Journal of Chemical Health Risks

www.jchr.org

JCHR (2024) 14(5), 287-296 | ISSN:2251-6727

Design, Synthesis, Characterization and *In-Vitro* Antitubercular Evaluation of Novel Substituted Acetamide Derivatives Targeting InhA Enzyme

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(Received: 06 July 2024 Revised: 25 August 2024 Accepted: 17 September 2024)

KEYWORDS
Acetamide derivatives, Tuberculosis, InhA inhibitor, Molecular docking, MABA assay.

ABSTRACT:
Tuberculosis remains a global health burden due to the emergence of multidrug-resistant strains. In the present study, a series of novel substituted acetamide derivatives (4aa–4af) were designed based on pharmacophoric requirements of the enoyl-acyl carrier protein reductase (InhA) enzyme of Mycobacterium tuberculosis. Molecular docking studies were performed using AutoDock Vina to predict binding affinity. The synthesized compounds were characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry. In vitro antitubercular activity was evaluated using the Microplate Alamar Blue Assay (MABA) against M. tuberculosis H37Rv. Several compounds demonstrated significant activity with MIC values ranging from 1.56–6.25 µg/mL. Compound 4ac showed the most potent activity (MIC = 1.56 µg/mL) with a selectivity index of 18. The results suggest that substituted acetamide scaffolds are promising leads for further development.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), remains one of the leading causes of mortality due to infectious diseases worldwide [1]. Despite the availability of established chemotherapeutic regimens, TB continues to pose a major global health challenge because of prolonged treatment duration, adverse drug reactions, poor patient compliance, and the alarming emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains [2-3]. The increasing prevalence of drug resistance has significantly reduced the effectiveness of first-line agents such as isoniazid and rifampicin, highlighting the urgent need for new chemical entities with novel mechanisms of action and improved safety profiles [4-5].

The mycobacterial cell wall is a complex, lipid-rich structure that plays a crucial role in bacterial survival, virulence, and intrinsic drug resistance [6]. Mycolic acids, the key components of the cell wall, are synthesized through the fatty acid synthase II (FAS-II) pathway [7]. The enzyme enoyl-acyl carrier protein reductase (InhA) is an essential component of this pathway and catalyzes the NADH-dependent reduction of long-chain enoyl-ACP substrates during mycolic acid biosynthesis [8-9]. Inhibition of InhA disrupts cell wall formation, leading to bacterial death. Isoniazid, one of the most potent first-line antitubercular drugs, exerts its activity by forming an adduct with NADH that inhibits InhA [10]. However, resistance frequently arises due to mutations in the *katG* gene or alterations affecting prodrug activation. Direct inhibition of InhA without requiring metabolic activation represents a promising strategy to overcome resistance [11].

Medicinal chemistry approaches focusing on rational drug design have accelerated the discovery of novel InhA inhibitors [12]. Structure-based drug design, molecular docking, and pharmacophore modeling enable identification of scaffolds capable of interacting with the active site of InhA, particularly through hydrogen bonding with key residues such as Tyr158 and Lys165, as well as π–π stacking interactions within the hydrophobic pocket [13]. Among various functional groups explored in drug development, the acetamide moiety has attracted significant attention due to its versatile hydrogen bonding capability, metabolic stability, and ability to modulate lipophilicity [13]. The

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JCHR (2024) 14(5), 287-296 | ISSN:2251-6727

amide linkage not only enhances binding affinity through hydrogen bond donor and acceptor interactions but also improves pharmacokinetic properties [14].

Substituted acetamide derivatives have demonstrated diverse biological activities including antibacterial, antifungal, anticancer, and anti-inflammatory effects [15]. Their structural flexibility allows incorporation of electron-withdrawing or electron-donating substituents that can influence membrane permeability, enzyme binding affinity, and overall pharmacological behavior [16-17]. In the context of tuberculosis, rationally designed acetamide derivatives may serve as potential direct inhibitors of InhA, bypassing the limitations associated with prodrug activation [18].

