DESIGN, SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF NOVEL PYRIDINE-THIADIAZOLE DERIVATIVES AS ANTITUBERCULAR AGENTS TARGETING *ATP SYNTHASE*

A Dissertation submitted to
THE TAMIL NADU Dr.M.G.R. MEDICAL UNIVERSITY
CHENNAI - 600 032

In partial fulfilment of the requirements for the award of the Degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL CHEMISTRY

Submitted by
L.KARTHIKEYAN
Reg. No : 261915706

Under the guidance of
Dr.A.JERAD SURESH M.Pharm., Ph.D., M.B.A.,
Principal, Professor & Head,
Department of Pharmaceutical Chemistry
College of Pharmacy, Madras Medical College

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
COLLEGE OF PHARMACY, MADRAS MEDICAL COLLEGE
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EXAMINERS

1.

2.

Acknowledgement

I thank god almighty for giving me the strength to successfully complete my M.Pharm project work.

I would like to express my gratitude towards My Family members for the encouragement which helped me in completion of this project.

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LIST OF ABBREVIATIONS

TB	Tubercle Bacillus
WHO	World Health Organization
HIV	Human Immuno Deficiency Syndrome
MDR-TB	Multi Drug Resistant TB
XRD-TB	Extensively Drug Resistant-TB
TDR- TB	Totally Drug Resistant – TB
INH	Isoniazid
AIDS	Acquired Immuno Deficiency Syndrome
FDA	Food and Drug Administration
DOTS	Directly Observed Treatment Short-Course
BCG	Bacilli Calmette Guerin
µg/ml	Microgram / millilitre
MTB	*Mycobacterium tuberculosis*
DNA	Deoxyribo Nucleic Acid
InhA	Enoyl acyl carrier protein reductase
ATP	Adenosine Tri Phosphate
ADP	Adenosine Di Phosphate
ADME	Absorption, Distribution, Metabolism, Excretion
CADD	Computer Aided Drug Design
HTS	High Throughput screening
SAR	Structural Activity Relationship
MABA	Microplate Alamar Blue Assay
OSIRIS	Optical, Spectroscopic and Infrared Remote Imaging System
PSA	Polar Surface Area
LBDD	Ligand Based Drug Design
SBDD	Structure Based Drug Design
TLC	Thin Layer Chromatography
LC-MS	Liquid Chromatography Coupled with Mass Spectrometry
NMR	Nuclear Magnetic Resonance Spectroscopy
PDB	Protein Data Bank

ADT	AutoDock Tools
Kcal	Kilo Calories
HPLC	High Performance Liquid Chromatography
MIC	Minimal Inhibitory Concentration
OECD	Organization for Economic Co-operation and Development
Rf	Retention Factor
BACTEC	Bactenecin
Log P	Partition Co-efficient
DMEM	Dulbecco's Modified Eagle Medium

INTRODUCTION

1. INTRODUCTION

TUBERCULOSIS:

Tuberculosis is caused by bacteria Mycobacterium tuberculosis and it most often affects the lungs. TB spreads through the air when people with pulmonary TB cough, sneeze or spit. A person needs to inhale only a few germs to become infected. In the late 1800s, tuberculosis (TB) was one of the leading causes of ill health and death in most of the parts in the world. On March 24, 1882, Dr. Robert Koch announced that *Mycobacterium tuberculosis*, is the cause for tuberculosis (TB) in a speech **"Die Aetiologie der Tuberculose"** at the Berlin Physiological Society conference. Common symptoms of TB disease include prolonged cough, chest pain, weakness or fatigue, weight loss, fever, night sweats. Diagnosis involves testing of sputum samples and for non-lung TB disease, body fluids and tissue can be tested for TB bacteria.

THE ETIOLOGICAL AGENT:

The Mycobacterium genus can be separated into two major groups. One group includes the Mycobacterium tuberculosis complex and the other includes non-tuberculous (also known as environmental) mycobacteria. The Mycobacterium tuberculosis complex includes M. tuberculosis (Mtb), M. canettii, M. africanum, M. microti, M. bovis, M.caprae and M.pinnipedii. Mycobacteria are facultative intracellular bacteria that multiply within phagocytic cells[1].

MYCOBACTERIAL CELL WALL:

The cell envelope of Mycobacterium tuberculosis consists of three main structural components: (a) the characteristic long-chain mycolic acids, (b) a highly branched arabinogalactan (AG) polysaccharide, and (c) a cross-linked network of peptidoglycan. The entire complex, referred to as mAGP, is essential for cell viability. In addition, an outer membrane segment that contains solvent-extractable lipids, such as inert waxes and glycolipids, intercalates the mycolate layer of the mAGP complex. Finally, an outermost capsule composed of polysaccharides and proteins completes the cell envelope of M. tuberculosis[2].

CO-INFECTION:

Tuberculosis, HIV coinfection with TB, emergence of multidrug-resistant TB, and extensively drug-resistant TB are the major causes of death from infectious diseases worldwide. An estimated 9.9 million people fell ill with TB worldwide in 2020. 1.5 million people died from TB including 214 000 people with HIV. TB remains one of the world's top infectious killers[3].

EPIDEMIOLOGY AND TB BURDEN IN DIFFERENT COUNTRIES:

Among all TB cases, 8.0% were among people living with HIV. The percentage of TB cases coinfected with HIV was highest in countries in the WHO African Region, surpassing 50% in parts of southern Africa.

TB threatens people of both sexes and all age group. The highest burden is in adult men, who reported for 56% of all TB cases in 2020, by comparison, adult women reported for 33% and children for 11%. The higher portion of TB cases among men is consistent with evidence from prevalence survey, which show that TB disease affects men more than women, and that gaps in case detection and reporting are higher among men.

BURDEN OF TB IN INDIA:

In India number of people newly diagnosed with TB is 5.8 million in 2020. Cumulative number of deaths averted by TB and TB/HIV interventions 2000–2020 (in millions), globally and by WHO region[3]

WHO REGION	HIV-NEGATIVE PEOPLE		HIV-POSITIVE PEOPLE		TOTAL	
	BEST ESTIMATE	UNCERTAINTY INTERVAL	BEST ESTIMATE	UNCERTAINTY INTERVAL	BEST ESTIMATE	UNCERTAINTY INTERVAL
African Region	6.6	5.5–7.7	8.2	6.9–9.5	15	13–17
Region of the Americas	1.8	1.7–2.0	0.34	0.31–0.38	2.3	2.0–2.3
South-East Asia Region	23	19–28	2.8	1.9–3.8	26	22–31
European Region	2.1	1.8–2.3	0.30	0.26–0.34	2.4	2.1–2.6
Eastern Mediterranean Region	4.7	4.1–5.3	0.08	0.06–0.10	4.8	4.2–5.4
Western Pacific Region	15	14–16	0.48	0.40–0.57	16	14–17
Global	54	47–60	12	11–14	66	59–73

CURRENT TREATMENT AGAINST TUBERCULOSIS:

DRUG RESISTANCE TB:

Since the prevalence of Multi drug resistant (MDR-TB), extremely drug resistant (XDR-TB) and totally drug resistant TB (TDR-TB) cases are increasing, it is important to introduce new anti-TB drugs with novel mechanism of action to treat all forms of TB. Since the past few decades, there are no new anti-TB drug has emerged except for bedaquiline and delamanid which are used for healing. pulmonary MDR-TB patients. Which is used in treatment of severe or life-threatening conditions.

(a) Multidrug-resistant tuberculosis (MDR TB):

MDR organisms are resistant to the front-line drugs which has been linked to mutations in at least 10 genes. Eg. Isoniazid and rifampicin which covers 5.3% of TB cases all over the world.

MDR-TB results from either infection with organisms which are already drug resistant or may develop in the course of a patient's treatment.

(b) Extensively drug resistant TB (XDR TB):

XDR Strains resistant to isoniazid and rifampicin as well as one of fluroquinolones and second line drugs eg.Capreomycin, kanamycin covers 2% of TB cases.

NEED FOR NEW ANTI-TB DRUGS:

- To expand the current treatment regimen of MDR-TB.
- To shorten the duration of tuberculosis treatment.
- To provide new drugs that are more active against latent Mtb organism.

VARIOUS TARGETS IN DRUG DISCOVERY:

Targeting the Transcription and Translation

Targeting the DNA replication and protein synthesis in Mtb is well-tested and many drugs from this category arebeing used in first line and second line treatments, including fluoroquinolones (DNA unwinding and replication), rifampin (RIF) (transcription), streptomycin, kanamycin, amikacin, and capreomycin (translation).

Targeting Cell Wall Biosynthesis

The cell wall of Mtb is a complex 3-dimensional structure that is essential for its pathogenesis and survival. The hydrophobic character of the complex cell wall offers a natural barrier for the passage of hydrophilic drugs, including many antibiotics. So inhibiting cell wall biosynthesis is also a recommended approach.

Targeting Energy Metabolism

Mycobacterium tuberculosis (Mtb) produces adenosine triphosphate (ATP) via 2 inter-linked metabolic pathways, substrate level phosphorylation and oxidative phosphorylation. In contrast to other bacteria, Mtb needs higher basal energy, so it depends on both phosphorylation methods. Mutagenetic studies have confirmed that oxidative phosphorylation is indispensable in Mtb. ATP synthase is an important target in this category[4].

ENZYME PROFILE:

Enzyme name: ATP synthase

Classification: Translocase

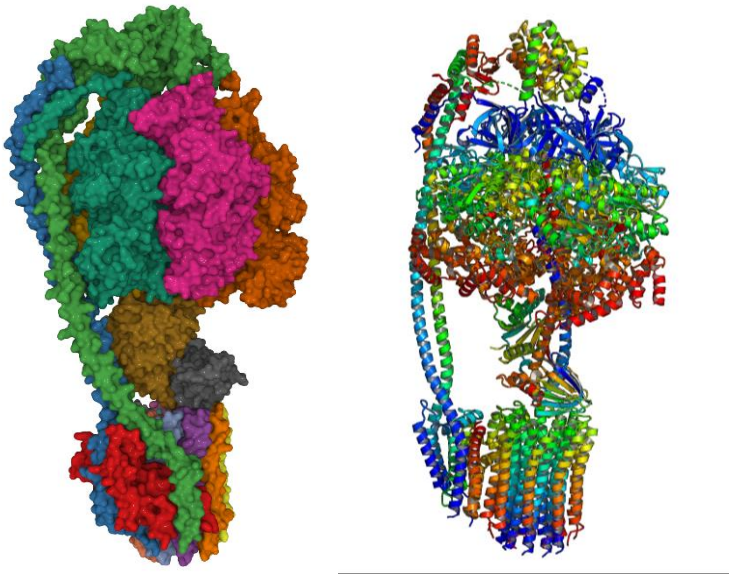
Type: Protein

Chains: A, B, C, D, E, F, G, H, I, J, K, L

Ligands: 2 Native ligands

Sequence Length: 548

Organism: Mycobacterium tuberculosis



Mycobacteria will able to respire and generate ATP via oxidative phosphorylation. Mycobacteria has many primary dehydrogenases to run Electron Transport Chain (ETC) and two terminal respiratory oxidases, cytochrome-c oxidase and cytochrome-bd-type menaquinol oxidase. Hypoxia causes decrease in key respiratory enzymes. Mycobacteria are obligate aerobes which has the ability to metabolize in the absence of oxygen and a number of reductases are present to produce reducing equivalents (e.g. nitrate reductase, succinate dehydrogenase/fumarate reductase). ATP synthesis is mediated by the membrane-bound ATP synthase in growing and non-growing organism and this enzyme is able to function over a wide range of oxygen levels (aerobic to hypoxic)[5]. ATP synthase is a omnipresent enzyme which is largely conserved across many kingdoms of life. In pathogenic bacteria which employ ATP synthase and deals with energetically unfavorable situations such as low oxygen in the human host, e.g., Mycobacterium tuberculosis which can survive inside macrophages for long time. This enzyme is validated as a druggable target for new antimycobacterial molecules[6].Bedaquiline is approved for treatment of drug-resistant *Mtb.* ATP synthesis

inhibition is effective against all states of organism like active, dormant, intracellular, extracellular, replicating and non-replicating.

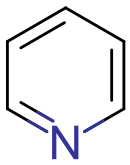
MECHANISM:

The Mycobacterium tuberculosis (which causes tuberculosis) can survive in low-energy conditions, allowing infections to remain dormant and decreasing their susceptibility to many antibiotics. The drug-free 3D structure proposes that hook-like extension from the α-subunit prevents the enzyme from running in reverse mode, thus inhibiting ATP hydrolysis and conserving energy in hypoxic conditions. Bedaquiline binding brings large conformational changes in the ATP synthase, creating tight binding pockets at the interface of subunits a and c which explains the potency of the drug as an anti-tuberculosis agent.

BASIC NUCLEUS: PYRIDYL-THIADIAZOLE

Pyridine:

Pyridine is a basic heterocyclic organic compound with the chemical formula C_5H_5N. In many aspects it can be related to well established and very fundamental aromatic molecule, benzene, with one C-H group replaced by a N atom. Pyridine has a conjugated system of six π-electrons exactly as benzene has, that are delocalized over the heterocyclic ring. The molecule is planar in nature and follows Huckel criteria for aromaticity.

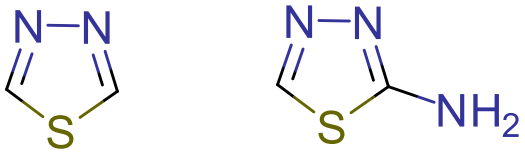


Structure of Pyridine

Molecular formula of pyridine is C_5H_5N. It is an Organic base which is flammable, toxic yellowish liquid with penetrating aroma and burning taste, soluble in water, alcohol, ether, benzene, and fatty oils and boils at 116°C. Pyridine and its derivatives have antimicrobial (isoniazid), anti-cancer, analgesic, and anti-depressant activity. Pyridine is used in the pharmaceutical industry as a starting material for various drugs, vitamins, and fungicide and as a solvent. Apart from thesr, Pyridine derivatives have following biological activities:
Antiviral activity, antichagastic activity, antifungal activity, antidiabetic activity.

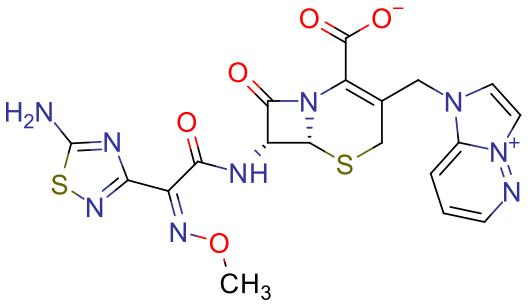
1,3,4-thiadiazole:

1,2,4-thiadiazole is a five-member heterocyclic moiety which represents an important class of core structures of great interest mainly because of their various biological activities and associated therapeutic applications.



Structure of 1,3,4-thiadiazole and 2-amino-1,3,4-thiadiazole

Thiadiazole scaffold acts as "hydrogen binding domain" and "two electron donor system." There are several isomers of thiadiazole, they are 1,2,3-thiadiazole, 1,2,4-thiadiazole, 1,2,5-thiadiazole, and 1,3,4-thiadiazole. 1,3,4-Thiadiazole is the important isomer which possess versatile pharmacological properties. 1,3,4-Thiadiazole was first described in 1882 by Fischer and developed further by Bush and coworkers, but true nature of the ring system was first demonstrated in 1956 by Goerdler et al. Thiadiazole has hydroxyl, amino and mercapto groups and exists in tautomeric forms. Many of the 2-amino-1,3,4-thiadiazole derivatives are taken as lead compounds for synthesis of drugs. One of the commercially available 1,2,4-thiadiazole drug is Cefozopran which is an antibiotic.



Structure of Antibiotic Cefozopran

The 1,3,4-thiadiazoles can be divided into three subclasses:

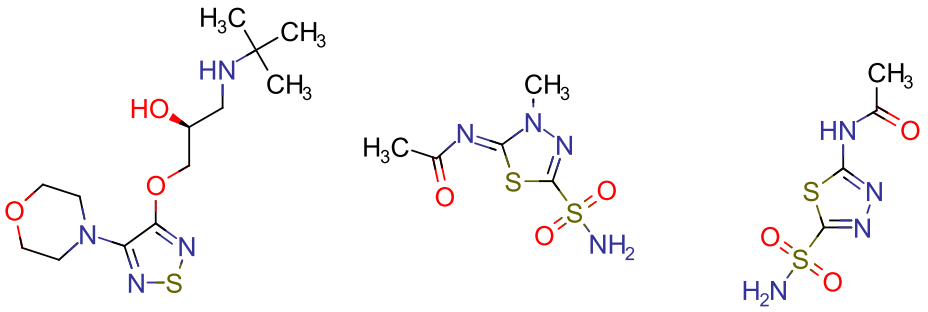
- Aromatic system like the neutral thiadiazole.
- Mesoionic systems which are defined as five-membered heterocycle which are not covalent or polar and possess a sextet of electrons in association with the five atoms in the ring.
- Non-aromatic system like the 1,3,4-thiadiazole and the tetrahydro 1,3,4-thiadiazole[7].

Different isomers of Thiadiazole

1,2,4-thiadiazole is bio-isostere of pyrimidine, and 1,3,4-thiadiazole is bio-isostere of pyridazine by the substitution of −CH=CH− by −S−. The thiadiazole ring is also a bio-isostere of oxadiazole, thiazole, oxazole, and benzene. The bio-isosteric replacement of a ring with another ring might lead to compounds with change in lipophilicity and improved biological activity. The thiadiazole derivatives, due to sulfur atom that gives high lipophilicity show good cell permeability[8].

Table 1 Bio-isosteres of thiadiazoles

Timolol	A non-selective beta-adrenergic blocker used in the treatment of elevated intraocular pressure in ocular hypertension or open angle glaucoma.
Methazolamide	A carbonic anhydrase inhibitor used to treat open angle glaucoma and acute angle closure glaucoma.
Acetazolamide	A carbonic anhydrase inhibitor used to treat edema from heart failure or medications, certain types of epilepsy, and glaucoma.



i) Timolol ii) Methazolamide iii) Acetazolamide

DRUG DISCOVERY PROCESS:

The current process of the drug discovery involves multiple disciplines such as chemical, structural biology, computational chemistry, organic synthesis, and pharmacology. And, includes different stages like:

(a) Target identification: It involves the discovery and isolation of individual targets like enzyme, receptors, ion channels and investigating the functions and its relation with the specific disease.

(b) Target validation stage shows where the drug target is linked to the disease of interest, as well as their capacity to regulate particular functions in the body after binding to a complementary molecule. Many studies are conducted to prove that the target macromolecule is linked to the diseased state.

(c) Lead identification involves the discovery of a compound that shows some degree of potency and specificity against a validated target and is assumed to have the properties of a drug which can ameliorate disease.

(d) Lead optimization stage involves the improvement of potency and other significant parameters through many cycles of evaluation of the lead compounds and their analogs. So, both *in-vitro* and *in-vivo* experiments are performed to prioritize and select the proper candidates with optimum potential for development as a safe and efficacious drug. Moreover, SARs are developed to optimize pharmacokinetic and pharmacodynamic properties.

(e) Preclinical stage includes drug synthesis and formulation research, *in-vivo* animal studies for potency and toxicity information, and identification of mechanism of toxicity.

