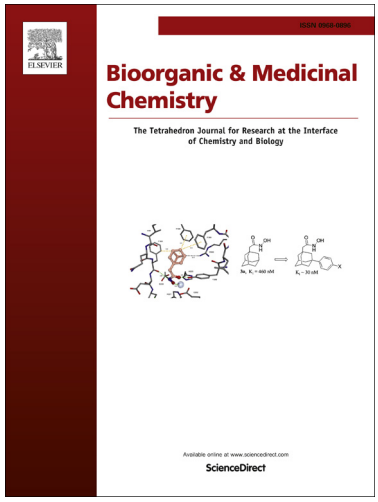

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Identification and development of 2-methylimidazo[1,2-a]pyridine-3-carboxamides as *Mycobacterium tuberculosis* Pantothenate synthetase inhibitors

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ABSTRACT

In the present study, we used crystal structure of mycobacterial pantothenate synthetase (PS) bound with 2-(2-(benzofuran-2-ylsulfonylcarbamoyl)-5-methoxy-1H-indol-1-yl) acetic acid inhibitor for virtual screening of antitubercular compound database to identify new scaffolds. One of the identified lead was modified synthetically to obtain thirty novel analogues. These synthesized compounds were evaluated for *Mycobacterium tuberculosis* (MTB) PS inhibition study, *in vitro* antimycobacterial activities and cytotoxicity against RAW 264.7 cell line. Among the compounds tested, N'-(1-naphthoyl)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (**5b**) was found to be the most active compound with IC_{50} of 1.90±0.12 µM against MTB PS, MIC of 4.53 µM against MTB with no cytotoxicity at 50 µM. The binding affinity of the most potent inhibitor **5b** was further confirmed biophysically through differential scanning fluorimetry.

Key words: Tuberculosis, Pantothenate synthetase, Cytotoxicity, 2-Methylimidazo[1,2-a]pyridine-3-carbohydrazide

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1. Introduction

The current treatment for tuberculosis (TB) consists of a multidrug regimen with rifampin, isoniazid, pyrazinamide, and ethambutol and several major problems are associated with these drugs. First, the duration and complexity of treatment itself result in non-compliance leading to suboptimal response, and emergence of resistance[1]. Secondly, the adverse events in response to anti-TB drugs also contribute to non-adherence to the regimen [2]. Third, increasing incidence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB also seem to be a serious concern[3] and fourth, co-infection of TB and HIV is a problem by itself. Combined treatment of TB and HIV involves high pill count associated with adherence problems, overlapping toxicity profiles, and drug-drug interactions[4]. Lastly, prophylactic therapy of latent TB with isoniazid also revealed problems of non-adherence[5]. Thus there is an urgent need to improve treatment by either enhancing the application of existing agents or introducing new drugs. Pantothenate synthetase (PS; EC 6.3.2.1) enzyme catalyzes the last step of pantothenate biosynthesis, an ATP-dependent condensation of D-pantoate, and ß-alanine to form pantothenate[6]. Pantothenate a major precursor of coenzyme A and acyl carrier protein, is essential for many intracellular processes including fatty acid metabolism, cell signalling, and synthesis of polyketides and non-ribosomal peptides[7,8]. There are reports that pantothenate biosynthetic pathway could be a potential drug target for persistent and virulent MTB[9]. In this work, we discovered novel 2-methylimidazo[1,2-a]pyridine-3-carboxylic acid derivatives as a novel scaffold as MTB PS inhibitors.

2. Results and discussion

2.1. Designing of the molecules

In the present study, crystal structure of the mycobacterial pantothenate synthetase (PS) in complex with 2-(2-(benzofuran-2-ylsulfonylcarbamoyl)-5-methoxy-1H-indol-1-yl)acetic acid inhibitor (PDB: 3IVX) having resolution of 1.73A° was used as a framework

for virtual screening of known antitubercular compound database to identify lead compounds against this enzyme. Based on the hit identified, we undertook synthesis of analogues and evaluated for its biological activity. Analysis of the crystal structure 3IVX revealed two key hydrogen-bonding interactions by the sulfone oxygen atom with the backbone amide group of Met40 and the side-chain nitrogen atom of His47. We also found other hydrogen bonding interactions with Val187, Ser196, Ser197 and His44. The protein crystal structure showed two hydrophobic pockets- one consisted of Met40, Leu50, Phe157, Thr39 and Pro38 whereas the other consisted of Lys160, His44 and Gly46. The reference ligand, 2-(2-(benzofuran-2-ylsulfonylcarbamoyl)-5-methoxy-1H-indol-1-yl) acetic acid was re-docked with the active site grid of the PS protein to validate the active site cavity. The ligand exhibited Glide score of -8.83 kcal/mol and was found in the vicinity of amino acids of HIE47, Met40, Val143, Val139, Pro38, Val142, Leu146, Asp161, Tyr82, Lys160, Ser196, Ser197, HIE44, Thr186, Val187, Leu50, Ala49, Gly46, and Gly158 residues. Re-docking results showed that the compound exhibited similar interactions as that of the original crystal structure with a RMSD of 1.18A$^{\circ}$. High-throughput virtual screening of known antitubercular compounds (100 compounds of TAACF and GSK with MIC of less than 6.25 µg/mL)[10–13] was performed using Glide XP (extra precision) docking and we identified 2,6-dimethyl-N-(thiophen-2-ylmethyl)-2H,3H- imidazo[1,2-a]pyridine-3-carboxamide **(Lead 1, GSK358607A**) and 6-acetyl-2-(thiophene-2-carboxamido)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxamide **(Lead 2, SID 92097880**) (**Fig 1**) as potential inhibitors in the PS site interacting with important amino acid residues like HIE44, Asp160, Ser196, Ser197 and HIE47 with a docking score of -6.98 and -7.89 respectively. Lead 1 was reported to have good MTB MIC of 0.19 µM; and we decided to take this lead as a starting point to develop various analogues to derive structure activity relationship. Docking of the Lead 1 (2,6-dimethyl-N-(thiophen-2-ylmethyl)-2H,3H-imidazo[1,2-a]pyridine-3-carboxamide) within the active site of the PS

protein is illustrated in **Fig 2**, where the following interactions were observed at the binding site. One hydrogen bonding interaction was observed between the oxygen atom of caboxamide and the NH group of the HIE44 amino acid side chain. Apart from hydrogen bonding; HIE44 was also involved in π-π stacking interaction with the thiophene ring. The N-(thiophen-2-ylmethyl) was found in the proximity of Met195, Gly46, Thr186, Leu50, Val187, and Lys106 where it showed hydrophobic interactions with Leu50 and Val187. The nitrogen atom of amide group was also found to interact with the Asp161. The bicyclic imidazo[1,2-a]pyridine ring was placed in the proximity of Gly41, Tyr82, Met40, Thr39, Pro38, Phe157 and Gly158 where it showed hydrophobic interaction with Tyr82, Met40 and Pro38. Further the compound was stabilized by the π-π stacking interaction with one of the important amino acid residues HIE47 of the active site.

2.2. Chemistry

The target molecules were synthesised by following a three step synthetic protocol (Scheme), wherein the first step of the reaction was of 2-aminopyridine (**1**) with 2-chloroethylacetoacetate in ethanol under reflux conditions to yield the bicyclic compound – "ethyl 2-methylimidazo[1,2-a]pyridine-3-carboxylate" (**2**) (**Fig 3**) in good yield. In the next step, two types of reactions were carried out on ester group, one was the conversion of ester group into carboxylic acid (**4**) using LiOH in Ethanol/Water (1:1), and the other was the direct conversion of ester into acid hydrazide (**3**) using 35% aqueous solution of hydrazine hydrate in ethanol under reflux conditions. Further the 2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (**3)** on reaction with various substituted aromatic/aliphatic carboxylic acids in presence of coupling agents EDCI, and HOBt produced the double amides (**5a-j**). Reaction of compound **3** with various substituted aldehydes in ethanol reflux conditions produced the acid hydrazones (**6a-j**) in excellent yields. This reaction was faster in presence of catalytic amount of con. H_2SO_4, as it formed the final molecules **6b, 6f,** and **6h** in less than 10 minutes

and for others the reaction time ranged from 30 to 60 minutes. During the reaction we observed the formation of desired product as a solid then reaction mixture was filtered directly and washed with distilled water, cold ethanol and hexane to obtain pure products without further purification steps. In the case of simple amides, 2-methylimidazo[1,2-a]pyridine-3-carboxylic acid (**4**) was treated with substituted aromatic/aliphatic primary amines in presence of peptide coupling agent EDCI to produce final compounds (**7a-j**). The purity of the synthesized compounds was checked by HPLC and elemental analyses and the structures were identified by spectral data. In the nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR), the signals of the respective protons of the prepared derivatives were verified on the basis of their chemical shifts, multiplicities, and coupling constants. The elemental analysis results were within ±0.4% of the theoretical values.

