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Study of acylhydrazone derivatives with deoxygenated seven-membered rings as potential β-ketoacyl-acyl carrier protein synthase III (FabH) inhibitors†‡

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Fatty acid biosynthesis is essential for bacterial survival. FabH, β-ketoacyl-acyl carrier protein (ACP) synthase III, is a particularly attractive target, since it is central to the initiation of fatty acid biosynthesis and highly conserved among Gram-positive and Gram-negative bacteria. Following previous studies, a series of acylhydrazone derivatives with deoxygenated rings were synthesized in this work. For all of the 36 analogues synthesized, studies using H NMR, C NMR, MS and elemental analyses were conducted. Their biological activities were also evaluated against two Gram-negative bacterial strains: *E. coli* and *P. aeruginosa*, and two Gram-positive bacterial strains: *B. subtilis* and *S. aureus* by the MTT method as potential FabH inhibitors. The resulting compound **F18** showed the highest antibacterial activity with MIC values of 1.56–3.13 μg mL^{-1} against the tested bacterial strains and was found to be the most potent *E. coli* FabH inhibitor with an IC_{50} value of 2.0 μM. Molecular modeling simulation studies were performed in order to predict the biological activities of compound **F18** into the *E. coli* FabH active site.

Introduction

Fatty acid synthesis (FAS) is a necessary metabolic process for the viability and growth of all living organisms.[1–3] The enzymes that catalyze fatty acid biosynthesis in prokaryotes and eukaryotes are classified into two types since they have some differences.[4] The type I system is found in animals and humans, and FAS involves a single polypeptide composed of several distinct enzyme domains.[5–7] The type II system is found in bacteria, plants and protozoa, and the FAS components are present as discrete proteins.[8,9] The corresponding enzymes of the two FAS systems are related in structure and function, but lack overall sequence homology. Thus, the FAS bacterial system represents a potential target for selective inhibition by broad-spectrum antibiotics.[10,11] Significant efforts and progress have been made in the search for small-molecule inhibitors of the type II FAS system. Fig. 1 illustrates several compounds, both natural products and synthetic analogues, that inhibit several steps in the FAS cycle.[9,10,12]

Due to these previous discoveries, serious attention has been paid to enzymes with key roles in the FAS cycle as potential targets for antibiotic inhibition. β-ketoacyl-acyl carrier protein synthase III (FabH) is the bacterial condensing enzyme in Gram-positive and -negative bacteria which initiates the FAB cycle by catalyzing the first condensation step between acetyl-CoA and malonyl-ACP. FabH uses acetyl-CoA as the primer for subsequent condensations, and cause the feedback inhibition of fatty acid synthesis by the final product palmitoyl-ACP to finally stop the circulation.[13–15] However, other condensing enzymes FabB and FabF, functioning later in the cycle, both use acyl-ACP as the primer for subsequent condensations and are hence non-

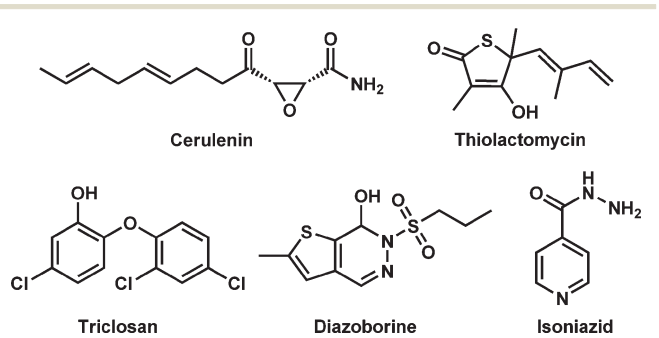

Fig. 1 Several reported inhibitors targeting the fatty acid synthesis (FAS) pathway.

