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Design, Synthesis and Evaluation of Halogenated Furanone Derivatives as Quorum Sensing inhibitors in *Pseudomonas aeruginosa*

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ABSTRACT: The biofilm formation of *Pseudomonas aeruginosa* (*P. aeruginosa*) is regulated by a phenomenon of quorum sensing (QS). With 5-hydroxyl-3,4-halogenated-5*H*-furan-2-ones as beginning, analogs bearing alkyl chains, vinyl bromide, or aromatic rings were designed and synthesized. The minimum inhibitory concentration (MIC) of the compounds against *P. aeruginosa* was assayed and the biofilm inhibition ratio was determined at different concentrations lower than the MIC. C-5 aromatic substituted furanones showed remarkable biofilm formation as well as inhibition of virulence factor production in *P. aeruginosa*. Fluorescence report analysis identified the QS regulatory mechanism of the most active compound 29. This study provides us a novel candidate for combating drug resistant bacteria strains

by merely inhibiting biofilm formation. Without suppressing the regular life cycle of the bacteria, bacterial resistance mechanisms may not be activated.

KEYWORDS: Furanones; Quorum Sensing; *Pseudomonas aeruginosa*; Biofilm.

1. Introduction

Since antibiotics were discovered in the early 20th century, many life-threatening diseases to human beings had an effective cure.[1] However, the indiscriminate and excessive usage of antibiotics during these decades has led to drug resistance and brought multiple drug resistant bacterial strains (MDR).[2] With 16 million people dying from infectious diseases caused by MDR strains every year, the urgent requirement for alternative therapies to fight against MDR admits no delay.[3,4] Among all infectious diseases, a noteworthy fact is that about 65% concern the proliferation of forming bacterial biofilms.[5] Biofilms are persistent and protective patterns of structured microbial cell communities that allow bacteria to survive in hostile circumstance by inserting in a matrix of extracellular polymeric substances. [6] The formation of bacterial biofilms is developed by a cell-to-cell signaling system of quorum sensing (QS), through which bacteria synchronize their behaviors by a series of signal molecules in a cell density dependent mode. In many species, this process results in various pathogenic events like biofilm formation as well as virulence factor production.[7,8] Efforts have been made to disrupt biofilms via inhibiting the QS system, many natural and synthetic molecules with QS inhibition have been identified as potential therapeutic approaches.[9-11] These quorum sensing inhibitors (QSIs) have the ability to competitively quench QS signaling systems, providing a new way for combating microbial infection.

P. aeruginosa, whose biofilm formation is based on quorum sensing system, is the most common Gram-negative bacterium found in nosocomial infections.[12,13] With a well-studied quorum sensing system regulating its biofilm formation[14,15], *P. aeruginosa* attracted many interest: two kinds of acyl homoserine lactones (AHLs) produced by *P. aeruginosa* serve as the QS system signaling molecules: N-3-oxododecanoyl homoserine lactone (3OC12) and N butanoyl homoserine lactone

(C4). These AHLs are received by the following receptors: QscR, LasR, and RhlR.[8,16] QscR and LasR normally sensed 3OC12 but regulate different regulons. RhlR operates its own regulon by recognizing C4 as well. QS systems control numerous genes expression, besides biofilm formation and virulence factor production; the production of extra cytoplasmic substances and other metabolites are also regulated. [17]

A lot of works have been made to search for novel natural QS inhibitors (QSI) from natural products.[15-17] Different categories of natural QSI of gram-negative bacteria have been identified, such as cyclic sulfur compounds from garlic,[18] halogenated furanones from *Delisea pulchra*,[19] patulin and penicillic acid from fungi,[20] and so forth. Among the above molecules, the QS inhibitory activities of furanone derivatives draw most attentions. Natural and synthetic furanone derivatives, as well as unsaturated lactones, have been shown to exhibit a wide range of biological activities including anticonvulsant, anti-inflammatory, analgesic, antitumor, antiviral, and anticancer activities.[21,22] Patulin can down-regulate QS genes in *P. aeruginosa* and halogenated furanones extracted from some marine plants also have antifouling activities.[19-20] Based on the furanone derivatives and our previous work[23], we designed a new series of halogenated furanone derivatives to investigate the structural requirements in inhibition of biofilm formation. Taking the natural products as promising lead compounds, 5-hydroxyl-3,4-halogenated-5*H*-furan-2-ones were obtained from 2-furaldehyde as the beginning of our chemical exploration (Figure 1).

[Figure 1]

2. Materials and methods

Bacterial strains

Pseudomonas aeruginosa strains ATCC27853, ATCC9027, and PAOA (clinical isolates) were used to study the effects of new compounds to biofilm assay. A bacterial suspension was made from fresh culture and aliquots were stored at -20^{o}C in glycerol and used within 2 weeks. Before used, bacterial suspensions were spread onto Mueller-Hinton solid medium and incubated at 37^{o}C for 18h.

2.1 Synthesis of halogenated furanone derivatives

2.1.1 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-carbaldehyde (1a)

In a 500 mL three-necked flask equipped with a condensed and stirred HCl exhaust gas absorption device, fresh distilled furfural (8 mL, 0.088 mol) and 320 mL of concentrated hydrochloric acid were added, 40 g of MnO_2 was added in portions with vigorous stirring (finished in two hours). After that, the mixture was heated to reflux for about 1 h, and another 8 g of MnO_2 was added until the black color did not fade, and the mixture was stirred at room temperature overnight. Directly add about 4 g of active charcoal to boil and decolorize, then filtered while hot. When the filtrate is cooled, a large amount of white fish scaly crystals are precipitated, then filtered and washed with ice water, dried to obtain 3.9 g of product. The filtrate was further concentrated twice to obtain 4.0 g and 2.1 g crystals respectively, the total yield was 10.0 g, yield 66.7%, and the mp was 124-125 ° C.

2.1.2 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl 4-chlorobutanoate (2)

5-hydroxy-3,4-dichloro-2(5H)-furanone (0.168 g, 1.0 mmol) was added into a 50 mL three-neck flask with N_2 protection, then 7 mL of anhydrous CH_2Cl_2 was added to dissolve below 0 °C. Then 0.15 mL of 4-chlorobutyryl chloride was slowly aadded. After 30 minutes, 0.5 mL of anhydrous Et3N was added, slowly increase to room temperature for 4 h, and monitored the reaction by TLC. Quenched with 4~5 mL of water, and extracted with CH_2Cl_2 (15 mL×3), washed with saturated brine and dried over anhydrous Na_2SO4. After filtration, the solvent was evaporated to dryness. Purified with flash column (eluting with 1:8 v/v ethyl acetate/petroleum ether) afforded yellow oil compound 2 for 0.232g, and yield was 85.3%. HRMS found m/z: 295.2 $[M+Na]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ6.92 (s, 1H, Furan-H), 3.62 (t, *J*= 6.3 Hz, 2H, CH_2Cl), 2.63 (m, 2H, $COCH_2$), 2.14 (m, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ170.4, 162.6, 146.9, 124.6, 91.3, 43.5, 30.7, 27.0.

2.1.3 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yloctanoate (3)

Same method as compound 2. White solid 0.262 g was obtained in a yield of 89.2%. HRMS found m/z: 317.3 $[M+Na]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ6.92 (s, 1H,

Furan-H), 2.33 (t, J = 7.5 Hz, 2H, $COCH_2$), 1.64 (m, 2H, CH_2), 1.28 (m, 8H, CH_2), 0.87 (t, J = 6.6 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ171.3, 162.6, 147.1, 124.4, 91.2, 33.7, 31.5, 28.9, 28.8, 24.5, 22.5, 14.0.

2.1.4 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yldecanoate (4)

Same method as compound 2. White solid 0.266 g was obtained in a yield of 82.7%, and purity was 97.0% (HPLC analysis conditions: octylsilane-bonded silica gel as a packed column; acetonitrile-water (40:60) as a mobile phase; detection wavelength of 254 nm). HRMS found m/z: 345.4 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ6.91 (s, 1H, Furan-H), 2.44 (t, J = 7.5 Hz, 2H, $COCH_2$), 1.66 (m, 2H, CH_2), 1.24 (m, 12H, CH_2), 0.86 (t, J = 6.3 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ171.2, 162.5, 147.1, 124.4, 91.1, 33.7, 31.8, 29.3, 29.2, 29.1, 28.8, 24.4, 22.6, 14.1.

2.1.5 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yldodecanoate (5)

Same method as compound 2. White solid 0.159 g was obtained in a yield of 79.4%, and purity was 93.8% (HPLC analysis, detection wavelength of 230 nm). HRMS found m/z: 373.2 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ6.92 (s, 1H, Furan-H), 2.44 (t, J = 7.5 Hz, 2H, $COCH_2$), 1.65 (m, 2H, CH_2), 1.28 (m, 16H, CH_2), 0.86 (t, J = 6.6 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ171.2, 162.5, 147.1, 124.3, 91.1, 33.7, 31.9, 29.5, 29.5, 29.3, 29.3, 29.1, 28.8, 24.4, 22.6, 14.1.

