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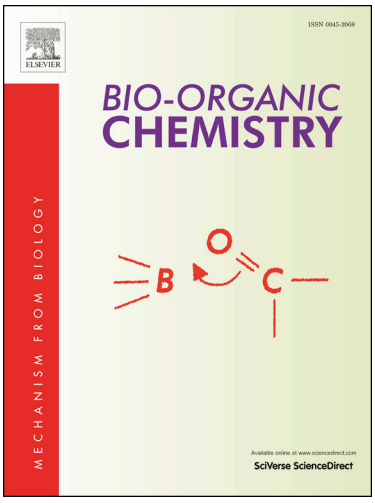

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Design and synthesis of aryl-substituted pyrrolidone derivatives as quorum sensing inhibitors

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ABSTRACT

Quorum sensing, a common cell-to-cell communication system, is considered to have promising application in antibacterial therapy since they are expected to induce lower bacterial resistance than conventional antibiotics. However, most of present quorum sensing inhibitors have potent cell toxicity, which limits their application. In this study we evaluated the diverse quorum sensing inhibition activities of different biaromatic furanones and brominated pyrrolones. On this basis, we further designed and synthesized a new series of aryl-substituted pyrrolones **12a**-**12f**. In the quorum sensing inhibition assay, compound **12a** showed improved characteristics and low toxicity against human hepatocellular carcinoma cell. In particular, it can inhibit the pyocyanin production and protease activity of *Pseudomonas aeruginosa* by 80.6 and 78.5%, respectively. Besides, in this series, some compounds exerted moderate biofilm inhibition activity. To sum up, all the findings indicate that aryl-substituted pyrrolidone derivatives is worth further investigation as quorum sensing inhibitors.

KEYWORDS *: Quorum sensing, Pseudomonas aeruginosa, Rubrolide, Pyocyanin*

1 Introduction

Drug-resistance bacteria have become a growing concern in the world. Patients with infections caused by drug-resistant bacteria are at increased risk of death. It is estimated that antimicrobial resistance induces seven hundred thousand to several million deaths per year according to the WHO global antimicrobial resistance surveillance report[1]. This trend will definitely continue to rise if no effective measures are carried out.

Quorum sensing (QS) is a mechanism of bacterial communication (a cell-to-cell communication), which regulates various phenotypes such as virulence factor expression and biofilm development [2, 3]. Up to now, there are mainly three different QS systems: acylated homoserine lactones (AHLs), peptide signals and autoinducer-2 (AI-2). Besides, there are also many other QS signals like Pseudomonas quinolone signal (PQS) and diffusible signal factor (DSF). It is believed that more QS systems will be discovered in the future[2, 4]. Biofilms enable bacteria to create a microenvironment that allows bacteria to attach to the surface of the host and evade its immune responses, even excluding standard antibiotic agent[5], which will result in the treatment of bacteria associated with biofilms to require approximately 10- to 1000-fold higher doses of antibiotic than that of planktonic bacteria[6]. In general, more than 80% of bacterial infections are related to biofilms. Since QS is not an essential process in bacteria cells, QS mutants in general don't show growth defects. Hence, preventing the production of virulence factors or biofilm formation by blocking or interfering with the communication between bacteria is an promising strategy to combat bacterial infections, thereby reducing the possibility of causing bacterial resistance[7]. An ideal quorum sensing inhibitor (QSI) must regulate bacterial virulence production without affect bacterial growth.

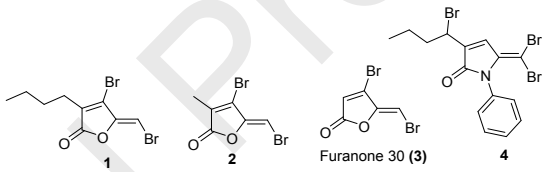


Figure 1. The structures of some quorum sensing inhibitors.

In 1993, compound **1** (Fig. 1) was firstly isolated from the marine red algae *Delisea Pulchara*[8], which was then proved to be a potent QSI[9, 10]. Since then, much focus has been put on this molecule. The structure-activity relationship study[11] shows that a conjugated exocyclic brominated vinyl group on the furanone ring is the most essential structural element for the inhibition activity against the biofilm formation of *Escherichia coli*. The mono-substituted bromine atom on the saturated carbon plays a negative role in biofilm inhibition[11], while the biofilm formation can be regulated through QS in *Pseudomonas aeruginosa*[2]. Furthermore, those furanones with the brominated groups on the saturated carbons also appear to be toxic[12]. Compound **2** and furanone 30 (**3**) (Fig. 1), designed and synthesized eventually as an optimized molecules, have been proved to be potent antagonists for LasR[13]. The predicted docking mode (Fig. 2) indicates that the carbonyl group in the molecule can perform as a hydrogen bond receptor with Trp60, and the other oxygen atom also forms a hydrogen bond with Arg61, thereby enhancing the binding affinity. However, all those brominated furanones possess various toxicity, which limits their clinical application[12]. In 2015, Wai-Kean *et.al* designed and synthesized various lactam analogues of furanones and tested for QS inhibition against the *E. coli* AHL-monitor strain JB357 *gfp* (ASV). Among them, compound **4** (Fig. 1) showed the best activity, which indicates that 1,5-dihydropyrrol-2-ones have the potential for a further development of new QS inhibitors[14].

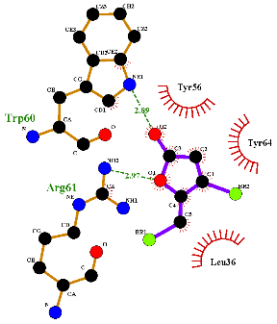


Figure 2. The predicted binding mode of furanone 30 with LasR (PDB ID: 2UV0).

In 2012, antimicrobial rubrolides (Fig. 3) with the furanone ring were extracted and identified from a South African Species of *Synoicum Tunicate*. Biological testing results indicated that they had moderate antibacterial properties against methicillin resistant *Staphylococcus epidermidis* and diverse growth inhibition against *Enterococcus faecalis* and *E. coli*[9]. Simultaneously, several antimicrobial cadiolide analogs (Fig. 3) were firstly isolated from the Ascidian *Synoicum sp.* Most of them were found to be broad-spectrum antibacterial agents. Besides, several compounds also exhibited significant inhibition of *Candida albicans*-derived isocitrate lyase and Na^+/K^+-ATPase, and moderate activity against bacterial sortase A[15].

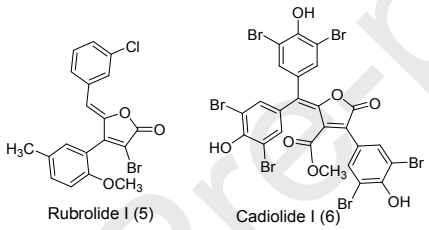


Figure 3. The structure of cadiolide I (MIC: 1.6 μg/mL against *S. enterica*) and rubrolide I (IC_{50} = 1.51 ± 0.116 μg/mL against *E. faecalis* while did not inhibit bacterial growth).

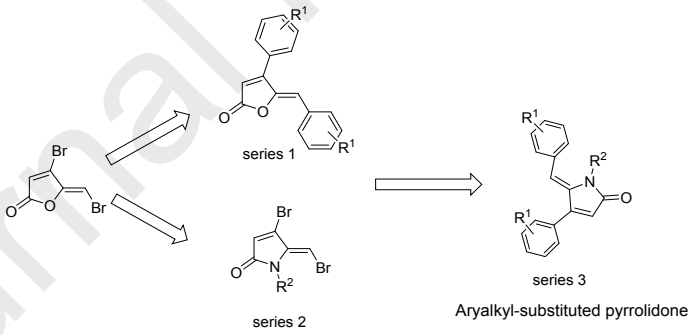


Figure 4. The design strategy of aryl-substituted pyrrolidone.

