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Hybrids of acylated homoserine lactone and nitric oxide donors as inhibitors of quorum sensing and virulence factors in *Pseudomonas aeruginosa*†

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Pseudomonas aeruginosa is an opportunistic pathogen causing a variety of life-threatening diseases such as cystic fibrosis and nosocomial infections in burn victims. The ability of *P. aeruginosa* to cause infection is attributed to the production of virulence factors such as pyocyanin and elastases. These virulence factors are under the control of quorum sensing (QS) a cell to cell communication process controlled by small diffusible signalling molecules based on *N*-acyl-homoserine lactones (AHLs) known as autoinducers. The inhibition of QS and thereby virulence factors is seen as a potential new anti-infective strategy. Additionally, the role of nitric oxide (NO) in downstream processes in bacteria such as biofilm dispersal, motility, virulence and antimicrobial defence systems is gaining attention and could be used to control bacterial. Herein we report the design and synthesis of hybrid compounds based on AHL signalling molecules and NO donors as anti-infective agents. A series of AHL-NO hybrids were synthesised and potent inhibitors of QS and virulence factors of *P. aeruginosa* were identified. This research has led to conversion of agonist AHLs to antagonist AHLs with dual properties of QS inhibition and NO release.

Introduction

Pseudomonas aeruginosa is a significant opportunistic pathogen causing a variety of life-threatening diseases and nosocomial infections. It is the predominant cause of chronic pulmonary infections in cystic fibrosis patients, as well as infections in AIDS and cancer patients leading to increased mortality rates. Additionally, nosocomial infections by *P. aeruginosa* during surgery and particularly in burn victims cause serious complications due to septicaemia. These infections are especially difficult to eradicate due to the increase in resistance of *P. aeruginosa* to many antibiotics.[1-4] The ability of *P. aeruginosa* to cause infection is dependent upon the production of virulence factors which cause damage to host tissues and also facilitate evasion of the host immune system.[3-5]

P. aeruginosa produces a wide range of virulence factors, including pyocyanin, elastases (Las B and Las A), alkaline protease and exotoxins.[5, 6] Pyocyanin (1-hydroxy-5-methyl-phenazine) is a redox-active phenazine shown to have multiple deleterious effects upon mammalian cells through the production of reactive oxygen species.[7] Pyocyanin binding to extracellular DNA in *P. aeruginosa* is of significance in biofilm formation and plays a critical role in causing lung infections and colonizing the airways of cystic fibrosis patients.[8-10] Elastase B (LasB) is a major extracellular elastolytic zinc metalloproteinase which plays a major role in *P. aeruginosa* pathogenesis.[11, 12] LasB makes the host vulnerable to infection through hydrolysis of the components of the extracellular matrix. Under *in vitro* experimental conditions, LasB can also degrade numerous components of innate and adaptive immune systems.[13] Overall, there is clear evidence that pyocyanin and elastase are crucial to the pathogenesis of *P. aeruginosa* infections. These virulence factors gene expression are often controlled in a cell-density dependent fashion through a mechanism known as quorum sensing (QS).[14]

QS is a cell to cell communication process controlled by small diffusible signalling molecules known as autoinducers. *P. aeruginosa* possesses two QS systems, LAS and RHL, which are regulated by autoinducers, *N*-acyl-homoserine lactones (AHLs) which control the production of multiple virulence factors including

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pyocyanin and elastase.[14, 15] The importance of QS in the virulence of *P. aeruginosa* has been studied in various animal models of corneal infection, burn wound infection and pneumonia.[16-18] The inhibition of QS and thereby virulence factor production and function is gaining interest as a potential new anti-infective strategy that would 'disarm' pathogens and allow the host immune system a better chance of clearing the infection before the bacteria cause too much damage.[19, 20] Targeting virulence should have a minimum impact upon the host's commensal microbiota as virulence factors are organism specific and being an inhibition approach not targeted at growth should lead to a weaker selective pressure for the development of resistant mutants relative to many traditional antibiotic treatments.[21, 22]

In order to generate inhibitors of the QS systems of Gram-negative bacteria, most research has focused on transforming an agonist (natural AHLs) into an antagonist (synthetic AHLs) by modification of the AHL structural motif.[23, 24] The natural signalling molecules of AHL systems across different species of Gram-negative bacteria share common structural motifs, including a common lactone (head) and an acylated chain (tail) (Figure 1a). However, subtle differences in the natural signalling molecules of one species promote antagonism in another species of bacteria. A wide range of strategies have been adopted to develop QS inhibitors based on AHL systems which have shown promising inhibition of QS and its virulence factors. Studies so far have indicated that the agonist or the antagonist activity of AHL analogues is very sensitive to the chain length, and incorporation of aryl functionality into AHLs generally yields analogues having the capability to act as universal inhibitors of the AHL QS system (Figure 1b).[24-27]

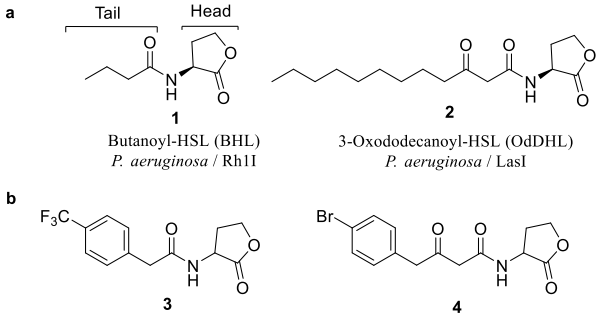


Figure 1: A) Natural autoinducers for *P. aeruginosa*; B) AHL based antagonist of QS system.

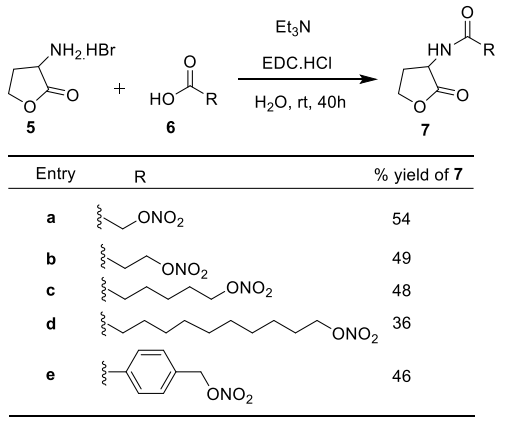
Nitric oxide (NO) is a ubiquitous signalling molecule in nature crucial to humans and is gaining recognition as a bacterial signalling molecule. NO regulates diverse downstream processes in bacteria such as biofilm dispersal, motility, virulence and antimicrobial defence systems. Exogenous NO, at sub-lethal concentrations, is able to induce the transition of bacteria in biofilms from a sessile mode of growth to a free-swimming, planktonic mode.[28] NO increases the sensitivity of various biofilms to antimicrobial treatments.[28, 29] Additionally, NO and QS are interconnected and NO influences QS in many bacterial systems such as in *Vibrio harveyi* where NO controls the QS circuit regulating motility and biofilm formation, or in *P. aeruginosa*, where NO derived from anaerobic respiration was found to modulate diverse QS systems and virulence.[30, 31] NO can exert its effects by selectively binding to sensor domains of signal transducing proteins or enzymes, such as the heme-nitric oxide/oxygen (H-NOX) domains which are part of a larger family of hemeprotein sensors for diatomic gases. Bacterial H-NOX proteins are often found in the same operon as signalling proteins, such as histidine kinases or diguanylate cyclases, suggesting HNOX roles as sensors in prokaryotic NO signalling pathways.[32] Binding of NO to HNOX-diguanylate cyclase/phosphodiesterase protein complexes controls the levels of the bacterial secondary messenger cyclic diguanosine monophosphate (c-di-GMP), which is known to be a crucial regulator for the transition between motile and sessile lifestyles in bacteria and the switch between chronic and acute infections.[32, 33] Therefore, NO can be used to regulate different pathways in bacteria and thereby control infection.

Research on both QS and NO signalling has opened up new approaches for the development of novel anti-infective drugs. NO-hybrids based on fimbrolides have shown to act as potent QS inhibitors compared to natural fimbrolides.[34] As a result, it is hypothesized that hybrids based on AHL and NO donors could also modulate QS and virulence factors in bacteria. Herein we report the design and synthesis of novel dual action compounds based on AHL signalling molecules and NO donors as anti-infective (anti-virulence) agents.

Results

Synthetic procedures

Synthesis of AHL and 3-oxo-AHL nitric oxide hybrids. In this work, AHL–NO hybrids were generated using homoserine lactone (HSL) and two different classes of NO donors; nitrates and diazeniumdiolates. The primary strategy for the syntheses of the nitrate class of AHL-NO hybrids involved 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling of the amine group on the homoserine lactone with the carboxylic acid group of the desired NO donor nitrate derivatives. Firstly, the required alkyl nitrate (nitroester carboxylic acid) derivatives **6a–e** were synthesized from the desired starting bromo-alkanoic acids by reaction with silver nitrate. The acid nitrates **6a-e** and EDC were added to a solution of *L*-HSL **5** containing triethylamine in water. The resulting reaction mixture was stirred for 40 h at room temperature (rt) followed by workup to generate the desired products **7a–e** (Scheme 1). The compounds were purified by washing with ether or by column chromatography, and were characterized using NMR and other spectroscopic techniques.

