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SPECIAL ISSUE

2,6-Difluorobenzamide Inhibitors of Bacterial Cell Division Protein FtsZ: Design, Synthesis, and Structure–Activity Relationships

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Dedicated to professor Luigi Villa, 1929–2007

A wide variety of drug-resistant microorganisms are continuously emerging, restricting the therapeutic options for common bacterial infections. Antimicrobial agents that were originally potent are now no longer helpful, due to their weak or null activity toward these antibiotic-resistant bacteria. In addition, none of the recently approved antibiotics affect innovative targets, resulting in a need for novel drugs with innovative antibacterial mechanisms of action. The essential cell division protein filamentous temperature-sensitive Z (FtsZ) has emerged as a possible target, thanks to its ubiquitous expression and its homology to eukaryotic β-tubulin. In the latest years, several compounds were shown to interact with this prokaryotic protein and selectively inhibit bacterial cell division. Recently, our research group developed interesting derivatives displaying good antibacterial activities against methicillin-resistant *Staphylococcus aureus*, as well as vancomycin-resistant *Enterococcus faecalis* and *Mycobacterium tuberculosis*. The aim of the present study was to summarize the structure–activity relationships of differently substituted heterocycles, linked by a methylenoxy bridge to the 2,6-difluorobenzamide, and to validate FtsZ as the real target of this class of antimicrobials.

Introduction

Antimicrobial resistance is one of the major health plagues. It began to emerge more than 70 years ago but has risen faster in recent years, prompting to the urgent need for innovative and efficient antibiotics. The search for antimicrobials is an ongoing problem. It started in the "Golden Age": the period of time from the 1940s until mid-1960s that led to the discovery of nearly all antibiotics in use today using the Waksman platform.[1] After the initial abundance of active molecules, scaffold redundancy, together with unrestrained expression and appearance of resistance, caused researchers to abandon this magic platform.

Pharmaceutical research then moved into the "Medicinal Chemistry Age." This second era, from the mid-1960s to 2000, was characterized by semisynthetic modifications of natural products, employing high-throughput screening (HTS) and rational drug design studies. Despite the initial expectations, no novel broad-spectrum derivatives were developed, and only daptomycin was discovered in 1986.[2]

This 40-year second era waned with the arrival of the current "Resistance Age" (2000-present), in which a wide variety of multidrug-resistant (MDR) superbugs are continuously appearing. Among these, bacterial strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci*

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(VRE), and penicillin-resistant *Streptococcus pneumoniae* have emerged, causing serious difficulties with therapy for common bacterial infections.

A second limitation is that the totality of clinically employed antibiotics acts only on old and known targets. If we consider all molecules that received approval for clinical use for the first time in any country in 2015,[3] only three were antibacterial agents: two quinolones and a β-lactamase inhibitor. Therefore, the emergency does not only refer to the development of novel antibacterial drugs, but also to the need for these compounds to possess innovative mechanisms of action.

During the last decade, the process of bacterial cell division has become an interesting and promising target.[4] This is due to multiple reasons, including that a good number of the components of the divisome are crucial for the replication and viability of bacteria. Furthermore, the most important division proteins are widely conserved among bacterial pathogens and are almost absent in eukaryotic cells.

In this context, the essential cell division protein filamentous temperature-sensitive Z (FtsZ) turned out to be an attractive target because it is the most conserved protein in bacteria and archea. In the bacterial cell division process, FtsZ undergoes polymerization in a GTP-dependent manner, forming a Z-ring at the midpoint of the cell. It recruits other cell division proteins, which enable cell constriction and formation of the mesosome and two daughter cells.[5] In addition, FtsZ is structurally and functionally homologous to mammalian β-tubulin,[6] suggesting a positive outcome in the discovery of antibacterial agents, as was the case for tubulin inhibitors in cancer therapy.

Until now, no drugs on the market have targeted FtsZ; nevertheless, in the last 10 years, several research groups studied and developed FtsZ inhibitors, demonstrating that inhibition of this protein results in a bactericidal effect. The initially discovered FtsZ inhibitors came from natural products,[7,8] which were further modified to increase their bactericidal activity; the best results were obtained with curcumin,[9] coumarins,[10] resveratrol,[11] berberine,[12,13] and cinnamaldehyde[14,15] derivatives.

On the other hand, significant results were achieved with synthetic small molecules, particularly with benzamide derivatives[16–21] After an assessment found that 3-methoxybenzamide (3-MBA, Figure 1) was able to stabilize the polymerization and disturb the GTPase activity of FtsZ,[22] albeit with poor potency (minimum inhibitory concentration [MIC] versus *S. aureus* of 4000 μg mL^{-1}), a large number of 3-MBA derivatives were designed.

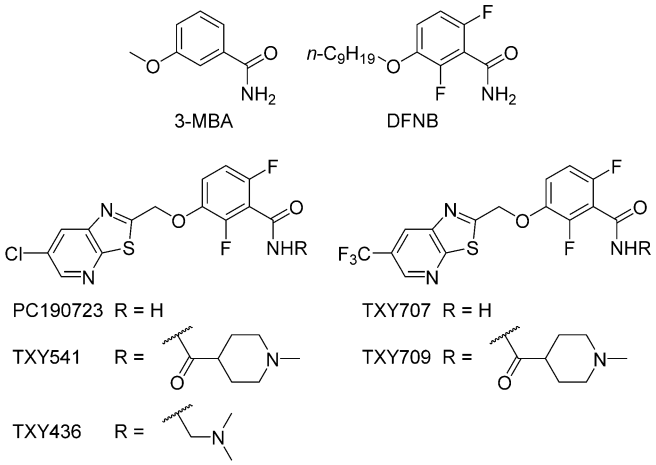


Figure 1. Structures of published reference compounds.

Among these, DFNB and PC190723 (Figure 1) were shown to potently inhibit FtsZ, having MIC values versus *S. aureus* of 2 and 1 μg mL^{-1}, respectively. PC190723 was further studied; in particular, analysis of the crystal structure of the *S. aureus* FtsZ–PC190723 complex determined that the binding site of PC190723 was structured as a groove that was only present if FtsZ was complexed. Consequently, it was observed that PC190723 increased the initial rate of GTP hydrolysis and caused FtsZ polymer stabilization in straight protofilaments.[17,23,24]

Nevertheless, PC190723 had suboptimal drug-like properties; its hydrophobic nature and metabolic instability limited formulation and clinical development, prompting further structural modifications. Several prodrugs of PC190723 were designed,[25] two of which (TXY436 and TXY541, Figure 1) were studied extensively, thanks to their enhanced formulation capability, but unfortunately, their development was limited by their poor pharmacokinetic properties.[26–28]

Consequently, a new prodrug (TXA709, Figure 1) of a different FtsZ-targeting benzamide compound (TXA707, Figure 1) arose in recent years and proved to have metabolic stability, good pharmacokinetic properties, and high in vivo potency versus MRSA.[29,30] In addition, combination of TXA709 with cefdinir was demonstrated to be effective and synergistic.[31]

In this context, in our attempt to design and develop novel antibacterial agents, we created several derivatives in recent years, replacing the thiazolopyridine of PC190723 with differently substituted 1,4-benzodioxane,[21,32] a heterocyclic scaffold extensively studied by our research group.[33–35] Among these, the most promising derivatives were **I**, **II**, and **III**, reported in Figure 2. In particular, we found that the unsubstituted benzodioxane-difluorobenzamide **I** had a potency similar to PC190723 and DFNB (MIC = 5 μg mL^{-1}); on the contrary, **II** and **III** were revealed to be more potent than **I** (MIC < 1 μg mL^{-1}).

Our efforts continued toward the development of antibacterial agents and elucidation of their structure–activity relationships (SAR); to that end, we designed a number of analogues of **I** and **III** through a series of isosteric, positional, or substituent modifications (Figure 3).

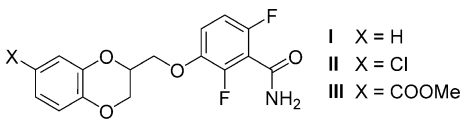


Figure 2. Structures of our reference compounds.

Herein we report the synthesis of difluorobenzamides **1**–**16** (Figure 4), their SAR, their antibacterial activity toward Gram-positive and Gram-negative strains, and their cytotoxicity

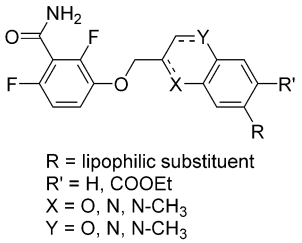


Figure 3. General FtsZ inhibitor structure.

toward human MRC-5 cells. Furthermore, to confirm the target, we performed two different biochemical assays aimed at studying GTPase and polymerization activities of *S. aureus* FtsZ following incubation with our compounds.

Results and Discussion

Chemistry

Compounds **1**–**16** were designed starting from the chemical structures of **I** and **III**; specifically, **1**–**12** resulted from modification of the 7-methoxycarbonyl functionality of **III**. In contrast, **13**–**16** derived from an isosteric substitution of the 1,4-benzodioxane ring of compound **I**, as a continuation of a study on the importance of the two oxygens of the 1,4-benzodioxane scaffold.

The syntheses of compounds **1**–**4** are shown in Scheme 1. The ethyl and isopropyl esters (**1** and **3**, respectively) were prepared in a similar way, starting from 3,4-dihydroxybenzoic acid. The acid was suitably esterified, affording derivatives **17** and **25**, and both underwent reaction with epibromohydrin, yielding 7-substituted intermediates **18** and **26**. Traces of the undesired 6-substituted derivatives were removed by purification using flash chromatography. Subsequent conversion into the corresponding mesylated derivatives (**19** and **27**) was accomplished prior to O-alkylation of 2,6-difluoro-3-hydroxy-benzamide, yielding **1** and **3**. Compound **2** was prepared from **17** following a six-step strategy that resulted in only the 6-substituted derivative. The first step, different from what was observed by Li and co-workers,[36] was selective *p*-benzylation, resulting in **20**. Condensation with epibromohydrin (resulting in **21**), hydrogenolysis (resulting in **22**), and cyclization yielded 6-ethoxycarbonyl-2-hydroxymethyl-1,4-benzodioxane (**23**). Mesylation (resulting in **24**) and O-alkylation of 2,6-difluoro-3-hydroxybenzamide afforded **2**. The 7-phenoxycarbonyl-substituted benzodioxane **4** was synthesized from 2-benzyloxymethyl-1,4-benzodioxan-7-carboxylic acid, detailed preparation of which was reported in our previous paper.[32] Subsequent esterification with phenol, using EDAC·HCl and HOBT, afforded **28**, which underwent hydrogenolysis (resulting in **29**), mesylation (resulting in **30**), and condensation with 2,6-difluoro-3-hydroxybenzamide, yielding derivative **4**.

The syntheses of 7-substituted benzodioxane derivatives **5**–**8** are shown in Scheme 2. The 7-methoxymethyl-substituted benzodioxane **5** was synthesized from 2-benzyloxymethyl-1,4-benzodioxan-7-carboxylic acid in five steps: 1) reduction with $LiAlH_4$ (yielding **31**), 2) O-methylation of the hydroxy group (yielding **32**), 3) debenzylation by hydrogenolysis (yielding **33**), 4) mesylation (yielding **34**), and 5) O-alkylation of 2,6-difluoro-3-hydroxybenzamide with mesylate **34**.

The 7-propionyl analogue **6** was also prepared from 2-benzyloxymethyl-1,4-benzodioxan-7-carboxylic acid, first converting the carboxylic functionality into a Weinreb amide (**35**) and then into ethyl ketone **36** through treatment with ethyl magnesium bromide. Debenzylation under acidic conditions (resulting in **37**) and mesylation yielded **38**, which was condensed with the 2,6-difluoro-3-hydroxybenzamide.

