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Lynngbyoic acid, a “tagged” fatty acid from a marine cyanobacterium, disrupts quorum sensing in *Pseudomonas aeruginosa*†

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Abstract

Quorum sensing (QS) is a mechanism of bacterial gene regulation in response to increases in population density. Perhaps most studied are QS pathways mediated by acylhomoserine lactones (AHLs) in Gram-negative bacteria. Production of small molecule QS signals, their accumulation within a diffusion-limited environment and their binding to a LuxR-type receptor trigger QS-controlled gene regulatory cascades. In *Pseudomonas aeruginosa*, for example, binding of AHLs to their cognate receptors (LasR, RhIR) controls production of virulence factors, pigments, antibiotics and other behaviors important for its interactions with eukaryotic hosts and other bacteria. We have previously shown that marine cyanobacteria produce QS-inhibitory molecules, including 8-*epi*-malyngamide C (**1**), malyngamide C (**2**) and malyngolide (**3**). Here we isolated a new small cyclopropane-containing fatty acid, lynngbyoic acid (**4**), as a major metabolite of the marine cyanobacterium, *Lynngbya* cf. *majuscula*, collected at various sites in Florida. We screened **4** against four reporters based on different AHL receptors (LuxR, AhyR, TraR and LasR) and found that **4** most strongly affected LasR. We also show that **4** reduces pyocyanin and elastase (LasB) both on the protein and transcript level in wild-type *P. aeruginosa*, and that **4** directly inhibits LasB enzymatic activity. Conversely, dodecanoic acid (**9**) increased pyocyanin and LasB, demonstrating that the fused cyclopropane “tag” is functionally relevant and potentially confers resistance to α -oxidation. Global transcriptional effects of **4** in some ways replicate the gene expression changes of *P. aeruginosa* during chronic lung infections of cystic fibrosis patients, with reduced *lasR* signaling, increased biofilm and expression of the virulence locus HSI-I. Compound **4** may therefore prove to be a useful tool in the study of *P. aeruginosa* adaption during such chronic infections.

†Electronic Supplementary Information (ESI) available: Figures S1–S4, Scheme S1, Tables S1–S4, Supplementary References and NMR Spectra for **4**. See DOI: 10.1039/b000000x/

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Introduction

Quorum sensing (QS) is a mechanism by which bacteria regulate their behavior in response to increases in their population density. Within a diffusion-limited environment, the local concentration of small molecule cues increases with bacterial population, acting to upregulate virulence genes in opportune situations (i.e., when the population of bacteria is large enough to overwhelm host defenses).¹ QS pathways, therefore, are an attractive target for antimicrobial defense. In the marine environment, QS also contributes to the formation of biofilms by bacteria, the first step in the process of colonization of abiotic surfaces (biofouling).²

In Gram-negative bacteria, acylhomoserine lactones (AHLs, Figure 1a) of varying alkyl chain lengths and oxidation states at C-3 are used in quorum signaling.³ AHLs with short side chains diffuse freely across cell membranes and can bind intracellularly with receptor proteins (R proteins), which typically act as transcriptional activators of target genes.⁴ One of these targets is generally the gene responsible for the synthesis of the signaling molecule itself. In this way QS is a positive feedback loop, and QS signaling molecules are sometimes referred to as “autoinducers”.⁵

Bacterial infections and the increase in antibiotic-resistant pathogens are an ever escalating problem. Such infections complicate and prolong hospital stays and increase the cost to individuals and society.⁶ Antibiotics exert strong selective pressure on bacteria, and consequently, resistance is strongly associated with increased antibiotic use.⁷ Agents that are not bacteriocidal, but instead modulate harmful bacterial behavior, could perhaps exert less selective pressure for resistance. It has been suggested that this may be a strategy used by marine organisms that lack cellular immune systems,⁸ due to the fact that the ecologically relevant concentrations of natural antibiotics are sometimes in the sub-lethal range.

One prime target for QS-based therapy is the opportunistic pathogen *Pseudomonas aeruginosa*. This organism is a particular problem in cystic fibrosis, where it can persistently establish itself in chronic lung infections.⁹ Persistence is largely due to the formation of antibiotic-resistant biofilms, a phenotype which is modulated by QS. *P. aeruginosa* can also cause serious eye infections in wearers of contact lenses,⁹ with extensive tissue damage mediated by the QS-controlled proteolytic enzymes, including the elastase LasB.⁹

P. aeruginosa has multiple QS pathways, mediated through two AHLs (C₄-HSL[‡] and 3-oxo-C₁₂-HSL)⁵ and a group of quinolone compounds (the *Pseudomonas* quinolone signaling pathway, PQS).¹⁰ The interplay of the two AHL pathways is shown in Figure 1b. One important feature of these is that the RhIR-RhII system is subordinate to the LasR-LasI system,¹¹ as expression of both RhIR and C₄-HSL synthesis (via RhII) is regulated by LasR/3-oxo-C₁₂-HSL (Figure 1a). Therefore both C₄-HSL and 3-oxo-C₁₂-HSL are required for expression of RhIR target genes. The expression of LasR is not under the control of AHL-mediated signaling,^{12,13} and it therefore represents an upstream target for QS inhibition in *P. aeruginosa*. Interplay of AHL signaling with the quinolone pathway is complex. While on the one hand, quinolone signaling is thought to be dependent on LasR-LasI,¹⁰ this pathway has been shown to act independently under some circumstances.¹⁴

[‡]Abbreviations used: CAD, Collisionally Activated Decomposition; CE, Collision Energy; COSY, Correlation Spectroscopy; CUR, CURtain gas; CXP, Collision-cell eXit Potential; DP, Declustering Potential; EP, Entrance Potential; ESIMS, ElectroSpray Ionization Mass Spectrometry; GS1, Gas 1; GS2, Gas 2; HMBC, Heteronuclear Multiple-Bond Correlation spectroscopy; HMQC, Heteronuclear Multiple-Quantum Correlation spectroscopy; HRESI/APCIMS, High Resolution ElectroSpray Ionization/Atmospheric Pressure Chemical Ionization Mass Spectrometry (dual probe); HRMS, High Resolution Mass Spectrometry; HSL, HomoSerine Lactone; HSQC, Heteronuclear Single-Quantum Correlation spectroscopy; IS, IonSpray voltage; MRM, Multiple Reaction Monitoring; ROESY, Rotating frame nuclear Overhauser Effect Spectroscopy; RT-qPCR, Real-time quantitative Polymerase Chain Reaction after Reverse Transcription; TEM, TEMperature

For some time, we have been involved in the search for novel and bioactive secondary metabolites. Such natural products have historically been a major source of pharmaceuticals, or have provided inspiration to medicinal chemists.¹⁵ Toxic compounds that are presumably used for chemical defense in competitive environments have found use as antibiotics or anticancer drugs.^{15,16} AHL signaling pathways are widely used among gram negative bacteria, and thus QS modulation by small molecules may be an effective strategy to alter competitor behavior in complex communities.¹⁷ Such small molecules could prove useful as drug candidates or tool compounds.

There is increasing evidence that various marine organisms can interfere with bacterial quorum sensing. Several halogenated furanones that inhibit AHL signaling have been isolated from the marine red alga *Delisea pulchra*.¹⁸ It has been shown that these compounds do not compete with AHLs but instead accelerate turnover of the LuxR protein.¹⁹ LuxR is the master regulator of three QS pathways in many luminescent *Vibrio* spp., and so furanones block all AHL signaling in these organisms.²⁰ Recently, tumonoic acids E–H from the cyanobacterium *Blennothrix cantharidosmum* were shown to reduce luminescence in wild-type *Vibrio harveyi*, although the mechanism of action was not determined.²¹ In a screen of 284 extracts of marine organisms, 23% were found to exhibit quorum sensing antagonism in a LuxR-based reporter.²² We have recently identified three other compounds that inhibit AHL-mediated quorum sensing in reporter systems, 8-*epi*-malyngamide C (**1**),²³ malyngamide C (**2**)²³ and malyngolide (**3**)²⁴ (Figure 1c). Therefore, it would seem that QS modulation may be a fairly widespread phenomenon amongst marine organisms for pathogen defense or for maintenance of bacterial symbionts. In addition, there is increasing evidence that interspecies crosstalk of quorum sensing is widespread,¹⁷ as are interactions between infection hosts and pathogenic QS pathways.²⁵ From a biomedical perspective, natural QS modulators may prove useful in the treatment and prevention of infection and be complementary to natural antibiotics, which have long provided benefit in the treatment of infections (e.g., penicillins, macrolides and glycopeptides).

