Small Polar Hits against *S. aureus*: Screening, Initial Hit Optimization, and Metabolomic Studies

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Supporting Information

ABSTRACT: The global prevalence of antibacterial resistance requires new antibacterial drugs with novel chemical scaffolds and modes of action. It is also vital to design compounds with optimal physicochemical properties to permeate the bacterial cell envelope. We described an approach of combining and integrating whole cell screening and metabolomics into early antibacterial drug discovery using a library of small polar compounds. Whole cell screening of a diverse library of small polar compounds against *Staphylococcus aureus* gave compound 2. Hit expansion was carried out to determine structure–activity– relationships. A selection of compounds from this series, together with other screened active compounds, was subjected to an initial metabolomics study to provide a metabolic fingerprint of the mode of action. It was found that compound 2 and its analogues have a different mode of action from some of the known antibacterial compounds tested. This early study highlighted the potential of whole cell screening and metabolomics in early antibacterial drug discovery. Future works will require improving potency and performing orthogonal studies to confirm the modes of action.

INTRODUCTION

Antibacterial resistance, an issue under the umbrella term of antimicrobial resistance (AMR), is becoming a major global public health issue, affecting both developed and developing countries alike, although the impact in low and middle income countries is disproportionately high.1−3 It has been estimated that the global death toll associated with AMR could rise to 10 million and the cumulative economic cost to U.S. $100 trillion by the year 2050 if AMR is not tackled urgently.4

Maintaining a healthy pipeline of new antibiotics is a key strategy in combating AMR as bacterial resistance against antibiotics is inevitable. An evaluation of the antibiotic pipeline by the Pew Charitable Trust5 in September 2017 found that there were 48 antibiotics in various phases in clinical development. Despite these seemingly healthy numbers, there are several caveats to this observation. First, there is the risk of attrition in each phase of the clinical development of antibiotics.5 Second, it was observed that most of the antibiotics in the pipeline were iterations of some of the major classes of antibiotics such as β-lactams, quinolones, tetracyclines, and oxazolidinones. This strategy, while maintaining a stream of new antibiotics, may suffer from the impact of cross-resistance, in which resistance against one antibiotic confers cross-resistance to other antibiotics of similar class, either through mutation of the molecular target or some common means of efflux or drug inactivation (e.g., β-lactamases).

As such, it is vital to maintain a healthy pipeline of antibiotics with either new modes of action or new chemical scaffolds. There have been a very few new classes of antibiotic uncovered since the golden age of antibiotic discovery (in the 1950s and 1960s),6 and the introduction of new classes of antibiotics post-2000 (such as oxazolidinones, lipopeptides, and mutilins) can be traced to their discovery before 1990.7

A key issue in the development of new antibiotics is obtaining sufficient compound levels in cells, due to restricted permeation of the bacterial cell envelope and ejection of compounds through efflux pumps. The physicochemical properties of compounds play a role in compound uptake. The wealth of discussions8−12 on the physicochemical properties of antibiotics and the comparison to other nonantibiotics highlights the challenges of designing an antibiotic with favorable physicochemical properties. The precise physicochemical properties required for cell entry are poorly understood. However, analysis suggests that antibacterial compounds tend to be more polar than other drugs.10,12

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with compounds acting against Gram-negative bacteria tending to be more polar/charged than those acting against Gram-positive bacteria. As such, the choice of libraries for screening bacteria is crucial to enhance the odds of finding hit compounds with the appropriate antibacterial physicochemical properties.

Current antibiotics target a limited set of enzymes or RNA structures within key bacterial cellular processes, such as cell wall biosynthesis, protein biosynthesis, and DNA biosynthesis. Although these targets are validated, there should be a search for other targets to treat bacteria resistant to current antibiotics.

There are two main routes to drug discovery, target-based and whole cell (phenotypic) approaches. Target-based discovery is hindered by the challenges of compound uptake/efflux, which means that compounds active in an enzyme assay are not necessarily active against intact bacteria. Further there is a risk that compounds that inhibit a single target can be subject to high rates of resistance. Whole cell approaches can also suffer from low hit rates due to compound uptake/efflux issues; however, compounds that are active hit essential targets and are able to accumulate to sufficient levels at those molecular targets. Whole cell hit discovery requires some degree of target deconvolution to ensure that whole cell actives are targeting a novel and/or progressible target. Knowledge of the binding mode of the lead in the protein is also valuable for compound optimization and to overcome issues such as poor pharmacokinetics.

Whole cell and target-based drug discovery processes are complementary and can be merged into a hybrid model (Figure 1). In this hybrid model, a library of compounds is screened for whole cell activity. Hits from the screen are then expanded to give various analogues to probe for any indicative structure−activity relationship (SAR). Target deconvolution can then be used to identify the molecular target. If the target is suitable for development, target-based approaches can be used in optimization and scaffold hopping. This approach has the advantage of starting with compounds that are cell penetrant and which give rise to the desired whole cell response. The identified molecular targets possess a higher degree of validation, as it has been demonstrated that a druglike compound acting against this molecular target can have a desired whole cell effect on the bacteria.

We decided to screen against intact whole bacteria with a diverse library of small polar compounds, which should address the physicochemical properties to some extent and being low molecular weight should cover a wide range of chemical space as well. Furthermore, small polar fragment-like molecules would facilitate optimization toward lead-like molecules through the process of adding complexity.

We decided to apply whole cell metabolomics as a way to compare modes of action, to be able to identify compounds that have a novel mechanism of action. Metabolomics can be described as a comprehensive bioanalytical technique to characterize and quantify metabolites of a biological system. Metabolites reflect the biochemical status of the biological system. Since biochemical changes in a biological system lead directly to changes in its whole cell response, it follows that metabolomics can facilitate the understanding of the genotypic–phenotypic link, as changes in metabolite levels can be ascribed to changes in protein activity, which is linked to genes as well as to environmental status, where precursor metabolites are acquired by the cell. As such, the whole cell response upon exposure to an antibacterial compound is a function of the change in the metabolites. In some cases, this can be deconvoluted to reveal affected metabolic pathways and individual target enzymes. This should provide a fingerprint to allow comparison of the modes of action of different compounds and potentially, in some cases, ultimately the protein target. Some examples illustrate the use of metabolomics in this scenario.

In this paper, we aim to describe the experience of using our in-house small, fragment-like polar compound library as the starting point in early antibacterial drug discovery. First, we aim to see if low-molecular-weight “fragment-like” compounds are a viable starting point for a drug discovery program and second can we use metabolomic fingerprints at this early stage of the discovery process to compare modes of action. We will present some early results, alongside thoughts and challenges that arose during the process.

**RESULTS AND DISCUSSION**

**Whole Cell Screening of In-house Library.** An in-house library of small, fragment-like polar molecules comprising of almost 1300 compounds has been described in detail by Ray et al. The library consisted of a diverse set of small molecules, with physicochemical properties with low molecular weight [heavy atom count (HAC) 5−18]; low lipophilicity (log D ≤2.5 and log P ≤2.5), and aromatic rings ≤3 (these represent the upper limits; the median values are much lower). These physicochemical properties (low molecular weight and high polarity) should increase the chances of entry into the bacteria. Further, the low molecular weight means that it is possible to cover a similar chemical space to libraries with much larger numbers of leadlike or druglike compounds. The library was curated to remove known structural alerts (toxophores, reactives, and pan-assay interference compounds). The library was mainly composed of commercially obtained fragments but also contained some proprietary compounds. The whole cell assay was based on measuring the turbidity of bacterial culture (as OD600nm) as a proxy of growth. The screening was performed using 384-well plates to enable high-throughput screening. As an example of Gram-

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**Figure 1.** Steps involved in the hybrid whole cell/target-based drug discovery.
positive bacteria, Staphylococcus aureus RN4220, which was used in previous in-house screenings, was utilized in this whole cell assay. Compounds were initially screened at a single concentration of 600 μM to reflect the low molecular weight of these small fragment-like molecules.

From the single-point whole cell assay, 23 compounds were returned as hits, defined as having inhibitory activity at three robust standard deviations from the median. To remove false positives from the set of hits, a 10-point dose−response assay, with the top concentration of 600 μM, was performed at the whole cell level to seek any dose-dependent inhibitory activity and to estimate the potency of these hits. Of the 23 hits forwarded into the dose−response assay, two compounds, 1 and 2 (Figure 2), gave clear dose−response curves. Given their small size, they had a relatively low potency \( pEC_{50} < 4 \), where \( pEC_{50} = -\log_{10}(EC_{50}) \), which is not unexpected. However, the ligand efficiencies (LE = determined as \( 1.37 \times pEC_{50}/\text{heavy atom count} \)) of the key hits were very promising, 0.30 and 0.35. By analogy with fragment-based drug discovery processes, the small size allows scope for optimization, while retaining ligand efficiency.

Compound 1 has been described in the literature as EX-527, a SIRT1 inhibitor.\textsuperscript{23,24} Literature investigation revealed compound 2 to be a ligand of the adenosine A\textsubscript{2A} receptor discovered using fragment-based drug discovery.\textsuperscript{25,26} It was also described as a noncompetitive inhibitor of the human divalent metal transporter DMT1/SLC1 1A2, with the \( K_i \) of approximately 20 μM.\textsuperscript{27} Interestingly, the pyrazolopyrimidine scaffold of 2 was described as having fungicidal activity.\textsuperscript{28,29} Additionally, 2 was described to be active against Mycobacterium tuberculosis.\textsuperscript{30}

Hit expansion of 2 was carried out, as 2 has lower molecular weight and lipophilicity than 1 and a greater chemical tractability, thus allowing greater complexity to be added during the hit expansion process.

