Efficient Synthesis of Amine-Linked 2,4,6-Trisubstituted Pyrimidines as a New Class of Bacterial FtsZ Inhibitors

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ABSTRACT: We have recently identified a new class of filamenting temperature-sensitive mutant Z (FtsZ)-interacting compounds that possess a 2,4,6-trisubstituted pyrimidine–quinuclidine scaffold with moderate antibacterial activity. Employing this scaffold as a molecular template, a compound library of amine-linked 2,4,6-trisubstituted pyrimidines with 99 candidates was successfully established by employing an efficient convergent synthesis designed to explore their structure–activity relationship. The results of minimum inhibitory concentration (MIC) assay against Staphylococcus aureus strains and cytotoxicity assay against the mouse L929 cell line identified those compounds with potent antistaphylococcal properties (MIC ranges from 3 to 8 μg/mL) and some extent of cytotoxicity against normal cells (IC₅₀ ranges from 6 to 27 μM). Importantly, three compounds also exhibited potent antibacterial activities against nine clinically isolated methicillin-resistant S. aureus (MRSA) strains. One of the compounds, 14av_amine16, exhibited low spontaneous frequency of resistance, low toxicity against Galleria mellonella larvae, and the ability to rescue G. mellonella larvae (20% survival rate at a dosage of 100 mg/kg) infected with a lethal dose of MRSA ATCC 43300 strain. Biological characterization of compound 14av_amine16 by saturation transfer difference NMR, light scattering assay, and guanosine triphosphatase hydrolysis assay with purified S. aureus FtsZ protein verified that it interacted with the FtsZ protein. Such a property of FtsZ inhibitors was further confirmed by observing iconic filamentous cell phenotype and mislocalization of the Z-ring formation of Bacillus subtilis. Taken together, these 2,4,6-trisubstituted pyrimidine derivatives represent a novel scaffold of S. aureus FtsZ inhibitors.

1. INTRODUCTION

The inexorable rise in the incidence of serious bacterial infections caused by multiple antibiotic-resistant bacteria in health care- and community-associated settings has become a pressing threat of public health worldwide.1 Of particular concern is the rise in the incidence of methicillin-resistant Staphylococcus aureus (MRSA) infections. MRSA can cause a wide range of illnesses, from mild skin and wound infections to pneumonia and bloodstream infections that cause sepsis and death. The Centers for Disease Control and Prevention of the United States estimates that over 80 000 severe MRSA infections occur annually, resulting in 11 000 deaths.2 This scenario has driven the search for novel classes of antistaphylococcal agents which act on novel bacterial drug targets.

The bacterial cell division machinery has been considered as an important field for exploring potential novel drug targets of antibacterial agents.3 The filamenting temperature-sensitive mutant Z (FtsZ) protein undoubtedly represents one of the well-characterized and exploitable antibacterial drug targets.4−10 FtsZ is a cytoplasmic protein and highly conserved tubulin-like guanosine triphosphatase (GTPase), playing an important role in bacterial cell division. In order for bacteria to carry out cell division, FtsZ monomers are required to localize mid cells through the precise positioning of cell division site positioning proteins and self-polymerize into single stranded straight protofilaments by means of head-to-tail association that curve upon the hydrolysis of guanosine triphosphate (GTP) molecules.11 Consecutive lateral contacts between FtsZ protofilaments produce FtsZ bundles, which eventually lead
to the formation of a contractile ring called Z-ring at the mid cell. Following the subsequent involvement of other down-stream cell division proteins, Z-ring contraction and depolymerization complete the cell division process to furnish identical daughter cells. Small molecules interfering the initial stage of the FtsZ polymerization are capable of blocking bacterial cell division, causing the abrogation of bacterial cell viability eventually. These types of compounds have great potential to be developed as efficacious antimicrobial agents with a novel mode of action for clinical applications. Several high-resolution X-ray crystal structures of FtsZ homologues have been reported.12 These results contributed to the knowledge regarding the general organization of the FtsZ protein structure, which is known to comprise two independent folding domains (Figure 1). The N-terminal domain forms a nucleotide-binding site (GTP-binding site, the upper red circle of Figure 1), whereas the C-terminal domain contains a flexible loop (T7 loop). Both the domains were interconnected via a long central helix 7 (H7) of high rigidity.

