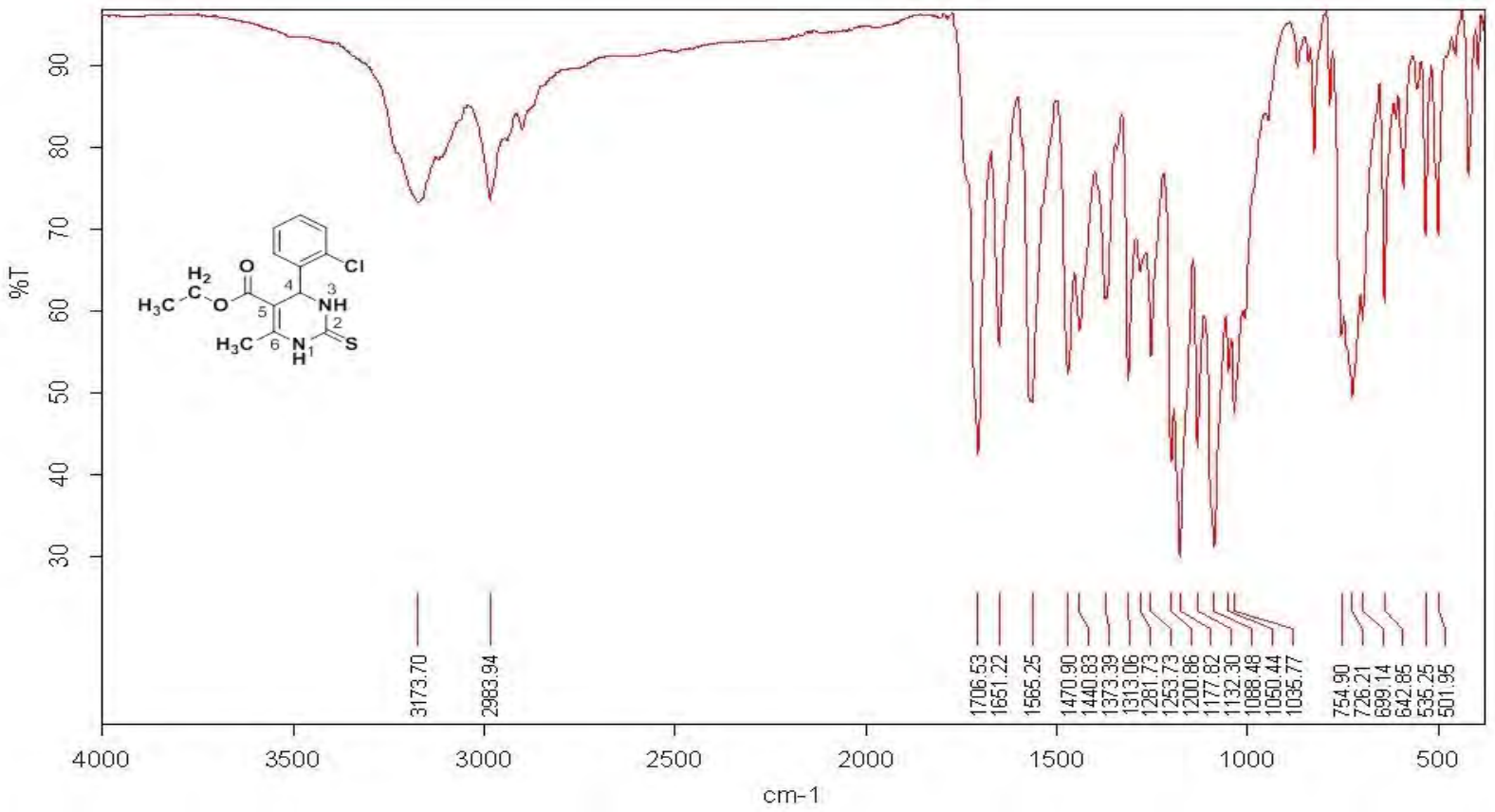
APPENDIX



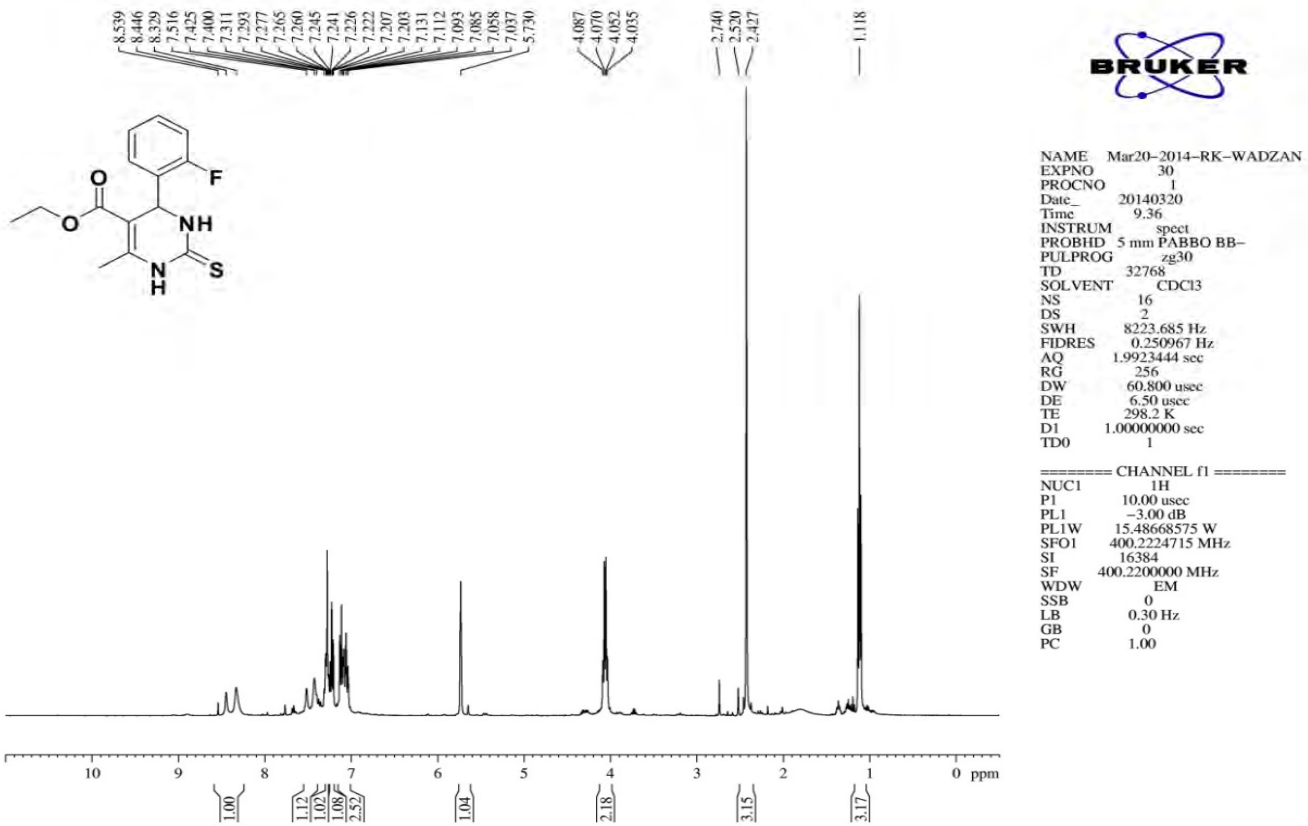
Spectrum 1.[1]HNMR of compound **3a**



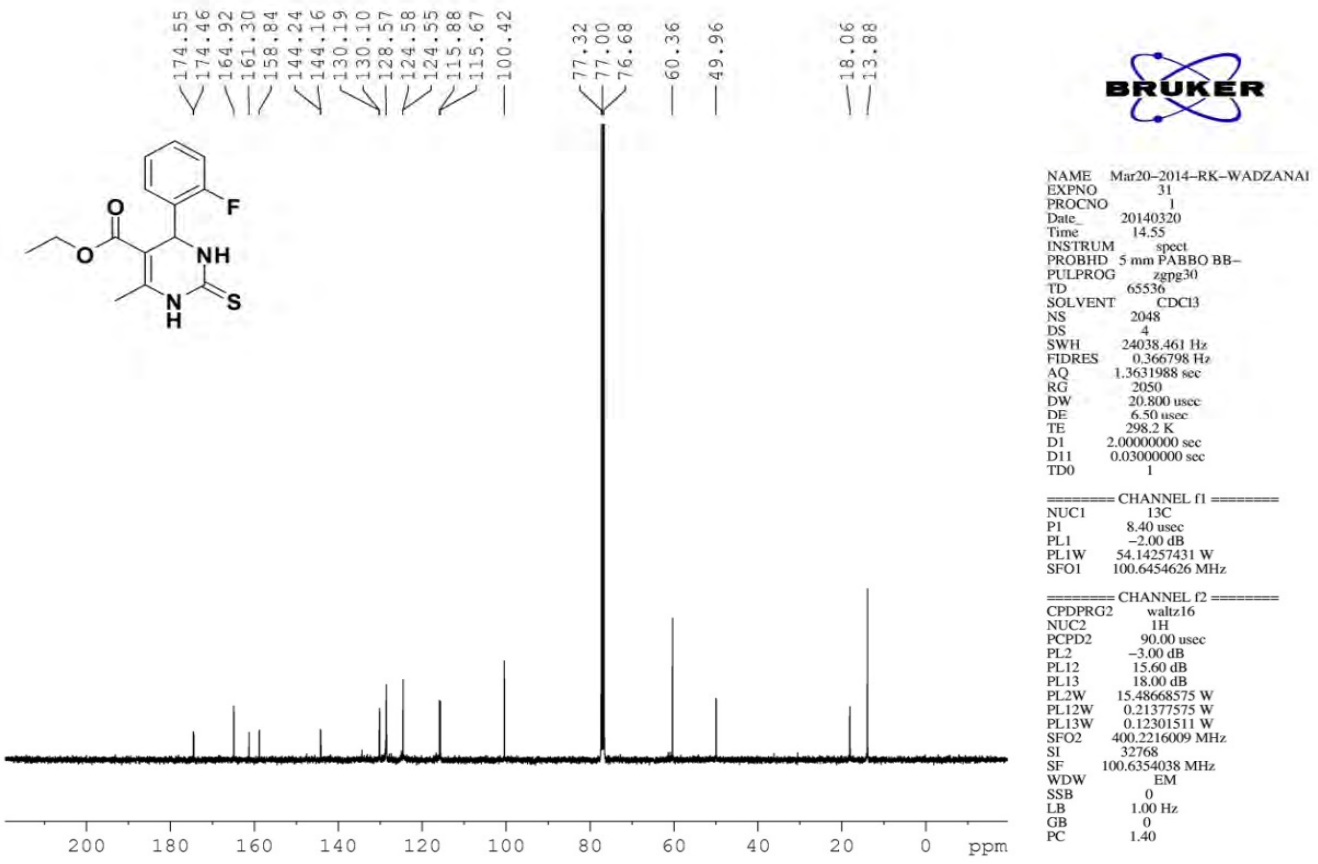
Spectrum 2.^{13}CNMR of compound **3a**



Spectrum 3.IR spectrum of compound **3a**



Spectrum 4.[1]HNMR of compound **3b**



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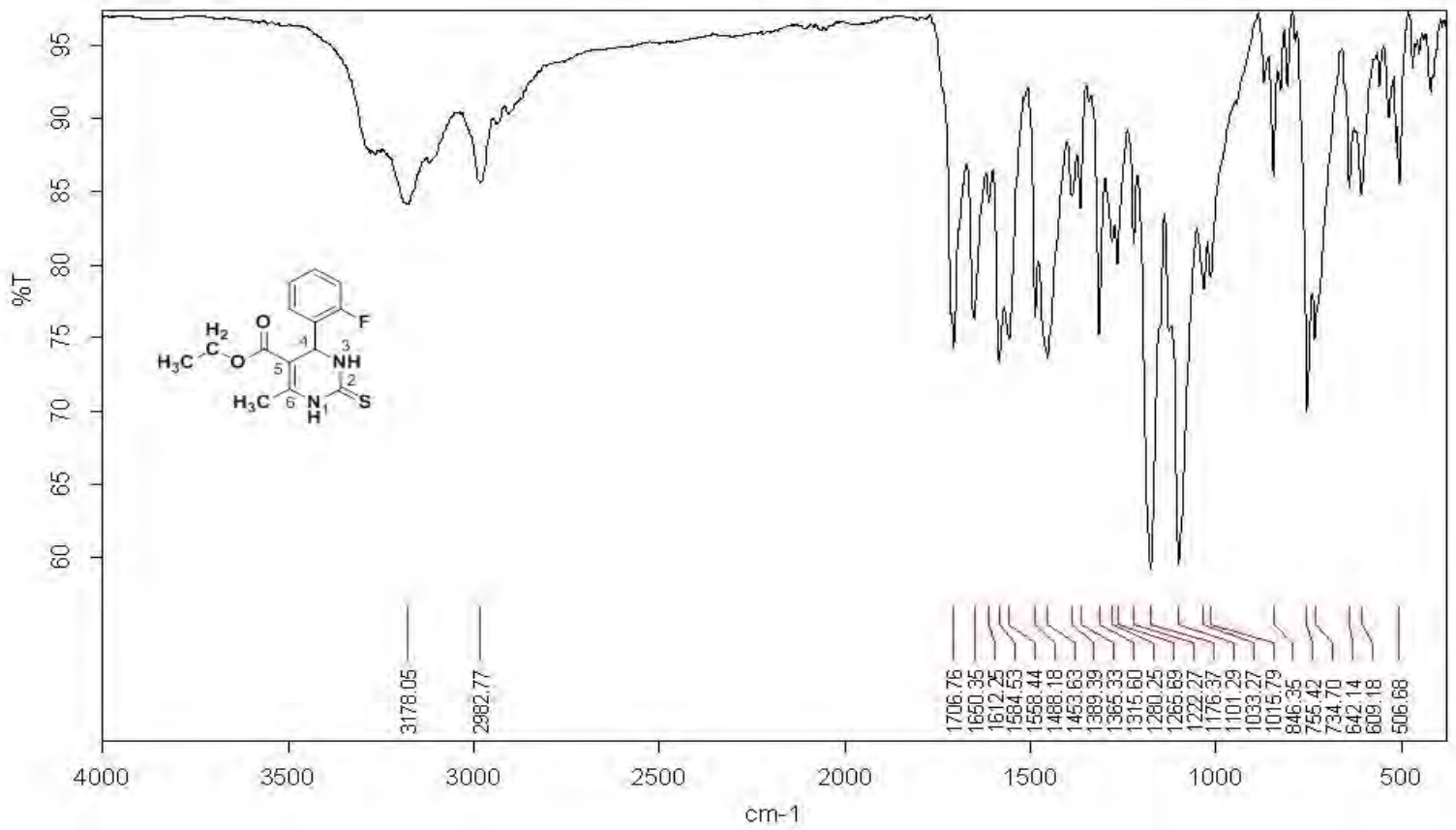
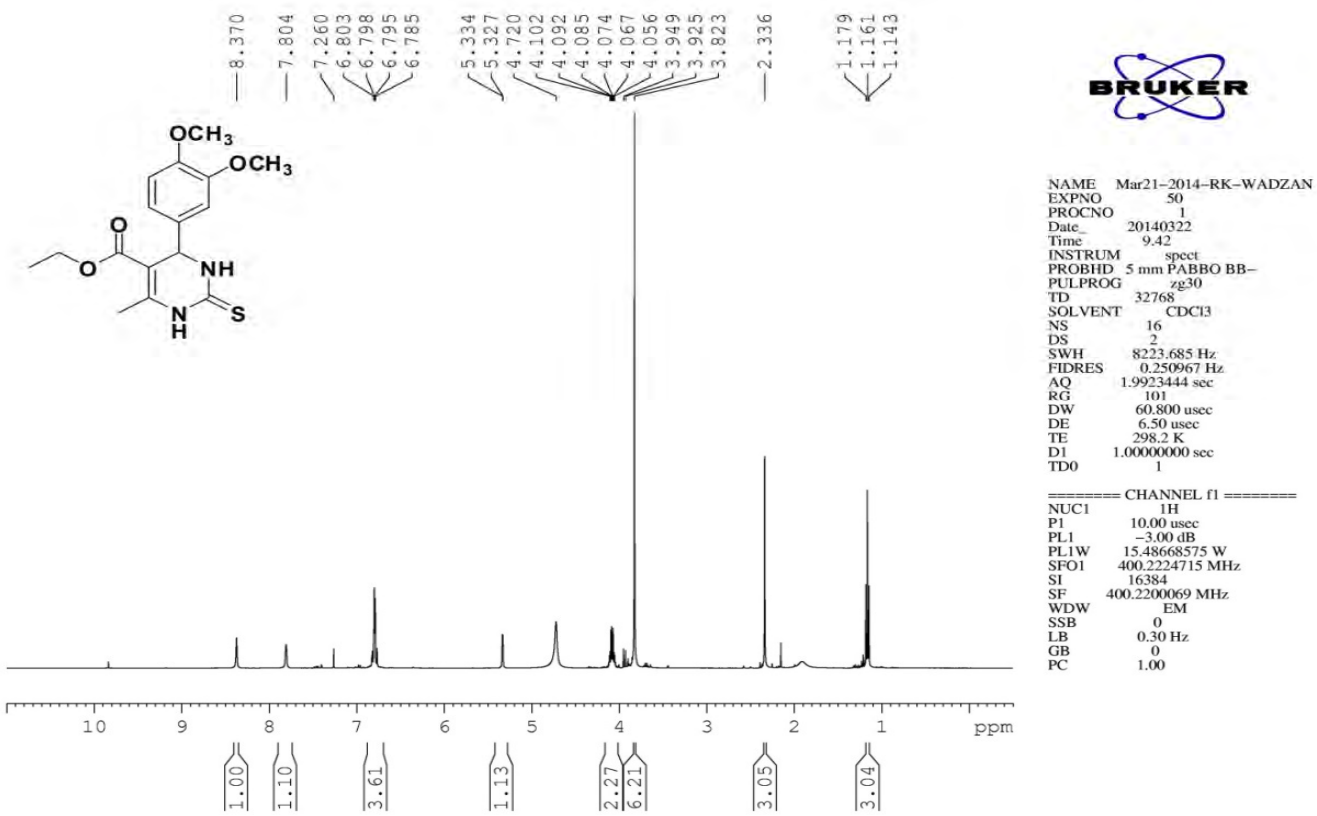
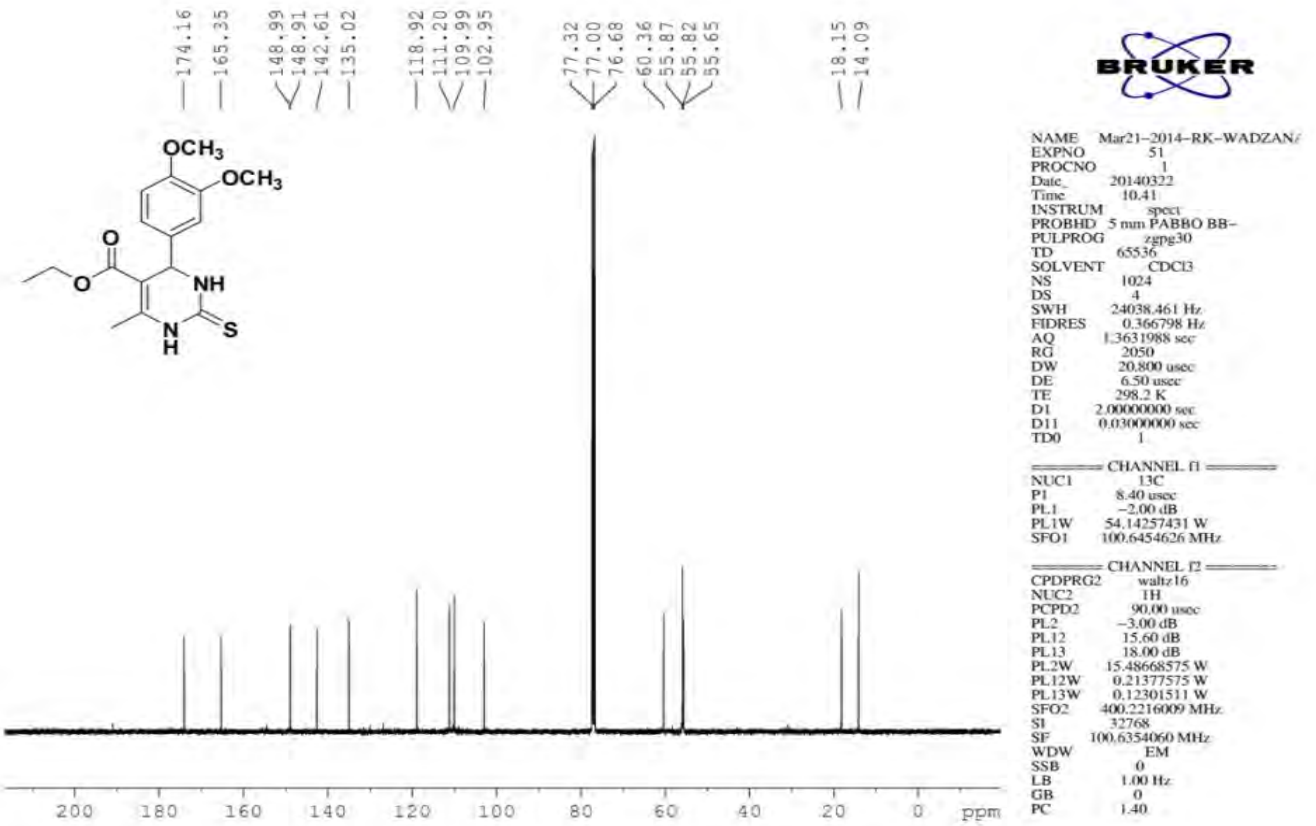


