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Synthesis and Evaluation of 1,2,4-Triazolo[1,5-*a*]pyrimidines as Antibacterial Agents Against *Enterococcus faecium*

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KEYWORDS: triazolopyrimidines, *Enterococcus faecium*, antibacterial activity

ABSTRACT: Rapid emergence of antibiotic resistance is one of the most challenging global public health concerns. In particular, vancomycin-resistant *Enterococcus faecium* infections have been increasing in frequency, representing 25% of enterococci infections in intensive care units. A novel class of 1,2,4-triazolo[1,5-*a*]pyrimidines active against *E. faecium* is reported herein. We used a three-component Biginelli-like heterocyclization reaction for the synthesis of a series of these derivatives based on reactions of aldehydes, β-dicarbonyl compounds and 3-alkylthio-5-amino-1,2,4-triazoles. The resulting compounds were assayed for antimicrobial activity against the ESKAPE panel of bacteria, followed by investigation of their *in vitro* activities. These analyses identified a subset of 1,2,4-Triazolo[1,5-*a*]pyrimidines that had good narrow-spectrum antibacterial activity against *E. faecium*, and exhibited metabolic stability with low intrinsic clearance. Macromolecular synthesis assays revealed cell-wall biosynthesis as the target of these antibiotics.

INTRODUCTION

Antibacterial resistance is on the increase, becoming one of the most serious public health challenges.[1,2,3] The ESKAPE bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are important pathogens, not only because they account for nearly one third of all nosocomial infections in the United States,[4,5] but also because they are broadly resistant to existing antibiotics.[4,6] Enterococci have been important clinically for some time. They are either intrinsically resistant to antibiotics or have acquired resistant mechanisms to many commonly used antibiotics.[7,8,9] This is especially true for bloodstream infections, for which they are the third most common bacterial pathogen.[10] *E. faecalis* and *E. faecium* are the major enterococcal species.[11] Although *E. faecium* is known to be a harmless commensal inhabitant of the gastrointestinal tract, it causes invasive diseases such as neonatal meningitis,[12] endocarditis,[13] urinary tract infections,[14] surgical wound infections[15] and bloodstream infections,[16] accounting for 20% of enterococcal infections in the United States.[11] It shows high resistance to many commonly used antibacterial reagents.[17] Vancomycin-resistant *E. faecium* (VRE) emerged in the 1980s in the United Kingdom and France.[18,19] The VRE strains disseminated globally rapidly.[19] Infections by VRE represent 25% of enterococci infections in intensive-care units,[20] and in some medical centers more than 70% of *E. faecium* isolates are vancomycin-resistant.[11,21] There is a genuine need for new antibacterial agents against this organism and agents selective for this organism are especially sought. The draw of selective agents is that they would be useful in treatment of infections by *E. faecium*, yet they would spare other organisms in the microbiota, mitigating the opportunity for rapid emergence of resistance clinically.

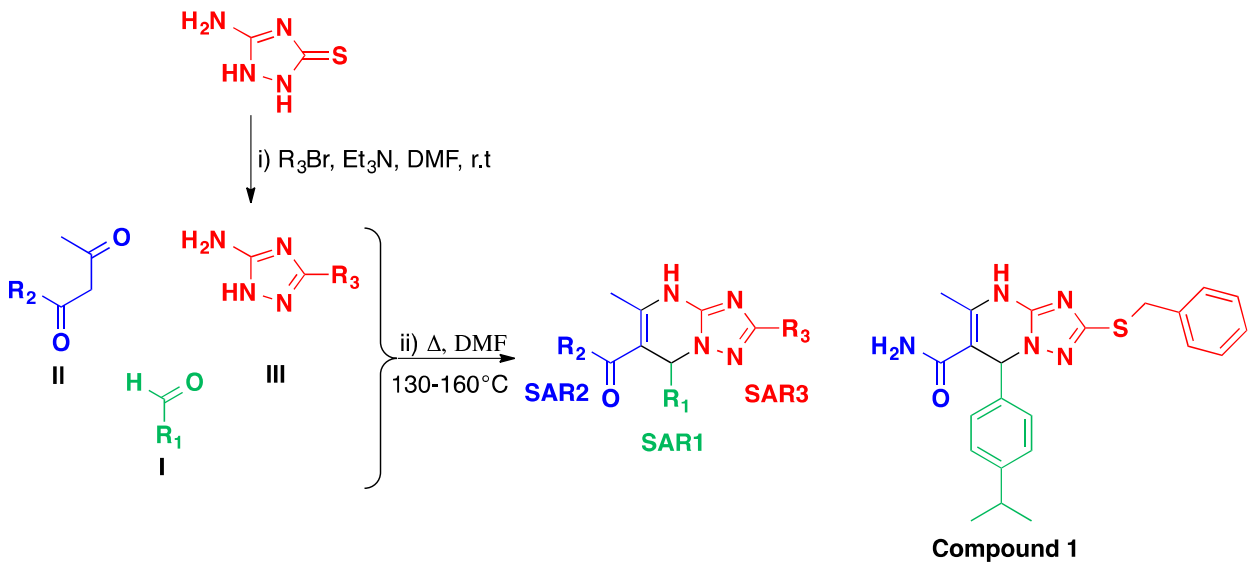
We report herein the discovery of a novel class of antibacterial agents, the triazolopyrimidines, which emerged from an *in silico* screening against a penicillin-binding protein (PBP), which is an essential enzyme for peptidoglycan biosynthesis.[22] The *in silico* screening was performed against 1.2 million compounds. Of the top 2,500 hits, we purchased 118 compounds, which were then evaluated against the living bacteria of the ESKAPE panel. The triazolopyrimidine emerged as a template with antibacterial activity, of which we synthesized 68 analogs. These derivatives, along with 18 additional compounds that could be purchased commercially, were evaluated for antibacterial activity. We disclose herein the structural template and describe the structure-activity relationship (SAR) for antibacterial activity for this set of molecules.

RESULTS AND DISCUSSION

Discovery of the lead template. We used *in silico* docking and scoring of 1.2 million drug-like compounds from the ChemDiv subset of the ZINC database[23] against the X-ray structure of PBP2a (PDB ID: 1VQQ),[24] in discovery of antibiotics. We reasoned that identification of the leads from the *in silico* screening should result in discovery of compounds that would have the ability to cross-inhibit more than one PBP, as the active sites of these enzymes are very similar. Whereas these *in silico* methodologies have come a long way in sophistication in the past few years, there still exists a degree of uncertainty in identifying molecules that indeed bind to the target site for which they were screened. As such, as will be described, experimental verification is critical. We analyzed the top 2,500 best scoring compounds and clustered them in groups according to structural similarity using hierarchical clustering. From these, we selected 118 compounds for purchase from ChemDiv and screened them for antibacterial activity against the ESKAPE panel of organisms. Therefore, the bar was set high from the outset, as demonstration of antibacterial activity with living bacteria was selected as the minimal criterion for a molecule

to move forward. This is in contrast to the possibility that the compound inhibiting avidly the recombinant target *in vitro*, which might turn out to be devoid of any activity with living bacteria. Compound **1** (Scheme 1) emerged from this screening, with an MIC of 8 μg/mL against *E. faecium*. No antibacterial activity was observed against the other bacteria in the ESKAPE panel. Hence, this compound fits the narrow-spectrum antibiotic definition for enterococci, which is desirable, as described earlier. However, as will be elaborated below—and as a cautionary note on *in silico* drug discovery—compound **1** did not inhibit PBP2a, which was the basis of the screening. Hence, notwithstanding the prediction by computation that this compound should inhibit PBP2a, it does not. Yet, it possesses antibacterial activity, as activity against living bacteria was set as the minimal criterion for moving forward. Optimization of the antibacterial activity became the goal of the diversification of structure that we undertook.

