GAZİ UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

**DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY**

**STUDIES ON SOME NOVEL ACYL HOMOSERINE**

**LACTONE ANALOGUES**

Master of Science Thesis

Pharm. Anooshirvan M. Miandji

Advisor

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# ACKNOWLEDGEMENTS

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**FIGURES**

**ABBRIVATIONS**

AHL          *N*-acyl-L-homoserine lactone

EF-2         Elongation factor 2

LuxI         LuxI enzyme synthesizes LuxR

LuxR         LuxR receptor is the site of interaction with quorum-sensing
             signals and inhibitors

QS           Quorum Sensing

QSI          Quorum Sensing Inhibitor

QSP          Quorum Sensing Binding Protein
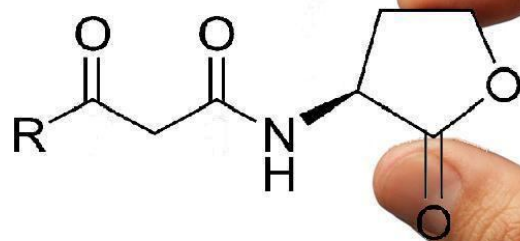
The Language of Bacteria
is The language of Life.

miandji

## 1. INTRODUCTION

"The majority of the cells in our body are not our own, nor are they even human. They are bacterial. Since the hidden strands of fungi waiting to grow among our toes, to the kilo of bacterial material in our intestines, we are finest observed as walking "super organisms", most complex collections of human cells, bacteria, fungi and viruses.

That's the opinion of researchers at Imperial College London who published a paper in *Nature Biotechnology* Oct. 6 describing how these microbes interact with the body. The scientists concentrated on bacteria. More than 500 different species of bacteria exist in our bodies, making up more than 100 trillion cells. Because our bodies are made of only some several trillion human cells, we are somewhat out numbered by the aliens. It follows that most of the genes in our bodies are from bacteria, too.

Luckily for us, the bacteria are on the whole commensal, sharing our food but doing no real harm. In fact, they are often beneficial: Our commensal bacteria protect us from potentially dangerous infections. They do this through close interaction with our immune systems.

We have known for some time that many diseases are influenced by a variety of factors, including both genetics and environment, but the concept of this superorganism could have a huge impact on our understanding of disease processes. The approach could apply to research on insulin-resistance, heart disease, some cancers and perhaps even some neurological diseases.

Following the sequencing of the human genome, scientists quickly saw that the next step would be to show how human genes interact with environmental factors to influence the risk of developing disease, the aging process and drug action. But because environmental

factors include the gene products of trillions of bacteria in the gut, they get very complex indeed. The information in the human genome itself, 3 billion base pairs long, does not help to reduce the complexity.

The human genome provides only scant information. The discovery of how microbes in the gut can influence the body's responses to disease means that we now need more research into this area. Understanding these interactions will extend human biology and medicine well beyond the human genome and help elucidate novel types of gene-environment interactions, with this knowledge ultimately leading to new approaches to the treatment of disease.

Human "super-organism" concept could have a huge impact on how we develop drugs, as individuals can have very different responses to drug metabolism and toxicity.

The microbes can influence things such as the pH levels in the gut and the immune response, all of which can have influences on the effectiveness of drugs." [1,2]

Researches focused on bacterial behaviors and communication tools will lead us to understand how their communities interact. We know that Quorum sensing (QS) is bacterial communication phenomenon. QS in pathogens is the core of these studies. QS signaling systems of pathogens are central regulators for the expression of virulence factors and represent highly attractive targets for the development of novel therapeutics. For example, in *P. aeruginosa,* QS systems are also involved in elevated antibiotic tolerance of biofilms as well as elevated tolerance to the activity of the innate immune system. The topic of biofilm is essential to consider in researches focused over patogen bacteria.

"Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili." [3]

"The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together. Some species are not able to attach to a surface on their own but are often able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via QS using such products as *N*-acyl homoserine lactones (AHL).

Gram-negative bacteria commonly use AHL as QS signal molecules. The use of signal molecule based drugs to attenuate bacterial pathogenecity rather than bacterial growth is attractive for several reasons, particularly considering the emergence of increasingly antibiotic-resistant bacteria. Compounds capable of this type of interference have been termed anti-pathogenic drugs. A large variety of synthetic AHL analogues and natural product libraries have been screened and a number of QS inhibitors (QSI) have been identified. Promising QSI compounds have been shown to make biofilms more susceptible to antimicrobial treatments, and are capable of reducing mortality and virulence as well as promoting clearance of bacteria in experimental animal models of infection."[4]

Concern about the synthesis of some novel AHL analogues has been the main subject of discussion for two years in the department of Pharmaceutical Chemistry . We were wondering how we could mimic AHL molecules which would inhibit binding sites without the effects of

antibiotics. The interface between these two activities is not clear yet and still constitutes a topic of discussion. Early this year we tried some novel techniques to synthesize some designed models by using a microwave synthesizer.

The general strategy of our design for the potentiation of Quorum Sensing Inhibitors (QSIs) has been to stay very close to the general structure of AHL with minimal modifications in some functional groups. (Fig. 1)



n: 5, 6, 8

$R^1$: H, $CH_3$, Cl

$R^2$: H, $CH_3$
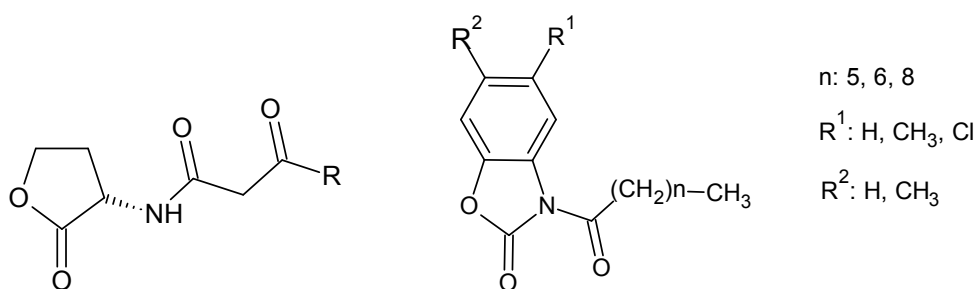
**Figure 1 :** The  general structure of AHL and designed molecules.

We preferred to stay as close as possible to the lactone-acyl scaffold to design some novel QSI candidates (Fig. 2). Structure activity relationships (SAR) calculations theoretically supported the molecules that were subsequently synthesized.

Synthesized analogues were tested for their anti QSI activities. Modifying the site of the functional groups would help us to sort out the possibilites which would lead to the discovery of  the pharmacophore shape of  the AHL binding site and design the best possible inhibitors.
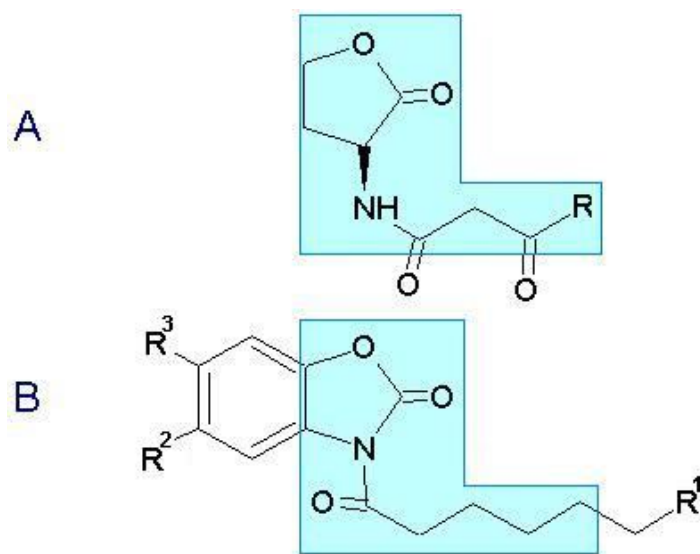
$R^1$= CH$_3$, C$_2$H$_5$, C$_4$H$_9$      $R^2$=Cl,CH$_3$   $R^3$=CH$_3$

