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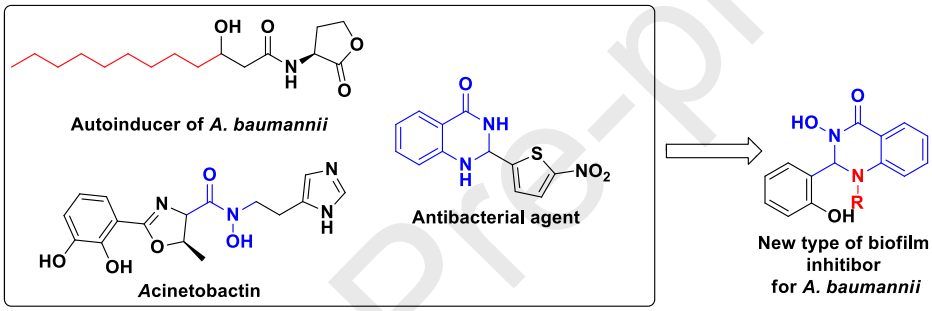

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ABSTRACT

The emergence of multidrug resistant microorganisms has triggered the impending need for new aitimicrobial strategies. The antivirulence strategy with the benefit of alleviating the drug resistance becomes the focus of research. In this study, 22 quorum sensing inhibitors were synthesized by mimicking the structure of autoinducer and acinetobactin and up to 34% biofilm inhibition was observed with 5u. The biofilm inhibition effect was further demonstrated with extracellular polysaccharides inhibition and synergism with Gentamycin sulphate.

1. Introduction

The increasing in antibacterial resistance is cause for great concern worldwide.[1] This situation is highlighted by the opportunistic gram-negative bacteria *Acinetobacter baumannii*, which has emerged over the past decades as one of the most notorious microorganism for infections. Statistically, up to 58% of *A. baumannii* isolates are multidrug resistant and up to 30% of *A. baumannii* isolates are pan-drug resistant.[2] *A. baumannii* is also a leading cause of hospital acquired infection especially in the intense care unit with the lethality rate as high as 43%.[3]

The resistance of *A. baumannii* is majorly due to the presence of bacterial biofilms. Bacterial biofilms are defined as surface-attached communities of bacteria encased in an extracellular matrix of biomolecules, deoxyribonucleic acid, and lipid, represent a significant hurdle for infectious disease treatment and difficult to be eradicated. In the biofilm state, bacteria are upward of 1000-fold more resistant to antibiotics than in the planktonic state.[4] Moreover, bacterial biofilms are also the cause of chronic infections and recurrences.[5] Bacteria have to determine their cell density and to coordinate their population behavior, which is known as quorum sensing (QS).[6] This process was realized by assessing the concentration of small extracellular signal molecules released into the environment acting as autoinducers. The QS system of *A. baumannii* includes an *abaI* synthase and an *abaR* receptor. The autoinducer produced by the *abaI* synthase was diffused into the environment. Once a certain threshold of autoinducer concentration is reached, which is monitored by the *abaR* receptor, cells start the production of virulence factors including biofilm formation, bioluminescence and so on. The autoinducer of *A. baumannii* was shown in Fig. 1, which is an N-acyl homoserine lactone (AHL) derivative.[7] The structure of autoinducer contains a hydrophobic side and a hydrophilic side, and this surfactant-like structure is important for the autoinducer to get through the bacteria cell wall.

To date, the development of new types of synthetic quorum sensing inhibitors (QSI) to counteract virulence factor including biofilm formation is a new strategy to tackle bacterial infection.[8-10] By disrupting the cell to cell communication instead of killing the bacteria, will applying less pressure on the development of drug resistance compared with the traditional antimicrobial agents.

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[†]G. Yang and C. Cheng made equal contribution to this work.

The focus of current investigation.[11-13] Several types of QSIs were developed by mimicking the autoinducer structure, non-native AHLs, 2-aminoimidazole derivatives and fusaric acids et al were identified as the quorum sensing inhibitors (QSI).[14-16] These small molecules binding to the abaR receptor in competence with native autoinducer and maintain the bacteria in the planktonic form. Although different types of QSIs were identified as biofilm inhibitor, the efficiency in reducing the overall biofilm formation was not satisfactory. Thus, the quest for more efficient QSI is still an existing challenging.

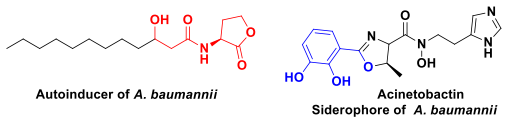


Figure 1. Structures of autoinducer and acinetobactin.

On the other hand, all bacteria (excepte *Borrelia burgdorferei*) require micro-molar levels of iron (Fe^{2+}/Fe^{3+}) for growth and reproduction, since iron serves as a cofactor in numerous biochemical processes.[17] Under iron restricted conditions, pathogens like *A. baumannii* overcome this iron limitation via the synthesis of small-molecule high-affinity iron chelators known as siderophores (Figure 1), which was secreted by bacteria and reimported from the external milieu after successfully iron chelating. The iron was most likely chelated to the hydroxyl groups or even the carbonyl group and reimported by the bacteria. In this regard molecules with hydroxyl group, carbonyl group, hydroxamic acid group or a combination of them may competing the iron chelating and retard the bacteria growth and reproduction.[18]

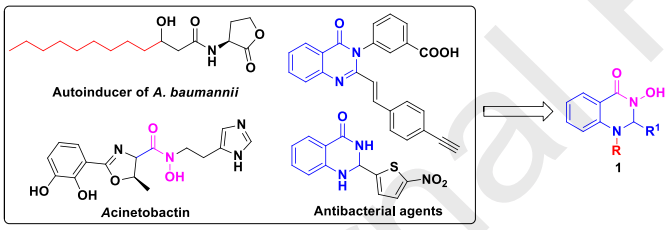
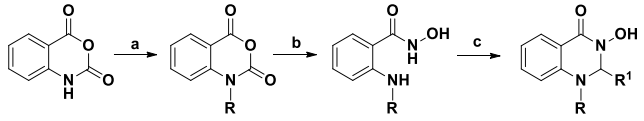


Figure 2. Homologous structure of 1 and acinetobactin toward the QS signal molecule.

The quinazolinone moiety is well known for its antimicrobial property, and SAR study reveals that the 3-aryl group is important for the antimicrobial property[19-21]. Recently, Benazza and coworkers designed artificial iron chelators with adhesion inhibiting functions contains multiple copies of the hydroxamic acid group, which is important for iron chelating.[22] Prompted by these observations and in combination with our researches on QSI study, we developed a new type of 3-hydroxy-2,3-dihydroquinazolin-4(1*H*)-one derivatives, bearing at the position 3 the hydroxamic acid moiety, with the aim to attenuate the antimicrobial activity while enhance their antibiofilm activity (Figure 2).



