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# Boosting the efficacy of anti-MRSA β-lactam antibiotics via an easily accessible, non-cytotoxic and orally bioavailable FtsZ inhibitor

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**Abstract**

The rapid emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) strains has undermined the therapeutic efficacy of existing β-lactam antibiotics (BLAs), prompting an urgent need to discover novel BLAs adjuvants that can potentiate their anti-MRSA activities. In this study, cytotoxicity and antibacterial screening of a focused compound library enabled us to identify a compound, namely **28**, which exhibited low cytotoxicity against normal cells and robust *in vitro* bactericidal synergy with different classes of BLAs against a panel of multidrug-resistant clinical MRSA isolates. A series of biochemical assays and microscopic studies have revealed that compound **28** is likely to interact with the *S. aureus* FtsZ protein at the T7-loop binding pocket and inhibit polymerization of FtsZ protein without interfering with its GTPase activity, resulting in extensive delocalization of Z-ring and morphological changes characterized by significant enlargement of the bacterial cell. Animal studies demonstrated that compound **28** had a favorable pharmacokinetic profile and exhibited potent synergistic efficacy with cefuroxime antibiotic in a murine systemic infection model of MRSA. Overall, compound **28** represents a promising lead of FtsZ inhibitor for further development of efficacious BLAs adjuvants to treat the staphylococcal infection.

## Introduction

β-Lactam antibiotics (BLAs), the life-saving drugs that have long been widely used to treat lethal bacterial infection, are arguably one of the most important classes of therapeutic drugs in the history of human medicine. Although there is currently a rich collection of BLAs available for clinical use, the twin threats of global overuse of BLAs and rapid emergence of multidrug-resistant pathogenic bacteria have led to dramatic erosion of the therapeutic efficacy of the entire classes of BLAs including penicillins, cephalosporins, and even carbapenems.[1-3] Indeed, some examples of these multidrug-resistant pathogenic bacteria include the community- and healthcare-associated methicillin-resistant *Staphylococcus aureus* (MRSA), which cause an alarming patient mortality of over 11,000 deaths in the United States annually.[4] Consequently, the scarcity of effective treatment options of BLAs has created an urgent need not only for the development of next generation BLAs but also for the discovery of BLAs adjuvants that can make recalcitrant multidrug-resistant MRSA more susceptible to existing BLAs. Augmenting BLAs with a second agent has been proven clinically as one of the most effective strategies to restore the efficacy and extend the lifespan of this important class of antibiotics.[5-6] The well-known examples include the combination of FDA-approved β-lactamase inhibitors, such as clavulanic acid, sulbactam, tazobactam, avibactam and vaborbactam, with BLAs, providing highly effective treatment options in restoring the efficacy of BLAs against Gram-negative bacteria that have acquired diverse β-lactamase enzymes.[7] Clinical BLAs resistance in MRSA, however, is primarily mediated by acquiring another penicillin-binding protein Pbp2a with markedly reduced affinity for all classes of BLAs. Development of new BLAs combination treatment paradigm to boost the clinical efficacy of these important drugs against MRSA would undoubtedly strengthen current infectious disease management.

The bacterial cell division machinery involves many essential proteins that are extremely sensitive to perturbation by small molecules.[8-9] Among those cell division proteins, the filamenting temperature-sensitive mutant Z (FtsZ) protein has been extensively studied as a drug target for the discovery of antibacterial agents.[10-11] Over the past decade, different classes of FtsZ inhibitors have been discovered, such as natural product chrysophaentin A,[12] berberine,[13] naphthol analogues,[14] guanosine triphosphate (GTP) derivatives[15] and benzimidazoles.[16] During the process of cell division, monomeric FtsZ proteins undergo self-activating GTP-dependent polymerization to produce FtsZ filaments and contractile Z-ring at the mid-cell, followed by the constriction and depolymerization to give rise to two identical daughter cells.[17]

Pioneering studies by Tan[18] have nicely demonstrated that a FtsZ-specific inhibitor PC190723 (**1**)[19-20] acts synergistically with imipenem both *in vitro* and in a murine model of MRSA infection (**Figure 1**). The underlying mechanism of synergy was unclear. However, it was proposed to be driven by the initial delocalization of FtsZ filaments after the treatment of **1**, resulting in subsequent delocalization of the penicillin-binding proteins, which are important bacterial enzymes that are involved in peptidoglycan biosynthesis of bacterial cell wall.[18, 21] Combined with the recent findings that the treadmilling of FtsZ filaments controls both the location and activity of the septal peptidoglycan synthesizing enzymes,[22-23] these findings thus provide a rational basis for exploring much wider chemical space of FtsZ inhibitors that can be developed as efficacious anti-MRSA BLAs adjuvants. Despite the highly hydrophobic nature and suboptimal drug-like properties of **1**, its structurally similar derivatives TXA707 (**2**)[24], TXA6101 (**3**)[25-26], and *N*-Mannich type prodrugs, such as TXY541 (**4**),[27-28] TXY436 (**5**)[29] and

TXA709 (**6**),[24, 30-31] as well as succinate prodrug **7**[32] with enhanced *in vitro* and *in vivo* activities, have been further pursued as anti-staphylococcal agents for clinical evaluation (**Figure 1**). Although such prodrug approach has partially improved the aqueous solubility and pharmacokinetic (PK) properties of the parental drugs **1** - **3**, the intrinsic chemical instabilities and multistep chemical synthesis of prodrugs **4** - **7** may remain a major obstacle for fully unleashing their potential to be used in clinical practice. Therefore, alternative strategies remain to be devised to exploit FtsZ inhibitors as anti-MRSA BLAs adjuvants.

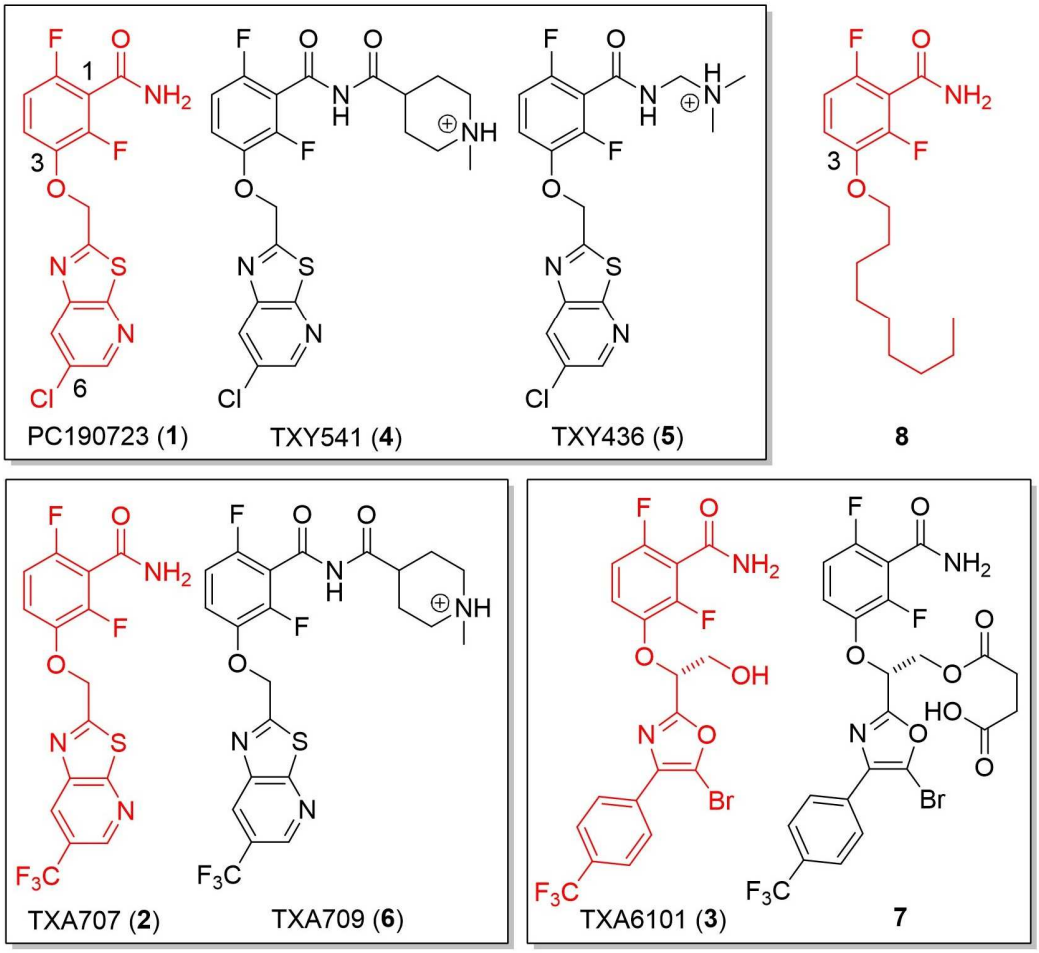


**Figure 1.** Chemical structures of FtsZ inhibitors. Parental drugs **1** - **3** and their prodrugs **4** - **7** are indicated in red and black respectively.

We have previously reported the identification of a series of novel FtsZ inhibitors through the computer-aided structure-based virtual screening[33-36] and the cell-based screening of natural product library.[37-39] Several quinuclidine-based FtsZ inhibitors were also found to exhibit strong synergistic effect against MRSA strains when combined with BLAs, suggesting that FtsZ protein maybe a desirable "potentiation drug target" of BLAs to boost their anti-staphylococcal activity.[40] In the present study, by use of compounds **1** and **8** as a starting template, we sought to systematically design, synthesize and screen a focused compound library of 47 candidates and identified a new class of FtsZ inhibitors which exhibited easy accessibility, low cytotoxicity and safe, favorable PK profile, and most importantly, potent *in vitro* and *in vivo* synergistic antimicrobial activity when used in combination with existing BLAs against MRSA.

## Results and Discussion

### 1. Compound design and chemical synthesis

As shown in **Figure 1**, PC190723 (**1**) was constructed from a 2,6-difluorobenzamide and a 6-chlorosubstituted thiazolopyridine moiety joined by an ether linkage at the C-3 position of the phenyl ring. Compound **8** possesses the same 2,6-difluorobenzamide warhead but with a different *n*-nonyloxy tail. For the sake of comparison, both compounds were also synthesized as positive controls according to the previous reports.[41-42] Resulted from the inspiration of their chemical structures as well as other related studies of FtsZ inhibitors,[43-48] our molecular design strategies are: (1) to replace the C-3 ether linkage with other functional groups such as secondary or tertiary amine, amide and triazole because these groups usually offer more favorable physicochemical properties than ether; (2) to replace the thiazolopyridine moiety with other

commercially available building blocks of low molecular weight for easy accessibility and rapid chemical synthesis; (3) to vary the position or reduce the number of fluorine group on the phenyl ring for investigating the influence of fluorine atom on antimicrobial potency; (4) to replace the amide group at C-1 position of phenyl ring with other bioisosteric functional groups for providing more potential hydrogen bonding interactions with the FtsZ protein.[49]

All newly designed compounds were synthesized as depicted in **Scheme 1** and **2**. As illustrated in **Scheme 1**, the chemical synthesis was initiated with the formation of amide and reduction of nitro group from a commercially available 2,6-difluoro-3-nitrobenzonic acid (**9**) following the reported procedures.[50-51] The key intermediate 2,6-difluoro-3-aminobenzamide (**10**) thus obtained in large quantity and high yield was further treated respectively with a wide range of commercially available aryl aldehydes, alkyl bromides, alkenyl bromides, substituted benzyl bromides or alkyl acid chloride to afford the desired products in one step with moderate to good yield, allowing a series of compounds to be prepared rapidly for biological study. Reductive alkylation of **10** with various commercially available aryl aldehydes in the presence of *p*-toluenesulfonic acid (*p*-TsOH) as a catalyst in methanol followed by treatment of sodium cyanoborohydride afforded the 3-aminobenzamide derivatives **11** - **21** in one-pot with good yield. Furthermore, alkylation of **10** under the basic condition with different fluoro- or chloro-substituted benzyl bromides using acetonitrile (ACN) as solvent furnished the mono- and di-benzyl substituted 3-aminobenzamide derivatives **22** - **25** in good yield. It is worthy to mention that these mono- and di-benzyl substituted 3-aminobenzamides can be easily purified by using flash column chromatography simply due to their large polarity difference. Mono-alkylation of **10** with different alkyl bromides or alkenyl bromides gave secondary alkyl or alkenyl substituted

144 3-aminobenzamide derivatives **26 - 30** and **32 - 35** in good yield. For amide **31**, a different
145 approach was used. It was successfully prepared in two steps with high yield via the conversion
146 of nonanoic acid to acid chloride by treating with oxalyl chloride followed by subsequent
147 reaction of the acid chloride with 3-aminobenzamide **10**. Further methylation of **27** and **28** with
148 dimethyl sulphate under basic condition using ACN as solvent afforded the tertiary 3-
149 aminobenzamide derivatives **36 - 37** in good yield. 4-Bromosubstituted 3-aminobenzamide
150 derivative **38** was prepared by the treatment of **28** with excess molecular bromine for 12 h at
151 room temperature in good yield. Similarly, a small group of 1,4-disubstituted 1,2,3-triazole
152 derivatives **40 - 42** was accessed in two steps with good yield by the initial formation of azide **39**
153 from 3-aminobenzamide **10** followed by regioselective Cu(I) catalyzed azide-alkyne
154 cycloaddition reaction in refluxing tetrahydrofuran (THF) with various terminal alkynes.[52]
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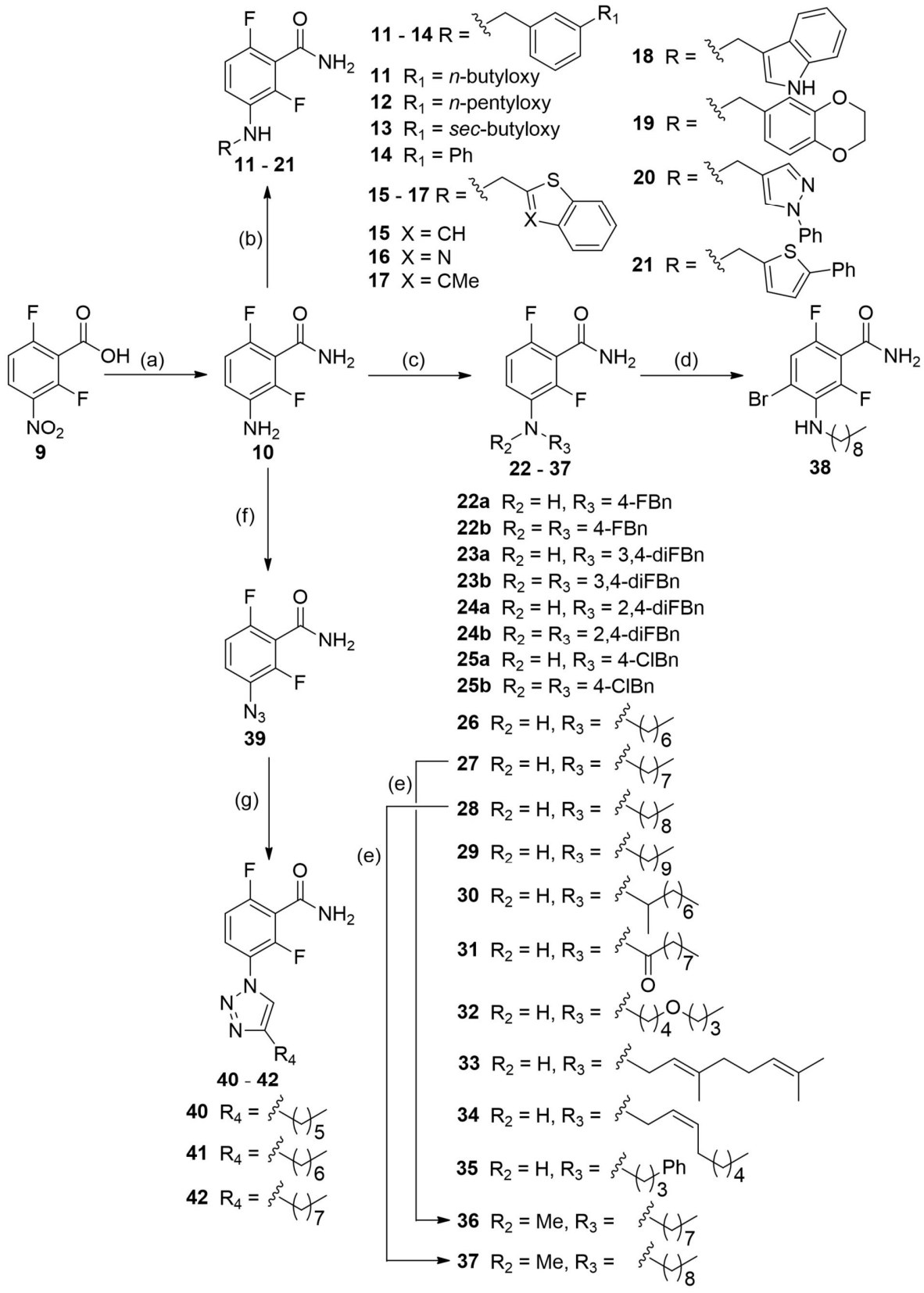
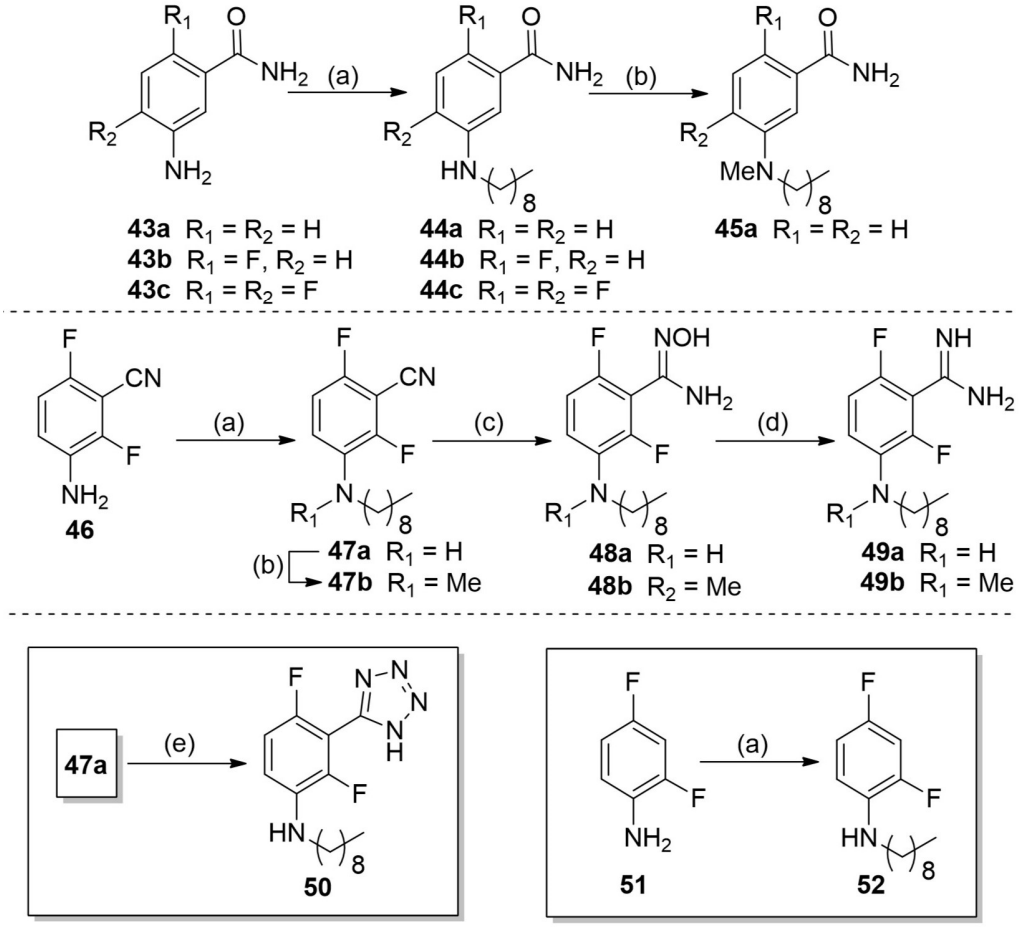

11 - 14 R =
11 R1 = n-butyloxy
12 R1 = n-pentyloxy
13 R1 = sec-butyloxy
14 R1 = Ph
15 - 17 R =
15 X = CH
16 X = N
17 X = CMe
18 R =
19 R =
20 R =
21 R =
11 - 21
(a)
(b)
(c)
(d)
(e)
(f)
(g)
9
10
22 - 37
38
39
40 - 42
22a R2 = H, R3 = 4-FBn
22b R2 = R3 = 4-FBn
23a R2 = H, R3 = 3,4-diFBn
23b R2 = R3 = 3,4-diFBn
24a R2 = H, R3 = 2,4-diFBn
24b R2 = R3 = 2,4-diFBn
25a R2 = H, R3 = 4-ClBn
25b R2 = R3 = 4-ClBn
26 R2 = H, R3 =
27 R2 = H, R3 =
28 R2 = H, R3 =
29 R2 = H, R3 =
30 R2 = H, R3 =
31 R2 = H, R3 =
32 R2 = H, R3 =
33 R2 = H, R3 =
34 R2 = H, R3 =
35 R2 = H, R3 =
36 R2 = Me, R3 =
37 R2 = Me, R3 =
40 R4 =
41 R4 =
42 R4 =

156

**Scheme 1**. (a) (i) $SOCl_2$, cat. DMF, reflux, 2 h; (ii) 30% $NH_3$ solution, $0^oC$, 1 h; (iii) $SnCl_2$, conc. HCl, $0^oC$ to r.t., 12 h; (b) aryl aldehydes, cat. *p*-TsOH, MeOH, r.t., 2 h, then $NaBH_3CN$, r.t., 12 h; (c) For **22** - **25**, various benzyl bromides, $K_2CO_3$, ACN, reflux, 4 h; For **26** - **30** and **32** - **35**, various alkyl or alkenyl bromides, $K_2CO_3$, cat. KI, ACN, reflux, 4 h; For **31**, nonanoyl chloride, Py/DCM, $0^oC$, 4 h; (d) **28**, $Br_2$, DCM, r.t., 12 h; (e) $Me_2SO_4$, $K_2CO_3$, ACN, reflux, 12 h; (f) conc. HCl, $NaNO_2$, $0^oC$, 0.5 h, then $NaN_3$, r.t., 4 h; (g) terminal alkynes, cat. $Cu(PPh_3)_3Br$, THF, reflux, 14 h.



**Scheme 2**. (a) **43**, **46** or **51**, 1-bromononane, cat. KI, $K_2CO_3$, DMF, reflux, 12 h; (b) For **45a**, $Me_2SO_4$, $K_2CO_3$, ACN, reflux, 12 h; For **47b**, methyl iodide, $K_2CO_3$, DMF, sealed tube, 60$^oC$, 24 h; (c) hydroxylamine hydrochloride, $NEt_3$, MeOH/THF, reflux, 12 h; (d) For **49a**, (i) acetic anhydride, AcOH, 0$^oC$, 12 h; (ii) $H_2$, Pd/C, MeOH, r.t., 12 h; (iii) conc. HCl, MeOH, reflux, 12 h; For **49b**, (i) 2-chloroacetyl chloride, DCM, 0$^oC$, 12 h; (ii) $H_2$, Pd/C, MeOH, r.t., 12 h; (e) $NaN_3$, $ZnCl_2$, DMF/$H_2O$, reflux 12 h.

