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Novel 2, 8-bit derivatives of quinolines attenuate *Pseudomonas aeruginosa* virulence and biofilm formation

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ABSTRACT

Signal molecules are stimulators of multiple quroum-sensing virulence and biofilm formation. Small molecule analogues have been suspected as a potent inhibitor in therapeutic strategy. Herein, we synthesized a series of small molecule compounds from the 2, 8-bit derivatives of quinoline by Suzuki coupling reaction. We found that these compounds have the biofilm inhibitory effect in normal condition instead of phosphate limitation state. Furthermore, *lacZ* reporter strain assay and rhamnolipids as well as pyocyanin experiments showed that these compounds did not affect *las* and *pqs* system but reduced the expression of *rhl*. All these results suggest that quinoline derivatives can be treated as potent inhibitors against biofilm and reduce virulence through the *rhl* system. This research will be useful in designing new quorum sensing inhibitors to attenuate the infection of bacteria.

In order to adapt the change of environment, especially the widespread use of antibiotics, bacteria develop multiple mechanisms to prevent themselves from kinds of adverse conditions and maintain their pathogenesis.[1] One of the crucial mechanisms is Quorum sensing (QS), which is a cell-to-cell communication that relies on cell density to control collective behavior.[2] With the regulation of QS, bacteria can form kinds of virulence such as elastase, pyocyanin, alginate, rhamnolipids and biofilm to survive in disadvantageous circumstance.[3–5]

Gram-negative bacteria *Pseudomonas aeruginosa* is a ubiquitous organism living on the surface of animals, plants and even human being.[6] It is a notorious opportunistic pathogen for inducing acute and persistent infection in the patients with cystic fibrosis, neutropenia and burns or wound.[7] Numerous articles revealed that QS plays an essential role in *P. aeruginosa* virulence and biofilm formation.[6,7] Some people suspected that QS in *P. aeruginosa* cause the prevalence of chronic lung infections in patients with cystic fibrosis.[8] To date, there are four QS system have been found in that pathogenic bacteria, including *las*, *rhl*, *iqs* and *pqs*. It is believed that QS exist a hierarchy network in bacteria. Traditional view thinks *las* system as the top of QS which controls the expression of *pqs* and *rhl*.[9–11] However, Lee et al. [12] proposed that *iqs* can replace the *las* system under the phosphate limitation conditions though the target is not yet clear. This hypothesis explains why so many clinically isolated strains with the lost of the *las* system are still pathogenic.[13] In *P. aeruginosa*, the expression of QS depends on the signal molecules which is called autoinducers (AIs). The signal molecules are N-(3-oxo-dodecanoyl)-l-homoserine lactone (OdHSL), *N*-butanoyl-homoserine lactone (BHL), 2-heptyl-3,4-dihydroxyquinoline (PQS), and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS) which are regulated by LasI, RhlI, PqsABCD and AmbBCDE respectively through their transcription factors LasR, RhlR and PqsR.[14] (Fig. 1) Biofilm is another critical reason for the bacterial resistance. Bacteria embed in a self-produced extracellular matrix that consists of polysaccharide matrix, fibrin, lipid protein that can reduce the injury from antibiotics. Solano et al. [15] supposed that QS regulates genes involved in biofilm development.

Recently, people found that signal molecule analogues exhibit excellent anti-biofilm activity and attenuate quorum-sensing virulence.[16,17] However, only few autoinducer type drugs are used in clinically treatment. In order to develop more effective drugs to use in the clinic for the inhibitions of the infection of *P. aeruginosa*, we designed and synthesized 2, 8-bit derivatives of quinolines based on the