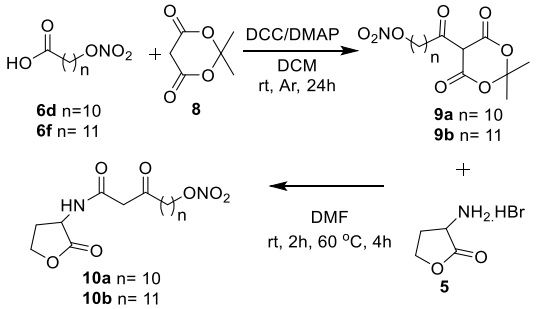


Entry	R	% yield of **7**
a	ONO_2	54
b	ONO_2	49
c	ONO_2	48
d	ONO_2	36
e	ONO_2	46

Scheme 1: Preparation of AHL nitrate analogues.

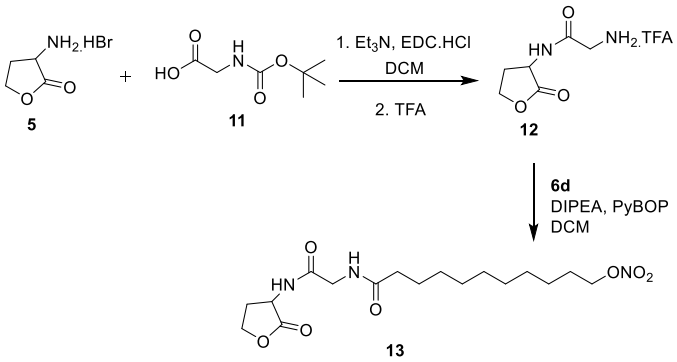
The structure of **7a** was additionally confirmed by X-ray crystallography, which showed the presence of the desired nitrate group (Figure 1, Supporting Information).

The next objective was the preparation of 3-oxo-AHL nitrate analogues using Meldrum's acid. We focused on the 3-oxo analogues bearing longer alkyl chain to mimic the natural autoinducer OdDHL **2**, which is at the top of QS regulatory hierarchy in *P. aeruginosa*. The acylation of Meldrum's acid **8** with nitrate acid **6d** or **6f** in anhydrous DCM with *N*,*N*-dicyclohexylcarbodiimide (DCC)/4-dimethylaminopyridine (DMAP) furnished the intermediate conjugates **9a** and **9b**, respectively. The crude reaction mixture was dissolved in DMF and HSL **5** was added, and the resulting mixture was stirred at room temperature for 2 h followed by stirring at 60 °C for 4 h. After workup, the desired products **10a–b** were isolated by column chromatography in 54–57% yields (Scheme 2).



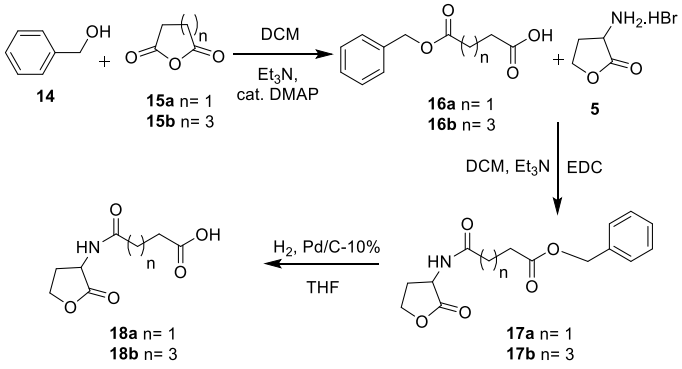
Scheme 2: Synthesis of 3-oxo-AHL nitrate derivatives.

Additionally, an AHL nitrate derivative with a glycine molecule between the HSL **5** and the nitrate acid linker **6d** was synthesized as illustrated in the Scheme 3. At first the Boc-glycine **11** was coupled to the HSL **5** *via* EDC coupling followed by deprotection of the Boc-group by trifluoroacetic acid. The HSL-glycine derivative **12** so obtained was then coupled with the alkanoic acid nitrate derivative **6d** using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) coupling conditions to yield the desired product **13**.



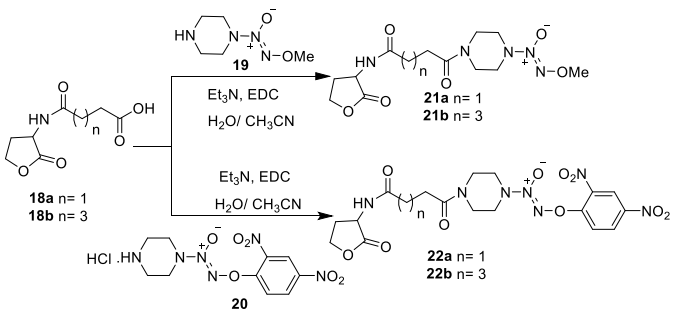
Scheme 3: Synthesis of glycine-linked AHL derivative **13**

Synthesis of AHL diazeniumdiolate derivatives. The second class of AHL-NO hybrid was based on diazeniumdiolates which were synthesized through coupling of easily accessible HSL acid derivatives with O^2-stabilized piperazine diazeniumdiolates. The required *L*-HSL acid derivatives **18** were synthesized by coupling the protected acids **16a–b**[35] with HSL **5** using EDC to afford the HSL amide derivatives **17a–b**. The removal of the benzyl protecting group was carried out using hydrogenation conditions (Pd/C, H_2, rt) to furnish the desired intermediate products **18a–b** in 64–69% overall yields (Scheme 4).



Scheme 4: Synthesis of AHL acid derivatives **18**.

In the final step, the AHL acids **18a–b** were reacted with piperazine derivatives **19** and **20** using EDC coupling in a water/acetonitrile mixture at room temperature for 40 h. Work-up followed by chromatography gave the desired diazeniumdiolate products **21a–b** and **22a–b** in 35–79% yield (Scheme 5).



Scheme 5: Synthesis of AHL diazeniumdiolate derivatives.

The structure of **22a** was further confirmed by X-ray crystallography (Figure 2, Supporting Information), which showed the presence of the AHL, piperazine and 2,4-dinitrophenyl motifs. The cisoid (*Z*)-type structural orientation of the diazeniumdiolate was also confirmed by X-ray crystallography.

Docking studies

NO-releasing AHL derivatives that mimic the natural QS autoinducers have been synthesized during the course of these studies. It is therefore important to evaluate the influence of the structural modifications on the binding of the novel AHL derivatives compared to the cognate signalling molecule (OdDHL) in the receptor binding site. Using Gold docking software through the Accelrys Drug Discovery Studio interface, different classes of AHL-NO donor hybrids were examined for their ability to bind to the receptor protein LasR (PDB code, 2UV0).[36] The docking study was initially carried out with the receptor agonist OdDHL in order to determine the reliability of the docking method. The docking protocol ranks the output on the basis of docking scores and higher docking score values indicate a stronger ligand binding affinity. The docking output was analyzed for the predicted binding interactions, including hydrogen bonding, electrostatic and hydrophobic interactions, between the compounds and the LasR receptor in the best scoring pose and results are presented in Table 1.

Table 1: Docking results of the AHL-NO donor hybrids with LasR protein.

Compound	Dock score	H Bond	Π and Hydrophobic	Unfavorable
7a	51.88	Tyr56, Trp60, Arg61, Tyr 64, Thr75, Ser129, Asp73, Leu110	Tyr64	Asp73
7b	53.31	Tyr56, Trp60, Arg61, Thr75, Ser129, Asp73, Leu110	Tyr64	—
7c	50.65	Tyr56, Thr75, Ser129, Asp73, Tyr47, Leu110	Tyr64	—
7d	60.76	Tyr56, Thr75, Ser129, Asp73	Tyr64	—
7e	54.28	Tyr56, Thr75, Ser129, Asp73, Tyr47, Leu110	Asp73, Tyr64	—
10a	67.72	Tyr56, Thr75, Thr115, Ser129, Asp73, Leu110	Tyr64	Asp73
10b	57.48	Tyr56, Tyr64, Thr115, Asp73, Val76	Tyr64	Asp73
13	63.62	Ser129, Cys79, Thr115, Leu110, Trp88, Ser129, Thr75, Tyr56, Asp73, Val76	Trp88	Ser129, Tyr56, Val76
21a	56.40	Tyr47, Asp73, Leu110	Trp88	Asp73, Ty64
21b	56.87	Tyr56, Tyr64, Ser129, Asp73, Leu110, Val76	Tyr64	Asp73
22a	56.37	Tyr56, Trp60, Arg61, Tyr93, Gly38, Gly126, Ser129	Tyr64, Trp88, Ala105, Leu110	Asp73
22b	26.82	Trp60, Arg61, Csy79, Thr115, Ser129, Gly126	Tyr64, Trp88, Ala105, Leu110	Asp73
OdDHL (2)[a]	61.70	Asp73, Tyr56, Trp60, Arg61, Ser129, Tyr93	Trp88, Tyr64	—

[a]LasR autoinducer (agonist)

Analysis of the docked structures indicated the presence of extensive hydrogen bonding interactions between the synthesized molecules and the ligand binding site. Compound **10b** had the highest dock score (67.72) of all the compounds studied, with hydrogen bonding to Tyr56, Thr75, Thr115, Ser129, Asp73, and Leu110. OdDHL **2** the LasR autoinducer had a lower score of 61.7 with hydrogen bonding with Asp73, Tyr56, Trp60, Arg61, Ser129 and Tyr93 (Figure 2). The docking results suggested that hydrogen bonding interactions to amino acid residues such as Tyr56, Thr75, Ser129, Asp73, Leu110 and π-interaction with Tyr64 andTrp88 played an important role in the binding of the AHL-NO hybrid compounds to the LasR receptor. It is also important to note that the attaching NO donor groups to AHL led to additional interaction with the binding site amino acid, suggesting the influence of these groups in transforming an agonist scaffold to an antagonist.

