Supplementary Information

Discovery and Evaluation of 3-(2-Isocyanobenzyl)-*1H*-indole Derivatives as Potential Quorum Sensing Inhibitors for the Control of *Pseudomonas aeruginosa* Infections *in vitro*

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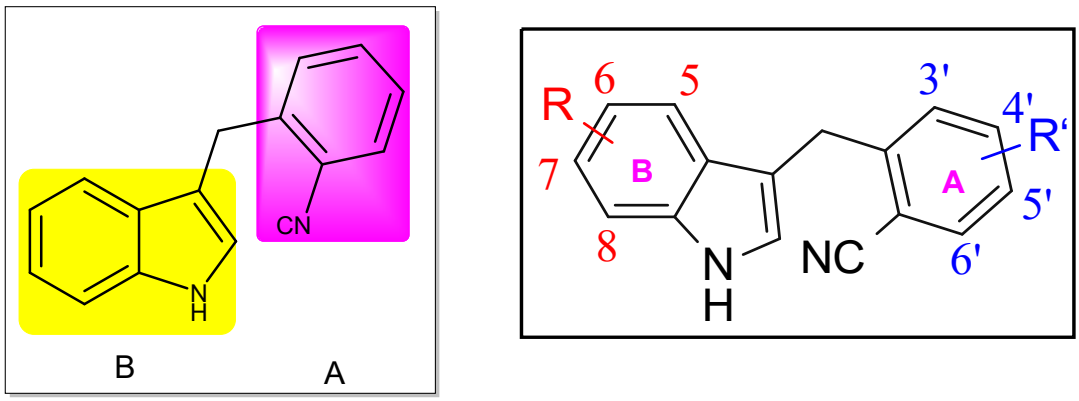
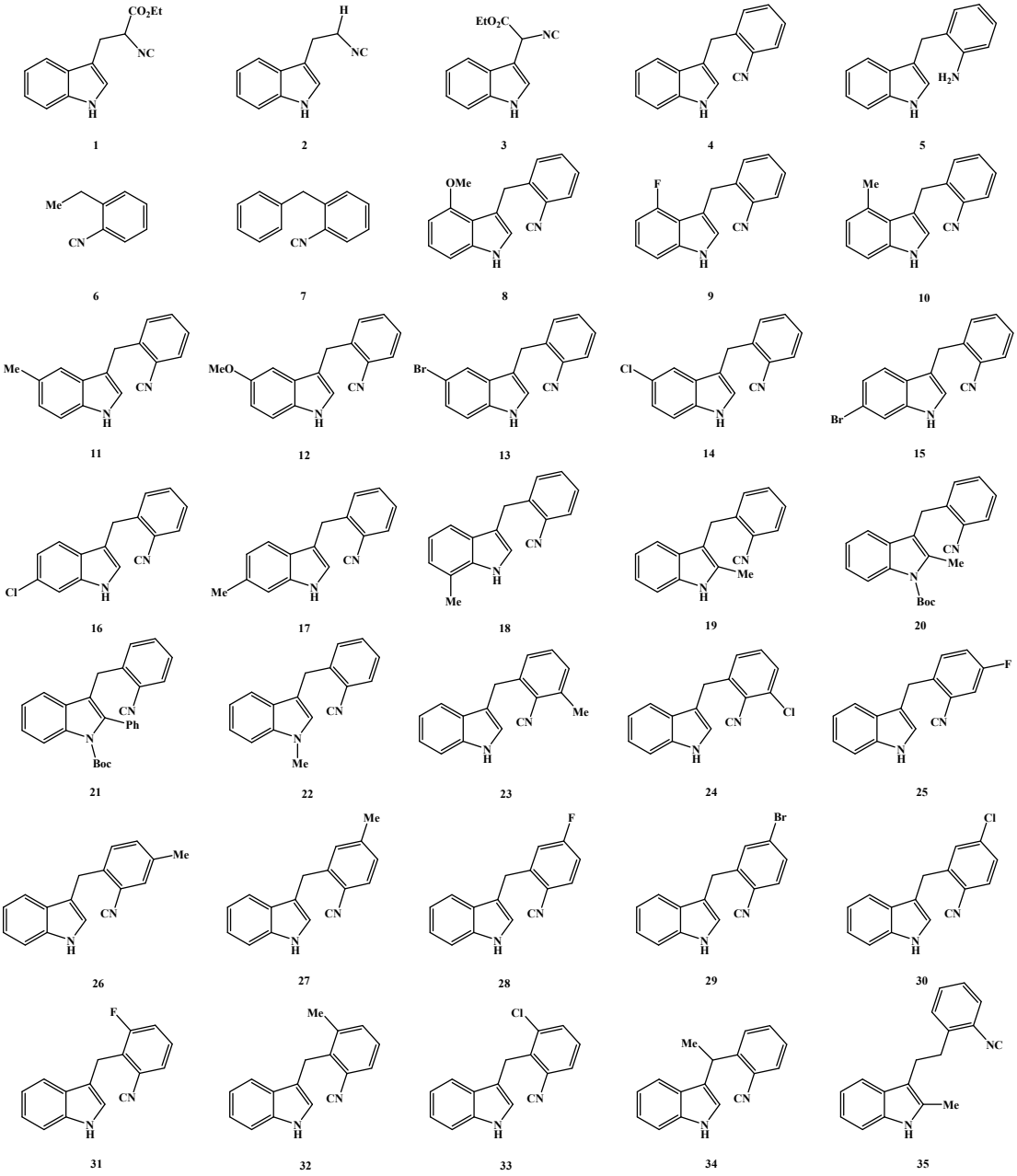
1 Chemicals

