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Pharmacophore Model Based Drug Designing, Synthesis and Pharmacological Evaluation of Potential Lipxygenase Inhibitors

by

Ammara Maqbool

A thesis submitted in partial fulfillment for the
degree of Master of Philosophy

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CERTIFICATE OF APPROVAL

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(Ammara Maqbool)

Abstract

This study aimed at utilizing Computer-Aided Drug Design (CADD) into synthetic medicinal chemistry for the design of new anti-inflammatory modulators by targeting lipoxygenase (LOX) pathway. A virtual screening of a designed chemical library was performed on the target model, AHHRRR, developed using Ligand Based pharmacophore modeling, followed by molecular docking analysis and AD-MET simulations to analyze the theoretical binding affinities, spatial alignment, and drug-like pharmacokinetic properties. These computational insights translated to a hypothesis for a series of 7 novel compounds named AM-1, AM-2, AM-3, AM-4, AM-5, AM-6 and AM-7 that are synthesized in a series of steps starting with an electrophilic chloroacetylated intermediate core, using a series of substituted aromatic and heterocyclic amines. The successful formation of the compounds was evident from the Fourier-Transform Infrared (FTIR) and Proton Nuclear Magnetic Resonance ($^1\text{H-NMR}$) spectroscopy. The top ranked compound AM-7 was selected for further in-vivo anti-inflammatory activity. To determine the anti-inflammatory efficacy of the compound, it was tested at three different doses - 10 mg/kg, 20 mg/kg, and 30 mg/kg - in a mouse paw edema model using carrageenan. Pharmacological results demonstrated dose dependent anti-inflammatory activity with compound showing significant anti-inflammatory effect which was in line with the trend predicted by the initial computational screening model. These preliminary results are promising, but are actually a preliminary exploratory rather than a therapeutic discovery stage. Additional extensive testing such as further in vivo testing of the remaining derivatives, careful in vitro enzymatic inhibition assays to establish accurate IC_{50} values,, tolerance testing in the stomach to evaluate ulcerogenicity potential, and chronic toxicity testing are all important to validate the therapeutic potential, safety margins, and definitive molecular mechanisms of this chemical series.

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Abbreviations

ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
ANOVA	Analysis of Variance
ATR-FTIR	Attenuated Total Reflection–Fourier Transform Infrared
BBB	Blood-Brain Barrier
BCS	Biopharmaceutics Classification System
BEDROC	Boltzmann-Enhanced Discrimination of Receiver Operating Characteristic
CADD	Computer-Aided Drug Design
CYP	Cytochrome P450
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
EF1%	Enrichment Factor at 1%
FTIR	Fourier-Transform Infrared
HTS	High-Throughput Screening
LBDD	Ligand-Based Drug Design
LBPM	Ligand-Based Pharmacophore Modeling
LOX	Lipoxygenase
MD	Molecular Dynamics
MM-GBSA	Molecular Mechanics Generalized Born Surface Area
MP	Melting Point
NMR	Nuclear Magnetic Resonance
PDB	Protein Data Bank
PPW	Protein Preparation Wizard
QSAR	Quantitative Structure-Activity Relationship

RMSD	Root-Mean-Square Deviation
SBDD	Structure-Based Drug Design
SBPM	Structure-Based Pharmacophore Modeling
SDF	Structure-Data File
TEA	Triethylamine
TLC	Thin-Layer Chromatography
TSV	Tab-Separated Values
Vd-	Volume of Distribution
XP	Extra Precision

Chapter 1

Introduction

Inflammation is the universal response of self-protection to protect the body against any damage or infection. One short-term phenomenon is acute inflammation which is triggered at the site of any injury or infection in the body, while the other is a persistent or long-term inflammation, which is the root cause of many inflammatory diseases including cancer [1]. Prostaglandin (PG) synthesis or cyclooxygenase (COX) pathway forms an integral part of inflammatory processes which produce a group of biologically active lipid compounds called prostanoids [2].

Most of the anti-inflammatory agents that are currently in the market are non-steroidal (NSAIDs) and their action involves the inhibition of COX enzymes. NSAIDs, however, have a number of side effects such as gastric and renal [3]. Aspirin was found to cause gastric damage in rats through the reduction in the level of the plasma membrane protein of tight junctions, occludin in the gut which was prevented by pretreatment with another drug Mosapride [4]. Short duration excess dose of ibuprofen may lead to jejunal ulcers [5]. Naproxen causes gastric antral ulcers and increased levels of lipid peroxides in the stomach. Likewise, NSAIDs have also been found to cause perforated jejunal ulcers [6] and small bowel ulcers [7].

These COX-2 selective anti-inflammatory drugs (COXIBs) were most preferred as they exhibited high anti-inflammatory activity and also some had low gastrointestinal toxicity [8]. However, it was soon discovered that COXIBs also exhibit cardiac side-effects when used for extended periods of time by arthritic patient [9]. In this way some of the COXIBs, such as Rofecoxib, were withdrawn from the market, but others, such as celecoxib, are still available for use with a warning label for their use in people with cardiovascular disorders [10].

Leukotrienes are formed by lipoxygenases (LOXs) from Arachidonic Acid (AA) including 5-LOX, 12-LOX and 15-LOX [11]. The enzyme 5-LOX is mainly responsible for the production of leukotrienes in the inflammation process. 5-LOX is one of non-heme iron enzymes which oxidize fatty acids to yield lipid hydroperoxides [12]. The presence of 5-LOX and its interactions with arachidonic acid are the first steps in the formation of leukotrienes (LTs), as shown in figure 2.1. This interaction mainly results in the production of lipid hydroperoxides which cause inflammation [13].

Additional research on the biochemical mechanisms of 5-LOX, however, has brought the pathophysiology of this enzyme and the importance of 5-LOX in respiratory and cardiovascular diseases to light. Under resting conditions, 5-LOX contains Fe(II) and under activation it contains Fe(III), physiological conditions induce the formation of lipid hydroperoxide, which activates 5-LOX, it is reported that the enzyme translocates in the cytosol to the nuclear membrane when it is activated, possibly in this same location it binds Ca^{2+} and/or is phosphorylated [4].

It has been found that leukotrienes are lipids that play a role in inflammation, and play a crucial role in cardiovascular disorders [6]. Leukotrienes have recently been found to play a role in the development of certain cancers, including prostate cancer, osteoporosis and leukemias. 5-LOX has already proven to be a great drug target for many diseases such as asthma, rheumatoid arthritis, osteoarthritis, allergies, cardiovascular diseases and certain cancers. More than three decades of research have seen significant developments towards searching for more efficient 5-LOX inhibitors. Nordihydroguaretic acid (polyphenolic compound isolated from creosote bush), curcumin (polyphenolic compound found in turmeric), eugenol

(polyphenolic compound from cloves) and quercetin (flavonoid from garlic) [14], Hyperforin (a phytochemical obtained from *Hypericum perforatum*) [8], and zileuton [15].

The 5-LOX inhibitors can be divided into four categories [16], (i) redox inhibitors (redox-active compounds disrupt the enzyme's redox cycle), (ii) iron-ligand inhibitors with low redox activity (inhibit by binding to the active site iron), (iii) non-redox inhibitors (compete with substrate AA at the active site), and (iv) allosteric inhibitors (inhibit by binding sites other than the active site).

LOX inhibitors are created at a slower pace than COX inhibitors because there is no specific structure for LOXs. 5-LOX is the primary isoform associated with inflammatory diseases. This isoform can be specifically aimed at to obtain an anti-inflammatory effect [17].

In the quest to design and develop novel therapeutic molecules that possess the highest number of pharmacophoric characteristics, computational methods have arisen as innovative instruments to reduce the time and resources needed for chemical syntheses, as well as for *in vitro* and *in vivo* evaluations.

Computer-aided Drug Design (CADD), or *in silico* drug design, primarily utilizes computational power to enhance and speed up the drug design process by identifying the most promising lead candidate for biological evaluation. The prevalent methods of CADD encompass Pharmacophore Modeling, Quantitative-structure Activity Relationship (QSAR), and Molecular Docking, among others [18, 19].

Pharmacophoric modeling relies on the concept that shared chemical functionalities and a comparable spatial arrangement result in biological activity on an identical target. Pharmacophore models can be created using two distinct methods, based on the input data used for model development, specifically "structure-based" and "ligand-based" pharmacophore modeling. The structure-based method utilizes the structural data of target proteins such as enzymes or receptors to pinpoint compounds that may serve as potential drugs. Conversely, the ligand-based method involves creating 3D pharmacophore models and modeling quantitative

structure-activity relationships (QSAR) or quantitative structure-property relationships (QSPR) by solely utilizing the physicochemical characteristics of established ligand compounds for drug development [20].

The pharmacophore modeling can be used to find the selective 5-LOX Inhibitors. The structure of 5-LOX is accessible on Protein Databank and the structure of compounds can be taken from ChEMBL. The software programs are available for these computational studies e.g. Schrodinger Suites for Pharmacophore Modeling, Molecular docking, Molecular Mechanics-Generalized Born Surface Area (MMG-BSA), etc.

In the present study, a ligand-based pharmacophore model will be established for the identification of significant pharmacophore features of 5-LOX inhibitors gathered from the literature search and ChEMBL database. On the basis of the predictive nature of the model, the best fit molecule(s) will be used for further analysis.

The inhibitors will be designed according to the pharmacophore model and best fit molecule(s) will be tested initially by *in-silico* methods such as molecular studies for further validation. After *in-silico* analysis, the optimized compound and its designed inhibitors will be synthesized, characterized, and tested for *in-vitro* and *in-vivo* biological activity.

1.1 Research Gap

Although much research has been carried out in the discovery of drugs aimed at inhibiting the action of 5-LOX enzyme, the only FDA-approved drug that effectively inhibits the 5-LOX enzyme in the treatment of asthma is zileuton whose usage is greatly hindered by its serious adverse effects which include poor potency, hepatotoxicity, and poor pharmacokinetic profile [21, 22]. These limitations highlight the need for discovery of new 5-LOX inhibitors which can be discovered and designed computationally to reduce time and resources, then synthesized and evaluated for potential pharmacological activity.

1.2 Rationale of the Study

Targeting LOX enzyme has emerged as a promising strategy against Inflammatory diseases, as its inhibition may result in reduced synthesis of leukotrienes which will help to reduce inflammation and can be a successful therapy against inflammatory diseases. This study aimed to design, synthesize, and evaluate novel 5-LOX Inhibitors using In-silico studies and experimental approaches.

1.3 Aim and Objectives

1.3.1 Aim

The aim of this study was to design and develop potential LOX inhibitors using pharmacophore modeling, chemical synthesis and pharmacological evaluation.

1.3.2 Objectives

- i. Identification of key chemical features necessary for 5-LOX inhibition by performing Pharmacophore Modeling.
- ii. Designing of potential 5-LOX inhibitors on the basis of pharmacophore model and performing molecular docking to evaluate binding affinity and selectivity.
- iii. Synthesis and characterization of the potential 5-LOX inhibitor(s).
- iv. Pharmacological evaluation of anti-Inflammatory activity of 5 - LOX inhibitors using Animal model.

Chapter 2

Literature Review

2.1 Introduction to Lipoxygenase Enzymes

Lipoxygenases (LOXs) are the family of non-heme, iron-containing dioxygenase enzymes that play a central role in human lipid metabolism. In humans, these enzymes catalyze the stereospecific oxidation of polyunsaturated fatty acids (PUFAs), like arachidonic acid, leading to the formation of lipid hydroperoxides. These primary products act as precursors for a wide range of the bioactive lipid mediators, including the leukotrienes, hydroxyeicosatetraenoic acids (HETEs), and lipoxins, which regulate inflammation, immunity, and the cellular signaling pathways [23]. LPOs play key roles in physiological and pathophysiological processes. Lipoxygenases have physiological functions including regulation of the immune system and tissue homeostasis, but their dysfunction has been associated with diseases of inflammation, including asthma, atherosclerosis, cancer and neurodegenerative diseases. Human lipoxygenase enzymes are of great interest as drug targets for treating inflammatory diseases because of their role in inflammation signaling [24].

2.1.1 Classification of Lipoxygenase Isoforms

There are two types of human lipoxygenases, depending on the site of oxygen incorporation into arachidonic acid. There are 3 main human lipoxygenases: 5-LOX, 12-LOX and the 15-LOX, which have different tissue distribution and functions[25].

5-LOX is mostly distributed in inflammatory cells including neutrophils, macrophages, and mast cells; it participates in the initial step in leukotriene production. Leukotrienes are highly active mediators of inflammation, and are responsible for the development of asthma and allergic diseases.

On the other hand, 12-LOX is localized mainly in platelets, and plays an essential role in platelet aggregation and vasoregulation. The 15-LOX isoform is expressed in airway epithelial cells, eosinophils and reticulocytes and is involved in the formation of inflammatory lipid mediators and the resolving lipid mediators, including lipoxins [23].

2.1.2 Structure of Lipoxygenase Enzymes

Human lipoxygenases are monomers having a molecular weight between 75 and 80 kDa [26]. Structurally, human lipoxygenases are made up of two domains; an N-terminal domain and the larger C-terminal catalytic domain [26].

The N-terminal domain is responsible for interaction with the membrane and substrate availability while the C-terminal domain contains all the catalytic machinery responsible for oxygenation reaction. The catalytic domain is highly conserved, and features the non-heme iron required for the enzyme's activity. Research results indicate that some differences in the amino acid residues within the active site domain lead to the selection of different substrates and product formation among the different isoforms [26].

2.1.3 Active Site and Iron Role in Lipoxygenase

Human lipoxygenase has a single non-heme Fe atom in its active site. Under resting conditions, it is in the oxidation state Fe^{3+} , and is essential for catalytic activity of the enzyme [23].

In catalysis, the iron changes from Fe_2 to Fe_3 and it is able to have the electrons transferred and to remove the hydrogen from the substrate. It is required to start the radical mechanism required for the incorporation of oxygen. Regio- and stereoselectivity of oxygenation are determined by the positioning of the substrate in the active site. Evidently, if any enzyme is disrupted in the coordination of iron and structure of the active site, the enzyme activity will decrease, and this can be utilized when designing lipoxygenase inhibitors [24].

2.1.4 Mechanism of Lipoxygenase Action

While the catalytic mechanism of human lipoxygenases shown in figure 2.1 is radical, the mechanism for human prostacyclin synthase is not. The enzyme first takes a hydrogen atom from the bis-allylic position on arachidonic acid creating a lipid radical. Catalytic iron is the catalyst [26].

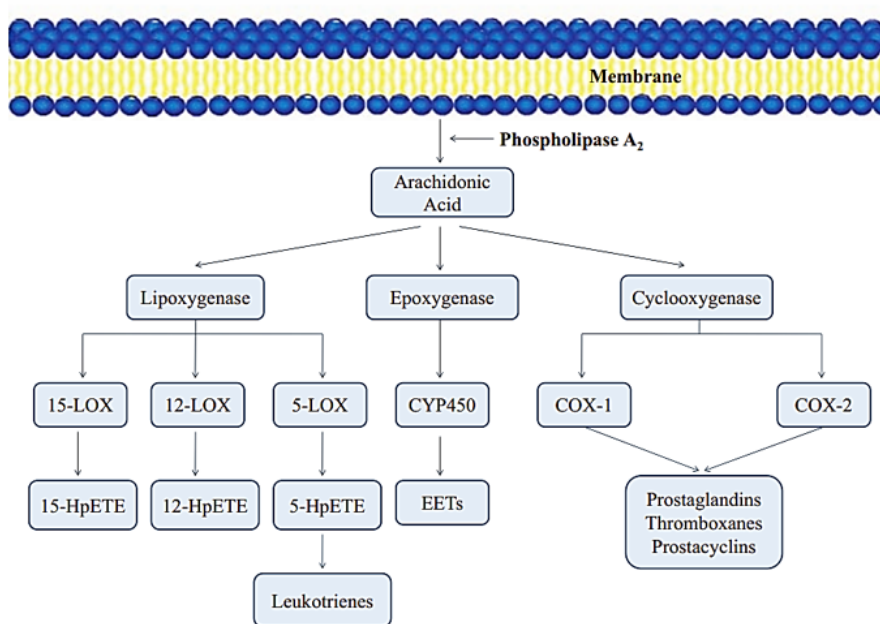


FIGURE 2.1: Mechanism of action of Lipoxygenase

The hydroperoxyeicosatetraenoic acids (HPETEs) are formed by the rearrangement of the lipid radical and the stereospecific incorporation of oxygen. These radicals are further enzymatically or otherwise reduced to HETEs or leukotrienes. This reaction is highly regioselective and the product formed is dependent on the type of lipoxygenase enzyme used [25].

2.1.5 Products of Lipoxygenase Pathway

Lipoxygenases generate a variety of biologically active lipid mediators, derived from arachidonic acid. These include mainly hydroperoxyeicosatetraenoic acids (HpETEs) that act as intermediate products in this pathway. They are then converted to hydroxyeicosatetraenoic acids (HETEs), leukotrienes and lipoxins [23].

Leukotrienes, mainly formed through the 5-LOX pathway, are highly inflammatory mediators, which promote bronchoconstriction, increased vascular permeability, and recruitment of leukocytes, thereby contributing to development of asthma and allergies.

In contrast, lipoxins that are mainly synthesized by 15-LOX are anti-inflammatory and pro-resolution mediators that function to clear inflammation and restore tissue to normalcy. HETEs are involved in the regulation of functions of vasculature and platelets, and are involved in the development of tumors [24].

2.1.6 Role of Lipoxygenase in Inflammation

Lipoxygenase (LOX) enzyme family plays an important role in regulation of the cellular homeostasis and development of different diseases by converting polyunsaturated fatty acids to active lipid mediators. **5-LOX**, **12-LOX** and **15-LOX** are molecular switches that regulate all kinds of processes from acute immune response to malignant cell growth.

LOX enzyme isoforms are a double-edged sword in the process of inflammation. The role of 5-LOX pathway in the initiation of inflammatory process is primarily the production of leukotrienes which recruit leukocytes and increase the permeability of blood vessels [27]. Other isoforms (12-LOX and 15-LOX) are very significant during the “class switching” phase in inflammation. Enzymes produce pro-resolving mediators (SPMs) of inflammation, like lipoxins and resolvins, which signalize about cessation of neutrophils infiltration and activate macrophage phagocytosis of the cellular remains. This way of functioning prevents the transformation of acute inflammation into the chronic one[28].

2.1.7 Role of Lipoxygenase in Cancer and Other Diseases

Lipoxygenases in oncology can also have a complex action that depends on the particular lipid or the specific enzyme isoform. Based on the data available, it has been demonstrated that the expression of 5-LOX and 12-LOX are often elevated in different types of cancer, including prostate, pancreatic and colorectal cancer [29]. These enzymes produce metabolites with a potent effect on cellular proliferation and inhibition of apoptosis via activation of the PI3K/Akt signaling pathway [30]. In contrast, 15-LOX-2 is a tumor suppressor, the activity of which is subdued during the growth of the tumor, and is linked to cell cycle arrest and differentiation [31].

Apart from proliferation process, LOX enzymes are implicated in regulating the epithelial-mesenchymal transition (EMT) and formation of tumor microenvironment during the development of metastasis. The increase in the expression of matrix metalloproteinases and cell adhesion molecules results in the enhancement of cell migration and invasion [32]. which helps them penetrate the basement membranes and enter the blood circulation. Besides this, eicosanoids made from LOX are involved in the neo angiogenesis of tumors [29].

The expansion of evidence shows that lipoxygenases have an important role in the pathogenesis of the cardiovascular diseases. The 12/15-LOX enzyme is also known to be involved in the oxidation of LDLs, which is one of the key steps in the

atherosclerosis pathology, within the cardiovascular system [33]. The oxidation of these lipoproteins results in the attraction of monocytes to the walls of blood vessels and foam cells formation, causing chronic remodeling of vessels and raising the risk of heart attacks [34].

High activity of 5-LOX has been associated with aging in the CNS and Alzheimer's disease - 5-LOX activity promotes increased secretion of amyloid beta peptide along with increased phosphorylation of tau protein leading to neuroinflammation and synapse destruction [35].

In addition, 12-LOX also plays a role in inflammation of adipose tissue and insulin resistance in people suffering from the Type 2 diabetes mellitus [36]. Therefore, LOX enzymes are a poly-functional therapeutic target for a number of medical specialties.

2.2 Overview of Known Lipoxygenase Inhibitors

A number of decades have seen the emergence of different scaffolds of inhibitors of LOXs grouped based on the type of inhibition, such as redox, iron-chelator and competitive non-redox inhibitors. Nordihydroguaiaretic acid (NDGA) is a typical example of a redox LOX inhibitor that by reducing the ferric iron to the inactive ferrous form. Unfortunately, most of these classes of inhibitors are commonly non-specific, and thus are accompanied by certain side effects [37].

The only clinically used 5-LOX inhibitor is zileuton (chemical structure is depicted in Figure 2.2), mostly used in treatment of chronic asthma patients. This inhibitor mechanism of action is through binding to the active site of LOXs and interacting with iron.

Besides synthetic compounds, certain natural compounds, like flavonoids (quercetin and baicalein), have also been demonstrated to be highly inhibitory with respect to 12-LOX and 15-LOX isoforms [38].

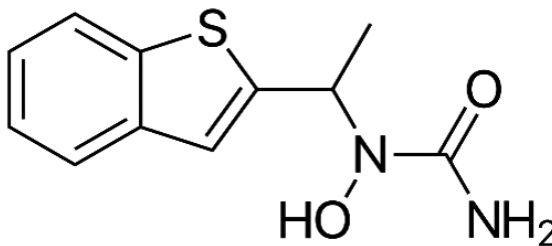


FIGURE 2.2: Chemical Structure of Zileuton

2.2.1 Limitations of Current Lipxygenase Inhibitors

Although there are clear benefits, there are a number of factors that limit the effectiveness of these lipxygenase inhibitors. The first is that most of the more or less known inhibitors, particularly the redox-active ones, are not very metabolically stable, and they show "off-target" effect, are methemoglobinogenic, or interfere with other metalloenzymes [39]. Second, zileuton is an effective drug but must be applied often due to its short half-life and has the potential to cause liver toxicity so it is only used when patient's liver function is carefully monitored. Third, many potent in vitro inhibitors do not show any efficacy in vivo due to high lipophilicity and strong plasma protein binding, which results in low ability to reach the target organs. Fourth, there is a so-called "leukotriene shunt", during which the blocking of one pathway of arachidonic acid can cause an increase in other inflammatory products, for example, prostaglandin [40].

2.2.2 Need for New and Safer Lipxygenase Inhibitors

Regarding the drug history of inhibition of LOX enzyme activity, one drug is so far outstanding - Zileuton, the only FDA approved 5-LOX inhibitor drug. Nonetheless, recent medical studies reveal several major disadvantages of this agent; first, due to the very low half-life time of Zileuton, it needs to be taken repeatedly, and secondly, there exists a proven risk of development of its hepatotoxic properties, requiring constant attention of patients [17]. In addition to the above health concerns, the efficacy of LOX inhibitors can be reduced by "leukotriene shunting,"

which occurs when an inhibitor of the COX pathway is used, and the metabolic flux is diverted towards the LOX pathway, resulting in increased leukotriene levels and worsening of diseases such as aspirin-exacerbated respiratory disease. [41].

Moreover, the need for the development of safer inhibitors has grown with the understanding of the function of LOX enzymes in ferroptosis - a kind of iron-dependent, oxidative cell death. Some specific 12/15-LOX isoforms were identified in 2025 and 2026 that specifically cause lipid peroxidation in Parkinson's and Alzheimer's diseases [42].

Future inhibitors should cross the blood-brain barrier, but should not cause systemic metabolic disruptions, as opposed to the first-generation inhibitors. Nowadays, the trend towards the development of inhibitors that act on 12/15-LOX isoforms (like ML-355 and Slug001), thus treating the pancreas from diabetes-induced damage more effectively than non-selective inhibitors, is taking place [43].

The incorporation of "The Rule of Four for Inflammation" - a recently developed structure-based approach to inhibitor design - is currently the best approach in creating potent and stable inhibitors [41].

2.3 Introduction to Computer - Aided Drug Design

Many people consider computer Aided Drug Design (CADD) technology to be one of the pillars of modern medicine that brings together the concepts of computational biology, quantum chemistry and systems pharmacology to accelerate the process of developing new drugs [44].

Typically, there are two main categories in computer-aided drug design technologies (CADD): structure-based drug design (SBDD), where three-dimensional structure of the target proteins is used; and ligand-based drug design (LBDD), where the focus is made on properties of active substances [45].

In this way, CADD technologies enable scientists to make accurate predictions about affinity and interaction of ligands and target proteins without any wet-lab experiments [44].

2.3.1 Importance of Computational Methods in Modern Drug Discovery

In the context of applying computational methods in 2026, the high development costs and long timeframes of traditional high-throughput screening (HTS) can be highlighted. Today, AI and ML are employed to deal with data heterogeneity in drug discovery, to find novel targets and to optimize lead compounds at an unprecedented speed. MD and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiling techniques were put in common use, reducing the median cost of developing new drugs [44].

2.4 Introduction to Pharmacophore Modeling

The concept of pharmacophore modeling (Figure 2.3) is one of the most advanced abstractions in computer-aided drug discovery and defines the necessary spatial and electronic properties of a molecule that can activate or inhibit some biological reaction.

Pharmacophore modeling has a few specific features: These are also known as pharmacophoric points and consist of hydrogen bond donors and acceptors, aromatic centers, hydrophobic patches, and ionizable sites. Such properties are not simply a set of functional groups, but they are defined by the exact 3D coordinates and tolerance spheres - which define the allowed geometrical deviation that allows for successful docking. Furthermore, one of the most fundamental properties is missing - the volumes of the atoms that exclude each other as a result of steric clashes are not included in basic properties [46].