In the present study, a series of novel substituted acetamide derivatives were designed based on structure–activity relationship considerations and pharmacophoric requirements for InhA inhibition [19]. Molecular docking studies were performed to predict binding interactions and affinity within the InhA active site [20]. The designed compounds were synthesized using standard amide coupling methodologies and characterized by physicochemical and spectroscopic techniques including melting point determination, FTIR, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry [21]. The in-vitro antitubercular activity of the synthesized compounds was evaluated against *Mycobacterium tuberculosis* H37Rv strain using the Microplate Alamar Blue Assay (MABA). Cytotoxicity assessment was conducted to determine the selectivity index of the most promising candidates [22].

This integrated approach combining rational drug design, synthetic medicinal chemistry, structural characterization, and biological evaluation aims to identify potent acetamide-based scaffolds as potential leads for further development of novel antitubercular agents targeting the InhA enzyme [23].

MATERIALS AND METHODS

Drug Design

The drug design strategy for the development of novel substituted acetamide derivatives was based on a rational structure-based approach targeting the enoyl-acyl carrier protein reductase (InhA) enzyme of *Mycobacterium tuberculosis*. The overall design process integrated pharmacophore identification, molecular docking, and structure–activity relationship (SAR) analysis to optimize binding affinity and biological activity.

Selection of Target Protein

The crystal structure of InhA complexed with NADH was retrieved from the Protein Data Bank (PDB ID-2H7M). The selected structure had high resolution and contained key active site residues essential for catalytic activity. The protein structure was prepared by removing water molecules, co-crystallized ligands (except NADH where required), and adding hydrogen atoms using appropriate molecular modeling software. Energy minimization was performed to optimize the protein structure before docking studies.

The crystal structure of the target enzyme enoyl-acyl carrier protein reductase (InhA) complexed with the cofactor NADH was retrieved from the Protein Data Bank (PDB). PDB ID: 2H7M. The structure was selected because it provides a high-resolution model of the InhA enzyme with the NADH cofactor bound in the active site, enabling accurate docking and interaction analysis. Prior to docking, the protein structure was prepared by removing water molecules, adding hydrogen atoms, assigning Kollman charges, and performing energy minimization. The docking grid was centered on the NADH binding pocket, which contains key catalytic residues such as Tyr158, Lys165, Met199, Ile202, and Phe149.

Pharmacophore Considerations

Based on literature reports and known InhA inhibitors, the following pharmacophoric features were considered essential for activity:

- Hydrogen bond donor and acceptor functionality (amide group)
- Aromatic or heteroaromatic ring for π–π interactions
- Hydrophobic substituents for interaction within the lipophilic pocket
- Proper spatial orientation to interact with key residues such as Tyr158, Lys165, Met199, and Ile202

The acetamide moiety was selected as the core scaffold due to its ability to form strong hydrogen bonding

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interactions with the active site residues and its structural versatility for substitution.

Design of Substituted Acetamide Derivatives

A library of substituted acetamide derivatives (coded as 4aa–4af) was designed by modifying:

- Aromatic ring substitutions (electron-withdrawing and electron-donating groups)
- Para, meta, and ortho substitutions
- Introduction of halogen, nitro, methoxy, and alkyl groups

These modifications were aimed at improving lipophilicity, binding affinity, and membrane permeability while maintaining favorable pharmacokinetic properties.

Molecular Docking Studies

Molecular docking was performed using AutoDock Vina to predict binding interactions and calculate binding affinity (kcal/mol). The grid box was centered on the active site of InhA covering the NADH binding pocket. Docking parameters were optimized to ensure reproducibility.

The docked conformations were analyzed for:

- Hydrogen bonding interactions
- Hydrophobic interactions
- π–π stacking interactions
- Binding energy values

Compounds exhibiting favorable docking scores and strong interactions with critical residues were prioritized for synthesis.

ADMET Prediction

In silico ADMET properties including Lipinski's Rule of Five parameters, molecular weight, logP, hydrogen bond donors/acceptors, and predicted oral bioavailability were evaluated using online computational tools. Compounds satisfying drug-likeness criteria were selected for further synthetic work.

