Design, synthesis and biological activity evaluation of novel 2,6-difluorobenzamide derivatives through FtsZ inhibition

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Design, synthesis and biological activity evaluation of novel 2,6-difluorobenzamide derivatives through FtsZ inhibition

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Abstract

Novel series of 3-substituted 2,6-difluorobenzamide derivatives as FtsZ inhibitors were designed, synthesized and evaluated for their *in vitro* antibacterial activity against various phenotype of Gram-positive and Gram-negative bacteria, and their cell division inhibitory activity against three representative strains. As a result, 3-chloroalkoxy derivative **7**, 3-bromoalkoxy derivative **12** and 3-alkyloxy derivative **17** were found to exhibit the best antibacterial activity against *Bacillus subtilis* with MICs of 0.25-1 μg/mL, and good activity (MIC<10 μg/mL) against both susceptible and resistant *Staphylococcus aureus*. Additionally, all the three compounds displayed potent cell division inhibitory activity with MIC values of below 1 μg/mL against *Bacillus subtilis* and *Staphylococcus aureus*.

Keywords: benzamide derivatives; design and synthesis; FtsZ inhibition; biological activity.

Bacterial resistance to current antibiotic therapies is a major global public health concern.[1, 2] The problem of antibiotic resistance is magnified by the lack of new therapeutic agents and the reduced investment of the pharmaceutical industry.[3, 4] There are two main strategies to overcome this problem. One is to extensively modify existing antibacterial drugs in order to maintain activity against their targets and the other is to develop novel antibacterial agents that can work by unique mode of action on known targets or by interacting with novel targets.

Filamentous temperature-sensitive protein Z (FtsZ), which is an essential cell division protein of bacteria, has been recently considered as an attractive target for the development of new antibacterial drugs.[5] When bacteria divide, FtsZ with bound GTP polymerizes into tubulin-like protofilaments at mid-cell that serves as a scaffold for the recruitment and organization of other cell division proteins, causing assembly of the septal ring.[6-8] The septal ring then constricts in concert with septal progression, enabling the daughter cells to separate.[9] The appeal of FtsZ as an antibacterial target lies in its essential role for bacterial viability, its highly conservation among important bacterial pathogens and its absence in higher eukaryotes.[7, 10] Accordingly, disrupting proper FtsZ assembly could result in bacterial growth inhibition and cell death.[11] To this end, various chemical classes of FtsZ inhibitors have been identified by different investigation approaches, such as 3-methoxybenzamide (3-MBA)[12], cinnamaldehyde[13], curcumin[14], benzimidazole[15], etc (Fig. 1).

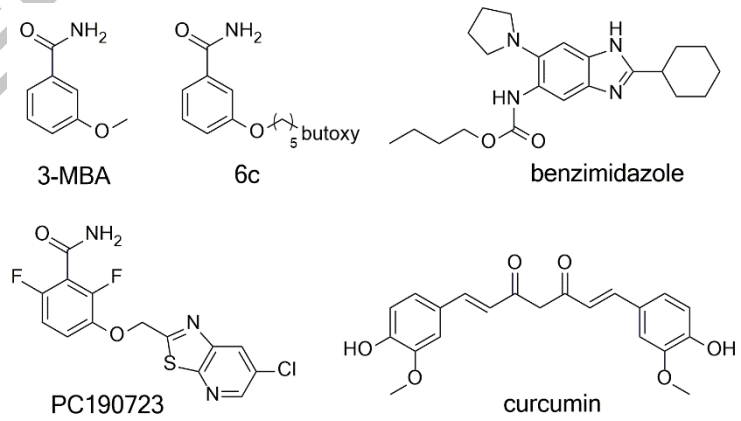


Fig. 1. Structures of some mentioned compounds inhibiting FtsZ

In particular, 3-MBA is one of the most attractive starting point for discovering FtsZ-targeting antibacterial compounds. For example, PC190723, a synthetic analogues of 3-MBA, has demonstrated potent activity against *B. subtilis* and various resistant *S. aureus* by disruption of FtsZ function.[16-18] The

