Synthesis and Antibacterial Activity of Cinnamaldehyde Acylhydrazone with a 1,4-Benzodioxan Fragment as a Novel Class of Potent β -Ketoacyl–Acyl Carrier Protein Synthase III (FabH) Inhibitor

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Fatty acid biosynthesis is essential for bacterial survival. β -Ketoacyl–acyl carrier protein (ACP) synthase III (FabH), is a particularly attractive antibacterial target, since it is central to the initiation of fatty acid biosynthesis. Three series of 21 cinnamaldehyde acylhydrazone derivatives, A3–9, B3–9, and C3–9, were synthesized and evaluated for FabH-inhibitory activity. Compound B6 showed the most potent biological activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis* (minimum inhibitory concentrations (MICs) values: 1.56–3.13 μ g/mL) and was comparable with the positive control. Docking simulation by positioning compound B6 in the FabH structure active site was performed to explore the possible binding model.

Key words cinnamaldehyde; acylhydrazone; benzodioxan; antibacterial; structure–activity relationship; FabH inhibitor

Bacteria has become a greater threat to human beings as antibiotic resistance sweeps the world.¹⁾ Fatty acid biosynthesis (FAB) is an essential metabolic process for prokaryotic organisms and is required for cell viability and growth.²⁾ The significant differences between human and prokaryotic FAB systems render prokaryotic FAB to be an attractive target.³⁾ The β -ketoacyl–acyl carrier protein (ACP) synthase III (FabH) has no homologs in human and is one of essential functional enzymes in bacterial FAB system. FabH initiates the FAB cycle by catalyzing the first condensation step between acetyl-CoA and malonyl-ACP, playing a key regulatory role in the bacterial FAB cycle.⁴⁾ Many kinds of compounds harbored Schiff base motif or hydrozone motif was screened for antimicrobial activity, some of which are potent inhibitors of FabH.⁵⁾ For example, compounds 1–8 (Fig. 1A) reported as inhibitors of *Escherichia coli* FabH showed the most potent FabH inhibitory activity in each article respectively.^{6–12)}

1,4-Benzodioxan is an important frame for medicine and widely found in natural products, e.g. silybin, purpureanol, 3,4-dihydroxystyrene dimmers (shown in Fig. 1B). It can also be acquired from more common natural sources.^{13–17)} It was reported that various derivatives of 1,4-benzodioxan show biological activities,^{18–20)} some of which are anticancer agents²¹⁾ or new anti-inflammatory compounds.¹⁸⁾ The aim of this study is to design a series of hydrazone-Schiff bases with a spectrum of antibacterial applications. In view of the findings mentioned above, we report in this present work the synthesis of a series of 1,4-benzodioxan acylhydrazone derivatives (showed in Fig. 1C) to achieve new potential antibacterial FabH inhibitors.

Results and Discussion

Chemistry In this study, twenty-one cinnamaldehyde-acylhydrazone derivatives were designed and synthesized. The synthesis of compounds A3–9; B3–9 and C3–9 fol-

lowed the general pathway outlined in Chart 1. All of them were prepared in three steps, which is based on our previous research.²²⁾ Esterification of the 2,3-dihydrobenzo[b][1,4]-dioxinecarboxylic acid A, B and C with ethanol and thionyl chloride afforded the corresponding ester A1, B1, C1. The hydrazone A2, B2, C2 was obtained by reacting A1, B1, C1 with 85% hydrazine monohydrate in ethanol. The synthesis of compounds A3–9, B3–9, C3–9 was accomplished by reacting the corresponding hydrazone with different substituted cinnamaldehyde in ethanol. The structure of synthesized compounds A3–9, B3–9, C3–9 was confirmed by spectral data.

Antibacterial Evaluation All the synthesized compounds A3–9, B3–9, C3–9 were evaluated for their antimicrobial activity. Two Gram-negative bacterial strains: *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 and two Gram-positive bacterial strains: *Bacillus subtilis* ATCC 6538 and *Staphylococcus aureus* ATCC 530 were tested with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The minimum inhibitory concentrations (MICs) of those compounds against these bacteria were presented in Table 1. Kanamycin B was screened under same conditions for comparison. We also took the starting material A, B, C as comparisons. The results reveal that the synthesized compounds exhibit antibacterial activity compared with the starting materials. Among the 21 synthesized compounds, compound B6 displays the most potent activity with MIC values of 1.53, 3.13, 6.25 and 6.25 μ g/mL against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*, respectively, which is similar to the positive control, broad-spectrum antibiotic kanamycin B with corresponding MIC values between 1.56 and 3.13 μ g/mL. C8 (3.13, 6.25, 6.25, 6.25 μ g/mL) demonstrated a relatively better activity among C3–C9 and also was comparable with kanamycin B. The A series of compounds show higher MIC values than the series of B and C (except compound A9), indicating backbone 2,3-dihydrobenzo[b][1,4]dioxine-2-carboxylic acid decreases the activity (5-carboxylic acid >6-carboxylic acid >2-carboxylic acid). It may be caused by the fact that

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Fig. 1. (A) Some Typical Reported Potential FabH Inhibitors Contained Schiff Base Motif or Hydrozone Motif; (B) Some Natural Products Contained Benzodioxan Motif; (C) The Design of Benzodioxan Cinnamaldehydeacyl-Hydrazone Derivatives

different carboxylic acid substitutions of 2,3-dihydrobenzo[*b*]-[1,4]dioxine orient the compounds differently. Compounds **A3**, **B3**, **C3** with no substituent in aromatic ring of cinnamaldehyde have little activity with MIC values of $>50\mu\text{g/mL}$ against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*, indicating the substituent on aromatic ring of cinnamaldehyde compounds is important.