Preliminary screening of anti-staphylococcal activity of these compounds revealed that the 2,6-difluoro-3-aminobenzamide derivatives **28** and **37** exhibited the most potent antimicrobial activity, implying that the amine groups of secondary *n*-nonyl amine and tertiary *n*-nonyl methylamine at C-3 position of phenyl ring are optimal substituents for the activity. Therefore, a subseries of 3-aminobenzamides and structurally related derivatives bearing these two important amino substituents was accessed next to investigate the effect of number and position of fluorine as well as the bioisosteric replacement of amide group at C-1 position on their antimicrobial activities. As shown in **Scheme 2**, mono-alkylation of aminobenzamides **43**, 3-amino-2,6-difluorobenzonitrile **46** and 2,4-difluoroaniline **51** with 1-bromononane under the basic condition in dimethylformamide (DMF) at elevated temperature afforded the corresponding monoalkylated aminobenzamides **44**, 3-amino-2,6-difluorobenzonitrile **47a** and 2,4-difluoroaniline **52** in good yield respectively. Methylated aminobenzamide **45a** and aminobenzonitrile **47b** were further prepared in good yield by treatment of **44a** and **47a** with dimethyl sulphate or methyl iodide under basic medium. 2,6-Difluorobenzamidoximes **48**, obtained from the reaction of hydroxylamine hydrochloride with 3-amino-2,6-difluorobenzonitriles **47**, were further converted to the desired 2,6-difluorobenzamidines **49** in two steps with moderate yield. Similarly, treatment

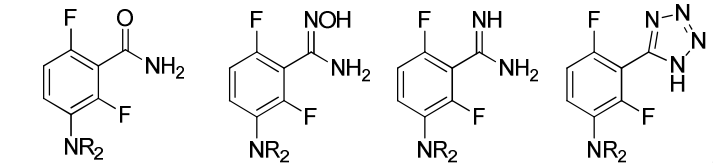
of 3-amino-2,6-difluorobenzonitrile **47a** with sodium azide in the presence of zinc(II) chloride at reflux temperature afforded the C-1 substituted tetrazole 2,6-difluoroaniline **50** in good yield. Collectively, these types of compounds were easily obtained within 3 to 4 synthetic steps with a reasonable overall yield by coupling various commercially available building blocks with 3-aminobenzamides or 3-aminobenzonitrile, allowing rapid construction of compound library for biological testing.

## 2. Evaluation of antibacterial and cytotoxic activities, SAR analysis and BLAs combination studies

With this compound library in hand, we next determined their antibacterial and cytotoxic activities simultaneously by measuring the minimal inhibitory concentrations (MICs) and the half-maximal growth inhibition concentration ($IC_{50}$) against two bacterial cells (*E. coli* 25922 and *S. aureus* 29213) and mouse fibroblasts L929 cell line respectively. The summarized results are presented in **Table 1**, in which only compounds with MIC values against *S. aureus* less than 20 μg/mL are shown. Compounds **1** and **8** were used as a positive control. Both compounds exhibited potent antibacterial activities against *S. aureus* with MIC of 0.5 to 1 μg/mL and low levels of cytotoxicity against L929 cells ($IC_{50} \geq 90$ μM), providing a relatively higher selectivity index (SI) value (Entry 1 and 2 of **Table 1**). They were, however, completely inactive against the Gram-negative *E. coli* even at a concentration of 64 μg/mL. These results were consistent with the previous reports.[19, 41] Time-kill curve evaluation of compound **1** at 2 × and 4 × its MIC against *S. aureus* ATCC BAA-41 confirmed its bactericidal mode of action, resulting in more than 4-log reduction of cell viability within 7 h of drug treatment (**Figure 2B**). After 24 h drug treatment, bacterial regrowth was not observed at all concentrations tested.

212

213 **Table 1**. Antibacterial and cytotoxic activities of selected compounds. [a]

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| Entry | Compound No. | $NR_2$ | MIC μg/mL [b] | | $IC_{50}$ μM | SI |
|---|---|---|---|---|---|---|
| | | | *E. coli* | *S. aureus* | L929 | |
| 1 | **1** | N.A. | > 64 | 0.5 (1.6) | > 100 | > 63 |
| 2 | **8** | N.A. | > 64 | 1 (3.1) | 90 ± 8 | 29 |
| 3 | **28** | nonylamino | > 64 | 1 (3.1) | > 100 | > 32 |
| 4 | **37** | methyl(nonyl)amino | > 64 | 1 (3.1) | 60 ± 10 | 19 |
| 5 | **30** | non-2-ylamino | > 64 | 4 (13) | > 100 | > 8 |
| 6 | **36** | methyl(octyl)amino | > 64 | 4 (13) | 92 ± 10 | 7 |
| 7 | **48b** | methyl(nonyl)amino | > 64 | 4 (13) | > 100 | > 8 |
| 8 | **25b** | bis(4-chlorobenzyl)amino | > 64 | 5 (13) | > 100 | > 8 |
| 9 | **34** | *cis*-non-2-en-1-ylamino | > 64 | 7 (25) | 99 ± 12 | 4 |
| 10 | **27** | octylamino | > 64 | 14 (50) | > 100 | > 2 |
| 11 | **49b** | methyl(nonyl)amino | > 64 | 15 (50) | > 100 | > 2 |
| 12 | **48a** | nonylamino | > 64 | 16 (50) | > 100 | > 2 |
| 13 | **50** | nonylamino | > 64 | 16 (50) | > 100 | > 2 |
| 14 | **11** | 3-(*n*-butyloxy)benzylamino | > 64 | 17 (50) | 99 ± 10 | 2 |
| 15 | **12** | 3-(*n*-pentyloxy)benzylamino | > 64 | 17 (50) | 87 ± 15 | 2 |
| 16 | **22b** | bis(4-fluorobenzyl)amino | > 64 | 19 (50) | > 100 | > 2 |

215 [a] N.A., not applicable. SI, selectivity index, it was calculated using the formula $IC_{50}$ (μM)
216 L929/MIC value of *S. aureus* (μM). All experiments were performed in at least triplicates. [b] μM
217 in the parentheses.

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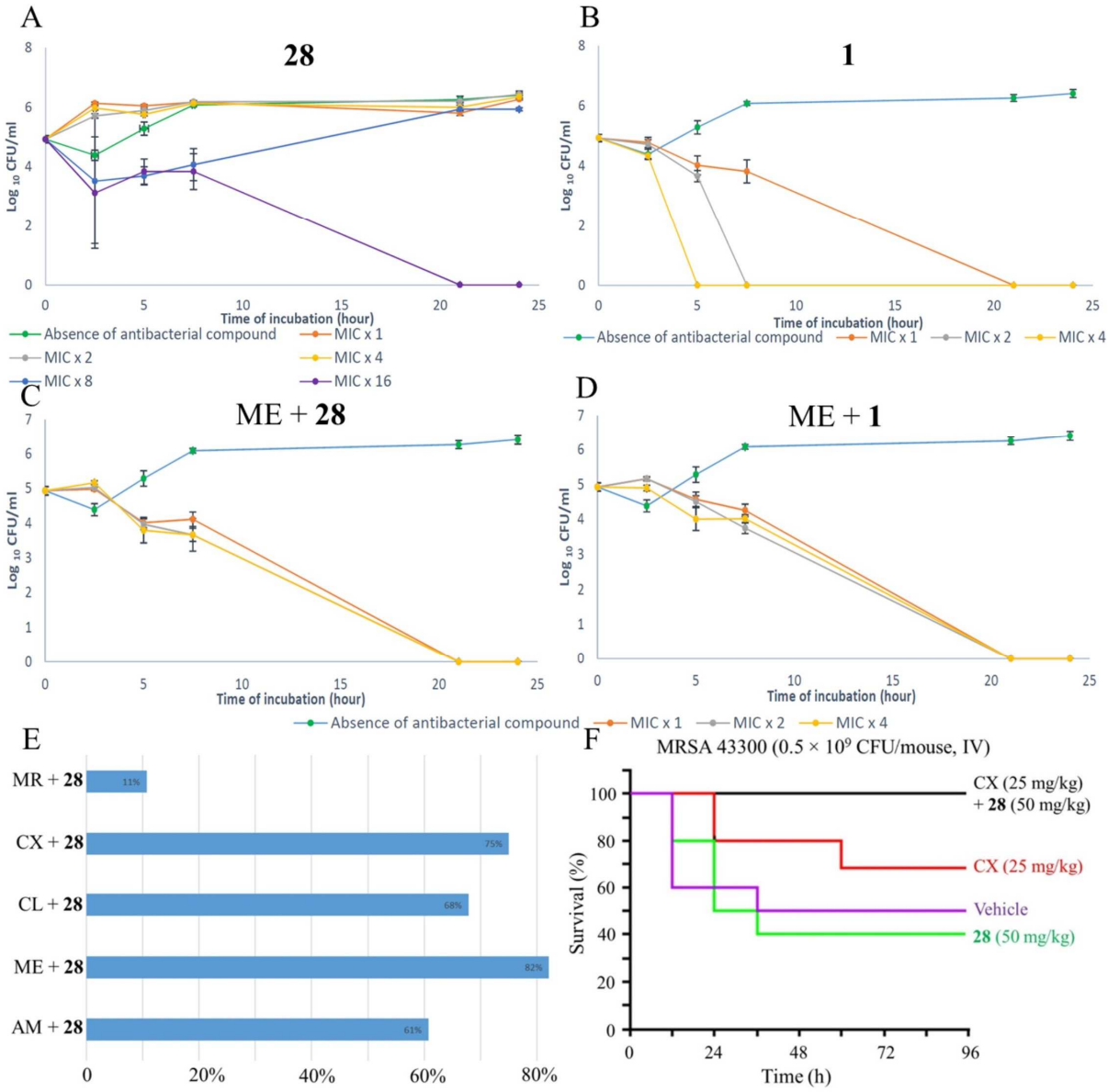



**Figure 2.** Time-kill curves of (A) **28**, (B) **1**, combinations of methicillin (ME) and (C) **28** or (D) **1** against *S. aureus* ATCC BAA-41. The error bars indicate standard derivations from measurements of triplicates. (E) Percentage of clinical MRSA isolates exhibiting synergistic effect (FIC index ≤ 0.5) to combinations of different BLAs with **28**. Twenty-eight MRSA strains were tested in total. MR, meropenem; CX, cefuroxime; CL, cloxacillin; ME, methicillin; AM, amoxicillin. (F) *In vivo* efficacy of intraperitoneal co-administering single agent of vehicle, CX,

**28** or combination of CX and **28** twice a day in a murine systemic infection model of MRSA ATCC 43300.

In general, among all newly synthesized compounds, low levels of cytotoxicity against normal cell L929 were observed with $IC_{50}$ values ranged from 60 µM to > 100 µM, implying that these compounds are potentially non-toxic and safe. Below 100 µM concentration, this class of compounds is unlikely to interact with other protein targets and induce cellular toxicity. Their $IC_{50}$ values are at least twice the observed MIC values, in particular, compound **28** displayed the highest SI of > 32 (Entry 3 of **Table 1**). Moreover, all newly synthesized compounds are also completely inactive against Gram-negative *E. coli* (MIC > 64 µg/mL), perhaps it is due to the intrinsic low permeability of compound itself to pass through the cell membrane of *E. coli* or the membrane efflux pumps presented in the *E. coli*, causing them far from reaching the drug target. More experiments on Gram-negative bacteria are required to test these hypotheses.

Among all the tested compounds, two compounds, namely **28** and **37**, displayed comparable anti-staphylococcal activity (MIC = 1 µg/mL) and selectivity index (≥ 19) with the positive controls. Interestingly, both compounds possess the common structural features of a warhead of 2,6-difluorobenzamide and a hydrophobic tail of *n*-nonylamino group. Detailed structure-activity relationships (SAR) analysis on the benzamide head and *n*-nonylamino tail revealed several structural features that are crucial to maintain the anti-staphylococcal activity. For the benzamide warhead, firstly, bioisosteric replacements of carboxamide group at C-1 position of compound **28** with other functional groups, such as *N*-hydroxycarboximidamide (compound **48a**), carboximidamide (compound **49a**) and tetrazole (compound **50**), weakened the antibacterial

activity. Similarly, replacement of carboxamide group of compound **28** with carbonitrile (compound **47a**) or hydrogen (compound **52**) even resulted in no antibacterial activity. Secondly, both the position and the number of fluorine atom on the phenyl ring play a very important role in the antibacterial potency. 2,6-Difluoro-substituted functional group of compound **28** exhibited the most potent antibacterial activity while reducing the number of fluorine atom to one (compound **44b**) or zero (compound **44a**) or varying the position of fluorine atoms to C-4 and C-6 positions (**44c**) lost their antibacterial activity. Thirdly, the secondary (compound **28**) or tertiary (compound **37**) amino groups at the C-3 position of the phenyl ring offered the most potent antibacterial activity. Installation of less freely rotatable substituents at this position, such as amide (compound **31**) and 1,4-disubstituted triazole moieties (compound **41**) dramatically reduced the antibacterial activity. On the other hand, for the *n*-nonylamino tail, several structural features, including the length, rigidity, bulkiness and lipophilicity, interfere the potency of antibacterial activity. Replacing the optimal *n*-nonylamino group with a longer *n*-decylamino group (compound **29**) or shorter *n*-heptyl (compound **26**) and *n*-octyl amino group (compound **27**) of straight alkyl chains or branched 2-nonylamino group (**30**) diminished sharply in the antibacterial activity. Moreover, increasing the chain rigidity by the introduction of alkene (compounds **33** and **34**) or benzyloxy ring (compounds **11** and **12**) in the amino tail also weakened their antibacterial activity significantly. Both decreasing the chain lipophilicity by introducing an oxygen atom (compound **32**) in the middle of the chain and increasing the chain bulkiness by installing a phenyl ring at the terminal position (compound **35**) of the alkyl chain lead to no antibacterial activity. Taken together, compound **28** demonstrated the most promising SI value among all tested compounds, it was selected for detailed biological characterization.

Surprisingly, time-kill curve evaluation of compound **28** clearly indicated that its mode of action is bacteriostatic (cells show arrested growth), but not bactericidal, because it required 16 × its MIC to kill the bacteria within 24 h (**Figure 2A**). After 24 h of drug treatment, bacterial regrowth was observed at concentrations below 16 × its MIC. We next assessed the synergistic effect of this compound in combination with a wide range of clinically used BLAs, including penicillin-type antibiotics methicillin (ME), cloxacillin (CL) and amoxicillin (AM), cephalosporin-type antibiotic cefuroxime (CX) and carbapenem-type antibiotic meropenem (MR), against a panel of twenty-eight clinical MRSA strains. As shown in **Table 2**, some of these strains exhibited a high level of drug resistance to multiple BLAs with MIC values ranging from 2 μg/mL to 1024 μg/mL. Encouragingly, combination studies revealed that compound **28** demonstrated strong synergistic effect with all tested BLAs against these three clinical MRSA , with calculated fractional inhibitory concentration (FIC) index as low as 0.1 (**Table 2**). Moreover, as shown in **Figure 2E**, 82%, 75%, 68%, 61% and 11% of clinical MRSA isolates exhibited synergistic effect (FIC index $\leq$ 0.5) to the combinations of ME, CX, CL, AM and MR antibiotics with compound **28** respectively (**Table S1**). These results suggested that compound **28** has a board spectrum for BLAs combination and is, therefore, an excellent BLAs adjuvant. In addition, time-kill curve evaluation of the combination of compound **28** and ME revealed that the mode of action is bactericidal (**Figure 2C**), which is similar to the combination of compound **1** and ME (**Figure 2D**). After 21 h of drug combination treatment, bacterial regrowth was not observed at all concentrations tested for both combinations.

**Table 2**. Combination studies of compound **28** with various BLAs against selected clinically isolated MRSA strains and calculated FIC index.[a]

| MRSA Strain No. | MIC (μg/mL) | | | | | | | | | | | FIC Index of combination | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **28** | ME | ME +**28** | CL | CL +**28** | CX | CX +**28** | AM | AM +**28** | MR | MR +**28** | ME +**28** | CL +**28** | CX +**28** | AM +**28** | MR +**28** |
| 417 | 32 | 1024 | 2 | 64 | 2 | 1024 | 2 | 512 | 8 | 64 | 4 | 0.1 | 0.1 | 0.1 | 0.3 | 0.2 |
| 2516 | 32 | 64 | 4 | 16 | 2 | 512 | 2 | 64 | 8 | 16 | 4 | 0.2 | 0.2 | 0.1 | 0.4 | 0.4 |
| 774 | 512 | 16 | 4 | 2 | 1 | 1024 | 2 | 512 | 8 | 32 | 4 | 0.3 | 0.5 | 0.1 | 0.1 | 0.1 |

[a] ME, Methicillin; CL, cloxacillin; CX, cefuroxime; AM, amoxicillin; MR, meropenem. FIC index is calculated by using the formulate FIC index = FIC (compound) + FIC (drug), where FIC (compound) is the (MIC of compound in combination with drug)/(MIC of compound alone) while FIC (drug) is the (MIC of compound in combination with drug)/(MIC of drug alone). The combination is considered synergistic if the FIC Index ≤ 0.5. All experiments were performed in at least triplicates.

3. *In vivo* efficacy of combination of CX and **28** against MRSA ATCC 43300

On the basis of *in vitro* data which show that compound **28** is broadly synergistic in combination with various BLAs against diverse clinically relevant MRSA strains and relatively non-cytotoxic to mouse peritoneal fibroblast L929 ($IC_{50}$ > 100 μM), we next pursued the synergistic efficacy of compound **28** in combination with CX when co-administered intraperitoneally (IP) to a murine systemic infection model of MRSA. The preclinical model of infection using MRSA ATCC 43300 has been frequently employed to predict the clinical antibiotic efficacy.[29] Among those BLAs that have been tested *in vitro*, CX was selected because it is an oral antibiotic, which would enjoy a higher patient acceptance. MIC studies demonstrated that combination of CX and **28** also exhibited strong synergistic effect against MRSA ATCC 43300 with a FIC index of 0.1, prompting us to carry out *in vivo* efficacy studies. Preliminary dose regime studies indicated that CX and compound **28** co-administered IP both at 50 mg/kg once a day provided a survival rate of 33%, but all the mice died at day 5 upon treatment by CX or compound **28** as a single agent (**Figure S52A**). These preliminary results suggested that such combination therapy is efficacious against MRSA ATCC 43300 although the survival rate was only moderate. We reasoned that such low survival rate is likely attributed to the hydrophobic

nature of compound **28** (cLogP = 5.0) that may cause high plasma protein binding and reduced potency. Nonetheless, an adjusted dose regime of compound **28** (50 mg/kg) and CX (25 mg/kg) at twice a day was tested next for improving the survival rate. As shown in **Figure 2F**, CX (25 mg/kg) and compound **28** (50 mg/kg) administered IP as a single agent only provided 70% and 40% survival rate respectively in treating mice with MRSA infection compared with the vehicle treatment (50% survival rate). Encouragingly, IP co-administering both compound **28** and CX at these dosages provided a significant increase in survival rate to 100% after 4 days of combination therapy. In addition, no compound **28**-CX-resistant mutants were identified among the CFU recovered from the *in vivo* study and no obvious trauma around the injection site of compound **28** was observed (**Figure S52B**). Collectively, these data provide strong evidence supporting the hypothesis that compound **28** may provide an alternative strategy to develop as a bactericidal BLAs combination agent that is efficacious against the clinical MRSA infection.

4. Validation of FtsZ protein as the drug target of compound **28**

PC190723 (**1**) has been shown to inhibit the bacterial cell division process through targeting the binding site at T7-loop of *S. aureus* FtsZ protein by using the protein-ligand crystal co-complex.[18, 53] Structurally, compound **28** also possesses the same 2,6-difluorobenzamide warhead, but with a more freely rotatable *n*-nonylamino substituent at the C-3 position of the phenyl ring. Due to their overall structural unlikeness, the next question we need to answer is whether this compound still bind to the same binding site at T7-loop of FtsZ protein and interfere the cell division process in a similar way. To address this question, we sought to conduct the following series of biochemical and microscopic studies to prove that the anti-staphylococcal

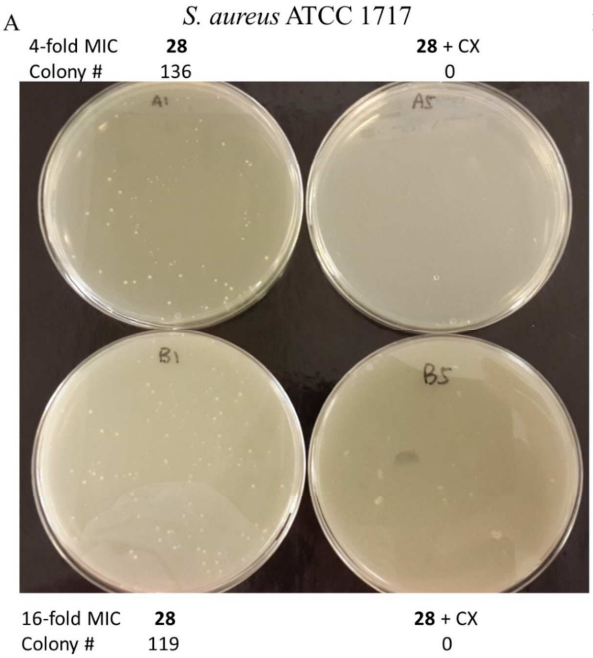
activity of compound **28** reflects its ability to target T7-loop of *S. aureus* FtsZ protein and interfere with the downstream cell division process.

4.1 Isolation of compound **28** resistant mutants for genetic studies and computational docking studies

The frequency of resistance (FOR) assays indicated that bacterial cells of *S. aureus* ATCC 1717 were able to grow even in the presence of 4-fold or 16-fold MIC of compound **28** as a single agent (**Figure 3A**), suggesting that potential genetic mutations in the target protein may have been induced, resulting in drug resistance. However, no colony was observed for the plates treated with the combination of **28** and CX after 48 h incubation, implying a reduced rate of drug resistance development. Therefore, the most definitive approach for *in vivo* target identification of compound **28** is through the drug resistance mapping analysis of compound **28**-resistant isolates, demonstrating that mutations in the target protein result in drug resistance. In this connection, we have employed a large-inoculum approach in an effort to raise spontaneous resistant mutants of *S. aureus* ATCC 29213 strains that are highly resistant to compound **28**. This approach successfully yielded three compound **28**-resistant strains with MIC values of 32 µg/mL, 64 µg/mL and 128 µg/mL respectively (**Figure 3B**, upper part). The genetic materials in each resistant strain as well as the wild-type strain were isolated and subjected to whole genome sequencing followed by sequence alignment to identify any nucleotide changes. Surprisingly, compared with the wild-type strain, the sequencing results indicated that all three compound **28**-resistant strains carried the same single nucleotide change of G786A, which is corresponding to the amino acid substitution of M262I that mapped to the *S. aureus* FtsZ protein (**Figure 3B**, upper part). Previous mutational analysis of PC190723 (**1**)-resistant mutants also identified

several major amino acid substitutions that mapped to FtsZ protein, including G193D, G196A and N263K (**Figure 3B**, lower part).[18-19] The amino acid substitution of M262I was found to be located exactly at the same binding pocket of PC190723 (**1**), suggesting that compound **28** is very likely to bind directly to the *S. aureus* FtsZ protein in the same manner as PC190723 (**1**). Our mutational analysis is, therefore, consistent with the FtsZ protein being the antibacterial drug target of compound **28**.

