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Original article

Design, synthesis and evaluation of second generation MurF inhibitors based on a cyanothiophene scaffold

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ABSTRACT

MurF ligase is a crucial enzyme that catalyses the ultimate intracellular step of bacterial peptidoglycan biosynthesis, and thus represents an attractive target for antibacterial drug discovery. We designed, synthesized and evaluated a new series of cyanothiophene-based inhibitors of MurF enzymes from *Streptococcus pneumoniae* and *Escherichia coli*. The target compounds had increased polarity compared to the first generation of inhibitors, with demonstrated enzyme inhibitory potencies in the low micromolar range. Furthermore, the best inhibitors displayed promising antibacterial activities against selected Gram-positive and Gram-negative strains. These results represent an important step towards the development of new antibacterial agents targeting peptidoglycan biosynthesis.

1. Introduction

The increasing emergence of pathogenic bacteria resistant to all known antibiotics has created an urgent need for development of new antibacterial agents [1–3]. Cell wall biosynthesis in bacteria is well characterized and thus represents an attractive target for the development of novel antibacterial drugs [4–6]. The biosynthetic pathway of peptidoglycan encompasses cytoplasmic, membrane and extracellular stages [4,6,7].

Mur ligases (MurC-F) are cytoplasmic ATP-dependent enzymes which catalyse the sequential addition of L-Ala, D-Glu and *meso*-DAP in Gram-negative or L-Lys in Gram-positive bacteria, and the dipeptide D-Ala-D-Ala to UDP-Mur*N*Ac, to form UDP-Mur*N*Ac-pentapeptide [7,8]. All Mur ligases have similar mechanisms of action, and share the same three-domain topology [9]. They are essential for the survival of bacteria and lack human counterparts; consequently, they represent interesting targets for the discovery of new antibacterials with selective toxicity [8]. There are only a few known classes of MurF inhibitors. The first classes were pseudo-tripeptide and pseudo-tetrapeptide aminoalkylphosphinic acids [10], followed by sulphonamides developed by Abbott Laboratories [11,12]. Thiazolylaminopyrimidines [13], 8-hydroxyquinolines [14] and diarylquinolines [15] were discovered by Johnson & Johnson. Recently, a triazine derivative was identified through structure-based virtual screening [16]. Additionally, ligand-based virtual screening was successfully employed by our group and one new compound with micromolar inhibitory activities against *S. pneumoniae* MurF ($MurF_{Sp}$) and *E. coli* MurF ($MurF_{Ec}$) was identified [17].

In our previous research, we also reported a series of cyanothiophene-based inhibitors of MurF enzymes from different pathogenic bacteria, where systematic structural modifications of the lead compound resulted in new potent nanomolar inhibitors of $MurF_{Sp}$ and micromolar inhibitors of $MurF_{Ec}$ and *Staphylococcus aureus* MurF ($MurF_{Sa}$). The crystal structures of $MurF_{Sp}$ in complex with two new inhibitors (PDB codes 3zm5 and 3zm6) revealed the binding modes of compounds, which allowed us to undertake structure-based design of novel, improved analogues with optimized physicochemical properties. Although some of the compounds from the first series showed good MurF inhibitory potency

Abbreviations: DAP, 2,6-diaminopimelic acid; Glc*N*Ac, *N*-acetyl glucosamine; MurC, UDP-*N*-acetylmuramate:L-Ala ligase; MurD, UDP-*N*-acetylmuramoyl-L-Ala:D-Glu ligase; MurE, UDP-*N*-acetylmuramoyl-L-Ala-D-Glu:*meso*-DAP ligase; MurF, UDP-*N*-acetylmuramoyl-L-Ala-γ-D-Glu-*meso*-DAP (or L-Lys):D-Ala-D-Ala ligase; Mur*N*Ac, *N*-acetyl muramic acid; UDP, uridine 5′-diphosphate; UMtri-L-Lys, UDP-*N*-acetylmuramoyl-L-Ala-γ-D-Glu-L-Lys; UMtri-mDAP, UDP-*N*-acetylmuramoyl-L-Ala-γ-D-Glu-*meso*-DAP.

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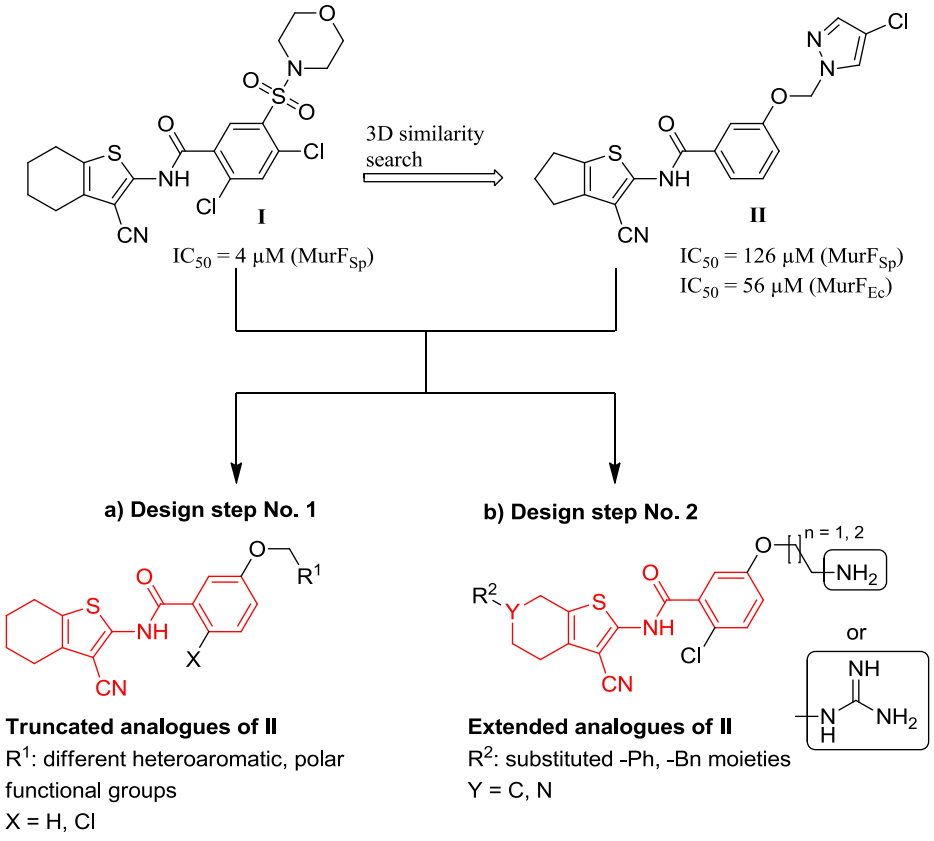


Fig. 1. Two-step design of new cyanothiophene MurF inhibitors. Overlapping scaffold essential for good inhibitory potency is shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and moderate antibacterial activity against *S. pneumoniae*, the solubility of the majority of assayed compounds in water was low. We postulated that the large planar lipophilic portion of the molecules associated with rather high molecular mass led to their high tendency for precipitation, making them inadequate drug candidates [18].

In order to present further insight into the structure–activity relationship of cyanothiophene-based MurF inhibitors, we report the design, synthesis and biological evaluation of a new series of inhibitors. Different structural modifications of the parent compounds resulted in a focused library of 37 new inhibitors, providing low micromolar inhibitors of MurF from *E. coli* and *S. pneumoniae*.

2. Results and discussion

2.1. Design

According to the results from Abbott Laboratories [11,12] and our previous research [18], 2-aminothiophene-3-carbonitrile and 5-sulfamoyl-2-chlorobenzoic acid linked together via an amide bond, are essential for good MurF inhibitory potency (*i.e.*, compound **I**, Fig. 1). Additionally, 3D similarity search revealed micromolar inhibitor **II**, where the cyclohexene ring in compound **I** was replaced by cyclopentene ring, sulfamoyl substituent was replaced by the 1-pyrazolylmethyloxy moiety and the chloro substituents of benzoic acid part were replaced by hydrogens [17].

Due to promising potencies of compounds **I** and **II**, the structural features of both inhibitors were combined. Overlapping both compounds yielded the key scaffold (in red, Fig. 1.), which was used as a starting point in the design of novel MurF inhibitors. The main problem observed with compounds **I** and **II** was their low aqueous solubility due to quite high lipophilicity (ClogP(**I**) = 2.82, and ClogP(**II**) = 3.40). It is well known that antibacterial agents have slightly different physiochemical properties than other drugs [19,20]. Average molecular weights are usually higher for antibacterials, with a defined cut-off at 600 Da. In addition, the lipophilicity of the Gram-positive and especially of Gram-negative antibacterials compared to “normal” drugs is reduced. These unique properties were taken into account during the design of the second generation of MurF inhibitors, where more polar compounds were targeted.

Two steps were performed in designing second-generation MurF inhibitors, as depicted in Fig. 1. In the first step, various alkyl or aryl substituents bearing mostly polar functional groups (R^1) were attached to the main scaffold in order to examine the inhibitory activities of compounds with different replacements of the 4-chloropyrazole moiety. In the second step, various phenyl and benzyl substituents were appended to position 6 (R^2 substituent) of the 4,5,6,7-tetrahydrobenzo[*b*]thiophene and 4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine, respectively. It is known from our previous study that extension of these ring systems significantly improves the potency of compounds against *S. pneumoniae* MurF and gains the potency against other MurF orthologes [18]. Although this extension increases lipophilicity, the presence of the protonated amine or guanidine moiety outweighs the contribution of these lipophilic residues so that the overall ClogP does not exceed the value of 2.8 (Table S1).

To predict the binding affinity of the designed compounds to MurF, a docking study was performed using the crystal structure of MurF enzyme from *S. pneumoniae* (PDB code: 3zm5) and