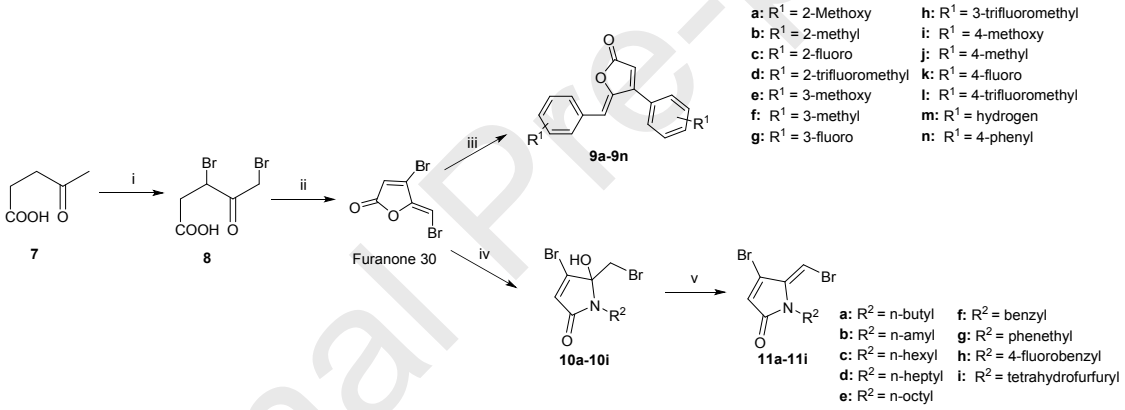
This study focuses on the structural optimization of brominated furanones and pyrrolidones. Firstly, various biaromatic furanone derivatives were designed, synthesized and evaluated to screen out suitable aromactic substituents. Pyocyanin is one of the virulence factors secreted by *P. aeruginosa*, which can regulate the ion transport of *P. aeruginosa* and interrupt the cell respiratory chain. Based on the various colors of different states of pyocyanin, we can quantify the pyocyanin of *P. aeruginosa*[16, 17]. Besides, we also use a common substrate "azocasein" to determine the activity of protease that can degrade immune components in *P. aeruginosa*[16]. Secondly, brominated pyrrolidones with various N-substituents were synthesized and evaluated to confirm suitable N-alkyl and N-aryl substituents. Finally, a new series of the aryl-substituted pyrrolidone derivatives as QSIs were designed and synthesized on the basis of the optimization results from the above two series of the furanones and pyrrolidones (Fig. 4).

2 Results and discussion

*2.1 Synthesis of compounds **9-12***

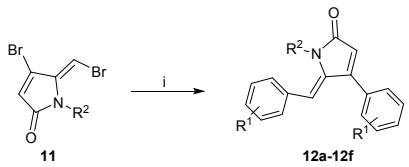
The biaromatic furanone derivatives were synthesized from commercially available levulinic acid as shown in Scheme 1[18, 19]. In this procedure, compound **7** was dibrominated by 2.2 eq bromine to give compound **8** in a yield of 78%. After that, furanone 30 was prepared in a moderate yield (63%) through the dehydration and cyclization of compound **8** under the condition of concentrated sulfuric acid. A subsequent Suzuki-Miyaura cross-coupling between furanone 30 and arylboronic acids in the presence of Na_2CO_3, tetrabutylammonium bromide and a catalytic amount of $Pd[P(C_6H_5)_3]_4$ resulted in the biaromatic furanone derivatives (**9a-9n**) in different yields (38-89%)[19, 20]. Seen from the reaction mechanism, electron-withdrawing groups arylboronic acids usually tend to be bad coupling partners, because they can be less nucleophilic and transmetalate more slowly than arylboronic acids with electron neutral or electron-donating groups[21]. In addition, the low conversions of the Suzuki cross-coupling reactions can be explained partly by the formation of the biphenyls.

Furanone 30 reacted with various amines in dichloromethane (DCM) to yield the corresponding hydroxy-lactams (**10a-10i**). Then the hydroxy-lactams were dehydrated by P_2O_5 to generate the brominated pyrrolidone derivatives (**11a-11i**) in various yields[22].



Scheme 1. Synthesis of rubrolide analogs **9a**-9**n** and **11a-11i**. Reagents and conditions: i: Br_2, HBr in acetic acid, petroleum ether (60-90 °C); iii: 98%H_2SO_4, 115 °C, 40 min; iii: arylboronic acid, Na_2CO_3, TBAB, $Pd[P(C_6H_5)_3]_4$, toluene, H_2O, reflux, 12 h; iv: amines, DCM, 0 °C, 40 min; v: P_2O_5, reflux, 3 h.

After initial activity screening, we roughly determined that 2-methoxyphenyl and the 3-tolyl groups were suitable aryl substituents for the biaromatic furanone derivatives. The compounds with the two substitutions exhibited good inhibitory activity on protease, and moderate activity on pyocyanin production. Since compounds **9** had a certain bacteriostatic effect, it was not certain what effect the compound would produce on pyocyanin and protease activity. Thus we chose three different types of side chains to study the activity of this series of compounds. On that basis, a new series of the aryl-substituted pyrrolidone derivatives **12a-12f** (Scheme 2) were designed and synthesized by further replacing the lactone of the furanone derivatives with a lactam. The compounds **11** and the arylboronic acid underwent a Suzuki-Miyaura cross-coupling to finally obtain the aryl-substituted pyrrolidone derivatives **12a-12f.**



a: R^1 = 3-methyl; R^2 = phenethyl
b: R^1 = 2-methoxy; R^2 = phenethyl
c: R^1 = 3-methyl; R^2 = n-hexyl
d: R^1 = 2-methoxy; R^2 = n-hexyl
e: R^1 = 3-methyl; R^2 = tetrahydrofurfuryl
f: R^1 = 2-methoxy; R^2 = tetrahydrofurfuryl

Scheme 2. Synthesis of aryl-substituted pyrrolidone derivatives **12a-12f**. Reagents and condition: i: arylboronic acid, Na_2CO_3, TBAB, $Pd[P(C_6H_5)_3]_4$, toluene: H_2O = 1:1, reflux for 12 h.

2.2 The result of the MIC assay

The results of the MIC assay[23, 24] (Table 1) indicate that the biaromatic furanone derivatives have no antibacterial activity against common Gram-positive and Gram-negative bacteria, which further demonstrates that this series of compounds do not interfere with the primary metabolism of bacterial cells and have little effect on the growth or reproduction of bacteria. In contrast, the brominated pyrrolidone derivatives have moderate inhibitory effects on Gram-positive bacteria such as *Staphylococcus aureus* ATCC25923 and *S. aureus* ATCC43300, and *Bacillus subtilis* ATCC9372, but do not have any inhibitory activity against two Gram-negative bacteria such as *E. coli* ATCC25922 and *P. aeruginosa* PAO1. Their inhibitory activity is probably attributed to the presence of multiple electron-deficient sites in their molecules, which are easily attacked by the protein's nucleophilic groups such as sulfhydryl or amino group in the cell membrane, thereby resulting in interaction of the molecules with the membrane proteins. Those pyrrolidone derivatives showed little inhibition activity against Gram-negative bacteria *E. coli* and *P. aeruginosa* as well since the cell wall of Gram-negative bacteria can protect the bacteria cells from antimicrobial agents.

