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Original article

Biological evaluation of halogenated thiazolo[3,2-a]pyrimidin-3-one carboxylic acid derivatives targeting the YycG histidine kinase

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ABSTRACT

With an intention to potent inhibitors of YycG histidine kinase, a series of halogenated thiazolo[3,2-*a*]pyrimidin-3-one carboxylic acid derivatives were synthesized and evaluated for their antibacterial, antibiofilm and hemolytic activities. The majority of the compounds showed good activity against *Staphylococcus epidermidis* and *Staphylococcus aureus*, with MIC values of 1.56–6.25 μM, simultaneously presented promising antiobifilm activity against *S. epidermidis* ATCC35984 at 50 μM. The test of inhibitory activity on YycG kinase suggested the antibacterial activities of these derivatives are based on inhibiting the enzyme activity of the YycG HK domain. The hemolytic activity test suggested these compounds exhibited in *vitro* antibacterial activity at non-hemolytic concentrations.

1. Introduction

Antibiotic-resistant bacteria have become a major concern for public health professionals since bacterial resistance has dramatically increased during the last two decades [1]. Indeed, bacteria growing as an adherent biofilm on catheters, orthopedic implants, and bones are inherently resistant to antibiotics and are poorly recognized by the host immune defenses [2]. Biofilm associated bacteria exhibit enhanced resistance to many conventional antibiotics [3]. *Staphylococcus epidermidis* has become a significant pathogen causing infections due to biofilm formation on surfaces of indwelling medical devices [4]. Consequently, it has an urgent need to design novel antibiotics against staphylococci infections, especially those can kill cells embedded in biofilm.

In recent years, some novel inhibitors of YycG histidine kinase had been reported which are of potential value as leads for developing new antibiotics against infecting staphylococci [5]. Among these inhibitors, a typical compound **A** (Fig. 1), which had a thiazolopyrimidinone core substituted by aromatic ring, showed potent antibacterial activity, moderate antibiofim activity, low cytotoxicity on mammalian cells and was not hemolytic. Our previous work on analogs of thiazolo[3,2-*a*]pyrimidin-3-ones **A** suggests that carboxylic acid fragment at C-2 position of A-ring was indispensable and B-ring opening analogs of thiazolo[3,2-*a*]pyrimidin-3-ones **A** maintained the antibiofilm potency [6,7]. However, improvement of antibacterial and antibiofilm potency of the compound **A** is still needed and its structure modification is a challenging task.

Herein, we describe further modifications that focused on preserving A-ring and carboxylic acid moieties at C-2 position of A-ring while simultaneously investigating the necessity of B-ring and variations to the B-ring substituents. Considering a significant number of antimycobacterial agents in clinical development are chlorinated structures [8], and the introduction of the strongly electron-withdrawing fluorine group into drugs or drug candidates can substantially enhanced binding interactions, metabolic stability, changes in physical properties, and selective reactivities [9], we paid attention to chlorine and fluorine substitution. A series of thiazolo[3, 2-*a*]pyrimidine-3-one derivatives were designed, accordingly (Fig. 1).

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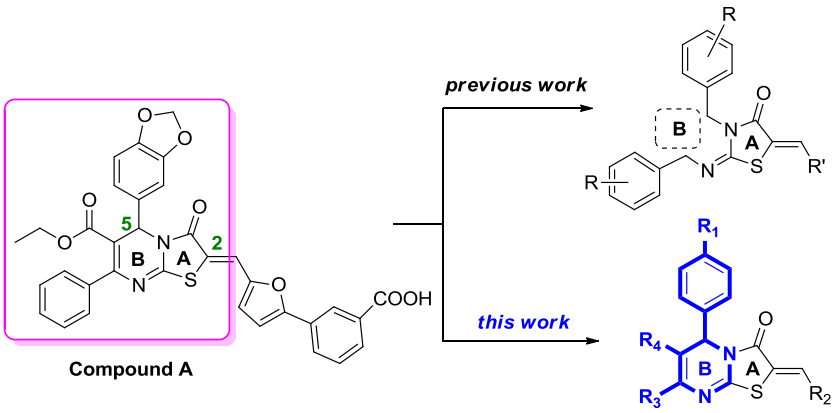


Fig. 1. Leading compound and structure-based design of the target compounds.

2. Chemistry

The synthetic route to **4a**–**o** is depicted in Scheme 1. A multiple-componentchemical reaction known as Biginelli reaction of aryl aldehyde or substituted aryl aldehyde, ethyl benzoylacetate and thiourea gave different dihydropyrimidine scaffolds (DHPMs, **2a**–**h**). Cyclization of **2a**–**h** with bromoacetic acid afforded **3a**–**h** [10], an aldol condensation of **3a**–**h** with equivalent amount substituted aromatic aldehydes [11] of was catalyzed by β-alanine to give **4a**–**o** in good yields [12]. As shown in Scheme 2, an aldol reaction of acetophenone and substituted phenylaldehyde gave **5a**–**c**, **5a**–**c** reacted with thiourea under base condition gave **6a**–**c** [13], cyclization of **6a**–**c** with bromoacetic acid afforded **7a**–**c**. **8a**–**c** were prepared via aldol condensation between **7a**–**c** and aromatic aldehydes. The identities of all of the synthesized compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and mass spectral.

3. Results and discussion

3.1. Evaluation of antibacterial activity

The *in vitro* antibacterial activity was evaluated with Gram-positive (*S. epidermidis* ATCC35984, *S. epidermidis* ATCC12228, *Staphylococcus aureus* ATCC25923) and Gram-negative (*Escherichia coli* and *Shigella flexneri* 301) bacterial strains, using the minimum inhibitory concentration (MIC). Cefazolin was used as positive control for bacteria.

As it can be concluded from the data in Table 1, most of the compounds were highly active against *S. epidermidis* ATCC35984, *S. epidermidis* ATCC12228 and *S. aureus* ATCC25923. Unlike the good activity of compounds against Gram positive bacterial strains, this class of compounds did not show any antibacterial activity *in vitro* against the Gram-negative (*E. coli* ATCC25922 and *S. flexneri* 301) strains at 200 μM.

