Mechanisms and Synthetic Modulators of AHL-Dependent Gene Regulation

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1. Introduction

Many different proteobacteria use a LuxR-acylated homoserine lactone (AHL) based quorum sensing (QS) system to control gene expression in response to population density. There is a high level of interest in determining whether methods can be developed to manipulate this bacterial cell−cell communication. Ideally, chemical signals would be added into an environment to stimulate beneficial functions of bacteria or curtail undesired activities. However, to artificially alter the QS response of bacteria through intelligent design of chemicals/drugs, a complete understanding of the molecular interactions between the AHL signal, the cognate regulatory proteins, target DNA, and RNA polymerase is necessary.

In this review, we discuss the key elements essential for LuxR-AHL QS. We will summarize the current literature describing the biological activities of the LuxR protein family in the context of its two-domain structure. This basic background will set the stage for an extensive discussion of the strategies used to design synthetic modulators, including AHL analogues and structurally unrelated compounds, and their ability to alter the activity of LuxR homologues. Although we do not yet have a full understanding of AHL-dependent QS-processes, by examining the current state of knowledge in the field from both biological and chemical perspectives, we hope to facilitate further analysis of these systems through more integrative and interdisciplinary approaches.

2. Basic Elements of AHL-Based Quorum-Sensing (QS) Systems

2.1. Discovery of AHL-Based QS in *Vibrio fischeri*

In Gram-negative proteobacteria, the AHL-dependent control of bioluminescence was first observed in the symbiotic marine bacterium *Vibrio fischeri* in the 1970s through some simple but elegant physiological experiments.[1,2] In 1983, molecular characterization of the regulation of the

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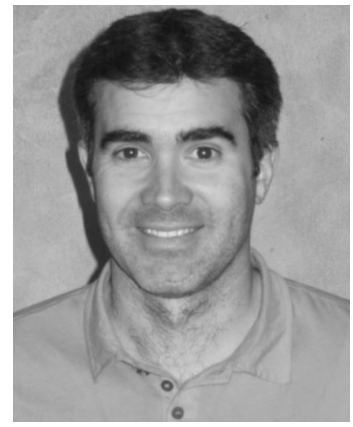
Ann M. Stevens (left) and Susanne von Bodman (right) have been research collaborators since 2001 but have known each other since their days at the University of Illinois. In 1993, Ann earned her Ph.D. from the Department of Microbiology at the University of Illinois at Urbana−Champaign in the laboratory of Abigail A. Salyers. For the next fours year, she served as a postdoctoral research associate under the supervision of E. Peter Greenberg, then in the Department of Microbiology at the University of Iowa. This is where she began studying aspects of QS in *Vibrio fischeri*. In 1997, she began a faculty position at Virginia Tech in the Department of Biological Sciences as an assistant professor and was promoted with tenure to the level of associate professor in 2004 and full professor in 2010. Research in her laboratory is currently focused on QS in the Vibrios and in the plant pathogen *Pantoea stewartii*; the latter work is done in cooperation with the von Bodman group. Susanne earned her Ph.D. from the University of Illinois at Urbana−Champaign in 1985 from the Department of Plant Pathology under the direction of Paul Shaw. She stayed at the University of Illinois in the positions of postdoctoral research associate in the Department of Biochemistry with Stephen Sligar, senior research associate in the Department of Plant Pathology with Stephen Farrand, and visiting assistant professor until 1997, when she moved to an assistant professor position at the University of Puerto Rico—Rio Piedras. In 1998, she accepted an assistant professor position in the Department of Plant Science at the University of Connecticut, where she was promoted to associate professor in 2004 and full professor in 2009. The major focus of research in her laboratory has been the QS control of virulence and biofilm formation in *Pantoea stewartii*. In 2010, she joined the National Science Foundation as a program officer.

Yves Queneau is Director of Research at the Centre National de la Recherche Scientifique (CNRS). After his Ph.D. in Paris-Orsay with Prof. A. Lubineau (1988) and a postdoctoral position at the Memorial Sloan Kettering Cancer Center in New York with Prof. S. J. Danishefsky (1991), he moved to INSA Lyon, a higher education school where he has since developed his research on carbohydrate and bioorganic chemistry. He was recently appointed as Honorary Professor at the University of Hull, U.K., and he is presently deputy-director of the Institut de Chimie et Biochimie Moléculaires et Supramoléculaires (ICBMS) at the University of Lyon.

luminescence system of *V. fischeri* MJ1 was made possible through the cloning of a 9-kb fragment of DNA that encodes all of the functions required for AHL-inducible luminescence



Laurent Soulère was born in 1973 in Toulouse, France, where he studied chemistry. After receiving his Ph.D. degree from the Paul Sabatier University in 2001, he worked from 2002 to 2003 in Dortmund, Germany, as postdoctoral fellow of the Von Humboldt foundation at the Max Plank Institute of Molecular Pharmacology under the guidance of Professor Herbert Waldmann. After working for one year at the University of Perpignan, in 2004, he joined the group of Professor Alain Doutheau as Maître de Conférences. His research interests are focused on the chemistry of bioactive molecules and molecular modeling studies.

Alain Doutheau received his Chemist Engineer Diploma at Ecole Nationale Supérieure of Chemistry of Caen in 1970 and his Ph.D. degree from the University Claude Bernard in Lyon in 1975 under the supervision of Prof. J. Gore. In 1976 he pursued postdoctoral research in the group of Professor P. Deslonchamps in Canada and was then appointed as Maître de Conférences in Lyon. Since 1986 he has been professor of organic chemistry in the Biosciences Department of the National Institute for Applied Sciences (INSA) in Lyon. His research interests are centered on the design and synthesis of biologically active compounds.

in the heterologous host *Escherichia coli*.[3] Although many other QS systems have been identified since, the *V. fischeri* luminescence system is considered a model for studies of the basic mechanism of QS in Gram-negative proteobacteria. In *V. fischeri*, *N*-(3-oxo-hexanoyl)-L-homoserine lactone (3-oxo-C_6-HSL) is the major diffusible signaling molecule produced by the LuxI autoinducer synthase.[4] It is used to self-sense population levels in a given environment. As the levels of this AHL signal rise, complexes form between it and an AHL-dependent activator of transcription, LuxR. The complex can activate transcription of the luminescence genes by binding to the *lux* box in the promoter upstream of the *lux* operon (reviewed in ref 5) as well as several other promoters in the regulon.[6]

There are now many proteobacteria known to have a LuxR-AHL based QS response, with each having minimally the same key components (Figure 1). A protein homologous to LuxI synthesizes an AHL signal in the L-configuration.[7]

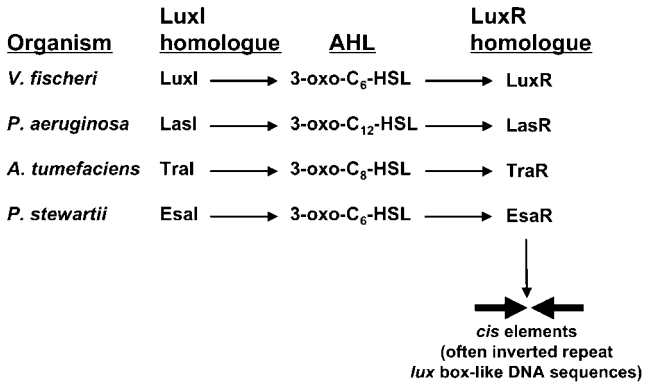


Figure 1. Key elements of an AHL-based QS response. A LuxI homologue or autoinducer synthase catalyzes the production of AHL, which in turn is recognized by a cognate LuxR homologue. Binding of the AHL ligand affects the ability of the LuxR homologue to bind to specific regions, often with dyad symmetry, upstream of promoters in the genes of its regulon. The binding site for LuxR upstream of the *lux* operon, the *lux* box, has the sequence ACCTGTAGGATCGTACAGGT. See the text for the full names of organisms.

At a critical threshold concentration, AHL signals form complexes more efficiently with LuxR homologue proteins. In most cases, these proteins become DNA-binding proficient upon forming a complex with AHL and they bind to DNA motifs of dyad symmetry in the promoter regions of target genes. However, there is a subset of LuxR homologues that are DNA binding competent in the absence of AHL. In the presence of AHL, this subset of LuxR homologues are unable to bind to the DNA and regulate transcription. There are also some exceptions to the rule of AHL being sensed by a cytoplasmic LuxR protein. For example, AHL is recognized by the sensor kinase LuxN in *Vibrio harveyi* (see accompanying review by Spring et al.). Some bacteria have complex, multitiered QS systems employing multiple AHL signals and receptors (for example, see refs 8−10). Here we will focus on basic interactions occurring in the *V. fischeri* LuxI-AHL-LuxR type of QS systems.

2.2. Identification of the Chemical Nature of the AHLs

The AHL molecule produced by LuxI, 3-oxo-C_6-HSL, was the first to have its structure elucidated.[4] It consists of a six carbon fatty acyl chain linked to a lactonized homoserine ring via an amide bond (Figure 2). The compound is synthesized from two substrates, *S*-adenosyl-L-methionine (SAM) from amino acid biosynthesis and an acylated-acyl carrier protein (acyl-ACP) from lipid metabolism.[11,12] LuxI and its homologous proteins synthesize AHL by binding the

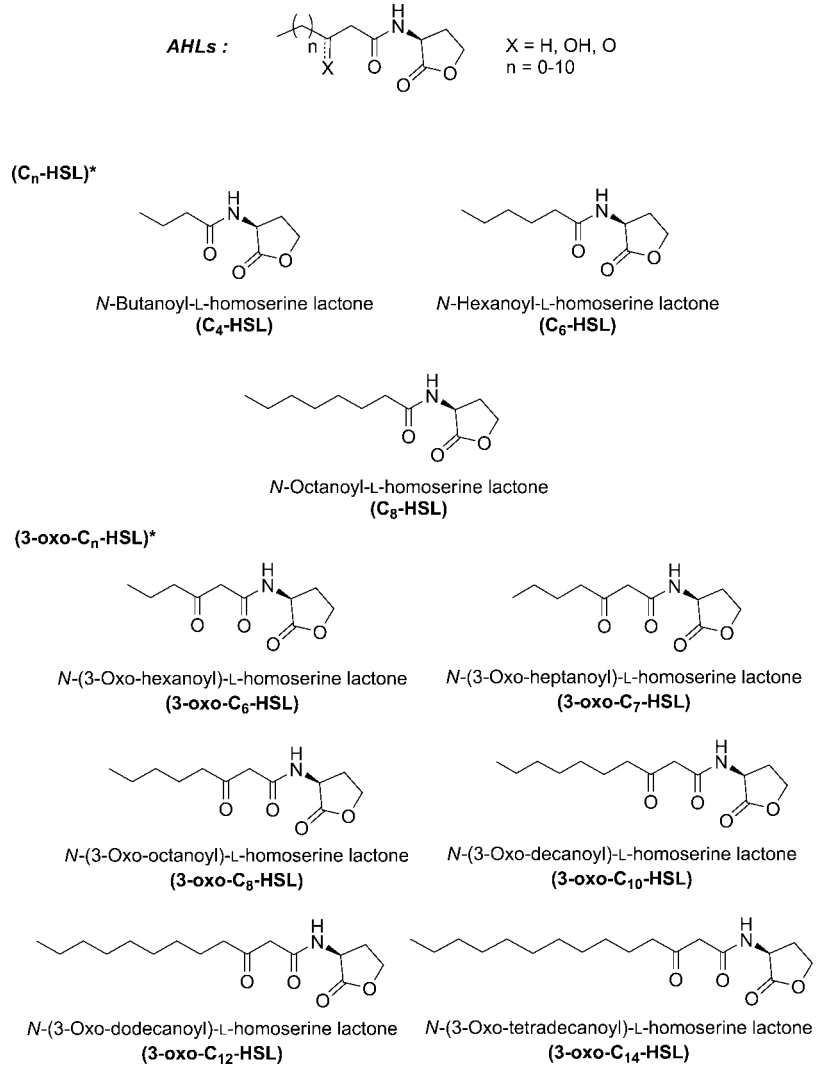


Figure 2. General structure of AHLs and the structure of some native AHLs discussed in this review. * indicates the format of AHL abbreviations used throughout.

A.

AHL Binding Domain

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LuxR    SILDNYPKKWRQYYDDANLIKYDPIVDYSNSNHSPINWN-I--FENNAVNKKSPNVIKEAKT 115
TraR    TAVTNYHRQWQSTYFDKKFEALDPVVKRARSRKHIFTWSGE--HERPTLSKDERAFYDHASD 107
LasR    FIVGNYPAAWREHYDRAGYARVDPTVSHCTQSVLPIFWE-P--SIYQTR--KQHEFFEEASA 107
RhlR    EVHGTYPKAWLERYQMQNYGAVDPAILNGLRSSEMVVWS-D--SLFDQSRMLWN----EARD 113
QscR    HFLSNYPGEWKSRYISEDYTSIDPIVRHGLLEYTPLIWN-GEDFQENRF------FWEEALH 107

EsaR    LIISSYPDEWIRLYRANNFQLTDPVILTAFKRTSPFAWD-E--NITLMSDLRFTKIFSLSKQ 103
ExpR    VIISNYPTEWVDIYRNNNYQHIDPVILTAINKISPFSWD-D--DLVISSKLKFSRIFNLSKD 103
SpnR    LIVSTYSDEWVELYRTNNFQLTDPVILTAFRRTSPFAWD-E--NITLMSDLKFTKIFSLAKH 103
                  54  58      67/68          82
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DNA Binding Domain

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LuxR    ANNKSNN---------DLTKREKECLAWACEGKSSWDISKILGCSERTVTFHLTNAQMKL 225
TraR    TPTAEDA---------AWLDPKEATYLRWIAVGKTMEEIADVEGVKYNSVRVKLREAMKRF 216
LasR    EHPVSKP---------VVLTSREKEVLQWCAIGKTSWEISVICNCSEANVNFHMGNIRRKF 219
RhlR    PMLMSNP---------VCLSHREREILQWTADGKSSGEIAIILSISESTVNFHHKNIQKKF 220
QscR    ---ESNV---------RLTARETEMLKWTAVGKTYGEIGLILSIDQRTVKFHIVNAMRKL 219

EsaR    TEGERAPALNQSADKTIFSSRENEVLYWASMGKTYAEIAAITGISVSTVKFHIKNVVVKL 223
ExpR    REMSRNRNNSKSQEADLFSQRENEILHWASMGKTYQEIALILGITTSTVKFHIGNVVKKL 222
SpnR    TSSSGRQRHHMDRVKPIFTPRENEVLYWSSMGKTYGDIAAIAGISISMVKFHMGNIVSKL 223
              171-178     185 189   195
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C-terminal Domain

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LuxR    NTTNRCQSISKAILTGAIDCPYFKN--- 250
TraR    DVRSKAHLTALAIRRKLI---------- 234
LasR    GVTSRRVAAIMAVNLGLITL-------- 239
RhlR    DAPNKTLAAAYAAALGL----------- 240
QscR    NSSNKAEATMKAYAIGLLN--------- 237

EsaR    GVSNARQAIRLGVELDLIRPA-ASAAR- 249
ExpR    GVLNAKHAIRLGVEMNLIKPVGPAKARS 250
SpnR    GVSNARQAIRLGVELELIK---RQRAR- 247
                      237-249
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B.

