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Research Letter

Synthesis and Biological Evaluation of Novel FtsZ-targeted 3-arylalkoxy-2,6-difluorobenzamides as Potential Antimicrobial Agents

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Novel series of 3-*O*-arylalkylbenzamide and 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives were synthesized and evaluated for their on-target activity and antibacterial activity. The results indicated that the 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives possessed much better on-target activity and antibacterial activity than the 3-*O*-arylalkylbenzamide derivatives. Among them, 3-*O*-chlorobenzyl derivative 36 was the most effective in antibacterial activity (0.5, 4, and 8 μg/mL) against Bacillus subtilis ATCC9372, methicillin-resistant Staphylococcus aureus ATCC29213, and penicillin-resistant Staphylococcus aureus PR, while 3-O-methylbenzyl derivative 41 only exhibited the most potent activity (2 μg/mL) against *Staphylococcus aureus* ATCC25923.

Key words: 2,6-difluorobenzamide, antibacterial activity, FtsZ inhibitors, on-target activity, synthesis

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The widespread misuse of antibiotics has resulted in the emergence and prevalence of bacterial resistance, which makes many originally powerful antibiotics in clinic become either weak or no activity (1). In particular, multidrug-resistant Gram-positive pathogens have evolved which are extremely difficult to eradicate (2,3). Accordingly, the critical human health outcome of antibiotic resistance among bacterial pathogens worldwide necessitates the development of structurally novel antibacterial agents possessing new mechanisms of action.

FtsZ (filamentous temperature-sensitive protein Z) is a widely distributed key cytoskeletal protein in archaea and bacteria, which offers an essential skeleton for the formation of Z-ring in the presence of guanosine triphosphate (GTP) during bacterial cell division (4). Thus, FtsZ is considered as an attractive target to develop antibacterial agents with selective toxicity to bacterial pathogens due to its absence in the mitochondria of higher eukaryotes and evolutionary distance from tubulin (5).

Various studies have been conducted in recent years for finding the FtsZ inhibitors that exert their antibacterial activity by disturbing the polymerization behavior or the activated GTPase of FtsZ or both of them crucial for the formation and contraction of Z-ring (6–8). The FtsZ inhibitors are mainly some natural products such as totarol and curcumin (Figure 1) or small synthesized molecules (9–11). Among them, 3-methoxybenzamide (3-MBA) (Figure 1) is one of most attractive starting point for discovering more potent FtsZ inhibitors. For example, PC190723 (Figure 1) as a 3-MBA derivative exhibits potent antibacterial activity against *Bacillus subtilis* and various resistant *staphylococci* by inhibiting the GTPase activity and localization of FtsZ (12). The preliminary structure-activity relationship (SAR) demonstrates that the replacement of the 3-methoxy group of 3-MBA with various groups could result in drug-like 3-MBA derivatives with a substantial improvement in on-target antibacterial activity (13–15). The docking model also predicts an enough space equivalent in length to at least 9–10 carbons for substitutions off the mother nucleus of benzamide (16).

In our previous work, we reported the 3-arylalkoxybenzamide derivatives possessing the 3-elongated side chains with two to nine atoms from 3-oxygen atom to the terminal groups such as halogen and heteroaryl groups, exhibiting potent antibacterial activity (17,18). In this study, to explore the influence of the mother nucleus of 2,6-difluorobenzamide as well as 3-shortened side chains with one atom from 3-oxygen atom to the terminal phenyl groups on on-target activity and antibacterial activity, we designed several

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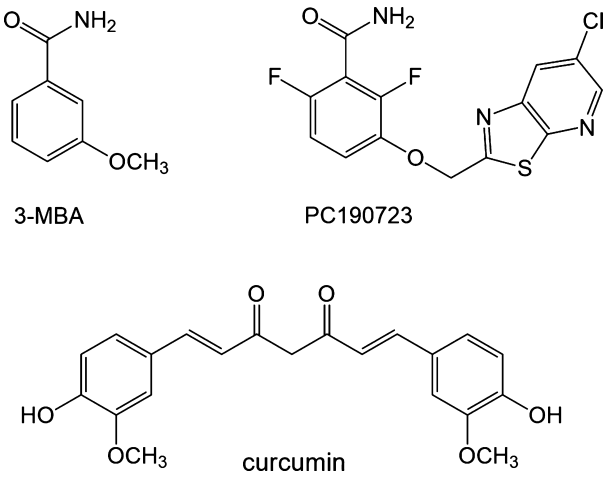


Figure 1: Structures of compounds inhibiting FtsZ polymerization Z-ring formation.

structural series of novel 3-*O*-arylalkylbenzamide and 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives that had mainly substituented benzyl side chain at the 3-position.