**Figure 2:** The structural similarity between (A) the main AHL molecule and (B) our candidates.

## 2. GENERAL INFORMATION

### 2.1 Antibiotics and Bacteria

"When bacteria overcome the cutaneous or mucosal barriers and penetrate body tissues, a bacterial infection sets in. Frequently the body succeeds in removing the invaders, without manifestations of disease, by mounting an immune response.If bacteria multiply faster than the body's defense can destroy them; infectious disease develops with manifestations, e.g., purulent wound infection or urinary tract infection. Appropriate treatment employs substances that injure bacteria and thereby prevent their further multiplication, without harming the cells of the host (Fig. 3). " [5]
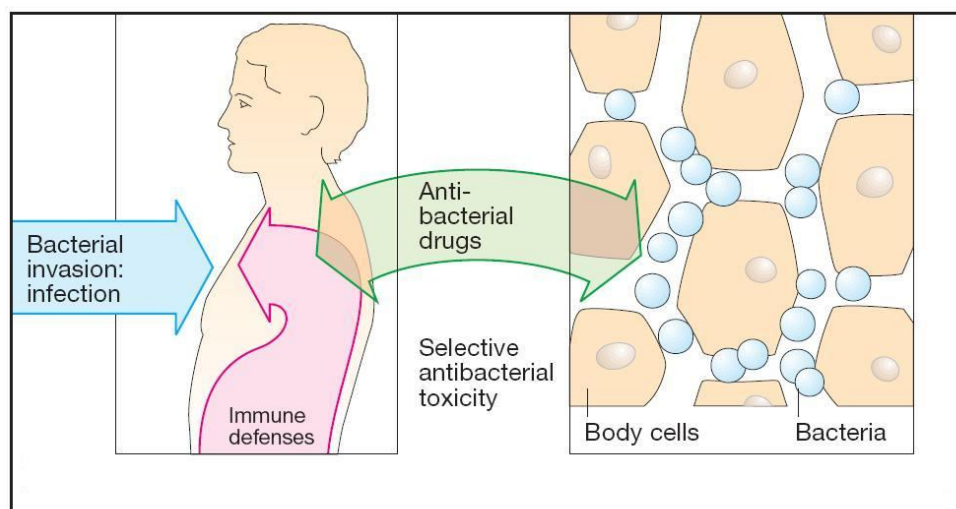


**Figure 3:** Appropriate treatment that injures bacteria without harming cells of the host.[5]

Antibiotics are produced by microorganisms (fungi, bacteria) and are directed "against life" at any phylogenetic level (prokaryotes, eukaryotes). Chemotherapeutic agents originate from chemical synthesis. This distinction has been lost in current usage. Specific damage to

bacteria is particularly practicable when a substance interferes with a metabolic process that occurs in bacteria but not in host cells.Clearly this applies to inhibitors of cell wall synthesis, because human and animal cells lack a cell wall. The points of attack of antibacterial agents are schematically illustrated in a grossly simplified bacterial cell, as depicted in (Fig. 4)
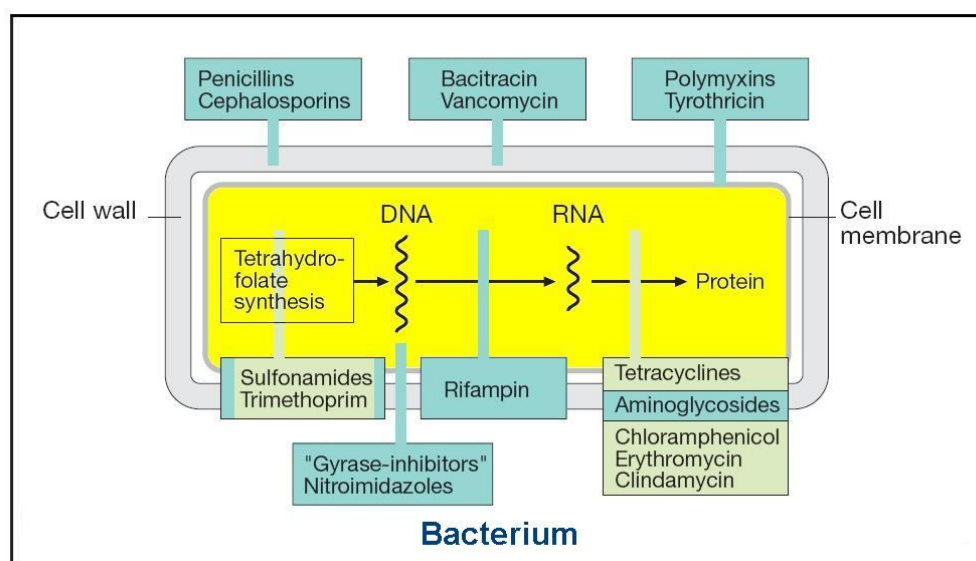


**Figure 4 :** Schematic illustration in a simplified bacterial cell and major antibiotic actions.[5]

For example, polymyxins and tyrothricin are polypeptide antibiotics enhance cell membrane permeability. Due to their poor tolerability, they are prescribed in humans only for topical use. The effect of antibacterial drugs can be observed *in vitro* (Fig. 5).
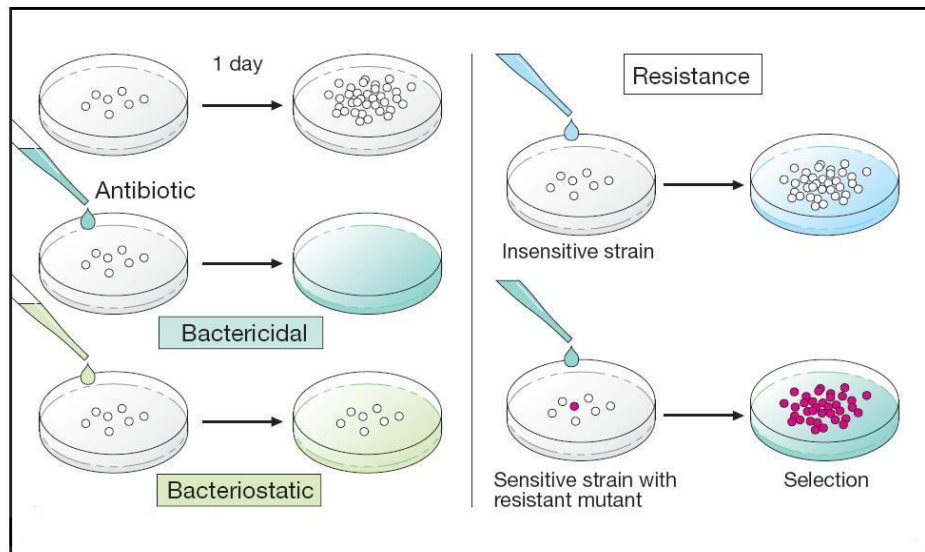
**Figure 5 :** in vitro applications of antibiotics. [5]

"Bacteria multiply in a growth medium under controlled conditions. If the medium contains an antibacterial drug, two results can be discerned: 1. bacteria are killed—bactericidal effect; 2. bacteria survive, but do not multiply—bacteriostatic effect. Although variations may occur under therapeutic conditions, different drugs can be classified according to their respective primary mode of action. When bacterial growth remains unaffected by an antibacterial drug, bacterial resistance is present. This may occur because of certain metabolic characteristics that confer a natural insensitivity to the drug on a particular strain of bacteria *(natural resistance).* Depending on whether a drug affects only a few or numerous types of bacteria, the terms narrow-spectrum (e.g., penicillin G) or broad-spectrum (e.g., tetracyclines) antibiotic are applied. Naturally susceptible bacterial strains can be transformed under the influence of antibacterial drugs into resistant ones *(acquired resistance),* when a random genetic alteration (mutation) gives rise to a resistant bacterium. Under the influence of the drug, the susceptible bacteria die off, whereas the mutant multiplies unimpeded. The more frequently a given drug is applied, the

more probable the emergence of resistant strains (e.g., hospital strains with multiple resistance!). Resistance can also be acquired when DNA responsible for nonsusceptibility (so-called *resistance plasmid*) is passed on from other resistant bacteria by conjugation or transduction." [5]

## 2.2 Quorum Sensing

"Quorum sensing (QS) is a type of decision-making process used by decentralized groups to coordinate behavior. Many species of bacteria use QS to coordinate their gene expression according to the local density of their population. Similarly, some social insects use QS to make collective decisions about where to nest. In addition to its function in biological systems, QS has several useful applications for computing and robotics.