In the compounds containing substituents on the phenyl ring, compounds **4e** (MIC = 1.56–6.25 μM), **4i** (MIC = 3.125–6.25 μM) and **4k** (MIC = 3.125 μM) with 4-Cl substituent, compounds **4f** (MIC = 3.125–6.25 μM) with 4-F substituent presented much more potent antibacterial activity, relative to the leading compound **A** and cefazolin. Compounds (**4b**–**f**, MIC = 1.56–50 μM) containing substituents on the phenyl ring showed higher levels of activity than non-substituted one (**4a**, MIC > 200 μM), Based on this fact, we inferred that the presence of 4-Cl or 4-F may have a good contribution to the activity. The derivatives bearing methyl group (**4g**, MIC = 6.25 μM and **4m**, MIC = 200 μM) on 6-position of B-ring showed lower levels of activity against *S. epidermidis* ATCC35984 than the corresponding derivatives bearing phenyl group (**4b** and **4k**, MIC = 3.125 μM), it revealed that π–π stacking effect of phenyl group might led thiazolo[3,2-*a*]pyrimidine-3-ones bind the target protein more easily. Moreover, in order to check out whether the ester moiety on 6-position of B-ring is essential for activity or not, compounds **8a**–**c** without 6-ester moiety were also synthesized. The biological results revealed that removing the ester moiety did not decrease the antibacterial activity.

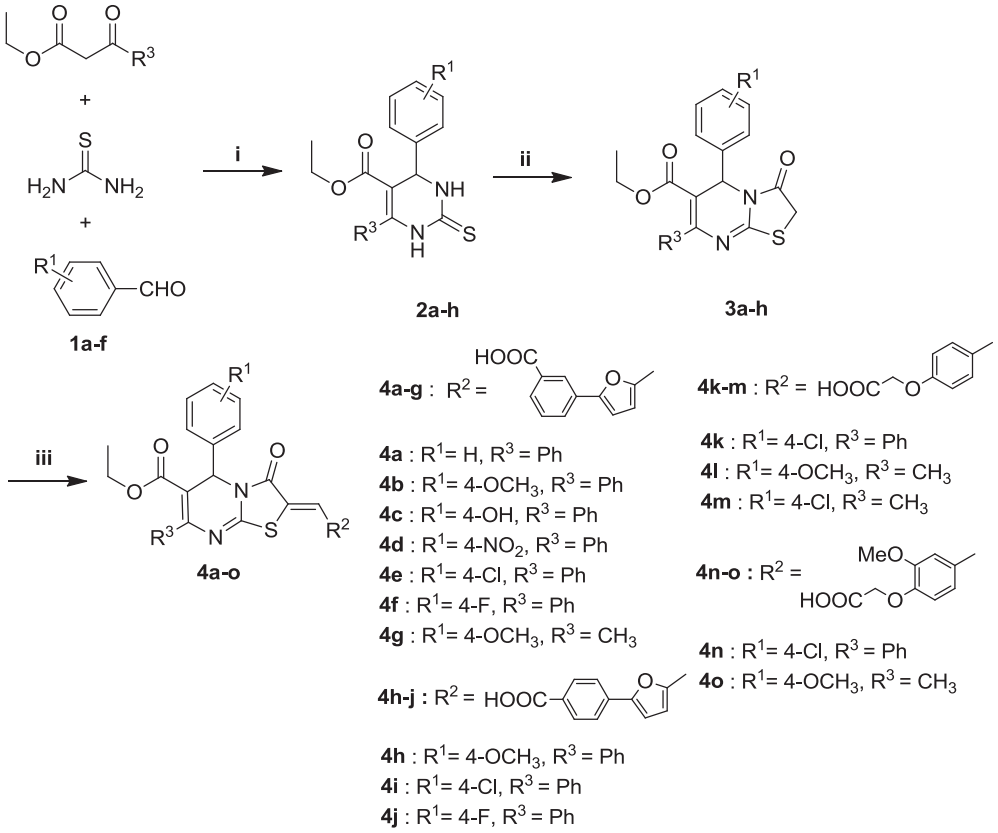
3.2. Antibiofilm and hemolysis activity of the derivatives

The newly synthesized compounds were further screened for their antibiofilm activities against *S. epidermidis* ATCC35984 and hemolytic activities of the derivatives on healthy human erythrocytes.

As indicated in Table 2, the compounds **4b**, **4e**, **4f**, **4i**, **4j**, **8b** and **8c** displayed antibiofilm activity at 50 μM. No compound showed hemolytic activity in human erythrocytes at 50 μM. Interestingly, in compare with the compound **A**, the antibiofilm activity of **8a** disappeared, although its antibacterial activity was improved (Table 1). While 4-Cl or 4-F substituted compounds **8b** and **8c** regained promising antibiofilm activity at 50 μM. In addition, compounds 4-Cl or 4-F substituted **4e**, **4f**, **4i**, **4j** also exhibited promising antibiofilm activity at 50 μM. All the results showed that an ester moiety at C-2 position of B-ring and the substituent phenyl moieties at C-5 position of B-ring might be two decisive factors influenced the antibiofilm activity.

3.3. Inhibition of YycG enzyme activity by the derivatives

The newly synthesized compounds are derivatives of lead compound **A** which is the inhibitor of YycG histidine kinase. To detect whether the derivatives still have the inhibitory activities on YycG kinase, the recombinant HATPase_c and HisKA domains of YycG were expressed in *E. coli* BL21 (DE3) and purified by the Ni-NTA column, then ATP-Luminescence assay kit (Promega) were used to measure the inhibitory activities of the compounds on YycG autophosphorylation activity. With good antibacterial activities as above mentioned, compounds **4e**, **8a** and **8b** were selected for the enzyme inhibitor assay. As it was envisaged, all of them displayed inhibition of phosphorylation of the recombinant YycG. The IC_{50} values were 12.65 μM, 49.92 μM and 55.61 μM, respectively, and the IC_{50} of prototype compound **A** was 14 μM (Table 3). This suggested that the bactericidal activity of these derivatives is based on inhibiting the enzyme activity of the YycG HK domain.