Reagents & conditions: a) R-Br, DMF, NaH, rt, 24h; b) NH_2OH, K_2CO_3, MeCN, 12h; c) MeCN, R^1CHO, AcOH, 80 °C, 3h.

Scheme 1. Procedure for the synthesis of **5**.

To study the influence of log P and substituent effect on the biofilm inhibitory activity, 3-hydroxy-2,3-dihydroquinazolin-4(1*H*)-one was modified at the positions 1 and 2. As depicted in Scheme 1, the important intermediate **3** was synthesized from isatoic anhydride by alkylation with alkyl halide and strong base. Then, intermediate **3** was subjected to hydroxylamine hydrochloride in the presence of base to give hydroxamic acid. The hydroxamic acid intermediates **4**, however, were not stable on silica gel condition and used for the next step after simple extraction. The ring closing step was realized by reacting hydroxamic acid with aldehyde in the presence of acetic acid under thermal condition. With this synthetic procedure, 3-hydroxy-2,3-dihydroquinazolin-4(1*H*)-one analogue **5** with different substitution groups at the positions 1 and 2 were realized.

With these synthetic derivatives in hand, the biofilm inhibitory effect against *A. baumannii* was examined using established crystal violet staining protocol.[23] The N-octyl-3-hydroxy-2,3-dihydroquinazolin-4(1*H*)-one subclass was the first group of analogues studied in mimicking the autoinducer of *A. baumannii*. Initially, the planktonic growth inhibition of the synthesized compounds was tested at 300 μM and no growth inhibition observed. Then, compound **5a** was screened at four different concentrations for anti-biofilm activity, which are 500 μM, 250 μM, 50 μM and 10 μM accordingly. Initial study reveals the dose dependent effect was not observed, we postulate that this may due to the restricted solubility of compound **5** in water so the rest of compounds were screened at 50 μM for anti-biofilm activity and (*S*)-2-(3-bromophenyl)-N-(2-oxotetrahydrofuran -3-yl)acetamide(3-BrBAHSL) developed by Blackwell group was used as positive control (table 1).[24] Moreover, Syto9/PI staining was used to see the live and dead bacteria of *A. baumannii* in the presence and absence of **5a**, and the results suggest that the compound **5a** didn't induce bacterial death at 50 μM level (Figure 3).

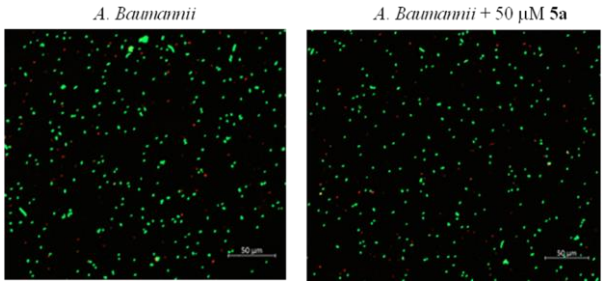


Figure 3. Laser confocal microscope observations of *A. baumannii* in the presence and absence of **5a**

Compound **5a** with a steric isopropyl group give moderate biofilm inhibitory effect. Changing the steric isopropyl to liner pentenyl (**5b**) decreased the inhibitory effect. We turned our attention to phenyl group as it may pose potential π-π staking with the target *abaR* receptor. However, with

unsu ... tion was achieved. Then, electron-donating and electron-withdrawing groups were introduced into the *para* position of the phenyl ring(**5c-5g**) and found out that *para*-hydroxy group is more efficient. This suggests additional hydrogen bonding donor is beneficial for the anti-biofilm activity. We carry on moving the hydroxyl group to the *ortho*-position of the phenyl ring(**5h**) also lead to increased efficiency, and we believe this increased efficiency may correlated with iron binding property of this molecule as the *ortho*-methoxy group(**5i**) lead to drastic decrease in biofilm inhibition rate. Moreover, other non-coordination group likes *ortho*-chloro also give lower biofilm inhibitory rate, which reinforces the iron coordination postulation.

Table 1. Biofilm inhibitory activity of 3-hydroxy-2,3-dihydroquinazolin-4(1*H*)-one analogues.

Cpd.	R	R_1	yield	Inhibition Rate(%)
5a	*n*-C_8H_{17}	*i*Pr	88%	10.2 ± 0.93
5b	*n*-C_8H_{17}	1-pentenyl	84%	2.4 ± 0.27
5c	*n*-C_8H_{17}	Ph	74%	5.5 ± 0.49
5d	*n*-C_8H_{17}	4-OMePh	75%	1.3 ± 0.37
5e	*n*-C_8H_{17}	4-MePh	78%	9.2 ± 1.06
5f	*n*-C_8H_{17}	4-ClPh	80%	5.2 ± 2.34
5g	*n*-C_8H_{17}	4-OHPh	87%	13.3 ± 4.66
5h	*n*-C_8H_{17}	2-OHPh	80%	23.5 ± 5.18
5i	*n*-C_8H_{17}	2-OMePh	78%	7.2 ± 1.12
5j	*n*-C_8H_{17}	2-ClPh	90%	8.1 ± 1.48
5k	*n*-$C_{16}H_{33}$	2-OHPh	84%	4.7 ± 6.71
5l	*n*-$C_{14}H_{29}$	2-OHPh	83%	20.9 ± 1.59
5m	*n*-$C_{12}H_{25}$	2-OHPh	78%	8.9 ± 7.11
5n	*n*-$C_{10}H_{21}$	2-OHPh	89%	30.7 ± 5.93
5o	*n*-C_6H_{13}	2-OHPh	76%	7.7 ± 2.01
5p	*n*-C_4H_9	2-OHPh	90%	29.7 ± 0.59
5q	Me	2-OHPh	88%	14.6 ± 6.62
5r	H	2-OHPh	86%	16.3 ± 2.63
5s	Bn	2-OHPh	78%	17.5 ± 2.16
5t	3-FBn	2-OHPh	85%	27.5 ± 4.22
5u	3-ClBn	2-OHPh	90%	34.1 ± 5.27
5v	2-CNBn	2-OHPh	85%	17.6 ± 2.03
3-BrBAHSL				7.9 ± 3.23

Investigation into how side chain on the nitrogen would affect activity was the next step in the SAR process for this region. This decision was based on the observation that different AHL autoinducer generally contains vary length of side chain. The chain length from *n*-$C_{16}H_{33}$ to H (**5k-5r**) was investigated and the best biofilm inhibition was observed when *n*-$C_{10}H_{21}$ was introduced, which suggests that the logP value is a cofactor in biofilm inhibition. Further effort to improve the biofilm inhibition rate by introducing benzyl group at the position 1 was also tested and the result was not better than *n*-$C_{10}H_{21}$ (**5s**). However, when electron-withdrawing group was introduced into the *meta*-position of the benzyl group the inhibition(**5t-5u**) rate increased , and makes it even better than the *n*-$C_{10}H_{21}$ side chain suggesting a potential π-π staking with an electron-rich moiety of the receptor. But electron-withdrawing group at the *ortho*-position did not(**5v**) improve the inhibition rate.