In the present work we describe the isolation and structure determination of a simple cyclopropane-containing fatty acid from a marine cyanobacterium, termed lyngbyoic acid (**4**, Figure 2). Because the compound was produced in relatively large amounts, and considering its small size, we hypothesized that it could be a signaling molecule. In view of the importance of QS to biofouling in the marine environment, we screened **4** against several AHL-responsive reporter constructs and found robust inhibitory activity against the 3-oxo-C₁₂-HSL-responsive reporter pSB1075. Further investigations using pSB1075 and related control plasmids aimed to 1) determine the role of the AHL-binding site of LasR to the inhibitory activity, and 2) to determine preliminary structure-activity relationships using several compounds with structures related to **4**. Subsequently, we investigated the inhibitory effect of **4** in the wild-type Gram-negative bacterium that expresses LasR, *P. aeruginosa*. Although this organism may not be an ecologically relevant target for the cyanobacterium, it is widespread in the environment,²⁶ including the sea²⁷ and it is a pathogen with an extremely broad host range, which includes protozoa, plants and humans.²⁸

Results and discussion

Isolation and structure determination

Samples of *Lyngbya* cf. *majuscula* were collected near Fort Pierce, Florida, in the Indian River Lagoon. Fractionation of the non-polar extract of this material yielded **4** (1.32% of extracted lipophilic material, Figure 2). Samples of *Lyngbya* cf. *majuscula* collected within the Dry Tortugas National Park, Florida, extracted and fractionated in a similar manner, afforded **4** (0.03% of extracted lipophilic material), malyngolide (**3**, Figure 1b) (0.007%) and lyngbic acid (**5**, Figure 2) (0.11%).

NMR data combined with a $[M - H]^-$ peak of 211.1702 in the HRESI/APCIMS of **4** suggested a molecular formula of $C_{13}H_{24}O_2$. Perusal of the 1H NMR spectrum revealed the presence of a carboxylic acid exchangeable proton (broad peak at δ_H 10.18) and a cyclopropane ring (shielded signals at δ_H 0.45 and 0.21). Analysis of 1D and 2D NMR data allowed the construction of the planar structure (Table 1). The relative configuration of the cyclopropane ring was assigned *trans*, as the H-5 methylene protons are magnetically equivalent due to “pseudo C_2 symmetry”, as in grenadamide (**6**, Figure 2), grenadadiene (**7**)²⁹ and majusculoic acid (**8**).³⁰ The absolute configuration could be determined as (4*R*,6*R*) from the optical rotation, which was equal magnitude and opposite sign to that of the synthetic enantiomer.³¹ Although **4** is essentially the fatty acid side chain of **6** and **7** it has not been previously reported as the free acid. Compounds **6** and **7** were previously shown to be moderately cytotoxic to brine shrimp and human cancer cell lines, respectively, and **6** binds to cannabinoid receptors.²⁹ Compound **8** was found to have some antifungal activity,³⁰ but no quorum sensing-related activities for **6–8** have been reported.

Initial AHL quorum sensing reporter screening

Compound **4** was found in relatively large amounts, similar to the tumonoic acids.³² Natural quorum sensing inhibitors generally exhibit IC_{50} s only in the micromolar range (including tumonoic acids,²¹ halogenated furanones¹⁸ and manoalides²²), and consequently they have to be present in high concentrations in the native organism. Interestingly, in addition to the main collection that produced **4** only, in another collection of *Lyngbya cf. majuscula* from Dry Tortugas, we co-isolated **4** with two other QS inhibitors, malyngolide²⁴ and lyngbic acid (**3** and **5**, respectively) in smaller amounts. With this in mind, and also with the consideration that **4** somewhat resembled some natural AHL disrupters, we screened it against three reporter plasmids in *E. coli* (pSB401, pSB536 and pSB1075, Figure 3, Table S1, Supporting Information).³³ Each plasmid encodes different R-proteins (that respond to different AHLs) and contains its cognate binding site within the QS-regulated promoter, cloned upstream of a promoterless light-producing *luxCDABE* cassette. In each, the R-protein is under the control of its native promoter.³³ Additionally, we tested **4** against an *Agrobacterium tumefaciens lacZ*-based reporter³⁵ that responds to 3-oxo-C₈-HSL.

Reporter strains were treated with compound **4** both in the presence and absence of the cognate AHL signaling molecule (Figure 3a and 3b), in order to detect antagonism or agonism of AHL signaling, respectively. Compound **4** antagonized 3-oxo-C₁₂-HSL mediated light production through LasR (pSB1075) with an apparent IC_{50} of approximately 100 μ M, and to a much lesser extent in the other two reporters (Figure 3a). Interestingly, **4** also reduced the baseline luminescence in all three reporters in the absence of cognate AHL (Figure 3b), perhaps indicating either an inverse-agonist type activity or an effect on expression of the R-protein, the *luxCDABE* cassette, or both. Compound **4** was not able to antagonize the production of blue pigment in the *A. tumefaciens* reporter in the presence of 3-oxo-C₈-HSL (see Figure S1, Supporting Information).

Investigation of dependence on the LasR AHL-binding site

To test whether the AHL-binding site was required for inhibition, we used a reporter (pTIM5319) that lacks this domain, but in other respects is identical to pSB1075³⁴ (Figure 4). Compound **4** reduced baseline luminescence in this reporter (Figure 5a), indicating that neither the cognate AHL, nor the AHL-binding domain of the AHL receptor is required. Additionally, we found that by varying the concentration of 3-oxo-C₁₂-HSL, **4** is not competitive with this ligand in pSB1075 (see Figure 5b). However, in a reporter that lacks the transcriptional repressor *rsaL* (pTIM505 pTIM5211), which in the PAO1 genome resides in the region between *lasR* and *lasI*, 3-oxo-C₁₂-HSL is clearly able to compete with **4** (Figure 5b). Taken together, these results suggest that the effects of **4** are exerted both

through the AHL-binding domain of LasR, and independently of it. The repressor *rsaL* is potentially implicated in the latter. To exclude a general effect on bacterial physiology or on the activity of the *lux* reporter cassette, we tested **4** on a reporter where the *lux* operon is under control of the constitutively active phage promoter (pTIM2442, Table S1, Supporting Information), and found no effect (Figure S2, Supporting Information).

Investigation of related compounds in pSB1075

We compared the effect of **4** on pSB1075 to that of compounds of related structure, such as dodecanoic acid (**9**) and the cyanobacterial metabolite lyngbic acid (**5**, see Figures 2 and 5c), which was co-produced with **4** by one of the *Lyngbya* cf. *majuscula* samples we investigated. The most closely related dodecanoic acid (**9**) exerted an inhibitory effect of similar magnitude to **4**, as did the previously identified quorum-sensing inhibitor malyngolide (**3**).²⁴ Similar to **4**, **9** was found to inhibit the AHL-binding deficient reporter pTIM5319 (Figure 5a), indicating that this site is non-essential for inhibition in both cases. We recently disclosed that 8-*epi*-malyngamide C (**1**) weakly inhibited pSB1075.²³ It can now be seen that compound **4** is more potent than **1**. Interestingly, both **1** and its free side chain, lyngbic acid (**5**), have similar potency. Considering that the fatty acids **4** and **9** inhibit stimulation of pSB1075 by 3-oxo-C₁₂-HSL, it could be that the ring-opened form of malyngolide is the active species. The methyl ester of dodecanoic acid (**10**) had only a small inhibitory effect, and butyric acid (**11**) was completely inactive, indicating a preference for free acids and longer alkyl chains, respectively (Figure 5c).

