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ORIGINAL RESEARCH

Design, synthesis, and biological evaluation of pyrimidine analogs as SecA inhibitors

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Abstract

SecA, a key component of the bacterial Sec-dependent secretion pathway, is an attractive target for the development of new antimicrobial agents. We have previously reported pyrimidine analogs as SecA inhibitors. Herein, we report an extension of the earlier work in the synthesis and evaluation of a series of 15 5-cyanothiouracil derivatives as SecA inhibitors. All the compounds have been evaluated for their inhibition of SecA ATPase (EcSecAN68) and for their antimicrobial activity against *Escherichia coli* NR698 (a leaky mutant) and *Bacillus anthracis* Sterne. Twelve compounds showed IC_{50} of less than 6.3 μM when tested against EcSecAN68. In antimicrobial studies against *E. coli* NR698, six compounds showed MIC of <12.5 μM with three being less than 6.3 μM. Against *B. anthracis* Sterne, three compounds showed MIC of <6.3 μM.

Keywords SecA inhibitor · Small molecule · Antimicrobial · Target · Drug-resistant

Introduction

Infectious diseases caused by bacterial pathogens have become a major public health problem in recent years due to the widespread occurrence of drug resistance. Therefore, there is an urgent need to develop new antimicrobial agents, especially those with a new drug target and/or with the ability to overcome drug resistance [1]. Along this line, we are interested in targeting SecA, which is a critical protein secretion machinery essential for bacterial survival [2–4]. SecA is a key component of the bacterial protein secretion (Sec) pathways. SecA ATPase is one of the essential components in the Sec machinery, which provides a major pathway to help protein translocation from the cytosol across or into the cytoplasmic membrane [5–11]. Because SecA is a conserved and essential protein in all bacteria and is absent in humans, it is considered as a promising antibacterial drug target. At present, small organic molecules that can inhibit SecA mainly include Rose Bengal [12], bisthiouracil [13], bistriazole [14] and their derivatives [15], thiazolo[4,5-d] pyrimidine derivatives [16], and others [17, 18].

As an extension of our earlier work [15, 19], we are working on optimizing existing SecA inhibitors of the substituted pyrimidine scaffold by exploring the chemistry space at the 2 and 4 positions of the pyrimidine core [13]. Below, we describe the results and implications in guiding future work in this area.

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Results and discussion

Chemistry

Earlier work has identified the compound in Fig. 1 as a lead for further optimization [15, 19].

To improve the potency, we focused our attention at the linker between the biphenyl rings and the *para* position of the benzylthio group. Specifically, the designed compounds were synthesized by introducing (a) linkers such as thioethers (–S–, $–CH_2–S–$), an inverted alkoxy ($–O–CH_2–$), an alkenyl group (–CH=CH–), an alkynyl (–C≡C–) group, and an amido group ($-CH_2$-NH-CO-) between the two phenyl rings and (b) substituents such as $–N_3$, –COOMe, $-CF_3$ at the *para* position of the benzylthioether moiety. Thus, a series of pyrimidine derivatives **3a**–**o** were synthesized. The synthetic route is shown in Scheme 1. Compounds **1a**, **b** were prepared by nucleophilic aromatic substitution reaction of 4-nitrobenzaldehyde [20] or 4-fluorobenzaldehyde [21] using the appropriate aryl thiols under basic conditions. Compound **1c** was obtained by following a published procedure [20] in Scheme 2. Palladium catalyzed Mizoroki-Hech reaction of 4-bromobenzaldehyde with styrene yielded **1d** [22]. Compound **1e** was prepared by palladium catalyzed Sonogoshira cross coupling of 4-bromobenzaldehyde with phenylacetylene [23]. Then 4-formylbenzoic acid was coupled with benzylamine using isobutyl chloroformate in the presence of triethylamine to provide amide **1f** by following a published procedure [24]. The reaction of the appropriate aromatic aldehydes, cyano ethyl acetate and thiourea with piperidine as catalyst in absolute ethanol at reflux temperature overnight afforded thiouracil **2a**–**f**. The target compounds **3a**–**o** were obtained by the *S*-benzylation in the presence of potassium carbonate in acetonitrile. Overall, 15 compounds have been prepared in overall yields ranging from 47–98 using readily available starting materials.