Recent work by Philip N. Rather *et al* has demonstrated that motility of *A. baumannii* on low-percentage (0.2 to 4%) agar plates was dependent on quorum sensing and the quorum sensing inhibitor will reduce the motility of the bacteria.[25] Using similar procedure, we found out that the motility of *A.* of synthetic compounds **5n** or **5u**, indicating these compounds are quorum sensing inhibitors (Figure 4).

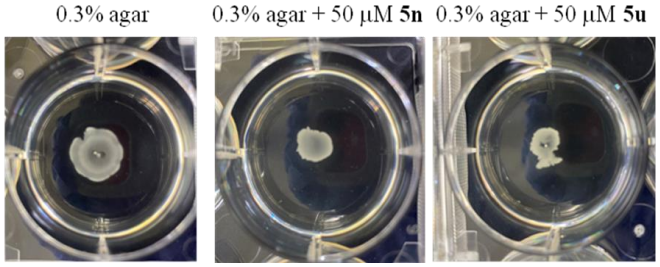


Figure 4. Reduction of motility in *A. baumannii* with 5n and 5u.

Once we had determined the effect that these 3-hydroxy-2,3-dihydroquinazolin-4(1*H*)-one had on *A. baumannii*, we determined their activity against a representative set of gram negative bacteria (Table 2). *A. baumannii* 1925052 is a clinical isolated drug resistant pathogen, to our delight, **5n** and **5u** exhibited better biofilm inhibitory effect comparing with the positive control. Moreover, our compounds are also prove to be effective biofilm inhibioors for *E. coli and P. aeruginosa*, especially with the tested strain of *P. aeruginosa*, 55% biofilm reduction was achieved.

Table 2. Test compound inhibition of biofilm formation at 50 μM.

	Acinetobacter baumannii (%)	*Escherichia coli* (%)	*Pseudomonas aeruginosa* (%)
3-BrBAHSL	28.22 ± 6.47	50.10 ± 3.26	-1.72 ± 2.16
5n	43.38 ± 4.74	40.83 ± 5.25	44.51 ± 9.57
5u	38.20 ± 9.43	27.46 ± 3.90	55.35 ± 4.29

Moreover, the extracellular polysaccharides are important components of biofilms and also help maintaining a structured multicellular bacterial community.[26] The reduction of extracellular polysaccharides would be an further index of biofilm inhibition.[27] With compounds 5n and 5u, the extracellular polysaccharides of *A. baumannii* was reduced by 31% and 34% respectively (Figure 5).

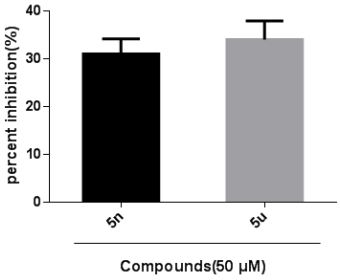


Figure 5. Extracellular polysaccharides inhibition test.

With the aim to develop a feasible medical application, two of the best QSIs (5n and 5u) was evaluated with Gentamycin sulphate by a checkerboard titration assay in microplates as recommended by the NCCLS and expressed as the sum of the fractional inhibitory concentration (FIC) index for each agent (Table 2).[28] As shown in Table 2, synergistic effect was clearly observed according to the FIC and FICI value (always less than 0.5).

Tab 55 –

	MIC alone (μg/mL)	MIC combination μg/mL	FIC (MIC^{comb}/MIC^{alone})	FICI
Gentamycin sulfate	0.25	0.0625	0.25	0.252
5n	1024	2	0.002	
Gentamycin sulfate	0.25	0.0625	0.25	0.252
5u	1024	2	0.002	

In conclusions, we demonstrated the biofilm formation of *A. baumannii* could be reduced with synthetic small molecules. And the 2-hydroxyphenyl at 2 position is crucial for the biofilm inhibitory effect, which would provide a guideline for further structure modification. The biofilm inhibitory effect of synthetic compounds was further demonstrated by its extracellular polysaccharides inhibitory ability. Moreover, the synergistic effect observed with Gentamycin sulphate prove its potential medical application.

General Information

All solvents and inorganic reagents were from commercial sources and used without purification unless otherwise noted. Starting material and products were prepared following the literature procedures.[1-3] Proton nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on INOVA 400MHz spectrometer (400MHz and 100MHz). Chemical shifts for protons are reported in parts per million downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent ($CDCl_3$: 0 δ). Chemical shifts for carbon are reported in parts per million downfield from tetramethylsilane and are referenced to the carbon resonances of the solvent ($CDCl_3$: δ 77.0). Data are represented as follows: chemical shift, integration, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constants in Hertz (Hz). All high resolution mass spectra were obtained on a Waters G2-XSQTof mass spectrometer. Melting points were determined on a Tektronix X-4 melting point apparatus. Analytical TLC was performed using EM separations percolated silica gel 0.2 mm layer UV 254 fluorescent sheets.

Pathogens used in this study, *Acinetobacter baumannii* S1 *Acinetobacter baumannii*1925052, *Escherichia coli* DH5α, and *Pseudomonas aeruginosa* ATCC 27853. All of the strains were maintained in Luria-Bertani (LB) at 37 ℃. All the stock cultures were kept at -80 ℃ in Nutrient broth with 40% of glycerol.

4.1.2 Synthesis of intermediate **3**

The isoacid anhydride (10 mmol) and 15 ml of a freshly distilled solvent DMF were added to a 100 ml round bottom flask, then 60% NaH (15 mmol) was added to the reaction mixture, and the temperature was maintained at 25 ℃. After 1 hour, the alkyl bromide (15 mmol) were added to the reaction mixture, and the reaction was monitored by TLC analysis. Upon completion, 80 ml of ice-water mixture was added to precipitate a solid. After filtration, the filter cake was washed with water and petroleum ether, and the filtrate was discarded and the filter cake was dried under vacuum to give a pale white solid and used for next step without further purification. 1-octyl-2H-benzo[d][1,3]oxazine-2,4(1H)-dione (3a)**:** White solid, 80% yield. mp: 70-72 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (dd, *J* = 7.8, 1.3 Hz, 1H), 7.88 – 7.80 (m, 1H), 7.36 (dd, *J* = 12.6, 5.0 Hz, 1H), 7.25 (d, *J* = 8.5 Hz, 1H), 0.96 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.5, 147.7, 141.3, 137.2, 130.9, 123.8, 113.9, 111.8, 45.0, 31.7, 29.2, 29.1, 26.8, 26.6, 22.6, 14.0. HRMS(ESI) calculated for $C_{19}H_{30}N_2O_2$ $[M+H]^+$:319.2390, found:319.2386;

4.1.3 Synthesis of intermediate **4**

The substituted isatoic anhydride (2 mmol), hydroxylamine hydrochloride (6 mmol), potassium carbonate (2 mmol), and 15 ml of dry acetonitrile solvent were added to a 100 ml round bottom flask. The reaction was stirred at 25 ℃, and the progress of the reaction was monitored by TLC analysis. Upon completion, saturate sodium bicarbonate 20 mL was added and the mixture was extracted with EA (2 × 20 mL). The organic phase was dried over anhydrous sodium sulfate for 10 min, concentrated, and dried under vacuum to obtain a crude light-colored solid.