**Resynthesis of 2 and Analogues.** Compound 2 is a pyrazolopyrimidine. The distribution of various tautomeric forms (pyrimidinone and hydroxypyrimidine) has been discussed\textsuperscript{31} using various theoretical methods, albeit in the gaseous phase. A computational study on complexes of 4-hydroxypyrimidine with water suggested that the pyrimidinone (4-oxopyrimidine) tautomeric form predominates over the 4-hydroxypyrimidine tautomeric form at a 3:1 ratio at room temperature (rt).\textsuperscript{32} Nevertheless, the hydroxypyrimidine representation will be used throughout this paper for consistency purposes and to aid visual comparison among the analogues synthesized.

A key early goal of the project was to validate the series and to try and increase its activity. The structure of 2 was amenable to derivatization, to probe the effect of various substituents at different locations on the scaffold, to increase potency (Figure 3). The choice of substituents for such a study was designed in

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Figure 2. Dose−response curves of 1 and 2, together with the corresponding structures and potency described as pEC\textsubscript{50}.

Figure 3. Possible strategies in generating analogues of 2.
a way that a simple change in the substituent can lead to changes in physicochemical properties such as lipophilicity, hydrogen bond donor and acceptor count, which will then impact compound activity. Various synthetic routes were generated to fulfill the various strategies highlighted in Figure 3.

Scheme 1. Synthesis of 2 and Analogues from 2,4-Dichloropyrimidine Derivatives

![Scheme 1](image)

“Reagent and conditions: (a) 3,5-dimethylpyrazole, dioxane, microwave (MW) 150 °C, 3 h, 24−35%; (b) 1 M NaOH (aq), tetrahydrofuran (THF), MW 150 °C, 0.5 h, 68−90%; (c) appropriate benzyl alcohol, Cs₂CO₃, MeCN, reflux, overnight, 36−79%; (d) 1:1 trifluoroacetic acid (TFA)−dichloromethane (DCM), rt, 1 h, 72%; (e) MeI, K₂CO₃, dimethylformamide (DMF), reflux, overnight, 75%.

Scheme 2. Comparison of the Calculated Regio-isomeric Ratio of the Synthesis of 3 and 3b by Inclusion of the Base

![Scheme 2](image)

“Calculated ratio was based on NMR integration.

Scheme 3. Synthesis of Analogues of 2 from 2-Chloropyrimidine Derivatives

![Scheme 3](image)

“Reagent and conditions: (a) 3,5-dimethylpyrazole, KOH, 18-crown-6, acetonitrile, room temperature, 1 day, 41−70%; or MW (80 °C, 15 min for 13; 120 °C, 30 min for 14), 32−47%; or for 17: 3,5-dimethylpyrazole, Cs₂CO₃, dioxane, MW 150 °C, 3 h, 64%; (b) LiCl, DMP, MW 160 °C, 30 min (3 h for 21), 24−97%; (c) LiOH−THF, rt, overnight, 87%; (d) NH₃ (for 23) or MeNH₂ (for 24) or Me₂NH (for 25), MeOH, rt, overnight, 99−100%; (e) Burgess reagent, THF, rt, overnight, 93%.
First, the pyrazolopyrimidine compounds were derived by reacting the pyrazole of interest with the appropriate pyrimidines. Substituted 2,4-dichloropyrimidines were used in the initial synthesis to generate the hit compound 2 and other analogues via the 4-chloro intermediate (for example, 3 and 4) (Scheme 1).

The first step in Scheme 1 suffered from regio-isomerism, in which the displacement by the pyrazole derivative could occur at either the 2-position or the 4-position of the pyrimidine ring. Using the reaction to generate 3 as an example, it was interesting to note that the formation of the desired regio-isomer 3 was favored and enhanced without the presence of a base (Scheme 2). However, the isolated yield was compromised by the difficulty in separating both regio-isomers. The structure of these regio-isomers was distinguished by performing 1H NMR experiments, in particular, nuclear Overhauser enhancement spectroscopy (Figures S1–S3).

Initially, it was envisaged that the synthesis of the hit compound 2 was carried out via the 4-methoxybenzyl ether derivative 5 through a TFA-mediated deprotection reaction. However, it transpired that direct hydroxylation of the 4-chloro derivatives (3 and 4) was also possible via microwave-assisted reaction with THF–NaOH solution, thus saving an additional step in generating analogues of 2. Other benzyl ether derivatives 6–9 were generated, thus increasing the diversity of analogues. Additionally, the hit compound 2 was further methylated to give the N-methyl product 11.

This synthetic scheme was extended to include 2-chloropyrimidine derivatives as precursors to other analogues with various substitutions on the 4- and 6-positions of the pyrimidine core (Scheme 3). For example, the methoxy derivatives 14–16 were demethylated to provide analogues 19–22.

The methyl ester analogue 17 provided another attractive starting point for rapid hit expansion as it could be readily derivatized. Hydrolysis provided the free acid 18, while displacement with simple amines provided various amidines 23–25. The primary amide was dehydrated to give the nitrile 26. Note that these simple chemical transformations allowed us to generate various analogues with different physicochemical properties, in particular, hydrogen bond donor/acceptor count, which allowed a good assessment of SAR.

The pyrazole moiety was modified by replacing one of the methyl groups with a hydroxyl group. This was performed by using aminoguanidine as the precursor and reacting it with a β-keto ester diketo ester such as ethyl acetoacetate to give a pyrazolone derivative 27 (Scheme 4). The similar synthetic methodology gave the 4-methylpyrazolone derivative 28. The β-keto ester precursor 27 was synthesized using a TiCl₄-mediated condensation reaction. The pyrazolone derivatives then underwent the ring formation reaction to create the desired pyrimidine core (30 and 31).

The pyrazole moiety was replaced entirely with cyclic saturated examples (Scheme 5). Three cyclic saturated structures were used as examples: pyrrolidine, 2-methylpyrrolidine, and morpholine. To achieve this, unsubstituted 1H-pyrazole-1-carboximidamide was used as a guanylation agent, attaching the guanyl group to the nitrogen atom of the cyclic saturated examples. These guanyl derivatives then underwent the usual ring formation reaction to give the analogues 35–37.

This ring formation strategy was useful in exploring the effect of substitution on the 5-position of the pyrimidine core. The starting compound 3,5-dimethyl-1H-pyrazole-1-carboximidamide can be reacted with either β-keto ester 27 or acrylates to give analogues 38, 39, and 42 (Scheme 6). As with in Scheme 2, basic functional group transformation allowed the generation of analogues with diverse physicochemical properties. It was interesting to note that the ethyl ester 39 underwent transesterification to give 40 upon treatment of methanolic ammonia; thus, a different route was required to obtain the amide 41. Direct synthesis of 42 gave a side reaction, which was documented in the literature, thus an alternative route was envisaged in which the amide 41 was dehydrated to give 42.

Attempts were also made to change the pyrimidine core for other heterocycles. At first, pyridine was used with 2,6-dichloropyrimidine derivatives as the starting point. The synthetic scheme (Scheme 7) is similar to Scheme 1, but the hydroxylation reaction required a copper-catalyzed reaction, modified from Yang et al. and Wang et al. There were limited number of pyridine examples, reasons of which will be discussed in the later sections.

All in all, these synthetic methodologies provided convenient methods to generate a diversity of analogues with varying pharmacophoric and physicochemical properties (examples shown in Figure S4). Furthermore, these synthetic methods can be applied in parallel synthesis, thus allowing rapid generation of analogues for SAR exploration.

**Biological Evaluation of 2 and Its Analogues.** The analogues synthesized (excluding intermediates 27–29 and 32–34) were then tested using the 10-point dose–response whole cell assay against *S. aureus*. Here, we noted the challenges in evaluating biological activity for some of these analogues. First, due to the low potency nature of small fragment-like polar molecules, high concentrations were used during the whole cell screening process. The dose–response assay was performed with the modified top concentration of 1.8 mM, diluted threefold across 10 concentration points, to better visualize the curve. As we have a maximum in-house dimethyl sulfoxide (DMSO) concentration limit of 1% in whole cell screening assays, the stock concentration for such compounds was set to 200 mM. Unfortunately, analogues with substituents on the 5-position of the pyrimidines (for example 16, 20, 39, 40, and 42) were found to be insoluble in DMSO at 200 mM.

The resynthesized 2 retained its inhibitory activity against *S. aureus* (Table 1). Some analogues have shown greater potency of almost a log fold than the original hit compound, in...
particular the benzyl ether analogues. Removal of the substituent groups on the pyrimidine core of the original hit 2 reduces the potency. The SAR on the pyrazole core is limited, with no other substituents having improved activity from the original 3,5-dimethyl substituents. The replacement of the pyrazole core with the saturated ring system removed any compound activity. The focused set of analogues that has been made cover a range of positions in the molecule and are sufficient to validate the hit and indicate potential ways to optimize the molecule. The overall SAR can be summarized in Figure 4.

