Quorum Sensing in Gram-Negative Bacteria: Small-Molecule Modulation of AHL and AI-2 Quorum Sensing Pathways

Warren R. J. D. Galloway,[†] James T. Hodgkinson,[†,‡] Steven D. Bowden,[‡] Martin Welch,[‡] and David R. Spring*,[†]

Department of Chemistry and Department of Biochemistry, University of Cambridge, Cambridge, CB2 1EW U.K.

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Contents

1. Introduction 28
  1.1. Advantages of the Chemical Biology Approach for the Study of Quorum Sensing Pathways 30
  1.2. Important Considerations When Comparing the Results of Different Studies 31
2. AHL-Based Quorum Sensing in Gram-Negative Bacteria 31
  2.1. Basics of the Quorum Sensing Circuit: LuxI/LuxR-Type Systems 31
  2.2. Modulating LuxI/LuxR-Type Quorum Sensing 33
    2.2.1. Targeting the Synthase 33
    2.2.2. Targeting the Signal 33
    2.2.3. Targeting the LuxR-Type Receptor 34
    2.2.4. Small Molecules Based around the AHL Scaffold 34
    2.2.5. Geske's Overview of the Use of AHL Analogues to Modulate Quorum Sensing 34
    2.2.6. Investigating Species Selectivity 36
    2.2.7. Additional AHL Analogue Studies 37
    2.2.8. Superagonists 40
  2.3. Beyond the AHL Framework 40
    2.3.1. Furanones and Related Structural Analogues 41
    2.3.2. Mechanism of Action of Furanone-Type Inhibitors 44
    2.3.3. Other (Nonfuranone) Quorum Sensing Modulators 45
    2.3.4. Natural Substances 45
    2.3.5. Synthetic Compounds 46
    2.3.6. Recognized Drugs 49
3. AI-2: Interspecies Communication 50
  3.1. AI-2 System: Synthesis of the Signal 51
  3.2. AI-2 System: Signal Detection and Gene Transcription 51
4. Small-Molecule Modulation of AI-2 Quorum Sensing Systems 51
  4.1. Targeting AI-2 Synthesis 51
    4.1.1. Modulating LuxS Activity—Substrate and Intermediate Analogues 52
    4.1.2. Modulating LuxS Activity—Other Structural Classes of Compounds 54
    4.1.3. Modulating LuxS Activity—MTAN Inhibition 55
  4.2. Targeting AI-2 Receptors 55
    4.2.1. Non-native Agonists 56
    4.2.2. Non-native Antagonists—DPD Analogues 58
    4.2.3. Non-native Antagonists—Mimicking the Borate Moiety 59
    4.2.4. Non-native Antagonists—Miscellaneous 60
  4.3. Modulating LuxR Activity 63
  4.4. Concluding Comments on AI-2 Signaling 63
5. Possible Allosteric Effects of Small-Molecule Modulators of AHL and AI-2-Based Quorum Sensing 63
6. General Concluding Remarks and Future Perspectives 63
7. Abbreviations 64
8. Acknowledgments 64
9. References 64

1. Introduction

Numerous species of bacteria employ a mechanism of intercellular communication known as quorum sensing. This signaling process allows the cells comprising a bacterial colony to coordinate their gene expression in a cell-density dependent manner.[1−3] Quorum sensing is mediated by small diffusible molecules termed autoinducers that are synthesized intracellularly (throughout the growth of the bacteria) and released into the surrounding milieu. As the number of cells in a bacterial colony increases, so does the extracellular concentration of the autoinducer. Once a threshold concentration is reached (at which point the population is considered to be "quorate"), productive binding of the autoinducer to cognate receptors within the bacterial cells occurs, triggering a signal transduction cascade that results in population-wide changes in gene expression.[4−6] Thus, quorum sensing enables the cells within a bacterial colony to act cooperatively, facilitating population-dependent adaptive behavior.[6]

Quorum sensing has been shown to play a critical role in both pathogenic and symbiotic bacteria−host interactions.[5] In symbionts, significant quorum sensing phenotypes include bioluminescence and root nodulation.[7−11] Several clinically relevant pathogens use quorum sensing systems to regulate processes associated with virulence; this enhances the survival prospects of the bacteria because a coordinated attack on the host is only made when the bacterial population reaches a high population density, increasing the likelihood that the hosts defenses will be successfully overwhelmed.[12,13] For example, in *Pseudomonas aeruginosa*, quorum sensing is involved in the formation of biofilms and their tolerance to antimicrobial agents[14−17] and the innate host immune

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* To whom correspondence should be addressed. Tel.: +44 (0)1223 336498. Fax: +44 (0)1223 336362. E-mail: spring@ch.cam.ac.uk.
[†] Department of Chemistry.
[‡] Department of Biochemistry.

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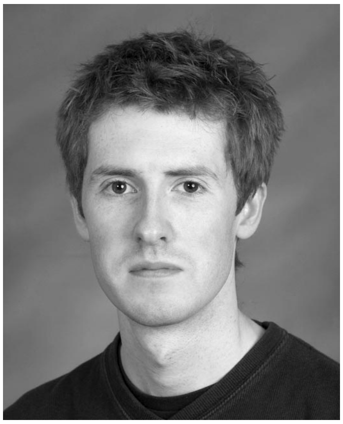


Warren Galloway was born in Dundee, Scotland, and attended the University of Cambridge for his undergraduate chemistry degree. He stayed at Cambridge for his Ph.D. studies under the supervision of Dr. David Spring, where he worked on the development of strategies for diversity-oriented synthesis. Warren received his Ph.D. in 2008 and is currently a postdoctoral research assistant for Dr. David Spring, working on the design and synthesis of small-molecule modulators of biological processes.

James Hodgkinson was born in Belfast, N. Ireland, and grew up in Killyleagh, County Down. He obtained his B.Sc. first class honours in Chemistry from Queens University Belfast in 2007. This course included a year working for GlaxoSmithKline based at Stevenage, England. James is currently working at the chemical/biological interface in the area of Quorum Sensing in the Spring and Welch groups as part of an Interdisciplinary Ph.D. funded by the Medical Research Council U.K.

Steven Bowden grew up in Hereford and later studied biochemistry at the University of Birmingham. In 2002 he began a Ph.D. at the University of Cambridge in the laboratory of Professor George Salmond to study the regulation of carbapenem antibiotic production in the plant pathogenic bacterium, *Pectobacterium carotovorum*. Upon finishing his Ph.D. in 2006, he joined the laboratory of Professor Jay Hinton and Dr. Arthur Thompson at the Institute of Food Research to study *Salmonella typhimurium* metabolism during infection of murine macrophages. This work demonstrated that glucose metabolism and glycolysis are essential metabolic pathways for Salmonella to infect macrophages and mice. In 2009 he joined the laboratory of Dr. Martin Welch, where he currently works on the stringent response of *Pectobacterium atrosepticum* to assess its potential role in virulence.

Martin Welch was raised near Newmarket and went on to study Biochemistry at Oxford University. In 1989 he started a Ph.D. at the Weizmann Institute of Science, where he worked on bacterial chemotaxis. In 1994 he took up a long-term EMBO fellowship and moved to Toulouse, where he solved the X-ray crystal structure of one of the bacterial chemotaxis proteins. In 1997 he returned to the U.K., where he carried out studies on bacterial virulence and quorum sensing. After a brief stint as a Royal Society University Research Fellow, he took up a University lectureship at Cambridge. His research interests lie in understanding how bacterial lifestyle (e.g., growth as biofilm or planktonic culture) and interaction with the environment influences microbial physiology.

system.[15,18−2021] This bacterium is one of the most prevalant pathogens in a range of life-threatening nosocomial infections and is a leading cause of mortality in cystic fibrosis (CF) sufferers.[21,22] Furthermore, in many bacterial species the production and secretion of virulence factors is regulated by quorum sensing.[11,23]

Given the diverse range of processes regulated by quorum sensing systems and the widespread effects these have upon human health and agriculture, it is unsurprising that the field has attracted significant interest in recent years.[12] Worthy of particular note is the possibility that quorum sensing systems may offer novel therapeutic targets for the treatment of a variety of bacterial infections.[16,24] Existing antibiotics generally inhibit bacterial cellular processes that are essential for microbial survival, thus stimulating bacterial evolution by creating a selective pressure for drug-resistant mutations.[25−27] Although quorum sensing systems are used by many bacterial pathogens to regulate virulence, they are not essential for survival.[21] Thus, disruption of quorum sensing (so-called "quorum quenching") should attenuate pathogenicity without imposing the level of selective pressure associated with antibacterial treatments.[16,28,29] Indeed, there is proof-of-concept from animal studies that the virulence of the Gram-negative bacterium *Pseudomonas aeruginosa* can be partially attenuated in vivo by the inhibition of quorum sensing (see section 2.3.1).[16,30]

From a chemist's perspective, the reliance of quorum sensing systems upon a language of small-molecule autoinducers offers the opportunity to investigate quorum sensing systems at the molecular level.[28,31] Thus, the discovery of non-native or unnatural small molecules that can modulate quorum sensing systems has attracted considerable interest.[32] Such compounds hold significant value as chemical probes for fundamental studies on this intercellular signaling pro-

David Spring is currently an EPSRC Advanced Fellow at the University of Cambridge Chemistry Department. Previous to this appointment, he spent two and a half years as a Wellcome Trust Postdoctoral Fellow and Fulbright Scholar at Harvard University with Professor Stuart L. Schreiber. He gained his D.Phil. for work on the proposed biosynthesis of the manzamine alkaloids at Oxford University under the supervision of Professor Sir Jack E. Baldwin FRS. David's research program is focused on diversity-oriented synthesis and the use of small molecules to probe biological processes.

cess,[12] which should facilitate our understanding of quorum sensing in general and consequently enhance our ability to manipulate such systems in a useful fashion.

Although the fundamental steps involved in all known bacterial quorum sensing systems are analogous, there is variation between different species in terms of one or more aspects of this signaling process, i.e., the exact nature of the chemical signals, receptors, mechanisms of signal transduction, and phenotypic expressions.[33] Of particular interest from a chemist's standpoint is the rich diversity in the signaling molecules used in quorum sensing, and the continued discovery of new autoinducers serves to increase our appreciation of the complexity of the bacterial chemical lexicon ever more.[2,33] Nevertheless, the majority of quorum sensing systems used by bacterial species can be classified into a limited number of categories based upon common structural features in the small-molecule autoinducers, that is, similarities in the chemical vocabulary used for cell−cell communication.[34,35]

A comprehensive analysis of the use of small molecules to modulate every class of quorum sensing system is not possible; notwithstanding the large number of autoinducers, some quorum sensing systems are poorly understood or not well characterized on a molecular level, or have yet to be explored to any real extent with unnatural chemical probes. In this manuscript we instead focus upon two types of quorum sensing systems in Gram-negative bacteria, each mediated by a distinct class of autoinducer, namely, (1) *N*-acylated-L-homoserine lactones (AHLs) and (2) autoinducer AI-2.

AHLs are the most common class of autoinducer used by Gram-negative bacteria; indeed quorum sensing mediated by AHLs represents one of the best-understood bacterial systems at the molecular level.[28] Cyclic peptides are the major class of cell−cell signaling molecules in Gram-positive bacteria; for a discussion of quorum sensing in these systems, the reader is directed toward several recent reviews covering this field.[11,36,37] It has been proposed that the AI-2 quorum sensing system is used by both Gram-negative and Gram-positive bacteria and that it may represent a possible means by which *different* bacterial species can communicate with each other, as opposed to the intraspecies communication typically mediated by other autoinducers discovered thus far.[38−41] Of particular interest is the fact that AI-2 and its synthase LuxS (see section 3) have been correlated with pathogenicity in a variety of organisms.[38,40,42−48] The discovery of antagonists of AI-2 quorum sensing may provide a possible means to achieve broad-spectrum antivirulence and has thus attracted significant attention in recent years.[49]

This review is split into two main sections according to the classes of quorum sensing system outlined above. Within each section we first provide a basic outline of the molecular basis of the quorum sensing system under discussion (that is, the structure of the autoinducer(s) and the nature of the cognate protein receptor(s) involved). We then provide a systematic overview of work pertaining to the use of small-molecule agents to modulate various aspects of the signaling system, with a primary focus upon studies carried out over the last 10 years. In these discussions, there is an emphasis placed upon the molecular basis of activity. Thus, we will highlight any structure−activity relationships (SARs) exhibited by small-molecule modulators of the pathway. Such insights into the molecular features required for small-molecule activation or inhibition of these quorum sensing systems should prove to be valuable in the rational design of next-generation chemical tools, with improved activities or selectivities, to study or manipulate these pathways.

1.1. Advantages of the Chemical Biology Approach for the Study of Quorum Sensing Pathways

In principal, the use of small molecules as molecular tools to probe and manipulate quorum sensing pathways (a chemical biology approach) should have several significant advantages over genetic techniques.[28,50] Small-molecule perturbation of protein function is generally reversible (due to metabolism or clearing), which allows studies to be carried out on the reversibility of the system; such experiments are difficult to perform using genetic techniques.[50] In addition, all small molecules can be used in a conditional manner; that is, they are added at any time point in the experiment, allowing for temporal control of a biological system. As the biological effects of small molecules are generally rapid, this allows the characterization of intermediate/early responses in the system. Genetic alterations only allow for steady-state observations.[28] Furthermore, small-molecule effects can be controlled by altering the concentration of the small molecule, thereby allowing the generation of dose−response data. However, as recently noted by Blackwell and coworkers, there are drawbacks to the use of small molecules to probe quorum sensing systems.[28] In particular, there are problems associated with the identification of highly active and specific agents, that is, small molecules that have a high degree of activity *and* specificity for the proteins involved in any particular quorum sensing system of a given bacterial species (see below). Furthermore, in whole-cell bioassays the membrane permeability of a compound is a complicating factor, as this plays a key role in determining efficacy. Nevertheless, a large variety of small molecules capable of modulating quorum sensing systems have been identified, which provides a chemical toolbox for the study of this signaling process.[28]

1.2. Important Considerations When Comparing the Results of Different Studies

In this review we provide a summary of the results of different studies into the use of small molecules to modulate quorum sensing pathways. However, a significant issue encountered in this context is the lack of standardization between the assays used in different reports to assess the biological effects of such agents.[12,28,34] Even in studies investigating modulation of the same protein, there is often variation in the bacterial strains, growth media, and assays used.[28] This is problematic, as it has been well established that not all compounds display similar activities in different strains; variation in membrane composition, secondary regulation of gene expression, and the presence of competing ligands may have a large impact on the observed biological effects of a small-molecule agent.[28,34] Therefore, a direct quantitative comparison of the levels of activities of small molecules (e.g., IC_{50} values) obtained from different studies can be misleading and is *not appropriate* in many cases. We would argue that a more qualitative approach in which the structures and *relative* biological effects (i.e., agonist or antagonistic) of small-molecule agents identified in different studies are compared is more suitable. From such analyses, it is possible to extract some general, if basic, SAR trends. Absolute activity values obtained within an individual study are, however, comparable, as are data resulting from different studies by the same research group, provided the exact same assay conditions are employed. Such information is valuable in terms of elucidating SAR trends within the particular compounds series under investigation.

An additional point to note is that bacterial species utilizing the same general type of quorum sensor (that is, the same general signals and receptors, for example, AHL-based signaling) should not be necessarily expected to respond in similar ways when exposed to a given chemical probe. That is, any structure−activity trends may be species-dependent rather than system-dependent. Such information is valuable for the identification of both selective and broad-spectrum, multispecies modulators of quorum sensing activity.

2. AHL-Based Quorum Sensing in Gram-Negative Bacteria

2.1. Basics of the Quorum Sensing Circuit: LuxI/LuxR-Type Systems

As noted previously, AHLs are the most common class of autoinducer used by Gram-negative bacteria. The majority of natural AHLs reported to-date share conserved structural characteristics, a homoserine lactone ring unsubstituted at the β- and γ-positions, which is N-acylated at the α-position with an acyl group derived from fatty acid biosynthesis (a fatty acyl group).[23] However, recent evidence suggests that nonfatty acyl groups can also be incorporated into homoserine lactone-based quorum sensing signals.[51]

The first AHL-based quorum sensing system was discovered in the bioluminescent marine bacterium *Vibrio fischeri*, which was observed to produce light at high, but not at low, cellular densities.[52,53] The quorum sensing circuit in question is composed of the autoinducer, *N*-3-oxohexanoylhomoserine lactone (OHHL), and the LuxI and LuxR proteins (Figure 1). OHHL is produced through the LuxI protein, whereupon it freely diffuses in and out of the cell. As the cell density of the population increases, so does the concentration of OHHL. LuxR is the receptor for OHHL. Though OHHL is generated at low basal levels throughout the growth of the bacteria, high cell densities are required to achieve an intracellular ligand concentration sufficient for productive LuxR binding.[28] Without the OHHL ligand, the LuxR protein is unstable and rapidly degrades; however, above a certain threshold concentration of OHHL, productive binding of OHHL to LuxR occurs.[4,28] The LuxR−OHHL protein complex acts as the transcriptional activator of the genes responsible for bacterial luminescence.[4] The expression of LuxI is also further stimulated by OHHL-bound LuxR. Thus, when the quorum sensing circuit engages, autoinducer production is induced, and the surrounding environment is flooded with the signal molecules, leading to more production of the autoinducer, i.e., an autoinduction positive feedback loop.[4]

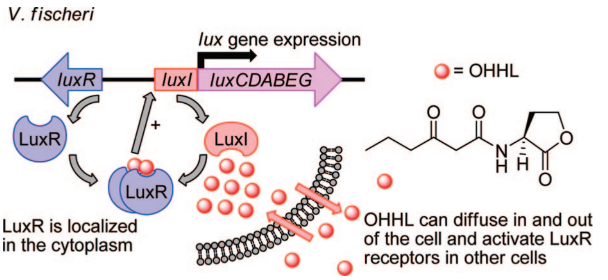


Figure 1. Schematic of the LuxI/LuxR quorum sensing system in *V. fischeri*.[31]

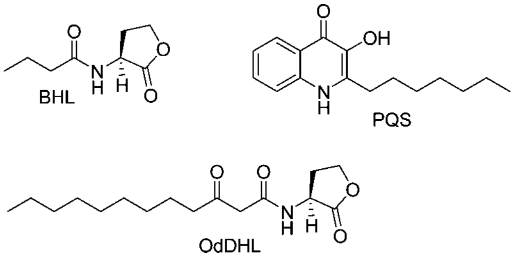
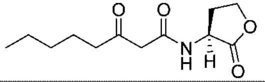
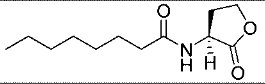
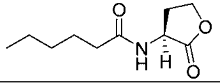
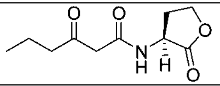
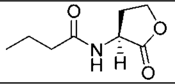
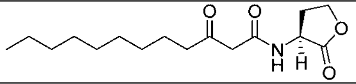
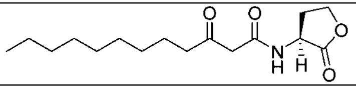
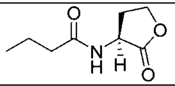
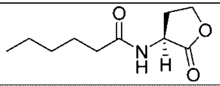
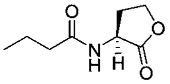
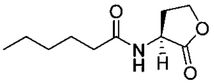
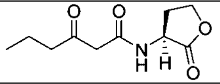
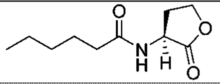
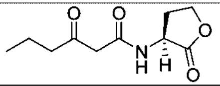
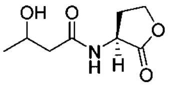


Figure 2. Native quorum sensing molecules in *P. aeruginosa*.

The quorum sensing circuit outlined in Figure 1 represents the basic paradigm for AHL-mediated signaling. Indeed, the proteins involved in the majority of AHL-based quorum sensing systems subsequently discovered have been termed LuxI-type synthases and LuxR-type receptors. It should be noted that the quorum sensing signaling process that occurs in some Gram-negative bacteria is often more complex than that outlined in Figure 1. Several bacterial species have been shown to use intricate network-type architectures for cell−cell signaling, involving two or more AHL signals, and even other types of quorum sensing pathways.[4,28] For example, *Pseudomonas aeruginosa* uses (at least) three types of quorum sensing signaling systems (Figure 2). Two of these are AHL-based. There are two discrete AHL molecules, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butanoyl-L-homoserine lactone (BHL). These are generated and sensed by two separate signaling systems, each involving a LuxI-type synthase and a LuxR-type receptor. OdDHL is generated by LasI with LasR being its cognate receptor. BHL is generated by the synthase RhlI and detected by the RhlR protein. These AHL-dependent signaling systems are integrated with a third system that employs a chemically distinct signal molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone (termed the *Pseudomonas* quinolone signal or PQS).[11] There exists

Table 1. AHL Systems Discussed in This Review (Adapted from the Report of Blackwell and Co-workers[28] and Information Provided by the University of Nottingham, England[59])

Bacterium	AHL(s) discussed in this review	Nomenclature used in this review	LuxI/R homologs
Agrobacterium tumefaciens[63,64]		OOHL, 3-oxo-C8-HSL	TraI/R
Burkholderia cenocepacia[65]		OHL, C8-HSL	CepI/R
Chromobacterium violaceum[66,67]		HHL, C6-HSL	CviI/R
Erwinia carotovora[68-70]		OHHL, 3-oxo-C6-HSL	ExpI/R; CarI/R
Pseudomonas aeruginosa[71-74]		BHL, C4-HSL	RhlI/R
		OdDHL, 3-oxo-C12-HSL	LasI/R; QscR[a]
Pseudomonas putida		OdDHL, 3-oxo-C12-HSL	PpuI/R
Serratia sp. ATC39006[75-77]		BHL, C4-HSL	SmaI/SmaR and CarR_{sma}
		HHL, C6-HSL	
Serratia liquefaciens MG1[78,79] (recently identified as *Serratia marcescens* MG1)[80,81]		BHL, C4-HSL	SwrI/SwrR
		HHL, C6-HSL	
Serratia marcescens AS-1[82]		OHHL, 3-oxo-C6-HSL	SpnI/SpnR
		HHL, C6-HHL	
Vibrio fischeri		OHHL, 3-oxo-C6-HSL	LuxI/R
Vibrio harveyi[b] [61,83,84]		3-hydroxy-C4-HSL	Generated by LuxM, detected by LuxN[b]

[a] QscR is an orphan receptor that responds to OdDHL produced by LasI (see section 2.2).[28] [b] This system is distinct from the typical *V. fischeri* regime as the genes coding for LuxM and LuxN show no homology with LuxI and LuxR.[60-62]

a hierarchy of quorum sensing regulation in *Pseudomonas* by which LasR activation triggers the successive activation of the RhlR and PQS systems.[54-57] Therefore, the LasR receptor is usually the main focus for activator or inhibitor development and biochemical studies in *P. aeruginosa* (see below).[31]

Nevertheless, the general principles of AHL-based quorum sensing outlined in Figure 1 are applicable to the majority of Gram-negative species. Thus, a LuxI-type protein produces a diffusible AHL ligand; above a certain threshold ligand concentration, productive binding of the ligand to its cognate cytoplasmic receptor (a LuxR-type protein) occurs and the resulting AHL–LuxR-type protein complex then modulates the expression of target genes that are involved in bacterial group processes.[4,13,28] Homologues of LuxI and LuxR have been identified in a large number of bacterial genomes with a variety of different AHLs regulating a range of physiological functions. In general, each bacterial species responds specifically to its own unique AHL autoinducer; the same general structure is maintained, but the length and functionality of the acyl chain vary.[58] A summary of the AHLs covered in this review is given in Table 1.

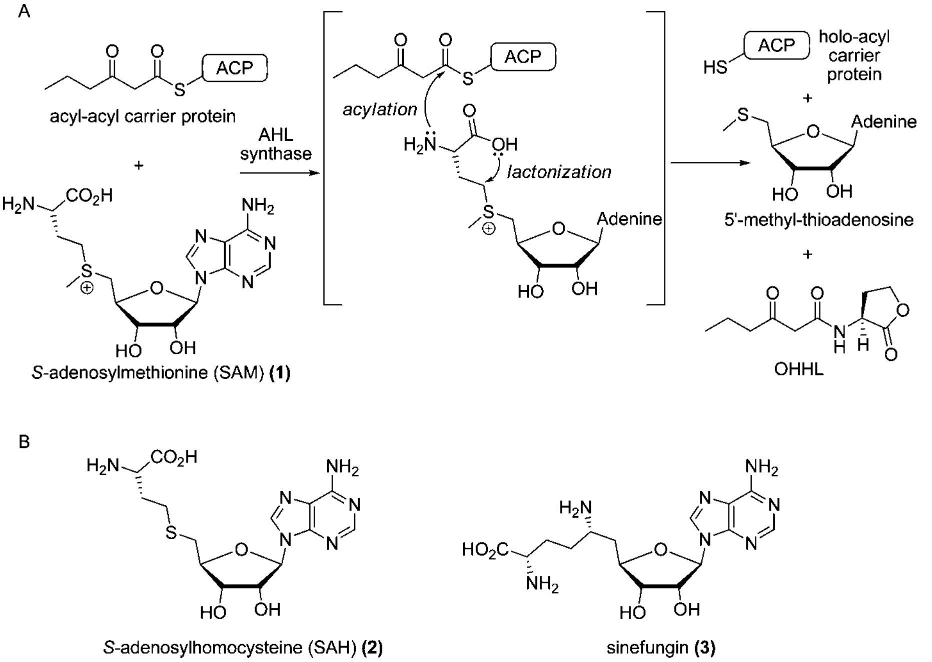


Figure 3. (A) Schematic diagram illustrating the general features of the AHL biosynthetic pathway. ACP = acyl carrier protein. SAM (**1**) and acyl-ACP bind the AHL synthase (a LuxI-type synthase), whereupon acylation and lactonization reactions occur. The AHL (in this example, OHHL) is then released, along with the byproduct holo-ACP and 5′-methylthioadenosineis. Adapted from the report of Watson et al.[90] (B) Two SAM analogues, **2** and **3**, that are known inhibitors of AHL synthesis in *P. aeruginosa*.[89,92]

2.2. Modulating LuxI/LuxR-Type Quorum Sensing

A large number of small molecules capable of modulating AHL-based quorum sensing systems have been identified over the last 30 years. For the purposes of this discussion, which is centered upon identifying any structure−activity trends associated with such small-molecule intervention, it is useful to analyze these data according to the component of the communication circuit that is affected. In principle, there are three such basic components and, thus, targets for external intervention in an AHL-mediated quorum sensing system: the LuxI-type synthase (the signal generator), the AHL ligand (the signal itself), and the LuxR-type receptor (the signal receptor).[24,28] It should be noted that this categorization is a generalization; in some bacterial species, the situation can be significantly more complex. For example, the opportunistic pathogenic bacterium *P. aeruginosa* has an AHL quorum sensing network composed of two interlinked systems based on LuxR-type receptors (LasR and RhlR) linked to AHL synthases (see above). However, recent studies have revealed the existence of a third LuxR-type protein in *P. aeruginosa* that is not linked to an AHL synthase.[85−87] This orphan receptor, termed QscR (quorum sensing control repressor), has been shown to play a regulatory role within the larger AHL quorum sensing network of this bacterium, including the control of virulence.[85,87] Recent studies suggest that QscR represents a valid target for the modulation of quorum sensing controlled genes in *P. aeruginosa*.[85] However, for the purposes of this discussion, we will focus upon modulators of the three basic components of AHL-based signaling discussed above, as these are recognized as the sites of intervention of the majority of non-native agents discussed in the literature.