A




B

| Strains | MIC of **28** (μg/mL) | Nucleotide at position 786 | Amino acid at position 262 |
|---|---|---|---|
| *S. aureus* 29213 | 1 | G | Met |
| Mutant32 | 32 | A | Ile |
| Mutant64 | 64 | A | Ile |
| Mutant128 | 128 | A | Ile |

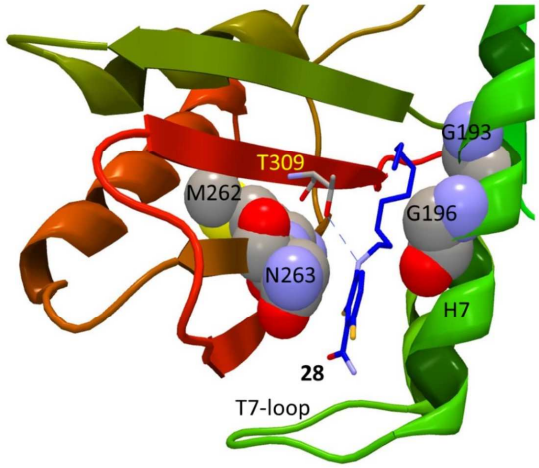



**Figure 3.** (A) FOR studies of compound **28** alone and combination of compound **28** and CX showing the number of colony and (B, upper part) Summary of MIC, DNA nucleotide changes and amino acid substitutions of compound **28**-resistant mutants and (B, lower part) Model of compound **28** (blue sticks) docked into the T7-loop cleft of FtsZ using the crystal structure of *S. aureus* FtsZ protein (PDB ID: 4DXD) with labelled helix 7 (H7), T7-loop and amino acid residues G193, G196, M262, N263 and T309. The grey dotted line indicates the potential

hydrogen bonding interaction between the C-3 amino group of compound **28** and the hydroxyl group of T309.

To gain more insights into the potential binding site and binding pose of compound **28** in the *S. aureus* FtsZ protein, computational docking studies of compound **28** using previously reported crystal structure of *S. aureus* FtsZ protein (PDB ID: 4DXD) was conducted next.[18] The results of docking studies revealed that the highest docking score positioned compound **28** into a cleft between the helix 7 (H7) and the C-terminal domain of FtsZ, which is in good agreement with PC190723 (**1**) (**Figure 3B**, lower part). The 2,6-difluorobenzamide warhead of **28** was well-situated in the hydrophobic pocket interacting with the T7-loop of FtsZ protein. A conventional hydrogen bonding interaction was predicted to be established between the C-3 amino group of compound **28** and the hydroxyl group of T309. The amino acid residues of M262, G193, G196 and N263 shown in **Figure 3B** were closely adjacent to the residues comprising the binding pocket of **28** proposed by the docking study. These results suggested that potential amino acid substitutions at this binding pocket are likely to be induced easily by small molecules that bind to this pocket. The resultant changes appear to alter slightly the overall shape of this binding pocket without interfering the normal function of FtsZ protein, resulting in compound **28** or PC190723 (**1**) no longer binding to FtsZ protein and causing drug resistance.

4.2 Effect on FtsZ protein polymerization and GTPase activity upon compound **28** treatment

Previous reports have shown that the antibacterial activities of PC190723 (**1**) are resulted from the overstimulation of FtsZ protein polymerization through stabilizing the nonfunctional FtsZ polymeric structures.[54] In order to confirm whether compound **28** would exert similar effect on

FtsZ protein polymerization, we next expressed and purified the *S. aureus* FtsZ protein for assessment of its polymerization dynamics in the absence or presence of compound **28** using an *in vitro* light scattering assay. In this assay, the monomeric FtsZ protein polymerization was continuously monitored in the presence of GTP by a time-dependent increase in light scattering as reflected by an increase in solution absorbance at 600 nm. The results of FtsZ protein polymerization in the presence of compound **28** at concentrations ranging from 12.5 μM to 100 μM were shown in **Figure 4A**. Surprisingly, compound **28** potently inhibited the FtsZ protein polymerization in a concentration-dependent manner, a behavior that is opposite to that of PC190723 (**1**), which stimulates FtsZ protein polymerization at concentrations of 12.5, 25 and 50 μM in a dose-dependent manner (**Figure S53**). Surprisingly, compound **1** at 100 μM completely inhibited FtsZ protein polymerization. On the other hand, compared with the vehicle control (1% DMSO) at 500 seconds, compound **28** at 25 μM, 50 μM and 100 μM exhibited about 34%, 39% and 47% inhibition of FtsZ protein polymerization respectively. These results suggested that compound **28** is able to perturb the FtsZ protein polymerization *in vitro*.

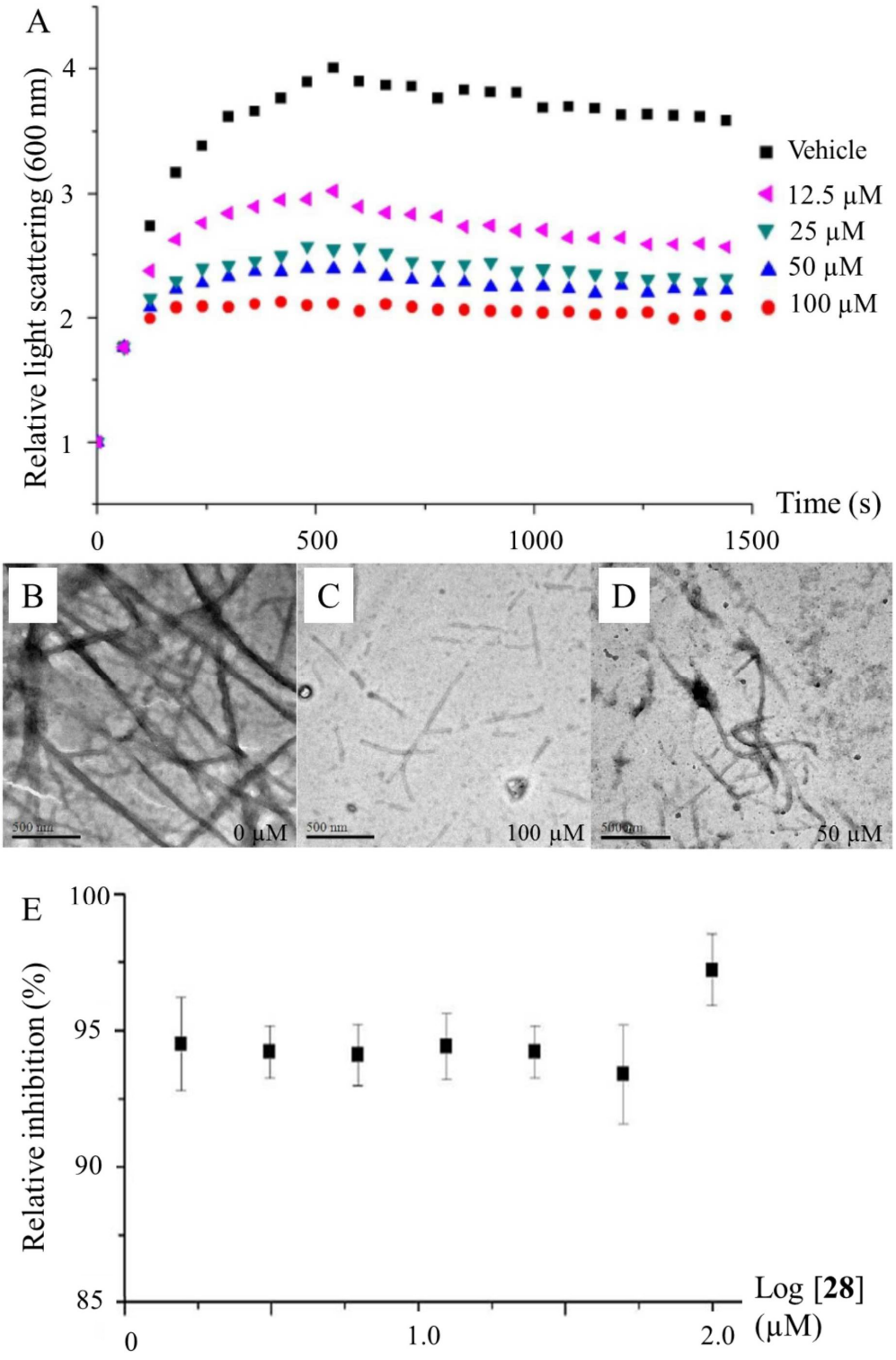



**Figure 4.** (A) Effect of compound **28** at different concentrations on the kinetics of *S. aureus* FtsZ polymerization. The experiments were performed in triplicate with the symbols indicating the

mean value. Electron micrographs of FtsZ polymer after the treatment of compound **28** at (B) 0 μM, (C) 100 μM and (D) 50 μM. The scale bar is 500nm. (E) Effect of compound **28** at various concentrations on the GTPase activity of *S. aureus* FtsZ protein.

To further demonstrate the effect of compound **28** on inhibition of FtsZ protein polymerization, transmission electron microscopy (TEM) imaging of the compound **28** treated and untreated *S. aureus* FtsZ protein was carried out to investigate the morphological change of FtsZ filaments. *S. aureus* FtsZ protein treated with compound **28** at concentrations of 0, 50 and 100 μM in the presence of GTP were visualized in **Figure 4B**, **4D** and **4C** respectively. As anticipated, there was considerable reduction in the extent of FtsZ filament formation upon treatment of compound **28** compared to the untreated FtsZ protein. The magnitude of these suppressing effects increases with the increasing concentration of compound **28**. At 100 μM of compound **28**, the density of *S. aureus* FtsZ filaments was substantially reduced, producing short, thin and single strand FtsZ filaments (**Figure 4C**), implying that compound **28** may block the FtsZ protein polymerization in a longitudinal and lateral manner. In a sharp contrast, the untreated *S. aureus* FtsZ protein showed a heavily dense network of FtsZ filaments (**Figure 4B**). These results clearly indicated the highly efficient inhibition of *S. aureus* FtsZ assembly to form filaments by compound **28** at a dose-dependent manner, which is consistent with the results of light scattering assay.

The GTPase activity of FtsZ protein also plays an important role of assembling monomeric FtsZ proteins by hydrolyzing GTP molecules as an important energy source for driving polymerization. Compound **1** has been reported to inhibit directly the GTPase activity of FtsZ in

441 a concentration-dependent manner with a half-maximal inhibitory concentration of 55 ng/mL.[19]
442 On the contrary, Our laboratory and others did not observe such inhibitory effect.[55] Compound **1**
443 at 30, 50 and 100 μM concentrations even increased the GTPase activity by 47%, 29% and 15%
444 respectively (**Figure S53E**). On the other hand, as shown in **Figure 4E**, there was no significant
445 change of the GTPase activity for compound **28** even at the concentration of 100 μM, suggesting
446 that compound **28** is likely to perturb FtsZ protein polymerization through binding to the T7-loop
447 of FtsZ protein without interfering its GTPase activity.
448
449 4.3 Microscopic studies of bacterial morphology and localization of the Z-ring of *B. subtilis* and
450 *S. aureus* cells
451 Formation of Z-ring at the appropriate site of cytokinesis is one of the most important
452 prerequisites for bacteria to carry out cell division properly.[56] Microscopic studies of previous
453 reports have demonstrated that small molecules, which block the Z-ring formation through
454 inhibition of FtsZ protein polymerization, at a sublethal concentration induced both iconic
455 elongated phenotype in rod-shaped *B. subtilis* cells and enlarged phenotype in spherical *S.*
456 *aureus* cells respectively. Moreover, an obvious septal delocalization of green fluorescent protein
457 (GFP)-tagged FtsZ polymers was also observed in both cells. As shown in **Figure 5** and **S54**,
458 such morphological changes and septal delocalization of GFP-tagged FtsZ polymer after
459 treatment of compound **28** were confirmed. Fluorescent microscopic studies indicated that
460 fluorescent foci at the mid cell were observed in the presence of 1% DMSO, implying the proper
461 formation and localization of Z-ring at the appropriate division septum (**Figure 5A** and **S54C**).
462 Upon treatment of compound **28** or **1**, multiple discrete foci throughout the whole elongated *B.*
463 *subtilis* 168 cells (**Figure S54E**), and enlarged *S. aureus* RN 4220 cells (**Figure 5B** and **5C**)

were observed respectively, demonstrating the markedly altered localization of Z-ring without being specifically restricted to the division septum. Moreover, for the bacterial morphology, elongated *B. subtilis* 168 cells (**Figure S54B**) and enlarged *S. aureus* ATCC BAA-41 cells (**Figure 5E** and **5F**) were observed respectively upon treatment of compound **28** or **1**. These results are consistent with other reported FtsZ inhibitors.

Combining all the studies related to compound **28**, we reasonably propose that *S. aureus* FtsZ protein is probably the drug target of compound **28** and it is likely to inhibit the *S. aureus* FtsZ protein polymerization process through binding to the T7-loop of FtsZ protein, causing subsequent delocalization of Z-ring and disruption of the cell division process.

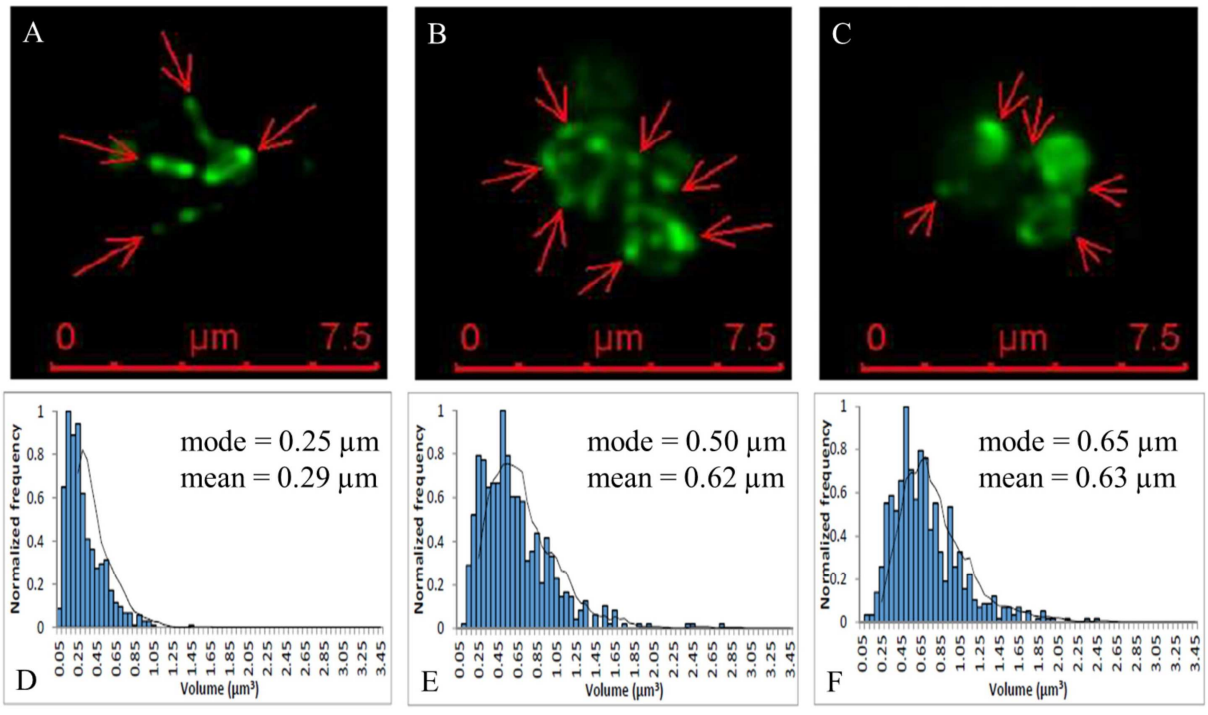



**Figure 5.** Fluorescent microscopic study (upper panel) of FtsZ-GFP fusion stain of *S. aureus* RN 4220 cells in the presence of (A) 1% DMSO, (B) 4 × MIC of compound **28** and (C) 4 × MIC of

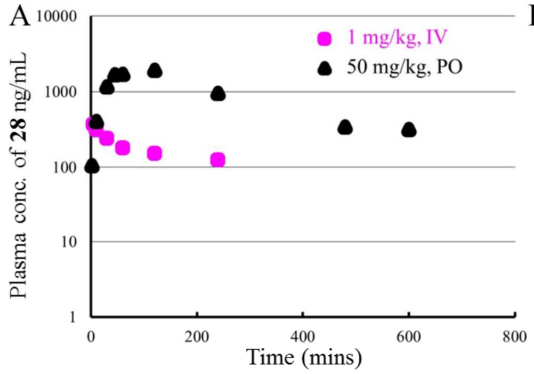
compound **1**. The scale bar is 7.5 μm and the red arrows indicated the fluorescent foci. Histograms (lower panel) showing the normalized frequency distribution of cell volume of *S. aureus* ATCC BAA-41 in the presence of (D) 1% DMSO, (E) 2 × MIC of compound **28** and (F) 2 × MIC of compound **1** with the indicated cell volumes at mode and mean respectively.

5. PK profile of compound **28**

Oral bioavailability is one of the key considerations for developing bioactive molecules as therapeutic agents. Lead compounds with poor oral bioavailability may result in low efficacy and unpredictable drug response. Previous study indicated that the small intestine of rat and human exhibit similar drug absorption profiles and transporter expression patterns, providing a more easier prediction of oral drug absorption potential in human.[57] In this connection, we sought to reveal the rat plasma concentration-time profile of compound **28** upon intravenous injection (IV) at a dose of 1 mg/kg and oral administration (PO) at a dose of 50 mg/kg (**Figure 6**, left). The rat PK parameters of compound **28** are listed in **Figure 6** (right). The results of PO indicated that compound **28** exhibits a fast absorption ($T_{max}$ = 2 h) with a peak plasma concentration ($C_{max}$) of 1.9 μg/mL. The terminal half-life ($t_{1/2}$) representing the time required for systemic level of compound **28** to reduce by half following PO and IV administrations were 3 and 5.5 h respectively. The area under the curve ($AUC_{0-\infty}$) representing the total systemic drug exposure for PO and IV were 10.3 and 1.6 mg/L·h respectively. Thus, the oral bioavailability (*F*) of compound **28**, which is the fraction of a compound that reaches systemic circulation following oral administration, was moderate at 13%. Taken together, these PK parameters indicated that compound **28** has moderate oral drug absorption in rat and compound **28** can serve as a lead for further structural optimization.

501

A




B

| PK parameters of **28** | Oral administration | Intravenous administration |
|---|---|---|
| Dosage (mg/kg) | 50 | 1 |
| $C_{max}$ (μg/mL) | 1.9 | - |
| $T_{max}$ (h) | 2 | - |
| $V_d$ (L/kg) | - | 4.8 |
| Cl (L/h/kg) | - | 0.6 |
| $t_{1/2}$ (h) | 3 | 5.5 |
| $AUC_{0-\infty}$ (mg/L·h) | 10.3 | 1.6 |
| $F$ % | 13 | - |

502

503 **Figure 6.** (A) The plasma concentration-time profile of compound **28** upon intravenous injection
504 (pink square) and oral administration (dark triangle) in rats and (B) PK parameters of compound
505 **28**.

506

507 **Conclusion**

508 In summary, a focused compound library of 3-aminobenzamides and structurally related
509 derivatives has been designed and synthesized for evaluation of their antibacterial and cytotoxic
510 activities against bacterial cells and normal cells. These compounds were easily obtained in 3 to
511 4 synthetic steps by coupling of various commercially available building blocks with 3-
512 aminobenzamides or 3-aminobenzonitrile, allowing rapid construction of the compound library
513 for SAR analysis. Our efforts have yielded a compound, **28**, which exhibits low cytotoxicity
514 against normal cells and robust *in vitro* bactericidal synergy with different classes of BLAs
515 against a panel of multidrug-resistant clinical MRSA isolates with FIC index as low as 0.1.
516 Further target identification and mechanistic studies employing a series of genetic study,
517 computational docking, biochemical assays and microscopic studies have revealed that
518 compound **28** is likely to interact with the *S. aureus* FtsZ protein at the T7-loop binding pocket

and inhibit the polymerization of FtsZ protein without interfering its GTPase activity, causing the subsequent extensive delocalization of Z-ring and enlarged morphological changes in *S. aureus*. Animal studies demonstrated that compound **28** has a favorable PK profile in rat and exhibits potent synergistic efficacy with cefuroxime antibiotic in a murine systemic infection model of MRSA, protecting infected mice with a 100% survival rate. Taken together, our findings indicated that compound **28** may serve a lead suitable for structural optimization into a BLA combination agent for the treatment of staphylococcal infection.