Quorum sensing can function as a decision-making process in any decentralized system, as long as individual components have a means of assessing the number of other components they interact with and a standard response once a threshold number of components is detected." [10]

Some of the best-known examples of QS come from studies of bacteria. Bacteria use QS to coordinate certain behaviors based on the local density of the bacterial population. Quorum sensing can occur within a single bacterial species as well as between diverse species, and can regulate a host of different processes, essentially serving as a simple communication network. A variety of different molecules can be used as signals. Common classes of signaling molecules are oligopeptides in Gram-positive bacteria, *N*-Acyl Homoserine Lactones (AHL) in Gram-negative bacteria and a family of autoinducers known as AI-2 in both Gram-negative and Gram-positive bacteria.[7]

Although QS was originally used to describe *N*-acylhomoserine lactone (AHL)-dependent cell-to-cell communication in Gram-negative bacteria, QS signal molecules exhibit significant chemical diversity (Fig. 6).

peptide thiolactone

cis-11-methyl-2-dodecenoic acid
diffusible signal factor (DSF)

autoinducer-2 (AI-2)

2-heptyl-3-hydroxy-4-quinolone (PQS)

*N*-acyl-L-HSL

*N*-(3-oxoacyl)-L-HSL

*N*-(3-hydroxyacyl)-L-HSL

*N*-(*p*-coumaroyl)-HSL
R = OH (*p*C-HSL)
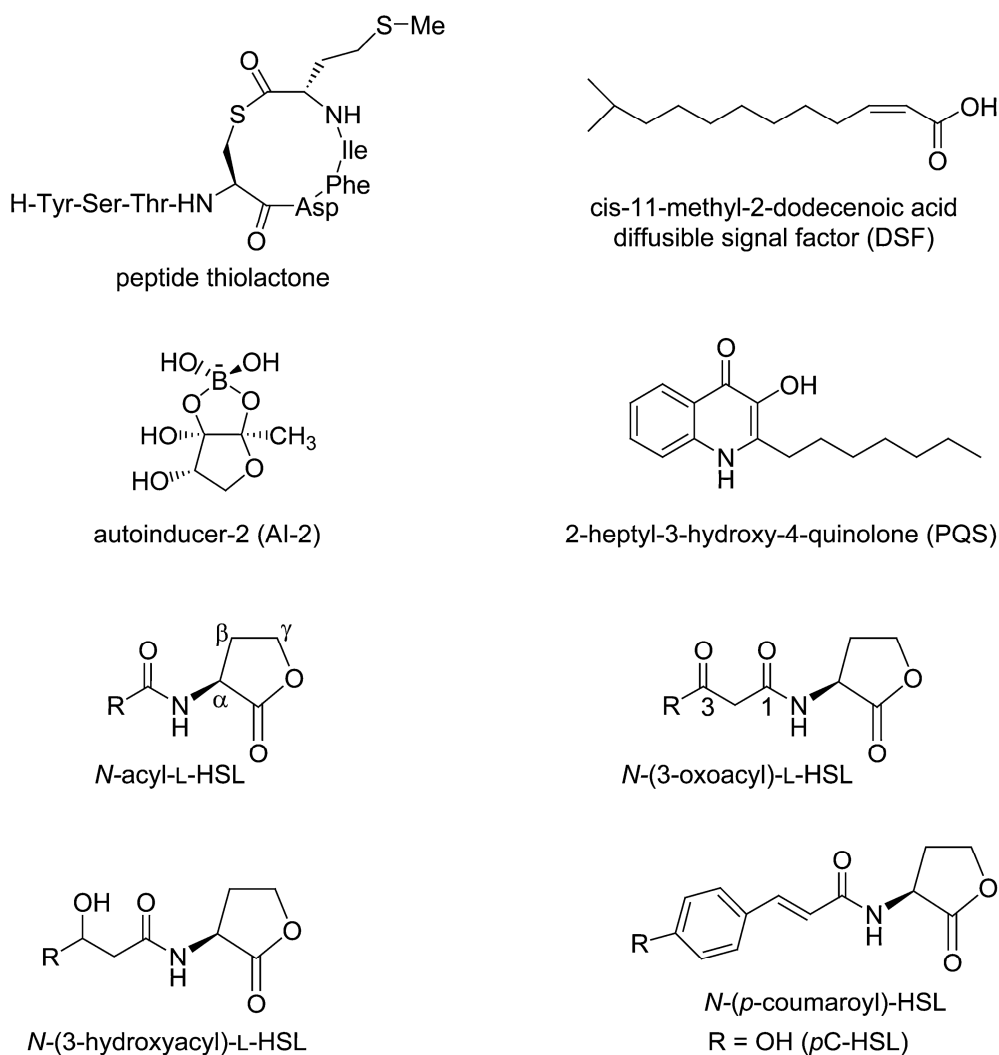
**Figure 6 :** Structures of some bacterial QS signal molecules

Where such molecules are responsible for inducing their own biosynthesis, they are referred to as "autoinducers". Although no "universal" bacterial QS language has yet been discovered, the

autoinducer-2(AI-2)/LuxS QS system is shared by both Gram-negative and Gram-positive bacteria. In addition, many bacteria employ more than one QS signal molecule from the same or a different chemical class, the activities of which may be coordinated via interacting QS systems, each of which incorporates a signal molecule synthase and a sensor/receptor.[8,9,10]

QS controls secondary metabolism, bioluminescence, protein secretion, motility, virulence factor production, plasmid transfer, and biofilm maturation in diverse bacteria.[8,10]

As a result, QS has attracted considerable industrial interest, particularly as a target for novel antibacterial agents that attenuate bacterial virulence rather than growth. [11] Because bacteria cohabit ecosystems with many other organisms, it is perhaps not surprising to discover that QS signals can modulate the behavior of both bacteria other than the QS signal producer itself and of higher organisms, in ways advantageous for bacterial survival.[10]

Conversely, higher organisms manipulate QS by producing signal mimics, by modulating QS pathways through the action of cytokines, by blocking QS through the deployment of inhibitors, or via the enzymatic inactivation of QS signals.

Two recent publications in this rapidly expanding field provide exciting new insights into the chemical diversity, evolution, and adaptability of AHL-dependent QS signal transduction pathways[12] and their effects on the host immune response[13]

## 2.2.1 N-Alkanoyl- and N-aroyl-Homoserine Lactone-Dependent QS

AHL-mediated QS appears to be employed exclusively by Gram-negative bacteria, including beneficial and pathogenic species, because no AHL-producing Gram-positive bacteria have yet been

identified.[10,14] Most AHL producers synthesize multiple AHLs that are characterized by a homoserine lactone ring, unsubstituted in the β and γ positions, which is *N*-acylated with an acyl chain incorporating variable saturation levels and oxidation states. AHLs are exemplified by compounds with an acyl chain of 4–18 carbons that belong to either the *N*-acyl, *N*-(3-oxoacyl), or *N*-(3-hydroxyacyl) classes AHLs with one or two double bonds in an acyl chain have also been described. The stereochemistry at the α-center of the homoserine lactone (HSL) ring is L, and the corresponding D-isomers are, in common with ring-open compounds, inactive. Consequently, it is the acyl chain moiety that confers QS signal specificity. [15]

## 2.2.2 AHL biosynthesis

AHL biosynthesis mostly depends on LuxI family proteins that use *S*-adenosylmethionine (SAM) and an acyl carrier protein (ACP) charged with the appropriate fatty acid as sources of the homoserine lactone (HSL) and acyl chain, respectively (Fig. 7).[16,17]