Subsequently preliminary structure–activity relationship (SAR) studies were conducted to deduce how the structure variation and modification could affect the activity. Within all of the compounds, the **B** series shows the more powerful inhibitory activity than the other series. We evaluated the effect of introducing different substitutions on aromatic ring of cinnamaldehyde in **B3**. Firstly, introducing nitro group on the aromatic ring of cinnamaldehyde in **B3** gives **B9** and **B6** with higher antimicrobial activity. **B6** with the nitro group on the *ortho*-position of aromatic ring of cinnamaldehyde shows MIC values of $1.56\text{--}6.25\mu\text{g/mL}$, which are better than those of **B9** ($6.25\text{--}12.5\mu\text{g/mL}$) with the nitro group on the *para*-position of aromatic ring of cinnamaldehyde. Secondly, compound **B7** bearing the bromine group on the *para*-position also shows potent activity ($3.13\text{--}6.25\mu\text{g/mL}$). It has been shown that introducing electron-withdrawing groups (halogen groups or NO_2) on the aromatic ring of cinnamaldehyde may increase the antimicrobial effects. Thirdly, introducing electron-

donating group (methoxy group) on the aromatic ring of cinnamaldehyde in **B3** gives **B4** and **B8** with little antimicrobial activity, indicating that the electron-donating methoxy group does not increase the antimicrobial effects. The **A** series (**A9** *p*- NO_2 $>$ **A7** *p*-Br $>$ **A4** *o*-F $>$ **A6** *o*- NO_2) also shows the introduction of the electron-withdrawing groups (halogen groups or NO_2) on the aromatic ring of cinnamaldehyde may increase the antimicrobial effects. The **C** series is different from **A** and **B** series, given the fact that **C8** *p*- OCH_3 shows the greatest activity of $3.13\text{--}6.25\mu\text{g/mL}$ and **C5** *o*- OCH_3 has activity in the range of $25\text{--}50\mu\text{g/mL}$.

Finally, the substituent on aromatic ring of cinnamaldehyde will dramatic contributed antimicrobial activity, and for the series **A** and **B** the electron-withdrawing groups may increase the antimicrobial effects.

***E. coli* FabH Inhibitory Activity** In addition, we selected the top 7 compounds **B6**, **C8**, **A9**, **B9**, **B7**, **C6**, **C7** and the bottom 3 compounds **B8**, **A3**, **B4** of the antibacterial activity according to their MIC values to test their activity as *E. coli* FabH inhibitors. The results are summarized in Table 2. All the bottom compounds show poor *E. coli* FabH inhibitory activity and the top compounds display potent *E. coli* FabH inhibitory activity. Among the tested compounds, **B6** shows the most potent inhibitory activity with IC_{50} of $2.5\mu\text{M}$, which is comparable to the positive control dichloro-1,2-dithiacyclop-

Series A	R ₁	R ₂	Series B	R ₁	R ₂	Series C	R ₁	R ₂
A3	H	H	B3	H	H	C3	H	H
A4	F	H	B4	F	H	C4	F	H
A5	OCH ₃	H	B5	OCH ₃	H	C5	OCH ₃	H
A6	NO ₂	H	B6	NO ₂	H	C6	NO ₂	H
A7	H	Br	B7	H	Br	C7	H	Br
A8	H	OCH ₃	B8	H	OCH ₃	C8	H	OCH ₃
A9	H	NO ₂	B9	H	NO ₂	C9	H	NO ₂

Reagents and conditions: i) EtOH, SOCl₂, reflux, overnight; ii) EtOH, 85% hydrazine hydrate, reflux, overnight; iii) EtOH, H₂O, glacial acetic acid 24h.

Chart 1. General Synthesis of Compounds (A3–9; B3–9; C3–9)

tenone (DDCP)²³ with IC₅₀ of 2.9 μM. The results indicate that **B6** has high binding affinity with *E. coli* FabH, which supports the observation. Then, analysis between the antibacterial activity against *E. coli* and the *E. coli* FabH inhibitory activity of the top 7 compounds (**B6**, **C8**, **A9**, **B9**, **B7**, **C6**, **C7**) indicates that there is a moderate correlation ($Y=0.870X+4.266$) between FabH inhibition (Y) and inhibition of *E. coli* cellular proliferation (X) with a correlation coefficient of 0.870 and R square value of 0.885. The compounds selected above were also detected for their hemolytic activity, and all lytic concentration 30% (LC₃₀) were >10 mg/mL.

Docking Study To gain better understanding on the po-

tency of the compounds **B6**, **C8**, **A9** and guide further SAR studies, we proceeded to examine the interaction of these compounds with molecular docking, which was performed by simulating the 21 compounds with a binding model based on the *E. coli* FabH–CoA complex structure (1HNJ.pdb²⁴). The binding modes of potent inhibitor **B6**, **C8**, **A9** with 1HNJ are shown in Figs. 2–4, respectively.

In the binding model (Fig. 2), compound **B6** is nicely bound to 1HNJ via π -sigma interactions and π -cation interactions. ARG36 (distance: 5.87 Å) forms a π -cation interaction with 5-carboxylic acid, and ALA246 (distance: 2.77 Å) engenders a π -sigma interaction with aromatic ring of cinnamaldehyde.