Preparation of 7-formyl derivative **7** started from methyl 2-hydroxymethyl-1,4-benzodioxan-7-carboxylate (see our previously published preparation method),[32] protecting the hydroxy functionality with *tert*-butyldimethylsilyl chloride (**39**). Reduction of the ester (resulting in **40**) and oxidation yielded aldehyde **41**, which underwent deprotection of the hydroxy functionality to yield **42** and condensation with 2,6-difluoro-3-hydroxybenzamide to yield **7** by Mitsunobu reaction. Ketal **8**

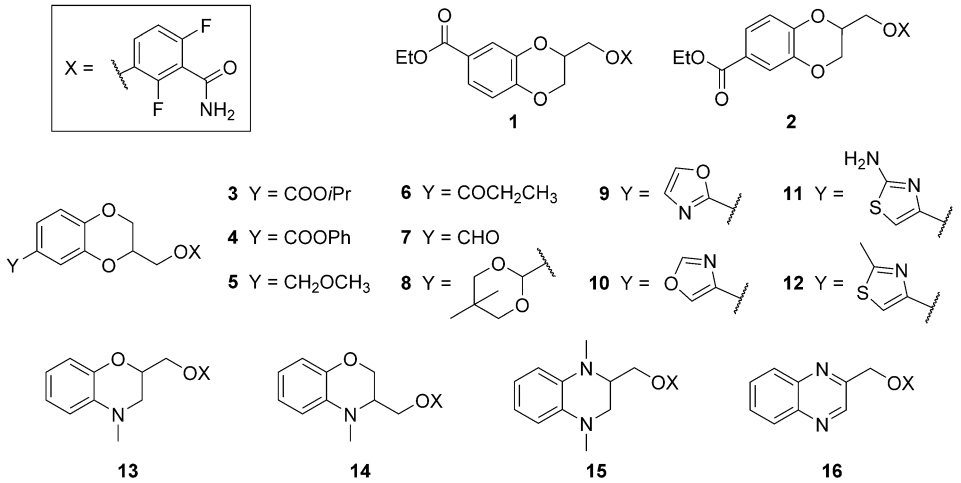
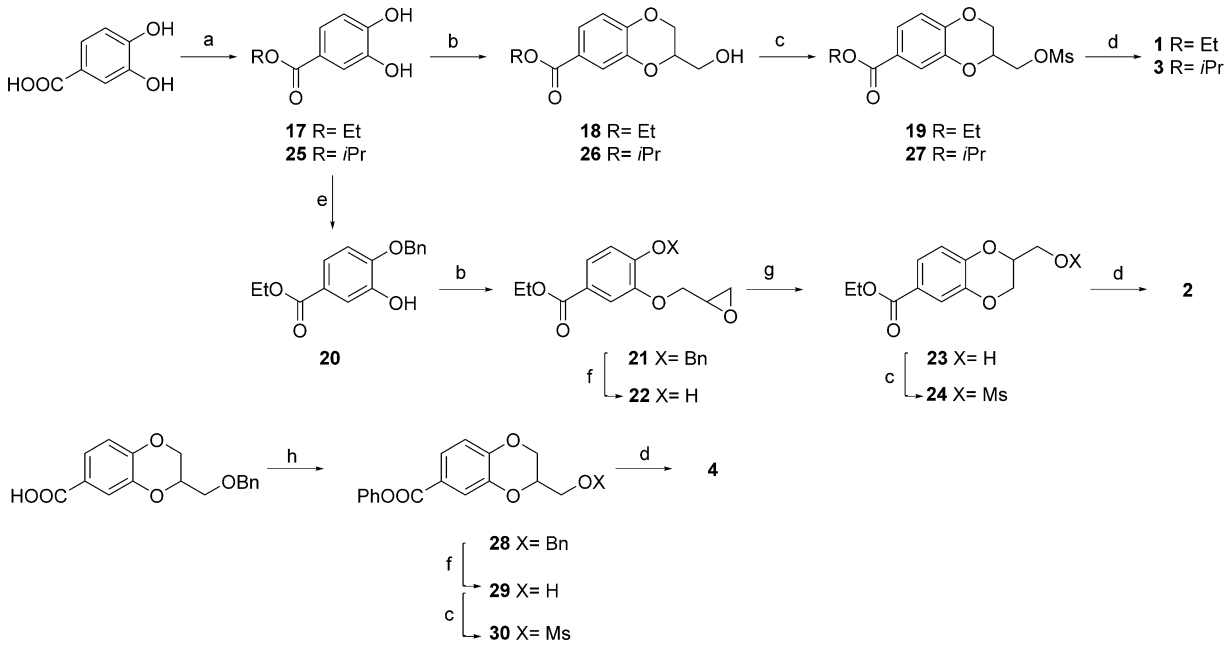


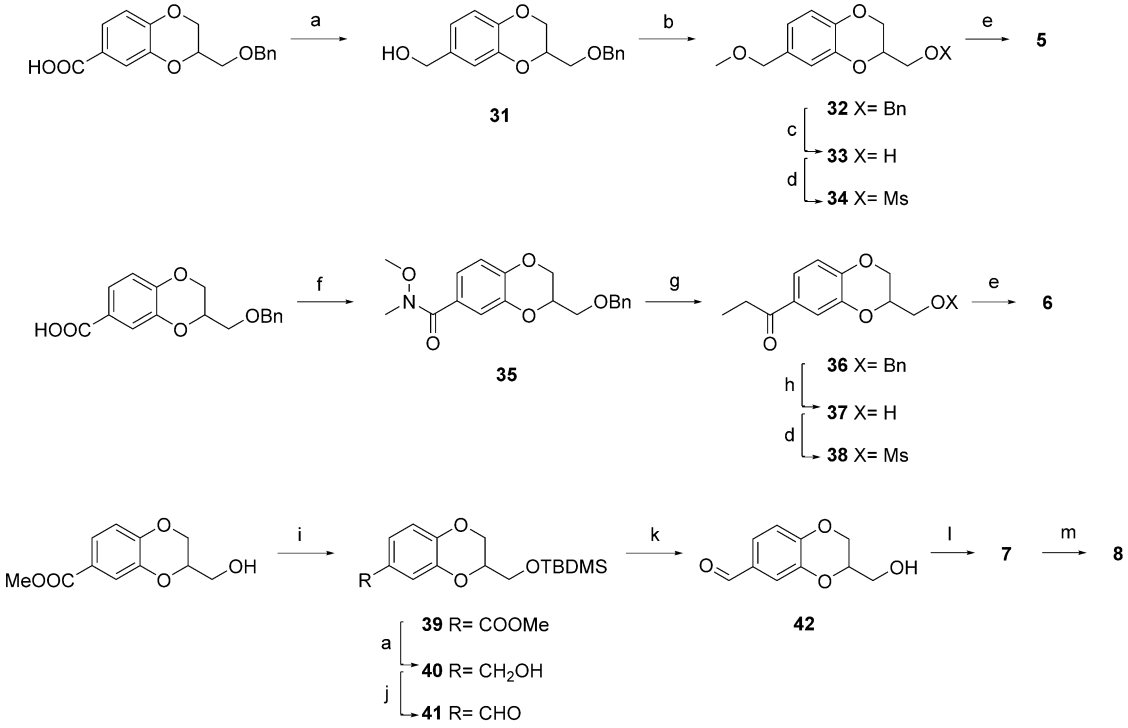
Figure 4. Structures of compounds **1**–**16**.

was prepared by reacting **7** with 2,2-dimethyl-1-propanol in the presence of zinc chloride.

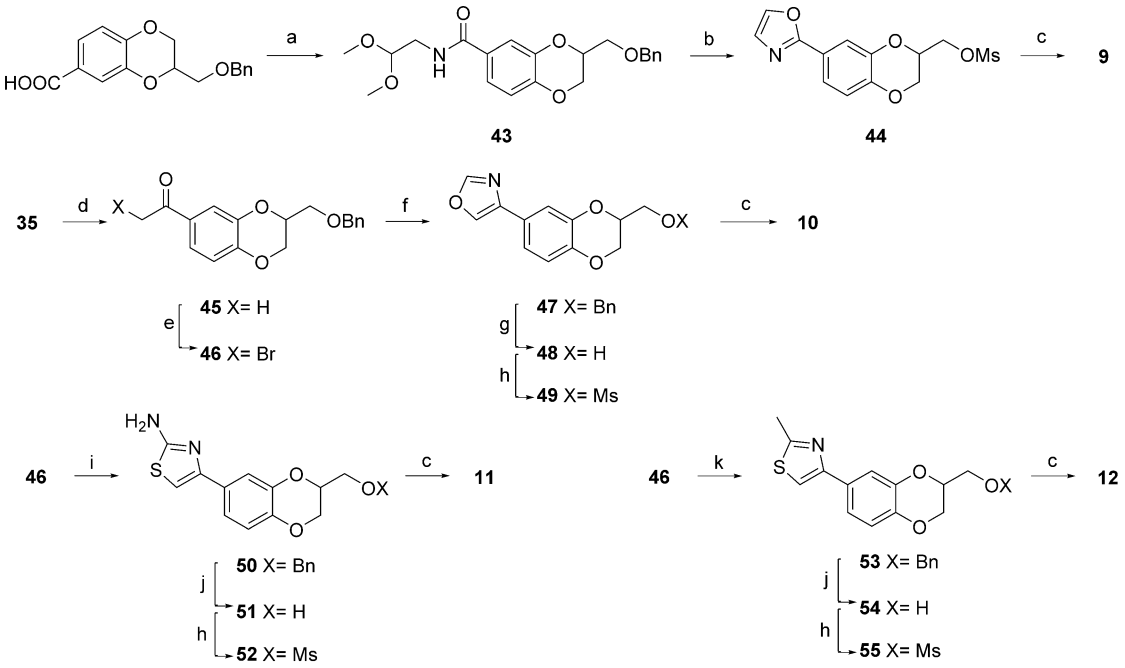
The syntheses of compounds **9**–**12** are shown in Scheme 3. The two 1,3-oxazole derivatives **9** and **10** were prepared following different methods: the synthesis of compound **9** started from 2-benzyloxymethyl-1,4-benzodioxan-7-carboxylic acid, with initial conversion into amide **43** following reaction with aminoacetaldehyde dimethylacetal. Treatment with Eaton's re-



Scheme 1. Synthesis of compounds **1**–**4**: a) H_2SO_4, 60–80 °C, 18 h, EtOH or IPA; b) epibromohydrin, K_2CO_3, 40 °C, 2–18 h, DMF; c) MsCl, TEA, RT, 1–3 h, CH_2Cl_2; d) 2,6-difluoro-3-hydroxybenzamide, K_2CO_3, 80 °C, 16 h, DMF; e) K_2CO_3, BnBr, 60 °C, 18 h, acetone; f) H_2, 5% Pd/C, RT, 2–3 h, EtOAc or MeOH; g) K_2CO_3, 50 °C, 3 h, DMF; h) phenol, DMAP, EDAC·HCl, HOBt, RT, 4 h, DMF.