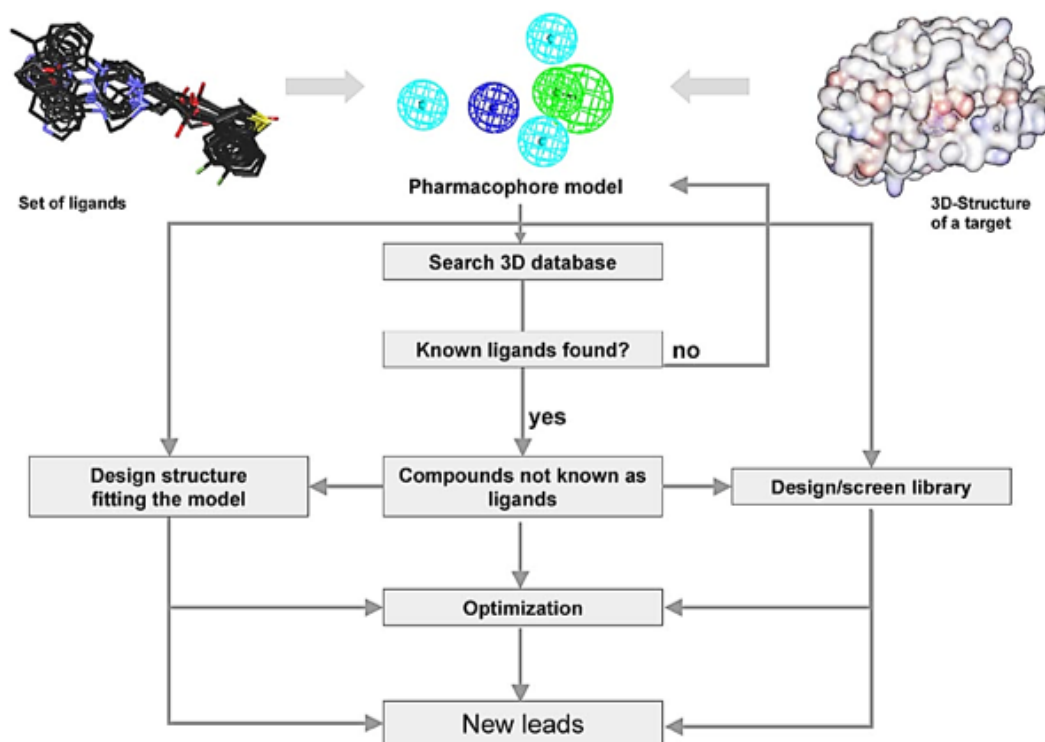


FIGURE 2.3: Pharmacophore Modeling Workflow

The advent of this methodology has brought about revolutionary changes in lead identification in terms of scaffold hopping by enabling different molecules that meet the pharmacophoric query to be considered as possible leads. Pharmacophore approach differs from SAR approach that tends to focus on replacing one of the atoms in the molecule. Thus, the method can be used to efficiently search large chemical databases and can connect structure biology and medicinal chemistry. In the modern virtual screening method, the pharmacophore model is a highly specific filter, as it is combined with geometrical restrictions. This virtual screening strategy includes geometrical restrictions, making the pharmacophore model a very precise filter, without the need for expensive in vitro testing [47].

2.4.1 Ligand-Based Pharmacophore Modeling Approach

There are basically two ways of Pharmacophore Modeling as indicated in Figure 2.4. Although the three dimensional structure of the biological target may be unknown or unavailable by X-ray crystallography or NMR techniques, the most

important method can be used is ligand-based pharmacophore modeling (LBPM) [48]. This approach was based on extracting the chemical functionality such as the hydrogen bond donors / acceptors, hydrophobic sites and aromatic moieties from a training set of multiple, already active ligands [49].

The procedure involves a detailed conformational analysis of the compounds to establish the favored conformation for the compounds that might be considered for biological activity and then alignment of the compounds to demonstrate the similarity in the spatial orientation of functional groups. In recent years, there have been some progress in implementation of 3D QSAR approach and AI latent space representation in LBPM, which expanded the prediction capacity of LBPM [50].

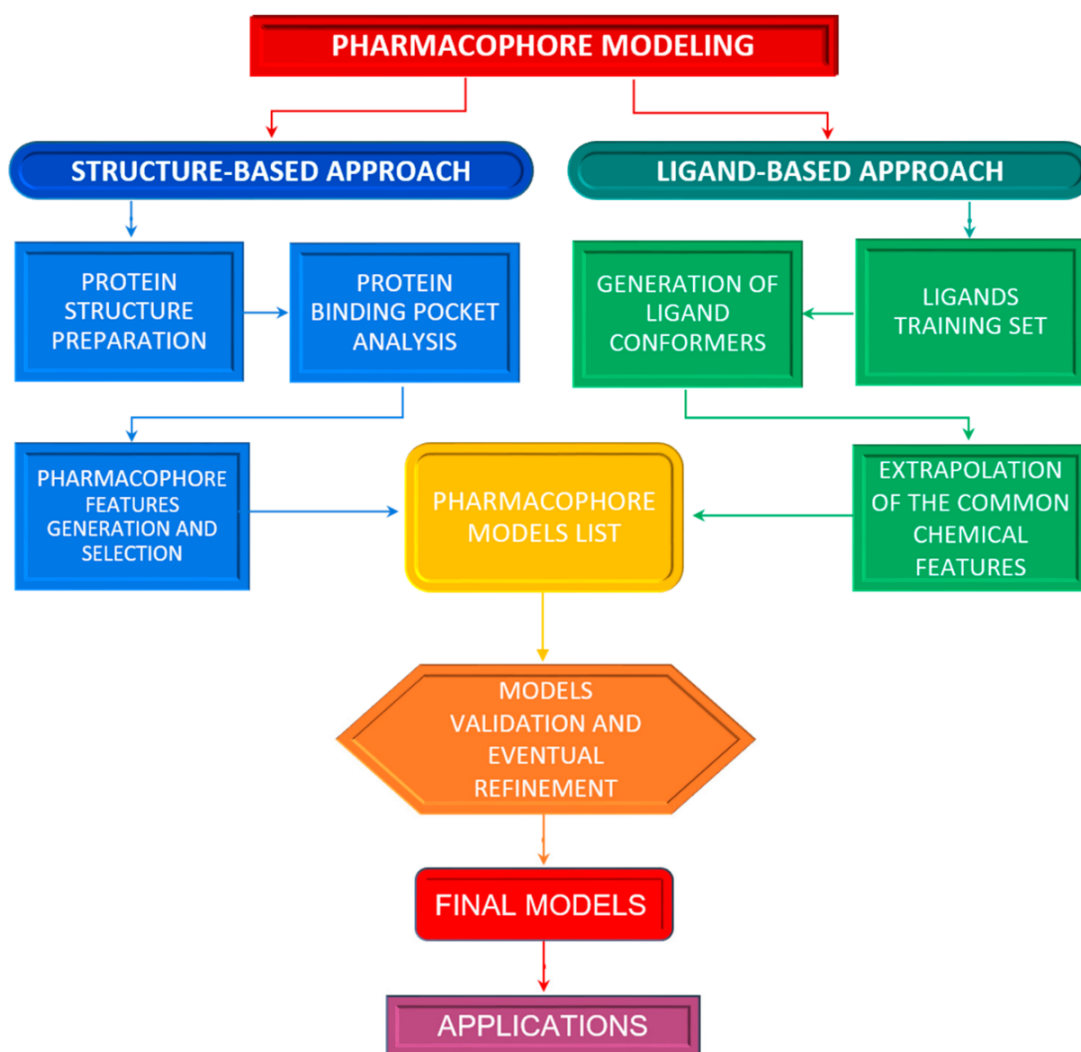


FIGURE 2.4: Approaches of Pharmacophore Modeling

2.4.2 Structure-Based Pharmacophore Modeling Approach

Structure-Based Pharmacophore Modeling (SBPM) is based on the atomistic 3D structure of the binding site of a target protein. It is used to build the pharmacophoric query based on the interactions between the target receptor and the ligand. SBPM can take into account the physicochemical properties of a binding pocket and determine which regions of contact it ought to be looking at, e.g., hydrogen bond acceptors/receptors or hydrophobic pockets. SBPM could easily detect all the limits of the binding site, including the excluded volume, which excludes steric clashes. In contemporary applications the flexible receptor is incorporated into diffusion-based generative models or molecular dynamics simulations and the SBPM is replaced by dynamic pharmacophores or dynophores instead of static ones [51].

2.4.3 Hybrid Pharmacophore Modeling

Hybrid pharmacophore modeling is a method based on using data from both known ligands and target receptor. The idea behind such modeling is to combine the strengths of both types of methods in order to get rid of their weaknesses, which are associated with bias caused by each particular type of method. Such hybrid model is especially effective when it comes to complex and/or flexible targets when a single hypothesis would not be enough to cover all possible aspects of molecular recognition, such as combining structure-based models based on different conformations of the receptor with ligand-based models based on different chemical scaffolds. A good screening strategy can then be developed - where diversity (a broader range of active scaffolds) and specificity (fewer false positives, from high structural constraint) are balanced [52].

2.4.4 Pharmacophore Model Generation and Optimization

There are various important processes in pharmacophore generation which include Conformational analysis, Feature extraction, and Hypothesis formation, and

dataset preparation. Proper preparation of the dataset is very important, since it greatly depends on the quality and diversity of the set of active compounds and determines the success of the pharmacophore model construction [53]. The problem of conformational flexibility can be solved by generating conformers of the ligands and their proper alignment with bioactive conformations. Common pharmacophoric features are commonly identified from the datasets by using genetic algorithm, clique-based method, or alignment method.

The optimization process is highly significant for improving the predictive power, and reducing the number of false positive predictions. It consists of feature types and parameters optimization such as spatial constraint and tolerances. Using the new machine learning and molecular dynamics simulation-based optimization procedures, flexible protein and interactions can be addressed and dynamic pharmacophore models created throughout different timepoints.

Multi-pharmacophore approach allows the diversity of binding modes and using the constructed models in complex biological systems. Pharmacophore modeling and QSAR modeling and docking simulation are also useful in improving the quality of the creation of the pharmacophore hypothesis [49].

2.4.5 Validation of Pharmacophore Models

The last validation stage is crucial to ensure that the pharmacophore model will be effective in subsequent application in virtual screening processes. Valid pharmacophore model needs to be able to distinguish actives from inactives. There are two types of validation procedures: internal validation and external validation. For internal validation, the evaluation of the pharmacophore model is done by using the training dataset, using statistical parameters such as root mean square deviation (RMSD), cost functions and correlation coefficients. To mention it is necessary, using just the internal validation may lead to overfitting the model. That's why it is very important to do external validation on the independent test set to estimate the robustness of the model [54].

One of the most popular validation approaches is the testing of the pharmacophore model to a mixture of known compounds that are active in the same target, and structurally diverse compounds that are not active (decoys). Typical metrics are the enrichment factor (EF), the receiver operating characteristics curve (ROC) and goodness-of-hit scores (GH). A high EF and AUC value indicates good discrimination.

The other important tool for validation of pharmacophore models is the Fischer's randomization test which is performed to determine the significance of the pharmacophore model, to see if it is generated by chance or not. There are also other methods to assess model stability, such as cross-validation and bootstrapping. To date, it has been shown that an invalidation of pharmacophore models results in poor generalization and misclassification of structurally varying molecules[55].

New advances in the field involve the use of hybrid validation methods using a combination of the pharmacophore-based screening approach, molecular docking, and molecular dynamics simulation. These techniques provide better evaluation of the analysis of ligand-receptor interaction and better accuracy of the virtual screening method [54].

2.5 Pharmacophore Model - Based Drug Designing

Pharmacophore modeling can be very useful and flexible in today's drug discovery efforts where there is limited structural information about the target protein. Pharmacophore models are used to search chemical databases for finding new molecules with the desired biological properties in a 3D query of the database. This approach saves time and testing expenses which are connected with the experimental screening process. A key utilization of pharmacophore modeling is virtual screening, a technique that involves scanning huge collections of chemical compounds for their pharmacophoric attributes so as to identify new medicines.

This approach has been applied to the identification of inhibitors, receptors, and drugs to target enzymes [56, 57].

Beyond the identification of hits, another important role of pharmacophore modelling is in the optimization of the hit, by structural modification to enhance the compound's potency, selectivity and pharmacological properties. The knowledge of the spatial arrangement of various features in a pharmacophore can enable medicinal chemists to design analogues that fit to the pharmacophoric hypotheses. Moreover, pharmacophore modeling is widely used in de novo drug designing for the synthesis of new molecular structures based on pharmacophoric hypotheses [58].

2.6 Pharmacophoric Features Required for LOX Inhibition

While creating a potential inhibitor of lipoxygenases, one needs to develop a pharmacophore model that will include all the necessary chemical functionalities that allow binding to the deep hydrophobic channel of the enzyme and interaction with catalytically active non-heme iron in its active site. A chelating/hydrogen-bonding functionality such as a hydroxamic acid, carboxylic acid or a catechol moiety capable of binding non-heme iron which di-oxygenates polyunsaturated fatty acids [59] is one of such features. The sequence of hydrophobic and aromatic groups similar to the arachidonic acid tail is frequently found in such an iron binding group [60].

Moreover, the geometry of hydrogen acceptors plays an important role in positioning of the ligands relative to the conserved residues, such as histidine and asparagine, which play a crucial role in positioning of the molecule to achieve proper inhibition. Recent computational analysis also shows the requirement for excluding volume spheres for taking into account the limited access of the catalytic site and make sure that the selected candidates do not contain bulky side chains

[59]. Therefore, using these parameters (hydrophobic tails, aromatic centers for π - π interactions and polar groups for iron coordination), researchers can distinguish selective and non-selective inhibitors and thus be able to design efficient drugs for anti-inflammatory action [60].

2.7 Design Strategies for Novel Lipoxygenase Inhibitors

Medicinal chemistry and computational design have greatly enhanced the design of novel lipoxygenases inhibitors. Conventional approaches were mainly based on substrate analogs and iron chelators; nowadays, structure- and pharmacophore-based approaches are preferred.

A strategy involves the use of iron-chelating functional groups (hydroxamic acids, catechols and N-hydroxyureas) to target the iron atom that is involved in LOX catalysis [61].

The second one is based on "hydrophobic interaction" in which a lipophilic aromatic substituent fits into the hydrophobic pocket of the enzyme.

The use of pharmacophore guided discovery has allowed the identification of the essential structural elements for the inhibition of the LOX enzyme, that include hydrogen bond donor/acceptor, hydrophobic group and metal binding properties. Several combinations of different pharmacophoric groups in a single molecule have led to higher activities and selectivities [61].

Bioisosteric replacement and scaffold hopping are other frequently used strategies that are applied to the improvement of the pharmacokinetic and pharmacodynamics properties of the inhibitors.

Recent advances include the use of artificial intelligence (AI) and machine learning (ML) methods to develop new inhibitors by predicting SARs.

2.8 Molecular Docking Studies of Lipoxygenase Inhibitors

Lipoxygenases (LOX) are non-heme iron proteins whose action leads to the oxidation of polyunsaturated fatty acids in order to produce biologically active lipid mediators that contribute to inflammatory processes and cancer development [62].

The study of the interaction of these enzymes and potential inhibitors has received significant importance with the use of molecular docking technique. The molecular docking enables predicting the orientation and mode of binding of the ligand molecules to the active site of LOX.

In the catalytic domain of lipoxygenase there is an iron ion bound by histidine amino acids along with a hydrophobic pocket where the substrate binds. The molecular docking studies indicate that efficient lipoxygenase inhibitors exhibit interactions with the iron centre both by direct chelation and through hydrogen bonds and hydrophobic interactions.

The interacting residues can be His367, His372, His550 or Ile673 (which will vary depending on the specific isoform) [63].

For the prediction of the binding energies and patterns of the candidate molecules, the docking programs such as AutoDock, Glide and GOLD have been widely used. A few studies have suggested using a combination of docking and pharmacophore modelling gives a greater level of confidence in the identification of hits [64].

Besides docking, the binding free energy estimation (MM-GBSA) and molecular dynamics simulation provides more information regarding the stability of the ligand-protein complex.

Useful as they are, docking predictions have a number of problems, such as protein flexibility, scoring functions, and the quality of the input structure [65].

2.9 Synthesis of Pharmacophore-Guided Compounds

Pharmacophore guided compound synthesis involves the computational methods of identifying pharmacophores to be synthesized chemically. Once the pharmacophore model is discovered, it is possible to carry out synthesis of the compounds having the required characteristics such as H-bond donors/acceptors, hydrophobic moiety and metal binding ability.

Some of the synthetic approaches can involve multi-step organic synthesis with the presence of functional group transformations, cyclizations and coupling reactions including amide bond formation and Suzuki-Miyaura cross-coupling.

For LOX inhibitors, benzothiazoles, quinolines, 2-furanones and indoles are some of the heterocycles used for the purpose of desired biological activity [66].

Structure modification for enhancing the biological activity and selectivity is made easy with the pharmacophore guided compound synthesis. The study on the SAR is essential for optimizing synthesized compounds by replacing the functional groups and for biological evaluation of the synthesized compounds.

One of the different methodology which was synthesized using new approach is green chemistry approach and microwave assisted synthesis [67].

2.10 Characterization Techniques for Synthesized Compounds

To ensure the chemical structure, purity and physicochemical properties of a synthetic compound, it is essential that it is properly identified. A variety of spectroscopic and analytical methods are used.

2.10.1 Melting point Determination

Determination of melting points is one of the significant techniques used for the characterization of medicinal chemicals. It involves determination of the exact temperature at which the substance melts. The reason why this procedure is important is because it gives important information regarding the identity and purity of the substance [68].

Pure substances have very sharp ranges of melting points whereas the presence of impurities may lead to broadening of melting points, either in the upward or the downward direction. The correct melting point of a molecule is crucial for its pharmaceutical development because it provides information on its identity and purity - characteristics of utmost importance in the case of medicinal products. Additionally, to ensure identity and consistency of drugs being developed, the melting point of each batch needs to be the same [69].

2.10.2 Fourier-Transform Infrared Spectroscopy

FTIR - Fourier transform infrared spectroscopy is one of the most useful methods for the characterization of the pharmaceutical products because of its ability to identify functional groups and chemical structures. FTIR measures absorption and transmission of infrared light by molecules which produces a unique fingerprint that explains the chemical nature of the drug.

FTIR can be useful in identification and validation of the nature and chemical structure of the pharmaceutical component, by checking the functional groups present in the pharmaceutical compounds.

The technique is important in assuring consistency in the formulation of drugs by detecting any contaminants or chemical variations in the drug. FTIR makes the comparison of spectra easy thus tracking and verifying chemical modifications in the drug during development [70, 71].

2.10.3 Nuclear Magnetic Resonance Spectroscopy

The nuclear magnetic resonance (NMR) is an analytical method that allows chemists to obtain the information on the structural and dynamic properties of molecules, as well as their composition. NMR detects the magnetic nature of some atomic nuclei, in particular of those having a (H1-NMR), carbon-13 (C13-NMR), and nitrogen-15 (N15-NMR). The nuclei absorb energy, and are flipped to another spin state, because of being in a strong magnetic field and the radiofrequency (RF) radiation. This results in the generation of an absorption signal which allows one to establish the way of connections of atoms in a molecule [72].

NMR applications are particularly important in the fields of pharmacy and biomedical research. This approach allows for determining the structure of the molecule, its stereochemistry and the presence of isomers and tautomers that are important for the drug discovery and development. Furthermore, it helps structural biology to explore the interactions between drugs and their targets, such as proteins and nucleic acids. To understand the molecular behavior of the drugs, 3D information on the atomic structure is required, which is given by NMR [73].

2.11 Pharmacological Evaluation of Lipoxygenase Inhibitors

Pharmacological evaluation of LOX inhibitors involves both in vitro and in vivo tests to assess the efficiency, specificity and toxicity of the inhibitors. Typically in vitro assays consist of measuring the effects of inhibitors on purified lipoxygenase enzymes or on cell systems [74].

The most common assays are those for hydroperoxide production and oxygen consumption assays. Cell experiments help to elucidate the effects of compounds on the inhibition of leukotriene production and inflammation. The pharmacodynamic and pharmacokinetic properties of the LOX inhibitors are evaluated in vivo using

animal models of inflammation, asthma or cancer. Such parameters as the availability, half-life, and toxicity of drugs are estimated. The LOX inhibitor, Zileuton, is a clinically tested compound that could be used as a reference compound for study of the other LOX inhibitors [75].

Even though there have been some promising findings, there is still need for solutions to problems such as poor selectivity, metabolism instability, and toxicology.

2.12 Bridging the Gap in Lipxygenase Inhibitors Discovery

Even though there have been great strides in developing inhibitors of lipxygenase enzymes, only a few inhibitors have managed to make their way to the clinic because of problems like poor selectivity, metabolism instability, toxicity and pharmacological properties.

Actually, during the past few years, the use of Computer-Aided Drug Design technique including molecular docking, pharmacophore modeling, QSAR analysis, scaffold hopping and virtual screening has been helpful in the search and optimization of lipxygenase inhibitors. These methodologies, in particular, have contributed to gaining a deeper understanding of the structure and physical-chemical properties that need to be fulfilled for successful enzyme inhibition.

Moreover, the use of multitarget-directed ligands is a promising approach to overcome the issue of pathway shunting and the related side effects of selective inhibition. However, additional investigations to improve bioavailability, decrease toxicity and validate biological activity are still needed [76].

The integration of these technologies, coupled with experiments such as organic synthesis, NMR and FTIR, are sure to bridge the drug discovery and development gap. Theoretical prediction and empirical data from structural and spectroscopic studies allow scientists to get a full picture of interactions at the molecular level. This enables rational design of novel drugs that are safer, more efficient and have

better pharmacokinetics. Hence, these approaches will prove to be useful for the faster and efficient drug discovery process [77].

Overall, continued developments of experimental and computational methods should help bring about a new generation of lipoxygenase inhibitors.

Chapter 3

Material and Methods

3.1 Materials

The computational Studies required advanced software tools that are reliable for drug discovery. The materials were carefully selected to ensure the consistent and reproducible results. Chemicals, laboratory materials, and the solvents were procured from the recognized providers to preserve uniformity across the synthetic, analytical, and biological processes.

3.1.1 Software and Tools

Maestro was used for computational studies; the graphical interface was Schrödinger Suites 2022-2024. The utilized different modules that were used are, LigPrep was used for Ligand Preparation, Protein Preparation Wizard for preparation of protein, Glide Module for Docking studies,

Prime Module for binding energy prediction using MMGBSA. Compounds are designed using ChemDraw Professional 21.0.0. Discovery Studio 2017 was used for visualization of compounds. Simulation Plus was used for *In-Silico* ADMET profiling of final potential inhibitors.

3.1.2 Databases

ChEMBL is a manually curated, freely available database of bioactive molecules with drug-like properties maintained by *European Molecular Biology Laboratory, European Bioinformatics Institute* (EMBL-EBI). DUD-E Decoys Database is used to generate decoys.

3.1.3 Chemicals

Chemicals used in this experiment are of Analytical Grade. Norfloxacin was purchased from *Luoyang Zhengmu Bio-Tech Co., Ltd.* Other chemicals like, Chloroacetyl chloride, Potassium Iodide 2-Amino-Benzothiazole, Aniline, Toluene, Anisidine, Phenylenediamine, para-Aminophenol, Sodium Chloride and Magnesium Sulfate were purchased from *Sigma-Aldrich*, and used without further purifications.

3.1.4 Solvents

The solvents used for the synthesis of compounds, their purification, and spectrum analysis were of Analytical Grade from Sigma Aldrich, including Triethylamine (TEA), Dichloromethane (DCM), Methanol (MeOH), and Dimethyl Sulfoxide (DMSO). All the solvents were analytical grade and employed directly in experiments with no further purification. For moisture sensitive processes, anhydrous solvents were used. Prior to usage in experiments, DCM was dried on activated molecular sieves.

3.1.5 Apparatus and Equipment

The compounds were synthesized, purified, and characterized using standard analytical equipment. An analytical balance, round bottom flasks, reflux condensers, magnetic stirrers, heating mantles, Hot plate, and micropipettes were used. TLC

plate (Silica Gel 60 F254) from Merck Pharma was used. FTIR was done using ATR-FTIR (Cary630) from Agilent Technologies. Gallenkamp melting point apparatus was used for melting point determination of the synthesized compounds.

3.2 Methods

3.2.1 Ligand Based Pharmacophore Modeling and Drug Designing

3.2.1.1 Extraction and preprocessing of Data

For pharmacophore modeling, a collection of known 5-LOX inhibitors was gathered from the ChEMBL database, which is a freely accessible chemical database that provides extensive information on bioactive compounds, including their chemical structures, interactions with targets, bioactivity data, and properties related to ADMET [78]. The target protein for this study was human 5-LOX (ChEMBL ID: ChEMBL215; UniProt ID: P09917).

A total of 4251 known inhibitors were extracted from ChEMBL. The list of compounds is filtered by pChEMBL value, the compounds having known pChEMBL values were selected which narrowed the dataset to 3476 compounds.

These compounds were organized into a Tab-Separated Values (TSV) file, which included details like molecular weight (Mol.Wt), pChEMBL values, ALogP, IC₅₀ (nM), and SMILES (Simplified Molecular Input Line Entry System) notations.

Then, the SMILES representations were changed into 3D SDF format using the Online SMILES Translator from the NCI/CADD Group (<https://cactus.nci.nih.gov/translate/>) which is suitable for molecular modeling and pharmacophore development [79].

3.2.1.2 Scaffold Decomposition

To classify and identify compounds based on their common scaffolds, the refined dataset underwent further analysis using the Schrödinger Suite Maestro [80]. During scaffold decomposition, compounds with a shared scaffold were grouped together into 2661 categories based on the number of rings or ring systems they have. They can further be filtered through parameters like Chirality, pIC50, etc. [81]. Researchers identified and classified a range of structurally different scaffolds by their ring system counts. One significant scaffold, 3-Phenylpyridine was selected to create a Pharmacophore Model further. Figure 3.1 shows the chemical structure of chosen scaffold.

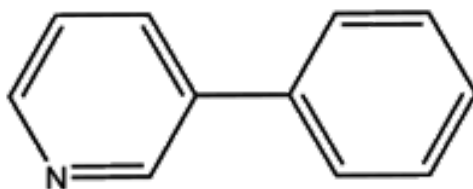


FIGURE 3.1: 3-Phenylpyridine Scaffold

3.2.1.3 Splitting into Training set and Test Set

After selecting the scaffold, the 60 compounds linked to that scaffold were split into distinct training and test sets. This was a crucial step in developing and validating the pharmacophore model. The compounds were divided randomly into training set and test set in 70:30 ratio. The training set included 70% of the molecules that were randomly selected, while the test set had 30% of the molecules. The training set particularly consisted of 42 randomly allocated compounds, while test set consisted of 18 randomly allocated compounds [82].

3.2.1.4 Ligand Preparation

Next, to improve the tautomeric and protonation states of each molecule within the physiological pH range of 7.0 ± 2 , LIGPREP and EPIK were used to process the inhibitors in both sets [83, 84]. The energy minimization process for each

molecule was made simpler by using the OPLS4 force field with its default settings [85]. Furthermore, tautomers and stereoisomers were generated, determined chirality from the 3D structure, generated at most 32 per ligand, and finally, the minimization of the drug molecule process was performed using the force field OPLS4 [86].

3.2.1.5 Development of Ligand-Based Pharmacophore Model

The pharmacophore model, which acts as 3D query, has been developed in order to identify the key chemical properties required for the molecule's biological activity [47], and it was developed using the PHASE module from the Schrödinger Suites [87]. Pharmacophore models were generated using a ligand-based approach using the training set, and a threshold of the 6.9 pIC₅₀ value was used to define the active molecules. Particularly, 30 molecules that possessed a pIC₅₀ value <6.9 have been considered inactive, whereas 18 molecules having pIC₅₀ value ≥ 6.9 were considered active. There are six crucial features of the PHASE module that have been taken into consideration for developing the pharmacophore model: Aromatic ring (R), Hydrogen bond Donor (D), Hydrogen bond Acceptor (A), Hydrophobic site (H), Positively ionizable (P), and the Negatively ionizable (N), bounded by a spatial tolerance radius of 2.0 Å, where each model consists of 4-6 features. Furthermore, a hypothesis difference criterion of 0.50 was implemented to enforce geometric variance among the outputs, and a maximum of 10 hypotheses were requested for each matching feature combination and "Excluded volume" was enabled.

