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Article

Synthesis and biological evaluation of new quinoline and anthranilic acid derivatives as potential quorum-sensing inhibitors

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Abstract: Inhibition of quorum sensing (QS)-dependent biofilm formation and virulence is extensively investigated as a novel approach to combat bacterial pathogens by impeding their ability to cause antibiotic-tolerant diseases. Such strategies are considered a promising alternative to conventional antibacterial therapy since interrupting the central cell-to-cell communication system imposes less selective pressure on the target pathogen. In this study, novel hybrid compounds composed of anthranilic acids substituted with halogens at different positions of the phenyl ring, and 4-(2-aminoethyl/4-aminobuthyl)amino-7-chloroquinoline linked via 1,3,4-oxadiazole were synthesized using standard procedures. Their anti-QS activities were evaluated using *Chromobacterium violaceum ATCC31532* (anti-biofilm and bactericidal activities) and *Pseudomonas aeruginosa* PAO1 (anti-biofilm/-virulence activities). The results showed that compounds **15–19** and **23** inhibited the production of violacein, the QS-inducible pigment, in *C. violaceum* to a similar extent as the model QS inhibitor - quercetin (83.5–90%). Compound **15** exhibited the strongest effect on *P. aeruginosa* in the anti-biofilm screening, reducing its ability to form biofilm by almost 50%, while compounds **16** and **19** were able to reduce the biofilm formation by approximately 30%. However, compound **23** did not demonstrate significant anti-biofilm activity and only inhibited pyocyanin production in *P. aeruginosa*. In conclusion, this study suggests that 1,3,4-oxadiazoles **15**, **16**, and **19** are the most promising compounds for future research as novel QS inhibitors against Gram-negative bacteria equipped with different QS signaling pathways.

Keywords: 4-amino-7-chloroquinoline; 1,3,4-oxadiazole; anthranilic acid; synthesis; quorum sensing; anti-biofilm; *P. aeruginosa*; PQS

1. Introduction

Bacterial pathogens use different strategies to cause disease and overcome obstacles faced in human hosts, including also strategies that allow increased viability in response to the use of traditional antibiotics. While efficiently killing the target bacteria, antibiotics promote selective pressure on the bacterial cell, thereby leading to the development of resistant species and complicating the successful treatment of infections caused by those bacteria [1]. Based on the data collected from 204 countries worldwide, an estimated 4.95 million deaths were associated with bacterial antimicrobial resistance (AMR) in 2019, including 1.27 million deaths directly attributable to bacterial AMR [2]. The decline in antibacterial drug discovery and development together with the rise of AMR represents one of the leading public health threats of the 21st century [3].

Quorum sensing (QS) is a mechanism of bacterial cell-to-cell communication and gene expression coordination and an important strategy performed by bacteria to enhance their virulence and promote host damage. It relies on signaling molecules called autoinducers (AI) [4] to activate community behaviors such as biofilm formation, which is a significant mechanism contributing to

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bacterial tolerance against antibiotics. Biofilm formation is also a key virulence factor enabling bacterial pathogens to establish chronic infections and promoting the development of antimicrobial resistance (AMR). In view of these findings, inhibition of QS represents a highly promising approach for anti-bacterial and anti-biofilm therapies, as it is expected to exert less selective pressure on bacterial pathogens compared to conventional antibiotics [5].

Molecular hybridization, *i.e.*, covalent linking of two or more bioactive scaffolds to form a single molecule with improved properties, was used to design the title compounds. The advantages of such compounds include increased efficacy, improved pharmacokinetics, and reduced risk of resistance development, side effects, and drug-drug interactions. In recent years, our group has successfully implemented this approach to obtain a harmine-based antimalarial compound library [6–11]. The concept of hybridization has already been used by other research groups in the subject area [12–14].

Several classes of quinoline derivatives were investigated as potential agents against QS and biofilm formation due to their structural similarity to *Pseudomonas* alkylquinolone AIs (e.g., 2-heptyl-3-hydroxy-4(1H)-quinolone, PQS, and its precursor 2-heptyl-4(1H)-quinolone, HHQ). Among these studies, the 4-amino-7-chloroquinoline scaffold emerged as a promising candidate for developing anti-QS and anti-biofilm agents targeting multidrug-resistant bacteria [15–19]. Additionally, anthranilic acid serves as a precursor in PQS biosynthesis [20], and halogenated anthranilic acids and their derivatives have been reported as inhibitors of PQS biosynthesis [21–25]. Thus, interrupting the PQS biosynthesis could lead to the discovery of novel anti-infective agents based on anti-virulence strategies.

Continuing our work on QS inhibitors [26], we synthesized a series of hybrid molecules incorporating the quinoline and anthranilic acid scaffolds (Figure 1). These compounds were screened for their anti-QS, antibacterial and antivirulence activities using Gram-negative *Chromobacterium violaceum* and *Pseudomonas aeruginosa* as the known biofilm models using a VioI/R (resembling LuxI/R-type QS) and several LuxI/R- dependent QS systems, respectively [27–29].

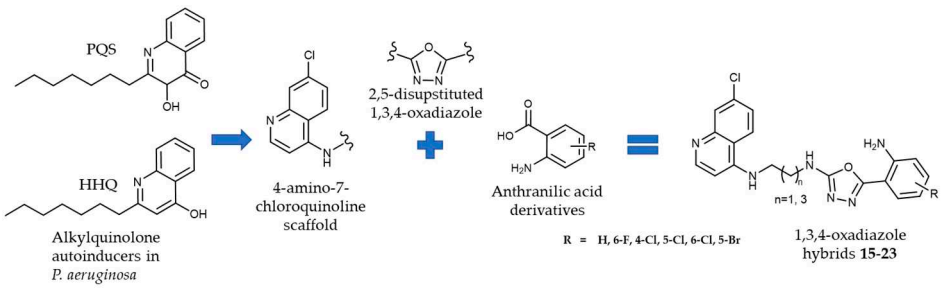

Figure 1. Novel 1,3,4-oxadiazoles as quorum sensing inhibitors.

2. Results and Discussion

2.1. Chemistry

In this study, we present the synthesis of novel hybrid compounds **15–23**, derived from anthranilic acids and 4-amino-7-chloroquinoline-based amines, linked via 1,3,4-oxadiazole ring, as well as the synthesis of their precursors. 1,3,4-Oxadiazole is a 5-membered heterocyclic ring, composed of one oxygen, two carbons, and two nitrogen atoms. It was selected as a linker due to the variety of favorable physicochemical and biological properties. It is often used as a bioisostere of carbonyl-containing functional groups, such as amides, esters, and carbamates. In addition, 1,3,4-oxadiazole-containing compounds have demonstrated increased aqueous solubility, decreased hERG inhibition, and improved metabolic stability when compared to 1,2,4-oxadiazole ones [30–32].

The structural variety of the title compounds was achieved by changing the length of the alkyl chain attached to the 4-amino-7-chloroquinoline scaffold (2 or 4 carbon atoms) and the position and the type of the halogen atom attached to the phenyl ring of the anthranilic acid scaffold (F, Cl or Br).

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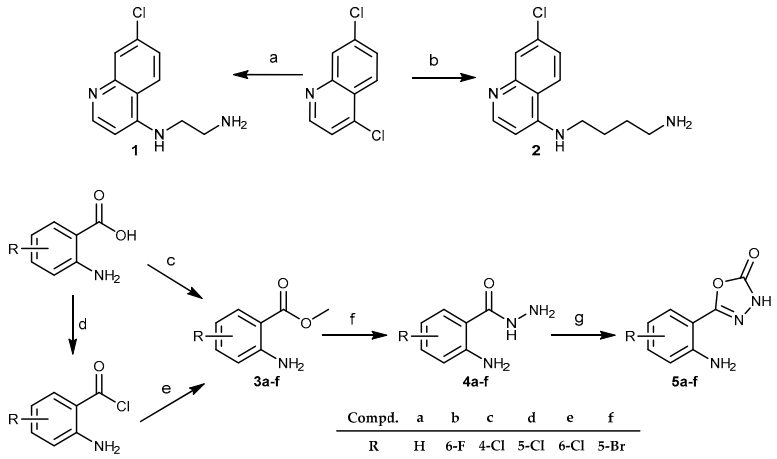
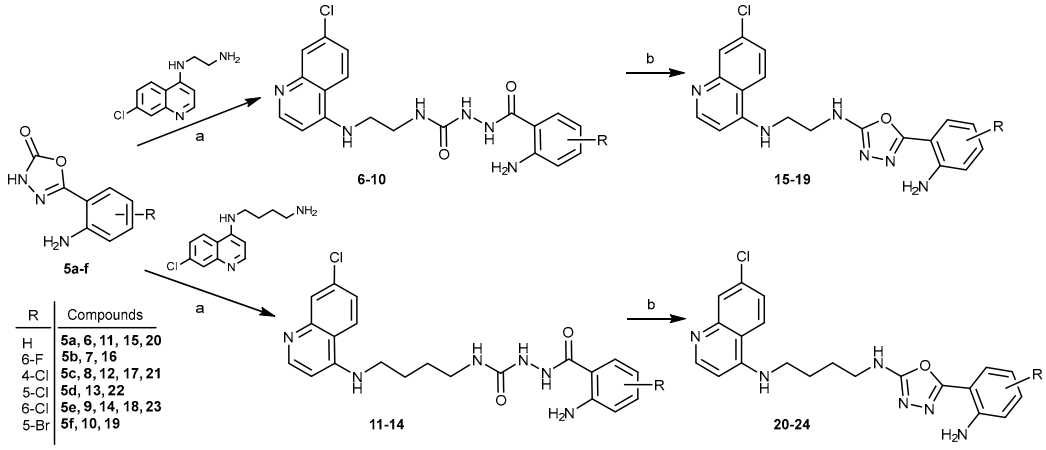
The compounds were obtained by standard synthetic procedures. The synthesis was divided into two parts. First, 4-amino-7-chloroquinoline- (**1** and **2**) and anthranilic acid-based (**5a–f**) building blocks were obtained, as outlined in Scheme 1.



