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NEW QUINOLINE ANDANTHRANILIC ACID DERIVATIVES ASPOTENTIAL QUORUM SENSING INHIBITORS

Gawade Baliram*

M. Pharmacy, Plot, No 4 Walchand Bapuji Nagar, Mohadi Up Nagar Dhule, Maharashtra, India.

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***Corresponding Author**

Gawade Baliram

M. Pharmacy, Plot, No 4 Walchand Bapuji Nagar, Mohadi Up Nagar Dhule, Maharashtra, India.

ABSTRACT

A promising approach that has been incorporated to fight bacterial pathogens are those which interfere with the Quorum sensing (QS), a main signal transduction system. Here, we have described new hybrids for the inhibition of QS mediated by PQS of P. aeruginosa one of the multidrug-resistant and highly virulent pathogens that desperately need new antibacterial therapeutic approaches. The synthesis of 12 compounds we performed according to the standard techniques with the help of which we established the connection between halogen- substituted anthranilic acids and 4-(2-aminoethyl/4-aminobuthyl) amino-7-chloroquinoline connected through 1,3,4-oxadiazole. The antibiofilm activities were firstly tested by Gram- negative Photobacterium violaceum-based reporter that distinguished compounds 15–19 and 23 as the compounds exerting the highest anti-QS and minimal bactericidal effects at the same time in one experiment. Subsequently, these five compounds were tested on P. aeruginosa PAO1 for their antibiofilm potential factors such as; their effectiveness in stopping biofilm formation, their effectiveness in eliminating prior biofilm formation as well as effectiveness in quenching bacterial virulence employing pyocyanin as a biomarker. The highest activities of antibiofilm effect of Compound 15 were reducing biofilm formation in 48 h by 49% and pre formed biofilm masses by 25%, respectively. In contrast, the compound 23 demonstrated the maximum ant virulence property and almost eliminated the pyocyanin production by more than70%. Therefore, the present investigation demonstrates that among the synthesized 1, 3, 4- oxadiazoles 15 and 23 could be further explored for antipseudomonal activity. Furthermore, more advanced interactive QS systems should be taken into account to have maximum anti-QSeffect against this clinically relevant species.

INTRODUCTION

The types of bacteria causing diseases possess intricate plans through the mechanism of pathogenesis and host adaptation to improve the prospects of existence and resistance to antibiotics. Antibiotics kill or inhibit bacterial species, but they also act selectively, thus promoting the evolution of articles' resistance. As such, this presents a problem in attaining a favorable therapeutic process for the infection.[1] Using the findings obtained from 204 countries, the approximate global prevalence rate of this condition was pegged at 4. It was indicated that bacterial AMR was responsible for 95 million deaths in 2019, out of which 1. Another cross-sectional study according to another source estimates that, the bacteria AMR isresponsible for about 27 million deaths.[2] Exponential increase in AMR collectively with the sharp decrease in antibacterial drug innovation and discovery is considered one of the biggest challenges to health in the twenty-first century.[3] This calls for the need to develop new classes of amicrobial agents or enhance the efficiency of old-fashioned antibiotic-based treatments.

Pseudomonas aeruginosa is a Gram- negative organism, an opportunistic pathogen, frequent in immuno-compromised patients, and the major source of morbidity/ mortality in cystic fibrosis.[4] This species has been identified as one of the most difficult-to-treat resistant species; with resistance being documented to piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, meropenem, imipenem-cilastatin, ciprofloxin and levofloxacin.[5] Thus, the pathogen has not only "acquired" genetic resistance to antibiotics of the Pseudomonas species, but can also enhance antibiotic tolerance by inhabiting a number of medical products and forming highly resistant biofilms in the conditions of a CF lung.[6] The biofilms are societies of multiple cells that find themselves enclosed within a protective layer they develop to shield them from dangers such as antibiotic threats. In bacteria, the biofilm formation and the production of many virulence factors are regulated through QS or the process through which cells are able to alter their behavior in a cell-density dependent manner. The QS signaling system requires the synthesis, secretion, and recognition cross the group of the autoinducers (AIs). AIs pile up in the environment and

once its concentration crosses some certain level; the QS responsible for biofilm formation and expression of virulence genes is triggered.[7] Inhibition of the central QS signaling system is regarded as one of the most effective and perspective strategies for pathogens control and subsequent minimalization; it includes P. aeruginosa, as with most bacteria, because an ideal anti-QS compound is believed to put lesser pressure on the pathogenthan traditional antibacterial agents.[8]

M. Pseudomonas aeruginosa PAO1, which is a chloramphenicol resistant derivative of the PAO wound isolate, is used in several studies, this strain utilizes four integrated QS circuits (Las-, Rhl-, PQS- and IQS- QS) for biofilm maturation and gene expression related to virulence factors such as elastase, rhamnolipids, and pyocyanin.[9-12] Some substances which hinder the formation of the biofilm of P. aeruginosa or other agents which can eliminate the existing biofilms may assist in managing of chronic and recurrent infections from this species. In this regard, several classes of quinoline derivatives have been explored for their prospects to act as the anti-QS compound that can inhibit biofilm formation due to their structural resemblance with AI, namely 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS), and the precursor 2-heptyl- 4(1H)-quinolone (HHQ) that is produced by P. Out of these, the 4-amino-7-chloroquinoline moiety appeared to be highly effective as an agent against QS/biofilm that affects MDR bacteria.[13–17] Also, it was reported that anthranilic acid is important for PQS biosynthesis[18], and halogenated anthranilic acids and their derivatives are known to inhibit PQS biosynthesis.[19–23]