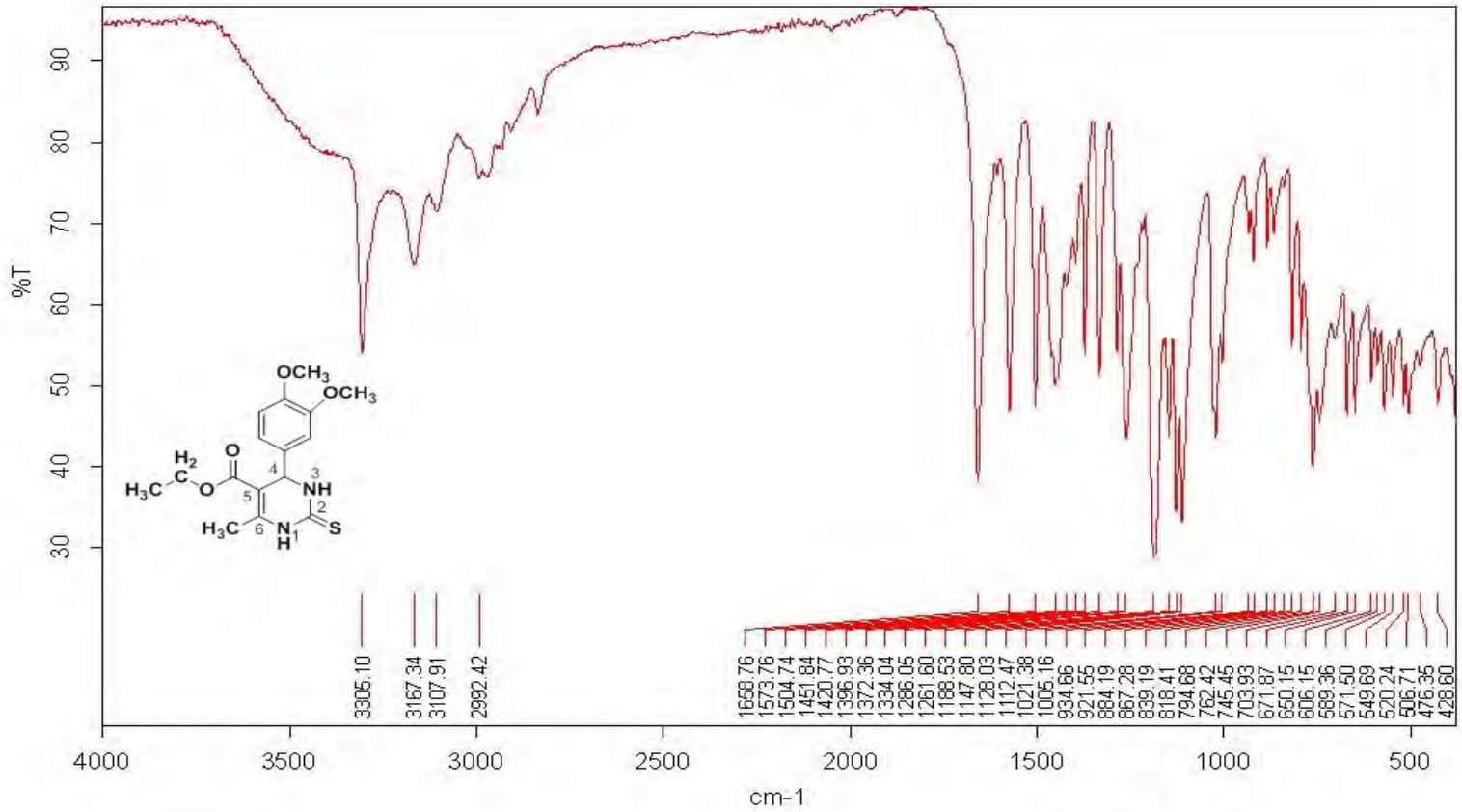
Spectrum 6.IR ofcompound **3b**



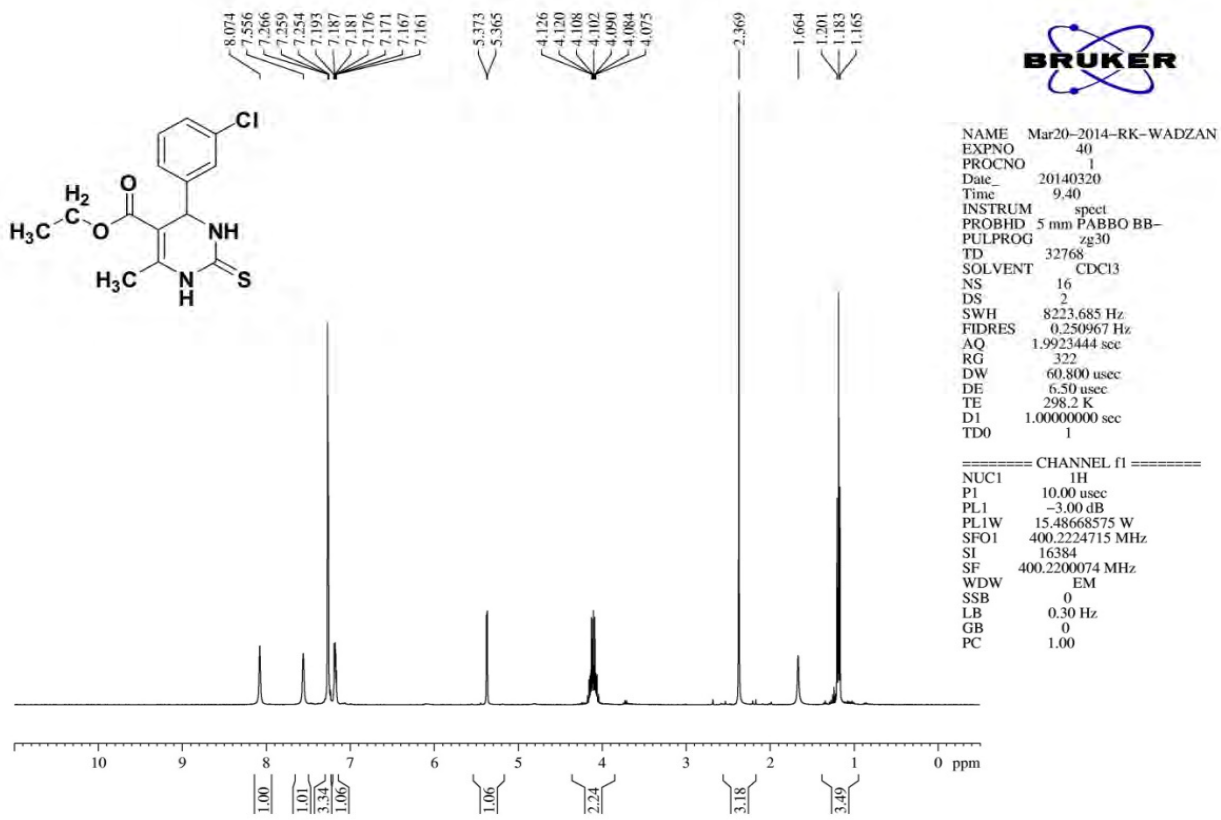
Spectrum 7.[1]HNMR of compound **3c**



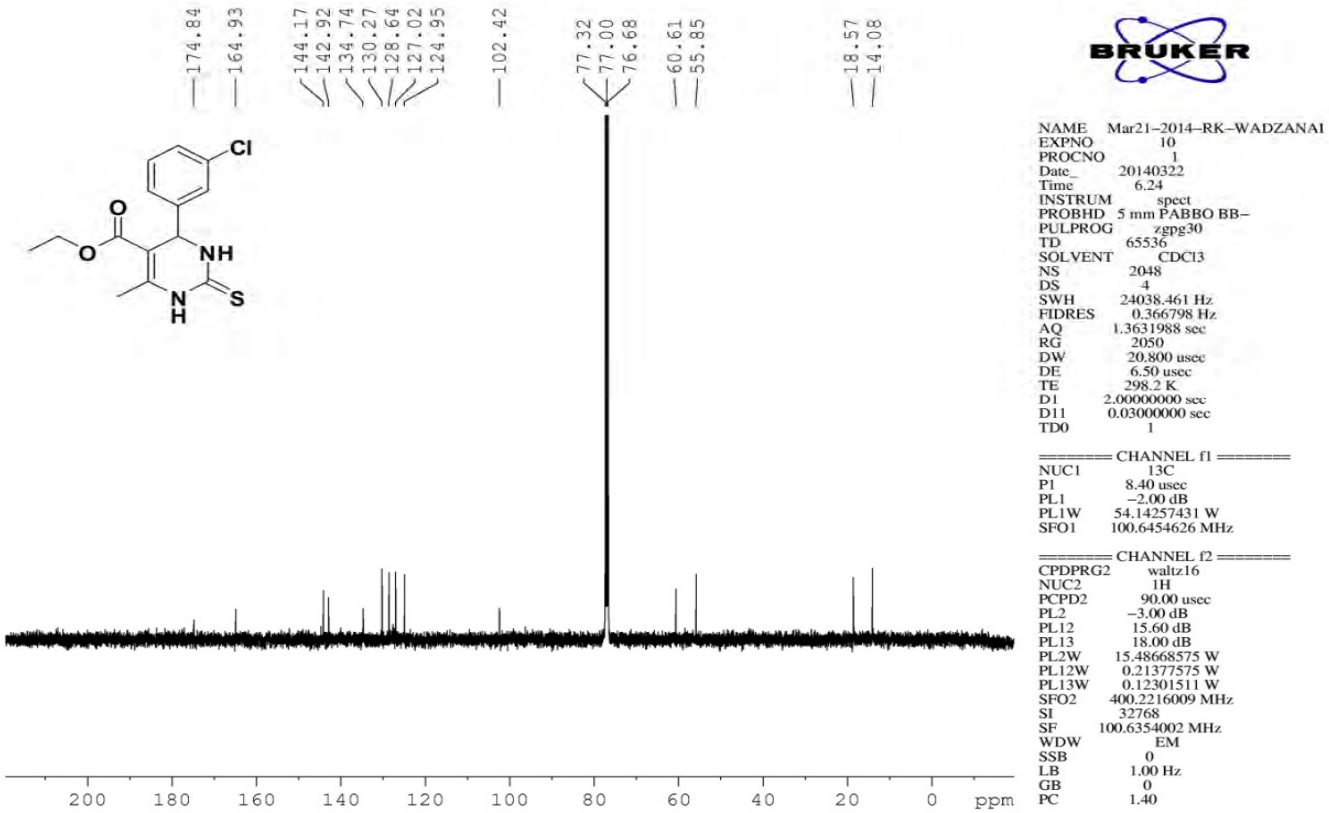
Spectrum 8.[13]CNMRof compound **3c**



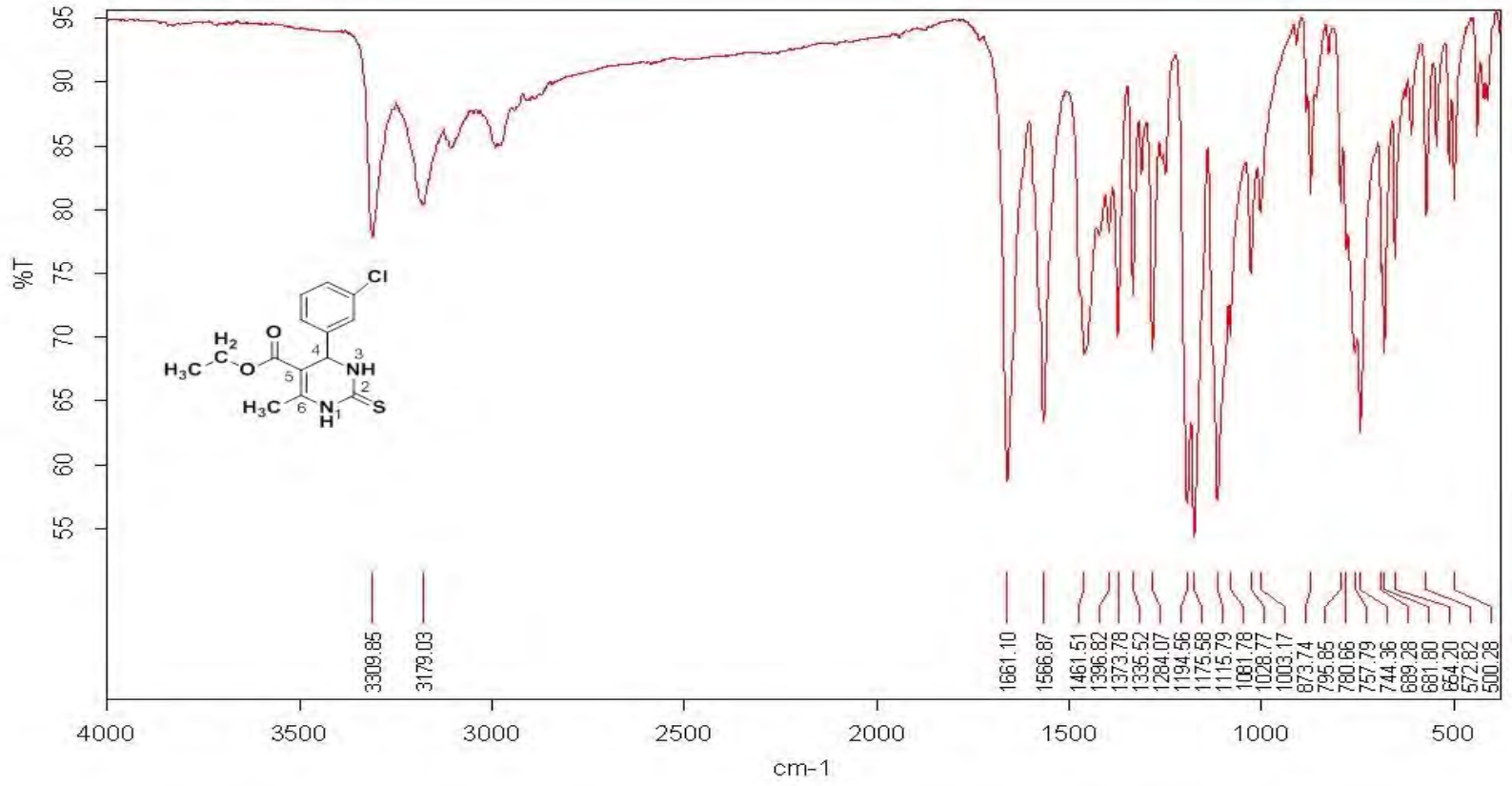
Spectrum 9.IR of compound **3c**



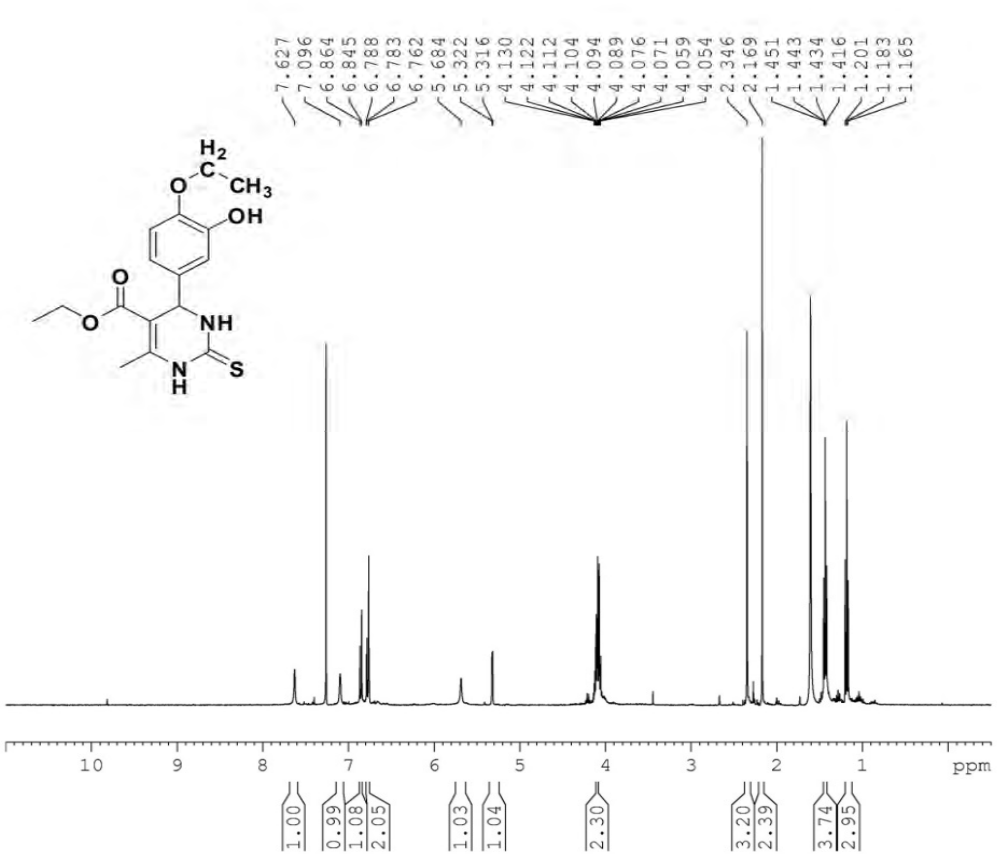
Spectrum 10.[1]HNMR of compound 3d



Spectrum 11.[13]CNMR of compound **3d**