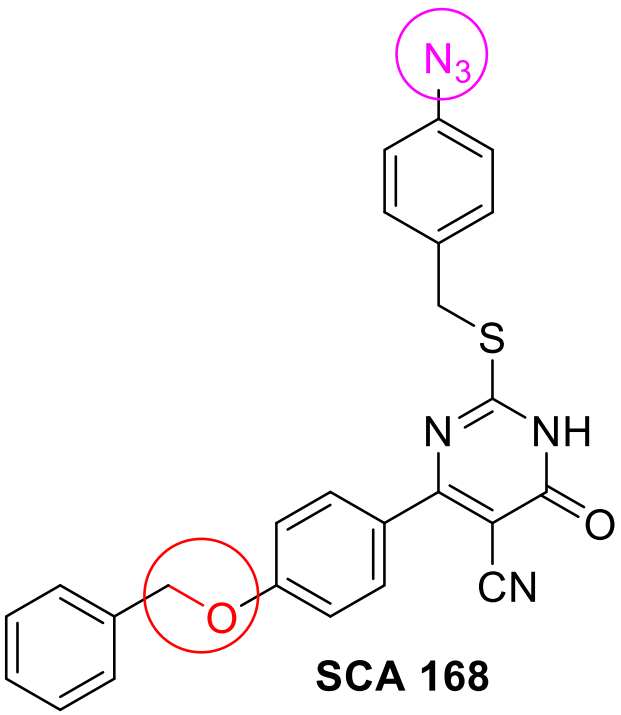
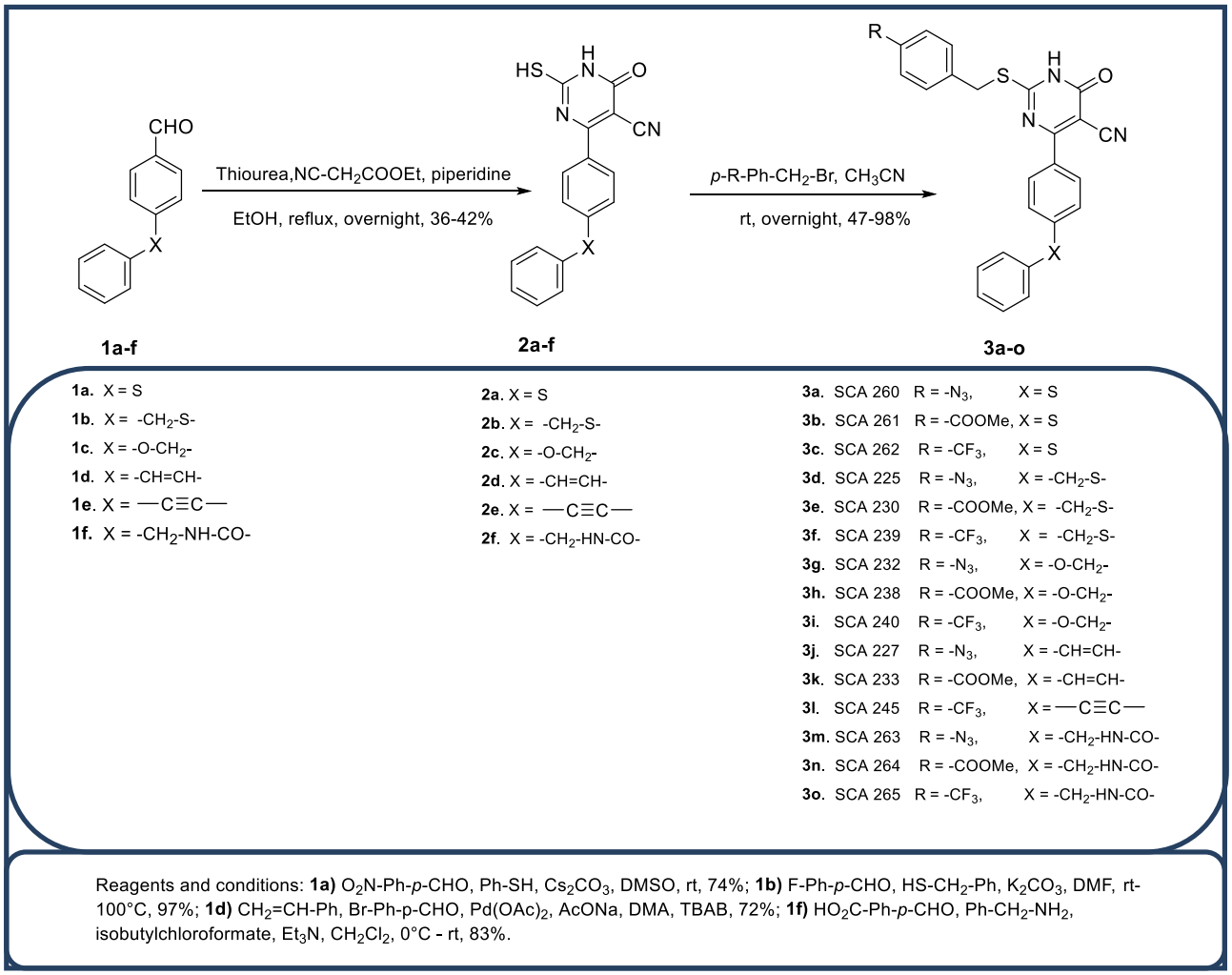