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) $SOCl_2$, MeOH, 60 °C; (d) $SOCl_2$, dry toluene, 120 °C; (e) MeOH, 0 °C; (f) N_2H_4/H_2O, 105 °C; (g) CDI, DMF, rt.

The 4-amino-7-chloroquinoline building blocks bearing terminal amino group (**1**,**2**) were prepared according to the previously published procedure, in the reaction of 4,7-dichloroquinoline and ethylenediamine or 1,4-diaminobuthane, respectively [33,34]. The anthranilic acid building blocks in the form of the corresponding 1,3,4-oxadiazol-2-ones (series **5**) were obtained in multiple reaction steps. First, anthranilic acid esters (series **3**) were either purchased (**3a–c**) or obtained from the corresponding anthranilic acids (**3d–f**). Hydrazides **4** were obtained from the anthranilic acid esters **3** and hydrazine hydrate**.** 3-*H*-1,3,4-oxadiazole-2-ones (series **5**) were obtained from the corresponding hydrazides **4** in the presence of 1,1-carbonyldiimidazole (CDI) following the previously published procedure [35,36]. The key reaction step was a nucleophilic attack of the primary amino group (**1** or **2**) on the carbonyl of the 3-*H*-1,3,4-oxadiazole-2-ones **5**, leading to the ring opening and merging of the two building blocks in the form of the corresponding acylsemicarbazides **6–14**. The reaction was conducted in ethanol at 100 °C in a sealed vial. In the case of acylsemicarbazides **7**, **10,** and **13**, we proceeded to the next reaction step without the purification of the product due to the low yields. The final reaction step presents intramolecular cyclization of the acylsemicarbazides **6–14**. Under the dehydration conditions (utilizing triphenylphosphine, carbon tetrachloride, and triethylamine) the desired 1,3,4-oxadiazoles **15–24** were obtained (Scheme 2).



Scheme 2. Synthesis of 2,5-disubstituted 1,3,4-oxadiazoles **15–19** and **20–24**. Reagents and conditions: (a) EtOH, 100 °C; (b) PPh_3, CCl_4, TEA, DCM, 46 °C.

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The reagents used in the cyclization reaction include triphenylphosphine, carbon tetrachloride, and triethylamine. Dichloromethane was used as a solvent, which proved to be suitable due to its dielectric constant that affects the reaction rate [37]. Triethylamine was also added to the reaction mixture as it promotes intra-molecular cyclization by deprotonating the hydroxyl group of the enol form of acylsemicarbazide [38,39].

The final reaction step progressed well, and the reaction yields were slightly higher for the butan-1,4-diamine 1,3,4-oxadiazoles (**20–23**), ranging from 41 to 73 %, as opposed to the ethan-1,2-diamine 1,3,4-oxadiazoles (**15–19**) (yields from 9 to 31 %). The lowest reactivity was observed in the case of the 5-Br derivative.

2.2. Anti-QS and bactericidal activity against the QS-reporter strain

Gram-negative C. violaceum ATCC 31532 was used as a reporter strain to indicate compounds with anti-QS and bactericidal activities. In this biofilm-forming pathogen, activation of QS induces the expression of genes contributing to biofilm formation and synthesis of a deep-purple violacein. This pigment, excreted out of the cells, acts as an indicator of induced QS in C. violaceum and its changes could be quantitatively monitored in 96-well microtiter plate format [40,41]. Since acylsemicarbazides and 1,3,4-oxadiazoles prepared in this study, are built from anthranilic acid derivatives and 4-amino-7-chloroquinoline, we decided to screen both types of compounds for their anti-QS and bactericidal activity against the C. violaceum reporter. Due to the low reaction yields for the acylsemicarbazides series **6**-**14**, only 3 compounds (**8**, **12,** and **14**) were tested. Quercetin was used as the positive control due to its known anti-QS effect and azithromycin as the positive control for bactericidal activity (cell viability) [40,41]. Inhibitory activity was first tested at 400 μM concentrations (Table 1). Six out of twelve tested compounds (**15–19** and **23**) inhibited violacein production in C. violaceum almost to the same extent as quercetin (indicated as red arrows in Figure 2a, 83.5–90 %). Interestingly, all ethan-1,2,diamine 1,3,4-oxadiazoles (**15**-**19**), and one butan-1,4-diamine 1,3,4-oxadiazole derivative (**23**) were active. Additionally, two compounds (**17** and **18**) also exerted a strong bactericidal effect on the reporter strain (Figure 2b, more than 70 %) which was measured as the ability of viable and metabolically active cells to reduce resazurin to fluorescent resorufin [42]. At the same time, acylsemicarbazides **8**, **12,** and **14** showed only weak or no effect on the violacein production as well as on the viability of the reporter strain. It is worth mentioning that in the acylsemicarbazide series, similarly to the 1,3,4-oxadiazoles, the ethan-1,2,diamine compound **8** was more potent when compared to the butan-1,4-diamine compounds **12** and **14**.

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 C. violaceum strain (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

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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 [40,41]

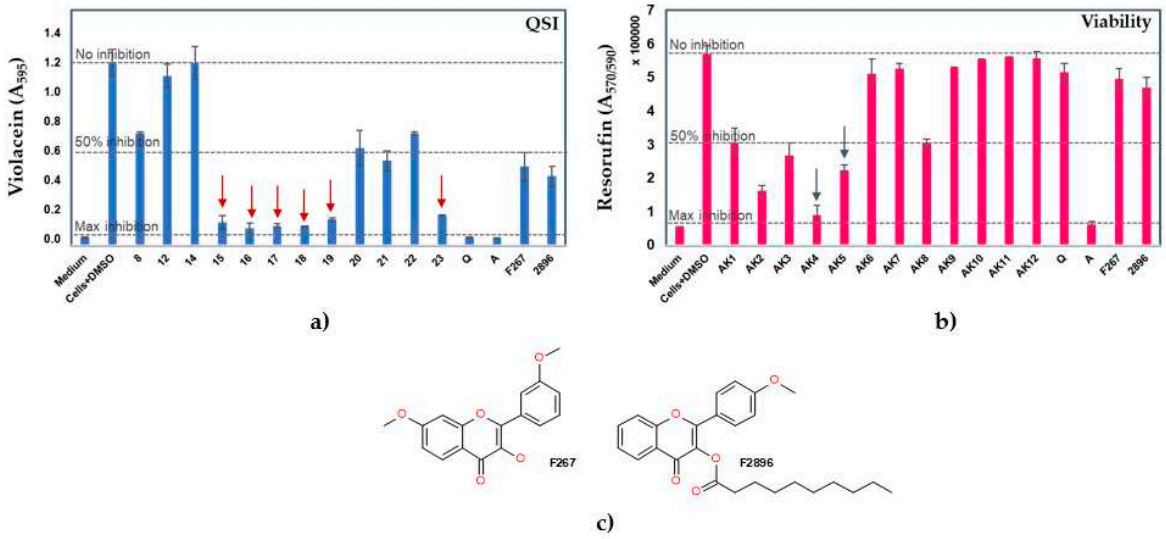

Figure 2. Comparison of violacein production (**a**) and cell viability (**b**) of *C. violaceum* ATCC 31523 cultures with and without the tested compounds. Medium – culture medium with 2 % DMSO; Cells+DMSO – *C. violaceum* cells with 2 % DMSO; Q – quercetin; AZ – azithromycin (both at 400 μM); (c) F267 and F2896 – previously identified flavonols with demonstrated anti-QS activity [40,41]. Red arrows in a) indicate the most active anti-QS compounds and blue arrows in b) indicate the compounds with the most pronounced bactericidal activity.