preliminary structure-activity relationship (SAR) indicates that the amide functional group is critical for inhibition of cell division, and 2,6-difluoro groups on the benzamide can substantially enhance inhibitory activity, while the 3-alkyloxy side chain could continue to be investigated for further improvement in the on-target activity and the antibacterial activity.[19-21] Accordingly, leaving the amino function unchanged and introducing a reasonably long hydrophobic side chain or a preferred aromatic heterocycle on the 3-oxygen, will coordinate with the hydrophobic pocket and enhance their interaction. We speculate that this rational design may result in a significant improvement in the on-target activity and the antibacterial activity. Actually, in our previous work, compound **6c** that just held the two simple pharmacophores exhibited good antibacterial activity against *B. subtilis* ATCC9372 and *S. aureus* ATCC29213 with MICs of 8 and 4 μg/mL. Furthermore, *in silico* docking model performed by AutoDock has further indicated that compound **6c** bound into SaFtsZ (3vob.pdb) in the same pocket as PC190723.[22] Its amide group and ether oxygen atom formed strong hydrogen bonds with the surrounding amino acid residues in the pocket, and its flexible ether side chain extended into a hydrophobic channel formed by the H7 helix and the C terminal sheet of the SaFtsZ (Fig. 2).

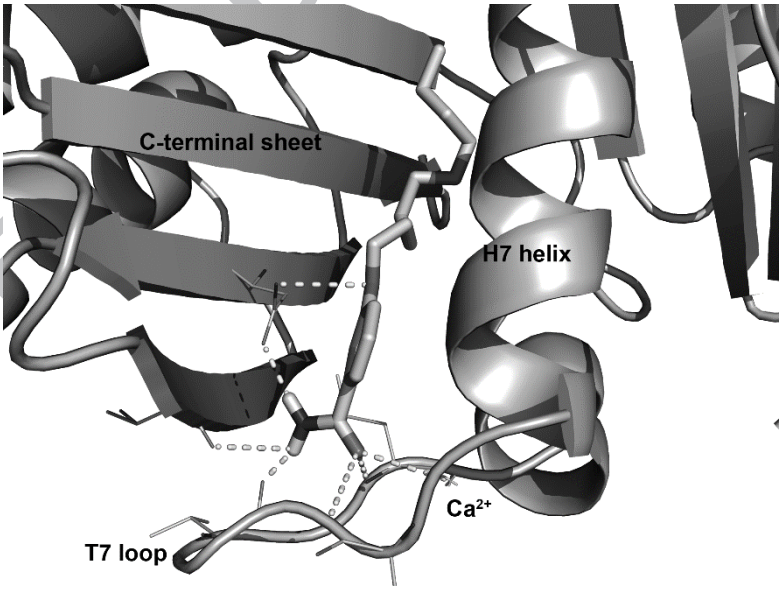
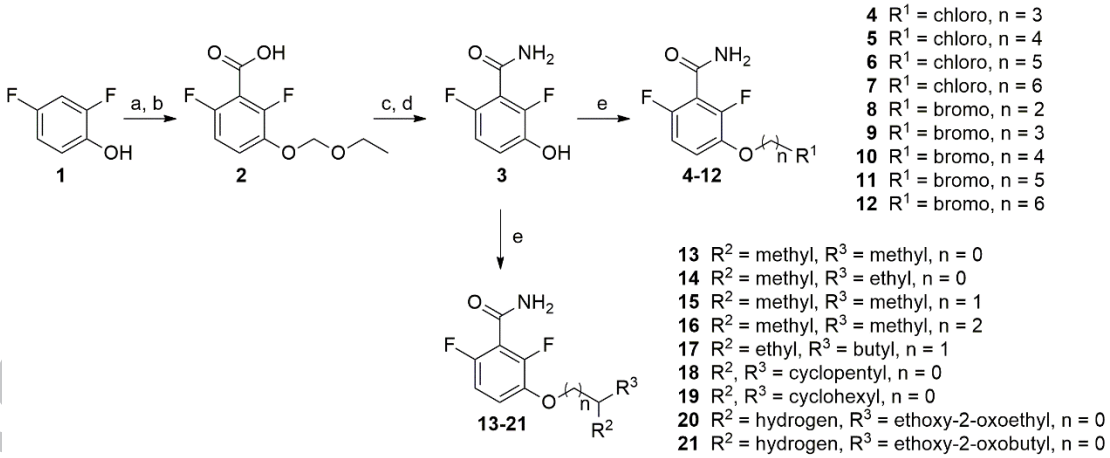


