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Long-Chain 4-Aminoquinolines as Quorum Sensing Inhibitors in *Serratia marcescens* and *Pseudomonas aeruginosa*

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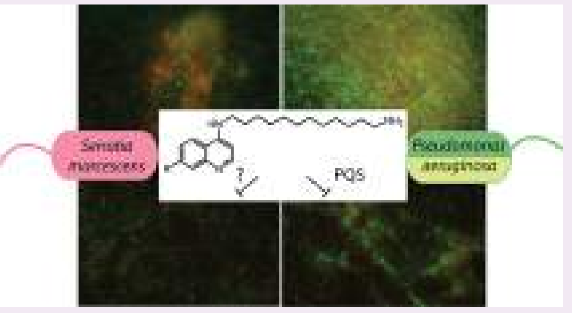
Supporting Information

ABSTRACT: Antibiotic resistance has become a serious global threat to public health; therefore, improved strategies and structurally novel antimicrobials are urgently needed to combat infectious diseases. Here we report a new type of highly potent 4-aminoquinoline derivatives as quorum sensing inhibitors in *Serratia marcescens* and *Pseudomonas aeruginosa*, exhibiting weak bactericidal activities (minimum inhibitory concentration (MIC) > 400 μM). Through detailed structure−activity study, we have identified 7-Cl and 7-CF_3 substituted *N*-dodecylamino-4-aminoquinolines (**5** and **10**) as biofilm formation inhibitors with 50% biofilm inhibition at 69 μM and 63 μM in *S. marcescens* and *P. aeruginosa*, respectively. These two compounds, **5** and **10**, are the first quinoline derivatives with anti-biofilm formation activity reported in *S. marcescens*. Quantitative structure−activity relationship (QSAR) analysis identified structural descriptors such as Wiener indices, hyper-distance-path index (HDPI), mean topological charge (MTC), topological charge index (TCI), and log $D(o/w)_{exp}$ as the most influential in biofilm inhibition in this bacterial species. Derivative **10** is one of the most potent quinoline type inhibitors of pyocyanin production described so far (IC_{50} = 2.5 μM). While we have demonstrated that **5** and **10** act as *Pseudomonas* quinolone system (PQS) antagonists, the mechanism of inhibition of *S. marcescens* biofilm formation with these compounds remains open since signaling similar to *P. aeruginosa* PQS system has not yet been described in *Serratia* and activity of these compounds on acylhomoserine lactone (AHL) signaling has not been detected. Our data show that 7-Cl and 7-CF_3 substituted *N*-dodecylamino-4-aminoquinolines present the promising scaffolds for developing antivirulence and anti-biofilm formation agents against multidrug-resistant bacterial species.



The rising problem of microbial resistance to current antibiotics and high spreading rate of resistant bacterial species has become a major public health concern. Resistance to most antibiotics has emerged only a few years after their introduction into clinical practice; therefore, improved strategies and new antimicrobials are urgently needed to control infectious diseases. Many bacteria employ a cell density-dependent communication system called quorum sensing (QS) to control their virulence factor production, motility, biofilm formation, bioluminescence, sporulation, and conjugation.[1] The quorum sensing system allows bacteria to monitor their cell density through the release of signaling molecules called autoinducers. At a high cell density, autoinducers reach threshold concentrations and initiate the signaling cascade that regulates expression of genes required for microbial pathogenicity.

Biofilms are complex communities of bacteria embedded in a self-produced matrix of polysaccharides, proteins, and extracellular DNA that strongly adhere to the surfaces of both organic and inorganic structures.[2] The biofilm-embedded cells are highly resistant to antimicrobial drug therapy, difficult to eradicate, and often cause serious life-threatening infections.[3] Biofilms formed in the tissues and medical devices associated with human body (e.g., catheters, naso-laryngeal tubes, or stents) account for 70% of nosocomial infections.[4] The colonization of a patient's tissue or the surfaces of indwelling medical devices with biofilm-forming bacteria usually leads to persistent infections that fail to resolve despite aggressive antibiotic therapies.

Biofilms play an important role in virulence of many bacteria including Gram-negative pathogens *Serratia marcescens* and *Pseudomonas aeruginosa*.[2] *S. marcescens* as an important nosocomial healthcare-associated pathogen has been recognized only in the last four decades.[5] These bacteria are particularly involved in catheter-associated bacteremia, urinary