2.3. Pantothenate synthetase enzyme inhibition studies

Synthesized compounds were assayed for MTB PS inhibition study that coupled the AMP produced in the condensation of β-alanine and pantoate with the oxidation of NADH to NAD+ through myokinase, pyruvate kinase and lactate dehydrogenase. The decrease in NADH can be monitored spectrophotometrically at 340 nm. In the initial screening at 25µM, twenty seven compounds showed more than 50% inhibition against MTB PS and were further studied for IC_{50} measurements. Compounds showed IC_{50} in the range of 1.90±0.12 µM to 9.20±0.96 µM. Compound N'-(1-naphthoyl)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (**5b**) emerged as the most active compound with an IC_{50} of 1.90±0.12 µM.

Further to support the activity we performed docking for these compounds. Ccompound **5b** showed highest docking score of -8.60 kcal/mol which correlates well with its potency in the enzyme assay. Closer analysis of this compound in the protein active site revealed showed three hydrogen bonding interactions with the important amino acid residues such as HIE44, Ser196, Ser197 and Asp161. In addition to hydrogen bonding interactions,

the compound was further stabilized by π-π stacking interaction with Hie44. The carbonyl oxygen of imidazole ring interacted with the side chain of HIE44. His44 showed a π-π stacking interaction with the imidazole ring, as well. The nitrogen atom of the amide group of the imidazole ring interacted with Asp161 and the carbonyl oxygen on the naphthalene moiety interacted with Ser196. Apart from hydrogen bonding interaction, the compound also showed hydrophobic interaction with Met40, Thr82, Met195, Pro38, Val139, Leu50 and Tyr82 and the napthyl ring was stabilized by the positively charged amino acid residues Arg198 and Arg278 (**Fig 4**). Compound **5b** displayed similar orientations as that of reference compound. While the compound **5e (**2-methyl-N'-(3-nitrobenzoyl)imidazo[1,2-a]pyridine-3-carbohydrazide) showed less activity IC_{50}=9.20 µM as compared to compound **5b** we found that the 3-nitrobenzohydrazide group was slightly away from the active site (**Fig 1, supplementary information**) thus it was not fully placed in the hydrophobic cavity and also the orientation of the molecule was different. This difference in the activity profile was also supported by docking score (-7.73 kcal/mol) which was slightly lower than the compound **5b** (-8.60 kcal/mol).

Similarly compounds **6a-j**, with substitutions at position 4 of the phenyl ring like electron withdrawing groups such as fluoro, bromo, trifluoromethyl and substitution with electron donating groups like hydroxyphenyl, methoxyphenyl, benzyloxyphenyl and trimethoxyphenyl moieties, showed IC_{50} range from 3.77 to 8.18 µM. The most active compound **6c** showed good docking score of -8.10 kcal/mol suggesting that this compound had been well-docked in the active site and showed good PS IC_{50} of 3.77 µM. It has showed three hydrogen bondings with Gln72, Gln164 and HIE47. The presence of fluoro group on the phenyl ring increased its hydrophobicity towards the protein and it was buried in the hydrophobic cavity surrounded by Val143, Pro38, Phe67, Leu146, val142, and Val139 (**Fig 2, supplementary information**). As the compound **6e** was inactive with IC_{50} of >25 µM,

also showed a low docking score of -4.90 and was found to make only one hydrogen bonding interaction with the protein (**Fig 3, supplementary information**). As shown in the figure, 4-nitrophenyl of compound **6e** was not fully occupied in the active site of the protein and apart from that nitro group of the molecule decreased the hydrophobicity and thus the activity was less.

Among the compounds **7a-j**, compound **7d** showed *in vitro* inhibition of PS enzyme and also showed a docking score of -6.23. The compound was found to fit very well in the active site pocket and was surrounded by many hydrophobic amino acid residues such as Met40, Tyr82, Val139, Val143, Pro38, Met195 and Phe197 (**Fig 4, supplementary information**). While the compound **7e** and **7j** substituted with 2-pyridyl and 4-ethoxyphenyl respectively were found to be inactive, and was to found to correlate well with their low docking scores of -5.48 and -3.88 respectively. Docking analysis of these compounds in the active site revealed that compound **7j** showed one hydrogen bonding interaction with the protein and also was not occupying the hydrophobic cavity of the protein. Even though 4-ethoxyphenyl belonged to hydrophobic class it was not involved in hydrophobic interaction which could be due to the bulkiness of the molecule which oriented the molecules away from the active site (**Fig 5, supplementary information**). In case of compound **7e**, 2-pyridyl group showed least docking score because of no hydrogen bonding interaction and showed only π-π stacking interaction (**Fig 6, supplementary information**). Analysis of docking with this series of compounds suggested that, interactions with side chains of residues Gln72, Ser196, Ser197, Asp161, HIE44 and HIE 47 could contribute to the binding strength. All the compounds substituted with hydrophobic groups showed bioactivity against the panC enzyme and the compounds having nitro group substitution had shown lesser activity as compared to hydrophobic substitution. This could be attributed to the fact that the protein has two

hydrophobic pockets as described earlier and hence suggest that hydrophobicity of a ligand could be playing a major role in retaining the activity of compounds.

2.4. In vitro MTB screening

All the synthesized compounds were also screened for their *in vitro* anti-tubercular activity against *Mycobacterium tuberculosis* H37Rv (ATCC27294) using an agar dilution method with drug concentrations from 50 µg/mL to 0.78µg/mL in duplicates. The minimum inhibitory concentration (MIC) was determined for each compound which was measured as the minimum concentration of compound required to completely inhibit the bacterial growth. Isoniazid, ethambutol, and GSK358607A were used as reference compounds for comparison. The MIC values of the synthesized compounds along with the standard drug for comparison are presented in **Table 1**. All the synthesized compounds showed activity against MTB with MIC ranging from 4.53 to 98.81 µM. Seven compounds (**5b-d, 5f, 5g, 5i** and **6d**) inhibited MTB with MIC of <20 µM. Compound **5b** was found to be the most active compound *in vitro* with MICs of 4.53 µM against log-phase culture of MTB and it was more potent than ethambutol (MIC 7.64 µM). All the synthesized compounds were less potent than standard antitubercular compounds like isoniazid and GSK lead compound. When compared to MTB PS activity; MIC results were found to be different. This might be due to MTB cell wall penetration problem with these compounds or involvement of MTB efflux pumps. With respect to structure-MTB activity relationship, the order of activity was double amides (**5a-j**) showed better activity followed by acid hydrazones (**6a-j**) and amides (**7a-j**). Among double amides, replacement of phenyl ring (**5a**) with napthyl ring (**5b**) enhanced (~10 times) the potency. Conversion of phenyl to cyclohexyl (**5c**) and furanyl ring (**5d**) yielded four times more potent compounds. Introduction of nitro, chloro, methoxy and benzyloxy groups on phenyl ring enhanced the activity, whereas 4-methyl group (**5h**) was found to be detrimental. In case of acid hydrazones, compound with 4-trifluoromethyl phenyl substituent (**6d**) showed

good potency indicated by its MIC of 9.01 μM. In the case of amides, replacement of phenyl ring (**7a**) with benzyl group (**7b**) enhanced potency up to eight times, but further enlargement with phenylethyl group (**7c**) reduced the activity.

2.5. In vitro cytotoxicity studies

Some of the selected compounds were also tested for *in vitro* cytotoxicity against RAW 264.7cells at 50 μM concentration using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Percentage inhibition of cells is reported in **Table 1**. The most promising anti-TB compound **5b** showed only 28.42% cytotoxicity at 50μM.

2.6. DSF studies

Furthermore the binding affinity of the most potent analogue was evaluated by measuring the thermal stability of the protein-ligand complex using the biophysical differential scanning fluorimetry. Differential scanning flourimetry (DSF) measure the thermal stability of a target protein and a subsequent increase in protein melting temperature indicate binding of a ligand to the protein. Protein complexes with ligand were heated from 25 to 95 ℃ in steps of 0.1 ℃ in the presence of a dye called sypro orange. The flourescence increased when the protein interacted with hydrophobic residues. Positive shift of T_m corresponding to native protein indicated that stability was increased due to inhibitor binding. The curves obtained in this are depicted in **Fig 5**. The protein MTB PS showed a melting temperature of 49.20 ℃, whereas with compound **5b** the corresponding T_m was found to be 51 ℃. The difference in the T_m indicated the stability of the native protein when it was bound with inhibitor.

3. Conclusion

In summary, we identified and synthesized a novel lead 2-methylimidazo[1,2-a]pyridine-3-carboxylic acid derivatives from a high-throughput virtual screening of known antitubercular compound database. Many of the compounds showed potent MTB PS

inhibition and MTB MIC. Compound **5i** showed potency, selectivity, and no cytotoxicity upto 50 μM and emerged as valid lead for further development. Further structure-activity, biophysical, pharmacokinetic and metabolism studies should prove fruitful.

