Bioorganic & Medicinal Chemistry 18 (2010) 1617–1625

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

The first low μM SecA inhibitors

Weixuan Chen [a], Ying-ju Huang [b], Sushma Reddy Gundala [b], Hsiuchin Yang [b], Minyong Li [a], Phang C. Tai [b,*], Binghe Wang [a,*]

[a] Department of Chemistry, Georgia State University, Atlanta, GA 30302-4098, USA
[b] Department of Biology, Georgia State University, Atlanta, GA 30302-4098, USA

ARTICLE INFO

Article history:
Received 7 October 2009
Revised 21 December 2009
Accepted 31 December 2009
Available online 6 January 2010

Keywords:
SecA inhibitor
2-Thiouracil

ABSTRACT

SecA ATPase is a critical member of the Sec family, which is important in the translocation of membrane and secreted polypeptides/proteins in bacteria. Small molecule inhibitors can be very useful research tools as well as leads for future antimicrobial agent development. Based on previous virtual screening work, we optimized the structures of two hit compounds and obtained SecA ATPase inhibitors with IC_{50} in the single digit micromolar range. These represent the first low micromolar synthetic inhibitors of bacterial SecA and will be very useful for mechanistic studies.

1. Introduction

With the rapid emergence of drug resistant bacteria, there is an urgent need for the development of new antimicrobial agents, especially those with a unique mechanism of action. With this ultimate goal in mind, we are interested in the development of inhibitors of bacterial protein translocation. Several protein transport mechanisms exist in bacteria.[1] Among them, the Sec machinery (or translocase) provides a major pathway of protein translocation from the cytosol across or into the cytoplasmic membrane. The Sec machinery has seven proteins including SecA, SecD, SecE, SecF, SecG, SecY, and YajC. Assembly and complex formation are required to yield the functional translocase. Among the Sec proteins, SecA is found both in the cytoplasm and bound to the inner membrane. When SecA is bound to the SecYEG complex, acidic phospholipids and a precursor protein such as proOmpA (the precursor of outer membrane protein A), it becomes fully active as an ATPase and a protein translocase.[2,3] Recently, several seminal papers described in intricate details as to how the SecA machinery functions in transporting proteins.[4–6]

It has been said that in any given organism, membrane and secreted polypeptides/proteins comprise more than 30% of the proteome; and no less than 10% of proteins cross a membrane before arriving at their final locations of function.[7,8] Such actions are often mediated by protein translocases. Therefore SecA is essential for bacterial survival. We envision that inhibitors of SecA can be very useful tools for studying bacterial protein transport and potential antimicrobial agents, especially because SecA has no human counterpart. We have previously reported effort in using virtual screening against the *Escherichia coli* SecA crystal structure[9] to search for possible structural features suitable for SecA inhibitor development.[10] In this paper, we describe our effort in optimizing the structural features of the initial hits for the development of bacterial SecA inhibitors. Several low μM inhibitors have been found. Considering the fact that currently inorganic azide, which is a SecA inhibitor with an IC_{50} value of about 3 mM, has cross reactivities against a number of enzymes,[11,12] and is the primary research tool for probing bacterial protein translocation, the newly discovered SecA inhibitors will be very important.

2. Results and discussions

2.1. Chemistry

In our earlier virtual screening efforts, two hits, **1** (SEW-05929) and **2** (HTS-12302), were shown to have modest SecA inhibitory activities (IC_{50} values of about 100 μM).[10,13] Since there were no other known SecA inhibitors except one natural product, for which the true inhibition mechanism was not known,[14] our effort to search for potent SecA inhibitors started with the optimization of these two modest inhibitors (Fig. 1).

Our optimization effort first started with the isoxazole carboxamide series (**1**) with the focus being on optimizing the aryl group attached to the amide. In this series, 14 analogs were synthesized. The synthesis started with conversion of halogenated benzaldehyde **3** to the corresponding oxime **4** (Scheme 1). Isoxazole acid **6** was prepared by reacting **5** with ethyl acetoacetate followed by hydrolysis.[15] Subsequent coupling/amidation reactions using EDCI

* Corresponding authors. Tel.: +1 404 413 5303; fax: +1 404 413 5301 (P.C.T.); tel.: +1 404 413 5544; fax: +1 404 413 5543 (B.W.).
E-mail addresses: biopct@langate.gsu.edu (P.C. Tai), wang@gsu.edu (B. Wang).

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.12.074

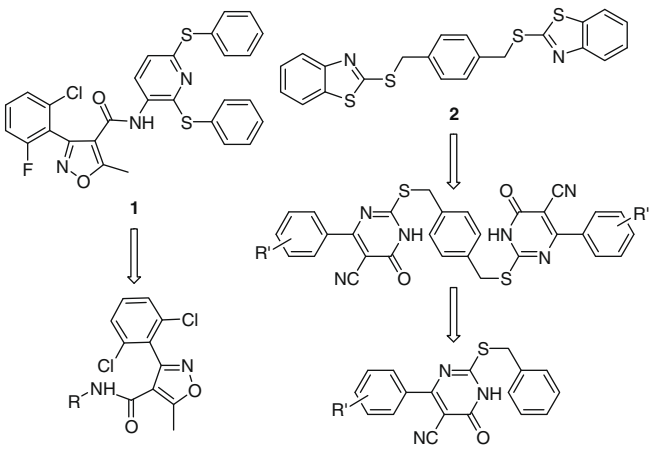


Figure 1. Two hit compounds and their derivatives.

and DMAP gave the final isoxazole carboxamide derivatives **7a–7n**. In this series, there were amides of aniline compounds **7a–g**, primary alkylamines **7h,i**, secondary alkyamines **7j–l**, and benzylamines **7m,n**.

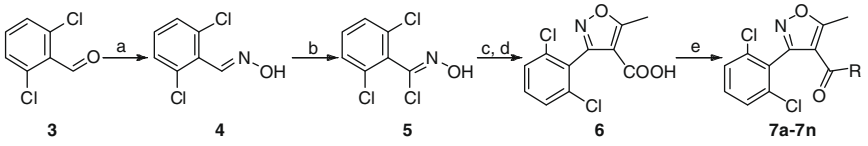
In optimizing the second series (**2,** Fig. 1), we first started by testing different aryl structures flanking the central ring. In our initial effort, 6-chloro-2-mercaptobenzothiazole and 2-mercaptobenzoxazole derivatives were prepared by reacting potassium ethylxanthate **8** with 2,4-dichloroaniline **9** or substituted 2-aminophenol **10** (Scheme 2). Further, 5-cyano-6-aryl-2-thiouracils were prepared 'by condensation of an aldehyde with ethyl cyanoacetate and thiourea in the presence of piperidine'.[16] The symmetrical compounds **15a–g** or **16a–i** were obtained by reacting 2 equiv of compounds **11a–g** or **14a–i** with *p*-xylylene dibromide in acetonitrile in the presence of K_2CO_3 (Scheme 3). One successful series of analogs was the 2,2′-(α,α′-xylene)bis(sulfanediyl)bis-(6-aryl-5-cyano-4-oxopyrimidine) **16a–i** (see below for biological results). For this series, we were interested in further simplifying the structure to understand the core structural need. Therefore, 'monomer' series **17d,e,g–i** was prepared by benzylation of compounds **14d,e,g–i** with benzyl chloride, and the difference in activities between the 'dimer' and 'monomer' series was also studied.

2.2. Biological evaluation

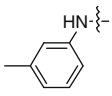
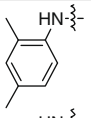
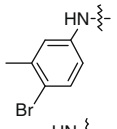
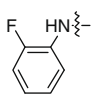
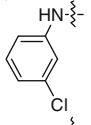
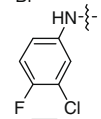
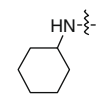
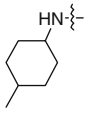
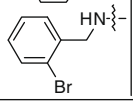
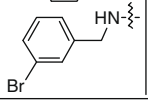
2.2.1. In vitro study

The synthesized compounds were first evaluated using EcN68 SecA, which is a truncated version without the C-terminal regulatory/inhibitory domain, by following procedures published earlier.[10] Briefly, ATPase activities were determined by the release of phosphate (Pi), which can be detected spectrophotometrically using malachite green.[14] For compounds **7a–n,** none of them showed improved activities over the original hit (**1**) or significant inhibition at 100 μM (Fig. 2). Such results coupled with the weak activities of the original hit compound led to the decision of not pursuing this class of compounds any further.