Scheme 1. Reagents and conditions: (i) $CeCl_3 \cdot 7H_2O$, EtOH, reflux; (ii) bromoacetic acid, NaOAc, AcOH/Ac_2O, reflux; (iii) β-alanine, acetic acid, reflux.

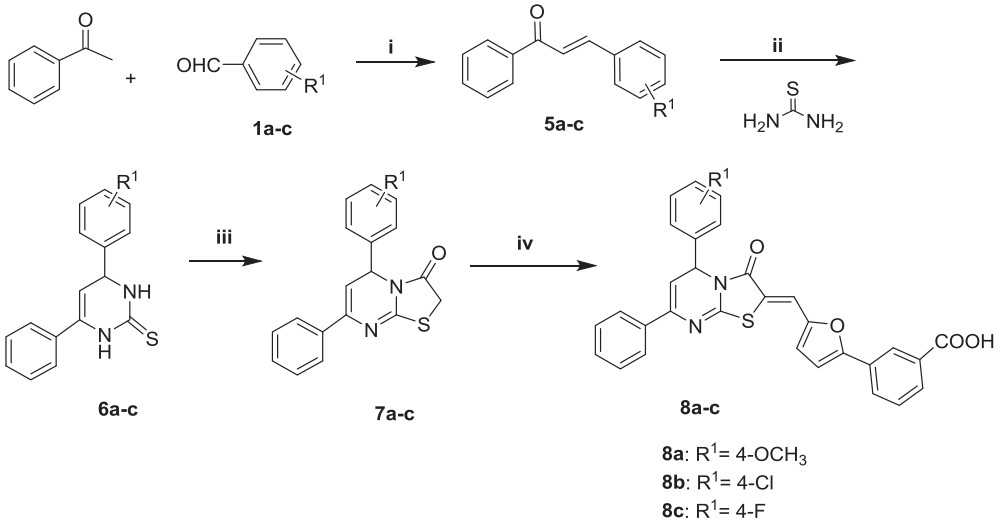
4. Conclusion

A series of thiazolo[3,2-*a*]pyrimidin-3-one carboxylic acid derivatives were synthesized and evaluated for antibacterial, antibiofilm and hemolytic activities. Most of the compounds showed good antibacterial activity and antibiofilm activity. In particular, compounds **4e**, **4f**, **8b** and **8c** exhibited stronger activity against *S. epidermidis* and *S. aureus* than the standard drug cefazolin. Compound **4e** bearing 4-Cl substituent exhibited higher level of inhibitory activity on YycG kinase. This suggested that chlorinated or fluorinated thiazolo[3,2-*a*]pyrimidin-3-ones might provide valuable leads for development of novel inhibitors of YycG histidine kinase. More modifications on compounds **4e**, **4f**, **8b** and **8c** and biological evaluation are on going in our laboratories.

5. Experimental protocols

5.1. Chemistry

All reagents and solvents were commercially available and used without further purification. Melting points were determined on an electrothermal digital apparatus model WRS-1B (Shanghai, China),



Scheme 2. Reagents and conditions: (i) methanol, 2 N NaOH (aq); (ii) KOH, ethanol; (iii) $BrCH_2COOH$, NaOAc, AcOH/Ac_2O; (iv) β-alanine, acetic acid, reflux.

Table 1
Antibacterial activities of the compounds **4a**–**o** and **8a**–**c**.

Compound	MIC (μM)[a]				
	S. epidermidis ATCC35984	*S. epidermidis* ATCC12228	*S. aureus* ATCC25923	*E. coli* ATCC25922	*Shigella flexneri* 301
A	6.25	6.25	6.25	>200	>200
4a	200	200	nd	>200	>200
4b	3.125	3.125	nd	>200	>200
4c	50	50	nd	>200	>200
4d	1.56	3.125	25	>200	>200
4e	1.56	3.125	6.25	>200	>200
4f	6.25	3.125	6.25	>200	>200
4g	6.25	nd	nd	>200	>200
4h	6.25	6.25	nd	>200	>200
4i	6.25	3.125	6.25	>200	>200
4j	6.25	6.25	6.25	>200	>200
4k	3.125	3.125	nd	>200	>200
4l	50	50	nd	>200	>200
4m	200	200	nd	>200	>200
4n	200	200	nd	>200	>200
4o	50	50	nd	>200	>200
8a	1.56	6.25	6.25	>200	>200
8b	1.56	6.25	3.125	>200	>200
8c	6.25	3.125	6.25	>200	>200
Cefazolin	17.6	17.6	17.6	–	–

nd = no detected.
[a] MIC assay was performed following the broth micro-dilution method (in tubes) of the CLSI of America. In this assay, the highest concentration of each compound was 200 μM.

Table 2
Antibiofilm and hemolysis activities of the compounds **4a**–**o** and **8a**–**c**.

Compound	Antibiofilm[a]	Hemolysis[b]
A	+	–
4a	–	–
4b	+	–
4c	–	–
4d	–	–
4e	+	–
4f	+	–
4g	–	–
4h	–	–
4i	+	–
4j	+	–
4k	–	–
4l	–	–
4m	–	–
4n	–	nd
4o	–	nd
8a	–	–
8b	+	–
8c	+	–

nd = no detected.
[a] Antibiofilm activities of the derivatives against *S. epidermidis* ATCC35984 were tested at 50 μM; +: active at 50 μM; –: no inhibition at 50 μM.
[b] Hemolytic activities of the derivatives on healthy human erythrocytes were tested at 50 μM; –: no hemolytic activity at 50 μM.

Table 3
Inhibition of YycG enzyme activities of the compound **4e** and **8a**.