The present study forms a sequence of the previous works done by the author on anti-QS agents against Gram-negative biofilm-forming pathogens.[24] Here, we describe a new series of hybrid molecules containing the quinoline and anthranilic acid cores (Figure 1). For compound design, molecular hybridization was used, this involves covalent connection of two or more biological active frameworks to be in a single molecule with better properties. It was to achieve together the usable features from different molecules to get a new compound for better potency or having more range of action. The benefits of such compounds include increased activity, better pharmacokinetic profile and lower propensity to deinking, resistance, side effects, and interactions with other agents.[25] Therefore, this method can be a valuable method to advance in new approaches to the management of bacterial infections and overcome problems connected to antibiotic resistance. We have applied this approach to isolate a harmane derived antimalarial compound library.[26-31] Moreover, the outer concept known as hybridization has been used prior to the research work of the selected number of research groups within the subject.[32–34] In this study, the generated hybrid compounds were first pre-screened for their anti-QS/biofilm and bactericidal activities using a Gram-negative Chromo-bacterium violaceum-based microscale screening platform as it enables to differentiate the compounds with genuine anti-QS effect from the bactericidal ones in the one and the same experiment.[24, 35–39] C. violaceum is also an opportunist pathogen which can present severe and sometimes fatal pathological conditions in both immune-depressed and immunocompetent patients[40] and, thus, it appears suitable for drug screening purposes. Here, we checked the most effective anti QS compounds for antibiofilm and ant virulence properties using PAO1, with four different QS systems interconnected. Based on our observations, it can be concluded that disruption of the PQS biosynthesis in P. aeruginosa could indeed result in identification of novel anti-infective agents against this organism. Thus, the bacterial models with less and more complex QS systems must be tested in order to discover that the potentially maximal efficacy of anti-QS compounds against Gram-negative bacteria would require targeting multiple and overlapping QS systems.

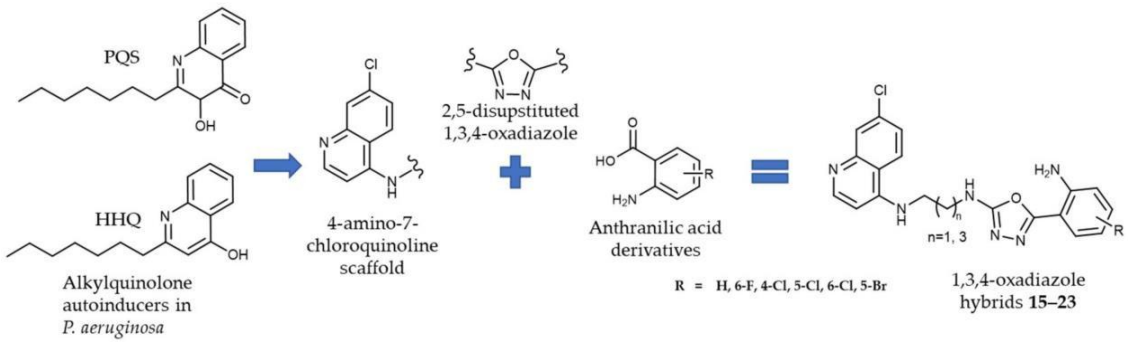


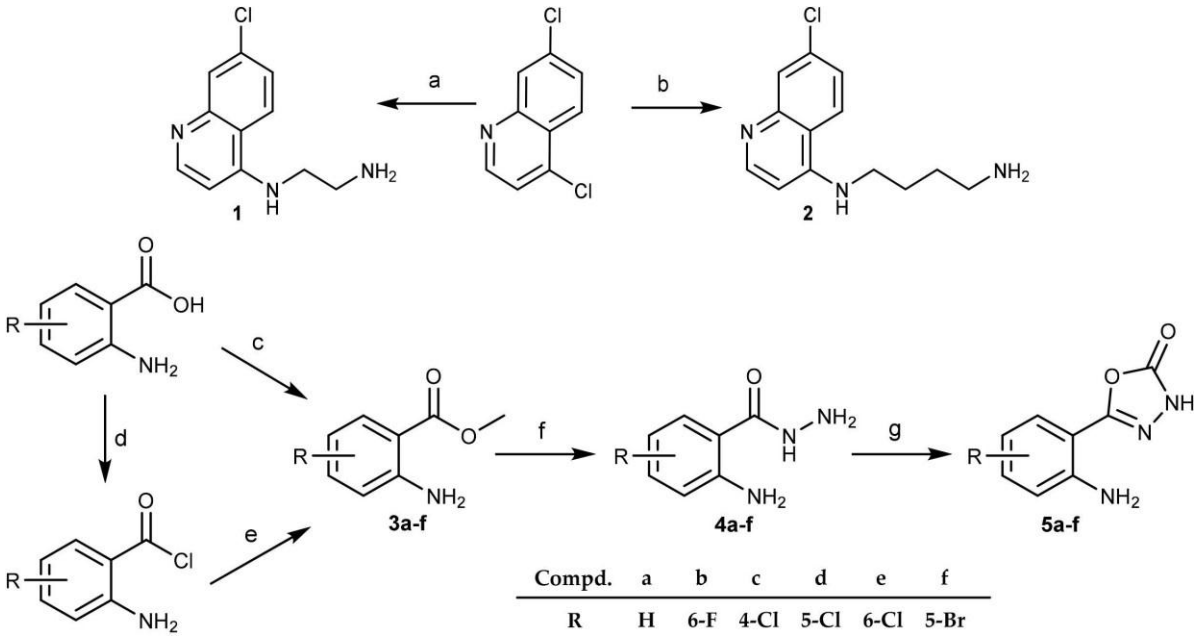
Figure 1: Novel 1,3,4-oxadiazoles as quorum sensing inhibitors.

