DESIGN AND SYNTHESIS OF 2-(N-SUBSTITUTED AMINO)-4,5,6,7-TETRA HYDRO BENZO[B]THIOPHENE-3-CARBOXYLIC ACID ETHYL ESTER DERIVATIVES AS NOVEL ANTI-TUBERCULAR COMPOUNDS

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Abstract:

A new series of 2-(N-substituted amino)-4,5,6,7-tetrahydro benzo[b]thiophene-3-carboxylic acid ethyl ester derivatives (a–g) has been synthesized and evaluated for their anti-tubercular activity. Furthermore, all derivatives were tested for in vitro anti-tubercular activity against Mycobacterium tuberculosis H37Rv. Isoniazid was employed as a positive control. The minimum inhibitory concentration values of the synthesized compounds vary from 3.121 µg/ml (ic) to 100 µg/ml (ia), while the MIC values of the Isoniazid is similar to ic compound, 3.121 µg/ml. Four compounds (**ic, ie, if and ig**) have demonstrated more potent activity than the others and it also been validated with molecular docking simulations and ADME profile estimations. These hit compounds with better performance in both in vitro and in silico investigations, have the potential to be further investigated for the development as anti-tuberculosis agents.

Keywords: Mycobacterium tuberculosis, Anti-tubercular activity, Benzothiophene, Molecular Docking.

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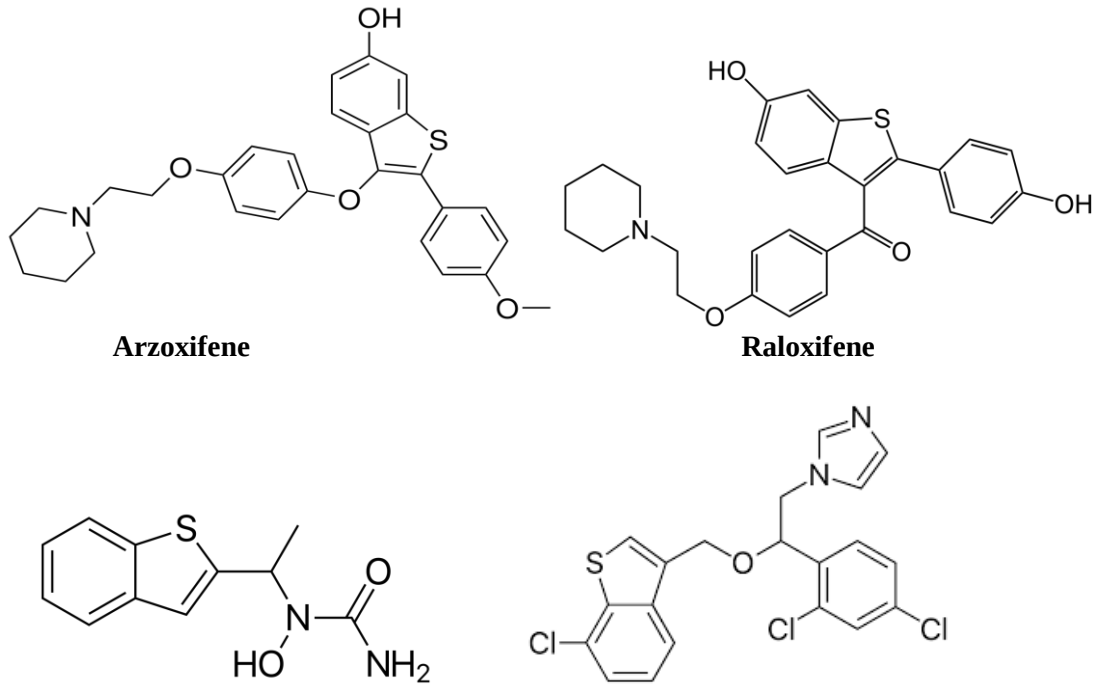
1. Introduction

Tuberculosis is a global infectious disease which pose a major challenge to the public health systems across the world leading to significant morbidity and mortality. It is caused by the bacteria Mycobacterium tuberculosis and predominantly affect the respiratory and other systems in the human body. TB still has a considerable impact on the people, communities and economies despite significant advances in the medical research. TB presents serious socioeconomic difficulties in the marginalized group of people with scarce health care facilities.

The emergence and spread of drug-resistant strains of Mycobacterium tuberculosis have led to significant hurdles in the battle against TB and such limitations with the conventional treatment approaches lead to the evolution of extensively drug-resistant TB (XDR-TB) and multidrug resistant TB (MDR-TB) which undermined the effectiveness of existing drugs and therefore new treatment strategies for the development of advanced anti-tubercular agents that targets the novel pathways critical for the survival bacteria should be explored [1].

There is an urgent need for the novel anti-tubercular drugs to provide alternative treatment options that will play a vital role in combating drug-resistant TB [2], offering hope for patients and improving overall treatment outcomes.

The central scaffold in the synthesized compounds is a sulphur containing heterocyclic compound called Benzothiophene, which is a versatile structure for drug discovery and reported to have various activities like anti-microbial [4,5], anti-tubercular [6,7], anti-diabetic [8], anti-cancer [9,10], anti-inflammatory [11], anti-convulsant [12]. Similarly, some drug molecules have this scaffold in their basic structure like Arzoxifene, Raloxifene, Zileuton and Sertaconazole.