As well as the potency of the compounds against S. aureus, various metrics were calculated. Ligand efficiency index (LEI) is normally calculated for binding to a protein. LEI, defined as $pEC_{50}$ divided by heavy atom count (HAC), is the simplification of LE in which the multiplier constant 1.37 was not applied in the equation. The value of LEI describes the extent of the improvement of the potency when more heavy atoms are added to the active compound. Originally used in the target-based fragment drug discovery, it can also be applied to whole cell screens where it measures how well each atom interacts with the molecular target and determines if substituents contribute significantly to the binding. Lipophilic ligand efficiency (LLE) indicates whether the interactions are driven by lipophilicity (Table 2).

Although compound 5 was the most potent compound, much of the binding compared to compound 2 was probably driven by lipophilicity of the benzyl group, as shown by the drop in LLE. Compound 10, on the other hand, had improved LEI and LLE compared to compound 2 and is the most efficient binder. It must be noted that compound 10 contains

Table 1: Activity of 2 and Its Analogues against S. aureus

<table>
<thead>
<tr>
<th>compound</th>
<th>mean $pEC_{50}$ (±SD)</th>
<th>compound</th>
<th>mean $pEC_{50}$ (±SD)</th>
<th>compound</th>
<th>mean $pEC_{50}$ (±SD)</th>
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<tr>
<td>2</td>
<td>3.9 (±0.05)</td>
<td>15</td>
<td>NA</td>
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</tr>
<tr>
<td>3</td>
<td>3.8 (±0.07)</td>
<td>16</td>
<td>NA</td>
<td>35</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>3.2 (±0.05)</td>
<td>17</td>
<td>NA</td>
<td>36</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>4.8 (±0.16)</td>
<td>18</td>
<td>NA</td>
<td>37</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>4.7 (±0.01)</td>
<td>19</td>
<td>3.2 (±0.20)</td>
<td>38</td>
<td>2.8 (±0.03)</td>
</tr>
<tr>
<td>7</td>
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<td>20</td>
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<tr>
<td>8</td>
<td>3.6 (±0.70)</td>
<td>21</td>
<td>NS</td>
<td>40</td>
<td>NS</td>
</tr>
<tr>
<td>9</td>
<td>4.6 (±0.07)</td>
<td>22</td>
<td>NS</td>
<td>41</td>
<td>NS</td>
</tr>
<tr>
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<td>4.4 (±0.04)</td>
<td>23</td>
<td>NS</td>
<td>42</td>
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<td>25</td>
<td>NA</td>
<td>44</td>
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</tr>
<tr>
<td>13</td>
<td>3.0 (±0.17)</td>
<td>26</td>
<td>NA</td>
<td>45</td>
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</tr>
<tr>
<td>14</td>
<td>NA</td>
<td>30</td>
<td>NA</td>
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*Expressed as the mean and its respective standard deviation from three replicates. *NA = no activity at 1.8 mM. *NS = not soluble in DMSO stock solution at 200 mM, hence not tested for compound activity.
an α-chloropyrimidine, which is potentially chemically reactive. In addition, compound 10 is less lipophilic than compound 5.

Metabolomics Study of 2 and Its Analogues and Other Relevant Compounds. The original hit 2 and the two selected compounds, 5 (highest potency) and 10 (highest LEI), were then subjected to metabolomics to elucidate any potential mode of action. Additionally, the other hit compound from the whole cell screening assay, 1, was included in this metabolomics study. Two approved antibiotics, ciprofloxacin and trimethoprim, were also included in this study for comparison.

The metabolomics study was based on the kill-kinetic studies similar to those used by Vincent et al.,17 in which the organism was exposed to an antibacterial agent and the effect monitored over time. The nature of the kill kinetics can be affected by the size of the starting inoculum and the dose of the compound administered at the beginning of the study.

*S. aureus* RN6390, which has been studied for its virulence and pathogenicity, was used, and it showed similar whole cell response and sensitivity to the RN4220 strain (examples in Table S1). Despite the subtle differences between the two strains RN6390 and RN4220, both were classified as methicillin sensitive and the lineage of these strains was traced back to NCTC8325 (also known as RN1) strain. This gave a hint of the whole cell similarity between the two strains and the capability of these compounds to target metabolic pathways, which are common and fundamental to these strains. The strain RN6390 has also been described to be virulent in various infection models39–41 and capable of producing α-toxins. On the other hand, the strain RN4220, although is useful in genetic manipulation studies, may contain mutations, which may affect virulence factors and strain fitness,42 and hence the change to the RN6390 strain for the metabolomics study.

The design of the metabolomics study was based on that of Vincent et al.17 The minimal inhibitory concentration (MIC) of each compound in this metabolomics study was determined by the microdilution method (Table 3).

Using 4 times the MIC as the starting dose, the bacteria were inoculated and incubated in fresh LB media for 2 h prior to the administration of the compound, thus coinciding with the exponential growth phase where bacterial metabolic activity is most active. The metabolome was sampled at intervals of 90 min to account for changes in metabolites due to cell death. The earlier time points are more likely to be representative of the specific mechanism of action.

The metabolome extraction protocol was adapted from that described by Vincent et al.,17 by including bead beating alongside chemical extraction (with 1:3:1 chloroform—methanol—water as the solvent) to obtain the internal metabolome.43 Glass beads were used as previously described43–46 for the extraction of metabolites from *S. aureus*.

Metabolome samples were analyzed by Glasgow Polyomics, University of Glasgow. The levels of metabolites and the identification of metabolites were determined based on the methods by Vincent et al.17 The comparison of metabolite
levels was initially performed using principal component analysis (PCA). A PCA plot is useful to reduce multidimensional data such as the data gathered in this study, as each data point consists of the various intensities of metabolites detected in each sample. By reducing multidimensional data to two- or three-dimensional data, a PCA plot presents a clear visual representation to identify any clustering of data, which gives an indication of similarity with one another. Figure 5 shows the effect of various compounds exhibiting antibacterial activity on the bacterial metabolome, visualized using PCA plots.

Ciprofloxacin has previously been shown to cause drastic changes to the nucleotide pool, and we also noted similar changes, in particular, in cytidine and guanine pool (Table S2). Trimethoprim inhibits dihydrofolate reductase (DHFR), but the folate pool was of insufficient abundance to be identified in the liquid chromatography–mass spectrometry platform we used. A significant increase in uridine is likely related to an accumulation of deoxyuridine monophosphate as its methylation to deoxoythymidine monophosphate (not identified) is prevented as DHFR is inhibited depriving the cells of the key methyl group donor used in DNA synthesis.

The observation that 2 and its two analogues have distinct clusters from the rest suggests that these compounds have the same/similar mode of action. However, although changes of metabolites were observed upon treatment with 2 and its two analogues, no metabolic pathways stood out as specific targets for these compounds based on clear changes in abundance across a single enzyme. This could indicate that 2 and its analogues act against a target, which has pleiotropic effects, against several targets, or the compound could act against targets with metabolites poorly represented in the database.

As ciprofloxacin and trimethoprim target nucleic acid metabolism (DNA and folate biosynthesis, respectively), it was interesting to observe that this general similarity in their mode of action translated to similar clustering in the PCA plot in Figure 5. Based on these clustering patterns, it can be suggested that the modes of action of 2 and its analogues are distinct compared to ciprofloxacin and trimethoprim. In addition, the clustering also suggests that compound 1 may have a distinct mode of action from the rest of the compounds tested. Nevertheless, the results from these early metabolomics studies must be supported by other orthogonal biological studies to generate or further strengthen a hypothesis on the mode of action.

These early results presented the challenge of determining the right moment in an early antibacterial drug discovery program for these compounds to be subjected to metabolomics study. As these compounds (excluding marketed antibiotics) are small polar molecules with low potency, there is always the possibility of their exerting activity at multiple targets across bacterial metabolism. In this case, during hit expansion to improve the potency of the analogues, the mode of action can shift. As seen in Figure 5, the more potent compound 5 seems to have a different metabolic fingerprint from its original hit 2. This may indicate that metabolomics can be useful to monitor whether SAR exploration may lead to a switch in the mechanism of action.

CONCLUSIONS

We have introduced the notion of small polar molecules as an attractive starting point in antibacterial drug discovery. This requires a well-constructed library to provide new chemical scaffolds with good physicochemical properties and novelty to allow efficient chemical expansion and optimization.

The whole cell screening process was designed to be amenable to high-throughput conditions, with straightforward set-up and easy-to-measure end points. From our in-house whole cell screening assay using our small polar library, we have identified two promising hit compounds, 1 and 2. The hit expansion of 2 has been illustrated in detail in this article, with diverse chemistry to probe the effect of various substitutions on the pyrazoloylpyrimidine core. This diverse chemistry can be amenable to parallel combinatorial chemistry to allow quick synthesis of analogues as well. Some initial SAR can be observed from these analogues synthesized, with compounds such as 5 and 10 gaining potency from the original hit. Although the original low-molecular-weight hit had relatively weak activity (but good ligand efficiency), it was possible to optimize it to a compound with much greater potency, not much less potent than trimethoprim, a clinically used antibiotic. As such, we believe that this series has the potential for further rounds of chemical optimization to improve potency and physicochemical properties, using ligand efficiency metrics as indicators. As physicochemical properties of these compounds are important in ensuring not only optimal entry into the bacteria but also pharmacokinetic properties, we would suggest using ligand efficiency metrics such as LEI and LLE to guide the direction of the chemical optimization.