## Experimental section

Chemical synthesis

All NMR spectra were recorded at room temperature on a Bruker Advance-III spectrometer at 400.13 MHz for $^{1}H$ and 100.62 MHz for $^{13}C$. All chemical shifts were reported as parts per million (ppm) in the unit relative to the resonance of $CDCl_3$, Acetone-$d_6$, DMSO-$d_6$. Low-resolution (LRMS) and high-resolution mass spectra (HRMS) were obtained on a Micromass Q-TOF-2 by electron spray ionization (ESI) mode. All organic solvents and reagents were reagent grade and were commercially available and they were used without further purification unless otherwise stated. The plates used for thin-layer chromatography (TLC) analysis were E. Merck Silica Gel $60F_{254}$ (0.25 mm thickness). They were visualized under short and long UV light (254 and 365 nm) and immersed in a 10% phosphomolybdic acid solution in ethanol followed by gentle heating with a heat gun. Chromatographic purifications were carried out using MN silica gel 60 (230−400 mesh) with gradient elution. Compound purity was determined by an Agilent 1100 series HPLC installed with a Prep-Sil Scalar column (4.6 mm × 250 mm, 5 µm) at UV detection of 254 nm (reference at 450 nm). All tested compounds were determined to have at

least 95% purity according to HPLC. Aryl aldehydes, such as 3-butoxybenzaldehyde, 3-(pentyloxy)benzaldehyde, 3-(*sec*-butoxy)benzaldehyde, [1,1'-biphenyl]-3-carbaldehyde, benzo[*b*]thiophene-2-carbaldehyde, benzo[*d*]thiazole-2-carbaldehyde, 3-methylbenzo[*b*]thiophene-2-carbaldehyde, 1*H*-indole-3-carbaldehyde, 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carbaldehyde, 1-phenyl-1*H*-pyrazole-4-carbaldehyde and 5-phenylthiophene-2-carbaldehyde, are commercially available. PC190723 (**1**) and **8** were prepared according to previous reports.[41-42]

**2,6-Difluoro-3-aminobenzamide (10)**. To a well-stirred mixture of 2,6-difluoro-3-nitrobenzonic acid (**9**) (44 g, 217 mmol) and excess thionyl chloride (100 mL) in the presence of few drops of DMF was heated to reflux under nitrogen atmosphere for 2 h. After that, the remaining thionyl chloride was removed under reduced pressure to afford the 2,6-difluoro-3-nitrobenzoyl chloride, which was used immediately for next step without further purification. To a well-stirred aqueous 30% ammonia solution (300 mL) at $0^{o}$C was added freshly prepared 2,6-difluoro-3-nitrobenzonic acid chloride dropwise. After the addition, the white precipitates were collected by suction filtration and washed twice with water to afford the 2,6-difluoro-3-nitrobenzamide (40 g, 91%), which was used for next step without further purification. To a well-stirred solution of tin (II) chloride (80 g, 421 mmol) in conc. hydrochloric acid (200 mL) at $0^{o}$C was added 2,6-difluoro-3-nitrobenzamide in portions. After the addition, the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was neutralized by pouring slowly to a potassium hydroxide solution until the pH reached 12 at $0^{o}$C. The alkaline solution was extracted with ethyl acetate (200 mL x 3). The combined organic layers were dried over anhydrous $MgSO_4$, filtered and evaporated to dryness to give the desired product (18 g,

565 53%) as a dark brown solid. $^{1}$H NMR (400 MHz, Acetone-$d_6$) δ 7.36 (br. s., 1H), 7.14 (br. s.,
566 1H), 6.85 - 6.90 (m, 1H), 6.77 (dd, $J$ = 8.0 Hz, 1H), 4.67 (br. s., 2H); $^{13}$C NMR (101 MHz,
567 Acetone-$d_6$) δ 162.3 (s, *C*ONH$_2$), 150.5 (dd, $J_{CF}$ = 238, 6.1 Hz, C6), 146.9 (dd, $J_{CF}$ = 244, 8.1 Hz,
568 C2), 132.9 (dd, $J_{CF}$ = 13, 2.0 Hz, C3), 116.3 (dd, $J_{CF}$ = 10, 5.1 Hz, C4), 115.3 (dd, $J_{CF}$ = 24, 20
569 Hz, C1), 110.8 (dd, $J_{CF}$ = 23, 4.0 Hz, C5); LRMS (ESI) *m/z* 173 ($M^+$ + H, 100); HRMS (ESI)
570 calcd for $C_7H_7F_2N_2O$ ($M^+$ + H) 173.1401, found 173.1405.
571
572 **2,6-Difluoro-3-((3-(*n*-butyloxy)benzyl)amino)benzamide (11)**. To a well stirred mixture of
573 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol) and 3-*n*-butoxybenzaldehyde (0.17 g,
574 1.0 mmol) in MeOH (10 mL) at 0$^{o}$C, was added *p*-toluenesulfonic acid monohydrate (0.02 g,
575 0.11 mmol) and the reaction mixture was stirred for 2 h. After that, excess sodium
576 cyanoborohydride (0.63 g, 10.0 mmol) was added in portions to the reaction mixture. After the
577 addition, the reaction mixture was stirred for further 12 h. The reaction was quenched by pouring
578 into a separating funnel containing 50 mL water and extracted with ethyl acetate (20 mL x 3).
579 The combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced
580 pressure to a crude product, which was subjected to purification by flash column
581 chromatography on silica gel with gradient elution (20 % to 50 % ethyl acetate in hexane) to
582 afford the titled compound (0.15 g) in 45% yield. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.26 (dd, $J$ =
583 7.8, 7.8 Hz, 1H), 6.86 - 6.97 (m, 2H), 6.73 - 6.86 (m, 2H), 6.62 - 6.63 (m, 1H), 6.56 (br. s., 1H),
584 6.16 (br. s., 1H), 4.27 - 4.40 (m, 3H), 3.96 (t, $J$ = 7.2 Hz, 2H), 1.71 - 1.81 (m, 2H), 1.44 - 1.57
585 (m, 2H), 0.99 (t, $J$ = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.1 (s, *C*ONH$_2$), 159.6,
586 152.2 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 149.2, 146.7 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 140.0, 133.7 (dd, $J_{CF}$
587 = 13, 2.7 Hz, C3), 129.8, 122.2 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 119.2, 113.5 (dd, $J_{CF}$ = 23, 23 Hz,

C1), 113.4, 111.2 (dd, $J_{CF}$ = 21, 3.6 Hz, C5), 67.7, 47.9, 31.3, 19.2, 13.9; LRMS (ESI) *m/z* 335 ($M^+$ + H, 60), 357 ($M^+$ + Na, 50); HRMS (ESI) calcd for $C_{18}H_{21}N_2O_2F_2$ ($M^+$ + H) 335.1571, found 335.1568.

**2,6-Difluoro-3-((3-(*n*-pentyloxy)benzyl)amino)benzamide (12)**. This compound (0.13 g, 38%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol), 3-(*n*-pentyloxy)benzaldehyde (0.19 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^1$H NMR (400 MHz, Acetone-$d_6$) δ 7.37 (br. s., 1H), 7.24 (dd, *J* = 7.8, 7.8 Hz, 1H), 7.10 (br. s., 1H), 6.93 - 7.01 (m, 2H), 6.72 - 6.85 (m, 2H), 6.65 (dd, *J* = 7.8, 7.8 Hz, 1H), 5.54 (br. s., 1H), 4.42 (d, *J* = 5.8 Hz, 2H), 3.92 - 4.02 (m, 2H), 1.71 - 1.82 (m, 2H), 1.34 - 1.49 (m, 4H), 0.87 - 0.97 (m, 3H); $^{13}$C NMR (101 MHz, Acetone-$d_6$) δ 162.1 (s, *C*ONH$_2$), 159.6, 152.9 (dd, $J_{CF}$ = 234, 8.2 Hz, C6), 149.3 (dd, $J_{CF}$ = 244, 8.2 Hz, C2), 141.3, 137.8 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 122.2 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 119.1, 116.5 (dd, $J_{CF}$ = 23, 23 Hz, C1), 113.3, 112.7, 110.4 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 67.5, 46.9, 28.4, 28.1, 22.2, 13.4; LRMS (ESI) *m/z* 349 ($M^+$ + H, 100), 371 ($M^+$ + Na, 50); HRMS (ESI) calcd for $C_{19}H_{23}N_2O_2F_2$ ($M^+$ + H) 349.1728, found 349.1739.

**3-((3-(*sec*-Butoxy)benzyl)amino)-2,6-difluorobenzamide (13)**. This compound (0.12 g, 34%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.18 g, 1.0 mmol), 3-(*sec*-butoxy)benzaldehyde (0.18 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.24 (dd, *J* = 7.2,

7.2 Hz, 1H), 6.72 - 6.82 (m, 5H), 6.58 - 6.64 (m, 1H), 6.26 (br. s, 1H), 4.37 (br. s, 1H), 4.27 - 4.32 (m, 3H), 1.58 - 1.77 (m, 2H), 1.29 (d, $J$ = 7.2 Hz, 3H), 0.97 (t, $J$ = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.3 (s, *C*$ONH_2$), 158.7, 152.2 (dd, $J_{CF}$ = 234, 8.2 Hz, C6), 149.1 (dd, $J_{CF}$ = 244, 8.2 Hz, C2), 140.1, 133.7 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 129.8, 119.1, 114.8, 114.6, 113.5 (dd, $J_{CF}$ = 23, 23 Hz, C1), 112.6 (dd, $J_{CF}$ = 23, 23 Hz, C1), 111.1 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 75.0, 47.9, 29.2, 19.2, 9.7; LRMS (ESI) *m/z* 335 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{18}H_{21}N_2O_2F_2$ ($M^+$ + H) 335.1571, found 335.1570.

**3-(([1,1'-Biphenyl]-3-ylmethyl)amino)-2,6-difluorobenzamide (14).** This compound (0.16 g, 45%) was prepared from 2,6-difluoro-3-aminobenzamide (**10)** (0.18 g, 1.0 mmol), [1,1'-biphenyl]-3-carbaldehyde (0.18 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.53 - 7.67 (m, 4H), 7.42 - 7.52 (m, 3H), 7.31 - 7.42 (m, 2H), 6.81 (dd, $J$ = 8.0, 8.0 Hz, 1H), 6.69 (dd, $J$ = 8.0, 8.0. Hz, 1H), 6.12 (br. s., 1H), 6.06 (br. s., 1H), 4.44 (br. s., 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.7 (s, *C*$ONH_2$), 152.3 (dd, $J_{CF}$ = 238, 6.1 Hz, C6), 149.9 (dd, $J_{CF}$ = 244, 8.1 Hz, C2), 141.9, 140.8, 138.9, 133.9 (dd, $J_{CF}$ = 13, 2.0 Hz, C3), 129.3, 128.8, 127.5, 127.2, 126.4, 126.1, 126.0, 113.3 (dd, $J_{CF}$ = 10, 5.1 Hz, C4), 113.2 (dd, $J_{CF}$ = 24, 20 Hz, C1), 111.5 (dd, $J_{CF}$ = 23, 4.0 Hz, C5); LRMS (ESI) *m/z* 339 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{20}H_{17}N_2OF_2$ ($M^+$ + H) 339.1309, found 339.1305.

**3-((Benzo[*b*]thiophen-2-ylmethyl)amino)-2,6-difluorobenzamide (15)**. This compound (0.10 g, 32%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol),

benzo[*b*]thiophene-2-carbaldehyde (0.16 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^{1}$H NMR (400 MHz, Acetone-$d_6$) δ 7.86 (d, $J$ = 7.8 Hz, 1H), 7.76 (d, $J$ = 7.8 Hz, 1H), 7.27 - 7.47 (m, 4H), 7.10 (br. s., 1H), 6.75 - 6.89 (m, 2H), 5.74 (d, $J$ = 5.8 Hz, 1H), 4.77 (d, $J$ = 5.8 Hz, 2H); $^{13}$C NMR (101 MHz, Acetone-$d_6$) δ 161.9 (s, *C*ONH$_2$), 150.4 (dd, $J_{CF}$ = 238, 8.4 Hz, C6), 146.8 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 145.2, 140.0, 132.8 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 139.5, 124.3, 124.0, 123.2, 122.2, 121.2, 116.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 112.5 (dd, $J_{CF}$ = 22, 22 Hz, C1), 110.5 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 43.1; LRMS (ESI) *m/z* 319 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{13}N_2OSF_2$ ($M^+$ + H) 319.0717, found 319.0718.

**3-((Benzo[*d*]thiazol-2-ylmethyl)amino)-2,6-difluorobenzamide (16)**. This compound (0.12 g, 38%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol), benzo[*d*]thiazole-2-carbaldehyde (0.16 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.99 (d, $J$ = 8.3 Hz, 1H), 7.84 (d, $J$ = 8.3 Hz, 1H), 7.49 (t, $J$ = 7.6 Hz, 1H), 7.33 - 7.44 (m, 1H), 6.65 - 6.83 (m, 2H), 6.59 (br. s., 1H), 6.26 (br. s., 1H), 4.96 (br. s., 1H), 4.78 (d, $J$ = 6.2 Hz, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 171.5, 162.8 (s, *C*ONH$_2$), 153.3, 150.5 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 146.8 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 134.9, 132.6 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 126.2, 125.2, 122.9, 121.9, 116.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.9 (dd, $J_{CF}$ = 23, 23 Hz, C1), 111.3 (dd, $J_{CF}$ = 21, 3.6 Hz, C5), 46.7; LRMS (ESI) *m/z* 320 ($M^+$ + H, 90); HRMS (ESI) calcd for $C_{15}H_{12}N_3OSF_2$ ($M^+$ + H) 320.0669, found 320.0672.

**2,6-Difluoro-3-(((3-methylbenzo[*b*]thiophen-2-yl)methyl)amino)benzamide (17)**. This compound (0.15 g, 48%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol), 3-methylbenzo[*b*]thiophene-2-carbaldehyde (0.17 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 7.8 Hz, 1H), 7.70 (d, *J* = 7.8 Hz, 1H), 7.38 - 7.45 (m, 1H), 7.31 - 7.38 (m, 1H), 6.80 - 6.88 (m, 1H), 6.71 - 6.80 (m, 1H), 6.14 (br. s., 1H), 6.06 (br. s., 1H), 4.60 (d, *J* = 3.9 Hz, 2H), 4.36 (br. s., 1H), 2.45 (s, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.6 (s, *C*$ONH_2$), 150.3 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 146.5 (dd, $J_{CF}$ = 242, 8.2 Hz, C2), 140.7, 138.6, 136.2, 133.9 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 128.4, 124.4, 124.1, 122.5, 121.6, 116.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.8 (dd, $J_{CF}$ = 23, 23 Hz, C1), 111.3 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 42.2, 11.7; LRMS (ESI) *m/z* 333 ($M^+$ + H, 90); HRMS (ESI) calcd for $C_{17}H_{15}N_2OSF_2$ ($M^+$ + H) 333.0873, found 333.0875.

**3-(((1*H*-indol-3-yl)methyl)amino)-2,6-difluorobenzamide (18).** This compound (0.16 g, 53%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.18 g, 1.0 mmol), 1*H*-indole-3-carbaldehyde (0.15 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^{1}$H NMR (400 MHz, Acetone-$d_6$) δ 10.18 (br. s., 1H), 7.73 (d, *J* = 7.8 Hz, 1H), 7.42 (d, *J* = 7.8 Hz, 1H), 7.27 - 7.40 (m, 2H), 7.00 - 7.20 (m, 3H), 6.93 (dd, *J* = 7.2, 7.2 Hz, 1H), 6.82 (dd, *J* = 7.2, 7.2 Hz, 1H), 5.05 (br. s., 1H), 4.59 (d, *J* = 4.8 Hz, 2H); $^{13}$C NMR (101 MHz, Acetone-$d_6$) δ 162.3 (s, *C*$ONH_2$), 151.4 (dd, $J_{CF}$ = 238, 6.1 Hz, C6), 148.4 (dd, $J_{CF}$ = 244, 8.1 Hz, C2), 137.0, 134.0 (dd, $J_{CF}$ = 13, 2.0 Hz, C3), 127.0, 123.6, 121.5, 118.9,

118.7, 112.7, 112.3 (dd, $J_{CF}$ = 10, 5.1 Hz, C4), 111.4, 110.7 (dd, $J_{CF}$ = 24, 20 Hz, C1), 110.5 (dd, $J_{CF}$ = 23, 4.0 Hz, C5); LRMS (ESI) *m/z* 302 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{14}N_3OF_2$ ($M^+$ + H) 302.1105, found 302.1101.

**3-(((2,3-Dihydrobenzo[*b*][1,4]dioxin-6-yl)methyl)amino)-2,6-difluorobenzamide (19)**. This compound (0.15 g, 45%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.18 g, 1.0 mmol), 2,3-dihydrobenzo[*b*][1,4]dioxine-6-carbaldehyde (0.17 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ 6.62 - 6.87 (m, 4H), 6.62 - 6.68 (m, 1H), 6.13 (br. s, 1H), 6.05 (br. s, 1H), 4.27 (s, 7H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.7 (s, *C*$ONH_2$), 152.3 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 148.5 (dd, $J_{CF}$ = 242, 8.2 Hz, C2), 143.7, 143.0, 134.9 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 131.5, 120.2, 117.5, 116.1, 115.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.4 (dd, $J_{CF}$ = 23, 23 Hz, C1), 111.5 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 64.4, 64.3, 47.4; LRMS (ESI) *m/z* 321 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{15}N_2O_3F_2$ ($M^+$ + H) 321.1051, found 321.1050.

**2,6-Difluoro-3-(((1-phenyl-1*H*-pyrazol-4-yl)methyl)amino)benzamide (20)**. This compound (95 mg, 30%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol), 1-phenyl-1*H*-pyrazole-4-carbaldehyde (0.17 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^1$H NMR (400 MHz, Acetone-$d_6$) δ 8.33 (s, 1H), 7.81 (dd, *J* = 0.8, 8.8 Hz, 2H), 7.73 (s, 1H), 7.45 - 7.53 (m, 2H), 7.40 (br. s., 1H), 7.26 - 7.33 (m, 1H), 7.14 (br. s., 1H), 6.79 - 6.93 (m, 2H), 4.40 (s, 2H); $^{13}$C NMR (101 MHz, Acetone-

$d_6$) δ 162.2 (s, *C*ONH$_2$), 150.4 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 147.0 (dd, $J_{CF}$ = 242, 8.2 Hz, C2), 140.4, 140.3, 133.5 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 129.4, 126.0, 125.8, 121.9, 118.3, 116.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 112.4 (dd, $J_{CF}$ = 23, 23 Hz, C1), 110.5 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 37.8; LRMS (ESI) *m/z* 329 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{17}H_{15}N_4OF_2$ ($M^+$ + H) 329.1214, found 329.1216.

**2,6-Difluoro-3-(((5-phenylthiophen-2-yl)methyl)amino)benzamide (21)**. This compound (0.15 g, 42%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.18 g, 1.0 mmol), 5-phenylthiophene-2-carbaldehyde (0.19 g, 1.0 mmol), *p*-toluenesulfonic acid monohydrate (0.02 g, 0.11 mmol), MeOH (10 mL) and sodium cyanoborohydride (0.63 g, 10 mmol) according to the preparation procedure of **11** described above. $^1$H NMR (400 MHz, Acetone-$d_6$) δ 7.62 (d, *J* = 7.2 Hz, 2H), 7.26 - 7.41 (m, 5H), 7.08 - 7.09 (m, 2H), 6.83 - 6.88 (m, 2H), 5.64 (br, s, 1H), 4.67 (d, *J* = 6.2 Hz, 2H); $^{13}$C NMR (101 MHz, Acetone-$d_6$) δ 162.0 (s, *C*ONH2), 150.8 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 147.9 (dd, $J_{CF}$ = 242, 8.2 Hz, C2), 143.6, 142.9, 134.4, 133.1 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 128.9, 127.3, 126.0, 125.3, 122, 115.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 112.6 (dd, $J_{CF}$ = 23, 23 Hz, C1), 110.5 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 42.6; LRMS (ESI) *m/z* 345 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{18}H_{15}N_2OSF_2$ ($M^+$ + H) 345.0873, found 345.0872.

**2,6-Difluoro-3-((4-fluorobenzyl)amino)benzamide (22a)** and **3-(bis(4-fluorobenzyl)amino)-2,6-difluorobenzamide (22b)**. To a well-stirred solution of 2,6-difluoro-3-aminobenzamide (**10**) (0.40 g, 2.3 mmol) and 4-fluorobenzyl bromide (0.55 g, 2.9 mmol) in ACN (20 mL), was added $K_2CO_3$ (0.40 g, 2.9 mmol). The reaction mixture was heated to reflux for 4 h. After the complete disappearance of starting material as indicated from TLC, the reaction

mixture was subjected to pass through a short pad of silica gel. The obtained filtrate was evaporated under reduced pressure and the crude mixture was subjected to purification by flash column chromatography on silica gel with gradient elution (10 % to 40 % ethyl acetate in hexane). Both of the titled compounds **22a** (0.15 g) and **22b** (0.29 g) were obtained in 23% and 32 % yield respectively.

**2,6-Difluoro-3-((4-fluorobenzyl)amino)benzamide (22a)**. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.32 (dd, *J* = 5.4, 7.8 Hz, 2H), 7.05 (dd, *J* = 8.0, 8.0 Hz, 2H), 6.78 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.58 - 6.63 (m, 1H), 6.48 (br. s., 1H), 6.12 (br. s., 1H), 4.34 (s, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.9 (s, *C*$ONH_2$), 162.4 (d, $J_{CF}$ = 246 Hz, C1'), 152.5 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 149.0 (dd, $J_{CF}$ = 254, 8.2 Hz, C2), 134.0 (d, $J_{CF}$ = 3.6 Hz, C4'), 133.4 (dd, $J_{CF}$ = 11, 2.7 Hz, C3), 128.8 (d, $J_{CF}$ = 7.3 Hz, C3'), 115.7 (d, $J_{CF}$ = 21 Hz, C2'), 113.5 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 112.7 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.3 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 47.3 (s, *C*$H_2$); LRMS (ESI) *m/z* 281 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{14}H_{12}F_3N_2O$ ($M^+$ + H) 281.2531, found 281.2525.

**3-(Bis(4-fluorobenzyl)amino)-2,6-difluorobenzamide (22b)**. $^{1}$H NMR (400 MHz, Acetone-$d_6$) δ 7.48 (br. s., 1H), 7.39 (dd, *J* = 8.0, 8.0 Hz, 4H), 7.20 (br. s., 1H), 7.01 - 7.13 (m, 5H), 6.82 (dd, *J* = 8.0, 8.0 Hz, 1H), 4.27 (s, 4H); $^{13}$C NMR (101 MHz, Acetone-$d_6$) δ 161.9 (d, $J_{CF}$ = 243 Hz, C1'), 161.7 (s, *C*$ONH_2$), 154.3 (dd, $J_{CF}$ = 244, 8.2 Hz, C6), 153.0 (dd, $J_{CF}$ = 250, 8.2 Hz, C2), 134.7 (dd, $J_{CF}$ = 12, 2.7 Hz, C3), 134.1 (d, $J_{CF}$ = 2.7 Hz, C4'), 130.2 (d, $J_{CF}$ = 7.3 Hz, C3'), 123.5 (dd, $J_{CF}$ = 9.1, 3.6 Hz, C4), 116.3 (dd, $J_{CF}$ = 24, 24 Hz, C1), 114.9 (d, $J_{CF}$ = 22 Hz, C2'); 110.6 (dd, $J_{CF}$ = 23, 3.6 Hz, C5), 55.4 (d, $J_{CF}$ = 2.0 Hz, *C*$H_2$); LRMS (ESI) *m/z* 389 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{21}H_{17}F_4N_2O$ ($M^+$ + H) 389.3661, found 389.3656.

**2,6-Difluoro-3-((3,4-difluorobenzyl)amino)benzamide (23a)** and **3-(bis(3,4-difluorobenzyl)amino)-2,6-difluorobenzamide (23b)**. These two compounds **23a** (0.20 g, 29%) and **23b** (0.31 g, 31 %) were prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.40 g, 2.3 mmol), 3,4-difluorobenzyl bromide (0.60 g, 2.9 mmol), ACN (20 mL) and $K_2CO_3$ (0.42 g, 3.0 mmol) according to the preparation procedure of **22** described above.

**2,6-Difluoro-3-((3,4-difluorobenzyl)amino)benzamide (23a).** $^1$H NMR (400 MHz, $CDCl_3$) δ 7.08 - 7.19 (m, 3H), 6.77 (dd, *J* = 9.2, 9.2 Hz, 1H), 6.50 - 6.58 (m, 2H), 6.16 (br. s, 1H), 4.44 (br, s, 1H), 4.34 (d, *J* = 5.6 Hz, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.8 (s, *C*$ONH_2$), 162.6 (dd, $J_{CF}$ = 246, 20 Hz, C1'), 152.4 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 150.6 (dd, $J_{CF}$ = 246, 20 Hz, C6'), 149.3 (dd, $J_{CF}$ = 254, 8.2 Hz, C2), 148.5 (d, $J_{CF}$ = 20, 8.2 Hz, C2'), 146.8 (dd, $J_{CF}$ = 20, 8.2 Hz, C5'), 135.6 (dd, $J_{CF}$ = 11, 2.7 Hz, C3), 122.8 (dd, $J_{CF}$ = 8.2, 2.7 Hz, C3'), 117.7 (dd, $J_{CF}$ = 8.2, 2.7 Hz, C4'), 113.5 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 111.5 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.2 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 46.9 (s, *C*$H_2$); LRMS (ESI) *m/z* 299 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{14}H_{11}F_4N_2O$ ($M^+$ + H) 299.0808, found 299.0803.