Fig. 2. Model of compound **6c** docked into *Sa*FtsZ (PDB code, 3vob). In the model, the predicted hydrogen bonds between compound **6c** and amino acid residues are indicated by dotted lines.

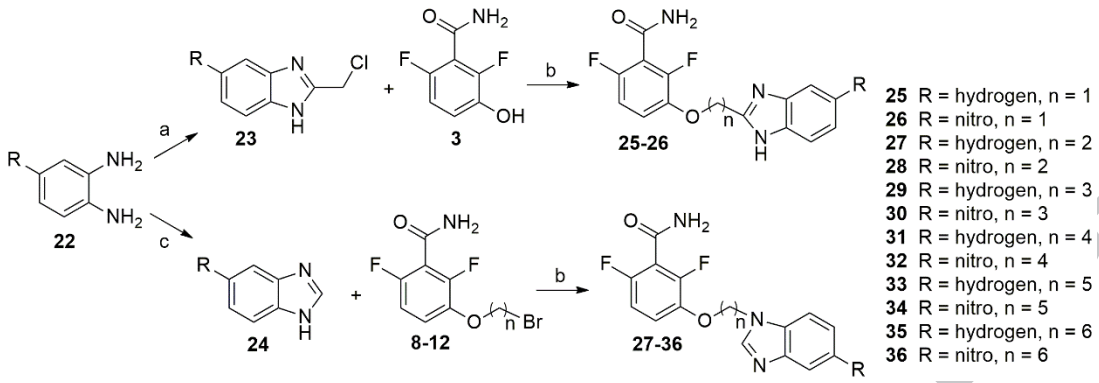
On the basis of the consideration detailed above, we designed a structural series of novel 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives by introducing fluorine atoms at the 2,6 positions of the benzene ring, the 3-*O*-arylalkyl side chain of which possessed various groups such as alkyl halide,

branched alkyl, ester, and heterocyclic groups. It's hoped that the 2,6-difluorobenzamide derivatives could exhibit more potent anti-FtsZ effect and higher antibacterial activity against various drug-susceptible and -resistant bacterial strains.

The synthesis of compounds **4**–**21** and **25**–**36** is shown in Schemes 1 and 2, respectively (Supplementary data, Chemical Synthesis). The commercially available 2,4-difluorophenol **1** was etherified with chloromethyl ethyl ether in the presence of DIPEA (N,N-diisopropylethylamine) and the resulting product was poured onto crushed carbon dioxide in the presence of n-BuLi (n-butyl lithium) to give protected 2,6-difluorobenzoic acid **2**. The acid was converted into amide in the presence of aqueous ammonia, DIPEA and ethyl chloroformate, and then deprotected to provide 3-hydroxy-2,6-difluorobenzamide **3**. The alkylation of **3** with different alkyl chloride or bromide in the presence of potassium carbonate afforded 3-*O*-alkyl-2,6-difluorobenzamide derivatives **4**–**21**. Similarly, the alkylation of **3** with 2-chloromethyl benzimidazole **23** gave 2-benzoimidazol-2,6-difluorobenzamide derivatives **25** and **26**. The nucleophilic substitution of the intermediates **8**–**12** with benzimidazole **24** provided 1-benzoimidazol-2,6-difluorobenzamide derivatives **27**–**36**.