2.1.6 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl undec-10-enoate (6)

Same method as compound 2. White oil 0.236g was obtained in a yield of 70.7%, and purity was 97.1% (HPLC analysis, detection wavelength of 254 nm). HRMS found m/z: 357.3 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ6.92 (s, 1H, Furan-H), 5.81 (m, 1H, =CH), 4.97 (m, 2H, CH_2=), 2.43 (t, J = 7.5 Hz, 2H, $COCH_2$), 2.07 (m, 2H, =C-CH_2), 1.65 (m, 2H, CH_2), 1.22 (m, 10H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ179.4, 163.1, 156.1, 139.1, 114.1, 101.7, 91.1, 33.7, 29.2, 29.0, 28.9, 27.5, 26.3, 24.7, 24.4.

2.1.7 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl tetradecanoate (7)

Same method as compound 2. White solid 0.288g was obtained in a yield of 76.3%, and purity was 95.0% (HPLC analysis, detection wavelength of 230 nm). HRMS found m/z: 401.4 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ6.93 (s, 1H, Furan-H), 2.45 (t, *J* = 7.2 Hz, 2H, $COCH_2$), 1.68 (m, 2H, CH_2), 1.28 (m, 20H, CH_2), 0.88 (t, *J* = 6.9 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ171.3, 162.4, 147.1, 124.3, 91.2, 33.7, 31.9, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.1, 28.9, 26.2, 24.4, 22.7, 14.1.

2.1.8 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl palmitate (8)

Same method as compound 2. Yellow oil 0.304g was obtained in a yield of 74.9%. HRMS found m/z: 429.5 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ6.93 (s, 1H, Furan-H), 2.46 (t, *J* = 7.5 Hz, 2H, $COCH_2$), 1.74 (m, 2H, CH_2), 1.28 (m, 24H, CH_2), 0.88 (t, *J* = 6.9 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ170.2, 163.9, 145.5, 126.4, 101.5, 31.9, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 28.9, 27.5, 26.3, 24.6, 24.5, 22.7, 14.1.

2.1.9 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl (E)-undec-2-enoate (9)

Same method as compound 2. Yellow liquid 0.276g was obtained in a yield of 63.9%, and purity was 92.6% (HPLC analysis, detection wavelength of 240 nm). HRMS found m/z: 455.4 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ6.92 (s, 1H, Furan-H), 5.32 (m, 2H, CH=CH), 2.44 (t, *J* = 7.2 Hz, 2H, $COCH_2$), 1.99 (m, 4H, =C-CH_2), 1.67 (m, 2H, CH_2), 1.24 (m, 20H, CH_2), 0.86 (t, *J* = 6.6 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ171.3, 162.7, 147.1, 130.1, 129.6, 124.4, 91.2, 33.9, 33.7, 31.9, 29.7, 29.7, 29.5, 29.3, 29.1, 29.0, 28.8, 27.2, 27.1, 24.4, 22.7, 14.1.

2.1.10 3-acetylphenyl 4-chlorobutanoate (10)

7 mL of anhydrous CH_2Cl_2 was added into 50 mL three-necked flask concluding M-hydroxyacetophenone (0.136 g, 1.0 mmol) with N_2 protection below -2 °C, after dissolved, 0.15 mL of 4-chloroprene was slowly added. After 30 min, 0.5 mL of anhydrous Et_3N was added and slowly increase to room temperature for 4 h. Then monitored the reaction by TLC. Quenched with 4~5 mL of water, extracted with CH_2Cl_2 (20 mL×3), washed with saturated brine and dried over anhydrous Na_2SO4.

The mixture was filtered and the solvent was evaporated to dryness. Purified with flash column (eluting with 1:6 v/v ethyl acetate/petroleum ether) afforded white solid compound 10 for 0.21g, yield 87.6%. HRMS found m/z: 263.0 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ7.83 (ddd, *J* = 7.8 Hz, 1.8 Hz, 1.2 Hz, 1H, ArH), 7.67 (t, *J* = 7.8 Hz, 1H, ArH), 7.49 (t, *J* = 8.4 Hz, 1H, ArH), 7.30 (ddd, *J* = 8.4 Hz, 2.4 Hz, 1.2 Hz, 1H, ArH), 3.69 (t, *J* = 6.3 Hz, 2H, $ClCH_2$), 2.81 (m, 2H, $COCH_2$), 2.60 (s, 3H, CH_3), 2.23 (m, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ196.9, 171.1, 150.8, 138.6, 129.7, 127.0, 125.9, 121.3, 43.9, 31.2, 27.4, 26.7.

2.1.11 3-acetylphenyl octanoate (11)

Same method as compound 10. White solid 0.239g was obtained in a yield of 91.4%. HRMS found m/z: 285.0 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ7.81 (ddd, *J* = 7.8 Hz, 1.5 Hz, 1.2 Hz, 1H, ArH), 7.66 (t, *J* = 1.8 Hz, 1H, ArH), 7.47 (t, *J* = 8.1 Hz, 1H, ArH), 7.29 (ddd, *J* = 8.1 Hz, 2.4 Hz, 1.2 Hz, 1H, ArH), 2.59 (s, 3H, CH_3), 2.57 (t, *J* = 7.5 Hz, 2H, $COCH_2$), 1.74 (m, 2H, CH_2), 1.28 (m, 8H, CH_2), 0.86 (t, *J* = 6.6 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ197.0, 172.1, 151.0, 138.5, 129.6, 126.5, 125.7, 121.4, 34.3, 31.6, 29.1, 28.9, 26.7, 24.9, 22.6, 14.1.

2.1.12 3-acetylphenyl decanoate (12)

Same method as compound 10. White solid 0.261g was obtained in a yield of 90.1%. HRMS found m/z:313.4 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ7.81 (ddd, *J* = 7.8 Hz, 1.2 Hz, 0.9 Hz, 1H, ArH), 7.66 (t, *J* = 2.1 Hz, 1H, ArH), 7.47 (t, *J* = 8.1 Hz, 1H, ArH), 7.29 (ddd, *J* = 8.1 Hz, 2.1 Hz, 0.9 Hz, 1H, ArH), 2.59 (s, 3H, CH_3), 2.35 (t, *J* = 7.5 Hz, 2H, $COCH_2$), 1.74 (m, 2H, CH_2), 1.27 (m, 12H, CH_2), 0.88 (t, *J* = 6.6 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ197.1, 172.2, 151.0, 138.5, 129.6, 126.5, 125.7, 121.4, 34.3, 34.0, 31.9, 29.4, 29.3, 29.1, 26.7, 24.9, 22.7, 14.1.

2.1.13 3-acetylphenyl dodecanoate (13)

Same method as compound 10. White solid 0.284g was obtained in a yield of 89.4%. HRMS found m/z:341.5 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ7.84 (ddd, *J* = 7.8 Hz, 1.5 Hz, 0.9 Hz, 1H, ArH), 7.68 (t, *J* = 2.1 Hz, 1H, ArH), 7.50 (t, *J* = 7.8

Hz, 1H, ArH), 7.31 (ddd, *J* = 8.1 Hz, 2.4 Hz, 0.9 Hz, 1H, ArH), 2.62 (s, 3H, CH_3), 2.58 (t, *J* = 7.5 Hz, 2H, $COCH_2$), 1.77 (m, 2H, CH_2), 1.28 (m, 16H, CH_2), 0.90 (t, *J* = 6.3 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ193.0, 172.2, 150.6, 137.6, 129.5, 126.7, 125.4, 121.4, 34.3, 32.0, 29.7, 29.6, 29.5, 29.3, 29.2, 29.1, 26.7, 24.9, 22.7, 14.1.

2.1.14 3-acetylphenyl undec-10-enoate (14)

Same method as compound 10. White solid 0.262g was obtained in a yield of 86.7%. HRMS found m/z: 325.0 $[M+Na]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ7.78 (dd, *J* = 7.8 Hz, 1.2 Hz, 1H, ArH), 7.65 (s, 1H, ArH), 7.43 (td, *J* = 7.8 Hz, 1.2Hz, 1H, ArH), 7.26 (m, 1H, ArH), 5.79 (m, 1H, =CH), 4.94 (m, 2H, CH_2=), 2.55 (m, 5H, CH_3), 2.03 (t, *J* = 7.5 Hz, 2H, $COCH_2$), 1.72 (m, 2H, CH_2), 1.30 (m, 10H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ193.9, 172.0, 151.0, 139.1, 138.5, 129.6, 126.4, 125.7, 121.4, 114.2, 34.3, 33.8, 29.3, 29.2, 29.1, 29.0, 28.9, 26.6, 24.8.

2.1.15 3-acetylphenyl tetradecanoate (15)

Same method as compound 10. White solid 0.315g was obtained in a yield of 91.1%. HRMS found m/z: 369.5 $[M+Na]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ7.84 (dt, *J* = 7.8 Hz, 1.5 Hz, 1H, ArH), 7.68 (m, 1H, ArH), 7.50 (t, *J* = 7.8 Hz, 1H, ArH), 7.31 (ddd, *J* = 8.1 Hz, 2.4 Hz, 0.9 Hz, 1H, ArH), 2.62 (s, 3H, CH_3), 2.58 (t, *J* = 7.5 Hz, 2H, $COCH_2$), 1.78 (m, 2H, CH_2), 1.37 (m, 16H, CH_2), 0.90 (t, *J* = 6.3 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ197.1, 172.2, 151.0, 138.5, 129.6, 126.5, 125.7, 121.5, 34.3, 32.0, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.1, 26.7, 24.9, 22.7, 14.1.