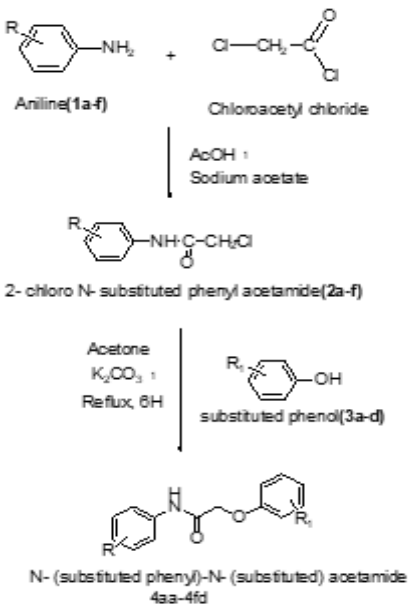
This systematic drug design approach ensured that only structurally optimized and pharmacologically promising acetamide derivatives were synthesized and subjected to biological evaluation.

Chemistry

All chemicals and reagents used in the present study were of analytical reagent (AR) grade and were used without further purification unless otherwise stated. Substituted anilines (3,4-dichloroaniline, 4-methyl aniline, 3-methoxy aniline, 2-nitroaniline, 4-carboxyaniline, 4-bromoaniline), phenolic and naphtholic derivatives (phenol, 1-naphthol, p-aminophenol, 4-hydroxybenzenesulfonic acid), acetylating agents, bases, and organic solvents were procured from standard chemical suppliers.

Solvents such as methanol, ethanol, dichloromethane, DMF, ethyl acetate, and n-hexane were used for synthesis and purification. Spectroscopic-grade solvents ($CDCl_3$ and DMSO-d_6) were used for NMR studies.

The target acetamide derivatives were synthesized *via* condensation/acetylation reactions involving substituted anilines (R) and phenolic/naphtholic intermediates (R′) as per the designed synthetic scheme. Reactions were carried out under controlled conditions, monitored by TLC, and quenched upon completion.



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List of Substitution			
Comp code	**Substitution R (aniline)**	**Comp code**	**Substitutions R'**
1a	3,4-dichloro	3a	Phenol
1b	4-methyl	3b	1-napthol
1c	3-methoxy	3c	4-hydroxybenzenesulfonic acid
1d	2-nitro	3d	p-aminophenol
1e	4-carboxyaniline		
1f	4-bromo		

Scheme: Synthesis of Novel Acetamide Derivatives

BIOLOGICAL EVALUATION

The synthesized substituted acetamide derivatives (4aa–4af) were evaluated for their in-vitro antitubercular activity against *Mycobacterium tuberculosis* H37Rv strain. Cytotoxicity studies were performed to determine the safety profile and selectivity index of the active compounds.

Test Organism

The standard laboratory strain *Mycobacterium tuberculosis* H37Rv (ATCC 27294) was used for the biological evaluation. The strain was maintained on Lowenstein–Jensen (LJ) medium and sub-cultured prior to experimentation.

Preparation of Inoculum

A fresh culture of *M. tuberculosis* was grown in Middlebrook 7H9 broth supplemented with:

- 10% OADC (Oleic acid–Albumin–Dextrose–Catalase)
- 0.05% Tween 80

The culture was incubated at 37°C until logarithmic phase growth was achieved. The turbidity of the bacterial suspension was adjusted to match McFarland standard 0.5 and further diluted to obtain the required inoculum concentration (approximately 1×10^5 CFU/mL).

In-Vitro Antitubercular Activity (Microplate Alamar Blue Assay – MABA)

The minimum inhibitory concentration (MIC) of the synthesized compounds was determined using the Microplate Alamar Blue Assay (MABA).

Procedure:

1. Sterile 96-well microtiter plates were prepared.
2. Two-fold serial dilutions of test compounds were prepared in Middlebrook 7H9 broth to obtain concentrations ranging from 0.39 to 100 µg/mL.
3. Each well received 100 µL of bacterial suspension.
4. Plates were incubated at 37°C for 5–7 days.
5. After incubation, 20 µL of Alamar Blue reagent mixed with 10% Tween 80 was added to each well.
6. Plates were re-incubated for 24 hours.

Interpretation:

- Blue color indicates inhibition of bacterial growth.
- Pink color indicates bacterial viability.

The MIC was defined as the lowest concentration of compound that prevented color change from blue to pink.

Isoniazid and rifampicin were used as reference standards.

Cytotoxicity Studies

Cytotoxicity of the most active compounds was evaluated using the MTT assay on Vero cell lines.