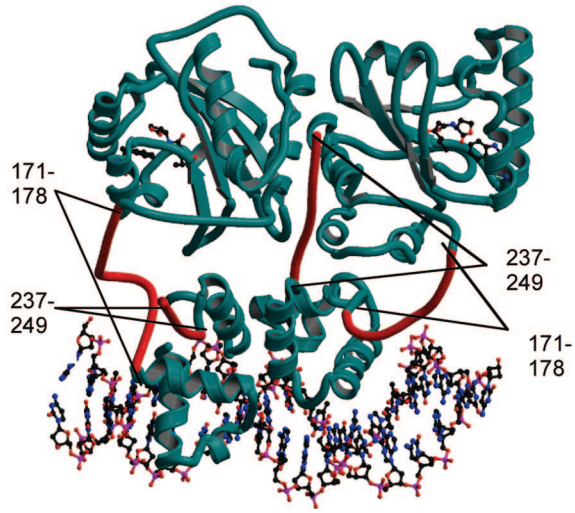


Figure 3. Domain structure of LuxR homologues. (A) Amino acid alignments of the indicated LuxR homologues demonstrate the existence of highly conserved residues in the AHL-binding domain and the DNA binding domain. Identical residues in this alignment are highlighted in blue; those found to be identical in ~95% of LuxR homologues[24] are numbered, with the specific residues corresponding to those in EsaR. The EsaR subfamily has an extended linker region between the AHL- and DNA-binding domains (171−178) and an extended C-terminus (237−249); these residues are highlighted in red. (B) EsaR homology model based on the AHL associated TraR dimer−DNA structure[38] kindly provided by A. Thode, D. Donham, and M. Churchill. Cyan colored regions were generated from a threaded model. Red colored regions are EsaR-subfamily specific, with residues 171−178 in the interdomain linker region and residues 237−249 at the C-terminus of EsaR.

substrates SAM and acyl-ACP in sequential order and catalyzing the transfer of the acyl group from the acyl-ACP to the amine of SAM. This leads to the release of ACP, followed by homoserine lactone ring formation and dissociation of the AHL from the synthase.[13] Besides the LuxI family of synthases, proteins homologous to AinS[14,15] and HdtS[16] have also been shown to be capable of AHL production.

As additional AHLs have been identified and their structures examined, it has been established that variation occurs between them, primarily within the acyl chain

(Figure 2). This is believed to provide specificity in recognition between a particular LuxR homologue and its cognate AHL (reviewed in ref 17). The length of the acyl chain varies between four and eighteen carbon atoms, generally in increments of two.[13,18] Further enhancing the natural AHL diversity among different proteobacteria is the oxidation/reduction state of the third carbon in the acyl chain, which may have a substituted hydroxyl or keto group. In addition, double bonds sometimes occur in the acyl side chain.[19]

2.3. Classes of LuxR Homologues

The LuxR protein subfamily, whose members detect and respond to AHL signals, is associated with a larger superfamily of regulatory proteins, including MalT, FixJ, NarL, GerE, and region 4 of the sigma-70 subunit of RNA polymerase, that are not involved in QS.[20,21] Some of these proteins utilize a mechanism whereby the N-terminal domain (NTD) prevents interactions between the C-terminal domain (CTD) and the target DNA until a ligand is bound. The region of shared homology among all of these polypeptides is located within the CTD and more specifically in a helix−turn−helix motif within a four-helix domain structure.[20,21] Genetic and biochemical studies of a number of LuxR homologues show them to feature a two-domain structure, with the NTD serving to recognize the AHL signal and the CTD recognizing its cognate binding site in the DNA (reviewed in refs 13, 22, and 23). Amino acid alignments reveal an overall low percentage (∼25%) of amino acid identity between pairwise comparisons of LuxR homologues. However, in multiprotein alignments, several well-conserved residues emerge in both the AHL-binding domain and the DNA binding domains[24] (Figure 3A).

The LuxR protein family was previously subdivided into three different classes.[25] However, we would like to suggest that at least five different classes exist based on the interactions of the LuxR homologues with AHL and their multimeric properties. TraR, LuxR, MrtR, EsaR, and SdiA are used to represent these five classes (Figure 4). Classes 1−3 include activators that become functional after interacting with the AHL either co- or post-translationally, such as TraR, LuxR, and MrtR. These are the LuxR homologues from *Agrobacterium tumefaciens*, *V. fischeri*, and *Mesorhizobium tianshanense*, respectively. Class I regulators such as TraR bind AHL cotranslationally, with the AHL becoming buried in the protein structure. In the absence of AHL, TraR is rapidly degraded by the Clp and Lon proteases.[26,27] Class 2 regulators such as LuxR exhibit enhanced stability in the presence of AHL, but ligand binding is readily reversible.[28] Class 3 regulators such as MrtR do not require AHL for protein folding, but the ligand is required for dimerization and activation.[29] The activity of the class 1−3 proteins is enhanced by the presence of AHL, although the speed with which allosteric regulation can occur varies depending on the stability of the protein and the accessibility of the binding site for the ligand. In contrast, class 4 homologues, such as EsaR from *Pantoea stewartii*, are only capable of functioning as dimeric DNA-binding proteins and regulating transcription in the absence of AHL.[30] Besides EsaR, class 4 only has a few members, most notably ExpR,[31,32] YenR,[33] and EanR.[34] The proteins in this group were first identified as repressors[35] and contain two unique regions in comparison to the rest of the LuxR homologues: (1) an extended linker region between the AHL binding NTD and DNA binding CTD and (2) extra

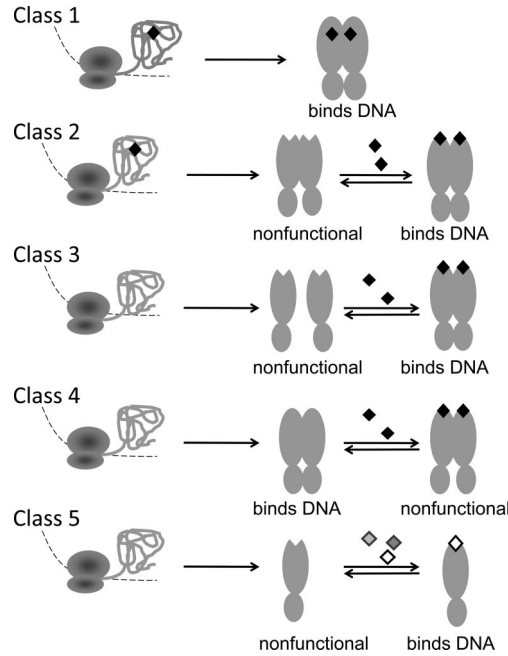


Figure 4. Subgroups of the LuxR protein family, based on their interactions with AHL and their multimeric properties. Class 1 regulators such as TraR associate with AHL cotranslationally, and the AHL is buried within the protein structure. Class 2 regulators such as LuxR are stabilized by AHL during translation; however, the AHL binding is reversible. Class 3 regulators such as MrtR are stable without AHL but require AHL to dimerize and activate transcription. Class 4 regulators such as EsaR fold properly and are capable of DNA binding in the absence of AHL; AHL binding inactivates these proteins. Class 5 regulators such as SdiA do not dimerize and are capable of recognizing multiple noncognate AHL signals. The cognate AHL for a given LuxR homologue is represented by a black diamond. White and gray diamonds represent noncognate AHLs.

residues at the C-terminus of the polypeptide (Figure 3B; A. Thode, D. Donham, and M. Churchill, personal communication). Finally, SdiA from *Escherichia coli* represents class 5. Class 5 proteins do not dimerize in response to AHL, and the AHLs they recognize are produced from neighboring cells; there is no self-produced/native AHL. Hence, they are considered to be a type of "orphan" LuxR homologue with no partner LuxI homologue.[24] Other orphan LuxR regulators do recognize self-produced AHLs and influence QS by the formation of heterodimers with other LuxR homologues.[24]

Detailed structural information is only available for a few members of the LuxR protein family (see accompanying review by Chen and Churchill; http://dx.doi.org/10.1021/cr1000817). NMR analysis showed that the N-terminal domain of SdiA from *Escherichia coli* is a monomer capable of binding C_8-HSL, although it has no known cognate AHL.[36] It is thought that SdiA functions as a detector of AHLs produced by other species of bacteria. The SdiA-AHL system exhibits a "folding switch" behavior, with the protein expressed as insoluble inclusion bodies in the absence of AHL. However, in the presence of AHL, the protein folds properly, with the AHL sequestered in a deep pocket of the hydrophobic core.[36]

Crystal structures have been solved for two LuxR homologues, TraR from *A. tumefaciens* and the LasR N-terminal domain from *Pseudomonas aeruginosa.* The first crystal structure was obtained for TraR with its cognate AHL, *N*-(3-oxo-octanoyl)homoserine lactone (3-oxo-C_8-HSL), at a resolution of 1.66 Å[37,38] when bound to its DNA recognition site.

The amino terminal domain is an alpha−beta−alpha sandwich that binds the AHL through four hydrogen bonds and numerous hydrophobic contacts. The carboxy terminus contains a helix−turn−helix motif that binds to the DNA. Overall, TraR has a dimeric conformation with a 2-fold symmetry axis within each monomer, but the two monomers are at an ∼90° angle to each other, producing overall asymmetry in the dimer. The residues critical for dimerization have been identified and appear to be necessary for both DNA binding and resistance to proteolytic degradation.[39] The crystal structure of LasR shows an overall alpha−beta−alpha structure similar to that of TraR, but it is a symmetrical dimer instead of asymmetrical.[40] This symmetry is believed to enable LasR to recognize a wide range of DNA binding sites, with or without dyad symmetry. The larger binding pocket in LasR (∼670 Å^3) accommodates the long-chain 3-oxo-C_{12}-HSL made by *P. aeruginosa*.[40] While the monomeric structures of SdiA, TraR, and LasR are similar, their quaternary structures vary, being a monomer, assymmetric dimer, or symmetric dimer, respectively. Efforts to obtain detailed structural information about additional LuxR homologues are in progress and will likely yield further insights into the elusive mechanisms that enable stimulation and destimulation of the biological activities of the LuxR homologues.

3. Biological Activities of LuxR Homologues

3.1. DNA Recognition by LuxR Homologues

LuxR homologues elicit physiological effects by controlling transcription rates from the genes in their regulons. The DNA binding site for LuxR, upstream of the *lux* operon, was the first to be identified. This so-called "*lux* box" consists of a twenty base pair region of dyad symmetry (ACCTGTAGGATCGTACAGGT). However, not all bases contribute equally to LuxR recognition; for example, the two distal bases of the *lux* box were found to be nonessential.[41] Subsequently, it was determined that some degree of conservation exists in the DNA binding sites of LuxR and its homologue LasR. LuxR, in the presence of its cognate AHL, recognizes the *lasB* promoter from *P. aeruginosa*, and conversely, LasR and its cognate AHL bind the *lux* operon promoter from *V. fischeri*.[42] Further, genetic analysis of the *lux* box revealed that both half sites are required for LuxR recognition[43] and that LuxR makes direct contact with the bases at positions 3−5 and 16−18.[44]

A detailed analysis of the interactions between TraR and its 18-bp DNA binding site has been facilitated by its crystal structure and genetic experiments. The consensus *tra* box is ATGTGCAGATCTGCACAT.[45] Like LuxR, TraR recognizes specific bases within its dyadic DNA binding sites, with each subunit of the TraR dimer using its helix−turn−helix motif to bind to one-half site of the *tra* box. The recognition helix (TraR residues 202−216) is perpendicular to the axis of the DNA and fits into the major groove.[38] Interestingly, this recognition helix does not contain all of the conserved amino acid residues identified through amino acid alignments of LuxR homologues (Figure 3A). The nucleotides at positions 4, 5, and 6 in the DNA recognition site of TraR make direct hydrogen bonds with TraR and are critical for its ability to bind DNA and activate transcription.[45] The six nucleotides at the center of the *tra* box are critical for high-affinity binding but do not appear to make direct contact with TraR. It is hypothesized that these nucleotides cause a bend in the DNA, which is thought to facilitate high-affinity TraR binding. Alterations of the outermost nucleotides in the *tra* box prevent transcription but do not prevent TraR binding, indicating that these bases may play a role in the ability of RNA polymerase to bind to the promoter.[45]

3.2. Interaction of LuxR Homologues with RNA Polymerase

In order for a LuxR homologue to activate transcription at promoters in its regulon, it must make appropriate contacts with the DNA and also interact properly with RNA polymerase (RNAP). In bacteria, RNAP has five essential subunits $\alpha_2\beta\beta'\sigma$ (Figure 5).[46] The β and β' subunits are responsible for the catalytic activity of RNA polymerase.[47] The alpha subunits are responsible for interactions with upstream promoter elements and trans-acting factors, including a large subset of activators of transcription.[48−50] Each alpha subunit consists of two domains, an N-terminal domain (αNTD) and a C-terminal domain (αCTD). At a class I promoter (Figure 5A), the primary interactions between RNAP and the activator occur due to interactions between the proximal side of the activator and the distal interface of the αCTD; the αCTD also simultaneously binds to the DNA using a second interface.[51,52] At a class II promoter (Figure 5B), the proximal interface of the αCTD interacts with RNAP by contacting positive control residues on the distal side of activators.[53,54] In this class of promoters, it is the αNTD that interacts with the proximal side of upstream-positioned activators.[55] The sigma subunit is responsible for recognition and binding to the −35 and −10 promoter sites,[56] although it can also make direct contact with some activators on their proximal side at class II promoters.[57]

For LuxR-dependent activation of transcription to be carried out correctly, LuxR must bind to the *lux* box centered at the −42.5 position of the *luxI* promoter, where it is in a position to make multiple contacts with RNAP (Figure 5B).[58,59] Thus, LuxR is considered to be an ambidextrous activator,[48] making contact with RNAP on both its distal and proximal sides at the class II *lux* operon promoter. To establish the role of the RNAP αCTD in LuxR-dependent transcriptional activation of the *lux* operon, a library of

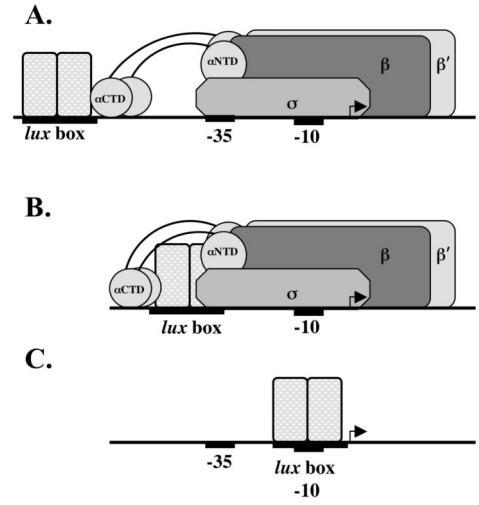


Figure 5. Model of interactions between LuxR homologues and RNA polymerase at different promoters. (A) Class I-activated, (B) class II-activated, and (C) repressed promoters are depicted; in each case the LuxR homologue is shown associated with a *lux* box. The LuxR homologue is represented by the speckled rectangles. See the text for additional details.

alanine substitution variants of αCTD were utilized to determine the functional role of individual amino acid residues in transcriptional activation. These experiments showed that residues 262, 265, 295, and 296 of the RNAP αCTD are important for DNA recognition while residue 290 appears to be in direct contact with LuxR.[58]

In the sigma subunit of RNAP, regions 2 and 4 of the polypeptide feature highly conserved, basic amino acid residues that are involved in DNA binding.[60,61] Region 2 is in the middle of the polypeptide sequence and binds the −10 site in promoters, whereas region 4 is near the C-terminus and binds the −35 site.[62] During activation, region 4 is positioned to interact with upstream transcriptional activators.[57] Indeed, region 4 of the σ^{70} subunit was shown to be involved in LuxR-dependent activation of the *luxI* promoter in vitro and in recombinant *E. coli*.[59] Moreover, variants of σ^{70} illustrated that some individual residues in region 4 increased and/or decreased LuxR-dependent transcription initiation. Accordingly, the region 4 residues appear to be critical for complex formation between RNAP, the activator, and the promoter, leading to activation of transcription at the *lux* operon.[59]

To further understand the interactions between the LuxR family of activators and RNAP, a genetic analysis of LuxR and TraR was used to identify critical residues required for activation. For LuxR, these studies showed that changing the C-terminal amino acid residues K198, W201, and I204 to alanine has a negative effect on transcriptional activation.[63] To this date, no amino acids in the N-terminal domain of LuxR have been reported to interact with RNAP and affect its ability to activate transcription. Indeed, just the C-terminal domain of LuxR, LuxRΔN, is capable of activating transcription of the *lux* operon in an AHL-independent manner.[64,65] These results suggest that, in the case of LuxR, only the C-terminal domain is required to interact with RNAP for activation to occur.