Many FtsZ-interacting compounds have been discovered and reported to bind either the GTP-binding site or a cleft formed by the H7, T7 loop, and C-terminal β sheet (Figure 1, black oval). Some exhibit potent antibacterial activity with minimum inhibitory concentration (MIC) at the micromolar range. PC190723 (Figure 1, top left)13−15 and its prodrugs 1a,16 1b,17−19 and benzamide 220 have been demonstrated to exhibit in vitro and in vivo efficacy in a murine infection model. Moreover, X-ray crystallographic analysis revealed that PC190723 binds to a narrow cleft formed by the H7, T7 loop, and C-terminal β sheet (Figure 1, black oval).21 However, analysis of PC190723 drug-resistant mutants across various MRSA strains revealed that all PC190723 drug-resistant isolates had multiple mutations, resulting in amino acid substitutions that mapped to the FtsZ protein.22 These mutations mainly occurred at amino acid positions 193, 196, and 263 (Figure 1), which accounted for over 90% of PC190723 drug-resistant mutants. These results suggested that amino acid substitutions can alter slightly the overall shape of the binding pocket without interfering the normal function of FtsZ. Nevertheless, this change resulted in PC190723 no longer binding to this pocket, therefore causing drug resistance. Such findings may hinder the potential of PC190723 and other related compounds23 from being developed into agents that exhibit the potential to target the same binding pocket for further clinical development. On the other hand, several compounds targeting the GTP-binding site of FtsZ have also been demonstrated to exhibit potent antibacterial activity, including zantrin Z3,24,25 natural product chrysophaentin A 3,26 C8-substituted GTP analogues 4,27,28 berberine analogues 5,29,30 and naphthol derivatives 631,32 (Figure 1, right). Surprisingly, among these inhibitors, no drug-resistant mutants have been reported in the literature so far, presumably because of the fact that the GTP-binding site is very important for recognizing the GTP molecules. Amino acid substitutions at this binding pocket may cause improper recognition of GTP molecules and thus hinder normal GTP hydrolysis process, therefore losing the energy source to drive the polymerization of FtsZ monomers. We reasoned that specially designed small molecules, which can mimic and compete with GTP molecules to bind the GTP-binding site of FtsZ, may have greater potential to be developed as antimicrobial agents without acquiring drug resistance.

To take advantage of this idea, we have employed a combined approach of high-throughput virtual screening and biological evaluation of natural product libraries to discover compounds with new chemical scaffolds that target the GTP-
binding site of the FtsZ protein. Our previous study identified a new class of FtsZ inhibitor 7 bearing a 2,4,6-trisubstituted pyrimidine and a chiral aminoquinuclidine moiety (Figure 1, bottom right). This class of compounds has been demonstrated to inhibit the GTPase hydrolysis activity of S. aureus FtsZ at a low micromolar IC50 value with moderate antimicrobial activity against S. aureus and Escherichia coli. Compound 7a also exhibited a strong synergistic effect against various MRSA and vancomycin-resistant Enterococcus faecium strains when combined with clinically used β-lactam antibiotics. However, the structural complexity of the chiral quinuclidine scaffold and limited compound availability from commercial sources have prevented further development. In the present study, we report a design and efficient synthesis of a novel amine-linked 2,4,6-trisubstituted pyrimidine compound library with the aims of simplifying the complex quinuclidine scaffold with simple amines and improving their antimicrobial activity. A promising compound exhibiting potent antimicrobial activity has been selected for further investigation regarding its interaction with the S. aureus FtsZ protein. Because of its structural novelty, potent antimicrobial activity, and high selectivity against S. aureus, a new series of such compounds may represent a promising starting point for further investigation.

2. RESULTS AND DISCUSSION

2.1. Compound Library Design and Synthesis.
Molecular docking study of compound 7 and GTP molecules using S. aureus FtsZ revealed that the 2,4,6-trisubstituted pyrimidine moiety of 7 occupied exactly the same binding pocket as the guanosine moiety of GTP molecules through an extensive network of hydrogen bonds with the FtsZ protein, suggesting that the pyrimidine moiety is crucial for binding. Therefore, our molecular design strategy is to retain the pyrimidine moiety and replace the chiral quinuclidine scaffold with simple amines, which offer...
desirable compound flexibility and physicochemical property. At the same time, various substituents will be installed at positions 2 and 6. As there was no structural information to guide the compound design, compound 7 was therefore divided into two parts (pyrimidine head and amine tail), as outlined in Figure 1. A compound library would then be designed and synthesized to sequentially interrogate the pyrimidine head and the amine tail using convergent synthesis. With the preliminary structure–activity relationship (SAR) in hand, compounds combining the promising structural motifs would then be synthesized by dedicated synthesis. On the basis of this approach, eight pyrimidine heads (Scheme 1) and 39 secondary amine tails (Chart 1) were designed and synthesized. Cyclic amine tails (for a seven-member homopiperazine ring, amine01−23, or for a six-member piperazine ring, amine24−34) and linear amine tails (amine35−39) were included in this study for comparison purpose. A general synthesis route of target compounds 14 is outlined in Scheme 1. The pyrimidine heads were easily obtained by the coupling of various acid chlorides 8a−d with silyl group-protected terminal alkyne 9 in the presence of triethylamine under Sonogashira conditions, followed by the subsequent addition of excess amidinium hydrochloride salts 10v−z in one pot, allowing a straightforward access to 2,4,6-trisubstituted pyrimidines 11 under mild conditions and in good yields. It is worthy to note that the use of unprotected terminal alkyne 9 should be avoided because of the relatively low yield of product. Deprotection of the silyl group of 11 by acidic treatment of concentrated hydrochloric acid.
Similarly, pyrimidines with 99 candidates was successfully constructed for SAR study. In ACN at room temperature. By employing this approach, a with a secondary amino group. Pyrimidines were synthesized in high yield, which were further acidi with excess 1-Boc-homopiperazine afforded Boc-protected pyrimidines 14 (14av_amine40 and 14az_amine40), respectively, in high yield, which were further acidified with trifluoroacetic acid (TFA) in dichloromethane (DCM) to furnish pyrimidines 14 (14av_amine41 and 14az_amine41) with a secondary amino group. Pyrimidines 14 (14av_amine42–47) with a sulfonamide group were successfully synthesized in good yield by treating various commercially available sulfonyl chlorides with amine 14az_amine41.