Methods and Materials

General

All necessary solvents were purified prior to use, unless noted otherwise. Reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (Qingdong Yumingyuan silica gel reagent factory, Shandong, China, YUYUAN). Flash column chromatography was performed with the indicated solvents using silica gel 60 (particle size 0.040–0.063 mm, Qingdong Yumingyuan silica gel reagent factory, Shandong, China, YUYUAN). Infrared spectra were recorded on KBr pellets using Nicolet Nexus 470FT-IR spectrometer (Denver, CO, USA). ^{1}H NMR spectra were recorded on Bruker Avance DRX 600 spectrometer (Bruker, Billerica, MA, USA) at ambient temperature (TMS as internal standard of chemical shifts). Mass spectra were recorded on API 4000 instrument (Applied Biosystems, Foster City, CA, USA). The C, H, N analyses were carried out on PE-2400 II elemental analyser (PerkineElmer, Waltham, MA, USA). Melting points are uncorrected and were determined on an X-6 melting point apparatus (Beijing Tianchengwode Biotech Co. Ltd, Beijing, China). Bacterial morphometric analysis was determined on Olympus CKX41 microscope (Center Valley, PA, USA).

Cell division inhibitory activity

Cell division inhibitory activity of the tested compounds was performed as described previously (19). Overnight cultures were grown in starvation medium supplemented with 1% hydrolyzed casein and then diluted in starvation medium supplemented with 3% hydrolyzed casein (*Bacillus subtilis* ATCC9372) or in (*Staphylococcus aureus* ATCC25923) and grown at 37 °C. The culture was diluted to A600 of ~0.06, and 10 μL of aliquots was added to transparent 96-well microtiter plates containing dilutions of the tested compounds, 3-MBA and curcumin as controls in 100 μL volumes of medium. After incubation for approximately 5 h (4–5 generations) at 37 °C, 20 μL of culture samples was transferred to poly-L-lysine-coated slides for microscopy. Cell morphology was assessed by phase-contrast light microscopy. The lowest concentration at which filamentation of *B. subtilis* ATCC9372 or ballooning of *S. aureus* ATCC25923 was recorded as the cell division inhibitory activity indicating on-target activity.