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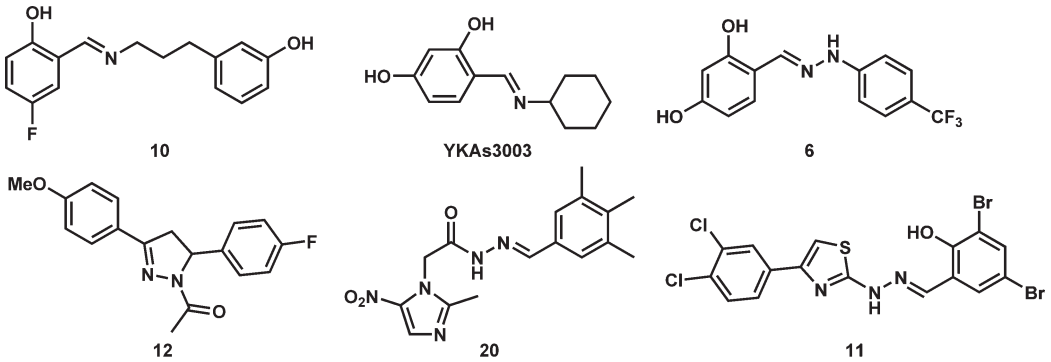
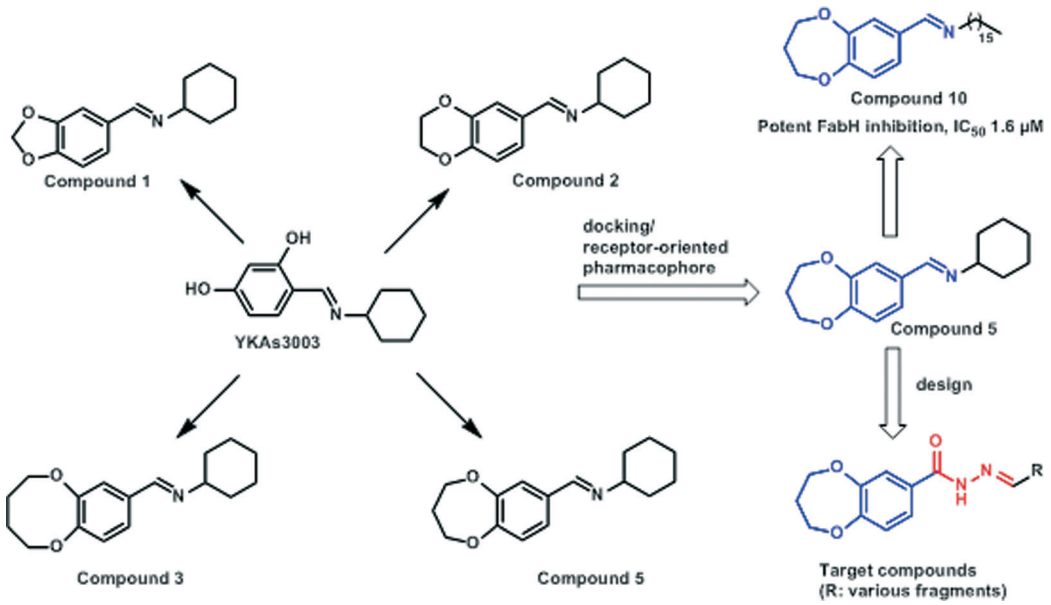


Fig. 2 Several reported antibacterial agents targeting FabH.

