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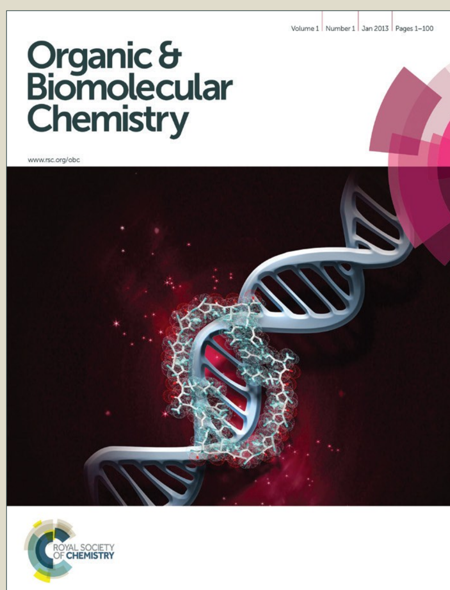

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Design, Synthesis and Evaluation of *N*-Aryl-Glyoxamide Derivatives as Structurally Novel Bacterial Quorum Sensing Inhibitors

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Bacteria cooperatively regulate the expression of many phenotypes through a mechanism called quorum sensing (QS). Many Gram-negative bacteria use an *N*-acyl homoserine lactone (AHL)-mediated QS system to control biofilm formation and virulence factor production. In recent years, quorum sensing inhibitors (QSIs) have become attractive tools to overcome antimicrobial resistance exhibited by various pathogenic bacteria. In the present study, we report the design and synthesis of novel *N*-arylisatin-based glyoxamide derivatives via the ring-opening reaction of *N*-aryl isatins with cyclic and acyclic amines, and amino acid esters. The QSI activity of the synthesized compounds was determined in the LasR-expressing *Pseudomonas aeruginosa* MH602 and LuxR-expressing *Escherichia coli* MT102 reporter strains. Compounds **31** and **32** exhibited the greatest QSI activity in *P. aeruginosa* MH602, with 48.7% and 42.7% reduction in QS activity at 250 µM, respectively, while compounds **31** and **34** showed 73.6% and 43.7% QSI activity in *E. coli* MT102. In addition, the ability of these compounds to inhibit the production of pyocyanin in *P. aeruginosa* (PA14) was also determined, with compound **28** showing 47% inhibition at 250 µM. Furthermore, computational docking studies were performed on the LasR receptor protein of *P. aeruginosa*, which showed that formation of a hydrogen bonding network played a major role in influencing the QS inhibitory activity. We envisage that these novel non-AHL glyoxamide derivatives could become a new tool for the study of QS and potentially for the treatment of bacterial infections.

Introduction

Antibiotic resistance has become an increasing problem in recent years due to the slackening rate of discovery of novel antibiotics, while at the same time antibiotic use is on the rise.[2] Traditional antibiotics exert their antibacterial activity by the inhibition of cell wall synthesis, DNA or RNA synthesis, protein synthesis, folate synthesis, or the depolarization of membrane potential.[3,4] Due to increased pressure, bacteria develop antibiotic resistance through biofilm formation, enzymatic degradation of antibiotics, reduced cell wall permeability, increased efflux mechanisms and mutation of the targets of antbiotics.[5] Moreover, the continued misuse and overuse of broad-spectrum antibiotics has accelerated the evolution of antibiotic resistance. Furthermore, the repeated outbreaks of new infectious diseases and the re-appearance of old infectious diseases has had a deleterious impact on human health and the global economy,[6,7] with infectious diseases also having a disproportionate impact on less economically developed countries. In this context, it is crucial to understand bacterial virulence and survival mechanisms in order to identify novel therapeutic approaches to combat bacterial infection.

Acyl homoserine lactone (AHL)-mediated quorum sensing (QS) is a process employed by a range of proteobacteria and archaea to regulate gene expression. QS has been implicated in the regulation of phenotypes such as bioluminescence, extracellular enzyme production and virulence factor production.[8] Bacteria release AHLs into their extracellular environment, and by surpassing of an AHL threshold concentration at high cell population density the expression or repression of target gene is triggered. This cooperative behaviour delivers a myriad of advantages to bacteria, including biofilm formation, access to nutrients and resistance to antibiotics. Different types of AHLs (**1**-**3**) have been identified in many Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Vibrio fischeri*, and *Vibrio harveyi* (**Fig. 1**).

The proteins responsible for the synthesis and recognition of the various autoinducers are the LuxI/LuxR (expressed in *V. fischeri*) and LasI/LasR (expressed in *P. aeruginosa*) systems.

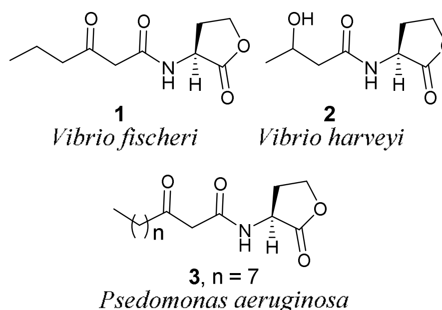

Fig. 1: Various natural autoinducers used by Gram-negative bacteria.

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The LuxI and LasI proteins are autoinducer synthases, which produce 3-oxohexanoyl homoserine lactone (OHHL, **1**) and 3-oxo-dodecanoyl homoserine lactone (OdDHL, **3**), respectively. These autoinducers are then recognized by the corresponding transcriptional receptor proteins, LuxR and LasR, respectively, thus giving a range of diverse AHL-mediated QS systems.[9-14]

One of the most widely studied Gram-negative, multidrug-resistant (MDR) opportunistic pathogens is *P. aeruginosa*, which is widespread in lung infections associated with cystic fibrosis.[15,16] *P. aeruginosa* controls pathogenicity through LasR/LasI and RhlR/RhlI-based QS systems that regulate biofilm formation and the production of virulence phenotypes, such as the production of pyocyanin, exotoxin and exoenzymes.[4]

Escherichia coli is another Gram-negative bacteria which can cause urinary tract infections, meningitis, sepsis and hemorrhagic colitis.[17] *E. coli* encodes the transcriptional regulator SdiA, which has been shown to bind AHLs to mediate QS regulated gene expression, despite the fact that *E. coli* does not have its own AHL synthase. Thus, *E. coli* responds to the AHLs produced by other bacteria in its local environment.[18]

Numerous synthetic QS agonists and antagonists have been investigated against the LasR QS system by modifications in the lactone ring and/or in the hydrophobic aliphatic side chain of natural AHLs.[19-28] In particular, QS inhibitors (QSIs) have the potential to interfere with bacterial communication, thereby inhibiting bacterial biofilm formation and virulence. However, QS antagonists based on AHLs are sensitive to both non-enzymatic hydrolysis at physiological pH and degradation by lactonases, yielding ring-opened products that usually lack biological activity. To overcome this problem, various non-AHL-based QSIs have recently been developed (**Fig. 2**).[29] A novel non-natural agonist **4** that binds to the LasR receptor protein of *P. aeruginosa* was identified by high throughput screening.[30,31] O'Brien *et al.* subsequently synthesized the related compounds **5** and **6**, which showed potent LasR antagonist activity in *P. aeruginosa* and also inhibited biofilm formation as well as the expression of the virulence factor pyocyanin in PAO1 and PA14 strains.[32] In addition, our research group recently reported novel indole-based QSIs with activity in the *P. aeruginosa* MH602 strain, of which compound **7** displayed the greatest activity.[33]

The basis of our approach to the synthesis of new QSI scaffolds lay in our appreciation of the unique reactivity of *N*-substituted isatins. Isatin derivatives with electron-withdrawing substituents attached to the nitrogen atom, such as *N*-acetylisatins, are very labile towards attack by nucleophiles such as amines and alcohols. These reactions are irreversible, clean and high-yielding. Previously, our group has synthesized *N*-acyl glyoxamide derivatives by the ring-opening reactions of *N*-acylisatins with various amines and amino acids[34], and evaluated these compounds as inhibitors of biofilm formation in *P. aeruginosa.* One such example is compound **8**, which was found to be the most active.[34] The use of the Isatin phenyl ring as a head group should improve the metabolic stability of the compounds, a strategy which has been successfully employed in the literature.[34] Furthermore, the glyoxamides offer increased capacity to form hydrogen bonds that might impact on their interactions with the LasR receptor protein and therefore their QSI activity.

Interestingly, the ring-opening reactions of *N*-arylisatins and the QSI activity of the resulting glyoxamides have not yet been explored. For the first time, we report herein the synthesis of *N*-aryl glyoxamide derivatives by the ring-opening reaction of *N*-arylisatins. The effect of the aryl substituents on the ring-opening reaction was investigated, as was the effect of using various acyclic and cyclic amines, or amino acid esters as nucleophiles. The Las-dependent QS inhibitory activity of these compounds was determined in the *P. aeruginosa* MH602 *las*B reporter strain and *E. coli* MT102 strain. Additionally, the ability of these compounds to inhibit pyocyanin production in the *P. aeruginosa* PA14 strain was also determined. Finally, molecular modelling studies were performed to investigate the binding of these compounds to the autoinducer receptor binding site of the LasR protein.

Results and discussion

Chemistry

While the ring-opening reactions of *N*-acylisatins have been previously reported, similar procedures based on *N*-arylisatins are largely unknown. To synthesize *N*-arylisatins, isatin **9** was subjected to Chan-Lam coupling[35] with various phenylboronic acids using $Cu(OAc)_2$ and Et_3N in dichloromethane to give *N*-arylisatins **10**-**14,** and purified by flash chromatography (ethylacetate-hexane) to obtain pure compounds in 27-62%

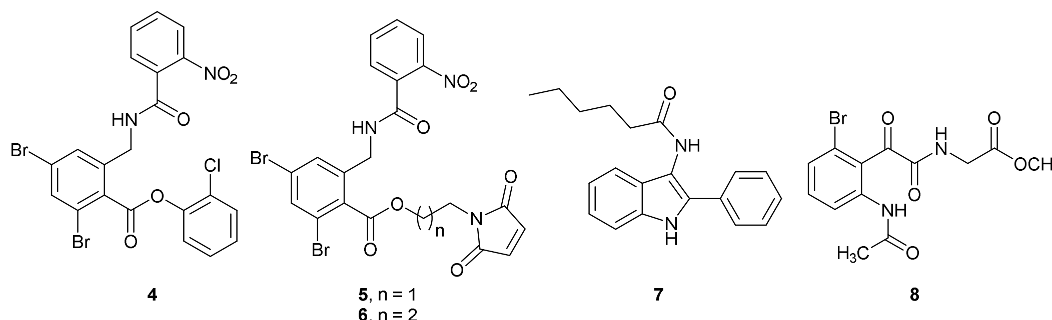

Fig. 2: Various non-native QSIs (**4**-**7**) and biofilm inhibitors (**8**).