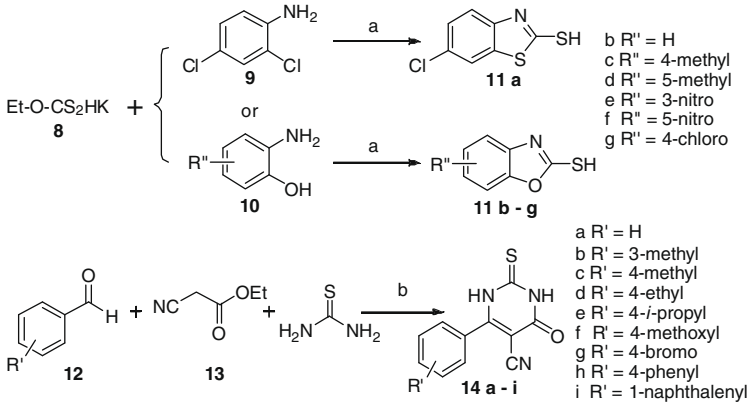
For analogs of **2,** compounds **15a–g** did not show significant improvement over the initial hit (data not shown). However, the substituted thiouracils (**16**) showed very significant activities when screened at 100 (data not shown) and 30 μM (Fig. 3). Those compounds that showed potent inhibition at 30 μM were further screened at 5 μM (Fig. 4). Within the symmetrical compound series 16a–**i**, there were two substitution patterns: one with a phenyl ring substituted at the 4-position and the other with a phenyl ring substituted at the 3-position. The results showed that the 4-substituted analogs were more potent than the 3-substituted class, which was in turn slightly more potent than the ones without phenyl substituent. For example the activities of the 4-methyl substituted (**16c,** Fig. 3) was higher than the 3-methyl analog (**16b,** Fig. 3), which was in turn higher than the unsubstituted one (**16a,** Fig. 3). With the initial indication that derivatives with a phenyl ring bearing a 4-substituent were more active, the subsequent effort was focused on optimizing this series of compounds. One approach adopted was to use relatively bulky alkyl groups at the



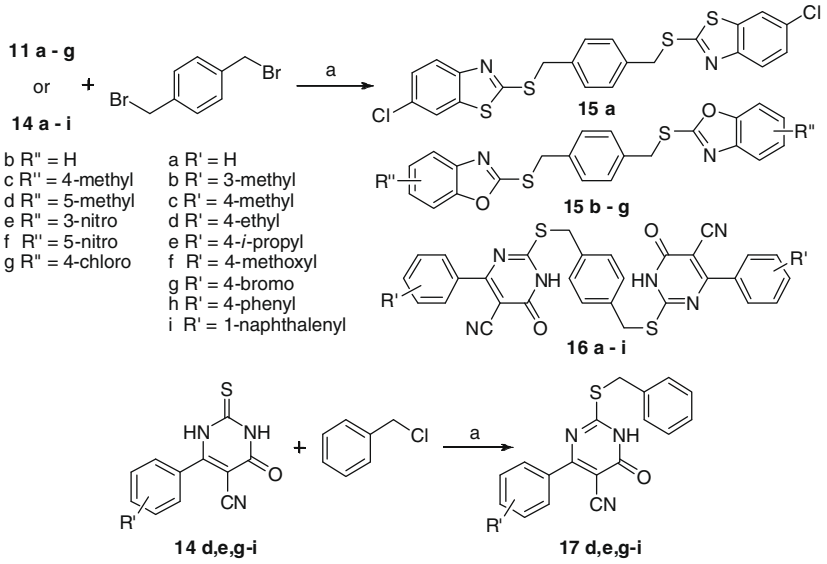
3 **4** **5** **6** **7a-7n**

Compound	R	Compound	R	Compound	R
7a		**7b**		**7c**	
7d		**7e**		**7f**	
7g		**7h**		**7i**	
7j		**7k**		**7l**	
7m		**7n**			

Scheme 1. Synthesis of isoxazole carboxamides **7a–n**. Reagents and conditions: (a) $HONH_2$·HCl, NaOH, EtOH, H_2O, reflux; (b) NCS, DMF; (c) ethyl acetoacetate, MeONa, THF; (d) NaOH, EtOH, H_2O; (e) EDCI, HOBt, DMAP, DMF.



Scheme 2. Synthesis of compounds **11a–g** and **14a–i**. Reagents: (a) EtOH, reflux; (b) piperidine, EtOH, reflux.



Scheme 3. Synthesis of compounds **15a–g**, **16a–i** and **17d,e,g–i**. Reagents: (a) K_2CO_3, CH_3CN, reflux.

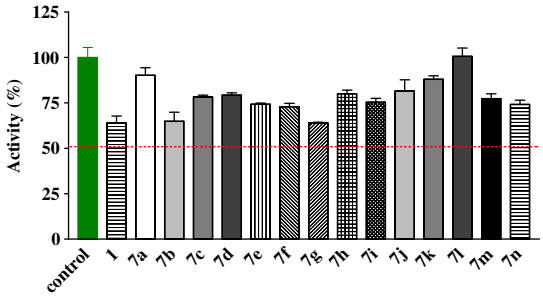


Figure 2. Inhibitory effect of compounds **1** and **7a–n** at 100 μM against EcN68 Sec A.

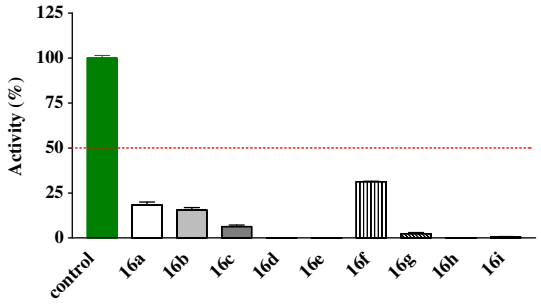


Figure 3. Inhibitory effect of compounds **16a–i** at 30 μM against EcN68 Sec A.

4-position. It turned out that these compounds were more potent than the corresponding 4-methyl substituted compounds. Among these compounds, those with an electron donating (e.g., methoxy) substituent seemed to be less active than the unsubstituted ones (e.g., **16f < 16a,** Fig. 3). At 5 μM, analogs with a halogen or aryl group substitution at the 4-position were more potent than the analogues with an alkyl substitution (e.g., **16g,h,i > 16c,d,e** Fig. 4). For the examination of the difference between the 'dimers' and 'monomers', *S*-benzyl-2-thiouracils analogues **17d,e,g–i** were also tested (Fig. 5). First of all, both thiouracil-based 'dimer' and 'monomer' compounds showed more potent inhibition than the benzothiazole or benzoxazole compounds **15a–g**. However, the 'dimer' series **16d,e,g–i** were more potent than the 'monomer' series **17d,e,g–i**, respectively. This higher potency for the "dimer" series seems to come from better fitting of the binding pocket of these compounds (see below). In the 'monomer' series, it was observed that a large sized R′ group seemed to confer high potency (e.g., **17h > 17g ≈ 17e > 17d**). However, when the substituent phenyl

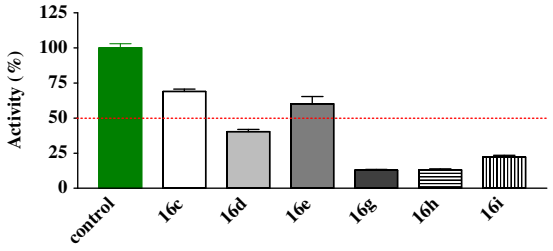


Figure 4. Inhibitory effect of compounds **16c–e,g–i** at 5 μM against EcN68 Sec A.

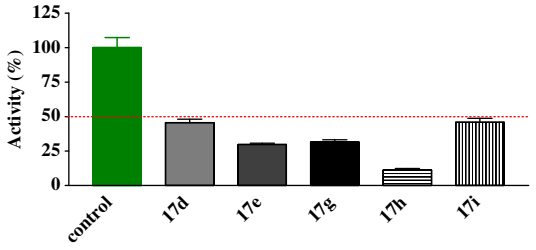


Figure 5. Inhibitory effect of compounds **17d,e,g–i** at 30 μM against EcN68 Sec A.

ring was replaced by a larger 1-naphthyl group, the activity seemed to decrease slightly.

We determined the IC_{50} values of compound **16g** and **16h** since they showed the most potent activities of all the compounds screened at 5 μM. The result showed they had low micro molar inhibition (IC_{50}: 2 μM, Fig. 6), which is 50-fold more potent than the hit compound **2** (IC_{50}: 100 μM).[10]

Inhibition tests using whole EcSecA gave similar results (**16g** IC_{50}: 20 μM, **16h** IC_{50}: 50 μM and **17h** IC_{50}: 60 μM, Fig. 7), which suggested that the EcN68 inhibition assay was more sensitive than the whole SecA inhibition assay. This is understandable since EcSecA contains a regulatory domain, which is essentially an inhibitor.

2.2.2. In vivo study

The biological activities of 'dimer' and 'monomer' compounds **16h** and **17h** were assessed against leaky mutant NR698 and wild type *E. coli* strain MC4100 by determining the minimum inhibition concentration (MIC) (Fig. 8). 'Monomer' compound **17h** exhibited the most potent inhibition effects against NR698, whereas 'dimer' compounds **16h** did not exhibit significantly antimicrobial activities. However, neither **17h** nor **16h** exhibited inhibition effects against wild type *E. coli* strain MC4100. Such results suggested that the permeability of **16h** against NR698 and **17h** against MC4100

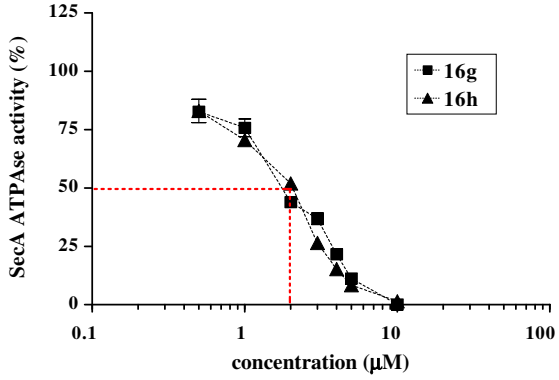


Figure 6. The inhibitory curves of the two most potent compounds, **16g** and **16h**, against EcN68 Sec A.

