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# Discovery of Novel Antibiotics as Covalent Inhibitors of Fatty Acid Synthesis

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**ABSTRACT:** The steady increase in the prevalence of multidrug-resistant *Staphylococcus aureus* has urged the search for novel antibiotics to combat this clinically important pathogen. In an effort to discover antibacterials with new chemical structures and mechanisms, we performed a growth inhibition screen of a synthetic library against *Staphylococcus aureus* and discovered a promising scaffold with 1,3,5-oxadiazin-2-one core. These compounds are potent against both methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* strains. Isolation of compound resistant strains followed by whole genome sequencing revealed its cellular target as FabH, a key enzyme in bacterial fatty acid synthesis. Detailed mechanism of action studies suggested the compounds inhibit FabH activity by covalently modifying its active site cysteine residue with high selectivity. A crystal structure of FabH protein modified by a selected compound Oxa1 further confirmed covalency and suggested a possible mechanism for reaction. Moreover, the structural snapshot provided an explanation for compound selectivity. Based on the structure, we designed and synthesized Oxa1 derivatives and evaluated their antibacterial activity. The structure-activity relationship supports the hypothesis that non-covalent recognition between compounds and FabH is critical for the activity of these covalent inhibitors. We believe further optimization of the current scaffold could lead to antibacterial with potentials to treat drug resistant bacteria in the clinic.

## Introduction

Antibiotics have been successfully used in the clinic to treat bacterial infections for more than 70 years, however, the emergence and spread of resistance to current antibiotics poses a serious threat to public health[1]. Of the Gram-positive pathogens, the incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) has increased globally in both healthcare and community settings. In a global report on antimicrobial resistance surveillance issued by the World Health Organization in 2014, most countries reported a proportion of MRSA exceeding 20% and, occasionally, up to 80%[1]. MRSA infection is normally associated with poorer clinical outcomes, increasing healthcare burdens globally. Recently, the glycopeptide vancomycin, which has been considered as the last line of defense against MRSA, started to lose its effectiveness against multi-resistant Gram-positive bacteria[2]. Therefore, identification and development of novel antibiotics to effectively treat life-threatening MRSA infections is in urgent need.

With the aim to identify antibiotics of new scaffolds and/or mechanisms of actions, we carried out a growth inhibition screen in *S. aureus*, with a library of synthetic chemicals. These chemicals mostly have novel structures and are relatively easy to synthesize. The screen led to the discovery of two compounds containing 1,3,5-oxadiazin-2-one with potent anti-*staphylococcus* activity, which we named Oxa1 and Oxa2. 1,3,5-oxadiazin-2-ones have not appeared in any known antibacterial structures, thus representing a novel scaffold for potential antibiotic development. The compounds can overcome methicillin resistance in *S. aureus* strains isolated from clinic, arguing for detailed analysis of their molecular mechanism.

We identified spontaneous mutations in *S. aureus* coding DNA sequences (CDS) by isolating compound resistant mutants followed by whole genome sequencing. With pathway confirmation, we revealed the molecular target of 1,3,5-oxadiazin-2-ones as $\beta$-ketoacyl-ACP synthase III (FabH), the enzyme initiating chain elongation in bacterial type II fatty acid synthesis (FAS II)[3, 4]. FabH is an essential enzyme for fatty acid synthesis in bacteria[5, 6] and has long been pursued as an antibacterial target[7-10]. FabH catalyzes the initial condensation reaction of acetyl-CoA with malonyl-ACP to form $\beta$-ketobutyryl-ACP, which is the rate limiting step in fatty acid biosynthesis[6]. The product is further catalyzed by FabG, FabA/FabZ, FabI/K/L/V, FabB/FabF to realize the extension of lipid chains which become the key components of phospholipids, lipoproteins, lipopolysaccharides, cell envelope and mycolic acid[11-13]. The divergence between mammalian and bacterial FAS II pathways, coupled with the multifaceted role in both the viability and virulence of many pathogenic bacteria, makes fatty acid inhibition an attractive strategy for the development of new antimicrobial agents[13-15]. A list of FASII

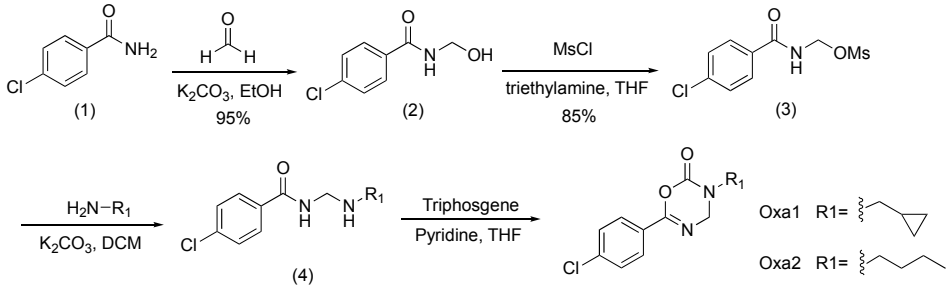
inhibitors, including FabF inhibitors platensimycin[16] and cerulenin[17], FabI inhibitors CG400549[18], MUT056399[19] and AFN-1252[20], FabH inhibitor platencin[21] and amycomycin[22] have been reported to be effective against *S. aureus in vitro* and in animal models. Although no inhibitors targeting *de novo* fatty acid synthesis in bacteria have been approved to use in clinic as anti-infectives and it requires further investigation to understand the essentiality of fatty acid biosynthesis in bacteria growth and virulence[23-25], compounds that specifically inhibits enzymes in the pathway would be desirable to facilitate this effort.

In this report, we investigated the detailed mechanism by which 1,3,5-oxadiazin-2-ones selectively inhibit FabH and explored their potential for further development as an antibacterial. We revealed by mass spectrometry that a selected compound Oxa1 inhibits FabH by reacting with its active site cysteine, resulting in a covalent adduct, which was further confirmed by a co-crystal structure of *S. aureus* FabH with Oxa1. Based on detailed analysis of FabH:Oxa1 structure, we designed and synthesized two series of Oxa1 derivatives and showed that their antibacterial activities could be optimized by improving the non-covalent interactions between the compounds and target protein. The work paves the way for further chemical optimization of the 1,3,5-oxadiazin-2-one series as novel antibiotics to treat life-threatening MRSA infections.