### 2.2. Evaluation of Antimicrobial Activities, SAR, and Cytotoxicity against Normal Cells

The compound library of 2,4,6-trisubstituted pyrimidines 14 was then evaluated simultaneously for (1) their antimicrobial activities against the Gram-positive *S. aureus* strain ATCC 29213 and the Gram-negative *E. coli* strain ATCC 25922 by measuring their MIC, which is the lowest concentration of a compound that prevents the visible growth of bacteria in a broth dilution susceptibility test according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, and (2) their cytotoxicity against normal mouse fibroblasts L929 by measuring their IC50, which is the concentration of a compound that is required for 50% inhibition. Parent compounds 7a and 7b (Figure 1, bottom right) were selected as a positive control. Their antimicrobial activities against both the bacterial strains ranged from 24 to 64 μg/mL, demonstrating only moderate antibacterial activity and poor selectivity of killing between the two bacterial strains (Table 1, entries 1 and 2). By employing progressive MIC screening together with cytotoxicity assay, we have successfully identified several compounds that displayed superior antimicrobial activity and selectivity against *S. aureus*. The results of

| entry | compound no. | R1            | R2            | amine no. | S. aureus (μg/mL) | E. coli (μg/mL) | L929 IC50 (μM) | SI      |
|-------|--------------|---------------|---------------|-----------|------------------|----------------|----------------|---------|
| 1     | 7a           | N.A.          | N.A.          | N.A.      | 24 (51)          | 32             | 36 ± 2         | 0.7     |
| 2     | 7b           | N.A.          | N.A.          | N.A.      | 64 (125)         | 64             | N.D.           |        |
| 3     | 14dv_amine16 | phenyl        | 4-Py          | 16        | 3 (5)            | >64            | 7 ± 1          | 1.4     |
| 4     | 14dv_amine06 | phenyl        | 4-Py          | 06        | 3 (5)            | >64            | 6 ± 1          | 1.2     |
| 5     | 14dv_amine20 | phenyl        | 4-Py          | 20        | 3 (5)            | >64            | 6 ± 1          | 1.2     |
| 6     | 14dv_amine07 | phenyl        | 4-Py          | 07        | 3 (5)            | >64            | 6 ± 1          | 1.2     |
| 7     | 14dv_amine08 | phenyl        | 4-Py          | 08        | 3 (5)            | >64            | 7 ± 1          | 1.4     |
| 8     | 14av_amine16 | 3-pentyl      | 4-Py          | 16        | 4 (8)            | >64            | 8 ± 1          | 1.0     |
| 9     | 14av_amine20 | 3-pentyl      | 4-Py          | 20        | 4 (8)            | >64            | 7 ± 1          | 0.9     |
| 10    | 14av_amine06 | n-pentyl      | 4-Py          | 06        | 6 (10)           | >64            | 7 ± 2          | 0.7     |
| 11    | 14av_amine16 | n-pentyl      | 4-Py          | 16        | 6 (10)           | >64            | 9 ± 1          | 0.9     |
| 12    | 14av_amine12 | phenyl        | 4-Py          | 12        | 6 (10)           | >64            | 9 ± 1          | 0.9     |
| 13    | 14av_amine10 | phenyl        | 4-Py          | 10        | 6 (10)           | >64            | 8 ± 1          | 0.8     |
| 14    | 14av_amine28 | 3-pentyl      | 4-Py          | 28        | 6 (10)           | >64            | 27 ± 1         | 2.7     |
| 15    | 14av_amine38 | 3-pentyl      | 4-Py          | 38        | 6 (10)           | >64            | 14 ± 1         | 1.4     |
| 16    | 14av_amine39 | 3-pentyl      | 4-Py          | 39        | 6 (10)           | >64            | 9 ± 1          | 0.9     |
| 17    | 14av_amine42 | 3-pentyl      | 4-Py          | 24        | 6 (12)           | >64            | 15 ± 2         | 1.3     |
| 18    | 14av_amine35 | 3-pentyl      | 4-Py          | 35        | 6 (12)           | >64            | 10 ± 1         | 0.8     |
| 19    | 14av_amine13 | 3-pentyl      | 4-Py          | 13        | 6 (13)           | >64            | 10 ± 1         | 0.8     |
| 20    | 14av_amine06 | 3-pentyl      | 4-Py          | 06        | 8 (16)           | >64            | 8 ± 2          | 0.5     |
| 21    | 14av_amine07 | 3-pentyl      | 4-Py          | 07        | 8 (16)           | >64            | 9 ± 2          | 0.6     |
| 22    | 14av_amine08 | 3-pentyl      | 4-Py          | 08        | 8 (16)           | >64            | 8 ± 2          | 0.5     |
| 23    | 14av_amine17 | 3-pentyl      | 4-Py          | 17        | 8 (16)           | >64            | 15 ± 1         | 0.9     |
| 24    | 14av_amine18 | 3-pentyl      | 4-Py          | 18        | 8 (16)           | >64            | 18 ± 2         | 1.1     |