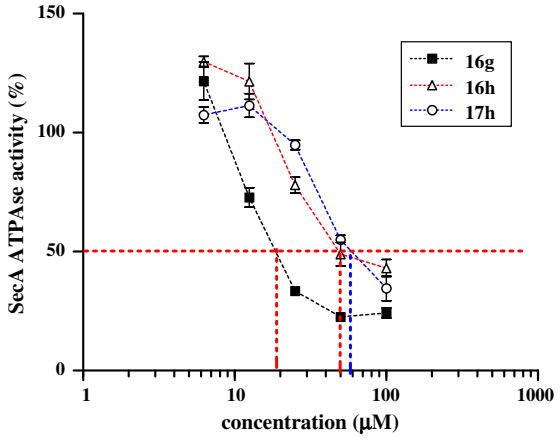


Figure 7. The inhibitory curves of **16g,h** and **17h** against EcSecA.

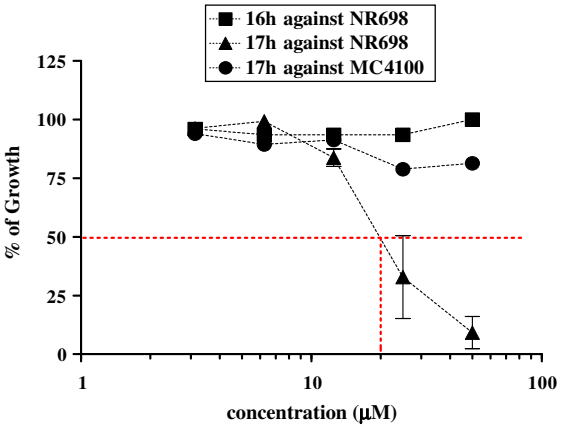


Figure 8. The inhibitory curves of **16g,h** and **17h** against bacterial growth.

might be a key factor and for in vivo applications future studies should focus on low molecular weight compounds such as **17h** for structural optimization.

2.3. Computational modeling

In order to achieve a detailed understanding the binding mode between SecA and our compounds, in silico modeling was conducted by using molecular simulation.[17–20] Herein, the parent compound, HTS-12302 and the most active compound, **16g**, were docked into the ATP-site of SecA using DOCK 5.4. The docked complexes were then optimized by molecular mechanics and molecular dynamics simulation implemented in AMBER 8. Finally, the possible ligand–protein interactions were examined by following similar procedures we used in previous studies.[10] After molecular simulation, compound HTS-12302 seems to bind SecA through interactions with Thr 104 by forming hydrogen bond and with Met 81, Phe 84, Gln 87, Gly 105, Glu 106, Gly 107, Lys 108, Thr 109, Leu 110, Gly 392 and Arg 509 through hydrophobic interactions (Fig. 9). Compound **16g** has a similar binding conformation and orientation, in which it seems to engage in more hydrogen bond interactions with Phe 84, Gln 87, Lys 108 and Glu 210. Moreover, compound **16g** still bears hydrophobic interactions with Met 81, Thr 104, Gly 105, Glu 106, Gly 107, Leu 110 and Arg 509. Upon analysis of the structural features of these two compounds, it seems that the inclusion of the thiouracil moiety may contribute to the inhibitory activity because of more hydrophobic interaction and hydrogen bonds when compared with lead compound

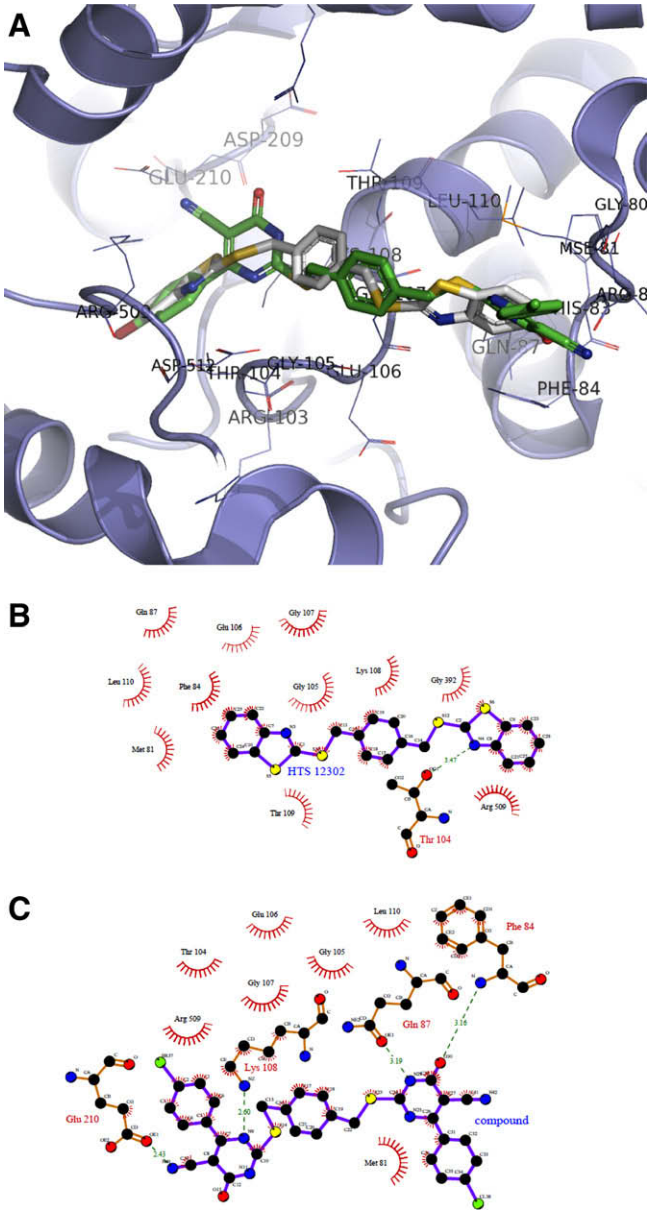


Figure 9. (A) The proposed docking conformation of HTS-12302 (white sticks) and compound **16g** (green sticks) around SecA ATP-site; (B) the proposed schematic interactions of HTS-12302 with SecA; and (C) the proposed schematic interactions of compound **16g** with SecA.

HTS-12302. Such structural insights will play a very critical role in future design of potent SecA inhibitors and in further structural optimizations.

2.4. Conclusion

Through optimization of two hit compounds **1** (SEW-05929) and **2** (HTS-12302) identified from virtual screening, we have found a series of thiouracil derivatives that are much more potent than the primary hits. The two most potent compounds, **16g** and **16h**, are 50-fold more active than the hit compounds. Results of antimicrobial tests suggest that future work should focus on low molecular weight analogs of **17h** for in vivo applications. These compounds are the first in its class and should be very useful as research tools in studying bacterial protein transport. The new inhibitory structural features identified should also be very useful for further structural optimization in search of even more potent inhibitors as potential antimicrobial agents.

3. Experimental

3.1. Chemistry

3.1.1. General chemical methods

All reagents and solvents were reagent grade or were purified by standard methods before use. Column chromatography was carried out on flash silica gel (Sorbent 230–400 mesh). TLC analysis was conducted on silica gel plates (Sorbent Silica G UV254). NMR spectra were recorded at ^{1}H (400 MHz) and ^{13}C (100 MHz) with a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz, respectively, using TMS (^{1}H NMR) and solvents (^{13}C NMR) as internal standards.

3.1.2. General procedure for the preparation of isoxazole carboxamide derivatives (7a–7n)

Under N_2 atmosphere, a solution of an isoxazole carboxylic acid (**6**, 0.1 mmol), amine (0.12 mmol), EDCI (23 mg, 0.12 mmol), DMAP (14.7 mg, 0.12 mmol) and HOBt (27 mg, 0.2 mmol) in DMF (2.5 mL) was stirred at room temperature overnight. Then most of the solvent was removed under reduced pressure. To the residue was added 10 mL H_2O and 10 mL EtOAc. Then the aqueous solution was extract by EtOAc (20 mL × 2). The organic layer was subsequently washed with brine (20 mL). The crude compound was purified by flash chromatography on silica gel using hexane and EtOAc (9:1) as the mobile phase to give **7a–7n**.

3.1.2.1. 3-(2,6-Dichlorophenyl)-5-methyl-N-m-tolylisoxazole-4-carboxamide (7a). Yield 76%; ^{1}H NMR ($CDCl_3$) δ 1.70 (s, 3H), 2.86 (s, 3H), 6.74 (br s, 1H), 7.00–7.07 (m, 2H), 7.18 (td, 1H, J = 1.6 Hz, 7.2 Hz), 7.44 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.50 (m, 2H), 7.90 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.7, 16.9, 112.0, 123.0, 125.4, 127.0, 127.3, 128.3, 129.1, 130.6, 132.7, 135.3, 136.6, 155.7, 159.0, 176.4. HRMS-ESI (+): Calcd for $C_{18}H_{15}N_2O_2Cl_2$: 361.0511. Found: 361.0527 $[M+H]^+$.

3.1.2.2. N-(3-Bromobenzyl)-3-(2,6-dichlorophenyl)isoxazole-4-carboxamide (7b). Yield 71%; ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H), 2.84 (s, 3H), 6.85 (m, 3H), 7.11 (m, 2H), 7.48 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.54 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 13.5, 21.7, 112.1, 117.1, 120.8, 125.8, 127.3, 129.0, 129.0, 132.7, 136.4, 137.2, 139.3, 155.9, 158.9, 175.7. HRMS-ESI (+): Calcd for $C_{18}H_{15}N_2O_2Cl_2$: 361.0511. Found: 361.0516 $[M+H]^+$.