Chemistry. The general procedure for the syntheses of triazolopyrimidine derivatives is outlined in Scheme 1. We made 68 analogs of compound **1** by this method. Compounds **III**, as key intermediates in these syntheses, were prepared by the reaction of 3-amino-5-thiol-1,2,4-triazole with alkyl bromides in the presence of triethyl amine as the base. Subsequently, equal amounts of derivatives **I**, **II**, and **III** were heated in DMF for roughly 15-20 min (with a temperature of 130-160 °C), which yielded the desired compounds after the work up. This three-component Biginelli-like heterocyclization method[25,26] afforded the rapid and efficient synthesis of a focused library of these compounds, using commercially available reagents. In light of the essentially single-pot reactions that led to these molecules, only the final compounds have been characterized in this report.



Scheme 1. Syntheses of the 1,2,4-triazolo[1,5-*a*]pyrimidine derivatives.

Antibacterial activity and SAR analysis. The SAR of the compound class was investigated by synthesizing structural variations at each of the three sites designated as SAR1, SAR2, and SAR3 (Figure 1) and by determination of their minimal-inhibitory concentration (MIC) values against *E. faecium*. Initial studies of the scaffold focused on modifications of the aromatic ring in the pyrimidine segment (SAR1). The antibacterial activities (as MICs) for these compounds are shown in Table 1. In the SAR1 study, the synthesis was driven by preserving the original functionalities for the SAR2 and SAR3 regions in the lead compound, while the SAR1 position was varied. Various functional groups, such as substituted phenyl and heterocycles, were introduced in SAR1. Substitution of the 4-*i*-propyl group with a hydrogen atom resulted in loss of antibacterial activity (**2**), which indicates addition of a substituent on the phenyl ring is necessary. An evaluation of derivatives with lipophilic steric bulk showed that the anti-*E. faecium* activity is linked with the size of the lipophilic group (-Ph-4-*i*-Bu (**5**)/-Ph-4-*i*-Pr (**1**)/ -Ph-4-Et (**4**) > -Ph-4-Me (**3**)/ -Ph-4-H (**2**)). Assessment of compounds with bulky oxygen and sulfur

substituents at the *para*-position also indicates a similar trend. Introduction of 4-O-*i*-Pr (**10**) and 4-SEt (**13**) into the scaffold showed 32-fold improvement of the anti-*E. faecium* activity, compared to the 4-OCH_3 (**7**) and 4-SMe (**12**) derivatives, which were devoid of activity. A series of analogs with nitrogen (or alkylated nitrogen) at this position, -Ph-4-NH_2 (**15**), -Ph-4-NMe_2 (**16**), -Ph-4-NEt_2 (**17**), -Ph-4-(1-pyrrolidinyl) (**18**) and -Ph-4-$NHCOCH_3$ (**19**), were prepared. Compound **16** with -Ph-4-NMe_2 was the best in this series. It showed 32-fold improved potency over compounds **15** (-Ph-4-NH_2) and **19** (-Ph-4-$NHCOCH_3$), 4-fold better antimicrobial activity than compound **18** (-Ph-4-(1-pyrrolidinyl)), and slightly better activity than compound **17** (-Ph-4-NEt_2); also 2-fold improved antibacterial activity over the lead compound **1**. Introduction of small electron-donating groups at the *para*-position of the phenyl ring resulted in loss of antimicrobial activity, as observed for compounds **6**, **7**, **8**, and **9**. On the other hand, introduction of electron-withdrawing groups at the *para*-position of the phenyl ring retained activity (**11**, **21** and **24**), but not in all cases (**14**, **25** and **26**), as compared to compound **1**. Analogs with halogen substituents did not show any activity (**22** and **23**). The variant **20** had no activity. From all the above findings, compounds with -Ph-4-*i*-Pr (**1**), -Ph-4-Et (**4**), -Ph-4-O-*i*-Pr (**10**), -Ph-4-OCF_3 (**11**), -Ph-4-NMe_2 (**16**), -Ph-4-NEt_2 (**17**) and -Ph-4-CF_3 (**21**) groups showed good activity (MIC of ≤ 8 μg/mL).

In general, the substituents that gave good activity at the *para*-position of the ring, gave poorer or no activity when placed at the *meta*- or *ortho*-positions. Compounds with -Ph-3-CF_3, -Ph-3-*i*-Pr, -Ph-3-Et, -Ph-3-O-*i*-Pr, and -Ph-3-OCF_3 substitutions (**28**, **29**, **30**, **31**, and **32**) were examples of these analogs. Also, compound **27** with CF_3 at the *ortho*-position of the phenyl group was inactive. Substitutions of the phenyl group with heterocyclic rings were also tried, including thiophene, furan, pyrrole, imidazole, indole, pyridine rings (compounds **33** to **41**); however,

these changes were generally not tolerated, resulting in loss of activity. The non-aromatic cyclohexyl moiety in the SAR1 position (**42**) was devoid of activity. Based on the SAR1 findings, the phenyl ring was necessary for activity. Furthermore, substituents at the *para*-position improved the activity. Introduction of bulky electron-donating groups proved useful in improving activity against *E. faecium*. We selected -Ph-4-NMe_2 (**16**) from SAR1 for its favorable logP value and for its MIC of 4 μg/mL.