The most promising compounds (**15**, **16**, **19,** and **23**) were selected for dose-response analyses at 400, 200, or 100 and 40 μM concentrations. The results indicated that each compound with concentrations up to 100 μM reduced the violacein production by more than 50 % compared to the control cells with DMSO, while the viability of the reporter under the same conditions was only marginally affected (Figure 3)

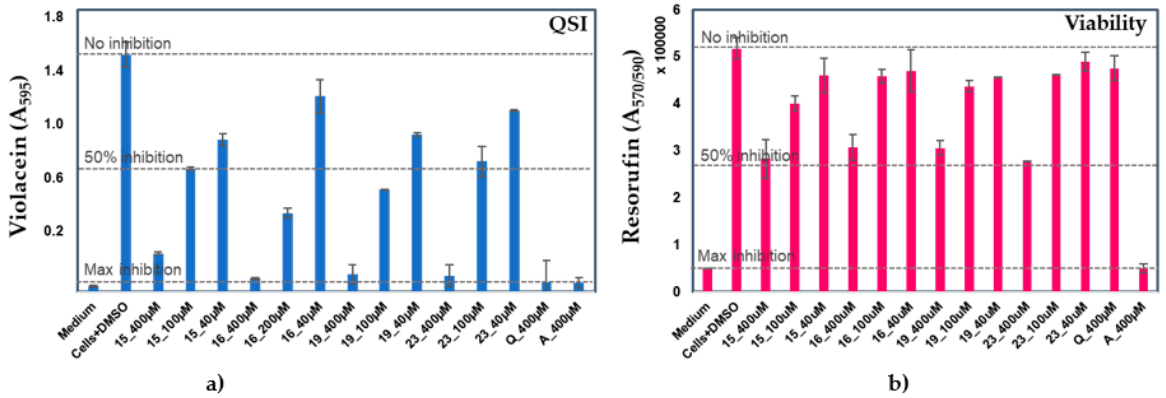

Figure 3. Dose-response analyses with the most promising anti-QS compounds (**15**, **16**, **19,** and **23**) at 400, 200, 100, and 40 μM concentrations of violacein production (**a**) and cell viability (**b**) of *C. violaceum* ATCC 31523 cultures with and without the tested compounds**.** Medium – culture medium with 2 % DMSO; Cells+DMSO – *C. violaceum* cells with 2 % DMSO; Q – quercetin; A – azithromycin (both at 400 μM).

For compound **19**, IC_{50} of 63.15 μM (95 % CI 47.62–83.75 μM) was measured. Compound **19** was one of the most active compounds in anti-QS screening since it reduced the violacein production in *C. violaceum* by more than 85 % at 400 μM concentration. The detected bactericidal effect under the

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same conditions was moderate (47 %, Table 1). At a concentration (100 μM), this compound was able to reduce the QS activity by more than 50 % (Figure 3a), while no effect on cell viability was observed (Figure 3b).

2.3. Effect of selected compounds on biofilm and pyocyanin production in Pseudomonas aeruginosa PAO1

P. aeruginosa is a Gram-negative pathogen conferring extremely difficult-to-treat infections due to its excellent ability to colonize a variety of medical materials (urinary catheters, implants, contact lenses, etc.) as well as to form resilient biofilms in the cystic fibrosis lung environment [43]. Widely studied *P. aeruginosa* PAO1 strain has been shown to consist of four interconnected systems (IQS, PQS, las, rhl) to coordinate biofilm-formation and virulence-related traits (*e.g.*, elastase, rhamnolipids, and pyocyanin) [44], and is considered a standard model to study QS-dependent biofilm formation within this species [45]. Agents that would prevent the biofilm formation, or disrupt them, could be useful in the treatment of chronic and recurring infections, such as bacterial diseases, including the clinically relevant *P. aeruginosa*, whose biofilm shows higher tolerance to antibiotics and host's immune cells [46,47]. In view of these findings, we tested the most promising compounds against *P. aeruginosa* PAO1. We determined the MIC and MBC of compounds **15**, **16**, **19,** and **23** that showed the best anti-QS activity on the *C. violaceum* reporter strain, with minimal influence on bacterial growth (Figure 3). We also included compound **18** which showed very strong anti-QS activity but also had a somewhat stronger bactericidal effect on the *C. violaceum* reporter strain. For *P. aeruginosa* PAO1, the MIC of all five compounds was 1600 μM and the MBC was 3200 μM. We tested the anti-QS activity of the selected compounds (**15**, **16**, **18, 19**, and **23**) only at the subinhibitory 100 μM concentration because at that concentration these compounds showed the optimal anti-QS activity *vs.* bactericidal effect on the *C. violaceum* reporter strain. Furthermore, 100 μM concentration was substantially below the MIC and MBC values determined for *P. aeruginosa* PAO1. In *P. aeruginosa*, the PQS system is involved in the regulation of biofilm formation and the production of virulence factor pyocyanin. Therefore, we wanted to assess whether the selected compounds interfere with these processes. We first incubated these compounds with bacteria to see if their presence will inhibit the formation of biofilm (results presented in Figure 4). The results revealed that all compounds reduced the formation of biofilm, with compound **19** seeming to be the most effective when compared to the control cells with DMSO (over 60% inhibition compared to non-treated cells, as shown in the BFI column of Table 2). However, we also observed that compound **19** showed the strongest inhibition of cell growth, compared to other tested compounds (column GI in Table 2). We, therefore, calculated the biofilm index that includes both the biofilm mass and the cell count (Table 2). We found out that compound **15** showed the most potent effect on *P. aeruginosa* and reduced its ability to form biofilm by almost 50%, followed by compounds **16** and **19** (approximately 30 % reduction), compound **18** (approximately 20 % reduction), and least effective compound (less than 10 % reduction).

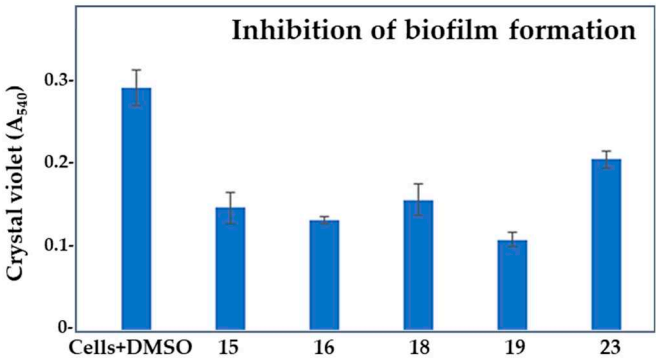

Figure 4. Effect of compounds **15**, **16**, **18**, **19,** and **23** at 100 μM concentrations on the biofilm formation of *P. aeruginosa* PAO1 compared to control cells treated with 1 % DMSO.

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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 μM 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)x100; BE – biofilm eradication (A540) ; PI – pyocyanin inhibition – (A520/A570)x100.

In the next experiment, we allowed P. aeruginosa to form biofilm for 24 hours and then treated established biofilm with selected compounds (Figure 5, Table 2 – column BE). Compound **16** was most effective and reduced the biofilm mass by 43%, then compound **19** with 35 % reduction, followed by compound **18** (almost 30% reduction) and compound **15** (almost 25 % reduction), while compound **23** was again the least effective with approximately 11 % reduction. Considering that compound **15** showed minimal inhibition of cell growth in the assay evaluating biofilm formation (less than 10 %, Table 2 - GI) we can assume that here the reduction in biofilm mass resulted from the interference with the exopolysaccharide, rather than from killing the cells within the biofilm. On the other hand, compounds **16**, **18,** and **19** showed more prominent inhibition of cell growth (in the range of 30–45 %, Table 2 - GI) and, therefore, appear to have a combined effect both on the biofilm matrix and cells within the biofilm.

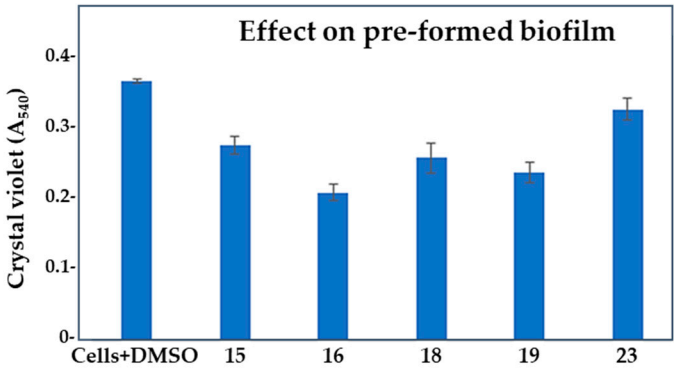

Figure 5. Effect of compounds **15**, **16**, **18**, **19,** and **23** in 100 μM concentrations on the established biofilm of *P. aeruginosa* PAO1 compared to control cells treated with 1% DMSO.

We also examined the effect of the compounds on the production of pyocyanin, a QS-controlled metabolite produced by *P. aeruginosa*. Again, the 100 μM concentration was selected, since it showed the strongest anti-QS activity without the prominent influence on the growth of the *C. violaceum* (Figure 3), and it was significantly below the MIC and MBC values for *P. aeruginosa*. Figure 6 and Table 2 (column PI) show that compound **23** was the most effective, reducing pyocyanin production by over 70 %. Compounds **15** and **19** similarly decreased the level of pyocyanin by approximately 40 %, while compounds **16** and **18** showed a decrease close to 20 %.

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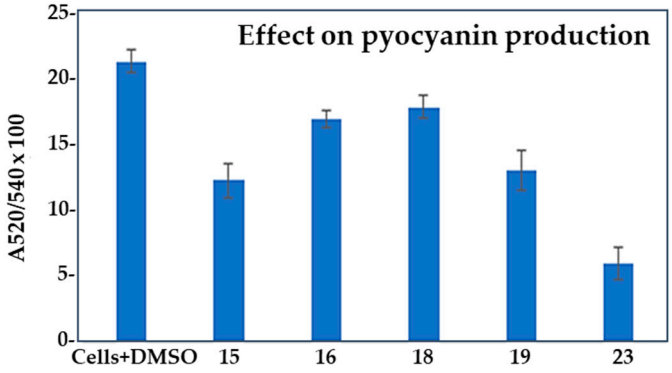

Figure 6. Effect of compounds **15**, **16**, **18**, **19,** and **23** at 100 μM concentrations on the production of pyocyanin by *P. aeruginosa* PAO1 compared to control cells treated with 1 % DMSO. Results are expressed as a ratio (A520/A570)x100 to normalize pyocyanin absorbance to the cell count.