3.1.2.3. (3-(2,6-Dichlorophenyl)-5-methylisoxazol-4-yl)(morpholino)methanone (7c). Yield 81%; ^{1}H NMR ($CDCl_3$) δ 1.63 (s, 3H), 2.18 (s, 3H), 2.80 (s, 3H), 6.62 (br s, 1H), 6.82 (s, 1H), 6.92 (d, 1H, J = 8.4 Hz), 7.37 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.44 (m, 2H), 7.66 (d, 1H, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$) δ 13.7, 16.9, 21.0, 112.0, 123.2, 127.4, 127.5, 128.6, 129.0, 131.2, 132.7, 135.3, 136.6, 155.7, 159.0, 176.2. HRMS-ESI (+): Calcd for $C_{19}H_{17}N_2O_2Cl_2$: 375.0667. Found: 375.0679 $[M+H]^+$.

3.1.2.4. (3-(2,6-Dichlorophenyl)-5-methylisoxazol-4-yl)(piperidin-1-yl)methanone (7d). Yield 67%; ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 3H), 2.78 (s, 3H), 6.70 (dd, 1H, J = 2.4 Hz, 8.4 Hz), 6.75 (br s, 1H), 7.12 (d, 1H, J = 2.4 Hz), 7.29 (d, 1H, 8.8 Hz), 7.43 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.49 (m, 2H), 7.62 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.6, 23.3, 111.9, 118.8, 120.1, 122.2, 127.2, 129.0, 132.8, 132.8, 136.3, 136.5, 139.0, 155.8, 158.8, 175.9. HRMS-ESI (+): Calcd for $C_{18}H_{14}N_2O_2Cl_2Br$: 438.9616. Found: 438.9633 $[M+H]^+$.

3.1.2.5. 3-(2,6-Dichlorophenyl)-N-(2,4-dimethylphenyl)-5-methylisoxazole-4-carboxamide (7e). Yield 55%; ^{1}H NMR ($CDCl_3$) δ 2.87 (s, 3H), 6.92–6.99 (m, 2H), 7.08 (t, 1H, J = 7.6 Hz), 7.20 (br s, 1H), 7.47 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.52 (m, 2H), 8.33 (td, 1H, J = 1.6 Hz, 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.7, 111.8, 114.7, 114.9, 121.6, 124.7, 124.8, 124.8, 124.8, 126.3, 129.1, 132.8, 136.3, 153.4, 155.9, 158.8, 176.4. HRMS-ESI (+): Calcd for $C_{17}H_{12}N_2O_2Cl_2F$: 365.0260. Found: 365.0269 $[M+H]^+$.

3.1.2.6. N-(3-Chlorophenyl)-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (7f). Yield 42%; ^{1}H NMR ($CDCl_3$) δ 2.79 (s, 3H), 6.81 (br s, 1H), 6.84 (m, 1H), 6.98 (m, 1H), 7.09 (t, 1H, J = 8.0 Hz), 7.30 (t, 1H, J = 1.6 Hz), 7.45(dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.50 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 90.2, 113.8, 128.2, 128.6, 132.0, 158.2, 160.2, 176.0. HRMS-ESI (+): Calcd for $C_{17}H_{12}N_2O_2Cl_3$: 380.9964. Found: 380.9962 $[M+H]^+$.

3.1.2.7. N-Cyclohexyl-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (7g). Yield 59%; ^{1}H NMR ($CDCl_3$) δ 2.83 (s, 3H), 6.83 (br s, 1H), 6.87 (m, 1H), 6.98 (t, 1H, J = 8.8 Hz), 7.42 (dd, 1H, J = 2.8 Hz, 6.8 Hz), 7.50 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.55 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 13.6, 111.7, 116.8, 117.0, 119.6, 119.7, 121.4, 121.6, 122.4, 127.1, 129.1, 132.9, 133.8, 136.3, 154.0, 155.7, 156.4, 158.9, 176.1. HRMS-ESI (+): Calcd for $C_{17}H_{11}N_2O_2FCl_3$: 398.9870. Found: 398.9885 $[M+H]^+$.

3.1.2.8. 3-(2,6-Dichlorophenyl)-5-methyl-N-o-tolylisoxazole-4-carboxamide (7h). Yield 79%; ^{1}H NMR ($CDCl_3$) δ 0.83 (m, 2H), 1.04 (m, 1H), 1.19–1.32 (m, 4H), 1.39 (m, 1H), 2.02 (m, 2H), 2.73 (s, 3H), 3.74 (m, 1H), 5.02 (m, 1H), 7.39 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.45 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 13.3, 24.1, 25.5, 32.5, 47.4, 112.0, 127.8, 128.7, 132.4, 136.3, 156.1, 159.9, 174.7. HRMS-ESI (+): Calcd for $C_{17}H_{19}N_2O_2Cl_2$: 353.0824. Found: 353.0838 $[M+H]^+$.

3.1.2.9. N-(4-Bromo-3-methylphenyl)-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (7i). Yield 82%; ^{1}H NMR ($CDCl_3$) δ 0.28 (m, 2H), 0.65–0.70 (m, 4H), 0.81 (d, 2H, J = 6.8 Hz), 0.94 (m, 2H), 1.16–1.25 (m, 2H), 1.31–1.44 (m, 4H), 1.49–1.58 (m, 4H), 1.75 (m, 2H), 3.63 (m, 1H), 4.10 (m, 1H), 7.39–7.51 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.2, 13.4, 22.2, 22.3, 29.6, 29.8, 31.5, 31.9, 32.9, 33.6, 44.3, 48.3, 111.8, 112.0, 127.7, 127.9, 128.7, 128.9, 132.3, 132.6, 136.2, 136.4, 156.0, 156.2, 159.8, 160.0, 174.5, 175.3. HRMS-ESI (+): Calcd for $C_{18}H_{21}N_2O_2Cl_2$: 367.0980. Found: 367.0991 $[M+H]^+$.

3.1.2.10. (3-(2,6-Dichlorophenyl)-5-methylisoxazol-4-yl)(thiomorpholino)methanone (7j). Yield 85%; ^{1}H NMR ($CDCl_3$) δ 1.34 (br s, 4H), 1.52 (m, 2H), 2.56 (s, 3H), 3.38 (br s, 4H), 7.32 (m, 1H), 7.40 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 12.4, 24.4, 25.9, 113.6, 127.7, 128.4, 131.5, 135.9, 157.5, 161.6, 169.6. HRMS-ESI (+): Calcd for $C_{16}H_{17}N_2O_2Cl_2$: 339.0667. Found: 339.0668[M+H]$^+$.

3.1.2.11. 3-(2,6-Dichlorophenyl)-N-(2-fluorophenyl)-5-methylisoxazole-4-carboxamide (7k). Yield 84%; ^{1}H NMR ($CDCl_3$) δ 2.52 (s, 3H), 3.36 (br s, 8H), 7.30 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.37 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 12.6, 66.7, 112.8, 127.4, 128.6, 131.8, 135.9, 157.3, 161.9, 170.4. HRMS-ESI (+): Calcd for $C_{15}H_{15}N_2O_3Cl_2$: 341.0460. Found: 341.0466 $[M+H]^+$.

3.1.2.12. N-(3-Chloro-4-fluorophenyl)-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (7l). Yield 72%; ^{1}H NMR ($CDCl_3$) δ 2.30 (br s, 4H), 2.52 (s, 3H), 3.62 (br s, 4H), 7.30 (dd, 1H, J = 6.4 Hz, 9.6 Hz), 7.37 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 12.6, 27.8, 113.0, 127.4, 128.6, 131.9, 135.9, 157.2, 162.2, 170.4. HRMS-ESI (+): Calcd for $C_{15}H_{15}N_2O_2SCl_2$: 357.0231. Found: 357.0237 $[M+H]^+$.

3.1.2.13. N-(2-Bromobenzyl)-3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxamide (7m). Yield 73%; ^{1}H NMR ($CDCl_3$) δ 2.73 (s, 3H), 4.37 (d, 2H, J = 6.0 Hz), 5.62 (br s, 1H), 7.04 (m, 1H), 7.16 (m, 2H), 7.26–7.33 (m, 3H), 7.38 (d, 1H, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 13.4, 43.9, 111.6, 123.9, 127.3, 127.9, 128.8, 129.5, 130.6, 132.3, 132.8, 136.2, 136.9, 156.1, 160.6, 175.2. HRMS-ESI (+): Calcd for $C_{18}H_{14}N_2O_2Cl_2Br$: 438.9616. Found: 438.9627 $[M+H]^+$.

3.1.2.14. 3-(2,6-Dichlorophenyl)-5-methyl-N-(4-methylcyclohexyl)isoxazole-4-carboxamide (mixture of cis and trans) (7n). Yield 93%; ^{1}H NMR ($CDCl_3$) δ 2.74 (s, 3H), 4.28 (d, 2H, J = 5.6 Hz), 5.27 (br s, 1H), 6.91 (d, 1H, J = 7.2 Hz), 7.04 (m, 2H), 7.29 (d, 2H, J = 7.6 Hz), 7.34 (d, 2H, J = 7.6 Hz); ^{13}C NMR ($CDCl_3$) δ 13.4, 42.9, 111.5, 122.9, 126.2, 127.3, 128.8, 130.2, 130.4, 132.5, 136.0, 139.8, 156.1, 160.7, 175.2. HRMS-ESI (+): Calcd for $C_{18}H_{14}N_2O_2Cl_2Br$: 438.9616. Found: 438.9635 $[M+H]^+$.