*N.A.: not applicable; N.D.: not determined; MIC, μg/mL for *S. aureus*, the number in the parentheses is the MIC value calculated in the unit of μM; * concentration of a compound that is required for 50% inhibition (*IC50*), μM, N = 1–3 independent experiments, and the values are presented as mean ± standard error of mean; and ^ SI, it was calculated using the formula L929 IC50 (μM)/MIC value of *S. aureus* (μM).
MIC screening and cytotoxicity assay are summarized in Table 1, in which only compounds with MIC values against S. aureus 29213 less than 8 μg/mL are shown. Compounds with MIC values higher than 8 μg/mL are shown in the Supporting Information (Table S1). In general, among all newly synthesized derivatives, most of them displayed potent inhibitory activities against the growth of S. aureus along with some extent of cytotoxicity against normal cells L929. The low IC_{50} values of L929 indicate that the compounds are very toxic and may have unselective interaction with protein targets other than FtsZ, causing high cytotoxicity against normal cells. However, all of them exhibited very weak inhibitory activities against the growth of E. coli even at a concentration of 64 μg/mL. Such a weak activity against E. coli may be attributed to their poor penetration ability into the cytoplasm of Gram-negative bacteria, which have an outer membrane of low permeability. From the compounds listed in Table 1, seven compounds displaying superior potency with MIC values at 3–4 μg/mL were identified, namely, 14av_amine16, 14dv_amine06, 14dv_amine20, 14dv_amine07, 14dv_amine08, 14av_amine16, and 14av_amine20 (Table 1, entries 3–9). Compared with compound 7b, their potencies were dramatically improved by 16–21-fold. Interestingly, these compounds possess the common structural features of a 4-pyridyl (4-Py) group at position 2 and a cyclic seven-member homopiperazine ring substituted with a benzyl group at position 4. Detailed SAR analysis of 2,4,6-trisubstituted pyrimidine 14 derivatives is depicted in Figure 2. For the pyrimidine head, the 4-Py group of R^2 of 14av_amine16 was very important for potent antibacterial activity. Replacing this functional group with others such as 3-pyridyl of 14av_amine16, 2-pyridyl of 14az_amine16, phenyl of 14ax_amine16, and methyl groups of 14av_amine16 resulted in a weak antibacterial activity, implying that the nitrogen atom of the 4-Py group is crucial for maintaining potent antibacterial activity. For R^1 group of the pyrimidine head, both the phenyl of 14dv_amine16 and the 3-pentyl group of 14av_amine16 were favorable substituents. The less bulky isopropyl group of 14bv_amine16 and the linear n-pentyl group of 14cv_amine16 both caused poor antibacterial activity. In general, pyrimidine head bearing 4-Py group of R^2 and phenyl or 3-pentyl groups of R^1 exhibited the most potent antibacterial activity. For SAR analysis of the amine tail (14av_amine01–14av_amine39), there were two important structural features that gave rise to potent antibacterial activity: (1) the cyclic seven-member homopiperazine ring with a substituted benzyl group and (2) the benzylic group substituted with a bulky group (tert-butyl of 14av_amine07, trifuoroethyl of 14av_amine16, isopropyl of 14av_amine06, and bromo of 14av_amine08) at the para position, but not at the meta or ortho position, of 14av_amine17–18. Replacing the amine tail with a cyclic six-member piperazine ring of 14av_amine24, linear 1,2-diamine of 14av_amine35–36, or 1,3-diamine of 14av_amine37–38 resulted in a poor antibacterial activity. It is also worthy to note that replacing the benzylic carbon with a rigid functional group, such as carbonyl group of 14av_amine19, sulfonyl group of 14az_amine43–44, or α,β-unsaturated ketone group of 14av_amine21–23, caused a poor antibacterial activity. These results suggested that a more flexible and freely rotatable functional group should be installed at this position. This observation was further supported by the low MIC value of compound 14av_amine20, in which the benzylic carbon was replaced with a freely rotatable CH₂CH₂ group.

Encouraged by these promising results, three compounds, namely, 14av_amine16, 14dv_amine16, and 14dv_amine06, were further selected to test against nine clinically isolated bacterial strains including S. aureus ATCC 29247, which is an ampicillin-resistant strain, S. aureus ATCC BAA-41, ATCC BAA-1717, ATCC BAA-1720, and ATCC 43300 which are methicillin-resistant strains, and four USA300 strains (#417, #757, #1799, and #2690), which are the predominant strain type of community-associated MRSA in the United States. PC190723 and compound 5 were also synthesized according the literature for a positive control. The MIC screening results are summarized in Table 2. All compounds were found to retain potent antibacterial activities against these antibiotic-resistant strains with MIC values ranging from 1 to 6.

Figure 2. Summary of the SAR study. The red arrows indicate unfavorable substituents. The green arrow and the green boxes indicate favorable substituents.