4. Experimental Section

4.1. Chemistry

Reagents and solvents obtained from commercial sources were used without further purification. All the reactions were monitored by thin layer chromatography (TLC) on silica gel 40 F254 (Merck, Darmstadt, Germany) coated on aluminium plates. All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 and 100 MHz spectrometer, Bruker BioSpin Corp., Germany. Chemical shifts are reported in parts per million (ppm) using tetramethyl silane (TMS) as an internal standard. Temperatures are reported in degrees Celsius and are uncorrected. Compounds were analysed for C, H, N and analytical results obtained were within ±0.4% of the calculated values for the formula shown. Molecular weights of the synthesised compounds were checked by (Shimadzu, LCMS-2020) ESI-MS method.

4.1.1. Preparation of ethyl 2-methylimidazo[1,2-a]pyridine-3-carboxylate (2)

2-aminopyridine (4.00 g, 42.50 mmol) and 2-chloroethylacetoacetate (7.08 mL, 51.00 mmol) were taken in 1,2-Dimethoxyethane (40 mL) and heated at 90 °C for 6 h. The reaction mixture was concentrated under reduced pressure, diluted with EtOAc (80 mL), washed the organic layer with H_2O (3 × 30 mL). The separated organic layer was dried over anhy Na_2SO_4 and concentrated under vacuo to get crude compound. The crude compound was purified by column chromatography using 15% EtOAc in Hexanes as eluent to get ethyl 2-methylimidazo[1,2-a]pyridine-3-carboxylate (**2**) (5.40 g, 62%) as an Off-white solid. ESI-MS showed 205 $[M+H]^+$ and carried to next step.

4.1.2. Preparation of 2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (3)

To the stirred solution of ethyl 2-methylimidazo[1,2-a]pyridine-3-carboxylate (**2)** (2.70 g) in Ethanol (30 mL) was added 35% aqueous solution of $N_2H_4.H_2O$ (25 mL) and refluxed for 3 h. The reaction mixture was concentrated to half volume and cooled on ice bath, the solids formed were filtered and dried in vacuum oven to get 2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (**3**) (2.25 g, 89%) as an Off-white solid. ESI-MS showed 191 $[M+H]^+$.

4.1.3. Preparation of 2-methylimidazo[1,2-a]pyridine-3-carboxylic acid (4)

To the stirred solution of ethyl 2-methylimidazo[1,2-a]pyridine-3-carboxylate (**2)** (2.00 g) in ethanol/Water (1:1) (30 mL) was added LiOH (4.00 g) and stirred at room temperature for 4 h. The reaction mixture was concentrated to half volume, and added 6N HCl at 0 °C till the reaction mixture turned to $_P$H ~ 6, the solids formed were filtered and dried in vacuum oven to get 2-methylimidazo[1,2-a]pyridine-3-carboxylic acid (**4**) (1.53 g, 88%) as an Off-white solid. ESI-MS showed 177 $[M+H]^+$.

4.1.4. General procedure for the synthesis of final molecules (5a-j)

To the stirred solution of carboxylic acid (1.0 equiv), EDCI (1.2 equiv), HOBt (1.2 equiv) and Et_3N (2.5 equiv) in Dichloromethane at 0 °C, was added compound **3** (1.05 equiv) and allowed stir at rt for 3 h. The reaction mixture was diluted with CH_2Cl_2 and washed with H_2O and the separated organic layer was concentrated under reduced pressure, purified by column chromatography using EtOAc/Hexanes as eluent.

4.1.5. N'-Benzoyl-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (5a): To the stirred solution of Benzoic acid (0.4 g, 3.27 mmol), in CH_2Cl_2 at 0 °C was added EDCI (0.76 g, 3.92 mmol), HOBt (0.53 g, 3.92 mmol), and Et_3N (1.02 mL, 7.19 mmol) stirred for few minutes then was added 2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (0.69 g, 3.60 mmol), and allowed stir at rt for 3 h, The reaction mixture was diluted with CH_2Cl_2 and washed with H_2O and the separated organic layer was concentrated under reduced pressure, purified by column chromatography using 25% EtOAc/Hexanes as eluent. MS(ESI) *m/z* 295 $[M+H]^+$. ^{1}H NMR

(400 MHz, DMSO-d_6): δ 10.33 (s, 2H, NH), 8.34 (d, J = 8.4 Hz, 1H, Ar), 8.10 (d, J = 8.0 Hz, 2H, Ar), 7.72–7.54 (m, 5H, Ar), 7.34 (t, J = 8.0 Hz, 1H, Ar), 2.61 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.7, 170.8, 153.6, 149.1, 136.8, 132.5, 126.6, 125.6(2C), 124.5, 121.4(2C), 120.6, 118.4, 116.1, 18.0. Anal. calcd for $C_{16}H_{14}N_4O_2$: C, 65.30; H, 4.79; N, 19.04 % Found C, 65.33; H, 4.89; N, 19.11%.

4.1.6. N'-(1-Naphthoyl)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (5b):

MS(ESI) *m/z* 345 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.44 (s, 2H, NH), 8.55 (d, J = 8.8 Hz, 1H, Ar), 8.19 (d, J = 8.4 Hz, 1H, Ar), 7.90–7.72 (m, 3H, Ar), 7.63–7.54 (m, 4H, Ar), 7.36 (t, J = 8.4 Hz, 1H, Ar), 7.29 (t, J = 8.4 Hz, 1H, Ar), 2.67 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.9, 167.8, 152.6, 150.1, 136.4, 133.4, 132.4, 130.6, 129.4, 128.4, 127.2, 126.9, 126.0, 125.6, 125.1, 124.2, 120.6, 119.2, 117.9, 19.2. Anal. calcd for $C_{20}H_{16}N_4O_2$: C, 69.76; H, 4.68; N, 16.27 % Found C, 69.83; H, 4.72; N, 16.31%.

4.1.7. N'-(Cyclohexanecarbonyl)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (5c):

MS(ESI) *m/z* 301 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.51 (s, 1H, NH), 10.42 (s, 1H, NH), 8.44 (d, J = 8.4 Hz, 1H, Ar), 7.72 (d, J = 8.4 Hz, 1H, Ar), 7.27 (t, J = 8.0 Hz, 1H, Ar), 7.02 (t, J = 8.4 Hz, 1H, Ar), 2.58 (s, 3H, CH_3), 2.22–2.19 (m, 1H, CH), 1.71–1.43 (m, 10H, $(CH_2)_5$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.1, 166.8, 151.6, 149.6, 133.4, 130.4, 127.4, 123.2, 118.8, 48.3, 27.9(2C), 26.3(2C), 26.1, 18.2. Anal. calcd for $C_{16}H_{20}N_4O_2$: C, 63.98; H, 6.71; N, 18.65 % Found C, 63.99; H, 6.73; N, 18.71%.

4.1.8. N'-(Furan-2-carbonyl)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (5d):

MS(ESI) *m/z* 285 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.53 (s, 2H, NH), 8.39 (d, J = 8.8 Hz, 1H, Ar), 8.22 (d, J = 8.8 Hz, 1H, Ar), 7.83–7.72 (m, 3H, Ar), 7.58–7.40 (m, 2H, Ar), 2.58 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.9, 169.2, 157.6, 153.1, 142.4, 139.4, 136.6, 135.2, 133.9, 128.6, 126.0, 123.3, 118.8, 19.1. Anal. calcd for $C_{14}H_{12}N_4O_3$: C, 59.15; H, 4.25; N, 19.71 % Found C, 59.23; H, 4.32; N, 19.91%.

4.1.9. 2-Methyl-N'-(3-nitrobenzoyl)imidazo[1,2-a]pyridine-3-carbohydrazide (5e):

MS(ESI) *m/z* 340 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.62 (s, 2H, NH), 8.91 (s, 1H, Ar), 8.32 (d, *J* = 8.4 Hz, 1H, Ar), 8.10–7.90 (m, 2H, Ar), 7.81 (t, *J* = 8.4 Hz, 1H, Ar), 7.72–7.60 (m, 3H, Ar), 2.58 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.2, 170.4, 166.6, 154.1, 145.7, 136.4, 135.6, 134.6, 133.9, 129.4, 127.4, 125.6, 123.3, 120.6, 118.8, 19.8. Anal. calcd for $C_{16}H_{13}N_5O_4$: C, 56.64; H, 3.86; N, 20.64 % Found C, 56.73; H, 3.92; N, 20.71%.

4.1.10. N'-(3,5-Dinitrobenzoyl)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (5f):

MS(ESI) *m/z* 385 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.10 (s, 2H, NH), 9.21 (s, 2H, Ar), 8.91 (s, 1H, Ar), 8.39 (d, *J* = 8.8 Hz, 1H, Ar), 7.72–7.54 (m, 2H, Ar), 7.30 (d, *J* = 8.4 Hz, 1H, Ar), 2.55 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.6, 171.4, 165.2, 156.2, 148.3(2C), 138.3, 137.1, 132.4(2C), 128.3, 126.6, 124.2, 119.2, 117.6, 20.5. Anal. calcd for $C_{16}H_{12}N_6O_6$: C, 50.01; H, 3.15; N, 21.87 % Found C, 50.03; H, 3.22; N, 21.91%.