4.1.4 Synthesis of **5k-5v**

The crude oxime (2 mmol) was dissolved in 10 mL of freshly distilled acetonitrile, then o-hydroxybenzaldehyde (2.5 mmol) and acetic acid (2 mmol) were added to the reaction mixture, and then the temperature was maintained at 80 ℃ and reacted for 2-5 hours, the reaction was monitored by TLC analysis. Upon completion the solvent was removed by rotary evaporator and the crude product was purified by flash column chromatography (petroleum ether)/ethyl acetate = 100:1 to 50:1 to yield the desired product, with a yield of 60-75%.

4.1.5 3-hydroxy-2-isopropyl-1-octyl-2,3-dihydroquinazolin-4(1*H*)-one (**5a**): White solid, 88% yield. mp: 75-76 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.73 (t, *J* = 5.1 Hz, 2H), 7.34 (ddd, *J* = 8.6, 7.2, 1.6 Hz, 1H), 6.67 (d, *J* = 8.5 Hz, 1H), 6.53 (t, *J* = 7.5 Hz, 1H), 3.16 (dd, *J* = 11.4, 6.8 Hz, 2H), 2.76 (dd, *J* = 13.7, 6.9 Hz, 1H), 1.70-1.59 (m, 2H), 1.42 (s, 2H), 1.32-1.23 (m, 8H), 1.19 (d, *J* = 6.9 Hz, 6H), 0.86 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ δ 166.4 , 163.8 , 151.9, 135.1, 131.0, 114.0, 111.4, 107.4, 42.9, 31.9, 29.8, 29.4, 29.2, 29.4, 27.3, 22.8, 19.9, 14.2. HRMS(ESI) calculated for $C_{19}H_{30}N_2O_2$ $[M+H]^+$: 319.2390, found: 319.2386;

4.1.6 (E)-3-hydroxy-1-octyl-2-(pent-1-en-1-yl)-2,3-dihydroquinazolin-4(1*H*)-one (**5b**): White solid, 84% yield. mp: 67-68 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.11 (d, *J* = 9.5 Hz, 1H), 7.88 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.74 (s, 1H), 7.36 (ddd, *J* = 8.6, 7.2, 1.7 Hz, 1H), 6.68 (d, *J* = 8.5 Hz, 1H), 6.58-6.51 (m, 1H), 6.44 – 6.35 (m, 1H), 6.31 (dd, *J* = 14.3, 7.7 Hz, 1H), 3.18 (dd, J = 11.7, 6.8 Hz, 2H), 2.23 (dd, *J* = 14.0, 7.1 Hz, 2H), 1.66 (dd, J = 14.9, 7.1 Hz, 2H), 1.50 (dd, *J* = 14.8, 7.4 Hz, 2H), 1.46-1.38 (m, 2H), 1.29 (ddd, *J* = 13.1, 9.6, 4.9 Hz, 8H), 0.94 (t, *J* = 7.4 Hz, 3H), 0.87 (t, *J* = 6.9 Hz, 3H; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.3, 157.4, 151.9, 148.0, 135.1, 131.0, 123.2, 114.1, 111.4, 107.3, 42.9, 35.1, 31.9, 29.5, 29.3, 29.1, 27.2, 22.8, 21.6, 14.2, 13.7; HRMS(ESI) calculated for $C_{21}H_{32}N_2O_2$ $[M+H]^+$: 345.2542, found: 345.2538.

4.1.7 3-hydroxy-1-octyl-2-phenyl-2,3-dihydroquinazolin-4(1*H*)-one (**5c**): White solid,74% yield. mp: 55-56 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.53 (s, 1H), 7.96 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.80 (dd, *J* = 5.1, 3.1 Hz, 2H), 7.76 (s, 1H), 7.49-7.40 (m, 3H), 6.71 (d, *J* = 8.6 Hz, 1H), 6.59 (t, *J* = 7.6 Hz, 1H), 3.20 (dd, *J* = 10.9, 6.8 Hz, 2H), 1.68 (dd, *J* = 14.8, 7.1 Hz, 2H), 1.50-1.40 (m, 2H), 1.31 (ddd, *J* = 10.7, 7.6, 3.6 Hz, 8H), 0.89 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.2, 156.1, 152.0, 135.3, 131.7, 131.1, 130.5, 129.0, 128.5, 114.1, 111.5, 107.2, 43.0, 32.0, 29.5, 29.3, 29.2, 27.3, 22.8, 14.2; HRMS(ESI) calculated for $C_{22}H_{28}N_2O_2$ $[M+H]^+$: 353.2229, found: 353.2224.

4.1.8 3-hydroxy-2-(4-methoxyphenyl)-1-octyl-2,3-dihydroquinazolin-4(1*H*)-one (**5d**): White solid, 75% yield. mp: 65-67 ℃. ^{1}H

NM… …6.53 = 8.1, 1.6 Hz, 1H), 7.76 (s, 1H), 7.76-7.72 (m, 2H), 7.43-7.33 (m, 1H), 6.99-6.89 (m, 2H), 6.70 (d, *J* = 8.6 Hz, 1H), 6.58 (ddd, *J* = 7.2, 6.6, 0.9 Hz, 1H), 3.85 (d, *J* = 3.0 Hz, 3H), 3.24-3.15 (m, 2H), 1.69 (dd, *J* = 10.0, 4.9 Hz, 2H), 1.50-1.38 (m, 2H), 1.30 (dd, *J* = 11.0, 7.1 Hz, 8H), 0.9-0.84 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.2, 162.3, 155.6, 151.8, 135.0, 131.0, 130.0, 122.7, 114.3, 114.0, 111.3, 107.3, 99.9, 55.4, 42.8, 31.8, 29.4, 29.2, 29.4, 27.2, 22.6, 14.1; HRMS(ESI) calculated for $C_{23}H_{30}N_2O_3$ [M+H]$^+$: 383.2335, found: 383.2334.