Scheme 1. Reagents and conditions: (a) chloromethyl ethyl ether, DIPEA, CH_2Cl_2, 0 °C, 2 h, 91.1%; (b) n-BuLi, CO_2, -78 °C, 2 h, 93.4%; (c) aqueous ammonia, DIPEA, ethyl chloroformate, rt, 6 h, 71.6%; (d) 6M HCl, CH_3OH (1:1), rt, 2 h, 70.1%; (e) alkyl chloride or alkyl bromide, K_2CO_3, DMF, 25–45 °C, 12–14 h, 48.2–67.0%.



Scheme 2. Reagents and conditions: (a) chloroacetic acid, 4M HCl, reflux, 4 h, 79.4%; (b) K_2CO_3, DMF, 60–65 °C, 12–14 h, 55.1–62.2%; (c) formic acid, 4M HCl, reflux, 4 h, 78.1%.

All the synthesized compounds **4**–**21** and **25**–**36** were determined for their biological activity, including the *in vitro* antibacterial activity and the cell division inhibitory activity (Supplementary data, Antibacterial Testing). The MIC value of *in vitro* antibacterial activity was determined with the application of the standard broth microdilution method recommended by NCCLS[23]. Curcumin and the key intermediate **3** were used as the FtsZ-targeted references. The tested strains included six Gram-positive strains of *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* PR, *B. subtilis* ATCC 9372, *S. pneumoniae* ATCC 49619 and *S. pyogenes* PS, two Gram-negative strains of *E. coli* ATCC 25922 and *P. aeruginosa* ATCC27853. The results of minimum inhibitory activity in units of μg/mL are shown in Table 1.

The cell division inhibitory activity of the synthesized compounds against three representative strains including *B. subtilis* ATCC9372, *S. aureus* ATCC25923 and *E. coli* ATCC25922 was assessed by analyzing the cell morphology with the application of phase-contrast light microscopy[24]. Lowest drug concentration at which filamentation of *B. subtilis* and *E. coli* or ballooning of *S. aureus* appeared was recorded as cell division inhibitory concentration indicating on-target activity. The results of cell division inhibitory activity in units of μg/mL are shown in Table 2.

Table 1

3-O-Arylalkyl-2,6-difluorobenzamide derivatives with their antibacterial activity.

Compd	Minimum inhibitory concentration/ MIC (μg/mL)							
	B. subtilis ATCC9372[a]	*S. aureus* ATCC25923[b]	*S. aureus* ATCC29213[c]	*S. aureus* PR[d]	*S. pyogenes* PS[e]	*S. pneumoniae* ATCC49619[f]	*E.coli* ATCC25922[g]	*P. aeruginosa* ATCC27853[h]
curcumin	32	2048	4096	4096	>4096	>4096	512	4096
3	1024	1024	512	1024	>4096	4096	512	512
4	32	16	32	32	128	>128	128	128

5	4	8	8	8	64	128	128	128
6	4	16	32	16	64	128	128	128
7	0.5	2	16	8	>128	128	256	256
8	16	16	64	32	64	>128	128	128
9	32	16	32	32	64	>128	128	128
10	4	4	32	8	128	>128	128	128
11	16	8	16	16	128	128	256	256
12	1	1	2	2	64	>128	128	128
13	256	256	256	256	>128	128	256	128
14	128	64	128	128	>128	128	128	128
15	64	32	128	64	>128	128	256	128
16	8	64	16	16	>128	128	128	128
17	0.25	1	8	8	>128	64	256	128
18	512	256	256	512	>128	128	128	128
19	64	128	256	256	>128	128	128	128
20	64	128	256	256	>128	128	128	128
21	64	64	128	256	>128	128	256	128
25	1024	1024	512	1024	1024	256	256	256
26	64	512	256	512	1024	256	512	256
27	1024	1024	512	1024	1024	512	512	1024
28	64	256	512	1024	512	256	256	512
29	1024	1024	512	1024	1024	512	512	512
30	256	1024	512	1024	512	512	1024	512
31	512	1024	512	1024	1024	512	1024	512
32	64	1024	256	512	256	128	512	512
33	512	512	512	512	1024	512	1024	512
34	512	512	1024	2048	1024	512	512	512
35	256	512	256	1024	512	256	512	512
36	1024	1024	512	1024	512	256	512	512

[a] *B. subtilis* ATCC9372: standard susceptible strain.