3. Materials and Methods

3.1. Chemistry

3.1.1. General information

Melting points of compounds **9** and **15**–**19** were determined by differential scanning calorimetry (DSC). Thermograms were recorded using DSC 822e (Mettler Toledo), and analyzed by STARe software (V16.20b) (Mettler-Toledo). Melting points of compounds **8**, **11**, **12**, **14,** and **20**-**23** were determined on a Stuart Melting Point Apparatus (Barloworld Scientific, UK) in open capillaries and were uncorrected. FTIR-ATR spectra were recorded using a Fourier-Transform Infrared Attenuated Total Reflection UATR Two spectrometer (PerkinElmer, Waltham, MA, USA) in the range from 450 to 4000 cm^{-1}. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 600, Bruker Avance DRX500, Bruker Avance AV400, and Bruker Avance DPX300 spectrometers operating at 300 or 400 MHz for the ^{1}H and 75, 101, or 151 MHz for the ^{13}C nuclei, BrukerDPX 300 MHz, Bruker AV400 MHz and Bruker DRX 500 MHz (Bruker, Billerica, MA, USA). ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance III 600, Bruker Avance DRX500, Bruker Avance AV400, and Bruker Avance DPX300 spectrometers (Billerica, MA, USA), Chemical shifts are reported in parts per million (ppm) using tetramethylsilane (TMS) as a reference in the ^{1}H and DMSO residual peak as a reference in the ^{13}C spectra (39.52 ppm). Data for ^{1}H NMR are described as follows: chemical shift (δ in ppm), multiplicity (s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet; bs: broad signal), integration, coupling constant J (Hz). Samples were measured in DMSO-d_6 solutions at 20 °C in 5 mm NMR tubes. Mass spectra were recorded on Agilent 1200 Series HPLC coupled with Agilent 6410 Triple Quad (Agilent Technologies, St. Clara, CA, USA). LCMS data were acquired on a Waters Acquity UPLC instrument using a Waters Acquity UPLC C18, (2.1x50 mm, 1.7um) column, water/MeCN gradient, and 0.1% (v/v) formic acid as an acidic modifier or 0.05% (v/v) NH_4OH as a basic modifier. The column eluent was analyzed using a Waters SQ mass spectrometer with ESI scanning in both positive and negative ion modes from 100 to 2000 Da and reaction conversions expressed as percentage (where applied) by using a UV detector at 254 nm.

All compounds were routinely checked by TLC with silica gel 60F-254 glass plates (Merck, Germany) using DCM/MeOH 7.5:2.5, 7:3, 8.5:1.5, 8:2, 9.5:0.5, 9:1, cyclohexane/EtOAc/MeOH 3:1:0.5 and 1:1:0.5 as the solvent system. Spots were visualized by UV light (λ = 254 nm; 365 nm) and iodine vapor. Column chromatography was performed on silica gel 0.063–0.200 mm (Sigma-Aldrich, USA) with the same eluents used for TLC. Flash chromatography was performed on Interchem Puriflash XS 520 Plus, Interchim Puriflash SiHC (12–25 g; 15 μm) columns. All chemicals and solvents were of analytical grade and purchased from commercial sources.

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Dry toluene was obtained by the following procedure: toluene was extracted with water and dried over anhydrated calcium chloride, distilled and stored over elemental sodium. Dry DCM: DCM was extracted with water and dried over anhydrated calcium chloride and distilled. Dry DMF was stored over activated molecular sieves.

3.1.1. Synthesis of 4-amino-7-chloroquinoline intermediates 1 and 2

N^1-(7-chloroquinolin-4-yl)butane-1,4-diamine (**1**)

Compound **1** was prepared according to the published procedure [34]. A mixture of 4,7-dichloroquinoline (5.00 g, 25.3 mmol) and 1,2-diaminoethane (13.50 g, 227.2 mmol) was gradually heated to 80 °C for 1 h while stirring. The reaction mixture was then heated to 120 °C and stirred for an additional 6 h and poured on iced water and stirred for 18 h. 5.2 g (93 %) of the white solid **1** was filtered off, washed with water, and vacuum-dried.

^{1}H NMR (DMSO-d_6, δ ppm, *J*/Hz) δ 8.38 (d, *J* = 4.83 Hz, 1H), 8.28 (d, *J* = 9.05 Hz, 1H), 7.78 (d, *J* = 3,02 Hz, 1H), 7.43 (d, *J* = 8.99 Hz, 1H), 7.29–7.20 (bs, 1H), 6.48 (d, *J* = 5.99 Hz, 1H), 3.25 (q, *J* = 5.99 Hz, 2H), 2.82 (t, *J* = 6.74 Hz, 2H).

N^1-(7-chloroquinolin-4-yl)butane-1,4-diamine (**2**)

Compound **2** was prepared according to the published procedure [33]. A mixture of 4,7-dichloroquinoline (0.400 g, 2.02 mmol) and 1,4-diaminobutane (1.78 g, 20,2 mmol) was stirred under microwave irradiation (300 W) at 95 °C for 1 h. The reaction mixture was then diluted with dichloro-methane (100 mL), extracted with 5 % NaOH (3 × 100 mL), and washed with water (1 × 100 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The crude product was triturated with diethyl ether to give 0.428 g (86 %) of white solid **2**.

^{1}H NMR (DMSO-d_6, δ ppm, *J*/Hz) δ 8.39 (d, *J* = 5.4 Hz, 1H), 8.27 (d, *J* = 9.0 Hz, 1H), 7.78 (d, *J* = 2,2 Hz, 1H), 7.43 (dd, *J* = 9.0, 2.2 Hz, 1H), 7.39 (s, 1H), 6.46 (d, *J* = 5.5 Hz, 1H), 3.26 (dd, *J* = 12.0, 6.9 Hz, 2H), 2.59 (t, *J* = 6.9 Hz, 2H), 1.69 (dt, *J* = 14.8, 7.4 Hz, 2H), 1.46 (dt, *J* = 14.0, 6.9 Hz, 2H).

3.1.2. Synthesis of methyl anthranilates 3 and hydrazides 4.

Methyl anthranilates **3a–f** were either purchased from commercial sources (**3a–c**) or prepared from the corresponding anthranilic acid (**3d–f**) according to the previously published procedures [35,36].

Hydrazides **4a–f** were obtained in the reaction of the corresponding methyl anthranilate (**3a–f**) and hydrazine hydrate according to the previously published procedure [35].

3.1.3. General procedure for the synthesis of 5-(2-aminophenyl)-l,3,4-oxadiazole-2(3H)-ones 5a–f

The compounds **5a–f** were obtained according to slightly modified procedures [35].

1,1′-carbonyldiimidazole (0.254 g, 1.6 mmol) was added to a solution of the corresponding anthranilic acid hydrazide **4a–f** (1.4 mmol) in dry DMF (1 mL). The reaction mixture was stirred for 4-18 hours at room temperature, followed by the addition of ethyl acetate (30 mL) and extraction with brine (3 × 30 mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The crude product was used in successive reaction steps with **(5d**) or without **(5a–c**, **5e**, **5f**) trituration with diethyl ether.

5-(2-aminophenyl)-1,3,4-oxadiazol-2(3*H*)-one (**5a**)

Hydrazide **4a**: 0.212 g; ^{1}H NMR (DMSO-d$_6$): δ 12.70–12.27 (bs, 1H), 7.44 (dd, 1H, *J* = 8.0, 1.6 Hz), 7.21 (dt, 1H, *J* = 7.2, 1.6 Hz), 6.84 (dd, 1H, *J* = 8.4, 0.8 Hz), 6.64 (t, 1H, *J* = 7.2, 1.2 Hz),6.37–6.29 (s, 2H).

5-(2-amino-6-fluorophenyl)-1,3,4-oxadiazol-2(3*H*)-one (**5b**)

Hydrazide **4b**: 0.236 g.

5-(2-amino-4-chlorophenyl)-1,3,4-oxadiazol-2(3*H*)-one (**5c**)

Hydrazide **4c**: 0.260 g.

5-(2-amino-5-chlorophenyl)-1,3,4-oxadiazol-2(3*H*)-one (**5d**)

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Hydrazide **4d**: 0.260 g; yield: 0.177 g (60 %); IR (ATR, ν/cm^{-1}) 3465, 3364, 3170, 1734, 1628, 1562, 1488, 1310, 1257, 1161, 1042, 813, 717; ^{1}H NMR (DMSO-d$_6$): δ 12.63 (bs, 1H), 7.39 (d, 1H, J = 2.6 Hz), 7.25 (dd, 1H, J = 8.9, 2.6 Hz), 6.88 (d, 1H, J = 8.9 Hz), 6.45 (s, 2H).