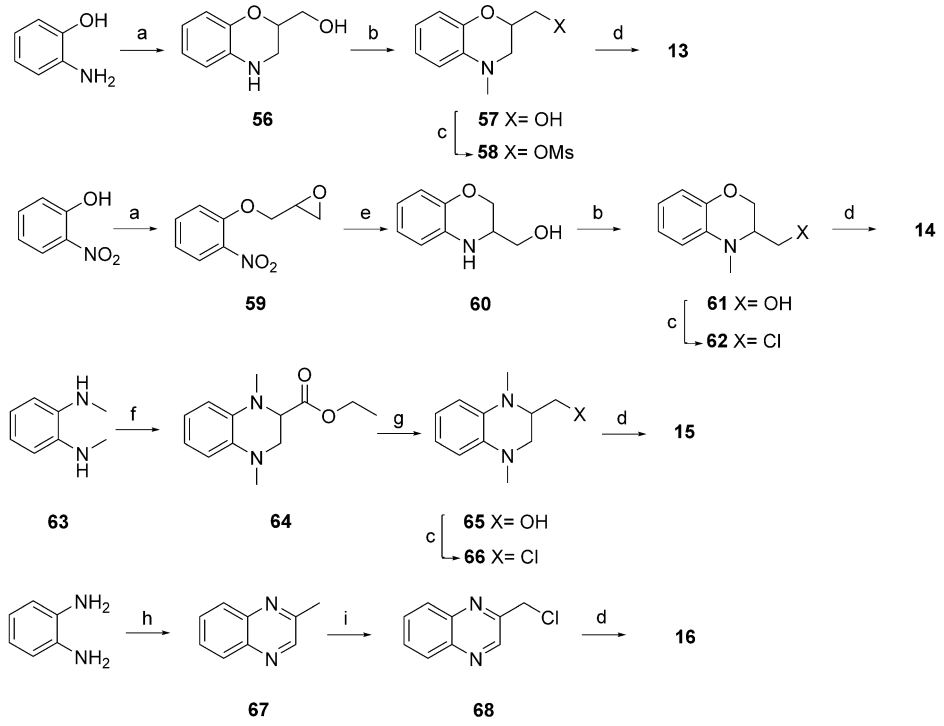


Scheme 2. Synthesis of compounds **5**–**8**: a) $LiAlH_4$, RT or 50 °C, 3 h, THF; b) MeI, NaH, RT, 4 h, DMF; c) H_2, 5% Pd/C, RT, 4 h, MeOH; d) MsCl, TEA, RT, 4–6 h, CH_2Cl_2; e) 2,6-difluoro-3-hydroxybenzamide, K_2CO_3, 100 °C, 2–16 h, DMF; f) CDI, *N*,*O*-dimethylhydroxylamine·HCl, RT, 18 h, CH_2Cl_2; g) EtMgBr, RT, 18 h, THF; h) 6 N HCl, 100 °C, 48 h, dioxanes; i) TBDMSCl, DIPEA, RT, 16 h, CH_2Cl_2; j) MnO_2, 60 °C, 2 h, $CHCl_3$; k) 10% aq. HCl, RT, 20 min, MeOH; l) 2,6-difluoro-3-hydroxybenzamide, triphenylphosphine, DIAD, RT, 18 h, THF; m) $ZnCl_2$, 2,2-dimethyl-1-propanol, 110 °C, 2 h, toluene.



Scheme 3. Synthesis of compounds **9**–**12**: a) Ethyl chloroformate, *N*-methylmorpholine, aminoacetaldehyde dimethylacetal, RT, 1 h, THF; b) Eaton's reagent (7.7% P_2O_5 in methanesulfonic acid), 110 °C, 5 h; c) K_2CO_3, 2,6-difluoro-3-hydroxybenzamide, 80–100 °C, 4–16 h, DMF; d) MeMgBr, RT, 18 h, THF; e) Br_2, 10 °C, 1 h, $CHCl_3$; f) formic amide, 130 °C, 2 h; g) H_2, 5% Pd/C, RT, 1 h, EtOH; h) MsCl, TEA, RT, 1–2 h, CH_2Cl_2; i) thiourea, 78 °C, 2 h, EtOH; j) BBr_3, −5 °C, 30 min, CH_2Cl_2; k) thioacetamide, 78 °C, 1 h, EtOH.

agent, freshly prepared, led to simultaneous formation of the 1,3-oxazol and mesylation, affording **44**. Reaction with 2,6-difluoro-3-hydroxybenzamide yielded the 7-(1,3-oxazol-2-yl) derivative **9**. On the other hand, preparation of **10** started from Weinreb amide **35**, which was first converted into methyl ketone **45** and then into α-bromo ketone **46**. Treatment with formic amide (yielding **47**), hydrogenolysis (yielding **48**), and mesylation afforded derivative **49**, which was reacted with 2,



Scheme 4. Synthesis of compounds **13**–**16**: a) Epichlorohydrin, RT, 2–18 h, aq. NaOH; b) K_2CO_3, MeI, 50 °C, 2–18 h, DMF; c) MsCl, TEA, RT, 1–3 h, CH_2Cl_2; d) K_2CO_3, 2,6-difluoro-3-hydroxybenzamide, 80–100 °C, 3–18 h, DMF; e) Fe, RT, 20 min, EtOH, acetic acid; f) ethyl 2,3-dibromopropionate, TEA, 80 °C, 18 h, toluene; g) $LiAlH_4$, RT, 3 h, THF; h) 1,3-hydroxypropan-2-one, $Pd(OAc)_2$, TEA, RT, 3 h, toluene, THF; i) trichloroisocyanuric acid, 60 °C, 1 h, $CHCl_3$.

6-difluoro-3-hydroxybenzamide to yield **10**. Thiazole derivatives **11** and **12** were prepared in a similar way, starting from 2-benzyloxymethyl-7-(2-bromoacetyl)-1,4-benzodioxane **46**, through treatment with thiourea (yielding **50**) or thioacetamide (yielding **53**), removal of the benzylic protection using BBr_3 (yielding **51** or **54**, respectively), mesylation (yielding **52** or **55**, respectively), and reaction with 2,6-difluoro-3-hydroxybenzamide to yield **11** and **12**, respectively.

The syntheses of compounds **13**–**16** are shown in Scheme 4. Starting from the 2-amino phenol, the benzomorpholine skeleton was constructed by treatment with epichlorohydrin[37] (yielding **56**); subsequent N-methylation (yielding **57**), mesylation (yielding **58**), and reaction with 2,6-difluoro-3-hydroxybenzamide afforded the 2-substituted benzoxazine **13**.

The 3-substituted benzoxazine **14**, in contrast, was obtained starting from the reaction of 2-nitrophenol with epichlorohydrin, affording 2-nitrophenoxymethyloxirane **59**. The following tandem reduction-oxirane-opening reaction[38] yielded **60**, which was converted into chloro derivative **61**, which was reacted with 2, 6-difluoro-3-hydroxybenzamide to yield **14**.

Tetrahydroquinoxaline **15** was prepared starting from *N*,*N*′-dimethylphenylendiamine **63**, as reported in literature,[39,40] through reaction with 2,3-dibromopropionate, leading to the formation of the tetrahydroquinoxaline ring (**64**). Reduction with $LiAlH_4$ (yielding **65**), conversion into the chloro derivative (yielding **66**), and condensation with 2,6-difluoro-3-hydroxybenzamide yielded **15**.

The 2-substituted quinoxaline **16** was accomplished from 1,2-diamino benzene and 1,3-hydroxypropan-2-one through a one-pot process using palladium acetate-catalyzed aerobic oxidation, followed by in situ trapping, as reported previously,[41] to yield **67**. Consequent conversion into the corresponding chloro derivative **68**, using trichloroisocyanuric acid,[42] and reaction with 2,6-difluoro-3-hydroxybenzamide afforded **16**.

Biochemical assays

Before determining the inhibitory activity of compounds **1**–**16** on Gram-positive and Gram-negative bacteria, our first aim was to investigate if FtsZ was the real target of this series of derivatives, which were shown to lead to bacterial death. Among all of the benzodioxane molecules previously synthesized, we chose compounds **II** and **III** (Figure 2) as candidates for this further evaluation. Validation of the target followed a procedure reported by Andreu,[23] similar in part to a recent procedure by Groundwater and co-workers.[43] Specifically, two different assays were performed to evaluate if our derivatives could induce significant changes in the biological activity of *S. aureus* FtsZ: a GTPase activity assay and a polymerization activity assay. Our goal was to assess whether **II** and **III** modified the GTPase activity of FtsZ and if they were able to stabilize FtsZ polymers, as PC190723 does, suppressing polymer dynamics and thus blocking bacterial cell division.

Polymerization assay

As *S. aureus* FtsZ can polymerize into single-stranded filaments in vitro in the presence of Mg^{2+} and GTP, we induced its assembly, as explained in the Experimental Section (4.5). Addition of increasing concentrations of **II** and **III** (5, 10, and 20 μg mL^{-1}) resulted in enhancement of the percentage of *S. aureus* FtsZ polymerized fraction (Figure 5 A).

These filaments sedimented in 20 min at 20000×*g*, allowing their quantification (Figure 5 B). We hence proved that, even at 5 μg mL^{-1}, our compounds were able to induce around 80% of *S. aureus* FtsZ polymerization; the percentage reached a maximum plateau from 10 to 20 μg mL^{-1} of **II** or **III**.

GTPase assay

The effect of **II** and **III** on GTPase activity of *S. aureus* FtsZ was determined by incubating each compound with *S. aureus* FtsZ

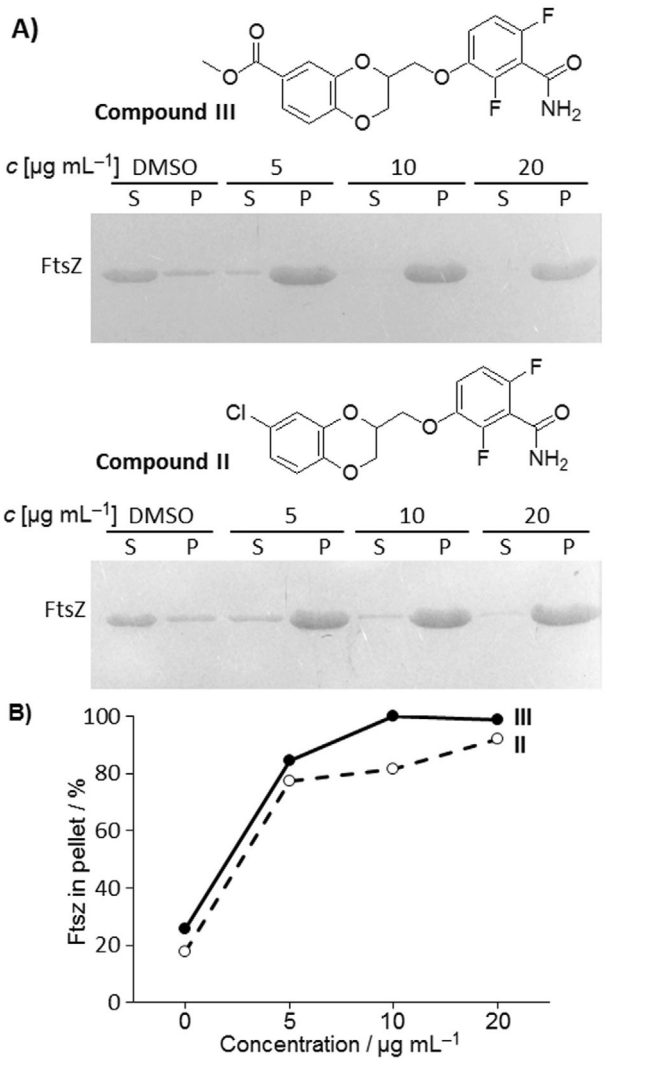


Figure 5. Compounds **II** and **III** induce FtsZ polymerization: SDS-PAGE gels of A) supernatant (S) and pellet (P) and B) quantification of the polymerization assay performed, incubating 1 mg mL^{-1} of *S. aureus* FtsZ protein with different concentrations of the two compounds as indicated.