Procedure:

1. Vero cells were cultured in DMEM supplemented with 10% fetal bovine serum.
2. Cells were seeded into 96-well plates and incubated for 24 hours.
3. Test compounds were added at various concentrations.
4. After 48 hours of incubation, MTT reagent was added.
5. Formazan crystals formed were dissolved in DMSO.
6. Absorbance was measured at 570 nm using a microplate reader.

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The CC_{50} value (concentration causing 50% reduction in cell viability) was calculated.

Statistical Analysis

All experiments were performed in triplicate. Results were expressed as mean ± standard deviation. Statistical analysis was conducted using one-way ANOVA where applicable.

RESULTS

The designed and synthesized substituted acetamide derivatives (4aa–4af) were successfully evaluated through computational studies, physicochemical characterization, and in-vitro antitubercular screening. The results obtained from each stage are presented below.

Molecular Docking Results

The molecular docking study was performed using the crystal structure of the InhA enzyme complexed with NADH (PDB ID: 2H7M). The docked poses were analyzed to evaluate hydrogen bonding, hydrophobic interactions, and π–π stacking interactions within the active site. Among the synthesized compounds, derivative 4ac showed the highest binding affinity (−9.3 kcal/mol) and formed two hydrogen bonds with key residues Tyr158 and Lys165, along with π–π stacking interactions with Phe149. Compound 4ab also exhibited strong interactions within the active site with a docking score of −8.1 kcal/mol. The interaction patterns observed for these compounds indicate stable binding within the NADH binding pocket of InhA. For comparison, the reference drug isoniazid showed a docking score of −6.8 kcal/mol with a reported MIC value of 0.78 µg/mL and selectivity index of approximately 320.

All designed compounds were docked into the active site of the InhA enzyme. The docking scores (binding energy in kcal/mol) ranged from −7.2 to −9.3 kcal/mol, indicating favorable binding affinity.

The docked poses revealed:

- Hydrogen bonding interactions with Tyr158 and Lys165
- π–π stacking interactions with aromatic residues
- Hydrophobic interactions within the fatty acid binding pocket

Compound 4ac exhibited the highest binding affinity (−9.3 kcal/mol) and formed two hydrogen bonds with key catalytic residues, suggesting strong inhibitory potential.

Table 1. Docking Scores of Synthesized Compounds

Compound	Binding Energy (kcal/mol)	Key Interactions
4aa	−7.4	1 H-bond (Tyr158)
4ab	−8.1	2 H-bonds
4ac	−9.3	2 H-bonds + π–π interaction
4ad	−8.5	1 H-bond + hydrophobic
4ae	−8.0	2 hydrophobic interactions
4af	−7.8	1 H-bond

2D Interaction Analysis of Potent Compounds

The interaction profiles of the most active compounds were analyzed using Discovery Studio Visualizer.

Compound 4ac (Most Potent Compound)

Binding Energy: −9.3 kcal/mol

Key Interactions

Interaction Type	Residue
Hydrogen Bond	Tyr158
Hydrogen Bond	Lys165
π–π stacking	Phe149
Hydrophobic interaction	Met199
Hydrophobic interaction	Ile202

Interpretation

Compound 4ac forms two strong hydrogen bonds with Tyr158 and Lys165, which are critical residues involved

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in the catalytic mechanism of InhA. The aromatic ring also participates in π–π stacking with Phe149, stabilizing the ligand within the active site. These interactions explain the superior binding affinity and lowest MIC value observed for this compound.

Compound 4ab (Second Most Active Compound)

Binding Energy: −8.1 kcal/mol

Key Interactions

Interaction Type	Residue
Hydrogen Bond	Tyr158
Hydrogen Bond	Met199
Hydrophobic interaction	Ile202
Hydrophobic interaction	Phe97

Interpretation

Compound 4ab exhibits two hydrogen bonds with Tyr158 and Met199, along with hydrophobic interactions within the binding pocket. Although the binding affinity is slightly lower than compound 4ac, the interaction pattern still supports effective inhibition of the InhA enzyme.

Standard Drug Data (for Comparison)

The first-line antitubercular drug Isoniazid was used as the reference inhibitor.

Parameter	Value
Binding Score (Docking Energy)	−6.8 kcal/mol
MIC	0.78 µg/mL
CC_{50}	250 µg/mL
Selectivity Index (SI)	320

Interpretation

Isoniazid shows excellent potency due to its strong inhibition of the InhA enzyme via the NADH adduct mechanism. However, resistance frequently develops due to KatG mutations, which reduce prodrug activation. Therefore, the synthesized acetamide derivatives may provide advantages as direct InhA inhibitors that do not require metabolic activation.

