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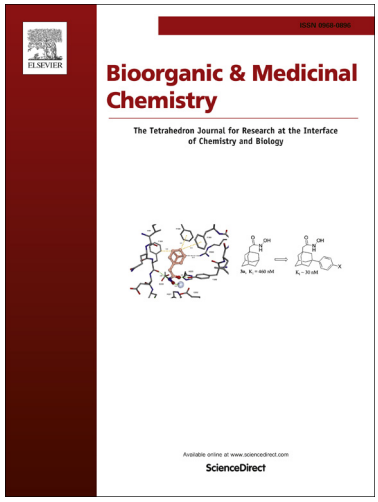

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Development of benzo[*d*]oxazol-2(3*H*)-ones derivatives as novel inhibitors of *Mycobacterium tuberculosis* InhA

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Abstract

A series of twenty seven substituted 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide derivatives were designed based on our earlier reported *Mycobacterium tuberculosis* (MTB) enoyl-acyl carrier protein reductase (InhA) lead. Compounds were evaluated for MTB InhA inhibition study, *in vitro* activity against drug-sensitive and -resistant MTB strains, and cytotoxicity against RAW 264.7 cell line. Among the compounds tested, 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(5-nitrothiazol-2-yl)acetamide (**30**) was found to be the most promising compound with IC_{50} of 5.12±0.44 µM against MTB InhA, inhibited drug sensitive MTB with MIC 17.11 µM and was non-cytotoxic at 100 µM. The interaction with protein and enhancement of protein stability in complex with compound **30** was further confirmed biophysically by differential scanning fluorimetry.

Key words: Tuberculosis, InhA, Enoyl acyl carrier protein reductase, Cytotoxicity

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1. Introduction

Today's tuberculosis (TB) drug regimen takes too long to be effective and requires too many medications. Treatment of drug sensitive disease requires 6-9 months whereas treatment of drug-resistant TB is even lengthier, taking 18-24 months or longer. Second-line drugs are also much more toxic and considerably more expensive than the standard first-line anti-TB regimen. Furthermore, current first-line treatment regimens are not compatible with the common antiretroviral therapies used to treat HIV/AIDS. Therefore, new drugs are needed which will be effective in treating active, resistant and latent TB infections, and will also be compatible with antiretroviral therapy. Among the most attractive molecular targets to design novel anti-tubercular agents are the fatty acid synthase (FAS) pathway enzymes.[1] The 2-*trans*-enoyl-acyl carrier protein (ACP) reductase (E.C.1.3.1.9) or MTB InhA, the fourth enzyme of the type II fatty acid synthase system (FAS II), is one of the key enzymes involved in the elongation cycle of fatty acids in MTB. InhA is essential to the mycolic acid biosynthesis in mycobacterium.[2] Its inactivation induces accumulation of saturated fatty acids, leading to cell wall alterations, lysis and consequently to death of the organism. Isoniazid, a first-line drug currently used to treat TB, is activated within the mycobacterial cell by KatG catalase. The activated molecule was thought to suppress mycolic acid biosynthesis through the inhibition of InhA, making it one of the best validated targets for the treatment of TB.[3] Isoniazid resistance is mediated through mutations in KatG that deters activation of the drug.[4] Therefore one should be able to attain same clinical efficacy as isoniazid and avoid much of the current resistance to isoniazid by bypassing the requirement for KatG activation and directly inhibiting InhA. Earlier we reported design of novel leads of MTB InhA inhibitors.[5] In the course of our studies on one of the lead *N*-(2,5-dichlorophenyl)-2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (**UPS 14**) (**Figure 1**), we

attempted to synthesize analogues around the lead to establish their biological activities and reported herein.

2. Results and discussion

2.1. Design and chemistry

In our earlier work we had generated and validated quantitative pharmacophore models using known set of reported MTB InhA inhibitors. The validated pharmacophore model was used as a query to screen an in-house/commercial database of 400,000 compounds and retrieved 25,000 hits. These hits were further ranked based on its shape and feature similarity with potent MTB InhA inhibitor using rapid overlay of chemical structures (ROCS) and subsequent hits were subjected for docking. Based on the pharmacophore, ROCS model and docking interactions, 32 compounds were selected finally and assayed for InhA inhibitory activity.[5] One of the lead **UPS 14** showed MTB InhA IC_{50} of 22.12±0.8 μM. Based on this and inputs from protein-ligand interactions observed in the structure of InhA with lead molecules, further modifications (and combinations thereof) were explored as a ligand expansion step. The lead molecule **UPS 14** enabled us convenient structure-activity relationship (SAR) investigations at both 6^{th} and N-1 positions of benzo[*d*]oxazol-2(3*H*)-one nucleus. Investigation of benzoxazolone pharmacophores has recently generated significant interest from a medicinal chemistry point of view and their synthesis has been well explored in the literature. The synthetic pathway used to achieve the lead modifications is delineated in **Schemes 1** and **2**. A library of 27 derivatives was synthesized (compound **8-25** and **28-36**, **Table 1**) and evaluated for their ability to inhibit InhA enzyme as step towards the derivation of SAR and hit optimization.

Construction of the target molecule was achieved by first treating the commercially available 2-aminophenol (**4**) with 1,1'-carbonyldiimidazole (CDI) in refluxing tetrahydrofuran to afford the corresponding benzoxazolone (**5**) in good yield. This benzoxazolone (**5**) was further alkylated at

the N-1 position with *tert*-butyl bromoacetate in presence of sodium hydride as base to give the corresponding acetate (**6**) in quantitative yield, which when subjected to acidic hydrolysis using trifluoroacetic acid (TFA) afforded the corresponding acid (**7**). The final library was developed by condensing this key intermediate acid derivative (**7**) with various substituted amines. Selection of amino substituents at N-1 position was based on our previous research experience in tuberculosis, in an effort to improve the anti-tubercular potency of these molecules. The final coupling was achieved by using EDC/HOBT as coupling agents. Though the reaction went smoothly for aromatic amines giving the desired products in good yield, a similar conversion was not observed in the case of heterocyclic amines especially with 2-amino-5-nitrothiazole, 2-aminobenzothiazole and 2-amino-6-nitrobenzothiazole, probably due to their low nucleophillicity and solubility issues. Hence, an alternative strategy was designed as depicted in **Scheme 2**, wherein the corresponding amine was first treated with chloroacetyl chloride in presence of triethylamine as base to give the corresponding 2-chloro-*N*-(aryl/heteroaryl)acetamide. This on further alkylation with the corresponding benzoxazolone derivative gave the desired amide derivative in good yields and purity. Both analytical and spectral data (^{1}H NMR, ^{13}C NMR and mass spectra) of all the synthesized compounds were in full agreement with the proposed structures.

2.2 MTB InhA inhibition assay and SAR

Recombinant MTB InhA was expressed in *E. coli* and subsequently purified according to a previously reported procedure.[6] The synthetic compounds were initially evaluated *in vitro* for the MTB InhA inhibition at 10 μM[6] and the results are presented in **Table 1**. Most of the synthesized compounds (except **16**) showed percentage inhibition ranging from 26.12 to 82.43 at 10 μM. Five compounds (**19**, **29-30**, **32-33**) showed more than 55 % inhibition and were subsequently tested for their IC_{50}s. Dose-response curves were plotted for these potent inhibitors using

GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) by taking log (inhibitor concentration) on the x-axis and response (% inhibition) on the y-axis as shown in **Figure 1**, **Supplementary data**. Compound 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(5-nitrothiazol-2-yl)acetamide (**30**) was found to be the most potent compound with IC_{50} of 5.12±0.44 μM.

To study the SAR, we carried out docking studies of all the synthesized compounds together with lead compound **UPS 14** for comparison. The crystal structure of MTB InhA complexed with reference inhibitor 1-cyclohexyl-*N*-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB:2H7M) having resolution of 1.62 Å was selected and docking results obtained with Glide, version 5.7, Schrodinger, LLC, New York, NY, 2012. Analysis of the crystal structure of 2H7M revealed that the reference inhibitor in the InhA active site formed hydrogen-bonding network between Tyr158, enzyme active site residues, and the NAD+ cofactor that probably served as the key feature that governed the orientation of the compound within the active site. Dual hydrogen bonding network was involved with the oxygen atom on the pyrrolidine carbonyl group, InhA catalytic residue Tyr158, and the NAD+. This hydrogen bonding network seemed to be a conserved feature among all the InhA-inhibitor complexes identified so far.[7] The reference 1-cyclohexyl-*N*-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide was re-docked with the active site residues of the MTB InhA to validate the active site cavity. The ligand exhibited highest Glide score of -8.02 kcal/mol and was found in the vicinity of amino acids Tyr158, Phe149, Met199, Ile215, Pro156, Leu218, Met155, Ala211, Ile202, Met103, Leu207, Ala157, Met161, Phe97, Met98, Gly96, Gly104 and Lys165 residues. The re-docking results showed that the compound exhibited similar interactions as that of the original crystal structure with RMSD of 0.87 Å suggesting reliability of the docking method in terms of. The superimposition of Glide docked conformation of co-crystal with co-crystal of 2H7M is shown in **Figure 2**.

The binding mode of lead compound **UPS14** with InhA enzyme was analysed in more detail. The binding orientation of lead compound *N*-(2,5-dichlorophenyl)-2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (**UPS14**) within the InhA binding pocket is represented in **Figure 3**. The predicted bound conformation of the lead compound **UPS14** showed that the carbonyl oxygen atom of benzo[*d*]oxazol-2(3*H*)-one ring formed hydrogen bond with the side chain of the Tyr158. The 2,5-dichlorophenyl ring attached to benzo[*d*]oxazol-2(3*H*)-one ring through an acetamide linker was surrounded by hydrophobic amino acids such as Phe149, Met199, Leu218, Ile215, Pro156, Ile202, Met103 and Ala157. The compound was very well fit into the active site cavity of the protein with a docking score of -7.52 kcal/mol.

Our SAR studies started from various substituted aryl/heteroaryl ring systems attached to benzo[*d*]oxazol-2(3*H*)-one ring through an acetamide linker. The effect of various aryl/heteroaryl substitutions at the R_2 position was explored. As shown in **Table 1**, the activity of most of the compounds exhibited % inhibition in the range of 26.12 to 82.43 % at 10 μM against InhA which was better than the lead compound **UPS14** (19.5 % at 10 μM).

The first subset of compounds (**8**, **11**, **14**, **17**, **20**, **23**, **28**, **31** and **34**), with H atom at R_1 with various substituted aryl/heteroaryl groups at R_2 position is shown in **Table 1**. Phenyl substitution at the R_2 position (compound **8**) displayed good InhA inhibitory activity. Compound with substitution at 2^{nd} and 5^{th} position of the phenyl ring (2-chloro-5-(trifluoromethyl)-2-phenyl group) (compound **11**) displayed moderate InhA inhibitory activity, similar was the introduction of 2-pyridyl group at R_2 position (compound **14**). The compounds substituted with 2-pyridylmethyl, 2-benzothiazolyl, 6-nitro-2-benzothiazoyl and 5-nitro-2-thiazolyl group (respective compounds **17, 28, 31,** and **34**) at R_2 position resulted in satisfactory *in vitro* InhA inhibitory activity in the range of 42.15 to 48.15 % inhibition at 10 μM, whereas substitution with 2-thiophenylmethyl ring (compound **23**) led to decreased potency compared to other

compounds from this subset. This was well supported by the interaction profile of all the molecules in the docking studies. The docking score of all the compounds from this subset was found to be in the range of -6.87 to -7.59 kcal/mol. All compounds, except compound **14**, were found to be associated with hydrogen bonding interactions with the side chain of Tyr158 and NAD^+. In most of the compounds the carbonyl oxygen atom on benzo[*d*]oxazol-2(3*H*)-one ring interacted with the side chain of Tyr158. Eventually, SAR from this subset of compounds revealed compound **28** as the most selective inhibitor of InhA and compound **23** to be comparatively least active. The bound conformation of compound **28** showed carbonyl oxygen atom of benzo[*d*]oxazol-2(3*H*)-one ring forming hydrogen bond with the side chain of Tyr158 and ribose hydroxyl group of NAD^+, which was also observed in the crystal ligand and also well correlated with the *in vitro* InhA inhibitory activity (**Figure 2**, **Supplementary data**). On the other hand binding analysis of compound **23** in the active site of protein revealed that the compound failed to produce any hydrogen bonding interaction with the side chain of Tyr158 residue of the protein and thus the inactivity of the compound could be correlated (**Figure 3**, **Supplementary data**).