2.1.16 3-acetylphenyl palmitate (16)

Same method as compound 10. White solid 0.331g was obtained in a yield of 88.4%. HRMS found m/z: 397.6 $[M+Na]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ7.84 (dt, *J* = 7.8 Hz, 1.2 Hz, 1H, ArH), 7.68 (m, *J* = 2.1 Hz, 1H, ArH), 7.50 (t, *J* = 7.8 Hz, 1H, ArH), 7.31 (ddd, *J* = 8.1 Hz, 2.4 Hz, 0.9 Hz, 1H, ArH), 2.62 (s, 3H, CH_3), 2.58 (t, *J* = 7.5 Hz, 2H, $COCH_2$), 1.78 (m, 2H, CH_2), 1.28 (m, 24H, CH_2), 0.90 (t, *J* = 6.3 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ197.0, 172.3, 151.0, 138.7, 129.6, 125.7, 121.6,

121.5, 34.3, 31.9, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 29.1, 26.7, 24.9, 22.7, 14.1.

2.1.17 3-acetylphenyl (E)-octadec-9-enoate (17)

Same method as compound 10. White solid 0.336g was obtained in a yield of 83.9%. HRMS found m/z: 423.6 $[M+Na]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ7.81 (dt, *J* = 7.8 Hz, 1.2 Hz, 1H, ArH), 7.66 (m, 1H, ArH), 7.47 (t, *J* = 8.1 Hz, 1H, ArH), 7.29 (ddd, *J* = 8.1 Hz, 2.4 Hz, 0.9 Hz, 1H, ArH), 5.36 (m, 2H, CH=CH), 2.59 (s, 3H, CH_3), 2.56 (t, *J* = 7.8 Hz, 2H, $COCH_2$), 2.03 (m, 4H, =C-CH2), 1.75 (m, 2H, CH_2), 1.28 (m, 20H, CH_2), 0.87 (t, *J* = 6.3 Hz, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ197.0, 172.1, 151.0, 138.5, 129.6, 126.5, 125.7, 121.4, 34.3, 31.9, 31.9, 31.5, 29.7, 29.5, 29.4, 29.3, 29.2, 29.1, 27.2, 26.6, 24.9, 22.7, 22.6, 14.1.

2.1.18 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl-4-(2-chloroethyl)benzoate (18)

7 mL of anhydrous CH_2Cl_2 was added into 50 mL three-necked flask concluding 5-hydroxy-3,4-dichloro-2(5H)-furanone (0.168 g, 1.0 mmol) with N2 protection below -2 °C, after dissolved, 0.21mg of p-chloromethylbenzoyl chloride was slowly added. After 30 min, 0.5 mL of anhydrous Et_3N was added and slowly increase to room temperature for 6 h. Then monitored the reaction by TLC. Quenched with 4~5 mL of water, extracted with CH_2Cl_2 (20 mL×3), washed with saturated brine and dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was evaporated to dryness. Purified with flash column (eluting with 1:5 v/v ethyl acetate/petroleum ether) afforded white solid compound 18 for 0.271g, and yield was 84.8%. HRMS found m/z: 343.3 $[M+Na]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ8.08 (dd, *J* = 6.6 Hz, 1.8 Hz, 2H, ArH), 7.52 (dd, *J* = 6.6 Hz, 1.8 Hz, 2H, ArH), 7.17 (s, 1H, Furan-H), 4.63 (s, 2H, CH_2Cl). ^{13}C NMR (75 MHz, $CDCl_3$): δ163.6, 162.4, 146.9, 144.3, 130.9, 129.1, 127.2, 123.3, 91.7, 44.9.

2.1.19 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl thiophene-2-carboxylate (19)

7 mL of anhydrous CH_2Cl_2 was added into 50 mL three-necked flask concluding 5-hydroxy-3,4-dichloro-2(5H)-furanone (0.168 g, 1.0 mmol) with N_2 protection below -2 °C, after dissolved, 0.13 ml of 2-thiophenecarbonyl chloride was slowly added.

After 30 min, 0.5 mL of anhydrous Et_3N was added and slowly increase to room temperature for 6 h. Then monitored the reaction by TLC. Quenched with 9~10 mL of water, extracted with CH_2Cl_2 (20 mL×3), washed with saturated brine and dried over anhydrous Na_2SO4. The mixture was filtered and the solvent was evaporated to dryness. Purified with flash column (eluting with 1:3 v/v ethyl acetate/petroleum ether) afforded white solid compound 19 for 0.250g, yield 90.1%. HRMS found m/z: 317.1 $[M+K]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ7.27 (d, *J* = 3.3 Hz, 1H, Thiophene-H), 7.00 (s, 1H, Thiophene-H), 6.99 (d, *J* = 3.0 Hz, 1H, Thiophene-H), 6.93 (s, 1H, Furan-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ168.3, 161.0, 146.9, 132.3, 127.6, 127.1, 125.8, 124.9, 91.4.

2.1.20 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl 2-(pyridin-2-yl)acetate (20)

2-pyridine acetic acid (0.521 g, 3.0 mmol) was added into a 50 mL three-necked flask, evacuated and filled with N_2 for 3 to 4 times, 7 mL of anhydrous CH_2Cl_2 and 0.05 mL of DMF were added below -5 °C. After dissolved, 0.5 mL of anhydrous Et3N was added, 0.6 mL of PivCl was added dropwise. After 30 minutes, 5-hydroxy-3,4-dichloro-2(5H)-furanone dissolved in CH2Cl2 was added (0.168 g, 1.0 mmol). after that, DMAP (0.086 g, 0.05 mmol) was added, the reaction was gradually returned to room temperature for 8 h. The reaction was monitored by TLC. Quenched with 10 mL of water, extract with CH_2Cl_2 (20 mL×3), wash with saturated NaHCO3 solution and saturated brine and dry with anhydrous Na_2SO_4. The mixture was filtered and the solvent was evaporated to dryness. Purified with flash column (eluting with 1:3 v/v ethyl acetate/petroleum ether) afforded pale yellow solid 20 for 0.146g, and yield was 50.9%. HRMS found m/z: 598.8 $[2M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ8.40 (ddd, *J* = 5.4 Hz, 1.8 Hz, 0.9 Hz, 1H, Pyridine-H), 7.93 (ddd, *J* = 8.7 Hz, 5.7 Hz, 1.8 Hz, 1H, Pyridine-H), 7.24 (m, 1H, Pyridine-H), 7.23 (m, 1H, Pyridine-H), 6.38 (s, 1H, Furan-H), 3.72 (dd, *J* = 14.1 Hz, 6.9 Hz, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ170.6, 157.1, 142.4, 138.6, 130.7, 127.6, 123.7, 120.4, 112.3, 95.6, 47.3.

2.1.21 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl 2-(3,4-dimethoxyphenyl)acetate

(21)

30 mL of anhydrous CH_2Cl_2 was added into 100 mL round bottom flask concluding 3,4-dimethoxyphenylacetic acid (0.588 g, 3.0 mmol), 0.1 mL of DMF and 0.6 mL of (COCl) $_2$ were added. At this moment, the color turned yellow. After 4 h, the reaction was stopped and the solvent was evaporated to give a yellow oil. After the oil was dissolved in CH_2Cl_2, 1.5 mL of anhydrous Et 3 N was added, followed by 5-hydroxy-3,4-dichloro-2(5H)-furanone (0.168 g, 1.0 mmol), and the reaction was stirred at room temperature overnight. The reaction was monitored by TLC, then the solvent was evaporated to dryness. The product was dissolved in ethyl acetate (10 mL), 10 ml Water was added. Then extracted with ethyl acetate (20 mL×3), washed with saturated $NaHCO_3$ saturated brine, dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was evaporated to dryness. Purified with flash column (eluting with 1:5 v/v ethyl acetate/petroleum ether) afforded yellow solid 21 for 0.238g, and yield was 68.7%. HRMS foundm/z: 348.2 $[M+H]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ7.61 (s, 1H, Furan-H), 6.80 (d, *J* = 8.1 Hz, 1H, ArH), 6.70 (dd, *J* = 8.4 Hz, 2.1 Hz, 1H, ArH), 6.63 (d, *J* = 2.1 Hz, 1H, ArH), 3.87 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.65 (s, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ173.3, 162.4, 152.6, 149.1, 148.1, 126.5, 125.0, 121.7, 112.5, 112.3, 103.4, 55.9, 55.8, 48.5.

2.1.22 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl-4-nitrobenzoate (22)

Same method as compound 21. Yellow solid 0.159g was obtained in a yield of 50.3%. HRMS found m/z: 356.4 $[M+K]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ8.32 (dd, *J* = 9.0 Hz, 1.2 Hz, 2H, ArH), 8.23 (dd, *J* = 9.3 Hz, 1.8 Hz, 2H, ArH), 6.54 (s, 1H, Furan-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ164.8, 163.2, 150.5, 146.0, 135.8, 130.7, 123.5, 117.7, 88.5.

2.1.23 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl 2-(4-nitrophenyl)acetate (23)

Same method as compound 21. Yellow solid 0.161g was obtained in a yield of 53.4%. HRMS found m/z: 370.1 $[M+K]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ8.19 (m, 2H, ArH), 7.48 (m, 2H, ArH), 6.10 (s, 1H, Furan-H), 3.70 (dd, *J* = 14.1 Hz, 7.2 Hz, 2H,

CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ175.5, 164.1, 150.5, 143.9, 139.6, 130.3, 129.2, 123.6, 117.2, 40.5.