Effects of lyngbyoic acid (**4**) and related compounds in wild-type *P. aeruginosa* and PAO-JP2

To assess whether **4** is able to inhibit a native quorum-sensing system, we treated wild-type *P. aeruginosa* cultures with **4** (1 mM) in a preliminary experiment measuring secreted pigments (assessed by UV absorption of culture supernatants at 360 nm), and LasB (assessed by degradation of elastin Congo red, see Figures 6a and 6b). It was observed that **4** was able to lower pigment production by the 6 h and 20 h timepoints, and that lower LasB activity levels were observed at 6 h only. Therefore the 6 h timepoint was used for subsequent experiments. To exclude an effect on the viability of PAO1, cultures were grown in the presence and absence of 1 mM **4**. No effect on growth, as assessed by OD₆₀₀, was observed (see Figure S3, Supporting Information). Because fatty acids can inhibit proteases,³⁶ we tested compound **4** for direct inhibition of purified LasB. Indeed, we found that **4** inhibited LasB with a K_i of 5.4 μM (see Figure S4, Supporting Information). However, we did not detect **4** by LC-MS in the filtered supernatants. Therefore, elastase activity in supernatants is a true reflection of expression and there is no direct inhibition of secreted enzyme by **4**. Since **4** is quite lipophilic it may be sequestered within cells, membranes, or membrane vesicles, or else it is degraded by cellular enzymes.

To assess potential differences between our reporter system and *P. aeruginosa*, we tested the complete set of compounds in PAO1 (see Figures 6c and 6d). Through extraction of pyocyanin from supernatants according to a published procedure,³⁷ and measurement of LasB activity, it could be seen that compound **4** reduced both pyocyanin and LasB by the greatest extent. The most striking contrast with results in reporter assays came from the effects of dodecanoic acid (**9**) in PAO1. This compound greatly increased pyocyanin compared to control, and LasB to a lesser extent (see Figure 6c). Intriguingly, this effect was not replicated in the *lasI rhII* mutant PAO-JP2,³⁸ indicating that it is dependent on either *lasI* or *rhII* genes, or their downstream targets. Plausibly, dodecanoic acid could act as a substrate for ω -oxidation pathways to produce the 3-oxo acid, which if bound to an acyl-carrier protein (ACP), is one of the substrates for LasI.³⁹ This would suggest that the cyclopropane of **4** precludes ω -oxidation at the 3-position and does not allow it to be utilized

by LasI. We therefore describe **4** as “tagged”, as the cyclopropane may allow the compound to persist in both the producing cyanobacterium and target organisms, by avoiding metabolism through α -oxidation. Gene expression studies by RT-qPCR revealed the effects on virulence factors due to compound treatments was largely duplicated in the transcript levels of *lasB* (PA3724) and *phzG1* (PA4216), a member of the pyocyanin biosynthetic operon (see Figure 6d).⁴⁰

Global gene expression analysis of lyngbyic acid (**4**)-treated *P. aeruginosa*

Some aspects of the reporter studies suggested that **4** may have effects on gene expression independent of AHL signaling. We investigated the effects of **4** on the transcriptome of PAO1 through microarray analysis, which revealed extensive gene expression changes (at 1.8-fold change with $p < 0.01$: 969 genes upregulated, 887 genes downregulated, see Scheme S1, Supporting Information). Importantly, comparison of microarray data revealed a high overlap with two landmark transcriptome studies of LasR-LasI and RhlR-RhlI controlled genes (Figure 7).^{12,13} This included downregulation of pyocyanin synthesis (*phzM*, *phzA1-G1*, *phzS* [PA4209–18], *phzA2-G2* [PA1899–1905] and *phzH* [PA0051]), secreted enzymes (*lasA* [PA1871], *lasB* [PA3724], *chiC* [PA2300] and *aprA* [PA1249]) and rhamnolipid production (*rhlA* and *B*, PA3479 and PA3480). Also, the *pqsABCDE* and *phnAB* operons (PA0996–PA1002), responsible for synthesis of QS quinolone signal molecules,¹⁰ were significantly decreased (–3.4 to –11.9-fold). These operons have previously been shown to be under the control of the Las system,^{12,13} but treatment with **4** did not affect the expression level of *lasR* (PA1430), *lasI* (PA1432), *rhlR* (PA3477) or *rhlI* (PA3476). Interestingly, expression of hydrogen cyanide production genes (*hcnA-C*, PA2193–5) was unaffected, even though they were previously identified as QS-controlled,^{12,13} and were downregulated by a previously described QS inhibitor.⁴¹

In addition to effects on quorum sensing, lyngbyic acid (**4**) reduced the expression of 36 genes previously identified as induced by iron-starvation (see Table S2, Supporting Information).⁴² For example the expression of some genes for the biosynthesis of pyoverdine⁴³ (*pvdA*, *D*, *I* and *J* [PA2386, PA2399 and PA2400/2401, respectively]), a fluorescent siderophore, were reduced (–2.9 to –4.5-fold) along with the outer membrane pyoverdine receptor *fvpA* (PA2398, –2.2-fold) and the regulatory σ -factor *pvdS* (PA2426, –4.7-fold). Genes for the biosynthesis of pyochelin (*pchABCDG*, PA4228–4231, –2.6 to –3.5-fold), another siderophore, were also reduced. Effects on both iron-regulated and QS-regulated genes potentially implicate an involvement of the regulator *vqsR*.^{44,45} A *vqsR* mutant was shown to have decreased expression of genes related to quorum sensing, as well as pyoverdine and pyochelin.⁴⁵

Transcript levels of some other regulators that have previously been implicated in quorum sensing were also affected in a complex manner. *rpoS* (PA3622),⁴⁶ *rsmA* (PA0905)⁴⁷ and *qscR* (PA1898)⁴⁸ were all decreased (–4.3, –2.4 and –2.0-fold, respectively), whereas *rpoN* (PA4462),⁴⁹ *mvfR* (PA1003)⁵⁰ and *pmpR* (PA0964)⁵¹ were all increased (+2.2, +1.8 and +2.8-fold, respectively). The downstream QS effects of **4** could perhaps in part depend on alteration of the balance of positive and negative regulators, as well as posttranscriptional/posttranslational effects. It is possible that **4** similarly modulates transcriptional regulators in *E. coli*, potentially accounting for the inhibitory effects seen in reporter systems that were independent of the *lasR* AHL-binding site (see Figures 5a and 5b). The effects in both systems could be due to regulators conserved between the two species, such as the GasS/A system, which is found in many Gram-negative bacteria.⁵²

The effect of **4** on biofilm genes was also complex. While some members of the *psl* operon (*pslA* and *B* [PA2231–2232] and *pslN* [PA2244]) were downregulated (–1.9 to –3.4-fold), the entire *pelABCDEFGHI* operon (PA3058–64) was upregulated (+2.0 to +7.2-fold). The *pel*

operon is required for the synthesis of a glucose-rich matrix exopolysaccharide that is an important component of biofilms, and its expression has been shown to be dependent on *lasI*.⁵³ Therefore, it would seem that in lyngbyoic acid (**4**)-treated cells, expression of *pel* is uncoupled from general QS effects, which are inhibited. Recently, it has been shown that *pel* genes are repressed by the tyrosine phosphatase TpbA (PA3885).⁵⁴ Lyngbyoic acid (**4**) paradoxically increased the expression of *tpbA* by 3.3 fold, perhaps suggesting a posttranscriptional or direct effect on the protein.

Compound **4** also increased the expression of a type VI secretion virulence locus, HSI-I⁵⁵ (PA0071–91, +1.8 to +5.7-fold). This locus expresses a secreted protein, Hcp1, along with its secretion apparatus. Hcp1 has been detected in CF patients that harbor chronic *P. aeruginosa* infections, and the expression of HSI-I is antagonistically regulated by RetS (repression) and LadS (activation). These two regulators also control exopolysaccharide production, are implicated in the control of virulence factor expression in acute (RetS) and chronic (LadS) infections,⁵⁵ and modulate the activity of the GasS/A pathway, that controls expression of QS post-transcriptionally.⁵⁶

We found that **4** caused extensive gene expression changes in *P. aeruginosa*. Similarly, using a more stringent cutoff (2.0-fold change) 437 genes were found by Son et al.⁵⁷ to be induced *in vivo* using *P. aeruginosa* isolates from CF patients, including many involved in general metabolism. This was attributed to nutrient sources within CF lungs, including lipid surfactants (e.g., phosphatidylcholine) and amino acids. As in Son et al.,⁵⁷ **4** affected many genes involved in central metabolism, for example the operons of the fatty acid sensors PsrA⁵⁸ and DesT⁵⁹ were upregulated and downregulated, respectively (see Scheme S1, Supporting Information). In another example, several genes involved in choline degradation (PA3933, *betA*, *betB*, *betI* and *betT* [PA5372–5375]), which were all upregulated by **4** (+9.6 to +18.1-fold) and also previously shown to be upregulated by phosphatidylcholine.⁵⁷ Importantly, although many of the gene expression changes could be as a result of involvement of **4** in central metabolism pathways, the quorum sensing effects are dependent on the cyclopropane moiety as evidenced by the QS-stimulatory effects seen after treatment with **9**.