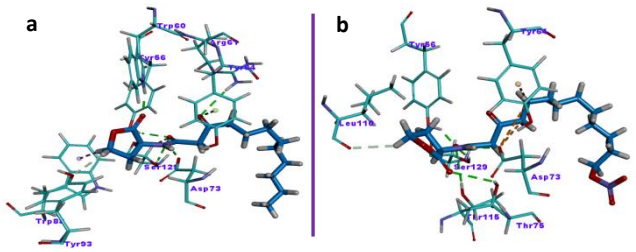


Figure 2: Docking poses and interactions with the LasR protein ligand binding domain of (A) natural AHL, OdDHL (**2**) and (B) highest dock score AHL NO donor hybrid **10a**. Selected interactions shown as dashed lines (green: H bonds; purple: hydrophobic interactions; orange and cream: interaction involving π systems).

Biological Screening

The synthesized AHL-NO hybrids were evaluated for their NO release, QS inhibition and anti-virulence properties and are discussed below.

Nitric Oxide measurement via the Griess reagent. Quantification of NO release was performed for the AHL nitrate derivatives via treatment of compounds at 37 °C for 1 h with xanthine oxidase, xanthine, and cysteine, which convert the nitrate into NO.[37] NO was subsequently converted into nitrite in aqueous media and was quantified using the Griess reagent. Due to the low percentage conversion of nitrate to nitrite (1−5%) under *in vitro* experimental conditions and the high detection limit of nitrite (≥1 µM) by the Griess reagent, relatively high concentration of 500 µM was required to generate signals within the detection limit. The Griess assay results indicated that the nitrate derivatives **7a** and **7e** had the highest nitrite (NO) release of 29.0 µM and 2.6 µM, respectively, at 500 µM of added compound. In comparison isosorbide dinitrate (ISDN) a known nitrate based NO donor showed NO release of 4 µM. On the other hand AHL substituted with longer alkyl nitrates didn't show any NO release compared to control and for AHL-diazeniumdiolates, there was no NO release even after 24 h of incubation.

QS Inhibition Assay. The QS inhibitory effects of the AHL−NO hybrids were evaluated using the QS reporter screening system developed by Hentzer *et al.*[18] The reporter strain *P. aeruginosa* MH602 P_{lasB}::*gfp*(ASV) has a green chromosomal unstable fluorescent protein (GFP-ASV) gene fused to the promoter region of the *lasB* QS gene of *P. aeruginosa*, which is induced during QS and normal growth of bacteria. An addition of a QS inhibitor to this bioreporter will result in a reduction in GFP expression to an extent that correlates with the efficacy of the inhibitor. In this study, P_{lasB}::*gfp*(ASV) bacteria were incubated with various concentrations of compounds, and half hourly measurements of GFP expression (relative fluorescence units, RFU) and cell growth (OD_{600}) were obtained. Representative data are shown for derivative **10a** in Figure 3. The percentage LasR inhibition of the compounds was calculated as the percentage difference in RFU value between the sample and the control at the time point when the fluorescence reached its maximum value in the control. The most active QS inhibitors in the nitrate series were **7d** and **10a**, which showed 70% and 75% QS inhibition at 266 µM, respectively (Table 2). By comparison, the AHL diazeniumdiolate hybrid derivatives **21a**–**b** and **22a** showed 42−58% QS inhibition activity at 266 µM (Table 2). The QS inhibitory activity of the synthesised compounds was concentration-dependent, and importantly, all the compounds tested had no effect on growth at concentrations tested.

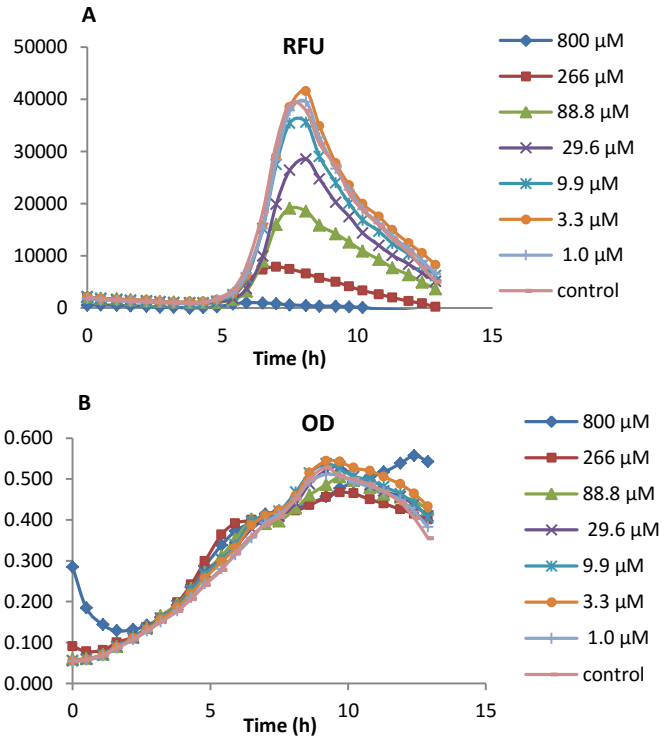


Figure 3: Biological screening assay for QS LasR inhibition by AHL−NO derivative **10a**. (A) Relative fluorescence units (RFU), and (B) optical density (OD) as function of time. The reporter strain *P. aeruginosa* MH602 harbouring the P_{lasB}::*gfp*(ASV) fusion plasmid was used to monitor QS LasR inhibition.

Table 2: Percentage LasR inhibition at different concentrations of AHL−NO derivatives as determined by reduction in GFP-ASV expression in the P_{lasB}::*gfp* reporter strain compared to control.

Compound	Concentration (µM)				
	266	**88.8**	**29.6**	**9.9**	**3.3**
7a	26.4	5.5	NA	NA	NA
7b	31.3	5.2	NA	NA	NA

7c	26.9	NA	NA	NA	NA
7d	70.1	25.2	NA	NA	NA
7e	62.2	14.2	NA	NA	NA
10a	74.5	47.1	29.9	8.7	NA
10b	59.9	20.1	1.1	NA	NA
13	62.3	20.4	4.1	NA	NA
21a	43.1	13.6	2.9	NA	NA
21b	41.7	13.8	2.5	2.1	NA
22a	57.8	25.4	11.7	3.3	NA
Fur 30[a]	GI	92.4[b]	61.9	29.8	3.7

NA - Not active (No reduction in RFU compared to control), [a]Fur 30, used as standard QS inhibitor,[18] GI - Growth inhibition >50%, [b]Growth inhibition 44%.

Further, the QS inhibitory effects of the AHL−NO hybrids were evaluated against the RHL system using the QS reporter strain, *P. aeruginosa* P_{rhlA}::*gfp*, were an increase in green fluorescent protein (GFP-ASV) production, as a consequence of QS induction, can be observed during the normal growth of the strain. None of the tested hybrid compounds showed QS inhibition against the RHL system (data not shown).

Pyocyanin and elastase assays. In order to determine the effect of the hybrids on virulence factors, the synthesised compounds were tested to determine their efficacy as inhibitors of elastase and pyocyanin production. Activity is given as percent inhibition compared to DMSO control for both the assay. Pyocyanin production inhibition studies were carried out using *P. aeruginosa* PA14 by incubating compounds at 100 µM overnight in the culture media. Compound **7a** was the most potent and showed 53% inhibition of pyocyanin production at 100 µM while not affecting the growth of *P. aeruginosa* PA14 compared to a DMSO control. Compounds **7d, 7e**, **10a**, **10b** and **22a** showed inhibition of pyocyanin production in the range of 15-20% at 100 µM (Figure 4). In order to determine whether these inhibitory effects were not due to direct interaction of these molecules with pyocyanin, an untreated *P. aeruginosa* PA14 overnight cultured was filtered to obtain a pyocyanin-containing cell-free culture fluid. This pyocyanin containing culture fluid was incubated with 100 µM of compounds and tested for pyocyanin and showed no altered pyocyanin levels. Elastase production inhibition studies were carried out against *P. aeruginosa* PAO1 at 200 µM, as compounds were found to have minimal activity at 100 µM. Compounds **10a** and **10b** with long hydrophobic alkyl chains and 3-oxo substitution were the most active with 16% and 23% inhibition of elastase production respectively (Figure 5).

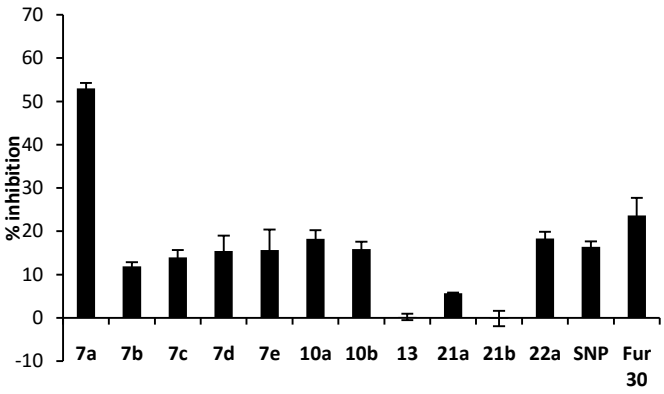


Figure 4: Percent inhibition of pyocyanin production by AHL−NO derivatives at 100 µM against *P. aeruginosa* PA14 compared to DMSO control. Sodium nitroprusside (SNP) used as reference NO donor (tested at 100 µM), Fur 30 as reference QS inhibitor (tested at 25 µM). No effect on growth was observed for any of the analogues. Error bars represent the standard error of the results (n = 3).