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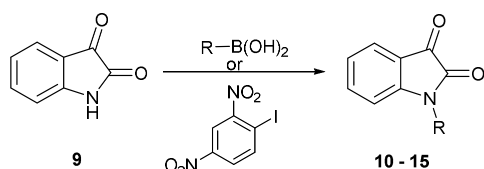
yields (**Scheme 1**). Generally, it was observed that electron-deficient boronic acids gave lower yields than electron-rich or electron-neutral systems (**Table 1**). Attempts to couple **9** with 3-cyanophenyl boronic acid, indole-6-boronic acid and 4-pyridylboronic acid were unsuccessful and corresponding *N*-arylisatins were not formed. In an alternative strategy, **9** was reacted with 2,4-dinitroiodobenzene to give **15** (**Scheme 1**).

Subsequently, *N*-arylisatins **10-15** were treated with a variety of cyclic and acyclic amines in acetonitrile at room temperature, giving glyoxamide derivatives **16**-**30** (**Scheme 2**). Reactions with cyclic amines were completed in 15-30 min and the products were obtained in 80-90% yields. In the case of acyclic amines, the reaction proceeded over much longer times (4-16 h) and gave the products in lower yields of 65-70%.



Scheme 1: Synthesis of *N*-arylisatins from isatin **9**. Reaction conditions for compounds **10-14**: **9** (1.0 equiv), boronic acid (1.5 equiv), $Cu(OAc)_2$ (1.5 equiv), Et_3N (2.0 equiv), dichloromethane, rt, 1 to 16 h. Reaction conditions for **15**: **9** (1.0 equiv), 2,4-dinitroiodobenzene (1.2 equiv), tetrabutylammonium bisulfate (1.0 equiv), chloroform, rt, 30 h.

Table 1: Synthesis of *N*-aryl isatins **10-15** with corresponding yields.

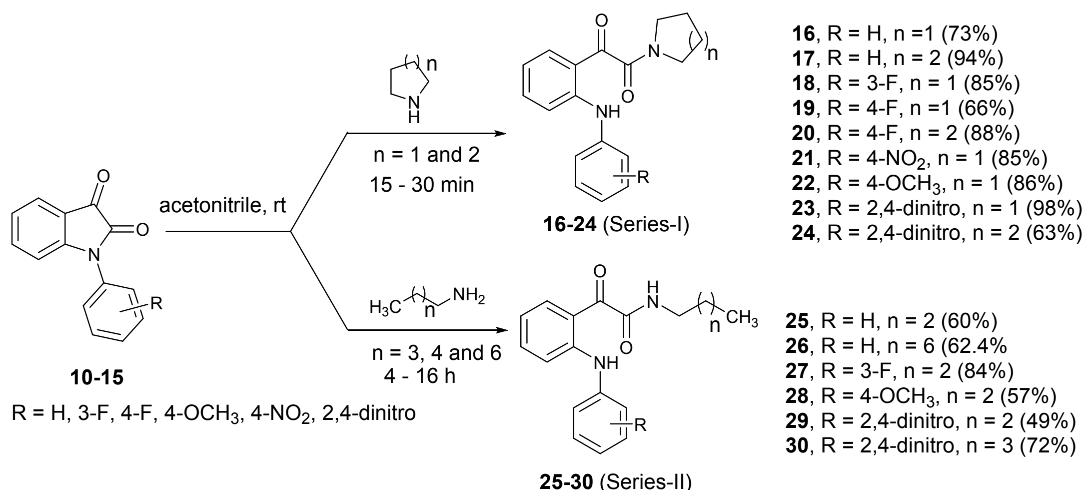
Entry	Compound	R	[a]Yield(%)
1	**10**	Ph-	55%
2	**11**	4-(OCH_3)Ph-	52%
3	**12**	3-FPh-	56%
4	**13**	4-FPh-	62%
5	**14**	4-(NO_2)Ph-	27%
6	**15**	2,4-(NO_2)Ph-	56%

[a]Isolated yields.

In order to perform ring-opening reactions of *N*-arylisatins with amino acid esters, further optimization of the method was required. *N*-Arylisatins **10**, **14** and **15** were reacted with glycine ethyl ester under a variety of conditions, primarily modifying the base (Et_3N or $NaHCO_3$) and solvent used (acetonitrile, MeOH or DCM). It was found that the reaction only proceeded with the 2,4-dinitro derivative **15**, when using Et_3N in DCM at room temperature for 16 h, producing compound **31** in 25% yield. This suggests that the ring-opening reaction with amino acid esters requires a highly electron-poor *N*-aryl substituent on the isatin scaffold. Under the optimized conditions, *N*-arylisatin **15** was ring-opened with different amino acid esters to obtain glyoxamide derivatives **32**-**36** in 24-58% yield (Scheme 3, Table 2).

Flash-column chromatography (ethyl acetate/hexane) proved to be an effective purification method for the glyoxamide derivatives (**16-36**). In particular, the purification of compounds **16-24** was relatively quick, with chromatography only necessary to remove baseline impurities. In the case of compounds **25-36**, purification was more involved, requiring longer times in order to separate the product from excess/unreacted starting material (**10-15**), resulting in their reduced yields compared to compounds **16-24**.

In general, the *N*-arylisatins (**10-14**) could be synthesized in moderate to good yields, with electron-poor aromatics being less favourable. This procedure offers an alternative method for the synthesis of such compounds.[35] We report the first example of conditions for the ring-opening of *N*-arylisatins with a variety of amines (cyclic, acyclic, and amino acid esters). In order to perform ring-opening reactions of *N*-arylisatins with amino acid esters, further optimization of the method was required. *N*-Arylisatins **10**, **14** and **15** were reacted with glycine ethyl ester under a variety of conditions, primarily modifying the base (Et_3N or $NaHCO_3$) and solvent used (acetonitrile, MeOH or DCM). It was found that the reaction only proceeded



Scheme 2: Synthesis of glyoxamide derivatives of *N*-arylisatins. Reaction conditions: aryl isatin (1.0 equiv), amine (1.0 equiv), acetonitrile, rt, 15 min to 16 h.

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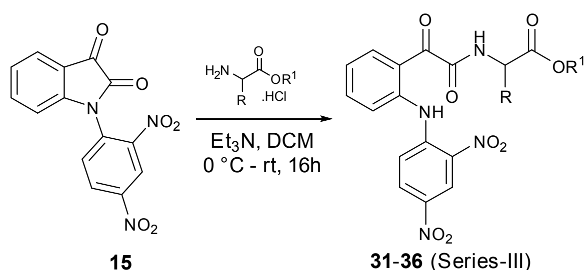

Scheme 3: Synthesis of amino acid-substituted glyoxamide derivatives from compound **15**. Reaction condition: **15** (1.0 equiv), amino acid (2.5 equiv), Et_3N (2.5 equiv), dichloromethane, rt, 16 h.

Table 2: Amino acid glyoxamide derivatives with yields.

Entry	[a]Amino acid-OR[1]	Product	[b]Yield
1	Glycine-OEt	**31**	25%
2	L-Alanine -OMe	**32**	58%
3	L-Valine-OMe	**33**	53%
4	L-Leucine-OMe	**34**	51%
5	L-Phenylalanine-OMe	**35**	24%
6	L-Tryptophan-OMe	**36**	24%

[a]As hydrochloride salts

[b]Isolated yields

with the 2,4-dinitro derivative **15**, when using Et_3N in DCM at room temperature for 16 h, producing compound **31** in 25% yield. This suggests that the ring-opening reaction with amino acid esters requires a highly electron-poor *N*-aryl substituent on the isatin scaffold. Under the optimized conditions, *N*-arylisatin **15** was ring-opened with different amino acid esters to obtain glyoxamide derivatives **32**-**36** in 24-58% yield (Scheme 3, Table 2).

This methodology has opened a new avenue for the development of *N*-arylglyoxamide derivatives, also allowing for the expansion of SAR data concerning isatin-based Quorum sensing inhibitors.

Quorum sensing inhibition assays

The synthesized compounds were tested for their QS inhibitory activity by following the protocol established by Hentzer *et al*.[36] The bacterial strains used were the *P. aeruginosa* MH602 *lasB* reporter strain (P_{lasB}::*gfp*(ASV)), which is in a wild-type, AHL positive background, and the *E. coli* MT102 reporter strain, which carries the *V. fischeri* luxR gene and the promoter for the luxI gene fused to the gfp reporter gene, such that the latter is under control of the AHL mediated QS system. These strains produce green fluorescent protein (GFP) as a function of an active QS system. Compounds that inhibit bacterial QS systems would be expected to reduce the expression of GFP in the reporter strain. In this assay, *P. aeruginosa* MH602 and *E. coli* MT102 cultures were incubated with various concentrations of the synthesized compounds at 37 °C. The fluorescence of GFP at λ = 535 nm and the optical density (OD) at 600 nm of the cultures were recorded every 30 min. The percentage QS inhibition of the compounds was calculated as the percentage difference of GFP intensity between the sample and the control at the time point when the fluorescence reached its maximum value in the control. The optical density (OD) of the cultures was utilized as a measure of bacterial cell growth. GFP inhibition and growth inhibition data for the most potent compound **31** are presented in **Figs. 3** and **4.** A well-known QS inhibitor, furanone 30 (**Fu-30**, **Fig. 5**) was used as a positive control.

The results showed that the synthesized compounds exhibited promising QS inhibitory activity as measured by the reduction in GFP fluorescence. The most active compound **31** inhibited GFP fluorescence by 48.7% at 250 µM in *P. aeruginosa* MH602 and 73.6% in *E. coli* MT102 (**Table 3**), values which are comparable with the positive control furanone 30 which inhibited GFP fluorescence by 83.9% at 250 µM in *P. aeruginosa* MH602 and 99.4% in *E. coli* MT102. Interestingly, the synthesized compounds inhibited bacterial growth by less than 10% at 250 µM against PAMH602 and 37% against *E. coli* MT102, whereas the positive control furanone 30 reduced bacterial cell growth by 51.9% against *P. aeruginosa* MH602 and 98.8% against *E. coli* MT102 (Table 1, supporting information).

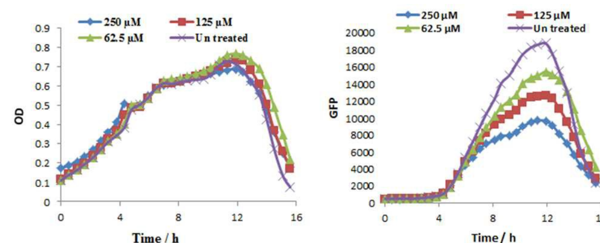

Fig. 3: Assay for LasR-based QS inhibition by compound **31** in *P. aeruginosa* MH602. (a) Inhibition of QS-regulated GFP production, (b) Optical density at 600 nm as a function of time.

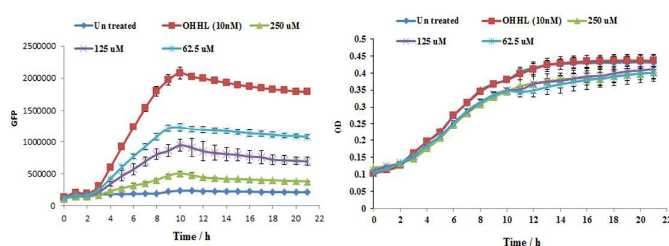

Fig. 4: Assay for LuxR-based QS inhibition by compound **31** in *E. coli* MT102, (a) Inhibition of QS-regulated GFP production, (b) Optical density at 600 nm as a function of time; negative control: untreated bacteria; OHHL: *N*–(3-oxohexanoyl) L-homoserine lactone.