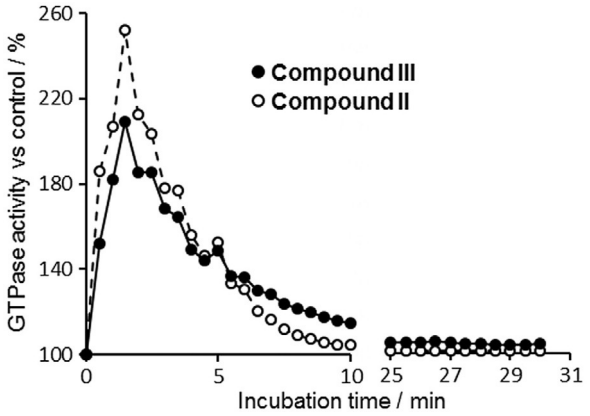


Figure 6. Compounds **II** and **III** enhance FtsZ GTPase rate of activity. GTPase activity measured using a MESG-based assay and incubating 1 mg mL^{-1} of *S. aureus* FtsZ protein with 5 μg mL^{-1} of the two compounds as indicated. Results are presented as rescaled values, setting the GTPase activity values of FtsZ protein incubated with DMSO (control) as 100%.

in a specific buffer and by kinetically measuring inorganic phosphate release as soon as GTP was added to the mixture. The results are shown in Figure 6 as the trend over time in FtsZ GTPase activity, with or without compounds **II** and **III**. Collected data evidenced that initial GTPase activity of FtsZ was extremely enhanced by the presence of compounds **II** or **III** at 20 μg mL^{-1}. This initially rapid GTPase activity should simply be the consequence, as already seen for PC190723 and as previously explained in the polymerization assay, of the large increase in FtsZ GTPase due to polymerization itself. Nevertheless, this initial enhancement rapidly decreased, reached the minimum level after 10 min, and maintained that value until the end of the experiment.

Antibacterial activity and cytotoxicity

Compounds **1**–**16** were tested on both Gram-positive and Gram-negative bacteria, in order to cover a broad spectrum of putative targets (Table 1). The chosen microorganisms, as a continuation of our previous studies, were an extended spectrum of ß-lactamase-positive (ESBL) *E. coli*, a methicillin-sensitive *S. aureus* (MSSA, ATCC 29213) and a methicillin-resistant *S. aureus* (MRSA, ATCC 43300). The inhibitory ability was determined through calculation of the minimal inhibitory concentration (MIC), that is, the lowest compound dose (μg mL^{-1}) at which growth is inhibited, and the minimal bactericidal concentration (MBC), that is, the minimal dose (μg mL^{-1}) at which cell growth is irreversibly blocked after compound removal. The compounds having promising MIC values in MRSA were also assessed for their cytotoxicity in human MRC-5 cells. TD_{90} values were determined as the concentration (μg mL^{-1}) able to decrease the viability of these cells by 90%; furthermore, we reported the therapeutic index (TI), expressed as the ratio between TD_{90} and MBC values. When tested on ESBL *E. coli*, none of the compounds inhibited cell growth at the highest dose (100 μg mL^{-1}); therefore, their MIC values are not listed in Table 1. On the other hand, interesting inhibition potency was reached by several derivatives when tested against MSSA and MRSA.

The present study was conceived as a dual SAR study: the first part of the work was focused on the modification of the carboxymethyl functionality of derivative **III** (derivatives **1**–**12**), whereas in the second section, the benzodioxane substructure of **I** was isosterically modified, as a continuation of our previous project[32] (derivatives **13**–**16**).

Initially, we evaluated if the benzodioxane ring of our FtsZ inhibitors targeted the protein in the same hydrophobic cleft as observed for PC190723.[17] Therefore, we transformed the

Table 1. Inhibitory activity of compounds DNFB, **I**–**III**, and **1**–**16** against MSSA, MRSA, and MRC-5.

Compd	MSSA ATCC 29213			MRSA ATCC 43300			MRC-5
	MIC [μg mL^{-1}][a]	MBC [μg mL^{-1}][a]	TI	MIC [μg mL^{-1}][a]	MBC [μg mL^{-1}][a]	TI	TD90 [μg mL^{-1}]
DNFB	1.0	1.0	>200	1.0	1.0	/	>200
I	5.0	80.0	n.d.	3.1	6.3	/	n.d.
II	0.5	0.5	>1600	0.4	0.8	/	>800
III	0.6	0.6	>1280	n.d.	n.d.	/	>800
1	0.6	1.3	>640	0.6	1.3	>640	>800
2	5.0	5.0	>160	2.5	5.0	160	>800
3	5.0	20.0	40	5.0	10.0	>80	>800
4	5.0	20.0	>40	5.0	10.0	>80	>800
5	5.0	10.0	40	5.0	10.0	40	400±8.78
6	2.5	5.0	>160	2.5	5.0	>160	>800
7	5.0	10.0	40	5.0	10.0	40	400±6.21
8	100.0	100.0	/	100.0	100.0	/	/
9	>100	>100	/	>100	>100	/	/
10	10.0	>10	/	10.0	20.0	/	/
11	>100	>100	/	>100	>100	/	/
12	10.0	>10	/	10.0	20.0	/	/
13	100.0	100.0	/	10.0	20.0	/	/
14	100.0	100.0	/	100.0	100.0	/	/
15	>100	>100	/	>100	>100	/	/
16	100.0	100.0	/	100.0	100.0	/	/

[a] Values are confirmed by three separate experiments, carried out in duplicate.

carboxymethyl functionality of **III**, firstly by enhancing the steric hindrance (compounds **1**–**3**), and secondly by converting it into a phenyl ester, where the introduction of a benzene ring could favor further lipophilic interactions (**4**).

Derivatives **1**–**4** were designed and prepared, taking into consideration our previous results, in which the introduction of hindered halogens (Br or I) on the benzodioxane scaffold was well-tolerated and resulted in potent bactericidal molecules.[32] In **1**, the methyl ester was elongated to an ethyl ester, and the resulting MIC value and cytotoxicity, were similar to that of **III** (see Table 1). In addition, we wanted to confirm whether 7-benzodioxane substitution was preferable to 6-substitution by preparing ethyl ester **2**. The results showed that the 7-substituted derivative **1** was more potent than the 6-substituted derivative **2**; therefore, all subsequent investigations used only 7-substituted derivatives. The loss in inhibitory and bactericidal activities of isopropyl derivative **3** and phenyl compound **4** suggested that the interaction between these compounds and FtsZ suffered from having substituents that were too hindered.

A second approach intended to investigate the importance of the two oxygen atoms of the methyl ester of **III** versus the interaction with the prokaryotic protein; we thus generated derivatives **5**, **6**, **7**, and **8**, in which the two oxygen atoms were partially substituted, removed, or had their distance from the aromatic ring changed. For **5** and **6**, we replaced one of the two oxygen atoms with isosteric groups; specifically, in methoxymethyl derivative **5**, the carbonyl was substituted by a methylene moiety, while in propionyl derivative **6**, the ester was converted into a ketone, replacing the oxygen on the chain with a methylene. The MIC and MBC values of **5** and **6** suggested the importance of keeping both oxygen atoms in order to retain potent antimicrobial activity. In addition, the higher inhibitory activity of **6** seemed to indicate that the carbonyl oxygen, and in particular the presence of the relative dipole moment, has a predominant effect on the interaction with the target. As a result, we designed derivative **7**, dismissing the alkyl chain and resulting in the unique aldehyde functionality.

Similar MIC and MBC values (see Table 1) confirmed that the maintenance of both oxygen atoms was mandatory. We thus designed **8**, in which both oxygens were retained without the polarity of the carbonyl group. Considering the substantial differences in antimicrobial activity between **5**–**7** and **8**, we hypothesized that the hindrance of the 5,5′-dimethyl-1,3-dioxane, as well as the absence of a dipole and the different benzylic carbon hybridization, highly affected the interaction with FtsZ.

In the third series, we introduced several small heterocycles, as suggested in a previous study[32] in which good results were achieved with substituents having modest to high lipophilicity. In particular, we converted the methyl ester into a cyclic imino ether, yielding oxazole derivatives **9** and **10**, linked by C(2) or C(4) of the heterocycles to the benzodioxane ring. Surprisingly, compound **9**, in which the carbonyl oxygen was replaced with a nitrogen atom, completely lost its activity against MRSA; on the contrary, compound **10** retained good inhibitory activity. To better understand these completely different behaviors, further investigations are required.

Starting from the promising derivative **10**, we introduce a 2-substituted thiazole in place of the oxazole, thus designing **11** and **12**. As expected, the MIC values of these two derivatives confirmed the possible isosteric replacement of the oxazole with a thiazole, together with the requirement of a lipophilic substituent. The last series of results referred to compound **13**–**16**, in which the benzodioxane substructure was isosterically modified. After replacing the oxygen atoms with methylene, we altered the benzodioxane scaffold, replacing one or both oxygen atoms with nitrogen atoms (**13**–**15**).

The clear demonstration of a preference for hydrogen bond acceptor (HBA) heteroatoms, confirmed by the dramatic loss of activity in the presence of hydrophilic and hydrogen bond donor (HBD) substituents, suggested methylation of the nitrogen atom. The inhibitory activity of compounds **13** (MIC = 10 $\mu g\,mL^{-1}$ against MRSA) and **14** (MIC = 100 $\mu g\,mL^{-1}$ against MRSA) once again emphasized the importance of benzodioxane O(1) for target interaction.[32] In addition, we hypothesized that the loss in activity of **14** could be due to N-methyl steric hindrance or to the basic properties of nitrogen, which limit FtsZ interaction. Our hypothesis was further confirmed by the complete inactivity of compound **15**. Lastly, to understand which of the two above-mentioned features played the primary role, we designed compound **16**, possessing basic properties and no hindered substituents. The modest but existing antimicrobial activity confirmed the need to avoid steric hindrance.

Conclusions

In this work, using two different biochemical assays, we assessed that the target of this class of derivatives is the prokaryotic cell division protein FtsZ. Such a statement is consistent with previous morphometric analyses[32] (Figure 7), revealing

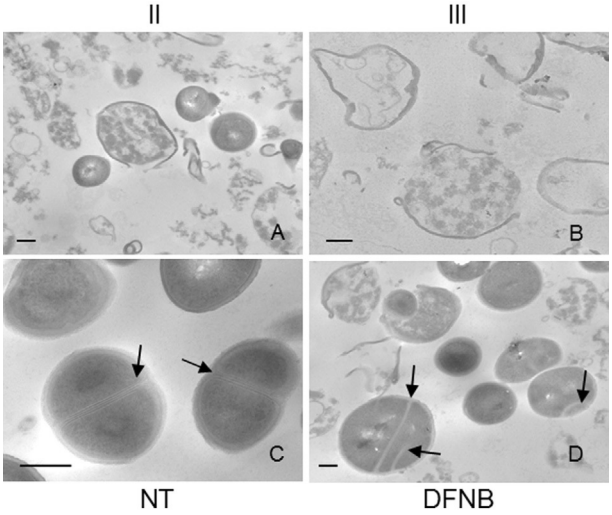


Figure 7. Morphological analysis of MSSA cultured in the presence of **II**, **III**, no compound (NT) as a negative control, or DFNB as a positive control. Cells were fixed and processed after overnight growth, and alterations were examined at the ultrastructural level by transmission electron microscopy. Arrows indicate septum formation. Scale bar: 0.5 μm.

that MSSA, treated with **II** and **III**, showed typical alterations in cell division inhibition. In addition, a series of 3-(benzodioxan-2-yl)-2,6-difluorobenzamides, substituted at the 1,4-benzodioxane or modified at the benzodioxane scaffold, were developed and tested for activity against a strain of MSSA, as well as against MRSA.

Within the substituted benzodioxane derivatives, 7-substitution proved to be preferred over 6-substitution; furthermore, we demonstrated that no hindered or hydrophilic substituents were allowed at the 7-position, as they resulted in progressive loss in inhibitory activity. Using compounds modified at the bicyclic scaffold, we concluded our incomplete previous study, assessing that both benzodioxane O(1) and O(4) are important to retain potent FtsZ inhibition.