The characterization of **4** may have biomedical significance. After initial infection of cystic fibrosis patients, *P. aeruginosa* adapts to the CF lung environment, acquiring a phenotype characterized by reduced quorum sensing, overproduction of exopolysaccharide (mucoid phenotype), and reduced motility.⁶⁰ This is often accompanied by a loss of *lasR*.⁶⁰ Compound **4** mimics many of these effects, including a general inhibition of quorum sensing and expression of the virulence determinant HSI-I in wild-type *P. aeruginosa*. It may therefore prove a valuable tool compound for modeling the process of adaption in CF, perhaps by replicating the response of *P. aeruginosa* to certain fatty acids present in CF sputum. In *P. aeruginosa*, quorum sensing circuits are known to cross-talk with environmental pressures independent of cell density, for example stringent starvation⁶¹ and membrane fluidity.⁶² The observed differential physiological and molecular responses to structurally related simple fatty acids suggest a major role of fatty acids to switch-on or switch-off certain pathways and adjust to environmental conditions.

Experimental

General experimental procedures

Optical rotation was measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on SpectraMax M5 (Molecular Devices). ¹H, ¹³C, and 2D NMR spectra were recorded in CDCl₃ on a Bruker Avance 500 MHz using residual solvent signals (¹H 7.26, ¹³C 77.0) as internal standards. HSQC experiments were optimized for ¹J_{C,H} = 145 Hz, and

HMBC experiments were optimized for $^nJ_{C,H} = 8$ Hz. HRMS data were obtained using an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector. Luminescence assays and OD₆₀₀ measurements were read on a Perkin-Elmer Victor3 microtiter plate reader. UV spectra of culture supernatants and elastase activity assays were read on a SpectraMax M5 (Molecular Devices). LC-MS data were obtained using a 3200 Q Trap LC/MS/MS system (Applied Biosystems). Real-time quantitative PCR experiments were carried out on an Applied Biosystems 7300 instrument. C₄-HSL and 3-oxo-C₆-HSL were obtained from Sigma Aldrich, 3-oxo-C₁₂-HSL was supplied by Cayman Biochemicals.

Extraction and isolation

Samples of *Lyngbya cf. majuscula* were collected in the Indian River Lagoon (IRL), near Fort Pierce (27°26.668 N, 80°18.095 W) on June 23, 2006. This was a recollection of the sample designated IRL1. A voucher sample is maintained at the Smithsonian Marine Station, Fort Pierce, FL. The freeze-dried material was extracted with EtOAc–MeOH (1:1) to furnish a crude non-polar extract, which was subsequently partitioned between 80% aqueous MeOH and hexanes. The H₂O–MeOH fraction was further partitioned between *n*-BuOH and H₂O. The *n*-BuOH extract (1.69 g) was subjected to silica gel chromatography, eluting fractions with increasing proportions of *i*-PrOH in CH₂Cl₂. The fraction eluting with 6% *i*-PrOH in CH₂Cl₂ was further purified by semi-preparative reversed-phase HPLC (Phenomenex Ultracarb 5u ODS column, 250 × 10 mm, 2.0 mL/min; UV detection at 220 and 240 nm) using a MeOH–H₂O linear gradient (60–100% MeOH over 50 minutes and then 100% MeOH for 20 min) to furnish compound **4**, *t*_R 49.0 min (42.4 mg). The yield was 1.32% of lipophilic material (the *n*-BuOH and hexanes fractions, excluding the H₂O fraction).

Samples of *Lyngbya cf. majuscula* were collected at Garden Key, within the Dry Tortugas National Park, FL on April 22, 2007. A voucher sample is maintained at the Smithsonian Marine Station, Fort Pierce, FL (DRTO0000003). The freeze-dried material was extracted with EtOAc–MeOH (1:1) and then subjected to solvent-solvent partitioning as with the IRL material. The *n*-BuOH extract (669 mg) was subjected to silica gel chromatography, eluting fractions with increasing proportions of *i*-PrOH in CH₂Cl₂. The fraction eluting with 10% *i*-PrOH in CH₂Cl₂ showed evidence by ¹H NMR of the presence of **3**, **4** and **5**, and so was further purified by semi-preparative reversed-phase HPLC (Phenomenex Synergi Hydro column, 250 × 10 mm, 2.0 mL/min; UV detection at 220 and 240 nm) using an ACN–0.1% HCOOH linear gradient (40–100% ACN over 20 min then 100% ACN for 30 min) to furnish compounds **3**, *t*_R 25.5 min (0.3 mg), **4**, *t*_R 26.5 (1.3 mg) and **5**, *t*_R 24.2 min (4.4 mg). Adjacent silica fractions also show the oxygenated methylene doublets (¹H 3.71 and 3.47) of malynolid (3) and the distinctive upfield cyclopropane (¹H 0.45 and 0.21) signals of **4** and so the total yield of these compounds is expected to be greater.

Lyngbyoic acid (4): Colorless oil; [α]_D²⁰ –15.5 (*c* 0.1, CHCl₃); UV (EtOH) λ_{max} (log ϵ) 202 (2.41), 230 (1.95), 260 (1.56); IR (film) λ_{max} 3400–2400 (br), 3061, 2923, 2854, 1710, 1541, 1456, 1414, 1283, 1213, 1120, 1079, 1021, 936, 772, and 722 cm⁻¹; ¹H NMR, ¹³C NMR, APT, COSY, HSQC and HMBC data, see Table 1; HRESI/APCIMS *m/z* [M – H]⁻ 211.1702 (calcd. for C₁₃H₂₃O₂, 211.1698).

Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are listed in Table S1, Supporting Information. Reporter strains were grown overnight in Luria-Bertani (LB) medium at 37 °C with agitation as previously described.⁶³ Briefly, overnight cultures were grown in the presence of the appropriate antibiotic (Table S1). The following day, cultures were diluted

100-fold with fresh LB and antibiotic, and incubated for 1 h, then diluted 100-fold again and incubated for a further 2 h. Cultures were diluted 10-fold with fresh LB and the appropriate antibiotic was added before cultures were used in assays. *P. aeruginosa* strain PAO1 was also grown using the same protocol, without added antibiotic.

luxCDABE-Based reporter assays—Test compounds and/or cognate AHL where appropriate were added to black 96-well plates, and the solvent was allowed to evaporate at room temperature. 100 μ L cultures of the appropriate reporter were added to each well, and the plates were incubated at 37 °C in a humid environment for 6.5 h before their luminescence was recorded. For each assay, untreated wells (+/- AHL where appropriate) were used as controls. The final concentrations used were 10 μ M (C₄-HSL), 10 μ M (3-oxo-C₆-HSL) and 1 nM (3-oxo-C₁₂-HSL), except for the 3-oxo-C₁₂-HSL competition experiment (see Figure 5b). These concentrations corresponded to the experimentally determined IC₅₀ of the AHLs against the relevant reporter strains under the same conditions as the assays.

A. tumefaciens reporter assay—The *A. tumefaciens lacZ*-based reporter was grown overnight in LB in the presence of gentamicin at 30 °C with shaking. The culture was diluted 100-fold then grown for a further 24 hours in M9 medium supplemented with sucrose (0.2% w/v), subcultured for 4 h and then mixed 1:1 with M9 sucrose containing 1.12% agar and immediately 100 μ L of this mixture was added to each well of a 96-well plate containing compound **4** with or without 3-oxo-C₈-HSL. The final concentration of 3-oxo-C₈-HSL used was 1 nM, a concentration found to produce ~half maximal blue coloration by visual inspection. Plates were incubated in a humidified atmosphere at 30 °C and then visually inspected for blue coloration.