Scheme S1 Design strategy and lead compound



Scheme S2. All the 3-(2-isocyanobenzyl)-*1H*-indole derivatives structures for QS inhibiting evaluation against *P. aeruginosa* PAO1.



2 Quorum sensing inhibitory initial screenings

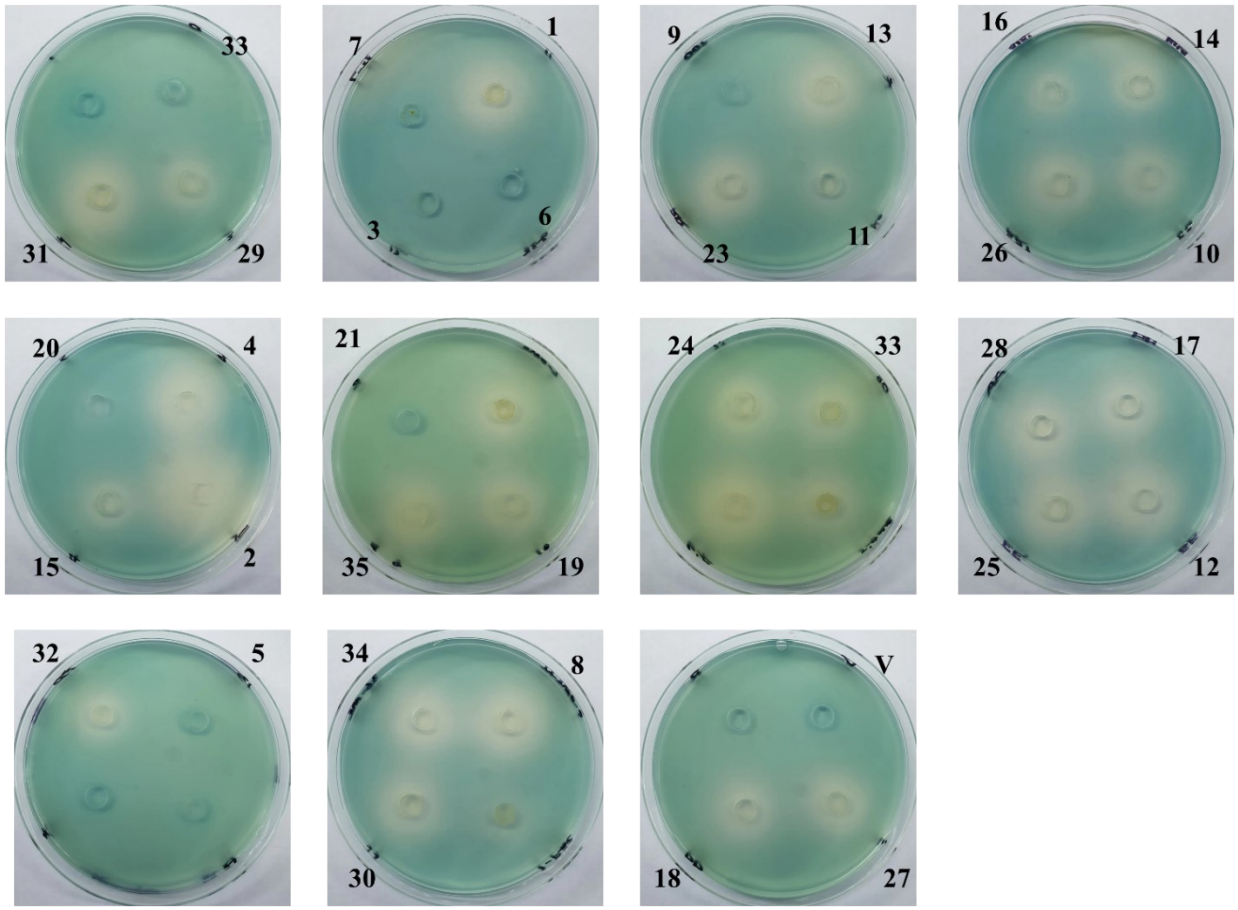


Fig. S1 QS inhibitory primary screenings of all the 3-(2-isocyanobenzyl)-*1H*-indole derivatives (100 mg/mL) against *P. aeruginosa* PAO1. The strain *P. aeruginosa* PAO1 was cultured in Luria Bertani (LB) broth (pH 7.0) medium at 37 °C. Compounds were dissolved in DMSO. DMSO used as the negative control. The resveratrol **(**RSV) served as the positive control. Data represent the means the standard deviations of three independent experiments.

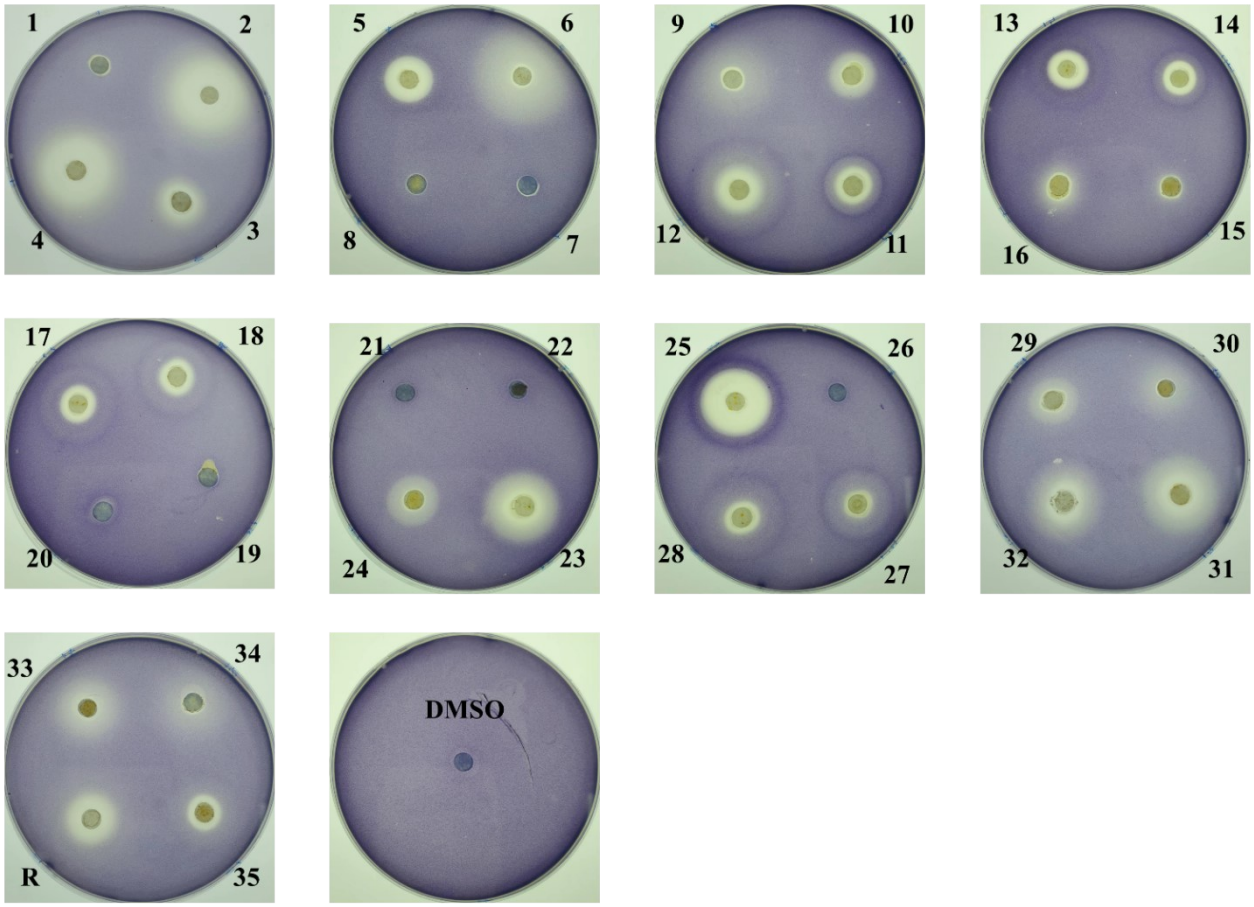


Fig. S2 QS inhibitory primary screening of all the 3-(2-isocyanobenzyl)-*1H*-indole derivatives (100 mg/mL) against *C. violaceum* CV026. The strain C. violaceum CV026 was cultured in LB broth (pH 7.0) medium at 37 °C. Compounds were dissolved in DMSO. DMSO used as the negative control. The resveratrol (RSV) served as the positive control. Data represent the means the standard deviations of three independent experiments.

