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Research paper

1,3-Oxazine-2-one derived dual-targeted molecules against replicating and non-replicating forms of *Mycobacterium tuberculosis*

Anand Babu Velappan [a], Dhanunjaya Kesamsetty [a], Dhrubajyoti Datta [b, 1], Rui Ma [c], Natarajan Hari [d], Scott G. Franzblau [c], Joy Debnath [a, *]

[a] Department of Chemistry, SCBT, SASTRA Deemed to Be University, Tamilnadu, 613401, India
[b] Department of Chemistry, Indian Institute of Science Education and Research, Pune, Maharashtra, 411008, India
[c] Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St, Chicago, IL, 60612, USA
[d] NMR Laboratory, SCBT, SASTRA Deemed to Be University, Tamilnadu, 613401, India

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ABSTRACT

The high mortality rate and increasing prevalence of resistant *Mtb* are the major concerns for the Tuberculosis (TB) treatment in this century. To curtail the prevalence of resistant *Mtb*, we have prepared 1,3-oxazine-2-one based dual targeted molecules. Compound **67** and **68** were found to be equally active against replicating and non-replicatiing form of *Mtb* (MIC_{MABA} 3.48 and 2.97 μg/ml; MIC_{LORA} 2.94 and 2.15 μg/ml respectively). They had found to suppress the biosynthesis of alfa, methoxy and keto-mycolate completely, as well as inhibit enzymatic activity of MenG (IC_{50} = 9.11 and 6.25 μg/ml respectively for H37Ra; IC_{50} = 11.76 and 10.88 μg/ml respectively for *M smegmatis*).

1. Introduction

Tuberculosis (TB) is one of the major threat for mankind, solely responsible for worldwide death of 1.6 million people in the year of 2017 including HIV positive and negative patients [1]. The new incidence caused by *Mycobacterium tuberculosis* (*Mtb*) is estimated about 10 million in 2017 [1]. The complete destruction of this pathogen is challenging because of the emergence of new resistance strains and their ability to survive in persisted form [2,3]. In an effort to shrink the morbidity and mortality rates different strategies have been adopted to confront the resistance issue; namely, virulence inhibition [4], combination therapy [5] and use of multi-targeted drugs or polypharmacology [6]. Among the others multi-targeted drug discovery programme has drawn significant attention for reducing the propensity of high-level endogenous resistance development [7]. In general, multi-targeted drug molecules work through three mechanisms; series inhibition, parallel inhibition and network inhibition [8].

Among the several other targets of anti-tuberculosis drugs, arguably the most important target is the cell wall biosynthesis for replicating *Mtb*. Isoniazid and ethambutol (1st line antibiotics) exhibit early bactericidal activity against actively metabolizing bacilli by inhibition of mycolic acid biosynthesis and arabinogalactan biosynthesis respectively [9]. However, it is not sufficient to arrest *Mtb* as it is capable to undergo in the dormant form and re-emerges under favorable condition [10]. Thus, electron transport system of *Mtb* is a vivid target for the development of new drugs to combat with non-replicating bacilli. Several drug like molecules like SQ109 [11], Piericidin A [12,13], Stigmatellin [14], Aurachin RE [15], Aurachin E [16], Aurachin C [15] and Polyalthidin [17] have been successfully tested for their inhibitory activity on the electron transport system. Among them SQ109 draw a significant attention, it inhibits the activity of MenA and MenG enzymes which in turn inhibit the biosynthesis of Menaquinone (MQ) [18]. Therefore, it is important to come up with molecules which are capable to eradicate both the replicating and non-replicating form of *Mtb*.

From our previous study, we observed that diaryl urea molecules are capable for selective inhibition of epoxy-mycolate biosynthesis [19]. However, these molecules did not show significant activity against the non-replicating *Mtb*. Therefore, in an effort to develop active molecular scaffold with dual activity, we

* Corresponding author.
E-mail address: joydebnath@scbt.sastra.edu (J. Debnath).
[1] Alnylam Pharmaceuticals, Inc. 675 W Kendall St., Cambridge, MA 02142, USA.

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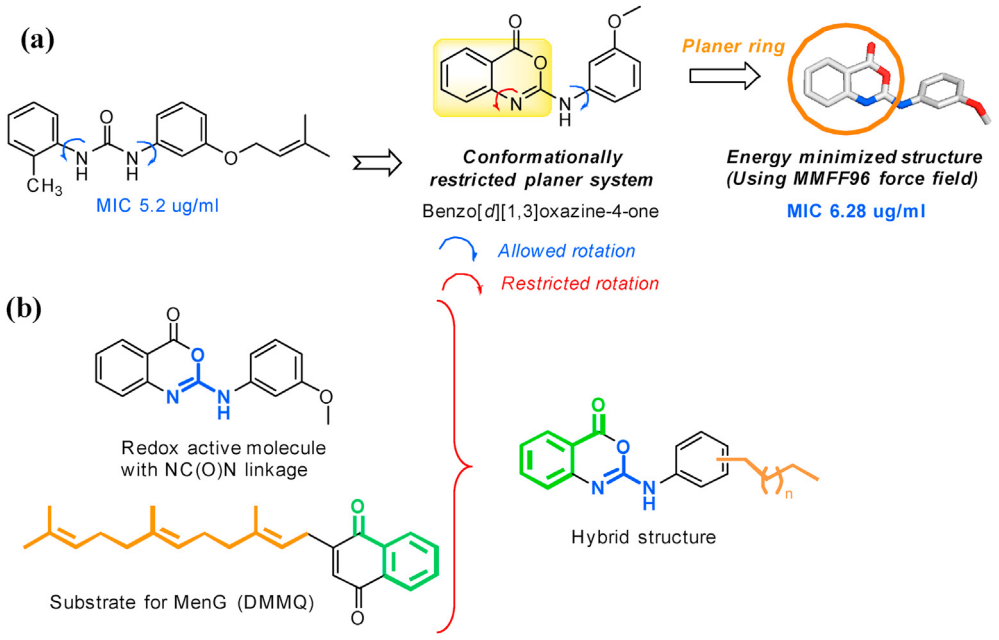
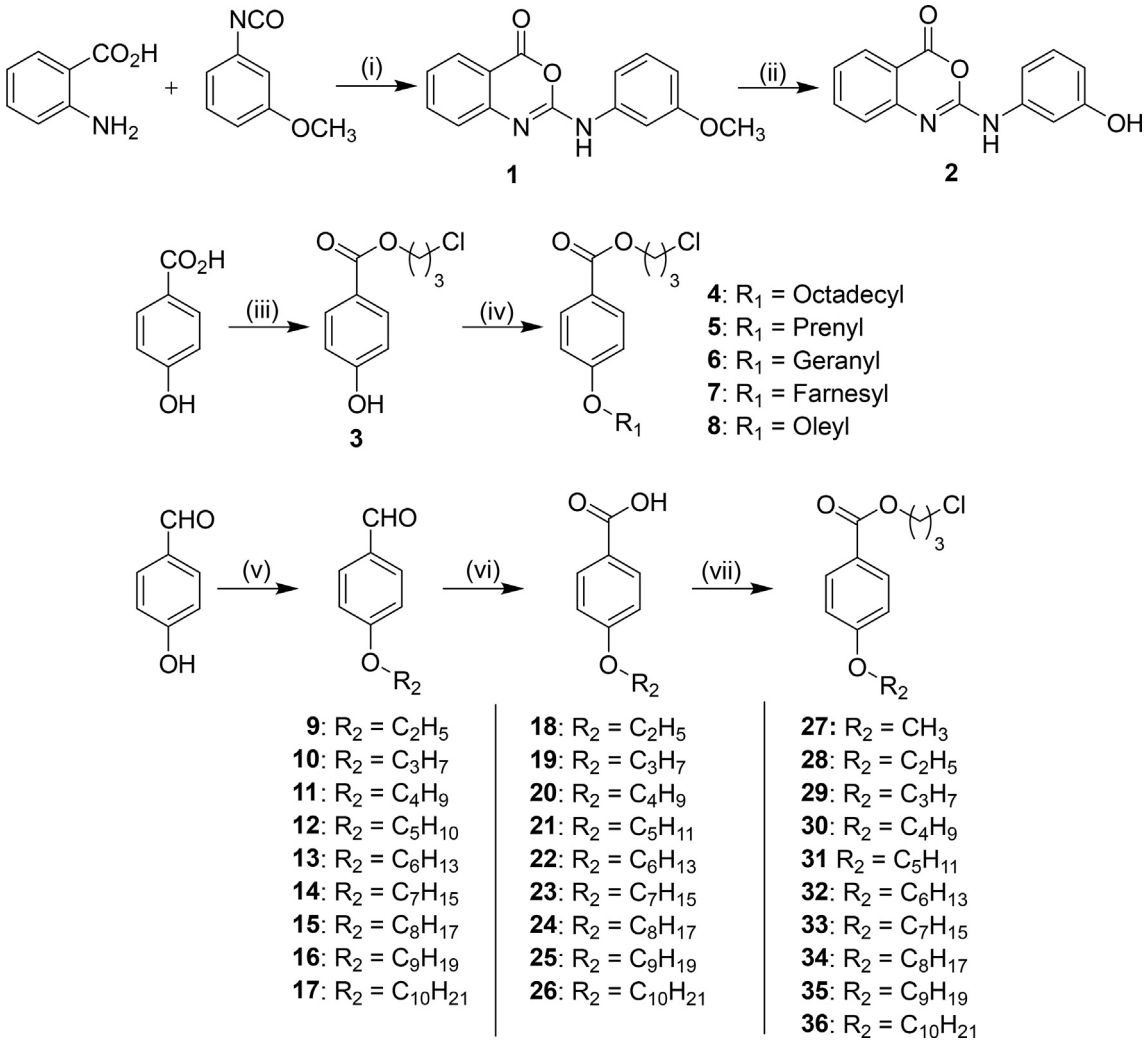
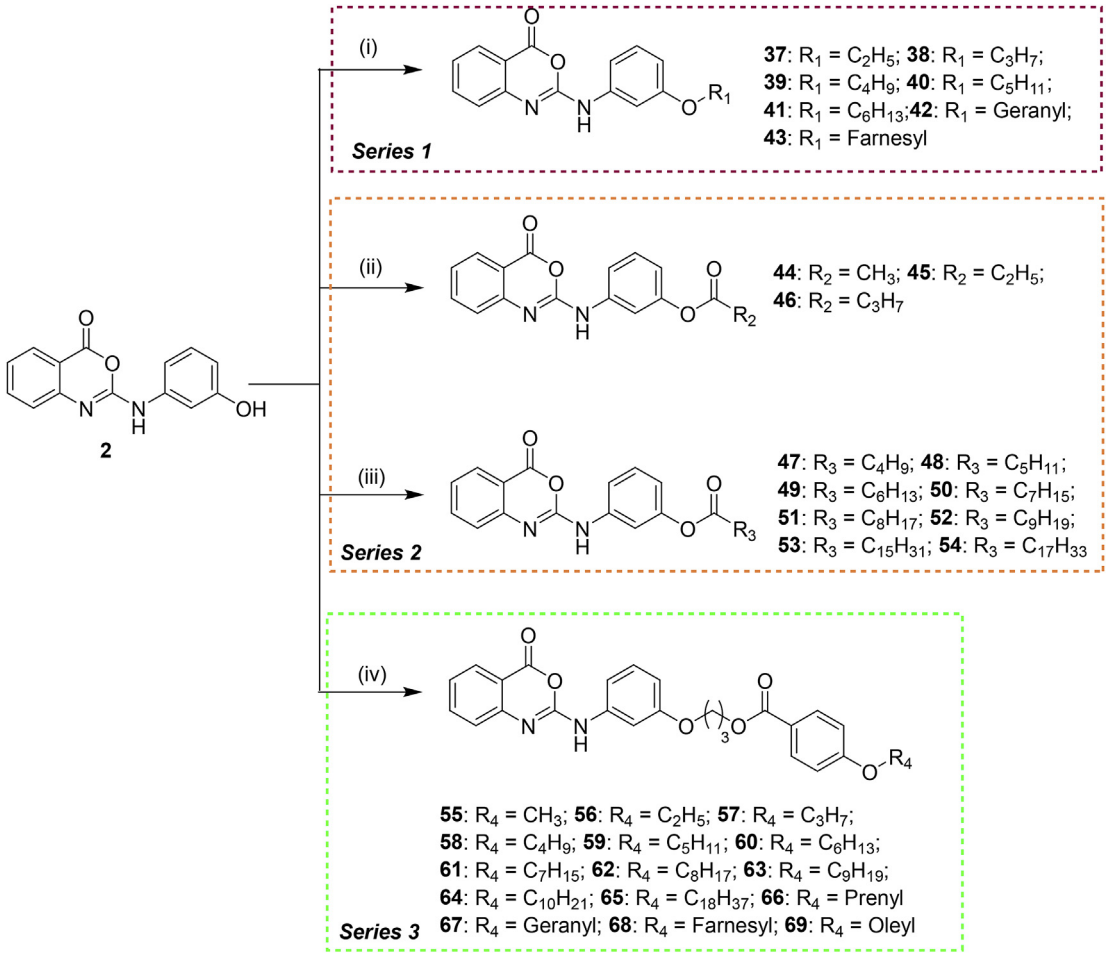


Fig. 1. (a) Energy minimized conformation of Benzo[*d*][1,3]oxazine-4-one. (b) Rationale of the designing.



Scheme 1. Reagents and condition: i) a. TEA, rt, 3 h; b. EDCI.HCl, rt, 3 h; ii) HBr/AcOH, 70 °C, 12 h; iii) 3-Chloro-1-propanol, TPP, DIAD, THF, rt, 6 h; iv) Corresponding alcohols, TPP, DIAD, THF, rt, 6–8 h; v) R_2Cl, K_2CO_3, DMF, 80 °C, 8–12 h; vi) $KMnO_4$, NaH_2PO_4, Acetonitrile/water, rt, 2 h; vii) 3-Chloro-1-propanol, DCC, DMAP, rt, 6 h.