3.1.3. General procedures for the preparation of and 2-mercaptobenzoxazole (11b–11g)

To a solution of a substituted 2-aminophenol (3 mmol) was added potassium ethylxanthate (484 mg, 3 mmol) in absolute ethanol (10 mL). The resulting mixture was heated under reflux overnight and then cooled to room temperature. The precipitate was dissolved in H_2O (10 mL) and washed with ethyl acetate (10 mL × 3) and the aqueous solution was then neutralized to pH 5 by slow addition of glacial acetic acid. Then the product precipitated (crystallized) out to give **11b–11g**.

3.1.3.1. 2-Mercaptobenzoxazole (11b). Yield 52%; ^{1}H NMR (DMSO-d_6) δ 7.29 (m, 3H), 7.53 (d, 1H, J = 7.6 Hz), 13.90 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 110.0, 110.5, 123.7, 125.1, 131.2, 148.1, 180.1. HRMS-ESI (+): Calcd for C_7H_6NOS: 152.0170. Found: 152.0170 $[M+H]^+$.

3.1.3.2. 2-Mercapto-5-methylbenzoxazole (11c). Yield 45%; ^{1}H NMR (DMSO-d_6) δ 2.37 (s, 3H), 7.05 (m, 2H), 7.34 (m, 1H), 13.73 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 20.8, 109.4, 110.4, 124.2, 131.2, 134.7, 146.3, 180.2. HRMS-ESI (+): Calcd for C_8H_8NOS: 166.0327. Found: 166.0333 $[M+H]^+$.

3.1.3.3. 2-Mercapto-6-methylbenzoxazole (11d). Yield 64%; ^{1}H NMR (DMSO-d_6) δ 2.39 (s, 3H), 7.10 (d, 1H, J = 7.6 Hz), 7.16 (t, 1H, J = 7.6 Hz), 7.31 (d, 1H, J = 7.6 Hz), 13.95 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 16.1, 107.2, 121.1, 123.6, 126.0, 130.4, 147.9, 180.1. HRMS-ESI (+): Calcd for C_8H_8NOS: 166.0327. Found: 166.0333 $[M+H]^+$.

3.1.3.4. 2-Mercapto-3-nitrobenzoxazole (11e). Yield 14%; ^{1}H NMR (DMSO-d_6) δ 7.44 (t, 1H, J = 8.0 Hz), 7.91 (d, 1H, J = 8.0 Hz), 8.06 (d, 1H, J = 8.0 Hz); ^{13}C NMR (DMSO-d_6) δ 115.8, 119.8, 123.4, 128.0, 131.4, 149.8, 181.7. HRMS-ESI (+): Calcd for $C_7H_5N_2O_3S$: 197.0021. Found: 197.0013 $[M+H]^+$.

3.1.3.5. 2-Mercapto-5-nitrobenzoxazole (11f). Yield 25%; ^{1}H NMR (DMSO-d_6) δ 7.17 (d, 1H, J = 8.8 Hz), 7.95 (d, 1H, J = 2.4 Hz), 7.99 (dd, 1H, J = 2.4 Hz, 8.8 Hz); ^{13}C NMR (DMSO-d_6) δ 101.8, 112.3, 119.8, 139.7, 150.3, 153.2, 188.4. HRMS-ESI (+): Calcd for $C_7H_5N_2O_3S$: 197.0021. Found: 197.0018 $[M+H]^+$.

3.1.3.6. 2-Mercapto-5-chlorobenzoxazole (11g). Yield 36%; ^{1}H NMR (DMSO-d_6) δ 7.30 (d, 2H, J = 7.6 Hz), 7.53 (d, 1H, J = 8.0 Hz), 14.04 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 110.3, 111.2, 123.5, 129.3,

132.6, 147.0, 180.8. HRMS-ESI (+): Calcd for C_7H_5NOSCl: 185.9780. Found: 185.9789 [M+H]+.

3.1.4. General procedures for the preparation of 2-thiouracils 14a–14i

To a solution of an aldehyde (RCHO, 10 mmol), ethyl cyanoacetate (1.0 mL, 10 mmol), and thiourea (0.76 g, 10 mmol) in absolute ethanol (50 mL) was added piperidine (2.0 mL, 20 mmol); the mixture was heated under reflux overnight and then cooled to room temperature. The precipitate was dissolved in 0.5 M NaOH (20 mL) and washed with ethyl acetate (10 mL × 3). The aqueous solution was then neutralized to pH 2 by slow addition of 1 M HCl. Then the product precipitated (crystallized) out to give **14a–14i**.

3.1.4.1. 5-Cyano-6-phenyl-2-thiouracil (14a). Yield 67%; ^{1}H NMR (DMSO-d_6) δ 7.62 (m, 5H), 13.19 (s, 1H), 13.32 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 90.2, 113.8, 128.2, 128.6, 132.0, 158.2, 160.2, 176.0. MS-ESI (+): 252.0 $[M+Na]^+$.

3.1.4.2. 5-Cyano-6-(3-tolyl)-2-thiouracil (14b). Yield 31%; ^{1}H NMR (DMSO-d_6) δ 2.39 (s, 3H), 7.46 (m, 4H), 13.17 (s, 1H), 13.26 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 20.9, 90.6, 114.7, 125.9, 128.4, 129.1, 129.2, 132.8, 137.9, 158.5, 160.9, 176.2. HRMS-ESI (+): Calcd for $C_{12}H_{10}N_3OS$: 244.0545. Found: 244.0555 $[M+H]^+$.

3.1.4.3. 5-Cyano-6-(4-tolyl)-2-thiouracil (14c). Yield 43%; ^{1}H NMR (DMSO-d_6) δ 2.45 (s, 3H), 7.41 (d, 2H, J = 7.6 Hz), 7.61 (d, 2H, J = 8.4 Hz); ^{13}C NMR (DMSO-d_6) δ 12.2, 81.7, 105.9, 118.4, 120.1, 121.2, 135.3, 150.9, 153.2, 168.2. HRMS-ESI (+): Calcd for $C_{13}H_{12}N_3OS$: 258.0701. Found: 258.0702 $[M+H]^+$.

3.1.4.4. 5-Cyano-6-(4-ethylphenyl)-2-thiouracil (14d). Yield 25%; ^{1}H NMR (DMSO-d_6) δ 1.22 (t, 3H, J = 7.6 Hz), 2.71 (q, 2H, J = 7.6 Hz), 7.42 (d, 2H, J = 8.0 Hz), 7.61 (d, 2H, J = 8.0 Hz), 13.15 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 15.2, 28.1, 90.4, 114.8, 126.6, 127.9, 128.9, 148.6, 158.5, 160.9, 176.2. HRMS-ESI (+): Calcd for $C_{14}H_{14}N_3OS$: 272.0858. Found: 272.0867 $[M+H]^+$.

3.1.4.5. 5-Cyano-6-(4-isopropylphenyl)-2-thiouracil (14e). Yield 37%; ^{1}H NMR (DMSO-d_6) δ 1.24 (d, 6H, J = 6.8 Hz), 3.00 (septet, 1H, J = 6.8 Hz), 7.46 (d, 2H, J = 8.0 Hz), 7.62 (d, 2H, J = 8.0 Hz), 13.15 (br s, 2H); ^{13}C NMR (DMSO-d_6) δ 23.6, 33.5, 90.3, 114.9, 126.5, 126.7, 129.0, 153.1, 158.6, 160.8, 176.2. HRMS-ESI (+): Calcd for $C_{14}H_{14}N_3OS$: 272.0858. Found: 272.0867 $[M+H]^+$.

3.1.4.6. 5-Cyano-6-(4-methoxyphenyl)-2-thiouracil (14f). Yield 21%; ^{1}H NMR (DMSO-d_6) δ 3.86 (s, 3H), 7.12 (d, 2H, J = 8.8 Hz), 7.68 (d, 2H, J = 8.8 Hz), 13.13 (br s, 2H); ^{13}C NMR (DMSO-d_6) δ 55.7, 89.9, 114.0, 115.2, 121.1, 131.0, 158.8, 160.6, 162.5, 176.3. HRMS-ESI (+): Calcd for $C_{12}H_{10}N_3O_2S$: 260.0494. Found: 260.0496 $[M+H]^+$.

3.1.4.7. 5-Cyano-6-(4-bromophenyl)-2-thiouracil (14g). Yield 39%; ^{1}H NMR (DMSO-d_6) δ 7.63 (d, 2H, J = 8.4 Hz), 7.80 (d, 2H, J = 8.4 Hz), 13.21 (s, 1H), 13.37 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 91.1, 114.6, 125.9, 128.5, 130.9, 131.6, 158.4, 160.0, 176.2. HRMS-ESI (+): Calcd for $C_{11}H_7N_3OSBr$: 307.9493. Found: 307.9504 $[M+H]^+$.

3.1.4.8. 5-Cyano-6-(biphenyl-4-yl)-2-thiouracil (14h). Yield 75%; ^{1}H NMR (DMSO-d_6) δ 7.45 (t, 1H, J = 7.2 Hz), 7.53 (t, 2H, J = 7.2 Hz), 7.78 (d, 4H, J = 8.4 Hz), 7.89 (d, 2H, J = 8.4 Hz) 13.19 (s, 1H), 13.35 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 90.6, 114.8, 126.6, 127.0, 128.1, 128.4, 129.2, 129.6, 138.7, 143.7, 158.5, 160.5, 176.2. HRMS-ESI (+): Calcd for $C_{17}H_{12}N_3OS$: 306.0701. Found: 306.0714 $[M+H]^+$.

