Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications


# Structures and Biofilm Inhibition Activities of Brominated Furanones for *Escherichia coli* and *Pseudomonas aeruginosa*

Gauri S. Shetye,[1] Nischal Singh,[1] Xiang Gao,[2] Debjyoti Bandyopadhyay,[1] Aixin Yan,[2]*

Yan-Yeung Luk[1]*

[1] *Department of Chemistry, Syracuse University*

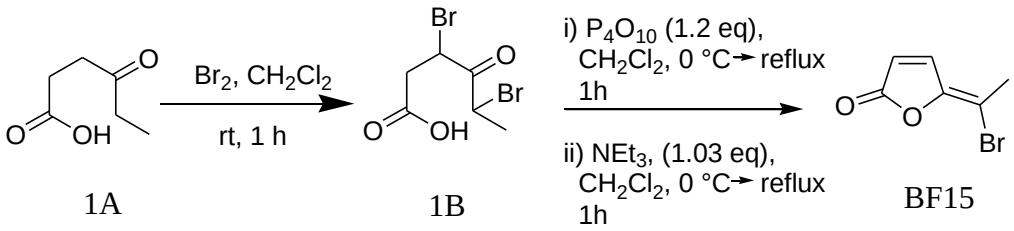
[2] *School of Biological Sciences, The University of Hong Kong, Hong Kong, China*

## Table of Contents

## General methods

The synthesis of BF15 was executed under an atmosphere of argon in flame dried glassware. 4-oxohexanoic acid and anhydrous $CH_2Cl_2$ ( > 99 % with sure seal)was bought from Sigma Aldrich. HRMS was obtained through positive ESI on Bruker 12 Tesla APEX-QE FTICR-MS with an Apollo II ion source at Cosmic instrumentation center, Virginia. TLC visualization was done under UV light (254 nm). Solvents were removed *in vacuo* using Bűchi rotary evaporator below 40$^{o}$C. EMD silica gel 60 F254 pre-coated plates (0.25-mm thickness) were used for TLC. Column chromatography was performed using Silicycle, silia-P flash silica gel with 40-63µ mesh size. $^{1}$H and $^{13}$ CNMR spectra were recorded on 300 MHz and 75 MHz Bruker instruments respectively. $^{1}$H chemical shifts are reported in ppm relative to CDCl3 δ 7.27. $^{13}$C chemical shifts are reported in ppm relative to $CDCL_3$ δ 77.0. FTIR was recorded on Nicolet IR 200 FTIR instrument (Thermo Scientific)

## Synthesis of brominated furanones (BF15)




1A 1B BF15

Step 1: 4-oxohexanoic acid (1A) (100 mg, 0.768 mmol) dissolved in dichloromethane ($CH_2Cl_2$) (2 mL) with 1 drop of HBr (40% v/v in water) was added to a flame dried round bottom flask under argon. Bromine (79 µL, 2 eq) was then added drop-wise. After complete addition of bromine the mixture was allowed to stir at room temperature for 1 h. After complete consumption of reactants (monitored by TLC, hexane:ethyl acetate, 2:1), the mixture was diluted with $CH_2Cl_2$ (4 mL), then successively washed with 1 N $Na_2S_2O_3$ (5 mL), brine (5 mL) and dried over anhydrous $Na_2SO_4$. The solvent was evaporated in *vacuo* to obtain a viscous yellow oily crude product 1B (dibromo-oxohexanoic acid, 0.150 g). This crude product was used in step 2 without any further purification.

Step 2: Step 1 crude product (1B) (0.150 g, 0.52 mmol) was dissolved in $CH_2Cl_2$ (1.5 mL) in an oven dried round bottom flask under argon. The reaction mixture was cooled to 0℃ and $P_4O_{10}$

(0.177 g, 0.62 mmol) was added. After stirring at 0℃ for 30 min the solution was allowed to warm to room temperature and then further heated at reflux for 1.5 h. The reaction mixture was then cooled to room temperature and transferred into a test tube and centrifuged (5 min, Metpath analytical centrifuge). The supernatant was decanted to yield a solid intermediate. The solid intermediate was dissolved in $CH_2Cl_2$ (2.0 mL) and transferred into an oven dried round bottom flask and cooled to 0℃. $Et_3N$ (0.79 µL, 1.5 mmol) was then added and the reaction mixture was allowed to stir at 0℃ for 1 h. The reaction mixture was then warmed to room temperature and later heated at reflux for 1 h. Further, the reaction mixture was allowed to cool to room temperature and aqueous $NH_4Cl$ (1.5 mL) was added. The aqueous phase was extracted with EtOAc ( 6 × 2 mL). The organic layers were combined and dried over anhydrous $Na_2SO_4$, and the solvent removed in *vacuo*. The impure compound was purified by column chromatography using a gradient of 20:1 to 10:1 of hexane:EtOAc to give title compound as a pale white solid (BF15) (yield – 0.065 g, 45 % (2 steps), $R_f$ – 0.51( 2:1, Hexane : EtOAc, mp 116-118℃). $^1$H NMR (300 MHz, $CDCl_3$): δ 7.61 (d, *J* = 4.8 Hz, 1H), 6.28 (d, *J* = 5.1 Hz, 1H), 2.53 (s, 3H). $^{13}$C NMR (75 MHz, $CDCl_3$): δ 168.9, 147.7, 138.1, 119.5, 108.7, 22.4. HRMS calcd for $(C_6H_5BrO_2)Na^+$ = 210.9365, found = 210.9365, difference < - 1.0 ppm (Positive ESI on Bruker 12 Tesla APEX-QE FTICR-MS with an Apollo II ion source). IR (KBr pellet): $\bar{\upsilon}$ = 3130, 3105, 1777, 1753, 1658, 1546, 1261, 1132, 902, 820, 712 $cm^{-1}$. The Z configuration of exo-cyclic double bond was proved by the NOE observed at H-3 (δ 7.61) while irradiating at H-6 (δ 2.53).