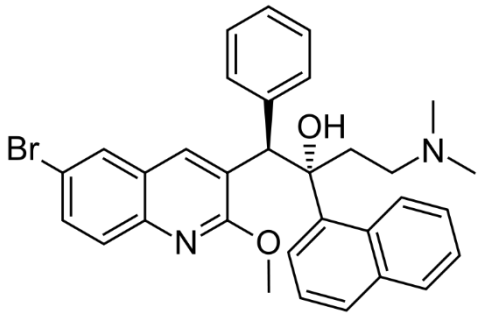
(f) Clinical trials include three phases that investigate safety, adverse side effects, dosage, efficacy, pharmacokinetic and pharmacological properties of the candidate drug on human volunteers.

LITERATURE REVIEW

2. LITERATURE REVIEW

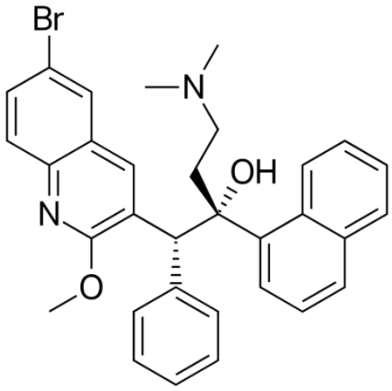
LITERATURE REVIEW RELATED TO ATP-SYNTHASE:

Lu et al. (2014) reported the importance of ATP synthase which is a ubiquitous enzyme in pathogenic bacteria which employ ATP synthase to deal with energetically unfavorable conditions such as low oxygen tensions in the human host, e.g., Mycobacterium tuberculosis. ATP synthase has been validated as a target for promising new antibacterial drugs[6].



TMC207

Sarathy et al. (2019) explained the two mechanisms of action of Bedaquiline that is both direct and indirect mechanism[9]



Bedaquilline

Montgomery et al. (2021) determined the 3D structure of ATP synthase from *Mycobacterium smegmatis*[10] by electron-cryo-microscopy

Segala et al. (2021) explained that TMC207 is a new antituberculosis agent which belongs to the diarylquinoline category which inhibit the ATP synthase of mycobacterium. and mapped the amino acid residues which are involved in the binding of TMC207[11].

LITERATURE RELATED TO MABA:

Franzblau et al. (1998) studied MIC determination by MABA. A colorimetric, Microplate Based Alamar Blue Assay (MABA) method was used to determine the MICs of first line antimycobacterials like Isoniazid, Rifampin, Streptomycin and Etambutol for 34 peruvian Mycobacterium tuberculosis isolates and the $H_{37}Rv$ strain. The MABA is a colorimetric assay which is simple, rapid, low cost, appropriate technology which does not require extensive instrumentation and which makes use of a nontoxic, temperature stable reagent[12].

Alba-Romero et al. **(2011)** studied the various applications of Alamar Blue indicator. Alamar Blue is a redox indicator which is used to evaluate metabolic function and cellular health. The Alamar Blue Bioassay is used to access cell viability and cytotoxicity in a number of cell types including bacteria, yeast, fungi, and protozoa.[39] 7. Jose de Jesus Alba-Romero et al. applied the Alamar Blue Assay to determine the susceptibility to anti-tuberculosis pharmaceuticals[13].

LITERATURE FOR TUBERCULOSIS:

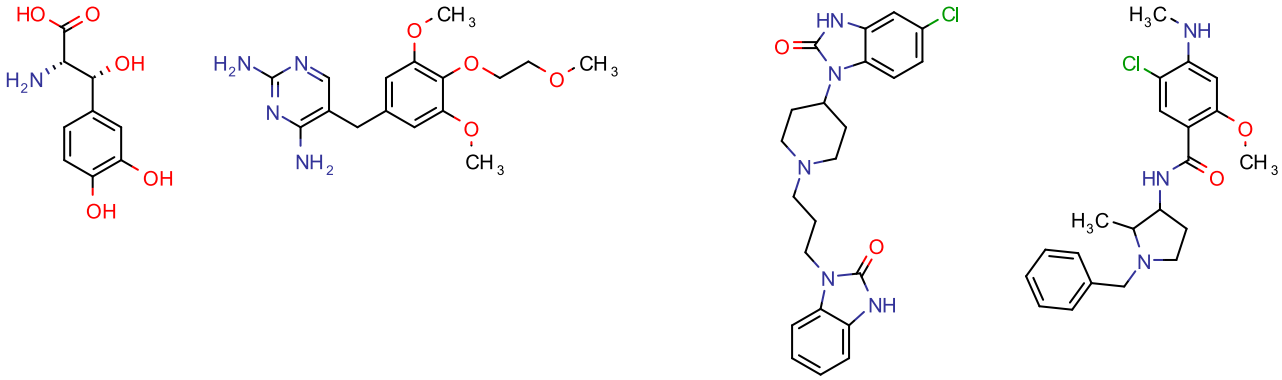
Smith et al. (2004) worked on TB drug discovery and addressed issues of persistence and resistance by studying the recent developments of some of the pathways involved in a persistent infection and pathogenesis of tuberculosis, which exposed new targets for drug development[14].

Kim et al. (2005) have shown that Tuberculosis (TB) is one of the most devastating diseases primarily due to several decades of neglect, and presents a global health threat of escalating proportions. TB is the second leading infectious causes of mortality today behind only HIV/AIDS[15].

LITERATURE REVIEW FOR DRUG DESIGN:

Balss, (2015) reported the basic Principles of Drug Discovery and Development and provided comprehensive explanations like high throughput screening, structure based drug design and molecular modeling[16].

Kaur et al. (2018) identified novel molecules including 4 FDA approved drugs (droxidrpa, tetroxoprim, domperidone and nemonapride) which can tackle the MDR Mtb[17].



Structure of Droxidopa, Tetroxoprim, Domperidone and Nemonapride

Shahbaaz et al. (2019) explained shortcomings in treatment of conventional tuberculosis and drug resistance in Mycobacterium tuberculosis. Bedaquiline was recently approved for the treatment of multidrug-resistant strains of tuberculosis, which targets the ATP synthases. The authors used Rv1311 as the target for M. tuberculosis and pharmacophore which revealed potential inhibitors present in ZINC database[18].

Jian et al. (2020) Studied the importance of thymidylate kinase (MtbTMPK) which is responsible for the synthesis of thymidine triphosphates and DNA synthesis. Synthesized cyanopyridone analogues and evaluated the anti-mycobacterial activity[19].

Mouchlis et al. (2021) reported how conventional drug designs like structure based and ligand-based drug design can be taken to next level using artificial neural networks. This approach can accelerate the drug discovery process many fold and reported some advances in de novo drug design using machine learning[20].

LITERATURE REVIEW FOR SPECTROSCOPY:

Kalsi, (2007) Text book on Spectroscopy of organic compounds[21].

Chatwal, (2008) Text book on Instrumental methods of chemical analysis[22].

Sharma, (2008) Text book on Elemental Organic Spectroscopy[23].

Kealey and Haines, (2010) Text book on Instant notes Analytical Chemistry[24].

LITERATURE RELATED TO PYRIDINE:

Nechipadappu and Trivedi (2017) synthesized six molecular salts of non-steroidal anti-inflammatory drugs and characterized the salts for their stability[25].

Bentzinger et al. (2020) Investigated the enantiopure substituted pyridines which can serve as new antimalarial drug candidates. Also, the author showed that 4-substituted pyridines show strong in-vitro antimalarial activity against *P. falciparum*[26].

Ismael et al. (2021) synthesised 12 new methylsulfonyl-containing imidazo[1,2-a]pyridines and evaluated for COX-1/2 inhibitory activity and in vivo anti-inflammatory activity[27].

LITERATURE RELATED TO 1,2,4-THIADIAZOLE:

Romagnoli et al. (2007) reported synthesis and biological activity of benzo[4,5]imidazo[1,2-d][1,2,4]thiadiazole and showed that these compounds suppress survival and proliferation by apoptosis. The apoptosis is induced by caspase-3 activation[28].

Hassanzadeh et al. (2020) showed that imine, amide derivatives of 1,2,4-thiadiazole derivatives possess antileishmanial effects[29].

Trafalis et al. (2021) synthesized compounds with fused 1,2,4-triazole and 1,3,4-thiadiazole rings and evaluated anticancer activity. They possesses potent in-vitro and in-vivo anti-cancer activity by inhibition of Akt Ser-473 phosphorylation. The compounds bind in ATP binding site in Akt1 and Akt2[30].

AIM AND OBJECTIVE

3. AIM AND OBJECTIVE

AIM:

To develop novel and potent anti-tubercular agents which targets ATP synthase enzyme of mycobacteria.

OBJECTIVE:

1. To design 500 molecules and predict ADMET properties, Druglikeness, Toxicity, anti-mycobacterial activity spectra using Molinspiration®, SwissADME, OSIRIS®, and WaytoDrug PASS online.

2. The molecules that possess druglikeness properties are evaluated for *in-silico* enzyme inhibition activity by molecular docking against *ATP Synthase* (PDB ID: 7JG5) and find high scoring compounds

3. To synthesize, purify and characterize the novel Pyridine-Thiadiazole derivatives.

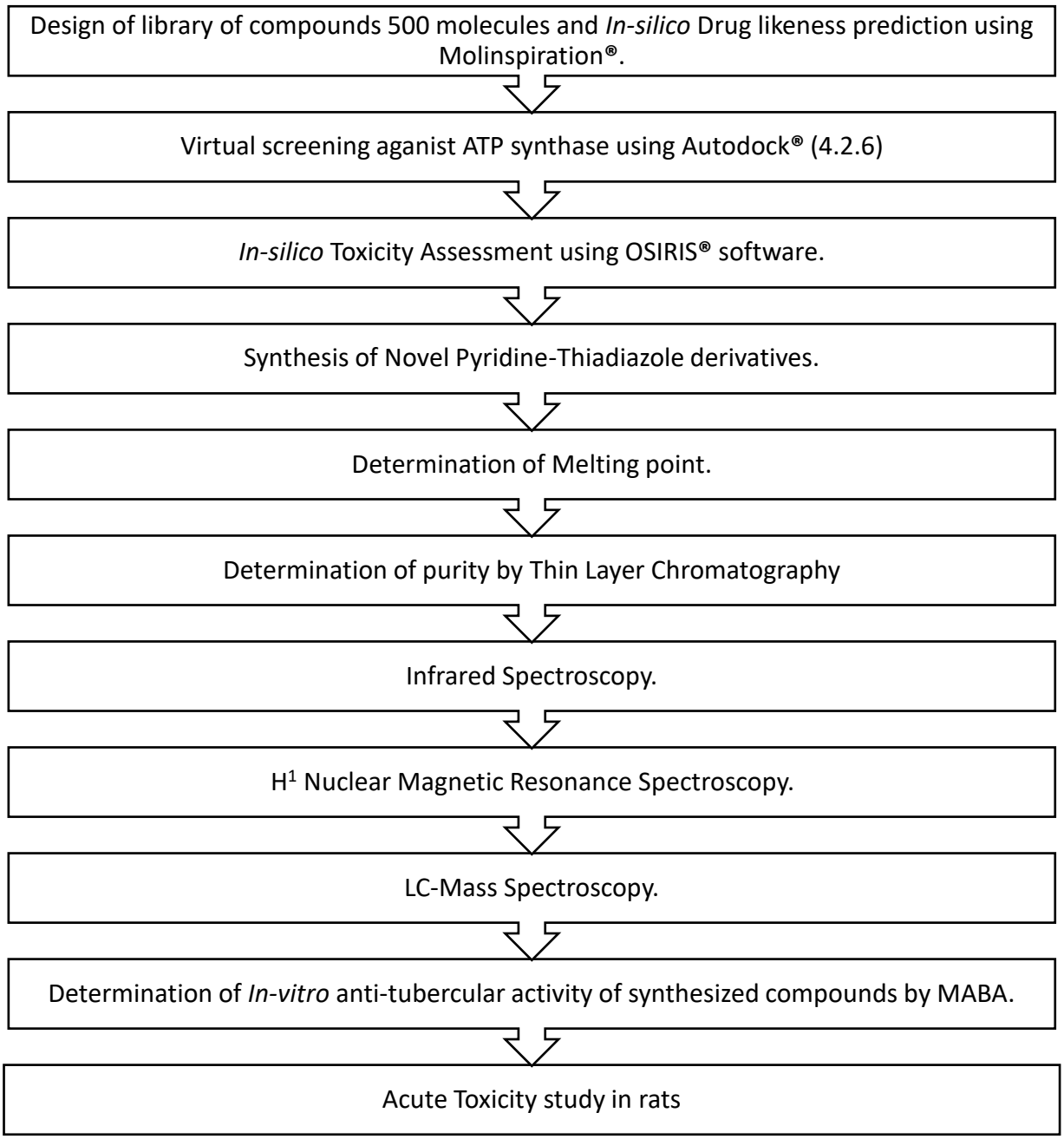
4. To evaluate anti-tubercular and acute toxicity of the novel Pyridine-Thiadiazole derivatives.

The plan of work includes:

- Design of library of compounds and *In-silico* Drug likeness prediction using Molinspiration® (https://www.molinspiration.com/)
- Virtual screening against ATP synthase by molecular docking using Autodock® (4.2.6 version) software.
- *In-silico* Toxicity Assessment using OSIRIS® software.
- Synthesis of Novel Pyridine-Thiadiazole derivatives.
- Characterization of the synthesized compounds by
    - Determination of melting point.
    - Infrared Spectroscopy.
    - H^1 Nuclear Magnetic Resonance Spectroscopy.
    - LC-Mass Spectroscopy.
- Determination of *In-vitro* anti-tubercular activity of synthesized compounds by MABA.
- Acute Toxicity study in rats by following OECD-423 guidelines

PLAN OF WORK

4. PLAN OF WORK



MATERIALS AND METHODS

5. MATERIALS AND METHODS

LIPINSKI'S RULE[31]

Lipinski's rule of five, also known as Pfizer's rule of five or simply rule of five, is a rule of thumb to predict druglikeness or determine if a molecule with a certain pharmacological activity has chemical and physical property that would make the compound orally active drug in humans. The rule was formulated by **Christopher A. Lipinski** in 1997. He proposed a rule that was based on the observation of orally administered drugs. Most of the orally available drugs are relatively small molecules and moderately lipophilic.

The rule defines those molecular properties which are important for a drug's pharmacokinetics, including their ADME. The rule does not predict if the compound is pharmacologically active but predicts if they can reach the site of action.

- Not more than 5 hydrogen-bond donors (the total number of nitrogens–hydrogen and oxygen–hydrogen bonds)
- Not more than 10 hydrogen-bond acceptors (all nitrogens or oxygen atoms)
- The molecular mass should be less than 500 daltons
- Octanol-water partition coefficient (log P) should not exceed 5.

many extensions to this rule have been developed Eg. Ghose filter, Veber's Rule.

IN-SILICO ASSESMENT OF DRUGLIKENESS – MOLINSPIRATION®[32]

Molinspiration is an online tool employed for predicting the Drug likeness & Bioactivity of the molecular compounds. Molecular properties of the designed ligands were predicted by drawing the structures in the online Molinspiration tool. Also, ADME prediction was carried out using free online web tool SWISS ADME (http://www.swissadme.ch/)

IN SILICO TOXICITY PREDICTION – OSIRIS® PROPERTY EXPLORER[33]

The prediction process is based on the precomputed structural fragments set. In all the toxicity class of compounds, the occurrences frequency of the fragments was searched. If the designed molecule contains any fragments which may be harmful [mutagenicity, tumorigenicity, irritation effects, reproductive effects], the tool detects the toxicity risk alerts and indicates the same in red color. The green color confirms the drug like behavior of the compounds without

any toxicity alerts. The *in-silico* toxicity of the designed compounds was predicted by drawing the structures using online tool.

ACTIVITY PREDICTION USING PASS ONLINE[34]

PASS Online predicts over 4000 classes of biological activity, including pharmacological effects, mechanisms of action, adverse effects and toxic effects. Also, interaction of compounds with metabolic enzymes, transporters and influence on the gene expression. PASS Online helps Finding most probable new leads with required activity spectra for the designed compounds

Pa – Probability of the compound being active

Pi – Probability of the compound being inactive

DOCKING

Docking programs are used to fit the ligand into the target 3D structure in different conformations. Docked conformation of ligand is known as pose. In order to identify the most favorable energetic pose, each pose is scored based on its complementarities to the target in terms of shape and electrostatic property.

SCORING FUNCTIONS:

To describe the binding energy of ligands to target the scoring functions are used. The Autodock used the following formula to calculate the docking score.

Estimated Free Energy of Binding = (1)+(2)+(3)-(4)

(1) Final Intermolecular Energy = (vdW + Hbond + desolv Energy and Electrostatic Energy)

(2) Final Total Internal Energy

(3) Torsional Free Energy

(4) Unbound System's Energy

MOLECULAR DOCKING BY AUTODOCK®[35]**:**

AutoDock® 4.2.6 is a software for predicting the interaction of ligands with targets. Progress in biomolecular x-ray crystallography provide the important protein and nucleic acid 3D

structures. These structures could be targets for animal and plant diseases. The understanding of the interaction of ligand molecules with the targets is crucial in drug discovery process.

In any docking scheme, two conflicting requirements must be balanced: the desire for speed and accuracy.

AutoDock® combines two methods to achieve these goals: rapid grid-based energy evaluation and efficient search of torsional freedom. The current version of AutoDock® uses the Lamarckian Genetic Algorithm and empirical free energy scoring function, typically will provide reproducible docking results for ligands with approximately 10 rotatable bonds. The quality of any docking result depends on the starting structure of both the protein and the ligand. The protein and ligand structure need to be prepared to achieve the best docking results.