3.1.4.9. 5-Cyano-6-(1-naphthyl)-2-thiouracil (14i). Yield 22%; ^{1}H NMR (DMSO-d_6) δ 7.64 (m, 3H), 7.74 (d, 1H, J = 6.8 Hz), 7.99 (dd, 1H, J = 6.0 Hz, 6.4 Hz), 8.06 (dd, 1H, J = 6.0 Hz, 6.4 Hz,), 8.15 (d, 1H, J = 8.4 Hz), 13.11 (s, 1H), 13.46 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 93.1, 114.5, 124.7, 125.2, 126.8, 127.2, 127.5, 128.3, 128.5, 129.5, 131.2, 132.8, 158.7, 161.1, 176.8. HRMS-ESI (+): Calcd for $C_{15}H_{10}N_3OS$: 280.0545. Found: 280.0554 $[M+H]^+$.

3.1.5. General procedure for the preparation of 2,2′-(α,α′-xylene)bis(sulfanediyl)bisbenzothiazole (15a), 2,2′-(α,α′-xylene)bis(sulfanediyl)bisbenzoxazole (15b–g), and 2,2′-(α,α′-xylene)bis(sulfanediyl)bis-4-oxopyrimidine (16a–i)

To a solution of the 2-mercaptobenzothiazole, 2-mercaptobenzoxazole, or 2-thiouracil derivatives (**11a–g or 10a–h**, 0.1 mmol) and α,α′-xylenedibromide (12 mg, 0.045 mmol) in acetonitrile (2.5 mL) was added K_2CO_3 (42 mg, 0.3 mmol). The mixture was heated under reflux overnight and then cooled to room temperature. The liquid was removed on a rotavapor and the residue was washed with 0.5 M NaOH (20 mL). Then the white solid residue was dried in vacuum oven at 40 °C overnight to give **15a–15g** or **16a–16h**.

3.1.5.1. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-chlorobenzothiazole) (15a). Yield 84%; ^{1}H NMR (DMSO-d_6) δ 4.62 (s, 4H), 7.45 (s, 4H), 7.47 (dd, 2H, J = 2.0 Hz, 8.4 Hz), 7.84 (d, 2H, J = 8.4 Hz), 8.11 (d, 2H, J = 2.0 Hz); ^{13}C NMR (DMSO-d_6) δ 36.3, 120.8, 121.7, 126.2, 128.7, 135.4, 135.9, 151.0, 166.6. HRMS-ESI (+): Calcd for $C_{22}H_{15}N_2S_4Cl_2$: 504.9495. Found: 504.9499 $[M+H]^+$.

3.1.5.2. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis(benzoxazole) (15b). Yield 35%; ^{1}H NMR (DMSO-d_6) δ 4.60 (s, 4H), 7.34 (m, 4H), 7.49 (m, 4H), 7.65 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 35.1, 110.2, 118.3, 124.3, 124.6, 129.2, 136.1, 141.2, 151.3, 163.8. HRMS-ESI (+): Calcd for $C_{22}H_{17}N_2O_2S_2$: 405.0731. Found: 405.0732 $[M+H]^+$.

3.1.5.3. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(5-methylbenzoxazole) (15c). Yield 57%; ^{1}H NMR (DMSO-d_6) δ 2.40 (s, 6H), 4.57 (s, 4H), 7.12 (d, 2H, J = 8.0 Hz), 7.46 (m, 8H); ^{13}C NMR (DMSO-d_6) δ 20.9, 35.1, 109.6, 118.3, 125.1, 129.2, 134.0, 136.2, 141.4, 149.5, 163.6. HRMS-ESI (+): Calcd for $C_{24}H_{21}N_2O_2S_2$: 433.1044. Found: 433.1041 $[M+H]^+$.

3.1.5.4. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-methylbenzoxazole) (15d). Yield 86%; ^{1}H NMR (DMSO-d_6) δ 2.49 (s, 6H), 4.59 (s, 4H), 7.18 (m, 4H), 7.43 (d, 2H, J = 7.6 Hz), 7.49 (s, 4H); ^{13}C NMR (DMSO-d_6) δ 16.0, 35.2, 107.5, 124.0, 125.1, 128.3, 129.3, 136.2, 140.4, 151.0, 162.6. HRMS-ESI (+): Calcd for $C_{24}H_{21}N_2O_2S_2$: 433.1044. Found: 433.1050 $[M+H]^+$.

3.1.5.5. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(4-nitrobenzoxazole) (15e). Yield 61%; ^{1}H NMR (DMSO-d_6) δ 4.66 (s, 4H), 7.50 (t, 4H, J = 8.0 Hz), 7.52 (s, 4H), 8.01 (d, 2H, J = 8.0 Hz), 8.08 (d, 2H, J = 8.0 Hz); ^{13}C NMR (DMSO-d_6) δ 35.3, 115.6, 119.8, 123.5, 128.8, 135.4, 136.9, 152.6, 168.1. HRMS-ESI (+): Calcd for $C_{22}H_{15}N_4O_6S_2$: 495.0433. Found: 495.0438 $[M+H]^+$.

3.1.5.6. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-nitrobenzoxazole) (15f). Yield 81%; ^{1}H NMR (DMSO-d_6) δ 4.67 (s, 4H), 7.53 (s, 4H), 7.85 (d, 2H, J = 8.4 Hz), 8.27 (dd, 2H, J = 2.0 Hz, 8.4 Hz), 8.62 (d, 2H, J = 2.0 Hz); ^{13}C NMR (DMSO-d_6) δ 35.4, 106.9, 118.2, 121.0, 129.4, 135.9, 143.9, 146.6, 150.6, 170.0. HRMS-ESI (+): Calcd for $C_{22}H_{15}N_4O_6S_2$: 495.0433. Found: 495.0431 $[M+H]^+$.

3.1.5.7. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(5-chlorobenzoxazole) (15g). Yield 49%; ^{1}H NMR (DMSO-d_6) δ 4.60 (s, 4H), 7.36 (dd, 2H, J = 2.0 Hz, 8.8 Hz), 7.48 (s, 4H), 7.67 (d, 2H, J = 8.8 Hz), 7.76 (d, 2H, J = 2.0 Hz); ^{13}C NMR (DMSO-d_6) δ 35.2, 111.5, 118.1, 124.3, 129.0, 129.3, 136.0, 142.5, 150.1, 165.9. HRMS-ESI (+): Calcd for $C_{22}H_{15}N_2O_2S_2Cl_2$: 472.9952. Found: 472.9974 $[M+H]^+$.

3.1.5.8. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-phenyl-5-cyano-4-oxopyrimidine) (16a). Yield 80%; ^{1}H NMR (DMSO-d_6) δ 4.26 (s, 4H), 7.31 (s, 4H), 7.44 (m, 6H), 7.77 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 33.6, 88.8, 119.9, 127.8, 127.9, 128.6, 129.3, 137.2, 137.7, 166.8, 170.3, 171.5. HRMS-ESI (−): Calcd for $C_{30}H_{19}N_6O_2S_2$: 559.1011. Found: 559.0989 $[M-H]^-$.

3.1.5.9. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-(3-tolyl)-5-cyano-4-oxopyrimidine) (16b). Yield 62%; ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 6H), 4.24 (s, 4H), 7.31 (m, 8H), 7.54 (s, 4H); ^{13}C NMR (DMSO-d_6) δ 21.0, 33.6, 88.9, 120.1, 125.3, 127.9, 128.6, 128.8, 130.2, 137.2, 137.4, 137.8, 167.2, 170.5, 171.6. HRMS-ESI (−): Calcd for $C_{32}H_{23}N_6O_2S_2$: 587.1324. Found: 587.1348 $[M-H]^-$.

3.1.5.10. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-(4-tolyl)-5-cyano-4-oxopyrimidine) (16c). Yield 80%; ^{1}H NMR (DMSO-d_6) δ 2.36 (s, 6H), 4.25 (s, 4H), 7.26 (d, 4H, J = 8.0 Hz), 7.32 (s, 4H), 7.68 (d, 4H, J = 8.0 Hz); ^{13}C NMR (DMSO-d_6) δ 21.0, 33.7, 88.6, 120.2, 128.1, 128.6, 128.9, 134.9, 137.4, 139.4, 166.9, 170.7, 171.7. HRMS-ESI (−): Calcd for $C_{32}H_{23}N_6O_2S_2$: 587.1324. Found: 587.1306 $[M-H]^-$.

3.1.5.11. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-(4-ethylphenyl)-5-cyano-4-oxopyrimidine) (16d). Yield 96%; ^{1}H NMR (DMSO-d_6) δ 1.21 (t, 6H, J = 7.6 Hz), 2.65 (q, 4H, J = 7.6 Hz), 4.26 (s, 4H), 7.29 (d, 4H, J = 8.0 Hz), 7.32 (s, 4H), 7.70 (d, 4H, J = 8.0 Hz); ^{13}C NMR (DMSO-d_6) δ 15.5, 28.1, 33.7, 88.6, 120.3, 127.5, 128.2, 128.9, 135.2, 137.4, 145.6, 166.9, 170.7, 171.7. HRMS-ESI (−): Calcd for $C_{34}H_{27}N_6O_2S_2$: 615.1637. Found: 615.1613 $[M-H]^-$.

