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Design, synthesis and evaluation of novel 2,5,6-trisubstituted benzimidazoles targeting FtsZ as antitubercular agents

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ABSTRACT

Filamenting temperature-sensitive protein Z (FtsZ), an essential cell division protein, is a promising target for the drug discovery of new-generation antibacterial agents against various bacterial pathogens. As a part of SAR studies on benzimidazoles, we have synthesized a library of 376 novel 2,5,6-trisubstituted benzimidazoles, bearing ether or thioether linkage at the 6-position. In a preliminary HTP screening against *Mtb* H37Rv, 108 compounds were identified as hits at a cut off concentration of 5 μg/mL. Among those hits, 10 compounds exhibited MIC values in the range of 0.63–12.5 μg/mL. Light scattering assay and TEM analysis with the most potent compound **5a** clearly indicate that its molecular target is *Mtb*-FtsZ. Also, the K_d of **5a** with *Mtb*-FtsZ was determined to be 1.32 μM.

1. Introduction

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis* (*Mtb*), remains a leading single infectious disease killer. In 2012, the World Health Organization (WHO) estimated that there were 8.6 million new cases of TB globally (13% co-infected with HIV) resulting in 1.3 million deaths.[1] In addition, multidrug-resistant (MDR-TB) and extensively drug resistant TB (XDR-TB) are a significant public health threat for TB control efforts.[2,3] Emergence of drug resistant strains of *Mtb* makes many of the currently available anti-TB drugs much less effective.[3] Despite efforts in last 50 years, development of new TB treatments have been limited to drug targets like cell wall biosynthesis, ATP synthesis, RNA synthesis etc., leading to resistance in these areas.[4,5] Hence, we need to discover novel drugs that target other bacterial processes in order to counter the developed bacterial resistance.

Filamenting temperature-sensitive protein Z (FtsZ), a tubulin homologue,[6] is an essential and the most abundant bacterial cell division protein. FtsZ polymerizes in the presence of GTP to form a highly dynamic structure, the Z-ring at the division site.[7,8] With the recruitment of several other cell division proteins, Z-ring constriction proceeds resulting in septum formation and subsequent cell division.[9] Due to the crucial role of FtsZ in bacterial cytokinesis, inactivation of FtsZ is an attractive target for novel drug discovery.[10,11]

Since FtsZ is a homologue of tubulin with less than 10% sequence identity[12], known tubulin inhibitors could be a good starting point for developing FtsZ specific inhibitor. Previously, various groups have explored known tubulin inhibitors based on the importance of FtsZ assembly in cell division to identify their ability to inhibit FtsZ polymerization or depolymerization.[10,13,14]

Following on this principle, albendazole and thiabendazole, known tubulin inhibitors, were tested for their anti-TB activities.[15] Slayden and co-workers found that these two compounds inhibit FtsZ polymerization that led to the absence of septum formation based on ultra structural analysis and gene expression profiling. As both of these compounds share a common benzimidazole moiety, we chose benzimidazole as the scaffold for development of novel anti-TB agents. In our previous work,[16,17] based on rational drug design, libraries of 2,5,6- and 2,5,7-trisubstituted benzimidazoles were synthesized and evaluated for anti-TB activities. A large number of compounds were identified with MICs in the range of 0.38–6.2 μg/mL against drug sensitive as well as drug resistant *Mtb* strains (Fig. 1). In the light scattering experiment, some of these novel lead compounds exhibited inhibition of FtsZ assembly in a dose dependent manner while enhancing the GTPase activity[18] of *Mtb*-FtsZ. These results confirmed the hypothesis that the lead benzimidazoles target FtsZ.

The preliminary SAR studies of lead compounds indicate that cyclohexyl group at the 2-position and diethyl amino/dimethyl

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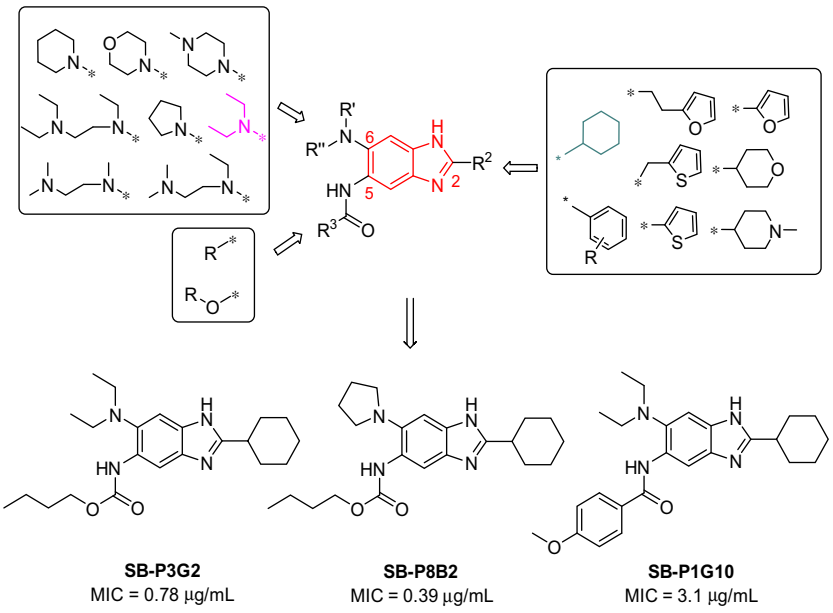


Figure 1. Previously reported anti-TB 2,5,6-trisubstituted benzimidazoles.

amino group at the 6-position play important role for antibacterial activity.[17,18] Building upon three representative compounds bearing alkyl carbamate or benzamide at the 5-position, we planned to expand our novel trisubstituted benzimidazole libraries with a substitution pattern different from the previous series for high throughput (HTP) screening.[19] [*Note*: In the 6-amino series, we have very recently found that the 6-dimethylamino series exhibit excellent activities up to the MIC value of 0.06 μg/mL.[17]]

In order to investigate the effect of substituents other than amines at the 6-postion on antibacterial activity, a new series of 2,5,6-trisubstituted benzimidazole library was designed and synthesized with ether/thioether groups at the 6-position (Fig. 2). Based on previous SAR studies, the cyclohexyl group at the 2-positon was fixed and various substituents at the 5-position were examined.

2. Chemical synthesis

General procedure for the synthesis of 2,5,6-trisubstituted benzimidazoles bearing an ether/thioether moiety at the 6-position is illustrated in Scheme 1.[20] The first step was the nucleophilic aromatic substitution of commercially available 2,4-dinitro-5-fluoroaniline (**1**) with various alkyl or aryl alcohol/thiols. Compounds **2a**, **2b** and **2e** were prepared by using 1 M KOH while **2c**, **2d** and **2f–2h** were obtained by using 1 M K_2CO_3 to afford compounds **2a–2h** in 91–100% yields. The acylation of compounds **2a–h** with the cyclohexanecarbonyl chloride gave **3a–h** in 82–89% yields.

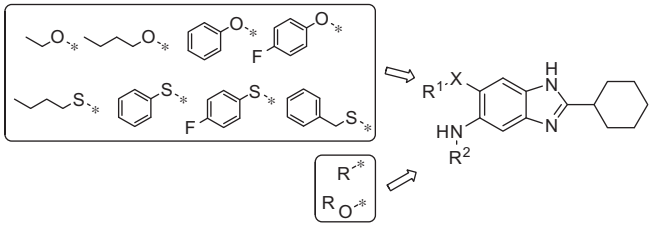


Figure 2. Novel 2,5,6-trisubstituted benzimidazoles bearing an ether or thioether substituent at the 6 position.

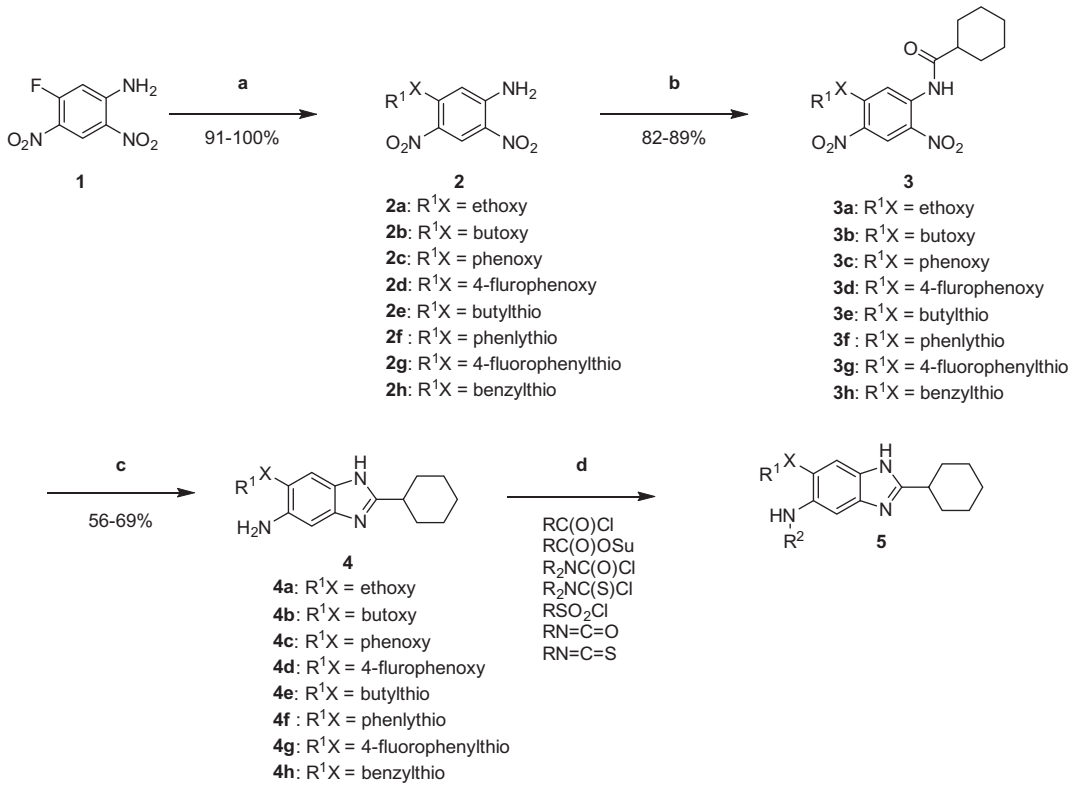
Compounds **3a–3d** were treated with tin(II) chloride dihydrate while **3e–3h** were reacted with tin(II) chloride dihydrate and 4 M HCl to afford benzimidazoles **4a–h** in 56–69% yields. 5-Aminobenzimidazoles **4a–h** (0.01 mM) were dissolved in dichloromethane and transferred into 96 well plates. Then, 47 different acyl chlorides, hydroxysuccinimide esters of chloroformates, isocyanates, isothiocyanates and sulfonyl chlorides (1.1 equiv) in dichloromethane were added to the individual wells. These 47 different reagents are shown in the Supporting information. The plates were gently shaken for a day. Then, aminomethylated polystyrene resin EHL/2% DVB (200–400 mesh) (10 equiv) was added to scavenge excess or unreacted acyl chloride, isocyanates, isothiocyanate and sulfonyl chlorides. After reacting for 24 h, the resin was filtered to afford a library of 376 novel 2,5,6-tribsustituted benzimidazoles **5**.