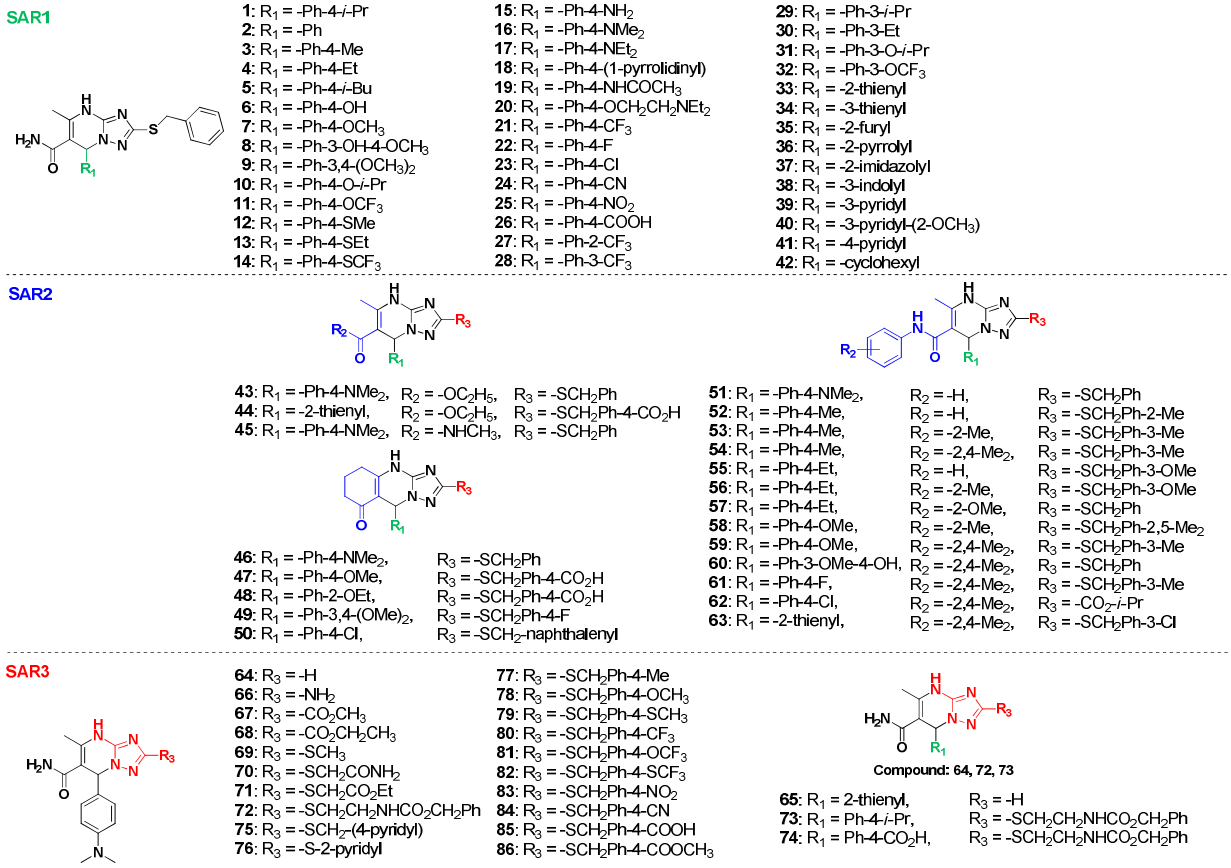


Figure 1. SAR of 1,2,4-triazolo[1,5-*a*]pyrimidine derivatives.

Next, our exploration shifted to modifications at the amide position within the bicyclic system SAR2 (Figure 1 and Table 1). Considering that the antibacterial activity favors the presence of

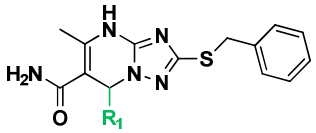
the -Ph-4-NMe_2 (of the SAR1), the subsequent analogs were investigated with this moiety retained. Replacing the carboxamide with an ester, *N*-methylcarboxamide, and *N*-phenylcarboxamide generated analogs **43**, **45**, and **51**, respectively. While the substituted amides (**45** and **51**) led to slight reduction of activity compared to compound **16**, the ester derivative (**43**) lost antimicrobial activity. Introduction of cyclohexenone next to the 1,6-dihydropyridine (**46**) abolished activity. This series of molecules shows that replacement of the carboxamide with an ester or cyclic ketone led to loss of activity. We also evaluated the commercially available derivatives **44**, **47**-**50**, and **52**-**63**. Unfortunately, all were devoid of antibacterial activity. These analyses indicated that the carboxamide segment of the molecular scaffolding was desirable for activity.

Our attention turned to the SAR3 series bearing the -Ph-4-NMe_2 (SAR1) and -$CONH_2$ (SAR2) moieties on the structural template (Figure 1 and Table 1). Replacement of the *S*-benzyl moiety with smaller groups (**64**-**71**) resulted in loss of activity. Introduction of longer R3 functionalities (than *S*-benzyl) in compounds **72**, **73** and **74** were not tolerated. Further exploration focused on the necessity of the phenyl ring and methylene of the *S*-benzyl group on the SAR3 region. Compound **75** was generated by replacing the phenyl ring with a pyridine ring; compound **76** was generated by removing the methylene from **75**. These changes resulted in loss of activity, which indicated the need for the phenyl ring and the methylene group. These results indicate the requirement for the *S*-benzyl group, which was retained in the next group of compounds that was prepared. A subset of analogs was made to investigate whether the antibacterial activity could be improved via substituents on the *S*-benzyl ring at the SAR3 position. A total of 10 *para*-substituted analogues (**77**-**86**) were made. While the ones with electron-donating groups at the *para*-position did not show good activity (**77**-**79**), compounds with electron-withdrawing groups

at the *para*-position, such as the trifluoromethylmercaptan (**82**) and the cyano (**84**) were as active as compound **1** and slightly less active than compound **16**. The nitro (**83**) analog was as good as compound **16**. Compounds **80** and **81** had no activity. The SAR3 revealed that the *S*-benzyl group and electron-withdrawing groups at the *para*-position are favored.

Table 1. Minimal-inhibitory concentrations (MICs, μg/mL) of SAR1, SAR2, and SAR3 compounds against *E. faecium* NCTC 7171[a]

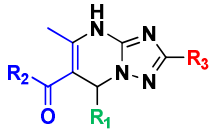
MICs of SAR1 compounds against *E. faecium* NCTC 7171



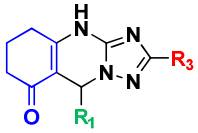
Compd	R_1	MIC	Compd	R_1	MIC
1	-Ph-4-*i*-Pr	8	**22**	-Ph-4-F	128
2	-Ph	>128	**23**	-Ph-4-Cl	>128
3	-Ph-4-Me	>128	**24**	-Ph-4-CN	16
4	-Ph-4-Et	8	**25**	-Ph-4-NO_2	64
5	-Ph-4-*i*-Bu	8	**26**	-Ph-4-COOH	>128
6	-Ph-4-OH	>128	**27**	-Ph-2-CF_3	>128
7	-Ph-4-OCH_3	>128	**28**	-Ph-3-CF_3	16
8	-Ph-3-OH-4-OCH_3	>128	**29**	-Ph-3-*i*-Pr	16
9	-Ph-3,4-$(OCH_3)_2$	>128	**30**	-Ph-3-Et	16
10	-Ph-4-O-*i*-Pr	4	**31**	-Ph-3-O-*i*-Pr	>128
11	-Ph-4-OCF_3	8	**32**	-Ph-3-OCF_3	16
12	-Ph-4-SMe	>128	**33**	-2-thienyl	>128

13	-Ph-4-SEt	4	**34**	-3-thienyl	>128
14	-Ph-4-SCF_3	>128	**35**	-2-furyl	>128
15	-Ph-4-NH_2	>128	**36**	-2-pyrrolyl	>128
16	-Ph-4-NMe_2	4	**37**	-2-imidazolyl	32
17	-Ph-4-NEt_2	8	**38**	-3-indolyl	>128
18	-Ph-4-(1-pyrrolidinyl)	16	**39**	-3-pyridyl	>128
19	-Ph-4-$NHCOCH_3$	128	**40**	-3-pyridyl-(2-OCH_3)	128
20	-Ph-4-$OCH_2CH_2NEt_2$	>128	**41**	-4-pyridyl	>128
21	-Ph-4-CF_3	8	**42**	-cyclohexyl	>128

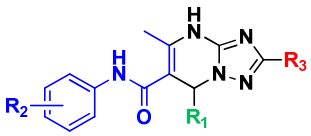
MICs of SAR2 compounds against *E. faecium* NCTC 7171



Compd	R_1	R_2	R_3	MIC
43	-Ph-4-NMe_2	-OC_2H_5	-SCH_2Ph	>128
44	-2-thienyl	-OC_2H_5	-SCH_2Ph-4-CO_2H	>128
45	-Ph-4-NMe_2	-$NHCH_3$	-SCH_2Ph	8

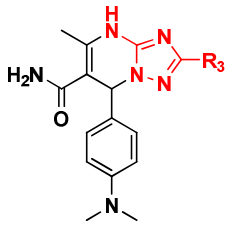


Compd	R_1	R_3	MIC	Compd	R_1	R_3	MIC
46	-Ph-4-NMe_2	-SCH_2Ph	>128	**49**	-Ph-3,4-$(OMe)_2$	-SCH_2Ph-4-F	>128
47	-Ph-4-OMe	-SCH_2Ph-4-CO_2H	>128	**50**	-Ph-4-Cl	-SCH_2-naphthalenyl	>128
48	-Ph-2-OEt	-SCH_2Ph-4-CO_2H	>128				