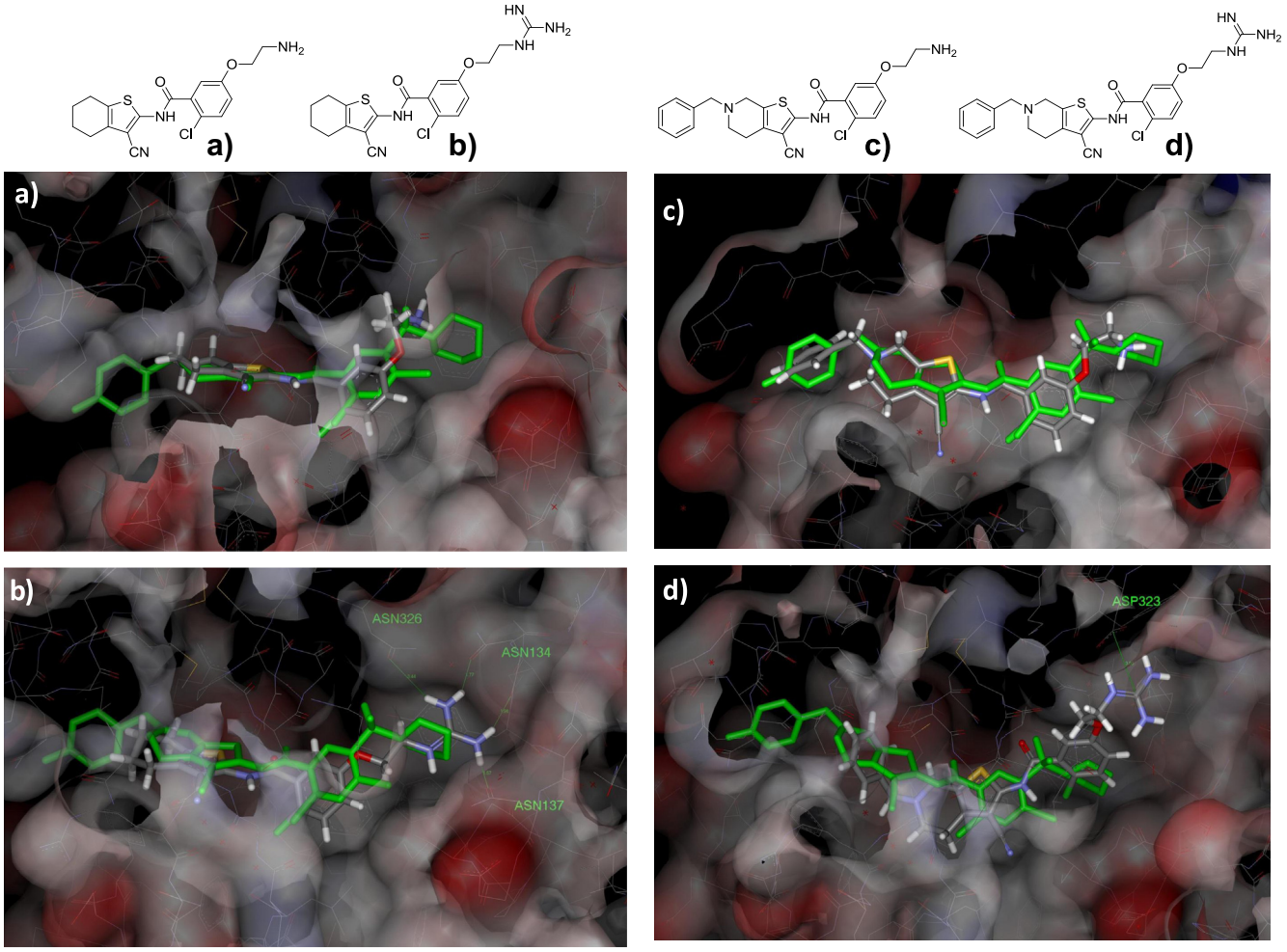


Fig. 2. New cyanothiophene MurF inhibitors (sticks rendering, coloured by atom) docked to $MurF_{Sp}$ (PDB code: 3zm5) with a co-crystallized inhibitor (sticks rendering, in green). The best ranked predicted binding mode can be seen for a) amine, and b) guanidine of truncated analogue of **II**; guanidine is predicted to fit into the "morpholine" pocket (binds morpholine moiety from the co-crystallized inhibitor) by forming 4 H-bonds with Asn134, Asn137 and Asn326 (in green, demonstrated by the distances between guanidine protons and aminoacid carbonyl oxygens), while the rest of the molecule overlaps the structure of a co-crystallized cyanothiophene scaffold. Virtually the same binding mode is predicted for c) amine with benzyl substituent attached to position 6 of 4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine, while its guanidine analogue d) moves towards the interior of the binding site to form close contact with Asp323 (in green, ionic bond and two H-bonds). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

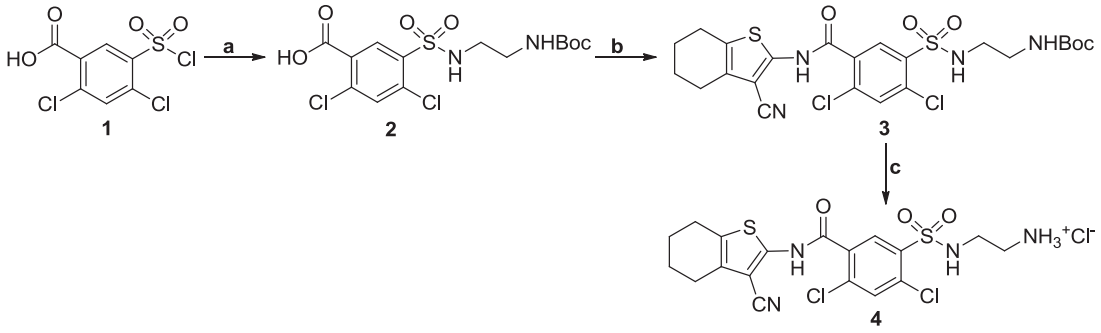
OEDocking software (Release 3.0.1, OpenEye Scientific Software, Inc.). This revealed the compounds with simple amine or guanidine at position R^1 as the ones with the highest predicted affinities (Fig. 2a–d). Furthermore, the replacement of H with Cl on the benzoic acid moiety was proposed to be beneficial for potent inhibition of MurF.

As all predicted binding poses were plausible and with good docking scores, we decided to incorporate both amino and guanidine groups into the structures of potential inhibitors. The design presented herein proved to be successful, as majority of amines/guanidines demonstrated low micromolar inhibition of MurF from *E. coli* and *S. pneumoniae*.

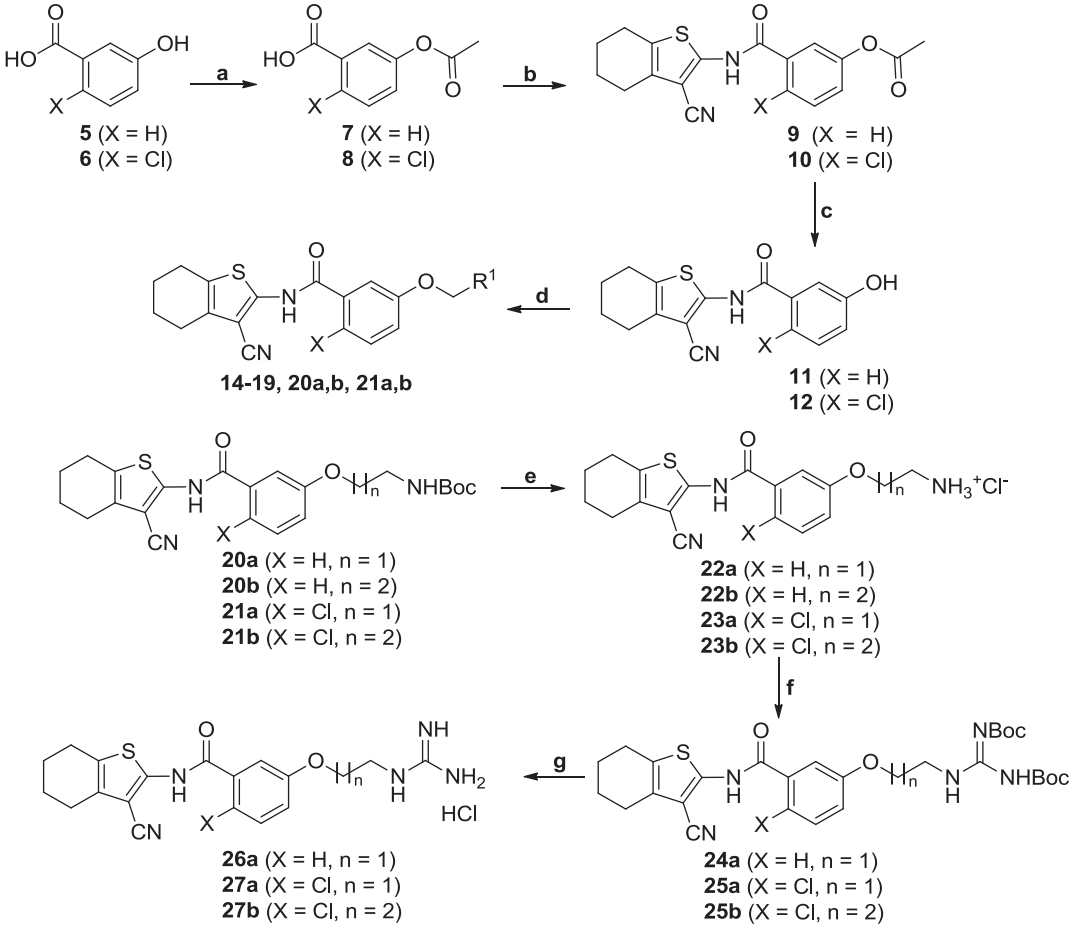
2.2. Chemistry

Compound **4** was synthesized as outlined in Scheme 1. In the first step, the chlorosulfonated 2,4-dichlorobenzoic acid (**1**) reacted with the *tert*-butyl (2-aminoethyl)carbamate to yield compound **2**. Then, the free acid was coupled with 2-aminothiophene through a two-stage process, including the conversion of the acid into acyl chloride in the presence of oxalyl chloride and catalytic amount of DMF, followed by the coupling in the presence of pyridine as a base, to obtain **3**. Compound **4** was prepared by the cleavage of the Boc protective group in the presence of CF_3COOH, and was subsequently transformed into hydrochloride salt with HCl in ethyl acetate.

The synthesis of target compounds **14**–**19**, **22a**, **22b**, **23a**, **23b**, **26a**, **27a** and **27b** is presented in Scheme 2. First, the hydroxyl group of compounds **5** and **6** was acetylated with acetic anhydride in pyridine. Next, compounds **9** and **10** were obtained using the same synthetic strategy as described for **3**. The acetoxy protective groups of compounds **9** and **10** were cleaved in the presence of K_2CO_3 in methanol. The diverse alkyl or aryl substituents were attached to the hydroxyl group of compounds **11** and **12** with two different approaches. In the first method, compounds **14**, **17**–**19**, **20a**, **20b**, **21a** and **21b** were obtained using K_2CO_3 or Cs_2CO_3 and different benzyl or alkyl halides. An alternative method (the Mitsunobu reaction using PPh_3, diisopropyl azodicarboxylate (DIAD) and the corresponding alcohol) was employed to give compounds **15** and **16**. As (1*H*-pyrazol-1-yl)methanol (**13**) was not commercially available, we prepared it by the already reported procedure [21]. Compounds **22a**, **22b**, **23a** and **23b** were obtained after the



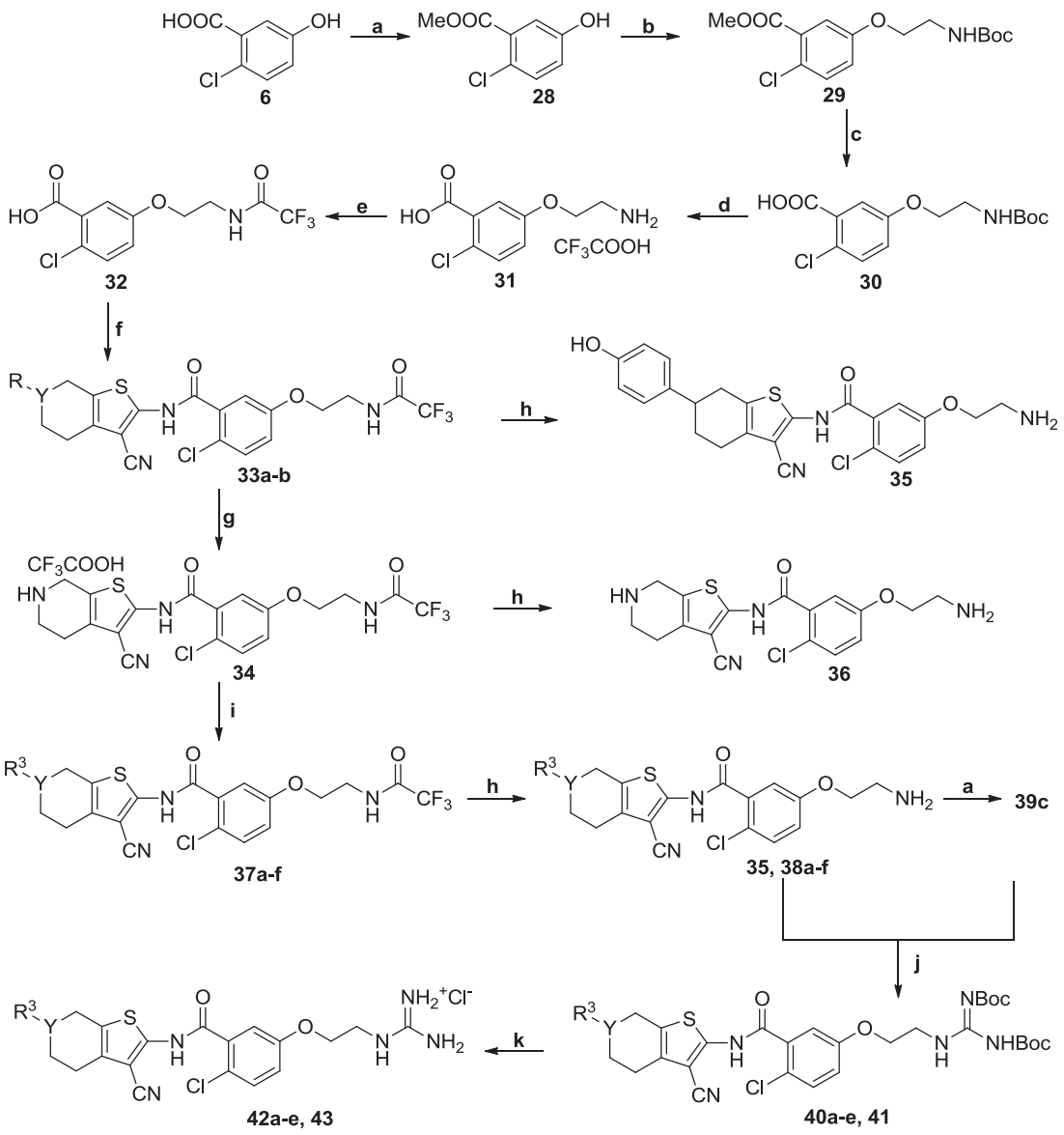
Scheme 1. Reagents and conditions: (a) *tert*-butyl (2-aminoethyl)carbamate, Et_3N, DCM, 0 °C to rt, 16 h; (b) 1. $(COCl)_2$, DMF, DCM, 0 °C to rt, 0.5 h; 2. 2-aminothiophene, pyridine, DCM, 0 °C to rt, 16 h; (c) 1. CF_3COOH, DCM, rt, 2 h; 2. 1 M HCl in EtOAc.