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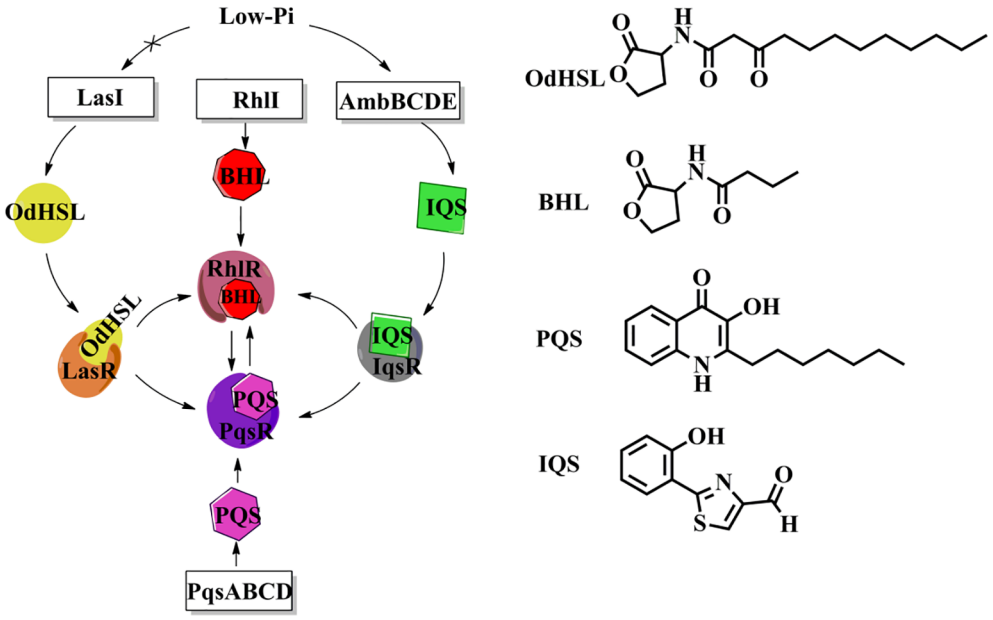


Fig. 1. A summary of quorum-sensing system networks in *P. aeruginosa* and the signal molecules.

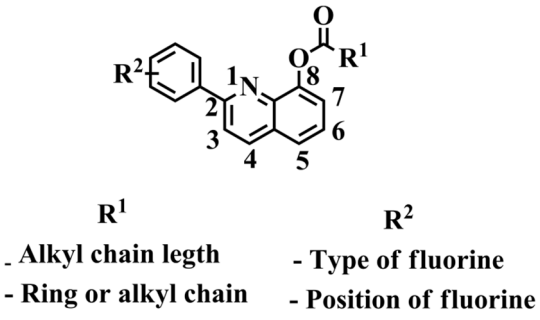
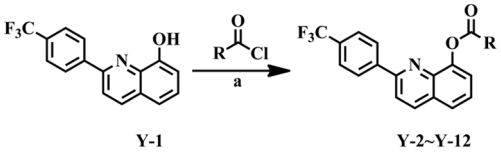


Fig. 2. Strategy for structural modification of quinoline derivatives.

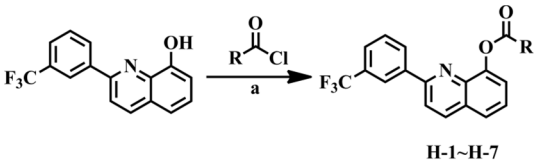
Table 1
Structure of 2-(4-(trifluoromethyl)phenyl)quinolin-8-ol derivatives.



Ragents and condition: (a) DMAP, ETA, DCM, rt, 2 h

Compound	R	Compound	R
Y-2		Y-8	
Y-3		Y-9	
Y-4		Y-10	
Y-5		Y-11	
Y-6		Y-12	
Y-7			

Table 2
Structure of 2-(3-(trifluoromethyl)phenyl)quinolin-8-ol derivatives.



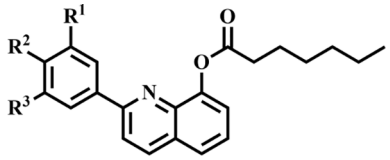
Ragents and condition: (a) DMAP, ETA, DCM, rt, 2 h

Compound	R	Compound	R
H-1		H-5	
H-2		H-6	
H-3		H-7	
H-4			

signal molecule. Considering the QS may involve in biofilm formation, we used crystal violet to evaluate the effect and found that some compounds decrease the biofilm formation of *P. aeruginosa* in rich medium instead of phosphate limitation condition. For further exploration, we tested the expression of *las*, *rhl* and *pqs* by the bioreporter assay based on the expression of *lacZ* in *P. aeruginosa* PAO1 and thus had detect these effective compounds took place via *rhl* system. The rhamnolipids and pyocyanin assay results confirmed our consequent.

Quinoline has a potent inhibitory activity towards bacteria virulence like pyocyanin, hydrogen cyanide and biofilm.[18] C-2 position in quinoline is an active site for antibacterial activity. It has been reported[19,20] that C-2 position fluorine-substituted phenyl groups can increase the antibacterial activities. The ester and amide bonds at the C-8 position can increase the biofilm inhibitory activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*. The quinoline derivatives play crucial effect on resisting both gram-negative and gram-

Table 3
Structure of 2-phenylquinolin-8-yl heptanoate derivatives.



