DESIGN, SYNTHESIS AND ANTI TUBERCULAR SCREENING OF TRIAZOLYL PYRAZOLES AS POSSIBLE MTB – CYP51 INHIBITORS

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

In partial fulfillment of the requirements for the award of the degree of
MASTER OF PHARMACY
IN
BRANCH- (Pharmaceutical Chemistry)

Submitted by

INDHUMATHI.L

261915102

Under the Guidance of

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COIMBATORE - 641 044.

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CERTIFICATES

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This is to certify that the M.Pharm dissertation entitled "**DESIGN, SYNTHESIS AND ANTI TUBERCULAR SCREENING OF TRIAZOLYL PYRAZOLES AS POSSIBLE MTB – CYP51 INHIBITORS"** being submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai was carried out by **INDHUMATHI.L (Reg. No.261915102)** in the Department of Pharmaceutical Chemistry, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, under my direct supervision and guidance to my fullest satisfaction.

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This is to certify that the M.Pharm dissertation entitled, "**DESIGN, SYNTHESIS AND ANTI TUBERCULAR SCREENING OF TRIAZOLYL PYRAZOLES AS POSSIBLE MTB – CYP51 INHIBITORS"** was carried out by **INDHUMATHI.L (Reg. No.261915102)** in the Department of Pharmaceutical Chemistry, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore which is affiliated to The Tamil Nadu Dr.M.G.R. Medical University, Chennai, under the guidance of **Dr. Sonia George, M. Pharm., Ph.D.,** Associate Professor, Department of Pharmaceutical Chemistry, College of Pharmacy, SRIPMS, Coimbatore.

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This is to certify that the M.Pharm dissertation entitled, "**Design, Synthesis And Anti Tubercular Screening Of Triazolyl pyrazoles As Possible mtb – cyp51 Inhibitors"** was carried out by **Mrs. Indhumathi.L (Reg. No.261915102)** in the Department of Pharmaceutical Chemistry, College of Pharmacy,Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore which is affiliated to TheTamil Nadu Dr. M.G.R. Medical University, Chennai, under the guidance of **Dr. Sonia George, M.Pharm., Ph.D.,** Associate Professor, Department of Pharmaceutical Chemistry, College of Pharmacy, SRIPMS, Coimbatore.

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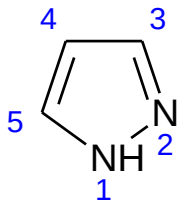
INTRODUCTION

INTRODUCTION

The synthesis of heterocyclic compounds is of huge attention in synthetic organic chemistry as it possesses a variety of therapeutic applications and their existence in numerous natural products like vitamins, hormones, antibiotics, and alkaloids. One of the most important fields of medicinal chemistry is the study of a heterocyclic bioactive molecule containing nitrogen atoms [1]. Various azole derivatives are now explored for their pharmacological and therapeutic potential aiming for the discovery of new drugs.

Pyrazole refers to the class of simple aromatic ring organic compounds of the heterocyclic series characterized by a 5-membered ring structure composed of three carbon atoms and two nitrogen atoms in adjacent positions. Pyrazole is a compound with the formula $C_3H_4N_2$. It is a weak base and a class of compounds with the ring C_3N_2 with adjacent nitrogen atoms. They are one of the most studied groups of compounds among the azole family. [2]



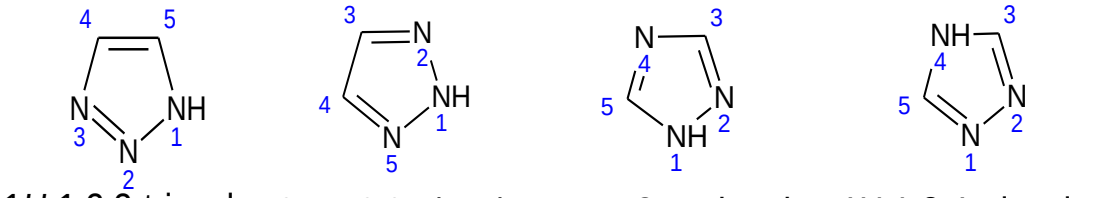
1*H*-pyrazole

The term pyrazole was given by Ludwig Knorr in 1883. Pyrazole derivatives have a long history of application in agrochemicals and the pharmaceutical industry as herbicides and active pharmaceuticals. The recent success of the pyrazole COX-2 inhibitor has further highlighted the importance of these heterocyclic rings in medicinal chemistry. A systematic investigation of this class of heterocyclic lead revealed that pyrazole-containing pharmacoactive agents play important role in medicinal chemistry. The prevalence of pyrazole cores in biologically active molecules has stimulated the need for elegant and efficient ways to make these heterocyclic leads. [3]

Pyrazole nucleus has been reported to show a broad spectrum of biological activity including anti-tuberculosis, antibacterial, antiviral, antitumor, anti-histaminic, anti-depressant, insecticidal, and fungicidal activity. [4]

Triazole has been found as a potential heterocyclic component in a wide range of drug scaffolds. It has a five-membered nitrogen heterocycle core with three nitrogen atoms and two carbon atoms. The core has a substantial impact on biological activity. [5]

It is otherwise known as pyrrodiazoles and is a five-membered, di unsaturated ring system. Two isomers of the triazole nucleus exist that differ in the 'position of nitrogen atoms' in the nucleus. These are 1, 2, 3 triazoles and 1, 2, 4 triazoles. It occurs as a pair of isomeric chemical compounds 1, 2, 3-triazole and 1, 2, 4-triazole with molecular formula $C_2H_3N_3$ and a molecular weight of 69.06. [6]



1*H*-1,2,3-triazole 2*H*-1,2,3-triazole 1*H*-1,2,4-triazole 4*H*-1,2,4-triazole

The name "triazole" was first used by Bladin in 1855 for describing the carbon-nitrogen ring system $C_2H_3N_3$. It is a white to pale yellow crystalline solid with a weak, characteristic odor, soluble in water and alcohol, melts at 120°C, and boils at 260°C. Novel triazole drugs were discovered and developed by applying bioisosteric replacement techniques with extending biological activities also captured special attention in medicinal chemistry. [7]

Among heterocyclic compounds, triazole has become an important one in the development of new drugs. A wide array of drugs comprising triazole nucleus display a wide range of pharmaceutical applications including anticonvulsants, antimalarial, antimicrobial, antitumor, antiviral, antiproliferative, anticancer, antioxidants, analgesics, antifungal, anti-plasmodial, antibacterial, immunostimulants, and anti-diabetic activity. This pharmacological significance of the triazole nucleus has driven the interest of researchers to develop potent triazole derivatives with auspicious biological activities. [8]

▪ DRUG DISCOVERY [9]

Drug discovery is considered as one of the fastest developing directions of high-tech investigations that rely on the achievements of molecular chemistry, biology, quantum physics, information technologies, and bio bioinformatics.

Drug discovery is a process, which aims at identifying a compound therapeutically useful in treating and curing disease. Typically a drug discovery effort addresses a biological target that has been shown to play a role in the development of the disease or starts from a molecule with interesting biological activities. The process of drug discovery involves the identification of candidates, synthesis, characterization, screening, and assays for therapeutic efficacy. Once a compound has shown its potential in these tests, the process of drug development can be initiated before clinical trials.

▪ DRUG DESIGN [10-15]

Drug design often referred to as rational drug design or simply rational design, is the inventive process of finding new medications based on the knowledge of a biological target. The drug is most commonly an organic small molecule that activates or inhibits the function of a biomolecule such as a protein, which in turn results in a therapeutic benefit to the patient. In the most basic sense, drug design involves the design of molecules that are complementary in shape and charge to the biomolecular target with which they interact and therefore will bind to it. Drug design for the knowledge of the three-dimensional structure of the biomolecular target is equant but not necessarily relies on computer modelling techniques. This type of modelling is sometimes referred to as computer-aided drug design.

Drug design that relies on the knowledge of the three-dimensional structure of the biomolecular target is known as structure-based drug design. In addition to small molecules, biopharmaceuticals and especially therapeutic antibodies are an increasingly important class of drugs, and computational methods for improving the affinity, selectivity, and stability of these protein-based therapeutics have also been developed. The major aim is to find whether the given molecule binds to the target and produces pharmacological actions or not.

The basic steps involved in CADD are:

1. Hit identification using virtual screening (structure- or ligand-based design)
2. Hit-to-lead optimization of affinity and selectivity (structure-based design, QSAR, etc.)

3. Lead optimization of other pharmaceutical properties while maintaining affinity

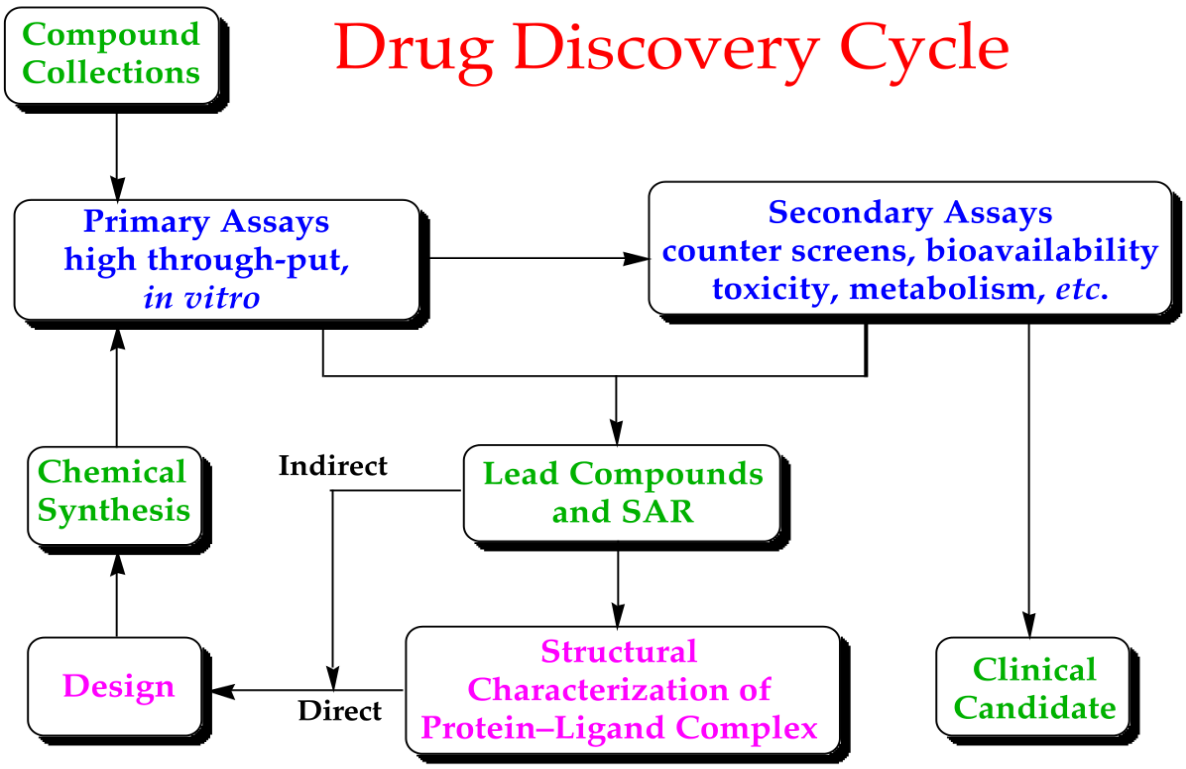


Fig1: Flowchart of Drug Discovery

Types of drug design

- Ligand-based drug design
- Structure-based drug design

Ligand Based Drug Design (LBDD)

It is also known as indirect drug design. In the absence of the structural information of the target, the ligand-based method is used to know about inhibitors for the target receptor. The biologically active lead molecule is detected by using structural or topological similarity or pharmacophoric similarity properties. There are several criteria for similarity comparisons such as structure, as well as the shape of individual fragments or electrostatic properties of the molecule. The generated lead molecules are ranked based on their similarity score or obtained by using different methods or algorithms.

Structure-based drug design (SBDD)

Structure-based drug design (or direct drug design) relies on knowledge or the three-dimensional structure of the biological target obtained through methods such as X-ray crystallography or NMR spectroscopy. If an experimental structure of a target is not available, it may be possible to create a homology model of the target based on the experimental structure of a related protein. Using the structure of the biological target, candidate drugs that are predicted to bind with high affinity and selectivity to the target may be designed using interactive graphics and the intuition of a medicinal chemist.

Alternatively, various automated computational procedures may be used to suggest new drug candidates. Currently, the use of SBDD has become a standard exercise as a part of drug discovery and development, both in academics and industry.

Typically, the process involves

- Selection and identification of the target.
- Search for lead or lead identification.
- Lead optimization.

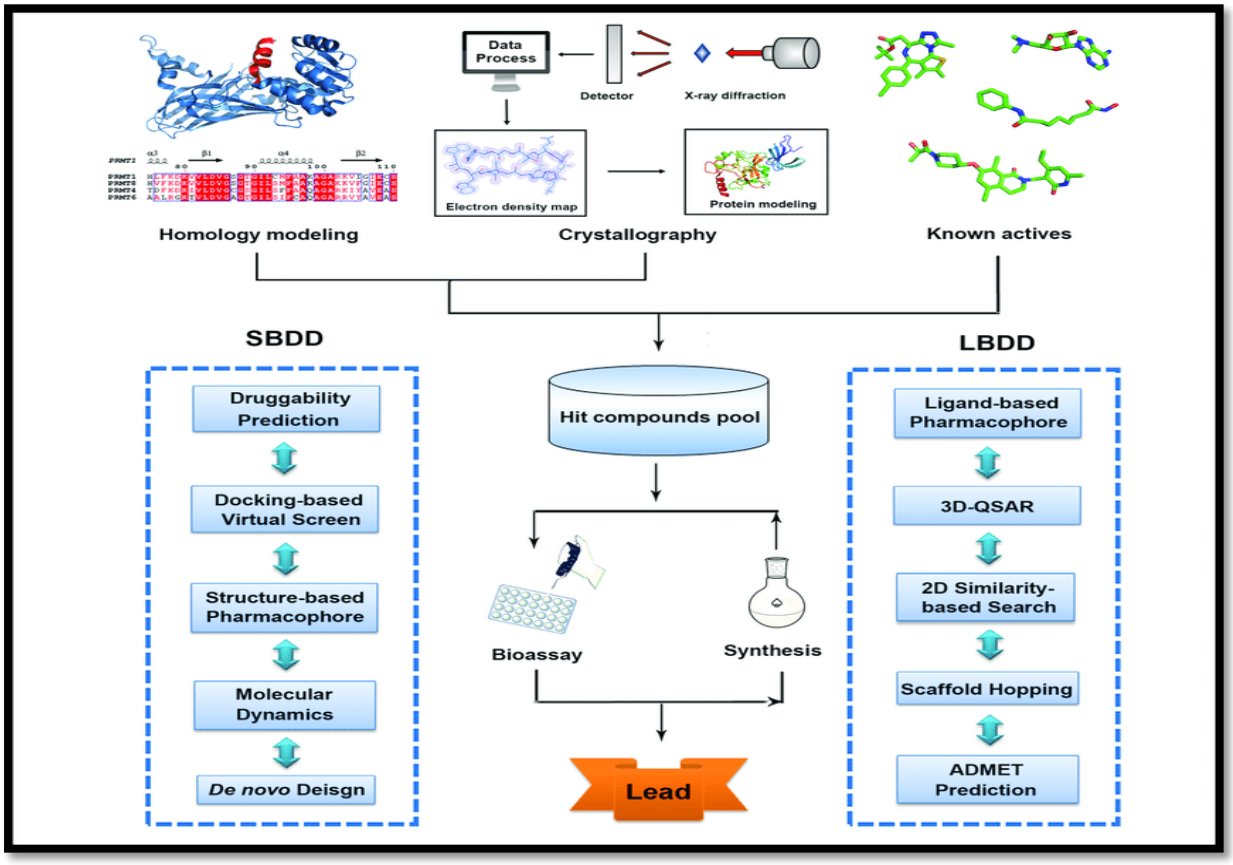


Fig2: Structure-Based Drug Design & Ligand Based Drug Design

▪ DRUG TARGET

The drug discovery process begins with the identification of a possible therapeutic target. The selected drug target must be a key molecule involved in a specific metabolic or cell signalling pathway that is known or believed to be related to a particular disease state.

Important drug targets include:

- Enzymes (inhibitor- reversible or irreversible)
- Receptors (agonist or antagonist)
- Nucleic acid inter collators or modifiers
- Ion channels (blockers or openers)
- Transporters (uptake inhibitors)

The 3D structure of the protein target is usually obtained by X-ray crystallography (crystal structures of different macromolecules are available from the Research Collaboratory for Structural

Bioinformatics (RCSB) Protein Database), Nuclear Magnetic Resonance (NMR), or homology modeling from a previously determined structure. Various other parameters like temperature factors, Vander Waals interactions, hydrogen bonding, etc., in the region of interest on the target, should be evaluated.

- **VIRTUAL SCREENING TECHNIQUES (VS) [16-18]**

Virtual screening (VS) is a computational technique used in drug discovery to search libraries of small molecules to identify those structures which are most likely to bind to a drug target, typically a protein receptor or enzyme.

Virtual screening has been defined as the "automatically evaluating very large libraries of compounds" using computer programs. As this definition suggests, VS has largely been a numbers game focusing on how the enormous chemical space of over 1060 conceivable compounds can be filtered to a manageable number that can be synthesized, purchased, and tested. Virtual screening has become an integral part of the drug discovery process.

These are two broad categories of screening techniques: Ligand-based and structure-based VS.

Ligand-based virtual screening technique

It is further divided into **ligand alignment, pharmacophoric approach, and machine learning algorithms.**

In **Ligand alignment,** a single 3D structure of a biologically active ligand is used as a template by ligand alignment for the super positioning and scoring of other 3D molecular structures from chemical libraries concerning the similarity of their characteristics like shape, interaction possibilities, or physicochemical properties.

In the **Pharmacophoric approach,** by use of a structurally diverse set of ligands that bind to the receptor, a coarse-grained 3D surrogate of the receptor is generated. These are usually done by calculating all of the possible super positions of predefined chemical groups which are recognized at the target binding site and are responsible for the biological activity. This pharmacophoric serves as a template for the selection of the molecules which fulfill the specified geometrical constraints in the VS queries.

Machine learning algorithms relays on QSARs which correlate biological data with molecular descriptors hence deriving statistical models used to predict the activity of novel compounds. Some of the examples of the machine learning techniques that are becoming popular

tools of model building and VS are: self-organizing maps (SOM), Binary QSAR, k-nearest neighbor approach (kNN), artificial neural network (ANN), etc.

Structure-based virtual screening technique [19, 20]

In silico or virtual screening (VS) of large compound collections to identify a subset of compounds that contains relatively many hits against the target. The compounds that are virtually screened can stem from corporate or commercial compound collections or virtual compound libraries. If a three dimensional (3D) structure or model of the target is available, a commonly used technique is structure-based virtual screening (SBVS)

Structure-based virtual screening involves docking of candidate ligands into a protein target followed by applying a scoring function to estimate the likelihood that the ligand will bind to the protein with high affinity.

Advantages of virtual screening

- Time and cost reduction of the screening process of millions of small molecules compared to HTS (High Through-put Screening).
- There is no need for physically existing compounds to perform the screening process, unlike HTS.
- A large number of docking programs and scoring functions.
- Different approaches of VS have been created for lead discovery depending.
- Each time on the availability of experimental information (SBVS Ligand-Based VS, Fragment-Based VS, etc.)

Limitations of virtual screening

- Many VS tools are applicable and successful to specific case studies (based on the training set) and not in general cases.
- Compounds being identified by HTS are usually more bioactive than compounds identified by VS.
- Weakness in perfect inclusion of receptor structural flexibility and water in docking computations due to computational cost and high complexity of its modeling.
- Scoring is still challenging in predicting accurately the correct binding pose and ranking of the compounds due to the difficulties in parameterizing the complexity of the ligand-

receptor binding interactions and the approximations in calculating desolvation and entropic terms.

By using various methods described above, the lead moiety is identified by using the software's **iGEMDOCK v.2, AUTODOCK, DOCK,** etc. The molecules which are docked well will be superimposed on one another. The ligands which are not docked well will be in different places. This lead molecule is being subjected to docking to know the interaction between the protein of interest and the lead. A graphical-automatic drug discovery system Called **iGEMDOCK v.2** is used for integrating docking. Post-analysis, screening, and visualization. To our best knowledge, **iGEMDOCK v.2** is the first system that combines structure-based Virtual screening and post-screening analysis.

The lead molecule which is identified by virtual screening is taken for lead optimization by knowing ADME data and drug likeliness properties.

- **ADME DATA [21]**

The high-throughput screening in drug discovery for absorption, distribution, metabolism, and excretion (ADME) properties has become the norm in the industry. Only a few years ago it was ADME properties were attributed to more failure of drugs than efficacy or safety in the clinical trials. With the realization of new techniques and refinement of existing techniques, better projections for the pharmacokinetic properties of compounds in humans are being made, shifting the drug failure attributes more to the safety and efficacy properties of drug candidates. There are tremendous numbers of tools available to discover scientists to screen compounds for optimization of ADME properties and selection of better candidates. However, the use of these tools has generally been to characterize these compounds rather than to select among them. This report discusses applications of the available ADME tools to better understand the clinical implication of these properties and to optimize these properties. It also provides tracts for the timing of studies concerning the stage of the compound during discovery, using a discovery assay by stage (DABS) paradigm. The DABS provides the team with a rationale for the types of studies to be done during the hit-to-lead, early, and late lead optimization stages of discovery, as well as outlining the deliverables (objectives) at those stages. DABS has proven to be optimal for efficient utilization of resources and helped the discovery team to track the progress of compounds and projects. Various medium and high throughput in vitro ADME screens are therefore now in use. In addition, there is an increasing need for good tools for predicting these properties to serve two key aims first, at the design stage of new compounds and compound libraries to reduce the risk of late-stage attrition; and second, to optimize the testing and

screening by looking at only the most promising compounds. Various software and servers are available; one such is the pharma algorithm server.

Drug Likeliness [22, 23]

Drug likeness is a qualitative concept used in drug design for how "drug-like" a substance is concerning factors like bioavailability. It is estimated from the molecular structure before the substance is even synthesized and tested. A drug like a molecule has properties such as

- Solubility in both water and fat, as an orally administered drug needs to pass through the intestinal lining after it is consumed, be carried in aqueous blood, and penetrate the lipid-based cell membrane to reach the inside of a cell.
- Potency at the biological target
- Several scoring methods can be used to express drug-likeness as a function of potency and physicochemical properties, for example, ligand efficiency and lipophilic efficiency.
- Since the drug is transported in aqueous media like blood and intracellular fluid, it has to be sufficiently water-soluble in the absolute sense (i.e., must have a minimum chemical solubility to be effective). Solubility in water can be estimated from the number of hydrogen bond donors vs. alkyl side chains in the molecule.
- Molecular weight: the smaller the better, because diffusion is directly affected. The great majority of drugs on the market have molecular weights between 200 and 600 Daltons.
- A traditional method to evaluate drug-likeness is to check compliance with Lipinski's Rule of Five, which covers the numbers of hydrophilic groups, molecular weight, and hydrophobicity. There are many online servers available for calculating the drug-likeness scores, one such is the molinspiration server.

Lipinski's Rule of Five [24]

Lipinski's rule of five also known as Pfizer's rule of five or simply the rule of five (RO5) is a rule of thumb to evaluate drug-likeness or determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans. The rule was formulated by Christopher A. Lipinski in 1997, based on the observation that most orally administered drugs are relatively small and moderately lipophilic molecules.

Lipinski's rule says that, in general, an orally active drug has not more than one violation of the following criteria:

- Not more than 5 hydrogen bond donors (sum of OHs and NHs).
- Not more than 10 hydrogen bond acceptors (sum of Ns and Os).
- Molecular weight is not greater than 500 Daltons.
- An octanol-water partition coefficient, log P, is not greater than 5.
- Number of violations less than 5

Improvements

To evaluate drug-likeness in a better way, the rules have spawned many extensions by Ghose et al. in 1999.

- Log P: -0.4 to +5.6 range.
- Molecular refractivity: 40-130
- Molecular weight: 160-480.
- A number of atoms: 20-70.
- The polar surface area must not be greater than 140 Å.

Over the past decade, Lipinski's profiling tool for drug-likeness has led to further investigations by scientists to extend profiling tools to lead-like properties of compounds in the hope that a better starting point in early discovery can save time and cost.

▪ DOCKING [25, 26]

Docking entails predicting the protein-ligand complex structure and is followed by scoring in SBVS to rank the compounds. Docking programs utilize various methods of conformational search to explore the ligand conformational space; these are categorized as follows:

a) Systematic methods, place ligands in the predicted binding site after considering all degrees of freedom.
b) Random or stochastic torsional searches about rotatable bonds, such as Monte Carlo Molecular and genetic algorithms to "evolve" new low energy conformers.
c) Molecular Dynamics simulation methods and energy minimization for exploring the energy landscape of a molecule.

Molecular docking may be defined as an optimization program, which would describe the "best-fit" orientation of a ligand that binds to a particular protein of interest. The focus of molecular docking is to computationally simulate the molecular recognition process.

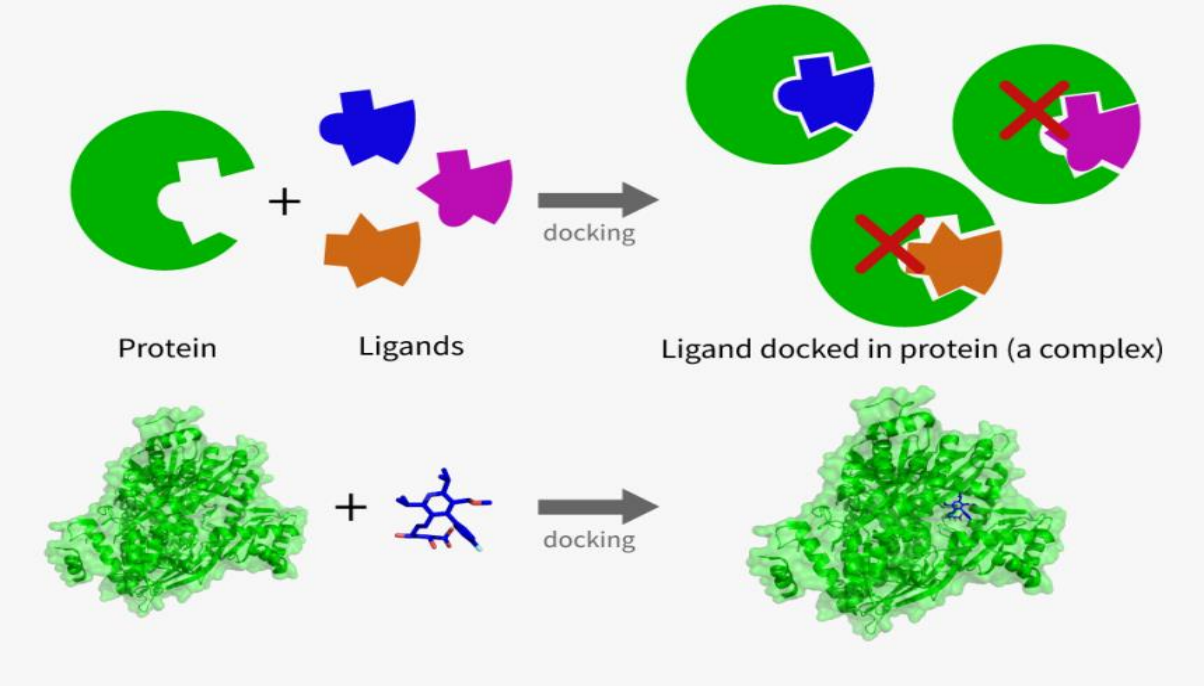


Fig.3: Docking

Molecular docking aims to achieve an optimized conformation for both the protein and ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized.