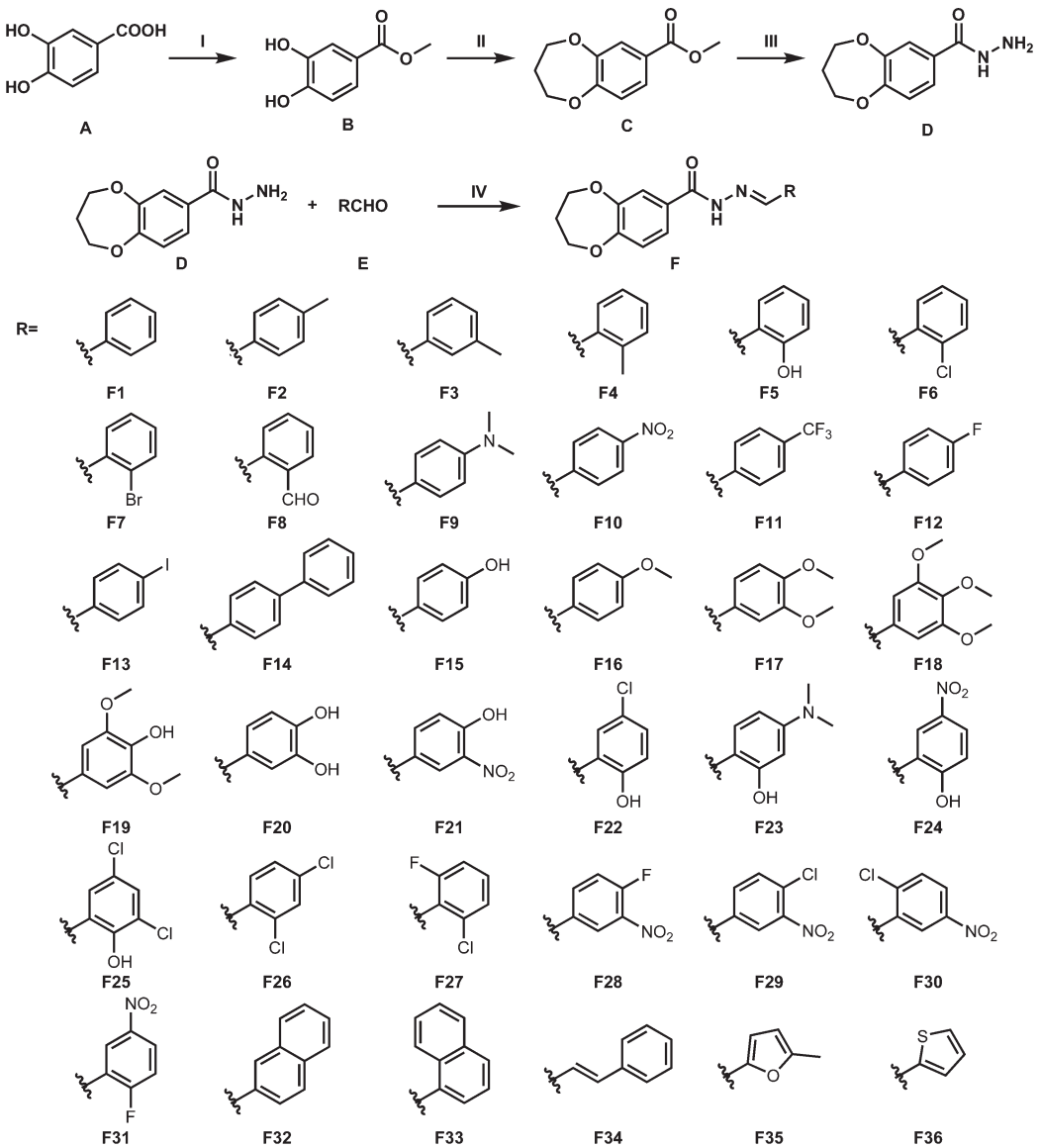


Scheme 1 Design strategy: a lead compound (YKAs3003) initiates a series of novel antibiotic agents with deoxygenated seven-membered rings and *N*-acylhydrazone.

redundant.[16,17] Furthermore, FabH proteins are highly conserved at the sequence and structural level in both Gram-positive and -negative bacteria, while there are no significantly homologous proteins in humans.[18] More importantly, there are essentially no differences between the amino acid residues of the FabH active sites of Gram-positive and Gram-negative bacteria. Thus, all lines of evidence indicate that FabH plays a critical role in the fatty acid biosynthesis pathway and provides a valid and unexploited target for the development of broad-spectrum antibiotics.