Scheme 2. Reagents and condition: i) R_1X, K_2CO_3, DMF, 70 °C, 8 h; ii) $(R_2CO)_2O$, Pyridine, Chloroform, rt, 5 h; iii) R_3COOH, EDCI.HCl, DMAP, THF, rt, 3 h; iv) **4**–**7** or **18**–**27**, K_2CO_3, DMF, 70 °C, 6 h.

restricted the conformational flexibility of urea linkage through 1,3-benzoxazin-2-one ring formation and chemically attached various aliphatic chain to mimic the structure of demethylmenaquinone (DMMQ) (Fig. 1a). The cyclization of −NC(O)N-linkage (1,3-oxazine-2-one ring system) induced a decent redox activity in the molecule (SI-II), which made it structurally and functionally resemble with DMMQ (Fig. 1b).

Based on these observations we had prepared several molecules with various flanking chains and checked their activity against *Mtb* (H37Rv, H37Ra) and *M. smegmatis* (mc^2155). We also determined their cytotoxic effect on PBMC cells. Among the three different sets of molecules, 10 molecules showed anti-tubercular activity <20 μg/ml. Out of them six best molecules against the replicating form of *Mtb* (comp. **1**, **39**, **40**, **41**, **48**, and **49** with MIC value < 10 μg/ml in MABA assay) were chosen to evaluate their effect on the biosynthesis of mycolic acid and menaquinone (MQ). For this purpose H37Ra (*Mtb*) was used as a model system.

2. Results and discussion

2.1. Synthesis of the target molecules

Benzo[*d*][1,3]oxazine-4-one is a biologically important scaffold and many methodologies have been reported for the synthesis of the same [20–23]. However, in the reported methods one need to go through multistep synthesis or need to use expensive catalyst. In an effort to obtain a convenient method to synthesize the active scaffold, we started with anthranilic acid and 3-methoxyphenyl isocyanate. Initially, we tied to form the urea linkage between anthranilic acid and isocyanate by stirring them together at room temperature as well as under heating. The yield of the reaction was found to be compromised because of the hydrolysis of isocyanate to the corresponding carbamic acid. Thereafter, we tried the reaction in presence of bases like pyridine, triethylamine and DBU at temperature 30–100 °C. The reaction was found slow and on prolonging the reaction time we observed significant amount of carbamic acid formation. So we tried one pot strategy to make the 1, 3-benzoxazin-4-one from anthranilic acid and 3-methoxyphenyl isocyanate in presence of triethylamine and EDCI.HCl. The urea formation and cyclization together were carried out in one pot reaction. However, immediate addition of EDCI.HCl was found to accelerate self coupling reaction of anthranilic acid, therefore, we added EDCI.HCl after 3 h to get a quantitative yield of the required 1,3-benzoxazin-4-one scaffold **1** (Scheme 1). Intermediate **2** was obtained by HBr/AcOH (33%) mediated demethylation of compound **1** at 80 °C [24].

Intermediate **3** was prepared by the esterification of 4-hydroxybenzoic acid with 3-chloro-1-propanol. To begin with we used DCC as the coupling reagent, but we observed formation of more than one product. So we tried Mitsunobu conditions for the esterification [25] and obtained the desired compound in quantitative yield. Thereafter, compounds **4** to **8** were prepared by making the ether linkage with their corresponding alcohols in presence of TPP/DIAD (Scheme 1). The third set of intermediates, *p*-

Table 1
The antitubercular activity and cytotoxicity (against PBMC cell) of the synthesized molecules.

Comp	Minimum Inhibitory Concentration (μg/mL)				Cytotoxicity IC_{50} (μg/mL)
	H37Rv (MABA)*	H37Rv (LORA)**	H37Ra (MABA)	mc^2155	
1	6.28	45.25	7.45	12.50	162.31
2	75.20	40.11	66.21	25.00	173.42
37	28.41	25.76	27.51	50.00	193.74
38	42.19	83.24	51.08	100.00	150.39
39	49.04	>100	68.32	>100	139.14
40	38.13	10.33	31.08	12.50	178.53
41	30.81	7.83	28.61	12.50	129.52
42	>100	5.14	>100	>100	195.27
43	>100	3.76	>100	100	168.35
44	>100	80.34	>100	100	131.43
45	>100	98.49	>100	100	179.81
46	>100	>100	>100	>100	199.42
47	>100	>100	>100	>100	201.39
48	>100	>100	>100	>100	186.43
49	>100	>100	>100	50.0	176.19
50	>100	>100	>100	>100	189.93
51	>100	>100	>100	>100	171.31
52	32.96	53.27	29.58	25.00	194.52
53	>100	>100	>100	100.0	181.63
54	47.38	68.93	51.43	50.00	178.56
55	9.71	40.0	8.16	>100	162.48
56	>100	>100	>100	>100	102.49
57	11.72	15.29	10.00	12.50	145.17
58	7.28	9.41	6.32	25.00	116.83
59	7.01	9.02	8.16	25.00	141.60
60	6.12	5.32	5.83	6.30	122.78
61	>100	>100	>100	>100	180.74
62	12.40	18.93	16.25	12.50	153.90
63	41.82	31.04	37.13	50.00	169.49
64	32.15	45.09	38.04	100.00	173.33
65	56.12	38.18	60.42	12.50	158.49
66	>100	>100	>100	12.50	189.96
67	3.48	2.94	3.93	6.30	134.25
68	2.97	2.15	2.71	3.20	169.29
69	18.72	35.92	19.58	12.50	149.76
INH[a]	0.43	>128	0. 30	6.10	71.68
RIF[b]	0.01	0.04	0.02	1.50	62.92

* Microplate Alamer Blue Assay (for replicating form of *Mtb*); ** Low Oxygen Recovery Assay (for non-replicating form of *Mtb*); [a]INH: Isoniazid; [b]RIF: Refampin.

alkoxybenzaldehydes **9**–**17** were synthesized by the nucleophilic substitution reaction of *p*-hydroxybenzaldehyde with the corresponding alkyl bromides in presence of K_2CO_3 in DMF at 70 °C (Scheme 1). Thereafter, **27**–**36** were prepared in two steps, oxidation of the aldehyde to corresponding carboxylic acid (**18**–**26**) in presence of $KMnO_4$ followed by esterification of the acid with 3-chloro-1-prpanol using Steglich esterification condition with DCC and catalytic amount of DMAP (Scheme 1).

The first set of hybrid molecules; the ether series (**37**–**43**) were prepared using the phenolic group of the intermediate **2**. In case of nucleophilic substitution reaction, there was a problem with the selectivity as both the −NH and the −OH groups are susceptible towards the alkylation reaction. Only at 60 °C the reaction was found to be favored towards O-alkylation than N-alkylation. We also tried Mitsunobu condition, but under this condition the yield was very low ~10–19% despite the use of different azodicarboxylates DIAD, DBAD, DNAD [26,27]. The part of second set of target molecules **44**–**46** were synthesized by treating the corresponding anhydrides with the phenolic intermediate **2** [28]. The reactions were carried out in chloroform and pyridine at room temperature (Scheme 2). Other molecules of this series (**47**–**54**) were prepared by esterification reaction in presence of EDCI.HCl and DMAP in THF (Scheme 2) [29]. The third set of target molecules **55**–**69** are the esters, where **2** was connected with the aliphatic chains via a linker. These molecules were prepared by nucleophilic substitution reaction between **2** and the intermediates **4**–**8** and **27**–**36** in presence of K_2CO_3 in DMF under heating (Scheme 2).

2.2. Evaluation of anti mycobacterial activity and cytotoxicity

The *in-vitro* anti-tubercular activity of the synthesized molecule was determined by its Minimum Inhibitory Concentration (MIC) [30]. For all the three series of 1, 3-benzoxazin-4-one derivatives, MIC was evaluated against *Mtb* (H37Rv and H37Ra strains) and *M. smegmatis* (mc^2155 strain). We determined the activity against the replicating *Mtb* by MABA (Microplate Alamar Blue Assay) [31] method, whereas, the activity on the non-replicating *Mtb* was determined by LORA (Low Oxygen Recovery Assay) method (Table 1) [32].

Cytotoxicity is an unavoidable criterion to access the utility of a molecule as a potential drug. The PBMC (Peripheral Blood Mononuclear Cells) cells majorly composed of lymphocytes and monocytes was used to determine the cytotoxicity. The cytotoxicity of these 1, 3-benzoxazin-4-one derivatives was evaluated by MTT based cell viability assay. The IC_{50} values were calculated from a dose-response curve plotted against percentage of viability versus $\log_{10}$ concentration (Table 1) [33].

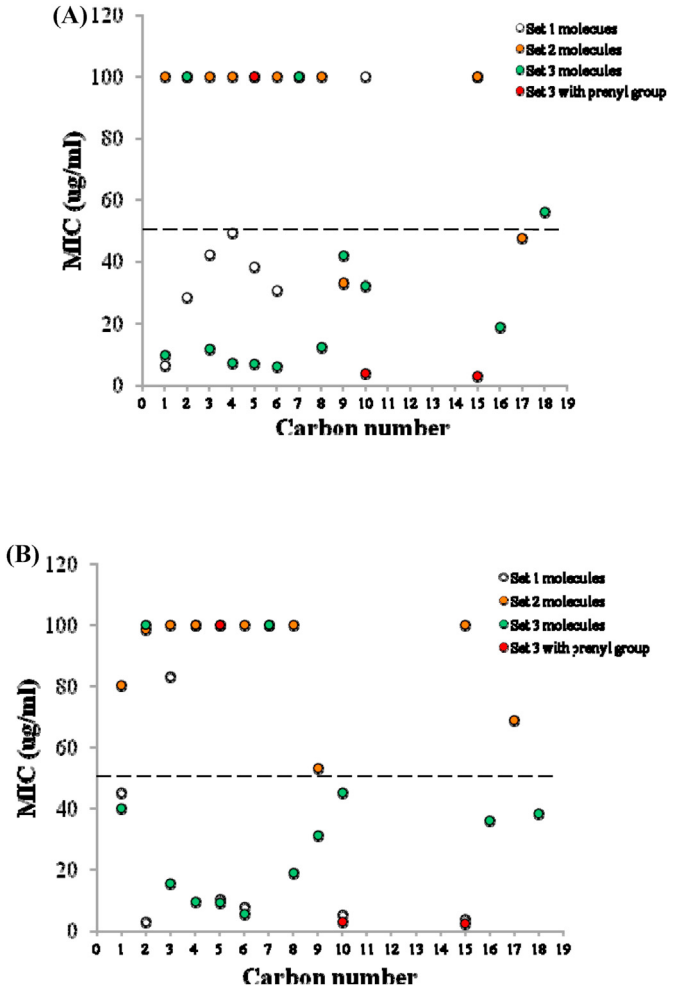


Fig. 2. Relation between the activity and the carbon number in the aliphatic chain. (A) Activity against H37Rv replicating form and (B) Activity against H37Rv non-replicating form.

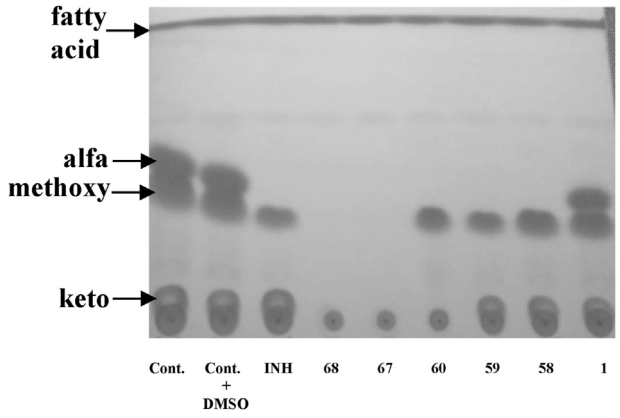


Fig. 3. Production of four different mycolic acids (for H37Ra) in presence of the synthesized molecules **68**, **67**, **60**, **59**, **58** and **1** (in DMSO). The concentration of the molecules was used 5 times of their corresponding MIC values against H37Ra.

For *series 1* molecules the alkyl chain was extended up to 6-carbon atoms (**37**–**41**) and for other two molecules (**42** and **43**) geranyl and farnesyl were attached through ether linkage. The MIC values were found in the range of 28.41 to >100 μg/ml (for replicating H37Rv), 27.51to >100 μg/ml (for replicating H37Ra) and 12.50 to >100 μg/ml (for replicating mc^{2}155). Interestingly, we observed improved activity against non-replicating H37Rv for compounds **40**, **41**, **42** and **43** (10.33, 7.83, 5.14 and 3.76 μg/ml respectively). Compound **42** and **43** were found to be active only against non-replicating form of *Mtb*, on the contrary **40** and **41** showed moderate activity against replicating *Mtb* but comparatively better activity was found against non-replicating *Mtb*. This observation indicated that the chain length plays a significant role for shifting the activity from replicating to non-replicating *Mtb*.

In *series 2*, we increased the chain length up to 9-carbon atoms (**44**–**52**) besides another two molecules with 15-carbon (**53**) and 17-carbon (**54**) chain length. Except compounds **52** and **54** all the ester derivatives were found almost inefficient against the both form of *Mtb*. We presume that the replacement of the ether linkage with ester was responsible for diminishing their activity against replicating as well as non-replicating *Mtb*.

