Chemical Communication–Do We Have a Quorum?

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Abstract: There are two types of bacterial communication systems, those in which the signal produced by bacteria is directed only at other organisms, and those where the signal is detected by others and self. The latter is involved in adaptation to the environment. The adaptation signals are autoinducers, the response is population density-dependent and has been termed "quorum sensing". Our current knowledge of bacterial signaling systems indicates that Gram positive bacteria use small peptides for both types of signaling, whereas Gram negative organisms use homoserine lactones as autoinducers. Gram- negative bacteria internalize the signals which act upon an intracellular receptor. Gram-positive bacteria use the signals as ligands for an extracellular receptor of a two-component signaling system. Inhibitors of quorum sensing compounds for both Gram positive and Gram negative bacteria are being explored. Signal inhibitors could be potentially effective in impeding biofilm formation, which might prolong the utility of the currently available antibiotics in this era of antibiotic resistance. In this review, we will explore both bacteria-host and bacteria-bacteria communication systems, with an emphasis on inhibitors of these systems both natural and synthetic.

INTRODUCTION

Cellular communication whether it is between nerve cells or bacterial cells occurs *via* chemical signaling. In bacteria this cell-cell communication is referred to as quorum sensing, since the changes in expression of cellular factors including virulence factors is population size dependent. Basically the larger the population size, the more chemical signal produced which interacts with bacterial cell surface receptors. When a sufficient number of receptors have been triggered (quorum has been reached), there is a phenotypic change in the population of cells which generally results in enhanced survival in the specific environment the organism occupies. Quorum sensing (QS) as molecules are produced by both Gram positive and Gram negative organisms, but the types of substances appear to be fundamentally different between the two groups. This means of regulating population behavior is also exploited by the microorganism hosts who produce quorum mimics in an effort to control bacterial population levels through co-opting at least part of the regulatory machinery which controls expression of coloni-zation factors. The focus of this review is to discuss the chemical nature of this communication system.

1. QS MIMICS OF NATURAL ORIGIN

Plants, including algae and higher plants, produce com-pounds that mimic bacterial quorum sensing compounds, presumably as a protective mechanism. The most studied of these bacterial QS mimics are halogenated furanones **1-4** (Fig. **1**) secreted by the marine red alga *Delisea pulcra*. Furanones secretion substantially alter the structure of the bacterial communities on the algal surface in the marine environment [1, 2]. It has been recognized [3] that this alga's furanones are similar in structure (Fig. **1**) to N-acyl homoserine lactones (AHLs), the most common QS signal among Gram-negative bacteria. It has been demonstrated that the *Delisea* furanones specifically inhibit AHL-regulated behaviors in multiple Gram negative species by binding to AHL bacterial receptor proteins and promoting proteolytic degradation of these receptors [4, 5]. Furanone AHL mimics have also been shown to have inhibitory effects on the *in vitro* formation of biofilm and virulence factors by *Pseudomonas aeruginosa* [6, 7].

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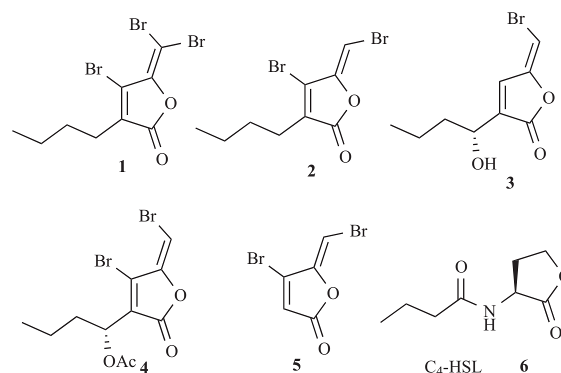

Fig. (1).

Like the algae, higher plants, such as pea, tomato, *Medicago truncatula* and rice, produce compounds that affect AHLs regulation of QS in bacteria [8, 9,10]. While all of the furanone AHL mimics in *D. pulcra* are inhibitory, most of the AHL mimics detected in higher plants and the unicellular green alga *Chlamydomonas reinhardtii*, stimulate gene expression of specific AHL receptors [9, 11]. Although the higher plant AHL mimic compound structures have not been determined, they do appear to have a different chemical

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structure as evidenced by their different organic solvent partitioning. Recent studies have demonstrated that plants such as *M. truncatula*, *C. reinhardtii* and *Chlorella sp.* also produce as yet unidentified compounds which regulate bacteria-host interactions by either stimulation or inhibition of the AI-2-specific receptor [9, 11, 17]. AI-2 is a furanosyl borate diester QS signal **7** (Fig. **2**) [14] used by *Vibrio harveyi, V. cholerae* and other enteric bacteria [12, 15, 16]. Regulation of QS by the bacterial host is not limited to the Plant Kingdom. Recently it has been reported that the mammalian hormone epinephrine **8** (Fig. **2**) stimulates the expression of virulence genes in *Escherichia coli* through the AI-3 QS receptor [19]. In this case, it appears that *E. coli* uses the hormonal signal to trigger an enhancement of their survival.