5-(2-amino-6-chlorophenyl)-1,3,4-oxadiazol-2(3*H*)-one (**5e**)

Hydrazide **4e**: 0.260 g.

5-(2-amino-5-bromophenyl)-1,3,4-oxadiazol-2(3*H*)-one (**5f**)

Hydrazide **4f**: 0.322 g.

3.1.4. General procedure for the synthesis of acylsemicarbazides (6–10)

N^1-(7-chloroquinolin-4-yl)athane-1,2-diamine **1** (0.213 g, 0,961 mmol) was added to a suspension of an appropriate 5-(2-aminophenyl)-l,3,4-oxadiazole-2(3*H*)-one **5** (0.915 mmol) in 5-10 mL of anhydrous EtOH. The reaction mixture was refluxed at 100 °C for 18-72 hours and cooled to room temperature. The crude product was filtered off and washed with EtOH and vacuum-dried (**6–9**) or purified using flash chromatography (**10**) (eluent DCM/MeOH 80:20).

2-(2-aminobenzoyl)-*N*-(2-((7-chloroquinolin-4-yl)amino)ethyl)hydrazine-1-carboxamide (**6**)

Oxadiazol-2-one **5a**: 0.162 g; reaction time: 48 h; yield 0.179 g (49 %); mp nd; ^{1}H NMR (DMSO-d$_6$): δ 9.93-9.72 (bs, 1H), 8.40 (d, 1H, J = 5.2 Hz), 8.16 (d, 1H, J = 8.8 Hz), 8.05 – 7.92 (bs, 1H), 7.79 (d, 1H, J = 2.8 Hz), 7.60 (d, 1H, J = 8.4 Hz), 7.43 (dd, 1H, J = 10.4, 2.4 Hz), 7.48–7.36 (m, 1H), 7.18 (dt, 1H, J = 8.4, 1.2 Hz), 6.86–6.75 (bs, 1H), 6.72 (d, 1H, J = 6.00 Hz), 6.57 (d, 1H, J = 5.6 Hz), 6.51 (t, 1H, J = 7.6 Hz), 6.44–6.32 (bs, 2H), 3.40–3.25 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ): δ 168.9, 159.3, 152.0, 150.1, 150.0, 149.0, 133.4, 132.2, 128.4, 127.5, 124.1, 123.8, 117.3, 116.3, 114.4, 112.4, 98.6, 43.4, 37.8 ppm; ESI-MS: m/z 399.14 (M+1)$^+$.

2-(2-amino-6-fluorobenzoyl)-*N*-(2-((7-chloroquinolin-4-yl)amino)ethyl)hydrazine-1-carboxamide (**7**)

Oxadiazol-2-one **5b**: 0.179 g, reaction time: 18 h; the structure of the product was indirectly confirmed in the successive reaction step.

2-(2-amino-4-chlorobenzoyl)-*N*-(2-((7-chloroquinolin-4-yl)amino)ethyl)hydrazine-1-carboxamide (**8**)

Oxadiazol-2-one **5c**: 0.194 g; reaction time: 72 h; yield: 0.177 g (45 %); mp 222.5–224 °C; IR (ATR, ν/cm^{-1}) 3344, 1661, 1630, 1613, 1587, 1539, 1449, 1370, 1255, 1235, 1146, 1081, 941, 877, 807; ^{1}H NMR (DMSO-d$_6$): δ 10.00–9.87 (bs, 1H), 8.41 (d, 1H, J = 5.4 Hz), 8.16 (d, 1H, J = 8.4 Hz), 8.02 (s, 1H), 7.79 (d, 1H, J = 2.4 Hz), 7.61 (d, 1H, J = 9,0 Hz), 7.44 (dd, 1H, J = 9.6, 2.4 Hz), 7.41 (t, 1H, J = 4.8 Hz), 6.88-6.80 (bs, 1H), 6.79 (d, 1H, J = 2.4 Hz), 6.73-6.65 (bs, 2H), 6.56 (d, 1H, J = 5.4 Hz), 6.54 (dd, 1H, J = 9.0, 1.8 Hz), 3.38-3.27 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 168.2, 159.2, 152.0, 151.2, 150.0, 149.0, 136.7, 133.3, 130.4, 127.5, 124.1, 123.8, 117.3, 115.0, 114.1, 111.2, 98.6, 43.3, 37.8 ppm; ESI-MS: m/z 432.97 (M+1)$^+$.

2-(2-amino-6-chlorobenzoyl)-*N*-(2-((7-chloroquinolin-4-yl)amino)ethyl)hydrazine-1-carboxamide (**9**)

Oxadiazol-2-one **5e**: 0.194 g; reaction time: 18 h; yield: 0.192 g (49 %); mp 213 °C; IR (ATR, ν/cm^{-1}) 3231, 1687, 1629, 1603, 1582, 1540, 1457, 1428, 1319, 1250, 1146, 907, 877, 811, 763; ^{1}H NMR (DMSO-d$_6$): δ 10.08–9.85 (bs, 1H), 8.56 (s, 1H), 8.41 (d, 1H, J = 6.0 Hz), 8.18 (d, 1H, J = 9.0 Hz), 7.79 (d, 1H, J = 2.0 Hz), 7.46 (dd, 1H, J = 4.5, 2.0 Hz), 7.40 (t, 1H, J = 5.0 Hz), 7.05 (t, 1H, J = 7.5 Hz), 6.63 (d, 1H, J = 9.0 Hz), 6.63 (s, 1H), 6.56 (d, 1H, J = 5.0 Hz), 6.54 (d, 1H, J = 6.5 Hz), 5.99 (s, 2H), 3.46-3.41 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 165.9, 159.2, 152.0, 150.0, 149.0, 147.9, 133.4, 130.6, 130.5, 127.5, 124.2, 123.9, 119.1, 117.3, 115.1, 113.2, 98.7, 43.3, 38.1 ppm; ESI-MS: m/z 433.1 (M+1)$^+$.

2-(2-amino-5-bromobenzoyl)-*N*-(2-((7-chloroquinolin-4-yl)amino)ethyl)hydrazine-1-carboxamide (**10**)

Oxadiazol-2-one **5f**: 0.234 g; reaction time: 18 h; the structure of the product was indirectly confirmed in the successive reaction step.

3.1.5. General procedure for the synthesis of acylsemicarbazides (11-14)

N^1-(7-chloroquinolin-4-yl)butane-1,4-diamine **2** (0.240 g, 0,961 mmol) was added to a suspension of an appropriate 5-(2-aminophenyl)-l,3,4-oxadiazole-2(3*H*)-one **5** (0.915 mmol) in 5 mL of anhydrous

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EtOH. The reaction mixture was refluxed at 100 °C for 24–48 hours and cooled at room temperature, the organic solvent was evaporated and the crude product was purified using column chromatography (DCM/MeOH 75:25).

2-(2-aminobenzoyl)-*N*-(4-((7-chloroquinolin-4-yl)amino)butyl)hydrazine-1-carboxamide (**11**)

Oxadiazol-2-one **5a**: 0.162 g; yield: 0.155 g (40%); mp 184–185 °C; IR (ATR, ν/cm^{-1}) 3328, 2934, 1645, 1613, 1580, 1532, 1450, 1368, 1251, 1137, 852, 806, 748; ^{1}H NMR (DMSO-d$_6$): δ 9.78 (s, 1H), 8.38 (d, 1H, *J* = 5,4 Hz), 8.28 (d, 1H, *J* = 9.0 Hz), 7.77 (d, 1H, *J* = 2.2 Hz), 7.69 (s, 1H), 7.58 (d, 1H, *J* = 7.9 Hz), 7.43 (dd, 1H, *J* = 9.0, 2.3 Hz), 7.34 (t, 1H, *J* = 5.4 Hz), 7.20 – 7.13 (m, 1H), 6.70 (dd, 1H, *J* = 8.4, 1.2 Hz), 6.52 – 6.46 (m, 3H), 6.41 (s, 2H), 3.28 (q, 2H, *J* = 7,1 Hz), 3.10 (q, 2H, *J* = 6.6 Hz), 1.66 (q, 2H, *J* = 7.3 Hz), 1.53 (q, 2H, *J* = 7.1 Hz) ppm; ^{13}C NMR (DMSO-d$_6$): δ 168.9, 158.7, 151.9, 150.1, 149.8, 149.0, 133.3, 132.1, 128.0, 127.4, 124.1, 124.0, 117.4, 116.2, 114.4, 112.7, 98.7, 42.2, 38.9, 27.6, 25.1 ppm; ESI-MS: *m*/*z* 427.1 (M+1)$^+$.