3 MICs determination

Table S1 MICs of compounds **1–35**

Compounds	MICs	Compounds	MICs
RSV	48	**18**	48
1	>96	**19**	48
2	12	**20**	>96
3	48	**21**	>96
4	96	**22**	>96
5	>96	**23**	24
6	>96	**24**	12
7	>96	**25**	48
8	6	**26**	24
9	>96	**27**	24
10	12	**28**	12
11	48	**29**	48
12	6	**30**	12
13	48	**31**	12
14	24	**32**	96
15	24	**33**	24
16	12	**34**	24
17	24	**35**	24

Positive control: the resveratrol (RSV). MICs of derivatives against *P. aeruginosa* PAO1 were determined using the 2-fold serially diluted method. The strain *P. aeruginosa* PAO1 was cultured in LB broth (pH 7.0) medium at 37 °C.

4 Biofilm inhibition

Table S2 Effects of 3-(2-isocyanobenzyl)-*1H*-indole derivatives on biofilm inhibition against *P. aeruginosa* PAO1.

Compounds	1/2 MICs	1/4 MICs
1	0.19±0.031	0.04±0.049
2	0.39±0.025	0.09±0.006
3	0.073±0.030	0.01±0.005
4	0.38±0.036	0.15±0.0159
5	0.33±0.008	0.21±0.022
6	0.67±0.045	0.12±0.025
7	0.16±0.028	0.13±0.021
8	0.21±0.058	0.15±0.002
9	0.35±0.013	0.23±0.043
10	0.17±0.025	0.01±0.004
11	0.50±0.035	0.25±0.025
12	0.07±0.005	0.04±0.008
13	0.39±0.006	0.16±0.014
14	0.11±0.029	0.00±0.014
15	0.21±0.073	0.03±0.001
16	0.26±0.059	0.20±0.013
17	0.07±0.019	0.00±0.009
18	0.40±0.053	0.31±0.036
19	0.13±0.012	0.04±0.016
20	0.38±0.001	0.29±0.023
21	0.47±0.054	0.33±0.032
22	0.24±0.020	0.08±0.031
23	0.27±0.011	0.07±0.016
24	0.01±0.012	0.03±0.005
25	0.19±0.044	0.06±0.006

26	0.01±0.014	0.01±0.014
27	0.24±0.013	0.12±0.020
28	0.19±0.060	0.03±0.022
29	0.21±0.037	0.05±0.047
30	0.56±0.016	0.21±0.063
31	0.49±0.005	0.16±0.039
32	0.52±0.038	0.49±0.024
33	0.04±0.033	0.03±0.015
34	0.34±0.011	0.16±0.064
35	0.24±0.051	0.18±0.023
RSV	0.48±0.048	0.29±0.026

Positive control: the resveratrol (RSV). For biofilm inhibition assay, *P. aeruginosa* PAO1 was grown overnight in LB medium at 37 °C. The biofilms were cultivated in LB broth supplemented with derivatives in 24-well plates, after 24 h of static incubation, cultures and planktonic cells were removed and sessile cells were stained with 0.05% crystal violet, and rinsed using distilled water. After dissolution with 95% ethanol, the absorbance of these solutions was measured at 520 nm.

5 Pyocyanin inhibition

Table S3 Effects of 3-(2-isocyanobenzyl)-*1H*-indole derivatives on pyocyanin inhibition against *P. aeruginosa* PAO1.