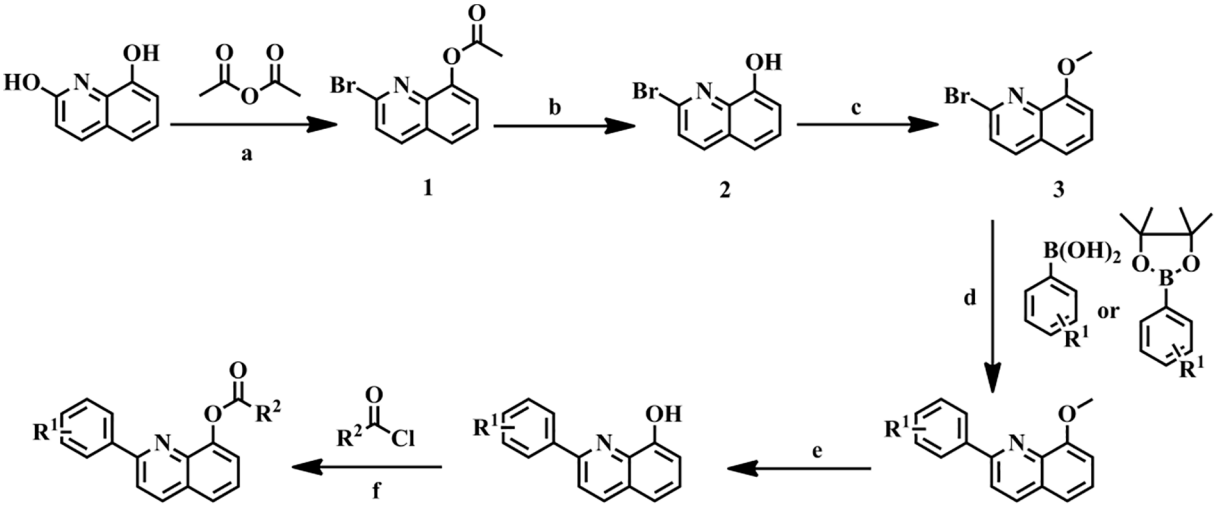
Compound	R^1	R^2	R^3
K-1	H	F	H
K-2	CF_3	H	CF_3

positive bacteria, and perform excellent activity in anti-quorum sensing and biofilm resistance. Therefore, the C-2 and C8 substituted quinoline derivatives were synthesized in this work. The strategy of the design of the library was illustrated in Fig. 2. In order to further elaborate on the structural features, our compounds are shown in Tables 1–3.

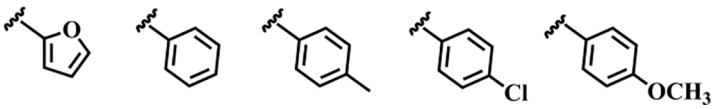
The synthesis of 2,8-substituted quinoline derivatives route was shown in Scheme 1. Selectively C-8 position mono-acetylation of 2,8-hydroxyquinoline with acetic anhydride followed by bromination at C-2 position with $POBr_3$ gave compound **1**. Then, hydrolysis of compound **1** with potassium hydroxide generated compound **2**. The resulting free alcohol was then methylation with CH_3I yielded compound **3**. Suzuki coupling of compound **3** with different Boron substrates generated corresponding 8-methoxy-2-phenylquinoline derivatives. After removal methyl group, 2, 8-quinoline derivatives were finally synthesized after acetylation with different acetyl chloride.

Antimicrobial drugs suppress the growth of bacteria. However, on the opposite side, bacteria develop multiple resistance mechanisms to survive. In order to avoid this result, quorum-sensing inhibitors take a new strategie to inhibit the virulence factor and biofilm formation instead of destroying bacteria. To make sure our compounds do not have influence on the growth of bacteria, we detected our compounds on the effect on the growth of *P. aeruginosa* PAO1 and the minimum inhibitory concentration (MIC) on two Gram-positive bacteria, the *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 8739) and two Gram-negative bacteria, the *Staphylococcus albus* (ATCC 8799), *Klebsiella Pneumoniae* (ATCC 13883) by measuring the optical density value at the wavelength of 600 nm (OD_{600}). As expected, *P. aeruginosa* PAO1 strain growth curve was not affected and all the MIC of bacteria were > 256 μM, which mean that our compounds have no influence on the growth of bacteria. The growth of bacteria assay and MIC assay (Fig. 3) confirmed these compounds are potent to be the quorum sensing inhibitors.