2-(2-amino-4-chlorobenzoyl)-*N*-(4-((7-chloroquinolin-4-yl)amino)butyl)hydrazine-1-carboxamide (**12**)

Oxadiazol-2-one **5c**: 0.194 g; yield 0.086 g (21 %); mp 174–175 °C; IR (ATR, ν/cm^{-1}) 3306, 2956, 2869, 1612, 1578, 1482, 1430, 1368, 1322, 1253, 1207, 1141, 1078, 920, 847, 809, 766, 643; ^{1}H NMR (DMSO-d$_6$): δ 9.86 (s, 1H), 8.39 (d, 1H, *J* = 5,4 Hz), 8.29 (d, 1H, *J* = 9.1 Hz), 7.78 (d, 1H, *J* = 2.2 Hz), 7.72 (s, 1H), 7.58 (d, 1H, *J* = 8.5 Hz), 7.44 (dd, 1H, *J* = 9.0, 2.3 Hz), 7.42 (t, 1H, *J* = 5.3 Hz), 6.77 (d, 1H, *J* = 2.2 Hz), 6.66 (s, 2H), 6.52 (dd, 2H, *J* = 8.5, 2.2 Hz), 6.49 (d, 1H, *J* = 5.5 Hz), 3.29 (q, 2H, *J* = 7,1 Hz), 3.09 (q, 2H, *J* = 6.6 Hz), 1.69 – 1.62 (m, 2H), 1.56 – 1.49 (m, 2H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 168.1, 158.6, 151.5, 151.1, 150.3, 148.6, 136.6, 133.5, 130.3, 127.1, 124.2, 124.1, 117.4, 115.0, 114.1, 111.5, 98.7, 42.2, 38.9, 27.6, 25.1 ppm; ESI-MS: *m*/*z* 461.1 (M+1)$^+$.

2-(2-amino-5-chlorobenzoyl)-*N*-(4-((7-chloroquinolin-4-yl)amino)butyl)hydrazine-1-carboxamide (**13**)

Oxadiazol-2-one **5d**: 0.194 g; the structure of the compounds was indirectly confirmed in the next reaction step.

2-(2-amino-6-chlorobenzoyl)-*N*-(4-((7-chloroquinolin-4-yl)amino)butyl)hydrazine-1-carboxamide (**14**)

Oxadiazol-2-one **5e**: 0.194 g; yield 0.085 g (22 %); mp 144–155.5 °C; IR (ATR, ν/cm^{-1}) 3345, 3229, 2934, 1651, 1601, 1579, 1532, 1469, 1450, 1368, 1332, 1279, 1250, 1206, 1137, 1081, 1041, 903, 852, 805, 644; NMR (DMSO-d$_6$): δ 9.92 (s, 1H), 8.39 (d, 1H, *J* = 5,4 Hz), 8.28 (d, 1H, *J* = 9.1 Hz), 8.17 (s, 1H), 7.77 (d, 1H, *J* = 2.2 Hz), 7.42 (dd, 1H, *J* = 9.0, 2.3 Hz), 7.32 (t, 1H, *J* = 5.4 Hz), 7.04 (t, 1H, *J* = 8.1 Hz), 6.60 (dd, 1H, *J* = 8.3, 1.0 Hz), 6.54 (dd, 2H, *J* = 7.8, 0.9 Hz), 6.49 (d, 1H, *J* = 5.5 Hz), 6.35 (t, 1H, *J* = 5.8 Hz), 5.93 (s, 2H), 3.29 (q, 2H, *J* = 7,1 Hz), 3.13 (q, 2H, *J* = 6.6 Hz), 1.72 – 1.64 (m, 2H), 1.59 – 1.51 (m, 2H) ppm; ^{13}C NMR (DMSO-*d*$_6$): δ 166.0, 158.6, 152.0, 150.1, 149.1, 147.9, 133.3, 130.6, 127.5, 124.1, 124.0, 119.1, 117.5, 115.1, 113.2, 98.7, 42.1, 39.1, 27.5, 25.1 ppm; ESI-MS: *m*/*z* 461.1 (M+1)$^+$.

3.1.6. General procedure for the synthesis of oxadiazoles (15-19)

Triphenylphosphine (0,168 g, 0,639 mmol for **15**,**17**-**19** or 0.223 g, 0.852 mmol for **16**), TEA (0.238 mL, 0.172 g, 1.704 mmol) and carbon tetrachloride (0,091 mL, 0,105 g, 0,682 mmol) were added to the solution of an appropriate acylsemicarbazide (**6**–**10**) (0.426 mmol) in dry dichloromethane (4 mL). The reaction mixture was refluxed 18 - 72 h at 46 °C. The reaction mixture was cooled to r.t. and 25 mL of dichloromethane was added. The organic layer was extracted with water (3 × 20 mL), dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The crude product was purified using flash chromatography (Interchim 12 g, 15 µm) and trituration with MeOH (**15**-**18**) or diisopropyl ether (**19**).

N'-(5-(2-aminophenyl)-1,3,4-oxadiazol-2-yl)-*N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (**15**)

Acylsemicarbazide **6**: 0.170 g; additional 0.056 g (0.213 mmol) of triphenylphosphine was added to the reaction mixture and refluxed at 46 °C for another 72 h; eluent: DCM/MeOH 9:1; yield 0.050 g (31 %); mp 219 °C; IR (ATR, ν/cm^{-1}) 3350, 1619, 1581, 1548, 1494, 1375, 1332, 1146, 1024, 806, 738; ^{1}H NMR (DMSO-d$_6$): δ 8.43 (d, 1H, *J* = 6.0 Hz), 8.22 (d, 1H, *J* = 9.6 Hz), 7.92 (t, 1H, *J* = 5.4 Hz), 7.79 (d, 1H, *J* = 4.2 Hz), 7.52 (t, 1H, *J* = 5.4 Hz), 7.45 (dd, 1H, *J* = 4.2Hz, 2.4 Hz), 7.38 (dd, 1H, *J* = 7.5, 1.2 Hz), 7.16

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(dt, 1H, J = 7.8, 1.8 Hz), 6.83 (d, 1H, *J* = 4.2 Hz), 6.64 (d, 1H, *J* = 3.0 Hz), 6.60 (t, 1H, *J* = 7.2 Hz), 6.58 – 6.52 (bs, 2H), 3.55 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 162.5, 158.7, 152.3, 150.6, 149.5, 147.2, 134.0, 131.4, 127.9, 126.6, 124.6, 124.5, 117.9, 115.9, 115.8, 105.6, 99.1, 41.9, 41.3 ppm; ESI-MS: *m*/*z* 380.93 (M+1)$^+$.

N′-(5-(2-amino-6-fluorophenyl)-1,3,4-oxadiazol-2-yl)-*N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (**16**)

Acylsemicarbazide **7**: 0.178 g; eluent: DCM/MeOH 0-20 %; yield 0.031 g (18 %); mp 191 °C; IR (ATR, ν/cm^{-1}) 3460, 3304, 1641, 1628, 1579, 1540, 1453, 1483, 1378, 1233, 1076, 1037, 998, 914, 867, 842, 810, 760; ^{1}H NMR (DMSO-d$_6$): δ 8.46 (d, 1H, *J* = 10.8 Hz), 8.30 (d, 1H, *J* = 9.6 Hz), 8.04 (t, 1H, *J* = 7.2 Hz), 8.03 – 7.93 (bs, 1H), 7.83 (d, 1H, *J* = 2.4 Hz), 7.53 (dd, 1H, *J* = 8.4, 1.8 Hz), 7.15 (q, 1H, *J* = 7.2 Hz), 6.85 (s, 2H), 6.71 (d, 1H, *J* = 5.4 Hz), 6.60 (d, 1H, *J* = 8.4 Hz), 6.44 – 6.40 (m, 1H), 3.63 (q, 2H), 3.53 (q, 2H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 163.1, 161.2, 159.5, 151.9, 150.2, 149.4, 135.0, 132.0 (d, *J* = 46.2 Hz), 125.9, 125.3, 124.9, 117.5, 111.7, 101.9 (d, *J* = 87.6 Hz), 99.1, 95.1 (d, *J* = 60.0 Hz), 42.0, 41.3 ppm; ESI-MS: *m*/*z* 399.16 (M+1)$^+$.

N′-(5-(2-amino-4-chlorophenyl)-1,3,4-oxadiazol-2-yl)-*N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (**17**)

Acylsemicarbazide **8**: 0.185 g; eluent: DCM/MeOH 0–20 %; yield 0.044 g (25 %); mp 208 °C; IR (ATR, ν/cm^{-1}) 3489, 3363, 1641, 1619, 1579, 1537, 1488, 1446, 1331, 1265, 1145, 1026, 844, 815, 787, 770, 737; ^{1}H NMR (DMSO-d$_6$): δ 8.42 (d, 1H, *J* = 5.4 Hz), 8.20 (d, 1H, *J* = 9.6 Hz), 7.97 (t, 1H, *J* = 5.4 Hz), 7.78 (d, 1H, *J* = 1.8 Hz), 7.46 (m, 1H), 7.45 (dd, 1H, *J* = 9.6, 2.4 Hz), 7.34 (d, 1H, *J* = 9.0 Hz), 6.89 (d, 1H, *J* = 2.4 Hz), 6.83–6.78 (bs, 2H), 6.62 (m, 2H), 3.54 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 162.6, 157.9, 152.4, 150.5, 148.6, 148.2, 135.7, 133.9, 128.2, 128.0, 124.6, 124.5, 118.0, 115.6, 114.8, 104.6, 99.1, 41.8, 41.2 ppm; ESI-MS: *m*/*z* 414.89 (M+1)$^+$

N′-(5-(2-amino-6-chlorophenyl)-1,3,4-oxadiazol-2-yl)-*N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (**18**)