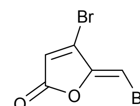

Fig. 5: Furanone 30 (**Fu-30**).

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Table 3: Percentage inhibition of GFP fluorescence by the synthesized compounds against the *P. aeruginosa* MH602 and *E. coli* MT102 strains at three different concentrations.

Compound	*P. aeruginosa* MH602			*E. coli* MT102		
	Concentrations (µM)					
	250	125	62.5	250	125	62.5
16	20.3±3.1[a]	11.4±2.7	7.2±2.9	12.2±1.4[c]	NA	NA
17	16.6±2.7[a]	10.3±1.9	7.1±1.9	11.1±1.3[c]	8.6±4.6	4.5±1.7
18	22.7±1.5[a]	10.0±1.8	3.4±3.6	17.5±0.4[c]	2.5±1.3	0
19	24±0.7[a]	14.5±0.7	4.2±0.6	20.5±1.2[c]	13.4±1.6	1.3±1.4
20	20.8±2.1[a]	8.8±0.3	8.6±1.8	11.1±0.8[c]	NA	NA
21	30.1±1.1[a]	19.7±1.5	9.6±0.6	17.8±2.3[c]	NA	NA
22	32.1±1.7[a]	16.0±1.9	8.1±2.2	12.2±1.4[c]	2.8±1.6	NA
23	22.7±0.9[a]	10.0±0.8	5.5±2.3	47.5±1.1[c]	37.7±0.4	26.5±0.7
24	23.1±1.8[a]	14.7±2.5	13.2±0.2	29.4±1.2[c]	27.1±0.8	21.7±1.6
25	17.9±2.8[a]	8.9±1.7	4.6±0.9	7.7±2.0[c]	NA	NA
26	13.8±2.8[a]	5.6±0.7	2.8±1.2	21.1±0.3[c]	9.5±1.9	NA
27	24.9±3.3[a]	14.5±2.8	4.2±2.6	5.4±1.2[c]	NA	NA
28	28.5±1.1[a]	18.1±4.8	2.8±6.2	15.8±0.8[c]	NA	NA
29	30.4±5.4[a]	20.8±5.9	13.1±4.2	19.3±0.7[c]	8.6±0.3	NA
30	25.7±1.8[a]	14.8±2.5	12.5±0.2	22.3±1.3[c]	17.5±0.7	NA
31	**48.7±0.2**[a]	32±0.6	16.7±0.9	**73.6±0.4**[c]	46.2±1.3	27.7±1.7
32	42.7±2.1[a]	27.8±2.4	14.7±3.4	35.1±0.8[c]	28.8±1.2	3.6±1.0
33	33.4±3.0[a]	8.3±2.6	10.9±2.2	18.6±1.5[c]	17.6±0.8	NA
34	37.3±0.9[a]	22.1±0.4	5.1±1.7	43.7±1.0[c]	29.2±0.3	5.1±0.3
35	31.3±0.6[a]	18.8±0.3	10.3±0.2	18.3±0.7[c]	13.1±1.4	NA
36	35.2±2.8[a]	21.3±1.8	12.9±1.2	27.9±0.7[c]	9.1±1.9	NA
Fu-30	83.9±0.1[b]	62.2±3.5	39.2±2.8	99.4±0.1[d]	99.2±0.1	44.1±0.1

[a] Growth inhibition less than 10%. [b] Growth inhibition greater than 50%. [c] Growth inhibition less than 40%. [d] Growth inhibition greater than 98%; ±standard deviation of the mean from at least two independent experiments. In each independent experiment, compounds were tested in triplicate; NA = no activity.

Pyocyanin assay

One of the key responsibilities of QS signalling is the regulation of virulence phenotypes, such as the production of pyocyanin in *P. aeruginosa*.[4] The LasR/LasI and RhlR/RhlI-based QS systems are the key mediators of this virulence factor production and it has been shown that antagonism of these receptors correlates to a reduction in pyocyanin production. Therefore, the ability of the synthesized compounds to inhibit the production of pyocyanin in *P. aeruginosa* PA14 was determined. The percentage inhibition was calculated by a comparison between the levels of pyocyanin in cultures treated with a 250 µM dose of the compounds for 24 h and a non-treated control (**Fig. 6**). The most active compound **28** showed 47% inhibition of pyocyanin production, while no growth inhibition was observed for any of the compounds against the P. *aeruginosa* (PA14) strain (Data not shown).

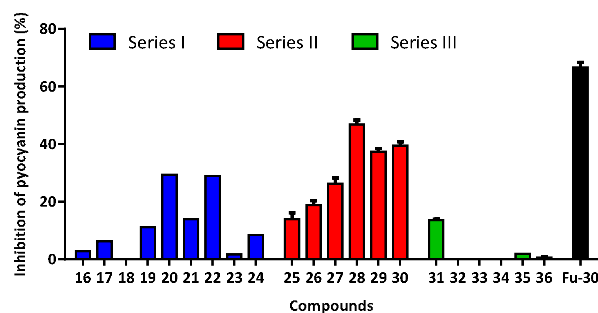

Fig. 6: Percentage inhibition of pyocyanin production in *P. aeruginosa* (PA14) following 24 h treatment with 250 µM of compounds. **Fu-30** was used as a reference compound. Error bars represent the standard error of three independent experiments (n=3).

Discussion of QSI activity

Bacteria communicate through a population-dependent QS system *via* autoinducer signalling molecules for the regulation of various virulence factors, biofilm formation, motility and antibiotic resistance. *P. aeruginosa* MH602 utilizes LasR-type receptors that recognize *N*-oxododecanoyl-L-homoserine lactone (OdDHL), whereas *E. coli* MT102 utilizes LuxR-type receptors that are regulated by the cognate *N*-(3-oxohexanoyl)-L-homoserine lactone (*OHHL*). In this work, three series of glyoxamide derivatives were generated by the ring-opening of *N*-aryl isatins, generating 21 novel compounds that were tested for their QSI activity (**Table 3**).

The series-I *N*-arylglyoxamide derivatives **16-24**, formed by the ring opening of various *N*-arylisatins with cyclic amines (pyrrolidine and piperidine), exhibited 16-32% and 11-47% QSI activities in *P. aeruginosa* MH602 and *E. coli* MT102 at 250µM,

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respectively. Amongst these, the *p*-nitro and *p*-methoxy derivatives with a pyrrolidine-substituted glyoxamide substituent (**21** and **22**) showed the highest QSI activites against *P. aeruginosa* MH602 with 30 and 32% inhibition, respectively. Meanwhile, the 2,4-dinitro derivative with a pyrrolidine substituent (**23**) exhibited the highest QSI activity against *E. coli* MT102, with 47.5% inhibition at 250 µM. However, increasing the ring size from a 5-membered ring (pyrrolidine, **23**) to a 6-membered ring (piperidine, **24**) reduced the QSI activity against *E. coli* MT102 to 29.4%. Compounds **16**, **18-20** and **23-24** showed similar levels of activity (20-24%) in *P. aeruginosa* MH602 at 250 µM, but lower activity (11-20%) in *E. coli* MT102 at the same concentration.

The series-II *N*-arylglyoxamide derivatives (**25**-**30**), formed by the ring-opening reaction of *N*-arylisatins with long chain aliphatic amines, displayed 13-30% QSI activity in *P. aeruginosa* MH602 at 250 µM. However, these compounds showed lower activities in *E. coli* MT102. The 2,4-dinitrophenyl derivative with butyl glyoxamide (**29**) exhibited the highest QSI activity in this series, with 30.4% and 22.3% inhibition in *P. aeruginosa* MH602 and *E. coli* MT102, respectively. As the chain length increased from 4-carbon (**29**) to 5-carbons (**30**), the QSI activity declined to 25.7% at 250 µM in *P. aeruginosa* MH602. Among series-II compound **30** with 2,4-dinitro- and pentyl glyoxamide showed the highest QSI activity of 22.3% at 250 µM in *E. coli* MT102.

Lastly, the series-III amino acid-substituted glyoxamide derivatives (**31**-**36**), having a common 2,4-dinitrophenyl group, exhibited the highest QSI activity amongst the three series of compounds. Glycine derivative **31** showed the greatest QSI activity among all compounds, with 73.6% and 48.7% inhibition at 250 µM against the *P. aeruginosa* MH602 and MT102 strains, respectively. Furthermore, **31** also showed moderate QSI activity at lower concentrations. The next-most active compound in this series was the L-alanine derivative **32**, which exhibited QSI activities of 42.7% and 35.1% against *P. aeruginosa* MH602 and *E. coli* MT102, respectively at 250 µM. Similar to **31**, this compound also showed moderate QSI activity at lower concentrations. Compound **34** (L-leucine) displayed significant QSI activity in *E. coli* MT102, with 43.7% and 29.2% inhibition at 250 and 125 µM, respectively. Compounds **33** (L-valine) and **35** (L-phenylalanine) also showed moderate QSI activity in *P. aeruginosa* MH602, with 33.4% and 31.3% inhibition, respectively, at 250 µM.

Pyocyanin plays a major role in the virulence of *P. aeruginosa* (PA14). Therefore, the effect of the glyoxamide derivatives on pyocyanin production was analysed in addition to their QS inhibitory activity. The series-I glyoxamide derivatives exhibited low to moderate (0-29%) pyocyanin inhibitory activity. The greatest levels of inhibition were observed for compounds **20** and **22**, which exhibited 28% and 29% inhibition at 250 µM respectively. Compounds from series-II showed the highest levels of inhibition (14-47%) amongst the three series of glyoxamide derivatives. Compound **28** exhibited the greatest inhibitory activity of 47%, while compounds **29** and **30** showed similar levels of inhibition, being 37% and 39% respectively. Surprisingly, the potent QS-inhibitory series-III glyoxamide derivatives showed little or no inhibition of pyocyanin production (≤14%). As our compounds were synthesized to target the LasR-based QS system, while *P. aeruginosa* mediates pyocyanin production through both the RhlR-based and LasR-based QS systems, it is possible that upon inhibition of LasR the bacteria compensate through increased activity of the RhlR system. Therefore, in our future work we would like to investigate the QSI activity of these compounds against the Rhl QS system of *P. aeruginosa*.

Overall, the glyoxamide derivatives formed by ring-opening reaction of 2,4-dinitrophenyl isatin exhibited significant QSI activity against both the QS systems. Furthermore, amino acid-based glyoxamide derivatives exhibited higher QSI activity against both *P. aeruginosa* MH602 (LasR) and *E. coli* MT102 (LuxR) QS systems. The glycine derivative **31** showed the highest QSI activity in both organisms, while the other amino acid derivatives (**32-36**) showed reduced QSI activity. The compounds with cyclic amines (series-I) and acyclic amines (series-II) showed moderate and low QSI activities in *E. coli* MT102, respectively, while both series showed moderate QSI activities in *P. aeruginosa* MH602.