3. Results and discussion

3.1. In vitro preliminary screening of the library of 2,5,6-trisubstituted benzimidazoles 5 against *Mtb*-H37Rv

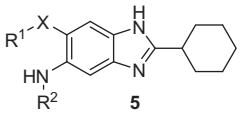
The library of 2,5,6-trisubstituted benzimidazoles **5** (376 compounds) was screened against drug sensitive *Mtb* H37Rv strain using 'Microplate Alamar Blue Assay (MABA)'[15] and then, growth inhibition was measured in percentage. Among these compounds, 108 compounds were identified to inhibit the growth of *Mtb* H37Rv by 22–79% at 5 μg/mL concentration and 22 compounds (see Table 1) exhibited 28–65% growth inhibition at 1.0 μg/mL concentration. From the preliminary screening, the butylthio group, followed by the benzylthio group at the 6 position appeared to be rather preferred, but 4-fluorophenoxy, 4-fluorophenylthio, and phenylthio groups did not seem to be much different. However, no compounds with a phenoxy group at the 6 position were included in the hit list. Also, no benzimidazoles bearing sulfoxide, urea or thiourea groups at the 5 position were found in the hit list. Thus, only amide or carbamate groups appear to be preferred at this position.

These hit compounds were resynthesized in analytically pure form and examined for their accurate MIC values. Then, it turned out that the MIC values did not necessarily correlate with the percent inhibition at the fixed concentration of the test compounds, as



Scheme 1. Library synthesis of 2,5,6-trisubstituted benzimidazoles. Reagents and conditions: (a) R^1OH/ R^1SH, 1 M KOH, THF, or K_2CO_3, Acetone, room temperature (rt), 1 h; (b) cyclohexanecarbonyl chloride, pyridine, reflux, overnight; (c) (i) $SnCl_2{\cdot}2H_2O$, EtOH, reflux, 1–6 h or (ii) $SnCl_2{\cdot}2H_2O$, 4 M HCl, EtOH, reflux, 1–6 h; (d) (i) RC(O)Cl, RC(O)OSu, $R_2NC(O)Cl$, $R_2NC(S)Cl$, RSO_2Cl, RN=C=O or RN=C=S (1.0 equiv), CH_2Cl_2, overnight, rt, aminomethylated polystyrene resin, filtration and concentration in vacuo.

Table 1
Hit compounds **5** from the preliminary screening against *Mtb* H37Rv strain at 1.0 μg/mL concentration



Compound	R^1X	R^2	% Growth inhibition	Compound	R^1X	R^2	% Growth inhibition
1	EtO	4-MeC_6H_4CO	65	**12**	BuS	$PH(CH_2)_2CO$	33
2	BuO	$CH_2{=}CH(CH_2)_2CO$	28	**13**	PhS	4-MeC_6H_4CO	31
3	4-FC_6H_4O	2,4-$F_2C_6H_3CO$	44	**14**	PhS	4-*t*-BuC_6H_4CO	42
4	4-FC_6H_4O	4-MeC_6H_4CO	36	**15**	PhS	$CH_2{=}CH(CH_2)_2CO$	56
5	4-FC_6H_4O	$CH_2{=}CH(CH_2)_2CO$	54	**16**	4-FC_6H_4S	$CH_3(CH_2)_2OCO$	46
6	BuS	2,4-$F_2C_6H_3CO$	51	**17**	4-FC_6H_4S	$PhSO_2$	30
7	BuS	$CH_3(CH_2)_2OCO$	38	**18**	4-FC_6H_4S	$CH_2{=}CH(CH_2)_2CO$	41
8	BuS	$PhSO_2$	42	**19**	$PhCH_2S$	$CH_3(CH_2)_2OCO$	28
9	BuS	4-*t*-BuC_6H_4CO	45	**20**	$PhCH_2S$	$PhSO_2$	30
10	BuS	4-MeC_6H_4CO	53	**21**	$PhCH_2S$	4-*t*-BuC_6H_4CO	52
11	BuS	$CH_2{=}CH(CH_2)_2CO$	52	**22**	$PhCH_2S$	$CH_2{=}CH(CH_2)_2CO$	64

anticipated. This would be due to, for example, inaccuracy in the actual weight and purity of a test compound in a 96-well plate, as well as false positives in the HTP screening. As Table 2 shows, some of the hit compounds with a 4-fluorophenoxy or buthylthio group exhibit promising activities, but those with a 6-phenylthio or 6-benzylthio group appear to be less potent among the compounds examined so far.

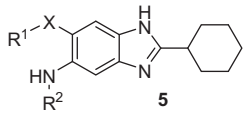
Although **5a**, bearing a *n*-butoxycarbonylamino group at the 5 position, was not among the 22 hit compounds, we added this compound for the MIC determination, since this carbamate group gave the best potency in the 6-dialkyamino series of 2,5,6-trisubstituted benzimidazoles in our another study,[17,18] Indeed, **5a** exhibited the best potency (MIC 0.63 μg mL) against *Mtb* H37Rv in this series (Table 2).

The cytotoxicity of **5a**–**5j** was evaluated in vitro against Vero cells using the MTT assay.[21] Compounds **5a**, **5b**, **5c** and **5j** showed cytotoxicity with IC_{50} values in the range of 26–75 μM. However, most of the analytically pure compounds did not show appreciable cytotoxicity against Vero cells.

3.2. FtsZ polymerization assay

Two benzimidazoles **5a** and **5d** were evaluated for their ability to inhibit the *Mtb*-FtsZ polymerization.[22] A light scattering assay

Table 2
MIC of selected hit compounds against *Mtb* H37Rv strain



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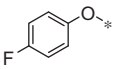
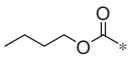
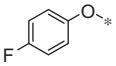
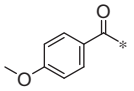
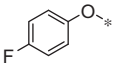
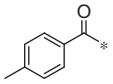
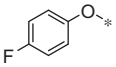
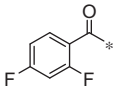
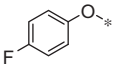
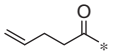
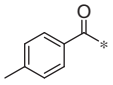
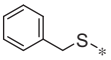
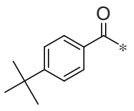
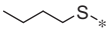
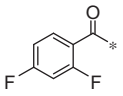
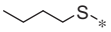
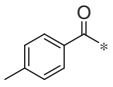
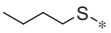
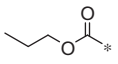
Compound	R^1X	R^2	MIC (μg/mL) *Mtb* H37Rv	Cytotoxicity (μM) vero cells
5a			0.63	60
5b			3.13	40
5c			1.56	26
5d			12.5	>200
5e			12.5	>200
5f			6.25	>200
5g			12.5	>200
5h			1.25	>200
5i			1.25	>200
5j			1.25	75

was carried out to examine the effect of these compounds on inhibition of the FtsZ polymerization.[17,18] The amount of the FtsZ polymer formed after addition of GTP was monitored by the intensity of light scattered by the sample. As Figure 3 shows, **5a** and **5d** inhibited FtsZ polymerization in a dose-dependent manner.

3.3. Transmission electron microscopy (TEM) imaging of FtsZ with compound 5a

Transmission electron microscopy (TEM) imaging of *Mtb*-FtsZ treated with **5a** exhibited the ability of the compound to inhibit FtsZ polymerization and aggregation.[17,18] *Mtb*-FtsZ (5 μM) incubated with **5a** at 40 μM and 80 μM concentrations in the presence of GTP (25 μM) showed shorter and thinner FtsZ polymers as compared to *Mtb*-FtsZ in the absence of compound **5a**. In the absence of inhibitor, *Mtb*-FtsZ formed a dense network of long polymers which tend to aggregate (Fig. 4A and B) while in the presence of **5a** (40 μM), the length, density and aggregation was visibly reduced (Fig. 4C and D). The effect was more apparent at 80 μM treatment where very short and dispersed FtsZ polymers were observed (Fig. 4E and F). Together with the light scattering assay, TEM images confirm the target of **5a** as *Mtb*-FtsZ and gives insight into the mode of action of the new series of trisubstituted benzimidazoles bearing an ether or thioether linkage at 6-postion.

3.4. Dissociation constant of compound 5a with FtsZ

The fluorescence anisotropy of **5a**, the most active compound, was used to determine its dissociation constant (K_d) with *Mtb*-FtsZ.[23] Compound **5a** has an emission maximum at 427.9 nm with the excitation maximum at 316 nm. The fluorescence anisotropy of **5a** was measured in the presence of increasing FtsZ concentrations to plot the resulting anisotropy profile (Fig. 5), which showed a concentration-dependent change. The change in fluorescence (ΔF) at 427.9 nm was applied to the standard equation $\Delta F = (\Delta F_{max} \times L)/(K_d + L)$ and the K_d of **5a** was determined to be 1.32 ± 0.5 μM.