Table 1. Antibacterial Activity of the Synthesized 1,4-Benzodioxan Derivatives Compounds (**A3–A9**, **B3–B9**, **C3–C9**, **A**, **B**, **C**)

Compound	Minimum inhibitory concentrations ($\mu\text{g/mL}$)			
	Gram-negative		Gram-positive	
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>B. subtilis</i>
A3	50	50	>50	>50
A4	25	25	50	>50
A5	25	25	25	25
A6	25	25	50	25
A7	25	25	25	12.5
A8	25	12.5	12.5	12.5
A9	3.13	3.13	6.25	6.25
B3	50	25	50	50
B4	>50	>50	>50	>50
B5	25	25	25	25
B6	1.56	3.13	6.25	6.25
B7	6.25	3.13	6.25	6.25
B8	50	50	50	50
B9	6.25	6.25	12.5	6.25
C3	50	50	>50	>50
C4	25	25	25	50
C5	25	50	25	25
C6	12.5	12.5	12.5	12.5
C7	12.5	12.5	12.5	12.5
C8	3.13	6.25	6.25	6.25
C9	25	12.5	25	25
A	>50	>50	>50	>50
B	>50	>50	>50	>50
C	>50	>50	>50	>50
Kanamycin B	1.56	1.56	3.13	3.13

Table 2. *E. coli* FabH Inhibitory Activity of the Selected Compounds

Compounds	<i>E. coli</i> FabH IC ₅₀ (μM)	Hemolysis LC ₃₀ ^{a)} (mg/mL)
B6	3.5 $\pm$ 0.31	>10
C8	6.6 $\pm$ 0.52	>10
A9	9.0 $\pm$ 0.13	>10
B9	9.4 $\pm$ 0.28	>10
B7	11.3 $\pm$ 0.47	>10
C6	14.7 $\pm$ 1.09	>10
C7	14.8 $\pm$ 0.43	>10
B8	59.1 $\pm$ 0.71	>10
A3	85.7 $\pm$ 0.92	>10
B4	>100	>10
DDCP	2.9	>10

a) Lytic concentration 30%.

It indicates that substituted electron-withdrawing groups enhance the binding between the receptor and the aromatic ring of cinnamaldehyde. As for compound **A9** (Fig. 3), 2,3-dihydrobenzo[*b*][1,4]dioxine-2-carboxylic acid scaffold contributes one hydrogen bond, and NO₂ substitution group makes an electronegativity interaction. The hydrogen of guanidyl in ARG36 (distance: 2.6 Å) contributes to the hydrogen bonding interaction (O...H-N: 2.37 Å, 143.280°) with the 4-oxygen atom of [1,4]dioxine-2-carboxylic acid. Furthermore, the *p*-NO₂ aromatic ring of cinnamaldehyde formed an electronegativity interaction with HIS244 (distance: 3.71 Å), which explains why **A9** (*p*-NO₂) substitution shows lower

MIC than other substitution group. And **C8** (Fig. 4) shows different binding to 1HNJ compared with **A** and **B** series. The 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carboxylic acid contributed two hydrogen bonds binding to the receptor. The oxygen atom of dioxine contributes to the hydrogen bonding interaction (O...H-N: 2.41 Å, 146.198°) with the backbone amino hydrogen atom of ASN210 (O...H-N: 2.01 Å, 143.280°) with the backbone amino hydrogen atom of GLY209. The steric hindrance of cinnamaldehyde substituted contributes more than electronegativity interaction.

Conclusion

To conclude, 3 series of compounds, **A3–9**, **B3–9** and **C3–9**, have been synthesized and evaluated for their antibacterial activities. Compound **B6** displayed the most potent activity against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*, respectively, with MIC values of 1.53, 3.13, 6.25 and 6.25 $\mu\text{g/mL}$, being comparable with the positive control. **A9**, **C8** also displayed potent activity 3.13–6.25 $\mu\text{g/mL}$ correlated with FabH inhibition. These new derivatives demonstrated strong broad-spectrum antimicrobial activity. Docking simulation was performed to position compound **B6**, **A9** and **C8** into the *E. coli* FabH structure active site to determine the probable binding model. It provided further insight into interactions between the enzyme and its ligands. This study shows that cinnamaldehydeacyl-hydrazone with 1,4-benzodioxan fragment are novel compounds that can be potent antimicrobial inhibitor of FabH and provides valuable information for the design of *E. coli* FabH inhibitors as antibacterial agents.

Fig. 2. (A) 2D Molecular Docking Modeling of Compound **B6** with 1HNJ; (B) 3D Model of the Interaction between Compound **B6** and 1HNJ Bonding Site

The π -cation interactions and π -sigma interaction are shown as orange lines with their corresponding amino acids labeled in yellow. (Color images were converted into gray scale.)

Experimental

Chemistry All chemicals used were purchased from J&K (China). The elution were monitored using Thin layer chromatography (TLC). TLC was run on the silica gel coated aluminum sheets (Silica Gel 60 Å GF254, E. Merck, Germany) and visualized in UV light (254nm). Separation of the compounds by column chromatography was carried out with silica gel 60 (200–300 mesh ASTM, E. Merck, Germany). Developed plates were visualized by Spectroline ENF 260C/F UV apparatus. The quantity of silica gel used was 50–100 times the weight charged on the column. Melting points (uncorrected) were determined on a XT4MP apparatus (Taikē Corp., Beijing, China). Electrospray ionization (ESI) mass spectra were obtained on a Mariner System 5304 mass spectrometer, and $^1\text{H-NMR}$ spectra were recorded on a DPX300 spectrometer at 25°C with TMS and solvent signals allotted as internal standards. Chemical shifts are reported in ppm (δ). Elemental analyses were performed on a CHN-O-Rapid instrument and were within 0.4% of the theoretical values.