Due to the above advantages of FabH, many research groups including our group have been committed to the development of antibacterial agents targeting FabH, and some potential FabH inhibitors with high antibacterial activity have been invented (Fig. 2).[19–24] Apparently, some of these compounds exhibited a hydrazine moiety (–NH–N=CH–) or a Schiff base moiety (–C=N–) in their core structures as shown in Fig. 2. Considering the instability of hydrazine and Schiff base moieties, the privileged structure, *N*-acylhydrazone (–CO–NH–N=CH–), was chosen as the basis for the design of novel FabH inhibitors. Moreover, acylhydrazones have aroused considerable attention due to their particular physical, chemical, and biological characteristics. Based on their unique properties and characteristics, they are known to possess a wide spectrum of biological activities, such as antibacterial activity.[25–27]

From our previous research, four novel Schiff bases derived from YKAs3003 (ref. 20) that retain the structure of the cyclohexylamine moiety were designed. The Schiff base with a deoxygenated seven-membered ring was the most suitable for the development of new FabH inhibitors *via* molecular docking. In this series of compounds, the compound containing 16 aliphatic substituents (compound 5 in Scheme 1) was found to be the most potent *E. coli* FabH inhibitor with an IC_{50} value of 1.6 μM.[28] Considering the excellent properties of acylhydrazones and our previous



Scheme 2 General synthesis of acylhydrazone derivatives (**F1**–**F36**). Reagents and conditions: I) H_2SO_4, MeOH, refluxing, overnight; II) 1,3-dibromopropane, K_2CO_3, 100 °C, 13 h; III) hydrazine hydrate, EtOH, refluxing, 1 day; IV) RCHO, AcOH, EtOH, refluxing, 5 h.

research results, our new design strategy is to replace the Schiff base moiety with an *N*-acylhydrazone moiety in the core structures, while keeping the deoxygenated seven-membered ring (Scheme 1).

Results and discussion

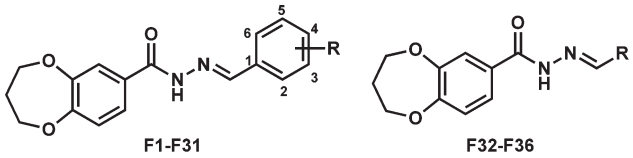
Chemistry

In this study, 36 acylhydrazone derivatives (**F1**–**F36**) were synthesized. The synthetic route to compounds **F1**–**F36** is shown in Scheme 2. These compounds were synthesized from 3,4-dihydroxybenzoic acid (**A**). Esterification of 4-hydroxy-3-methoxybenzoic acid by methanol and concentrated sulfuric acid afforded the corresponding ester **B** with 98% yield. Compound **C** was obtained from the reaction of ester **B** and 1,3-dibromopropane in DMF solvent and using K_2CO_3 as a base. Then, hydrazide **D** was yielded by reacting **C** with hydrazine hydrate in ethanol. The synthesis of compounds **F1**–**F36** was accomplished by reaction of **D** with various aldehydes **E** in ethanol with 75–98% yield. Details of the whole synthesis process are shown in the experimental section. The structures of all synthetic compounds were fully characterized by spectroscopic methods and elemental analysis. All the details of the compounds are in the ESI.‡

Biological activity

Ethics statement. These experiments were conducted in accordance with the guideline issued by the State Food and Drug Administration (SFDA of China). The cell lines were cultured and passaged in accordance with the guidelines established by the National Science Council of the Republic