2.1.24 3,4-dichloro-5-oxo-2,5-dihydrofuran-2-yl cinnamate(24)

7 mL of anhydrous CH_2Cl_2 was added into 50 mL three-necked flask concluding 5-hydroxy-3,4-dichloro-2(5H)-furanone (0.168 g, 1.0 mmol) with N_2 protection below -2 °C, after dissolved, 0.21mg of cinnamoyl chloride was slowly added. After 30 min, 0.5 mL of anhydrous Et_3N was added and slowly increase to room temperature for 6 h. Then monitored the reaction by TLC. Quenched with 4~5 mL of water, extracted with CH_2Cl_2 (20 mL×3), washed with saturated brine and dried over anhydrous Na_2SO_4. The mixture was filtered and the solvent was evaporated to dryness. Purified with flash column (eluting with 1:5 v/v ethyl acetate/petroleum ether) afforded yellow solid compound for 0.24g, and yield was 78.1%. HRMS found m/z: 337.3 $[M+K]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ7.85 (m, 1H, =CH), 7.59 (m, 2H, ArH), 7.46 (m, 2H, ArH), 7.43 (dd, *J* = 6.6 Hz, 3.0 Hz, 1H, ArH), 7.09 (s, 1H, Furan-H), 6.52 (m, 1H, CH=). ^{13}C NMR (75 MHz, $CDCl_3$): δ172.0, 162.6, 148.8, 147.1, 131.4, 130.8, 129.2, 129.0, 128.7, 128.4, 117.3, 116.8, 106.4.

2.1.25 3,4-dibromo-5-hydroxyfuran-2(5H)-one (1b)

In a 250 mL three-necked flask equipped with a condensed and stirred HBr tail gas absorbing apparatus, fresh distilled furfural (8.1 mL, 0.1 mmol) and 96 mL of water were added, and 25.2 mL of liquid bromine was added dropwise at -5 °C. After that, the reaction turned to room temperature and then slowly heated to reflux for about 30 minutes. Then, it was changed to a vacuum distillation system. At this time, a large amount of white solid was precipitated. Then, 0.9 g of $NaHSO_3$ dissolved in ice water was added to remove excess bromine, which was filtered with suction and washed with cold water and dried to give a white solid of 18.6 g. 73.4%, mp is 125~126 °C.

2.1.26 3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl 4-(2-chloroethyl)benzoate (25)

Same method as compound 18. Yellow solid 0.382g was obtained in a yield of 79.6%. HRMS found m/z: 431.4 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ8.15 (dd, *J*

= 8.4 Hz, 1.8 Hz, 2H, ArH), 7.56 (dd, *J* = 8.7 Hz, 1.8 Hz, 2H, ArH), 6.91 (s, 1H, Furan-H), 4.65 (s, 2H, CH_2Cl). ^{13}C NMR (75 MHz, $CDCl_3$): δ167.8, 161.7, 144.1, 131.0, 130.6, 129.0, 128.6, 114.2, 93.8, 45.1.

2.1.27 3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl thiophene-2-carboxylate (26)

Same method as compound 19. Yellow solid 0.317g was obtained in a yield of 86.8%. HRMS found m/z: 404.9 $[M+K]^+$. ^1H NMR(300 MHz, d6-DMSO): δ7.46 (dd, *J* = 4.8 Hz, 1.2 Hz, 1H, Thiophene-H), 7.12(s, 1H, Furan-H), δ7.02 (m, 2H, Thiophene-H). ^{13}C NMR (75 MHz, d6-DMSO): δ169.2, 164.5, 144.3, 134.2, 128.2, 127.3, 126.5, 118.3, 94.9.

2.1.28 3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl cinnamate (27)

Same method as compound 24. Yellow solid 0.30g was obtained in a yield of 76.3%. HRMS found m/z: 408.9 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ7.83 (m, 1H, =CH), 7.57 (m, 2H, ArH), 7.44 (m, 2H, ArH), 7.40 (dd, *J* = 7.2 Hz, 3.0 Hz, 1H, ArH), 7.07 (s, 1H, Furan-H), 6.50 (m, 1H, CH=). ^{13}C NMR (75 MHz, $CDCl_3$): δ171.9, 162.5, 148.7, 147.1, 134.1, 130.8, 129.1, 129.0, 128.6, 128.4, 117.3, 116.8, 108.6.

2.1.29 3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl 2-(4-nitrophenyl)acetate (28)

Same method as compound 21. Yellow solid 0.24g was obtained in a yield of 57.3%. HRMS found m/z: 441.4 $[M+Na]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ8.19 (m, 2H, ArH), 7.46 (m, 2H, ArH), 6.61 (s, 1H, Furan-H), 4.18 (dd, *J* = 14.4 Hz, 7.2 Hz, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ170.2, 163.3, 147.4, 144.5, 141.4, 130.3, 123.7, 112.8, 107.2, 41.1.

2.1.30 3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl 2-(3,4-dimethoxyphenyl)acetate (29)

Same method as compound 21. Yellow solid 0.242g was obtained in a yield of 55.7%. HRMS found m/z: 473.3 $[M+K]^+$. ^1H NMR(300 MHz, $CDCl_3$): δ7.08 (s, 1H, Furan-H), 6.83 (d, *J* = 8.4 Hz, 1H, ArH), 6.71 (dd, *J* = 8.1 Hz, 2.1 Hz, 1H, ArH), 6.64 (d, *J* = 1.8 Hz, 1H, ArH), 3.88 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.67 (s, 2H, CH_2).

^{13}C NMR (75 MHz, $CDCl_3$): δ172.2, 168.2, 149.1, 148.1, 131.6, 126.5, 121.7, 116.6, 112.5, 111.3, 100.8, 55.9, 55.8, 48.5.

2.1.31 3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl 4-nitrobenzoate(30)

Same method as compound 21. Yellow solid 0.193g was obtained in a yield of 47.7%. HRMS found m/z: 428.3 $[M+Na]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ8.31 (dd, *J* = 9.0 Hz, 1.8 Hz, 2H, ArH), 8.24 (dd, *J* = 9.3 Hz, 2.1 Hz, 2H, ArH), 6.54 (s, 1H, Furan-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ164.8, 163.2, 146.0, 135.8, 131.3, 130.7, 123.5, 117.7, 88.5.

2.1.32 3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl nicotinate (31)

Same method as compound 21. Yellow solid 0.167g was obtained in a yield of 46.3%. HRMS found m/z: 722.8 $[2M+H]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ8.78 (ddd, *J* = 4.5 Hz, 1.5 Hz, 0.6 Hz, 1H, Pyridine-H), 8.17(dt, *J* =7.8 Hz, 1.2 Hz, 1H, Pyridine-H), 7.88 (td, *J* =6.6 Hz, 1.5 Hz 1H, Pyridine-H), 7.51 (ddd, *J* = 6.0 Hz, 4.8 Hz, 1.2 Hz, 1H, Pyridine-H), 6.93 (s, 1H, Furan-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ168.1, 165.7, 149.8, 147.9, 137.1, 134.0, 127.0, 125.1, 117.8, 103.4.

2.1.33 3,4-dibromo-5-oxo-2,5-dihydrofuran-2-yl 2-(pyridin-2-yl)acetate (32)

Same method as compound 21. Yellow solid 0.197g was obtained in a yield of 52.5%. HRMS found m/z: 373.7 $[M-H]^+$. ^{1}H NMR(300 MHz, $CDCl_3$): δ9.36 (ddd, *J* = 8.7 Hz, 6.2 Hz, 1.5 Hz, 1H, Pyridine-H), 9.02 (ddd, *J* = 7.5 Hz, 6.3 Hz, 1.8 Hz, 1H, Pyridine-H), 8.40 (m, 1H, Pyridine-H), 7.93 (m, 1H, Pyridine-H), 6.38 (s, 1H, Furan-H), 3.72 (dd, *J* = 14.4 Hz, 7.2 Hz, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ170.7, 163.9, 142.6, 138.5, 130.7, 127.6, 123.7, 120.4, 112.3, 95.5, 39.7.

2.2 MIC determination

To define the biofilm inhibitory test concentration under MIC and make sure the effects of new compounds were via QS inhibition not that of inhibiting the bacteria themselves. The MICs of all compounds were to measure. It was performed on planktonic cultures using the two-fold dilution method according to clinical and

laboratory standards institute guidelines.[24] MICs were performed in 96-well sterile microplates (cosmo) and the results were recorded after 18h of incubation at 37^{o}C. 5-(dibromomethylene)furan-2(5H)-one was used as positive control.