Scheme 2. Reagents and conditions: (a) Ac_2O, pyridine, rt, 1 h; (b) 1. $(COCl)_2$, DMF, DCM, 0 °C to rt, 0.5 h; 2. amine, pyridine, DCM, 0 °C to rt, 16 h; (c) K_2CO_3, MeOH, rt, 1 h; (d) A: K_2CO_3 or Cs_2CO_3, corresponding halogenide, 50 °C, 16 h; B: (1*H*-pyrazol-1-yl)methanol (**13**) or cyclopentylmethanol, PPh_3, DIAD, THF, 0 °C to rt, 16 h; (e) CF_3COOH, DCM, rt, 2 h, 1 M HCl in EtOAc; (f) di-Boc-methyl-thiourea, $HgCl_2$, Et_3N, DMF, rt, 4 h; (g) 1. CF_3COOH, DCM, rt, 2 h; 2. 1 M HCl in EtOAc.

cleavage of the Boc protective group using CF_3COOH. Amine groups of compounds **22a**, **23a** and **23b** were converted into di-Boc protected guanidines in the presence of mercuric chloride and triethylamine. Finally, the cleavage of both Boc protective groups with CF_3COOH, followed by direct transformation to hydrochloride salts with HCl in ethyl acetate, gave target compounds **26a**, **27a** and **27b**.

The synthesis of target compounds **33**–**36**, **37a**–**f**, **38a**–**f**, **39c**, **42a**–**e** and **43** is outlined in Scheme 3. 2-Chloro-5-hydroxybenzoic (**6**) acid was first protected as methyl ester and then alkylated using *tert*-butyl (2-bromoethyl)carbamate and K_2CO_3 to yield **28**. Compound **31** was obtained after alkaline hydrolysis of the methyl ester with aqueous sodium hydroxide, followed by cleavage of Boc protective group using CF_3COOH. The amino group was further protected as trifluoroacetamide with trifluoroacetic anhydride and pyridine as a base to give **32**. Compounds **33a**–**b** were obtained from **32** and the corresponding 2-aminothiophenes using the same procedure as described for compound **3**. Compound **34** was obtained after the cleavage of the Boc group of **33a** with the CF_3COOH. Final compounds **35** and **36** were obtained by alkaline hydrolysis of the trifluoroacetamide group of **33b** and **34**, respectively. The



Scheme 3. Reagents and conditions: (a) $SOCl_2$, MeOH, rt, 3 h; (b) K_2CO_3, DMF, 80 °C, 16 h; (c) NaOH, dioxane/water, rt, 16 h; (d) CF_3COOH, DCM, rt, 2 h; (e) $(CF_3CO)_2O$, pyridine, DCM, 0 °C to rt, 2 h; (f) 1. $(COCl)_2$, DMF, DCM, 0 °C to rt, 1 h; 2. corresponding 2-aminothiophene, pyridine, DCM, 0 °C to rt, 16 h; (g) CF_3COOH, DCM, rt, 2 h; (h) 1 M NaOH, MeOH, rt, 16 h; (i) A: K_2CO_3, corresponding benzyl bromide, DMF, rt, 16 h; B: corresponding benzaldehyde, $Na(OAc)_3BH$, AcOH, THF, rt, 16 h; (j) di-Boc-methyl-thiourea, $HgCl_2$, Et_3N, DMF, rt, 4 h; (k) 1. CF_3COOH, DCM, rt, 2 h; 2. 1 M HCl in EtOAc.

variously substituted benzyl fragments were attached to compound **34** via two different strategies. The first method was a nucleophilic substitution, where K_2CO_3 and different benzyl bromides were used to yield compounds **37a**–**c**; in the second method, compounds **37d**–**f** were synthesized by sodium triacetoxyborohydride-promoted reductive amination of **34** with various benzaldehydes. In the next step, the removal of the trifluoroacetamide group of **37a**–**f** was achieved through alkaline hydrolysis, which led to target compounds **38a**–**f**. Finally, target compounds **42a**–**e** and **43** were obtained using the same strategy as described for **26a**.

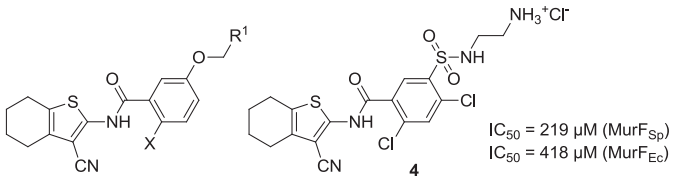
2.3. Inhibition of MurF

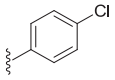
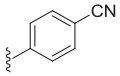
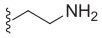
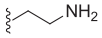
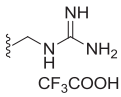
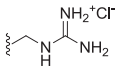
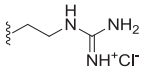
All target compounds were assayed for inhibition of MurF from *E. coli* and *S. pneumoniae*, using the Malachite green assay [22], which detects the orthophosphate generated during the enzymatic reaction. To avoid possible non-specific inhibition, all of the compounds were tested in the presence of detergent (0.005% Triton X-114) [23]. Results are presented as residual activities (RAs) of the respective enzyme in the presence of 250 or 100 μM of each compound. For the most active compounds, IC_{50} values were also determined.

First, a small series of compounds (Table 1, compounds **14**–**19**) was synthesized where the 4-chloropyrazole moiety was replaced with different aryl and alkyl fragments. None of these compounds significantly inhibited MurF. However, compounds with aminoethyl (**23a**) and aminopropyl substituents (**23b**) showed modest inhibitory activities against both MurF enzymes. As expected, the introduction of chlorine *ortho* to the amide bond was essential for the activity while the length of alkyl chain had little influence on the potency. Furthermore, compounds with the guanidino moiety (**26a**, **27a** and **27b**) were prepared and their potencies were 2- to 3-fold higher than potencies for the corresponding amine.

If we compare the compound **I** with the compounds **23a** and **27a**, we can notice that the morpholinosulfonyl fragment and chlorine next to sulfonamide linker are rather important for good

Table 1
Inhibitory activities of compounds **4, 14**–**19**, **22a**, **22b**, **23a**, **23b**, **26a**, **27a** and **27b** against MurF from *S. pneumoniae* and *E. coli*.



Compound	X	R^1	RA[a] (%) or IC_{50}[b] (μM)	
			$MurF_{Sp}$	$MurF_{Ec}$
14	-H		102%	97%
15	-H		81%	99%
16	-H		89%	85%
17	-Cl		81%	82%
18	-Cl		102%	97%
19	-Cl		87%[c]	74%[c]
22a	-H		98%	93%
22b	-H		53%[c]	75%[c]
23a	-Cl		170 μM	208 μM
23b	-Cl		128 μM	272 μM
26a	-H		167 μM	258 μM
27a	-Cl		75 μM	56 μM
27b	-Cl		78 μM	102 μM

[a] Residual activity (%) of the enzyme at 100 μM of the tested compound. Data are means of two independent experiments. Standard deviations were within±10% of the means.
[b] Concentration of the inhibitor for which the residual activity of the enzyme is 50%.
[c] Residual activity (%) of the enzyme at 250 μM of the tested compound.

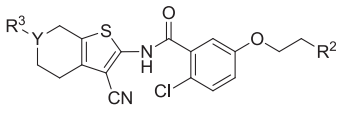
inhibition of $MurF_{Sp}$. This can be seen also in the co-crystal structure, where one of the sulfonyl oxygens of compound **I** makes two hydrogen bonds with the protein and the chlorine forms hydrophobic interactions [18]. These interactions are not present in our new inhibitors, resulting in their higher IC_{50} values against $MurF_{Sp}$. However, this interaction pattern is not necessary for inhibition of $MurF_{Ec}$, as compound **I** showed no inhibition of this orthologue. Furthermore, to examine the importance of the sulfonamido group, compound **4** was synthesized. By the comparison of compounds **4** and **23a**, we can see that the inhibition is in the same range as far as $MurF_{Sp}$ is concerned (**4**, IC_{50} = 219 μM; **23a**, IC_{50} = 170 μM). However, in the case of $MurF_{Ec}$ compound **4** was 2-fold less potent than **23b**, which makes sulfonamido group less favourable for inhibition of this enzyme.

In the next step, compounds with 4-hydroxyphenyl and different substituted benzyl substituents attached to position 6 of the 4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carbonitrile or 4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine-3-carbonitrile moiety were synthesized (Table 2), as it is known that substitution of this ring leads to greater potency [11,12,18]. First, compounds with the 4-hydroxyphenyl fragment appended to the saturated ring (**33b** and **35**) were prepared. The protected compound (**33b**) showed no inhibition against MurF while the compound with free amino and hydroxyl groups (**35**) inhibited both MurF enzymes in the low micromolar range (IC_{50} = 58 μM and 81 μM for $MurF_{Sp}$ and $MurF_{Ec}$, respectively). Compounds with different benzyl substituents and trifluoracetamide group (**37a**–**f**) showed modest inhibitory activities against MurF. The most potent inhibitor of $MurF_{Sp}$ and $MurF_{Ec}$ within this set was **37f** with the *p*-tetrazole group attached to the benzyl fragment, with IC_{50} values of 103 and 152 μM, respectively. Furthermore, inhibitors with a free amino group (**38a**–**f** and **49c**) were synthesized and most of them showed improved activity against both MurF enzymes. Exceptions were **38c** and **38f** with carboxylic acid and tetrazole on *para* position, respectively, which showed significantly lower inhibition of $MurF_{Sp}$ activity. The introduction of substituents to either *para* or *meta* position on the benzyl ring led to 2-fold lower potency against $MurF_{Sp}$ compared to **38a** (IC_{50} = 51 μM), while the *p*-substituents on the benzyl ring did not influence the activity against $MurF_{Ec}$ within this series. Finally, compounds with the guanidine substituent (**42a**–**e** and **43**) were prepared. The most potent inhibitor within this set was compound **43**, with the *p*-hydroxyphenyl group attached to the saturated ring: IC_{50} values were 20 and 25 μM against $MurF_{Sp}$ and $MurF_{Ec}$, respectively. Compounds **42a** and **42d** had similar inhibitory activities against both MurF orthologs as the corresponding amine precursors, while inhibitors **42b**, **42c** and **42e** showed about 2-fold better activity against $MurF_{Sp}$ than their corresponding amines (**38b**, **38c** and **38e**, respectively). Unfortunately, the introduction of the guanidine moiety does not improve the activity of inhibitors (**42a**–**e**) against $MurF_{Ec}$ compared to their respective amine precursors (**38a**–**e** and **39c**).