3.2.1.6 Validation of Pharmacophore Model

The performance of the created pharmacophore models was assessed to gauge their ability to identify active compounds utilizing the compounds from the testing set. To enhance the evaluation, a decoy set of 900 compounds was created from the test set using the DUD.E database [88], utilizing the SMILES representations of the compounds from the test set for the generation of decoys. The decoys were

created and employed alongside the Test set to comprehensively assess the effectiveness of the produced models [89]. An extensive evaluation of the Enrichment Reports related to each pharmacophore model was conducted. The enrichment viewer wizard from the PHASE module was employed to deliver a comprehensive evaluation of the model's capacity to differentiate between active compounds and decoys, supplying important insights into the anticipated accuracy and general effectiveness of the system.

3.2.1.7 Designing of Potential Inhibitor(s)

ChemDraw Professional 21.0.0 was used to design and draw 80 different potential inhibitors by using structure-based drug design based on the pharmacophore model features, considering the measure of bond distances, bond angles, etc. The designed inhibitors should match as many features of pharmacophore model as possible.

3.2.1.8 Screening and Alignment with Model

The designed compounds are then screened through the model to match the chemical features along with feature distances and angles [90]. The top 7 compounds with best Fitness Score, Vector score, Volume score, Phase Screen Score, and Align Score are selected for further confirmation of affinity with targeted 5-LOX through molecular docking and further studies.

3.2.1.9 Selection and Preparation of Protein

For Molecular Docking studies, the high-resolution X-ray crystal structure of the human 5-LOX protein (RCSB PDB ID: 3V99) in complex with Arachidonic Acid, with a resolution of 2.25Å, was retrieved from the *Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB)* (<http://www.rcsb.org>) [91].

Using the Protein Preparation Wizard (PPW), built into the Schrödinger interface, the protein was prepared. PPW has a three-step workflow in the following order: (1) import and refine, (2) review and modify and (3) minimize [92].

The first of protein preparation involves preprocessing in the workspace, where the protein structure is chosen, bond orders are assigned using the CCD Database, explicit hydrogens are added to the structure, zero-order bonds are established for metals, disulfide bonds are formed, and missing side chain atoms are optimized by executing a Prime job. Additionally, missing loops are filled in by running the Prime job, and water molecules located more than 5.00 Å away from any heteroatoms, including ions, are removed. The next step involves checking and adjusting when a new chain, water, or heteroatom is chosen to zoom so that the selection fits within the workspace, choose waters and hets within 5.0 Å of the selected chains, maintain the other residues and chains as the default setting, and create a state pH of 7.5.

The third step involves refining the chosen orientations of water samples along with other groups, setting the protonation states of residues and ligands using PROPKA at pH: 7.5, then simulating the precise experimental conditions and choosing to optimize hydroxyl, ASN, GLN, HIS states automatically with ProtAssign, while excluding water that is further than 3.0 Å beyond hets, along with ions. The restrained minimization of proteins was performed using OPLS4 force field [93].

3.2.1.10 Molecular Docking Studies

Molecular docking was performed using the GLIDE (Grid-based Ligand Docking with Energetics) module of Schrödinger [93]. During the receptor grid generation step, a docking grid was defined using the Grid Center coordinates: $x = 8.5014$, $y = -79.1715$, and $z = -25.2689$, which correspond to active binding site of 5-LOX. A scaling factor of 0.8 Å was applied to refine the Van der Waals radii of the receptor atoms for better accuracy. To achieve higher reliability and precision in ligand-receptor binding prediction, the Extra Precision (XP) docking mode [94] was used.

3.2.1.11 MMGBSA Binding Energy

MMGBSA (Molecular Mechanics Generalized Born Surface Area) analysis was carried out on the XP docked complexes through PRIME in the Schrödinger Suite [93]. This can be useful in assessing the binding free energy of the ligand with the use of molecular mechanics and solvation. The MMGBSA calculations were done using the pose viewer files generated after the docking process [95].

The calculations involved different computational techniques which included the use of the OPLS4 force field in the molecular mechanics, rotamer search algorithms to improve side chains, and VSGB solvation model [96].

Overall, this MM-GBSA continuum solvent model offered a deeper insight into the binding energetics by taking into account both the molecular interactions within the complex and the effects of the aqueous environment.

3.2.1.12 Drug Likeness and *In-Silico* ADMET Profiling

The selected potential inhibitor underwent *in-silico* ADMET profiling with the ADMET predictor 12.0 on the Simulation Plus (<https://www.simulations-plus.com/software/admetpredictor/>), an advanced computational tool [97].

Comprehensive assessments of various factors were performed at a physiological pH of 7.4. The ADMET profiling aimed to predict and examine the molecule's absorption, distribution, metabolism, excretion, and toxicity characteristics to offer crucial insights into its pharmacokinetic and safety attributes.

3.2.2 Scheme of Synthesis

The compounds are synthesized in two steps as shown in the Scheme of Synthesis in Figure 3.2.

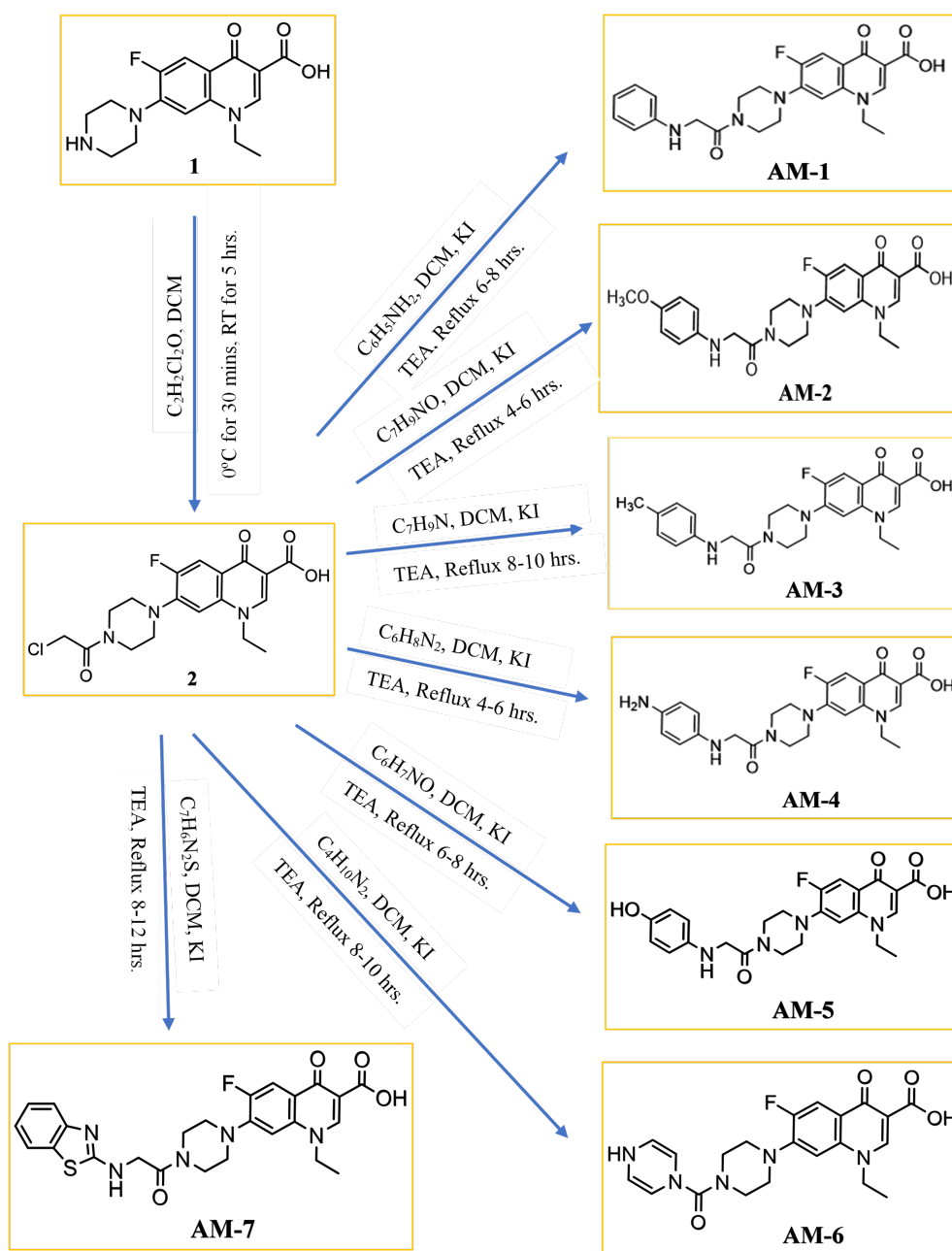


FIGURE 3.2: Scheme of Synthesis of Compounds AM-1 to AM-7

3.2.2.1 Step 1: General Procedure for Synthesis of Intermediate Compound 2

In the first step, the intermediate **Compound 2** (7-(4-(2-Chloroacetyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid) was synthesized following the already reported method [98, 99]. **Compound 1** i.e. Norfloxacin free base (3.84g, 12 mmol) was dissolved in 50 ml of dry Dichloromethane (DCM), followed by addition of Triethylamine (TEA) (1.7 ml, 12 mmol) with stirring for

20 mins at 0°C. Then Chloroacetyl Chloride (1.45 ml, 18 mmol) was slowly added over the period of 30 minutes, with additional stirring for 30 minutes at 0°C, followed up by TLC to confirm the reaction status, using Methanol (MeOH) and Dichloromethane (DCM) in 1:9 as Mobile Phase. The solution is then kept at room temperature for 5 hours. The reaction mixture was filtered, and the obtained solid was thoroughly washed with water and DCM. The aqueous layer was extracted with 3 portions of 30 ml DCM. The organic layer was dried over MgSO_4 and concentrated at room temperature.

3.2.2.2 Step 2: General procedure for synthesis of Final Compounds

To a solution of intermediate compound 2 (395.81 mg, 1 mmol) in dry Dichloromethane (30 mL), Triethylamine (140 μL , 1 mmol) was added at room temperature, with stirring for 15 minutes. For activation, Potassium Iodide (166 mg, 1 mmol) was added and stirred for additional 15 minutes at room temperature. After the activation, the equimolar amount (i.e. 1 mmol) of the selected Amines including 2-Aminobenzothiazole, Aniline, Toluidine, p-Anisidine, p-Aminophenol, piperazine, and phenylenediamine were added in the reaction mixture. The progress of the reaction was monitored by TLC with a suitable mobile phase. Upon completion, the reaction was quenched with saturated Brine solution. The reaction mixture was cooled and filtered. The residue is dried at room temperature to yield dried product.

3.2.3 Characterization

3.2.3.1 Fourier Transform Infrared Spectroscopy

The FTIR Spectra for all the samples were obtained by using Cary 630 FTIR spectrometer (Agilent technologies) using the transmittance modality. The spectra were recorded using a base setting of 2cm^{-1} resolution and a wave number range of $4000\text{--}650\text{cm}^{-1}$. All measurements were taken and recorded at 25°C.

3.2.3.2 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) served as a key analytical tool for determining molecular structures, as well as for quality control and research to evaluate the composition and purity of samples. This technique was based on the interaction between magnetic fields and the magnetic moments of atomic nuclei. The magnetic moment of a nucleus arose from its angular momentum, which was generated by the nucleus spin. The magnitude of this spin was quantified by a value known as the spin number which was vital for understanding the nuclear spin behavior. The properties of nuclear spin, including the spin number, were used to define the characteristics of the nucleus. In cases where a nucleus contained an equal number of protons and neutrons, both its nuclear spin and magnetic moment were effectively zero.

3.2.4 *In-Vivo* Anti-Inflammatory Activity

To evaluate the *in-vivo* anti-inflammatory potential of the synthesized compounds, the Acute Carrageenan-induced paw edema model was utilized.

3.2.4.1 Experimental Animal Grouping and Acclimatization

Female BALB/c mice, weighing between 20–25 g were housed under standard laboratory conditions with a controlled temperature environment (25°C), standard humidity environment, and free access to a diet and water ad libitum.

Prior to initialization, the animals were randomly assigned into five experimental groups (n = 6 animals per group) and allowed to acclimatize for one week. The animals were grouped as follows:

- i. Group I (Normal Control/ Blank): Received the vehicle baseline (Normal Saline, 2 mL, i.p.)

- ii. Group II (Negative Control): Received 0.04 ml of 1% Carrageenan in sub-plantar region of right hind paw of mice.
- iii. Group II (Positive Control): Treated with diclofenac sodium, 20 mg/kg, i.p. [100].
- iv. Group III (Low Dose): Treated with test compound at a dose of 10 mg/kg, p.o.
- v. Group IV (Medium Dose): Treated with test compound at a dose of 20 mg/kg, p.o. [101].
- vi. Group V (High Dose): Treated with test compound at a dose of 30 mg/kg, p.o.

3.2.4.2 Dose Preparation

The compound was dissolved in high-concentration pure DMSO, then diluted with 0.9% Normal Saline, so that the final DMSO Concentration remained below 10% (V/V). [102].

3.2.4.3 Procedure

The baseline thickness of the right hind paw of each mouse was measured precisely prior to any pharmacological intervention (0h) using a standard mechanical screw gauge. Following baseline documentation, the vehicle, reference standard, and varying doses of test compound were administered intraperitoneally to their designated cohorts.

Exactly 30 minutes post-injection, acute localized inflammation was induced by a subcutaneous injection of 0.04 mL of a 1% w/v carrageenan solution [103] in normal saline into the sub-plantar tissue of the right hind paw of each mouse.

3.2.4.4 Assessment and Calculation Metrics

Following induction, the subsequent progress of linear paw thickness was measured by Vernier Calipers [104] at regular intervals of 0, 1, 2, 4, and 6 hours post-carrageenan administration. The raw expansion index for each time increment was determined as the net change between the thickness recorded at the respective hour (T_t) and its initial baseline threshold at zero hours (T_0).

The percentage inhibition of edema, which reflects the systemic anti-inflammatory efficacy of test compound relative to the control, was calculated at each interval using the following standard equation:

$$\text{Percentage Inhibition (\%)} = \frac{\Delta T_0 - \Delta T_t}{\Delta T_0}$$

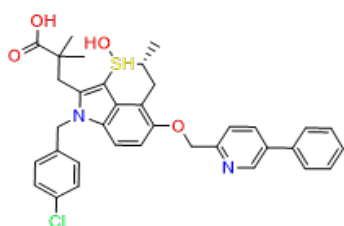
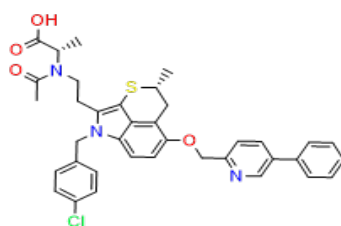
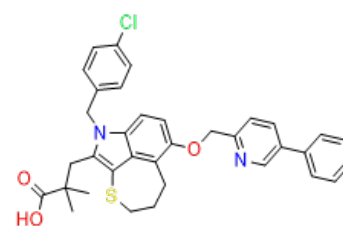
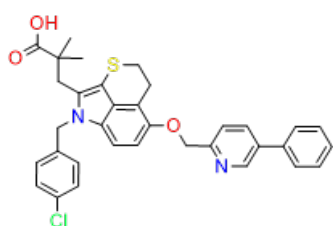
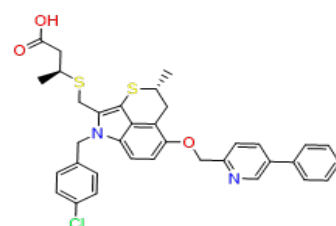
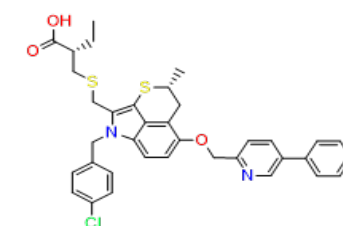
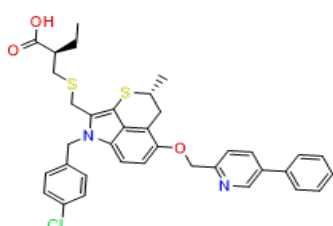
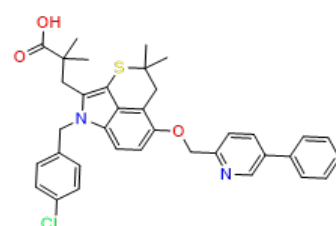
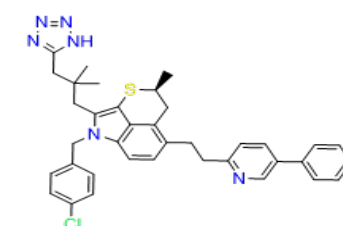
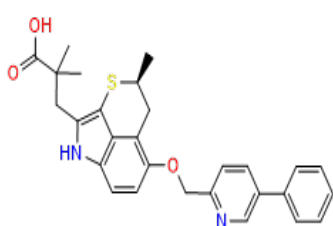
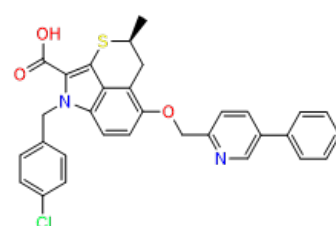
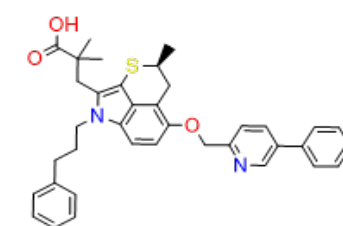
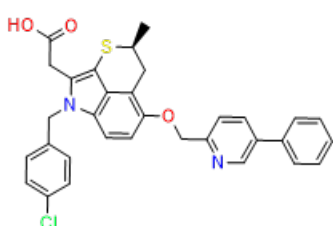
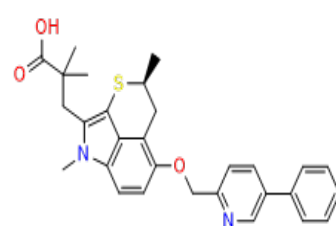
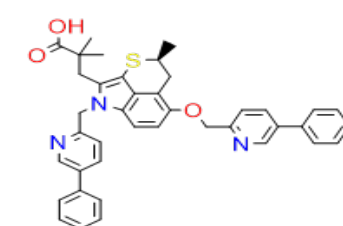
Two way ANOVA was used to systematically evaluate statistical significance of percentage inhibition of paw odema at different hours after inducing inflammation by carrageenan.

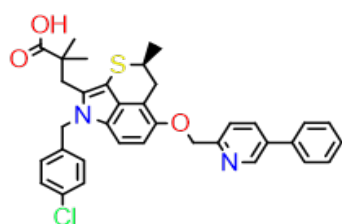
Chapter 4

Results and Discussion

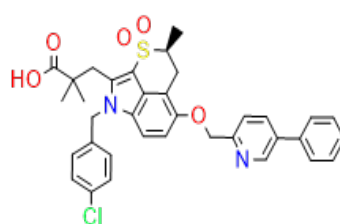
4.1 Processing of Training Set Compounds

The training set, consisted of 42 compounds, shown in the figure 4.1, were converted into a Phase database to create the stereoisomers by retaining some fixed chiral centers while modifying other chiral centers. The phase database consisted of up to one low energy stereo-conformation for 4 or 5 membered rings while preserving a maximum of 4 lowest possible energy stereoisomers. Utilizing the prepared Phase database and applying the criteria aforementioned in Chapter 3, a pharmacophore model was established. According to the preset criteria of threshold pIC₅₀ value i.e. ≥ 6.9 , 30 compounds out of 42 were considered as active compounds. However, 12 compounds whose pIC₅₀ values fall short of the threshold were considered as inactive compounds. So, compound 4, 5, 6, 7, 8, 9, 11, 13, 15, 16, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 31, 32, 33, 34, 37, 38, 39, 40, 41, and 42 are active. On the other hand, compound 1, 2, 3, 10, 12, 14, 17, 18, 29, 30, 35, and 36 are inactive.

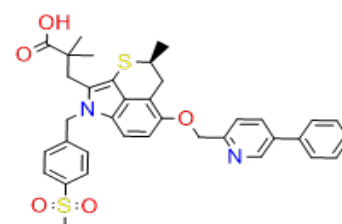
**Compound 1**pIC₅₀ Value: 6.08**Compound 2**pIC₅₀ Value: 6.05**Compound 3**pIC₅₀ Value: 6.80**Compound 4**pIC₅₀ Value: 7.00**Compound 5**pIC₅₀ Value: 7.16**Compound 6**pIC₅₀ Value: 7.40**Compound 7**pIC₅₀ Value: 6.92**Compound 8**pIC₅₀ Value: 7.06**Compound 9**pIC₅₀ Value: 8.52**Compound 10**pIC₅₀ Value: 6.30**Compound 11**pIC₅₀ Value: 7.01**Compound 12**pIC₅₀ Value: 6.52**Compound 13**pIC₅₀ Value: 7.43**Compound 14**pIC₅₀ Value: 6.52**Compound 15**pIC₅₀ Value: 7.80



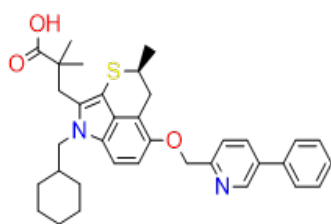
Compound 16
pIC₅₀ Value: 7.66



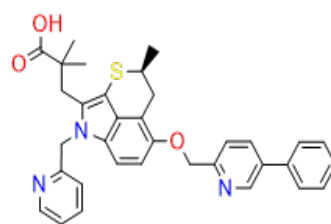
Compound 17
pIC₅₀ Value: 6.68



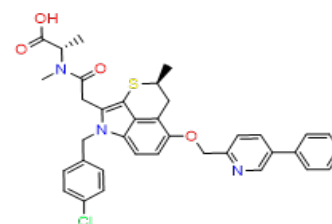
Compound 18
pIC₅₀ Value: 6.38



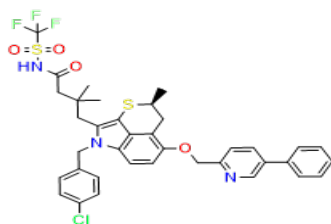
Compound 19
pIC₅₀ Value: 7.48



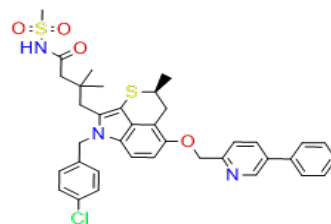
Compound 20
pIC₅₀ Value: 6.96



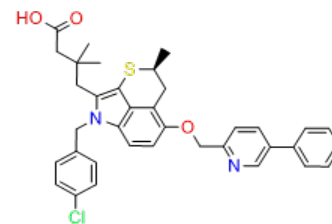
Compound 21
pIC₅₀ Value: 7.64



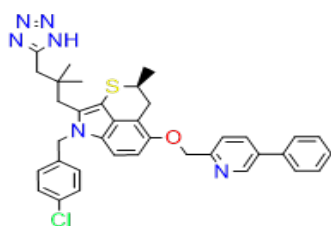
Compound 22
pIC₅₀ Value: 6.90



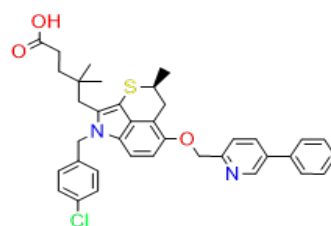
Compound 23
pIC₅₀ Value: 7.96



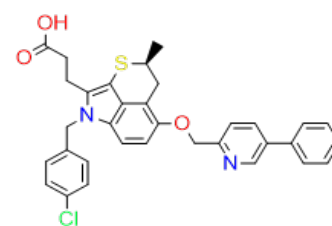
Compound 24
pIC₅₀ Value: 7.92



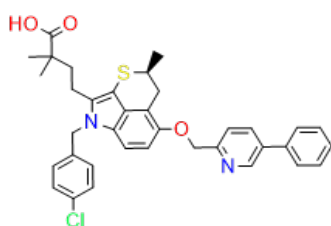
Compound 25
pIC₅₀ Value: 8.10



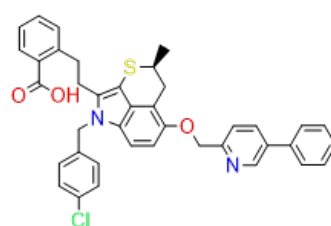
Compound 26
pIC₅₀ Value: 7.72



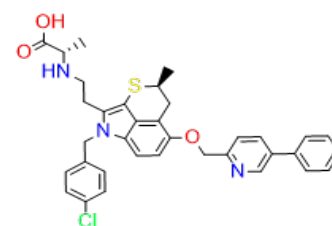
Compound 27
pIC₅₀ Value: 7.42



Compound 28
pIC₅₀ Value: 7.64



Compound 29
pIC₅₀ Value: 6.44



Compound 30
pIC₅₀ Value: 5.40

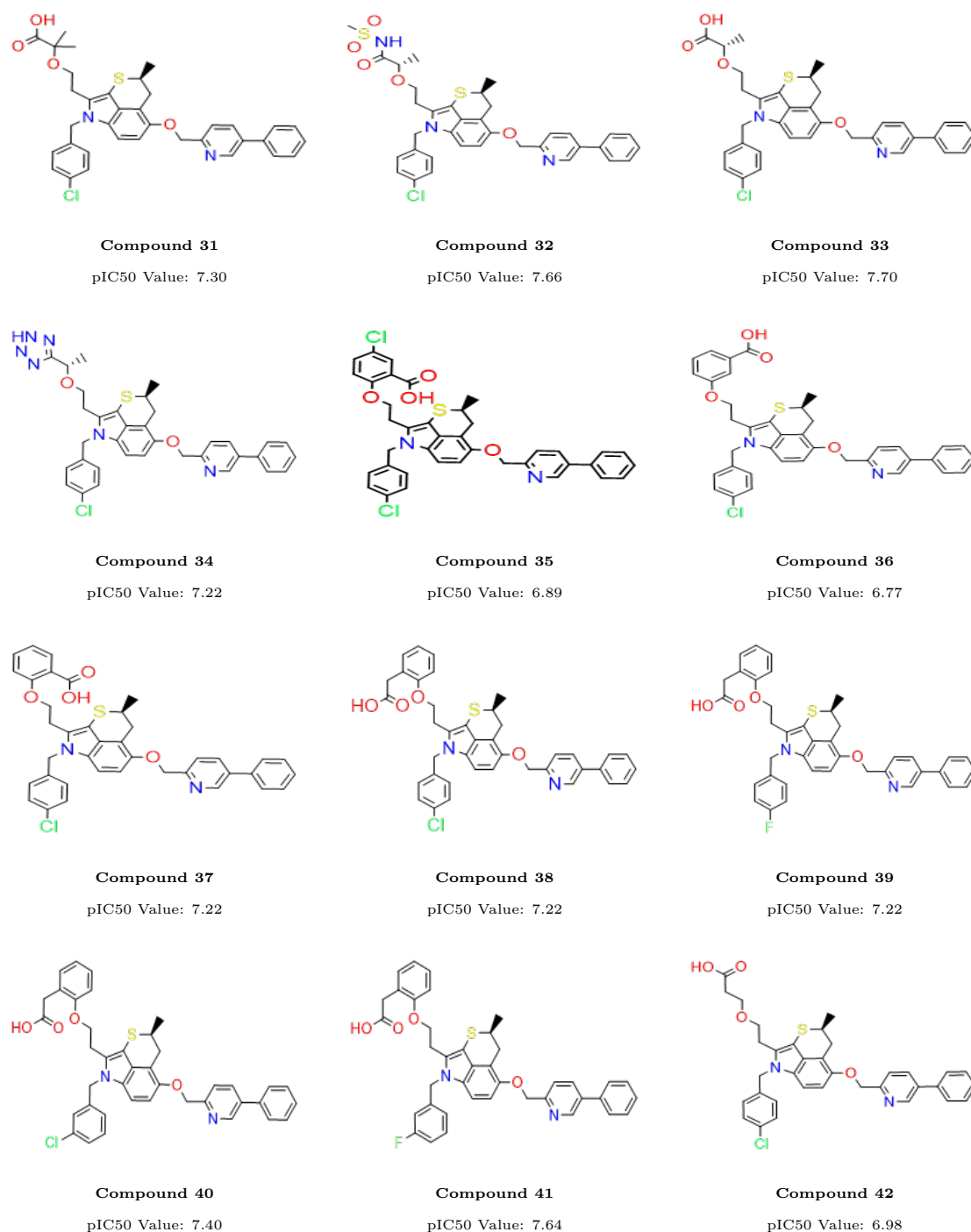


FIGURE 4.1: Chemical structures with corresponding pIC₅₀ Values of 42 Training Set

4.2 Development of Pharmacophore Models

The alignment and common feature extraction protocols successfully yielded a total of 30 distinct pharmacophore hypotheses. These models were categorized into

four separate chemical feature combinations (variants): HRRR, HHRR, HHRRR, and AHHRRR. The successful generation of highly complex 5- and 6-feature models indicates a high degree of structural conservation and shared spatial architecture among the active compounds within the training set. All the 30 models of pharmacophore hypothesis generated are categorized into four different structural classes based on the spatial structure and chemical composition of each model shown in Table 4.1.