3.1.5.12. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-(4-isopropylphenyl)-5-cyano-4-oxopyrimidine) (16e). Yield 87%; ^{1}H NMR (DMSO-d_6) δ 1.25 (d, 12H, J = 7.2 Hz), 2.98 (septet, 2H, J = 7.2 Hz), 4.52 (s, 4H), 7.36 (s, 4H), 7.38 (d, 4H, J = 8.4 Hz), 7.87 (d, 4H, J = 8.0 Hz); ^{13}C NMR (DMSO-d_6) δ 22.7, 32.7, 33.7, 91.8, 114.9, 125.8, 128.2, 128.4, 132.2, 135.3, 152.1, 161.1, 165.3, 166.4. HRMS-ESI (−): Calcd for $C_{36}H_{31}N_6O_2S_2$: 643.1950. Found: 643.1943 $[M-H]^-$.

3.1.5.13. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-(4-methoxyphenyl)-5-cyano-4-oxopyrimidine) (16f). Yield 46%; ^{1}H NMR (DMSO-d_6) δ 3.81(s, 6H), 4.28 (s, 4H), 6.97 (d, 4H, J = 8.8 Hz), 7.30 (s, 4H), 7.82 (d, 4H, J = 8.8 Hz); ^{13}C NMR (DMSO-d_6) δ 33.7, 55.3, 88.2, 113.5, 120.4, 128.9, 129.8, 130.0, 137.4, 160.6, 166.3, 170.6, 171.4. HRMS-ESI (−): Calcd for $C_{32}H_{23}N_6O_4S_2$: 619.1222. Found: 619.1230 $[M-H]^-$.

3.1.5.14. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-(4-bromophenyl)-5-cyano-4-oxopyrimidine) (16g). Yield 95%; ^{1}H NMR (DMSO-d_6) δ 4.25 (s, 4H), 7.31 (s, 4H), 7.67 (d, 4H, J = 8.4 Hz), 7.72 (d, 4H, J = 8.8 Hz); ^{13}C NMR (DMSO-d_6) δ 33.7, 88.9, 119.9, 123.3, 128.9, 130.2, 131.1, 136.8, 137.3, 165.9, 170.2, 171.8. HRMS-ESI (−): Calcd for $C_{30}H_{18}Br_2N_6O_2S_2$: 714.9221. Found: 714.9213 $[M-H]^-$.

3.1.5.15. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-(biphenyl-4-yl)-5-cyano-4-oxopyrimidine) (16h). Yield 68%; ^{1}H NMR (DMSO-d_6) δ 4.28 (s, 4H), 7.35 (s, 4H), 7.40 (t, 2H, J = 7.2 Hz), 7.49 (t, 4H, J = 7.6 Hz), 7.75 (m, 8H), 7.88 (d, 4H, J = 8.4 Hz); ^{13}C NMR (DMSO-d_6) δ 33.7, 88.8, 120.3, 126.3, 126.8, 127.8, 128.7, 128.9, 129.0, 136.7, 137.4, 139.5, 141.3, 166.4, 170.4, 171.8. HRMS-ESI (−): Calcd for $C_{42}H_{27}N_6O_2S_2$: 711.1637. Found: 711.1661 $[M-H]^-$.

3.1.5.16. 2,2′-(α,α′-Xylene)bis(sulfanediyl)bis-(6-(1-naphthyl)-5-cyano-4-oxopyrimidine) (16i). Yield 92%; ^{1}H NMR (CD_3OD) δ 4.37 (s, 4H), 7.32 (s, 4H), 7.44–7.57 (m, 8H), 7.74 (d, 2H, J = 8.0 Hz), 7.91 (d, 2H, J = 8.2 Hz), 7.96 (dd, 2H, J = 2.0 Hz, 7.0 Hz); ^{13}C NMR (CD_3OD) δ 35.7, 94.1, 119.1, 126.2, 126.5, 127.4, 127.8, 127.9, 129.5, 130.3, 130.9, 132.0, 135.2, 136.6, 138.5, 171.6, 174.0, 175.0. HRMS-ESI (−): Calcd for $C_{38}H_{23}N_6O_2S_2$: 659.1324. Found: 659.1343 $[M-H]^-$.

3.1.6. General procedure for the preparation of *S*-benzyl-2-thiouracils (17d,e,g–i)

To a solution of the 2-thiouracil derivatives (**10d**,**e**,**g–i**, 2 mmol) and benzylchloride (253 mg, 2 mmol) in acetonitrile (10 mL) was added K_2CO_3 (829 mg, 6 mmol). The mixture was heated under reflux for 8 h and then cooled to room temperature. The liquid was removed on a rotavapor, and the residue was washed by H_2O (20 mL). Then the solid was dried in a vacuum oven at 40 °C overnight to give **17d**,**e**,**g–i**.

3.1.6.1. S-Benzyl-5-cyano-6-(4-ethylphenyl)-2-thiouracil (17d). Yield 28%; ^{1}H NMR (DMSO-d_6) δ 1.27 (t, 3H, J = 7.6 Hz), 2.71 (q, 2H, J = 7.6 Hz), 4.40 (s, 2H), 7.21 (m, 1H), 7.28 (m, 4H), 7.41 (d, 2H, J = 7.2 Hz), 7.74(d, 2H, J = 8.4 Hz); ^{13}C NMR (DMSO-d_6) δ 16.0, 29.8, 36.0, 90.6, 120.1, 128, 128.8, 129.2, 129.4, 129.8, 130.1, 136.0, 139.8, 148.2, 170.2, 174.9. HRMS-ESI (+): Calcd for $C_{20}H_{18}N_3OS$: 348.1171. Found: 348.1185 $[M+H]^+$.

3.1.6.2. S-Benzyl-5-cyano-6-(4-isopropylphenyl)-2-thiouracil (17e). Yield 40%; ^{1}H NMR (DMSO-d_6) δ 1.23 (d, 6H, J = 6.8 Hz), 2.94 (septet, 1H, J = 6.8 Hz), 4.30 (s, 2H), 7.22 (t, 1H, J = 6.8 Hz), 7.31 (m, 4H), 7.40 (d, 2H, J = 7.2 Hz), 7.73 (d, 2H, J = 7.6 Hz); ^{13}C NMR (DMSO-d_6) δ 23.7, 33.3, 33.8, 88.7, 120.3, 126.0, 126.7, 128.2, 128.3, 128.8, 135.3, 139.0, 150.2, 166.8, 170.5, 171.5. HRMS-ESI (+): Calcd for $C_{21}H_{20}N_3OS$: 362.1327. Found: 362.1335 $[M+H]^+$.

3.1.6.3. S-Benzyl-5-cyano-6-(4-bromophenyl)-2-thiouracil (17g). Yield 33%; ^{1}H NMR (DMSO-d_6) δ 4.27 (s, 2H), 7.22 (t, 1H, J = 7.2 Hz), 7.29 (t, 2H, J = 7.2 Hz), 7.39 (d, 2H, J = 7.2 Hz), 7.67 (d, 2H, J = 8.4 Hz), 7.71 (d, 2H, J = 8.4 Hz); ^{13}C NMR (DMSO-d_6) δ 33.8, 88.9,120.0, 123.2, 126.7, 128.3, 128.9, 130.2, 131.1, 136.9, 139.0 165.8, 170.1, 171.8. HRMS-ESI (+): Calcd for $C_{18}H_{13}N_3OSBr$: 397.9963. Found: 397.9950 $[M+H]^+$.

3.1.6.4. S-Benzyl-5-cyano-6-(biphenyl-4-yl)-2-thiouracil (17h). Yield 37%; ^{1}H NMR (DMSO-d_6) δ 4.32 (s, 2H), 7.23 (t, 1H, J = 7.6 Hz), 7.31 (t, 2H, J = 7.6 Hz), 7.40 (m, 3H), 7.50 (t, 2H, J = 7.6 Hz), 7.74 (d, 2H, J = 8.0 Hz), 7.77 (d, 2H, J = 8.4 Hz), 7.90 (d, 2H, J = 8.0 Hz); ^{13}C NMR (DMSO-d_6) δ 33.8, 89.1, 126.4, 126.8, 126.8, 127.9, 128.3, 128.8, 128.9, 129.0, 136.6, 139.0, 139.4, 141.4, 166.4, 169.7, 171.3. HRMS-ESI (+): Calcd for $C_{24}H_{18}N_3OS$: 396.1171. Found: 396.1187 $[M+H]^+$.

3.1.6.5. S-Benzyl-5-cyano-6-(1-naphthyl)-2-thiouracil (17i). Yield 43%; ^{1}H NMR (DMSO-d_6) δ 4.26 (s, 2H), 7.23 (t, 1H, J = 7.2 Hz), 7.29 (t, 2H, J = 7.2 Hz), 7.39 (d, 2H, J = 7.2 Hz), 7.55 (m, 5H), 7.78 (d, 1H, J = 8.0 Hz), 7.99 (t, 2H, J = 6.8 Hz); ^{13}C NMR (DMSO-d_6) δ 33.8, 92.4, 119.3, 125.2, 125.4, 126.0, 126.2, 126.4, 126.8, 128.2, 128.3, 128.9, 130.1, 133.1, 135.9, 138.9, 168.7, 167.6, 171.4. HRMS-ESI (+): Calcd for $C_{22}H_{16}N_3OS$: 370.1014. Found: 370.1015 $[M+H]^+$.