Alanine-scanning mutagenesis of TraR revealed that single substitutions at positions W184, V187, K189, E193, V197, and D217 in the C-terminal domain have a negative effect on its ability to activate transcription of both class I and class II promoters.[66] These residues are likely to make contact with the αCTD of RNAP. In contrast to LuxR, TraR uses two specific amino acids (N10, G123) in the N-terminal region for transcriptional activation.[67] More recent studies identified additional amino acids in the NTD[68,69] and CTD[69] of TraR that play a role in positive control. These studies involved an analysis of TraR variants from both nopaline and octopine producing *A. tumefaciens* strains. Collectively, a model has emerged showing that TraR has three surface exposed patches of positive control residues; two patches (I and III) are near the DNA binding domain on both lateral faces of the dimer, while patch II is positioned near the top of the molecule, as viewed down the DNA axis. Interestingly, patches I and II involve residues from both subunits of the TraR dimer.[69] Residues in these patches that are involved in positive control of both class I (*traM*) and class II (*traI*) promoters are hypothesized to contact the RNAP αCTD, while those required only for class II promoter activation likely contact other regions of the RNAP.[68]

TraR has a significantly higher affinity for the alpha and sigma subunits of RNAP from *A. tumefaciens* compared to RNAP from *E. coli.* Nine residues in the alpha subunit of *A. tumefaciens* are required for TraR-mediated activation.[69] Indeed, most of the positive control residues in TraR play a role in interactions with the alpha subunit of RNAP at class II promoters. Therefore, while some similarities exist in the manner with which RNAP interacts with either LuxR or TraR, there are also unique features, which are indicative of a divergence in the mechanism of activation among the LuxR family of proteins.

Not all members of the LuxR family function as activators. A subset of LuxR homologues related to EsaR was initially identified as repressors of transcription. These transcription factors function by binding to a site in the promoter near the −10 and thereby interefere with promoter recognition by RNAP (Figure 5C). EsaR functions in the absence of AHL by binding to DNA as a dimer and thereby blocking transcription by RNA polymerase at the *esaR* and *rcsA* promoters.[70,71] However, in 2003, it was determined that EsaR retains the ability to function as a weak activator of the *V. fischeri lux* operon in recombinant *E. coli*.[72] The physiological relevance of EsaR as an activator has been established through the identification of a native promoter of *P. stewartii* that controls expression of a sRNA.[73] Sequence alignment of EsaR with LuxR and homology modeling of EsaR to TraR enabled the mapping of previously characterized positive control variants on EsaR. A combination of deletion and site-directed mutagenesis strategies identified three critical residues of EsaR, which are required for activation. Like TraR, these residues are in both the NTD and CTD.[73]

3.3. Binding of AHL by LuxR Homologues

Early efforts to define the AHL binding domain of LuxR focused on genetic studies where the effect of random mutations in *luxR* was studied to determine which residues were essential for its function.[74,75] Thus, the residues located in the N-terminal domain between 79 and 127 of LuxR were predicted to play a role in AHL recognition. Deletion analysis of LuxR determined that the C-terminal domain of the protein could function in an AHL independent manner, further implicating the N-terminal of the protein in AHL recognition.[64,76] Alignments of LuxR homologues have highlighted several conserved residues in the AHL binding region (Figure 3).[5,24] This degree of conservation is thought to reflect in part the concurrent invariant nature of the homoserine lactone moiety across AHL ligands. Indeed, early studies using synthetic AHLs demonstrated the essential nature of the homoserine lactone ring for signal recognition.[77] However, it was not until the detailed structural analysis of SdiA, TraR, and LasR was completed that a more complete picture of the interactions occurring between these LuxR homologues and native AHLs emerged.

SdiA has a more spacious binding pocket than TraR and LasR, which may explain its ability to bind to a broader range of ligands.[36] TraR and LasR have specificity in their ability to recognize 3-oxo-C_8-HSL and 3-oxo-C_{12}-HSL, repectively. In TraR, the recognition of AHL is believed to occur through three direct hydrogen bonds and one water mediated hydrogen bond, as well as additional hydrophobic interactions.[37,38] TraR variants that have disrupted water-mediated hydrogen bonds to the 3-oxo moiety of 3-oxo-C_8-HSL recognize oxo-AHLs and 3-unsubstituted AHLs equally well, while TraR variants with bulkier residues than the wild-type protein in the AHL binding site preferentially recognize AHLs with six or seven carbon atoms instead of eight.[78] In comparison to the case of TraR, there are significant differences in the H-bonding network used in LasR to recognize the cognate AHL, 3-oxo-C_{12}-HSL[40] (see accompanying review by Chen and Churchill; http://dx.doi.org/10.1021/cr1000817). Thus, the ability of

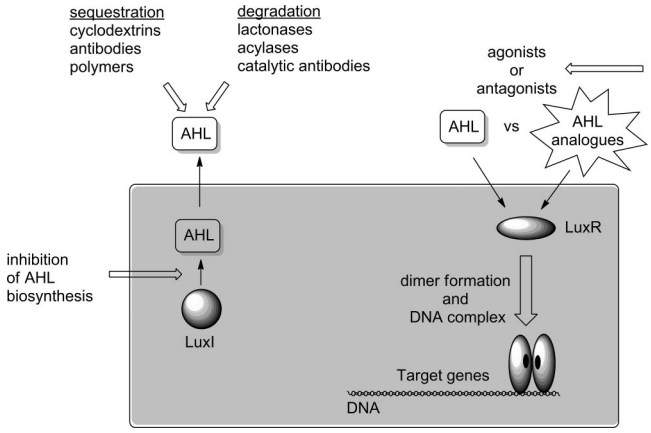


Figure 6. Schematic view of various strategies for QS modulation as indicated by the open arrows. See the text for details.

each LuxR homologue to recognize its cognate AHL has unique qualities. Studies of the ability of synthetic modulators of LuxR homologues to serve as agonists or antagonists have provided further insights into the critical points of recognition between the protein and its ligand, as described below.

4. Strategies for Designing QS Inhibitors

AHL-mediated QS-dependent gene regulation lends itself to intervention by several strategies, including the design of inhibitors for the LuxI-type AHL synthases[79] and the sequestration of AHLs by free or immobilized cyclodextrins,[80,81] by antibodies,[82,83] or by adsorption on polymers[84] (Figure 6). The AHLs themselves are inactivated by enzymes such as lactonases and acylases[85,86] and catalytic antibodies.[87] Other strategies involve the use of AHL antagonists directed against the AHL LuxR-type protein receptors to block the QS biological response (Figure 6) by impacting dimer formation, protein/DNA binding or interactions with RNAP.

AHL-mediated QS offers an ideal target for pharmacological intervention of many important animal and plant diseases, as well as for other environmentally or industrially detrimental bacterial impacts. This realization has prompted considerable efforts to design potential QS inhibitors based on the AHL scaffold (for recent reviews, see refs 17 and 88−92).

In the following sections, we will focus on a discussion of AHL agonists and antagonists. Agonists and antagonists are compounds which both bind the target receptor. Agonists induce the same biological effects as natural ligands do, whereas antagonists block the action of the natural ligand. Aspects of the other modulation strategies depicted in Figure 6 are covered in accompanying review articles in this thematic issue.

Ligand-specific agonists or antagonists can be designed based on the structures of the native ligands and their impact on protein receptor activity. Although it can be anticipated that structural analogues of native ligands would show a satisfactory affinity for cognate target proteins, it is difficult to predict *a priori* whether they will display an agonistic or antagonistic activity. Modeling the active site of the receptor proteins and the molecular interactions occurring within it is also an essential approach. However, in the field of the design of AHL analogues for QS modulation, most of the investigations reported to date have been based on systematic structural variations, with only a few of them incorporating modeling studies, often performed *a posteriori*. Though AHLs are structurally rather simple

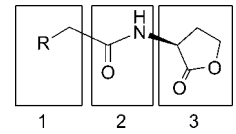


Figure 7. The three signature components of AHLs include (1) the acyl chain including the characteristic carbonyl or hydroxyl substitution or lack thereof at the C-3 position, (2) the central amide connective function, and (3) the lactone moiety.

molecules compared to many other hormones and biomolecules, one can delimit three areas ideal for chemical modification when considering the variations which can be addressed: (1) the acyl chain including the characteristic carbonyl or hydroxyl substitution or lack thereof at the C-3 position, (2) the central amide connective function, and (3) the lactone moiety (Figure 7). Overall, this makes the AHL amphiphilic. The lipophilic acyl chain is often quite long and can aid in diffusion through the membranes and contribute to ligand binding within the receptor pocket. The amide function provides a strongly polar moiety to AHLs, with a high potential to serve as a H-bond donor or acceptor. The lactone ring is subject to degradation by lactonases released into the environment by neighboring bacteria. Interestingly, it has been shown that AHLs can spontaneously rearrange to tetramic acids.[93,94] Besides the design and synthesis of structural analogues, a complementary approach for discovering ligand agonists or antagonists is the screening of libraries of synthetic compounds or natural extracts.

We will address the impact of active compounds identified from these approaches on QS, using assays conducted in *V. fischeri* and *P. aeruginosa*, and, to a lesser extent, in *A. tumefaciens* and *Erwinia carotovora.* Many investigations on the structure activity relationship (SAR) of AHL analogues have been reported in the last 25 years. The salient results are reviewed in section 5, which is organized with respect to the chemical structure of AHL analogues. The three areas of the AHL structure depicted in Figure 7 are discussed. Active compounds, which are structurally unrelated to AHLs, are described in section 6. A table summarizing the representative most active compounds and their level of activity with respect to QS modulation in the main bacterial strains is given in section 7.1.

5. AHL Analogues

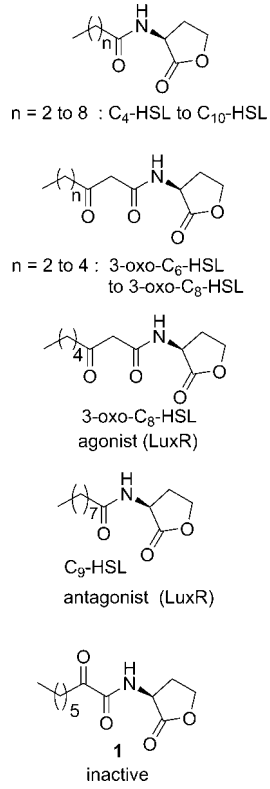
5.1. Variations in the Acyl Chain

5.1.1. Closest Structural Analogues of Natural AHLs

One of the key AHL specificity determinants identified early on was the length and character of the AHL acyl side chain. Various bacteria produce AHLs that either stimulate or inhibit QS in other bacteria.[95] Long side-chain AHLs are often antagonists of short-chain AHLs. For example, *N*-decanoyl-L-homoserine lactone (C_{10}-HSL) inhibits the production of the antibiotic pigment violacein by *Chromobacterium violaceum*, an *N*-hexanoyl-L-homoserine lactone (C_6-HSL)-dependent function.[96] These realizations prompted a number of efforts to screen for chemically synthesized AHL analogues with the desired inhibitory effects. The syntheses of native AHLs or of close analogues were performed either by classical solution phase chemistry[77,95−105] or solid phase chemistry, which is more appropriate for accessing libraries of compounds.[106−113]

Eberhardt and co-workers were the first to synthesize and test a small collection of 17 analogues of 3-oxo-C_6-HSL

Chart 1. Variations in Chain Length and Carbonyl Position in AHLs

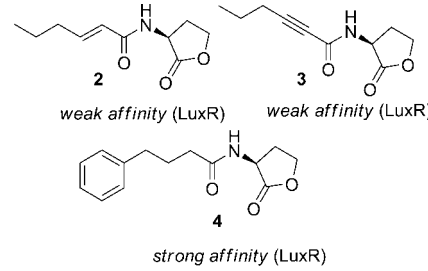


using solution phase chemistry.[95] Apart from a few other structural alterations, the compounds were mainly acyl-chain-modified AHL analogues varying in the chain length and the presence or absence of the 3-oxo function. In addition, this group also synthesized the 2-oxo-variant AHL compound **1** (Chart 1). These analogues were tested for their ability to activate or inhibit luminescence in various *V. fischeri* strains, including strain B-61, from which the native AHL was originally isolated. A lesson learned from this study is that a certain level of variability in the acyl chain is acceptable for binding to the LuxR protein, since several compounds displayed some agonistic or antagonistic activity. However, only one of the analogues showed important agonist activities, 3-oxo-C_8-HSL. Among the compounds lacking agonistic activities, some proved to be quite efficient antagonists, especially C_9-HSL. Finally, displacing the oxo group from C-3 to C-2 (in compound **1**) led to no activity.