**3-(Bis(3,4-difluorobenzyl)amino)-2,6-difluorobenzamide (23b)**. $^1$H NMR (400 MHz, $CDCl_3$) δ 6.99 - 7.13 (m, 7H), 6.85 - 6.91 (m, 1H), 6.73 (dd, *J* = 9.2, 9.2 Hz, 1H), 6.39 (br. s, 1H), 4.15 (s, 4H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.0 (s, *C*$ONH_2$), 162.6 (dd, $J_{CF}$ = 246, 20 Hz, C1'), 151.6 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 156.2 (dd, $J_{CF}$ = 246, 20 Hz, C6'), 149.1 (dd, $J_{CF}$ = 254, 8.2 Hz, C2), 151.5 (d, $J_{CF}$ = 20, 8.2 Hz, C2'), 148.4 (dd, $J_{CF}$ = 20, 8.2 Hz, C5'), 134.6 (dd, $J_{CF}$ = 11, 2.7 Hz, C3), 124.8 (dd, $J_{CF}$ = 8.2, 2.7 Hz, C3'), 117.3 (dd, $J_{CF}$ = 8.2, 2.7 Hz, C4'), 117.7 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 114.5 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.3 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 55.6 (s, *C*$H_2$); LRMS (ESI) *m/z* 425 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{21}H_{15}F_6N_2O$ ($M^+$ + H) 425.1089, found 425.1087.

**2,6-Difluoro-3-((2,4-difluorobenzyl)amino)benzamide (24a)** and **3-(bis(2,4-difluorobenzyl)amino)-2,6-difluorobenzamide (24b)**. These two compounds **24a** (0.15 g, 22%) and **24b** (0.31 g, 31 %) were prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.40 g, 2.3 mmol), 2,4-difluorobenzyl bromide (0.60 g, 2.9 mmol), ACN (20 mL) and $K_2CO_3$ (0.42 g, 3.0 mmol) according to the preparation procedure of **22** described above.

**2,6-Difluoro-3-((2,4-difluorobenzyl)amino)benzamide (24a).** $^1$H NMR (400 MHz, $CDCl_3$) δ 7.28 - 7.33 (m, 1H), 6.76 - 6.86 (m, 3H), 6.60 - 6.66 (m, 2H), 6.20 (br. s, 1H), 4.37 (br. s, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.7 (dd, $J_{CF}$ = 246, 6.2 Hz, C5'), 163.0 (s, *C*ONH$_2$), 161.2 (dd, $J_{CF}$ = 246, 6.2 Hz, C1'), 152.4 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 150.0 (dd, $J_{CF}$ = 244, 8.2 Hz, C2), 133.3 (dd, $J_{CF}$ = 8.2, 8.2 Hz, C3'), 130.0 (dd, $J_{CF}$ = 24, 2.7 Hz, C4'), 121.3 (dd, $J_{CF}$ = 24, 2.7 Hz, C3), 113.4 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 112.9 (dd, $J_{CF}$ = 24, 20 Hz, C1), 111.5 (dd, $J_{CF}$ = 24, 2.7 Hz, C5), 111.3 (dd, $J_{CF}$ = 22, 3.6 Hz, C2'), 104.3 (d, $J_{CF}$ = 24, 24 Hz, C6'), 41.1 (d, $J_{CF}$ = 6.0 Hz, *C*H$_2$); LRMS (ESI) *m/z* 299 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{14}H_{11}F_4N_2O$ ($M^+$ + H) 299.0808, found 299.0807.

**3-(Bis(2,4-difluorobenzyl)amino)-2,6-difluorobenzamide (24b)**. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.34 (d, *J* = 8.0 Hz, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 6.90 - 6.93 (m, 1H), 6.74 - 6.83 (m, 5H), 6.56 (br. s, 1H), 6.08 (br. s, 1H), 4.27 (s, 4H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.5 (dd, $J_{CF}$ = 246, 6.2 Hz, C5'), 162.6 (s, *C*ONH$_2$), 161.0 (dd, $J_{CF}$ = 246, 6.2 Hz, C1'), 156.4 (dd, $J_{CF}$ = 238, 6.1 Hz, C6), 153.0 (dd, $J_{CF}$ = 244, 8.1 Hz, C2), 134.5 (dd, $J_{CF}$ = 8.2, 8.2 Hz, C3'), 131.3 (dd, $J_{CF}$ = 24, 2.7 Hz, C4'), 124.9 (dd, $J_{CF}$ = 10, 5.1 Hz, C4), 120.3 (dd, $J_{CF}$ = 13, 2.0 Hz, C3), 113.9 (dd, $J_{CF}$ = 24, 20 Hz, C1), 111.4 (dd, $J_{CF}$ = 23, 4.0 Hz, C5); 111.2 (dd, $J_{CF}$ = 22, 3.6 Hz, C2'), 103.7

(d, $J_{CF}$ = 24, 24 Hz, C6'), 49.3 (s, *C*$H_2$); LRMS (ESI) *m/z* 425 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{21}H_{15}F_6N_2O$ ($M^+$ + H) 425.1089, found 425.1083.

**2,6-Difluoro-3-((4-chlorobenzyl)amino)benzamide (25a)** and **3-(bis(4-chlorobenzyl)amino)-2,6-difluorobenzamide (25b)**. These two compounds **25a** (0.18 g, 26%) and **25b** (0.29 g, 30 %) were prepared from 2,6-difluoro-3-aminobenzamide (0.40 g, 2.3 mmol), 4-chlorobenzyl bromide (0.60 g, 2.9 mmol), ACN (20 mL) and $K_2CO_3$ (0.42 g, 3.0 mmol) according to the preparation procedure of **22** described above.

**2,6-Difluoro-3-((4-chlorobenzyl)amino)benzamide (25a)**. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.26 - 7.39 (m, 4H), 6.78 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.56 - 6.60 (m, 1H), 6.22 (br. s., 1H), 6.06 (br. s., 1H), 4.36 (s, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.7 (s, *C*$ONH_2$), 155.2 (dd, $J_{CF}$ = 248, 6.4 Hz, C6), 154.6 (dd, $J_{CF}$ = 257, 4.5 Hz, C2), 136.8 (s, C1'), 134.9 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 133.3 (s, C4'), 128.9 (s, C2'), 128.4 (s, C3'), 118.9 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 114.3 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.2 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 47.3 (s, *C*$H_2$); LRMS (ESI) *m/z* 297 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{14}H_{12}ClF_2N_2O$ ($M^+$ + H) 297.7077, found 297.7075.

**3-(Bis(4-chlorobenzyl)amino)-2,6-difluorobenzamide (25b)**. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.10 - 7.36 (m, 8H), 6.85 - 6.89 (m, 1H), 6.72 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.68 (br. s., 1H), 6.12 (br. s., 1H), 4.18 (s, 4H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.8 (s, *C*$ONH_2$), 155.0 (dd, $J_{CF}$ = 248, 6.4 Hz, C6), 153.6 (dd, $J_{CF}$ = 257, 4.5 Hz, C2), 135.9 (s, C1'), 134.9 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 133.1 (s, C4'), 129.6 (s, C2'), 128.6 (s, C3'), 124.4 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 114.3 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.3 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 55.7 (s, *C*$H_2$); LRMS (ESI) *m/z* 422 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{21}H_{17}Cl_2F_2N_2O$ ($M^+$ + H) 422.2753, found 422.2756.

**2,6-Difluoro-3-(heptylamino)benzamide (26)**. To a well-stirred solution of 2,6-difluoro-3-aminobenzamide (**10**) (0.70 g, 4.1 mmol) and 1-bromoheptane (0.80 g, 4.4 mmol) in ACN (50 mL) was added $K_2CO_3$ (0.60 g, 4.4 mmol) and catalytic amount of NaI (0.08 g). The reaction mixture was heated to reflux for 4 h. After the complete disappearance of starting material as indicated by TLC, the reaction mixture was subjected to pass through a short pad of silica gel. The brown filtrate obtained was evaporated under reduced pressure and subjected to purification by flash column chromatography on silica gel with gradient elution (10 % to 40 % ethyl acetate in hexane) to afford the titled compound (0.40 g) in 36% yield. $^1$H NMR (400 MHz, $CDCl_3$) δ 6.85 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.66 - 6.70 (m, 1H), 6.14 (br. s., 1H), 6.05 (br. s., 1H), 3.12 (t, *J* = 7.2 Hz, 2H), 1.59 - 1.69 (m, 2H), 1.27 - 1.45 (m, 8H), 0.91 (t, *J* = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.9 (s, *C*$ONH_2$), 149.6 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 146.8 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 134.2 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 116.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.1 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.4 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 43.9, 31.8, 29.3, 29.1, 27.0, 22.6, 14.1; LRMS (ESI) *m/z* 271 ($M^+$ + H, 100), 293 ($M^+$ + Na, 60); HRMS (ESI) calcd for $C_{14}H_{21}N_2OF_2$ ($M^+$ + H) 271.1622, found 271.1612.

**2,6-Difluoro-3-(octylamino)benzamide (27)**. The titled compound **27** (0.26 g, 39%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.40 g, 2.3 mmol), 1-bromooctane (0.45 g, 2.3 mmol), NaI (0.04 g), ACN (20 mL) and $K_2CO_3$ (0.40 g, 2.9 mmol) according to the preparation procedure of **26** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ 6.83 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.65 - 6.70 (m, 1H), 6.36 (br. s., 1H), 6.09 (br. s., 1H), 3.81 (br. s., 1H), 3.06 - 3.17 (m, 2H), 1.59 - 1.69 (m, 2H), 1.23 - 1.43 (m, 10H), 0.90 (t, *J* = 6.60 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.0 (s, *C*$ONH_2$), 151.9 (dd, $J_{CF}$ = 241, 6.4 Hz, C6), 148.5 (dd, $J_{CF}$ = 247, 6.4

Hz, C2), 134.1 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 113.0 (dd, $J_{CF}$ = 9.1, 5.4 Hz, C4), 112.5 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.3 (dd, $J_{CF}$ = 23, 3.6 Hz, C5), 43.9, 31.8, 29.4, 29.3, 29.2, 27.0, 22.6, 14.1; LRMS (ESI) *m/z* 285 ($M^+$ + H, 100), 307 ($M^+$ + Na, 20); HRMS (ESI) calcd for $C_{15}H_{23}N_2OF_2$ ($M^+$ + H) 285.1778, found 285.1773.

**2,6-Difluoro-3-(nonylamino)benzamide (28)**. The titled compound **28** (0.49 g, 38%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.74 g, 4.3 mmol), 1-bromononane (1.20 g, 5.8 mmol), NaI (0.08 g), ACN (50 mL) and $K_2CO_3$ (1.20 g, 8.7 mmol) according to the preparation procedure of **26** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ 6.77 - 6.94 (m, 1H), 6.66 - 6.70 (m, 1H), 6.12 (br. s., 1H), 6.05 (br. s., 1H), 3.82 (br. s., 1H), 3.12 (t, *J* = 7.2 Hz, 2H), 1.58 - 1.73 (m, 2H), 1.23 - 1.46 (m, 12H), 0.90 (t, *J* = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.8 (s, *C*$ONH_2$), 149.5 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 146.7 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 134.2 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 116.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.1 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.2 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 43.9, 31.9, 29.5, 29.4, 29.3, 29.2, 27.0, 22.7, 14.1; LRMS (ESI) *m/z* 299 ($M^+$ + H, 97), 321 ($M^+$ + Na, 100); HRMS (ESI) calcd for $C_{16}H_{24}N_2OF_2Na$ ($M^+$ + Na) 321.1754, found 321.1756.

A hydrochloride salt of compound **28** was prepared by mixing a solution of compound **28** in DCM and excess concentrated hydrochloric acid followed by evaporation under high vacuum to dryness. This compound was used for *in vivo* PK and efficacy studies.

**3-(Decylamino)-2,6-difluorobenzamide (29)**. The titled compound **29** (0.27 g, 37%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.40 g, 2.3 mmol), 1-bromodecane (0.56 g, 2.5 mmol), NaI (0.04 g), ACN (20 mL) and $K_2CO_3$ (0.40 g, 2.9 mmol) according to the

preparation procedure of **26** described above. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.83 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.67 - 6.71 (m, 1H), 6.29 (br. s., 1H), 6.10 (br. s., 1H), 3.75 - 3.89 (m, 1H), 3.12 (t, *J* = 7.2 Hz, 2H), 1.59 - 1.68 (m, 2H), 1.21 - 1.49 (m, 14H), 0.90 (t, *J* = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.0 (s, *C*$CONH_2$), 151.9 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 147.0 (dd, $J_{CF}$ = 242, 8.2 Hz, C2), 134.1 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 116.2 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.1 (dd, $J_{CF}$ = 22, 22 Hz, C1), 111.1 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 57.9, 43.9, 31.9, 31.9, 29.6, 29.5, 29.3, 27.0, 22.7, 14.1; LRMS (ESI) *m/z* 313 ($M^+$ + H, 28), 335 ($M^+$ + Na, 95); HRMS (ESI) calcd for $C_{17}H_{26}N_2OF_2Na$ ($M^+$ + Na) 335.1911, found 335.1923.

**2,6-Difluoro-3-(nonan-2-ylamino)benzamide (30)**. The titled compound **30** (0.23 g, 33%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.40 g, 2.3 mmol), 2-bromononane (0.47 g, 2.3 mmol), NaI (0.04 g), ACN (20 mL) and $K_2CO_3$ (0.40 g, 2.9 mmol) according to the preparation procedure of **26** described above. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.83 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.65 - 6.70 (m, 1H), 6.18 (br. s., 1H), 6.05 (br. s., 1H), 3.63 (br. s., 1H), 3.35 - 3.47 (m, 1H), 1.14 - 1.62 (m, 15H), 0.90 (t, *J* = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.9 (s, *C*$CONH_2$), 152.0 (dd, $J_{CF}$ = 241, 6.4 Hz, C6), 149.0 (dd, $J_{CF}$ = 247, 6.4 Hz, C2), 134.0 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 113.3 (dd, $J_{CF}$ = 9.1, 5.4 Hz, C4), 112.4 (dd, $J_{CF}$ = 24, 24 Hz, C1), 111.4 (dd, $J_{CF}$ = 23, 3.6 Hz, C5), 48.8, 37.0, 31.8, 29.6, 29.3, 26.1, 22.7, 20.7, 14.1; LRMS (ESI) *m/z* 299 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{25}N_2OF_2$ ($M^+$ + H) 299.1935, found 299.1934.

**2,6-Difluoro-3-nonanamidobenzamide (31)**: To a well-stirred solution of 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol) in DCM (5 mL) and pyridine (5 mL) at 0$^{o}$C, was added nonanoyl chloride (0.23 g, 1.3 mmol) dropwise. The reaction mixture was stirred for 4 hr at 0$^{o}$C.

The reaction was then quenched by pouring into a separating funnel containing 1 M HCl (50 mL) and extracted with DCM (20 mL x 3). The combined organic layers was washed with $NaHCO_3$, dried over $MgSO_4$, filtered and evaporated under reduced pressure to give a crude reaction mixture, which was further subjected to purification by flash column chromatography on silica gel with gradient elution (10 % to 40 % ethyl acetate in hexane) to afford the desired compound (0.11 g, 36%). $^1$H NMR (400 MHz, Acetone-$d_6$) δ 8.95 (br. s., 1H), 8.14 - 8.19 (m, 1H), 7.51 (br. s., 1H), 7.19 (br. s., 1H), 7.02 (dd, $J$ = 8.0, 8.0 Hz, 1H), 2.47 (t, $J$ = 7.2 Hz, 2H), 1.66 - 1.74 (m, 2H), 1.25 - 1.43 (m, 10H), 0.81 - 0.98 (m, 3H); $^{13}$C NMR (101 MHz, Acetone-$d_6$) δ 171.7, 161.2 (s, *C*ONH$_2$), 151.8 (dd, $J_{CF}$ = 234, 8.2 Hz, C6), 146.0 (dd, $J_{CF}$ = 245, 8.2 Hz, C2), 134.1 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 123.6 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 116.8 (dd, $J_{CF}$ = 23, 23 Hz, C1), 110.7 (dd, $J_{CF}$ = 21, 3.6 Hz, C5), 36.3, 31.7, 28.4, 25.3, 22.4, 19.1, 18.5, 13.4; LRMS (ESI) *m/z* 313 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{22}N_2O_2F_6$ ($M^+$ + H) 313.1728, found 313.1726.

**3-((4-Butoxybutyl)amino)-2,6-difluorobenzamide (32)**. The titled compound **32** (0.05 g, 17%) was prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol), 1-bromo-4-butoxybutane (0.21 g, 1.0 mmol), NaI (0.03 g), ACN (20 mL) and $K_2CO_3$ (0.15 g, 1.1 mmol) according to the preparation procedure of **26** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ 6.83 (dd, $J$ = 8.0, 8.0 Hz, 1H), 6.65 - 6.70 (m, 1H), 6.32 (br. s., 1H), 6.09 (br. s., 1H), 3.95 (br. s., 1H), 3.41 - 3.48 (m, 4H), 3.16 (br. s., 2H), 1.65 - 1.76 (m, 4H), 1.53 - 1.61 (m, 2H), 1.32 - 1.44 (m, 2H), 0.93 (t, $J$ = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.0 (s, *C*ONH$_2$), 151.1 (dd, $J_{CF}$ = 234, 8.2 Hz, C6), 146.7 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 134.1 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 116.2 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.0 (dd, $J_{CF}$ = 23, 23 Hz, C1), 111.2 (dd, $J_{CF}$ = 21, 3.6 Hz, C5),

907 70.8, 70.3, 43.7, 31.8, 27.2, 26.2, 19.4, 13.9; LRMS (ESI) *m/z* 301 ($M^+$ + H, 40); HRMS (ESI)
908 calcd for $C_{15}H_{23}N_2O_2F_2$ ($M^+$ + H) 301.1728, found 301.1716.
909
910 **(*E*)-3-((3,7-Dimethylocta-2,6-dien-1-yl)amino)-2,6-difluorobenzamide (33)**. The titled
911 compound **33** (0.29 g, 48%) were prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.34 g,
912 2.0 mmol), geranyl bromide (0.42 g, 2.0 mmol), NaI (0.04 g), ACN (20 mL) and $K_2CO_3$ (0.29 g,
913 2.1 mmol) according to the preparation procedure of **26** described above. $^1$H NMR (400 MHz,
914 $CDCl_3$) δ 6.83 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.65 - 6.70 (m, 1H), 6.48 (br. s., 1H), 6.11 (br. s., 1H),
915 5.30 (t, *J* = 6.11 Hz, 1H), 5.03 - 5.13 (m, 1H), 3.83 (br. s., 1H), 3.72 (d, *J* = 7.2 Hz, 2H), 2.02 -
916 2.16 (m, 4H), 1.66 - 1.75 (m, 6H), 1.62 (s, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.1 (s,
917 *C*$CONH_2$), 152.1 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 146.8 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 139.9, 134.1 (dd,
918 $J_{CF}$ = 14, 2.7 Hz, C3), 131.8, 123.8, 120.7, 116.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.4 (dd, $J_{CF}$ = 24,
919 24 Hz, C1), 111.4 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 41.8, 39.5, 26.3, 25.7, 17.7, 16.4; LRMS (ESI) *m/z*
920 309 ($M^+$ + H, 100), 321 ($M^+$ + Na, 6); HRMS (ESI) calcd for $C_{17}H_{23}N_2OF_2$ ($M^+$ + H) 309.1778,
921 found 309.1779.
922
923 **(*Z*)-2,6-Difluoro-3-(non-2-en-1-ylamino)benzamide (34)**. The titled compound **34** (0.13 g,
924 44%) were prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.17 g, 1.0 mmol), (*Z*)-1-
925 bromonon-2-ene (0.21 g, 1.0 mmol), NaI (0.04 g), ACN (20 mL) and $K_2CO_3$ (0.15 g, 1.1 mmol)
926 according to the preparation procedure of **26** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ
927 6.84 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.67 – 6.71 (m, 1H), 6.28 (br. s., 1H), 6.08 (br. s., 1H), 5.52 - 5.61
928 (m, 1H), 5.32 - 5.43 (m, 1H), 3.88 (br. s., 1H), 3.09 - 3.18 (m, 2H), 2.40 (q, *J* = 7.2 Hz, 2H), 2.06
929 (q, *J* = 7.2 Hz, 2H), 1.24 - 1.41 (m, 6H), 0.90 (t, *J* = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ

930 162.9 (s, *C*ONH$_2$), 152.0 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 149.6 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 133.9
931 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 133.4, 125.4, 116.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.3 (dd, $J_{CF}$ = 24,
932 24 Hz, C1), 111.4 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 43.4, 31.5, 29.3, 27.3, 27.0, 22.5, 14.0; LRMS
933 (ESI) *m/z* 297 ($M^+$ + H, 100), 319 ($M^+$ + Na, 35); HRMS (ESI) calcd for $C_{16}H_{23}N_2OF_2$ ($M^+$ + H)
934 297.1778, found 297.1768.
935
936 **2,6-Difluoro-3-((3-phenylpropyl)amino)benzamide (35)**. The titled compound **35** (0.16 g,
937 53%) were prepared from 2,6-difluoro-3-aminobenzamide (**10**) (0.18 g, 1.0 mmol), (3-
938 bromopropyl)benzene (0.21 g, 1.0 mmol), NaI (0.04 g), ACN (20 mL) and $K_2CO_3$ (0.15 g, 1.1
939 mmol) according to the preparation procedure of **26** described above. $^1$H NMR (400 MHz,
940 $CDCl_3$) δ d 7.27 - 7.36 (m, 2H), 7.15 - 7.27 (m, 3H), 6.75 - 6.89 (m, 1H), 6.70 (br. s., 1H), 6.62
941 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.18 (br. s., 1H), 3.86 (br. s., 1H), 3.15 (t, *J* = 7.0 Hz, 2H), 2.75 (t, *J* =
942 7.0 Hz, 2H), 1.94 - 2.01 (m, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.3 (s, *C*ONH$_2$), 151.9 (dd,
943 $J_{CF}$ = 238, 6.1 Hz, C6), 149.2 (dd, $J_{CF}$ = 244, 8.1 Hz, C2), 141.3, 133.9 (dd, $J_{CF}$ = 13, 2.0 Hz,
944 C3), 128.5, 128.4, 126.1, 113.0 (dd, $J_{CF}$ = 10, 5.1 Hz, C4), 112.6 (dd, $J_{CF}$ = 24, 20 Hz, C1), 111.4
945 (dd, $J_{CF}$ = 23, 4.0 Hz, C5), 43.1, 33.1, 30.7; LRMS (ESI) *m/z* 291 ($M^+$ + H, 100); HRMS (ESI)
946 calcd for $C_{16}H_{17}N_2OF_2$ ($M^+$ + H) 291.1309, found 291.1308.
947
948 **2,6-Difluoro-3-(methyl(octyl)amino)benzamide (36)**: To a well-stirred solution of 2,6-
949 difluoro-3-(octylamino)benzamide (**27**) (0.12 g, 0.4 mmol) and dimethyl sulphate (0.27 g, 2.1
950 mmol) in ACN (10 mL) was added $K_2CO_3$ (0.30 g, 2.1 mmol). The reaction mixture was heated
951 to reflux for 14 h. After the complete disappearance of starting material as indicated by TLC, the
952 reaction mixture was subjected to pass through a short pad of silica gel. The filtrate obtained was

evaporated under reduced pressure and subjected to purification by flash column chromatography on silica gel with gradient elution (10 % to 40 % ethyl acetate in hexane) to afford the titled compound (0.03 g) in 24% yield. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.93 - 6.96 (m, 1H), 6.80 - 6.90 (m, 1H), 6.63 (br. s., 1H), 6.11 (br. s., 1H), 3.04 (t, $J$ = 7.2 Hz, 2H), 2.79 (s, 3H), 1.47 - 1.58 (m, 2H), 1.22 - 1.35 (m, 10H), 0.89 (t, $J$ = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.2 (s, *C*$ONH_2$), 154.9 (dd, $J_{CF}$ = 236, 8.2 Hz, C6), 152.4 (dd, $J_{CF}$ = 242, 8.2 Hz, C2), 137.5 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 121.1 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 113.9 (dd, $J_{CF}$ = 22, 22 Hz, C1), 111.1 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 55.6, 40.0, 31.8, 29.5, 29.3, 27.2, 27.0, 22.6, 14.1; LRMS (ESI) *m/z* 299 ($M^+$ + H, 100), 321 ($M^+$ + Na, 26); HRMS (ESI) calcd for $C_{16}H_{25}N_2OF_2$ ($M^+$ + H) 299.1935, found 299.1928.