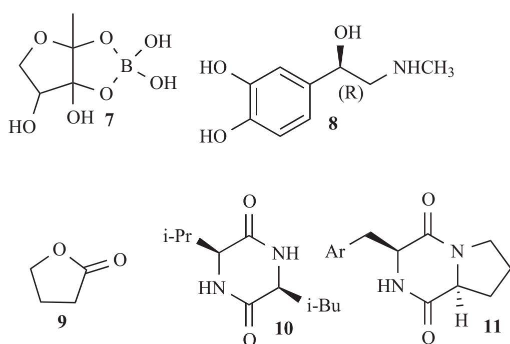

Fig. (2).

Like their plant counterparts, AHLs produced by bacteria are not homogeneous [12, 13, 20, 21] and can exert external interference in QS. For example, butyrolactones (2(3H) –furanones) **9** (Fig. **3**) from *Streptomyces* species are structurally related to the AHLs and also participate in QS signaling (Fig. **2**) [1, 18]. Another mechanism of interference is production of side chain variant AHLs which can interfere with QS as in the case of *Vibrio fischeri* [22, 23]. Alternatively, in mixed biofilms AHL signals from *P. aeruginosa* have been shown to act on *Burkholderia cepacia* system but not *vice versa* [24]. In other *Pseudomonas* sp. diketopiperazines **10**, **11** (Fig. **3**) interact with the AHL dependent signaling system [25] while *Staphyloccocus aureus* responds to *S. epidermis* QS signals [26].

2. SYNTHETIC QS COMPOUNDS – DERIVATIVES OF AHLS

N-acylhomoserine lactones' action is based on the gene products of the *luxR* gene analogs [27-32]. Different bacterial species may produce various AHL analogs that differ in N-acyl chain length (1 to 4 carbons) and in the substitution at the 3-position of the side chain (Fig. **3**) [34-35].

Autoinducer (AI) N-(3-oxododecanoyl)-L-homoserine lactone **(3-oxo-C$_{12}$-HSL) 12** (Fig. **3**) activates LasR, a master regulatory transcription factor which controls a number of individual genes involved in *P. aeruginosa* virulence. In addition RhIR and its agonist N-butyryl-L-homoserine comprise a second regulator-autoinducer complex making *P. aeruginosa,* which is a significant pathogen in immunocompromised individuals, e.g. those with cystic fibrosis, and burn wounds [36], one of the few recognized microorganisms with two sets of QS proteins [37]. Mutation in one or more components of these regulatory systems results in decreased virulence in mouse model studies [38, 39] and ability to colonize as indicated by depressed *in vitro* adherence to human bronchial epithelial cells [40].

2a. AHL Analogs as Agonists

Studies on the molecular interaction between regulatory (R) proteins and their autoinducers (AIs) from *V .fischeri*

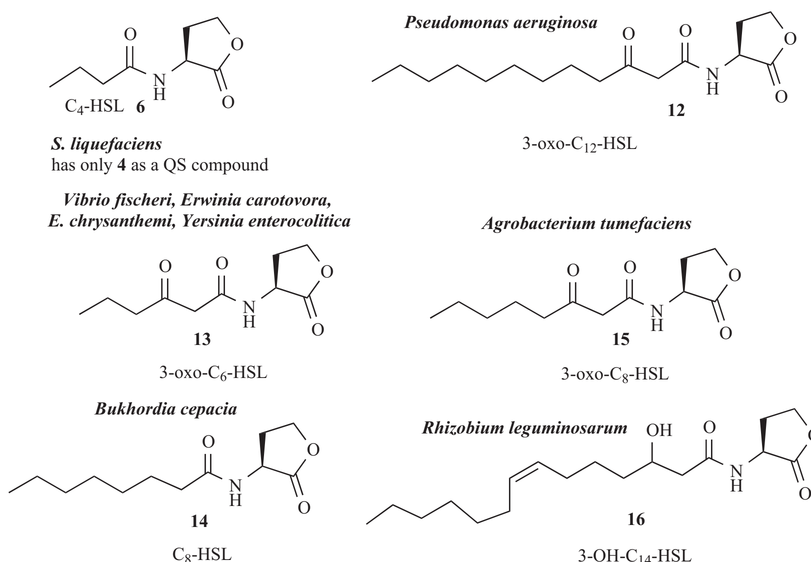

Fig. (3).