A molecular docking calculation consists of the following steps:

- Optimization of the ligand geometry, calculation of p^H-dependent partial charges, and identification of rotatable bonds.
- Calculation of electrostatic properties of the protein of interest and defining the ligand-binding region.
- Calculation of ligand-protein interaction by a scoring function that includes terms and equations that describe the intermolecular energies.

Docking produces plausible candidate structures. These candidates must be ranked by using scoring functions and to identify structures that are most likely to occur in nature.

Rigid-body docking and flexible docking

If the bond angles, torsion angles, and bond lengths of the components are not modified at any stage of complex generation, then they are known as rigid-body docking. A rigid-body docking is sufficiently good for most docking when substantial change occurs within the components at rigid-body docking. Docking procedures that permit flexible docking procedures or conformational change must intelligently select a small subset of possible conformational changes for consideration.

Mechanics of docking

To perform a docking screen, the first requirement is the structure of the interesting protein. Usually, the structure has been measured using a biophysical technique such as X-ray crystallography or NMR spectroscopy. The protein structure and a database of potential ligands serve as inputs to a docking program. The success of a docking program is based on two components:

Search algorithm

The search space includes all possible orientations and conformations of the protein paired with the ligand. With present computing resources, it is impossible exhaustively explore the search space; which involves enumerating all possible distortions of each molecule (molecules are dynamic and exist in an ensemble of conformational states) and all possible rotational and translational orientations of ligand relative to the protein at a given level of granularity. Most docking programs account for a flexible ligand, and several are attempting to model a flexible protein receptor. Each "snapshot" of the pair is referred to as a pose.

There are many conditions for sampling the search space. Here are some examples:

- Use a coarse-grained molecular dynamics simulation to propose energetically reasonable poses stimulation. (Direct search-simplex method; gradient-based search-steepest descent, Fletcher-Reeves method, Newton Raphson method; least square methods-Marquardt method).
- Simulated annealing (Monte Carlo search of the parameter space).
- Use a "linear combination" of multiple structures determined for the same protein to emulate receptor flexibility.
- Use a genetic algorithm to "evolve" new poses that are successively more fragment-based construction.

Scoring function

The scoring function takes a pose as input, returns a number indicating the likelihood that the pose represents the favorable binding interaction.

Most scoring functions are physics-based molecular mechanics force fields that estimate the energy of the pose; a low (negative) energy indicates a stable system and thus likely for a binding interaction. It is an alternative approach to derive a statistical potential for interactions from a large database of protein-ligand complexes, such as the Protein Data Bank. This evaluates the fit of the pose according to this inferred potential.

There are a large number of structures from X-ray crystallography for complexes between proteins and high-affinity ligands. It is comparatively fewer for low-affinity ligands as the later complexes tend to be less stable and therefore more difficult to crystallize. Scoring function trained with this data can dock hits (ligands predicted to bind to the protein and do not when placed together in a test tube).

Various software used for docking studies are:

Auto Dock, Gold, Vega, Glide, Flexi dock, Flex, Fred, Hint, etc.

Autodock 4.2

Autodock is a suite of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D structure and combines a rapid energy evaluation through pre-calculated grids of affinity potentials with a variety of search algorithms to find suitable finding positions. Autodock uses the Monte Carlo method and simulated annealing in combination with a genetic algorithm for building the possible conformations. The genetic algorithm is used for global optimization. Autodock works in the Linux platform. Cygwin is used as a user-friendly interface.

The local search method is energy minimization and the Amber "force field" model helps in the evaluation of binding positions compatible with several scoring functions based on the free energy. The atomic affinity grids can be visualized. This is helpful to guide organic synthetic chemists to design better binders. Autodock consists of two main programs:

- Auto Grid pre-calculates the grids.
- Auto Dock performs the docking of the ligand to a set of grids describing the target protein.

It also has got capabilities to visualize atomic affinity grids and its graphical user interfaces Auto Dock Tools (ADT), thus to supports the analysis of docking results. It has the advantage of getting a free academic license, at the same time parallel computations are not supported.

TUBERCULOSIS (TB)

Tuberculosis (TB) is an infection caused by the bacterium *Mycobacterium tuberculosis*, first discovered in 1882 by Robert Koch. TB most commonly occurs in the lungs but can sometimes also affect other organs, including the skin, bones, lymph nodes, liver, digestive tract, and central nervous system (brain and spinal cord).[27]

Tuberculosis is a leading cause of infectious disease mortality in the world. In the past 25-28 years, the incidence of microbial infection has increased on alarming levels over the world as a result of microbial resistance it is because of an increase in the number of patients suffering from TB worldwide.[28] Approximately 32% of the world population is infected with *Mycobacterium Tuberculosis*. HIV-positive patients are more susceptible to *Mycobacterium tuberculosis* with a fifty-fold risk increase over HIV-negative patients. The rate of the progression of latent TB to active disease in HIV-positive patients is higher than in non-HIV-infected individuals. An additional concern is a rise in multi-drug resistance. [29]

According to the World Health Organization (WHO), nearly one-third of the world's population has been exposed to the tuberculosis pathogen [30]. Several known factors make people more susceptible to tuberculosis infection worldwide, the most important of which is the human immunodeficiency virus (HIV). [31]

WHO has recently launched its innovative "End TB Strategy" [32], supporting the TB elimination strategy and the vision of a TB-free world with zero death, disease, and suffering due to TB. The new strategy supports universal access to high-quality MDR-TB diagnosis and treatment. However, since the market launch of rifampicin in the early 1960s, no new anti-TB drug has been specifically developed until recently. The need for new drugs and regimens is obvious [33].

Biology of Mycobacterium tuberculosis [34]

The common causative organism for TB, *Mycobacterium tuberculosis* (Mtb) is a major component of the microbiological history and is also referred to as the tubercle bacillus.

- Mtb is a small, non-motile, gram-positive slow-growing bacillus with a generation time of 12 to 20 hours and a prolonged culture period on agar of up to 21 days.

- Mtb possesses a unique cell wall highly rich in lipid content, which includes mycolic acids and other glycolipids. It has an unusual waxy coating of mycolic acid on its cell surface making it impervious to gram staining. It is detected by the acid-fast technique.
- It is highly aerobic and does not produce toxins.
- It can survive for long periods under adverse conditions.

While in humans, Mycobacterium tuberculosis is the main cause of the infection, several other Mycobacterium species including Mycobacterium Bovis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium Canetti, Mycobacterium caprae, and Mycobacterium pinnipedii are also known to cause the disease.

ANTITUBERCULAR DRUGS

Chemical Classification of anti-tubercular drugs

- Salicylic Acid Derivatives - Para Amino Salicylic Acid
- Pyridine Derivatives - Isoniazid, Ethionamide, Prothionamide
- Pyrazine Derivatives - Pyrazinamide
- Ethylene diamino butanol Derivatives - Ethambutol
- Antibiotics – Streptomycin, Rifampin, Kanamycin
- Miscellaneous Drugs - Fluoroquinolones, Ofloxacin, Ciprofloxacin, Clarithromycin, Azithromycin

Pharmacological Classification of anti-tubercular drugs

- **First-line drugs**

  Isoniazid (INH), Rifampicin (RIF), Ethambutol (ETH), Pyrazinamide (PZA), Streptomycin (STR)

- **Second-line drugs**

  Amikacin, Kanamycin, Capreomycin, Ciprofloxacin, Levofloxacin, Prothionamide, Cycloserine, Para aminosalicylic acid, Moxifloxacin, Ethionamide

- **Third line drugs**

  Rifabutin, Clarithromycin, Linezolid, Thioacetazone, Thioridazine

DRUG RESISTANCE IN TUBERCULOSIS [35, 36]

TB organisms resistant to the drugs used in its treatment are widespread and occur in all countries. Drug resistance emerges as a result of inadequate treatment and organisms acquire resistance they can spread from person to person in the same way as drug-sensitive TB strains. Individuals with drug-resistant TB disease can also transmit the resistant strain of the disease directly to others.

- **Multi Drug Resistant Tuberculosis (MDR-TB)**

Multi-drug-resistant (MDR) TB refers to simultaneous resistance to at least two or more of the five first-line anti-TB drugs (INH, RIF, PZA. ETH, and STR), caused by sharing of genes between different species or genera of the pathogen. Generally mediated by small pieces of extra-chromosomal DNA, known as transposons or plasmids. According to WHO, about 3.6% of new TB patients in the world have MDR TB strains. Treatment for multi-drug-resistance tuberculosis is prolonged, less effective. Costly and therapeutically poorly tolerated.

- **Extensively Drug-Resistant Tuberculosis (XDR-TB)**

Extensively-drug-resistant (XDR) TB is defined as resistance to at least INH and RIF, in addition to any fluoroquinolones and at least one of the three injectable second-line agents, i.e. Capreomycin, Amikacin, or kanamycin. The principles used for MDR-TB and XDR-TB treatment are the same. The main difference is that XDR-TB is associated with a much higher mortality rate than MDR-TB, because of the reduced number of effective treatment options.

ENZYME INHIBITORS

Enzyme as targets for drug design

Enzyme inhibition is a promising approach for the rational discovery of new leads or drugs. Target enzymes selected for rational drug design are those whose inhibition in vivo would lead to the desired therapeutic effect. The two general categories of target enzymes are

- A potential drug is designed for an enzyme whose inhibition is known to produce a specific pharmacological effect, but existing inhibitors have certain undesirable properties such as lack of potency or specificity or exhibit side effects.
- Approach to design inhibitors of an enzyme whose inhibition has not yet been established to lead to a desired therapeutic effect.

MYCOBACTERIUM TUBERCULOSIS CYTOCHROME P450 STEROL 14α DEMETHYLASE (MT-CYP51)

For aerobic bacteria such as Mycobacterium tuberculosis (MT), the architectural requirements of the cell membrane can be satisfied by either sterol surrogates, e.g., pentacyclic hopanoids synthesized directly from squalene by an anaerobic pathway, or sterols synthesized from squalene by an aerobic pathway. However, sterols play a dual role in eukaryotic cell physiology at vastly different cellular concentrations, structurally as bulk inserts to affect permeability and hormonally to regulate growth, reproduction, and other processes, the level of cellular sterols in bacteria may be substantially less than even the hormonal level required in eukaryotes. In bacteria, sterol concentration was found to be two or more orders of magnitude less than the level of eukaryotic cells, which ranges from ca. 30 to 3,000fg/cell depending on the size of the cell. Although most bacteria are reported not to contain sterols, chemical and biochemical studies have shown the occurrence and biosynthesis of distinct sterols in several nonphotosynthetic and photosynthetic bacteria.

Steps involved in the oxygen-requiring portion of sterol biosynthesis in eukaryotic organisms are still unknown in bacteria. Recently, Lamb et al. showed that Mycobacterium smegmatis, a closely related species to MT, is able to synthesize cholesterol from radiolabeled mevalonic acid, indicating the presence of genes encoding sterol biosynthetic enzymes in some oxygenic bacteria.

The discovery of mycobacterial sterol biosynthesis led to consider the potential for azole and other sterol-inhibiting therapies against these pathogens. Anti-mycobacterial compounds are urgently required to target the serious increase in frequency of multi-drug-resistant tuberculosis (M-DRTB) emerging across the globe. Current therapy involves mainly isoniazid, and resistance to this antibiotic and other limited treatment options have become a major problem.

Lanosterol 14 α-demethylase (MT-CYP51) catalyzes an essential early step in sterol metabolism whereby it removes a methyl group, from lanosterol. This leads to an accumulation of methylated sterol precursors. Inhibition of this enzyme leads to the disruption of cell membrane which results in inhibition of growth and/or cellular death.

Recent studies have confirmed that azole derivatives which inhibit fungal CYP51, also inhibits the growth of *Mycobacterium bovis* and *Mycobacterium smegmatis*, accepted models for the study of *M.tuberculosis* at nanomolar concentration and were more active than the most used drug isoniazid and other inhibitors were identified experimentally through high throughput screening. MT-CYP51 may also be a potential M. tuberculosis therapeutic target on its own. It is one of the 20 different CYP enzymes encoded by the M. tuberculosis genome. The susceptibility of M. tuberculosis to the

azole antifungal agents that target these enzymes suggests their important roles in M. tuberculosis physiology and, hence, their potential use as therapeutic targets. MT-CYP51 is the only M. tuberculosis CYP enzyme whose catalytic function has been demonstrated. Although due to the absence of the complete sterol biosynthetic pathway, M. tuberculosis cannot synthesize sterols de novo, MT-CYP51 can demethylate the sterols lanosterol, dihydrolanosterol, and obtusifoliol. It has been found that for MT-CYP51 there are about fifteen residues essential for ligand binding namely Tyr 76, Phe 78, Met 79, Ileu 82, Phe 83, His 101, Thr 176, Ser 252, Phe 255, Ala 256, His 259, Ile 321, Met 433, and Val 434. These residues were considered to be neighbouring the ligand as long as it was in its 3.5Å proximity. At the base of the binding pocket, there is a heme porphyrin where the iron binds with the N of the azole.

MYCOBACTERIUM TUBERCULOSIS CYTOCHROME P450 STEROL 14α-DEMETHYLASE MTB-CYP51 INHIBITORS

The discovery that the M. tuberculosis genome encodes a CYP-51 enzyme immediately raised interest in its potential utility as a drug target. The CYP-51 family of cytochrome P450 enzymes consists of substrate-specific enzymes that catalyze the 14α-demethylation of sterols such as that of lanosterol in the cholesterol biosynthetic pathway (Fig) [37]. The 14α-methyl sterols that accumulate in the membrane when the 14α-demethylase is inhibited, cause membrane disruptions that lead to cell death. Indeed, the proliferation of azole drugs such as ketoconazole and posaconazole stems from their efficacy in the treatment of fungal infections [39].

Cholesterol is important for the infectivity of Mtb into macrophages [40]. However, Mtb itself does not produce cholesterol intrinsically due to the absence of a de novo sterol metabolic pathway [41], but it uses cholesterol from the host's membrane, likely breaking it down as an energy source while engulfed in the host macrophage. [42]

The most likely explanation for the roles of CYP-51B1 in Mtb is that it could function in host-cholesterol metabolism upon infectivity. [43] This appears to be the main reason that clouds the efforts to uncover the primary role of CYP-51B1 in Mtb [38].

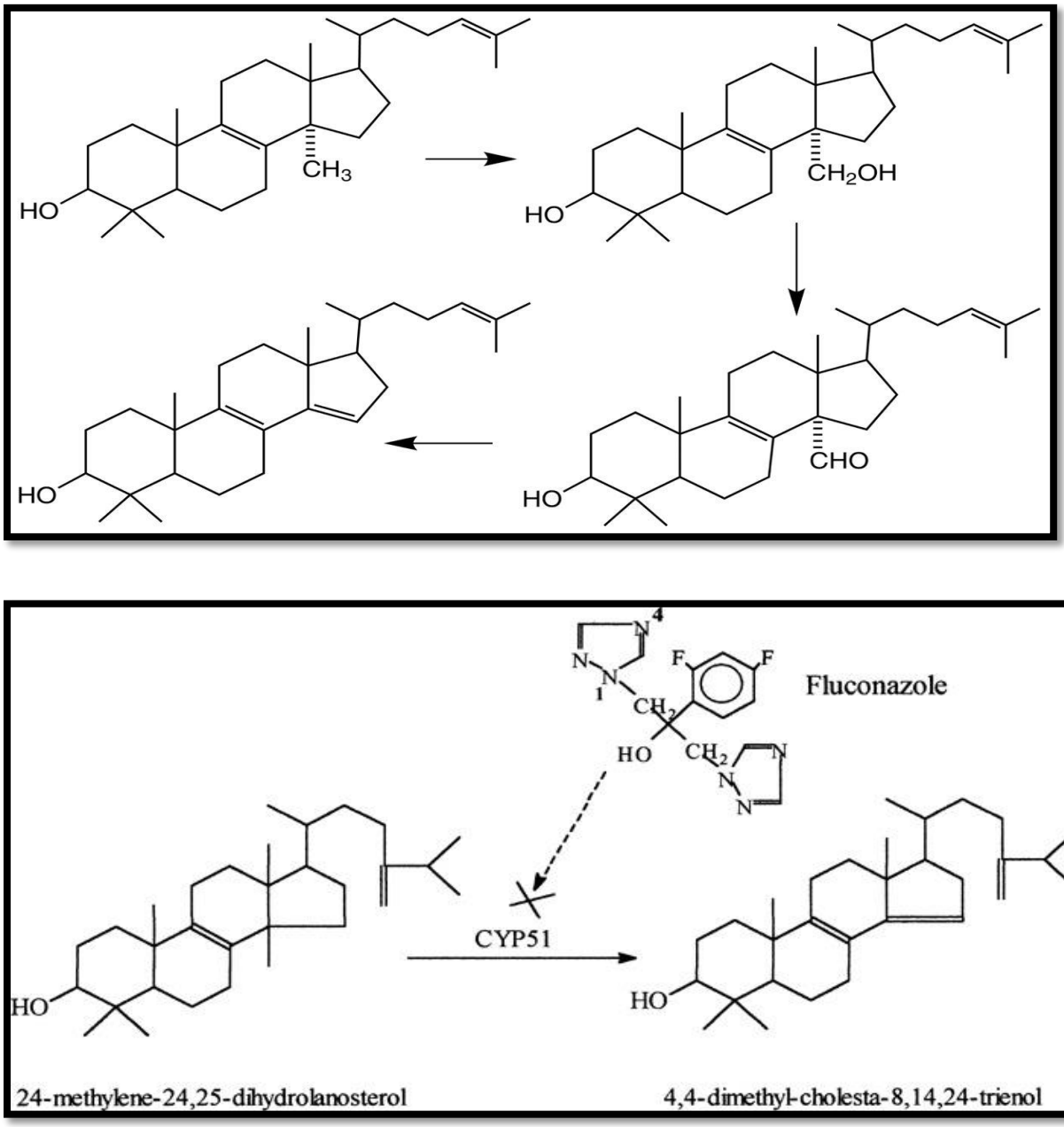


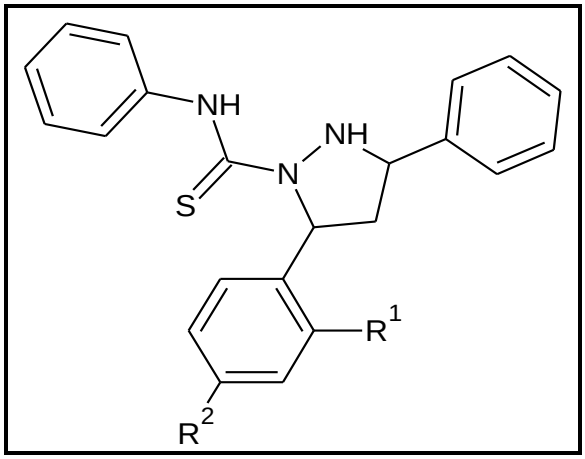
Fig 4: The three sequential reactions of the cholesterol biosynthetic pathway catalyzed by CYP51

REVIEW OF LITERATURE

REVIEW OF LITERATURE

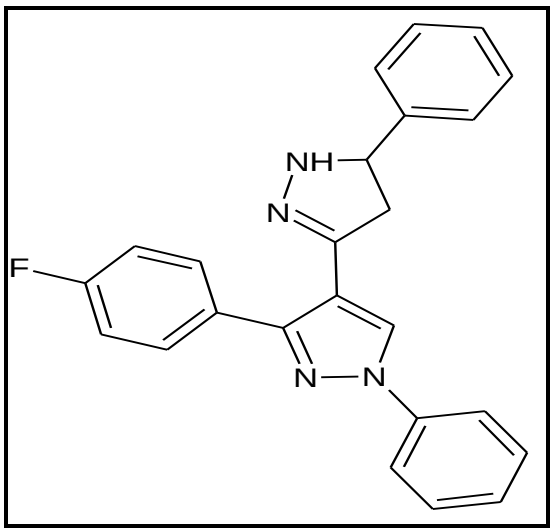
PYRAZOLE

- **Kok Tong Wong *et al.*,** [44] (2021) reported 3, 5-Disubstituted-Pyrazoline derivatives that were tested for anti TB activity against *Mycobacterium tuberculosis* $H_{37}Ra$. The molecular docking at the active site of cytochrome P450 14 alpha-sterol demethylase (CYP51) was performed. The Compounds have shown the highest docking and highest activity.



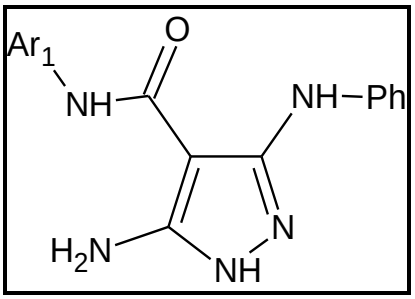
(1)

- **T. Prabha *et al.*,** [45] (2021) reported a series of chalcone annulated pyrazoline conjugates that were designed, synthesized, and evaluated for their anti-mycobacterial activity against *Mycobacterium tuberculosis* $H_{37}Rv$.



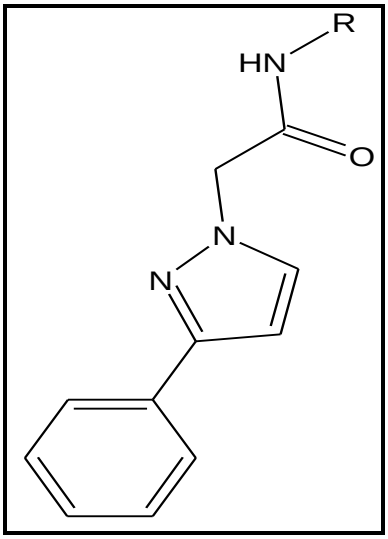
(2)

- **Shorouk S. Mukhtar *et al.*, [46]** (2021) reported a series of Schiff bases linked with heterocyclic moiety pyrazole-azomethines that were designed, synthesized, and evaluated for their in vitro anti-tubercular activities. The compounds exhibited moderate activities.



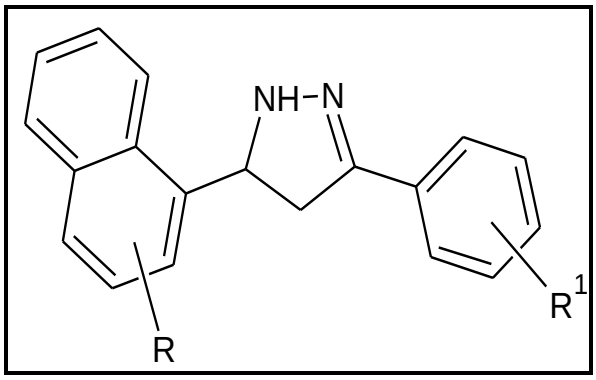
(3)

- **Nikhil B. Gaikwad *et al.*, [47]** (2020) reported a series of new 3- phenyl pyrazole derivatives that were designed, synthesized, and evaluated for their anti-mycobacterial potential. The biological evaluation revealed that synthesized compounds exhibited selective and potent inhibitory activity against *Mycobacterium tuberculosis.*



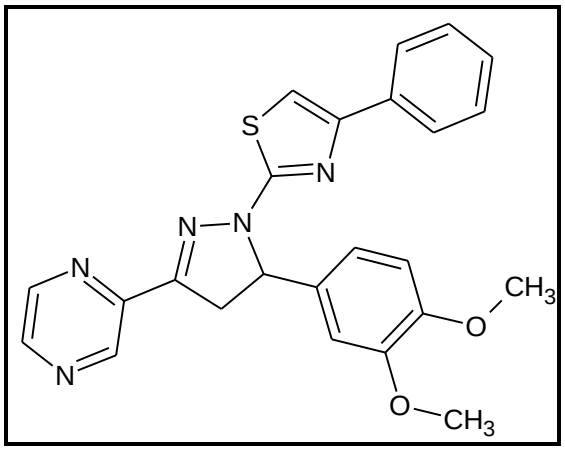
(4)

- **Shivani pola *et al.*, [48]** (2020) reported a new series of naphthyl chalcones and their pyrazoline derivatives that were designed, synthesized, and evaluated for their *anti-mycobacterial* and *anti-bacterial* activities.



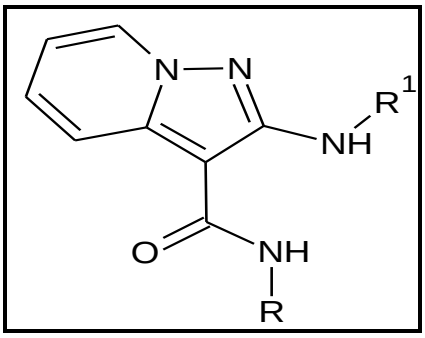
(5)

- **Nayera W. Hassan *et al.*, [49]** (2020) synthesized a series of novel hybrid molecules. The compounds were screened *in vitro* for their activity against *mycobacterium tuberculosis* $H_{37}Rv$ strain using MABA method. Six compounds displayed significant activity against *Mycobacterium tuberculosis* with MIC values ≤6.25 μg/ml versus 6.25 μg/ml.



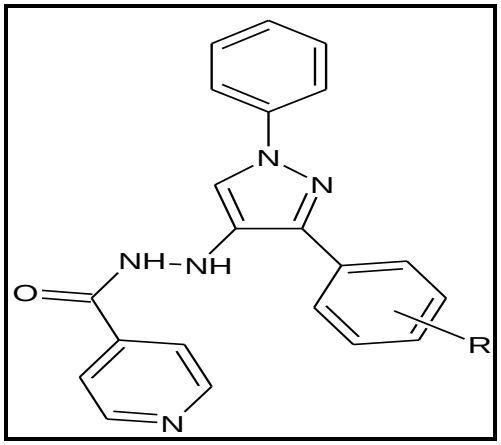
(6)

- **Palmi Modi *et al.*,** [50] (2019) reported novel pyrazolo [1, 5-*a*] pyrimidine analogues that were designed and synthesized in good yields. All the synthesized compounds were evaluated for their *in-vitro* anti-tubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$ strain by the alamar blue assay method. Most of the synthesized compounds displayed potent anti-tubercular activities.