Compounds	1/2 MICs
RSV	0.38±0.012
1	0.15±0.001
2	0.21±0.030
3	0.08±0.060
4	0.43±0.015
5	0.42±0.025
6	0.06±0.060
7	0.11±0.029
8	0.15±0.002
9	0.11±0.052
10	0.48±0.049
11	0.14±0.004
12	0.47±0.018
13	0.64±0.015
14	0.04±0.060
15	0.27±0.009
16	0.19±0.032
17	0.53±0.008
18	0.21±0.008
19	0.20±0.019
20	0.06±0.0406
21	0.49±0.006
22	0.15±0.013
23	0.27±0.042

24	0.23±0.029
25	0.58±0.009
26	0.60±0.010
27	0.17±0.021
28	0.46±0.046
29	0.17±0.023
30	0.29±0.045
31	0.24±0.033
32	0.63±0.030
33	0.59±0.007
34	0.16±0.038
35	0.12±0.078

Positive control: the resveratrol (RSV). For pyocyanin production assay, *P. aeruginosa* PAO1 was grown overnight in LB medium at 37 °C. Then test tubes containing 5 mL aliquot of culture and test compounds were grown for 18 h at 37 °C (200 rpm). To extract pyocyanin, the culture was centrifuged at 10 000 rpm for 5 min and the supernatant was collected and extracted with 3 mL of chloroform. Then, 1 mL of 0.2 M HCl was mixed with the chloroform layer, and the red upper aqueous phase was collected by centrifugation at 5 000 rpm for 10 min. The absorbance of these solutions was measured at 520 nm.

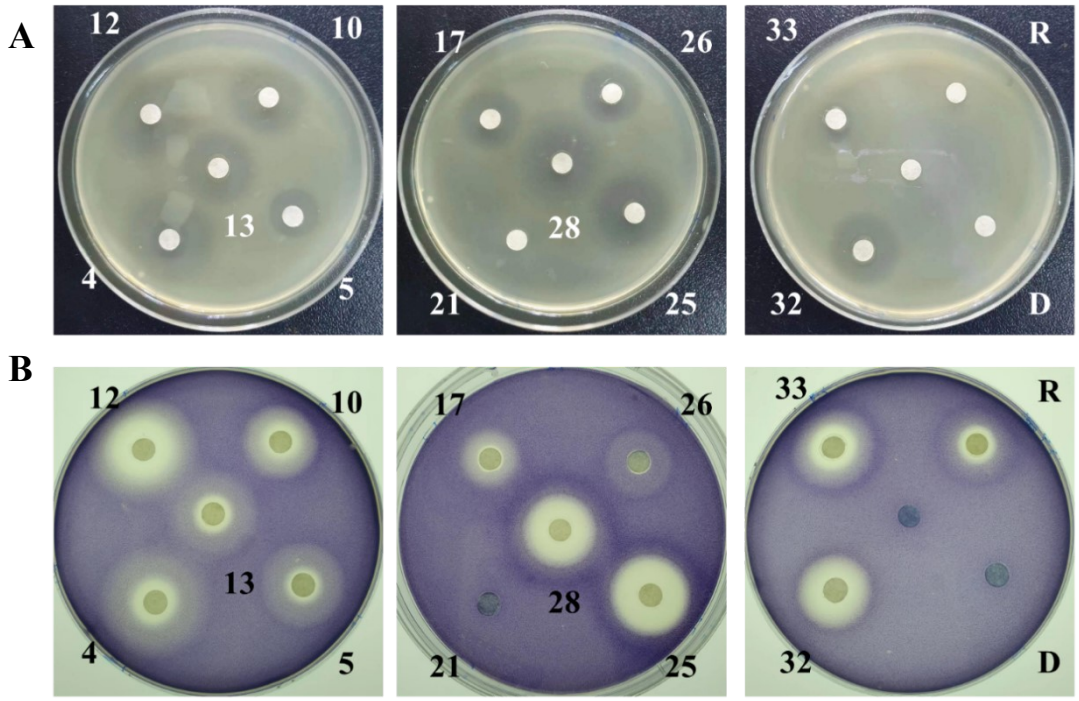


Fig. S3 Screenings for quorum sensing inhibitory activities of of 3-(2-isocyanobenzyl)-*1H*-indole derivatives against *P. aeruginosa* PAO1. Representative derivatives (**4, 5, 10, 12, 13, 17, 21, 25, 26, 28, 32,** and **33**) underwent final screenings for QS inhibition against *P. aeruginosa* PAO1 **(A)** and *C. violaceum* CV026. **(B)** The screening concentration of the compounds was 10 mg/mL. *P. aeruginosa* PAO1 was cultured in LB broth (pH 7.0) medium at 37 °C. DMSO served as the solvent, and the compounds were dissolved in DMSO. Resveratrol (RSV) was utilized as positive control. All data were presented with the standard deviation from three independent experiments.