4.1.9 3-hydroxy-1-octyl-2-(p-tolyl)-2,3-dihydroquinazolin-4(1*H*)-one (**5e**): White solid, 78% yield. mp: 60-61 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.50 (s, 1H), 7.96 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.76 (s, 1H), 7.69 (d, *J* = 8.1 Hz, 2H), 7.39 (ddd, *J* = 8.6, 7.1, 1.6 Hz, 1H), 7.24 (s, 1H), 6.71 (d, *J* = 8.6 Hz, 1H), 6.59 (ddd, *J* = 8.0, 7.2, 0.9 Hz, 1H), 3.20 (dd, *J* = 11.7, 6.8 Hz, 2H), 2.40 (s, 3H), 1.68 (dd, *J* = 14.8, 7.1 Hz, 2H), 1.49-1.39 (m, 2H), 1.38-1.21 (m, 9H), 0.88 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.3, 156.1, 152.0142.2, 135.2, 131.1, 129.7, 128.4, 127.6, 114.1, 111.5, 107.3, 43.0, 32.0, 29.5, 29.3, 29.2, 27.3, 22.8, 21.7, 14.2; HRMS(ESI) calculated for $C_{23}H_{30}N_2O_2$ [M+H]$^+$: 367.2386, found: 367.2380.

4.1.10 2-(4-chlorophenyl)-3-hydroxy-1-octyl-2,3-dihydroquinazolin-4(1*H*)-one (**5f**): White solid, 80% yield. mp: 53-54 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.30 (s, 1H), 8.00 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.75 (s, 1H), 7.69 (d, *J* = 8.1 Hz, 2H), 7.41 (ddd, *J* = 8.6, 7.1, 1.6 Hz, 1H), 7.24 (s, 1H), 6.71 (d, *J* = 8.6 Hz, 1H), 6.59 (ddd, *J* = 8.0, 7.2, 0.9 Hz, 1H), 3.20 (dd, *J* = 11.7, 6.8 Hz, 2H), 1.68 (dd, *J* = 14.8, 7.1 Hz, 2H), 1.49-1.39 (m, 2H), 1.38-1.21 (m, 9H), 0.89 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.1, 154.9, 152.1, 137.8, 135.4, 131.1, 129.6, 129.4, 129.0, 114.2, 111.6, 107.0, 43.0, 32.0, 29.5, 29.3, 29.2, 27.3, 22.8, 14.2; HRMS(ESI) calculated for $C_{22}H_{27}ClN_2O_2$ [M+H]$^+$: 387.1839, found: 387.1835.

4.1.11 3-hydroxy-2-(4-hydroxyphenyl)-1-octyl-2,3-dihydroquinazolin-4(1*H*)-one (**5g**): White solid, 87% yield. mp: 62-64 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.45 (d, *J* = 1.3 Hz, 1H), 7.95 (dt, *J* = 8.1, 1.5 Hz, 1H), 7.71 (s, 1H), 7.61 (dd, *J* = 8.6, 1.4 Hz, 2H), 7.39 (ddd, *J* = 7.1, 6.4, 1.6 Hz, 1H), 6.92-6.86 (m, 2H), 6.71 (d, *J* = 8.7 Hz, 1H), 6.59 (ddd, *J* = 8.2, 2.3, 1.1 Hz, 1H), 3.19 (s, 2H), 1.73-1.62 (m, 2H), 1.48-1.37 (m, 2H), 1.36-1.20 (m, 8H), 0.87 (dt, *J* = 7.1, 3.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.0, 159.4, 156.1, 152.0, 135.4, 131.2, 130.4, 122.4, 116.2, 114.3, 111.6, 107.2, 43.0, 31.9, 29.5, 29.3, 29.2, 27.3, 22.8, 14.2; HRMS(ESI) calculated for $C_{22}H_{28}N_2O_3$ [M+H]$^+$: 369.2178, found: 369.2173.

4.1.12 3-hydroxy-2-(2-hydroxyphenyl)-1-octyl-2,3-dihydroquinazolin-4(1*H*)-one (**5h**): White solid, 80% yield. mp: 73-75 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz):δ 10.08 (s, 1H), 8.58 (s, 1H), 7.95 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.67 (t, *J* = 4.5 Hz, 1H), 7.40 (qd, *J* = 7.2, 1.6 Hz, 2H), 7.28 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.08 (d, *J* = 8.2 Hz, 1H), 6.96 (td, *J* = 7.5, 0.8 Hz, 1H), 6.72 (d, *J* = 8.5 Hz, 1H), 6.63-6.58 (m, 1H), 3.21 (dd, *J* = 12.1, 7.0 Hz, 2H), 1.74-1.65 (m, 2H),1.50-1.40 (m, 2H), 1.36-1.26 (m, 8H), 0.89 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.0, 158.5, 157.2, 152.1, 135.5, 133.1, 132.0, 131.1, 119.8, 117.5, 115.3, 114.3, 111.6, 106.6, 43.0, 32.0, 29.5, 29.3 29.2, 27.3, 22.8, 14.3; HRMS(ESI) calculated for $C_{22}H_{28}N_2O_3$ [M+H]$^+$: 369.3564, found: 369.3560.

4.1.13 3-hydroxy-2-(2-methoxyphenyl)-1-octyl-2,3-dihydroquinazolin-4(1*H*)-one (**5i**): White solid, 78% yield. mp: 52-54 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.95 (s, 1H), 8.07 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.98 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.78 (s, 1H), 7.50-7.34 (m, 1H), 3.89 (s, 3H), 3.20 (td, J = 6.9, 5.1 Hz, 2H), 1.68 (dd, *J* = 14.9, 7.1 Hz, 2H), 1.51-1.39 (m, 2H), 1.31 (ddd, *J* = 10.4, 7.9, 3.6 Hz, 8H), 0.89 (dd, *J* = 9.3, 4.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.4, 158.5, 152.2, 152.0, 135.1, 133.1, 131.2, 127.7, 121.0, 118.9, 114.1, 111.4, 111.2, 107.4, 55.7, 43.0, 32.0, 29.5, 29.3, 29.2, 27.3, 22.8, 14.2; HRMS(ESI) calculated for $C_{23}H_{30}N_3O_3$ [M+H]$^+$: 383.2335, found: 383.2331.

4.1.14 2-(2-chlorophenyl)-3-hydroxy-1-octyl-2,3-dihydroquinazolin-4(1*H*)-one (**5j**): White solid, 90% yield. mp: 53-55 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.97 (s, 1H), 8.17 (dd, *J* = 7.7, 1.4 Hz, 1H), 7.98 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.74 (t, *J* = 4.4 Hz, 1H), 7.46-7.37 (m, 3H), 7.36 -7.30 (m, 1H), 6.72 (d, *J* = 8.6 Hz, 1H), 6.65-6.58 (m, 1H), 3.20 (dd, *J* = 11.8, 6.9 Hz, 2H), 1.68 (dd, *J* = 14.9, 7.1 Hz, 2H), 1.49-1.40 (m, 2H), 1.37-1.24 (m, 8H), 0.88 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 166.1, 153.0, 152.1, 135.4, 135.1, 132.6, 131.2, 130.1, 128.4, 127.3, 114.2, 111.5, 107.0, 43.0, 32.0, 29.5, 29.3, 29.2, 28.3, 22.8, 14.2; HRMS(ESI) calculated for $C_{22}H_{27}ClN_2O_2$ [M+H]$^+$: 387.1839, found: 387.1835.