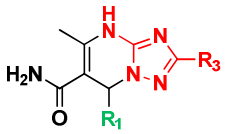
Compd	R_1	R_2	R_3	MIC
51	-Ph-4-NMe_2	-H	-SCH_2Ph	8
52	-Ph-4-Me	-H	-SCH_2Ph-2-Me	>128
53	-Ph-4-Me	-2-Me	-SCH_2Ph-3-Me	>128
54	-Ph-4-Me	-2,4-Me_2	-SCH_2Ph-3-Me	>128
55	-Ph-4-Et	-H	-SCH_2Ph-3-OMe	>128
56	-Ph-4-Et	-2-Me	-SCH_2Ph-3-OMe	>128
57	-Ph-4-Et	-2-OMe	-SCH_2Ph	>128
58	-Ph-4-OMe	-2-Me	-SCH_2Ph-2,5-Me_2	>128
59	-Ph-4-OMe	-2,4-Me_2	-SCH_2Ph-3-Me	>128
60	-Ph-3-OMe-4-OH	-2,4-Me_2	-SCH_2Ph	16
61	-Ph-4-F	-2,4-Me_2	-SCH_2Ph-3-Me	>128
62	-Ph-4-Cl	-2,4-Me_2	-CO_2-*i*-Pr	16
63	-2-thienyl	-2,4-Me_2	-SCH_2Ph-3-Cl	>128

MICs of SAR3 compounds against *E. faecium* NCTC 7171



Compd	R_3	MIC	Compd	R_3	MIC
64	-H	>128	**77**	-SCH_2Ph-4-CH_3	>128
66	-NH_2	>128	**78**	-SCH_2Ph-4-OCH_3	>128
67	-CO_2CH_3	>128	**79**	-SCH_2Ph-4-SCH_3	>128

68	-$CO_2CH_2CH_3$	>128	**80**	-SCH_2Ph-4-CF_3	>128
69	-SCH_3	>128	**81**	-SCH_2Ph-4-OCF_3	>128
70	-SCH_2CONH_2	>128	**82**	-SCH_2Ph-4-SCF_3	8
71	-SCH_2CO_2Et	>128	**83**	-SCH_2Ph-4-NO_2	4
72	-$SCH_2CH_2NHCO_2CH_2Ph$	>128	**84**	-SCH_2Ph-4-CN	8
75	-SCH_2-(4-pyridyl)	128	**85**	-SCH_2Ph-4-COOH	>128
76	-S-2-pyridyl	>128	**86**	-SCH_2Ph-4-$COOCH_3$	>128



Compd	R_1	R_3	MIC
65	-2-thienyl	H	>128
73	-Ph-4-*i*-Pr	-$SCH_2CH_2NHCO_2CH_2Ph$	>128
74	-Ph-4-CO_2H	-$SCH_2CH_2NHCO_2CH_2$ Ph	>128

[a]Compounds with MIC ≤ 16 μg/mL are highlighted in gray background.

The most active compounds (**1**, **10**, **13**, **16**, **83** and **84**) were further evaluated for their MICs with several additional Enterococci strains, both from commercial as well as the clinical sources (Table 2). These included strains that were resistant to erythromycin, tetracycline, vancomycin and teicoplanin.

Table 2. Minimal-inhibitory concentrations (MICs, μg/mL) of compounds **1**, **10**, **13**, **16**, **83**, **84** and Vancomycin with additional *E. faecium* strains

	MIC (μg/mL)						
	1	**10**	**13**	**16**	**83**	**84**	**Vancomycin**
E. faecium ATCC BAA 2127[a]	4	16	16	4	32	8	1
E. faecium 119-39A (Van S)[b]	4	4	8	4	16	8	0.5
E. faecium ATCC 700221(Van R)[c]	4	8	8	4	8	8	16
E. faecium C9[d]	16	4	8	4	8	4	2
E. faecium C38[d]	16	32	16	16	32	16	64
E. faecium C68[d,e]	16	16	16	4	32	4	64

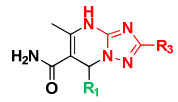
[a]Resistant to copper, erythromycin, and tetracycline, and sensitive to vancomycin and teicoplanin. [b]Vancomycin-susceptible clinical isolate. [c]Isolated from human feces, *vanA* positive, resistant to vancomycin and teicoplanin. [d]Clinical strains isolated in Cleveland hospitals, gifts from Dr. Robert A. Bonomo. [e]Most prevalent VRE clinical strain from Cleveland hospitals.

***In vitro* metabolic stability, plasma-protein binding, and cytotoxicity**. The most active compounds (**1**, **10**, **13**, **16**, **83**, and **84**) were evaluated for metabolic stability using human liver S9, which contains microsomal and cytosolic fractions capable of phase I and II metabolism. In

general, the compounds were metabolically stable, with half-lives of around 1 h or longer (Table 3). Replacement of the -Ph-4-*i*-Pr (**1**) with -Ph-4-O-*i*-Pr (**10**) did not change the half-life, however -Ph-4-SEt (**13**) and -Ph-4-NMe_2 (**16**) resulted in increased metabolic stability and lower intrinsic clearance. Retention of -Ph-4-NMe_2 at R_1 and -SCH_2Ph-4-NO_2 at R_3 (**83**) and -SCH_2Ph-4-CN at R_3 (**84**) also increased the half-lives, and consequently resulted in the lowest intrinsic clearances. We also ascertained plasma-protein binding for these compounds in human plasma using equilibrium dialysis (Table 3). Compound **1** showed 98.7 ± 0.7% plasma-protein binding. Plasma-protein binding of compounds **10**, **13**, and **83** was high. Compound **16** had 97.6 ± 0.1% plasma-protein binding, while compound **84** had the lowest protein binding at 96.8 ± 0.4%. Forty three percent of the 1,500 frequently prescribed drugs on the market show protein binding greater than 90%, and 27% of anti-inflammatory drugs have protein binding above 99%.[27] Moreover, many marketed antibiotics, including daptomycin,[28] oxacillin,[29] teicoplanin,[30] rifampicin,[31] and clindamycin[31] have plasma protein binding of >91%. Compounds **1**, **10**, **13**, **16**, **83** and **84** were evaluated for *in vitro* toxicity in the hemolysis assay (Table 3). The most active of these compounds (e.g., compounds **16** and **84**) showed 1-2% hemolysis of red blood cells at a fixed concentration of 64 μg/mL for the antibiotic.

The minimal-bactericidal concentrations (MBCs) of compounds **1**, **10**, **13**, **16**, **83** and **84** were determined using *E. faecium* NCTC7171. The MBC values were 32, 16, 16, 32, 16, and 16 μg/mL for the given antibiotics, respectively. This reveals that cidal effects are seen at 2- to 4-fold above the MIC values.

Table 3. Metabolic stability, apparent intrinsic clearance, plasma-protein binding and hemolysis of red blood cells of selected antibiotics.