**Bacteria strains and growth media**

*E. coli* RP437 and *E. coli* RP437 (pRSH103) (constitutively expresses red fluorescence proteins) were provided by Dr. Dacheng Ren (Syracuse University). 10 µg/mL of tetracycline was added to maintain the plasmid pRSH103 in all the bacterial cultures. Plasmids p*lasI*-LVAgfp and p*rhlI*-LVAgfp were provided by Dr. Hiroaki Suga (The University of Tokyo). 300 µg/mL of carbenicillin was added to maintain the plasmids of PAO1-GFP, PAO1 (p*lasI*-LVAgfp), PAO1 (p*rhlI*-LVAgfp). All the bacterial strains were grown in Luria-Bertani (LB) medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl at 37 $^{o}$C. Single trial values reported for each experiment (Figures 1, 2, 4, 5, 6, S2) were similar to values obtained from replicate (3 or more) experiments.

**Construction of AY1297 (*E. coli* RP437 but Δ*sdiA*)**

Deletion of the *sdiA* gene in *E. coli* RP437 chromosome was achieved by P1 transduction. To prepare the donor P1 lysate, overnight culture of the donor strain (JW1901) was diluted 1:100 into 5 ml fresh LB medium. The culture was grown at 37°C aerobically with shaking at 220 rpm till the $OD_{600}$ reaches 0.1~0.2. 50 µl of 1 M $CaCl_2$ and 100 µl P1*vir* stock were added and the bacterial culture was continued to shake for ~3 hrs until cells lyse. After lysis, 1 drop of chloroform was added and the cell culture was subject to continuous shaking for 10 min. 1 ml of this suspension was removed and subject to centrifugation for 2 min at maximum speed (16000 rcf). The supernatant was collected and a few drops of chloroform were added. This P1 *vir* lysate was stored at 4°C.

To perform P1 transduction, overnight culture of the recipient strain (*E. coli* RP437) was diluted 1:100 into 5 ml fresh LB medium. The culture was grown at 37°C with shaking at 220 rpm till the $OD_{600}$ reaches 0.7. 50 µl of 1M $CaCl_2$ was added to the culture followed by continuous shaking for 15 min. Then 200 µl of the recipient cells were transferred to a sterile 1.5 ml eppendorf tube. P1 lysate from the donor strain prepared as above was added into the recipient cells with varying volumes. Following P1 *vir* infection of the recipient cells by shaking the tubes at 37°C for 20 min. 100 µl 30% citrate was added to stop the infection and 500 µl fresh LB medium was added for cell recovering. The culture was incubated for 1 hr at 37°C with aeration. The cells were then spun down, resuspended in 30 µL 30% citrate and 70 µL LB and spread on LB plates containing aitibiotic (kanamycin) and 4 mM citrate. Transductants were streaked twice on LB plates containing aitibiotic and 4 mM citrate to completely remove the P1*vir*. Single colonies were selected and positive transductants were screened by colony PCR and subsequently verified by DNA sequencing.

**Electrporation method for transferring plasmid**

Plasmid in *E.coli* RP437 (pRSH103) was extracted using Qiagen plasmid extraction kit and was then transferred to *E.coli* AY1297 (Δ*sdiA* mutant) by electroporation. Briefly, overnight culture of AY1297 was subcultured in 25 mL LB broth and was grown to reach the $OD_{600}$ of 0.5 to 0.8 by shaking the culture at 37 °C. Flask containing subculture was cooled on ice for 30 min. Cell pallet was obtained by centrifugation at 4500 rpm for 5 min. Supernatant was removed and cell pellet was resuspended in 20 mL ice-cold 10 % glycerol. Cells were centrifuged at 4500 rpm for 5 min. Supernatant was discarded and cell pellet was resuspended in 1 mL ice-cold 10 % glycerol. Cell suspension was transferred to a new 1.5 mL ice-cold microcentrifuge tube and spun down at 13,000 rpm for 30 sec. Supernatant was discarded and cell pellet was resuspended in 500 µL ice-cold 10 % glycerol. Last time cells were spun down at 13,000 rpm for 30 sec. After removal of the supernatant, cell pellet was resuspended in 100 µL ice-cold 10 % glycerol. 50 µL of the competent cell suspension along with 1 µL of plasmid DNA in TE buffer was transferred to the cold 0.1 cm electroporation cuvette. Electroporator was set to Ec1 and pulse was passed