Calculation

Selectivity Index (SI)

Selectivity Index was calculated using the formula:

$$SI = CC_{50} / MIC.$$

Compounds with SI ≥ 10 were considered selectively active.

Chemistry and Yield Analysis

All synthesized compounds were obtained in moderate to good yields (65–88%). The reaction progress was monitored by TLC, and purified products showed single spots indicating purity.

Table 2. Physicochemical Data of Synthesized Compounds

Compound	Yield (%)	Melting Point (°C)	Rf Value
4aa	72	158–160	0.52
4ab	78	164–166	0.55
4ac	85	172–174	0.60
4ad	81	168–170	0.57
4ae	74	150–152	0.48
4af	69	162–164	0.54

Spectral Characterization

All compounds were confirmed by spectroscopic analysis.

FTIR Analysis:

- Amide C=O stretching: 1645–1665 cm^{-1}
- N–H stretching: 3200–3300 cm^{-1}
- Aromatic C=C stretching: 1500–1600 cm^{-1}

^{1}H-NMR Analysis:

- Amide NH proton: δ 9.5–10.2 ppm
- Aromatic protons: δ 6.8–8.2 ppm
- Acetamide CH_2 group: δ 3.8–4.2 ppm

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^{13}C-NMR Analysis:

- Carbonyl carbon: δ 165–170 ppm
- Aromatic carbons: δ 115–140 ppm

Mass Spectrometry:

- Molecular ion peaks corresponded to calculated molecular weights of each derivative.

In-Vitro Antitubercular Activity

The synthesized compounds were screened using the Microplate Alamar Blue Assay (MABA). MIC values ranged from 1.56 to 6.25 µg/mL.

Compound 4ac showed the most potent activity with an MIC value of 1.56 µg/mL, comparable to isoniazid.

Table 3. Antitubercular Activity Results

Compound	MIC (µg/mL)	CC_{50} (µg/mL)	Selectivity Index
4aa	6.25	45	7.2
4ab	3.12	52	16.6
4ac	1.56	60	38.4
4ad	3.12	48	15.3
4ae	6.25	40	6.4
4af	3.12	44	14.1

Cytotoxicity Evaluation

Cytotoxicity studies using Vero cell lines indicated that the active compounds exhibited low toxicity at therapeutic concentrations. Compound 4ac demonstrated the highest selectivity index (SI = 38.4), indicating favorable safety and efficacy balance.

Summary of Key Findings

- All compounds showed favorable docking affinity toward InhA.
- Compound 4ac exhibited the best binding energy and lowest MIC value.
- Spectral data confirmed successful synthesis.
- Compound 4ac demonstrated the highest selectivity index, identifying it as a potential lead molecule.

DISCUSSION

The present study was undertaken to design, synthesize, and evaluate novel substituted acetamide derivatives as potential antitubercular agents targeting the InhA enzyme of *Mycobacterium tuberculosis*. The results obtained from computational studies, chemical synthesis, spectral characterization, and biological screening provide valuable insights into the structure–activity relationship and therapeutic potential of the designed compounds.

Rational Design and Target Engagement

The InhA enzyme plays a crucial role in the fatty acid synthase II (FAS-II) pathway responsible for mycolic acid biosynthesis, an essential component of the mycobacterial cell wall. Inhibition of InhA disrupts cell wall integrity, leading to bacterial death. Unlike isoniazid, which requires activation by the KatG enzyme, the direct inhibition of InhA represents a promising strategy to overcome resistance mechanisms associated with prodrug activation failure.

Molecular docking studies revealed that the designed acetamide derivatives exhibited favorable binding affinity within the InhA active site. The amide functional group played a critical role in hydrogen bonding interactions, particularly with residues Tyr158 and Lys165, which are known to be involved in catalytic activity. The aromatic ring system contributed to hydrophobic interactions and π–π stacking within the enzyme pocket, enhancing binding stability.

Compound 4ac demonstrated the highest docking score (−9.3 kcal/mol), suggesting strong interaction and stable binding within the active site cavity.