Fig. 1 Lead compound for the synthesis of novel SecA inhibitors

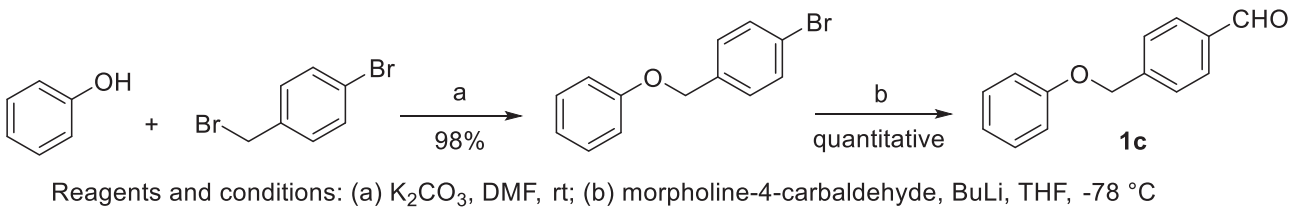
Biological evaluation

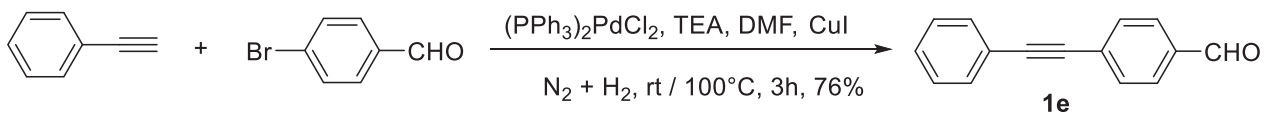
The activities of all newly synthesized compounds **3a**–**o** were first screened using a truncated version of *E. coli* SecA, EcSecAN68, at 6.25 μM [15]. As it can be seen in Fig. 2, most of these 15 new compounds showed more than 50% inhibition of the SecA ATPase activity at this concentration, suggesting potent inhibitory activity. Some of the compounds seemed to be more potent than the lead compound, SCA168, and showed more than 90% inhibition at 6.25 μM such as SCA225, 227, 230, 232, 233, 239, 245, 260, and 262. The overall results from the SecA inhibition studies seem to suggest that the "linker" part can accommodate thioethers (SCA225, 230, 239, and SCA260–262), an inverted oxo ether (SCA232, 238, 240), an alkenyl linker (SCA227, 233), and alkynyl linker (SCA245) as well as variations in the length by one methylene group (1–2 atoms) among those with a thioether linker. Among all these variations, it is especially interesting to see the accommodation of the linearization of the linker with an alkynyl group (SCA245). It also seems that the para-position of the thiobenzyl group of the 4-position of the pyrimidine ring can tolerate various groups. From this limited set of compounds, it is also clear that a three-atom linker in the form of an amido group is not well-tolerated.

All compounds were also evaluated for their antimicrobial activity against a "leaky" outer membrane mutant of Gram-negative *E. coli* NR698 [25]. Among them, four compounds showed minimum inhibitory concentration (MIC) at about 12.5 μM (SCA225, 232, 240, 261) and two compounds showed MIC at about 6.3 μM (SCA260, 262) against *E. coli* NR698 (Table 1). Compounds were also evaluated for their antimicrobial activity against Gram positive *B. anthracis* Sterne (Table 2). Three of them showed MIC at about 6.3 μM (SCA225, 227, 232) against *B. anthracis* Sterne, which are comparable to some of our best compounds in this class [15]. With the limited data set, it seems that the potency against *B. anthracis* is higher than that of *E. coli*. Such results are understandable because *E. coli* is Gram-negative strain. Even with a compromised outer membrane, it still has an outer membrane, which presents a permeability barrier [26].



Scheme 1 Synthesis of compound **3a–o**





Scheme 2 Synthesis of compound **1c–e**

Overall, the results provide very useful information for those who might be interested in SecA inhibitor design for both improved potency and for avoiding the chemical space that would not be productive.

Conclusion

We have described the design, syntheses, and biological evaluation of a series of 15 small-molecule SecA inhibitors

with μM inhibition. The results provide information on tolerable structural features of the linker part between the two phenyl rings at the 2-position and the para-substitution of the benzyl group at the 4-position of the pyrimidine ring.