Although one or two compounds usually predominate, most LuxI homologs produce a range of AHLs, indicating that they exhibit some flexibility and can accept a number of different acyl-ACP donors[18] In LuxI-driven AHL biosynthesis, two separate chemical events occur. The first is the transfer of the acyl group from the ACP to the amino group of SAM, followed by lactonization and the release of the AHL and methylthioadenosine. In this reaction mechanism, SAM acts as a homoserine rather than as a methyl donor[16]. Structural studies of EsaI and LasI (which produce predominantly *N*-[3-oxohexanoyl]- l -homoserine lactone and *N*-[3-oxododecanoyl]- l -homoserine lactone [3-oxo-C12-HSL], respectively) have highlighted a substrate-binding pocket for SAM and a

common binding site for the acyl-ACP phosphopantetheine group, which constitutes a tunnel passing through the enzyme in LasI. This is occluded in EsaI, a finding that provides a partial explanation for the acyl chain specificities of the two enzymes.[19,20]
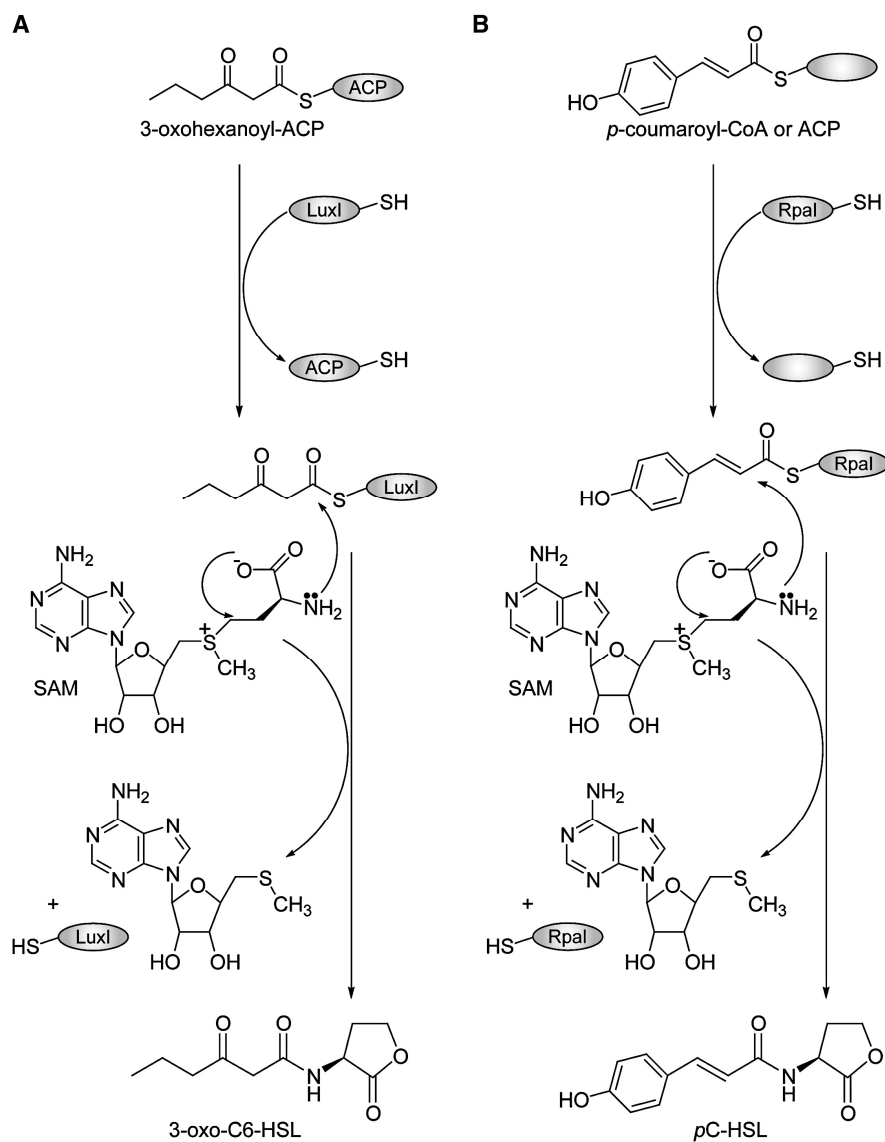


**Figure 7 :** Biosynthesis of 3-oxo-C6-HSL and pC-HSL (A) Biosynthesis of *N*-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL) via the charged acyl carrier protein and either LuxI or RpaI. (B) Biosynthesis of pC-HSL via the charged acyl carrier protein and either LuxI or RpaI.

Once produced, AHLs diffuse across the cell envelope and subsequently accumulate within the extracellular environment until a feedback concentration sufficient to activate a target LuxR family protein has been achieved. AHLs bind to and activate LuxR proteins directly such that the LuxR/AHL complex activates or represses one or more target genes that often include the AHL synthase gene, thus establishing a positive autoinduction circuit in which the AHL signal also controls its own biosynthesis.[21] (Fig. 8)
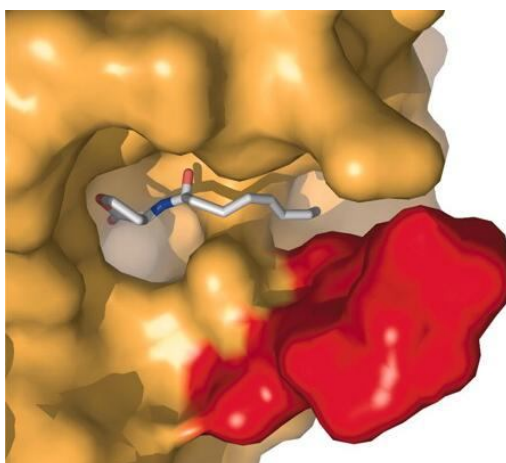


**Figure 8 :** AHL in binding site target LuxR family protein [22]

## 2.2.3 Biofilm Formation

The formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili.

The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together. Some species are not able to attach to a surface on their own but are often able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via QS using such products as AHL. Once colonization has begun, the biofilm grows through a combination of cell division and recruitment. The final stage of biofilm formation is known as development, and is the stage in which the biofilm is established and may only change in shape and size. The development of a biofilm may allow for the aggregate cell colony(ies) to be increasingly antibiotic resistant.(Fig. 9)
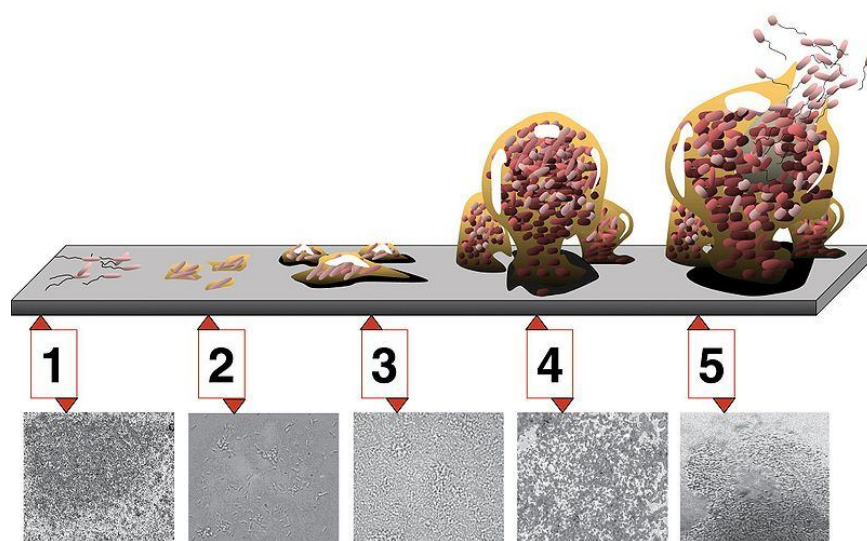


**Figure 9:** The five stages of biofilm development.

Each stage of development in the diagram is paired with a photomicrograph of a developing *P. aeruginosa* biofilm. All photomicrographs are shown to the same scale. [23]

## 2.2.4 Biofilms and infectious diseases

Biofilms have been found to be involved in a wide variety of microbial infections in the body, by one estimate 80% of all infections. [24] Infectious processes in which biofilms have been implicated include common problems such as urinary tract infections, catheter infections, middle-ear infections, formation of dental plaque, gingivitis, coating contact lenses, and less common but more lethal processes such as endocarditis, infections in cystic fibrosis, and infections of permanent indwelling devices such as joint prostheses and heart valves. More recently it has been noted that bacterial biofilms may impair cutaneous wound healing and reduce topical antibacterial efficiency in healing or treating infected skin wounds.