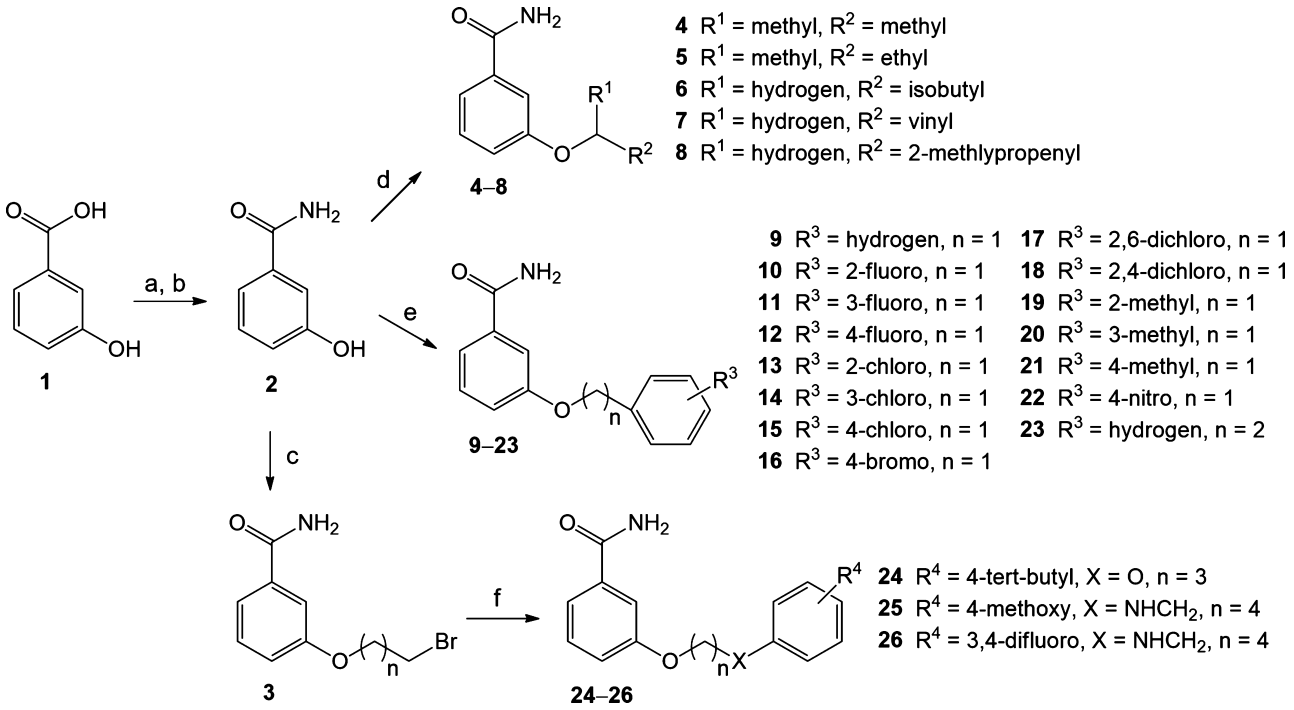
Antibacterial activity

The minimum inhibitory concentration (MIC) of the tested compounds was determined applying tube dilution method recommended by NCCLS (20). Bacterial strains were incubated on MHA (Mueller–Hinton Agar) medium at 37 °C for 24 h. The bacteria solution was prepared by suspension in 10 mL of sterile water for colonies from culture on MHA medium. MHB (Mueller–Hinton Broth) was used for bacteria in this test. The cell density of each inoculum was adjusted in sterile water of a 0.5 McFarland standard. In this method, various concentrations of the tested compounds were prepared from 128 to 0.25 μg/mL in sterile tubes No. 1 to 10. Hundred microliters of sterile Mueller–Hinton broth (MHB) was poured in each sterile tube followed by addition of 200 μL test compound in tube 1. Twofold serial dilutions were carried out from tube 1 to tube 10, and excess broth (100 μL) was discarded from the last tube No. 10. To each tube, 100 μL of standard inoculum (1.5×10^8 cfu/mL) was added. 3-MBA and curcumin were used as controls. Turbidity was observed after incubating the inoculated tubes at 37 °C for 24 h. The last tube with no growth of micro-organism was recorded to represent the MIC value expressed in μg/mL. The tested bacteria were four penicillin-susceptible strains of *B. subtilis* ATCC9372, *S. aureus* ATCC25923, *Streptococcus pneumonia* ATCC49619, and *Streptococcus pyogenes* PS, two resistant strains of methicillin-resistant *S. aureus* and penicillin-resistant *S. aureus* PR.