2.2.1. Targeting the Synthase

Interfering with AHL autoinducer production is a conceptually simple method for modulating quorum sensing pathways; if no signal molecule is produced, no signaling can occur. However, there are relatively few reports detailing the use of small-molecule agents to target LuxI-type synthase proteins.[28] AHL synthesis by LuxI-type synthases generally proceeds via a sequentially ordered reaction mechanism utilizing *S*-adenosylmethionine (SAM, **1**) as the amino donor for the formation of the homoserine lactone ring moiety and a charged (acylated) carrier protein (ACP) as the precursor to the acyl side chain (Figure 3).[24,88−90] The majority of studies on the chemical modulation of AHL synthesis to date are based on the use of various analogues of SAM; for example, *S*-adenosyl-homocysteine (SAH, **2**), sinefungin (**3**), and butyryl SAM have proved to be potent inhibitors of the *P. aeruginosa* AHL synthase RhlI in vitro, presumably acting directly at the level of the synthase (Figure 3).[24,88] Other autoinducer synthase blocking compounds have been examined including homologues and analogues of purine nucleotides (e.g., thiol derivatives and alkylated thio derivatives) and homoserine lactone derivatives.[91]

Interestingly, certain macrolide antibiotics have been shown to repress the *P. aeruginosa* AHL synthesis when applied at subminimal growth-inhibitory concentrations,[88,93−95] but such effects are generally thought to be mediated at the ribosomal level rather than via direct interaction with the synthase protein itself.[88,96] Recently, several crystal structures for LuxI-type proteins have been reported that could potentially be exploited in the rational design of synthetic ligands.[28,97,98] This is an area that is expected to garner significant interest in the near future.[28]

2.2.2. Targeting the Signal

The second basic target for external intervention in AHL-mediated quorum sensing is the AHLs themselves; any modulation (typically degradation) of these signaling molecules should interfere with the normal communication pathway. It is difficult to envisage how small-molecule chemistry can be utilized directly to achieve this goal, though an "indirect" approach based on the use of small-molecule agents to elicit catalytic antibodies capable of hydrolyzing AHLs has attracted significant interest in recent years.[99,100] Nature is known to have evolved quorum quenching enzymes that are capable of hydrolyzing both the amide and lactone moieties of AHL signaling molecules.[101,102] For example, a class of enzymes known as paraoxanases has been identified in several mammals, which are capable of inactivating OdDHL and thus attenuating *P. aeruginosa* quorum sensing in cell cultures and in vivo.[103]

2.2.3. Targeting the LuxR-Type Receptor

The majority of work carried out on small-molecule modulation of AHL-mediated quorum sensing has focused upon identifying agents that can interact with the LuxR-type receptor protein. Given the critical role that quorum sensing has been shown to play in the pathogenicity of many bacterial species, it is unsurprising that the discovery of potent antagonists of AHL binding to LuxR homologues has been most intensively investigated. However, selective activators of this receptor are also desirable in the context of certain agricultural applications.[28] For the purposes of this discussion, it is useful to categorize small molecules that modulate LuxR-type receptors according to basic structural characteristics.

2.2.4. Small Molecules Based around the AHL Scaffold

At this point, it is useful to outline how we define a molecule as being based around an AHL framework. For the purposes of this discussion, we will take compounds that exhibit "global" structural similarity to native AHLs as being AHL analogues, that is, compounds that contain a carbon chain (of any functionality or substitution) or aryl group linked to an amide group (or derivative thereof), the nitrogen atom of which is directly attached to a ring system at a chiral carbon. This definition is somewhat arbitrary but nevertheless covers the majority of compounds that are typically referred to as AHL analogues in the literature.

Small-molecule agents capable of modulating a given quorum sensing pathway via interaction with the relevant LuxR-type receptor have traditionally been discovered through a design and synthesis process utilizing the structure of the known natural AHL signaling molecule as a template. The X-ray crystal structures of a limited number of LuxR-type receptors complexed to their natural AHL ligands have been reported,[104−109] and such information has been used to guide the design of synthetic AHL ligands in recent years.[12,56] Computational pharmacophore modeling has also proven valuable for providing an understanding of how different AHL structural features engender various biological activities.[56,110−112] Until very recently (see below),[113] there were no known reports describing the X-ray crystal structures of the binding domains of LuxR-type receptors with non-native ligands, which has hindered the rational de novo design of synthetic ligands that are not based around the generalized AHL structure. Thus, non-native AHLs still represent the most extensively studied class of synthetic quorum sensing modulators to date, and there is a significant body of work pertaining to SARs associated with these derivatives.[12,28,110] High-throughput synthesis has proven to be a particularly valuable tool in identifying biologically active synthetic AHL mimics and the elucidation of structure−activity trends, as libraries of AHL analogues with systematic modifications can be synthesized and tested in a time- and cost-efficient fashion.[7,12,31,114]

2.2.5. Geske's Overview of the Use of AHL Analogues to Modulate Quorum Sensing

In 2008 Geske et al. published a comprehensive review of the SARs for non-natural AHL analogues in several bacterial species, with a particular focus on quorum sensing systems utilizing five different LuxR-type proteins: LuxR from *V. fisheri*, LasR and RhlR from *P. aeruginosa*, TraR in *A. tumefaciens*, and CarR in *E. carotovora*.[28] A summary of the main conclusions from this report is discussed in the following section, and an overview of the AHL analogues covered in the report is given in Table 2. For a full analysis of the effects of various AHL mimics upon each of these bacterial species, the reader is directed to the original review. In sections 2.2.7 and 2.2.8 of this manuscript, we provide an overview of the use of AHL analogues to modulate LuxR-type quorum sensing systems in a variety of bacterial species published since the review of Geske et al. in 2008. In addition, selected significant examples in this area that are not covered in Geske's review are discussed.

As a result of their analysis, Blackwell and co-workers were able to draw some general conclusions about the structural features of AHL molecules that are necessary for activity against LuxR-type proteins. It should be noted that clear distinctions between antagonist and agonist activity could not always be made; many AHL-based compounds were found to be able to both slightly activate and inhibit a quorum sensing circuit depending upon their concentration. The authors note that it is therefore valuable to consider activity against LuxR-type proteins as a continuum from activation and inhibition, and for the purposes of their analysis, they grouped activators and inhibitors together as necessary and simply discussed trends in terms of "activity". It should be noted that similar observations had previously been made by Rasmussen and Givskov.[21]

Blackwell and co-workers delineated five broad activity trends from their studies:

(1) The length of the acyl chain was generally found to be critical for the activity of AHL mimics with compounds whose chain lengths were close to that of the natural AHL having heightened activity.

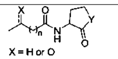
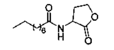
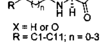
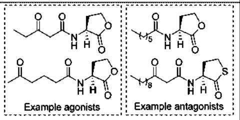
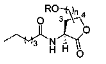
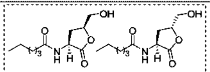
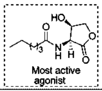
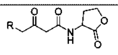
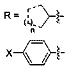
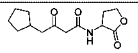
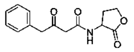
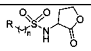
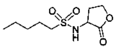
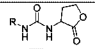
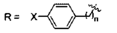
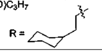
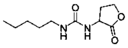
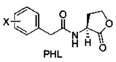
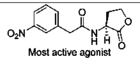
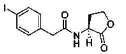
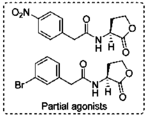
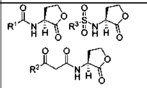
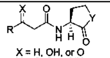
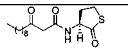
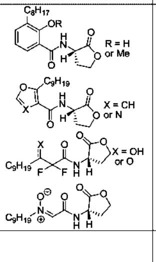
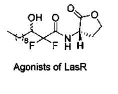
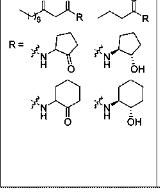
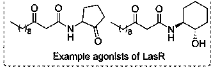
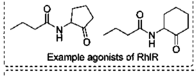
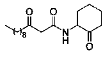
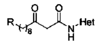
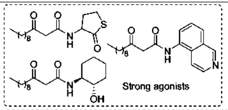
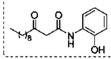
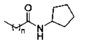
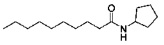
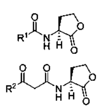
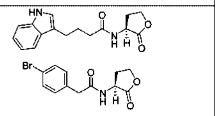
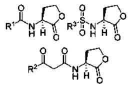
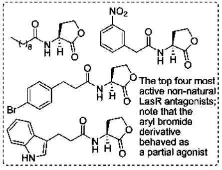
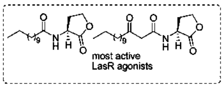
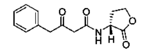
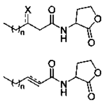
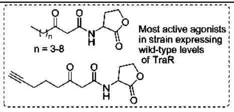
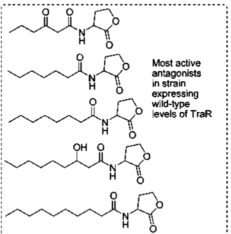
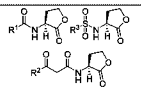
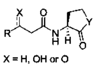
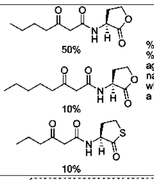
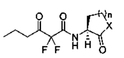
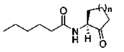
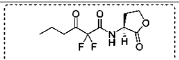
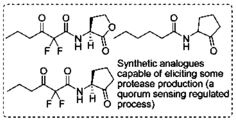
(2) Where the natural AHL possesses a modification at the 3-carbon of the acyl chain (e.g., a carbonyl), this group was important, but not essential, for activity. The removal of this group typically results in AHL mimics with inhibitory, rather than agonistic, activity.

(3) In general, the natural L-stereoisomer of the lactone ring is needed for activity. However, there are exceptions, and a relatively small number of D-AHLs have been studied to date.

(4) In some, but not all, systems, direct modifications to the lactone ring are tolerated but typically result in compounds that are less active. However, lactone replacements that mimic the parent headgroup in terms of retaining hydrogen bond acceptors (e.g., a thiolactone) are often most active.

(5) The incorporation of aromatic functionality into AHLs, as either lactone ring replacements or substituents in the "tail" section, generally yields analogues with inhibitor activity.

Table 2. Summary of the AHL Analogue Studies Recently Reviewed by Geske et al.[28] (For More Detail, the Reader Is Directed Toward the Aforementioned Review and the Relevant Primary Literature)

Entry; primary literature reference	General structural features of AHL analogues tested	Significant compounds identified in the study
1; Eberhard *et al.*[115]	 X = H or O Y = O, S, or $(CH_2)_2NH$ R = C2-C7; n = 0-3	 Most active agonist
2;Schaefer *et al.*[116]	 X = H or O R = C1-C11; n = 0-3	 Example agonists Example antagonists
3;Olsen *et al.*[117]	 Various subsitutents at 3- or 4-positions; n = 0 or 1 R = H or $(CO)NHCH_2CH_3$	 Most active antagonists  Most active agonist
4; Reverchon *et al.*[118]	 Cycloalkyl, branched and aryl subsituted AHL analogues  R = ; n = 1 or 2 X = H, F, Cl, Br OMe, CF_3, Ph	 Displays agonist activity comparable to that of native autoinducer (3-oxo-C6-HSL)  Most active antagonist
5;Castang *et al.*[119]	 R = CH_3, n = 3-8 R= Ph, n =1-3	 Most active antagonist
6;Frezza *et al.*[120]	  R = n = 0, 1, 2, 3, 4, or 5 and X = H n = 1 and X = CF_3, Cl or CH_3 R = C_4H_9, C_5H_{11}, C_3H_7, C_6H_{13}, $(C{=}O)C_3H_7$ R = 	 Most active antagonist
7;Geske *et al.*[12,110,121]	 PHL PHL = phenylacetyl homoserine lactone X = various groups	 Most active agonist  Most active antagonist  Partial agonists See also Section 2.2.6
8;Geske *et al.* (LasR, TraR and LuxR)[12]	 Large number and variety of R groups used ~ 90 compounds evaluated in total	See Sections 2.2.6 and 2.2.8
9; Passador *et al.* (LasR)[122]	 X = H, OH, or O Y = O, S, or NH R = C1-C12	 A functional agonist of LasR and the best competitive LasR binder
10; Kline *et al.* (LasR)[123]	 R = H or Me X = CH or N X = OH or O	 Agonists of LasR
11; Smith *et al.* (LasR and RhlR)[124]	 R =	 Example agonists of LasR  Example agonists of RhlR  Inhibits both LasR and RhlR systems; strongly inhibits pyocyanin and elastase production and biofilm growth in *P. aeruginosa*
12; Smith *et al.* (LasR)[125]	 R = CH_3 or CH_2CH_2OH Het = heterocycle (various)	 Strong agonists  most potent antagonist; inhibitor of *P. aeruginosa* elastase B production
13; Ishida *et al.* (LasR and RhlR)[126]	 n = 1-10	 Most active antagonist
14; Geske *et al.* (LasR)[114]	 R^1 or R^2 = C_3H_7, C_5H_{11}, C_7H_{15}, C_9H_{19}, $C_{11}H_{23}$, $C_{13}H_{27}$ C_3H_7, C_7H_{15} or $C_{11}H_{23}$	 Most active antagonists; capable of strongly inhibiting static *P. aeruginosa* biofilm growth in a dose-dependent manner (Note that some cross-species effects were also seen: see Section 2.2.6)
15; Geske *et al* (LasR, TraR and LuxR)[12]	 Large number and variety of R groups used. ~ 90 compounds evaluated in total	 The top four most active non-natural LasR antagonists; note that the aryl bromide derivative behaved as a partial agonist  most active LasR agonists  Showed similar agonistic activity to the natural ligand for LasR at the same concentration; demonstrates that not all D-stereoisomers of AHL-type ligands are inactive Note that some cross-species effects were also seen: see Section 2.2.6
16; Zhu *et al.*[127]	 X = H, OH n = 0-8 n = 0-6	 n = 3-8 Most active agonists in strain expressing wild-type levels of TraR  Most active antagonists in strain expressing wild-type levels of TraR
17; Geske *et al.* (LasR, TraR and LuxR)[12]	 Large number and variety of R groups used. ~ 90 compounds evaluated in total	See also Sections 2.2.6 and 2.2.8
18; Chhabra *et al.*[128]	 X = H, OH or O Y = O [S or NH for X = O and R = $(CH_2)_2CH_3$] R = C1-C9, Ph, $CH(CH_3)_2$	 50% 10% 10% % values refer to % activities as agonists relative to natural autoinducer which is given a value of 100%
19; Welch *et al.* (CarR and EccR)[129]	 n = 1, X = CH_2 n = 2, X = CH_2 n = 3, X = CH_2 n = 1, X = O  n = 1 or n = 2	 Only synthetic analogue able to elicit some production of the antibiotic carbapenem (a quorum sensing regulated process)  Synthetic analogues capable of eliciting some protease production (a quorum sensing regulated process) There was an excellent correlation between antibiotic production *in vivo* and *in vitro* binding to CarR. However, no such correlation was found between protease production and analogue binding to EccR.

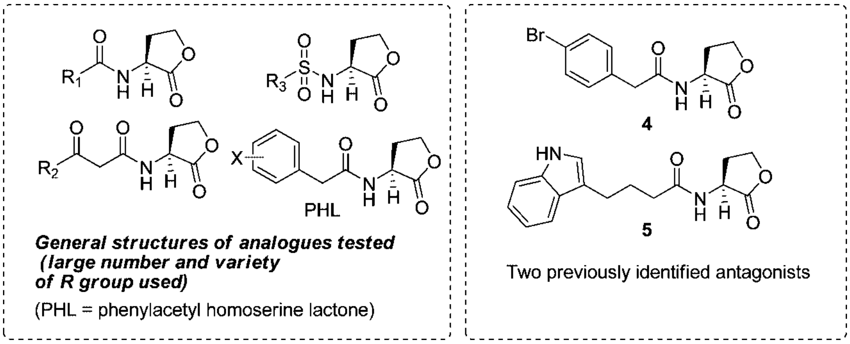


Figure 4. General structural features of AHL analogues tested by Geske et al.[12] The structure of two antagonists **4** and **5** identified from a previous study by Blackwell and co-workers[114] (see Table 1, entry 14) are also shown.

2.2.6. Investigating Species Selectivity

In general, known antagonists and agonists of quorum sensing have typically been examined primarily in one bacterial species; the general SARs outlined above were discerned (at least in part) from literature studies that each individually focused upon one organism at a time or looked at a limited subset of AHL analogues.[31,110] The lack of comparative investigations of non-native AHL function across multiple bacterial species has resulted in a relative paucity of information pertaining to the molecular features that confer selectivity or broad-range activity to synthetic quorum sensing modulators in Gram-negative bacteria.[12] In recent seminal reports, Blackwell and co-workers presented a comprehensive and systematic study that directly compared the activities of ~90 AHL analogues across three Gram-negative bacterial species: the pathogens *A. tumefaciens* and *P. aeruginosa* and the symbiont *V. fischeri*.[12,31] Their strategy was based around the synthesis of four focused combinatorial libraries of non-native AHLs, using the natural AHL ligands of these species together with the structures of previously identified synthetic antagonists and the X-ray crystal structure of the TraR protein[107] (i.e., the ligand binding site) to guide initial ligand design (Figure 4).

The major conclusion from this study was the identification of sets of ligands that selectively modulate one, two, or all three of the LuxR-type proteins investigated. This is illustrated by the Venn diagrams shown in Figure 5.[31] Thus, for the first time, both broad-spectrum and species-selective antagonists of LuxR-type proteins, and therefore quorum sensing responses in Gram-negative bacteria, had been identified. In contrast, broad-spectrum agonists with high activity were not identified. Indeed, the authors noted that far fewer LuxR-type protein agonists were identified relative to antagonists, and those agonists that were discovered typically showed exquisite selectivity for individual LuxR-type proteins.

Although a more comprehensive SAR analysis of synthetic AHL ligands has subsequently been reported by Geske and colleagues (see section 2.2.5), this earlier study still remains one of the few sources of SAR information in the context of species selectivity. The researchers noted eight such SAR trends:

(1) In general, AHLs with acyl groups up to eight atoms long, containing either aromatic functionality with electron-withdrawing groups or straight-chain aliphatic functionality, displayed broad-spectrum antagonist activity against all three receptors, TraR, LasR, and LuxR.

(2) Ligands containing a phenylacetanoyl (PHL) group in the acyl chain displayed a wide range of antagonistic and agonistic activities across all three proteins in the study. Thus, the PHL group appears to be a "privileged" scaffold for LuxR-type protein modulation. PHLs with electron-withdrawing and lipophilic substituents in the 4-position on the phenyl group display the strongest antagonistic activities against TraR and LuxR. The same trend holds true in LasR for PHLs with substituents in the 3-position.

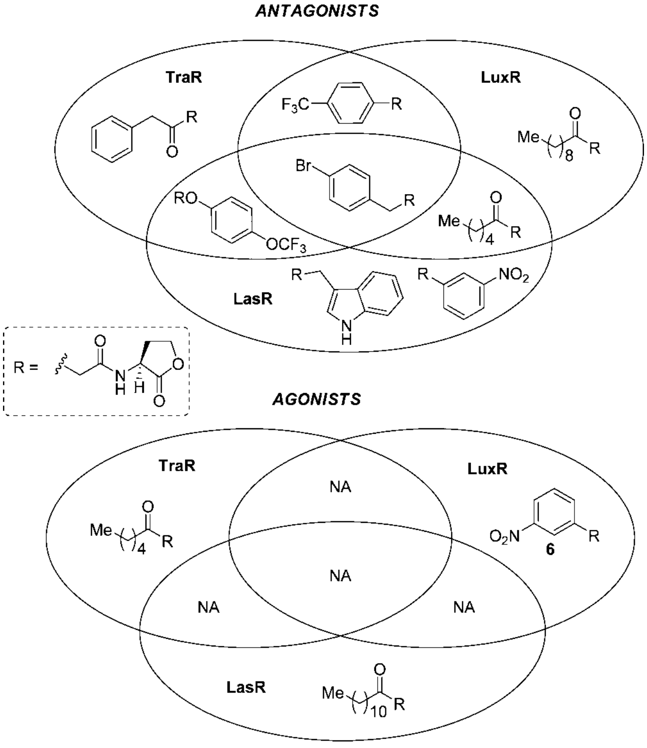


Figure 5. Venn diagrams illustrating the structures of a selection of the most potent LuxR-type protein antagonists and agonists identified and their selectivities for different LuxR-type proteins from *V. fischeri* (LuxR), *A. tumefaciens* (TraR), and *P. aeruginosa* (LasR). Ligands in the intersection of the circles have significant activity for two or more proteins.[12] NA = no applicable ligands identified. For a full version of the Venn diagrams, the reader is directed to the primary text.[12]

(3) Of the AHLs structurally related to 4-bromo PHL **4** (a known antagonist identified in a previous study[114]), a flexible carbon spacer of at least one carbon between the lactone ring and an aromatic acyl group and a 4-bromo substituent on the phenyl group engender the strongest antagonistic activity.

(4) A three-carbon spacer between the lactone ring and an aromatic acyl group is optimal for inhibition in ligands structurally related to indole AHL **5** (a known antagonist identified in a previous study[114]).

(5) The carbonyl groups on aliphatic AHL TraR and LuxR antagonists can be replaced with sulfonyl groups without significant loss in activity. The sulfonyl-based AHLs in this study were most active against TraR yet virtually inactive against LasR.

(6) The TraR protein was found to be the most sensitive to the length of the acyl group on AHLs; inhibitory activity was found to drop off dramatically for AHLs with acyl tails longer than eight atoms. This implies that the TraR receptor may have a sterically more congested ligand-binding site, which is in accordance with the X-ray crystal structure of TraR, assuming that synthetic AHLs target the same binding site on TraR.

(7) The LasR protein was the most tolerant of varying functionality on the AHL acyl chain, acyl chain length, and the stereochemistry of the homoserine lactone ring. This implies that the LasR receptor has a larger ligand-binding site than TraR, which is in accordance with the X-ray crystal structure of LasR, assuming that synthetic AHLs target the same binding site on LasR (as with TraR).

(8) The LuxR receptor was most strongly inhibited by AHLs with medium to long (6−14 carbons) 3-oxo-aliphatic acyl groups and most strongly *activated* by PHL ligands with electron-withdrawing substituents in the 3-position.

The authors hypothesized that these synthetic AHLs are all bona fide ligands for the protein ligand-binding sites (that is, they all target the same site of a given receptor), and that the inhibition of activation is based on the specific binding mode, and therefore affinity, of the AHL, suggesting that not all of the molecules are binding in the same way as the natural ligand. This binding flexibility will dramatically complicate any rational structure-based design of further selective/broad-spectrum agents based on these hits.[31] Nevertheless, it is anticipated that the molecules identified in this study will prove useful as chemical probes to study quorum sensing, particularly in natural environments harboring multiple species.[12] This study also marked the discovery of the dependence of AHL analogue activity upon concentration, which was highlighted in Geske's 2008 review discussed previously, that is, many of the most potent LuxR-type "antagonists" identified are actually better described as "partial agonists" because, at high concentrations, they were able to activate the transcriptional regulators. In addition, this study also marked the first reported discovery of a superagonist of *V. fischeri* (**6** in Figure 5, see section 2.2.8).

2.2.7. Additional AHL Analogue Studies

As noted previously, the study by Geske et al.[28] highlighted potential pitfalls associated with attributing a definite type of biological effect (i.e., always acting as an antagonist or always an agonist) to any given small-molecule quorum sensing modulator. These researchers thus considered activity against LuxR-type proteins as a continuum from activation to inhibition and grouped activators and inhibitors together as necessary, discussing trends in terms of "activity". However, this terminological approach is not used in the majority of the literature in the field and thus will not be adopted for the remainder of this review. Therefore, we will utilize the terms agonist/activator and antagonist/inhibitor as specified in the relevant primary literature.

Geske et al. have reported the synthesis of a focused library of AHL analogues based around known lactone leads from previous studies(s) (Figure 6).[130] Compounds **7**−**13** represent some of the most potent agonists and antagonists of LuxR-type receptors previously reported by this research group.[12,110] The compounds are classified as phenylacetyl homoserine lactones (PHLs, **7**−**11**), phenoxyacetyl homoserine lactone (POHL, **12**), or phenylpropionyl homoserine lactones (PPHLs, **13**−**14**). The newly synthesized analogues were evaluated for antagonistic and agonistic activity against quorum sensing receptors in *A. tumefaciens*, *P. aeruginosa*, and *V. fischeri*, and several compounds were identified with significantly higher activities relative to the lead compounds. For example, **15**, **16**, and **17** were antagonists in TraR and LuxR with markedly improved activities. In addition, PHLs **18** and **19** were identified as strong activators of LuxR.

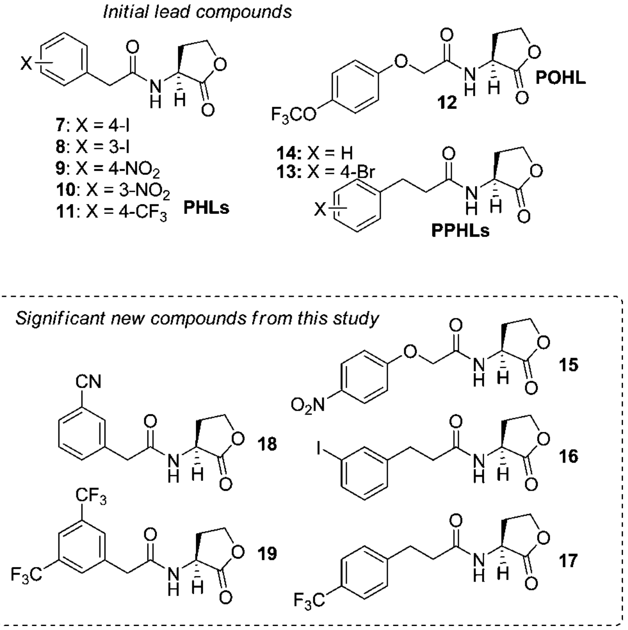


Figure 6. Focused library of AHL analogues was designed around known lactone leads and evaluated for activity against quorum sensing systems.[130]

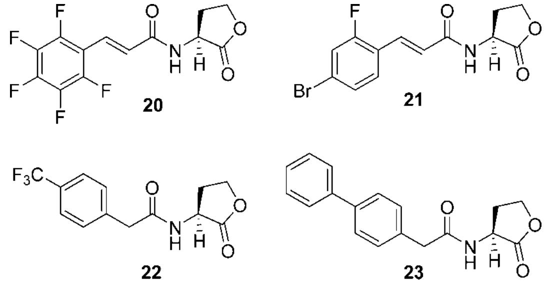


Figure 7. Quorum sensing modulators synthesized and evaluated using small-molecule macroarrays.[7]

Blackwell and co-workers have recently reported the synthesis and evaluation of quorum sensing modulators using small-molecule macroarrays.[7] Several new quorum sensing antagonists were identified in *V. fischeri* (**20** and **21**) and *C. violaceum* (**22** and **23**). These are shown in Figure 7.