2.4. Antibacterial activity

For antibacterial evaluation of selected compounds, typical representatives of Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) were chosen (Table 3). In the case of *E. coli*, compounds were further evaluated against efflux-deficient strains (*E. coli* SM1411 and ES100), and those in which the outer membrane had been artificially permeabilized with polymyxin B (PMBN). Compounds **23a**–**b**, **26a** and **27a**–**b** showed modest antibacterial activities against *S. aureus* and *E. coli*, which increased against the latter species upon deletion of the AcrAB-TolC efflux transporter. Compounds **33a**, **34**, **36** and **37a**–**f** showed no activity against *E. coli* and *S. aureus*; this was expected since none of these compounds showed potent inhibition of $MurF_{Ec}$ and $MurF_{Sp}$. Compound **35** was inactive against *S. aureus* and *E. coli*, although antibacterial activity was observed against PMBN-permeabilized and/or efflux-deficient strains of *E. coli*. Compounds **38a**–**f** and **39c** showed modest antibacterial activities against both bacterial species. **38c** showed limited inhibition of both MurF enzymes (IC_{50} ~ 500 μM), and no antibacterial activity was observed. On the other hand, compounds **38b**, **38d**, **38e** and **39c** demonstrated antibacterial activities against *S. aureus* and *E. coli* strains, and inhibited isolated enzymes more

Table 2
Inhibitory activities of compounds **33**–**38f**, **39c**, **42a**–**e** and **43** against MurF from *S. pneumoniae* and *E. coli*.



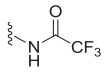
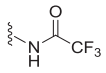
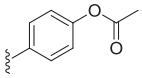
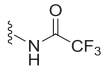
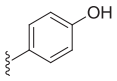
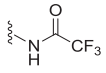
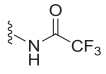
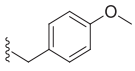
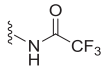
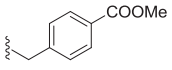
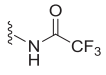
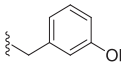
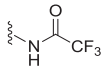
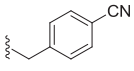
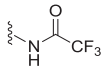
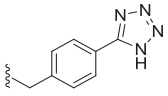
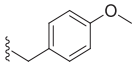
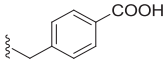
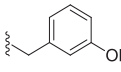
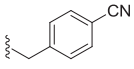
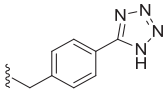
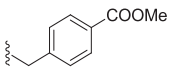
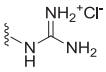
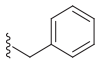
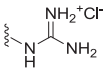
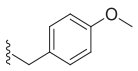
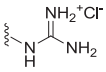
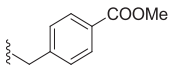
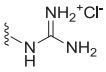
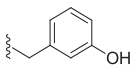
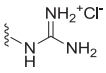
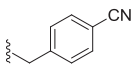
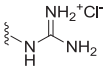
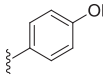
Compound	Y	R^2	R^3	RA[a] (%) or IC_{50}[b] (μM)	
				$MurF_{Sp}$	$MurF_{Ec}$
33	N			96%	102%
33b	C			98%	101%
34	N		$-H_2^+CF_3COO-$	99%	91%
35	C	$-NH_2$		58 μM	81 μM
36	N	$-NH_2$	-H	85%	65%
37a	N			172 μM	46%
37b	N			154 μM	81%
37c	N			55%	255 μM
37d	N			71%	56%
37e	N			55%	45%
37f	N			103 μM	152 μM
38a	N	$-NH_2$		51 μM	84 μM
38b	N	$-NH_2$		125 μM	73 μM
38c	N	$-NH_2$		663 μM	336 μM
38d	N	$-NH_2$		119 μM	150 μM
38e	N	$-NH_2$		107 μM	76 μM
38f	N	$-NH_2$		454 μM	83 μM
39c	N	$-NH_2$		109 μM	88 μM

Table 2 (*continued*)

Compound	Y	R^2	R^3	RA[a] (%) or IC_{50}[b] (μM)	
				$MurF_{Sp}$	$MurF_{Ec}$
42a	N			55 μM	57 μM
42b	N			61 μM	78 μM
42c	N			37 μM	80 μM
42d	N			117 μM	143 μM
42e	N			61 μM	66 μM
43	C			20 μM	25 μM

[a] Residual activity (%) of the enzyme at 250 μM of the tested compound. Data are means of two independent experiments. Standard deviations were within±10% of the means.
[b] Concentration of the inhibitor for which the residual activity of the enzyme is 50%.

potently (IC_{50} around or below 100 μM). The most potent inhibitor of MurF within this series was **38a**, which has 2-fold lower MIC values against all strains compared to its analogues. Finally, compounds with the guanidine moiety **42a**–**e** and **43** showed promising antibacterial activities against *S. aureus* with MICs ranging between 4 and 32 μg/mL. The latter compounds also demonstrated a modest antibacterial activity against *E. coli*. All compounds with antibacterial activity against *E. coli* exhibited increased activity against efflux-deficient strains and strains with increased membrane permeability.

Additional studies were performed to determine whether the antibacterial activities of the compounds are the consequence of peptidoglycan biosynthesis inhibition or non-specific effect. The *Bac*Light™ assay was used to assess the membrane integrity of *S. aureus* SH1000 at 4 × MIC values of inhibitors [24]. Unfortunately, all of the tested compounds (**38b**, **42a**, **42c**, **42e** and **43**) significantly decreased the membrane integrity.

3. Conclusion

We have designed, synthesized and evaluated a second generation of 37 new MurF inhibitors with cyanothiophene scaffold. The major improvement of the present compounds over the first-generation inhibitors is their reduced lipophilicity and increased solubility, with conserved enzyme inhibitory potency in micromolar range. The most potent inhibitors have well balanced inhibitory properties against both MurF from *Streptococcus pneumoniae* and MurF from *E. coli* (the best inhibitor **42** inhibits $MurF_{Sp}$ and $MurF_{Ec}$ with IC_{50} values of 20 μM and 25 μM, respectively). Furthermore, the most potent compounds within this series demonstrate antibacterial activities. As these compounds achieve part of their antimicrobial action by damaging the bacterial cytoplasmic membrane, they are unlikely to be developed as antibacterial drug candidates until this non-specific effect is diminished, without compromising their inhibitory effect on MurF.

Table 3
Antibacterial activities of compounds **4**, **23a**–**b**, **26a**, **27a**–**b**, **33a**, **34**–**38f**, **39c**, **42a**–**e** and **43** against *S. aureus* and *E. coli* bacterial strains.

Compound	MIC (μg/mL)						
	S. aureus	*E. coli*					
	SH1000	1411[a]	1411 + PMBN[d]	AB734[a]	AB734 + PMBN[d]	SM1411[b]	ES100[c]
4	>256	>256	128	>256	128	128	64
23a	128	128	32	128	32	32	32
23b	128	64	32	64	32	32	32
26a	64	64	64	64	>256	16	16
27a	64	16	8	16	8	8	8
27b	>256	32	32	32	32	16	8
33a	>256	>256	>256	>256	>256	>256	>256
34	>256	>256	>256	>256	>256	>256	>256
35	>256	>256	16	>256	16	32	8
36	>256	>256	>256	>256	>256	>256	>256
37a	>256	>256	>256	>256	>256	>256	>256
37b	>256	>256	>256	>256	>256	>256	>256
37c	>256	>256	>256	>256	>256	>256	>256
37d	>256	>256	>256	>256	>256	>256	>256
37e	>256	>256	>256	>256	>256	>256	>256
37f	>256	>256	>256	>256	>256	>256	64
38a	64	64	32	64	32	32	16
38b	128	128	64	128	64	64	32
38c	>256	>256	>256	>256	>256	>256	>256
38d	>256	128	64	128	64	64	64
38e	128	<256	64	<256	64	64	32
38f	>256	>256	>256	>256	>256	>256	>256
39c	128	>256	64	>256	64	64	32
42a	8	32	16	32	16	32	8
42b	32	32	32	32	32	32	8
42c	8	64	32	64	32	16	8
42d	32	128	64	128	64	64	32
42e	16	128	32	256	32	16	16
43	4	64	16	64	16	16	4

[a] *E. coli* 1411 and AB734 are parental strains of SM1411 and ES100, respectively.
[b] SM1411 is 1411 deleted for *acrAB*.
[c] ES100 is AB734 deleted for *tolC*.
[d] PMBN, polymyxin B nonapeptide.

4. Experimental section

4.1. Inhibition assay

The inhibition of MurF ligases was determined using the Malachite green assay with slight modifications. The mixture with final volume of 50 μL contained:

***S. pneumoniae* MurF**: 50 mM Hepes, pH 8.0, 50 mM $MgCl_2$, 0.005% Triton X-114, 100 μM D-Ala-D-Ala, 50 μM UMtri-L-Lys, 250 μM ATP, purified $MurF_{Sp}$ [17], and 250 or 100 μM of each tested compound dissolved in DMSO.

***E. coli* MurF**: 50 mM Hepes, pH 8.0, 50 mM $MgCl_2$, 0.005% Triton X-114, 600 μM D-Ala-D-Ala, 100 μM UMtri-mDAP, 500 μM ATP, purified $MurF_{Ec}$ [25], and 250 or 100 μM of each tested compound dissolved in DMSO.

In both cases, the final concentration of DMSO was 5% (v/v). After incubation for 15 min at 37 °C, the enzyme reaction was stopped by adding Biomol®reagent and the absorbance was measured at 650 nm. All of the experiments were run in duplicate. Residual activities (RAs) were calculated with respect to similar assays without the tested compounds and with 5% DMSO. The IC_{50} values, which were determined by measuring the residual activities at seven different compound concentrations, represented the concentration for which the RA was 50%.

4.2. Microbiological evaluation

Minimum inhibitory concentrations (MICs) for selected compounds were determined by broth microdilution in Mueller-Hinton broth (MHB), against *S. aureus* SH1000 [26], and *E. coli* strains 1411, SM1411 (*acrAB* derivative of 1411) [27], AB734 and ES100 (*tolC* derivative of AB734) [28], according to CLSI guidelines [29]. To assess the impact of outer-membrane permeability on antimicrobial activity against *E. coli*, MICs were also determined against strains 1411 and AB734 in the presence of 4 μg/mL polymyxin B nonapeptide (PMBN) [30]. The BacLight™ assay was used to measure membrane damage in *S. aureus* SH1000 induced by compounds at 4 × MIC, compared to a drug free control [24].