Spectrum 12. IR of compound**3d**



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FIDRES	0.250967 Hz
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DW	60.800 usec
DE	6.50 usec
TE	298.2 K
D1	1.00000000 sec
TD0	1
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P1	10.00 usec
PL1	–3.00 dB
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SI	16384
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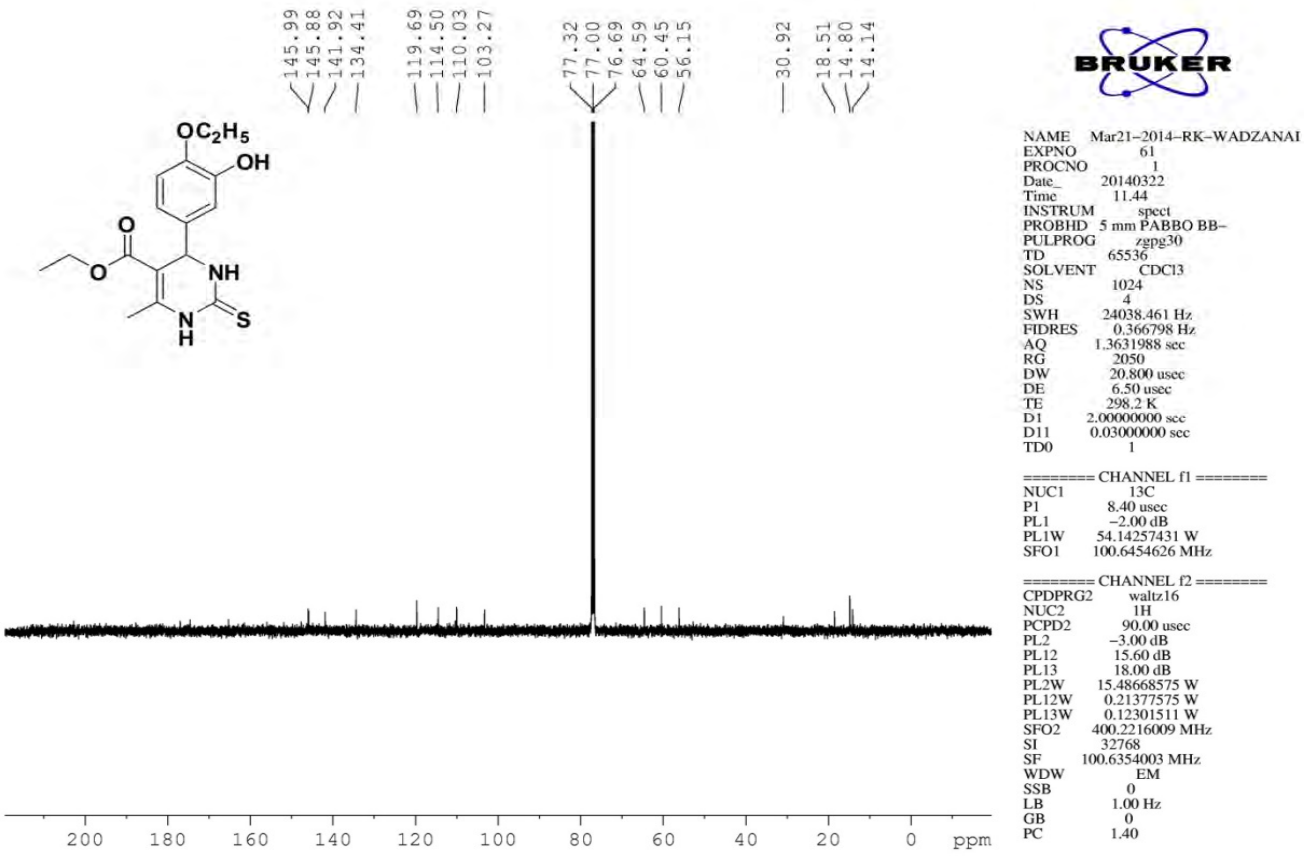
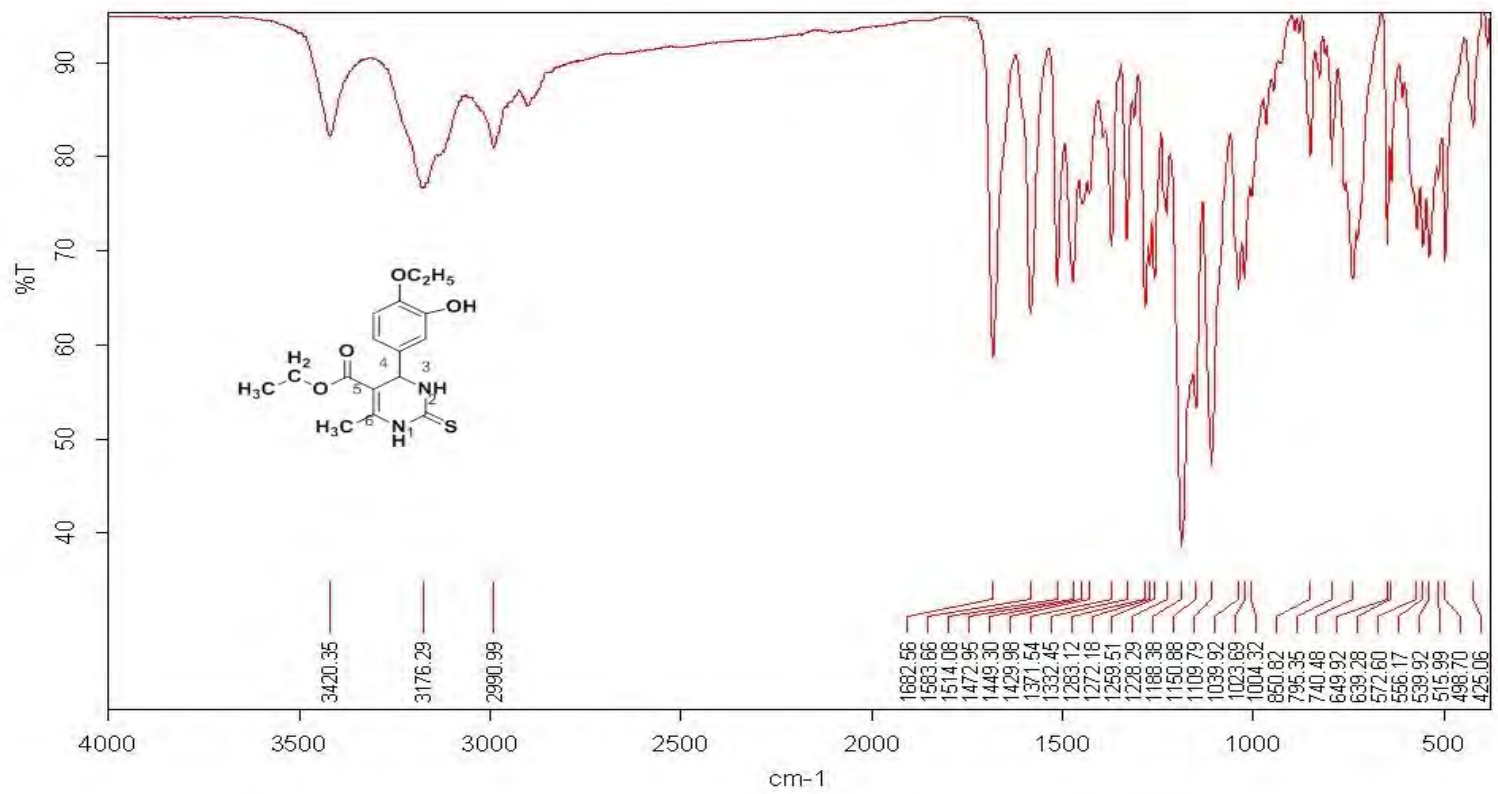
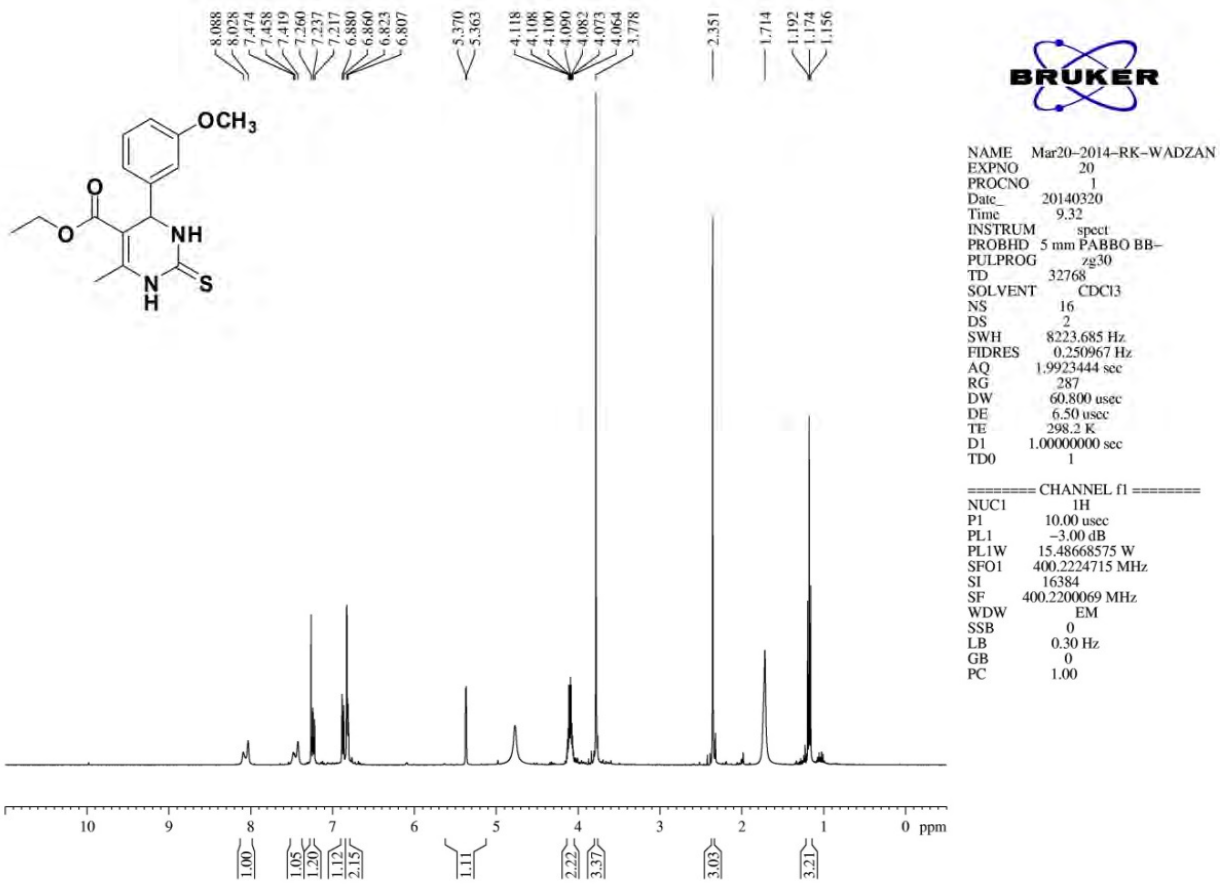


Spectrum 14.[13]CNMR of compound **3e**



Spectrum 15. IR of compound **3e**



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DE 6.50 usec
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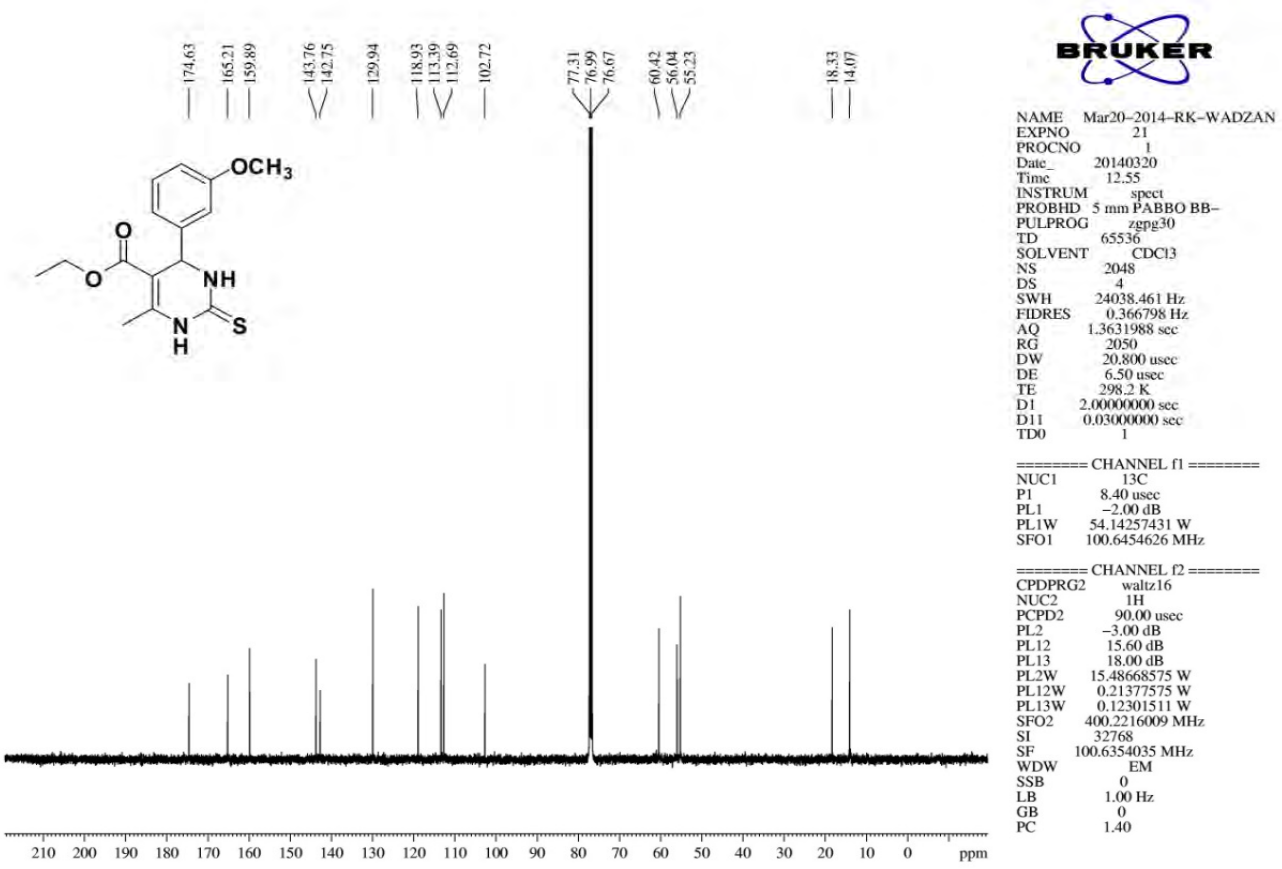
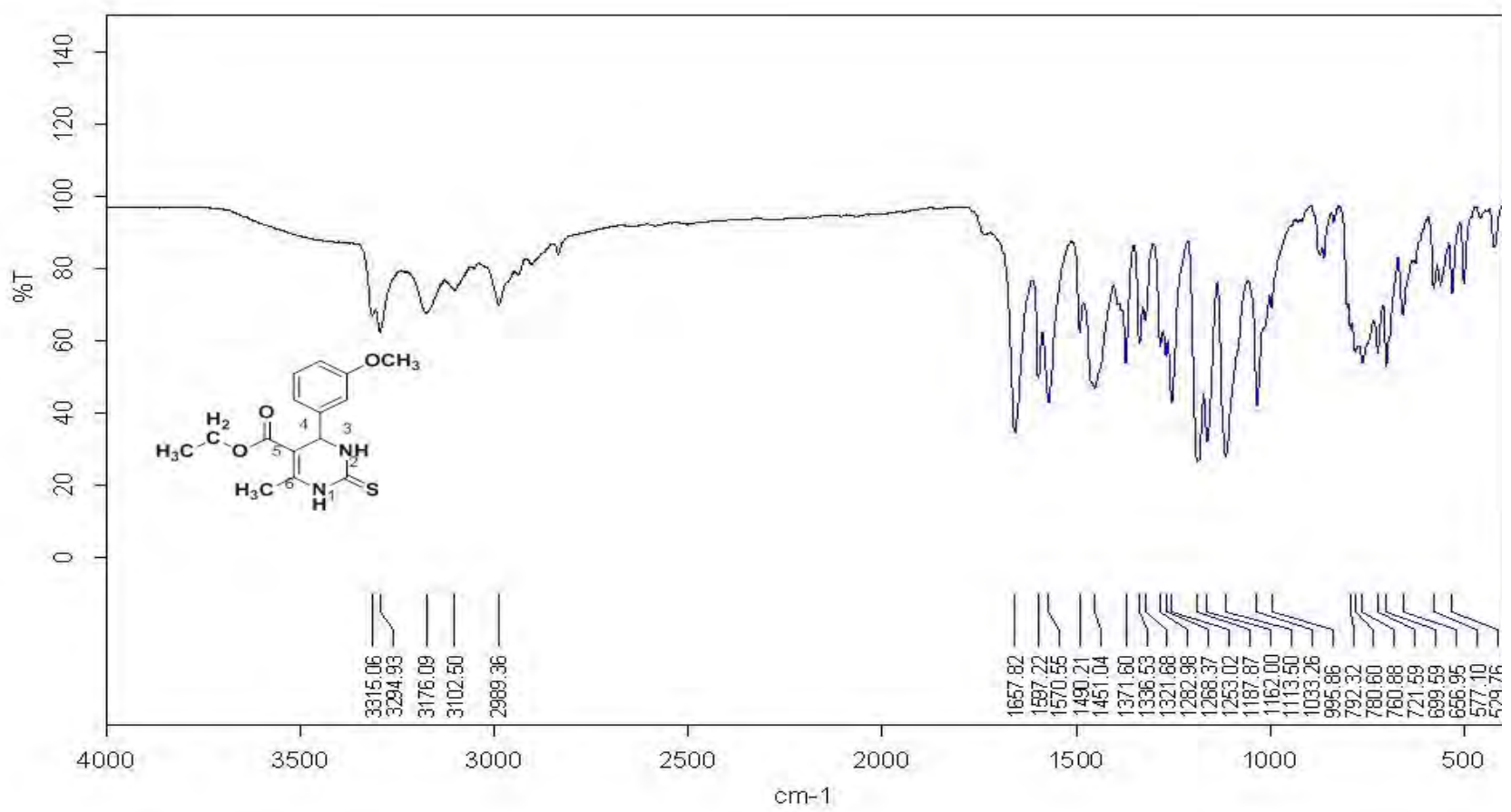