Compound	IC_{50}[a] (μM)
A	14
4e	12.65
8a	49.92
8b	55.61

[a] IC_{50} represents half maximal inhibitory concentration of the derivatives, which inhibit half of the autophosphorylation of recombinant YycG.

without correction. All ^1H NMR (300 or 400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker AM-300 or 400 MHz spectrometer (Bruker) as solutions in DMSO-d_6 or $CDCl_3$ with TMS as an internal standard. Mass spectra (MS) were recorded on an HP-5973 instrument (HP). IR spectra were recorded (in KBr) on an FTIR 1730. The purity of the final compounds was determined by HPLC on a DIONEX-P680 instrument (DIONEX) at 254 nm and all final compounds have a purity of above 95%.

5.2. General procedure for the preparation of compounds *2a*–*h*

To a solution of phenylaldehyde or substituted phenylaldehyde **1a**–**f** (3 mmol), ethyl benzoylacetate (3 mmol), thiourea (9 mmol) and $CeCl_3 \cdot 7H_2O$ (0.75 mmol) in absolute ethanol (10 ml) was refluxed for 5 h. Then the mixture was poured into 200 ml ice-water and solid was precipitated. The crude products were filtered and recrystallized in methanol to obtain pure compound as a white solid.

5.3. General procedure for the preparation of compounds *3a*–*h*

To a solution of **2a**–**h** (3 mmol), bromoacetic acid (3 mmol), NaOAc (3 mmol) in 20 ml HAc/Ac_2O (3:1 v/v) was refluxed for 6 h. Then the mixture was cooled to room temperature and poured into 200 ml water and solid was precipitated. The crude products were filtered and recrystallized in methanol to obtain pure compound as a golden crystal **3a**–**h**.

5.4. General procedure for the preparation of compounds *4a*–*o*

The corresponding aldehyde (1 equiv), β-alanine (1 equiv), and **3a**–**h** (1 equiv) were heated at 100 °C for 1 h in glacial acetic acid. Upon completion of the reaction, the mixture was cooled, the reaction was quenched with water, and the precipitate was filtered off. The solid products were filtered and recrystallized in methanol to obtain pure compound.

*5.4.1. 3-(5-((6-(Ethoxycarbonyl)-3-oxo-5,7-diphenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl)furan-2-yl)benzoic acid (**4a**) [6]*

Yellow solid: yield 93%; mp: 284–285 °C; IR (KBr) cm^{-1}: 3387 (CO–OH), 1698 (C=O), 1549 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.07 (s, 1H, CH), 8.40 (s, 1H, CH), 8.13–8.05 (d, J = 6 Hz, 1H, furan-H), 7.93–7.91 (d, J = 6 Hz, 1H, furan-H), 7.69 (d, J = 7.9 Hz, 2H, Ar–H), 7.48–7.40 (m, 10H, Ar–H), 7.29 (d, J = 3.8 Hz, 2H, Ar–H), 6.16 (s, 1H, CH), 3.86–3.81 (q, J = 7.0 Hz, 2H, CH_2), 0.82–0.80 (t, J = 7.0 Hz, 3H, CH_3); ESI-MS m/z 575.2 $[M-H]^-$.

*5.4.2. 3-(5-((6-(Ethoxycarbonyl)-5-(4-methoxyphenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4b**) [6]*

Orange solid: yield 91.4%; mp: 252.3–253.4 °C; IR (KBr) cm^{-1}: 3371 (CO–OH), 1608 (C=O), 1547 (C=C); ^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 8.34 (s, 1H, CH), 8.05–8.03 (d, J = 6 Hz, 1H, furan-H), 7.93–7.91 (d, J = 6 Hz, 1H, furan-H), 7.64–6.92 (m, 13H, Ar–H), 6.06 (s, 1H, CH), 3.85–3.78 (q, J = 6 Hz, 2H, CH_2), 3.69 (s, 3H, OCH_3), 0.79–0.75 (t, J = 6 Hz, 3H, CH_3); ESI-MS m/z 607.2 $[M+H]^+$.

*5.4.3. 3-(5-((6-(Ethoxycarbonyl)-5-(4-hydroxyphenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4c**)*

Orange solid: yield 91.6%; mp: 273.3–274.6 °C; IR (KBr) cm^{-1}: 3431 (CO–OH), 1608 (C=O), 1547 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.05 (s, 1H, CH), 8.38 (s, 1H, CH), 8.11–8.03 (d, J = 6 Hz, 1H, furan-H), 7.96 (d, J = 6 Hz, 1H, furan-H), 7.66–7.27 (m, 13H, Ar–H), 6.16 (s, 1H, CH), 4.17 (s, 1H, OH); 3.83–3.79 (q, J = 7 Hz, 2H, CH_2), 0.80–0.77 (t, J = 7 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6,100 MHz, ppm): δ 169.04, 166.67, 165.37, 163.93, 156.02, 155.91, 150.53, 149.83, 149.14, 138.55, 137.32, 131.82, 129.65, 129.52, 128.95, 128.70, 128.27, 127.64, 124.72, 122.22, 121.69, 118.76, 116.57, 110.63, 109.67, 60.16, 55.10, 13.24; ESI-MS m/z 593.1 $[M+H]^+$.

*5.4.4. 3-(5-((6-(Ethoxycarbonyl)-5-(4-nitrophenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4d**)*

Orange solid: yield 93.6%; mp: 232.3–233.4 °C; IR (KBr) cm^{-1}: 3492 (CO–OH), 1709 (C=O), 1630 (C=N), 1528 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.15 (s, 1H, COOH), 8.39 (s, 1H, CH), 8.08 (d, J = 7.8 Hz, 1H, furan-H), 8.00–7.93 (d, J = 7.8 Hz, 1H, furan-H), 7.79–7.43 (m, 12H, Ar–H), 7.29 (d, J = 3.8 Hz, 1H), 6.27 (s, 1H, CH), 3.83 (q, J = 6.9 Hz, 2H, CH_2), 0.80 (t, J = 6.9 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 166.67, 165.13, 163.89, 156.29, 156.15, 150.55, 149.12, 147.47, 146.62, 138.46, 131.84, 129.69, 129.58, 129.07, 128.94, 128.83, 128.29, 127.66, 124.74, 124.04, 123.87, 121.94, 118.98, 116.33, 110.71, 108.64, 60.26, 56.00, 13.22; ESI-MS m/z 622.1 $[M+H]^+$.