			MIC of *E. faecium* C68 (μg/mL)	Half-life in human liver S9 (min)	Apparent intrinsic clearance ($mLmin^{-1}kg^{-1}$)	Plasma-protein binding (%)	Hemolysis at 64 μg/mL (%)
Compd	R_1	R_3					
1	-Ph-4-*i*-Pr	$-SCH_2Ph$	16	56 ± 1	11.1	98.7 ± 0.7	22%
10	-Ph-4-O-*i*-Pr	$-SCH_2Ph$	16	57 ± 8	10.9	99.9 ± 0.2	<1%
13	-Ph-4-SEt	$-SCH_2Ph$	16	>60	<10.4	99.9 ± 0.4	46%
16	$-Ph-4-NMe_2$	$-SCH_2Ph$	4	>60	<10.4	97.6 ± 0.1	2%
83	$-Ph-4-NMe_2$	$-SCH_2Ph-4-NO_2$	32	>60	<10.4	99.9 ± 0.5	23%
84	$-Ph-4-NMe_2$	$-SCH_2Ph-4-CN$	4	>60	<10.4	96.8 ± 0.4	1%

Mechanism of Action. Compound **16** was selected to investigate the mode of action by using macromolecular synthesis assays with *E. faecium* NCTC7171. Incorporation of radiolabeled precusors [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine, L-[4,5-^{3}H]-leucine, or D-[2,3-^{3}H]-alanine into DNA, RNA, protein, or peptidoglycan, respectively, was monitored in the logarithmic phase of growth of this bacterium. Ciprofloxacin, rifampicin, tetracycline, and meropenem, which are known inhibitors of each corresponding pathway, were used as positive controls. Compound **16** showed notable inhibition of peptidoglycan biosynthesis. It did not inhibit replication, transcription, or translation appreciably by these assays (Figure 2). These experiments with living bacteria were followed up by additional *in vitro* transcription and translation assays. A

transcriptAid T7 high-yield transcription kit was used for *in vitro* transcription assay; an *E. coli* S30 extract for circular DNA kit plus a β-galactosidase assay were used for a tandem *in vitro* transcription and translation assay. Compound **16** did not show any effect on transcription and/or translation (Figure 3) in these assays.

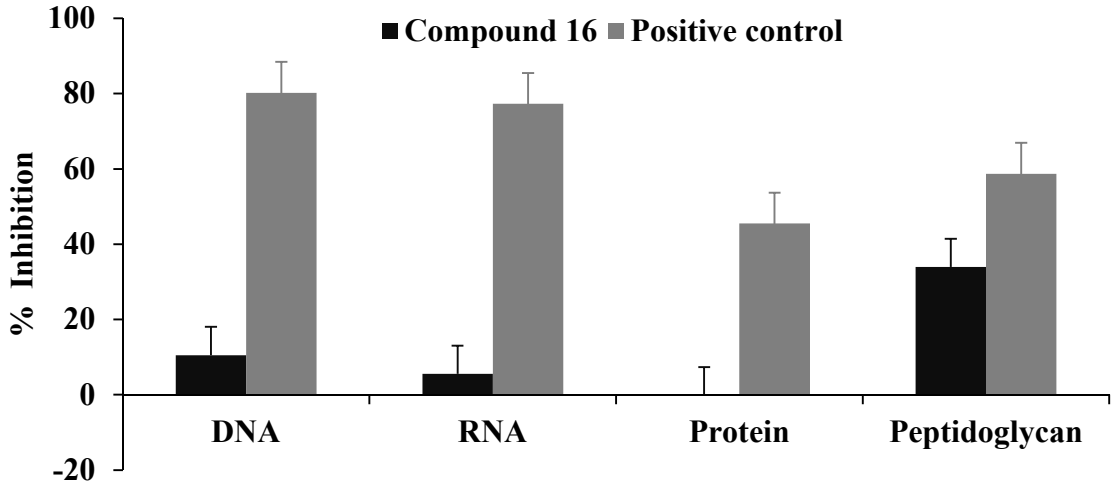


Figure 2. Macromolecular synthesis assays. Compound **16** at 2 × MIC. Positive controls for DNA, RNA, protein, and peptidoglycan syntheses are ciprofloxacin (4 μg/mL), rifampicin (4 μg/mL), tetracycline (248 ng/mL) and meropenem (4 μg/mL), respectively. The inhibition reported for compound **16** is at 120 min of the incubation; other time points of incubation are provided in the Supporting Information.

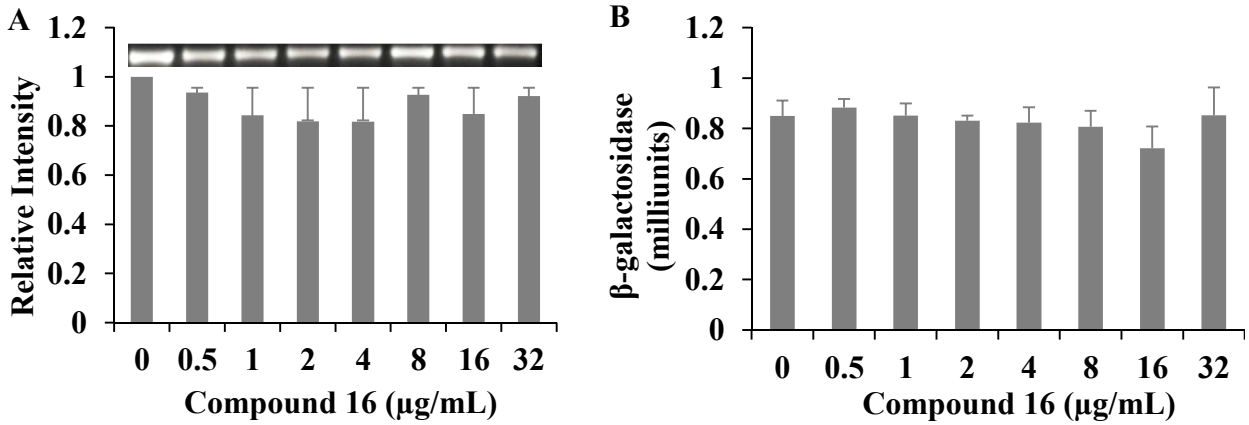


Figure 3. (A) *In vitro* transcription assay of compound **16**. (B) *In vitro* transcription and translation assay of compound **16** to evaluate β-galactosidase expression levels.

Binding assays for PBP2a of *S. aureus* and PBP5fm of *E. faecium*. Compound **1** was conceived from *in silico* screening of PBP2a of *S. aureus*. We explored if recombinant purified PBP2a was actually inhibited by this compound. At concentrations of up to 100 μg/mL we observed no inhibition. We then turned to PBP5fm, an important PBP of *E. faecium*, as these compounds had activity against this organism. Similarly, at up to 100 μg/mL we saw no inhibition of this enzyme. We also investigated compound **84**, an active compound that emerged from the SAR analyses. This compound also lacked the ability to inhibit both the recombinant proteins. As a last experiment, we also investigated if other PBPs in the *E. faecium* membrane fractions were inhibited in the presence of these compounds. The data from the membrane fractions were less clear due to the low copy numbers of PBPs in this organism. Regardless, no inhibition was apparent in membranes exposed to these antibiotics. It would appear that triazolopyrimidines are not inhibitors of PBPs.

CONCLUSION

In summary, we synthesized 68 compounds and investigated the SAR of these and 18 additional compounds that were purchased. The most active compounds were evaluated in several *E. faecium* strains, including clinical VRE isolates, as well as assessed for metabolic stability, plasma-protein binding, and cytotoxicity. Compound **16** has promising activity against *E. faecium*, it is metabolically stable, has low intrinsic clearance, and the lower plasma-protein binding.

Notwithstanding the fact that the *in silico* segment of screening was done against the PBP2a structure, compounds **1** and **84** did not inhibit this enzyme *in vitro*, nor the PBP5fm of *E. faecium* or likely the PBPs in the *E. faecium* membranes. This exercise reveals the limitations of *in silico* search. Experimental verification is critical. We state that by setting the bar high at the outset for the experimental verification, we went directly for documentation of the antibacterial activity with living bacteria, rather than the *in vitro* exploration of inhibition of recombinant PBP2a. If the bar had not been set high, by mere inhibition assays for PBPs the triazolopyrimidine class of antibacterials would not have been discovered.

An important aspect of the work with the triazolopyrimidine template is that a large set of molecules of this class can be prepared essentially in single-pot three-component reactions, using the Biginelli-like heterocyclization reaction. This ease in preparation should lend itself to further exploration of the SAR. Notwithstanding our observation that cell-wall biosynthesis is inhibited by this class of antibiotics, the identification of the actual target in the pathway is open for elucidation.