In addition, all derivatives were investigated for their growth inhibition of the two bacterial strains. These compounds had minimal effects on growth of the bacteria, with our most active compound **31** displaying <10% of *P. aeruginosa* MH602 and <20% of *E. coli* MT102 growth inhibition (supporting information), while the reference compound (Furanone-30) inhibited growth of *E. coli* MT102 and *P. aeruginosa* MH602 by 98.8 and 51.9%, respectively. This suggests that the novel compounds might be unlikely to exert selective pressure on bacteria to develop resistance.

Docking studies

To predict the binding mode of the synthesized glyoxamides to the active site of the LasR protein (PDB code 2UV0)[16], docking studies of compounds **16**-**36** were performed using the GOLD docking software, accessed *via* the Accelrys Discovery Studio software package. Firstly, the protein and ligands were prepared for docking by adding hydrogens and minimizing the ligands with a CHARMM forcefield. The co-crystallized ligand (OdDHL, **37**) was then docked back into the protein as a control (**Table 4**; Last 2 entries). The GOLD docking software was then be employed to optimize a protocol for obtaining possible binding modes of the proposed scaffolds within the LasR binding site, as well as to generate Goldscore values to indicate the relative affinity of each potential ligand. Predicted binding interactions (such as hydrogen bonding, electrostatic π and hydrophobic interactions) between the highest scored pose for each compound and the LasR receptor are presented in Table 4. The docking poses and interactions formed of both ODdHL and compound **31** are shown in **Fig. 6**.

From our docking studies, we found that hydrogen bonding interactions to residues such as Arg61, Asp73, Ser129, Thr75, Thr115, Trp88, Trp60, Tyr56, and Tyr93 are predicted to play an important role in the binding of the synthesized compounds

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Table 4: Docking study of the glyoxamide-based QS inhibitors to the LasR receptor protein of *P. aeroginosa.*

Compound	Goldscore fitness	Interaction from selected pose	
		H-bond and electrostatic Interactions	Hydrophobic and π Interactions
16	49.94	Ser129, Thr75	Tyr64, Gly126, Ala127, Trp88, Leu36, Val76, Cys79, Leu125
17	50.09	Tyr56, Ser129, Asp73	Gly126, Ala127, Trp88, Leu36, Val76, Cys79
18	49.39	Gly126	Tyr64, Trp88, Ala70, Val76 Cys79, Leu125, Ala127
19	49.96	Gly126, Asp73	Tyr64, Gly126, Ala127, Trp88, Leu36, Val76, Cys79
20	47.68	Thr75, Ser129, Leu110	Tyr64, Trp88, Tyr56, Ala50, Ala70, Val76, Ala127, Leu36, Leu110
21	48.58	Thr75, Leu39, Asp73	Tyr64, Gly126, Trp88, Ala127, Ala50, Val76, Leu36
22	51.19	Thr75, Gly38	Tyr64, Trp88, Val76, Cys79, Leu125, Ala127
23	44.32	Gly126, Asp73, Trp60, Ala127, Gly38, Leu39, Asp73	Tyr64, Val76, Cys79, Leu125, Ala127, Ala50, Ile52, Ala70, Leu36
24	43.13	Tyr64, Asp73, Trp60, Arg61, Thr75, Thr115, Ser129, Tyr64, Arg61, Tyr64	Tyr64, Val76, Cys79, Leu125, Ala127, Tyr47, Ala50, Ile52
25	68.13	Tyr56, Thr115, Asp73	Tyr64, Gly126, Ala127, Ala105, Leu110, Trp88, Leu36, Val76, Cys79
26	67.64	Trp60, Arg61, Asp73	Trp88, Val76, Cys79, Leu125, Tyr47, Leu36, Ala127, Ala105, Leu110
27	66.80	Tyr56, Ser129, Thr115, Asp73	Tyr64, Gly126, Ala105, Leu110, Trp88, Leu36, Val76, Ala127, Cys79
28	70.52	Asp73, Thr115, Tyr56	Tyr64, Gly126, Ala127, Ala105, Leu110, Trp88, Tyr93, Phe101, Leu36, Val76, Cys79
29	51.38	Thr75, Ser129	Ala127, Tyr64, Gly126, Ala105, Leu110, Trp88, Trp93, Leu36,Vla76, Cys79, Asp73
30	55.40	Tyr56, Arg61, Trg88, Ser129	Tyr56, Trp88, Leu40, Tyr47, Leu36, Val76, Ala127, Ala105
31	59.78	Tyr56, Tyr64, Thr75, Val76, Asp73	Tyr64, Gly126, Aal105, Trp88
32	55.10	Arg61, Asp73, Leu40, Tyr56, Thr115, Leu39	Tyr64, Ala105,Trp60, Phe101
33	49.63	Thr75, Ser129, Asp73	Thr75, Ser129,Asp73,Tyr64,Trp88, Tyr56, Ala50, Ala70, Tyr64, Tyr47
34	53.56	Trp88, Thr115, Gly126, Ser129	Trp88, Thr115, Gly126, Ser129, Trp88, Tyr56, Ala50, Ala70, Ala127,Tyr64, Typ64
35	57.98	Trp88, Asp73, Tyr93, Arg61, Trp60	Ala70, Tyr64, Gly38, Tyr47,Trp88
36	65.82	Trp60, Arg61, Arg61, Asp73	Trp88, Gly38, Leu39, Ala70, Tyr64, Leu36, Ala127, Ala105, Leu110, Leu40,Ala50, Vla76, Ala127
37 (OdDHL) docked	68.32	Tyr56, Trp60, Arg61, Ser129, Asp73, Tyr93	
37 (OdDHL) crystal structure	-	Tyr56, Trp60, Asp73	Trp 88

to the LasR receptor. These results are consistent with Stacy *et al.*,[37] who showed that hydrogen bonding interactions play a significant role for the binding of ligands to LasR. The presence of numerous hydrogen bond donor and acceptors, as well as two to three aromatic rings in the synthesized compounds, allow for multiple hydrogen bonding and hydrophobic interactions with the LasR protein. Interestingly, compounds which showed hydrogen bonding interactions similar to the natural ligand OdDHL showed relatively higher QSI activities.

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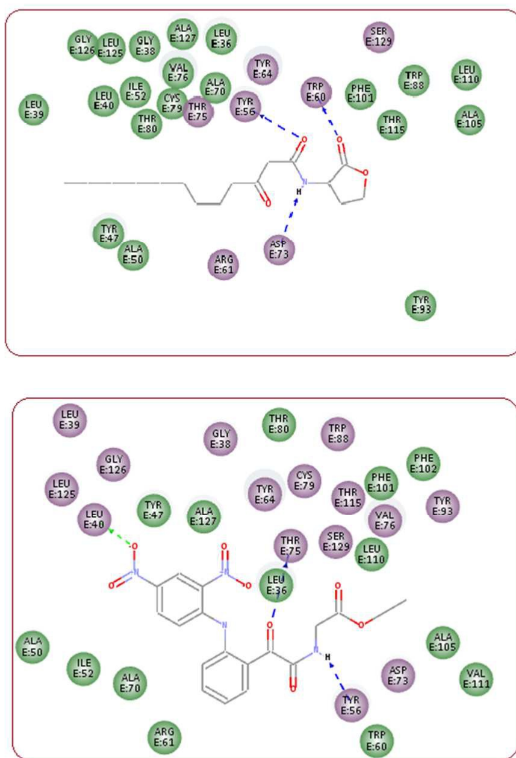

Fig. 6: 2D-representation of highest scoring pose of ODdHL **37** (top); and compound **31** (bottom) with LasR protein; blue dashed lines represent the hydrogen bonding interactions between LasR residues (Purple and Green) and either ODdHL **37** or compound **31**. The green dashed line represents a hydrogen bond between compound **31** and the amide backbone of the LasR receptor.

Amongst series-I, *p*-nitro-aniline and *p*-methoxy-aniline glyoxamide derivatives containing a pyrrolidine substituent (**24** and **25**) showed significant QSI activities of 30 and 32% in PAMH602, respectively. This was consistent with their docking scores of 48.58 and 51.19, respectively, which were the highest amongst this series. The series-II compounds showed high docking scores to LasR, possibly due to the presence of their aliphatic acyclic long chain that is also found in natural AHLs such as OdDHL. However, the docking scores of the series-II compounds did not correlate well with their QSI activities. Interestingly, for the series-III compounds, the docking scores were well correlated with the QSI activity, particularly for compounds (**31**-**34**) that interacted with LasR *via* important hydrogen bond interactions rather than hydrophobic interactions. Exceptions were compounds **35** and **36**, which showed higher docking scores due to the hydrophobic interactions conferred by their phenyl (from L-Phe) and indolyl (L-Trp) groups, respectively, but these showed relatively low QSI activities amongst this series.

Conclusion

A simple, mild and highly efficient set of conditions have been developed for the facile ring-opening reaction of *N*-arylisatins with a variety of amines and amino acids to yield glyoxamide derivatives. Using these conditions, we synthesized 21 novel glyoxamide derivatives which have synthesized in moderate to high yields. These compounds were found to act as novel non-native and non-AHL inhibitors of quorum sensing in Gram- negative bacteria. The QSI activity of the novel compounds was tested in two QS systems using the bacterial strains *P. aeruginosa* MH602 and *E. coli* MT102. The glycine-substituted glyoxamide **31** exhibited the highest QSI activity of 48.7% and 73.6% at 250 µM concentration in *P. aeruginosa* MH602 and *E. coli* MT102 respectively. The ability of these compounds to inhibit production of the bacterial toxin pyocyanin was also determined using the *P. aeruginosa* PA14 strain. Compound 28 exhibited the greatest reduction in pyocyanin production of 47% following 24 h treatment at 250 µM. Furthermore, most compounds do not prevent the growth of the bacteria, which could reduce the likelihood of engendering antibiotic resistance. Lastly, docking studies compounds to the LasR receptor protein of *P. aeruginosa* showed that hydrogen bonding interactions are predicted to play an important role in determining the QSI activity. This was demonstrated by compound **31**, which exhibited the highest QSI in *P. aeruginosa* MH602 and also the showed important hydrogen bonding interactions with a docking score of 59.78 amongst amino acid-substituted glyoxamide derivatives. By acting *via* a different mechanism to traditional antibiotics, these QS inhibitory compounds might represent a new avenue for combating bacterial infections.