Arzoxifene **Raloxifene**

Zileuton **Sertaconazole**

Figure 1: Drugs with the Benzothiophene scaffold in their molecular structures

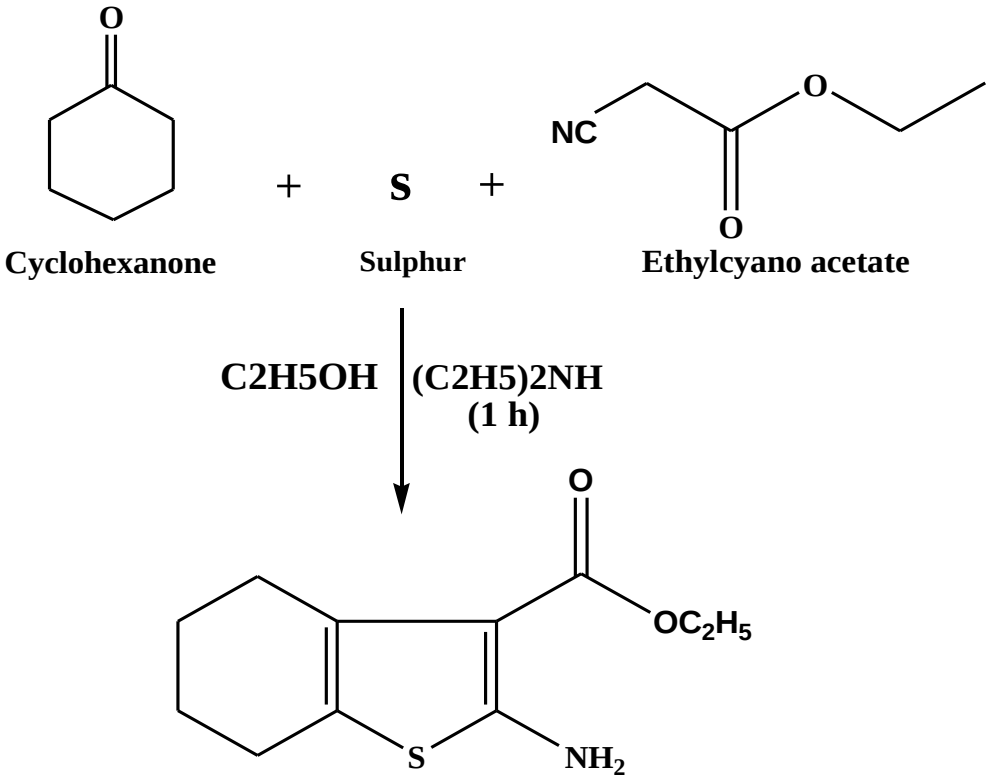
Experimental

Chemistry

1. Synthesis of 2-amino-3-carbethoxy-4,5,6,7-tetrahydro-benzo[b]thiophene

Cyclohexanone (0.04 mol, 3.75 g), ethylcyanoacetate (0.04 mol, 4.25 ml) and sulphur (0.04 mol, 1.28 g) in ethanol (40 ml) were taken in a round bottomed flask and warmed with stirring meanwhile diethylamine (0.0386 mol, 4 ml) was added drop by drop (maintaining the reaction at a temperature 40 ^{0}C) until sulphur dissolves into solution completely. After completion of the reaction, the solid gets separated out. The resultant precipitated solid was filtered, dried and recrystallized from chloroform.

Step 1:



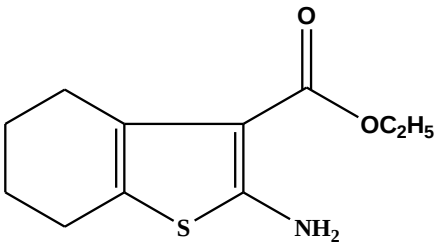
2-amino-3-carbethoxy-4,5,6,7-tetrahydro-benzo[*b*]thiophene (i)

Figure 2. Synthetic scheme of 2-amino-3-carbethoxy-4,5,6,7-tetrahydro-benzo[b]thiophene

Synthesis of 2-benzoylamino-4,5,6,7-tetrahydro-benzo[*b*]thiophene-3-carboxylic acid ethyl ester

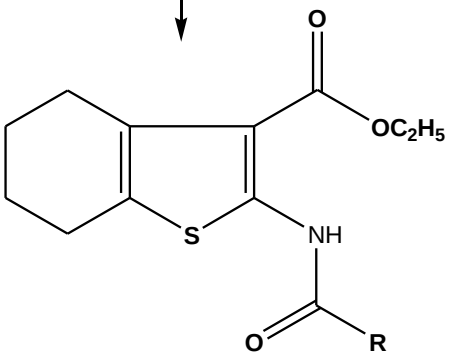
2-Amino-3-carbethoxy-4,5,6,7-tetrahydro-benzo[*b*]thiophene **(i)** (0.004 mol, 1 g) and dry pyridine (10 ml) were taken in a 100 ml two necked round bottomed flask and kept for stirring on a magnetic stirrer at 0 ^{0}C. Benzoyl chloride (0.0026 mol, 0.306 ml) and derivatives of benzoyl chloride (nitro, amino, chloro, bromo, iodo and fluro) was added drop by drop for about 30-40 minutes .The progress of the reaction was checked by using TLC. After completion of the reaction, the reaction mixture was poured into a beaker containing crushed ice. The precipitated solid was filtered, dried and then recrystallized using ethanol.