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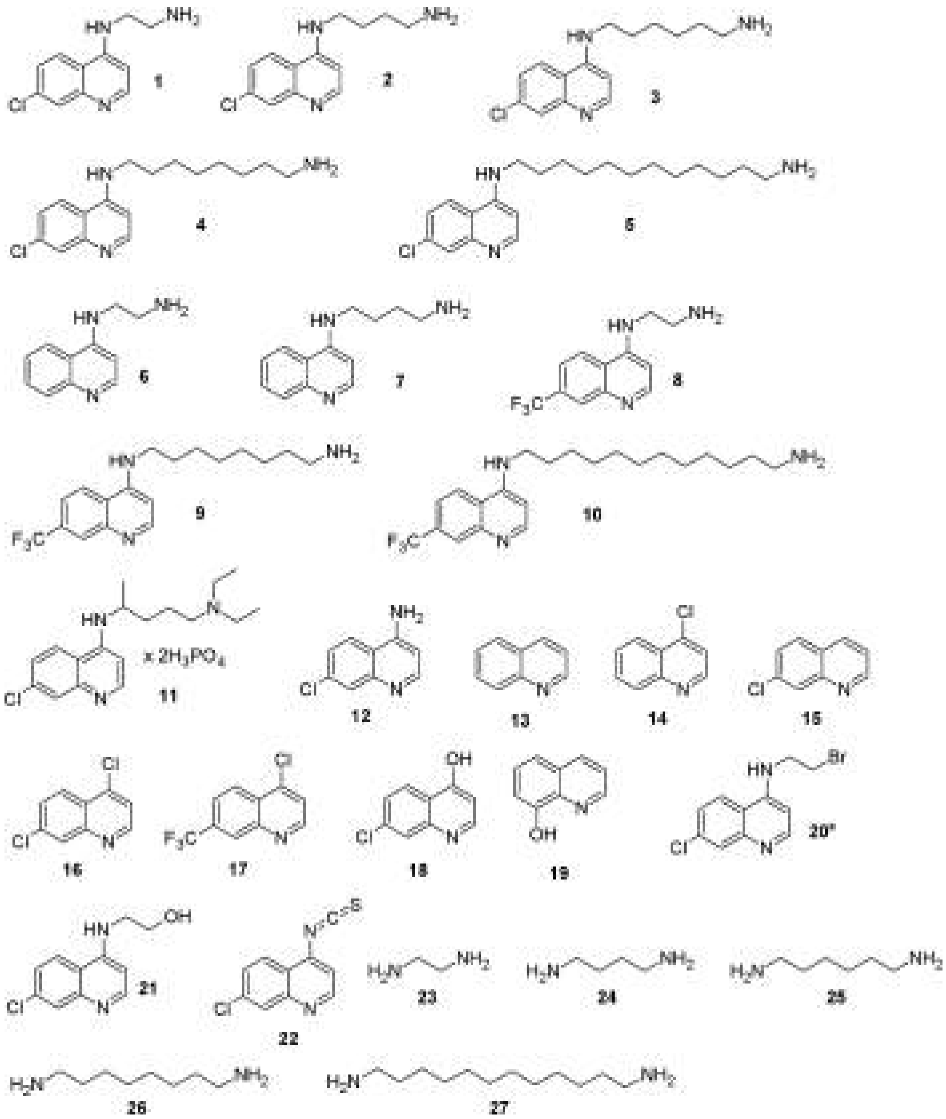

Figure 1. Chemical structures of tested compounds.

tract infections, and wound infections. Infections caused by this opportunistic pathogen may be difficult to treat due to their resistance to multiple antimicrobial agents such as β-lactams, aminoglycosides, and fluoroquinolones.[6] On the other hand, *P. aeruginosa* is a well-known opportunistic pathogen that poses a significant health threat for immunocompromised patients such as burn victims or cancer, cystic fibrosis (CF), or AIDS patients.[7] Therapeutics against *P. aeruginosa* are increasingly limited due to the continued emergence and spreading of antibiotic resistant strains causing high mortality.[8] At the same time, multidrug-resistant strains of *S. marcescens* have been isolated from both clinical and environmental settings,[9] suggesting that antibiotic usage should be restricted and an alternative therapeutic approach should be implemented to treat these infections. Both *S. marcescens* and *P. aeruginosa* control their virulence factor production including biofilm formation using a QS system. Therefore, attenuation of virulence and biofilm formation through interference with QS could be a strategy of choice to treat these multidrug-resistant bacterial infections. Disrupting cell-to-cell communication instead of killing the bacteria would result in less selective pressure compared to conventional antibacterial agents and thus could circumvent the problem of resistance.[10]

Numerous small organic molecules with QS inhibitory activity influencing bacterial virulence in tissue culture and in animal models have been described.[11,12] The majority of these compounds are non-natural synthetic or semisynthetic derivatives of acylhomoserine lactone (AHL). The most active biofilm inhibitors are synthetic or semisynthetic antimicrobial peptides,[13] but they show low stability and significant toxicity, thus making small organic QS inhibitors more attractive therapeutic options against persistent bacterial infections.

The quinoline moiety is a well-known pharmacophore responsible for a broad spectrum of biological activities[14] including antimalarial and inhibitory activity against botulinum neurotoxins.[15] A number of quinolone derivatives with strong bactericidal activity have been synthesized, affecting both planktonic and biofilm-associated bacteria.[16−20] Some of them have demonstrated QS inhibitory activity such as the antibiotic nitroxoline, which in micromolar concentrations reduces formation of *P. aeruginosa* biofilm up to 80%.[19] Recently, new quinolines have been described with strong anti-QS[21,22] or anti-biofilm activities, including halogen derivatives[16] and nitroxo-

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line amino derivatives,[17] quinoline β-amino alcohol,[18] imines,[23] phthalazine-quinoline[24] or 2-alkyl-4(3*H*)-quinazolinone derivatives.[25]

Considering that various quinoline derivatives affect both Gram(−) and Gram(+) bacterial species, together with their good anti-QS and anti-biofilm properties, in this study we have examined antimicrobial activity of a structurally less complex series of 4,7-disubstituted aminoquinoline derivatives.

RESULTS AND DISCUSSION

4-Aminoquinoline derivatives (4-AQs) are clinically used antimalarial drugs. While their antibacterial activity has been investigated to some extent,[26,27] to the best of our knowledge their antivirulence activity has not yet been reported. Numerous small inhibitors of *P. aeruginosa* virulence factor production have been synthesized but reports on synthetic biofilm inhibitors of *S. marcescens* remain scarce.[28] Here we address antibacterial and antivirulence potentials of a series of 22 4,7-disubstituted AQs (Figure 1) against these two common nosocomial opportunistic pathogens. Two derivatives (**9** and **10**) were synthesized for the first time in this study (Figure 1). Synthesis procedures with corresponding spectral data and copies of NMR spectra are given in Supporting Information 1 (SI1, Scheme 1S).