Table 1 Antibacterial activity of synthesized compounds



		MIC [μg mL^{-1}]			
		Gram-negative		Gram-positive	
Compd[a]	R	*E. coli*	*P. aeruginosa*	*B. subtilis*	*S. aureus*
F1	H	50	50	>100	>100
F2	4-CH_3	50	50	50	>100
F3	3-CH_3	50	25	50	>100
F4	2-CH_3	>100	50	>100	>100
F5	2-OH	50	50	12.5	25
F6	2-Cl	25	50	>100	50
F7	2-Br	50	25	25	50
F8	2-CHO	25	25	50	25
F9	4-$N(CH_3)_2$	6.25	12.5	6.25	6.25
F10	4-NO_2	25	25	25	50
F11	4-CF_3	25	12.5	25	12.5
F12	4-F	12.5	25	50	25
F13	4-I	25	25	12.5	6.25
F14	4-Ph	3.13	6.25	6.25	12.5
F15	4-OH	50	25	25	12.5
F16	4-OCH_3	25	12.5	50	25
F17	3,4-OCH_3	3.13	3.13	3.13	6.25
F18	3,4,5-OCH_3	1.56	3.13	1.56	3.13
F19	3,5-OCH_3; 4-OH	3.13	6.25	3.13	6.25
F20	3,4-OH	12.5	12.5	25	25
F21	3-NO_2; 4-OH	6.25	12.5	12.5	25
F22	2-OH; 5-Cl	12.5	25	25	50
F23	2-OH; 4-$N(CH_3)_2$	12.5	12.5	50	25
F24	2-OH; 5-NO_2	6.25	3.13	3.13	6.25
F25	2-OH; 3,5-Cl	12.5	6.25	6.25	12.5
F26	2,4-Cl	6.25	6.25	12.5	6.25
F27	2-Cl; 6-F	12.5	25	50	25
F28	3-NO_2; 4-F	12.5	6.25	6.25	6.25
F29	3-NO_2; 4-Cl	3.13	6.25	3.13	6.25
F30	3-NO_2; 6-Cl	3.13	3.13	6.25	3.13
F31	2-F; 5-NO_2	6.25	12.5	12.5	12.5
F32	β-Naphthalene	6.25	3.13	3.13	6.25
F33	α-Naphthalene	3.13	6.25	6.25	6.25
F34	Allyl benzene	50	25	50	>100
F35	5-Methylfuran	>100	>100	>100	>100
F36	Thiophene	>100	>100	>100	>100
Kanamycin B	—	3.13	3.13	1.56	1.56
DMSO	—	>100	>100	>100	>100

[a] The compounds tested for antibacterial activity are consistent with the description in the Experimental section.

of China. The primary cell lines were purchased from ATCC. All experimental protocols were approved by the Academic Committee of Nanjing University.

All the synthesized acylhydrazone derivatives **F1–F36** were evaluated for their antimicrobial activity against two Gram-negative bacterial strains: *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, and two Gram-positive bacterial strains: *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 530 utilizing the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The minimum inhibitory concentrations (MIC) of these compounds against these bacteria are presented in Table 1. The results were compared with those provided by the known antibiotic kanamycin under identical conditions, which exhibited significant antibacterial activities among most synthesized compounds. DMSO was added as the negative control within the biological testing. All tested compounds were divided into three groups according to their biological activity: high activity (**F14**, **F17–F19**, **F24**, **F29**, **F30**, **F32**, **F33**), medium activity (**F9–F13**, **F15**, **F16**, **F20–F23**, **F25–F28**, **F31**), and low activity

