N-Benzylanilines as Fatty Acid Synthesis Inhibitors against Biofilm-related Methicillin-resistant *Staphylococcus aureus*Jing Zhang,^{†,‡} Hao Huang,^{§,‡} Xueting Zhou,[‡] Yingying Xu,[‡] Baochun Chen,[†] Wenjian Tang,^{*,‡,§} and Kehan Xu^{*,§}[†]Prevention and Treatment Center for Occupational Disease, Anhui No. 2 Provincial People's Hospital, Hefei 230022, China[‡]College of Pharmacy, Anhui Medical University, Hefei 230032, China[§]College of Basic Medical, Anhui Medical University, Hefei 230032, China

Supporting Information

ABSTRACT: Bacterial fatty acid synthase system is a well validated target for the development of novel antimicrobial agents. This study reports the synthesis of Schiff bases and their reductive N-benzylanilines. Most N-benzylanilines were active against Gram-positive bacteria, among which compound **4k** performed best against both *S. aureus* and MRSA with the MIC value at 0.5 mg/L. Moreover, we identified the strong antibacterial activity for compound **4k** against 19 clinical MRSA strains isolated from different specimen, which indicated its potential in clinical application. *In vitro* biofilm inhibition and microscopy assay revealed compound **4k** inhibits biofilm formation and eradicates preformed biofilm effectively. The size-exclusion chromatography and docking study indicated that compound **4k** mimics the binding mode of triclosan with saFabI. The efficiency of the protein–inhibitor interaction was evaluated by measuring NADPH reduction using *trans*-2-octenoyl-CoA as substrate. Overall, our data demonstrate that N-benzylaniline is a promising scaffold for anti-staphylococcal drug development.

KEYWORDS: Schiff base, N-benzylaniline, antibacterial, MRSA, saFabI

Antimicrobial resistance (AMR) is a global threat to public health, which largely reduces the antibiotic efficacies and increases health care costs, and the situation is getting worse due to the emergence of multidrug-resistant (MDR) bacterial pathogens, such as *Staphylococcus aureus*, which is frequently involved in biofilm in wounds and indwelling medical devices.¹ As a consequence, there is now an urgent need to develop novel and useful antibiotics, which could be addressed by developing new antibacterial agents with unique chemical scaffolds.^{2,3}

Bacterial fatty acid biosynthesis is carried out by cyclical reactions catalyzed by a collection of enzymes, and two carbon units are assembled at each round of elongation.⁴ The enoyl-ACP reductase (FabI) catalyzes the last step in each cycle and plays the determinant role in regulating the rate of fatty acid synthesis,⁵ which is receiving increasing attention as an effective antibacterial target.^{6,7} 2-Hydroxydiphenyl ethers with a broad-spectrum antibacterial activity, such as triclosan (TCS), have been identified to interrupt the function of FabI enzyme by occupying the active site of FabI protein in the presence of NAD⁺/NADP⁺.^{8,9} The synthesis is initiated from the condensation of acetyl-coenzyme A (acetyl-CoA) with malonyl-acyl carrier protein (malonyl-ACP) catalyzed by β -ketoacyl-ACP synthase III (FabH), which is another key component involved in the reaction cycle.¹⁰ Schiff base, a

scaffold that exhibits a broad range of biological activities, including antifungal, antibacterial, antimalarial, antiproliferative, anti-inflammatory, antiviral, and antipyretic properties,^{11–17} was suggested to inhibit fatty acid synthesis by interfering with FabH.^{18,19} Moreover, the salicylaldehyde Schiff base showed potent antimicrobial activity in both “free” form or as ligand in metallic complex, and the halogenations of the salicylic moiety largely improved the antibacterial and antifungal activity.^{20,21} All of these prompt us to further understand the antibacterial activity of Schiff base analogs derived from halogeno-salicylaldehyde.