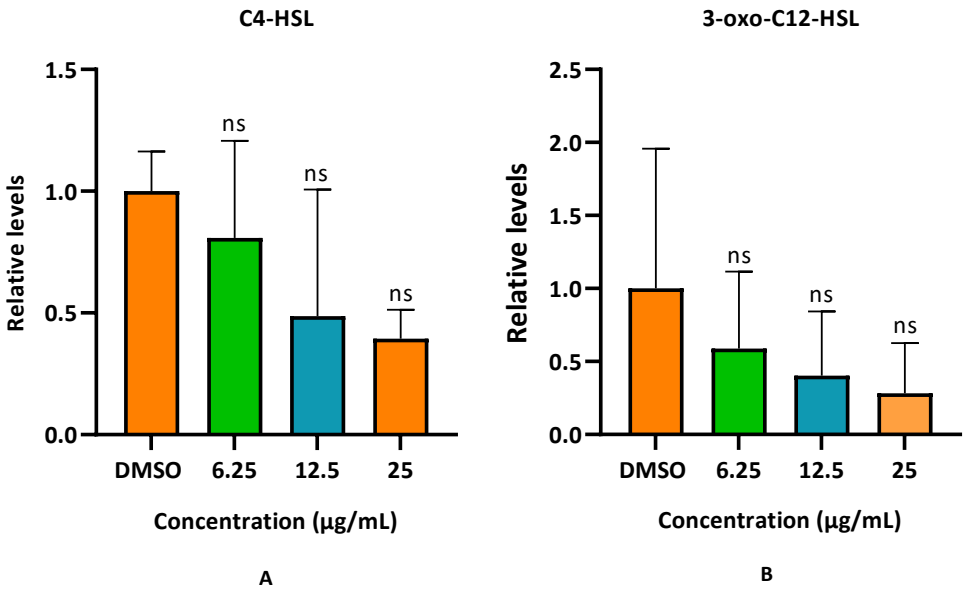


Fig. S4 Effects of **32** on C4-HSL and 3-oxo-C12 HSL against *P. aeruginosa* PAO1. **(A)** Quantitative analysis of **32** (25, 12.5 and 6.25 µg/mL) on C4-HSL of *P. aeruginosa* PAO1 using LC−MS/MS chromatograms. **(B)** Quantitative analysis of **32** (25, 12.5 and 6.25 µg/mL) on 3-oxo-C12 HSL of *P. aeruginosa* PAO1 using LC−MS/MS chromatograms. All the data represent the means and the standard deviations of three independent experiments. Statistical differences were determined by ANOVA followed by Tukey-Kramer test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. the DMSO control. "ns": no significance or no label $p > 0.05$.

6 PCR primers for RT-qPCR

Table S4 PCR primers for RT-qPCR

genes	primer directions	sequences (5′ → 3′)
lasI	Forward	GGCTGGGACGTTAGTGTCAT
	Reverse	AAAACCTGGGCTTCAGGAGT
lasR	Forward	ACGCTCAAGTGGAAAATTGG
	Reverse	TCGTAGTCCTGGCTGTCCTT
rhlI	Forward	AAGGACGTCTTCGCCTACCT
	Reverse	GCAGGCTGGACCAGAATATC
rhlR	Forward	CATCCGATGCTGATGTCCAACC
	Reverse	ATGATGGCGATTTCCCCGGAAC
pqsR	Forward	AACATGTTCCTCCAGGTCAT
	Reverse	GTTGAGATTGAAGGCGATGT
sdhB	Forward	AGCTGGACGGTCTGTACGAGTG
	Reverse	CGCTCTTCGGTCTTGGTGTCAC
sucD	Forward	GGCTCCAACTTCATCGACATCCTG
	Reverse	CGGCTTCTTCTTCGGCGGAAC
sodB	Forward	CTGCTGACCGTTGACGTGTGG
	Reverse	CGCTGCGAAGGTCCAGTTGAC
A5439	Forward	GCAGATCAGCGTCAACGAGACC
	Reverse	GCAGAGCAACTGGAGCAGATGG