Step-2:



2-amino-3-carbethoxy-4,5,6,7-tetrahydro-benzo[*b*]thiophene (i)

Pyridine | Acid chloride
0 ^{0}C



2-(N-substituted amino)-4,5,6,7-tetrahydro benzo[*b*]thiophene-3-carboxylic acid ethyl ester derivatives (ia-ig)

R= a: Phenyl (-C_6H_5); b: 4-Nitro phenyl (-$C_6H_5NO_2$);

c: 4-Amino phenyl (-$C_6H_5NH_2$); d: 4-Chloro phenyl (-$C_6H_5Cl_2$)

e: 4-Bromo phenyl (-C_6H_5Br); f- 4-Iodo phenyl (-C_6H_5I)

g: 4-Fluro phenyl (-C_6H_5F)

Figure 3. Synthetic scheme of 2-benzoylamino-4,5,6,7-tetrahydro-benzo[b]thiophene-3-carboxylic acid ethyl ester

Biological Evaluation

In-vitro anti-tubercular activity:

Microplate Alamar Blue assay:

The anti-mycobacterial activity of the compounds was determined against M. tuberculosis using micro plate Alamar Blue assay (MABA). This method involves the preparation of 100 µl of Middlebrook 7H9 broth was added into required number of wells of the 96-microtiter plate. 100 µl of the stock concentration (test compound ia-ig) is added to the first well, mixed thoroughly to carry out serial dilution to get series of concentration (200, 100, 50, 25, 12.5, 6.25, 3.125, …….0.39µg in the respective well) and then 100 µl of test culture (M. tuberculosis H37Rv grown in Middlebrook 7H9 broth) containing 0.5 × 10 6 cells /ml equivalent to 0.5 McFarland standards is added to wells containing broth and a specific concentration of test compound. Maintenance of another well that contains test culture and standard antitubercular drug for reference. Another well was maintained that contains test culture and standard antitubercular drug for reference. Positive control well (medium + test organism) and negative control well (sterile medium) was maintained and then additional well can be maintained by including alamar blue solution and test compound to check the

interaction between them. Microtitre plates were covered and sealed with parafilm and incubated at 37 ◦C for 2-3 weeks. Then, 40 µl Alamar Blue solution was added to the plate and incubated for 24 h. A blue colour in the well was interpreted as no bacterial growth and pink colour was scored as growth. The MIC was defined as the lowest drug concentration, which prevented colour change from blue to pink [13].

Table 1- Antitubercular activity of of 2-(N-substituted amino)-4,5,6,7-tetrahydro benzo[b]thiophene-3-carboxylic acid ethyl ester compounds (ia-ig) and standard drug

Sr.no	Synthesized Compounds	MIC (µg/ml)
1	ia	100
2	ib	50
3	ic	3.125
4	id	25
5	ie	12.5
6	if	12.5
7	ig	6.25
8	**Standard (Isoniazid)**	3.125

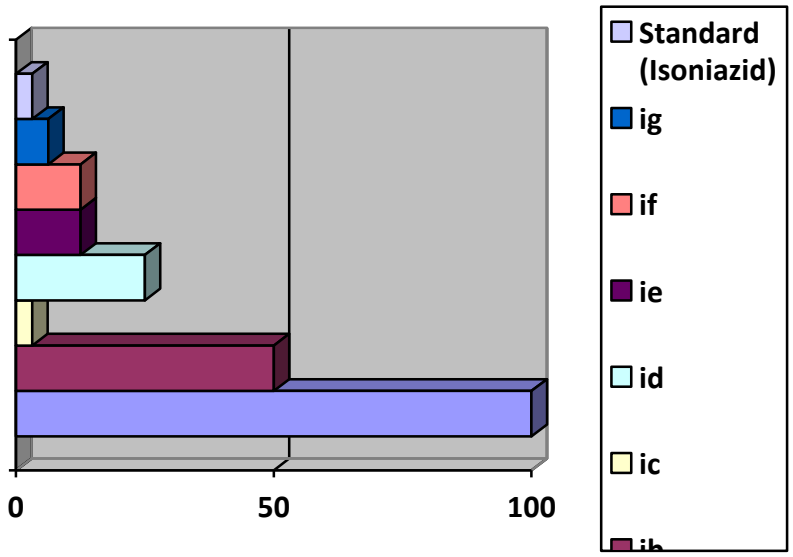


Figure 4. Plot of Minimum inhibitory concentration (MIC/µg/mL) of compounds ia-ig and isoniazid required to stop the growth of Mycobacterium tuberculosis H37Rv

Molecular Docking:

Docking simulations were performed to assess the binding strengths and molecular interactions, aiming to comprehend the structural mechanisms underlying the in vitro anti-tubercular effects of the compounds.

They were performed by using Glide module [14] integrated in the Schrodinger suite [1] to identify compounds with high binding affinity. Insilco docking simulation studies to evaluate the molecular interactions of ia-ig compounds were done with M. tuberculosis enoyl-CoA hydratase, EchA6 (PDB ID: **5DU4**) [15] and M. tuberculosis Decaprenyl-phosphoryl-ribose 2′-epimerase, DprE1 (PDB ID: **6G83**) [16] and both these chosen proteins had the co-crystallized inhibitors.

In the present docking study, we mainly focused on Mtb **EchA6 and DprE1** as the targets to investigate the molecular interactions of the compounds. These are the novel targets and plays an important role for the survival of the Mtb. Therefore, we find it

intriguing to explore and evaluate the compounds further through the present in silico studies.

Protein structures were obtained from the protein data bank (PDB) and processed to ensure an optimized structure for docking studies and it was executed with UCSF Chimera Dock Prep module and that includes the following steps: elimination of water molecules and other ligands, addition of missing atoms and residues, energy minimization done with OPLS force field and assigning charges and polar hydrogens. The 2D structure of the ligands was drawn with BIOVIA Draw2022 software and the structures were optimized with LigPrep wizard then energy minimization with done with Optimized Potentials for Liquid Simulations-2005 (OPLS-2005) force parameters. A grid box is generated with default parameters in the co-crystallized inhibitor active site. Post docking analysis and visualization of binding poses and molecular interactions were done with Maestro and Chimera X

Docking scores and molecular interaction profile of the compounds were compared with the standard drug INH used for the invitro anti-tubercular activity.

Table 2: Docking score of 2-(N-substituted amino)-4,5,6,7-tetrahydro benzo[b]thiophene-3-carboxylic acid ethyl ester compounds (ia-ig) in the binding pocket of M. tuberculosis enoyl-CoA hydratase, EchA6 and Decaprenyl-phosphoryl-ribose 2′-epimerase, DprE1 (PDB ID: 6G83).

