

Article

Halogen-furan-2(5H)-one derivatives decrease biofilm production in *Pseudomonas aeruginosa*.

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Abstract:

Clinical evidence has shown that bacterial infections are more difficult to eradicate when forming a biofilm aggregate than when are produced by bacteria in planktonic form. Therefore, compounds that inhibit biofilm formation could be used against severe infections. It has been reported that bromo 2-(5H) furanones inhibited biofilm formation by their anti-quorum sensing properties. To determine if the 2-(5H) furanone moiety is essential to induce inhibition of biofilm formation, we evaluated ten halogen 2-(5H) furanones derivatives previously synthesized. Besides evaluating the inhibition of biofilm formation, we assessed pyocyanin production, swarming motility, and transcription of essential QS genes: *rsaL*, *rhlA*, *pqsA* and *phz1* genes. Our results showed that although three bromo-furan-2(5H)-one-type derivatives (A1-A3) and two bromo-4-(phenylamino)-furan-2(5H)-one-type compounds (B2 and B6) inhibited the biofilm formation in both *P. aeruginosa* PA14 (reference) and PA64 (drug-resistant) strains only the furanones A1-A3 were efficient to inhibit QSS.

Keywords: quorum sensing; furanones; biofilm

1. Introduction

Multiple reports in the specialized literature indicate that bacterial multidrug resistance (MDR) is an emerging problem of universal dimensions' public health. To find new therapies against these resistance microorganisms, the World Health Organization (WHO) announced a list of priority microbes that is urgent to prevent due to high pathogenicity. One of these is *Pseudomonas aeruginosa* [1].

P. aeruginosa is the most common Gram-negative pathogen causing nosocomial pneumonia. For example, in Mexico, from 2007 to 2013, forty-six nosocomial infections produced by *P. aeruginosa* were detected at a pediatric hospital with a significant mortality rate (17.39%) [2].

The pathogenicity of *P. aeruginosa* can be explained by the production of several virulence factors such as biofilm formation, swarming motility, pyocyanin, and rhamnolipids production; these factors are coordinated by quorum sensing systems (QSS) which

depend on population density. Nowadays, three well-organized and hierarchy established quorum sensing pathways have been reported in *P. aeruginosa*, two LuxI/R-type systems (LasI/R and RhII/R) and pseudomonas quinolone signal (PQS). It is generally accepted that LasI/R is at the top of the hierarchy in the QSS cascade except in conditions of limiting phosphate concentrations [3]. QSS is characterized by the conjugation of gene regulation and the synthesis of diffusible chemical signals called autoinducers (AIs). *P. aeruginosa* synthesized acyl-homoserine lactone (AHL) derivatives as AIs. When the cell density rises, reaching a certain concentration threshold, AI concentration increases, allowing the interaction between AHLs and the LuxR-type receptor. This complex binds to the corresponding promoter region and turn on the expression of several genes encoding diverse virulence factors. Thus, interrupting *P. aeruginosa* QSS could decrease bacterial virulence. It could become a new therapy to fight MDR bacteria. Furthermore, because this therapy does not exert a direct selection pressure, therefore, it may have a low probability of developing resistance [4].

It has been published that those infections caused by *P. aeruginosa* in its single, free-floating planktonic form can be eradicated with adequate antibiotic treatment. However, the infection is complicated to eradicate when this bacterium forms part of an aggregate known as biofilm [5]. Secreted proteins, polysaccharides, nucleic acids, and cellular debris are fundamental parts of bacterial biofilms. This aggregate could be attached to abiotic or biotic surfaces. The immersion of the bacteria in the biofilm gives it a better resistance against the antibiotics and the defenses of the infected host's immune system. The relationship between QSS and biofilm formation arises because *lasI*, which encodes for the synthetase responsible for 3O-C12-HSL production, is critical for mature biofilm formation in *P. aeruginosa* PAO1 [6].