**Growth Inhibition Screen in *Staphylococcus aureus*.** An in-house collection of synthetic compounds was screened for inhibition of bacterial growth against *S. aureus* ATCC29213 (methicillin sensitive). At least two compounds, named Oxa1 and Oxa2, were capable of blocking bacterial growth with an MIC of 1 and 0.25 μg/mL, respectively. Both compounds contain a 1,3,5-oxadiazin-2-one group, a *para*-chlorophenyl group and an aliphatic chain (**Scheme 1**). A search of the 1,3,5-oxadiazin-2-one group in the CA SciFinder chemical database showed no significant similarity to any known antibiotics, which may indicate a mechanism not exploited before.

We re-synthesized these two compounds via a 4-step route outlined in **Scheme 1** and confirmed their chemical structures by $^{1}$H NMR, $^{13}$C NMR and high-resolution mass spectrometry. The target compounds could be obtained in a final yield of 83-95% and are stable at room temperature for at least 15 days. We then measured the antibacterial activity of these compounds in 10 clinically isolated *S. aureus* strains with medium to high resistance to methicillin, as well as *E. coli* ATCC8739 and *P. aeruginosa* ATCC27953. These compounds showed similar activity against all tested *S. aureus* strains but are not active against the two Gram-negative strains (**Table 1**). The compounds' antibacterial activity regardless of methicillin-resistance status may indicate a mechanism non-overlapping with existing antibiotics and could potentially offer an alternative solution in treating infections caused by MRSA.

## Results and Discussion




**Scheme 1.** Synthetic route for compound Oxa1 and Oxa2.

**Table 1.** MICs of compound Oxa1 and Oxa2 against clinically isolated MRSA strains.

| Strains | MICs (μg/mL) | | | | |
|---|---|---|---|---|---|
| | Methicillin | Vancomycin | Linezolid | Oxa1 | Oxa2 |
| *S. aureus* ATCC29213 | 1 | 1 | 2 | 1 | 0.25 |
| *S. aureus* ATCC25923 | 1 | 1 | 1 | 1 | 0.25 |
| MRSA219236 | 8 | 1 | 1 | 0.5 | 0.25 |
| MRSA305026 | 8 | 1 | 1 | 1 | 0.25 |
| MRSA214026 | 8 | 0.5 | 1 | 1 | 0.5 |
| MRSA216054 | 16 | 0.5 | 1 | 1 | 0.25 |
| MRSA814039 | 16 | 0.5 | 2 | 0.5 | 0.25 |
| MRSA212014 | >64 | 1 | 1 | 1 | 0.5 |
| MRSA831024 | >64 | 1 | 1 | 0.5 | 0.5 |
| MRSA503025 | >64 | 1 | 2 | 0.25 | 0.5 |
| MRSA614019 | >64 | 1 | 1 | 1 | 0.25 |
| MRSA919025 | >64 | 1 | 1 | 1 | 0.25 |
| *P. aeruginosa* ATCC27953* | >64 | - | - | >64 | >64 |
| *E. coli* ATCC8739 | >64 | >64 | >64 | >64 | >64 |

- Not tested.
* MIC for polymyxin B is 1 μg/mL.

**Target Identification by Isolation of Compound-Resistant Mutants and Whole Genome Sequencing.** In order to unveil the molecular target of these compounds, we used the traditional method to isolate compound-resistant mutants and then analyzed spontaneous mutants for mutations by whole genome sequencing. First, through screening *S. aureus* on agar plates containing inhibitory concentrations of Oxa1 or Oxa2, we were able to obtain six clones resistant to compound Oxa2 (we were not able to obtain Oxa1-resistant clones) (**Figure 1A**). All six clones were characterized for their level of resistance and we found their MICs for Oxa2 were elevated 8-32 fold relative to their parental strain (**Table S1**). Next, we performed whole genome sequencing on clone 1 together with the wild type strain ATCC29213 using Illumina high-throughput sequencing platform. After analyzing all the missense mutations in CDS, the gene encoding *β*-ketoacyl-ACP synthase III (FabH) was proposed as our top target candidate because FabH is an essential enzyme in bacterial fatty acid synthesis[3, 4] and has been pursued as an antibacterial target[5-10]. Moreover, the identified mutation site (A81V) is close to the catalytic triad in 3D structure, making it likely to affect the binding of an active site inhibitor. We confirmed this point mutation (C to T) in *fabH* (**Figure 1B, Table S2**) by sequencing the PCR product of the *fabH* gene. We further analyzed the PCR products of *fabH* from rest of the resistant mutants and found both single point mutations resulting in A81V substitution and double mutations resulting in A81V and A111T/S substitutions in *fabH* gene product (**Figure 1C**).