It has recently been shown that biofilms are present on the removed tissue of 80% of patients undergoing surgery for chronic sinusitis. The patients with biofilms were shown to have been denuded of cilia and goblet cells, unlike the controls without biofilms who had normal cilia and goblet cell morphology. [25]

Biofilms were also found on samples from two of 10 healthy controls mentioned. The species of bacteria from interoperative cultures did not correspond to the bacteria species in the biofilm on the respective patient's tissue. In other words, the cultures were negative though the bacteria were present.[26]

Biofilms can also be formed on the inert surfaces of implanted devices such as catheters, prosthetic cardiac valves and intrauterine devices. [27]

New staining techniques are being developed to differentiate bacterial cells growing in living animals, e.g. from tissues with allergy-inflammations.[28]

### 2.2.5 *P. aeruginosa* biofilms

The achievements of medical care in industrialised societies are markedly impaired due to chronic opportunistic infections that have become increasingly apparent in immunocompromised patients and the aging population. Chronic infections remain a major challenge for the medical profession and are of great economic relevance because traditional antibiotic therapy is usually not sufficient to eradicate these infections. One major reason for persistence seems to be the capability of the bacteria to grow within biofilms that protects them from adverse environmental factors. *P. aeruginosa* is not only an important opportunistic pathogen and causative agent of emerging nosocomial infections but can also be considered a model organism for the study of diverse bacterial mechanisms that contribute to bacterial persistence. In this context the elucidation of the molecular mechanisms responsible for the switch from planktonic growth to a biofilm phenotype and the role of inter-bacterial communication in persistent disease should provide new insights in *P. aeruginosa* pathogenicity, contribute to a better clinical management of chronically infected patients and should lead to the identification of new drug targets for the development of alternative anti-infective treatment strategies. [29]

In recent years, the micro-organism on which most QS related studies have been initiated is *P. aeruginosa*. *P. aeruginosa* is an important human pathogen which is responsible for opportunistic infections in cancer, AIDS and cystic fibrosis (CF) patients (10-12). A wide variety of extracellular enzymes contribute to the virulence of *P. aeruginosa*. These include elastase, protease, hemolysins, exotoxin A, rhamnolipid biosurfactants and phospholipase. These exofactors are collectively capable of causing extensive tissue damage in humans and other mammals. [30]

### 2.2.6 Host defense and biofilm

Elongation factor 2 is a protein that in humans is encoded by the *EEF2* gene. This gene encodes a member of the GTP-binding translation elongation factor family. This protein is an essential factor for protein synthesis. It promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome. This protein is completely inactivated by EF-2 kinase phosporylation. It is the target of diphtheria toxin and exotoxin A. The Pseudomonas exotoxin or exotoxin A  is an exotoxin produced by *P. aeruginosa*. It acts at elongation factor-2. (The mechanism of the toxin is similar to that of Diphtheria toxin.) Diphtheria toxin is a single polypeptide chain of 535 amino acids consisting of two subunits linked by disulfide bridges. Binding to the cell surface of the less stable of these two subunits allows the more stable part of the protein to penetrate the host cell. It catalyzes the ADP-ribosylation of eukaryotic elongation factor-2 (eEF2), inactivating this protein. It does so by ADP-ribosylating the unusual amino acid diphthamide. In this way, it acts as a RNA translational inhibitor. The exotoxin A of *P. aeruginosa* uses a similar mechanism of action.[31, 32, 33]

### 2.3 Inhibitors Activity

As the population of bacteria increases so too does the QS molecules (QSMs) concentration, until some apparent threshold is reached at which all the bacteria switch on a behavioural trait; the population at this stage is sometimes referred to as being in a quorate state. On a more biochemical level, QSMs diffusing from the external media bind with an appropriate protein (QSP), located within the bacterium, to form a complex (Fig. 10). This QSM-QSP complex in turn binds to a region of DNA on the bacterium's chromosome (known as a *lux*-box) (Fig. 10), inducing or enhancing behavioural characteristics, as well

as the up-regulation of QSM production. In view of this self-induced up-regulation in QSM production, QSMs are often referred to as autoinducers. The *lux*-box bound QSM-QSP complex provides a stable base for the complexes involved in gene expression, namely RNA polymerase (RNAP), to activate the QS genes However, even with an empty *lux*-box the RNAP can activate these genes, providing background production of QSMs, but this process is considerably more unstable. The system illustrated in (Fig. 10), represents the relatively simple QS process involved in bioluminescence in *V. fischeri*, for which there is only a single QSM. Our experimental work towards the verification of the model discussed in this thesis focuses on *P. aeruginosa*, whose QS system is more complex, involving two or more QSMs. Here, the primary QSM, together with the primary QSP, up-regulates production not only of itself and some virulence factors, but also of the secondary QSM and QSP, the latter system being involved in the upregulation of a number of other virulence factors.

We considered the model system illustrated in (Fig. 10), which focuses on on the response of a single cell to external QSM concentrations. We proposed that QS works by the switching between two stable steady-state solutions, reflecting relatively low and high rates of QSM production, whereby increasing the population density causes the shift from low to high production rates.

The most promising mechanism for inhibiting QSP activation is the use of AHL analogues that act as antagonists for 3O-C12-HSL and C4-HSL. These molecules would most likely be similar in structure to the natural AHLs produced by *P. aeruginosa* and would compete for binding to LasR proteins . Structural variations of the 3O-C12-HSL and C4-HSL molecules have revealed epitopes that are important for the activation and inhibition of LasR activity.[34] (Fig. 10)
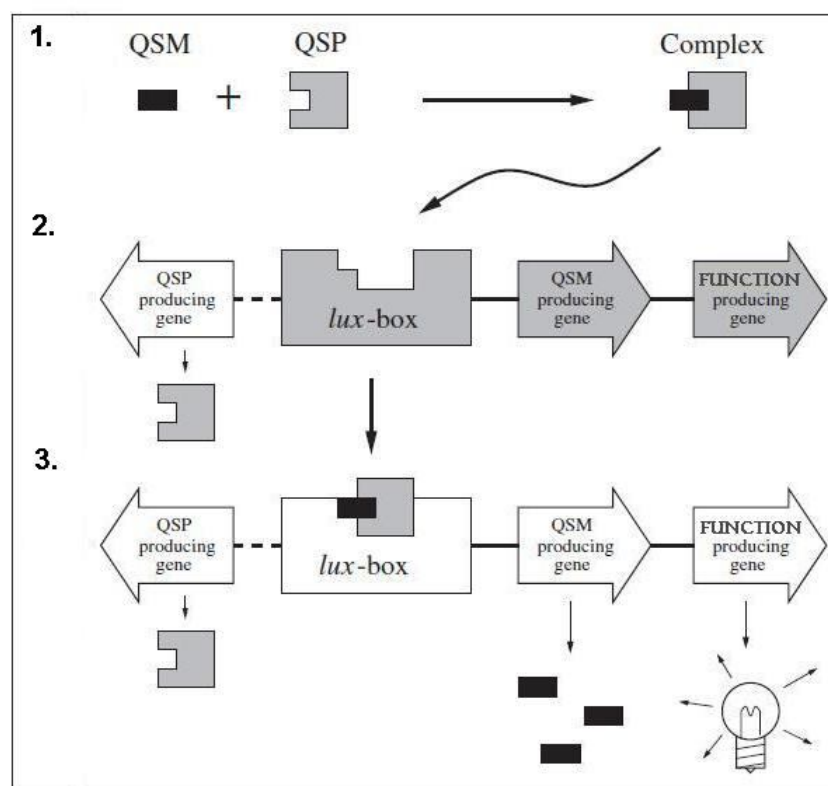
**Figure 10 :** The schematic illustration of QS regulation inside the bacterium cell. **(1)** the binding of the QSI and QSP ( LuxR) **(2)** the binding of the QSI-QSP complex *to lux*-box induces activation of the quorum sensing genes from a down-regulated state (grey background) to **(3)** an up-regulated one (white background).