General Method of Synthesis of Ethyl 2,3-Dihydrobenzo[b]-[1,4]dioxinecarboxylate (A1, B1 and C1) 2,3-Dihydrobenzo[b]-[1,4]dioxinecarboxylic acid **A**, **B** and **C** (10mmol) in ethanol (200mL) was treated with thionyl chloride (5mL) under reflux

Fig. 3. (A) 2D Molecular Docking Modeling of Compound **A9** with 1HNJ; (B) 3D Model of the Interaction between Compound **A9** and 1HNJ Bonding Site

The one H-bond (green line) is displayed as dotted lines and the amino acid it acts on is labeled in green. (Color images were converted into gray scale.)

overnight. Excess ethanol was distilled out and the solid left was dissolved in ethyl acetate (150mL), washed with saturation sodium bicarbonate (200mL) and then sodium chloride (200mL). After drying the organic layer with anhydrous Na_2SO_4 and evaporating the solvent under reduced pressure and solid appeared. The solid was recrystallized from ethanol to obtain the compound **A1**, **B1** and **C1**. The completion of the reaction was monitored on TLC by using silica gel-G coated plates by using ethyl acetate and petroleum ether (1 : 1) as the elution and observed in UV light.

General Method of Synthesis of Ethyl 2,3-Dihydrobenzo[b]-[1,4]dioxinecarbohydrazide (A2, B2 and C2) A stirred solution of compound **A1**, **B1** and **C1** (5mmol) in ethanol (200mL) was treated with the hydrazine hydrate (85%), under reflux overnight. Excessive ethanol was distilled out and the contents were allowed to cool. The crystals formed was filtered, washed thoroughly with water and dried to obtain the compound **A2**, **B2** and **C2**.

General Procedure for Synthesis of the Target Compounds To dissolve hydrazide **A2**, **B2** and **C2** (1mmol) and Cinnamaldehydes (1mmol) in ethanol (15mL), water (1mL) was added followed by dropwise addition of glacial acetic acid (0.2mL). The resulting mixture was stirred at room temperature until the target product precipitated from the solvent, which was collected using suction filtration and dried, followed by recrystallization in ethanol.

(E)-N'-((E)-3-Phenylallylidene)-2,3-dihydrobenzo[b][1,4]-

Fig. 4. (A) 2D Molecular Docking Modeling of Compound **C8** with 1HNJ; (B) 3D Model of the Interaction between Compound **C8** and 1HNJ Bonding Site

The two H-bonds (green line) are displayed as dotted lines and the amino acid it acts on is labeled in green. (Color images were converted into gray scale.)

dioxine-2-carbohydrazide (A3) Light yellow powder, yield 73%; mp 169–171°C. $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ : 4.47 (1H, s), 4.73 (1H, s), 5.01 (1H, s), 6.86 (1H, s), 6.92–6.98 (4H, m), 7.23–7.26 (1H, m), 7.33–7.40 (3H, m), 7.58–7.63 (3H, m), 10.63 (1H, s). $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ : 64.2 (t), 70.1 (d), 117.5 (d), 117.9 (d), 120.5 (d), 122.4 (d), 127.4 (d), 128.1 (d), 128.9 (d), 129.1 (d), 137.1 (d), 144.0 (d), 146.2 (d), 151.5 (d), 165.9 (s). ESI-MS m/z : 309.1 ($\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_3$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_3$: C, 70.12; H, 5.23; N, 9.09; Found: C, 70.17; H, 5.25; N, 9.12%.

(E)-N'-((E)-3-(2-Fluorophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-2-carbohydrazide (A4) Light yellow powder, yield 76%; mp 171–173°C. $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ : 4.48 (1H, s), 4.72 (1H, s), 5.00 (1H, s), 6.70 (1H, s), 6.88–6.92 (4H, m), 7.10–7.15 (3H, m), 7.34 (1H, d, $J=5.74$), 7.52 (1H, d, $J=4.47$), 7.62–7.66 (1H, m), 10.57 (1H, s). $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ : 64.3 (t), 70.2 (d), 115.6 (d, $J=20.1$ C20-F coupling), 117.4 (d), 117.9 (d), 120.5 (d), 122.3 (d), 122.6 (d, $J=7.6$ C17-F coupling), 124.6 (d, $J=21.3$ C18-F coupling), 125.3 (d), 126.6 (d), 128.3 (d), 129.0 (d, $J=7.2$ C21-F coupling), 144.0 (d), 146.1 (d), 151.1 (d), 162.1 (d, $J=251.2$ C19-F coupling), 167.1 (s). ESI-MS m/z : 327.1 ($\text{C}_{18}\text{H}_{15}\text{FN}_2\text{O}_3$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{FN}_2\text{O}_3$: C, 66.25; H, 4.63; N, 8.58; Found: C, 66.21; H, 4.59; N, 8.62%.

(E)-N'-((E)-3-(2-Methoxyphenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-2-carbohydrazide (A5) Light

yellow powder, yield 78%; mp 172–174°C. $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ : 3.76 (3H, s), 4.46 (1H, s), 4.77 (1H, s), 5.10 (1H, s), 6.68–6.70 (1H, m), 6.90–7.02 (7H, m), 7.22 (1H, t, $J=7.68$ Hz), 7.55 (1H, d, $J=5.09$ Hz), 7.68 (1H, d, $J=4.31$ Hz), 10.35 (1H, s). $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ : 57.1 (q), 64.3 (t), 71.0 (d), 113.1 (d), 117.4 (d), 117.9 (d), 120.5 (d), 121.2 (d), 122.2 (d), 127.5 (d), 128.4 (d), 130.1 (d), 132.9 (d), 144.0 (d), 146.8 (d), 151.5 (d), 157.1 (d), 165.9 (s). ESI-MS m/z : 339.2 ($\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$: C, 67.44; H, 5.36; N, 8.28; Found: C, 67.42; H, 5.31; N, 8.30%.