Results and Discussion

Chemistry

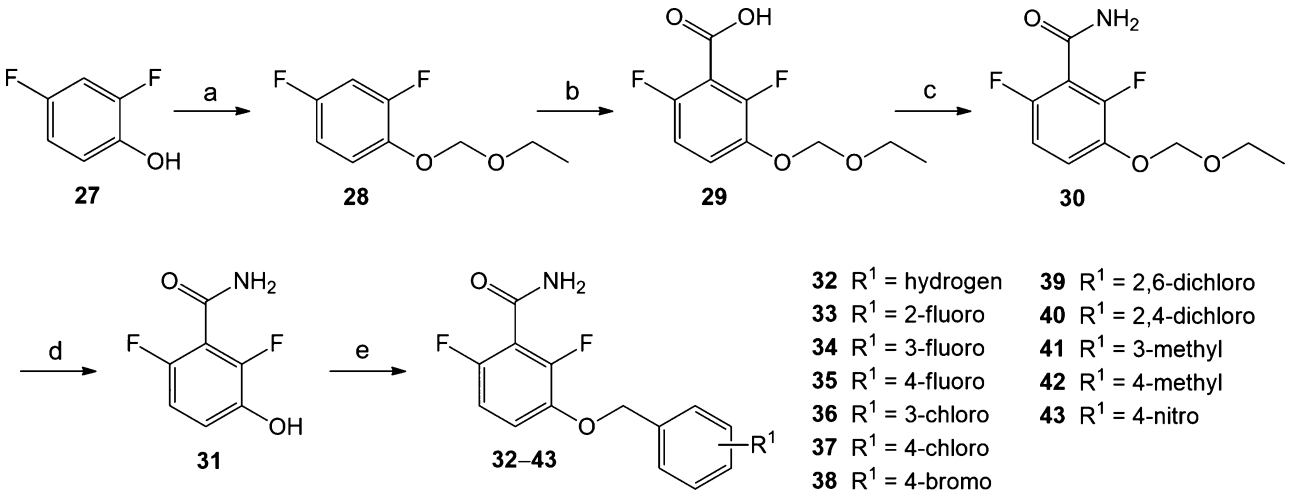
The 3-*O*-arylalkylbenzamide derivatives (**4**–**26**) were synthesized from the commercially available 3-hydroxybenzoic acid (**1**) as outlined in Scheme 1. The chlorination of **1** with thionyl chloride was followed by reaction with aqueous ammonia to give 3-hydroxybenzamide (**2**) in a good yield. The alkylation of **2** with alkyl chloride or bromide (sodium iodide was not needed for the reaction with alkyl bromide) in the presence of sodium iodide and potassium carbonate afforded 3-*O*-alkylbenzamide derivatives (**4**–**8**) in 57–63% yields. Similarly, the alkylation of **2** with phenylalkyl chloride or bromide provided 3-*O*-arylalkylbenzamide



Scheme 1: Reagents and conditions: (a) $SOCl_2$, toluene, reflux, 4.5 h; (b) aqueous ammonia, rt, 18 h, 71% for two steps; (c) 1,3-dibromopropane (or 1,4-dibromobutane), K_2CO_3, CH_3CN, 60 °C, 18 h, 65–67%; (d) alkyl chloride and KI (or alkyl bromide), K_2CO_3, DMF, 60–65 °C, 16–18 h, 57–63%; (e) phenylalkyl chloride (or bromide), K_2CO_3, DMF, 60–65 °C, 18–20 h, 70–77%; (f) substituted phenol (or benzylamine), K_2CO_3, DMF, 45–60 °C, 12–14 h, 52–65%.

derivatives (**9**–**23**) in 70-77% yields. The alkylation of **2** with alkyl dibromide was subsequent reaction with substituted phenol or benzylamine to produce 3-*O*-elongated arylalkyl-benzamide derivatives (**24**–**26**) in yields ranging from 52 to 65%.

The 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives (**32**–**43**) were synthesized from the commercially available 2,4-difluorophenol (**27**) as outlined in Scheme 2. The etherification of **27** with chloromethyl ethyl ether in the presence of DIPEA (N,N-diisopropylethylamine) was followed by pouring onto freshly crushed carbon dioxide in the presence of n-BuLi (n-butyl lithium) to provide 1-(ethoxymethoxy)-2,6-difluorobenzoic acid (**29**) in an excellent yield. The ammoniation of **29** in the presence of DIPEA and ethyl chloroformate was subsequently



Scheme 2: Reagents and conditions: (a) chloromethyl ethyl ether, DIPEA, CH_2Cl_2, 0 °C, 2 h, 91%; (b) *n*-BuLi, CO_2, −78 °C, 2 h, 93%; (c) aqueous ammonia, DIPEA, ethyl chloroformate, rt, 6 h, 72% (d) 6M HCl: CH_3OH (1:1), rt, 2 h, 70% (e) substituted benzyl chloride and KI (or substituted benzyl bromide), K_2CO_3, DMF, 40–45 °C, 12–14 h, 52–72%.

subjected to deprotection of ethoxymethyl group to afford 3-hydroxy-2,6-difluorobenzamide (**31**) in good yield. The alkylation of **31** with substituted benzyl chloride or bromide (sodium iodide was not needed for the benzyl bromide) in the presence of sodium iodide and potassium carbonate produced the target compounds **32**–**43** in 61-66% yields.