The mechanism of action of our designed molecules is in the shape of making a interaction with QSP which lead to structural change and result in a stereochemical abnormality that blocks *lux*-box regulation. As it is shown in (Fig. 11) the antagonistic analogues of the cognate AHLs compete for binding to QSP but do not result in the activation of the protein.This pair with *lux-Box* inhibits gene translation and thus protein production. This step is important from the standpoint of the inhibition of the virulence factor production caused by the biofilm formation in pathogenic microorganisms such as *P. aeruginosa*.
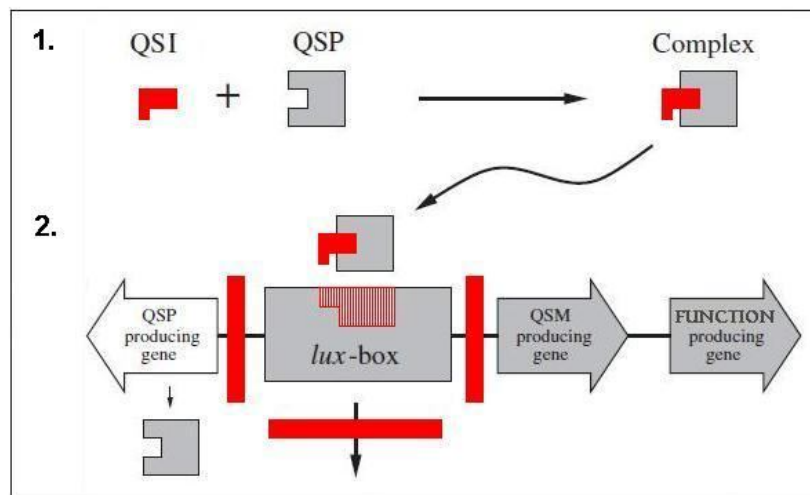
**Figure 11:** Schematic illustration of QSI inside the bacterium cell.
**(1)** the binding of the QSI and QSP ( LuxR) and  the formation of the
abnormal complex **(2)** the binding  the QSI-QSP complex *to lux*-box blocks
lux-box regulation.

## 2.4 Microwave Assisted Synthesis

It has long been known that molecules undergo excitation
with electromagnetic radiation. (Fig. 12)[35]

This effect is utilized in household microwave ovens to heat
up food. However, chemists have only been using microwaves as a
reaction methodology for a few years. Some of the first examples gave
amazing results, which led to a flood of interest in this novel technique.
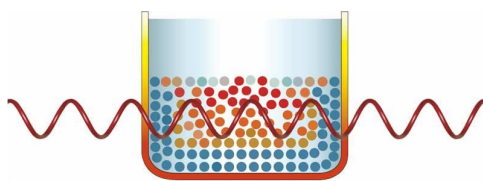
**Figure 12 :**   A schematic illustration of electromagnetic radiation in a microwave synthesizer, in which the red dots represent higher enthalpy/ entropy.

The water molecule is the target for microwave ovens in the home; like any other molecule with a dipole, it absorbs microwave radiation. Microwave radiation is converted into heat with high efficiency, so that "superheating" becomes possible at ambient pressure. Enormous accelerations in reaction time can be achieved, if superheating is performed in closed vessels under high pressure; a reaction that takes several hours under conventional conditions can be completed over the course of minutes.[36]

### 2.4.1 Thermal vs. Nonthermal Effects

Excitation with microwave radiation results in the molecules aligning their dipoles within the external field. Strong agitation, provided by the reorientation of molecules, in a phase with the electrical field excitation, causes an intense internal heating. The question of whether a nonthermal process is operating can be answered simply by comparing the reaction rates between the cases where the reaction is carried out under irradiation versus  conventional heating. In fact, no nonthermal effect has been found in the majority of reactions, and the acceleration is attributed to superheating alone. It is clear, though, that nonthermal effects do play a role in some reactions.[37]

The principle of microwave synthesis offers three major benefits to chemistry development: speed, reproducibility, and a scalable approach to compound synthesis. Additionally, the significant advantages of our technical solution are ease of use, safety, verified synthesis methods to enable new users, and an efficient work flow that fits the drug discovery process.

To gain the greatest advantages offered by microwave synthesis, Personal Chemistry has implemented a system called Coherent Synthesis. Incorporating planning systems, automation, distributed system access, searchable databases and reaction optimisation kits. The technology has already proved itself to dramatically improve the quality of output from medicinal chemistry for lead optimisation in all the major pharmaceutical and biotech companies.[38]

## 2.5. DESIGN OF THE ACTIVE SPECIES

### 2.5.1. Scope of the study and design of the active species

A human body carries $10^{12}$ microorganisms versus $10^{10}$ host cells. This means that the human body is in perfect balance with the microorganism domain. To use antibiotics particularly when not properly justified may turn out to be hurting this balance. The most suitable way to treat a bacterial infection should be through host system's defence forces which would selectively identify the invaders and destroy them. But then external help is needed. This help must not interfere with the rest of the microorganisms but keep the invaders from being harmful until the immunsystem can reach them. As it is explained above in detail, one way to achieve this strategy can be through QSIs.

This study aimed to design and synthesize molecules that would be competitive inhibitors for the natural QS process of some certain bacteria.

Among the QS mediators the best targets may be the ones for which the formulae have been given below. (Fig. 13)



3-oxo-C12-HSL                3-oxo-C6-HSL
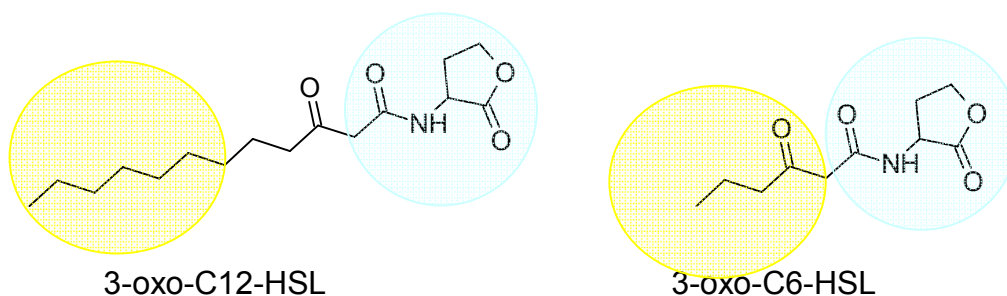
**Figure 13:** There are many sites on these molecules that can be modified subsequently turning them into inhibitors.

We decided to keep the side chain mainly as it was on the original molecule making some changes such as the length, and the number of oxo groups. AHLs were inserted into the site of activity. Any bulky change would decrease this insertion. As the number of the atoms on the side chain vary between 7 and 13 we decided to stick to numbers 7 - 10 incorporating one of the oxo groups into the ring system. The main impetus for this application was finding that the total energy of the AHLs and the inhibitors that were designed seemed to be very close to each other. When we also added the distances and charge distributions that were also very close to each other, the new design appeared to be reasonable.

We decided to modify the cyclic head of the molecule still keeping the main characteristics of the original structure. The exocyclic nitrogen was pushed into the ring just at the attachment point. One of the ways to potentiate the binding of the new molecules was to enhance the

liphophilicity. The best way to do that would be to incorporate a phenyl ring. The results of the theoretical log P calculations suggested an increase in the liphophilicity of the projected molecules. As the models suggest, there are two modes that the incorporation can be made, either at the end of the straight side chain or at the other end of the molecule on/in the lactone ring. The benzene ring can be incorporated into the molecule either as phenyl group or fused. Structural studies revealed the fact that when the benzene ring was inserted as a phenyl ring into any corner of the lactone ring the geometry was drastically disturbed and the independable free rotation of the benzene ring impeded the binding. When fused to the lactone ring this free rotation was canceled and a smooth geometry was obtained. Therefore we preferred the latter case imitating benzo furan and by taking the nitrogene in as benzoxazolone.

The final molecule was benzoxazoloneamide provided it would dock with the site of activity. Studies revealed that the removal of one of the oxo groups would lead to easier synthesis if the activity was not altered. In fact good activity was observed with better results. The compounds that were synthesized in this study appear in (Table 1).

**Table 1 :** List of derivatives of synthesized candidates and substitutions.
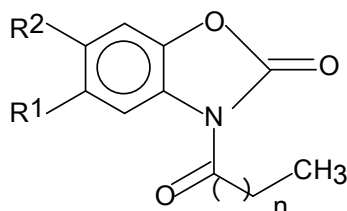


| Code Name | n | $R_1$ | $R_2$ |
|---|---|---|---|
| AMM33 | 8 | -H | -H |
| AMM34 | 6 | -H | -H |
| AMM40 | 5 | -H | -H |
| AMM37 | 8 | -Cl | -H |
| AMM38 | 6 | -Cl | -H |
| AMM39 | 5 | -Cl | -H |
| AMM43 | 8 | -H | $-CH_3$ |
| AMM44 | 6 | -H | $-CH_3$ |
| AMM45 | 5 | -H | $-CH_3$ |
| AMM46 | 5 | $CH_3$ | -H |

The following phase of design was used to adjust the overall physicochemical properties through suitable substitutions on the benzene ring. Meanwhile the results of the QSI activity were also taken into consideration, e.g. the activity of the derivative that carried the methyl substitution at position 5 did not turn out to be positive. This mode of substitution did not continue and changed into position 6. Only one of these derivatives turned out to show activity. The others exhibited either antibacterial activity or no activity at all.