[b] *S. aureus* ATCC25923: standard susceptible strain.

[c] *S. aureus* ATCC29213: methicillin-resistant strain.

[d] *S. aureus* PR: penicillin-resistant strain isolated clinically, not characterized.

[e] *S. pyogenes* PS: penicillin-susceptible strain.

[f] *S. pneumoniae* ATCC49619: standard susceptible strain.

[g] *E.coli* ATCC25922: standard susceptible strain.

[h] *P. aeruginosa* ATCC27853: standard susceptible strain.

Table 2

3-O-Arylalkyl-2,6-difluorobenzamide derivatives with their cell division inhibitory activity.

Compd	Cell division inhibition[a] (μg/mL)		
	B. subtilis ATCC937	*S. aureus* ATCC25923	*E.coli* ATCC25922
curcumin	16	1024	256
3	1024	2048	512
4	16	32	64
5	2	4	64
6	2	16	64
7	0.25	0.5	128
8	2	16	32
9	16	16	64
10	1	4	64
11	2	4	64
12	0.5	1	64
13	128	128	64

14	128	32	64
15	32	16	64
16	4	4	64
17	0.125	0.25	64
18	256	256	64
19	64	128	64
20	32	64	64
21	32	128	64
25	OT[b]	512	128
26	64	128	128
27	OT	OT	256
28	32	256	128
29	512	256	512
30	128	1024	512
31	OT	OT	512
32	OT	512	256
33	OT	OT	512
34	32	OT	256
35	128	128	256
36	64	OT	256

[a] Lowest concentration at which filamentation of *B. subtilis* and *E.coli* or ballooning of *S. aureus* is observed indicating cell division inhibitory activity.

[b] off-target activity: no effect on morphology at 2048-4096 μg/mL.

All the synthesized 3-alkyloxy derivatives **4**–**21** displayed significantly improved antibacterial activity against all the tested strains in comparison with curcumine and their precursor **3**. Especially for *B. subtilis* ATCC9372 and three *S. aureus* strains of *S. aureus* ATCC25923, *S. aureus* ATCC29213 and *S. aureus* PR, the most potent compounds exhibited 4096-, 1024-, 256- and 512-fold higher efficacy against those four strains than the precursor **3** respectively, indicating a successful optimization in this program.

In the subseries of the 3-chloroalkoxy derivatives **4**–**7** and 3-bromoalkoxy derivatives **8**–**12**, there was a clear trend that the longer carbon chain from the 3-oxygen atom to the terminal halogen resulted in the better antibacterial activity. For instance, compounds **7** and **12** possessing a 6 carbon atoms chain exhibited much stronger antibacterial activity against *B. subtilis* (MICs, 0.5 and 1 μg/mL) than their analogs **4** and **9** with a 3 carbon atoms chain (MICs, 32 and 32 μg/mL). In the subseries of the branched alkyl substituted derivatives **13**–**19**, those compounds also showed an improved trend in the antibacterial activity with elongation of their 3-alkoxy side chain. Among them, compound **17** with 3-*O*-(2-ethyl) hexyl group exhibited the most potent activity with MIC values of 0.25, 1, 8 and 8 μg/mL against above four *B. subtilis* and *S. aureus* strains, respectively. For the cycloalkyl substituted derivatives **18** and **19**, however, the improvement of their antibacterial activity was not obvious. This could be because the hydrophobic channel tolerating the 3-substituents was volume-limited. When the elongated 3-*O*-alkyl side chain was changed to 3-*O*-ester side chain containing five or seven atoms length, the antibacterial

activity was decreased. For example, compounds **20** and **21** showed relatively weak activity against *B. subtilis* with same MIC values of 64 μg/mL, indicating that the interaction sites could not prefer the ester chains to the alkyl chains.