4.1.11. N'-(2,4-Dichlorobenzoyl)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (5g):

MS(ESI) *m/z* 363 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.57 (s, 1H, NH), 10.06 (s, 1H, NH), 8.97 (d, *J* = 6.8 Hz, 1H, Ar), 7.76 (s, 1H, Ar), 7.71–7.57 (m, 3H, Ar), 7.43 (t, *J* = 7.2 Hz, 1H, Ar), 7.07 (t, *J* = 6.8 Hz, 1H, Ar), 2.67 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.9, 160.5, 146.7, 145.5, 135.3, 133.3, 131.7, 130.7, 129.5, 127.4, 127.0, 126.9, 116.2, 114.2, 113.3, 15.6. Anal. calcd for $C_{16}H_{12}Cl_2N_4O_2$: C, 52.91; H, 3.33; N, 15.43 % Found C, 52.94; H, 3.49; N, 15.48%.

4.1.12. 2-Methyl-N'-(4-methylbenzoyl)imidazo[1,2-a]pyridine-3-carbohydrazide (5h):

MS(ESI) *m/z* 309 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.56 (s, 2H, NH), 8.53 (d, *J* = 9.2 Hz, 1H, Ar), 8.01–7.74 (m, 3H, Ar), 7.63 (d, *J* = 8.4 Hz, 2H, Ar), 7.47 (t, *J* = 8.0 Hz, 1H, Ar), 7.36 (t, *J* = 8.0 Hz, 1H, Ar), 2.60 (s, 3H, CH_3), 2.41 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.8, 169.2, 158.6, 152.1, 137.3, 136.3, 134.6(2C), 132.4, 127.6(2C), 126.2,

123.6, 120.4, 118.2, 22.5, 17.9. Anal. calcd for $C_{17}H_{16}N_4O_2$: C, 66.22; H, 5.23; N, 18.17 % Found C, 66.28; H, 5.29; N, 18.29%.

4.1.13. N'-(2-Methoxybenzoyl)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (5i): MS(ESI) *m/z* 325 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.72 (s, 2H, NH), 8.39 (d, *J* = 8.8 Hz, 1H, Ar), 7.99–7.81 (m, 3H, Ar), 7.69–7.54 (m, 3H, Ar), 7.39 (t, *J* = 8.4 Hz, 1H, Ar), 3.96 (s, 3H, OCH_3), 2.61 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.8, 167.6, 157.4, 151.9, 144.7, 139.7, 136.2, 134.2, 130.6, 127.4, 125.4, 123.5, 121.6, 119.5, 117.9, 61.2, 18.3. Anal. calcd for $C_{17}H_{16}N_4O_3$: C, 62.95; H, 4.97; N, 17.27 % Found C, 62.98; H, 5.02; N, 17.34%.

4.1.14. 2-Methyl-N'-(4-phenoxybenzoyl)imidazo[1,2-a]pyridine-3-carbohydrazide (5j): MS(ESI) *m/z* 387 $[M+H]^+$. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.71 (s, 2H, NH), 8.61 (d, *J* = 8.8 Hz, 1H, Ar), 7.92–7.81 (m, 4H, Ar), 7.69 (d, *J* = 8.0 Hz, 2H, Ar), 7.54–7.36 (m, 4H, Ar), 7.33–7.20 (m, 2H, Ar), 2.66 (s, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.4, 170.3, 168.5, 160.6, 158.1, 155.4, 144.9, 141.4, 136.6, 134.5, 133.2(2C), 132.9, 132.1(2C), 130.2(2C), 128.5, 126.4, 121.6, 119.6, 18.7. Anal. calcd for $C_{22}H_{18}N_4O_3$: C, 68.38; H, 4.70; N, 14.50 % Found C, 68.44; H, 4.79; N, 14.58%.

4.1.15. General procedure for the synthesis of final molecules (6a-j)

2-methylimidazo[1,2-a]pyridine-3-carbohydrazide **(3)** (1.0 equiv)**,** aldehyde (1.1 equiv), conc. H_2SO_4 (cat) were taken in Ethanol and refluxed for 3 min to 1 h. The formed solids were filtered, dried and triturated with CH_2Cl_2/Hexanes to get pure products.

4.1.16. N'-Benzylidene-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (6a): 2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (0.4 g, 2.10 mmol), Benzaldehyde (0.23 mL, 2.31 mmol), conc. H_2SO_4 (3 drops) were taken in Ethanol (7 mL) and refluxed for 30 min. The solids in the reaction mixture were filtered, washed with Water, cold Ethanol, Hexanes and dried in vacuum oven to get (0.49 g, 84%) title compound MS(ESI) *m/z* 279 $[M+H]^+$. ^{1}H

NMR (400 MHz, DMSO-d_6): δ 12.33 (s, 1H, NH), 8.91 (s, 1H, Ar), 8.21 (d, *J* = 8.4 Hz, 1H, Ar), 7.99 (d, *J* = 8.4 Hz, 2H, Ar), 7.63–7.44 (m, 5H, Ar), 7.27 (t, *J* = 8.0 Hz, 1H, Ar), 2.63 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.6, 158.8, 154.2, 144.9, 134.8, 132.9, 129.6(2C), 127.6, 124.3(2C), 123.2, 119.4, 117.8, 116.1, 17.1. Anal. calcd for $C_{16}H_{14}N_4O$: C, 69.05; H, 5.07; N, 20.13 % Found C, 69.13; H, 5.12; N, 20.19%.

4.1.17. N'-(4-Bromobenzylidene)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (6b): MS(ESI) *m/z* 357 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.06 (s, 1H, NH), 9.03 (s, 1H, Ar), 8.31 (d, *J* = 9.2 Hz, 1H, Ar), 8.03–7.72 (m, 3H, Ar), 7.63 (d, *J* = 8.4 Hz, 2H, Ar), 7.47 (t, *J* = 8.0 Hz, 1H, Ar), 7.29 (t, *J* = 8.4 Hz, 1H, Ar), 2.56 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.5, 162.8, 152.4, 146.2, 136.4, 135.8, 133.2(2C), 128.4(2C), 126.9, 125.2, 124.4, 120.6, 118.2, 19.2. Anal. calcd for $C_{16}H_{13}BrN_4O$: C, 53.80; H, 3.67; N, 15.68 % Found C, 53.93; H, 3.72; N, 15.79%.

4.1.18. N'-(4-Fluorobenzylidene)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (6c): MS(ESI) *m/z* 297 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.12 (s, 1H, NH), 8.78 (s, 1H, Ar), 8.26 (d, *J* = 8.8 Hz, 1H, Ar), 7.92 (d, *J* = 8.4 Hz, 2H, Ar), 7.81–7.72 (m, 2H, Ar), 7.54 (d, *J* = 8.8 Hz, 2H, Ar), 7.39 (t, *J* = 8.0 Hz, 1H, Ar), 2.62 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.2, 163.5, 156.3, 150.3, 144.7, 138.3, 136.3, 135.3(2C), 133.5, 129.7(2C), 125.4, 123.3, 119.6, 18.8. Anal. calcd for $C_{16}H_{13}FN_4O$: C, 64.86; H, 4.42; N, 18.91 % Found C, 64.93; H, 4.52; N, 18.99%.

4.1.19. 2-Methyl-N'-(4-(trifluoromethyl)benzylidene)imidazo[1,2-a]pyridine-3-carbohydrazide (6d):

MS(ESI) *m/z* 347 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.91 (s, 1H, NH), 8.68 (s, 1H, Ar), 8.34 (d, *J* = 8.4 Hz, 1H, Ar), 7.83 (d, *J* = 8.4 Hz, 2H, Ar), 7.64 (d, *J* = 8.8 Hz, 2H, Ar), 7.31–7.22 (m, 2H, Ar), 7.02 (t, *J* = 8.0 Hz, 1H, Ar), 2.58 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.3, 160.5, 153.9, 144.7, 138.2, 136.4, 135.6, 133.6, 132.4(2C), 130.4,

128.2(2C), 126.1, 121.6, 118.4, 17.1. Anal. calcd for $C_{17}H_{13}F_3N_4O$: C, 58.96; H, 3.78; N, 16.18 % Found C, 58.99; H, 3.82; N, 16.29%.