Acknowledgements

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Experimental

5.1. Chemistry

All chemical reagents were purchased from commercial sources (Alfa-Aesar and Sigma Aldrich) and used without further purification. Solvents were commercial and used as obtained. Reactions were performed using oven-dried glassware under an atmosphere of nitrogen and in anhydrous conditions (as required). Room temperature refers to the ambient temperature. Yields refer to chromatographically and spectroscopically pure compounds unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC) precoated with Merck silica gel 60 F_{254}. Visualization was performed by the quenching of short or long wavelength UV fluorescence. Flash chromatography was carried out using Grace Davison LC60A 6-35 µm silica gel. Infrared spectra were recorded using a Cary 630 ATR spectrophotometer. High-

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resolution mass spectrometry was performed by the Bioanalytical Mass Spectrometry facility, UNSW. Melting points were obtained using a Mel-Temp melting point apparatus and are uncorrected. Proton and Carbon NMR spectra were recorded in the solvents specified using a Bruker DPX 300 or a Bruker Avance 300 spectrometer as designated. Chemical shifts (δ) are quoted in parts per million (ppm), to the nearest 0.01ppm and internally referenced relative to the solvent nuclei. ^{1}H NMR spectral data are reported as follows: [chemical shift in ppm; multiplicity in br, broad; s, singlet; d, doublet; t, triplet; q, quartet; quint, quintet; sext, sextet; sept, septet; m, multiplet; or as a combination of these (*e.g.* dd, dt *etc.*)]; coupling constant (*J*) in hertz, integration, proton count and assignment.

General procedure (A) for the synthesis of *N*-arylisatins (**10-15**): To a suspension of isatin (1 mmol) in dry dichloromethane (10 mL) was added anhydrous $Cu(OAc)_2$ (1 mmol), triethylamine (2 mmol) and the arylboronic acid (2 mmol) under an argon atmosphere. The reaction mixture was stirred at room temperature for 16 h. After completion of the reaction, the mixture was filtered through a pad of Celite and washed with dichloromethane. The filtrate was concentrated *in vacuo* to yield the crude compound, which was subjected to flash chromatography on silica. The compounds were eluted in 10-15% of ethyl acetate-hexane.

General procedure (B) for the synthesis of Glyoxamide derivatives (19-33): To a suspension of the *N*-arylisatin (1 mmol) in dichloromethane (10 mL) was added the amine (1 mmol). The reaction mixture was stirred at room temperature for 15 min to 4h. After completion of the reaction, water was added and the mixture was extracted with dichloromethane. The organic layer was separated and dried over sodiumsulfate, filtered and concentrated *in vacuo* to yield the crude compound, which was subjected to flash chromatography on silica. The compounds were eluted with 20-40% of ethyl acetate-hexane.

General procedure (C) for the synthesis of Glyoxamide derivatives (34-39): To a suspension of 2,4-dintro-phenylisatin (1 mmol) in dichloromethane (10 mL) was added the amine (1 mmol) and trimethylamine (2 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 16h. After completion of the reaction, water was added and the mixture was extracted with dichloromethane. The organic layer was separated and dried over sodiumsulphate, filtered and concentrated *in vacuo* to give the crude compound, which was subjected to flash chromatography on silica. The compounds were eluted in 20-50% of ethyl acetate-hexane.

1-Phenylindoline-2,3-dione (10): The title compound **10** was prepared from isatin (1.0 g, 6.79 mmol) and phenylboronic acid (0.34 g, 8.15 mmol) according to general procedure **A**. [35] The product **10** was obtained by flash chromatography, eluting with 15% ethyl acetate-hexane to yield an orange solid (0.60 g, 55%); Mp 129–132 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.72 (d, *J* = 8.0 Hz, 1H), 7.62–7.52 (m, 3H), 7.51–7.42 (m, 3H), 7.19 (t, *J* = 7.66 Hz, 1H), 6.92 (d, *J* = 8.07 Hz, 1H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 184.3, 158.7, 153.1, 139.7, 134.3, 131.4, 130.2, 127.4, 127, 125.7, 118.9, 112.7; HRMS (ESI): *m/z* calcd for $C_{14}H_{10}NO_2$ $[M+H]^+$ 224.0705, found 224.0706; IR (ATR): v_{max} 3452, 3058, 2305, 2086, 1732, 1604, 1358 cm^{-1}.

1-(4-Methoxyphenyl)indoline-2,3-dione (11): The title compound **11** was prepared from isatin (1.00 g, 6.79 mmol) and 4-methoxy-phenylboronic acid (1.54 g, 10.0 mmol) according to general procedure **A**. The product **11** was obtained by flash chromatography, eluting with 15% ethyl acetate-hexane to yield an orange solid (0.89 g, 52%); Mp 129–132 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.72–7.67 (m, 1H), 7.55–7.51 (dt, *J* = 1.4, 7.8 Hz, 1H), 7.38–7.31 (m, 2H), 7.21–7.14 (dt, *J* = 8.2, 7.5 Hz, 1H), 7.11–7.05 (m, 2H), 6.85 (*J* = 8.0 Hz, 1H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 183.1, 159.7, 157.6, 152.0, 138.3, 127.4, 125.4, 125.3, 124.1, 117.4, 116.0, 115.2, 114.8, 111.1, 55.6; HRMS (ESI): *m/z* calcd for $C_{15}H_{11}NO_3Na$ $[M+Na]^+$ 276.0630, found 276.0631. IR (ATR): v_{max} 3452, 2943, 2842, 1731, 1606, 1509, 1461, 1365, 1294, 1251 cm^{-1}.

1-(3-Fluorophenyl)indoline-2,3-dione (12): The title compound **12** was prepared from isatin (0.50 g, 3.40 mmol) and 3-fluoro-phenylboronic acid (0.72 g, 5.10 mmol) according to general procedure **A**. The product **12** was obtained by flash chromatography, eluting with 15% ethyl acetate-hexane to yield an orange solid (0.46 g, 56%); Mp 168–170 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.78–7.70 (m, 1H), 7.65–7.51 (m, 2H), 7.32–7.15 (m, 4H), 7.00–6.65 (m, 1H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 182.3, 164.8, 161.5, 157.0, 151.0, 138.4, 125.8, 124.6, 121.6, 117.5, 115.9, 113.9, 111.2; HRMS (ESI): *m/z* calcd for $C_{14}H_8FNO_2Na$ $[M+Na]^+$ 264.0430, found 264.0431; IR (ATR): v_{max} 3448, 3100, 2919, 1727, 1603, 1492, 1360 cm^{-1}.

1-(4-Fluorophenyl)indoline-2,3-dione (13): The title compound **13** was prepared from isatin (2.00 g, 13.6 mmol) and 4-fluoro-phenylboronic acid (2.28 g, 16.3 mmol) according to general procedure **A**. The product **13** was obtained by flash chromatography, eluting with 15% ethyl acetate-hexane to yield an orange solid (2.0 g, 63%); Mp 224– 226 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.75–7.70 (m, 1H), 7.61–7.54 (m, 1H), 7.48–7.38 (m, 2H), 7.32–7.24 (m, 3H), 7.24–7.18 (td, *J* = 0.84, 7.54 Hz, 1H), 6.90–6.84 (m, 1H). ^{13}CNMR ($CDCl_3$, 300 MHz): δ 182.5, 160.6, 157.3, 151.5, 138.3, 128.0, 125.7, 124.4, 117.5, 117.2, 116.9, 111.0; HRMS (ESI): *m/z* calcd for $C_{14}H_8FNO_2Na$ $[M+Na]^+$ 264.0430, found 264.0431; IR (ATR): v_{max} 3447, 3078, 2921, 1733, 1599, 1506, 1223 cm^{-1}.

1-(4-Nitrophenyl) indoline-2,3-dione (14): The title compound **14** was prepared from isatin (0.20 g, 1.36 mmol) and 4-nitro-phenylboronic acid (0.34 g, 2.00 mmol) according to general procedure **A**. The product **14** was obtained by flash chromatography, eluting with 10% ethyl acetate-hexane to yield an orange solid (0.10 g, 27%); Mp 240–242 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.50–8.41 (m, 2H), δ 7.84–7.75 (m, 2H), 7.75–7.62 (m, 2H), 7.30–7.21 (m, 1H), 7.06 (d, *J* = 8.09 Hz, 1H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 182.5, 157.7, 150.3, 146.7, 139.7, 138.3, 127.4, 125.51, 125.39, 124.6, 118.5, 111.4; HRMS (ESI): *m/z* calcd for $C_{14}H_8N_2O_4Na$ $[M+Na]^+$ 291.0375, found 291.0376; IR (ATR): v_{max} 3452, 3084, 2320, 1734, 1609, 1461, 1177 cm^{-1}.

1-(2,4-Dinitrophenyl)indoline-2,3-dione (15): A solution of isatin (0.50 g, 3.40 mmol), 2,4-nitroiodobenzene (1.16 g, 3.40 mmol) and tetrabutylammonium hydrogensulphate (0.12 g, 0.34 mmol) in chloroform (100 ml) was stirred till a clear

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solution was obtained. Then KOH (50%, 10ml) was added drop wise and stirred at room temperature for 24h. The product **15** was obtained by flash chromatography, eluting with 20% ethyl acetate-hexane to yield an orange solid (0.60 g, 56%); Mp 161–164 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 9.09 (d, *J* = 2.54 Hz, 1H), 8.70 (dd, *J* = 2.53, 8.65 Hz, 1H), 7.89 (d, *J* = 8.69 Hz, 1H), 7.85–7.89 (m, 1H), 7.65 (td, *J* =1.43, 7.83Hz, 1H), 7.33 (td, *J* = 0.79, 7.62 Hz, 1H), 6.83–6.78 (m, 1H); ^{13}CNMR (CDCl$_3$, 300 MHz): δ 180.0, 156.5, 149.2, 147.4, 145.4, 138.8, 132.0, 130.5, 128.8, 126.6, 125.6, 122.2, 118.0, 110.4; HRMS (ESI): *m/z* calcd for $C_{14}H_7N_3O_6$ [M+H]$^+$ 314.0406 found 314.0408; IR (ATR): v_{max} 3113, 2321, 1736, 1602, 1528, 1345 cm^{-1}.

1-(2-(Phenylamino) phenyl)-2-(pyrrolidin-1-yl) ethane-1,2-dione (16): The title compound **19** was prepared from *N*-phenylisatin (0.10 g, 0.44 mmol) and pyrrolidine (0.06 g, 0.89 mmol) according to general procedure **B**. The product **16** was obtained by flash chromatography, eluting with 30% ethyl acetate-hexane to yield a yellow solid (0.10 g, 73%); Mp 116–118 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.2 (bs, 1H), 7.62 (dd, *J* = 1.56, 8.11 Hz, 1H), 7.45–7.28 (m, 5H), 7.27–7.16 (m, 2H), 6.78–6.71 (m, 1H); ^{13}CNMR (CDCl$_3$, 300 MHz): δ 194.3, 165.2, 149.8, 139.4, 136.1, 134.0, 129.4, 124.8, 123.8 1H), 116.9, 114.5, 113.9, 46.7, 45.1, 25.8, 24.1; HRMS (ESI): *m/z* calcd for $C_{19}H_{21}N_2O_3$ [M+H]$^+$ 295.1443, found 295.1441; IR (ATR): v_{max} 3256, 3054, 2975, 2877, 1717, 1613, 1512, 1220 cm^{-1}.