Due to the Hydrophobic (H) and Aromatic Ring (R) properties, the binding structure has been determined as mainly Lipophilic and Structural stacking. Models with fewer features, namely HRRR (7 models) and HHRR (3 models), provide the basic structure of the active set of compounds and are very useful as permissive high throughput screening models. The highest complexity was obtained for the 5-feature (HHRRR) and 6-feature (AHHRRR) classes, each of which was able to generate 10 models. While the HHRRR framework maps out an extensive hydrophobic network using two lipophilic centers and three aromatic rings to accommodate the extended scaffolds of the active compounds, the AHHRRR class represents the most chemically sophisticated and selective variant by integrating a critical, directional Hydrogen Bond Acceptor (A) into that same expansive hydrophobic matrix. Ultimately, this structural hierarchy transitions from general core features to highly specialized, 6-feature spatial arrangements.

TABLE 4.1: Distribution and Features of Generated Pharmacophore Hypotheses

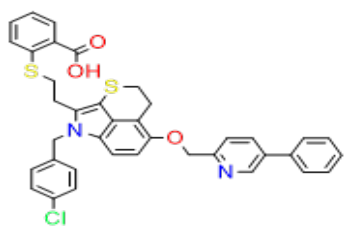
Hypothesis Class	Models Generated	Constituent Features	Description / Structural Significance
HRRR	7 (HRRR_1 to HRRR_7)	1 Hydrophobic (H) 3 Aromatic Rings (R)	Captures a highly localized, pi-system dominated binding core; suitable for broader, less-stringent database filtration.
HHRR	3 (HHRR_1 to HHRR_3)	2 Hydrophobic (H) 2 Aromatic Rings (R)	Establishes a balanced, purely lipophilic and aromatic framework mapping the central hydrophobic core of the ligands.

Table 4.1 continued from previous page

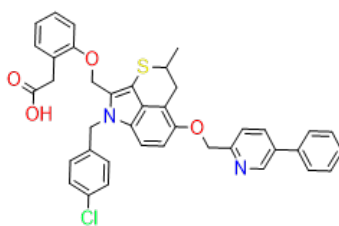
Hypothesis Class	Models Generated	Constituent Features	Description / Structural Significance
HHRRR	10 (HHRRR_1 to HHRRR_10)	2 Hydrophobic (H) 3 Aromatic Rings (R)	Represents an extended 5-feature hydrophobic network capable of mapping extensive lipophilic interactions across the target pocket.
AHHRRR	10 (AHHRRR_1 to AHHRRR_10)	1 Acceptor (A) 2 Hydrophobic (H) 3 Aromatic Rings (R)	The most comprehensive 6-feature variant; combines an explicit electrostatic anchor (Acceptor) with an expansive hydrophobic scaffold.

4.3 Validation and Statistical Evaluation of Pharmacophore Models

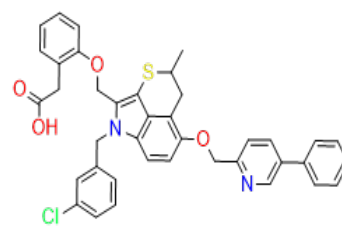
To rigorously assess the predictive reliability, robustness, and screening efficiency of the 30 generated pharmacophore models, statistical validation workflows were evaluated. For this purpose, a test set (structures shown in Figure 4.2) comprising 18 known active LOX inhibitors and 900 diverse decoy (inactives) compounds was utilized. Essentially, the evaluation was carried out with the following three important parameters: The Phase Hypo Score, used for calculating the quality and geometric fit of the alignment; The Enrichment Factor at 1% (EF1%), used to assess the early active recovery; The Boltzmann-Enhanced Discrimination of Receiver Operating Characteristic (BEDROC $\alpha=160.9$), used to assess the early recognition ability. In addition to this, the tolerance of partial mapping was measured based on the ratio of the matched features to hypothesis features and also ensuring that the excluded volumes were activated.



Compound 1
pIC50 Value: 7.52



Compound 2
pIC50 Value: 7.58



Compound 3
pIC50 Value: 8.00

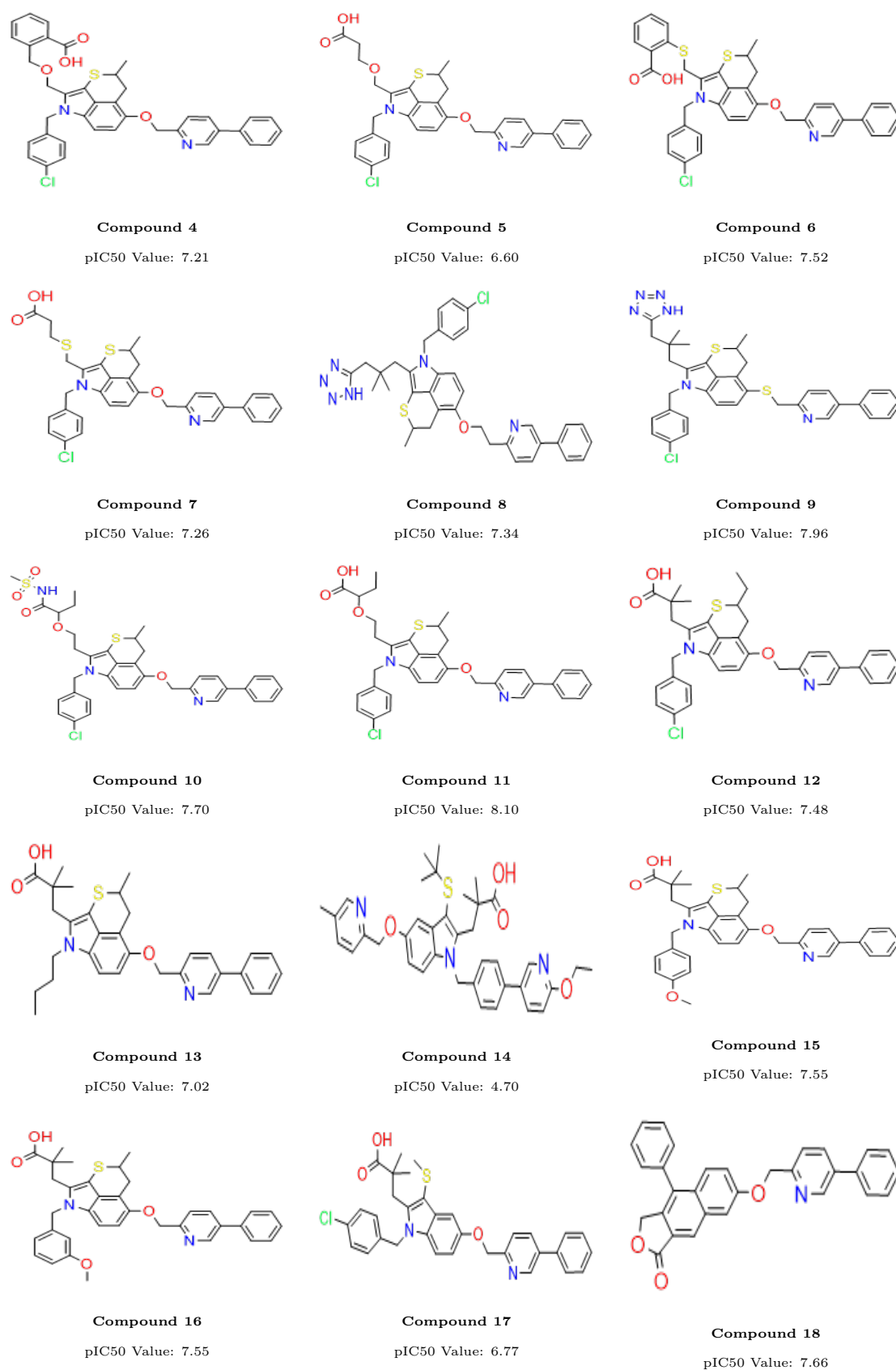


FIGURE 4.2: Chemical structures with corresponding pIC₅₀ Values of 18 Test Set Molecules

Table 4.2 shows that there is a clear relationship between complexity of structural features and early enrichment parameters when analysing the statistics. In the entire ensemble, the Phase Hypothesis Scores remained at high levels (1.26 - 1.35). This consistency is evidence that all molecules are good geometric fit with conformations of the active core. There is however some noticeable discrepancy in enrichment statistics. The lowest early active retrieval (EF1%) was obtained by the 4-feature systems with HRRR_1, HHRR_1, HRRR_3, and HHRR_3, with 27.11% EF1% and BEDROC value of 0.54. Such results mean that even though 4-feature systems are able to provide necessary geometric requirements, they lack necessary spatial restrictions for aggressive rejection of the false positive hits at an early stage of virtual screening procedure. However, adding an additional feature to the model, to go from 5-features (HHRRR) to 6-features (AHHRRR), resulted in a surprising optimization of early active enrichment. In these more advanced versions, all models were able to perform an optimized early recovery and all of them achieved an EF1% of 61.00%. Perhaps most significant, their BEDROC scores significantly improved and remained above 0.90. Two hypotheses, AHHRRR_3 and AHHRRR_9, showed superior performance with a score of 0.97 BEDROC score, and the remaining two, HHRRR_2 and HHRRR_7, with a score of 0.96 BEDROC score. These sophisticated models have a very high ability to discriminate true actives at the top rank with near perfect BEDROC scores.

TABLE 4.2: Validation Profiles of Generated Pharmacophore Hypotheses

S. No.	Hypothesis	Phase Hypo Score	EF1% (%)	BEDROC ($\alpha = 160.9$)
1.	HRRR_7	1.34	61.00	0.89
2.	HRRR_6	1.34	47.44	0.76
3.	HRRR_5	1.34	47.44	0.76
4.	HRRR_4	1.34	54.22	0.81
5.	HRRR_3	1.34	27.11	0.54
6.	HRRR_2	1.34	33.89	0.63
7.	HRRR_1	1.34	27.11	0.54
8.	HHRR_3	1.26	27.11	0.54
9.	HHRR_2	1.27	40.67	0.70
10.	HHRR_1	1.32	27.11	0.54
11.	HHRRR_9	1.34	47.44	0.88

Table 4.2 continued from previous page

S. No.	Hypothesis	Phase Hypo Score	EF1% (%)	BEDROC ($\alpha = 160.9$)
12.	HHRRR_8	1.34	61.00	0.91
13.	HHRRR_7	1.34	61.00	0.96
14.	HHRRR_6	1.35	61.00	0.93
15.	HHRRR_5	1.35	61.00	0.89
16.	HHRRR_4	1.35	61.00	0.92
17.	HHRRR_3	1.35	61.00	0.94
18.	HHRRR_2	1.35	61.00	0.96
19.	HHRRR_10	1.34	61.00	0.94
20.	HHRRR_1	1.35	61.00	0.92
21.	AHHRRR_9	1.32	61.00	0.97
22.	AHHRRR_8	1.35	54.22	0.83
23.	AHHRRR_7	1.33	61.00	0.93
24.	AHHRRR_6	1.33	40.67	0.79
25.	AHHRRR_5	1.33	61.00	0.92
26.	AHHRRR_4	1.35	61.00	0.94
27.	AHHRRR_3	1.35	61.00	0.97
28.	AHHRRR_2	1.35	61.00	0.95
29.	AHHRRR_10	1.32	61.00	0.94
30.	AHHRRR_1	1.33	54.22	0.82

4.4 Strategic selection of Model for Rational Drug Designing

The AHHRRR_3 model with 6 features (as shown in figure 4.3) has been selected as the final structural prototype for virtual screening procedures and rational design of new drug-like chemicals after the detailed comparison of the statistics validation features. The AHHRRR_3 is the best point in terms of screening accuracy of all the models developed, with a BEDROC value of 0.97, and with the highest enrichment factor at 1% (EF1%) of 61.00%, it mathematically ensures the best rank of active substances in the top part of the screened database. For the geometric aspect, the AHHRRR_3 model consists of one Hydrogen Bond Acceptor (A), two Hydrophobic

regions (H) and three Aromatic Rings (R), which contribute the maximum van der Waals interactions and pi-pi stacking interactions with the polar receptor.

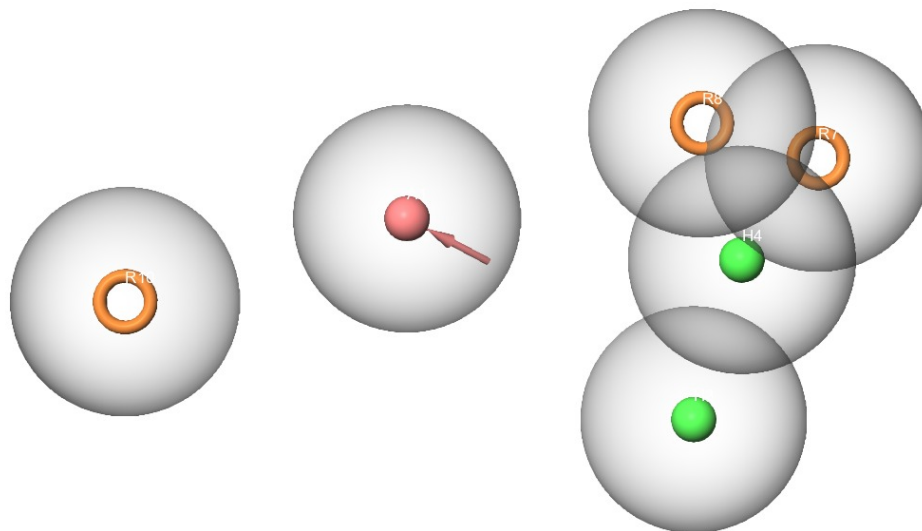


FIGURE 4.3: AHRRR_3 Model

4.4.1 Spatial Geometry and Inter-Feature Distance Analysis

The detailed analysis of spatial geometry of the selected 3D pharmacophore hypothesis AHRRR_3 was performed by calculating distances between coordinates of the features. All the unique distances are illustrated in the integrated Table 4.3. and show the exact shape of the molecules that should be designed to interact with the target. The table clearly indicates that there are two distinct regions of the pharmacophore, both with Hydrogen Bond Acceptor (A1) features as the key polar interaction sites. On one hand, the extremely short distances in the right-hand cluster such as R7-R8 (2.16 Å) and H4-R7.

(2.24 Å) indicate that this area is very compact and requires the presence of the rigidly bound ring structures that would fit in without going beyond the forbidden excluded volume area. However, the distances between the different atoms in the entire model, such as A1-R7 (12.41 Å) and H4-R10 (10.56 Å), clearly indicate that the whole binding site is relatively long.

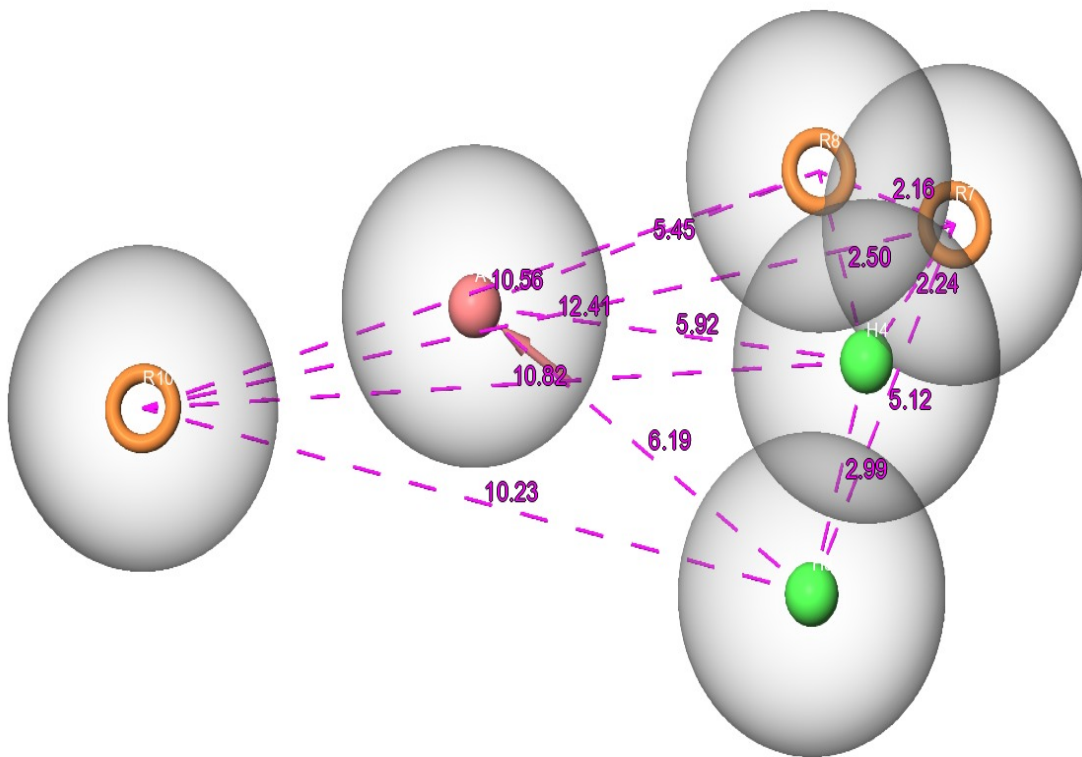


FIGURE 4.4: Inter-Feature Distances of AHRRR_3 Model

TABLE 4.3: Inter-Feature Distances of AHRRR_3 Model

Feature Pair	Distance (Å)	Feature Pair	Distance (Å)
A1 - R10	5.14	H3 - R10	10.23
A1 - R8	5.45	H4 - R7	2.24
A1 - H3	6.19	H4 - R8	5.26
A1 - H4	10.82	H4 - R10	10.56
A1 - R7	12.41	R7 - R8	2.16
H3 - H4	2.99	R7 - R10	7.31
H3 - R7	5.12	R8 - R10	2.50

4.4.2 Angular geometry and Inter-Feature Angles Analysis

Some of the key angles between features within the molecule were also computed and are shown in Figure 4.5, to provide more information beyond the distance measurements to complete the three dimensional geometry of the AHRRR_3 model.

The information is essential for setting up the geometric constraints that must be fulfilled by the 85 novel derivatives so that they can assume their stable conformation when fitting the pharmacophore. The critical angles of interaction between the central polar moiety, the left aromatic chain and the right hydrophobic domain are listed below in Table 4.4.

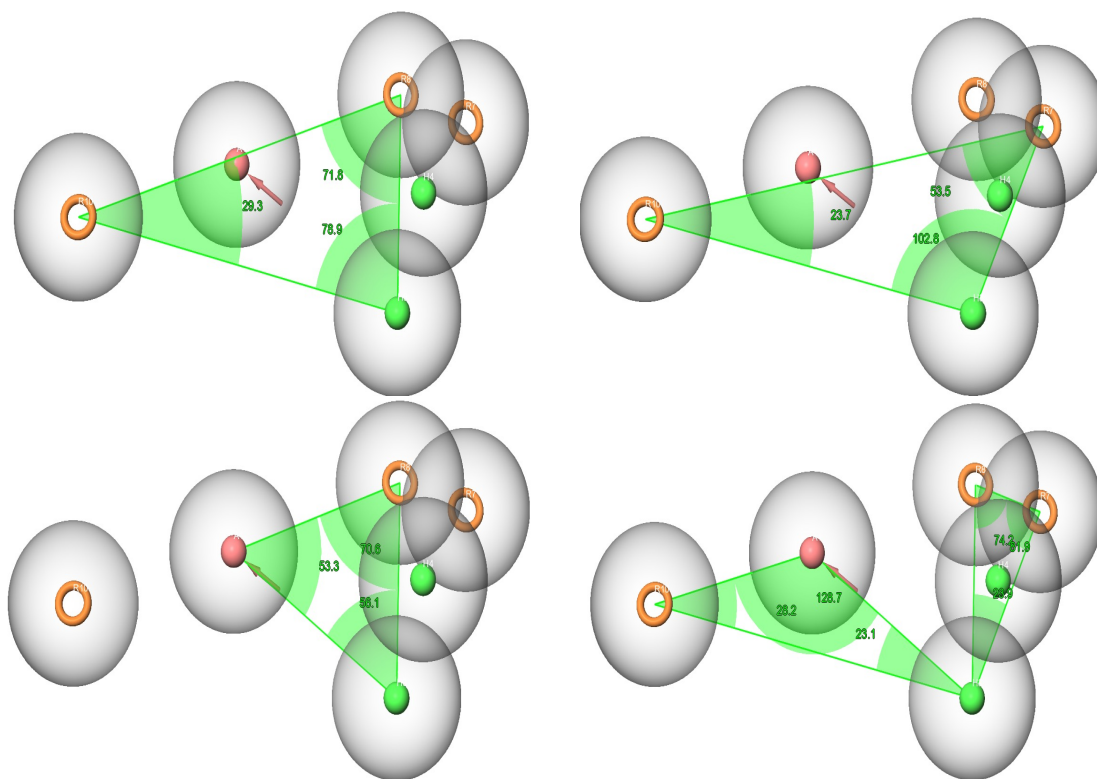


FIGURE 4.5: Inter-Feature Angles in Model AHRRR_3

TABLE 4.4: Inter-Feature Angles of AHRRR_3 Model

Feature Triangle	Measured Angle (180°C)	Feature Triangle	Measured Angle (180°C)
R10 - A1 - H3	29.3	A1 - H4 - H3	80.7
R10 - A1 - H4	28.2	R8 - H4 - R7	23.9
H3 - A1 - H4	28.5	H4 - R7 - R8	69.2
R8 - A1 - H4	25.0	H3 - R8 - A1	71.8
R8 - A1 - H3	53.3	A1 - R8 - H4	88.3
R10 - H3 - A1	23.7	A1 - R8 - H3	66.7
R10 - H3 - R8	23.1	A1 - R10 - H3	128.7
A1 - H3 - H4	70.9	A1 - H3 - R8	56.1

4.5 Designing of Potential Inhibitors

A de novo design was carried out on the validated pharmacophore hypothesis AHRRR_3 (6 features) and a new library of 85 derivatives was generated. While designing the compounds, effort was made to maximize the complementation between these designed compounds with the target binding site by making changes in the structure of functional groups. In this context, it was attempted to introduce the appropriate electronegative functional groups to satisfy the need to have one Hydrogen Bond Acceptor (A) feature, and various side lipophilic and aromatic functional groups were introduced to satisfy the need to have two hydrophobic centers (H) and three Aromatic Rings (R).

4.6 Screening and Alignment with Model

The aligned ligand structure shows good correspondence with the developed pharmacophore hypothesis AHRRR_3, as shown in the Figure 4.6, demonstrating proper spatial fitting of the key chemical features.

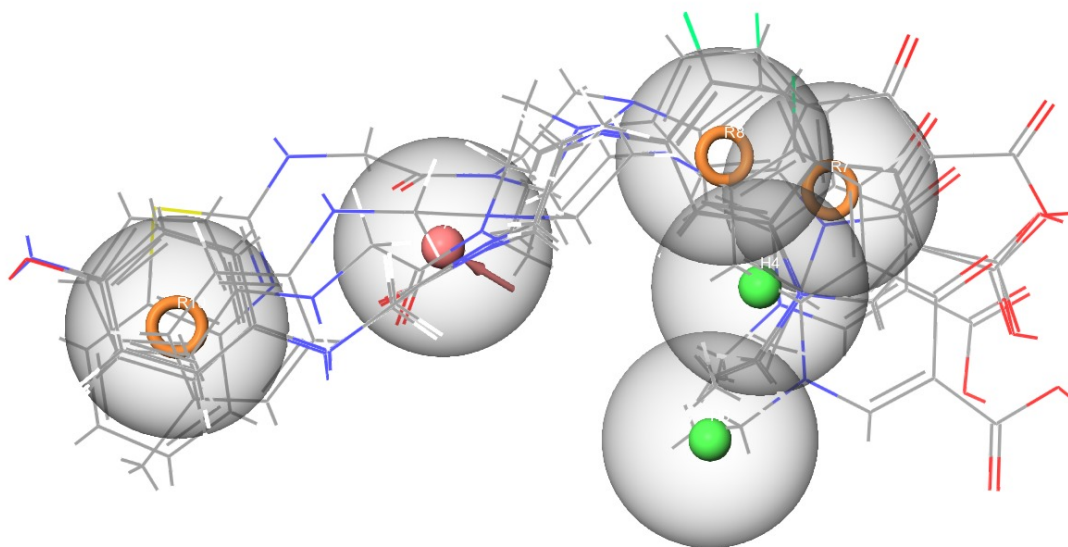


FIGURE 4.6: Screening and Alignment of designed compounds with Model

The screening criteria evaluated each candidate's capability to geometrically match the 3D arrangement of the model's essential polar and lipophilic sub-pockets. Every compound was quantitatively ranked using multi-factor computational parameters, specifically scoring their Geometrical fitness (Phase Screen Score), spatial overlay accuracy (Align Score), the alignment of their directional chemical bonds (Vector Score), and their spatial volume intersection within the pocket (Volume Score). These metrics were combined to generate an overall Phase Fitness Score representing the structural compatibility of each derivative.

Following this screening process, the final seven compounds, AM-1, AM-2, AM-3, AM-4, AM-5, AM-6, AM-7 (shown in Figure 4.7), exhibiting the highest overall fitness values and strongest alignment criteria (Table 4.5) were selected as the prioritized leads for further molecular docking.