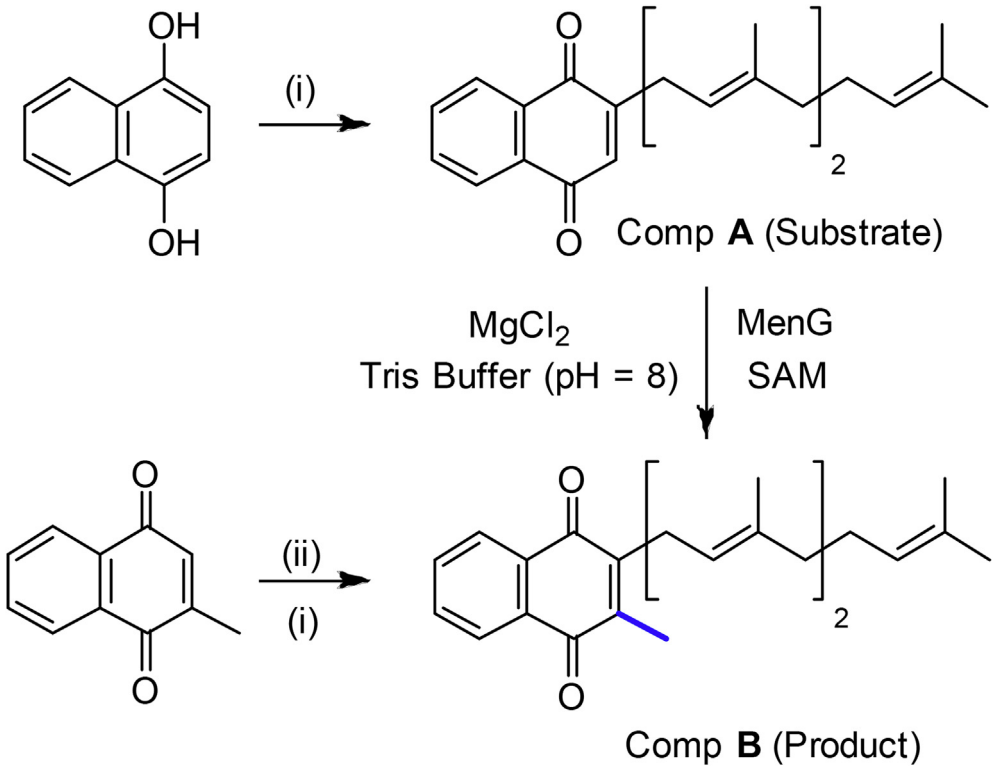
Series 3 molecules (**55**–**69**), where we connected the benzoxazinone core to 4-alkoxybenzoic acids through a 1, 3-dioxopropyl linkage showed much better activity compared to *series 1* and *series 2* molecules. This set of molecules showed improved activity against H37Rv in both MABA and LORA method. Improved efficacy against mc^{2}155 strain was also observed for these molecules. Compounds **67** and **68** showed the best activity profile against both replicating and non-replicating *Mtb* (H37Rv and H37Ra) and *M. smegmatis* (mc^{2}155). Altogether, we obtained 7 molecules from this series where the MIC values were <20 μg/ml for all the different mycobacterium species.

2.3. Structure-activity relationship

The anti-tubercular activity of the synthesized molecules was found to be dependent on the carbon numbers in the aliphatic chain and also on the type of linkage with benzo[*d*][1,3]oxazine-4-one scaffold.

For the molecules belonging to *series 1*, the activity against replicating *Mtb* was found to increase with concomitant increase of carbon number till 4-carbon atoms. Thereafter, further increment to 5-carbon and 6-carbon improved the activity to moderate level, but the activity completely diminished for 10-carbon and 15-carbon (MIC > 100 μg/ml) (Fig. 2A). On the other hand, none of the molecules except **52** and **54** showed MIC <50 μg/ml with 9-carbon and 17-carbon atoms respectively. This poor activity is presumably because of the alteration of the polarity caused by ester linkage. Compared to *series 1* molecules, *series 3* molecules showed better activity against the replicating *Mtb*. In this set of molecules the activity was found <50 μg/ml for all the chain length (1-carbon to 18-carbon atoms) except 2-carbon (**56**), 7-carbon (**61**) and 18-carbon (**65**). The best activity was found for compound **68** with farnesyl group (15-carbon) (Fig. 2A). For H37Ra and mc^{2}155 strains we also observed similar type of correlation between the activity and carbon number in the aliphatic chain.

The activity profile of the compounds of *series 1* showed a definite trend against non-replicating *Mtb*. Compounds **40**, **41**, **42** and **43** with increasing number of carbon atom showed gradual improvement in their activity and reached to the limiting activity for 10-carbon (**42**) and 15-carbon (**43**) (Fig. 2B). However, for *series 2* molecules we did not observed any activity (<50 μg/ml) against the non-replicating *Mtb*, irrespective of the various chain lengths. On the contrary, most of the molecules of *series 3* showed activity at a concentration <20 μg/ml. The activity improved significantly by increasing the chain length from 1-carbon to 6-carbon atoms (except **56**), then it again diminished with increase of carbon number 8-carbon to 10-carbon. Finally, it reached to a static level for 16-carbon (**69**) and 18-carbon (**65**) chains. However, it is



Scheme 3. Reagents and conditions: (i) $BF_3 \cdot Et_2O$, EtOAc/Dioxane (1:1), 70 °C, 4 h; (ii) 10% aqueous $Na_2S_2O_4$, rt, 3 h. Conversion of A (substrate) to B (product) was achieved in presence of the membrane fraction of H37Ra and SAM as a methyl transferring agent.

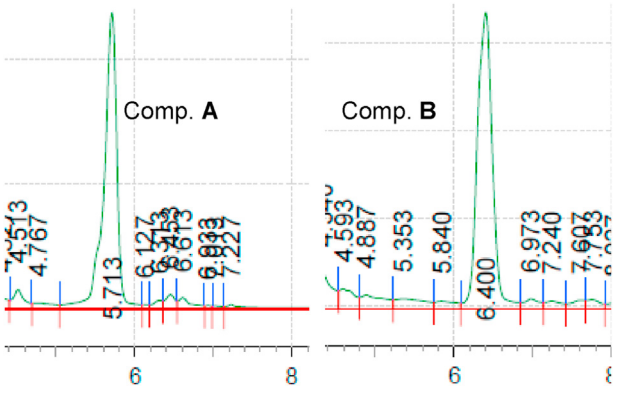


Fig. 4. Retention time for compounds A and B measured at 250 nm with a flow rate of 1 ml/min in the solvent system of ACN/H_2O (90:10).

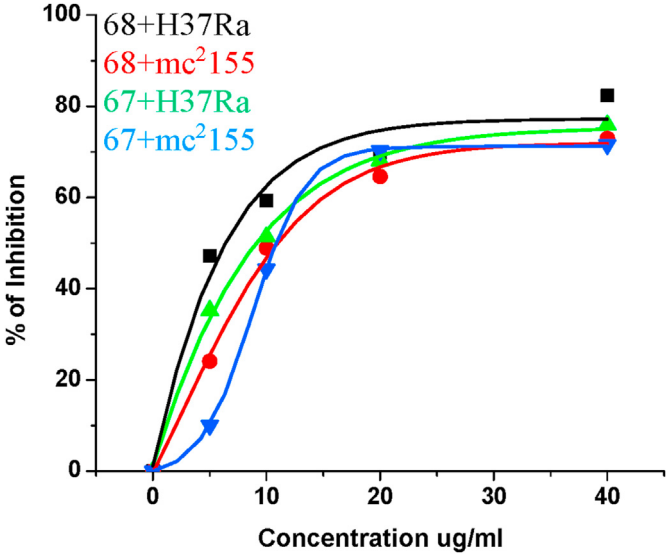


Fig. 5. The IC_{50} curve for compound 49 and 48 with H37Ra and mc^2155. Substrate A (500 μM) was mixed with 500 μM SAM and 100 μl membrane fraction (H37Ra and M smegmatis separately) and incubated for 3 h at 37 °C.

pertinent to note that for 10-carbon (**67**, geranyl) and 15-carbon (**68**, farnesyl) they showed the optimum activity (Fig. 2B).

From the structure activity relationship, we can conclude that the length of aliphatic side chain along with its position from the main scaffold play a crucial role to offer the activity against replicating and non-replication *Mtb*. Comparing the activity of **1** with **42**, **43**, **67** and **68** it is clear that geranyl and farnesyl chains plays a definite role to kill the *Mtb* in its latent state.

2.4. Effect of the hybrid molecules on mycolic acid biosynthesis

We had selected six best molecules **1**, **55**, **58**, **59**, **60**, **67** and **68** from all the three series which showed MIC <10 μg/ml (MABA) against replicating *Mtb*. They were subjected for the evaluation of their effect on the mycolic acid biosynthesis. For this purpose H37Ra (*Mtb*) was used as the model system. In this assay we had used a chromophoric benzyl moiety instead of ^{14}C-radio labled acetate (Fig. 1) [34]. The individual mycolic acids were characterised by MALDI-TOF in presence of a reference molecule $C_{83}H_{154}O_3$ (Mass = 1200.2; Benzyl ester of mycolic acid $C_{76}H_{148}O_3$) (shown in SI–I) [35]. Molecules belong to *series 3* (**67** and **68**) completely inhibit the production of alfa, methoxy and keto-mycolates. Whereas, compound **60** abolished the production of alfa and keto-mycolates

Table 2
Effect of the synthesized molecules on the MenG inhibition.

Compound	Men G Inhibition; IC_{50} (μg/ml)	
	H37Ra	mc^2155
1	-	30.59
41	26.47	22.03
58	20.06	29.71
59	20.88	27.19
60	13.53	16.33
67	9.11	11.76
68	6.25	10.88

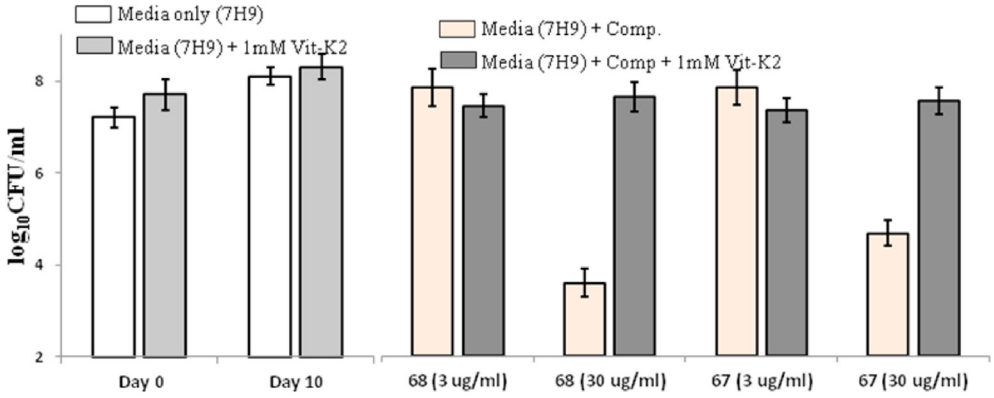


Fig. 6. Effect of the comp. **68** and **69** against non-replicating H37Ra: Growth of the non-replicating H37Ra in absence and in presence of Vit-K2 (1 mM) at 0th and 10th day (Left panel); Growth of the non-replicating H37Ra in absence and in presence of Vit-K2 (1 mM) and comp. **67** and **68** at 3 and 30 μg/ml on 10th day followed by 27 days after CFU counting on 7H10 agar plate (Right panel).

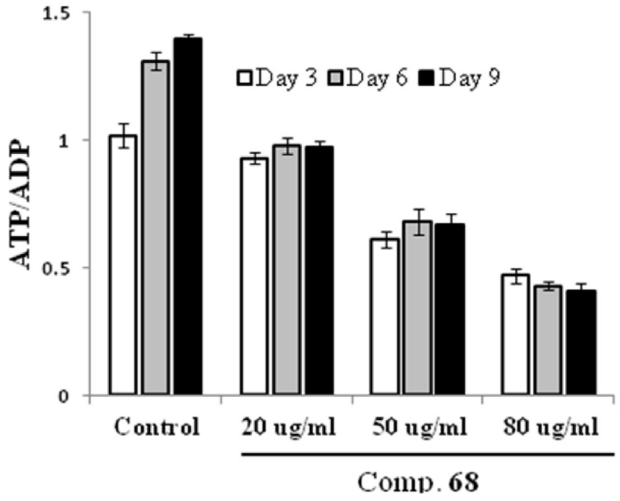


Fig. 7. Effect of Comp. **68** on the ATP synthesis against non-replicating H37Ra on 3rd, 6th and 9th day at 20, 50 and 80 μg/ml.

along with 61% reduction of methoxy-mycolates (calculated using ImageJ software) compared with the control set. For compound **58** and **59**, we observed complete inhibition of alfa-mycolate biosynthesis and partial inhibition of methoxy (48% for **59**; 36% for **58**) and keto-mycolates (37% for **59**; 31% for **58**). However, for the base molecule **1**, unlike the others we observed partial inhibition of alfa

Table 3
The MIC values of the compounds **67** and **68** against H37Rv drug resistance strains.

Entry	Strains	MIC (μg/ml)			
		67	68	INH[a]	RIF[b]
1	H37Rv (ATCC 27294)	3.5	2.9	0.4	0.01
2	H37Rv−INH−R (ATCC 35822)	4.4	2.3	>8	0.03
3	H37Rv-RIF-R (ATCC 35838)	2.6	1.8	0.25	>2

[a] INH: Isoniazid.
[b] RIF: Refampin, The MIC value were calculated by agar plate dilution method.

(51%), methoxy (29%) and keto-mycolates (68%) (Fig. 3). Comparing the effect of these molecules on mycolic acid biosynthesis it is pertinent to note that the presence of geranyl and fernasyl group enhances their inhibitory effect on mycolic acid biosynthesis. However, these molecules showed insignificant or no effect on the fatty acid biosyjthesis similar to our previous study with unsymmetrical diaryl derivatives [19]. It may be attributed because of their selective interaction with FAS-II enzymes rather than FAS-I [36].