Table 1. The result of minimum inhibitory concentration (μg/mL)

Comp	*B. subtilis* ATCC9372	*S. aureus* ATCC25923	*E. coli* ATCC25922	*P. aeruginosa* PAO1	*S. aureus* ATCC43300	*S. pneumoniae*	*S. epidermidis*
3	32	32	>128	128	128	64	32
9a	>128	>128	>128	>128	>128	>128	>128
9b	>128	>128	>128	>128	>128	>128	>128
9c	>128	>128	>128	>128	>128	>128	>128
9d	>128	>128	>128	>128	>128	>128	128
9e	>128	>128	>128	>128	>128	>128	>128
9f	>128	>128	>128	>128	>128	>128	>128
9g	>128	>128	>128	>128	>128	>128	>128
9h	>128	>128	>128	>128	>128	>128	>128
9i	>128	>128	>128	>128	>128	128	32
9j	>128	>128	>128	>128	>128	>128	128
9k	>128	>128	>128	>128	>128	>128	64
9l	>128	>128	>128	>128	>128	>128	64
9m	>128	>128	>128	>128	>128	>128	>128
9n	>128	>128	>128	>128	>128	128	128
11a	16	8	>128	128	>128	8	32
11b	16	4	>128	128	4	4	64

11c	8	4	>128	128	2	2	8
11d	8	4	>128	128	16	4	4
11e	4	4	>128	128	8	4	64
11f	16	4	>128	>128	16	16	16
11g	16	4	>128	128	8	4	128
11h	64	32	>128	128	128	32	64
11i	32	16	>128	128	32	32	16
12a	>128	>128	>128	>128	>128	>128	>128
12b	>128	>128	>128	>128	128	>128	>128
12c	>128	>128	>128	>128	>128	>128	>128
12d	>128	>128	>128	>128	>128	>128	>128
12e	>128	>128	>128	>128	>128	>128	>128
12f	>128	>128	>128	>128	>128	>128	>128
CLA	<0.25	<0.25	32	64	16	128	128
CIP	2	8	>128	4	4	2	2

CIP: ciprofloxacin; CLA: clarithromycin.

2.3 Growth Inhibition

For the growth inhibition assay[25] (Fig. S1-S6 in the supporting information), the biaromatic furanone derivatives (1 mM) did not inhibit the growth of *P. aeruginosa* PAO1, while brominated pyrrolidone derivatives had a similar activity as the positive control furanone 30 at 1 mM. This is potent growth inhibition activity against *P. aeruginosa* PAO1 but does not kill the bacteria. As for the aryl-substituted pyrrolidone derivatives, compounds **12a** and **12e** showed no inhibition activity against *P. aeruginosa* PAO1 at 1 mM, while compounds **12b** and **12f** inhibited its growth slightly, and in particular, compounds **12c** and **12d** strongly inhibited its growth.

As for *E. coli* ATCC25922, the biaromatic furanone derivatives (except compounds **9a** and **9m**) did not inhibit the bacterial growth at 1 mM, while compounds **9a** and **9m** only exhibited moderate growth inhibition without bactericidal activity. The brominated pyrrolidone derivatives (except compound **11f**) did not also inhibit the growth of *E. coli* ATCC25922 at 1 mM, while compound **11f** showed sustained growth inhibition without bactericidal activity. Among those aryl-substituted pyrrolidone derivatives, compounds **12a**, **12d**, **12e**, **12b** and **12f** did not inhibit any growth of *E. coli* ATCC25922 at 1 mM. In contrast, compound **12c** potently inhibited the growth of *E. coli* ATCC25922 but did not kill the bacteria.

As for *S. aureus* ATCC25923, most biaromatic furanone derivatives showed no growth inhibition at 1 mM, while compounds **9c**, **9d** and **9e** only slightly inhibited its growth. The brominated pyrrolidone derivatives had a significant growth inhibitory activity against *S. aureus* ATCC25923 at 1 mM, compound **11f** exhibited a moderate growth inhibitory activity, and compounds **11a**, **11b**, **11c**, **11d** and **11i** exhibited bactericidal activity.

Although the aryl-substituted pyrrolidone derivatives did not have any bactericidal activity at 1 mM, they exhibited varying degrees of inhibition effect, among which, compounds **12c**, **12d** and **12f** had moderate inhibitory effect on *S. aureus*.

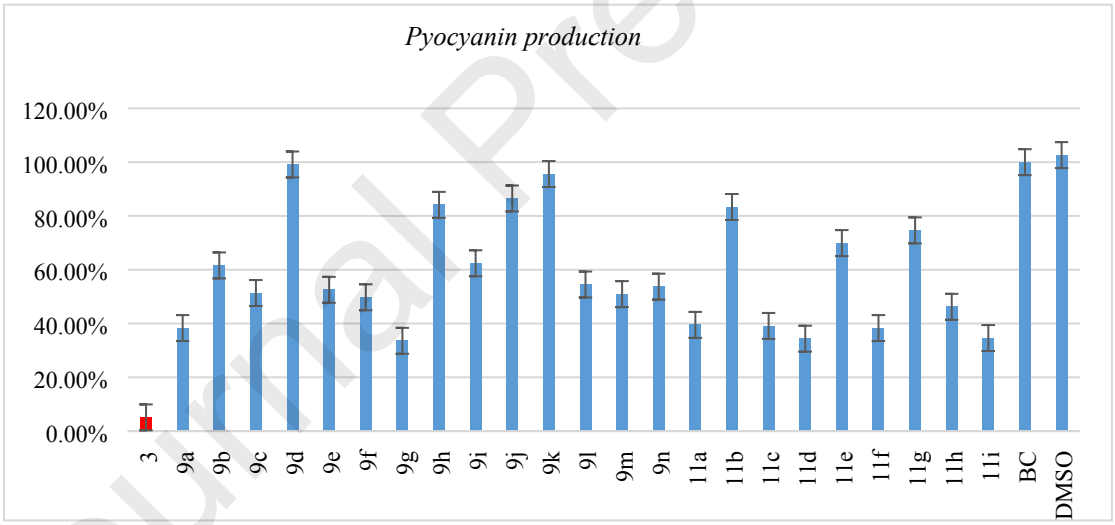
As for *S. pneumoniae*, the biaromatic furanone derivatives did not have potent inhibitory activity of the growth at 1 mM. For example, compounds **9a** and **9f** only inhibited the growth of bacteria without bactericidal activity. In the brominated pyrrolidone derivatives, compound **11d** had no inhibitory effect on the growth of *S. pneumoniae* at 1 mM, while compounds **11e** and **11h** had moderate inhibition activity, and the other brominated pyrrolidone derivatives were potent growth inhibitors of *S. pneumoniae*. The aryl-substituted pyrrolidone derivatives (except **12d**) did not inhibit the growth of *S. pneumoniae* at 1 mM, while compound

12d inhibited its growth moderately. In particular, compound **12c** inhibited the growth of *S. pneumoniae* potently, retaining the density of *S. pneumoniae* at a low range (OD_{620} < 0.075).

As for *S. epidermidis*, the biaromatic furanone derivatives (1 mM) significantly inhibited bacterial growth without bactericidal activity. In the brominated pyrrolidone derivatives, compounds **11a**, **11b**, **11f** and **11h** had a bactericidal activity, while the other biaromatic furanone derivatives had an inhibitory effect on the growth of *S. epidermidis*, but the bactericidal activity was not obvious. All of the aryl-substituted pyrrolidone derivatives had potent and significant growth inhibitory activity, but only compound **12b** had a bactericidal activity.

As for *B. subtilis* ATCC9372, the biaromatic furanone **9g** (1 mM) had a potent growth inhibitory effect, while compounds **9a**, **9b**, **9k** and **9n** did not display obvious inhibition activity. Additionally, the other compounds showed slight effects on promoting bacterial growth. The brominated pyrrolidone derivatives were potent growth inhibitors except **11f** and **11h**. Besides, compounds **11a**, **11b**, **11d** and **11e** and **11i** were even bactericidal agents. Among the aryl-substituted pyrrolidone derivatives, compounds **12a 12b** and **12d** were potent inhibitors, while the other compounds had no growth inhibition activity.

In general, the biaromatic furanone derivatives had little growth inhibition activity against the tested bacterial strains. The brominated pyrrolidone derivatives possessed less potent inhibitory effect on Gram-negative bacteria than Gram-positive strains, in which some brominated pyrrolidone derivatives had bactericidal activity. As for the aryl-substituted pyrrolidone derivatives, compounds **12c** and **12d** had potent growth inhibitory activity against some tested Gram-positive strains without bactericidal activity.