4. Conclusion

New library of 2,5,6-trisubstituted benzimidazoles bearing sulfide and ether linkage at the 6 position have been synthesized. A number of hit compounds have been identified against *Mtb* H37Rv strain with good MIC values in the range of 0.63–12.5 μg/mL. Compound **5a** and **5d** were chosen for FtsZ polymerization assays and compound **5a** was used for TEM images for target validation. These results showed that the two selected compounds inhibit FtsZ assembly in a dose dependent manner. The dissociation constant (K_d) of **5a** was determined to be 1.32 μM based on its fluorescent

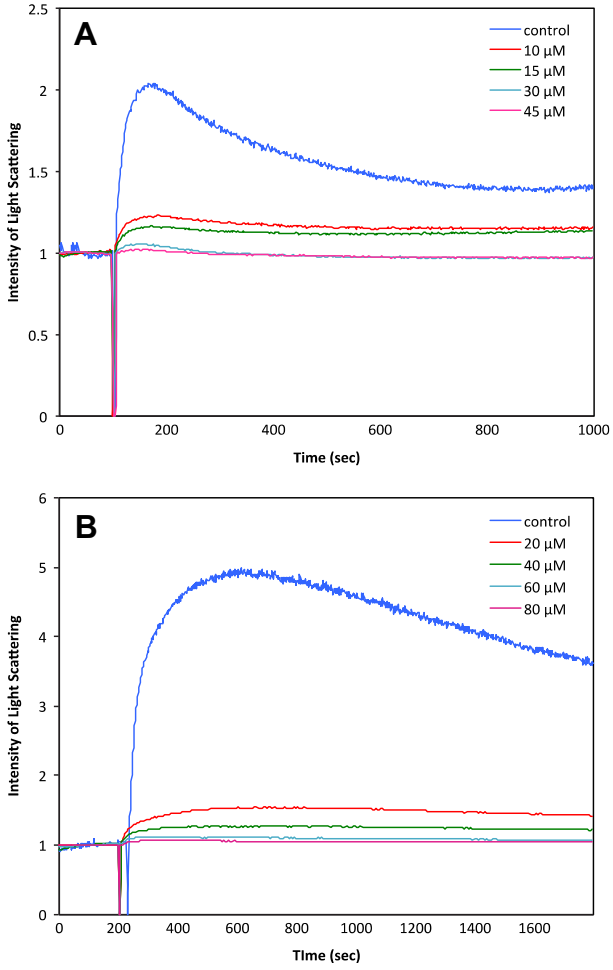


Figure 3. Inhibition of FtsZ polymerization by (A) **5a**, (B) **5d**.

anisotropy. This result provides direct evidence for the binding interaction between this benzimidazole and *Mtb*-FtsZ protein, which could be applicable to all potent benzimidazoles in this series. Further SAR study is necessary to obtain more detailed information for the substituent effects at 5 and 6 positions of the 2,5,6-trisubstituted benzimidazoles in this series. Nevertheless, 4-fluorophenoxy and butylthio groups were found to be preferred substituents at the 6 position. For the compounds, bearing a 4-fluorophenoxy group at the 6 position, a carbamate group at the 5 position gave the most potent compound (**5a**), but there is no difference between a carbamate group and benzamide groups for their potency (**5j** vs **5h** and **5i**) for the compounds, bearing a butylthio group at the 6 position. This is a unique feature in this series of benzimidazoles since a carbamate group at the 5 position provides, in general, more potent compounds than the corresponding 5-amidobenzimidazoles in the 5-dialkylamino-benzimidazole series.[17,18] Further optimization of the lead compounds for their anti-TB activities is actively underway in our laboratory. Also biological evaluations of the hit/lead compounds of this series against various other pathogens will be carried out to investigate their pathogen specific as well as broad spectrum antibacterial activities.

5. Experimental

The chemicals were purchased from Sigma Aldrich Co., Synquest Inc., Alfa Aesar and purified before use by standard methods. Tetrahydrofuran was freshly distilled from sodium metal and benzophenone. Dichloromethane was also distilled immediately prior to use under nitrogen from calcium hydride. ^{1}H and ^{13}C NMR spectra were measured on a Bruker 400 or 500 MHz NMR spectrometer. Melting points were measured on a Thomas Hoover Capillary melting point apparatus and are uncorrected. TLC was performed on Sorbtech with UV254 and column chromatography was carried out on silica gel 60 (Merck; 230–400 mesh ASTM). High-resolution mass spectra were obtained on Agilent-TOF instrument. The optical density was determined from the resulting solutions using the Acsent Multiskan optical density reader. Purity of synthesized compounds was determined by HPLC analysis at 244 and 303 nm wavelengths with a Shimadzu LC-2010A HT series HPLC assembly or Agilent 1100 series HPLC assembly. For the HPLC analysis, an Adsorbosphere silica 5 μm, 250 mm × 4.6 mm column was used with isopropanol–hexanes (5–50% isopropanol in gradient) as eluent at the flow rate of 1 mL/min, t = 0–40 min. Light scattering assays were performed using a PTI-QM4 spectrofluorimeter. FEI Tecnai12 BioTwinG transmission electron microscope with an AMT XR-60 CCD digital camera system was used to acquire transmission electron microscopy images.

5.1. 2,4-Dinitro-5-ethoxyaniline (2a)

To a magnetically stirred solution of 2,4-dinitro-5-fluoroaniline (**1**) (4.0 g, 19.9 mmol) in 50 mL of THF and excess ethanol was added 1 M KOH aqueous solution dropwise until a yellow precipitate appeared. The reaction mixture was stirred for additional 1 h. The solution was concentrated to dryness and then extracted with ethyl acetate. The organic layer was collected, dried over anhydrous magnesium sulfate and concentrated in vacuo to give **2a** as a yellow solid (4.8 g, 100% yield): mp 162–165 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.53 (t, 3H, J = 7.0 Hz), 4.18 (q, 2H, J = 7.0 Hz), 6.23 (s, 1H), 8.95 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.3, 66.1, 99.7, 127.2, 148.8, 158.1; HRMS (ESI) m/z calcd for $C_8H_{10}N_3O_5^+$ 228.0615 found: 228.0615 (Δ = 0.0 ppm).

In a similar manner, intermediate **2b**, **2e** were synthesized and characterized.

5.2. 5-Butoxy-2,4-dinitroaniline (2b)

Yellow solid; 76% yield; mp 176–178 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.99 (t, 3H, J = 7.4 Hz), 1.21–1.33 (m, 3H), 1.41–1.46 (m, 2H), 1.53 (dd, 2H, J = 15.1, 7.5 Hz), 1.63–1.66 (m, 2H), 1.75–1.78 (m, 2H), 1.86 (t, 2H, J = 7.6 Hz), 1.92–1.95 (m, 2H), 2.32–2.36 (m, 1H), 4.10 (t, 2H, J = 6.4 Hz), 6.24 (s, 1H), 8.95 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.7, 19.0, 25.3, 25.7, 28.8, 30.6, 42.8, 70.0, 99.7, 124.5, 127.2, 130.2, 148.8, 158.2; HRMS (ESI) m/z calcd for $C_{10}H_{14}N_3O_5^+$ 256.0928 found: 256.0931 (Δ = 1.17 ppm).

5.3. 2,4-Dinitro-5-phenoxyaniline (2c)

To a magnetically stirred solution of 2,4-dinitro-5-fluoroaniline **1** (3.0 g, 14.9 mmol) in 45 mL of acetone were added phenol (1.68 g, 17.9 mmol) and anhydrous K_2CO_3 (4.12 g, 29.8 mmol). The reaction mixture was stirred mechanically at room temperature for at least 16 h until the total disappearance of **1** was confirmed by MS (FIA) analysis. The solution was concentrated to dryness and then extracted with ethyl acetate. The organic layer was collected, dried over anhydrous magnesium sulfate, and concentrated in vacuo to give **2c** as a yellow solid (3.7 g, 91% yield): mp 148–150 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.97 (s, 1H), 7.56 (m, 3H), 7.60 (dd, 2H, J = 7.57, 1.83 Hz), 9.05 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 114.6, 126.4, 128.4, 129.7, 130.5, 130.9, 134.7, 136.2, 146.3, 148.7; HRMS (ESI) m/z calcd for $C_{12}H_{10}N_3O_5^+$ 276.0615 found: 275.0358 (Δ = −29.0 ppm).