(E)-N'-((E)-3-(2-Nitrophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-2-carbohydrazide (A6) Yellow powder, yield 53%; mp 185–187°C. $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ : 4.36 (1H, s), 4.43 (1H, s), 4.99 (1H, s), 6.82–6.90 (5H, m), 7.35 (1H, d, $J=4.37$), 7.53 (1H, d, $J=5.12$), 7.81–7.94 (2H, m), 8.15–8.21 (2H, m), 10.58 (1H, s). $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ : 64.3 (t), 70.9 (d), 117.4 (d), 117.9 (d), 120.5 (d), 122.1 (d), 126.4 (d), 126.9 (d), 128.7 (d), 130.4 (d), 131.1 (d), 131.6 (d), 132.9 (d), 144.0 (d), 146.8 (d), 147.0 (d), 151.5 (d), 166.2 (s). ESI-MS m/z : 354.1 ($\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$: C, 61.19; H, 4.28; N, 11.89; Found: C, 61.21; H, 4.18; N, 12.16%.

(E)-N'-((E)-3-(4-Bromophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-2-carbohydrazide (A7) Brown powder, yield 75%; mp 201–203°C. $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ : 4.32 (1H, s), 4.61 (1H, s), 4.91 (1H, s), 6.94–7.06 (5H, m), 7.21 (1H, d, $J=2.58$ Hz), 7.56–7.65 (5H, m), 10.79 (1H, s). $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ : 64.2 (t), 70.1 (d), 117.5 (d), 117.9 (d), 120.5 (d), 122.4 (d), 123.1 (d), 128.1 (d), 128.9 (d), 129.1 (d), 131.5 (d), 137.5 (d), 144.0 (d), 146.2 (d), 151.5 (d), 165.9 (s). ESI-MS m/z : 387.0 ($\text{C}_{18}\text{H}_{15}\text{BrN}_2\text{O}_3$, $[\text{M}+\text{H}]^+$); 389.0 ($[\text{M}+2+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{BrN}_2\text{O}_3$: C, 55.83; H, 3.90; N, 7.23; Found: C, 55.82; H, 3.93; N, 7.21%.

(E)-N'-((E)-3-(4-Methoxyphenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-2-carbohydrazide (A8) Light yellow powder, yield 88%; mp 169–171°C. $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ : 3.92 (3H, s), 4.27–4.33 (1H, m), 4.57–4.62 (1H, m), 4.87–4.90 (1H, m), 6.89–6.96 (7H, m), 7.32 (1H, d, $J=2.37$ Hz), 7.38–7.42 (1H, m), 7.61–7.65 (2H, m), 10.80 (1H, s). $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ : 56.4 (q), 64.4 (t), 71.0 (d), 114.4 (d), 117.6 (d), 117.9 (d), 120.5 (d), 122.1 (d), 128.1 (d), 128.8 (d), 129.6 (d), 130.3 (d), 144.0 (d), 146.2 (d), 151.5 (d), 160.4 (d), 165.9 (s). ESI-MS m/z : 339.1 ($\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$: C, 67.44; H, 5.36; N, 8.28; Found: C, 67.42; H, 5.36; N, 8.32%.

(E)-N'-((E)-3-(4-Nitrophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-2-carbohydrazide (A9) Brown powder, yield 70%; mp 189–191°C. $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ : 4.43 (2H, s), 5.06 (1H, s), 6.85–6.89 (3H, m), 6.91–7.04 (1H, m), 7.14 (1H, d, $J=3.72$ Hz), 7.35–7.45 (2H, m), 8.10 (2H, d, $J=2.40$ Hz), 8.30 (2H, d, $J=2.43$ Hz), 10.49 (1H, s). $^{13}\text{C-NMR}$ (75 MHz, $\text{DMSO-}d_6$) δ : 64.3 (t), 71.0 (d), 117.5 (d), 117.9 (d), 120.6 (d), 122.2 (d), 124.4 (d), 128.1 (d), 128.3 (d), 128.8 (d), 144.1 (d), 146.2 (d), 147.3 (d), 151.5 (d), 166.1 (s). ESI-MS m/z : 354.2 ($\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$: C, 61.19; H, 4.28; N, 11.89; Found: C, 61.20; H, 4.30; N, 11.85%.

(E)-N'-((E)-3-Phenylallylidene)-2,3-dihydrobenzo[b][1,4]dioxine-5-carbohydrazide (B3) Light yellow powder, yield 71%; mp 145–147°C. $^1\text{H-NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ : 4.54 (4H, s), 6.93 (1H, t, $J=7.50$ Hz), 7.00–7.11 (2H, m), 7.33–7.40

(4H, m), 7.48–7.51 (2H, m), 7.63–7.87 (2H, m), 10.55 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.6 (t), 63.3 (t), 119.7 (d), 121.0 (d), 122.4 (d), 122.7 (d), 127.4 (d), 128.1 (d), 128.8 (d), 137.1 (d), 144.4 (d), 150.1 (d), 150.8 (d), 151.5 (d), 163.5 (s). ESI-MS m/z : 309.1 ($\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_3$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_3$: C, 70.12; H, 5.23; N, 9.09; Found: C, 70.10; H, 5.27; N, 9.10%.

(E)-N'-((E)-3-(2-Fluorophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-5-carbohydrazide (B4) Light yellow powder, yield 74%; mp 143–145°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 4.35 (4H, s), 6.78–6.95 (2H, m), 7.15–7.20 (3H, m), 7.35–7.40 (2H, m), 7.53–7.65 (3H, m), 10.89 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.5 (t), 63.3 (t), 70.2 (d), 115.6 (d, $J=20.2$ C20-F coupling), 119.3 (d), 121.1 (d), 122.4, 122.8 (d), 124.7 (d, $J=21.1$ C18-F coupling), 125.4 (d), 126.5 (d), 128.3 (d, $J=8.6$ C23-F coupling), 129.0 (d, $J=7.6$ C21-F coupling), 141.5 (d), 149.9 (d), 151.1 (d), 162.1 (d, $J=251.8$ C19-F coupling), 163.6 (s). ESI-MS m/z : 327.1 ($\text{C}_{18}\text{H}_{15}\text{FN}_2\text{O}_3$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{FN}_2\text{O}_3$: C, 66.25; H, 4.63; F, 5.82; N, 8.58. Found: C, 66.27; H, 5.90; N, 8.60%.