Sr.no	Compounds	Docking Score 5DU4	Docking Score 6G83
1	ia	-6.5	-7.7
2	ib	-6.8	-7.9
3	ic	-7.9	-8.8
4	id	-7.0	-8.1
5	ie	-7.5	-8.6
6	if	-7.2	-8.2
7	ig	-7.3	-8.3
8	Isoniazid	-5.4	-4.8

Molecular interaction analysis:

Among the compounds explored through the current docking simulations, ic compound with a 4-amino benzoyl substitution had a better docking score within this series and the amino group is displaying a H-bond interaction with GLU207 active site residue in EchA6 protein where as in DprE1 protein, it had three H-bond interactions with TYR60, GLY117, LYS134 residues. The structural moieties of ic that are contributing to these interactions are -NH2, -C=O of the benzoyl at 2nd position and carboxylate on 3rd position of the tetrahydrobenzo[b]thiophene ring and the respective interactions are represented in the figure 5 and 8 respectively.

Compound ie, which had a 4-benzoyl Bromine substitution on the scaffold had a hydrogen bond interaction with the EchA6 active site residue, ASP131. The amino group on the 2nd position with a differentiating halogen (Br) substitution is involved in the H-bond interaction. In DprE1 protein, ie compound is engaged in a H-bond interaction with LYS134 through the -C=O moiety of carboxylate and it also had a pi-cation interaction with LYS418. These observations can be visually represented in figure 6 and 9 respectively.

Similar H-bond interaction with LYS134 is observed with both the ic and ie compounds in DprE1 protein and these compounds exhibited better docking score with both the proteins.

The interaction profile of compound ig with 4-benzoyl Fluro substitution involves the Hydrophobic interactions in the active site of EchA6 protein, which observed in the corresponding Figure 7.

Compound if with 4-benzoyl Iodo substitution is displaying one H-bond interaction with the LYS418 through the carboxylate -C=O group

in the DprE1 protein and it can be viewed from the figure 10.

Molecular interactions of Isoniazid indicates that it had a hydrogen bond interaction with GLN107 and GLN336 active site residues of EchA6 and DprE1 proteins respectively and these interactions as well as binding modes of isoniazid was represented in Figure 11&12.

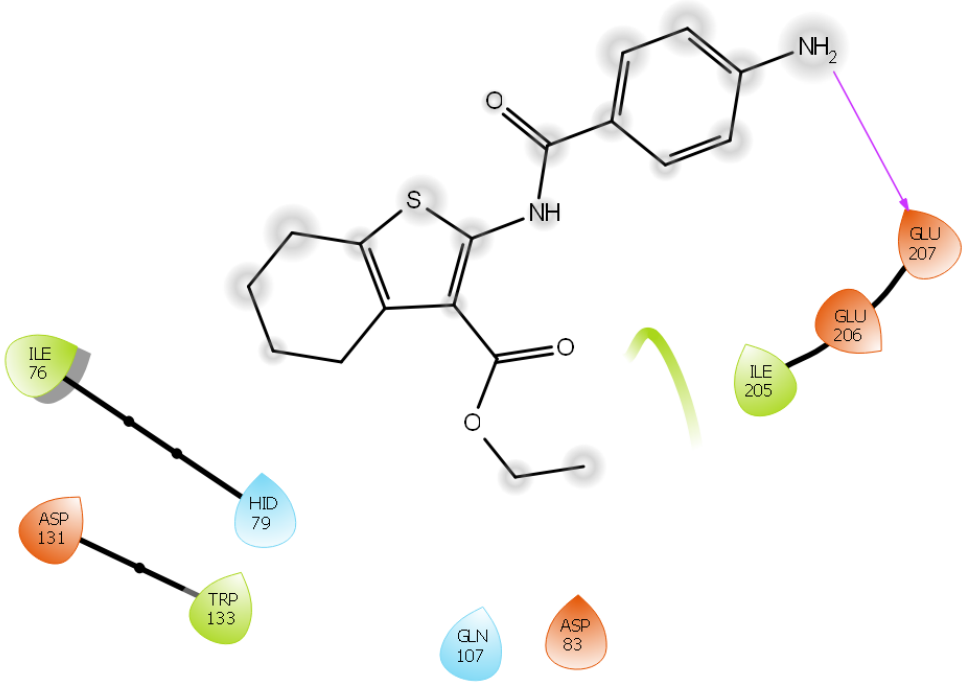


Figure 5: Compound ic molecular interactions with Mycobacterium tuberculosis enoyl-CoA hydratase, EchA6

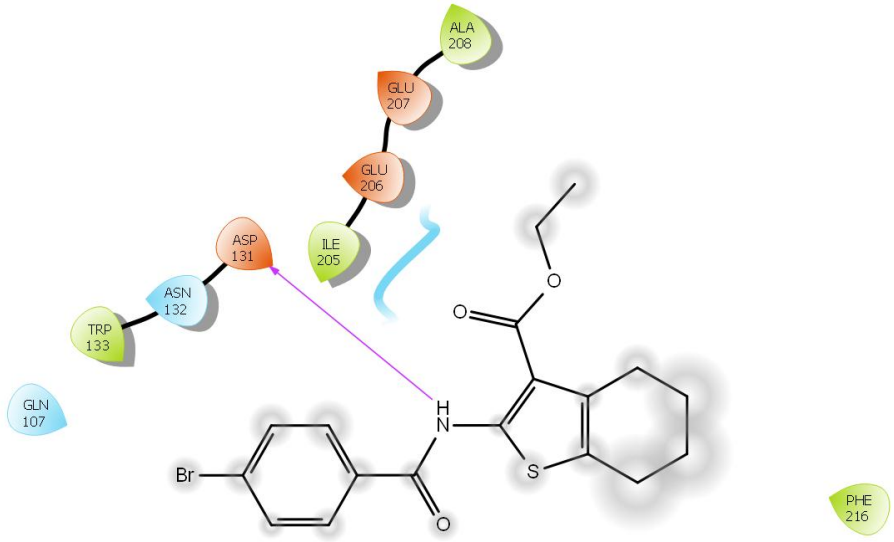


Figure 6: Compound ie molecular interactions with Mycobacterium tuberculosis enoyl-CoA hydratase, EchA6