Pigment and elastase production in *P. aeruginosa*

Cultures (1 mL) of strain PAO1 were grown in 15 mm diameter glass tubes with shaking at 37 °C in the presence of 1 mM test compounds, added directly to the cultures as 10 μ L of 100 mM stocks (in EtOH), for 6 h. Negative controls consisted of PAO1 cultures with 10 μ L EtOH added, while positive controls were PAO-JP2 cultures with 10 μ L EtOH added. To obtain an estimate of the pigment production, cultures were spun down and the absorbance of the supernatant at 360 nm was measured, corresponding to one of the UV maxima reported for pyocyanin.⁶⁴ The supernatants were then passed through a 0.2 μ m filter. Following the procedure of Müh et al.³⁷ 100 μ L of each supernatant was added to 900 μ L of a 5 mg/mL suspension of elastin Congo red (ECR, Elastin Products Company, Inc.).⁶⁵ The mixtures were incubated in 15 mm plastic tubes at 37 °C with shaking for 18 h, at which point the reaction was stopped by addition of 100 μ L 0.12 M EDTA. Soluble reaction product was quantified by UV absorption of the supernatants at 495 nm after centrifugation. Pyocyanin was quantified according to the procedure of Müh et al.,³⁷ with some differences. A portion (500 μ L) of the filtered culture supernatants was extracted with 500 μ L CHCl₃ in an Eppendorf tube. The CHCl₃, which took on a visible blue color in cultures with high levels of pyocyanin, was transferred to a new tube and back-extracted with 150 μ L of 0.2 N HCl. Under acidic conditions the UV spectrum of pyocyanin changes and solutions take on a visible red color in high-pyocyanin samples.⁶⁴ A portion (100 μ L) of the aqueous layer was transferred to a 384-well plate, and the absorbance at 385 nm was measured. The UV maximum at 385 nm has a higher than the maximum at 520 nm⁶⁴ used elsewhere,³⁷ and thus is more suitable for small-scale cultures.

LC-MS of PAO1 culture supernatants

A portion (1 μ L) of supernatants from the elastase activity assay were subjected to an LC separation step followed by MS detection (Phenomenex Luna 5u C₈ column, 4.6 \times 50 mm,

0.5 mL/min; detection by ESIMS, MRM scan in negative mode) using an isocratic solvent system (90:10 MeOH–H₂O, both with 0.1% HCOOH). Samples were compared to a standard solution of **4** at the expected concentration, and spiked control cultures. Compound **4** was detected in spiked control cultures containing non-denatured excreted LasB and other proteins, and thus specific and non-specific protein binding did not significantly affect detection. Authentic lyngbyoic acid (**4**) eluted at t_R 6.3 min. The MS parameters were as follows: MRM ion pair 211–193, DP –66, EP –6.3, CEP –12, CE –25, CXP –3.4, CUR 30, CAD Medium, IS –4500, TEM 500, GS1 50, GS2 60.

***In vitro* inhibition of LasB**—The *in vitro* inhibition of LasB (Elastin Products Company, Inc.) was assessed using BODIPY-FL casein (Invitrogen). 2 μ L stock solutions of lyngbyoic acid (**4**) were added to a mixture of 1 μ L LasB (10 μ g/ml), 20 μ L H₂O and 77 μ L assay buffer (10 mM Tris-HCl, pH 7.8), and incubated at 37 °C for 30 min. 100 μ L BODIPY-FL was then added (10 μ g/mL), and the reaction was followed by fluorescence ($\lambda_{ex}/\lambda_{em}$ 505/589 nm). EDTA (10 mM in H₂O), a zinc chelating compound known to inhibit LasB, was used as a positive control. Similar results were seen using ECR as the substrate.

The K_i of lyngbyoic acid (**4**) was determined according to the protocol recommended by Copeland.⁶⁶ First, the K_M of the substrate BODIPY-FL casein was determined by measuring the slope of reactions in the presence of different substrate concentrations. Reaction mixtures consisted of 189 μ L 10 mM Tris-HCl, pH 7.8, 1 μ L of 10 μ g/mL LasB, and 10 μ L substrate solution. Initial slope was plotted against substrate concentration and the substrate concentration that gave half-maximal rate (the K_M) was calculated by non-linear curve fitting in Graphpad to be 20 μ g/mL. The IC_{50} of lyngbyoic acid (**4**) at this substrate concentration was then determined under the same conditions to be 4.3 μ M. The K_i was then determined by running reactions in the presence of different substrate concentrations (10 \times , 5 \times , 2.5 \times , 1.25 \times , 0.625 \times , 0.3125 \times and 0.1563 \times K_M) and different inhibitor concentrations (0, 1 μ M, 3.16 μ M and 10 μ M). Best fit was obtained by assuming a noncompetitive inhibition model in Graphpad, and the K_i was calculated to be 5.4 μ M.

Transcriptome analysis

Cultures (1 mL) of PAO1 were grown either in the presence or absence of 1 mM **4** (added as 10 μ L of a 100 mM stock solution in EtOH), for 6 h at 37 °C with shaking in 15 mm diameter glass tubes. Parallel cultures of each condition were grown alongside, and after ~5.5 h these were spun down and the UV absorbance of their supernatants was measured to confirm differential pyocyanin expression. RNA was stabilized *in vivo* by use of RNAprotect bacteria reagent (Qiagen) according to the manufacturer's instructions. RNA was extracted using the RNeasy Kit (Qiagen) according to the manufacturer's instructions.

RNA samples were quantified by UV absorbance (Nanodrop 8000, Thermo), and DNA contamination was quantified by RT-qPCR of the samples using a primer/probe set for *rpsL* (PA4268, see Table S3, Supporting Information). To reduce DNA contamination, Turbo DNA-free (Ambion) was used according to the manufacturer's "stringent" treatment protocol. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) and samples were reverse transcribed, fragmented and labeled according to Affymetrix's protocol for prokaryotic target preparation.⁶⁷ Samples were hybridized with rotation at 50 °C for 16 h to the Affymetrix GeneChip® *P. aeruginosa* Genome Arrays. The arrays were washed and stained with the reagents supplied in GeneChip® Hybridization Wash and Stain Kit (Affymetrix, Inc.) on an Affymetrix Fluidics Statino 450, and scanned with a GeneChip® 7G Scanner (Affymetrix, Inc.). Statistical tests were performed using Bioconductor statistical software (<http://www.bioconductor.org>) and the R program (R: A language and environment for statistical computing).⁶⁸ Raw data were normalized by

Robust Multichip Analysis (RMA) approach.⁶⁹ Then the probe set's detection call was estimated using the Wilcoxon signed rank-based algorithm. Probe sets that were absent in all the study samples were removed from further analysis. A linear modeling approach and empirical Bayes statistics as implemented in the limma package⁷⁰ in the R software were employed for differential expression analysis. The *p*-values obtained were controlled for multiple testing (FDR, false discovery rate) using the Benjamini-Hochberg method.⁷¹ Differentially expressed genes were then ranked by their *p*-values, and genes with *p*-value less than 0.01 and with greater than or equal to 1.8-fold change were considered as differentially expressed genes at a statistically significant level.

The microarray data were validated by realtime quantitative PCR using probes for *lasB*, *phzG1*, *retS*, *fadA5* and *lasR* (vide infra). Comparison of real-time PCR and microarray data is shown in Table S4, Supporting Information. The global transcriptome data have been deposited in NCBI's Gene Expression Omnibus⁷² and are accessible through GEO Series accession number GSE22999.