- Protein preparation
- Ligand preparation
- Receptor grid generation
- Ligand docking (screening)

DOCKING PROCEDURE

Preparation of protein:

- Read molecule from the file (allows reading of PDB coordinate files)
- Edit → Charges → Compute Gasteiger (for arbitrary molecules)
- Edit → Hydrogen → Merge non polar

Preparation of Ligand:

- Ligand → Input from file
- Ligand → Torsion → choose torsion: Rotatable bonds are shown in green, and nonrotatable bonds are shown in red.
- Ligand → Torsion → set number of torsions: sets the number of rotatable bonds in the ligand by leaving the specified number of bonds as rotatable.
- Ligand → Output → save as ligand.pdbqt in working folder

Grid generation:

- Grid → Macromolecule → choose (choose the protein file that has been prepared and then save it in protein.pdbqt extension in working folder).
- Grid → Set map types – open/choose ligand: tools to define the atom types for the grids that will be calculated
- Grid → Grid box – launches interactive commands for setting the grid dimensions and center (Set required dimension in the center on macromolecule)
- File → Close saving current
- Grid → Output – save as grid.gpf file (grid parameter file)
- Open command prompt and give the command [**autogrid4.exe –p grid.gpf –l grid.glg]**

Preparation of Docking Parameters:

- Docking → docking parameters: opens a panel for setting the parameters used during the docking calculation.
- Docking → Open the macromolecules → set rigid file name.
- Docking → ligand → open/choose the ligand.
- Docking → search parameters → genetic algorithm parameters: this command opens a panel for setting the parameters used by the search algorithm.
- Docking → output → Lamarkian GA –save as dock.dpf file (docking parameter file)
- Open command prompt and give the command **[autodock4.exe –p dock.dpf –l dock.dlg]**

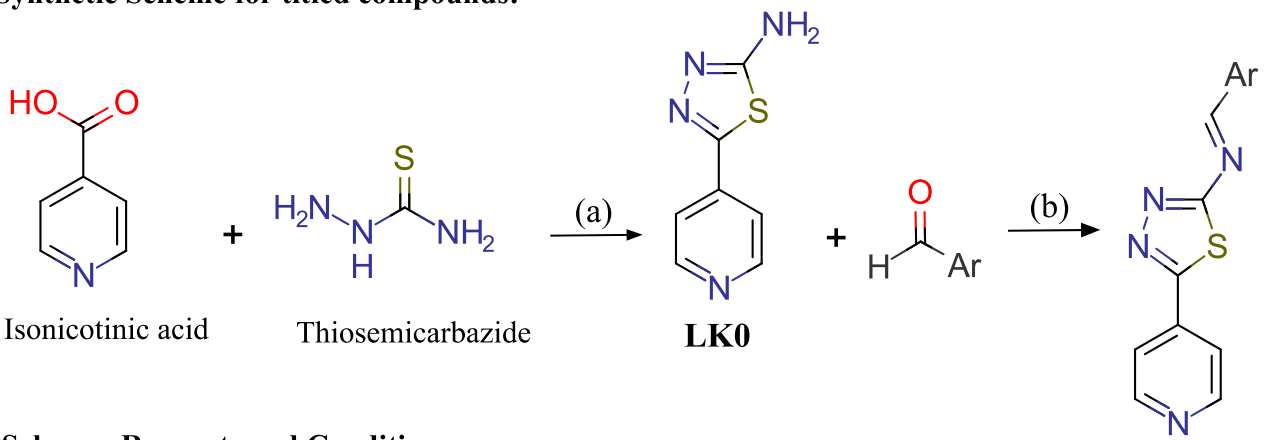
Visualization / Interpretation of Docking:

- Analysis → Docking → open dock.dlg (docking log file) file
- Analysis → open the macromolecule
- Analysis → Confirmation → Play and Play ranked by energy: Play → will use the order of conformations as they were found in the docking calculations, and Play Ranked By Energy.
- Analysis → Load: Information on the predicted interaction energy is shown at the top and the individual conformations
- Analysis → Docking → show interaction: specialized visualization to highlight interactions between the docked conformation of the ligand and the receptor.

PROCEDURE FOR SYNTHSIS OF PROPOSED TITLED COMPOUNDS

PROCEDURE:

Synthetic Scheme for titled compounds:



Scheme: Reagents and Conditions
(a) - $POCl_3$ reflux, 3h
(b) - C_2H_5OH, reflux, 6h

Step1 General procedure for preparation of 2-amino-5-(substituted)-1,3,4-thiadiazole (LK0):

A mixture of corresponding carboxylic acid (0.1 mol) and thiosemicarbazide (0.1 mol) was treated with $POCl_3$ (0.3 mol) dropwise at 0–5 °C and maintained at this temperature for 30 minutes. The reaction mixture was refluxed with strring for 4h. After cooling, 50 mL water was added to the reaction mixture. The pH of the mixture was adjusted to the range of 8–9 with the solution of 50% NaOH. The crude product is precipitated, was filtered, washed with water, dried, and recrystallized from ethanol to give the product[36].

Step2 General procedure for preparation of imines of 2-amino-5-(substituted)-1,3,4-thiadiazole (LK1-5)

A solution of 1 (0.01 mol) was prepared in 20 ml alcohol in a round bottom flask. Required aromatic aldehydes (0.01 mol) were dissolved in 15 ml alcohol, was then added to it. The mixture was refluxed for 4 hr. The volume of alcohol was reduced to half by distillation under reduced pressure. The resulting solution was poured on crushed ice. The precipitate which got separated was dried and recrystallized from ethanol [37].

REACTANT PROFILE

1. **ISO-NICOTINIC ACID:**

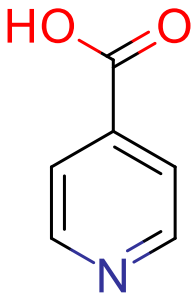


Table 2 Reactant profile of iso-nicotinic acid

SYNONYM	iso-nicotinic acid, Pyridine-4-carboxylic acid
MOLECULAR FORMULA	CH_5NO_2
MOLECULAR WEIGHT	123.11 g/mol
MELTING POINT	310ºC
DESCRIPTION	White powder, odourless

2. **THIOSEMICARBAZIDE**

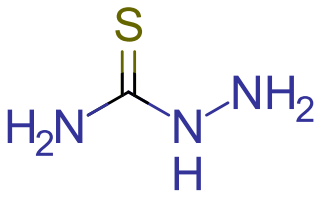


Table 3 Reactant profile of Thiosemicarbazide

SYNONYM	Thiosemicarbazide, N-amino urea
MOLECULAR FORMULA	CH_5N_3S
MOLECULAR WEIGHT	91.13 g/mol
MELTING POINT	183ºC
DESCRIPTION	White odorless solid

3. PHOSPHORUS OXY CHLORIDE

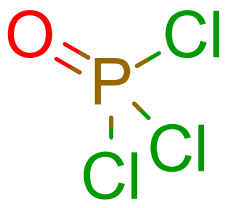


Table 4 Reactant profile of Phosphorous oxy chloride

SYNONYM	Phosphorous oxy chloride, Phosphorylchloride
MOLECULAR FORMULA	$POCl_3$
MOLECULAR WEIGHT	153.33 g/mol
MELTING POINT	1.25ºC
DESCRIPTION	Colourless liquid, fumes in moist air

4. N,N-DIMETHYL-AMINO-BENZALDEHYDE

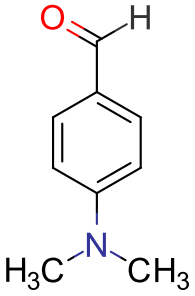


Table 5 Reactant profile of N,N-dimethyl-amino-benzaldehyde

SYNONYM	N,N-dimethyl-amino-benzaldehyde
MOLECULAR FORMULA	$C_9H_{11}NO$
MOLECULAR WEIGHT	149.19 g/mol
MELTING POINT	74ºC
DESCRIPTION	Off white to brown crystals

5. SALICYLALDEHYDE

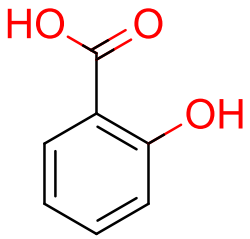


Table 6 Reactant profile of Salicylaldehyde

SYNONYM	Salicylaldehyde, 2-hydroxy-benzaldehyde
MOLECULAR FORMULA	$C_7H_6O_2$
MOLECULAR WEIGHT	122.123 g/mol
BOILING POINT	197^0C
DESCRIPTION	Colourless oily liquid

6. CINNAMALDEHYDE

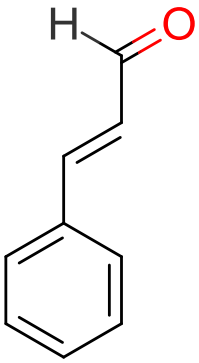


Table 7 Reactant profile of Trans- cinnamaldehyde

SYNONYM	Trans- cinnamaldehyde
MOLECULAR FORMULA	C_9H_8O
MOLECULAR WEIGHT	132.16 g/mol
BOILING POINT	248^0C
DESCRIPTION	Yellow oil

7. VERATRALDEHYDE

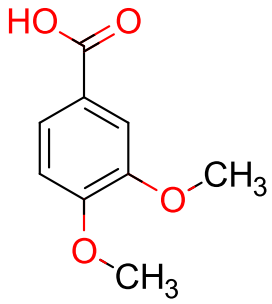


Table 8 Reactant profile of veratraldehyde

SYNONYM	Veratraldehyde, 3,4-dimethoxybenzaldehyde
MOLECULAR FORMULA	$C_9H_{10}O_3$
MOLECULAR WEIGHT	166.176 g/mol
BOILING POINT	281ºC
DESCRIPTION	Colourless solid

8. p-ANISALDEHYDE

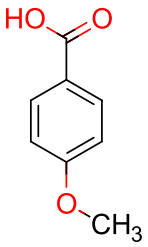


Table 9 Reactant profile of p-anisaldehyde

SYNONYM	p-anisaldehyde**,** 4-Methoxy benzaldehyde
MOLECULAR FORMULA	$C_8H_8O_2$
MOLECULAR WEIGHT	136.150 g/mol
BOILING POINT	248ºC
DESCRIPTION	Colourless liquid

CHARACTERIZATION

MELTING POINT

The melting point of the synthesized compound was determined by one end sealed capillary tube method. The temperature at which the compound starts losing its crystallinity and changes from solid to liquid form was found and recorded.

THIN LAYER CHROMATOGRAPHY

The reactants and products were dissolved in ethanol. It was spotted on the TLC plate. A single principal spot for the product and the absence of secondary spots for parent compounds and intermediates confirmed the purity of the compound. Stationary phase: pre-coated silica gel GF and appropriate mobile phase was used. The detection was done using UV chamber

IR SPECTROSCOPY

IR spectroscopy helps to determine the presence of the functional groups. Infrared spectroscopy is a technique based on the vibrations of bonds in a molecule. An infrared spectrum is commonly obtained by passing infrared radiation through a sample and determining what fraction of the incident radiation is absorbed at a particular energy. The energy at which any peak in an absorption spectrum appears corresponds to the frequency of a vibration of a part of a sample molecule. The synthesized compound was made into a pellet with potassium bromide by pressed pellet technique using KBr pellet press. The pellet was mounted on the pellet disc and percentage transmittance was recorded in ABB-MB-3000 IR Spectrophotometer.

NMR SPECTROSCOPY

Proton NMR Spectroscopy helps to study the number of equivalent protons and their environment thereby the structure of molecule can be ascertained. The NMR spectra was recorded on 300 MHz BRUKER Advance III NMR Spectrometer. DMSO was used as solvent. The NMR phenomenon is based on the fact that nuclei of atoms have magnetic properties that can be utilized to yield chemical information. The chemical shift of a particular nucleus can be correlated with its chemical environment. The scalar coupling (or J-coupling) indicates an indirect interaction between individual nuclei, mediated by electrons in a chemical bond. Under suitable conditions, the area of a resonance is related to the number of nuclei giving rise to it.

The possible characteristic peaks are in the following δ value range:

- Aromatic and Hetero aromatic proton 6 – 8 ppm
- Phenolic proton 4 – 12 ppm
- Aliphatic proton 1.5 – 4.5 ppm

LC - MASS SPECTROMETRY

Mass spectrometry is a powerful analytical technique that is used to elucidate unknown compounds, to quantify known compounds, and to identify the structure and chemical properties of molecules. Detection of compounds is done with very minute quantities. Mass spectra was recorded on Shimadzu HPLC-MS using Electron Spray Ionization Technique and was quantified using Lab Solutions Software 7.0, Samples were prepared by dissolving a minute quantity of pure compounds in methanol. Fragmentation pattern was reported in m/z values.

BIOLOGICAL EVALUATION

EVLUATION OF ANTI TUBERCULAR ACTIVTIY

There are various high through put assays available for screening of new chemical entities against Tuberculosis. They are

1. Micro Plate Alamar Blue Assay
2. BACTEC Assay
3. Luciferous Reporter Phage Assay
4. REMA assay
5. Broth Dilution Assay
6. Middle Brook (7H9,7H10,7H11) Agar, Dilution Assay

MICROPLATE ALAMAR BLUE ASSAY[12,38]

The Micro Plate Alamar Blue Assay (MABA) method was used to evaluate anti-tubercular activity of the synthesized compounds against mycobacterial strain *Mycobacterium tuberculosis* $H_{37}Rv$. The medium used for this evaluation is Middlebrook 7H9broth was used as the medium.

PRINCIPLE

Alamar Blue Cell Viability Reagent is a ready-to-use resazurin-based solution that functions as a cell health indicator by using the reducing power of living cells which is used to quantitatively measure viability of the cell. Resazurin is the active ingredient of alamarBlue reagent which is a non-toxic, cell-permeable compound. Resazurin is blue in color and non-fluorescent. When entering the living cell, resazurin is reduced to resorufin, which is red in color and highly fluorescent. Changes in viability can be effortlessly detected using either an absorbance- or fluorescence- based reader. This Reagent has broad applications and can be used with various human and animal cell lines, bacteria and fungi.

$$\% \text{ Inhibition} = \frac{\text{Test well fluorescence}}{\text{Mean fluorescence of triplicate wells}}$$

PROCEDURE

1. The anti-mycobacterial activity of compounds was evaluated against *M. tuberculosis* using Micro Plate Alamar Blue assay (MABA).

2. This method is non-toxic and uses a thermally stable reagent.

3. 200µl of sterile de-ionized water was added to all outer perimeter wells of sterile 96 wells plate to minimize evaporation of medium in the test wells during incubation.

4. The 96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilution of compounds was made directly on plate.

5. The final drug concentrations tested were 100 to 0.8µg/ml.

6. Plates were covered and sealed with parafilm and incubated at 37ºC for five days.

7. After this period of time, 25µl of freshly prepared 1:1 mixture of AlamarBlue reagent and 10% Tween 80 was added to each plate and incubated for 24 hrs.

8. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth MIC is defined as lowest drug concentration, which prevents the color change from blue to pink**.**

ADVANTAGES

- It has accurate time-course measurement
- It has high sensitivity and linearity
- It involves no cell lysis
- It is ideal for use with post measurement functional assays

PHARMACOLOGICAL EVALUATION

ACUTE TOXIC STUDY

Acute toxicity is defined as those adverse effects which occur after the oral administration of a single dose of test substance or multiple doses administered within 24 hours.

Animal: Female Albino rat

PROCEDURE

- The acute oral toxicity of the synthesized compounds was determined by acute toxic class method.
- As per the OECD guidelines No:423 the dosage of (2000mg/kg) was selected
- Healthy young adult female albino rats were selected in the age range of 8-12 weeks with weight of 100-150g.
- All rats were fastened prior to dosing by withholding food (not water) for 3-4 hrs.
- Following the period of fasting, the animals were weighed and the synthesized compounds should be administered orally at the dose of 2000mg/kg body weight.
- The animals were observed individually for every 30 minutes after dosing 2000 mg /kg, (3 animals per each dose) periodically for the first 24 hours.
- Animals were observed 14 days for any signs of morbidity and mortality.

RESULTS AND DISCUSSION

6. RESULTS AND DISCUSSION

PREDICTION OF DRUG LIKENESS:

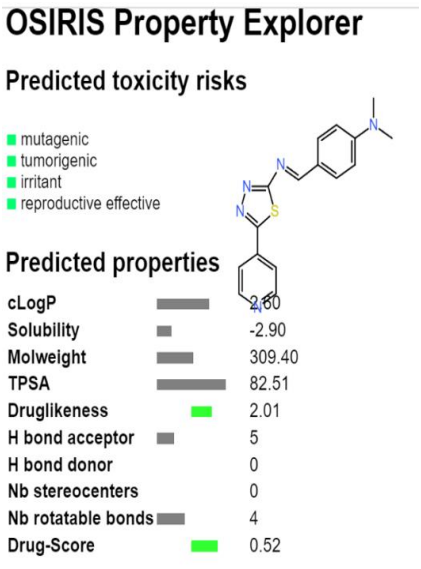
The drug likeness results of molecules are presented in below table for the selected compounds. All the compounds complied with Lipinski's rule and were selected for synthesis.

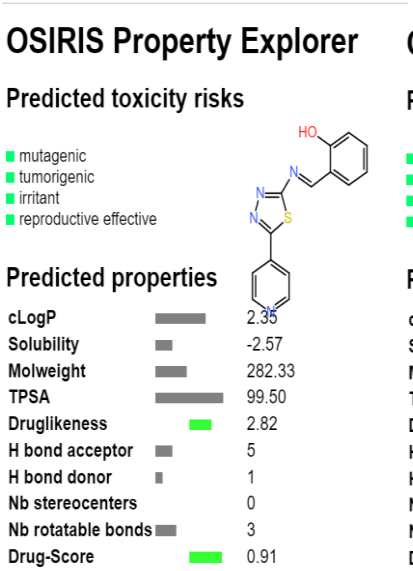
Table 10 prediction of drug likeness

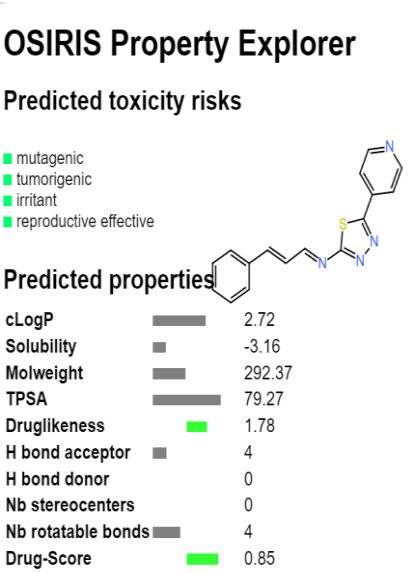
Cpd ID	MW	Log P O/W	H-bond donor	H-bond acceptor	TPSA	Lipinski rule	Log S	Rotatable bonds
LK01	309.39	2.99	0	4	82.51	0	-3.77	4
LK02	282.32	2.03	1	5	99.50	0	-2.43	3
LK03	292.36	2.99	0	4	79.27	0	-3.63	4
LK04	326.37	2.93	0	6	97.73	0	-3.67	5
LK05	296.35	2.20	0	5	92.41	0	-3.14	3

PREDICTION OF TOXICITY:

- The snapshots of the selected compounds toxicity prediction using OSIRIS® Property explorer is presented below.







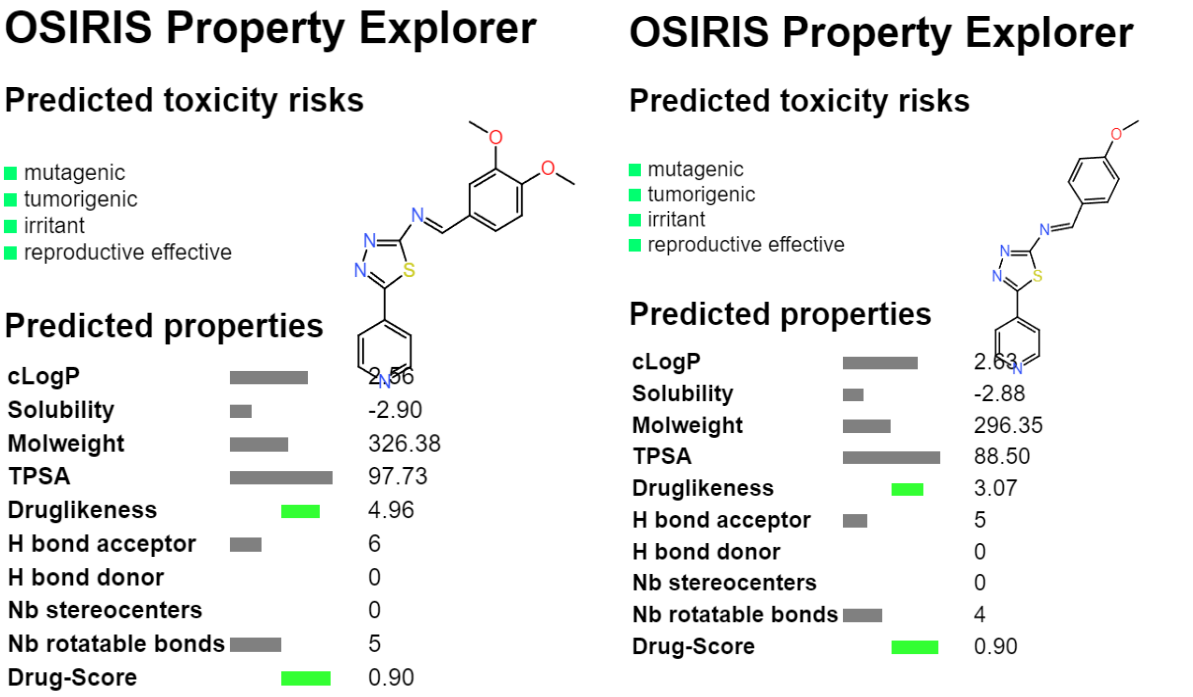


Fig. 5.1 Prediction of toxicity using OSIRIS® tool

Red colour indicated toxicity and green colour indicates there is no toxicity for the compound. Also the druglikeness also presented in the snapshots. It shows that the selected compounds possess no mutagenic, tumorigenic, irritant effect. They have no effect on the reproductive system also.