RESULTS AND DISCUSSION

2.1 Chemistry

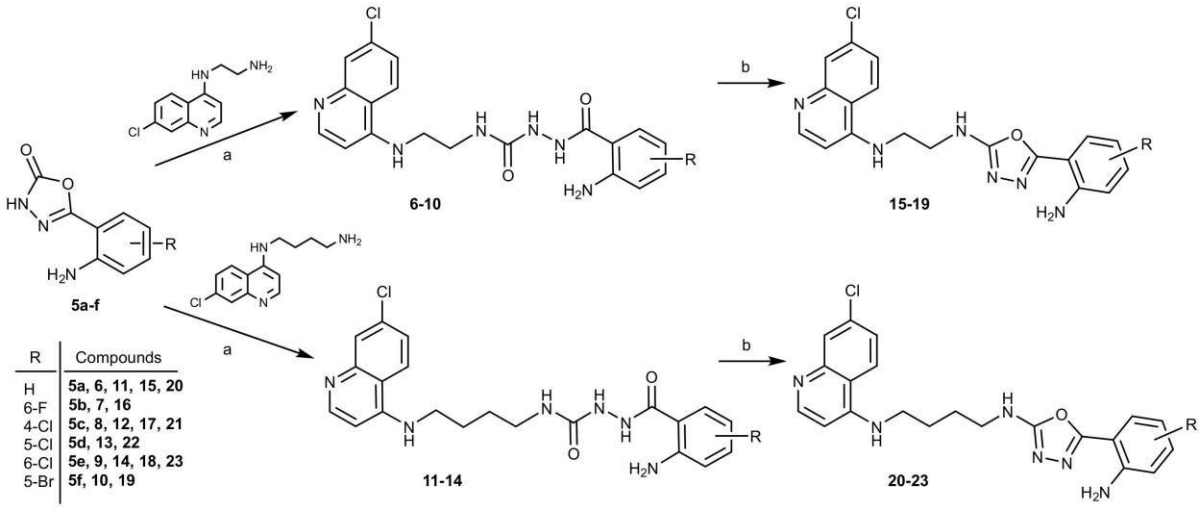
The compounds can with standard synthetic procedures that were followed in the laboratory. This synthesis was sectionalized and was made up of two syntheses. First of all, the synthesis of 4-amino-7-chloroquinoline- (1 and 2) and anthranilic acid-based (5a–f) building blocks is described in Scheme 1. The terminal amino functionalized 4-amino-7-chloroquinoline building blocks were obtained in the reaction of 4,7-dichloroquinoline with ethylenediamine (1) or 1,4-diaminobutane (2).[41, 42] The anthranilic acid building blocks in the form of the

corresponding 1,3,4-oxadiazol-2- ones (series 5) can be prepared in many steps. First, Anthranilic acid esters (series 3) included 3a–c was bought from the market while 3d–f was synthesized from Anthranilic acids. Thus, the synthesis of the required hydrazides 4 occurred when anthranilic acid esters 3 were reacted with hydrazine hydrate. Series 5 – 3-H-1,3,4- oxadiazole-2-ones were synthesized from the corresponding hydrazides 4 using 1,1- carbonyldiimidazole (CDI) according to the literature method.[43,44]



Scheme 1. Synthesis of 4-amino-7-chloroquinoline and anthranilic acid intermediates 1, 2, and 5. Reagents and conditions: (a) ethylenediamine, 120 _C; (b) 1,4-butylenediamine, 95 _C, MW; (c) SOCl2, MeOH, 60 _C; (d) SOCl2, dry toluene, 120 _C; (e)MeOH, 0 _C; (f) N2H4/H2O, 105 _C; (g) CDI, DMF, rt.

The reaction step proving to be pivotal was the nucleophilic attack of the primary amino group (either 1 or 2) on the carbonyl of the 3-H-1,3,4-oxadiazole-2-ones 5 thereby causing the ring opening and joining of the two constituents in the form of the acyl semi-carbazides 6–14. The reaction was carried out in ethanol at 100 ◦C in an experiment vial. In the case of acyl semi- carbazides 7, 10 and 13, further reaction was carried out without isolating the product since the yields were low. The last reaction step describes intramolecular cyclization of acyl semi- carbazides 6–14. Treating the amidines 15–23 under the dehydration conditions using triphenylphosphine, carbon tetrachloride as well as triethylamine gave the desired 1,3,4- oxadiazoles 15–23 as shown in scheme 2.



Scheme 2. Synthesis of 2,5-disubstituted 1,3,4-oxadiazoles 15–19 and 20–23. Reagents and conditions:(a) EtOH, 100 _C; (b) PPh3, CCl4, TEA, DCM, 46 ℃.