Kim et al. have recently reported the development of inhibitors against TraR quorum sensing in *A. tumefaciens.*[131] A series of structural analogues of the native autoinducer OOHL in which the carboxamide bond was replaced with a nicotinamide or a sulphonamide bond (i.e., *N*-nicotinyl and *N*-sulfonyl homoserine lactones) were designed by in silico molecular modeling to exhibit tight binding to the TraR receptor (Figure 8). These analogues were then synthesized using solid-phase methodology.

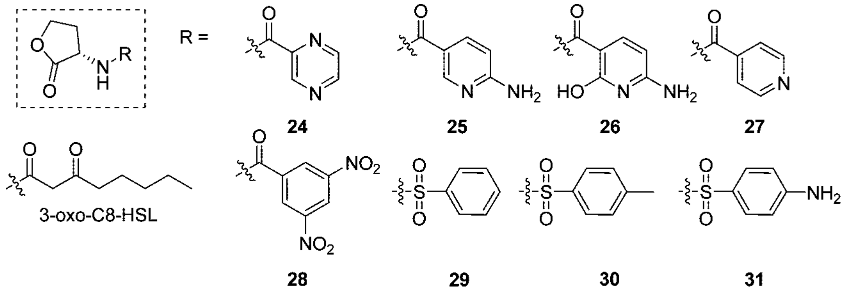


Figure 8. Quorum sensing inhibitors investigated by Kim et al.[131] The structure of the natural autoinducer OOHL is shown for comparison.

The in vivo inhibitory activities of these compounds against quorum sensing in *A. tumefaciens* were assayed using a variety of reporter strain systems; all eight compounds (**24**−**31**) were found to disrupt AHL-based quorum sensing and inhibit biofilm formation. There was a fairly good correlation of the inhibitory activities of the compounds with the estimated binding energies from the modeling study, suggesting that the in silico interpretation of ligand−receptor structures can be a valuable tool for the design of new quorum sensing antagonists. The compounds were also assayed for their effects upon biofilm formation in *P. aeruginosa*. The authors used an adhesion-based assay that examines the effect of an external agent upon the number of bacterial cells (colony forming units or CFU) that are bound to a glass surface after incubation under continuous flow. The general principle underpinning the assay is that a decrease in biofilm formation will lead to a decrease in the number of adhesive *P. aeruginosa* CFU.[132] The compounds were found to significantly reduce the number of adhered *P. aeruginosa* cells. On the basis of this result, the authors concluded that the compounds inhibit *P. aeruginosa* biofilm formation presumably through the inhibition of quorum sensing. However, it is important to note that there are reports which demonstrate that quorum sensing is *not* always required for biofilm formation in *P. aeruginosa* (for example, the impact of quorum sensing upon biofilm formation is known to be dependent upon the growth conditions used).[133] It has been shown that the production of extracellular genomic DNA (eDNA) by *P. aeruginosa* is dependent upon quorum sensing.[134] eDNA is believed to act as a structural component of the *P. aeruginosa* biofilm matrix.[134] Therefore, inhibition of quorum sensing in *P. aeruginosa* may be expected to lead to a decrease in biofilm stability. Thus, rather than inhibiting biofilm formation, the effect of the compounds tested by Kim et al. may have been to decrease the structural stability of the biofilm aggregates via interference with quorum sensing systems of the bacterium. The LasI−LasR system, employing OdDHL as the autoinducer, is at the top of the hierarchical quorum sensing tree of *P. aeruginosa*. Because the structures of OOHL and OdDHL differ, it is initially surprising that the synthetic ligands are capable of modulating both *A. tumefaciens* and *P. aeruginosa* quorum sensing. However, the authors rationalized this observation on the basis of the structures of the protein receptors. The binding site of TraR has two main “pockets” that vary in size, termed the “large” and “small” pockets accordingly.[131] The large pocket is where the acyl tail of the native ligand is thought to be accommodated. Because the structural difference between OOHL and OdDHL is the length of the acyl tail, the structures of TraR and LasR must differ significantly in the large pocket where the acyl tails are thought to be positioned. However, the modeling studies indicated that the synthesized inhibitors mostly interact with residues in the small binding pocket and they have no long protrusion toward the large pocket. This suggests that the inhibitors are insensitive to the structural differences in the large pockets of the TraR and LasR proteins and, thus, are able to interact with, and modulate, both of these proteins. The authors therefore propose that the small pocket of quorum sensing receptor proteins may be a better target for the development of inhibitors with broad-spectrum activity.

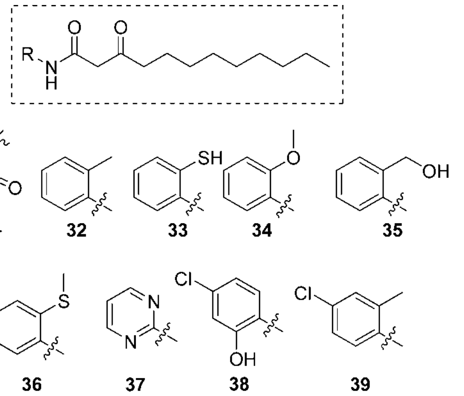
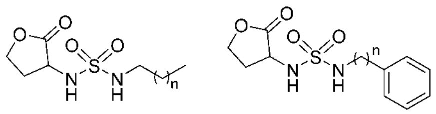


Figure 9. Analogues of OdDHL synthesized and tested for their ability to inhibit quorum sensing in *P. aeruginosa*.[56] **38** and **39** showed lower in vivo activity than **32**−**37**. This was in agreement with modeling studies which suggested that the polar *p*-Cl substituent interfered with the binding of the headgroup to the small pocket of the receptor. Indeed, **38** and **39** were predicted to exhibit a different binding mode from **32**−**37**. **33** and **39** showed the strongest and poorest inhibition, respectively, which is also consistent with the modeling results.

The same researchers have also reported the use of molecular modeling to design inhibitors of the LasR system in *P. aeruginosa*.[56] A set of inhibitors (**32**−**39**) with modification in the head part of the native autoinducer OdDHL were designed, and their docking poses and scores (binding energies) against the LasR receptor were predicted by in silico modeling (Figure 9). All compounds gave better scores than OdDHL. The in vivo activities of the compounds were assessed by measuring β-galactosidase activity in a recombinant *E. coli* bioassay reporter strain carrying a LasR expression plasmid and a *lasI::lacZ* fusion reporter plasmid (note that *lacZ* codes for β-galactosidase). The activity measured in the presence of OdDHL only (set as 100%) was compared to that obtained in the presence of OdDHL and an inhibitor (experiments were carried out using 0.1 μM OdDHL and an equimolar amount of inhibitor or a 10-fold excess of inhibitor). In principle, an inhibitor of LasR quorum sensing should repress the LasR-driven expression of the *lasI::lacZ* fusion, thereby leading to a reduction in β-galactosidase activity. All eight compounds successfully inhibited the activity of LasR by >50% at both concentrations examined. The authors state that the inhibitors compete with



n = 2 = **40**
n = 3 = **41**
n = 4 = **42**
n = 6 = **43**
n = 8 = **44**

n = 1 = **45**
n = 2 = **46**
n = 3 = **47**
n = 4 = **48**

Figure 10. Sulfonylurea AHL analogues synthesized by Frezza et al.[135]

OdDHL for binding to LasR. In addition, all eight compound were found not to be toxic to the host strain. The overall correlation between the docking scores and the extent of inhibition was described as "fair" (although it should be noted that the docking score means the affinity of the ligand−receptor binding rather than the inhibition strength itself). Because all the compounds tested had modifications in the head region, the biological data implies that the head part of the native ligand OdDHL contributes significantly to forming the active conformation of the LasR−OdDHL complex; the inhibitor compounds may act by binding to, and thus altering, the LasR conformation into an inactive form. The authors suggest that the head part of OdDHL is therefore a good target moiety for the development of novel *Pseudomonas* inhibitors. In addition, this study helps to validate the use of a modeling approach for the design of such compounds.

Frezza et al. prepared nine homoserine lactone-derived sulfamide derivatives (**40**−**48**) substituted with either an alkyl chain or a phenyl group (Figure 10).[135] All of these compounds inhibited the action of the *V. fischeri* quorum sensing regulator OHHL, with the aliphatic compounds showing higher levels of activity. Compounds **41** and **45**, which showed the best antagonist activity in the alkyl and phenyl series, respectively, were selected for molecular modeling in the ligand-binding site of TraR. These studies used this ligand-binding site as a model for the supposed ligand-binding site of the LuxR protein. The authors state that this model is appropriate because the docking of the natural ligand of either LuxR (OHHL) or TraR (OOHL) within the ligand-binding site led to very similar binding modes. The modeling studies showed that the ligand-binding site of this protein can readily accommodate the synthetic derivatives. In view of the structural similarities of the sulfamide derivatives to the native autoinducer, the authors thus postulate that these compounds target the LuxR ligand binding site. The modeling studies also indicated that the presence of the sulfamide group perturbs the hydrogen bond network in the proximity of the amide−lactone moiety in the ligand−protein complexes. The authors hypothesize that the antagonism displayed by the sulfonylurea derivatives could be related to this effect and that only a relatively slight perturbation is enough to induce significant antagonist activity.[135]

The research group of Ishida et al. has studied the use of *N*-acyl cyclopentylamine (Cn-CPA) derivatives as quorum sensing inhibitors (Figure 11). In a study published in 2007, they synthesized Cn-CPA derivatives with a variety of acyl chain lengths ($x = 1-10$ in Figure 11) and reported that C10-CPA (**49**, acyl side chain length of 10 carbons) was the most effective inhibitor of the LasR and RhlR quorum sensing systems in *P. aeruginosa* (see Figure 11 and Table 2, entry 13).[126] Later that year, Morohoshi et al. reported the effects of Cn-CPA ($x = 1-10$) on Spn quorum sensing in *Serratia marcescens* AS-1, with C9-CPA (**50**) showing the strongest inhibitory effect.[82] The authors note that the length of the acyl chain of the most effective Cn-CPA inhibitors for *P. aeruginosa* and *S. marcescens* differed considerably from the lengths of the acyl chains of their own AHLs. In a more recent study, the same research group reported on the inhibitory effects of Cn-CPA ($x = 2-9$) on the *V. fischeri* Lux quorum sensing system.[136] The most effective inhibitors in this bacterium were C6-, C7-, and C8-CPA (compounds **51**, **52**, and **53**, respectively). Thus, Cn-CPA compounds are representative of a general class of AHL analogues capable of antagonizing the LuxR, LasR, Rhl, and Spn quorum sensing systems. The researchers also demonstrated that the inhibitory effects of C9-CPA (**50**) on quorum sensing in *S. marcescens* and those of C6-CPA (**51**) on the LuxR quorum sensing system were better than those of halogenated furanone **54**, a naturally occurring compound known to inhibit quorum sensing in a number of bacteria (see below).

Nonenzymatic lactone hydrolysis is significant for AHL degradation in vivo, which is a potential drawback to the use of AHL analogues in a therapeutic context (see below). Suga and co-workers[124,125,137] and Spring and co-workers[138] have reported the synthesis of nonhydrolyzable BHL analogues **55** and **56** (Figure 12). Compound (*S*,*S*)-**56** was found to be a weak agonist, whereas (*S*)-**55** was more potent than BHL in a *P. aeruginosa* pigmentation assay (BHL-RhlR signaling system). However, a disadvantage associated with compound **55** is its tendency to slowly epimerize under physiological conditions. Thus, Spring and co-workers became interested in the development of configurationally stable BHL analogues.[77] The authors hypothesized that the weak activity of **56** was due to either an unfavorable hydrogen bond donation from the hydroxyl group to a suitable acceptor in the LuxR-type receptor or an unfavorable spatial preference of the *trans*-amino alcohol. It was postulated that a methoxy substituent in the hydroxyl position

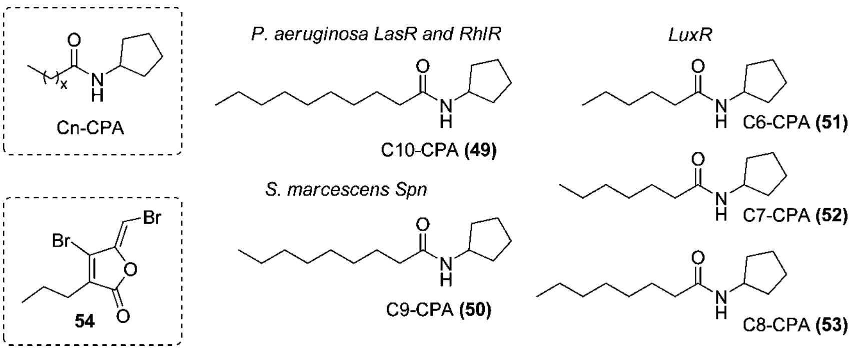


Figure 11. *N*-Acyl cyclopentylamides found to inhibit a variety of bacterial quorum sensing systems.[82,126,136]

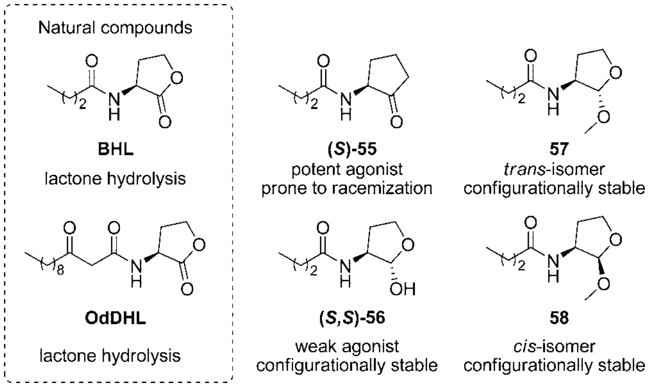


Figure 12. BHL analogues investigated by Spring and co-workers.[77]

would act as a hydrogen bond acceptor (for a conserved tryptophan residue in the LuxR-type binding pocket), while not being a hydrogen bond donor. Thus, the authors targeted *trans*- and *cis*-methoxy analogues of BHL (**57** and **58**, respectively). The compounds were synthesized and evaluated for biological activity in quorum sensing-regulated phenotypic assays with *P. aeruginosa* and *Serratia* (strain ATCC39006). The diastereomeric compounds were quorum sensing agonists but were significantly less active relative to (*S*)-**55** and BHL. Additional assays showed that **57** and **58** did not display any significant inhibitory activity. The authors conclude that the weak activity of **56**−**58** is not simply due to either an unfavorable hydrogen bond donation or an unfavorable spatial preference of the oxygen lone pair. Instead, it is possible that there is an unfavorable steric interaction due to the methyl group, or that the ketone may be hydrated in the binding pocket.

2.2.8. Superagonists

AHL analogues that display heightened activities relative to native AHLs (so-called superagonists) are of significant interest.[121] Such compounds, which are able to provoke the same response as the natural signal, but at a lower concentration, could potentially initiate bacterial group behaviors at lower cell numbers than those required in natural environments, which would be of value for the study, and potential manipulation, of bacteria−host interactions.[58,121] For example, a pathogen could be stimulated to initiate infection earlier than would naturally occur (i.e., at a lower population density), increasing the likelihood of successful clearance by the host's immune system.[121] However, it should be noted that there are experimental observations that cast some doubt upon the concept of developing superagonists capable of acting as early inducers of quorum sensing. Hentzer et al. have compared the expression profiles of AHL-induced genes in a signaling-deficient *P. aeruginosa* mutant (a *lasI rhlI* double mutant) with a wild-type strain.[16] Despite growing the mutants in the presence of saturating concentrations of OdDHL and BHL, the temporal expression profile and absolute expression levels of quorum-induced genes were similar to those observed in the wild-type. The fact that gene expression in the mutant could not be induced prematurely at low cell densities despite the presence of high doses of exogenous signaling molecules indicates that the onset of induction is not simply dictated by the signal concentration. That is, there is evidence that the timing of the onset of quorum sensing controlled induction (at least in the case of *P. aeruginosa*) is regulated by other factors in addition to the autoinducer.[16,139] Despite these observations, there are several reports in the literature regarding the discovery of superagonists of quorum sensing, including the LasR system of *P. aeruginosa*.

The structures of known non-native AHL analogues that have been reported to display superagonist activity (**6**, **59**, **60**, and **61**) are shown in Figure 13. Some of these compounds were identified in studies discussed in detail elsewhere in this review but are summarized here for the sake of clarity.

2.3. Beyond the AHL Framework

Despite the significant advancements made in the identification of synthetic AHL-based modulators of quorum sensing, there are still some drawbacks associated with the potential use of this class of molecules in real-world scenarios. The rational design of AHL-based quorum sensing compounds is innately hindered by the fact that most LuxR-type receptors show a very high specificity for their cognate autoinducers.[34] Thus, there are significant structural constraints placed upon any non-native small-molecule modulators of any given receptor, which are based on the AHL scaffold, with relatively little deviation from the parent framework generally tolerated without a relatively large loss in affinity.[34,142] As a consequence, the deliberate, rational optimization of any AHL "hits" to improve various molecular properties, for example, efficacy and selectivity, is also difficult. The high level of specificity displayed by LuxR-type proteins for their natural ligand may be one of the reasons why relatively few synthetic AHL-based derivatives capable of exhibiting heightened activities relative to native AHLs have been identified (although there are some examples of superagonist derivatives; see above). In the context

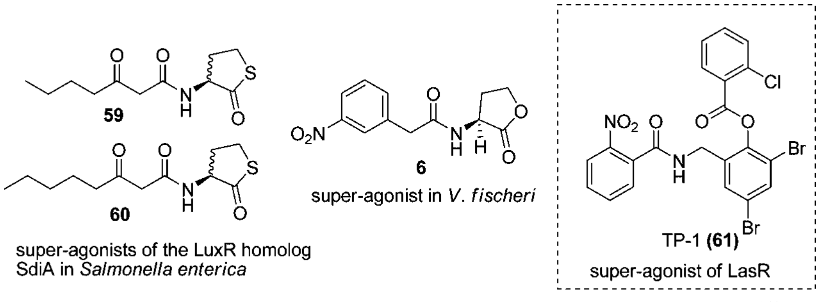
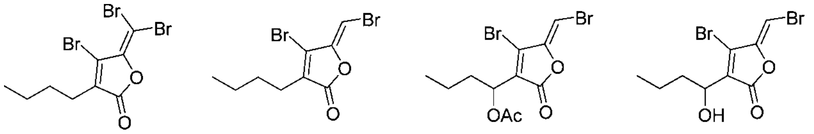


Figure 13. Known superagonists of quorum sensing. **59** and **60** come from the work of Janssens et al.,[140] and **16** comes from studies by Blackwell and co-workers.[12,121] TP-1 (**61**) is not based on the AHL scaffold and is discussed in more detail in section 2.3.5. TP-1 comes from the work of Muh et al.[141] Note that the original structure proposed for TP-1 (**61**)[141] was subsequently revised to that shown above.[113] Blackwell and co-workers have also noted that a native ligand for the SdiA receptor is yet to be reported; therefore, terming compounds **59** and **60** as superactivators must be done with caution.[12]



62 **63** **65** **64**

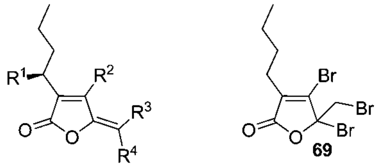
Figure 14. Some examples of furanones isolated from extracts of *D. pulchra*.

of the design of biologically useful synthetic AHL antagonists, this is of particular concern; most known AHL-based inhibitors are presumed to act in a competitive fashion (that is, they target the binding site occupied by the natural AHL ligand) and would only really be useful if they displayed a higher affinity for the receptor than the natural AHL. In addition, the homoserine lactone moiety is known to be unstable at alkaline pH and is readily degraded by mammalian lactonases.[138,143,144] Thus, the efficacy of any AHL-based pharmaceutical agent incorporating this structural feature is limited.[142]

As a result, there have been significant efforts in recent years toward the identification of new classes of small-molecule modulators of LuxR-type proteins that are structurally distinct from the native homoserine lactone autoinducers. Despite this interest, this field is still in its infancy. The most widely used method for the discovery of non-AHL based modulators has been the screening of either natural product isolates or synthetically derived chemical libraries; the use of rational design methods has yet to be exploited to the same extent, though these are expected to play an increasingly prominent role in forthcoming years as more knowledge is garnered regarding the structure of LuxR-type receptors (see below). In addition, the active agents identified from different studies tend to vary considerably in the nature of their core scaffolds. Because of the relatively limited number of ligands from each "scaffold class" examined to date, general SARs, including comparative assessments across more than one bacterial strain, for non-AHL based quorums sensing modulators are yet to be established. Nevertheless, a discussion of known non-AHL based ligands is valuable in terms of providing additional insights into the regions of chemical space spanned by small-molecule agents capable of modulating quorum sensing systems.

2.3.1. Furanones and Related Structural Analogues

In 1993 Denys et al. reported the isolation of >20 halogenated furanone compounds from extracts of marine algae *Delisea pulchra* (Figure 14).[145] These furanones, believed to be secondary metabolites produced by the algae, were known to have antimicrobial properties[146] and were subsequently shown to be capable of inhibiting surface colonization (biofouling) by bacteria.[147,148] In a landmark paper, Givskov et al. hypothesized that the biological effects of the furanones may be due to their ability to interfere with AHL-regulated quorum sensing systems.[32] The authors demonstrated that purified samples of the *D. pulchra* furanones **62** and **63** inhibit various AHL-controlled processes in prokaryotes without affecting their growth, specifically the swarming motility of *Serratia liquefaciens* and the bioluminescence produced by the bacterial strains *V. fischeri* and *Vibrio harveyi*.[32]

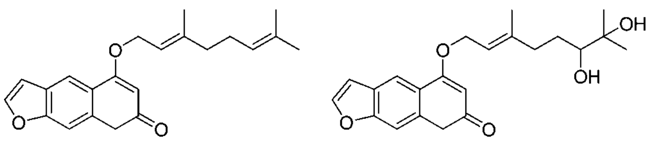


Furanone	R^1	R^2	R^3	R^4
62	H	Br	Br	Br
63	H	Br	H	Br
65	OAc	Br	H	Br
64	OH	Br	H	Br
66	OAc	Br	H	I
67	H	H	Br	Br
68	OAc	Br	Br	Br

Figure 15. General structure of natural and synthetic furanones used in AHL regulation bioassay systems by Kjelleberg et al.[154]

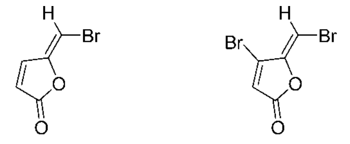
Gram et al. found that pure samples of natural furanone **64** were capable of inhibiting swarming motility in *Proteus mirabilis* at concentrations that did not affect growth rate and swimming motility, although compounds **62**, **63**, and **65** had no affect on this phenotype.[149] However, it should be noted that the regulatory target in this system is not known (although the authors state that these results are consistent with the involvement of a quorum sensing system). Natural furanone **63** inhibits swarming and biofilm formation in *Escherichia coli*,[150] although production of AHLs has never been observed in *E. coli*.[11,151] Furanone **63** has been shown to inhibit siderophore biosynthesis in *Pseudomonas putida*; this process is known to be regulated by a quorum sensing system, and the organism has been shown to produce AHLs.[152] Interestingly, stimulation of siderophore biosynthesis is seen in *P. aeruginosa*.[152] *D. pulchra* furanone **64** was found to inhibit quorum sensing controlled virulence and the OHHL-regulated production of the antibiotic carbapenem in *E. carotovora*.[153] In addition, Kjelleberg et al. have reported that several natural and synthetic halogenated furanones are capable of interfering, in a specific and nongrowth inhibitory fashion, with exoenzyme production (*S. liquefaciens*, *V. harveyi*, *P. aeruginosa*) and pigment production (*C. violaceum*), which are all AHL-regulated phenotypes (Figure 15).[154] Within the compound set analyzed, two structural prerequisites for this activity were identified: the presence of an exocyclic double bond at the carbon 5-position as well as an acetyl or hydroxyl group at the carbon 1′-position.

The natural *D. pulchra* furanone compounds are unable to inhibit the quorum sensing systems of *P. aeruginosa*; given the clinical importance of this pathogen, it is perhaps unsurprising that there has been considerable research directed toward the identification of new natural compounds and synthetic furanone analogues capable of inhibiting quorum sensing in this bacterium.[24,88] Girennavar et al. isolated the furocoumarins bergamottin (**70**) and dihydroxybergamottin (**71**) from grapefruit juice. These compounds



Bergamottin **(70)** Dihydroxybergamottin **(71)**

Figure 16. Compounds isolated from grapefruit juice capable of inhibiting quorum sensing regulated phenotypes.[155]



72 **73**

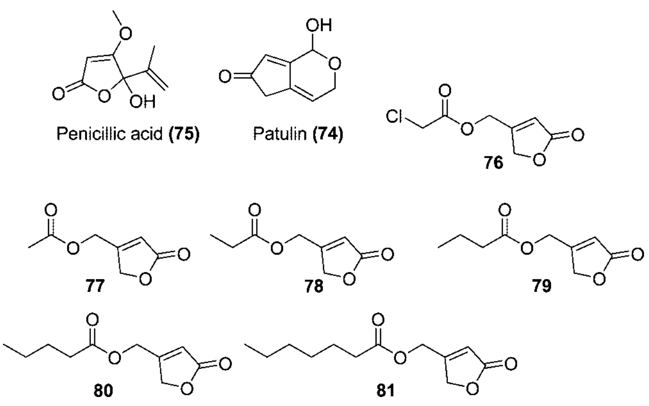
Figure 17. Synthetic furanone derivatives with quorum sensing inhibitory activity.[16,30,156]

are structurally reminiscent of the *D. pulchra* furanones and were found to inhibit a range of quorum sensing regulated activities in a variety of bacterial species, including the inhibition of biofilm formation in *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *P. aeruginosa* (Figure 16).[155] Inhibition of both AHL and AI-2 quorum sensing systems in *V. harveyi* was also observed (see below).