*5.4.5. 3-(5-((5-(4-Chlorophenyl)-6-(ethoxycarbonyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4e**)*

Orange solid: yield 92.5%; mp: 256.2–257.1 °C; IR (KBr) cm^{-1}: 3431 (CO–OH), 1710 (C=O), 1547 (C=C); ^{1}H NMR (DMSO-d_6, 300 MHz, ppm) δ 13.08 (s, 1H, COOH), 8.38 (s, 1H, CH), 8.10–8.07 (d, J = 7.8 Hz, 1H, furan-H), 7.97–7.95 (d, J = 7.8 Hz, 1H, furan-H), 7.70–7.67 (d, J = 9 Hz, 2H, Ar–H), 7.46–7.41 (m, 10H, Ar–H), 7.31–7.29 (d, J = 3.9 Hz 1H, Ar–H), 6.16 (s, 1H, CH), 3.86–3.79 (q, J = 6.9 Hz, 2H, CH_2), 0.82–0.78 (t, J = 6.9 Hz 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 166.77, 165.42, 164.03, 156.13, 155.96, 149.87, 149.25, 138.79, 138.62, 131.94, 129.76, 129.06, 128.78, 128.65, 128.34, 128.28, 127.74, 127.66, 124.82, 121.79, 118.81, 116.71, 115.87, 115.66, 110.74, 109.80, 60.23, 56.10, 13.34; ESI-MS m/z 611.1 $[M+H]^+$.

*5.4.6. 3-(5-((6-(Ethoxycarbonyl)-5-(4-fluorophenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4f**)*

Yellow solid: yield 92.1%; mp: 232.1–233.5 °C; IR (KBr): ν (cm^{-1}): 3436 (CO–OH), 1712 (C=O), 1546 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.06 (s, 1H, COOH), 8.38 (s, 1H, CH), 8.12–8.04 (d, J = 8.4 Hz, 1H, furan-H), 7.97 (d, J = 8.4 Hz, 1H, furan-H), 7.67–7.27 (m, 13H, Ar–H), 6.16 (s, 1H, CH), 3.84–3.80 (q, J = 7.2 Hz, 2H, CH_2), 0.81–0.78 (t, J = 7.2 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 166.68, 165.33, 163.94, 156.04, 155.86, 150.17, 149.77, 149.15, 138.69, 138.52, 131.84, 129.53, 128.97, 128.69, 128.56, 128.25, 128.19, 127.64, 127.57, 124.72, 121.70, 118.71, 116.61, 110.65, 109.70, 108.47, 60.14, 56.00, 13.25; ESI-MS m/z 595.2 $[M+H]^+$.

*5.4.7. 3-(5-((6-(Ethoxycarbonyl)-5-(4-methoxyphenyl)-7-methyl-3-oxo-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4g**)*

Orange solid: yield 91.3%; mp: 276.5–277.4 °C; IR (KBr) cm^{-1}: 3441 (CO–OH), 1709 (C=O), 1633 (C=N), 1533 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.21 (s, 1H, COOH), 8.37 (s, 1H, CH), 8.07–8.05 (d, J = 7.8 Hz, 1H, furan-H), 7.97–7.95 (d, J = 7.8 Hz 1H, furan-H), 7.69–6.88 (m, 8H, Ar–H), 5.96 (s, 1H, CH), 4.08–4.01 (q, J = 7.2 Hz, 2H, CH_2), 3.71 (s, 3H, OCH_3), 2.39 (s, 3H, CH_3), 1.17–1.12 (t, J = 7.2 Hz 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 166.69, 164.88, 163.95, 159.20, 155.93, 155.90, 150.99, 149.14, 132.56, 131.81, 129.64, 129.50, 128.96, 128.69, 128.25, 124.62, 121.55, 118.53, 116.67, 113.93, 110.60, 108.65, 60.06, 55.06, 54.24, 22.33, 13.88; ESI-MS m/z 545.1 $[M+H]^+$.

*5.4.8. 4-(5-((6-(Ethoxycarbonyl)-5-(4-methoxyphenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4h**)*

Orange solid: yield 88.2%; mp: 252.3–253.4 °C; IR (KBr) cm^{-1}: 3452 (CO–OH), 1710 (C=O), 1536 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.05 (s, 1H, COOH), 8.06 (d, J = 6 Hz, 2H, furan-H, Ar–H), 7.92 (d, J = 6 Hz, 1H, furan-H, Ar–H), 7.65 (s, 1H, CH), 7.42 (m, 6H, Ar–H), 7.36–7.29 (m, 2H, Ar–H), 7.26 (d, J = 3.8 Hz, 1H, Ar–H), 6.95 (d, J = 8.8 Hz, 2H, Ar–H), 6.09 (s, 1H, CH), 3.85–3.78 (q, J = 7.1 Hz, 2H, CH_2), 3.73 (s, 3H, OCH_3), 0.80–0.76 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 166.71, 165.42, 163.93, 159.40, 155.82, 155.62, 149.68, 149.41, 138.59, 132.20, 131.90, 130.54, 130.22, 128.84, 128.59, 128.22, 127.62, 124.12, 121.49, 118.37, 117.37, 114.13, 111.76, 110.18, 60.09, 55.09, 13.28; ESI-MS m/z 607.2 $[M+H]^+$.