Several compounds have been found which disrupt the quorum-sensing system. Historically, the first inhibitors of QSS have been synthesized using the 2-furanone structural template, the basic unit of acylated homoserine lactones (AHLs). It has been reported that AHLs controls both the virulence genes of bacteria and the compartment of host cells. For example, 3-oxo-C12 HSL, besides its QS regulation, is also responsible for the immunomodulation of the host immune system [7]. Among the anti-QSS furanones, C30 (5Z-4-bromo-5-(bromomethylidene) furan-2-one) is one of the more relevant [8,9]. C30 possess a compelling profile against several virulence factors in such a way that it has become the usually reference compound in evaluating new anti-QSS compounds [8]. Besides, *in vivo* studies, C30 diminishes the bacterial population in pulmonary infection in mice [9].

On the other hand, recently, there has been an interest in evaluating anticancer drugs as antibacterial compounds [10]. So, we decided to evaluate two series of cytotoxic halogen-furan-2[5H]-one derivative [11] as an inhibitor of *Pseudomonas aeruginosa* QSS. The structural diversity of the different derivatives, together with the results of their biological activities, will allow the finding of critical structural components for the inhibition of virulence factors.

2. Results

2.1 Synthesized furanones.

Three bromofuran-2(5H)-one-type derivatives (A1-A3), and seven bromo-4-(phenyl-amino)-furan-2(5H)-one-type compounds (B1-B7), were synthesized as previously reported [11] and evaluated for their anti-QSS activities (Figure 1).

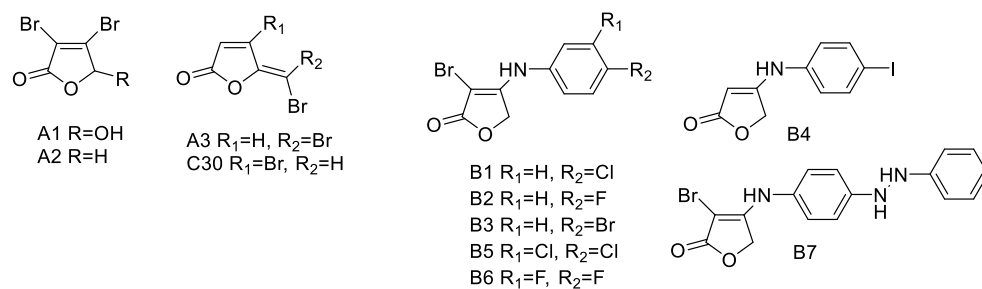


Fig. 1. The A1-A3 and B1-B7 furanone structures. A= bromofuran-2(5H)-one-type derivatives. B= bromo-4-(phenylamino)-furan-2(5H)-one-type compounds.

2.1 Growth of PA14 and INP64 *P. aeruginosa* strains in the presence of furanones

Our results showed that, in PA14 bacteria growth, is slightly diminished by A3, although it was not statically significant. In the PA64 strain, the furanones did not inhibit the growth of the bacteria (data not shown). Furanones A1-A3 were evaluated at 100 μ M, while B1-B7 furanones were assessed at 1 mM.

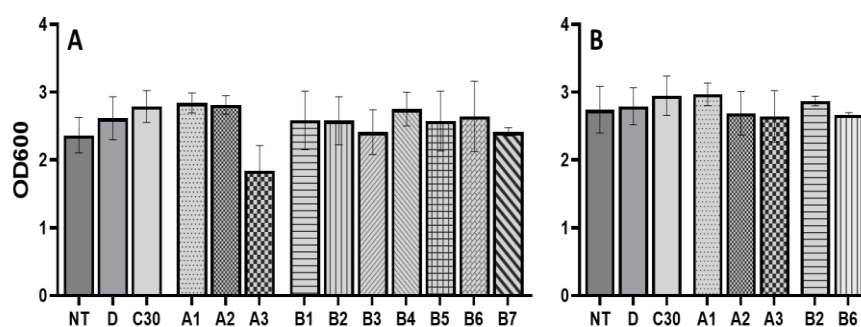
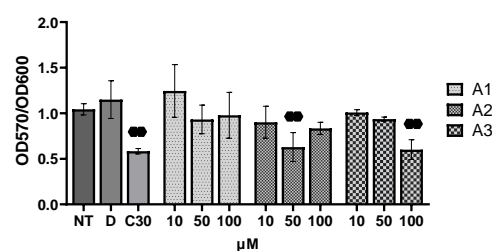


Fig 2. A. Growing of *P. aeruginosa* PA14 in A1-A3 or B1-B7 presence. B. Growing of *P. aeruginosa* INP64 in the A1-A3 or B2 and B6 presence. Furanones were added at the beginning of culture at different concentrations. Cultures were incubated for 18 h.