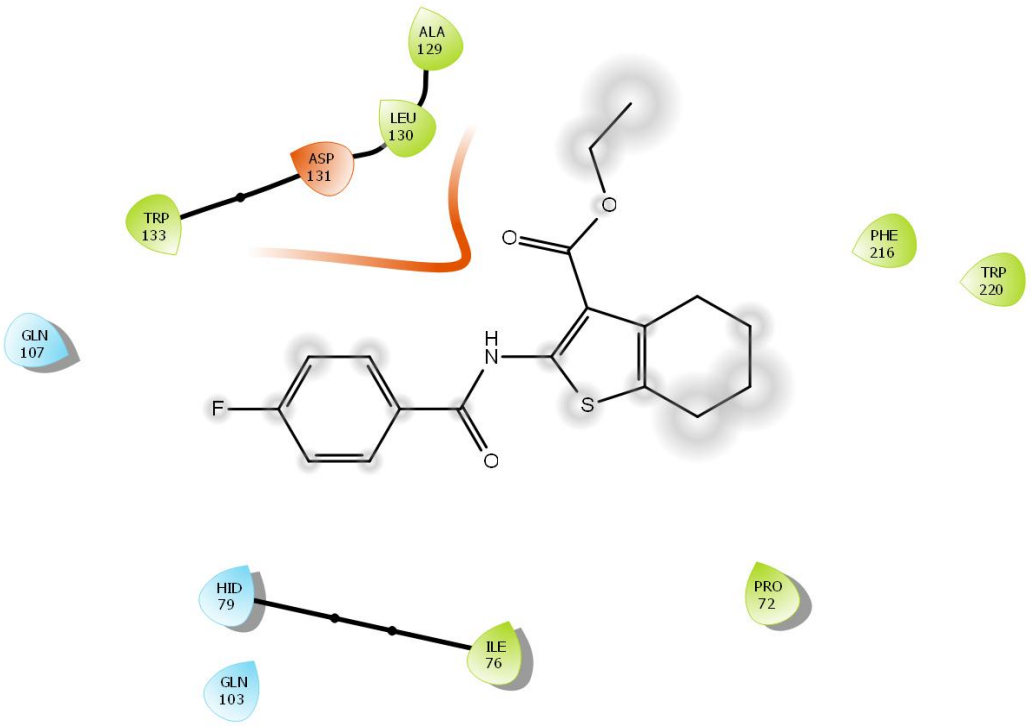


Figure 7: Compound ig molecular interactions with Mycobacterium tuberculosis enoyl-CoA hydratase, EchA6

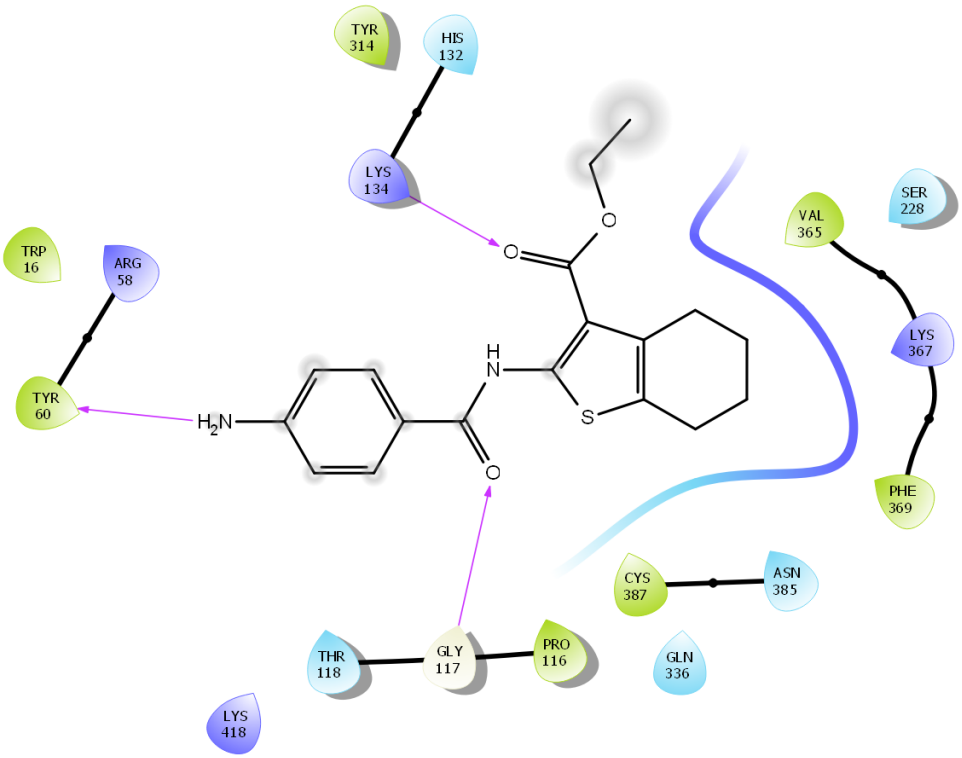


Figure 8: Compound ic molecular interactions with Mycobacterium tuberculosis Decaprenyl-phosphoryl-ribose 2′-epimerase, DprE1