4.1.15 1-hexadecyl-3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydroquinazolin-4(1*H*)-one (**5k**): White solid, 84% yield. mp: 67-69 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.59 (s, 1H), 7.94 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.66 (s, 1H), 7.39 (ddd, *J* = 8.9, 6.1, 1.6 Hz, 2H), 7.08 (d, *J* = 8.2 Hz, 1H), 6.99- 6.95 (m, 1H), 6.73 (d, *J* = 8.5 Hz, 1H), 6.60 (t, *J* = 7.5 Hz, 1H), 3.22 (dd, *J* = 11.1, 6.2 Hz, 2H), 1.72-1.68 (m, 2H), 1.44 (dd, *J* = 10.7, 4.9 Hz, 2H), 1.26 (d, *J* = 10.3 Hz, 26H), 0.88 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 164.9, 158.5, 157.2, 152.0, 135.4, 134.7, 133.0, 132.5, 131.9, 131.0, 119.7, 117.5, 115.2, 114.3, 114.2, 111.5, 111.3, 106.6, 42.9, 31.9, 29.7-29.1, 27.2, 22.7, 14.1; HRMS(ESI) calculated for $C_{30}H_{44}N_2O_3$ [M+H]$^+$: 481.4522, found: 481.4518.

4.1.16 3-hydroxy-2-(2-hydroxyphenyl)-1-tetradecyl-2,3-dihydroquinazolin-4(1*H*)-one (**5l**): Yellow solid, 83% yield. mp: 71-72 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.58 (s, 1H), 7.95 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.68 (d, *J* = 4.9 Hz, 1H), 7.50-7.34 (m, 2H), 7.34-7.22 (m, 1H), 7.08 (dd, *J* = 4.9, 4.5 Hz, 1H), 7.02-6.90 (m, 1H), 6.72 (d, *J* = 9.0 Hz, 1H), 6.60 (ddd, *J* = 8.1, 4.6, 1.0 Hz, 1H), 3.30-3.15 (m, 2H), 2.05 (s, 1H), 1.71 (dd, *J* = 15.2, 7.5 Hz, 2H), 1.43 (dd, *J* = 15.5, 7.3 Hz, 3H), 1.27 (d, *J* = 9.9 Hz, 19H), 0.88 (dd, *J* = 8.2, 7.1 Hz, 3H; ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.0, 158.5, 157.2, 152.1, 135.5, 133.1, 132.0, 131.1, 119.8, 117.5, 115.3, 114.3, 111.6, 106.6, 43.0, 32.1, 29.8-29.2, 27.3, 22.8, 14.3; HRMS(ESI) calculated for $C_{28}H_{40}N_2O_3$ [M+H]$^+$: 453.3535, found: 453.3534.

4.1.17 1-dodecyl-3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydroquinazolin-4(1*H*)-one (**5m**): Yellow solid, 78% yield. mp: 69-71 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.59 (s, 1H), 7.95 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.76- 7.63 (m, 1H), 7.57- 7.34 (m, 2H), 7.34-7.17 (m, 1H), 7.08 (d, *J* = 8.6 Hz, 1H), 7.03-6.91 (m, 1H), 6.73 (d, *J* = 8.9 Hz, 1H), 6.60 (td, *J* = 8.1, 0.9 Hz, 1H), 3.21 (dd, *J* = 12.9, 7.5 Hz, 2H), 2.05 (d, *J* = 0.9 Hz, 1H), 2.01- 1.64 (m, 3H), 1.45 (dd, *J* = 14.7, 7.5 Hz, 3H), 1.39-0.93 (m, 14H), 0.88 (dd, *J* = 7.7, 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.0, 158.5, 157.2, 152.1, 135.5, 133.1, 132.0, 131.1, 119.8, 117.5, 115.3, 114.3, 111.6, 106.6, 100.0, 43.0, 32.0, 29.8 - 29.5, 29.2, 27.3, 22.8, 21.2, 14.3; HRMS(ESI) calculated for $C_{26}H_{36}N_2O_3$ [M+H]$^+$: 425.4334, found: 425.4330.

4.1.18 1-decyl-3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydroquinazolin-4(1*H*)-one (**5n**): White solid, 89% yield. mp:70-71 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.59 (s, 1H), 7.95 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.68 (d, *J* = 5.4 Hz, 1H), 7.57-7.35 (m, 2H), 7.32-7.22 (m, 1H), 7.08 (d, *J* = 8.6 Hz, 1H), 7.01-6.86 (m, 1H), 6.73 (d, *J* =

8.9 [illegible] *J* = 2.05 (d, *J* = 0.9 Hz, 1H), 2.01-1.64 (m, 3H), 1.45 (d, *J* = 7.2 Hz, 3H), 1.34-1.21 (m, 10H), 0.88 (dd, *J* = 7.7, 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.0, 158.5, 157.2, 152.0, 135.5, 133.1, 132.0, 131.1, 119.8, 117.5, 115.3, 114.3, 111.6, 106.6, 43.0, 32.0, 29.7, 29.2, 28.4, 27.3, 22.8, 14.3; HRMS(ESI) calculated for $C_{24}H_{32}N_2O_3$ $[M+H]^+$: 397.3544, found: 397.3539.

4.1.19 1-hexyl-3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydro quinazolin-4(1*H*)-one (**5o**): Yellow solid, 76% yield. mp: :68-70 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.58 (s, 1H), 7.95 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.68 (d, *J* = 5.1 Hz, 1H), 7.40 (dtd, *J* = 9.1, 7.3, 1.8 Hz, 2H), 7.31- 7.21 (m, 1H), 7.14-7.02 (m, 1H), 6.96 (td, *J* = 7.5, 1.2 Hz, 1H), 6.77- 6.68 (m, 1H), 6.60 (ddd, *J* = 8.3, 7.1, 1.2 Hz, 1H), 3.27- 3.08 (m, 2H), 2.05 (s, 1H), 1.77- 1.64 (m, 2H), 1.45 (d, *J* = 7.0 Hz, 2H), 1.40- 1.29 (m, 3H), 1.26 (t, *J* = 7.2 Hz, 1H), 0.90 (ddd, *J* = 8.3, 6.5, 2.4 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.0, 158.5, 157.2, 152.1, 135.5, 133.1, 132.0, 131.1, 120.0, 117.5, 115.3, 114.3, 111.6, 106.6, 43.0, 31.7, 29.2, 27.0, 22.7, 14.2; HRMS(ESI) calculated for $C_{20}H_{24}N_2O_3$ $[M+H]^+$: 341.2843, found: 341.2839.