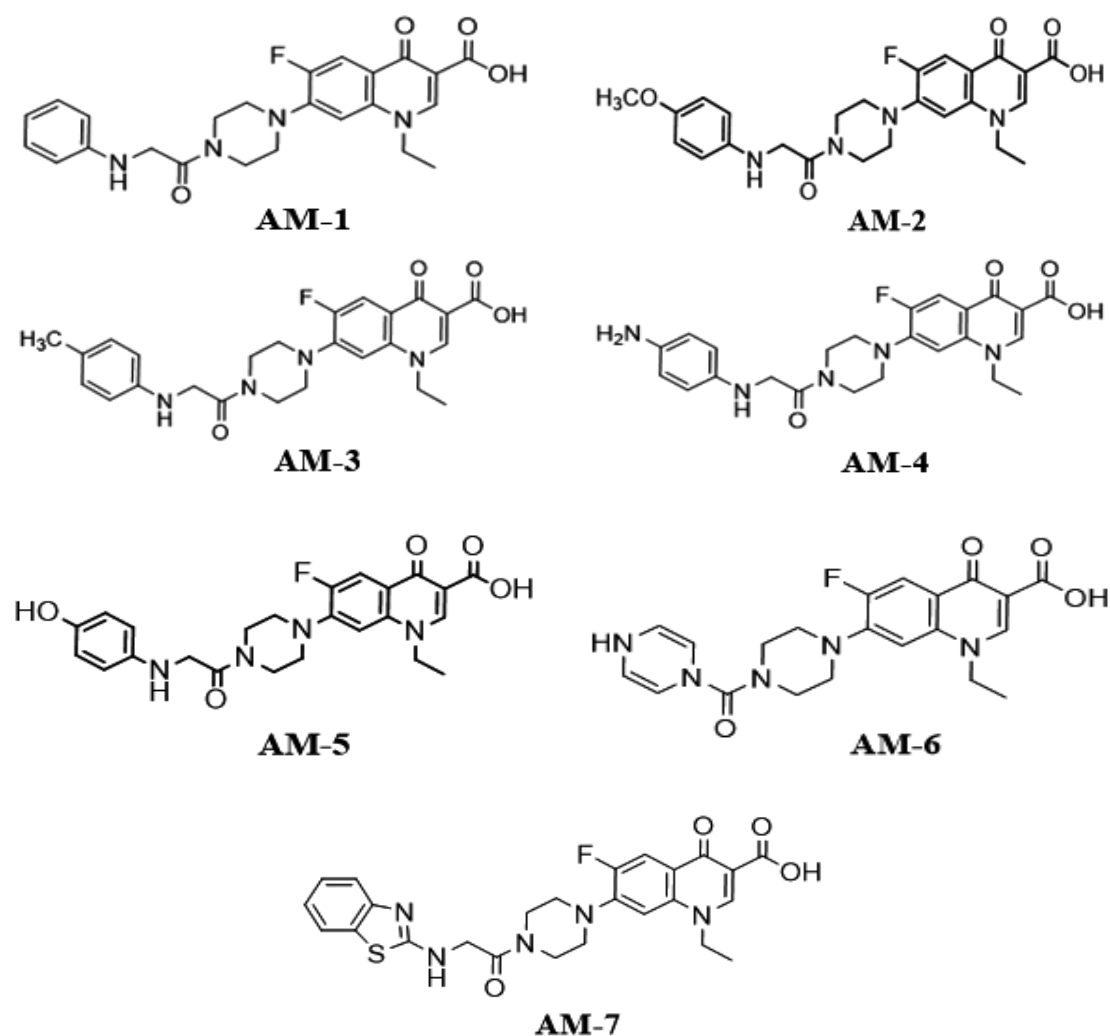


FIGURE 4.7: Compounds AM-1 to AM-7 showing highest ranks after screening

TABLE 4.5: Compounds AM-1 to AM-7 showing highest ranks after screening

Candidate	Vector	Volume	Align	Phase Screen	Fitness
Compound	Score	Score	Score	Score	Score
AM-1	0.882	0.466	1.040	1.458	1.458
AM-2	0.903	0.333	0.993	1.347	1.347
AM-3	0.885	0.443	0.995	1.438	1.438
AM-4	0.886	0.486	1.071	1.461	1.461
AM-5	0.901	0.484	1.092	1.46	1.46
AM-6	0.908	0.350	1.001	1.365	1.365
AM-7	0.767	0.468	1.408	1.088	1.088

4.7 Molecular Docking Studies

Molecular docking was performed using Schrödinger Maestro Glide algorithm in Extra Precision (XP) mode to test the binding properties and specific interaction modes of selected lead molecules into the binding pocket of the target 5-LOX protein receptor (PDB ID: 3V99).

In order to make sure that the entire region of catalytically active pocket and conformational flexibility of the designed ligand structures were taken into consideration, the search region was surrounded by a precisely defined 3D grid box. This grid box's orientation and central point were set to the absolute coordinates: 8.5014 on the X axis, -79.1715 on the Y axis, and -25.2689 on the Z axis.

The binding profiles generated were utilized for thermodynamic energetics analysis and it was found that all 7 of the selected compounds showed very favourable binding energy characteristics in the 5-LOX active site (Table 4.6). Compound AM-7, with the highest docking score and Glide GScore of -5.860 kcal/mol, was the most preferred compound as evidenced by the high Glide Emodel value (-63.875 kcal/mol), which showed high stability and low energy of the conformation of the compound. The next two biggest binding energy values were for AM-1 (-4.789 kcal/mol), and AM-3 (-4.599 kcal/mol). All other lead compounds demonstrated

reasonable binding energy levels of -4.427 kcal/mol (AM-5), -4.323 kcal/mol (AM-4), -3.885 kcal/mol (AM-6) and -3.793 kcal/mol (AM-2).

TABLE 4.6: Molecular Docking Scores and MMGBSA Scores for designed compounds

Candidate Compound	Docking Score (kcal/mol)	Glide Emodel (kcal/mol)	Glide XP GScore (kcal/mol)	MM - GBSA dG Bind (kcal/mol)
AM-1	-4.789	-56.03	-4.789	-38.32
AM-2	-3.793	-59.313	-3.793	-33.78
AM-3	-4.599	-59.339	-4.599	-17.41
AM-4	-4.323	-69.723	-4.323	-40.75
AM-5	-4.427	-69.918	-4.427	-33.11
AM-6	-3.885	-50.02	-3.885	-29.95
AM-7	-5.86	-63.875	-5.86	-26.55

The Quantitative distribution of the scoring terms revealed differences in the intermolecular binding mode of the different compounds. The binding of AM-7 and AM-1 was predominantly based on a good global shape complementarity and hydrophobic binding with the 5-LOX pocket with negligible contribution of direct hydrogen bond, other compounds have a very secure binding network with directionally driven hydrogen bonds. Direct hydrogen bond contributions of AM-2 and AM-6 with the residues of the binding pocket were very high with XP HBond scoring -1.433 and -1.268, respectively, followed by AM-4 with -1.135 and AM-5 with -0.960.

Besides, the ligand efficiency scores were in the range from -0.108 to -0.163 that indicated that the structural modification from AM-1 to AM-7 is optimized toward atomic contributions to the target with minimum molecular weight.

The interaction Diagrams (Figure 4.8 to 4.14) from 2D interaction for each lead compounds (AM-1 to AM-7) inside the 5-LOX binding pocket were examined to provide molecular recognition insight. AM-1 was positioned linearly on the surface of hydrophobic channel (ALA-606, LEU-607, PHE-610) by a directional hydrogen bond between secondary amide linkage (-NH-) and the main chain carbonyl group

of VAL-671. AM-2 was located in horseshoe like shape by the interacting network that composed of pi-cation interaction between LYS-409, perpendicular pi-pi stacking interaction between PHE-555 and two hydrogen bondings with ASN-554 and VAL-671.

The molecule of AM-3 occupied a parallel orientation within the primary hydrophobic channel that interacted with LYS-409 via pi-cation and connected to GLN-413 with sharp hydrogen bonding between terminal carboxylic acid hydroxyl (-OH) and it.

Two derivatives AM-4 and AM-5 adopted similar horizontal orientation in the two opposite sides of the pocket.

Both compounds had formed hydrogen bond between their left-hand carboxylic acid (-COOH) with ASN-554 near LEU-607, whereas the central amide nitrogen (-NH-) linked with GLU-614.

The right-hand side interacted through a polar bond between the phenolic hydroxyl group (-OH) and ALA-398 (in AM-4) and between the terminal amine group (-NH) and the same residue in AM-5.

Compound AM-6 is positioned in proximity to PHE-555 due to placement of its quinolone part there, and is solely anchored by one hydrogen bond of its carbonyl oxygen atom to the side chain of GLN-557 because of positioning of its piperidine part toward the hydrophobic pocket formed by ALA-672.

The highest scored AM-7, has perfectly matched to the extended cavity, through overall geometrical complementarity; because it had no any single point hydrogen bonding; its van der Waals interactions were used maximally in terms of the entire surface area, with methoxy-phenyl part having fitted into the hydrophobic pocket formed by PHE-555, ASN-554, GLN-557, and TYR-558 residues and fluorinated quinolone part perfectly filling into the hydrophobic cavity created by ALA-410 and ILE-406.

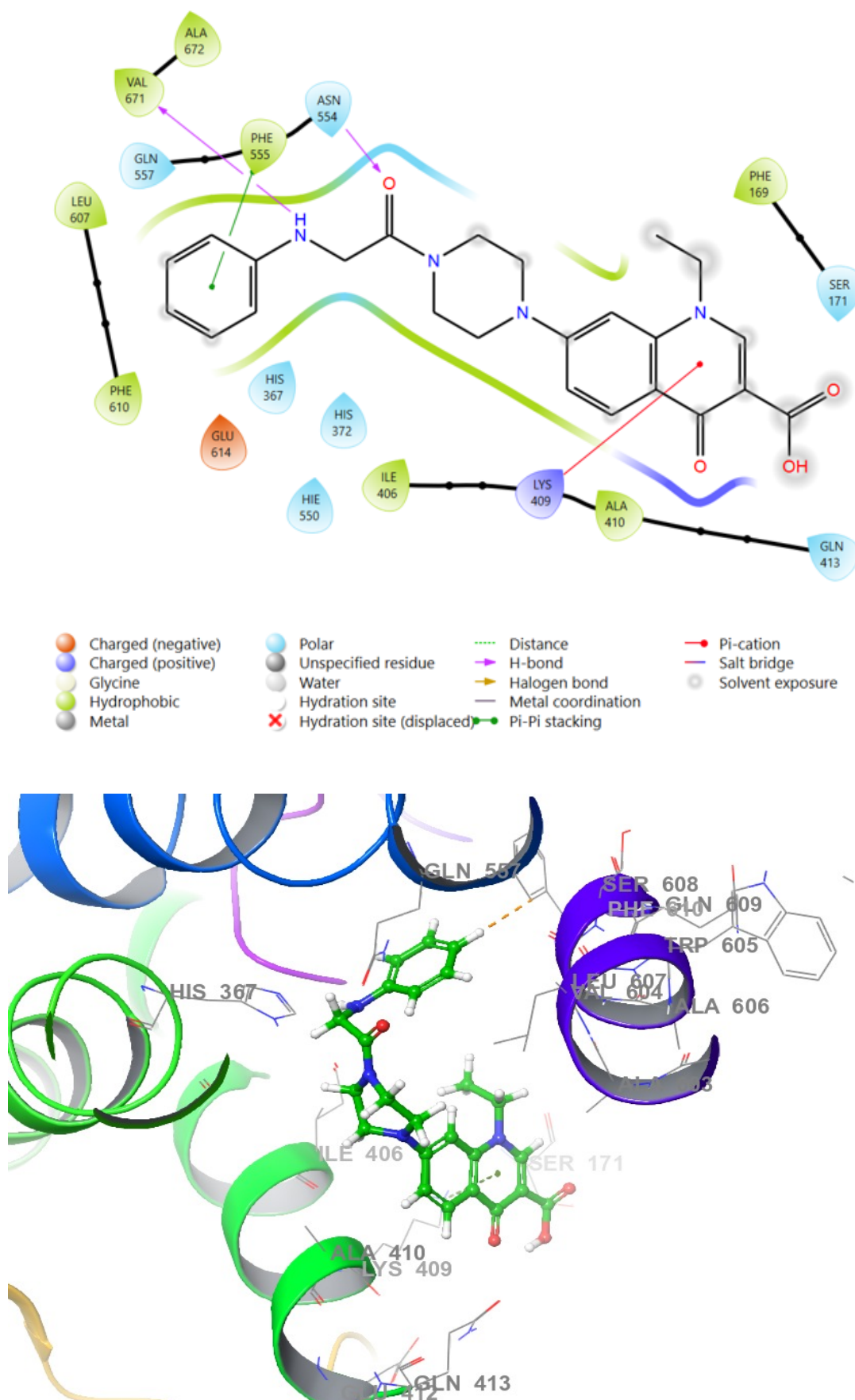


FIGURE 4.8: 2D Ligand Interaction Diagram for compound AM-1

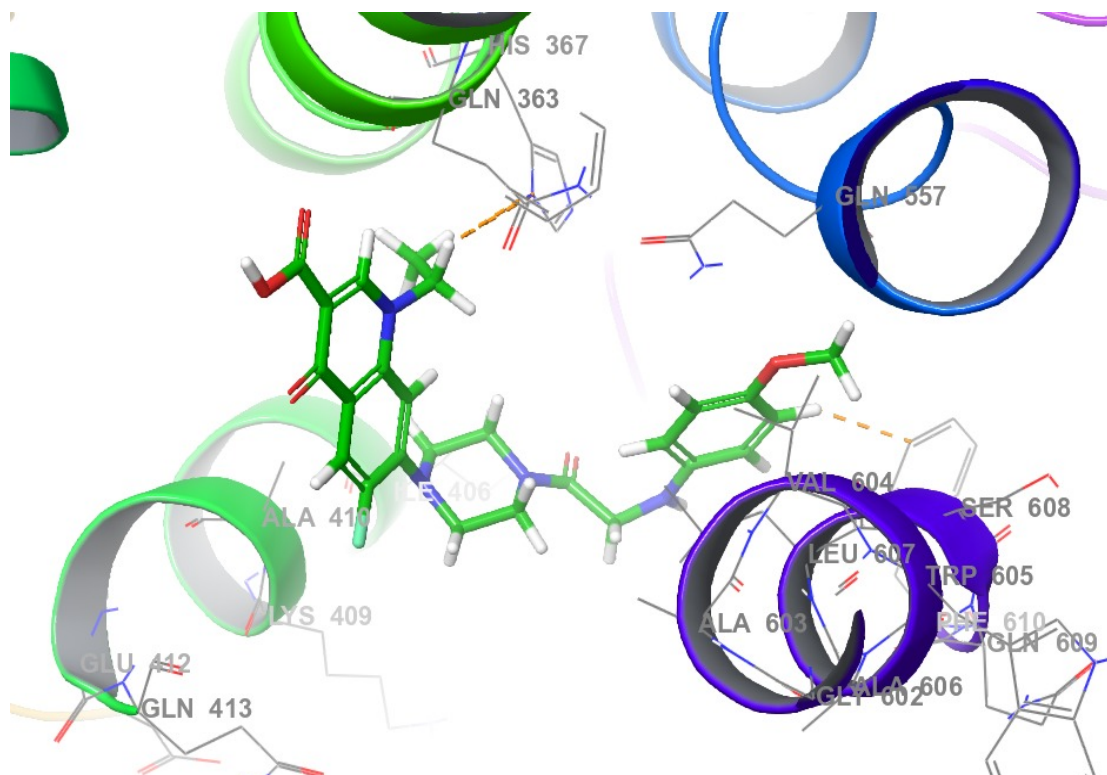
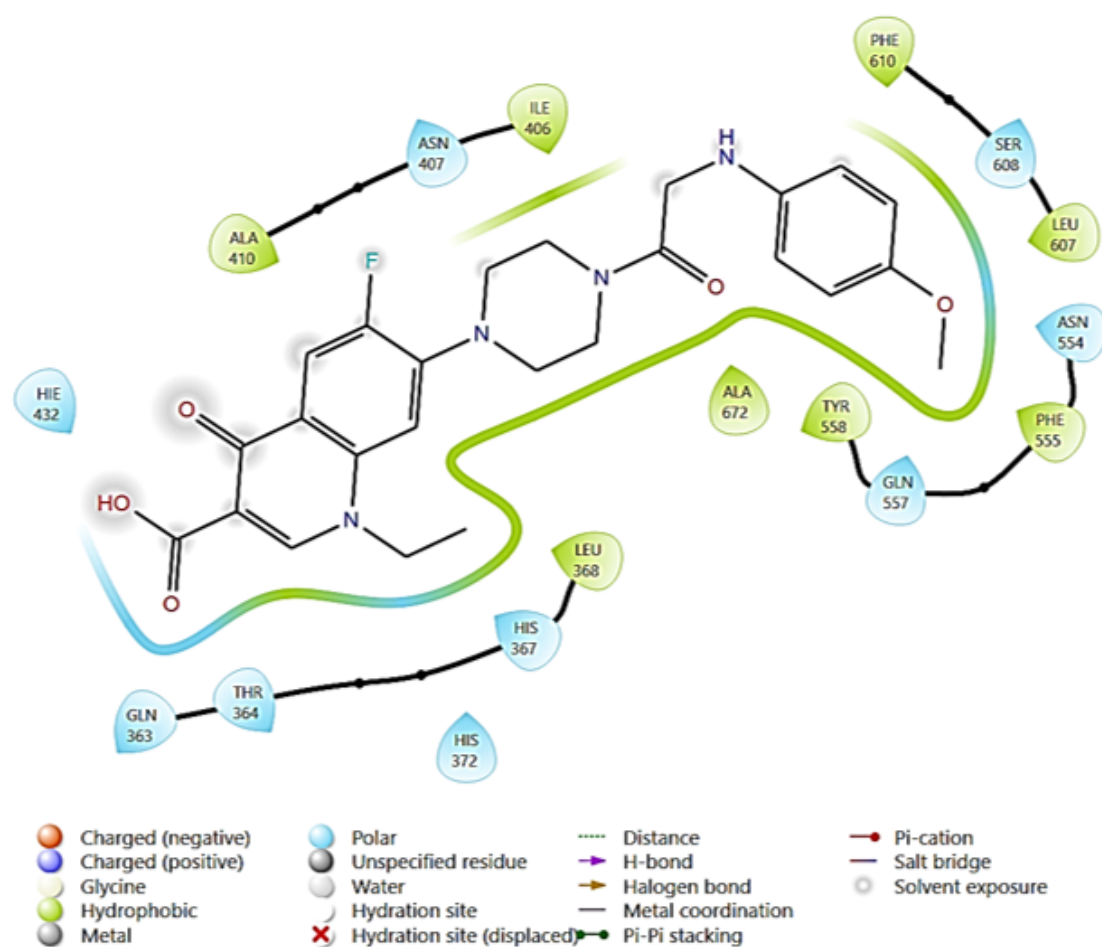


FIGURE 4.9: 2D Ligand Interaction Diagram for compound AM-2

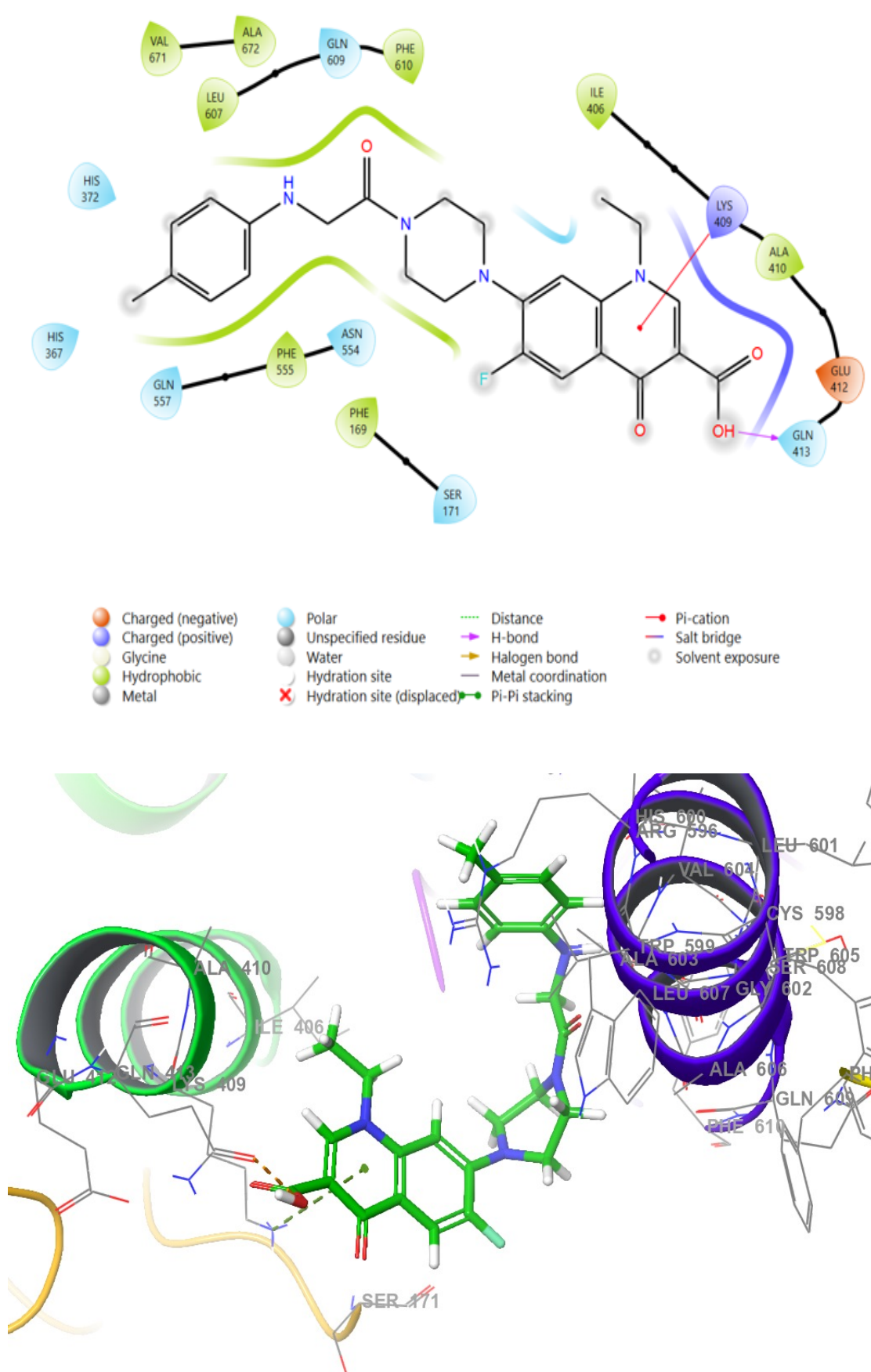


FIGURE 4.10: 2D Ligand Interaction Diagram for compound AM-3

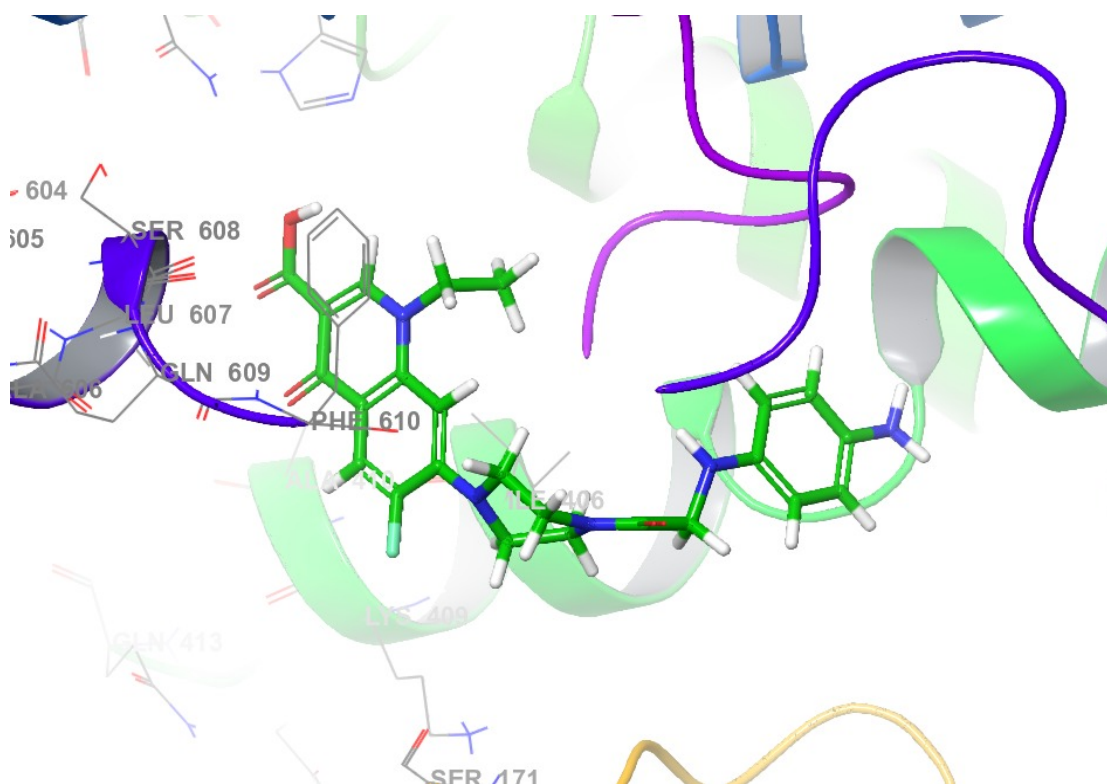
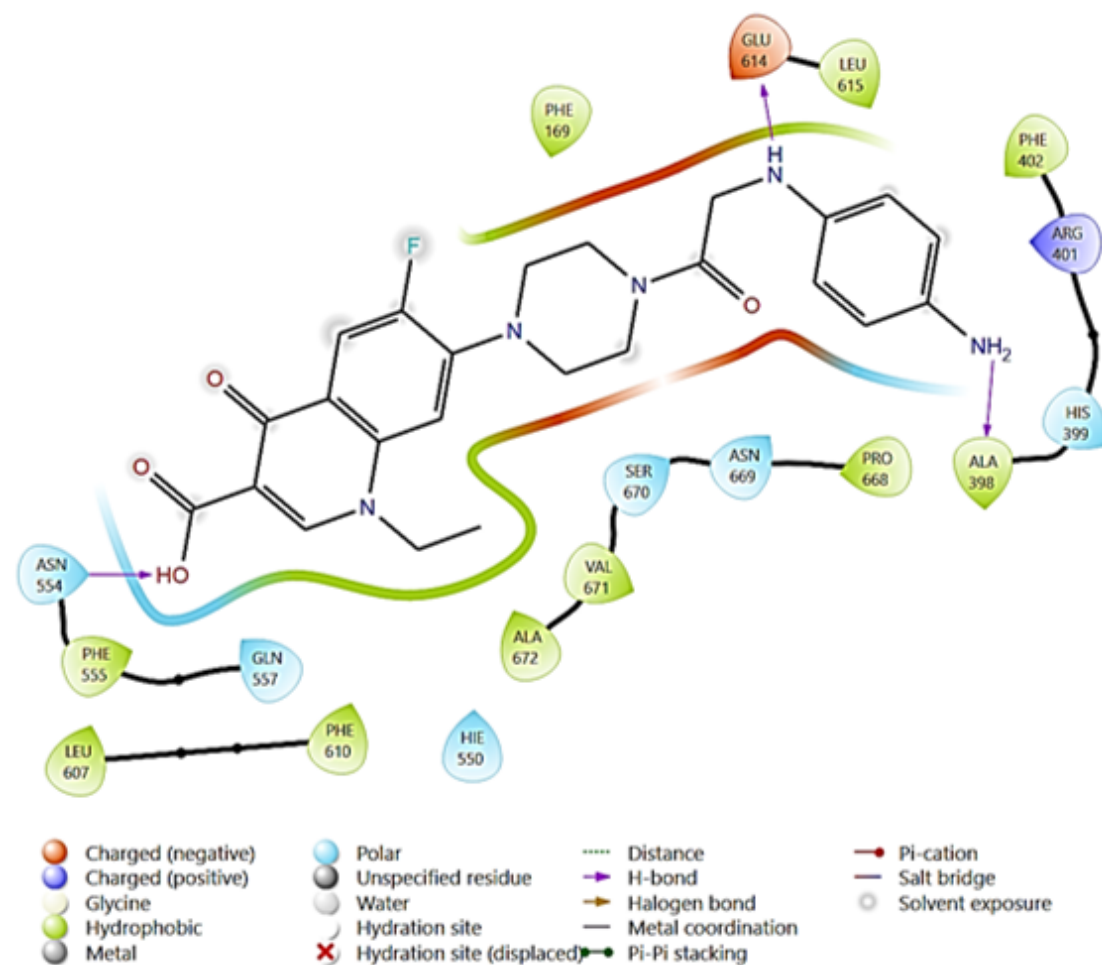


