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Research paper

Design, synthesis and antimicrobial activities of thiouracil derivatives containing triazolo-thiadiazole as SecA inhibitors

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ABSTRACT

A series of novel thiouracil derivatives containing a triazolo-thiadiazole moiety (**7a-7l**) have been synthesized by structural modifications on a lead SecA inhibitor, **2**. All the compounds have been evaluated for their antibacterial activities against *Bacillus amyloliquefaciens*, *Staphylococcus aureus*, and *Bacillus subtilis*. Compounds **7d** and **7g** were also tested for their inhibitory activities against SecA ATPase due to their promising antimicrobial activities. The inhibitory activity of compound **7d** was found to be higher than that of **2**. Molecular docking work suggests that compound **7d** might bind at a pocket close to the ATPase ATP-binding domain.

1. Introduction

Since the 1980s, bacterial drug resistance has become a major problem because of the widespread use, even abuse, of antibiotics. With Gram-positive bacteria, many drug resistant strains including *methicillin-resistant Staphylococcous aureurs* (MRSA), *methicillin-resistant Staphylococcus epidermidis* (MRSE), *penicillin-resistant Streptococcus pneumonia* (PRSP) and *vancomycin-resistant Enterococci* (VRE) are posing serious public health problems [1–4]. As a result, there is an urgent need for new types of antibacterial drugs, especially important is the search for new targets or mechanisms to achieve antimicrobial effects [5–12]. Along this line [13–16], we are interested in targeting SecA, which is a critical protein secretion machinery essential for bacterial survival.

In bacterial cells, over 30% proteins become functional after they are transported outside of the cytoplasm. *Trans*-membrane movement of most of these proteins is *via* the Sec pathway (i.e. secretion pathway). 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 [17–20]. Because SecA is a conserved and essential protein in nearly all bacteria, it is considered as a promising antibacterial drug target [21–25]. In recent years, studies have confirmed that inhibiting SecA can lead to bacteriostatic and bactericidal effects [26,27]. Therefore, developing innovative antibacterial drugs by targeting SecA has drawn much attention. It is expected that such inhibitors may have the intrinsic ability to minimize side effects because there is no counterpart of SecA in human. At present, small organic molecules that can inhibit SecA mainly include Rose Bengal **1**, thiouracil **2**, and triazole **3** derivatives (Fig. 1) [28–31]. In order to find new SecA inhibitors to better understand the SAR and the biological mechanism of SecA, it is worthwhile to explore the chemistry space of known inhibitor to increase structural diversity.

The compounds containing triazole or thiadiazole have a wide range of biological activity. Such two pharmacophore were fused to a triazolo-thiadiazole moiety, which also has a variety of biological and pharmacological activity [32–34], such as antibacterial and anti-tumor. Due to their strong ability to form hydrogen bond with macro biomoleculars [35], we would like to report the design and synthesis of compound **7** containing both thiouracil and triazolo-thiadiazole motifs (Scheme 1). Such compounds are based on the