[44-47], *Agrobacterium tumefaciens* [43] and *P. aeruginosa* [48, 49] are focused primarily on modification of the acyl chain. QS assays demonstrate that most R proteins respond to analogs with the natural acyl chain length plus or minus two carbons. More drastic changes in the length of the acyl chain lead to a loss of AI activity. Reduced AI activity also occurs when bulky groups [43] or unsaturated bonds [43, 47] (Fig. **3**) are added to the acyl chains. AI activity is also diminished upon reduction of 3-oxo functionality to 3-hydroxyl or methylene [43, 44, 45, 49]. Replacement of the N-acyl-homoserine lactone moiety (AHL moiety) of *V. fischeri* AI (**3-oxo-C6-HSL, 15,** Fig. **3**) with homo-cysteine-derived lactone analog results in AI activity reduction by one order of magnitude [45] indicating that AHL moiety is important for R activation.

Replacement of the AHL moiety with lactam or oxazoline eliminates the AI activity [50], however its replacement with homocysteine thiolactone does not affect *las* AI activity (3-oxo-C12-HSL, 5, Fig. **2**).

A more extensive study of *las* AI analogs showed that LasR and RhIR respond differently to AI analogs with substitutions in the AHL moiety [51]. Further findings of this study led to discovery of two new structural elements that stimulate the *las* QS signaling. The most active AHL substituent has been identified as 2-aminocyclohexanol, and its 3-oxo-C_{12}- derivative, **24** (Fig. **4**) both of which demonstrated activity similar to their natural analog 3-oxo-C_{12}-AHL **12** (Fig. **3**). In contrast, structural analogs **21-23** (Fig. **4**) of *las* QS system have not proven to be good agonists.

Structures with the C4 side chain (**17**-**20**, Fig. **4**) have a different activity profile for the *rhl* QS circuit. The most active compound is C4-(2-aminocyclopentanone) **17** (Fig. **4**) while compound **20** exhibited little activity [51]. X-ray structure of 3-oxo-C8-HSL-TraR complex [52, 53] indicated the functionality of activity studies, i.e., the carbonyl group of the ester bond in AHL forms a hydrogen bond with the H-atom of the indole NH group of Trp57; however, the bridging ester oxygen does not participate in a hydrogen bond. Therefore, the hydroxyl- and keto group of compounds **24** and **17**, respectively, can be AHL keto-group mimics as hydrogen bond acceptors and the ether oxygen can be replaced with C-atom if the correct conformation and ring size are maintained. These observations have clearly demonstrated that the binding sites of the AHLs in LuxR system and its homolog in *P. aeruginosa*, LasR and RhlR differ.

2b. Antagonists of QS

Antagonists of QS regulatory pathways in addition to mutation can be used to regulate pathogen virulence. These include the longer chain AHLs which have been described as antagonists for the relatively shorter chain AHL QS systems of *V. fischeri* [41], *Serratia liquefaciens* [42], *A. tumefaciens* [43] and *Chromobacterium violaceum* [34]. Conversely, shorter chain AHLs do not appear capable of being antagonists for the longer chained AHLs.

Natural brominated furanones **1** and **2** (Fig. **1**) from *D. pulcra* disrupt *S. liquefaciens* QS by disrupting the SwR and C4-HSL [54] interaction and inhibiting interactions of LuxR with 3-oxo-C6-HSL [55] and CarR with 3-oxo-C_6-HSL [56]. However these brominated furanones have minimal effect on the interaction of LasR with 3-oxo-C_{-12} -HSL. An antagonist analog, that lacks the natural furanone alkyl side chain [57] **5** (Fig. **1**), has considerable *P. aueruginosa* QS system inhibitory activity. Furanone **5** inhibited QS-controlled reporter genes and virulence factor expression in a QS mutant with exogenous AIs, but was not as active in the wild-type *P. aeruginosa* with natural levels of AI. Although furanone **5** did not inhibit wild type biofilm formation, it did affect biofilm structure and aided in the process of bacterial