Hentzer et al. have reported the synthesis of **72**, an analogue that lacks the alkyl side-chain common to the natural *D. pulchra* furanones (Figure 17).[156] In vitro studies demonstrated that this compound was capable of interfering with AHL-mediated quorum sensing in *P. aeruginosa*; quorum sensing controlled reporter genes and virulence factors were inhibited, and although the compound did not inhibit the formation of biofilms, it did affect biofilm architecture.[29,156] Hentzer et al.[16] and Wu et al.[30] subsequently examined the effects of **72** and the bromo-derivative **73** on *P. aeruginosa* lung infections in mice (Figure 17). These compounds reduced the severity of lung pathology[30] and accelerated bacterial clearance from the lungs by the host.[16,30] In mice with lethal *P. aeruginosa* infections, treatment with either **72** or **73** resulted in significantly increased survival times.[30] These results indicate that both compounds function as quorum sensing inhibitors in vivo, which is in accordance with previous in vitro studies.[156] These studies are significant; they clearly demonstrate that bacterial virulence can be partially attenuated by the inhibition of quorum sensing and provide a proof-of-concept for the treatment of Gram-negative bacterial infections by quorum quenching.[16,30]

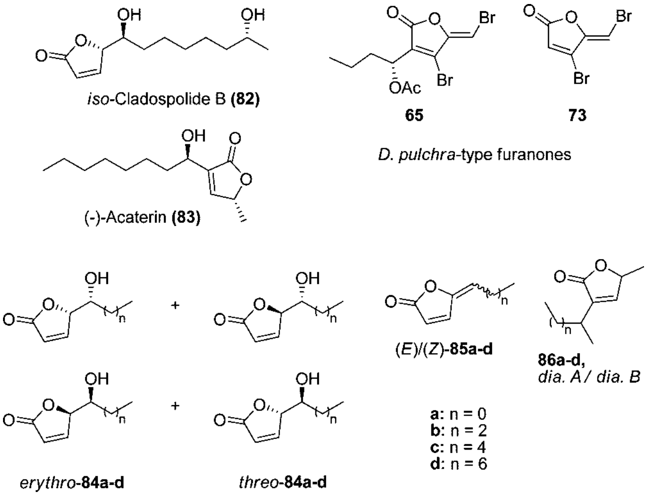
In 2005 Rasmussen et al. identified the naturally occurring compounds patulin (**74**) and penicillic acid (**75**), produced by *Penicillium coprobium* and *P. radicicola*, respectively, as inhibitors of quorum sensing in *P. aeruginosa*.[157] The ability of **74** and **75** to inhibit quorum sensing was verified by DNA microarray transcriptomics, which suggested that they taget the RhlR and the LasR proteins. In addition, in a mouse pulmonary infection model, patulin was found to accelerate the clearance of *P. aeruginosa* from the lungs of infected mice (when compared with the placebo group).

Kim et al. have since synthesized several synthetic furanone compounds **76**−**81** based on the structure of the natural products **74** and **75**; each has a common hydroxymethylfuranone core structure but a variable-length acyl side-chain or modified derivative thereof (Figure 18).[158] The compounds were assayed for their effects upon LasR quorum sensing using a recombinant *E. coli* bioassay reporter strain carrying a LasR expression plasmid and a *lasI::lacZ* fusion reporter plasmid (see section 2.2.7). The compounds were all found to significantly inhibit the OdDHL-dependent activity of LasR (as shown by repression of the LasR-driven expression of the *lasI::lacZ* fusion). This inhibition was found to be concentration-dependent, which led the authors to propose that these compounds most likely compete with native OdDHL for binding to the same binding site of LasR, i.e., they are competitive inhibitors. Compounds **78** and **76** showed the strongest inhibition of quorum sensing, with **81** being the weakest; all compounds were less effective inhibitors than patulin itself.



Penicillic acid **(75)** Patulin **(74)** **76** **77** **78** **79** **80** **81**

Figure 18. Furanone derivatives based on the natural products penicillic acid (**75**) and patulin (**74**).[158]



iso-Cladospolide B **(82)**

(-)-Acaterin **(83)**

65 **73**

D. pulchra-type furanones

(*E*)/(*Z*)-**85a-d**

86a-d, *dia. A / dia. B*

a: n = 0
b: n = 2
c: n = 4
d: n = 6

erythro-**84a-d** *threo*-**84a-d**

Figure 19. Natural products iso-Cladospolide B (**82**) and Acaterin (**83**). Three scaffolds (**84**, **85**, and **86**) based on these natural products were targeted and various analogues were synthesized for biological evaluation as quorum sensing inhibitors.[159] *dia.* = diastereoisomer.

A natural-product-guided approach for the discovery of furanone-based quorum sensing inhibitors was also utilized by Hjelmgaard et al.[159] They recognized structural similarities between the known quorum sensing antagonist furanones **65** and **73** and the natural products iso-Cladospolide B (**82**) and acaterin (**83**), which have biological activity unrelated to quorum sensing (Figure 19). These two natural products served as structural templates for the synthesis of target compounds based around three target scaffolds, namely, the *erythro/threo*-**84a**−**d** series (isolated either as individual isomers or as mixtures) and (*E*)/(*Z*)-**85b**−**d** and **86a**−**d**, both diastereoisomers (Figure 19).

The authors report a study of the structure−activity relationship of these furanone-based natural product analogues toward two quorum sensing systems: a *luxR*, *PluxI*-

gfp(ASV)-based monitor *E. coli* (pJBA89) in which fluorescent GFP expression can be induced by exogenous OHHL (the natural autoinducer)[160] and the quorum sensing dependent swarming motility of *S. liquefaciens*. For the *erythro/threo*-**84a**−**d** series, the compounds with the shorter alkyl chains were found to show greater antagonistic effect in the *E. coli* assay than compounds with the longer alkyl chains. The most active compound in this assay was *erythro/threo*-**84a**, whereas no effect was seen for *threo*-**84c**, *erythro*-**84d**, and *threo*-**84d**. The same effect was seen for **85b**−**d** where **85b** was the most effective of these compounds and **85d** showed no activity. For **86a**−**d** the most active compound was **86b**, *diameter A*, which was also the most active compound in general for the *E. coli* assay. This result is in good accordance with the structure of known active furanones **65** and **73** because these compounds, as well as **86b**, all contain an alkyl chain of butyl length in the 3-position. The synthetic analogues were found to be much less potent inhibitors in this assay than the halogenated furanone compound **65** and **73**, which the authors attribute to the presence of additional reactive groups on the furanones that are capable of interacting with LuxR-type proteins (see below). In the swarming assay, for the *erythro/threo*-**84a**−**d** series and **85b**−**d**, shorter alkyl chain length was again correlated with increased antagonistic effect, with only *erythro/threo*-**84a**, *erythro*,*threo*-**84b**, and **85b** showing an effect. Notably, the most active compound, *erythro*-**84b**, also has an alkyl chain of butyl length, though this time at the 5-position (c.f., **86b**). Compounds **86a**−**d** were largely inactive, with **86d**, *diameter A* showing some effect. Overall, the authors concluded that, in both assays, 5*H*-furan-2-ones substituted with short alkyl chains were in general more active quorum sensing antagonists than their longer-chain counterparts, and in both cases, differences in the activities between the different diastereoisomers were observed. However, there was little coherence between the relative activities for the different compounds in the two assays. The most active synthetic antagonists in this study both had a butyl chain in the 3- or 5-position, which is consistent with the biological activity of the natural *D. pulchra* furanone **65** and analogues thereof.

Estephane et al. have reported the synthesis of a variety of AHL−furanone "hybrid" molecules, that is, AHL-type derivatives that incorporate a furanone moiety rather than a homoserine lactone.[161] In total, six compounds **87**−**92** were synthesized, three of which incorporated halogen atoms at the 4-position and are thus structurally reminiscent of the *D. pulchra* furanones (Figure 20).

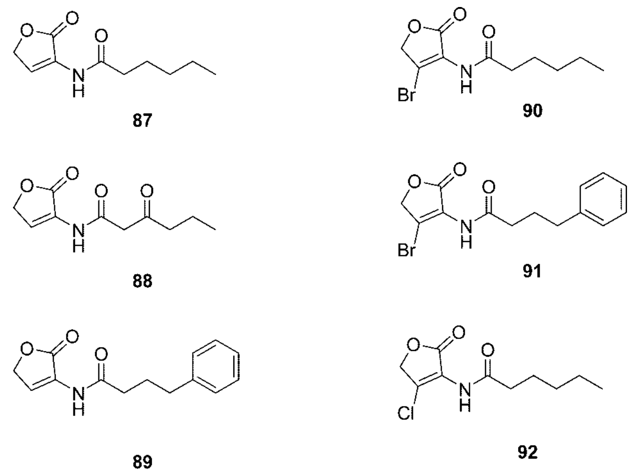


Figure 20. AHL derivatives with headgroups reminiscent of *D. pulchra* furanones.[161]

Both types of analogue proved to be inhibitors of LuxR-dependent quorum sensing in *V. fischeri*, with the halogenated compounds being significantly more active than their hydrogenated counterparts. Molecular modeling studies were carried out with compounds **87** and **90** and a LuxR ligand-binding site defined as a docking box with the natural ligand OHHL. The experiments suggest that the conjugated enamide group present in **87** and **90** induces a conformational change as compared to the saturated lactone; two preferential binding conformations leading to specific binding modes are now available. The two binding modes were designated I and II. The binding mode I for compounds **87** and **90** involved the same hydrogen-bond network as the natural ligand. However, the hydrogen-bond network of binding mode II for compounds **87** and **90** was different from that seen with the natural ligand. The authors postulate that this could be a plausible explanation for the antagonist activity of the synthesized compounds. The comparison of binding modes (both I and II) between compounds **87** and **90** showed a different orientation of the lactone moiety for **90** due to specific hydrophobic and electrostatic interactions of the bromine atom with residues strictly conserved or conservatively replaceable in the LuxR-type family of proteins. The authors postulate that these specific interactions may provide an explanation for the increased antagonistic activity of halogenated derivatives **90**−**92** relative to **87**−**89**.

Furanone analogues have also been utilized to facilitate the discovery of a structurally unrelated class of inhibitors of *P. aeruginosa* quorum signaling. Taha et al. have reported the discovery of three potent inhibitors of pseudomonal quorum sensing via pharmacophore modeling and in silico screening.[162] In this work, four brominated furanones with known activity against pseudomonal quorum sensing (**62**, **63**, **72**, and **73**) were used as "training" compounds to derive a hypothetical pharmacophore model for LasR-based quorum sensing inhibitors. The model was then employed to screen a selected range of known compounds (219 240) from the National Cancer Institute database to identify compounds that possessed chemical functionalities that spatially overlapped with corresponding features within the pharmacophore model. The 86 001 hits were subsequently fitted against the pharmacophore, and 19 of the highest ranking compounds (in terms of "tightness" of fit) were assessed for their effects of pseudomonal production of pyocyanin and pyoverdin, both phenotypes that are regulated by a hierarchical quorum sensing system. Most of the tested hits were inactive. However, one of the hits, **93**, was found to exhibit nanomolar inhibitory activity (IC_{50} values < 1 μM in the pyocyanin and pyoverdin assays, Figure 21).

The authors hypothesized that this activity was related to the presence of the lead atom and was mediated by an additional mechanism that complemented pharmacophore recognition within the binding pocket of LasR. The fact that mercury and lead have similar chemical reactivities and biological profiles prompted the authors to evaluate the antiquorum sensing activity of the known mecurial bromides thimerosal (**94**) and phenyl mercuric nitrate (**95**). Both compounds fit tightly onto the pharmacophoric model and exhibited significant quorum sensing inhibitory activity against *P. aeruginosa*. The authors postulate that these compounds are acting as active-site directed irreversible inhibitors of LasR, that is, initial selective and reversible

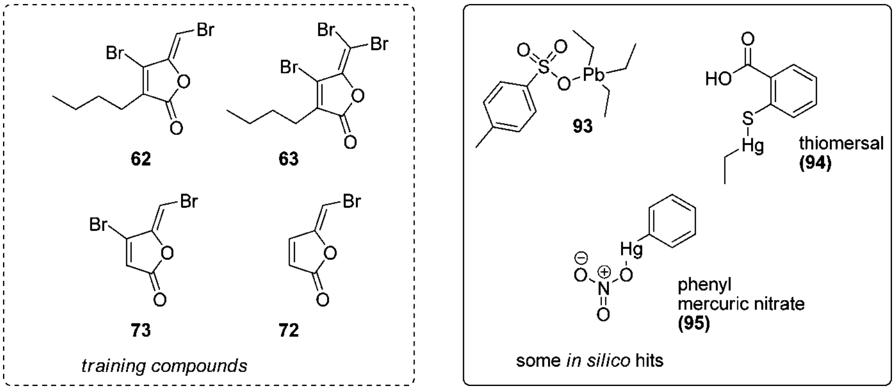


Figure 21. Furanone analogues **62**, **63**, **72**, and **73** were used as “training” compounds to derive a hypothetical pharmacophore model for LasR-based quorum sensing inhibitors.[162] In silico screening identified compounds that fit this model. One such molecule, **93**, exhibited nanomolar inhibitory activity against *P. aeruginosa* quorum signaling. **94** and **95** also fit the pharmacophore model but were approximately 2.5 and 10 times less active, respectively.

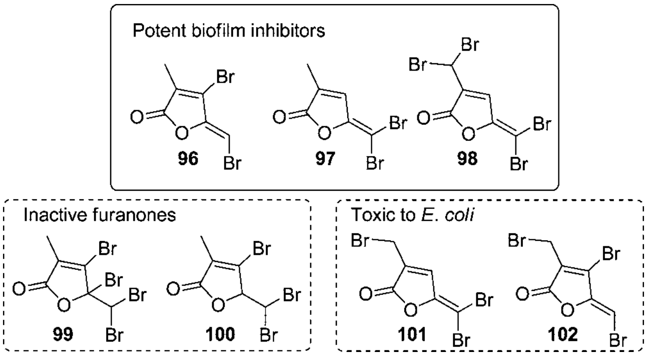


Figure 22. Structures of brominated furanones examined by Han et al.[163]

binding within the binding pocket occurs (i.e., pharmacophore recognition) followed by covalent bonding that connects a certain nucleophilic center within the binding pocket (probably a thiol group) with the metallic core of the inhibitors.

Han et al. recently reported the results of a study designed to identify the important structural elements of brominated furanones for inhibiting biofilm formation in *E. coli*.[163] It should be noted that, although *E. coli* does not have an AHL-dependent quorum sensing system, it does have a LuxR-type receptor (SdiA).[164,165] Thus, the bacterium may be expected to respond to AHLs (and compounds thought to modulate AHL-based signaling, such as the *D. pulchra* furanones) even though it does not produce AHLs itself.[164,165] In the work of Han et al., a series of structurally closely related brominated furanones **96**−**102** were synthesized and examined for their toxicity and ability to inhibit biofilm formation in this bacterium (Figure 22). The results indicated that there is a strong correlation between the structure and activity (both potency and toxicity) of this class of brominated furanones. **101** and **102** were found to be toxic to *E. coli* cells (bactericidal effects), with both compounds bearing monosubstituted bromides on an exocyclic methyl group. A comparison of the structures of the active and inactive compounds revealed a structural motif critical for biofilm inhibition, namely, a vinyl bromide at the δ-position of the extended conjugation of the furanone ring. The vinyl bromide on the furanone ring does not appear to be critical, and the monosubstitution of bromide on saturated carbon appears to decrease biofilm inhibition activity.

2.3.2. Mechanism of Action of Furanone-Type Inhibitors

The ability of *D. pulchra* furanones and synthetic analogues to disrupt quorum sensing in Gram-negative bacterial strains is well documented. However, the molecular targets and precise mode of action of such compounds remain elusive, and this is a topic of some debate in the literature.[153,166,167] The structural similarities between AHL molecules and furanones has led some to hypothesize that these compounds disrupt AHL-mediated quorum sensing in LuxI/LuxR-type systems by competitively binding to the AHL receptor site on the LuxR-type protein.[167] There is significant experimental evidence for this model.[32,88,154,156,168,169] For example, Givskov and co-workers demonstrated that the inhibitory effect of the *D. pulchra* furanones upon swarming motility in *S. liquefaciens* can be reversed by the addition of increasing concentrations of exogenous BHL, a native AHL signal in this species.[32,169] Manefield et al. have shown the furanone-mediated displacement of radiolabeled AHL molecules from LuxR.[88,168] However, Koch et al.[105] have studied the interactions between LuxR and halogenated furanones and could not conclude that these compounds bind to the AHL-binding site, instead suggesting that the furanones do not compete in a classical way with the signaling molecules.[167] Manefield et al. have also reported a study on the inhibition of quorum sensing in *E. carotovora* by furanone **64**. Their data suggest that furanone **64** cannot compete with the natural AHL, OHHL, for the LuxR-type receptor CarR.[153] It has also been demonstrated that halogenated furanones promote rapid turnover of the LuxR-type receptor protein, reducing the amount of the receptor able to interact with AHL and, thus, the amount of the subsequent complex able to act as a transcription regulator.[167,170] Thus, it has been hypothesized that halogenated furanones inhibit quorum sensing by inducing the degradation of the LuxR-type protein.[170] Some have suggested a model in which both factors are important, that is, one in which halogenated furanones inhibit AHL-mediated gene expression by occupying the AHL binding site of LuxR homologues *and* initiating accelerated turnover of the regulatory protein.[153] Defoirdt et al. have recently postulated that furanone **63** blocks quorum sensing in the bacterium *V. harveyi* by decreasing the DNA-binding activity of the transcriptional regulator protein LuxR.[167] The authors hypothesize that the furanone reacts with the LuxR protein in some unspecified way, thereby altering it in such a way that it cannot bind DNA anymore, either by altering the structure of the DNA-binding domain or the regions involved in dimer formation.[167] Furanones have also been demonstrated to interfere with the autoinducer-2 (AI-2) bacterial systems in some Gram-negative bacteria (see below).[150,166,171]

2.3.3. Other (Nonfuranone) Quorum Sensing Modulators

A variety of other compounds, structurally distinct from both AHLs and furanones, that are capable of modulating Gram-negative quorum sensing have been identified. In some cases, the mode of action of such agents, in terms of which quorum system is modified (e.g., AHL or AI-2) or which part of the system is targeted (e.g., synthase or receptor), is not specified; therefore, it may be the case that some of the compounds discussed in the following section do not effect AHL-based signaling via LuxR-type receptor modulation. However, for the sake of completeness, it is useful to summarize such agents in this section, even if a mode of action has not been delineated; it is hoped that such information will prove valuable to researchers within the field. The screening of natural extracts and synthetic compound libraries have been the most extensively used methods to discover non-AHL based agents structurally distinct from the furanone products discussed previously.

2.3.4. Natural Substances

Over the course of the past decade, a wide range of naturally occurring substances, particularly extracts from plants and foods, have been evaluated for their ability to modulate LuxR-type quorum sensing in Gram-negative bacteria. In many cases, desired activity has been observed; however, data pertaining to the precise structure of the bioactive molecular component(s) in such substances is not always available. In addition, the mechanism of action of many of these compounds is poorly understood; when structures can be obtained, a significant deviation from the AHL framework is usually observed and there is little structural correlation with any other quorum sensing-modulating agent whose molecular target is known. For example, the following natural substances are known to modulate various AHL-mediated quorum sensing systems or AHL-regulated phenotypes: clove oil (*C. violaceum* and *P. aeruginosa*),[172] dietary phytochemicals (secondary metabolites of plants, *C. violaceum* and *P. aeruginosa*),[173] honeys (*E. carotovora*, *Yersinia enterocolitica*, *Aeromonas hydrophilia*, and *C. violaceum*),[174,175] cranberry juice (*V. harveyi*),[176] extracts of *Tremella fuciformis* (white jelly mushroom, *C. violaceum*),[177] extracts of various medicinal plants from the Indian subcontinent (*P. aeruginosa*),[178] extracts of pea (*Psium sativum*) seedlings (*C. violaceum*),[179] extracts of *Medicago truncatula* seedlings (*P. putida* CepR reporter, *E. coli* LuxR reporter, *E. coli* LasR reporter, and *C. violaceum*),[180] and extracts of various South Florida plants (*P. aeruginosa*).[181] Ellagitannin natural products from various medicinal plants have also shown antiquorum sensing activity (various Gram-negative bacteria including *P. aeruginosa*).[182,183] Subinhibitory concentrations of phenyl lactic acid, known to be produced by *Lactobacillus* probiotic strains, have been shown to attenuate *P. aeruginosa* virulence and pathogenicity by interfering with different processes regulated by quorum sensing.[184] Tannic acid, a plant polyphenol, has been shown to inhibit quorum sensing systems in various Gram-negative bacteria.[185] Ethanolic extracts of the plants *Sonchus oleraceus* and *Laurus nobilis* have been shown to inhibit quorum sensing in the Gram-negative bacterium *C. violaceum*.[186]

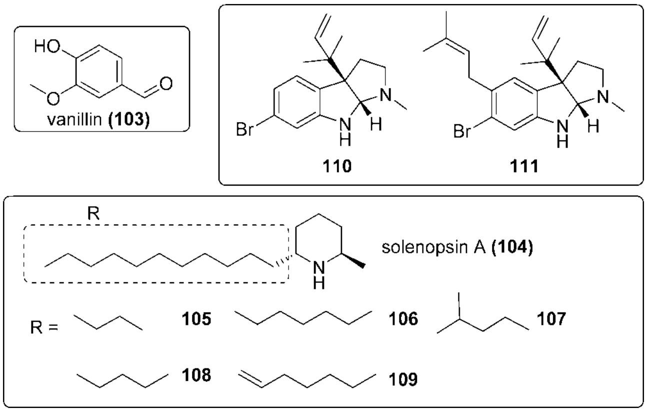


Figure 23. Quorum sensing inhibitors identified by Choo et al.[187] and Ponnusamy et al.[188] (vanillin **103**), Peters et al.[190] (alkaloids **110** and **111**), and Park et al.[189] (solenopsin A and analogues **104**−**109**).

Vanillin extracts were demonstrated by Choo et al. to have antiquorum sensing activity in *C. violaceum*,[187] and recent studies by Ponnusamy et al. suggest that vanillin (**103**) itself may be the active agent (Figure 23).[188] Park et al. have reported that solenopsin A (**104**), a venom alkaloid from the fire ant *Solenopis invicta*, inhibits quorum sensing in *P. aeruginosa*.[189] This compound is structurally reminiscent of OdDHL (the natural AHL of the LasR system) in that both contain a long hydrocarbon chain attached to a nitrogen-containing heterocycle, via a chiral carbon; however, both structures contain distinct molecular frameworks. Interestingly, exogenously added BHL, but not OdDHL, restored *P. aeruginosa* quorum sensing signaling, suggesting that solenopsin A actually targets the BHL-dependent *rhl* quorum sensing system. Several solenopsin analogues **105**−**109** were investigated, but none demonstrated increased quorum sensing inhibition relative to the parent compound. Analysis of the secondary metabolites of the North Sea bryozoan *Flustra foliacea* led to the isolation of a variety of brominated alkaloids, two of which (**110** and **111**) were found to specifically block AHL-regulated gene expression.[190] Both compounds caused a reduction in the signal intensities in biosensor strains of *P. putidia* and *E. coli*. In addition, compound **110** was capable of suppressing the production of extracellular proteases in *P. aeruginosa*, a phenotype associated with the virulence of this pathogen, which is under the stringent control of AHL-dependent quorum sensing systems.

Park and co-workers have reported that extracts of the red alga *Ahnfeltiopsis flabelliformis* inhibited quorum sensing mediated by OHL and the TraR transcriptional activator protein.[191,192] Using activity-guided fractionation, they isolated an active fraction containing betonicine (**112**), floridoside (**113**), and isethionic acid (**114**) (Figure 24). Individual samples of each of these compounds were obtained, and their quorum sensing inhibition activities were examined. None of the three compounds exhibited inhibition activity when tested individually. In contrast, a complex of floridoside and isethionic acid revealed a dose-dependent inhibition on OHL activity, suggesting that these two compounds are responsible for the inhibition activity of red algae extract. Unexpectedly, betocine and *cis*-betocine showed a dose-dependent stimulatory effect in TraR-mediated quorum sensing responses.

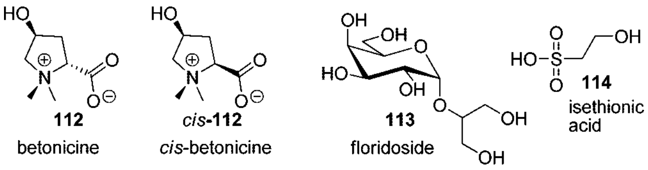


Figure 24. Compounds isolated from the alga *Ahnfeltiopsis flabelliformis* by Park and co-workers that were tested for antiquorum sensing activity.[191,192]

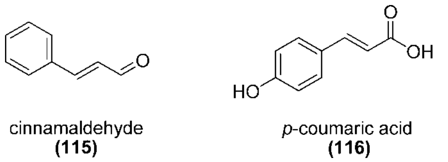


Figure 25. Cinnamaldehyde (**115**)[194] and *p*-coumaric acid (**116**)[195] have been found to have antiquorum sensing activity.

Niu and co-workers screened plant essential oil components and isolated those that modulated biofilm formation in *E. coli*.[193] One of these components was identified as cinnamaldehyde (**115**), which was subsequently reported to be an effective inhibitor of 3-oxo-C6-HSL (OHHL) quorum sensing in *E. coli* and 3-hydroxy-C4-HSL quorum sensing in *V. harveyi* at subinhibitory concentrations (Figure 25).[194] In the case of *V. harveyi*, this inhibition was not selective for AHL quorum sensing, with the AI-2 system also being affected (see below). Interestingly, cinnamaldehyde (**115**) had minimal effect on *lasR* promoter activity, induced by OdDHL, in an *E. coli* strain containing a LasR biosensor (OdDHL inducible). From these results, the authors postulate that the 3-carbon aliphatic side chain of cinnamaldehyde (**115**) may interfere with the binding of the smaller 3-hydroxy-C4- and 3-oxo-C6-HSL AHLs to their cognate receptors but was not sufficiently long enough to substantially reduce the binding of OdDHL to LasR. Bodini et al. have recently reported that *p*-coumaric acid (**116**), a natural compound primarily produced by plants, has putative antagonistic activity against *Chromobacterium*, *Agrobacterium*, and *Pseudomonas* quorum sensing.[195] *p*-Coumaric acid (**116**) and cinnamaldehyde (**115**) share a common structural motif, namely, an $\alpha-\beta$-unsaturated carbonyl functionality connected to a phenyl ring system.

Recently, Vandeputte et al. screened extracts of the bark of *Combretum albiflorum* for their capacity to inhibit the production of extracellular virulence factors in *P. aeruginosa*.[196] Several active fractions containing flavonoid-like compounds were isolated; purification and structural characterization of one of the active compounds led to the identification of the flavan-3-ol catechin (**117**), which was also found to have a negative impact on the transcription of several other quorum sensing-related genes in this bacterium (Figure 26). Catechin (**117**) is thought to possibly interfere with the perception of the native AHL by the LuxR homologue RhlR, though little is known about the precise mechanism of action.

Rasmussen et al. screened a library of synthetic compounds and a selection of extracts from food sources and herbal medicines for quorum sensing inhibitors and reported that 4-nitropyridine-*N*-oxide (**118**) and crude toluene extracts of garlic specifically inhibit quorum sensing-regulated gene expression in *P. aeruginosa*, as judged from DNA microarray-based transcriptome analysis (Figure 26).[17] Subsequent in vitro studies on *P. aeruginosa* biofilms demonstrated that garlic extract significantly reduced the tolerance of the bacteria to the antibiotic tobramycin.[17] Bodini et al. have also found that garlic extracts can antagonize the activity of the quorum sensing receptors AhyR and TraR.[195] Rasmussen and co-workers have recently demonstrated that garlic extract promotes rapid clearing of mice pulmonary *P. aeruginosa* infections in vivo.[197] Through bioassay-guided fractionation, six sulfur-containing compounds from the garlic extract, **119**−**124**, were identified that inhibited quorum sensing in a LuxR monitor system.[198] Compounds **119**−**122** antagonized LuxR but were toxic to the bacteria. Compounds **123** and **124** possessed quorum sensing activity exclusively, but only in the LuxR monitor system; none of the sulfur-containing compounds **119**−**124** have activity against *P. aeruginosa* quorum sensing. A number of AHL derivatives that incorporated sulfide, sulfinyl, and sulfonyl motifs were subsequently synthesized; a significant portion of these compounds were found to inhibit either one or both of the LuxR and LasR quorum sensing systems.