In the second subset of compounds (**9**, **12**, **15**, **18**, **21**, **24**, **29**, **32** and **35**), the effect of chloro substitution on the benzo[*d*]oxazol-2(3*H*)-one ring at R_1 position was investigated to understand the effect of similar aryl/heteroaryl substituent's at R_2 position as that of the previous set of compounds. SAR study revealed that, chloro group at R_1 position increased hydrophobicity of the compounds due to hydrophobic interactions with the active site which was crucial for inhibition. This subset of compounds displayed more inhibitory potency than the previous subset. Here, all the compounds showed satisfactory *in vitro* InhA inhibitory activity in the range of 28.16 to 72.60 % inhibition at 10 μM concentrations. The compounds **12**, **15** and **35** with 2-chloro-5-(trifluoromethyl)-2-phenyl, 2-pyridyl and 6-nitro-2-benzothiazoyl groups respectively

as R_2 substituents, showed good InhA inhibitory activity, whereas compound **29** with 5-nitro-2-thiazolyl group as R_2 substituent was more active than compound **32** with 2-benzothiazolyl group as R_2 substituent. It was observed that phenyl substitution at R_2 (**9**) was somewhat less favoured than the other substitutions. Additionally, 2-furylmethyl, 2-pyridylmethyl and 2-thiophenylmethyl ring substitutions at R_2 position (respective compounds **21**, **18** and **24**) also displayed good activity in InhA enzyme inhibition studies. In this subset, compounds **29** and **32** were demonstrated to be effective InhA inhibitors with good activity. Both the compounds displayed docking scores of -7.12 and -7.073 kcal/mol respectively. The predicted binding pose of the most active compounds (**29** and **32**) suggested that the observed potency may be due to the extensive hydrophobic interactions predicted to be formed with the side chains of Met199, Leu218, Met155, Pro156, Ala157, Ile202, Met103, Phe149 and Met161 along with hydrogen bonding interactions with side chain of Tyr158 as well as with the ribose hydroxyl group of NAD^+ (**Figure 4**). Ligand binding analysis of one of the less active derivative from this subset (**9**) showed hydrophobic interactions between the phenyl ring and some hydrophobic amino acid residues. It also displayed hydrogen bonding interaction between carbonyl oxygen atom of 6-chlorobenzo[*d*]oxazol-2(3*H*)-one ring and side chain of Tyr158 amino acid residue. However, the orientation in the active site cavity of InhA pushed the chloro group away from the cavity, which might be the reason for its lesser enzyme inhibition (**Figure 4**, **Supplementary data**).

In the third subset of compounds (**10**, **13**, **16**, **19**, **22**, **25**, **30**, **33** and **36**), R_1 consisted of nitro group and R_2 was substituted with various aryl/heteroaryl groups similar to previous two subsets as shown in **Table 1**. The compounds **30, 33** and **19** with 5-nitro-2-thiazolyl, 2-benzothiazolyl and 2-pyridylmethyl substitution at R_2 respectively, emerged as most promising compounds from this subset showing satisfactory *in vitro* InhA inhibitory activity in the range of 60.80 to 82.43 % inhibition at 10 μM with IC_{50}s of 5.12 to 8.23 μM. This was well supported by their interaction

profile in docking studies where docking score of these compounds were found in the range of -7.11 to -7.48 kcal/mol. Closer analysis of compounds **30, 33** and **19** in the binding site revealed that the carbonyl oxygen atom on 6-nitrobenzo[*d*]oxazol-2(3*H*)-one ring was involved in hydrogen bonding interactions with side chain of Tyr158 residue as well as with ribose hydroxyl group of NAD^+ (**Figure 5**). In this subset of compounds, 2-pyridyl at R_2 position (**16**) showed least activity among the whole series of compounds with 15.63 % inhibition at 10 μM concentration. *In silico* analysis of this compound indicated that the molecule oriented in a different manner than that of other active derivatives and failed to demonstrate any interaction with Tyr158 residue as well as with NAD^+ resulting in less enzyme inhibition potency (**Figure 5**, **Supplementary data**).

From the docking results, it was evident that the formation of hydrogen bonds with side chain of Tyr158 and hydroxyl group of NAD^+ along with hydrophobic interactions with the active site were predicted to be the most crucial factors affecting the inhibitory potency of these compounds. The overall trend in enzyme inhibition activity showed that substitution at R_1 position with nitro group was more favoured than chloro group whereas H substitution was least favoured. In case of R_2 position, substitutions with heteroaryl groups such as 5-nitro-2-thiazolyl, 2-benzothiazolyl, 6-nitro-2-benzothiazolyl, 2-methylpyridyl, 2-furylmethyl and 2-thiophenylmethyl group were more favoured than with 2-chloro-5-(trifluoromethyl)-2-phenyl, phenyl and 2-pyridyl group.

2.3 *In vitro* MTB activity studies

All the synthesized compounds were also screened for their *in vitro* anti-tubercular activity against MTB H37Rv (ATCC27294) using agar dilution method[8] with drug concentrations from 50 μg/mL to 0.78 μg/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, rifampicin and ofloxacin 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 15.04 to 82.58 µM. Six compounds (**25, 29-32** and **36**) inhibited MTB with MIC of <20 µM. Compound **30** which showed IC_{50} of 5.12±0.44 µM in the MTB InhA enzyme also exhibited MTB MIC of 17.11 µM. In general, compounds which possessed (sub)-thiazolyl and –benzothiazolyl showed good MICs. Some of the molecules showed good potency in the enzyme inhibition study but failed to exhibit equally potent inhibition of MTB, and this seem to be a major challenge in the arena of TB drug discovery. Among other parameters, efflux mechanisms contributed in a major way to intrinsic resistance to drugs.[9] Efflux pumps of MTB belong to the ABC (ATP-binding cassette) transporters and major facilitator superfamily (MFS) proteins,[10] or antibiotic-modifying and -degrading enzymes,[11] and SMR (small multidrug resistance) family. Efflux-mediated drug resistance in MTB could be due to one or more efflux pumps working alone or in coordination. This was possible because of the redundancy of their functions, which overlapped extensively.[12] In the current work, we carried out MTB screening of the synthesized compounds in the presence of reported efflux pump inhibitors verapamil (50 µg/mL) and piperine (8 µg/mL).[13] In most of the cases MIC decreased 2 to 10 fold when compared to the absence of an efflux pump inhibitor. Compound **30** showed MTB MIC of 8.55 µM in the presence of verapamil and piperine and its MTB InhA IC_{50} was very well correlated. Encouraged by their promising *in vitro* anti-tubercular activity, the more potent analogues were further evaluated for their *in vitro* potency against the clinical isolate of XDR-TB strain (strain resistant to Isoniazid, Rifampicin, Ofloxacin and Kanamycin). All the compounds displayed mycobacterial kill against the XDR strain and five compounds (**20, 25, 29-30** and **36**) showed XDR-TB MIC of <20 µM (Table 1).

2.4. Cytotoxicity study

All the compounds were further tested for cytotoxicity in a RAW 264.7 cell line (mouse leukemic monocyte macrophage) at 50 and 100 μM. We selected macrophage cell line to test toxicity as it was known that MTB reside inside the macrophages and drug molecules should not possess any toxicity against these macrophages. After 72 h of exposure, viability of the cells was assessed on the basis of cellular conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into a formazan product using the Promega Cell Titer 96 non-radioactive cell proliferation assay.[14] Most of the tested compounds were not cytotoxic (less than 50% inhibition at 50 μM) to RAW 264.7 cells and their percentage growth inhibitions are reported in **Table 1**. The most active MTB InhA inhibitor (**30)** showed lesser toxicity of 20.24 and 43.79 % inhibition at 50 and 100 μM drug concentrations respectively.

2.5. Biophysical characterization

One of the active compounds from this series of chemical class of molecules was further investigated using a biophysical technique, differential scanning fluorimetry (DSF). The ability of the compounds to stabilize the catalytic domain of the InhA protein was assessed utilizing the DSF technique by which the thermal stability of the catalytic domain of InhA native protein and of the protein bound with the ligand was measured.[15] Complex with compound **30** was heated stepwise from 25 to 95 °C in steps of 0.1 °C rise in the presence of a fluorescent dye (sypro orange), whose fluorescence increased as it interacted with hydrophobic residues of the InhA protein. As the protein was denatured, the amino acid residues became exposed to the dye. A right side positive shift of T_m in comparison to native protein meant higher stabilization of the protein-ligand complex, which was a consequence of the inhibitor binding. In our study, compound **30** showed significant positive T_m shift of 1.9 °C confirming the stability of the

protein-ligand complex as shown in **Figure 6** which depicts the curves obtained in the DSF experiment for the MTB InhA protein (pink) and protein-compound **30** complex (green).

3. Conclusion

In present work, we reported synthesis and screening results of twenty seven substituted 2-(2-oxobenzo[d]oxazol-3(2H)-yl)acetamide derivatives against MTB InhA as well as drug sensitive and resistant MTB strains. Most of the synthesized compounds showed better InhA inhibition as compared to lead molecules, and 2-(6-nitro-2-oxobenzo[d]oxazol-3(2H)-yl)-N-(5-nitrothiazol-2-yl)acetamide (compound **30**) was found to be the most active compound with IC_{50} of 5.12±0.44 µM against MTB InhA, inhibited drug sensitive MTB with MIC of 17.11 µM and was non-cytotoxic at 100 µM.

4. Experimental

4.1. Chemistry

4.1.1. General

The melting points were determined by capillary melting point apparatus and were not corrected. Homogeneity of the compounds was monitored by thin layer chromatography (TLC) on silica gel 60 F_{254} coated on aluminium plates, visualized by UV light and $KMnO_4$ treatment. All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-300 (300.12 MHz, 75.12 MHz) NMR spectrometer, Bruker BioSpin Corp, Germany. Chemical shifts were reported in ppm (δ) with reference to the internal standard TMS. Molecular weights of the synthesized compounds were checked by LCMS 6100B series Agilent Technology. Elemental analyses were carried out on an automatic Flash EA 1112 Series, CHN Analyser (Thermo). All commercially available chemicals and solvents were used without further purification.

4.1.2. General procedure for the synthesis of 6-substituted benzo[*d*]oxazol-2(3*H*)-ones (5a-c)

To a stirred solution of appropriate aminophenol (**4a-c**) (1 mmol) in dry tetrahydrofurane (THF) (20 mL) was added 1,1'-carbonyldiimidazole (CDI) (1.1 mmol) at room temperature (rt). The solution was refluxed for about 4 h (monitored by TLC & LCMS for completion), and solvent evaporated under reduced pressure.[16] The residue was further diluted with water (20 mL) and ethyl acetate (20 mL) and the layers separated. The organic layer was washed with 2 N hydrochloric acid (15 mL), water (10 mL), dried over anhydrous sodium sulphate and evaporated under reduced pressure. The residue was purified by silica gel column chromatography using hexane: ethyl acetate as eluent to give the corresponding benzoxazolone (**5a-c**) in good yield.