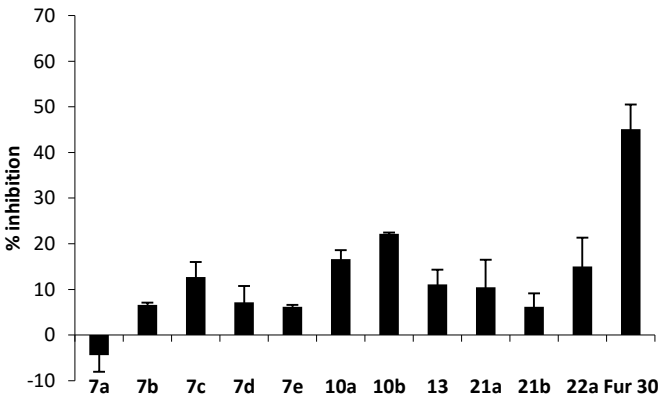


Figure 5: Percent inhibition of elastase production by AHL−NO derivatives at 200 µM against *P. aeruginosa* PAO1 compared to DMSO control. Fur 30 used as reference QS inhibitor (tested at 50 µM due to bactericidal effect at higher concentrations). No effect on growth was observed for any of the analogues. Error bars represent the standard error of the results (n = 3).

Discussion

A series of AHL based NO hybrids comprising different NO donor groups, such as nitrates, and diazeniumdiolates have been successfully synthesized under standard amide coupling reaction conditions in good to moderate yields. These compounds were further evaluated to determine their NO releasing, QS and virulence factors modulatory effects.

The comparison of QS inhibitory results indicated that **10a** was the most potent AHL derivative in the series with 74.5% QS inhibition, followed by **7d** (70% QS inhibition), **7e** (62.2% QS inhibition) and **13** (62.3% QS inhibition). Significantly, compound **10a** had no effect on growth even at a high concentration of 800 µM (Figure 3 and Table 2). This result is consistent with docking studies where compound **10a** had the highest score of 67.72 followed by **13** and **7d** with scores of 63.62 and 60.76, respectively (Table 1). In addition, 3-oxo-AHL analogue **10a** followed by AHL analogue **7d** most closely resemble the natural signalling molecule OdDHL **2** recognized by the LasR receptor protein, further suggesting why these compounds have better activity and docking results. This highlights the significance of the terminal substitution of nitrates on the alkyl chain in transforming an agonist to an antagonist, which is in accordance with the literature where terminal substitution of isothiocyanates and chloroacetamides led to potent QS inhibitor activity. Further, in comparison the activity of **10a** (IC_{50}: 107 µM) is similar to the activity of itc-12 (IC_{50}: 113 µM) but lower than itc-13 (IC_{50}: 45.2 µM) against respective wild-type reporter strain.[38] The activity of **7e** is also in accordance with literature studies, which have reported acylated phenyl homoserine lactone derivatives exhibit potent QS inhibitory activities.[23, 27] Not surprisingly these hybrid molecules do not affect the Rhl controlled gene expression when testes against *P. aeruginosa* P_{rhlA}::*gfp* system. Hybrids with shorter alkyl chain nitroesters had lower QS inhibitor effects, which also correlates to the low docking score seen for these AHL-NO hybrids. Additionally, increasing the carbon chain to greater than 11 carbons (compound **10b**) led to lower QS inhibition effect.

In the case of the diazeniumdiolate AHL derivatives, the bulkier O^2-protected diazeniumdiolate derivative **22a** displays better QS inhibition compared to the other diazeniumdiolate derivatives. Again, the activities of the daizeniumdiolate AHL hybrids correlated with the docking score obtained. In summary, the results show that bulkier hydrophobic substitution on the acylated homoserine lactone scaffold is more conducive to inhibitory activity, due to better complementarities with the hydrophobic pocket of the LasR receptor protein, leading to superior inhibition of the AHL-mediated QS pathway. Also, an 11 carbon alkyl chain was considered optimal for high docking score and for QS inhibition activity.

As mentioned earlier, virulence factors are crucial for bacteria in overcoming host immune responses and their pathogenicity. The synthesized compounds were therefore evaluated to determine the inhibitory effect on two virulence factors, pyocyanin and elastase, both of which are known to create severe damage in disease conditions. In case of inhibition of pyocyanin production, compound **7a** had the highest inhibitory effect, followed by **10a**. The inhibitory activity of **7a** was interesting, as we expected the most active QS inhibitory compound **10a** to be more active. One plausible reason for the higher activity could be that in *P. aeruginosa* the RHL system which produces and responds to BHL **1** has shown to be involved in the regulation of pyocyanin production. Therefore, compound **7a** was also tested against the RHL based QS system, but the compound did not show any antagonist effect for the RHL system. Further, NO donor SNP was tested for its inhibition of pyocyanin production activity, which showed 16% inhibition. Based on the Griess assay, compound **7a** showed 26 µM NO release. Thus, the pyocyanin production inhibitory activity could be partially attributed to the NO releasing property of compound **7a**. Although the effect of NO on controlling pyocyanin production is not known, there is a literature report which shows nitrite in presence of H_2O_2 could interact with pyocyanin.[39] This could explain the effect of **7a** towards pyocyanin. The effect could also be partially related to its QS inhibition of the LasB system as a hierarchical relationship exists between the two QS systems of *P. aeruginosa,* were the LAS system positively regulates the expression of both *rhlR* and *rhlI*. In case of the elastase production inhibition assay, compounds had moderate to weak inhibitory effect. Compounds **10a** and **10b** were the most active in inhibiting elastase production, which correlates with the LasB based QS assay, were **10a** had the most potent inhibitory effects. This is in accordance with the literature, as the production of elastase (LasB and LasA) is controlled by the LAS system which responds to OdDHL.

Conclusions

In conclusion, several AHL-NO hybrids were synthesized using facile coupling reactions. AHL-NO hybrids of different NO donors, namely nitrates and diazeniumdiolates, were prepared and further evaluated for their biological roles. Overall, the nitrate based AHL hybrids have the most promising activity, inhibiting QS and related virulence factors. Additionally, these compounds were non-bactericidal at the concentrations tested, which is ideal for overcoming resistance issues associated with modern antibiotics. This work demonstrates a simple, elegant way to convert a natural autoinducer to not just an antagonist, but also with additional NO donor properties which could lead to synergistic effects as exhibited by the activities of these compounds.

Materials and methods

All reactions requiring anhydrous conditions were performed under an argon atmosphere. Methanol (MeOH), ethanol (EtOH), and ethyl acetate were obtained from commercial sources. Anhydrous dichloromethane (DCM), ether, and tetrahydrofuran (THF) were obtained using a PureSolv MD Solvent Purification System. Commercially available reagents were purchased from Fluka, Aldrich, Acros Organics, Alfa Aesar, and Lancaster and used without further purification. Nitric oxide gas (99.9%) was purchased from Linde Gas and used without further purification. Reactions were monitored using thin layer chromatography, performed on Merck DC aluminum plates coated with silica gel GF254. Compounds were detected by short and long wavelength ultraviolet light. Vacuum column chromatography was carried out using Grace Davison LC60A 6–35 µm silica gel. Preparative thin layer chromatography was carried out on 3 × 200 × 200 mm glass plates coated with Merck 60GF254 silica gel.

NMR spectra were obtained in the designated solvents on a Bruker DPX 300 or a Bruker Avance 400 spectrometer as designated. Chemical shifts (δ) are in parts per million and internally referenced relative to the solvent nuclei. ^{1}H NMR spectral data are reported as follows: chemical shift measured in parts per million (ppm) and internally referenced relative to the solvent nuclei (δ); multiplicity; observed coupling constant (J) in hertz (Hz); proton count; assignment. Multiplicities are assigned as singlet (s), doublet (d), doublet of doublet (dd), doublet of triplet (dt), triplet, (t), quartet (q), quintet (p), doublet of doublet of doublets (ddd), multiplet (m), and broad singlet (bs) where appropriate. ^{13}C NMR spectra were recorded in the designated solvents, and chemical shifts are reported in ppm and internally referenced relative to the solvent nuclei. Melting points were measured using a Mel-Temp melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Spotlight 400 FTIR microscope. Ultraviolet spectra were measured using a Perkin-Elmer Lambda 35 UV–visible spectrometer in the designated solvents and data reported as wavelength (λ) in nm and adsorption coefficient (ε) in $M^{-1}cm^{-1}$. High-resolution mass spectrometry was performed by the Bioanalytical Mass Spectrometry unit, UNSW. Microanalysis was performed on a Carlo Erba Elemental Analyzer EA 1108 at the Campbell Microanalytical Laboratory, University of Otago, New Zealand. The NO donor derivatives **6a–e**, **19** and **20** were synthesized using literature procedures.[40-43] The characterization data were in agreement with those reported in the literature