In the C-8 position, we tried to find the relationship between the activity and the length of optimal chain. Thus, we synthesized 2-(4-(trifluoromethyl)phenyl)quinolin-8-ol with different chains (Table 1, **Y-1** to **Y-7**) and investigated the biofilm inhibition by crystal violet.[21] As shown in Fig. 4, anti-biofilm activities increased as the alkyl chain lengthened to 4, 6 or 7, indicating that biofilm inhibitor should keep a long chain like the AI. Comparing **Y-1** to **Y-12** and **H-1** to **H-7**, we found that the anti-biofilm activities are better when the chain is alkyl instead of aromatic ring. These results may relate to the function of alkyl group contributes to the van der Waals interaction in the QS receptor hydrophobic subpocket while ring structure has no such effect. Since we



Ragents and conditions: (a) i) Acetic anhydride, 140 °C, overnight, reflux. ii) $POBr_3$, $CHCl_3$, 2h, refiux
(b) KOH, EtOH, rt, 1h
(c) CH_3I, NaH, THF 0 °C-rt, 12h
(d) Pd $(PPh_3)_4$ or $PdCl_2$ (dppf), PhMe, EtOH, H_2O,$CsCO_3$, 24 h, reflux
(e) BBr_3, DCM , 2h, rt
(f) DMAP, TEA, DCM, 2h, rt
R^1 = 3'-CF_3; 4' -CF_3; 3', 5'-CF_3; 4'-F
R^2 = CH_3; CH_2CH_3; $(CH_2)_2CH_3$; $(CH_2)_3CH_3$; $(CH_2)_4CH_3$; $(CH_2)_5CH_3$;



Scheme 1. Synthesis of 2, 8-quinoline derivatives.

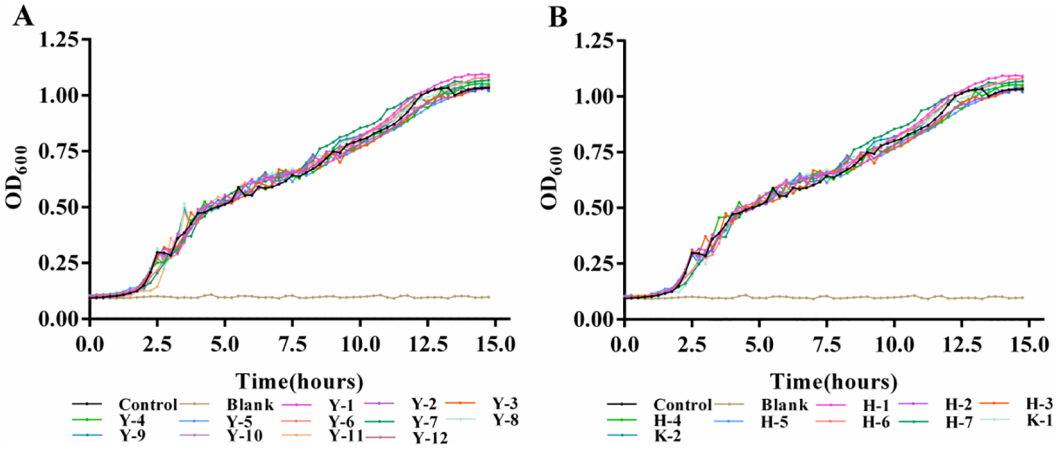


Fig. 3. Growth curve of *P. aeruginosa* (A) without treated (control) or treated with 10 μM **Y-1 to Y-12**. (B) without treated (control) or treated with 10 μM **H-1** to **H-7**, **K-1**, **K-2**.

found that the fluorine-substituted phenyl groups at the C-2 position can increase the antibacterial and antibiosis effects of quinoline compounds, a series of C-2 fluorinated compounds were designed and tested.[19,20] The graphic of Fig. 4 reveal that the 4′-position were more favorable for inhibition of biofilm.