Our venture into studying the lipid pathway inhibitors was initially promoted by efforts to identify new drugs that are able to target on both FabH and FabI enzymes. Therefore, 16 Schiff base derivatives were designed based on the architecture of FabH and FabI inhibitors, for the Schiff base and triclosan (TCS), respectively (Table 1). Considering the rigidity features of the C=N bond of Schiff base may constrain the torsion of two benzene rings, the corresponding N-benzylanilines (**4a–4p**) were also synthesized. Schiff bases (**3a–3p**) and N-benzylanilines were obtained from the corresponding

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Table 1. Structure of Compounds 3a–3p and 4a–4p

compd	R ₁	R ₂	R ₃	R ₄	R ₅
3a	H	Cl	F	H	CF ₃
3b	H	Cl	Cl	H	CF ₃
3c	H	Cl	H	OCF ₃	H
3d	H	Br	Cl	H	CF ₃
3e	H	Br	F	H	CF ₃
3f	H	Br	H	H	OCF ₃
3g	Cl	Cl	Cl	H	CF ₃
3h	Br	Cl	4-fluoro-phenylethylamine		
3i	Br	Cl	4-bromo-phenylethylamine		
3j	Br	Cl	H	H	CF ₃
3k	Br	Cl	Cl	H	CF ₃
3l	Br	Cl	H	H	OH
3m	Br	Br	F	H	CF ₃
3n	Br	Br	H	OH	H
3o	Br	Br	H	H	CF ₃
3p	Br	Br	Cl	H	CF ₃
4a	H	Cl	F	H	CF ₃
4b	H	Cl	Cl	H	CF ₃
4c	H	Cl	H	OCF ₃	H
4d	H	Br	Cl	H	CF ₃
4e	H	Br	F	H	CF ₃
4f	H	Br	H	H	OCF ₃
4g	Cl	Cl	Cl	H	CF ₃
4h	Br	Cl	4-fluoro-phenylethylamine		
4i	Br	Cl	4-bromo-phenylethylamine		
4j	Br	Cl	H	H	CF ₃
4k	Br	Cl	Cl	H	CF ₃
4l	Br	Cl	H	H	OH
4m	Br	Br	F	H	CF ₃
4n	Br	Br	H	OH	H
4o	Br	Br	H	H	CF ₃
4p	Br	Br	Cl	H	CF ₃

salicylaldehyde (1) and amine (2) (Scheme 1) according to literature procedures.²² The imines (3a–3p) were synthesized

Scheme 1. Synthesis of Compounds 3a–3p and 4a–4p^a

^aReagents and conditions: (i) methanol; (ii) NaBH₄ solution, ethanol.

from the condensation of primary amines (2) and salicylaldehydes (1) via the nucleophilic addition. An imine (3) can be reduced to an amine (4) via treatment with sodium borohydride.

The antimicrobial potential of all compounds was tested against a panel of bacteria and fungi in Mueller–Hinton broth (MHB), including four Gram-positive bacteria, *Bacillus cereus*, *Enterococcus faecium*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* (MRSA); two Gram-negative

bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*; and two fungi, *Candida tropicalis* and *Candida albicans*, respectively.

As shown in Table S1, all compounds had quite low antimicrobial effects against Gram-negative bacteria and fungi, with most of the MIC values ≥ 64 mg/L. To validate if the efflux pump present on the outer membrane of Gram-negative bacteria was responsible for the resistance, we analyzed the *in vitro* effects against both *E. coli* and *P. aeruginosa* by using *N*-benzylanilines in combination with efflux pump inhibitors (EPI) as suggested in the literature.²³ However, no significant antibacterial activity improvement (synergistic effect) was observed in the presence of CCCP or NMP (data not shown).²⁴ A moderate to strong level of antibacterial activity against Gram-positive bacteria were observed for most *N*-benzylaniline derivatives, while the corresponding Schiff bases showed poor bacteriostasis effects. Compounds 4b, 4d, 4g, 4j, 4k, 4o, and 4p performed best against Gram-positive bacteria with the MIC values ≤ 2 mg/L, which were better than other tested front-line antibiotics including TCS, with the exception for *E. faecium*, which was less susceptible to all tested compounds (Table S1). Moreover, the methicillin-resistant *Staphylococcus aureus* (MRSA) was sensitive to most of the *N*-benzylaniline derivatives as well and displayed a similar susceptibility pattern with standard *S. aureus* strain (Table S1). Moreover, we tested the effect of compound 4k on 19 clinical *S. aureus* isolated from different specimen, which are resistant to multiple antibiotics, using broth dilution assay (Table S2). All tested strains had an MIC ≤ 1 mg/L (Table S2 last panel), confirming the significant potential of these *N*-benzylaniline derivatives for development of agents to treat resistant infections.