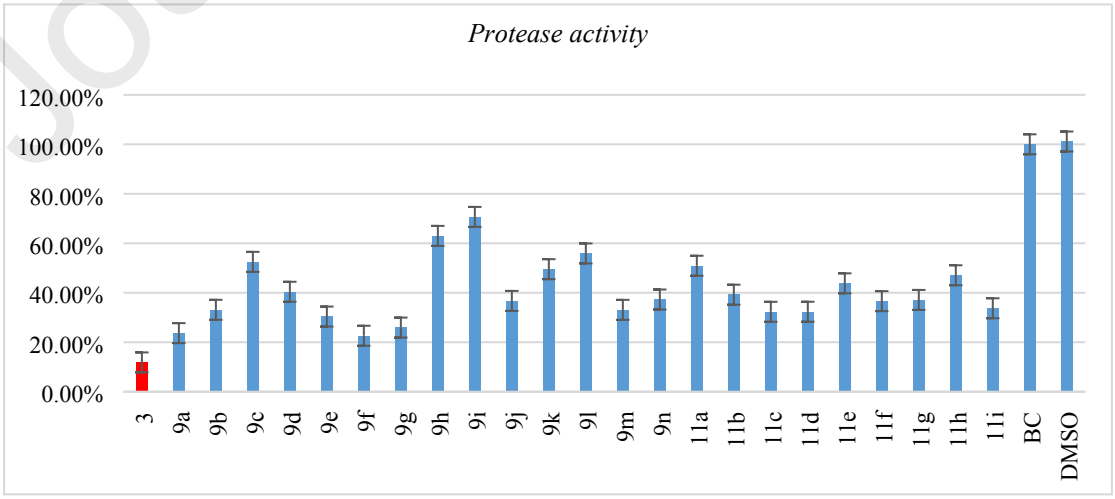


Figure 5. Pyocyanin production and protease inhibition activities of compounds **9** and **11** (1 mM). (*P. aeruginosa* PAO1, BC: blank control, DMSO: DMSO only).

*2.4 Pyocyanin production and protease inhibition of compounds **9** and **11***

In the pyocyanin expression inhibition assay of *P. aeruginosa* PAO1[16] (Fig. 5), compound **9g** showed the strongest inhibition of pyocyanin production with an inhibition ratio of 66.4%, which is narrowly followed by compound **9a** with an inhibition ratio of 61.7%. However, all of the other biaromatic furanone derivatives were proved to be less effective. Among them, compounds **9c**, **9e**, **9f**, **9l**, **9m** and **9n** had similar activity and inhibited the production of pyocyanin by more than 40%. Compounds with a fluorine in the *ortho* or *meta* positions of the benzene ring possessed considerable activity, but the compounds possessing a fluorine in the *para* position displayed extremely poor activity and hardly inhibited the production of pyocyanin. Excitingly, the results of the protease activity assay [16] indicated that compounds **9a**, **9f** and **9g** also showed good inhibition of protease activity, the inhibition percentages of which reached 76.3, 77.4 and 74.1%, respectively. In addition, the other compounds in this series displayed varying degrees of protease inhibition. Given the fact that the above compounds have little effects on bacterial growth, it can be concluded that their protease inhibition and pyocyanin production inhibition effects do not interfere with the growth and reproduction of bacteria. Therefore, they can be used as ideal QSIs. In the series of the brominated pyrrolidone derivatives, most of them can inhibit pyocyanin production moderately, such as compounds **11a, 11c, 11d, 11f** and **11i** with the inhibition ratios of pyocyanin production by more than 60%. However, compounds **11b**, **11e** and **11g** seem to be ineffective with inhibition ratios of 16.8, 30.1 and 25.4%, respectively. It appears that the length of the alkyl substituents on the nitrogen atom should not exceed 7 atoms. The protease activity assay results show similar pattern as well. The most active compound **11c** is inhibition percentage of 66.68%. Octyl-substituted compound **11e** has less potent protease inhibition activity than heptyl-substituted compound **11d**. Besides, benzyl-substituted compound **11f** and tetrahydroindenyl-substituted compound **11i** exhibit excellent protease inhibitory activity with inhibition percentages of 63.4 and 66.3%, respectively. Overall, the inhibitory effect of the compound on protease activity is a little better than that on the production of pyocyanin. Thus we selected the compounds **9f** and **11c** with the best protease activity in their respective series to determine their IC_{50} values. The result indicated that compound **11c** showed better activity on pyocyanin production with an IC_{50} value of 0.3918 mM than compound **9f**. Moreover, both compounds had moderate inhibitory activity against proteases activity (Table 2).

Table 2. The IC_{50} values against protease inhibition and pyocyanin production (*P. aeruginosa* PAO1) of compounds **9f** and **11c**.

Comp	Pyocyanin production		Protease activity	
	IC_{50} (mM)	R^2	IC_{50} (mM)	R^2
9f	0.9710	0.9333	0.5344	0.9305
11c	0.3918	0.9651	0.6645	0.9784

R^2: coefficient of determination.

*2.5 evaluation of compounds **12a-12f***

Seen from the MIC results shown in Table 1, all of the above six compounds don't have any antibacterial activity against either Gram-positive or Gram-negative bacteria. Similarly, they have little inhibition effect on

P. aeruginosa growth. This indicates that those compounds do not participate in the primary metabolic process of common bacteria and do not affect the growth and reproduction of *P. aeruginosa* as well.

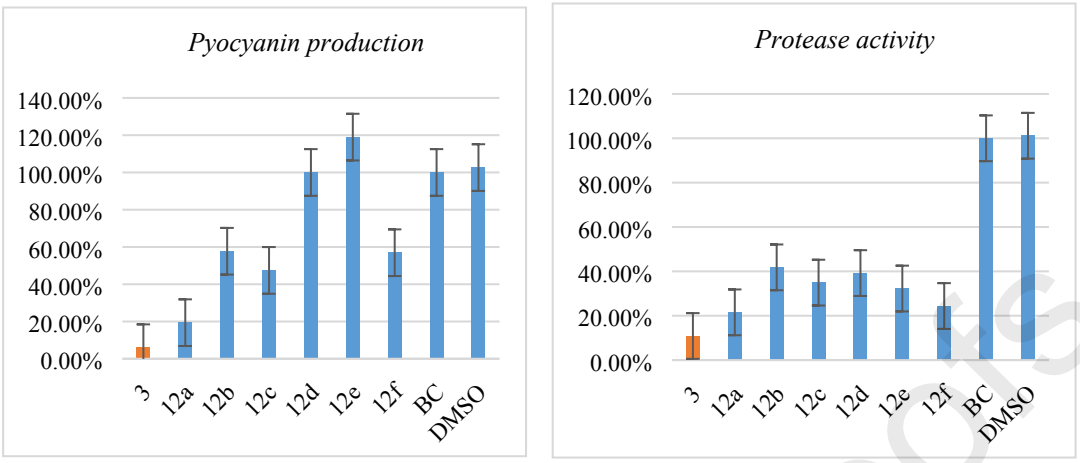


Figure 6. Pyocyanin production and protease inhibition activities of compounds **9** and **11** (1 mM). (*P. aeruginosa* PAO1, BC: blank control, DMSO: DMSO only).