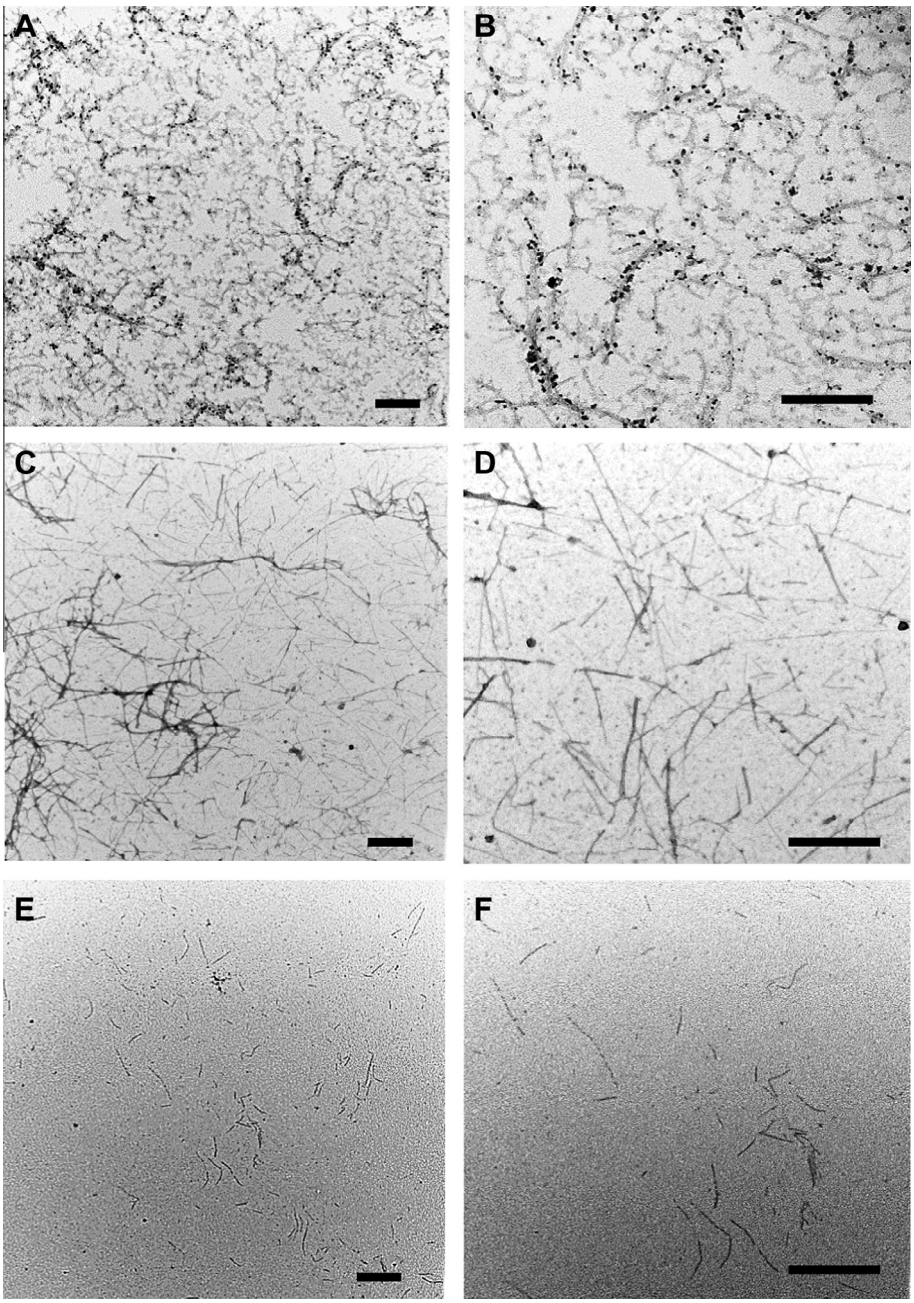


Figure 4. Transmission electron microscopy (TEM) Images of FtsZ. FtsZ (5 μM) was polymerized by GTP (25 μM) in the absence (A and B) and presence of **5a** at 40 μM (C and D) and 80 μM (E and F). Images (A, C, E) are at 23,000× magnification (scale bar 500 nm) and (B, D, F) are at 49,000× magnification (scale bar 500 nm).

In a similar manner, intermediate **2d**, **2f**, **2g**, and **2h** were synthesized and characterized.

5.4. 2,4-Dinitro-5-(4-fluorophenoxy)aniline (2d)

Yellow solid; 93% yield; mp 164–165.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.01 (s, 1H), δ 7.11–7.19 (m, 4H), δ 9.05 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 103.7, 117.2, 117.4, 122.5, 122.6, 127.6, 148.5, 149.2, 157.8, 159.5, 161.5; HRMS (ESI) m/z calcd for $C_{12}H_{9}FN_3O_5^+$ 294.0521 found: 294.0521 (Δ = 0.0 ppm).

5.5. 5-(Butylthio)-2,4-dinitroaniline (2e)

Yellow solid; 99% yield; mp 148–149 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.99 (t, 3H, J = 7.5 Hz), 1.53–1.57 (m, 2H), 1.74–2.05 (m, 2H), 2.01 (t, 2H, J = 7.5 Hz), 6.55 (s, 1H), 9.18 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.7, 22.3, 29.2, 32.4, 112.6, 126.6, 127.8, 135.4, 146.3, 147.8; HRMS (ESI) m/z calcd for $C_{10}H_{14}N_3O_4S^+$ 272.0700 found: 272.0701 (Δ = 0.35 ppm).

5.6. 2,4-Dinitro-5-(phenylthio)aniline (2f)

Yellow solid; 100% yield; mp 214–217 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.97 (s, 1H), 7.21–7.24 (m, 3H), 7.28–7.32 (m, 2H), 9.02 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 126.4, 127.2, 127.5, 129.1, 130.5, 130.8, 136.2, 137.0; HRMS (ESI) m/z calcd for $C_{12}H_{10}N_3O_4S^+$ 292.0387 found: 292.0387 (Δ = 0.0 ppm).

5.7. 2,4-Dinitro-5-(4-fluorophenylthio)aniline (2g)

Yellow solid; 100% yield; mp 217–219 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.96 (s, 1H), 7.04 (t, 1H, J = 8.6 Hz), 7.27 (d, 1H, J = 8.53),

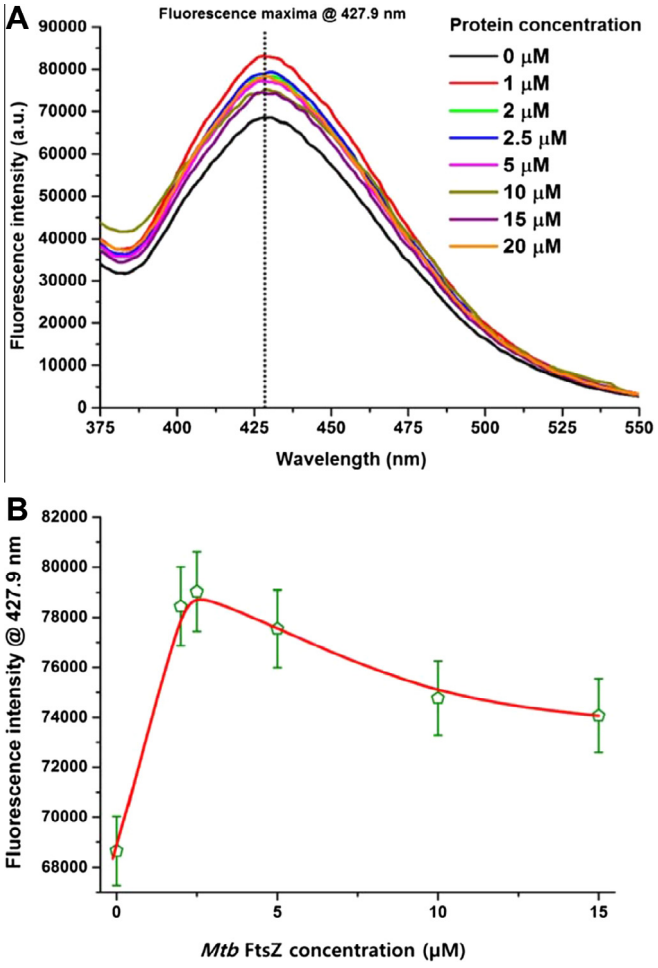


Figure 5. Determination of binding parameter of **5a** with *Mtb*-FtsZ. (A) Fixed concentration of **5a** (100 μM) was excited at 316 nm with varying concentration of *Mtb*-FtsZ (as shown in the graph) and fluorescence monitored at 427.9 nm and plotted against wavelength. Increase in fluorescence intensity observed on addition of increasing concentration of protein. (B) Fluorescence profiles of emission intensity at 427 nm for the titration of *Mtb*-FtsZ. The peak saturation was observed at 2.5 μM and then there is a progressive decrease in fluorescence emission. The K_d of **5a** was calculated to be 1.32 ± 0.5 μM/L.

7.49–7.46 (m, 1H), 7.62 (dd, 1H, *J* = 8.5, 5.3 Hz), 9.23 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 114.4, 116.2, 116.4, 117.8, 118.0, 126.5, 131.2, 131.3, 138.3, 138.4, 146.3; HRMS (ESI) *m*/*z* calcd for $C_{12}H_9FN_3O_4S^+$ 310.0292 found: 310.0292 (Δ = 0.0 ppm).

5.8. 5-(Benzylthio)-2,4-dinitroaniline (2h)

Yellow solid; 100% yield; mp 188.5–190 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.17 (s, 2H), δ 6.62 (s, 1H), δ 7.36–7.44 (m, 5H), 9.19 (s, 1H); ^{13}C NMR (100 MHz, CDCl3) δ 37.8, 113.0, 126.5, 128.2, 129.0, 129.1, 133.7; HRMS (ESI) *m*/*z* calcd for $C_{12}H_{12}N_3O_4S^+$ 306.0543 found: 306.0543 (Δ = 0.0 ppm).

5.9. 1-(Cyclohexanecarboxamido)-5-ethoxy-2,4-dinitro-benzene (3a)

To a solution of **2a** (0.71 g, 3.13 mmol) in 12 mL of pyridine was added cyclohexanecarbonyl chloride (0.54 mL, 1.3 equiv), and the mixture was magnetically stirred and refluxed overnight. After completion of the reaction by TLC analysis, the reaction mixture was concentrated under reduced pressure, diluted with ethyl acetate, and then washed with $CuSO_4$ solution twice to get rid of the leftover pyridine. The reaction mixture was washed with brine, diluted with ethyl acetate, and washed with water three times. The organic layers were dried over sodium sulfate, filtered, and concentrated to afford **3a** as a yellow solid (0.9 g, 89% yield): mp 109–109.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.28–1.31 (m, 1H), 1.38–1.42 (m, 2H), 1.51 (t, 3H, *J* = 7.41 Hz), 1.59–1.60 (m, 2H), 1.76–1.79 (m, 1H), 1.88–1.93 (m, 2H), 2.05–2.09 (m, 2H), 2.44 (tt, 1H, *J* = 11.6, 3.5 Hz), 3.12 (q, 2H, *J* = 7.44 Hz), 9.19 (s, 1H), 9.24 (s, 1H), 10.9 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.2, 25.4, 25.5, 27.1, 29.4, 47.4, 116.9, 124.9, 130.7, 138.1, 149.7, 176.0; HRMS (ESI) *m*/*z* calcd for $C_{15}H_{20}N_3O_6^+$ 338.1347 found: 338.1351 (Δ = 1.18 ppm).

In a similar manner, compounds **3b**–**3h** were synthesized and characterized.