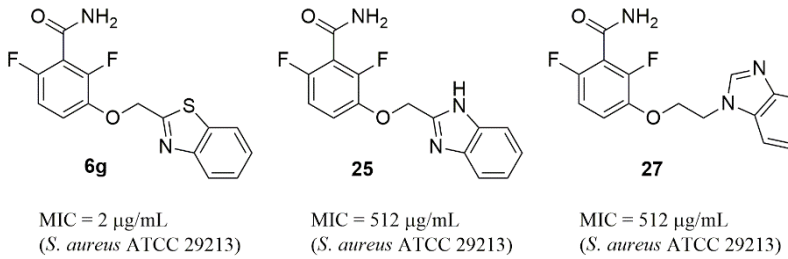


Fig. 3. Structures of compounds **6g**, **25** and **27**

By contrast, the benzimidazolyl substituted derivatives **25**–**36** just showed slightly improved antibacterial activity against those tested strains compared to curcumine and their precursor **3**. In previous studies of Haydon, D.J, compound **6g** containing a benzothiazole (Fig. 3) as a key precursor leading to the discovery of PC190723, displayed potent antibacterial and cell division inhibitory activity against *S. aureus* with levels of 2 and 4 μg/mL, respectively.[25] Benzimidazole and benzothiazole share similar molecular skeleton, which are widely applied as versatile and important pharmacophores in medicinal chemistry due to their good antitumor, antiviral and antimicrobial activities.[26-28] Given that, we replaced the benzothiazole with benzimidazole to obtain compound **25** (Fig. 3). However, this modification did not achieve a desired result. We suspected that the polar secondary amine of benzimidazole was not suitable for interaction with the hydrophobic channel. In order to reduce the hydrophilicity of benzimidazole, different length side chains were introduced into the polar secondary amine, providing compounds **27**–**36**. But none of this subseries could showed comparable antibacterial activity to compound **6g**. We attributed this contrast to speculation that the stereostructure of this hydrophobic binding site might be very strict with the 3-substituted groups, especially with the rigid heterocyclic moieties while the flexible side chains could fit comfortably into the critical hydrophobic pocket. This was consistent with the results that the 3-*O*-alkyl substituted compounds **4**–**17** are generally much better activity than the 3-*O*-heterocyclic substituted compounds **25**–**36**.

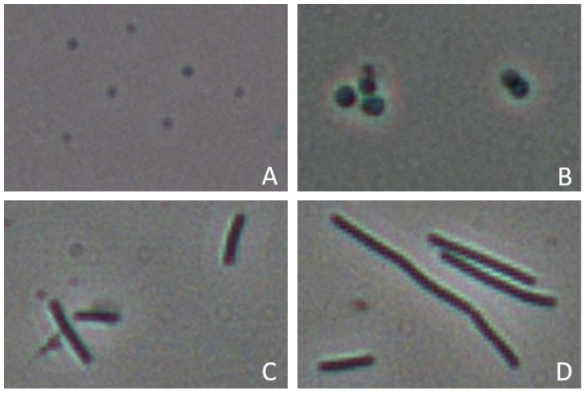


Fig. 4. Compound **17** inhibits cell division in *S. aureus* ATCC 25923 (A and B) and *B. subtilis* ATCC9372 (C and D) were grown in the absence (A and C) or presence (B and D) of 0.25μg/mL of **17** and these phenotypes were analyzed by phase-contrast light microscopy at a magnification of ×400.