Spectrum 17.[13]CNMR of compound **3f**



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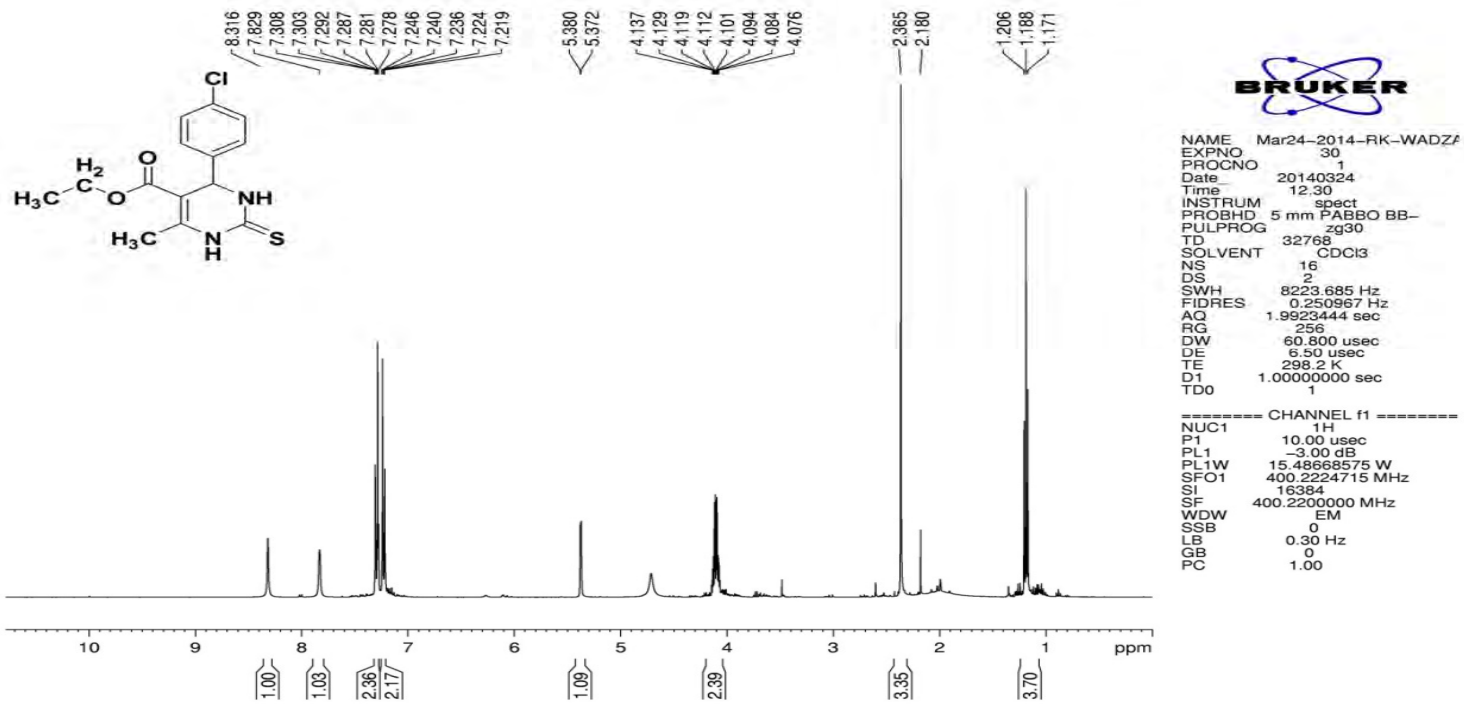

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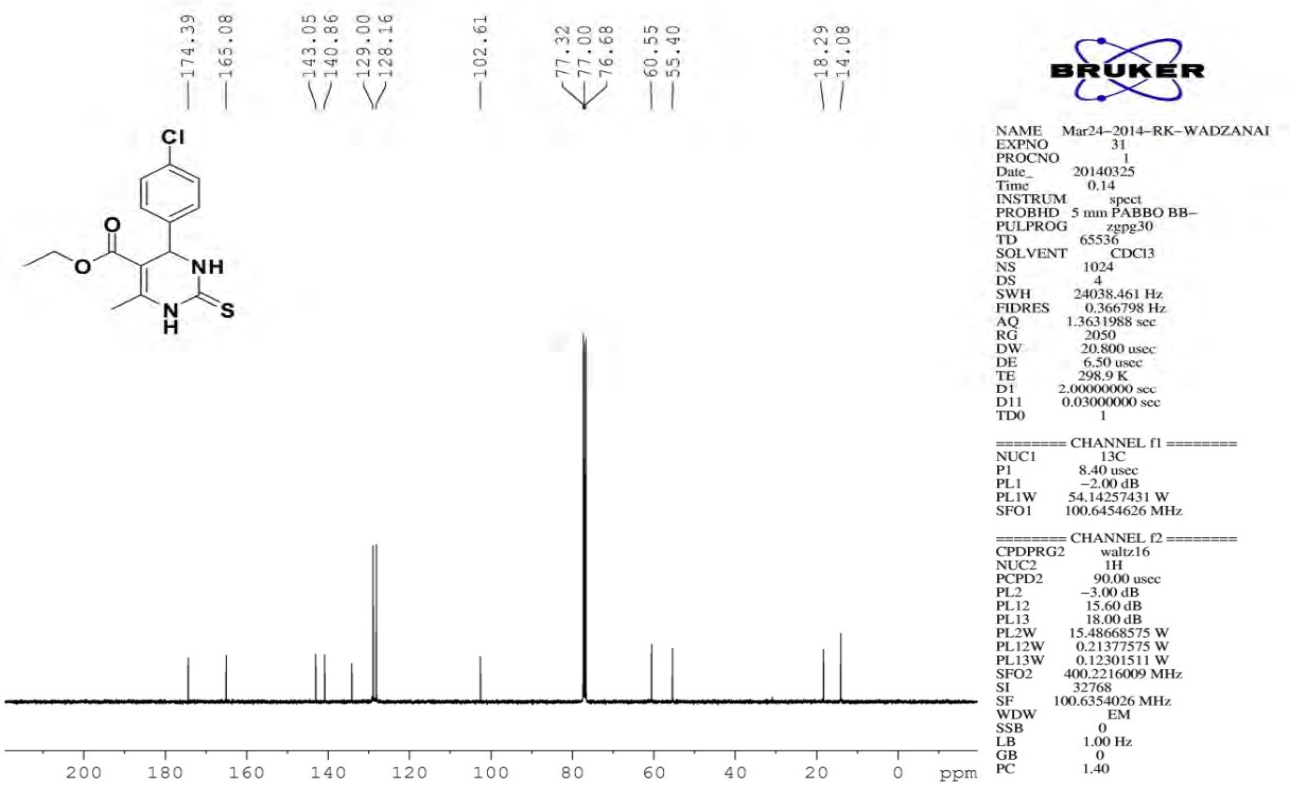

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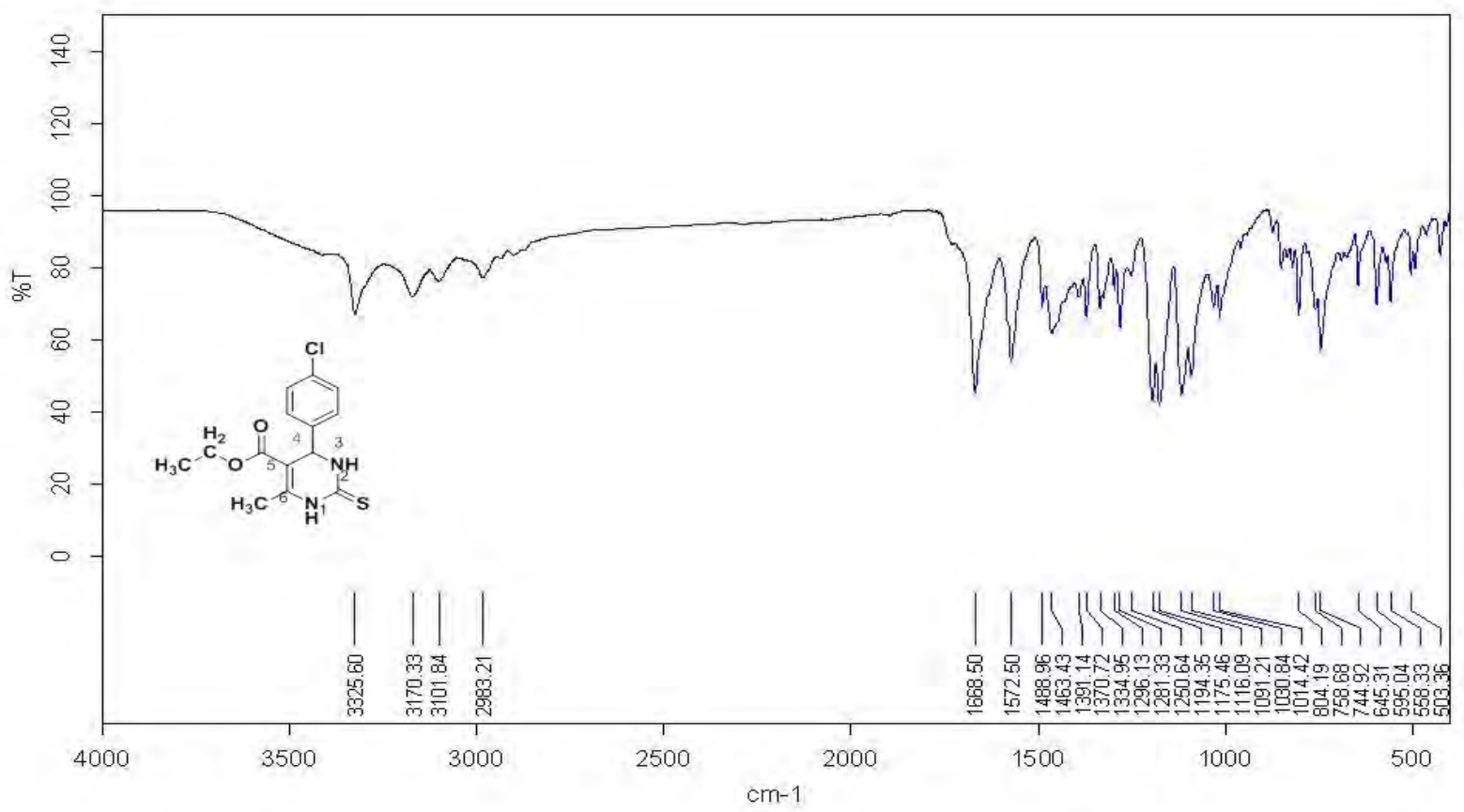
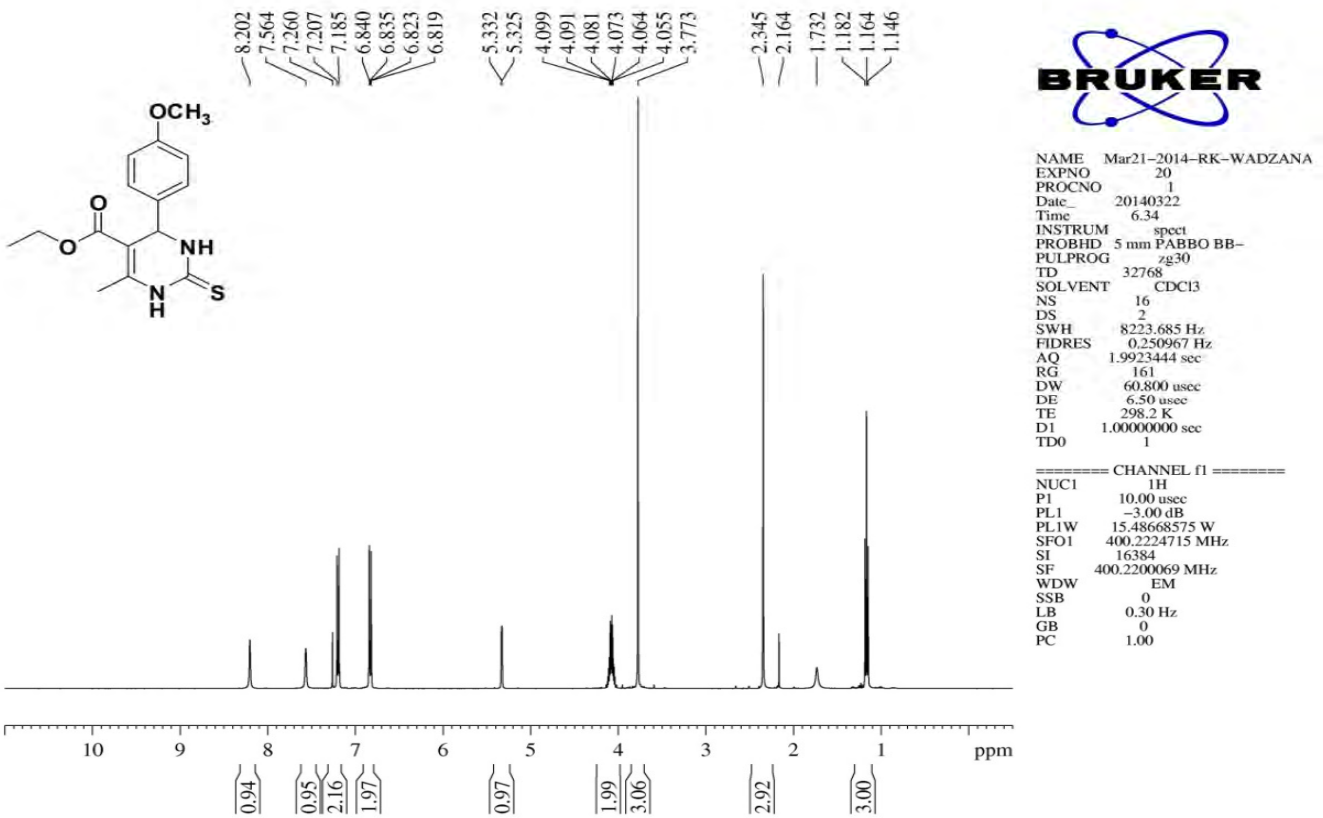
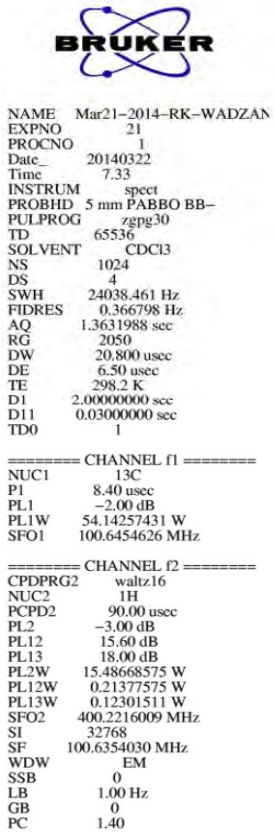