5.10. 1-(Cyclohexanecarboxamido)-5-butoxy-2,4-dinitro-benzene (3b)

Yellow solid; 100% yield; mp 187.5–189 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.99 (t, 3H, *J* = 7.4 Hz), 1.21–1.33 (m, 3H), 1.45 (d, 2H, *J* = 11.5 Hz), 1.50–1.56 (m, 2H), 1.6401.66 (m, 1H), 1.75–1.78 (m, 2H), 1.86 (dd, 2H, *J* = 8.4, 6.8 Hz), 1.93 (d, 2H, *J* = 12.8 Hz), 2.32–2.36 (m, 1H), 4.09 (t, 2H, *J* = 6.4 Hz), 6.24 (s, 1H), 8.95 (s, 1H);^{13}C NMR (125 MHz, $CDCl_3$) δ 13.7, 19.0, 25.3, 25.3, 28.8, 30.6, 42.8, 70.0, 99.7, 124.5, 127.2, 130.2, 148.8, 158.2; MS (ESI) *m*/*z* 366.1 (M+1).

5.11. 1-(Cyclohexanecarboxamido)-5-phenoxyphenyl-2,4-dinitro-benzene (3c)

Yellow solid; 97% yield; mp 150.5–152 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21 (tt, 1H, *J* = 12.3, 3.2 Hz), 1.26–1.35 (m, 2H), 1.43 (qd, 2H, *J* = 12.3, 3.1), 1.68–1.72 (m, 1H), 1.81 (dt, 2H, *J* = 13.1, 3.3 Hz), 1.95 (dd, 2H, *J* = 13.5, 1.9 Hz), 2.23–2.33 (m, 1H), 7.15–7.17 (m, 2H), 7.35 (t, 1H, *J* = 7.5 Hz), 7.49–7.52 (m, 2H), 8.53 (s, 1H), 9.06 (s, 1H), 10.8 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.5, 29.3, 47.3, 108.8, 120.6, 121.6, 125.6, 126.7, 128.8, 129.4, 130.6, 133.2, 140.4, 153.2, 157.7, 175.4; HRMS (ESI) *m*/*z* calcd for $C_{19}H_{20}N_3O_6^+$ 386.1347 found: 386.1345 (Δ = −0.52 ppm).

5.12. 1-(Cyclohexanecarboxamido)-5-(4-fluorophenoxy)-2,4-dinitrobenzene (3d)

Yellow solid; mp 147–149 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.18–1.20 (m, 1H), 1.27–1.32 (m, 2H), 1.40 (d, 2H, *J* = 11.6 Hz), 1.71 (dd, 1H, *J* = 1.69, 1.42 Hz), 1.78–1.83 (m, 2H), 1.90–1.94 (m, 2H), 2.22–2.30 (m, 1H), 7.23–2.30 (m, 2H), 7.58–7.62 (m, 2H), 8.47 (s, 1H), δ 9.21 (s, 1H), δ 10.6 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.2, 25.4, 29.1, 29.3, 47.2, 117.9, 118.1, 119.0, 123.9, 124.1, 124.7, 138.1, 138.2,150.4, 165.8, 174.8; MS (ESI) *m*/*z* 404.0 $(M+1)^+$.

5.13. 1-(Cyclohexanecarboxamido)-5-(butylthio)-2,4-dinitro-benzene (3e)

Yellow solid; 100% yield; mp 118–119 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.99 (t, 3H, *J* = 7.3 Hz), 1.25–1.32 (m, 1H), 1.33–1.42 (m, 2H), 1.55–1.59 (m, 4H), 1.74–1.78 (m, 1H), 1.80–1.83 (m, 2H), 1.88 (dt, 2H, *J* = 13.3, 3.4 Hz), 2.05 (dd, 2H, *J* = 13.4, 2.2 Hz), 2.42 (tt, 1H, *J* = 11.7, 3.5 Hz), 3.07 (t, 2H, *J* = 7.3 Hz), 9.16 (s, 1H), 9.21 (s, 1H), 10.9 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.7, 22.1, 25.5, 29.5, 32.7, 47.5, 117.0, 124.9, 138.1, 150.0, 175.9; MS (ESI) *m*/*z* 382.1 $(M+1)^+$.

5.14. 1-(Cyclohexanecarboxamido)-5-(phenylthio)-2,4-dinitrobenzene (3f)

Yellow solid; 100% yield; mp 217–218 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.18–1.22 (m, 2H), 1.24–1.32 (m, 2H), 1.37(qd, 2H,

J = 12.2, 2.8 Hz), 1.67 (dd, 1H, J = 12.8, 0.8 Hz), 1.78–1.82 (m, 2H), 1.89–1.92 (m, 2H), 2.24 (tt, 1H, J = 11.7, 3.5 Hz), 7.60 (q, 5H, J = 6.1 Hz), 8.46 (s, 1H), 9.22 (s, 1H), 10.6 (s, 1H); ^{13}C NMR (100 MHz, CDCl3) δ 25.4, 29.3, 30.9, 47.3, 119.1, 124.7, 128.6, 130.6, 131.1, 135.9, 137.5, 138.0, 150.7, 174.8; MS (ESI) m/z 402.1 $(M+1)^+$.

5.15. 1-(Cyclohexanecarboxamido)-5-(4-fluorophenylthio)-2,4-dinitrobenzene (3g)

Yellow solid; 96% yield; mp 186–189 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.16–1.23 (m, 1H), 1.25–1.31 (m, 2H), 1.34–1.42 (m, 2H), 1.68–1.74 (m, 1H), 1.79–1.83 (m, 2H), 1.94 (dd, 2H, J = 12.9, 2.1 Hz), 2.25–2.31 (m, 1H), 7.25–7.29 (m, 2H), 7.62 (dd, 2H, J = 8.8, 5.2 Hz), 8.49 (s, 1H), 9.24 (s, 1H), 10.6 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.5, 29.3, 31.0, 47.3, 119.1, 124.7, 128.7, 130.6, 131.1, 131.5, 135.9, 137.6, 138.1, 150.8, 174.8; MS (ESI) m/z 420.1 $(M+1)^+$.

5.16. 1-(Cyclohexanecarboxamido)-5-(benzylthio)-2,4-dinitrobenzene (3h)

Yellow solid; 96% yield: mp 150–153 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.27–1.30 (m, 1H), 1.37–1.44 (m, 2H), 1.52–1.62 (m, 2H), 1.74–1.77 (m, 1H), 1.87–1.90 (m, 2H), 2.04–2.07 (m, 2H), 2.39–2.47 (m, 1H), 4.30 (s, 2H), 7.26–7.37 (m, 3H), 7.45 (d, 2H, J = 6.8 Hz), 9.21 (s, 1H), 9.29 (s, 1H), 10.9 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.4, 25.5, 29.4, 38.1, 43.3, 47.4, 117.1, 124.8, 127.4, 128.2, 128.5, 128.8, 129.4, 129.6, 130.9, 133.5, 137.4, 138.1, 138.2, 149.1, 175.9; MS (ESI) m/z 416.0 $(M+1)^+$.

5.17. 5-Amino-6-ethoxy-2-cyclohexyl-1*H*-benzo[*d*]imidazole (4a)

A solution of **3a** (100 mg, 0.30 mmol), tin(II) chloride dihydrate (0.47 g, 2.1 mmol) in 10 mL of EtOH was magnetically stirred and refluxed at 90 °C under nitrogen for 1 h. The reaction mixture was cooled, quenched with 30% KOH, and pH adjusted to ~13. The solution was diluted with dichloromethane and washed with water three times. The organic layers were dried over sodium sulfate, filtered, and concentrated. The residue was purified by flash chromatography on silica gel (gradient 20–40% ethyl acetate/hexanes) to afford compound **4a** as a pale red color solid (69 g, 89% yield): mp 89–90 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.23 (ddd, 1H, J = 14.2, 10.8, 3.3 Hz), 1.32 (ddd, 2H, J = 14.2, 11.1, 3.1 Hz), 1.37–1.42 (m, 3H), 1.60 (qd, 2H, J = 12.4, 3.1 Hz), 1.69–1.71 (m, 1H), 1.79–1.81 (m, 2H), 2.08 (d, 2H, J = 12.4 Hz), 2.80 (tt, 1H, J = 11.8, 3.51 Hz), 3.79 (br, 2H), 3.99 (dd, 2H, J = 8.3, 3.3 Hz), 6.78 (s, 1H), 6.96 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.0, 25.9, 26.1, 32.0, 38.5, 64.4, 132.9, 133.0, 144.5, 157.0; HRMS (ESI) m/z calcd for $C_{15}H_{22}N_3O^+$ 260.1757 found: 260.1758 (Δ = 0.38 ppm).

In a similar manner, compounds **4b**–**4d** were synthesized and characterized.

5.18. 5-Amino-6-butoxy-2-cyclohexyl-1*H*-benzo[*d*]imidazole (4b)

Pale red solid; 69% yield; mp 113–115 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.97 (t, 3H, J = 7.4 Hz), 1.23–1.28 (m, 1H), 1.35–1.40 (m, 2H), 1.50 (dd, 2H, J = 15.0, 7.5 Hz), 1.60 (qd, 2H, J = 12.4, 3.2 Hz), 1.70–1.75 (m, 1H), 1.76–1.86 (m, 4H), 2.08–2.12 (m, 2H), 2.78–2.84 (m, 1H), 3.76 (br, 2H), 3.98 (t, 2H, J = 6.5 Hz), 6.80 (s, 1H), 7.00 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.9, 19.4, 25.9, 26.1, 31.4, 32.0, 38.4, 68.6, 133.1, 144.6, 156.7; HRMS (ESI) m/z calcd for $C_{17}H_{26}N_3O^+$ 288.2070 found: 288.2071 (Δ = 0.35 ppm).