4.3. Chemistry

All of the chemicals used were obtained from commercial sources (Acros, Sigma–Aldrich, Alfa Aeser, Apollo Scientific, Fluka and Merck), and were used without further purification. Solvents were used without purification or drying, unless otherwise stated. Reactions were monitored using analytical thin-layer chromatography plates (Merck, silica gel 60 F_{254}, 0.25 mm), and compounds were visualized with ultraviolet light and ninhydrin or ferric chloride. Silica gel grade 60 (particle size 0.040–0.063 mm, Merck, Germany) was used for flash column chromatography. ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 400 MHz spectrometer in acetone-d_6, $CDCl_3$, DMSO-d_6, CD_3OD or pyridine-d_5, with TMS as the internal standard. Mass spectra were obtained with a VG-Analytical Autospec Q mass spectrometer (Centre for Mass Spectrometry, Institute Jožef Stefan, Ljubljana). Infrared spectra were recorded on a Perkin–Elmer FTIR 1600 spectrometer. Melting points were determined using a Reichert hot-stage microscope and are uncorrected. Microanalyses were performed on a 240 C Perkin Elmer elemental analyser. Analyses indicated by the

symbols of the elements were within 0.4% of the theoretical values. HPLC analyses were performed on an Agilent Technologies HP 1100 instrument with a G1365B UV–VIS detector (220 and 254 nm), using a Luna C18 column (4.6 × 250 mm) at a flow rate of 1 mL/min. The eluent was a mixture of 0.1% CF_3COOH in water (A) and acetonitrile (B). The gradient was 10%–90% B in 19 min.

4.3.1. 5-(N-(2-((tert-butoxycarbonyl)amino)ethyl)sulfamoyl)-2,4-dichlorobenzoic acid (**2**)

To a solution of *tert*-butyl (2-aminoethyl)carbamate (0.450 g, 2.81 mmol) in DCM (15 mL), Et_3N (0.59 mL, 4.21 mmol) was added. The reaction mixture was cooled on an ice bath and **1** (0.813 g, 2.81 mmol) was added portion wise. The reaction mixture was stirred for 16 h, diluted with 30 mL of DCM, and washed with 1 M HCl (2 × 40 mL) and brine (1 × 50 mL), and dried with Na_2SO_4. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography.

Yield = 15%; white needles, mp = 192–193 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.34 (s, 9H, 3 × CH_3), 2.90 (q, J = 5.6 Hz, 2H, SO_2–NH–C$\underline{H}_2$), 2.96 (q, J = 6.4 Hz, 2H, CO–NH–C$\underline{H}_2$), 6.72 (t, J = 5.6 Hz, 1H, SO_2–NH), 8.01 (s, 1H, Ar–H), 8.17 (t, J = 6.4 Hz, 1H, O–CO–NH), 8.34 (s, 1H, Ar–H) ppm. ^{13}C NMR (100 MHz, CD_3OD): δ 28.77, 28.83, 40.74, 41.08, 41.23, 43.57, 80.26, 80.35, 132.19, 133.93, 134.67, 136.52, 137.01, 138.40, 158.46, 158.60, 167.88 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 555.1447, found 555.1448. IR (KBr) ν_{max}: 3377, 2980, 2935, 1694, 1586, 1526, 1454, 1385, 1367, 1336, 1277, 1253, 1163, 1086 cm^{-1}.

4.3.2. tert-Butyl (2-(2,4-dichloro-5-((3-cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)carbamoyl) phenylsulfonamido) ethyl)carbamate (**3**)

To a suspension of **2** (0.305 g, 0.74 mmol) in anhydrous DCM (15 mL), catalytic amounts of DMF and $(COCl)_2$ (0.190 mL, 2.21 mmol) were added drop-wise, and the mixture was stirred for 0.5 h at room temperature. The solvent was evaporated to dryness and the residue was dissolved in DCM (20 mL), the mixture of 2-aminothiophene (0.125 g, 0.70 mmol) and pyridine (0.180 mL, 2.21 mmol) in DCM (15 mL) was added drop-wise, and the reaction was stirred overnight at room temperature. The reaction mixture was washed with 1 M HCl (2 × 40 mL), saturated aqueous $NaHCO_3$ (1 × 40 mL) and brine (1 × 40 mL), and dried (Na_2SO_4). The solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography.

Yield = 24%; white crystals, mp = 196–198 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.43 (s, 9H, 3 × CH_3), 1.85–1.90 (m, 4H, CH_2–C$\underline{H}_2$–C$\underline{H}_2$–CH_2), 2.63 (t, J = 5.6 Hz, 2H, C$\underline{H}_2$–CH_2–CH_2–CH_2), 2.70 (t, J = 5.6 Hz, 2H, CH_2–CH_2–CH_2–C$\underline{H}_2$), 3.06 (q, J = 4.8 Hz, 2H, SO_2–NH–C$\underline{H}_2$), 3.23 (q, J = 4.8 Hz, 2H, SO_2–NH–CH_2–C$\underline{H}_2$), 4.95 (t, J = 4.8 Hz, 1H, SO_2–N$\underline{H}$), 6.21 (t, J = 4.8 Hz, 1H, SO_2–NH–CH_2–CH_2–N$\underline{H}$), 7.61 (s, 1H, Ar–H), 8.31 (s, 1H, Ar–H), 10.04 (bs, 1H, N$\underline{H}$–CO–Ar) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.10, 23.05, 24.01, 24.06, 28.35, 40.24, 43.44, 79.99, 94.88, 114.59, 129.62, 131.57, 132.12, 132.61, 132.90, 134.42, 136.12, 136.57, 145.83, 156.34, 161.00 ppm. ESI HRMS calcd. for $[M - (H)]^-$ 571.0643, found 571.0644. IR (KBr) ν_{max}: 3414, 3077, 2974, 2227, 1683, 1637, 1616, 1579, 1481, 1447, 1400, 1383, 1369, 1347, 1327, 1286, 1259, 1167, 1105, 1078 cm^{-1}.

4.3.3. 5-[(2-Aminoethyl)sulfamoyl]-2,4-dichloro-N-(3-cyano-4,5,6,7-tetrahydro-1-benzothiophen-2-yl)benzamide hydrochloride (**4**)

To a solution of **3** (0.095 g, 0.166 mmol) in DCM (10 mL), CF_3COOH (0.6 mL, 8.0 mmol) was added. The reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure and the product was dissolved in 1 M HCl in EtOAc (3 mL). EtOAc was removed and the product was dried in a desiccator overnight.

Yield = 100%; pale brown crystals, mp = 204–206 °C; ^{1}H NMR (400 MHz, MeOD): δ 1.85–1.93 (m, 4H, CH_2–C$\underline{H}_2$–C$\underline{H}_2$–CH_2), 2.62 (t, J = 5.6 Hz, 2H, C$\underline{H}_2$–CH_2–CH_2–CH_2), 2.72 (t, J = 5.6 Hz, 2H, CH_2–CH_2–CH_2–C$\underline{H}_2$), 3.10 (t, J = 6.0 Hz, 2H, SO_2–NH–C$\underline{H}_2$), 3.22 (t, J = 6.0 Hz, 2H, SO_2–NH–CH_2–C$\underline{H}_2$), 7.91 (s, 1H, Ar–H), 8.33 (s, 1H, Ar–H) ppm, NH_3^+ and CONH are exchanged. ^{13}C NMR (100 MHz, MeOD): δ 23.31, 24.23, 24.91, 25.09, 40.77, 41.13, 97.21, 114.61, 131.01, 133.05, 133.15, 134.25, 134.82, 135.66, 137.55, 137.99, 146.59, 164.12 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 473.0276, found 473.0268. IR (KBr) ν_{max}: 3437, 2928, 2361, 2228, 1671, 1582, 1448, 1334, 1295, 1174, 1084, 1034 cm^{-1}. HPLC t_R = 11.977 min (100% at 220 nm, 100% at 254 nm).

4.3.4. General procedure for synthesis of compounds **7** and **8**

To a solution of 5-hydroxybenzoic acid (**5**) (17.4 mmol) in pyridine (10 mL), acetic anhydride was added dropwise (6.6 mL, 70.0 mmol). The reaction mixture was stirred at room temperature for 1 h and was subsequently poured into water (50 mL). The water phase was acidified to pH 2 and extracted with EtOAc (3 × 40 mL). The combined organic phases were washed with 0.1 M HCl (1 × 100 mL), brine (1 × 100 mL), and dried with Na_2SO_4. The solvent was evaporated under reduced pressure and the crude product was used without further purification.

*4.3.4.1. 3-Acetoxybenzoic acid (**7**).* Yield = 89%; white crystals, mp = 127–129 °C (lit [31]. 128.7–131.3 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.28 (s, 3H, CO–C$\underline{H}_3$), 7.39 (ddd, J_1 = 8.1, J_2 = 2.4, J_3 = 1.1 Hz, 1H, Ar–H), 7.52–7.56 (m, 1H, Ar–H), 7.65–7.69 (m, 1H, Ar–H), 7.80–7.86 (m, 1H, Ar–H), 13.18 (bs, 1H, COOH) ppm.

4.3.5. General procedure for synthesis of **9** and **10**

To a solution of **7** (14.0 mmol) in anhydrous DCM (30 mL), DMF (catalytic amount) and $(COCl)_2$ (3.60 mL, 42 mmol) were added drop-wise, and the mixture was stirred for 0.5 h at room temperature. The solvent was evaporated to dryness and the residue was dissolved in DCM (20 mL). A mixture of 2-aminothiophene (2.245 g, 12.6 mmol) and pyridine (3.30 mL, 42 mmol) in DCM (30 mL) was added drop-wise, and the reaction was stirred overnight at room temperature. The reaction mixture was washed with 1 M HCl (40 mL), saturated aqueous $NaHCO_3$ (40 mL) and brine (40 mL), and dried (Na_2SO_4). The solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography and recrystallized from ethanol.

*4.3.5.1. 3-((3-Cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)carbamoyl)phenyl acetate (**9**).* Yield = 65%; white crystals, mp = 161–162 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.82–1.88 (m, 4H, CH_2–C$\underline{H}_2$–C$\underline{H}_2$–CH_2), 2.34 (s, 3H, OCO–C$\underline{H}_3$), 2.59–2.71 (m, 4H, C$\underline{H}_2$–CH_2–CH_2–C$\underline{H}_2$), 7.35 (ddd, J_1 = 8.1, J_2 = 2.3, J_3 = 1.0 Hz, 1H, Ar–H), 7.54 (t, J = 8.0 Hz, 1H, Ar–H), 7.65 (t, J = 2.0 Hz, 1H, Ar–H), 7.78 (ddd, J_1 = 7.8, J_2 = 1.7, J_3 = 1.0 Hz, 1H, Ar–H), 8.99 (s, 1H, CO–N$\underline{H}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.11, 22.12, 23.09, 23.98, 94.26, 114.54, 121.12, 124.93, 126.26, 128.94, 130.07, 131.25, 133.41, 146.53, 150.95, 162.87, 169.30 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 341.0960, found 341.0966. IR (KBr) ν_{max}: 3237, 3202, 3074, 2936, 2842, 2365, 2345, 2223, 2212, 1765, 1671, 1572, 1545, 1458, 1369, 1325, 1291, 1272, 1206, 1146, 1094,1010 cm^{-1}. Anal. Calcd. for $C_{18}H_{16}N_2O_3S \times 0.1H_2O$: C, 63.18; H, 4.77; N, 8.19. Found C, 62.90; H, 4.45; N, 8.05.

4.3.6. General procedure for synthesis of **11** and **12**

To a suspension of **9** (3.0 g, 8.0 mmol) in methanol (20 mL), K_2CO_3 (1.69 g, 12.0 mmol) was added. The reaction mixture was stirred at room temperature for 1 h, and the solvent was evaporated

under reduced pressure. The residue was dissolved in 1 M HCl (40 mL) and extracted with EtOAc (3 × 40 mL). The combined organic phases were washed with brine, dried with Na_2SO_4, and the solvent was removed under reduced pressure. The product was used without further purification.