*5.4.9. 4-(5-((5-(4-Chlorophenyl)-6-(ethoxycarbonyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4i**)*

Orange solid: yield 92.5%; mp: 256.2–257.1 °C; IR (KBr) cm^{-1}: 3441 (CO–OH), 1706 (C=O), 1556 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.05 (s, 1H, COOH), 8.38 (s, 1H, CH), δ 8.10–8.07 (d, J = 7.8 Hz, 1H, furan-H), 7.97–7.95 (d, J = 7.8 Hz, 1H, furan-H), 7.70–7.29 (m, 13H, Ar–H), 6.16 (s, 1H, CH), 3.87–3.80 (q, J = 7.2 Hz, 2H, CH_2), 0.82–0.78 (t, J = 7.2 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm) δ 166.70, 165.25, 163.87, 155.93, 155.91, 149.92, 149.64, 138.77, 138.48, 133.32, 132.16, 130.58, 130.22, 129.45, 128.85, 128.72, 128.25, 127.62, 124.13, 121.68, 118.61, 117.09, 111.79, 109.40, 60.18, 55.12, 13.24; ESI-MS m/z 611.1 $[M+H]^+$.

*5.4.10. 4-(5-((6-(Ethoxycarbonyl)-5-(4-fluorophenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) furan-2-yl)benzoic acid (**4j**)*

Yellow solid: yield 92.1%; mp: 237.1–238.5 °C; IR (KBr) cm^{-1}: 3444 (CO–OH), 1706 (C=O), 1637 (C=N), 1533 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.08 (s, 1H, COOH), 8.07 (d,

J = 8.5 Hz, 2H, furan-H, Ar–H), 7.93 (d, J = 8.5 Hz, 2H, furan-H, Ar–H), 7.67 (s, 1H, CH), 7.53–7.38 (m, 8H, Ar–H), 7.32–7.17 (m, 3H, Ar–H), 6.15 (s, 1H, CH), 3.83 (q, J = 7.0 Hz, 2H, CH_2), 0.80 (t, J = 7.0 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 166.71, 165.30, 163.90, 160.78, 155.88, 155.83, 149.74, 149.66, 138.49, 132.18, 130.58, 130.23, 129.81, 129.73, 128.69, 128.24, 127.63, 124.14, 121.63, 117.18, 115.79, 115.57, 111.79, 109.72, 60.15, 55.03, 13.25; ESI-MS m/z 595.2 $[M+H]^+$.

*5.4.11. 2-(4-((5-(4-Chlorophenyl)-6-(ethoxycarbonyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) phenoxy)acetic acid (**4k**)*

Yellow solid: yield 85.6%; mp:156.8–157.9 °C; IR (KBr) cm^{-1}: 3445 (CO–OH), 1715 (C=O), 1690 (C=N), 1598 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.11 (s, 1H, COOH), 7.78 (s, 1H, CH), 7.59 (d, J = 8.9 Hz, 2H, Ar–H), 7.50–7.36 (m, 9H, Ar–H), 7.09 (d, J = 8.9 Hz, 2H, Ar–H), 6.16 (s, 1H, CH), 4.78 (s, 2H, CH_2), 3.81 (q, J = 7.0 Hz, 2H, CH_2), 0.79 (t, J = 7.0 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 169.67, 165.35, 164.35, 159.73, 155.52, 149.93, 138.75, 138.44, 133.05, 132.11, 129.49, 128.85, 128.74,128.23,127.66, 125.70, 116.65, 115.50, 109.46, 64.54, 60.19, 55.11, 13.24; ESI-MS m/z 575.1 $[M+H]^+$.

*5.4.12. 2-(4-((6-(Ethoxycarbonyl)-5-(4-methoxyphenyl)-7-methyl-3-oxo-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) phenoxy)acetic acid (**4l**)*

Yellow solid: yield 85.6%; mp: 189.8–191.7 °C; IR (KBr) cm^{-1}: 3445 (CO–OH), 1710 (C=O), 1596 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.13 (s, 1H, COOH), 7.74 (d, J = 6.6 Hz, 1H, CH), 7.56 (t, J = 7.3 Hz, 2H, ArH), 7.22 (d, J = 8.7 Hz, 2H, ArH), 7.13–7.02 (m, 2H, ArH), 6.89 (t, J = 5.7 Hz, 2H, ArH), 5.99 (s, 1H, CH), 4.78 (s, 2H, CH_2), 4.09–3.98 (q, J = 7.1 Hz, 2H, CH_2), 3.71 (d, J = 1.9 Hz, 3H, CH_3), 2.39 (s, 3H, CH_3), 1.13 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm) δ 169.67, 164.89, 164.41, 159.65, 159.23, 155.49, 151.11, 132.79, 132.49, 132.01, 128.76, 125.71, 116.80, 115.44, 113.92, 108.64, 64.53, 60.07, 55.05, 54.25, 22.35, 13.86; ESI-MS m/z 509.1 $[M+H]^+$.

*5.4.13. 2-(4-((5-(4-Chlorophenyl)-6-(ethoxycarbonyl)-7-methyl-3-oxo-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl) phenoxy)acetic acid (**4m**)*

Yellow solid: yield 87.6%; mp:164.3–166.4 °C; IR (KBr) cm^{-1}: 3453 (CO–OH), 1712 (C=O), 1587 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.09 (s, 1H, COOH), 7.76 (d, J = 6.6 Hz, 1H, CH), 7.61 (m, 2H, ArH), 7.22–7.02 (m, 4H, ArH), 6.89 (m, 2H, ArH), 6.08 (s, 1H, CH), 4.78 (s, 2H, CH_2), 4.11–3.97 (q, J = 7.0 Hz, 2H, CH_2), 2.41 (s, 3H, CH_3), 1.09 (t, J = 7.0 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 169.42, 164.64, 164.16, 159.40, 158.98, 155.24, 150.86, 132.54, 132.24, 131.76, 128.51, 125.46, 116.55, 115.19, 113.67, 108.39, 64.28, 59.82, 54.00, 22.10, 13.61; ESI-MS m/z 513.1 $[M+H]^+$.