Table 2. MIC Values of Selected Pyrimidines against Nine Clinically Isolated S. aureus Strains

| compound no. | strain 1 | strain 2 | strain 3 | strain 4 | strain 5 | strain 6 | strain 7 | strain 8 | strain 9 |
|--------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| PC190723     | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 1       | 1       |
| 5            | 2       | 2       | 2       | 2       | 2       | 2       | 2       | 2       | 2       |
| 14av_amine16 | 4       | 4       | 4       | 6       | 6       | 6       | 6       | 6       | 6       |
| 14dv_amine16 | 3       | 3       | 6       | 6       | 6       | 6       | 6       | 6       | 6       |
| 14dv_amine06 | 3       | 3       | 6       | 6       | 6       | 6       | 6       | 6       | 6       |
| methicillin  | N.D.    | >64     | >64     | >64     | >64     | >64     | >64     | 64      | 64      | 32      |

N.D.: not determined; strain 1: ATCC 29247; strain 2: ATCC BAA-1717; strain 3: ATCC BAA-1720; strain 4: ATCC BAA-41; strain 5: ATCC 43300; strain 6: USA300 #417; strain 7: USA300 #757; strain 8: USA300 #1799; and strain 9: USA300 #2690.
µg/mL. Compounds 14av_amine16, 14dv_amine16, and 14dv_amine06 displayed a potent antibacterial activity against MRSA, with MIC values of 3–6 µg/mL, which are very close to those of reported FtsZ inhibitors (S and PC190723). Compared with methicillin, which is the clinically used antibiotic with MIC values higher than 64 µg/mL against most of the MRSA, these pyrimidine derivatives exhibited significantly lower MIC values and thus exhibited the potential to be developed into new antistaphylococcal agents in the future. It is worthy to mention that compound 7 did not exhibit any antibacterial activity against these clinically isolated S. aureus strains (MIC > 48 µg/mL).

2.3. Investigation of the Potential for the Development of Resistance and in Vivo Biological Evaluation. A crucial attribute of potential antimicrobial agents is that the spontaneous development of drug resistance is not easily attained. To evaluate the spontaneous resistance rate of these compounds in MRSA, compound 14av_amine16 was selected for the study against the MRSA strain ATCC 43300. We plated 1 × 10^6 cells on agar plates containing 1% dimethyl sulfoxide (DMSO) or 8x the MIC of compound 14av_amine16 and observed the degree of bacterial growth. As shown in Figure 3A (left), almost confluent bacterial growth was observed for the DMSO-treated bacterial cells, yet no colony was observed for the plates treated with compound 14av_amine16 (Figure 3A, right) after 48 h incubation, implying that the rate of emergence of spontaneous resistant mutants was as low as <1 × 10^9. This low frequency of spontaneous resistance implies a reduced probability of rapid development of resistance against compound 14av_amine16 in clinical practice.

As a preliminary measure of in vivo toxicity and efficacy, we next tested our lead compound 14av_amine16 in a Galleria mellonella model, which is an easy and inexpensive in vivo model with no ethical constraints for investigating the antibacterial activity of a compound.39 To examine the feasibility of using G. mellonella for compound 14av_amine16 toxicity study, we examined the ability of compound 14av_amine16 to kill G. mellonella larvae. Various concentrations of compound 14av_amine16 dissolved in 50% poly(ethylene glycol) (PEG) in saline (0, 50, and 100 mg/kg) were injected into the hemocoel of last-instar G. mellonella larvae, and survival was scored over time. All concentrations of compound 14av_amine16 tested did not kill the G. mellonella larvae during a 48 h period (Figure 3B). These data indicate that the formulation of 50% PEG in saline and compound 14av_amine16 are nontoxic and safe against G. mellonella larvae.

Next, we investigated the efficacy of compound 14av_amine16 against G. mellonella larvae infected with MRSA ATCC 43300 (Figure 3C). Inoculation of lethal dose of 2.5 × 10^6 CFU/larva of MRSA ATCC 43300 led to a significant death rate. All larvae injected with 50% PEG in saline (0 mg/kg) died within a 24 h infection period. Injection of compound 14av_amine16 at a dose of 50 mg/kg to the infected larvae was found to prolong the survival time of the infected larvae to 36 h. Encouragingly, we observed 20% survival rate of the infected larvae over the infection period with increased dosage with this agent at 100 mg/kg. Compared to the vehicle group, it was found to be highly significant (p < 0.05). Further increased dosage did not lead to improved efficacy (data not shown). These data indicated that compound 14av_amine16 is capable of preventing or delaying the lethal effect of MRSA ATCC 43300 in G. mellonella larvae. This suggests that compound 14av_amine16 has strong potential for animal studies in future.

2.4. Saturation Transfer Difference NMR Study. Saturation transfer difference (STD) NMR spectroscopy is a powerful and unique tool that can detect the magnetization that was transferred from a protein to a bound ligand proton. It is commonly used to detect the interaction between low-molecular-weight compounds and large biomolecules.40 To get further insights into the interaction between compound 14av_amine16 and S. aureus FtsZ protein, STD NMR spectroscopy was employed to characterize the binding properties and identify the epitopes of small molecules that interact with a protein receptor. S. aureus FtsZ protein was expressed and purified as described in a previous report.29 STD NMR spectroscopy was performed, and the relative degrees of saturation for individual protons of compound 14av_amine16 without (B) and with (C) lethal dose of MRSA ATCC 43300 inoculation. Larvae were considered dead if they did not respond to physical stimuli. Data presented are the mean of three independent experiments.
is bound to the FtsZ protein. The largest amount of saturation transfer was observed for H1, indicating that the pyrimidine ring of compound 14av_amine16 is making more intimate contacts with the FtsZ protein.