Acylsemicarbazide **9**: 0.185 g; reaction time: 72 h; eluent: DCM/MeOH 0-17 %; yield 0.041 g (23 %); mp 210 °C; IR (ATR, ν/cm^{-1}) 3311, 1627, 1611, 1578, 1453, 1428, 1331, 1281, 1140, 1019, 907, 852, 778, 725; ^{1}H NMR (DMSO-d$_6$): δ 8.68–8.58 (bs, 1H), 8.50 (d, 1H, *J* = 6.6 Hz), 8.41 (d, 1H, *J* = 8.4 Hz), 8.01 (t, 1H, *J* = 5.4 Hz), 7.89 (s, 1H), 7.62 (d, 1H, *J* = 9.0 Hz), 7.14 (t, 1H, *J* = 7.8 Hz), 6.82 (d, 1H, *J* = 7.2 Hz), 6.75 (d, 1H, *J* = 9.0 Hz), 6.66 (d, 1H, *J* = 8.4 Hz), 6.22–6.12 (bs, 2H), 3.70 (q, 2H, *J* = 3.6 Hz), 3.55 (q, 2H, *J* = 3.5 Hz) ppm; ^{13}C NMR (DMSO-d$_6$): δ 163.4, 154.4, 151.7, 150.7, 149.8, 148.8, 136.5, 133.5, 132.7, 131.8, 127.1, 124.2, 124.1, 117.4, 116.5, 114.1, 105.7, 98.6, 41.4, 40.9 ppm; ESI-MS: *m*/*z* 415.12 (M+1)$^+$

N′-(5-(2-amino-5-bromophenyl)-1,3,4-oxadiazol-2-yl)-*N*-(7-chloroquinolin-4-yl)ethane-1,2-diamine (**19**)

Acylsemicarbazide **10**: 0.204 g; an additional 0.056 g (0.213 mmol) of triphenylphosphine was added to the reaction mixture and refluxed at 46 °C for another 72 h; flash chromatography eluent: DCM/MeOH 0-15%; yield 0.018 g (9 %); mp 213 °C; IR (ATR, ν/cm^{-1}) 3456, 3304, 1627, 1604, 1579, 1541, 1452, 1333, 1282, 1231, 1075, 1039, 866, 841, 810, 779; ^{1}H NMR (DMSO-d$_6$): δ 8.46 (d, 1H, *J* =6.6 Hz), 8.26 (d, 1H, *J* = 9.6 Hz), 8.00 (t, 1H, *J* = 6.0 Hz), 7.82 (d, 1H, *J* = 2.4 Hz), 7.86–7.73 (m, 1H), 7.49 (dd, 1H, *J* = 8.4, 2.4 Hz), 7.47 (d, 1H, *J* = 3.6 Hz), 7.29 (dd, 1H, *J* =9.0, 2.4 Hz), 6.82 (d, 1H, *J* = 9.6 Hz), 6.75–6.70 (bs, 2H), 6.69 (d, 1H, *J* = 5.4 Hz), 3.60–3.55 (m, 4H) ppm; ESI-MS: *m*/*z* 461.0 (M+1)$^+$

3.1.7. General procedure for the synthesis of oxadiazoles (20–23)

PPh_3 (0.168 g, 0.639 mmol), TEA (0.238 mL, 0.172 g, 1.704 mmol), and carbon tetrachloride (0.091 mL, 0.105 g, 0.682 mmol) were added to the solution of an appropriate acylsemicarbazide (**11–14**) (0.426 mmol) in dry dichloromethane (4 mL). The reaction mixture was stirred for 18 h at 46 °C. The reaction mixture was cooled to r.t. and 30 mL of dichloromethane was added. The organic layer was extracted with water (3 × 30 mL) and evaporated under reduced pressure. The crude product was purified using column chromatography and trituration with diethyl ether.

N^1-(5-(2-aminophenyl)-1,3,4-oxadiazol-2-yl)-*N*^{4}-(7-chloroquinolin-4-yl)butane-1,4-diamine (**20**)

Acylsemicarbazide **11**: 0.182 g; column chromatography eluent: DCM/MeOH 85:15; yield 0.128 g (74 %); mp 127.0–128.0 °C; IR (ATR, ν/cm^{-1}) 3646, 3448, 3319, 3195, 2948, 1636, 1585, 1610, 1547, 1494,

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1453, 1367, 1332, 1268, 1199, 1147, 1025, 897, 870, 814, 764, 739, 645; ^{1}H NMR (DMSO-d$_6$): δ 8.40 (d, 1H, J = 5.5 Hz), 8.30 (d, 1H, J = 9.1 Hz), 7.81 – 7.75 (m, 2H), 7.51 – 7.42 (m, 3H), 7.19 – 7.14 (m, 1H), 6.84 (dd, 1H, J = 8.3, 1.1 Hz), 6.62 (t, 1H, J = 8.1 Hz), 6.57 (s, 2H), 6.52 (d, 1H, J = 5,6 Hz), 3.36 – 3.28 (m, 4H), 1.78 – 1.69 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 162.1, 158.0, 151.2, 150.5, 148.3, 146.7, 133.7, 130.8, 126.8, 126.1, 124.2, 117.3, 115.4, 115.3, 105.2, 98.7, 42.3, 42.1, 26.5, 25.1 ppm; ESI-MS: m/z 409.0 (M+1)$^+$.

N^1-(5-(2-amino-4-chlorophenyl)-1,3,4-oxadiazol-2-yl)-*N*^{4}-(7-chloroquinolin-4-yl)butane-1,4-diamine (**21**)

Acylsemicarbazide **12**: 0.196 g; column chromatography eluent: DCM/MeOH 85:15; yield 0.115 g (62 %); mp 123.0–124.5°C; IR (ATR, ν/cm^{-1}) 3454, 3319, 2949, 1644, 1610, 1585, 1546, 1489, 1426, 1369, 1350, 1335, 1262, 1201, 1141, 1085, 1029, 901, 864, 844, 816, 791, 764; ^{1}H NMR (DMSO-d$_6$): δ 8.40 (d, 1H, J = 5.5 Hz), 8.30 (d, 1H, J = 9.1 Hz), 7.83 (t, 1H, J = 5.6 Hz), 7.79 (d, 1H, J = 2.1 Hz), 7.50 (t, 1H, J = 4.9 Hz), 7.46 (dd, 1H, J = 9.0, 2.1 Hz), 7.41 (d, 1H, J = 8.5 Hz), 6.90 (d, 1H, J = 2.0 Hz), 6.81 (s, 2H), 6.64 (dd, 1H, J = 8.5, 2.0 Hz), 6.53 (d, 1H, J = 5.6 Hz), 3.80 – 3.08 (m, 4H), 1.94 – 1.51 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 162.6, 157.7, 151.6, 151.0, 148.6, 148.2, 135.6, 134.2, 128.2, 127.1, 124.7, 117.7, 115.6, 114.8, 104.7, 99.1, 42.8, 42.6, 26.9, 25.5 ppm; ESI-MS: m/z 443.1 (M+1)$^+$.

N^1-(5-(2-amino-5-chlorophenyl)-1,3,4-oxadiazol-2-yl)-*N*^{4}-(7-chloroquinolin-4-yl)butane-1,4-diamine (**22**)

Acylsemicarbazide **13**: 0.196 g; column chromatography eluent: DCM/MeOH 85:15; yield 0.104 g (55 %); mp 125 °C; IR (ATR, ν/cm^{-1}) 3395, 3097, 2947, 2869, 1672, 1613, 1581, 1547, 1492, 1471, 1450, 1357, 1322, 1254, 1212, 1138, 1058, 873, 721; ^{1}H NMR (DMSO-d$_6$): δ 8.39 (d, 1H, J = 5.5 Hz), 8.29 (d, 1H, J = 9.0 Hz), 7.84 (t, 1H, J = 5.7 Hz), 7.78 (d, 1H, J = 2.3 Hz), 7.48 – 7.44 (m, 2H), 7.39 (d, 1H, J = 2.5 Hz), 7.20 (dd, 1H, J = 8.8, 2.5 Hz), 6.87 (d, 1H, J = 8.8 Hz), 6.71 (s, 2H), 6.52 (d, 1H, J = 5.5 Hz), 3.40 – 3.31 (m, 4H), 1.77 – 1.70 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 162.3, 156.9, 151.3, 150.4, 148.4, 145.5, 133.7, 130.5, 126.9, 124.9, 124.2, 124.2, 118.4, 117.3, 117.2, 106.3, 98.7, 42.3, 42.1, 26.5, 25.1 ppm; ESI-MS: m/z 443.1 (M+1)$^+$.