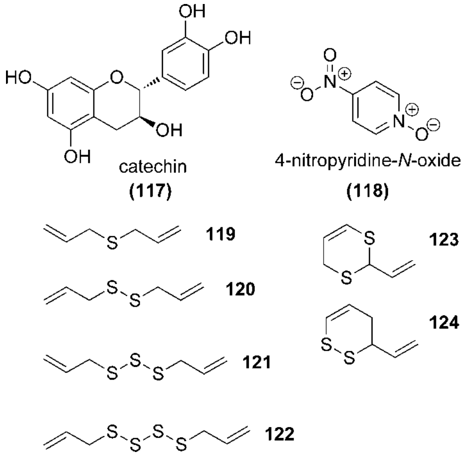


Figure 26. Compounds with antiquorum sensing activity identified by Vandeputte et al.[196] (catechin) and Rasmussen and co-workers[17,198] (4-nitropyridine-*N*-oxide and **119**−**124**).

2.3.5. Synthetic Compounds

An ultrahigh throughput cell-based assay was developed by Muh et al. and used to screen a library of ~20 000 compounds for inhibitors of LasR-dependent gene expression.[199] The two most active compounds identified were **125** and **126**, designated V-06-018 and PD12, respectively (Figure 27). Both compounds contain a 12-carbon aliphatic chain, which is analogous to that present in the native Las-dependent signaling molecule OdDHL; nevertheless, they are examples of quorum sensing inhibitors with a non-AHL core scaffold. A focused library of 66 compounds was synthesized to explore the SAR of PD-12 (**126**); compounds were designed to investigate the effect of various groups at the C-5 position of the tetrazole and also the effects of varying

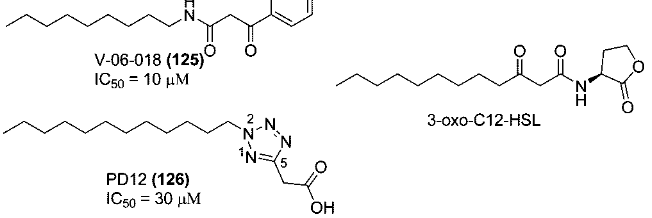


Figure 27. V-06-018 (**125**) and PD12 (**126**) were found to act as inhibitors of *P. aeruginosa* quorum sensing. The native LasR signaling molecule, OdDHL, is shown for comparison.[199]

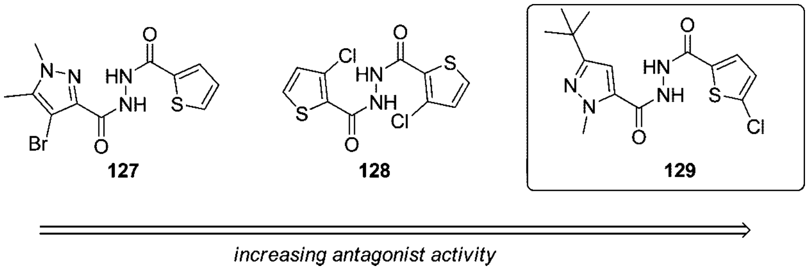


Figure 28. Evolution of quorum sensing inhibitor compound **129**.[200] Compound **127** was an initial hit. The activity of this compound was improved by iterative rounds of virtual screening and activity testing, yielding compound **128** and finally compound **129**, which represented the end point of the optimization procedure.

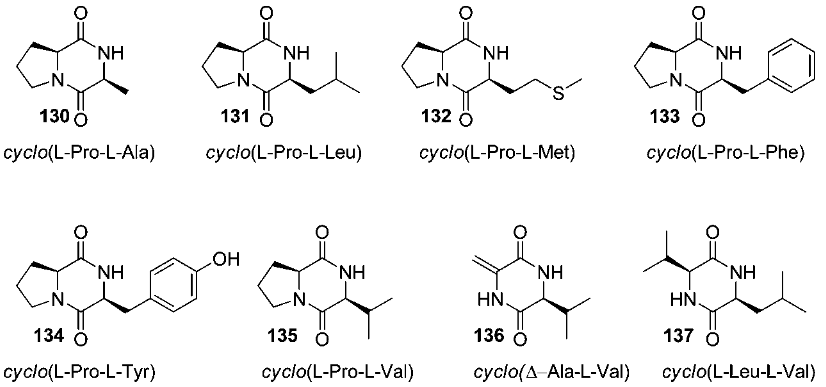


Figure 29. DKPs reported to modulate LuxR-type protein activity.

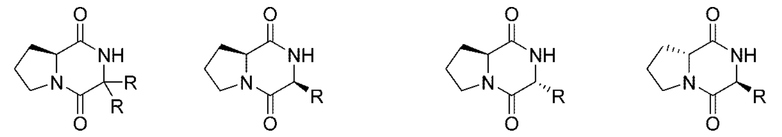
alkyl chain length and regioisomeric substitution at either the N-1 or N-2 position of the tetrazole. The optimal tetrazole inhibitor carried an acetic acid moiety at C-5 and was substituted with an alkyl chain of C_{12} (PD12, **126**) or C_{14} at the N-2 position. The authors suggest that compounds **125** and **126** might function as inhibitors by interacting directly with the native-AHL-binding site on LasR. That is, the inhibitors compete with OdDHL for binding to LasR but do not provoke whatever conformational change is needed in LasR to activate gene transcription.

In 2006, Riedel et al. reported the computer-aided design of molecules completely unrelated structurally to AHLs that are capable of inhibiting the *cep* quorum sensing system of *Burkholderia cenocepacia*; the system employs the autoinducer OHL and the LuxR homologue CepR (Figure 28).[200] Virtual screening of a compound database was carried out to identify those that were most similar to the reference molecules OdDHL and OHL in terms of shape and possible molecular interactions. Compounds from this initial screen were tested in various bioassays, and on the basis of these results, more focused virtual combinatorial libraries were generated. Iterative cycles of virtual screening and testing were used to improve the biological activities of the compounds. After several rounds proceeding via compounds **127** and **128**, compound **129** was identified as a novel specific inhibitor of the *cep* quorum sensing system in *B. cenocepacia*, interfering with a variety of quorum sensing regulated functions (including swarming motility, biofilm formation, and the expression of the virulence factor AidA) without affecting bacterial growth. Exogenous addition of OHL reversed the inhibitory effect of **129**, suggesting a competitive inhibition mechanism.

Over the course of the last 20 years, a set of cyclic dipeptides (2,5-diketopiperazines, or DKPs) have been isolated, either individually or as mixtures from culture supernatants of a range of bacterial species. Eight DKPs (**130**−**137**) have been reported to modulate LuxR-type receptor activity in sensitive AHL biosensor strains previously considered specific for AHLs (Figure 29).[201−203] For example, **134** and **136** were reported to be weak competitive inhibitors of the OHHL-mediated activation of LuxR in *E. coli*.[203]

However, recent work by Blackwell and co-workers has challenged the accepted hypothesis that DKPs modulate Gram-negative quorum sensing through interaction with the LuxR-type proteins.[204] Blackwell and colleagues designed and synthesized a collection of non-natural DKPs (**138**, **139**, **140**, and **141**) to determine the structural features necessary for LuxR-type protein activation and inhibition and to probe their mechanism of action (Figure 30). These DKPs, together with previously reported natural DKPs **130**−**135**, were examined for their ability to agonize and antagonize well-characterized LuxR-type proteins (TraR, LasR, and LuxR itself) using both sensitive biosensor strains and reporter strains with native protein levels.

The previously reported DKPs **130**−**135**, and all of the synthetic DKPs derived from natural α-amino acids, failed to exhibit either antagonistic or agonistic activities in the native protein level reporter strains examined. However, two synthetic DKPs derived from non-natural amino acids (**139b** and **139c**) were shown to be capable of inhibiting, but not activating, luminescence in *V. fisheri*. Further work indicated that this inhibition does not occur through interaction with the LuxR protein; that is, although these DKPs are capable of modulating the LuxR quorum sensing system, they do not compete with the natural ligand OHHL for LuxR. Thus, questions still remain as to the mode of action of DKPs in bacterial systems. Campbell and Blackwell have also recently reported the use of a macroarray format to efficiently synthesize a library of over 400 structurally more complex DKPs.[205] In a solution-based cell-based assay, the authors identified six DKPs **142**−**147** capable of inhibiting the



cyclo(L-Pro-Xxx)

138a-b;
a: Aib
b: Gly

cyclo(L-Pro-L-Xxx)

139a-i;
a: Ile
b: 4-Cl-Phe
c: 4-I-Phe
d: 3-NO$_2$-Phe
e: 4-NO$_2$-Phe
f: Pro
g: Ser
h: Thr
i: Trp

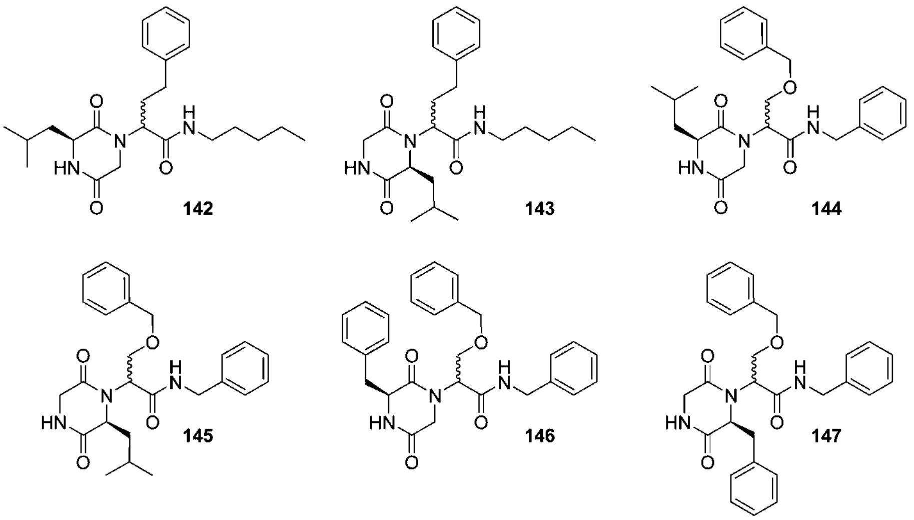
cyclo(L-Pro-D-Xxx)

140a-f;
a: Leu
b: Met
c: Phe
d: Pro
e: Tyr
f: Val

cyclo(D-Pro-L- Xxx)

141a-f;
a: Leu
b: Phe
c: 4-Cl-Phe
d: Trp
e: Tyr
f: Val

Figure 30. Structures of the four focused DKP sublibraries synthesized and evaluated in the study by Blackwell and co-workers.[204]



142 **143** **144** **145** **146** **147**

Figure 31. Structures of the six most active DKPs identified in a study by Campbell and Blackwell.[205]

quorum sensing-modulated luminescence phenotype of *V. fisheri* by at least 80% at a concentration of 500 μM (Figure 31).

Muh et al. employed a library of >200 000 compounds in an in vitro screen designed to detect compounds capable of modulating the LasR quorum sensing system of *P. aeruginosa*.[141] This led to the identification of a novel triphenyl scaffold-based compound TP-1 (**61**), which was shown to be a potent activator of LasR-dependent signaling despite the lack of any appreciable structural similarities to the LasR autoinducer OdDHL (Figure 32). Indeed, TP-1 (**61**) was found to be a superagonist of LasR, a more potent activator of the receptor than the natural signal.[141] Four derivatives of TP-1 (**61**) were synthesized: TP-2 (**148**), TP-3 (**149**), TP-4 (**150**), and TP-5 (**151**). TP-1–TP-4 were also found to activate the same signaling pathway, whereas TP-5 (**151**) was found to be an antagonist. Subsequent research established that TP-1–TP-4 act directly through the LasR receptor in a highly selective fashion. Zou and Nair have recently reported the high-resolution structure of the ligand-binding domain of the LasR receptor in complex with TP-1–TP-4.[113] Analyses of these crystal structures allowed delineation of the process of recognition of these novel compounds by LasR at a molecular level, which represents a significant advancement in the understanding of the molecular basis of quorum sensing in *P. aeruginosa*.[142] Such data should facilitate the rational design of novel inhibitors, based around the triphenyl scaffold (and perhaps other frameworks), that target intercellular signaling in this pathogenic bacterium. In addition, this work provides a molecular rationale for understanding how structurally distinct classes of compounds can interact with the same highly selective receptor; this information should provide a framework for a deeper understanding of the molecular basis behind the activity of previously identified small-molecule modulators of LasR-dependent signaling. This knowledge may assist in the rational modification of such agents to improve various properties (e.g., efficacy and selectivity).[142]

Peng et al. have reported the identification of a range of sulfone-type derivatives capable of modulating both the AHL and AI-2 quorum sensing systems of *V. harveyi* (see below).[206]

In a recent report, Amara et al. described a novel mode of quorum sensing inhibition based around the use of small-molecule agents (termed covalent probes) to covalently modify, and thus inactivate, LuxR-type receptors.[34] The basic rationale behind this method is the use of small molecules that are carefully designed such that they fulfill two criteria. First, they should present only a minimal deviation in structure from the parent autoinducer and consequently should bind with high affinity and specificity to the cognate receptor (see above). Second, the probes should contain a small reactive moiety that can react with, and thus form a covalent bond to, a residue in the LasR-type protein binding pocket. Such covalent probes would be expected to compete effectively with the native AHL for binding to LasR-type protein; once they are bound, a chemical reaction occurs, causing a conformational change in the protein complex such that it binds its target DNA less effectively. Thus, quorum sensing-regulated gene expression is specifically inhibited.

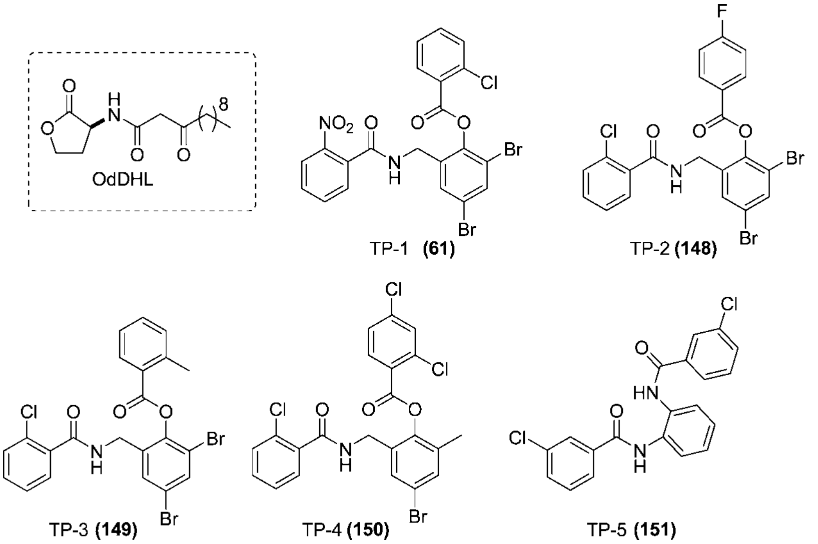


Figure 32. Chemical structure of OdDHL, the native ligand for the LasR receptor, together with the structures of the non-native TP-ligands developed by Muh and co-workers.[113,141,142,199] Note the original structures proposed for TP-1−TP-4 in 2006[141] were subsequently revised to give those shown previously.[113]

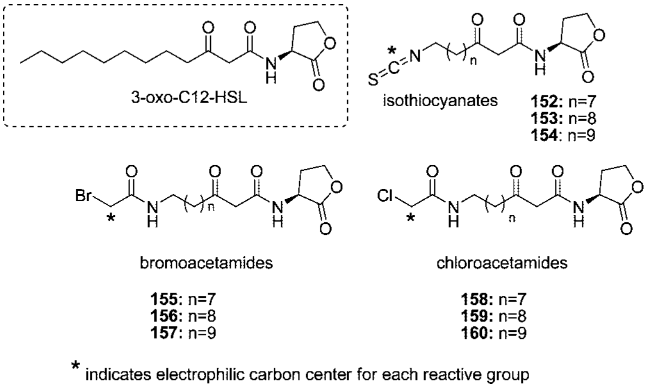


Figure 33. Structure of the natural *P. aeruginosa* LasR autoinducer OdDHL and nine synthetic analogues designed to be "covalent probes" for the LasR receptor. The electrophilic carbon center for each class of probe molecule is indicated.[34]

In this report, the authors focused on the LasR system in *P. aeruginosa*. The crystal structure of the ligand-binding domain of LasR complexed to its natural ligand had previously been determined; LasR is known to contain a nucleophilic cysteine residue in close proximity to the end of the alkyl chain of the bound natural ligand.[109] Therefore, a series of small molecules with electrophilic functionality (isothiocyanates **152**−**154**, bromoacetamides **155**−**157**, and chloroacetamides **158**−**160**) were designed (Figure 33). Through the use of well-characterized reporter strains, the influence of these probes on quorum sensing-related gene expression in *P. aeruginosa* was evaluated. Ambiguous biological effects were seen for the haloacetamides, with **156** showing strong activity. No covalent interactions between any of the haloacetamides and LasR were observed. This suggested that the inhibitory effects of the haloacetamides may be mediated in a manner similar to other strong inhibitors, namely, via binding to nascent LasR followed by protein misfolding and precipitation.[34] The isothiocyanate analogues were shown to inhibit a variety of quorum sensing-regulated activities including the production of virulence factors and biofilm formation. Further research established that the most potent inhibitors were actually acting as partial agonists, effectively inhibiting these quorum sensing-regulated phenotypes at moderate concentrations. In addition, the authors demonstrated that these isothiocyanate-based probes covalently and selectively bound Cys79, found in the LasR binding pocket. Covalent modification of the quorum sensing receptor LasR was thus demonstrated to be an effective means of attenuating *P. aeruginosa* quorum sensing with small molecules, and potentially this method could be applied in other bacterial systems. However, the rational design of suitable covalent probes for a particular quorum sensing system is dependent upon detailed knowledge of the structure of the ligand-binding domain of cognate receptor. Such information is not known for a large number of LuxR-type receptors, which may limit studies using deliberately designed agents and instead encourage a high-throughput screening approach utilizing small-molecule libraries.

2.3.6. Recognized Drugs

A variety of recognized drugs have been shown to have quorum sensing activities in addition to the biological effects for which they are marketed. For example, some macrolide antibiotics have been shown to inhibit AHL-mediated quorum sensing, possibly at the level of the ribosome (see above). Nonmacrolide antibiotics have also been reported to have effects upon quorum sensing systems in Gram-negative bacteria. Skindersoe et al. discovered that the antibiotics iprofloxacin (**161**) and ceftazidime (**162**) are capable of decreasing the expression of a range of quorum sensing-regulated virulence factors in *P. aeruginosa* at subinhibitory concentrations (Figure 34).[57] In silico docking experiments suggested that these compounds have a low affinity for the LasR receptor site and led the authors to postulate that they may exert their quorum sensing regulatory effects through novel, as yet undetermined, mechanisms. Conversely, through structure-based virtual screening of known drugs, Yang et al. identified that three compounds, salicylic acid (**163**), nifuroxazide (**164**), and chlorzoxazone (**165**), that inhibit *P. aeruginosa* quorum sensing are believed to act via interaction with the LasR protein.[207] The authors speculate that competitive binding of these quorum sensing inhibitors to LasR occurs with the protein in a more open

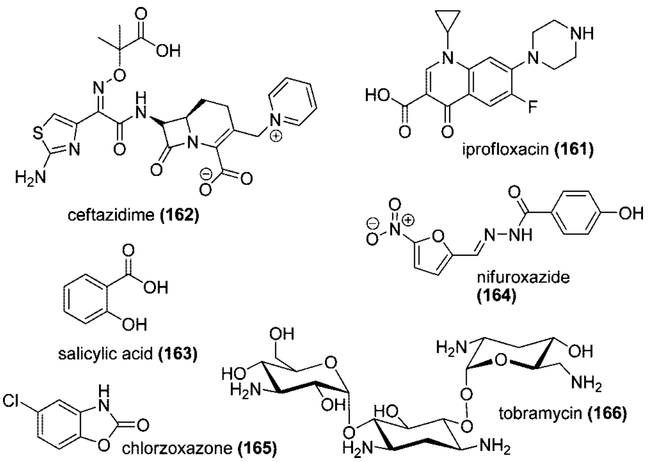


Figure 34. Structures of some known drug molecules that have been shown to be able to modulate AHL-mediated quorum sensing.

conformation than that which is associated with the binding of the native AHL, where the ligand is deeply buried in a cavity inside the protein. Garske et al. have reported that the antibiotic tobramycin (**166**) is capable of reducing virulence factor (elastase) production in *P. aeruginosa*.[208] The capability of known drug molecules to attenuate bacterial quorum sensing highlights the potential of using these compounds in multiple medical applications and suggests that we have not yet fully explored and exploited their therapeutic potential.

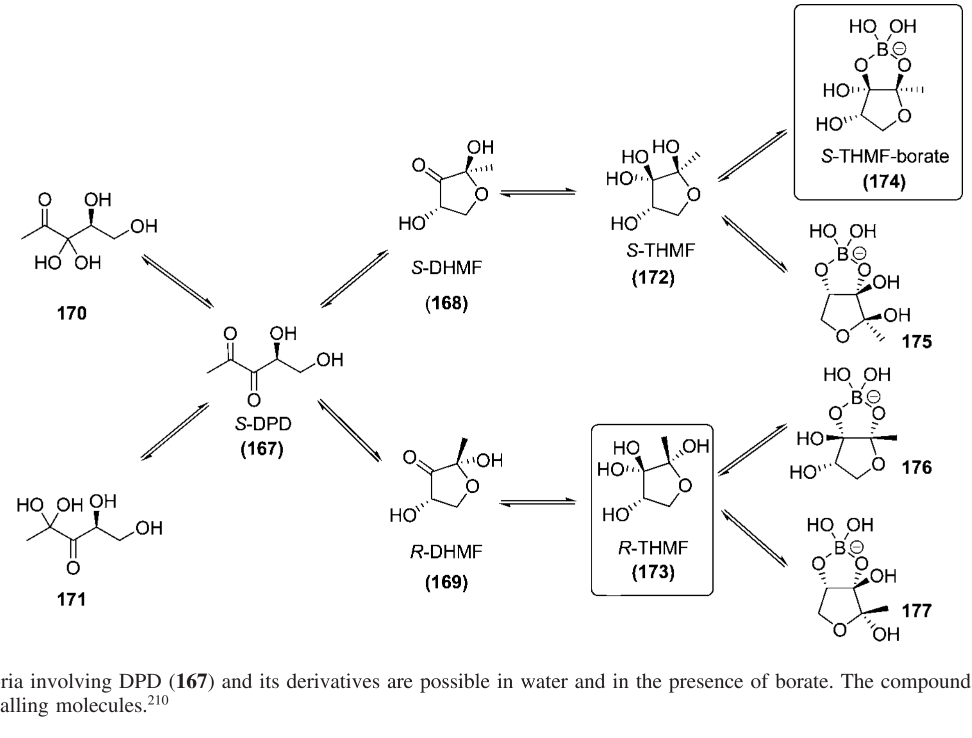
3. AI-2: Interspecies Communication

AHLs and peptides represent the two major classes of known bacterial signaling quorum sensing molecules, used by Gram-negative and Gram-positive bacteria, respectively, for intraspecies communication. Recently, a family of molecules generically termed autoinducer-2 (AI-2) has been found (Scheme 1).[209] It has been proposed that AI-2 is a nonspecies-specific autoinducer that mediates intra- and interspecies communication among Gram-negative and Gram-positive bacteria.[40,209,210]

AI-2-based quorum sensing was first identified in the early 1990s in the Gram-negative bacterium *V. harveyi*.[211] It was observed that an AHL-deficient strain of the bacterium remained capable of producing bioluminescence even in the absence of the natural AHL autoinducer 3-hydroxy-C4-HSL.[212] This suggested that a second quorum sensing pathway, employing a different signaling molecule, was operating. This novel autoinducer, whose structure at the time was unknown (although has since been elucidated, see section 3.2), was termed AI-2. It was subsequently shown that cell-free culture fluids from a number of bacterial species were capable of stimulating activity in a *V. harveyi* AI-2 reporter strain.[213] This suggested that the AI-2 signal may be produced by numerous bacterial species. Later work demonstrated that the same gene was responsible for AI-2 biosynthesis in *V. harveryi*, *E. coli*, and *S. typhimurium*.[214] This gene, designated *luxS*, has since been found in over 70 bacterial species;[212] in all known cases, if a bacterium produces AI-2, it possesses a *luxS* gene, and if this gene is inactivated, AI-2 production is eliminated.[40] These observations have led to the proposal that AI-2 is a universal signaling molecule for interspecies communication.[38] It should be noted that the product of the *luxS* gene, the enzyme LuxS, is thought to have a metabolic role in cells, in addition to being responsible for AI-2 biosynthesis.[38,212] This may provide an alternative explanation for the widespread conservation of *luxS*.[38] In spite of this controversy, there is a growing body of evidence that AI-2 does indeed represent a universal language for interspecies communication.[38,40,212]

AI-2 signaling is known to be much more complex than that mediated by AHLs. The pathways involved in AI-2-based quorum sensing have been reviewed extensively recently.[38] In this manuscript, we focus on work pertaining to the use of small-molecule agents to modulate various aspects of the AI-2 signaling process. The AI-2 system has been correlated with pathogenicity in a variety of organisms

Scheme 1. AI-2 Pool[a]



[a] Complex equilibria involving DPD (**167**) and its derivatives are possible in water and in the presence of borate. The compounds highlighted in boxes are known AI-2 signalling molecules.[210]

and is known to regulate a host of bacterial processes including virulence and biofilm formation.[49,215,21638,40,42−48] Thus, the identification of small-molecule agents that are capable of interfering with AI-2-mediated signaling may provide a possible means to achieve broad-spectrum antivirulence and has thus attracted significant attention in recent years.[49]

3.1. AI-2 System: Synthesis of the Signal

The enzyme LuxS, the product of the gene *luxS*, which is widely conserved throughout the bacterial kingdom, is responsible for AI-2 biosynthesis.[217] LuxS synthesizes 4,5-dihydroxy-2,3-pentanedione (DPD, **167**), which undergoes spontaneous rearrangements to form a variety of DPD derivatives that interconvert and exist in equilibrium (known as the AI-2 pool). Cyclization of DPD generates compounds *S*-DHMF (**168**) and *R*-DHMF (**169**), hydration generates **170**−**173**, and boronoate ester formation from DPD occurs if enough borate is present in solution to generate **174**−**177** (Scheme 1).[209,210] Different bacterial strains recognize different DPD derivatives; the interconversion of molecules within the AI-2 pool therefore presumably allows bacteria to respond to their own AI-2 and also to AI-2 produced by other bacterial species.[217] It has thus been proposed that AI-2 may serve as a universal signal for interspecies cell−cell communication.[209,217]

3.2. AI-2 System: Signal Detection and Gene Transcription

AI-2 responses in different bacterial species can be triggered by different members of the AI-2 compound pool (see above). Because the chemical nature of the active signaling molecule from this pool varies between species,[217] it is unsurprising that the nature of the AI-2 receptor for these signals is also variable. To date, only three proteins that bind AI-2 signaling components have been characterized.[218] In 2002, Chen et al. solved the crystal structure of the receptor protein involved in AI-2 signaling in *V. harveyi*, LuxP, complexed to its native DPD-derived ligand.[209] This ligand was thus identified as *S*-THMF-borate (**174**).[209,219] However, in the bacteria *S. typhimurium* and *E. coli*, the protein LsrB is the AI-2 signaling molecule binding protein, and crystal structure analysis has revealed that the active AI-2 signal itself in *S. typhimurium* is *R*-THMF (**173**).[219,220] In 2006, James et al. proposed that the AI-2 receptor in the bacterium *Actinobacillus actinomycetemcomitans* was RbsB, although the structure of the active AI-2 signaling molecule has not yet been determined.[221] It should be noted that *R*-THMF (**173**) and *S*-THMF-borate (**174**) are distinct despite the fact that both are derived from DPD (**167**) and rapidly convert in solution.[222] Studies on the use of non-native small molecules to modulate AI-2-based quorum sensing have focused on those AI-2 systems that are best characterized at a molecular level, namely, the LsrB-based system of *S. typhimurium*, which regulates β-galactosidase activity, and, primarily, the LuxP-based system of *V. harveyi*, which plays an important role in bioluminescence.