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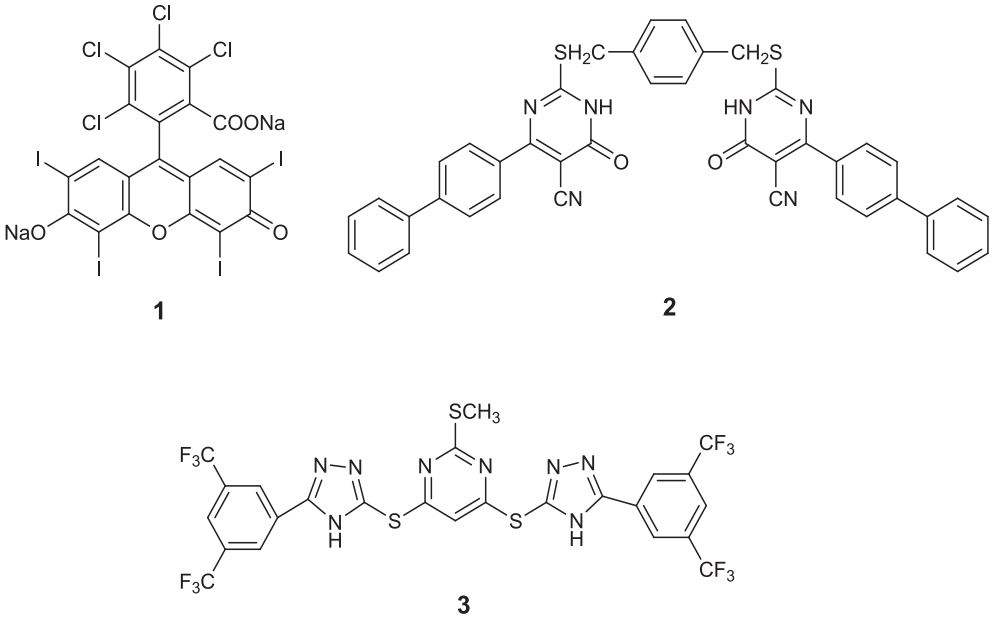
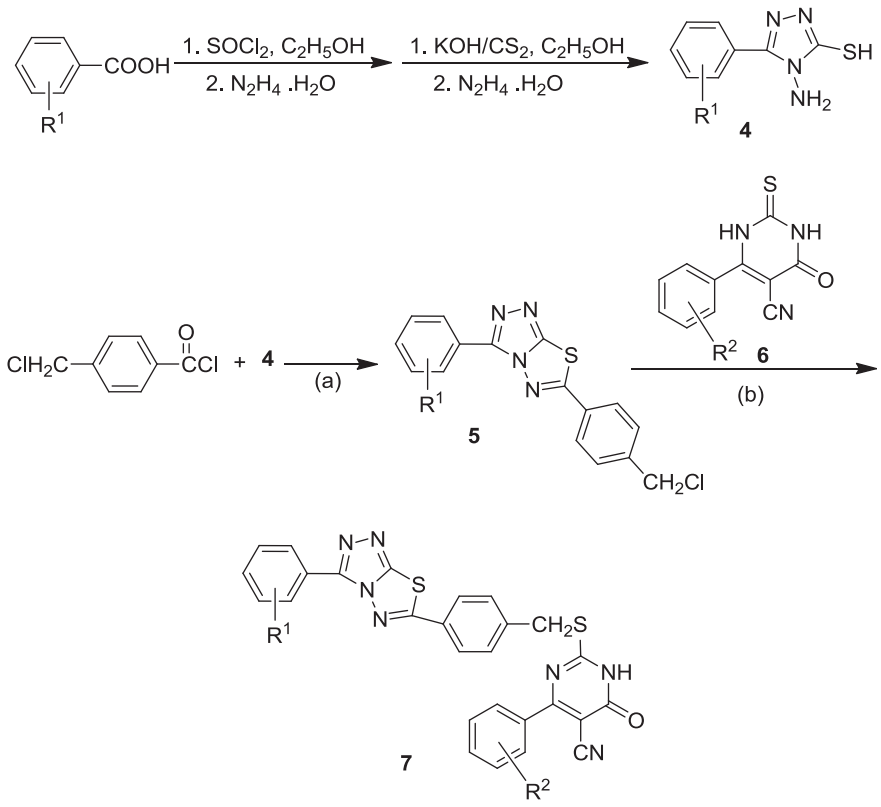


Fig. 1. The structures of SecA inhibitors.



7a-c: R^1 = H, R^2 = H, 2-Cl, 4-Cl;
7d-f: R^1 = H, R^2 = 2,4-diCl, 2,6-diCl, 4-Ph;
7g-i: R^1 = 2-Cl, R^2 = 2,4-diCl, 2,6-diCl, 4-Ph;
7 j-l: R^1= 2,6-diCl, R^2 = 2,4-diCl, 2,6-diCl, 4-Ph

Scheme 1. Synthesis of compounds **7a-7l**. *Conditions and reagents*: (a) $POCl_3$, reflux; (b) K_2CO_3, acetonitrile, reflux.

parent compound **2** (Fig. 1). We hypothesize that the triazolo-thiadiazole scaffold would have the capability to form more hydrogen-bond interactions with the target protein SecA, and would be beneficial to improving the antibacterial activity.

2. Results and discussions

2.1. Chemistry

In this study, a series of novel thiouracil derivatives (**7a-l**) containing a triazolo-thiadiazole moiety were synthesized. The synthetic route for **7** is shown in Scheme 1. The reaction of aromatic aldehydes, cyano ethyl acetate and thiourea with pyridine as catalyst in absolute ethanol at reflux temperature for 8 h afforded thiouracil **6** [28]. Intermediate **4**, prepared by using aromatic acids as starting materials through multi-step reactions [30], was reacted with 4-(chloromethyl)benzoyl chloride in $POCl_3$ to give compound **5**. The target compounds **7** were obtained by reacting **5** and thiouracil **6** in acetonitrile at reflux temperature.