The reagents that are employed in cyclization reaction are triphenylphosphine, carbon tetrachloride and triethylamine. Solvent used in this method was Dichloromethane; this was suitable because of its Dielectric constant influencing the rate of the reaction.[45] Triethylamine was also used in the reaction mixture because through deprotonating, it increasesintramolecular cyclization through the participation of the hydroxyl group of the enol form of the acyl semi-carbazide.[46,47] The final reaction step came out well, the reaction yields of butan-1,4-diamine 1,3,4-oxadiazoles (20–23) were in the range of 41- 73 % and ethan-1,2- diamine 1,3,4-oxadiazoles (15–19) the yields was 9- 31%. As it can be seen, the lowest reactivity of the compound under study was registered in case of the 5-Br derivative.

Table 1. Quorum sensing inhibition and bactericidal effect of acylsemicarbazide (**8**, **12** and **14**) and 1,3,4-oxadiazole (**15**–**23**) derivatives at 400 µM concentrations on the *C. violaceum* ATCC 31532.

Compd.	QSI (%)	Bactericidal Effect (%)
8	38.6 ± 0.9	7.2 ± 0.0
12	ne	2.9 ± 0.1
14	ne	1.8 ± 0.2
15 *	87.4 ± 3.6	52.9 ± 6.3
16 *	90.5 ± 2.8	60.6 ± 2.5
17 *	89.6 ± 0.4	84.0 ± 4.8
18 *	89.3 ± 0.8	71.4 ± 2.8
19 *	85.8 ± 0.9	46.0 ± 7.1
20	46.5 ± 9.7	10.6 ± 7.8
21	53.7 ± 5.5	7.6 ± 2.7
22	38.6 ± 0.7	2.6 ± 3.6
23 *	83.5 ± 0.03	46.9 ± 2.1
Q	95.8 ± 0.4	9.8 ± 4.7
AZ	95.9 ± 0.2	89.1 ± 1.5
F267	56.8 ± 7.7	13.1 ± 5.3
F2896	62.1 ± 5.6	17.8 ± 5.6

QSI—quorum sensing inhibition; Q—quercetin; AZ—azithromycin; ne—no effect; F267 and F2896—previously identified flavonols with demonstrated anti-QS effects [37,38]. *, compounds showing significant change (unpaired *t*-test with Welch's correction, $p < 0.05$) in comparison to control cells with DMSO.

Thus, using their precursors (4-amino-7-chloroquinoline 1 and 2, and anthranilic acid-based building blocks 5a–f) as well as unique hybrid compounds 15–23, which are formed from anthranilic acids and 4-amino-7-chloroquinoline-based amines, linked via a 1,3,4-oxadiazole ring, were created. One oxygen, two carbons, and two nitrogen atoms make up the five- membered heterocyclic ring 1,3,4-oxadiazole, which was chosen as a linker because of its many advantageous physicochemical and biological characteristics. Interestingly, 1,3,4- oxadiazole is frequently employed as a bio isostere of functional groups that contain carbonyl, like carbamates, esters, and amides. Furthermore, compared to compounds containing 1,2,4- oxadiazole, those containing 1,3,4-oxadiazole have shown higher metabolic stability, lower inhibition of hERG (the human Ether-a-go-go-Related Gene), and greater water solubility.[48–50] The total length of the alkyl chain attached to the 4-amino-7-chloroquinoline scaffold (two or four carbon atoms) and the type and location of the halogen atom attached to the phenyl ring of the anthranilic acid scaffold (F, Cl, or Br) were changed to produce the structural diversity of the title compounds. The Supplementary Materials display the compounds' 1H and 13C NMR spectroscopy data.

2.2 Anti-QS and Bactericidal Activity against the QS-Reporter Strain

First, the created compounds were pre-screened using the *C. violaceum* ATCC 31532 strain as the reporter and the previously defined ideal circumstances for 96-well format to determine their anti-QS and antibacterial properties.[39] The activation of QS in this reporter strain results in the simultaneous the development of a deep-purple violacein[35] that can be quantitatively monitored[37,38] and the expression of genes involved in biofilm formation. This high-throughput screening approach enables the separation of real anti-QS medication from those with bactericidal action in a single experiment when paired with a parallel resazurin staining.[37,38,51] Only three compounds (8, 12, and 14) from the acyl semi- carbazides series 6–14 were evaluated against the *C. violaceum* reporter due to their low reaction yields (Table 1). The violacein synthesis in C. violaceum was suppressed by six out of twelve investigated compounds (ethan-1,2 diamine 1,3,4-oxadiazoles 15–19 and butan- 1,4-diamine 1,3,4-oxadiazole derivative 23) at 400 µM, practically to the

same extent as quercetin (shown as red arrows in Figure 2a, 83.5–90%). Two of these chemicals, 17 and 18, also had a significant bactericidal impact on the strain of the reporter (Figure 2b, greater than 70%). Meanwhile, neither the reporter strain's viability nor the synthesis of QS-inducible violacein was affected by acyl semi-carbazides 8, 12, or 14. Recall that in the acyl semi-carbazide series, compound 8 (ethan-1,2 diamine) was more effective than compounds 12 and 14 (butan-1,4-diamine), much like in the case of the 1,3,4-oxadiazoles.