2-Oxo-2-(2-oxotetrahydrofuran-3-ylamino)ethyl nitrate (7a). To a stirred solution of L-homoserine lactone hydrobromide **5** (0.36 g, 3.56 mmol) in water (4.00 mL), triethylamine (0.54 mL, 3.92 mmol) was added followed by the addition of acid **6a** (0.64 g, 5.34 mmol) and l-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (1.09 g, 5.70 mmol). The mixture was stirred at room temperature for 40 h and then evaporated *in vacuo* to dryness. The residue was partitioned between water (20 mL) and ethyl acetate (50 mL) and the organic layer successively washed with 5% sodium bicarbonate ($NaHCO_3$) solution (2 × 20 mL), 1 M potassium hydrogen sulfate ($KHSO_4$) solution (2 × 20 mL) and brine. The organic layer was dried over sodium sulfate ($NaSO_4$) and evaporated to dryness to yield the title compound **7a** as a white solid (0.21 g, 54%). M.p. 96-98 °C; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 2.16-2.28 (m, 1H, H4a), 2.82-2.91 (m, 1H, H4b), *δ* 4.27-4.36 (m, 1H, H5a) 4.51 (t, *J* = 9.1 Hz, 1H, H5b), 4.56-4.65 (m, 1H, H3), 4.95 (s, 2H, CH_2ONO_2), 6.57 (bs, 1H, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) *δ* 30.1(CH_2), 49.1 (CH), 66.1 (CH_2), 69.0 (CH_2), 165.4 (C=O), 174.4 (C=O); IR (neat): ν_{max} 3351, 3000, 2948, 2922, 1764, 1688, 1646, 1546, 1421, 1380, 1288, 1247, 1224, 1195, 1181, 1144, 1046, 1010, 982, 953, 938, 848, 815, 768, 739, 720 cm^{-1}; HRMS (ESI) *m/z* Calcd. for $C_6H_8N_2O_6Na$ $(M + Na)^+$ 227.0280. Found 227.0271.

3-Oxo-3-(2-oxotetrahydrofuran-3-ylamino)propyl nitrate (7b). The title compound synthesis was carried out as described for the preparation of compound **7a** using acid **6b** (0.72 g, 5.34 mmol) to give desired product **7b** as an off white solid (0.21 g, 49%). M.p. 74-76 °C; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 2.13-2.28 (m, 1H, H4a), 2.67-2.71 (dt, *J* = 3 and 9, 2H, $COCH_2$), 2.73-2.82 (m, 1H, H4b), 4.25-4.34 (m, 1H, H5a), 4.44-4.51 (dt, *J* = 9.0, 3.0 Hz, 1H, H5b), 4.56-4.65 (m, 1H, H3), 4.77 (t, *J* = 6 Hz, 2H, CH_2ONO_2), 6.85 (bd, *J* = 6 Hz, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) 29.5 (CH_2), 33.0 (CH_2), 49.1 (CH), 66.1 (CH_2), 68.4 (CH_2), 169.2 (C=O), 175.5 (C=O); IR (neat): ν_{max} 3307, 3098, 2938, 1774, 1649, 1614, 1558, 1456, 1408, 1376, 1355, 1268, 1212, 1170, 1007, 951, 895, 862, 792, 760, 688 cm^{-1}; HRMS (ESI) *m/z* Calcd. for $C_7H_{10}N_2O_6Na$ $(M + Na)^+$ 241.0437. Found 241.0426.

4-Oxo-4-(2-oxotetrahydrofuran-3-ylamino)hexyl nitrate (7c). The title compound synthesis was carried out as described for the preparation of compound **7a** using acid **6c** (0.94 g, 5.34 mmol) to give desired product **7c** as an off white solid (0.24 g, 48%). M.p. 50 °C; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 1.42-1.50 (m, 2H, CH_2), 1.62-1.80 (m, 4H, CH_2), 2.06-2.21 (m, 1H, H4a), 2.27 (t, *J* = 6, 2H, $COCH_2$), 2.79-2.88 (m, 1H, H4b), 4.43-4.50 (m, 3H, CH_2ONO_2 and H5b merge), 4.52-4.59 (m, 1H, H3), 6.06 (bd, *J* = 3 Hz, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) *δ* 24.6 (CH_2), 25.1 (CH_2), 26.4 (CH_2), 30.43 (CH_2), 35.5 (CH_2), 49.2 (CH), 66.0 (CH_2), 72.8 (CH_2), 172.9 (C=O), 175.3 (C=O); IR (neat): ν_{max} 3309, 3074, 2954, 2865, 1775, 1639, 1622, 1543, 1493, 1451, 1382, 1360, 1279, 1225, 1167, 1107, 1014, 998, 976, 947, 899, 865, 759, 700, 684, 657 cm^{-1}; HRMS (ESI) *m/z* Calcd. for $C_{10}H_{16}N_2O_6Na$ $(M + Na)^+$ 283.0906. Found 283.0900.

11-Oxo-11-(2-oxotetrahydrofuran-3-ylamino)undecyl nitrate (7d). The acid **6d** (0.67 g, 2.67 mmol) was coupled to HSL (0.18 g, 1.78 mmol) using the method described for compound **7a** to give the title compound **7d** as a white solid (0.11 g, 36%). M.p. 62 °C; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 1.28-1.43 (m, 12H, 6 × CH_2), 1.58-1.75 (m, 4H, 2 × CH_2), 2.05-2.20 (m, 1H, H4a), 2.24 (t, *J* = 6.0, 2H, $COCH_2$), 2.80-2.89 (m, 1H, H4b), 4.23-4.32 (m, 1H, H5a), 4.43 (t, *J* = 6.0 Hz, 2H, CH_2ONO_2), 4.46 (dt, *J* = 12.0, 3.0 Hz, 1H, H5b), 4.52-4.58 (m, 1H, H3), 6.04 (bs, 1H, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) *δ* 25.2 (CH_2), 25.5 (CH_2), 26.6 (CH_2), 28.9 (CH_2), 29.0 (CH_2), 29.12 (CH_2), 29.13 (CH_2), 29.16 (CH_2), 30.5 (CH_2), 36.0 (CH_2), 49.1 (CH), 66.0 (CH_2), 73.3 (CH_2), 173.6 (C=O), 175.4 (C=O); IR (neat): ν_{max} 3317, 3073, 2924, 2852, 1775, 1642, 1622, 1545, 1468, 1381, 1361, 1278, 1225, 1170, 1107, 1012, 999, 946, 865, 803, 758, 722 cm^{-1}; HRMS (ESI) *m/z* Calcd. for $C_{15}H_{26}N_2O_6Na$ $(M + Na)^+$ 353.1689. Found 353.1681; Anal. Calcd. for $C_{15}H_{26}N_2O_6$: C, 54.53; H, 7.93; N, 8.48. Found: C, 54.98; H, 8.15; N, 8.31.

4-(2-Oxotetrahydrofuran-3-ylcarbamoyl)benzyl nitrate (7e). The acid **6e** (0.73 g, 3.71 mmol) was coupled to HSL (0.25 g, 2.47 mmol) using the method described for compound **7a** to give the title compound **7e** as a white solid (0.17 g, 46%). M.p. 152-154 °C; ^{1}H NMR (300 MHz, $CDCl_3$) *δ* 2.15-2.27 (m, 1H, H4a), 2.87-2.96 (m, 1H, H4b), *δ* 4.25-4.34 (m, 1H, H5a) 4.50 (dt, *J* = 9.0, 1.2 Hz, H5b), 4.62-4.70 (m, 1H, H3), 5.40 (s, 2H, CH_2ONO_2), 6.67 (bs, 1H, NH), 7.41 (d, *J* = 10.2 Hz, 2H, 2 × ArH), 7.82 (d, *J* = 10.2 Hz, 2H, 2 × ArH); ^{13}C NMR (75.6 MHz, $CDCl_3$) *δ* 30.1 (CH_2), 49.8 (CH), 66.3 (CH_2), 73.6 (CH_2), 127.7 (ArCH), 128.9 (ArCH), 133.8 (ArC), 136.4 (ArC), 167.0 (C=O), 175.4 (C=O); IR (neat): ν_{max} 3299, 2925, 1775, 1614, 1577, 1533, 1506, 1449, 1384, 1342, 1281, 1221, 1184, 1167, 1115, 1024, 992,

975, 955, 882, 850, 756, 732, 696 cm^{-1}; UV (MeOH): λ_{max} 228 nm (ε 12933 $M^{-1}cm^{-1}$); HRMS (ESI) *m/z* Calcd. for $C_{12}H_{12}N_2O_6Na$ $(M + Na)^+$ 303.0593. Found 303.0587.