PREDICTION OF ACTIVITY SPECTRA USING PASS ONLINE TOOL:

- PASS Online tool predicts biological activity, mechanisms of action, adverse effects and toxic effects.
- PASS Online helps find the most probable new leads with required activity spectra (anti-mycobacterial activity) for the designed compounds

Table 11 Anti-tuberculosic activity prediction using pass online

Compound	Pa	Pi
Isoniazid	0.810	0.003
Rifampicin	0,642	0,005
Pyrazinamide	0.549	0.008
Ethambutol	0,192	0,180
Streptomycin	0,995	0,000
LK01	0.606	0.005
LK02	0.761	0.004
LK03	0.715	0.004
LK04	0.700	0.004
LK05	0.742	0.004

Pa – Probability of the compound being active

Pi – Probability of the compound being inactive

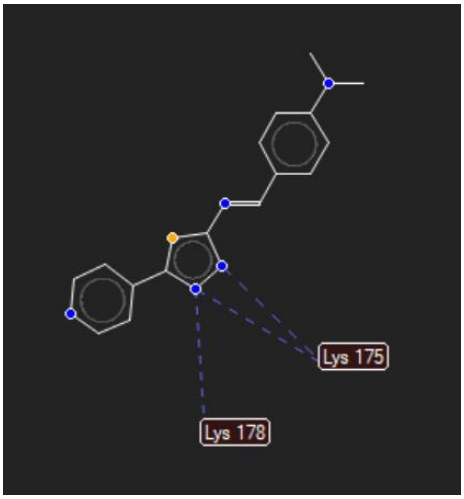
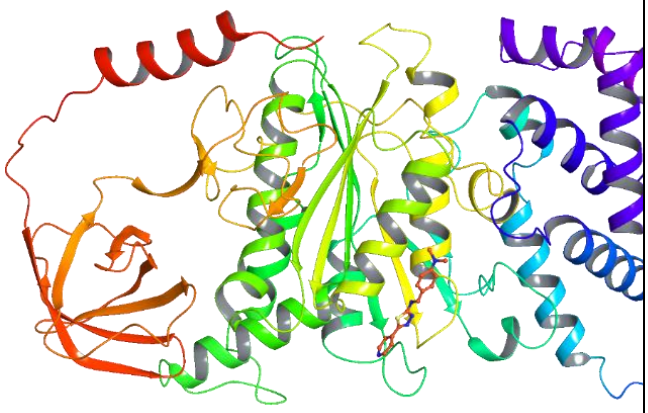
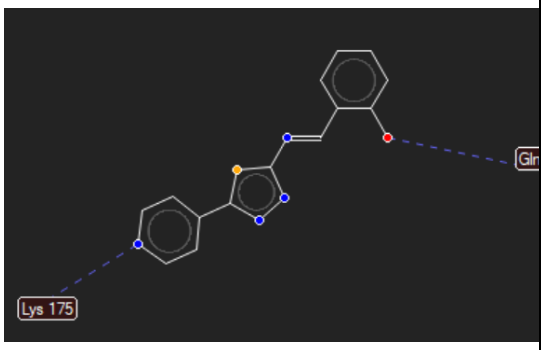
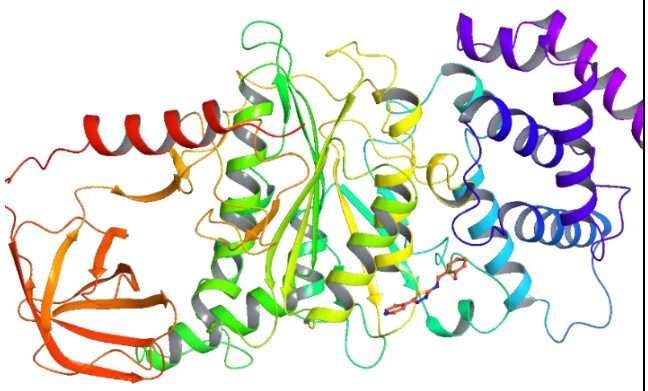
Thus, the activity spectra prediction tells that the compounds have 60% to 76% probability of being active as an anti-tubercular agent. The values of standard drugs Isoniazid and Streptomycin have 80% and 99% probability respectively.

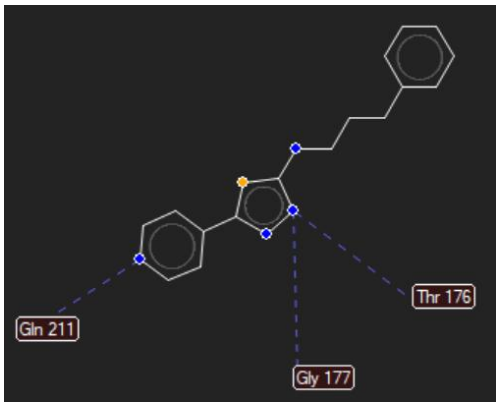
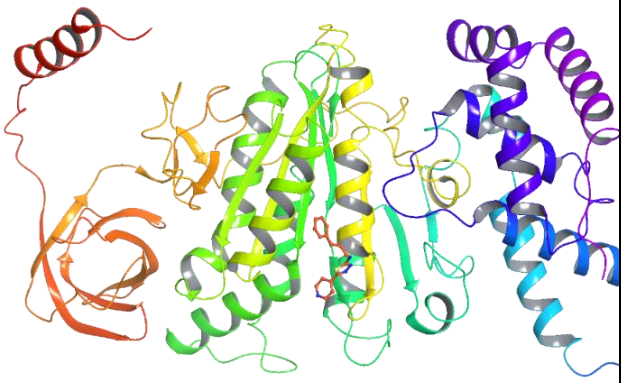
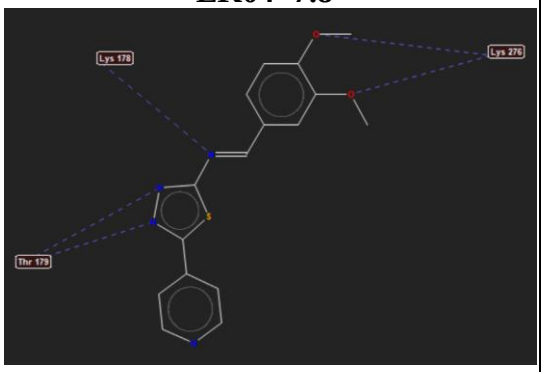
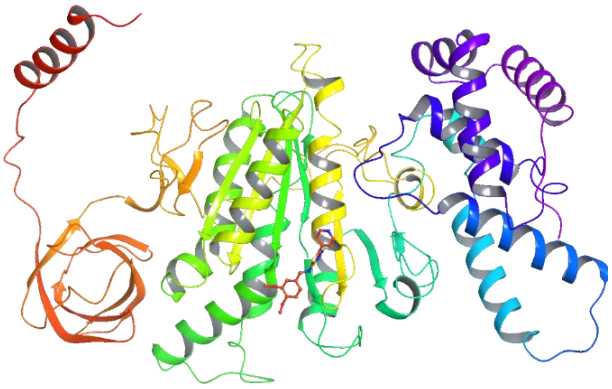
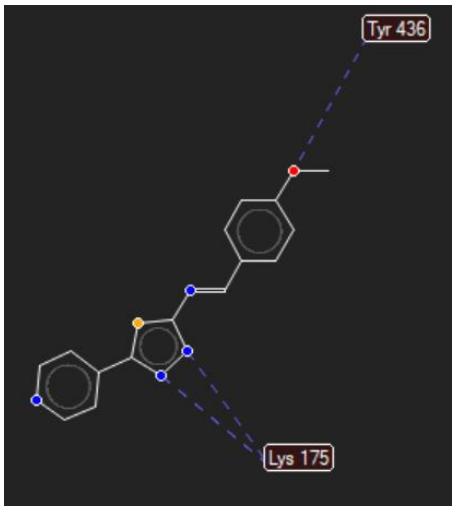
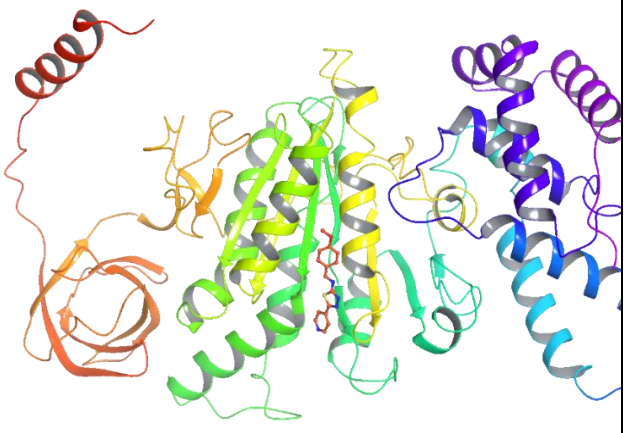
DOCKING STUDIES:

Interactions of the selected compounds with the enzyme ATP synthase is shown in 2D and 3D views. The results show that the selected compounds bind with enzyme in similar sites as shown in 3D images. The docking scores range between -7.8 KJ/mol to -9.4 KJ/mol. From the 2D view, they interact with residues Lys175, Thr176, Gly177, Lys178, Thr179 and Gln211,

Lys276, Gln435, Tyr436. Mostly they interact with Lys175, Thr176, Gly177, Lys178, Thr179 residues.

Table 12 Docking Score and interactions Autodock®

Dock score (KJ/mol) and 2D interactions	3D interactions
LK01 -8.1 	
LK02 -9.3 	

LK03 -9.4 	
LK04 -7.8 	
LK05 -7.9 	

RESULTS OF SYNTHESIS:

For the selected 5 compounds the synthetic scheme was developed. Then they were synthesized using appropriate procedures and purified by recrystallisation. The melting point and TLC was performed to check for purity. The Rf value, compound code, molecular weight, melting point and percentage yield was presented in below table.

Table 13 Compounds yield and Melting point

S.No	Cpd code	Molecular weight	Percentage Yield	R_f value	Melting point (°C)
1	LK01	309.39	60%	0.53	175 °C
2	LK02	282.32	65%	0.61	215 °C
3	LK03	292.36	65%	0.65	238 °C
4	LK04	326.37	61%	0.57	268 °C
5	LK05	296.35	57%	0.62	295 °C

By *in-silico* toxicity studies the designed molecules were found to be nontoxic is then synthesised and recrystallised. The reaction completion was observed by TLC in which Rf value of reactant was compared with Rf value of product. The Rf value of product was found to be different from reactants.

PRODUCT PROFILE

COMPOUND CODE: LK01

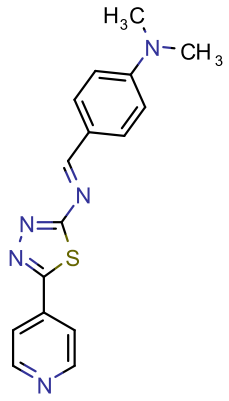


Table 14 Product profile LK01

Molecular Formula:	$C_{16}H_{15}N_5S$
Formula Weight:	309.3888
Composition:	C(62.11%) H(4.89%) N(22.64%) S(10.36%)
Molar Refractivity:	92.24 ± 0.5 cm^3
Molar Volume:	247.0 ± 7.0 cm^3
Parachor:	655.3 ± 8.0 cm^3
Index of Refraction:	1.669 ± 0.05
Surface Tension:	49.5 ± 7.0 dyne/cm
Density:	1.25 ± 0.1 g/cm^3
Polarizability:	36.57 ± 0.5 $10^{-24}cm^3$

IR SPECTRUM OF LK01

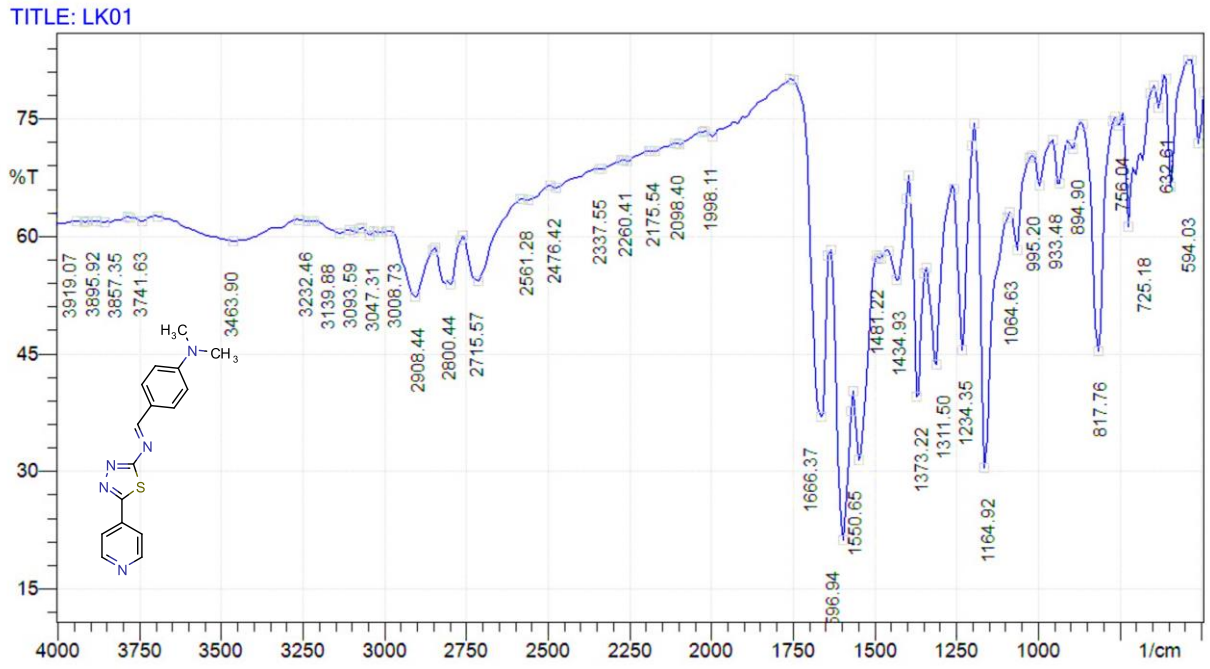


Table 15 INTERPRETATION OF IR SPECTRUM OF LK01

S.No	Wavenumber (cm^{-1})	Functional group
1	1666	-C=N- imine
2	3047	SP_2 -C-H Stretching

NMR SPECTRUM OF LK01

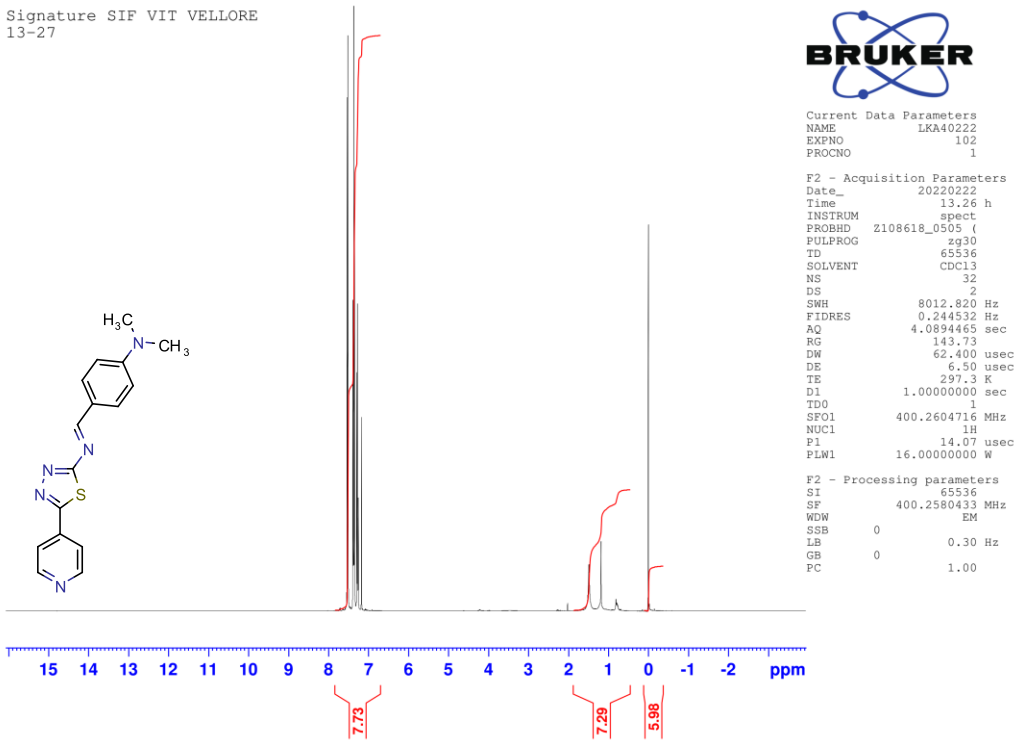
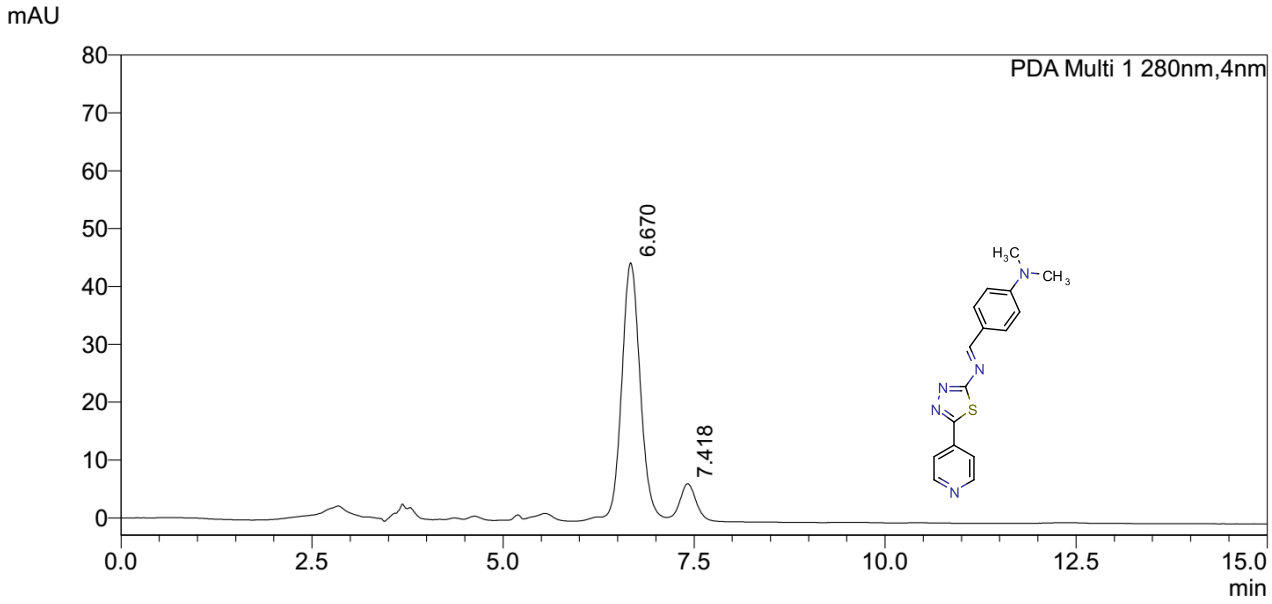