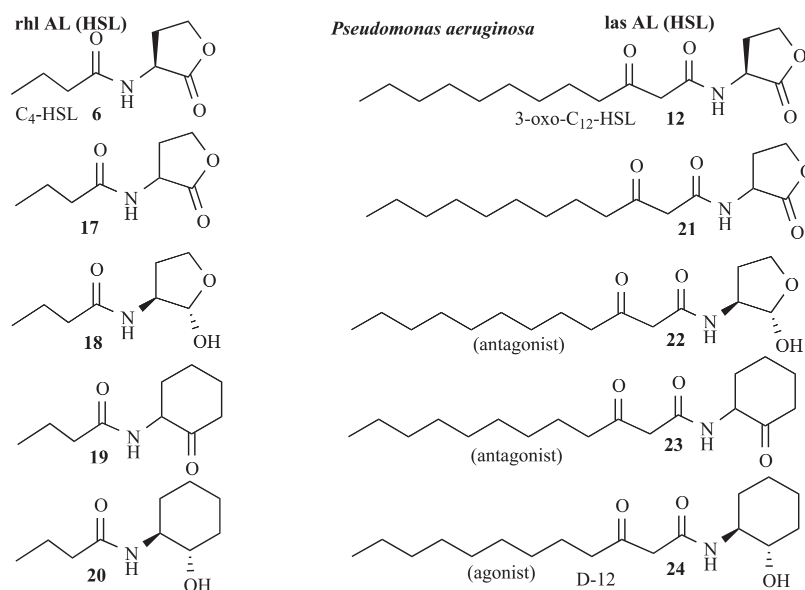

Fig. (4).

detachment which may have important therapeutic considerations [57].

Halogenated furanones **3** and **4** isolated from *D. pulchra* and their synthetic analogs antagonize the swarming motility of *S. lucifaciens* MGI [42] and biofilm formation and virulence factor expression in *P. aeruginosa*, as well as rapid proteolytic degradation of LuxR receptor [55] all of which are under QS controlled gene expression. Recently, two new natural products, iso-cladospolide B (**25**) and acaterin (**26**) (Fig. **5**) which are structurally similar to furanones **3**, **4**, **5** have been isolated from a marine sponge [58] and from *Pseudomonas sp.* A92 [59-61], respectively. Analogs of **25** have extra-QS activity which includes tumor cell cytotoxicity [62] while **26** analogs appear to inhibit acyl-CoA (cholesterol acyltransferase) [59]. Based on the structural similarities between compounds **3, 4, 25, 26**, the **25** and **26** scaffold have been used in the synthesis of QS antagonist analogs **27a-d, 28a-d** and **29a-d** (Fig. **5**). In general it appears that compounds with shorter alkyl chains are better anta-gonists than compounds with the longer chains when screened using luxR, PluxI-gfp (ASV) based monitor *Escherichia coli* (pJBA89) [63]. *Erythro/threo*-**27a** is the best antagonist of the *erythro/threo* **27a-d** series, while *threo*-**27c**, *erythro*-**27d** and *threo*-**27d** have no anti-QS activity. The same pattern occurs for **28b-d**, where **28b** is the most effective antagonist and **28d** has no anti-QS activity. However, the limited shelf stability of these compounds could have influenced results [64]. From the **29a-d** series, the most active compound was one of the diastereomers of **29b** which has a butyl chain length in the 3-position, similar to the naturally occurring compounds **3** and **4** (Fig. **1**). However, in contrast to the natural compounds **3** and **4**, even the most potent of synthesized compounds have inferior antagonist activity. This is perhaps due to nucleophilic substitutions in the receptor by the reactive groups of the brominated furanones, which in turn makes the protein prone to degradation [55].

Screening of synthetic AI substituted AHL libraries has led to discovery of a novel class of QS inhibitors [51, 65]. It should be noted that three antagonists **2**, **5** and **30** are structurally related to the synthetic agonist **24** (Fig. **4**). Structural changes of the 2-aminocyclohexanol group in the ring size 6 to 5 membered ring **22** (Fig. **4**), type of ring cyclohexanol to phenol in **30** (Fig. **6**) and ring substituents, i.e., hydroxyl- to keto group **23** (Fig. **4**) alter the activity dramatically from agonist to antagonist. It is very likely that these antagonists bind to LasR, but are unable to activate it, thus act as strong competitive inhibitors.