Experimental

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

General procedure for the synthesis of (2a–f)

Our previously published [15] procedure was followed. Briefly, to a solution of ethanol (25 ml) and appropriate aldehyde (RCHO, 5 mmol) was added ethyl cyanoacetate (0.5 ml, 5 mmol), thiourea (0.38 g, 5 mmol) and piperidine (1.0 ml, 10 mmol). The mixture was heated at reflux overnight and then cooled to room temperature. The precipitate was dissolved in 0.5 M NaOH (20 ml) and washed with ethyl acetate (15 ml × 3). Then the aqueous solution was acidified to pH ~2 by slow addition of 1 M HCl. This caused the product to precipitate, which was then filtered using vacuum filtration.

General procedure for the synthesis of (3a–o)

To a solution of 2-mercapto-6-oxo-4-(4-(phenylthio)phenyl)-1,6-dihydropyrimidine-5-carbonitrile derivatives (1.36 mmol) in CH_3CN (10 ml), K_2CO_3 (6.79 mmol) was added and the resulting mixture was stirred for 10–15 min. To this was added the appropriate (bromomethyl)phenyl derivatives (1.22 mmol) and the reaction was stirred at room temperature for 16–18 h.

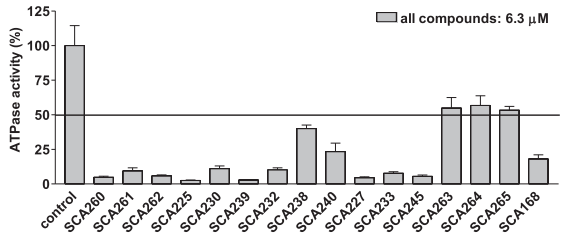


Fig. 2 Screening for inhibition of the ATPase activity of EcSecAN68

Upon completion, the reaction mixture was cooled to ambient temperature and the solvent removed in vacuo. The dried residue was washed by H_2O (pH = 9–10, 20 ml × 2) and brine (15 ml × 2) followed by product extraction in ethyl acetate (20 ml). The solvent was evaporated in vacuo to obtain a crude product, which was purified using silica gel column chromatography.

2-Mercapto-6-oxo-4-(4-(phenylthio)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (2a) Yield: 58%

^{1}H NMR (DMSO-d_6): δ 13.26 (bs, 1H), 12.91 (s, 1H), 7.63 (d, J = 8.4 Hz, 2H), 7.50 (m, 5H), 7.31 (d, J = 8.4 Hz, 2H); ^{13}C NMR (DMSO-d_6): δ 177.6, 161.5, 159.5, 142.3, 133.8, 132.0, 131.9, 131.2, 130.8, 130.5, 130.1, 130.1, 129.5, 128.5, 127.9, 115.8, 90.1 ppm. HRMS (ESI-TOF) (m/z): Calcd. for $C_{17}H_{10}N_3OS_2$, [M–H$^+$]: 336.0260; found: 336.0263.

4-(4-(Benzylthio)phenyl)-2-mercapto-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (2b) Yield: 45%

^{1}H NMR (DMSO-d_6): δ 13.81 (s, 1H), 13.17 (bs, 1H), 7.61 (d, J = 8.8 Hz, 2H), 7.47 (d, J = 8.4 Hz, 2H), 7.44 (d, J = 7.2 Hz, 2H), 7.33 (t, J = 7.6 Hz, 2H), 7.25 (m, 1H), 4.37 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 178.1, 162.1, 159.9, 142.4, 137.3, 129.7, 129.3, 129.0, 128.1, 127.7, 126.6, 116.2, 89.5, 35.8. HRMS (ESI-TOF) (m/z): Calcd. for $C_{18}H_{12}N_3OS_2$, [M-H$^+$]: 350.0416; found: 350.0420.

2-Mercapto-6-oxo-4-(4-(phenoxymethyl)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (2c) Yield: 36%

^{1}H NMR (DMSO-d_6): δ 13.31 (bs, 1H), 13.14 (s, 1H), 7.71 (d, J = 8.4 Hz, 2H), 7.63 (d, J = 8.0 Hz, 2H), 7.31 (t, J = 7.6 Hz, 2H), 7.04 (d, J = 8.0 Hz, 2H), 6.96 (t, J = 7.2 Hz, 1H), 4.37 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 176.8, 161.3, 159.2, 158.6, 142.1, 130.0, 129.5, 127.8, 121.5, 115.4, 91.1, 68.9. HRMS (ESI-TOF) (m/z): Calcd. for $C_{18}H_{12}N_3O_2S$, [M-H$^+$]: 334.0645; found: 334.0645.