2.2 Biofilm inhibition.

Our findings showed that in *P. aeruginosa* PA14 strain, A1 at 50 and 100 μ M doses decreased biofilm formation. However, it was not statistically significant. Unexpectedly, furanone A2 became more active at 50 μ M dose than at 100 μ M dose, achieving inhibition of approximately 50% in biofilm formation. A3 reduces the biofilm by 50% at 100 μ M dose. Concerning the derivatives of the bromo-4-(phenylamino)-furan-2 (5H)-one type, the furanones B2 and B6 showed biofilm inhibition from the 250 μ M dose. It should be noted that the inhibitory activities of type B furanone are shown at higher doses than those shown for type A (Figure 3).



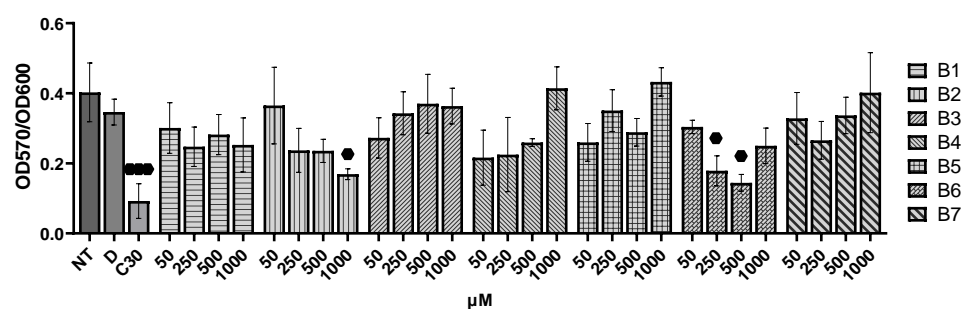


Figure 3. Effect of furanones A1-A3 and B1-B7 on the production of biofilms of *P. aeruginosa* PA14. The bacteria were incubated for 18 h at 37 °C and 200 rpm in the presence of furanones. Subsequently, the biofilm was stained and quantified by spectrophotometry. NT: no treatment, D: dimethyl sulfoxide, C30: C30 furanone. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$.

On the other hand, our results indicated that in *P. aeruginosa* INP64, drug-resistant strain, A1 and A3 inhibit biofilm formation from 10 μM dose, while furanone A2 did it from 50 μM (Figure 4a). In contrast, B2 and B6 inhibit biofilm formation from 500 μM (Figure 4b). Again, note the difference between the small doses of active furanones of type A and those of type B active furanones.

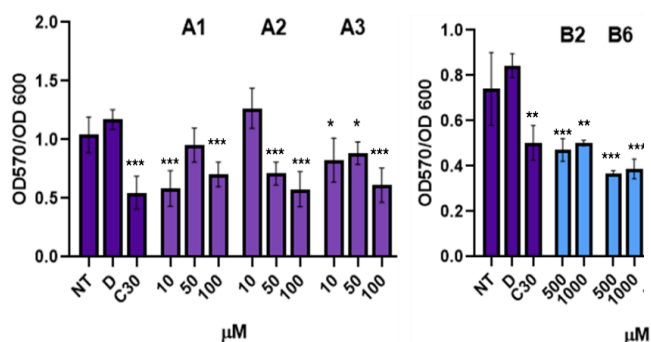


Figure 4. Effect of furanones A1-A3 (Fig. 4a) as well as furanones B2 and B6, (Fig. 4b) on the biofilm production in the *P. aeruginosa* INP64 strain (resistant drug).

2.3 Pyocyanin production.

The A1-A3 furanones inhibited the pyocyanin production on the *P. aeruginosa* PA14 strain. In general, these furanones showed a decrease from 35% at 10 μM to 50% at 100 μM doses. On the other hand, group B furanones did not inhibit the pyocyanin generation (data not shown) (Fig. 5).