11,13-Dioxo-13-(2-oxotetrahydrofuran-3-ylamino)tridecyl nitrate (10a). *N,N*-(dimethylamino)pyridine (DMAP) (0.22 g, 1.96 mmol), *N,N*-dicyclohexylcarbodiimide (DCC) (0.40 g, 1.18 mmol), the acid **6d** (0.40 g, 1.64 mmol) and Meldrum's acid **8** (0.23 g, 1.64 mmol) were dissolved in 20 mL of dichloromethane. The resulting solution was stirred overnight, cooled to r.t. and then filtered to remove *N,N*-dicyclohexyl urea formed in the reaction. The filtrate was concentrated in *vacuo*. The resulting residue was dissolved in DMF (10 mL) and HSL **5** (0.30 g, 1.64 mmol) was added. The mixture was stirred at room temperature for 1 h and at 60 °C for an additional 4 h. The resulting solution was diluted with ethyl acetate 50 mL, and washed with saturated sodium carbonate solution, 1 M sodium hydrogen sulfate solution and brine. The organic phase was dried over sodium sulfate, filtered and concentrated in *vacuo*. Further purification by vacuum chromatography using ethyl acetate/ methanol (9.5:0.5) gave the title compound **10a** as a yellow solid (0.32 g, 54%). M.p. 58 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28-1.43 (m, 12H, 6 × CH_2), 1.53-1.58 (m, 2H, CH_2), 1.65-1.74 (m, 2H, CH_2), 2.19-2.28 (m, 1H, H4a), 2.49 (t, *J* = 6.0, 2H, $COCH_2$), 2.66-2.76 (m, 1H, H4b), 3.45 (s, 2H, $COCH_2CO$) 4.21-4.28 (m, 1H, H5a), 4.40 (t, *J* = 6.0 Hz, 2H, CH_2ONO_2), 4.45 (dt, *J* = 12.0, 3.0 Hz, 1H, H5b), 4.56-4.62 (m, 1H, H3), 7.65 (bs, 1H, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 23.2 (CH_2), 25.5 (CH_2), 26.7 (CH_2), 28.9 (CH_2), 29.0 (CH_2), 29.21 (CH_2), 29.25 (CH_2), 29.3 (CH_2), 29.6 (CH_2), 43.7 (CH_2), 48.3 (CH_2), 49.0 (CH), 65.9 (CH_2), 73.4 (CH_2), 166.4 (C=O), 174.9 (C=O), 206.4 (C=O). IR (neat): ν_{max} 3265, 2923, 2853, 1783, 1722, 1647, 1642, 1548, 1467, 1421, 1383, 1348, 1280, 1167, 1033, 999, 976, 866, 721, 702 cm^{-1}; UV (MeOH): λ_{max} 249 nm (ε 12943 $M^{-1}cm^{-1}$); HRMS (ESI) *m/z* Calcd. for $C_{17}H_{29}N_2O_7$ $(M + H)^+$ 373.1975. Found 373.1959.

12,14-Dioxo-14-(2-oxotetrahydrofuran-3-ylamino)tetradecyl nitrate (10b). The title compound was synthesized as described for **10a** by reacting acid **6f** (0.42 g, 1.64 mmol) with Meldrum's acid **8** followed by HSL **5**, the resulting crude mixture was purified by vacuum chromatography to afford the title product **10b** as a light yellow solid (0.36 g, 57%). M.p. 62 °C; ^{1}H NMR (300 MHz, CDCl3): 1.25-1.39 (m, 14H, 7 × CH_2), 1.53-1.58 (m, 2H, CH_2), 1.67-1.72 (m, 2H, CH_2), 2.18-2.27 (m, 1H, H4a), 2.51 (t, *J* = 7.3 Hz, 2H, $COCH_2$), 2.67-2.76 (m, 1H, H4b), 3.45 (s, 2H, $COCH_2CO$), 4.21-4.30 (m, 1H, H5a), 4.42 (t, *J* = 6.6 Hz, 2H, CH_2ONO_2), 4.46 (dt, *J* = 9.0, 1.4 Hz, 1H, H5b), 4.53-4.62 (m, 1H, H3), 7.65 (bd, *J* = 6.5 Hz, 1H); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 23.3 (CH_2), 25.6 (CH_2), 26.7 (CH_2), 28.9 (CH_2), 29.0 (CH_2), 29.30 (CH_2), 29.36 (CH_2), 29.4 (CH_2), 29.7 (CH_2), 43.8 (CH_2), 48.2 (CH_2), 49.0 (CH), 65.9 (CH_2), 73.4 (CH_2), 166.4 (C=O), 174.9 (C=O), 206.5 (C=O). IR (neat): ν_{max} 3290, 2919, 2850, 1776, 1715, 1642, 1625, 1543, 1468, 1418, 1381, 1342, 1278, 1225, 1173, 1016, 1000, 949, 865, 720, 699 cm^{-1}; UV (MeOH): λ_{max} 251 nm (ε 11285 $M^{-1}cm^{-1}$); HRMS (ESI) *m/z* Calcd. for $C_{18}H_{30}N_2O_7Na$ $(M + Na)^+$ 409.1951. Found 409.1931.

11-Oxo-11-(2-oxo-2-(2-oxotetrahydrofuran-3-ylamino)ethylamino)undecyl nitrate (13). The preparation of the title compound involved the synthesis of 2-amino-*N*-(2-oxotetrahydrofuran-3-yl)acetamide **12** by coupling HSL **5** (0.5 g, 2.73 mmol) with *N*-Boc-glycine **11** (0.57 g, 3.28 mmol) in the presence of EDC (0.783 g, 4.10 mmol) and triethylamine (0.41 mL, 3.07 mmol) in DCM. The mixture was stirred at room temperature for 24 h and then evaporated in *vacuo* to dryness. The residue was partitioned between water (20 mL) and ethyl acetate (50 mL) and the organic layer successively washed with 5% sodium bicarbonate ($NaHCO_3$) solution (2 x 20 mL), 1M potassium hydrogen sulfate ($KHSO_4$) solution (1 x 20 mL) and brine. The organic layer was dried over sodium sulfate (Na_2SO_4) and evaporated to dryness to yield the HSL-Boc glycine derivative as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40 (s, 9H, 3 × CH_3), 2.20-2.28 (m, 1H, H4a), 2.59-2.68 (m, 1H, H4b), 3.81-3.83 (m, 2H, $COCH_2NH$) 4.20-4.29 (m, 1H, H5a) 4.41 (dt, *J* = 8.9, 1.1 Hz, H5b), 4.56-4.64 (m, 1H, H3), 5.59 (bs, 1H, NH), 7.35 (bs, 1H, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 28.2 (3 × CH_3) , 29.3 (CH_2), 48.8 (CH), 66.0 (CH_2), 156.2 (C=O), 170.6 (C=O), 175.5 (C=O). The product was treated with trifluoroacetic acid (5 mL) to remove the Boc group to give the desired product **12** as yellow oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.13-2.24 (m, 1H, H4a), 2.40-2.46 (m, 1H, H4b), 3.63 (s, 2H, $COCH_2NH$) 4.20-4.28 (m, 1H, H5a) 4.37 (dt, *J* = 1.5 and 8.7 Hz, H5b), 4.67-4.73 (m, 1H, H3), 8.11 (bs, 1H, NH), 8.94 (bd, *J* = 7.8, 1H, NH); ^{13}C NMR (75.6 MHz, DMSO-d_6) δ 28.7 (CH_2), 40.5 (CH_2), 48.6 (CH), 65.8 (CH_2), 166.6 (C=O), 175.2 (C=O).

The crude Boc deprotected compound **12** (0.19 g, 0.69 mmol) was then coupled with 11-(nitrate)undecanoic acid **6d** (0.17 g, 0.69 mmol) using PyBop (0.36 g, 0.69 mmol) and DIPEA (0.36 mL, 2.09 mmol) in DCM at room temperature for 18 h. The reaction mixture was concentrated in *vacuo*, diluted with ethyl acetate (50 mL), and washed with saturated sodium carbonate solution, 1 M sodium hydrogen sulfate solution and brine. The organic phase was dried over sodium sulfate, filtered and concentrated in *vacuo* to give the title compound **13** as an off white solid (0.26 g, 98%). M.p. 76-78 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.26-1.39 (m, 12H, 6 × CH_2), 1.48-1.52 (m, 2H, CH_2), 1.64-1.78 (m, 2H, CH_2), 2.12 (t, *J* = 7.3 Hz, 2H, $COCH_2$), 2.16-2.23 (m, 1H, H4a), 2.34-2.43 (m, 1H, H4b), 3.70 (d, *J* = 5.9 Hz, 2H, $COCH_2NH$), 4.18-4.240 (m, 1H, H5a), 4.35 (dt, *J* = 1.4 and 9.0 Hz, 1H, H5b), 4.52 (t, *J* = 6.6 Hz, 2H, CH_2ONO_2), 4.54-4.64 (m, 1H, H3), 8.06 (t, *J* = 5.8 Hz, NH), 8.33 (d, *J* = 8.0 Hz, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 25.3 (CH_2), 26.4 (CH_2), 28.6 (CH_2), 28.9 (CH_2), 29.1 (CH_2), 29.2 (CH_2), 35.6 (CH_2), 42.1 (CH_2), 48.3 (CH), 65.7 (CH_2), 74.3 (CH_2), 169.7 (C=O), 173.0 (C=O), 175.6 (C=O). IR (neat): ν_{max} 3284, 3078, 2922, 2852, 1775, 1661, 1643, 1546, 1465, 1416, 1375, 1353, 1277, 1175, 1017, 1000, 950, 858, 758, 697 cm^{-1}; HRMS (ESI) *m/z* Calcd. for $C_{17}H_{29}N_3O_7Na$ $(M + Na)^+$ 410.1903. Found 410.1881.

4-(Benzyloxy)-4-oxobutanoic acid (16a). Succinic anhydride (5 g, 50.0 mmol) was dissolved in anhydrous DCM (40 mL). Benzyl alcohol (5.69 mL, 55.0 mmol), triethylamine (7.50 mL, 55.0 mmol), and a catalytic amount of DMAP were added to this solution. The resulting clear solution was stirred at room temperature for 18h,

after which the, all the volatiles were removed under vacuum. The crude residue was taken up in diethyl ether (200 mL) and was extracted with 2N NaOH (2 × 75 mL). The aqueous extracts were carefully acidified to pH 2 with concentrated HCl and then extracted with diethyl ether (2 × 100 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated to give the title acid **16a** as a white solid (8.84 g, 85 %). M.p. 52-54 °C, lit.[44] 56-57 °C; ^{1}H NMR (300 MHz, acetone-d_6): δ 2.68-2.71 (m, 4H, 2 × CH_2), 5.14 (s, 2H, CH_2Ar), 7.34-7.36 (m, 5H, ArH).