2.5. Effect of the hydride molecules on MenG

To understand the plausible reason of their activity against non-replication *Mtb*, we tested active molecules against the respiratory system of dormant *Mtb*. Hence, our synthesized molecules have structural resemblance with MenG substrate, we performed the MenG inhibition study with compounds **41**, **58**, **59**, **60**, **67** and **68**

(LORA MIC < 10 μg/ml; MIC_{MABA} < 10 μg/ml). The MenG inhibition values (IC_{50}) were determined by HPLC based assay, which is quite similar to the ManA inhibition assay where we used DMMQ and SAM (methyl transferring agent) [10]. MenG is the final enzyme in the menaquinone biosynthesis pathway, which converts the DMMQ to MQ in presence of SAM. The MQ accepts two electrons from NADH and get converted to its reduced form, finally it again reversibly converted to MQ by transferring the electrons to the other electron acceptors present in the electron transport system. Therefore, inhibition of this enzyme is expected to perturb the respiratory system of *Mtb* through which it harnesses its required energy in the latent state [37].

The substrate and the product were prepared separately (Scheme 3) [38–40] for calibration and identification purpose. The retention times for comp. **A** and comp. **B** were 5.713 min and 6.400 min respectively, which was found good enough to determine the IC_{50} value using HPLC system (Fig. 4).

The membrane fraction (according to the reported protocol) [18] from H37Ra and mc^2155 was used as a source of membrane bound MenG. However, no significant conversion of **A** to **B** was observed under *in vitro* condition and it was apparently because of the use of **A** in oxidized form. Therefore, to overcome this issue we had used a reducing agent DTT to convert **A** in the reduced form and it makes **A** chemically feasible to undergo the methylation (*i. e.*, comp. **B**). Representative IC_{50} profile for comp. **67** and **68** is shown in Fig. 5. The IC_{50} calculation was based on the conversion of **A** with respect to the control set (without inhibitor molecule) and given in Table 2.

In this assay the conversion of the model substrate (comp. **A**) by MenG to the corresponding product (comp. **B**) was monitored at 250 nm (by HPLC) in presence of 4 different concentrations of the synthesized molecules (**41**, **58**, **59**, **60**, **67** and **68**) and the corresponding IC_{50} values were calculated by non-linear regression analysis within the error limit of ±0.5% (Table 2).

To probe the significance of the MQ biosynthesis inhibition in the dormant form, we had prepared the dormant H37Ra following the reported protocol [41] and performed the Vit-K2 rescue study. In this experiment we observed the growth of the H37Ra (dormant form) did not significantly affected in presence of Vit-K2 (1 mM) till 10 days of exposure (Fig. 6; left panel). However, in presence of compound **67** and **68** (30 μg/ml) the growth was diminished by 2.9 and 4.1 fold respectively (logCFU/ml) compared to the set with 1 mM Vit-K2 (Fig. 6; right panel). This provided strong evidence that these molecules inhibit the biosynthesis of MenG and in turn reduced their growth in dormant state by blocking the respiratory chain of H37Ra.

Next, we determined the ATP production in presence of comp. **68** in the dormant state for H27Ra. The ATP/ADP ratio was varied in the range of 1.02–1.40 for the control set from day 3 to day 9 (Fig. 7). Whereas, for the co-culture of the dormant H37Ra with comp. **68** (20, 50 and 80 μg/ml), we observed continuous reduction in their ATP production level. It confirms that the compound **68** eradicates *Mtb* in the dormant state by inhibiting the MenG biosynthesis and as a whole by reducing its ATP production.

2.6. Effect on resistant strains of Mtb

Finally, we tested the best molecules **67** and **68** (MIC < 4 μg/ml) against the isoniazide and refampin resistant H37Rv strains (ATCC-35822 and ATCC-35838 respectively). Both of these compounds showed slightly better MIC values against the resistance strains as compared to the non-resistance strain (Table 3).

3. Conclusion

In this study, we have designed and synthesized 33 hybrid molecules to target the cell wall biosynthesis and the respiratory system of *Mtb* simultaneously. Out of them compound **67** and **68** showed the best activity where the 1,3-oxazine-2-one and the aliphatic chains are connected through a linker. The relatively weaker activity of compound **1** against the non-replicating *Mtb* proved the necessity of prenyl chains for the recognition by the MenG. In Vit-K2 rescue study compound **67** and **68** with geranyl and farnesyl group respectively, clearly showed their inhibitory effect on the electron transport system which get revived in presence of Vit-K2. Similarly, the ATP/ADP ratio get diminished in presence of these compounds, which plausibly confirms that these molecules kill the *Mtb* in its non-replication form by affecting its electron transport system. On the other hand 1,3-oxazine-2-one scaffold was found to be responsible for the inhibition of mycolic acid biosynthesis. The dual-targeted best molecules **67** and **68** are also active against the INH and RIF resistant strains. This work shows that by customizing the designing of hybrid molecules we can perturb more than one target of *Mtb*, in an effort to counteract the emergence of resistant strain.

4. Experimental

4.1. Reagents and instrumentation

All the reagents were purchased from sigma-Aldrich, Alfa-Aesar and Merck chemicals. Solvents were dried according to the standard protocols. TLC was performed on Merck silica gel 60, f_{254} precoated aluminium plates and spots were visualized under UV lamp or stained using 10% PMA in Ethanol, 5% Sulfuric acid in methanol or Iodine. Column chromatographic separations were performed using silica gel (100–200 mesh). NMR spectra were run on Bruker AVANCE II instrument using TMS as internal standard for ^1H (300 and 400 MHz) and ^{13}C (75 and 100 MHz) experiments. Chemical shifts were given in ppm (δ scale), UV–vis measurements were made using a PerkinElmer UV–vis spectrophotometer (Model Lambda 25). Mass spectra had been recorded using Waters Mass Spectrometer (model XevoG2QTof). HPLC experiments were performed using an Agilent 1200 infinity series with silica C_{18} column (4.6 × 250mm and 5 μm). ImageJ app was used for calculating the TLC intensity profile from the image.

4.1.1. Synthesis of 2-((3-ethoxyphenyl)amino)-4H-benzo[d][1,3] oxazin-4-one (**37**)

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.163 g, 1.18 mmol) was added under stirring condition. Then bromoethane (0.035 mL, 0.47 mmol) was added and the reaction mass was heated at 70 °C for 8 h. After completion of reaction (checked by TLC), the reaction mass was added into water and extracted with ethyl acetate (3 × 30 mL). Then the combined organic layer was washed with water followed by brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.063 g, 57.3%, sticky white solid, ^1H NMR (400 MHz, DMSO-d_6): δ 1.25 (t, J = 7.2 Hz, 3H), 4.16 (q, J = 7.2 Hz, 2H), 6.71 (d, J = 8.8 Hz, 2ArH), 6.82–6.85 (m, 1ArH), 7.21–7.33 (m, 2ArH), 7.55 (d, J = 8.4 Hz, 1ArH), 7.81 (t, J = 7.2 Hz, 1ArH), 8.07 (dd, J = 1.6, 8.0 Hz, 1ArH), 9.65 (s, 1 N–H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 12.9, 38.8, 114.9, 115.6, 116.1, 116.6, 119.8, 123.1, 128.7, 129.9, 136.0, 137.5, 140.3, 150.6, 158.2, 161.7. HRMS (ESI^+): m/z calculated for $C_{16}H_{15}N_2O_3$ $[M+H]^+$: 283.1083; found: 283.1088.

4.1.2. Synthesis of 2-((3-propoxyphenyl)amino)-4H-benzo[d][1,3] oxazin-4-one (**38**)

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.163 g, 1.18 mmol) was

added under stirring condition. Then 1-bromopropane (0.043 mL, 0.47 mmol) was added and the reaction mass was heated at 70 °C for 8 h. After completion of reaction (checked by TLC), the reaction mass was added into water and extracted with ethyl acetate (3 × 30 mL). Then the combined organic layer was washed with water followed by brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.061 g, 52.6%, white solid, m.p: 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.96 (t, J = 7.6 Hz, 3H), 1.64–1.77 (m, 2H), 4.06 (t, J = 7.6 Hz, 1H), 6.70 (d, J = 8.8 Hz, 2ArH), 6.82–6.85 (m, 1ArH), 7.21–7.33 (m, 2ArH), 7.55 (d, J = 8.4 Hz, 1ArH), 7.82 (t, J = 7.6 Hz, 1ArH), 8.07 (dd, J = 1.6, 7.6 Hz, 1ArH), 9.64 (s, 1N–H). ^{13}C NMR (100 MHz, DMSO-d_6): δ11.4, 20.7, 45.0, 115.1, 115.6, 116.0, 116.5, 119.8, 123.1, 129.9, 136.0, 140.5, 150.6, 158.2, 161.7. HRMS (ESI$^+$): m/z calculated for $C_{17}H_{17}N_2O_3$ [M+H]$^+$: 297.1239; found: 297.1240.

4.1.3. Synthesis of 2-((3-butoxyphenyl)amino)-4H-benzo[d][1,3] oxazin-4-one (**39**)

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.163 g, 1.18 mmol) was added under stirring condition. Then 1-Bromobutane (0.051 mL, 0.47 mmol) was added and the reaction mass was heated at 70 °C for 8 h. After completion of reaction (checked by TLC), the reaction mass was added into water and extracted with ethyl acetate (3 × 30 mL). The combined organic layer was washed with water followed by brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.065 g, 53.7%, white solid, m.p: 166–168 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.93 (t, J = 7.2 Hz, 3H), 1.39–1.47 (m, 2H), 1.61–1.66 (m, 2H), 4.10 (t, J = 7.6 Hz, 2H), 6.72 (d, J = 8.4 Hz, 2ArH), 6.82–6.84 (m, 1ArH), 7.21–7.33 (m, 2ArH), 7.53 (d, J = 8.4 Hz, 1ArH), 7.82 (t, J = 7.6 Hz, 1ArH), 8.07 (dd, J = 1.6, 7.6 Hz, 1ArH), 9.63 (s, 1 N–H). ^{13}C NMR (100 MHz, DMSO-d_6):δ14.2, 29.5, 31.2, 43.3, 115.1, 115.6, 116.1, 116.5, 119.8, 123.1, 128.7, 129.9, 136.0, 137.6, 140.4, 150.6, 158.2, 161.6. HRMS (ESI$^+$): m/z calculated for $C_{18}H_{19}N_2O_3$ [M+H]$^+$: 311.1395; found: 311.1396.

4.1.4. Synthesis of 2-((3-(pentyloxy)phenyl)amino)-4H-benzo[d] [1,3]oxazin-4-one (**40**)

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.163 g, 1.18 mmol) was added under stirring condition. Then 1-bromopentane (0.056 mL, 0.47 mmol) was added and the reaction mass was heated at 70 °C for 8 h. After completion of reaction (checked by TLC), the reaction mass was added into water and extracted with ethyl acetate (3 × 30 mL). Then the combined organic layer was washed with water followed by brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.069 g, 54.3%, waxy solid, ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.88 (t, J = 6.8 Hz, 3H), 1.32–1.38 (m, 4H), 1.66 (quintet, J = 7.2 Hz, 2H), 4.09 (t, J = 8.0 Hz, 2H), 6.71 (d, J = 8.4 Hz, 2ArH), 6.82–6.84 (m, 1ArH) 7.21–7.35 (m, 2ArH), 7.53 (d, J = 8.8 Hz, 1ArH), 7.79–7.84 (m, 1ArH) 8.07 (dd, J = 1.6, 7.6 Hz, 1ArH), 9.62 (s, 1 N–H). ^{13}C NMR (100 MHz, DMSO-d_6): δ14.3, 22.4, 28.2, 28.8, 43.5, 115.1, 115.6, 116.0, 116.5, 119.8, 123.1, 128.7, 129.9, 136.0, 137.6, 140.4, 150.6, 158.2, 161.6. HRMS (ESI$^+$): m/z calculated for $C_{19}H_{21}N_2O_3$ [M+H]$^+$: 325.1552; found: 325.1555.

4.1.5. Synthesis of 2-((3-(hexyloxy)phenyl)amino)-4H-benzo[d][1,3] oxazin-4-one (**41**)

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.163 g, 1.18 mmol) was added under stirring condition. Then 1-Bromohexane (0.066 mL, 0.47 mmol) was added and the reaction mass was heated at 70 °C for 8 h. After completion of reaction (checked by TLC), the reaction mass was added into water and extracted with ethyl acetate (3 × 30 mL). Then the combined organic layer was washed with water followed by brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.076 g, 57.6%, white solid, m.p: 140–142 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.88 (t, J = 6.4 Hz, 3H), 1.18–1.39 (m, 6H), 1.62–1.67 (m, 2H), 4.09 (t, J = 7.2 Hz, 2H), 6.71 (d, J = 8.4 Hz, 2ArH), 6.82–6.84 (m, 1ArH) 7.23–7.30 (m, 1ArH), 7.53 (d, J = 8.4 Hz, 1ArH), 7.82 (t, J = 7.6 Hz, 1ArH), 8.07 (d, J = 7.6 Hz, 1ArH), 9.63 (s, 1 N–H). ^{13}C NMR (100 MHz, DMSO-d_6): δ14.4, 22.5, 27.2, 29.1, 31.4, 43.5, 115.1, 115.6, 116.0, 116.5, 119.8, 123.1, 128.7, 129.9, 136.0, 137.6, 140.4, 150.6, 158.3, 161.6. HRMS (ESI$^+$): m/z calculated for $C_{20}H_{23}N_2O_3$ [M+H]$^+$: 339.1708; found: 339.1711.