2.5. S. aureus FtsZ Polymerization and GTPase Hydrolysis Assay. Next, we sought to determine whether the binding of compound 14av_amine16 to S. aureus FtsZ protein led to a change in the polymerization activity and GTPase activity of the protein itself. To assay the polymerization activity, an in vitro light scattering assay, in which FtsZ polymerization was detected in solution by a time-dependent increase in light scattering as reflected by an increase in solution absorbance at 600 nm, was employed. Figure 5 shows the relative time-dependent absorbance at 600 nm of S. aureus FtsZ in the presence of compound 14av_amine16 at concentrations ranging from 0 to 30 μM. It was clearly demonstrated that compound 14av_amine16 potently suppressed the self-polymerization of S. aureus FtsZ protein, with the magnitude of these suppressing effects increasing with increasing compound concentration. Compared with the vehicle (1% DMSO), complete inhibition of FtsZ polymerization was observed at 30 μM of compound 14av_amine16. In addition, we also investigated the inhibitory effect of compound 14av_amine16 on GTPase hydrolysis activity according to the protocol described previously.33 Compounds 7a and 7b inhibited the GTPase hydrolysis activity with IC50 values at 73 and 189 μM, respectively. However, compound 14av_amine16 at 50 and 75 μM displayed only moderate inhibition of the GTPase hydrolysis activity at about 20 ± 3% and 25 ± 4%, respectively. GTPase hydrolysis activity at higher compound concentrations (>80 μM) cannot be measured because of the poor compound solubility, which causes precipitation in aqueous medium. Nonetheless, it seems very likely that compound 14av_amine16 suppresses the self-polymerization of the FtsZ protein, probably via disrupting the GTPase hydrolysis activity of the FtsZ protein.

2.6. Effects on the Bacillus subtilis 168 Cell Morphology and Localization of the Z-Ring. The underlying mode of action of the antibacterial activity of compound 14av_amine16 was further investigated by the microscopic observation of a rod-shaped B. subtilis 168 cell morphology. Compound 14av_amine16 exhibited antibacterial activity against B. subtilis 168 with an MIC value at 12 μM. Treatment of B. subtilis cells at a sublethal concentration of compound 14av_amine16 significantly increased the cell length with average cell length > 20 μm (Figure 6B) as compared with the DMSO-treated cells (cell length < 5 μm, Figure 6A). Interestingly, such a phenomenon of cell elongation was also observed for other FtsZ inhibitors reported, strongly suggesting that 14av_amine16 interacts with the FtsZ protein in vivo. The iconic filamentous cell phenotype of FtsZ inhibitors was
believed to be caused by the disruption and mislocalization of the Z-ring. To further confirm that, a fluorescence microscopic analysis of dynamic Z-ring formation in *B. subtilis* 168 was carried out by using a functional green fluorescent protein-tagged FtsZ (C,E), and their corresponding phase-contrast microscopic pictures (D,F).

**Figure 6.** Effects of DMSO or compound 14av_amine16 treated on the cell morphology of *B. subtilis* 168 (A,B), *B. subtilis* 168 with a functional green fluorescent protein-tagged FtsZ (C,E), and their corresponding phase-contrast microscopic pictures (D,F).

3. CONCLUSIONS

In this study, a total of 99 amine-linked 2,4,6-trisubstituted pyrimidines, many displaying potent antistaphylococcal activity and some extent of cytotoxicity against normal cells, has been synthesized systematically by varying the substitutions in 2, 4, and 6 positions. These compounds also exhibited potent antibacterial activities against clinically isolated MRSA strains. These promising results led to the efficacy testing of the lead compound 14av_amine16, which revealed a very low spontaneous FOR and limited toxicity against *G. mellonella* larvae. Investigation of the mode of action suggests that compound 14av_amine16 exerted its antibacterial activity by interacting with *S. aureus* FtsZ protein and suppressing its polymerization, resulting in obvious mislocalization of the Z-ring formation. In summary, these newly synthesized 2,4,6-trisubstituted pyrimidine derivatives represent a novel scaffold of FtsZ inhibitors.

4. EXPERIMENTAL SECTION

4.1. Chemical Synthesis. All NMR and mass spectra were recorded on a Bruker Avance-III spectrometer and a Micromass Q-TOF-2 spectrometer by the electrospray ionization (ESI) mode, respectively. STD NMR experiments were performed on a Bruker Avance III 600 NMR spectrometer equipped with a QCI cryoprobe at 298 K. All reagents and solvents were of reagent grade and were used without further purification unless otherwise stated. The thin-layer chromatography (TLC) plates (silica gel 60F254, 0.25 mm thickness) were purchased from E. Merck. Flash column chromatographic purifications were performed on MN silica gel 60 (230–400 mesh). Compound purity was determined by an Agilent 1100 series high-performance liquid chromatography (HPLC) system installed with a Prep-Sil column (4.6 mm × 250 mm, 5 μm) at an UV detection of 254 nm (reference at 450 nm). All tested compounds were shown to have >95% purity according to HPLC.

**4.2. General Procedure I for the Synthesis of Pyrimidines 11.** To a well-stirred solution of Pd(PPh3)4Cl2 (0.4 mmol) and Cul (0.8 mmol) in degassed THF (150 mL) under a nitrogen atmosphere, were added NET3 (13 mmol), acid chloride 8 (10 mmol), and alkyne 9 (10 mmol) successively. The reaction mixture was stirred for 3 h until complete conversion as monitored by TLC. After that, Na2CO3 (34 mmol) and amidinium hydrochloride salt 10 (12 mmol) were added to the reaction mixture. The resulting suspension was heated to reflux for 14 h. After cooling to room temperature, the reaction mixture was filtered through a short pad of silica gel to remove excess Na2CO3. The obtained pale brown filtrate was evaporated under reduced pressure to crude oil which was further subjected to flash column chromatography on silica gel with gradient elution (5% ethyl acetate (EA) in Hex to 30% EA in Hex) to furnish the desired product 11.