*4.3.6.1. N-(3-Cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)-3-hydroxybenzamide (**11**).* Yield = 87%; small white crystals, mp = 249–250 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 1.80–1.90 (m, 4H, CH_2–C$\underline{H}_2$–C$\underline{H}_2$–CH_2), 2.53–2.71 (m, 4H, C$\underline{H}_2$–CH_2–CH_2–C$\underline{H}_2$), 7.07–7.13 (m, 1H, Ar–H), 7.35–7.42 (m, 1H, Ar–H), 7.45–7,54 (m, 2H, 2 × Ar–H), 8.85 (s, 1H, CO–N$\underline{H}$), 10.52 (s, 1H, Ar–OH) ppm. ^{13}C NMR (100 MHz, acetone-d_6): δ 22.92, 23.84, 24.46, 24.67, 96.20, 114.52, 115.62, 119.87, 120.44, 129.47, 130.70, 132.17, 134.96, 147.33, 158.47, 165,45 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 299.0854, found 299.0856. IR (KBr) ν_{max}: 3380, 3244, 3077, 2992, 2943, 2922, 2840, 2345, 2222, 1660, 1616, 1587, 1576, 1559, 1490, 1458, 1399, 1327, 1306, 1283, 1260, 1216, 1147, 1078, 1029 cm^{-1}. Anal. Calcd. for $C_{16}H_{14}N_2O_2S$: C, 64.41; H, 4.73; N, 9.39. Found C, 64.19; H,4.56; N, 9.27.

*4.3.7. General procedure for synthesis of **14**–**29**, **20a**–**b** and **21a**–**b***

Method A: To a solution of *N*-(3-cyano-4,5,6,7-tetrahydrobenzo [*b*]thiophen-2-yl)-3-hydroxybenzamide (**12**) (0.270 g, 0.90 mmol) in DMF (5 mL), K_2CO_3 or Cs_2CO_3 (1.35 mmol) was added. After 15 min, the corresponding bromide or chloride (1.35 mmol) was added and the reaction mixture was stirred for 16 h at 50 °C. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc (40 mL). The organic phase was washed with water (2 × 30 mL), 1 M HCl (1 × 30 mL), brine (1 × 30 mL), and dried with Na_2SO_4. The solvent was removed under reduced pressure and the crude product was purified by flash chromatography.

*4.3.7.1. 3-((4-Chlorobenzyl)oxy)-N-(3-cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)benzamide (**14**).* Yield = 44%; white crystals, mp = 195–196 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.79–1.90 (m, 4H, CH_2–C$\underline{H}_2$–C$\underline{H}_2$–CH_2), 2.58–2.70 (m, 4H, C$\underline{H}_2$–CH_2–CH_2–C$\underline{H}_2$), 5.11 (s, 2H, O–C$\underline{H}_2$-Ar), 7.17–7.21 (m, 1H, Ar–H), 7.34–7.42 (m, 4H, 4 × Ar–H), 7.42–7.48 (m, 2H, 2 × Ar–H), 7.51–7.55 (m, 1H, Ar–H), 8.85 (s, 1H, CO–N$\underline{H}$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 22.12, 23.10, 24.00, 69.46, 94.08, 113.64, 114.49, 119.42, 120.07, 128.87, 128.92, 130.26, 131.12, 133.29, 134.06, 134.74, 146.57, 159.04, 163.27 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 423.0934, found 423.0939. IR (KBr) ν_{max}: 3446, 3252, 3200, 3076, 2932, 2855, 2366, 2217, 1889, 1670, 1602, 1595, 1573, 1558, 1490, 1470, 1456, 1411, 1399, 1378, 1327, 1296, 1279, 1229, 1164, 1153, 1116, 1095, 1082, 1054, 1032, 1014 cm^{-1}. Anal. Calcd. for $C_{23}H_{19}ClN_2O_2S \times 0.3H_2O$: C, 64.49; H, 4.61; N, 6.54. Found C, 64.29; H, 4.32; N, 6.44.

Method B: To a solution of *N*-(3-cyano-4,5,6,7-tetrahydrobenzo [*b*]thiophen-2-yl)-3-hydroxybenzamide (**12**) (0.250 g, 0.838 m mol) in anhydrous THF (5 mL), the corresponding alcohol (0.838 mmol) and PPh_3 (0.330 g, 1.25 mmol) were added. The reaction mixture was cooled on ice bath and DIAD (0.25 mL, 1.25 mmol) was added. The reaction was stirred overnight at room temperature. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography.

*4.3.7.2. 3-((1H-pyrazol-1-yl)methoxy)-N-(3-cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)benzamide (**16**).* Yield = 26%; pale yellow crystals, mp = 86–88 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.69–1.79 (m, 4H, CH_2–C$\underline{H}_2$–C$\underline{H}_2$–CH_2), 2.44 (t, *J* = 5.2 Hz, 2H, C$\underline{H}_2$–CH_2–CH_2–CH_2), 2.56 (t, *J* = 4.8 Hz, 2H, CH_2–CH_2–CH_2–C$\underline{H}_2$), 6.09 (s, 2H, C$\underline{H}_2$–O), 6.37 (t, *J* = 2.4 Hz, 1H, CH–C$\underline{H}$–CH-pyrazol), 6.84 (ddd, J_1 = 8.0, J_2 = 2.8, J_3 = 0.8 Hz, 1H, Ar–H), 6.92 (dt, J_1 = 7.6, J_2 = 1.6 Hz, 1H, Ar–H), 6.98 (dd, J_1 = 2.4, J_2 = 1.6 Hz, 1H, Ar–H), 7.10 (t, *J* = 8.0 Hz, 1H, Ar–H), 7.54 (dd, J_1 = 1.6, J_2 = 0.4 Hz, 1H, CH_A–CH–CH_B-pyrazole), 7.62 (bs, 1H, CO–NH), 7.84 (d, *J* = 2.4 Hz, 1H, CH_A–CH–C$\underline{H}_B$-pyrazole) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.62, 22.62, 24.12, 24.62, 64.25, 107.30, 110.01, 112.66, 115.25, 118.54, 119.74, 129.46, 131.28, 134.42, 134.79, 136.96, 140.30, 148.79, 156.04, 170.75 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 379.1229, found 379.1220. IR (KBr) ν_{max}: 3418, 2937, 2225, 1668, 1599, 1449, 1376, 1291, 1248, 1194, 1128, 1086, 1054 cm^{-1}. HPLC t_R = 12.696 min (100% at 220 nm, 100% at 254 nm).

*4.3.8. General procedure for synthesis of **22a**–**b** and **23a**–**b***

To a solution of Boc-protected amine (**20a**–**b** and **21a**–**b**) (0.1 mmol) in DCM, CF_3COOH (3–5 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 2 h. Solvent was evaporated and some products were converted into hydrochloride salts with HCl in EtOAc.

*4.3.8.1. 3-(2-Aminoethoxy)-N-(3-cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)benzamide (**22a**).* Yield = 76%; white crystals, mp = 158–160 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.73–1.84 (m, 4H, CH_2–C$\underline{H}_2$–C$\underline{H}_2$–CH_2), 2.54–2.67 (m, 4H, C$\underline{H}_2$–CH_2–CH_2–C$\underline{H}_2$), 3.25–3.31 (m, 2H, NH–C$\underline{H}_2$–CH_2–O), 4.22–4.30 (m, 2H, NH–CH_2–C$\underline{H}_2$–O), 7.25–7.31 (m, 1H, Ar–H), 7.50–7.57 (m, 2H, 2 × Ar–H), 7.58–7.64 (m, 1H, Ar–H), 7.93–8.14 (bs, 3H, NH_3^+) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.67, 22.57, 23.45, 23.59, 38.32, 64.63, 96.29, 114.18, 114.30, 118.81, 121.07, 128.84, 129.82, 131.45, 133.80, 146.16, 157.74, 164.79 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 342.1276, found 342.1277. IR (KBr) ν_{max}: 3434, 3235, 3195, 3080, 2936, 2220, 1669, 1576, 1559, 1511, 467, 1450, 1326, 1297, 1282, 1230, 1203, 1183, 1128, 1076, 1025 cm^{-1}. Anal. Calcd. for $C_{18}H_{19}N_3O_2S \times CF_3COOH$: C, 52.74; H, 4.43; N, 9.23. Found C, 52.56; H, 4.22; N, 9.13.

*4.3.9. General procedure for synthesis of compounds **24a**, **25a**–**b**, **40a**–**e** and **41***

To a solution of amine (**22a**, **23a**–**b**, **35**, **38a**–**e** or **39c**) (0.150 mmol) in anhydrous DMF (5 mL), di-Boc-*S*-methyl-isothiourea (0.150 mmol), Et_3N (0.300 mmol) and $HgCl_2$ (0.150 mmol) were added. The reaction mixture was stirred for 2 h at room temperature. The formed precipitate was filtered off and the solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc (30 mL), washed with water (3 × 30 mL), saturated $NaHCO_3$ (30 mL), brine (30 mL), and dried with Na_2SO_4. The crude product was purified by flash chromatography.

*4.3.9.1. tert-Butyl(tert-butoxycarbonylamino)(2-(3-(3-cyano-4,5,6,7-tetrahydrobenzo[b] thiophen-2-ylcarbamoyl)phenoxy)ethylamino) methylencarbamate (**24a**).* Yield = 51%; white crystals, mp = 165–166 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.53 (dd, J_1 = 6.8, J_2 = 1.2 Hz, 16H, 2 × COO–($C\underline{H}_3$)$_3$), 1.81–1.94 (m, 4H, CH_2–C$\underline{H}_2$–C$\underline{H}_2$–CH_2), 2.61–2.74 (m, 4H, C$\underline{H}_2$–CH_2–CH_2–C$\underline{H}_2$), 3.90 (q, *J* = 5.3 Hz, 2H, NH–C$\underline{H}_2$–CH_2–O), 4.20 (t, *J* = 5.2 Hz, 2H, NH–CH_2–C$\underline{H}_2$–O), 7.18–7.27 (m, 1H, Ar–H), 7.41–7.50 (m, 2H, 2 × Ar–H), 7.50–7.56 (m, 1H, Ar–H), 8.78 (t, *J* = 5.3 Hz, 1H, C–N$\underline{H}$–CH_2), 8.90 (s, 1H, CO–N$\underline{H}$–C), 11.20 (s, 1H, C–N$\underline{H}$-Boc) ppm. ^{13}C NMR (100 MHz, acetone-d_6): δ 14.37, 22.92, 23.30, 23.83, 24.46, 24.68, 28.14, 28.47, 32.32, 40.70, 67.21, 79.02, 83.84, 96.29, 114.50, 114.93, 119.77, 121.46, 129.56, 130.78, 132.27, 135.00, 159.79 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 584.2543, found 584.2545. IR (KBr) ν_{max}: 3341, 3285, 3080, 2976, 2934, 2362, 2344, 2219, 1741, 1656, 1623, 1576, 1546, 1493, 1459, 1434, 1415, 1397, 1362, 1330, 1300, 1276, 1223, 1146, 1086, 1062, 1046, 1027 cm^{-1}. Anal. Calcd. for $C_{29}H_{37}N_5O_6S \times 0.9H_2O$: C, 58.06; H, 6.52; N, 11.67. Found C, 58.33; H, 6.44; N, 11.27.

*4.3.10. General procedure for synthesis of compounds **26a**, **27a–b**, **42a–e** and **43***

To a solution of Boc-protected guanidine (**24a**, **25a–b**, **40a–e** and **41**) (0.095 mmol) in DCM (5 mL), CF_3COOH (0.210 mL, 2.84 mmol) was added, and the mixture was stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and 1 M HCl in EtOAc (3 mL) was added to the residue. The formed precipitate was collected by filtration.