Bycroft and co-workers examined a set of AHL analogues, among them several acyl-chain-modified compounds, as agonists of 3-oxo-C_6-HSL. 3-Oxo-C_6-HSL is the cognate AHL of the LuxR-type CarR QS regulator found in the plant pathogen *E. carotovora*.[99] Subtle alteration of the acyl chain, such as removing one carbon atom or the 3-oxo carbonyl, resulted in a drastic reduction in agonistic activity compared to the case of the native ligand. This indicated that CarR is highly selective for 3-oxo-C_6-HSL.

Schaefer et al. synthesized a new series of native AHLs and corresponding analogues a decade after the first study by Eberhard et al.[95] and tested their effects in terms of (i) competitive inhibition of tritiated 3-oxo-C_6-HSL binding to LuxR-containing *E. coli*, (ii) activation of the *V. fischeri* luminescence genes in *E. coli*, and (iii) competitive inhibition of 3-oxo-C_6-HSL-dependent induction of luminescence in recombinant *E. coli*.[77] These studies revealed that the C_5 to C_{14} analogues, differing from 3-oxo-C_6-HSL only in the acyl chain length, competitively inhibited native AHL binding to LuxR. In this series, the C_6 and C_8 derivatives were the most active, while the C_4 analogue had no appreciable effect. Studies comparing the inhibitory potential of the 3-oxo and corresponding 3-methylene-substituted derivatives showed that the 3-oxo derivatives were the most efficient competitors of tritiated 3-oxo-C_6-HSL-LuxR binding. Further, competitor activity decreased by introducing an unsaturation adjacent to the amide bond (compound **2**, Chart 2), and an even stronger effect was observed for a carbon−carbon triple bond (compound **3**) at the same position. Most interesting, perhaps, was the observation that the 3-oxo-C_4-derivative bearing a terminal phenyl group on the acyl chain (compound **4**, Chart 2) was capable of efficiently outcompeting the binding of the tritiated 3-oxo-C_6-HSL to LuxR. This suggested that provoking steric hindrance by the introduction of bulky substituents to the acyl chain is relevant for designing active analogues, a key aspect which was confirmed by several other studies (see below).[104,111,114]

Chart 2. Incorporation of Unsaturated Motifs in the Side Chain of AHLs Analogues



Analogues showing evidence of LuxR binding were also tested as activators or inhibitors of luminescence. None of the compounds analyzed were as active as the native 3-oxo-C_6-HSL, although several showed considerable inducing potential, particularly those equipped with the C-3 carbonyl function. The general trend emerging from these studies was that the most effective analogue competitors were also the most effective inducers of luminescence. Four of the analogues (C_7-HSL, C_9-HSL, C_{10}-HSL, and 3-oxo-C_{12}-HSL) did inhibit 3-oxo-C_6-HSL activity by 80%, although at higher effective concentrations.

Passador et al. prepared a series of structural analogues of 3-oxo-C_{12}-HSL.[115] These authors used *E. coli* MG4 pKDT17 as a reporter strain to assay their predicted agonistic activity. This heterologous strain expresses *lasR* but not *lasI* and reports LasR activity as a function of β-galactosidase expression. Parallel experiments assayed the ability of the 3-oxo-C_{12}-derivatives to exclude tritiated 3-oxo-C_{12}-HSL from binding to LasR in an *E. coli* MG4 background. These studies showed that the most potent agonists were those with an acyl chain length either longer (3-oxo-C_{14}-HSL) (Figure 2) or shorter (3-oxo-C_{10}-HSL) (Figure 2) than the native AHL chain, and preferably bearing the native C-3 carbonyl function. Further, it was observed that the introduction of an (*E*) carbon−carbon double bond at the C6−C7 position (compound **5**, Chart 3) was of limited effect, as this analogue was only slightly less active than 3-oxo-C_{12}-HSL, with an

Chart 3

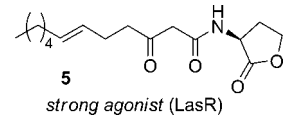
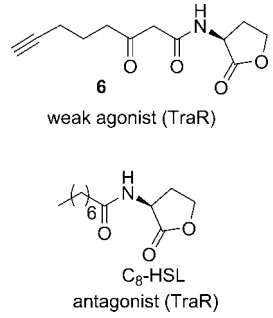


Chart 4



EC_{50} (effective concentration required for half-maximal activation) of 10 and 7 nM, respectively.

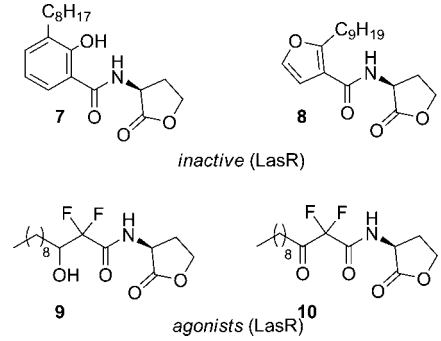
By contrast, increasing the carbon chain length to 15 carbon atoms or shortening it to 6 carbon atoms rendered the analogue compounds about 300 times less active in binding to the LasR regulator than the native inducer. Not surprisingly, the analogues that were the best competitors of tritiated 3-oxo-C_{12}-HSL-LasR binding were also the best agonists for LasR. For instance, efficient agonist **5** was nearly as good a competitor as the native AHL, decreasing binding of tritiated 3-oxo-C_{12}-HSL by approximatively 80%, compared to native AHL.[115]

Zhu et al. compared the ability of 3-oxo-C_8-HSL and 32 acyl-chain-modified compounds,[116] similar to those tested by Schaefer et al. in LuxR[77] and Passador et al. in LasR,[115] to activate the expression of a TraR-regulated promoter in *A. tumefaciens*. Only 3-oxo-C_{12}-HSL was a strong activator of the reporter gene carried by *A. tumefaciens* strain WCF47 (pCF372). However, four compounds having acyl chains of seven carbons or more and containing the C-3 carbonyl moiety [3-oxo-C_7-HSL, 3-oxo-C_{11}-HSL, 3-oxo-C_{12}-HSL (Figure 2), and the alkynyl 3-oxo-C_8 analogue **6** (Chart 4)] displayed a detectable activity, though only at higher concentrations. The remaining 28 compounds[116] were inactive as inducers but proved to be potent antagonists, in particular those in which the C-3 carbonyl function was replaced by a methylene or a hydroxyl group, or compounds featuring a C2−C3 carbon−carbon double bond in the acyl side chain. Interestingly, the best antagonist (C_8-HSL) only lacks the C-3 carbonyl group in comparison to the native AHL (3-oxo-C_8-HSL), suggesting that this 3-oxo function is essential for converting TraR to an active conformation, though not for binding to TraR.

5.1.2. More Diversified AHL Analogues

The biological activities of several new, more diversified AHLs analogues were reported by Kline et al.[117] These authors sought to evaluate the QS modulatory ability of constrained analogues of 3-oxo-C_{12}-HSL, focusing on the importance of the keto−enol equilibrium of the β-ketoamide linkage in 3-oxo-AHLs. Among other compounds, they notably synthesized salicylamide compound **7** and furan compound **8** (Chart 5), rigid mimics of the (*Z*) and (*E*) enol tautomeric forms, respectively. They also prepared α-difluoro analogues such as compounds **9** and **10** (Chart 5), in which the keto−enol tautomerization is impossible. These analogues were tested for their ability to stimulate or diminish the AHL effects on the 3-oxo-C_{12}-HSL-LasR and C_4-HSL-RhlR interactions. The assay utilized *P. aeruginosa* strain PAO-JP2, deficient in *lasI* (for 3-oxo-C_{12}-HSL production) and *rhlI* (for C_4-HSL production) but containing fully functional

Chart 5. Enol Mimics and Nonenolizable AHL Analogues



LasR and RhlR proteins. The results showed that, while the difluoro derivatives **9** and **10** (Chart 5) displayed moderate agonistic activity for LasR, these compounds were highly effective competitors capable of reducing tritiated 3-oxo-C_{12}-HSL binding to LasR by 85−90% of control levels. These findings indicate that the agonistic activity of compounds **9** and **10** could be attributed to direct agonist activity at LasR. The fact that analogues **7** and **8**, which possess a cyclic appendage within the acyl chain, are inactive supports the hypothesis that retaining an extended chain geometry is necessary for a compound to be recognized by LasR.

Reverchon et al. prepared a series of 22 novel racemic synthetic C-4-substituted analogues of 3-oxo-C_6-HSL (compounds **11**, Chart 6) with ramified alkyl, cycloalkyl, or aryl substitutions at the C-4 position, as well as three 4-aryl-substituted analogues (compounds **12**, **13**, and **14**, Chart 6) of the secondary AHL, C_6-HSL, in *V. fischeri*.[104] These compounds were first screened for their ability to induce LuxR-dependent bioluminescence in the recombinant *E. coli* strain NM522, harboring the sensor plasmid pSB401, which contains the *luxR* gene from *V. fischeri* and the *V. fischeri luxI* promoter coupled to the entire *lux* structural operon (*luxCDABE*) from *Photorhabdus luminescens*.[118]

These studies showed that, apart from the two analogues bearing a very bulky substituent (**11a**, R = *tert*-butyl, and **11b**, R = adamantyl, Chart 6), which were inactive, all other alkyl-substituted analogues displayed agonistic activities, with the cyclopentyl derivative **11c** being slightly more active than 3-oxo-C_6-HSL. Compounds that failed to show significant agonist activity were tested as potential antagonists against 3-oxo-C_6-HSL. With the exception of compound **11b**, these molecules all displayed antagonistic activity, measured as a function of luminescence. This activity was influenced to some degree by the C4-phenyl substitution, with an IC_{50} of 10 μM for the 4-trifuoromethyl-substituted compound **11e** compared to 2 μM for the unsubstituted parent compound

Chart 6. Ramified Alkyl, Cycloalkyl, and Aryl C-4-Substituted AHL Analogues

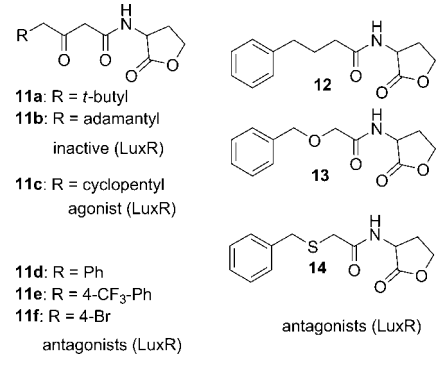
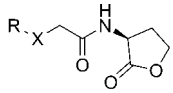


Chart 7. Sulfide, Sulfoxide, and Sulfone AHL Analogues



X = S, R = C_7H_{15} (**15**)
X = S, R = 4-CH_3-Ph (**16**)
X = SO, R = C_7H_{15} (**17**)
*antagonists (*LasR*)*

X = SO_2, inactive *(*LasR*)*

11d. Reverchon et al.[104] tested the hypothesis that the antagonist activity of aryl-substituted AHL analogues would result from a specific interaction with aromatic amino acids of the ligand receptor pocket to prevent LuxR from adopting an active dimeric form. This hypothesis was supported by the cocrystallized TraR-3-oxo-C_8-HSL X-ray structure,[38] which revealed the presence of two aromatic amino acid residues (Y53; W57) in the area of the N-terminal domain ligand pocket facing the alkyl chain.

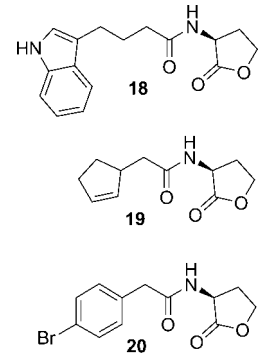
In a study aimed at evaluating the influence of sulfur atoms in AHL analogues, in relation with the observation that some sulfur-containing compounds found in garlic were QS inhibitors,[119] Givskov and colleagues synthesized a set of 32 novel AHL analogues. These compounds featured the native homoserine lactone and C-3 modifications including either a sulfur atom, a sulfoxide, or a sulfone and alkyl chains of various lengths.[120] This group also prepared compounds equipped with an aromatic moiety linked either directly to the sulfur atom or to distant carbon-atoms on the alkyl chain. Their biological activity was examined using the QS inhibitor selector system QSIS1,[119] which reports whether a compound is able to block LuxR mediated QS in the presence of 3-oxo-C_6-HSL. All sulfide and all but one sulfoxide derivative displayed the anticipated inhibitory activities, while the sulfones were all inactive. The active compounds were further assayed for activity against the LasR system based on the expression of a *lasB::gfp* promoter gene fusion (ASV) expressed in *P. aeruginosa* PAO1.[121] These studies identified the thioethers **15** and **16** and the sulfoxide **17** as the most potent QS inhibitors (Chart 7). The authors suggested that the strong inhibition observed for sulfide **15** could be related to its incapacity to interact with the β-sheet but its ability to form important contacts inside the binding-pocket, notably by van der Waals interactions between the α-helices and the lipophilic acyl group.

5.1.3. Libraries of Aromatic Substituted Compounds

Blackwell and co-workers conducted a series of important studies focused on the design and the synthesis of a large collection of acyl-chain-modified AHL analogues, and they evaluated these compounds for activity against LuxR-type proteins in a range of Gram-negative bacteria.[110,111,113,114,122−124] Their synthesis approach was innovative and highly efficient in preparing native and analogue AHLs based on solid-phase chemistry and microwave technology.[108,110,111] These technologies allowed them to develop small (∼20 compounds) to medium (∼90 compounds) sized combinatorial libraries, which enabled them to establish the most comprehensive set of structure−function relationships of AHL-derived QS modulators across different bacterial species.