4.1.6. Synthesis of (E)-2-((3-((3,7-dimethylocta-2,6-dien-1-yl)oxy) phenyl)amino)-4H-benzo[d][1,3]oxazin-4-one (**42**)

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.163 g, 1.18 mmol) was added under stirring condition. Then Geranylchloride (0.087 mL, 0.47 mmol) was added and the reaction mass was heated at 70 °C for 8 h. After completion of reaction (checked by TLC), the reaction mass was added into water and extracted with ethyl acetate (3 × 30 mL). Then the combined organic layer was washed with water followed by brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.098 g, 64.4%, white solid, m.p: 178–180 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.63–1.64 (m, 6H), 1.84 (s, 3H), 2.01–2.09 (m, 4H), 4.81 (d, J = 6.0 Hz, 2H), 5.02 (t, J = 6.6 Hz, 1H), 5.22 (t, J = 6.0 Hz, 1H), 6.49 (bs, 1 N–H), 6.63 (t, J = 2.1 Hz, 1ArH), 6.79 (td, J = 1.8, 7.8 Hz, 2 ArH), 7.21–7.29 (m, 1ArH), 7.30–7.35 (m, 2ArH), 7.71 (td, J = 1.5, 7.5 Hz, 1ArH), 8.27 (dd, J = 1.5, 7.8 Hz, 1ArH) ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.9, 17.9, 25.9, 26.4, 39.7, 42.7, 114.6, 116.0, 116.2, 116.7, 118.3, 119.9, 123.4, 123.8, 129.7, 130.5, 132.1, 135.7, 136.2, 140.5, 140.8, 151.1, 157.7, 162.4. HRMS (ESI$^+$): m/z calculated for $C_{24}H_{27}N_2O_3$ [M+H]$^+$: 391.2022; found: 391.2026.

4.1.7. Synthesis of 2-((3-(((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)oxy)phenyl)amino)-4H-benzo[d][1,3]oxazin-4-one (**43**)

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.163 g, 1.18 mmol) was added under stirring condition. Then farnesyl chloride (0.123 g, 0.47 mmol) was added and the reaction mass was heated at 70 °C for 8 h. After completion of reaction (checked by TLC), the reaction mass was added into water and extracted with ethyl acetate (3 × 30 mL). Then the combined organic layer was washed with water followed by brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.121 g, 68.0%, white solid, m.p: 76–78 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.57–1.58 (m, 6H), 1.63–1.67 (m, 3H), 1.85 (s, 3H), 2.00–2.10 (m, 8H), 4.81 (d, J = 6.0 Hz, 2H), 5.05 (t, J = 1.2 Hz, 2H), 5.18–5.23 (m, 1H) 6.61–6.62 (m, 2ArH), 6.75–6.80 (m, 2H), 7.21–7.34 (m, 3ArH), 7.64–7.72 (m, 1ArH), 8.27 (d, J = 7.8 Hz, 1ArH) ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.3, 16.9, 17.9, 23.6, 26.4, 26.9, 32.2,39.9, 42.7, 114.5, 116.0, 116.2, 116.7, 118.2, 118.3, 119.9, 123.3, 123.7, 124.5, 129.7, 130.5, 135.7, 135.9, 136.3, 140.5, 141.0, 151.1, 157.7, 162.4. HRMS (ESI$^+$): m/z calculated for $C_{29}H_{35}N_2O_3$ [M+H]$^+$: 459.2648; found: 459.2650.

*4.1.8. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl acetate (**44**)*

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in chloroform and stirred at room temperature. To that pyridine (0.063 mL, 0.78 mmol) was added and cooled to 0 °C–5 °C and stirred at that temperature for 15 min. Acetic anhydride (0.056 mL, 0.59 mmol) was added dropwise and stirred at room temperature for 5 h. After completion of reaction water was added and neutralized with 5% hydrochloric acid and the organic layer collected (3 × 30 ml). Combined organic layers washed with water and brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.071 g, 61.7%, white solid, m.p: >240 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.29 (s, 3H), 7.18 (t, J = 1.8 Hz, 1ArH), 7.20–7.26 (m, 4ArH), 7.51 (t, J = 7.8 Hz, 1ArH), 7.71 (td, J = 1.5, 9.1 Hz, 1ArH), 7.95 (dd, J = 1.2, 9.1 Hz, 1ArH), 11.60 (bs, 1 N–H). ^{13}C NMR (100 MHz, $CDCl_3$+ DMSO-d_6): δ 20.0, 113.3, 114.6, 120.7, 121.4, 121.7, 125.2, 126.9, 128.4, 134.1, 135.2, 138.9, 149.4, 149.9, 161.4, 167.6. HRMS (ESI^+): m/z calculated for $C_{16}H_{13}N_2O_4$ $[M+H]^+$: 297.0875; found: 297.0879.

*4.1.9. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl propionate (**45**)*

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in chloroform and stirred at room temperature. To that pyridine (0.063 mL, 0.78 mmol) was added and cooled to 0 °C–5 °C and stirred at that temperature for 15 min. Propionic anhydride (0.075 mL, 0.59 mmol) was added dropwise and stirred at room temperature for 5 h. After completion of reaction water was added and neutralized with 5% hydrochloric acid and the organic layer collected (3 × 30 ml). Combined organic layers washed with water and brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.092 g, 76.0%, m.p: > 240 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.14 (t, J = 7.5 Hz, 3H), 2.62 (q, J = 7.5 Hz, 2H), 7.17 (t, J = 2.1 Hz, 1ArH), 7.20–7.26 (m, 4ArH), 7.51 (t, J = 7.8 Hz, 1ArH), 7.71 (td, J = 1.5, 8.7 Hz, 1ArH), 7.94 (dd, J = 1.5, 8.1 Hz, 1ArH), 11.59 (bs, 1 N–H). ^{13}C NMR (100 MHz, $CDCl_3$+ DMSO-d_6): δ 7.5, 25.8, 112.9, 114.0, 120.2, 121.1, 124.9, 126.3, 127.9, 133.6, 134.9, 138.4, 148.8, 149.4, 160.8, 170.6. HRMS (ESI^+): m/z calculated for $C_{17}H_{15}N_2O_4$ $[M+H]^+$: 311.1032; found: 311.1033.

*4.1.10. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl butyrate (**46**)*

The phenolic intermediate **2** (0.1 g, 0.39 mmol) was dissolved in chloroform and stirred at room temperature. To that pyridine (0.063 mL, 0.78 mmol) was added and cooled to 0 °C–5 °C and stirred at that temperature for 15 min. Butanoic anhydride (0.096 mL, 0.59 mmol) was added dropwise and stirred at room temperature for 5 h. After completion of reaction water was added and neutralized with 5% hydrochloric acid and the organic layer collected (3 × 30 ml). Combined organic layers washed with water and brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 30% ethyl acetate in hexanes. Yield: 0.089 g, 70.6%, m.p: 232–234 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.98 (t, J = 7.5 Hz, 3H), 1.67 (sextet, J = 7.5 Hz, 2H), 2.58 (t, J = 7.2 Hz, 2H), 7.18 (t, J = 1.8 Hz, 1ArH), 7.19–7.26 (m, 4ArH), 7.52 (t, J = 8.1 Hz, 1ArH), 7.71 (td, J = 1.5, 8.1 Hz, 1ArH), 7.94 (dd, J = 1.2, 8.1 Hz, 1ArH), 11.59 (bs, 1 N–H). ^{13}C NMR (100 MHz, $CDCl_3$+ DMSO-d_6): δ 12.4, 17.0, 34.7, 113.2, 114.4, 120.6, 121.3, 121.5, 125.1, 126.7, 128.2, 134.0, 135.1, 138.7, 149.2, 149.8, 161.2, 170.1. HRMS (ESI^+): m/z calculated for $C_{18}H_{17}N_2O_4$ $[M+H]^+$: 325.1188; found: 325.1190.

*4.1.11. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl pentanoate (**47**)*

Valeric acid (0.052 mL, 0.47 mmol) was dissolved in THF. To that DMAP (0.005 g, 0.04 mmol) and EDCI.HCl (0.09 g, 0.47 mmol) were added and stirred at room temperature. After 30 min the phenolic intermediate **2** (0.1 g, 0.39 mmol) was added and stirred for 3 h at room temperature. After completion of reaction (monitored by TLC), volatiles evaporated and the crude mass was dissolved in ethyl acetate and washed with 10% sodium bicarbonate, water and finally with brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 25% ethyl acetate in hexanes. Yield: 0.102 g, 77.9%, white solid, m.p: 190–192 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.95 (t, J = 7.2 Hz, 3H), 1.43 (sextet, J = 7.2 Hz, 2H), 1.73 (quintet, J = 7.6 Hz, 2H), 2.56 (t, J = 7.2 Hz, 2H), 6.89 (d, J = 8.0 Hz, 1ArH), 7.15 (t, J = 1.6 Hz, 1ArH), 7.18–7.27 (m, 3ArH), 7.52 (t, J = 8.0 Hz, 2ArH), 8.11 (d, J = 7.6 Hz, 1ArH), 10.45 (s, 1 N–H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.7, 22.2, 26.9, 34.1, 114.6, 115.7, 122.1, 122.3, 123.5, 126.0, 128.5, 129.3, 135.6, 138.8, 151.3, 151.7, 162.4, 171.8. HRMS (ESI^+): m/z calculated for $C_{19}H_{19}N_2O_4$ $[M+H]^+$: 339.1345; found: 339.1347.

*4.1.12. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl hexanoate (**48**)*

Hexanoic acid (0.059 mL, 0.47 mmol) was dissolved in THF. To that DMAP (0.005 g, 0.04 mmol) and EDCI.HCl (0.09 g, 0.47 mmol) were added and stirred at room temperature. After 30 min the phenolic intermediate **2** (0.1 g, 0.39 mmol) was added and stirred for 3 h at room temperature. After completion of reaction (monitored by TLC), volatiles evaporated and the crude mass was dissolved in ethyl acetate and washed with 10% sodium bicarbonate, water and finally with brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 25% ethyl acetate in hexanes. Yield: 0.099 g, 72.2%, white solid, m.p: 196–198 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.92 (t, J = 6.9 Hz, 3H), 1.36–1.41 (m, 4H), 1.67–1.77 (m, 2H), 2.55 (t, J = 7.5 Hz, 2H), 7.02 (d, J = 8.1 Hz, 1ArH),7.14 (t, J = 1.8 Hz, 1ArH), 7.18–7.21 (m, 1ArH), 7.24–7.27 (m, 2ArH), 7.53 (t, J = 8.1 Hz, 1ArH), 7.60 (td, J = 1.2, 8.4 Hz, 1ArH), 8.14 (d, J = 7.8 Hz, 1ArH), 9.82 (bs, 1NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.9, 22.3, 24.6, 31.2, 34.4, 114.7, 115.6, 122.1, 122.3, 123.6, 126.0, 128.6, 129.8, 135.5, 135.6, 138.8, 151.3, 151.6, 162.4, 171.8. HRMS (ESI^+): m/z calculated for $C_{20}H_{21}N_2O_4$ $[M+H]^+$: 353.1501; found: 353.1504.

*4.1.13. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl heptanoate (**49**)*

Heptanoic acid (0.067 mL, 0.47 mmol) was dissolved in THF. To that DMAP (0.005 g, 0.04 mmol) and EDCI.HCl (0.09 g, 0.47 mmol) were added and stirred at room temperature. After 30 min the phenolic intermediate **2** (0.1 g, 0.39 mmol) was added and stirred for 3 h at room temperature. After completion of reaction (monitored by TLC), volatiles evaporated and the crude mass was dissolved in ethyl acetate and washed with 10% sodium bicarbonate, water and finally with brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 25% ethyl acetate in hexanes. Yield: 0.111 g, 78.2%, white solid, m.p: 196–198 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, J = 6.9 Hz, 3H), 1.28–1.42 (m, 6H), 1.72 (q, J = 7.5 Hz, 2H), 2.55 (t, J = 7.5 Hz, 2H), 7.03 (d, J = 8.1 Hz, 1ArH), 7.14 (t, J = 1.8 Hz, 1ArH), 7.17–7.22 (m, 1ArH), 7.25–7.26 (m, 2ArH), 7.53 (t, J = 8.1 Hz, 1ArH), 7.61 (td, J = 1.5 Hz, 8.1 Hz, 1ArH), 8.14 (d, J = 8.1 Hz, 1ArH), 9.61 (s, 1NH). ^{13}C NMR (75 MHz, $CDCl_3$):

δ 14.0, 22.4, 24.7, 29.6, 31.3, 34.3, 114.5, 115.5, 121.7, 122.1, 122.8, 125.9, 128.2, 129.5, 135.1, 135.9, 139.7, 150.8, 151.2, 162.6, 171.5. HRMS (ESI^+): *m/z* calculated for $C_{20}H_{23}N_2O_4$ $[M+H]^+$: 367.1658; found: 367.1659.

4.1.14. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl octanoate (**50**)

Octanoic acid (0.074 mL, 0.47 mmol) was dissolved in THF. To that DMAP (0.005 g, 0.04 mmol) and EDCI.HCl (0.09 g, 0.47 mmol) were added and stirred at room temperature. After 30 min the phenolic intermediate **2** (0.1 g, 0.39 mmol) was added and stirred for 3 h at room temperature. After completion of reaction (monitored by TLC), volatiles evaporated and the crude mass was dissolved in ethyl acetate and washed with 10% sodium bicarbonate, water and finally with brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 25% ethyl acetate in hexanes. Yield: 0.106 g, 71.6%, white solid, m.p: 202–204 °C. ^1H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, *J* = 6.9 Hz, 3H), 1.25–1.59 (m, 8H), 1.74 (q, *J* = 6.9 Hz, 2H), 2.55 (t, *J* = 7.5 Hz, 2H), 7.03 (d, *J* = 7.8 Hz, 1ArH), 7.4 (t, *J* = 1.8 Hz, 1ArH), 7.20–7.22 (m, 1ArH), 7.25–7.27 (m, 2ArH), 7.53 (t, *J* = 8.1 Hz, 1ArH), 7.62 (td, *J* = 1.2, 8.4 Hz, 1ArH), 8.15 (dd, *J* = 1.2, 8.1 Hz, 1ArH), 9.49 (bs, 1NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.0, 22.4, 24.7, 28.7, 28.8, 31.5, 34.2, 114.4, 115.5, 121.7, 122.1, 122.7, 125.9, 128.1, 129.5, 135.1, 135.9, 139.7, 150.6, 151.1, 162.5, 171.4. HRMS (ESI^+): *m/z* calculated for $C_{22}H_{25}N_2O_4$ $[M+H]^+$: 381.1814; found: 381.1817.