**2,6-Difluoro-3-(methyl(nonyl)amino)benzamide (37)**: The titled compound **37** (0.03 g, 19%) were prepared from 2,6-difluoro-3-(nonylamino)benzamide (**28**) (0.15 g, 0.5 mmol), dimethyl sulphate (0.15 g, 1.2 mmol), acetone (20 mL) and $K_2CO_3$ (0.15 g, 1.1 mmol) according to the preparation procedure of **36** described above. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 6.90 - 7.02 (m, 1H), 6.79 - 6.90 (m, 1H), 6.39 (br. s., 1H), 6.05 (br. s., 1H), 3.00 - 3.08 (m, 2H), 2.79 (s, 3H), 1.53 (br. s., 2H), 1.27 (br. s., 12H), 0.89 (t, $J$ = 6.6 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.0 (s, *C*$ONH_2$), 154.9 (dd, $J_{CF}$ = 234, 8.2 Hz, C6), 148.9 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 137.6 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 121.2 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 116.9 (dd, $J_{CF}$ = 22, 22 Hz, C1), 111.2 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 55.6, 55.5, 40.0, 31.9, 29.6, 29.3, 27.2, 27.0, 22.7, 14.1; LRMS (ESI) *m/z* 313 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{17}H_{27}N_2OF_2$ ($M^+$ + H) 313.2091, found 313.2083.

**4-Bromo-2,6-difluoro-3-(nonylamino)benzamide (38)**. To a well-stirred solution of 2,6-difluoro-3-(nonylamino)benzamide (**28**) (0.3 g, 1.0 mmol) in DCM (20 mL) at room temperature was added excess bromine (1 mL) and stirred for 12 h. After the complete disappearance of starting material as indicated by TLC, the reaction mixture was poured into a separating funnel containing saturated sodium thiosulfate solution (30 mL) and extracted with ethyl acetate (20 mL x 3). The combined organic layers were dried over $MgSO_4$, filtered and evaporated to give a crude product which was further subjected to purification by flash column chromatography on silica gel with gradient elution (10 % to 40 % ethyl acetate in hexane) to furnish the titled compound (0.28 g, 74%). $^1$H NMR (400 MHz, $CDCl_3$) δ 7.11 (dd, $J$ = 1.96, 8.80 Hz, 1H), 6.76 (br. s., 1H), 6.19 (br. s., 1H), 3.74 (br. s., 1H), 3.28 (t, $J$ = 6.0 Hz, 2H), 1.52 - 1.61 (m, 2H), 1.24 - 1.38 (m, 12H), 0.83 - 0.92 (m, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 162.5 (s, *C*$ONH_2$), 152.9 (dd, $J_{CF}$ = 234, 8.2 Hz, C6), 148.4 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 133.0 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 115.8 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 114.9 (dd, $J_{CF}$ = 21, 21 Hz, C1), 113.4 (dd, $J_{CF}$ = 21, 3.6 Hz, C5), 47.3, 47.2, 31.9, 31.6, 29.5, 29.2, 26.8, 22.7, 14.1; LRMS (ESI) *m/z* 377 ($M^+$ + H, 96), 399 ($M^+$ + Na, 16); HRMS (ESI) calcd for $C_{16}H_{24}N_2OF_2Br$ ($M^+$ + H) 377.1040, found 377.1049.

**3-Azido-2,6-difluorobenzamide (39)**. To a well-stirred mixture of 2,6-difluoro-3-aminobenzamide (**10**) (2.90 g, 16.8 mmol) in water (5 mL) at 0$^o$C, was added conc. HCl (5 mL) dropwise and the reaction mixture was stirred for 10 minutes. After that, a solution of $NaNO_2$ (1.30 g, 18.8 mmol) in water (5 mL) was added dropwise to the reaction mixture while keeping the internal temperature below 5$^o$C. After the addition of $NaNO_2$, the reaction mixture was stirred for further 30 minutes. Then a solution of $NaN_3$ (1.20 g, 18.4 mmol) in water (2 mL) was added dropwise to the reaction mixture while keeping the internal temperature below 5$^o$C and

stirred for 4 h. The reaction was quenched by pouring into a separating funnel containing 50 mL water and extracted with ethyl acetate (20 mL x 3). The combined organic layers were dried over $MgSO_4$, filtered and evaporated under reduced pressure to a crude product, which was subjected to flash column chromatography to afford the titled compound (2.61 g, 78 %). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.19 (br. s., 1H), 7.94 (br. s., 1H), 7.37 - 7.43 (m, 1H), 7.14 - 7.28 (m, 1H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 161.1 (s, *C*ONH$_2$), 155.9 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 150.3 (dd, $J_{CF}$ = 242, 8.2 Hz, C2), 124.4 (dd, $J_{CF}$ = 13, 2.7 Hz, C3), 122.6 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 117.7 (dd, $J_{CF}$ = 23, 23 Hz, C1), 113.0 (dd, $J_{CF}$ = 22, 3.6 Hz, C5); LRMS (ESI) *m/z* 221 ($M^+$ + Na, 100); HRMS (ESI) calcd for $C_7H_4N_4OF_2Na$ ($M^+$ + Na) 221.0251, found 221.0250.

**2,6-Difluoro-3-(4-hexyl-1*H*-1,2,3-triazol-1-yl)benzamide (40)**: To a well stirred solution of 3-azido-2,6-difluorobenzamide (**39**) (0.26 g, 1.3 mmol) and oct-1-yne (0.16 g, 1.4 mmol) in THF (20 mL), was added catalytic amount of $Cu(PPh_3)_3Br$ (0.08 g, 0.09 mmol). The reaction mixture was heated to reflux for 14 h. After the complete disappearance of starting material as indicated from TLC, the reaction was subjected to pass through a short pad of silica gel. The obtained filtrate was evaporated under reduced pressure and subjected to purification by flash column chromatography on silica gel to afford the titled compound (0.25 g) was obtained in 62% yield. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.95 - 7.99 (m, 1H), 7.81 (br. s., 1H), 7.13 (t, *J* = 8.3 Hz, 1H), 6.58 (br. s., 1H), 6.40 (br. s., 1H), 2.79 (t, *J* = 6.8 Hz, 2H), 1.67 - 1.78 (m, 2H), 1.24 - 1.45 (m, 6H), 0.82 - 0.98 (m, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 160.9 (s, *C*ONH$_2$), 157.7, 155.5 (dd, $J_{CF}$ = 238, 8.2 Hz, C6), 150.3 (dd, $J_{CF}$ = 243, 8.2 Hz, C2), 139.0, 127.0 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 122.6 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 117.7 (dd, $J_{CF}$ = 23, 23 Hz, C1), 113.9 (dd, $J_{CF}$ = 22, 3.6

Hz, C5), 31.5, 29.1, 28.9, 25.5, 22.5, 14.0; LRMS (ESI) *m/z* 309 ($M^+$ + H, 100), 331 ($M^+$ + Na, 20); HRMS (ESI) calcd for $C_{15}H_{19}N_4OF_2$ ($M^+$ + H) 309.1527, found 309.1531.

**2,6-Difluoro-3-(4-heptyl-1*H*-1,2,3-triazol-1-yl)benzamide (41)**. This compound **41** (0.28 g, 66%) was prepared from 3-azido-2,6-difluorobenzamide (**39**) (0.26 g, 1.3 mmol), non-1-yne (0.18 g, 1.4 mmol), THF (20 mL) and catalytic amount of $Cu(PPh_3)_3Br$ (0.08 g, 0.09 mmol) according to the preparation procedure of **40** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.89 - 8.04 (m, 1H), 7.78 (br. s., 1H), 7.13 (t, *J* = 8.8 Hz, 1H), 6.57 (br. s., 1H), 6.40 (br. s., 1H), 2.79 (t, *J* = 7.6 Hz, 2H), 1.65 - 1.77 (m, 2H), 1.37 (br. s., 8H), 0.90 (t, *J* = 6.4 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 160.9 (s, *C*$ONH_2$), 157.8, 155.5 (dd, $J_{CF}$ = 234, 8.2 Hz, C6), 151.3 (dd, $J_{CF}$ = 240, 8.2 Hz, C2), 138.4, 127.0 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 122.7 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C4), 117.7 (dd, $J_{CF}$ = 23, 23 Hz, C1), 113.0 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 31.7, 29.2, 29.1, 29.0, 25.5, 22.6, 14.0; LRMS (ESI) *m/z* 323 ($M^+$ + H, 100), 345 ($M^+$ + Na, 20); HRMS (ESI) calcd for $C_{16}H_{21}N_4OF_2$ ($M^+$ + H) 323.1683, found 323.1697.

**2,6-Difluoro-3-(4-octyl-1*H*-1,2,3-triazol-1-yl)benzamide (42)**. This compound **42** (0.30 g, 68%) was prepared from 3-azido-2,6-difluorobenzamide (**39**) (0.26 g, 1.3 mmol), dec-1-yne (0.20 g, 1.4 mmol), THF (20 mL) and catalytic amount of $Cu(PPh_3)_3Br$ (0.08 g, 0.09 mmol) according to the preparation procedure of **42** described above. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.33 (s, 1H), 8.27 (br., s, 1H), 8.01 (s, 1H), 7.91 (dd, *J* = 8.0, 8.0 Hz, 1H), 7.41 (dd, *J* = 8.0, 8.0 Hz, 1H), 2.72 (t, *J* = 7.2 Hz, 1H), 1.65 - 1.67 (m, 2H), 1.26 - 1.32 (m, 10H), 0.86 (t, *J* = 7.2 Hz, 3H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 160.7 (s, *C*$ONH_2$), 159.8 (dd, $J_{CF}$ = 234, 8.2 Hz, C6), 152.6 (dd, $J_{CF}$ = 240, 8.2 Hz, C2), 148.2, 127.6 (dd, $J_{CF}$ = 14, 2.7 Hz, C3), 123.9, 122.3 (dd, $J_{CF}$

= 9.1, 5.5 Hz, C4), 117.7 (dd, $J_{CF}$ = 23, 23 Hz, C1), 113.3 (dd, $J_{CF}$ = 22, 3.6 Hz, C5), 31.7, 29.2, 29.2, 29.1, 29.0, 25.3, 22.5, 14.4; LRMS (ESI) *m/z* 337 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{17}H_{23}N_4OF_2$ ($M^+$ + H) 337.1840, found 337.1839.

**3-(Nonylamino)benzamide (44a)**. To a well-stirred solution of 3-aminobenzamide (**43a**) (0.20 g, 1.4 mmol) and 1-bromononane (0.32 g, 1.5 mmol) in ACN (20 mL) was added $K_2CO_3$ (0.23 g, 1.6 mmol). The reaction mixture was heated to reflux for 4 h. After the complete disappearance of starting material as indicated by TLC, the reaction mixture was subjected to pass through a short pad of silica gel. The filtrate obtained was evaporated under reduced pressure and subjected to purification by flash column chromatography on silica gel. The titled compound (0.15 g) was obtained in 39% yield. $^1$H NMR (400 MHz, $CDCl_3$) δ 7.23 (dd, *J* = 7.8, 7.8 Hz, 1H), 7.11 (d, *J* = 1.9 Hz, 1H), 7.03 (d, *J* = 7.3 Hz, 1H), 6.75 (dd, *J* = 2.2, 7.6 Hz, 1H), 6.15 (br. s., 1H), 5.99 (br. s., 1H), 3.15 (t, *J* = 7.2 Hz, 2H), 1.63 (quin, *J* = 7.2 Hz, 2H), 1.25 - 1.45 (m, 12H), 0.84 - 0.95 (m, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 170.2, 148.8, 134.4, 129.3, 116.1, 115.3, 111.6, 43.9, 31.9, 29.6, 29.4, 29.3, 27.1, 22.7, 14.1; LRMS (ESI) *m/z* 263 ($M^+$ + H, 100), 285 ($M^+$ + Na, 8); HRMS (ESI) calcd for $C_{16}H_{27}N_2O$ ($M^+$ + H) 263.2123, found 263.2122.

**2-Fluoro-5-(nonylamino)benzamide (44b)**: To a well-stirred solution of 2-fluoro-5-aminobenzamide (**43b**) (0.20 g, 1.3 mmol) and 1-bromononane (0.30 g, 1.4 mmol) in ACN (20 mL) was added $K_2CO_3$ (0.25 g, 1.8 mmol). The reaction mixture was heated to reflux for 4 h. After the complete disappearance of starting material as indicated by TLC, the reaction mixture was subjected to pass through a short pad of silica gel. The filtrate obtained was evaporated under reduced pressure and subjected to purification by flash column chromatography on silica

gel. The titled compound (0.11 g) was obtained in 30% yield. $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.23 - 7.34 (m, 1H), 6.94 (d, *J* = 8.8 Hz, 1H), 6.76 (s, 1H), 6.62 - 6.71 (m, 1H), 6.28 (br. s., 1H), 3.70 (br. s., 1H), 3.11 (t, *J* = 7.0 Hz, 2H), 1.61 (quin, *J* = 7.0 Hz, 2H), 1.22 - 1.44 (m, 12H), 0.89 (t, *J* = 6.6 Hz, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 165.6 (s, *C*$ONH_2$), 153.7 (d, $J_{CF}$ = 232 Hz, C2), 145.4 (d, $J_{CF}$ = 2.0 Hz, C5), 120.1 (d, $J_{CF}$ = 26 Hz, C1), 117.4 (d, $J_{CF}$ = 9.1 Hz, C4), 116.5 (dd, $J_{CF}$ = 12 Hz, C3), 114.3 (d, $J_{CF}$ = 9.1 Hz, C6), 44.4, 31.9, 29.5, 29.4, 29.3, 27.1, 22.7, 14.1; LRMS (ESI) *m/z* 281 ($M^+$ + H, 100), 303 ($M^+$ + Na, 50); HRMS (ESI) calcd for $C_{16}H_{26}N_2OF$ ($M^+$ + H) 281.2029, found 281.2033.

**2,4-Difluoro-5-(nonylamino)benzamide (44c)**: To a well-stirred solution of 2,4-difluoro-5-aminobenzamide (**43c**) (0.20 g, 1.1 mmol) and 1-bromononane (0.28 g, 1.4 mmol) in ACN (20 mL) was added $K_2CO_3$ (0.23 g, 1.7 mmol). The reaction mixture was heated to reflux for 4 h. After the complete disappearance of starting material as indicated by TLC, the reaction mixture was subjected to pass through a short pad of silica gel. The obtained filtrate was evaporated under reduced pressure and subjected to purification by flash column chromatography on silica gel. The titled compound (0.09 g) was obtained in 26% yield: $^{1}$H NMR (400 MHz, $CDCl_3$) δ 7.54 - 7.34 (m, 1H), 6.75 - 6.92 (m, 1H), 6.60 - 6.75 (m, 1H), 6.23 (br. s., 1H), 3.79 (br. s., 1H), 3.45 - 3.09 (m, 2H), 1.56 - 1.71 (m, 2H), 1.19 - 1.44 (m, 14H), 0.80 - 0.96 (m, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 164.9 (s, *C*$ONH_2$), 154.0 (dd, $J_{CF}$ = 238, 8.2 Hz, C4), 151.4 (dd, $J_{CF}$ = 242, 8.2 Hz, C2), 133.3 (dd, $J_{CF}$ = 14, 2.7 Hz, C5), 118.0 (dd, $J_{CF}$ = 22, 3.6 Hz, C1), 113.3 (dd, $J_{CF}$ = 23, 23 Hz, C3), 110.3 (dd, $J_{CF}$ = 9.1, 5.5 Hz, C6), 43.8, 40.1, 31.9, 29.5, 29.4, 29.2, 27.0, 22.7, 14.1; LRMS (ESI) *m/z* 299 ($M^+$ + H, 100), 321 ($M^+$ + Na, 85); HRMS (ESI) calcd for $C_{16}H_{25}N_2OF_2$ ($M^+$ + H) 299.1935, found 299.1939.

**3-(Methyl(nonyl)amino)benzamide (45a)**: To a well stirred solution of 3-(nonylamino)benzamide (**44a**) (0.09 g, 0.3 mmol) and dimethyl sulfate (0.06 g, 0.5 mmol) in ACN (10 mL) was added $K_2CO_3$ (0.06 g, 0.4 mmol). The reaction mixture was heated to reflux for 12 h. After the complete disappearance of starting material as indicated by TLC, the reaction mixture was diluted with ethyl acetate (20 mL) and subjected to pass through a short pad of silica gel. The filtrate obtained was evaporated under reduced pressure and subjected to purification by flash column chromatography on silica gel. The titled compound (0.04 g) was obtained in 42% yield: $^1$H NMR (400 MHz, $CDCl_3$) δ 7.23 - 7.32 (m, 1H), 7.21 (s, 1H), 6.99 (d, *J* = 7.3 Hz, 1H), 6.84 (dd, *J* = 2.4, 8.3 Hz, 1H), 6.15 (br. s., 1H), 5.95 (br. s., 1H), 3.30 - 3.41 (m, 2H), 2.96 (s, 3H), 1.53 - 1.64 (m, 2H), 1.22 - 1.37 (m, 12H), 0.83 - 0.95 (m, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 170.5, 149.5, 134.3, 129.2, 115.3, 113.8, 111.1, 52.7, 38.4, 31.9, 29.6, 29.5, 29.3, 27.1, 26.7, 22.7, 14.1; LRMS (ESI) *m/z* 277 ($M^+$ + H, 100), 299 ($M^+$ + Na, 7); HRMS (ESI) calcd for $C_{17}H_{29}N_2O$ ($M^+$ + H) 277.2280, found 277.2271.

**2,6-Difluoro-3-(nonylamino)benzonitrile (47a).** A round-bottom flask was charged with 3-amino-2,6-difluorobenzonitrile (**46**) (1.0 g, 6.5 mmol), 1-bromononane (1.6 g, 7.7 mmol), $K_2CO_3$ (1.4 g, 10.1 mmol), KI (1.1 g, 6.6 mmol) and DMF (10.0 mL). The reaction mixture was stirred at 110 °C for 14 h. After cooling to room temperature, the reaction was quenched by addition of water (50 mL). The mixture was extracted with ethyl acetate (20 mL × 3). The combined organic layers were washed twice with brine and dried over anhydrous $MgSO_4$. The organic layer was filtered, concentrated in vacuum and subjected to purification by flash column chromatography on silica gel with gradient elution (hexane/ethyl acetate from 200:1 to 50:1) to obtain the

unreacted starting material (0.73 g) and desired product (0.39 g) as pale yellow oil in 79% recovery yield. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 7.13 - 7.18 (m, 1H), 7.01 - 7.07 (m, 1H), 5.86 (t, $J$ = 4.8 Hz, 1H), 3.06 (q, $J$ = 6.8 Hz, 2H), 1.53 (quin, $J$ = 7.0 Hz, 2H), 1.24 - 1.28 (m, 12H), 0.85 (t, $J$ = 6.4 Hz, 3H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 152.1 (dd, $J_{CF}$ = 245, 4.0 Hz, C2), 150.0 (dd, $J_{CF}$ = 254, 4.0 Hz, C6), 134.8 (dd, $J_{CF}$ = 8.1, 6.1 Hz, C4), 117.3 (dd, $J_{CF}$ = 19, 4.0 Hz, C3), 112.5 (dd, $J_{CF}$ = 19, 4.0 Hz, C5), 110.7 (d, $J_{CF}$ = 2.0 Hz, *C*N), 90.7 (dd, $J_{CF}$ = 20, 17 Hz, C1), 43.1, 31.8, 29.4, 29.3, 29.1, 28.7, 26.9, 22.6, 14.4; LRMS (ESI) *m/z* 281 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{23}F_2N_2$ ($M^+$ + H) 281.1824, found 281.1833.

**2,6-Difluoro-3-(methyl(nonyl)amino)benzonitrile (47b).** A 35 mL Ace pressure tube was charged with 2,6-difluoro-3-(nonylamino)benzonitrile (**47a**) (0.59 g, 2.12 mmol), $K_2CO_3$ (0.59 g, 4.24 mmol), DMF (5.0 mL) and MeI (1.20 g, 8.48 mmol). The pressure tube was sealed and the reaction mixture was stirred at 60 °C for 24 h. When TLC indicated complete consumption of the starting material, water (20 mL) was added to the mixture and extracted with ethyl acetate (20 mL × 3). The combined organic layer was washed twice with brine and dried over anhydrous $MgSO_4$. The organic layer was evaporated in vacuum and subjected to purification by flash column chromatography on silica gel with gradient elution (hexane/ethyl acetate from 200:1 to 100:1) to afford the desired product (0.34 g) as brown oil in 54% yield. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 7.35 - 7.39 (m, 1H), 7.23 - 7.28 (m, 1H), 3.08 (t, $J$ = 7.3 Hz, 2H), 2.78 (s, 3H), 1.45 - 1.49 (m, 2H), 1.22 (br. s., 12H), 0.83 - 0.86 (m, 3H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 155.5 (dd, $J_{CF}$ = 251, 4.0 Hz, C2), 153.8 (dd, $J_{CF}$ = 259, 4.0 Hz, C6), 137.4 (dd, $J_{CF}$ = 19, 4.0 Hz, C3), 125.4 (dd, $J_{CF}$ = 9.1, 6.1 Hz, C4), 112.5 (dd, $J_{CF}$ = 19, 4.0 Hz, C5), 110.4 (s, *C*N), 92.0 (dd, $J_{CF}$ =

21, 19 Hz, C1), 54.7, 54.7, 31.7, 29.4, 29.2, 29.1, 26.9, 26.7, 22.5, 14.3; LRMS (ESI) *m/z* 295 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{17}H_{25}F_2N_2$ ($M^+$ + H) 295.1980, found 295.1985.