4.1.20. 2-Methyl-N'-(4-nitrobenzylidene)imidazo[1,2-a]pyridine-3-carbohydrazide (6e):

MS(ESI) *m/z* 324 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.82 (s, 1H, NH), 8.62 (s, 1H, Ar), 8.55 (d, *J* = 8.8 Hz, 1H, Ar), 8.23 (d, *J* = 8.8 Hz, 2H, Ar), 7.94 (d, *J* = 8.4 Hz, 2H, Ar), 7.54–7.42 (m, 2H, Ar), 7.09 (t, *J* = 8.4 Hz, 1H, Ar), 2.61 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.3, 162.3, 158.8, 148.2, 144.7, 137.6, 136.3, 134.2, 129.3(2C), 126.6, 123.5(2C), 121.5, 119.1, 16.9. Anal. calcd for $C_{16}H_{13}N_5O_3$: C, 59.44; H, 4.05; N, 21.66 % Found C, 59.49; H, 4.12; N, 21.69%.

4.1.21. N'-(4-Hydroxybenzylidene)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (6f):

MS(ESI) *m/z* 295 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.22 (s, 1H, NH), 9.61 (s, 1H, Ar), 8.71 (s, 1H, Ar), 8.58 (d, *J* = 8.4 Hz, 1H, Ar), 7.93 (d, *J* = 8.8 Hz, 2H, Ar), 7.72–7.54 (m, 2H, Ar), 7.04 (d, *J* = 8.4 Hz, 2H, Ar), 6.99 (t, *J* = 8.4 Hz, 1H, Ar), 2.56 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.5, 166.8, 156.3, 144.9, 142.9, 136.4, 133.2, 130.9, 124.2(2C), 123.9, 120.5(2C), 117.5, 116.1, 18.1. Anal. calcd for $C_{16}H_{14}N_4O_2$: C, 65.30; H, 4.79; N, 19.04 % Found C, 65.40; H, 4.82; N, 19.09%.

4.1.22. N'-(4-Methoxybenzylidene)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (6g):

MS(ESI) *m/z* 309 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.92 (s, 1H, NH), 8.49 (s, 1H, Ar), 8.39 (d, *J* = 8.4 Hz, 1H, Ar), 7.89 (d, *J* = 8.4 Hz, 2H, Ar), 7.80 (d, *J* = 7.6 Hz, 1H, Ar), 7.69–7.32 (m, 4H, Ar), 3.94 (s, 3H, OCH_3), 2.56 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 166.8, 156.3, 144.9, 142.9, 136.4, 133.2, 130.9, 124.2(2C), 123.9, 120.5(2C), 117.5, 116.1, 63.7, 18.1. Anal. calcd for $C_{17}H_{16}N_4O_2$: C, 66.22; H, 5.23; N, 18.17 % Found C, 66.26; H, 5.27; N, 18.29%.

4.1.23. N'-(4-(Benzyloxy)benzylidene)-2-methylimidazo[1,2-a]pyridine-3-carbohydrazide (6h):

MS(ESI) *m/z* 385 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.72 (s, 1H, NH), 8.61 (s, 1H, Ar), 8.52 (d, *J* = 8.8 Hz, 1H, Ar), 8.12–7.82 (m, 6H, Ar), 7.72 (d, *J* = 7.6 Hz, 2H, Ar), 7.67–7.45 (m, 3H, Ar), 7.36 (t, *J* = 8.4 Hz, 1H, Ar), 5.22 (s, 2H, CH_2), 2.62 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.4, 164.3, 154.1, 148.5, 138.9, 135.2, 134.1, 133.6, 133.0, 130.9, 130.4(2C), 128.5(2C), 127.2, 126.6, 125.1(2C), 123.3(2C), 121.5, 118.7, 16.9. Anal. calcd for $C_{23}H_{20}N_4O_2$: C, 71.86; H, 5.24; N, 14.57 % Found C, 71.96; H, 5.34; N, 14.60%.

4.1.24. 2-Methyl-N'-(3,4,5-trimethoxybenzylidene)imidazo[1,2-a]pyridine-3-carbohydrazide (6i):

MS(ESI) *m/z* 369 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.92 (s, 1H, NH), 8.47–8.35 (m, 2H, Ar), 7.62 (d, *J* = 8.4 Hz, 1H, Ar), 7.36 (s, 2H, Ar), 7.22–7.15 (m, 2H, Ar), 3.94 (s, 9H, $(OCH_3)_3$), 2.55 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.3, 166.1, 152.6, 149.2, 146.6, 144.7, 141.5, 137.4, 136.2, 134.3, 133.9, 132.8, 125.1(2C), 119.1, 63.9(2C), 63.0, 17.8. Anal. calcd for $C_{19}H_{20}N_4O_4$: C, 61.95; H, 5.47; N, 15.21 % Found C, 62.01; H, 5.53; N, 15.29%.

4.1.25. 2-Methyl-N'-(4-methylbenzylidene)imidazo[1,2-a]pyridine-3-carbohydrazide (6j):

MS(ESI) *m/z* 293 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.88 (s, 1H, NH), 8.54 (s, 1H, Ar), 8.41 (d, *J* = 8.0 Hz, 1H, Ar), 7.81 (d, *J* = 8.4 Hz, 2H, Ar), 7.74 (d, *J* = 7.2 Hz, 1H, Ar), 7.64–7.48 (m, 3H, Ar), 7.24 (t, *J* = 7.6 Hz, 1H, Ar), 2.58 (s, 3H, CH_3), 2.29 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.4, 163.8, 152.4, 142.6, 139.5, 135.2, 132.9, 129.5, 125.6, 123.7(2C), 121.5(2C), 119.5, 118.1, 22.5, 17.7. Anal. calcd for $C_{17}H_{16}N_4O$: C, 69.85; H, 5.52; N, 19.17 % Found C, 69.96; H, 5.67; N, 19.29%.

4.1.26. General procedure for the synthesis of final molecules (7a-j)

To the stirred solution of carboxylic acid (1.0 equiv), EDCI (1.2 equiv), HOBt (1.2 equiv) and Et_3N (2.5 equiv) in Dichloromethane at 0 °C, was added compound **4** (1.05 equiv) and allowed stir at room temperature for 4 h. The reaction mixture was diluted with CH_2Cl_2 and

washed with H_2O and the separated organic layer was concentrated under reduced pressure to get crude compound. The crude product was purified by column chromatography using EtOAc/Hexanes as eluent.

4.1.27. 2-Methyl-N-phenylimidazo[1,2-a]pyridine-3-carboxamide (7a): To the stirred solution of 2-methylimidazo[1,2-a]pyridine-3-carboxylic acid (0.30 g, 1.70 mmol), in CH_2Cl_2 at 0 °C was added EDCI (0.39 g, 2.04 mmol), HOBt (0.27 g, 2.04 mmol), and Et_3N (0.53 mL, 3.74 mmol) stirred for few minutes then was added Aniline (0.17 mL, 1.87 mmol), and allowed stir at rt for 4 h, The reaction mixture was diluted with CH_2Cl_2 and washed with H_2O and the separated organic layer was concentrated under reduced pressure, purified by column chromatography using 20% EtOAc/Hexanes as eluent to get (0.33 g, 78%) title compound. MS(ESI) *m/z* 252 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.91 (s, 1H, NH), 8.50 (d, *J* = 8.4 Hz, 1H, Ar), 7.91–7.72 (m, 3H, Ar), 7.53–7.26 (m, 5H, Ar), 2.67 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.6, 164.9, 156.3, 141.1, 135.1, 133.0, 132.4, 132.0, 127.4, 126.1, 125.6, 123.3, 120.8, 119.1, 18.1. Anal. calcd for $C_{15}H_{13}N_3O$: C, 71.70; H, 5.21; N, 16.72 % Found C, 71.78; H, 5.38; N, 16.79%.

4.1.28. N-Benzyl-2-methylimidazo[1,2-a]pyridine-3-carboxamide (7b):

MS(ESI) *m/z* 266 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.42 (d, *J* = 9.6 Hz, 1H), 7.56 (d, *J* = 9.6 Hz, 1H), 7.39–7.29 (m, 5H), 6.91–6.82 (m, 3H), 4.72 (d, *J* = 5.6 Hz, 2H), 2.68 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.2, 163.6, 154.7, 144.6, 136.3, 130.9(2C), 128.4(2C), 127.0, 124.4, 121.9, 121.3, 120.4, 50.4, 17.8. Anal. calcd for $C_{16}H_{15}N_3O$: C, 72.43; H, 5.70; N, 15.84 % Found C, 72.49; H, 5.68; N, 15.92%.

4.1.29. 2-Methyl-N-phenethylimidazo[1,2-a]pyridine-3-carboxamide (7c):

MS(ESI) *m/z* 280 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.03 (s, 1H, NH), 8.62 (d, *J* = 8.0 Hz, 1H, Ar), 7.69–7.48 (m, 6H, Ar), 7.38–7.27 (m, 2H, Ar), 3.44 (t, *J* = 8.4 Hz, 2H, CH_2), 2.76 (t, *J* = 8.4 Hz, 2H, CH_2), 2.56 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.2,

164.8, 156.9, 144.6, 134.6, 132.2(2C), 127.9(2C), 127.3, 125.7, 121.4, 120.5, 119.6, 44.4, 38.4, 18.9. Anal. calcd for $C_{17}H_{17}N_3O$: C, 73.10; H, 6.13; N, 15.04 % Found C, 73.19; H, 6.18; N, 15.12%.