Biological activity

Cell division inhibitory activity and *in vitro* antibacterial activity for the 3-*O*-arylalkylbenzamide derivatives **4**–**26** are shown in Table 1. Almost all of them showed greatly improved cell division inhibitory activity against *B. subtilis* and *S. aureus*, and significantly enhanced antibacterial activity against *B. subtilis* and three tested strains of *S. aureus*, but failed to exhibit an improvement in antibacterial activity against *S. pyogenes* and *S. pneumonia* compared with 3-MBA or their precursor **2**. In the subseries of the 3-*O*-alkylbenzamide derivatives **4**–**8**, compound **6** with isobutyl group was found to possess not only the most greatly improved cell division inhibitory activity against *B. subtilis* ATCC9372 and *S. aureus* ATCC25923, being 128- and 256-fold better than its precursor **2**, but also the most remarkably antibacterial activity against *B. subtilis* ATCC9372, *S. aureus* ATCC25923, *S. aureus* ATCC29213, and *S. aureus* PR, exhibiting 64-, 256-fold, 128-, and 256-fold higher activity than 3-MBA, respectively. In the subseries of the 3-*O*-arylalkylbenzamide derivatives **9**–**23**, compound **14** with 3-*O*-chlorobenzyl group was the most effective in cell division inhibitory activity against *B. subtilis* ATCC9372 and antibacterial activity against *B. subtilis* ATCC9372 and *S. aureus* PR, showing 512-, 32-, and 256-fold better activity than their precursor **2**, respectively. In contrast, compound **20** with 3-*O*-methylbenzyl group was the most active in cell division inhibitory activity against *S. aureus* ATCC25923 and antibacterial activity against *S. aureus* ATCC25923, *S. aureus* ATCC29213, and *S. aureus* PR, being 1024-, 512-, 256-, and 256-fold better than their precursor **2**, respectively. As for the subseries of the 3-*O*-elongated arylalkylbenzamide derivatives **24**–**26**, however, they did not displayed markedly improved on-target activity and antibacterial activity.

Cell division inhibitory activity and *in vitro* antibacterial activity for the 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives **32**–**43** are shown in Table 2. This series presented an apparent improvement in cell division inhibitory activity against *B. subtilis* and *S. aureus* and antibacterial activity against all the tested strains in comparison with their precursor **31** and curcumin. In particular, compound **36** bearing 3-*O*-chlorobenzyl group showed the best cell division inhibitory activity (0.5 μg/mL) against *B. subtilis* ATCC9372 and the strongest antibacterial activity (0.5, 4, and 8 μg/mL) against *B. subtilis* ATCC9372, *S. aureus* ATCC29213, and *S. aureus* PR, displaying 2048-, 128-, and 64-fold better activity than its precursor **31**, respectively, while compound **41** bearing 3-*O*-methylbenzyl group exhibited the most effective cell division inhibitory activity (2 μg/mL) and antibacterial activity (2 μg/mL) against *S. aureus* ATCC25923, revealing 1024- and 512-fold higher activity than its precursor **31**, respectively. Furthermore, compounds **36** and **41** exerted the most powerful activity against the above-mentioned bacterial strains among all of the tested compounds **4**–**26** and **32**–**43** as well. Although 3-chloro and 3-methyl groups of the two compounds shared conflicting electrical characters to each other, they showed electron-donating groups on the benzene ring in the structures and belonged to hydrophobic groups. The two hydrophobic groups might display the hydrophobic interactions with the binding site of the thiazolopyridine moiety of PC190723, formed by the amino acid residues (21). Besides, compounds **34**, **37**, **38** and **41** against *B. subtilis* ATCC9372 and compound **36** against *S. aureus* ATCC25923 were also found to possess potent antibacterial activity (8, 8, 8 and 4 μg/mL).