(E)-N'-((E)-3-(2-Methoxyphenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-5-carbohydrazide (B5) Light yellow powder, yield 71%; mp 169–171°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 3.81 (3H, s), 4.35 (4H, s), 6.78–7.05 (5H, m), 7.36–7.43 (3H, m), 7.55 (1H, d, $J=4.56$ Hz), 7.67 (1H, d, $J=4.03$ Hz), 10.80 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 57.1 (q), 61.5 (t), 63.3 (t), 113.0 (d), 119.7 (d), 121.0 (d), 121.3 (d), 122.4 (d), 122.7 (d), 127.4 (d), 128.4 (d), 130.1 (d), 132.9 (d), 141.3 (d), 149.5 (d), 150.9 (d), 157.5 (d), 163.6 (s). ESI-MS m/z : 339.2 ($\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$: C, 67.44; H, 5.36; N, 8.28. Found: C, 67.49; H, 5.22; N, 8.40%.

(E)-N'-((E)-3-(2-Nitrophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-5-carbohydrazide (B6) Brown powder, yield 57%; mp 189–191°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 4.37 (4H, s), 6.75 (1H, d, $J=2.73$ Hz), 7.20 (1H, d, $J=3.42$ Hz), 7.35–7.50 (4H, m), 7.80–7.86 (2H, m), 8.13–8.21 (2H, m), 10.55 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.6 (t), 63.3 (t), 119.6 (d), 121.1 (d), 122.4 (d), 122.8 (d), 126.5 (d), 126.9 (d), 128.4 (d), 130.5 (d), 131.1 (d), 131.5 (d), 132.9 (d), 141.3 (d), 149.8 (d), 150.9 (d), 161.9 (s). ESI-MS m/z : 354.3 ($\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$: C, 61.19; H, 4.28; N, 11.89; Found: C, 61.32; H, 4.33; N, 11.95%.

(E)-N'-((E)-3-(4-Bromophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-5-carbohydrazide (B7) Brown powder, yield 79%; mp 186–188°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 4.54 (4H, s), 6.97–7.05 (2H, m), 7.09–7.12 (1H, m), 7.40–7.48 (3H, m), 7.56–7.64 (4H, m), 10.94 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.6 (t), 63.3 (t), 119.6 (d), 121.1 (d), 122.4 (d), 122.6 (d), 122.8 (d), 128.1 (d), 128.4 (d), 129.8 (d), 131.6 (d), 137.5 (d), 141.4 (d), 149.8 (d), 150.9 (d), 161.8 (s). ESI-MS m/z : 387.0 ($\text{C}_{18}\text{H}_{15}\text{BrN}_2\text{O}_3$, $[\text{M}+\text{H}]^+$); 389.0 ($[\text{M}+2+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{BrN}_2\text{O}_3$: C, 55.83; H, 3.90; Br, 20.63; N, 7.23; Found: C, 55.79; H, 3.91; N, 7.33%.

(E)-N'-((E)-3-(4-Methoxyphenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-5-carbohydrazide (B8) Light yellow powder, yield 81%; mp 160–162°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 3.80 (3H, s), 4.31 (4H, s), 6.51–6.55 (2H, m), 6.92–6.95 (1H, m), 7.05–7.10 (1H, m), 7.16–7.19 (1H, m), 7.39–7.45 (2H, m), 7.60–7.66 (3H, m), 10.94 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 56.2 (q), 61.6 (t), 63.3 (t),

114.5 (d), 119.6 (d), 121.1 (d), 122.4 (d), 122.7 (d), 128.1 (d), 128.4 (d), 129.8 (d), 130.6 (d), 141.4 (d), 149.8 (d), 150.9 (d), 160.7 (d), 161.8 (s). ESI-MS m/z : 339.3 ($\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$: C, 67.44; H, 5.36; N, 8.28; Found: C, 67.41; H, 5.32; N, 8.33%.

(E)-N'-((E)-3-(4-Nitrophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-5-carbohydrazide (B9) Yellow powder, yield 85%; mp 210–212°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 4.36 (4H, s), 7.13–7.17 (2H, m), 7.34–7.36 (2H, m), 7.48–7.51 (2H, m), 8.10 (2H, d, $J=5.12$), 8.35 (2H, d, $J=5.21$), 10.92 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.6 (t), 63.3 (t), 119.6 (d), 121.1 (d), 122.4 (d), 122.7 (d), 124.5 (d), 128.1 (d), 128.3 (d), 128.9 (d), 141.4 (d), 144.3 (d), 147.6 (d), 149.8 (d), 150.9 (d), 161.9 (s). ESI-MS m/z : 354.4 ($\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{N}_3\text{O}_5$: C, 61.19; H, 4.28; N, 11.89; Found: C, 61.12; H, 4.33; N, 11.96.

(E)-N'-((E)-3-Phenylallylidene)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (C3) Light yellow powder, yield 87%; mp 172–174°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 4.32 (4H, s), 6.90–6.95 (2H, m), 7.22 (1H, d, $J=8.22$ Hz), 7.40–7.54 (5H, m), 7.60–7.68 (3H, m), 11.34 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.8 (t), 111.5 (d), 119.6 (d), 121.9 (d), 122.4 (d), 122.9 (d), 127.5 (d), 128.1 (d), 128.3 (d), 128.8 (d), 137.2 (d), 145.7 (d), 149.5 (d), 150.9 (d), 163.1 (s). ESI-MS m/z : 309.3 ($\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_3$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_3$: C, 70.12; H, 5.23; N, 9.09; Found: C, 70.03; H, 5.21; N, 9.14%.