1-(2-(Phenylamino)phenyl)-2-(piperidin-1-yl)ethane-1,2-dione (17): The title compound **17** was prepared from *N*-phenylisatin (0.15 g, 0.67 mmol) and piperidine (0.12 g, 1.34 mmol) according to general procedure **B**. The product **17** was obtained by flash chromatography, eluting with 30% ethyl acetate in hexane to yield an yellow solid (0.20 g, 94%); Mp 81–83 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.3 (bs, 1H), 7.58 (dd, *J* = 1.52, 8.13 Hz, 1H), 7.45–7.27 (m, 6H), 7.26–7.15 (m, 2H), 6.80–6.70 (m, 1H), 3.81–3.61 (m, 1H), 3.41–3.33 (m, 2H), 1.78–1.69 (m, 4H), 1.66 –1.55 (m, 2H); ^{13}CNMR (CDCl$_3$, 300 MHz): δ 194.6, 165.3, 149.5, 139.4, 136.1, 133.9, 129.4, 124.7, 123.7, 116.9, 115.0, 113.9, 47.2, 42.0 26.2, 25.4, 24.4; HRMS (ESI): *m/z* calcd for $C_{19}H_{20}N_2O_2Na$ [M+Na]$^+$ 331.1415, found 331.1417; IR (ATR): v_{max} 3273, 3037, 2942, 1622, 1589, 1322 cm^{-1}.

1-(2-((3-Fluorophenyl)amino)phenyl)-2-(pyrrolidin-1-yl)ethane-1,2-dione (18): The title compound **18** was prepared from 1-(3-fluorophenyl) indoline-2, 3-dione (0.05 g, 0.20 mmol) and pyrrolidine (0.03 g, 0.41 mmol) according to general procedure **B**. The product **18** was obtained by flash chromatography eluting with 30% ethyl acetate-hexane to yield a yellow solid (0.06 g, 85%); Mp 61– 64°C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.4 (bs, 1H), 8.30–8.20 (m, 2H), 7.71–7.70 (m, 1H), 7.58–7.51 (m, 2H), 7.41–7.32 (m, 2H), 7.04–6.95 (m, 1H), 3.69 (t, *J* = 7.1 Hz, 2H), 3.46 (t, *J* = 6.6 Hz, 2H), 2.05–1.95 (m, 4H); ^{13}CNMR (CDCl$_3$, 300 MHz): δ 194.5, 165.0, 161.7, 148.8, 141.4, 136.2, 134.1, 130.6, 118.6, 117.8, 115.1, 114.2, 111.2, 111.0, 46.7, 45.1, 25.8, 24.1; HRMS (ESI): *m/z* calcd for $C_{18}H_{17}FN_2O_2Na$ [M+Na]$^+$ 335.1165, found 335.1166; IR (ATR): v_{max} 3271, 2951, 2872, 1621, 1563, 1509, 1324 cm^{-1}.

1-(2-((4-Fluorophenyl)amino)phenyl)-2-(pyrrolidin-1-yl)ethane-1,2-dione (19): The title compound **19** was prepared from 1-(4-fluorophenyl) indoline-2,3-dione (0.10 g, 0.41 mmol) and pyrrolidine (0.06 g, 0.83 mmol) according to general procedure **B**. The product **19** was obtained by flash chromatography, eluting with 30% ethyl acetate-hexane to yield a yellow solid (0.09 g, 66%); Mp 160– 162 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.1 (bs, 1H), 7.66–8.20 (m, 2H), 7.40–7.32 (m, 1H), 7.31–7.21 (m, 3H), 7.15–7.02 (m, 3H), 6.78–6.70 (m, 1H), 3.69 (t, *J* = 6.9 Hz, 2H), 3.46 (t, *J* = 5.5 Hz, 2H), 2.0–1.95 (m, 4H); ^{13}CNMR (CDCl$_3$, 300 MHz): δ 194.2, 165.2, 161.7, 158.5, 150.4, 36.2, 135.3, 134.0, 126, 116.9, 116.4, 116.1, 114.3, 113.5, 46.7, 45.1, 24.8, 24.1; HRMS (ESI): *m/z* calcd for $C_{18}H_{17}FN_2O_2Na$ [M+Na]$^+$ 335.1167, found 335.1166; IR (ATR): v_{max} 3258, 3077, 2962, 1618, 1600, 1503, 1209 cm^{-1}.

1-(2-((4-Fluorophenyl)amino)phenyl)-2-(piperidin-1-yl)ethane-1,2-dione (20): The title compound **20** was prepared from 1-(4-fluorophenyl) indoline-2,3-dione (0.10 g, 0.41 mmol) and piperidine (0.07 g, 0.82 mmol) according to general procedure **B**. The product **20** was obtained by flash chromatography, eluting with 30% ethyl acetate-hexane to yield a yellow solid (0.12 g, 88%); Mp 82–85 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.1 (bs, 1H), 7.56 (dd, *J* = 1.48, 8.05 Hz, 1H), 7.39–7.31(m, 1H), 7.30–7.20 (m, 2H), 7.17–7.02 (m, 3H), 6.78–6.70 (m, 1H), 3.79–3.68 (m, 2H), 3.36 (t, *J* = 5.54 Hz, 2H), 1.78–1.67 (m, 4H), 1.66–1.54 (m, 2H); ^{13}CNMR (CDCl$_3$, 300 MHz): δ 194.6, 165.3, 161.7, 158.5, 150.1, 136.2, 135.3, 133.9, 126.3, 116.8, 116.4, 116.1, 114.8, 113.5, 47.2, 42.0, 26.2, 25.4, 24.2. HRMS (ESI): *m/z* calcd for $C_{19}H_{19}FN_2O_2Na$ [M+Na]$^+$ 349.1322, found 349.1323; IR (ATR): v_{max} 3276, 3060, 2922, 2853, 1626, 1563, 1507, 1444, 1320 cm^{-1}.

1-(2-((4-Nitrophenyl)amino)phenyl)-2-(pyrrolidin-1-yl)ethane-1,2-dione (21): The title compound **21** was prepared from 1-(4-nitrophenyl) indoline-2, 3-dione (0.08 g, 0.12 mmol) and pyrrolidine (0.02 g, 2.23 mmol) according to general procedure **B**. The product **21** was obtained by flash chromatography, eluting with 30% ethyl acetate-hexane to yield a yellow solid (0.08 g, 85%); Mp 160– 162 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.2 (bs, 1H), 7.68–7.61 (m, 1H), 7.45–7.37 (m, 1H), 7.36–7.29 (m, 2H), 7.11–7.00 (m, 2H), 6.91–6.76 (m, 2H), 3.69 (t, *J* = 6.9 Hz, 2H), 3.46 (t, *J* = 6.7 Hz, 2H), 2.0–1.95 (m, 4H); ^{13}CNMR (CDCl$_3$, 300 MHz): δ 194.7, 164.5, 146.5, 146.0, 142.4, 136.1 1H), 134.4, 125.7, 120.2, 119.4, 117.3, 115.8, 46.7, 45.2, 25.8, 24.0; HRMS (ESI): *m/z* calcd for $C_{18}H_{17}N_3O_4Na$ [M+Na]$^+$ 362.1109, found 362.1111; IR (ATR): v_{max} 3295, 307, 2958, 2638, 1740, 1625, 1579, 1301 cm^{-1}.

1-(2-((4-Methoxyphenyl)amino)phenyl)-2-(pyrrolidin-1-yl)ethane-1,2-dione (22): The title compound **22** was prepared from 1-(4-methoxyphenyl) indoline-2,3-dione (0.11 g, 0.42 mmol) and pyrrolidine (0.06 g, 0.83 mmol) according to general procedure **B**. The product **25** was obtained by flash chromatography, eluting with 30% ethyl acetate-hexane to yield a yellow solid (0.12 mg, 86%); Mp 94– 96 °C; ^{1}H NMR (CDCl$_3$, 300 MHz): δ 10.1 (bs, 1H), 7.58 (dd, *J* = 1.47, 8.04 Hz, 1H), 7.36–7.28 (m, 1H), 7.25–7.18 (m, 2H), 7.02–6.92 (m, 3H), 6.72–6.65 (m, 1H), 3.85 3H), 3.69 (t, *J* = 6.99 Hz, 2H), 3.48 (t, *J* = 6.70 Hz, 2H), 2.05–1.93 (m, 4H); ^{13}CNMR (CDCl$_3$, 300 MHz): δ 194.2, 165.4, 157.3, 151.2, 136.1, 133.9, 132.0, 126.5, 116.2, 114.7, 113.8, 113.6, 55.5, 46.7, 45.0, 25.8, 24.1; HRMS (ESI):

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m/z calcd for $C_{19}H_{21}N_2O_3$ $[M+H]^+$ 325.1545, found 325.1547; IR (ATR): ν_{max} 3125, 2290, 1733, 1608, 1365 cm^{-1}.

1-(2-((2,4-Dinitrophenyl)amino)phenyl)-2-(pyrrolidin-1-yl)ethane-1,2-dione (23): The title compound **23** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.15 g, 0.47 mmol) and pyrrolidine (0.07 g, 0.96 mmol) according to general procedure **B**. The product **23** was obtained by flash chromatography, eluting with 30% ethyl acetate-hexane to yield a yellow solid (0.18 g, 98%); Mp 129–132 °C; Mp 201–204 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.61 (d, *J* = 2.6 Hz, 1H), 8.35–8.25 (m, 1H), 7.91 (dd, *J* = 1.38, 7.91 Hz, 1H), 7.71–7.56 (m, 3H), 7.39–7.29 (m, 1H), 3.69–3.49 (m, 4H), 2.04–1.94 (m, 4H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 192.2, 163.7, 144.0, 140.3, 139.0, 135.1, 134.5, 133.9, 129.3, 125.0, 124.6 123.7, 121.8, 118.1, 46.9, 45.6, 25.9, 23.9; HRMS (ESI): *m/z* calcd for $C_{18}H_{17}N_4O_6$ $[M+H]^+$ 385.1141, found 385.1143; IR (ATR): ν_{max} 3257, 3063, 2882, 2639, 1636, 1587, 1328 cm^{-1}.

1-(2-((2,4-Dinitrophenyl)amino)phenyl)-2-(piperidin-1-yl)ethane-1,2-dione (24): The title compound **24** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.15 g, 0.47 mmol) and pyrrolidine (0.08 g, 0.95 mmol) according to general procedure **B**. The product **24** was obtained by flash chromatography, eluting with 30% ethyl acetate-hexane to yield a yellow solid (0.12 g, 63%); Mp 180– 182 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.3 (bs, 1H), 7.58 (dd, *J* = 1.52,8.13 Hz, 1H), 7.45–7.27 (m, 6H), 7.26–7.15 (m, 2H), 6.80–6.70 (m, 1H), 3.81–3.61 (m, 1H), 3.41–3.33 (m, 2H), 1.78–1.69 (m, 4H), 1.66–1.55 (m, 2H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 194.6, 165.3, 149.5, 139.4, 136.1, 133.9, 129.4, 124.7, 123.7, 116.9, 115.0, 113.9, 47.2, 42.0, 26.2, 25.4, 24.4; HRMS (ESI): *m/z* calcd for $C_{19}H_{20}N_2O_2Na$ $[M+Na]^+$ 421.1117, found 421.1119; IR (ATR): ν_{max} 3273, 3037, 2942, 1622, 1589, 1322 cm^{-1}.