The structure–activity relationship (SAR) analysis showed that (i) *N*-benzylanilines (4a–4p) had better antibacterial activity than the corresponding Schiff bases (3a–3p) except for 3h and 3i, which performed slightly better than 4h and 4i; (ii) compounds 4h and 4i with aliphatic amine had very low antibacterial activity against Gram-positive bacteria; (iii) *N*-benzylanilines with a CF₃ and at least one Cl substituent generally exhibited better antibacterial activity (such as 4a, 4b, 4d, 4g, 4j, 4k, 4m, 4o, and 4p), while the compounds with an OCF₃ substituent had decreased activity (such as 4c and 4f); (iv) for *N*-benzylanilines, F substituent on benzene ring reduced the antibacterial activity, e.g., for *S. aureus* and MRSA (MIC values), 4a (4, 32 mg/L) < 4b (1, 1 mg/L); 4e (16, 16 mg/L) < 4d (2, 1 mg/L); 4m (4, 4 mg/L) < 4p (1, 1 mg/L); (v) Schiff bases 3m, 3n, 3o, and 3p with dibromo substituent exhibited moderate antibacterial activity.

S. aureus is able to live together in the form of biofilm to avoid elimination by the host. Therefore, a strategy to counteract the development of biofilms would be useful in the treatment of *S. aureus* infections, especially for MRSA, which is frequently involved in biofilm formation in wounds and indwelling medical devices.¹ Here, the inhibition of biofilm formation by active compounds against MRSA was first evaluated. As shown in Figure 1, there is a trend of biomass decrease along with the compounds concentration increase from 0.5 to 4 mg/L (Figure 1A–H), especially for compound 4k, which was able to inhibit the biofilm formation effectively at 0.5 mg/L and almost entirely at 2 mg/L (Figure 1E). To provide direct evidence, scanning electron microscopy (SEM) was utilized to take the views of live biofilm and the effects by compound 4k (Figure 1I–M). It was shown that no biofilm was observed at the concentration up to 1 mg/L, confirming

Figure 1. Biofilm elimination by *N*-benzylanilines. (A–H) Inhibition of biofilm growth of MRSA (ATCC 43300) by compounds **4b**, **4d**, **4g**, **4j**, **4k**, **4o**, **4p**, and DMSO control, respectively. (I–M) Scanning electronic microscopic images of the 24 h biofilm of MRSA (ATCC 43300) for (I) untreated and (J–M) treated with gradually increased compound **4k**. (N–W) Disruption of the 24 h preformed biofilms of 10 selected MRSA clinical strains (N–W for clinical strain No. 2, 3, 4, 5, 6, 11, 12, 16, 17, and 18, respectively) by compound **4k**. Statistically significant *p*-values of control vs. experimental were indicated with **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

that bacteria were not able to attach and form biofilm at the bottom of the cultivation plates in the presence of compound **4k**. Moreover, the antibiofilm capability of compound **4k** was also analyzed against 10 of the isolated clinical MRSA strains, for which biofilm formation was observed during bacterial cultivation. The treatment of compound **4k** in the concentration up to 2 mg/L resulted in the disruption of performed biofilm by most of the clinical MRSA strains (Figure 1N–W), suggesting compound **4k** could not only inhibit the formation of the biofilm but also effectively eradicate the already existing biofilm.

Triclosan is one of the most effective FabI inhibitors that acts by binding at the active site with cofactors to form stable FabI–NAD⁺/NADP⁺–TCS ternary complex. Kinetics study suggested that the FabI from *S. aureus* (saFabI) prefers NADPH rather than NADH as its cofactor.²⁵ Moreover, structural analysis on saFabI revealed major conformational change upon TCS and NADP⁺ binding.²⁶ The binding fixes the disordered region and results in the dimer–tetramer transition of saFabI in solution (Figure 2A).^{26,27} To investigate the binding mechanism of the newly synthesized compounds, the protein saFabI was incubated with NADP⁺ and compound **4k** in a molar ratio of 1:10:10 and analyzed on size-exclusion chromatography as previous described.²⁸ The result revealed

Figure 2. SaFabI inhibition by *N*-benzylanilines. (A) Dimer–tetramer transition of saFabI (previously reported PDB code: left panel, 3GNT, and middle and right panels, 4ALI). Apo-saFabI was colored in black; saFabI structure in dimer and tetramer conformation was colored in white. (B) Size-exclusion chromatography analysis of saFabI protein alone (in blue curve), mixture with compound **4k** and NADP⁺ (in black curve), or mixture with TCS and NADP⁺ (in green curve). (C) Inhibition of saFabI by **4k** *in vitro*. (D) Close-up of the interactions between compound **4k**, NADP⁺, and saFabI. (E) Schematic view of the interactions. (F) Comparison of the TCS and compound **4k** binding modes. Compound **4k** and cofactor were both shown as sticks in black, and the protein was shown as a space filling model in gray. TCS was shown in white. The structures were all displayed using pymol (Schrödinger, LLC).