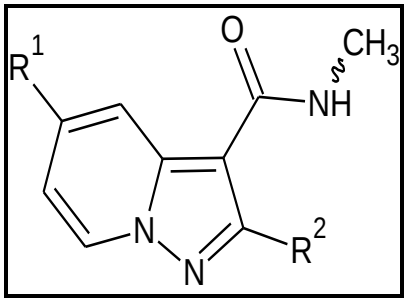
(7)

- **Sameer I. Shaikh *et al.*,** [51] (2017) reported a series of new classes of pyrazole derivatives that were designed, synthesized, and evaluated for their anti-mycobacterial activity against *Mycobacterium tuberculosis* $H_{37}Rv$ strain by alamar blue assay method. The compound displayed significant activity with MIC of 12.30 µg/ml.



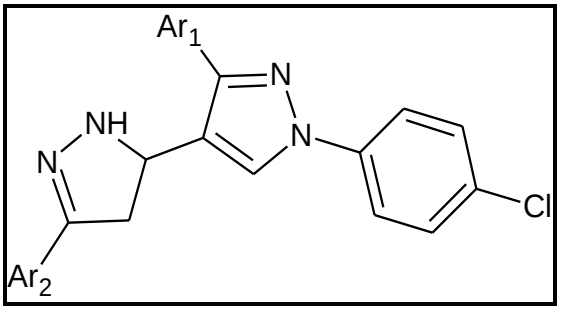
(8)

- **Xiaoyun Lu *et al.*,** [52] (2017) reported a series of pyrazolo [1, 5-a] pyridine-3-carboxamide hybrids that were designed and evaluated as novel anti-tubercular agents. The compounds exhibited promising in vitro activity against *Mycobacterium tuberculosis* $H_{37}Rv$ strain with MIC values of 0.006 mg/mL.



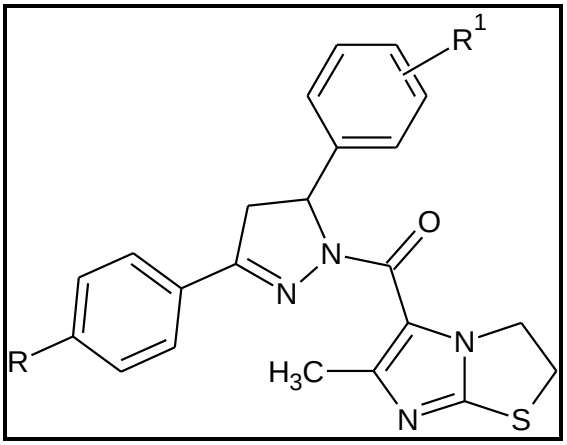
(9)

- **Nandam Harikrishna *et al.*,** [53] (2015) reported a new series of 1′-(4-chlorophenyl)-5- substituted aryl)-3′- (substituted aryl)-3, 4-dihydro-2*H*, 1′*H*-[3, 4′] bipyrazolyl derivatives that were synthesized, characterized, and screened against the antimicrobial and anti-tubercular activity.



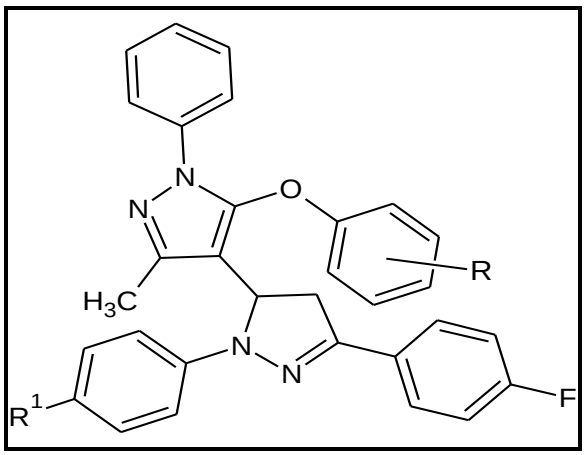
(10)

- **S. G. Alegaon *et al.*,** [54] (2014) reported a class of Novel 3, 5-diaryl-4, 5-dihydro-1 *H* –pyrazole derivatives that were efficiently synthesized and evaluated for their in vitro anti-tubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$. Most of the title compounds have exhibited significant anti-tubercular activity.



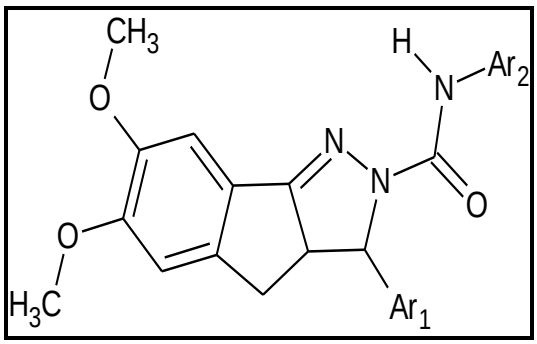
(11)

- **Sharad C. Karad *et al.,*** [55] (2014) synthesized a series of fluoro substituted pyrazolyl pyrazolines. The compounds were screened for their anti-tuberculosis activity against *Mycobacterium tuberculosis* $H_{37}Rv$. The compounds showed significant anti-tubercular activity. Some of them also exhibited superior antibacterial activity as compared to the first-line drugs.



(12)

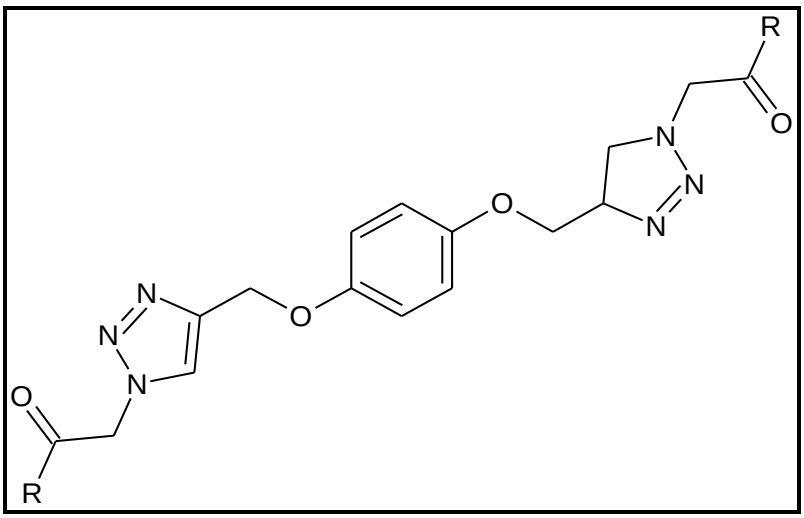
- **Mohamed Jawed Ahsan *et al.*, [56]** (2011) reported a series of 3-substituted-N-aryl-6, 7-dimethoxy-3a, 4-dihydro-3Hindeno [1, 2-c] pyrazole-2-carboxamide analogues were synthesized and evaluated for anti-tubercular activity by two-fold serial dilution technique. All the newly synthesized compounds showed moderate to high inhibitory activities against *Mycobacterium tuberculosis* $H_{37}Rv$.



(13)

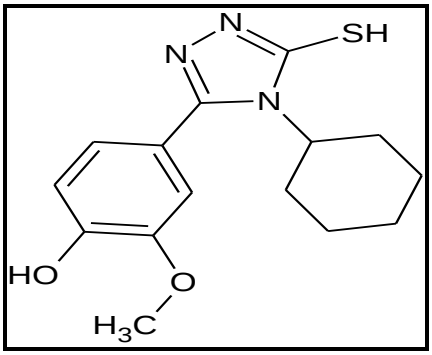
TRIAZOLE

- **Tejshri R. Deshmukh *et al.*, [57]** (2019) done their work on design, docking, synthesis, and anti-tubercular screening of novel aryloxy-linked dimeric 1, 2, 3-triazoles as possible inhibitors of CYP-51 enzyme.



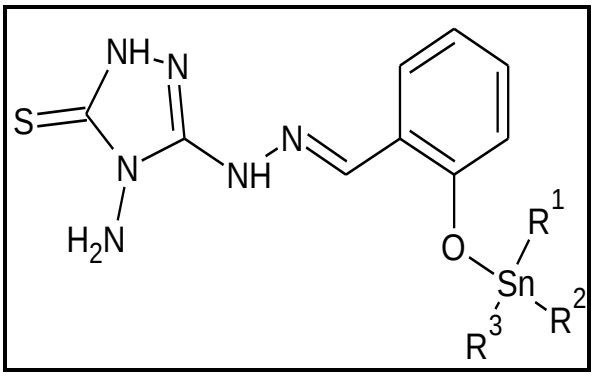
(14)

- **Neethu Dasan *et al.*, [58]** (2019) reported a series of 3, 4 – Di substituted Triazoles as possible inhibitors of CYP-51 (sterol 14α- demethylase). All the newly synthesized compounds were evaluated for anti-TB activity against *Mycobacterium tuberculosis*.



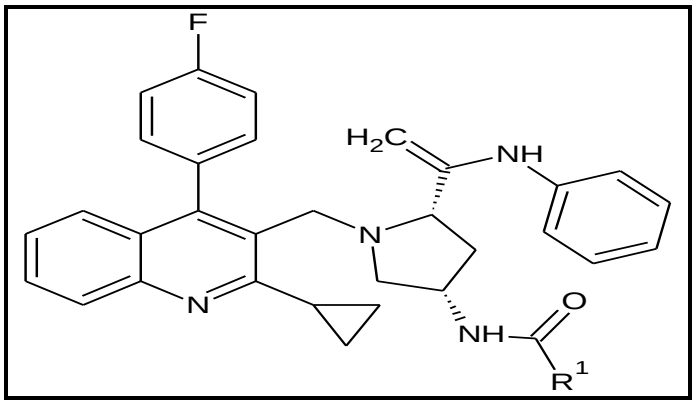
(15)

- **Rachana Joshi *et al.*,** [59] (2019) synthesized triorganotin(IV) complexes of schiff base (E)-4-amino-3-(2-(2-hydroxy benzylidene) hydrazinyl)-1H-1,2,4-triazole-5(4H)-thione) as possible inhibitors of CYP-51 (sterol 14α-demethylase). All the synthesized compounds were evaluated for anti-tubercular activity against *Mycobacterium tuberculosis* H_{37} Rv. Most of the compounds exhibited mild to moderate anti-tubercular activity.



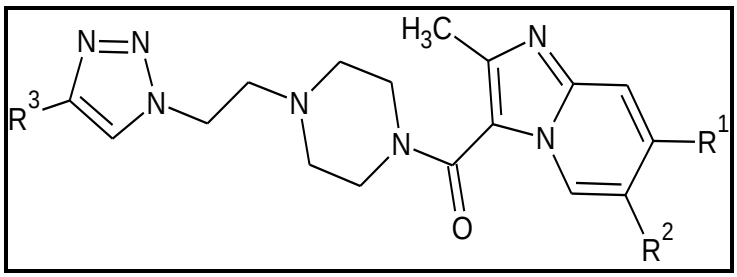
(16)

- **Moorthiamma Sarathy Ganesan *et al.*,** [60] (2021) synthesized a series of novel quinoline-proline hybrids and quinoline-proline- 1, 2, 3-triazole hybrids. All the titled target compounds were tested for anti-tubercular activity by MABA and they exhibited significant activity against the tested *Mycobacterium tuberculosis* H_{37}Rv strain.



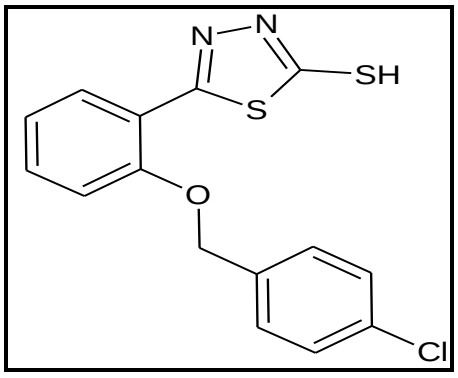
(17)

- **Adinarayana Nandikolla *et al.*, [61]** (2021) synthesized novel 1, 2, 3-triazole analogues of imidazo-[1, 2-a]-pyridine-3-carboxamide. The final compounds are screened *in vitro* for anti-tubercular activity using microplate Alamar blue assay (MABA) method.



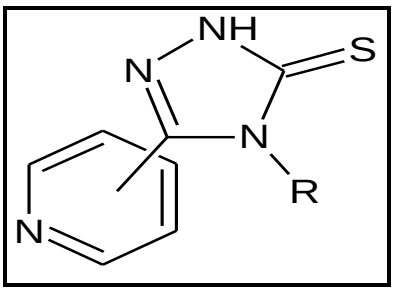
(18)

- **Katharigatta N. Venugopala *et al.*, [62]** (2020) reported 1, 2, 4-triazole (4H)-thione derivatives that were tested for anti-tubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$, and the compound showed significant activity with MIC of 0.08 µg/mL. And the docking studies were carried out.



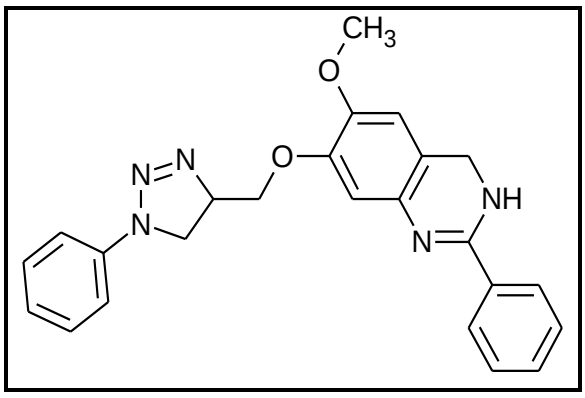
(19)

- **Zbigniew Karczmarzyk *et al.*, [63]** (2020) reported a series of 1, 2, triazoles derivatives as inhibitors of CYP121 having moderate antitubercular activity with percentage inhibition respectively, at MIC of 0.976 µg/mL.



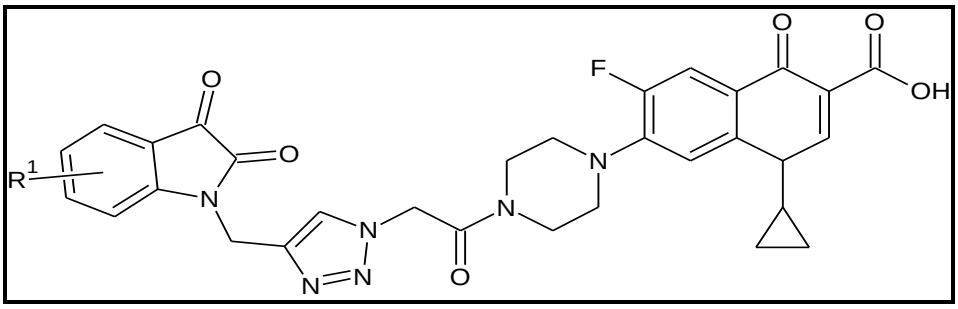
(20)

- **Narendra Kumar Maddali *et al.*, [64]** (2019) reported a series of 1, 4-disubstituted triazoles that were designed and synthesized by the Cu-catalyzed azide-alkyne cycloaddition. The target compounds were screened for antitubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$ by the Broth microdilution method using Lowenstein Jensen medium (LJ). The compounds displayed good anti-tubercular activity with MIC 7–11 µg/ml.



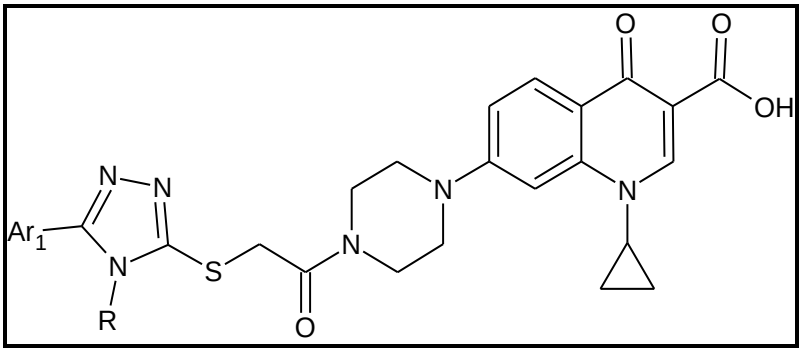
(21)

- **Rongxing Chen *et al.*,** [65] (2019) reported novel amide tethered ciprofloxacin-1, 2, 3-triazole-isatin hybrids and evaluated for their *in vitro* anti-mycobacterial activity. The synthesized hybrids showed considerable *in vitro* activity against *Mycobacterium tuberculosis* $H_{37}Rv$.



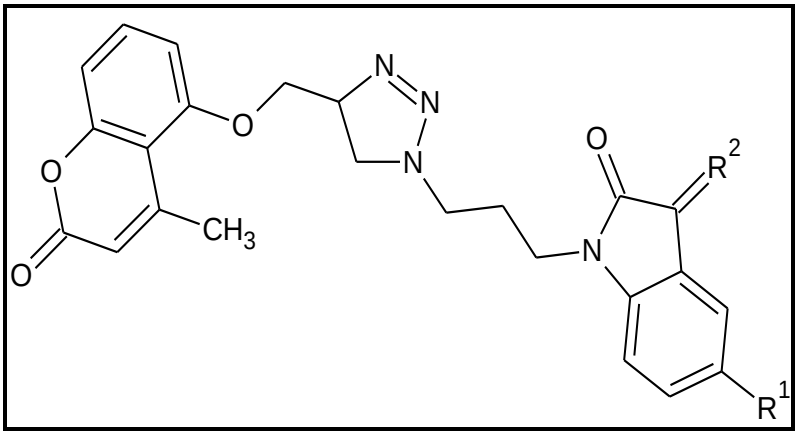
(22)

- **Hamada H.H. Mohammed *et al.*,** [66] (2019) synthesized a new *N*-4-piperazinyl ciprofloxacin-triazole hybrids. The *in vitro* anti-mycobacterial activity revealed that the compound exhibited promising anti-mycobacterial activity against *Mycobacterium smegmatis* compared with the reference isoniazid (INH). Additionally, compound **6a** exhibited broad-spectrum antibacterial activity against all the tested strains.



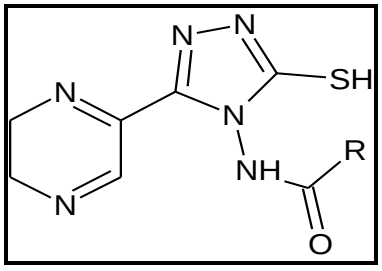
(23)

- **Guo-Cheng Huang *et al.,*** [67] (2018) reported a series of novel propylene-1H-1, 2, 3-triazole-4-methylene-tethered isatin-coumarin hybrids that were designed, synthesized, and assessed for their in vitro anti-tubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$.



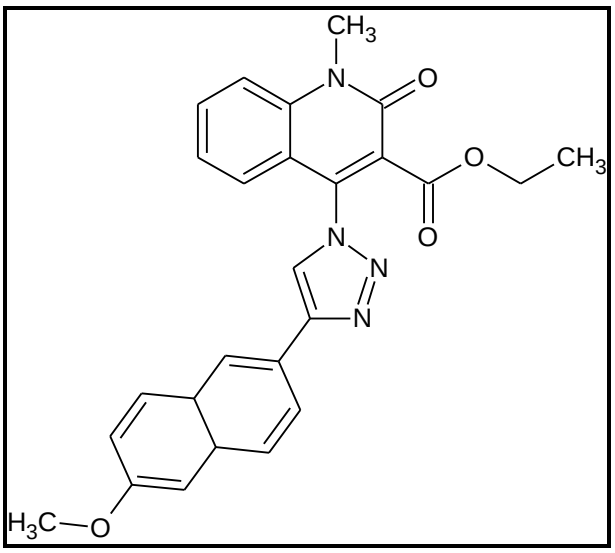
(24)

- **T. N. V. Ganesh Kumar *et al.,*** [68] (2018) reported a series of novel 1, 2, 4-triazole derivatives that were designed, synthesized, and evaluated for in vitro anti-tubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$.



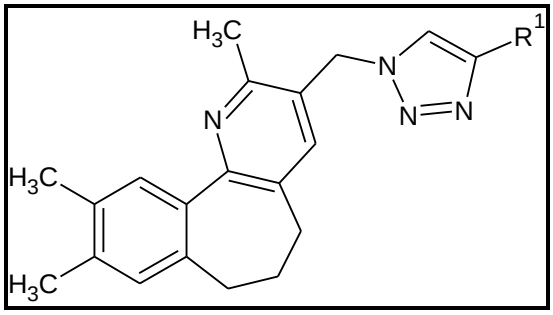
(25)

- **Saleha Banu *et al.*, [69]** (2018) reported a series of dihydroquinoline derivatives possessing triazolo substituents that were efficiently synthesized using click chemistry. The newly synthesized compounds were evaluated for their *in vitro* anti-tubercular activity against *Mycobacterium tuberculosis* $H_{37}Rv$. The compounds showed promising activity when compared to first-line line drug such as ethambutol.



(26)

- **Yasodakrishna Sajja *et al.*, [70]** (2017) synthesized a series of novel benzo-cyclohepta-pyridine-1, 2, 3-triazole hybrids. The newly synthesized compounds were evaluated for their in vitro anti-mycobacterial activity against *Mycobacterium tuberculosis* $H_{37}Rv$. The compounds displayed the most potent activity with a MIC value of 1.56 mg/mL.[69]



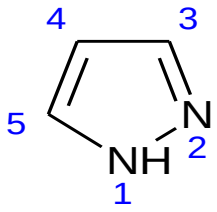
(27)

CHEMISTRY

CHEMISTRY

PYRAZOLES [70-75]

Pyrazoles are an important class of five-membered heterocyclic compounds. It is also known as 1, 2 Diazole. Pyrazoles are widely found as the core structure in a large variety of compounds that possess important agrochemical and pharmaceutical activities. Pyrazoles have been the recent target of numerous methodologies, mostly due to their prevalence as scaffolds in the synthesis of bioactive compounds and reactions in different media. Pyrazoles are colorless solids, soluble in water.



Chemical formula: C3H4N2

Molecular weight: 68.07 g/mol

Boiling point: 186 to 188 °C

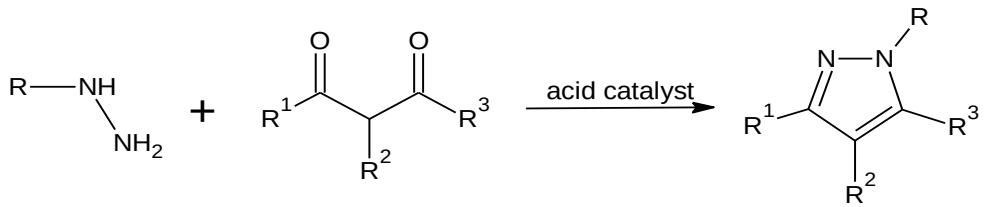
Melting point: 66 to 70 °C

METHODS OF PREPARATION

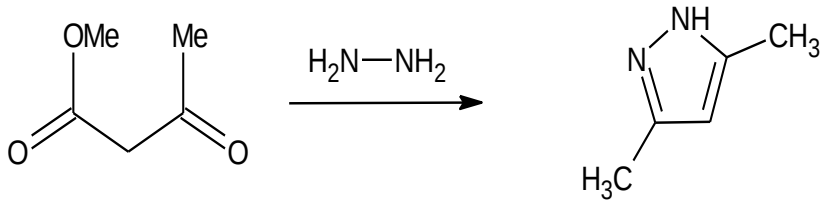
▪ **KNORR PYRAZOLE SYNTHESIS (**from dicarbonyl compounds)

The Knorr pyrazole synthesis is an organic reaction used to convert a hydrazine or its derivatives and a 1, 3- dicarbonyl compound to a pyrazole using an acid catalyst.

A simple pyrazole is obtained with 1, 3 dicarbonyl compounds such as acetylacetone treatment with hydrazine or phenylhydrazine.

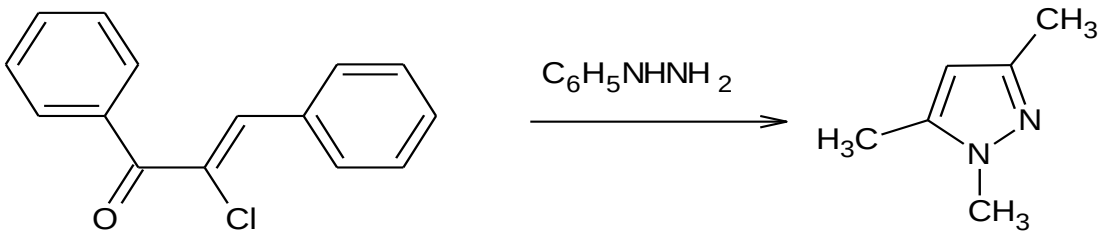


R – H, Alkyl, Aryl, Acyl.



- **FROM α, β -ETHYLENE CARBONYL COMPOUNDS**

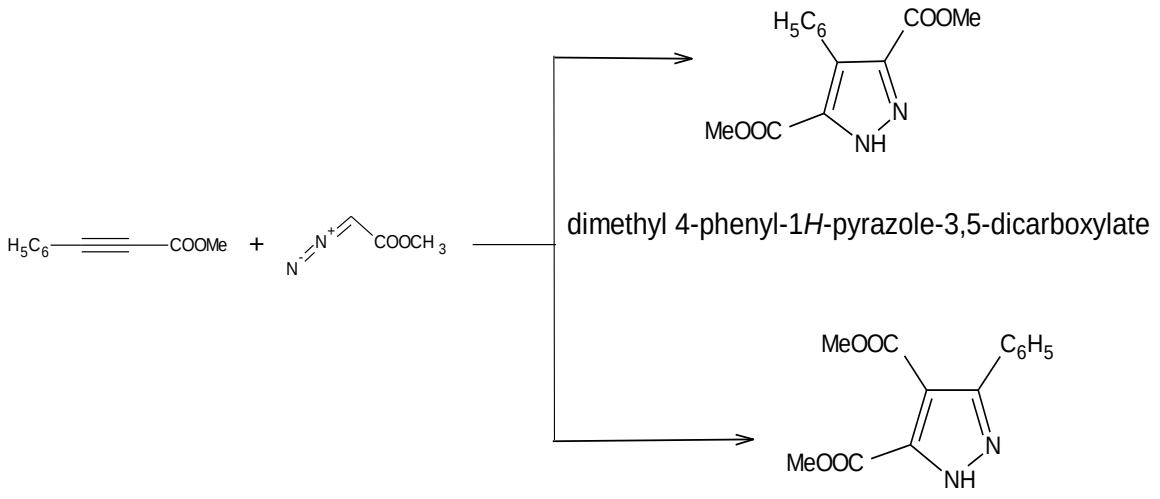
An important method consists of a reaction between a, ß ethylene carbonyl derivative and hydrazine. The former must contain an easily replaceable group at the 2 or ß position.