5.19. 5-Amino-6-phenoxy-2-cyclohexyl-1*H*-benzo[*d*]imidazole (4c)

Pale yellow solid; 46% yield; mp 136–138 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25–1.32 (m, 1H), 1.35–1.44 (m, 2H), 1.56–1.66 (m, 2H), 1.72–1.77 (m, 1H), 1.83–1.88 (m, 2H), 2.09–2.14 (m, 2H), 2.80–2.86 (m, 1H), 3.73 (br, 2H), 6.93–6.97 (m, 2H), 7.01–7.05 (m, 1H), 7.12 (s, 1H), 7.26–7.29 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.9, 26.0, 31.9, 38.5, 116.7, 122.4, 129.7, 135.3, 140.3, 158.1; HRMS (ESI) m/z calcd for $C_{19}H_{22}N_3O^+$ 308.1757 found: 308.1758 (Δ = 0.32 ppm).

5.20. 5-Amino-6-(4-fluorophenoxy)-2-cyclohexyl-1*H*-benzo[*d*]imidazole (4d)

Beige solid; 70% yield; mp 195–196 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26–2.30 (m, 1H), 1.38–1.43 (m, 2H), 1.61 (dd, 2H, J = 12.4, 3.1 Hz), 1.72–1.77(m, 1H), 1.86 (dt, 2H, J = 13.1, 3.3 Hz), 2.12 (dd, 2H, J = 13.6, 2.0 Hz), 2.83 (tt, 1H, J = 11.8, 3.6 Hz), 3.74 (br, 2H), 6.95 (m, 5H), 8.89 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.8, 26.0, 31.8, 38.5, 116.0, 116.2, 118.1, 118.2, 154.0, 157.1, 159.5; HRMS (ESI) m/z calcd for $C_{19}H_{23}FN_3O^+$ 326.1663 found: 326.1667 (Δ = 1.2 ppm).

5.21. 5-Amino-6-(butylthio)-2-cyclohexyl-1*H*-benzo[*d*]imidazole (4e)

A solution of **3e** (1.97 g, 5.16 mmol), tin(II) chloride dihydrate (15.5 g, 36.1 mmol), and conc. HCl (80 mL) in 200 mL of ethanol was magnetically stirred and refluxed for 4 h. The reaction mixture was cooled, quenched with 1 M NaOH, and pH was adjusted to ~10. Tin salts precipitated in solution upon addition of 1 M NaOH. The reaction mixture was filtered to remove the tin salts. The solution was diluted with ethyl acetate and washed with water three times. The organic layers were dried over magnesium sulfate, filtered, and concentrated in vacuo. The residue was purified by flash chromatography on silica gel (gradient 20–40% ethyl acetate/hexanes) to afford compound **4e** as a pale greenish gray solid (0.413 g): 71% yield; mp 110–112 °C; ^{1}H NMR 500 MHz, $CDCl_3$) δ 0.88 (t, 3H, J = 7.3 Hz), 1.25–1.30 (m, 1H), 1.36–1.42 (m, 4H), 1.55 (dt, 2H, J = 14.0, 7.4 Hz), 1.64 (qd, 2H, J = 12.4, 3.2 Hz), 1.72–1.76 (m, 1H), 1.85 (dt, 2H, J = 13.2, 3.3 Hz), 2.13 (dd, 2H, J = 13.7, 1.9 Hz), 2.72 (t, J = 7.4 Hz), 2.86 (tt, 1H, J = 11.8, 3.5 Hz), 4.35 (br, 2H), 6.83 (s, 1H), 7.66 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.7, 21.9, 25.8, 26.0, 31.6, 31.9, 35.5, 38.5, 114.5, 144.0, 158.8; HRMS (ESI) m/z calcd for $C_{17}H_{26}N_3S^+$ 304.1842 found: 304.1842 (Δ = 0.0 ppm).

In a similar manner, compounds **4f**–**4h** were synthesized and characterized.

5.22. 5-Amino-6-(phenylthio)-2-cyclohexyl-1*H*-benzo[*d*]imidazole (4f)

Pale brown solid; 57% yield; mp 116–118 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.27–1.35 (m, 1H), 1.42 (qt, 2H, J = 12.79, 3.29 Hz), 1.66 (qd, 2H, J = 12.4, 3.29 Hz), 1.76–1.80 (m, 1H), 1.89 (dt, 2H, J = 13.3, 3.38 Hz), 2.16 (dd, 2H, J = 13.7, 2.01 Hz), 2.88 (tt, 1H, J = 11.8, 3.55 Hz), 4.22 (br, 2H), 6.92 (s, 1H), δ 7.08–7.13 (m, 3H), δ 7.20–7.23 (m, 2H), δ 7.67 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.8, 26.0, 31.8, 38.5, 110.6, 125.3, 126.1, 128.9, 137.6, 144.6; HRMS (ESI) m/z calcd for $C_{19}H_{22}N_3S^+$ 324.1529 found: 324.1529 (Δ = 0.0 ppm).

5.23. 5-Amino-6-(4-fluorophenylthio)-2-cyclohexyl-1*H*-benzo[*d*]imidazole (4g)

Pale green solid; 61% yield; mp 107–108 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.25 (ddd, 1H, J = 13.8, 7.1, 3.6 Hz), 1.33–1.40 (m, 2H), 1.62

(qd, 2H, J = 12.4, 3.1 Hz), 1.71–1.74 (m, 1H), 1.82–1.84 (m, 2H), 2.85 (tt, 1H, J = 11.8, 3.5 Hz), 6.86–6.89 (m, 3H), 7.02–7.05 (m, 2H), 7.70 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.8, 26.0, 31.8, 38.5, 98.8, 111.2, 115.9, 116.1, 124.2, 128.2, 132.4, 144.4, 159.3, 160.2, 162.1; HRMS (ESI) m/z calcd for $C_{19}H_{22}N_3S^+$ 342.1435 found: 342.1435 (Δ = 0.0 ppm).

5.24. 5-Amino-6-(benzylthio)-2-cyclohexyl-1*H*-benzo[*d*]imidazole (4h)

Pale beige solid; 56% yield; mp 129.5–131 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.18–1.23 (m, 1H), 1.27–1.35 (m, 2H), 1.60 (qd, 2H, J = 12.4, 3.2 Hz), 1.69 (d, 1H, J = 12.7 Hz), 1.78 (dt, 2H, J = 13.1, 3.0 Hz), 2.07 (dd, 2H, J = 14.2, 2.5 Hz), 2.80–1.85 (m, 1H), 3.86 (s, 2H), 6.77 (s, 1H), 7.10–7.12 (m, 2H), 7.17 (td, 3H, J = 6.5, 2.8 Hz), 7.49 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.7, 26.0, 31.7, 38.4, 40.7, 64.4, 98.4, 114.0, 123.1, 126.9, 128.3, 128.7, 132.8, 138.2, 139.1, 144.2, 159.1, 176.3; HRMS (ESI) m/z calcd for $C_{20}H_{24}N_3S^+$ 338.1685 found: 338.1686 (Δ = 0.3 ppm).

5.25. 5-Butoxycarbonylamino-2-cyclohexyl-6-(4-fluorophenoxy)-1*H*-benzo[*d*]imidazole (5a)

To a solution of **4a** (100 mg, 0.31 mmol) in 6 mL of dichloromethane was added *N*-butoxycarbonyloxysuccinimide (68 mg, 0.31 mmol) in 6 mL of dichloromethane and the mixture was magnetically stirred under nitrogen atmosphere in an ice bath. The reaction mixture was slowly warmed up to room temperature and stirred for 16 h. The solution was diluted with dichloromethane and basified with $NaHCO_3$ and then washed with water three times. The organic layers were dried over sodium sulfate, filtered, and concentrated. The residue was purified by flash chromatography on silica gel (gradient 20–40% ethyl acetate/hexanes) to afford compound **5a** as an off-white solid (54 mg, 47% yield): mp 91–92 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 0.97 (t, 3H, J = 7.4 Hz), 1.28–1.30 (m, 1H), 1.39–1.45 (m, 4H), 1.61–1.65 (m, 2H), 1.67 (t, 2H, J = 7.5 Hz), 1.76 (d, 1H, J = 12.5 Hz), 1.85–1.88 (m, 2H), 2.12 (d, 2H, J = 12.5 Hz), 2.83–2.88 (m, 1H), 4.19 (t, 2H, J = 6.7 Hz), 6.96–7.05 (m, 4H), 7.13 (s, 1H), 8.23 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.8, 19.1, 25.8, 26.0, 31.0, 31.8, 38.5, 65.3, 116.5, 119.5, 125.6, 142.3, 153.1, 154.0, 157.8, 159.5, 159.6, 159.7; HRMS (ESI) m/z calcd for $C_{24}H_{29}FN_3O_3^+$ 426.2187 found: 426.2187 (Δ = 0.0 ppm). HPLC: t = 7.2 min, purity >98%.

In a similar manner, compound **5j** was synthesized and characterized.

5.26. 6-(Butylthio)-2-cyclohexyl-5-propoxycarbonylamino-1*H*-benzo[d]imidazole (5j)

Off-white solid; 62% yield; mp 133–134.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (t, 3H, J = 7.32 Hz), 1.00 (t, 3H, J = 7.43 Hz), 1.30 (dt, 1H, J = 3.51, 12.5 Hz), 1.38–1.43 (m, 3H), 1.50–1.53 (m, 2H), 1.59–1.67 (m, 4H), 1.74 (q, 2H, J = 7.11 Hz), 1.88 (dt, 2H, J = 3.41, 13.3 Hz), 2.13 (dd, 2H, J = 2.29, 13.4 Hz), 2.69 (t, 2H, J = 7.35 Hz), 2.84–2.89 (m, 1H), 4.26 (t, 2H, J = 6.74 Hz), 7.88 (s, 1H), 8.20 (s, 1H), 8.27 (s, 1H), 8.95 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.4, 13.6, 21.8, 22.3, 25.8, 26.0, 29.7, 31.4, 31.7, 36.9, 38.4, 66.8, 98.5, 100.2, 126.5, 126.6, 134.9, 153.9; HRMS (ESI) m/z calcd for $C_{21}H_{32}N_3O_2S^+$ 390.2210 found: 390.2214 (Δ = 1.0 ppm). HPLC: t = 11.3 min, purity >94%.