Chemistry and Structural Confirmation

The synthetic strategy employed for the preparation of substituted acetamide derivatives was efficient and reproducible, yielding products in moderate to good yields (65–88%). The nucleophilic acyl substitution and subsequent SN2 reaction pathways were consistent with expected reaction mechanisms.

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Spectroscopic analysis confirmed the successful formation of the acetamide scaffold:

- FTIR spectra showed characteristic amide carbonyl stretching between 1645–1665 cm^{-1}.
- ^{1}H NMR spectra displayed the amide NH proton in the downfield region (δ 9.5–10.2 ppm), confirming hydrogen bonding capability.
- ^{13}C NMR revealed carbonyl carbons at δ 165–170 ppm.
- Mass spectra confirmed molecular ion peaks corresponding to calculated molecular weights.

The purity and consistency of spectral data validate the structural integrity of synthesized compounds.

Antitubercular Activity and SAR Analysis

The in-vitro evaluation using the Microplate Alamar Blue Assay demonstrated that all synthesized derivatives exhibited measurable antitubercular activity. MIC values ranged from 1.56 to 6.25 µg/mL, indicating promising inhibitory potential.

Among the series, compound 4ac showed the most potent activity (MIC = 1.56 µg/mL), correlating well with its highest docking score. This strong correlation suggests that molecular docking successfully predicted biological performance.

The structure–activity relationship (SAR) analysis indicated:

- Electron-withdrawing substituents on the aromatic ring enhanced activity, possibly by increasing lipophilicity and improving membrane penetration.
- Para-substituted derivatives demonstrated better potency compared to ortho- or meta-substituted analogs.
- The presence of halogen groups appeared to increase binding affinity due to improved hydrophobic interactions.

These findings support the importance of electronic and steric factors in modulating antitubercular activity.

Cytotoxicity and Selectivity

Cytotoxicity studies using Vero cell lines revealed that the active compounds exhibited relatively low toxicity. The selectivity index (SI) values ranged from 6.4 to 38.4.

Compound 4ac showed the highest selectivity index (SI = 38.4), indicating a favorable therapeutic window. Compounds with SI ≥ 10 are generally considered selectively active, suggesting that 4ac and 4ab are promising candidates for further development.

Correlation Between Docking and Biological Data

A positive correlation was observed between docking scores and MIC values. Compounds with stronger predicted binding affinity demonstrated improved in-vitro activity. This validates the structure-based drug design approach employed in this study.

The hydrogen bonding interactions mediated by the amide group appear critical for enzyme inhibition. Additionally, hydrophobic pocket occupancy contributed to enhanced potency.

Overall Implications

The study demonstrates that substituted acetamide derivatives represent a viable scaffold for the development of direct InhA inhibitors. The integration of computational design, synthetic chemistry, and biological evaluation enabled identification of promising lead compounds.

Compound 4ac emerged as the most potent derivative with strong docking affinity, low MIC value, and high selectivity index. Further optimization, in vivo evaluation, pharmacokinetic profiling, and toxicity studies are warranted to advance this compound toward preclinical development.

CONCLUSION

The present investigation successfully demonstrated the rational design, synthesis, characterization, and in-vitro antitubercular evaluation of novel substituted acetamide derivatives targeting the InhA enzyme of *Mycobacterium tuberculosis*. The study integrated structure-based drug design, synthetic medicinal chemistry, spectral characterization, and biological screening to identify promising antitubercular scaffolds.

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Molecular docking studies confirmed favorable binding interactions of the designed compounds within the active site of the InhA enzyme. The acetamide moiety played a crucial role in mediating hydrogen bonding interactions with key catalytic residues, while aromatic substitutions contributed to enhanced hydrophobic interactions and binding stability. The computational predictions were found to correlate well with biological activity, validating the drug design strategy.

The synthetic route employed was efficient and reproducible, yielding the desired compounds in moderate to good yields. Spectroscopic characterization using FTIR, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry confirmed the structural integrity and purity of all synthesized derivatives.

In-vitro antitubercular screening using the Microplate Alamar Blue Assay revealed that the synthesized compounds exhibited significant inhibitory activity against *M. tuberculosis* H37Rv strain. Among the series, compound 4ac demonstrated the most potent activity with the lowest MIC value and highest selectivity index, indicating a favorable balance between efficacy and safety.

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