Experimental Section

Biochemical assays

All reagents were purchased from commercial suppliers, as was the *Staphylococcus aureus* FtsZ protein (Cytoskeleton, FTZ02). Absorbance was detected using a VERSAmax tunable microplate reader.

Polymerization assay: *S. aureus* FtsZ protein was diluted at a final concentration of 1 mg mL^{-1} in PIPES-KOH pH 6.8 (50 mM), KCl (250 mM), and $MgOAc_2$ (5 mM). Compounds **II**, **III,** or DMSO, used as negative control, were added to each reaction tube at the specified concentration (5, 10, or 20 μg mL^{-1}), and polymerization was initiated by adding GTP (final concentration = 0.5 mM) and incubating the obtained reaction mixtures at 37 °C for 15 min. The samples were then centrifuged at 20000 × *g* for 20 min, then the supernatants were immediately removed, and the pellet was resuspended in an equivalent volume of Laemmli sample buffer. Pellets and supernatants were then loaded on a 12% SDS-PAGE gel. Gels were stained with Coomassie Brilliant Blue G-250 and, after scanning, the intensity of each band was estimated by densitometric quantification using ImageJ software.

GTPase assay: GTPase activity was assessed using an 2-amino-6-mercapto-7-methylpurine riboside (MESG)-based assay using the GTPase Kinetic ELIPA Assay Kit (Cytoskeleton, BK052). In particular, *S. aureus* FtsZ protein was diluted at a final concentration of 1 mg mL^{-1} in HEPES-KOH pH 7.5 (50 mM), potassium acetate (300 mM), and magnesium acetate (5 mM). According to the protocol, the MESG and the purine nucleoside phosphorylase were added to the protein. The reaction was then aliquoted in half-area 96-well plates and incubated with DMSO or compound **II** or **III** (5 μg mL^{-1}). The kinetics of the reaction were monitored in real-time using a pre-warmed microplate reader at 37 °C, and the absorbance at λ = 360 nm was detected every 30 sec for 30 min.

Chemistry

All starting materials were obtained from commercial suppliers and used without further purification. ^{1}H and ^{13}C NMR spectra were taken on a Varian 300 Mercury NMR spectrometer operating at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR. Chemical shifts (δ) are reported in ppm relative to residual solvent ($CDCl_3$ or [D_6]DMSO) as an internal standard. Signal multiplicity was used according to the following abbreviations: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, td = triplet of doublets, q = quadruplet, m = multiplet, sept = septuplet, and bs = broad singlet. Elemental analyses of the new substances were within 0.40% of theoretical values. Silica gel F_{254} was used in analytical thin-layer chromatography (TLC), and silica gel (particle size = 40–63 μm, Merck) was used in flash chromatography; visualization was accomplished with UV light (λ 254 nm). Melting points were determined by differential scanning calorimetry (DSC; TA Instruments) or a Büchi melting point apparatus. For NMR data, elemental analyses, and melting points, see the Supporting Information.

Ethyl 3,4-dihydroxybenzoate (17): H_2SO_4 (98%, 0.20 mL) was added dropwise to a solution of 3,4-dihydroxybenzoic acid (1.0 g, 6.49 mmol) in EtOH (10 mL) at 0 °C. Once added, the reaction mixture was warmed to room temperature, then to 60 °C, and was stirred overnight. The reaction mixture was then concentrated under reduced pressure, redissolved in EtOAc (20 mL), and washed with $NaHCO_3$ (10%, 15 mL) and brine (15 mL). The organic layer was then dried over Na_2SO_4, filtered, and concentrated under reduced pressure, yielding 920 mg (77.8%) of **17** as a brown solid.

Ethyl 2-hydroxymethyl-1,4-benzodioxan-7-carboxylate (18): Potassium carbonate (0.87 g, 6.31 mmol) was added portionwise to **17** (0.92 g, 5.05 mmol) in DMF (9 mL) at room temperature, and the mixture was stirred for 30 min. Then, epibromohydrin (0.54 mL, 6.31 mmol) was slowly added dropwise, and the reaction mixture was heated at 40 °C and stirred overnight. The mixture was then diluted with EtOAc (15 mL) and washed with brine (3 × 15 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by flash chromatography on silica gel, eluting with cyclohexane/EtOAc (75:25), yielding 630 mg (52.3%) of **18** as a light yellow oil.

Ethyl 2-mesyloxymethyl-1,4-benzodioxan-7-carboxylate (19): Mesyl chloride (0.27 mL; 3.43 mmol) was slowly added to a mixture of **18** (0.63 g, 2.64 mmol) and TEA (0.48 mL, 3.43 mmol) in CH_2Cl_2 (6 mL) at 0 °C. The mixture was brought to room temperature and stirred for 1 h. Once the reaction mixture reached completion, it was quenched with NaCl (10%, 10 mL), washed with $NaHCO_3$ (10%, 10 mL) and brine (10 mL), dried over Na_2SO_4, filtered, and concentrated, under vacuum, yielding 800 mg (95.8%) of **19** as a yellow oil.

2,6-Difluoro-3-(7-ethoxycarbonyl-1,4-benzodioxan-2-yl)methoxybenzamide (1): Potassium carbonate (0.38 g, 2.78 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (0.48 g, 2.78 mmol) in DMF (4 mL). After stirring at room temperature for 20 min, a solution of **19** (0.80 g, 2.53 mmol) in DMF (4 mL) was added, and the mixture was heated, stirring at 80 °C for 16 h. Upon reaching completion, the mixture was concentrated under vacuum and diluted with water (15 mL) and EtOAc (15 mL). The organic layer was washed with brine (3 × 15 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by flash chromatography on silica gel, eluting with cyclohexane/EtOAc (6:4), affording 600 mg of a white wax that was further slurried from isopropyl ether, yielding 500 mg (50.2%) of **1** as a white solid.

Ethyl 4-benzyloxy-3-hydroxybenzoate (20): Potassium carbonate (0.99 g, 7.14 mmol) was added portionwise to a solution of **17** (1.3 g, 7.14 mmol) in acetone (15 mL) at room temperature. After stirring for 30 min, the mixture was cooled to 0 °C, and benzyl bromide (0.85 mL; 7.14 mmol) was slowly added dropwise. The mixture was warmed to room temperature and then heated at reflux and stirred overnight. After reaching completion, the mixture was concentrated, redissolved with EtOAc (20 mL), and washed with brine (3 × 10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The crude was purified by flash chromatography

on silica gel, eluting with cyclohexane/ethyl acetate (9:1), yielding 1.13 g (58.1%) of **20** as a light yellow oil.

Ethyl 4-benzyloxy-3-(2,3-epoxypropyl)benzoate (21): Potassium carbonate (0.75 g, 5.39 mmol) was added portionwise to **20** (1.13 g, 4.15 mmol) in DMF (15 mL) at room temperature. After stirring for 30 min, epibromohydrin (0.46 mL, 5.39 mmol) was slowly added dropwise. The reaction mixture was then heated at 40 °C and stirred for 2 h. After completion, the mixture was concentrated under vacuum, diluted with EtOAc (15 mL), washed with brine (3× 10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The oily residue was further crystallized from chloroform, yielding 1.22 g (89.7%) of **21** as a white solid.

Ethyl 4-hydroxy-3-(2,3-epoxypropyl)benzoate (22): A solution of **21** (0.24 g, 0.73 mmol) in EtOAc (10 mL) was added to 5% Pd/C (2.4 mg), and the mixture was vigorously shaken under hydrogen at room temperature for 3 h. The catalyst was removed by filtration, and the filtrate was concentrated to dryness, affording 170 mg (quantitative yield) of **22** as a white waxy solid.

Ethyl 2-hydroxymethyl-1,4-benzodioxan-6-carboxylate (23): Potassium carbonate (0.15 g, 1.05 mmol) was added portionwise to **22** (0.25 g, 1.05 mmol) in DMF (2 mL) at room temperature. The reaction mixture was heated at 50 °C; after stirring for 3 h, it was diluted with EtOAc (10 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The crude residue was purified by flash chromatography on silica gel, eluting with toluene/EtOAc (7:3), yielding 160 mg (64%) of **23** as a white oil.

Ethyl 2-mesyloxymethyl-1,4-benzodioxan-6-carboxylate (24): Mesyl chloride (93 μL, 1.2 mmol) was slowly added dropwise into a mixture of **23** (0.22 g, 0.92 mmol) and TEA (0.17 mL, 1.2 mmol) in CH_2Cl_2 (2 mL) at 0 °C. After this addition, the mixture was brought to room temperature and stirred for 3 h. After completion, the mixture was quenched with NaCl (10%, 3 mL), washed with brine (3 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The crude was purified by flash chromatography on silica gel, eluting with toluene/EtOAc (8:2), to yield 170 mg (58.4%) of **24** as a sticky solid.

2,6-Difluoro-3-(6-ethoxycarbonyl-1,4-benzodioxan-2-yl)methoxybenzamide (2): Potassium carbonate (49 mg, 0.35 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (55 mg, 0.32 mmol) in DMF (1 mL). After stirring at room temperature for 20 min, a solution of **24** (100 mg, 0.32 mmol) in DMF (2 mL) was added, and the mixture was heated at 90 °C while stirring for 5 h. After completion, it was concentrated under vacuum, diluted with water (10 mL), and EtOAc (10 mL); the organic layer was washed with brine (3×5 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by flash chromatography on silica gel, eluting with toluene/EtOAc (8:2), affording 71 mg (56.4%) of **2** as a white solid.

Isopropyl 3,4-dihydroxybenzoate (25): Starting from 3,4-dihydroxybenzoic acid (1.0 g, 6.49 mmol) and following the procedure used for **17**, using isopropyl alcohol instead of EtOH, yielded 660 mg (51.8%) of **25** as a brown solid.

Isopropyl 2-hydroxymethyl-1,4-benzodioxan-7-carboxylate (26): Starting from **25** (0.66 g, 3.36 mmol), and following the procedure used for **18**, yielded compound **26** as a light yellow oil (610 mg, 72%).

Isopropyl 2-mesyloxymethyl-1,4-benzodioxan-7-carboxylate (27): Starting from **26** (0.61 g, 2.42 mmol) and following the procedure used for **19**, yielded compound **27** as a light yellow oil (520 mg, 65%).

2,6-Difluoro-3-(7-isopropoxycarbonyl-1,4-benzodioxan-2-yl)methoxybenzamide (3): Potassium carbonate (0.24 g, 1.73 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (0.30 g, 1.73 mmol) in DMF (3 mL). After stirring at room temperature for 20 min, a solution of **27** (0.52 g, 1.57 mmol) in DMF (3 mL) was added, and the mixture was heated at 80 °C while stirring for 3 h. After completion, the mixture was concentrated under vacuum and diluted with water (15 mL) and EtOAc (15 mL). The organic layer was washed with brine (3×15 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by flash chromatography on silica gel, eluting with cyclohexane/EtOAc (6:4), affording 510 mg of a white waxy solid that was further slurried from isopropyl ether to yield 300 mg (47.1%) of **3** as a white solid.

Phenyl 2-benzyloxymethyl-1,4-benzodioxan-7-carboxylate (28): DMAP (140 mg, 1.16 mmol), EDAC·HCl (210 mg, 1.07 mmol), 2-benzyloxymethyl-1,4-benzodioxan-7-carboxylic acid (0.27 g, 0.89 mmol; see Chiodini et al. for preparation[21]), and HOBt (150 mg, 1.07 mmol) were stirred in dry DMF at room temperature under a nitrogen atmosphere. After 30 min, phenol (80 mg, 89 μmol) was added to the suspension, and the mixture was stirred for 4 h. After completion, it was concentrated, resumed with EtOAc (15 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified by flash chromatography on silica gel, eluting with cyclohexane/EtOAc (6:4) to give 200 mg (71.5%) of **28** as a colorless oil.