Compounds **23** (Fig. **4**) and **30** (Fig. **6**) have virulence factor inhibitory activity. Compound **28** inhibits biofilm formation in both QS mutants (in the presence of exogenous AIs) and wild type *P. aeruginosa*. The phenol derivative **30** alters the biofilm structure, but does not inhibit biofilm formation. Therefore compounds **23** and **30** appear to be promising leads for libraries of QS inhibitors.

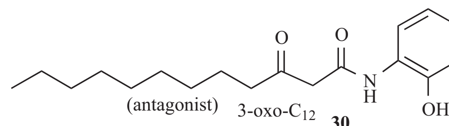

Fig. (6).

Based on these leads, the design and synthesis of a library of compounds where the common structural element of various Gram-negative bacteria, HSL, has been replaced with various amines has been recently accomplished (personal communication with Geetanjali J. Jog, Department of Chemistry, SUNY Buffalo). This study led to the identification of new scaffolds of QS antagonists such as ones having pyridine ring (with *p*-substitutions) or ring(s) containing heteroatom(s) with 2-3 carbon-alkyl spacer. These amine-containing analogs possibly interact with critical residues involved in the binding of the natural autoinducer.

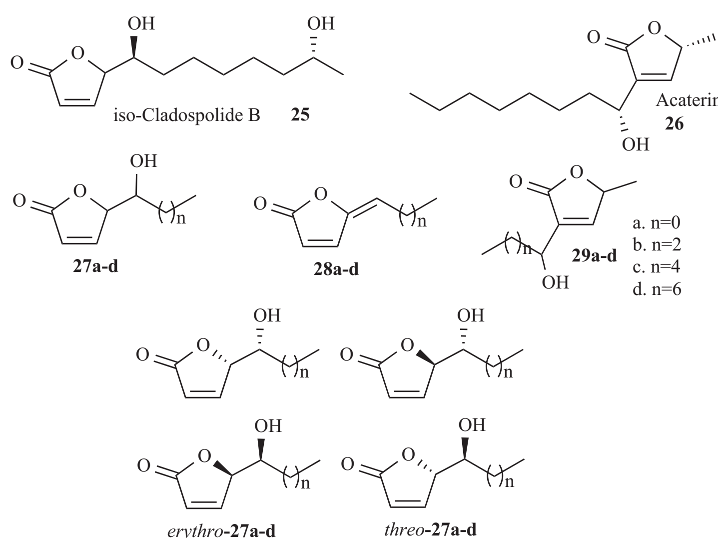

Fig. (5).

Earlier studies showed that analogs of AHLs (AHLs such as **31** and **13**, Fig. **7**) having aromatic rings, at the extreme opposite to the furanone end, display antagonist activity the most active of which being compounds **32** and **33** [68]. Recently a series of novel AHLs analogs have been synthesized where the carboxamide bond is replaced by a sulfonamide bond [66]. The sulfonamide analogs showed good antagonistic activity using the *V. fischeri* assay when the carboxamide function in AI **31** was replaced by a sulfonamide group in compound **34** [66]. Similarly analog **42** showed significant inhibitory activity. In contrast, analog **43** of AI **13** and analog **44** were inactive. Therefore the presence of a sulfonamide function in alkyl substituted derivatives led to a strong antagonist activity, while the combination of 3-oxo and sulfonamide groups yielded compounds with no inhibitory effect.

The number of carbon atoms in the alkyl chain of the sulfonamide derivatives also affects their activity as exemplified by the activities of compounds **34**-**40** [66]. The poorest antagonist was analog **35** with a butyl group. The analog with the highest activity was antagonist **34** with a pentyl chain. Increasing the chain length from five to nine carbon atoms (compound **34**, **36** and **37**-**39**, Fig. **7**) resulted in a corollary decrease in antagonist activity.

The influence of the distance between the sulfonamide and the phenyl group on antagonist activity was also explored since compound **42** showed inhibitor activity. When the two groups have one carbon atom between them (compound **40**, Fig. **7**), no significant inhibitory activity could be detected. However, when the two groups are spaced by two carbon atoms activity increased so that compound **41** was more active than **42** and almost as active as **34**. Although no synergy between the sulfonamide and phenyl group was observed, the position of the phenyl group appears important for activity. Molecular modeling studies indicate [66] that the strongest antagonists **34** and **41** best fit the active site of LuxR, due to the fact that their alkyl chains are firmly packed at the entrance of the pocket while their lactone group is tightly bound to the receiver domain. Sulfonamides **42** and **43** appear to poorly fit the protein cavity of LuxR, most likely due to the orientation of the alkyl groups which is different when attached to the tetragonal sulfonamide group, as compared to the trigonal geometry of carboximide. The former does not permit either the phenyl group in **42** or the keto group in **43** to be correctly positioned in the pocket [66].