4.1.2.1. Benzo[*d*]oxazol-2(3*H*)-one (5a)

The compound was synthesized according to the general procedure using 2-aminophenol (**4a**) (2.0 g, 18.32 mmol) and CDI (3.2 g, 20.15 mmol) to afford **5a** (1.9 g, 76.7 %) as white solid. M.p: 139-141 °C. ^{1}H NMR (DMSO-d_6): δ_H 8.18 (s, 1H), 7.47 – 6.91 (m, 4H). ^{13}C NMR (DMSO-d_6): δ_C 155.3, 144.6, 129.8, 125.4, 124.6, 110.1, 109.6. ESI-MS *m/z* 136 $(M+H)^+$. Anal. Calcd. for $C_7H_5NO_2$: C, 62.22; H, 3.73; N, 10.37; Found: C, 62.25; H, 3.70; N, 10.40.

4.1.2.2. 6-Chlorobenzo[*d*]oxazol-2(3*H*)-one (5b)

The compound was synthesized according to the general procedure using 2-amino-5-chlorophenol (**4b**) (2.0 g, 13.93 mmol) and CDI (2.4 g, 15.32 mmol) to afford **5b** (1.8 g, 78.4 %) as white solid. M.p: 193-195 °C. ^{1}H NMR (DMSO-d_6): δ_H 8.25 (s, 1H), 7.83 – 7.41 (m, 3H). ^{13}C NMR (DMSO-d_6): δ_C 155.6, 140.3, 131.7, 128.5, 125.9, 123.2, 122.8. ESI-MS *m/z* 168 $(M-H)^-$. Anal. Calcd. for $C_7H_4ClNO_2$: C, 49.58; H, 2.38; N, 8.26; Found: C, 49.61; H, 2.40; N, 8.29.

4.1.2.3. 6-Nitrobenzo[*d*]oxazol-2(3*H*)-one (5c)

The compound was synthesized according to the general procedure using 2-amino-5-nitrophenol (**4c**) (2.0 g, 12.97 mmol) and CDI (2.3 g, 14.27 mmol) to afford **5c** (1.8 g, 79.3 %) as pale yellow solid. M.p: 250-252 °C. ^{1}H NMR (DMSO-d_6): δ_H 8.29 (s, 1H), 8.31 – 8.01 (m, 3H). ^{13}C NMR

(DMSO-d_6): δ_C 155.7, 144.0, 140.1, 136.6, 121.1, 117.5, 115.1. ESI-MS *m/z* 179 $(M-H)^-$. Anal. Calcd. for $C_7H_4N_2O_4$: C, 46.68; H, 2.24; N, 15.55; Found: C, 46.65; H, 2.21; N, 15.58.

4.1.3. General procedure for the synthesis of 6-substituted *tert*-butyl 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetate (6a-c)

To a well stirred suspension of sodium hydride (60% dispersion in mineral oil, 1.2 mmol) in *N*,*N*-dimethylformamide (DMF) (10 mL) at 0 °C was added drop wise a solution of the corresponding benzoxazolone (**5a-c**) (1 mmol) in DMF (10 mL), followed by *tert*-butyl bromoacetate (1.1 mmol). The solution was slowly warmed to rt and stirred at rt for about 1 h (monitored by TLC & LCMS for completion), quenched with saturated ammonium chloride solution (15 mL) and concentrated under reduced pressure. The residue was partitioned between ethyl acetate (20 mL) and water (10 mL) and the organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using hexane: ethyl acetate as eluent to give the corresponding product (**6a-c**).

4.1.3.1. *tert*-butyl 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetate (6a)

The compound was synthesized according to the general procedure using benzo[*d*]oxazol-2(3*H*)-one (**5a**) (1.5 g, 11.10 mmol) and *tert*-butyl bromoacetate (2.3 g, 12.21 mmol) to afford **6a** (1.9 g, 69.4 %) as white solid. M.p: 150-152 °C. ^{1}H NMR (DMSO-d_6): δ_H 8.15 – 7.42 (m, 4H), 4.27 (s, 2H), 1.39 (s, 9H). ^{13}C NMR (DMSO-d_6): δ_C 169.7, 154.8, 139.2, 132.5, 125.7, 124.6, 116.3, 116.0, 83.1, 53.6, 28.2. ESI-MS *m/z* 250 $(M+H)^+$. Anal. Calcd. for $C_{13}H_{15}NO_4$: C, 62.64; H, 6.07; N, 5.62; Found: C, 62.58; H, 6.04; N, 5.65.

4.1.3.2. *tert*-butyl 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetate (6b)

The compound was synthesized according to the general procedure using 6-chlorobenzo[*d*]oxazol-2(3*H*)-one (**5b**) (1.5 g, 8.84 mmol) and *tert*-butyl bromoacetate (1.8 g,

9.73 mmol) to afford **6b** (1.37 g, 82.1 %) as white solid. M.p: 197-199 °C. ^{1}H NMR (DMSO-d_6): δ_H 7.87 – 7.31 (m, 3H), 4.23 (s, 2H), 1.34 (m, 9H). ^{13}C NMR (DMSO-d_6): δ_C 169.6, 154.7, 140.5, 131.7, 130.1, 125.9, 123.6, 122.8, 82.3, 53.2, 28.5. ESI-MS *m/z* 284 $(M+H)^+$. Anal. Calcd. for $C_{13}H_{14}ClNO_4$: C, 55.04; H, 4.97; N, 4.94; Found: C, 55.07; H, 4.99; N, 4.96.

4.1.3.3. *tert*-butyl 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetate (6c)

The compound was synthesized according to the general procedure using 6-nitrobenzo[*d*]oxazol-2(3*H*)-one (**5c**) (3.0 g, 16.65 mmol) and *tert*-butyl bromoacetate (3.5 g, 18.32 mmol) to afford 6c (2.1 g, 68.6 %) as white solid. M.p: 182-184 °C. ^{1}H NMR (DMSO-d_6): δ_H 8.28 – 7.98 (m, 3H), 4.31 (s, 2H), 1.37 (s, 9H). ^{13}C NMR (DMSO-d_6): δ_C 169.5, 154.5, 144.2, 140.3, 138.6, 123.1, 121.2, 117.5, 82.0, 53.6, 28.4. ESI-MS *m/z* 295 $(M+H)^+$. Anal. Calcd. for $C_{13}H_{14}N_2O_6$: C, 53.06; H, 4.80; N, 9.52; Found: C, 53.09; H, 4.78; N, 9.49.

4.1.4. General procedure for the synthesis of 6-substituted 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acids (7a-c)

Trifluoroacetic acid (TFA) (2 mmol) was added drop wise over 5 min to a stirred solution of corresponding 6-substituted *tert*-butyl 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetate (**6a-c**) (1 mmol) in anhydrous dichloromethane (DCM) (15 mL) under nitrogen at 0 °C. The solution was slowly warmed to the rt and stirred at rt for about 4 h (monitored by TLC & LCMS for completion) and concentrated under reduced pressure. The residue was dissolved in toluene and concentrated under reduced pressure (twice), and finally triturated with ether to give a corresponding product (**7a-c**).

4.1.4.1. 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (7a)

The compound was synthesized according to the general procedure using *tert*-butyl 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetate (**6a**) (1.5 g, 6.0 mmol), and TFA (1.3 g, 12.0 mmol) to

afford **7a** (0.9 g, 83.1 %) as white solid. M.p: 226-228 °C. ^{1}H NMR (DMSO-d_6): δ_H 11.17 (s, 1H), 7.82 – 7.18 (m, 4H), 4.12 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 176.6, 154.7, 139.2, 132.5, 125.9, 124.8, 116.4, 116.1, 56.1. ESI-MS *m/z* 192 $(M-H)^-$. Anal. Calcd. for $C_9H_7NO_4$: C, 55.96; H, 3.65; N, 7.25; Found: C, 56.00; H, 3.67; N, 7.23.

4.1.4.2. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (7b)

The compound was synthesized according to the general procedure using *tert*-butyl 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetate (**6b**) (1.35 g, 4.75 mmol), and TFA (1.08 g, 9.51 mmol) to afford **7b** (0.91 g, 87.7 %) as white solid. M.p: 268-270 °C. ^{1}H NMR (DMSO-d_6): δ_H 11.20 (s, 1H), 8.14 – 7.31 (m, 3H), 4.14 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 176.2, 154.8, 140.1, 131.6, 130.4, 126.0, 123.6, 122.7, 56.3. ESI-MS *m/z* 226 $(M-H)^-$. Anal. Calcd. for $C_9H_6ClNO_4$: C, 47.49; H, 2.66; N, 6.15; Found: C, 47.52; H, 2.65; N, 6.13.

4.1.4.3. 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (7c)

The compound was synthesized according to the general procedure using *tert*-butyl 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetate (**6c**) (1.5 g, 5.09 mmol), and TFA (1.16 g, 10.19 mmol) to afford **7c** (0.95 g, 78.7 %) as white solid. M.p: 261-263 °C. ^{1}H NMR (DMSO-d_6): δ_H 11.22 (s, 1H), 8.31 – 7.98 (m, 3H), 4.17 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 176.3, 154.6, 144.2, 140.0, 138.5, 123.2, 121.1, 117.7, 55.4. ESI-MS *m/z* 237 $(M-H)^-$. Anal. Calcd. for $C_9H_6N_2O_6$: C, 45.39; H, 2.54; N, 11.76; Found: C, 45.42; H, 2.52; N, 11.78.

4.1.5. General procedure for the synthesis of substituted 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamides (8-25)

A solution of corresponding 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7a-c**) (1 mmol), substituted amine (1.2 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (1.2 mmol), N-hydroxybenzotriazole (HOBT) (1.2 mmol), and triethylamine (TEA) (2.2 mmol) were

stirred for 6 h at rt in DCM (4 mL) (monitored by TLC & LCMS for completion). The reaction mixture was further diluted with water (10 mL) and DCM (10 mL), and the layers separated. The organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using hexane: ethyl acetate as eluent to give the corresponding product (**8-25**).

4.1.5.1. 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-phenylacetamide (8)

The compound was synthesized according to the general procedure using 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7a**) (0.1 g, 0.51 mmol), aniline (0.057 g, 0.62 mmol), EDC (0.12 g, 0.62 mmol) and HOBT (0.083 g, 0.62 mmol) to afford **8** (0.092 g, 66.6 %) as white solid. M.p: 190-191 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.25 (s, 1H), 8.16 – 7.04 (m, 9H), 4.76 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.5, 155.6, 139.0, 138.7, 132.1, 128.3, 128.0, 125.7, 124.6, 121.2, 116.4, 115.9, 54.1. ESI-MS *m/z* 269 $(M+H)^+$. Anal. Calcd. for $C_{15}H_{12}N_2O_3$: C, 67.16; H,4.51; N, 10.44; Found: C, 67.12; H, 4.52; N, 10.42.

4.1.5.2. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-phenylacetamide (9)

The compound was synthesized according to the general procedure using 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid **(7b**) (0.1 g, 0.43 mmol), aniline (0.049 g, 0.52 mmol), EDC (0.10 g, 0.52 mmol) and HOBT (0.070 g, 0.52 mmol) to afford **9** (0.097 g, 73.2 %) as white solid. M.p: 181-183 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.26 (s, 1H), 8.22 - 7.05 (m, 8H), 4.77 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.6, 154.8, 140.1, 138.7, 131.3, 130.6, 128.7, 128.3, 125.4, 123.5, 122.7, 121.6, 54.2. ESI-MS *m/z* 301 $(M-H)^-$. Anal. Calcd. for $C_{15}H_{11}ClN_2O_3$: C, 59.52; H, 3.66; N, 9.25; Found: C, 59.55; H, 3.64; N, 9.28.