Inhibition of Quorum Sensing in *Serratia marcescens* by 7-Cl-Aminoquinolines. First we have tested antibacterial activity of N-substituted derivatives of 4-amino-7-chloroquinoline containing aliphatic aminoalkyl normal chains with different lengths, that is, C2, C4, C6, C8, and C12 (**1**−**5**, Figure 1). All tested compounds showed low antibacterial activities against *S. marcescens* with minimal inhibitory concentrations (MICs) ranging from 565 μM for **1** to 1800 μM for **3** (Table 1). Lengthening the aliphatic chain to C8 or C12 (**4** and **5**, respectively) slightly increased antibacterial effects of the compounds and observed order of bactericidal activity was **1** > **2** > **3** < **4** < **5** (Table 1). Obtained MIC values were similar to MIC values reported for 1-methyl-3-sulfonylthio-4-aminoquinolinium salts against *S. marcescens*[26] but were at least 1 order of magnitude higher than MIC values reported for C(2) substituted bromo-quinolines against *Staphylococcus aureus* and *Staphyloccous epidermidis*[16,17] or quinoline amino alcohols against *Vibrio cholera*.[18]

Since bactericidal effects were negligible, we have next examined anti-quorum-sensing (anti-QS) activity of these compounds. *S. marcescens* has an *N*-acylhomoserine lactones (AHL)-dependent QS system and produces at least four AHLs (3-oxo-C6-HSL, C6-HSL, C7-HSL, and C8-HSL), which regulate production of prodigiosin, carbapenem antibiotic resistance, extracellular and cell-associated enzymes, and virulence factors such as motility and biofilm formation.[29] The interference of *N*-alkylamino-7-Cl-AQ-derivatives with QS in *S. marcescens* was examined by measuring inhibition of prodigiosin production in a disk assay, and inhibitory effect of tested compounds was detected as appearance of a colorless halo around a disk. All compounds except **5** caused inhibition of prodigiosin synthesis. The zones of prodigiosin inhibition were larger in the presence of derivatives with shorter aliphatic chains ranging from 14 to 22 mm in following order **1** > **2** = **3** = **4** (Table 2, Table 1S). All the compounds (except **7**) also inhibited violacein production in *Chromobacterium violaceum* CV026 disk assay, suggesting that 4-AQ derivatives can interfere with AHL signaling (Table 1S).

Table 1. Minimal Inhibitory Concentration of Tested Compounds against *Serratia marcescens* and *Pseudomonas aeruginosa*

	MIC[a] (μM)	
compd	*S. marcescens*	*P. aeruginosa*
1	565	565
2	1001	1000
3	1800	1800
4	1635	1635
5	690	1380
6	1335	1335
7	2320	2320
8	980	1960
9	185	1475
10	315	1265
11	970	970
12	700	1400
13	3870	3870
14	3055	3055
15	765	3055
16	2525	2525
17	2160	2160
18	1390	2785
19	430	860
20	1750	1750
21	2245	2245
22	1130	1130
23	8320	8320
24	5670	5670
25	4300	4300
26	3465	3465
27	2495	2495

[a]MIC indicates concentration of tested compound that cause 100% inhibition of bacterial growth.

Therefore, we have then tested whether 7-Cl-AQ derivatives show anti-biofilm formation activity, since biofilm formation is the major virulence factor regulated by QS in *S. marcescens*.[30] Surprisingly, only derivatives without prodigiosin inhibitory activity, **5** and **10**, exhibited anti-biofilm formation activity with $BFIC_{50}$ (concentration of compound that inhibited biofilm formation by 50%) of 69 and 63 μM, respectively (i.e., 50% inhibition at 25 μg/mL, Table 2). More importantly, **5** showed significant difference between anti-biofilm formation and antibacterial activities exhibiting 10-fold lower $BFIC_{50}$ than MIC value. Together, these results indicated that *N*-alkylamino-7-Cl-AQ derivatives could target different processes and thus exhibit different activities against *S. marcescens*.

SAR Analysis of Quinoline Derivatives As Biofilm Formation Inhibitors in *Serratia marcescens*. To investigate an effect of structural changes to observed bioactivities, we examined influence of substituents at C(7) and C(4) on bactericidal, anti-QS, and anti-biofilm formation activities. Replacing the chlorine atom at C(7) with hydrogen resulted in the reduction of bactericidal activities as demonstrated by 2-fold decrease in activities of derivatives **6** (MIC = 1335 μM) and **7** (MIC = 2320 μM) in comparison to their 7-chloro analogues **1** and **2**, respectively (Table 1). These weak bactericidal activities fulfilled the requirements of ideal anti-QS agents exhibiting low selective pressure on bacteria and thus reducing the possibility to develop resistance. However, these des-chloro derivatives demonstrated low effect on prodigiosin

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Table 2. Inhibition of Prodigiosin Synthesis and Biofilm Formation in *Serratia marcescens* in the Presence of Quinoline Derivatives and Experimentally Determined log *D* values