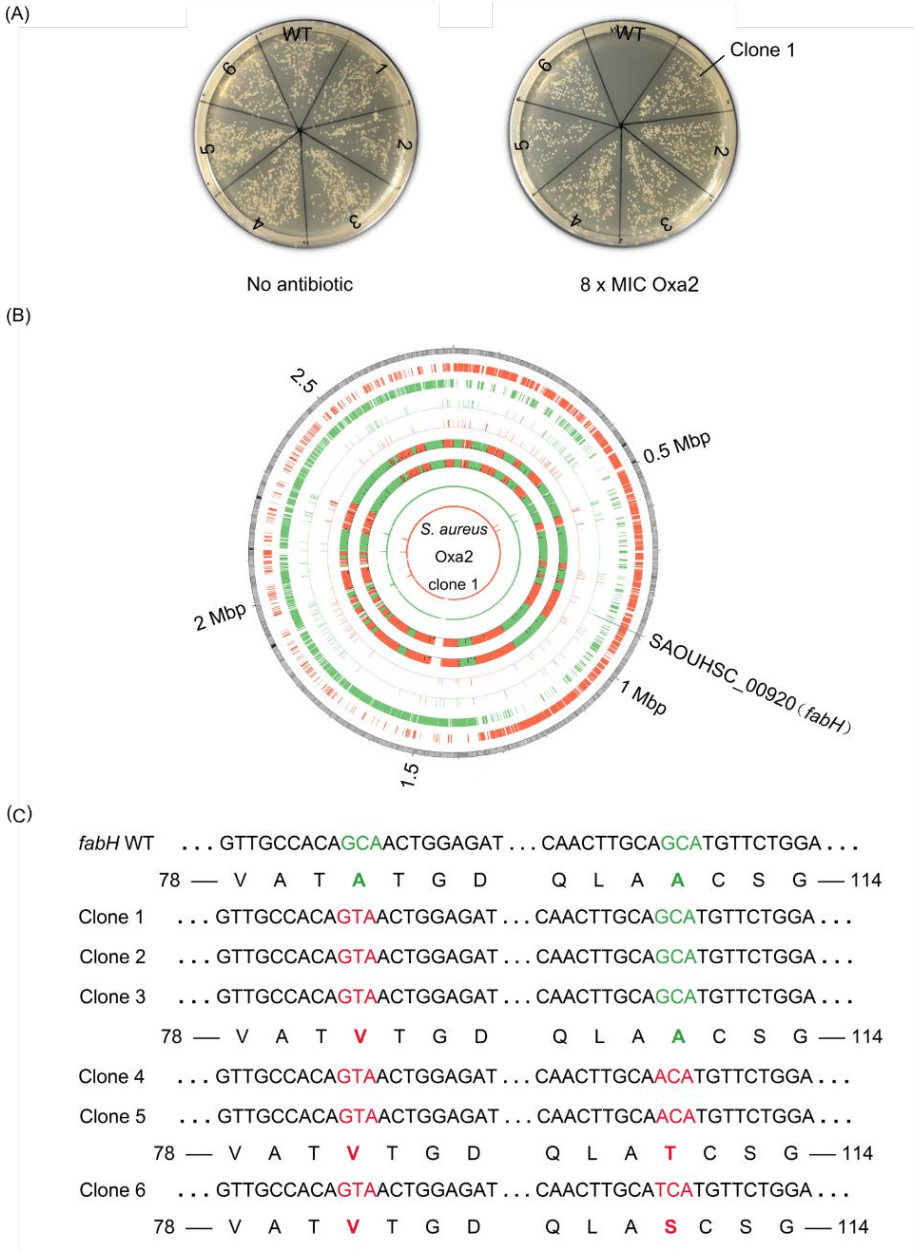



**Figure 1.** (A) Wild type and Oxa2 resistant clones on LB agar plate with (right) and without Oxa2 (left). (B) Circular representation of ATCC29213 WT and Oxa2-resistant clone 1 draft genome. Circles from the outermost are as follows: reference genome *S. aureus* NCTC8325 and GC (grey); forward CDS (red); reverse CDS (green); Mutations in clone 1 from reference (green); Mutations in

ATCC29213 from reference (red); contigs of clone 1; contigs of ATCC29213; sequencing depth of clone 1; sequencing depth of ATCC29213. (C) Sequencing results of *fabH* gene PCR product from all 6 collected Oxa2 resistant clones. A81V mutations was found in all 6 clones and A111T/S was found in 3 clones. Mutated codons colored in red while corresponding wild type colored in green.

One of the common mechanisms for acquired resistance to antibiotics include alterations in the target protein that confer drug resistance while retaining catalytic activity. Both A81 and A111 reside close to the enzyme's substrate binding pocket, so if the compound binds next to the substrate pocket it is possible that these mutations could affect compound binding significantly. Therefore, the sequencing results strongly suggest FabH as the molecular target of 1,3,5-oxadiazin-2-ones, however, it still requires further investigations for confirmation.

**Unsaturated Fatty Acids Protect *S. aureus* Against Oxa1 and Oxa2.** Bacterial FabH catalyzes the initiation of fatty acid biosynthesis by condensing malonyl-ACP with acetyl-CoA, while FabB and FabF are involved in the condensation of malonyl-ACP with a growing acyl-ACP chain to form *β*-ketoacyl-ACP, which is subsequently reduced by *β*-ketoacyl-ACP reductase, *β*-hydroxyacyl-ACP dehydrase and enoyl-ACP reductase. The bacterial type II fatty acid biosynthesis system is a novel target that has engendered considerable attention[13-15].

It has been shown that unsaturated fatty acid such as oleic acid or palmitoleic acid can protect *S. aureus* from antibiotics targeting FAS II *in vitro*[23]. This is because *S. aureus* has the molecular machinery to incorporate external fatty acids into its membrane and the unsaturated fatty acid could partially replace the function of its naturally existing branched saturated fatty acid in membrane phospholipids[23]. To confirm that our compounds target *de novo* fatty acid synthesis in *S. aureus,* we studied the effect of exogenous fatty acids on the antibacterial activity of these compounds. We found that in the presence of commercially available oleic acid (18:1) as well as linoleic acid (18:2), but not saturated fatty acid, the MICs of Oxa1 and Oxa2 against *S. aureus* increased to higher than 64 μg/mL (**Table 2**). Platencin, which is a dual inhibitor of FabH and FabF, showed a similar trend. The results strongly suggest that these compounds potentially target fatty acid biosynthesis in *S. aureus*.

**Table 2.** MICs of compound Oxa1 and Oxa2 against *S. aureus* with fatty acids supplementation in medium.

| Growth medium | MICs (μg/mL) | | | |
|---|---|---|---|---|
| | Kanamycin | Oxa1 | Oxa2 | Platencin |
| LB | 2 | 1 | 0.25 | 1 |
| LB+BSA* | 2 | 1 | 0.25 | 2 |
| LB+BSA+18:1 | 4 | >64 | >64 | 32 |
| LB+BSA+18:2 | 4 | >64 | >64 | 16 |
| LB+BSA+16:0 | 2 | 1 | 0.25 | 4 |
| LB+BSA+18:0 | 2 | 1 | 0.25 | 4 |

*Addition of BSA is to help dissolve fatty acid in growth media.

**Oxa1 Inhibits FabH Activity by Covalently Modifying Its Catalytic Residue.** We then sought to understand the mechanism of inhibition in further details. *S. aureus* FabH catalyzes the Claisen-condensation of malonyl-ACP and isobutyryl-coenzyme A to produce 3-keto-4-methyl-pentanoyl-ACP and CoA. In order to measure the inhibitory activity of the compounds against FabH, we established a FabH activity assay by quantifying the production of CoA with 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM), which becomes fluorescent after reaction (**Figure 2A**). Oxa1 and Oxa2 inhibit FabH in the activity assay with an $IC_{50}$ value of 2.6 μM and 1.2 μM, respectively (**Figure 2B**). This result confirmed that the compounds are *bona fide* FabH inhibitors, as suggested by whole genome sequencing and fatty acid protection experiment.