4.1.5.3. 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-phenylacetamide (10)

The compound was synthesized according to the general procedure using 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7c**) (0.1 g, 0.41 mmol), aniline (0.046 g, 0.50 mmol), EDC (0.098 g, 0.50 mmol) and HOBT (0.067 g, 0.50 mmol) to afford **10** (0.096 g, 73.3 %) as pale yellow solid. M.p: 222-223 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.28 (s, 1H), 7.92 – 7.06 (m, 8H), 4.83 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.6, 154.9, 143.9, 140.2, 138.6, 138.4, 129.0, 128.2, 122.8, 121.7, 120.9, 117.7, 54.4. ESI-MS *m/z* 312 $(M-H)^-$. Anal. Calcd. for $C_{15}H_{11}N_3O_5$: C, 57.51; H, 3.54; N, 13.41; Found: C, 57.55; H, 3.52; N, 13.44.

4.1.5.4. *N*-(2-chloro-5-(trifluoromethyl)phenyl)-2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (11)

The compound was synthesized according to the general procedure using 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7a**) (0.1 g, 0.51 mmol), 2-chloro-5-(trifluoromethyl)aniline (0.12 g, 0.62 mmol), EDC (0.12 g, 0.62 mmol) and HOBT (0.083 g, 0.62 mmol) to afford **11** (0.13 g, 69.6 %) as pale yellow solid. M.p: 157-159 °C. ^{1}H NMR (DMSO-d_6): δ_H 9.97 (s, 1H), 8.10 – 7.19 (m, 7H), 4.82 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.7, 168.6, 139.1, 137.9, 132.2, 129.5, 129.3, 125.9, 125.7, 124.8, 124.3, 122.2, 118.7, 116.6, 116.1, 54.2. ESI-MS *m/z* 369 $(M-H)^-$. Anal. Calcd. for $C_{16}H_{10}ClF_3N_2O_3$: C, 51.84; H, 2.72; N, 7.56; Found: C, 51.87; H, 2.70; N, 7.53.

4.1.5.5. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(2-chloro-5-(trifluoromethyl)phenyl)acetamide (12)

The compound was synthesized according to the general procedure using 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid **(7b)** (0.1 g, 0.43 mmol), 2-chloro-5-(trifluoromethyl)aniline (0.10 g, 0.52 mmol), EDC (0.10 g, 0.52 mmol) and HOBT (0.070 g, 0.52 mmol) to afford **12** (0.13 g, 74.3 %) as white solid. M.p: 139-140 °C. ^{1}H NMR (DMSO-d_6): δ_H

9.98 (s, 1H), 8.11 – 7.19 (m, 6H), 4.82 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.5, 154.0, 140.8, 137.3, 131.7, 130.5, 129.6, 129.4, 125.9, 125.6, 124.4, 123.7, 122.8, 122.2, 118.9, 54.5. ESI-MS *m/z* 404 $(M-H)^-$. Anal. Calcd. for $C_{16}H_9Cl_2F_3N_2O_3$: C, 47.43; H, 2.24; N, 6.91; Found: C, 47.45; H, 2.25; N, 6.94.

4.1.5.6. *N*-(2-chloro-5-(trifluoromethyl)phenyl)-2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (13)

The compound was synthesized according to the general procedure using 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7c**) (0.1 g, 0.41 mmol), 2-chloro-5-(trifluoromethyl)aniline (0.097 g, 0.50 mmol), EDC (0.098 g, 0.50 mmol) and HOBT (0.067 g, 0.50 mmol) to afford **13** (0.13 g, 78.5 %) as pale yellow solid. M.p: 127-129 °C. ^{1}H NMR (DMSO-d_6): δ_H 9.98 (s, 1H), 8.12 – 7.19 (m, 6H), 4.83 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.4, 154.6, 143.9, 140.1, 138.5, 137.7, 129.5, 129.3, 126.1, 124.3, 122.9, 122.4, 121.0, 118.8, 117.7, 54.2. ESI-MS *m/z* 414 $(M-H)^-$. Anal. Calcd. for $C_{16}H_9ClF_3N_3O_5$: C, 46.23; H, 2.18; N, 10.11; Found: C, 46.19; H, 2.16; N, 10.14.

4.1.5.7. 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(pyridin-2-yl)acetamide (14)

The compound was synthesized according to the general procedure using 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7a**) (0.1 g, 0.51 mmol), pyridin-2-amine (0.058 g, 0.62 mmol), EDC (0.12 g, 0.62 mmol) and HOBT (0.083 g, 0.62 mmol) to afford **14** (0.094 g, 68.2 %) as white solid. M.p: 221-223 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.42 (s, 1H), 8.67 – 7.02 (m, 8H), 4.87 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.5, 154.7, 151.8, 146.7, 139.1, 138.5, 132.1, 127.6, 124.8, 124.3, 116.5, 116.1, 115.8, 52.4. ESI-MS *m/z* 270 $(M+H)^+$. Anal. Calcd. for $C_{14}H_{11}N_3O_3$: C, 62.45; H, 4.12; N, 15.61; Found: C, 62.42; H, 4.10; N, 15.59.

4.1.5.8. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(pyridin-2-yl)acetamide (15)

The compound was synthesized according to the general procedure using 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid **(7b)** (0.1 g, 0.43 mmol), pyridin-2-amine (0.048 g, 0.52 mmol), EDC (0.10 g, 0.52 mmol) and HOBT (0.070 g, 0.52 mmol) to afford **15** (0.086 g, 65.3 %) as white solid. M.p: 243-245 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.44 (s, 1H), 8.67 – 7.10 (m, 7H), 4.88 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.6, 154.8, 151.6, 146.6, 140.2, 138.5, 131.4, 130.4, 125.7, 124.3, 123.6, 122.7, 116.6, 52.3. ESI-MS *m/z* 302 $(M-H)^-$. Anal. Calcd. for $C_{14}H_{10}ClN_3O_3$: C, 55.37; H, 3.32; N, 13.84; Found: C, 55.34; H, 3.35; N, 13.87.

4.1.5.9. 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(pyridin-2-yl)acetamide (16)

The compound was synthesized according to the general procedure using 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7c**) (0.1 g, 0.41 mmol), pyridin-2-amine (0.047 g, 0.50 mmol), EDC (0.098 g, 0.50 mmol) and HOBT (0.067 g, 0.50 mmol) to afford **16** (0.088 g, 67.1 %) as pale yellow solid. M.p: 253-255 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.51 (s, 1H), 8.68 – 7.11 (m, 7H), 4.89 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 168.9, 154.9, 151.8, 146.7, 144.0, 139.9, 138.8, 138.4, 124.6, 122.9, 121.2, 117.2, 115.9, 52.6. ESI-MS *m/z* 313 $(M-H)^-$. Anal. Calcd. for $C_{14}H_{10}N_4O_5$: C, 53.51; H, 3.21; N, 17.83; Found: C, 53.48; H, 3.18; N, 17.86.

4.1.5.10. 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(pyridin-2-ylmethyl)acetamide (17)

The compound was synthesized according to the general procedure using 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7a**) (0.1 g, 0.51 mmol), pyridin-2-ylmethanamine (0.067 g, 0.62 mmol), EDC (0.12 g, 0.62 mmol) and HOBT (0.083 g, 0.62 mmol) to afford **17** (0.095 g, 65.2 %) as white solid. M.p: 162-164 °C. ^{1}H NMR (DMSO-d_6): δ_H 9.921 (s, 1H), 8.37 – 7.01 (m, 8H), 4.75 (s, 2H), 4.54 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.6, 155.8, 154.2, 148.5,

139.5, 139.2, 132.0, 125.9, 124.8, 124.2, 121.1, 116.4, 116.1, 54.3, 45.2. ESI-MS *m/z* 284 $(M+H)^+$. Anal. Calcd. for $C_{15}H_{13}N_3O_3$: C, 63.60; H, 4.63; N, 14.83; Found: C, 63.57; H, 4.60; N, 14.81.

4.1.5.11. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(pyridin-2-ylmethyl)acetamide (18)

The compound was synthesized according to the general procedure using 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid **(7b**) (0.1 g, 0.43 mmol), pyridin-2-ylmethanamine (0.056 g, 0.52 mmol), EDC (0.10 g, 0.52 mmol) and HOBT (0.070 g, 0.52 mmol) to afford **18** (0.089 g, 64.4 %) as white solid. M.p: 182-184 °C. ^{1}H NMR (DMSO-d_6): δ_H 9.92 (s, 1H), 8.41 – 7.01 (m, 7H), 4.81 (s, 2H), 4.55 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.7, 156.2, 154.3, 148.8, 140.7, 139.9, 131.8, 130.1, 125.7, 124.3, 126.3, 122.2, 121.1, 54.4, 45.5. ESI-MS *m/z* 316 $(M-H)^-$. Anal. Calcd. for $C_{15}H_{12}ClN_3O_3$: C, 56.70; H, 3.81; N, 13.23; Found: C, 56.66; H, 3.78; N, 13.20.

4.1.5.12. 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(pyridin-2-ylmethyl)acetamide (19)

The compound was synthesized according to the general procedure using 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7c**) (0.1 g, 0.41 mmol), pyridin-2-ylmethanamine (0.054 g, 0.50 mmol), EDC (0.098 g, 0.50 mmol) and HOBT (0.067 g, 0.50 mmol) to afford **19** (0.092 g, 66.7 %) as white solid. M.p: 187-189 °C. ^{1}H NMR (DMSO-d_6): δ_H 9.92 (s, 1H), 8.42 – 7.02 (m, 7H), 4.83 (s, 2H), 4.55 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.5, 156.3, 154.9, 148.3, 144.2, 139.9, 139.6, 138.4, 124.5, 123.1, 121.1, 120.8, 117.7, 54.4, 45.4. ESI-MS *m/z* 327 $(M-H)^-$. Anal. Calcd. for $C_{15}H_{12}N_4O_5$: C, 54.88; H, 3.68; N, 17.07; Found: C, 54.87; H, 3.70; N, 17.10.

4.1.5.13. *N*-(furan-2-ylmethyl)-2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (20)

The compound was synthesized according to the general procedure using 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7a**) (0.1 g, 0.51 mmol), furan-2-ylmethanamine (0.060 g, 0.62 mmol), EDC (0.12 g, 0.62 mmol) and HOBT (0.083 g, 0.62 mmol) to afford **20** (0.101 g, 72.2 %) as white solid. M.p: 110-112 °C. ^{1}H NMR (DMSO-d_6): δ_H 9.76 (s, 1H), 7.45 – 6.79 (m, 7H), 4.79 (s, 2H), 4.33 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.3, 154.2, 149.3, 142.5, 132.1, 130.3, 124.5, 122.6, 119.2, 116.1, 110.4, 108.6, 51.3, 35.0. ESI-MS *m/z* 273 $(M+H)^+$. Anal. Calcd. for $C_{14}H_{12}N_2O_4$: C, 61.76; H, 4.44; N, 10.29; Found: C, 61.73; H, 4.42; N, 10.26.