EXPERIMENTAL SECTION

General chemistry. All chemicals, reagents, and solvents were used directly as purchased without further purification. Chemical shifts in ^{1}H NMR and ^{13}C NMR spectra are reported in parts per million (ppm) relative to tetramethylsilane (TMS). ^{1}H and ^{13}C NMR spectra were recorded on Bruker AVANCE III HD 400 or Bruker AVANCE III HD 500 spectrometers (Bruker Daltonik, Bremen, Germany) equipped with BBFO probes and operating at ^{1}H resonance frequencies of 400.13 and 500.13 MHz, respectively, or using Varian UnityPlus 300 spectrometer (Varian Inc., Palo Alto, CA, USA). High-resolution mass spectra were measured using a Bruker micrOTOF/Q2 mass spectrometer in ESI ionization. Compounds **1**, **44**, **47-50**, **52-63** were purchased from ChemDiv with >95% purity. All synthetic compounds had >95% purity as determined by high-performance liquid chromatography.

General procedure for syntheses of the 1,2,4-triazolo[1,5-*a*]pyrimidine analogs.

The 1,2,4-triazolo[1,5-*a*]pyrimidine analogs were synthesized by using a modification of a known method.[26] Equimolar mixtures of compounds **I**, **II**, and **III** (0.80-2.0 mmol), were heated in 0.30-0.50 mL of DMF within the temperature range of 130-160 °C for 15-20 min. After cooling, acetone (10-25 mL) was added to the solution. The mixture was allowed to stand for a minimum of 2 h or overnight (depending on the nature of the derivative), at which time the product precipitates. The precipitate was filtered to obtain the desired product in the range of 6.0-82% of yield. Spectral data for the six active compounds (**1**, **10**, **13**, **16**, **83**, and **84**) are given below. The spectral data of the remaining 63 compounds are given in Supporting Information.

Compounds **III** were synthesized by a modification of a known method.[32] 3-Amino-5-thiol-1,2,4-triazole (6.0 mmol) was stirred in anhydrous DMF (10 mL) for 5 min. Triethylamine (7.0 mmol) was added dropwise to this solution, and the mixture was stirred under a nitrogen

atmosphere at room temperature for 15 min. Alkyl bromide (7.0 mmol) was added to the solution, followed by stirring under a nitrogen atmosphere at room temperature for 3 h. The solvent was evaporated to dryness, and the resulting residue was partitioned between ethyl acetate (30 mL) and water (20 mL). The organic layer was washed with brine (10 mL), dried over anhydrous $MgSO_4$, and it was concentrated under reduced pressure. The crude product was typically purified by column chromatography with hexane/ethyl acetate.

2-(Benzylthio)-7-(4-isopropylphenyl)-5-methyl-4,7-dihydro[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide (**1**). Compound **1** was prepared from 4-isopropyl-benzaldehyde (0.30 g, 2.0 mmol), acetoacetamide (0.20 g, 2.0 mmol) and 3-amino-5-(benzylthio)-1,2,4-triazole (0.41 g, 2.0 mmol) in 44% yield as a white solid (0.37 g). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.09 (s, 1H, NH), 7.29–7.12 (m, 10H, 9ArH + NH), 6.96 (s, 1H, NH), 6.31 (s, 1H, CHN), 4.24 (A of AB, 1H, J = 13.4 Hz, one of SCH_2), 4.16 (B of AB, 1H, J = 13.4 Hz, one of SCH_2), 2.86 (m, 1H, C*H*$(CH_3)_2$), 2.17 (s, 1H, =C–CH_3), 1.18 (d, J = 7.1 Hz, 6H, CH(C*H*$_3)_2$). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 167.8, 157.2, 148.5, 148.1, 138.2, 138.1, 136.0, 128.8, 128.3, 127.2, 127.0, 126.4, 103.4, 59.7, 34.8, 33.2, 23.9, 17.4. HRMS (ESI) *m/z*: 442.1672 calcd for $C_{23}H_{25}N_5NaOS$ $[M+Na]^+$; 442.1697 obsd

2-(Benzylthio)-7-(4-isopropoxyphenyl)-5-methyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide (**10**). Compound **10** was prepared from 4-isopropoxy-benzaldehyde (0.16 g, 1.0 mmol), acetoacetamide (0.10 g, 1.0 mmol) and 3-amino-5-(benzylthio)-1,2,4-triazole (0.21 g, 1.0 mmol) in 80% yield as a white solid (0.35 g). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.08 (s, 1H, NH), 7.41–7.18 (m, 6H, 5ArH + NH), 7.13 (d, 2H, J = 8.7 Hz, ArH), 6.97 (s, 1H, NH), 6.85 (d, 2H, J = 8.7 Hz, ArH), 6.28 (s, 1H, CHN), 4.56 (m, 1H, OC*H*$(CH_3)_2$), 4.23 (A of AB, 1H, J = 13.2 Hz, one of SCH_2), 4.15 (B of AB, 1H, J = 13.2 Hz,

one of SCH_2), 2.17 (s, 3H, =C–CH_3), 1.25 (d, 6H, J = 5.9 Hz, OCH(CH_3)$_2$). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 167.7, 157.3, 157.0, 148.3, 138.1, 135.8, 132.5, 128.7, 128.5, 128.2, 127.0, 115.1, 103.4, 69.1, 59.4, 34.8, 21.8, 17.3. HRMS (ESI) *m/z*: 458.1621 calcd for $C_{23}H_{25}N_5NaO_2S$ $[M+Na]^+$; 458.1644 obsd.

2-(Benzylthio)-7-(4-ethylthiophenyl]-5-methyl-4,7-dihydro[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide (**13**). Compound **13** was prepared from 4-methylthio-benzaldehyde (0.20 g, 1.2 mmol), acetoacetamide (0.12 g, 1.2 mmol) and 3-amino-5-(benzylthio)-1,2,4-triazole (0.25 g, 1.2 mmol) in 79% yield as a light-yellow solid (0.42 g). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.13 (s, 1H, NH), 7.27–7.15 (m, 10H, 9ArH + NH), 6.99 (s, 1H, NH), 6.31 (s, 1H, CHN), 4.24 (A of AB, 1H, J = 13.4 Hz, one of SCH_2Ph), 4.16 (B of AB, 1H, J = 13.4 Hz, one of SCH_2Ph), 2.97 (q, 2H, J = 7.3 Hz, S$CH_2$$CH_3$), 2.17 (s, 3H, =C–$CH_3$), 1.23 (t, 3H, J = 7.3 Hz, SCH_2CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 167.5, 157.2, 148.4, 138.0, 137.8, 136.3, 136.0, 128.6, 128.1, 127.8, 127.4, 126.9, 103.1, 59.5, 34.7, 25.8, 17.3, 14.1. HRMS (ESI) *m/z*: 460.1236 calcd for $C_{22}H_{23}N_5NaOS_2$ $[M+Na]^+$; 460.1260 obsd.