The bioluminescence phenotype in *V. harveyi* is actually regulated by two main quorum sensing systems.[223,224] The first is a Gram-negative-like system that employs an AHL (3-hydroxy-C4-HSL) as the autoinducer that binds to the LuxN receptor. The second system is the AI-2 pathway. *V. harveyi* is also known to respond to a third autoinducer, CAI-1, although the mechanism of action is less well-defined.[225,226] In the case of *V. harveyi*, the detection of AI-2 requires two proteins, LuxP (a periplasmic binding protein) and LuxQ (a two-component hybrid sensor kinase embedded in the bacterial inner membrane).[209] As discussed previously, LuxP is thought to be the primary receptor with *S*-THMF-borate (**174**) as the autoinducer.[209,227] LuxP and LuxQ are thought to associate to form a complex (termed LuxPQ).[227] When the concentration of *S*-THMF-borate (**174**) exceeds a threshold level, *S*-THMF-borate (**174**) binding to LuxP modifies the activity of this LuxPQ complex, ultimately leading to the production of LuxR, the quorum sensing master regulator that controls expression of the genes in the quorum sensing regulon.[225,227] It is important to note that *V. harveyi*-type LuxR proteins are not related to *V. fischeri*-type LuxR proteins discussed previously in the context of AHL-mediated quorum sensing in Gram-negative bacteria.[225] LuxR production also occurs upon detection of AHL and CAI-1 autoinducers.[225]

There is evidence that, regardless of the presence or absence of AI-2 pool molecules, the LuxP and LuxQ proteins of *V. harveyi* exist in a complex.[227] Thus, although small molecules that modulate AI-2 sensing through direct interaction at the receptor level are more often described as LuxP binders, it may be more appropriate to refer to them as LuxPQ binders. However, because ligands interact primarily with the LuxP component of the complex, the molecular targets of compounds described as either LuxP or LuxPQ binders are essentially identical.

4. Small-Molecule Modulation of AI-2 Quorum Sensing Systems

4.1. Targeting AI-2 Synthesis

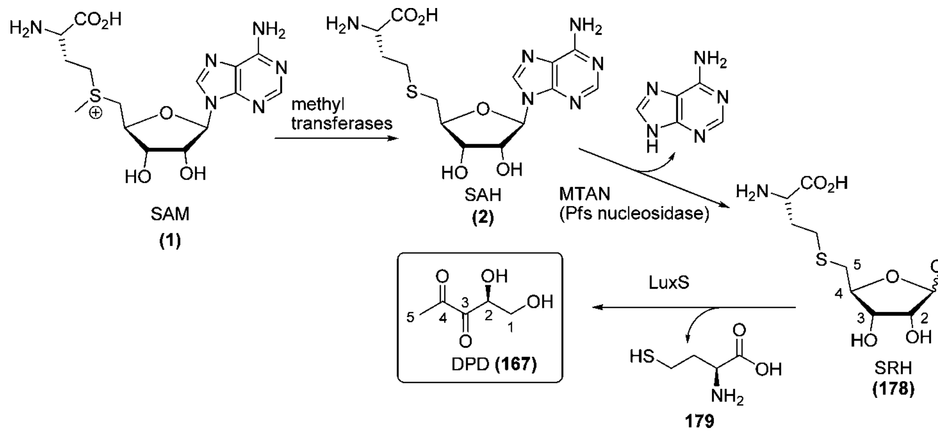
AI-2 synthesis is dependent upon the activity of the enzyme LuxS. The substrate for the LuxS enzyme is *S*-ribosyl-L-homocysteine (SRH, **178**), which is derived from SAM (**1**) (Scheme 2).

SAM (**1**) is converted to SAH (**2**) via the action of SAM-dependent methyl transferases.[36,228,229] SAH (**2**) is hydrolyzed to SRH (**178**) by the 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN) enzyme (also known as Pfs nucleosidase). SRH (**178**) is then cleaved by LuxS to form L-homocysteine (Hcy, **179**) and DPD (**167**).

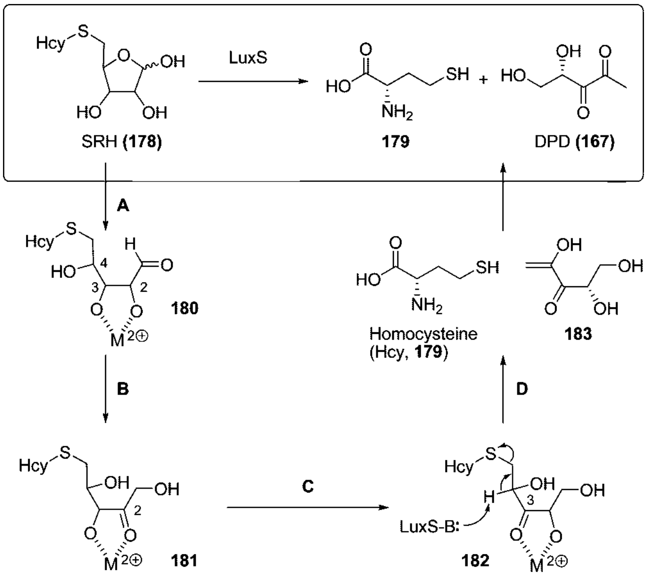
Several different groups have proposed mechanisms for the LuxS-catalyzed cleavage of SRH (**178**) and, while there is some slight variation in precise details, all agree in terms of the major steps of the transformation and their order.[215,230−236] An overview of this process is outlined in Scheme 3.

LuxS is a metalloenzyme containing a divalent metal in the active site; the type of metal cation present is presumed to vary during the course of the reaction.[215,231] The metal ion is believed to play a key role in stabilizing varying intermediates in the pathway by binding to them in a bidentate manner.[215] In the initial steps of the reaction (steps A−C, Scheme 3), the metal ion acts as a Lewis acid, facilitating two consecutive aldose−ketose isomerization steps, converting SRH (**178**) to **180**, **181**, and ultimately compound **182** with a ketone at the C3 position.[215,231,235] A base in LuxS abstracts the C4 proton from **182** and eliminates the homocysteinyl thiol (**179**) (Scheme 3, step D). The resultant enol intermediate **183** spontaneously rearranges into DPD (**167**).[235]

Scheme 2. Enzymatic Synthesis of DPD (167)



Scheme 3. Outline of the Proposed Mechanism for LuxS-Catalyzed Cleavage of SRH (178) to Form DPD (167)



Small molecules that modulate AI-2 synthesis can intervene at various points in this cycle. As the AI-2 system has been correlated with pathogenicity in a variety of organisms, the discovery of AI-2 antagonists has, unsurprisingly, attracted more attention than agonist identification.[49]

4.1.1. Modulating LuxS Activity—Substrate and Intermediate Analogues

In theory, small molecules that resemble SRH or enzyme-associated intermediates produced during LuxS-mediated SRH cleavage should be able to interact with, and therefore possibly modulate, LuxS activity. Thus, the design and synthesis of such compounds has attracted interest.

Zhou and co-workers have reported the synthesis of two LuxS substrate analogues **184** and **185** that function as inhibitors and mechanistic probes of AI-2-mediated signaling (Figure 35).[235]

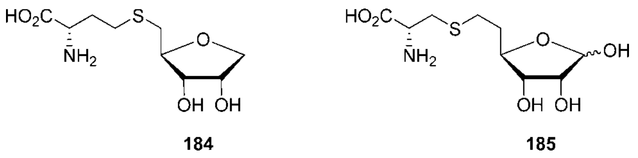


Figure 35. LuxS substrate analogues tested by Zhou and co-workers.[235]

The first substrate analogue, **184**, replaced the hemiacetal of SRH (**178**) with an ether group, which the authors reasoned would prevent the initial aldo-ketose isomerization that occurs in SRH (**178**) hydrolysis. On the other hand, **184** still possessed the 2,3-diol for ligation to the active site metal ion; thus, it was hoped that **184** would bind to the active form of LuxS in a similar fashion as the substrate SRH (**178**) but would not be hydrolyzed to form DPD (**167**), thereby acting as a LuxS inhibitor. The second compound, **185**, replaced the C5-carbon−sulfur bond of SRH (**178**) with a C5−C6 carbon−carbon bond, effectively making carbon−sulfur bond cleavage impossible. However, because of the fact that the ribose moiety and the amino acid moiety of **185** and SRH (**178**) are connected by the same number of C−C and C−S bonds, **185** was expected to be able to bind to LuxS in a productive orientation, i.e., **185** should still be able to undergo isomerization. Preliminary studies showed that LuxS did not cleave the C−S bonds of **184** and **185**; moreover, both compounds were found to inhibit the LuxS enzyme. In a previous study, Zhou and co-workers had shown that SRH analogues **186**−**189** have no activity when assayed as LuxS inhibitors (Figure 36).[230] Taken together, the results from these two studies suggest that the amino acid moiety in ribosylhomocysteine is crucial for substrate binding and activity.[230]

Shen et al. have reported the design and synthesis of a range of structural analogues of SRH (**178**) and a postulated 2-ketone intermediate **181** involved in the LuxS catalyzed cleavage of SRH (**178**) (compounds **190**−**196**, Figure 37).[215] Kinetic studies indicated that the compounds acted as reversible, competitive inhibitors against LuxS, with the most potent compounds in this series, **190** and **191**, having K_I values in the submicromolar range (0.72 and 0.37 μM, respectively). In **190** and **191**, the possible unstable enediolate moieties resulting from tautomerization of 2-ketone intermediate **181** have been replaced with a planar hydroxamate

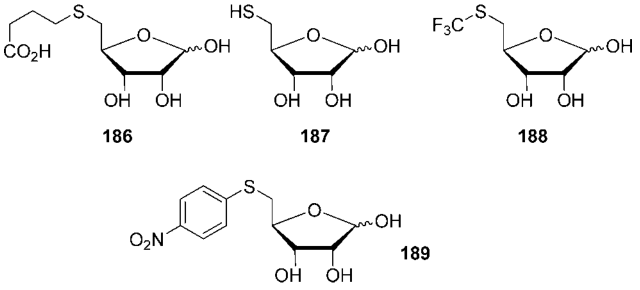


Figure 36. SRH analogues prepared by Zhao et al. that had no activity when assayed as LuxS inhibitors.[230]

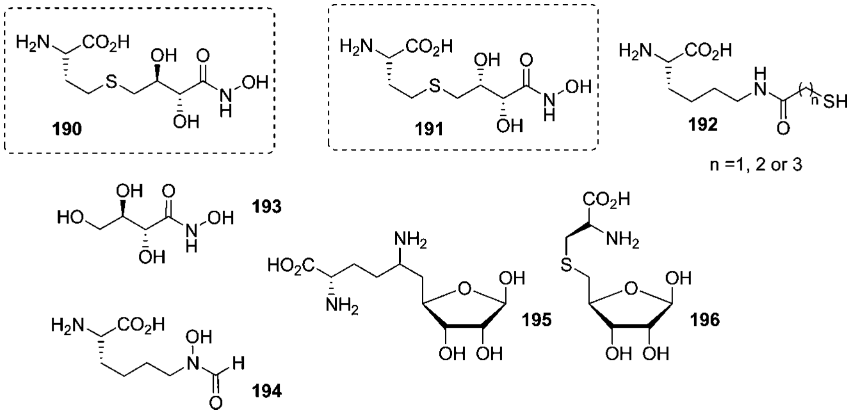


Figure 37. Structures of LuxS inhibitors identified by Shen et al.[215]

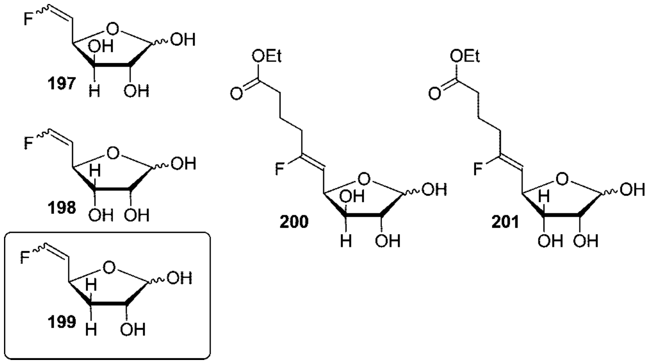


Figure 38. SRH analogues prepared and tested by Wnuk et al.[237]

group, which the authors presume should result in stable isosteres with high affinity to LuxS. That is, the hydroxamate group closely mimics the endiolate intermediates, whose tighter binding to the metal ion would slow or prevent catalytic turnover. Co-crystal structures of LuxS bound to **190** and **191** provided evidence that a high-affinity inhibitor should be able to bind to both the homocysteine-binding pocket and the metal ion of the LuxS active site. Interestingly, **190** and **191** were found to coordinate with the metal center of LuxS using the O2 and O3 atoms, instead of bidentate interaction via the hydroxamate (O1 and O2) as the authors initially expected.

Wnuk et al. have reported the syntheses of SRH (**178**) analogues with the carbon-5 and sulfur atoms replaced by (fluoro)vinyl motifs (compounds **197**−**201**, Figure 38).[237] The authors' supposition was that LuxS may be capable of adding water across the double bonds of these analogues and that the resulting adduct, or derivative thereof, may cause covalent modification and inactivation of the enzyme; that is, the compounds may act as suicide substrates.

These alkenyl analogues were evaluated for inhibition of *Bacillus subtilis* LuxS; one of the compounds, **199**, acted as a competitive inhibitor of a moderate potency.[237] In a later report from the same research group, several SRH (**178**) analogues modified at the ribose C3-position were synthesized and evaluated for their activity against *B. subtilis* LuxS (compounds **202**−**209**, Figure 39).[238] The compounds all lacked a hydroxyl group at the C3 position, replaced by either a proton or a methoxy group. It was thought that such derivatives would bind LuxS but would be unable to undergo the second enolization step to produce the 3-keto intermediate (**182**) that is known to occur in LuxS-catalyzed SRH hydrolysis (step C in Scheme 3).

Removal or methylation of the C3-OH resulted in simple competitive inhibitors of LuxS of moderate potency. However, inversion of the C3 stereochemistry or substitution of fluorine for the C3-OH resulted in slow-binding inhibitors of improved potency. The most potent compound identified

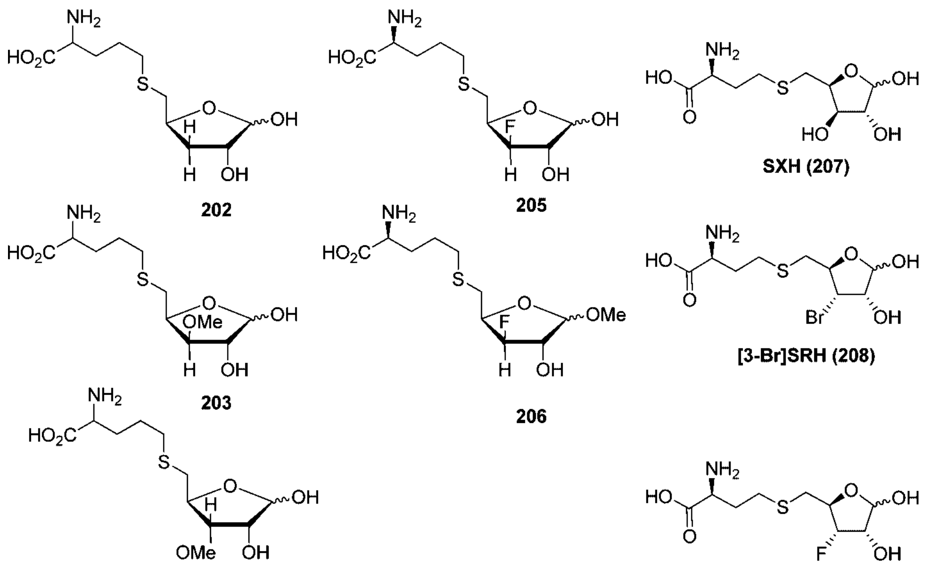
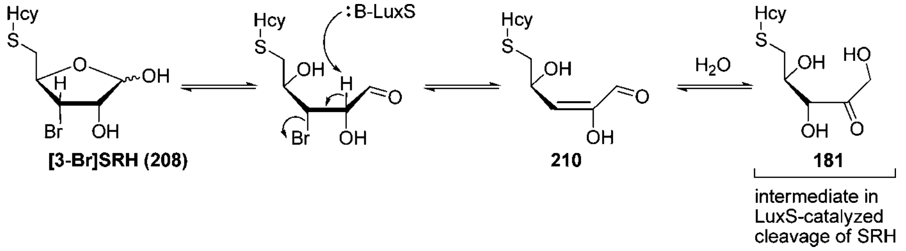
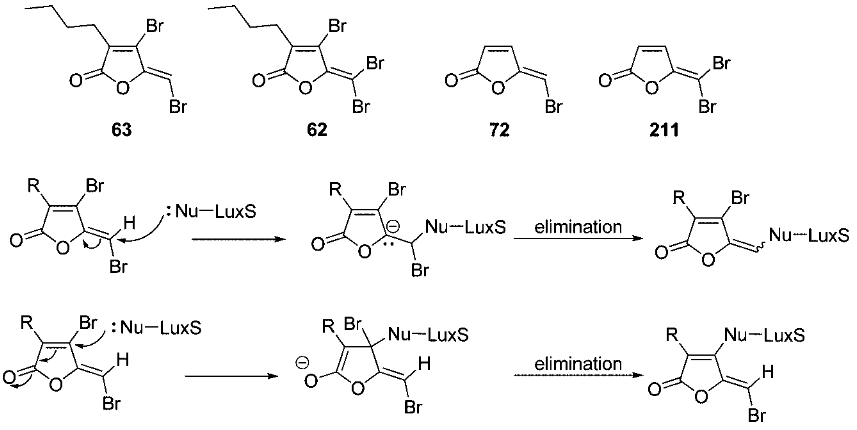


Figure 39. SRH analogues tested by Wnuk and co-workers. All compounds lacked an enolizable hydroxyl group at the carbon 3-position.[237,238]

Scheme 4. Simplified Overview of a Possible Mechanism of Time-Dependent LuxS inhibition by [3-Br]SRH (208) and [3-F]SRH (209)[231]



Scheme 5. Structure of Brominated Furanones Investigated by Zang et al.[166] [a]



[a] **63** and **72** were found to display high levels of inhibition activity against LuxS. Two possible mechanisms for this inhibition have been postulated.[166]

was **207** (termed SXH). Two SRH analogues halogenated at the C3-position of the ribose ring (termed [3-Br]SRH and [3-F]SRH, compounds **208** and **209**, respectively)[231] were also evaluated and also found to act as time-dependent inhibitors of LuxS. The time dependence was thought to be due to enzyme-catalyzed elimination of the halide ions via an E1cB mechanism.[231] The resulting $\alpha-\beta$-unsaturated aldehydes **210** could then tautomerize and undergo the addition of a water molecule to generate the 2-ketone intermediates **181** normally found in the SRH (**178**) cleavage pathway, which can then react to generate DPD (**167**) and thus activate AI-2 signaling (Scheme 4).[231] The authors postulated that compounds **207** and **205** may also undergo similar structural changes in the LuxS active site (i.e., the formation of 2-ketone intermediate-like species). It is presumed that the inverted stereochemistry at the C3-position in SXH (**207**) would prevent the conversion of the intermediate into products.[238]

4.1.2. Modulating LuxS Activity—Other Structural Classes of Compounds

Zang et al. have recently reported the biological evaluation of four pure samples of naturally occurring brominated furanones **62**, **63**, **72**, and **211** (also known as fimbrolide natural products) for their ability to inhibit LuxS (Scheme 5).[166] Furanones **63** and **72** were found to inhibit LuxS in a concentration-dependent manner, with **63** displaying the higher level of activity. Until recently, **63** was described as the "gold standard" with regards to antagonists of AI-2-based quorum sensing (see below).[239] In contrast, **62** and **211** displayed much weaker inhibition. The authors postulated that structural differences between the two pairs of compounds were responsible for these differing levels of activities; **63** and **72** both contain a vinyl monobromide, whereas **62** and **211** are substituted with a vinyl gem-dibromide. Further mechanistic studies were performed with **63**, which led the authors to conclude that the compound covalently modifies and inactivates LuxS. An addition–elimination for this process was proposed in which a nucleophile in LuxS adds directly to either the exocyclic or ring vinyl bromide (Scheme 5). Although the authors could not differentiate between these two pathways, they note that all known naturally occurring brominated furanones display either an exocyclic vinyl monobromide or dibromide moiety but do not require the ring vinyl bromide moiety for activity. In addition, the exocyclic vinyl bromide groups have been determined as essential structural elements for the inhibition of *E. coli* biofilm formation by synthetic brominated furanone analogues (see above). The authors conclude that LuxS is a molecular target for halogenated furanones and that the subsequent disruption of the AI-2 pathway may be the mechanism by which these biologically active compounds disrupt bacterial quorum sensing. Earlier genetic studies on *E. coli* also support this supposition.[171]

Benneche et al. synthesized a range of furanone derivatives **212**–**218** and evaluated them for their ability to inhibit quorum sensing in a variety of systems (Figure 40).[240] All synthesized furanones (and a reference compound **72**) reduced biolumi-

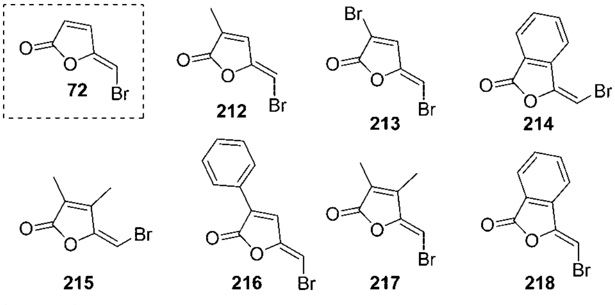


Figure 40. Structures of furanones investigated for antiquorum sensing activity by Benneche et al.[240]

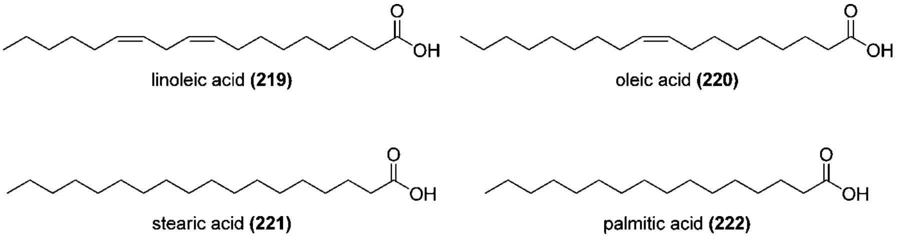

linoleic acid **(219)** oleic acid **(220)** stearic acid **(221)** palmitic acid **(222)**

Figure 41. Four fatty acids that were demonstrated to act as inhibitors of AI-2-mediated signaling.[241,242]

nescence in *V. harveyi* BB170 significantly, with **218** being slightly more effective than **72**. Because this strain lacks the receptor for the native AHL (as discussed previously), it only responds to intermicrobial communication via the AI-2 system. Therefore, the authors assumed that the tested furanones interfered with AI-2 quorum sensing. The most effective furanones, **218** and **72**, also reduced biofilm formation by *Staphylococcus epidermidis* without affecting growth.

Widmer et al. have identified a variety of poultry meat-derived fatty acids **219**−**222** that each act as inhibitors of AI-2-mediated signaling (Figure 41).[241] The authors speculate that the acid functionality of the compounds described in this study may be binding to LuxS, impeding its function. A similar set of compounds has also been isolated from ground-beef extracts.[242]

4.1.3. Modulating LuxS Activity—MTAN Inhibition

Rather than targeting the LuxS enzyme directly, a conceptually different approach toward modulating LuxS enzyme activity is to inhibit the production of its substrate SRH (**178**). This process is mediated by the enzyme MTAN, which catalyzes the hydrolytic deadenylation of SAH (**2**) to form adenine and SRH (**178**).[243] MTAN is the only enzyme known to generate SRH (**178**); therefore, inhibition of MTAN is expected to inhibit AI-2 synthesis. Singh et al. have reported the design and synthesis of a range of small molecules designed to be transition state analogues of this cleavage process.[243] A number of the resulting compounds were found to be powerful inhibitors of *Streptococcus pneumoniae* MTAN activity, and in preliminary studies, selected analogues demonstrated the ability to block the synthesis of quorum sensing molecules in cultured *S. pneumoniae* (the four most potent compounds **223**−**226** are shown in Figure 42).