compd	prodigiosin inhibition[a]	biofilm formation (%)			$\log D(o/w)_{exp}$
		at 10 μg/mL compd	at 25 μg/mL compd	at 50 μg/mL compd	
1	20 ± 2	147 ± 10	135 ± 8	95 ± 10	1.03
2	14 ± 2	109 ± 7	107 ± 10	140 ± 12	1.22
3	14 ± 1	137 ± 12	137 ± 12	147 ± 10	1.43
4	14 ± 1	98 ± 10	98 ± 10	99 ± 10	1.81
5	*b*	88 ± 15	48 ± 5	39 ± 5	2.81
6	10 ± 1	138 ± 12	125 ± 10	134 ± 15	0.45
7	*b*	128 ± 10	117 ± 8	169 ± 15	0.71
8	*b*	93 ± 10	96 ± 2	90 ± 10	1.17
9	12 ± 1	103 ± 15	70 ± 5	80 ± 10	1.91
10	*b*	110 ± 6	43 ± 2	40 ± 4	2.94
11	16 ± 2	118 ± 10	130 ± 15	144 ± 15	1.73
12	*b*	124 ± 15	110 ± 15	110 ± 8	1.17
13	22 ± 2	155 ± 15	165 ± 8	116 ± 6	1.62
14	*b*	100 ± 12	100 ± 15	126 ± 7	1.51
15	*b*	129 ± 15	136 ± 12	158 ± 20	1.43
16	*b*	122 ± 12	101 ± 5	170 ± 15	1.91
17	*b*	138 ± 8	133 ± 7	110 ± 10	2.57
18	*b*	88 ± 10	95 ± 8	99 ± 10	1.77
19	28 ± 3	125 ± 10	119 ± 10	96 ± 8	1.13
20	28 ± 2	139 ± 25	159 ± 10	136 ± 15	1.81
21	*b*	150 ± 10	170 ± 20	132 ± 15	1.43
22	8 ± 1	134 ± 10	140 ± 15	139 ± 12	1.70
23	*b*	98 ± 12	104 ± 10	108 ± 15	*c*
24	*b*	89 ± 12	85 ± 10	94 ± 15	*c*
25	*b*	104 ± 6	119 ± 10	139 ± 10	*c*
26	*b*	94 ± 10	85 ± 12	112 ± 15	*c*
27	14 ± 1	105 ± 10	124 ± 12	141 ± 10	*c*

[a]Zones of inhibition (mm). Inhibition of pigment production was determined in the presence of 250 μg of tested compound per disk. [b]Not active. [c]Not determined.

production and stimulated biofilm formation (Table 2). Introduction of the strong electron withdrawing CF_3-group at the C(7) position was followed by the increase of bactericidal activity of **8** (MIC = 980 μM) in comparison to des-chloro derivative **6**, but still **8** was two times less active in comparison to **1**. However, while biofilm formation was stimulated in the presence of small concentrations of **1** (147% and 135% at 10 and 25 μg/mL, respectively; Table 2), biofilms remained at the same level (around 95%) in the presence of all three tested concentrations of **8**. Introduction of C8 and C12 chains to 7-CF_3 derivatives caused significant increase in bactericidal activity of the compounds against *S. marcescens* (Table 1). Derivatives **9** and **10**, with MIC values of 184 and 316 μM were 5 and 3 times, respectively, more active than **8**. Furthermore, we have shown that 7-CF_3 derivatives with long aminoalkyl chain were significantly more active in comparison to 7-Cl derivatives. These results suggest that C(7) substituents have a strong influence on bactericidal activity of long chain C(4)-amino(alkylamino) substituted quinoline derivatives, with **9** being the most active derivative within the group. Inhibition of prodigiosin production was detected only in the presence of **9** (Table 2, Table 1S), while **10** was the sole compound that inhibited biofilm formation in *S. marcescens* (Table 2), with a $BFIC_{50}$ value (69 μM) that was five times lower than its MIC value. Importantly, the inhibition of biofilm formation with derivatives **5** and **10** occurs without effect on bacterial viability as confirmed by fluorescence and scanning electron microscopy (Figure 1S).

Only 7-Cl and 7-CF_3 derivatives substituted with a C12 alkyl chain showed effective inhibition of biofilm formation in *S. marcescens*. Derivatives with shorter diaminoalkyl chains either showed no effect or stimulated biofilm formation. These results have indicated that the length of aminoalkyl chain rather than the type of the substituent at C(7) is the key parameter for efficient anti-biofilm formation activity of 4-AQ derivatives against *S. marcescens*.

The importance of the presence of a substituent at C(4) and its character was explored using a range of quinoline derivatives (Tables 1 and 2). Substantial changes in the structure, from derivatives without substituent, that is, with hydrogen at C(4), like **13** and **15**, through 4-chloroquinolines (**14**, **16**, **17**), 4-hydroxyquinoline (**18**), 4-amino-7-chloroquinoline (**12**), and 4-amino-*N*-(substituted) derivatives (**11**, **20**, **21** and **22**) did not significantly affect either bactericidal activity or prodigiosin production and biofilm formation in *S. marcescens*. The strongest bactericidal activity exhibited derivative **19** with MIC = 430 μM, already known as good Fe(III), Zn(II), and Ca(II) N,O-chelating ligand.[31−33]

Diaminoalkanes **23**, **24**, **25**, **26**, and **27** did not display any antibacterial (MIC > 2500 μM), anti-QS, or anti-biofilm formation activity (Tables 1 and 2), thus demonstrating that the quinoline core has been an indispensable component in establishing QS and biofilm formation inhibitory activities within the tested group of compounds.

Quantitative Structure−Activity Relationship (QSAR) Modeling of Biofilm Formation in *Serratia marcescens*

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Table 3. Statistical Performance of QSAR Models Connecting the Most Contributing Molecular Descriptors with Inhibition of the Biofilm Formation in *Serratia marcescens*

dependent variable	statistical performance parameters	most contributing variables (VIP scores > 1)
log BI_{50}	$RMSE_{Cal}$ = 0.037, $RMSE_{CV}$ = 0.080, $RMSE_{Pred}$ = 0.100; R^2_{Cal} = 0.950, R^2_{CV} = 0.784, R^2_{Pred} = 0.645; n(PLS components) = 4% of variance captured by each latent variable in X and Y domain respectively	QPlogBB, W, SMT, MDDD, GMT, GMTV, AVDD, UP, CENT, VAR, ECCc, ECC, QW, FM, TCI8−10, MTCI9,10, HDPI, Whet(Z, e, m, v, and p), logD
	PLS1: 56.10% and 62.20%	
	PLS2: 11.71% and 19.34%	
	PLS3: 6.60% and 11.67%	
	PLS4: 6.12% and 1.76%	
log BI_{25}	$RMSE_{Cal}$ = 0.064, $RMSE_{CV}$ = 0.081, $RMSE_{Pred}$ = 0.084; R^2_{Cal} = 0.817, R^2_{CV} = 0.720, R^2_{Pred} = 0.690; n(PLS components) = 2% of variance captured by each latent variable in X and Y domain respectively	IP, Dipole_PC_X, Dipole_S_X, W, MW, MSDB, SMT, SMTV, MDDD, GMT, GMTV, AVDD, UP, CENT, VAR, ECCc, ECC, AECC, DECC, AvX5, QW, FM, SM, HDPI, Whet(Z, e, m, v, and p), ACIX2, KAMS3, RSIpw5
	PLS1: 63.47% and 67.53%	
	PLS2: 14.26% and 14.13%	