2.2. Antibacterial activity

All the newly synthesized compounds were evaluated for their antibacterial activities against three strains of Gram-positive organisms including *Bacillus amyloliquefaciens*, *Staphylococcus aureus*, and *Bacillus subtilis* [36,37]. Norfloxacin and the parent compound **2** (Fig. 1) were used as the positive controls. The tests were carried out in accordance with the plate colony counting protocol. The results are shown in Table 1 (the concentration is 25 μg/mL). The results show that compounds **7** (**a-l**) have good to potent inhibitory activity against the three tested bacterial strains. Among them, compounds **7d** and **7g** have the strongest antibacterial activities against *B. subtilis* and their inhibitory rates were above 90%, higher than that of approved drug norfloxacin. Their inhibitory rates exceeded 85% against *S. aureus*, similar with that of norfloxacin. Although their *anti-B. amyloliquefaciens* activities were less than that of norfloxacin, their inhibitory rates were also above 80%, which suggested that both compounds had potent antibacterial activities. Compounds **7i-7l** also had broad antibacterial activities against the tested three organisms with above 80% inhibition except **7j** against *B. amyloliquefaciens*, similar with that of norfloxacin against *B subtilis*. The anti *B. subtilis* activities of compounds **7d, 7g** and **7i-l** were superior to that of compound **2**, a known SecA inhibitor as antibacterial agent. Additionally, the activities of **7i** and **7k** against *B. amyloliquefaciens*, **7d** against *S. aureus*, and **7h** against *B. subtilis* were slightly higher than that of **2**. Such results indicate that the introduction of the triazolo-thiadiazole pharmacophore to **2** could increase and broaden its antibacterial activity. When R^1 is H or 2-Cl, compound **7d** and **7g** (R^2 = 2,4-dichloro) have relatively higher antibacterial activities than those of the tested compounds, such results indicate that two Cl atoms on the phenyl ring near thiouracil seemed to be favorable for their antibacterial activities. The activities of compounds **7j-l** improved significantly when R^1 is 2,6-diCl on the phenyl near triazolo-thiadiazole group and R^2 is fixed as 2,4-dichloro, 2,6-dichloro and 4-phenyl, respectively. The above structure activity relationships (SAR) analysis suggested that the introduction of additional Cl atoms on the marginal phenyls would be beneficial for antibacterial activities. Such insights should help to guide further design and synthesis of the more potent antibacterial agents derived from compound **2**.

2.3. SecA inhibitory activity

Two potent compounds **7d** and **7g** with relatively high antibacterial activities were chosen for evaluation SecA inhibitory activities [28–31]. The results indicated that compound **7d** showed significant inhibitory activity as shown in Fig. 2. For example, the inhibitory activity of **7d** was 51% at 10 μg/mL and 72% at 20 μg/mL. The IC_{50} (50% inhibitory concentrations) value of compound **7d** was calculated as 9.7 μg/mL, which is lower than that of compound **2** (20.8 μg/mL). Unfortunately, the IC_{50} value of compound **7g** was not accurately measured due to its very poor solubility under assay conditions.

2.4. Computational modeling

In order to achieve some initial insights into the binding interactions, the newly synthesized compound **7d** was docked into the SecA crystal structure. Two possible binding pockets were selected: the ATPase ATP-binding site and the pocket between IRA2 and NBD domain [38]. The second pocket is close to the ATP binding site as we proposed before (the closest distance of ATP and docked

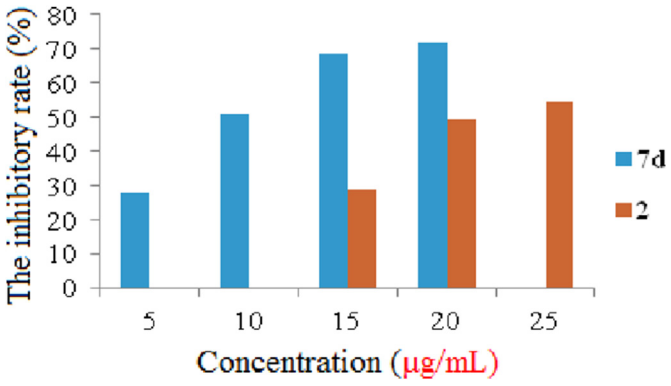


Fig. 2. SecA inhibitory activities of **7d** and **2**.

Table 1
Antibacterial activities of **7a-7l** (Inhibitory rate/% at 25 μg/mL).

Compounds	R^1	R^2	*Bacillus amyloliquefaciens*	*Staphylococcus aureus*	*Bacillus subtilis*
7a	H	H	57%	44%	48%
7b	H	2-Cl	66%	46%	55%
7c	H	4-Cl	62%	49%	51%
7d	H	2,4-dichloro	82%	88%	**90%**
7e	H	2,6-dichloro	60%	56%	49%
7f	H	4-Ph	58%	68%	57%
7g	2-Cl	2,4-dichloro	80%	85%	**92%**
7h	2-Cl	2,6-dichloro	78%	56%	63%
7i	2-Cl	4-Ph	81%	83%	88%
7j	2,6-dichloro	2,4-dichloro	78%	81%	86%
7k	2,6-dichloro	2,6-dichloro	86%	82%	84%
7l	2,6-dichloro	4-Ph	82%	84%	86%
2			85%	87%	62%
Norfloxacin			89%	86%	85%

molecule is 9.3 Å) [31]. 20 outputs were generated and the docking scores 3.56–5.12 at the ATP-binding site and 4.62–5.97 at pocket that was identified for the lead structures [31]. Thus it is reasonable to suggest that the new inhibitor binds at a similar position as the lead structure. The interactions are shown in Fig. 3: molecule **7d** generated hydrogen-bond interactions with ARG566, GLN570, ARG642 and two water molecules. It also forms cation-pi interactions with ARG642 and hydrophobic interaction with VAL131 and ILE216.