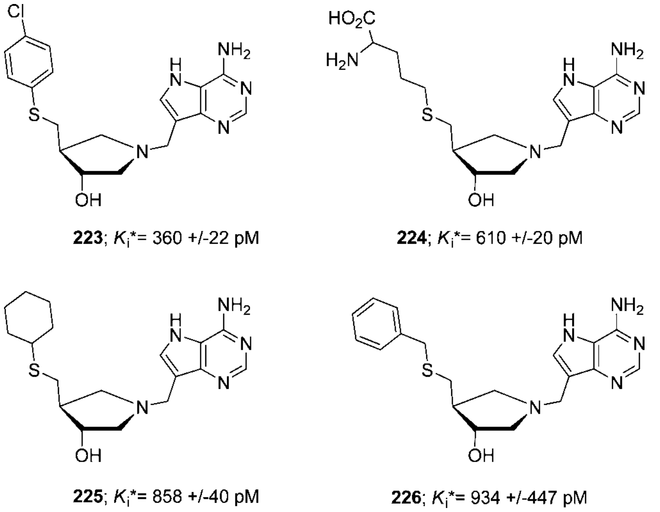

223; K_i^*= 360 +/-22 pM
224; K_i^*= 610 +/-20 pM
225; K_i^*= 858 +/-40 pM
226; K_i^*= 934 +/-447 pM

Figure 42. Structures and inhibition constant for the four most potent MTAN inhibitors identified by Singh et al.[243]

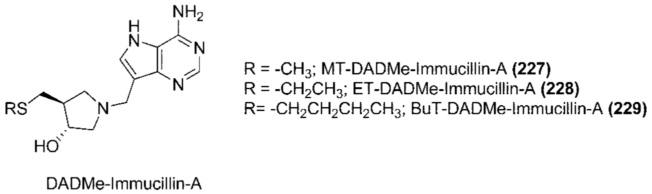

R = $-CH_3$; MT-DADMe-Immucillin-A **(227)**
R = $-CH_2CH_3$; ET-DADMe-Immucillin-A **(228)**
R= $-CH_2CH_2CH_2CH_3$; BuT-DADMe-Immucillin-A **(229)**
DADMe-Immucillin-A

Figure 43. MTAN inhibitors identified by Gutierrez et al.[244]

Gutierrez et al. have recently developed several MTAN transition state analogues capable of inhibiting MTAN activity and thus modulating AI-2 quorum sensing signaling.[244] For example, MT-DADMe-Immucillin-A (**227**), EtT-DADMe-Immucillin-A (**228**), and BuT-DADMe-Immunicillin-A (**229**) were found to be tight-binding inihibitors of *Vibrio cholerae* MTAN, and the compounds disrupted autoinducer production in a dose-dependent manner without affecting growth (Figure 43). MT- and BuT-DADMe-Immucillin-A (compounds **227** and **229**) also inhibited AI-2 production in enterohemorrhagic *E. coli*. BuT-DADMe-Immucillin-A (**229**) inhibition of AI-2 production in both strains persisted for several generations and caused reduction in biofilm formation.

4.2. Targeting AI-2 Receptors

Studies on the use of non-native small molecules to interact with AI-2 receptors have focused on those AI-2 systems that are best characterized at a molecular level, namely, the LsrB-based system of *S. typhimurium*, which regulates β-galactosidase activity, and, primarily, the LuxP-based system of *V. harveyi*, which plays an important regulatory role in bioluminescence. Investigations into the AI-2 systems in other bacterial species have been reported, although they are hampered by a lack of knowledge of the nature of the active AI-2 signal and cognate protein. Studies on the selective modulation of AI-2-mediated quorum sensing in *V. harveyi* are typically designed so that the activity of the native AHL system is decoupled from expression of the bioluminescence phenotype; e.g., the AHL system has been blocked or accounted for in some way such that AHL stimulation does not affect the assay outcome. Usually this is achieved through the use of mutant strains of *V. harveyi*. Two strains of *V. harveyi* that are widely employed to investigate AI-2-based quorum sensing, following the protocol reported by Schauder and co-workers, are BB170 and MM30.[218,223] *V. harveyi* strain BB170 lacks the native LuxN receptor (and thus cannot respond to the AHL signal) but does contain the LuxP receptor to sense AI-2. Strain MM30 is a LuxS mutant that is unable to synthesize DPD (**167**). These mutants show an increase in bioluminescence upon addition of *S*-THMF-borate (**174**) and DPD (**167**), which can generate *S*-THMF-borate (**174**) in situ. Another strain, MM32, is commonly employed; MM32 lacks LuxS and the LuxN receptor.[245] For precise details of the biological methods employed in the studies discussed below, the reader is directed to the relevant primary literature. It should be noted that information regarding the degree of selectivity of compounds for modulation of the AI-2 system versus the AHL system in *V. harveyi* is not always available.

There is undoubtedly a relative paucity of work pertaining to small-molecule modulation of AI-2 receptors in comparison to the extensive studies on LuxR-type receptors involved in AHL-based quorum sensing. Because of this dearth of information, the rational design of new ligands for AI-2 receptors has not really been pursued to date. The majority of studies have focused on the use of non-native analogues of known signaling molecules or precursors thereof: i.e., *R*-THMF (**173**), *S*-THMF (**172**), or DPD (**167**) derivatives. In addition, the biological screening of small-molecule libraries has proven to be a successful means to discover non-native compounds capable of modulating AI-2 receptor activity. The limited amount of structural data that is available on the nature of the molecular interactions involved in AI-2-ligand−receptor interactions have been used in virtual screening experiments to identify active compounds also. The field of small-molecule modulation of AI-2 receptor activity is still in its infancy but, nevertheless, constitutes a rapidly growing research area of significant interest.

4.2.1. Non-native Agonists

McKenzie et al. have investigated the role of various metals other than boron on AI-2 signaling in *V. harveyi*.[246] A variety of metal salts were chosen and evaluated for their ability to induce light production in *V. harveyi* strain BB170 in boron-free media. Positive results were obtained as a result of the addition of metal carbonates, leading the authors to conclude that a hitherto unknown compound, *S*-THMF-carbonate (**230**), arising from the reaction of a furanosyl form of *S*-DPD with carbonate, is capable of modulating AI-2 quorum sensing in *V. harveyi*, presumably through interaction with the binding pocket of LuxP (Figure 44).[246] However, no biophysical data on the postulated *ortho*-carbonate species have been obtained.

Semmelhack et al. have reported that the natural product Laurencione (**231**) and cyclic compound **232** (MHF) are capable of inducing bioluminescence in *V. harveyi*, albeit with an activity 100-fold less than that of enzymatically prepared DPD (**167**) (Figure 45).[247] Ribose and compounds **233**−**235** were inactive in the *V. harveyi* assay.

The majority of work relating to small-molecule modulation of AI-2-based signaling (both agonist and antagonist identification) has focused on the synthesis of non-native derivatives of DPD (**167**). As discussed previously, DPD (**167**) is in a rapid equilibrium with the compounds comprising the AI-2 pool, including the active signaling compound for any given bacterial system (Scheme 1). Therefore, it is logical to assume that non-native DPD (**167**) analogues will also have the capacity to exist in an equilibrium mixture of non-native DPD derivatives. In general, it is assumed that the biologically active agent (if any) in such a mixture is likely to be the corresponding derivative of the natural signaling compound, i.e., an *S*-THMF-type analogue in the case of *V. harveyi*. However, the precise structure of any biologically active molecule in such non-native equilibrium mixtures is difficult to establish with certainty. Furthermore, the mode of action of such agents is also difficult to delineate. Although it is generally assumed that an active compound derived from a DPD derivative will interact with the AI-2 receptor that normally binds to the native active AI-2 signaling molecules, this is not always likely to be the case (see below). Given these considerations, the development of a detailed understanding of the fundamental molecular basis behind modulation of AI-2 pathways using DPD derivatives is challenging. SAR analyses on DPD derivatives are proving valuable in attempts to achieve this goal.


S-THMF-borate **174** *S*-THMF-carbonate **230**

Figure 44. *S*-THMF-borate and *S*-THMF-carbonate.[246]

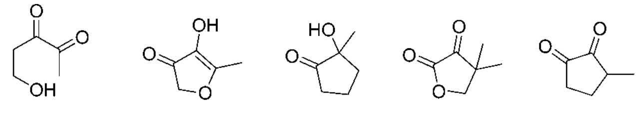
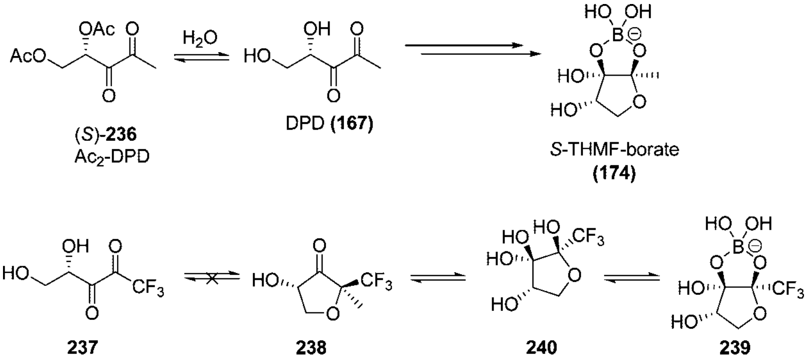

Laurencione **(231)** MHF **(232)** **233** **234** **235**

Figure 45. Some compounds investigated by Semmelhack et al. for their ability to modulate AI-2-mediated bioluminescence in *V. harveyi*.[247]

Despite the structural simplicity of DPD (**167**), its chemical synthesis has proven to be nontrivial.[248] The compound is only stable at dilute concentration, and it has been shown that at higher concentrations dimerization occurs to furnish a biologically inactive triacetal derivative.[248,249] Frezza et al. synthesized Ac_2-DPD (**236**) in the hope that it would act as a convenient stable precursor of DPD (**167**) (and, thus, AI-2 signaling pool components).[250] The authors anticipated that Ac_2-DPD (**236**) would have the same activity as DPD (**167**) by releasing the latter compound after in situ hydrolysis of the ester groups (Scheme 6). This was indeed found to be the case; Ac_2-DPD (**236**) induced the same biological effects as DPD (**167**) on the Gram-negative bacteria *V. harveyi* and *S. enterica* and on the Gram-positive bacterium *Bacillus cereus*. The authors reasoned that it was very unlikely that Ac_2-DPD (**236**) itself is the active component and that the observed biological activity does indeed result through the in situ release of DPD (**167**) and its subsequent rearrangement in to the active AI-2 signaling molecules in these bacterial strains. The same research group has also reported studies toward the synthesis of **237**, a trifluoromethyl analogue of DPD (**167**) (Scheme 6).[251] In contrast to DPD (**167**), which exists in both the hemiketal (80%) and open-chain (20%) forms, **237** was found to exist only in the hemiketal form **238** due to the strong electronegative effect of the trifluoromethyl group. However, as with DPD (**167**), compound **238** could not be isolated because of its instability on concentration, and therefore, the crude product solution was used for biological studies. The AI-2 activity of **238** was compared with that of *rac*-DPD (*rac*-**167**) using a *V. harveyi* bioluminesecence assay. **238** was found to exhibit agonist activity, although it was 10-fold less active than *rac*-DPD (*rac*-**167**) with an IC_{50} value of ~30 μM compared to ~3 μM for *S*-DPD (**167**). The authors postulated that this variation in activity could be attributed to structural differences in the actual *V. harveyi* AI-2 signaling molecule that is formed in the presence of **167** (or **236**) and **238**, that is, differences between the furanosyl boranoate diesters **174** and **239** resulting from **167** (or **236**) and **238** (via **240**), respectively (Scheme 6). Analysis of the crystal structure of the native

Scheme 6. Freeza and Co-workers Identified DPD Analogues 236 and 237 as Agonists of the *V. harveyi* AI-2 Quorum Sensing System[250,251]



S-THMF-LuxP complex suggests that **239** should have a decreased affinity for the LuxP binding site relative to **174** because the presence of the CF_3 group in **239** should make the two adjacent oxygen atoms less prone to developing hydrogen bonds with the receptor.

In 2005, Lowery et al. reported the results of a study designed to probe the specificity of the LuxP binding site through the synthesis and biological evaluation of a series of natural and non-natural analogues of DPD (**167**) or DPD-derived compounds (compounds **167**, (*R*)-**167**, **231**, **232**, and **241**−**244**, Figure 46).[252] The progression of molecules that were investigated included enantiomeric variants, carbon-chain extension, and hydroxyl-functional group additions/deletion of DPD. Also, several compounds with similar structural features to DPD or DPD-derived agents were included. These compounds were evaluated for their ability to induce bioluminescence in *V. harveyi* strain MM30. From the obtained activities, the authors concluded that the LuxP binding cleft can accommodate a number of different structural variants of DPD-derived active signaling compounds, albeit with invariably considerably lower activities than that found for the native DPD (**167**). The data indicated that *both* the chelation of boron and the position of the hydroxyl moiety in *V. harveyi* AI-2 (*S*-THMF-borate (**174**)) are crucial in its binding to LuxP.

Later work from the same research group involved the synthesis of a range of C1-alkyl-substituted DPD analogues (compounds **245**−**250**, Figure 47).[222] The compounds were evaluated for modulation of quorum sensing in two established biological assays: induction of β-galactosidase activity in *S. typhimurium* and bioluminescence production in *V. harveyi*. These phenotypes are both regulated by AI-2 signaling systems.

No agonists of AI-2-based *S. typhimurium* quorum sensing were uncovered from the agonist assays, which were all performed in the absence of DPD (**167**). Interestingly, however, all compounds were found to act as antagonists in the presence of DPD (**167**) (the most active compounds being **246** and **247**). When the compounds were evaluated for agonist activity in *V. harveyi* (assay in the absence of DPD (**167**)), only **245** exhibited weak agonistic activity. However, when the test compounds were incubated with *V. harveyi* and DPD (**167**) to monitor antagonism, a synergistic *agonistic* effect was observed. That is, across the whole compound

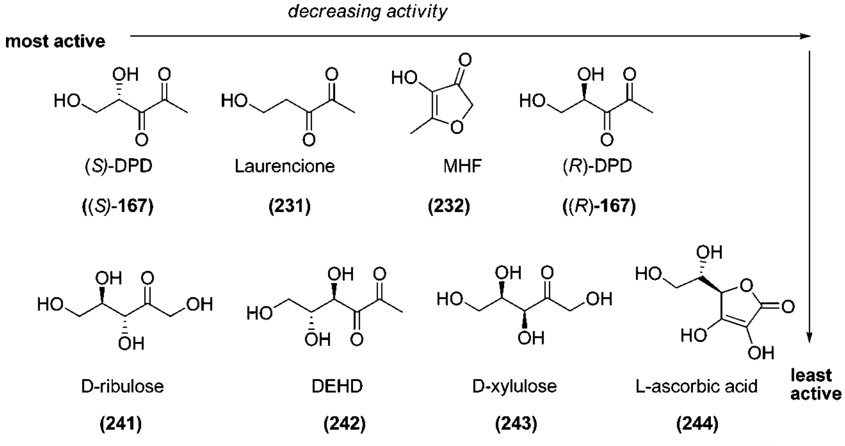


Figure 46. Structures and order of agonist activities of DPD analogues synthesized by Lowery et al.[252]

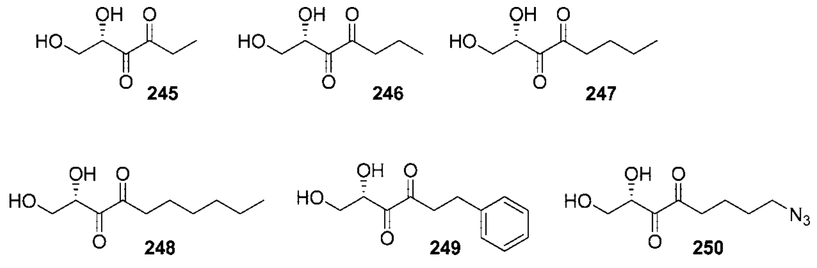


Figure 47. C1-Alkylated DPD analogues examined by Lowery et al.[222]

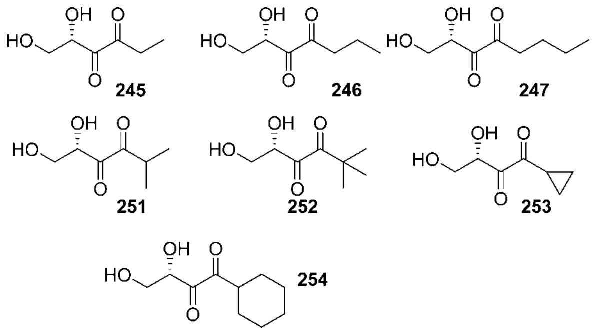


Figure 48. Summary of C1-alkylated DPD analogues prepared by Smith et al.[248] The most active synergistic agonist in this study was **254**. Compounds were synthesized and tested as racemates.

series, an enhancement of bioluminescence was observed when a compound was present with DPD compared to the bioluminescence observed in the presence of DPD (**167**) alone (i.e., DPD-enhanced AI-2 induced bioluminescence). Ganin et al. have recently reported very similar findings in *V. harveyi*; both studies were in agreement in that there was a decrease in synergistic activity with increasing chain length of the DPD analogue.[218] Smith et al. have observed activity when using C1 branched and cyclic alkyl chain DPD analogues (compounds **245**−**247** and **251**−**254**, Figure 48).[248] These compounds were evaluated for their ability to affect bioluminescence in *V. harveyi* strain MM32. The compounds were unable to induce bioluminescence on their own. However, the authors found that diverse shapes and sizes of the C1-alkyl chain of DPD (and thus of the resulting equilibrium compounds) are all able to synergistically induce bioluminescence in *V. harveyi* in the presence of DPD (**167**). This suggests that the receptors that mediate the DPD and DPD-analogue synergistic agonism display marked promiscuity of ligand binding. It should be noted that the synergistic agonism assays carried out by Lowery et al. were done without the addition of boric acid to the media. The authors rationalized this decision on the basis that the presence of boric acid itself is known to induce quorum sensing activity, rendering *V. harveyi* less sensitive to different concentrations of DPD (**167**). However, Smith et al. did add boric acid to the culture media for the bioluminescence assays, as they believed that if this was not done then the analogues might scavenge for adventitious borate, thereby affecting the results of different assays that contained different amounts of adventitious borate. Nonetheless, the results of both studies are in agreement.[248] The origin of the concentration-dependent synergistic enhancement of DPD-induced bioluminescence remains unknown, although Smith et al.[248] and Ganin et al.[218] have postulated various explanations.

Aharoni et al. have discovered compounds that are capable of synergistic enhancement of bioluminescence induction in *V. harveyi* that are structurally distinct from the DPD analogues discussed above.[253] In this study, the authors identified a structural relationship between *V. harveyi* *S*-THMF-borate (**174**) and oxazaborolidine derivatives **255**−**259**; they are heterocyclic hydrated complexes, containing a negatively charged tetra-coordinated boron atom that has the ability to form hydrogen bonds (Figure 49). Therefore, the authors postulated that these compounds might selectively bind to LuxP, thereby triggering an agonistic response.

Of the five compounds, **255** and **259** most strongly induced the bioluminescence of the reporter strain *V. harveyi* BB170, with **255** being the most active. However, no effect on bioluminescence was seen when the compounds were examined in a *V. harveyi* mutant (BB886) capable of synthesizing native AHL but lacking AI-2. Using a mutant strain that produced neither native autoinducer, the authors showed that the presence of synthetic DPD (**167**) or spent medium containing *S*-THMF-borate (**174**) was essential for the activity of **255** and **259**. Thus, the authors concluded that these compounds most probably interact with the LuxP receptor and that their mode of action on bioluminescence in *V. harveyi* is of a coagonist category (i.e., synergistic enhancement of AI-2 signal transduction). Some possible SAR trends for this compound series were discussed.[253] The fact that **255** and **259** demonstrated specific activity in AI-2 signaling despite the fact that the structures are only slightly reminiscent of *V. harveyi* *S*-THMF-borate (**174**) indicated to the authors that the presence of a five-membered heterocyclic ring containing tetrahedral boron bearing a hydroxyl group is sufficient for specific interaction with LuxP. The finding that **255** and **259** induced bioluminescence but **256**−**258** did not may indicate that the methyl on the nitrogen is an important factor in activation of quorum sensing in *V. harveyi*. The fact that **255** was more active than **259** may indicate that the aromatic group attached to the boron in **255** interacts favorably at the active site of the receptor.[253]

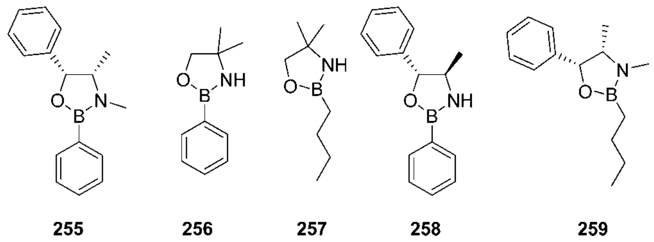
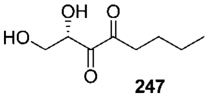


Figure 49. Structures of oxazaborolidine derivative investigated by Aharoni et al.[253]

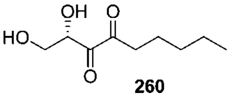
4.2.2. Non-native Antagonists—DPD Analogues

The studies by Lowery et al.[222] and Ganin et al.[218] discussed previously, which led to the identification of synergistic agonists of bioluminescence in *V. harveyi*, also provided compounds capable of *antagonizing* quorum sensing systems. All the C1-substituted DPD analogues prepared by Lowery (Figure 47) were found to act as antagonists of AI-2-based quorum sensing in *S. typhimurium* (as determined by a β-galactosidase activity assay) without affecting bacterial growth.[222] Notably, compounds **246** and **247** were potent inhibitors with IC$_{50}$ values 10-fold below the concentration of the natural DPD (**167**) signal. Thus, this compound series was shown to elicit strikingly different biological effects in two different bacterial species with known AI-2 quorum sensing systems, a phenomenon which the authors declare could not be predicted solely on the basis of the crystal structures of the AI-2 signaling molecules nor the receptor proteins. Ganin et al. demonstrated that two of their DPD analogues, **247** and **260**, inhibit the production of the virulence factor pyocyanin in *P. aeruginosa*, a phenotype which is regulated by quorum sensing (Figure 50).[218] The authors conclude that this inhibition most likely occurs through interference with the LasR system.

Lowery et al. have recently presented a direct comparison of the AI-2 inhibition activity of their alkyl-DPD analogues (Figure 47)[222] and naturally occurring fimbrolide natural product furanone **63**.[239] All DPD analogues displayed inhibitor activity; as a general trend, increasing the length of the



247



260

Figure 50. Two DPD analogues prepared by Ganin et al. that were found to inhibit the production of the virulence factor pyocyanin in *P. aeruginosa*.[218]

alkyl chain corresponded to an increase in activity. Hexyl-DPD (**248**) was identified as the most potent inhibitor of bioluminescence of *V. harveyi* BB170 cells, ~4 times more active than the furanone **63**. Interestingly, the authors demonstrated that, while *V. harveyi* was able to overcome the effects of hexyl-DPD (**248**) after a 2 h incubation time, the inhibitory effects of furanone **63** remained. In the case of assays in the presence of hexyl-DPD (**248**), the luminescence could be "turned-off" again by the addition of further hexyl-DPD (**248**) to the cultures after the 2 h time period. These results suggested that there was a difference in mechanism of action between the furanone **63** and hexyl-DPD (**248**). The authors propose that there is a covalent interaction between furanone **63** and its target protein (presumed to be LuxS[166]), whereas a noncovalent mechanism operates for hexyl-DPD (**248**), thus allowing for on−off control of quorum sensing using this compound and rendering it an effective probe for the temporal study of AI-2 signaling.[239] Furthermore, the alkyl-DPD analogues have previously been shown to inhibit AI-2 quorum sensing in *S. typhimurium*,[222] whereas furanone **63** was found to be inactive.[239] Thus, the alkyl-DPD analogues represent the only reported compounds to date that are effective against both *V. harveyi* and *S. typhimurium* AI-2 quorum sensing systems. In addition, the authors propose that their DPD analogues represent a viable alternative to the widely accepted use of fimbrolide-derived compounds as "gold standard" antagonists of AI-2-based quorum sensing.

In a recent patent application, Miller et al. describe the structures of various analogues of the monocyclic forms of DPD and hydrated DPD, which exhibit antagonist/agonist activity in AI-2 signaling.[219] However, further biological data is not available at this time.

4.2.3. Non-native Antagonists—Mimicking the Borate Moiety

On the basis of the concept of molecular mimicry, Ni et al. envisioned that boronic acids could serve as excellent candidates for binding to LuxP because of the structural similarities between *S*-THMF-borate (**174**) and the boronic acid functional group.[254] Therefore, the authors screened a series of ~50 boronic acid compounds for their ability to inhibit AI-2-mediated quorum sensing in *V. harveyi* strain MM32, which produces no endogenous AI-2 signal. Five compounds showed significant inhibitory activities with IC_{50} values in the single-digit micromolar range (first series of compounds **261**−**265**, Figure 51). Seventeen compounds showed no/minimal inhibitory activities below a compound concentration of 100 μM and were considered inactive.

From these data, some general SARs were drawn. First, the active boronic acids were all phenylboronic acids. Second, boronic acids directly attached to a sp^3 carbon were generally not active, which the authors presumed was due to the relatively high p*K*a of such alkylboronic acids (i.e., relatively electron-rich boron centers). Boronic acids with a low p*K*a may represent the best chance of mimicking *S*-THMF-borate (**174**) because of their high tendency to exist in the anionic tetrahedral form upon binding to LuxP. Third, boronic aids with additional ionizable functional groups (under physiological conditions) tended to be less active. None of the most active compounds were found to exhibit

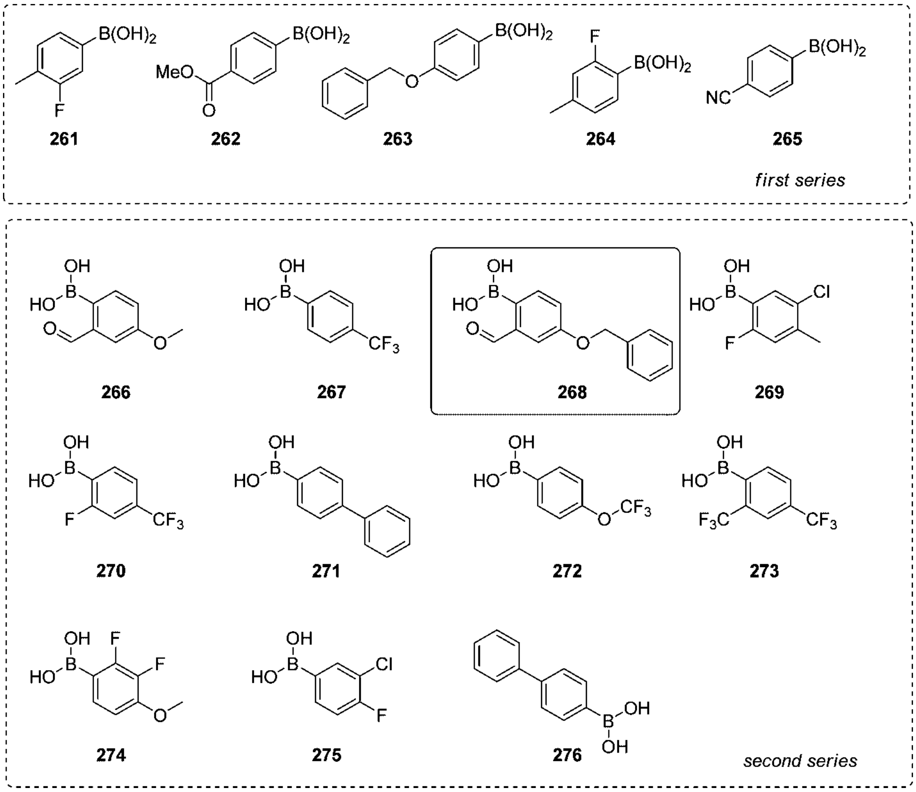
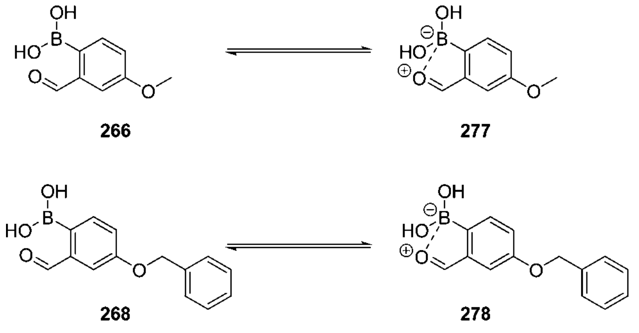


Figure 51. Structures of boronic acids found by Ni et al. to have inhibitory activities (IC_{50} at or below single-digit micromolar range) against AI-2-mediated luminescence in *V. harveyi* (strain MM32).[254] The most active antagonist, **268**, is highlighted. Assays carried out in the presence of 5 μM DPD (**167**).