7 Cytotoxicity

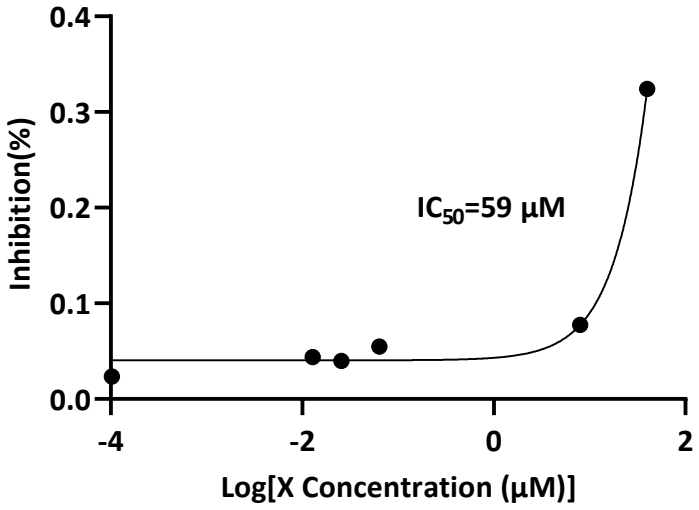


Fig. S5 The cytotoxic effects of **32** were tested via CCK-8 assay. The HEK 293T cells in logarithmic growth phase were collected and inoculated into 96-well plates with 1 × 10^4 cell density. The cells were maintained in an incubator at 37 °C in a humidified incubator involving 5% carbon dioxide atmosphere for 1 d. The HEK 293T cells were labelled using CCK-8 solution. The experiments were conducted three times, and the value obtained indicated the 50% inhibitory concentration (IC_{50}) of total cells/mL in the HEK 293T cells from three separate experiments with three samples.

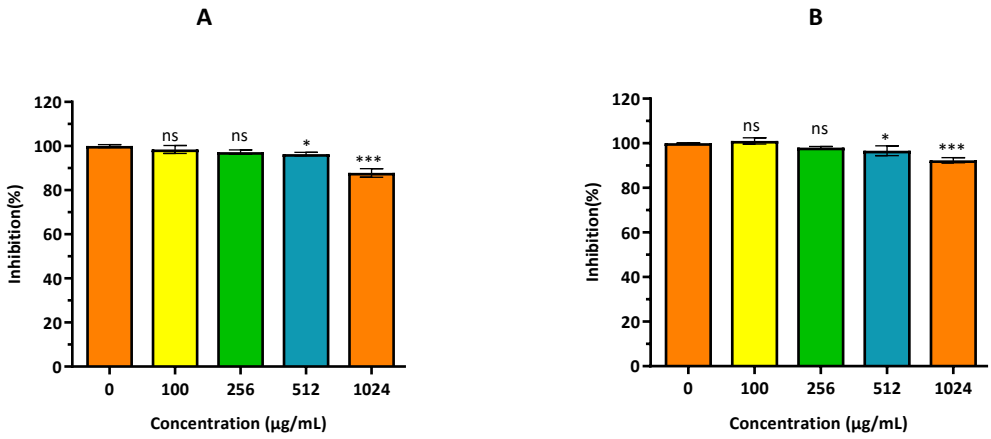


Fig. S6 Mouse embryonic fibroblasts (MEFs) (**A**) and mouse erythrocytes (**B**) tests. Each experiment was performed in triplicate and at least 400 cells were counted for each treatment. Compounds were dissolved in DMSO, and the DMSO was used as the negative control. All the data were represented with standard deviation of three independent experiments. Statistical differences were determined by one-way ANOVA test. The error bars represent standard deviation of three biological replicates. p-values derived from these comparisons are highlighted with asterisks, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs the DMSO control. ns: no significance or no label $p > 0.05$.

MATERIALS AND METHODS

Molecular docking

The chemical structures of all ligands were constructed by using ChemDraw 15.1. Molecular docking was performed using the AutoDock program [1]. Nacyl-L-homoserine lactones (AHLs), which are secreted by gram-negative bacteria, serve as the main QS signaling molecules [2]. C4 to C12-HSL synthesis is involved in the pathogenesis of *P. aeruginosa* PAO1, which mediates biofilm formation, motility, and the production of extracellular components [3].The two QS systems are known to be hierarchically organized, with the las system controlling both rhlR and rhlI transcription [4]. LasR, a crucial component of the circuit, requires 3-oxo-C12-HSL for proper folding to control the entire QS system [3]. EsaL, in consider of Esal/EsaR system in QS to regulate EPS secretion, is also investigated in this study [5]. The three-dimensional structures of RhlI (PDB ID: 4NG2), RhlR(PDB ID: 7KGW), LasR (PDB ID: 3RKR), EsaL [6] and PqsR (PDB ID: 7NBW), were retrieved from the Protein Data Bank (PDB). These target proteins were individually docked with **32** and their native ligands. After docking, the energy of the ligands docked to the proteins was minimized for MD analysis.