3. Conclusions

A series of novel thiouracil derivatives containing a triazolothiadiazole moiety **7** (**a-l**) were designed, synthesized, and evaluated for their antibacterial activities. The results showed that most of the compounds have strong antibacterial activity. Additionally, compound **7d** expressed better inhibitory activity against SecA ATPase than that of the known inhibitor **2**, suggesting a beneficial role for the newly introduced triazole-thiadiazole moiety. Molecular simulations suggest that compound **7** might bind to a pocket close to the ATPase ATP-binding domain.

4. Experimental section

4.1. General

Column chromatography was carried out on flash silica gel (300–800 mesh). TLC analysis was conducted on silica gel plates (Silica G UV254). Melting points were measured in an open capillary on a SGW X-4 melting point apparatus and are uncorrected. Element analysis was performed using a Heraeus (CHNO, rapid) elemental analyzer. IR spectra were determined on a WQF-510 as KBr tablets for solid samples and were expressed in cm^{-1} scale. NMR spectra were recorded at 600 MHz for ^1H on a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and hertz, respectively, using TMS (^1H NMR) solvents as internal standards. ESI-MS spectra were determined using an Agilent G6300 ion Trap mass spectrometer, and signals were recorded in m/z. Absorption spectra were recorded on a FLUOstar Omega MicroplateReader.

4.2. General procedure for the synthesis of compounds *7(a-l)*

To a solution of aromatic acid (10 mmol) in absolute ethanol (50 mL) was added $SOCl_2$ (15 mmol); then the mixture was heated at 60 °C for 3 h. Upon completion, the reaction mixture was cooled to ambient temperature and the solvent was removed in vacuo. The residue was dissolved in ethyl acetate and washed with saturated aqueous sodium bicarbonate (10 mL) and water (10 mL × 2), then the organic layer was dried with sodium sulfate for 2 h. After filtration, the solvent was removed in vacuo to give the aromatic ester. The crude ester (5 mmol) and 30 mL 80% hydrazine hydrate were added into a 100 mL flask; then the mixture were refluxed for about 5 h. After completion of the reaction as monitored by TLC, the mixture was cooled to room temperature, and precipitate was filtered and dried to give the aryl hydrazide product. To a solution of KOH (6 mmol) in absolute ethanol (40 mL) was added the aryl hydrazide (3 mmol). Then CS_2 (10 mL) was added into the mixture dropwise, leading to light yellow precipitates, which were filtered and dried to give the intermediate. A solution of this intermediate (2 mmol) in hydrazine hydrate (20 mL) were heated under reflux for about 4 h. Upon completion, the mixture was poured into cold water (150 mL). Then the aqueous solution was acidified to pH ~5 by slow addition of 1 M HCl. This caused the formation of precipitates, which were filtered using vacuum filtration to give compound **4**.

A solution of compound **4** (1 mmol) and 4-(chloromethyl) benzoyl chloride (1.2 mmol) in $POCl_3$ (30 mL) were heated under reflux for about 5 h. Upon completion, the solvent was removed in vacuo and then 30 mL water was added into the flask. The solution was neutralized by slow addition of 1 M HCl, leading to formation of precipitates, which were filtered using vacuum filtration to give compound **5**.

To a solution of aromatic aldehyde (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 acidified to pH 2 by slow addition of 1 M HCl. Then the product precipitated (crystallized) out to give compound **6**.

A solution of compound **5** (1.1 mmol), compound **6** (1 mmol) and K_2CO_3 (3 mmol) in acetonitrile (30 mL) were heated under

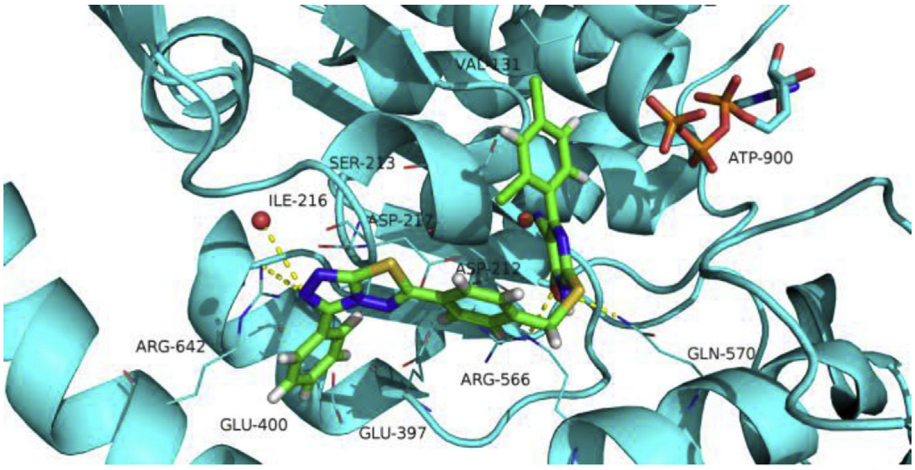


Fig. 3. The proposed docking conformation of **7d** within the pocket between IRA2 and NBD domain. The crystal structure is shown in cyan ribbon and sticks; **7d** is shown in green sticks; and water is shown in red spheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reflux. After the completion of the reaction as indicated by TLC, the solvent was evaporated under vacuum to obtain crude **7**, which was washed with 0.2 mol/L NaOH and dried in vacuo. Subsequent purification using column chromatography (ethyl acetate/methanol = 15:1) gave the target products **7a-l**.