N^1-(5-(2-amino-6-chlorophenyl)-1,3,4-oxadiazol-2-yl)-*N*^{4}-(7-chloroquinolin-4-yl)butane-1,4-diamine (**23**)

Acylsemicarbazide **14**: 0.196 g; column chromatography eluent: DCM/MeOH 75:25; yield 0.077 g (41 %); mp 133.0–134.0 °C; IR (ATR, ν/cm^{-1}) 3278, 2945, 2866, 1645, 1610, 1584, 1465, 1357, 1334, 1282, 1233, 1140, 1017, 900, 855, 811, 774, 726; ^{1}H NMR (DMSO-d$_6$): δ 8.41 (d, 1H, J = 5.7 Hz), 8.35 (d, 1H, J = 9.1 Hz), 7.81 (dd, 2H, J = 10.3, 3.9 Hz), 7.74 (s, 1H), 7.50 (dd, 1H, J = 9.0, 2.2 Hz), 7.14 (t, 1H, J = 8.1 Hz), 6.77 (dd, 1H, J = 7.8, 0.7 Hz), 6.56 (d, 1H, J = 5.8 Hz), 6.20 (s, 2H), 3.46 – 3.23 (m, 4H), 1.80 – 1.68 (m, 4H) ppm; ^{13}C NMR (DMSO-d$_6$): δ 162.9, 154.7, 151.5, 150.7, 150.2, 147.6, 134.6, 133.1, 132.2, 126.4, 125.0, 124.9, 117.6, 117.0, 114.5, 106.3, 99.1, 42.8, 42.7, 26.9, 25.6 ppm; ESI-MS: m/z 443.0 (M+1)$^+$.

3.2. Anti-QS and bactericidal activity screening

The Chromobacterum violaceum ATCC31532 (ATCC; Wesel, Germany) was used as the indicator reporter strain to screen the title compounds for anti-QS/-biofilm or bactericidal activities [19,40,48]. Shortly, the reporter strain grown overnight at 27 °C on Luria-Bertani agar (Fischer Scientific, Leicestershire, UK) was suspended in PDYT (0.5% peptone, 0.3 % D-glucose, 0.25 % yeast extract, 0.05 % L-tryptophan, m/v) to achieve OD_{600} = 0.02. Then, 200 µL of the obtained cell suspension with 2 % DMSO (control) or with the indicated compounds dissolved in DMSO at varying concentrations (400, 200, 100, and 40 µM) were added into the wells in two parallel 96-well plates (Tissue Culture Treated, polystyrene, flat-bottom, Becton Dickinson). Quercetin [49] and azithromycin (Sigma-Aldrich) at 400 µM dissolved in DMSO were used as positive controls for QS inhibition and cell viability (bactericidal agent), respectively. The plates were incubated at 27 °C under aerobic conditions (200 rpm) for 22 hours. Resazurin, a redox-sensitive dye that is reduced to fluorescent resorufin only by viable cells, was added at 200 µM per well in the first 96-well plate to assess the bactericidal effects of the compounds [50,51]. The 96-well plates, with/without the resazurin, were shaken for an additional 30 min (pm) in the dark and then centrifuged (4,000 rpm, for 20 min, 20 °C) to pellet insoluble violacein and cells. Resorufin containing supernatants (100 µL) were transferred into a new plate and the produced/remaining fluorescence was recorded with a

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PerkinElmer Victor3 multilabel microtiter plate reader using an excitation/emission wavelengths of 550/590 nm. Supernatants from the 96-well plate without resazurin were removed and the pelleted violacein was dissolved in 96 % (v/v) ethanol. The 96-well plate was centrifuged (4,000 rpm for 20 min at 20 °C) and the supernatants with soluble violacein (100 µL) were transferred into a new 96-well plate. Changes in the violacein yields were monitored at 595 nm using the PerkinElmer Vic-tor3 reader. Both the anti-QS and bactericidal screening experiments were repeated twice with at least three technical replicates in each plate. Statistical parameters (Z′, S/N, and S/B) [52,53] for each assay were calculated to confirm the assay performance and high quality of the obtained results. Potency (half inhibitory concentrations, IC_{50}) calculation was conducted using the GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA, USA).

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

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for Pseudomonas aeruginosa PAO1 (Leibnitz Institute DSMZ 1707, Braunschweig, Germany) were determined for compounds **15**, **16**, **18**, **19,** and **23** (initially dissolved in DMSO) by the broth dilution method [54]. For MIC assessment P. aeruginosa PAO1 was grown overnight in Luria-Bertani (LB) medium, diluted with LB medium to 10^5 CFU/mL, and added to the 96-well plate with serially diluted compounds (from 3200 to 100 µM). After incubation at 37 °C for 24 h, the MIC value was determined as the minimum compound concentration that prevented visible growth. The MBC was evaluated by transferring 10 µL from the wells showing no bacterial growth after the MIC assay on the LB agar plate. After 24 h of incubation at 37 °C, the MBC was determined as the lowest concentration of the compound that showed no growth on LB agar plates. Three independent experiments with three technical replicates per experiment were performed for both the MIC and MBC assay.

3.4. Anti-biofilm assay

The effect of compounds on biofilm formation and on the eradication of preformed biofilm was studied on Pseudomonas aeruginosa PAO1 (Leibnitz Institute DSMZ 1707, Braunschweig, Germany). We performed three independent experiments with 8 technical replicates per experiment.

For the inhibition of biofilm formation, P. aeruginosa PAO1 was grown overnight in Luria-Bertani (LB) medium and diluted with LB medium to the optical density of 0.5 McFarland. To initiate QS-dependent biofilm formation, we performed an additional 1:100 dilution step to obtain the optical density of approximately 0.01 at 600 nm. In this second step 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) at 100 µM concentration and 100 µL was transferred to the 96 well microtiter plate with U-bottom in 8 technical replicates. Plates were incubated aerobically at 37°C for 24 h. Biofilm was detected as described in O'Toole G. A., 2011 [55]. Bacterial growth was measured at A570 (Wallac Victor 2 1420, Perkin Elmer), planktonic cells were removed, and biofilm was stained with 125 µL of 0.1 % crystal violet for 15 minutes. The microtiter plate was rinsed three times with H_2O, dried and biofilm was extracted using 150 µL of 30 % acetic acid. 125 µL of solubilized crystal violet was transferred to the new flat-bottom microtiter plate and absorbance was measured at 540 nm (Wallac Victor 2 1420, Perkin Elmer). Biofilm index was calculated as a ratio (A540/A570)x100. To study the effect of selected compounds (**15**, **16**, **18**, **19,** and **23**) on already formed biofilm, P. aeruginosa PAO1 was grown overnight in LB medium and diluted with LB medium to the optical density of 0.5 McFarland. Bacterial culture was then diluted 1:100 in M63 medium with 0.4 % Arg and 100 µL was transferred to the 96-well microtiter plate with a U-bottom and incubated aerobically at 37°C for 24 h to form biofilm. The bacterial suspension was removed, and wells were washed twice with M63 medium with 0.4 % Arg. 100 µL of 1 % DMSO in the M63 medium with 0.4 % Arg (control) or 100 µM indicated compounds in the M63 medium with 0.4 % Arg was added to each well in 8 technical replicates. Plates were further incubated at 37 °C for 24 h and biofilm was stained as described above.

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3.5. Effect on pyocyanin production

P. aeruginosa PAO1 cells were grown overnight in LB medium, adjusted to 0.5 McFarland with LB medium, and diluted 1:100 in 5 mL LB medium containing either 1 % DMSO (control) or 100 μM compounds **15**, **16**, **18**, **19,** and **23**. The culture was grown aerobically for 24 h at 37 °C and bacterial growth was measured at A570. Pyocyanin was extracted as described in Essar et al., 1990 [56]. Cells were pelleted and pyocyanin was extracted from the supernatant with 3 mL of chloroform, centrifuged for 10 minutes at 5,000 rpm (Thermo Jouan BR4i) and the chloroform layer was reextracted with 1 mL of 0.2 M HCl. Absorbance was measured at 520 nm and the results were expressed as a ratio (A520/A570)x100 to normalize pyocyanin values to cell growth. We performed three independent experiments with 2 technical replicates per experiment.

3.6. Statistical analysis

In anti-biofilm assays and the test on the pyocyanin production the data are presented as mean ± SD for the indicated number of independent experiments. Significance ($p < 0.05$) was determined using a one-way analysis of variance (ANOVA).

4. Conclusions

As the prevalence of antimicrobial resistance increases, there is an urgent need for new innovative therapeutic approaches. With this objective in mind, we have chosen to tackle the central cell-to-cell communication system (QS) and QS-dependent biofilm formation of Gram-negative pathogens by applying a molecular hybridization approach. We successfully synthesized and characterized a series of nine novel 1,3,4-oxadiazole hybrid compounds, incorporating 4-(2-aminoethyl/4-aminobuthyl)amino-7-chloro- quinoline and anthranilic acid scaffolds. Subsequently, their anti-QS and antibiofilm activities were evaluated using C. violaceum and P. aeruginosa as the biofilm models, representing Gram-negative bacteria using different QS signaling pathways to coordinate virulence and biofilm formation. Among the tested compounds, ethan-1,2-diamine 1,3,4-oxadiazoles **15**-**19** significantly reduced violacein production of the C. violaceum reporter strain, while only one butan-1,4-diamine 1,3,4-oxadiazole (**23**) exhibited comparable activity. Furthermore, we examined the effects of these compounds on biofilm formation, disruption of pre-formed biofilms, and pyocyanin production using P. aeruginosa PAO1 as the model. Once again, ethan-1,2-diamine 1,3,4-oxadiazoles showed significant anti-biofilm activity and inhibitory effect on pyocyanin production, while compound **23** was most effective only in blocking the production of this virulence factor. In our future studies, we will focus on expanding the chemical library around the most active compounds to establish a reliable SAR and to obtain more potent compounds.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1.

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