(E)-2-Mercapto-6-oxo-4-(4-styrylphenyl)-1,6-dihydropyrimidine-5-carbonitrile (2d) Yield: 56%

^{1}H NMR (DMSO-d_6): δ 13.32 (bs, 1H), 12.93 (s, 1H), 7.78 (d, J = 8.4 Hz, 2H), 7.71 (d, J = 8.4 Hz, 2H), 7.66 (d,

Table 2 SecA inhibitors with MIC of <50 μM against *B. anthracis* Sterne

	SCA225	SCA227	SCA230	SCA232	SCA168
MIC (μM)	6.25	6.25	25	6.25	6.25

Table 1 SecA inhibitors with MIC of <50 μM against *E. coli* NR698

	SCA225	SCA232	SCA238	SCA239	SCA240	SCA260	SCA261	SCA262	SCA168
MIC (μM)	12.5	12.5	50	50	12.5	6.25	12.5	6.25	12.5

$J = 7.2$ Hz, 2H), 7.37–7.48 (m, 4H), 7.33 (d, $J = 6.0$ Hz, 1H); ^{13}C NMR (DMSO-d_6): δ 177.5, 161.7, 159.5, 141.1, 137.0, 131.5, 129.7, 129.4, 129.2, 128.9, 128.7, 127.7, 127.3, 126.6, 115.8, 90.0. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{19}H_{12}N_3OS$, [M-H$^+$]: 330.0696; found: 330.0698.

2-Mercapto-6-oxo-4-(4-(phenylethynyl)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (2e) [15] Yield: 58%

^{1}H NMR (DMSO-d_6): δ 10.03 (s, 1H), 7.87 (d, $J =$ 8.0 Hz, 2H), 7.69 (d, $J = 8.0$ Hz, 2H), 7.57 (t, $J = 8.0$ Hz, 2H), 7.39 (t, $J = 8.0$ Hz, 2H); ^{13}C NMR (DMSO-d_6): δ 171.4, 135.4, 132.1, 131.8, 129.6, 129.0, 128.5, 122.5, 93.4, 88.5. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{19}H_{10}N_3OS$, [MH-]: 328.0545; found: 328.0541.

***N*-Benzyl-4-(5-cyano-2-mercapto-6-oxo-1,6-dihydropyrimidin-4-yl)benzamide (2f)** Yield: 58%

^{1}H NMR (DMSO-d_6): δ 13.41 (s, 1H), 13.20 (s, 1H), 9.26 (t, $J = 6.0$ Hz, 1H), 8.04 (d, $J = 8.4$ Hz, 2H), 7.78 (d, $J =$ 8.0 Hz, 2H), 7.34 (d, $J = 4.4$ Hz, 4H), 7.28 (m, 1H), 4.51 (d, $J = 6.0$ Hz, 2H); ^{13}C NMR (DMSO-d_6): 176.6, 165.8, 160.7, 158.8, 139.8, 137.8, 132.1, 129.5, 129.4, 128.7, 127.8, 127.6, 127.2, 115.0, 91.6, 43.2. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{19}H_{13}N_4O_2S$, [M-H$^+$]: 361.0754; found: 361.0756.

2-((4-Azidobenzyl)thio)-6-oxo-4-(4-(phenylthio)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (3a) Yield: 90%

^{1}H NMR (DMSO-d_6): δ 7.83 (d, $J = 8.0$ Hz, 2H), 7.49 (m, 3H), 7.43 (d, $J = 8.4$ Hz, 2H), 7.32 (d, $J = 8.4$ Hz, 2H), 7.04 (d, $J = 8.0$ Hz, 2H), 4.51 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 169.2, 166.3, 166.6, 166.4, 140.4, 138.6, 135.2, 134.8, 133.2, 132.8, 131.0, 130.3, 129.8, 129.0, 128.6, 119.5, 118.4, 91.1, 33.9. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{24}H_{15}N_6OS_2$, [M-H$^+$]: 467.0749; found: 467.0751.

Methyl4-(((5-cyano-6-oxo-4-(4-(phenylthio)phenyl)-1,6-dihydropyrimidin-2-yl)thio)methyl)benzoate (3b) Yield: 97%

^{1}H NMR (DMSO-d_6): δ 7.87 (d, $J = 8.4$ Hz, 2H), 7.77 (d, $J = 8.0$ Hz, 2H), 7.54 (d, $J = 8.0$ Hz, 2H), 7.41–7.49 (m, 5H), 7.31 (d, $J = 8.4$ Hz, 2H), 4.42 (s, 2H), 3.83 (s, 3H); ^{13}C NMR (DMSO-d_6): δ 170.0, 166.6, 166.4, 144.6, 139.7, 135.2, 133.0, 130.3, 129.7, 129.6, 129.6, 128.9, 128.8, 128.6, 119.0, 90.5, 52.5, 34.0. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{26}H_{18}N_3O_3S_2$, [M-H$^+$]: 484.0795; found: 484.0774.