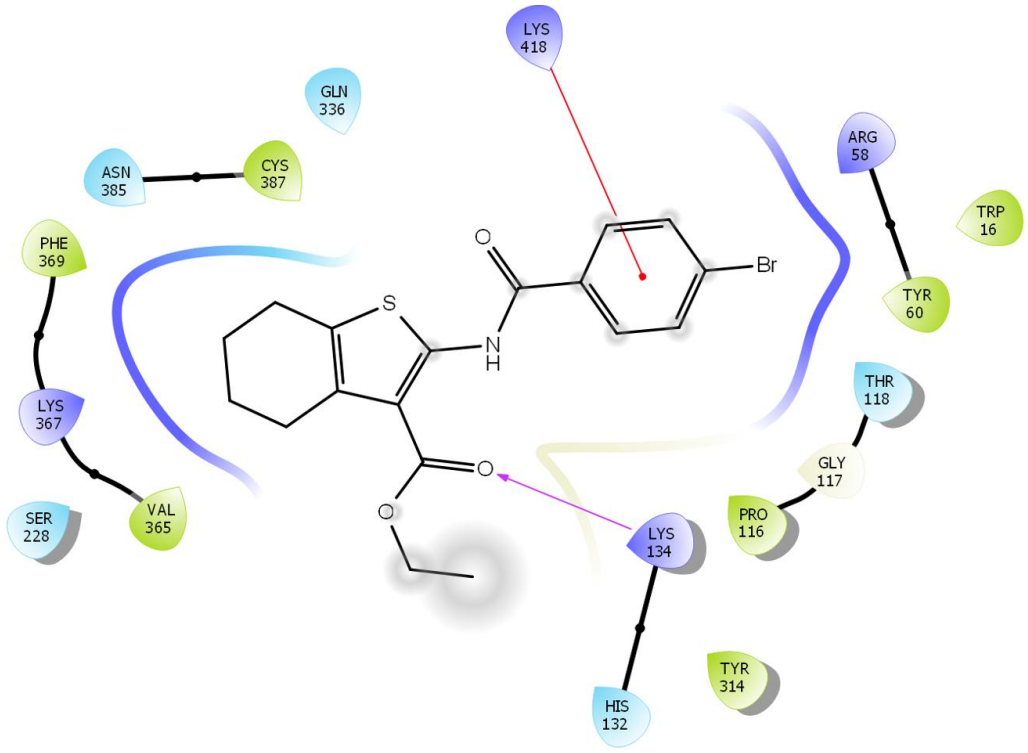


Figure 9: Compound ie molecular interactions with Mycobacterium tuberculosis Decaprenyl-phosphoryl-ribose 2′-epimerase, DprE1

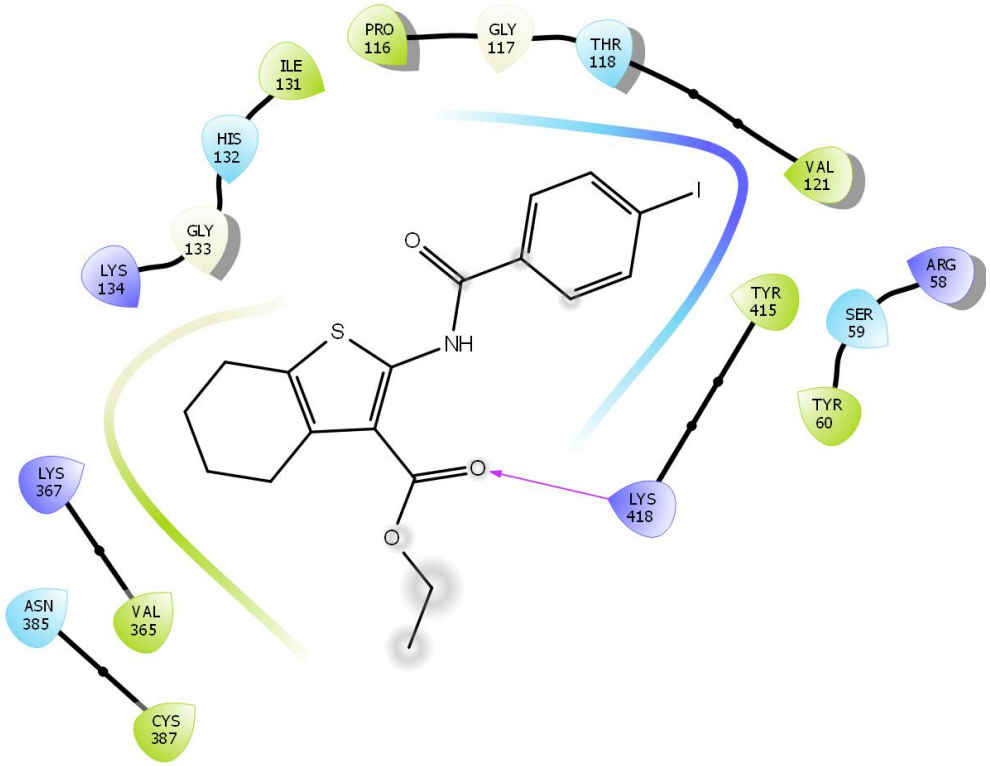


Figure 10: Compound if molecular interactions with Mycobacterium tuberculosis Decaprenyl-phosphoryl-ribose 2′-epimerase, DprE1