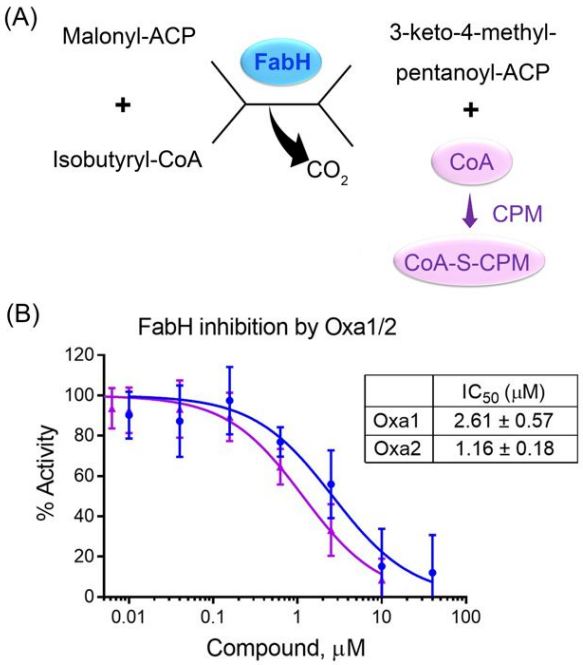



**Figure 2.** (A) FabH activity assay scheme. *S. aureus* FabH catalyzes malonyl-ACP and isobutyryl-CoA to produce 3-keto-4-methyl-pentanoyl-ACP and CoA, the later when reacted with CPM to form CoA-S-CPM, which can be detected at Ex384/Em470. (B) FabH inhibition by Oxa1 and Oxa2, $IC_{50}$ of Oxa1 and Oxa2 are 2.61 μM and 1.16 μM, respectively.

Interestingly, we observed variations in $IC_{50}$ numbers measured with different pre-incubation times during the FabH assay, which prompted us to look into the possibility of covalent mechanism. MALDI-TOF-MS analysis of FabH incubated with Oxa1 shows a +268 adduct, implying a possible covalent modification of FabH by one molecule of compound Oxa1 **(Figure 3A)**. Increasing compound concentration does not give additional peaks, suggesting only one site on FabH could be modified. In order to identify the site of modification, Oxa1-treated FabH was subjected to trypsin digest and subsequent LC-MS analysis. The peptide with molecular weight

corresponding to one Oxa1 adduct was identified, and MS/MS data suggests the site of modification on cysteine 112 (**Figure 3B**). To further confirm the site for covalent modification, we mutated cysteine 112 to alanine by site-directed mutagenesis. The purified C112A mutant is soluble and exists as dimer, the same as wild type FabH, analyzed by size exclusion chromatography (**Figure S1**), suggesting it is likely a well folded protein. However, FabH C112A mutant could not form Oxa1 adduct under the same conditions as wild type FabH, consistent with Cys112 being the only site for covalent modification as suggested by LC-MS (**Figure 3C**). Cys112 is the invariant residue in FabH and is indispensable for the transfer of acetyl- or acyl- group from its CoA primer to malonyl-ACP, which explains the inhibitory effect of Oxa1 by targeting Cys112.