1,3,5 - triphenyl pyrazole

- **FROM 1, 3 DIPOLAR ADDITION**

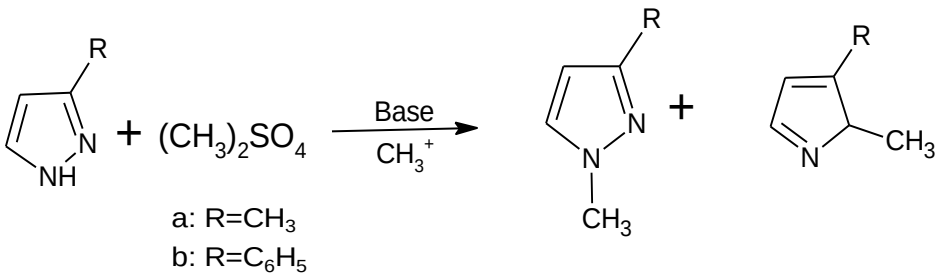
A diazo compound adds to an acetylenic derivative which has its triple bond activated by an electron-withdrawing substituent. The reaction is usually carried out in a suitable solvent at room temperature. Diazomethane or methyl or ethyl diazoacetate are commonly employed. Thus methyl diazoacetate and phenylpropionate yield the following isomeric pyrazoles in equal amounts. The reaction between an allene and diazomethane yields 3-methyl pyrazole.



CHEMICAL REACTIONS

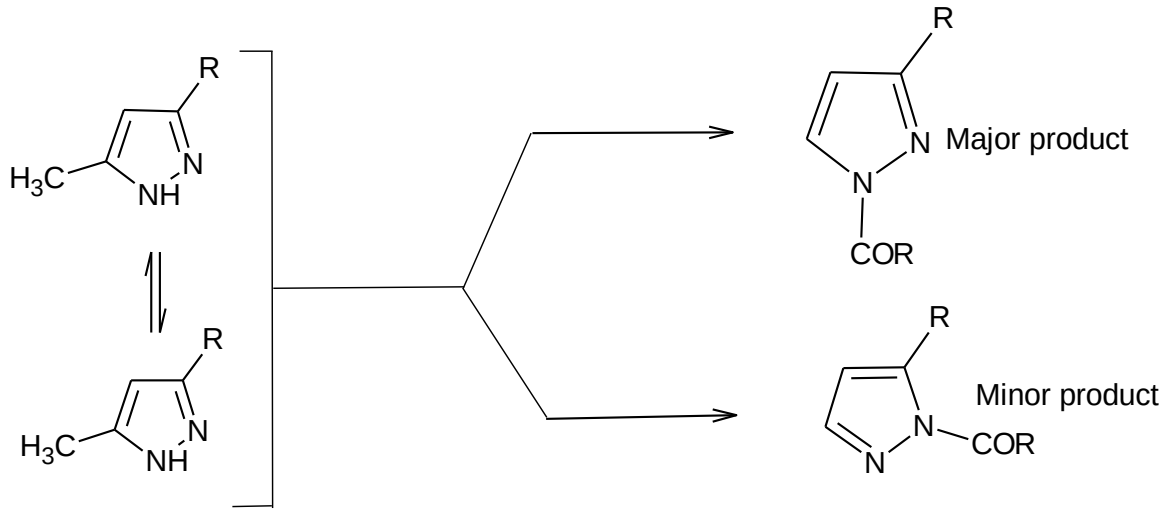
- **N - Alkylation**

The alkylation of the free NH group of pyrazole proceeds with alkylating agents such as alkyl halides, diazomethane, or dimethyl sulphate, substituted pyrazoles undergo alkylation to give a mixture of two isomeric products. Excess of alkylating agent causes quarterisation



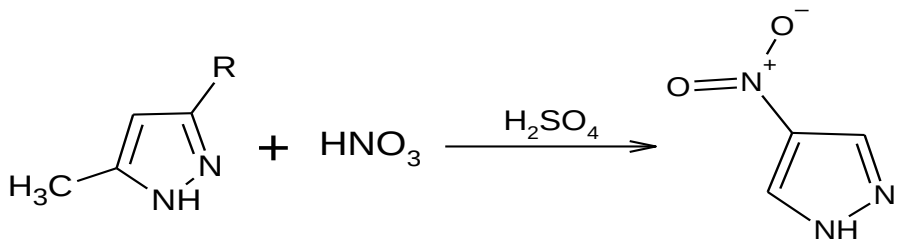
- **N – Acylation**

Pyrazole with free N-H group undergoes acylation when treated with acetyl chloride (alone in the presence of pyridine) or acetic anhydride.

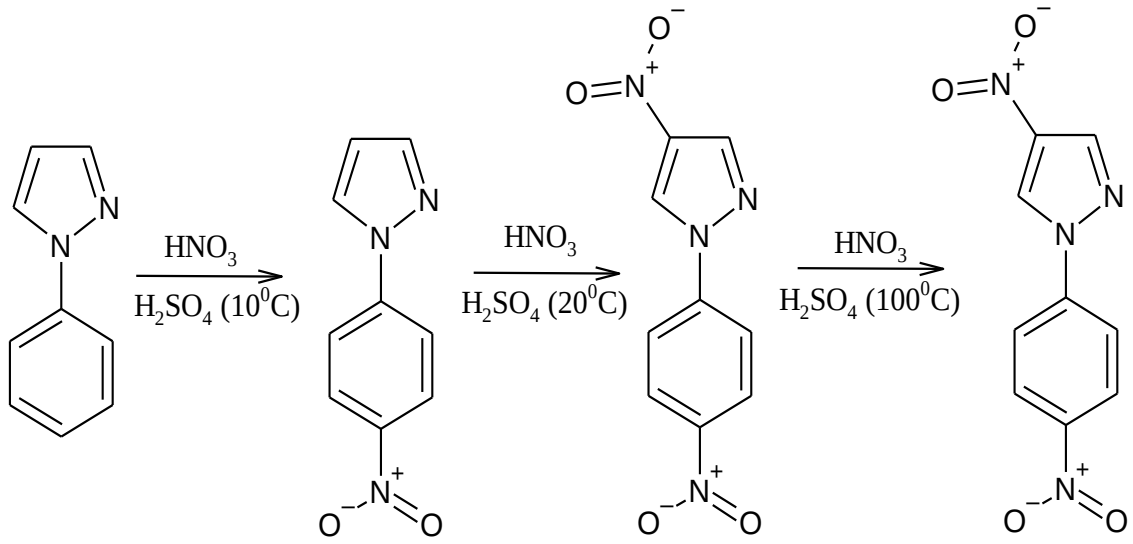


- **Nitration**

Nitration of pyrazole with a nitrating mixture of concentrated nitric acid and sulphuric acids occurred at position-4.

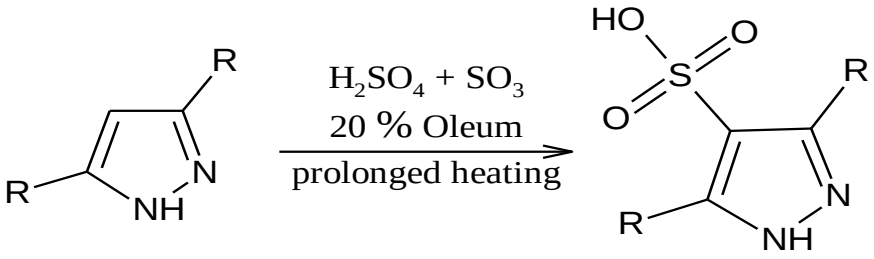


If the pyrazole is substituted with a phenyl group at position-1, it competes with the pyrazole ring and nitration occurs at para-position.



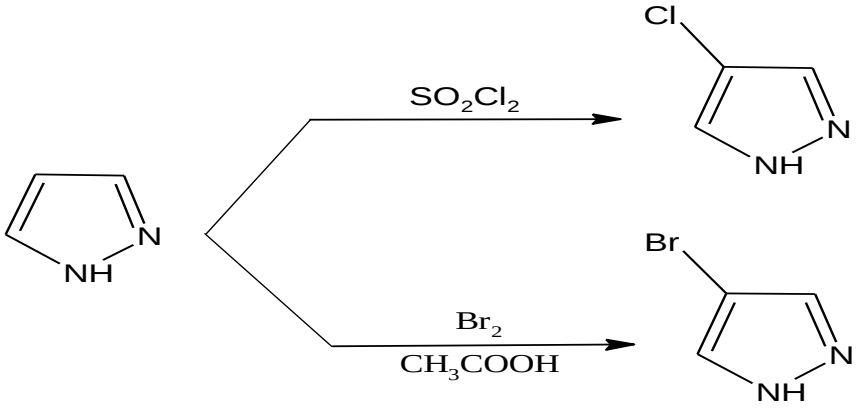
- **Sulphonation**

Pyrazole undergoes sulphonation only under various reaction conditions with the introduction of the sulfonic acid group the position- 4.



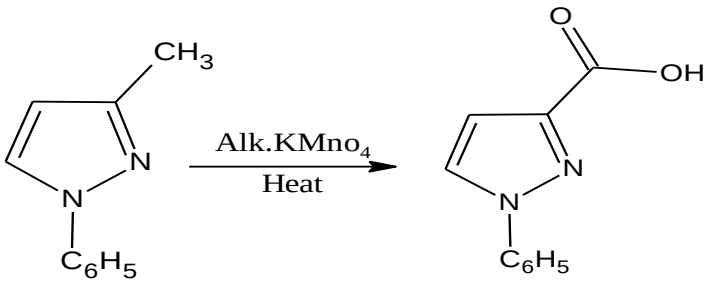
- **Halogenation**

Halogenation of pyrazole usually occurs at position-4. Pyrazole can be chlorinated by chlorinating reagents such as chlorine water, chlorine in carbon, and chlorine in acetic acid. Pyrazole is brominated by bromine in chloroform and bromine in acetic acid.

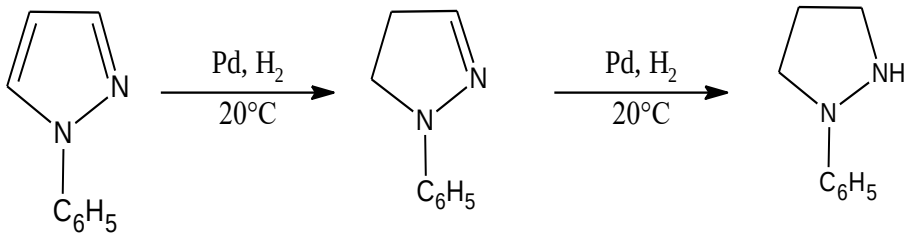


- **Reaction with oxidizing and reducing agents**

The pyrazole ring is remarkably stable to the action of oxidizing agents but the side chain may be oxidized to the carboxylic function. The oxidation proceeds well in the presence of alkaline potassium permanganate. Pyrazole and its derivatives have been reduced under a variety of conditions. Thus with Na/C_2H_5OH, 2- pyrazoline in obtained.



The catalytic reduction of 1- phenyl pyrazole yields both phenyl pyrazoline and 1- phenyl pyrazoline.

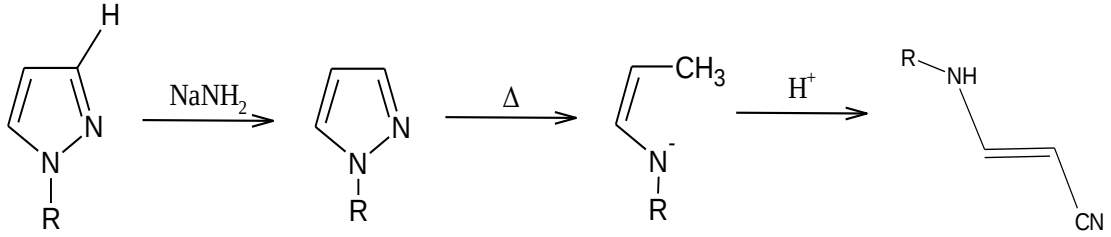


- **Nucleophilic reactions**

Pyrazole is reactive, but the electron-withdrawing substituent attached at α - to the halogen atom makes it reactive towards nucleophilic substitutions. Pyrazoles exist partly as anions and thus react with electrophiles as phenols and undergo diazo coupling, nitrosation, and Mannich reaction.

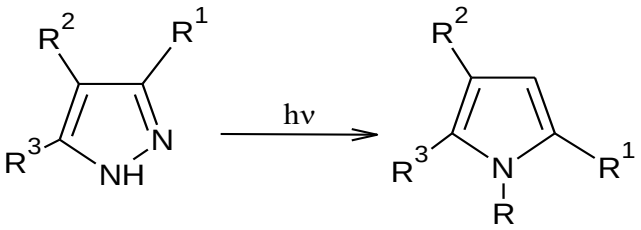
- **Ring cleavage via deprotonation**

Pyrazole ring unsubstituted at position – 3 is cleaved by a strong base ($NaNH_2$) via deprotonation at C-3.



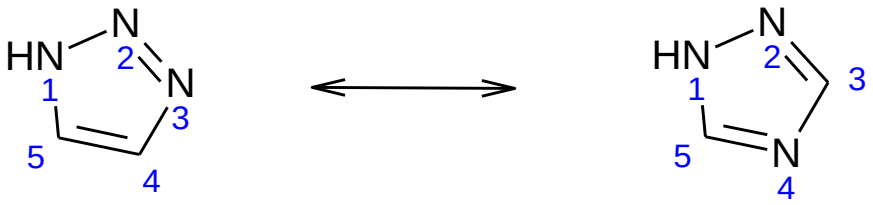
- **Photochemical reaction**

Pyrazole photochemically transformed into imidazole's involving the exchange of positions N-2 and C-3 of pyrazole with the positions C-2 and N-3 of imidazole.



TRIAZOLES [76-79]

Triazoles are an important class of heterocyclic compounds exhibiting a wide range of pharmacological activities. It is also known as pyrrodiazoles, and is a five-membered, di unsaturated ring system containing three nitrogen atoms in a heterocyclic core and occurs in two possible isomeric forms 1,2,3 triazoles and 1,2,4 triazoles. Triazoles are white to pale yellow crystals soluble in water and alcohol.



Chemical formula: C2H2N3

Molecular weight: 69.06 g/mol

Boiling point: 260 °C0

Melting point: 120 to 121°C

Structure and properties

- **Aromaticity and stability**

Aromaticity is the main reason for the stability of the triazole nucleus. An aromatic sextet is formed by the donation of one π electron from each atom connected by double bonds, in addition to the remaining two electrons from a nitrogen atom. Also, the triazole nucleus is stabilized by the resonance that it can be represented by tautomeric forms.

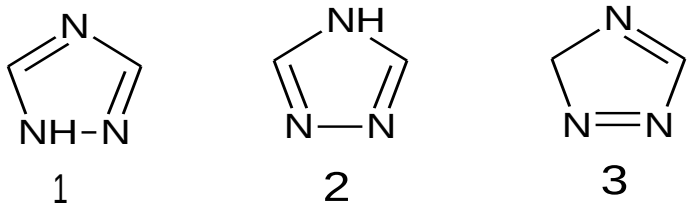
- **Tautomerism in triazoles**

Tautomerism is possible in both the structural isomers of triazole.

1, 2, 4- TRIAZOLE

1, 2, 4 – Triazole is one of the pairs of isomeric chemical compounds with molecular formula $C_2H_3N_3$ called triazoles, which have a five-membered ring of two carbon atoms and three nitrogen atoms. 1, 2, 4 – Triazole derivatives find use in a wide variety of applications, most notably as antifungals such as fluconazole and itraconazole. It can be arranged in two combinations to give

either 1, 2, 3 – triazole or 1, 2, 4 – triazole. Although two NH (1 and 2) and one CH2 (3) tautomeric forms are possible for 1, 2, 4- triazole, this structure is best represented as a positively charged hydrogen associated with the resonance stabilized triazole anion. 3- Substituted and 3, 5 - disubstituted 1, 2, 4- triazoles are usually indexed as s- triazoles and 1, 2, 3- triazoles as v- triazoles.

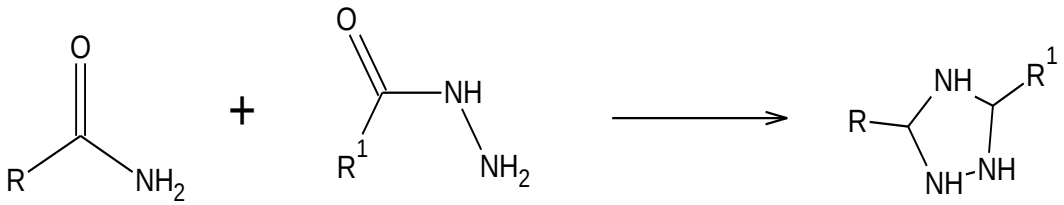


The 1, 2, 3 -1H triazole notation is used to describe a 1 N – substituted triazole, whereas 1, 2, 4 – 4H – triazole is used to describe a 4N – substituted triazole.

METHODS OF PREPARATION

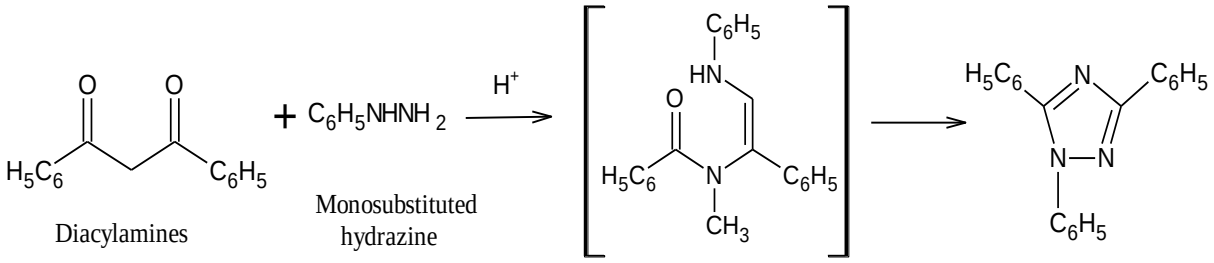
- **Pellizzari Reaction**

The pellizzari reaction is the chemical reaction of an amide and a hydrazide to form a 1, 2, 4-triazole.



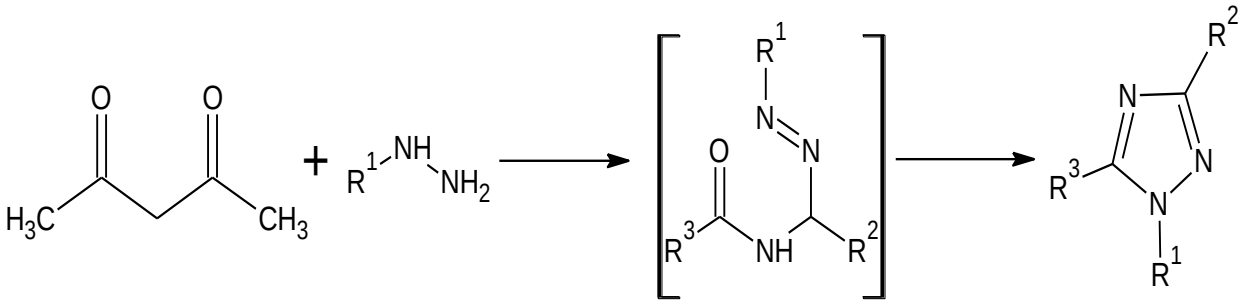
- **Einhorn – Brunner Reaction**

This reaction involves the condensation of diacylamines with monosubstituted hydrazine in presence of weak acid and proceeds via an amidrazone intermediate.



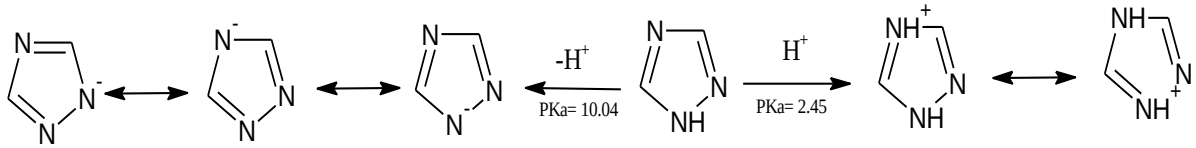
- **From Hydrazine Derivatives**

Synthesis of 1, 2, 4 – triazoles involves the use of Hydrazine



CHEMICAL REACTIONS

1, 2, 4 – triazole is slightly acidic (pKa = 10.04 for proton loss). The basicity of 1, 2, 4 – triazole is attributed to the mesomeric stabilization of imidazolium type cation formed on protonation, the cation is more stable.

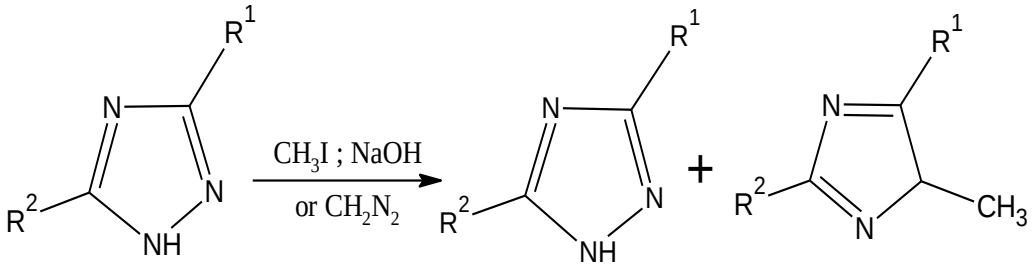


- **Reaction with Electrophile**

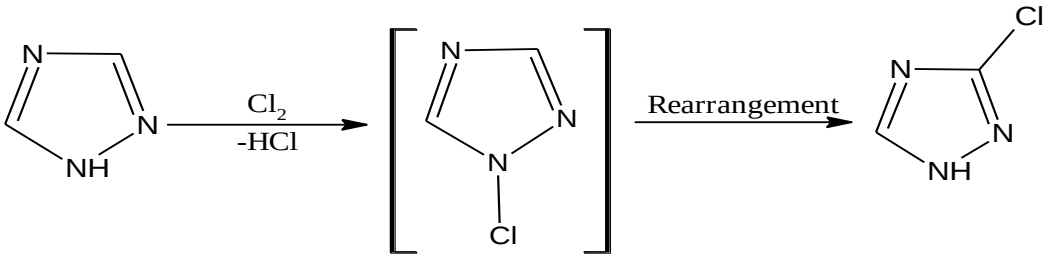
1, 2, 4 – triazole nearly unreactive towards electrophile cause fail to undergo sulphonation, nitration, and N – oxidation. But it undergoes alkylation and acylation very readily.

- **Electrophilic attack at nitrogen**

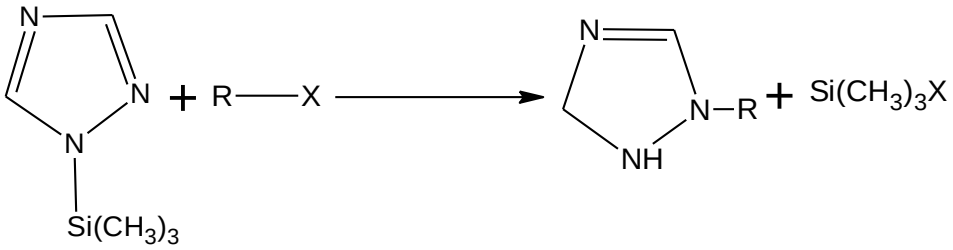
Alkylation of N - substituted 1, 2, and 4 – triazoles generally occur at N-1 rather than at N-4. If there is any choice of alkylation between N-1 and N-2 due to the nature of substituted at positions -3 and -5, the alkylation occurs in both the positions (N-1 and N-2).



The alkylation of 3- halo-1, 2, 4-triazoles with dimethyl sulphate in the absence of a base occurs at N-1, N-2, and N-4.

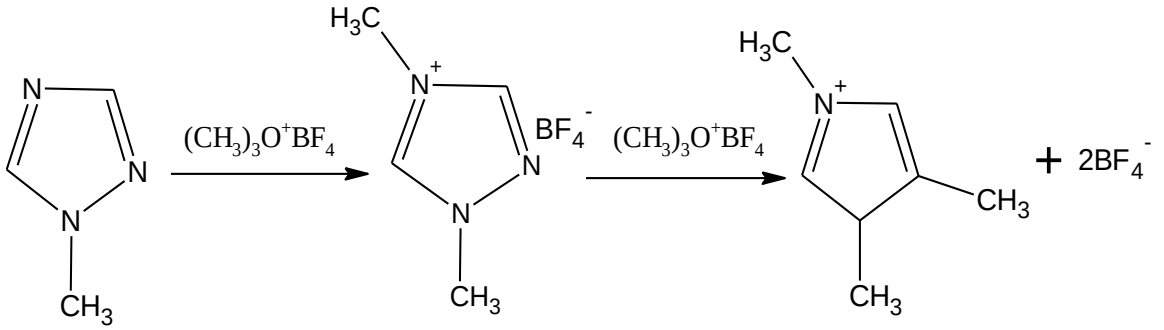


If the trimethylsilyl group is present at N-1, the alkylation occurs selectively at N-2 with the removal of the trimethylsilyl group.



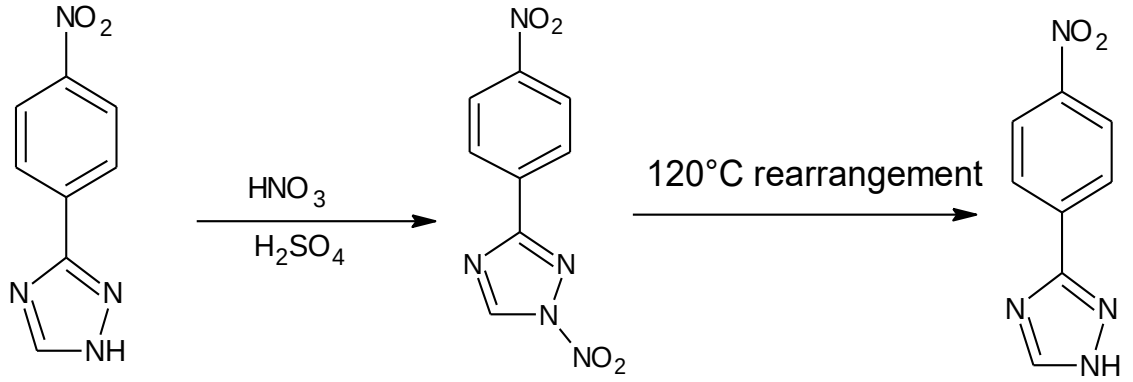
- **Quaternization**

1, 2, 4 – triazoles substituted with alkyl, aryl, or acyl substituents on N-1 or N-4 undergo quaternization when treated with powerful quaternizing agents i.e., trialkyloxonium tetrafluoro borates.