In their initial study, these researchers prepared 11 native AHLs and a small test library of non-natural analogues (Chart 8).[110] These compounds were screened into two well-characterized bacterial reporter strains for antagonism of QS:

Chart 8. Variations in the Carbocyclic and Aromatic Substitution in AHL Analogues



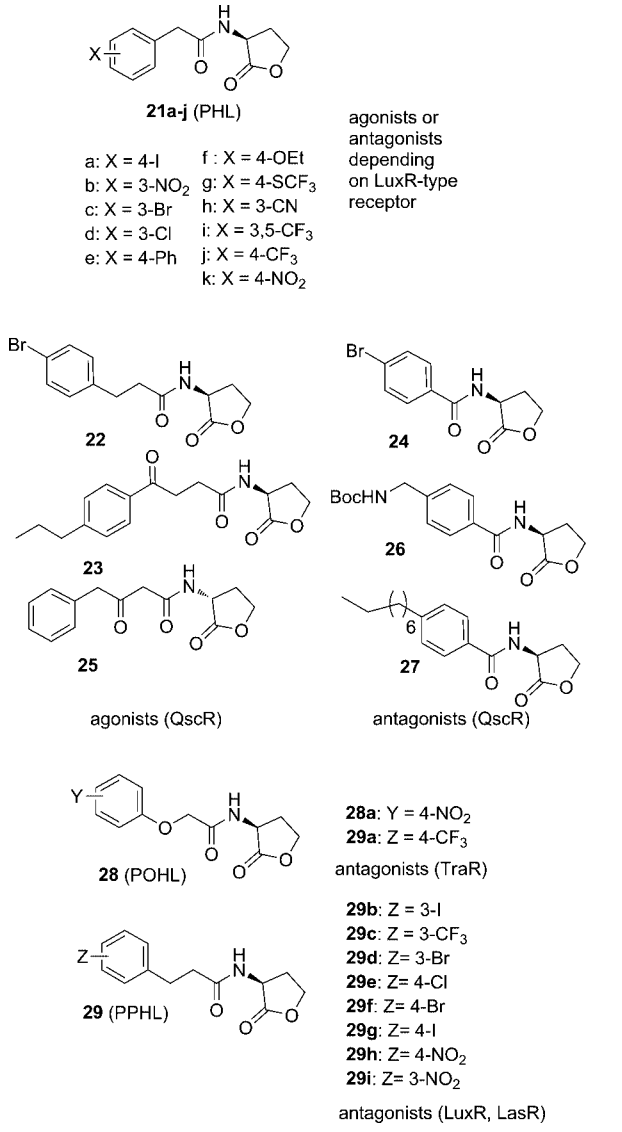
antagonists (LasR, TraR)

P. aeruginosa PAO-JP2(p*las*I-LVAgfp)[125] and *A. tumefaciens* WCF47(pCF372).[116] Both of these reporter strains lack their native AHL synthases yet retain active LuxR-type receptors (LasR and TraR, respectively). Exogenous ligand is required for receptor activation, which can be measured by fluorescence (green fluorescence protein (GFP) for LasR) or absorbance (via β-galactosidase activity for TraR) outputs. In this series, **18**, **19**, and **20** (Chart 8) showed significant antagonistic activity (1 to 2 orders of magnitude more active than other established antagonists) against LasR in *P. aeruginosa* as well as TraR in *A. tumefaciens*.

Blackwell and co-workers extended these studies through synthesis of a larger library of new non-native *N*-acylated homoserine lactones of four specific structural types.[111,122] This library was designed to probe the role of key features of AHL structure on QS activity, including the acyl chain length, the lactone stereochemistry, as well as the general functionality on the acyl group. A first sublibrary contained, among other compounds, the native C_4-, C_6-, C_8-, C_{10}-, C_{12}-, and C_{16}-HSL, plus 3-oxo-C_{10}-HSL and 3-oxo-C_{14}-HSL. A second sublibrary concerned the absolute configuration of the stereogenic lactonic carbon atom, the acyl group aromaticity, and the importance of the distance between the aromatic group and the lactone ring. A third sublibrary consisted of 25 *N*-phenylacetyl-L-homoserine lactones (PHL, **21**) (Chart 9), while a fourth was composed of 21 structurally diverse synthetic AHLs analogues. A more focused library of PHLs was further prepared.[123] All these AHL analogues were evaluated across three Gram-negative bacterial species: *A. tumefaciens*, *P. aeruginosa*, and *V. fischeri.*

These combined studies revealed key structure−activity relationship (SAR) trends. First, AHL analogues with acyl groups up to eight carbons, most of them having the terminal carbon−carbon bonds of the acyl chain involved in an aromatic system substituted by an electron-withdrawing group, antagonize the regulatory activities of TraR, LasR, and LuxR. Of these, the 3-(4-bromophenyl)-C_3-HSL (**22**; Chart 9) has the highest broad-spectrum antagonist activity.[111] Second, the members of the PHL sublibrary (compounds of type **21** in Chart 9), particularly the 3- and 4-phenyl-substituted compounds, display a wide range of antagonistic and agonistic activities. However, interpretation of SARs was delicate, as minuscule structural alterations led to significant changes in ligand activity. For instance, 4-I PHL compound **21a** is a potent antagonist across all three "R" receptor proteins and the 3-bromo (**21c**) and the 3-chloro (**21d**) PHLs are antagonists against LasR, while some 3-substituted compounds proved to be potent LuxR agonists, particularly 3-NO_2-PHL (**21b**) and 3-CN-PHL (**21h**) (Chart 9).

Chart 9. Libraries of PHL, POHL, and PPHL Analogues



The 3-NO_2-PHL compound **21b** was identified as the first non-native superagonist of the QS regulated phenotype in *V. fischeri* exhibiting a 10-fold lower EC_{50} than 3-oxo-C_6-HSL (0.35 μM vs 3 μM) (for other types of superagonists, see refs 104, 126, and 127). Since the most potent R protein antagonists are able to act as LuxR weak agonists when used in high concentration, the authors hypothesized that these compounds exert their antagonistic activity through a partial agonistic mechanism.[128,129]

Because QscR, a third LuxR-type protein in *P. aeruginosa*,[130] also responds to various AHLs including C_{12}-HSL, Blackwell and co-workers tested the same libraries of synthetic *N*-acylated HSL[111,122,123] for modulatory activity of the QscR function,[124] using a previously reported recombinant *E. coli* strain that reports QscR activity *via* the production of β-galactosidase from a promoter fusion.[131] Of these compounds, 11% of the compounds were able to activate QscR to ≥50% of native activity while 6% inhibited QscR by ≥75%. Some of the most active QscR agonists and antagonists are depicted in Chart 9. Among the agonists were found closely related analogues to 3-oxo-C_{12}-HSL, such as C_8-HSL, C_{10}-HSL, and 3-oxo-C_9-HSL, the (D)-configurated AHL derivatives **25** and compounds bearing an aromatic group, such as and **22**, **23**, and **25**. Among the antagonists were found various PHL derivatives **21b**−**f** as well as other aromatic AHL analogues, such as **24**, **26**, and **27** (Chart 9). Interestingly, compound **22**, which was found to be a moderate activator of QscR, was previously shown to strongly inhibit LasR.[111] Thus, this substance may have a synergistic virulence inhibitory effect in *P. aeruginosa* because QscR is a QS repressor and LasR a QS activator. Compounds displaying antagonistic activity (**21c**−**f**, **24**, **26**, and **27**; Chart 9) all contain aromatic acyl groups and native (L)-lactone stereochemistry. Further studies demonstrated that the antagonistic effect was more likely due to the inhibition of the 3-oxo-C_{12}-HSL-dependent QscR/DNA binding.[124]

Blackwell and co-workers widened the scope of their study[114] by preparing a new library of compounds derived from the lead compounds and testing their agonistic and antagonistic effects on the three transcriptional regulators, TraR, LasR, and LuxR, in *A. tumefaciens, P. aeruginosa*, and *V. fischeri*, respectively, using established assay methods.[111,122,123] This included ∼40 compounds of three basic structures (Chart 9): phenylacetyl-L-homoserine lactones (PHL, **21**), phenoxyacetyl-homoserine lactones (POHL, **28**), and phenylpropionyl-homoserine lactones (PPHL, **29**), with various substitutions on the aromatic ring. This study highlighted that, most often, the most active antagonists were the POHL and PPHL compounds. Among the active compounds, 4-NO_2-POHL (**28a**) and 4-CF_3-PPHL (**29a**) were the most potent TraR antagonists (capable of inhibiting TraR by 50% at a ∼1:1 ratio with the native ligand 3-oxo-C_8-HSL). Against LasR, the most potent antagonists were 3-I-PPHL (**29b**) and 4-Cl-PPHL (**29e**), whereas the most potent LuxR antagonists were **29b**, 3-CF_3-PPHL (**29c**), and 3-Br-PPHL (**29d**).

Regarding the agonist activity, 3-CN-PHL (**21h**) and 3,5-CF_3-PHL (**21i**) (Chart 9) were identified as highly potent LuxR agonists with EC_{50} values ∼10-fold lower than that of 3-oxo-C_6-HSL, whereas none of the tested analogues were active against TraR. Four compounds showed weak LasR agonistic activity, with 4-SCF_3-PHL (**21g**) being the most active.

The activity of PHLs substituted at the 4-position as QS antagonists was confirmed in *Chromobacterium violaceum*, with the most active compounds being the 4-Ph-PHL (**21e**) and 4-CF_3-PHL (**21j**) (Chart 9).[113] This recent observation was made possible through the use of macroarray platforms which facilitate both synthesis and screening of molecules.[132] In the same study, Blackwell and co-workers discovered a new family of active compounds, referred to as cinnamyl-homoserine lactones (CHLs, compounds of type **30**; Chart 10) and previously suggested as candidates for QS modulation activity.[133] In this series, F_5-CHL (compound **30a**) and 4-Br-2-F-CHL (compound **30b**) were found to be capable of inhibiting QS regulated responses in *V. fischeri* by 50% at 1:1 against 3-oxo-C_6-HSL.

5.1.4. Miscellaneous AHL Analogues

Sulfur-containing compounds and perfluoroalkyl AHL analogues inspired by Kline's and Givskov's studies[117,120] were prepared by Yoon and co-workers using solid-phase synthesis.[112] In this work, *A. tumefaciens* A136 (pTiA136, pCF218, and pCF372) was used as the reporter strain for antagonistic activity screening.[134] All compounds were potent QS antagonists, with the most active being compound **31** (Chart 11), which has a thioester linkage within the acyl chain. Not surprisingly, when the binding energy of tested

Chart 10. Cinnamyl-homoserine Lactone Analogues

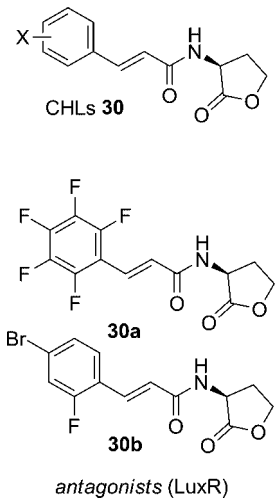


Chart 11

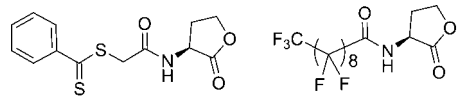
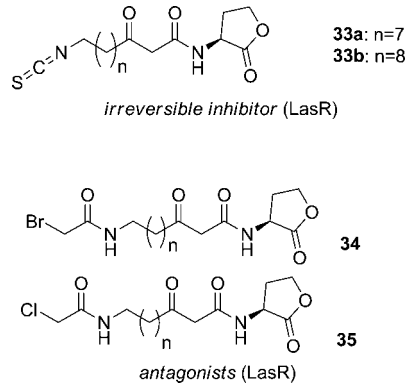


Chart 12. Analogues Designed for Covalently Binding to the Receptor Active Site



compounds with the ligand-pocket of TraR was calculated by molecular docking, dithioester **31** was identified as the one showing the lowest binding energy (−20.17 kcal/mol) as compared to −12.78 kcal/mol for 3-oxo-C_8-HSL. In contrast, the perfluorinated compound **32** (Chart 11), which exhibits the highest energy (−5.93 kcal/mol), proved to be the weakest inhibitor. Favorable hydrophobic vs repulsive fluorophobic interactions within the active site were thus suggested to be responsible for the observed variations.

The first example of QS inhibition based on covalent binding to the active site of the reporter was described in 2009 by Meijler and co-workers.[135] The authors targeted LasR with a set of electrophilic probes: isothiocyanates **33**, bromoacetamides **34**, and chloroacetamides **35** (Chart 12) designed to covalently react with the cysteine residue (C79) of the AHL binding pocket. This residue had been localized in close proximity to the end of the alkyl chain of 3-oxo-C_{12}-HSL.[40] When the LasR ligand-binding domain (LBD) (overexpressed and purified from *E. coli* BL-21 cell) was incubated with isothiocyanates **33a** and **33b** (Chart 12), LC-MS measurements revealed that the binding domains had been covalently modified. These modified domains were then purified and submitted to trypsin digestion, revealing that C79 was the place of the new covalent bond. Further confirmation was given using a variant protein missing the cysteine residue for which no covalent modification was observed.

α-Haloacetamide probes **34** and **35** (targeting the same residue) did not covalently bind to the protein, but several of them were shown to display strong antagonist activity for *P. aeruginosa* QS. This means that even though they displayed good affinity for the binding pocket, the desired covalent reaction was unsuccessful due to either lower electrophilic character or unfavored orientation with respect to the cysteine residue. Finally, it was observed that biofilm formation and pyocyanin production in wild type *P. aeruginosa* PAO1 were significantly inhibited by compound **33b**.

5.2. Substitution of the Amide Function by Bioisosteric Ones

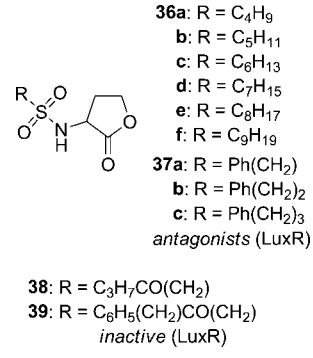
Replacing an amide function by bioisoteric ones is a well documented approach in peptide chemistry for designing compounds displaying agonistic or antagonistic activity with improved stability (in a biological medium) as compared to the parent peptides.[136]

5.2.1. Sulfonamides

The substitution of the amide bond by a sulfonamide function was reported independently by the groups of Giskov and co-workers, in a patent[137] covering a huge variety of substituted structures and possible applications, and Doutheau and co-workers,[138] who focused on the activity of a series of racemic *n*-alkyl- and phenylalkyl-sulfonyl HSL analogues (**36**−**39**) (Chart 13) in *V. fischeri*. Tested as agonists or antagonists of 3-oxo-C_6-HSL against LuxR, alkyl- and phenylalkyl-substituted sulfonamides (compounds **36**−**37**) exhibited a pronounced antagonist activity while their 3-oxo analogues (**38** and **39**) were not active.[138] Concerning the antagonist activity, the number of carbon atoms in the alkyl chain proved to be a key parameter, with butyl-sulfonamide **36a** (the shortest analogue in the series) being poorly active while the maximum activity was found for the pentyl derivative **36b**. Further increasing the chain length up to nine carbon atoms (compound **36c**−**f**) resulted in a consistent decrease of antagonist activity.