***N*-Butyl-2-oxo-2-(2-(phenylamino)phenyl)acetamide (25):** The title compound **25** was prepared from *N*-phenylisatin (0.10 g, 0.45 mmol) and butylamine (0.07 g, 0.90 mmol) according to general procedure **B**. The product **25** was obtained by flash chromatography, eluting with 15-20% ethyl acetate-hexane to yield a yellow solid (0.08 g, 60%); Mp 62–64 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.2 (bs, 1H), 8.48 (dd, *J* = 1.56, 8.29 Hz 1H), 7.45–7.33 (m, 3H), 7.32–7.14 (m, 4H), 6.87 (bs, 1H), 6.81– 6.72 (m, 1H), 3.44 (q, *J* = 6.84 Hz, 2H), 1.70–1.55 (m, 2H), 1.52–1.36 (m, 2H), 0.99 (t, *J* = 7.28 Hz, 3H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 189.9, 150.0, 139.6, 136.1, 134.9, 129.4, 124.6, 123.5, 116.9, 115.5, 114.0, 39.2, 31.4, 20.0, 13.7; HRMS (ESI): *m/z* calcd for $C_{18}H_{21}N_2O_2$ $[M+H]^+$ 297.1599, found 297.1598; IR(ATR): ν_{max} 3232, 3076, 2955, 2869, 1737, 1630, 1556, 1323 cm^{-1}.

***N*-Octyl-2-oxo-2-(2-(phenylamino)phenyl)acetamide (26):** The title compound **26** was prepared from *N*-phenylisatin (0.10 g, 0.45 mmol) and octylamine (0.12 g, 0.90 mmol) according to general procedure **B**. The product **26** was obtained by flash chromatography, eluting with 15-20% ethyl acetate-hexane to yield a yellow solid (0.10 g, 62%); Mp 56–58 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.2 (bs, 1H), 8.48 (dd, *J* = 1.56, 8.25 Hz,1H), 7.46–7.32 (m, 3H), 7.32–7.14 (m, 4H), 6.92–6.81 (bs, 1H), 6.80–6.72 (m, 1H), 3.44 (q, *J* = 6.84 Hz, 2H), 1.70–1.55 (m, 2H), 1.48–1.30 (m, 11H), 0.99 (t, *J* = 6.92 Hz, 3H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 189.9, 163.8, 150.0, 139.6, 136.1, 134.9, 129.4, 124.6, 123.5, 116.9, 115.5, 114.0, 39.5, 31.7, 29.3, 29.2, 29.1, 26.9, 22.6, 14.0; HRMS (ESI): *m/z* calcd for $C_{22}H_{28}N_2O_2$ $[M+H]^+$ 353.2220, found 353.2220; IR(ATR): ν_{max} 3233, 3077, 2921, 2850, 1664, 1615, 1557, 1508, 1444, 1323 cm^{-1}.

***N*-Butyl-2-(2-((3-fluorophenyl)amino)phenyl)-2-oxoacetamide (27):** The title compound **27** was prepared from 1-(3-fluorophenyl) indoline-2, 3-dione (0.05 g, 0.20 mmol) and butylamine (0.02 g, 0.31 mmol) according to general procedure **B**. The product **27** was obtained by flash chromatography, eluting with 15-20% ethyl acetate-hexane to yield a yellow solid (0.06 g, 84%); Mp 62–64°C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.17 (bs, 1H), 8.47 (dd, *J* = 1.57, 8.24 Hz, 1H), 7.46–7.35 (m, 1H), 7.37–7.26 (m, 2H), 7.07–6.96 (m, 1H), 6.91 (bs, 1H), 6.88–6.79 (m, 2H), 3.43 (q, *J* = 6.98 Hz, 2H), 1.70–1.55 (m, 2H), 1.52–1.36 (m, 2H), 0.99 (t, *J* = 7.28 Hz, 3H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 190.2, 165.0, 161.8, 148.8, 136.1, 141.6, 136.1, 135.0, 130.6, 118.1, 117.8, 116.3, 114.4, 110.9, 109.6, 39.2, 31.3, 20.0, 13.7; HRMS (ESI): *m/z* calcd for $C_{18}H_{20}FN_2O_2$ $[M+H]^+$ 315.1504, found 315.1503; IR (ATR): ν_{max} 3265, 3091, 2930, 2867, 1634, 1564, 1446, 1282, 1206 cm^{-1}.

***N*-Butyl-2-(2-((4-methoxyphenyl)amino)phenyl)-2-oxoacetamide (28):** The title compound **28** was prepared from 1-(4-methoxyphenyl) indoline-2,3-dione (0.11 g, 0.43 mmol) and butylamine (0.06 g, 0.87 mmol) according to general procedure **B**. The product **28** was obtained by flash chromatography, eluting with 15-20% ethyl acetate-hexane to yield a yellow solid (0.080 g, 57%); Mp 92– 94 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.1 (bs, 1H), 8.47 (dd, *J* = 1.52, 8.30 Hz, 1H), 7.36–7.28 (m, 1H), 7.23–7.58 (m, 2H), 7.00–6.90 (m, 3H), 6.86 (bs, 1H), 6.75– 6.65 (m, 1H), 3.84 (s, 3H), 3.44 (q, *J* = 6.89 Hz, 2H), 1.69–1.56 (m, 2H), 1.51–1.36 (m, 2H), 0.98 (t, *J* = 7.38 Hz, 3H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 189.7, 164.0, 149.8, 157.3, 151.5, 136.2, 134.8, 132.2, 126.4, 116.1, 114.7, 114.6, 113.6, 55.5, 39.2, 31.4, 20.0, 13.7; HRMS (ESI): *m/z* calcd for $C_{19}H_{23}N_2O_3$ $[M+H]^+$ 327.1702, found 327.1703; IR (ATR): ν_{max} 3269, 3081, 2951, 1660, 1558, 1509, 1207 cm^{-1}.

***N*-Butyl-2-(2-((2,4-dinitrophenyl)amino)phenyl)-2-oxoacetamide (29):** The title compound **29** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.15 g, 0.47 mmol) and butylamine (0.07 g, 0.96 mmol) according to general procedure **B** . The product **29** was obtained by flash chromatography, eluting with 15-20% ethyl acetate-hexane to yield a yellow solid (0.09 g, 49%); Mp 102– 105 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.4 (bs, 1H), 9.17 (d, *J* = 2.65 Hz, 1H), 8.36 (dd, *J* = 1.52, 7.95 Hz, 1H), 8.30–8.22 (m, 1H), 7.71–7.61 (m, 1H), 7.60–7.48 (m, 2H), 7.42–7.33 (m, 1H), 7.22 –7.07 1H), 3.41 (q, *J* = 6.78 Hz, 2H), 1.67–1.54 (m, 2H), 1.48–1.34 (m, 2H), 0.97 (t, *J* = 7.26 Hz, 3H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 189.3, 160.9, 144.5, 139.4, 138.6, 134.4, 134.3, 133.8, 129.5, 126.3, 125.2, 123.8, 122.7, 117.4, 39.5, 31.2, 20.0, 13.6; HRMS (ESI): *m/z* calcd for $C_{18}H_{19}N_4O_6$ $[M+H]^+$ 387.1299, found 387.1299; IR (ATR): ν_{max} 3260, 3070, 2865, 1650, 1582, 1272 cm^{-1}.

2-(2-((2,4-Dinitrophenyl)amino)phenyl)-2-oxo-*N*-pentylacetamide (30): The title compound **30** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.10 g, 0.32 mmol) and pentylamine (0.10 g, 0.64 mmol) according to general procedure **B** outlined above. The product **30** was

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obtained by flash chromatography, eluting with 15-20% ethyl acetate-hexane to yield a yellow solid (0.09 g, 72%); Mp 83–85 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.4 (bs, 1H), 9.17 (d, *J* = 2.62 Hz, 1H), 8.36 (dd, *J* = 1.52, 8.0 Hz, 1H), 8.30–8.22 (m, 1H), 7.71–7.61 (m, 1H), 7.60–7.48 (m, 2H), 7.42–7.33 (m, 1H), 7.22–7.07 1H), 3.41 (q, *J* = 6.82 Hz, 2H), 1.67–1.54 (m, 2H), 1.44–1.32 (m, 4H), 0.93 (t, *J* = 6.92 Hz, 3H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 189.3, 160.9, 144.5, 139.4, 138.6 134.6, 134.3, 133.8, 129.5, 126.3, 125.2, 123.8, 122.7, 117.4, 39.7, 28.9, 28.8, 22.2, 13.9; HRMS (ESI): *m/z* calcd for $C_{19}H_{20}N_4O_6Na$ $[M+Na]^+$ 423.1272, found 423.1275; IR (ATR): v_{max} 3337, 3243, 2922, 2857, 1585, 1513, 1450, 1308, 1280, 1205 cm^{-1}.

Ethyl (2-(2-((2,4-dinitrophenyl)amino)phenyl)-2-oxoacetyl)glycinate(31): The title compound **31** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.20 g, 0.64 mmol) and glycine ethyl ester hydrochloride (0.22 g, 1.59 mmol) according to general procedure **C**. The product **31** was obtained by flash chromatography, eluting with 30-40% ethyl acetate-hexane to yield a yellow solid (0.16 g, 58%); Mp 140–142 °C; ^{1}HNMR ($CDCl_3$, 300 MHz): δ 11.3 (bs, 1H), 9.17 (d, *J* = 2.68 Hz, 1H), 8.33 (dd, *J* = 1.51 , 7.94 Hz, 1H), 8.25 (dd, *J* = 2.65, 9.39 Hz, 1H), 7.71–7.62 (m, 1H), 7.62–7.54 (m, 1H), 7.53–7.48 (m, 1H), 7.42–7.34 (m, 1H), 7.22–7.07 1H), 4.22 (q, *J* = 7.17 Hz, 2H), 4.17 (d, *J* = 5.62, Hz, 2H), 1.33 (t, *J* = 7.17 Hz, 3H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 188.3, 168.6, 161.2, 144.5, 139.5, 138.7, 134.8, 134.2, 133.9, 129.5, 126.1, 125.3, 123.8, 122.8, 117.4, 61.9, 41.4, 14.1; HRMS (ESI): *m/z* calcd for $C_{18}H_{17}N_4O_8$ $[M+H]^+$ 417.1036, found 417.1036; IR (ATR): v_{max} 3339, 3090, 2952, 1738, 1656, 1584, 1334 cm^{-1}.

Methyl (2-(2-((2,4-dinitrophenyl)amino)phenyl)-2-oxoacetyl)-L-alaninate (32): The title compound **32** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.20 g, 0.63 mmol) and L-Alanine methyl ester hydrochloride (0.18 g, 1.29 mmol) according to general procedure **C**. The product **32** was obtained by flash chromatography, eluting with 30-40% ethyl acetate-hexane to yield a yellow solid (155 mg, 53%); Mp 82–84 °C; $[a]^{25}/_{589}$ −25.0 (MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.4 (bs, 1H), 9.18 (d, *J* = 2.63 Hz, 1H), 8.32 (dd, *J* =1.46, 7.99 Hz, 1H), 8.29–8.22 (m, 1H), 7.71–7.62 (m, 1H), 7.60–7.48 (m, 3H), 7.41–7.34 (m, 1H), 4.64–4.56 (m, 1H), 3.80 (s, 3H), 2.41–2.22 (m, 1H), 1.00 (t, *J* = 6.72, Hz, 6H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 188.5, 171.2, 160.9, 144.5, 139.5, 138.7, 134.8, 134.2, 133.8, 129.5, 126.1, 125.3, 123.8, 122.8, 117.5, 57.6, 52.4, 31.3, 19.0, 17.1; HRMS (ESI): *m/z* calcd for $C_{20}H_{21}N_4O_8$ $[M+H]^+$ 417.1038, found 445.1041; IR (ATR): v_{max} 3384, 3248, 3101, 2955, 1740, 1681, 1586, 1334, 1273 cm^{-1}.