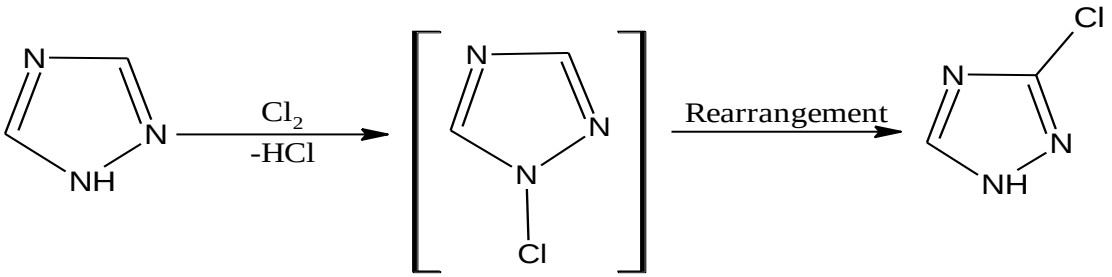


- **Electrophilic attack at carbon**

1, 2, 4 – triazole and its C- monoalkyl derivatives fail to undergo nitration. If 1, 2, 4 – triazole is substituted with an aryl group on carbon, nitration occurs in the benzene ring. But further nitration occurs to give C- nitro derivative of triazole.

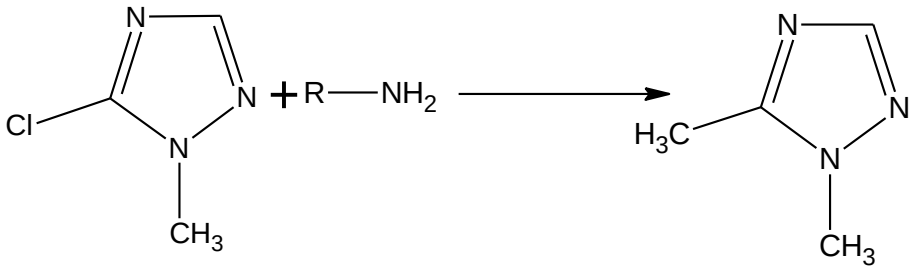


Halogenation of 1, 2, 4- triazole is considered to proceed via N- halo 1, 2, and 4 with the formation of 3- halo – 1, 2, 4- triazole.



- **Reaction with nucleophile:**

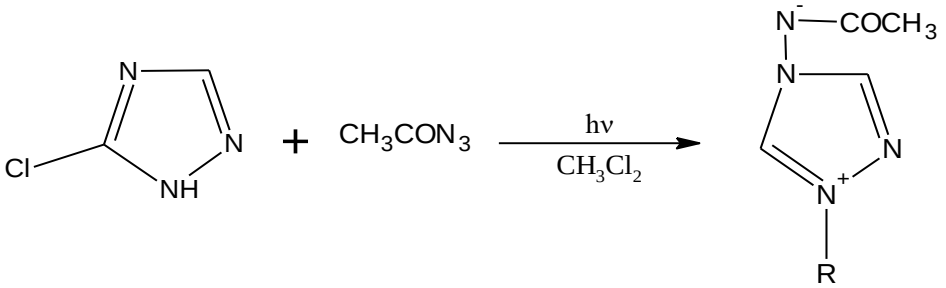
1, 2, 4- Triazoles substituted with halo group at position 3 and undergo nucleophilic substitution reaction.



- **Reaction with electron–deficient species:**

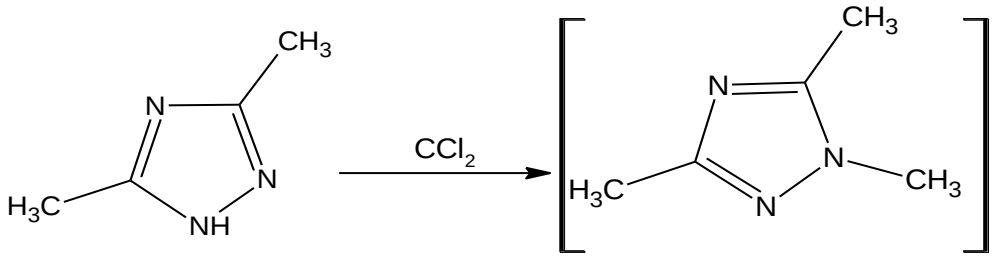
- **Reaction with nitrines:**

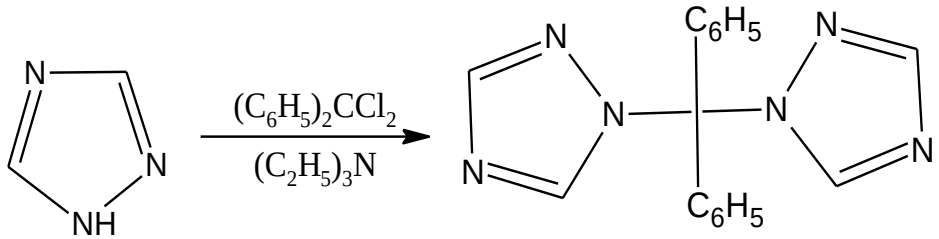
The reaction of 1-alkyl – 1, 2, 4 – triazoles with nitrines, generated by irradiation of azides, results in the formation of N- imines.



- **Reaction with carbenes:**

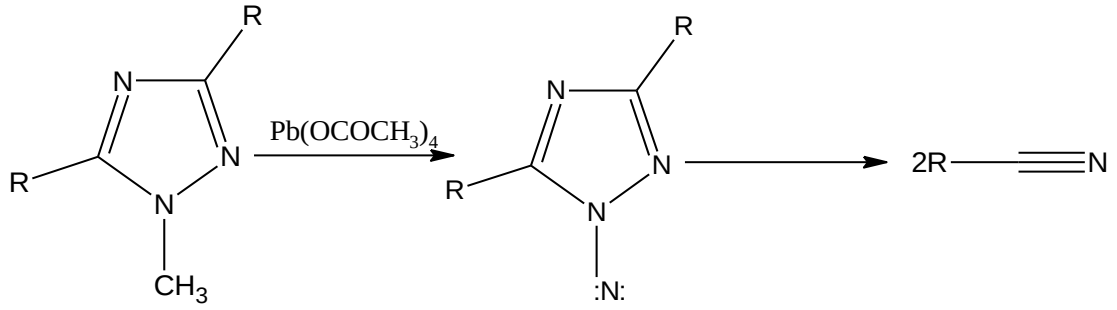
The reaction of 1, 2, 4 – triazoles with dichloro carbine does not proceed with the ring expansion as in pyrrole and imidazole, but result in the formation of bis- or tri- 1, 2, 4- triazoles.





- **Oxidation**

1, 2, 4 – triazole is resistant to oxidation, but N-amino triazoles undergo oxidative fragmentation via nitrenes intermediate when treated with lead tetraacetate. The formation of nitrene intermediate from N-amino triazole causes destabilization of the triazole ring.



- **Tautomerism**

  The tautomerism of 1, 2, 4-triazoles may involve one or more of the following possibilities.

  - Annular phototrophy
  - Tautomerism restricted to the substituents
  - Annular tautomerism involves the moment of a proton between two angular nitrogen atoms. Angular form of "s" triazoles.

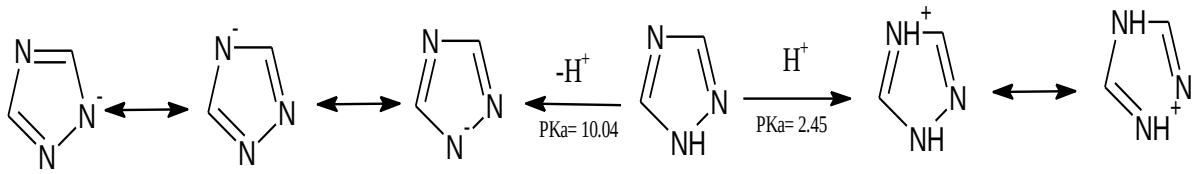
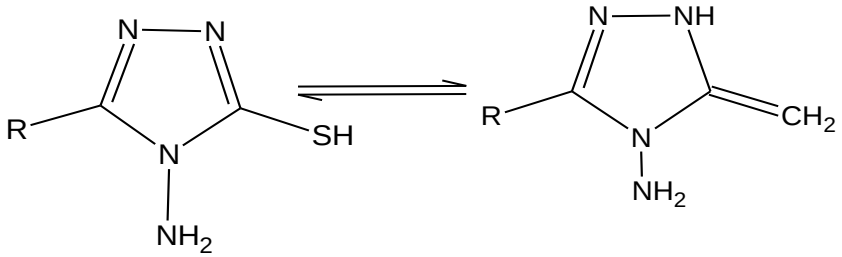


Photo trophy in triazoles is particularly complex when substituents such as –OH, -SH, -NHR are available to donate protons to angular nitrogen, for example,



Here, the SH group donates a proton to angular nitrogen. Hence 5-Mercapto – 1, 2, 4- triazole are found to undergo thiol-thione tautomerism.

PURPOSE OF THE STUDY

PURPOSE OF THE STUDY

Drug design is an important tool in the field of medicinal chemistry wherein the synthesis of new medicinal compounds is done by molecular or chemical manipulation of the lead moiety in order to produce a highly active compound with minimum steric effect. New drugs discovery may be considered broadly in terms of two kinds of investigational activities, "Exploration" and “Exploitation” of the leads. In this, the former involves the search for new ligands and the latter the assessment, improvement, and extension of the lead. The sole objective of these alternations is to improve efficacy, potency and to minimize or remove untoward side effects

Tuberculosis (TB) is the most common cause of death from infectious disease worldwide, which affects mainly the poorest countries of the world. Novel anti-tubercular drugs are urgently needed because TB remains a global health priority

The present work is to design a drug in such a way that it can be used clinically to treat diseases. Recent literature shows that the search for new drugs is now focused on the enzyme targets which can play a crucial role in the etiology of the disease.

The current research is aimed at the design of enzyme inhibitors for the drug discovery. Literature review suggests that the azole-based drugs inhibit the enzyme, CYP 51 (sterol 14α-demethylase). From literature and virtual screening techniques, azoles like pyrazoles, triazoles, thiazoles, thiadiazole, imidazoles, etc. were found to possess promising anti-tubercular activity by inhibiting the enzyme Mtb sterol 14α-demethylase in Mycobacterium species. Linkers like Schiff base (imine), hydrazones, amides, mannich bases were also reported to enhance the activity profile.

Research on pyrazoles, as anti-tubercular agents, is in pipeline and is found to be very active in anti-tubercular drug discovery and many of them have been reported recently. This helped the researchers to design new pyrazole-based anti-tubercular agents having linkers. It was also found that pyrazole derivatives are potential in inhibiting CYP 51 (sterol 14α- demethylase). Triazoles are also identified as potential CYP 51 inhibitors. The significance of triazoles as antifungal, anti-tubercular agents have been reported and the inhibition of CYP 51 is gaining importance in the anti-tubercular drug discovery.

The purpose of the present work was to design and synthesize new triazolyl imino pyrazole derivatives to explore the extent of their anti-tubercular activity. The compounds were designed by the *in-silico* method using *Mycobacterium tuberculosis*-CYP51 as the target molecule.

PLAN OF WORK

PLAN OF WORK

The present work has been carried out under the following sections.

Phase I - *In-silico* Methods

Phase II - Synthetic Studies

Phase III - Spectral Characterization

Phase IV - Anti Mycobacterial Screening

PHASE I: *IN-SILICO* METHODS

Step I - Selection of drug target as sterol 14α demethylase (CYP-51)

Step II - Hit selection through virtual screening using iGEMDOCK v.2

Step III - Lead Optimization using SWISSadme and Molinspiration server

Step IV - Docking of the lead molecules with target enzymes using Autodock 4.2

PHASE II: SYNTHESIS OF PYRAZOLYL IMINO TRIAZOLE DERIVATIVES

Scheme I – Synthesis of triazole amine and its derivatives

Scheme II – Synthesis of pyrazole aldehyde

Scheme III – Synthesis of six triazolyl imino pyrazole derivatives

PHASE III: SPECTRAL CHARACTERIZATION

Spectral characterization of synthesized compounds was done using UV, IR, proton NMR, and Mass spectroscopy.

PHASE IV: ANTI MYCOBACTERIAL SCREENING

The anti-tubercular activity was evaluated for the synthesized compounds by the Microplate Alamar Blue assay method.

EXPERIMENTAL WORK

EXPERIMENTAL WORK

PHASE I – *IN-SILICO* METHODS

Software and databases used

- iGEMDOCK v.2
- ZINC database
- AutoDock 4.2
- Python
- Cygwin
- Mgltools 1.5.6
- Discovery studio visualizer
- Molinspiration server
- Swiss ADME
- Pre ADMET
- RCSB protein data bank
- Online smiles translator

All the *in-silico* experiments are carried out in the Department of Pharmaceutical Chemistry, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore.

DRUG TARGET SELECTION

Database used

- RCSB protein data bank
    - Sterol 14α -demethylase of *Mycobacterium tuberculosis* (Mtb) was selected as the drug target from the literature data. The corresponding enzyme was obtained from the RCSB protein data bank and their accession code is **IEA1**.

Table 1: Enzyme description as in Protein Data Bank

NAME	CRYSTAL STRUCTURE	PDB ID	RESOLUTION
Sterol 14α -demethylase		**1EA1**	**2.21Å**

VIRTUAL SCREENING

Virtual screening is a computational technique used in drug discovery to search libraries of small molecules to identify those structures which are most likely to bind to a drug target, typically a protein receptor or enzyme. It is generally used for the selection of the lead from hits.

Procedure:

Virtual screening is done by using iGEMDOCKv.2.

- Using ZINC, the free database of around thirteen million commercially available compounds, a small molecule library consisting of 50 compounds was constructed. The protein with the

accession code 1EA1 corresponding to 14α Sterol-demethylase of *mycobacterium tuberculosis* was selected from the RCSB protein data bank.

- The protein was uploaded in the iGEMDOCK v.2 & the binding site was chosen.
- Similarly, ligands were uploaded in the iGEMDOCK v.2
- Click start virtual screening.
- After completion of the process, click view docked pose and post analyze.
- Save the fitness value and take a snapshot.

LEAD OPTIMIZATION

Lead optimization was done by evaluating the

- Drug likeliness potential
- ADME screening

Computation of Drug likeliness potential

The drug-likeness score will help to evaluate the better oral absorption of the ligands. The score includes solubility, diffusion, Log P, hydrogen bond acceptors, hydrogen bond donors, molecular weight, etc. One of the ideal methods for this is using Lipinski's rule of five with the molinspiration server.

Steps involved in molinspiration server (Lipinski's rule of five)

- Draw the structures in chemsketch and generate smiles notation (to generate smiles notation click tools → go to generate → smiles notation)
- Open the molinspiration home page.
- Click calculation of molecular properties and drug-likeness.
- Draw the structure of TP1-TP6 in the JME window or paste the smile notation of the compounds.
- Click the calculate properties module.
- The data is generated for the compounds.
- Take a snapshot of the properties.
- Save the properties.
- JAVA program is required in the computer for the calculation of the properties.

The calculation for the rest of the compounds is observed in the same manner.

***In-silico* ADME screening of Ligand**

The compounds selected as the lead should possess optimal absorption, distribution, metabolism, and excretion. The ADME studies performed by using SwissADME will help in the computational evaluation of the ADME scores.

Procedure

2D structures were directly introduced into SwissADME for carrying out ADME study by using **Edit chemistry module** of the software, then the structure is subjected to **Function module** and the data for descriptors like blood-brain barrier penetration (BBB), human intestinal absorption (HIA), plasma protein binding and aqueous solubility were calculated.

DOCKING STUDIES

From the virtual screening and literature review, Sterol 14α-demethylase (CYP-51) were selected as the drug target for the present study. X-ray crystallographic structure of CYP-51 has been selected from RCSB Protein Data Bank and the docking studies were performed with the AutoDock 4.2 version.

STEP I: SELECTION FROM PROTEIN DATA BANK (PDB)

Mycobacterium tuberculosis CYP-51(PDB accession code is 1EA1.pdb)

The corresponding enzyme was downloaded from RCSB Protein Data Bank for the docking studies were performed.

STEP II: PROTEIN STRUCTURE REFINEMENT

Protein (1EA1) downloaded from the protein data bank as such cannot be used for the docking process. It has to be refined before docking. Refinement of downloaded protein involves the removal of water and bound ligand if any.

Steps involved are

- Open Discovery studio visualizer
- File → open → Protein (downloaded from PDB)
- View → Hierarchy.
- Click water molecule.

- Ctrl + shift and click the last water molecule (select all the water molecules).
- Give right click and cut.
- Select the ligand, which is unnecessary, Give a right click and cut.
- Save the molecule in our desired area.

Proteins were refined by the above method.

STEP III: LIGAND FILE FORMAT CONVERSION

The ligands TP1-TP6 were drawn in Chemsketch.

- Tools → Generate → SMILES notation (Simplified Molecular Input Line Entry System, which is a file format)
- Save the SMILES in a word document.
- Open the online smile translator – catus.nci.nih.gov/services/translate/.
- Upload the SMILES.
- By choosing the required file format we can save the file. Here, we are saving it as PDB format in Cygwin/usr/local/bin.

Online smile translator allows the user to convert SMILES format into PDB, MOL, SDF, and smile text file format. Thus the selected ligand molecule of canonical smile formats was converted to PDB formats. The protein and ligand files which are prepared by above said procedures were taken for docking.

STEP IV: DOCKING

Docking was performed using Auto Dock and requires a refined protein and the ligand in PDB format and files like autogrid4 and autodock4.

The docking process is done with AutoDock 4.2 Steps involved are

Saving and Refining the Enzyme

Open autodock – file – read molecule – open from saved location (bin) – click on enzyme – open

Select – Select from string – atom – type *HOH*- add – no changes – ok – dismiss

Edit – hydrogens – add – polar only – ok

Edit – charges – add Kollman charges – ok

File – save – write PDB – browse – save in location (bin) – replace old enzyme – ok – overwrite – ok

Edit – delete – delete all molecules – continue

Saving and Refining the Ligand

Click ligand – input – open (from the bin) – add gastiger charges – ok

Ligand – torsion tree – detect the root

Ligand – torsion tree – show root expansion

Ligand – torsion tree – choose torsions – done

Ligand – torsion tree – set no/ of torsions – dismiss

Ligand – torsion tree – hide root expansion

Ligand – torsion tree – show/hide root markers

Ligand – output – save as .pdbqt – saved in location (bin)

Edit – delete – delete all molecules – done

Grid – macromolecule (enzyme) – open from location (bin) – ok – save as .pdbqt file in location (bin)

Grid – set map types – open ligand – open from location (bin)

Grid – grid box – X- 60, Y-60, Z-60 – file – close saving current

Grid – output – save as .gpf file – in location (bin)

Edit – delete - delete all molecules - continue

Docking – macromolecule – set rigid file name – open from a location – click enzyme – (nothing will be seen on-screen full blank only)

Docking – ligand – open from location (bin) – accept (default)

Docking – search parameters – a genetic algorithm – accept (default)

Docking – docking parameters – accept (default)

Docking – output Lamarckian GA – file name .dpf – save in location (bin)

Comments

Open cygwin or cygwin terminal

cd c: - enter

cd cygwin or cygwin 64(laptop) - enter

cd usr - enter

cd local - enter

cd bin - enter

ls - enter

autogrid4.exe -p filename.gpf -l filename.glg - enter

Check the bin for the glg file

autodock4.exe -p filename.dpf -l file name.dlg - enter

It takes a long time, so check bin anCygwinin at an interval of time

After successful completion of the .dlg file

Auto dock – edit – delete – delete all molecules- continue

Analyze the Result

Analyze – docking – open - .dlg file from bin

Analyze – confirmations – play (don't close place at side)

Analyze – confirmations – load – click the first one

Analyze – docking – show interaction

Analyze – macro molecule – open from the bin (enzyme)

Analyse – docking – show interaction – don't close place at side – unclick display msms

On atoms unclick close contact

Set backgrounds

Ligand colouring

File – save – save as image – saved in a location (bin) – ok

Docking studies for all the ligands TP1-TP6 and standard, Fluconazole were carried out in the same manner.

PHASE II – SYNTHETIC STUDIES

Based on the results obtained from the docking results and the availability of the chemicals, compounds having a good docking score and good ligand interaction were synthesized using conventional synthetic methods.

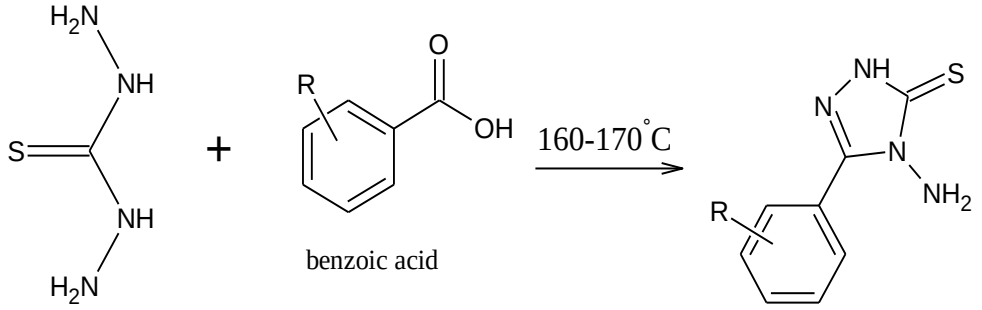
Chemicals and Reagents used:

- Thiocarbohydrazide, Benzoic acid, 4-Chloro benzoic acid, 4-Fluoro benzoic acid, 4-Nitro benzoic acid, 2-Chloro 5-Nitro benzoic acid, 3,5-Dinitro benzoic acid, and Liq. paraffin.
- Phenyl hydrazine, Ethyl acetoacetate, Acetic acid, Ether, Phosphorus oxychloride, Dimethylformamide, Glacial acetic acid, and Ethanol.

All the reagents and chemicals were procured from Sigma Aldrich, Loba Chem, and Sd fine chemicals. The procured compounds were used for the synthetic procedure.

SYNTHETIC SCHEME

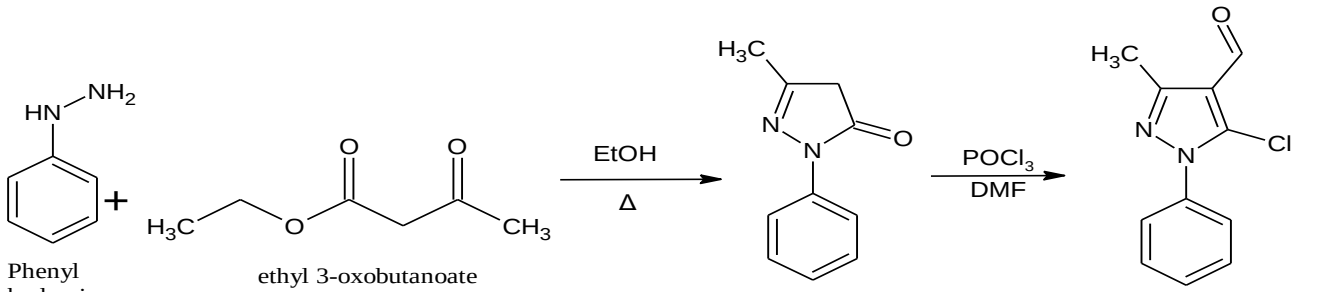
SCHEME – 1



Thiocarbohydrazide

4-amino-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione

SCHEME – 2



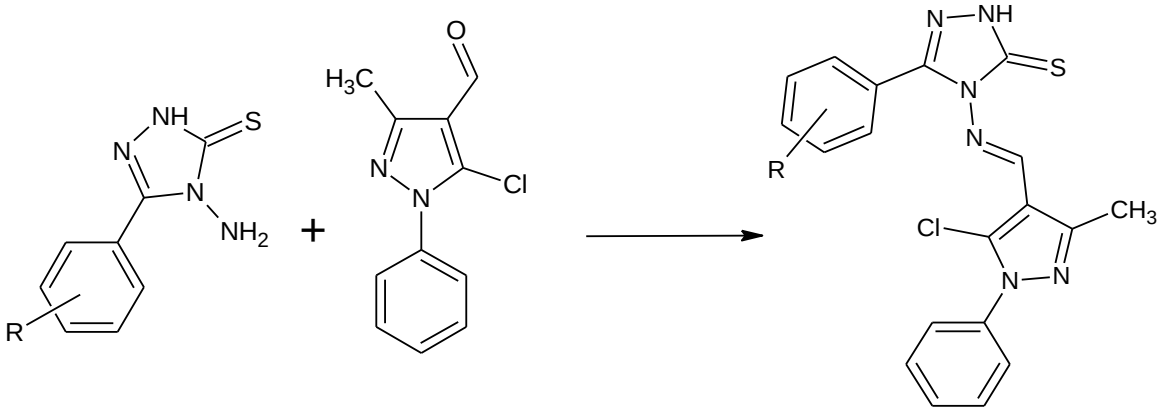
Phenyl hydrazine

ethyl 3-oxobutanoate

5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-one

5-chloro-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde

SCHEME – 3



4-{(*E*)-[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methylidene] amino}-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione

COMPOUND	R
TP1	2-Cl,5-NO_2
TP2	4-F
TP3	3,5-$(NO_2)_2$
TP4	4-Cl
TP5	4-NO_2
TP6	H

PROCEDURE:

SCHEME 1 - SYNTHESIS OF TRIAZOLE AMINE AND ITS DERIVATIVES

A mixture of thiocarbohydrazide (0.1 mol) and benzoic acid derivatives (0.1 mol) was heated in an oil bath at 160-170^{0}C for 2hrs. The fused mass thus obtained was dispersed with hot water to obtain the triazole. The product was recrystallized from methanol.

SCHEME 2 - SYNTHESIS OF PYRAZOLE ALDEHYDE

I) 5-Methyl-2-phenyl-2,4-dihydro-3H-pyrazole-3-one

A mixture of phenylhydrazine (0.01 mol) and ethyl acetoacetate (0.01 mol) with a few drops of acetic acid was refluxed until the orange color solution becomes a thick liquid. The reactant mixture was allowed to cool at room temperature and 15 ml of ether was added to it. The yellow color precipitate was filtered, dried, and recrystallized from ethanol.