In order to optimize the interactions of the designed molecules with the site of activity the following studies were also executed: Liphophilic/hydrophilic balance, Stereochemical suitability, Charge distribution suitability,

# 3. MATERIALS AND METHODS

All chemicals and solvents were purchased locally from Merck AG and Aldrich Chemicals.

## 3.1 Chemistry

Microwave reaction was carried out in MicroSYNTH Microwave Labstation at 1600 W (2 magnetrons 800W x2) (Milestone S.r.l. Italy). ^{1}H NMR spectra were recorded in DMSO-$d_6$ on a Varian Mercury 400, 400 MHz High Performance Digital FT-NMR spectrometer using tetramethylsilane as the internal standard at the NMR facility of the Faculty of Pharmacy, Ankara University. All chemical shifts were recorded as σ (ppm). Microanalyses for C, H, and N were performed on a Leco-932 at the Faculty of Pharmacy, Ankara University, Ankara, Turkey, and they were within the range of ± 0.4% of the theoretical value.

The synthesis of 2(3H)-benzoxazolone[39,40], 5-methyl-2(3H)-benzoxazolone[41], 6-methyl-2(3H)-benzoxazolone[42] and 3-decanoyl-2(3H)-benzoxazolone[46] was previously reported.

## 3.1.1 General procedure for the synthesis of 2(3H)-benzoxazolone derivatives [40]

The mixture of 2-aminophenol (0.1 mol) (or 4-methyl-2-aminophenol, or 5-methyl-2-aminophenol) and urea (0.3 mol) was heated to 140 $^{\circ}$C and stirred for 10 min under microwave irradiation. The reaction mixture was poured onto crushed ice. The precipitated solid product was

collected by suction filtration, washed with water, dried and recrystallized from appropriate solvents.

## 3.1.2 General procedure for the synthesis of 3-acyl-2(3H)-benzoxazolone derivatives

The acyl chloride (11 mMol) was added dropwise over 15 min to a solution of 2(3H)-benzoxazolone derivatives (10 mMol) and dry TEA (11 mMol) in 10 mL of dry THF cooled at -4°C. The reaction mixture was heated to 100 $^{\circ}$C for 20 min under microwave irradiation, added to 200 mL of ice water and stirred for 1 h. The resulting precipitate was filtered, washed with water, dried, and recrystallized from appropriate solvents.

## 3.2 Antibacterial Activity

### 3.2.1 Microdilution Method

Standard strains of *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, *Klebsiella pneumoniae* RSHM 574 and clinical isolates of the microorganisms that are known to be resistant to various antimicrobial agents were included in the study. *E.coli* isolate and *K. pneumoniae* isolate have Extended Spectrum β-Lactamase (ESBL) enzyme and are resistant to beta lactam antibiotics and *P.aeruginosa* isolate is resistant to gentamycin.

Standard powders of ampicillin, gentamycin sulphate, ofloxacin, tetracycline, ceftriaxon, meropenem, amoxicillin/clavulonic acid and ampicillin/sulbactam were obtained from the manufacturers.

Stock solutions of the tested compounds were dissolved in DMSO. Standard antibiotic solutions were dissolved in appropriate solvents recommended by CLSI guidelines.

**Table 2 :** Standard antibiotic solutions were dissolved in appropriate solvents recommended by CLSI guidelines.

| Antibiotics | Solvent | Diluent |
|---|---|---|
| ampicillin | 0.1mol/L pH:8 PBS | 0.1mol/L pH:6 PBS |
| ampicillin/sulbactam | 0.1mol/L pH:8 PBS | 0.1mol/L pH:6 PBS |
| amoxicillin/clavulonic acid | 0.1mol/L pH:6 PBS | 0.1mol/L pH:6 PBS |
| gentamycin | Distiled water | Distiled water |
| tetracycline | Distiled water | Distiled water |
| ceftriaxon | Distiled water | Distiled water |
| ofloxacin | 0.1 mol/L NaOH with distiled water | Distiled water |
| meropenem | Distiled water | Distiled water |

All bacterial isolates were subcultured in Mueller Hinton Agar (MHA) plates and incubated overnight at 37 °C.

Stock solutions of the tested compounds and standard drugs were diluted two-fold in the wells of the microplates so the solution of the synthesized compounds were prepared at 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 μg/mL concentrations and standard drugs were prepared at 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03, 0.015, 0.008, 0.004, 0.002, 0.001 μg/mL concentrations.

Bacterial susceptibility testing was performed according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) M100-S16[1]. Mueller Hinton Broth (MHB) was added to each well of the microplates. The bacterial suspensions used for inoculation were prepared at $10^5$ CFU/mL by diluting fresh cultures at McFarland 0.5 density ($10^7$CFU/mL). Suspensions of the bacteria at $10^5$CFU/mL concentration were inoculated to the two fold-diluted solution of the compounds. There were $10^4$ CFU/mL bacteria in the wells after inoculations. A 10 µl bacteria inoculum was added to each well of the microplates. Microplates were incubated at 37 °C overnight. After incubation, the lowest concentration of the compounds that completely inhibits macroscopic growth was determined and reported as minimum inhibitory concentrations (MICs).

All solvents and diluents, pure microorganisms and pure media were used in control wells. All the experiments were done in 3 parallel series.[44]

## 3.3  Quorun Sensing Inhibitory Activity

### 3.3.1 Test for the inhibition of violacein production in *Chromobacterium violaceum*

The quorum sensing inhibitory activity of QS analogues was assayed by the agar well-diffusion test. Violacein production was determined on agar plates employing *C. violaceum* ATCC 12472, *C. violaceum* CV026 (a mini-Tn5 mutant) and *C. violaceum* VIR07 indicator organisms. In *C. violaceum* ATCC 12472, production of a purple pigment, violacein, is under the control of a QS system. This wild-type strain produces and responds to the cognate autoinducer molecules C6-AHL and C4-AHL. *C violaceum* CV026 is a mutant of the wild-type strain that is unable to synthesize its own C6-HSL, but it retains the ability to respond

C4-AHL and C6-AHL [12]. QSI compounds inhibit production of violacein in both cases, making these strains ideal for screening.

Five mL of molten LB agar (0.3% w/v) were inoculated with 50 of a culture of either the *C. Violaceum* ATCC 12472, *C. violaceum* CV026 or *C. violaceum* VIR07 grown overnight in LB. *C. violaceum* CV026 and *C. violaceum* VIR07 were supplemented with C6-AHL (Fluka) and C12-AHL (Fluka) respectively. The agar culture solution was immediately poured over the surface of prewarmed LB agar plates. Up to 75 of the extracts to be tested were pipetted into wells punched in the solidified agar with a sterile cork borer. The plates were incubated overnight at 30 °C and examined for violacein production. QS inhibition was detected by a colorless, opaque, but viable, halo around the wells. Organic solvents themselves were also used as controls.

### 3.3.2 Violacein extraction

Violacein extraction was carried out according to Choo et al. 2006.[45] Briefly, 1 ml culture from flasks containing test compounds was centrifuged at 13000 rpm for 10 minutes to precipitate the violacein. The supernatant was discarded and the pellet was solubilised in 1 ml of DMSO. The solution was vortexed to completely solubilize violacein and centrifuged at 13000 rpm to remove the cells. The extracted violacein was quantified using spectrophotometer (optical density at 585 nm wavelength [OD585]).