2-(Benzylthio)-7-(4-dimethylaminophenyl)-5-methyl-4,7-dihydro[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide (**16**). Compound **16** was prepared from 4-dimethylamino-benzaldehyde (0.22 g, 1.5 mmol), acetoacetamide (0.15 g, 1.5 mmol) and 3-amino-5-(benzylthio)-1,2,4-triazole (0.31 g, 1.5 mmol) in 33% yield as an off-white solid (0.21 g) ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.02 (s, 1H, NH), 7.30–7.17 (m, 6H, 5ArH + NH), 7.05 (d, 2H, J = 8.6 Hz, ArH), 6.94 (s, 1H, NH), 6.65 (d, 2H, J = 8.6 Hz, ArH), 6.21 (s, 1H, CHN), 4.23 (A of AB, 1H, J = 13.2 Hz, one of SCH_2), 4.15 (B of AB, 1H, J = 13.2 Hz, one of SCH_2), 2.87 (s, 6H, N(CH_3)$_2$), 2.16 (s, 3H, =C–CH_3). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 167.8, 156.8, 150.2, 148.3,

138.1, 135.6, 128.7, 128.2, 128.0, 127.0, 112.0, 103.6, 59.5, 40.1, 34.8, 17.3. HRMS (ESI) *m/z*: 421.1805 calcd for $C_{22}H_{25}N_6OS$ $[M+H]^+$; 421.1808 obsd.

2-(4-Nitrobenzylthio)-7-(4-dimethylaminophenyl)-5-methyl-4,7-dihydro[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide (**83**). Compound **83** was prepared from 4-dimethylamino-benzaldehyde (0.22 g, 1.5 mmol), acetoacetamide (0.15 g, 1.5 mmol) and 3-amino-5-[4-nitro(benzylthio)]-1,2,4-triazole (0.38 g, 1.5 mmol) in 20% yield as a light-yellow solid (0.14 g). ^{1}H NMR (DMSO-*d*6, 400 MHz): δ 10.00, (s, 1H, NH), 7.99(d, 2H, *J* = 8.7 Hz, ArH), 7.47 (d, 2H, *J* = 8.7 Hz, ArH), 7.16 (s, 1H, NH), 7.04 (d, 2H, *J* = 8.7 Hz, ArH), 6.93 (s, 1H, NH), 6.64 (d, 2H, *J* = 8.7 Hz, ArH), 6.20 (s, 1H, CHN), 4.34 (A of AB, 1H, *J* = 14.0 Hz, one of SCH_2), 4.23 (B of AB, 1H, *J* = 14.0 Hz, one of SCH_2), 2.89 (s, 6H, $N(CH_3)_2$), 2.16 (s, 3H, $=C-CH_3$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 167.7, 155.9, 150.1, 148.4, 146.7, 146.2, 135.5, 129.9, 128.0, 123.1, 111.8, 103.5, 59.5, 39.9, 33.7, 17.2. HRMS (ESI) *m/z*: 466.1656 calcd for $C_{22}H_{24}N_7O_3S$ $[M+H]^+$; 466.1631 obsd.

2-(4-Cyanobenzylthio)-7-(4-dimethylaminophenyl)-5-methyl-4,7-dihydro-[1,2,4]triazolo[1,5-*a*]pyrimidine-6-carboxamide (**84**). Compound **84** was prepared from 4-dimethylamino-benzaldehyde (0.12 g, 0.80 mmol), acetoacetamide (0.08 g, 0.80 mmol) and 3-amino-5-(4-cyanobenzylthio)-1,2,4-triazole (0.19 g, 0.80 mmol) in 15% yield as a yellow solid (53 mg). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 9.99 (s, 1H, NH), 7.62 (d, 2H, *J* = 8.7 Hz, ArH), 7.43 (d, 2H, *J* = 8.7 Hz, ArH), 7.15 (s, 1H, *J* = 8.7 Hz, NH), 7.03 (d, 2H, *J* = 8.7 Hz, ArH), 6.92 (s, 1H, NH), 6.65 (d, 2H, *J* = 8.7 Hz, ArH), 6.19 (s, 1H, CHN), 4.28 (A of AB, 1H, *J* = 13.8 Hz, one of SCH_2), 4.20 (B of AB, 1H, *J* = 13.8 Hz, one of SCH_2), 2.89 (s, 6H, $N(CH_3)_2$), 2.16 (s, 3H, $=C-CH_3$). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 167.8, 156.1, 150.2, 148.4, 144.5, 135.6, 132.0,

129.8, 128.14, 128.07, 118.8, 112.0, 109.6, 103.6, 59.6, 40.1, 34.2, 17.3. HRMS (ESI) *m/z*: 446.1758 calcd for $C_{23}H_{24}N_7OS$ $[M+H]^+$; 446.1752 obsd.

Bacterial strains. *S. aureus* ATCC 29213 , *K. pneumonia* ATCC 700603, *A. baumanii* ATCC 17961, *P. aeruginosa* ATCC 27853, *E. aerogenes* ATCC 35029, *E. coli* ATCC 25922, *E. faecium* NCTC 7171 (ATCC 19734), *E. faecium* ATCC 700221, and *E. faecium* ATCC BAA 2127 were used for the initial screen and were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Strain *E. faecium* 119-39A was collected from Wayne State University School of Medicine. The strains *E. faecium* C9, C38, and C68 were generously supplied by Dr. Robert A. Bonomo.

Determination of Minimal-inhibitory concentrations (MICs) and Minimal-Bactericidal Concentraions (MBCs). The MIC assays of each compound were conducted in triplicate in 96-well plates with cation-adjusted Mueller Hinton II Broth (MH) according to broth microdilution method described by the Clinical and Laboratory Standards Institute.[33] Bacteria were grown to OD_{600} of approximately 0.60-0.80, then the suspension was diluted 100-fold with fresh MH-medium. The final bacterial innoculum was 5×10^5 CFU/mL. Each compound was prepared from a 5 mg/mL DMSO stock solution. The stock solution was diluted with MH-medium to give various concentrations of antibiotics in a manner that the final concentration of DMSO was kept to 2% (v/v) with a supplement of 0.25% (v/v) glacial acetic acid. After the plates were incubated at 35 °C for 16-20 h, MICs were read. All MIC determinations were performed in triplicate. Determination of MBCs was performed by a reported method.[34] *E. faecium* NCTC 7171 was used for the determination of MBCs.

***In vitro* metabolic stability**. Pooled human liver S9 (1 mg/mL, BD Biosciences, Woburn, MA), NADPH (1 mM), and a given synthetic compound (10 μM) in sodium phosphate buffer

(PBS, 0.20 M sodium phosphate, 0.15 M NaCl, pH 7.4) were incubated at 37 °C for 60 min. Aliquots were taken at each of six time points, and the reaction was terminated by addition of two volumes of acetonitrile containing the internal standard (5 μM of **24**). The mixture was centrifuged (20 min, 20,000 *g*) and the supernatant was analyzed by ultra-performance liquid chromatography (UPLC) with multiple reaction monitoring (MRM). Half-lives of the compounds in human liver S9 were calculated based on the first-order rate constants that were measured. Intrinsic clearance was calculated by the following equation:[35]

CL_{int} = (0.693/in vitro $t_{1/2}$) × (mL incubation /mg microsomal protein) × (45 mg microsomal protein/gram liver) × (20 g liver/kg body weight)

The metabolic stability for each compound was determined in duplicates.

Determination of plasma-protein binding. The plasma-protein binding of compounds **1**, **10**, **13**, **16**, **83**, and **84** was determined in a rapid equilibrium dialysis device (Pierce Biotechnology, ThermoFisher Scientific, Waltham, MA). Human plasma was thawed and centrifuged at 1,200 *g* for 10 min to remove any particulates. The compounds were dissolved in DMSO and incubated in human plasma at a final concentration of 10 μM and 0.1% DMSO. A 200-μL aliquot was added into the sample chamber and 350 μL of 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 mM NaCl was added into the adjacent chamber. The compounds were dialyzed at 37 °C in an orbital shaker overnight. A 150-μL-aliquot from the buffer side was mixed with 300 μL internal standard solution (**24** with final concentration of 1 μM in acetonitrile), and an equivalent sample volume from the plasma side was also mixed with 300 μL internal standard solution. The solutions were vortexed and centrifuged (20 min, 20,000 *g*). A 400-μL aliquot of the supernatant was concentrated to dryness, and then dissolved in 120 μL of acetonitrile,

followed by centrifugation. A 100-μL aliquot of the supernatant was analyzed by UPLC-MRM. The plasma-protein binding (B%) was calculated according to the following equation:

B% = $(C_p-C_f)/C_p \times 100$, where C_p and C_f are the total plasma concentration and the free concentration of the compound.