FIGURE 4.11: 2D Ligand Interaction Diagram for compound AM-4

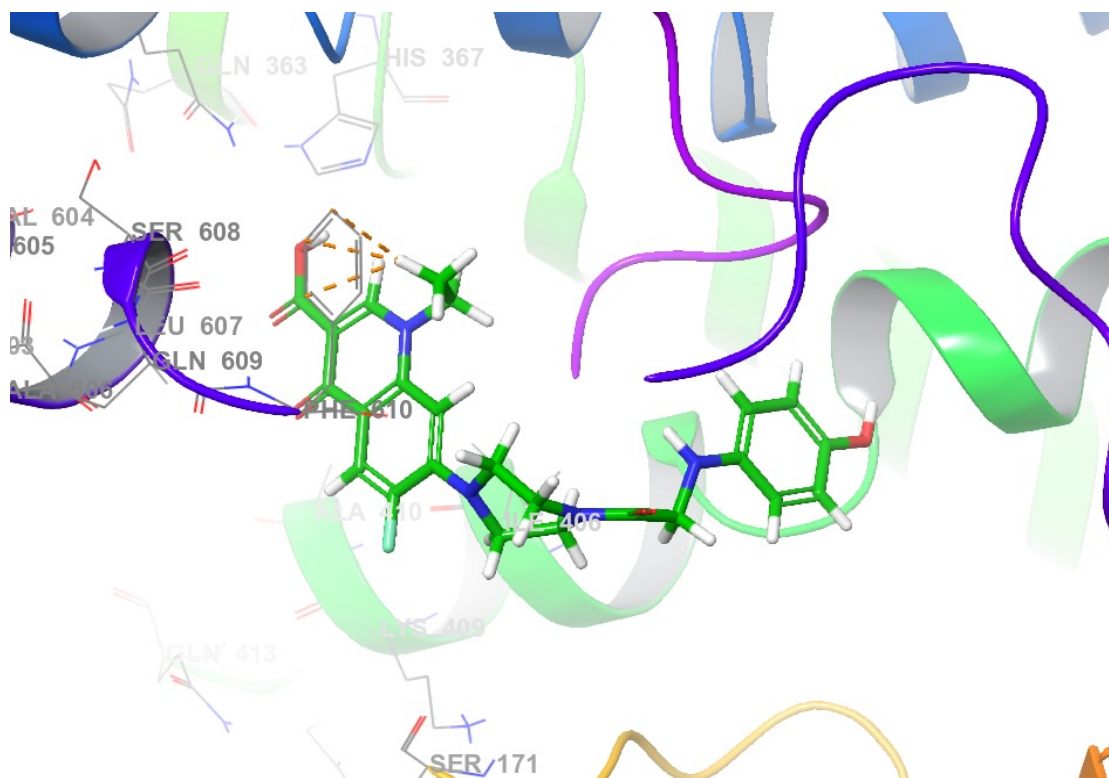
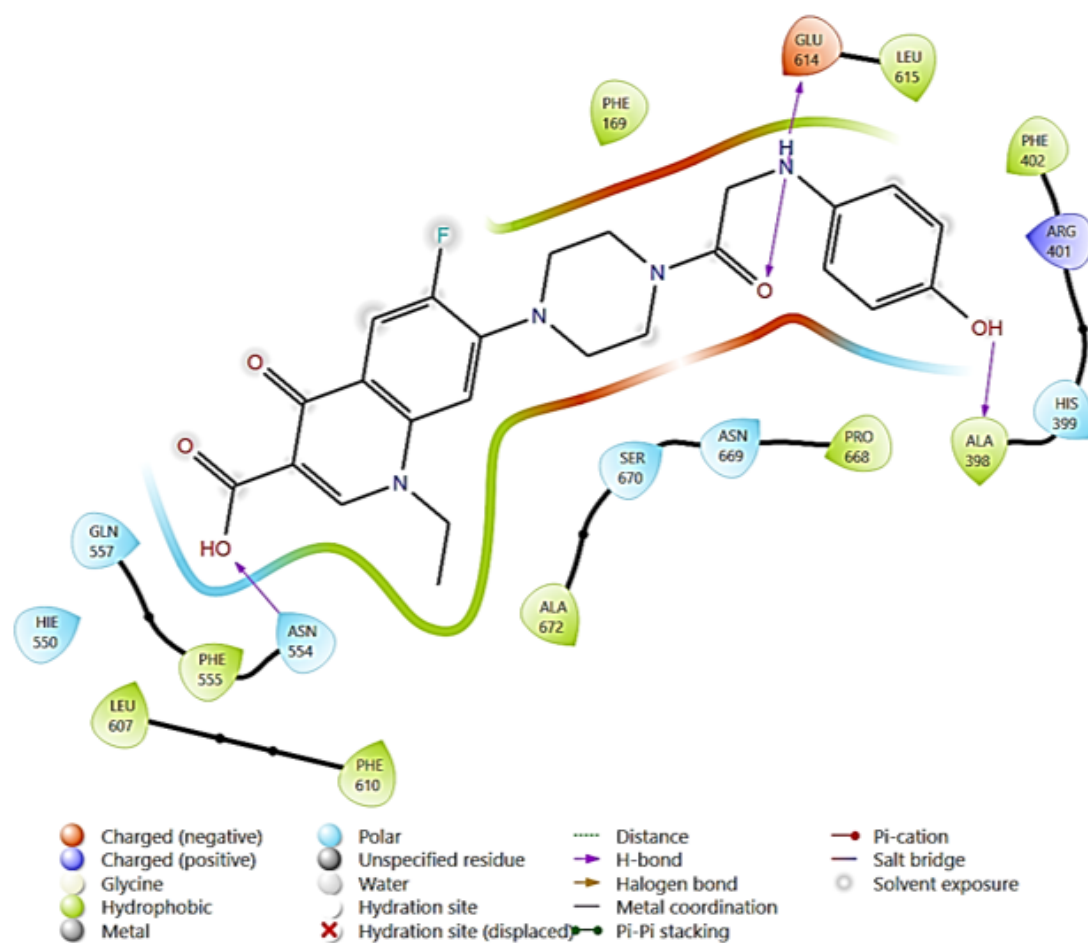


FIGURE 4.12: 2D Ligand Interaction Diagram for compound AM-5

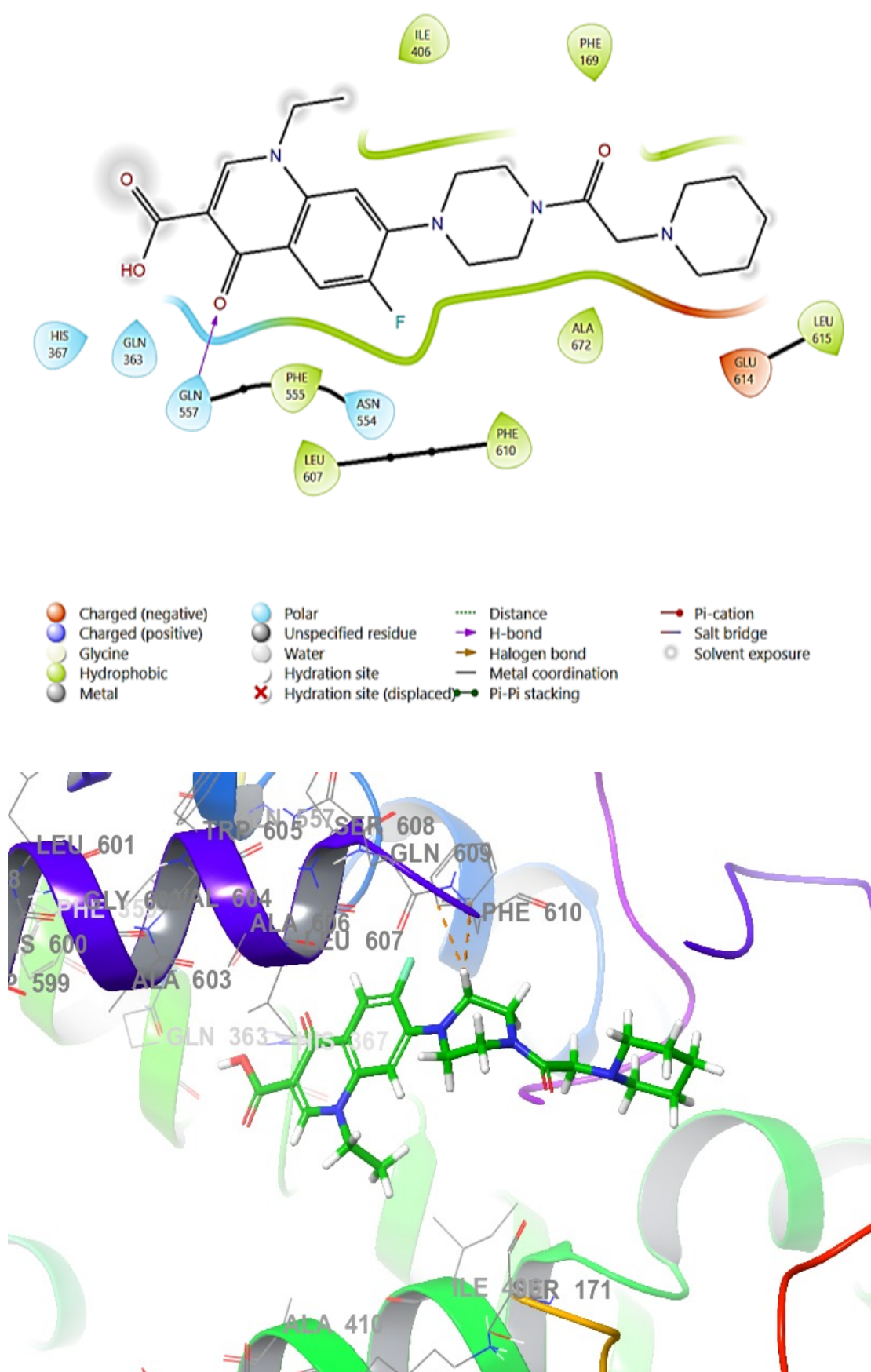


FIGURE 4.13: 2D Ligand Interaction Diagram for compound AM-6

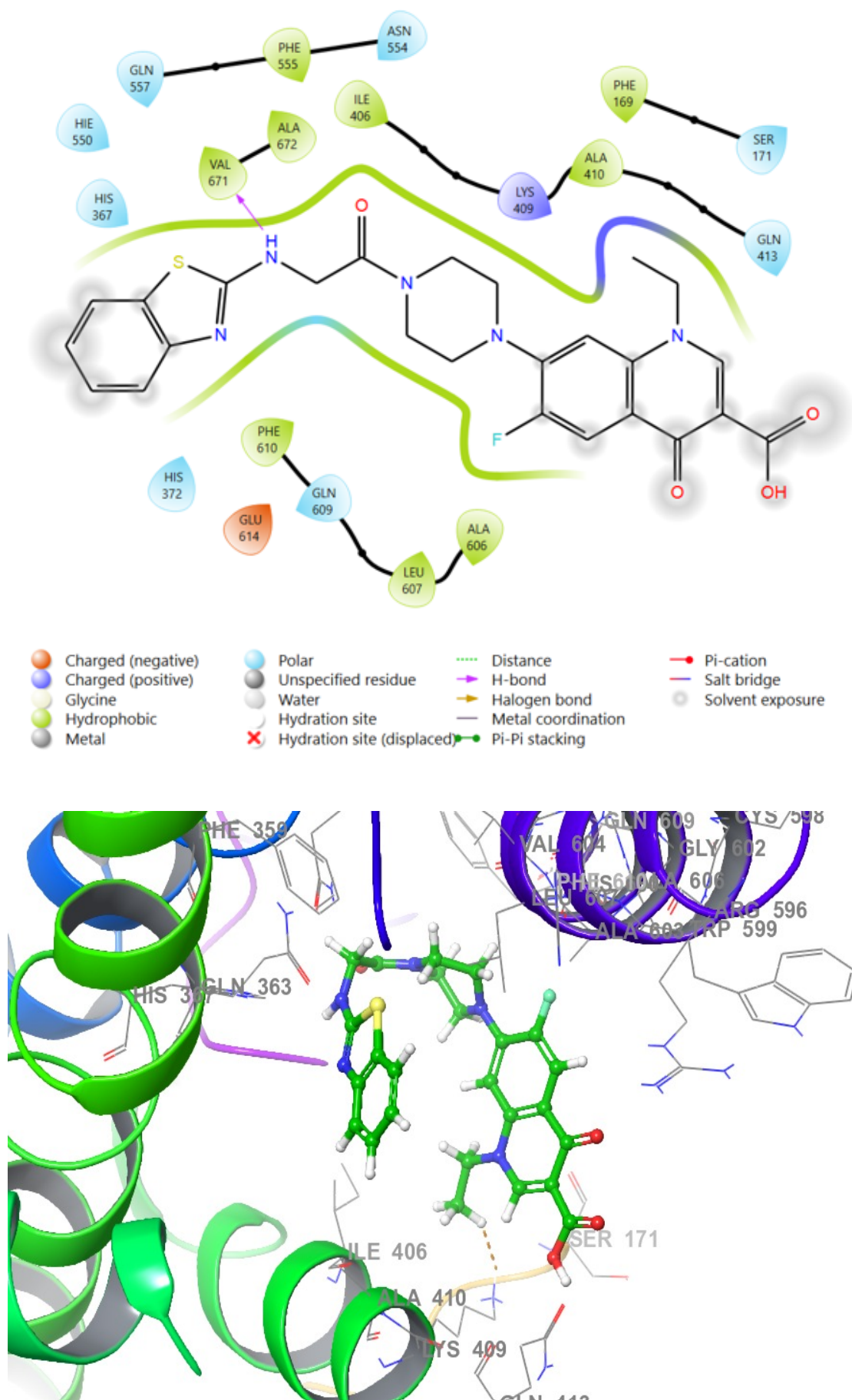


FIGURE 4.14: 2D Ligand Interaction Diagram for compound AM-7

4.8 MMGBSA Binding Energy

The MMGBSA endpoint calculation was performed in the Prime Module to validate the thermodynamically stable docked poses and to determine more accurate relative binding affinities. This analysis also accounts for complex minimization, gas-phase energies and desolvation penalties to give a more robust thermodynamic indicator than a simple empirical docking score.

Highly spontaneous and stable binding profile was confirmed by the Prime MMGBSA results for the whole set of prioritized compounds listed in Table 4.6. The most thermodynamically favorable free energy of binding was observed for AM-4 (-40.75 kcal mol⁻¹), indicating that it had the most favorable surface area coverage and a well-balanced distribution of polar and non-polar interactions. This was closely followed by AM-1 at -38.32 kcal/mol, AM-2 at -33.78 kcal/mol and AM-5 at -33.11 kcal/mol, all with strong structural stabilization. AM-6 at -29.95 kcal/mol and AM-7 at -26.55 kcal/mol produced a good baseline score and AM-3 completed the group at -17.41 kcal/mol. The very sharp decrease of the total energy shows that these structural changes allow for an efficient fit in the catalytic environment of the 5-LOX pocket, without a high desolvation cost.

4.9 In-Silico ADMET Profiling

A thorough *in silico* ADMET Characterization was performed to facilitate systematic studies on drug-likeness, physiological feasibility and margin of safety with respect to the designed compounds AM-1 to AM-7. The data of the simulation was sorted sequentially by each of the individual features of the ADMET (as shown below).

4.9.1 Physicochemical Properties

Selected physicochemical descriptors were evaluated (Table 4.7) in order to establish the basic structural and physical features of the analogs (AM-1 to AM-7).

The basic molecular properties are the basis of membrane border entry, basic dissolution and binding properties.

4.9.1.1 Molecular Weight and Thermal Properties

The formula weight (AP_FWeight) range is 444.509 to 509.562 g/mol for the smaller AM-1 to the heavier methoxy-phenyl substitution, AM-7. Proximity to 500 g/mol is required to retain the molecular stability with an increase in number of the specific binding points.

The calculated melting points (S+MP) show that all seven analogs have stable molecular structures between a minimum of 196.436 °C for AM-2 and a maximum of 219.875 °C for AM-7, which indicated strong intermolecular crystal lattices in all seven of the analogs.

4.9.1.2 Lipophilicity

There is a strong dependence on differences in topology between the two interrelationships of lipophilicity and aqueous ionization. Lipophilic distribution index (S+logP) ranges from very high hydrophilic value of 0.500 in case of aliphatic piperidine variant AM-6 to the maximum lipophilic value of 2.331 in case of AM-3. When taking into account physiological pH condition, the distribution coefficients (S+logD) fall down considerably, reaching the lowest negative value of -0.126 for AM-4.

These directly relate to aqueous solubility (S+Sw); the most hydrophilic of which being AM-6 with an aqueous solubility index of 0.041 mg/mL while the most hydrophobic being AM-3 with an aqueous solubility index of 0.004 mg/mL and AM-7 with an aqueous solubility index of 0.003 mg/mL.

The macroscopically identified functional states have been found to correspond to structure states with the constant high acidic range (S+Acidic_pKa) from 5.94 to 6.36, and the distinct basic states (S+Basic_pKa) with high values of 7.45 for AM-6.

TABLE 4.7: Physicochemical Properties of AM-1 to AM-7

Parameter	AM-1	AM-2	AM-3	AM-4	AM-5	AM-6	AM-7
AP.FWeight (g/mol)	452.498	482.515	466.515	467.503	468.488	444.509	509.562
S+MP (180°C)	202.795	196.436	201.795	208.166	209.517	209.638	219.875
S+logP	1.812	1.794	2.331	0.798	1.454	0.5	2.252
S+logD	0.799	0.58	1.166	-0.126	0.225	0.263	0.919
S+Sw (mg/mL)	0.007	0.008	0.004	0.02	0.015	0.041	0.003
S+Acidic_pKa	6.34	6.14	6.22	6.36	10.77	5.94	6.06
S+Basic_pKa	3.21	3.45	3.24	5.46	3.36	7.45	4.29

4.9.2 Absorption Profile and Membrane Permeability

The chemical modification on candidates AM-1 to AM-7 are directly associated with their lipophilicity and membrane permeability as displayed in Table 4.8. Their total absorption risk (Absn.Risk) is in an extremely favorable range, far below their critical values, from 0.800 in AM-5 to 2.136 in AM-4. It implies that the physical changes that have been implemented will be able to remedy the basic oral absorption challenges.

Thus, the chemicals will be greatly different based on the Biopharmaceutics Classification System (BCS_Class). Aromatic compounds (AM-2, AM-3, AM-4 and AM-7) with lower hydrophilicity are classified as BCS Class II (low solubility, high permeability); the smaller compound AM-1 and the highly polar compounds AM-5 and AM-6 are more hydrophilic and will not have the initial oral dissolution problem.

Physiological membrane models also can be used to verify the permeability capabilities at tissue boundaries. In the case of AM-3, the membrane transit indices across the corneal tissue (Perm_Cornea) are found to have advanced value of 107.745, however in the case of membrane transmission indices across the skin layer tissues (Perm_Skin), they are varying from lowest value of 0.356 for AM-6 to maximum value of 8.660 for AM-3. This variation highlights the balance between aromaticity and polar function groups.

TABLE 4.8: Absorption and Membrane permeability Profile

Parameter	AM-1	AM-2	AM-3	AM-4	AM-5	AM-6	AM-7
BCS Class	Class I	Class II	Class II	Class II	Class I	Class I	Class II
Absn.Risk	0.912	1.394	1.8	2.136	0.8	1.131	2
Perm.Cornea	96.611	78.136	107.745	25.193	67.612	37.335	83.335
Perm.Skin	3.676	3.094	8.66	2.343	3.387	0.356	4.436

4.9.3 Distribution and Tissue Compartmentalization

The distribution properties computed show how well the compounds can spread through the vascular system and distribute in the peripheral tissues, shown in Table 4.9 below. The aliphatic piperidine substitution increases tissue distribution with steady state volume of distribution (Vd) ranging from 0.762 L/kg for AM-1 to 1.326 L/kg for AM-6. Plasma fractions (hum.fup%) vary widely: AM-6 has a relatively low plasma protein binding and high unbound fraction of 31.060% while the aromatic analogs have low unbound fractions as high as 1.879% in AM-7. Successful central nervous system restriction is attained in order to limit any nervous side effects. The partition coefficients (LogBB) into the brain fall in the safe negative range from -0.915 to -1.297 with the BBB filter (BBB_Filter) which classifies all seven compounds as low permeation structure.

TABLE 4.9: Distribution and Compartmentalization Profiles

Parameter	AM-1	AM-2	AM-3	AM-4	AM-5	AM-6	AM-7
BBB.Filter	Low	Low	Low	Low	Low	Low	Low
hum.fup%	4.512	4.316	3.764	6.75	5.812	31.06	1.879
LogBB	-1.146	-1.176	-1.143	-1.297	-1.139	-0.915	-1.08
Vd (L/kg)	0.762	1.02	0.949	1.076	0.911	1.326	0.82

TABLE 4.10: Metabolism and Clearance Profile

Compound	HEP_hCLi	S+CL_Mech	S+CL_Met	S+CL_Ren	S+CL_Upt
AM-1	18.976	HepUptake	No	No	Yes
AM-2	18.222	HepUptake	No	No	Yes
AM-3	24.466	HepUptake	No	No	Yes

Table 4.10 continued from previous page

Compound	HEP_hCLi	S+CL_Mech	S+CL_Met	S+CL_Ren	S+CL_Upt
AM-4	7.507	Renal	No	Yes	Yes
AM-5	17.655	HepUptake	No	No	Yes
AM-6	10.662	Renal	No	Yes	No
AM-7	19.413	HepUptake	No	No	Yes

4.9.4 Toxicity Profiles

As evident from Table 4.11, the toxicity index TOX_Risk reflects the fact that compounds AM-1 to AM-5 have a basic toxicity score of 0 and hence show no toxicity whereas AM-6 and AM-7 have a score of 1. The remarkable similarity of the cardiotoxicity profile of all seven compounds is evident, none of the compounds block the hERG potassium channel (hERG_Filter). None of the derivatives have Androgen receptor toxicity (Andro_Filter) and Estrogen receptor toxicity (Estro_Filter) except AM-6 which can potentially have androgen receptor toxicity. The mutagenicity risk index values (MU_Risk) are fairly low ranging from 0 to 0.6 throughout the chemical matrix. The safety index RePro_Tox shows that the methoxy-phenyl derivative (AM-7) has a nontoxic reproductive safety profile.

TABLE 4.11: Toxicity Profiles

Parameter	AM-1	AM-2	AM-3	AM-4	AM-5	AM-6	AM-7
TOX_Risk	0	0	0	0	0	1	1
Andro_Filter	Nontoxic	Nontoxic	Nontoxic	Nontoxic	Nontoxic	Toxic	Nontoxic
Estro_Filter	Nontoxic	Nontoxic	Nontoxic	Nontoxic	Nontoxic	Nontoxic	Nontoxic
hERG_Filter	No	No	No	No	No	No	No
MUT_Risk	0.3	0	0	0.3	0	0.6	0.6
Repro_Tox	Toxic	Toxic	Toxic	Toxic	Toxic	Toxic	Nontoxic

4.10 Synthesis of Compounds

The final compounds **AM-1** (1-ethyl-6-fluoro-4-oxo-7-(4-(2-(phenylamino)acetyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid), **AM-2** (1-ethyl-6-fluoro-

7-(4-(2-((4-methoxyphenyl)amino)acetyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid), **AM-3** (1-ethyl-6-fluoro-4-oxo-7-(4-(2-((4-methylphenyl)amino)acetyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid), **AM-4** (7-(4-(2-((4-aminophenyl)amino)acetyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid), **AM-5** (1-ethyl-6-fluoro-7-(4-(2-((4-hydroxyphenyl)amino)acetyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid), **AM-6** (1-ethyl-6-fluoro-4-oxo-7-(4-(2-(piperazin-1-yl)acetyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid), and **AM-7** (7-(4-(2-(1,3-benzothiazol-2-ylamino)acetyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid), were synthesized by following the scheme shown in Figure 3.2.

The successful formation of these compounds was confirmed by changing position of chromatogram over the TLC plate and through FTIR. The purity of compounds was confirmed by TLC, and structures were elucidated through FTIR.

The physical characteristics of synthesized compounds, including molecular formula, solubility, physical color, and state are presented in Table 4.12.

TABLE 4.12: Physical Properties of Synthesized Compounds

Compound	Molecular Formula	Molecular Weight	Physical Color	Physical State	Solubility
AM-1	C ₂₄ H ₂₆ FN ₄ O ₄	452.48	Light yellow	Solid	DMSO*
AM-2	C ₂₅ H ₂₇ FN ₄ O ₅	482.515	Pale yellow to light yellow	Solid	DMSO*
AM-3	C ₂₅ H ₂₇ FN ₄ O ₄	466.515	Off-white / light yellow	Solid	DMSO
AM-4	C ₂₄ H ₂₆ FN ₅ O ₄	467.503	Brownish Green	Solid	DMSO*
AM-5	C ₂₄ H ₂₅ FN ₄ O ₅	468.488	Off-white to pale yellow	Solid	DMSO*
AM-6	C ₂₂ H ₂₈ FN ₅ O ₄	444.509	White to off-white	Solid	DMSO*
AM-7	C ₂₅ H ₂₄ FN ₅ O ₄ S	509.562	Dark Yellow	Solid	DMSO*

*Soluble upon heating and sonication

4.11 Characterization

4.11.1 Fourier - Transform Infrared Spectroscopy Analysis

An in-depth analysis of each FTIR spectra was carried out in order to prove successful formation of target compounds (AM-1, AM-3, AM-5, and AM-7). Different aromatic and heterocyclic amines were used in the nucleophilic substitution of the N-chloroacetyl fluoroquinolone intermediate compound, which led to the characteristic changes of the functional group vibrational frequencies. The presence of the large secondary amine (N-H) stretching band at 3410.5 cm^{-1} and the well-defined zwitterionic hydrogen bonding bands from $2500\text{--}2800\text{ cm}^{-1}$ indicates that the terminal piperazinyll N is not modified.