Reverse transcription and real-time quantitative PCR

RNA for use in RT-qPCR experiments was extracted and treated with DNase as for the GeneChip[®] samples. Samples were reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and random primers (Invitrogen). TaqMan primers/probes were custom designed by Applied Biosystems, using FAM as the fluorescent reporter and NFQ as the quencher. The sequences of primers and probes used is shown in Table S3, Supporting Information, and in all experiments the housekeeping gene *ipoD* (PA0576) was used as the endogenous control, as previously it has been found to have very stable expression, suitable for its use as a control in RT-qPCR experiments.⁷³ Additionally, this gene was found to not be affected by lnyngbyoic acid (**4**) in the microarray experiment. Real-time PCR was performed by using 12.5 μ L of TaqMan 2 \times gene expression master mix (Applied Biosystems), 1.25 μ L of 20 \times TaqMan gene expression assay mix (see Table S3, Supporting Information), 0.5 μ L of cDNA and 10.75 μ L of sterile water, in a total volume of 25 μ L per well reaction in a 96-well plate (Applied Biosystems). The thermocycler program consisted of 2 min at 50 $^{\circ}$ C, 10 min at 95 $^{\circ}$ C, and 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min. Each assay was carried out in triplicate.

Conclusions

We have described a new cyclopropane-containing fatty acid that we termed lnyngbyoic acid (**4**). This compound was found to inhibit the response of LasR-based QS reporter plasmids to 3-oxo-C₁₂-HSL. The AHL-binding site of LasR was not essential to this effect, but competition experiments indicated that **4** likely has a dual mechanism, acting both through the AHL-binding site and independently of it. Comparison of **4** with related compounds revealed a structure–activity relationship. While dodecanoic acid (**9**) had a similar potency in pSB1075 compared to **4**, either esterification (**10**) or shortening of the alkyl chain (**11**) reduced activity.

In an organism that possesses native quorum sensing circuitry, *P. aeruginosa*, we found **4** to reduce downstream pigment and elastase production. This was reflected by reduced expression of genes required for the biosynthesis of the pigment pyocyanin and the elastase LasB. Additionally, we found that **4** is able to directly inhibit purified LasB. Strikingly, despite minimal structural differences, dodecanoic acid (**9**) had opposite effects in *P. aeruginosa*, increasing pyocyanin and LasB on the transcript level. This indicates the functional relevance of the “tag” in **4**.

Global gene expression analysis revealed that **4** downregulates the majority of genes previously identified as controlled by quorum sensing. In addition, differential expression of known QS regulators was noted, as well as complex effects on biofilm genes. Compound **4** also had various effects on central metabolism and upregulated the CF virulence locus HSI-I.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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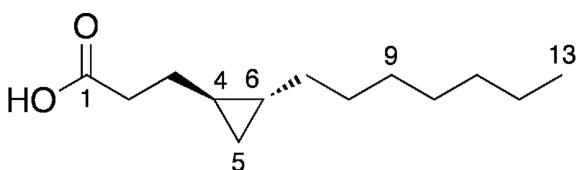
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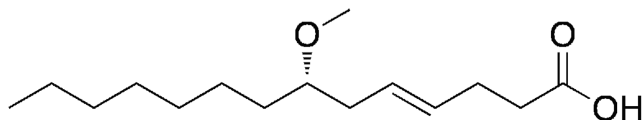
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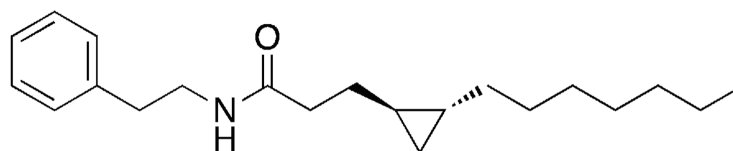
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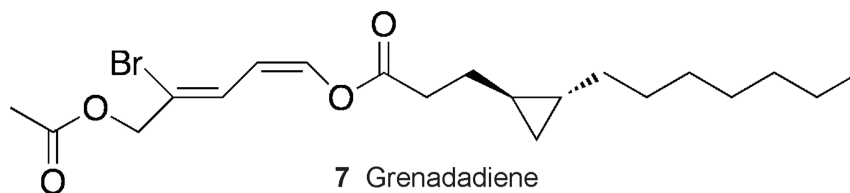
4 Lyngbyoic acid



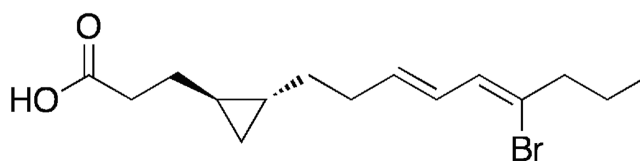
5 Lyngbic acid



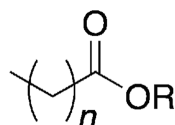
6 Grenadamide



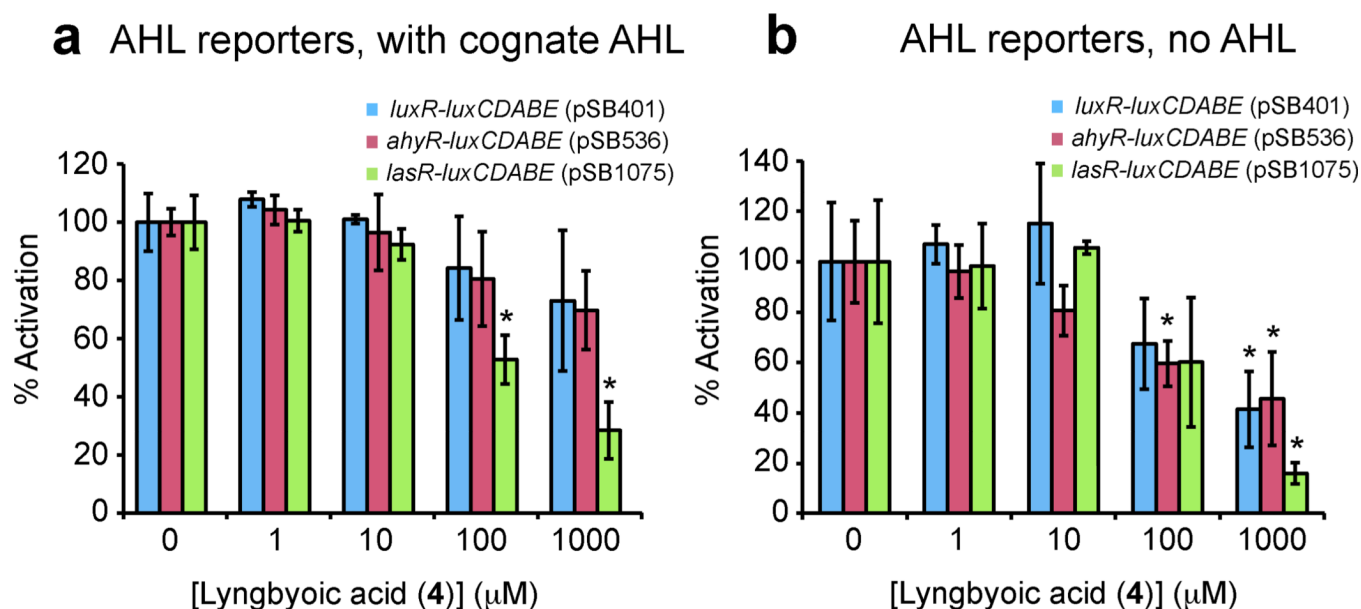
7 Grenadiene



8 Majusculoic acid

9 R = H, $n = 10$ Dodecanoic acid10 R = Me, $n = 10$ Methyl dodecanoate11 R = H, $n = 2$ Butanoic acid**Figure 2.**

Structures of lyngbyoic acid (**4**), lyngbic acid (**5**), grenadamide (**6**), grenadiene (**7**), majusculoic acid (**8**), dodecanoic acid (**9**), methyl dodecanoate (**10**) and butanoic acid (**11**). Note that the depicted absolute configurations for **7** and **8** are arbitrary because only relative configurations have been determined.

**Figure 3.**

Determination of inhibitory activity of compound **4** in *luxCDABE* reporter constructs expressed in *E. coli* (see Table S1, Supporting Information). (*) Indicates downregulation with statistical significance of $P < 0.05$ (t -test), compared with untreated controls, and error bars depict standard deviation. a) Effect of lyngbyic acid (**4**) on luminescence of the reporter strains pSB401, pSB536 and pSB1075 in the presence of 3-oxo-C₆-HSL, C₄-HSL and 3-oxo-C₁₂-HSL, respectively. Compound **4** and the appropriate cognate AHL were added to a 96-well plate and the solvent was allowed to dry off before 100 μL cultures of the appropriate reporter strain were added to each well. Plates were incubated for 6.5 h at 37 °C before fluorescence was measured. Results are expressed as % activation compared to control wells treated with cognate AHL alone (100%). Compound **4** was able to inhibit 3-oxo-C₁₂-HSL mediated luminescence in pSB1075 and to a lesser extent in the other reporters. b) Effect of lyngbyic acid (**4**) on background luminescence, in the absence of cognate AHL. The same protocol as for panel a) was used, except that cognate AHLs were not added to the plate. Results are expressed as % activation compared to untreated control (100). Compound **4** reduced background luminescence in all reporters.