The compounds that showed the greatest promise (15, 16, 19, and 23) were then chosen for dose-response studies. In comparison to the control cells treated with DMSO, Figure 3 shows that each chemical at concentrations up to 100 µM decreased the synthesis of violacein by approximately 50%. However, under the same conditions, the reporter's viability was only slightly impacted. An IC50 of 63.15 µM ($p < 0.05$, confidence CI 47.6–83.8 µM) was determined for compound 19. Compound 19 had the highest level of anti-QS action; at 400 µM concentration, it inhibited the synthesis of violacein in *C. violaceum* by almost 85% and had a modest bactericidal impact (47%) under the same conditions. This chemical was able to inhibit QS activity by over 50% at a concentration of 100 µM (Figure 3a), but it had no effect on cell viability (Figure 3b). When combined, our results showed that at 100 µM doses, chemicals 15, 16, 19, and 23 showed strong anti-QS activity and just a slight bactericidal impact against *C. violaceum.*

2.3 Effect of Selected Compounds on Biofilm and Pyocyanin Production in P. aeruginosaPAO1

Using *P. aeruginosa* PAO1 as the model, we aimed to determine whether the compounds (15, 16, 19, and 23) with true anti-QS activity against *C. violaceum* could disrupt these cellular processes since the PQS-dependent QS system controls both the formation of biofilms and the synthesis of pyocyanin in *P. aeruginosa.*[11,12] These analyses also included Compound 18, which demonstrated the strongest bactericidal impact on *C. violaceum*. The compounds were examined for their anti-QS activity at 100 µM since compounds 15, 16, 19, and 23 displayed the best anti-QS activity compared to their bactericidal effect on the *C. violaceum* reporter at that dose. Initially, we examined the compounds' capacity to prevent *P. aeruginosa* PAO1 from forming biofilms (BFI). All compounds exhibited a reduction in biofilm development, as seen in Figures 4 and 2. Compound 19 showed the strongest antibiofilm impact (>60%, $p < 0.05$) when compared to the control cells treated with 1% DMSO. Compound 19 did, however, also have the largest inhibitory effect on cell growth (Table 2—GI), indicating that it may primarily function as a bactericidal agent against the strain of P. aeruginosa. Considering this, we next computed the biofilm index for every component (Table 2), which encompasses the biofilm mass and the number of cells.

Table 2: Parameters of cell growth inhibition, inhibition of biofilm formation, biofilm eradication, pyocyanin inhibition, and biofilm index of P. aeruginosa PAO1 cells treated with 100 _M compounds compared to control cells treated with 1% DMSO.

Compd.	GI (%)	BFI (%)	Biofilm Index	BE (%)	PI (%)
Ø *			60.25		
15 *	8.13 ± 2.76	48.6 ± 1.81	33.72	24.86 ± 1.13	42.34 ± 1.37
16 *	31.85 ± 1.64	53.72 ± 0.43	40.9	43.08 ± 2.15	20.45 ± 0.69
18 *	28.34 ± 2.57	45.26 ± 1.88	46.02	29.74 ± 1.47	16.02 ± 0.87
19 *	43.7 ± 2.24	61.57 ± 0.84	41.13	35.36 ± 1.62	38.87 ± 1.57
23 *	23.38 ± 3.03	29.08 ± 1.03	55.75	10.89 ± 1.29	72.02 ± 1.25

Ø—1% DMSO; GI—growth inhibition (A570); BFI—biofilm formation inhibition (A540); biofilm index—(A540/A570) × 100; BE—biofilm eradication (A540); PI—pyocyanin inhibition—(A520/A570) × 100. *, compounds showing statistically significant change in comparison to the control cells with DMSO (1-ANOVA, $p < 0.05$).

Consequently, of all the compounds that were evaluated, compound 15 had the highest effectiveness against *P. aeruginosa*, lowering its capacity to produce biofilm by about 50% while having a little impact on cell growth ($p < 0.05$), suggesting that this drug is anti-QS.

In the following experiment, we treated the pre-formed biofilm with the chosen five compounds after letting P. aeruginosa PAO1 build a biofilm for 24 hours (Figure 5, Table 2- column BE). The compounds 16 and 19 had the highest efficacy in eliminating biofilm, with a 43% and 35% reduction in biofilm mass, respectively ($p < 0.05$). Compound 23 was shown to be the least efficient biofilm eradicator, with an 11% reduction in biofilm mass. chemical 15 was shown to treat pre-formed biofilms with about 25% less biofilm mass, suggesting that in addition to its expected anti-QS efficacy (around 50% BHI), this chemical may also have an effect on mature biofilms. Furthermore, an analysis that contrasts all cell growth inhibitory values (GI) with those that represent the effectiveness of biofilm eradication for every investigated substance reinforces the hypothesis that compound 15 is a true anti-QS agent that has activity against biofilms that have developed.

Compounds 16 and 19 exhibited the strongest inhibitory effects on cell development, with all compounds exhibiting much stronger inhibitory effects (by 30% to 45%, Table 2—GI). We propose that these chemicals, which were also more effective in inhibiting the formation of biofilms, primarily functioned by eliminating PAO1 cells, which may comprise both planktonic and biofilm cells, instead of disrupting the PQS-QS system. In addition to these analyses, we determined the five recommended drugs' minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC). For every drug, the MBC was 3200 µM and the MIC was 1600 µM. Since the compounds were first dissolved in DMSO, we also looked at the inhibitory effects of DMSO by itself at similar concentrations (1% to 32% v/v). The results showed the following effects: 4.82 0.95% for 1%, 12.53 1.12% for 2%, 18.72 1.76% for 4%, 28.12 1.97% for 8%, 78.13 2.23% for 16%, and 97.21 2.11% for 32% DMSO were the results. Therefore, the real MIC and MBC values for the drugs are estimated to be much larger when accounting for the effects of DMSO. These results also show that the concentration used to test the chosen compounds, 100 µM, was significantly lower than their MIC and MBC values, confirming that the observed antibiofilm effect—rather than antibacterial or bactericidal activity-was probably the consequence of interfering with the PQS-QS system.