3.2. Biological evaluation

3.2.1. General in vitro biological methods

EcN68, the N-terminal fragment of SecA from *E. coli* without the C-terminal regulatory domain, and EcSecA, the full length SecA from *E. coli*, were over-expressed from pIMBB-8[21] and pT7-SecA,[22] respectively, and purified as described.[23,24] EcN68 was used for screening because it has higher intrinsic activity and is more sensitive to inhibitors.

All potential inhibitors were dissolved in 100% DMSO. The ATPase activity was determined by the release of phosphate (Pi) detected by malachite green as described[3] in a modified procedure[15] and in the presence of 10% DMSO. Inhibitory effect was determined by the percentage of the remaining ATPase activity as compare to the controls without test compounds. Briefly, 50 μL reaction mixture was prepared so that it contained 2.25 μg N68 or 5 μg SecA, 2 mM ATP, 50 mM Tris–HCl (pH7.6), 20 mM KCl, 20 mM NH_4Cl, 1 mM DTT, and 2 mM $Mg(OAc)_2$. Reactions took place at 40 °C for 20 min (for N68) or 40 min (for SecA) then were stopped by adding 800 μL of malachite green and then 100 μL of 34% citric acid within 1 min. The mixtures were incubated at room temperature for 40 min and then the absorption at 660 nm was measured. All assays were done at least in triplicate, and the results were presented as bar graphs with standard error of the mean.

3.2.2. General in vivo biological methods

Log-phase growing cells (O.D. 600 nm ~ 0.5–1.0) were diluted to an absorbance of 0.05 at O.D. 600 nm, added with indicated compounds, and followed by culturing in an Eppendorf Thermomixer R (Brinkmann instruments, Inc.) at 37 °C,1050 rpm for 10–12 h. All cultures contain 5% DMSO with a final volume of 100 μl. All tested compounds were dissolved in 100% DMSO (Sigma).

Bacterial strain: NR698 (MC4100 *imp*4213)[25] with increased outer membrane permeability. MC4100, an *E. coli* K-12 wild-type strain.[26]

3.3. Computational method

3.3.1. Molecular simulation of ligand-SecA complexes

The 3D structures for these compounds were refined using the PM3 method in the MOPAC 7 program[27] and assigned with AM1-BCC partial charges[28–30] by the QUACPAC program. All partial charges on the atoms of the homology model were derived from AMBER 8 parameters. Docking of the ligands into SecA around the active site (included residues Gly80, Mse81, Arg82, His83, Phe84, Gln87, Arg103, Thr104, Gly105, Glu106, Gly107, Lys108, Thr109, Leu110, Arg138, Asp209, Glu210, Arg509 and Gln578) was performed by using DOCK 5.4.[31] After docking, MD simulations were conducted with the ligand–receptor complexes following similar procedures we reported before.[17–20] In brief, the docked complexes were solvated by using the TIP3P water model,[32] subjected to 500-steps of molecular mechanics minimization and molecular dynamics simulations at 300 K for 1.0 ns using the SANDER module in the AMBER 8 program.[33] The resulting structures were then analyzed using PyMOL 1.0,[34] HBPLUS 3.06[35] and Ligplot 4.22[36] to identify specific contacts between ligands and SecA. During the computation, molecular docking (DOCK 5.4), binding analysis (HBPLUS 3.06 and Ligplot 4.22) and visualization (PyMOL 1.0) were carried out on a Xeon-based Linux workstation. Molecular mechanics calculations and molecular dynamics simulations (AMBER 8) were performed on URSA, a 160-processor computer based on the Power5+ processor and IBM's P series architecture.

Acknowledgments

We gratefully acknowledge the financial support by the Georgia Research Alliance, Georgia Cancer Coalition, NIH Grant GM34766, and the Molecular Basis of Disease Program at GSU.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.074.

References and notes

1. Saier, M. H. *J. Membr. Biol.* **2006**, *214*, 75.
2. van Klompenburg, W.; Ridder, A. N.; van Raalte, A. L.; Killian, A. J.; von Heijne, G.; de Kruijff, B. *FEBS Lett.* **1997**, *413*, 109.
3. Lill, R.; Dowhan, W.; Wickner, W. *Cell* **1990**, *60*, 271.
4. Erlandson, K. J.; Miller, S. B.; Nam, Y.; Osborne, A. R.; Zimmer, J.; Rapoport, T. A. *Nature* **2008**, *455*, 984.
5. Tsukazaki, T.; Mori, H.; Fukai, S.; Ishitani, R.; Mori, T.; Dohmae, N.; Perederina, A.; Sugita, Y.; Vassylyev, D. G.; Ito, K.; Nureki, O. *Nature* **2008**, *455*, 988.
6. Zimmer, J.; Nam, Y.; Rapoport, T. A. *Nature* **2008**, *455*, 936.
7. Mori, H.; Ito, K. *Trends Microbiol.* **2001**, *9*, 494.
8. Vrontou, E.; Economou, A. *Biochim. Biophys. Acta* **2004**, *1694*, 67.
9. Papanikolau, Y.; Papadovasilaki, M.; Ravelli, R. B.; McCarthy, A. A.; Cusack, S.; Economou, A.; Petratos, K. *J. Mol. Biol.* **2007**, *366*, 1545.
10. Li, M.; Huang, Y. J.; Tai, P. C.; Wang, B. *Biochem. Biophys. Res. Commun.* **2008**, *368*, 839.
11. Bowler, M. W.; Montgomery, M. G.; Leslie, A. G.; Walker, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 8646.
12. Stoddard, B. L.; Ringe, D.; Petsko, G. A. *Protein Eng.* **1990**, *4*, 113.
13. In our earlier screening, these two compounds were found to have IC_{50} values of about 30 μM. However, upon more rigorous studies, **2** was found to have IC_{50} of 100 μM. **1** showed similar inhibition activities as **2**, but started having solubility problems when approaching 100 μM.
14. Sugie, Y.; Inagaki, S.; Kato, Y.; Nishida, H.; Pang, C. H.; Saito, T.; Sakemi, S.; Dib-Hajj, F.; Mueller, J. P.; Sutcliffe, J.; Kojima, Y. *J. Antibiot. (Tokyo)* **2002**, *55*, 25.
15. Maloney, P. R.; Parks, D. J.; Haffner, C. D.; Fivush, A. M.; Chandra, G.; Plunket, K. D.; Creech, K. L.; Moore, L. B.; Wilson, J. G.; Lewis, M. C.; Jones, S. A.; Willson, T. M. *J. Med. Chem.* **2000**, *43*, 2971.
16. Abdou, I. M.; Strekowski, L. *Tetrahedron* **2000**, *56*, 8631.
17. Li, M.; Wang, B. *Biochem. Biophys. Res. Commun.* **2006**, *347*, 662.
18. Li, M.; Wang, B. *J. Mol. Model.* **2007**, *13*, 1237.
19. Li, M.; Ni, N.; Chou, H.-T.; Lu, C.-D.; Tai Phang, C.; Wang, B. *ChemMedChem* **2008**, *3*, 1242.
20. Zheng, S.; Kaur, G.; Wang, H.; Li, M.; Macnaughtan, M.; Yang, X.; Reid, S.; Prestegard, J.; Wang, B.; Ke, H. *J. Med. Chem.* **2008**, *51*, 7673.
21. Karamanou, S.; Vrontou, E.; Sianidis, G.; Baud, C.; Roos, T.; Kuhn, A.; Politou, A. S. *Mol. Microbiol.* **1999**, *34*, 1133.
22. Cabelli, R. J.; Chen, L.; Tai, P. C.; Oliver, D. B. *Cell* **1988**, *55*, 683.
23. Chen, X.; Brown, T.; Tai, P. C. *J. Bacteriol.* **1998**, *180*, 527.
24. Chen, X.; Xu, H.; Tai, P. C. *J. Biol. Chem.* **1996**, *271*, 29698.
25. Ruiz, N.; Falcone, B.; Kahne, D.; Silhavy, T. J. *Cell* **2005**, *121*, 307.
26. Casadaban, M. J. *J. Mol. Biol.* **1976**, *104*, 541.
27. Stewart, J. J. *J. Comput. Aided Mol. Des.* **1990**, *4*, 1.
28. Jakalian, A.; Bush, B. L.; Jack, D. B.; Bayly, C. I. *J. Comput. Chem.* **2000**, *21*, 132.
29. Jakalian, A.; Jack, D. B.; Bayly, C. I. *J. Comput. Chem.* **2002**, *23*, 1623.
30. Tsai, K.-C.; Wang, S.-H.; Hsiao, N.-W.; Li, M.; Wang, B. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3509.
31. Moustakas, D. T.; Lang, P. T.; Pegg, S.; Pettersen, E.; Kuntz, I. D.; Brooijmans, N.; Rizzo, R. C. *J. Comput. Aided Mol. Des.* **2006**, *20*, 601.
32. Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. *J. Chem. Phys.* **1983**, *79*, 926.
33. Case, D. A.; Cheatham, T. E., 3rd; Darden, T.; Gohlke, H.; Luo, R.; Merz, K. M., Jr.; Onufriev, A.; Simmerling, C.; Wang, B.; Woods, R. J. *J. Comput. Chem.* **2005**, *26*, 1668.
34. DeLano, W. L.; DeLano Scientific, San Carlos, CA, USA, 2006, http://www.pymol.org.
35. McDonald, I. K.; Thornton, J. M. *J. Mol. Biol.* **1994**, *238*, 777.
36. Wallace, A. C.; Laskowski, R. A.; Thornton, J. M. *Protein Eng.* **1995**, *8*, 127.