Sulfide QS inhibitors which include compounds **45 a-s** (Fig. **8**), sulfoxides **46a-e** and sulfones **47a-e** (Fig. **5**) prepared either by one-pot or a sequential approach have also been reported [69]. These compounds were designed as competitive inhibitors of transcriptional QS regulators LuxR and LasR. The design was based on critical interactions within the binding site and structural motifs of QS compounds isolated from garlic. Garlic (*Allium sativum*, L.) possesses intrinsic antibiotic activity [70]. This activity is principally attributed to 2-propenesulfenic acid, which after cleaving gives allicin, a thiosulfinate [71]. Additional compounds isolated from garlic extracts [69] include compounds **48** and **49**, (Fig. **10**) which while devoid of antimicrobial activity do act as QS inhibitors. Structurally these compounds differ from **45**, thus inferring affinity for another AHLs binding site likely located in close proximity. The possibility that two individual low-affinity binders can produce a new ligand with high affinity has also been explored [72] by incorporating elements of the garlic QS compounds in the AHL's scaffold.

Sulfinyl- and sulfonyl functionalities are isosters of ketones and have analogous reactivity to sulfides and ketones due to the presence of acidic α-proton. These properties have made analogs of **45** with the aforementioned functionalities an interesting target [69]. The less specific LuxR QS system in *V. fischeri* was used for primary compound screening prior to testing their activity as inhibitors for the *P. aeruginosa* LasR QS circuit [69]. The highest activities were observed for compounds **45c** and **46c**, which shows that the optimal chain length is two carbons shorter than in the natural ligand. This is not necessarily due to pharmacodynamics, since it is well accepted that the efficiency of diffusion through membranes of molecules with a long carbon chain is to a

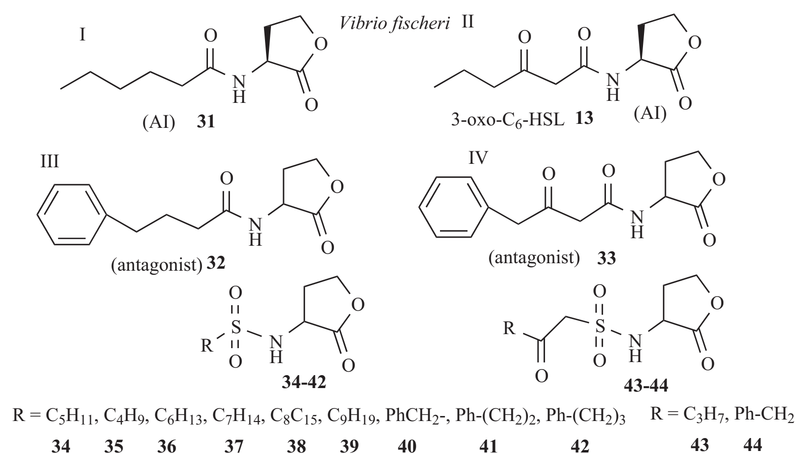

Fig. (7).