Since the PQS-QS system in *P. aeruginosa* regulates the formation of pyocyanin, an extracellular virulence factor, we also looked into the potential effects of the five compounds that were chosen (15, 16, 18, 19, and 23). Since this concentration produced statistically significant variations in biofilm formation and pre-formed biofilm assays, the effect of substances on the synthesis of this pigment was examined at the 100 µM level. Compound 23was the most efficient anti-pyocyanin drug, as seen in Figure 6 and Table 2 (column PI), lowering the synthesis of this virulence factor by more than 70% when compared to the controlcells ($p < 0.05$). Compounds 15 and 19 were the second best by decreasing the pyocyanin level by ca. 40% ($p < 0.05$). Compounds 16 and 18, with predicted bactericidal effects, were the least effective, which is likely the consequence of the reduced cell growth detected for the two compounds. Since compound 23 displayed only a minor antibiofilm effect, we conclude that this could be a genuine anti-virulence compound against *P. aeruginosa* PAO1.

2.4 Factors Limiting the Efficacy of Compounds Targeting PQS-QS of P. aeruginosa

Given that no investigated chemical was able to totally stop the formation of biofilms, remove pre-formed biofilms, or stop the synthesis of pyocyanin, we propose that the results obtained can be explained by *P. aeruginosa* PAO1's four interrelated QS systems (Figure 7). At the summit of the QS hierarchy, the Las-QS system regulates the Rhl- and PQS-based QS systems and stimulates the production of alginate, a negatively charged polysaccharide crucial to the creation of biofilms.[11] Apart from alginate, *P. aeruginosa* PAO1 also produces lectin, rhamnolipids, Psl, and Pel, which are lipopolysaccharides. These polysaccharides are crucial for the formation, development, stabilization of the biofilm matrix, and defense of the mature biofilm against external agents.[52] Among them, the PQS-QS system regulates lectin, Psl, and Pel, whereas Las- and Rhl-dependent QS stimulates the synthesis of rhamnolipids.[11] It's probable that compound 15's mode of action involved interfering with the synthesis of lectin, Psl, Pel, and rhamnolipids rather than directly destroying the cells within the biofilm, given the close relationship between the PQS- and Rhl-QS systems in *P. aeruginosa* (Figure 7). However, unlike the other chemicals, this one did not result in decreased cell proliferation, which is what led to the eradication of pre-formed biofilms. As a result, we cannot rule out the possibility that compound 15 also inhibited the production of one or more of the charged polysaccharides[53–55] or other factors associated with or secreted from the cell surface (such as proteins, extracellular DNA/eDNA, pyocyanin, etc.)[56,57] that aid in the development of a stable biofilm matrix. However, given that compound 15 could only partially block the biofilm formation—by 50%—and that *P. aeruginosa* can also use Las- and Rhl-QS to drive biofilm formation, we propose that one or both of these systems may have also played a role in the biofilm formation.

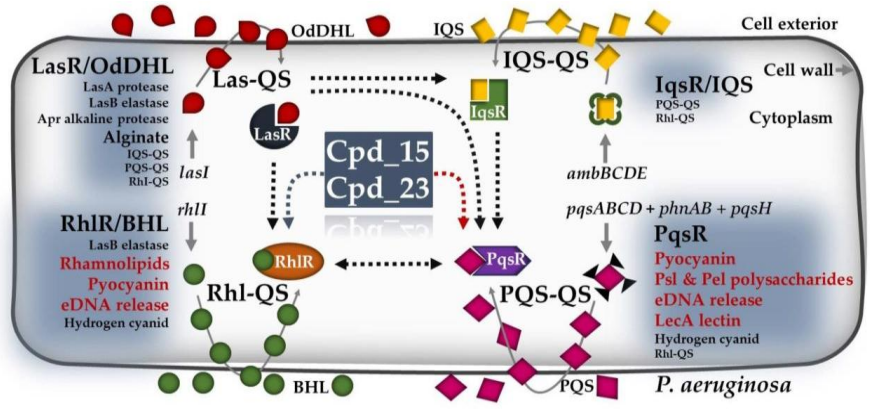


Fig. Schematic representation of the four QS signaling networks present *in P. aeruginosa,* with indicated QS-mediated pathways likely to be affected by compounds.

Our results suggest that compound 23 preferentially targets the PQS-QS-mediated pyocyanin synthesis rather than having a substantial antibiofilm effect. It has been demonstrated that 95% of P. aeruginosa isolates, including strain PAO1, secrete this virulence factor.[60] The release of eDNA, a vital component of the biofilm matrix that provides structural support and facilitates biofilm formation, is one of its primary functions, in addition to giving the biofilm cells enhanced virulence.[57] Compound 23 probably does not completely block pyocyanin synthesis in P. aeruginosa because both the PQS- and Rhl-QS systems regulate pyocyanin synthesis (Figure 7). Given that this chemical only had a negligible antibiofilm effect on PAO1, we hypothesize that the pyocyanin-stimulated biofilm development may be offset by the enhanced synthesis of other matrix-associated components through PQS-QS-independent pathways.