Early metabolomics study indicated that 2 and its analogues, alongside the other whole cell hit 1, have distinct mode of actions compared to other antibiotics tested (ciprofloxacin and trimethoprim), even though the mode of actions for 2 and its analogues (alongside 1) cannot be precisely determined. It is also interesting to note the shift in the mode of action as the compound is optimized. This is perhaps not surprising. A
weak, although ligand efficient, low-molecular-weight compound may inhibit several targets weakly. However, during compound optimization, the profile of which molecular targets is inhibited and by how much, may change.

Determining the precise mode of action of a molecular target can be very challenging. A future study to derive the metabolomic fingerprint of key antibiotics will be important as a reference to compare to new compounds would be very valuable. Orthogonal mode of action studies such as chemical proteomics or resistance generation followed by whole genome sequencing can also be proposed to support any hypothesis from this untargeted metabolomics approach.

Despite the limitations discussed, we are optimistic that whole cell screening coupled with metabolomic can be an attractive route to finding new starting points with novel modes of action in early antibacterial drug discovery. The work of Pogliano has shown that there is a distinct phenotype associated with compounds, which target particular pathways within bacteria. There is a scope for extending this current work, by seeing if there is a correlation between the metabolome fingerprint and the morphological effect on the bacteria. This whole cell-based platform highlights the capability for finding new hits with cellular activity and potential new mode of action. The mode of action can then be validated at the early stages of hit discovery, which then increases the odds of success for the drug development process.

**EXPERIMENTAL SECTION**

**Whole Cell Screening.** The whole cell screening was performed using the in-house small polar library. The composition of the library and some illustrated examples of synthesis of such molecules were described by Ray et al. Compounds from the library were stamped into each well from column 1 to column 22 of 384-well plates with a Echo 550 Liquid Handler (Labcyte Inc.). *S. aureus* RN4220 (Division of Molecular Microbiology, University of Dundee) was used for whole cell screening. All cultures, which require shaking, were shaken at 200 rpm. An overnight culture was prepared by culturing a colony in 3 mL (for *S. aureus* RN4220) of fresh LB media (Central Technical Services, University of Dundee) at 37 °C for 12−15 h. The culture was dispensed into white-walled, clear flat-bottom 384-well plates (Greiner Bio-One) by a Multidrop Combi Reagent Dispenser (Thermo Scientific). Plates were read using PHERAsart FS (BMG LABTECH). 50 μL of the culture (prepared by diluting an overnight culture of *S. aureus* RN4220 with fresh LB media at 1:400 ratio) was added to columns of each well, depending on the type of study: single-point study (columns 1−22 of the plate) or (columns 1−10 and 13−22 of the plate). The positive control (gentamicin 0.5 mg/mL) was filled into column 23 (for single-point study) or columns 11 and 23 (for dose−response study), while the negative control (no drug) was filled in the remaining columns. The plates were sealed with air-permeable seals (AeraSeal, Excel Scientific, Inc.) and incubated still at 37 °C for 6 h. The plate was then put into a plate reader to measure OD_{600nm}. All calculations were performed on ActivityBase XE (IDBS) using the raw data obtained from the plate reader. The raw data correspond to the OD_{600nm} measured on each well. PA positive control is defined as a completely active compound with 100% inhibitory effect against bacteria. The negative control is defined as a completely inactive compound with 0% inhibitory effect against bacteria. Hit analysis was performed and visualized using Vortex (Dotmatics Limited). Graphic visualization and statistical analysis were performed and visualized using Vortex and GraphPad Prism 6 (GraphPad Software Inc.).

**Chemistry. General Procedures.** Chemicals and solvents were purchased from Aldrich Chemical Co., Acros, TCI, and Fluorochem. Analytical thin-layer chromatography (TLC) was performed on precoated TLC plates (layer 0.20 mm silica gel 60 with fluorescent indicator UV 254, from Merck). Developed plates were air-dried and analyzed under a UV lamp (UV 254/365 nm) or sprayed with a potassium permanganate solution to visualize oxidizable substances. Flash column chromatography was performed using prepackaged silica gel cartridges (Teledyne ISCO) using CombiFlash R200i (Teledyne ISCO). Microwave-assisted chemistry was conducted using a Biotage initiator microwave synthesizer. 1H and 13C NMR spectra were recorded on a Bruker ARX-500 spectrometer (500, 470, and 125 MHz for 1H, 19F, and 13C NMR, respectively). Spectra were acquired using DMSO-d_6, D_2O, and CDCl_3 as solvents. Chemical shifts (δ) are reported in parts per million relative to the residual solvent peak as the internal reference, and coupling constants (J) are reported in hertz (Hz). Coupling constants are assumed to be H−H coupling constants unless stated otherwise. The spin multiplicity is reported as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. LC−MS analyses were performed with either an Agilent HPLC 1100 series connected to a Bruker Daltonics MicroTOF or an Agilent Technologies 1200 series HPLC connected to an Agilent Technologies 6130 quadrupole spectrometer, where both instruments were connected to an Agilent diode array detector. LC−MS chromatographic separations were conducted with a Waters X bridge C18 column, 50 mm × 2.1 mm, 3.5 μm particle size; mobile phase, H_2O/MeCN + 0.1% HCOOH, or H_2O/MeCN + 0.1% NH_3; linear gradient from 80:20 to 5:95 over 3.5 min and then held for 1.5 min; flow rate of 0.5 mL/min. High-resolution electrospary measurements were performed on a Bruker Daltonics MicroTOF mass spectrometer. All assay compounds had a measured purity of ≥95% (by TLC and UV) as determined using both LC−MS and NMR spectroscopy. Melting points were determined on the Griffin melting point apparatus and were uncorrected.

**General Procedure A for the Coupling of Chloropyrimidines and 3,5-Dimethylpyrazoles. Method 1 (Compounds 3, 4, and 17).** To a solution of appropriate chloropyrimidine (1 equiv) in dioxane (0.2 M) was added 3,5-dimethylpyrazole (1 equiv), KOH (1 equiv), and 18-crown-6 (0.1 equiv). The reaction mixture was irradiated at various temperatures. The reaction mixture was quenched with 2 M HCl, and the aqueous layer was extracted with ethyl acetate. The organic layer was then separated and concentrated in vacuo to give the residue, which was then purified using flash column chromatography (DCM/heptane or EtOAc/heptane) to give the intended product.

**Method 2 (Compounds 12−16).** To a solution of appropriate chloropyrimidine (1 equiv) in acetonitrile (0.33 M) were added 3,5-dimethylpyrazole (1 equiv), KOH (1 equiv), and 18-crown-6 (0.1 equiv). The reaction mixture was left to stir at rt for 1 day or was subjected to microwave irradiation at 150 °C for 3 h. The solvent was removed, and the crude residue was purified using flash column chromatography (DCM/heptane or EtOAc/heptane) to give the intended product.
4-Chloro-2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine (3). General procedure A, method 1, from 2,4-dichloro-6-methylpyrimidine (163 mg, 1.0 mmol). Chromatographic purification (50% DCM in heptane to 100% DCM) gave compound 3 as white solid (79 mg, 35%): mp: 58–60 °C (lit. mp 57 °C);

1H NMR (CDCl₃, 500 MHz) δ: 7.06 (s, 1H, Ar H), 6.07 (s, 1H, Ar H), 2.68 (s, 3H, CH₃), 2.65 (s, 3H, CH₃), 2.63 (s, 3H, CH₃), 13C NMR (CDCl₃, 125 MHz) δ: 170.9 (C), 161.6 (C), 156.8 (C), 152.1 (C), 143.5 (C), 117.0 (CH), 110.9 (CH), 24.2 (CH₂), 15.5 (CH₃), 14.0 (CH₃); MS [electrospray ionization (ESI)] m/z: 223 [M + H]+, 151 [M + Cl]+.

2-(3,5-Dimethyl-1H-pyrazol-1-yl)-4,6-dimethoxypyrimidine (15). General procedure A, method 2, from 2-chloro-4,6-dimethoxypyrimidine (524 mg, 3.0 mmol) and stirred at rt. Chromatographic purification gave compound 15 as white solid (492 mg, 70%): mp: 102–104 °C; 1H NMR (CDCl₃, 500 MHz) δ: 6.03 (s, 1H, Ar H), 5.93 (s, 1H, Ar H), 4.03 (s, 3H, CH₃), 2.69 (s, 3H, CH₃), 2.34 (s, 3H, CH₃); 13C NMR (CDCl₃, 125 MHz) δ: 172.1 (2x C), 156.1 (C), 151.3 (C), 142.8 (C), 110.3 (CH), 86.7 (CH), 54.5 (2x OCH₃), 15.6 (CH₃), 14.0 (CH₃); MS (ESI) m/z: 235 [M + H]+; HRMS (ESI) m/z: calcld for C₁₁H₁₂N₄O⁺ [M + H]+, 235.1190; found, 235.1208.