(E)-N'-((E)-3-(2-Fluorophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (C4) Light yellow powder, yield 68%; mp 178–180°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 4.31 (4H, s), 6.88 (1H, d, $J=4.77$ Hz), 7.00–7.04 (2H, m), 7.12 (2H, m), 7.36 (1H, m), 7.48–7.60 (4H, m), 11.05 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.6 (t), 111.9 (d), 115.7 (d, $J=20.9$ C20-F coupling), 122.0 (d), 122.2 (d), 122.4 (d, $J=7.7$ C17-F coupling), 122.9 (d), 124.5 (d, $J=20.1$ C18-F coupling), 125.4 (d), 126.5 (d), 128.3 (d, $J=8.6$ C23-F coupling), 129.0 (d, $J=7.2$ C21-F coupling), 145.4 (d), 149.6 (d), 150.9 (d), 161.3 (d, $J=251.9$ C19-F coupling), 163.2 (s). ESI-MS m/z : 327.3 ($\text{C}_{18}\text{H}_{15}\text{FN}_2\text{O}_3$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{18}\text{H}_{15}\text{FN}_2\text{O}_3$: C, 66.25; H, 4.63; N, 8.58; Found: C, 66.27; H, 4.64; N, 8.63%.

(E)-N'-((E)-3-(2-Methoxyphenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (C5) Yellow powder, yield 75%; mp 171–173°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 3.82 (3H, s), 4.31 (4H, s), 6.81 (1H, d, $J=8.76$ Hz), 6.96–7.03 (3H, m), 7.20–7.25 (2H, m), 7.48–7.53 (2H, m), 7.64 (1H, s), 7.71 (1H, d, $J=8.58$ Hz), 11.16 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 56.9 (q), 61.8 (t), 111.4 (d), 113.0 (d), 121.6 (d), 121.9 (d), 122.4 (d), 122.9 (d), 127.5 (d), 128.3 (d), 131.8 (d), 133.2 (d), 145.5 (d), 149.5 (d), 150.9 (d), 159.4 (d), 163.5 (s). ESI-MS m/z : 339.2 ($\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$, $[\text{M}+\text{H}]^+$). *Anal.* Calcd for $\text{C}_{19}\text{H}_{18}\text{N}_2\text{O}_4$: C, 67.44; H, 5.36; N, 8.28; Found: C, 67.49; H, 5.37; N, 8.21%.

(E)-N'-((E)-3-(2-Nitrophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (C6) Brown powder, yield 82%; mp 191–193°C. ^1H -NMR (300 MHz, DMSO- d_6) δ : 4.33 (4H, s), 6.73 (1H, d, $J=3.09$ Hz), 7.15 (1H, d, $J=4.76$ Hz), 7.35–7.38 (1H, m), 7.47–7.53 (2H, m), 7.63 (1H, s), 7.80–7.84 (2H, m), 8.03 (1H, d, $J=6.93$ Hz), 8.21 (1H, d, $J=7.02$ Hz), 10.96 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.8 (t), 111.4 (d), 121.9 (d), 122.4 (d), 122.9 (d), 126.4 (d), 128.7 (d), 130.5 (d), 131.2 (d), 131.5 (d), 133.0 (d), 145.5

(d), 146.4 (d), 149.5 (d), 150.9 (d), 163.3 (s). ESI-MS m/z : 354.2 ($C_{18}H_{15}N_3O_5$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{15}N_3O_5$: C, 61.19; H, 4.28; N, 11.89; Found: C, 61.17; H, 4.24; N, 11.97%.

(E)-N'-((E)-3-(4-Bromophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (C7) Brown powder, yield 69%; mp 210–212°C. 1H -NMR (300 MHz, DMSO- d_6) δ : 4.32 (4H, s), 6.97 (1H, d, $J=8.40$ Hz), 7.17–7.25 (2H, m), 7.48–7.55 (4H, m), 7.65 (1H, s), 7.70–7.74 (2H, m), 10.93 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.6 (t), 111.5 (d), 121.9 (d), 122.4 (d), 122.9 (d), 128.1 (d), 128.9 (d), 129.6 (d), 131.6 (d), 137.2 (d), 145.7 (d), 149.4 (d), 150.9 (d), 163.4 (s). ESI-MS m/z : 387.0 ($C_{18}H_{15}BrN_2O_3$, $[M+H]^+$); 389.0 ($[M+2+H]^+$). Anal. Calcd for $C_{18}H_{15}BrN_2O_3$: C, 55.83; H, 3.90; N, 7.23; Found: C, 55.71; H, 3.98; N, 7.27%.

(E)-N'-((E)-3-(4-Methoxyphenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (C8) Yellow powder, yield 80%; mp 178–180°C. 1H -NMR (300 MHz, DMSO- d_6) δ : 3.85 (3H, s), 4.26 (4H, s), 6.80 (1H, d, $J=5.42$ Hz), 6.91 (2H, d, $J=8.40$ Hz), 7.26–7.29 (2H, m), 7.46–7.52 (5H, m), 11.42 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 56.1 (q), 61.6 (t), 111.5 (d), 114.1 (d), 121.9 (d), 122.4 (d), 122.9 (d), 128.1 (d), 128.9 (d), 129.6 (d), 131.0 (d), 145.5 (d), 149.4 (d), 151.0 (d), 161.3 (d), 163.4 (s). ESI-MS m/z : 339.2 ($C_{19}H_{18}N_2O_4$, $[M+H]^+$). Anal. Calcd for $C_{19}H_{18}N_2O_4$: C, 67.44; H, 5.36; N, 8.28; Found: C, 67.36; H, 5.29; N, 8.42%.