Compounds having an aromatic residue at the extremity on the alkyl chain (**37a**−**c**) (Chart 13) exhibited variations in activity with respect to the distance from the aromatic to the sulfonamide linkage, ranging from weak activity for compound **37a**, in which the phenyl and sulfonamide groups are spaced by one carbon atom, to significant activity for compound **37b** (two carbon atom spacer), almost as active

Chart 13



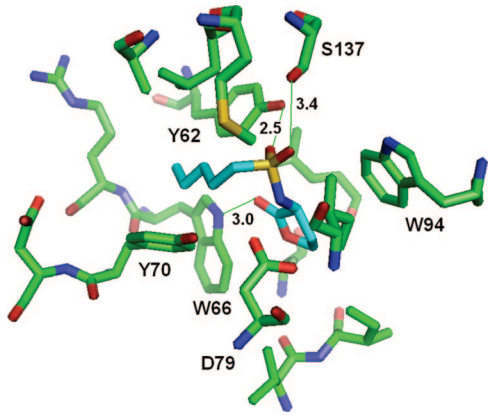


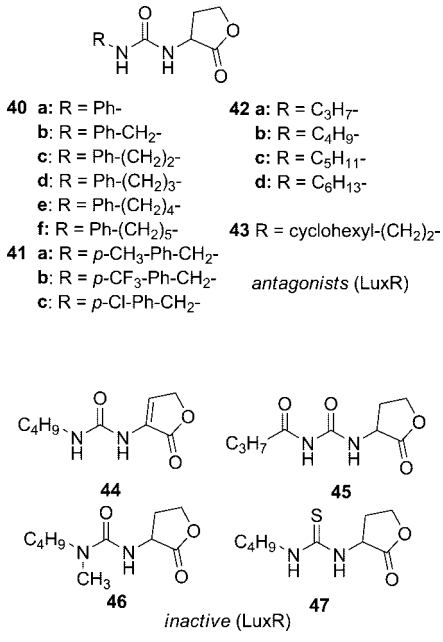
Figure 8. Proposed binding mode of *n*-pentylsulfonamide **36b** showing theoretical interactions within the binding site of LuxR and the putative hydrogen bond network. Named residues are conserved in LuxR-type proteins; numbering corresponds to their position in LuxR.

as the pentyl analogue **36b**, with compound **37c** (three-carbon spacer) exhibiting intermediate activity.[138] An anticipated synergistic effect resulting from combining the sulfonamide and the aromatic motifs, which were previously shown to provide antagonist activity,[104] was not observed. Molecular modeling established by docking compound **36b** using a LuxR model based on the X-ray structure of TraR[38] suggested an interaction of the alkyl sulfonamide AHL analogue through additional hydrogen bonding with Y62 and S137 residues (Figure 8). The authors suggested that these interactions might affect the dimerization process.

5.2.2. Ureas

Doutheau and co-workers extended the approach further with a series of 18 racemic *N*-substituted ureas **40**−**47** derived from homoserine lactone (Chart 14).[139] The study included alkylderivatives of various chain length, aryl derivatives with the aromatic moiety being at variable distance from the urea function, and benzyl ureas variously substituted on the aromatic ring. Also, compounds varying in their structure with an unsaturation in the lactone ring, *N*-methylation, an additional oxo function on the chain, or thiourea subtitution in place of the urea were all evaluated for their ability to interfere with the induction of luminescence in a *V. fischeri* QS system.[104] Among the phenyl-substituted ureas, **40a**−**f**, with four to nine σ-bonds between the aromatic and the lactone rings, compounds **40b** and **40d**−**f**, in which the phenyl group is distant from the lactone ring by five, seven, eight, and nine bonds, respectively, displayed inhibitory activity with an IC_{50} ranging from 4 to 7 μM. Urea **40c**, in which this distance is six bonds, is significantly less active. The strongest activity was observed for the seven-bond spacing (compound **40d**), as in the case of the sulfonlyamide **36b**. Analogues with substitutions on the aromatic ring (**41a**−**c**) displayed inhibitory activity in the same range as their unsubstituted parent compound **40b**. It was slightly enhanced ($IC_{50} \approx 3$ μM) when the phenyl group was substituted with an electron-donating susbtituent (methyl group (**41a**)) or a chlorine atom (**41c**), whereas the strongly electron-withdrawing trifluoromethyl group in **41b** did not affect the activity.[139]

Chart 14



The *n*-alkyl ureas **42b**−**d** (Chart 14) displayed increasing antagonist activity with increasing chain length, up to a maximum for **42c** (pentyl) and **42d** (hexyl) ($IC_{50} \approx 1$ μM), similar to that of compound **43**, bearing a cyclohexyl substituent. Alkylation of the urea nitrogen with a methyl group (urea **46**) resulted in a drastic decrease in antagonist activity, and compounds **44**, **45**, and **47** were deprived of any inhibitory activity.[139]

In an electrophoretic mobility experiment with the N-terminal domain of ExpR, the LuxR homologue in *E. chrysanthemi*, an increase of urea **42b** (Chart 14) concentration inhibited the dimerization of the protein in the presence of 3-oxo-C_6-HSL (20 μM), suggesting a competition between the native AHL and **42b** for the ligand-binding site of ExpR. Furthermore, docking studies (using the LuxR model as described for sulfonamide derivatives) revealed that this urea fits well in the binding site, with hydrophobic interactions of the alkyl chain and the lactone ring in the corresponding hydrophobic pockets. This modeling study also suggested that a possible new hydrogen bond might exist between D79, a conserved residue in this family of proteins, and the supplementary NH group, possibly explaining why the *N*-methylated analogue **46** was inactive (Figure 9).[139]

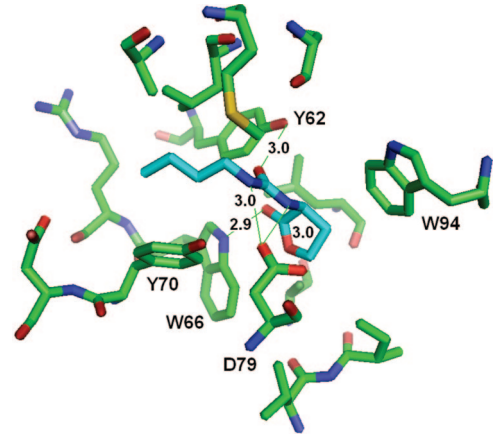
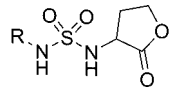


Figure 9. Proposed binding mode of urea **42b** showing theoretical interactions within the binding site of LuxR and the putative hydrogen bond network. Named residues are conserved in LuxR-type proteins; numbering corresponds to their position in LuxR.

Chart 15



48-49

48 a: R = C_4H_9-
b: R = C_5H_{11}-
c: R = C_6H_{13}-
d: R = C_8H_{17}-
e: R = $C_{10}H_{21}$-
antagonists (LuxR)

49 a: R = Ph-CH_2-
b: R = Ph-$(CH_2)_2$-
c: R = Ph-$(CH_2)_3$-
d: R = Ph-$(CH_2)_4$-
weaker antagonists (LuxR)

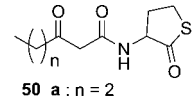
5.2.3. Sulfonylureas

AHL-derived sulfonylureas of type **48** and **49** (Chart 15), whose structures combine structural features of the urea and sulfonamide of their two previous studies (namely two NH groups and the tetrahedral sulfur center), were then reported by Doutheau and co-workers.[140] All the compounds were active when tested for their ability to inhibit the induction of luminescence by 3-oxo-C_6-HSL.[104] For aliphatic compounds having a chain ranging from 4 to 10 carbon atoms (**48a**−**e**), the activity varied slightly with chain length; the *n*-pentyl-sulfonylurea **48b** was the most active, with an IC_{50} value of 2 μM. Sulfonyl-ureas **49a**−**d** bearing a phenyl substituent at the extremity of the alkyl chain are less active than the alkyl ones, with the most active being the *N*-benzyl sulfonylurea **49a**. A molecular modeling study performed on the most active alkyl and phenylalkyl compounds suggested that the antagonist activity could be related to the perturbation of the hydrogen bond network in the ligand−protein complexes. The antagonist activity of the most active *n*-pentyl-sulfonylurea **48b** was also compared to that of the closest analogues in the sulfonyl-amide and urea series, namely the *n*-pentyl-sulfonylamide **36b** (see Chart 13) and the *n*-butyl urea **42b** (see Chart 14), exhibiting IC_{50} values within the same micromolar range, i.e. 6, 3, and 5 μM, respectively. Thus, no synergistic effect on the antagonist activity of the tetrahedral arrangement of the SO_2 group and the urea function was observed. Molecular modeling suggests that this could result from the incapacity of the sulfonyl-urea to establish more than two (for aliphatic ureas) or three (for phenyl-substituted ureas) hydrogen bonds, as donor or acceptor, within the ligand-binding site.[140]

5.3. Alteration of the Lactone Ring

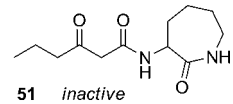
In addition to a systematic structural investigation, there are two reasons that inspire the design of compounds in which the HSL ring structure is replaced by either carbocyclic or heterocyclic rings. First, these compounds would not be susceptible to degradation by AHL-degrading enzymes. Second, since the lactone headgroup is a common motif in all AHLs (unlike the acyl chain which varies from one strain to another), active compounds modified at the lactone moiety would exhibit a broader application range.

Chart 16

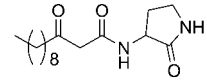


50 a : n = 2
b : n = 8
c : n = 3
d : n = 4

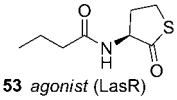
50 a: *antagonist* (LuxR)
50 b: *agonist* (LasR)
50 a-d: *all strong agonists* (SdiA)



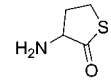
51 *inactive*



52 *weak agonist* (LasR)



53 *agonist* (LasR)



54 *weak agonist* (LuxR)

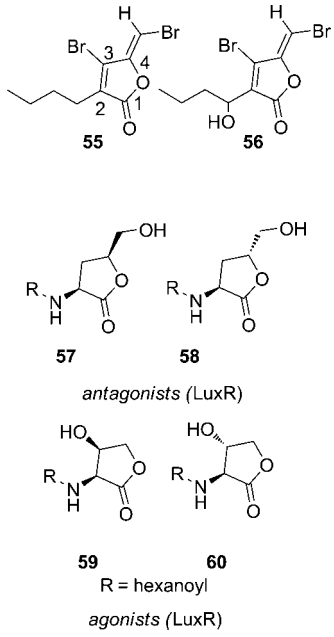
5.3.1. Thiolactones and Lactams

Acyl-homoserine-thiolactone 3-oxo-C_6-HTL (**50a**) and the seven-membered lactam **51** were prepared by Eberhardt and co-workers (Chart 16).[95](Homoserine thiolactones could actually be referred to as homocysteine lactones.) In a *V. fischeri* bacterial reporter, compound **50a** is a very weak agonist and compound **51** is totally deprived of agonistic activity. However, compound **50a** proved to be a quite powerful inhibitor of 3-oxo-C_6-HSL induction whereas compound **51** was a weak inhibitor. Schaeffer et al. tested these same compounds, as well as the longer C_{12} thiolactone **50b**.[77] They confirmed the inactivity of compound **51** (Chart 16) and found a similar poor agonist activity for thiolactones **50a** and **50b**. By contrast, of the two thiolactones **50a** and **50b**, only the C_{12} thiolactone **50b** proved to be a good inhibitor, at least at the highest concentration. When these compounds were evaluated with LasR,[115] the thiolactone **50b** was found to be an agonist, as potent as the native 3-oxo-C_{12}-HSL, while its lactam counterpart **52** was considerably less active. Again in this assay, only compound **50b** proved to be a strong competitor of the tritiated AHL.

The synthesis of optically pure (L and D) enantiomers of *N*-acyl-HTL was reported by Ikeda et al.[80] Bioassays revealed that the L-isomer of *N*-butanoyl-HTL (**53**, Chart 16) acted as an AHL for QS in *P. aeruginosa* while no effects were observed with the D-isomers (same as for AHLs).

Janssens et al. synthesized and screened a small library of AHL analogues which included the homocysteine lactone **54** (HTL) and four of its acylated derivatives: 3-oxo-C_6-DL-HTL (**50a**), 3-oxo-C_7-DL-HTL (**50c**), 3-oxo-C_8-DL-HTL (**50d**), and 3-oxo-C_{12}-DL-HTL (**50b**) (Chart 16).[127] These analogues were tested on LuxR of *V. fischeri* and SdiA of *S. enterica* serovar Typhimurium. The thiolactone **54** exhibited weak QS-inducing properties in *V. fischeri*, while the 3-oxo-acyl-HTLs **50a**−**d** were found to be stronger activators of SdiA than their lactone counterpart. For instance, 3-oxo-C_6-DL-HTL (**50a**) activated SdiA at a 2.5-fold-lower con-

Chart 17



centration than 3-oxo-C_6-HSL. No inhibitory activity was found in competition assays with 3-oxo-C_6-HSL in *E. coli* JM109/pSB401 or *S. enterica* serovar Typhimirium 14028/pJNS25 for the compounds that had shown little or no activity during the induction experiments. Thiolactone analogues were also tested on *C. violaceum*.[96]

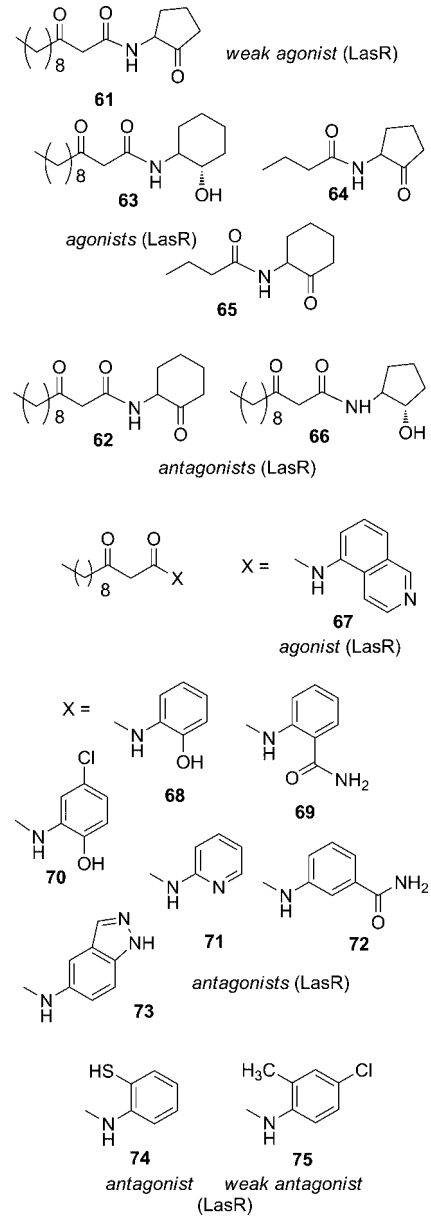
5.3.2. Substituted γ-Lactone

Halogenated furanones such as compounds **55** and **56** (Chart 17), isolated from the red marine alga *Delisea pulchra*, display inhibitory activity against various QS systems (see refs 141 and 142). These compounds have some structural analogy with AHLs but are substituted at the 3- and 4-positions of the lactonic ring. Olsen et al. designed other AHL analogues **57**−**60** (Chart 17) featuring this structural characteristic with *cis* or *trans* relationships to the amide,[143] and they screened them for their ability to activate or inhibit a *V. fischeri* LuxI/LuxR-derived QS reporter system using C_6-HSL as positive control. Compounds substituted at position 4 of the lactone ring (**57** and **58**) were only weak activators whereas the 3-substituted analogues **59** and **60** (Chart 17) were significantly more potent, with **59** being able to activate LuxR as efficiently as C_6-HSL. Compounds **57** and **58** were found to inhibit QS at the maximum concentration (50 μM), though less efficiently than the furanone inhibitor **56**.