6-Oxo-4-(4-(phenylthio)phenyl)-2-((4-(trifluoromethyl)benzyl)thio)-1,6-dihydropyrimidine-5-carbonitrile (3c) Yield: 90%

^{1}H NMR (DMSO-d_6): δ 7.79 (d, $J = 8.4$ Hz, 2H), 7.64 (qd, $J = 8.4$ Hz, 4H), 7.43–7.51 (m, 5H), 7.31(d, $J =$ 8.0 Hz, 2H), 4.49 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 168.6, 166.7, 166.0, 143.4, 140.7, 134.5, 133.3, 130.3, 130.0, 129.8, 129.1, 128.5, 128.2, 127.9, 126.0, 125.6, 123.3, 118.0, 91.4, 55.3, 33.8. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{25}H_{15}F_3N_3OS_2$, [M-H$^+$]: 494.0614; found: 494.0598.

2-((4-Azidobenzyl)thio)-4-(4-(benzylthio)phenyl)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (3d) Yield: 40%

^{1}H NMR (DMSO-d_6): δ 7.90 (d, $J = 8.0$ Hz, 2H), 7.50 (d, $J = 8.4$ Hz, 2H), 7.44 (d, $J = 7.6$ Hz, 4H), 7.33 (t, $J =$ 8.0 Hz, 2H), 7.26 (m, 1H), 7.06 (d, $J = 8.4$ Hz, 2H), 4.52 (s, 2H), 4.39 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 166.6, 165.7, 161.5, 144.8, 142.8, 139.0, 137.2, 133.9, 132.1, 131.1, 129.3, 128.9, 127.7, 126.8, 119.6, 116.4, 92.8, 35.7, 34.1. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{25}H_{17}N_6OS_2$, [M-H$^+$]: 481.0905; found: 481.0911.

Methyl 4-(((4-(4-(benzylthio)phenyl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio)methyl) benzoperoxoate (3e) Yield: 68%

^{1}H NMR (DMSO-d_6): δ 7.88 (d, $J = 8.0$ Hz, 2H), 7.69 (d, $J = 8.0$ Hz, 2H), 7.53 (d, $J = 8.0$ Hz, 2H), 7.42 (t, $J =$ 6.4 Hz, 4H), 7.31 (t, $J = 7.2$ Hz, 2H), 7.24 (m, 1H), 4.34 (s, 2H), 4.32 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 171.6, 170.8, 166.6, 166.5, 145.6, 139.3, 137.6, 135.0, 129.6, 129.3, 129.2, 129.0, 128.9, 128.4, 127.6, 127.2, 127.0, 120.5, 89.1, 52.5, 36.3, 33.8. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{27}H_{20}N_3O_3S_2$, [M-H$^+$]: 498.0946; found: 498.0953.

4-(4-(Benzylthio)phenyl)-6-oxo-2-((4-(trifluoromethyl)benzyl)thio)-1,6-dihydropyrimidine-5-carbonitrile (3 f) Yield: 59%

^{1}H NMR (DMSO-d_6): δ 7.83 (d, $J = 8.4$ Hz, 2H), 7.68 (d, $J = 8.4$ Hz, 2H), 7.63 (d, $J = 8.0$ Hz, 2H), 7.48 (d, $J =$ 8.0 Hz, 4H), 7.44 (d, $J = 7.2$ Hz, 2H), 7.32 (t, $J = 7.6$ Hz, 2H), 7.25 (m, 1H), 4.59 (s, 2H), 4.38 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 166.7, 165.8, 142.5, 137.2, 132.2, 130.1, 129.6, 129.3, 128.9, 127.7, 126.8, 125.8, 125.7, 117.02, 92.9, 35.8, 33.9. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{26}H_{17}F_3N_3OS_2$, [M-H$^+$]: 508.076; found: 508.0773.

2-((4-Azidobenzyl)thio)-6-oxo-4-(4-(phenoxymethyl)phenyl)-1,6-dihydropyrimidine-5-carbonitrile (3 g) Yield: 66%

^{1}H NMR (DMSO-d_6): δ 7.96 (d, $J = 8.0$ Hz, 2H), 7.65 (d, $J = 7.6$ Hz, 2H), 7.45 (d, $J = 8.0$ Hz, 4H), 7.31 (t, $J =$ 7.6 Hz, 2H), 7.05 (t, $J = 8.8$ Hz, 4H), 6.96 (t, $J = 6.8$ Hz, 1H), 5.22 (s, 2H), 4.53 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 167.4, 166.01, 161.6, 158.5, 141.6, 139.1, 135.0, 133.8, 131.1, 130.0, 129.4, 127.9, 127.8, 121.3, 119.6, 116.3, 115.2, 93.6, 68.8, 34.1. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{25}H_{17}N_6O_2S$, [M-H$^+$]: 465.1134; found: 465.1149.