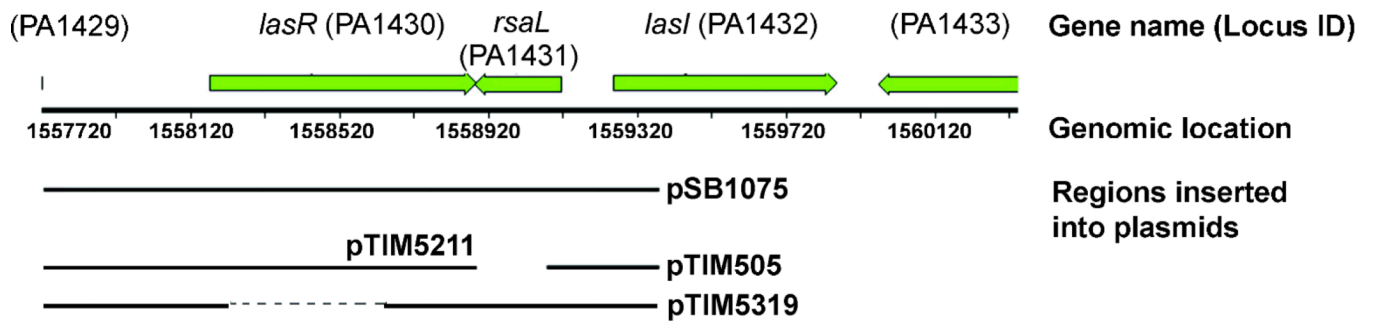
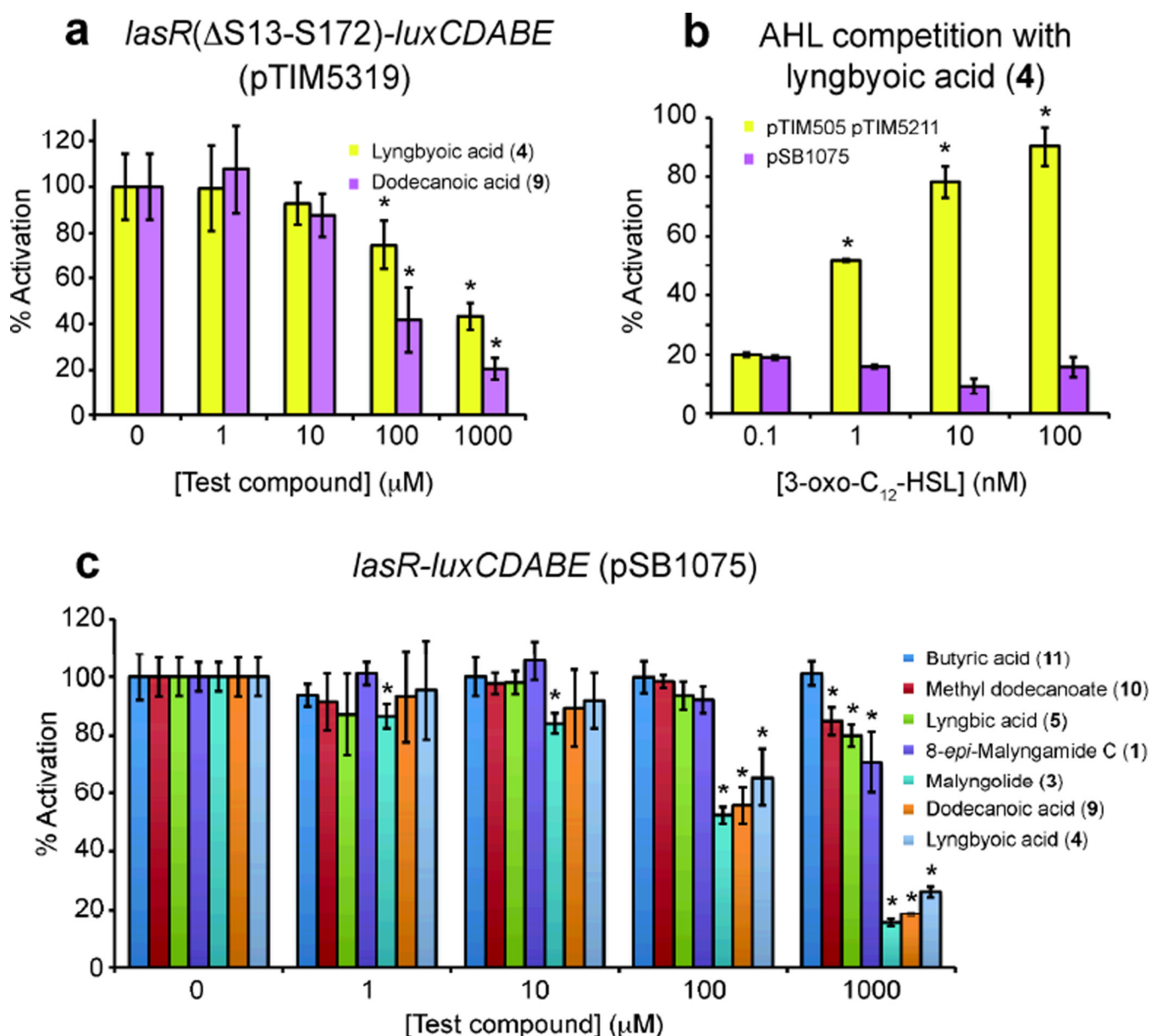
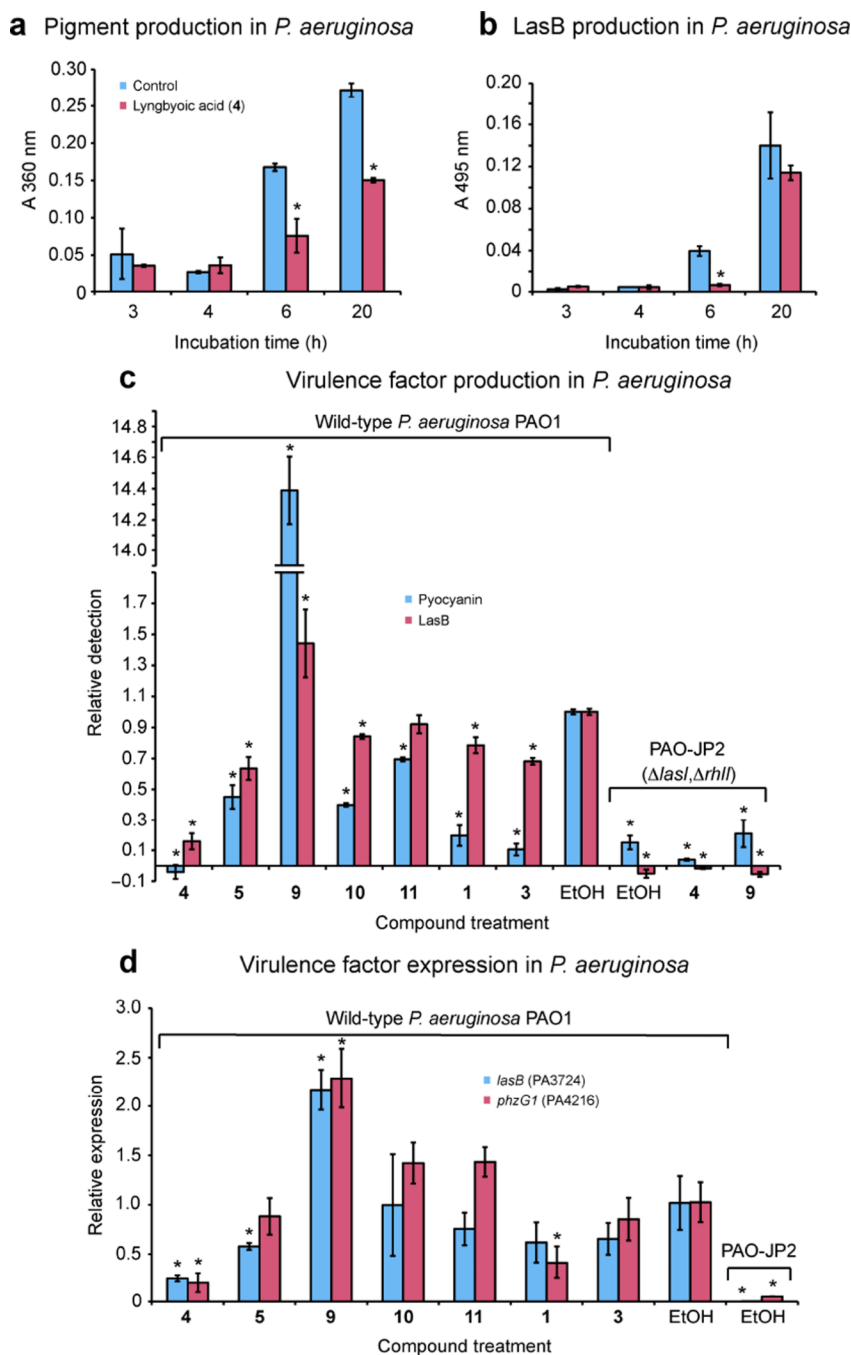