Table 2 *E. coli* FabH inhibitory activity of synthesized compounds

Compd[a]	*E. coli* FabH IC_{50} [μM]	PSA[b] [Å^2]	Hemolysis LC[c] [mg mL^{-1}]	Cytotoxicity IC_{50} [μM]
F9	7.2 ± 0.4	62.646	>10	134.8 ± 11.5
F10	17.6 ± 1.5	102.117	>10	185.1 ± 9.8
F11	12.4 ± 0.9	59.294	>10	104.2 ± 6.5
F12	32.5 ± 3.1	59.294	>10	125.9 ± 11.7
F13	6.9 ± 0.5	59.294	>10	200.6 ± 12.6
F14	4.7 ± 0.3	59.294	>10	146.1 ± 8.5
F15	38.7 ± 2.7	80.110	>10	178.3 ± 10.4
F16	24.1 ± 1.6	68.224	>10	131.6 ± 12.8
F17	4.1 ± 0.3	77.154	>10	125.8 ± 6.4
F18	2.0 ± 0.1	122.933	>10	120.5 ± 8.8
F19	3.9 ± 0.3	97.970	>10	147.3 ± 13.1
F20	7.8 ± 0.6	100.925	>10	101.8 ± 5.2
F21	8.9 ± 0.5	122.933	>10	198.0 ± 14.7
F22	13.2 ± 0.8	80.110	>10	110.6 ± 10.3
F23	6.7 ± 0.2	83.462	>10	203.3 ± 16.9
F24	3.5 ± 0.1	122.933	>10	156.8 ± 11.4
F25	5.4 ± 0.4	80.110	>10	184.2 ± 10.6
F26	4.3 ± 0.2	59.294	>10	197.5 ± 13.9
F27	20.6 ± 1.8	59.294	>10	163.6 ± 15.1
F28	5.1 ± 0.3	102.117	>10	130.9 ± 9.6
F29	4.4 ± 0.2	102.117	>10	147.1 ± 12.0
F30	3.9 ± 0.3	102.117	>10	172.5 ± 10.4
F31	9.5 ± 0.6	102.117	>10	168.3 ± 7.9
F32	4.6 ± 0.2	59.294	>10	114.4 ± 10.3
F33	5.2 ± 0.3	59.294	>10	129.7 ± 9.5
Kanamycin B	5.6 ± 0.4	282.615	>10	139.6 ± 12.3

[a] The compounds tested for antibacterial activity are consistent with the description in the Experimental section. [b] Molecular polar surface area (PSA); calculated with Discovery Studio 3.1.[29] [c] Lytic concentration 30%.

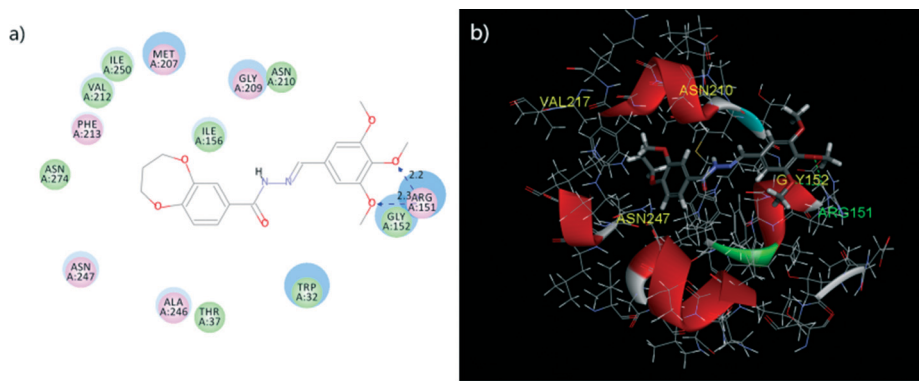


Fig. 3 (a) 2D model of the interaction between compound **F18** and the 1HNJ binding site. (b) 3D molecular docking model of compound **F18** with 1HNJ. The H-bond was displayed as a dotted line and the amino acid it acts on was labeled in green. Other important residues were labeled in yellow.

(**F1–F8**, **F34–F36**). All compounds in the high-activity group demonstrated excellent inhibitory activity against Gram-negative and Gram-positive bacteria. Especially, compound **F18** showed comparable activity to the positive control kanamycin B (MIC = 3.13, 3.13, 1.56 and 1.56 μg mL^{-1}, respectively; Table 1). Most small molecules of the medium-activity group showed modest antibacterial activities against the four pathogenic strains, and the remaining compounds of the last group had little or no bioactivity for any strains.