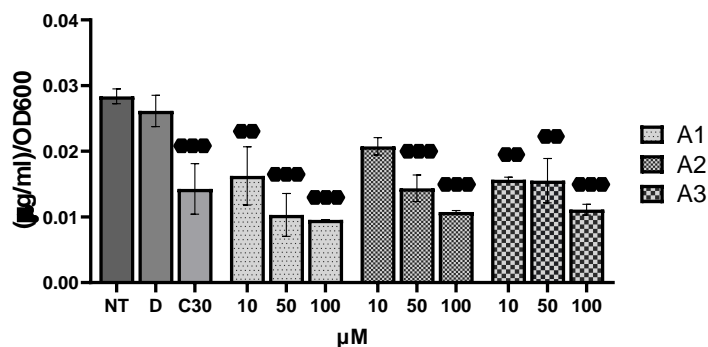


Fig 5. Effect of furanones over the pyocyanin production in *P. aeruginosa* PA14 strain. NT: not treatment, D: dimethyl sulfoxide, C30: furanone C30. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$.

In the INP64 strain, the furanones A1-A3 decreased the pyocyanin production from 10 to 100 μM doses (Fig. 6). In this strain, the furanones B type did not reduce the pyocyanin production (data not shown).

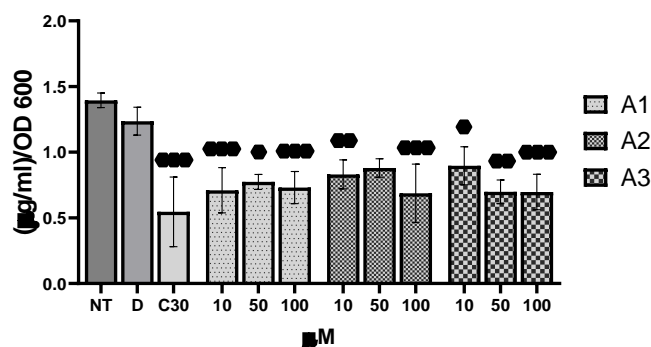


Figure 6. Effect of A1-A3 furanones over the pyocyanin production in *P. aeruginosa* INP64 strain. NT: not treatment, D: dimethyl sulfoxide, C30: furanone C30. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$.

2.4 Swarming motility

In the PA14 strain A1 decrease swarming motility (SM) from 35% at 10 μM to 50% at 50 and 100 μM doses, while furanone A2 only diminishes SM at 100 μM dose. Furanone A3 showed dose-dependent SM inhibition, 70% at 10 μM dose, 80% at 50 μM dose, while at 100 μM dose, it decreases 90% (Fig 5a). On the other hand, in the INP64 strain, A1 only showed activity at 100 μM , while A2 and A3 showed dose-dependent activity with almost 100% inhibition of SM at 100 μM doses. The furanones of the B group did not affect this factor (Fig. 5b).

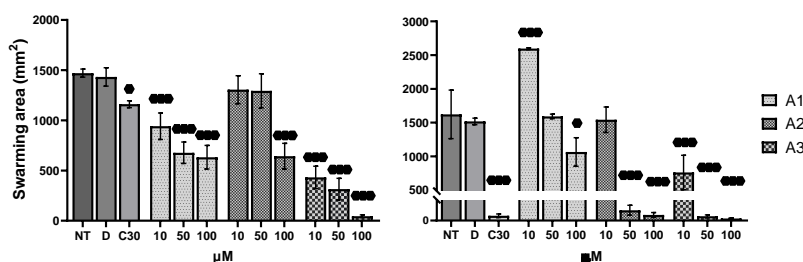


Fig 5. The effect of A1-A3 furanones on swarming motility in *P. aeruginosa* PA14 strain (Fig. 5a) and INP64 strain. Bacteria were incubated in BM2 medium in plates at 37°C in the presence of furanones for 18 h. Later, the swarming motility was quantified by

Kodiak software. NT: not treatment, D: dimethyl sulfoxide, C30: furanone C30. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$.

2.5 Rhamnolipids production

The results showed that in the PA14 strain, A1, A2 and A3 at 100 μM , while B2 and B6 at 1 mM decrease rhamnolipids production. However, in the INP64 strain, there have not been results because in cultures of 24 h rhamnolipids have not been detected.