In the inhibition of pyocyanin production assay of *P. aeruginosa* PAO1 (Fig. 6), compound **12a**, with an OD_{520} value of 0.0082, showed the most potent inhibition activity among the six compounds, which could inhibit over 80% of pyocyanin production in *P. aeruginosa* compared with blank control. Besides, compounds **12b**, **12c** and **12f** also exerted potent inhibition activity with inhibition rates of 43.3, 52.6 and 43.1%, respectively. However, the other two compounds **12d** and **12e** had no activity in the pyocyanin production assay. The protease activity assay results indicate that all the six compounds possess inhibition effects on the protease activity of *P. aeruginosa*. Among them, compound **12a** has the most potent inhibitor with an inhibition ratio of 78.5%, which is narrowly followed by compound **12f** with an inhibition ratio of 75.6%. The other four compounds display similar inhibition activity with inhibition ratios of around 65% against protease activity. Then, we determined the IC_{50} valuesof compounds **12a** and **12c**, the inhibitory activity of which reached more than 50% against pyocyanin production and protease activity (Table 3). For instance, compound **12a** could inhibit the production of pyocyanin by 50% at a concentration of 0.2897 mM, but it needed a concentration of 0.4364 mM to inhibit 50% of protease activity. In contrast, compound **12c** exhibited weak activity with the IC_{50} values of 0.7492 and 0.7060 mM on pyocyanin production and protease activity, respectively.

Table 3. The IC_{50} values on protease inhibition and pyocyanin production (*P. aeruginosa* PAO1) of compounds **12a** and **12c**.

Comp	Pyocyanin production		Protease activity	
	IC_{50} (mM)	R^2	IC_{50} (mM)	R^2
12a	0.2897	0.9464	0.4364	0.9540
12c	0.7492	0.9020	0.7060	0.9390

R^2: coefficient of determination.

According to the dose-inhibition curve （ Fig. 7 ） of compound **12a**, its inhibition rate on pyocyanin production and protease activity were found to increase with the rise of concentration, but there were also obvious differences between their inhibition rates. The inhibition rate of the compound on the protease activity

increased significantly at high concentration while the inhibition rate of the compound on pyocyanin production increased rapidly at low concentration. For example, at a concentration of 0.2909 mM, compound **12a** suppressed the expression of pyocyanin and the protease activity by 80.20 and 58.11%, respectively.

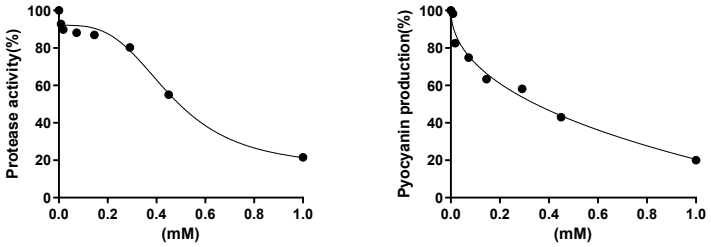


Figure 7. Protease inhibition and pyocyanin production inhibition activity (*P. aeruginosa* PAO1, 18 h) of compound **12a** in different concentration.

Furthermore, *in vitro* cytotoxicity of compound **12a** to Hela human cancer cell was measured. The result indicated that no toxicity was observed for **12a** up to 100 μM while furanone 30 was evaluated as a high-toxic substance (up to 20 μM on HeLa human cancer cells) in previous study[26, 27]. Further, the degree of inhibition of this series of compounds on the biofilms were determined to confirm whether they were biofilm inhibitors[28]. The results showed that this series had different degrees of biofilm inhibition activity (Table 4). In particular, compound **12a** exhibited an IC_{50} value of 0.2599 mM with R^2 value of 0.885, which implied this compound inhibited the biosynthesis of biofilms in *P. aeruginosa* to some extent. What's more, the other compounds such as **12b** and **12c** displayed better biofilm inhibition activity with the IC_{50} values of 0.219 and 0.242 mM than compound **12a**, which can be used as potential biofilm inhibitors. In remarkable contrast, compounds **12e** and **12f** were found to have no inhibition activity against biofilm formation in *P. aeruginosa.*

Table 4. The biofilm inhibition activity of the aryl-substituted pyrrolidone derivatives **12a-12f**.

Comp	IC_{50} (mM)	R^2
12a	0.260 ± 0.035	0.885
12b	0.219 ± 0.008	0.7983
12c	0.242 ± 0.032	0.7292
12d	0.281 ± 0.012	0.6931
12e	NI	-
12f	NI	-

NI: no inhibition activity found (IC_{50}≥1mM). R^2: coefficient of determination.

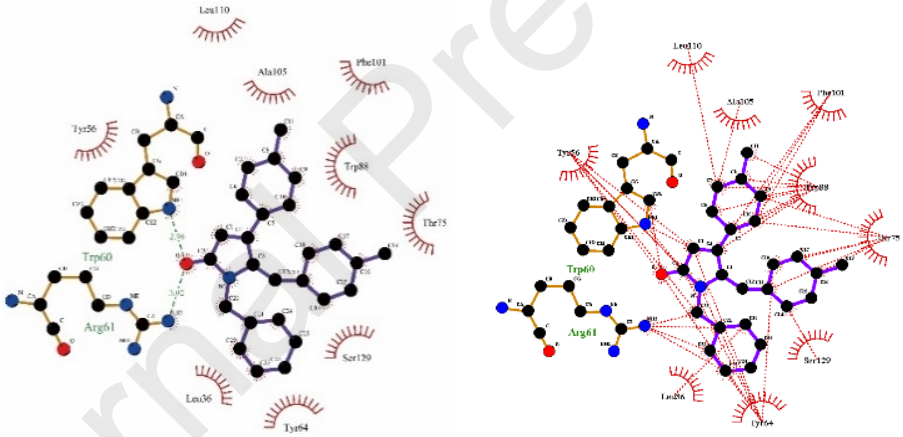
Therefore, it is reasonable that compound **12a** is a promising QSI. Then we explored the inhibition activity of compound **12a** combined with antibiotics against *P. aeruginosa* PAO1. The results indicated that this compound at 10 μM improved the antibacterial activity of ciprofloxacin and clarithromycin (Table 5). When compound **12a** (10 μM) were added to ciprofloxacin (0.60 μM) or clarithromycin (4.28 μM), their inhibition rates against *P. aeruginosa* were 60.4 and 50.6%, respectively, while ciprofloxacin and clarithromycin had only inhibition rates of 26.1 and 21.6% against *P. aeruginosa* when used alone in the same concentrations. On basis of the fact that it has no inhibition activity against *P. aeruginosa*, we speculate that compound **12a** interferes with bacterial biosynthesis process of bacterial biofilm, which increases the proportion of the above antibiotics into the bacterial cells, thereby resulting in the enhanced effectiveness of the antibiotics.

Table 5. The drug combination results.

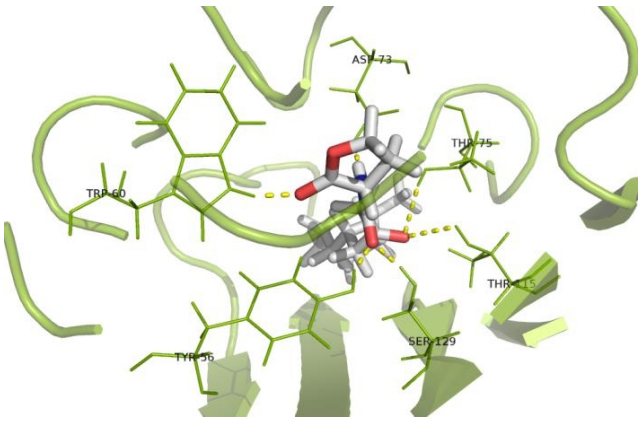
Drug combination	Inhibition rate (%)
CIP (0.60 μM)	32.4 ± 4.4
CLA (4.28 μM)	24.6 ± 4.7
12a (10 μM)	NI
12a (10 μM) + CIP (0.60 μM)	60.4 ± 1.9
12a (10 μM) + CLA (4.28 μM)	50.6 ± 2.6

NI: no inhibition activity found.