*4.2.1. 6-Oxo-4-phenyl-2-[4-(3-phenyl- [1,2,4]triazolo [3,4-b] [1,3,4] thiadiazol-6-yl)-benzylsulfanyl]-1,6-dihydropyrimidine -5-carbonitrile (**7a**)*

Light yellow solid, Yield 55%; mp 225–228 °C; IR (KBr, ν, cm^{-1}): 3390 (N–H), 2364 (–CN) and 1652 (C=O); ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.33 (d, J = 7.2 Hz, 2H, ArH), 7.99 (d, J = 7.8 Hz, 2H, ArH), 7.75 (t, J = 3.6 Hz, 2H, ArH), 7.69 (d, J = 8.4 Hz, 2H, ArH), 7.63 (t, J = 7.8 Hz, 2H, ArH), 7.57 (t, J = 7.8 Hz, 1H, ArH), 7.47 (t, J = 3.6 Hz, 3H, ArH), 4.40 (s, 2H, CH_2); MS (ESI) m/z: 518.2 ([M - H]$^+$). Anal. Calcd. for $C_{27}H_{17}N_7OS_2$: C, 62.41; H, 3.30; N, 18.87. Found: C, 62.34; H, 3.25; N, 18.81.

*4.2.2. 4-(2-Chloro-phenyl)-6-oxo-2-[4-(3-phenyl- [1,2,4] triazolo [3,4-b] [1,3,4]thiadiazol-6-yl)-benzylsulfanyl]-1,6-dihydro-pyrimidine-5-carbonitrile (**7b**)*

Light yellow solid, Yield 65%; mp 204–206 °C; IR (KBr, ν, cm^{-1}): 3380 (N–H), 2362 (–CN) and 1652 (C=O); ^{1}H NMR (600 MHz, CD_3OD) δ (ppm): 8.08 (d, J = 7.2 Hz, 2H, ArH), 7.99 (d, J = 7.8 Hz, 2H, ArH), 7.75 (t, J = 3.6 Hz, 2H, ArH), 7.69 (d, J = 8.4 Hz, 2H, ArH), 7.63 (d, J = 7.8 Hz, 2H, ArH), 7.57 (t, J = 7.2 Hz, 1H, ArH), 7.47 (t, J = 3.6 Hz, 2H, ArH), 4.40 (s, 2H, CH_2); MS (ESI) m/z: 551.3 ([M - 3H]$^+$). Anal. Calcd for $C_{27}H_{16}ClN_7OS_2$: C, 58.53; H, 2.91; N, 17.70. Found: C, 58.60; H, 2.85; N, 17.61.

*4.2.3. 4-(4-Chloro-phenyl)-6-oxo-2-[4-(3-phenyl- [1,2,4] triazolo [3,4-b] [1,3,4]thiadiazol-6-yl)-benzylsulfanyl]-1,6-dihydro-pyrimidine-5-carbonitrile (**7c**)*

Light yellow solid, Yield 65%; mp 220–223 °C; IR (KBr, ν, cm^{-1}): 3372 (N–H), 2360 (–CN) and 1662 (C=O); ^{1}H NMR (600 MHz, CD_3OD) δ (ppm): 8.04 (d, J = 7.2 Hz, 2H, ArH), 7.97 (d, J = 7.8 Hz, 2H, ArH), 7.78 (t, J = 3.6 Hz, 2H, ArH), 7.71 (d, J = 8.4 Hz, 2H, ArH), 7.64 (d, J = 7.2 Hz, 2H, ArH), 7.55 (t, J = 7.8 Hz, 1H, ArH), 7.47 (t, J = 3.6 Hz, 2H, ArH), 4.40 (s, 2H, CH_2); MS (ESI) m/z: 553.4 ([M - H]$^+$). Anal. Calcd for $C_{27}H_{16}ClN_7OS_2$: C, 58.53; H, 2.91; N, 17.70. Found: C, 58.59; H, 2.82; N, 17.61.