2.3 *In vitro* determination of biofilm formation

All the synthetic compounds above were assayed for their *in vitro* bacterial biofilm formation inhibitory activities against *P. aeruginosa* ATCC27853, ATCC9027 and PAOA. Method of crystal violet staining was used. *P. aeruginosa* was diluted with LB broth at OD_{600}=0.05. A concentrated compound solution was transferred into each well, except those used as controls, to achieve a final test concentration of 64 μg/mL, and each well was filled to a final volume of 200μL. After 20h of incubation at 35^{o}C and being washed twice with PBS (Phosphate Buffered Saline) solution, the biofilm remained on the wells was fixed with 200μL of 99% methanol for 15 min. The solution was discarded and the microplate was put in super clean bench. When the wells were dried, each of them was added with 250 μL of 1% solution of crystal violet (CV). After 15 min staining at room temperature, the wells were washed twice carefully with distilled solution. Then 250 μL 95% ethanol was added to each well, as to dissolve the stain and biofilm. 15 min later, the absorbance of plates was determined at 570 nm in a spectrophotometer. The percentage of biofilm inhibition was calculated using the following formula: inhibition percentage=[($OD_{\text{negative control}}$−$OD_x$) /$OD_{\text{negative control}}$]×100, where x refers to the tested halogenated compounds.[25-30]

To find out the rule of concentration affecting QS system, some of the synthesized compounds were chose to determine the biofilm inhibition percentages at concentrations of MIC, 1/2MIC, 1/4MIC, 1/8MIC and 1/16MIC using the same method described above.

2.4 Observation of *P. aeruginosa* biofilm formation with scanning electron microscopy (SEM)

The *P. aeruginosa* inoculums were centrifuged (10000 rpm, 5 min) and washed with saline twice to acquire planktonic cultures. It was diluted with sterile saline at 0.5MCF (Mcfarland standard), and then diluted 25 fold with sterile saline. 1mLof the prepared cultures was added to each test tube, as well as 1mL compound solution

(dissolved by LB broth, 128 μg/mL). The final concentration of compounds was 64μg/mL. After the polyvinyl chloride catheter (0.5 cm×0.5 cm) was set on the bottom of each tube, cultures were incubated in shaking bath at 37°C for 7 days. Following washed twice with PBS solution, the bacteria on the catheter was fixed by placing in 2.5% glutaraldehyde/cacodylate (v/v) buffer for 5h. The samples were then dried with ethanol steps—50% aqueous ethanol (v/v) for 10min, 75% aqueous ethanol (v/v) for 10min, 85% aqueous ethanol (v/v) for 10min, 95% aqueous ethanol (v/v) for 10min, 100% aqueous ethanol (v/v) for 10min, and isoamyl acetate for 10 min. They were frozen in a freezer at -65°C and dried at the critical point of vacuum pressure at -53°C, and then covered with gold. Biofilms on the catheter were then observed with a SEM.[31,32]

2.5 Pyocyanin Quantification Assay

The OD_{600} value of overnight culture of *P. aeruginosa* PAOA was adjusted to 0.1 and diluted 100 times with fresh King's medium B with a final volume of 5 mL. Then, compounds were added to the bacteria suspensions with a final concentration of 64 μg/mL and cultured for 48 h at 37°C in a shaking-bath (200 rpm). After that, bacteria cultures were centrifuged for 10 min and the supernatants were collected. Pyocyanin in the supernatants were extracted using 1 mL chloroform and 0.3 mL HCl (0.2 M). The content of the extracted pyocyanin was quantified by measuring the OD value at 520 nm. The absorbance of this solution was measured at 520 nm. Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying the optical density at 520 nm (OD_{520}) by 17.072. [33]

2.6 *P. aeruginosa* QS Inhibition Assays.

Test compounds were mixed with ABTGC medium and serial diluted to give a final concentration of 20 μM in the first well. An overnight culture of PAO1-*lasB-gfp* strain (grown in LB medium at 37°C, 200 rpm) was diluted in ABTGC medium to a final optical density at 600 nm (OD_{600}) of 0.02 (2.5 × 108 CFU/mL). An equal amount of the bacterial suspension was added to the wells to reach final inhibitor concentration

of 10 μM. DMSO control (0.1% final concentration) and blank control were used. The microtiter plate was incubated at 37°C in Tecan Infinate 200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland) to measure the cell density (OD_{600}) and GFP fluorescence (excitation at 485 nm, emission at 535 nm) with 15min intervals for at least 15 h. The inhibition assay for all test compounds and controls were done in triplicate manner. For *P. aeruginosa* Rhl and Pqs inhibition assay, a similar method was performed as the LasB inhibition assay.[34]

2.7 Cytotoxicity and plasma stability study

The cell viability was measured as described in a previous report[1]. Briefly, RAW264.7 cells were grown in DMEM media supplements with 10% FBS, counted using a hemocytometer. Cells were then serially diluted in a clear cell culture plate and incubated for 4 hours with MTT reagent at 37°C. After incubation, cells were treated with MTT solvent for 15 minutes at room temperature. Absorbance was measured at OD_{590}.[35]

Plasma and compound 29 (100 μM in DMSO) are added to the individual wells of a 96-well microtiter plate. The plate is incubated at 37 °C with gentle agitation. During the incubation, aliquots are withdrawn at 0, 15, 30, 60, and 120 minutes time points and quenching solution (25/50 ng/mL terfenadine/tolbutamide in ACN/MeOH (1:1, v/v)) is added. After mixing, the quenched aliquots are centrifuged and supernatant is withdrawn for analysis by HPLC-UV.[36]

3. Results

3.1 Synthesis of Furanone Derivatives

Alkyl chains with different length were first considered as a potent key structure in QS and biofilm inhibition according to AHLs and 3OC12. Referring to the length of alkyl chains in native signal molecule, a range of C3 to C15 alkyl acids were acylated at C-5 hydroxyl in 5-hydroxyl-3,4-halogenated-5*H*-furan-2-ones. Meanwhile, molecules with aromatic ring substituted furanone rings drew our attention for their reported activity in inhibiting LasR at low micromolar concentrations and reducing the production of various virulence factors. Thus, a series of compounds with an

aromatic core structure instead of furanone rings were also synthesized. The same alkyl chains were acylated to 3-hydroxy acetophenone to obtain compounds **10-17**. A hypothesis of a key π-π interaction driven us thinking to replace the alkyl chain by an aromatic substituent, hence different aromatic acids with electron-withdrawing or electron-donating substituents were linked to chloro or bromo 5*H*-furan-2-ones to obtain compounds **24-32**. The detailed procedures and yields are shown in the method section.

[Scheme 1]

[Scheme 2]

3.2 MIC of inhibitors

The results (Table 1) showed that most of compounds we synthesized have MICs higher than 1024 μg/mL except compounds **20** and **21**, whose MIC against strain ATCC 9027 is 32 μg/mL.

[Table 1]

3.3 Biofilm inhibition

Biofilm inhibition ratio was evaluated at concentrations lower than the MIC by crystal violet staining quantitative determination. The assays were carried out at 1/2, 1/4, 1/8 and 1/16 the concentration of MIC to investigate the effect on biofilm inhibition under a safe dose for the *P. aeruginosa* life cycle. The results were shown in Table 2, 3, 4, 5 and indicated a concentration-dependent relation between the compounds and biofilm inhibition.

[Table 2]

[Table 3]

[Table 4]

[Table 5]

3.4 SEM Observation of compound 29

The effect of compound 29 on biofilm formation was further confirmed by scanning electron microscopy (SEM). As shown in Figure 2, compared with the **blank**,

incubation of *P. aeruginosa* PAOA with compounds **29** at different sub-MIC resulted in obvious reduction of the biofilm.

[Figure 2]

3.5 Pyocyanin Quantification Assay

P. aeruginosa also produces a blue-green phenazine pigment called pyocyanin, a secondary metabolite whose expression is under the control of QS. To verify the anti-biofilm and anti-bacteria activity of the obtained compounds, pyocyanin production assays were established. As shown in Figure 3, a significant decrease in pyocyanin production of *P. aeruginosa* was observed after treatment with phenolic compounds and their derivatives. Among which compound 29 showed obvious reduction in pyocyanin formation, which is consistent with the biofilm formation inhibitory activity.

[Figure 3]

3.6 *P. aeruginosa* QS Inhibition Assays

To further confirm the QS inhibitory activity of compound 29, reporter strain PAO1-*lasB-gfp* was introduced to perform the screening.[37] As shown in Figure 4, the decrease of GFP fluorescence indicated the presence of 3-oxo-C12-HSL antagonist and the inhibition of the *lasB* gene. The GFP expression was normalized by dividing the GFP values with the OD_{600} measured at the corresponding time points. Compound 29 were found to be effective in inhibiting the *lasB* expression in a dose-dependent manner.

[Figure 4]

3.7 Cytotoxicity and plasma stability of compound 29

The cytotoxicity of compound 29 was determined using MTT assay in RAW264.7 cells. As shown in Figure 5, the cell viability remained basically at 100% after treatment with the compounds, indicating the low toxicity of compound 29. Additionally, compound 29 in Table 6 showed relatively stable in the plasma stability assay.