The crystal structure (PDB code: 2uv0) of LasR with the auto-inducer N-(3-oxododecanoyl)-L-homoserine (OdDHL) was used to analyze the interaction mode of those compound with the quorum sensing LasR receptor. The proposed binding mode of compound **12a** indicates that the oxygen atom in the molecule can form two different hydrogen bonds with Trp60 and Arg61 in protein LasR which is similar with furanone 30. Compared with the results of previous studies on the interaction between the autoinducer OdDHL and LasR[29], the amide and ketone functional groups in OdDHL can form multiple hydrogen bonds with the residues in the LasR ligand binding site (for example, Arg 61, Trp 60 and Ser129). Thus, we speculate that compound **12a** may inteact the same binding site on LasR as OdDHL. The formation of hydrogen bonds between compounds with Trp60 and Arg61 seem to be the key to the formation of antagonistic activity. In addition, there are also hydrophobic interactions between compound**12a** and LasR as shown in Fig. 8.



(A)



(B)

Figure 8. (A) Proposed hydrogen bonds and hydrophobic interactions between compound **12a** and LasR (PDB ID: 2UV0); (B) The crystal structure of LasR with autoinducer N-(3-oxododecanoyl)-L-homoserine (OdDHL) (PDB code: 2UV0)

2.6 Conclusion and discussion

In summary, we explored the appropriate substituents on the benzene ring of biaromatic furanones and found that the compounds with electron-donating groups had the better inhibition activity against pyocyanin production and protease activity in *P. aeruginosa*. The bioevaluation results of the brominated pyrrolones indicated the compounds with benzyl and n-hexyl substituents had good quorum sensing inhibition activity. Based on the above findings and the computational docking result, a new series of aryl-substituted pyrrolidone derivatives were designed and synthesized. Further investigation indicated that compound **12a**, as a potent quorum sensing inhibitor, effectively inhibited pyocyanin production (by 80.6%) and protease activity (by 78.5%) in *P. aeruginosa* without affecting the growth and reproduction of the tested bacteria. Moreover, it blocked the biofilm formation of *P. aeruginosa* to some extent, which makes it possible to improve the effects of antibiotics. In the cytotoxicity assay this compound showed little toxicity against human hepatocellular carcinoma cell. Finally, molecular docking method was used to visualize possible binding mode of compound **12a** and protein LasR. Taken together, compound **12a** is believed to serve as a novel structure with low toxicity for further investigation as a quorum sensing inhibitor. Moreover, researches on aryl-substituted pyrrolidone derivatives can be carried out for enhanced their activity.

Supporting informations

Growth inhibition results; Growth curves; Experimental methods; ^{1}H NMR, ^{13}C NMR, HRMS and MS spectra of representative compounds can be found in supporting informations.

Acknowledgements

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Conflicts of interest

The authors declare no competing financial interest.

ABBREVIATIONS

QSIs: quorum sensing inhibitors
QS: quorum sensing
DCM: dichloromethane
BC: blank control
CIP: ciprofloxacin
CLA: clarithromycin

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The captions for Figures and Schemes

Figure 1. The structures of some quorum sensing inhibitors.

Figure 2. The predicted binding mode of furanone 30 with LasR (PDB ID: 2UV0).

Figure 3. The structure of cadiolide I (MIC: 1.6 μg/mL against *S. enterica*) and rubrolide I (IC_{50} = 1.51 ± 0.116 μg/mL against *E. faecalis* while did not inhibit bacterial growth).

Figure 4. The design strategy of aryl-substituted pyrrolidone.

Figure 5. Pyocyanin production and protease inhibition activities of compounds **9** and **11** (1 mM). (*P. aeruginosa* PAO1, BC: blank control, DMSO: DMSO only).

Figure 6. Pyocyanin production and protease inhibition activities of compounds **9** and **11** (1 mM). (*P. aeruginosa* PAO1, BC: blank control, DMSO: DMSO only).

Figure 7. Protease inhibition and pyocyanin production inhibition activity (*P. aeruginosa* PAO1, 18 h) of compound **12a** in different concentration.

Figure 8. (A) Proposed hydrogen bonds and hydrophobic interactions between compound **12a** and LasR (PDB ID: 2UV0); (B) The crystal structure of LasR with autoinducer N-(3-oxododecanoyl)-L-homoserine (OdDHL) (PDB code: 2UV0)

Scheme 1. Synthesis of rubrolide analogs **9a**-9**n** and **11a**-**11i**. Reagents and conditions: i: Br_2, HBr in acetic acid, petroleum ether (60-90 °C); iii: 98%H_2SO_4, 115 °C, 40 min; iii: arylboronic acid, Na_2CO_3, TBAB, $Pd[P(C_6H_5)_3]_4$, toluene, H_2O, reflux, 12 h; iv: amines, DCM, 0 °C, 40 min; v: P_2O_5, reflux, 3 h.

Scheme 2. Synthesis of aryl-substituted pyrrolidone derivatives **12a-12f**. Reagents and condition: i: arylboronic acid, Na_2CO_3, TBAB, $Pd[P(C_6H_5)_3]_4$, toluene: H_2O = 1:1, reflux for 12 h.