*4.2.4. 4-(2,4-Dichloro-phenyl)-6-oxo-2-[4-(3-phenyl- [1,2,4] triazolo [3,4-b] [1,3,4]thiadiazol-6-yl)-benzylsulfanyl]-1,6-dihydro-pyrimidine-5-carbonitrile (**7d**)*

Light yellow solid, Yield 54%; mp 214–217 °C; IR (KBr, ν, cm^{-1}): 3370 (N–H), 2360 (–CN) and 1655 (C=O); ^{1}H NMR (600 MHz, CD_3OD) δ(ppm): 7.94 (d, J = 7.8 Hz, 2H, ArH), 7.79 (d, J = 8.4 Hz, 2H, ArH), 7.62 (d, J = 7.2 Hz, 1H, ArH), 7.55 (d, J = 8.4 Hz, 2H, ArH), 7.53 (d, J = 8.4 Hz, 2H, ArH), 7.30 (d, J = 6.6 Hz, 1H, ArH), 7.23 (m, 2H, ArH), 4.37 (s, 2H, CH_2); ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 177.6, 159.3, 131.7, 130.1, 129.3, 128.9, 126.9, 115.5, 113.9, 52.2; MS (ESI) m/z: 587.4 ([M - H]$^+$). Anal. Calcd. for $C_{27}H_{15}Cl_2N_7OS_2$: C, 55.11; H, 2.57; N, 16.66. Found: C, 55.19; H, 2.50; N, 16.57.

*4.2.5. 4-(2,6-Dichloro-phenyl)-6-oxo-2-[4-(3-phenyl- [1,2,4] triazolo [3,4-b] [1,3,4]thiadiazol-6-yl)-benzylsulfanyl]-1,6-dihydro-pyrimidine-5-carbonitrile (**7e**)*

Light yellow solid, Yield 50%; mp 229–231 °C; IR (KBr, ν, cm^{-1}): 3374 (N–H), 2362 (–CN) and 1665 (C=O); ^{1}H NMR (600 MHz, CD_3OD) δ (ppm): 7.98 (d, J = 7.8 Hz, 2H, ArH), 7.81 (d, J = 8.4 Hz, 2H, ArH), 7.66 (d, J = 7.2 Hz, 1H, ArH), 7.58 (d, J = 7.8 Hz, 2H, ArH), 7.54 (d, J = 7.8 Hz, 2H, ArH), 7.34 (d, J = 6.6 Hz, 1H, ArH), 7.26 (m, 2H, ArH), 4.38 (s, 2H, CH_2); MS (ESI) m/z: 587.3 ([M - H]$^+$). Anal. Calcd for $C_{27}H_{15}Cl_2N_7OS_2$: C, 55.11; H, 2.57; N, 16.66. Found: C, 55.17; H, 2.50; N, 16.57.

*4.2.6. 4-Biphenyl-4-yl-6-oxo-2-[4-(3-phenyl- [1,2,4]triazolo [3,4-b] [1,3,4]thiadiazol-6-yl)-benzylsulfanyl]-1,6-dihydro-pyrimidine -5-carbonitrile (**7f**)*

Light yellow solid, Yield 59%; mp 232–235 °C; IR (KBr, ν, cm^{-1}): 3360 (N–H), 2360 (–CN) and 1654 (C=O); ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.33 (d, J = 7.2 Hz, 2H, ArH), 8.02 (d, J = 8.4 Hz, 2H, ArH), 7.89 (d, J = 8.4 Hz, 2H, ArH), 7.78 (d, J = 7.8 Hz, 2H, ArH), 7.74 (d, J = 7.8 Hz, 2H, ArH), 7.71 (d, J = 8.4 Hz, 2H, ArH), 7.64 (t, J = 7.2 Hz, 2H, ArH), 7.58 (t, J = 7.8 Hz, 1H, ArH), 7.49 (t, J = 7.2 Hz, 2H, ArH), 7.40 (t, J = 7.8 Hz, 1H, ArH), 4.42 (s, 2H, CH_2); MS (ESI) m/z: 595.1 ([M - H]$^+$). Anal. Calcd for $C_{33}H_{21}N_7OS_2$: C, 66.54; H, 3.55; N, 16.46. Found: C, 66.46; H, 3.50; N, 16.38.

*4.2.7. 2-{4-[3-(2-Chloro-phenyl)- [1,2,4]triazolo [3,4-b] [1,3,4] thiadiazol-6-yl]-benzylsulfanyl}-4-(2,4-dichloro-phenyl)-6-oxo-1,6-dihydro-pyrimidine-5-carbonitrile (**7g**)*

Light yellow solid, Yield 65%; mp 201–204 °C; IR (KBr, ν, cm^{-1}): 3360 (N–H), 2360 (–CN) and 1640 (C=O); ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 7.98 (d, J = 7.2 Hz, 2H, ArH), 7.90 (d, J = 7.8 Hz, 2H, ArH), 7.75 (t, J = 3.6 Hz, 2H, ArH), 7.69 (d, J = 8.4 Hz, 1H, ArH), 7.63 (t, J = 7.8 Hz, 2H, ArH), 7.57 (d, J = 7.8 Hz, 1H, ArH), 7.47 (t, J = 3.6 Hz, 1H, ArH), 4.40 (s, 2H, CH_2); ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 177.6, 159.3, 137.3, 132.1, 131.9, 131.7, 130.0, 129.2, 128.9, 117.1, 115.7, 113.9, 52.5; MS (ESI) m/z: 622.0 ([M - H]$^+$). Anal. Calcd. for $C_{27}H_{14}Cl_3N_7OS_2$: C, 52.06; H, 2.27; N, 15.74. Found: C, 52.14; H, 2.20; N, 15.61.