Phenyl 2-hydroxymethyl-1,4-benzodioxan-7-carboxylate (29): A solution of **28** (0.20 g, 0.53 mmol) in MeOH (10 mL) was added with 5% Pd/C (20 mg), and the mixture was vigorously shaken under hydrogen atmosphere at room temperature for 2 h. The catalyst was removed by filtration, and the filtrate was concentrated to dryness, affording 140 mg (92.3%) of **29** as a yellow oil.

Phenyl 2-mesyloxymethyl-1,4-benzodioxan-7-carboxylate (30): Mesyl chloride (0.04 mL, 0.54 mmol) was slowly added to a mixture of **29** (0.14 g, 0.49 mmol) and TEA (0.08 mL, 0.54 mmol) in CH_2Cl_2 (2 mL) at 0 °C. After the addition, the mixture was brought to room temperature and was stirred for an additional 3 h. After completion, the mixture was quenched with NaCl (10%, 3 mL), washed with brine (3 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum, yielding 180 mg (quantitative yield) of **30** as a yellow oil.

2,6-Difluoro-3-(7-phenoxycarbonyl-1,4-benzodioxan-2-yl)methoxybenzamide (4): Potassium carbonate (70 mg, 0.54 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (93 mg, 0.54 mmol) in DMF (2 mL). After stirring at room temperature for 20 min, a solution of **30** (180 mg, 0.49 mmol) in DMF (2 mL) was added, and the mixture was heated at 80 °C while stirring for 3 h. After completion, the mixture was concentrated under vacuum and diluted with water (10 mL) and EtOAc (10 mL). The organic layer was washed with brine (3×5 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by flash chromatography on silica gel, eluting with cyclohexane/EtOAc (6:4), to afford 80 mg (36.7%) of **4** as a white solid.

2-Benzyloxymethyl-7-hydroxymethyl-1,4-benzodioxane (31): $LiAlH_4$ (410 mg, 10.84 mmol) was suspended in dry THF (10 mL) at 0° C under nitrogen atmosphere. A solution of 2-(benzyloxymethyl)-1,4-benzodioxan-7-carboxylic acid (1.63 g, 5.42 mmol; see Chiodini et al.[21]) in THF (20 mL) was added. The mixture was heated at

50 °C and stirred for 3 h; after completion, it was cooled to 0 °C and slowly quenched with 10% aqueous HCl (10 mL). EtOAc (10 mL) was then added, the organic layer was washed with brine (3×10 mL), dried over Na_2SO_4, and concentrated under vacuum to give 1.52 g (98.0%) of **31** as a colorless oil.

2-Benzyloxymethyl-7-methoxymethyl-1,4-benzodioxane (32): NaH (113 mg, 4.72 mmol) was suspended in dry DMF (2 mL) at 0° C under nitrogen atmosphere. A solution of **31** (1.04 g, 3.63 mmol) in DMF (18 mL) was added. After 30 min of stirring, the mixture was cooled to 0 °C, and methyl iodide (0.90 mL, 14.54 mmol) was slowly added dropwise. The mixture was then warmed to room temperature and stirred for 4 h. After completion, it was concentrated under vacuum and redissolved in EtOAc (10 mL). The organic layer was washed with 10% aqueous $NaHCO_3$ (3×10 mL) and brine (3×10 mL), dried over Na_2SO_4, and concentrated under vacuum to give 1.09 g (quantitative yield) of **32** as an orange oil.

2-Hydroxymethyl-7-methoxymethyl-1,4-benzodioxane (33): A solution of **32** (730 mg, 2.43 mmol) in MeOH (20 mL) was added to 5% Pd/C (73 mg), and the mixture was vigorously shaken under hydrogen at room temperature for 4 h. The catalyst was removed by filtration, and the filtrate was concentrated to give 510 mg (quantitative yield) of **33** as a colorless oil.

2-Mesyloxymethyl-7-methoxymethyl-1,4-benzodioxane (34): Mesyl chloride (0.27 mL, 3.49 mmol) was added dropwise to a solution of **33** (490 mg, 2.33 mmol) and TEA (0.49 mL, 3.49 mmol) in CH_2Cl_2 (10 mL) at 0° C. The mixture was stirred at room temperature for 6 h, diluted with CH_2Cl_2 (20 mL), washed with 10% aqueous NaCl (10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield an oily residue. The crude residue was purified by flash chromatography on silica gel, eluting with cyclohexane/EtOH (85:15), giving 380 mg (56.7%) of **34** as a colorless oil.

3-(7-Methoxymethyl-1,4-benzodioxan-2-yl)methyloxy-2,6-difluorobenzamide (5): Potassium carbonate (200 mg, 1.46 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (240 mg, 1.40 mmol) in dry DMF (3 mL). After stirring at room temperature for 30 min, a solution of **34** (380 mg, 1.33 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 100° C for 16 h, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to give a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (1:1) gave a white solid, which was further slurried from isopropyl ether to yield 180 mg (37.0%) of **5** as a white solid.

***N*-methyl-*N*-methoxy-2-benzyloxymethyl-1,4-benzodioxan-7-carboxamide (35)**: Carbonyldiimidazole (87 mg, 0.55 mmol) was added portionwise to a solution of 2-(benzyloxymethyl)-1,4-benzodioxan-7-carboxylic acid (150 mg, 0.5 mmol) in CH_2Cl_2 (5 mL) at room temperature. After stirring at room temperature for 30 min, *N*,*O*-dimethyl-hydroxylamine hydrochloride (81 mg, 0.5 mmol) was added slowly. The mixture was stirred at room temperature for 18 h, then diluted with CH_2Cl_2 (15 mL), washed with 10% aqueous HCl (3×10 mL), 10% aqueous $NaHCO_3$, and brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (7:3) gave 130 mg (76.0%) of **35** as a colorless oil.

2-Benzyloxymethyl-7-propionyl-1,4-benzodioxane (36): Ethyl magnesium bromide (1.46 mL, 3.0 M) in Et_2O (1.46 mL, 4.37 mmol) was added dropwise to a solution of **35** (1.00 g, 2.91 mmol) in dry THF (10 mL) at 0 °C under nitrogen atmosphere. The mixture was stirred at room temperature for 18 h and poured into 10% aqueous HCl (20 mL) and EtOAc (20 mL) at 0 °C. The organic layer was washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (8:2) gave 0.64 g (70.4%) of **36** as a colorless oil.

2-Hydroxymethyl-7-propionyl-1,4-benzodioxane (37): Compound **36** (0.70 g, 2.24 mmol) was dissolved in HCl (6 N, 14 mL) and dioxane (14 mL) at room temperature and stirred at reflux for 48 h. It was then diluted with H_2O, buffered to neutral pH, and extracted with EtOAc (3×20 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under vacuum to yield a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (7:3) gave 0.44 g (88.4%) of **37** as a yellow oil.

2-Mesyloxymethyl-7-propionyl-1,4-benzodioxane (38): Mesyl chloride (0.013 mL, 0.17 mmol) was added dropwise to a solution of **37** (30 mg, 0.13 mmol) and TEA (0.023 mL, 0.17 mmol) in CH_2Cl_2 (0.6 mL) at 0 °C. The mixture was stirred at room temperature for 4 h, diluted with CH_2Cl_2 (20 mL), washed with 10% aqueous HCl (3×10 mL), 10% aqueous $NaHCO_3$ (3×10 mL), and brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield 20 mg (51.3%) of **38** as a brown oil.

3-(7-Propionyl-1,4-benzodioxan-2-yl)methyloxy-2,6-difluorobenzamide (6): Potassium carbonate (140 mg, 1.02 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (170 mg, 0.98 mmol) in DMF (3 mL). After stirring at room temperature for 30 min, a solution of **38** (280 mg, 0.93 mmol) in DMF (3 mL) was added. The reaction mixture was stirred at 100 °C for 2 h, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to give a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (1:1) gave a white solid that was crystallized from chloroform, yielding 150 mg (42.8%) of **6** as a white solid.

Methyl 2-*tert*-butyldimethylsilyloxymethyl-1,4-benzodioxan-7-carboxylate (39): *Tert*-butyldimethylsilyl chloride (1.01 g, 6.69 mmol) was added portionwise to a solution of methyl 2-hydroxymethyl-1,4-benzodioxan-7-carboxylate (1.0 g, 4.46 mmol) and DIPEA (1.17 mL, 6.69 mmol) in CH_2Cl_2 (10 mL) at room temperature. The reaction mixture was stirred for 16 h; after completion, it was diluted with CH_2Cl_2 (20 mL), washed with 10% aqueous HCl (3×10 mL), 10% aqueous $NaHCO_3$, and brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (7:3) gave 910 mg (60.3%) of **39** as a colorless oil.

2-*tert*-Butyldimethylsilyloxymethyl-7-hydroxymethyl-1,4-benzodioxane (40): $LiAlH_4$ (48 mg, 1.27 mmol) was suspended in dry THF (2 mL) at 0 °C under nitrogen atmosphere. A solution of **39** (430 mg, 1.27 mmol) in THF (5 mL) was added slowly. The mixture was warmed to room temperature and stirred for 3 h; after completion, it was cooled to 0 °C and slowly quenched with 10% aqueous HCl (3 mL). EtOAc (10 mL) was then added and the organic layer was washed with brine (3×10 mL), dried over Na_2SO_4, and concentrated under vacuum to give 380 mg (96.4%) of **40** as a yellow oil.

2-*tert*-Butyldimethylsilyloxymethyl-7-formyl-1,4-benzodioxane (41): Manganese dioxide (1.06 g, 12.24 mmol) was added portionwise to a solution of **40** (380 mg, 1.22 mmol) in chloroform (5 mL) at room temperature. The mixture was heated at reflux and stirred

for 2 h, then cooled to room temperature and diluted with CH_2Cl_2 (15 mL). The suspension was filtered over a Celite pad and concentrated under vacuum to yield 340 mg (90.4%) of **41** as a colorless oil.

2-Hydroxymethyl-7-formyl-1,4-benzodioxane (42): HCl (10% aqueous solution, 1 mL) was added dropwise to a solution of **41** (340 mg, 1.10 mmol) in MeOH (10 mL) at room temperature, and the mixture was stirred for 20 min. After completion, the reaction mixture was concentrated under vacuum to yield a residue that was redissolved with H_2O, buffered to pH 7, and extracted with EtOAc (3×10 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under vacuum to give 210 mg (quantitative yield) of **42** as a brown oil.

3-(7-Formyl-1,4-benzodioxan-2-yl)methyloxy-2,6-difluorobenzamide (7): Triphenylphosphine (530 mg, 2.01 mmol) and 2,6-difluoro-3-hydroxybenzamide (230 mg, 1.34 mmol) were added to a solution of **42** (260 mg, 1.34 mmol) in dry THF (5 mL). A solution of DIAD (0.400 mL, 2.01 mmol) in THF (5 mL) was added dropwise at 0 °C. After stirring overnight at room temperature, the mixture was concentrated, and water (30 mL) and EtOAc (30 mL) were added to the residue. The aqueous layer was separated and extracted with EtOAc (3×20 mL). The combined organic phases were dried over Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel; elution with cyclohexane/EtOAc (1:1) and successive slurrying from isopropyl ether gave 167 mg (35.7%) of **7** as a white solid.