5-Chloro-2-(3,5-dimethyl-1H-pyrazol-1-yl)-4-methoxypyrimidine (16). General procedure A, method 2, from 2,5-dichloro-4-methoxypyrimidine (263 mg, 1.47 mmol) and stirred at rt. Chromatographic purification gave compound 16 as white solid (144 mg, 41%): 1H NMR (CDCl₃, 500 MHz) δ: 8.50 (s, 1H, Ar H), 6.07 (s, 1H, Ar H), 4.15 (s, 3H, OCH₃), 2.70 (s, 3H, CH₃), 2.35 (s, 3H, CH₃); 13C NMR (CDCl₃, 125 MHz) δ: 165.2 (C), 156.8 (CH), 154.7 (C), 151.9 (C), 142.7 (C), 113.2 (C), 110.7 (CH), 55.4 (CH₃), 15.5 (CH₃), 13.9 (CH₃); MS (ESI) m/z: 239 [M + H]+; HRMS (ESI) m/z: calcld for C₁₀H₁₁N₄O⁺ [M + H]+, 239.0725; found, 239.0725.

Methyl 2-(3,5-Dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-carboxylate (17). General procedure A, method 1, from methyl 2-chloro-6-methylpyrimidine-4-carboxylate (1.88 g, 10.1 mmol). Chromatographic purification (0–25% EtOAc in heptane) gave compound 17 as yellow solid (1.60 g, 64%): 1H NMR (CDCl₃, 500 MHz) δ: 7.70 (s, 1H, Ar H), 6.08 (s, 1H, Ar H), 4.03 (s, 3H, OCH₃), 2.73 (s, 3H, CH₃), 2.73 (s, 3H, CH₃), 2.37 (s, 3H, CH₃); 13C NMR (CDCl₃, 125 MHz) δ: 171.5 (C), 164.0 (C), 156.9 (C), 155.1 (C), 151.3 (C), 142.8 (C), 116.2 (CH), 110.0 (CH), 52.6 (CH₃), 24.1 (CH₃), 14.7 (CH₃), 13.4 (CH₃); MS (ESI) m/z: 247 [M + H]+; HRMS (ESI) m/z: calcld for C₁₂H₁₄N₄O⁺ [M + H]+, 247.1150; found, 247.1215.

2-(3,5-Dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-carboxylate (2). To a solution of 4,6-dichloro-2-(3,5-dimethyl-1H-pyrazol-1-yl)pyrimidine (3) (179 mg, 0.80 mmol) in THF (4 mL) was added 1 M NaOH (4 mL) and then subjected to microwave irradiation at 150 °C for 30 min. Excess base was neutralized with 2 M HCl, and the reaction mixture was extracted with ethyl acetate. The organic phase was separated and concentrated in vacuo. The residue was then purified using flash column chromatography (0–3% MeOH in DCM) to give 3 as a white solid (148 mg, 90%): mp: 126–126 °C; 1H NMR (CDCl₃, 500 MHz) δ: 10.40 (s, 1H, CH₉0), 6.08 (s, 1H, Ar H), 6.05 (s, 1H, Ar H), 2.69 (s, 3H, CH₃), 2.30 (s, 3H, CH₃), 2.27 (s, 3H, CH₃); 13C NMR (CDCl₃, 125 MHz) δ: 165.1 (C), 161.9 (C), 151.9 (C), 147.5 (C), 143.6 (C), 111.5 (CH₉0), 108.9 (CH₉0), 24.0 (CH₃), 15.1 (CH₉3), 13.6 (CH₃); MS (ESI) m/z: 205 [M + H]+; HRMS (ESI) m/z: calcld for C₁₂H₁₄N₄O⁺ [M + H]+, 205.1084; found, 205.1091.

Alternative Procedure Using TFA–DCM. To 2-(3,5-dimethyl-1H-pyrazol-1-yl)-4-(4-methoxybenzyl)oxy-6-methylpyrimidine (5) (49 mg, 0.15 mmol) was added excess volume of trifluoroacetic acid (200 equiv) and an equivalent volume of DCM. The reaction mixture was left to stir for 1 h at rt. The reaction mixture was evaporated in vacuo, and the residue was purified by reverse-phase UV-HPLC to give 2 as a white solid (22 mg, 72%).
6-Chloro-2-(3,5-dimethyl-1H-pyrazol-1-yl)pyrimidin-4-ol (10). To a solution of 4,6-dichloro-2-(3,5-dimethyl-1H-pyrazol-1-yl)pyrimidine (4) (100 mg, 0.41 mmol) in THF (2.05 mL) were added 1 M NaOH (0.82 mL) and additional water (1.23 mL) and then subjected to microwave irradiation at 150 °C for 30 min. Excess base was neutralized with 2 M HCl, and the reaction mixture was extracted with ethyl acetate. The organic phase was separated and concentrated in vacuo. The reaction mixture was then re

General Procedure B for the Formation of Benzyl Ether Analogues (Compounds 5–9). To a solution of 4-chloro-2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine (3) (1 equiv) in acetonitrile (0.3 M) were added the appropriate benzyl alcohol (1.2 equiv) and Cs2CO3 (1.2 equiv). The reaction mixture was then re

General Procedure B, from 4-fluorobenzyl alcohol (70 μL, 0.65 mmol) to give 8 as white solid (132 mg, 78%): 1H NMR (CDCl3, 500 MHz) δ: 7.33 (dd, J1 = 8.8 Hz, J2 = 5.4 Hz, 2H, 2x Ar H), 7.00 (dd, J1 = 8.7 Hz, J2 = 8.7 Hz, 2H, 2x Ar H), 6.41 (1H, Ar H), 5.95 (s, 1H, Ar H), 5.33 (s, 2H, CH2), 2.57 (s, 3H, CH3), 2.46 (s, 3H, CH3), 2.27 (s, 3H, CH3); 13C NMR (CDCl3, 125 MHz) δ: 170.1 (C), 169.6 (C), 162.7 (d, JCF = 247.2 Hz, C), 156.5 (C), 151.3 (C), 142.5 (C), 131.8 (C), 129.9 (d, JCF = 8.2 Hz, 2x CH), 115.6 (dd, J1 = 21.7 Hz, 2x CH), 110.3 (CH), 103.5 (CH), 67.8 (CH2), 24.3 (CH3), 15.4 (CH3), 14.0 (CH3); 19F NMR (CDCl3, 470 MHz) δ: −113.6 (Ar F); MS (ESI) m/z: calcd for C18H16F6O+ [M + H]+; HRMS (ESI) m/z: found, 313 [M + H]+; HRMS (ESI) m/z: calcd for C18H16F6O+ [M + H]+; HRMS (ESI) m/z: found, 313.1393.

4-(Benzyloxy)-2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine (9). General procedure B, from benzyl alcohol (56 μL, 0.54 mmol) to give 9 as white solid (71 mg, 54%): 1H NMR (CDCl3, 500 MHz) δ: 7.45–7.35 (3H, 5x Ar H), 6.52 (s, 1H, Ar H), 6.04 (s, 1H, Ar H), 5.47 (s, 2H, CH2), 2.66 (s, 3H, CH3), 2.36 (s, 3H, CH3), 2.01 (s, 3H, CH3); 13C NMR (CDCl3, 125 MHz) δ: 170.1 (C), 169.8 (C), 162.7 (d, JCF = 247.2 Hz, C), 156.5 (C), 151.3 (C), 142.5 (C), 131.8 (C), 129.9 (d, JCF = 8.2 Hz, 2x CH), 115.6 (dd, J1 = 21.7 Hz, 2x CH), 110.3 (CH), 103.5 (CH), 67.8 (CH2), 24.3 (CH3), 15.4 (CH3), 14.0 (CH3); MS (ESI) m/z: 295 [M + H]+; HRMS (ESI) m/z: calcd for C17H15N4O+ [M + H]+, 295.1553; found, 295.1553.

2-(3,5-Dimethyl-1H-pyrazol-1-yl)-3,6-dimethylpyrimidin-4(3H)-one (11). To a solution of 2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-ol (2) (51 mg, 0.25 mmol) and K2CO3 (69 mg, 0.50 mmol) in DMF (2.77 mL) was added methyl iodide (62 μL, 1.00 mmol). The reaction was then refluxed overnight. Water was added into the reaction mixture, and the reaction mixture was washed with ethyl acetate. The organic phase was then separated, dried, and concentrated in vacuo to give a residue, which was purified using flash column chromatography (10–25% ethyl acetate in heptane) to give 11 as a off-brown solid (41 mg, 75%): 1H NMR (CDCl3, 500 MHz) δ: 6.24 (s, 1H, Ar H), 5.94 (s, 1H, Ar H), 3.34 (s, 3H, NCH3), 2.28 (s, 3H, CH2), 2.22 (s, 3H, CH2), 2.02 (s, 3H, CH3); 13C NMR (CDCl3, 125 MHz) δ: 164.1 (C), 162.1 (C), 154.7 (C), 149.3 (C), 142.4 (C), 111.4 (CH), 97.5 (CH), 73.4 (CH), 68.4 (CH2), 55.3 (CH3), 48.4 (CH3), 24.3 (CH3), 15.4 (CH3), 14.0 (CH3); MS (ESI) m/z: 219 [M + H]+; HRMS (ESI) m/z: calcd for C17H15N4O+ [M + H]+, 295.1240; found, 219.1243.