(E)-N'-((E)-3-(4-Nitrophenyl)allylidene)-2,3-dihydrobenzo[b][1,4]dioxine-6-carbohydrazide (C9) Brown powder, yield 77%; mp 211–214°C. 1H -NMR (300 MHz, DMSO- d_6) δ : 4.31 (4H, s), 7.13–7.17 (2H, m), 7.38–7.40 (2H, m), 7.50–7.56 (2H, m), 8.10 (2H, m), 8.26 (2H, d, $J=7.68$ Hz), 11.34 (1H, s). ^{13}C -NMR (75 MHz, DMSO- d_6) δ : 61.6 (t), 111.5 (d), 121.9 (d), 122.4 (d), 122.9 (d), 124.6 (d), 128.1 (d), 128.4 (d), 128.9 (d), 145.5 (d), 147.3 (d), 149.4 (d), 151.0 (d), 163.4 (s). ESI-MS m/z : 354.3 ($C_{18}H_{15}N_3O_5$, $[M+H]^+$). Anal. Calcd for $C_{18}H_{15}N_3O_5$: C, 61.19; H, 4.28; N, 11.89; Found: C, 61.09; H, 4.40; N, 11.95%.

Biology: Bacterial Suppressive Assay The antibacterial activity of the synthesized compounds was tested against *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 530 using MH medium (Mueller–Hinton medium: casein hydrolysate 17.5 g, soluble starch 1.5 g, beef extract 1000 mL). The MIC values of the tested compounds were determined by a colorimetric method using the dye MTT. A stock solution of the synthesized compound (100 μ g/mL) in dimethyl sulfoxide (DMSO) was prepared and graded quantities of the test compounds were incorporated in specified quantity of sterilized liquid MH medium. A specified quantity of the medium containing the compound was poured into microtitration plates. Suspension of the microorganism was prepared to contain approximately 10^5 cfu/mL and applied to microtitration plates with serially diluted compounds in DMSO to be tested and incubated at 37°C for 24 h. After the MICs were visually determined on each of the microtitration plates, 50 μ L of phosphate buffered saline (PBS) 0.01 mol/L, pH 7.4: $Na_2HPO_4 \cdot 12H_2O$ 2.9 g, KH_2PO_4 0.2 g, NaCl 8.0 g, KCl 0.2 g, distilled water 1000 mL) containing 2 mg of MTT/mL was added to each well. Incubation was continued at room temperature for 4–5 h. The content of each well was removed, and 100 μ L of isopropanol containing 5% 1 mol/L HCl was added to extract the dye. After 12 h of incubation at room

temperature, the optical density (OD) was measured with a microplate reader at 550 nm.

E. coli FabH Inhibitory Assay Native *E. coli* FabH protein was overexpressed in *E. coli* BL21 (DE3) cells using the pET30 vector and purified to homogeneity in three chromatographic steps (Q-Sepharose, MonoQ, and hydroxyapatite) at 4°C. The selenomethionine-substituted protein was expressed in *E. coli* BL21 (DE3) cells and purified in a similar way. Harvested cells containing FabH were lysed by sonication in 20 mM Tris, pH 7.6, 5 mM imidazole, 0.5 M NaCl and centrifuged at 20000 rpm for 30 min. The supernatant was applied to a Ni-NTA agarose column, washed, and eluted using a 5–500 mM imidazole gradient over 20 column volumes. Eluted protein was dialyzed against 20 mM Tris, pH 7.6, 1 mM dithiothreitol (DTT), and 100 mM NaCl. Purified FabHs were concentrated up to 2 mg/mL and stored at –80°C in 20 mM Tris, pH 7.6, 100 mM NaCl, 1 mM DTT, and 20% glycerol for enzymatic assay. In a final 20 μ L reaction, 20 mM Na_2HPO_4 , pH 7.0, 0.5 mM DTT, 0.25 mM $MgCl_2$, and 2.5 μ M holo-ACP were mixed with 1 nM FabH, and H_2O was added to 15 μ L. After 1 min incubation, a 2 μ L mixture of 25 μ M acetyl-CoA, 0.5 mM reduced nicotinamide adenine dinucleotide (NADH), and 0.5 mM NADPH was added for FabH reaction for 25 min. The reaction was stopped by adding 20 μ L of ice-cold 50% trichloroacetic acid (TCA), incubating for 5 min on ice, and centrifuging to pellet the protein. The pellet was washed with 10% ice-cold TCA and resuspended with 5 μ L of 0.5 M NaOH. The incorporation of the 3H signal in the final product was read by liquid scintillation. When determining the inhibition constant (IC_{50}), inhibitors were added from a concentrated DMSO stock such that the final concentration of DMSO did not exceed 2%.

Docking Study The three-dimensional structures of test compounds were constructed using Chem. 3D ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft Corporation, U.S.A. (2010)], then they were energetically minimized by using MMFF94 with 5000 iterations and minimum RMS gradient of 0.10. The crystal structures of *E. coli* FabH (PDB code: 1HNJ) complex were retrieved from the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>). All bound waters and ligands were eliminated from the protein and the polar hydrogen was added to the proteins.

Molecular docking of compounds **C14** and **D6** into the three-dimensional X-ray structure of *E. coli* FabH (PDB code: 1HNJ) was carried out using the Discovery Studio (version 3.1) as implemented through the graphical user interface DS-CDocker protocol. CDOCKER is an implementation of a CHARMM based molecular docking tool using a rigid receptor, including the steps in literature.²⁵⁾

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