In the cell division inhibitory assay, the same trend as the *in vitro* antibacterial activity was observed. Most of the 3-alkyloxy derivatives **4**–**21** showed improved cell division inhibitory activity against the three tested strains compared to the curcumine and their precursor **3**. The most active compounds were **7**, **12** and **17** with effective concentrations of 0.25, 0.5 and 0.125 μg/mL against *B. subtilis* ATCC9372, respectively (Fig. 4). And they were also the three best compounds standing out from the antibacterial assay. In the benzimidazolyl substituted derivatives **25**–**36**, many of them showed off-target effects, which suggested that the large groups could hinder the interaction with target FtsZ, thereby resulting in poor antibacterial activity. Generally, the cell inhibitory activity was accordant with the *in vitro* antibacterial activity, indicating that these compounds inhibited the proliferation of bacteria via influencing the function of FtsZ.

In summary, a series of novel 3-*O*-arylalkyl-2,6-difluorobenzamide derivatives were designed, synthesized and evaluated for their antibacterial activity and cell division inhibitory activity against various pathogen strains. In all the tested compounds, the 3-*O*-alkyl derivatives showed much more potent cell division inhibitory and antibacterial activity than the 3-*O*-heteroaromatic derivatives. In particular, compounds **7**, **12** and **17** that possessed 3-*O*-flexible side chains displayed the best antibacterial activity (MICs, 0.25–8 μg/mL) against both susceptible and resistant strains including *B. subtilis* ATCC9372, *S. aureus* ATCC25923, *S. aureus* ATCC29213, *S. aureus* PR. For the 3-*O*-rigid heteroaryl substituted derivatives, however, their improved activity was not outstanding, although some derivatives had very similar structure to previously discovered compound **6g**. Further optimization of 2,6-difluorobenzamide derivatives wiht potent FtsZ-targeted antibacterial activity should continue to be

investigated.

Conflict of interest

The authors declare that this study was carried out only with public funding. There is no funding or no agreement with commercial for profit firms.

Acknowledgements

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version,

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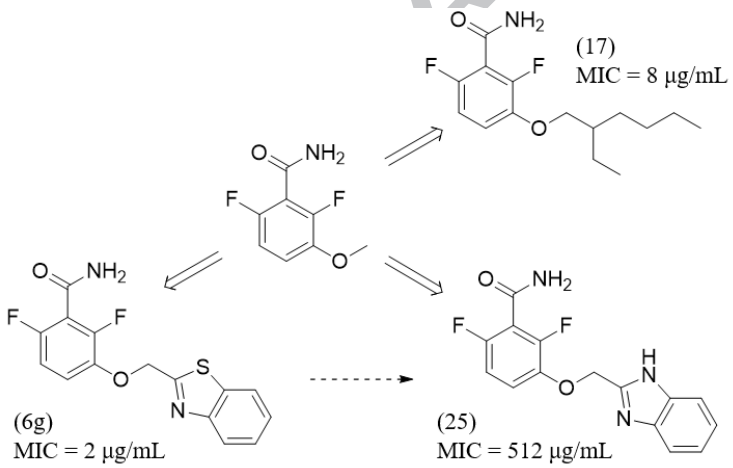
Graphical abstract:

Design, synthesis and biological activity evaluation of novel 2,6-difluorobenzamide derivatives through FtsZ inhibition

Fangchao Bi [a], Liwei Guo [a], Yinhu Wang [a], Henrietta Venter [b], Susan J. Semple [b], Fang Liu [a], Shutao Ma [a,*]

The 2,6-difluorobenzamide derivative (**17**) bearing 3-*O*-flexible alkyl side chains exhibited modest *in vitro* antibacterial and cell division inhibitory activities, while the the 3-*O*-rigid heteroaryl substituted derivative (**25**) showed weak antibacterial activity although it had very similar structure to previously discovered compound **6g**.



Research Highlights

> Novel 2,6-difluorobenzamide derivatives were synthesized and evaluated. > Some compounds showed potent activity against both susceptible and resistant strains. > They exerted their effects by inhibition of bacterial cell division protein FtsZ. > Replacing the benzothiazole with benzimidazole lost its antibacterial activity.