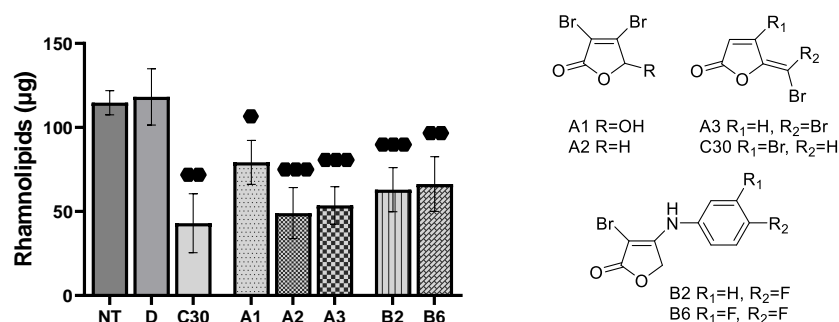


Figure 6. Effect of different furanones on rhamnolipids production of *P. aeruginosa* PA14 strain. Bacteria were incubated in liquid LB medium at 37°C in the presence of furanones for 18 h. Later, the rhamnolipids production was quantified with orcinol. NT: not treatment, D: dimethyl sulfoxide, C30: furanone C30. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$.

2.6 Effect of furanones on regulators of QSS.

The PA14 strain with plasmids carrying a transcriptional fusion of β -gal with *rsaL*, *rhlA*, *pqsA* and *phz1* genes was donated by Dr Gloria Soberón. The data shows that A1 decreases the transcription of *rsaL*; however, A2 and A3 did not have activity on this gene. Nevertheless, the A1-A3 furanones showed a transcriptional decrease on *rhlA*, *pqsA* and *phz1* genes. Plasmid vector 220 was used as control (Fig. 7). The furanones B type did not show activity.

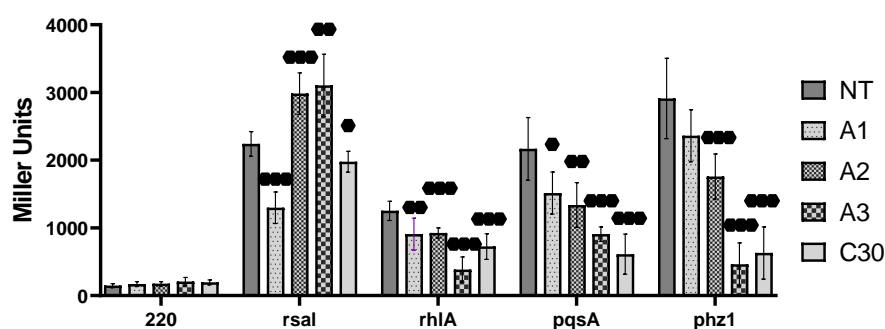


Figure 7. Transcriptional decrease on *rsaL*, *rhlA*, *pqsA* and *phz1* genes by the A1-A3 furanones.

DISCUSSION.

The acyl-homoserine lactones (AHLs) that function as autoinducers (AI) in *P. aeruginosa* QSS have a 2-furanone moiety as their base structure. Considering the above, C30

and C56, two potent QSS inhibitors, have been synthesized, which in addition to having 2-furanone fragments, both have bromine atoms. In this paper, we showed the results of anti-QSS activity of two series of halogen 2-furanone derivatives. Series A: three bromo-furan-2(5H)-one-type derivatives (A1-A3) and series B: seven bromo-4-(phenylamino)-furan-2(5H)-one-type (B1-B7). All compared to C30 anti-QSS activity.

Our results indicate that A1, A2, A3, B2 and B6 inhibited the biofilm formation in both *P. aeruginosa* PA14 (reference) and INP64 (drug-resistant) strains. However, A1-A3 inhibited biofilm production, in both *P. aeruginosa* strains, with one order of magnitude less of concentration than B2 and B6, furanones. For example, A3, in the PA 14 reference strain, decreases biofilm formation 50% at 100 mM, while B2 inhibits approximately 60% at a dose of 1000 μ M. The results showed that in the PA14 strain, A1, A2, A3 at 100 μ M dose decrease rhamnolipids production. At the same time, B2 and B6 decreased it at 1 mM doses.