*4.2.8. 2-{4-[3-(2-Chloro-phenyl)- [1,2,4]triazolo [3,4-b] [1,3,4] thiadiazol-6-yl]-benzylsulfanyl}-4-(2,6-dichloro-phenyl)-6-oxo-1,6-dihydro-pyrimidine-5-carbonitrile (**7h**)*

Light yellow solid, Yield 65%, mp 224–227 °C; IR (KBr, ν, cm^{-1}): 3370 (N–H), 2360 (–CN) and 1664 (C=O); ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 7.99 (d, J = 7.8 Hz, 2H, ArH), 7.88 (d, J = 7.8 Hz, 2H, ArH), 7.74 (t, J = 3.6 Hz, 2H, ArH), 7.66 (d, J = 7.8 Hz, 1H, ArH), 7.60 (t, J = 7.2 Hz, 2H, ArH), 7.54 (d, J = 7.8 Hz, 1H, ArH), 7.43 (t, J = 4.8 Hz, 1H, ArH), 4.41 (s, 2H, CH_2); MS (ESI) m/z: 622.1 ([M - H]$^+$). Anal. Calcd. for $C_{27}H_{14}Cl_3N_7OS_2$: C, 52.06; H, 2.27; N, 15.74. Found: C, 52.00; H, 2.32; N, 15.67.

*4.2.9. 4-Biphenyl-4-yl-2-{4-[3-(2-chloro-phenyl)- [1,2,4] triazolo [3,4-b] [1,3,4]thiadiazol-6-yl]-benzylsulfanyl}-6-oxo-1,6-dihydro-pyrimidine-5-carbonitrile (**7i**)*

Light yellow solid, Yield 65%, mp 231–234 °C; IR (KBr, ν, cm^{-1}): 3360 (N–H), 2364 (–CN) and 1662 (C=O); ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.01 (s, 2H, ArH), 7.92 (d, J = 7.2 Hz, 4H, ArH), 7.77 (d, J = 8.4 Hz, 2H, ArH), 7.69 (m, 4H, ArH), 7.48 (t, J = 7.8 Hz, 2H, ArH), 7.40 (t, J = 7.2 Hz, 1H, ArH), 7.25 (m, 2H, ArH), 4.52 (s, 2H, CH_2); MS (ESI) m/z: 629.7 ([M - H]$^+$). Anal. Calcd. for $C_{33}H_{20}ClN_7OS_2$: C, 62.90; H, 3.20; N, 15.56. Found: C, 62.80; H, 3.16; N, 15.50.

*4.2.10. 4-(2,4-Dichloro-phenyl)-2-{4-[3-(2,6-dichloro-phenyl) -[1,2,4]triazolo [3,4-b] [1,3,4]thiadiazol-6-yl]-benzylsulfanyl} -6-oxo-1,6-dihydro-pyrimidine-5-carbonitrile (**7j**)*

Light yellow solid, Yield 65%; mp 217–219 °C; IR (KBr, ν, cm^{-1}): 3370 (N–H), 2360 (–CN) and 1650 (C=O); ^{1}H NMR (600 MHz, CD_3OD) δ (ppm): 7.99 (s, 1H, ArH), 7.93 (d, J = 7.8 Hz, 1H, ArH), 7.65 (d, J = 7.8 Hz, 2H, ArH), 7.63 (s, 1H, ArH), 7.50 (d, J = 7.2 Hz, 2H, ArH), 7.48 (s, 1H, ArH), 7.42 (d, J = 8.4 Hz, 1H, ArH), 7.36 (t, J = 7.8 Hz, 1H, ArH), 4.42 (s, 2H, CH_2); ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 166.1, 135.9, 134.2, 132.1, 131.5, 131.4, 129.3, 129.2, 129.0, 128.9, 128.7, 128.6, 127.7, 127.4, 127.3, 126.6, 44.5; MS (ESI) m/z: 656.3 ([M - H]$^+$). Anal. Calcd. for $C_{27}H_{13}Cl_4N_7OS_2$: C, 49.33; H, 1.99; N, 14.91. Found: C,

49.25; H, 1.96; N, 14.84.

*4.2.11. 4-(2,6-Dichloro-phenyl)-2-{4-[3-(2,6-dichloro-phenyl) -[1,2,4]triazolo [3,4-b] [1,3,4]thiadiazol-6-yl]-benzylsulfanyl} -6-oxo-1,6-dihydro-pyrimidine-5-carbonitrile (**7k**)*

Light yellow solid, Yield 65%; mp 230–233 °C; IR (KBr, ν, cm^{-1}): 3360 (N–H), 2360 (–CN) and 1655 (C=O); ^1H NMR (600 MHz, DMSO-d_6) δ (ppm): 7.98 (d, J = 7.8 Hz, 2H, ArH), 7.67 (d, J = 7.2 Hz, 2H, ArH), 7.63 (d, J = 7.8 Hz, 2H, ArH), 7.50 (d, J = 7.8 Hz, 2H, ArH), 7.42 (d, J = 8.4 Hz, 1H, ArH), 7.36 (t, J = 7.8 Hz, 1H, ArH), 4.42 (s, 2H, CH_2); ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 166.0, 132.1, 131.8, 131.4, 131.0, 130.8, 130.5, 129.1, 129.0, 128.4, 127.8, 127.3, 52.3; MS (ESI) m/z: 656.1 ([M - H]$^+$). Anal. Calcd. for $C_{27}H_{13}Cl_4N_7OS_2$: C, 49.33; H, 1.99; N, 14.91. Found: C, 49.20; H, 1.98; N, 14.82.