II)5-Chloro-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde

Phosphorus oxychloride (13 ml) was taken in a 250 ml round bottom flask, cooled at 0-5 ^{0}C and dimethylformamide (6 ml) was added dropwise. The reaction mixture was allowed to stir at the same temperature for 10-15 mins and (5-methyl-2-phenyl-2, 4-dihydro-3H-pyrazole-3-one) was added to it. The reaction mixture was allowed to stir at room temperature and heated at reflux temperature for 5-6 hrs. The progress of the reaction was monitored at TLC. After completion of the reaction, the mass was poured into cold water and filtered off, washed with cold water, and dried under reduced pressure at 80-90 ^{0}C to obtain pure product.

SCHEME 3 - SYNTHESIS OF TRIAZOLYL IMINO PYRAZOLE DERIVATIVES

Triazole amine derivatives (0.005 mol) were dissolved in 30 ml of ethanol and 0.9 ml of glacial acetic acid was added. Pyrazole aldehyde (0.005 mol) was added to the above mixture and was refluxed for 6 hrs. After cooling, the mixture was added to crushed ice by stirring. The separated product was filtered, washed, dried, and recrystallized from ethanol.

CHARACTERIZATION

INSTRUMENTS:

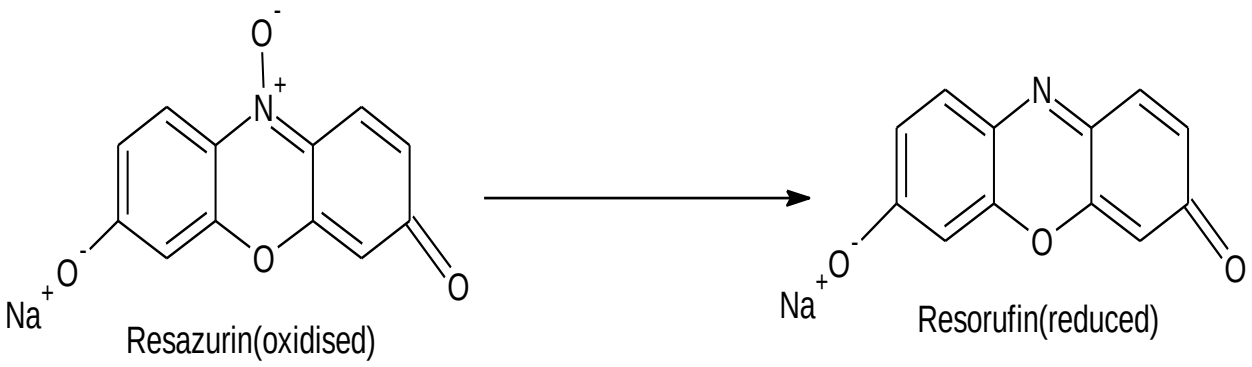
- Mechanical stirrer (REMI Elektrotechnik Ltd) in the Department of Pharmaceutical Chemistry, College of Pharmacy, SRIPMS, Coimbatore.
- The melting point was determined using Visible Range melting point apparatus (LABINDIA) in the Department of Pharmaceutical Chemistry, College of Pharmacy, SRIPMS, Coimbatore.
- UV Spectra was recorded using (JASCO V 530) - UV Visible Spectrophotometer in the Department of Pharmaceutical Analysis, College of Pharmacy, SRIPMS, Coimbatore.
- IR Spectra was recorded using (SHIMADZU) - IR spectrophotometer in the Department of Pharmaceutical Analysis, College of Pharmacy, PSG Institutions, Coimbatore.
- ^{1}H-NMR Spectroscopy-(BRUKER) NMR 300MHz in Department of Applied Chemistry, Karunya Institute of Technology and Sciences, Coimbatore.
- Mass Spectroscopy in Interdisciplinary Institute of Indian System of Medicine, SRM Institute of Science and Technology, Kattankulathur, Chennai.

PHASE IV – ANTI MYCOBACTERIAL SCREENING

All the synthesized compounds were screened for anti-tubercular activity in Maratha Mandal's Central Research Laboratory, Belgaum, Karnataka.

MICROPLATE ALAMAR BLUE ASSAY (MABA) METHOD

Alamar Blue is a cell viability assay reagent that contains the cell-permeable, non-toxic, and weakly fluorescent blue indicator dye called resazurin. It is an oxidation-reduction indicator used for the screening of cell growth, particularly in various cell toxicity studies. The dye changes its color from blue to pink and becomes fluorescent when reduced to resorufin by oxidoreductases within viable cells.



Alamar Blue is used for the screening of anti-tubercular activity. Since Mycobacterium is an aerobic organism, its presence of growth turns Alamar Blue to pink color. Hence, the pink colour indicates the presence of growth (no anti-tubercular activity) and the blue colour indicates the absence of growth (inhibitory activity of agents tested).

Standard Strain used: ***Mycobacterium tuberculosis*** (Vaccine strain, H37 RV strain): ATCC No- 27294.

PROCEDURE

The anti-mycobacterial activity of compounds was assessed against *M. tuberculosis* using Microplate Alamar Blue Assay (MABA). This methodology is non-toxic, uses a thermally stable reagent, and shows a good correlation with proportional and BACTEC radiometric methods. To the outer perimeter of sterile 96 wells plates, 200µl of sterile deionized water was added to minimize

evaporation of medium in the test wells during incubation. The 96 wells plates received 100 µl of the Middlebrook 7H9 broth (inoculated with *Mycobacterium tuberculosis* of H37RV Strain) and serial dilution of compounds was made directly on the plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as the lowest drug concentration which prevented the color change from blue to pink.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

PHASE I

DRUG TARGET SELECTION

- The enzyme involved is ***Mycobacterium tuberculosis*** **CYP-51 (sterol 14α-demethylase).**
- The corresponding enzyme was obtained from the protein data bank and their accession code is **IEA1.**

VIRTUAL SCREENING

Among the 50 hits screened, PYRAZOLE and TRIAZOLE moieties were identified as the lead. The results are tabulated in the table and the snapshots of ligands binding with 1EA1.pdb are given in fig 5.

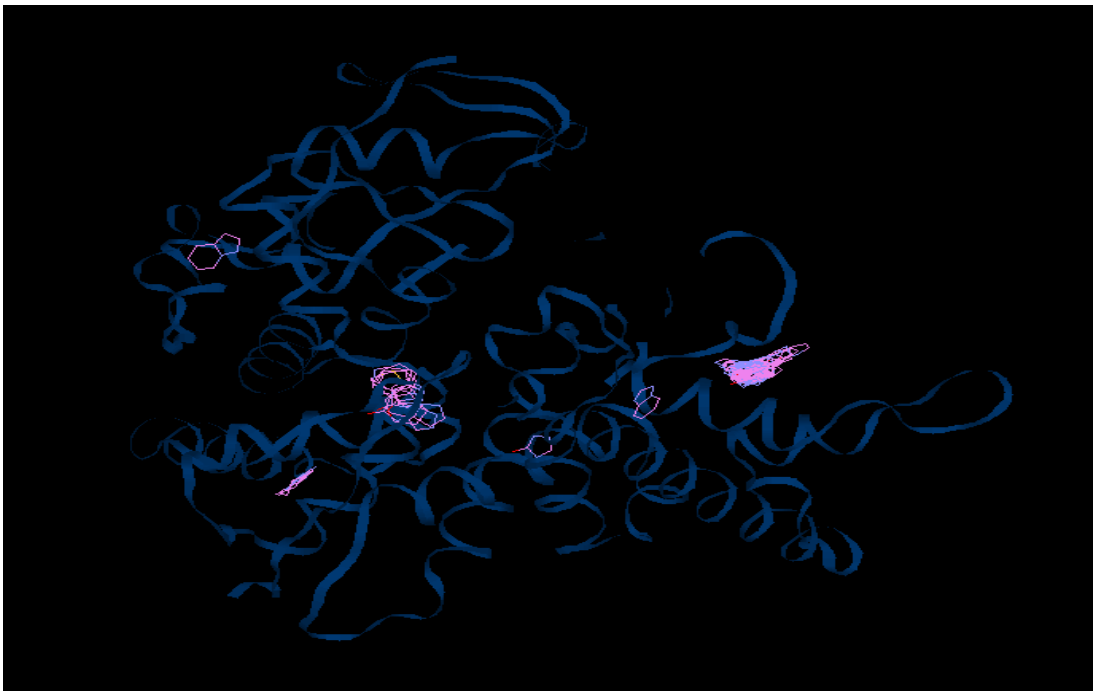
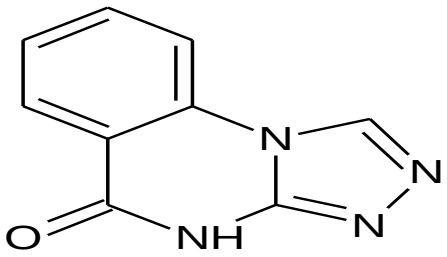


Fig 5: Binding of ligands with CYP-51 (1EA1.pdb)

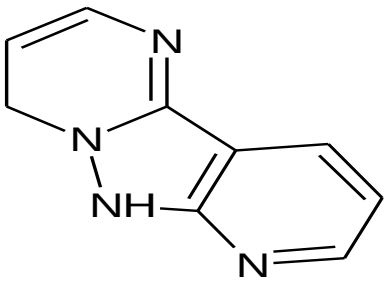
Table 2: Fitness value of 50 ZINC compounds

S.NO	COMPOUND CODE	FITNESS VALUE 1EA1
1	ZINC56381073	-52.0508
2	ZINC1048526	-69.5539
3	ZINC723967	-66.7783
4	ZINC450607	-64.637
5	ZINC427807	-77.9462
6	ZINC374341	-52.5766
7	ZINC238808	-60.1253
8	ZINC207112	-60.6482
9	ZINC117373	-65.7554
10	ZINC99745	-59.1563
11	ZINC93101	-63.3437
12	ZINC67854	-76.6326
13	ZINC54491	-72.6524
14	ZINC53166	-67.256
15	ZINC48003	-65.777
16	ZINC45892	-76.2098
17	ZINC45115	-60.7976
18	**ZINC38307**	**-78.3053**
19	ZINC25571	-71.6113
20	ZINC25485	-72.3509
21	ZINC23474	-74.2967
22	**ZINC21237**	**-82.9321**
23	**ZINC20794**	**-80.262**
24	ZINC20039	-66.3103
25	**ZINC16904**	**-86.2466**
26	ZINC20899397	-55.658
27	ZINC8149964	-59.0748

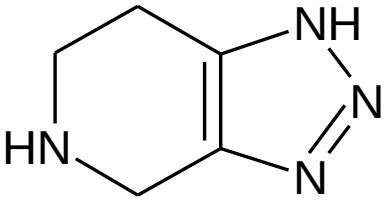
28	ZINC917344	-76.5168
29	ZINC902498	-63.1267
30	ZINC810480	-67.7082
31	ZINC542217	-69.2295
32	ZINC509609	-70.4308
33	ZINC418775	-69.2088
34	ZINC311343	-61.6063
35	ZINC307806	-61.5023
36	ZINC305135	-61.8331
37	ZINC217694	-73.0644
38	ZINC213153	-73.2246
39	ZINC196850	-61.7948
40	ZINC126372	-65.4068
41	**ZINC118099**	**-81.1849**
42	**ZINC106386**	**-79.2415**
43	ZINC95354	-75.0996
44	ZINC90553	-69.7196
45	**ZINC84848**	**-86.8796**
46	**ZINC77450**	**-83.067**
47	ZINC70672	-71.9183
48	**ZINC64695**	**-79.1584**
49	**ZINC58303**	**-79.0237**
50	ZINC44663	-72.2485



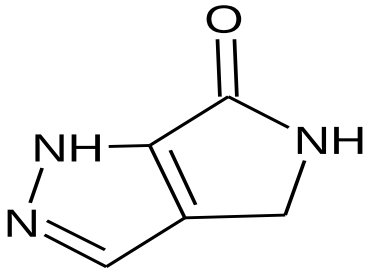
ZINC84848



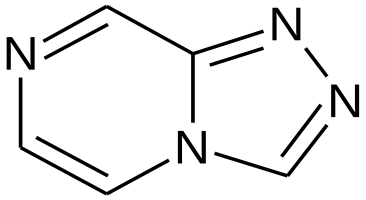
ZINC16904



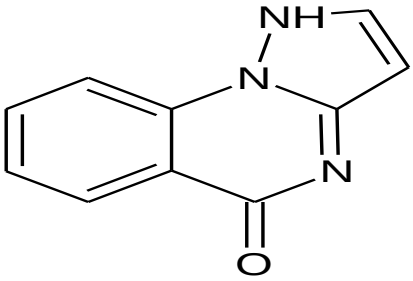
ZINC77450



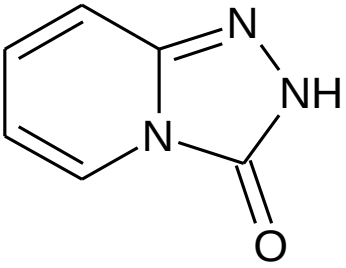
ZINC21237



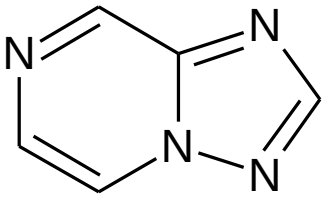
ZINC21237



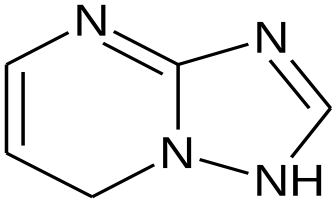
ZINC118099



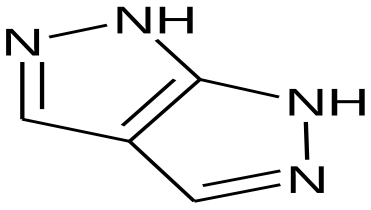
ZINC106386



ZINC64695



ZINC58303



ZINC38307

By analyzing the results, compounds containing **PYRAZOLE** and **TRIAZOLE (ZINC84848, ZINC16904, ZINC77450, ZINC21237, ZINC118099, ZINC20794, ZINC106386, ZINC64695, ZINC58303, and ZINC38307)** nucleus were found to have good fitness values. Therefore, based on the virtual screening performed in iGEMDOCK and the literature, **pyrazole-linked triazole** is taken as a lead for CYP-51 inhibitor in the present study.

LEAD OPTIMIZATION

COMPUTATION OF DRUG-LIKE PROPERTIES

The drug-likeness properties and oral bioavailability profile were analyzed by utilizing the Lipinski filter in the molinspiration server. The results are tabulated in Table 3.

Table 3: Drug likeliness scores of TP1-TP6 ligands

S.NO	COMP CODE	M log p	Mol wt	No of H acceptors	No of H donors	No of violations
01	**TP 1**	4.24	474.33	9	1	0
02	**TP 2**	3.83	412.88	6	1	0
03	**TP 3**	3.52	484.88	12	1	1
04	**TP 4**	4.35	429.34	6	1	0
05	**TP 5**	3.63	439.89	9	1	0
06	**TP 6**	3.67	394.89	6	1	0

The lead optimization of the ligands shows that none of the derivatives had any violations of the rule. Therefore it can be predicted that these compounds when administered orally will more likely have a good absorption or permeation. Most of them satisfy the rule; which indicates all the ligands TP1-TP6 have good absorption.

IN SILICO ADME STUDIES OF THE LIGAND

The ADME properties were analyzed by utilizing the SwissADME server. The results are tabulated in Table 4.

Table 4: ADME data of TP1-TP6

S. No	COMP CODE	TPSA	SOL LOGS	BBB	ALOGP 98	PPB	HIA	GI Abs	SKIN PER	CYP2D6 inhibitor	Solvation
1	**TP 1**	141.7	-6.16	0.10	5.87	90.78	99.00	Low	-2.56	Non inhibitor	-5.24
2	**TP 2**	95.88	-5.68	0.32	5.55	89.93	97.02	High	-2.89	Non inhibitor	-4.49
3	**TP 3**	187.52	-5.63	0.08	4.45	86.71	96.44	Low	-2.85	Non inhibitor	-9.64
4	**TP 4**	95.88	-6.11	0.30	5.97	90.90	97.30	High	-2.55	Non inhibitor	-2.15
5	**TP 5**	141.7	-5.57	0.52	5.20	89.58	98.98	Low	-2.65	Non inhibitor	-4.96
6	**TP 6**	95.88	-5.53	0.69	4.98	89.07	96.41	High	-2.75	Non inhibitor	-5.47

The six newly synthesized compounds exhibited optimum ADME values. The leads were found to have good oral absorption, intestinal absorption, solubility, and less interaction. Hence, the synthesized compounds may show good oral bioavailability.

DOCKING STUDIES

To analyze the binding interactions and predict the feasibility of newly designed TRIAZOLYL IMINO PYRAZOLE derivatives, the docking study was performed utilizing the AUTODOCK 4.2 version.

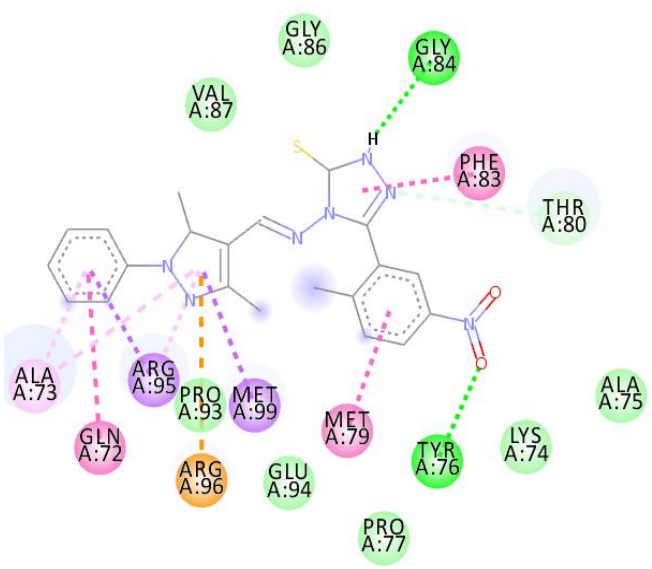
The docking results of CYP-51 (1EA1.pdb) with the ligands TP1-TP6 are reported below. The best-docked structures should have binding energy lower to the standard. The binding site and the active sites are represented in the snapshots and the binding energy was found to be more when compared to the standard. The results have been tabulated in table 5 followed by the snapshots.

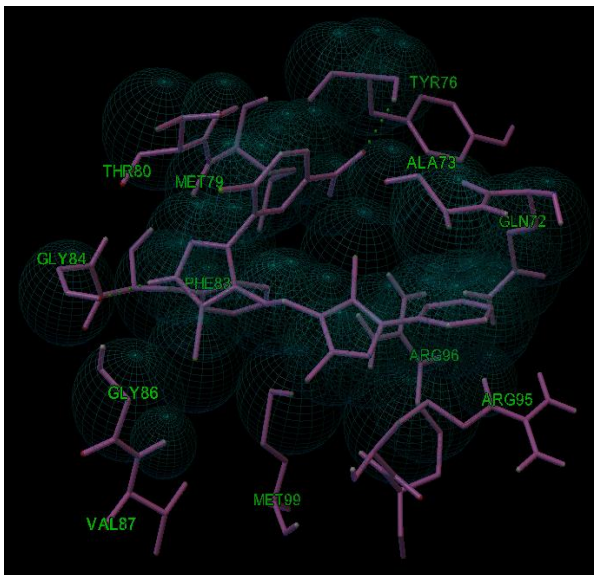
Table 5: Binding energies of TP1-TP6 with CYP-51 (1EA1.pdb)

S.NO	COMPOUND CODE	BINDING ENERGY (KJ/MOL) ΔG 1EA1.pdb
01	TP1	-8.93
02	TP2	-8.26
03	TP3	-8.38
04	TP4	-8.68
05	TP5	-8.02
06	TP6	-8.13
07	FLUCONAZOLE	-6.07

Binding interactions of TP1 with CYP-51 (1EA1.pdb)

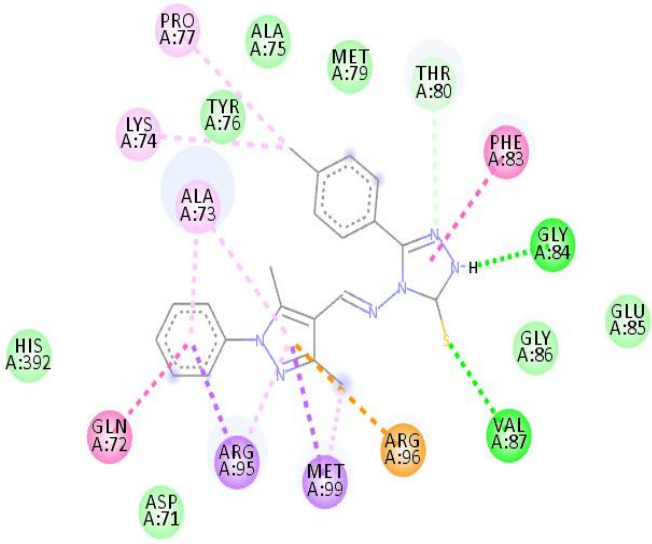
TP1 interacts with **sterol 14α-demethylase** at Gln 72, Ala 73, Tyr 76, Met 79, Thr 80, Phe 83, Gly 84, Gly 86, Val 87, Arg 95, Arg 96, and Met 99. The binding energy was found to be **-8.93 kcal/mol.**

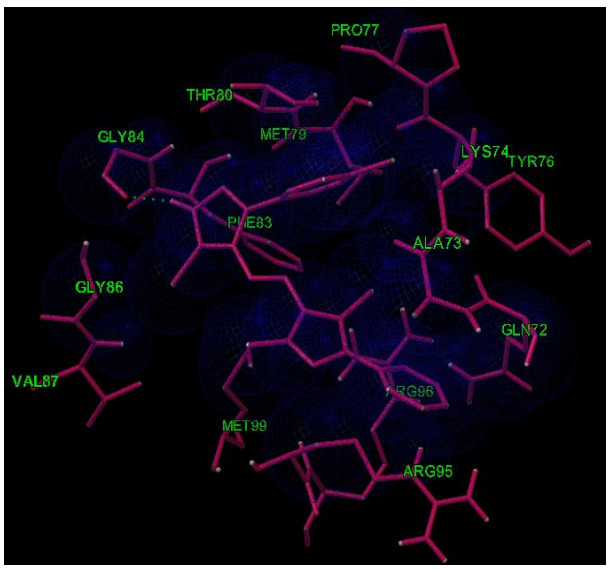




Binding interactions of TP4 with CYP-51 (1EA1.pdb)

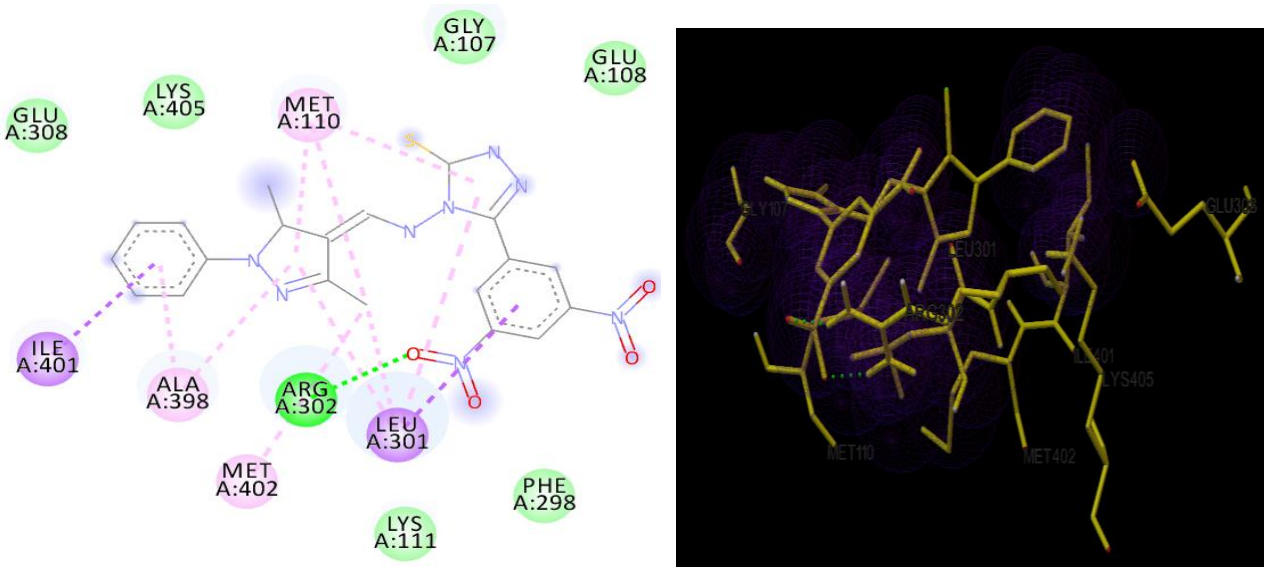
TP4 interacts with **sterol 14α-demethylase** at Gln 72, Ala 73, Lys 74, Tyr 76, Pro 77, Met 79, Thr 80, Phe 83, Gly 84, Gly 86, Val 87, Arg 95, Arg 96, and Met 99. The binding energy was found to be **-8.68 kcal/mol.**





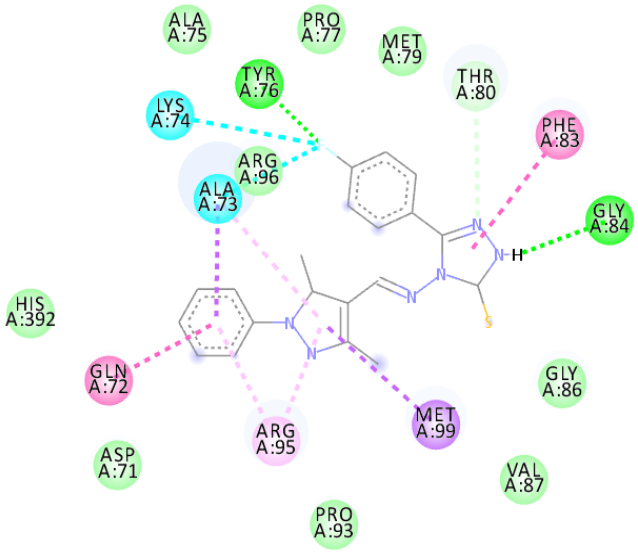
Binding interactions of TP3 with CYP-51 (1EA1.pdb)