the peak shift due to the tetramer transition of partial dimeric saFabI protein upon ligand binding (Figure 2B), which is consistent with previous report, suggesting that compound **4k** mimics the binding mode of TCS with that of saFabI. Furthermore, the efficiency of these compounds as a FabI inhibitor was compared with TCS by measuring the initial rates of the reaction using *trans*-2-octenoyl-CoA as substrate. In general, the *N*-benzylanilines (**4b**, **4d**, **4g**, **4j**, **4k**, **4o**, and **4p**), which possess good antibacterial activity, also performed better saFabI inhibitory effects than the corresponding Schiff bases (**3b**, **3d**, **3g**, **3j**, **3k**, **3o**, and **3p**) (Figures 2C and S1). Compound **4k** inhibits saFabI with an IC₅₀ value of approximately 4 μM, making it an effective FabI inhibitor as TCS (Figure 2C). Docking study of compound **4k** using saFabI–NADP⁺–TCS complex structure (PDB code: 4ALI) as the model identified strong potential interactions (Figure 2D,E). To be noted, a torsion of ~80° between two benzene

rings was observed, which is also found in TCS binding model (Figure 2F).²⁶ This may explain the much lower saFabI inhibition efficiency observed for Schiff bases was possibly due to the C=N bond restricting the bond angle and further affecting the proper fitting into the binding pocket.

In conclusion, we designed and synthesized a set of Schiff base and *N*-benzylaniline analogs, aiming to target on the lipid synthesis pathway. However, the Schiff base derivatives (3a–3p) exhibited quite a weak antibacterial activity and were not included in later biological analysis. All compounds were not active against Gram-negative bacteria, and we suspected this may due to the efflux pump present on the outer membrane of Gram-negative bacteria, which has been suggested as a ubiquitous mechanism associated with resistance to numbers of antimicrobial agents.²⁴ However, no significant antibacterial activity improvement (synergistic effect) against both *E. coli* and *P. aeruginosa* was observed when using *N*-benzylanilines in combination with CCCP or NMP (data not shown).^{23,24} It implied that Gram-negative bacteria may combat these synthesized compounds not by pumping them out but through other mechanisms.²⁹ Nineteen MRSA strains isolated from various human samples, which are all resistant to multiple front-line antibiotics, were included to test the antibacterial capability of compound 4k. Besides, compound 4k showed low cytotoxicity (50% reduction of cell viability concentrations at approximately 80 mg/L) (Figure S3). The results confirmed its potential for development of agents to treat resistant infections. Lipid is one of the major components of biofilm and essential in biofilm formation of Gram-positive bacteria by participating in the construction of lipoteichoic acid (LTA), which acts as lipid anchor to link cell membrane.³⁰ The compounds targeting lipid synthesis pathway are supposed to interrupt biofilm formation. Here, we tested the antibiofilm effects using 10 MRSA strains producing biofilm and confirmed that compound 4k, which disrupts saFabI function efficiently, also possesses the capability to inhibit the formation of biofilm and eradicate already existing biofilm. However, considering the complicated composition of bacterial biofilm, whether these compounds have other cellular targets needs to be further investigated. In conclusion, we developed a series of *N*-benzylanilines targeting fatty acid synthesis and provide a novel scaffold for the design of the next generation of inhibitors.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.8b00612.

Experimental details and characterization data for the reported compounds, NMR spectra, and biological data. MIC of Schiff base and *N*-benzylaniline derivatives against bacteria and fungi. MIC of the front-line antibiotics and 4k against clinical *Staphylococcus* strains. In vitro assay of saFabI inhibition. SAR of *N*-benzylanilines. Cell cytotoxicity assay of selected *N*-benzylanilines to GES-1 and LO2 cells (PDF)

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#These authors contributed equally. W.J.T. and K.H.X. conceived the project. All the authors conducted the experiments and analyzed the data. K.H.X. wrote the paper. All the authors have given approval to the final version of the manuscript.

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Notes

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■ ABBREVIATIONS

MRSA, methicillin-resistant *Staphylococcal aureus*; TCS, Triclosan; MIC, minimal inhibitory concentration; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; NMP, 1-(1-naphthylmethyl)-piperazine

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