Figure 1

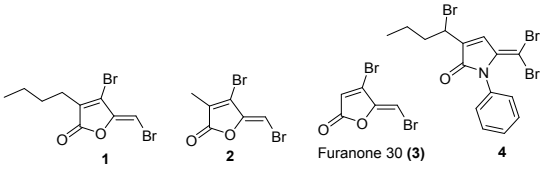


Figure 2

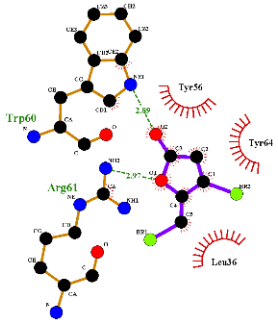


Figure 3

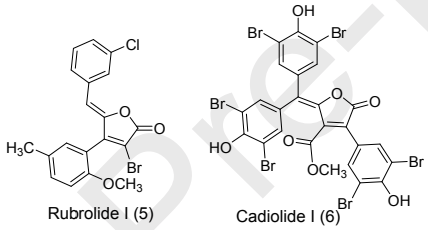


Figure 4

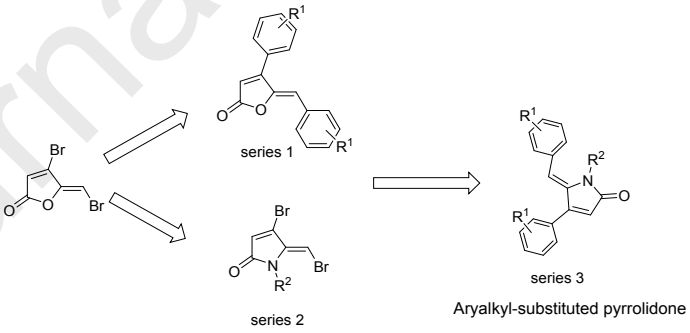


Figure 5

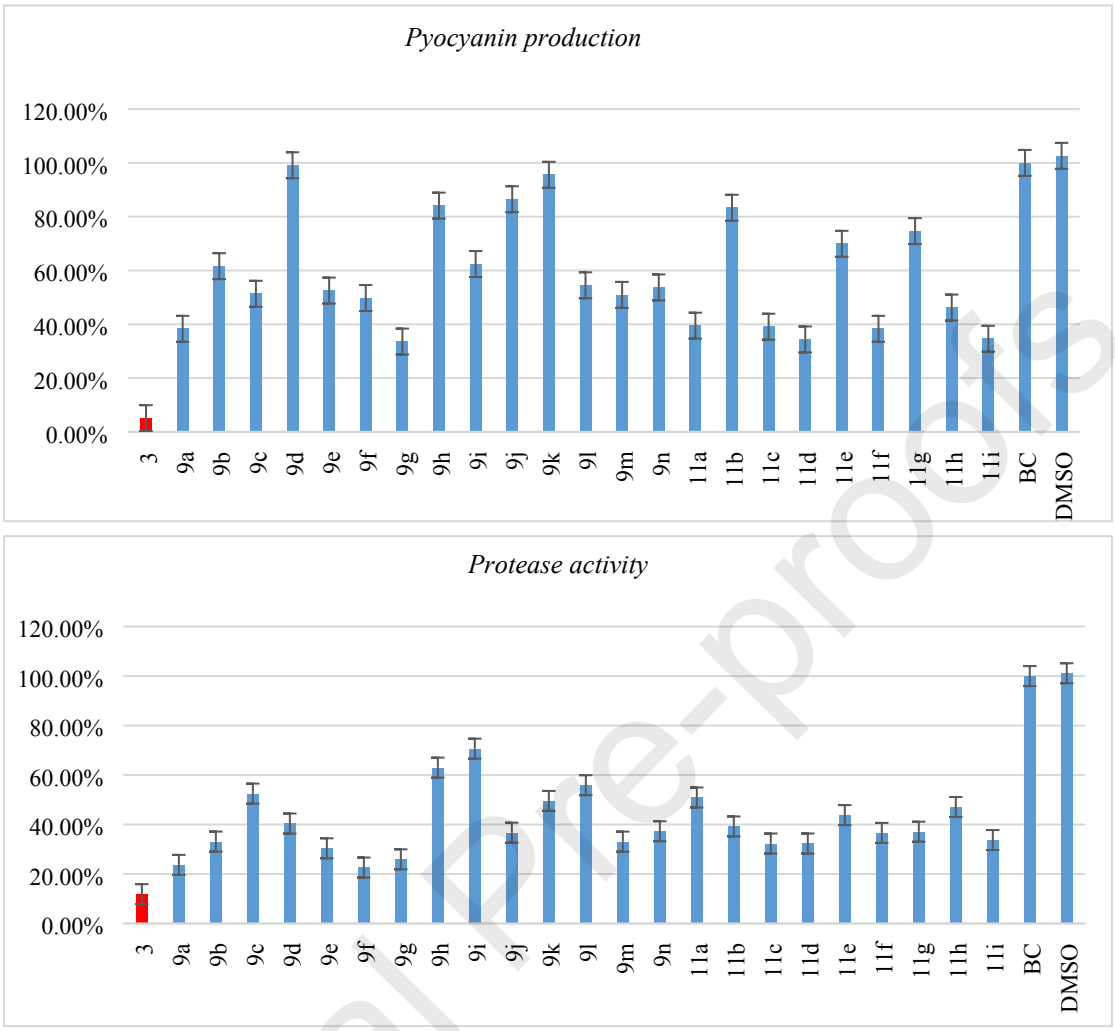


Figure 6

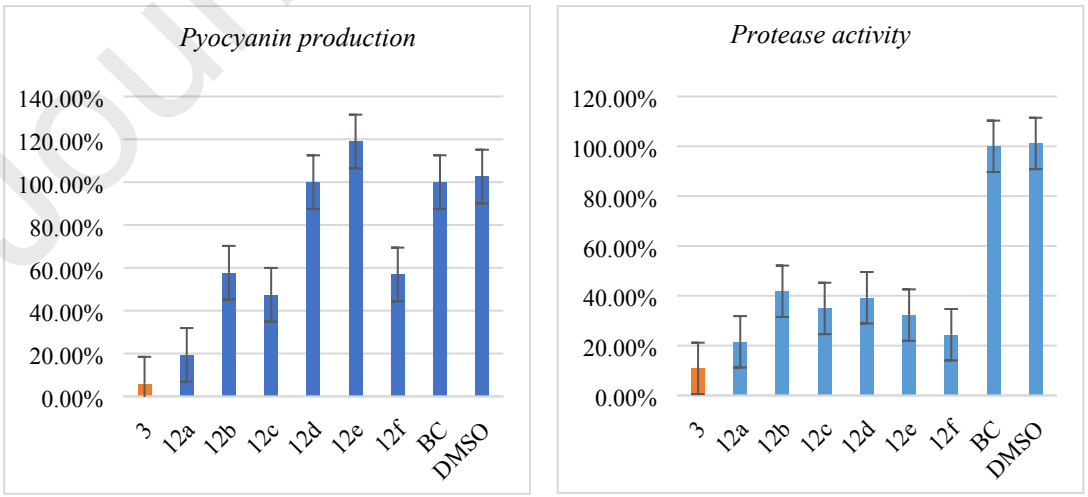


Figure 7

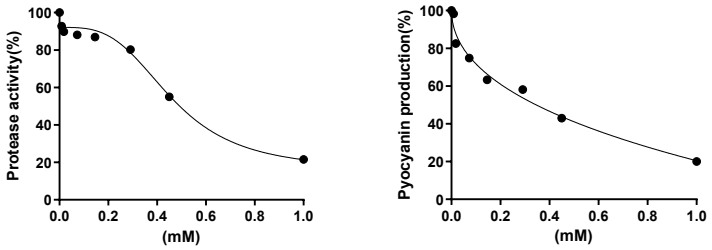
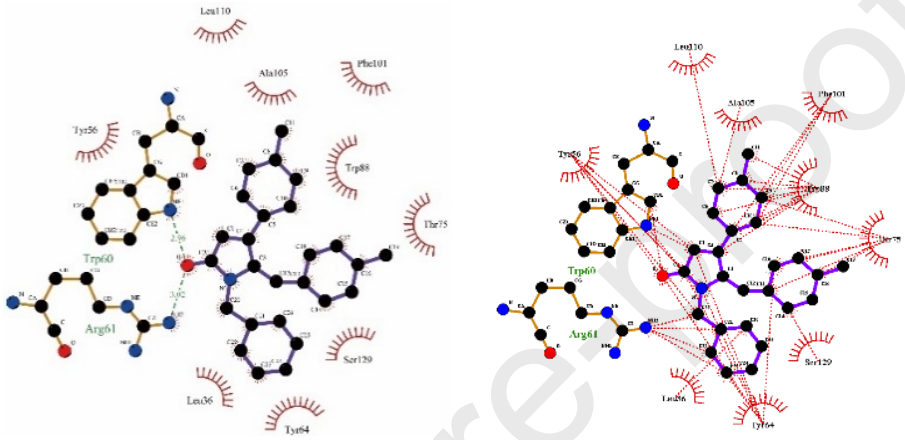


Figure 8



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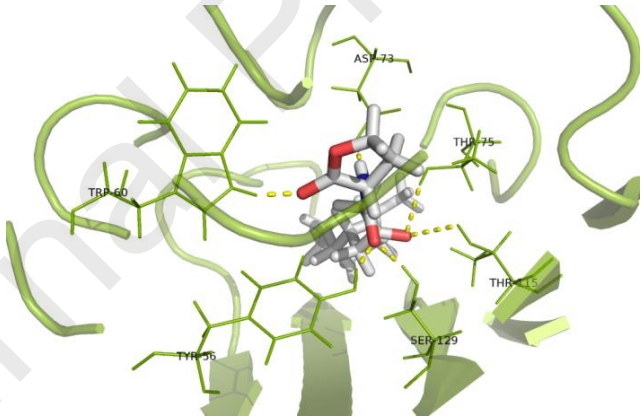
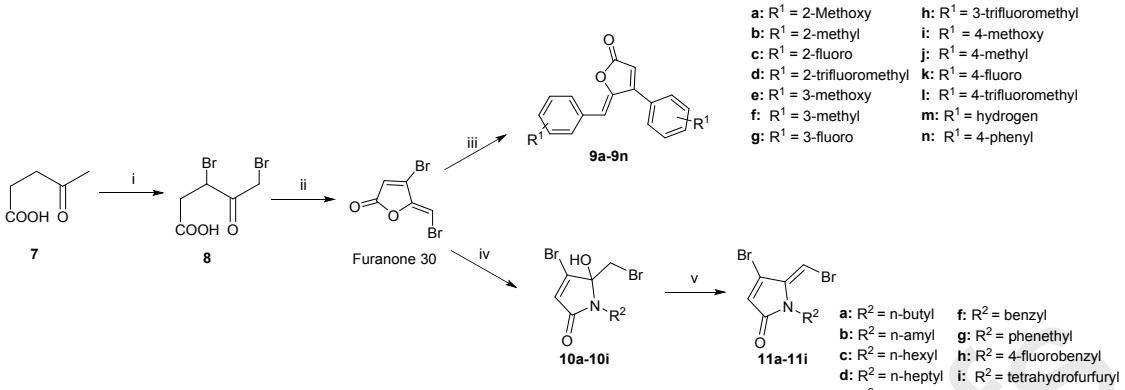