Methyl (2-(2-((2,4-dinitrophenyl)amino)phenyl)-2-oxoacetyl)-L-valinate (33): The title compound **33** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.20 g, 0.64 mmol) and L-valine methyl ester hydrochloride (0.21 g, 1.25 mmol) according to general procedure **C**. The product **33** was obtained by flash chromatography, eluting with 30-40% ethyl acetate-hexane to yield a yellow solid (0.07 g, 51%); Mp 122–124 °C; $[a]^{25}/_{589}$ −13.6 (MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.4 (bs, 1H), 9.17 (d, *J* = 2.63 Hz, 1H), 8.33 (dd, *J* = 1.52, 8.05 Hz, 1H), 8.29–8.22 (m, 1H), 7.71–7.63 (m, 1H), 7.62–7.54 (m, 2H), 7.54–7.48 (m, 1H), 7.42–7.33 (m, 1H), 4.66(1H), 3.81(s, 3H), 1.54 (d, *J* = 7.20, Hz, 3H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 188.5, 172.1, 160.5, 144.5, 139.4, 138.7, 134.8, 134.2, 133.8, 129.5, 126.1, 125.3, 123.8, 122.8, 117.4, 52.7, 48.4, 17.9; HRMS (ESI): *m/z* calcd for $C_{18}H_{17}N_4O_8$ $[M+H]^+$ 445.1356, found 445.1354; IR (ATR): v_{max} 3293, 3096, 2952, 2649, 1738, 1655, 1582, 1500, 1332 cm^{-1}.

Methyl (2-(2-((2,4-dinitrophenyl)amino)phenyl)-2-oxoacetyl)-L-leucinate (34): The title compound **37** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.20 g, 0.64 mmol) and L-leucine methyl ester hydrochloride (0.21 g, 1.59 mmol) according to general procedure **C**. The product **37** was obtained by flash chromatography, eluting with 30-40% ethyl acetate-hexane to yield a yellow solid (0.08 g, 24%); Mp 80–82 °C; $[a]^{25}/_{589}$ 45.4 (MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.4 (bs, 1H), 9.18 (d, *J* = 2.64 Hz, 1H), 8.33 (dd, *J* = 1.48, 8.04 Hz, 1H), 8.29–8.23 (m, 1H), 7.71–7.63 (m, 1H), 7.60–7.54 (m, 1H), 7.54–7.48 (m, 1H), 7.46–7.34 (m, 2H), 4.73–4.36 (m, 1H), 4.26 (q, *J* = 7.19, Hz, 2H), 1.82–1.62 (m, 3H), 1.32 (t, *J* = 7.16 Hz, 3H), 0.99 (d, *J* = 6.20, Hz, 6H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 188.5, 171.7, 160.7, 144.5, 139.5, 138.7, 134.8, 134.3, 129.5, 126.1, 125.2, 123.8, 122.7, 117.5, 61.7, 51.0, 41.3, 24.9, 22.7, 21.7, 14.1; HRMS (ESI): *m/z* calcd for $C_{21}H_{23}N_4O_8$ $[M+H]^+$ 459.1496, found 459.1510; IR (ATR): v_{max} 3294, 3102, 2957, 1735, 1655, 1583, 1500,1332 cm^{-1}.

Methyl (2-(2-((2,4-dinitrophenyl)amino)phenyl)-2-oxoacetyl)-L-phenylalaninate (35): The title compound **38** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.20 g, 0.64 mmol) and L-phenyl alanine methyl ester hydrochloride (0.32 g, 1.27 mmol) according to general procedure **C**. The product **37** was obtained by flash chromatography, eluting with 30-40% ethyl acetate-hexane to yield a yellow solid (0.08 g, 24%); Mp 78–80 °C; $[a]^{25}/_{589}$ −25.0 (MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.3 (bs, 1H), 9.17 (d, *J* = 2.67 Hz, 1H), 8.29–8.22 (m, 1H), 8.16 (dd, *J* = 1.92, 7.99 Hz, 1H), 7.69–7.61 (m, 1H), 7.58–7.40 (m, 3H), 7.38–7.26 (m, 4H), 7.21–7.13 (m, 2H), 5.00–4.89 (m, 1H), 3.78 (s, 3H), 3.33–3.09 (m, 2H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 188.4, 170.8, 160.7, 144.5, 139.5, 138.7, 135.2, 134.8, 134.2, 133.9, 129.5, 129.1, 128.8, 127.4, 125.9, 125.2, 123.8, 122.7, 117.5, 53.5, 52.6, 38.0; HRMS (ESI): *m/z* calcd for $C_{24}H_{21}N_4O_8$ $[M+H]^+$ 493.1362, found 493.1354; IR (ATR): v_{max} 3307, 3100, 2919, 1738, 1655, 1503, 1334 cm^{-1}.

Methyl (2-(2-((2,4-dinitrophenyl)amino)phenyl)-2-oxoacetyl)-L-tryptophanate (36): The title compound **39** was prepared from 1-(2,4-dinitrophenyl)indoline-2,3-dione (0.20 g, 2.04 mmol) and L-tryptophan methyl ester hydrochloride (0.32 g, 1.27 mmol) according to general procedure **C**. The product **37** was obtained by flash chromatography, eluting with 30-40% ethyl acetate-hexane to yield a yellow solid (0.08 g, 24%); Mp 123–125 °C; $[a]^{25}/_{589}$ −33.3 (MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.3 (bs, 1H), 9.16 (d, *J* = 2.64 Hz, 1H), 8.27–8.20 (m, 1H), 8.20–8.11 (m, 1H), 7.69–7.60 (m, 1H), 7.59–7.50 (m, 3H), 7.49–7.44 (m, 1H), 7.39–7.30 (m, 2H), 7.22–7.14 (m, 1H), 7.13–7.04 (m, 2H), 5.04–4.93 (m, 1H), 3.76 3H), 3.42 (d, *J* = 5.78 Hz, 2H); ^{13}CNMR ($CDCl_3$, 300 MHz): δ 188.5, 171.2, 160.8, 144.5, 139.3, 138.6, 136.1, 134.7, 134.1, 133.7, 129.5, 127.2, 126.1, 125.3, 123.8, 122.8, 122.8, 122.4, 119.8, 118.4, 117.4, 111.3, 109.4, 53.0, 52.6, 27.7; HRMS (ESI): *m/z* calcd for $C_{26}H_{22}N_5O_8$ $[M+H]^+$

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532.1462, found 532.1463; IR (ATR): v_{max} 3327, 3086, 2950, 1736, 1659, 1583, 1502, 1333 cm^{-1}.

5.2 Quorum sensing inhibition assay for PAMH602

To assess the effectiveness of the synthesized glyoxamide derivatives on QS signaling, the *P. aeruginosa* MH602 P_{lasB}::*gfp*(ASV) reporter strain, which harbors a chromosomal fusion of the *lasB* promoter to an unstable *gfp* gene and responds to the AHL 3-oxo-dodecanoyl homoserine lactone (3oxo-C12-HSL), was used[36]. To each well of the top row in a 96-well plate, 160 µL of Luria-Bertani (LB_{10}) broth medium and 40 µL of 5 mM test compound solution in DMSO were added. This was followed by 2 times dilution each time in LB_{10} broth medium in all subsequent wells. Then 100 µL of a diluted (100 times diluted in LB_{10} broth) overnight culture of *P. aeruginosa* MH602 were added to all wells and the final volume in each well was 200 µL. The plates were incubated for 15 h in a microplate reader (Wallac Victor, Perkin-Elmer) heated at 37 ºC and every 30 min were briefly shaken and measured for GFP expression (fluorescence: excitation 485 nm, emission 535 nm) and cell growth (OD 600). The inhibitory effect of a DMSO control (1% of total volume) was examined in similar fashion but no inhibitory effect either for GFP expression or OD was observed.

5.3 Quorum sensing inhibition assay for *E. coli* MT102

The AHL dependent GFP expressing *E. coli* MT102 strain (harbouring *p*JBA132 expressing Gfp(ASV) in response to AHLs) was cultured overnight in LB_{10}. The culture was diluted (1:100) in AB medium supplemented with 0.25% tryptone and 0.13% yeast extract and 200 µl aliquots were dispensed to flat bottom 96-well plate wells (Sarstedt Australia). As *E. coli* does not produce its own AHL, the cultures were supplemented with 10 nM 3-oxo-hexanoyl HSL dissolved in methanol and air dried prior to addition of the culture. The cultures were supplemented with varying concentrations of synthetic compounds dissolved in DMSO. Control culture was supplemented with equal amounts of DMSO (1%). Plates were sealed with self-adhesive microplate sealers (TopSeal-A, PerkinElmer) to allow air diffusion and to prevent condensation, and incubated in a plate reader (EnSight Multimode Plate Reader, PerkinElmer) at 37 ºC with shaking briefly, prior to each reading of fluorescence (excitation, 485 nm; emission, 535 nm) and OD_{600} every 30 min over 22 h. Ampicillin (100 µg/ml) was added to the *E.coli* MT102 culture.

5.4 Pyocyanin inhibition assay for PA14

The pyocyanin producing *P. aeruginosa* PA14 strain was cultured overnight in LB_{10} medium supplemented with 250 µM of the synthetic compounds in DMSO. Bacterial cells were removed by centrifugation at maximum speed over 5 minutes and absorbance of the supernatants were recorded at 691 nm by Bio-Rad Smartspec 3000 (Bio-Rad Laboratories Pvt Ltd, USD). The control culture was supplemented with solvent only (DMSO). All cultures were prepared in triplicates.

5.5 Docking

Possible binding sites and poses of the compounds within the quorum sensing receptor LasR were predicted by docking these compounds into the LasR receptor (PDB code, 2UV0, resolution 1.8 Å) using the docking software GOLD 5.2.2 (Cambridge Crystallography Data Centre, UK) in its implementation through the Discovery Studios (Accelrys) interface. The compounds were sketched and hydrogens were added to all ligands and the receptor prior to performing the docking runs. All ligands were also briefly minimized under the CHARMm forcefield. The binding pocket was defined from the binding site of agonist OdDHL in the crystal structure. The number of docking runs was set to 100, the "Detect Cavity" and "Early Termination" options were set to be "False". All other parameters were left at their default values. Gold scores, hydrogen bonds, hydrophobic and π-interactions of the ligands were analyzed for the first pose with the highest Gold score.

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