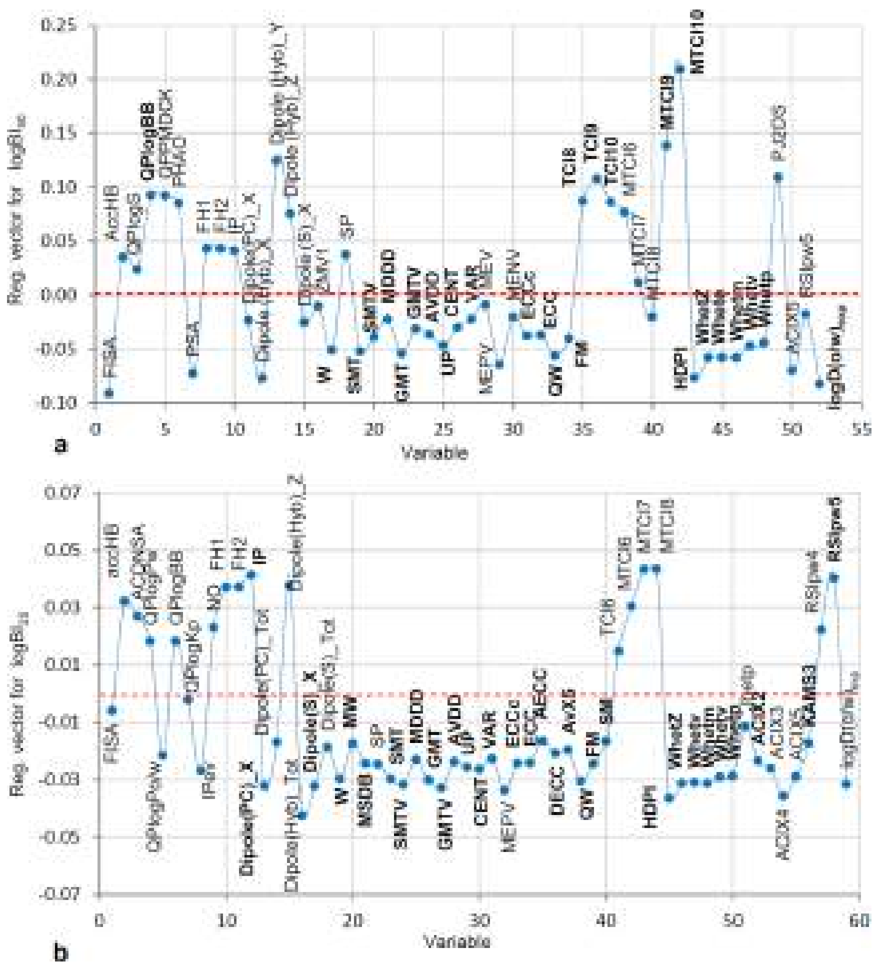

Figure 2. Regression coefficients corresponding to the QSAR models of log BI_{50} (a) and log BI_{25} (b). Variables with VIP score >1 are marked in bold.

with 4-Aminoquinolines. Chromatographic parameters obtained under reversed-phase (RP) conditions can be correlated with biological activity of compounds, such as transport through cell membranes, binding to plasma proteins, or drug−target interactions.[34,35] Most of the investigated 4-AQ derivatives exhibit strong retention on octadecyl (C18) stationary phase, using mobile phase pH ≥ 5. On the contrary, with mobile phase of pH ≤ 1, their retentions were much weaker, and a typical RP mechanism of retention, based on hydrophobic interactions with the nonpolar stationary phase, was established. The partition coefficients of examined quinoline derivatives, denoted as log $D(o/w)_{exp}$ (Table 2), were determined using reversed-phase thin-layer (RP-TLC) method as described in SI1.

In order to quantitatively correlate structural features of 4-AQ derivatives with their potential to affect biofilm formation in *S. marcescens*, QSAR models were built using partial least square (PLS) regression.[36] The entire set of 180 molecular descriptors (SI2) and log $D(o/w)_{exp}$ values were used as independent variables, while logarithms of biofilm formation

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values measured at different concentration (10, 25, and 50 μg/mL), denoted as log BI_{10}, log BI_{25}, and log BI_{50}, respectively, were used as dependent ones. The number of variables in the final models was reduced to 52−59 by a double fold PLS regression, hence significantly increasing predictive performance. Predictive ability and optimal model complexity (number of PLS components) were estimated through the double cross-validation procedure proposed by Warmuza and Filzmoser.[37] Detailed computation and modeling procedures are described in SI1 and SI2. In the case of log BI_{50} and log BI_{25}, models of satisfactory predictive ability were achieved with R^2 values not falling below 0.645 for prediction and 0.817 for calibration purposes and errors not exceeding 0.1 log units (Table 3). On the other hand, in the case of log BI_{10}, a narrow range of biofilm formation percentages (0.25 log units) was insufficient to build a reliable QSAR (R^2_{Cal} = 0.649, R^2_{CV} = 0.304, R^2_{Pred} = 0.371).