4.1.5.14. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(furan-2-ylmethyl)acetamide (21)

The compound was synthesized according to the general procedure using 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid **(7b)** (0.1 g, 0.43 mmol), furan-2-ylmethanamine (0.050 g, 0.52 mmol), EDC (0.10 g, 0.52 mmol) and HOBT (0.070 g, 0.52 mmol) to afford **21** (0.098 g, 73.4 %) as white solid. M.p: 142-144 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.42 (s, 1H), 7.60 – 6.36 (m, 6H), 4.62 (s, 2H), 4.33 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.5, 154.6, 154.1, 149.2, 142.6, 132.3, 130.0, 122.7, 119.0, 116.3, 110.6, 108.3, 51.5, 34.9. ESI-MS *m/z* 305 $(M-H)^-$. Anal. Calcd. for $C_{14}H_{11}ClN_2O_4$: C, 54.83; H, 3.62; N, 9.13; Found: C, 54.79; H, 3.60; N, 9.15.

4.1.5.15. *N*-(furan-2-ylmethyl)-2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (22)

The compound was synthesized according to the general procedure using 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7c**) (0.1 g, 0.41 mmol), furan-2-ylmethanamine (0.048 g, 0.50 mmol), EDC (0.098 g, 0.50 mmol) and HOBT (0.067 g, 0.50 mmol) to afford **22** (0.097 g, 73.2 %) as pale yellow solid. M.p: 165-167 °C. ^{1}H NMR (DMSO-d_6): δ_H 11.04 (s, 1H), 7.75 – 6.80 (m, 6H), 4.81 (s, 2H), 4.33 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.7, 154.5, 144.2, 149.2, 142.4, 132.2, 130.0, 122.5, 119.2, 116.4, 110.7, 108.5, 51.2, 34.9. ESI-MS *m/z* 316 $(M-H)^-$. Anal. Calcd. for $C_{14}H_{11}N_3O_6$: C, 53.00; H, 3.49; N, 13.24; Found: C, 52.97; H, 3.46; N, 13.22.

4.1.5.16. 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(thiophen-2-ylmethyl)acetamide (23)

The compound was synthesized according to the general procedure using 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7a**) (0.1 g, 0.51 mmol), thiophen-2-ylmethanamine (0.070 g, 0.62 mmol), EDC (0.12 g, 0.62 mmol) and HOBT (0.083 g, 0.62 mmol) to afford **23** (0.111 g, 74.4 %) as white solid. M.p: 128-130 °C. ^{1}H NMR (DMSO-d$_6$): δ_H 9.87 (s, 1H), 7.47 – 6.81 (m, 7H), 4.79 (s, 2H), 4.34 (s, 2H). ^{13}C NMR (DMSO-d$_6$): δ_C 169.6, 154.7, 153.2, 138.2, 128.8, 128.7, 127.2, 126.8, 126.3, 123.3, 119.1, 116.6, 51.6, 36.3. ESI-MS *m/z* 287 $(M-H)^-$. Anal. Calcd. for $C_{14}H_{12}N_2O_3S$: C, 58.32; H, 4.20; N, 9.72; Found: C, 58.35; H, 4.18; N, 9.73.

4.1.5.17. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(thiophen-2-ylmethyl)acetamide (24)

The compound was synthesized according to the general procedure using 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid **(7b**) (0.1 g, 0.43 mmol), thiophen-2-ylmethanamine (0.058 g, 0.52 mmol), EDC (0.10 g, 0.52 mmol) and HOBT (0.070 g, 0.52 mmol) to afford **24** (0.102 g, 72.3 %) as white solid. M.p: 166-168 °C. ^{1}H NMR (DMSO-d$_6$): δ_H 10.32 (s, 1H), 7.52 – 6.24 (m, 6H), 4.79 (s, 2H), 4.35 (s, 2H). ^{13}C NMR (DMSO-d$_6$): δ_C 169.4, 154.6, 153.0, 139.6, 139.4, 129.5, 128.3, 126.7, 126.0, 124.5, 120.4, 115.6, 51.4, 36.3. ESI-MS *m/z* 321 $(M-H)^-$. Anal. Calcd. for $C_{14}H_{11}ClN_2O_3S$: C, 52.10; H, 3.44; N, 8.68; Found: C, 52.13; H, 3.42; N, 8.66.

4.1.5.18. 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(thiophen-2-ylmethyl)acetamide (25)

The compound was synthesized according to the general procedure using 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetic acid (**7c**) (0.1 g, 0.41 mmol), thiophen-2-ylmethanamine (0.056 g, 0.50 mmol), EDC (0.098 g, 0.50 mmol) and HOBT (0.067 g, 0.50 mmol) to afford **25** (0.104 g, 74.7 %) as white solid. M.p: 220-222 °C. ^{1}H NMR (DMSO-d$_6$): δ_H 11.06 (s, 1H), 7.76 – 6.97 (m, 6H), 4.81 (s, 2H), 4.50 (s, 2H). ^{13}C NMR (DMSO-d$_6$): δ_C 169.2, 154.4, 152.5, 146.1,

137.9, 130.2, 128.0, 127.4, 126.8, 126.2, 114.2, 111.1, 51.2, 36.4. ESI-MS *m/z* 332 $(M-H)^-$. Anal. Calcd. for $C_{14}H_{11}N_3O_5S$: C, 50.45; H, 3.33; N, 12.61; Found: C, 50.42; H, 3.35; N, 12.64.

4.1.6. General procedure for the synthesis of 2-chloro-*N*-(aryl/heteroaryl)acetamides (27a-c)

Chloroacetyl chloride (2 mmol) was added drop wise to a well stirred suspension of corresponding amine (**26**) (1 mmol) and TEA (3 mmol) in DMF (15 mL) at 0 °C. The reaction mixture was then slowly warmed to rt and stirred at rt for about 3 h (monitored by TLC & LCMS for completion). The solvent was then evaporated under reduced pressure and the residue was further diluted with water (20 mL) and ethyl acetate (30 mL), and the layers separated. The aqueous layer was re-extracted with ethyl acetate (2 × 30 mL) and the combined organic layer was washed with brine (20 mL), dried over anhydrous sodium sulphate and evaporated under reduced pressure. The residue was then purified by silica column chromatography using hexane: ethyl acetate as eluent to give corresponding product (**27a-c**).

4.1.6.1. 2-chloro-*N*-(5-nitrothiazol-2-yl)acetamide (27a)

The compound was synthesized according to the general procedure using chloroacetyl chloride (1.55 g, 13.77 mmol) and 5-nitrothiazol-2-amine (1.0 g, 6.88 mmol) to afford **27a** (1.03 g, 67.7 %) as pale yellow solid. M.p: 252-254 °C. ^{1}H NMR (DMSO-d_6): δ_H 9.94 (s, 1H), 8.34 (s, 1H), 4.45 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.4, 166.3, 148.1, 138.6, 43.1. ESI-MS *m/z* 220 $(M-H)^-$. Anal. Calcd. for $C_5H_4ClN_3O_3S$: C, 27.10; H, 1.82; N, 18.96; Found: C, 27.13; H, 1.79; N, 18.99.

4.1.6.2. *N*-(benzo[*d*]thiazol-2-yl)-2-chloroacetamide (27b)

The compound was synthesized according to the general procedure using chloroacetyl chloride (1.50 g, 13.31 mmol) and benzo[*d*]thiazol-2-amine (1.0 g, 6.65 mmol) to afford **27b** (0.92 g, 66.2 %) as pale yellow solid. M.p: 276-278 °C. ^{1}H NMR (DMSO-d_6): δ_H 11.78 (s, 1H), 8.21 – 7.63 (m, 4H), 4.39 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 173.7, 168.8, 153.4, 130.9, 125.5, 124.7, 121.5, 118.4, 42.9. ESI-MS *m/z* 225 $(M-H)^-$. Anal. Calcd. for $C_9H_7ClN_2OS$: C, 47.69; H, 3.11; N, 12.36; Found: C, 47.72; H, 3.13; N, 12.39.

4.1.6.3. 2-chloro-*N*-(6-nitrobenzo[*d*]thiazol-2-yl)acetamide (27c)

The compound was synthesized according to the general procedure using chloroacetyl chloride (1.15 g, 10.24 mmol) and 6-nitrobenzo[*d*]thiazol-2-amine (1.0 g, 5.12 mmol) to afford **27c** (0.92 g, 66.2 %) as pale yellow solid. M.p: 273-275 °C. ^{1}H NMR (DMSO-d_6): δ_H 12.62 (s 1H), 9.11 (s, 1H), 8.34 – 7.97 (m, 2H), 4.55 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 173.9, 168.5, 159.6, 144.8, 131.6, 121.5, 119.2, 117.1, 43.0. ESI-MS *m/z* 270 $(M-H)^-$. Anal. Calcd. for $C_9H_6ClN_3O_3S$: C, 39.79; H, 2.23; N, 15.47; Found: C, 39.76; H, 2.22; N, 15.50.

4.1.7. General procedure for the synthesis of substituted 2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamides (28-36)

To a solution of corresponding 2-chloro-*N*-(aryl/heteroaryl)acetamides (**27a-c**) (1.2 mmol) in DMF (3 mL) was added anhydrous potassium carbonate (1.5 mmol) and corresponding benzo[*d*]oxazol-2(3*H*)-one (**5a-c**) (1 mmol). The reaction mixture was heated at 60 °C for 3 h (monitored by TLC & LCMS for completion). The residue was further diluted with water (10 mL) and ethyl acetate (20 mL) and the layers separated. The aqueous layer was re-extracted with ethyl acetate (2 x 15 mL) and the combined organic layer was washed with brine (15 mL), dried over anhydrous sodium sulphate and evaporated under reduced pressure and the residue was

purified by silica column chromatography using hexane: ethyl acetate as eluent to give the desired product (**28-36**).

4.1.7.1. *N*-(5-nitrothiazol-2-yl)-2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (28)

The compound was synthesized according to the general procedure using 2-chloro-*N*-(5-nitrothiazol-2-yl)acetamide (**27a**) (0.137 g, 0.62 mmol) and benzo[d]oxazol-2(3H)-one (**5a**) (0.1g, 0.51 mmol) to afford **28** (0.11 g, 72.2 %) as pale yellow solid. M.p: 193-195 °C. ^{1}H NMR (DMSO-d_6): δ_H 13.60 (s, 1H), 8.67 (s, 1H), 7.41 – 7.15 (m, 4H), 4.95 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.5, 162.8, 154.4, 145.7, 138.8, 135.5, 132.5, 125.8, 124.7, 116.6, 116.1, 53.2. ESI-MS *m/z* 319 $(M-H)^-$. Anal. Calcd. for $C_{12}H_8N_4O_5S$: C, 45.00; H, 2.52; N, 17.49; Found: C, 45.04; H, 2.49; N, 17.46.

4.1.7.2. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(5-nitrothiazol-2-yl)acetamide (29)

The compound was synthesized according to the general procedure using 2-chloro-*N*-(5-nitrothiazol-2-yl)acetamide (**27a**) (0.156 g, 0.70 mmol) and 6-chlorobenzo[*d*]oxazol-2(3*H*)-one (**5b**) (0.1g, 0.58 mmol) to afford **29** (0.148 g, 70.9 %) as pale yellow solid. M.p: 268-270 °C. ^{1}H NMR (DMSO-d_6): δ_H 13.51 (s, 1H), 8.65 (s, 1H), 7.89 – 7.12 (m, 3H), 4.81 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.6, 162.6, 154.7, 147.1, 140.0, 135.6, 131.5, 130.4, 125.9, 123.5, 122.8, 53.4. ESI-MS *m/z* 353 $(M-H)^-$. Anal. Calcd. for $C_{12}H_7ClN_4O_5S$: C, 40.63; H, 1.99; N, 15.79; Found: C, 40.60; H, 2.01; N, 15.81.