4.1.20 1-butyl-3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydroqui nazolin-4(1*H*)-one (**5p**): Yellow solid, 90% yield. mp: 69-71 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.58 (s, 1H), 7.99-7.91 (m, 1H), 7.67 (s, 1H), 7.39 (ddd, *J* = 12.8, 9.4, 5.0 Hz, 2H), 7.32- 7.21 (m, 2H), 7.08 (d, *J* = 8.3 Hz, 1H), 6.96 (t, *J* = 7.5 Hz, 1H), 6.73 (d, *J* = 8.6 Hz, 1H), 6.60 (t, *J* = 7.5 Hz, 1H), 3.26-3.19 (m, 2H), 1.73-1.66 (m, 2H), 1.48 (dd, *J* = 15.0, 7.5 Hz, 2H), 0.98 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.1, 158.6, 157.3, 152.2, 135.6, 133.1, 132.0, 131.1, 119.9, 117.6, 115.4, 114.4, 111.7, 106.7, 42.7, 31.3, 20.5, 14.0; HRMS(ESI) calculated for $C_{18}H_{20}N_2O_3$ $[M+H]^+$: 313.3345, found: 313.3344.

4.1.21 3-hydroxy-2-(2-hydroxyphenyl)-1-methyl-2,3-dihydroqui nazolin-4(1*H*)-one (**5q**): Red solid, 88% yield. mp: 88-90 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.61-8.55 (m, 1H), 7.96 (ddd, *J* = 8.2, 4.2, 1.3 Hz, 1H), 7.66 (s, 1H), 7.50-7.34 (m, 2H), 7.32- 7.21 (m, 1H), 7.08 (d, *J* = 8.5 Hz, 1H), 7.00- 6.92 (m, 1H), 6.72 (d, *J* = 8.8 Hz, 1H), 6.66- 6.55 (m, 1H), 2.94 (d, *J* = 5.0 Hz, 3H), 2.92 (d, *J* = 1.0 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 164.9, 158.5, 157.1, 152.8, 135.6, 133.1, 132.0, 131.0, 119.9, 117.5, 115.3, 114.5, 111.2, 106.9, 29.7; HRMS(ESI) calculated for $C_{15}H_{14}N_2O_3$ $[M+H]^+$: 271.1975, found: 271.1970.

4.1.22 3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydroquinazolin-4(1*H*)-one (**5r**): White solid, 86% yield. mp: 129-131 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.06 (s, 1H), 8.58 (s, 2H), 7.98-7.86 (m, 1H), 7.44-7.26 (m, 2H), 7.13-7.02 (m, 1H), 6.97 (td, *J* = 7.6, 1.3 Hz, 1H), 6.79- 6.60 (m, 2H), 5.83 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 164.4, 158.5, 157.4, 151.4, 135.0, 133.2, 132.0, 130.6, 119.9, 117.5, 117.0, 116.4, 115.2, 107.8; HRMS(ESI) calculated for $C_{14}H_{12}N_2O_3$ $[M+H]^+$: 257.1774, found: 257.1771.

4.1.23 1-benzyl-3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydroqui nazolin-4(1*H*)-one (**5s**): White solid, 78% yield. mp: 115-116 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.06 (s, 1H), 8.60 (s, 1H), 8.18 (d, *J* = 5.9 Hz, 1H), 7.99 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.37 (tdd, *J* = 13.0, 5.8, 2.0 Hz, 6H), 7.31-7.20 (m, 2H), 7.08 (d, *J* = 8.5 Hz, 1H), 7.02- 6.89 (m, 1H), 6.74- 6.55 (m, 2H), 4.50 (d, *J* = 6.1 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.0, 158.5, 157.4, 151.7, 138.5, 135.6, 133.2, 132.0, 131.1, 128.9, 127.4, 127.1, 119.9, 117.7, 115.1, 112.2, 107.3, 47.0; HRMS(ESI) calculated for $C_{21}H_{18}N_2O_3$ $[M+H]^+$: 347.1825, found: 347.1824.

4.1.24 1-(3-fluorobenzyl)-3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydroquinazolin-4(1*H*)-one (**5t**): White solid, 85% yield. mp: 88-89 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.03 (s, 1H), 8.14 (t, *J* = 5.3 Hz, 1H), 7.99 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.42-7.33 (m, 3H), 10.2, 9.5, 6.7 Hz, 3H), 6.97 (td, *J* = 7.6, 1.0 Hz, 1H), 6.72-6.63 (m, 2H), 4.56 (d, *J* = 5.8 Hz, 2H), 1.56 (d, *J* = 60.5 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 164.9, 161.6, 159.9, 158.5, 157.4, 151.5, 135.5, 133.1, 131.9, 131.0, 128.9, 125.4, 124.4, 119.8, 117.5, 115.5, 115.3, 111.9, 107.5, 40.4; HRMS(ESI) calculated for $C_{21}H_{17}FN_2O_3$ $[M+H]^+$: 365.4432, found: 365.4428.

4.1.25 1-(3-chlorobenzyl)-3-hydroxy-2-(2-hydroxyphenyl)-2,3-dihydroquinazolin-4(1*H*)-one (**5u**): White solid, 90% yield. mp: 84-86 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ10.02 (s, 1H), 8.60 (s, 1H), 8.19 (t, *J* = 5.3 Hz, 1H), 7.99 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.38 (ddd, *J* = 15.6, 8.6, 4.4 Hz, 2H), 7.32-7.21 (m, 5H), 7.08 (d, *J* = 8.2 Hz, 1H), 6.99- 6.93 (m, 1H), 6.70- 6.58 (m, 2H), 4.48 (d, *J* = 5.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 165.0, 158.5, 157.4, 151.4, 140.8, 135.5, 134.7, 133.1, 131.9, 131.1, 130.1, 127.5, 127.1, 125.0, 119.8, 117.5, 115.4, 115.1, 112.0, 107.6, 46.5; HRMS(ESI) calculated for $C_{21}H_{17}ClN_2O_3$ $[M+H]^+$: 381.4533, found: 381.4528.

4.1.26 2-((3-hydroxy-2-(2-hydroxyphenyl)-4-oxo-3,4-dihydro quinazolin-1(2*H*) yl)methyl)benzonitrile (**5v**): Yellow solid, 85% yield. mp: 76-77 ℃. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.01 (s, 1H), 8.61 (s, 1H), 8.10 (t, *J* = 5.3 Hz, 1H), 7.89 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.35 (ddd, *J* = 15.6, 8.6, 4.4 Hz, 2H), 7.31-7.18 (m, 5H), 7.05 (d, *J* = 8.2 Hz, 1H), 7.01-6.93 (m, 1H), 6.74- 6.53 (m, 2H), 4.45 (d, *J* = 5.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 164.9, 161.5, 158.5, 157.3, 151.4, 135.5, 133.0, 131.9, 131.0, 128.8, 125.4, 124.3, 119.7, 117.5, 115.4, 115.2, 111.8, 107.4, 40.4; HRMS(ESI) calculated for $C_{22}H_{17}N_3O_3$ $[M+H]^+$: 372.3456, found: 372.3450.