Obtained QSAR models showed that branching and the size of the molecules are the key topological descriptors responsible for modulation of biofilm formation. The most contributing variables (VIP > 1) for log BI_{50} and log BI_{25} were Wiener topological index (W), MW, WhetZ, Whete, Whetm, Whetv, and Whetp, GMT and GMTV, MTCI9 and MTCI10, ECCECCc, HDPI, UP, and molecular centrality (CENT) (for abbreviation definitions and complete list see SI2). Common contributing variables for log BI_{50} and log BI_{25} are the Wiener indices associated with molecular branching and size. They are reciprocally related to branching degree, that is, molecules with smaller values have more branched substituents.[38] All of the Wiener indices have negative regression coefficients (Figure 2), which means that introduction of long unbranched hydrocarbon chains to 4-AQs core results in an increase of anti-QS activity. Derivatives **5** and **10** have significantly higher values of Wiener indices (SI2), corresponding to other examined 4-AQs, which coincide with the highest biofilm formation inhibition, even when they are compared to structurally closest derivatives **4** and **9**. Similarly, HDPI show negative contribution to log BI_{50} and log BI_{25}. Again, the most active derivatives **5** and **10** have the highest values of HDPI. The highest positive contribution on log BI_{50} was MTCI10 index. The lowest values of MTCI10 (SI2) are found for the most potent biofilm inhibitors **5** and **10**, with **4** and **9** as closest followers. Lipophilicity (log $D(o/w)_{exp}$) may also be a significant contributor to anti-QS and biofilm formation inhibitory activity of 4-AQ derivatives. Although less important than most of the topological descriptors, log $D(o/w)_{exp}$ negatively affects log BI_{50} (Figure 2a), which means that the increase of the lipophilicity of 4-AQs leads to increased anti-biofilm formation potency. As it was expected, linear dependence between alkyl-chain length and log $D(o/w)_{exp}$ was found. In addition, compounds with strong electron withdrawing CF_3-group at C(7) are more lipophilic than the corresponding 7-chloro derivatives, and following order of lipophilicity was observed: 7-CF_3 > 7-Cl > 7-H. Derivative **10** has the highest lipophilicity value (Table 2), while **6** have the lowest. Indices TCI (order 8, 9, and 10; Figure 2a) and MTCI (order 9 and 10; Figure 2a) have a positive impact on log BI_{50}. Those indices describe the ability of charge transfer throughout the molecule. Derivatives with 7-CF_3 group have 1.5−2 times higher values of TCI indices in comparison to corresponding 7-Cl analogues, and obtained order 7-CF_3 > 7-Cl > 7-H clearly indicates stronger influence of substituent in C(7) position than length of alkyl chain on TCI values. That is additionally confirmed with very low values of TCIs for diaminoalkanes. On the other hand, MTCI values are more influenced by the length of alkyl chain than by the nature of C(7) substituents, that is, derivatives with longer diaminoalkyl chain have lower MTCI values. However, correlation between TCIs and either anti-QS or anti-biofilm formation values have not been observed. Descriptors defining specific interactions such as accptHB, NO, FISA and PSA, QPlogS, and QPlogBB, have significantly lower impact on log BI. This clearly implies that shape and size of the molecule, especially introduction of long unbranching substituents, are the most important factors in establishing ligand−receptor interactions that affects QS signaling pathways.

Interference of 7-Cl-Aminoquinolines with Quorum Sensing in *Pseudomonas aeruginosa*. To investigate whether there is species-specific response to tested compounds and to explore their mechanism(s) of action, selected compounds were tested for bactericidal, anti-QS, and anti-biofilm formation activities against another clinically relevant pathogen, *Pseudomonas aeruginosa* PAO1 strain. Tested compounds failed to perform any substantial bactericidal activity against *P. aeruginosa* (Table 1), and showed MIC values in the range of or significantly higher than previously reported 1-methyl-3-sulfonylthio-4-aminoquinolinium salts.[26]

Two compounds with anti-biofilm formation activity in *S. marcescens*, derivatives **5** and **10**, were examined for their anti-QS activity in *P. aeruginosa*. Derivative **4**, which demonstrated weak anti-QS activity and had no effect on biofilm formation in *S. marcescens*, was also tested for comparison. QS inhibition in *P. aeruginosa* was followed in biofilm formation and pyocyanin production assays in the presence of these derivatives. Derivative **4** inhibited *P. aeruginosa* biofilm formation by approximately 60%, even at 10 μg/mL (33 μM, Table 4).

Table 4. Effects of Selected Quinoline Derivatives on Biofilm Formation and Inhibition of Pyocyanin Production in *Pseudomonas aeruginosa*

	biofilm formation (%)			
compd	at 10 μg/mL compd	at 25 μg/mL compd	at 50 μg/mL compd	pyocyanin production[a] (%)
4	44 ± 2	48 ± 5	38 ± 2	155 ± 10
5	79 ± 2	58 ± 7	60 ± 6	50 ± 3
10	67 ± 3	46 ± 5	52 ± 5	10 ± 1
17	83 ± 8	82 ± 10	98 ± 10	103 ± 1

[a]Production of pyocyanin was measured in the presence of a compound at concentration of 50 μg/mL.