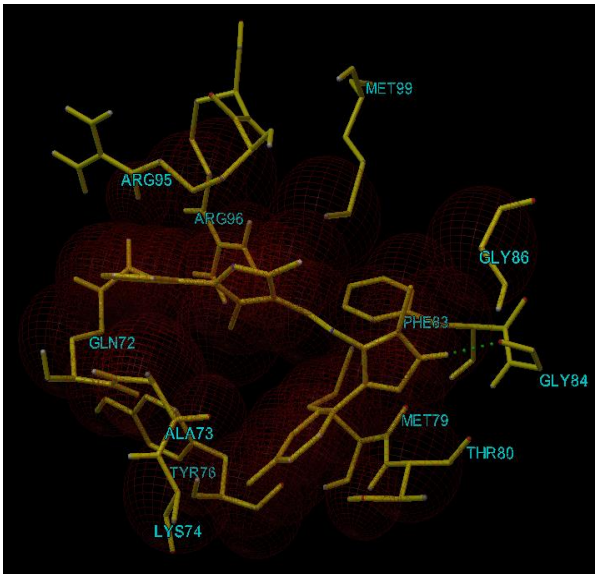
TP3 interacts with **sterol 14α-demethylase** at Gly 107, Met 110, Leu 301, Arg 302, Glu 308, Ile 401, Met 402, and Lys 405. The binding energy was found to be **-8.38 kcal/mol.**



Binding interactions of TP2 with CYP-51 (1EA1.pdb)

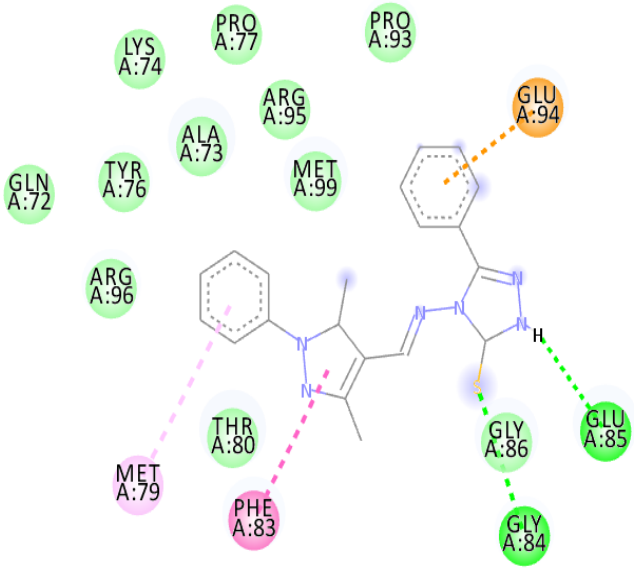
TP2 interacts with **sterol 14α-demethylase** at Gln 72, Ala 73, Lys 74, Tyr 76, Met 79, Thr 80, Phe 83, Gly 84, Gly 86, Arg 95, Arg 96, and Met 99. The binding energy was found to **be -8.26 kcal/mol.**

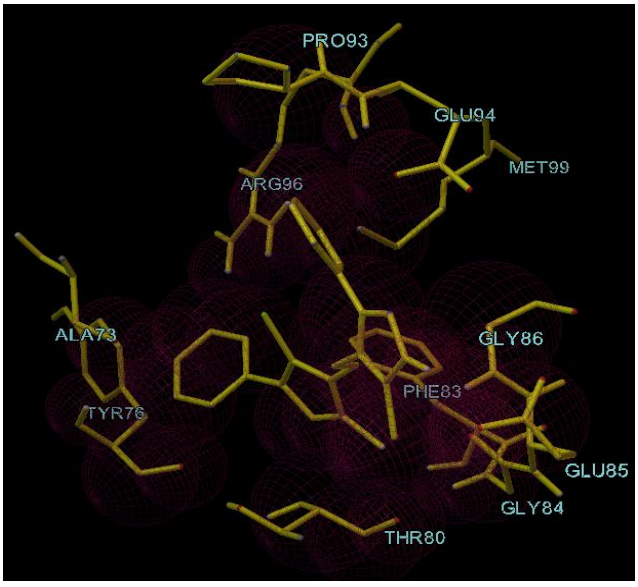




Binding interactions of TP6 with CYP-51 (1EA1.pdb)

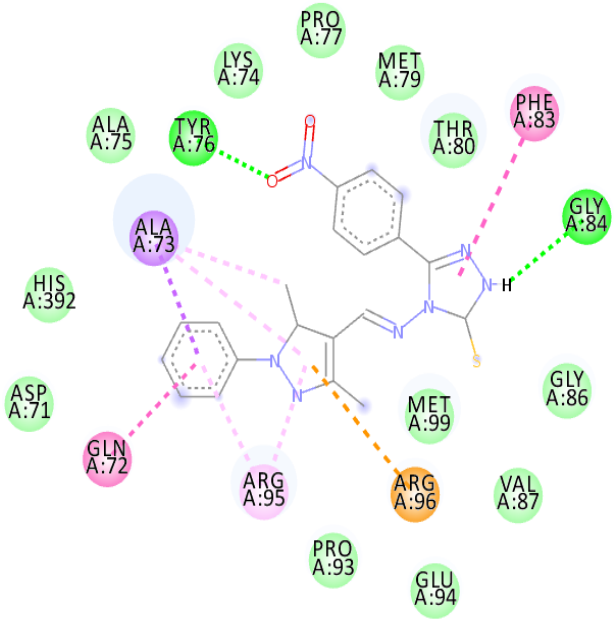
TP6 interacts with **sterol 14α-demethylase** at Ala 73, Tyr 76, Thr 80, Phe 83, Gly 84, Glu 85, Gly 86, Pro 93, Glu 94, Arg 96, and Met 99. The binding energy was found to be **-8.13 kcal/mol.**

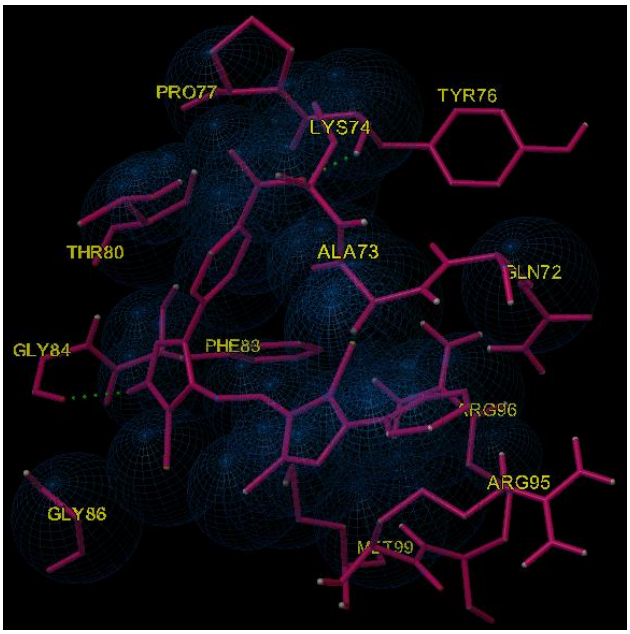




Binding interactions of TP5 with CYP-51 (1EA1.pdb)

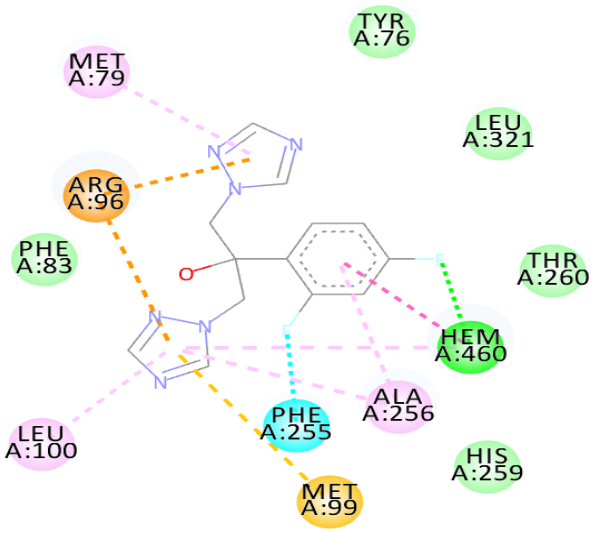
TP5 interacts with **sterol 14α-demethylase** at Gln 72, Ala 73, Lys 74, Tyr 76, Pro 77, Thr 80, Phe 83, Gly 84, Gly 86, Arg 95, Arg 96, and Met 99 .The binding energy was found to be **-8.02 kcal/mol.**

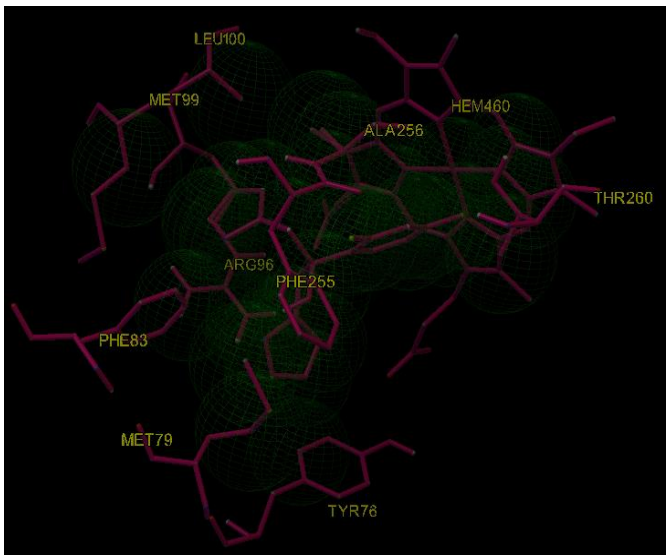




Binding interactions of Fluconazole with CYP-51 (1EA1.pdb)

Fluconazole binds to the active site of **sterol 14α-demethylase** via coordination of the N atom of the azole nucleus with iron of the heme group. Then it interacts with Tyr 76, Met 79, Phe 83, Arg 96, Met 99, Leu 100, Phe 255, Ala 256, and Thr 260. The binding energy was found to be **-6.07 kcal/mol.**





All the ligands TP1-TP6 showed excellent binding interactions with CYP-51 (1EA1.pdb) through Tyr 76, Met 79, Phe 83, Arg 96, and Met 99. Among the triazolyl pyrazole derivatives **TP1, TP4, TP3, TP2** had shown the highest binding energies, **-8.93, -8.68, -8.38, -8.26 kcal/mol** when compared to the standard, **fluconazole.**

PHASE II – SPECTRAL STUDIES

All the six new compounds TP1-TP6 obtained through two different schemes were prepared in good yield and evaluated by their physical characterization (Melting point and TLC) and spectral characterization (UV, IR, MASS, and NMR) data.

Recrystallization Solvent: Ethanol

Solvent system: Acetone: Benzene (2:8)

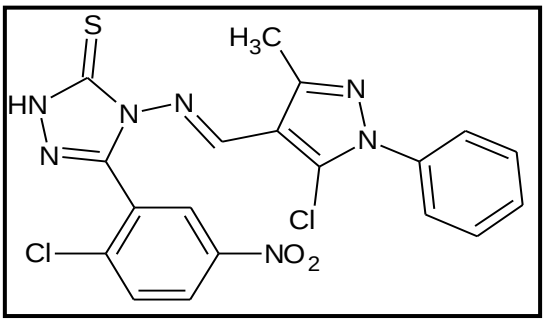
Visualizing agent: Iodine Vapour

Table 6 - Physical characterization of triazolyl pyrazole derivatives (TP1-TP6)

S.NO	Comp code	R	Molecular formula	Molecular weight	% Yield	Melting point ˚C	R_f value	UV (λ_{max})
1	**TP1**	2-Cl-5NO_2	$C_{19}H_{13}Cl_2N_7O_2S$	474.32	65	132-134˚C	0.74	341 nm
2	**TP2**	4-F	$C_{19}H_{14}ClN_6S$	412.87	72	130-132˚C	0.6	253 nm
3	**TP3**	3,5- NO_2	$C_{19}H_{13}ClN_8O_4S$	484.87	60	113-115˚C	0.54	253 nm
4	**TP4**	4-Cl	$C_{19}H_{14}Cl_2N_6S$	429.32	65	135-137˚C	0.72	247 nm
5	**TP5**	4-NO_2	$C_{19}H_{14}ClN_7O_2S$	439.87	75	128-131˚C	0.65	341 nm
6	**TP6**	H	$C_{19}H_{15}ClN_6S$	394.88	70	134-136˚C	0.61	252 nm

PHASE III - SPECTRAL CHARACTERIZATION

Compound Code: TP1



Chemical name : 4-[(*E*)-[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl) methylidene] amino]-5-(2-chloro-5-nitrophenyl)-2, 4-dihydro-3*H*-1, 2, 4-triazole-3-thione

UV Spectrum

Solvent used : DMSO

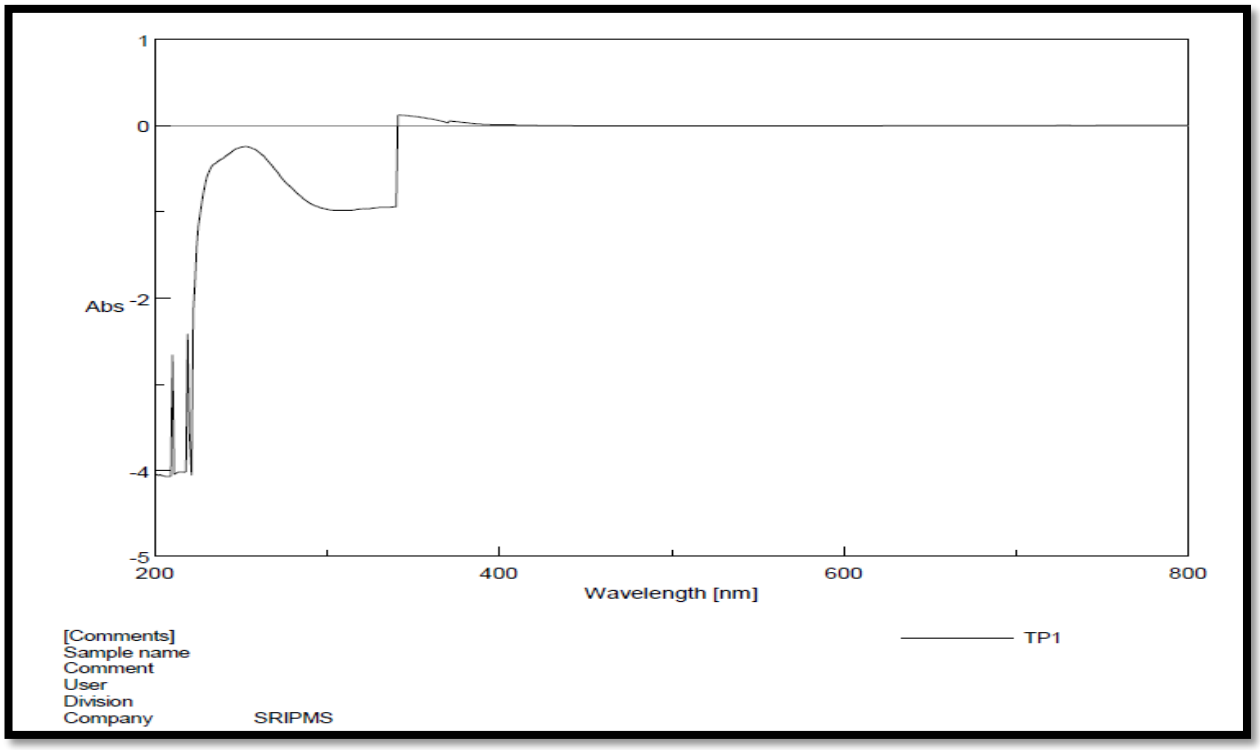
λ_{max} : 341 nm

IR (KBr, νmax in cm^{-1}) : 3043.77 (NH), 2903.93 (Aromatic C-H), 1680.05 (CH=N), 1517.06 (C=N), 1343.46 (N-NH), 1249.91 (C=S), 1046.42 (C-Cl)

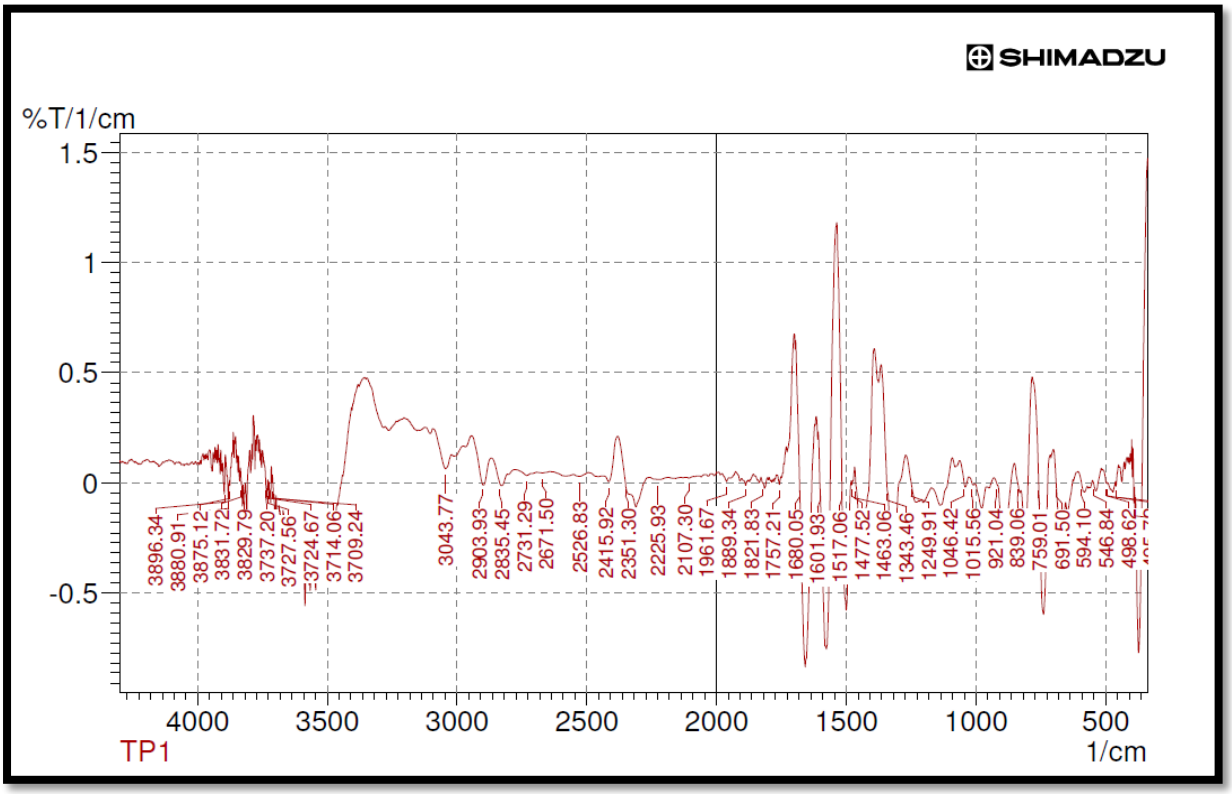
^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm): 2.4043 (s, 3H, CH_3), 7.5572-7.5842 (s, 1H, CH=N, m, 8H, Ar-H), 9.9094 (s, 1H, NH triazole)

Mass : 475 (M^+ ion peak)

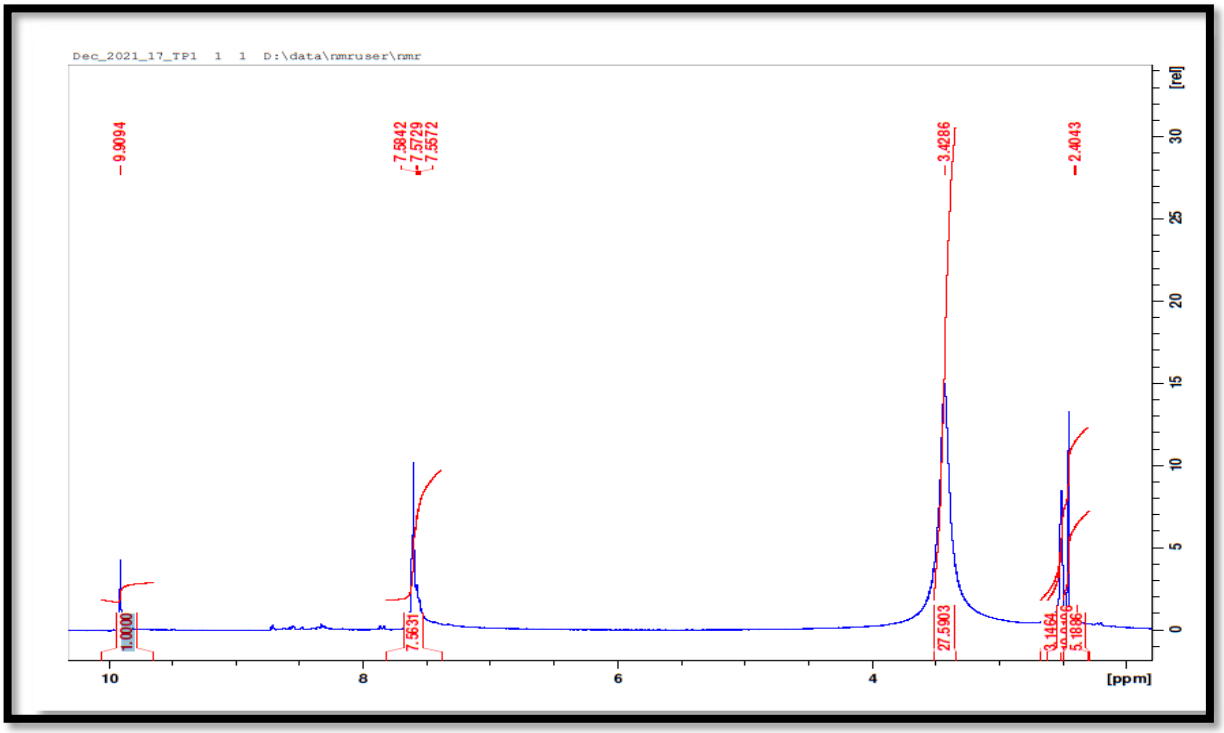
UV SPECTRUM



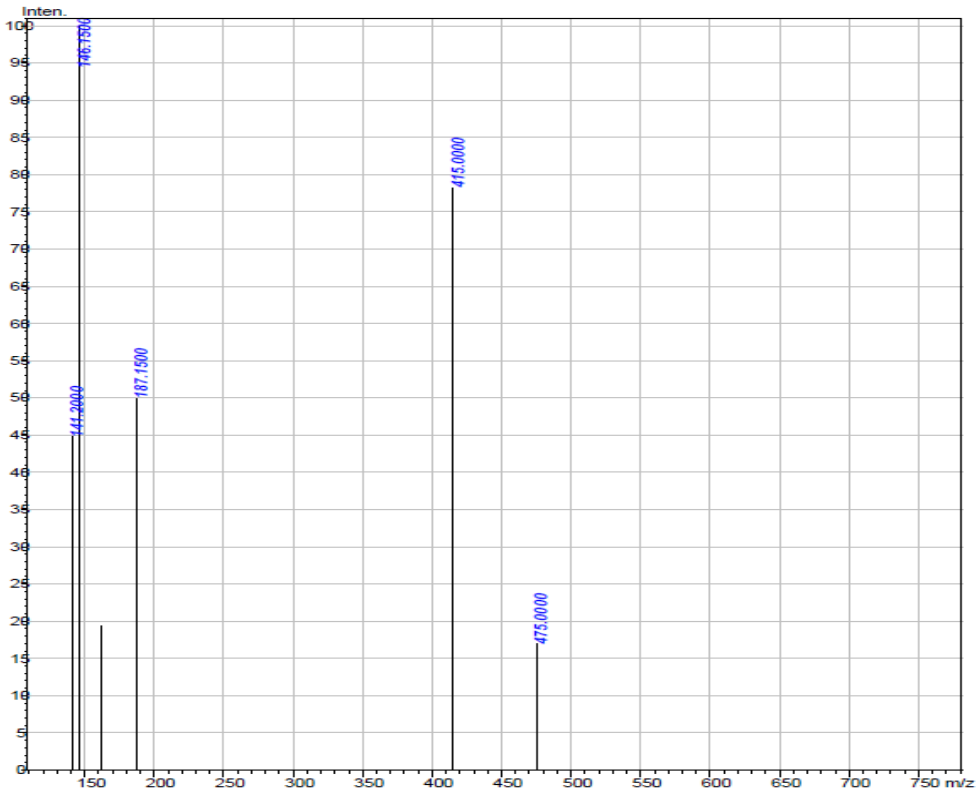
IR SPECTRUM



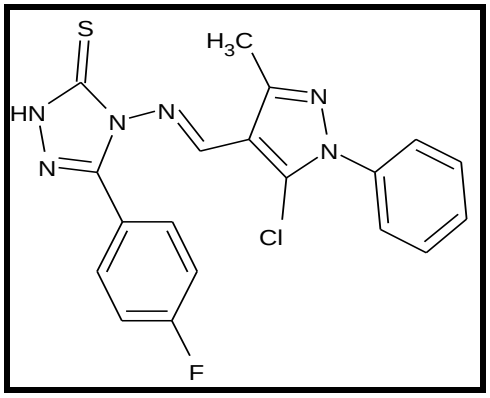
NMR SPECTRUM



MASS SPECTRUM



Compound code TP2



Chemical name : 4-[(*E*)-[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl) methylidene] amino]-5-(4-fluorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione

UV Spectrum

Solvent used : DMSO

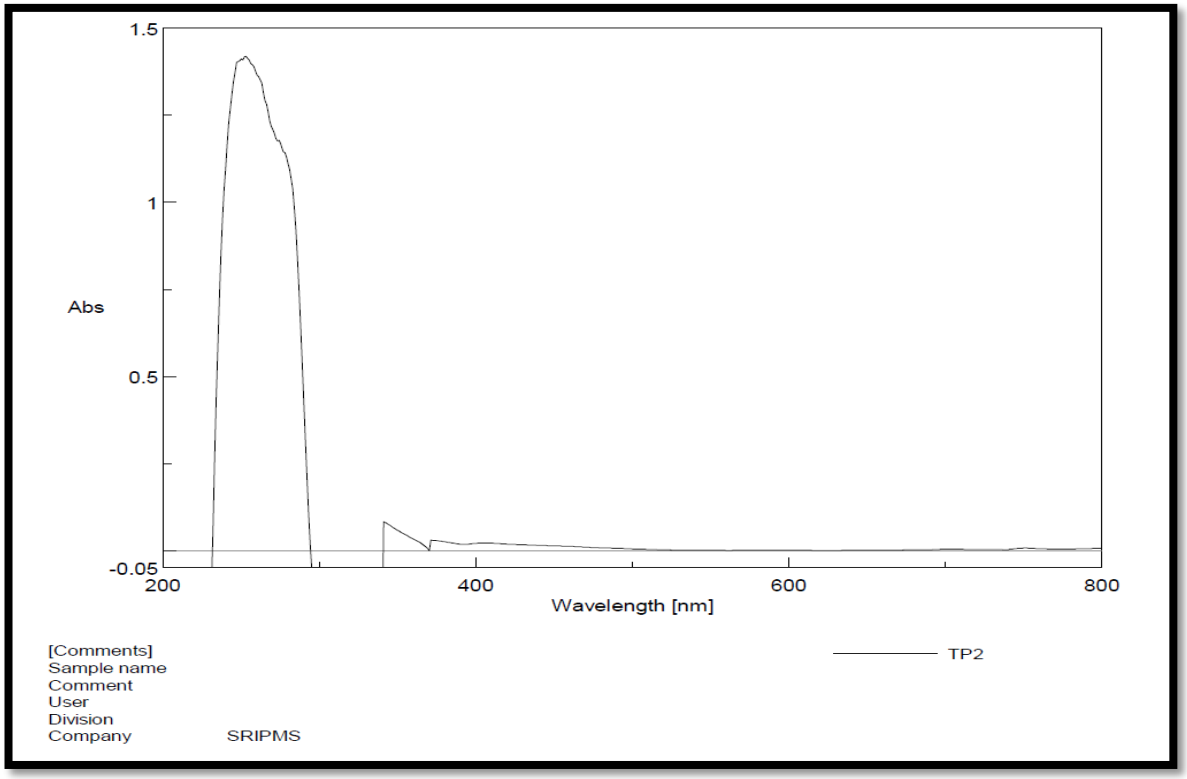
λ_{max} : 253 nm

IR (KBr, νmax in cm^{-1}) : 3049.56 (NH), 2926.11 (Aromatic C-H), 1673.30 (CH=N), 1507.42 (C=N), 1339.61 (N-NH), 1254.74 (C=S), 1030.99 (C-NO_2)