In order to discuss the structure–activity relationships (SARs) of these compounds, the compounds were classified into two groups: compounds (**F1–F31**) which contain various substituents on an aromatic ring, and compounds (**F32–F36**) which contain a naphthalene ring/heterocyclic ring. To determine how the substituents on the benzene ring affected the antimicrobial activity, compound **F1** without any substituent groups on the benzene ring was first evaluated, which showed lower antimicrobial activity with MIC values of 50, 50, more than 100 μg mL^{-1} and more than 100 μg mL^{-1} against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*, respectively. Introducing electron-donating groups like the methyl group (**F2**, **F3**, **F4**) was disadvantageous to the biological activity, regardless of

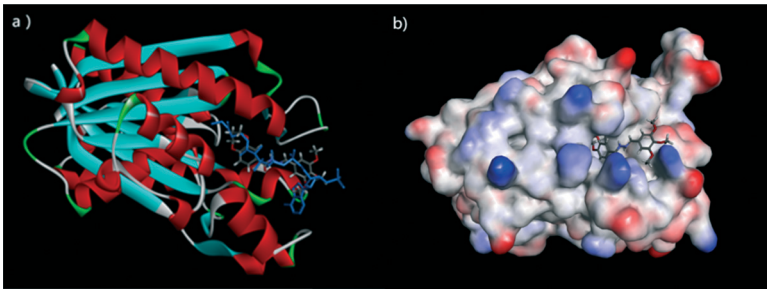


Fig. 4 (a) Compound **F18** mimicking the original ligand in steric conformation. (b) The receptor surface model of compound **F18** embedded in FabH.

the position of the substituent on the benzene ring. Moreover, compounds **F5–F8** with only an *-ortho* substituent with a slightly lower activity suggest that this *-ortho* substitution weakened the inhibitory activity. *Para*-substitution on the ring strengthened the activity (**F9–F16**), while electron-withdrawing groups at the *para*-position had no distinct effect on the activity. Compounds **F9** and **F14** with larger steric substituents on the *para*-position presented higher activities than others. By increasing the number of $-OCH_3$ substituents on the benzene ring, the activities of the compounds (**F16–F19**) were enhanced. This caused compound **F18** owing 3 $-OCH_3$, to have the maximum activity with MIC values of 1.56, 3.13, 1.56 and 3.13 μg mL^{-1} against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*, respectively. Besides, this phenomenon still occurred when the substituent was –Cl (**F6**, **F26**) or –OH (**F5**, **F15**, **F20**). When the *ortho*-position of the benzene ring had a –OH (**F22–F25**) or –Cl (**F26** and **F27**) substituent, while the other positions were substituted by other electron-withdrawing groups, the activities were not obviously enhanced. Furthermore, the MIC values of compounds with $-NO_2$ on the *meta*-position were in the range of 3.13–12.5 μg mL^{-1}; these results indicated that introducing strong electron-withdrawing groups like $-NO_2$ (**F21**, **F24**, **F28–F31**) into the *meta*-position of benzene increased the probability of possessing antibacterial activity.

For the other group, compounds with α- and β-naphthalene rings (**F32**, **F33**) strengthened the antimicrobial effects, which suggests that the large steric substituents contributed to the antibacterial activity of our compounds. Finally, the benzene ring of **F1** was replaced with allyl benzene, a furan ring or a thiophene ring (**F34–F36**, respectively), which resulted in a significant decrease in potency with MIC values of 25 to >100 μg mL^{-1}. This suggested that the benzene ring contributed to the antibacterial activity of the synthesized compounds.