*5.4.14. 2-(4-((5-(4-Chlorophenyl)-6-(ethoxycarbonyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene) methyl)-2-methoxyphenoxy)acetic acid (**4n**)*

Yellow solid: yield 87.2%; mp:160.8–161.9 °C; IR (KBr) cm^{-1}: 3454 (CO–OH), 1720 (C=O), 1667 (C=N), 1589 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 7.76 (s, 1H, CH), 7.52–7.31 (m, 9H, ArH), 7.22 (d, J = 1.9 Hz, 1H, ArH), 7.17 (dd, J = 8.5, 1.9 Hz, 1H, ArH), 7.02 (d, J = 8.6 Hz, 1H, ArH), 6.16 (s, 1H, CH), 4.79 (s, 2H, CH_2), 3.89–3.74 (m, 5H, CH_2, CH_3), 0.80 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 169.66, 165.32, 164.28, 155.53, 149.91, 149.46, 148.98, 138.75, 138.39, 133.40, 133.35, 129.48, 128.85, 128.77, 128.26, 127.64, 126.01, 123.53, 116.89, 113.63, 113.20, 109.41, 64.77, 60.20, 55.58, 55.10, 13.24; ESI-MS m/z 605.1 $[M+H]^+$.

*5.4.15. 2-(4-((6-(Ethoxycarbonyl)-5-(4-methoxyphenyl)-7-methyl-3-oxo-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl)-2-methoxyphenoxy)acetic acid (**4o**)*

Yellow solid: yield 90.2%; mp:167.3–168.4 °C; IR (KBr) cm^{-1}: 3444 (CO–OH), 1709 (C=O), 1595 (C=C); ^{1}H NMR (DMSO-d_6, 400 MHz, ppm): δ 13.10 (s, 1H, COOH), 7.72 (d, J = 8.3 Hz, 1H), 7.31–7.18 (m, 3H, Ar–H), 7.14 (d, J = 8.5 Hz, 1H, Ar–H), 7.01 (d, J = 8.5 Hz, 1H, Ar–H), 6.89 (d, J = 8.7 Hz, 2H, Ar–H), 5.99 (s, 1H), 4.77 (s, 2H, CH), 4.04 (q, J = 7.0 Hz, 2H, CH_2), 3.83 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 2.39 (s, 3H, CH_3), 1.13 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): δ 169.73, 164.90, 164.35, 159.23, 155.56, 151.10, 149.51, 148.95, 133.19, 132.50, 128.72, 125.95, 123.39, 117.02, 113.94, 113.66, 113.17, 108.65, 64.98, 60.08, 55.59, 55.06, 54.22, 22.34, 13.87; ESI-MS m/z 539.1 $[M+H]^+$.

5.5. General procedure for the preparation of compounds **5a–c**

Chalcones **5a–c** were prepared by condensing substituted benzaldehydes **1a–c** with acetophenone in the presence of sodium hydroxide under the Clasein–Schmidt reaction conditions.

5.6. General procedure for the preparation of compounds **6a–c**

A mixture of chalcones **5** (1 mol) and thiourea (1.5 mmol) in ethanolic potassium hydroxide (60 g in 50 ml) was heated under reflux for 4 h. The volume of the reaction mixture was reduced to half of its original volume, diluted with ice cold water, then acidified with dilute acetic acid and kept overnight. The crude products were filtered and recrystallized to obtain pure compound **6a–c**.

5.7. General procedure for the preparation of compounds **7a–c**

A mixture of thione **6** (3 mmol), bromoacetic acid (3 mmol), anhydrous sodium acetate (3 mmol), glacial acetic acid (15 ml) and acetic anhydride (3 ml) was heated under reflux for 3 h. Then the reaction mixture was cooled and poured onto crushed ice with vigorous stirring. The separated solid was filtered and washed with water, recrystallized from methanol to give **7a–c**.

5.8. General procedure for the preparation of compounds **8a–c**

The corresponding aldehyde (1 mmol), β-alanine (1 mmol) and **7** (1 mmol) were heated at 100 °C for 1 h in glacial acetic acid. Upon completion of the reaction, the mixture was cooled, the reaction was quenched with water, and the precipitate was filtered off. The solid products were filtered and recrystallized in methanol to obtain **8a–c**.

*5.8.1. 3-(5-((5-(4-Methoxyphenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl)furan-2-yl)benzoic acid (**8a**)*

Yellow solid: yield 90.1%; mp: 267.3–268.4 °C; IR (KBr) cm^{-1}: 3434(CO–OH), 1706 (C=O), 1595 (C=C); ^{1}H NMR (DMSO-d_6, 300 MHz, ppm): δ 13.08 (s, 1H, COOH), 8.41 (s, 1H, CH), 8.11–8.08 (d, J = 7.8 Hz, 1H, furan-H), 7.99–7.96 (d, J = 7.8 Hz, 1H, furan-H), 7.84–6.93 (m, 13H, Ar–H), 6.13–6.11 (d, J = 4.5 Hz, 1H), 5.93–5.92 (d, J = 4.5 Hz, 1H), 3.73 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 100 MHz, ppm): 166.75, 164.01, 160.86, 156.11, 150.25, 149.23, 138.77, 138.60, 136.25, 131.92, 129.89, 129.80, 129.74, 129.61, 129.04, 128.32, 128.26, 127.72, 127.64, 121.77, 118.79, 116.69, 115.86, 110.72, 108.55, 60.21, 56.08; ESI-MS m/z 535.1 $[M+H]^+$.