One point to consider is that furanones of B series did not inhibit pyocyanin production and swarming motility in both *P. aeruginosa* strains evaluated. These results suggest that furanones of type B inhibit biofilm formation and rhamnolipids production in the strains of *P. aeruginosa* by a different mechanism than QSS, which could explain the high doses used compared to those of A1-A3. This proposal is supported by the fact that the furanones of the B series had no activity on the transcription of the *rsaL*, *rhlA*, *pqsA* and *phz* genes, all involved in QSS.

A feature that attracts attention is that the A2 and A3 did not reduce the transcription of *rsaL* as A1 did. The *rsaL* gene is located between the *lasR* and *lasI* genes. Overexpression of *rsaL* in *P. aeruginosa* PA01 resulted in a 20-fold reduction of 3-O-C12-HSL production [12]. Conversely, 3-O-C12-HSL production is dramatically enhanced in a *rsaL* mutant. Unexpectedly, the *rsaL* is among the genes most strongly activated by the LasR-3-OC12-HSL complex [13]. Therefore, the *rsaL* gene could be considered a repressor of QSS. It has been reported that due to their vicinity RsaL and LasR binding sites, these two proteins compete for attaching to the *rsaL-lasI* bidirectional promoter. Consequently, LasR would triggers *lasI* transcription by outcompeting the transcriptional repressor RsaL at high cell densities when the 3OC12-HSL levels reach a certain threshold. The fact that *rsaL* expression itself is also dependent upon LasR adds further complexity to this regulatory mechanism.

In our case, the administration of A1-A3 inhibited biofilm formation and reduced pyocyanin production and swarming both virulence factors governed by QSS. These results indicated that the inhibition of *rsaL* gene transcription by A1 did not affect the expression of virulence factors.

Considering that biofilm formation is a multifactorial development involving processes, such as producing exopolysaccharides that QS does not regulate, furanones B1 and B6 may affect biofilm production without affecting the QS response [14]. In the case of the rhamnolipids production, the *rhlA* promoter expression is dependent on *RhlR*, and if the expression of this promoter is not decreased, but the production of rhamnolipids does a decrease in the presence of furanones B1-B7, it may be that because these compounds affected the manufacture of the rhamnolipids by other mechanism [15]

Even though all the synthesized derivatives share a base system of 2-(5H) furanone core and halogen atoms, the reactivity that gives them the size and conformation of their structures makes only A1-A3 show anti-QSS.

4. Materials and Methods

4.1 Strains and compounds.

The PA14 strain were kindly provided by Dr. Ausubel and were used as reference strain and INP64 strain was provided by Dr. Rafael Coria Jimenez of National Institute of Pediatrics. The bacteria were kept in glycerol and frozen at -70°C. The furanones were

previously synthesized as described [11] (Table 1). The furanones were dissolved in DMSO at a stock of 1 M and stored at -20°C.

4.2 Bacteria growth in the presence of furanones.

Overnight of bacteria was diluted in fresh LB medium at OD₆₀₀= 0.05. The furanones were added, at beginning of culture, in different concentrations: 10, 50, 100, 250, 500 and 1000 µM. The cultures were incubated for 18 h at 37°C/200 rpm. At 18 h samples were taken to measure growth on a spectrophotometer at OD 600.

4.3 *P. aeruginosa* pyocyanin production.

Overnight of bacteria was diluted in 5 ml of fresh LB medium at OD 600=0.05. The furanones were added at beginning of culture in different concentrations: 10, 50, 100, 250, 500 and 1000 µM. The cultures were incubated for 18 at 37°C/200 rpm. Later, pyocyanin was extracted and quantified as reported previously [16]. Briefly, one milliliter of culture was centrifuged, and supernatant was used for extraction. Pyocyanin was extracted with chloroform and HCl and quantified on a spectrophotometer OD520 using HCl 0.2 N as blank.

4.4 *P. aeruginosa* biofilm formation.