6-(Benzyloxy)-6-oxohexanoic acid (16b). The title compound was synthesized following the procedure described for compound **16a** using adipic anhydride and benzyl alcohol, the resulting crude mixture was purified by washing to afford the title product **16b** as a white waxy oil (7.45, 81%).^{1}H NMR (300 MHz, $CDCl_3$): δ 1.65-1.70 (m, 4H, 2 × CH_2) 2.32-2.39 (m, 4H, 2 × CH_2), 5.11 (s, 2H, CH_2Ar), 7.31-7.36 (m, 5H, ArH); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 24.0 (CH_2), 24.3 (CH_2), 33.1 (CH_2), 33.8 (CH_2), 66.2 (CH_2), 128.2 (ArH), 128.5 (ArH), 173.1 (C=O), 178.7 (C=O).

4-Oxo-4-(2-oxotetrahydrofuran-3-ylamino)butanoic acid (18a). To a stirred solution of L-HSL **5** (0.5 g, 2.73 mmol) in DCM (10 mL), triethylamine (0.42 mL, 3.00 mmol) was added followed by the addition of acid **16a** (0.62 g, 3.00 mmol) and EDC (0.78 g, 4.10 mmol). The mixture was stirred at room temperature for 30h and then evaporated in *vacuo* to dryness. The residue was partitioned between water (20 mL) and ethyl acetate (50 mL) and the organic layer successively washed with 5% sodium bicarbonate ($NaHCO_3$) solution (2 x 20 mL), 1M potassium hydrogen sulfate ($KHSO_4$) solution (1 x 20 mL) and brine. The organic layer was dried over sodium sulfate and evaporated to dryness to yield the benzyl protected HSL derivative **17a** as a colorless oil (0.53 g, 66%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.05-2.20 (m, 1H, H4a), 2.53-2.58 (m, 2H, CH_2), 2.68-2.73 (m, 3H, CH_2 and H4b), 4.18-4.27 (m, 1H, H5a), 4.40 (dt, *J* = 9.0, 1.3 Hz, 1H, H5b), 4.49-4.53 (m, 1H, H3), 5.11 (s, 2H, CH_2Ar), 6.68 (bd, *J* = 6.3 Hz, 1H, NH), 7.32 (m, 5H, ArH). The compound's benzyl group was removed by hydrogenation using 10% Pd/C (0.20 g) and H_2 gas at atmospheric pressure in THF at reflux for 30h. The crude reaction mixture was filtered through a column of Celite and silica to remove Pd/C. The filtrate was evaporated to dryness to give the title compound **18a** as a white solid (0.30 g, 84%). M.p. 112-114 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.07-2.18 (m, 1H, H4a), 2.33-2.51 (m, 5H, 2 × CH_2 and H4b), 4.15-4.24 (m, 1H, H5a), 4.33 (dt, *J* = 8.7, 1.8 Hz, 1H, H5b), 4.49-4.58 (m, 1H, H3), 6.68 (bd, *J* = 7.9 Hz, 1H, NH), 12.09 (bs, COOH); ^{13}C NMR (75.6 MHz, DMSO-d_6) δ 28.7 (CH_2), 29.3 (CH_2), 30.1 (CH_2), 48.3 (CH), 65.7 (CH_2), 171.5 (C=O), 174.1 (C=O), 175.7 (C=O); IR (neat): ν_{max} 3352, 2920, 2519, 1771, 1715, 1611, 1556, 1442, 1427, 1384, 1353, 1277, 1238, 1179, 1131, 1103, 1022, 999, 952, 919, 845, 754, 699, 676 cm^{-1}; HRMS (ESI) *m/z* Calcd. for $C_8H_{11}NO_5Na$ (M + Na)$^+$ 224.0535. Found 224.0524; Anal. Calcd. for $C_8H_{11}NO_5$: C, 47.76; H, 5.51; N, 6.96. Found: C, 47.87; H, 5.49; N, 6.91.

6-Oxo-6-(2-oxotetrahydrofuran-3-ylamino)hexanoic acid (18b). The title compound was synthesized following the procedure for compound **18a** using acid **16b** (0.64 g; 3.00 mmol) first to synthesize protected acid **17b** (0.5 g, 58%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.67 (m, 4H, 2 × CH_2), 2.05-2.20 (m, 1H, H4a), 2.23-2.38 (m, 4H, 2 × CH_2), 2.75-2.78 (m, 1H, H4b), 4.18-4.31 (m, 1H, H5a), 4.43 (dt, *J* = 9.0, 1.1 Hz, 1H, H5b), 4.49-4.53 (m, 1H, H3), 5.10 (s, 2H, CH_2Ar), 6.26 (bd, *J* = 5.2 Hz, 1H, NH), 7.32 (m, 5H, ArH). The deprotection of compound **17b** gave the desired product **18b** as a white solid (0.27 g, 78%). M.p. 142-144 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.48-1.52 (m, 4H, 2 × CH_2), 2.05-2.19 (m, 5H, H4a and 2 × CH_2), 2.33-2.42 (m, 1H, H4b), 4.15-4.23 (m, 1H, H5a), 4.33 (dt, *J* = 8.8, 1.8 Hz, 1H, H5b), 4.47-4.56 (m, 1H, H3), 8.32 (bd, *J* = 7.9 Hz, 1H, NH). ^{13}C NMR (75.6 MHz, DMSO-d_6) δ 24.5 (CH_2), 25.1 (CH_2), 28.7 (CH_2), 33.9 (CH_2), 35.2 (CH_2), 48.2 (CH), 65.6 (CH_2), 172.4 (C=O), 174.8 (C=O), 175.8 (C=O); IR (neat): ν_{max} 3342, 3291, 2955, 2934, 2872, 1763, 1692, 1640, 1536, 1467, 1429, 1407, 1359, 1282, 1251, 1223, 1190, 1171, 1103, 1080, 1015, 999, 945, 920, 803, 734, 716 cm^{-1}; HRMS (ESI) *m/z* Calcd. for $C_{10}H_{15}NO_5Na$ (M + Na)$^+$ 252.0848. Found 252.0840; Anal. Calcd. for $C_{10}H_{15}NO_5$: C, 52.40; H, 6.60; N, 6.11. Found: C, 52.42; H, 6.73; N, 6.17.

***O*^{2}-Methyl-1-(4-(4-oxo-4-(2-oxotetrahydrofuran-3-ylamino)butanoyl)piperazin-1-yl)diazen-1-ium-1,2-diolate (21a).** To a solution of acid **18a** (0.15 g, 0.75 mmol) and diazeniumdiolate **19** (0.14 g, 0.90 mmol) in water/acetonitrile (5:1) (6 mL) EDC (0.21g, 1.12 mmol) was added. The reaction mixture was allowed to stir at r.t. for 40 h. The solvent was concentrated under *vacuo*, and extracted with EtOAc (2 × 50 mL). The organic layer was washed with brine (2 × 25 mL), dried over anhydrous Na_2SO_4 and evaporated to give a crude residue. Purification by vacuum chromatography using ethyl acetate/methanol (9.5:0.5) gave the title compound **21a** as a yellow oil (0.11 g, 43%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.12-2.26 (m, 1H, H4a), 2.57-2.62 (m, 2H, CH_2), 2.66-2.73 (m, 3H, CH_2 and H4b), 3.36-3.44 (m, 4H, 2 × CH_2), 3.65-3.78 (m, 4H, 2 × CH_2), 4.00 (s, 3H, OCH_3), 4.20-4.29 (m, 1H, H5a), 4.43 (dt, *J* = 9.0, 1.3 Hz, 1H, H5b), 4.49-4.54 (m, 1H, H3), 6.86 (bd, *J* = 6.6 Hz, 1H, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 28.1 (CH_2), 29.8 (CH_2), 30.7 (CH_2), 40.4 (CH_2), 43.9 (CH_2), 49.0 (CH), 51.1 (CH_2), 61.2 (OCH_3), 65.9 (CH_2), 170.3 (C=O), 172.7 (C=O), 175.2 (C=O); IR (neat): ν_{max} 3309, 2919, 2866, 1775, 1637, 1534, 1494, 1442, 1380, 1281, 1213, 1170, 1112, 1028, 946, 914, 813, 727 cm^{-1}; UV (MeOH): λ_{max} 244 nm (ε 5012 M^{-1}cm^{-1}); HRMS (ESI) *m/z* Calcd. for $C_{13}H_{21}N_5O_6Na$ (M + Na)$^+$ 366.1390. Found 366.1379.