4.2 Determination of Minimun Inhibitory Concentration (MIC)

Compounds were dissolved in biological grade DMSO as 10 mM stocks and stored at -20 ℃. Bacteria were cultured for 6-8 hours in MHB this culture was used to inoculate fresh media at 5×10^5 CFU/mL. Aliquots of the resulting bacterial suspension were distributed to culture tubes and compound was added from stock solutions to give the final testing concentration. Bacteria not treated with compound served as the control. An aliquot of each sample was transferred to a new culture tube and antibiotic was added. Rows B-H of a 96-well microtiter plate were filled (100 µL per well) from the remaining bacterial subcultures, allowing the concentration of compound to be kept uniform throughout the antibiotic dilution procedure. Aliquots (200 µL) of the samples containing antibiotic were distributed to the corresponding first-row wells of the microtiter plate. Row A wells were mixed six to eight times, and then 100 µL were transferred to row B. This procedure was repeated to serially dilute the rest of the rows of the microtiter plate, with the exception of the final row, to which no antibiotic was added (to check for growth of bacteria in the presence of compound alone). The plate was then covered and incubated under stationary conditions at 37 ℃. After 16-18 h, MICs were recorded as the lowest concentration of antibiotic at which there was no visible growth of bacteria.

4.3 Biofilm Inhibition Assays

Compounds were dissolved in biological grade DMSO as 10 mM stocks and stored at -20 ℃. The biofilm assays utilized here were adapted from literature procedure. Bacteria were cultured overnight in LB broth, then subcultured to an OD_{600} of 0.01 in the same media. Compounds were added from stock solutions to give the desired concentrations in triplicate. Inoculums with no compound added served as the untreated control. Samples were aliquot (100 µL) into the wells of the 96-well PVC microtiter plate. Followed by an incubation under stationary conditions for 24 h at 37 ℃. After incubation the medium was discarded from

the [illegible] and Add 95% ethanol and discard after 30 minutes. Plates were then stained with 110 μL of 0.1% aqueous crystal solution violet (CV) and then incubated at ambient temperature for 10 min. Plates were washed with water again(3 times), and treated with 33% glacial acetic acid to release the stain. Biofilms were quantified by recording the optical density of the released dye (590 nm).The OD_{590} of each well was measured and biofilm inhibition was quantified by calculating the OD_{590} of treated wells as a percentage of untreated control wells.

4.4 Laser confocal microscope observations of *A. baumannii* in the presence and absence of **5a**

Grow 30 mL cultures of *A. baumannii* bacteria to late log phase in LB broth with or without 5a. Then, concentrate 25 mL of the bacterial culture by centrifugation at 10,000 × g for 10–15 minutes. Remove the supernatant and resuspend the pellet in 2 mL of 0.85% NaCl. Add 1 mL of this suspension to each of two 30–40 mL centrifuge tubes containing 20 mL of 0.85% NaCl. Incubate both samples at room temperature for 1 hour, mixing every 15 minutes. Pellet both samples by centrifugation at 10,000×g for 10–15 minutes. Resuspend the pellets in 20 mL of 0.85% NaCl and centrifuge again 10,000×g for 10–15 minutes. Resuspend both pellets in separate tubes with 10 mL of 0.85% NaCl each. Determine the optical density at 670 nm (OD_{670}) of a 3 mL aliquot of the bacterial suspensions in glass or acrylic absorption cuvettes (1 cm pathlength). Combine a sample of the 2X stock Syto9/PI staining solution with an equal volume of the bacterial suspension. The final concentration of each dye will be 6 μM SYTO 9 stain and 30 μM propidium iodide. Mix thoroughly and incubate at room temperature in the dark for 15 minutes. Trap 5 μL of the stained bacterial suspension between a slide and an 18 mm square coverslip. Observe in a fluorescence microscope and the excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide. The background remains virtually nonfluorescent.

4.5 Motility in the presence of 5n and 5u[25]

The motility of *A. baumannii* is shown on modified LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 0.3% Eiken either unsupplemented or containing 50 μM of 5n or 5u. *A. baumannii* was grown overnight in 3 ml of modified LB broth, and a 1-μL drop was placed on the surface of each plate. Plates were photographed after incubated at 37 ℃ for 24 hours.

4.6 Determination of Extracellular Polysaccharides in Biofilms[6]

Add 1 ml of medium to a 24-well plate, culture 190 μL of bacterial solution and 10 μL of QSI with different concentrations in triplicate and incubate at 30 ℃ for 24 h. The upper layer of bacterial solution is aspirated and discarded. Rinse 24 wells with 0.9% NaCl, then add 0.25 ml of 5% phenol and 1.25 mL of concentrated sulfuric acid (containing 0.2% hydrazine sulfate) to a 24-well plate, and incubate at 30 ℃ for 1 h in the dark, then measure OD_{490}. Extracellular polysaccharides were quantified by recording the optical density of the released dye (490 nm). The OD_{490} of each well was measured and extracellular polysaccharides inhibition was quantified by calculating the OD_{490} of treated wells as a percentage of untreated control wells.

4.7 Checkerboard Assays

The synergism of the QSIs (5n and 5u) with Gentamycin sulfate was evaluated by a checkerboard titration assay in microplates as recommended by the NCCLS and expressed as the sum of the fractional inhibitory concentration (FIC) index for each agent. In brief, checkerboards were set up with double dilutions of QSIs (512-2 μg/mL) in the vertical wells. Then, a 190 mL *Acinetobacter baumannii* S1 adjusted to 5×10^5 CFU/mL was added to each well and cultured at 37 ℃ for 24 h in MHB.

After incubation, plates were visually inspected for turbidity to determine growth. Visual readings of the MIC were measured. Experiments were performed in triplicate on different days. Each checkerboard test generates many different combinations and, by convention, the FIC value of the most effective combination was used in calculating the fractional inhibitory concentration index (FICI). FICI was calculated by adding both FICs:

$FICI = FIC_A + FIC_B = C_A^{comb}/MIC_A^{alone} + C_B^{comb}/MIC_B^{alone}$

Where MIC_A alone and MIC_B alone are the MICs of compound A and B when acting alone and C_A^{comb} and C_B^{comb} are concentrations of compounds A and B at the isoeffective combinations. The FICI was interpreted as synergistic when it was<0.5, antagonistic when it was >4.0, and any value between was interpreted as indifferent.

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