4.1.15. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl nonanoate (**51**)

Nonanoic acid (0.083 mL, 0.47 mmol) was dissolved in THF. To that DMAP (0.005 g, 0.04 mmol) and EDCI.HCl (0.09 g, 0.47 mmol) were added and stirred at room temperature. After 30 min the phenolic intermediate **2** (0.1 g, 0.39 mmol) was added and stirred for 3 h at room temperature. After completion of reaction (monitored by TLC), volatiles evaporated and the crude mass was dissolved in ethyl acetate and washed with 10% sodium bicarbonate, water and finally with brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 25% ethyl acetate in hexanes. Yield: 1.11 g, 76.0%, white solid, m.p: 210–212 °C. ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J* = 6.9 Hz, 3H), 1.23–1.45 (m, 10H), 1.74 (q, *J* = 7.2 Hz, 2H), 2.55 (t, *J* = 7.5 Hz, 2H), 7.02 (d, *J* = 8.1 Hz, 1ArH), 7.15 (t, *J* = 1.8 Hz, 1ArH), 7.18–7.22 (m, 1ArH), 7.24–7.26 (m, 2ArH), 7.53 (td, *J* = 0.9, 9.0 Hz, 1ArH), 7.61 (td, *J* = 1.2, 8.1 Hz, 1ArH), 7.14 (dd, *J* = 0.9, 7.8 Hz, 1ArH), 9.67 (bs, 1NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1, 21.1, 22.6, 24.8, 29.1, 29.6, 31.8, 34.4, 114.5, 115.6, 121.8, 122.1, 122.9, 125.9, 128.3, 129.6, 135.2, 135.9, 139.6, 150.9, 151.1, 162.6, 171.5. HRMS (ESI^+): *m/z* calculated for $C_{23}H_{27}N_2O_4$ $[M+H]^+$: 395.1971; found: 395.1974.

4.1.16. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl decanoate (**52**)

Decanoic acid (0.091 mL, 0.47 mmol) was dissolved in THF. To that DMAP (0.005 g, 0.04 mmol) and EDCI.HCl (0.09 g, 0.47 mmol) were added and stirred at room temperature. After 30 min the phenolic intermediate **2** (0.1 g, 0.39 mmol) was added and stirred for 3 h at room temperature. After completion of reaction (monitored by TLC), volatiles evaporated and the crude mass was dissolved in ethyl acetate and washed with 10% sodium bicarbonate, water and finally with brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 25% ethyl acetate in hexanes. Yield: 0.102 g, 64.2%, white solid, m.p: 222–224 °C. ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J* = 6.9 Hz, 3H), 1.26–1.45 (m, 12H), 1.69–1.76 (m, 2H), 2.55 (t, *J* = 7.5 Hz, 2H), 7.04 (d, *J* = 8.1 Hz, 1ArH), 7.14 (t, *J* = 1.8 Hz, 1ArH), 7.18–7.22 (m, 1ArH), 7.24–7.26 (m, 2ArH), 7.53 (t, *J* = 8.1 Hz, 1ArH), 7.62 (td, *J* = 1.5, 8.7 Hz, 1ArH), 8.15 (d, *J* = 7.2 Hz, 1ArH), 9.56 (bs, 1NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1, 22.7, 24.9, 29.1, 29.3, 29.4, 29.7, 31.9, 34.4, 114.7, 115.6, 122.1, 122.3, 123.6, 126.0, 128.6, 129.8, 135.5, 135.6, 138.8, 151.3, 151.7, 162.4, 171. HRMS (ESI^+): *m/z* calculated for $C_{24}H_{29}N_2O_4$ $[M+H]^+$: 409.2127; found: 409.2128.

4.1.17. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl palmitate (**53**)

Palmitic acid (0.121 g, 0.47 mmol) was dissolved in THF. To that DMAP (0.005 g, 0.04 mmol) and EDCI.HCl (0.09 g, 0.47 mmol) were added and stirred at room temperature. After 30 min the phenolic intermediate **2** (0.1 g, 0.39 mmol) was added and stirred for 3 h at room temperature. After completion of reaction (monitored by TLC), volatiles evaporated and the crude mass was dissolved in ethyl acetate and washed with 10% sodium bicarbonate, water and finally with brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 25% ethyl acetate in hexanes. Yield: 0.132 g, 68.8%, white solid, m.p: 192–194 °C ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J* = 6.9 Hz, 3H), 1.25–1.39 (m, 22H), 1.69–1.79 (m, 4H), 2.55 (t, *J* = 7.5 Hz, 2H), 7.04 (d, *J* = 8.1 Hz, 1ArH), 7.14 (t, *J* = 1.8 Hz, 1ArH), 7.18–7.20 (m, 1ArH), 7.22–7.25 (m, 2ArH), 7.53 (t, *J* = 8.1 Hz, 1ArH), 7.62 (td, *J* = 1.2, 8.1 Hz, 1ArH), 8.15 (d, *J* = 4.2 Hz, 1ArH), 10.05 (bs, 1NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1, 22.7, 24.9, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 29.7, 31.9, 34.4, 114.6, 115.7, 122.1, 122.3, 123.5, 126.0, 128.5, 129.8, 135.6, 138.8, 151.3, 151.7, 162.4, 171.8. HRMS (ESI^+): *m/z* calculated for $C_{30}H_{41}N_2O_4$ $[M+H]^+$: 493.3066; found: 493.3067.

4.1.18. Synthesis of 3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl)amino) phenyl oleate (**54**)

Oleic acid (0.148 mL, 0.47 mmol) was dissolved in THF. To that DMAP (0.005 g, 0.04 mmol) and EDCI.HCl (0.09 g, 0.47 mmol) were added and stirred at room temperature. After 30 min the phenolic intermediate **2** (0.1 g, 0.39 mmol) was added and stirred for 3 h at room temperature. After completion of reaction (monitored by TLC), volatiles evaporated and the crude mass was dissolved in ethyl acetate and washed with 10% sodium bicarbonate, water and finally with brine solution. It was dried over sodium sulfate and evaporated. The compound was purified by column chromatography where the product was eluted at 25% ethyl acetate in hexanes. Yield: 0.156 g, 77.2%, white waxy solid, ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, *J* = 6.9 Hz, 3H), 1.25–1.41 (m, 20H), 1.60–1.79 (m, 4H), 2.02–2.06 (m, 2H), 2.55 (t, *J* = 7.5 Hz, 2H), 5.33–5.38 (m, 2H), 7.00 (d, *J* = 8.1 Hz, 1ArH), 7.14 (t, *J* = 1.8 Hz, 1ArH), 7.15–7.27 (m, 3ArH), 7.53 (t, *J* = 8.1 Hz, 1ArH), 7.60 (td, *J* = 1.5, 7.2 Hz, 1ArH), 8.13 (d, *J* = 7.8 Hz, 1ArH), 10.09 (bs, 1NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.1, 22.7, 24.9, 27.2, 29.1, 29.2, 29.3, 29.5, 29.7, 31.9, 34.4, 114.7, 115.5, 122.1, 122.3, 123.6, 125.9, 128.1, 128.6, 129.8, 130.0, 135.5, 138.7, 151.3, 151.6, 162.3, 171.7. HRMS (ESI^+): *m/z* calculated for $C_{32}H_{43}N_2O_4$ $[M+H]^+$: 519.3223; found: 519.3225.

4.1.19. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-methoxybenzoate (**55**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **27** (0.089 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica

gel (100–200 mesh) using 25% ethyl acetate in hexanes to afford the compound. Yield: 0.123 g, 70.1%, white solid, m.p: 210–212 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.27 (m, 2H), 3.85 (s, 3H), 4.37 (t, J = 7.2 Hz, 2H), 4.45 (t, J = 6.0 Hz, 2H), 6.30 (s, 1H N–H), 6.64 (t, J = 2.1 Hz, 1ArH), 6.75 (m, 1ArH), 6.83 (dd, J = 2.4, 8.4 Hz, 1ArH), 6.87–6.93 (m, 2ArH), 7.26–7.35 (m, 3ArH), 7.68 (td, J = 1.5, 8.7 Hz, 1ArH), 7.91–7.98 (m, 2ArH), 8.26 (dd, J = 1.5, 7.8 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 26.4, 40.9, 52.2, 62.0, 113.5, 115.5, 116.5, 119.5, 121.0, 122.7, 128.9, 129.6, 131.2, 135.3, 136.4, 139.7, 150.6, 157.3, 161.5, 163.3, 165.8. HRMS (ESI$^+$): m/z calculated for $C_{25}H_{23}N_2O_6$ $[M+H]^+$: 447.1556; found: 447.1558.

4.1.20. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-ethoxybenzoate (**56**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **28** (0.095 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 25% ethyl acetate in hexanes to afford the compound. Yield: 0.116 g, 64.4.0%, white wax, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (t, J = 6.9 Hz, 3H), 2.27 (quintet, J = 6.9 Hz, 2H), 4.07 (q, J = 6.9 Hz, 2H), 4.36 (t, J = 6.9 HZ, 2H), 4.44 (t, J = 6.0 Hz, 2H), 6.90 (bs, 1H N–H), 6.63 (t, J = 2.1 Hz, 1ArH), 6.72–6.75 (m, 1ArH), 6.80–6.83 (m, 1ArH), 6.86–6.89 (m, 2ArH), 7.26–7.33 (m, 3ArH), 7.67 (td, J = 1.5, 7.5 Hz, 1ArH), 7.92 (dt, J = 2.1, 6.9 Hz, 2ArH), 8.26 (dd, J = 1.5, 7.8 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.9, 27.0, 41.6, 62.4, 64.0, 113.7, 114.4, 116.0, 116.3, 116.7, 120.0, 122.2, 123.5, 129.9, 130.5, 131.8, 135.8, 136.2, 140.1, 151.2, 157.5, 162.1, 163.2, 166.4. HRMS (ESI$^+$): m/z calculated for $C_{26}H_{25}N_2O_6$ $[M+H]^+$: 461.1713; found: 461.1716.

4.1.21. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-propoxybenzoate (**57**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **29** (0.1 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 25% ethyl acetate in hexanes to afford the compound. Yield: 0.118 g, 63.8%, white solid, m.p: 134–136 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.97 (t, J = 2.8 Hz, 3H), 1.74 (m, 2H), 2.18 (m, 2H), 3.88 (t, J = 6.8 Hz, 2H), 4.28 (t, J = 7.2 Hz, 2H), 4.36 (t, J = 6.0 Hz, 2H), 6.56 (t, J = 2.0 Hz, 1ArH), 6.64 (d, J = 8.0 Hz, 1ArH), 6.73 (d, J = 2.0 Hz, 1ArH), 6.80–6.82 (m, 3ArH), 7.19–7.23 (m, 3ArH), 7.59–7.60 (m, 1ArH), 7.84 (dd, J = 2.0, 7.2 Hz, 2ArH), 8.18 (d, J = 6.0 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 10.6, 22.6, 26.9, 41.5, 62.3, 69.9, 113.7, 114.2, 115.9, 116.2, 116.6, 119.8, 122.0, 123.4, 129.8, 130.4, 131.7, 135.8, 136.1, 140.0, 151.2, 157.6, 162.1, 163.3, 166.4. HRMS (ESI$^+$): m/z calculated for $C_{27}H_{27}N_2O_6$ $[M+H]^+$: 475.1865; found: 475.1867.

4.1.22. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-butoxybenzoate (**58**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **30** (0.106 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 25% ethyl acetate in hexanes to afford the compound. Yield: 0.126 g, 66.3%, white wax, ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.98 (t, J = 7.2 Hz, 3H), 1.44–1.51 (m, 2H), 1.74–1.83 (m, 2H), 2.27 (quintet, J = 6.9 Hz, 2H), 4.00 (t, J = 6.6 Hz, 2H), 4.36 (t, J = 6.9 Hz, 2H), 4.44 (t, J = 6.0 Hz, 2H), 6.47 (bs, 1H N–H), 6.63 (t, J = 2.1 Hz, 1ArH), 6.74 (d, J = 7.8 Hz, 1ArH), 6.82 (dd, J = 1.5, 8.1 Hz, 1ArH), 6.86–6.91 (m, 2ArH), 7.25–7.34 (m, 3ArH), 7.67 (td, J = 1.8, 7.5 Hz, 1ArH), 7.89–7.94 (m, 2ArH), 8.26 (dd, J = 1.5, 8.1 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.0, 19.4, 27.0, 31.3, 41.6, 62.4, 68.2, 113.8, 114.4, 116.0, 116.3, 116.7, 120.0, 122.1, 123.5, 129.9, 130.5, 131.7, 135.8, 136.2, 140.1, 151.2, 157.5, 162.1, 163.4, 166.5. HRMS (ESI$^+$): m/z calculated for $C_{28}H_{29}N_2O_6$ $[M+H]^+$: 489.2026; found: 489.2028.