In general, the two subseries of the 3-*O*-arylalkylbenzamide derivatives **9**–**22** and the 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives **32**–**43** had the similar trend in cell division inhibitory activity and antibacterial activity. In addition, the most active compounds **14** and **36** in their respective subseries had the same side chain of 3-*O*-chlorobenzyl group, and similarly, the most active compounds **20** and **41** also had the same side chain of 3-*O*-methylbenzyl group. However, the 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives **32**–**43** showed much more potent on-target activity and antibacterial activity than the 3-*O*-arylalkylbenzamide derivatives **9**–**22.** For example, compound **36** was 32-, 4-, and 8-fold more effective than compound **14** in antibacterial activity against *B. subtilis* ATCC9372, *S. aureus* ATCC25923, and *S. aureus* ATCC29213, respectively, while compound **41** was 8- and 4-fold more active than compound **20** in antibacterial activity against *B. subtilis* ATCC9372 and *S. aureus* ATCC25923, respectively. The difference in the antibacterial activity could be attributed to the mother nucleus of 2,6-difluorobenzamide that was more helpful in a remarkable improvement in on-target activity and antibacterial activity than that of the benzamide. For instance, compounds **14** and **36** might bind to the binding pocket of PC190723 on FtsZ due to their structural similarities. The crystal structure of PC190723 with *S. aureus* FtsZ has indicated that two fluoro groups in its scaffold display important hydrophobic interactions with the surrounding amino acid residues (21). Accordingly, the hydrophobic interactions also make compound **36** share better activity than compound **14**. These results described above made us conclude that (i) the introduction of the 3-monosubstituented benzyl side chain would be more beneficial for increasing cell division inhibitory activity against *B. subtilis* and *S. aureus* and antibacterial activity against *B. subtilis* ATCC9372, *S. aureus* ATCC25923, *S. aureus* ATCC29213, and *S. aureus* PR than other monosubstituented or multisubstituented benzyl side chain and especially, 3-*O*-chloro or 3-*O*-methyl group on the benzyl

Table 1: Cell division inhibitory activity and antibacterial activity for the 3-*O*-arylalkoxybenzamide derivatives

	Cell division inhibition[a] (μg/mL)		Minimum inhibitory concentration/MIC(μg/mL)						
Compound	*B. subtilis* ATCC9372[b]	*S. aureus* ATCC25923[c]	*B. subtilis* ATCC9372	*S. aureus* ATCC25923	*S. aureus* ATCC29213[d]	*S. aureus* PR[e]	*S. pyogenes* PS[f]	*S. pyogenes* PR[g]	*S. pneumoniae* ATCC49619[h]
3-MBA	512	WT[i]	4096	2048	2048	4096	>128	>128	>128
2	4096	4096	512	4096	4096	4096	>128	>128	>128
4	256	WT	512	>128	>128	>128	>128	>128	>128
5	64	256	128	64	64	64	>128	>128	>128
6	32	16	32	8	16	16	>128	>128	>128
7	128	256	256	64	64	128	>128	>128	>128
8	128	32	32	32	64	64	>128	>128	>128
9	64	128	128	>128	>128	>128	>128	>128	>128
10	128	512	256	>128	>128	>128	>128	>128	>128
11	64	128	128	>128	>128	>128	>128	>128	>128
12	1024	1024	2048	>128	>128	>128	>128	>128	>128
13	1024	1024	2048	>128	>128	>128	>128	>128	>128
14	8	64	16	16	32	16	>128	>128	>128
15	256	WT	2048	>128	>128	>128	>128	>128	>128
16	WT	256	1024	>128	>128	>128	>128	>128	>128
17	512	516	1024	>128	>128	>128	>128	>128	>128
18	2048	WT	2048	>128	>128	>128	>128	>128	>128
19	512	WT	512	>128	>128	>128	>128	>128	>128
20	16	4	32	8	16	16	>128	>128	>128
21	1024	512	1024	>128	>128	>128	>128	>128	>128
22	WT	512	2048	>128	>128	>128	>128	>128	>128
23	16	4096	16	>128	>128	>128	>128	>128	>128
24	256	1024	256	>128	>128	>128	>128	>128	>128
25	WT	WT	1024	>128	>128	>128	>128	>128	>128
26	WT	1024	1024	>128	>128	>128	>128	>128	>128

[a]Lowest concentration at which filamentation of *B. subtilis* or ballooning of *S. aureus* is observed indicating on-target activity.
[b]*B. subtilis* ATCC9372: penicillin-susceptible strain.
[c]*S. aureus* ATCC25923: penicillin-susceptible strain.
[d]*S. aureus* ATCC29213: methicillin-resistant strain.
[e]*S. aureus* PR: penicillin-resistant strain isolated clinically, not characterized.
[f]*S. pyogenes* PS: penicillin-susceptible strain.
[g]*S. pyogenes* RP: penicillin-resistant strain.
[h]*S. pneumoniae* ATCC49619: penicillin-susceptible strain.
[i]No effect on morphology at 2048–4096 μg/mL.