4.2.1. 4-(2-((tert-Butyldimethylsilyl)oxy)ethyl)-6-(pentan-3-yl)-2-(pyridin-4-yl)pyrimidine (11av). The titled compound 11av was obtained as a colorless oil (0.32 g, 83% yield) according to the general procedure I described above: 1H NMR (400 MHz, CDCl3): δ 8.76 (d, J = 4.40 Hz, 2H), 8.35 (d, J = 5.87 Hz, 2H), 7.03 (s, 1H), 4.10 (t, J = 6.11 Hz, 2H), 3.01 (t, J = 6.11 Hz, 2H), 2.50–2.64 (m, 1H), 1.69–1.87 (m, 4H), 0.77–0.88 (m, 15H), –0.02 (s, 6H); 13C NMR (101 MHz, chloroform-d): δ 173.8, 168.2, 161.9, 150.3, 145.7, 122.2, 119.9, 61.9, 51.0, 41.2, 27.5, 25.8, 18.2, 12.0, –5.5; LRMS (ESI) m/z: 386 (M+ + H, 100); HRMS (ESI) calcd for C22H24N6O6Si (M+ + H) 386.2628; found, 386.2621.
dried over MgSO₄, filtered, and evaporated to afford the desired product 12, which is pure enough for the next step.

4.3.1. 2-(6-(Pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)-ethanol (12av). The titled compound 12av was obtained as a colorless oil (0.27 g, quantitative yield) according to the general procedure II described above: ¹H NMR (400 MHz, CDCl₃): δ 8.46 (d, J = 4.40 Hz, 2H), 8.15 (d, J = 5.87 Hz, 2H), 6.96 (s, 1H), 5.00 (br, s, 1H), 4.00 (t, J = 4.84 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 175.3, 182.1, 181.0, 145.8, 122.1, 118.0, 60.1, 40.7, 30.5, 27.9, 12.0; LRMS (ESI) calcd for C₁₆H₂₂N₃O + H, 272.1750; found, 272.1750.

4.4. General Procedure III for the Synthesis of Bromides 13. To a well-stirred solution of slightly excess (3 mmol) and PPh₃ (3.6 mmol) in THF (50 mL) at room temperature, was added carbon tetrabromide (3.6 mmol) once. The reaction mixture was stirred for 3 h until complete conversion as monitored by TLC. The reaction mixture was filtered through a short pad of silica gel. The obtained pale brown filtrate was evaporated under reduced pressure to crude oil which was further subjected to flash column chromatography on silica gel with gradient elution (1% MeOH in DCM to 5% MeOH in DCM) to furnish the desired product 13.

4.4.1. 4-(2-Bromoethyl)-6-(pentan-3-yl)-2-(pyridin-4-yl)-pyrimidine (13av). The titled compound 13av was obtained as a colorless oil (0.27 g, 80% yield) according to the general procedure IV described above: ¹H NMR (400 MHz, CDCl₃): δ 8.42 (m, 2H), 8.38 (d, J = 8.29 Hz, 2H), 7.52−7.41 (m, 2H), 7.34 (m, 1H, J = 5.89, 8.38 Hz), 3.36 (m, 4H, J = 6.04, 7.55 Hz); ¹³C NMR (100 MHz, CDCl₃): δ 174.5, 166.5, 162.2, 150.3, 145.4, 122.2, 119.4, 51.0, 40.4, 30.1, 27.5, 12.0; LRMS (ESI) m/z: 272 (M⁺ + H, 100); HRMS (ESI) calcd for C₁₆H₂₁N₃Br + H, 334.0919; found, 334.0914.

4.5. General Procedure IV for the Synthesis of Pyrimidines 14. To a well-stirred solution of slightly excess amine (1.1 mmol) in ACN (10 mL) at room temperature, was added a freshly prepared bromide 13 (1 mmol) solution in ACN (10 mL) once. The reaction mixture was stirred for 14 h until complete conversion as monitored by TLC. The reaction mixture was evaporated under reduced pressure to crude oil which was further subjected to flash column chromatography on silica gel with gradient elution (1% MeOH in DCM to 5% MeOH in DCM) to furnish the desired product 14.