4.1.23. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-(pentyloxy)benzoate (**59**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **31** (0.111 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 25% ethyl acetate in hexanes to afford the compound. Yield: 0.126 g, 64.3%, white solid, m.p: 138–140 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.86 (t, J = 5.1 Hz, 3H), 1.31–1.37 (m, 4H), 1.71–1.7 (m, 2H), 2.17–2.21 (m, 2H), 3.92 (t, J = 6.4 Hz, 2H), 4.29 (t, J = 6.4 Hz, 2H), 4.37 (t, J = 6.0 Hz, 2H), 6.55 (t, J = 2.0 Hz, 1ArH), 6.66 (d, J = 8.0 Hz, 1ArH), 6.73 (d, J = 2.0 Hz, 1ArH), 6.79–6.82 (m, 3ArH), 7.20–7.25 (m, 3ArH), 7.60 (t, J = 6.0 Hz, 1ArH), 7.84 (d, J = 6.8 Hz, 2ArH), 8.18 (dd, J = 1.2, 7.2 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.6, 26.9, 28.3, 28.9, 41.6, 62.3, 68.4, 113.7, 114.3, 115.9, 116.2, 116.6, 119.9, 122.0, 123.4, 129.8, 130.5, 131.7, 135.8, 136.1, 140.0, 151.2, 157.5, 162.1, 163.3, 166.4. HRMS (ESI$^+$): m/z calculated for $C_{29}H_{31}N_2O_6$ $[M+H]^+$: 503.2182; found: 503.2186.

4.1.24. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-(hexyloxy)benzoate (**60**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **32** (0.117 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 22% ethyl acetate in hexanes to afford the compound. Yield: 0.132 g, 65.7%, white solid, m.p: 130–132 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.85 (t, J = 5.6 Hz, 3H), 1.18–1.25 (m, 2H), 1.26–1.28 (m, 2H), 1.68–1.73 (m, 4H), 2.18 (sextet, J = 6.4 Hz, 2H), 3.91 (t, J = 6.4 Hz, 2H), 4.28 (t, J = 6.8 Hz, 2H), 4.36 (t, J = 6.0 Hz, 2H), 6.55 (t, J = 2.0 Hz, 1ArH), 6.64 (d, J = 8.0 Hz, 1ArH), 6.72 (d, J = 2.0 Hz, 1ArH), 6.79–6.81 (m, 3ArH), 7.19–7.23 (m, 3ArH), 7.59 (t, J = 6.0 Hz, 1ArH), 7.83 (dd, J = 2.0, 6.8 Hz, 2ArH), 8.18 (dd, J = 1.2, 7.6 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 22.7, 25.8, 26.9, 29.2, 31.7, 41.5, 62.3, 68.4, 113.7, 114.2, 115.9, 116.2, 116.6, 119.7, 122.0, 123.4, 129.8, 130.4, 131.6, 131.7, 135.8, 136.0, 140.0, 151.2, 157.7, 162.1, 163.3, 166.4. HRMS (ESI$^+$): m/z calculated for $C_{30}H_{33}N_2O_6$ $[M+H]^+$: 517.2338; found: 516.2343.

*4.1.25. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-(heptyloxy)benzoate (**61**)*

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **33** (0.122 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 22% ethyl acetate in hexanes to afford the compound. Yield: 0.138 g, 66.7%, white solid, m.p: 114–116 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, J = 6.6 Hz, 3H), 1.25–1.38 (m, 4H), 1.40–1.46 (m, 4H), 1.80 (quintet, J = 6.6 Hz, 2H), 2.27 (quintet, J = 6.3 Hz, 2H), 4.00 (t, J = 6.6 Hz, 2H), 4.36 (t, J = 7.2 Hz, 2H), 4.44 (t, J = 5.7 Hz, 2H), 6.13 (bs, 1 N–H), 6.64 (t, J = 2.1 Hz, 1ArH), 6.76 (dd, J = 1.2, 7.8 Hz, 1ArH), 6.84 (dd, J = 1.8, 8.4 Hz, 1ArH), 6.88 (d, J = 9.0 Hz, 2ArH), 7.25–7.36 (m, 3ArH), 7.68 (td, J = 1.8, 8.7 Hz, 1ArH), 7.92 (d, J = 8.7 Hz, 2ArH), 8.26 (dd, J = 1.5, 7.8 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.1, 22.6, 26.0, 26.8, 29.0, 30.3, 31.8, 41.4, 62.1, 68.3, 113.6, 114.2, 115.8, 116.1, 116.6, 119.6, 121.8, 123.3, 129.7, 130.4, 131.5, 135.7, 135.9, 139.9, 151.1, 157.5, 162.0, 163.2, 166.3. HRMS (ESI$^+$): m/z calculated for $C_{31}H_{35}N_2O_6$ $[M+H]^+$: 531.2495; found: 531.2498.

*4.1.26. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-(octyloxy)benzoate (**62**)*

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **34** (0.127 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 22% ethyl acetate in hexanes to afford the compound. Yield: 0.156 g, 73.6%, colorless liquid, ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, J = 6.9 Hz, 3H), 1.25–1.32 (m, 8H), 1.40–1.48 (m, 2H), 1.75–1.84 (m, 2H), 2.27 (q, J = 6.6 Hz, 2H), 3.99 (t, J = 6.6 Hz, 2H), 4.36 (t, J = 7.5 Hz, 2H), 4.44 (t, J = 6.0 Hz, 2H), 6.32 (bs, 1NH), 6.64 (t, J = 2.1 Hz, 1ArH), 6.75 (d, J = 7.8 Hz, 1ArH), 6.83 (dd, J = 1.8, 8.1 Hz, 1ArH), 6.87–6.91 (m, 2ArH), 7.25–7.35 (m, 3ArH), 7.68 (td, J = 1.5, 8.7 Hz, 1ArH), 7.90–7.93 (m, 2ArH), 8.26 (dd, J = 1.5, 6.3 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 22.9, 26.2, 26.9, 29.3, 29.4, 29.5, 32.0, 41.6, 62.3, 68.5, 113.8, 114.4, 115.9, 116.3, 116.7, 119.9, 122.0, 123.5, 129.9, 130.5, 131.7, 135.8, 136.1, 140.1, 151.2, 157.6, 162.2, 163.4, 166.5. HRMS (ESI$^+$): m/z calculated for $C_{32}H_{37}N_2O_6$ $[M+H]^+$: 545.2652; found: 545.2655.

*4.1.27. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-(nonyloxy)benzoate (**63**)*

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **35** (0.133 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 20% ethyl acetate in hexanes to afford the compound. Yield: 0.132 g, 60.8%, white solid, m.p: 120–122 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, J = 6.9 Hz, 3H), 1.26–1.28 (m, 10H), 1.40–1.46 (m, 2H), 1.75–1.84 (m, 2H), 2.27 (q, J = 6.9 Hz, 2H), 4.00 (t, J = 6.6 Hz, 2H), 4.36 (t, J = 7.2 Hz, 2H), 4.44 (t, J = 6.0 Hz, 2H), 6.29 (bs, 1NH), 6.65 (t, J = 2.1 Hz, 1ArH), 6.76 (dt, J = 0.9, 6.9 Hz, 1ArH), 6.83–6.91 (m, 3ArH), 7.25–7.36 (m, 3ArH), 7.68 (td, J = 1.5, 7.2 Hz, 1ArH), 7.91–8.02 (m, 2ArH), 8.26 (dd, J = 1.5, 6.3 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 22.9, 26.2, 27.0, 29.3, 29.5, 29.6, 29.7, 32.1, 41.6, 62.3, 68.5, 113.8, 114.4, 115.9, 116.3, 116.7, 119.9, 122.0, 123.5, 129.9, 130.5, 131.7, 135.9, 136.1, 140.1, 151.2, 157.6, 162.2, 163.4, 166.5. HRMS (ESI$^+$): m/z calculated for $C_{33}H_{39}N_2O_6$ $[M+H]^+$: 559.2808; found: 559.2811.

*4.1.28. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-(decyloxy)benzoate (**64**)*

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **36** (0.138 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 20% ethyl acetate in hexanes to afford the compound. Yield: 0.141 g, 63.2%, white solid, m.p: 122–124 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.81 (t, J = 6.0 Hz, 3H), 1.20–1.25 (m, 10H), 1.36–1.38 (m, 2H), 1.67–1.73 (m, 4H), 2.16–2.19 (m, 2H), 3.91 (t, J = 6.4 Hz, 2H), 4.28 (t, J = 6.0 Hz, 2H), 4.36 (t, J = 6.0 Hz, 2H), 6.54 (t, J = 2.0 Hz, 1ArH), 6.64 (d, J = 8.0 Hz, 1ArH), 6.71 (d, J = 2.0 Hz, 1ArH), 6.79–6.81 (m, 3ArH), 7.19–7.24 (m, 3ArH), 7.59 (t, J = 6.4 Hz, 1ArH), 7.83 (d, J = 6.8 Hz, 2ArH), 8.17 (d, J = 7.2 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 22.8, 26.1, 27.0, 29.2, 29.4, 29.5, 29.7, 32.0, 41.6, 62.3, 68.4, 113.7, 114.3, 115.9, 116.2, 116.7, 119.7, 122.0, 123.4, 129.8, 130.5, 131.6, 135.8, 136.0, 140.0, 151.2, 157.7, 162.1, 163.3, 166.4. HRMS (ESI$^+$): m/z calculated for $C_{34}H_{41}N_2O_6$ $[M+H]^+$: 573.2965; found: 573.2966.

*4.1.29. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-(octadecyloxy)benzoate (**65**)*

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **4** (0.171 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 20% ethyl acetate in hexanes to afford the compound. Yield: 0.148 g, 57.8%, white solid, m.p: 112–114 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, J = 6.9 Hz, 3H), 1.18–1.38 (m, 28 H), 1.42–1.46 (m, 2H), 1.75–1.83 (m, 2H), 2.26–2.32 (m, 2H), 4.01 (t, J = 6.6 Hz, 2H), 4.37 (t, J = 7.8 Hz, 2H), 4.45 (t, J = 6.0 Hz, 2H), 6.73–6.78 (m, 2ArH), 6.89–6.95 (m, 3ArH), 7.10 (bs, 1NH), 7.25–7.28 (m, 1ArH), 7.31–7.37 (m, 2ArH), 7.68 (td, J = 1.5, 8.7 Hz, 1ArH), 7.95 (d, J = 8.7 Hz, 2ArH), 8.26 (dd, J = 1.5, 7.8 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 22.9, 26.2, 29.3, 29.6, 29.7, 29.8, 29.9, 32.1, 41.6, 62.3, 68.5, 113.8, 114.4, 115.9, 116.3, 116.7, 120.0, 122.0, 123.5, 129.9, 130.6, 131.7, 135.8, 136.2, 140.1, 151.2, 157.5, 162.1, 163.4, 166.5. HRMS (ESI$^+$): m/z calculated for $C_{42}H_{57}N_2O_6$ $[M+H]^+$: 685.4217; found: 685.4221.

*4.1.30. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-((3-methylbut-2-en-1-yl)oxy)benzoate (**66**)*

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **5** (0.11 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with

water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 20% ethyl acetate in hexanes to afford the compound. Yield: 0.125 g, 64.1%, white solid, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.76 (s, 3H), 1.81 (s, 3H), 2.27 (q, J = 6.0 Hz, 2H), 4.36 (t, J = 6.6 Hz, 2H), 4.44 (t, J = 5.7 Hz, 2H), 4.56 (d, J = 6.9 Hz, 2H), 5.48 (td, J = 1.5, 5.4 Hz, 1H), 6.59 (bs, 1NH), 6.63 (t, J = 2.1 Hz, 1ArH), 6.74 (td, J = 0.9, 7.8 Hz, 1ArH), 6.80–6.83 (m, 1ArH), 6.87–6.91 (m, 2ArH), 7.25–7.34 (m, 3ArH), 7.67 (dt, J = 1.5, 8.7 Hz, 1ArH), 7.89–7.96 (m, 2ArH), 8.26 (dd, J = 1.5, 7.8 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 18.5, 26.0, 27.0, 41.6, 62.4, 65.2, 113.8, 114.6, 116.0, 116.3, 116.7, 119.2, 120.1, 122.2, 123.5, 129.9, 130.5, 131.7, 135.8, 136.2, 139.1, 140.1, 151.2, 157.5, 162.1, 163.1, 166.4. HRMS (ESI$^+$): m/z calculated for $C_{29}H_{29}N_2O_6$ $[M+H]^+$: 501.2026; found: 501.2029.

4.1.31. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl (E)-4-((3,7-dimethylocta-2,6-dien-1-yl)oxy) benzoate (**67**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **6** (0.137 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 20% ethyl acetate in hexanes to afford the compound. Yield: 0.156 g, 70.6%, white wax, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61 (s, 3H), 1.70 (s, 3H), 1.75 (s, 3H), 2.06–2.12 (m, 4H), 2.27 (q, J = 6.0 Hz, 2H), 4.37 (t, J = 7.8 Hz, 2H), 4.45 (t, J = 6.0 Hz, 2H), 4.59 (d, J = 6.3 Hz, 2H), 5.09–5.1 (m, 1H), 5.48 (t, J = 8.4 Hz, 1H), 6.00 (bs, 1NH), 6.63–6.70 (m, 1ArH), 6.77 (d, J = 7.5 Hz, 1ArH), 6.85–6.90 (m, 1ArH), 6.91–6.93 (m, 2ArH), 7.25–7.37 (m, 3ArH), 7.68 (td, J = 1.5, 7.2 Hz, 1ArH), 7.92–7.95 (m, 2ArH), 8.27 (dd, J = 1.5, 7.8 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.0, 17.9, 25.3, 25.9, 26.5, 39.8, 62.3, 65.34, 113.8, 114.7, 116.0, 116.4, 116.9, 119.0, 120.4, 122.2, 123.4, 123.9, 129.9, 130.6, 131.7, 135.8, 136.2, 139.2, 140.1, 151.4, 157.6, 162.0, 163.4, 166.5. HRMS (ESI$^+$): m/z calculated for $C_{34}H_{37}N_2O_6$ $[M+H]^+$: 569.2652; found: 569.2655.