*4.3.10.1. N-(3-Cyano-4,5,6,7-tetrahydrobenzo[b]thiophen-2-yl)-3-(2-guanidinoethoxy) benzamide (**26a**).* Yield = 86%; white crystals, mp = 179–181 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 1.84–1.89 (m, 4H, CH_2–CH_2–CH_2–CH_2), 2.57–2.61 (m, 2H, CH_2–CH_2–CH_2–CH_2), 2.68–2.72 (m, 2H, CH_2–CH_2–CH_2–CH_2), 3.78–3.83 (m, 2H, NH–CH_2–CH_2–O), 4.33 (t, J = 5.2 Hz, 2H, NH–CH_2–CH_2–O), 7.25–7,29 (m, 1H, Ar–H), 7.48–7.53 (m, 1H, Ar–H), 7.62–7.69 (m, 2H, 2 × Ar–H), 7.79 (bs, 3H, C–NH_3^+), 8.68 (bs, 1H, NH), 10.72 (bs, 1H, NH) ppm. ^{13}C NMR (100 MHz, MeOD): δ 22.91, 23.82, 24.47, 24.68, 41.68, 41.79, 67.71, 96.50, 114.50, 114.94, 119.87, 121.62, 129.64, 130.74, 132.31, 135.02, 147.20, 159.52, 165.34 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 384.1494, found 384.1493. IR (KBr) ν_{max}: 3425, 3366, 3179, 2936, 2855, 2363, 2344, 2225, 1674, 1579, 1558, 1454, 1397, 1326, 1304, 1272, 1206, 1186, 1134, 1063, 1028 cm^{-1}. HPLC t_R = 11,836 min (98.2% at 220 nm, 100% at 254 nm).

*4.3.11. Methyl 2-chloro-5-hydroxybenzoate (**28**)*

To a cooled (0 °C) solution of 2-chloro-5-hydroxybenzoic acid (6) (4 g, 23.6 mmol) in methanol (50 mL), thionyl chloride (2.5 mL, 35 mmol) was added drop-wise. The reaction mixture was stirred at room temperature for 3 h. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc (100 mL). The organic phase was washed with saturated $NaHCO_3$ (3 × 50 mL), water (50 mL) and brine (50 mL), dried with Na_2SO_4 and evaporated under reduced pressure.

Yield = 99%; white crystals, mp = 96–97 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.91 (s, 3H, O–CH_3), 6.91 (dd, J_1 = 8.7, J_2 = 3.1 Hz, 1H, Ar–H), 7.27 (d, J = 8.7 Hz, 1H, Ar–H), 7.31 (d, J = 3.1 Hz, 1H, Ar–H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 52.79, 118.15, 120.33, 124.67, 130.25, 132.14, 154.51, 166.77 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 187.0162, found 187.0155. HPLC t_R = 10.618 min (100% at 220 nm, 100% at 254 nm).

*4.3.12. Methyl 5-(2-((tert-butoxycarbonyl)amino)ethoxy)-2-chlorobenzoate (**29**)*

To a solution of **28** (1.00 g, 5.36 mmol) in DMF (15 mL), K_2CO_3 (1.11 g, 8.04 mmol) was added. After 10 min at room temperature, *tert*-butyl 2-bromoethylcarbamate (1.56 g, 6.97 mmol) was added and the reaction mixture was stirred at 80 °C for 16 h. DMF was evaporated, the crude residue was dissolved in EtOAc (50 mL), washed with water (2 × 30 mL), 1 M NaOH (2 × 30 mL), water (1 × 50 mL), brine (10 mL), and dried with Na_2SO_4. The crude residue was purified by flash chromatography.

Yield = 73%; colourless oil; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.47 (s, 9H, 3 × CH_3), 3.56 (q, J = 5.6 Hz, 2H, O–CH_2–CH_2–NH), 3.95 (s, 3H, O–CH_3), 4.05 (t, J = 4.8 Hz, 2H, O–CH_2–CH_2–NH), 4.98 (bs, 1H, NH–CO), 6.98 (dd, J_1 = 8.8, J_2 = 2.8 Hz, 1H, Ar–H), 7.36 (s, 1H, Ar–H), 7.37 (d, J = 6.0 Hz, 1H, Ar–H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 28.38, 39.95, 52.53, 67.71, 79.72, 116.72, 119.22, 125.39, 130.56, 131.95, 155.83, 156.88, 165.87 ppm. ESI HRMS calcd. for $[M+(H)]^+$ 330.1108, found 330.1103.

*4.3.13. 5-(2-(tert-Butoxycarbonylamino)ethoxy)-2-chlorobenzoic acid (**30**)*

To a solution of **29** (1.00 g, 3.03 mmol) in a mixture of dioxane and water (1/1, 30 mL), NaOH (0.24 g, 6.00 mmol) was added. The reaction mixture was stirred at room temperature for 4 h. Dioxane was evaporated under reduced pressure and the remaining water was acidified with 1 M HCl to pH 3. The water phase was extracted with EtOAc (3 × 30 mL). The combined organic phases were washed with brine (1 × 50 mL), dried with Na_2SO_4 and evaporated under reduced pressure.

Yield = 98%; white crystals, mp = 117–119 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.48 (s, 9H, 3 × CH_3), 3.58 (q, J = 5.6 Hz, 2H, O–CH_2–CH_2–NH), 4.07 (t, J = 4.8 Hz, 2H, O–CH_2–CH_2–NH), 5.03 (bs, 1H, NH–CO), 7.03 (dd, J_1 = 8.0, J_2 = 2.4 Hz, 1H, Ar–H), 7.40 (d, J = 8.8 Hz, 1H, Ar–H), 7.52 (d, J = 2.8 Hz, 1H, Ar–H), 7.64 (bs, 1H, COOH) ppm. ^{13}C NMR (100 MHz, acetone-d_6): δ 28.63, 40.51, 68.35, 78.98, 117.93, 119.83, 124.93, 132.34, 132.64, 156.77, 158.37, 166.56 ppm. ESI HRMS m/z calcd. for $[M - (H)]^+$ 314.0795, found 314.0791. IR (KBr) ν_{max}: 3403, 2981, 2576, 1717, 1669, 1605, 1572, 1527, 1478, 1464, 1430, 1394, 1366, 1275, 1252, 1207, 1162, 1110, 1077, 1053, 1037 cm^{-1}.

*4.3.14. 2-(3-Carboxy-4-chlorophenoxy)ethan ammonium trifluoroacetate (**31**)*

To a solution of **30** (1.00 g, 3.17 mmol) in DCM, CF_3COOH (7.0 mL, 91.4 mmol) was added and stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the crude product was used without further purification.

Yield = 100%; mp = 147–149 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 4.29 (t, J = 5.6 Hz, 2H, O–CH_2–CH_2–NH_2), 4.51 (t, J = 4.8 Hz, 2H, O–CH_2–CH_2–NH_2), 7.17 (dd, J_1 = 8.8, J_2 = 3.2 Hz, 1H, Ar–H), 7.42 (d, J = 3.2 Hz, 1H, Ar–H), 7.43 (d, J = 8.8 Hz, 1H, Ar–H) ppm.

*4.3.15. 2-Chloro-5-(2-(2,2,2-trifluoroacetamido)ethoxy)benzoic acid (**32**)*

To a suspension of **31** (1.00 g, 4.64 mmol) in anhydrous DCM (15 mL), anhydrous pyridine (1.12 mL, 13.9 mmol) was added and the reaction mixture was cooled to 0 °C. $(CF_3CO)_2O$ (0.72 mL, 5.10 mmol) was added drop-wise and the mixture was stirred for 2 h at 0 °C then allowed to warm to room temperature. The reaction mixture was diluted with DCM (30 mL), washed with 1 M HCl (2 × 30 mL), water (30 mL), and saturated $NaHCO_3$ (3 × 30 mL). The combined alkaline water phases were acidified with concentrated HCl to pH = 1 and extracted with EtOAc (3 × 50 mL). The combined organic phases were washed with water (50 mL), brine (50 mL) and dried with Na_2SO_4. The solvent was evaporated under reduced pressure.

Yield = 76%; white crystals, mp = 141–143 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 3.79 (q, J = 5.6 Hz, 2H, O–CH_2–CH_2–NH), 4.28 (t, J = 5.2 Hz, 2H, O–CH_2–CH_2–NH), 7.15 (dd, J_1 = 8.8, J_2 = 3.2 Hz, 1H, Ar–H), 7.43 (d, J = 2.8 Hz, 1H, Ar–H), 7.45 (d, J = 8.4 Hz, 1H, Ar–H), 8.79 (bs, 1H, NH–CO) ppm. ^{13}C NMR (100 MHz, acetone-d_6): δ 39.98 (d, $^4J_{F,C}$ = 12 Hz, CH_2–NH), 67.01, 117.08 (q, $^1J_{F,C}$ = 285.8 Hz, CF_3), 117.89, 119.86, 125.21, 132.46, 132.70, 157.93 (qd, $^2J_{F,C}$ = 36.5 Hz, $^2J_{F,C}$ = 8 Hz, CO–CF_3), 158.07, 166.52 ppm. ESI HRMS m/z calcd. for $[M - (H)]^+$ 310.0094, found 310.0088. IR (KBr) ν_{max}: 3414, 3316, 3110, 2927, 2633, 1710, 1597, 1560, 1486, 1469, 1432, 1414, 1384, 1374, 1349, 1321, 1277, 1260, 1235, 1206, 1182, 1116, 1061, 1052, 1039 cm^{-1}. HPLC t_R = 10.996 min (100% at 220 nm, 100% at 254 nm).

*4.3.16. General procedure for synthesis of compounds **33a** and **33b***

To a solution of **32** (2.15 g, 6.88 mmol) in anhydrous DCM (30 mL), DMF (catalytic amount) and $(COCl)_2$ (1.77 mL, 20.6 mmol) were added drop-wise, and the mixture was stirred for 0.5 h at room temperature. The solvent was evaporated to dryness and the residue was dissolved in DCM (20 mL). A mixture of the corresponding 2-aminothiophene (1.73 g, 6.19 mmol) and pyridine (1.63 mL, 20.6 mmol) in DCM (20 mL) was added drop-wise, and

the reaction was stirred overnight at room temperature. The reaction mixture was washed with 1 M HCl (30 mL), saturated aqueous $NaHCO_3$ (30 mL) and brine (30 mL), and dried (Na_2SO_4). The solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography.

*4.3.16.1. tert-Butyl 2-(2-chloro-5-(2-(2,2,2-trifluoroacetamido) ethoxy)benzamido)-3-cyano-4,5-dihydrothieno [2,3-c]pyridine-6(7H)-carboxylate (**33a**).* Yield = 79%; orange crystals, mp = 89–91 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.46 (s, 9H, 3 × CH_3), 2.67 (t, J = 5.6 Hz, 2H, C$\underline{H}_2$–CH_2–N), 3.68 (t, J = 5.6 Hz, 2H, CH_2–C$\underline{H}_2$–N), 3.78 (q, J = 5.4 Hz, 2H, O–CH_2–C$\underline{H}_2$–NH), 4.13 (t, J = 5.1 Hz, 2H, O–C$\underline{H}_2$–CH_2–NH), 4.51 (s, 2H, C$\underline{H}_2$–N–CO), 6.98 (bs, 1H, N$\underline{H}$–CO–CF_3), 7.01 (dd, J_1 = 8.9, J_2 = 3.1 Hz, 1H, Ar–H), 7.37 (d, J = 8.7 Hz, 1H, Ar–H), 7.48 (d, J = 3.1 Hz, 1H, Ar–H), 9.84 (s, 1H, N$\underline{H}$–CO-thiophene) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.07, 28.39, 31.45, 36.56, 39.18, 66.27, 80.65, 94.18, 113.65, 114.32, 116.95, 117.18, 119.95, 122.99, 131.64, 131.98, 147.04, 154.54, 157.21, 157.57 (q, $^2J_{C,F}$ = 37.4 Hz), 161.82, 162.62 ppm. ESI HRMS m/z calcd. for $[M + (H)]^+$ 573.1186, found 573.1201. IR (KBr) ν_{max} 3261, 2982, 2935, 2217, 1666, 1545, 1459, 1416, 1367, 1287, 1237, 1210, 1153, 1118, 1063, 1038, 1005 cm^{-1}. HPLC t_R = 16.299 min (100% at 220 nm, 100% at 254 nm).