through the cells. LB medium (1 mL) was immediately added to the electroporator cuvette and cell suspension was transferred to a sterile microcentrifuge tube and incubated for 1 h at 37 °C with shaking at 180 rpm. Cells were then spread on the LB plates supplemented with respective antibiotics and incubated over night at 37 °C. Plasmids p*lasI*-LVAgfp and p*rhlI*-LVAgfp were transferred to the PAO1 using the same electroporation method except that glycerol was replaced by sucrose in the preparation of competent cells.

**Stock solution of brominated furanones**

Stock solution of all brominated furanones (10 mM) were prepared in DMSO, sterilized by filtering through 0.2 µm syringe filter, and stored at -20 °C in sealed vials. Appropriate amount of DMSO was added to controls in all assays to eliminate solvent effect.

**Antimicrobial activities of BF against planktonic growth**

Optical density was measured using Biotek ELx800 ™ absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT) using Gen5™ data analysis software. The OD values were taken in sterile conditions at 0, 2, 4, 6, 8, 10, 12, and 24 h.

**Crystal violet based-biofilm inhibition assay**

Inhibitory effect of four brominated furanones on bioilm formation by *E.coli* and its sdiA knockout mutant was determined by biofilm inhibition assays. Overnight culture of both the E.coli strains was sub cultured to an $OD_{600}$ of 0.01 into the Luria-Bertani (LB) media.[1] 200 µL of the sub culture was aliquoted into the wells of 96 well plate PVC microtiter plate when it reached the $OD_{600}$ of 0.05. Stock solutions of all the test compounds were made in DMSO (biology grade, autoclaved). Predetermined concentrations of the test compounds were then added to the respective wells of 96 well plate containing sub culture. Sample plates were wrapped in GLAD Press n' Seal® followed by incubation under stationary conditions for 24 h at 37 °C. After incubation the media was discarded and the plates were washed with water. The sample plates were then stained with 200 µL of 0.1% aqueous solution of crystal violet (CV) and then incubated at ambient temperature for 30 min.[2] The CV stain was then discarded and the plates were washed with water. The remaining stain was solubilized with 200 µL of 95% ethanol. After the stain was dissolved (15 minutes) 100 µL of the solubilized CV was transferred from each well into the corresponding wells of a polystyrene microtiter dish. Biofilm inhibition was quantified by measuring the $OD_{540}$ of each well in which a negative control lane wherein no biofilm was formed served as a background and was subtracted out. The percent inhibition was calculated by the comparison of the $OD_{540}$

for biofilm grown in the absence of compound (control) versus biofilm grown in the presence of compound under identical conditions.

**Confocal scanning laser microscopy (CLSM)**

Biofilms were grown by inoculating the bacteria on 316 L stainless steel coupons (ca. 3/8 in. × 3/8 in., from McMaster-Carr) with or without BFs in a 24-well microtiterplate. Saran wrapped plate was then incubated at 37 $^{o}$C for 24 h without shaking. Each steel coupon was then washed gently by dipping into 0.85 w/v% aqueous NaCl solutions 3 times (fresh NaCl solution was used for each dipping) and then placed upside down on a microscope cover glass (50 x 24mm, No. 2, Fisher Scientific, Pittsburgh, PA). The biofilms were visualized using a Zeiss LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss, Jena, Germany). A 488 nm laser line was used to visualize biofilms formed by PAO1-GFP. Z-stacks from four randomly picked spots were taken for each steel coupon. Quantification of analysis of biomass, mean thickness, and surface area of the biofilms formed in the absence and presence of brominated furanones were obtained from fluorescence image using COMSTAT software. Values are normalized by that of the brominated furanone-free control.

**Visual semiquantification of biofilm on SAM patterned gold slides using scanning electron microscopy**

Semitransparent gold film of ~ 280 Å thickness was deposited onto the piranha-cleaned glass substrates with an electron beam evaporation system from Thermionics (Port Townsend, WA). About 80 Å of titanium was deposited before depositing gold to enhance the adhesion of gold on glass substrate (angle of deposition - 45°, rate of deposition - 0.2 Å/s). Gold slides were soaked in 2mM solution of (1-Mercapto-n-yl)-(triethylene glycols) for 15 h after microcontact printing 1-pentadecanethiol using (PDMS) stamp. Gold slides were taken out of solution, washed with ethanol and dried with nitrogen gas before placing them in bacterial cell culture.