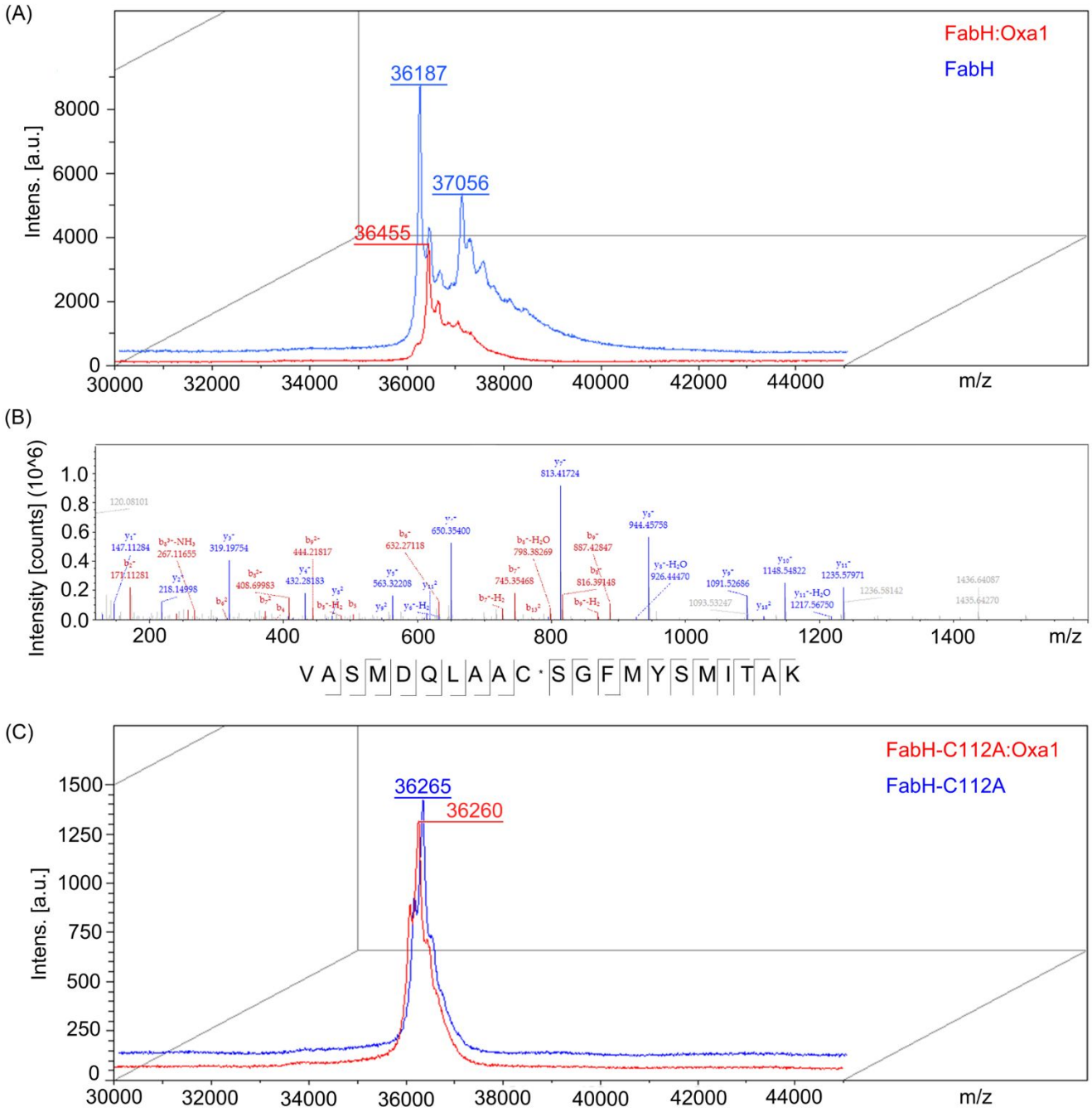



**Figure 3.** (A) MALDI-TOF-MS results of covalent modification of FabH by Oxa1 and FabH alone. (B) LC-MS results for site identification of trypsin digested FabH modified by Oxa1. (C) MALDI-TOF-MS results of FabH C112A incubated with or without Oxa1.

Selectivity is a key factor to successful drug discovery. And it is especially important for the development of covalent inhibitors due to their potential toxicities from irreversible modification of target proteins. To understand the selectivity of 1,3,5-oxadiazin-2-ones, we first synthesized a short peptide containing reactive amino acids such as cysteine, lysine and serine. Surprisingly, incubation of Oxa1 with the peptide at various conditions did not produce any modifications analyzed

by mass spectrometry (**Figure S2**). We then asked if Oxa1 could react with glutathione which is the most abundant reactive thiol in cells. Within the time frame of our experiments, we did not observe any reaction between Oxa1 and glutathione (**Figure S3**). These experiments suggest that Oxa1 does not modify solvent-exposed cysteines or serines or lysines non-specifically. We next probed Oxa1 selectivity with TEV protease, a known cysteine protease containing a reactive cysteine at its active site. However, Oxa1 does not inhibit TEV protease activity at concentrations up to 100 μM (**Figure S4**). Finally, we tested if Oxa1 could react with *S. aureus* FabF, a close homolog of FabH, which catalyzes the condensation reaction between malonyl-ACP and acyl-ACP in fatty acid synthetic pathway. Unexpectedly, Oxa1 does not covalently modify FabF under the same conditions (**Figure S5**). Taken together, these results provided strong evidence for Oxa1 to be a specific covalent inhibitor of FabH, but how it achieves selectivity requires further investigation.

**Crystal Structure Determination and Structure and Activity Studies.** In order to understand the mechanism for this selective covalent modification at molecular level, we performed co-crystallization of *S. aureus* FabH and Oxa1. Initial molecular replacement with apo FabH structure (PDB1zow) with iterative rounds of refinement revealed an unresolved electron density extended from the sulfur terminal of Cys112 (**Figure 4A**). This density can be resolved very well with a linearized Oxa1 covalently attached to γS of Cys112 and extended into the CoA binding pocket toward the entrance (**Figure 4B**). This is consistent with our previous finding from LC-MS analysis, confirming Cys112 as the site for modification. The final Oxa1 modified FabH structure is refined to 2.3 Å and have been deposited in the Protein Data Bank under accession codes 6KVS (**Table S3**).