Spectrum 21.IR of compound **3g**



Spectrum 22. ^{1}HNMR of compound **3h**



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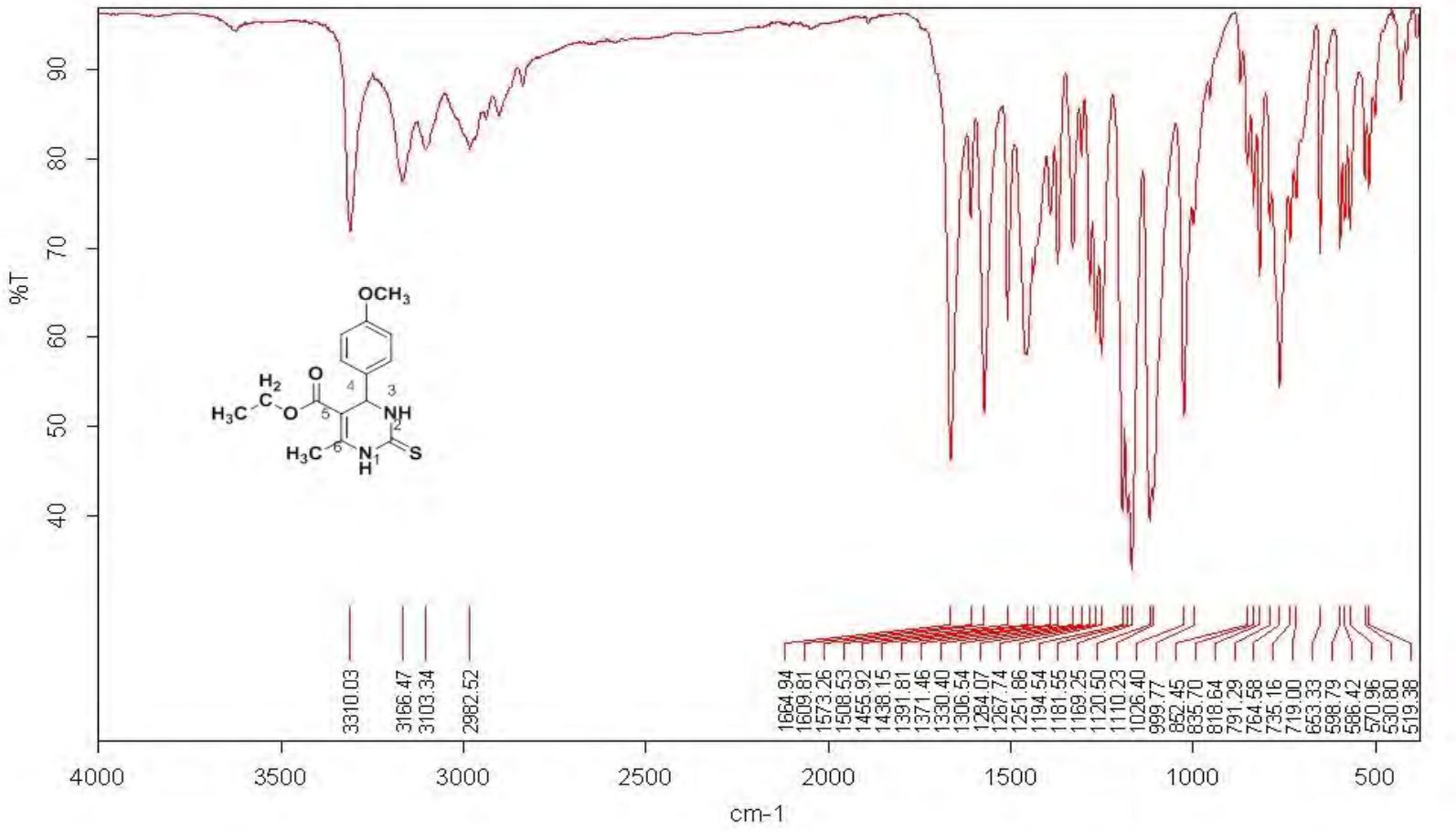

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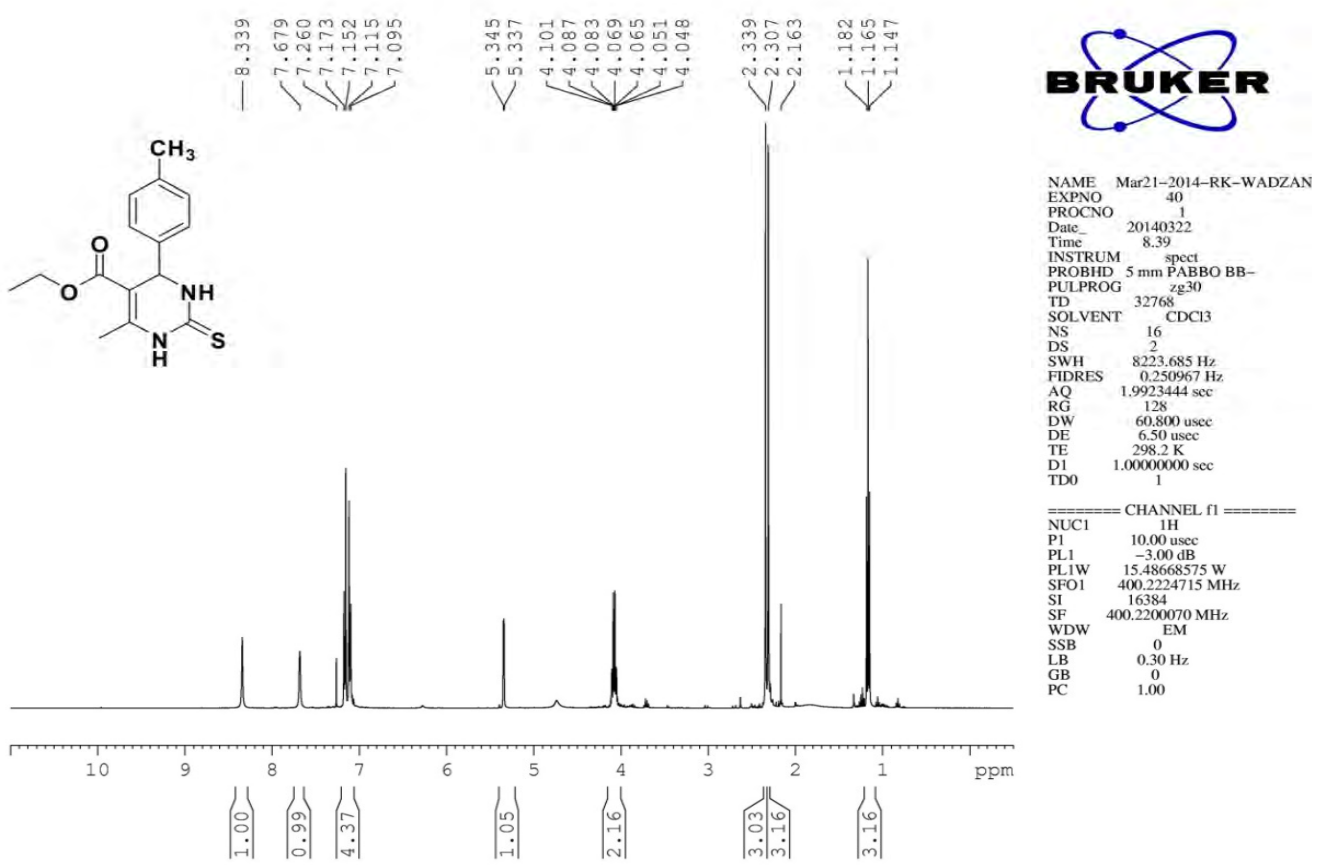
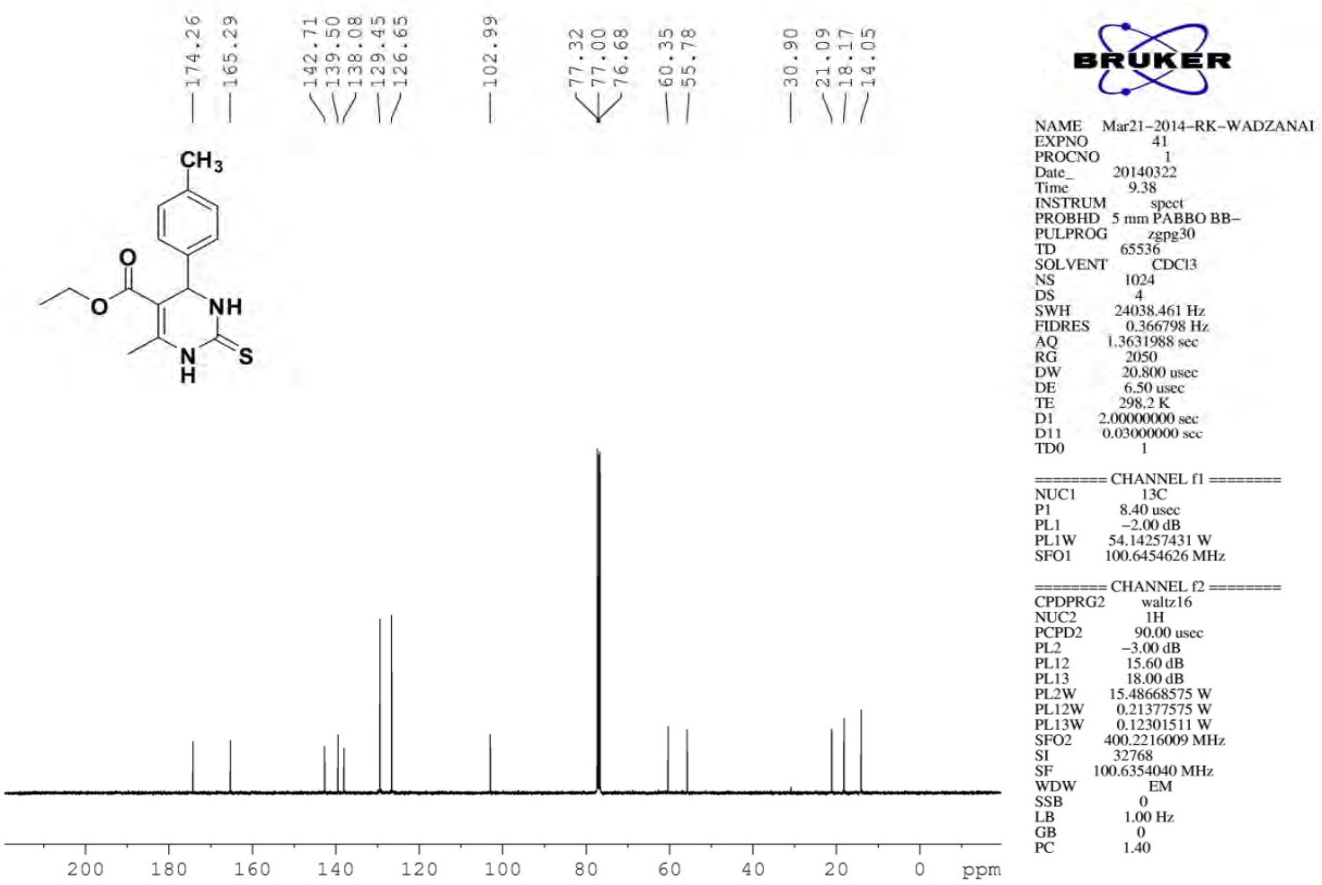
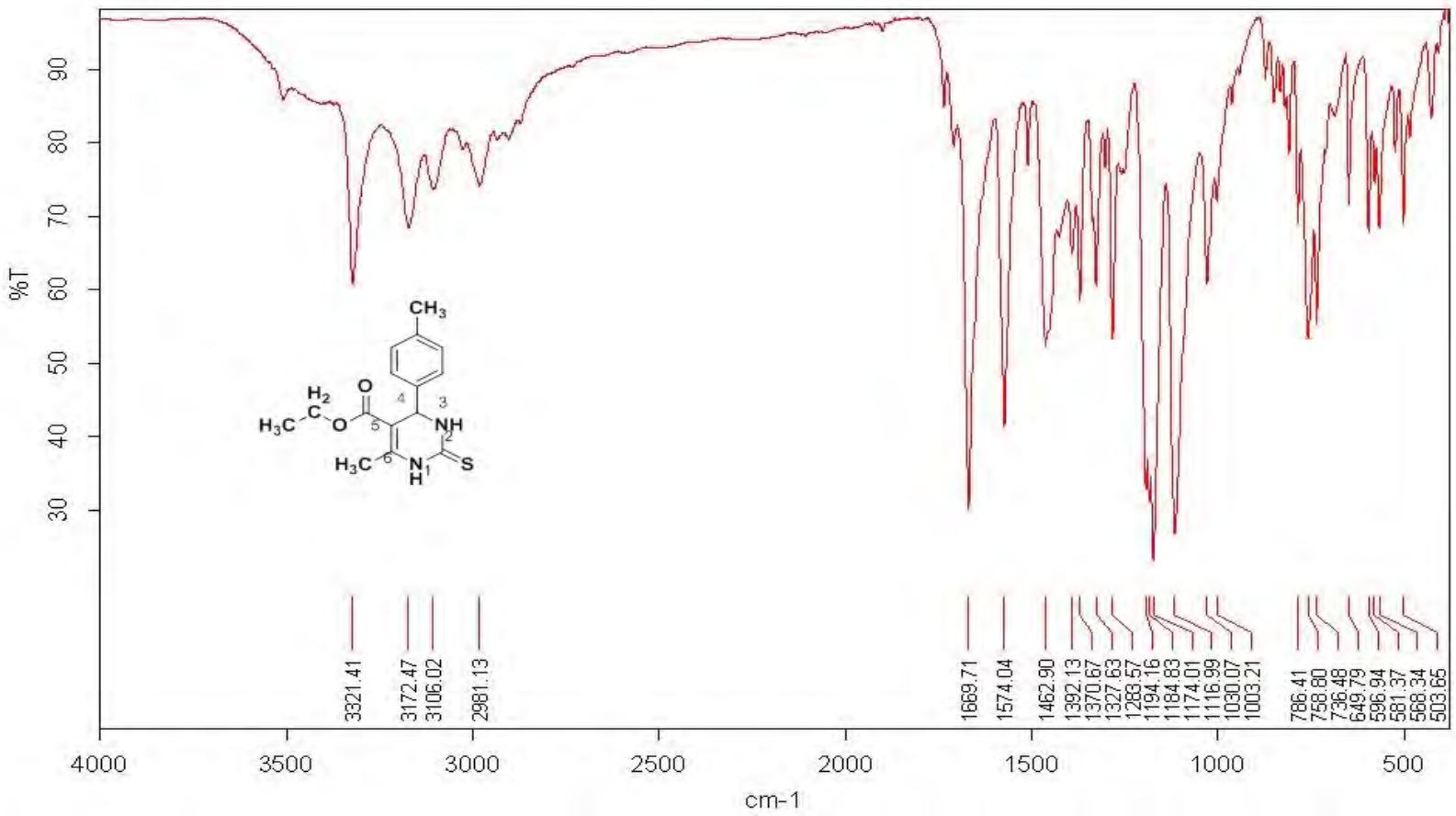