4.5.1. 1-(2-(Pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)-ethyl)-4-(4-(trifluoromethyl)benzyl)-1,4-diazepane (14av-_amine16). The titled compound 14av-_amine16 was obtained as a colorless oil (0.27 g, 53% yield) according to the general procedure IV described above: ¹H NMR (400 MHz, CDCl₃): δ 8.77−8.80 (m, 2H), 8.31−8.38 (m, 2H), 7.52−7.60 (m, J = 8.31 Hz, 2H), 7.30−7.40 (m, J = 8.31 Hz, 2H), 6.99 (s, 1H), 3.67 (s, 2H), 2.96−3.08 (m, 4H), 2.83−2.90 (m, 2H), 2.77−2.83 (m, 2H), 2.66−2.72 (m, 4H), 2.57 (tt, J = 5.81, 8.38 Hz, 1H), 1.73−1.87 (m, 6H), 0.83 (t, J = 7.58 Hz, 6H); ¹³C NMR (100 MHz, CDCl₃): δ 173.9, 169.0, 161.9, 150.3, 143.7, 143.9, 128.8, 125.6, 125.2, 125.1, 125.1, 122.9, 122.2, 119.1, 122.1, 57.2, 57.2, 55.3, 55.2, 54.3, 53.9, 51.0, 35.9, 27.8, 27.5, 12.0; LRMS (ESI) m/z: 512 (M⁺ + H, 100), 534 (M⁺ + Na, 13); HRMS (ESI) calcd for C₂₉H₂₅N₃F₃ + H, 512.3001; found, 512.2986.

4.6. Antimicrobial Susceptibility Tests. The MIC values of all compounds were determined using the broth micro-dilution procedure in accordance with the CLSI guidelines³⁷ and our previous reports.⁷⁹,⁴⁴

4.7. Cytotoxicity (IC₅₀) Assay toward the L929 Cell Line. Standard MTS assay was performed to determine the cytotoxicity of all compounds toward the L929 cells. Briefly, 10 000 cells were mixed with compounds at different concentrations in a final volume of 100 μL in each well of a 96-well plate, followed by 3 days incubation at 37 °C. DMSO at 1% was used as a solvent control. The half-maximal inhibition of the compounds was determined using a CellTiter 96 AQueous assay (Promega). An aliquot of the freshly prepared MTS/phenazine methosulfate mixture at a ratio of 20:1 was added into each well, followed by 2 h incubation at 37 °C. Optical absorbance at 490 nm was measured with a microplate reader. The IC₅₀ values were determined from the dose−response curves of the MTS assay (Prism 4.0). All experiments were performed in triplicates, and the results will be presented as the average of the three independent measurements.

4.8. Expression and Purification of S. aureus FtsZ Protein. Expression and purification of S. aureus FtsZ protein were performed as previously reported, which was stored at −20 °C as a lyophulated powder.⁷⁹,⁴⁴ A stock solution of the FtsZ protein was then prepared from the lyophulated powder for subsequent STD NMR study, light scattering assay, and GTPase activity assay.

4.9. STD NMR Study. The STD NMR study of compound 14av-_amine16 was performed according to our previous report.⁴² Group epitope mapping was performed by integrating the STD signal of the individual protons with respect to the strongest STD signal, which was assigned to a value of 100%. 

4.10. Light Scattering Assays and GTPase Activity Assays. The light scattering assays and GTPase activity assays were performed as previously described.

4.11. FOR Determination. MRSA ATCC 43300 cells were grown to the late-exponential phase (1 × 10⁶ CFU/mL) and spread on agar plates containing 1% DMSO or compound 14av-_amine16 at 8 folds of MIC level. The plates were incubated for 48 h to allow resistant mutants to grow. The spontaneous FOR was calculated as the number of resistance colonies divided by the number of CFUs originally plated. The assay was performed in triplicates.

4.12. Evaluation of Toxicity and Antimicrobial Efficacy Using a G. mellonella Model of Infection. A G. mellonella model of S. aureus infection was adopted to test the in vivo toxicity and efficacy of compound 14av-_amine16 according to previous reports.⁴¹,⁴³ Groups of G. mellonella larvae (N = 10) weighing 200−300 mg were used in these assays. All larvae were incubated at 37 °C, and the mortality rates were assessed at 12 h interval for 48 h after different treatments. All larvae were scored as dead if they showed no signs of movement to physical stimuli. All injections were carried out into the last left proleg of the larvae using a 10 μL Hamilton syringe. For toxicity evaluation, the larvae were injected with 0 (vehicle), 50, and 100 mg/kg compound 14av-_amine16 only. For antimicrobial efficacy, the larvae were inoculated with a lethal dose of 10 μL of S. aureus 43300 (2.5 × 10⁶ CFU). All larvae received compound 14av-_amine16 of different dosages (0, 50, and 100 mg/kg) 1 h before bacterial inoculation. All data were analyzed for statistical significance using a log rank and χ² squared test with 1° of freedom.
4.13. Bacterial Morphology of B. subtilis 168. The bacterial morphology study of B. subtilis 168 was performed under a phase-contrast optical microscope as previously described.29,44

4.14. Z-Ring Visualization of B. subtilis 168. The study of Z-ring visualization of B. subtilis 168 was performed under a fluorescence and phase-contrast microscope using the Olympus FSX100 bio imaging navigator software as previously described.29,44

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Notes

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ABBREVIATIONS

MRSA, methicillin-resistant Staphylococcus aureus; FtsZ, filamenting temperature-sensitive mutant Z; GTPase, guanosine triphosphatase; GTP, guanosine triphosphate; MIC, minimum inhibitory concentration; VRE, vancomycin-resistant Enterococcus faecium; SAR, structure–activity relationship; ACN, acetonitrile; TFA, trifluoroacetic acid; DCM, dichloromethane; STD, saturation transfer difference; CLSI, Clinical and Laboratory Standards Institute; CA-MHB, cation-adjusted Mueller Hinton broth; DMSO, dimethyl sulfoxide; TLC, thin-layer chromatography; 4-Py, 4-pyridyl

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