**2,6-Difluoro-*N*'-hydroxy-3-(nonylamino)benzimidamide (48a).** A round-bottom flask was charged sequentially with 2,6-difluoro-3-(nonylamino)benzonitrile (**47a**) (0.42 g, 1.50 mmol), $Et_3N$ (0.76 g, 7.50 mmol), MeOH (4 mL), THF (1 mL) and hydroxylamine hydrochloride (0.42 g, 6.06 mmol). The reaction mixture was stirred at 80 °C for 5 h. When TLC indicated complete consumption of the starting material, the mixture was cooled and the organic solvents were removed in vacuum. Addition of water (30 mL) followed by extraction with ethyl acetate (20 mL × 3) to give an organic layer, which was washed twice with brine and dried over anhydrous $MgSO_4$. The organic layer was concentrated in vacuum and subjected to purification by flash column chromatography on silica gel with gradient elution (DCM/MeOH from 100:1 to 10:1) to afford the desired product (0.24 g) as pale yellow oil in 51% yield. $^1$H NMR (400 MHz, $CDCl_3$) δ 6.81 (dd, *J* = 8.2, 8.2 Hz, 1H), 6.61 - 6.69 (m, 1H), 4.97 (br. s., 1H), 1.60 - 1.67 (m, 2H), 1.51 - 1.58 (m, 2H), 1.28 (br. s., 12H), 0.88 - 0.91 (m, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 163.5 (s, HON=*C*$NH_2$), 151.6 (dd, $J_{CF}$ = 241, 5.1 Hz, C6), 148.5 (dd, $J_{CF}$ = 246, 8.1 Hz, C2), 144.5 (dd, $J_{CF}$ = 24, 3.6 Hz, C3), 133.9 (dd, $J_{CF}$ = 23, 4.0 Hz, C5), 112.3 (dd, $J_{CF}$ = 9.1, 5.1 Hz, C4), 111.0 (dd, $J_{CF}$ = 23, 23 Hz, C1), 62.9, 44.0, 32.7, 31.9, 31.8, 27.1, 25.7, 22.6, 14.1; LRMS (ESI) *m/z* 314 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{26}F_2N_3O$ ($M^+$ + H) 314.2038, found 314.2045.

**2,6-Difluoro-*N*'-hydroxy-3-(methyl(nonyl)amino)benzimidamide (48b).** This compound **48b** (0.24 g, 77%) was prepared from 2,6-difluoro-3-(methyl(nonyl)amino)benzonitrile (**47b**) (0.28 g, 0.95 mmol), $Et_3N$ (0.48 g, 4.77 mmol), MeOH (1 mL), THF (4 mL) and hydroxylamine

hydrochloride (0.26 g, 3.82 mmol) according to the preparation procedure of **48a** described above. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.51 (s, 1H), 6.95 - 7.06 (m, 2H), 5.91 (s, 1H), 2.97 - 3.02 (m, 2H), 2.71 (s, 3H), 1.47 (br. s., 2H), 1.25 (br. s., 12H), 0.86 (t, $J$ = 6.8 Hz, 3H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 162.4 (s, HON=*C*NH$_2$), 154.7 (dd, $J_{CF}$ = 242, 6.1 Hz, C6), 153.1 (dd, $J_{CF}$ = 251, 6.1 Hz, C2), 137.2 (dd, $J_{CF}$ = 13, 2.0 Hz, C3), 120.3 (dd, $J_{CF}$ = 10, 5.1 Hz, C4), 113.1 (dd, $J_{CF}$ = 22, 4.0 Hz, C5), 110.9 (dd, $J_{CF}$ = 22, 20 Hz, C1), 55.3, 55.2, 31.7, 29.5, 29.4, 29.1, 27.1, 27.0, 22.6, 14.4; LRMS (ESI) *m/z* 328 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{17}H_{28}F_2N_3O$ ($M^+$ + H) 328.2195, found 328.2201.

**2,6-Difluoro-3-(nonylamino)benzimidamide (49a).** To a well-stirred solution of 2,6-difluoro-*N*'-hydroxy-3-(nonylamino)benzimidamide (**48a**) (0.12 g, 0.38 mmol) in acetic acid (1.0 mL), was added acetic anhydride (0.16 g, 1.53 mmol) at 0 °C and stirred for 12 h. The mixture was diluted with water (30 mL) and extracted with ethyl acetate (20 mL × 3). The organic layer was dried over anhydrous $MgSO_4$ and concentrated in vacuum to furnish a crude product for next step. Then the crude product was dissolved in MeOH (2 mL) and 10% Pd/C (30 mg) was added into the mixture. The mixture was stirred under hydrogen atmosphere for 12 h. The mixture was filtered to remove the Pd catalyst and the obtained filtrate was added conc. HCl (1 mL). The mixture was stirred at reflux for 12 h. The reaction was quenched by addition of saturated $Na_2CO_3$ solution and extracted with ethyl acetate (20 mL × 3). The organic layer was dried over anhydrous $MgSO_4$, filtered and evaporated to give a crude mixture, which was subjected to purification by flash column chromatography on silica gel with gradient elution (DCM/MeOH from 100:1 to 10:1) to afford the desired product (34 mg) as a pale yellow oil in 30%. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 6.90 (dd, $J$ = 8.0, 8.0 Hz, 1H), 6.70 – 6.64 (m, 1H), 5.36 (s, 1H), 3.01 -

3.06 (m, 2H), 1.52 – 1.55 (m, 2H), 1.26 (br. s., 12H), 0.86 (t, $J$ = 8.0 Hz, 3H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 156.8 (s, HN=*C*NH$_2$), 149.2 (dd, $J_{CF}$ = 236, 6.1 Hz, C6), 146.7 (dd, $J_{CF}$ = 244, 7.1 Hz, C2), 134.3 (dd, $J_{CF}$ = 13, 3.0 Hz, C3), 115.1 (dd, $J_{CF}$ = 9.1, 6.1 Hz, C4), 111.5 (dd, $J_{CF}$ = 22, 3.0 Hz, C5), 111.2 (dd, $J_{CF}$ = 23, 19 Hz, C1), 43.31, 31.8, 29.5, 29.4, 29.2, 28.9, 27.0, 22.6, 14.4; LRMS (ESI) *m/z* 298 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{26}F_2N_3$ ($M^+$ + H) 298.2095, found 298.2099.

**2,6-Difluoro-3-(methyl(nonyl)amino)benzimidamide (49b).** To a well-stirred solution of 2,6-difluoro-*N*'-hydroxy-3-(methyl(nonyl)amino)benzimidamide (**48b**) (0.10 g, 0.30 mmol) in DCM (1.0 mL) was added 2-chloroacetyl chloride (0.04 g, 0.37 mmol) at 0 °C and stirred for 12 h. Addition of water (30 mL) followed by extraction with DCM (20 mL × 3) to give the organic layer, which was washed twice with brine and dried over anhydrous $MgSO_4$. The organic layer was concentrated in vacuum to obtain a crude product for next step. The crude product was dissolved in MeOH (2 mL) and 10% Pd/C (20 mg) was added. The reaction mixture was stirred under hydrogen atmosphere for 12 h. The mixture was filtered to remove the Pd catalyst and the filtrate was concentrated in vacuum. The crude product was subjected to purification by flash column chromatography on silica gel with gradient elution (DCM/MeOH from 100:1 to 15:1) to afford the desired product (18 mg) as a pale yellow oil in 19% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.69 (br. s., 3H), 7.17 - 7.27 (m, 2H), 3.03 - 3.06 (m, 2H), 2.76 (s, 3H), 1.49 (br. s., 2H), 1.25 (br. s., 12H), 0.84 - 0.87 (m, 3H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 158.5 (s, HN=*C*NH$_2$), 152.2 (dd, $J_{CF}$ = 245, 4.0 Hz, C6), 150.7 (dd, $J_{CF}$ = 253, 6.1 Hz, C2), 137.5 (dd, $J_{CF}$ = 10, 6.1 Hz, C3), 122.8 (dd, $J_{CF}$ = 9.1, 6.1 Hz, C4), 111.9 (dd, $J_{CF}$ = 21, 4.0 Hz, C5), 109.6 (dd,

$J_{CF}$ = 19, 19 Hz, C1), 55.1, 55.0, 31.7, 29.5, 29.4, 29.1, 27.1, 26.9, 22.6, 14.4; LRMS (ESI) *m/z* 312 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{17}H_{28}F_2N_3$ ($M^+$ + H) 312.2246, found 312.2251.

**2,4-Difluoro-*N*-nonyl-3-(1*H*-tetrazol-5-yl)aniline** (**50**). To a mixture of 2,6-difluoro-3-(nonylamino)benzonitrile (**47a**) (0.18 g, 0.64 mmol), sodium azide (0.10 g, 1.61 mmol), zinc(II) chloride (0.11 g, 0.77 mmol) in DMF (2.0 mL) and water (2.0 mL), was stirred at reflux for 12 h. The reaction mixture was then cooled and acidified to pH 2 by using 3M hydrochloric acid. The reaction mixture was then extracted with ethyl acetate for 3 times. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, filtered and evaporated to give a crude mixture, which was subjected to purification by flash column chromatography on silica gel with ethyl acetate as eluent to obtain the desired product (0.11 g) as pale yellow oil in 53% yield. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.12 - 7.17 (m, 1H), 6.90 - 6.96 (m, 1H), 5.65 (s, 1H), 3.10 (t, *J* = 13.2 Hz, 2H), 1.53 - 1.60 (m, 2H), 1.25 (s, 12H), 0.85 (t, *J* = 13.2 Hz, 3H); $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 149.6 (dd, $J_{CF}$ = 238, 6.1 Hz, C6), 148.0 (dd, $J_{CF}$ = 244, 8.1 Hz, C2), 134.8 (dd, $J_{CF}$ = 13, 2.0 Hz, C3), 114.2 (dd, $J_{CF}$ = 10, 5.1 Hz, C4), 112.2 (dd, $J_{CF}$ = 24, 20 Hz, C1), 111.9 (dd, $J_{CF}$ = 23, 4.0 Hz, C5), 102.9, 43.2, 31.8, 29.5, 29.3, 29.1, 28.8, 27.0, 22.6, 14.4; LRMS (ESI) *m/z* 324 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{16}H_{24}F_2N_5$ ($M^+$ + H) 324.1994, found 324.2006.

**2,4-Difluoro-*N*-nonylaniline (52)**. The titled compound **52** (0.09 g, 35%) were prepared from 2,4-difluoroaniline (**51**) (0.13 g, 1.0 mmol), 1-bromononane (0.21 g, 1.0 mmol), NaI (0.04 g), ACN (20 mL) and $K_2CO_3$ (0.15 g, 1.1 mmol) according to the preparation procedure of **26** described above. $^1$H NMR (400 MHz, $CDCl_3$) δ 6.74 - 6.82 (m, 2H), 6.59 - 6.65 (m, 1H), 3.67 (br, s, 1H), 3.12 (t, *J* = 7.2 Hz, 2H), 1.58 - 1.69 (m, 2H), 1.30 - 1.44 (m, 12H), 0.91 (t, *J* = 7.2 Hz,

3H); $^{13}$C NMR (101 MHz, $CDCl_3$) δ 155.2 (dd, $J_{CF}$ = 238, 6.1 Hz, C2), 152.9 (dd, $J_{CF}$ = 238, 6.1 Hz, C4), 133.6 (dd, $J_{CF}$ = 24, 2.0 Hz, C1), 111.8 (dd, $J_{CF}$ = 6.1, 6.1 Hz, C6), 110.6 (dd, $J_{CF}$ = 24, 2.0 Hz, C5), 103.3 (dd, $J_{CF}$ = 24, 24 Hz, C3), 44.1, 31.9, 29.5, 29.5, 29.4, 29.3, 27.1, 22.7, 14.1; LRMS (ESI) *m/z* 173 ($M^+$ + H, 100); LRMS (ESI) *m/z* 256 ($M^+$ + H, 100); HRMS (ESI) calcd for $C_{15}H_{24}F_2N$ ($M^+$ + H) 256.1877, found 256.1874.

Antimicrobial (MIC) testing

MIC of BLAs and compounds alone, as well as different combinations of BLAs and compounds, were determined by using the broth microdilution method according to guidelines of the Clinical and Laboratory Standards Institute.[58] All compounds were dissolved in DMSO for MIC testing as previously described.[34, 59] All tests were performed in duplicate and inhibition of bacterial growth was determined by naked eyes.

Cytotoxicity ($IC_{50}$) testing

Standard MTS assay was employed to determine the cytotoxicity of each compound towards the L929 cells as previously described.[34] All experiments were performed in triplicates and results were presented as the average of the three independent measurements.

Time-kill assay

A single colony of *S. aureus* BAA-41 was picked from TSB agar plate and inoculated in 5 mL of CA-MH broth at 37 $^{o}$C with shaking at 250 rpm for 16 h. This culture was diluted 100-fold in 5 mL fresh CA-MH broth and the cells were further incubated to achieve mid-log phase with $OD_{595}$ of 0.8. The cell culture was diluted to a standard inoculum of $5 \times 10^5$ CFU/mL in a fresh

CA-MH broth and then transferred into incubation tubes. Compound **28**, PC190723 (**1**) and combination of ME with **28** or **1** were added at concentrations of 1×, 2×, 4×, 8× and 16× MIC. Control experiment was conducted in the presence of DMSO. The bacterium-antibacterial compound mixtures were incubated at 37 $^{o}$C with shaking at 250 rpm. The inoculum was sampled at 0, 2.5, 5, 7.5, 21 and 24 h. The samples were diluted with the appropriate fractions and then sub-cultured on the CA-MH agars without antibacterial compounds and the agars were further incubated at 37$^{o}$C for 24 h. Colony counting was carried out by imaging system with Quantity One® 1-D Analysis Software.

*In vivo* efficacy study

The animal study was conducted in full compliance with the standard protocol approved by the animal research ethics committee of the National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention. Five-week-old BALB/C male mice were used in this study. All mice were housed under constant temperature (22 $^{o}$C) and relative humidity (60%). They were kept in a photoperiod of 12 h light/dark cycle and a constant supply of drinking water along with grain-supplemented standard rodent pellets. MRSA ATCC 43300 was grown overnight at 37°C in brain-heart infusion broth. The overnight culture was diluted 1:100 using fresh TSB medium and incubated at 37°C with shaking (200 rpm) for 3 h. Log phase cells were collected, washed with phosphate-buffered saline (PBS) twice and suspended in PBS for further use. Mice were randomly divided into groups with 10 mice per group. To establish the infection, mice were injected IV via the lateral tail vein at a lethal dose of MRSA ATCC 43300 suspended in PBS. A solution of compound **28** hydrochloride salt was freshly prepared in the formulation of 5% CremophorEL, 5% ethanol, 90% saline at a

concentration of 2 mg/mL. Different treatment groups, including vehicle (5% CremophorEL, 5% ethanol, 90% saline), compound **28** alone (50 mg/kg), CX alone (25 mg/kg), a combination of compound **28** (50 mg/kg) and CX (25 mg/kg), were administered IP twice a day after bacterial challenge. A group of mice received vancomycin at 30 mg/kg twice a day post-infection was use a positive control. Death of mice was recorded at 12 h interval for 4 days after infection. Survival curves were plotted and analyzed by using a non-parametric Log-rank (Mantel-Cox) test. P values less than 0.05 were considered statistically significant.

Frequency of resistance (FOR) study

To evaluate the frequency of resistance to compound **28** or CX-compound **28** combination that arises spontaneously in a tested organism, an inoculum of $10^9$ *S. aureus* ATCC 1717 were plated on Muller-Hinton agar (MHA) containing compound **28** or a combination of CX-compound **28** at a concentration of 4- and 16-fold of MIC. The plates were incubated at 37°C for 48 hr. FOR was calculated by dividing the number of colonies growing on the agar plates over the number of the initial inoculation.

Isolation of compound **28** resistant mutants for sequencing

Cells of *S. aureus* ATCC 29213 were cultured in LB with constant shaking at 250 rpm at 37°C. Cells were initially grown in medium without addition of compound **28**. Then, 50 μL of cell culture in the stationary-phase was transferred into 3 mL of LB broth in the absence or presence of compound **28** at a final concentration of half the MIC and cultured for 20 h with shaking at 250 rpm to obtain 2 samples T(0) and T(1) respectively. The regrown bacterial cells in T(1) were thereafter transferred to a broth containing a 2-fold concentration of **28** and cultured as above

method. If the bacterial cells could not grow in T(1), bacterial cells in T(0) were transferred to another fresh T(1) culture until the bacterial cells could grow in T(1). The experiments were repeatedly conducted with an escalating concentration of **28** from 1 μg/mL to 128 μg/mL. compound **28** resistant mutants at MIC values of 32 μg/mL (Mutant32), 64 μg/mL (Mutant64) and 128 μg/mL (Mutant128) along with wild type *S. aureus* ATCC 29213 were obtained respectively for subsequent DNA isolation and whole-genome NDA sequencing using the Illumina NextSeq platform (NextSeq 500/550 Kits v2; 2 × 151 cycles). Reference sequence of the *ftsz* gene was downloaded from NCBI GenBank. The genome sequences were BLAST against the *ftsz* gene using CLC workbench software. Relative sequences were extracted from the genome sequences and were aligned against the reference *ftsz* gene sequence to locate the difference.

Docking study

CLC Drug Discovery Workbench (Version 2.5, QIAGEN) software was used for docking study. The 2D structures of compound **28** was generated from SIMLES and imported into the software. The X-ray crystal structure of *S. aureus* FtsZ in complex with **1** (PDB ID: 4DXD) was downloaded from Protein Data Bank (https://www.rcsb.org/) and used directly for docking without any changes. Using the software function of "Find Binding Pockets", the software was able to identify two potential binding pockets such as GDP binding site and compound **1** binding site. The identification of ligand binding modes was done iteratively by evaluating 10,000 ligand conformations and estimating the binding energy of their interactions with these binding pockets. The binding pose with the top 5% highest scores were returned for further visual inspection. The

highest scores positioned compound **28** into the binding site of **1** with potential binding pose shown in **Figure 3B** (lower part).

*S. aureus* FtsZ Protein purification

*S. aureus* FtsZ protein was expressed and purified according to our previous reports.[35, 60]

Light scattering assay and GTPase activity assay

These two assays were performed as previously described.[60]

Bacterial morphology and microscopic studies

TEM studies of FtsZ filaments were performed as previously described.[60] The bacterial morphology studies and Z-ring visualization studies of *B. subtilis* and *S. aureus* were performed as previously described.[60-61]

PK studies of compound **28**

The animal study was conducted in full compliance with the standard protocol approved by the Animal Subjects Ethics Sub-committee (ASESC) of The Hong Kong Polytechnic University (ASESC Case No. 14-15/16-ABCT-R-GRF). Male Sprague–Dawley (SD) rats (body weight 250-280 g) were obtained from the Centralised Animal Facilities of The Hong Kong Polytechnic University. Animals were kept in a temperature and humidity-controlled environment with 12 h light-dark cycle with standard diet and water. Right jugular vein cannulation was preformed one day in advance of the experiment. Animals were fasted overnight and given free access to water throughout the experiment. A solution of compound **28** hydrochloride salt was freshly prepared

in the formulation of 5% CremophorEL, 5% ethanol, 90% saline at a concentration of 2 mg/mL. This solution was prepared on the day of use and used for animal study within 0.5 h. In the current study, compound **28** was administered through passive oral feeding (oral) and intravenous (IV) injection respectively. Blood samples (approx. 500 µL) were collected in heparinzied tubes (20 units of heparin salt/tube) via jugular vein at 5, 10, 30, 45, 60, 120, 240 and 420 minutes post administration for IV study. For oral study, plasma samples were collected at 2, 10, 30, 45, 60, 120, 240, 480 and 600 minutes. Blood plasma samples were obtained by centrifugation at 16,100 G for 10 minutes. Plasma sample were stored at -20$^{o}$C until further analysis. One hundred (100 µL) of plasma was retrieved for quantification. For all plasma samples 300 µL of methanol was added for protein precipitation. The supernatant was filtered using 0.22 µM syringe filter and 10 µL was injected for UPLC-MS/MS analysis. The UPLC-MS/MS system consists of an Acquity Waters UPLC interfaced with triple quadrupole mass spectrometer (Micromass model Quattro Ultima) equipped with an electrospray ionization source in positive mode. Chromatographic separation was performed on ACQUITY UPLC BEH C18 1.7 µm (2.1 × 50 mm) column. The mobile phase consists of methanol + 0.1% formic acid (solvent B) and Milli-Q water + 0.1% formic acid (solvent A). Multiple reaction monitoring (MRM) was set to monitor the transition for compound **28** $[M + H]^+$ at 299 *m/z* to 142 *m/z*. The collision energy, cone voltage, source temperature, desolvation temperature and capillary voltage are 25, 30, 150 $^{o}$C, 350 $^{o}$C and 3 Kv respectively. The flow rates of the cone gas and desolvation gas were 150 L/h and 600 L/h respectively. PK parameters were generated from the plasma concentration-time profile using non-compartmental analysis (PK Solutions 2.0, Summit Research Services, Montrose, CO, USA). The PK parameters determined include maximal plasma concentration ($C_{max}$), time to reach maximal plasma concentration ($T_{max}$), volume of

distribution ($V_d$), clearance (Cl), half-life ($t_{1/2}$), area under the concentration-time curve ($AUC_{0-\infty}$) and oral bioavailability ($F$).

ASSOCIATED CONTENT

**Supporting Information.** The following files are available free of charge.

Word document containing **Figures S1-S55** and **Table S1** (docx)

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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ABBREVIATIONS

MRSA, methicillin-resistant *Staphylococcus aureus*; BLAs, β-lactam antibiotics; FtsZ, filamenting temperature-sensitive mutant Z; GTP, guanosine triphosphate; PK, pharmacokinetic; ACN, acetonitrile; *p*-TsOH, *p*-toluenesulfonic acid; THF, tetrahydrofuran; MICs, minimal inhibitory concentrations; SI, selectivity index; SAR, structure-activity relationships; ME, methicillin; CL, cloxacillin; AM, amoxicillin; CX, cefuroxime; MR, meropenem; FIC index, fractional inhibitory concentration index; IP, intraperitoneally; FOR, frequency of resistance; TEM, transmission electron microscopy; IV, intravenous injection; PO, oral administration; Cl, clearance; $V_d$, volume of distribution; *F*, oral bioavailability; AUC, area under the curve.

References

1. Poole, K., Resistance to β-lactam antibiotics. *Cellular and Molecular Life Sciences CMLS* **2004,** *61* (17), 2200-2223.

2. Fisher, J. F.; Meroueh, S. O.; Mobashery, S., Bacterial Resistance to β-Lactam Antibiotics: Compelling Opportunism, Compelling Opportunity. *Chemical Reviews* **2005,** *105* (2), 395-424.

3. Brown, E. D.; Wright, G. D., Antibacterial drug discovery in the resistance era. *Nature* **2016,** *529*, 336-343.

4. https://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf (accessed April 18, 2018).

5. Wright, G. D., Antibiotic Adjuvants: Rescuing Antibiotics from Resistance. *Trends in Microbiology* **2016,** *24* (11), 862-871.

6. Wright, G. D., Opportunities for natural products in 21st century antibiotic discovery. *Natural Product Reports* **2017,** *34* (7), 694-701.

7. Drawz, S. M.; Bonomo, R. A., Three Decades of β-Lactamase Inhibitors. *Clinical Microbiology Reviews* **2010,** *23* (1), 160-201.

8. Lock, R. L.; Harry, E. J., Cell-division inhibitors: new insights for future antibiotics. *Nature Reviews Drug Discovery* **2008,** *7*, 324-338.