Table 16 Interpretation of NMR spectrum of LK01

S.No	δ value	Nature of the protons	Nature of the peaks	No. of protons
1	6.8-7.8	Aromatic C-H	multiplet	8
2	1.2	Imine C-H	singlet	1
3	1.5	Methyl C-H	singlet	6

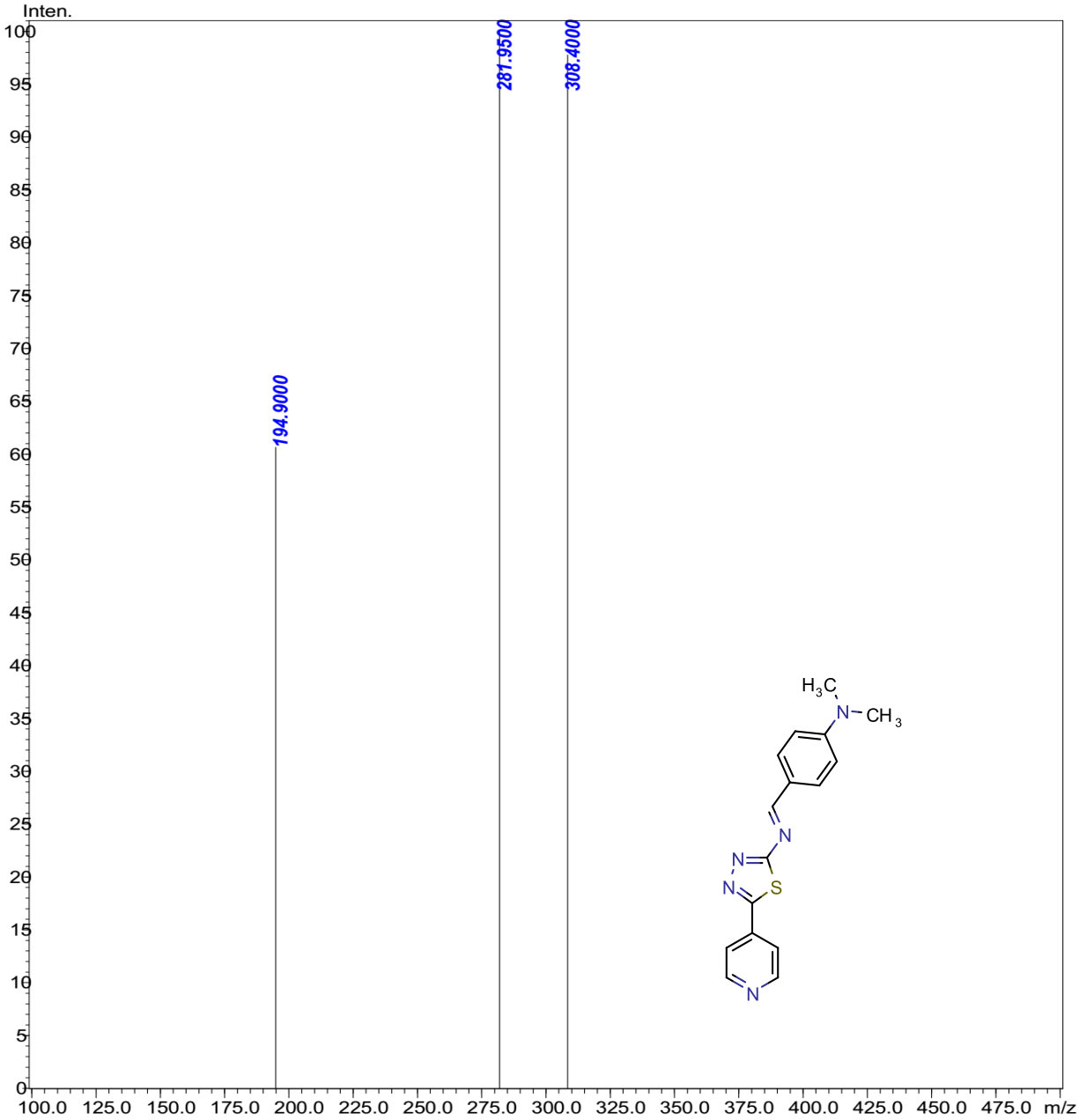
LC-MS OF LK01



Peak Table

PDA Ch1 280nm

Peak#	Ret. Time	Area	Height	Area%
1	6.670	717709	43959	93.135
2	7.418	52899	4818	6.865
Total		770608	48777	100.000


Inten.
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
281.9500
308.4000
194.9000
100.0 125.0 150.0 175.0 200.0 225.0 250.0 275.0 300.0 325.0 350.0 375.0 400.0 425.0 450.0 475.0 m/z
H3C
N
CH3
N
N
N
S
N

COMPOUND CODE: LK02

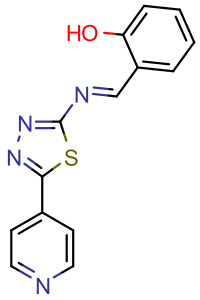


Table 17 Product profile LK02

Molecular Formula:	$C_{14}H_{10}N_4OS$
Formula Weight:	282.3204
Composition:	C(59.56%) H(3.57%) N(19.85%) O(5.67%) S(11.36%)
Molar Refractivity:	80.29 ± 0.5 cm^3
Molar Volume:	203.1 ± 7.0 cm^3
Parachor:	564.7 ± 8.0 cm^3
Index of Refraction:	1.720 ± 0.05
Surface Tension:	59.7 ± 7.0 dyne/cm
Density:	1.38 ± 0.1 g/cm^3
Polarizability:	31.83 ± 0.5 $10^{-24}cm^3$

IR SPECTRUM OF LK02

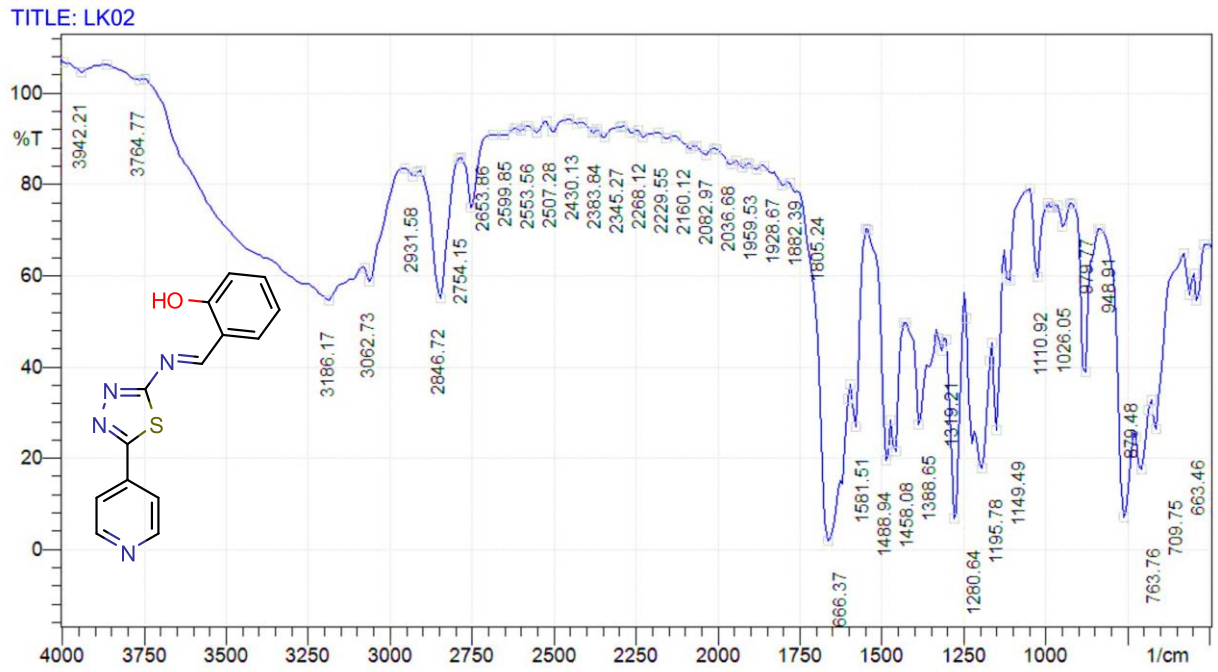


Table 18 INTERPRETATION OF IR SPECTRUM OF LK02

S.No	Wavenumber (cm^{-1})	Functional group
1	1666	-C=N- imine
2	3764	-OH
3	3062	SP_2-C-H Stretching

NMR SPECTRUM OF LK02

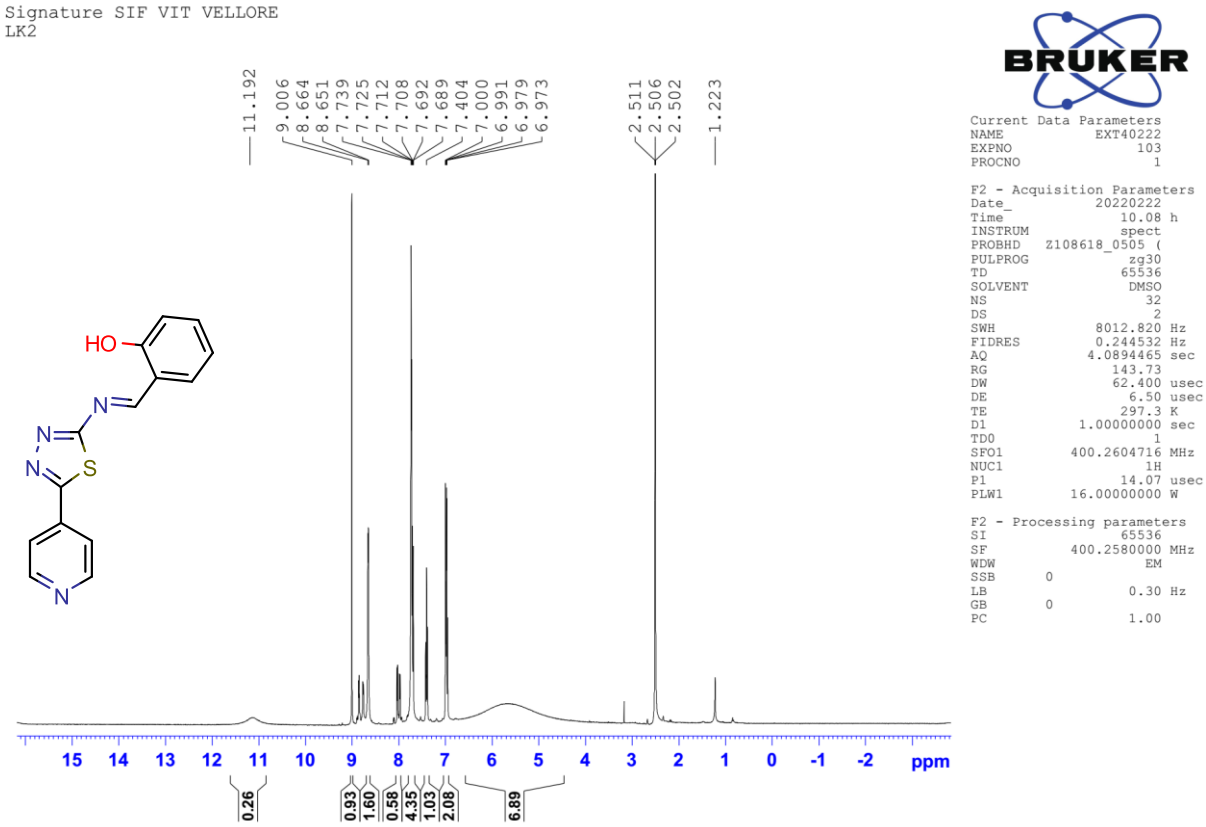
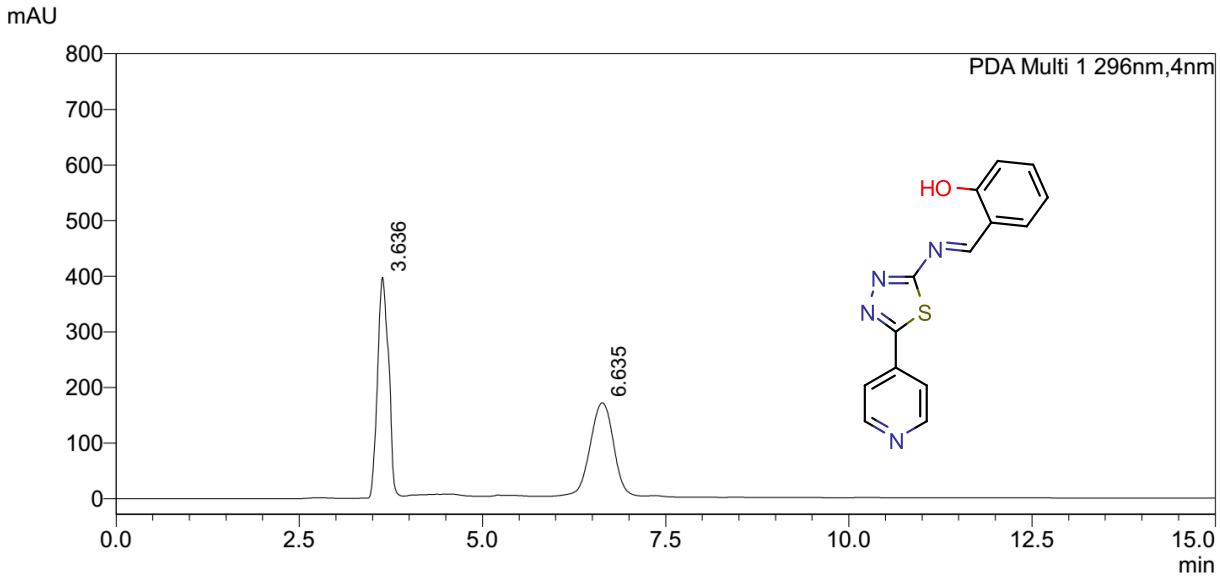


Table 19 Interpretation of NMR spectrum of LK02

S.No	δ value	Nature of the protons	Nature of the peaks	No. of protons
1	6.97-8.66	Aromatic C-H	multiplet	8
2	2.50	Imine C-H	singlet	1
3	1.22	Hydroxyl O-H	singlet	1

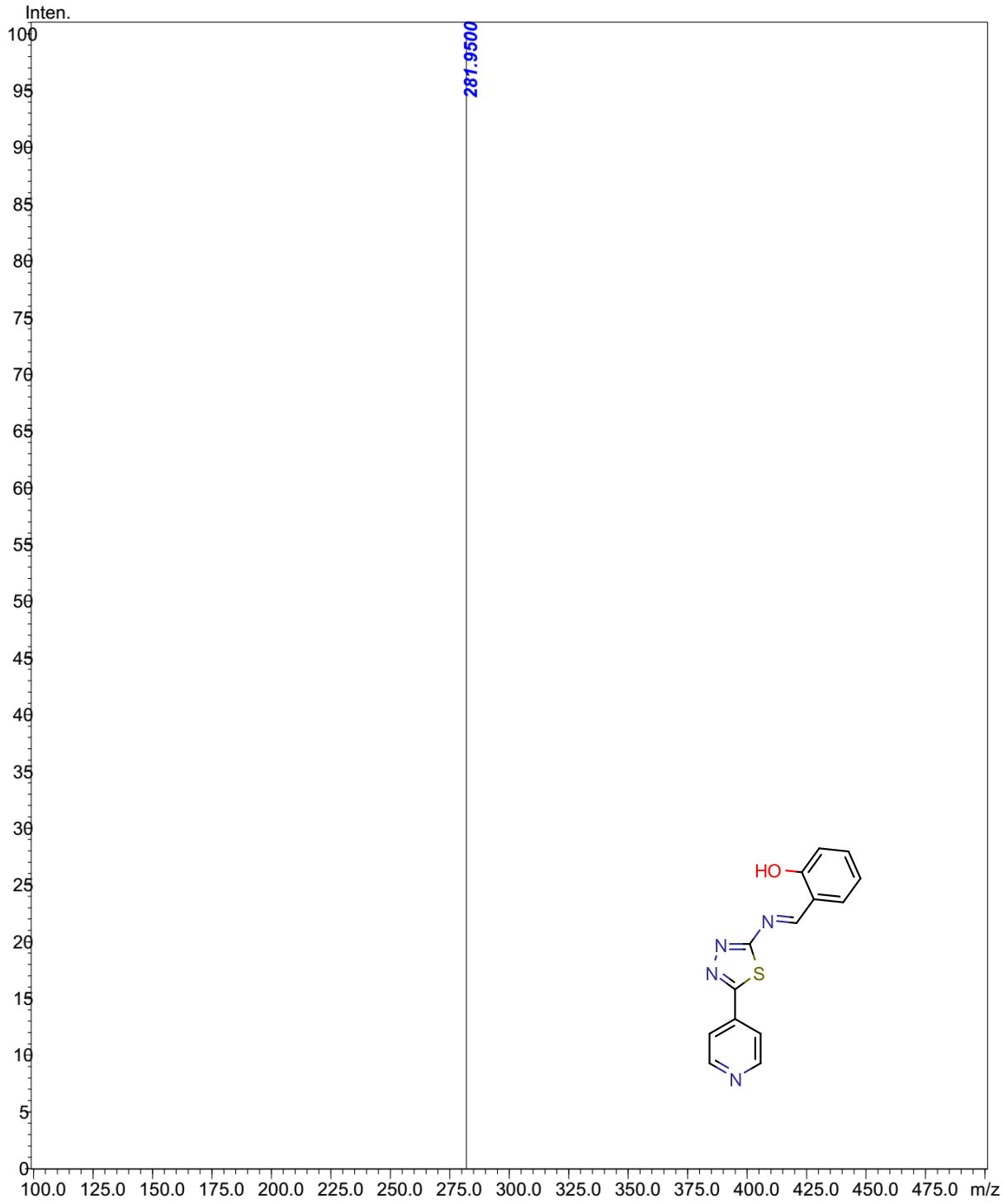
LC-MS OF LK02



Peak Table

PDA Ch1 296nm

Peak#	Ret. Time	Area	Height	Area%
1	3.636	2679716	314186	41.606
2	6.635	3761042	168181	58.394
Total		6440758	482367	100.000