Macromolecular Synthesis Assays. Macromolecular synthesis assays were performed according to a previously reported method.[36] *E. faecium* NCTC 7171 was grown in brain-heart infusion culture at 37 °C until early-log phase was reached. Radiolabeled precursor (15 μL, 1 μCi/mL of [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine, L-[4,5-^{3}H]-leucine, or D-[2,3-^{3}H]-alanine) was added into each Falcon tube of cell culture, followed by incubation at 37 °C for 5 min. The radiolabeled precursors were purchased from Perkin Elmer (Waltham, MA, USA). Negative control (DMSO) and positive controls (ciprofloxacin, rifampicin, tetracycline, and meropenem), as well as compound **16** (8 μg/mL) were added into each tube and allowed to growth for an additional 2 h. The positive controls were purchased from Sigma Aldrich (Sigma-Aldrich, MO, USA). Samples (650 μL) were collected every 20 min and were mixed with 650 μL of 20% ice-cold trichoroacetic acid (TCA), and allowed to sit on ice for 1 h. The sample (1.3 mL) in each case was transferred to a glass microfiber filter (GF/A grade, whatman), which was washed with 5% TCA (2×) and with 95% ethanol (2×), before letting it dry. The microfiber filter was placed in 20-mL scintillation vials with 5 mL of scintillation cocktail for radioactive measurement. Scintillation vials were counted using a Beckman LS-100 liquid scintillation counter (Beckman Coulter, Inc., Brea, CA, USA). Duplicates aliquots were taken at each time point for measurements.

***In Vitro* Transcription Assay**. *In vitro* transcription and the tandem transcription/translation assays were performed using a previous reported method.[36]

High performance liquid chromatography (HPLC). The HPLC instrument consisted of a Perkin Elmer series 200 autosampler, series 200 UV/VIS detector, series 200 pump, using TotalChrom Navigator software (PerkinElmer, Shelton, CT USA). The HPLC conditions consisted of isocratic elution on a ZORBAX Rx-C8 5 μm, 4.6×250 mm column (Agilent Technologies , Santa Clara, CA, USA) at a flow rate of 1 mL/min with 50% A/50% B or 60% A/40% B for 15 min (A = 0.1% trifluoroacetic acid/water; B = 0.1% trifluoroacetic acid/acetonitrile). Effluent was detected at 254 nm. All synthetic compounds were ≥95% pure.

Mass spectrometry and sample analysis. Mass spectra were acquired on a Waters Acquity UPLC Binary Solvent Manager (Waters Corporation, Milford, Massachusetts, USA), using MASSLYNX V4.1 software. The instrument consists of a Sample Manager, PDA Detector, and Triple Quadrupole Detector. The experiments were performed in the ESI positive mode with MRM. The parameters of capillary voltage, cone voltage, extractor voltage, RF lens voltage were set at 3.6 kV, 30 V, 3 V, 0.3 V, respectively. The source temperature was 150 °C, desolvation temperature was 350 °C. Desolvation gas flow rate (N_2) was 650 L/hour and the cone gas (Xe) flow rate was 10 L/hour. The dwell time was 0.1 s and collision energy was 20 V. Samples were analyzed by UPLC-MRM on a Waters Acquity UltraPerformance Thermo Scientific Acclaim™ RSLC 120 C18 2.2 μm, 120 Å, 2.1×100 mm column at 40 °C. Compounds **1**, **10**, and **13** were eluted at 0.4 mL/min with 80% A/20% B for 2 min, followed by a 8-min linear gradient to 10% A/90% B.(A = 0.1% formic acid/water; B = 0.1% formic acid/acetonitrile). For compounds **16**, **83**, **84** the flow was changed to 0.5 mL/min; the other conditions were same. The transitions monitored for each compound were: 420 → 403 for **1**, 437 → 378 for **10**, 439 → 422 for **13**, 421 → 404 for **16**, 466 → 122 for **83**, 446 → 122 for **84**, and 403 → 91 for the internal standard **24**.

Purification of PBP5fm. The *pbp5* gene corresponding to amino acids 34 to 678 for PBP5 from *E. faecium* D63r was custom synthesized (GenScript) for optimal expression in *E. coli*. This synthetic gene was cloned into plasmid pET-28a(+) with a His-tag in the *N*-terminal position to give the recombinant plasmid pET28-PBP5fm. This construct lacks the signal peptide for delivery to the periplasm, hence the recombinant protein is expresses in the cytoplasm. For the enzyme purification, *E. coli* BL21 (DE3) harboring the pET28-PBP5fm plasmid was grown at 37 °C in Luria-Bertani (LB) supplemented with kanamycin (50 μg/mL) to OD_{600} of 0.6. Protein expression was then induced using 0.4 mM IPTG and the culture incubated at 25 °C for an additional 6 h. The protein was purified using a Macro-Prep High Q anion exchange column (BioRad) equilibrated with 50 mM Tris pH 7.5 and eluted using a gradient from 0-0.5 M NaCl in 50 mM Tris pH 7.5. The fractions containing PBP5fm were loaded onto a HiTrap Chelating column (GE Healthcare) and eluted in a 0.5 M imidazole gradient. After removing the imidazole, protein concentration was determined spectrophotometrically using the predicted extinction coefficient ($\Delta\varepsilon_{280}$ = 57760 M^{-1} cm^{-1}) and purity was assessed by resolution on 12% SDS-PAGE. The enzyme was stored at 4 °C in 50 mM Tris pH 7.5, 0.5 M NaCl.

Binding Assays for PBP2a and PBP5fm. Purification of PBP2a was reported earlier and was followed in the present study.[37] The assay is identical for PBP5fm, so we only explain the procedure for PBP2a here.

BOCILLIN FL, a fluorescent penicillin (Molecular Probes, Inc., CA), is used in this assay as a labeling reagent for the active sites of PBP2a.[38] A 19-μL reaction solution was prepared on ice by adding 1 μL PBP2a (final concentration 2 μM) into 18 μL of the inhibitor (**1** and **83**) in 25 mM Hepes, 1 M NaCl (pH 7.0). The mixture was incubated at 37 °C for 10 min. A 1-μL BOCILLIN FL solution was added into the reaction mixture to give the final concentration of 20

μM, and the mixture was allowed to incubate for an additional 10 min at 37 °C. A total of 15 μL of a SDS sample buffer was added to the reaction mixture, and it was boiled for 5 min. The samples at different antibiotic concentrations were loaded onto 12% SDS-PAGE and the gel was developed at 125 V for 120 min. The gels were scanned using Storm840 Fluorimager and the bands were quantified with IMAGEQUANT 5.2 software. A control sample without inhibitor was also prepared at the same time.

The assay for PBP5fm is similar to that for PBP2a. The differences were the final concentration of PBP5fm (40 μM) and BOCILLIN FL (30 μM), and the incubation time was extended from 10 min to 20 min.

Supporting Information.

Additional experimental information and ^{1}H and ^{13}C NMR spectra of all the other compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DMSO, dimethyl sulfoxide; ESI, electrospray ionization; MRM, multiple-reaction monitoring; MS, mass spectrometry; UPLC, ultraperformance liquid chromatography.

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