9. Melander, R. J.; Melander, C., Antibiotic Adjuvants. Springer Berlin Heidelberg: Berlin, Heidelberg, pp 1-30.

10. Sass, P.; Brötz-Oesterhelt, H., Bacterial cell division as a target for new antibiotics. *Current Opinion in Microbiology* **2013,** *16* (5), 522-530.

11. Hurley, K. A.; Santos, T. M. A.; Nepomuceno, G. M.; Huynh, V.; Shaw, J. T.; Weibel, D. B., Targeting the Bacterial Division Protein FtsZ. *Journal of Medicinal Chemistry* **2016,** *59* (15), 6975-6998.

12. Plaza, A.; Keffer, J. L.; Bifulco, G.; Lloyd, J. R.; Bewley, C. A., Chrysophaentins A−H, Antibacterial Bisdiarylbutene Macrocycles That Inhibit the Bacterial Cell Division Protein FtsZ. *Journal of the American Chemical Society* **2010,** *132* (26), 9069-9077.

13. Domadia, P. N.; Bhunia, A.; Sivaraman, J.; Swarup, S.; Dasgupta, D., Berberine Targets Assembly of Escherichia coli Cell Division Protein FtsZ. *Biochemistry* **2008,** *47* (10), 3225-3234.

14. Artola, M.; Ruiz-Avila, L. B.; Vergoñós, A.; Huecas, S.; Araujo-Bazán, L.; Martín-Fontecha, M.; Vázquez-Villa, H.; Turrado, C.; Ramírez-Aportela, E.; Hoegl, A.; Nodwell, M.; Barasoain, I.; Chacón, P.; Sieber, S. A.; Andreu, J. M.; López-Rodríguez, M. L., Effective GTP-Replacing FtsZ Inhibitors and Antibacterial Mechanism of Action. *ACS Chemical Biology* **2015,** *10* (3), 834-843.

15. Marcelo, F.; Huecas, S.; Ruiz-Ávila, L. B.; Cañada, F. J.; Perona, A.; Poveda, A.; Martín-Santamaría, S.; Morreale, A.; Jiménez-Barbero, J.; Andreu, J. M., Interactions of Bacterial Cell Division Protein FtsZ with C8-Substituted Guanine Nucleotide Inhibitors. A Combined NMR, Biochemical and Molecular Modeling Perspective. *Journal of the American Chemical Society* **2013,** *135* (44), 16418-16428.

16. Awasthi, D.; Kumar, K.; Knudson, S. E.; Slayden, R. A.; Ojima, I., SAR Studies on Trisubstituted Benzimidazoles as Inhibitors of Mtb FtsZ for the Development of Novel Antitubercular Agents. *Journal of Medicinal Chemistry* **2013,** *56* (23), 9756-9770.

17. Margolin, W., FtsZ and the division of prokaryotic cells and organelles. *Nature Reviews Molecular Cell Biology* **2005,** *6*, 862-871.

18. Tan, C. M.; Therien, A. G.; Lu, J.; Lee, S. H.; Caron, A.; Gill, C. J.; Lebeau-Jacob, C.; Benton-Perdomo, L.; Monteiro, J. M.; Pereira, P. M.; Elsen, N. L.; Wu, J.; Deschamps, K.; Petcu, M.; Wong, S.; Daigneault, E.; Kramer, S.; Liang, L.; Maxwell, E.; Claveau, D.; Vaillancourt, J.; Skorey, K.; Tam, J.; Wang, H.; Meredith, T. C.; Sillaots, S.; Wang-Jarantow, L.; Ramtohul, Y.; Langlois, E.; Landry, F.; Reid, J. C.; Parthasarathy, G.; Sharma, S.; Baryshnikova, A.; Lumb, K. J.; Pinho, M. G.; Soisson, S. M.; Roemer, T., Restoring Methicillin-Resistant Staphylococcus aureus Susceptibility to β-Lactam Antibiotics. *Science Translational Medicine* **2012,** *4* (126), 126ra35-126ra35.

19. Haydon, D. J.; Stokes, N. R.; Ure, R.; Galbraith, G.; Bennett, J. M.; Brown, D. R.; Baker, P. J.; Barynin, V. V.; Rice, D. W.; Sedelnikova, S. E.; Heal, J. R.; Sheridan, J. M.; Aiwale, S. T.; Chauhan, P. K.; Srivastava, A.; Taneja, A.; Collins, I.; Errington, J.; Czaplewski, L. G., An Inhibitor of FtsZ with Potent and Selective Anti-Staphylococcal Activity. *Science* **2008,** *321* (5896), 1673-1675.

20. Haydon, D. J.; Bennett, J. M.; Brown, D.; Collins, I.; Galbraith, G.; Lancett, P.; Macdonald, R.; Stokes, N. R.; Chauhan, P. K.; Sutariya, J. K.; Nayal, N.; Srivastava, A.; Beanland, J.; Hall, R.; Henstock, V.; Noula, C.; Rockley, C.; Czaplewski, L., Creating an Antibacterial with in Vivo Efficacy: Synthesis and Characterization of Potent Inhibitors of the Bacterial Cell Division Protein FtsZ with Improved Pharmaceutical Properties. *Journal of Medicinal Chemistry* **2010,** *53* (10), 3927-3936.

21. Ferrer-González, E.; Kaul, M.; Parhi, A. K.; LaVoie, E. J.; Pilch, D. S., β-Lactam Antibiotics with a High Affinity for PBP2 Act Synergistically with the FtsZ-Targeting Agent TXA707 against Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **2017,** *61* (9), e00863-17.

22. Bisson-Filho, A. W.; Hsu, Y.-P.; Squyres, G. R.; Kuru, E.; Wu, F.; Jukes, C.; Sun, Y.; Dekker, C.; Holden, S.; VanNieuwenhze, M. S.; Brun, Y. V.; Garner, E. C., Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. *Science* **2017,** *355* (6326), 739-743.

23. Yang, X.; Lyu, Z.; Miguel, A.; McQuillen, R.; Huang, K. C.; Xiao, J., GTPase activity–coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall synthesis. *Science* **2017,** *355* (6326), 744-747.

24. Lepak, A. J.; Parhi, A.; Madison, M.; Marchillo, K.; VanHecker, J.; Andes, D. R., In Vivo Pharmacodynamic Evaluation of an FtsZ Inhibitor, TXA-709, and Its Active Metabolite, TXA-707, in a Murine Neutropenic Thigh Infection Model. *Antimicrobial Agents and Chemotherapy* **2015,** *59* (10), 6568-6574.

25. Stokes, N. R.; Baker, N.; Bennett, J. M.; Chauhan, P. K.; Collins, I.; Davies, D. T.; Gavade, M.; Kumar, D.; Lancett, P.; Macdonald, R.; MacLeod, L.; Mahajan, A.; Mitchell, J. P.; Nayal, N.; Nayal, Y. N.; Pitt, G. R. W.; Singh, M.; Yadav, A.; Srivastava, A.; Czaplewski, L. G.; Haydon, D. J., Design, synthesis and structure–activity relationships of substituted oxazole–benzamide antibacterial inhibitors of FtsZ. *Bioorganic & Medicinal Chemistry Letters* **2014,** *24* (1), 353-359.

26. Fujita, J.; Maeda, Y.; Mizohata, E.; Inoue, T.; Kaul, M.; Parhi, A. K.; LaVoie, E. J.; Pilch, D. S.; Matsumura, H., Structural Flexibility of an Inhibitor Overcomes Drug Resistance Mutations in Staphylococcus aureus FtsZ. *ACS Chemical Biology* **2017,** *12* (7), 1947-1955.

27. Kaul, M.; Mark, L.; Zhang, Y.; Parhi, A. K.; LaVoie, E. J.; Pilch, D. S., Pharmacokinetics and in vivo antistaphylococcal efficacy of TXY541, a 1-methylpiperidine-4-carboxamide prodrug of PC190723. *Biochemical Pharmacology* **2013,** *86* (12), 1699-1707.

28. Kaul, M.; Zhang, Y.; Parhi, A. K.; LaVoie, E. J.; Tuske, S.; Arnold, E.; Kerrigan, J. E.; Pilch, D. S., Enterococcal and streptococcal resistance to PC190723 and related compounds: Molecular insights from a FtsZ mutational analysis. *Biochimie* **2013,** *95* (10), 1880-1887.

29. Kaul, M.; Mark, L.; Zhang, Y.; Parhi, A. K.; LaVoie, E. J.; Pilch, D. S., An FtsZ-Targeting Prodrug with Oral Antistaphylococcal Efficacy In Vivo. *Antimicrobial Agents and Chemotherapy* **2013,** *57* (12), 5860-5869.

30. Kaul, M.; Mark, L.; Zhang, Y.; Parhi, A. K.; Lyu, Y. L.; Pawlak, J.; Saravolatz, S.; Saravolatz, L. D.; Weinstein, M. P.; LaVoie, E. J.; Pilch, D. S., TXA709, an FtsZ-Targeting Benzamide Prodrug with Improved Pharmacokinetics and Enhanced In Vivo Efficacy against Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **2015,** *59* (8), 4845-4855.

31. Kaul, M.; Mark, L.; Parhi, A. K.; LaVoie, E. J.; Pilch, D. S., Combining the FtsZ-Targeting Prodrug TXA709 and the Cephalosporin Cefdinir Confers Synergy and Reduces the Frequency of Resistance in Methicillin-Resistant Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **2016,** *60* (7), 4290-4296.

32. Stokes, N. R.; Baker, N.; Bennett, J. M.; Berry, J.; Collins, I.; Czaplewski, L. G.; Logan, A.; Macdonald, R.; MacLeod, L.; Peasley, H.; Mitchell, J. P.; Nayal, N.; Yadav, A.; Srivastava, A.; Haydon, D. J., An Improved Small-Molecule Inhibitor of FtsZ with Superior In Vitro Potency, Drug-Like Properties, and In Vivo Efficacy. *Antimicrobial Agents and Chemotherapy* **2013,** *57* (1), 317-325.

33. Chan, F.-Y.; Sun, N.; Neves, M. A. C.; Lam, P. C.-H.; Chung, W.-H.; Wong, L.-K.; Chow, H.-Y.; Ma, D.-L.; Chan, P.-H.; Leung, Y.-C.; Chan, T.-H.; Abagyan, R.; Wong, K.-Y., Identification of a New Class of FtsZ Inhibitors by Structure-Based Design and in Vitro Screening. *Journal of Chemical Information and Modeling* **2013,** *53* (8), 2131-2140.

34. Chan, K.-F.; Sun, N.; Yan, S.-C.; Wong, I. L. K.; Lui, H.-K.; Cheung, K.-C.; Yuan, J.; Chan, F.-Y.; Zheng, Z.; Chan, E. W. C.; Chen, S.; Leung, Y.-C.; Chan, T. H.; Wong, K.-Y., Efficient Synthesis of Amine-Linked 2,4,6-Trisubstituted Pyrimidines as a New Class of Bacterial FtsZ Inhibitors. *ACS Omega* **2017,** *2* (10), 7281-7292.

35. Sun, N.; Chan, F.-Y.; Lu, Y.-J.; Neves, M. A. C.; Lui, H.-K.; Wang, Y.; Chow, K.-Y.; Chan, K.-F.; Yan, S.-C.; Leung, Y.-C.; Abagyan, R.; Chan, T.-H.; Wong, K.-Y., Rational Design of Berberine-Based FtsZ Inhibitors with Broad-Spectrum Antibacterial Activity. *PLOS ONE* **2014,** *9* (5), e97514.

36. Fang, Z.; Zheng, S.; Chan, K.-F.; Yuan, W.; Guo, Q.; Wu, W.; Lui, H.-K.; Lu, Y.; Leung, Y.-C.; Chan, T.-H.; Wong, K.-Y.; Sun, N., Design, synthesis and antibacterial evaluation of 2,4-disubstituted-6-thiophenyl-pyrimidines. *European Journal of Medicinal Chemistry* **2019,** *161*, 141-153.

37. Sun, N.; Zheng, Y.-Y.; Du, R.-L.; Cai, S.-Y.; Zhang, K.; So, L.-Y.; Cheung, K.-C.; Zhuo, C.; Lu, Y.-J.; Wong, K.-Y., New application of tiplaxtinin as an effective FtsZ-targeting chemotype for an antimicrobial study. *MedChemComm* **2017,** *8* (10), 1909-1913.

38. Sun, N.; Du, R.-L.; Zheng, Y.-Y.; Huang, B.-H.; Guo, Q.; Zhang, R.-F.; Wong, K.-Y.; Lu, Y.-J., Antibacterial activity of N-methylbenzofuro[3,2-b]quinoline and N-methylbenzoindolo[3,2-b]-quinoline derivatives and study of their mode of action. *European Journal of Medicinal Chemistry* **2017,** *135*, 1-11.

39. Sun, N.; Du, R.-L.; Zheng, Y.-Y.; Guo, Q.; Cai, S.-Y.; Liu, Z.-H.; Fang, Z.-Y.; Yuan, W.-C.; Liu, T.; Li, X.-M.; Lu, Y.-J.; Wong, K.-Y., Antibacterial activity of 3-methylbenzo[d]thiazol-methylquinolinium derivatives and study of their action mechanism. *Journal of Enzyme Inhibition and Medicinal Chemistry* **2018,** *33* (1), 879-889.

40. Chan, F.-Y.; Sun, N.; Leung, Y.-C.; Wong, K.-Y., Antimicrobial activity of a quinuclidine-based FtsZ inhibitor and its synergistic potential with β-lactam antibiotics. *The Journal of Antibiotics* **2015,** *68*, 253-258.

41. Czaplewski, L. G.; Collins, I.; Boyd, E. A.; Brown, D.; East, S. P.; Gardiner, M.; Fletcher, R.; Haydon, D. J.; Henstock, V.; Ingram, P.; Jones, C.; Noula, C.; Kennison, L.; Rockley, C.; Rose, V.; Thomaides-Brears, H. B.; Ure, R.; Whittaker, M.; Stokes, N. R., Antibacterial alkoxybenzamide inhibitors of the essential bacterial cell division protein FtsZ. *Bioorganic & Medicinal Chemistry Letters* **2009,** *19* (2), 524-527.

42. Sorto, N. A.; Olmstead, M. M.; Shaw, J. T., Practical Synthesis of PC190723, an Inhibitor of the Bacterial Cell Division Protein FtsZ. *The Journal of Organic Chemistry* **2010,** *75* (22), 7946-7949.

43. Hu, Z.; Zhang, S.; Zhou, W.; Ma, X.; Xiang, G., Synthesis and antibacterial activity of 3-benzylamide derivatives as FtsZ inhibitors. *Bioorganic & Medicinal Chemistry Letters* **2017,** *27* (8), 1854-1858.

44. Straniero, V.; Pallavicini, M.; Chiodini, G.; Zanotto, C.; Volontè, L.; Radaelli, A.; Bolchi, C.; Fumagalli, L.; Sanguinetti, M.; Menchinelli, G.; Delogu, G.; Battah, B.; De Giuli Morghen, C.; Valoti, E., 3-(Benzodioxan-2-ylmethoxy)-2,6-difluorobenzamides bearing hydrophobic substituents at the 7-position of the benzodioxane nucleus potently inhibit methicillin-resistant Sa and Mtb cell division. *European Journal of Medicinal Chemistry* **2016,** *120*, 227-243.

45. Straniero, V.; Zanotto, C.; Straniero, L.; Casiraghi, A.; Duga, S.; Radaelli, A.; De Giuli Morghen, C.; Valoti, E., 2,6-Difluorobenzamide Inhibitors of Bacterial Cell Division Protein FtsZ: Design, Synthesis, and Structure–Activity Relationships. *ChemMedChem* **2017,** *12* (16), 1303-1318.

46. Artola, M.; Ruiz-Avila, L. B.; Ramirez-Aportela, E.; Martinez, R. F.; Araujo-Bazan, L.; Vazquez-Villa, H.; Martin-Fontecha, M.; Oliva, M. A.; Martin-Galiano, A. J.; Chacon, P.; Lopez-Rodriguez, M. L.; Andreu, J. M.; Huecas, S., The structural assembly switch of cell

division protein FtsZ probed with fluorescent allosteric inhibitors. *Chemical Science* **2017,** *8* (2), 1525-1534.

47. Bi, F.; Ji, S.; Venter, H.; Liu, J.; Semple, S. J.; Ma, S., Substitution of terminal amide with 1H-1,2,3-triazole: Identification of unexpected class of potent antibacterial agents. *Bioorganic & Medicinal Chemistry Letters* **2018,** *28* (5), 884-891.

48. Bi, F.; Guo, L.; Wang, Y.; Venter, H.; Semple, S. J.; Liu, F.; Ma, S., Design, synthesis and biological activity evaluation of novel 2,6-difluorobenzamide derivatives through FtsZ inhibition. *Bioorganic & Medicinal Chemistry Letters* **2017,** *27* (4), 958-962.

49. Chiodini, G.; Pallavicini, M.; Zanotto, C.; Bissa, M.; Radaelli, A.; Straniero, V.; Bolchi, C.; Fumagalli, L.; Ruggeri, P.; De Giuli Morghen, C.; Valoti, E., Benzodioxane–benzamides as new bacterial cell division inhibitors. *European Journal of Medicinal Chemistry* **2015,** *89*, 252-265.

50. Wang, J.; Wang, X.; Li, H.; Ji, D.; Li, Y.; Xu, Y.; Zhu, Q., Design, synthesis and biological evaluation of novel 5-fluoro-1H-benzimidazole-4-carboxamide derivatives as potent PARP-1 inhibitors. *Bioorganic & Medicinal Chemistry Letters* **2016,** *26* (16), 4127-4132.

51. Bellamy, F. D.; Ou, K., Selective reduction of aromatic nitro compounds with stannous chloride in non acidic and non aqueous medium. *Tetrahedron Letters* **1984,** *25* (8), 839-842.

52. Meldal, M.; Tornøe, C. W., Cu-Catalyzed Azide−Alkyne Cycloaddition. *Chemical Reviews* **2008,** *108* (8), 2952-3015.

53. Matsui, T.; Yamane, J.; Mogi, N.; Yamaguchi, H.; Takemoto, H.; Yao, M.; Tanaka, I., Structural reorganization of the bacterial cell-division protein FtsZ from Staphylococcus aureus. *Acta Crystallographica Section D* **2012,** *68* (9), 1175-1188.

54. Elsen, N. L.; Lu, J.; Parthasarathy, G.; Reid, J. C.; Sharma, S.; Soisson, S. M.; Lumb, K. J., Mechanism of Action of the Cell-Division Inhibitor PC190723: Modulation of FtsZ Assembly Cooperativity. *Journal of the American Chemical Society* **2012,** *134* (30), 12342-12345.

55. Anderson, D. E.; Kim, M. B.; Moore, J. T.; O'Brien, T. E.; Sorto, N. A.; Grove, C. I.; Lackner, L. L.; Ames, J. B.; Shaw, J. T., Comparison of Small Molecule Inhibitors of the Bacterial Cell Division Protein FtsZ and Identification of a Reliable Cross-Species Inhibitor. *ACS Chemical Biology* **2012,** *7* (11), 1918-1928.

56. Haeusser, D. P.; Margolin, W., Splitsville: structural and functional insights into the dynamic bacterial Z ring. *Nature Reviews Microbiology* **2016,** *14*, 305.

57. Cao, X.; Gibbs, S. T.; Fang, L.; Miller, H. A.; Landowski, C. P.; Shin, H.-C.; Lennernas, H.; Zhong, Y.; Amidon, G. L.; Yu, L. X.; Sun, D., Why is it Challenging to Predict Intestinal Drug Absorption and Oral Bioavailability in Human Using Rat Model. *Pharmaceutical Research* **2006,** *23* (8), 1675-1686.

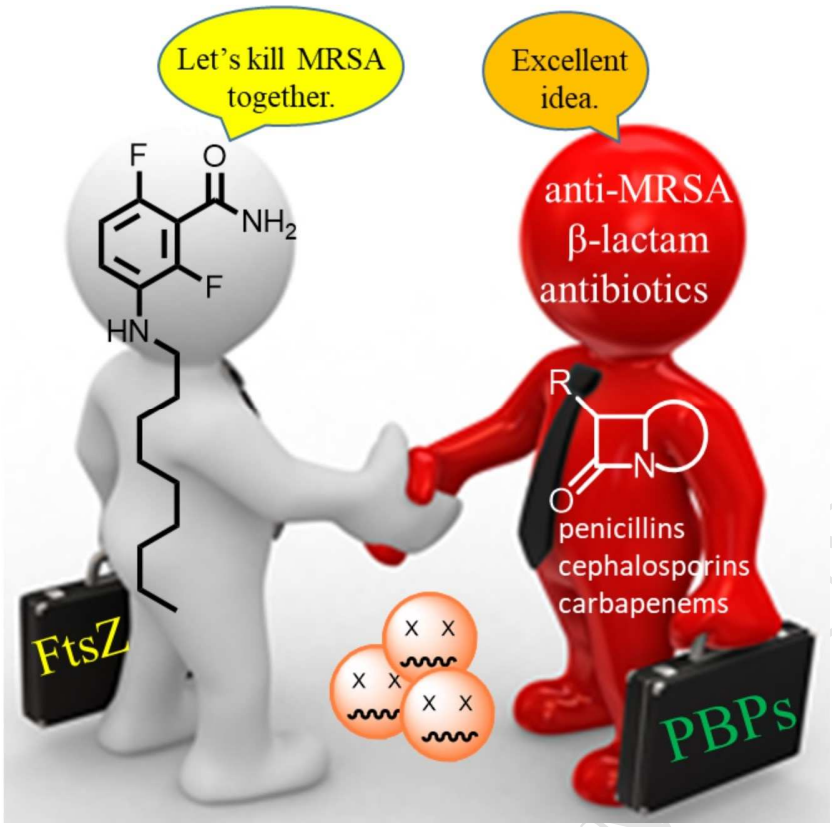
58. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard, 7th ed.; CLSI document M07-A7; Clinical and Laboratory Standards Institute: Wayne, PA, 2006.

59. Jin, W. B.; Xu, C.; Cheng, Q.; Qi, X. L.; Gao, W.; Zheng, Z.; Chan, E. W. C.; Leung, Y.-C.; Chan, T. H.; Wong, K.-Y.; Chen, S.; Chan, K.-F., Investigation of synergistic antimicrobial effects of the drug combinations of meropenem and 1,2-benzisoselenazol-3(2H)-one derivatives on carbapenem-resistant Enterobacteriaceae producing NDM-1. *European Journal of Medicinal Chemistry* **2018,** *155*, 285-302.

60. Lui, H. K. Alkoxy- and amino-benzamides as inhibitors of the bacterial cell division protein FtsZ and antibacterial agents. M. Phil. Thesis, The Hong Kong Polytechnic University, Hong Kong, **2014**.

61. Cheung, K. C. The effect of FtsZ inhibitors on the B-lactam resistant activity in methicillin-resistant staphylococcus aureus. M. Phil. Thesis, The Hong Kong Polytechnic University, Hong Kong, **2017**.

SYNOPSIS

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**Highlights**

- A focused compound library of 3-aminobenzamide derivatives was designed and synthesized.
- Compound **28** was found to exhibit strong synergistic activity *in vitro* and *in vivo* when combined with various anti-MRSA β-lactam antibiotics.
- Compound **28** is likely to interact with the *S. aureus* FtsZ at the T7-loop binding pocket and inhibit polymerization of FtsZ protein, resulting in extensive delocalization of Z-ring and morphological changes.