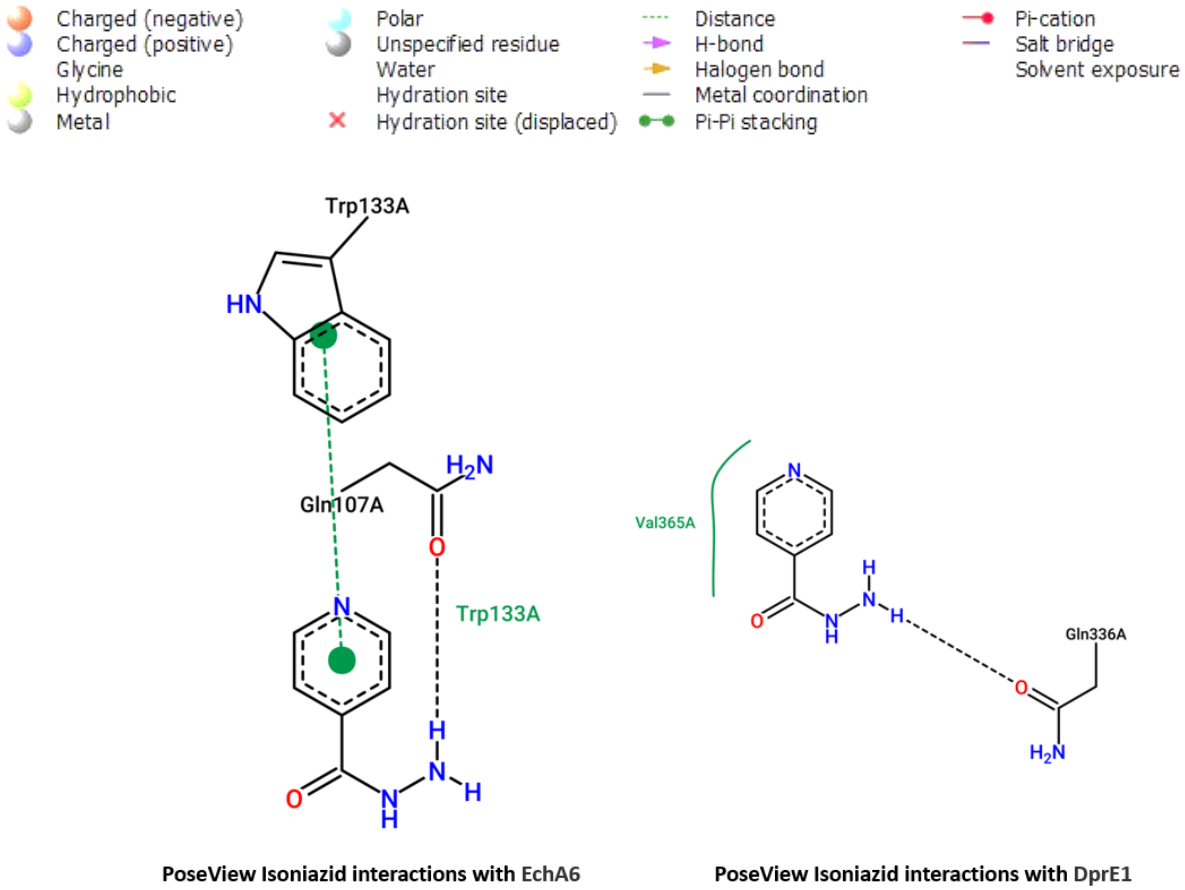


Figure 11: PoseView interactions of Isoniazid with EchA6 and DprE1 proteins

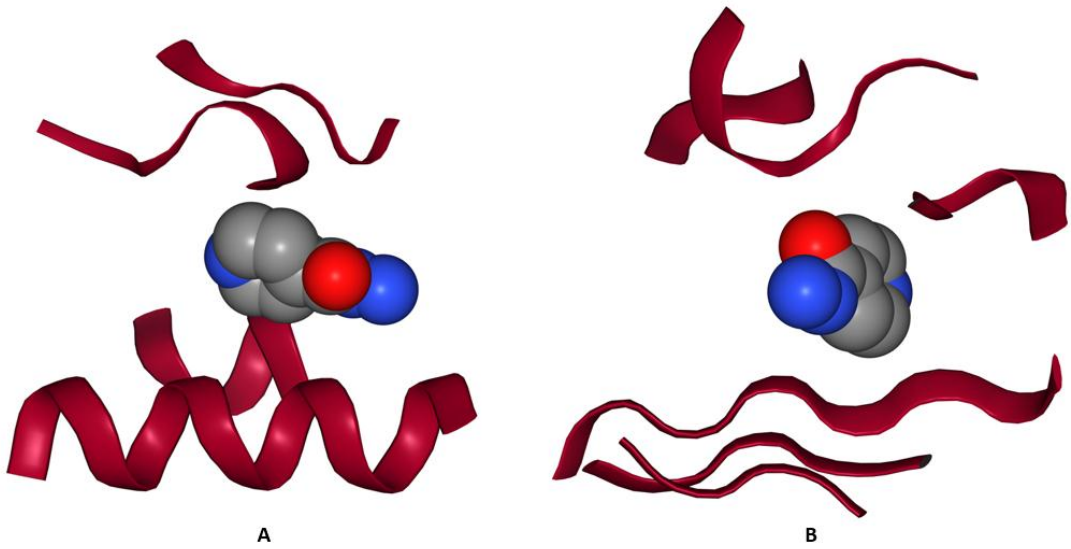


Figure 12: Binding modes of Isoniazid with A) EchA6 and B) DprE1 proteins

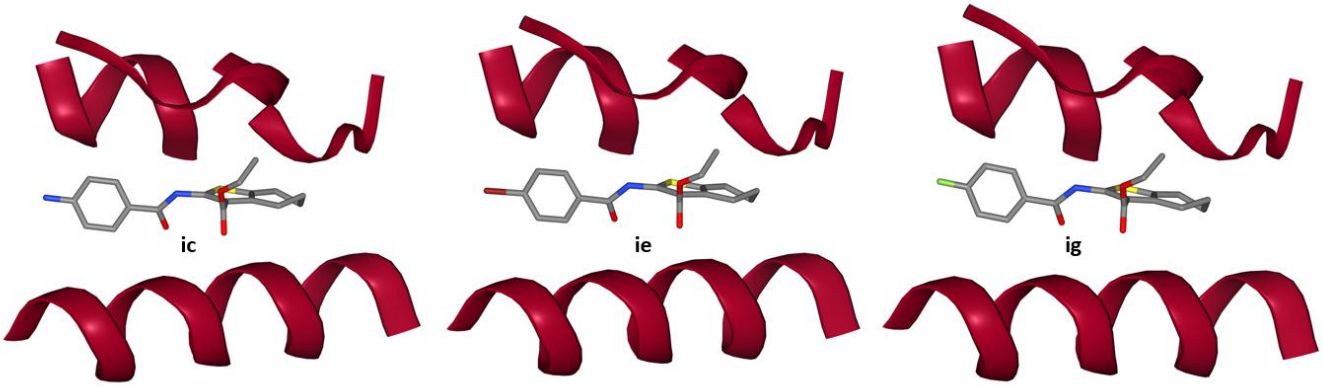


Figure 13: 3D representation of the binding orientations of ic, ie, and ig compounds in the active site

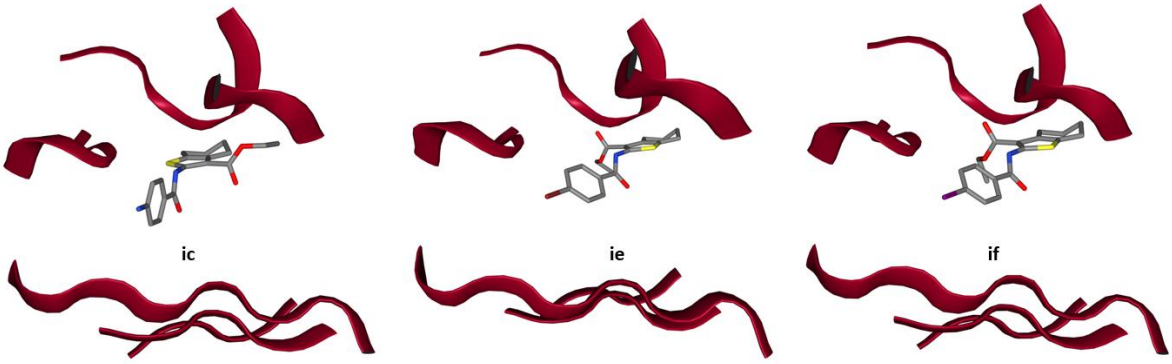


Figure 14: 3D representation of the binding orientations of ic, ie, and if compounds in the active site of M. tuberculosis Decaprenyl-phosphoryl-ribose 2′-epimerase.

In-silico ADME prediction:

ADME Properties: SWISS ADME webserver [4] (http://www.swissadme.ch/) had been used for the evaluation of physico-chemical, metabolism and drug likeliness properties of compounds which are found to be promising in the *in vitro* and *in silico* assessments.

Table 3: Physico-chemical properties and drug-likeness prediction of compounds with better invitro and insilico performance using SWISS ADME.

Parameters	ic	ie	if	ig
Molecular Weight (g/mol)	344.43	408.31	455.31	347.40
Log P o/w	3.42	4.60	4.63	4.33
No. of. H-bond Donors	2	1	1	1
No. of H-bond Acceptors	3	3	3	4
Solubility	Moderate	Poor	Poor	Poor
TPSA ($Å^2$)	109.66	83.64	83.64	83.64
GI absorption	High	High	High	High
BBB permeation	No	No	No	No
P-gp substrate	No	No	No	No
Drug likeness (Lipinski)	Yes	Yes	Yes	Yes
Bioavailability score	0.55	0.55	0.55	0.55
CYP450 isoforms inhibition	CYP1A2/2C19/ 2C9/3A4	CYP1A2/2C19/2C9/ 3A4	CYP1A2/2C19/2 C9/3A4	CYP1A2/2C19/2C9/ 3A4

In summary, the ADME predictions reveal that compound ic has moderate solubility, while compounds ie, if, and ig possess poor solubility. All of the compounds exhibit high