4.1.32. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl 4-(((2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)oxy)benzoate (**68**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **7** (0.163 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 15% ethyl acetate in hexanes to afford the compound. Yield: 0.158 g, 63.7%, white wax, ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.60 (s, 6H), 1.65 (s, 3H), 1.68 (s, 3H), 1.95–2.18 (m, 8H), 2.27 (q, J = 6.0 Hz, 2H), 4.36 (t, J = 7.5 Hz, 2H), 4.44 (t, J = 6.0 Hz, 2H), 4.58 (d, J = 6.6 Hz, 2H), 5.06–5.11 (m, 2H), 5.48 (t, J = 6.3 Hz, 1H) 6.31 (bs, 1NH), 6.64 (t, J = 2.1 Hz, 1ArH), 6.75 (dt, J = 0.9, 7.8 Hz, 1ArH), 6.82–6.85 (m, 1ArH), 6.87–6.92 (m, 2ArH), 7.25–7.35 (m, 3ArH), 7.68 (td, J = 1.5, 7.5 Hz, 1ArH), 7.93 (d, J = 9.0 Hz, 2ArH), 8.26 (dd, J = 1.5, 6.3 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 16.3, 17.0, 17.9, 25.9, 27.0, 29.9, 39.9, 41.6, 62.4, 65.3, 113.8, 114.6, 116.0, 119.0, 120.2, 122.2, 123.4, 123.8, 124.5, 129.9, 130.5, 131.7, 135.8, 136.3, 140.1, 151.2, 157.3, 162.1, 163.1, 166.4. HRMS (ESI$^+$): m/z calculated for $C_{39}H_{45}N_2O_6$ $[M+H]^+$: 637.3278; found: 637.3279.

4.1.33. Synthesis of 3-(3-((4-oxo-4H-benzo[d][1,3]oxazin-2-yl) amino)phenoxy)propyl (E)-4-(octadec-9-en-1-yloxy)benzoate (**69**)

Intermediate **2** (0.1 g, 0.39 mmol) was dissolved in DMF and to that potassium carbonate (0.162 g, 1.17 mmol) was added under stirring. Finally intermediate **8** (0.181 g, 0.39 mmol) was added and maintained at 70 °C for 8 h. After completion of reaction, the reaction mass was poured into water and extracted with ethyl acetate (3 × 150 ml). Combined organic layer was thoroughly washed with water followed by brine solution. The organic portion was then evaporated and purified by column chromatography on silica gel (100–200 mesh) using 15% ethyl acetate in hexanes to afford the compound. Yield: 0.145 g, 54.5%, white solid, m.p: 90–92 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.88 (t, J = 6.9 Hz, 3H), 1.26–1.32 (m, 22H), 1.43–1.46 (m, 2H), 1.80 (q, J = 6.6 Hz, 2H), 2.01–2.02 (m, 2H), 2.23–2.32 (m, 2H), 4.00 (t, J = 6.6 Hz, 2H), 4.37 (t, J = 7.5 Hz, 2H), 4.45 (t, J = 6.0 Hz, 2H), 5.35 (td, J = 1.2, 7.8 Hz, 2H), 5.62 (bs, 1NH), 6.67 (t, J = 2.1 Hz, 1ArH), 6.79 (qd, J = 0.9, 7.8 Hz, 1ArH), 6.87–6.91 (m, 3ArH), 7.28–7.32 (m, 2ArH), 7.35 (t, J = 7.8 Hz, 1ArH), 7.68 (td, J = 1.8, 7.5 Hz, 1ArH), 7.91–7.95 (m, 2ArH), 8.27 (dd, J = 1.5, 7.8 Hz, 1ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.3, 22.9, 26.2, 27.4, 29.5, 29.7, 29.7, 30.0, 32.1, 41.6, 62.3, 68.5, 113.8, 114.4, 115.9, 116.3, 116.7, 119.8, 122.0, 123.5, 129.9, 130.0, 130.2, 130.5, 131.7, 135.8, 136.1, 140.1, 151.2, 157.7, 162.2, 163.3, 165.5. HRMS (ESI$^+$): m/z calculated for $C_{42}H_{55}N_2O_6$ $[M+H]^+$: 683.4060; found: 683.4061.

4.2. Drug susceptibility on replicating M. tuberculosis H37Rv by MABA assay

The evaluation of MIC for the synthesized molecules against replicating H37Rv was performed by MABA assay. Briefly the bacteria were grown using the 7H12 media instead of the 7H9 media supplemented with glycerol, casitone and OADC. Luciferase reporter strains of H37Rv were used for compounds with intrinsic background fluorescence and the intracellular ATP levels measured. Cultures were incubated in 200 μl medium in 96-well plates for 7 days at 37 °C. Alamar Blue and Tween 80 were added and incubation was continued for 24 h at 37 °C. Fluorescence was determined at excitation/emission wavelengths of 530/590 nm respectively. The MIC was calculated as the lowest concentration effecting a reduction in fluorescence (or luminescence) of 90% relative to controls. Isoniazid and rifampin were run as the reference drug.

4.3. Drug susceptibility on non replicating M. tuberculosis H37Rv by LORA assay

Low Oxygen Recovery Assay was used to determine the anti mycobacterial efficiency of the synthesized molecule against the non replicating bacilli. The strain used for LORA is a recombinant H37Rv strain pFCA-LuxAB which makes direct luminescence measurements to determine the MIC. Briefly the strain was cultured in 7H12 media under humidified atmosphere (37 °C, 5% CO_2). Cultures were incubated in 200 μl medium in 96-well plates evacuated in an anaerobic jar for producing hypoxic environment using anaerobic gas mixture of 10% H_2, 5% CO_2 and 85% N_2 for 10 days at 37 °C. Then the culture was subjected to a recovery phase in 5% CO_2 and 95% humidity in a CO_2 incubator. Then 1% n-decanal was added and luminescence measured in a luminometer.

4.4. Drug susceptibility on replicating M. tuberculosis H37Ra by MABA assay

The MIC evaluation of the synthesized molecules against replicating H37Ra was performed by MABA assay. Briefly the bacteria were grown using the 7H9 media supplemented with OADC. at

37 °C. For minimum inhibitory concentration (MIC), we prepared different drug concentration in optically clear round bottom 96-well plates. An equivalent volume of mid loge phase H37Ra culture (diluted to an optical density at 570 nm of 0.01 was added to achieve a final drug concentration range of 100−0.78 μg/ml in 7H9 broth, with a control. Plates were incubated in ambient air at 37 °C for 7 day. Thereafter, Alamar Blue and Tween 80 were added and incubation was continued for 24 h at 37 °C. Fluorescence was determined at excitation/emission wavelengths of 530/590 nm respectively. The MIC was calculated as the lowest concentration effecting a reduction in fluorescence (or luminescence) of 95% relative to controls. Isoniazid and rifampin were run as the reference drug.

4.5. Drug susceptibility on M. Smegnatis mc^{2}155 strain

M. smegmatis (mc^{2}155) was cultured in Middlebrook 7H9 broth supplemented with 10% (v/v) albumin−dextrose complex and 0.03% (v/v) tween 80 at 37 °C. For minimum inhibitory concentration (MIC) testing, two fold serial dilutions of compounds were prepared in 100 μl in optically clear, round bottom 96-well plates. 100 μl of mid log−phase mc^{2}155 culture (diluted to an optical density at 570 nm of 0.01 was added to achieve a final drug concentration range of 100−0.78 μg/ml in 7H9 broth, with a control. Plates were incubated in ambient air at 37 °C for 48 h, at which point MICs were recorded at the lowest concentration of compound that prevented any visual growth.

4.6. Cytotoxicity study

Cytotoxicity studies were performed against the PBMC cells isolated from blood. The PBMC cells were propagated in RPMI media in a humidified incubator (37 °C, 5% CO_2). After scrapping the cells with a cell scraper, they were collected by centrifugation (1000 rpm for 5 min), re-suspended in fresh medium at ~1 × 10^6 cells/mL, dispensed into 96-well microplates (100 μl/well) and incubated for 24 h at 37 °C before being used for cytotoxicity assays. Test compounds were subsequently added at concentrations ranging from 400 to 0.2 μg/mL and incubation continued for another 72 h before the cytopathic effects of compounds was determined using the MTT Cell Proliferation Assay. The cytotoxic IC_{50} defined as the concentration causing 50% reduction in PBMC cell viability, was obtained from a dose−response curve plotted from percentage activity versus $\log_{10}$ concentration.

4.7. Preparation of the dormant H37Ra

To obtain the dormant H37Ra, we had taken 2 × 10^6 bacteria per ml in a flat bottom glass vial with 30 ml capacity (sample volume 28 ml). Thereafter, we sealed those glass vials (24 individual vials) and incubated at 37 °C for total 24 days with 25 rpm. We took 1 ml bacterial culture from each vial for all the 24 days and measures the OD_{600} and plated the same on agar plate (7H10 admixed with 5% ADC) and calculated the CFU.

4.8. Inhibition of mycolic acid biosynthesis

The inhibitory capability of these 1,3-Benzoxazi-4-one derivatives was evaluated by comparing the production of the different mycolic acid components of *Mtb* (H37Ra) bacilli in control and drug treated culture of H37Ra. In all the available literature methods, it was done by radio-labelled [1−^{14}C] acetate assay or converting the mycolic acid to its methyl esters or evaluating them in TLC by PMA staining. However the use of scintillation counters for radio-labelled assay or the PMA staining for TLC method are tedious. In order to avoid the difficulties we modified the methyl esterification protocol with benzyl and thereby the production of UV active mycolic acid esters became visible on the TLC plate which was then quantified using ImageJ software.

The H37Ra cultures were grown on 7H9 media supplemented with OADC till the mid log phase. 5 ml cultures of $OD_{570} = 0.5$ were incubated with the test molecules for 7 days at 37 °C. The concentration of the molecules was used 5 times of their MIC value. One control experiment and one set with DMSO treated experiment were also carried out. After 7 days of incubation, the bacilli were collected by centrifugation (3000 rpm for 10 min) followed by washing with PBS buffer (3 times). Then the bacilli were subjected to cell wall hydrolysis using 40% tetrabutyl ammonium hydroxide solution (5 mL) at 100 °C for overnight. Then dichloromethane was added and stirred, to that 20 μL benzylbromide was added and stirred further at room temperature for 3 h. The organic layer collected from each experiment, dried and evaporated. Stock solutions of the benzyl esters were made by dissolving them in 15 μL of DCM. Exactly 5 μL of the samples were used for TLC, and the TLC plates were eluted 3 times with 3% ethyl acetate in hexanes. Finally the images were recorded using a Nikon camera and the images were quantified by using ImageJ app.

4.9. MenG inhibition assay

The inhibitory activity of the synthesized molecules against MenG was evaluated by a HPLC based assay by monitoring S-Adenosyl Methionine (SAM) mediated methylation of Comp **A** (representative molecule for DMMQ) in presence of the molecule under investigation. In brief for 200 μl assay mixture, 500 μM Comp **A** (20 μl) was incubated with 5 μM (20 μl) $MgCl_2$, 5 mM DTT (20 μl) and 0.1% CHAPS (20 μl) in 100 mM Tris buffer (pH = 8). To that 500 μM SAM (10 μl) was added along with the inhibitor molecule with varing concentration (10 μl) and the reaction was initiated by adding 100 μl of H37Ra membrane bound protein (obtained by disrupting H37Ra cell using probe sonicator) and 100 μl of M smegmatis membrane bound protein separately. The mixture was incubated for 3 h and then quenched by adding 0.1 M acetic acid in methanol. The reaction mixture was then extracted with ether and injected to the HPLC (Agilent) and quantified the conversion of Comp **A** to Comp **B**. The same experiment was repeated with the synthesized molecules in increasing concentrations (5, 10, 20 and 40 μg/ml) and the corresponding IC_{50} was determined from non-linear regression analysis. We had used HyperClone 5 μm BDS C18 130 Å column from Phenomenex (250 × 4.6 mm) with the solvent system ACN/Water (90:10) with 0.05% TFA. The flow rate was maintained at 1 ml/min.

4.10. Vit-K2 rescue assay

The activity of Comp. **67** and **68** against non-replicating *Mtb* (H37Ra) was determined by using resazurin reduction assay. The effects of Vit-K2 supplementation were investigated using medium supplemented with 1 mM Vit-K2 (as a control set). The growth was measured by plating and counting the CFU from 0 to 10 days of culture. Similarly, the growth in presence of comp. **67** and **68** (3 and 30 μg/ml) was measured at 10th day with 1 mM Vit-K2 supplementation. In this case the cultures (incubated with **67** and **68** individually + 1 mM Vit-K2) were grown for 10 days followed by 27 days after CFU counting on 7H10 agar plates.

4.11. Determination of the ATP production

H37Ra was cultured for 12 days (with 25 rpm at 37 °C) in 7H9 broth till OD_{600} = 0.8. Thereafter, we incubated H37Ra

($OD_{600} = 0.8$; 2 ml) with three different concentrations of comp. **68** (20, 50 and 80 μg/ml along with a control set). We took 10 μl of the inoculums on 3rd, 6th and 9th day (from the day of drug addition) and measured the conversion of ADP to ATP using microplate reader.

In brief, the total content of ATP and ADP obtained form 100 μl of inoculums (from 3rd, 6th and 9th day separately) by adding trichloroacetic acid (0.5%). After 5 min it was neutralize by addition of TEA buffer and diluted to 5 fold with the same. Then we used 100 μl of reaction mix (containing 10 μl ATP monitoring enzyme and 90 μl nucleotide releasing buffer) with 10 μl of the solution containing ADP and ATP and incubated for 2 min and take the reading using a plate reader. Thereafter, 10 μl ADP converting enzyme was added and luminescence was recorded using the plate reader. The ATP/ADP ratio was determined according to the manufacturer protocol.

Declaration of competing interest

There is no conflict of interest with others.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112835.

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