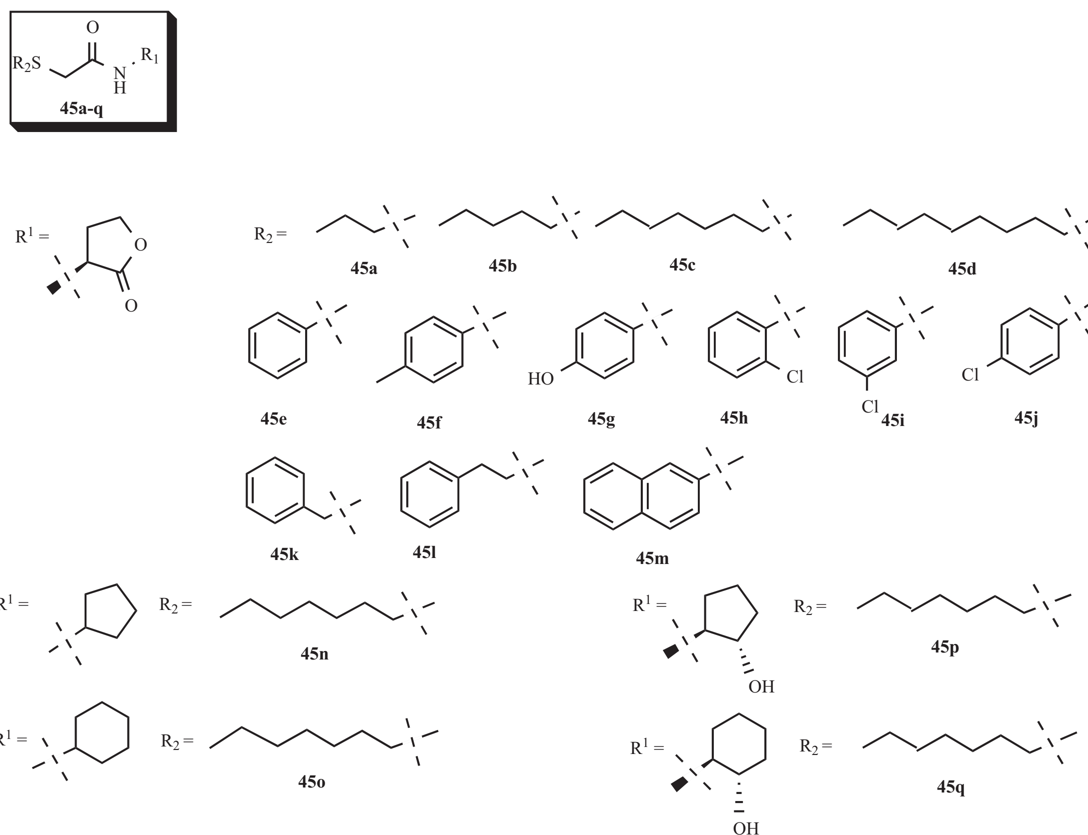

Fig. (8).

great extent determined by their length [73]. After initial biological screening of compounds **45a-e**, **46a-e** and **47a-e**, it was concluded that the most potent of the three types of functionalities is the thioether. The impact of the thioether functionalities as a part of the scaffold could be further evaluated by comparing all of the 4-substituted phenyl derivatives. Due to the fact that the activity of 4-chlo derivative **45j** disappeared as compared to non-substituted **45e**, and both **45f** and **45g** had higher potency, these outcomes were attributed to – σ effect [74]. Whether the mechanism for the higher potency of **45f** as compared to **45g** is due to the +π effect or the – σ effect is unclear at this time.

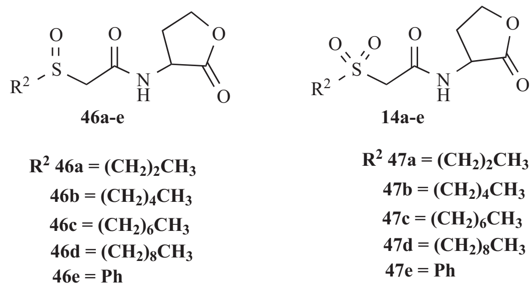

Fig. (9).

For derivatives **45n-q**, the lactone moiety was altered. This change resulted in antagonistic activity for the LuxR system [69]. The design of **45p-q** was based on the acyl chain in the highly active **45c** as well as on the QS inducer structures observed earlier [75], which have the same cyclic moiety as do **45p-q** but with the acyl chain of **12** (Fig. **2**). Their analogs **45p** and **45q** have antagonist activity toward LasR with the same relative potency as **45p** and **45q**. A thioether group with a shorter acyl chain resulted in **45r** and displaying antagonism of the LasR system. Analogs **45n** and **45o** lacking the 1-oxo-group, which leads to their inability to form hydrogen bonds to residue Trp57 in LasR, have also shown to be antagonists of this QS system. However, none of the aforementioned compounds proved to be a more effective antagonist than **45c**, which is several times more potent than the previously reported **45d** and **45l** [68].