Scheme 7. Two Compounds 266 and 268 Probably Can Exist in the Ionized Forms 277 and 278, Respectively, at Neutral pH Due to an Intramolecular Interaction from the *ortho*-Formyl Groups[a]



[a] These ionized forms, which contain an anionic tetrahedral boron center, would be expected to mimic closely the native LuxP autoinducer *S*-THMF-borate (**174**) in terms of binding capability.[255]

cytotoxic effects. In a subsequent study by the same research group, an additional 30 *para*-subsituted arylboronic acids were screened for their ability to inhibit AI-2-mediated quorum sensing in *V. harveyi*.[255] Among these new compounds, 11 showed IC_{50} values in the single-digit micromolar range (second series of compounds **266**−**276**, Figure 51), with compound **268** having a submicromolar IC_{50} value. None of these 11 compounds exhibited significant inhibition of bacterial growth when compared with the control group (no boronic acid). In general, a low p*K*a of the boronic acid functional group was found to favor inhibition activity, in line with the arguments discussed previously. Thus, compounds with an electron-donating *para*-substituent were generally weakly active or inactive because the formation of the anionic tetrahedral borate form is disfavored relative to compounds with electron-withdrawing groups and, thus, a relatively more electron-poor boron center. Compounds **266** and **268** stood out as nonconformers, having electron-donating groups yet exhibiting excellent inhibitory activities. This effect was attributed to the presence of the formyl group at the *ortho*-position. It is thought that the oxygen is appropriately positioned to donate a lone pair of electrons to boron and thus convert the boron atom to its tetrahedral form. Thus, these two compounds probably can exist in the ionized forms **277** and **278** with a formal negative charge on the boron atom at neutral pH; therefore, they would be expected to function as close mimics of the native LuxP autoinducer in terms of their binding capability (Scheme 7).[255] Some SAR trends from this series were delineated: (i) a bulky and hydrophobic group at the *para*-position is favorable for inhibition activities; (ii) low p*K*a of the boronic acid functional group favors inhibition activities; (iii) nitrogen on the aryl ring is unfavorable for activities; (iv) polar and hydrophilic groups are undesirable; and (v) an *ortho*-substituent can be used to enhance the acidity of the boronic acid group through coordination and, thus, enhance the inhibition activity. Interestingly, most of the second series of boronic acids showed moderate inhibition of *V. harveyi* strain BB886 (which responds only to AHL autoinducer stimulation and not AI-2), thus implying that the boronic acids are also capable of inhibiting AHL-based signaling.[255] However, IC_{50} values for AHL inhibition were generally 2- to 4-fold higher than that against MM32.[255] The authors have yet to suggest a mechanism through which AHL quorum sensing inhibition occurs.

Related work by Ni et al. focused upon the screening of diol-containing compounds in an effort to identify antagonists of AI-2 quorum sensing in *V. harveyi*.[256] The authors reasoned that diol-containing compounds, when complexed with boric acid, should be molecular mimics of *S*-THMF-borate (**174**) and, therefore, have the potential to bind to LuxP. Toward this end, a variety of aromatic-containing diols and two five-membered ring diols were screened to establish their ability to inhibit AI-2-regulated bioluminescence in *V. harveyi*. Of the 15 compounds tested, 5 showed IC_{50} values at single-digit micromolar concentrations (compounds **279**−**283**, Figure 52). No compounds showed general cytotoxic effects. The 5 most active compounds were all based around a pyrogallol-type scaffold (i.e., a benzene ring with three hydroxyl groups attached). The only nonaromatic diol tested had no effect. Catechols showed much lower activities than the pyrogallols, indicating that the third hydroxyl group is important in binding interactions. In general, the presence of ionizable groups (under normal physiological conditions) at the 4-position of the pyrogallol scaffold resulted in a decrease in activity relative to pyrogallol itself. Molecular modeling studies suggested that side-chain ionizable functional groups on pyrogallol, either positive or negative, may engage in either attractive or repulsive interactions with a specific aspartic acid residue in the binding pocket of LuxP. This can move the entire complex away from an otherwise ideal binding position.

4.2.4. Non-native Antagonists—Miscellaneous

Li et al. have reported the high-throughput structure-based virtual screening of 1.7 million small molecules from various commercial databases against the *V. harveyi* LuxP crystal structure; the molecules were each docked into the binding site of the receptor, and scoring functions were used to evaluate their potential complementarity.[49] Of the top 42 hits, 27 were obtained from commercial vendors and evaluated biologically for their ability to modulate AI-2-mediated bioluminescence in *V. harveyi*. Two compounds, **284** and **285**, were found to antagonize this quorum sensing system without displaying cytotoxic effects (Figure 53). The authors postulated that these compounds may interact with LuxP using the sulfone group at the borate position of the natural ligand. Specifically, the oxygen atoms of the sulfone may mimic the borate oxygen atoms in terms of their interactions with two arginine residues in the binding pocket.[206] The IC_{50}

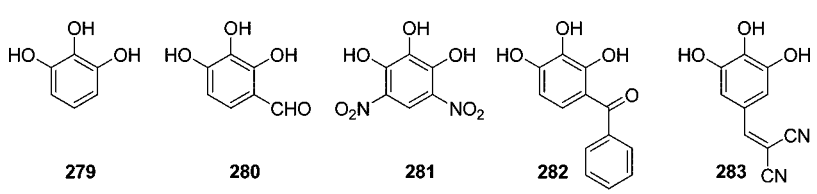
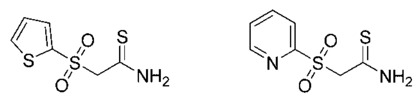


Figure 52. Structures of diol compounds found by Ni et al. to have inhibitory activities (IC_{50} at or below single-digit micromolar range) against AI-2-mediated luminescence in *V. harveyi* strain MM32.[256] Assays carried out in the presence of 5 μM DPD (**167**) and 1 mM boronic acid.

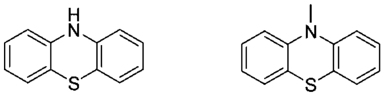


284 **285**

Figure 53. Structures of the two compounds identified by library screening by Li et al. to have the highest inhibitory activities against AI-2-mediated luminescence in *V. harveyi* strain MM32.[49] The most active compound was **284** (~1.5 times more active than **285**). Assays carried out in the presence of 5 μM DPD (**167**) and 1 mM boronic acid.

values obtained for **284** and **285** were less than the top boronic acid derivatives identified by the same research group and also the pyrogallol-based derivatives (see above); the authors therefore suggest that the interactions of the sulfone group with LuxP may be weaker than those of the boronic acid and complexed boronic acid functionalities (though, as discussed previously, care must be taken when directly comparing absolute activity values obtained in different studies). A comparison of compounds **284** and **285** with other inactive sulfones highlighted some structural characteristics associated with inhibition activity: (i) the aryl ring is important for activity (presumably involved in some sort of hydrophobic interactions); (ii) the sulfone group should be directly attached to an aryl group; and (iii) the thioamide group should be separated from the sulfone group by one atom.

In an effort to identify sulfone-based inhibitors with higher levels of activity, the same research group synthesized 39 analogues of compounds **284** and **285**, varying four general parts of the core structure (parts A–D, Figure 54).[206] The analogues were tested for their ability to inhibit AI-2 quorum sensing in *V. harveyi* strain MM32 (which does not respond to AHL signaling) and also for their ability to inhibit AHL-based quorum sensing in *V. harveyi* strain BB886 (which lacks the AI-2 receptor and, thus, does not respond to AI-2). Twelve of these new analogues (**286**–**297**) showed equal or better inhibitory activities than the lead compounds (IC_{50} < 40 μM), 4 of which showed single-digit micromolar IC_{50} values (**293**, **294**, **296**, and **297**), while 5 of the 12 (**288**, **289**, **293**, **295**, and **297**) possessed good selectivity toward AI-2 quorum sensing over AHL-mediated signaling with IC_{50} values for AHL inhibition >200 μM. A selection of these compounds were shown not to exhibit cytotoxic effects. Overall, the following structural features were found to be beneficial for AI-2 inhibition activity: a sulfone group (part B), "thiation" of the carbonyl group of part C, a hydrophobic group of modest size in part D, and a biphenyl system in part A.



298
Inhibits *both* AHL and AI-2 signalling

299
Inhibits AHL signalling; no activity against the AI-2 system

Figure 55. Structures of the two compounds identified by library screening by Li et al. to have selective inhibitory activities against quorum sensing in *V. harveyi*.

Through the random screening of compounds against two mutant strains of *V. harveyi*, Ni et al. identified two compounds (**298** and **299**) based on a phenothiazine scaffold that were capable of modulating the expression of the bioluminescence phenotype without exhibition of general cytotoxic effects (Figure 55).[257] Compound **299** was found to be a selective inhibitor against the AHL-based quorum sensing system in *V. harveyi*, while compound **298** was equally active against both the AHL and AI-2 systems.

The reasons for this difference in inhibition selectivity are not known at this time. There are other small-molecule agents that show similar inhibition promiscuity in *V. harveyi*. For example, the furocoumarins dihydroxybergamottin (**71**) and bergamottin (**70**) isolated from grapefruit juice have been found to inhibit both AHL and AI-2 activities, which is thought to possibly occur through competitive binding with the autoinducer receptors in both cases.[155] Cinnamaldehyde (**115**) has also been shown to inhibit both AHL and AI-2-regulated quorum sensing systems in *V. harveyi*, although it is thought that this inhibition is mediated via modulation of LuxR activity (see below).[194]

In a recent study, Brackman et al. screened a small panel of nucleoside analogues for their ability to disturb AI-2-based quorum sensing.[258] A variety of inhibitors were identified (selected examples **300**–**305** shown in Figure 56); the most

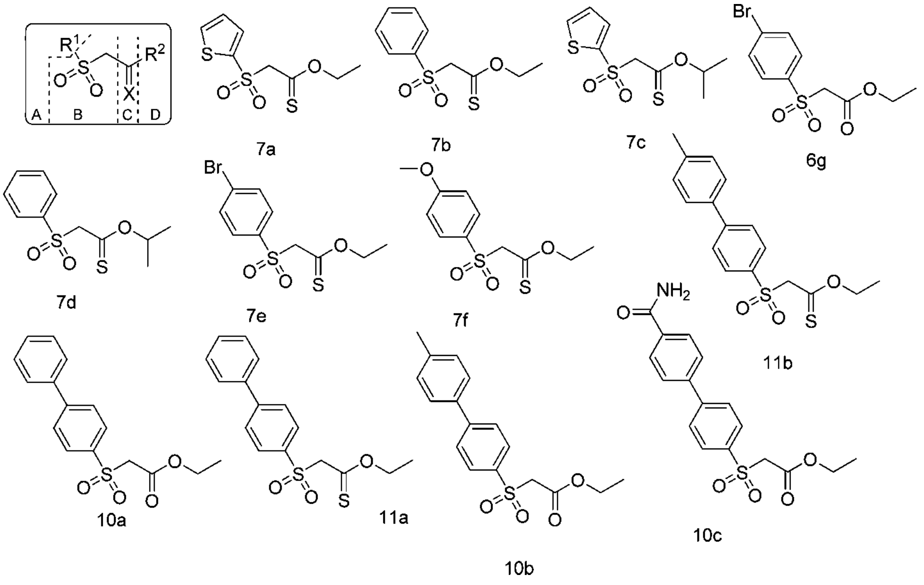


Figure 54. General core structure of sulfone "hits" **284** and **285** identified by Li et al.[49] together with the structures of the 12 most active analogues of these lead compounds.[206]

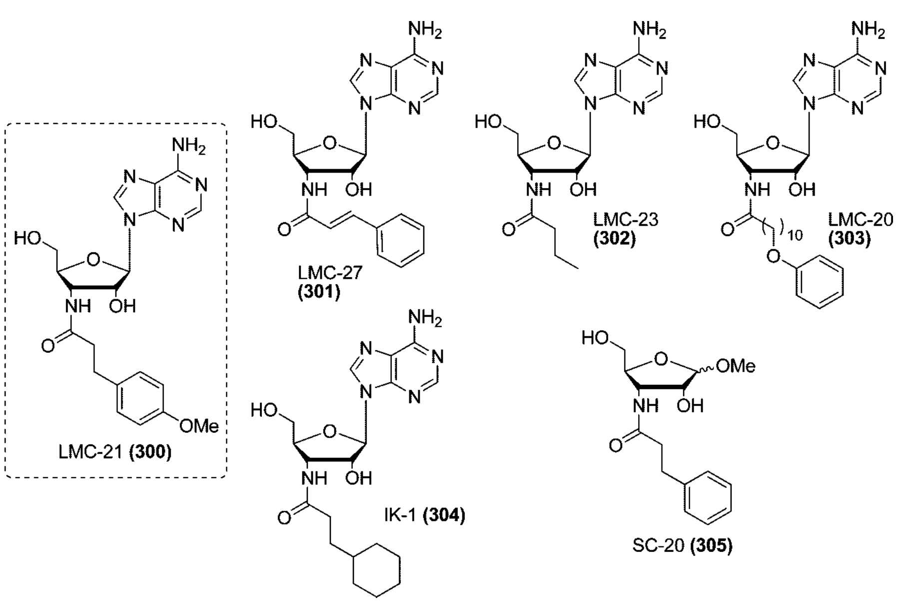


Figure 56. Selected examples of analogues used in the study of Brackman et al.[258]

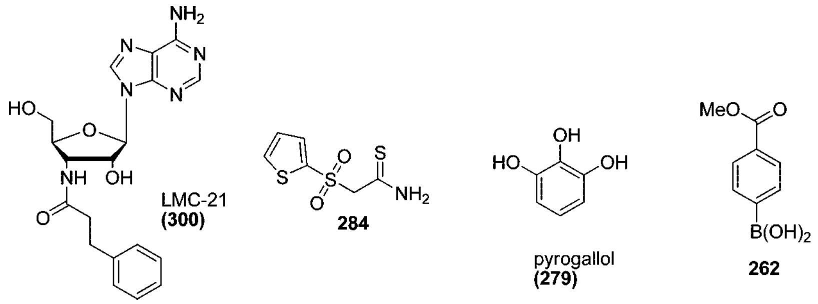


Figure 57. Compounds evaluated by Brackman et al. for their ability to inhibit quorum sensing regulated virulence phenotypes in *Vibrio* spp.[258]

active was the adenosine derivative **300** termed LMC-21. Its mechanism of inhibition was elucidated by measuring the effect on bioluminescence in a series of *V. harveyi* AI-2 quorum sensing mutants. The results indicated that this compound, as well as a truncated analogue lacking the adenine base (**305**, termed SC-20), blocked AI-2-based quorum sensing at the level of LuxPQ without interfering with bacterial growth. SC-20 (**305**) was a significantly weaker inhibitor than LMC-21 (**300**).

The authors noted that this was an unexpected result; on the basis of structural similarities between LMC-21 (**300**) and SAM (**1**), it was originally supposed that LMC-21 (**300**) would interfere at the level of AI-2 synthesis (i.e., DPD (**167**) biosynthesis) rather than at the level of AI-2 signal transduction. The authors noted important structural elements for achieving quorum sensing inhibition in this compound series. Minor changes, e.g., moving the methoxy group of LMC-21 (**300**) from the *para*- to the *meta*-position, or the insertion of an extra CH_2 group between the phenylpropionamido substituent and the ribose moiety of LMC-21 (**300**), resulted in decreased activity. Other molecules strongly resembling LMC-21 (**300**) (e.g., LMC-20 (**303**), LMC-23 (**302**), LMC-27 (**301**), and IK-1 (**304**)) failed to inhibit AI-2 quorum sensing, pointing toward a specific, receptor-mediated effect. The ribofuranose moiety was found to be essential for activity. Although an adenine group was not essential for activity, its presence resulted in more active compounds. In addition to the identification of LMC-21 (**300**), the authors also evaluated LMC-21 (**300**) and pyrogallol (**279**), boronic acid **262**, and sulfone **284** (compounds previously identified by other researchers as AI-2 inhibitors of bioluminescence in *V. harveyi*, see above) for their ability to inhibit quorum sensing-regulated virulence phenotypes in vitro in *Vibrio* species. The specific virulence phenotypes examined were virulence factor production (pigment production and protease activity) and biofilm formation (Figure 57).

LMC-21 (**300**) was the only compound to significantly inhibit pigment production by *V. anguillarum*. Addition of LMC-21 (**300**), **262**, or pyrogallol (**279**) resulted in a significantly decreased *V. anguillarum* protease activity, with LMC-21 (**300**) being at least as active as the other agents. LMC-21 (**300**) decreased the biofilm biomass of *V. anguillarum* and *V. vulnificus*, without reducing the number of viable cells present in the biofilms. Pyrogallol (**279**) only decreased biofilm biomass in *V. vulnificus* but to a higher extent than LMC-21 (**300**). *Vibrio* species are known to regulate stress adaptation by means of their quorum sensing systems; AI-2 is capable of regulating different stress responses including starvation in *V. cholerae*, *V. vulnificus*, *V. anguillarum*, and *V. angustum*. LMC-21 (**300**) was found to suppress the quorum sensing-regulated starvation response in all *Vibrio* species examined, whereas the other compounds increased susceptibility to starvation-associated stress conditions in some *Vibrio* species only, and to a lesser extent than LMC-21 (**300**). LMC-21 (**300**) was also shown to be a potent suppressor of *V. harveyi* BB120 virulence in vivo; high mortality rates were observed when *Artemia* shrimps were exposed to *V. harveyi* BB120, but LMC-21 (**300**) at a compound concentration of 40 μM was able to completely protect *Artemia* during bacterial challenge. LMC-21 was found not to exhibit cytotoxic effects against murine and human cell

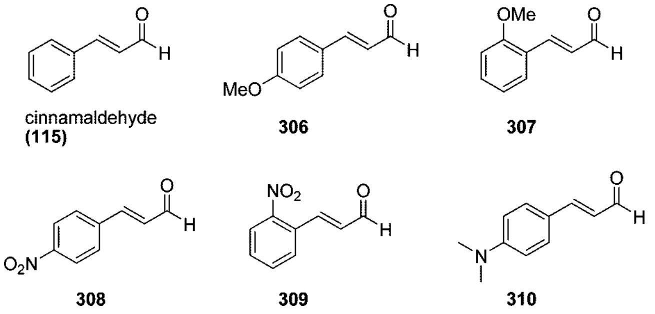


Figure 58. Structures of cinnamaldehyde and cinnamaldehyde derivatives from the study of Brackman et al.[259]

lines when used at a compound concentration of 40 μM. **262**, **284**, and pyrogallol (**279**) were able to inhibit AI-2-mediated bioluminescence in *V. harveyi* BB170. The authors postulate that **262** and **284** target LuxPQ.

4.3. Modulating LuxR Activity

Cinnamaldehyde (**115**) has been shown to inhibit both AHL and AI-2 regulated quorum sensing systems in *V. harveyi* (see above).[194] Recent work by Brackman et al. attempted to elucidate the mechanism of this AI-2 inhibition in *Vibrio* spp.[259] In this report, a range of cinnamaldehyde and substituted cinnamaldehyde derivatives were screened for their ability to interfere with AI-2-based quorum sensing in a variety of *V. harveyi* mutant strains (compounds **306**−**310**, Figure 58). The study identified several non-native derivatives that interfere with AI-2-based quorum sensing by decreasing the ability of LuxR to bind to its target promoter sequence. The use of these compounds at subinhibitory concentrations resulted in several marked phenotypic changes in a variety of *Vibrio* spp., including reduced virulence and an increased susceptibility to stress. More specifically, some of the target compounds were shown to (i) inhibit biofilm formation in several *Vibrio* spp. (**115**, **309**, and **306**), (ii) result in a reduced ability to survive starvation and antibiotic treatment (**115** only), (iii) reduce pigment and protease production in *Vibrio anguillarum* (**115** and **309**), and (iv) protect gnotobiotic *Artemia* shrimp against virulent *Vibrio harveyi* BB120 (**115** and **309**).

4.4. Concluding Comments on AI-2 Signaling

The use of a chemical biology approach to study AI-2 quorum sensing has attracted significant interest in recent years. This can be primarily attributed to the fact that AI-2 signaling operates within, and between, a range of bacterial species, raising the possibility that non-native small molecules can be employed to achieve broad-spectrum modulation of AI-2 controlled phenotypes. However, the vast majority of studies in this field to date have been limited to the only two well-defined AI-2 signaling pathways, namely, the LsrB-based system of *S. typhimurium* and the LuxP-based system of *V. harveyi*. Though non-native small-molecule modulators of these systems, together with some valuable SAR data, have been obtained, it is clear that significant work needs to be done to develop our understanding of AI-2 signaling manipulation in other bacterial species and thus more fully exploit the potential rewards offered by small-molecule manipulation of this "universal" communication system. Toward this end, future experiments should focus upon the delineation of the dictates of AI-2 signaling in a wide range of bacteria species, i.e., characterization of the identity of the active AI-2 signaling molecules and determination of the receptors and detection cascades involved in specific cases.[39] Such information should provide a deeper understanding of the molecular basis behind AI-2 quorum sensing and facilitate the rational design of more active species specific and broad-spectrum AI-2 modulators. In addition, the majority of work on AI-2 thus far has been carried out in vitro, and it is well established that in vitro behavior cannot always be paralleled in vivo. Thus, although the small-molecule modulation of AI-2 systems offers significant potential in a therapeutic context, real-life applications remain a distant goal.

5. Possible Allosteric Effects of Small-Molecule Modulators of AHL and AI-2-Based Quorum Sensing

As noted previously, the majority of work carried out on small-molecule modulation of AHL and AI-2-mediated quorum sensing has focused on identifying agents that can interact with the relevant autoinducer receptor. In the vast majority of cases, it is thought that agents that bind at the receptor level do so at the binding site of the cognate autoinducer. In some reports, this is supported by crystallographic analysis or molecular modeling studies. Typically, however, there is little experimental or computational data regarding the binding modes of such agents; interaction with the receptor at the binding site of the cognate autoinducer is often assumed simply on the basis of structural similarities to the autoinducer. It is possible that many modulators of quorum sensing systems that act at receptor level act in an allosteric fashion; that is, they bind to the receptor at distant sites from the binding pocket of the cognate autoinducer and induce conformation changes in the receptor that impact the activity of the quorum system. Allosteric modulation of quorum sensing by a naturally occurring regulatory protein has been proposed, supported by crystallographic data.[260] Evidence for allosteric-based modulation of quorum sensing by *small molecules* is limited, although it has been used as a rationale in some cases. For example, in section 4.2.1 it was noted that C1-alkyl-substituted DPD analogues have a synergistic agonistic effect upon *V. harveyi* AI-2 quorum sensing when they are incubated with DPD. That is, increased agonist activity is observed when the compounds were incubated in the presence of DPD. Ganin and co-workers have proposed that the C1-alkyl-substituted DPD analogues are interacting allosterically with the AI-2 receptor LuxP, but only in the presence of DPD.[218] Overall, however, there is a relative dearth of information pertaining to allosteric modulation of quorum sensing systems using small molecules.

6. General Concluding Remarks and Future Perspectives

The use of small molecules to modulate bacterial quorum sensing systems has attracted significant interest over the course of the last 15 years. A large number of structurally diverse non-native activators and inhibitors have been discovered, providing researchers with an expansive set of chemical tools to study this form of intercellular communication.[12] Ultimately, strategies based upon the chemical modulation of bacterial quorum sensing may prove to be of value in a wide range of fields, including medicinal, agricultural, and environmental.[12] However, real-life applications (e.g., chemotherapeutics and antifouling coatings) remain a long way off. Achieving a combination of efficacy *and* selectivity (in which the small-molecule modulation of a specific quorum sensing-regulated phenotype in a given bacterial species is achieved) presents a significant challenge.[31] Toward this end, significant advancements need to be made in both the theoretical and practical aspects of the field. As alluded to previously, there is a significant need for the standardization of the assays used by different researchers to study small-molecule modulation of quorum sensing pathways. This would facilitate the elucidation of more accurate (and indeed, more meaningful) SAR data for quorum sensing modulators, which should enhance our understanding of the molecular features necessary for desired biological activity. In this context, there is a definite need for more detailed fundamental studies into the molecular basis of quorum sensing modulation, that is, the mechanisms of action of small-molecule activators and inhibitors in terms of the fundamental bonding interactions involved. Such information would provide a framework for a deeper understanding of the behavior of existing small-molecule modulators on a molecular level and also facilitate the rational de novo design of new next-generation agents with improved molecular properties (i.e., efficacy and selectivity). Overall, the field of small-molecule modulation of quorum sensing can be considered, in many regards, to still be in its infancy. There is, thus, considerable scope for further exciting developments to be made in this area; the reliance of quorum sensing upon a language of small molecules undoubtedly means that chemists will play an integral role in such progress.

7. Abbreviations

Ac	acetyl
ACP	acyl carrier protein
AHL	*N*-acylated-L-homoserine lactone
AI-2	autoinducer-2
B	unspecified Lewis base
CFU	colony forming units
Cn-CPA	*N*-acyl cyclopentylamine
dia.	diastereoisomer
DKP	2,5-diketopiperazine
DNA	DNA
DPD	4,5-dihydroxy-2,3-pentanedione
E1cB	elimination, unimolecular, conjugate base
eDNA	extracellular genomic DNA
GFP	green fluorescent protein
Het	heterocycle
Hcy	homocysteine
IC_{50}	inhibitor concentration that produces 50% enzyme inhibition in the presence of substrate
K_I	equilibrium (dissociation) constant of the reversible combination of an enzyme with a competitive inhibitor
K_i^*	final equilibrium dissociation constant
M	molar
M^{2+}	unspecified divalent metal
Me	methyl
MTAN	5′-Methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (also known as Pfs nucleosidase)
NA	no applicable ligands identified
Nu	unspecified nucleophile
p	*para*
Ph	phenyl
PHL	phenylacetanoyl
POHL	phenoxyacetyl homoserine lactone
PPHL	phenylpropionyl homoserine lactone
PQS	*Pseudomonas* quinolone signal
QscR	quorum sensing control repressor
rac	racemic
SAH	*S*-adenosyl homocysteine
SAM	*S*-adenosylmethionine
SAR	structure−activity relationship
SRH	*S*-ribosyl-L-homocysteine

For a list of the nomenclature used to describe AHLs, consult Table 1.

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