Gold slides with circular patterns of 1-pentadecanethiol surrounded with (1-Mercapto-n-yl)-(triethylene glycols) SAM were placed in a 24 well plate containing cultures of either *E.coli* RP437 or *E.coli* AY1297 ($OD_{600}$ =0.05) with 200 µM BF8 for 24 h at 37 °C. Control experiments for both the bacterial strains were carried out under the same conditions but without BF8. 24 well plates were covered and wrapped in GLAD Press n' Seal® followed by incubation under stationary conditions for 24 h at 37 °C. Biofilm on gold slides was fixed with 4% paraformaldehyde solution, dehydrated with sequential soaking in solutions containing 10%, 30%, 50%, 70%, 90% ethanol, and pure ethanol, and sputter coated with gold-palladium alloy. Topographic features of the patterned biofilm on gold surface and effect of BF8 were examined under a JEOL JSM-5800LV Scanning Electron Microscope (SEM).

**Gene reporter assays for *las* and *rhl* quorum sensing systems of *P. aeruginosa*** [3]

GFP used in this study is LVAgfp. LVAgfp is a modified form of GFP which is unstable (estimated half-life of 40–60 min) as compared to native GFP (estimated half life of one day).[4] The short half life of LVAgfp makes the real time detection of gene expression possible. Since modified GFP (LVAgfp) has an excitation wavelength of 501 nm (excitation wavelength for wild type GFP is 395 nm) and emission wavelength of 511 nm (similar to wild type GFP).[5]

An overnight culture of *P.aeruginosa* PAO1/*plasI*-LVAgfp in LB broth (supplemented with 300 µg/mL carbenicillin) was grown from a single colony from an LB agar plate supplemented with 300 µg/mL carbenicillin. The overnight culture was diluted and grown to $OD_{600}$ of 0.1 in LB broth containing 300 µg/mL of carbenicillin. Bacteria culture (200 µL) was added to each well of a polystyrene 96-well microplate (Costar 3370) containing appropriate amount of brominated furanones or DMSO as a control. The plate was incubated at 37 °C for 24 h in a rotary shaking incubator (250 rpm). The culture from each well was then transferred to a flat-bottom, 96-well plate with black wall (µClear, Greiner-One 655096). The fluorescence and OD absorbance in each well was measured by Synergy 2 multi-mode microplate reader with Gen5 data analysis software. Background signals from LB broth were deducted from all samples. Fluorescence was measured at an excitation wavelength of 500 nm and an emission wavelength of 540 nm.

**Elastase B assay**

Elastase B activity in *P. aeruginosa* was measured as previously reported,[6] with some modifications: Bacteria were grown overnight in LB media (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl) at 37 $^{o}$C, diluted and grown to midlog phase, and subcultured to LB at $OD_{600}$ of 0.05. The culture was then aliquoated to test tubes containing brominated furanones to the desired final concentrations. The tubes were incubated for 24 h at 37 °C with shaking (250 rpm). Culture supernatants were recovered by centrifugation at 3000 rpm (Galaxy 5D centrifuge, VWR) for 10 min at room temperature and then passed through a 0.45 µm PVDF syringe filter (Santa Cruz Biotechnology). A 100 µL aliquot of the supernatant was added to 900 µL of Elastin-Congo red (ECR) buffer (100 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.2) containing 4.5 mg of Elastin-Congo red (Sigma-Aldrich) and incubated for 24 h at 37 $^{o}$C with shaking (250 rpm). After incubation, 0.2 mL of 0.12 M EDTA was added to stop the reaction. Insoluble ECR was removed by centrifugation and the absorbance of the supernatant ($OD_{490}$) was measured. Elastase B activity was represented by $OD_{490}$ of the samples treated with brominated furanones after subtracting the $OD_{490}$ of the samples incubated in the absence of cell culture filtrate.