The spectrum of intermediate 2, after functionalization, shows the complete disappearance of the mentioned (N-H) free band and the disappearance of the zwitterionic background giving a clear view of the bands corresponding to aliphatic (C-H) stretching bands at $2800\text{--}3000\text{ cm}^{-1}$ [105]. The above mentioned loss of the native zwitterion is accompanied by the appearance of a new intense carbonyl (C=O) stretching band at 1656.8 cm^{-1} and the appearance of the corresponding carbonyl band at 1709 cm^{-1} [105], which corresponds to the core carbonyl group of carboxylic acid.

Important, a new aliphatic C-Cl stretching absorption band was observed at 788.5 cm^{-1} [102] after the introduction of a new chlorinated electrophile modifier into the structure of Norfloxacin and was proved to be the same. All the above mentioned spectral changes are very clear to see that the molecule has undergone covalent change at the Nitrogen atom of the piperazine ring.

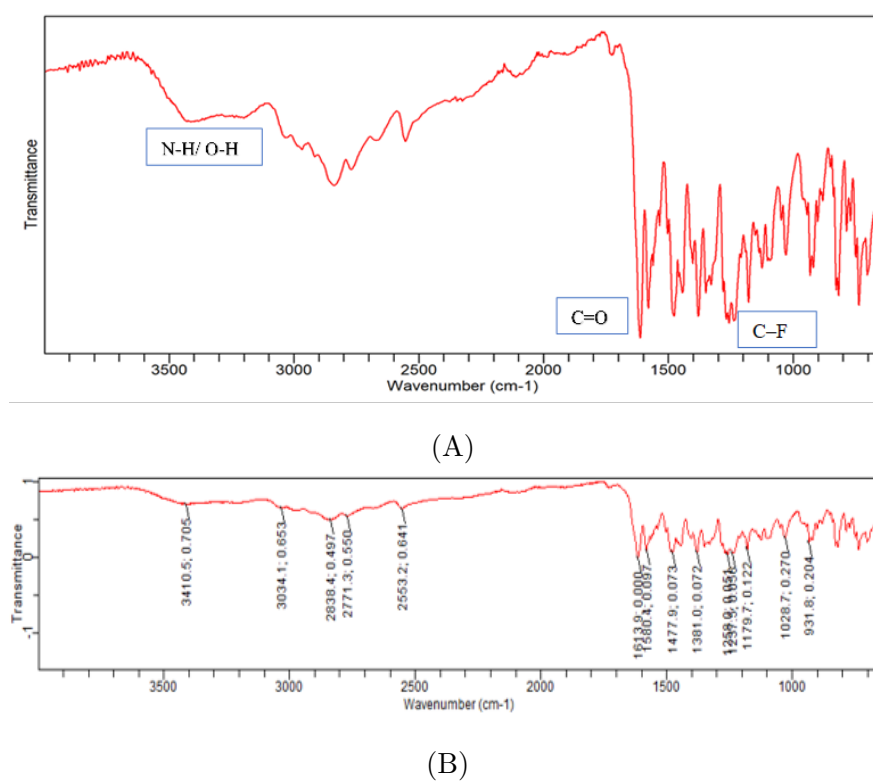


FIGURE 4.15: FTIR Spectrum of Norfloxacin, Unlabeled clear peaks (A), Labeled peaks (B)

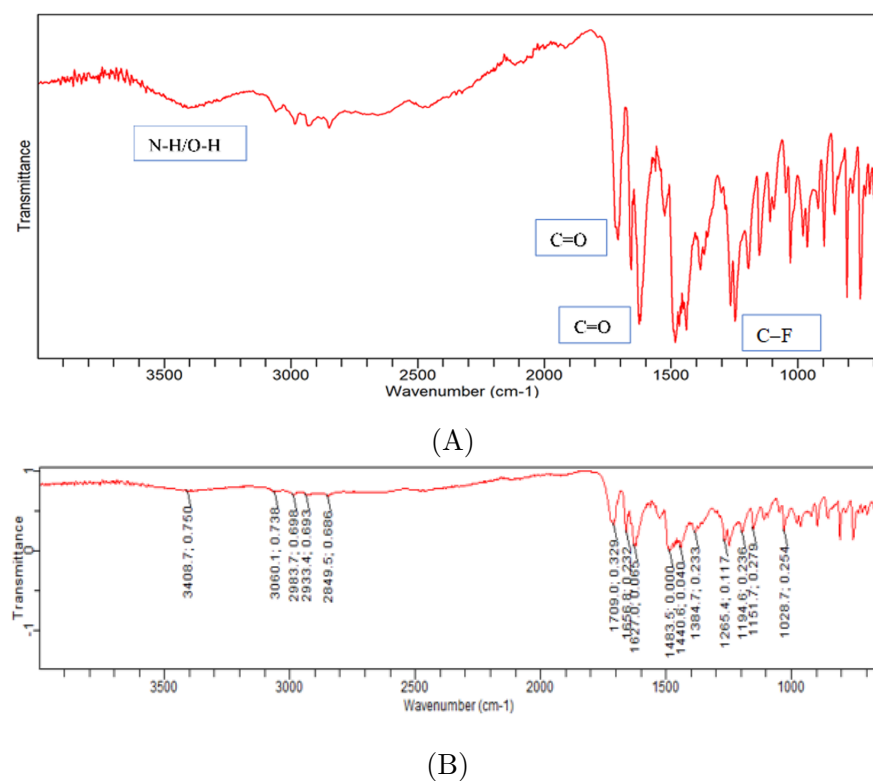


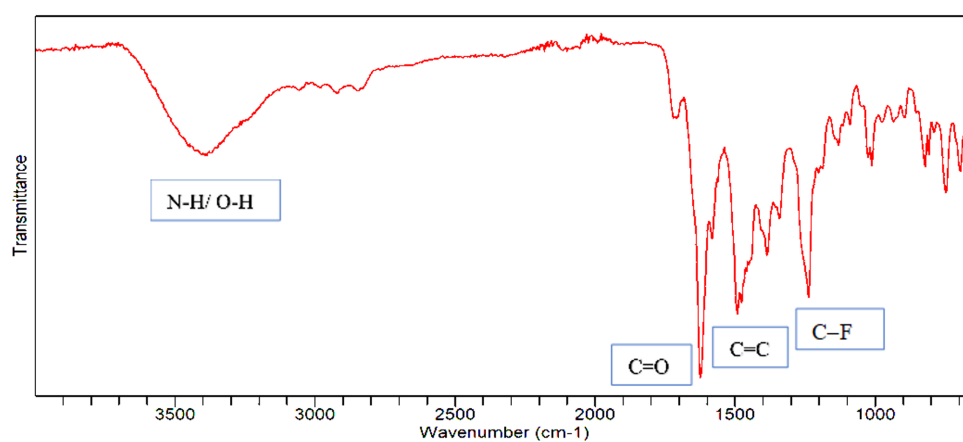
FIGURE 4.16: FTIR Spectrum of Intermediate, Unlabeled clear peaks (A), Labeled peaks (B)

4.11.1.1 FTIR Spectral Analysis of Compound AM-1

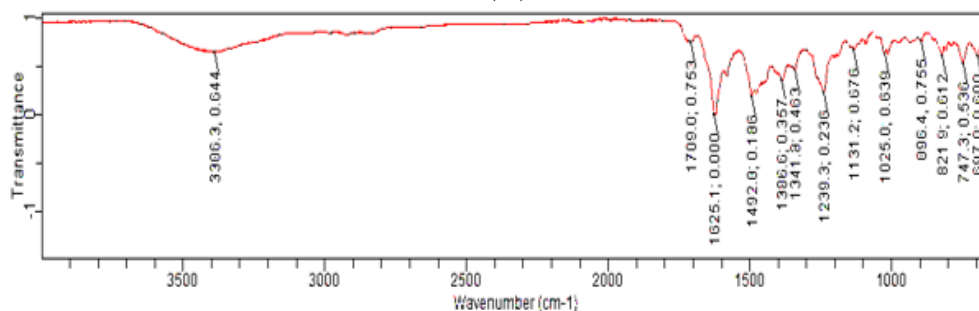
TABLE 4.13: FTIR Spectral Data of Compound AM-1

Observed Wavenumber (cm ⁻¹)	Functional Group Assignment
3386 (Broad)	N-H/O-H Stretch
1709	C=O Stretch
1625	Amide C=O Stretch
1492	Aromatic C=C Stretch
1239	C-F Stretch

The spectrum of AM-1 (shown in Figure 4.17) confirms the replacement of the aliphatic chlorine atom of the intermediate in spectrum shown in Figure 4.16 with the nucleophilic aniline group. The most descriptive diagnostic marker is the appearance of a sharp secondary amine (N-H) stretching band, overlapping with O-H band at 3386 cm⁻¹ [106].



(A)



(B)

FIGURE 4.17: FTIR Spectrum of Compound AM-1, Unlabeled clear peaks (A), Labeled peaks (B)

The structural consolidation of the acetamide link is verified by a strong Amide I (C=O) carbonyl stretch at 1625 cm^{-1} [107], contrasting with the core framework's carboxylic/quinolone ketone (C=O) stretch resolved at 1709 cm^{-1} [105]. The aromatic ring system introduced by the aniline fragment is evidenced by classic C-H bending and sharp skeletal aromatic (C=C) stretching frequencies at 1492 cm^{-1} . The principle FTIR absorption bands are shown in Table 4.13.

4.11.1.2 FTIR Spectral Analysis of Compound AM-2

The summary of spectral profile of AM-2, presented in Table 4.14 shows a single, broad, intense absorption peak, shown in Figure 4.18. centered near 3365 cm^{-1} , which represents the overlapping stretching vibrations of both the core 3-carboxylic acid hydroxyl (O-H) group and the newly introduced secondary amide N-H network [106].

Successful substitution is further substantiated by a powerful asymmetric aryl ether (C-O-C) stretching band localized at 1341 cm^{-1} along with a sharp methoxy C-H stretching signature near 2849 cm^{-1} .

The core carboxylic acid carbonyl vibrates at 1722 cm^{-1} [105], while the newly established acetamide carbonyl bridge vibrates cleanly as an Amide I band at 1619 cm^{-1} [107].

Aromatic ring skeletal stretches (C=C) shift to 1511 cm^{-1} , influenced by the attached aromatic system, while the core aliphatic stretching networks remain cleanly resolved between $2800\text{-}3000\text{ cm}^{-1}$.

TABLE 4.14: FTIR Spectral Data of compound AM-2

Observed Wavenumber (cm^{-1})	Functional Group
3365 (Broad)	N-H/O-H Stretch (Overlapping)
1619	Amide C=O Stretch (Amide I)
1511	Aromatic C=C Stretch
1341	C-O-C (Methoxy) Stretch
2849, 2849	Aliphatic C-H Stretch (Methoxy)

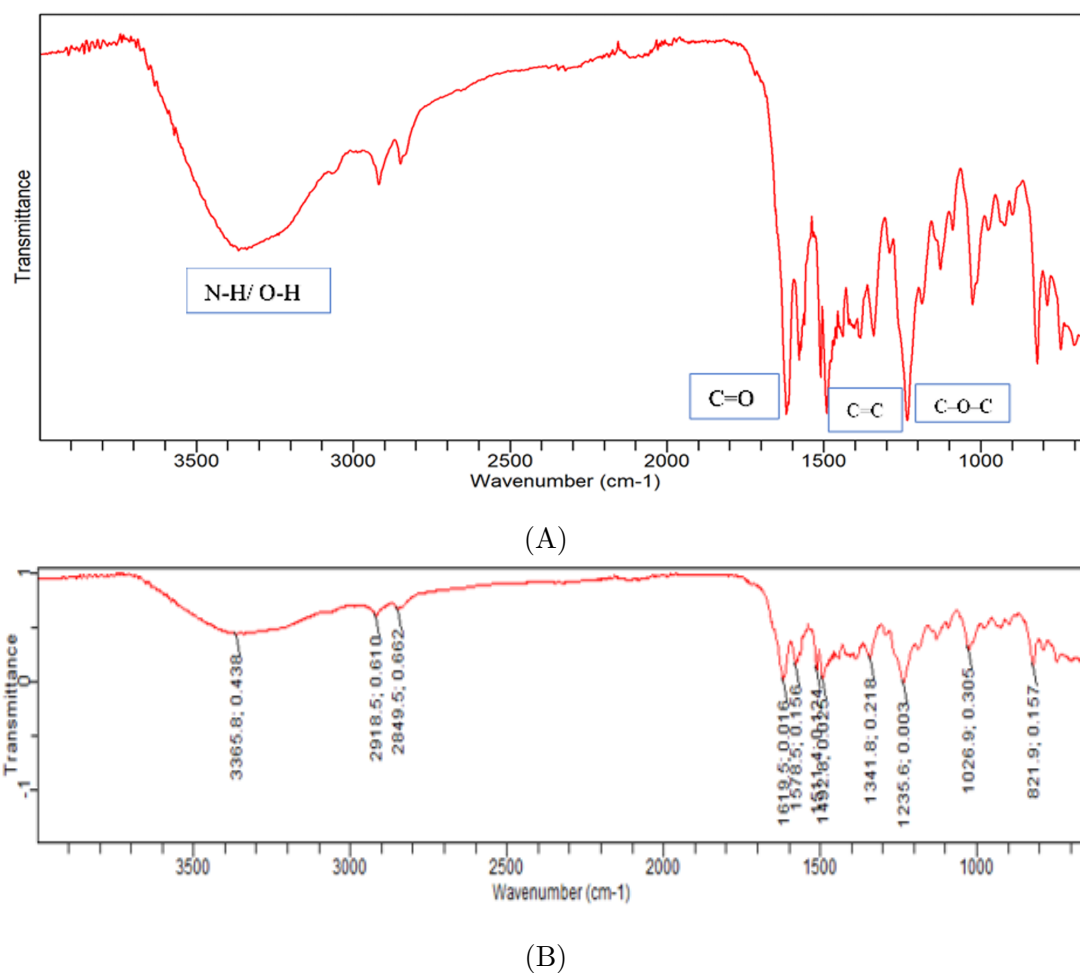


FIGURE 4.18: FTIR Spectrum of Compound AM-2, Unlabeled clear peaks (A), Labeled peaks (B)

4.11.1.3 FTIR Spectral Analysis of Compound AM-3

The spectral profile of AM-3, presented in Table 4.15, highlights the successful formation of Amide linkage between p-Toluidine and intermediate. A well-defined secondary amide/amine (N-H) stretching frequency appears at 3408 cm^{-1} [106].

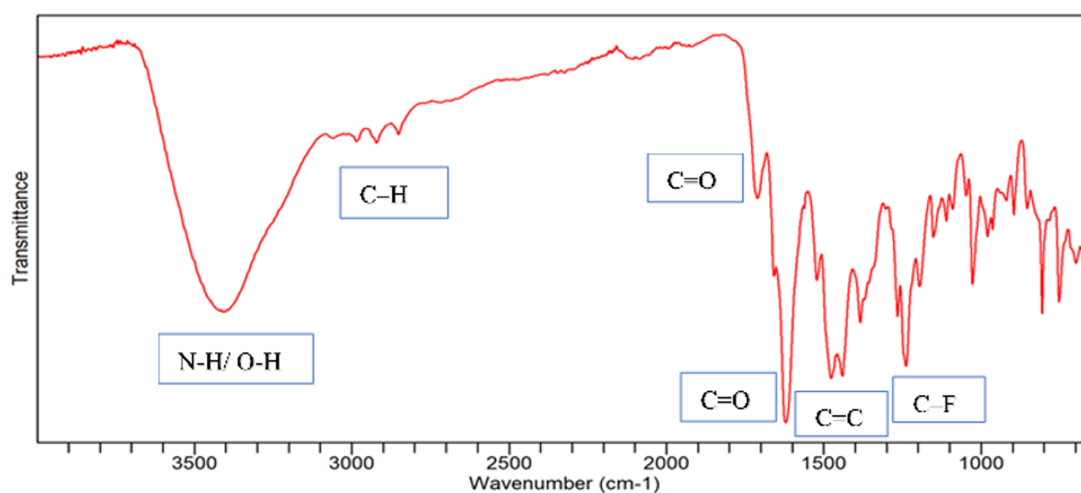
To verify presence of terminal methyl group ($-\text{CH}_3$) attached to the newly added benzene ring, distinct aliphatic symmetric and asymmetric C-H stretching bands are observed at 2922 cm^{-1} and 2853 cm^{-1} .

TABLE 4.15: FTIR Spectral Data of compound AM-3

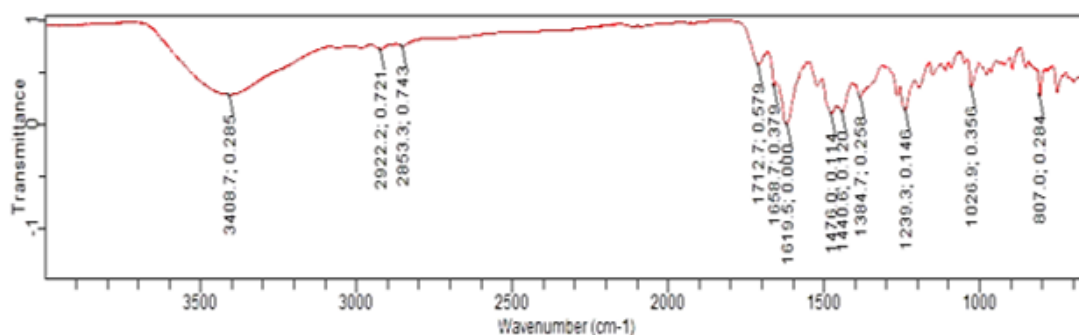
Observed Wavenumber (cm^{-1})	Functional Group
3408 (Broad)	N-H/O-H Stretch
2922, 2853	Aliphatic C-H Stretch

Table 4.15 continued from previous page

Observed Wavenumber (cm^{-1})	Functional Group
1712	C=O Stretch
1619	Amide C=O Stretch (Amide I)
1476, 1440	Aromatic C=C Stretch
1239	C-F Stretch



(A)



(B)

FIGURE 4.19: FTIR Spectrum of Compound AM-3, Unlabeled clear peaks (A), Labeled peaks (B)

The dual carbonyl topology maps accurately, showcasing the secondary amide (N-H) stretch at 1619 cm^{-1} [107] and the core keto-carbonyl stretch at 1712 cm^{-1} [105]. The strong para-substituted aromatic skeletal vibrations reveal a sharp peak at 1476 , and 1440 cm^{-1} , whereas the primary C-F stretching indicator remains stable at 1239 cm^{-1} , shown in Figure 4.19.

4.11.1.4 FTIR Spectral Analysis of Compound AM-4

TABLE 4.16: FTIR Spectral Data of compound AM-4

Observed Wavenumber (cm^{-1})	Functional Group
3390 (Broad)	N-H/O-H Stretch (Overlapping)
1735, 1701	Carboxylic Acid C=O Stretch
1617	Amide C=O Stretch (Amide I)
1511	Aromatic C=C Stretch
1239	C-F Stretch

The spectral profile of AM-4, presented in Table 4.16, shows a broad, complex absorption envelope, shown in Figure 4.20, centered near 3390 cm^{-1} representing the overlapping core carboxylic acid hydroxyl (O-H) and secondary amide N-H stretching vibrations [106]. The split carbonyl system expression is confirmed by the core carboxylic acid carbonyl stretch at 1735 and 1701 cm^{-1} and the sharp acetamide linker (Amide I) band at 1617 cm^{-1} [107]. The aromatic system is further validated by a sharp C=C ring skeletal stretch near 1511 cm^{-1} .

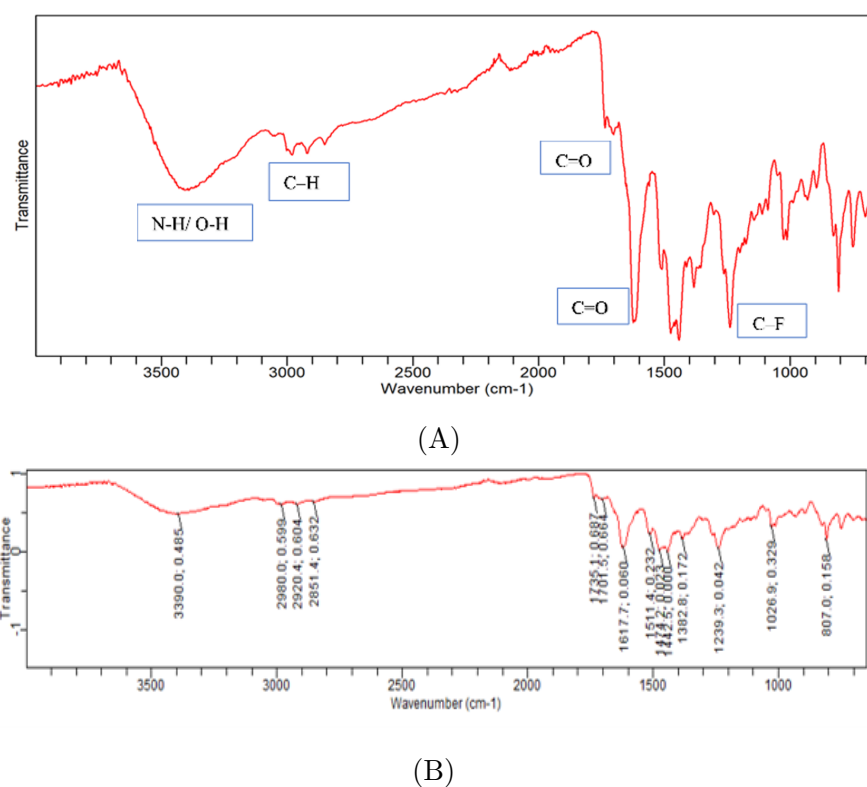


FIGURE 4.20: FTIR Spectrum of compound AM-4, Unlabeled clear peaks (A), Labeled peaks (B)

4.11.1.5 FTIR Spectral Analysis of Compound AM-5

The infrared spectrum of AM-5, shown in Figure 4.21, provides definitive validation for the coupling of p-aminophenol. The FTIR absorption bands are shown in Table 4.17.

TABLE 4.17: FTIR Spectral Data of compound AM-5

Observed Wavenumber (cm ⁻¹)	Functional Group
3391 (Broad)	N-H/O-H Stretch (Overlapping)
1619	Amide C=O Stretch (Amide I)
1490	Aromatic C=C Stretch
1239	C-F Stretch
2983, 2838	C-H Stretch

The diagnostic high-frequency cluster is marked by a single, broad, intense absorption envelope centered at 3391 cm⁻¹ [106], which represents the overlapping stretching vibrations of both the secondary amide N-H and the free phenolic hydroxyl (O-H) group participating in intermolecular hydrogen bonding.

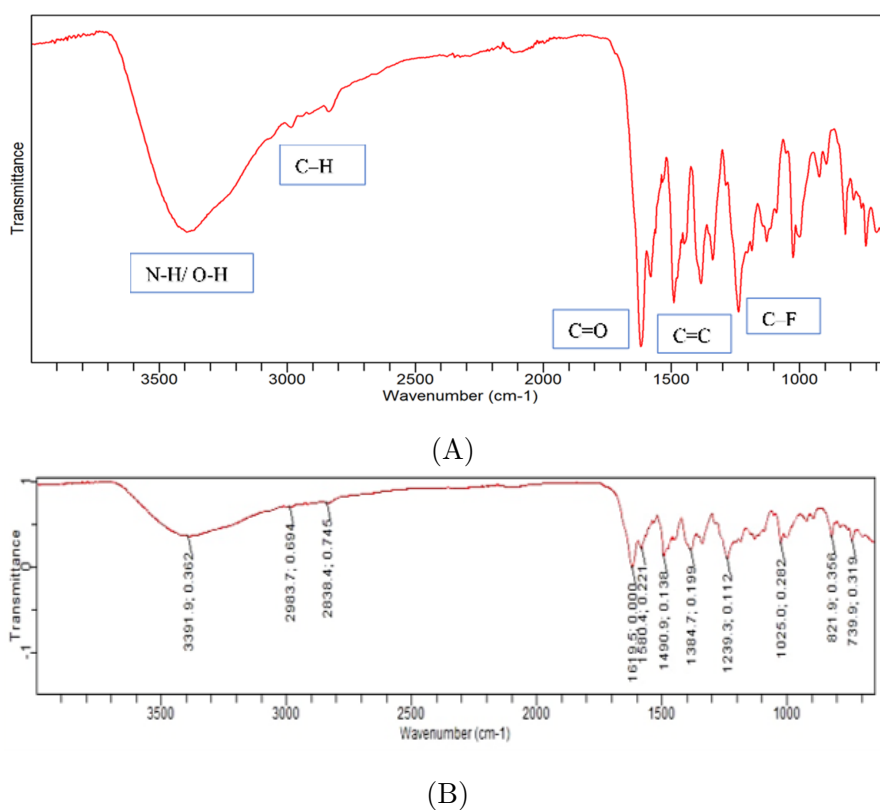


FIGURE 4.21: FTIR Spectrum of compound AM-5, Unlabeled clear peaks (A), Labeled peaks (B)

Successful substitution is further substantiated by a powerful phenolic C-O stretching band localized at 1195 cm^{-1} . The newly established acetamide carbonyl bridge vibrates cleanly as an Amide I band at 1619 cm^{-1} [107]. Aromatic frame stretches (C=C) shift to 1490 cm^{-1} , influenced by the electron-donating properties of the hydroxyl group. distinct aliphatic symmetric and asymmetric C-H stretching bands are observed at 2983 cm^{-1} and 2838 cm^{-1} .

4.11.1.6 FTIR Spectral Analysis of Compound AM-6

TABLE 4.18: FTIR Spectral Data of compound AM-6

Observed Wavenumber (cm^{-1})	Functional Group
3442 (Broad)	N-H / O-H Stretch (Overlapping)
3201	N-H of piperazine
1735	Carboxylic Acid C=O Stretch
1653, 1627	Amide C=O Stretch (Amide I)
2890, 2829	Aliphatic C-H Stretch (Increased Intensity)
1263	C-F Stretch

The broad baseline envelope near 3442 cm^{-1} representing the core carboxylic acid (O-H) stretch overlapping with the secondary amide linkage (Figure 4.22), out of which a sharp heterocyclic secondary amine stretching peak (N-H) resolves at 3402 cm^{-1} [106].

This environment is structurally supported by a distinct, lower-frequency piperazine secondary amine stretching ring shoulder at 3201 cm^{-1} . Below this region, the sharp aliphatic C-H stretches $2800\text{-}3000\text{ cm}^{-1}$ demonstrate a noticeable increase in intensity due to the additional methylene groups of the secondary ring.