*4.3.17. 2-(2-Chloro-5-(2-(2,2,2-trifluoroacetamido)ethoxy) benzamido)-3-cyano-4,5,6,7-tetra-hydrothieno [2,3-c]pyridin-6-ium trifluoroactetate (**34**)*

To a solution of **33a** (2.13 g, 3.72 mmol) in DCM (30 mL), CF_3COOH (8.60 mL, 112 mmol) was added and stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the residue was dried in a desiccator overnight.

Yield = 96%; orange crystals, mp = 90–92 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.07 (t, J = 6.2 Hz, 2H, C$\underline{H}_2$–CH_2–N), 3.60 (t, J = 6.2 Hz, 2H, CH_2–C$\underline{H}_2$–N), 3.81 (q, J = 5.1 Hz, 2H, O–CH_2–C$\underline{H}_2$–NH), 4.16 (t, J = 5.1 Hz, 2H, O–C$\underline{H}_2$–CH_2–NH), 4.39 (s, 2H, C$\underline{H}_2$–NH_2^+), 6.71 (t, J = 5.7 Hz, 1H, N$\underline{H}$–CO–CF_3), 7.07 (dd, J_1 = 8.8, J_2 = 3.1 Hz, 1H, Ar–H), 7.44 (d, J = 8.8 Hz, 1H, Ar–H), 7.56 (d, J = 3.1 Hz, 1H, Ar–H), 10.00 (s, 1H, N$\underline{H}$–CO-thiophene) ppm. ^{13}C NMR (100 MHz, MeOD): δ 22.26, 27.78, 40.29, 42.35, 42.76, 67.46, 95.33, 113.83, 116.08, 116.57, 118.94, 119.78, 121.38, 124.03, 130.43, 132.32, 135.79, 149.83, 158.83, 161.78 (q, J = 36.8 Hz), 166.53 ppm. ESI HRMS m/z calcd. for $[M + (H)]^+$ 473.0662, found 473.0668. IR (KBr) ν_{max} 2992, 2842, 2221, 1711, 1667, 1547, 1462, 1419, 1292, 1135, 1067, 1039 cm^{-1}. HPLC t_R = 9.914 min (96.6% at 220 nm, 97.1% at 254 nm).

*4.3.18. General procedure for synthesis of compounds **35**, **36** and **38a–f***

To a solution of **33b**, **34** or **37a–f** (0.17 mmol) in methanol (2 mL), 1 M NaOH (2 mL) was added. The reaction mixture was stirred overnight. The methanol was evaporated and the residue was diluted with water (20 mL). The water phase was extracted with EtOAc (3 × 20 mL) and the combined organic phases were washed with water (30 mL), brine (30 mL) and dried with Na_2SO_4. The solvent was evaporated under reduced pressure and the residue was purified by flash chromatography.

*4.3.18.1. 5-(2-Aminoethoxy)-2-chloro-N-(3-cyano-6-(4-hydroxyphenyl)-4,5,6,7-tetrahydro benzo[b]thiophen-2-yl)benzamide (**35**).* Yield = 100%; white crystals, mp = 162–164 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.81–1.90 (m, 1H, CH–C$\underline{H}_A$H$_B$–CH_2), 1.97–2.02 (m, 1H, CH–CH_AH$_B$–CH_2), 2.61–2.68 (m, 3H, CH_2–C$\underline{H}$–CH_2–C$\underline{H}_2$), 2.80–2.92 (m, 2H, C$\underline{H}_2$–CH), 3.22 (t, J = 4.8 Hz, 2H, O–CH_2–C$\underline{H}_2$–NH_2), 4.22 (t, J = 4.8 Hz, 2H, O–C$\underline{H}_2$–CH_2–NH_2), 6.72 (d, J = 8.4 Hz, 2H, Ar–H), 7.09–7.13 (m,, 3H, 3 × Ar–H), 7.24 (d, J = 3.2 Hz, 1H, Ar–H), 7.45 (d, J = 8.8 Hz, 1H, Ar–H), 9.25 (bs, 1H, NH–CO) ppm. ^{13}C NMR (100 MHz, pyridine-d_6): δ 26.76, 32.06, 34.27, 41.92, 42.18, 69.31, 97.49, 116.97, 117.96, 118.38, 120.29, 125.17, 130.44, 130.72, 133.03, 133.68, 138.26, 138.39, 149.53, 159.40, 159.57, 167.05 ppm. ESI HRMS calcd. for $[M + (H)]^+$ 468.1149, found 468.1143. IR (KBr) ν_{max}: 3549, 3475, 3413, 3237, 3084, 3012, 2922, 2224, 2032, 1677, 1656, 1638, 1617, 1574, 1556, 1514, 1460, 1408, 1384, 1317, 1296, 1234, 1201, 1178, 1156, 1139, 1074, 1017 cm^{-1}. HPLC t_R = 11.363 min (100% at 220 nm, 100% at 254 nm).

*4.3.19. General procedure for synthesis of compounds **37a–f***

Method A: To a solution of **34** (0.30 g, 0.511 mmol) in DMF (3 mL), Et_3N (1.5 mmol) was added. After 10 min of stirring at room temperature, the corresponding benzyl bromide (0.766 mmol) was added. The reaction mixture was stirred overnight at room temperature. DMF was then evaporated, the crude residue was dissolved in EtOAc (20 mL) and washed with water (3 × 10 mL), brine (10 mL) and dried with Na_2SO_4. The crude product was purified by flash chromatography.

Method B: To a solution of **34** (0.50 g, 0.852 mmol) in THF (5 mL), the corresponding benzaldehyde (1.28 mmol) and $Na(OAc)_3BH$ (0.45 g, 2.13 mmol) were added, and the reaction mixture was stirred overnight at room temperature. To quench the reaction, saturated aqueous $NaHCO_3$ (10 mL) was used, and the water phase was extracted with EtOAc (3 × 20 mL). The combined organic phases were washed with brine (30 mL) and dried with Na_2SO_4. The crude residue was purified by flash chromatography.

*4.3.19.1. N-(6-Benzyl-3-cyano-4,5,6,7-tetrahydrothieno [2,3-c]pyridin-2-yl)-2-chloro-5-(2-(2,2,2-trifluoroacetamido)ethoxy)benzamide (**37a**).* Yield = 35%; pale brown crystals, mp = 71–73 °C; ^{1}H NMR (400 MHz, acetone-d_6): δ 2.68 (t, J = 5.6 Hz, 2H, C$\underline{H}_2$–CH_2–N), 2.85 (t, J = 5.6 Hz, 2H, CH_2–C$\underline{H}_2$–N), 3.61 (s, 2H, CH_2–N), 3.76–3.79 (m, 4H, N–C$\underline{H}_2$-Ar + O–CH_2–C$\underline{H}_2$–NH), 4.26 (t, J = 5.1 Hz, 2H, O–C$\underline{H}_2$–CH_2–NH), 7.11 (dd, J_1 = 8.9, J_2 = 3.1 Hz, 1H, Ar–H), 7.25–7.29 (m, 2H, 2 × Ar–H), 7.33–7.37 (m, 2H, 2 × Ar–H), 7.39–7.44 (m, 3H, 3 × Ar–H), 8.79 (bs, 1H, N$\underline{H}$–CO–CF_3), 11.01 (s, 1H, N$\underline{H}$–CO-thiophene) ppm. ^{13}C NMR (100 MHz, acetone-d_6): δ 24.98, 40.04, 50.15, 51.41, 62.04, 67.18, 95.27, 114.08, 115.69, 116.38, 118.54, 119.28, 132.30, 127.55, 128.05, 129.21, 129.77, 131.14, 131.85, 135.97, 139.53, 147.26, 158.29, 164.51 ppm. ESI HRMS m/z calcd. for $[M + (H)]^+$ 563.1132, found 563.1137. IR ν_{max} 3319, 3081, 2923, 2807, 2214, 1714, 1667, 1545, 1456, 1405, 1366, 1288, 1209, 1154, 1057, 1036, 1007 cm^{-1}. HPLC t_R = 11.907 min (100% at 220 nm, 100% at 254 nm).

4.4. Molecular docking studies

A docking study was performed using the crystal structure of MurF enzyme from *S. pneumoniae* (PDB code: 3zm5) and OEDocking software (Release 3.0.1, OpenEye Scientific Software, Inc.).

4.4.1. Ligand preparation

The molecules were built with ChemBioDraw Ultra 12.0 (CambridgeSoft). The ligand geometries were optimized with ChemBio 3D Ultra 12.0 (CambridgeSoft) using MM2 force field until a minimum 0.100 root mean square (RMS) gradient was reached. The optimized structure was refined with GAMESS interface using the semi-empirical AM1 method, QA optimization algorithm and Gasteiger Hückel charges for all atoms for 100 steps. Amines and guanidines were kept in their ionized state, corresponding to pH 7.4. FRED requires a set of input conformers for each ligand, which were generated with OMEGA (OMEGA version 2.4.6. OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com), with maximum number of conformations set to 1000, and using the RMS cut-off value (Root Square (RMS) cartesian distance below

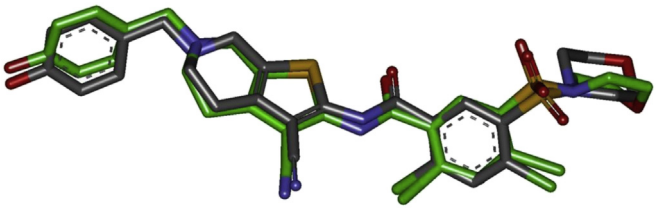


Fig. 3. Crystal structure of the ligand (2,4-dichloro-*N*-[3-cyano-6-[(4-hydroxyphenyl) methyl]-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridin-6-ium-2-yl]-5-morpholino sulfonyl-benzamide, shown as green sticks) and top docked pose of the ligand, as predicted by FRED (shown as stick coloured by atom type). The top docked ligand pose had an RMSD of 1.15 Å. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which two conformers are duplicates) of 0.3 Å. All the other options were left as default values [32,33].

4.4.2. Receptor preparation & docking protocol

The crystal structure of MurF enzyme from *S. pneumoniae* in complex with the ligand (PDB code: 3zm5) was taken as a starting-point. The ligand (2,4-dichloro-*N*-[3-cyano-6-[(4-hydroxyphenyl) methyl]-4,5,6,7-tetrahydrothieno [2,3-*c*]pyridin-6-ium-2-yl]-5-morpholinosulfonyl-benzamide) was taken as a reference structure and a grid box including all active-site atoms (including hydrogens) with a volume of 32562 Å, dimensions of 36.22 Å × 36.33 Å × 24.67 Å, and outer contour of 2953 Å, was created around it as a "docking receptor" using Make Receptor 3.0.1.

The docking software FRED (OEDocking version 3.0.1. OpenEye Scientific Software, Santa Fe, NM. http://www.eyesopen.com) was used for docking studies with the default settings, except for dock resolution, which was set to "high", and number of poses, which was set to 50 [34,35]. The proposed 10 binding modes with the highest rank of the docked inhibitors were evaluated using final score and root mean square deviation (RMSD) as a tool to explore relative structural differences between proposed binding modes. The graphical representations of the proposed binding positions of all the molecules were obtained using Accelrys Discovery Studio 2.5 (Accelrys Software Inc.).

4.4.3. Validation of the docking protocol

The docking procedure should be able to correctly predict the binding poses of the molecules in the PDB database *i.e.*, the crystal structures of ligands in complex with proteins. We have attempted to reproduce the pose of the ligand as seen in its X-ray complex with the target MurF enzyme from *S. pneumoniae* (PDB code: 3zm5). The top 10 docking poses of the docked ligand were within 1.5 Å RMSD of the ligand crystal structure. The predicted top score pose was the one that best fitted the crystal structure of the ligand, overlapping it almost completely, which offers the proof of the protocol validation (Fig. 3).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.11.031.

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