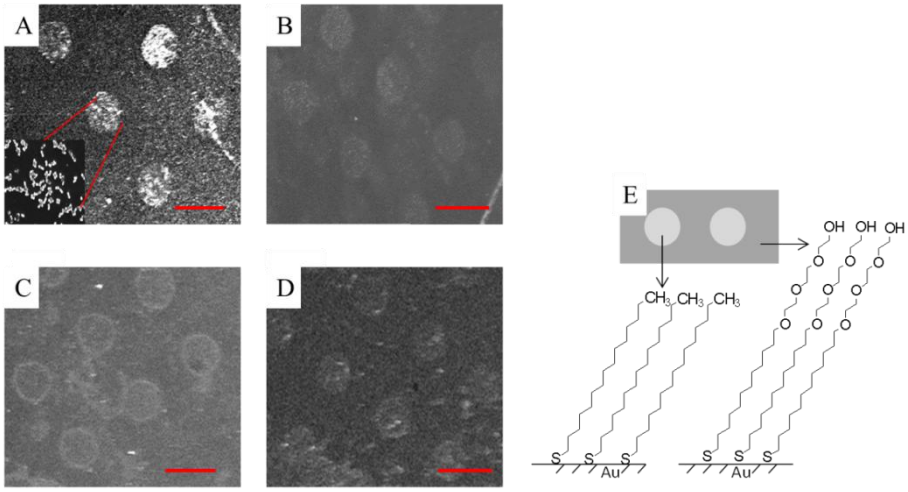



**Figure S1.** Scanning electron micrographs of biofilm formed by parent strain *E. coli* RP437 without brominated furanones (A) and with 200 µM **BF8** (C); and by *sdiA* knockout *E. coli* AY1297 without **BF** (B) and with 200 µM **BF8** (D) on patterned monolayer for 24 hr. Scale bar = 200 µm. The insert show the details of the *E. coli* in the pattern. Schematic representation of patterned SAMs of pentadecanethiol, $HS(CH_2)_{14}CH3$, surrounded by tri(ethylene glycol)-terminated alkanethiols, $HS(CH_2)_{11}(OCH_2CH_2)_3OH$, was shown to the right (E).