5.27. 2-Cyclohexyl-6-(4-fluorophenoxy)-5-(4-methoxybenzamido)-1*H*-benzo[*d*]imidazole (5b)

To a solution of **4d** (100 mg, 0.31 mmol) in 6 mL of dichloromethane was added 4-methoxybenzoyl chloride (42 μL, 0.31 mmol) in 6 mL of dichloromethane, and magnetically stirred in the ice bath. The reaction mixture was slowly warmed up to room temperature and stirred for 16 h. The solution was diluted with dichloromethane, basified with $NaHCO_3$ and then washed with water three times. The organic layers were dried over sodium sulfate, filtered, and concentrated. The residue was purified by flash chromatography on silica gel (gradient 20–40% ethyl acetate/hexanes) to afford compound **5b** as an off-white solid (152 mg, 92% yield): mp >230 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22–1.33 (m, 3H), 1.59 (dd, 2H, J = 12.1, 2.9 Hz), 1.68–1.71 (m, 1H), 1.78–1.81 (m, 2H), 2.01–2.08 (m, 2H), 2.76–2.82 (m, 1H), 3.87 (s, 3H), 6.96 (d, 2H, J = 8.8 Hz), 7.03 (d, 3H, J = 6.3 Hz), 7.78 (d, 2H, J = 8.8 Hz), 8.55 (s, 1H), 8.83 (s, 1H), 9.81 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.8, 19.1, 25.8, 26.0, 31.0, 31.8, 38.5, 65.3, 116.5, 119.5, 125.6, 142.3, 153.1, 154.0, 157.8, 159.5, 159.6, 159.8; HRMS (ESI) m/z calcd for $C_{27}H_{27}FN_3O_3^+$ 460.2031 found: 460.2028 (Δ = −0.7 ppm). HPLC: t = 9.4 min, purity >99%.

In a similar manner, compounds **5c**–**5g** were synthesized and characterized.

5.28. 2-Cyclohexyl-6-(4-fluorophenoxy)-5-(4-methxylbenzamido)-1*H*-benzo[*d*]imidazole (5c)

Off-white solid; 47% yield; mp 166–168 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.81–1.23 (m, 1H), 1.26–1.34 (m, 2H), 1.56–1.64 (qd, 2H, J = 12.4, 2.6 Hz), 1.71 (m, 1H), 1.78–1.81 (d, 2H, J = 12.8 Hz), 2.01 (dd, 2H, J = 12.5, 0.6 Hz), 2.45 (t, 3H), 2.79 (t, 1H, J = 11.5 Hz), 7.05 (d, 3H, J = 6.4 Hz), 7.24 (s, 1H), 7.31 (d, 2H, J = 7.8 Hz), 7.74 (d, 2H, J = 8.1 Hz), 8.63 (s, 1H), 8.90 (s, 1H), 10.2 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.5, 25.7, 25.9, 29.7, 31.7, 38.5, 116.4, 116.6, 119.4, 119.5, 125.3, 126.9, 132.2, 142.6, 153.0, 153.1, 137.9, 159.9, 160.2, 165.8; HRMS (ESI) m/z calcd for $C_{27}H_{27}FN_3O_2^+$ 442.2082 found: 442.2082 (Δ = 0.0 ppm). HPLC: t = 7.7 min, purity >99%.

5.29. 2-Cyclohexyl-5-(2,4-difluorobenzamido)-6-(4-fluorophenoxy)-1*H*-benzo[*d*]imidazole (5d)

Off-white solid; 92% yield; mp 184–185 °C; ^{1}H NMR (500 MHz, $CDCl_3$) 0.83–0.88 (m, 1H), 1.25–1.32 (m, 2H), 1.59 (dd, 2H, J = 12.3, 3.0 Hz), 1.68–1.70 (m, 1H), 1.78–1.80 (m, 2H), 2.07 (d, 2H, J = 12.8 Hz), 2.80–2.84 (m, 1H), 6.90 (ddd, 1H, J = 11.7, 8.8, 2.6 Hz), 6.99 (d, 3H, J = 6.3 Hz), 7.02–7.06 (m, 1H), 7.21 (s, 1H), 8.21 (td, 1H, J = 8.9, 6.6 Hz), 8.81 (s, 1H), 9.26 (d, 1H, J = 15.7 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.9, 26.2, 31.9, 38.2, 101.1, 105.2, 112.5, 112.6, 112.7, 116.5, 116.7, 120.8 120.9, 126.4, 128.8, 138.4, 144.8, 151.7, 158.4, 161.0, 163.1; HRMS (ESI) m/z calcd for $C_{26}H_{23}F_3N_3O_2^+$ 466.1737 found: 466.1743 (Δ = 1.29 ppm). HPLC: t = 4.6 min, purity >96%.

5.30. 2-Cyclohexyl-6-(4-fluorophenoxy)-5-(pent-4-enimido)-1*H*-benzo[*d*]imidazole (5e)

White solid; 63% yield; mp 180.5–181.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.24–1.30 (m, 2H), 1.36–1.44 (m, 2H), 1.59–1.64 (m, 2H), 1.73–1.76 (m, 1H), 1.86 (dq, 2H, J = 3.34, 9.98 Hz), 2.11 (dd, 2H, J = 2.28, 13.3 Hz), 2.48 (dt, 3H, J = 5.88, 11.7 Hz), 2.82–2.87 (m, 1H), 4.99 (d, 1H, J = 10.2 Hz), 5.07 (d, 1H, J = 16.1 Hz), 5.82 (ddt, 1H, J = 6.27, 10.5, 16.9 Hz), 6.95–7.04 (m, 3H), 7.17 (s, 1H), 7.87 (s, 1H), 8.58 (s, 1H), 9.58 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.2, 25.8, 26.0, 29.5, 31.7, 37.3, 38.5, 60.4, 102.7, 108.0, 116.1, 116.3, 116.5, 119.5, 125.2, 136.4, 142.4,157.9, 159.8, 170.7; HRMS (ESI) m/z calcd for $C_{24}H_{26}FN_3O_2^+$ 408.2082 found: 408.2090 (Δ = 1.96 ppm). HPLC: t = 10.3 min, purity >97%.

5.31. 2-Cyclohexyl-5-(4-methxylbenzamido)-6-(phenylthio)-1*H*-benzo[*d*]imidazole (5f)

Off-white solid; 79% yield; mp 184.5–186 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.06–1.20 (m, 3H), 1.53–1.63 (m, 3H), 1.70 (d, 2H, J = 9.5 Hz), 2.00 (d, 2H, J = 11.3 Hz), 2.44 (s, 3H), 2.73 (t, 1H, J = 9.6 Hz), 7.14 (t, 2H, J = 7.62 Hz), 7.22–7.27 (m, 4H), 7.59 (d, 2H, J = 8.0 Hz), 8.06 (s, 1H), 9.01 (s, 1H), 9.39 (s, 1H), 11.0 (s, 1H);^{13}C NMR (125 MHz, $CDCl_3$) δ 21.5, 25.6, 25.9, 29.7, 31.6, 38.5, 103.3, 113.6, 126.0, 126.3, 126.4, 126.9, 129.0, 129.3, 129.6, 132.2, 134.2, 136.6, 142.6, 160.9, 166.1; HRMS (ESI) m/z calcd for $C_{26}H_{34}N_3OS^+$ 436.2417 found: 436.2417 (Δ = 0.0 ppm). HPLC: t = 4.8 min, purity >99%.

5.32. 6-(Benzylthio)-2-cyclohexyl-5-(4-*tert*-butylbenzamido)-1*H*-benzo[*d*]imidazole (5g)

Off-white solid; 92% yield; mp 173–173.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.06–1.23 (m, 3H), 1.40 (s, 9 H), 1.51–1.59 (m, 3H), 1.68 (d, 2H, J = 11.3 Hz), 1.97 (d, 2H, J = 11.8 Hz), 2.67–2.72 (m, 1H), 3.90 (s, 2H), 6.98 (dd, 2H, J = 7.1, 2.3 Hz), 7.04 (dd, 3H, J = 5.0, 1.9 Hz), 7.52 (d, 2H, J = 8.4 Hz), 7.70 (d, 2H, J = 8.4 Hz), 7.96 (s, 1H), 8.93 (s, 1H), 9.38 (s, 1H), 11.1 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.6, 25.9, 31.2, 31.7, 35.1, 38.5, 43.4, 102.2, 116.4, 125.8, 126.9, 127.1, 127.3, 128.5, 132.3, 135.3, 138.0, 140.2, 155.5, 160.8, 165.8; HRMS (ESI) m/z calcd for $C_{31}H_{36}N_3OS^+$ 498.2574 found: 498.2574 (Δ = 0.0 ppm). HPLC: t = 13.8 min, purity >95%.

5.33. 6-(Butylthio)-5-(2,4-difluorobenzamido)-2-cyclohexyl-1*H*-benzo[*d*]imidazole (5h)

Off-white solid; 93% yield; mp 170–170.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83 (t, 3H, J = 7.33 Hz), 1.17–1.29 (m, 3H), 1.36 (dd, 2H, J = 15.0, 7.37 Hz), 1.49–1.60 (m, 4H), 1.67 (d, 1H, J = 12.1 Hz), 1.77 (d, 2H, J = 12.3 Hz), 2.06 (d, 2H, J = 11.9 Hz), 2.73 (t, 2H, J = 7.42 Hz), 2.79 (m, 1H), 6.96–7.09 (m, 2H), 7.93 (s, 1H), 8.23 (td, 1H, J = 8.85, 6.54 Hz), 8.87 (s, 1H), 10.1 (s, 1H), 10.3 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 13.6, 21.7, 25.7, 25.9, 31.3, 31.7, 37.0, 38.5, 104.7, 112.5, 112.7, 117.8, 118.3, 118.4, 133.8, 134.3, 160.5; HRMS (ESI) m/z calcd for $C_{24}H_{28}F_2N_3OS^+$ 444.1916 found: 444.1922 (Δ = 1.35 ppm). HPLC: t = 7.0 min, purity >97%.