2-(3,5-Dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-carboxylic Acid (18). To a solution of methyl 2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-carboxylate (17) (231 mg, 0.94 mmol) in 1:1 THF/H2O (14 mL) was added lithium hydroxide (79 mg, 1.88 mmol), and the reaction mixture was allowed to stir overnight at rt. The reaction mixture was concentrated in vacuo, and the residue was purified using mass-directed HPLC to give 18 as a white solid (190 mg,
87%).: $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$: 7.82 (s, 1H, Ar H), 7.07 (s(b), 1H, OH), 6.10 (s, 1H, Ar H), 2.74 (s, 3H, CH$_3$), 2.70 (s, 3H, CH$_3$), 2.35 (s, 3H, CH$_3$); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 172.3 (C), 164.3 (C), 156.5 (C), 155.5 (C), 152.0 (C), 143.7 (C), 116.2 (CH), 111.0 (CH), 24.6 (CH$_3$), 15.6 (CH$_3$), 13.6 (CH$_3$); MS (ESI) m/z: 233 [M + H]$^+$; HRMS (ESI) m/z: calcld for C$_{11}$H$_9$N$_2$O$_2$ [M + H]$^+$, 225.0538; found, 225.0536.

**General Procedure D for the Formation of Substituted Amides (Compounds 23−25).** Methyl 2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-carboxylate (17) (1 equiv) was dissolved in suitable amines (5 equiv) in methanol, and the reaction mixture was left to stir overnight at rt. The reaction mixture was concentrated in vacuo to give the intended product.

2-(3,5-Dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-carboxamide (23). General procedure D, using 7 M NH$_3$ in methanol (1.54 mL) to give 23 as off-yellow solid (493 mg, 99%): $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$: 7.85 (s, 1H, Ar H), 7.79 (s(b), 1H, NH), 6.10 (s, 1H, Ar H), 6.00 (s(b), 1H, NH), 2.72 (s, 3H, CH$_3$), 2.70 (s, 3H, CH$_3$), 2.37 (s, 3H, CH$_3$); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 172.4 (C), 164.9 (C), 157.1 (C), 156.6 (C), 151.9 (C), 142.7 (C), 115.0 (CH), 110.8 (CH), 24.8 (CH$_3$), 15.5 (CH$_3$), 13.9 (CH$_3$); MS (ESI) m/z: 232 [M + H]$^+$; HRMS (ESI) m/z: calcld for C$_{11}$H$_9$N$_2$O$^+ [M + H]$^+$, 232.1193; found, 232.1212.

2-(3,5-Dimethyl-1H-pyrazol-1-yl)-N,6-dimethylpyrimidine-4-carboxamide (24). General procedure D, using 2 M trichloroacetonilidene in methanol (1.29 mL) to give 24 as white solid (126 mg, 100%): $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$: 8.24 (s(b), 1H, NH), 7.84 (s, 1H, Ar H), 6.09 (s, 1H, Ar H), 3.06 (d, $J$ = 5.1 Hz, 3H, NCH$_3$), 2.70 (s, 3H, CH$_3$), 2.69 (s, 3H, CH$_3$), 2.37 (s, 3H, CH$_3$); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 171.9 (C), 163.3 (C), 157.9 (C), 156.4 (C), 151.7 (C), 143.0 (C), 114.7 (CH), 110.6 (CH), 26.3 (CH$_3$), 24.6 (CH$_3$), 15.5 (CH$_3$), 13.8 (CH$_3$); MS (ESI) m/z: 246 [M + H]$^+$; HRMS (ESI) m/z: calcld for C$_{11}$H$_9$N$_2$O$^+ [M + H]$^+$, 246.1349; found, 246.1374.

2-(3,5-Dimethyl-1H-pyrazol-1-yl)-N,N,6-trimethylpyrimidine-4-carboxamide (25). General procedure D, using 2 M dimethylamino in methanol (1.34 mL) to give 25 as off-yellow solid (138 mg, 99%): $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$: 7.18 (s, 1H, Ar H), 5.97 (s, 1H, Ar H), 5.06 (s, 3H, NCH$_3$), 3.02 (s, 3H, NCH$_3$), 2.58 (s, 3H, CH$_3$), 2.56 (s, 3H, CH$_3$), 2.27 (s, 3H, CH$_3$); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 171.3 (C), 166.7 (C), 162.3 (C), 156.4 (C), 151.7 (C), 143.0 (C), 116.0 (CH), 38.7 (CH$_3$), 35.5 (CH$_3$), 24.7 (CH$_3$), 15.4 (CH$_3$), 14.0 (CH$_3$); MS (ESI) m/z: 260 [M + H]$^+$; HRMS (ESI) m/z: calcld for C$_{11}$H$_9$N$_2$O$^+ [M + H]$^+$, 260.1506; found, 260.1510.

2-(3,5-Dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-carboxamid (26). To a solution of 2-(3,5-dimethyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-carboxamide (23) (50 mg, 0.22 mmol) in THF (1 mL) was added Burgess reagent (258 mg, 1.08 mmol) in two portions, and the reaction mixture was allowed to stir overnight at rt. Water was added to the reaction, and the reaction mixture was washed with DCW. The organic phase was separated, dried, and concentrated in vacuo to give a residue, which was purified using flash column chromatography (0−50% ethyl acetate in heptane) to give 26 as a white solid (43 mg, 93%): $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$: 7.34 (s, 1H, Ar H), 6.10 (s, 1H, Ar H), 2.72 (s, 3H, CH$_3$), 2.69 (s, 3H, CH$_3$), 2.37 (s, 3H, CH$_3$); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$: 172.3 (C), 157.5 (C), 152.8 (C), 143.9 (C), 141.6 (C), 120.2 (CH), 115.3 (C), 111.5 (CH), 24.7 (CH$_3$), 15.6 (CH$_3$), 14.0 (CH$_3$); MS (ESI) m/z: 214 [M + H]$^+$; HRMS (ESI) m/z: calcld for C$_{11}$H$_9$N$_2$O$^+ [M + H]$^+$, 214.1087; found, 214.1101.

**Ethyl 2-Methyl-3-oxopropanoate (27).** To a stirred solution of ethyl propionate (2.30 mL, 20.4 g, 20 mmol),
methyl formate (3.70 mL, 3.60 g, 60 mmol) in DCM (20 mL) at 0 °C was added 1.0 M TiCl₄ solution in DCM dropwise (40 mL). After 30 min, triethylamine (6.97 mL, 5.06 g, 50 mmol) was added dropwise. After 1 h, the reaction mixture was warmed to rt over the period of 3 h. The reaction was then quenched with water and extracted with DCM. The organic phase was then separated, dried, and concentrated in vacuo to give a residue, which was purified using flash column chromatography (DCM) to give 27 as a light yellow solid (717 mg, 85%): ¹H NMR (CDCl₃, 500 MHz) δ: 2.53 (s, 1H, Ar H), 2.81 (s, 1H, Ar H), 1.95 (s, 3H, CH₃); ¹³C NMR (CDCl₃, 125 MHz) δ: 141.0771; found, 141.0757.

2-Methylpyrrolidine-1-carboximidamide Hydrochloride (33). General procedure F, using 2-methylpyrrolidine (530 μL, 442 mg, 5.19 mmol) to give 33 as a light yellow solid (717 mg, 85%): ¹H NMR (DMSO-d₆, 500 MHz) δ: 7.35 (s (b), 4x NH), 4.09–4.03 (m, 1H, CH), 3.47–3.43 (m, 1H, CH from CH₂), 3.28–3.23 (m, 1H, CH from CH₂), 2.06–1.88 (m, 3H, CH₂ and CH from CH₂), 1.69–1.62 (m, 1H, CH from CH₂), 1.10 (d (J = 6.3 Hz, 3H, CH₃)); ¹³C NMR (DMSO-d₆, 125 MHz) δ: 154.6 (C), 53.9 (CH₂), 32.5 (CH₂), 22.8 (CH₂), 19.2 (CH₂); HRMS (ESI) m/z: calc'd for C₅H₁₀N₂O⁺ [M + H]+, 128.1182; found, 128.1193.

Morpholine-4-carboximidamide Hydrochloride (34). General procedure F, using morpholine (451 μL, 456 mg, 5.23 mmol) to give 34 as a light yellow solid (633 mg, 74%): ¹H NMR (CDCl₃, 500 MHz) δ: 2.76 (s (b), 4x NH), 3.45–3.63 (m, 4H, 2x CH₂), 3.44–3.42 (m, 4H, 2x CH₂); ¹³C NMR (DMSO-d₆, 125 MHz) δ: 157.1 (C), 65.7 (2x CH₂), 45.6 (2x CH₂); HRMS (ESI) m/z: calc'd for C₆H₁₀N₂O⁺ [M + H]+, 130.0975; found, 130.0975.

General Procedure G for the Ring Formation of Substituted Pyrimidines from Carboximidamides (Compounds 30, 31, 33, 36, and 37). To a solution of the suitable carboximidamides (1 equiv) in ethanol (0.6 M) were added NaOEt (1 equiv) and ethyl acetoacetate (1 equiv). The reaction mixture was refluxed overnight. Work-up and purification described individually in each example gave the intended product.

2-(5-Hydroxy-3-methyl-1H-pyrazol-1-yl)-6-methylpyrimidine-4-ol (30). General procedure G, from 5-hydroxy-3-methyl-1H-pyrazole-1-yl)-6-methylpyrimidine-4-ol (28) (315 mg, 2.25 mmol). The precipitate was filtered and washed with a minimal volume of cold water to give the intended product.