Overnight of bacteria was diluted in 2 ml of fresh LB medium at OD₆₀₀ =0.05 in tubes and incubated for 18 h at 37°C/200rpm. Later, the biofilm formation was quantified as reported previously [17] with slightly modifications. Briefly, the culture was discarded, and the tube was washed 2 times with water to remove the remaining planktonic cells. Subsequently 2.5 ml of violet crystal dye at 0.1% was added and left for 15 min. The excess of dye was removed rinsing with distilled water. Later, 3 ml of ethanol 70% was added and left for 15 min. The violet crystal concentration was measured in a spectrophotometer OD570 using EtOH 70% as blank.

4.5 Swarming motility.

Overnight of bacteria was diluted in 1 ml of fresh LB medium at OD₆₀₀ =0.05 and were inoculated on BM2 medium plates (K₂HPO₄ 62 mM, MgSO₄ 2 mM, FeSO₄ 10 µM, glucose 0.4%, casamino acids 0.1% and agar 0.5%) [18]. Furanones A1, A2 y A3 were separately added at 100 µM and B2, B6, C1 and C7 at 1000 µM. The plates were incubated at 37°C during 18 h. Later, the plates were photographed in Gel Logic 200 Imaging System by Kodak. The measure of swarming area was performed in Molecular Imaging Software v. 4.0.3 by Kodak.

4.6 Rhamnolipids production.

The PA14 strain was incubated in liquid LB medium. The rhamnolipids production was quantified as reported previously [19] with some modifications. Briefly, cultures were centrifuged to remove bacteria and supernatant was used to extract rhamnolipids. The extraction was performed with diethyl ether (1:1) twice, let dry at room temperature. Rhamnolipids were dissolved in 100 µl of distilled water and added 900 µl of orcinol 1.6% (dissolved in 60% H₂SO₄). A standard curve of rhamnose was used to quantify rhamnolipids (1µg of rhamnose equals to 2.5 µg of rhamnolipids).

4.7 β-galactosidase assay.

Plasmids had the genes *rhlA*, *rsaL*, *pqsA*, and *phz1* fused to the *lacZ* reporting gene were incorporated into the PA14 strain by electroporation. The transformed bacteria were selected with X-gal and tetracycline or chloramphenicol for *rhlA*, *rsaL*, *pqsA* and *phz1* genes. The strains were grown in LB medium in the presence of furanones at 100 µM for 4 h. Later, a sample was taken to quantify the activity of β-galactosidase as reported [20].

4.8 Statistical analyses.

All assays were done at least in triplicate. The data were analyzed by One-way ANOVA followed by Dunnett's multiple comparisons test performed in GraphPad Prism software version 8.1.2 for Windows.

5. Conclusions

Three bromofuran-2(5H)-one-type derivatives (A1-A3) were able to inhibit the production of virulence factors and biofilm formation by anti-QSS mechanism and were effective against a reference strain and a drug-resistant *P. aeruginosa* strain. None of these was found to be toxic to the bacterium. However, B2 and B6 derivatives of bromo-4-(phenylamino)-furan-2(5H)-one-type inhibited, at high doses compared with those showed by A1-A, the biofilm formation and rhamnolipids production in *P. aeruginosa* but not QS regulatory genes. These results suggest that B2 and B6 inhibited the rhamnolipids production and biofilm formation by a mechanism independent of QS. Even though mucobromic acid (A1) has been used to obtain compounds with anti-QSS activity, as far as we know, this is the first time that A1 has been evaluated as an inhibitor of the quorum-sensing system [21, 22]

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

Author Contributions: “Conceptualization, M.M.V.; R.G.C. and N.A.A.R.; methodology, N.A.A.R. and R.G.C.; validation, I.C.J. formal analysis, M.M.V.; writing—original draft preparation, N.A.A.R. and R.G.C. writing—review and editing, M.M.V.; project administration, M.M.V.; funding acquisition, M.M.V. All authors have read and agreed to the published version of the manuscript

Funding: This research was funded by DGAPA-UNAM, grant number PAPITT IN211819.

Acknowledgments: Nelly Araceli Aburto Rodríguez is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and has received CONACyT fellowship 288715. We greatly acknowledge the technical support of PhD Toshiko Takahashi Iñiguez.

Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the compounds are available from the authors.

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