***O*^{2}-Methyl-1-(4-(4-oxo-4-(2-oxotetrahydrofuran-3-ylamino)hexanoyl)piperazin-1-yl)diazen-1-ium-1,2-diolate (21b).** HSL acid **18b** (0.2 g, 0.87 mmol) was coupled with diazeniumdiolate **19** (0.15 g, 0.95 mmol) as described for compound **21a** to give the title compound **21b** as a yellow oil (0.11 g, 35%).^{1}H NMR (300 MHz, $CDCl_3$): δ 2.16-2.21 (m, 1H, H4a), 2.23-2.35 (m, 4H, 2 × CH_2), 2.64-2.73 (m, 1H, H4b), 3.34-3.40 (m, 4H, 2 × CH_2), 3.60-3.77 (m, 4H, 2 × CH_2), 4.00 (s, 3H, OCH_3), 4.20-4.29 (m, 1H, H5a), 4.43 (dt, *J* = 8.9, 1.4 Hz, 1H, H5b), 4.53-4.62 (m, 1H, H3), 6.94 (bd, *J* = 6.7 Hz, 1H, NH); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 24.2 (CH_2), 24.8 (CH_2), 29.7 (CH_2), 32.5 (CH_2), 35.5 (CH_2), 40.1 (CH_2), 43.9 (CH_2), 48.9 (CH), 51.2 (CH_2), 61.2

(OCH_3), 65.9 (CH_2), 171.3 (C=O), 173.3 (C=O), 175.4 (C=O); IR (neat): ν_{max} 3299, 2943, 2869, 2247, 1774, 1629, 1534, 1494, 1439, 1380, 1280, 1218, 1175, 1111, 1064, 1031, 947, 915, 727 cm^{-1}; UV (MeOH): λ_{max} 242 nm (ε 5420 $M^{-1}cm^{-1}$); HRMS (ESI) *m/z* Calcd. for $C_{15}H_{25}N_5O_6Na$ $(M + Na)^+$ 394.1703. Found 394.1693.

***O*²-(2,4-Dinitrophenyl)-1-(4-(4-oxo-4-(2-oxotetrahydrofuran-3-ylamino)butanoyl)piperazin-1-yl)diazen-1-ium-1,2-diolate (22a).** The title compound was synthesized following the procedure for compound **21a** using the HSL acid **18a** (0.1 g; 0.50 mmol) and diazeniumdiolate **20** (0.19 g, 0. 55 mmol) to give desired product **22a** as a yellow solid (0.17 g, 79%). M.p. 112-114 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.15-2.27 (m, 1H, H4a), 2.63-2.66 (m, 2H, CH_2), 2.69-2.76 (m, 3H, CH_2 and H4b), 3.36-3.87 (m, 8H, 4 × CH_2), 4.22-4.31 (m, 1H, H5a), 4.46 (dt, *J* = 9.0 and 1.2 Hz, 1H, H5b), 4.49-4.57 (m, 1H, H3), 6.61 (bd, *J* = 6.5 Hz, 1H, NH), 7.92 (d, *J* = 9.0 Hz, ArCH), 8.54 (dd, *J* = 9.0 and 1.2 Hz, ArCH), 8.87 (d, *J* = 1.2 Hz, ArCH); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 28.1 (CH_2), 29.8 (CH_2), 30.8 (CH_2), 40.2 (CH_2), 43.8 (CH_2), 49.1 (CH), 50.5 (CH_2), 60.4 (CH_2), 65.9 (CH_2), 117.8 (ArCH), 122.1 (ArCH), 129.1 (ArCH), 138.3 (ArC), 140.9 (ArC), 158.2 (ArC), 170.3 (C=O), 172.6 (C=O), 175.1 (C=O); IR (neat): ν_{max} 3313, 3053, 2916, 2857, 1780, 1674, 1605, 1527, 1472, 1447, 1343, 1264, 1239, 1209, 1158, 1114, 1068, 1012, 959, 909, 850, 835, 731, 699 cm^{-1}; UV (MeOH): λ_{max} 297 nm (ε 58484 $M^{-1}cm^{-1}$); HRMS (ESI) *m/z* Calcd. for $C_{18}H_{21}N_7O_{10}Na$ $(M + Na)^+$ 518.1248. Found 518.1240.

***O*²-(2,4-Dinitrophenyl)-1-(4-(4-oxo-4-(2-oxotetrahydrofuran-3-ylamino)hexanoyl)piperazin-1-yl)diazen-1-ium-1,2-diolate (22b).** The title compound was synthesized following the procedure for compound **21a** using the HSL acid **18b** (0.1 g, 0.43 mmol) and diazeniumdiolate **20** (0.16 g, 0.47 mmol) to give desired product **22b** as a yellow solid (0.16 g, 72%). M.p. 108-110 °C, ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.17-2.23 (m, 1H, H4a), 2.27-2.44 (m, 4H, 2 × CH_2), 2.64-2.73 (m, 1H, H4b), 3.58-3.65 (m, 4H, 2 × CH_2), 3.81-3.93 (m, 4H, 2 × CH_2), 4.20-4.29 (m, 1H, H5a), 4.43 (dt, *J* = 1.4 and 8.9 Hz, 1H, H5b), 4.53-4.62 (m, 1H, H3), 6.94 (bd, *J* = 6.7 Hz, 1H, NH), 7.68 (d, *J* = 9.0 Hz, ArCH), 8.47 (dd, *J* = 9.0 and 1.2 Hz, ArCH), 8.89 (d, *J* = 1.2 Hz, ArCH); ^{13}C NMR (75.6 MHz, $CDCl_3$) δ 22.7 (CH_2), 24.8 (CH_2), 29.8 (CH_2), 31.9 (CH_2), 35.5 (CH_2), 40.1 (CH_2), 43.9 (CH_2), 48.9 (CH), 51.2 (CH_2), 60.2 (CH_2), 65.9 (CH_2), 118.3 (ArCH), 122.3 (ArCH), 129.6 (ArCH), 138.1 (ArC), 141.2 (ArC), 157.7 (ArC), 171.3 (C=O), 173.3 (C=O), 181.6 (C=O); HRMS (ESI) *m/z* Calcd. for $C_{20}H_{25}N_7O_{10}Na$ $(M + Na)^+$ 546.1561. Found 546.1554.

Docking Study

The crystal structure of the QS protein LasR was retrieved from the protein data bank (PDB) (PDB code, 2UV0, resolution 1.8 Å).[36] Possible binding sites and poses of the compounds within QS receptor LasR were predicted by docking synthesised compounds into the LasR receptor using Ligand Docking (GOLD) (Cambridge Crystallography Data Centre, UK) in its implementation through the Discovery Studio (Accelrys) interface. Hydrogens were added to all ligands and the receptor prior to performing the docking runs. All ligands were also minimised 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 10, 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, and π-interactions of the ligands were analysed for the first pose with the highest Gold score.

QS Inhibition Assay

To assess the impact of the compounds on QS signalling, the *P. aeruginosa* P_{lasB}::*gfp*(ASV) reporter strain (MH602), which harbors a fusion of the *lasB* promoter to an unstable green fluorescent protein gene, *gfp*(ASV) and responds to the AHL 3-oxo-dodecanoyl homoserine lactone (OdDHL), was used. To the 96-well plate was added 160 µL of Luria Bertani (LB10) medium. A 3-fold serial dilution row of the pure compound was made, leaving the last well for control. Finally 40 µL of overnight culture of P_{lasB}::*gfp*(ASV) diluted 10 times in fresh LB10 medium was added to the wells. The GFP expression (fluorescence, excitation 485 nm, emission 535 nm) and cell growth (OD_{600}) were measured every 30 min at 37 °C by the use of a microplate reader (Wallac Victor, Perkin-Elmer).

Nitrite Measurement by the Griess Assay

The release of NO from compounds was assessed colorimetrically using the Griess assay. Compounds were diluted to 500 µM in phosphate-buffered saline (PBS), pH 7.4, supplemented with 500 µM L-cysteine (Sigma), 100 µM xanthine (Sigma), and 1 U/mL xanthine oxidase. 100 µL aliquots were added in triplicate to a microtiter plate, and incubated for 1 h at 37 °C with shaking at 180 rpm.[37] Then, 150 µL of Griess reagent (Molecular Probes-G7921) containing 0.07% sulphanilamide and 0.007% N-naphthylethylenediamine dihydrochloride in PBS were added to the wells. After incubation for 30 min at room temperature, the absorbance was measured at OD_{550} using a microplate reader (Wallac Victor, Perkin-Elmer).

Solutions of 0 to 100 µM sodium nitrite were used to prepare a standard curve of nitrite absorbance versus concentration under the same experimental conditions. The concentration of NO released (quantitated as nitrite ions) from the compounds was calculated from the standard curve.

Pyocyanin assay

Overnight *P. aeruginosa* cultures of PA14 were subcultured 1:1,000 into 5 mL fresh LB10 medium. AHL-NO hybrid compounds were assayed at 100 µM following 17 h of aerobic growth with shaking (200 rpm) at 37 °C. Cells were separated from culture fluids via centrifugation at 10,000 g for 15 min. Culture fluids were passed through 0.22 µm syringe-driven filters (Millipore). Cell-free culture fluids were analyzed for pyocyanin on a Perkin Bio100 spectrophotometer from 200 to 800 nm; 695 nm was chosen for graphical representation.

Elastase assay

Elastase activity was assayed as described previously by Ohman et al. 1980.[45] Briefly, overnight cultures of *P. aeruginosa* PA01 were subcultured 1:100 into 5 mL LB10 medium and incubated with AHL-NO hybrid at 200 µM for 6 h with shaking (200 rpm) at 37 °C. One milliliter of the culture fluid was centrifuged and 100 µl of supernatant was incubated with 900 µl of Tris-HCl buffer (pH ~7.0), and 20 mg of elastin-Congo Red at 37 °C for 3 h. The reaction was terminated by adding 350 µl of 0.7 M sodium phosphate buffer (pH 6.0). The insoluble elastin-Congo Red was removed by centrifugation and the absorbance of the supernatant was measured at 490 nm to determine the elastase activity.

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