Methyl-4-(((5-cyano-6-oxo-4-(4-(phenoxymethyl)phenyl)-1,6-dihydropyrimidin-2-yl)thio)methyl)benzoate (3h) Yield: 68%

^{1}H NMR (DMSO-d_6): δ 7.90 (d, $J = 8.0$ Hz, 4H), 7.62 (d, $J = 8.0$ Hz, 2H), 7.57 (d, $J = 8.0$ Hz, 2H), 7.31 (t, $J =$ 7.6 Hz, 2H), 7.04 (d, $J = 7.6$ Hz, 2H), 6.96 (t, $J = 7.2$ Hz, 1H), 5.02 (s, 2H), 4.57 (s, 2H), 3.84 (s, 3H); ^{13}C NMR

(DMSO-d_6): δ 171.4, 167.5, 166.4, 158.6, 143.1, 141.5, 135.2, 130.0, 129.7, 129.3, 127.9, 121.3, 115.2, 108.6, 93.4, 68.9, 52.6, 34.2. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{27}H_{20}N_3O_4S$, [M-H$^+$]: 482.1175; found: 482.1175.

6-Oxo-4-(4-(phenoxymethyl)phenyl)-2-((4-(trifluoromethyl)benzyl)thio)-1,6-dihydropyrimidine-5-carbonitrile (3i) Yield: 65%

^{1}H NMR (DMSO-d_6): δ 7.90 (d, $J = 8.0$ Hz, 2H), 7.62–7.69 (m, 6H), 7.31 (t, $J = 7.6$ Hz, 2H), 7.04 (d, $J =$ 8.0 Hz, 1H), 6.95 (t, $J = 7.2$ Hz, 1H), 5.22 (s, 2H), 4.61 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 168.6, 167.4, 165.9, 158.5, 142.4, 141.6, 135.0, 130.2, 130.0, 129.3, 127.9, 125.8, 125.7, 121.3, 116.4, 115.2, 93.7, 68.8, 33.9. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{26}H_{17}F_3N_3O_2S$, [M-H$^+$]: 492.0994; found: 492.0989.

^{1}H NMR (DMSO-d_6): δ 8.00 (d, $J = 8.0$ Hz, 2H), 7.81 (d, $J = 8.4$ Hz, 2H), 7.66 (d, $J = 7.6$ Hz, 2H), 7.48 (d, $J =$ 8.4 Hz, 2H), 7.40 (m, 3H), 7.33 (d, $J = 6.4$ Hz, 2H), 7.08 (d, $J = 5.2$ Hz, 2H), 7.32 (d, $J = 8.4$ Hz, 2H), 5.55 (s, 2H); ^{13}C NMR (DMSO-d_6): 167.6, 166.9, 165.9, 141.1, 139.1, 137.1, 134.0, 131.5, 131.1, 129.7, 129.2, 128.9, 128.6, 128.3, 127.8, 127.3, 126.9, 119.7, 93.0, 34.1. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{26}H_{17}N_6OS$, [M-H$^+$]: 461.1185; found: 461.1179.

(E)-Methyl 4-(((5-cyano-6-oxo-4-(4-styrylphenyl)-1,6-dihydropyrimidin-2-yl)thio)methyl) benzoate (3k) Yield: 34%

^{1}H NMR (DMSO-d_6): δ 7.94 (d, $J = 8.4$ Hz, 2H), 7.91 (d, $J = 7.6$ Hz, 2H), 7.79 (d, $J = 7.6$ Hz, 2H), 7.66 (d, $J =$ 7.6 Hz, 2H), 7.59 (d, $J = 7.6$ Hz, 2H), 7.42 (d, $J = 6.4$ Hz, 2H), 7.39 (d, $J = 5.2$ Hz, 2H), 7.32 (d, $J = 8.4$ Hz, 2H), 5.61 (s, 2H), 3.84 (s, 3H); ^{13}C NMR (DMSO-d_6): δ 167.0, 166.5, 165.9, 162.1, 143.2, 141.1, 137.1, 134.4, 131.4, 129.7, 129.2, 129.0, 128.7, 127.7, 127.2, 126.9, 116.7, 93.1, 52.6, 34.1. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{28}H_{20}N_3O_3S$, [M-H$^+$]: 478.1225; found: 478.1240.