Molecular dynamic studies on binding stability and interaction energy

On an Ubuntu (18.06) Linux operating system with the NVIDIA CUDA-supported GPU hybrid parallel computation platform, the Groningen Machine for Chemical Simulation (GROMACS, 2020.03) was used to perform molecular dynamic analyses. In the analysis, the Amber99SB force field was used. Each target protein was positioned in the center of a dodecahedron unit cell, with a spacing of 3.0 nm between the proteins and the edge. The cells were filled with H_2O, then neutralized by replacing H_2O with Na^+ and Cl^- with finally concentrations of 0.1 M. After a brief energy minimization and temperature and pressure equilibration, the analysis was carried out using the Amber99SB force field, which is optimized for ab-initio calculation of protein three-dimensional structure, and the TIP3P explicit water model. Each analysis has 50,000,000 time steps, generating 8 Gb data approximately. The root mean square

deviation (RMSD) of the position of the heavy atoms in a ligand was analyzed to evaluate the binding stability of the ligand by equation (1):

$$RMSD_{t_1,t_2} = \left[\frac{1}{M}\sum_{i=1}^{N} m_i ||r_i(t_1) - r_i(t_2)||^2\right]^{\frac{1}{2}} \quad (1)$$

where **r** is the position vector, t time, and M the mass of all atoms.

The interaction energy between a pair of atoms (i,j) is the sum of Lennards-Jones and Coulombic potential between atom pairs by equation (2) and (3):

$$V_{LJ(ij)} = \frac{C_{ij}^{(12)}}{r_{ij}^{12}} - \frac{C_{ij}^{(6)}}{r_{ij}^{6}} \quad (2)$$

$$V_{C(ij)} = f\frac{q_i q_j}{\varepsilon_r r_{ij}} \quad (3)$$

where q is the elementary charge which euqals to $1.602176565\times10^{-19}$C, and r the vector length, and f the electric conversion factor.

Cytotoxicity

Cell lines and cell culture

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37 °C in a humidified incubator involving 5% carbon dioxide atmosphere. When the cells entered the growth phase, they were carefully washed two times with PBS and digested with trypsin. After rounding the cells, they were maintained in 96-well plates prior to use in subsequent experiments. Finally, 0.1% DMSO was used as the negative control group.

CCK-8 assay

It was tested via CCK-8 assay following the manufacturer's instructions. The HEK 293T cells in logarithmic growth phase were collected and inoculated into 96-well plates with $1x10^4$ cell density. The cells were maintained in an incubator at 37 °C in a humidified incubator involving 5% carbon dioxide atmosphere for 1 d. The HEK 293T cells were labelled using CCK-8 solution. For each well, their absorbance at 450 nm was determined. The experiments were conducted three times, and the value obtained indicated the 50% inhibitory concentration (IC_{50}) of total cells/mL in the HEK 293T cells from three separate experiments with three samples.

Mouse embryonic fibroblasts (MEFs) or mouse erythrocytes test

Mouse embryonic fibroblasts (MEFs) or mouse erythrocytes were purchased from Type Culture Collection (ATCC, Rockefeller, Maryland, USA). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen, Shanghai, China) and 10 U/mL penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. The effects of compounds on cell viability were assessed after a 24 h treatment by using MTT (Beyotime, Cat# C0009, Chengdu, China) assay. After treatment, cells were incubated with 0.5 $mg \cdot ml^{-1}$ MTT at 37 °C for 4 h, the supernatant was discarded, and cells were resolved in DMSO solution for the measurement of the absorbance at 490 nm. The relative cell viability was calculated.

References

[1] S. Nam, S.-Y. Ham, H. Park, Y. Byun, Discovery and Characterization of Pure RhlR Antagonists against *Pseudomonas aeruginosa* Infections, Journal of Medicinal Chemistry **2020** 63 8388–8407.

[2] J.-H. Lee, H.S. Cho, J. Lee, Indole and 7-benzyloxyindole attenuate the virulence of *Staphylococcus aureus*, Applied Microbiology and Biotechnology **2013** 97 4543–4552.

[3] D. Bakkiyaraj, C. Sivasankar, S.K. Pandian, Inhibition of quorum sensing regulated biofilm formation in *Serratia marcescens* causing nosocomial infections, Bioorganic & Medicinal Chemistry Letters **2012** 22 3089–3094.

[4] S. Ramanathan, D. Ravindran, K. Arunachalam, V.R. Arumugam, Inhibition of quorum sensing-dependent biofilm and virulence genes expression in environmental pathogen *Serratia marcescens* by petroselinic acid, Antonie van Leeuwenhoek **2018** 111 501–515.

[5] Carlier, A., Burbank, L., & Von Bodman, S. B.. Identification and characterization of three novel EsaI/EsaR quorum-sensing controlled stewartan exopolysaccharide biosynthetic genes in *Pantoea stewartii ssp. stewartii.* Molecular microbiology, **2009**, *74*(4).

[6] Latifi, Amel, et al. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Molecular microbiology **1995** 17.2 333-343.