*4.2.12. 4-Biphenyl-4-yl-2-{4-[3-(2,6-dichloro-phenyl)- [1,2,4] triazolo [3,4-b] [1,3,4]thiadiazol-6-yl]-benzylsulfanyl}-6-oxo-1,6-dihydro-pyrimidine-5-carbonitrile (**7l**)*

Light yellow solid, Yield 65%; mp 238–241 °C; IR (KBr, ν, cm^{-1}): 3364 (N–H), 2360 (–CN) and 1645 (C=O); ^1H NMR (600 MHz, DMSO-d_6) δ(ppm): 7.95 (s, 2H, ArH), 7.94 (s, 2H, ArH), 7.77 (d, J = 8.4 Hz, 2H, ArH), 7.72 (d, J = 7.8 Hz, 2H, ArH), 7.68 (d, J = 8.4 Hz, 2H, ArH), 7.50 (s, 1H, ArH), 7.47 (d, J = 3.6 Hz, 2H, ArH), 7.47 (s, 1H, ArH), 7.40 (d, J = 7.2 Hz, 1H, ArH), 7.35 (t, J = 7.8 Hz, 1H, ArH), 4.54 (s, 2H, CH_2); ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 166.1, 156.7, 140.4, 139.4, 138.8, 134.9, 134.1, 133.9, 132.1, 131.5, 131.4, 128.9, 128.8, 128.6, 127.6, 127.3, 127.1, 126.7, 126.6, 126.5, 47.9; MS (ESI) m/z: 663.5 ([M – H]$^+$). Anal. Calcd. for $C_{33}H_{19}Cl_2N_7OS_2$: C, 59.64; H, 2.88; N, 14.75. Found: C, 59.56; H, 2.81; N, 14.69.

4.3. Antibacterial test

Antibacterial activity was measured by the plate colony counting protocol. Three strains of Gram-positive organisms such as *Bacillus amyloliquefaciens*, *Staphylococcus aureus*, and *Bacillus subtilis* were tested. After sterilization of NA (Nutrient Agar) medium, the tested compounds were added at 25 μg/mL and poured into Petri dishes. A suspension of each organism was prepared from fresh colonies on NA medium slant after 24 h incubation. Serial ten fold dilutions for each aliquot were prepared with sterilized water. Duplicate aliquot (70 μL) of 10^{-5} and 10^{-6} bacterial suspension dilutions were spread to each plate containing 25 μg/mL tested compounds. Control plates contained only NA medium. The plates were incubated overnight at 35 °C and bacterial colony counting was performed.

4.4. SecA inhibitory activity test

The SecA ATPase was sourced from BL21.19 [secA (am)supFtstrp (am)zch::Tn10recA::catclpA::kan], which was supplied by Dr. Tai at Georgia State University. The buffer was prepared from 0.5 M Tris-HCl (pH = 7.6), 0.2 M KCl, 0.2 M NH_4Cl, 10 mM DTT, 20 mM $Mg(OAc)_2$. Mix MG (0.45% Malachite Green) and AM (4.2% ammonium molybdate in 4 N HCl) in 3:1 ratio and filtrate. Then Triton X-100 (0.1%) was added to give he final color reagent. The mixture of 3 μL of the buffer, 2 μg ATPase (SecA), 1 μL ATP (10 mM), 1 μL of the sample was diluted to 10 mL with distilled water to get the reaction mixture (1 μL distilled H_2O for blank control). The reaction mixture was incubated at 37 °C for 30 min and then 160 μL color reagent was added. After incubation for 1 min, 20 μL 34% citrate was added before absorption at 660 nm was measured using a FLUOstar Omega MicroplateReader. The inhibition rate of SecA was calculated according to the absorption. The IC_{50} values for the tested samples were calculated by Origin 8.

4.5. Computational modeling

The molecular structure of the compounds **7d** was drawn with Chemoffice Ultra 8.03 (CambridgeSoft, USA). Then the 3D structure was optimized with Omega2 [38–40] on GSU cluster Orion [41]. Finally, the crystal structure of the SecA (PDBID: 2FSG [42]) was docked with the compound **7d** using Surflex-Dock [43]. We picked the pocket in two methods: designating the ATPase ATP binding site as the pocket using the co-crystalized ATP molecule and following our previous procedures [31]. 20 outputs were generated for docking score analysis.

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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.2016.12.053.

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