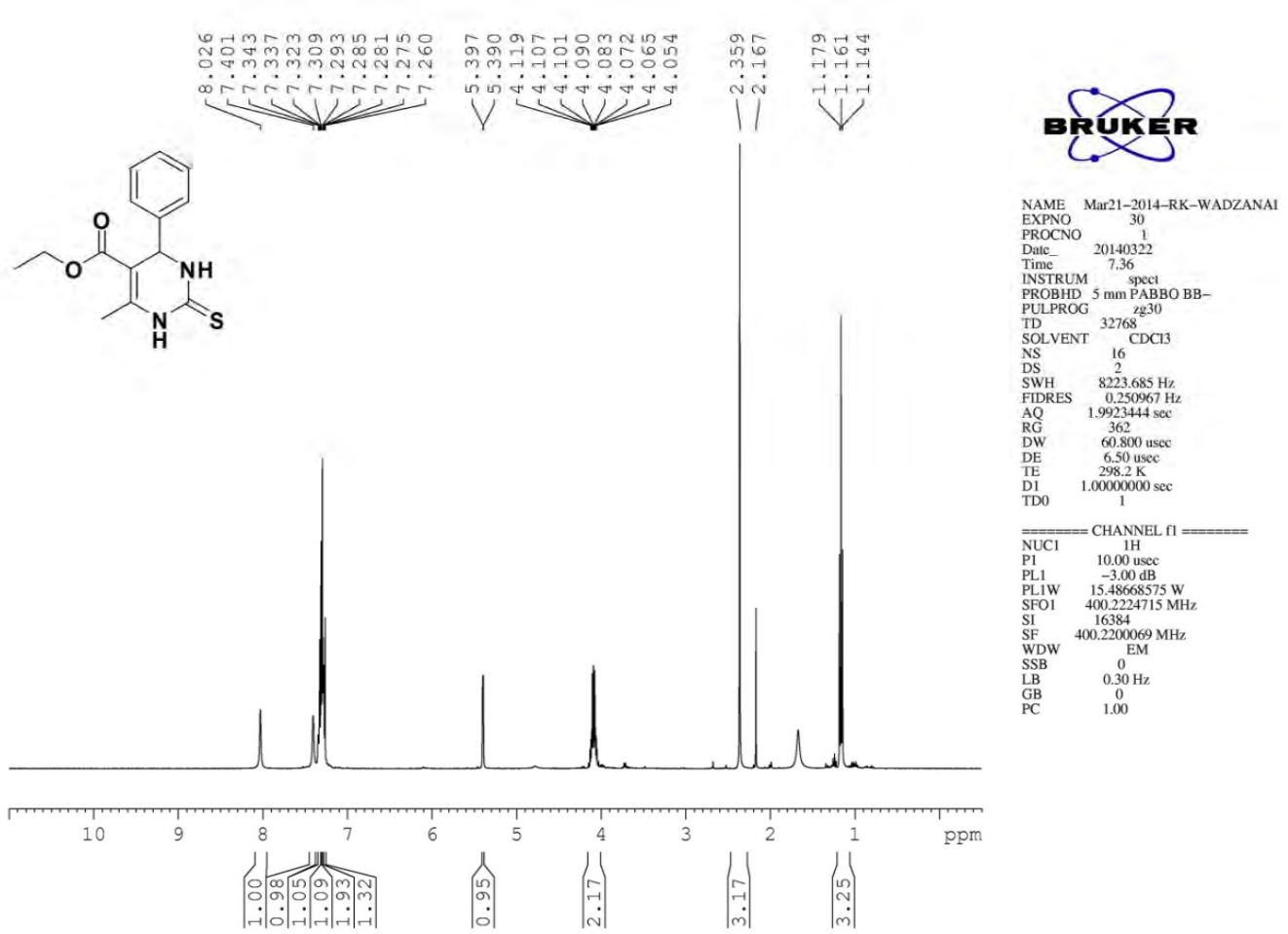
Spectrum 25.[1]HNMR of compound **3i**



Spectrum 26. ^{13}CNMR of compound **3i**



Spectrum 27. IR of compound **3i**



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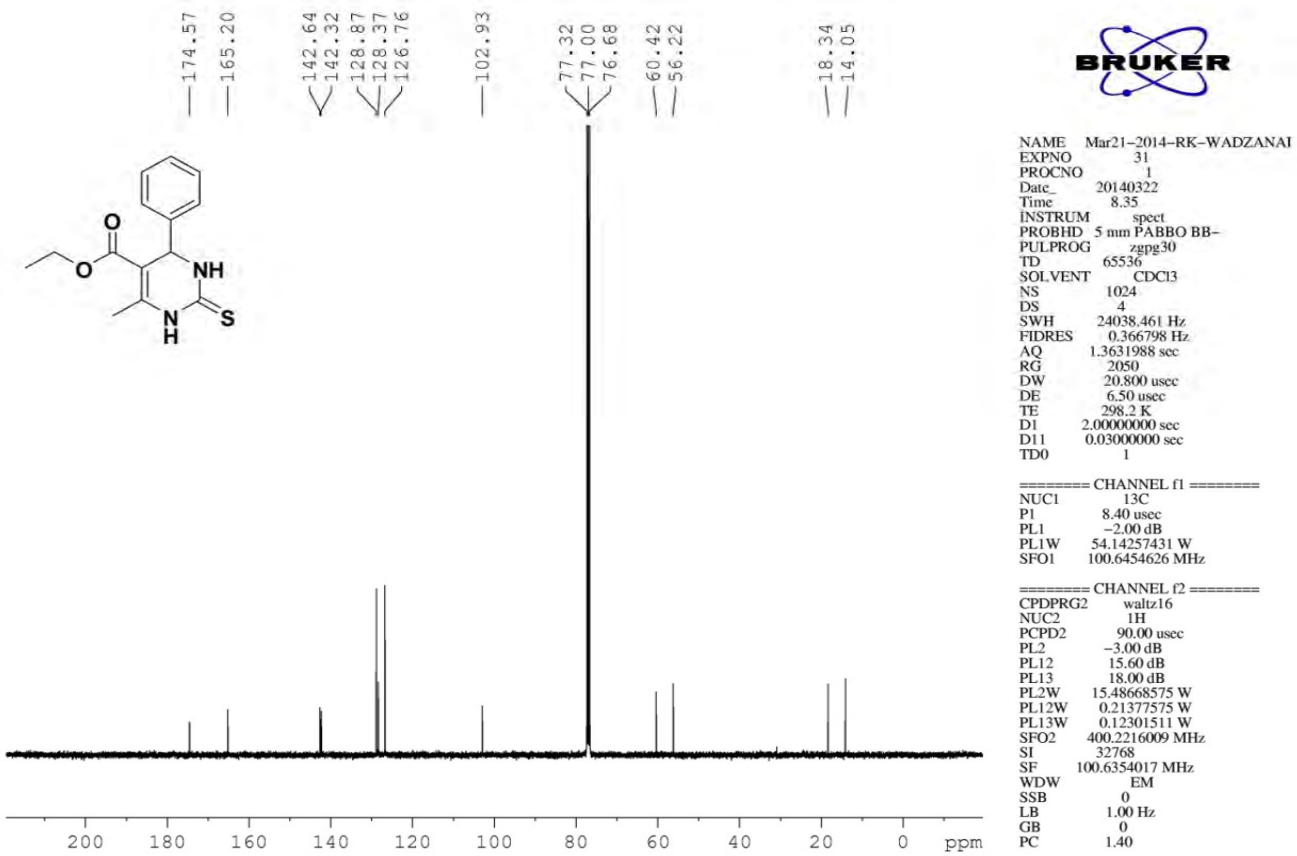