The compounds synthesized were then evaluated for their *E. coli* FabH inhibitory activity (**F9–F33**), and the results are listed in Table 2. All compounds showed potent *E. coli* FabH inhibition, and compound **F18** showed the highest activity against *E. coli* FabH with an IC_{50} value of 2.0 μM. This result supports the potent antibacterial activities of compound **F18**, which is the most potent FabH inhibitor in this series of compounds. Compound **F18** possessing three methoxyl groups on the benzene ring which induce larger steric effects, are more beneficial for inhibitory activity as compared to two or one methoxyl group (**F16**, **F17**). Nitro ($-NO_2$) on the *meta*-position of the benzene ring strengthened the FabH inhibitory activity and the IC_{50} values of compounds **F21**, **F24** and **F28–F31** were in the range of 3.5–9.5 μM. Moreover, compounds **F14**, **F32** and **F33** bearing a diphenyl or naphthalene ring also presented higher activity with IC_{50} values of 4.7, 4.6 and 5.2 μM, respectively. Developing various compounds with $-NO_2$ on the *meta*-position of the benzene ring or large steric groups was another strategy to produce more potent compounds with antibacterial activity. Molecular polar surface area (PSA) is also calculated in Table 2, which is a very useful parameter for prediction of drug transport properties, related to the binding affinity and the cell permeability of FabH inhibitors.[30] Because the molecular polar surface areas (PSA) of these compounds were in the range of 59.294–122.933 $Å^2$, therefore this molecular property cannot significantly account for the results obtained. The PSA value for compound **F18** is 122.933 $Å^2$, which meets Veber rules (<140 $Å^2$). In summary, the results for the *E. coli* FabH inhibitory activity of the test compounds described above generally corresponded to the SARs of their antibacterial activities. This demonstrates that the potent antibacterial activities of the selected compounds are probably correlated with their FabH inhibitory activities.

The compounds selected above were also tested for their hemolytic activity. Additionally, they were also tested for their cytotoxic activity on a mouse embryonic fibroblast cell line (NIH-3 T3) using the MTT assay.[31] The results are summarized in Table 2. All the compounds displayed low hemolytic activities. The cytotoxicity assay determined the selectivity of our compounds for bacterial over mammalian cells. Interpretation of the cytotoxicity data indicates that the compounds possessing inhibitory activity were shown to have low toxicity.

To determine the interaction binding mode between the target protein and small molecules, molecular docking of the most active compound **F18** and *E. coli* FabH was performed on a binding model based on the *E. coli* FabH–CoA complex structure (PDB: 1HNJ).[32] The corresponding result is shown in Fig. 3, which was composed of two interaction maps. The docking study was performed using the CDOCKER protocol.[29]

The 2D optimal conformation and the 3D diagram of the interaction with the FabH active site are presented in Fig. 3a and b, respectively. The main chain of ARG151 forms two hydrogen bonds (HE–O, 2.27 Å, 111.676°; HE–O, 2.18 Å, 165.173°) with two oxygen atoms of compound **F18**.

These interactions were vital for the stabilization of the binding mode. As shown in Fig. 4a, inheriting the advantage of mimicking the original ligand malonyl-CoA, compound **F18** exhibited more powerful interactions with the active site. The receptor surface model in Fig. 4b also suggests that **F18** embedded deeply into the active site of FabH. Thus, compound **F18** could be expected as a potential inhibitor of *E. coli* FabH with potent antibacterial activity.

Conclusions

In this paper, we synthesized 36 novel derivatives with *N*-acylhydrazone and deoxygenated seven-membered fused rings, and evaluated their antibacterial activities against *E. coli* FabH for the first time. Several compounds (**F14**, **F17–F19**, **F24**, **F29**, **F30**, **F32**, **F33**) presented potent anti-Gram-negative and -positive bacterial activities. Compound **F18** showed the most potent antibacterial activity with MIC values of 1.56–3.13 μg mL^{-1} against the tested bacterial strains and exhibited the most potent *E. coli* FabH inhibitory activity with an IC_{50} of 2.0 μM. Preliminary structure–activity relationships and molecular modeling studies provided further insight into the interactions between the enzyme and its ligands. This study showed **F18** was a potential inhibitor of *E. coli* FabH with potent antibacterial activity. Moreover, it provided valuable information for the designing *E. coli* FabH inhibitors as antibacterial agents in the future. With these promising results, we will be utilizing the structure of compound **F18** as the basis for further compound design and SAR development in the future. Moreover, the development of similar novel compounds based on this core structure containing nitrogen heterocyclic or alkane chains instead of a benzene ring on the right side will be investigated in future studies.

Experimental section

All laboratory instruments, methods and data of this article are in the ESI.‡

Acknowledgements

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