5.3.3. Cycloalkanols, Cycloalkanones, and Aromatic Rings

Suga and co-workers examined the activity of AHL analogues against LasR and RhlR, in which the 3-oxo-C_{12}- or C_4-acyl chain is retained but the γ-lactone is replaced with carbocyclic or heterocyclic rings (Chart 18).[107,144,145] For 3-oxo-C_{12}-substituted compounds, the authors used the reporter strain *P. aeruginosa* PAO-JP2 (*lasI*, *rhlI*),[146] harboring plasmid p*las*I-LVAgfp,[125] for rapid screening of LasR agonist activity by measuring the level of green fluorescent protein (GFP) expression. The same reporter strain was also used to find compounds that could compete against 3-oxo-C_{12}-HSL and reduce GFP expression. The biological activity of C_4-alkyl chain compounds was evaluated using PAO-JP2

Chart 18. Libraries of AHL Analogues Featuring Carbocycles and Heterocycles in the Place of the Lactone Ring



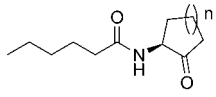
(p*rhlI*-LVAgfp). The first study[107] revealed that *trans* 3-oxo-C_{12}-(2-aminocyclohexanol) (**63**; Chart 18) showed strong agonist activity, with GFP induction levels only 2-fold less than those of native 3-oxo-C_{12}-HSL. 3-Oxo-C_{12}-(2-aminocyclopentanone) (**61**), though structurally more similar to 3-oxo-C_{12}-HSL than the 6-membered ring **63**, induced the reporter only at high concentrations. Even closer analogues, *trans*-3-oxo-C_{12}-(2-aminocyclopentanol) (**66**) and 3-oxo-C_{12}-(2-aminocyclohexanone) (**62**), were almost devoid of agonist activity. Concerning the C_4-alkyl chain compounds, it was observed that the two ketones C_4-(2-aminocyclopentanone) (**64**) and C_4-(2-aminocyclohexanone) (**65**) showed strong agonist activity, inducing the reporter at concentrations as low as 10 μM. Using solid phase synthesis, this work was extended to a larger series of compounds[144] in which the lactone was replaced by various aromatic cycles. The 2-quinolin derivative analogue **67** appeared as a strong agonist of LasR while monocyclic aromatic compounds **68**−**72** and indazole derivative **73** (Chart 18) inhibited reporter gene expression by greater than 50%.

The main trends emerging from the studies of Suga and co-workers[107,144] are the following. First, the AHL analogues that agonize LasR are all compounds having a hydroxyl or a keto group (playing the role of the carbonyl group in the parent γ-lactone) adjacent to the amino group, whenever they possess a five- or six-membered ring structure. Second, anilines having an appropriate H bond acceptor at the *ortho* or *meta* position are antagonists. Thus, the main difference between LasR inhibitors and activators is the nature of the ring being aromatic or not. However, it was observed that very subtle changes in the structure transformed an agonist into an antagonist. For instance, replacing the hydoxyl group in the agonist cyclohexanol **63** by a ketone in compound **62** (Chart 18), or the saturated ring of **63** by an aromatic benzene ring in phenol **68**, or the six-membered ring of **63** by its five-membered ring counterpart **66** made them antagonists. Also, the absolute and relative stereochemistries were found to influence the activity.[145] For instance, with respect to cycloalkanol analogues, it has been observed that an appropriate combination of absolute and relative stereochemistries of the amide and hydroxyl groups is essential for agonist activity.

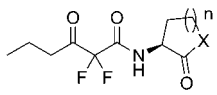
Using solid phase synthesis, Kim et al. prepared eight other 3-oxo-C_{12}-HSL analogues[147] in which the lactone ring is replaced by different aromatic rings. The free binding energies for the ligand binding site of these compounds were calculated using in silico modeling (FlexX docking[148] within LasR). All eight compounds gave better docking scores than 3-oxo-C_{12}-HSL (−8.93 kcal/mol) and inhibited the activity of LasR in *P. aeruginosa.* The docking scores better reflect the binding affinities of the compounds for the ligand binding site rather than the degree of inhibitory effects. However, the modeling and biological evaluations gave consistent results, since the strongest inhibitor, the thiophenol **74** (Chart 18), had the best FlexX docking score (−16.38 kcal/mol) and the weakest inhibitor **75** had the poorest docking score (−13.83 kcal/mol).

Six acylated 2-aminocycloalkanones (**76**−**78**) (Chart 19), some of them equipped with an α-difluoro-β-ketoamide motif, were prepared by Welch et al.[149] The effect of these compounds on QS-dependent carbapenem and protease production was investigated in *E. carotovora*. Only **77d**, the gem-difluoro analogue of the native AHL 3-oxo-C_6-HSL, was capable of eliciting some production of the antibiotic at

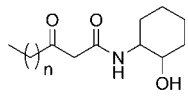
Chart 19. Cycloalkanones and Cyclohexanol AHL Analogues



76 **a**: n = 1 *weak agonist* (CarR)
b: n = 2 *inactive*



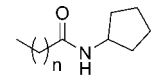
77 **a**: n = 1, X = CH_2
b: n = 2, X = CH_2
c: n = 3, X = CH_2
d: n = 1 X = O
variable activities



78 **a**: n = 2
b: n = 3
c: n = 4

agonists (LuxR, SdiA)

Chart 20



C_n-CPA

n = 1 to 10: C_3 to C_{12}-CPA

n = 3 : **79a**
n = 4 : **79b**
n = 5 : **79c**
n = 6 : **79d**
n = 7 : **79e**
n = 8 : **79f**

antagonists (LasR, LuxR)

the highest concentration applied. The protease production proved to be less sensitive to lactone ring modification, since the cyclopentanone derivative **77a** showed some protease-inducing activity. The cyclopentanone **76a** showed weak protease-inducing activity while the corresponding cyclohexanone **76b** was devoid of any activity. When this library of AHL analogues was further analyzed using additional reporters,[127] it was determined that the six-membered ring analogues *N*-(3-oxo-acyl)-2-aminocyclohexanols **78a**−**c** were good agonists of LuxR in *V. fischeri* and SdiA in the *S. enterica* serovar Typhimurium.[149]

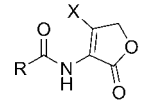
5.3.4. Cyclopentanes

Ishida et al.[150] also observed that *N*-octanoyl cyclopentyl amide (**79d**, C_8-CPA) (Chart 20) moderately inhibited expression of a *lasB-lac*Z transcriptional fusion gene in *P. aeruginosa* PAO1. Building on this observation, they prepared a series of structural analogues of C_8-CPA, with alkyl chain length varying from 3 to 12 carbon atoms, and demonstrated their ability to inhibit QS in *P. aeruginosa* by displaying antagonistic activity against LasR.[150] The decanoyl cyclopentyl amine (**79f**, C_{10}-CPA) was the most active compound. The same cyclopentyl-amides were then tested in the Gram-negative bacterium *Serratia marcescens* AS1, in which QS is controlled by several AHLs, among them C_6-HSL and 3-oxo-C_6-HSL.[151] In this assay, the more active compound was C_9-CPA (**79e**) (Chart 20), which was able to inhibit QS at a concentration only four times that of the exogenous C_6-HSL. Very recently, Ikeda and co-workers[150,152] also tested a series of C_n-CPA (Chart 20) in *V. fischeri* and observed that C_5- to C_{10}-CPA (**79a**−**f**) showed similar strong inhibitory activity on the LuxR QS system.[152] The most effective inhibitors were C_6-, C_7-, and C_8-CPA (**79b**−**d**), with an IC_{50} of about 4 μM.

5.3.5. Enaminolactones

Doutheau and co-workers reported the synthesis and biological evaluation of the *N*-acyl-3-amino-*5H*-furanone derivatives **80a**−**c** and the 3-halogeno derivatives **80d**−**f** (Chart 21) in *V. fischeri*.[153] Compounds **80a**−**f** were designed to evaluate the influence of conformational and H-bonding

Chart 21



80 **a**: R = -$(CH_2)_3$-Ph, X = H
inactive (LuxR)

b: R = -C_5H_{11}, X = H
c: R = -CH_2-CO-C_3H_7, X = H
d: R = -C_5H_{11}, X = Br
e: R = -$(CH_2)_3$-Ph, X = Br
f: R = -C_5H_{11}, X = Cl
antagonists (LuxR)

modifications in AHL analogues, resulting from the replacement of the amide by an enamide function, on the modulation of the antagonist activity. These compounds are also analogues of the natural brominated furanone **55** (depicted in Chart 17). Furanones **80a**−**f** were evaluated for their ability to inhibit the induction of luminescence obtained with 3-oxo-C_6-HSL in a *V. fischeri* QS system.[104] Analogues **80b** and **80c**, closest in structure to the native AHL, displayed an IC_{50} of 85 and 55 μM, respectively, whereas compound **80a**, bearing a phenyl group on the terminal alkyl chain, was inactive. This latter result contrasted with previous ones obtained with phenyl-substituted 3-oxo-C_6-HSL analogues.[104,122] However, it was subsequently shown by molecular modeling that the enamide function induces significant conformational change as compared to the saturated lactone.[153] This would significantly modify the orientation of the side chain, preventing the aromatic ring from interfering with key residues in the receptor binding pocket. The brominated analogues **80d** and **80e** appeared to be stronger inhibitors compared to the unsubstituted counterparts, which are either inactive (**80c**) or about 10 times less active (**80a**), and the chlorinated enamide **80f** was found to be as active as bromide **80d**. Molecular modeling suggests that the conjugated enamide group induces two preferential conformations leading to specific binding modes. In addition, the presence of the halogen atom could enhance the fit of the lactone ring through specific interactions with strictly conserved or conservatively replaceable residues in the LuxR protein family, namely the D79, W94, and I81 residues in LuxR.[153]

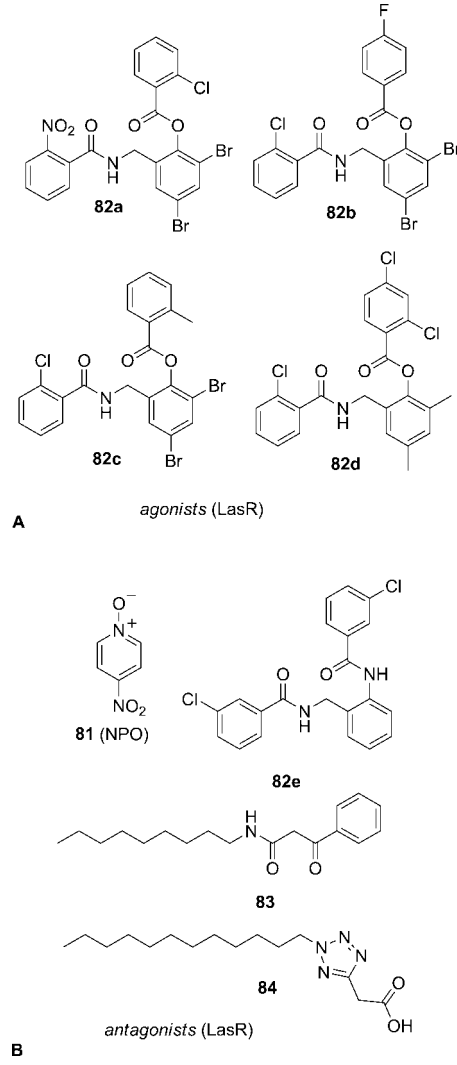
6. Modulators Unrelated to AHLs Identified by Screening

6.1. Low- and High-Throughput Screening

High-throughput screening (HTS) has been used to discover QS modulators. Givskov and co-workers[119] reported the development of recombinant bacteria with two screening systems for QS inhibitors (QSI). The first system is based on the regulation of a gene encoding a lethal protein regulated by a QS-controlled promoter. Thus, bacteria are unable to grow in the presence of AHLs unless an efficient, functional, and nontoxic QSI compound is present. The second system uses an antibiotic resistance gene controlled by a repressor, whose own expression was driven by a QS-regulated promoter. Consequently, bacterial growth can occur in the presence of the appropriate antibiotic because AHL antagonists prevent the native AHLs from repressing the expression of the antibiotic resistance gene. The authors used these two systems to screen libraries of either pure compounds or extracts from food sources and herbal medicine. They identified 4-nitropyridine-*N*-oxide (4-NPO, compound **81**; Chart 22B) and garlic extracts as concentration-dependent inhibitors of QS and the pathogenicity of *P. aeruginosa* PAO1 based on studies in a *C. elegans* nematode model.

In 2006, Greenberg and co-workers[126] reported the screening of a library of 200,000 potential QSI compounds in *P. aeruginosa*. The authors identified the AHL structurally unrelated triphenyl derivative (compound **82a**,[154] Chart 22A) as an activator of LasR. Molecular modeling studies suggested that compound **82a** and the native 3-oxo-C_{12}-HSL bind to LasR in a similar fashion. In silico modeling of **82a**, analogues led to the discovery of QS modulators, able to activate (**82b**, **82c**, and **82d**) (Chart 22A) or inhibit (**82e**) LasR (Chart 22B).

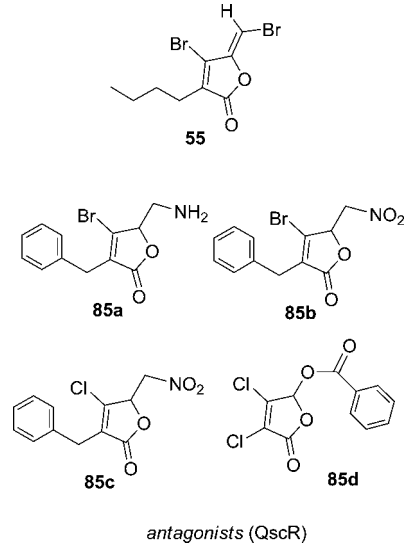
Chart 22. QS Modulators Identified from Low- and High-Throughput Screening Investigations



This group also developed an ultra-high-throughput cell-based assay to screen approximately 200,000 compounds for LasR dependent gene expression.[155] While the library consists of very diverse chemical families, the two best inhibitors identified were compounds **83** and **84** (Chart 22B), with AHL structural analogy and displaying an IC_{50} of 10 μM and 30 nM, respectively. The authors then prepared a small focused library of compounds derived from **84**, but none of them showed increased activity as compared to the parent compound.

In a recent study, Liu et al. screened a library of about 100 analogues of the natural QS inhibitor furanone **55** for their ability to inhibit QscR.[156] In addition to parent furanone **55**, four other compounds, all bearing an aromatic appendage at various positions of the furanone backbone (**85a**−**d**) (Chart 23), displayed moderate to significant inhibitory activities. The furanone **85a** bearing a pendant amino group proved to be the most active and was able to completely inhibit QscR activity *in vitro* at a concentration of 50-fold molar excess over the native 3-oxo-C_{12}-HSL. The presence of the amino group proved to be important, as its nitro counterpart **85b** showed much weaker activity. Since the other nitro derivative **85c** exhibited weak acivity also, a strongly electron-withdrawing function was considered as undesired for inhibition activity.

Chart 23. QS Inhibitor Furanones



6.2. Virtual Screening

Virtual screening methods consist of essentially two main approaches, which can be combined: (1) structural alignment with a natural ligand or a known active compound and (2) molecular docking within the binding site of a crystallized or modeled receptor. The databases are often freely available on the web, from commercial or institutional Web sites, and can be focused using various parameters (H-bond donor/acceptor ability, molecular weight, log *P*, etc.) and/or according to a specific target by 2D-alignment with a known substrate.