Derivatives **5** and **10** also inhibited biofilm formation, with decreases ranging from 20% to 40% and 30% to 50%, respectively (Figure 1Sc,d), depending on applied dose and C(7) substituent. On the other hand, while **5** and **10** were able to reduce pyocyanin production with respective IC_{50} values of 140 and 2.5 μM, in comparison to DMSO treated controls, **4** induced pyocyanin overproduction (Table 4 and Figure 2S). Pyocyanin inhibitory activity of derivative **10** was similar to recently reported activity of 3-carboxamido-2-heptyl-4-quinolinon antagonist (IC_{50} = 2 μM),[39] but 20 times stronger than 2-alkyl-4(3*H*)-quinazolinone derivatives.[25] However, derivative **10** was 10-fold less potent in pyocyanin inhibition than benzamide−benzimidazole derivatives, which are one of the most potent pyocyanin inhibitors described so far.[40] Derivative **17**, which was also used as a control compound, exhibited no QS inhibitory activity. Similar results showing the influence of alkyl chain length on *P. aeruginosa* QS system with agonistic or

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antagonistic activities were reported for some 4-quinolone analogues.[21,41]

The QS network of *P. aeruginosa* is organized in a multilayered hierarchy consisting of at least four interconnected signaling pathways: Las, Rhl, the PqsR-controlled quinolone system (PQS), and Integrated QS system (IQS). Three autoinducer synthases LasI, RhlI, and PqsABCDH produce autoinducers 3-oxo-C12-homoserine lactone (HSL), C4-HSL, and 2-heptyl-3-hydroxy-4-quinolone (PQS), respectively, which regulate formation of biofilms and production of virulence factors.[42] Inhibition of specific QS pathways in *P. aeruginosa* PAO1 by **4**, **5**, and **10** was quantified using three biosensors: *P. aeruginosa* PA14-R3, used to measure 3OC12-HSL production (LasI activity), *P. aeruginosa* PAOJP2/pKD-*rhlA*, used for measurements of C4-HSL (RhlI activity), and *P. aeruginosa* PAO1Δ*pqsA* (CTX *lux*::*pqsA*), used for evaluation of PQS production (PqsABCDH activity). Compound **17** with no effect on pyocyanin production and biofilm formation was used as negative control.

Derivative **4** reduced Las signaling by 45%, while C12-amino derivatives **5** and **10** induced overproduction of C12-AHL by 316% and 118%, respectively (Table 5). Three-fold stronger effect of **5** over **10** on LasI activity is due to different C(7)-substituent. Derivatives **5** and **10** did not have any effect on prodigiosin production in *S. marcescens* (Table 2), which is regulated by short-chain AHLs,[43] suggesting the absence of interference with C4-AHL signaling. The assumption was confirmed by their weak effect on RhlI activity, manifested as 10% and 20% inhibition, respectively. Derivative **4**, which showed a small zone of prodigiosin inhibition (Table 1), reduced RhlI activity by 23%.

Table 5. Influence of Tested Derivatives on *Pseudomonas aeruginosa* QS Pathways

compound[a]	production of autoinducers in *P. aeruginosa* PAO1 (%)		
	3oxoC12-HSL	C4-HSL	PQS
4	55 ± 6	77 ± 5	91 ± 8
5	316 ± 16	91 ± 15	16 ± 2
10	118 ± 10	80 ± 12	26 ± 4
17	148 ± 12	86 ± 9	69 ± 3

[a]Bacteria were incubated with tested compounds at 50 μg/mL; values are given relative to control samples where bacteria were grown with DMSO and are average of three independent experiments ± SD.

Significant differences between tested derivatives were observed when comparing their activities against *P. aeruginosa* PQS. While **4** showed a minor effect on quinolone signaling with only 9% inhibition, **5** and **10** inhibited PQS by 85% and 75%, respectively. Derivative **17** showed 1.5-fold stimulatory effect on LasI activity and caused 30% reduction of PQS signaling.

Taken together, these results suggest that 4-AQs with *N*-dodecylamino substituents inhibit biofilm formation and virulence factor production in *P. aeruginosa* through inhibition of PQS signaling. These data are consistent with previous reports showing that PQS signaling regulates the production of diverse virulence factors including pyocyanin, in addition to affecting biofilm formation.[44] Although **4** exhibited stronger anti-biofilm formation activity in *P. aeruginosa* than **5** and **10**, it also had stimulatory effect on pyocyanin production (Table 4). Considering the detrimental effects that pyocyanin can cause in infected host tissues through interference with cellular functions such as electron transport, cellular respiration, energy metabolism, gene expression, and innate immune mechanisms,[45] our results show that C12 substituted derivatives have more preferred antivirulence characteristics comparing to compound **4**.

Conclusion. In this study, we have examined 22 quinoline derivatives as potential antimicrobial agents against two pathogens, *S. marcescens* and *P. aeruginosa*, and identified new quinoline derivatives with strong anti-QS activity inhibiting biofilm formation and without bactericidal effect. We have demonstrated that the efficient QS inhibition by these compounds depends on the presence of a C(7)-substituent, a basic amino group, and the long-chain *N*-alkylamino substituent at C(4) of quinoline core, with C12 chain derivatives being the most active against biofilm formation. Consistent with a previous report from Klein and co-workers,[46] differences observed for C8 and C12 AQs related to inhibition of bacterial pigment production and biofilm formation in both examined bacteria clearly showed that relatively small structural changes cause significant differences in QS modulating activity. Derivative **10** reduced *P. aeruginosa* pyocyanin production with a potency belonging to the group of the most active quinoline type inhibitors described so far. Notably, the compounds **5** and **10** are the first quinoline derivatives with demonstrated anti-biofilm formation activity in *S. marcescens.* The important question opened in this study but not yet clarified is the mechanism of *S. marcescens* biofilm formation inhibition with C12 4-AQs since signaling similar to *P. aeruginosa* PQS system has not been described in *S. marcescens* and activity of these compounds on AHL signaling was not detected.

METHODS

Compounds Synthesis. Compounds **11**, **13**, **14**, **15**, **16**, **17**, **19**, **23**, **24**, **25**, **26**, and **27** were obtained from Sigma-Aldrich Co (Sigma-Aldrich, Germany) and used without further purification. Compound **18** was obtained according to a procedure described by Terzić and co-workers,[47] and all spectra were identical. Compounds **9** and **10** were synthesized for the first time, while other tested compounds were synthesized according to previously published procedures (SI1).