Notably, PQS has also been demonstrated to carry out QS-independent activities, pointing to a pleiotropic role for this AI in *P. aeruginosa*. These activities include iron uptake, cytotoxicity, outer membrane vesicle biogenesis, and host immunological modulation.[61,62] Additionally, research has shown that QS Las R mutants readily arise in vivo during cystic fibrosis.[62–65] emphasizing the role that PQS-QS plays in making up for the absence of the Las-QS system. Las R also activates the Rhl R-activated genes, which include genes involved in biofilm formation and virulence (such as pyocyanin and rhamnolipids) (Figure 7).

By promoting the expression of the Rhl-QS system and the synthesis of downstream virulence factors, PQS could thereby circumvent the reliance on Las R[59] (Figure 7). Given this, the best course of action for treating *P. aeruginosa* infections in clinical settings may actually involve strategies that target both the PQS-dependent and -independent pathways.

When combined, the 1,3,4-oxadiazoles 15 and 23 described in this work held the most potential for creating fresh, potent anti-QS tactics against P. aeruginosa. Our findings also emphasize the significance of interaction QS systems, which should be taken into account when trying to maximize efforts against this clinically significant pathogen, in order to achieve that goal.

Anti-QS and Bactericidal Activity Screening

The indicator reporter strain, *Chromobacterium violaceum* ATCC31532 (ATCC; Wesel, Germany), was employed to test compounds 8, 12, and 14–23 for bactericidal or anti- QS/biofilm properties.[17,35,37] Achieving OD600 = 0.02 required suspending the reporter strain, which had been cultivated overnight at 27 ◦C on Luria-Bertani agar (Fischer Scientific, Leicestershire, UK), in PDYT (0.5% peptone, 0.3% D-glucose, 0.25% yeast extract, and 0.05% L-tryptophan, m/v). Next, 200 µL of the resulting cell suspension was applied to each of the two parallel 96-well plates (polystyrene, flat-bottom, Becton Dickinson, tissue culture treated) that had been previously treated with 2% DMSO (control) or with the relevant compounds dissolved in DMSO at different doses (400, 200, 100, and 40 µM). One set of positive controls for QS suppression was azithromycin (Sigma-Aldrich) dissolved in DMSO at 400 µM, and another set was quercetin[66] (bactericidal drug) for cell viability. The plates were incubated for 22 hours at 27 ◦C in an aerobic environment at 200 rpm. To evaluate the bactericidal effects of the compounds, 200 µM of resazurin, a redox-sensitive dye that is converted to fluorescent resorufin only by live cells, was applied per well in the first 96-well plate.[67,68] In order to pellet insoluble violacein and cells, the 96-well plates—with or without the resazurin—were centrifuged at 4000 rpm for 20 min at 20 ◦C after being agitated for a further 30 min (pm) in the dark. Supernatants containing resorubifin (100 µL) were then transferred to a new plate, and the fluorescence that was created and what was left over was measured using a PerkinElmer Victor3 multilabel microtiter plate reader at 550 and 590 nm for excitation and emission, respectively. After removing the supernatants from the 96-well plate sans resazurin, 96% (v/v) ethanol was used to dissolve the pelleted violacein. After centrifuging the 96-well plate at 4000 rpm for 20 minutes at 20 ◦C, the supernatants containing soluble violacein (100 µL) were moved onto another 96-well plate. The PerkinElmer Victor3 reader was used to track variations in the violacein yields at 595 nm. With a minimum of three technical duplicates for every plate, the anti-QS and bactericidal screening assays were conducted twice. Using cells with DMSO as the control and the previously mentioned experimental conditions, the inhibitory action of the most potent anti-QS compounds exhibiting little bactericidal activity against C. violaceum was confirmed in 96-well plates with six separate replica samples.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Using the broth dilution method, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for compounds 15, 16, 18, 19, and 23 (first dissolved in DMSO) of Pseudomonas aeruginosa PAO1 (Leibnitz Institute DSMZ 1707, Braunschweig, Germany) were found.[69] In order to achieve a concentration of 105 CFU/mL for the MIC experiments, PAO1 cells were diluted in fresh Luria-Bertani (LB) broth after being cultured overnight in LB broth. The cells were then separated into 200 µL aliquots on 96-well plates, either with serially diluted chemicals (from 3200 to 100 µM) or without (DMSO alone). The MIC value—which represents the lowest chemical concentration that inhibited observable growth—was ascertained by measuring the cell density at 570 nm after 24 hours of incubation at 37 °C. After the MIC test, 100 µL from the bacterial cultures that showed no discernible growth was transferred onto LB agar plates to assess the MBC. Following a 24-hour incubation period at 37 °C, the MBC was found to be the chemical concentration

with the lowest rate of growth in these circumstances. The initial concentration of 105 CFU/mL was lowered to less than 10 CFU/mL because the method's limit of detection is 10 CFU/mL. Consequently, the molecule that kills 99.9% of bacteria at the lowest concentration was identified as the MBC.[70] Three separate experiments and three technical duplicates were used for each of the threetriplicate MIC and MBC assays.

Antibiofilm Assay

The study examined the impact of several chemicals on the development of biofilm and the removal of pre-existing biofilm on *Pseudomonas aeruginosa* PAO1 (Leibnitz Institute DSMZ 1707, Braunschweig, Germany). We carried out three separate studies, each involving eight technical replicates.