[Figure 5]

[Table 6]

4. Discussion

As mentioned above, our goal is to achieve a new approach to combat *P. aeruginosa* without disturbing bacteria's life cycle. A safe concentration must be determined before the evaluation of biofilm inhibition. The MIC assay results suggest that the compounds do not disturb the normal life cycle of *P. aeruginosa* at high concentrations as most antibiotics do.[38] For the drug resistance are caused on occasion in eliminating bacteria, those compounds might coexist with *P. aeruginosa* and do not trigger the mechanism of resistance. On the basis of the MIC data, biofilm inhibition ratio was evaluated at concentrations lower than the MIC. The assays were carried out at 1/2, 1/4, 1/8 and 1/16 the concentration of MIC to investigate the effect on biofilm inhibition under a safe dose for the *P. aeruginosa* life cycle. And the results showed a concentration-dependent relation between the compounds and biofilm inhibition. At 1/2 and 1/4 MIC most of the furanone compounds inhibit biofilm formation well in drug resistant strains, including *P. aeruginosa* strains ATCC27853, ATCC9027, and PAOA (clinical isolates). At 1/8 MIC, the inhibition declined but most chlorofuranone compounds still exhibit considerable inhibition. However, at 1/16 MIC the inhibition ratios declined to a quite low level, and only compound **9** showed a promising effect with 45.4% inhibition on PAOA. Some compounds even demonstrated a promotion effect on biofilm formation. Although the biofilm inhibitory effects at low concentrations are not as promising as compound **9**, compounds with activity superior to positive control still account for a large proportion of those tested. The overall analysis revealed that compounds with furanone as core structure are in general more potent than those with a 3-hydroxyacetophenone core. The hydrophobic alkyl chain of a length above C-10 is desirable. Chloro-substitutuents seem more active at high concentration, whereas at low concentrations they resemble bromo substituted furanone derivatives. This structure information emphasizes the halogenate furanone and the hydrophobic side chain.

After comparing the furanone and acetophenone derivatives, we returned our

focus to the alkyl chain. As we know, the long alkyl chain normally contributes no polar interaction in binding with acceptors, we wondered if this alkyl chain could be replaced by other hydrophobic groups of less flexibility. Fortunately, the AHLs bearing such a long alkyl chain have been well studied by X-ray crystallography. The interaction mode between AHLs and LasR was depicted from an X-ray crystallography 4NG2. [39-43] As shown in Figure 6, the two key contacts with LasR are a) hydrogen bonding interactions with protein at residues Asp73, Ser 1**29** and Tyr56, b) van der Waals contact deep in the hydrophobic pocket via the alkyl group.

[Figure 6]

Having investigated the conformation of alkyl group and hydrophobic pocket consisted of Tyr64, Val76 and Tyr47, we noted that the pocket is not a narrow channel but a cavity surrounded by residues with aromatic rings. The pocket wraps curly alkyl chains. A hypothesis occurred to us: if the alkyl chain was replaced by an aromatic substituent, the π-π interaction would help improve binding affinity and biofilm inhibition. To verify this hypothesis compounds **24-32** were synthesized and evaluated too.

Among compounds **24-32**, the MIC values are not that stagnant for a multitude of compounds can inhibit strain ATCC 9027 at 32 μg/mL, except for compounds **25** and **29** which are of desired tolerance. The biofilm inhibition ratio was still evaluated in concentrations at 1/2, 1/4, 1/8 and 1/16 MIC. We are overjoyed by the excellent performance of compound 29 in biofilm inhibition against ATCC 9027 whose inhibitory rate at 1/16 MIC was 63%. With other strains and concentrations compound 29 also demonstrated adorable activities. The effect of compound 29 on biofilm formation was further confirmed by scanning electron microscopy (SEM). These assays exhibited the outstanding biofilm inhibitory effects of compound 29 at every level of concentration. The pyocyanin assay also indicated compound 29 with obvious reduction in pyocyanin formation, which is consistent with the biofilm formation inhibitory activity. Considering its prominent effect in reducing pyocyanin production and the desired MIC value, compound 29 showed the most promising potency in biofilm inhibition. To explore the possible mechanism of compound 29

inhibiting the LasR, a brief molecular docking study was conducted[29] (Figure 7). Superimposition of AHLs and compound 29 reveled similar interactions and the aromatic substituent laid exactly where the alkyl chain is, which conformed to our hypothesis.

[Figure 7]

According to the QS Inhibition assays, compound 29 can inhibit the *lasB* expression in a dose-dependent manner. In addition to this, *rhlA-gfp* and *pqsA-gfp* bioreporters in the same stain which correlate rhamnolipid and PQS molecules respectively were also studied and the results were proven to be affirmative. The cytotoxicity and ADME stability assay also give promising result in the future development of compound 29.

5. Conclusion

In conclusion, we have used natural and chemically synthesized halogenated furanones as beginning to prepare analogs bearing alkyl chain, vinyl bromide key or aromatic ring. Optimization efforts established the furanone core structure and hydrophobic side chain. Combination of these findings ultimately led to the discovery of compound 29, a potent new approach to combat the drug resistant bacteria strains. On the basis of our findings, compound 29 was advanced into a promising lead in combating drug resistant bacteria. As potent QSI candidates, compound 29 which have no effect in suppressing the regular life cycle of the bacteria, may bring us a new anti-bacterial mechanism that trigger no drug resistance system while the pathogenicity and virulence factors decline.

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■ ABBREVIATIONS

QS, quorum sensing; MIC, minimum inhibitory concentration; MDR, multiple drug resistant; QSIs, quorum sensing inhibitors; AHLs, acyl homoserine lactones; 3OC12, N-3-oxododecanoyl homoserine lactone; SEM, scanning electron microscopy.

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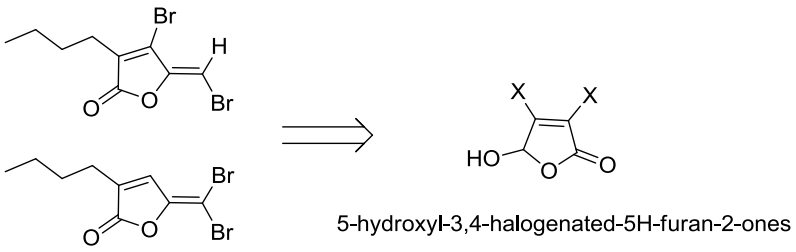


Figure 1. Natural products to 5-hydroxyl-3,4-halogenated-5*H*-furan-2-ones.

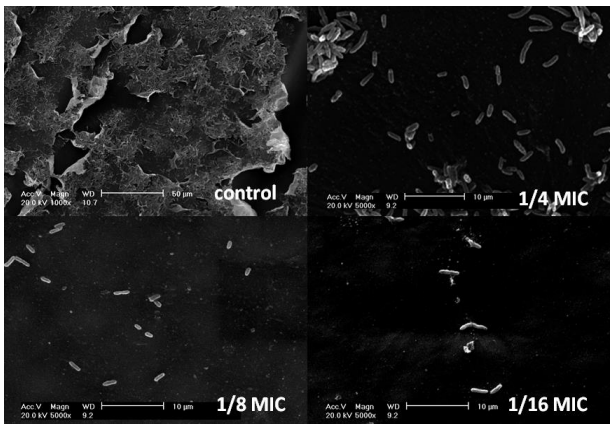


Figure 2. Scanning electron micrographs (5000×) of PAOA on the surface of catheter for seven days in the absence (blank) or presence of compound **29**.

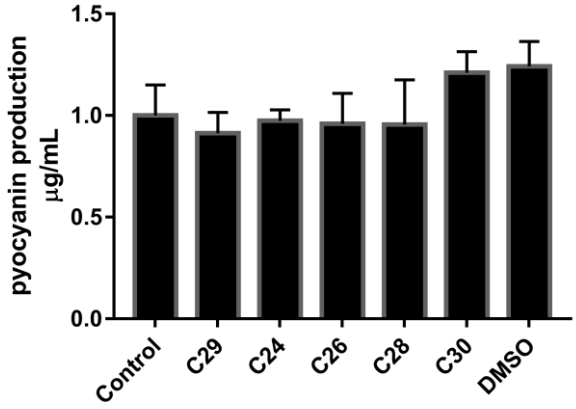


Figure 3. Pyocyanin quantification with phenolic derivatives. The concentration of compounds is 64 µg/mL. Experiments were done in triplicate.

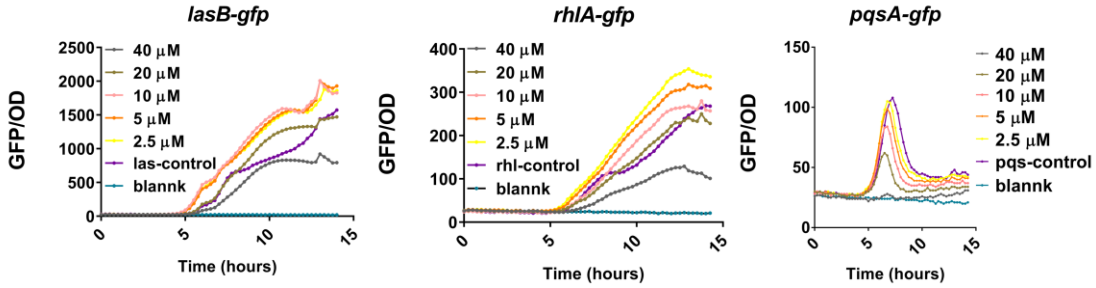


Figure 4. Dose-dependent inhibition curves of compound **29** with the following QS monitors PAO1-*lasB-gfp*, *rhlA-gfp*, and *pqsA-gfp*. Experiments were done in triplicate.