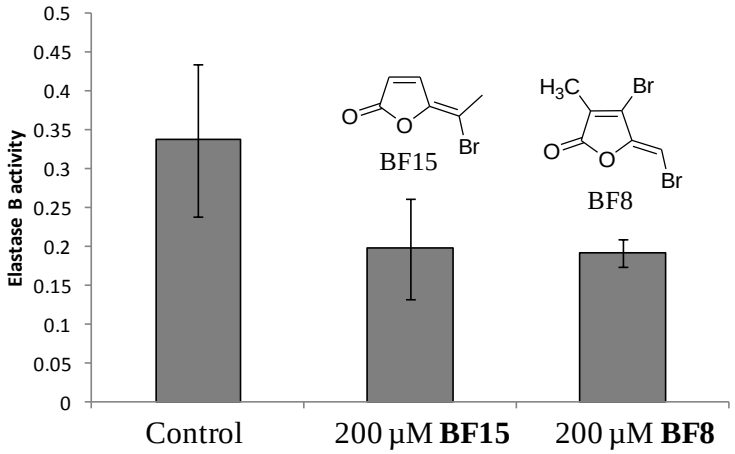
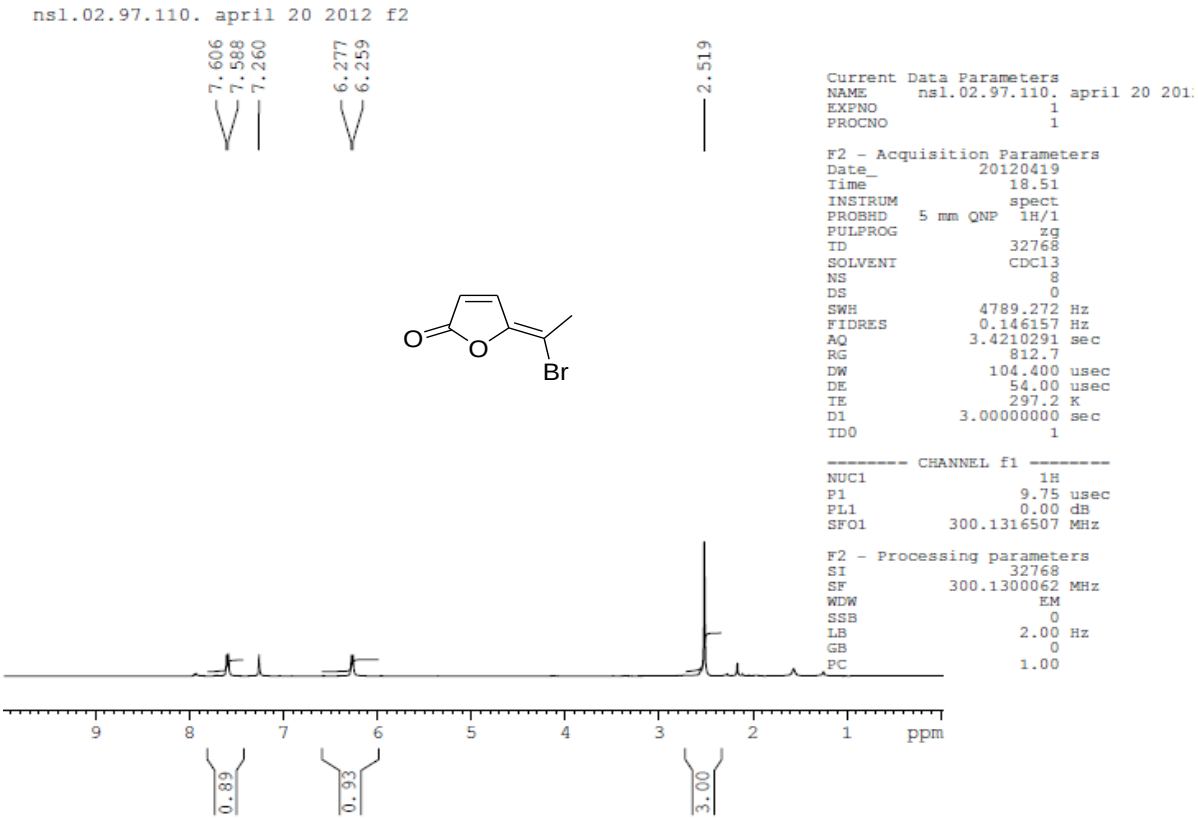
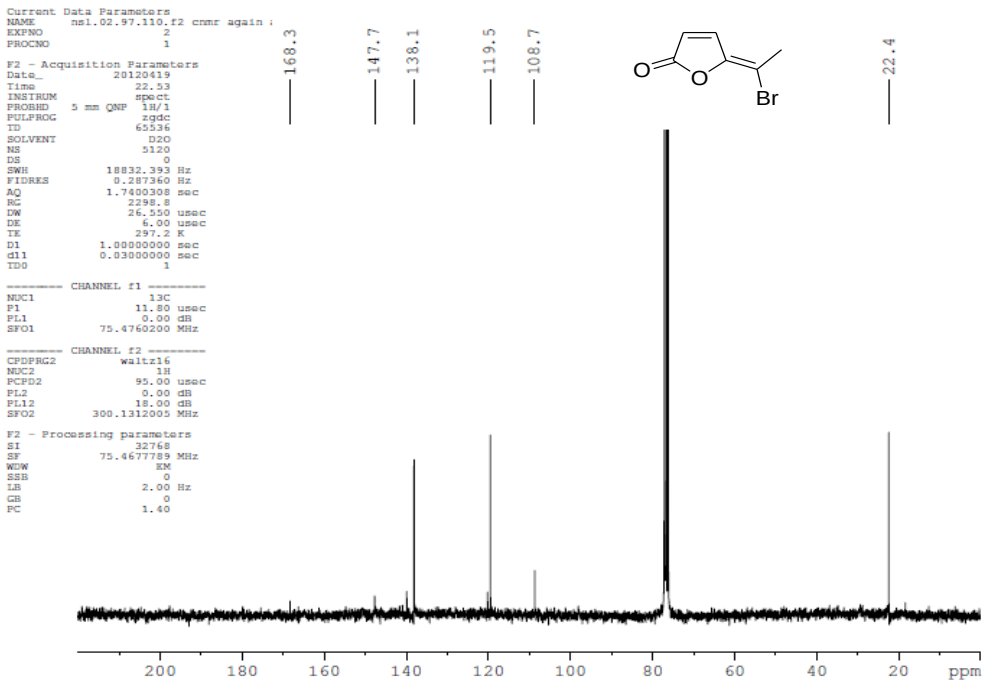
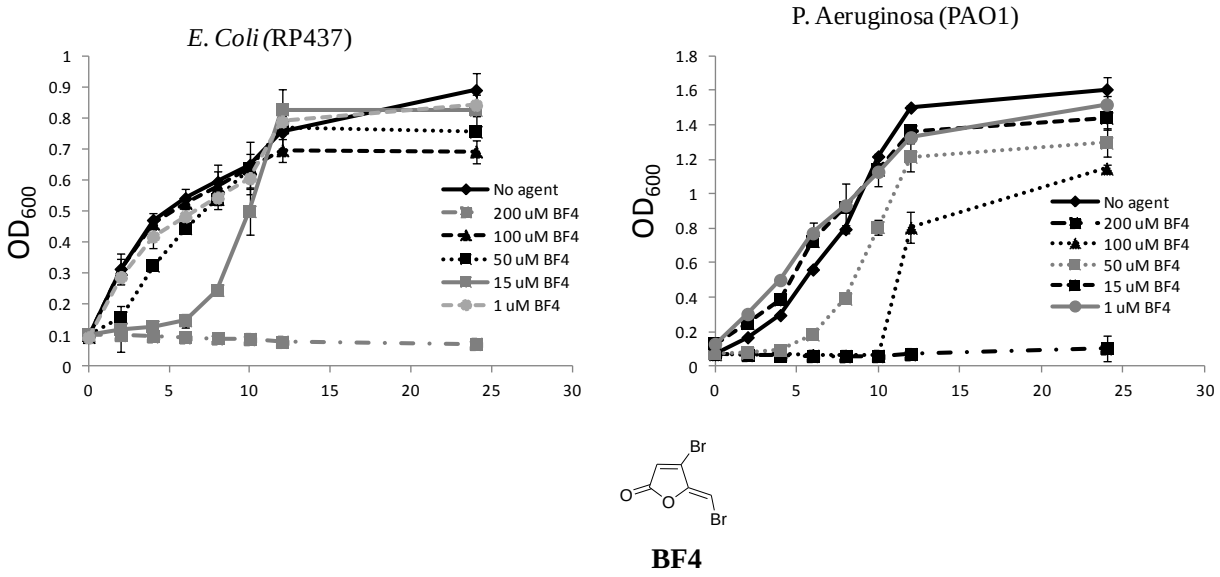



**Figure S2**. Elastase B activity produced by *P. aeruginosa* PAO1 in the absence (control) and presence of 200 µM of **BF15** or **BF8**. Error bar is standard error of the mean from 4 replicates.