Figure 4.

Schematic of selected reporter plasmids and controls used (see Table S1, Supporting Information, and text). Regions cloned upstream of the promoterless *luxCDABE* cassette are indicated by a thick line. pTIM5319 carries a truncated *lasR* in which the AHL binding pocket (S13-S172, indicated by a thin dashed line) was removed using primers GCGTGGCGATGGGCCGACAGTG and GCGTTCCAGCTCAAGAAAACCGTC; AAACCGGTGGTTCTGACCAGCCGG and CACTAACGTCCCAGCCTTTGCGCTC, as described in Rajamani et al., 2008.³⁴

**Figure 5.**

Probing of the mechanism of inhibition by **4** by use of *luxCDABE* reporter constructs, and comparison of **4** with compounds of related structure. (*) Indicates downregulation with statistical significance of $P < 0.05$ (*t*-test), compared with untreated controls, and error bars depict standard deviation. a) Effect of lyngbyoic acid (**4**) and dodecanoic acid (**9**) on a reporter that lacks a functional AHL-binding domain (pTIM5319).³⁴ Cultures of the reporter were grown in 96-well plates in the presence of varying concentrations of **4** or **9**. Results are expressed as % activation compared to untreated control (set to 100%). b) 3-oxo-C₁₂-HSL is able to compete with **4** (1 mM) in a reporter strain lacking *rsaL* (pTIM505 5211), but not in pSB1075. c) Effect of lyngbyoic acid (**4**) and other compounds on luminescence in pSB1075.

**Figure 6.**

Effect of lyngbyoic acid (**4**) and other compounds on wild-type *P. aeruginosa* and the *lasI rhlI* mutant PAO-JP2. In all graphs (*) indicates downregulation with statistical significance of $P < 0.05$ (t -test) compared to untreated controls. a) Lyngbyoic acid (**4**) is able to reduce pigment production by PAO1. Cultures (500 μ L containing 1 mM **4**) were incubated at 37 $^{\circ}$ C with shaking for the appropriate time before they were spun down and the absorbance of the supernatant at 360 nm was measured. b) Lyngbyoic acid (**4**) is able to reduce LasB production in PAO1. A portion of supernatants (50 μ L) was incubated for 5 h at 37 $^{\circ}$ C with shaking in the presence of elastin Congo red (200 μ L of a 12.5 mg/mL

suspension in 30 mM Tris-HCl, pH 7.2), then spun down and the absorbance at 495 nm was measured. c) Effect of lyngbyoic acid (**4**) and other compounds (1 mM) on pyocyanin and LasB production in PAO1 and PAO-JP2. Cultures (1 mL) were grown in the presence of test compounds or EtOH for 6 h at 37 °C with shaking, before being spun down. A portion (100 µL) of each supernatant was added to 900 µL 5 mg/mL elastin Congo red and incubated at 37 °C with shaking for 18 h, then the mixtures were spun down and the absorbance of the supernatant was measured at 495 nm to measure LasB activity. Another portion (500 µL) of culture supernatants was extracted with 500 µL CHCl₃ and then back-extracted with 0.2 N HCl. The absorbance of this acidic aqueous layer was measured at 385 nm to quantify pyocyanin production. d) Effect of lyngbyoic acid (**4**) and other compounds on the gene expression of *lasB* and *phzG1* as assessed by RT-qPCR. Cultures were grown under the same conditions as in c).

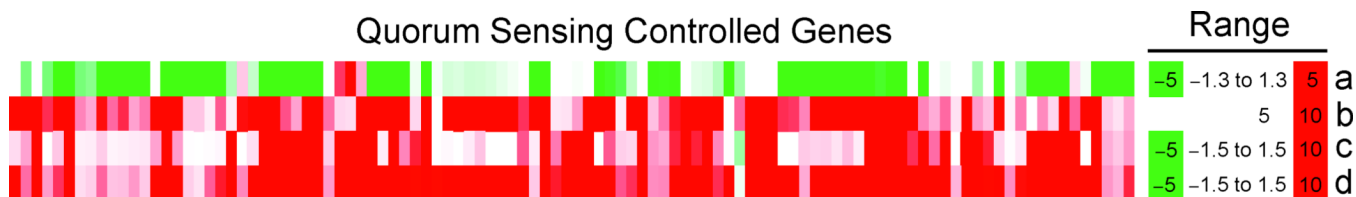


Figure 7.

Comparison of lyngbyoic acid (**4**) induced changes in gene expression (a) with previous studies of quorum-sensing controlled genes, Wagner et al.¹³ (b) and Schuster et al.¹² (c and d). a) Cultures of PAO1 were treated with 1 mM **4** for 6 h at 37 °C with shaking. b) PAO-JP2 treated with exogenous 3-oxo-C₁₂-HSL and C₄-HSL. c) PAO-MW1 treated with 3-oxo-C₁₂-HSL. d) PAO-MW1 treated with 3-oxo-C₁₂-HSL and C₄-HSL.

Table 1

NMR data for lyngbyoic acid (**4**) in CDCl₃ (500 MHz)

C/H No.	C, mult. ^a	ν (J in Hz)	COSY	HMBC ^b
OH		10.18, br		
1	180.7, s			
2	34.5, t	2.42, t (7.5)	H-3a, H-3b	1, 3, 4
3a	29.6, t	1.56, m	H ₂ -2, H-4	1, 2, 4, 6
3b		1.52, m	H ₂ -2, H-4	1, 2, 4, 5, 6
4	18.3, d	0.45, m	H ₂ -5, H-3a, H-3b	5, <i>c</i> <i>7c</i>
5	12.0, t	0.21, m (2H)	H-4, H-6	3, 4, 6, 7
6	19.2, d	0.45, m	H ₂ -5, H-7a, H-7b	5, <i>c</i> <i>7c</i>
7a	34.3, t	1.21, m	H-6, H-7b, H ₂ -8	4, 5, 6, 8
7b		1.13, m	H-6, H-7a, H ₂ -8	4, 5, 6, 8
8	29.8, t	1.33, m	H-7a, H-7b, H ₂ -9	6, 7
9	29.7, <i>d</i> _t	1.25, m	H ₂ -8	7
10	29.5, <i>d</i> _t	1.25, m		12
11	32.1, t	1.25, m		12
12	22.2, t	1.27, m	H ₂ -11, H ₃ -13	11, 13
13	14.0, q	0.88, t (7.0)	H ₂ -12	11, 12

^aMultiplicity is derived from APT and HSQC spectra.^bProtons showing long-range correlation to indicated carbon.^cIt could not be distinguished which proton shows HMBC correlations to C-5 and C-7.^dAssignment of these carbons is interchangeable.