4.1.7.3. 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(5-nitrothiazol-2-yl)acetamide (30)

The compound was synthesized according to the general procedure using 2-chloro-*N*-(5-nitrothiazol-2-yl)acetamide (**27a**) (0.147 g, 0.66 mmol) and 6-nitrobenzo[*d*]oxazol-2(3*H*)-one (**5c**) (0.1g, 0.55 mmol) to afford **30** (0.149 g, 73.6 %) as pale yellow solid. M.p: 273-275 °C. ^{1}H

NMR (DMSO-d_6): δ_H 13.50 (s, 1H), 8.55 (s, 1H), 7.89 – 7.19 (m, 3H), 4.83 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 169.6, 162.8, 154.2, 147.1, 143.3, 140.2, 138.5, 135.6, 123.2, 121.3, 117.7, 53.2. ESI-MS *m/z* 364 $(M-H)^-$. Anal. Calcd. for $C_{12}H_7N_5O_7S$: C, 39.46; H, 1.93; N, 19.17; Found: C, 39.49; H, 1.94; N, 19.20.

4.1.7.4. *N*-(benzo[*d*]thiazol-2-yl)-2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (31)

The compound was synthesized according to the general procedure using *N*-(benzo[*d*]thiazol-2-yl)-2-chloroacetamide (**27b**) (0.140 g, 0.62 mmol) and benzo[*d*]oxazol-2(3*H*)-one (**5a**) (0.1g, 0.51 mmol) to afford **31** (0.11 g, 67.9 %) as white solid. M.p: 250-252 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.68 (s, 1H), 7.85 – 7.37 (m, 8H), 4.79 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 173.5, 168.6, 154.7, 153.3, 139.2, 132.4, 130.9, 125.8, 125.5, 124.7, 124.5, 121.9, 118.7, 116.6, 116.0, 54.2. ESI-MS *m/z* 326 $(M+H)^+$. Anal. Calcd. for $C_{16}H_{11}N_3O_3S$: C, 59.07; H, 3.41; N, 12.92; Found: C, 59.04; H, 3.38; N, 12.89.

4.1.7.5. *N*-(benzo[*d*]thiazol-2-yl)-2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (32)

The compound was synthesized according to the general procedure using *N*-(benzo[*d*]thiazol-2-yl)-2-chloroacetamide (**27b**) (0.158 g, 0.70 mmol) and 6-chlorobenzo[*d*]oxazol-2(3*H*)-one (**5b**) (0.1g, 0.58 mmol) to afford **32** (0.108 g, 68.4 %) as white solid. M.p: 267-269 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.73 (s, 1H), 8.02 – 7.34 (m, 7H), 4.81 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 173.4, 168.5, 154.2, 153.5, 140.8, 131.7, 130.8, 130.5, 125.9, 125.5, 124.3, 123.5, 122.8, 121.9, 118.8, 54.4. ESI-MS *m/z* 358 $(M-H)^-$. Anal. Calcd. for $C_{16}H_{10}ClN_3O_3S$: C, 53.41; H, 2.80; N, 11.68; Found: C, 53.39; H, 2.78; N, 11.71.

4.1.7.6. *N*-(benzo[*d*]thiazol-2-yl)-2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (33)

The compound was synthesized according to the general procedure using *N*-(benzo[*d*]thiazol-2-yl)-2-chloroacetamide (**27b**) (0.135 g, 0.66 mmol) and 6-nitrobenzo[*d*]oxazol-2(3*H*)-one (**5c**) (0.1g, 0.55 mmol) to afford **33** (0.105 g, 67.6 %) as pale yellow solid. M.p: 256-258 °C. ^{1}H NMR (DMSO-d_6): δ_H 10.75 (s, 1H), 8.12 – 7.41 (m, 7H), 4.81 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 173.7, 168.7, 154.8, 153.1, 144.2, 139.8, 138.6, 130.9, 125.5, 124.9, 123.3, 121.9, 121.0, 118.4, 117.7, 54.2. ESI-MS *m/z* 369 $(M-H)^-$. Anal. Calcd. for $C_{16}H_{10}N_4O_5S$: C, 51.89; H, 2.72; N, 15.13; Found: C, 51.86; H, 2.70; N, 15.16.

4.1.7.7. *N*-(6-nitrobenzo[*d*]thiazol-2-yl)-2-(2-oxobenzo[*d*]oxazol-3(2*H*)-yl)acetamide (34)

The compound was synthesized according to the general procedure using 2-chloro-*N*-(6-nitrobenzo[*d*]thiazol-2-yl)acetamide (**27c**) (0.168 g, 0.62 mmol) and benzo[*d*]oxazol-2(3*H*)-one (**5a**) (0.1g, 0.51 mmol) to afford **34** (0.124 g, 64.8 %) as pale yellow solid. M.p: >280 °C. ^{1}H NMR (DMSO-d_6): δ_H 13.08 (s, 1H), 8.46 (s, 1H), 8.23 – 6.99 (m, 6H), 4.78 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 173.5, 168.6, 159.5, 154.7, 144.5, 139.2, 132.5, 131.5, 125.8, 124.4, 121.1, 119.6, 117.4, 116.4, 116.1, 54.4. ESI-MS *m/z* 369 $(M-H)^-$. Anal. Calcd. for $C_{16}H_{10}N_4O_5S$: C, 51.89; H, 2.72; N, 15.13; Found: C, 51.86; H, 2.74; N, 15.10.

4.1.7.8. 2-(6-chloro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(6-nitrobenzo[*d*]thiazol-2-yl)acetamide (35)

The compound was synthesized according to the general procedure using 2-chloro-*N*-(6-nitrobenzo[*d*]thiazol-2-yl)acetamide (**27c**) (0.190 g, 0.70 mmol) and 6-chlorobenzo[*d*]oxazol-2(3*H*)-one (**5b**) (0.1g, 0.58 mmol) to afford **35** (0.156 g, 65.7 %) as pale yellow solid. M.p: 269-271 °C. ^{1}H NMR (DMSO-d_6): δ_H 13.00 (s, 1H), 8.46 (s, 1H), 8.24 – 7.11 (m, 5H), 4.89 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 173.3, 168.7, 160.1, 154.6, 144.7, 140.4, 131.7, 131.5, 130.6, 125.7,

123.3, 122.5, 121.7, 119.4, 117.7, 54.5. ESI-MS *m/z* 403 $(M-H)^-$. Anal. Calcd. for $C_{16}H_9ClN_4O_5S$: C, 47.47; H, 2.24; N, 13.84; Found: C, 47.44; H, 2.22; N, 13.82.

4.1.7.9. 2-(6-nitro-2-oxobenzo[*d*]oxazol-3(2*H*)-yl)-*N*-(6-nitrobenzo[*d*]thiazol-2-yl)acetamide (36)

The compound was synthesized according to the general procedure using 2-chloro-*N*-(6-nitrobenzo[*d*]thiazol-2-yl)acetamide (**27c**) (0.179 g, 0.66 mmol) and 6-nitrobenzo[*d*]oxazol-2(3*H*)-one (**5c**) (0.1g, 0.55 mmol) to afford **36** (0.157 g, 68.1 %) as pale yellow solid. M.p: 265-267 °C. ^{1}H NMR (DMSO-d_6): δ_H 13.02 (s, 1H), 8.46 (s, 1H), 8.24 – 7.82 (m, 5H), 4.89 (s, 2H). ^{13}C NMR (DMSO-d_6): δ_C 174.0, 168.8, 160.0, 154.9, 144.9, 144.3, 140.2, 138.6, 131.7, 123.1, 121.4, 121.2, 119.4, 117.7, 117.5, 54.6. ESI-MS *m/z* 414 $(M-H)^-$. Anal. Calcd. for $C_{16}H_9N_5O_7S$: C, 46.27; H, 2.18; N, 16.86; Found: C, 46.24; H, 2.17; N, 16.84.

4.2. Biological activity

4.2.1. InhA protein expression, isolation and purification

MTB *inhA* gene that encodes InhA was cloned and transformed into *E. coli* BL21 (DE3) cells.[6] The transformed colonies were grown in LB broth at 37 ºC with constant aeration, in the presence of 100 µg/ml of ampicillin. Later, the transformed colonies were grown at 37 ºC to reach an optical density of 0.6 (A595). Exponentially growing cultures were induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and further the cells were grown at 18 ºC for 12-16 h. Cells were harvested and lysed by sonication in lysis buffer containing 50 mM Tris-HCl, 200 mM NaCl, 0.05 % β-mercaptoethanol, 0.1 mM PMSF and 8 % glycerol, pH-7.5. The cell lysate containing His 6-fusion protein was equilibrated with Ni-NTA affinity resins for 3-4 h, and washed with wash buffer containing 50 mM Tris-HCl, 200 mM NaCl, 20 mM imidazole

and 8% Glycerol. Later the tagged proteins were eluted with buffer containing different concentration of imidazole ranging from 100 mM - 300 mM. Fractions were pooled and the InhA protein isolated was identified using Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE).

4.2.2. Enzyme inhibition studies

The substrate 2-*trans*-decenoyl-CoA (DD-CoA) was synthesized from 2-*trans*-decenoic acid using the mixed anhydride method and purified according to the procedure described by Goldman and Vagelos.[17] The absorption of each reaction mixture was determined with a PerkinElmer VICTOR X3 spectrophotometer. The Kinetic assays using DD-CoA and wild-type InhA were performed as described.[18] For the inhibition assays with InhA the pre-incubation reactions were performed in 80 µl (total volume) of 30 mM PIPES buffer, 150 mM NaCl, pH 6.8 at 25 °C containing 70 nM InhA and inhibitor (at 10 µM). DMSO was used as co-solvent and its final concentration was 0.5 %. After 2 h of pre-incubation, the addition of 35 mM substrate (DD-CoA) and 200 mM cofactor (NADH) initiated the reaction which was followed at 340 nm (oxidation of NADH) and at 25 °C using PerkinElmer VICTOR X3 spectrophotometer. Control reactions were carried out under the same conditions as described above but without ligands. The inhibitory activity of each compound tested was expressed as the percentage inhibition of InhA activity (initial velocity of the reaction) with respect to the control experiments. All activity assays were performed in triplicate.

4.2.3. Bacterial growth inhibition with *M. tuberculosis*

Two-fold serial dilutions of each test compound/drug were prepared and incorporated into Middlebrook 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 MTB H37Rv ATCC 27294/XDR-TB 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 ~10^{7} cfu/mL. 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 mg/mL) were determined after 28 days. This method is similar to that recommended by the National Committee for Clinical Laboratory Standards for the determination of MIC in triplicate.

4.2.4. Molecular docking studies

Glide XP (extra precision) module of Schrödinger 9.2 (Glide, version 5.7, Schrödinger, LLC, New York, NY, 81 2011) was utilized for docking. The crystal structure of MTB InhA complexed with 1-cyclohexyl-N-(3,5-dichlorophenyl)-5-oxopyrrolidine-3-carboxamide (PDB:2H7M) having resolution of 1.62 Å was retrieved from the Protein Data Bank (www.rcsb.org) and further used for docking of synthesized compounds. The reported crystal structure is a monomer, having only one chain A with the inhibitor bound to it. The protein was prepared using protein preparation wizard and glide energy grids were generated for prepared protein complex. The binding site was defined by a rectangular box surrounding the X-ray ligand. Ligand was refined using the "Refine" option in Glide, and the option 70 to output Glide XP descriptor information was chosen (Glide 71 v5.7, Schrodinger, LLC, New York, NY). For the refinement and docking calculations, the default settings as available in the software package were used.