6-Oxo-4-(4-(phenylethynyl)phenyl)-2-((4-(trifluoromethyl)benzyl)thio)-1,6-dihydropyrimidine-5-carbonitrile (3l) Yield: 51%

^{1}H NMR (DMSO-d_6): δ 7.93 (d, $J = 8.0$ Hz, 2H), 7.64–7.75 (m, 6H), 7.65 (m, 2H), 7.46(m, 3H), 4.60 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 166.6, 162.5, 142.6, 135.6, 132.0, 131.9, 130.2, 129.6, 129.4, 129.3, 125.9, 125.8, 122.3, 116.5, 93.7, 92.3, 89.1, 33.9. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{27}H_{16}F_3N_3OS$, [M-H$^+$]: 486.0888; found: 486.0884.

4-(2-((4-Azidobenzyl)thio)-5-cyano-6-oxo-1,6-dihydropyrimidin-4-yl)-N-benzylbenzamide (3m) Yield: 55%

^{1}H NMR (DMSO-d_6): δ 9.24 (t, $J = 6.0$ Hz 1H), 8.04 (d, $J = 8.4$ Hz, 2H), 7.98 (d, $J = 8.4$ Hz, 2H), 7.45 (d, $J = 8.4$ Hz, 2H), 7.34 (d, $J = 4.0$ Hz, 4H), 7.26 (m, 1H), 7.12 (t, $J =$ 7.6 Hz, 1H), 7.07 (d, $J = 8.4$ Hz, 2H), 4.51 (m, 4H); ^{13}C NMR (DMSO-d_6): δ 167.1, 166.7, 166.0, 162.0, 139.9, 139.0, 138.2, 137.3, 134.1, 131.1, 129.2, 128.7, 127.9, 127.2, 119.6, 116.3, 93.9, 43.2, 34.1. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{26}H_{18}N_7O_2S$, [M-H$^+$]: 492.1248; found: 492.1232.

Methyl 4-(((4-(4-(benzylcarbamoyl)phenyl)-5-cyano-6-oxo-1,6-dihydropyrimidin-2-yl)thio) methyl)benzoate (3n) Yield: 63%

^{1}H NMR (DMSO-d_6): δ 9.24 (t, $J = 6.0$ Hz 1H), 7.98 (d, $J = 8.0$ Hz, 2H), 7.88 (dd, $J = 8.0$ Hz, 4H), 7.55 (d, $J =$ 8.0 Hz, 2H), 7.34 (d, $J = 4.4$ Hz, 4H), 7.25 (m, 1H), 4.51 (d, $J = 6.0$ Hz, 2H), 4.44 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 170.3, 168.0, 166.9, 166.5, 166.1, 144.7, 139.9, 139.7, 136.2, 129.6, 128,7, 128.6, 127.6, 127.2, 118.9, 91.0, 52.5, 43.1, 34.0. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{28}H_{21}N_4O_4S$, [M-H$^+$]: 509.1289; found: 509.1271.

***N*-Benzyl-4-(5-cyano-6-oxo-2-((4-(trifluoromethyl)benzyl)thio)-1,6-dihydropyrimidin-4-yl)benzamide (3o)** Yield: 68%

^{1}H NMR (DMSO-d_6): δ 9.21 (t, $J = 6.0$ Hz 1H), 8.02 (d, $J = 8.0$ Hz, 2H), 7.92 (d, $J = 8.4$ Hz, 2H), 7.66 (qd, $J =$ 8.0 Hz, 4H), 7.34 (d, $J = 4.4$ Hz, 4H), 7.26 (m, 1H), 4.58 (s, 2H), 4.52 (d, $J = 6.0$ Hz, 2H); ^{13}C NMR (DMSO-d_6): δ 167.2, 167.1, 166.0, 163.1, 142.8, 139.9, 138.5, 137.1, 130.2, 129.1, 128.7, 128.4, 128,1, 127.8, 127.2, 126.0, 125.7, 123.3, 116.8, 93.5, 43.2, 33.9. HRMS (ESI-TOF) (*m/z*): Calcd. for $C_{27}H_{18}F_3N_4O_2S$, [M-H$^+$]: 519.1097; found: 519.1091.

ATPase assays

Inhibition on ATPase activity of EcSecAN68 was determined by malachite green colorimetric assay as previously described [15]. IC_{50} is defined as the concentration of the compound that inhibits 50% of ATPase activity.

Bacteriostatic effects

Bacteriostatic effects were evaluated at 37 °C in 96-well microtitier plates as previously described [15]. Minimum inhibitory concentration (MIC) is the lowest concentration of compounds at which bacterial cells were not able to grow at tested condition.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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