3-(7-(5,5-Dimethyl-1,3-dioxan-2-yl)-1,4-benzodioxan-2-yl)methyloxy-2,6-difluorobenzamide (8): Zinc chloride (3.12 mg, 23 μmol) was added to a solution of **7** (80 mg, 0.23 mmol) and 2,2-dimethyl-1-propanol (190 mg, 1.84 mmol) in dry toluene (5 mL). The mixture was heated at reflux for 2 h, with azeotropic removal of water. After completion, the mixture was concentrated under vacuum, and water (10 mL) and EtOAc (10 mL) were added to the residue. The aqueous layer was separated and extracted with EtOAc (2×10 mL). The combined organic phases were dried over Na_2SO_4 and concentrated. The residue was purified by chromatography on silica gel; elution with toluene/EtOAc (6:4) gave 50 mg (50.0%) of **8** as a white waxy solid.

***N*-(2-Dimethoxyethyl)-2-benzyloxymethyl-1,4-benzodioxan-7-ylcarboxamide (43)**: Ethyl chloroformate (0.33 mL, 3.5 mmol) was added dropwise to a solution of 2-benzyloxymethyl-1,4-benzodioxan-7-carboxylic acid (1.0 g, 3.33 mmol) and *N*-methylmorpholine (0.4 mL, 3.66 mmol) in dry THF (20 mL) at 0 °C under nitrogen atmosphere. The reaction mixture was stirred for 1 h at 0 °C; then aminoacetaldehyde dimethyl acetal (0.40 mL, 3.66 mmol) was added dropwise to the mixture. After stirring for 1 h at room temperature, the mixture was diluted with Et_2O (20 mL) and $NaHCO_3$ (20 mL). The organic layer was further washed with brine (15 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield 760 mg (58.9%) of **43** as a yellow oil.

2-Mesyloxymethyl-7-(1,3-oxazol-2-yl)-1,4-benzodioxane (44): Compound **43** (0.76 g, 1.96 mmol) was combined with freshly prepared Eaton's reagent (7.7% P_2O_5 in methanesulfonic acid, 10 mL) at 0 °C. The mixture was warmed to room temperature and heated at 110 °C, and stirring was retained for 5 h; after completion, it was cooled to 0 °C and slowly poured into ice. After stirring for 30 min, CH_2Cl_2 (30 mL) was added, the mixture was filtered over a Celite pad, and the organic layer was washed with brine (3×10 mL), dried over Na_2SO_4, and concentrated under vacuum. The residue was treated with concentrated CH_2Cl_2 (5 mL), resulting in the precipitation of 250 mg (40.9%) of **44** as a white solid.

3-(7-(1,3-Oxazol-2-yl)-1,4-benzodioxan-2-yl)methyloxy-2,6-difluorobenzamide (9): Potassium carbonate (0.23 g, 1.68 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (0.15 g, 0.88 mmol) in dry DMF (5 mL). After stirring at room temperature for 30 min, a solution of **44** (0.25 g, 0.8 mmol) in DMF (3 mL) was added. The reaction mixture was stirred at 80 °C for 5 h, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated, to give a residue that was slurried from CH_2Cl_2/MeOH to yielding 140 mg (45.0%) of **9** as a white solid.

2-Benzyloxymethyl-7-acetyl-1,4-benzodioxane (45): Methyl magnesium bromide (1.06 mL, 3.0 M) in Et_2O (1.06 mL, 4.36 mmol) was added dropwise to a solution of **35** (1.25 g, 3.64 mmol) in dry THF (10 mL) at 0 °C under nitrogen atmosphere. The mixture was stirred at room temperature for 18 h and poured into 5% aqueous HCl (20 mL) and EtOAc (20 mL) at 0 °C. The organic layer was washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield 960 mg (89.0%) of **45** as an orange oil.

2-Benzyloxymethyl-7-(2-bromoacetyl)-1,4-benzodioxane (46): Bromine (0.17 mL, 3.22 mmol) was added dropwise to a solution of **45** (0.96 g, 3.22 mmol) in chloroform (10 mL) at 10 °C. After stirring at 10 °C for 1 h, the mixture was quenched with 10% aqueous $Na_2S_2O_5$, and the organic layer was washed with brine (10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield 1.17 g (96.3%) of **46** as a brown oil.

2-Benzyloxymethyl-7-(1,3-oxazol-4-yl)-1,4-benzodioxane (47): Compound **46** (0.34 g, 0.90 mmol) was diluted in formic amide (5 mL) and heated at 130 °C; the mixture was stirred for 2 h, then cooled to room temperature and diluted with EtOAc (20 mL). After 20 min of quenching with 10% aqueous $Na_2S_2O_5$ (10 mL), the organic layer was washed with brine (10 mL), dried over Na_2SO_4, and concentrated under vacuum. The residue was purified by flash chromatography on silica gel, eluting with cyclohexane/EtOAc (9:1), affording 100 mg (34.0%) of **47** as a yellow oil.

2-Hydroxymethyl-7-(1,3-oxazol-4-yl)-1,4-benzodioxane (48): A solution of **47** (100 mg, 0.31 mmol) in EtOH (20 mL) was added to 5% Pd/C (10 mg), and the mixture was vigorously shaken under hydrogen at room temperature for 1 h. The catalyst was removed by filtration, and the filtrate was concentrated to give 80 mg (quantitative yield) of **48** as a colorless oil.

2-Mesyloxymethyl-7-(1,3-oxazol-4-yl)-1,4-benzodioxane (49): Mesyl chloride (0.04 mL, 0.51 mmol) was added dropwise to a solution of **48** (100 mg, 0.43 mmol) and TEA (0.07 mL, 0.51 mmol) in CH_2Cl_2 (3 mL) at 0 °C. The mixture was stirred at room temperature for 2 h, diluted with CH_2Cl_2 (20 mL), washed with 10% aqueous NaCl (10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield 130 mg (quantitative yield) of **49** as a yellow oil.

3-(7-(1,3-Oxazol-4-yl)-1,4-benzodioxan-2-yl)methyloxy-2,6-difluorobenzamide (10): Potassium carbonate (63 mg, 0.46 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (79 mg, 0.46 mmol) in dry DMF (3 mL). After stirring at room temperature for 30 min, a solution of **49** (130 mg, 0.42 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at 80 °C for 16 h, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to give a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (7:3) gave a white solid, which was further slurried from isopropyl ether to yield 100 mg (61.3%) of **10** as a white solid.

2-Benzyloxymethyl-7-(2-aminothiazol-4-yl)-1,4-benzodioxane (50): Thiourea (0.20 g, 2.62 mmol) was added to a solution of **46** (0.90 g, 2.39 mmol) in EtOH (12 mL) at room temperature. The mixture was heated at reflux and stirred for 2 h, then concentrated under vacuum. The crude was redissolved in EtOAc (20 mL), washed with 10% aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to give 730 mg (87.0%) of **50** as a pink solid.

2-Hydroxymethyl-7-(2-aminothiazol-4-yl)-1,4-benzodioxane (51): BBr_3 (2.18 mL, 1.0 M) in CH_2Cl_2 (2.18 mL) was added dropwise to a solution of **50** (0.7 g, 1.98 mmol) in CH_2Cl_2 (15 mL) at −5 °C. The mixture was stirred at −5 °C for 30 min, then it was diluted with CH_2Cl_2 (10 mL) and quenched with 10% aqueous $NaHCO_3$ (20 mL), stirring at 0 °C for 20 min. The organic layer was washed with brine (10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield 450 mg (86.0%) of **51** as a yellow oil.

2-Mesyloxymethyl-7-(2-aminothiazol-4-yl)-1,4-benzodioxane (52): Mesyl chloride (0.17 mL, 2.16 mmol) was added dropwise to a solution of **51** (0.44 g, 1.67 mmol) and TEA (0.30 mL, 2.16 mmol) in CH_2Cl_2 (10 mL) at 0 °C. The mixture was stirred at room temperature for 1.5 h, diluted with CH_2Cl_2 (10 mL), washed with 10% aqueous $NaHCO_3$ (20 mL) and brine (10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to yield 550 mg (96.2%) of **52** as a brown oil.

3-(7-(2-Aminothiazol-4-yl)-1,4-benzodioxan-2-yl)methyloxy-2,6-difluorobenzamide (11): Potassium carbonate (0.25 g, 1.83 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (0.30 g, 1.75 mmol) in DMF (6 mL). After stirring at room temperature for 30 min, a solution of **52** (0.57 g, 1.66 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 100 °C for 16 h, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to give a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc gave 200 mg (24.5%) of **11** as a beige solid (DMF solvate).

2-Benzyloxymethyl-7-(2-methylthiazol-4-yl)-1,4-benzodioxane (53): Thioacetamide (0.26 g, 3.41 mmol) was added to a solution of **46** (1.17 g, 3.10 mmol) in EtOH (12 mL) at room temperature. The mixture was heated at reflux and stirred for 1 h, then concentrated under vacuum. The crude was redissolved in EtOAc (20 mL), washed with 10% aqueous $NaHCO_3$ (10 mL) and brine (10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum. The residue was purified by flash chromatography on silica gel, eluting with cyclohexane/EtOAc (8:2) to give 600 mg (55.0%) of **53** as a colorless waxy solid.

2-Hydroxymethyl-7-(2-methylthiazol-4-yl)-1,4-benzodioxane (54): Starting from **53** (0.6 g, 1.70 mmol) and following the same procedure described for **51** yielded **54** as a yellow oil (450 mg, quantitative yield).

2-Mesyloxymethyl-7-(2-methylthiazol-4-yl)-1,4-benzodioxane (55): Starting from **54** (0.45 g, 1.70 mmol) and following the same procedure described for **52** yielded **55** as a brown oil (570 mg, quantitative yield).

3-(7-(2-Methylthiazol-4-yl)-1,4-benzodioxan-2-yl)methyloxy-2,6-difluorobenzamide (12): Potassium carbonate (0.24 g, 1.77 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (0.29 g, 1.69 mmol) in DMF (5 mL). After stirring at room temperature for 30 min, a solution of **55** (0.55 g, 1.61 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at 80 °C for 4 h, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to give a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (1:1) gave 290 mg (43.0%) of **12** as a white solid.

2-Hydroxymethylbenzomorpholine (56): NaOH (2.5 N, 5.13 mL, 12.82 mmol) was added dropwise to a solution of 2-aminophenol (1.00 g, 9.16 mmol) in H_2O (10 mL). After stirring at room temperature for 30 min, epichlorohydrin (0.86 mL, 11.00 mmol) was added dropwise, and the mixture was stirred for 2 h, then buffered to neutral pH and extracted with EtOAc (3×15 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated, and the resulting residue was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (6:4) gave 0.75 g (49.6%) of **56** as a brown oil.

***N*-methyl-2-hydroxymethylbenzomorpholine (57)**: Potassium carbonate (0.53 g, 3.84 mmol) was added to a solution of **56** (0.53 g, 3.20 mmol) in DMF (15 mL). After stirring at room temperature for 20 min, methyl iodide (0.24 mL, 3.84 mmol) was added at 0 °C. The reaction mixture was heated at 50 °C and stirred for 2 h, concentrated, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to yield 0.43 g (75.0%) of **57** as a brown oil.

***N*-methyl-2-mesyloxymethylbenzomorpholine (58)**: Mesyl chloride (0.30 mL, 3.91 mmol) was added dropwise to a solution of **57** (0.54 g, 3.01 mmol) and TEA (0.54 mL, 3.91 mmol) in CH_2Cl_2 (10 mL) at 0 °C. The mixture was stirred at room temperature for 1 h, diluted with CH_2Cl_2 (15 mL), and washed with H_2O (15 mL). The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated to yield a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (8:2) gave 0.55 g (71.0%) of **58** as a blue oil.