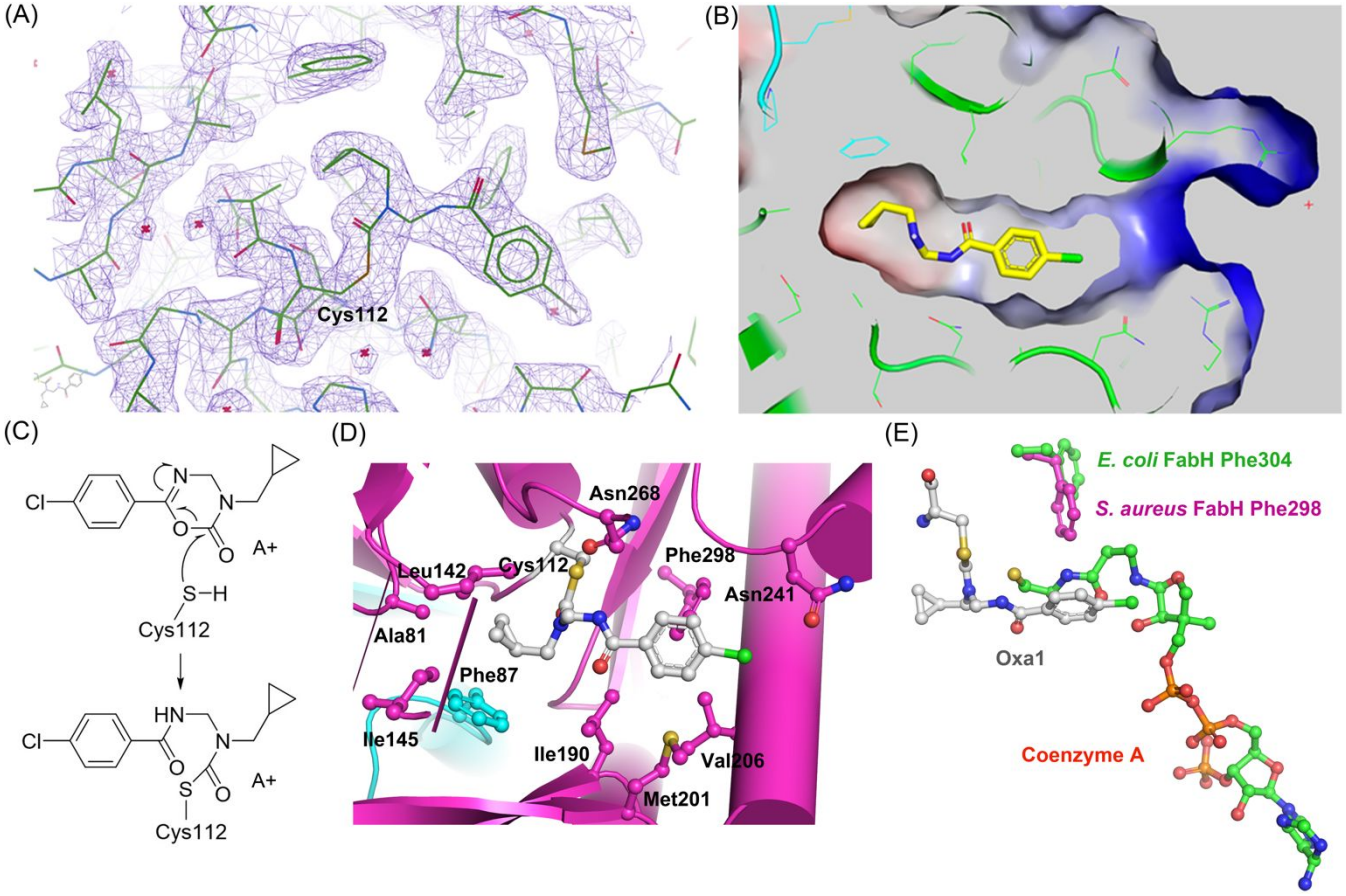


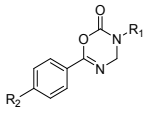

**Figure 4.** Analysis of co-crystal structure of *S. aureus* FabH:Oxa1 and the proposed reaction mechanism. (A) Electron density map (purple wire) near FabH Cys112 is fitted very well with a linearized Oxa1. (B) Surface representation of FabH illustrating Oxa1 (shown in sticks) in substrate binding tunnel and extending toward entrance. Red represents acidic amino acids, blue represents basic amino acids and white represents hydrophobic amino acids. (C) Proposed mechanism for Oxa1 covalent reaction. The sulfur of Cys112 makes nucleophilic attack at Oxa1 carbonyl group nearby, and electron rearrangement causes ring-opening with possible assistance of a Lewis acid. (D) Structural view of Oxa1 binding interactions with FabH. Protein is show in a cartoon representation with one monomer colored in magenta and the other in cyan; Oxa1 attached to Cys112 is shown in ball and stick representation and colored by atom type (carbon, white; nitrogen, blue; oxygen, red); Key side chains lining the binding pocket are also shown in ball and stick representation and colored by atom (carbon, magenta; nitrogen, blue; oxygen, red) with Phe87 from the other monomer colored in cyan. All structural figures were created by CCP4 and Pymol. (E) Overlay of Oxa1 attached to Cys112 with CoA from the structure of *E. coli* FabH: CoA complex. Structures of FabH:Oxa1 and *E. coli* FabH: CoA (PDB 1ebl) were superimposed in Pymol; Oxa1 attached to Cys112 and CoA are both shown in ball and stick representation, colored by atom type (carbon, white; nitrogen, blue; oxygen, red).

The overall structure of Oxa1 modified FabH is almost identical to FabH apo structure (PDB 1zow), with an RMSD of 0.455Å (all protein atoms included in the dimer structure). The major differences reside in the compound binding site. Both His238 and Asn268 shifted slightly aside to accommodate the adduct molecule. Phe298, which controls the entrance of the substrate binding channel, has its side chain rotated in a way that the aromatic ring almost 90-degree stacks against that of Oxa1. According to the resolved structure, we propose a possible mechanism for this covalent reaction (**Figure 4C**). Basically, Cys112 makes nucleophilic attacks at the carbonyl group of Oxa1, followed by electron rearrangement which resulted in the opening of the 1,3,5-oxadiazin-2-one ring structure. From the resolved structure, His238 sits very close to the carbonyl group in Oxa1 and so may likely have facilitated the reaction by protonating the carbonyl group as a Lewis acid. However, it requires experimental verification by biochemical analysis of the appropriate mutants.

Moreover, Oxa1 makes specific interactions with residues along the substrate binding tunnel, potentially contributing to its high activity. First of all, the cyclopropylmethyl group of Oxa1 fits snuggly in a hydrophobic pocket mainly made by Ala 81, Phe87, Ile145, Leu142 and Leu190 (**Figure 4D**). It is the proposed primer binding pocket of *S. aureus* FabH where the branched acyl chain binds. In the co-crystal structure, the cyclopropyl group is only 4 Å away from Ala81, which might explain how the A81V mutation confers resistance to both Oxa1 and Oxa2 (**Table S1**). It is also worth noting that the cyclopropyl moiety directly stacks against the phenyl ring of Phe87 from another molecule of the dimer. To verify the contribution of these hydrophobic interactions to compounds' activity, we replaced cyclopropyl- methyl ($R_1$) with aliphatic substituents of 2-5 carbon lengths, both straight and branched chain structures, and measured their antibacterial activities. MIC values of these compounds showed that n-butyl group (Oxa2) at $R_1$ position gave optimal activity, followed by cyclopropylmethyl (Oxa1) and cyclobutylmethyl (Oxa6), while the isopropyl substituent (Oxa5) is less active (Table 3). This result supports the notion that optimal hydrophobic interactions from $R_1$ with the target result in higher activities. Although both Oxa1 and Oxa2 have 4 carbon length in the aliphatic chain, Oxa2 is consistently more active than Oxa1. It could be that the linear acyl chain is more flexible, thus able to adopt an ideal conformation for binding. As expected from the structure, this part of the pocket is of limited size, introduction of larger groups such as benzyl in Oxa4 and isopentyl in Oxa3 abolished their antimicrobial activity completely.

Secondly, the *para*-chlorophenyl group occupies the CoA channel as we overlay the structure with that of *E. coli* FabH: CoA complex (**Figure 4E**). The phenyl ring is only 1-1.7 angstrom away from corresponding atoms in CoA and it almost stacks perpendicularly against the side chain of Phe298, which is the proposed residue for constraining substrate entry (Phe304 in *E. coli* FabH). Apart from Phe298, this channel is made of both polar and non-polar residues, including Met201, Val206, Leu156 and Asp268, Asn241 as well as backbones of three surrounding loops (**Figure 4D**), leaving opportunity to pick up additional specific interactions. We first introduced different substitutions at $R_2$ position, including pyridyl, 2-methoxylbenzyl, 2-nitrophenyl, Br and F. The introduction of a halogen group of a different size at $R_2$ does not affect its activity, a methoxyl or ethoxyl substitution could also be tolerated, however, a hexatomic ring such as pyridyl resulted in complete loss of antibacterial activity (**Table 3**). We speculated that the linkage between a hexatomic ring and benzyl is rigid in nature that it creates steric hindrance when binds in the CoA pocket of FabH. However, we could not exclude the possibility that introducing phenyl groups at $R_2$ might have changed the compounds' permeability into the cell. Overall, although this FabH complex structure gives a post-reaction snapshot, it could still provide some information for the rational design of more potent inhibitors of this novel antibacterial class.