In order to determine the quorum sensing activity of our synthesized compounds, we investigated the effect of our compounds with 10 μM concentration on the expression of *lasB-lacZ*, *rhlA-lacZ*, *pqsA-lacZ* in the *P. aeruginosa*.[22] If the *lacZ* is down regulated, the production of *β*-galactosidase will decrease and then we can detect it by *o*-Nitrophenyl-*β*-D-Galactopyranoside (ONPG). Based on that result, we detected the relation between our compounds and the promoter of *las*, *rhl* and *pqs* system. To our surprise, the *rhlA-lacZ* had obviously been inhibited while *lasB-lacZ*, *pqsA-lacZ* were not affected. So, we speculated that the anti-biofilm activities of our synthesized compounds are realized through the *rhl* system. Since **Y-7** and **H-7** showed the best inhibition activity of QS report strains among the synthesized compounds, they were used as our model molecules in the mechanism study (See Fig. 5).

To verify our speculation, we tested the effect of our compounds on *rhl* system by the rhamnolipids and pyocyanin assay.[23,24] Rhamnolipids is known as a condition for the development of chronic infection which were detected in the sputum from cystic fibrosis (CF) patients.[25] By regulating the enzymes for the biosynthesis of rhamnolipids, *rhl* system controls the rhamnolipids virulence factor directly. So, we monitor the rhamnolipids through orcinol. Consistent with our speculation, the production of rhamnolipids was significantly reduced by **Y-7** and **H-7**. Then we can make a conclusion that our compounds indeed inhibit the expression of *rhl* system. To further evidence, we tried to detect the production of pyocyanin strictly adjusted via *pqs* and *rhl* pathway.[26]

Since pyocyanin can be synthesized by *P. aeruginosa*, *pqs* genes must be functionalized. *Pqs* is a gene which produces a transcription factor that activates *phnAB* genes. These genes produce the molecule quinolone. However, through our study, a strange phenomenon that we can observe is **Y-7** and **H-7** had no effect on the production of pyocyanin as shown in Fig. 6. The possible reason pyocyanin is controlled by both *pqs* and *rhl* system. If we just inhibit one of two systems, bacteria can improve the other pathway to maintain its virulence. Combined the results of rhamnolipids and pyocyanin assay, we realized that our compounds only repressed the *rhl* system thus the production of rhamnolipids was

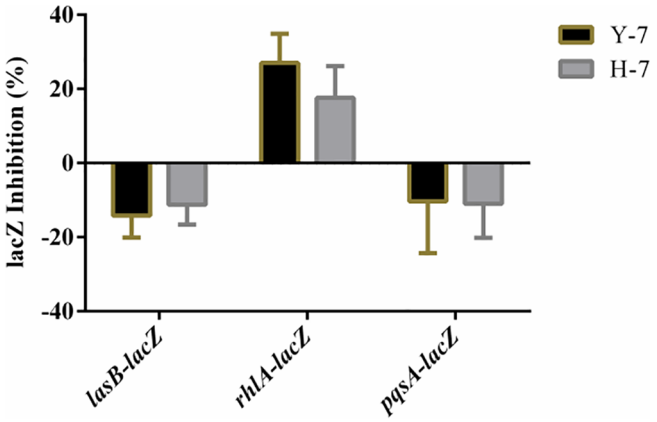


Fig. 5. Effect of **Y-7** (10 μM) and **H-7** (10 μM) on the different QS monitors, PAO1-*lasB-lacZ*, PAO1-*rhlA-lacZ*, PAO1-*pqsA-lacZ*. Experiments were done in triplicate.

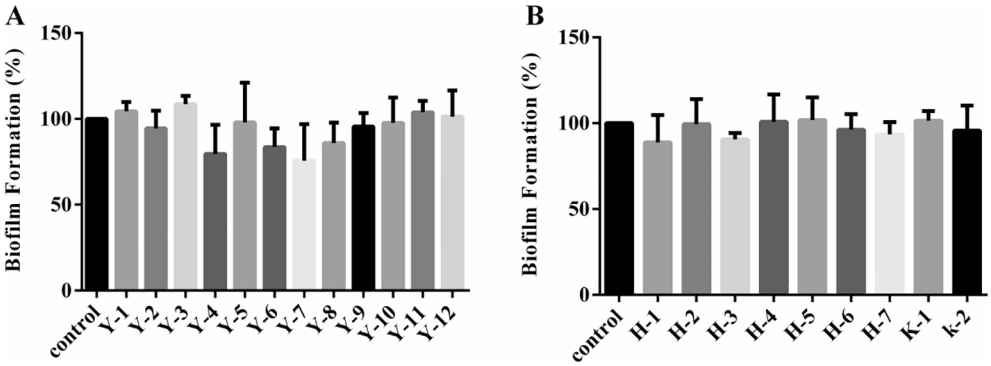


Fig. 4. Biofilm formation of *P. aeruginosa* in ABTGC medium (A) without treated (control) or treated with 10 μM **Y-1** to **Y-12**. (B) Without treated (control) or treated with 10 μM **H-1 to H-7**, **K-1** to **K-2**.