Compound **45l** has potent LuxR system inhibitory activity [68], but low potency in the LasR assay [69]. The key feature for this type of structure/function activity appears to be a shorter acyl chain than in **12** and a 3'-oxo group substituent with a sulfur atom [69]. The ability of a sulfide group to stabilize α-anions is considered to be significant since electronic factors should have influence on the binding caused by thioethers. Otherwise, it would be difficult to explain the similar potencies of the aromatic analogs **45e-n** and **45a-d** despite having different geometry. Surprisingly,

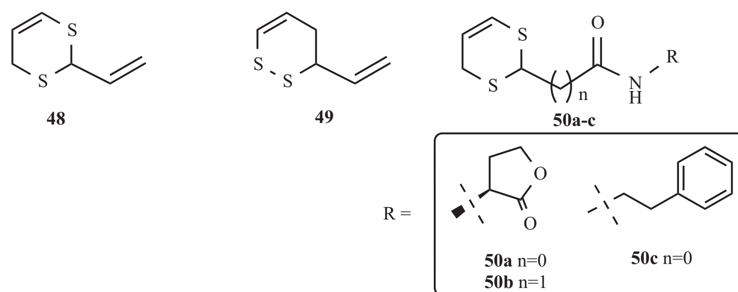

Fig. (10).

the conformationally restricted garlic-derived analogs **50a-c** and especially **50a** which has two sulfur atoms in β-position, are not better antagonists than their individual parts [69], i.e., **50a** and **50b** are antagonists of LuxR but not LasR. This finding also holds for the naturally occurring garlic compounds **48** and **49**. Unfortunately, the combination of the two individually potent compounds **48** and **45c** resulting in compound **50c** proved inactive against both QS monitor systems. The reason for the lack of **50c** activity is presumed to be due to either a wrong linker between compounds **48** and **45c** or the lack of the double bond in **50a-c**, which is present in **48** [69]. Compound **5** remains the most potent inhibitor of the LasR system identified to date, however its chemical instability under the conditions of the assay makes it an unsuitable candidate for further development.

Early investigation of the antimicrobial compounds produced by the Gram positive *Streptomyces antibioticus* TÜ 99 established the presence of butenolides (furanones) **51**-**54** (Fig. **11**) amongst other antibacterial compounds [76]. Initial screening indicated that these four butenolides exhibit weak anti-pseudomonal activity and weak inhibition of chitinase from *S. marcescens* [76]. However these findings could not be confirmed [77]. The absolute configuration of compounds **51**-**54** was recently established and non-stereoselective synthetic approach has been sought to prepare analogs of these compounds as stereoisomers [77]. Despite this, some of the compounds did prove to inhibit QS signaling in the *C. violaceum* mutant CV026 [78].

Furanones **56c**, **d**, **e** have also been found to inhibit the production of violacin, when administered with the natural signaling compound of *C. violaceum,* N-hexanoyl-homoserine lactone **6** (Fig. **5**). In contrast, metabolites **53** and **54** and their diastereomers **56b** and **55** have agonist activity, showing a synergistic effect when administered together with a suboptimal concentration of lactone **6** [77]. These findings present yet another class of furanones for further QS activity investigation.

CONCLUSION

The ability to use quorum analogs to exogenously regulate microbial physiology represents a potent tool for controlling virulence factor expression and environmental bio-fouling, especially in the face of increasing resistance to both antibiotics and disinfectants. Although we are beginning to understand the structure-function relationship for quorum compounds from Gram negative bacteria, much less is known concerning the quorum peptides which regulate the physiology of Gram positive organisms. Clearly, this field is still in its infancy and much work still needs to be done.

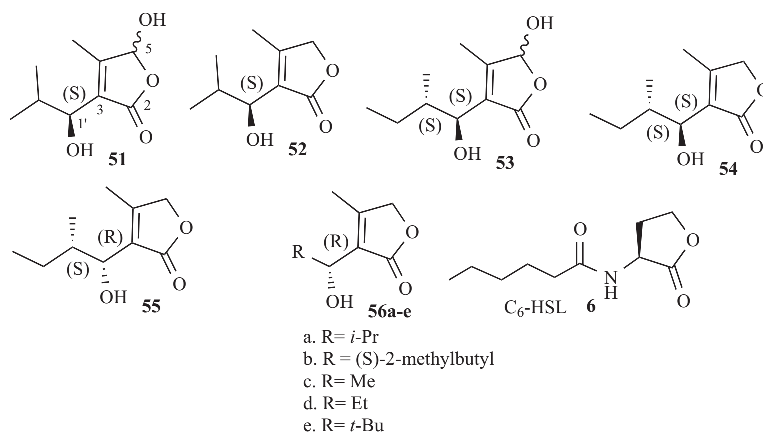

Fig. (11).

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Received: September 19, 2005 Revised: February 07, 2006 Accepted: February 10, 2006

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