Inten.
281.9500
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
100.0 125.0 150.0 175.0 200.0 225.0 250.0 275.0 300.0 325.0 350.0 375.0 400.0 425.0 450.0 475.0 m/z
HO
N
N
N
S
N

COMPOUND CODE: LK03

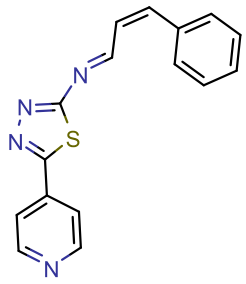


Table 20 Product profile LK03

Molecular Formula:	$C_{16}H_{12}N_4S$
Formula Weight:	292.35828
Composition:	C(65.73%) H(4.14%) N(19.16%) S(10.97%)
Molar Refractivity:	88.66 ± 0.5 cm^3
Molar Volume:	238.0 ± 7.0 cm^3
Parachor:	636.2 ± 8.0 cm^3
Index of Refraction:	1.667 ± 0.05
Surface Tension:	51.0 ± 7.0 dyne/cm
Density:	1.22 ± 0.1 g/cm^3
Polarizability:	35.14 ± 0.5 $10^{-24}cm^3$

IR SPECTRUM OF LK03

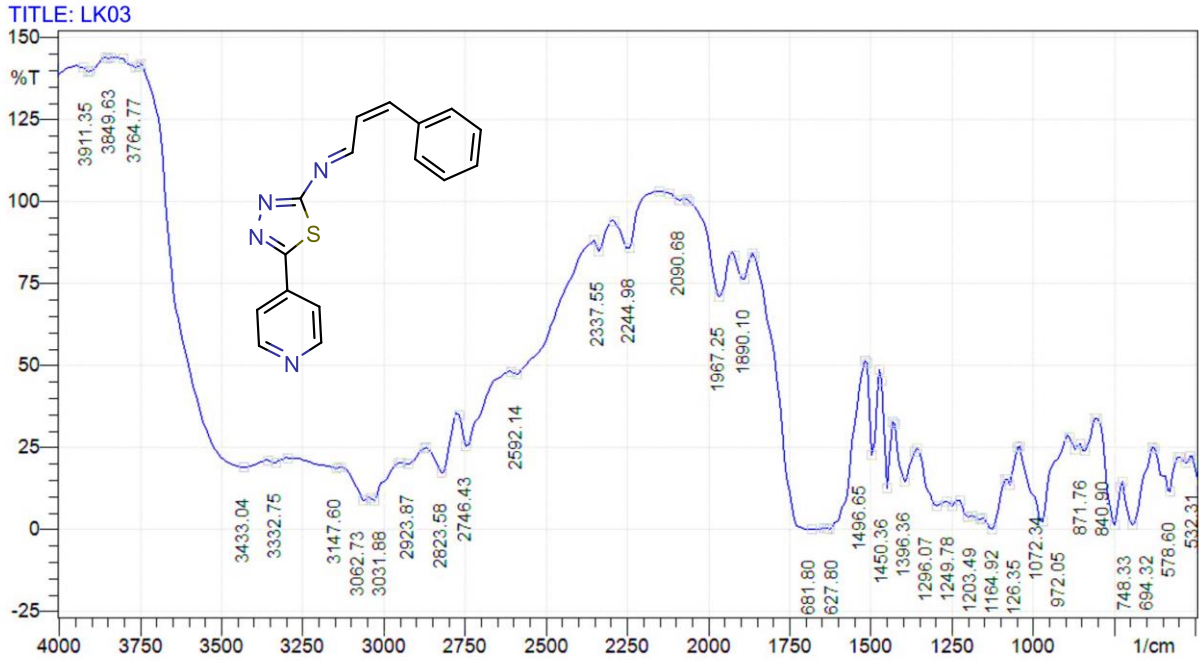


Table 21 INTERPRETATION OF IR SPECTRUM OF LK03

S.No	Wavenumber (cm^{-1})	Functional group
1	1627	-C=N- imine
2	1681	-C=C-
3	3031	SP_2 -C-H Stretching

NMR SPECTRUM OF LK03

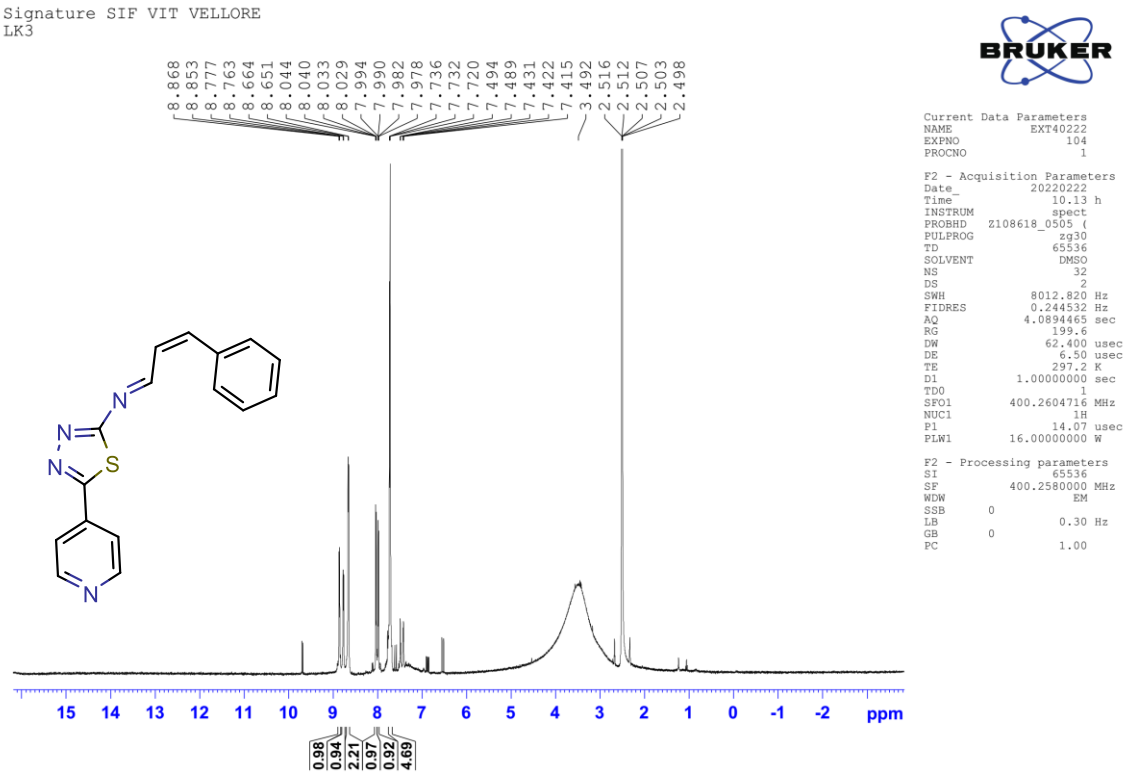
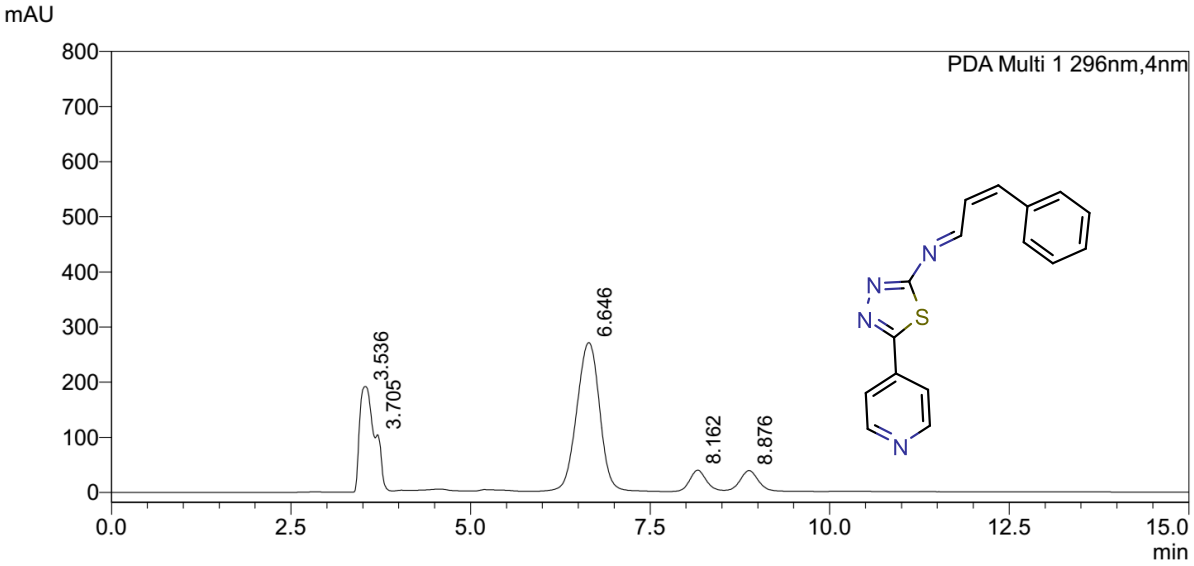


Table 22 Interpretation of NMR spectrum LK03

S.No	δ value	Nature of the protons	Nature of the peaks	No. of protons
1	7.72-8.86	Aromatic C-H	multiplet	9
2	2.57	Imine C-H	doublet	1
3	7.41-7.43	Conjugated alkene C-H	triplet	1
4	7.48-7.49	Conjugated alkene C-H	doublet	1

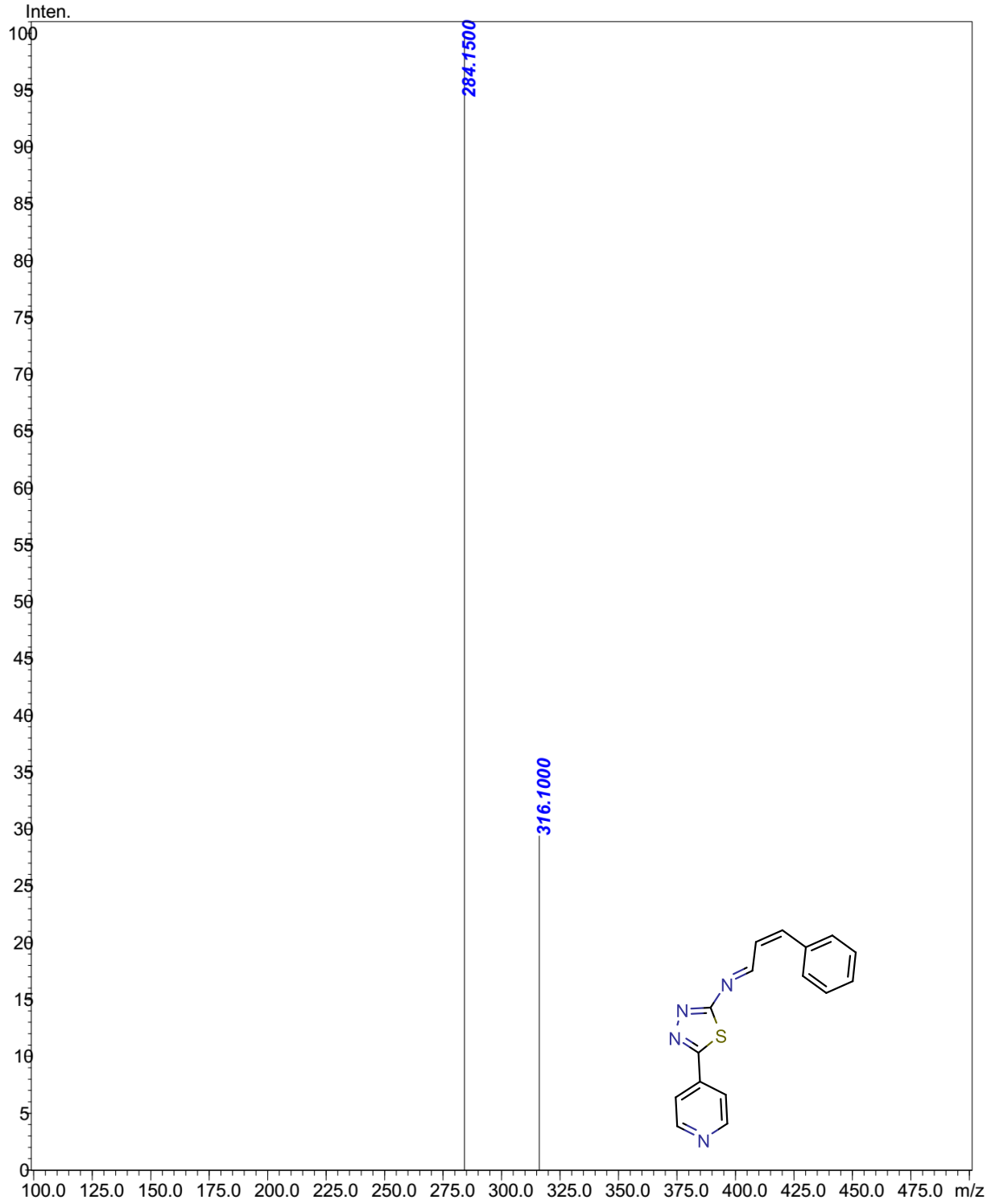
LC-MS OF LK03



Peak Table

PDA Ch1 296nm

Peak#	Ret. Time	Area	Height	Area%
1	3.536	1457090	140260	18.063
2	3.705	82993	21489	1.029
3	6.646	5955234	269609	73.823
4	8.162	259341	24510	3.215
5	8.876	312229	25773	3.870
Total		8066887	481642	100.000


Inten.
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
284.1500
316.1000
N
N
N
S
N
100.0 125.0 150.0 175.0 200.0 225.0 250.0 275.0 300.0 325.0 350.0 375.0 400.0 425.0 450.0 475.0 m/z

COMPOUND CODE: LK04

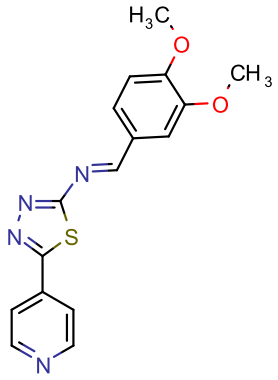


Table 23 Product profile LK04

Molecular Formula:	$C_{16}H_{14}N_4O_2S$
Formula Weight:	326.37296
Composition:	C(58.88%) H(4.32%) N(17.17%) O(9.80%) S(9.82%)
Molar Refractivity:	91.07 ± 0.5 cm^3
Molar Volume:	249.2 ± 7.0 cm^3
Parachor:	659.5 ± 8.0 cm^3
Index of Refraction:	1.651 ± 0.05
Surface Tension:	49.0 ± 7.0 dyne/cm
Density:	1.30 ± 0.1 g/cm^3
Polarizability:	36.10 ± 0.5 $10^{-24}cm^3$

IR SPECTRUM OF LK04

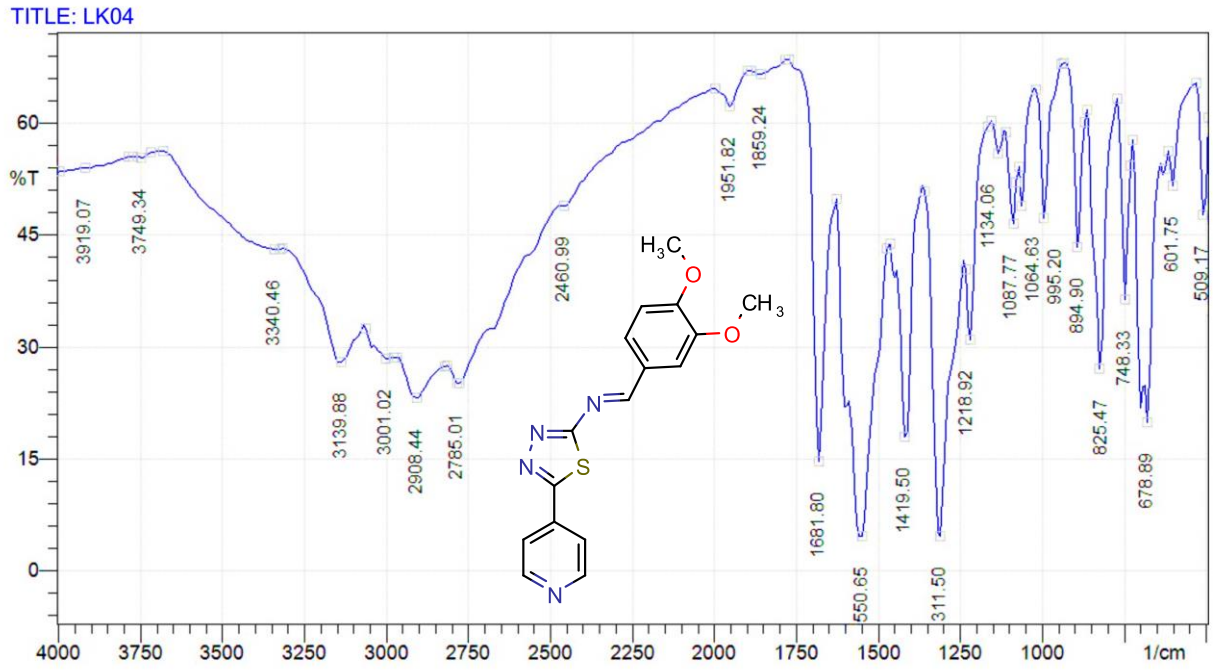


Table 24 INTERPRETATION OF IR SPECTRUM OF LK04

S.No	Wavenumber (cm^{-1})	Functional group
1	1681	-C=N- imine
2	1218	-C-O-C Phenyl alkyl ether
3	1064	-C-O-C Phenyl alkyl ether
3	3001	SP_2 -C-H Stretching