Successful assembly is confirmed in the carbonyl region by the core carboxylic acid carbonyl stretching at 1735 cm^{-1} [105] and the acetamide linker (Amide I) band at 1653 and 1627 cm^{-1} , while strong alicyclic C-N stretching vibrations resolve clearly in the fingerprint region between 1263 cm^{-1} [107]. The principle FTIR absorption bands are shown in Table 4.18.

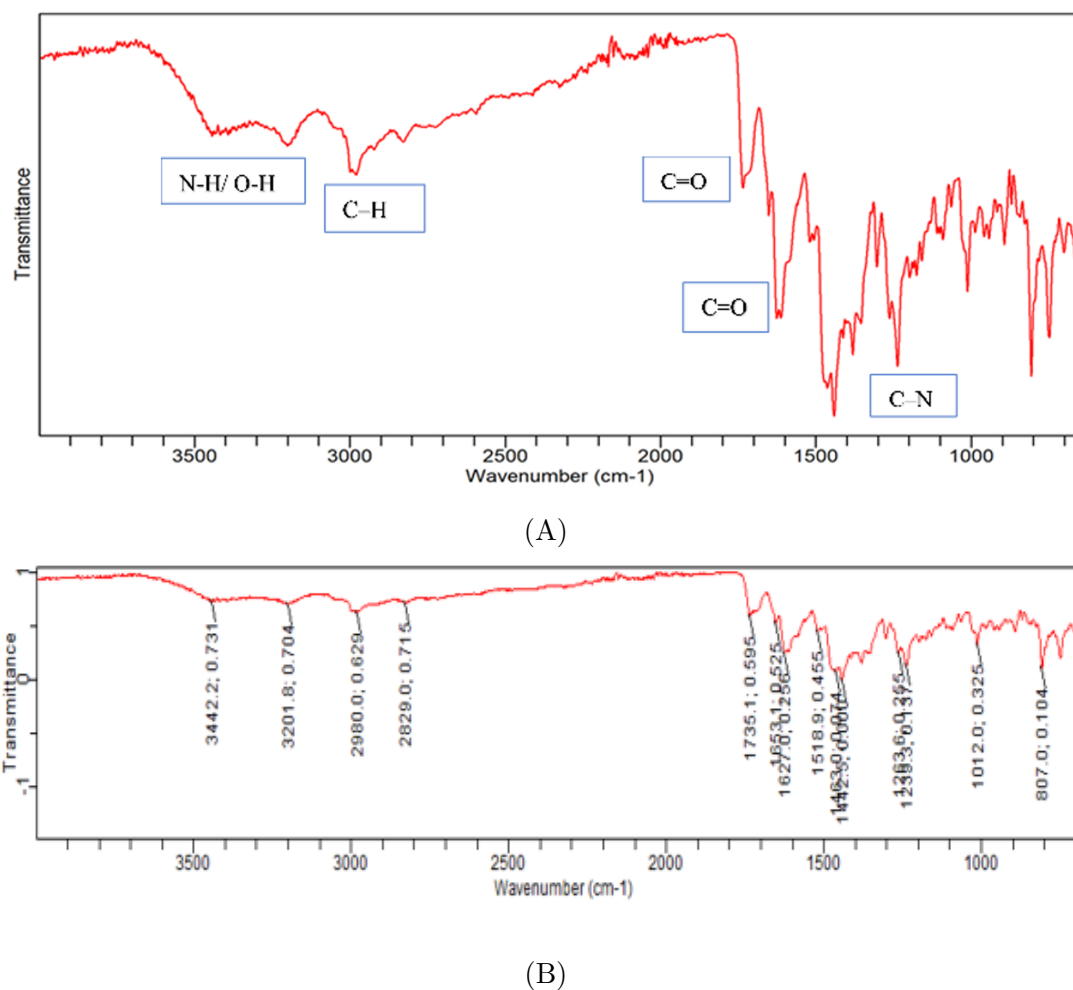


FIGURE 4.22: FTIR Spectrum of compound AM-6, Unlabeled clear peaks (A), Labeled peaks (B)

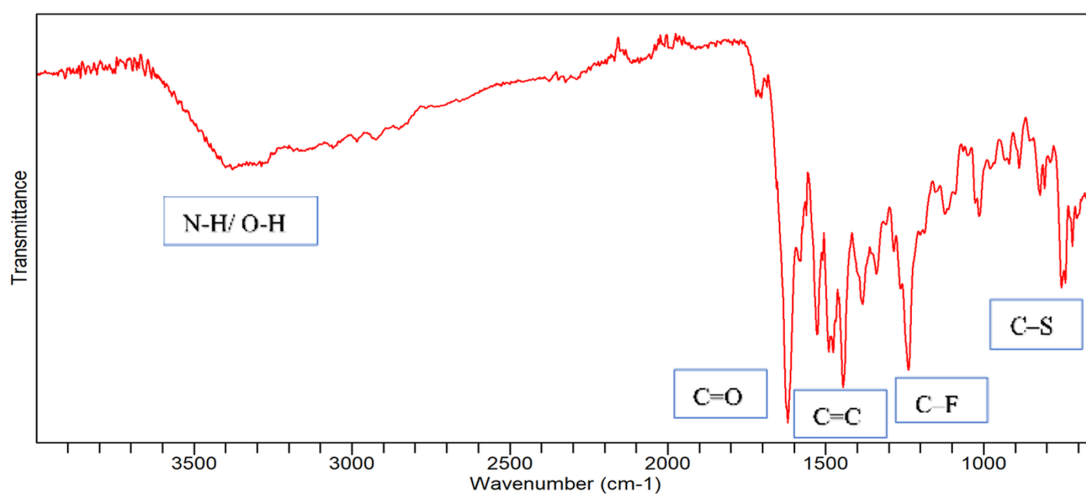
4.11.1.7 FTIR Spectral Analysis of Compound AM-7

The FTIR spectrum for AM-7, shown in Figure 4.23, provides highly convincing evidence of the introduction of the heteroaromatic 2-aminobenzothiazole system. The secondary amine (N-H) stretching band is resolved as a sharp peak at 3401 cm^{-1} [106]. Because of the dense electronic nature of the fused benzothiazole ring, unique diagnostic markers emerge: a prominent heterocyclic C=N stretching vibration is recorded at 1528 cm^{-1} , which couples with the neighboring aromatic ring stretches at 1490 , and 1444 cm^{-1} . The newly constructed secondary amide linkage exhibits its characteristic carbonyl absorption at 1619 cm^{-1} [107], while the original central carbonyl system records at 1730 cm^{-1} [105]. Additionally, the presence of the thioether network is confirmed by a clear C-S stretching frequency

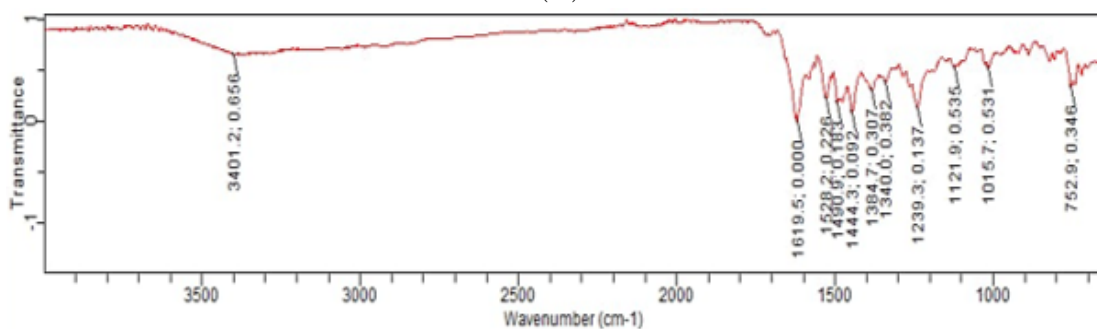
deep in the fingerprint region at 752 cm^{-1} . The principle FTIR absorption bands are shown in Table 4.19.

TABLE 4.19: FTIR Spectral Data of compound AM-7

Observed Wavenumber (cm^{-1})	Functional Group
3401	N-H/O-H Stretch
1730	C=O Stretch
1619	Amide C=O Stretch (Amide I)
1528	Heterocyclic C=N Stretch
1490, 1444	Aromatic C=C Stretch
1239	C-F Stretch
752	C-S Stretch



(A)



(B)

FIGURE 4.23: FTIR Spectrum of compound AM-7, Unlabeled clear peaks (A), Labeled peaks (B)

4.11.2 Nuclear Magnetic Resonance Spectroscopic Analysis

AM-7 was characterized by ^1H -NMR Spectroscopy (400 MHz, DMSO- d_6) (Refer to Appendix A). Successful covalent anchoring is confirmed by the complete disappearance of the characteristic chlorinated methylene singlet $-\text{COCH}_2\text{Cl}$ at δ 4.44 ppm [108] alongside the absolute absence of the broad exocyclic primary amine signal $-\text{NH}_2$ of the 2-aminobenzothiazole precursor [109] typically observed at δ 4 ppm (s, 2H, $-\text{NH}_2$), validating quantitative displacement at the exocyclic amine site.

This coupling is corroborated by a shielding shift of the spacer methylene protons upfield into a complex multiplexed aliphatic envelope at δ 3.44- 3.99 ppm (m, 10H, piperazinyl protons and $-\text{COCH}_2\text{N}-$), alongside the appearance of a new aromatic fingerprint region at δ 7.64 ppm (d, $J=7.8$ Hz, ^1H , benzothiazole C_7-H), δ 7.24 ppm (d, $J=8.0$ Hz, ^1H , benzothiazole C_4-H), δ 7.18 ppm (t, $J=7.6$ Hz, ^1H , benzothiazole C_6-H), and δ 6.99 ppm (t, $J=7.5$ Hz, ^1H , benzothiazole C_5-H).

Furthermore, the spectrum displays a clean coexistence of the intact native fluoroquinolone core fragments, demonstrating a sharp two-proton quartet centered at δ 4.59-4.60 (q, $J=7.1$ Hz, 2H, $\text{N}_1-\text{CH}_2\text{CH}_3$) cleanly separated from the adjacent shifted acetamide linker at δ 3.97-3.99 ppm (s, 2H, $-\text{COCH}_2\text{N}$). While the remaining downfield architecture is confirmed by the sharp isolated vinyl singlet at δ 8.96 ppm (s, ^1H , C_2-H) and the highly deshielded carboxylic acid proton at δ 15.33 ppm (s, ^1H , $-\text{COOH}$).

^1H -NMR (400 MHz, DMSO- d_6) δ (ppm): 15.33 (singlet, ^1H , carboxylic acid proton), 8.96 (singlet, ^1H , quinolone C-2 proton), 7.90 (doublet, $J = 13.0$ Hz, ^1H , quinolone C-5 proton), 7.64 (doublet, $J = 7.8$ Hz, ^1H , benzothiazole C-7 proton), 7.24 (doublet, $J = 8.0$ Hz, ^1H , benzothiazole C-4 proton), 7.18 (triplet, $J = 7.6$ Hz, ^1H , benzothiazole C-6 proton), 7.17 (doublet, $J = 7.0$ Hz, ^1H , quinolone C-8 proton), 6.99 (triplet, $J = 7.5$ Hz, ^1H , benzothiazole C-5 proton), 4.59-4.60 (quartet, $J = 7.1$ Hz, 2H, N-1 methylene protons), 3.97-3.99 (singlet, 2H, acetamide linker

methylene protons), 3.44-3.85 (multiplet, 8H, piperazinyl protons), 1.42 (triplet, $J = 7.1$ Hz, 3H, ethyl terminal methyl protons).

4.12 *In-Vivo* Anti-Inflammatory Activity

The *in vivo* anti-inflammatory potential of the newly synthesized norfloxacin derivatives was systematically evaluated using the classic carrageenan-induced mouse paw edema model, with statistical variances determined via a Two-Way Mixed ANOVA design.

TABLE 4.20: Anti-Inflammatory Effect of AM-1 on Carrageenan-Induced Paw Edema in Mice

Group	Paw Thickness (mm) & Percentage Inhibition				
	0 h	1 h	2 h	4 h	6 h
Normal Saline Control (G1)	2.06 ± 0.04	2.08 ± 0.04	2.10 ± 0.04	2.12 ± 0.04	2.09 ± 0.04
Negative Control (G2)	1.90 ± 0.01	2.80 ± 0.02	3.60 ± 0.02	4.10 ± 0.02	3.60 ± 0.02
Positive Control (G3, 20 mg/kg)	1.93 ± 0.02	2.72 ± 0.01	3.21 ± 0.02**	3.32 ± 0.02***	2.82 ± 0.02***
		-2.86%	-10.83%	-39.00%	-21.67%
Test Compound (G4, 10 mg/kg)	2.01 ± 0.01	2.82 ± 0.02	3.60 ± 0.02	4.10 ± 0.03	3.22 ± 0.03*
		(-0.71%)	0.00%	-0.30%	-10.56%
Test Compound (G5, 20 mg/kg)	1.91 ± 0.02	2.82 ± 0.02	3.61 ± 0.04	4.01 ± 0.02	3.31 ± 0.03*
		(-0.71%)	(-0.28%)	-1.20%	-8.06%
Test Compound (G6, 30 mg/kg)	1.91 ± 0.02	2.81 ± 0.02	3.60 ± 0.02	3.91 ± 0.04*	3.20 ± 0.02**
		(-0.36%)	0.00%	-9.00%	-11.11%

Note: Values are expressed as Mean ± SEM ($n = 6$ animals per group). Statistical significance was evaluated relative to the Negative Control group (G2) utilizing a Two-Way Mixed ANOVA followed by Bonferroni's post-hoc test: $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*). Values enclosed within parentheses represent the calculated percentage inhibition of edema at each respective time interval.

Injection of the Inflammation inducing agent carrageenan into the subplantar region of the negative control group (G2) successfully elicited an acute localized inflammatory response. Paw edema in this group escalated from a baseline value of 1.90 ± 0.01 mm to a maximum thickness of 4.10 ± 0.02 mm at the 4-hour mark, verifying the expected kinetics of the assay. Intraperitoneal administration of the standard reference drug, Diclofenac (G3, 20 mg/kg) [110], validated the experimental protocol by demonstrating early onset protection at 1 hour (2.86%), progressing to clear statistical significance by Hour 2 (10.83%, $P < 0.01$), and culminating in highly significant anti-edematous preservation at the 4-hour peak interval (3.32 ± 0.02 mm, $P < 0.001$), which represents a maximum percentage inhibition of 39.0%.

The groups treated with varying doses of the novel test compound displayed a distinct, dose-dependent anti-inflammatory profile with a characteristic delayed onset of action. During the early phase of edema development (Hours 1 and 2), no significant mitigation was observed in the 10 mg/kg (G4), 20 mg/kg (G5), or 30 mg/kg (G6) treatment groups, with mean paw volumes hovering close to or slightly exceeding control levels due to initial autacoid release patterns. However, at the critical 4-hour peak inflammatory threshold, the highest dosage tier of 30 mg/kg (G6) manifested a statistically significant reduction in paw edema (3.91 ± 0.04 mm, $P < 0.05$), presented graphically in Figure 4.24. yielding a target-specific percentage inhibition of 9.0%. At the same post-injection interval, the lower dosage groups of 10 mg/kg and 20 mg/kg achieved marginal, statistically non-significant inhibition values of 0.3% and 1.2%, respectively, shown in Table 4.20.

From a pathophysiological standpoint, the 4-hour post-injection milestone serves as the primary benchmark for screening target-specific anti-inflammatory agents. Carrageenan-induced inflammation follows a classic biphasic profile; the early phase (0-2 hours) is mediated by the release of histamine, serotonin, and bradykinin, whereas the delayed second phase (2-4 hours) is driven by the localized overproduction of prostaglandins and leukotrienes via cyclooxygenase and lipoxygenase

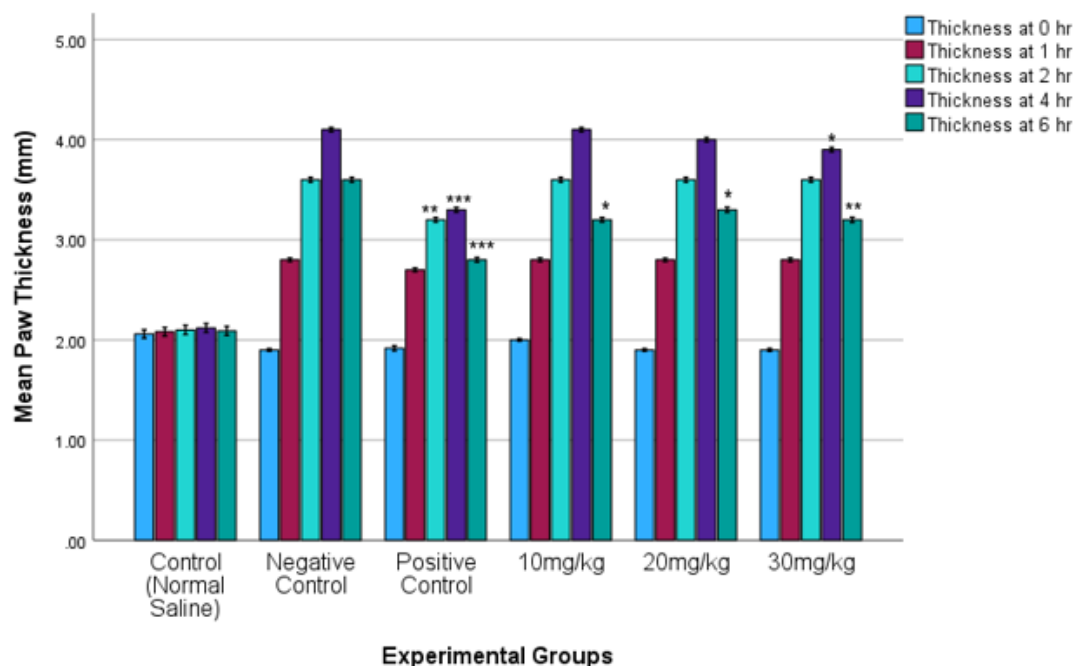


FIGURE 4.24: Graphical representation of Effect of different doses of AM-7 on Paw Thickness

Note: Values are expressed as Mean $\pm$ SEM ($n = 6$), $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*) compared to the negative control group.

pathways. Because the vehicle control group reaches its maximum physiological expansion at the 4-hour mark, this interval provides the most accurate and sensitive metric to determine the active enzyme-inhibitory capacity of the test derivatives.

Conversely, the data gathered at the 6-hour interval was deliberately excluded from peak efficacy considerations, despite showing apparent percentage inhibitions of 10.56%, 8.06%, and 11.11% for G4, G5, and G6, along with associated post-hoc significance ($P < 0.05$ and $P < 0.01$). This exclusion is methodologically necessary because the untreated carrageenan control group (G2) naturally enters a phase of spontaneous resolution by Hour 6, with paw volume declining from 4.10 mm back down to 3.60 mm. Consequently, the downstream significance observed at 6 hours reflects a combination of endogenous tissue recovery and passive clearance rather than active therapeutic intervention. Isolate screening at the 4-hour peak ensures that the statistical conclusions rigorously validate the true, active anti-inflammatory potency of the target derivatives.

Chapter 5

Conclusion and Recommendations

In this study, the application of Computer-Aided Drug Design (CADD) together with synthetic chemistry for the investigation of seven novel compounds AM-1 to AM-7 as potential anti-inflammatory agents acting on the LOX pathway has been investigated. The pharmacophore modeling is used for the design of these compounds and then subjected to molecular docking and ADMET analysis to determine the favorable binding properties and the drug-like nature. The chemical modifications were carried out in the laboratory in accordance with the computational results, and confirmed by FTIR and ¹H-NMR spectroscopy. Anti-inflammatory activity was found to be dose dependent in the carrageenan induced mouse paw edema test with doses of 10, 20 and 30mg/kg of AM-7 in the preliminary *in-vivo* study. Although these preliminary results seem promising, additional research is needed in order to validate the therapeutic value, mode of action, and safety of these compounds. The future work will need to include in vitro studies of LOX inhibition to find out IC₅₀ values, gastric tolerance and ulceration studies in animals to study their gastrointestinal safety, and in vivo toxicological and pharmacokinetics studies. Exact Molecular weight of compounds can be determined through Mass Spectrometry in future. Furthermore, it would be beneficial to expand the chemical library with different ring substitutions to examine

the structure-activity relationships, to test for dual inhibition of COX and LOX enzymes, to carry out molecular dynamics studies to check the stability of the binding and to perform co-crystallization studies.

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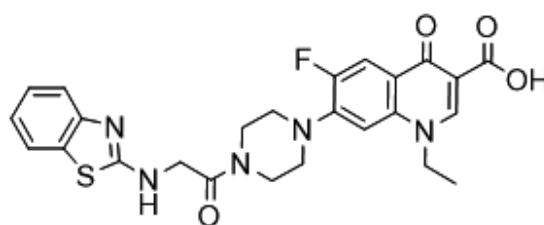
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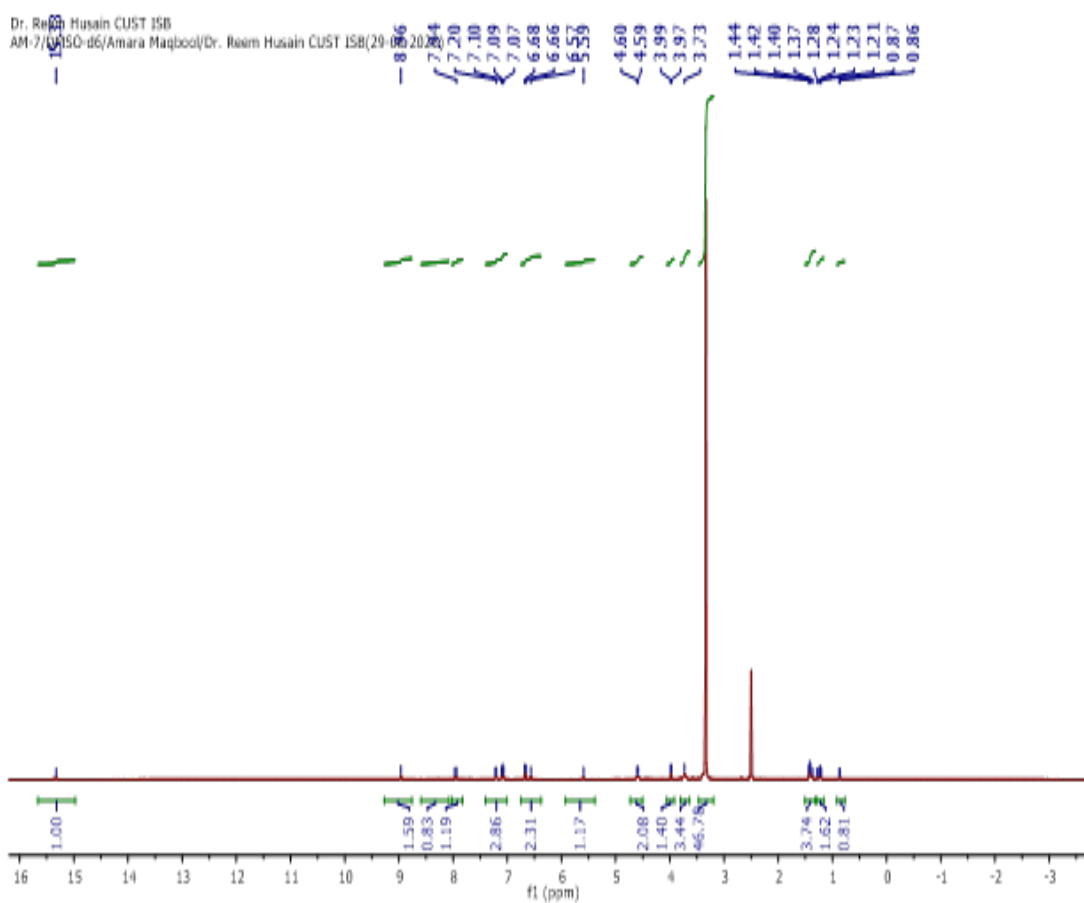
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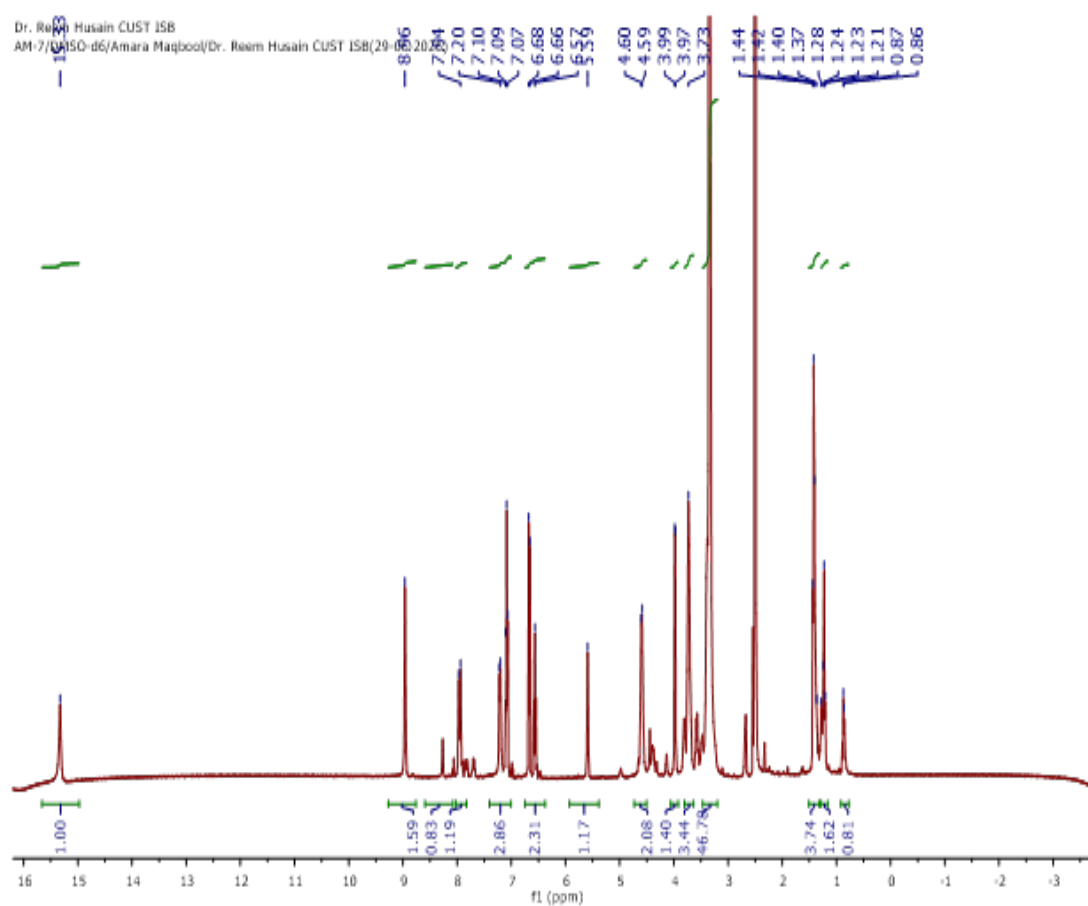
Appendix A



AM-7



(A)



(B)

¹H-NMR of compound AM-7 (C₂₅H₂₄FN₅O₄S), (A) Normal peaks (B) Zoomed peaks