4.1.30. N-Cyclohexyl-2-methylimidazo[1,2-a]pyridine-3-carboxamide (7d):

MS(ESI) *m/z* 258 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.93 (s, 1H, NH), 8.58 (d, *J* = 8.0 Hz, 1H, Ar), 7.89–7.74 (m, 2H, Ar), 7.12 (t, *J* = 8.0 Hz, 1H, Ar), 3.39–3.31 (m, 1H, CH), 2.58 (s, 3H, CH_3), 1.68–1.11 (m, 10H, $(CH_2)_5$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.8, 160.8, 155.3, 142.6, 133.9, 129.3, 123.3, 120.4, 49.5, 36.0(2C), 27.4(2C), 26.9, 17.1. Anal. calcd for $C_{15}H_{19}N_3O$: C, 70.01; H, 7.44; N, 16.33 % Found C, 70.11; H, 7.48; N, 16.42%.

4.1.31. 2-Methyl-N-(pyridin-2-yl)imidazo[1,2-a]pyridine-3-carboxamide (7e):

MS(ESI) *m/z* 253 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.97 (s, 1H, NH), 8.63 (d, *J* = 8.0 Hz, 1H, Ar), 8.11 (d, *J* = 8.0 Hz, 1H, Ar), 7.81–7.64 (m, 3H, Ar), 7.27–7.02 (m, 3H, Ar), 2.61 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.8, 161.1, 158.6, 144.7, 139.3, 136.7, 135.0, 129.4, 127.8, 124.3, 121.5, 120.6, 118.9, 17.7. Anal. calcd for $C_{14}H_{12}N_4O$: C, 66.65; H, 4.79; N, 22.21 % Found C, 66.68; H, 4.88; N, 22.29%.

4.1.32. N-(Furan-2-ylmethyl)-2-methylimidazo[1,2-a]pyridine-3-carboxamide (7f):

MS(ESI) *m/z* 256 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.64 (s, 1H, NH), 8.49 (d, *J* = 8.4 Hz, 1H, Ar), 7.72–7.58 (m, 3H, Ar), 7.44 (d, *J* = 8.0 Hz, 1H, Ar), 7.38–7.20 (m, 2H, Ar), 5.02 (d, *J* = 8.0 Hz, 2H, CH_2), 2.56 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.9, 161.1, 157.6, 145.4, 139.4, 132.4, 130.5, 128.4, 126.1, 120.3, 119.6, 118.8, 42.1, 18.4. Anal. calcd for $C_{14}H_{13}N_3O_2$: C, 65.87; H, 5.13; N, 16.46 % Found C, 65.93; H, 5.22; N, 16.51%.

4.1.33. N-(4-Bromophenyl)-2-methylimidazo[1,2-a]pyridine-3-carboxamide (7g):

MS(ESI) *m/z* 330 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.07 (s, 1H, NH), 8.64 (d, *J* = 8.4 Hz, 1H, Ar), 7.81 (d, *J* = 9.2 Hz, 2H, Ar), 7.69–7.54 (m, 4H, Ar), 7.18 (t, *J* = 8.0 Hz, 1H, Ar), 2.62 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.4, 158.5, 149.9, 139.6, 137.3,

133.6(2C), 132.4, 129.6, 127.6, 125.9(2C), 121.5, 119.8, 18.7. Anal. calcd for $C_{15}H_{12}BrN_3O$: C, 54.56; H, 3.66; N, 12.73 % Found C, 54.63; H, 3.72; N, 12.79%.

4.1.34. N-(4-Chlorophenyl)-2-methylimidazo[1,2-a]pyridine-3-carboxamide (7h):

MS(ESI) *m/z* 286 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.27 (s, 1H, NH), 8.67 (d, *J* = 8.4 Hz, 1H, Ar), 7.90 (d, *J* = 9.2 Hz, 2H, Ar), 7.68 (d, *J* = 9.2 Hz, 2H, Ar), 7.63–7.56 (m, 2H, Ar), 7.21 (t, *J* = 8.4 Hz, 1H, Ar), 2.64 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 163.6, 160.4, 151.2, 139.3, 138.2, 134.4(2C), 133.6, 127.4, 126.2(2C), 124.8, 121.9, 120.4, 18.9. Anal. calcd for $C_{15}H_{12}ClN_3O$: C, 63.05; H, 4.23; N, 14.71 % Found C, 63.13; H, 4.32; N, 14.78%.

4.1.35. 2-Methyl-N-(3-(trifluoromethyl)phenyl)imidazo[1,2-a]pyridine-3-carboxamide (7i):

MS(ESI) *m/z* 320 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.21 (s, 1H, NH), 8.54 (d, *J* = 8.4 Hz, 1H, Ar), 8.17 (s, 1H, Ar), 7.78–7.39 (m, 5H, Ar), 7.18 (t, *J* = 8.4 Hz, 1H, Ar), 2.66 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.9, 160.2, 156.4, 142.5, 138.4, 135.2, 133.9, 130.6, 128.3, 126.4, 125.6, 124.4, 123.9, 121.5, 120.9, 16.9. Anal. calcd for $C_{16}H_{12}F_3N_3O$: C, 60.19; H, 3.79; N, 13.16 % Found C, 60.28; H, 3.88; N, 13.29%.

4.1.36. N-(4-Ethoxyphenyl)-2-methylimidazo[1,2-a]pyridine-3-carboxamide (7j):

MS(ESI) *m/z* 296 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.47 (s, 1H, NH), 8.58 (d, *J* = 8.4 Hz, 1H, Ar), 7.72 (d, *J* = 8.8 Hz, 2H, Ar), 7.58–7.49 (m, 2H, Ar), 7.42–7.26 (m, 3H, Ar), 4.19 (q, *J* = 7.2 Hz, 2H, CH_2), 2.68 (s, 3H, CH_3), 1.38 (t, *J* = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.4, 158.5, 149.9, 139.6, 137.3, 133.6(2C), 132.4, 129.6, 127.6, 125.9(2C), 121.5, 119.8, 69.1, 18.7, 16.2. Anal. calcd for $C_{17}H_{17}N_3O_2$: C, 69.14; H, 5.80; N, 14.23 % Found C, 69.23; H, 5.92; N, 14.39%.

4.2. Biological activity

4.2.1. MTB PS screening

The MTB panC gene (Rv3602c) encoding the pantothenate synthetase was cloned and transformed into BL21 (DE3) cells and the expression of the protein was performed as reported in literature.[7] For the assay, in a 96-well plate, 60 mL of PS reagent mix containing NADH, pantoic acid, β-alanine, ATP, phosphoenol pyruvate, $MgCl_2$, myokinase, pyruvate kinase, and lactate dehydrogenase in buffer was added. Compounds were then added to plates in 1-mL volumes. The reaction was initiated with the addition of 39 mL of PS, diluted in buffer. The test plate was immediately transferred to a micro plate reader, and absorbance was measured at 340 nm every 12 s for 120 s.[7] Percentage inhibition was calculated using following formula, 100 *1 –compound rate – background rate/full reaction rate – background rate.

4.2.2. In vitro MTB screening

Two-fold serial dilutions of each test compound/drug were prepared and incorporated into Middle- brook 7H11 agar medium with oleic acid, albumin, dextrose, and catalase (OADC) growth supplement to get final concentrations of 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 μg/mL. Inoculum of *M. tuberculosis* H37Rv ATCC 27294 was prepared from fresh Middlebrook 7H11 agar slants with OADC (Difco) growth supplement adjusted to 1 mg/ mL (wet weight) in Tween 80 (0.05%) saline diluted to 10^{-2} to give a concentration of $\sim 10^7$ cfu/mL[14]. Five microliters of this bacterial suspension was spotted onto 7H11 agar tubes containing different concentrations of the drug as discussed above. The tubes were incubated at 37 °C, and final readings (as MIC in μg/mL) were determined after 28 days. The MIC is defined as the minimum concentration of compound required to give complete inhibition ofbacterial growth. This method is similar to that recommended by the National Committee for Clinical Laboratory Standards for the determination of MIC in triplicate.

4.2.3. In vitro cytotoxicity screening

Some compounds were further examined for toxicity in a RAW 264.7 cell line at the concentration of 50 µM. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 non-radioactive cell proliferation assay.