gastrointestinal (GI) absorption. None of the top compounds are predicted to permeate the blood-brain barrier (BBB) or act as substrates for P-glycoprotein (P-gp). Overall, the estimated ADME predictions indicate a favourable pharmacokinetic profile for all of the top compounds and structural optimization might be required to enhance the solubility.

All of the top compounds investigated in this study adhered to Lipinski's rule of five, suggesting their drug-like properties without any violations. Furthermore, the compounds were predicted to have the potential to inhibit isoforms CYP2C9, CYP2C19, CYP1A2, and CYP3A4.

Based on the docking simulations conducted M. tuberculosis enoyl-CoA hydratase and Decaprenyl-phosphoryl-ribose 2′-epimerase**,** it is evident that compounds ic, ie, if, and ig exhibited better docking score and interaction profiles compared to other compounds analysed in this in silico study. These promising compounds, which demonstrated favourable performance in both in vitro and in silico investigations, have the potential to be further investigated as potential hit compounds for the development as anti-tubercular agents.

4. Conclusion

In conclusion, we have design and synthesized a new series of 2-(N-substituted amino)-4,5,6,7-tetrahydro benzo[b]thiophene-3-carboxylic acid ethyl ester derivatives (**ia-ig**). Further, all derivatives were assessed for their *in vitro* anti-tubercular activity properties against Mycobacterium tuberculosis H37Rv. The minimum inhibitory concentration values of the synthesized compounds vary from 3.121 µg/ml (ic) to 100 µg/ml (ia). Overall, four compounds (**ic, ie, if and ig**) have demonstrated more potent activity than the others and these hit compounds with better performance in both in vitro and in silico investigations, have the potential to be further investigated for the development as anti-tuberculosis agents.

Authorship contribution statement

Declaration of competing interest

Acknowledgments

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