In the first search for QS inhibitors using structure-based virtual screening (SBVS), Taha et al.[157] used the HipHop6refine module of CATALYST[158] software, which models ligand−receptor interaction using information derived only from the ligand structure. As a training set for pharmacophore modeling, they employed the brominated furanones **86**−**88** (Chart 24), as well as the already mentioned compound **55**

Chart 24. Heavy Metal-Containing Active Compounds (Compared to Known Furanone QS Inhibitors)

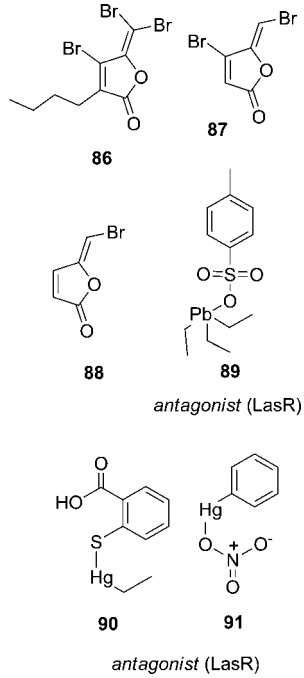
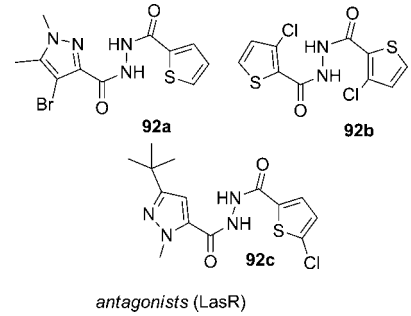


Chart 25. Heteroaromatic Hydrazide QS Inhibitors



(see Chart 23), all known as QS antagonists, and screened a library of about 230,000 compounds selected from the NCI structure database using compounds with molecular weights ranging from 100 to 500 Da. The pharmacophore search captured about 86,000 hits, and of the top 40 compounds suggested, 19 were evaluated for QS inhibitory activity, measuring the production of pyocyanin and pyoverdin.[120] After biological evaluation, only the Pb containing compound **89** proved to be a potent QS inhibitor (IC_{50} 800 nM). The authors suggested that the activity was due to possible interaction of the Pb atom with thiol functions. A similar QS inhibitory activity was observed for compounds **90** and **91** (Chart 24), in which a mercury atom is present.[157] However, the presence of heavy metals limits the potential use of such compounds as future drugs.

A comparable approach was described by Riedel et al. in an attempt to find inhibitors of QS for the opportunistic pathogen *Burkholderia cenocepacia*.[159] Using molecular alignment scores for molecules with structural similarity to 3-oxo-C_{12}-HSL, the authors screened an in-house database containing 1.1 million commercially available compounds. They used the 4SCan software, which combines a molecular alignment tool with an iterative database screening and prioritization procedure.[160] The hundred compounds with the highest alignment scores were tested as QS antagonists with the aid of two GFP-based AHL biosensors (*Pseudomonas putida* F117(pKr-C12) and *P. putida* F177 (pAS-C8).[161] The hydrazide compound **92a** (Chart 25) was the most effective in inhibiting the C_8-HSL based biosensors and was therefore chosen for a structural design of a potent QS blocker specific for the *Burkholderia cep* QS system. A combinatorial library was synthesized based on the structure of compound **92a** and tested using the biosensor *P. putida* (pAS-C8). Several iterative rounds of molecule design and testing of over 400 substances ultimately yielded hydrazide compounds **92b** and **92c**. Compound **92c** proved to be particularly capable of efficiently blocking the expression of pathogenic traits in *Burkholderia*.

Zeng et al. reported a docking based virtual screening (DBVS) for QS inhibitors,[159] using the DOCK structure-based drug design software package, which employs flexible ligand minimization to evaluate proposed binding modes.[162,163] They evaluated a small library of 51 active components of Traditional Chinese Medicines with antibacterial activities. These compounds as well as the brominated furanone **86** (Chart 24) were docked as rigid ligands with a maximum of 30,000 orientations in the TraR binding site (pdb 1H0M). The selected benzopyranic and coumarinic compounds **93**−**95** inhibited biofilm formation of *P. aeruginosa*, and compound **93b** induced TraR degradation in vitro (Chart 26).

Yang et al.[164] selected a library of 147 compounds from the SuperNatural and SuperDrug databases[165,166] based on

Chart 26. Benzopyranic and Coumarinic QS Inhibitors

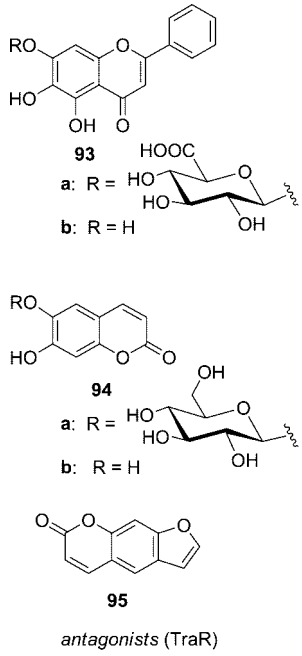
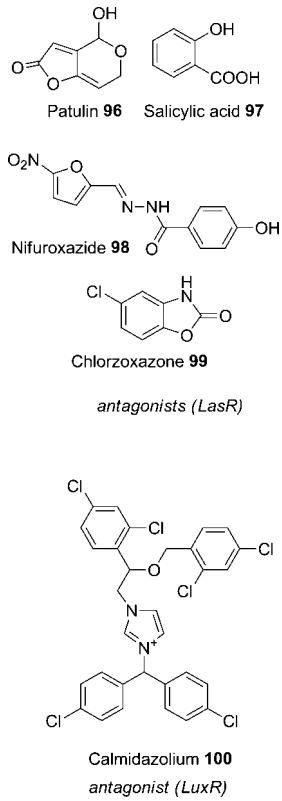


Chart 27. Aromatic QS Antagonists Identified by Virtual Screening



2D structural similarity with 3-oxo-C_{12}-HSL, as well as known agonists (compound **82a**,[126,154] Chart 22) or antagonists, furanone **86** (Chart 24), and patulin (compound **96**; Chart 27). This library was then docked with LasR (after selection of poses that showed similarity with 3-oxo-C_{12}-HSL) using a Molegro Virtual docker,[167] resulting in a series of six top-ranking QS modulator candidates with docking scores better than that of the known QS-inhibitor 4-nitropyridine-*N*-oxide (**81**; Chart 22).[119] Biological evaluation of these six candidates led to the identification of effective antagonists of the LasR system in *P. aeruginosa*, namely salicylic acid (compound **97**; Chart 27), a natural compound and the main metabolite of aspirin, the synthetic antimicrobial agent, nifuroxazide (compound **98**; Chart 27), and the muscle relaxant chlorzoxazone (compound **99**; Chart 27). In addition, these compounds also inhibited the production of common virulence factors and biofilm development in *P. aeruginosa*. Recently, the polyaromatic imidazolium compound **100** (Calmidazolium) was identified as a new QS inhibitor in *Vibrio fischeri* by a virtual screening, involving flexible docking sequences within the LuxR, TraR, and LasR binding sites.[168]

7. Concluding Thoughts

7.1. Main Points

The LuxR family of proteins continues to grow. While there is overall similarity in the domain organization of the proteins and some highly conserved residues have been identified, there is also noticeable diversity across the protein family, with several recognizable subfamilies emerging. Even within these subfamilies, there are unique qualities to each LuxR-type protein with regard to its ability to bind to the AHL, to bind to the DNA, and, for activators, to interact appropriately with RNAP. Because of these multiple inherent activities, attention must be paid to which activities are being examined when assaying for the effect of agonists and antagonists on specific LuxR homologues. Limited detailed structural information about the protein family has restricted the capacity to use that information toward the design of synthetic modulators. Thus, the vast majority of efforts to date have concerned design strategies focused on the AHL ligand itself and concurrent structure−activity relationship (SAR) studies.

Several hundred close AHL analogues have been designed, prepared, and experimentally tested. In addition, other more chemically diverse modulators have been discovered through screening assays. Several families of compounds have proven to be agonists or antagonists of the different LuxR homologues (Table 1). Ligand recognition by these receptors accommodates molecules with surprising structural diversity. For AHL-like analogues, the lactone can be substituted by 5- or 6-membered rings, and the amide linkage can be substituted by classical bioisostere functions (sulfonamides, ureas, sulfonylureas). The alkyl chain can also undergo significant substitution and branching. In respect to AHL-unrelated compounds, structures as different as triaromatic amides and the tetraaromatic-substituted calmidazolium, as well as natural bromofuranones and their analogues, were found to be active. The EC_{50}/IC_{50} values reach the nanomolar range for some active agonists and antagonists, which is not far from what is considered to be an acceptable dosage level for potential drugs.[169,170]

The diversity of chemical structures of the active compounds and assay protocols used to study them precludes a meaningful global comparison of the results obtained for the various SAR studies. Structural studies of a few LuxR homologues have enabled the determination of specific amino acids that are critical for AHL binding. A comparison of the structures of cocrystallized complexes of LasR with its cognate AHL or three AHL-unrelated agonists has provided a molecular rationale for understanding how this one receptor is activated by varied ligands.[154] However, a rationale behind why some compounds are agonists and others are antagonists remains elusive, especially due to the lack of any comparable

Table 1. Representative QS Agonists and Antagonists of Three Bacterial Strains[a]

	V. fischeri		*P. aeruginosa*		*A. tumefaciens*		
compound	EC_{50}	IC_{50}	EC_{50}	IC_{50}	EC_{50}	IC_{50}	ref
C_7-HSL		1.36				0.69	111
C_8-HSL		0.77		1.75		0.77	111
C_9-HSL		0.74					77, 95
C_{10}-HSL		0.40		0.25		1.05	111, 116
C_{12}-HSL			0.04				111
C_{14}-HSL		0.74	0.01				111
3-oxo-C_6-HSL	3.0 (AI)						111
3-oxo-C_8-HSL				0.11	0.20 (AI)		95, 111
3-oxo-C_{12}-HSL		0.40	0.007 (AI)				111, 115
5			0.01				115
11c	0.25						104
11d		2	0.54				104, 111
12		2				1.12	104, 111
15				6			119
16				50			119
17				50			119
20		3.70		3.89		4.73	111
21a		0.86		1.72		1.25	111, 114
21b	0.35			0.61			111, 114
21c				4.06			111
21d				3.97			111
21g		1.3	3.4			4.7	114
21h	0.3						114
21i	0.37						114
21j		0.61				0.81	111
21k		0.96				2.25	111, 114
22		1.35		0.34		0.92	111
28a						0.29	114
29a		2.2				0.21	111
29b		0.39		1.8			114
29c		0.50					114
29d		0.53			3.3		114
29e		0.97		2.2		3.3	114
29f		1.35		0.34		0.92	114
29g		0.63				2.1	114
29h		0.99		3.0		1.6	114
29i		0.88		3.4			114
33a				300			135
33b				113			135
36b		2					138
36c						0.83	111
36d						3.49	111
36f		1.03					111
40b, 40d−f		4−7					139
42d		1.0					139
43		1.8					139
48a−e		2−7.8					140
50b			0.007				115
56		*					142
63			*				107, 144, 145
66				*			107, 144, 145
74				*			147
79f				80			150
80a		6.5					153
82a				0.014			126
82b			0.24				126
82c			0.054				126
82d			0.92				126
82e				50			126
83				10			155
84				0.03			155
89				0.8			157
98				*			164
100		7					168

[a] When reported, EC_{50} (for agonists) and IC_{50} (for antagonists) values are given in μM. AI stands for the native autoinducer. * indicates reported as significantly or very active.

structural information for antagonist-receptor interactions. Very subtle structural variations (i.e., changing either the nature or even only the position of a substituent on an aromatic ring, or changing cyclohexanol to cyclohexanone) may convert an agonist that is active on one LuxR homologue to an antagonist, on either that same protein or a different member of the family. Some antagonists may also serve as weak agonists, which further complicates the issue.

Nevertheless, systematic modifications of just one structural signature component in the AHL backbone at a time have demonstrated that some families of compounds exhibit consistent activities. In the case of *V. fischeri*, most aliphatic alkylamides compounds were found to be agonists, whereas aromatic substituted ones proved to be antagonists. In the case of sulfonamides, aliphatic and aromatic ones were found to be antagonists. However, no synergy came from the addition of cumulative chemical modifications. The impact of the presence of an aromatic moiety is important as evidenced by results from screening libraries of aromatic containing AHL analogues (PHL, POHL and PPHL). Whereas most POHLs and PPHLs proved to be antagonists, the activity of PHLs was found to be much more sensitive to the nature and the position of the substituent on the aromatic moiety. It is noticeable that among aromatic PHLs agonists, some are even significantly more potent than the native AHLs. These "super agonists" may be useful for QS activation of bacterial in biotechnology applications.

7.2. Future directions

From the biological perspective, our understanding of the LuxR protein family is limited due to the relatively few members of the family for which detailed structural information exists. Information from additional NMR-or crystallography-based structures of other LuxR homologues will facilitate the modeling of new modulators. Further, our understanding of the mechanism of responsiveness of the LuxR-type proteins to their AHL ligands is still largely a "black box". To date all known structures of LuxR homologues have been solved in the presence of AHL. Structural analysis of members of the LuxR family that are more stable in the absence of AHL (i.e., MrtR or EsaR) may finally reveal how the AHL ligand impacts the structure and activity of the LuxR proteins. These efforts would enable additional molecular-based modeling approaches toward the design of agonists/antagonists and a greater understanding of the effect of these compounds on protein structure/function.

From the chemical perspective, progress has been made in using in vivo tests to identify QS modulators that can decrease the expression of virulence factors and disrupt biofilm formation. If we are to reach the ultimate goal of using these LuxR-AHL modulators in therapeutic applications, new approaches for identifying additional agonists/antagonists of interest must be pursued. As described above, additional structural information about the LuxR proteins will facilitate further applications of this approach. For instance, a molecular-based modeling approach has been recently used to design an irreversible inhibitor. However for AHL-related analogues, most of the possible structural variations have already been performed, limiting the prospect for finding more potent active agonists or antagonists from this approach. Fortunately, the identification of new AHL-unrelated antagonists from screening existing libraries of compounds still holds future promise. Further, it is quite likely that new natural antagonists will be identified from animals, plants, or microorganisms that have evolved mechanisms to protect themselves from pathogens or competitors. An increasing number of such natural compounds, found in extracts, have been reported in the recent literature. Once the structure of these active compounds is elucidated, new groups of related analogues will be designed and examined for their effectiveness in modulating AHL-LuxR-based QS responses. This

will also contribute to the identification of pertinent interactions within the active sites of the receptors with regards to the antagonistic activity of select ligands, building upon the knowledge that exists for some agonists.

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