IR spectra were recorded on a PerkinElmer spectrophotometer FT-IR 1725X. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-200 spectrometer (at 200 and 50 MHz, respectively), and on a Bruker Ultrashield Advance III spectrometer (at 500 and 125 MHz, respectively) employing indicated solvents (*vide infra*) using TMS as the internal standard. Chemical shifts are expressed in ppm (δ) values and coupling constants (J) in Hz. ESI-MS spectra were recorded on Agilent Technologies 6210 time-of-flight LC-MS instrument in positive ion mode with CH_3CN/H_2O 1/1 with 0.2% HCOOH as the carrying solvent solution. Samples were dissolved in CH_3CN or MeOH (HPLC grade purity). The selected values were as follows: capillary voltage = 4 kV, gas temperature = 350 °C, drying gas = 12.1 min^{-1}, nebulizer pressure = 45 psig, fragmentator voltage = 70 V. The elemental analysis was performed on the Vario EL III C,H,N,S/O elemental analyzer (Elementar Analysensysteme GmbH, Hanau-Germany). Thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F254 and RP-18 F254 plates.

Microbial Strains and Growth Conditions. *Pseudomonas aeruginosa* PAO1 NCTC 10332 and *Serratia marcescens* ATCC 27117 were used in this study. Bacteria were grown in Luria−Bertani (LB) broth on a rotary shaker at 180 rpm.

Antimicrobial Susceptibility Tests for Planktonic Cells. The minimum inhibitory concentrations of 4-AQ derivatives were determined according to standard broth microdilution assays recommended by the Clinical and Laboratory Standards Institute (M07-A9). Stock solutions of 4-AQ derivatives were prepared in

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DMSO (50 g/L, w/v). The highest tested concentration of any compound was 500 mg/L. The inoculums were 10^5 colony forming units (CFU)/mL. The MIC value corresponds to the lowest concentration that inhibited the growth after 20 h at 30 °C for *S. marcescens* or 37 °C for *P. aeruginosa*.

Antimicrobial Susceptibility Tests for Biofilms. Biofilm quantification assays were performed in 96-well microtiter plates using a crystal violet (CV) method to stain adherent cells.[48] Biofilms formed for 24 h in the presence or absence of compound at 30 °C for *S. marcescens* or 37 °C for *P. aeruginosa* were washed, and adherent cells were stained with 0.1% (v/v) CV. Each biofilm formation assay was performed in six wells and repeated three times.

***Serratia marcescens* Disk Assay.** Overnight culture of *S. marcescens* was diluted 100-fold in molten semisolid LB agar (0.3% w/v) and poured over solid LB medium. Cellulose disks containing compounds (250 μg/disk) were placed on solidified agar and incubated for 24 h at 30 °C. Inhibition of prodigiosin synthesis was identified by the absence of red color around the disk.

Pyocyanin Assay. The pyocyanin assay was performed with *P. aeruginosa* PA14 indicator strain as reported previously.[12] Pyocyanin in the supernatant was quantified using UV−vis spectrophotometer Ultrospec 3300pro (Amersham Biosciences, USA) at 695 nm. All experiments were performed in triplicate and repeated at least three times.

AHL Production Assays. Production of 3OC12-HSL and C4-HSL were determined in supernatants of *P. aeruginosa* PAO1 culture grown for 6 h in the presence of selected compounds or DMSO as previously reported.[49] Aliquots of *P. aeruginosa* PAO1 supernatants were added to *P. aeruginosa* PA14-R3 (Δ*lasI Prsal::lux*)[50] or PAOJP2/pKD-*rhlA* (Δ*rhlA* P*rhlA::lux*)[51] biosensor cultures, and cell density (A_{600}) and bioluminescence (light counts per second, LCPS) were simultaneously measured after 4 h of incubation using Tecan Infinite200 multiplate-reader (Tecan Group Ltd., Switzerland). Luminescence values were normalized per cell density.

PQS Measurements. PQS measurements were performed according to Fletcher et al.[52] with some modifications. *P. aeruginosa* PAO1 stationary phase cultures (10 mL) grown with selected compounds or DMSO were extracted with the same volume of acidified ethyl acetate. After vortexing and centrifugation, organic phase was transferred to a fresh tube and dried out under a stream of nitrogen gas. The residue was resuspended in 50 μL of methanol for subsequent PQS measurements. Overnight cultures of *P. aeruginosa* PAO1 Δ*pqsA* (CTX *lux*::*pqsA*)[52] were diluted 1:1000 in fresh LB medium, and 0.2 mL of cultures were grown in microtiter plates in the presence of 5 μL of extracts. Cell density and bioluminescence were measured as described above. All assays were carried out in triplicate at least two times.

Molecular and QSAR Analysis. All structures were built using the Maestro 10.1 from Schrödinger Suite 2015-1 (Maestro, version 10.1, Schrödinger, LLC, New York, 2015). QikProp, version 4.3 (Schrödinger, LLC, New York, 2015), was used for the calculation of physically significant molecular descriptors and pharmaceutically relevant properties. Single point calculations using the RM1 method[53] from Semiempirical NDDO module of Schrödinger Suite 2015-1 was used for semiempirical parameters. QSAR models were built by Partial Least Square regression using PLS_Toolbox software package (v. 5.7 Eigenvectors Inc.) for MATLAB (v. 7.8.0 R2009; MathWorks, Natick, USA). Detailed procedure is described in SI1.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b01149.

Supporting Tables S1 and S2, Figures S1 and S2, Scheme S1, experimental and synthetic procedures, compounds characterizations, QSAR computations, and NMR spectra (PDF)

Molecular descriptors and details of PLS model (XLSX)

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Notes

The authors declare no competing financial interest.

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