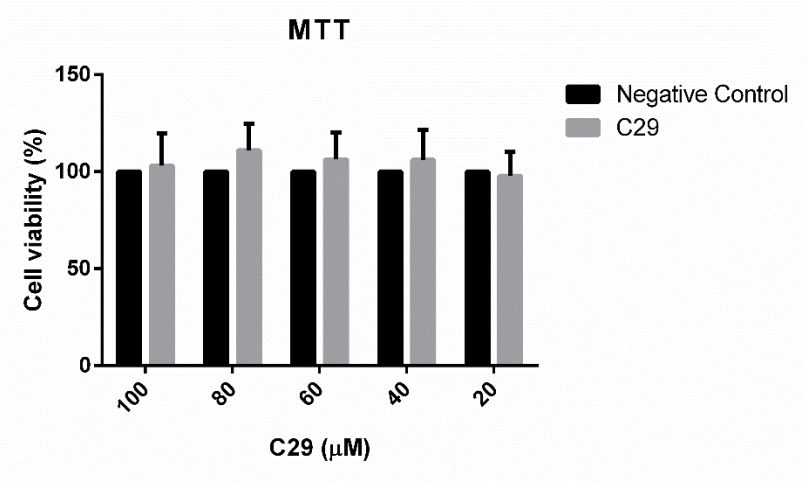


Figure 5 The cytotoxicity of derivatives of C29. DMSO (0.1%) was used as control group.

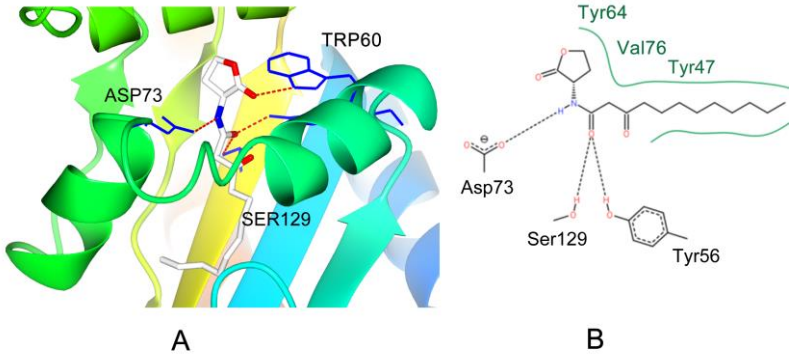


Figure 6. Interaction between AHLs and LasR. A) 3D interactive model; B) 2D structure-diagrams of protein-ligand complexes.

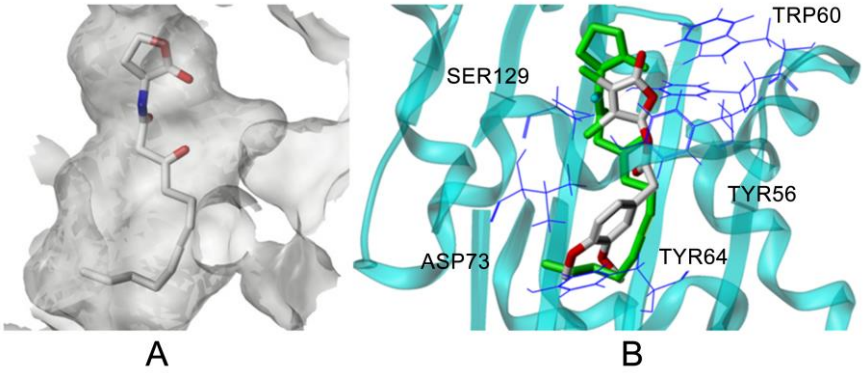


Figure 7. Molecular docking of compound **29**: A) Shape of AHL pocket in LasR receptor; B) superimposition of AHLs and compound **29** in the active site.

Table 1 MIC of compounds 2-32

compound	MIC (μg/mL)		
	ATCC 27853	ATCC 9027	PAOA
2	1024	1024	1024
3	1024	1024	1024
4	>1024	1024	1024
5	>1024	>1024	>1024
6	>1024	>1024	>1024
7	>1024	>1024	>1024
8	>1024	>1024	>1024
9	>1024	>1024	>1024
10	>1024	>1024	>1024
11	>1024	>1024	>1024
12	>1024	>1024	>1024
13	>1024	>1024	>1024
14	>1024	>1024	>1024
15	>1024	>1024	>1024
16	>1024	>1024	>1024
17	>1024	>1024	>1024
18	>1024	>1024	512
19	>1024	>1024	>1024
20	>1024	32	>1024
21	>1024	32	>1024
22	>1024	1024	>1024
23	>1024	>1024	>1024
24	>1024	>1024	>1024
25	>1024	512	>1024
26	>1024	>1024	>1024
27	>1024	32	>1024
28	>1024	32	>1024
29	512	256	512
30	1024	32	1024
31	>1024	32	512
32	1024	32	512
control	512	512	512

Control: 5-(dibromomethylene)furan-2(5H)-one.

Table 2. Biofilm inhibition rate in 1/2 MIC

compound	Biofilm Inhibition（%）		
	ATCC 27853	ATCC 9027	PAOA
2	65.5±3.4△	68.6±2.7*	79.4±2.0△△
3	69.8±6.0△	73.8±2.8*	74.4 ±3.3△△
4	45.9±7.8△	68.9±2.3*	81.4±5.3△△
5	67.9±2.4△	56.6±3.9△	60.6±1.8*
6	67.9±4.8△	57.9±6.1*	61.3±5.3△△
7	67.8±1.9△	64.9±4.2*	60.9±2.6*
8	68.6±1.4△	67.8±.1.2*	58.8±2.5*
9	69.2±3.2△	78.5±5.1*	68.8±3.6△△
10	42.9±7.5△	43.5±1.7△	67.7±3.1*
11	30.5±1.4△	43.9±7.8△	57.3±1.7*
12	44.1±7.6△	51.9±4.7△	52.9±2.1△
13	55.2±5.0△	58.2±2.8*	58.9±1.9*
14	46.5±3.3△	46.1±4.3△	42.6±1.5△
15	45.4±3.1△	48.7±1.7△	53.5±1.8*
16	51.5±5.6△	45.0±3.4△	42.9±4.2△
17	54.3±4.8△	42.7±4.2△	56.7±6.4*
18	6.66±1.1△	33.0±2.6△	35.1±2.0△
19	58.0±6.1△	44.4±1.6△	26.4±1.3△
20	20.5±2.5△	47.3±1.9△	61.7±1.5*
21	33.5±1.3△	41.9±2.8△	56.5±7.3*
22	14.0±5.2△	42.8±4.7△	39.4±5.9*
23	12.8±3.9△	26.5±1.9△	47.3±6.6*
24	40.1±2.2△	44.0±5.1△	53.8±5.4*
25	6.04±2.1△	36.6±1.9△	47.7±2.8*
26	46.0±2.5△	46.2±9.7△	48.2±5.9*
27	46.1±1.1△	47.4±2.5△	55.8±6.2*
28	45.5±4.4△	44.2±1.6△	47.6±1.2*
29	61.0±7.7△	73.8±1.9*	74.4±6.4△△
30	43.6±3.6△	46.5±1.9△	35.4±6.2△
31	53.8±3.3△	53.3±2.3△	34.8±4.7△
32	58.3±3.8△	58.2±6.9*	63.6±4.6△△
control	78.8±2.5	72.0±8.4	54.9±9.1

△: P <0.05 & Biofilm Inhibition < control　△△: P <0.05 & Biofilm Inhibition > control

*: P >0.05. Control: 5-(dibromomethylene)furan-2(5H)-one.

Table 3. Biofilm inhibition rate in 1/4 MIC

compound	Biofilm Inhibition（%）		
	ATCC 27853	ATCC 9027	PAOA
2	55.5±2.8△	62.3±2.1△	42.0±6.7*
3	59.1±5.1△	51.7±7.7△	43.3 ±7.8*
4	37.6±3.6△	64.5±8.6△△	42.0±4.8*
5	59.8±7.6△	45.2±5.5△	51.9±6.5△△
6	70.3±4.4△△	38.6±3.5△	36.9±2.8*
7	53.9±3.1△	56.4±2.6△	53.7±2.0△△
8	60.0±2.1△	54.0±.1.7△	44.3±3.6*
9	44.7±2.5△	54.6±1.5*	58.8±3.2△△
10	33.0±4.2△	35.1±5.6*	56.5±7.1△△
11	10.6±5.7△	24.8±8.5△	49.8±2.5△△
12	33.7±7.2△	43.5±3.1△	38.4±1.8*
13	51.9±1.3△	42.1±3.8△	49.4±1.8△△
14	35.6±1.8△	26.6±8.9△	38.4±3.2△
15	35.1±6.8△	42.4±1.6△	43.3±1.6*
16	44.4±8.5△	33.8±7.9△	32.4±2.6*
17	37.5±2.5△	27.0±3.9△	47.0±4.0△
18	-21.8±2.1△	18.7±3.1△	20.9±3.0*
19	22.9±5.0△	38.2±4.0△	21.8±1.6*
20	15.4±1.2△	22.9±6.9△	51.1±2.1△△
21	22.5±4.0△	36.5±2.6△	48.0±3.6*
22	10.9±4.0△	30.8±5.1△	28.5±6.0*
23	11.0±4.7△	16.1±2.2△	32.1±4.7*
24	35.3±5.1△	33.9±7.6△	39.5±2.1*
25	-6.01±8.2△	20.0±4.2△	34.3±2.1△
26	12.3±7.3△	20.5±6.4△	29.8±2.1*
27	33.1±3.9△	36.5±3.4△	44.8±3.8*
28	37.9±1.9△	40.6±2.7△	37.2±2.2*
29	53.3±2.8△	70.0±3.8△△	72.6±5.0△△
30	7.41±2.0△	33.9±8.4△	30.8±3.7*
31	38.2±6.3△	36.1±2.7△	30.4±4.1*
32	37.5±5.3△	42.0±4.5△	47.2±5.8△△
control	74.2±3.7	65.7±8.7	47.7±5.0