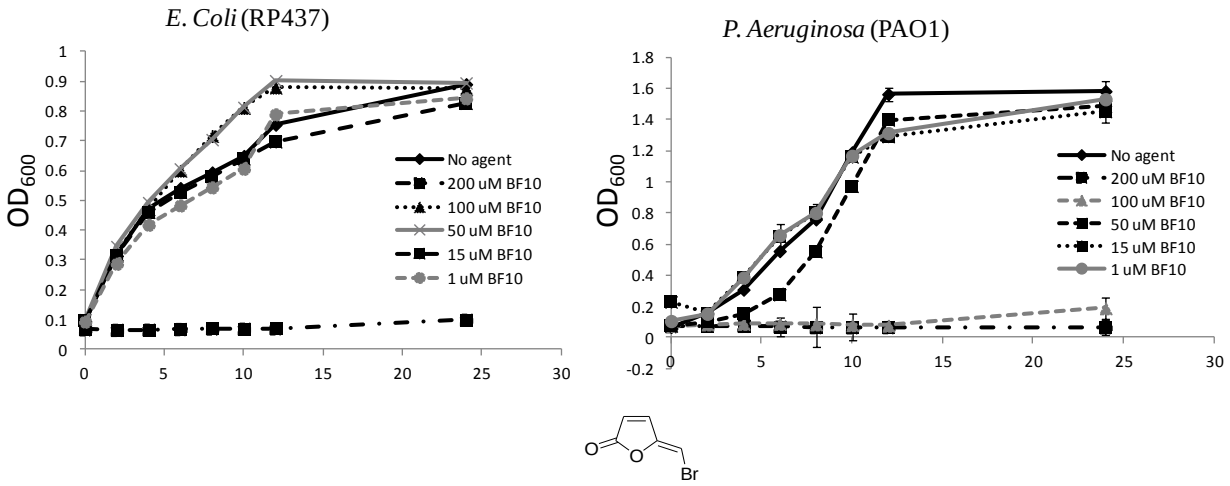

nsl.02.97.110. april 20 2012 f2
7.606
7.588
7.260
6.277
6.259
2.519
O
O
Br
Current Data Parameters
NAME nsl.02.97.110. april 20 201
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20120419
Time 18.51
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zg
TD 32768
SOLVENT CDCl3
NS 8
DS 0
SWH 4789.272 Hz
FIDRES 0.146157 Hz
AQ 3.4210291 sec
RG 812.7
DW 104.400 usec
DE 54.00 usec
TE 297.2 K
D1 3.00000000 sec
TD0 1
CHANNEL f1
NUC1 1H
P1 9.75 usec
PL1 0.00 dB
SFO1 300.1316507 MHz
F2 - Processing parameters
SI 32768
SF 300.1300062 MHz
WDW EM
SSB 0
LB 2.00 Hz
GB 0
PC 1.00
9
8
7
6
5
4
3
2
1
ppm
0.89
0.93
3.00


Current Data Parameters
NAME nsl.02.97.110.f2 cnmr again
EXPNO 2
PROCNO 1
F2 - Acquisition Parameters
Date_ 20120419
Time 22.53
INSTRUM spect
PROBHD 5 mm QNP 1H/1
PULPROG zgdc
TD 65536
SOLVENT D2O
NS 5120
DS 0
SWH 18832.393 Hz
FIDRES 0.287360 Hz
AQ 1.7400308 sec
RG 2298.8
DW 26.550 usec
DE 6.00 usec
TE 297.2 K
D1 1.00000000 sec
d11 0.03000000 sec
TD0 1
CHANNEL f1
NUC1 13C
P1 11.80 usec
PL1 0.00 dB
SFO1 75.4760200 MHz
CHANNEL f2
CPDPRG2 waltz16
NUC2 1H
PCPD2 95.00 usec
PL2 0.00 dB
PL12 18.00 dB
SFO2 300.1312005 MHz
F2 - Processing parameters
SI 32768
SF 75.4677789 MHz
WDW EM
SSB 0
LB 2.00 Hz
GB 0
PC 1.40
168.3
147.7
138.1
119.5
108.7
22.4
O
O
Br
200
180
160
140
120
100
80
60
40
20
ppm




**BF4**

**Figure S3.** Effect of brominated furanone **BF4** on the growth of wt *E. coli* (RP437) and wt *P. aeruginosa* PAO1. Error bar is standard error of the mean from 6 replicates.