*5.8.2. 3-(5-((5-(4-Chlorophenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl)furan-2-yl)benzoic acid (**8b**)*

Yellow solid: yield 91.3%; mp: 257.1–258.4 °C; IR (KBr) cm^{-1}: 3441 (CO–OH), 1710 (C=O), 1546 (C=C); ^1H NMR (DMSO-d_6, 300 MHz, ppm): δ 13.00 (s, 1H, COOH), 8.42 (s, 1H, CH), 8.12–8.09 (d, J = 7.8 Hz, 1H, furan-H), 7.99–7.96 (d, J = 7.8 Hz, 1H, furan-H), 7.83–7.24 (m, 13H, Ar–H), 6.15–6.13 (d, J = 4.5 Hz, 1H), 6.02–6.01 (d, J = 4.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz, ppm): δ 167.22, 164.84, 160.62, 156.00, 153.35, 149.88, 138.90, 137.52, 136.59, 132.30, 130.08, 129.79, 129.62, 129.47, 129.36, 128.79, 128.54, 125.66, 125.14, 120.97, 117.41, 116.27, 115.98, 110.88, 106.77, 55.81; ESI-MS m/z 539.1 $[M+H]^+$.

*5.8.3. 3-(5-((5-(4-Fluorophenyl)-3-oxo-7-phenyl-3,5-dihydro-2H-thiazolo[3,2-a]pyrimidin-2-ylidene)methyl)furan-2-yl)benzoic acid (**8c**)*

Orange solid: yield 89.6%; mp: 267.1–268.1 °C; IR (KBr) cm^{-1}: 3429 (CO–OH), 1710 (C=O), 1528 (C=C); ^1H NMR (DMSO-d_6, 300 MHz, ppm): δ 13.08, (s, 1H, COOH), 8.12–8.09 (d, J = 7.8 Hz, 1H, furan-H), 7.99–7.96 (d, J = 7.8 Hz, 1H, furan-H), 7.87–7.81 (m, 4H, Ar–H), 7.70 (s, 1H, CH), 7.38–7.24 (m, 9H, Ar–H), 6.14–6.13 (d, J = 4.5 Hz, 1H), 6.02–6.01 (d, J = 4.5 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz, ppm): δ 166.60, 164.22, 160.00, 155.37, 152.73, 149.26, 138.28, 136.90, 135.97, 131.68, 129.46, 129.17, 129.00, 128.85, 128.74, 128.17, 127.92, 125.04, 124.52, 120.35, 116.79, 115.65, 115.36, 110.25, 106.15, 55.19; ESI-MS m/z 523.1 $[M+H]^+$.

5.9. Evaluation of biological activity assay

5.9.1. Minimal inhibitory assay

MIC assays for the antibacterial activities of the derivatives were performed according to the broth micro-dilution (in tubes) method of the Clinical and Laboratory Standards Institute (CLSI) of America [14]. In brief, a serial twofold dilution of derivatives was added to eight tubes containing 4 ml Mueller-Hinton Broth (OXOID, England), making the final concentration from 200 μM to 0.78 μM. The turbidity of 6 h strain cultures is adjusted to match that of a 0.5 McFarland standard (approximately 108,142 CFU/ml), and then 0.02 ml of the bacterial inoculum was added to each tube. An inoculated broth containing no antibiotic was included as a bacterial growth control and a tube of un-inoculated broth was used as a sterility control. These bacteria were incubated at 37 °C for 12–16 h, the lowest concentration that completely inhibits visible growth of the organism as detected by the unaided eye is recorded as the MIC.

5.9.2. Antibiofilm activity assay

An overnight culture of *S. epidermidis* strain ATCC35984 was diluted 1:200 in TSB containing 0.25% glucose, and then 200 μl bacterial suspension was added into the wells of sterile 96-well polystyrene microtiter plates (Falcon) incubated at 37 °C for 6 h. The plates with young biofilm were washed gently four times with sterile phosphate buffered saline (PBS) before adding fresh tryptic soy broth medium (TSB) containing the various derivatives at gradient dilution, and incubated at 37 °C for 18 h. The plates were washed again four times with PBS, and 99% methanol was added for 15 min. After air-dried, the biofilm was dyed by crystal violet, then the plates were scanned at 570 nm using a 96-well plate spectrophotometer (DTX880, Beckman Coulter, USA) to determine the optical density of stained biofilms. The well with young biofilm washed by sterile PBS then dried was served as negative control; The cut-off OD value (ODc) is defined as ODc = average OD570 of negative control+3 × SD of negative control [15]. According to the OD570 of the negative control, the ODc was calculated as 0.28 in this paper. Each assay was performed in triplicate.

5.9.3. Erythrocyte hemolysis

To determine the hemolytic activities of the derivatives on erythrocytes, 5% (v/v) healthy human erythrocytes resuspended in NS were co-incubated with the derivatives at 100 μm for 1 h at 37 °C in 96-well microtiter plates. After the incubation, the suspensions were centrifuged at 350 × g for 10 min, and the level of hemolysis was determined by measuring the absorbance of the supernatant at 570 nm. Cells treated with DMSO (0.1%) and Triton-X100 (1%) were used as negative and positive controls, respectively [16].

5.9.4. Inhibition assay for YycG autophosphorylation activity

To detect whether the compound still have the inhibitory activities on YycG kinase, the recombinant HATPase_c and HisKA domains of YycG were expressed in *E. coli* BL21 (DE3) and purified by the Ni-NTA column, then ATP-Luminescence assay kit (Promega, Madison, WI, USA) were used to measure the inhibitory activities of the compounds on YycG autophosphorylation activity [5]. Briefly, 0.13 μmol/L recombinant YycG was pre-incubated with serial dilutions of the derivatives in reaction buffer (40 mmol/L Tris Ph 8.0, 20 mmol/L $MgCl_2$, and 0.1 mg/mL BSA) at 25 °C for 30 min. Then, 4 μmol/L ATP was added and the plates were incubated for 30 min at 25 °C, and Kinase-Glo™ Reagent was added to detect the remaining ATP, recorded by luminescence measurement (RLU). The rate of protein phosphorylation (Rp) inhibition by the derivatives was calculated using the equation [5]. The half maximal inhibitory concentration (IC_{50}, the concentration of the derivatives required to inhibit half of the autophosphorylation of the recombinant YycG) was determined by Origin 8.0 software (OriginLab, Northampton, USA) [5].

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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.2014.09.096.

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