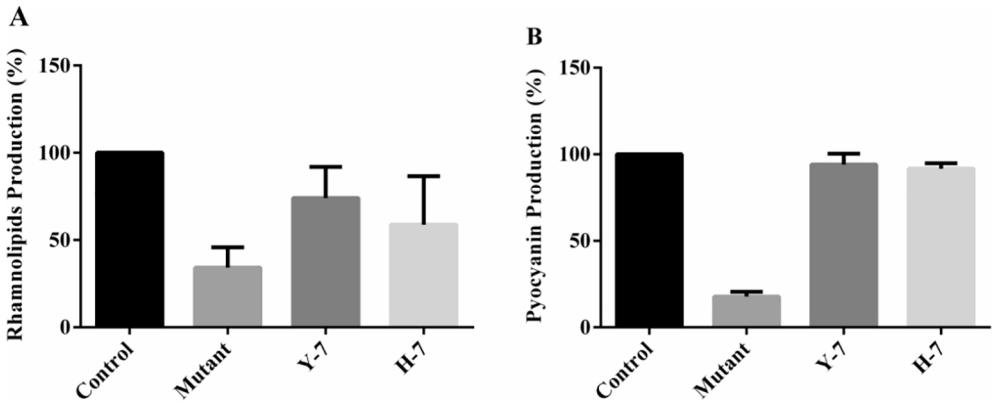


Fig. 6. The virulenc assay in *P. aeruginosa* without treated (control), *P aeruginosa lasI rhlI* double mutant (Mutant), *P. aeruginosa* treated with **Y-7** (10 μM) and **H-7** (10 μM) (A) rhamnolipids assay (B) pyocyanin assay. Experiments were done in triplicate.

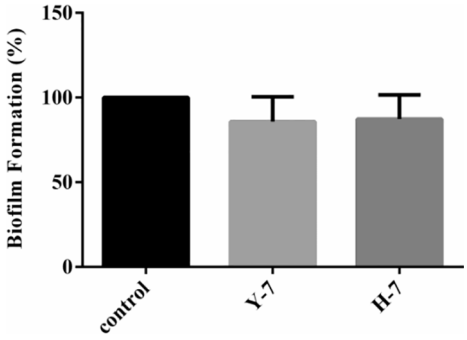


Fig. 7. Biofilm formation of *P. aeruginosa* in phosphate limitation medium without treated (control) or treated with 10 μM **Y-7 to H-7**.

down regulated while the pyocyanin was not affected.

Actually, there are four quorum sensing systems in *P.* aeruginosa. From the above experiment, we can conclude that our compounds can inhibit the QS through *rhl* system in normal condition. However, we can't exclude the probability that our compounds may take place through the fourth pathway, *iqs*. To exam this hypothesis, we used phosphate limitation medium to culture *P. aeruginosa* and detect the biofilm formation. As we can see in the Fig. 7, the biofilm is not affected. Thus, we can confirm that our compounds is not dependent on *iqs* system.

In our experiment, a series compounds derived from quinoline were designed and synthesized. Their anti-biofilm activity, QS inhibition and reduction of virulence were evaluated by kinds of reporter strain. From the above result, we made a conclusion that C-8 position of quinoline should keep a long chain where 7 carbons is the best like the AI and the alkyl group is better than the ring structure. The C-2 position with the 4′-position benzene ring replacement are more favorable than the 3′, 5′-position for inhibition of biofilm and quorum sensing. These phenomenon reveals the alkyl group may contribute to the van der Waals interaction in the QS receptor hydrophobic subpocket. We further explored the mechanism of our compounds and found that the effect against *P. aeruginosa* is through the *rhl* pathways, as evidenced by the QS system report strains and virulence assay. Among these compounds, **Y-7** and **H-7** showed the most potent to be quorum sensing inhibitors.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2018.12.068.

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