5-Hydroxy-3-methyl-1H-pyrazole-1-carboximidamide (28). General procedure E, using ethyl acetoacetate (5.46 mL, 56.1 g, 63.1 mmol) and stirred overnight to give 28 as an off-white solid (3.87 g, 64%): ¹H NMR (DMSO-d₆, 500 MHz) δ: 10.3 (s (b), 1H, OH), 7.72 (s (b), 3H, NH₂ and NH), 4.43 (s, 1H, Ar H), 1.97 (s, 3H, CH₃); ¹³C NMR (DMSO-d₆, 125 MHz) δ: 168.1 (C), 156.5 (C), 153.5 (C), 82.6 (CH), 15.4 (CH₂); HRMS (ESI) m/z: calc'd for C₆H₁₂N₂O⁺ [M + H]+, 207.0877; found, 207.0899.

5-Hydroxy-4-methyl-1H-pyrazole-1-carboximidamide (29). General procedure E, using ethyl 2-methyl-3-oxopropionate (27) (381 mg, 2.93 mmol) and stirred for 7 days to give 29 as an off-white solid (214 mg, 52%): ¹H NMR (DMSO-d₆, 500 MHz) δ: 10.5 (s (b), 1H, OH), 7.83 (s (b), 3H, NH₂ and NH), 7.28 (s, 1H, Ar H), 1.64 (s, 3H, CH₃); ¹³C NMR (DMSO-d₆, 125 MHz) δ: 165.8 (C), 154.0 (C), 148.5 (CH), 88.8 (C), 80 (CH₂); HRMS (ESI) m/z: calc'd for C₅H₁₂N₂O⁺ [M + H]+, 141.0771; found, 141.0757.

General Procedure F for the Formation of Carboximidamides (Compounds 32–34). To a solution of appropriate amine (1.01 equiv) in acetonitrile (2.3 M) were added triethylamine (1.01 equiv) and 1H-pyrazole-1-carboximidamide (1 equiv). The reaction mixture was refluxed overnight. The reaction mixture was then cooled down to rt, and the precipitate was filtered from the reaction mixture to give the product as a hydrochloride salt.

Pyrrolidine-1-carboximidamide Hydrochloride (32). General procedure F, using pyrrolidine (132 μL, 113 mg, 1.58 mmol) to give 32 as a light yellow solid (177 mg, 75%): ¹H NMR (DMSO-d₆, 500 MHz) δ: 7.35 (s (b), 4x NH), 3.34–3.31 (m, 4H, 2x CH₂), 1.92–1.89 (m, 4H, 2x CH₂); ¹³C NMR (DMSO-d₆, 125 MHz) δ: 155.1 (C), 47.4 (2x CH₂), 25.2 (2x CH₂); HRMS (ESI) m/z: calc'd for C₆H₁₂N₂⁺ [M + H]+, 114.1026; found, 114.1042.
6-Methyl-2-(2-methylpyrrolidin-1-yl)-pyrimidin-4-ol (36). General procedure G, from 2-methylpyrrolidin-1-carboximidamide hydrochloride (33) (195 mg, 1.19 mmol). Water was added to the reaction, and the mixture was washed with DCM. The organic phase was then separated, dried, and concentrated in vacuo to give a residue, which was purified using flash column chromatography (0–5% MeOH/NH3 in DC) to give 36 as a light brown solid (167 mg, 72%): 1H NMR (CDCl3, 500 MHz) δ: 11.0 (s(b), 1H, OH), 5.60 (s, 1H, Ar H), 2.66 (s, 3H, CH3), 2.26 (s, 3H, CH3), 2.09 (s, 3H, CH3); 13C NMR (CDCl3, 125 MHz) δ: 167.8 (C), 165.9 (C), 151.9 (C), 99.3 (CH), 53.6 (CH), 19.4 (CH3); MS (ESI) m/z: calcd for C9H14N3O+ [M + H]+, 180.1131; found, 180.1136.

Ethyl 2-(3,5-dimethyl-1H-pyrazol-1-yl)-4-hydroxypyrimidine-5-carboxylate (41). General procedure H, using ethyl 2-cyano-3-ethylacrylate (433 mg, 2.56 mmol). The reaction mixture was then cooled down to rt, and the precipitate was filtered and washed with a minimal volume of cold ethanol. The precipitate was then redissolved in methanol and the solution was passed through an ion exchange column to remove any side products and give 42 as an off-white solid (142 mg, 28%): 1H NMR (CDCl3, 500 MHz) δ: 10.9 (s(b), 1H, OH), 8.31 (s, 1H, Ar H), 6.15 (s, 1H, Ar H), 2.68 (s, 3H, CH3), 2.30 (s, 3H, CH3); 13C NMR (CDCl3, 125 MHz) δ: 162.1 (CH), 157.5 (C), 154.7 (C), 150.5 (C), 145.0 (C), 113.6 (C), 113.4 (CH), 15.2 (CH3), 13.7 (CH3); MS (ESI) m/z: 216 [M + H]+; HRMS (ESI) m/z: calcd for C10H15N3O+ [M + H]+, 216.0880; found, 216.0900.

Alternative Procedure Using Burgess Reagent. To a solution of 2-(3,5-dimethyl-1H-pyrazol-1-yl)-4-hydroxypyrimidine-5-carboxamide (41) (112 mg, 0.48 mmol) in THF (1 mL) was added Burgess reagent (286 mg, 1.20 mmol). The reaction mixture was stirred overnight at rt. The reaction mixture was concentrated in vacuo, and the residue was purified using mass-directed HPLC to give 42 as a white solid (21 mg, 20%).

General Procedure L for the Formation of 5-Substituted Pyrimidine Analogues (Compounds 38, 39, and 42). To a solution of 3,5-dimethyl-1H-pyrazole-1-carboximidamide nitrate (1 equiv) in ethanol (0.65 M) were added the appropriate 1,3-dicarboxyls or acrylates (1.1 equiv) and NaOH (1.1 equiv). The reaction mixture was heated under reflux at 100 °C. Work-up and purification described individually in each example gave the intended product.

Ethyl 2-(3,5-Dimethyl-1H-pyrazol-1-yl)-4-hydroxypyrimidine-5-carboxylate (39). General procedure H, using diethyl 2-(ethoxymethylene)malonate (2.00 mL, 2.14 g, 9.89 mmol). The reaction mixture was then cooled down to rt, and the precipitate was filtered and washed with a minimal volume of cold ethanol to give 39 as a white solid (1.17 g, 50%): 1H NMR (D2O, 500 MHz) δ: 8.64 (s, 1H, Ar H), 6.09 (s, 1H, Ar H), 4.28 (q, J = 7.1 Hz, 2H, CH2), 2.45 (s, 3H, CH3), 2.18 (s, 3H, CH3), 1.30 (s, J = 7.2 Hz, 3H, CH3); 13C NMR (D2O, 125 MHz) δ: 172.5 (C), 168.1 (C), 160.1 (C), 158.2 (CH), 151.7 (C), 143.4 (C), 110.4 (C), 109.1 (CH), 61.6 (CH3), 13.5 (CH3), 12.8 (CH3); MS (ESI) m/z: 263 [M + H]+; HRMS (ESI) m/z: calcd for C12H15N4O3+ [M + H]+, 263.1139; found, 263.1161.

The reaction mixture was stirred overnight at 75 °C. The reaction mixture was then concentrated in vacuo, and the residue was purified using mass-directed HPLC to give 40 as a white solid (125 mg, 102%): 1H NMR (D2O, 500 MHz) δ: 8.64 (s, 1H, Ar H), 6.09 (s, 1H, Ar H), 4.28 (q, J = 7.1 Hz, 2H, CH2), 2.45 (s, 3H, CH3), 2.18 (s, 3H, CH3), 1.30 (s, J = 7.2 Hz, 3H, CH3); 13C NMR (D2O, 125 MHz) δ: 172.5 (C), 168.1 (C), 160.1 (C), 158.2 (CH), 151.7 (C), 143.4 (C), 110.4 (C), 109.1 (CH), 61.6 (CH3), 13.5 (CH3), 12.8 (CH3), 12.4 (CH3); MS (ESI) m/z: 263 [M + H]+; HRMS (ESI) m/z: calcd for C10H13N4O2+ [M + H]+, 263.1161.

Ethyl 2-(3,5-Dimethyl-1H-pyrazol-1-yl)-4-hydroxypyrimidine-5-carboxamide (41). Ethyl 2-(3,5-dimethyl-1H-pyrazol-1-yl)-4-hydroxypyrimidine-5-carboxylate (39) (183 mg, 0.70 mmol) was suspended in NH4OH (3 mL). The suspension was then stirred at 75 °C overnight, of which the suspension became clear. The reaction mixture was then cooled to rt and was concentrated in vacuo to give 41 as a white solid (140 mg, 86%): 1H NMR (MeOD, 500 MHz) δ: 8.77 (s, 1H, Ar H), 6.04 (s, 1H, Ar H), 2.60 (s, 3H, CH3), 2.24 (s, 3H, CH3); 13C NMR (D2O, 125 MHz) δ: 166.1 (C), 157.0 (CH), 150.5 (C), 148.6 (C), 143.3 (C), 121.0 (C), 111.2 (CH), 14.9 (CH3), 13.6 (CH3), 12.8 (CH3); MS (ESI) m/z: 216 [M + H]+; HRMS (ESI) m/z: calcd for C10H15N4O3+ [M + H]+, 216.0880; found, 216.0900.