NMR SPECTRUM OF LK04

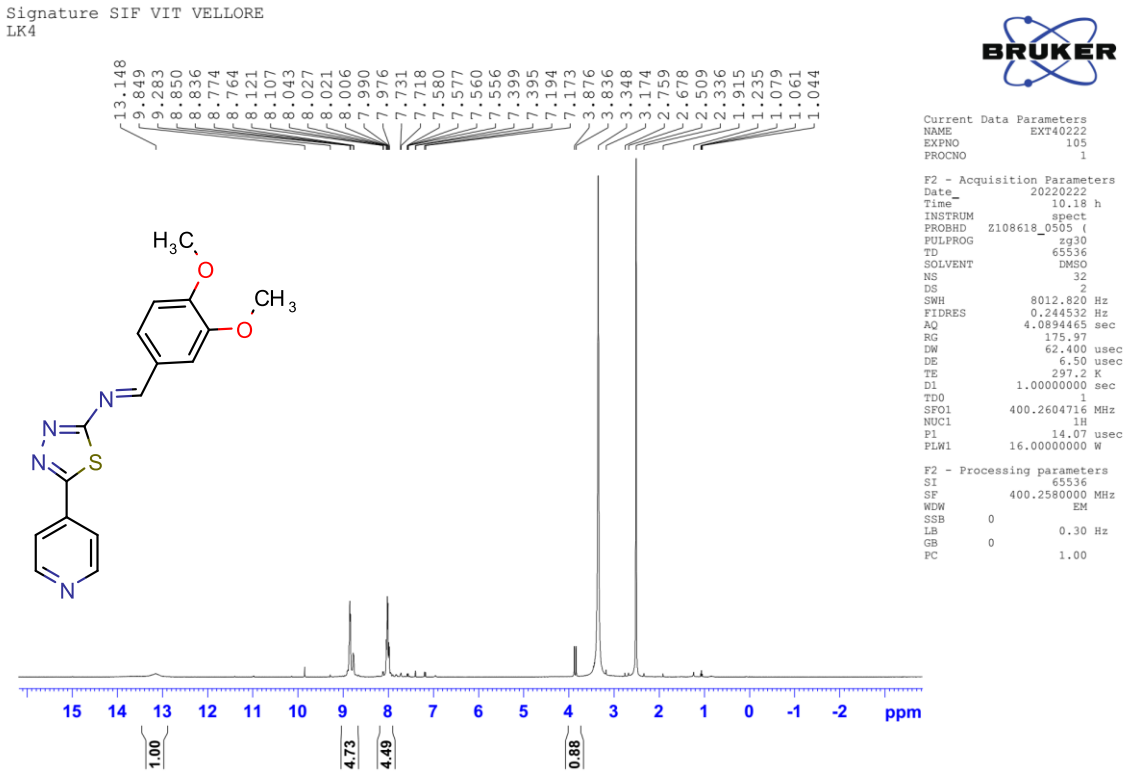
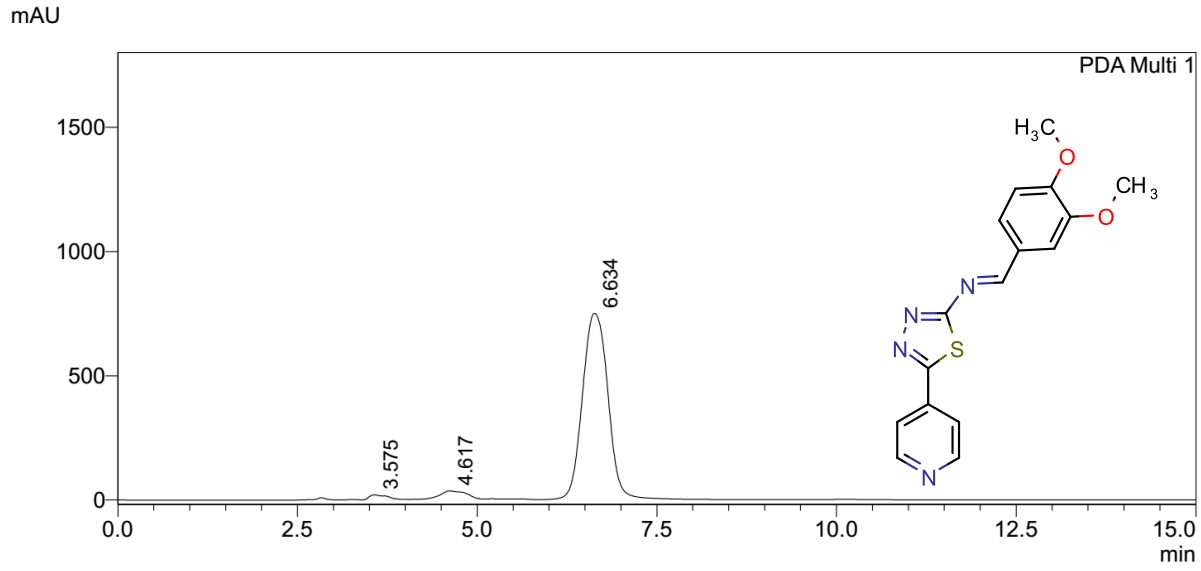


Table 25 Interpretation of NMR spectrum LK04

S.No	δ value	Nature of the protons	Nature of the peaks	No. of protons
1	7.17-8.85	Aromatic C-H	multiplet	7
2	2.5	Imine C-H	singlet	1
3	3.34	Ether O-CH_3	singlet	6

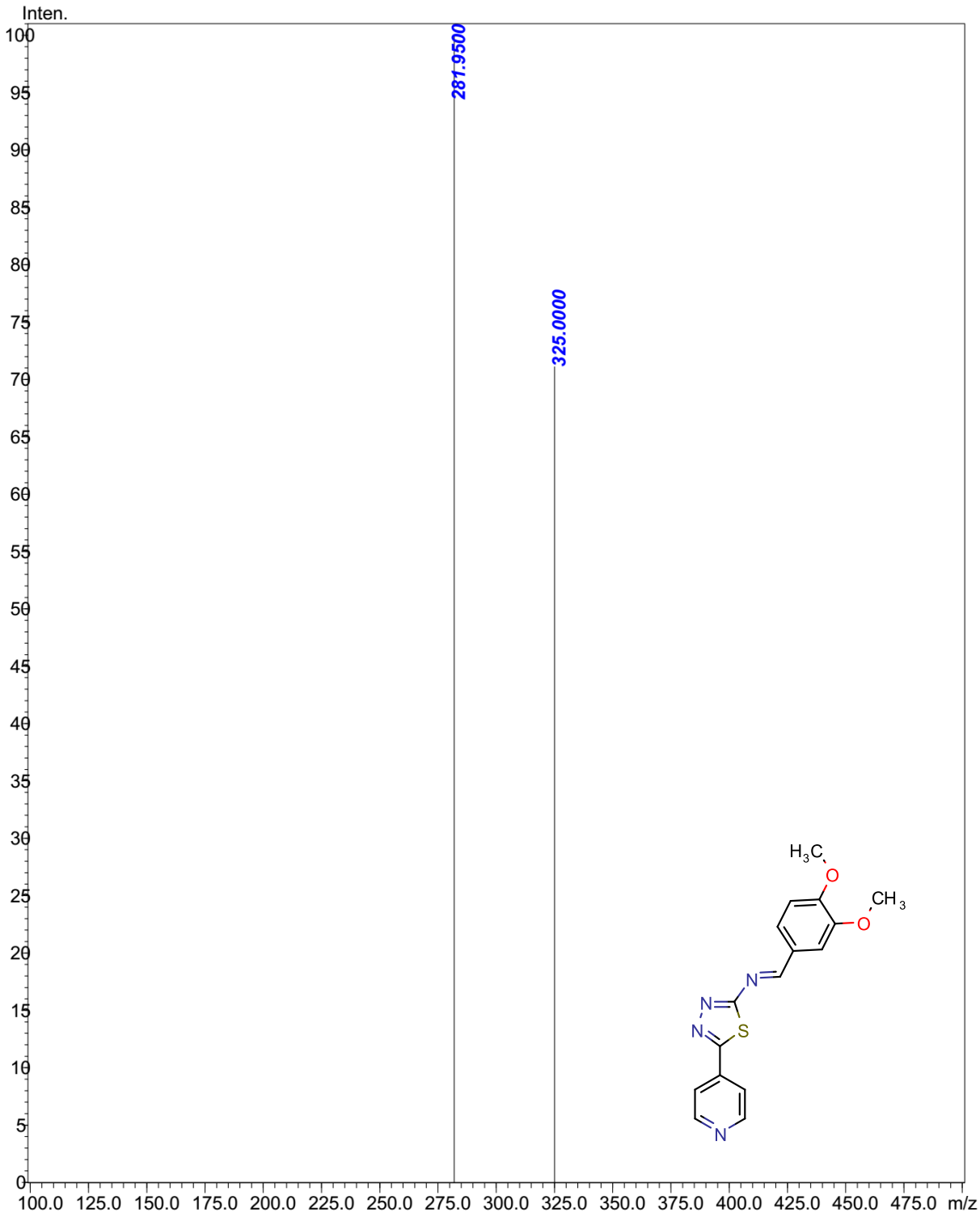
LC-MS OF LK04



Peak Table

PDA Ch1 296nm

Peak#	Ret. Time	Area	Height	Area%
1	3.575	51792	6458	0.280
2	4.617	197578	15009	1.068
3	6.634	18253940	747735	98.652
Total		18503310	769202	100.000


Inten.
100
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90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
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281.9500
325.0000
100.0 125.0 150.0 175.0 200.0 225.0 250.0 275.0 300.0 325.0 350.0 375.0 400.0 425.0 450.0 475.0 m/z
H3C
O
CH3
O
N
N
N
S
N

COMPOUND CODE: LK05

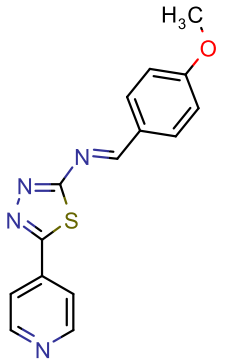


Table 26 Product profile LK05

Molecular Formula:	$C_{15}H_{12}N_4OS$
Formula Weight:	296.34698
Composition:	C(60.79%) H(4.08%) N(18.91%) O(5.40%) S(10.82%)
Molar Refractivity:	85.25 ± 0.5 cm^3
Molar Volume:	227.5 ± 7.0 cm^3
Parachor:	609.3 ± 8.0 cm^3
Index of Refraction:	1.672 ± 0.05
Surface Tension:	51.4 ± 7.0 dyne/cm
Density:	1.30 ± 0.1 g/cm^3
Polarizability:	33.79 ± 0.5 $10^{-24}cm^3$

IR SPECTRUM OF LK05

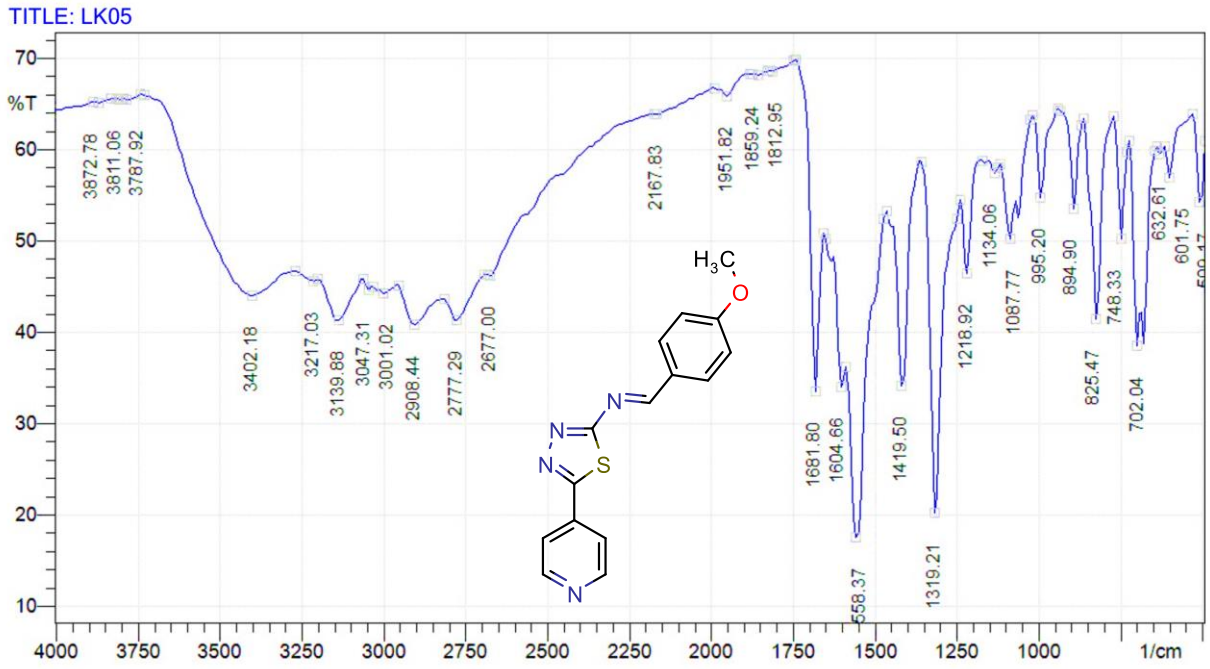


Table 27 INTERPRETATION OF IR SPECTRUM OF LK05

S.No	Wavenumber (cm^{-1})	Functional group
1	1681	-C=N- imine
2	1218	-C-O-C- Phenyl alkyl ether
3	1087	-C-O-C- Phenyl alkyl ether
4	3001	SP_2 -C-H Stretching

NMR SPECTRUM OF LK05

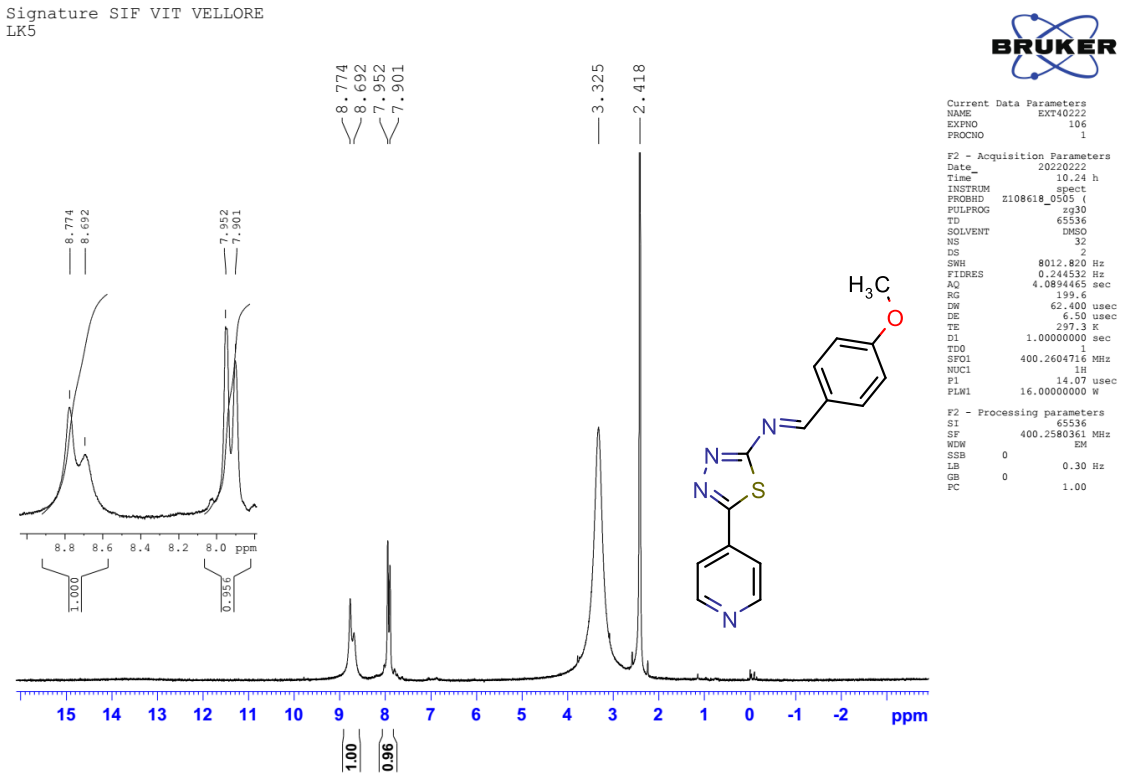
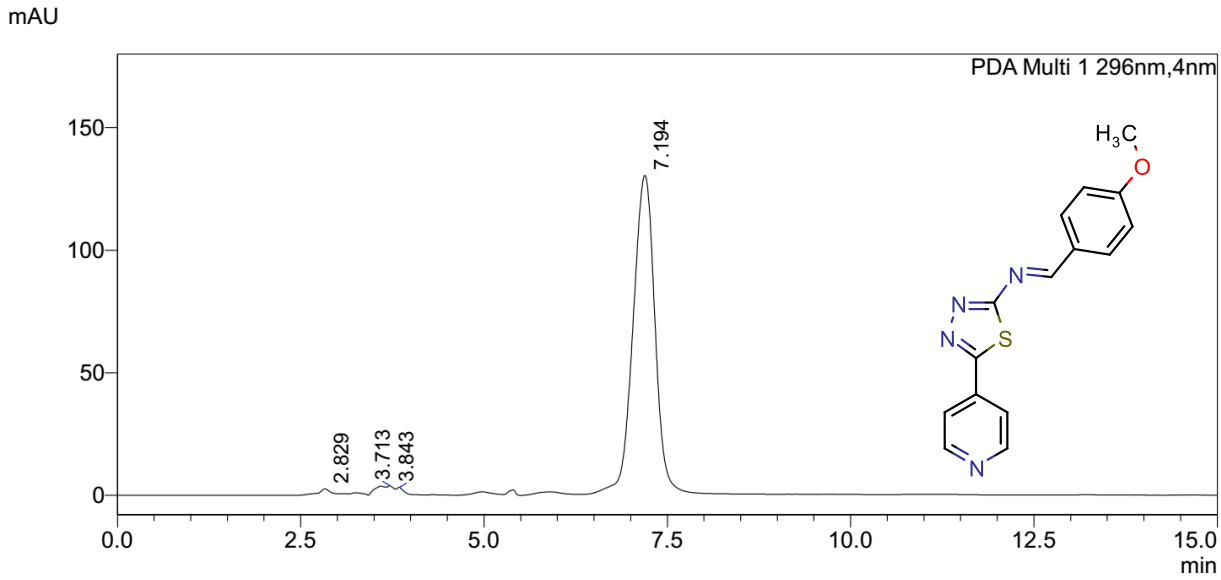


Table 28 Interpretation of NMR spectrum of LK05

S.No	δ value	Nature of the protons	Nature of the peaks	No. of protons
1	7.90-8.77	Aromatic C-H	multiplet	8
2	2.41	Imine C-H	singlet	1
3	3.32	Ether O-CH_3	singlet	3

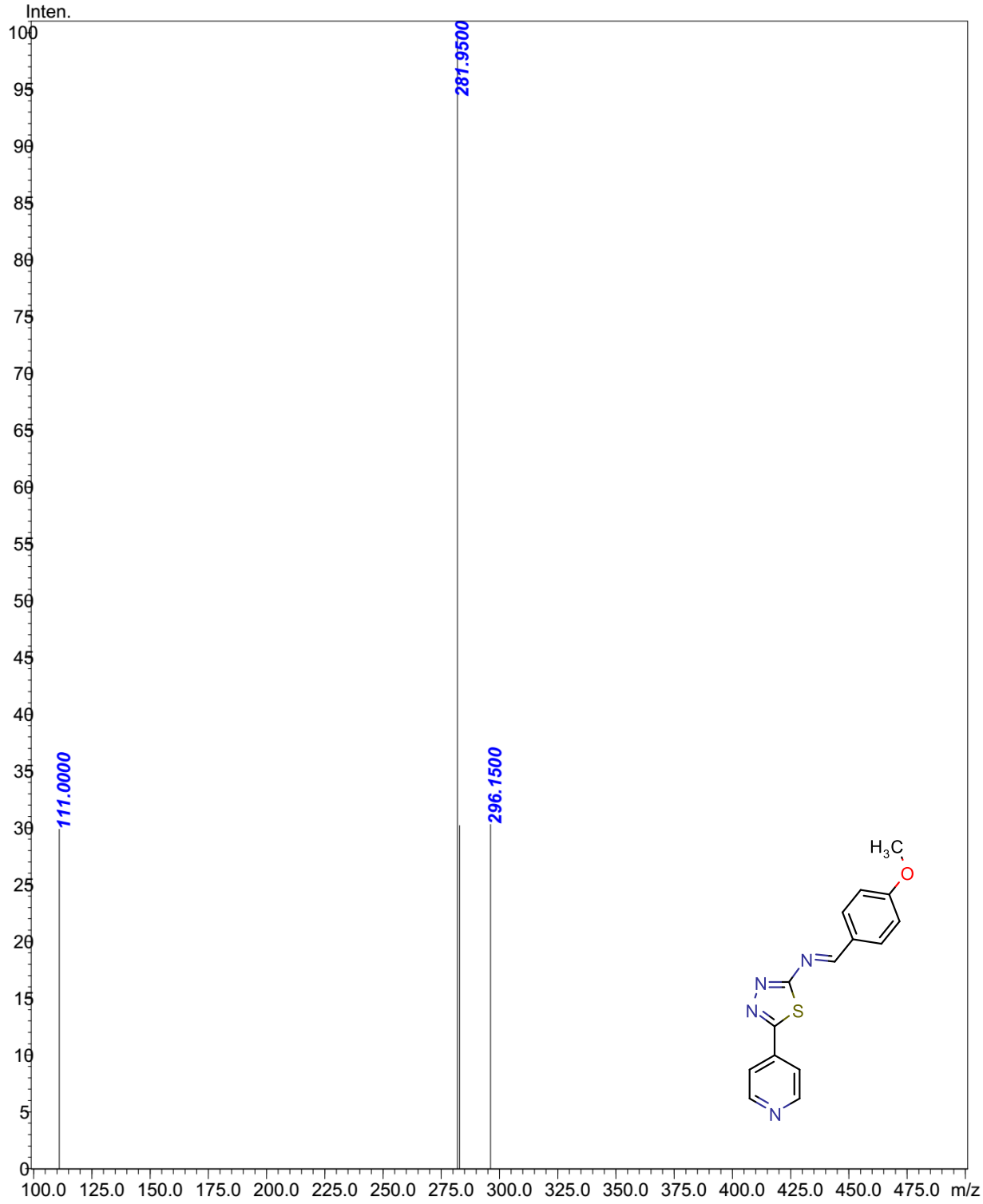
LC-MS OF LK05



Peak Table

PDA Ch1 296nm

Peak#	Ret. Time	Area	Height	Area%
1	2.829	11481	1775	0.418
2	3.713	2128	781	0.078
3	3.843	5166	1324	0.188
4	7.194	2725339	130134	99.316
Total		2744114	134014	100.000


Inten.
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
281.9500
296.1500
111.0000
100.0 125.0 150.0 175.0 200.0 225.0 250.0 275.0 300.0 325.0 350.0 375.0 400.0 425.0 450.0 475.0 m/z
H3C
O
N
N
N
S
N

BIOLOGICAL EVALUATION:

The anti-tubercular activity of the compounds LK00-LK05 was determined using MABA. The organism used in the study is Mycobacterium tuberculosis H37Rv. All the synthesized compounds show varying activity between 12.5µg/ml and 1.6µg/ml. Among the 6 compounds, compound LK05 (anisaldehyde derivative) shows activity at concentration of 1.6µg/ml. The intermediate (pyridyl-thiadiazolyl-amine) shows least activity at 25µg/ml.