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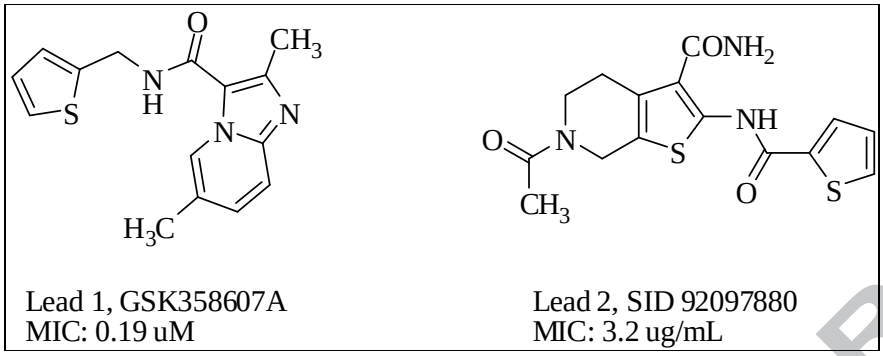


Figure 1: Identified lead compounds from high-throughput virtual screening

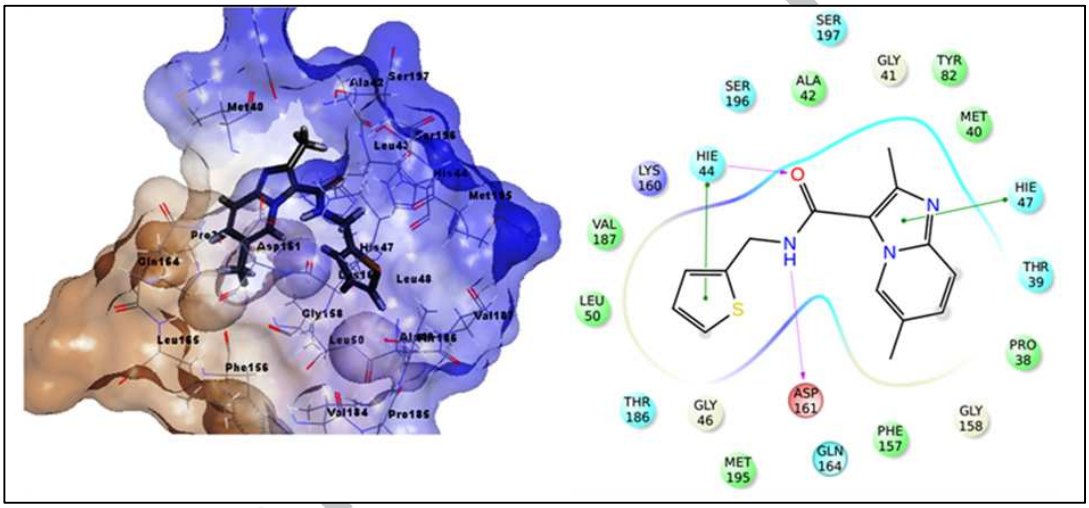
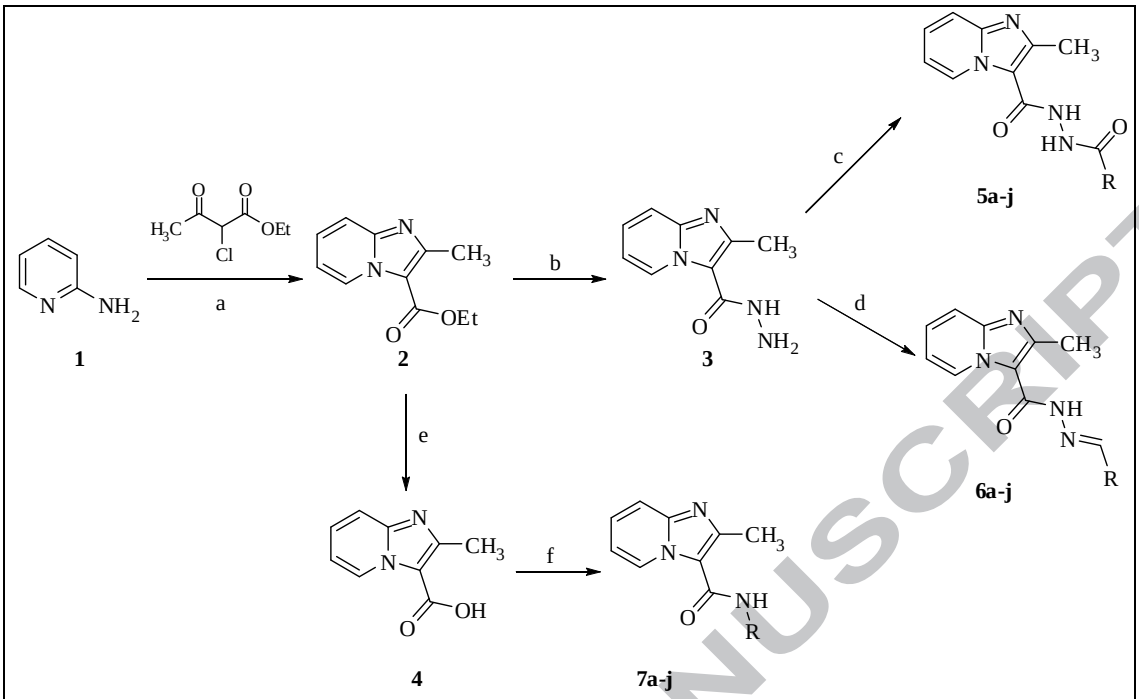


Figure 2: Binding mode of the (**Lead 1**) and the interacting pattern in the active site of the PS protein.



Reagents & Conditions: a: 1,2-Dimethoxyethane, 90 °C, 6 h; b: 35% $N_2H_4.H_2O$, Ethanol. reflux, 3 h; c: RCOOH, EDCI, HOBt, Et_3N, CH_2Cl_2, 0 °C - rt, 3 h; d: RCHO, Ethanol, reflux, 3 to 60 min; e: LiOH, Ethanol/H_2O (1:1), rt, 6 h; f: RNH_2, EDCI, HOBt, Et_3N, CH_2Cl_2, 0 °C - rt, 4 h:

Scheme: Synthetic protocol of compounds

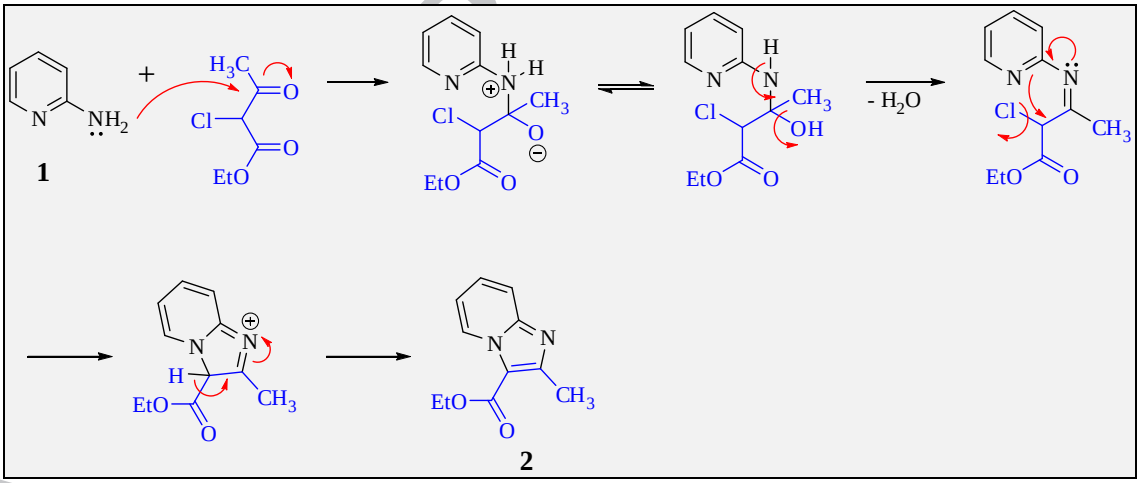


Figure 3: Mechanism of conversion of compound **1** to **2**

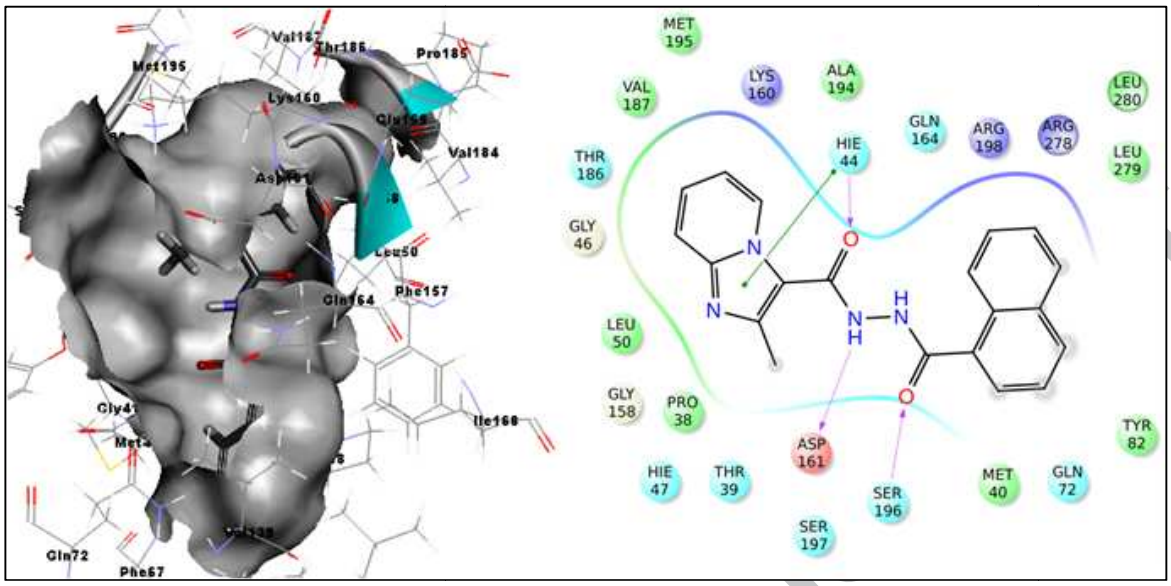


Figure 4: Binding mode of the most active compound **5b** and the interacting pattern in the active site of the panC protein.