**BF10**

**Figure S4.** Effect of brominated furanone **BF10** on the growth of wt *E. coli* (RP437) and wt *P. aeruginosa* PAO1. Error bar is standard error of the mean from 6 replicates.

**BF4** and **BF10** were tested for their biofilm inhibition activities at the concentrations which were not toxic to the growth of *E. coli* and *P. aeruginosa*.

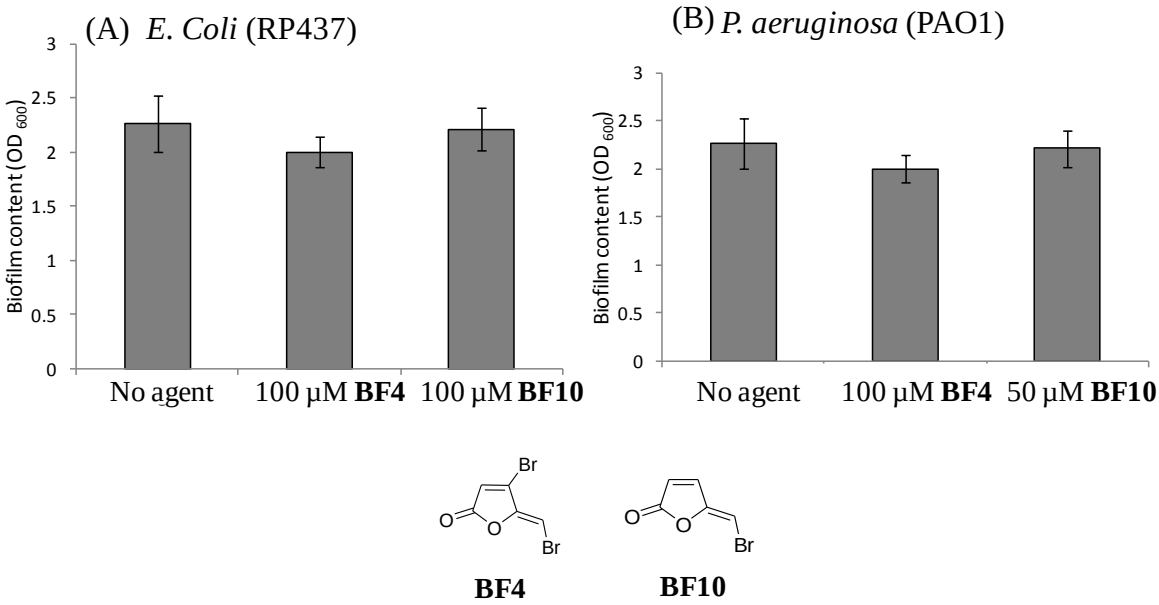



**Figure S5**. Biofilm inhibition of *E. coli* RP437 by 100 uM **BF4** and 100 uM **BF10** (A); biofilm inhibition of *P. aeruginosa* by 100 uM **BF4** and 50 uM **BF10**

## References

1. J. F. Sambrook, D. W. Russell and Editors, *Molecular cloning: A laboratory manual, third edition*2000.
2. H. Kanamaru and O. Yoshida, *Urological research*, 1989, **17**, 259-264.
3. R. Frei, A. S. Breitbach and H. E. Blackwell, *Angew. Chem. Int. Ed.*, 2012, **51**, 5226-5229.
4. J. B. Andersen, C. Sternberg, L. K. Poulsen, S. P. Bjørn, M. Givskov and S. Molin, *Applied and Environmental Microbiology*, 1998, **64**, 2240-2246.
5. R. Frei, A. S. Breitbach and H. E. Blackwell, *Angew. Chem., Int. Ed.*, 2012, **51**, 5226-5229, S5226/5221-S5226/5254.
6. J. P. Pearson, E. C. Pesci and B. H. Iglewski, *Journal of Bacteriology*, 1997, **179**, 5756-5767.

1. J. F. Sambrook, D. W. Russell and Editors, *Molecular cloning: A laboratory manual, third edition*2000.
2. H. Kanamaru and O. Yoshida, *Urological research*, 1989, **17**, 259-264.
3. R. Frei, A. S. Breitbach and H. E. Blackwell, *Angew. Chem. Int. Ed.*, 2012, **51**, 5226-5229.
4. J. B. Andersen, C. Sternberg, L. K. Poulsen, S. P. Bjørn, M. Givskov and S. Molin, *Applied and Environmental Microbiology*, 1998, **64**, 2240-2246.
5. R. Frei, A. S. Breitbach and H. E. Blackwell, *Angew. Chem., Int. Ed.*, 2012, **51**, 5226-5229, S5226/5221-S5226/5254.
6. J. P. Pearson, E. C. Pesci and B. H. Iglewski, *Journal of Bacteriology*, 1997, **179**, 5756-576