Table 2: Cell division inhibitory activity and antibacterial activity for the 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives

	Cell division inhibition[a] (μg/mL)		Minimum inhibitory concentration/MIC(μg/mL)						
Compound	*B. subtilis* ATCC9372[b]	*S. aureus* ATCC25923[c]	*B. subtilis* ATCC9372	*S. aureus* ATCC25923	*S. aureus* ATCC29213[d]	*S. aureus* PR[e]	*S. pyogenes* PS[f]	*S. pyogenes* PR[g]	*S. pneumoniae* ATCC49619[h]
Curcumin	16	1024	32	2048	4096	4096	>4096	>4096	>4096
31	1024	2048	1024	1024	512	1024	4096	>4096	4096
32	16	32	16	32	64	64	>128	>128	128
33	64	128	64	128	128	>128	>128	>128	32
34	8	128	8	32	32	64	>128	>128	32
35	32	128	64	128	128	128	>128	>128	>128
36	0.5	4	0.5	4	4	8	32	64	>128
37	8	64	8	64	>128	64	128	>128	32
38	8	64	8	64	>128	128	>128	>128	64
39	1024	256	>128	>128	>128	>128	>128	>128	>128
40	1024	256	>128	>128	>128	>128	>128	>128	>128
41	2	2	4	2	32	32	>128	>128	128
42	8	32	32	>128	>128	>128	>128	>128	>128
43	64	64	64	>128	128	128	>128	>128	>128

[a]Lowest concentration at which filamentation of *B. subtilis* or ballooning of *S. aureus* is observed indicating on-target activity.
[b]*B. subtilis* ATCC9372: penicillin-susceptible strain.
[c]*S. aureus* ATCC25923: penicillin-susceptible strain.
[d]*S. aureus* ATCC29213: methicillin-resistant strain.
[e]*S. aureus* PR: penicillin-resistant strain isolated clinically, not characterized.
[f]*S. pyogenes* PS: penicillin-susceptible strain.
[g]*S. pyogenes* RP: penicillin-resistant strain.
[h]*S. pneumoniae* ATCC49619: penicillin-susceptible strain.
[i]No effect on morphology at 2048–4096 μg/mL.

side chain could be the best 3-monosubstituented group in exerting the antibacterial activity, (ii) the mother nucleus of 2,6-difluorobenzamide could play an more important role than that of the benzamide in enhancing cell division inhibitory activity and antibacterial activity against the bacterial strains mentioned above. Notably, the findings also clearly revealed that the 3-monosubstituented benzyl side chain might be a suitable for interaction with the hydrophobic cleft in FtsZ while 2,6-difluoro groups on the benzamide might further enhanced affinity for FtsZ.

Conclusion

Several structural series of novel 3-*O*-arylalkylbenzamide and 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives were designed, synthesized, and evaluated for their cell division inhibitory activity and antibacterial activity. They presented greatly improved on-target activity and antibacterial activity. In particular, the 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives showed much more potent cell division inhibitory activity and antibacterial activity than the 3-*O*-arylalkylbenzamide derivatives**.** Among all of the tested compounds, compound **36** displayed the best cell division inhibitory activity (0.5 μg/mL) against *B. subtilis* ATCC9372 and the strongest antibacterial activity (0.5, 4, and 8 μg/mL) against *B. subtilis* ATCC9372, *S. aureus* ATCC29213, and *S. aureus* PR, while compound **41** exerted the most effective cell division inhibitory activity (2 μg/mL) and the most active antibacterial activity (2 μg/mL) against *S. aureus* ATCC25923. Moreover, compounds **36** and **41** possessing 3-*O*-chlorobenzyl group and 3-*O*-methylbenzyl group on 2,6-difluorobenzamide, respectively, belonged to the series of 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives. It is worth noting that the introduction of the 3-monosubstituented benzyl side chain into the mother nucleus of 2,6-difluorobenzamide could greatly increase cell division inhibitory activity against *B. subtilis* and *S. aureus* and antibacterial activity against *B. subtilis* ATCC9372, *S. aureus* ATCC25923, *S. aureus* ATCC29213, and *S. aureus* PR than that of other monosubstituented or multisubstituented benzyl side chain.

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Conflict of Interest

All authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Experimental procedures for synthesis and spectral data of intermediates and final compounds.