P. aeruginosa PAO1 was cultivated overnight in Luria-Bertani (LB) medium and diluted with LB medium to an optical density of 0.5 Mc-Farland in order to limit the formation of biofilms. In order to achieve an optical density of roughly 0.01 at 600 nm, we carried out an extra 1:100 dilution step to start QS-dependent biofilm growth. In this second step, 100 µL of the bacterialculture was transferred to a 96-well microtiter plate with a U-bottom in 8 technical replicates.The bacterial culture was diluted with 1% DMSO in the M63 medium with 0.4% Arg (control) or with the M63 medium with 0.4% Arg containing indicated compounds (15, 16, 18, 19, and 23; initially dissolved in DMSO). For twenty-four hours, plates were incubated aerobically at 37 °C. Biofilm was found in the manner mentioned in O'Toole, G. A. (2011).[71] After removing planktonic cells and measuring bacterial growth at 570 nm (Wallac Victor 2 1420, Perkin Elmer), biofilm was stained for 15 minutes using 125 µL of 0.1% crystal violet. After drying and rinsing the microtiter plate three times with water, 150 µL of 30% acetic acid was used to extract the biofilm. To measure absorbance at 540 nm, 125 µL of solubilized crystal violet was added to a fresh flat-bottom microtiter plate (Wallac Victor 2 1420, Perkin Elmer). The biofilm index was computed as a ratio of 100 (A540/A570). In order to investigate the impact of certain compounds (15, 16, 18, 19, and 23) on pre-existing biofilm, P. aeruginosa PAO1 was cultured in LB medium for a whole night and then diluted to an optical density of 0.5 McFarland using LB medium. Following a 1:100 dilution in M63 medium with 0.4% Arg, 100 µL of the bacterial culture was added to a 96-well microtiter plate featuring a U-bottom. The plate was then incubated aerobically at 37°C for a whole day in order to create biofilm. After removing the bacterial solution, the wells were thrice cleaned with M63 medium containing 0.4% Arg. Eight technical duplicates were made, with 100 µL of 1% DMSO in the M63 medium with 0.4% Arg (control) or 100 µM of the relevant compounds in the M63 medium with 0.4% Arg added to each well. After a 24-hour incubation period at 37 °C, the plates were dyed with biofilm using the previously mentioned method.

Effect on Pyocyanin Production

P. aeruginosa PAO1 cells were cultured in LB medium for an entire night, adjusted to 0.5 McFar- land using LB medium, and then diluted 1:100 in 5 mL LB media that contained 100 µM compounds 15, 16, 18, 19, and 23 or 1% DMSO (control). Bacterial growth was assessedusing A570 after the culture was cultivated aerobically for 24 hours at 37 °C. Extracting pyocyanin was done in accordance with Essar et al., 1990.[72] After the cells were pelleted and 3 mL of chloroform was used to extract pyocyanin from the supernatant, the mixture was centrifuged for 10 minutes at 5000 rpm using a Thermo Jouan BR4i, and the chloroform layerwas extracted again using 1 mL of 0.2 M HCl. In order to standardize pyocyanin readings to cell growth, absorbance was measured at 520 nm, and the results were represented as a ratio (A520/A570) 100. Three separate experiments were conducted, each with two technical replicates.

Statistical Analysis

The screening window coefficient (Z'), as reported in[73], was computed to track theeffectiveness of C. violaceum-based screening results. Using a paired t-test (two-tailed) and a 95% confidence interval as criterion, potency (half inhibitory concentrations, IC50) calculations were carried out using GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA, version 8.0). GraphPad Software's unpaired t-test with Welch's correction was used to further validate the most effective QS inhibitors against C. violaceum. A p-value of less than 0.05 was set as the threshold for substantial inhibition. In the antibiofilm and anti- pyocyanin experiments involving P. aeruginosa, a significant alteration was defined as a one- way analysis of variance (1-ANOVA) with $p < 0.05$.

CONCLUSION

Applying a molecular hybridization method, we designed nine new chemicals in this study to prevent *P. aeruginosa* from forming biofilms and developing virulence. In order to differentiatebetween true QS inhibitors and those with bactericidal effects in a single experiment, the generated 1,3,4-oxadiazole hybrid compounds, which included 4-(2-aminoethyl/4- aminobuthyl) amino-7-chloro-quinoline and anthranilic acid scaffolds, were first pre-screened using a Gram-negative *C. violaceum*-based microscale screening system. The most promising anti-QS activity was shown by ethan-1,2-diamine 1,3,4-oxadiazoles 15–19 and butan-1,4- diamine 1,3,4-oxadiazole 23 from the tested compounds. These compounds were then evaluated for their capacity to inhibit pyocyanin production, disrupt pre-formed biofilms, and prevent biofilm formation using P. aeruginosa PAO1 as the model. The largest anti-QS/biofilm effect (cutting biofilm formation by 50%) and some biofilm eradication activity (lowering thebiomass by 25%) were shown by ethan-1,2-diamine 1,3,4-oxadiazole 15, out of these five anti- QS agents, but cell growth was only little affected. The most effective anti-virulence chemical was found to be butan-1,4-diamine 1,3,4-oxadiazole 23, which had a negligible

antibiofilm impact but inhibited the production of pyocyanin by over 70%. Because *P. aeruginosa* harbours four highly interconnected QS systems, including the PQS-QS system, our compounds were unable to totally stop the formation of biofilms or the synthesis of pyocyanin in the PAO1 biofilm model. Based on these results, we therefore propose that in order to establish a dependable SAR and achieve a maximal anti-QS effect against this clinically significant pathogen, the chemical library should be expanded around the two most active compounds (15 and 23) and the AIs employed by other interactive QS systems used by P. aeruginosa should be taken into consideration.

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