△: P <0.05 & Biofilm Inhibition < control　△△: P <0.05 & Biofilm Inhibition > control

*: P >0.05. Control: 5-(dibromomethylene)furan-2(5H)-one

Table 4. Biofilm inhibition rate in 1/8 MIC

compound	Biofilm Inhibition（%）		
	ATCC 27853	ATCC 9027	PAOA
2	53.5±3.5△△	37.2±5.1△△	-3.19±1.8△
3	27.7±6.1*	38.3±1.7△△	41.0 ±3.2△△
4	-19.2±3.5△	2.35±1.5△	27.1±2.2△△
5	35.6±5.9△△	37.8±4.0△△	47.1±3.8△△
6	35.7±5.2△△	36.3±4.4△△	30.2±2.6△△
7	46.9±2.2△△	42.2±3.7△△	30.4±5.6△△
8	34.6±1.8△△	46.9±1.9△△	34.8±2.4△△
9	37.1±3.9△△	36.9±2.5△△	57.4±2.5△△
10	15.1±2.9△	21.6±3.1*	48.8±4.6△△
11	-3.04±4.1△	11.3±2.2△	37.0±1.7△△
12	15.5±1.1△	23.5±4.3*	24.7±3.4△△
13	35.2±5.5*	33.1±3.5△△	45.6±4.4△△
14	15.3±3.4△	14.4±1.2*	14.8±2.8*
15	15.2±6.1△	29.1±1.6△△	24.2±1.4△△
16	35.1±2.4△△	13.1±5.0△	17.6±2.6*
17	18.3±3.4*	17.9±6.1*	35.2±5.6△△
18	-29.3±2.8△	4.02±1.4△	5.25±2.6△
19	3.46±1.9△	22.7±1.3*	3.82±2.2△
20	7.18±1.5△	20.6±1.4*	43.3±2.7△△
21	11.5±3.1△	22.6±2.7*	40.8±3.1△△
22	-7.97±1.7△	13.4±3.1△	11.5±4.4*
23	3.94±4.2△	3.79±1.4△	16.8±2.2*
24	24.4±4.5*	34.8±4.7△△	26.1±4.8△△
25	-7.15±1.7△	4.80±4.6△	18.3±3.8*
26	2.96±1.8△	12.7±1.7△	20.3±3.3*
27	13.5±4.3△	16.0±1.8*	42.1±3.0△△
28	18.7±6.3*	25.2±6.1*	16.5±2.5*
29	47.5±4.1△△	66.3±4.0△△	34.4±4.9△△
30	4.75±2.3△	26.8±4.2△△	15.0±5.5*
31	3.40±4.8△	29.1±3.3△	24.0±5.9*
32	10.3±4.5△	19.4±3.5*	23.9±3.9△△
control	24.1±4.6	20.5±4.2	15.7±1.8

△: P <0.05 & Biofilm Inhibition < control　△△: P <0.05 & Biofilm Inhibition > control

＊: P >0.05. Control: 5-(dibromomethylene)furan-2(5H)-one

Table 5. Biofilm inhibition rate in 1/16 MIC

compound	Biofilm Inhibition（%）		
	ATCC 27853	ATCC 9027	PAOA
2	14.4±1.5*	26.4±4.1△△	-19.8±2.8△
3	19.4±8.4*	19.5±2.1*	19.7 ±2.3△△
4	-34.8±6.1△	-27.2±7.6△	8.28±5.1*
5	11.8±3.8*	22.5±2.5△△	33.5±2.1△△
6	10.3±3.3*	21.5±3.1*	17.0±5.1△△
7	33.5±4.4△△	33.1±3.2△△	10.4±4.4*
8	11.5±2.6*	35.5±4.5△△	29.5±1.1△△
9	23.1±5.7△△	24.1±6.6△△	45.4±2.8△△
10	-33.6±6.3△	16.3±4.5*	42.4±1.8△△
11	-15.8±5.9△	1.13±1.6△	24.4±2.8△△
12	-11.2±1.5△	7.32±4.6△	12.9±5.2*
13	-14.6±5.4△	15.2±4.8*	21.2±3.8△△
14	0.30±0.3△	2.86±1.3*	7.76±1.7*
15	8.49±1.1*	-26.0±1.5△	9.93±5.3*
16	25.2±3.4△△	-8.95±8.5△	14.7±2.8△△
17	3.52±3.2△	15.3±3.4*	23.4±4.0△△
18	-65.3±1.6△	-18.2±7.2△	-21.8±6.1△
19	-11.7±1.1△	-7.60±4.3△	-22.2±4.2△
20	-7.77±2.3△	17.2±3.5*	29.6±2.1△△
21	-59.2±1.4△	-5.55±3.8△	32.8±1.2△△
22	-22.7±3.1△	-9.43±3.8△	-2.43±3.2△
23	-38.7±2.0△	-20.9±3.7△	-9.45±3.7△
24	15.8±5.6*	23.6±3.7△△	12.9±3.3△△
25	-16.7±1.8△	-12.1±6.5△	-9.43±1.4△
26	-15.3±2.0△	2.90±2.5△	1.98±1.4*
27	-42.0±5.4△	-8.43±5.3△	31.9±1.8△△
28	-47.9±5.9△	8.13±6.3*	-35.3±2.7△
29	30.9±3.3△△	63.0±1.8△△	6.62±3.9*
30	-63.0±2.3△	-12.6±2.5△	-21.2±3.9△
31	-8.45±1.3△	-3.93±2.0△	-5.11±1.7△
32	-3.49±5.1△	8.42±2.4*	5.63±3.7*
control	7.33±3.7	11.6±6.8	4.65±3.3

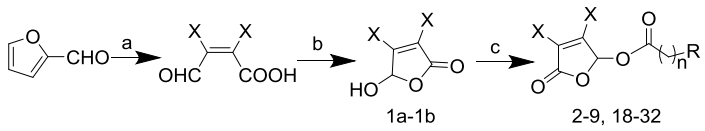
△: P <0.05 & Biofilm Inhibition < control　△△: P <0.05 & Biofilm Inhibition > control

*: P >0.05. Control: 5-(dibromomethylene)furan-2(5H)-one

Table 6. Plasma stability of compound 29

Cpd	Concentration (μM)	Mean remaining parent comp (%)
Warfarin	5	96.4
29	5	91.2

Control: Warfarin



1a X=Cl

1b X=Br

2 X=Cl, n=3, R=H

3 X=Cl, n=6, R=CH_3

4 X=Cl, n=8, R=CH_3

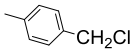
5 X=Cl, n=10, R=CH_3

6 X=Cl, n=8, R=CH_2CH

7 X=Cl, n=12, R=CH_3

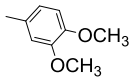
8 X=Cl, n=14, R=CH_3

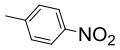
9 X=Cl, n=7, R=$CHCH(CH_2)_7CH_3$

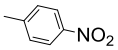
18 X=Cl, n=0, R= 

19 X=Cl, n=0, R= 

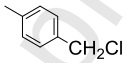
20 X=Cl, n=1, R= 

21 X=Cl, n=1, R= 

22 X=Cl, n=0, R= 

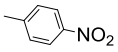
23 X=Cl, n=1, R= 

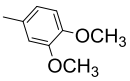
24 X=Cl, n=0, R= 

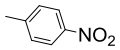
25 X=Br, n=0, R= 

26 X=Br, n=0, R= 

27 X=Br, n=0, R= 

28 X=Br, n=1, R= 

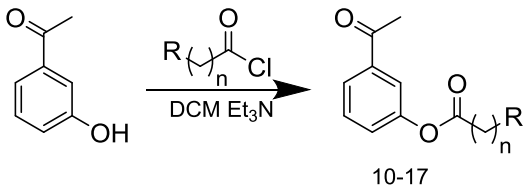
29 X=Br, n=1, R= 

30 X=Br, n=0, R= 

31 X=Br, n=0, R= 

32 X=Br, n=0, R= 

Scheme 1. Synthesis of compounds **1-9** and **18-32**. Reagents and conditions: (a) MnO_2, HCl/HBr, reflux, 1h; (b) rt overnight 66.7%; (c) alkyl acid, Et_3N, 0 °C, r.t., 18 h.



10-17

10 n=3, R=Cl
11 n=6, R=CH_3
12 n=8, R=CH_3
13 n=10, R=CH_3
14 n=8, R=CH_2CH
15 n=12, R=CH_3
16 n=14, R=CH_3
17 n=7, R=$CHCH(CH_2)_7CH_3$

Scheme 2. Synthesis of Compounds **10-17**.

Graphical Abstract