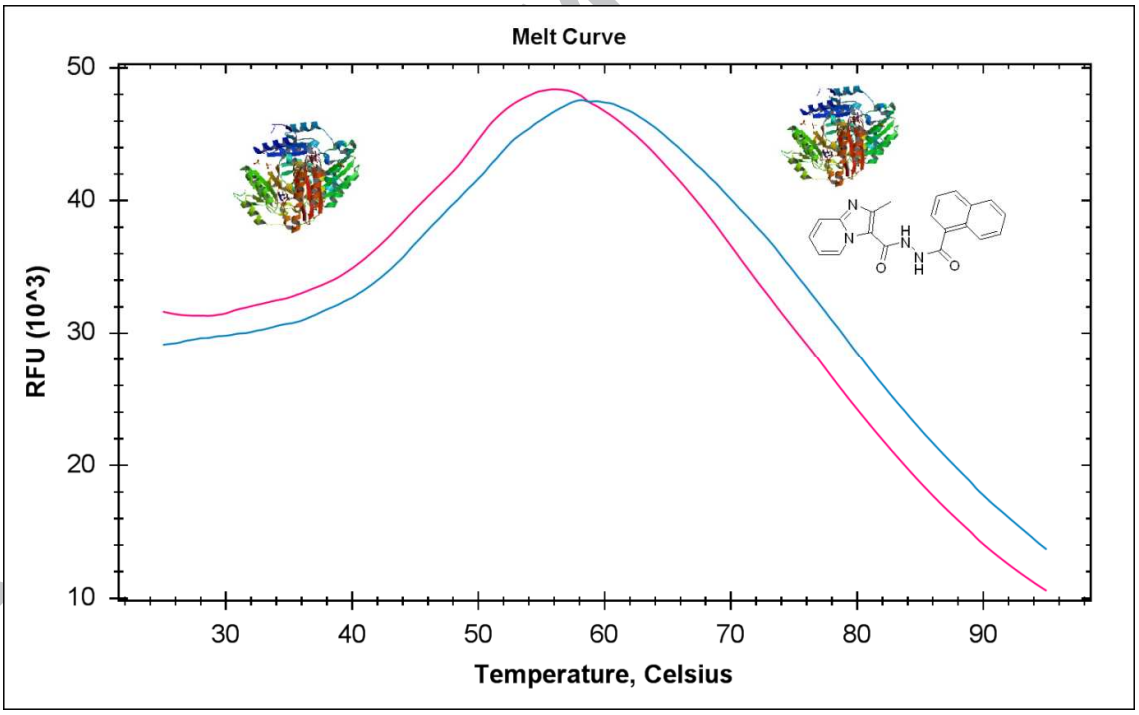


Figure 5: DSF experiment for compound **5b** (protein-ligand complex, blue) showing an increase in the thermal shift of 1.8 ℃ when compared to the native PS protein (red).

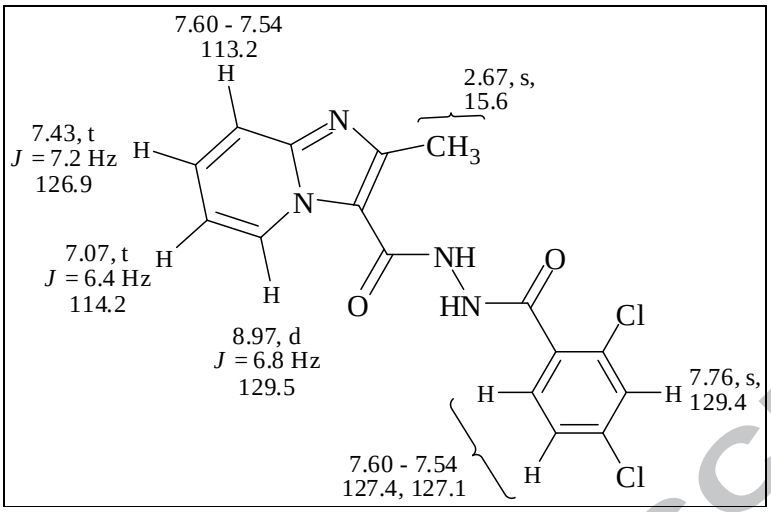
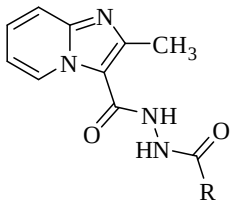
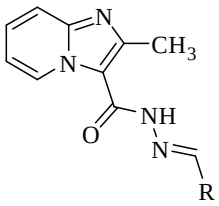


Figure 6: ^{1}H and ^{13}C chemical shifts in one of the compounds

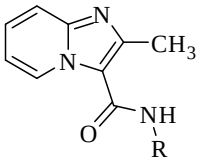
Table 1: Biological activities of synthesized compounds



5a-j



6a-j



7a-j

Compound	R	Yield (%)	MP (°C)	PanC IC_{50} in µM	MTB MIC in µM	Cytotoxicity at 50 µM (RAW 264.7 cells) % inhibition
5a	Phenyl	81	180-181	3.54±0.18	42.37	NT
5b	Naphthyl	79	260-261	1.90±0.12	4.53	28.42
5c	Cyclohexyl	88	251-252	7.70±0.67	10.38	31.67
5d	2-Furyl	69	186-187	8.93±0.53	10.96	16.76
5e	3-Nitrophenyl	76	220-221	9.20±0.96	36.76	NT
5f	3,5-Dinitrophenyl	80	138-139	6.48±0.26	16.23	40.62
5g	2,4-Dichlorophenyl	87	251-252	5.13±0.24	17.22	20.12
5h	4-Tolyl	74	189-190	3.35±0.32	80.9	NT
5i	2-Methoxyphenyl	69	162-163	8.21±0.42	19.23	18.96
5j	4-Phenoxyphenyl	89	142-143	7.22±0.29	32.30	NT
6a	Phenyl	84	184-186	4.86±0.61	179.2	NT
6b	4-Bromophenyl	90	274-275	4.43±0.12	35.01	NT
6c	4-Fluorophenyl	88	250-252	3.77±0.08	21.04	20.94
6d	4-Trifluromethylphenyl	76	223-224	8.18±0.14	9.01	24.56
6e	4-Nitrophenyl	92	268-269	>25	38.58	NT
6f	4-Hydroxyphenyl	87	277-278	7.49±0.22	21.19	19.42
6g	4-Methoxyphenyl	93	201-202	6.37±0.12	80.91	NT
6h	4-Benzyloxyphenyl	90	172-173	5.37±0.36	32.47	NT
6i	3,4,5-Trimethoxyphenyl	91	206-207	7.05±0.47	67.75	NT
6j	4-Tolyl	82	238-240	7.46±0.45	21.33	16.66
7a	Phenyl	78	184-185	12.83±0.19	187.9	NT
7b	Benzyl	82	>300	2.74±0.05	23.50	24.50
7c	Phenethyl	84	120-121	5.77±0.03	89.29	NT
7d	Cyclohexyl	76	155-156	1.99±0.01	96.90	NT
7e	2-Pyridyl	69	163-164	>25	98.81	NT
7f	2-Furanylmethyl	63	141-142	2.81±0.02	24.41	20.70
7g	4-Bromophenyl	81	155-156	6.95±0.34	37.88	NT
7h	4-Chlorophenyl	83	191-192	2.60±0.03	87.4	NT
7i	3-Trifluromethylphenyl	72	152-153	7.01±0.04	78.1	NT
7j	4-Ethoxyphenyl	81	160-161	>25	42.23	NT
	Isoniazid			>25	0.72	NT
	Ethambutol			>25	7.64	NT
	GSK358607A			8.12±0.03	0.19	NT

Graphical Abstract

Identification and development of 2-methylimidazo [1,2-a]pyridine-3-carboxamides as *Mycobacterium tuberculosis* pantothenate synthetase inhibitors

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