**Table 3.** Antibacterial activities of 1,3,5-oxadiazin-2-one derivatives Oxa1-13.



| | | | MICs (μg/mL) | |
|---|---|---|---|---|
| Compound | $R_1$ | $R_2$ | S. aureus | E. coli |
| Oxa1 |  | Cl | 1 | >64 |
| Oxa2 |  | Cl | 0.25 | >64 |
| Oxa3 |  | Cl | >64 | >64 |
| Oxa4 |  | Cl | >64 | >64 |
| Oxa5 |  | Cl | >64 | >64 |
| Oxa6 |  | Cl | 2 | >64 |
| Oxa7 |  | Br | 1 | >64 |
| Oxa8 |  | F | 2 | >64 |
| Oxa9 |  |  | >64 | >64 |
| Oxa10 |  |  | >64 | >64 |
| Oxa11 |  |  | >64 | >64 |
| Oxa12 |  |  | 8 | >64 |
| Oxa13 |  |  | 16 | >64 |
| Kanamycin | - | - | 4 | 16 |

**Discussion**. Despite the vigorous rate of publications emphasizing the spread of antibiotic resistance, the same cannot be said for the numbers of approved new drugs reaching the market. And most of them are improved iterations of old scaffolds for which there are already underlying resistance mechanisms[26]. In another word, there remains a paucity of novel agents active against novel targets. There has been much debate over how to replenish the antibiotic pipeline. Phenotypic screen

is one way to identify hits with whole cell activity, however, without a quality library, these efforts often lead to rediscovery of old compounds or natural products that are synthetically challenging for further development[27, 28]. To overcome the potential problems, we started our growth inhibition screen with a collection of synthetic compounds that are novel in structure and easy to prepare derivatives chemically. Together with isolation of compound resistant clones and whole genome sequencing, it is likely to pinpoint the molecular target with relative ease.

In this report, we described the discovery of a series of FabH inhibitors containing a new chemical scaffold 1,3,5-oxadiazin-2-one. The compounds are active against clinically isolated methicillin-sensitive and methicillin-resistant *S. aureus*, thus could potentially be further optimized into drugs to treat life threatening MRSA infections. Detailed mechanism of action studies suggested the compounds inhibit FabH activity by covalently modifying its active site cysteine residue with high selectivity. A crystal structure of FabH protein modified by compound Oxa1 confirmed the covalent nature of inhibition. Cys112 is a catalytic residue in *S. aureus* FabH and together with His238 and Asn268 is indispensable for the enzyme's condensation activity. FabH operates via a ping–pong mechanism utilizing an acetyl-S-Cys-enzyme intermediate. It was proposed that this active site cysteine in FabH has unusual reactivity due to its location at the N terminus of a long alpha helix[4, 29], which could generate a strong dipole effect. In addition, the cysteine is ideally positioned at the bottom of the substrate binding tunnel to receive the full benefit of a half unit of positive charge generated by the strong dipole of an alpha helix. The cysteine thus becomes a strong enough nucleophile to catalyze the thioester exchange reaction responsible for the formation of the acetyl-FabH intermediate. So FabH Cys112's enhanced reactivity makes it an ideal target for covalent inhibitors as it does not require highly reactive warhead, which could cause potential toxicity.

The crystal structure also indicated that apart from the covalent attachment to the target, the compound makes specific interactions with residues in the target active site and these non-covalent interactions could contribute to its antibacterial activity. Structure and activity study with chemical derivatives support this hypothesis and shed a light for further activity optimization. We believe that further improvement from the1,3,5-oxadiazin-2-one scaffold could lead to highly efficient and selective antibacterial with potentials to treat drug resistant pathogens.

## Methods

**Reagents and Strains.** *S. aureus* ATCC29213, *S. aureus* ATCC25923, *E. coli* ATCC8739 and *P. aeruginosa* ATCC27953 were purchased from Guangdong Microbial Culture Collection Center (GDMCC). Methicillin-resistant *S. aureus* strains were provided by the clinical laboratory of Qiao Liuhua hospital. TEV protease was purchased from Beyotime Biotechnology. Substrate for TEV protease was provided by Dr. Huihao Zhou in Sun Yat-sen University. Platencin was provided by Dr. Yong Huang in Central South University. Chemical reagents and growth medium unless otherwise noted were purchased from Sigma. The whole gene sequencing was conducted by Guangzhou Ige Biotechnology Limited Company.

**Chemistry.** General methods. Unless otherwise noted, all purchased reagents were used as received without further purification. $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded on Ascend TM 500 spectrometers. MS analyses were carried out on Waters UPLC-MS instrument. The final compounds were all purified by C18 reverse phase HPLC column with solvent A (0.1% TFA in $H_2O$) and solvent B (0.1% TFA in $CH_3CN$) as eluents. The purities of all the final compounds were confirmed to be > 95% by UPLC-MS or UPLC. For compound details, see Supplemental Methods.

**Growth Inhibition Screen in *S. aureus*.** Inoculate 2 mL of LB media with a single colony of *S. aureus* ATCC29213 and grow to saturation by shaking at 37 °C overnight. Dilute bacteria culture to ca. $2 \times 10^6$ CFU/mL each in LB media. Dispense 80 μL of LB into 96-well and transfer 1.6 μL each compound from DMSO stock (1 mg/mL) into duplicate plates, to give a screening concentration of 10 μg/mL. Kanamycin serves as positive control. Add 80 μL of $2 \times 10^6$ CFU/mL bacteria culture to all wells in plates. Stack the plates and cover the top plate with a low evaporation lid. Place the plates in a 37 °C incubator overnight (16-24 h). Using a plate reader, measure the optical density of each well at 600 nm.

**Minimal Inhibitory Concentrations.** The Minimal inhibitory concentrations (MICs) for compound Oxa1-13 were determined using broth microdilution method against *S. aureus*, MRSA strains, *E. coli* and *P. aeruginosa.* Three to five colonies from the agar plate culture grown overnight were inoculated into 5 mL LB, and the culture was incubated at 37°C. After 4-6 h, dilute culture until $OD_{600} = 0.1$, then further diluted to inoculate wells of a 96-well plate containing compound dilutions in growth media, with $5 \times 10^5$ cells each. The plate was incubated at 37℃ for 20 h and read at 600 nm.