Table 29 MIC of 6 compounds LK00-LK05 (µg/ml)

Sl. No.	Sample	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.12 µg/ml	1.6 µg/ml	0.8 µg/ml
01	LK-0	S	S	S	R	R	R	R	R
02	LK-1	S	S	S	S	R	R	R	R
03	LK-2	S	S	S	S	R	R	R	R
04	LK-3	S	S	S	S	R	R	R	R
05	LK-4	S	S	S	S	S	R	R	R
06	LK-5	S	S	S	S	S	S	S	R

Note:

S - Sensitive

R - Resistant

Standard Strain used: Mycobacteria tuberculosis (Vaccine strain, H37 RV strain):

ATCC No- 27294.

Standard values for the Anti-Tb molecules in use is as follows

Isoniazid – 1.6 µg/ml

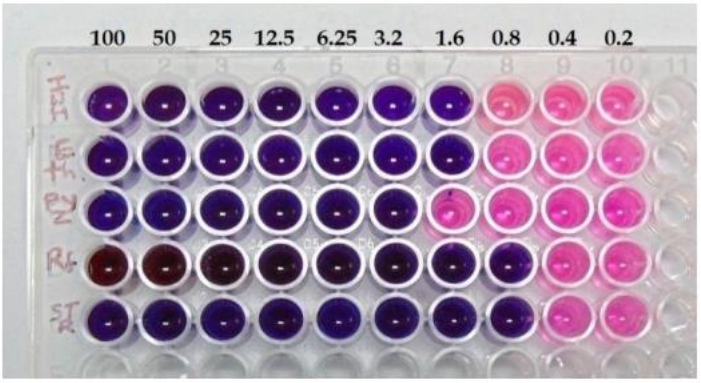
Ethambutol – 1.6 µg/ml

Pyrazinamide- 3.125µg/ml

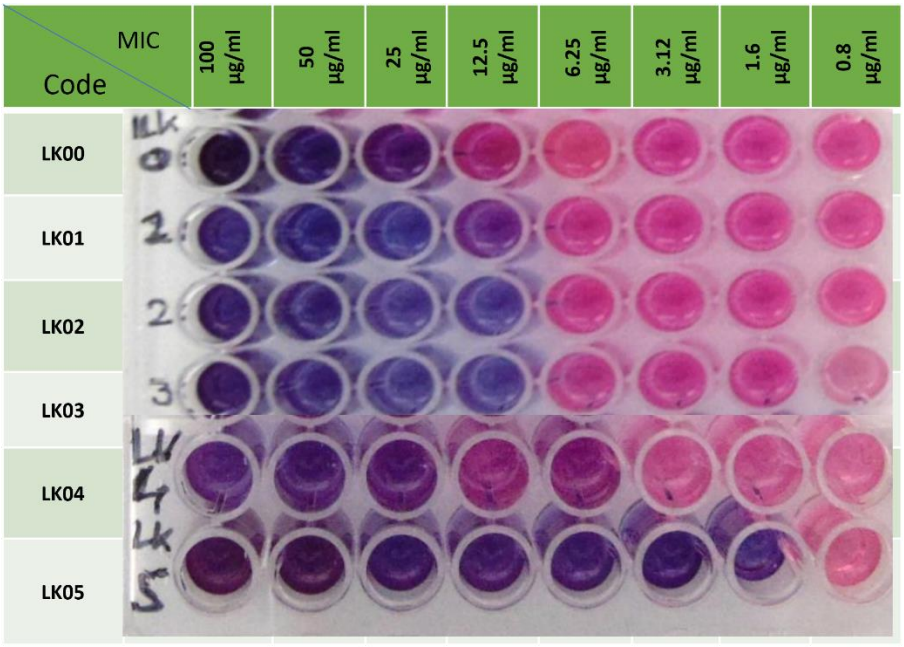
Rifampicin – 0.8 µg/ml

Streptomycin- 0.8µg/ml

Standard Drug Photograph



Compounds LK00 – LK05 Photograph



Compound LK04 possess MIC of 6.25µg/ml and LK05 possess 1.6µg/ml. The standard drugs Streptomycin and Rifampicin show activity at 0.8µg/ml. INH and Ethambutol at 1.6µg/ml, Pyrazinamide at 3.125µg/ml concentrations in same assay procedure. MIC values indicates that LK05 is more potent than Pyrazinamide and equipotent to INH and Ethambutol.

ACUTE ORAL TOXICITY STUDY:

Table 30 Acute oral toxicity parameters

S.No	PARAMETERS	RESULTS
1	Toxic signs	Absent
2	Pre-terminal deaths	Nil
3	Body weight	No specific change
4	Motor activity	Normal
5	Tremors	Absent
6	Convulsion	Absent
7	Straub reaction	Absent
8	Righting reflex	Present
9	Lacrimation and salivation	Normal
10	Unusual vocalization	Absent
11	Sedation	Absent
12	Body temperature	Normal
13	Analgesia	Absent
14	Ptosis	Absent
15	Diarrhoea	Absent
16	Skin colour	Normal
17	Respiration	Normal
18	Scratching	Absent
19	Aggressiveness and restlessness	Absent

Animals were observed for behavioural signs of toxicity like motor activity, tremor etc., and no significant toxic signs were observed during 14 days. The results of the acute toxicological studies showed that the administration of 5 molecules by oral route up to 2000mg/kg/body weight did not produce any mortality and was tolerated.

SUMMARY

SUMMARY

On the basis of literature survey, ATP synthase was selected as the antitubercular target for the study. The mechanism of action and reason for selecting ATP synthase was discussed in enzyme profile.

A database of 500 molecules were designed based on literature review.

ADME and *In-silico* druglikeness properties of the designed molecules were determined by using the MOLINSPIRATION® tool.

The bioactivity prediction was determined using PASS online tool.

The molecules were subjected to toxicity assessment by OSIRIS® property explorer tool.

Then molecular docking was performed for the 500 molecules against the target protein ATP synthase using AutoDock 4.2.6®.

Five molecules with good docking score [lower binding energy] and interactions were taken and optimized for the synthesis.

The scheme for the synthesis was developed and the compounds were synthesized with satisfactory yield.

Purity of the synthesized compounds was improved by doing recrystallization repeatedly and the purity was evaluated by TLC and melting point for the individual compounds.

The characterization of the synthesized compounds was done using Infra-red spectroscopy, Nuclear Magnetic Resonance [^{1}H NMR] spectroscopy methods and Liquid Chromatography-Mass spectrometric methods [LC-MS].

The compounds were screened for *in-vitro* anti-mycobacterial activity by Microplate Alamar Blue Assay [MABA].

The synthesized compounds showed sensitivity [Minimum Inhibitory Concentration] between 12.5µg/ml to 1.6µg/ml. Compound LK04 possess MIC of 6.25µg/ml and LK05 possess 1.6µg/ml.

Standard drugs Streptomycin and Rifampicin show activity at 0.8µg/ml. INH and Ethambutol at 1.6µg/ml, Pyrazinamide at 3.125µg/ml concentrations in same assay procedure.

MIC values indicates that LK05 is more potent than Pyrazinamide and equipotent to INH and Ethambutol.

All compounds were found to be safe as per the acute toxicity study.

CONCLUSION

CONCLUSION:

The work concludes that the novel pyridine-thiadiazole derivatives inhibit the enzyme ATP synthase which is important for energy metabolism of Mycobacterium tuberculosis.

All the 5 compounds gave docking score between -7 to -9 Kcal/mol which shows good binding affinity to ATP synthase

The minimum inhibitory concentration of the 5 synthesized compounds against $H37R_V$ ranged from 12.5 to 1.6 μg/ml. The intermediate compound shows 25 μg/ml.

The acute toxicity studies revealed that all the compounds found to be safe and non-toxic.

Further, structural modification of LK05 is expected to yield promising molecules against the pathogen Mycobacterium tuberculosis.

The assay using pathogenic strain and chronic toxicity are the future prospects of the work.

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BIBLIOGRAPHY

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ANNEXURE

MADRAS MEDICAL COLLEGE, CHENNAI – 600003

INSTITUTIONAL ANIMAL ETHICS COMMITTEE

PROCEEDINGS

PRESENT: Dr. A. JERAD SURESH, M.Pharm., Ph.D., MBA

Roc. No: 5/AEL/IAEC/MMC/2022 Dated: 01-11-2021

Sub: IAEC, MMC, Ch-3 – Approval of Laboratory Animals – Regarding

Ref: IAEC Meeting held on 21-10-2021

This order is issued based on the approval by the Institutional Animal Ethics Committee Meeting held on 21-10-2021, Thursday.

Project Proposal ID Number	16/2021-2022
CPCSEA Registration Number	1917/GO/ReBi/2016/CPCSEA Valid till 19-9-2026
Name of the Researcher with ID Number	L. KARTHIKEYAN 261915706
Name of the Guide	Dr. A. Jerad Suresh, M.Pharm., Ph.D., MBA
Project Title	Design, Synthesis, Characterization and Biological Evaluation of Novel Pyridine-Thiadiazole derivatives as Anti tubercular Agents Targeting ATP Synthase.
Date of submission of proposal to IAEC	07-10-2021
Date of IAEC meeting	21-10-2021
Date of submission of modified proposal to IAEC	22-10-2021
Date of Approval	21-10-2021
Validity of the Approved Proposal	One Year
Number & Species of Laboratory Animals Approved	30 Wistar Rats Approved

Chairperson 5/11/21
Institutional Animal Ethics Committee
Madras Medical College
Chennai-600003

PRINCIPAL
COLLEGE OF PHARMACY
MADRAS MEDICAL COLLEGE
CHENNAI-600 003

To
Dr. A. Jerad Suresh, M.Pharm., Ph.D, MBA.
Principal, Prof. & Head, Dept. of Pharmaceutical Chemistry,
College of Pharmacy,
MMC, Ch-3.

Copy to:
Special Veterinary Officer, Animal Experimental Laboratory,
Madras Medical College, Ch-3. .

CSIR-SUMMER RESEARCH TRAINING PROGRAM (CSIR-SRTP)2020 ONLINE

CSIR-SUMMER RESEARCH TRAINING PROGRAM (CSIR-SRTP) 2020 ONLINE HAS THE FOLLOWING ACTIVITIES

Eminent Scientist Lectures, Special Sessions, Project specific classes, Elocution video, Poster designing, Essay writing, Assignments / Project works given by mentor/coordinator & Summer Research Project Completion Report

CANDIDATE'S NAME: KARTHIKEYAN SL. NO.: CSIR/SRTP/2020/NEIST/2260

GRADE: A

HOST INSTITUTE: CSIR-NEIST

MENTOR'S NAME: MUNAGALA GAYATRI RAMYA

REMARKS

.....

MENTOR

CSIR-SUMMER RESEARCH TRAINING PROGRAM
(CSIR-SRTP)2020 ONLINE

CERTIFICATE

Name: KARTHIKEYAN **Sl. No.:** CSIR/SRTP/2020/NEIST/2260

has completed all the requirements of the CSIR-Summer Research Training Program (CSIR-SRTP) 2020 online during June to August, 2020 coordinated by CSIR-NEIST, Jorhat

DR. G. NARAHARI SASTRY **DIRECTOR** **CSIR-NORTH EAST INSTITUTE OF SCIENCE AND TECHNOLOGY**	PROF. ALOK DHAWAN **DIRECTOR** **CSIR-INDIAN INSTITUTE OF TOXICOLOGY RESEARCH**	DR. SHEKHAR C. MANDE **DIRECTOR GENERAL, CSIR** **SECRETARY, DSIR, GOVT. OF INDIA**

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Roll No: NPTEL20CY34S71330233

To
KARTHIKEYAN L
137,AMMAN KOVIL ST,
THEVUR (PO),
SANKARI(TK), SALEM (DT)
TAMIL NADU - 600003
PH. NO :9944674743

Score	Type of Certificate
>=90	Elite+Gold
75-89	Elite+Silver
>=60	Elite
40-59	Successfully Completed
<40	No Certificate

No. of credits recommended by NPTEL:3
An additional 1 credit may be awarded if the University deems it fit, based on the actual student effort involved.

Elite

NPTEL Online Certification

(Funded by the Ministry of HRD, Govt. of India)

This certificate is awarded to

KARTHIKEYAN L

for successfully completing the course

Principles of Organic Synthesis

with a consolidated score of **62** %

Online Assignments	14.38/25	Proctored Exam	48/75

Total number of candidates certified in this course: **49**

Prof. Hemant B Kaushik
Head, Centre for Educational Technology
& NPTEL Coordinator
IIT Guwahati

Sep-Dec 2020
(12 week course)

Indian Institute of Technology Guwahati

FREE ONLINE EDUCATION swayam शिक्षित भारत, उन्नत भारत

Roll No: NPTEL20CY34S71330233 To validate and check scores: https://nptel.ac.in/noc

This certificate is computer generated and can be verified by scanning the QR code given below. This will display the certificate from the NPTEL repository, https://nptel.ac.in/noc/

Roll No: NPTEL21CY24S23352849

To
KARTHIKEYAN L
137,AMMAN KOVIL ST,
THEVUR (PO),
SANKARI(TK), SALEM (DT)
TAMIL NADU - 637104
PH. NO :9944674743

Score	Type of Certificate
>=90	Elite+Gold
75-89	Elite+Silver
>=60	Elite
40-59	Successfully Completed
<40	No Certificate

No. of credits recommended by NPTEL:3
An additional 1 credit may be awarded if the University deems it fit, based on the actual student effort involved.

NPTEL Online Certification

(Funded by the Ministry of HRD, Govt. of India)

This certificate is awarded to

KARTHIKEYAN L

for successfully completing the course

Chemistry of Main Group Elements

with a consolidated score of **52** %

Online Assignments	18.5/25	Proctored Exam	33.25/75

Total number of candidates certified in this course: **55**

Jan-Apr 2021
(12 week course)

Prof. Sridhar Iyer
Head CDEEP & NPTEL Coordinator
IIT Bombay

Indian Institute of Technology Bombay

FREE ONLINE EDUCATION swayam शिक्षित भारत, उन्नत भारत

Roll No:NPTEL21CY24S23352849

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Cid: 668.0582131753287

Office of the Principal Scientific Advisor to the Government of India

MoE's INNOVATION CELL (GOVERNMENT OF INDIA)

Ministry of Education Government of India

DRUG DISCOVERY HACKATHON 2020 Innovate4NewDrugs

DRUG DISCOVERY HACKATHON 2020

Certificate

This Certificate is awarded to

L Karthikeyan

for Participating in

Drug Discovery Hackathon 2020

Prof. Vijay Raghavan
Principal Scientific Adviser
Government of India

Prof. Anil Sahasrabudhe
Chairman
AICTE

Prof. B. Suresh
President
Pharmacy Council of India

Dr. Abhay Jere
Chief Innovation Officer
Ministry of Education Innovation Cell

MHRD

MHRD'S INNOVATION CELL (GOVERNMENT OF INDIA)

India First Leadership Talk

Certificate
of Participation

This is to certify that

Karthikeyan L

from COP, Madras Medical College has

Successfully attended the India's First Leadership Talk

with Shri Shashi Shekhar (CEO- Prasar Bharti)

Held on 11 July, 2020 by MHRD's Innovation Cell.

Dr. Abhay Jere

Chief Innovation Officer, MHRD's Innovation Cell

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This is to certify that Prof./Dr./Ms./Mr. **KARTHIKEYAN L** has participated in the Webinar on **"COVID-19 and Ayurveda: Principle, Practice and Policy,"** & **Inauguration of Amity Ayurveda Research Center** in New York, USA by **Sh. Shripad Yesso Naik,** Hon'ble Minister of AYUSH (I/C) and Minister of State for Defence (Chief Guest) & **Padamshree Vaidya Rajesh Koteja**, Secretary AYUSH (Guest of Honor), held on **May 21, 2020**, organized by Amity Science, Technology & Innovation Foundation (ASTIF), Amity University Uttar Pradesh, Noida (INDIA).

Dr. Swati Madan
Asst. Director - AIISM

Prof. (Dr.) B.C. Das
Dean - Health & Allied Sciences

Dr. W. Selvamurthy
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This is to certify that Mr.Karthikeyan L *has participated in* ***AN INTERNATIONAL WEBINAR ON "GUIDANCE TO WRITE A SCIENTIFIC PAPER & IMPORTANCE OF CITATION & REFERENCING OF PUBLICATIONS"*** *on* ***19th June, 2020*** *organised by* ***Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Guntur*** *in Association with* ***Indian Pharmaceutical Association, Education Division.***

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President,
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Dr. Vasudev Rao Avupati
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