(B)

Scheme 1



Scheme 2

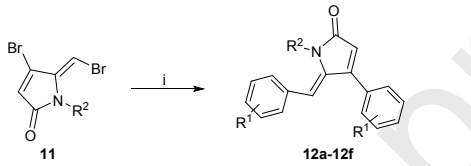


Table 1. The result of minimum inhibitory concentration (μg/mL)

Comp	*B. subtilis* ATCC9372	*S. aureus* ATCC25923	*E. coli* ATCC25922	*P. aeruginosa* PAO1	*S. aureus* ATCC43300	*S. pneumoniae*	*S. epidermidis*
3	32	32	>128	128	128	64	32
9a	>128	>128	>128	>128	>128	>128	>128
9b	>128	>128	>128	>128	>128	>128	>128
9c	>128	>128	>128	>128	>128	>128	>128
9d	>128	>128	>128	>128	>128	>128	128
9e	>128	>128	>128	>128	>128	>128	>128
9f	>128	>128	>128	>128	>128	>128	>128
9g	>128	>128	>128	>128	>128	>128	>128
9h	>128	>128	>128	>128	>128	>128	>128
9i	>128	>128	>128	>128	>128	128	32
9j	>128	>128	>128	>128	>128	>128	128
9k	>128	>128	>128	>128	>128	>128	64
9l	>128	>128	>128	>128	>128	>128	64
9m	>128	>128	>128	>128	>128	>128	>128
9n	>128	>128	>128	>128	>128	128	128
11a	16	8	>128	128	>128	8	32
11b	16	4	>128	128	4	4	64
11c	8	4	>128	128	2	2	8
11d	8	4	>128	128	16	4	4

11e	4	4	>128	128	8	4	64
11f	16	4	>128	>128	16	16	16
11g	16	4	>128	128	8	4	128
11h	64	32	>128	128	128	32	64
11i	32	16	>128	128	32	32	16
12a	>128	>128	>128	>128	>128	>128	>128
12b	>128	>128	>128	>128	128	>128	>128
12c	>128	>128	>128	>128	>128	>128	>128
12d	>128	>128	>128	>128	>128	>128	>128
12e	>128	>128	>128	>128	>128	>128	>128
12f	>128	>128	>128	>128	>128	>128	>128
CLA	<0.25	<0.25	32	64	16	128	128
CIP	2	8	>128	4	4	2	2

CIP: ciprofloxacin; CLA: clarithromycin.

Table 2. The IC_{50} values against protease inhibition and pyocyanin production (*P. aeruginosa* PAO1) of compounds **9f** and **11c**.

Comp	Pyocyanin production		Protease activity	
	IC_{50} (mM)	R^2	IC_{50} (mM)	R^2
9f	0.9710	0.9333	0.5344	0.9305
11c	0.3918	0.9651	0.6645	0.9784

R^2: coefficient of determination.

Table 3. The IC_{50} values on protease inhibition and pyocyanin production (*P. aeruginosa* PAO1) of compounds **12a** and **12c**.

Comp	Pyocyanin production		Protease activity	
	IC_{50} (mM)	R^2	IC_{50} (mM)	R^2
12a	0.2897	0.9464	0.4364	0.9540
12c	0.7492	0.9020	0.7060	0.9390

R^2: coefficient of determination.

Table 4. The biofilm inhibition activity of the aryl-substituted pyrrolidone derivatives **12a-12f**.

Comp	IC_{50} (mM)	R^2
12a	0.260 ± 0.035	0.885
12b	0.219 ± 0.008	0.7983
12c	0.242 ± 0.032	0.7292
12d	0.281 ± 0.012	0.6931
12e	NI	-
12f	NI	-

NI: no inhibition activity found (IC_{50}≥1mM). R^2: coefficient of determination.

Table 5. The drug combination results.

Drug combination	Inhibition rate (%)
CIP (0.60 μM)	32.4 ± 4.4
CLA (4.28 μM)	24.6 ± 4.7
12a (10 μM)	NI
12a (10 μM) + CIP (0.60 μM)	60.4 ± 1.9
12a (10 μM) + CLA (4.28 μM)	50.6 ± 2.6

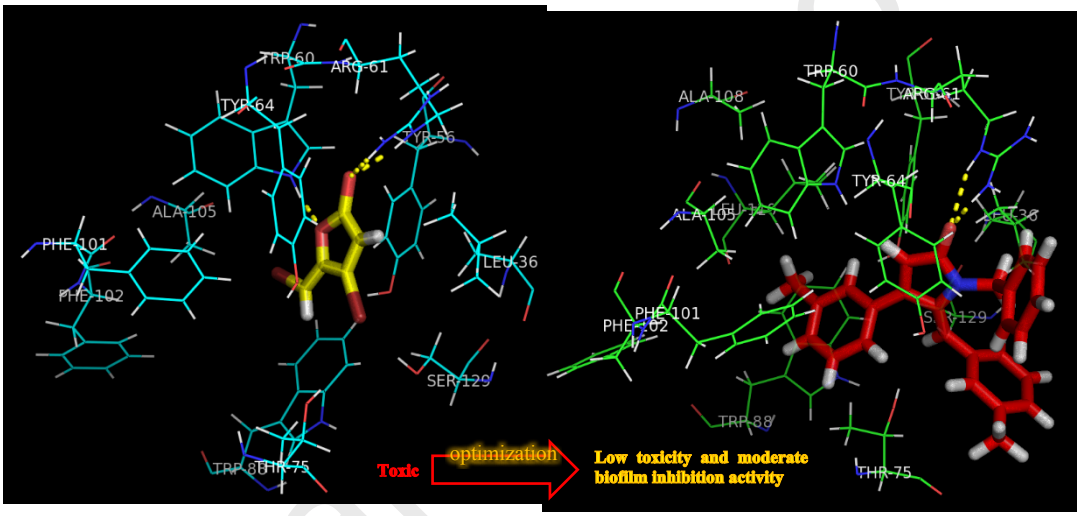
NI: no inhibition activity found.

Graphical Abstract:

Design and synthesis of aryl-substituted pyrrolidone derivatives as quorum sensing inhibitors

Zhiyang Liu [a,1], Panpan Zhang [a,1], Yinhui Qin [a], Nan Zhang [a], Yuetai Teng [a], Henrietta Venter [b], Shutao Ma [a,*]

A novel series of aryl-substituted pyrrolones were confirmed to be quorum sensing inhibitors against *P. aeruginosa*. In particular, compound **12a** was found to inhibit the pyocyanin production and protease activity of *P. aeruginosa* by 80.6 and 78.5%, respectively.



Research Highlights

> A novel series of aryl-substituted pyrrolidone were designed and synthesized. > They were evaluated as quorum sensing inhibitors against *P. aeruginosa*. > Compound **12a** inhibited the pyocyanin production and protease activity by 80.6 and 78.5%, respectively. > Compound **12a** is worth further investigation as a quorum sensing inhibitor.

Conflicts of interest

The authors declare that this study was carried out only with public funding. There is no funding or no agreement with commercial for profit firms.