5.34. 6-(Butylthio)-5-(4-metylbenzamido)-2-cyclohexyl-1*H*-benzo[*d*]imidazole (5i)

Off-white solid; 34% yield; mp 155–156 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (t, 3H, J = 7.38 Hz), 1.05–1.15 (m, 3H), 1.24 (s, 1H), 1.34 (q, 2H, J = 7.34 Hz), 1.49–1.60 (m, 4H), 1.66 (d, 2H, J = 12.1 Hz), 1.97 (d, 2H, 11.9 Hz), 2.46 (s, 3H), 2.71–2.75 (m, 3H), 7.36 (d, 2H, J = 7.79 Hz), 7.92 (d, 3H, J = 7.73 Hz), 8.99 (s, 1H), 9.84 (s, 1H), 11.4 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ; 13.5, 21.5, 21.8, 25.5, 25.8, 29.6, 31.4, 31.6, 37.2, 38.4, 102.7, 117.1, 126.2, 127.0, 129.7, 132.3, 133.8, 135.2, 140.1, 142.6, 160.7, 165.7; HRMS (ESI) m/z calcd for $C_{25}H_{32}N_3OS^+$ 422.2261 found: 422.2265 (Δ = 0.9 ppm). HPLC: t = 5.2 min, purity >97%.

5.35. Bacterial strains and growth

For evaluation of drug sensitivity, *Mtb* H37Rv was grown in 7H9 media containing 10% oleic acid/albumin/catalase (OADC) enrichment and 0.05% Tween-80 and assessed at mid log phase growth.

5.36. Antibacterial activity

The minimum inhibitory concentration (MIC) was determined by the microplate Alamar Blue assay (MABA)[19] as described previously. Briefly, stock solutions of the compounds were prepared in DMSO and were serially diluted 2-fold in 96-well microtiter plates, and *Mtb* H37Rv strain was added to each well to an OD600 of 0.005. Plates were incubated for 6 days at 37 °C. Alamar Blue (Invitrogen) was added to the plates, and the plates were incubated for an additional 24 h at 37 °C. Plates were monitored for color change, and MIC_{99} was determined in triplicate.

5.37. Cytotoxicity assay

The cytotoxicity of the compounds was tested against Vero cells using MTT assay.[21,24] The cells were grown in DMEM media supplemented with 5% Bovine Serum and 1% Penn Strip and incubated at 37 °C with 5% CO_2. Then, 5000 cells were added to each well of a 96-well plate in 200 μL aliquots. The cells were incubated at 37 °C for 1–2 days. A serial dilution of benzimidazoles 5 dissolved in sterile DMSO was added to the 96-well plates in 200 μL aliquots. The plates were incubated at 37 °C for 3 days. The medium was aspirated and then 40 μL of 0.5 mg/mL MTT in DPBS was added to each well. The plates were then incubated at 37 °C for 3 h. Then, 40 μL of 0.8 M HCl solution were added to dissolve the remaining crystals. Each experiment was run in triplicate. The optical density data was used to calculate IC_{50} values using the Hill slope equation. The IC_{50} values and their standard errors were calculated from the viability-concentration curve using the Four Parameter Logistic Model of Sigmaplot.

5.38. *Mtb*-FtsZ protein expression and preparation[18,25]

Escherichia coli expression plasmid encoding the *ftsz* gene (pET 15b vector) was transformed into 100 μL of BL21 (DE3) cells. The transformed cells were plated onto LB plates, containing 100 μg/mL ampicilin. The antibiotic concentration was kept the same for the following steps. The plates were incubated overnight at 37 °C. The colonies were picked and grown in 10 mL of LB media at 37 °C at 250 rpm. The inoculum was transferred to 1 L of LB media in a 4 L flask and grown to an OD of 0.6 at A600. Then, 1 mM IPTG was added to induce protein expression overnight at 20 °C at 250 rpm. Cells were then pelleted by centrifugation at 3000*g*, flash frozen in liquid nitrogen, and stored at −80 °C until further purification steps. Thawed cells were suspended in 40 mL 50 mM Tris pH 7.5, 500 mM NaCl, 100 mM KCl, 0.1% NP-40 per liter cell culture growth and passed through 3 rounds of cell disrupter (French press) at 27 psi to disrupt cells. Lysed cells were centrifuged at 27,000*g* for 20 min to clear insoluble cellular components and cell wall fractions.

For polymerization assay, protein was purified as follows. Thawed cells were re-suspended in 40 mL of buffer containing 50 mM sodium phosphate pH 7.5, 300 mM sodium chloride, 10 mM imidazole, per liter cell culture growth and sonicated at 15 W 6 times for 30 s each with 1 min pauses in between to disrupt cells. Lysed cells were centrifuged at 44,000*g* for one hour to clear insoluble cellular components. Cleared lysate was applied to Ni^{+2} charged His-bind resin and washed with double the volume of resuspension buffer of 50 mM sodium phosphate pH 7.5, 300 mM sodium chloride, then double the volume of re-suspension buffer of 50 mM sodium phosphate pH 7.5, 300 mM sodium chloride, 60 mM imidazole. FtsZ protein was eluted with 4 × 5 mL portions of 50 mM sodium phosphate pH 7.5, 300 mM NaCl, 500 mM imidazole. Protein was loaded onto a Sephadex G25 size exclusion column to remove excess imidazole and to exchange protein into 25 mM HEPES pH 7.2, 1 mM DTT, 0.1 mM EDTA, 10% glycerol (or other buffer as indicated). Resulting protein fractions were pooled and the N-terminal 6×His affinity tag was removed by biotin tagged thrombin treatment overnight at 4 °C (0.25 Units biotinylated thrombin per mg tagged FtsZ protein). Successive passes

through streptavidin agarose and fresh Ni^{+2} charged His-bind resin removed biotinylated thrombin, uncut FtsZ protein, and free cut off affinity tag. A final cleanup was performed through an Akta driven Sephadex 200 60/16 size exclusion column in 25 mM HEPES pH 7.2, 1 mM DTT, 0.1 mM EDTA, 10% glycerol or other buffer as indicated. Protein was then concentrated to 10 mg/mL (~250 μM) with centrifugal 30 kDa molecular weight cutoff filters, aliquoted 150 μL, flash frozen in liquid nitrogen, and stored at −80 °C.

For K_d studies the protein was purified as follows. Cleared lysate was applied to Ni^{+2} charged His-bind resin and made to equilibrate for one hour followed by washing in two volumes of wash buffer (50 mM Tris pH 7.5, 300 mM NaCl, 100 mM KCl, 0.1% NP-40, and 10 mM Imidazole). Bound protein was eluted with 10 mL of Elution buffer (50 mM Tris pH 7.5, 500 mM NaCl, 100 mM KCl, and 500 mM imidazole). The eluted protein was first dialyased against 2 L Storage buffer (50 mM Tris pH 7.8, 200 mM NaCl, 100 mM KCl), overnight followed by 3 h dialysis against Storage buffer with 10% glycerol. Post dialysis, the concentration of the protein was checked by Bradford assay and purity of the purified protein determined by SDS page. If necessary, protein was further concentrated to 10 mg/mL (~250 μM) with centrifugal 10 kDa molecular weight cutoff filters, aliquoted 500 μL, and flash frozen in liquid nitrogen, and stored at −80 °C till further use.

For TEM analysis the following procedure was followed for protein purification. The cells were suspended in approximately 20–30 mL binding buffer (500 mM NaCl, 20 mM sodium phosphate, pH 7.8) and lysed using cell disruptor. The lysate was centrifuged in an ultracentrifuge at 126,603g, 4 °C for 90 min. The supernatant was filtered and loaded onto Ni^{2+}-NTA column, washed with 50 mL of binding buffer and eluted using a gradient of binding buffer with 30–500 mM imidazole. The eluted protein was dialyzed against buffer containing 50 mM Tris, 5 mM $MgCl_2$, 50 mM KCl, pH 7.2 followed by buffer containing 10% v/v glycerol. The protein after dialysis was concentrated and stored at −80 °C for further use. Since the number of aromatic residues in *Mtb*-FtsZ protein are low (Tyr: 1, Trp: 0), it is not reliable to follow concentration of protein by scanning at A280. The concentration of protein was therefore ascertained using the Bradford kit from Sigma.

5.39. *Mtb*-FtsZ polymerization inhibitory assay

The inhibitory activity of lead benzamidazoles for *Mtb*-FtsZ polymerization was determined by means of light scattering on a PTI-QM4 Fluorescence Master system. The 90° light scattering was measured at 30 °C, using excitation and emission wavelength of 400 nm with slit width of 2 nm. The gain was set at 875 V. *Mtb*-FtsZ (15 μM) was incubated in the polymerization buffer (50 mM MES pH 6.5, 100 mM KCl, 5 m M $MgCl_2$) for up to 300 s. Polymerization was initiated with 100 μM GTP and monitored for up to 30 min. Benzimidazole stocks were prepared in DMSO and incubated with FtsZ enzyme prior to initiation of polymerization with GTP.

5.40. Transmission electron microscopy (TEM) analysis

Stock solution of compound **5a** was prepared in ethanol. *Mtb*-FtsZ (5 μM) was incubated with 40 or 80 μM of **5a** in the polymerization buffer (50 mM MES, 5 mM $MgCl_2$, 100 mM KCl, pH 6.5) for 15 min on ice. To each solution was added GTP to the final concentration of 25 μM. The resulting solution was incubated at 37 °C for 30 min. The incubated solution was diluted 2 times with the polymerization buffer and immediately transferred to carbon coated 300 mesh formvar copper grid and negatively stained with 1% uranyl acetate. The samples were viewed with a FEI Tecnai12 BioTwinG transmission electron microscope at 80 kV. Digital images were acquired with an AMT XR-60 CCD digital camera system.[18]

5.41. Binding studies of 5a with *Mtb*-FtsZ

The fluorescence anisotropy of the compound was measured in the presence of increasing FtsZ concentrations, in 50 mM MES, 100 mM KCl, 5 mM $MgCl_2$ in absence of GTP by exciting the compound at 316 nm and monitoring the change of fluorescence at 427.9 nm using a PTI-QM4 spectrofluorometer. The change in fluorescence (ΔF) at 427.9 nm was fitted into the equation $\Delta F = (\Delta F_{max} \times L)/(K_d + L)$.[23]

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Supplementary data

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