The MICs for Oxa1 and Oxa2 against *S. aureus* with fatty acids supplements were determined as follows: growth medium was made in LB containing 20 mg/mL BSA with or without 800 μM specified fatty acids (a16:0, a18:0, a18:1 or a18:2) [25]. *S. aureus* ATCC29213 is incubated in LB at 37°C. After 4-6 h, dilute culture until $OD_{600} = 0.1$, followed by 100-fold dilution into each specified medium. 50 μL aliquots of diluted cells were added to a U-bottomed, 96-well plate containing 50 μL of fatty acid containing medium with an appropriate concentration of compounds. The plate was incubated at 37 °C for 20 h and read at 600 nm.

**Compound-resistant Mutant Generation and Whole Genome Sequencing.** *Staphylococcus aureus* ATCC29213 strains cultured at 37℃ on LB agar plates were collected and resuspended in LB broth to an $OD_{600}$ of approximately 3.0, which corresponds to a density of approximately $10^9$ CFU/mL. 100 μL volumes of this suspension were spread evenly in triplicate onto agar plates containing compound at concentrations 4 × MIC and incubated at 37℃. Meanwhile a serial dilution of this suspension was spread onto regular LB agar plates to determine the exact number of CFU. Resistant

variants were enumerated after 2 days of incubation at 37 °C. The initial screened Oxa2-resistant colonies were spread onto agar plates containing Oxa2 at concentrations 8 × MIC to screen further resistant colonies. 2 days later, resistant clones were harvested individually, and DNAs were purified and analyzed by agarose gel electrophoresis. Covaris ultrasonic breaker was used to fragment the DNA sample, and the whole library preparation was completed through the steps of terminal repair, A-tail addition, sequencing joint addition, purification and PCR amplification. After the construction of the library, Qubit 2.0 was used for preliminary quantification and dilution of the library, and then Agilent 2100 was used to detect the inserted fragments of the library. Next, Q-PCR method was used to accurately quantify the effective concentration of the library. Illumina high-throughput sequencing platform was used for the sequencing job. Genome assembly and data analysis was done using SPAdes, breseq and svmu.

**FabH Activity Assay.** Preparation of FabH, FabD and malonyl-ACP are described in reference[30] and SI text. Each FabH reaction contains: 100 mM phosphate buffer (pH 7.0), 20 μM isobutyryl-CoA, 0.05 μg FabH, 8 μM malonyl-ACP in a final volume of 10 μL. The reaction was quenched after 5 min with 40 μL of 10 mM 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoum- arin (CPM) in 100% DMSO solution. Fluorescence was measured at Ex384/Em470 using a monochromator-based multimode microplate reader. The microplate reader was calibrated by measuring CoA at concentrations ranging from 0 to 5 μM in the DMSO-based quench solution. All experiments were performed in sextuplicate. For $IC_{50}$ measurement, compounds were added to each well in serial dilutions to a final DMSO concentration of 0.5% and incubated with FabH for 15 min before adding malonyl-ACP to initiate FabH reaction. $IC_{50}$ data fitting were done with 4-parameter logistic model using GraphPad 7.0.

**Site Identification by LC-MS.** 100 μM Oxa1 was used to react with 50 μM FabH protein for 4 h at room temperature. For FabH protein digestion, a total protein of 7.5 μg was diluted into 50 mM $NH_4HCO_3$ solution and 0.15 μg trypsin (1:50 mass ratio) was added for an overnight incubation at 37℃. A 2-layer stage-tips (C18/C18) was used to perform peptide purification according to the following protocol: (1) Wetting the tip: add 100 μL 100% methanol and centrifugation at 1200 xg for 10 min for 3 times, then add 100 μL 50% MeOH 0.1% TFA and centrifugation at 4000 xg for 4 min for 3 times. (2) Equilibration: add 100 μL 0.1% TFA and centrifugation at 6000 xg for 4 min for 3 times. (3) Load sample and centrifugation at 2000 xg for 12 min for 2 times. (4) Wash off salts: add 100 μL 0.1% TFA and centrifugation at 6000 xg for 4 min. (5) Wash off TFA: add 100 μL 0.1% formic acid and centrifugation at 6000 xg for 4 min for 2 times. (6) Elution: add 50 μL 50% MeOH 0.1% formic acid and centrifugation at 2000 xg for 4min for 2 times. (7) Dried in speed-vac for 20 min, then lyophilized to dryness. Dissolve the dried in 40 μL 0.1% formic acid for LC-MS analysis.

**Co-crystallization of *S. aureus* FabH with Oxa1.** For co-crystallization of FabH and compound Oxa1, Ni-NTA purified FabH was further purified by size exclusion on a superdex 200 10/300 G column. Protein eluted at about 66kDa was collected and concentrated to 10 mg/ml in a storage buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM sodium chloride, 3 mM DTT and 10% glycerol. FabH was then mixed with Oxa1 in a molar ratio of 1:2 and crystallization was set up using the hanging drop method. Reservoir solution is composed of 25% PEG4000, 0.1 M sodium citrate (pH = 5.4), 0.2 M ammonium acetate. Crystals appeared after 3 days at 18°C and were harvested for data collection. All data were collected at 100 K using X-ray diffractometer (Xcalibur Nova). Data were integrated, reduced and scaled using the CCP4 program suite. Structure of *S. aureus* FabH:Oxa1 was determined by molecular replacement using CCP4 suite with apo FabH structure (PDB 1zow) followed by refinement using Refmac[6]. Data and refinement statistics are in **Table S3**.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supplemental Methods including protein production, chemical synthesis of compounds, NMR spectra and HRMS of compounds, whole genome sequencing analysis, glutathione quantification and TEV protease gel assay.

Supplemental Figures and Tables including Figure S1- S5 and Table S1- S3.

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### Author Contributions

Y.Y. designed the experiments and supervised all research activities. J.L. and M.S. contributed to the identification of Oxa1 as an antibacterial compound and initial target identification. J.W., Y.C and Q.Y. designed and synthesized all the compounds; J.W., X.Y., X.Y. S.W. and J.T. performed all other experiments; Y.Y., W.H. and J.W. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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