Ethyl 2-(3,5-Dimethyl-1H-pyrazol-1-yl)-4-hydroxypyrimidine-5-carboxylate (39). General procedure H, using diethyl 2-(ethoxymethylene)malonate (2.00 mL, 2.14 g, 9.89 mmol). The reaction mixture was then cooled down to rt, and the precipitate was filtered and washed with a minimal volume of cold ethanol to give 39 as a white solid (1.17 g, 50%): 1H NMR (D2O, 500 MHz) δ: 8.64 (s, 1H, Ar H), 6.09 (s, 1H, Ar H), 4.28 (q, J = 7.1 Hz, 2H, CH2), 2.45 (s, 3H, CH3), 2.18 (s, 3H, CH3), 1.30 (s, J = 7.2 Hz, 3H, CH3); 13C NMR (D2O, 125 MHz) δ: 172.5 (C), 168.1 (C), 160.1 (C), 158.2 (CH), 151.7 (C), 143.4 (C), 110.4 (C), 109.1 (CH), 61.6 (CH3), 13.5 (CH3), 12.8 (CH3), 12.4 (CH3); MS (ESI) m/z: 263 [M + H]+; HRMS (ESI) m/z: calcd for C12H15N4O3+ [M + H]+, 263.1161.

General Procedure L for the Coupling of Chlororopridines and 3,5-Dimethylpyrazoles (Compounds 43 and 44). To a solution of appropriate chlororopidine (1 equiv) in acetonitrile (0.6 M) were added 3,5-dimethylpyrazole (1 equiv), KOH (1
2-Chloro-6-(3,5-dimethyl-1H-pyrazol-1-yl)-4-methylpyridine (43). General procedure I, from 2,6-dichloro-4-methylpyridine (486 mg, 3.0 mmol), and subjected to microwave irradiation for 1.25 h. Chromatographic purification gave compound 43 as a white solid (331 mg, 50%): mp 45–46 °C; 1H NMR (CDCl₃, 500 MHz) δ: 7.65 (s, 1H, Ar H), 7.01 (s, 1H, Ar H), 6.00 (s, 1H, Ar H), 2.67 (s, 3H, CH₃), 2.41 (s, 3H, CH₃), 2.31 (s, 3H, CH₃); 13C NMR (CDCl₃, 125 MHz) δ: 152.9 (C), 152.5 (C), 150.3 (C), 148.7 (C), 121.3 (CH), 114.0 (CH), 109.5 (CH), 21.0 (CH₃), 14.7 (CH₃), 13.6 (CH₃); MS (ESI) m/z: calc for C₁₁H₁₃N₃Cl⁺ [M + H]⁺, 222.0793; found, 222.0803.

2-Chloro-6-(3,5-dimethyl-1H-pyrazol-1-yl)pyridine (44). General procedure I, from 2,6-dichloropyridine (444 mg, 3.0 mmol), and subjected to microwave irradiation for 2.25 h. Chromatographic purification gave compound 44 as a white solid (345 mg, 55%): mp 68–69 °C; 1H NMR (CDCl₃, 500 MHz) δ: 7.83 (d, J = 8.1 Hz, 1H, Ar H), 7.73 (dd, J₁ = J₂ = 7.9 Hz, 1H, Ar H), 7.16 (d, J = 7.7 Hz, 1H, Ar H), 6.01 (s, 1H, Ar H), 2.69 (s, 3H, CH₃), 2.31 (s, 3H, CH₃); 13C NMR (CDCl₃, 125 MHz) δ: 153.1 (C), 150.5 (C), 148.7 (C), 142.2 (C), 140.5 (CH), 120.3 (CH), 113.2 (CH), 109.7 (CH), 14.8 (CH₃), 13.6 (CH₃); MS (ESI) m/z: 208 [M + H⁺]; HRMS (ESI) m/z: calc for C₁₀H₁₁N₃Cl⁺ [M + H]⁺, 208.0636; found, 208.0642.

General Procedure J for the Copper-Catalyzed Hydroxylation of Aryl Halides (Compounds 45 and 46). To a solution of the suitable aryl halide (1.0 equiv) in water (0.06 M) were added 55% tetrabutylammonium hydroxide (4.0 equiv), Cul (0.1 equiv), and 8-hydroxyquinoline 1-oxide (0.4 equiv). The reaction mixture was then subjected to microwave irradiation at 180 °C for 3 h. The reaction mixture was washed with ethyl acetate, and the organic phase was separated, dried, and concentrated in vacuo to give a residue, which was purified using flash column chromatography (0–25% EtOAc in heptane) to give the desired product.

6-(3,5-Dimethyl-1H-pyrazol-1-yl)-4-methylpyridin-2-ol (45). General procedure J, from 2-chloro-6-(3,5-dimethyl-1H-pyrazol-1-yl)-pyridine (44) (42 mg, 0.20 mmol) to give compound 45 as off-white solid (33 mg, 87%): mp 107–108 °C; 1H NMR (CDCl₃, 500 MHz) δ: 9.83 (s(b), 1H, OH), 7.52 (dd, J₁ = J₂ = 8.9 Hz, J₃ = 7.6 Hz, 1H, Ar H), 6.49 (d, J = 8.7 Hz, 1H, Ar H), 6.47 (d, J = 7.2 Hz, 1H, Ar H), 6.03 (s, 1H, Ar H), 2.50 (s, 3H, CH₃), 2.27 (s, 3H, CH₃); 13C NMR (CDCl₃, 125 MHz) δ: 162.2 (C), 150.7 (C), 143.4 (C), 141.3 (CH), 140.5 (C), 113.9 (CH), 110.2 (CH), 97.5 (CH), 13.9 (CH₃), 13.5 (CH₃); MS (ESI) m/z: 190 [M + H⁺]; HRMS (ESI) m/z: calc for C₁₀H₁₂N₂O⁺ [M + H⁺], 190.0975; found, 190.0966.

Metabolomics. General. Compounds tested were obtained from commercial suppliers (marketed antibiotics) or in-house (hit compounds and analogues). S. aureus RN6390 was from in-house laboratory collection. LB were prepared in-house. All procedures were performed under aseptic conditions.

Determination of MIC via Microdilution Method. A series of dilution was prepared from a stock solution of compounds (dissolved in water/DMSO for known antibacterial drugs, DMSO for other synthetic in-house compounds) by diluting the stock solution with LB media. From each dilution, 100 μL was added to the wells of a 96-well plate. At the same time, the inoculum was prepared by making a 0.5 McFarland standard culture by diluting an overnight culture of S. aureus RN6390 with the appropriate media. The standard culture was then further diluted 100-fold with the same media. This diluted culture (100 μL) was added into the wells of a 96-well plate, bringing the final volume to 200 μL. The plate was covered with the lid provided and was incubated at 35–37 °C in air for 18–20 h. The MIC of the compound (averaged from two independent replicates) was defined as the lowest concentration of the compound, at which there was no visible growth.

Metabolomics Study of S. aureus RN6390 upon Exposure to Various Compounds with Antibacterial Activity. The metabolite extraction was adapted from Vincent et al. Briefly, a 0.5 mL overnight culture of S. aureus RN6390 was inoculated into 49.5 mL of LB and the culture was incubated with shaking at 200 rpm at 37 °C. At 2 h, antibacterial compounds were added at 4X MIC. Samples were taken at 0, 1.5, and 3 h after the addition in 10 mL aliquots. Samples were cooled to 4 °C in a dry-ice-ethanol bath to quench metabolism before they were temporarily stored in ice. Cells (10 mL) were pelleted at 3894 relative centrifugal force (RCF), washed in 10 mL of cold 0.85% saline, and then resuspended in 1 mL of 0.85% saline. The OD₆₀₀nm of the cell suspension was taken and adjusted to 1. A 1 mL aliquot of cells was pelleted and resuspended in 200 μL of chloroform–methanol–water mixture (1:3:1, by volume) in a microcentrifuge tube. Acid-washed glass beads (200 μL) with the particle size ≤106 μm (Sigma-Aldrich) were added to the suspension, and the tube was shaken at 2000 rpm at 4 °C for 45 min (Eppendorf ThermoMixer C). The tube was subjected to a final centrifugation at 15871 RCF of which the supernatant was then taken and kept at −80 °C prior to data acquisition. Nine independent samples were generated for the untreated control, and three independent samples were generated for each treatment group. The data acquisition was similar to Vincent et al. A 10 μL aliquot of each sample was run in a randomized order on a ZIC-hydrophilic interaction chromatography column (Merck SeQuant) coupled to an Orbitrap mass spectrometer (Thermo Scientific) according to previously published methods. Data analysis was performed using the MzMatch and IDEOM software packages for untargeted, initial analyses. PiMP was used for further pathway analysis, generating log, fold changes and peak verification. The PCA plot was generated using the MetaboAnalyst software. Meteorological data have been deposited to the EMBL-EBI MetaboLights database (DOI: 10.1093/nar/gks1004. PubMed PMID: 23109552) with the identifier MTBLS788.
Notes on analysis of the data from the whole cell screening, NMR data to show the structure of key compounds, physicochemical data of key compounds, activity data of selected compounds against different strains of *S. aureus*, further metabolomic data on ciprofloxacin and trimethoprim (PDF)

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