***N*-methylbenzomorpholin-2-yl)methyloxy-2,6-difluorobenzamide (13)**: Potassium carbonate (180 mg, 1.28 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (200 mg, 1.16 mmol) in DMF (3 mL). After stirring at room temperature for 30 min, a solution of **58** (300 mg, 1.16 mmol) in DMF (3 mL) was added. The reaction mixture was stirred at 80 °C for 3 h, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to give a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (6:4) yielded a residue that was crystallized from chloroform to give 0.12 g (31.0%) of **13** as a white solid.

1,2-Epoxy-3-(2-nitrophenoxy)propane (59): 2-Nitrophenol (500 mg, 3.59 mmol) was added to a solution of NaOH (4.31 mmol) in H_2O (5.4 mL), and the mixture was stirred at room temperature for 30 min. Epichlorohydrin (0.42 mL, 5.28 mmol) was added, and the mixture was stirred for 18 h, diluted with 10% aqueous NaOH (20 mL), and extracted with CH_2Cl_2 (3×10 mL). The collected organic layers were washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to yield a residue that was purified by flash chromatography. Elution with cyclohexane/EtOAc (7:3) gave 270 mg (38.6%) of **59** as a brown oil.

3-Hydroxymethylbenzomorpholine (60): Metallic iron (3.77 g, 67.63 mmol) was added portionwise to a solution of **59** (2.64 g, 13.52 mmol) in a solution of EtOH (2.64 mL), H_2O (5.28 mL), and acetic acid (2.64 mL). After stirring at room temperature for 20 min, the mixture was poured into a mixture of EtOAc (30 mL) and 10% aqueous NaOH (30 mL) at 0 °C and filtered. The organic layer was

dried over Na_2SO_4, filtered, and concentrated to give 1.62 g (72.6%) of **60** as a dark oil.

***N*-methyl-3-hydroxymethylbenzomorpholine (61)**: Potassium carbonate (2.71 g 19.60 mmol) was added to a solution of **60** (1.62 g, 9.80 mmol) in DMF (30 mL). After stirring the mixture at room temperature for 30 min, methyl iodide (1.21 mL, 19.60 mmol) was added dropwise at 0 °C. The mixture was stirred at 50 °C for 18 h, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, filtered, and concentrated to yield **61** (1.75 g, 99.6%) as a dark oil that was used in the next step without further purification.

***N*-methyl-3-chloromethylbenzomorpholine (62)**: Mesyl chloride (0.10 mL, 1.30 mmol) was added dropwise to a solution of **61** (90 mg, 0.5 mmol) and TEA (0.19 mL, 1.30 mmol) in CH_2Cl_2 (10 mL) at 0 °C. The mixture was stirred at room temperature for 3 h, diluted with CH_2Cl_2 (10 mL), and washed with brine (15 mL). The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated to yield a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (8:2) gave 70 mg (70.8%) of **62** as a dark oil.

(*N*-methylbenzomorpholin-3-yl)methyloxy-2,6-difluorobenzamide (14): Potassium carbonate (50 mg, 0.38 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (64 mg, 0.37 mmol) in DMF (1 mL). After stirring at room temperature for 30 min, a solution of **62** (70 mg, 0.35 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at 80 °C overnight, concentrated under vacuum, diluted with EtOAc (10 mL), washed with brine (3×5 mL), dried over Na_2SO_4, filtered, and concentrated to give a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (6:4) gave 37 mg (32.0%) of **14** as a brown oil.

Ethyl *N*,*N*′-dimethyl-1,2,3,4-tetrahydroquinoxaline-2-carboxylate (64): Ethyl 2,3-dibromopropionate (4.19 g, 16.12 mmol) was added to a solution of *N*,*N*′-dimethyl-phenylendiamine (2.19 g, 16.12 mmol) and TEA (4.94 mL, 35.46 mmol) in toluene (45 mL). After stirring the mixture at 80 °C for 18 h, it was washed with brine (30 mL), 10% aqueous HCl (30 mL), 10% aqueous NaOH (30 mL), 10% aqueous $Na_2S_2O_5$ (30 mL), and H_2O. The organic layer was dried over Na_2SO_4, filtered, and concentrated under vacuum to give 1.75 g (46.4%) of **64** as a brown oil.

***N*,*N*′-dimethyl-2-hydroxymethyl-1,2,3,4-tetrahydroquinoxaline (65)**: A solution of **64** (1.13 g, 4.82 mmol) in THF (10 mL) was added to a suspension of $LiAlH_4$ (0.37 g, 9.65 mmol) in dry THF (5 mL) at 0 °C under nitrogen atmosphere. After stirring at room temperature for 3 h, 10% aqueous HCl (10 mL) and EtOAc (10 mL) were slowly added. The organic layer was washed with brine (3× 10 mL), dried over Na_2SO_4, filtered, and concentrated under vacuum to give 0.92 g (quantitative yield) of **65** as a yellow oil.

***N*,*N*′-dimethyl-2-chloromethyl-1,2,3,4-tetrahdroquinoxaline (66)**: Mesyl chloride (0.51 mL, 6.56 mmol) was added dropwise to a solution of **65** (0.63 g, 3.28 mmol) and TEA (0.91 mL, 6.56 mmol) in CH_2Cl_2 (10 mL) at 0 °C. The mixture was stirred at room temperature for 2 h, diluted with CH_2Cl_2 (20 mL), and washed with H_2O (15 mL) and 20% aqueous EDTA (15 mL). The organic layer was separated, dried over Na_2SO_4, and concentrated under vacuum to yield 0.67 g (97.0%) of **66** as a dark oil.

(*N*,*N*′-dimethyl-1,2,3,4-tetrahydroquinoxalin-2-yl)methyloxy-2,6-difluorobenzamide (15): Potassium carbonate (110 mg, 0.78 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (130 mg, 0.75 mmol) in DMF (2 mL). After stirring at room temperature for 30 min, a solution of **66** (150 mg, 0.71 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at 100 °C overnight, concentrated under vacuum, diluted with EtOAc (20 mL), washed with brine (3×10 mL), dried over Na_2SO_4, and concentrated to give a residue that was purified by flash chromatography on silica gel. Elution with cyclohexane/EtOAc (6:4) gave 80 mg (32.4%) of **15** as a brown waxy solid.

2-Methylquinoxaline (67): 1-Hydroxypropan-2-one (0.63 mL, 9.25 mmol) was added to a solution of toluene (40 mL), dry THF (6 mL), and TEA (1.30 mL, 0.925 mmol). After 5 min of stirring at room temperature, 1,2-diamino benzene (1.0 g, 9.25 mmol) and $Pd(OAc)_2$ (50 mg) were added. After stirring at room temperature for 3 h, the mixture was filtered through a Celite pad and washed with 5% aqueous HCl (2×15 mL) and 10% aqueous NaCl (15 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated to yield 0.80 g (60%) of **67** as a dark oil.

2-Chloromethylquinoxaline (68): Trichloroisocyanuric acid (0.13 g, 0.55 mmol) was added to a solution of **67** (0.18 g, 1.25 mmol) in $CHCl_3$ (2 mL) at reflux. After stirring for 1 h, the mixture was cooled to room temperature and quenched with ice water (20 mL); the pH was adjusted to 9, and the aqueous layer was further extracted with CH_2Cl_2 (3×20 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuum, giving 0.17 g (76.2%) of **68** as a yellow oil.

(Quinoxalin-2-yl)methyloxy-2,6-difluorobenzamide (16): Potassium carbonate (0.14 g, 0.99 mmol) was added to a solution of 2,6-difluoro-3-hydroxybenzamide (0.16 g, 0.94 mmol) in DMF (2 mL). After stirring at room temperature for 20 min, a solution of **68** (0.16 g, 0.90 mmol) in DMF (2 mL) was added. The reaction mixture was stirred at 80 °C for 16 h, concentrated, and diluted with water. The aqueous layer was extracted with EtOAc (3×20 mL). The combined organic layers were washed with brine (15 mL), dried over Na_2SO_4, filtered, and concentrated. The resulting solid residue was crystallized from chloroform to give 200 mg (70.5%) of **16** as a yellow solid.

Chemistry abbreviations: *Solvents*: DMF = *N*,*N*-dimethylformamide, DMSO = dimethyl sulfoxide, Et_2O = diethyl ether, IPA = isopropyl alcohol, IPE = isopropyl ether, THF = tetrahydrofuran. *Reagents*: CDI = 1,1′-carbonyldidimidazole, DIAD = diisopropyl azodicarboxylate, DIPEA = *N*,*N*-diisopropylethylamine, DMAP = 4-(dimethylamino)pyridine, EDAC·HCl = *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, EDTA = ethylenediaminetetraacetic acid, HOBt = 1-hydroxybenzotriazole hydrate, TEA = triethylamine.

Cells

Normal human lung fibroblasts (MRC-5) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin in an incubator at 5% CO_2 atmosphere and 37 °C. The Gram-positive *S. aureus* (MSSA ATCC 29213, and MRSA ATCC 43300) and Gram-negative *E. coli* (ESBL, extended-spectrum beta-lactamase-positive *E. coli*) bacterial cells were grown in Luria-Bertani Broth (LB) medium at 37 °C under constant shaking at 300 rpm.

Antibacterial activity

The antibacterial activities of compounds **1**–**16** and DFNB were tested using both a methicillin-sensitive and a methicillin-resistant *S. aureus* strain, and an ESBL *E. coli* clinical isolate. All the compounds were dissolved to a final concentration of 20 mg mL^{-1} in

dimethyl sulfoxide (DMSO) and serially diluted in LB medium. After incubation at 37 °C for 16 h in aerobic culture tubes, the concentration of prokaryotic cells was determined by optical density measurement at 600 nm (OD_{600}) using a SmartSpec 3000 spectrophotometer (Bio-Rad, Oceanside, CA, USA). Fresh cell cultures were used at 10^3 cells mL^{-1} in a final volume of 2 mL. Each bacterial sample was grown with different compound concentrations that ranged from 0.1 to 100 μg mL^{-1} for the initial screening of all compounds. For compounds **1**–**7**, **10**, and **12**, which were active at 10 μg mL^{-1} but not at 0.1 μg mL^{-1}, further analyses with intermediate concentrations were performed. After incubation of each sample overnight at 37 °C, an aliquot was collected under sterile conditions, and the OD_{600} was measured to determine the MIC value. To also determine the MBC value, the bacteria were then washed three times with LB, centrifuged at 900×*g* for 10 min at 4 °C, and the pellet was resuspended in fresh LB. After overnight incubation at 37 °C, the absence of growth was confirmed by OD_{600} measurement. Antibacterial tests were performed in triplicate twice for each series of experiments. Both positive (no compounds) and negative (no bacteria) controls were included.

Cytotoxicity assay

Compounds **1**–**7**, which all showed antibacterial activity at concentrations lower than 10 μg mL^{-1}, were serially diluted in DMEM and tested in MRC-5 cells using the thiazolyl blue tetrazolium bromide (MTT) cytotoxicity assay (Sigma, St. Louis, MO, USA). Cells (10^4 cells per well) were tested in a 96-well plate using serially twofold-diluted concentrations of the compound in 100 μL DMEM medium. After 24 h incubation, the compound was removed, and the cells were overlaid with 1 mg mL^{-1} MTT in 100 μL serum-free DMEM for 3 h at 37 °C. The MTT solution was then replaced with DMSO for 10 min, and the absorbance was measured at 570 nm. The percentage of cytotoxicity was calculated by the formula 100−(sample OD/untreated cells OD) ×100. The compound concentration reducing cell viability by 50 or 90% was defined as the TD_{50} or TD_{90} toxic dose. The therapeutic index (TI) was also determined and defined as the ratio between TD_{90} and the MBC values. In the MRC-5 cytotoxicity activity assays, all of the compounds were tested twice in triplicate. Standard deviations were calculated, when applicable, and are shown in Table 1.

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Conflict of interest

The authors declare no conflict of interest.

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