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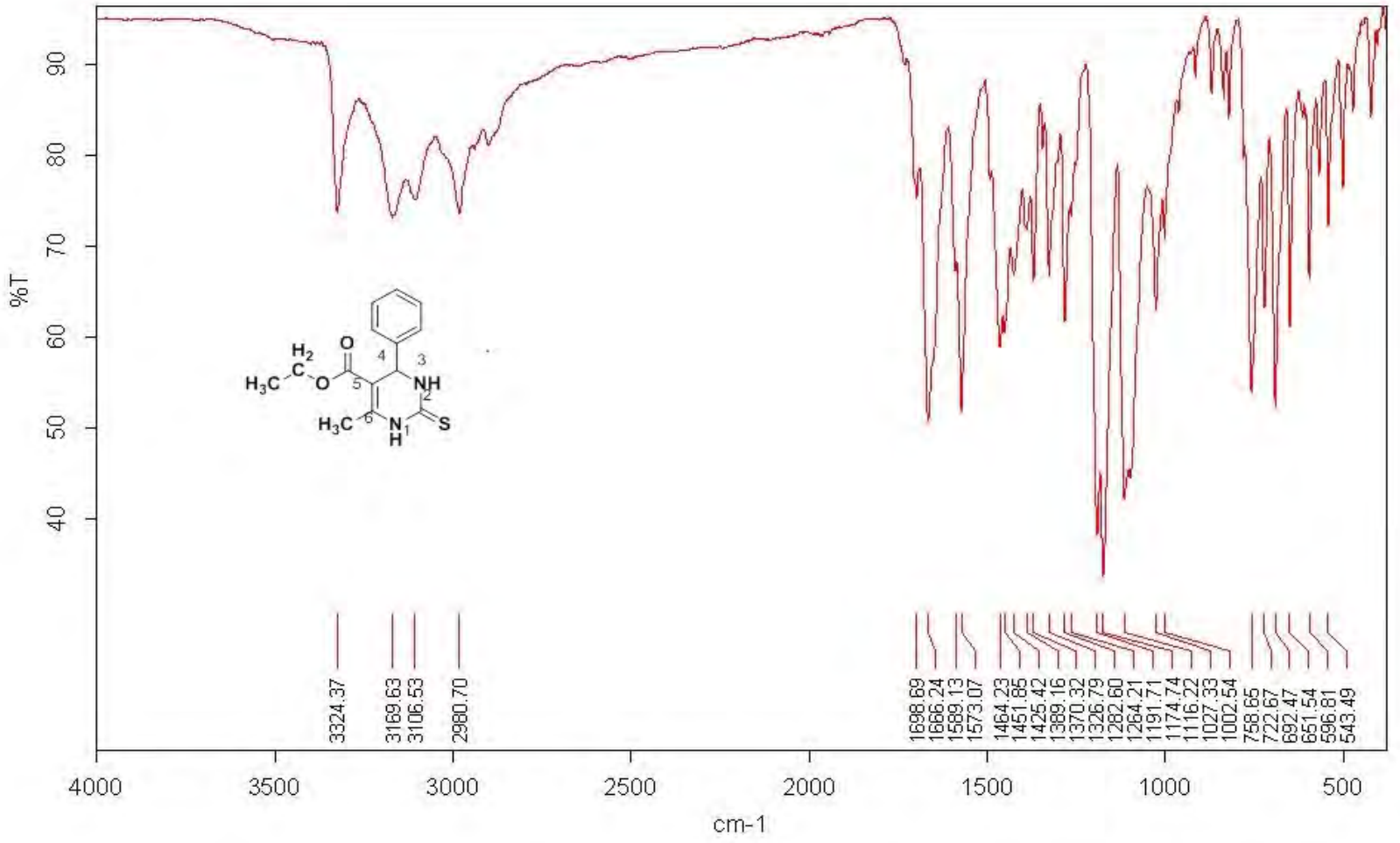

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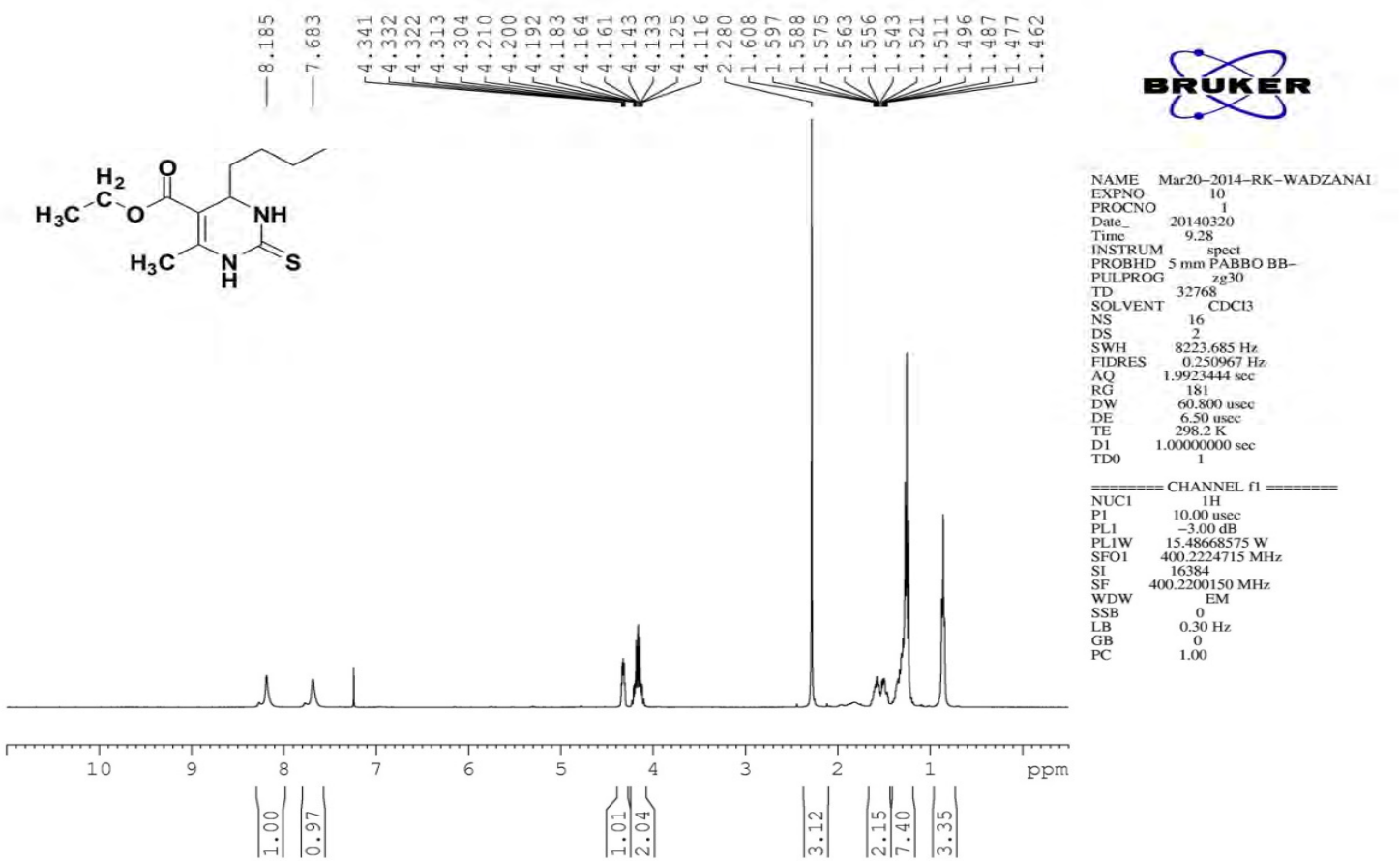

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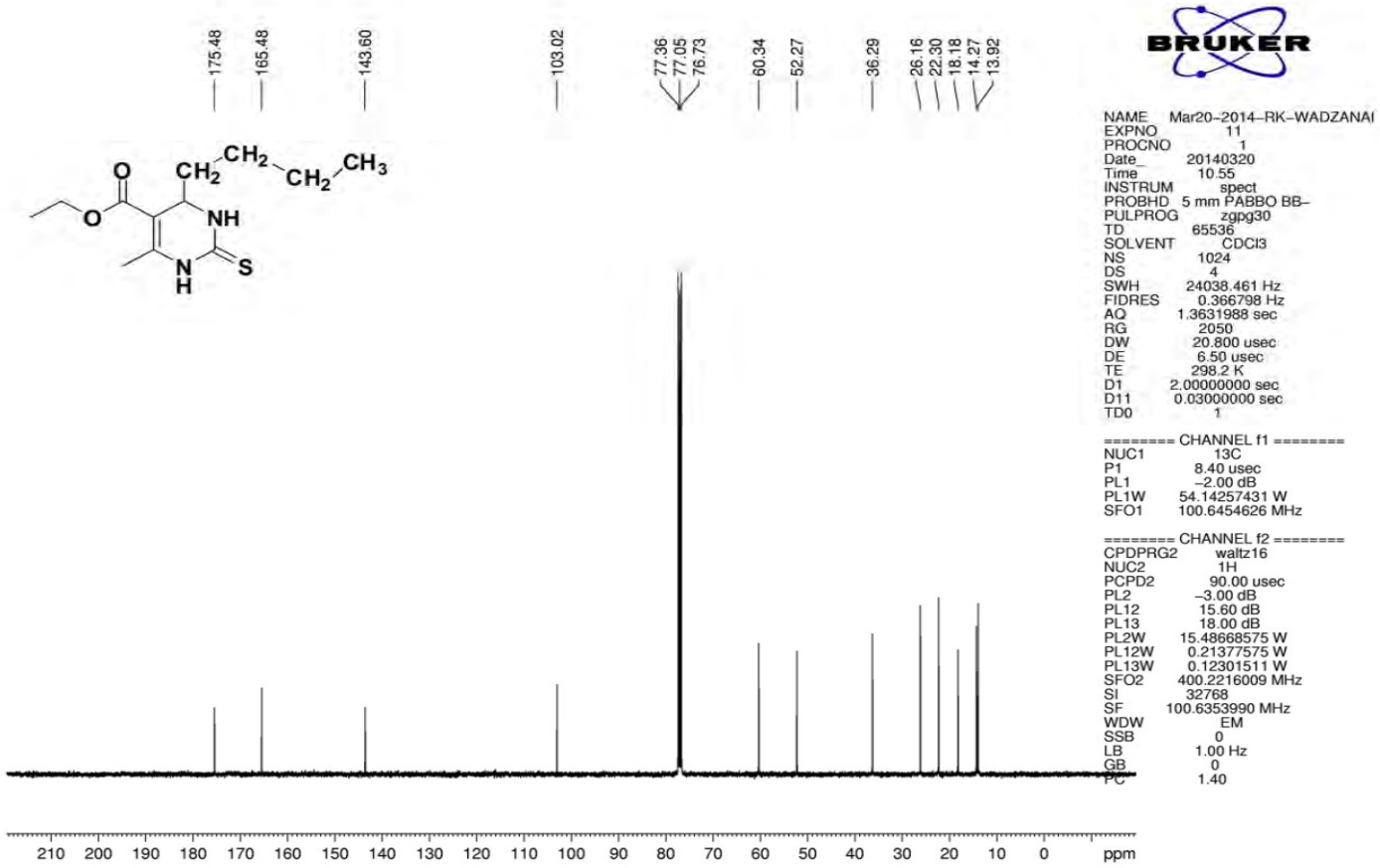

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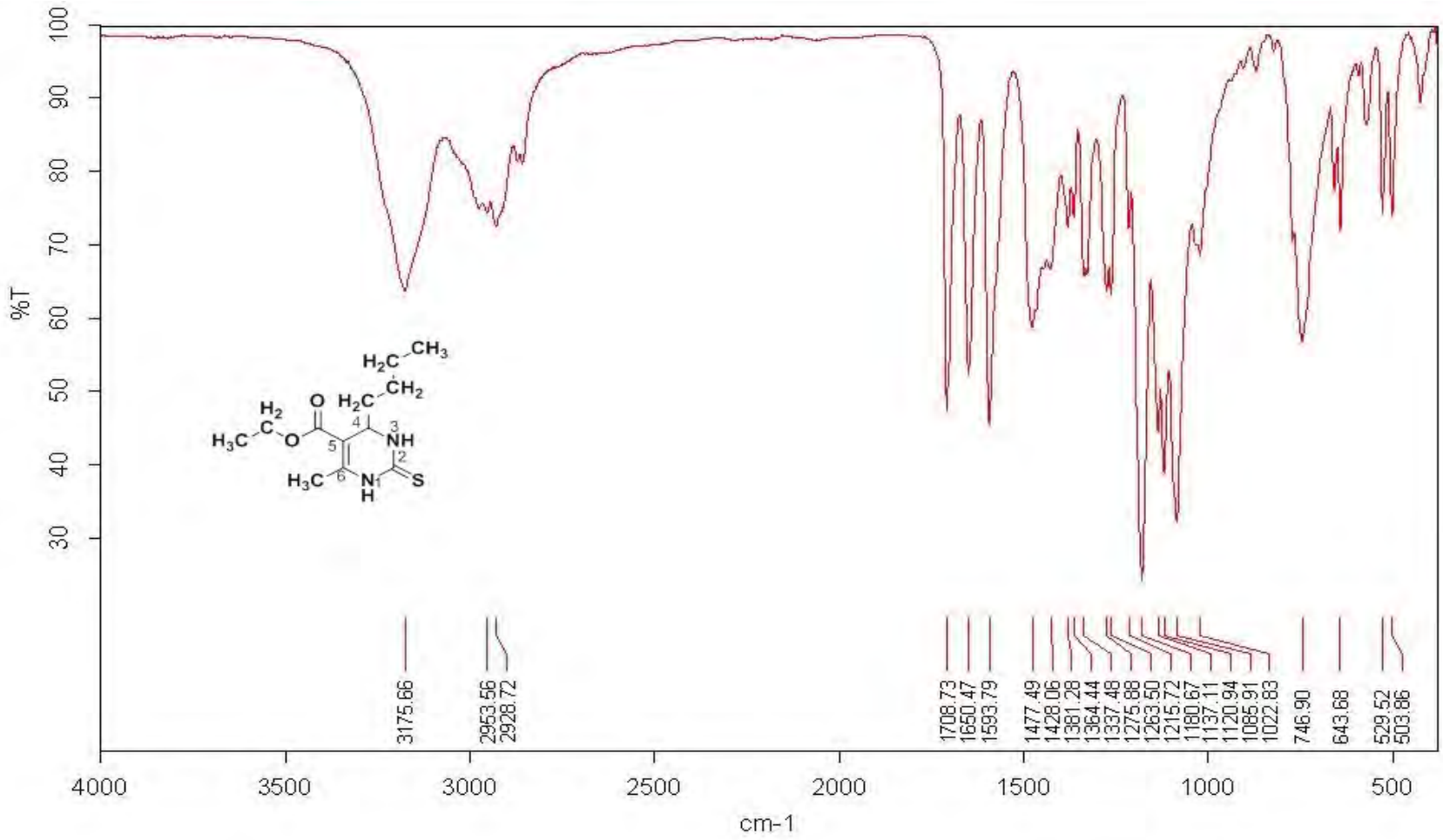

Spectrum 33. IR of compound **3k**