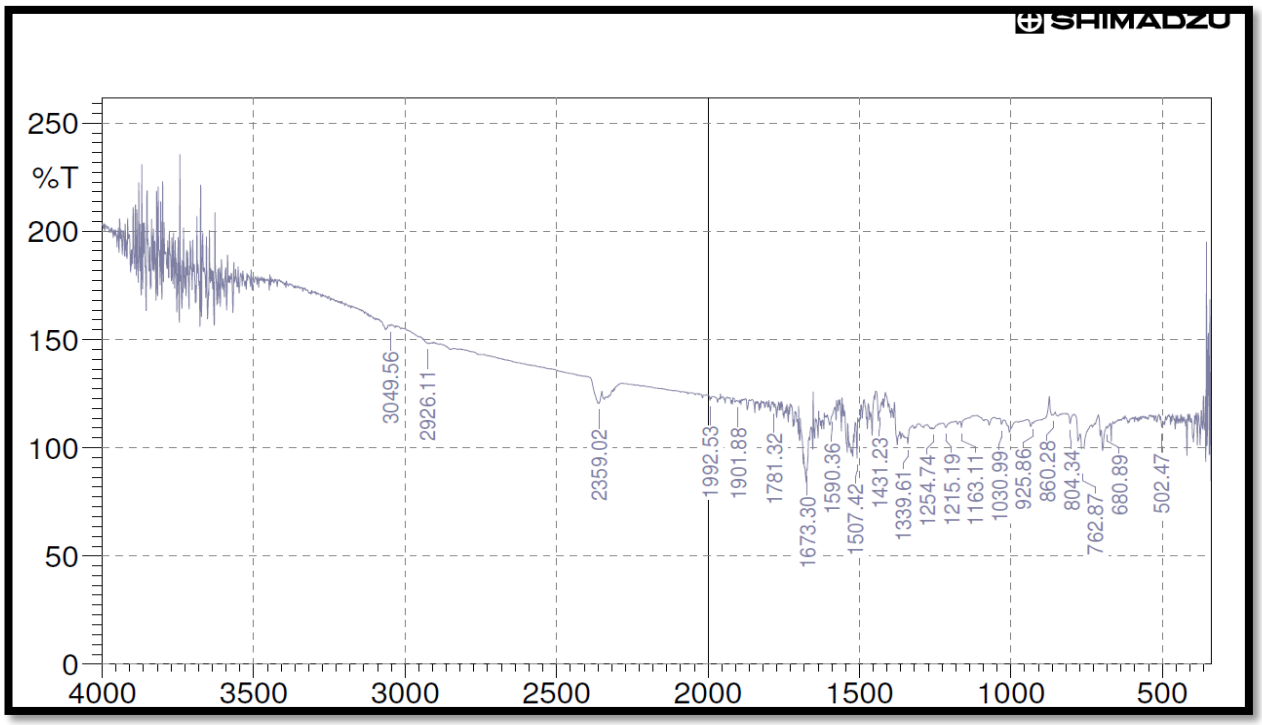
^{1}H-NMR (300 MHz, DMSO-d_6, δ ppm) : 2.5667 (s, 3H, CH_3), 7.1322-7.5170 (s, 1H, CH=N, m, 9H, Ar-H), 9.9989 (s, 1H, NH triazole)

Mass : 412 (M^+ ion peak)

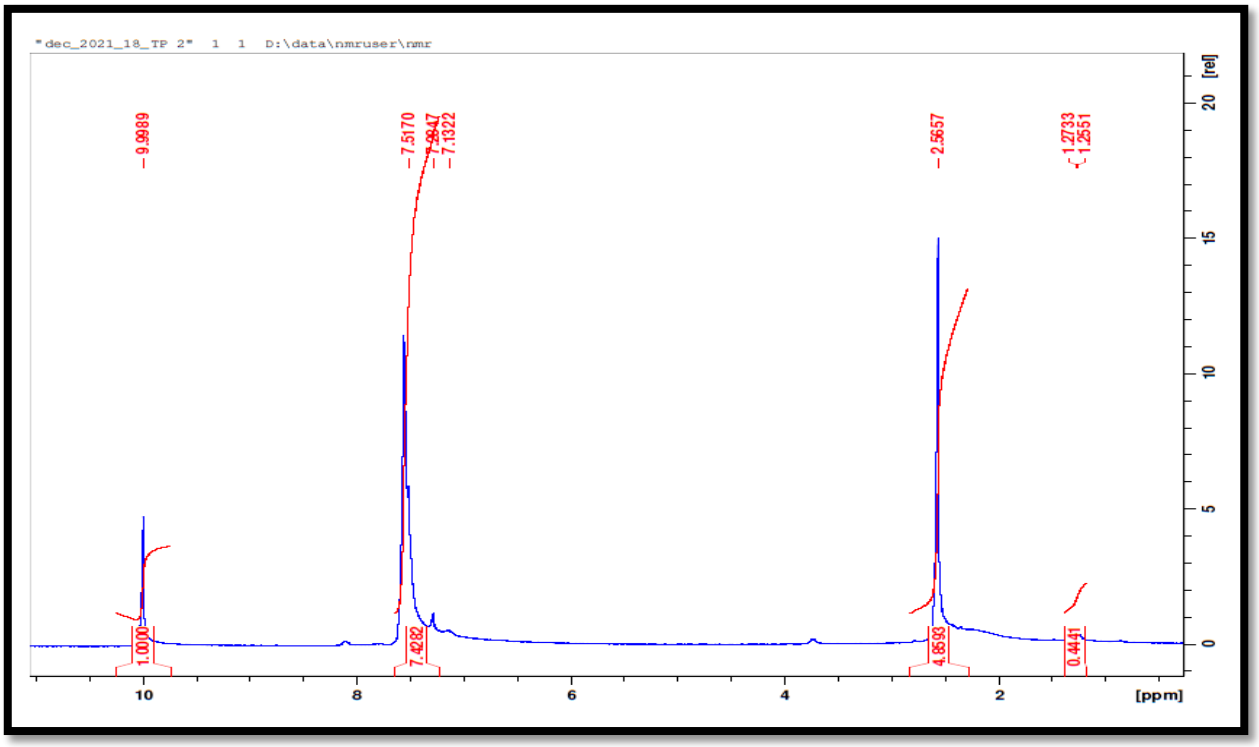
UV SPECTRUM



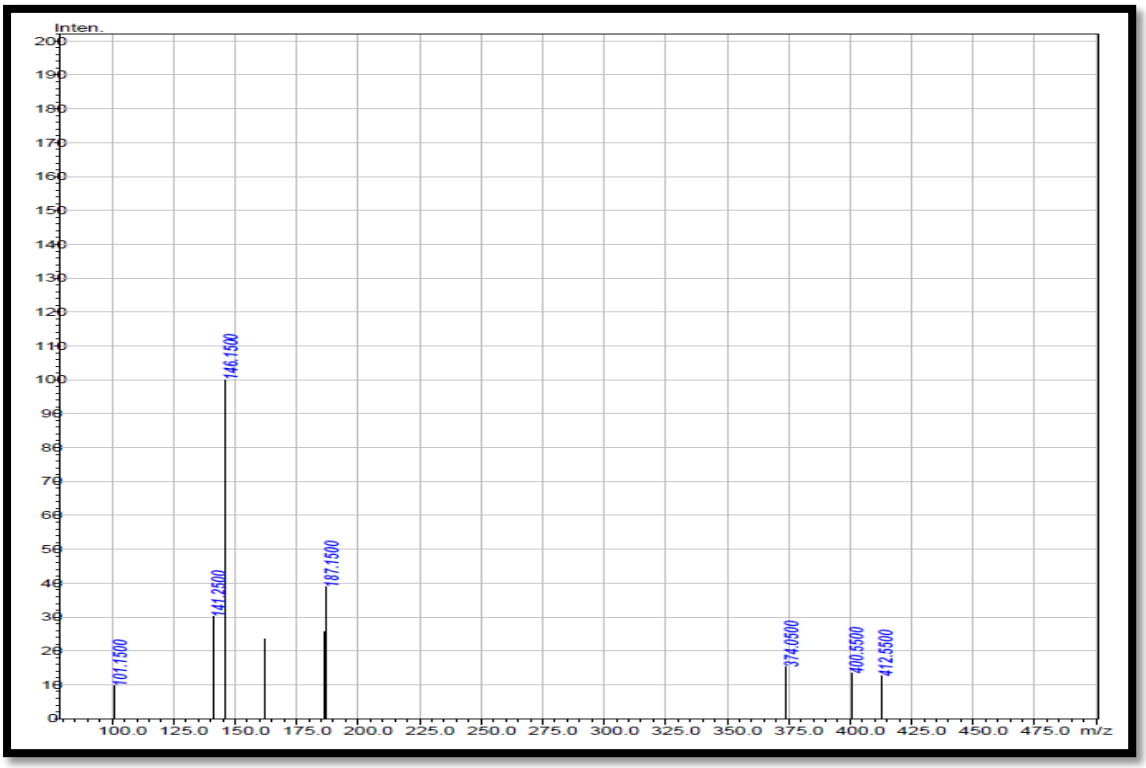
IR SPECTRUM



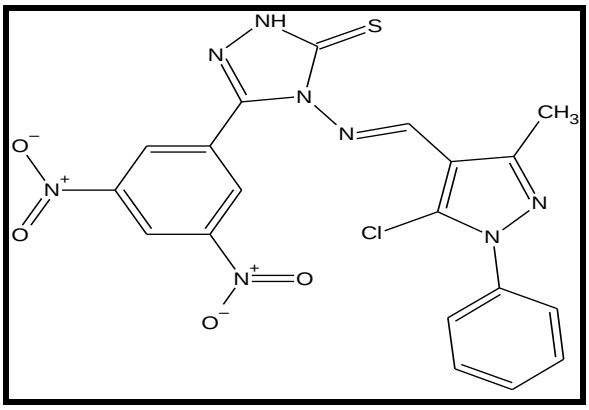
NMR SPECTRUM



MASS SPECTRUM



Compound code TP3



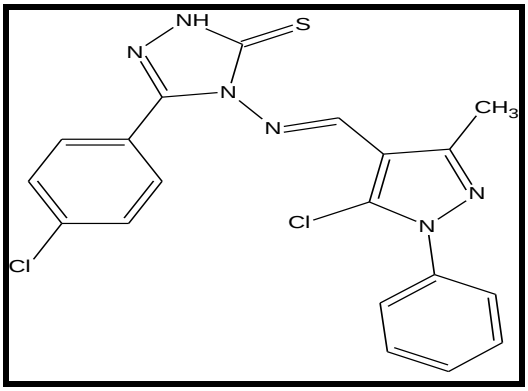
Chemical name : 4-{(*E*)-[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methylidene]amino}-5-(3,5-dinitrophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione

UV Spectrum

Solvent used : DMSO

λ_{max} : 253 nm

Compound code TP4



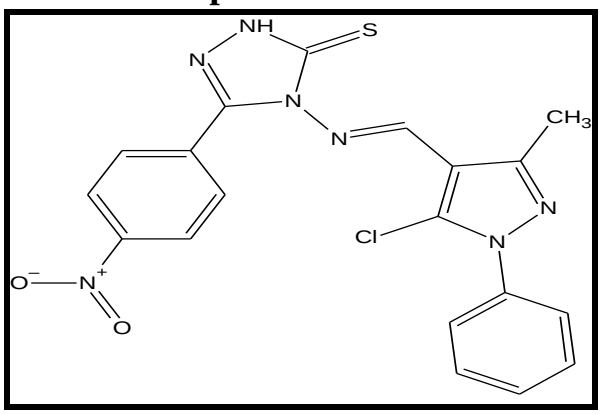
Chemical name : 4-{(*E*)-[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl) methylidene]amino}-5-(4-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione

UV Spectrum

Solvent used : DMSO

λ_{max} : 247 nm

Compound code TP5



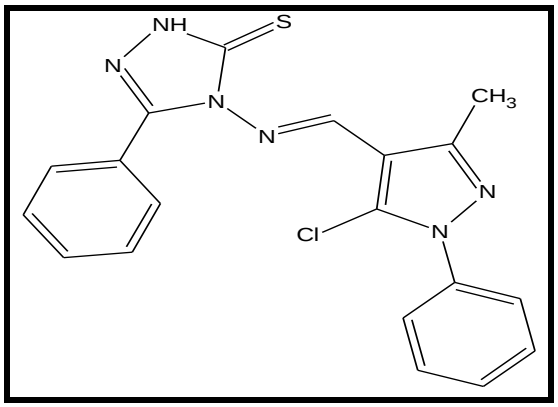
Chemical name : 4-{(*E*)-[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methylidene]amino}-5-(4-nitrophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione

UV Spectrum

Solvent used : DMSO

λ_{max} : 253 nm

Compound code TP6



Chemical name : 4-{(*E*)-[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methylidene]amino}-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione

UV Spectrum

Solvent used : DMSO

λ_{max} : 253 nm

PHASE IV – ANTI MYCOBACTERIAL SCREENING

MICROPLATE ALAMAR BLUE ASSAY (MABA)

The synthesized compounds were evaluated for *in-vitro* anti-tubercular activity by the Alamar Blue assay method. The results are shown in figure 6.

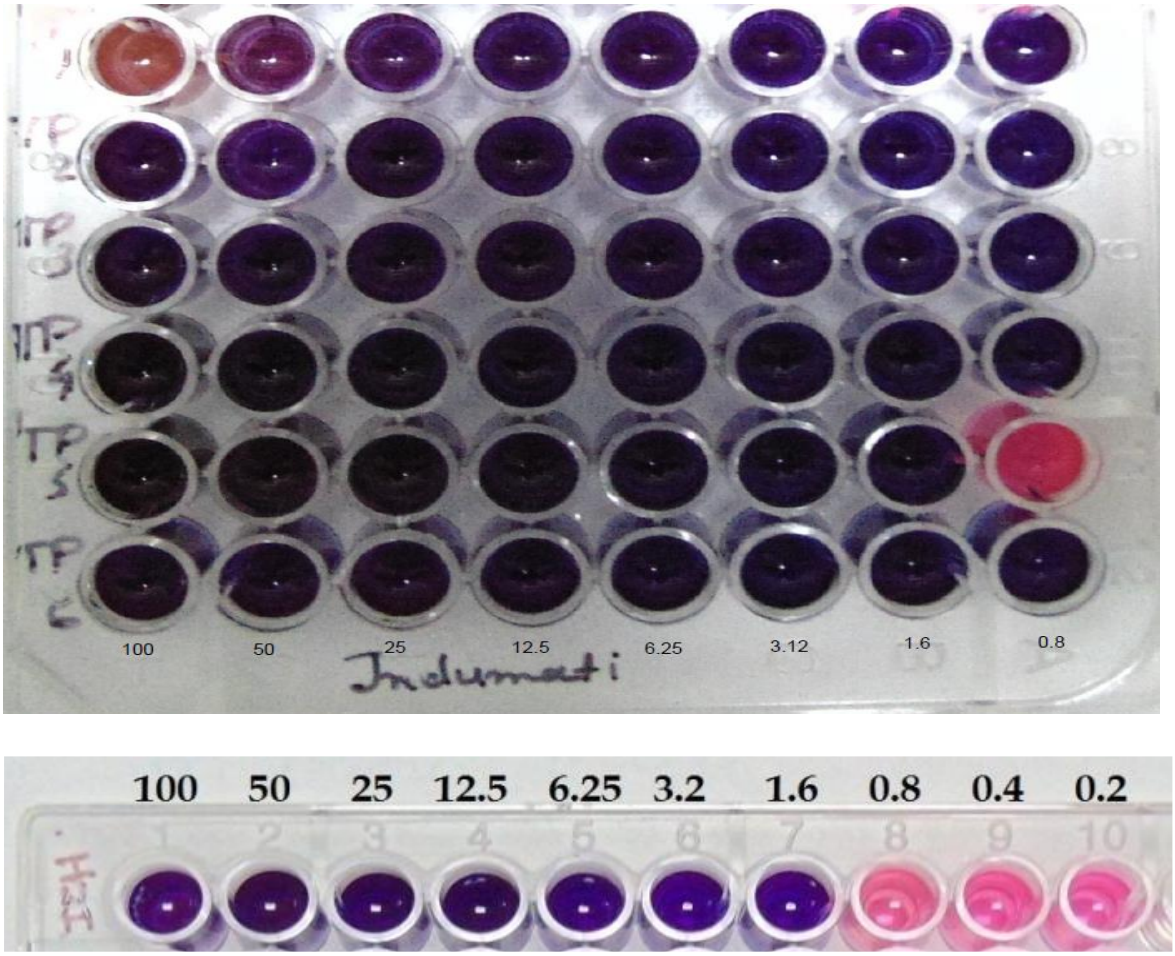


Figure 6: Anti-mycobacterial screening of TP1-TP6 using Alamar Blue Assay method

The results have shown that all the triazolyl pyrazole derivatives (TP1-TP6) were found to inhibit the growth of *Mycobacterium tuberculosis* when compared to the standard, Isoniazid (**MIC: 1.6 µg/ml**). All the derivatives of triazolyl pyrazoles (**TP1 to TP6)** were found to inhibit the growth of *Mycobacterium tuberculosis*. The compound **TP1 (2-Cl, 5-NO_2 triazolyl pyrazole)** had shown minimum inhibitory concentration at **50 µg/ml** and **TP5 (4-NO_2 triazolyl pyrazole)** showed MIC of

1.6 µg/ml. The nitro derivative is found to show the activity owing to its strong electron withdrawing nature.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

SUMMARY

The present work was focused on the design, docking, synthesis, and evaluation of the anti-tubercular activity of triazole-linked pyrazole derivatives as possible CYP-51 (sterol 14α-demethylase) inhibitors.

Phase I- *In-silico* studies

- **Selection of the target**

CYP-51 (sterol 14α-demethylase) was selected as the drug target for the anti-tubercular activity. The corresponding enzyme was obtained from the RCSB protein data bank **(PDB ID: 1EA1).**

- **Selection of lead by virtual screening**

Virtual screening was performed by iGEMDOCK v.2. Fifty hits were obtained from the ZINC database, from which **triazole** and **pyrazole** were selected as the lead for inhibiting CYP-51 (sterol 14α-demethylase).

- **Lead optimization**

The six modified ligands TP1-TP6 were subjected to *in-silico* lead optimization. Lead optimization was done by observing *in-silico* ADME studies and computation of drug-like properties. Lead optimization revealed that all the six selected derivatives possess good ADME properties and hence were eligible for further study. The ligands were optimized for evaluating oral bioavailability by utilizing the Molinspiration server and SWISSadme server.

- **Docking**

The optimized leads were subjected to docking studies using Autodock4.2 and the interactions of the derivatives with active sites of the enzyme were studied. The derivatives were subjected to interaction with CYP-51. Fluconazole was used as standard ligand. The binding energy was found to be superior for all the compounds when compared to the standard (fluconazole). Among the triazolyl pyrazole derivatives, **TP1, TP4, TP3, TP2** showed maximum binding energies, **-8.93, -8.68, -8.38, -8.26 kcal/mol** respectively. They were interacting well with the active sites on the enzyme i.e., Tyr 76, Met 79, Phe 83, Arg 96, and Met 99.

PHASE II-SYNTHESIS AND PHYSICAL CHARACTERIZATION

- **Synthesis of the designed compounds**

In this present work, six new compounds were synthesized. In the **first scheme** triazole amine and its derivatives were synthesized by the reaction of thiocarbohydrazide with substituted and unsubstituted benzoic acid. Two steps were involved in the **scheme 2**. The first step involves the synthesis of 5-Methyl-2-phenyl-2, 4-dihydro-3H-pyrazole-3-one by the reaction of phenylhydrazine with ethyl acetoacetate. The second step involves the synthesis of 5-Chloro-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde. The resulting compound from the first step was treated with phosphorus oxychloride and dimethylformamide.

Scheme **3** involves the synthesis of triazolyl pyrazole derivatives. Schiff bases were prepared from triazole amine derivatives with pyrazole aldehyde to obtain desired products.

- **Physical characterization**

The melting point of newly synthesized compounds were determined. R_f values were determined by fixing various suitable solvent system on precoated silica gel G plates. The solvent system used was Acetone: Benzene (2:8).

PHASE III: SPECTRAL CHARACTERIZATION

The structures of the synthesized compounds were established on the basis of UV, IR, ^{1}H NMR, and MASS spectral data.

PHASE IV: ANTI MYCOBACTERIAL SCREENING

MICROPLATE ALAMAR BLUE ASSAY (MABA) METHOD

The anti-tubercular activity was performed by microplate alamar blue assay method by using *mycobacterium tuberculosis* H_{37}Rv strain. All the derivatives of triazolyl pyrazoles (**TP1 to TP6)** were found to inhibit the growth of *Mycobacterium tuberculosis*. The compound **TP1 (2Cl-5NO_2 triazolyl pyrazole)** had shown minimum inhibitory concentration at **50 µg/ml** and **TP5 (4-NO_2 triazolyl pyrazole)** showed MIC of **1.6 µg/ml**.

CONCLUSION

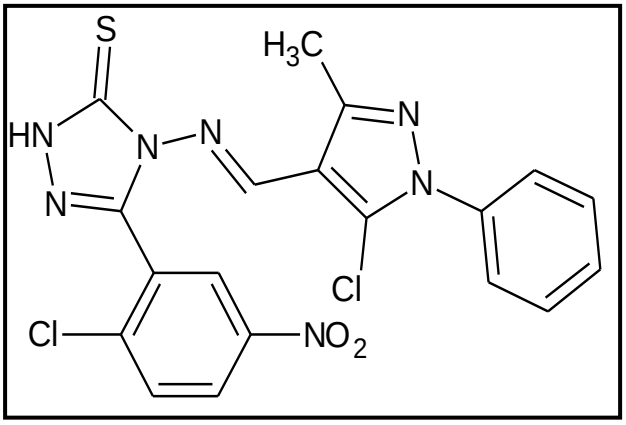
The present study has proved to be a tool in minimizing the tedious process of drug discovery process over the traditional methods of discovery. Virtual screening was utilized for filtering the compounds and selecting the lead compounds. The *In-silico* ADME & drug-likeness scores of the ligands showed the compound to be promising as a good orally bioactive drug. The binding energy obtained from the docking study further confirmed the affinity of the selected leads towards the enzyme, CYP-51 (sterol 14α-demethylase) from *Mycobacterium tuberculosis.* Various triazolyl imino pyrazole derivatives were synthesized with good yield utilizing three schemes. The structure of the synthesized compounds were confirmed by melting point, TLC, UV, IR, NMR and mass spectra. The compounds were screened for antimycobacterial activity which establishes the correlation of activity with the docking study. The present study includes the design, docking, synthesis and screening of various triazole incorporated pyrazole imines as possible CYP-51 inhibitors and the design has paved the way to establish the lead triazolyl pyrazole as antimycobacterial drug choice. Among the synthesized compounds, 2-chloro5-nitro-triazolyl pyrazole imine was found to be potent as CYP-51 inhibitor due to superior dock result and biological activity result. The docking study reveals that the triazolyl imino prazole has excellent interaction with CYP51, and therefore can be probed as possible MtbCYP-51 inhibitors.

FUTURE PERSPECTIVE

Since the most potential derivative was the chloro and nitro substituted triazolyl imino pyrazole derivative, more focus can be given on electron withdrawing substituents for future synthesis. The present novel derivatives are found to exhibit antimycobacterial activity, the mechanism of action can be confirmed by performing enzyme inhibitory assay as a future perspective.

OUTCOME OF THE STUDY

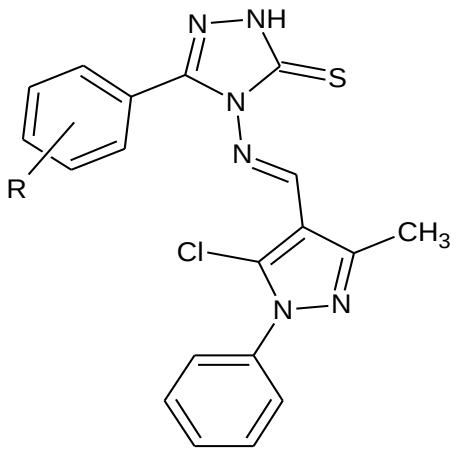
From the present study, the most significant compound were found to be



4-[[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol- 4-yl) methylidene] amino]-5-(2-chloro-5-nitrophenyl)-2, 4-dihydro-3*H*-1, 2, 4-triazole-3-thione (**TP1**)

LIST OF NEWLY SYNTHESIZED COMPOUNDS

TRIAZOLYL PYRAZOLE DERIVATIVES



COMPOUND CODE	R	COMPOUND NAME
TP1	2Cl5NO_2	4-{[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol- 4-yl) methylidene] amino}-5-(2-chloro-5-nitrophenyl)-2, 4-dihydro-3*H*-1, 2, 4-triazole-3-thione
TP2	4- F	4-{[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl) methylidene] amino}-5-(4-fluorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione
TP3	3,5-di NO_2	4-{[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methylidene]amino}-5-(3,5-dinitrophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione
TP4	4- Cl	4-{[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl) methylidene]amino}-5-(4-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione
TP5	4- NO_2	4-{[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methylidene]amino}-5-(4-nitrophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione
TP6	H	4-{[(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)methylidene]amino}-5-phenyl-2,4-dihydro-3*H*-1,2,4-triazole-3-thione

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