4.2.5. Cytotoxicity studies

All the synthesized compounds were examined for cytotoxicity in a RAW 264.7 cell line (mouse leukaemic monocyte macrophage) at concentrations of 50 and 100 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.[14] The percentage inhibition of cells was reported in **Table 1** and it's in range of 1.61 – 43.54 % at 50 µM, and 12.08 – 6.10 % at 100 µM. The most active MTB InhA inhibitor **30** has shown cytotoxicity of 20.24 and 43.79 % inhibition at 50 and 100 µM respectively.

4.2.6. Biophysical characterization

The binding affinity of the one active ligand was evaluated by measuring fluorescence of the native protein and the protein–ligand complex in the presence of a fluorescent dye (sypro orange) whose fluorescence increases when exposed to non-polar residues of the protein and reaches a maximum when the protein denatures.[15] In brief, the native protein (7.5 µL (1.5 mg/mL) of protein + 3.5 µL of buffer [50 mM Tris (pH 7.4), 1 mM EDTA, 5 mM DTT]) was subjected to stepwise heating in a PCR instrument (Bio-Rad iCycler5) from 25°C to 100°C with an increment of 0.6°C/min in the presence of the fluorescent dye sypro orange [2.5 µL (1:100); Sigma, St Louis, MO]. As the temperature increases, the stability of the protein decreases and becomes zero at equilibrium, where the concentrations of folded and unfolded protein become equal. This temperature was noted as the melting temperature (T_m). The dye exhibited maximum fluorescence at this point as it was exposed to the hydrophobic portion of the protein as a result of protein denaturing. A higher or positive shift in T_m of the protein–ligand complex compared with the native protein signifies better stabilization of the protein–ligand complex, which in turn is a reflection of inhibitor binding.

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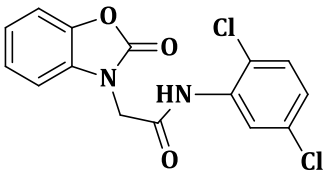
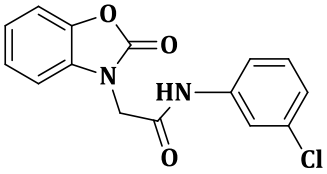
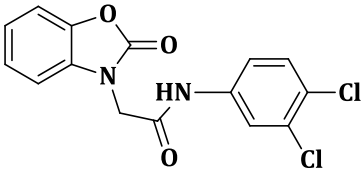


UPS 14 (1)
19.5% inhibition at 10µM
IC_{50}= 22.12±0.8 µM



UPS 15 (2)
17% inhibition at 10µM
IC_{50} > 25 µM



UPS 16 (3)
16.5% inhibition at 10µM
IC_{50} > 25 µM

Figure 1: Earlier identified lead compounds

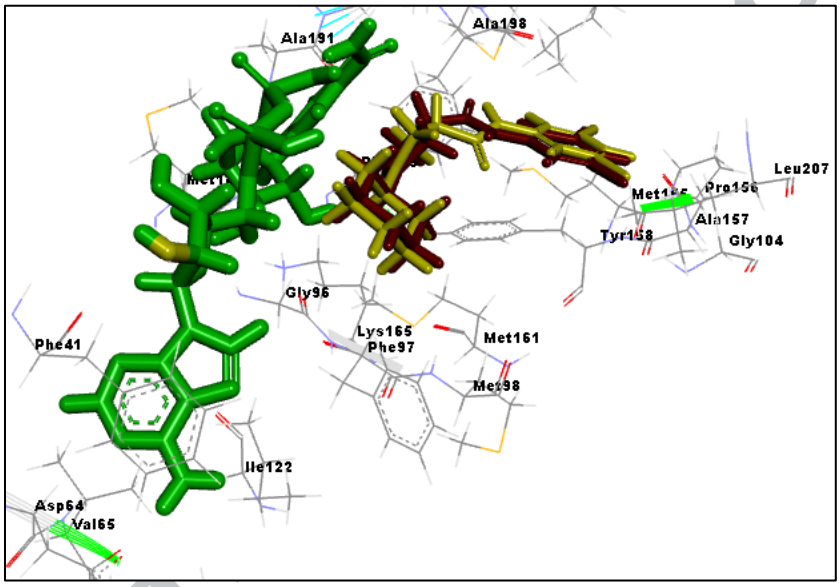


Figure 2: Superimposition of docked pose of the reported reference inhibitor (Yellow) to the original pose of the reference inhibitor (Red).

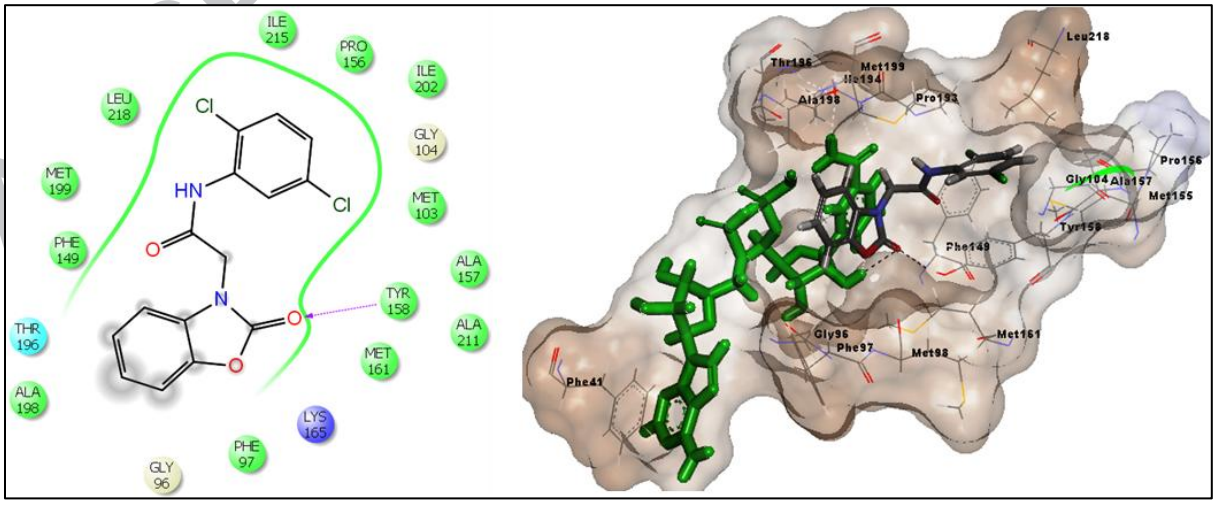


Figure 3: Binding pose and interaction pattern of lead molecule **UPS14**.

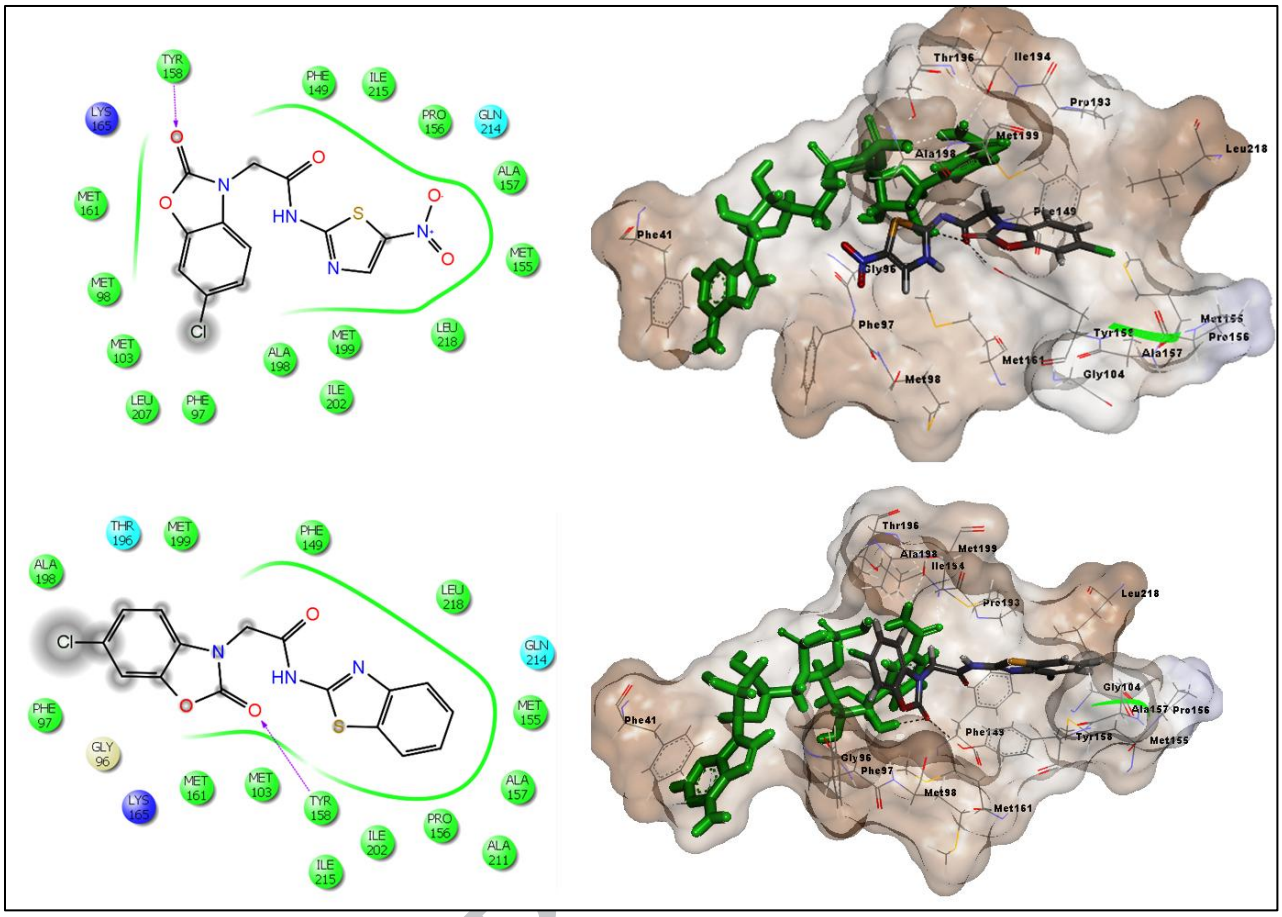


Figure 4: Binding pose and its interaction pattern of the compound **29** and **32**.

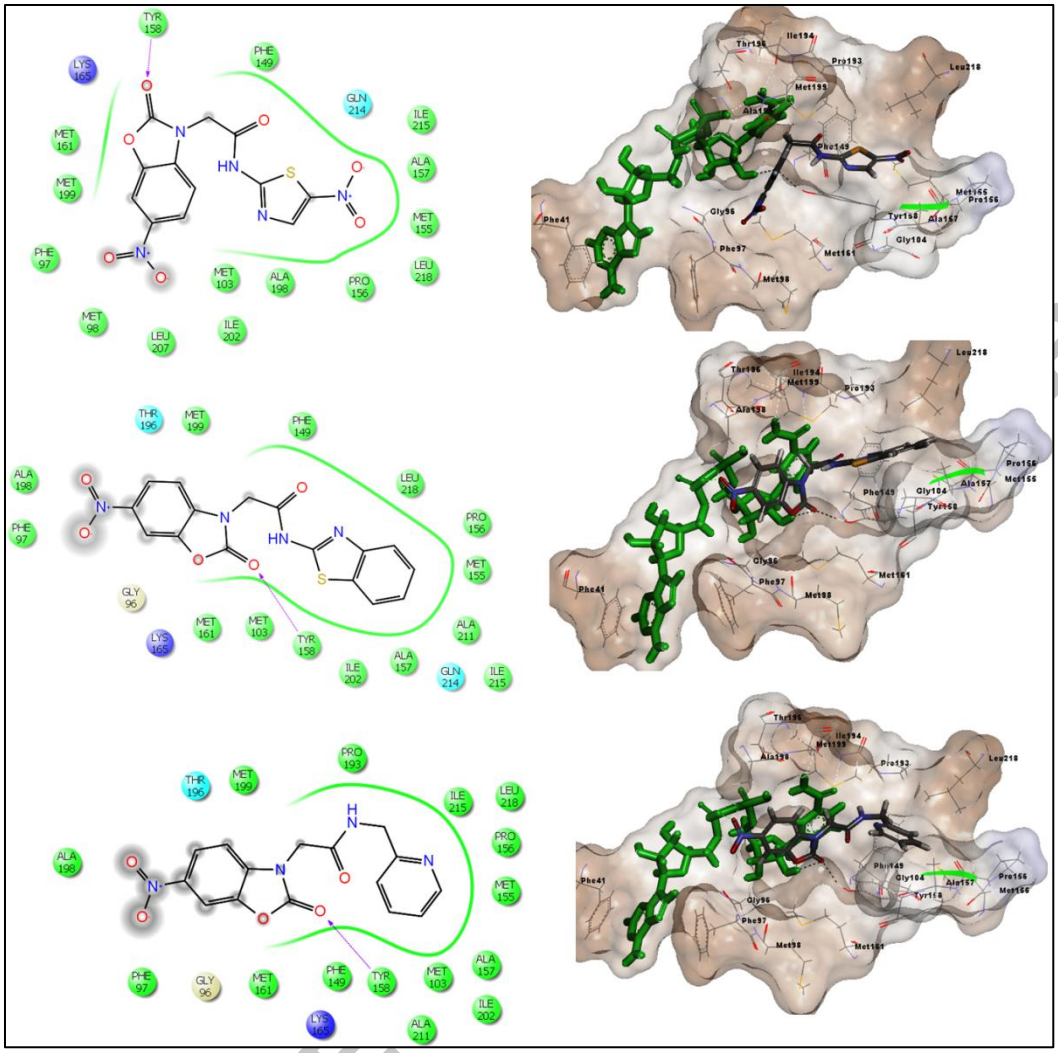


Figure 5: Binding pose and its interaction pattern of the compound **30, 33** and **19**.

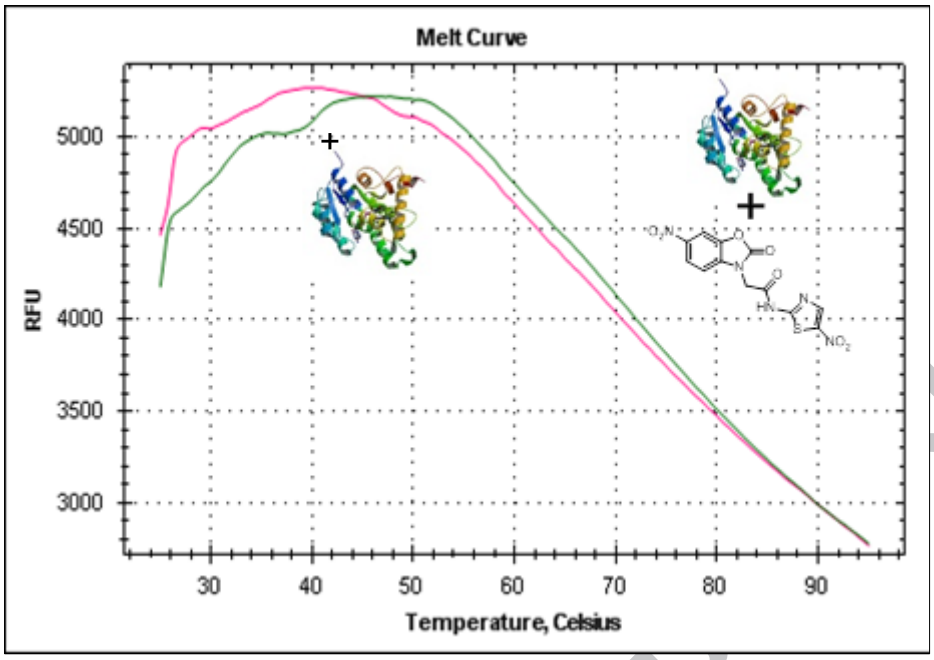
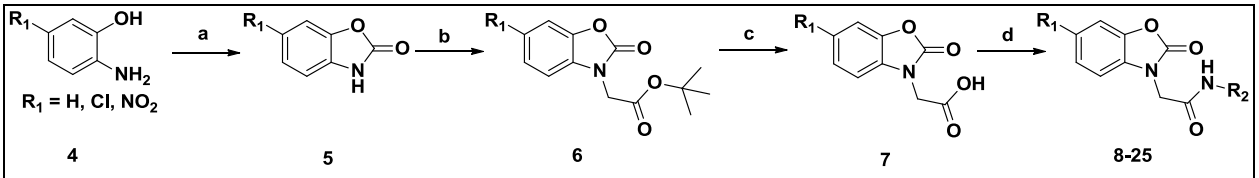
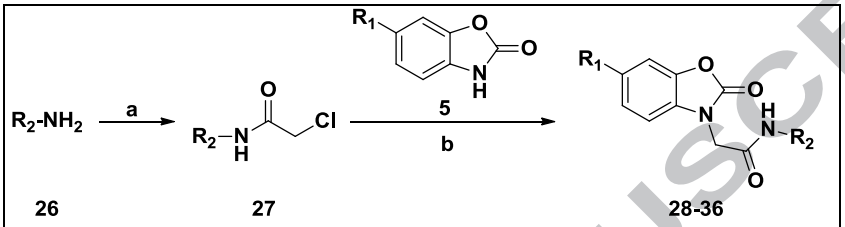


Figure 6: DSF experiment for compound **30** showing an increase in thermal stability between the native InhA protein (pink) and InhA protein−compound **30** complex (green)



Reagents and conditions: a.CDI, THF, reflux; b. t-butyl bromoacetate, NaH, DMF, 0°C; c.TFA, DCM, rt; d. R_2-NH_2,TEA, EDC, HOBT,DCM 0°C-rt.

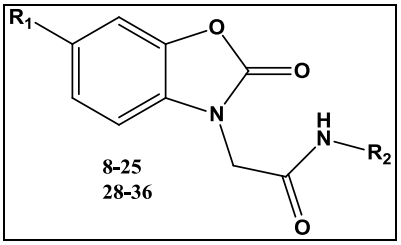
Scheme 1: Synthetic protocol of compounds **8-25**



Reagents and conditions: a.$ClCOCH_2Cl$, TEA, DMF, 0°C - rt; b.K_2CO_3, DMF, 60°C.

Scheme 2: Synthetic protocol of compounds **28-36**

Table 1: Biological results of synthesized compounds



Comp	R_1	R_2	% of InhA inhibition at 10 µM (IC_{50} in µM)[a]	MIC (µM)[b]				Cytotoxicity[c] (% inhib)	
				MTB	MTB (Verapamil)	MTB (Piperine)	XDR-MTB	at 100µM	at 50µM
8	H	Phenyl	32.41±0.23	46.59	23.29	23.29	NT	39.82	35.10
9	Chloro	Phenyl	28.16±0.31	82.58	20.64	41.29	NT	20.23	15.79
10	Nitro	Phenyl	46.12±1.34	79.80	19.95	9.97	NT	26.35	18.04
11	H	2-Chloro-5-(trifluoromethyl)-2-phenyl	26.80±0.95	67.43	33.71	16.86	NT	15.36	12.30
12	Chloro	2-Chloro-5-(trifluoromethyl)-2-phenyl	40.12±1.52	61.70	15.42	15.42	NT	12.08	1.61
13	Nitro	2-Chloro-5-(trifluoromethyl)-2-phenyl	32.16±0.63	60.13	15.03	30.06	NT	31.59	12.80
14	H	2-Pyridyl	26.12±0.55	46.42	4.64	9.28	NT	28.42	20.16
15	Chloro	2-Pyridyl	51.60±0.44	82.31	10.29	10.29	NT	43.68	33.02
16	Nitro	2-Pyridyl	15.63±0.79	39.77	19.88	9.94	NT	47.28	38.61
17	H	2-Pyridylmethyl	42.18±1.63	88.25	22.06	44.12	NT	38.68	37.26
18	Chloro	2-Pyridylmethyl	43.12±1.79	39.34	4.91	4.91	NT	40.03	36.15
19	Nitro	2-Pyridylmethyl	68.12±0.92 (8.23±0.65)	76.15	38.07	9.51	NT	33.35	21.81
20	H	2-Furylmethyl	30.12±0.85	22.95	22.95	22.95	11.47	33.83	32.82
21	Chloro	2-Furylmethyl	47.48±0.66	40.75	20.37	10.18	NT	50.32	27.75
22	Nitro	2-Furylmethyl	36.86±0.54	78.80	39.40	39.40	NT	60.10	26.78
23	H	2-Thiophenylmethyl	26.12±0.84	21.66	10.83	5.41	21.67	49.13	23.39
24	Chloro	2-Thiophenylmethyl	45.62±2.93	38.72	9.64	9.64	NT	53.60	20.31
25	Nitro	2-Thiophenylmethyl	35.71±0.86	18.75	18.75	18.75	18.75	40.95	36.55
28	H	5-Nitrothiazol-2-yl	48.15±1.34	78.05	39.02	19.51	NT	44.62	24.98

Comp	R_1	R_2	% of InhA inhibition at 10 µM (IC_{50} in µM)[a]	MIC (µM)[b]				Cytotoxicity[c] (% inhib)	
				MTB	MTB (Verapamil)	MTB (Piperine)	XDR-MTB	at 100µM	at 50µM
29	Chloro	5-Nitrothiazol-2-yl	72.60±2.73 (6.82±0.53)	17.61	8.81	17.61	17.61	50.64	14.26
30	Nitro	5-Nitrothiazol-2-yl	82.43±0.83 (5.12±0.44)	17.11	8.55	8.55	17.11	43.79	20.24
31	H	2-Benzothiazolyl	42.10±0.63	19.21	9.60	9.60	38.42	59.64	43.54
32	Chloro	2-Benzothiazolyl	58.16±0.72 (8.90±0.38)	17.37	8.68	17.37	34.74	52.52	38.68
33	Nitro	2-Benzothiazolyl	60.80±0.88 (7.62±0.59)	33.75	33.75	33.75	NT	56.07	22.69
34	H	6-Nitro-2-benzothiazolyl	45.12±1.72	33.75	8.43	4.21	NT	19.19	12.41
35	Chloro	6-Nitro-2-benzothiazolyl	48.60±0.82	30.88	7.72	7.72	NT	14.23	12.49
36	Nitro	6-Nitro-2-benzothiazolyl	51.70±0.84	15.04	3.75	3.75	15.04	38.97	14.77
		Isoniazid		0.72	NT	NT	45.57	NT	NT
		Ethambutol		7.64	NT	NT	24.47	NT	NT
		Rifampicin		0.15	NT	NT	30.37	NT	NT
		Ofloxacin		2.16	NT	NT	34.59	NT	NT

IC_{50}, 50% inhibitory concentration; MTB, *Mycobacterium tuberculosis*; MIC, minimum inhibitory concentration; XDR, extensively drug-resistant; NT, not tested

a MTB InhA enzyme inhibition activity
b *In vitro* activity against MTB H37Rv
c Against RAW 264.7 cells

Graphical Abstract

Development of benzo[*d*]oxazol-2(3*H*)-ones derivatives as novel inhibitors of *Mycobacterium tuberculosis* InhA

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