## 4. RESULTS

### 4.1 Chemical Results

**3-Decanoyl-1,3-benzoxazol-2(3$H$)-one (AMM33)**
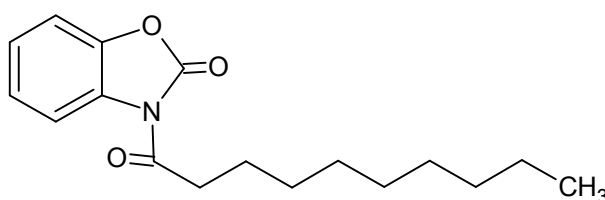
Recrystallized from ethanol to yield 68%; FT-IR (KBr), cm−1: 1788, 1726 (C=O),^{1}H NMR (DMSO-d6) δ 7.97–7.95 (1H, m, benzoxazolone H[7]), 7.42–7.40 (1H, m, benzoxazolone H[5]), 7.31–7.26 (2H, m, benzoxazolone H[4], H[6]), 3.01 (2H, t, –CH2–CO–), 1.64 (2H, m, -CH2CH2CO-), 1.34-1.26 (12H, m, CH2), 0.86 (3H, t, CH3). Anal. Calc. for $C_{17}H_{23}NO_3$: C, 70.56; H, 8.01; N, 4.84. Found: C, 70.20; H, 8.14; N, 4.91%.

**3-Octanoyl-1,3-benzoxazol-2(3$H$)-one (AMM34)**
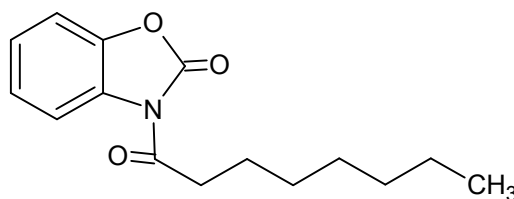
Recrystallized from ethanol to yield 66%; FT-IR (KBr), cm$^{-1}$: 1789, 1726 (C=O),^{1}H NMR (DMSO-d6) δ 7.97–7.95 (1H, m, benzoxazolone H[7]), 7.42–7.40 (1H, m, benzoxazolone H[5]), 7.31–7.26 (2H, m, benzoxazolone H[4], H[6]), 3.01 (2H, t, –CH$_2$–CO–), 1.64 (2H, m, -CH$_2$CH$_2$CO-), 1.36-1.27 (8H, m, CH$_2$), 0.87 (3H, t, CH$_3$). Anal. Calc. for $C_{15}H_{19}NO_3$: C, 68.94; H, 7.33; N, 5.36. Found: C, 68.42; H, 7.33; N, 5.42%.

## 3-Heptanoyl-1,3-benzoxazol-2(3*H*)-one (AMM40)
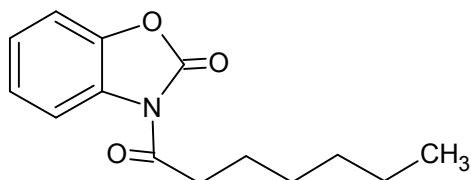


Recrystallized from ethanol to yield 67%; FT-IR (KBr), cm$^{-1}$: 1803, 1730 (C=O),$^{1}$H NMR (DMSO-d6) δ 7.94–7.92 (1H, m, benzoxazolone H$^{7}$), 7.39–7.37 (1H, m, benzoxazolone H$^{5}$), 7.29–7.23 (2H, m, benzoxazolone H$^{4}$, H$^{6}$), 2.99 (2H, t, –CH$_2$–CO–), 1.62 (2H, m, -CH$_2$CH$_2$CO-), 1.35-1.24 (6H, m, CH$_2$), 0.85 (3H, t, CH$_3$). $^{13}$C NMR (DMSO-d6) δ172.44 (C=O),150.78 (C=O),141.82 (aromatic H),127.85 (aromatic H),124.82 (aromatic H),124.36 (aromatic H),115.08 (aromatic H), 109.72 (aromatic H), 35.82 (CH$_2$), 30.98 (CH$_2$), 28.00 (CH$_2$), 23.09 (CH$_2$), 21.92 (CH$_2$),13.84 (CH$_3$) Anal. Calc. for  C$_{14}$H$_{17}$NO$_3$: C, 68.00; H, 6.93; N, 5.66. Found: C, 67.89; H, 6.97; N, 5.74%.

## 5-Chloro-3-decanoyl-1,3-benzoxazol-2(3*H*)-one (AMM37)
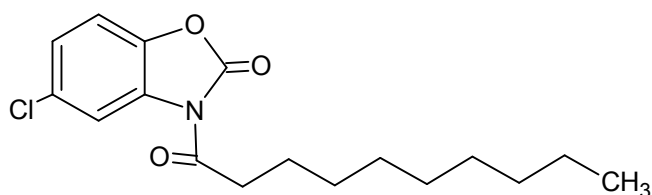


Recrystallized from ethanol to yield 85%; FT-IR (KBr), cm$^{-1}$: 1796, 1724 (C=O),$^{1}$H NMR (DMSO-d6) δ 7.93 (1H, d, 5-chlorobenzoxazolone H$^{4}$ J=2.4 Hz), 7.46 (1H, d, 5-chlorobenzoxazolone H$^{7}$ J=8.8 Hz), 7.36 (1H, dd, 5-chlorobenzoxazolone H$^{6}$ J=8.6 Hz,2 Hz), 2.99 (2H, t, –CH$_2$–CO–), 1.63 (2H, m, -CH$_2$CH2CO-), 1.34-1.25 (12H, m, CH$_2$), 0.85 (3H, t, CH$_3$). Anal. Calc. for  C$_{17}$H$_{22}$ClNO$_3$: C, 63.06; H, 6.85; N, 4.33. Found: C, 62.93; H, 7.21; N, 4.43%.

**5-Chloro-3-octanoyl-1,3-benzoxazol-2(3*H*)-one (AMM38)**
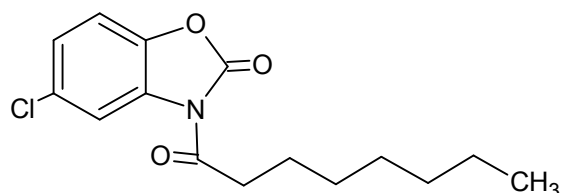


Recrystallized from ethanol to yield 66%; FT-IR (KBr), cm$^{-1}$: 1793, 1724 (C=O),$^{1}$H NMR (DMSO-d6) $\delta$ 7.92 (1H, d, 5-chlorobenzoxazolone H$^4$ J=2.4 Hz), 7.45 (1H, d, 5-chlorobenzoxazolone H$^7$ J=8.4 Hz), 7.35 (1H, dd, 5-chlorobenzoxazolone H$^6$ J=8.4 Hz ,2.4 Hz), 2.99 (2H, t, –C̲H̲$_2$–CO–), 1.63 (2H, m, -C̲H̲$_2$CH$_2$CO-), 1.36-1.27 (8H, m, CH$_2$), 0.87 (3H, t, CH$_3$). Anal. Calc. for C$_{15}$H$_{18}$ClNO$_3$: C, 60.91; H, 6.13; N, 4.74. Found: C, 60.50; H, 6.46; N, 4.84%.

**5-Chloro-3-heptanoyl-1,3-benzoxazol-2(3*H*)-one (AMM39)**
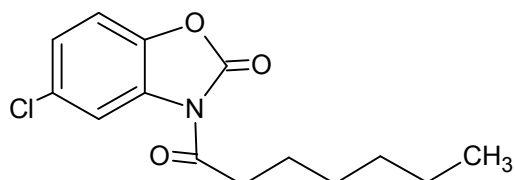


Recrystallized from ethanol to yield 71%; FT-IR (KBr), cm$^{-1}$: 1793, 1723 (C=O),$^{1}$H NMR (DMSO-d6) $\delta$ 7.91 (1H, d, 5-chlorbenzoxazolone H$^4$ J=2.4 Hz), 7.45 (1H, d, 5-chlorobenzoxazolone H$^7$ J=8.4 Hz), 7.35 (1H, dd, 5-chlorobenzoxazolone H$^6$ J=8.4 Hz, 2.4 Hz), 2.99 (2H, t, –C̲H̲$_2$–CO–), 1.63 (2H, m, -C̲H̲$_2$CH$_2$CO-), 1.39-1.27 (6H, m, CH$_2$), 0.88 (3H, t, CH$_3$). Anal. Calc. for C$_{14}$H$_{16}$ClNO$_3$: C, 59.68; H, 5.72; N, 4.97. Found: C, 59.31; H, 5.72; N, 5.11%.

### 3-Decanoyl-6-methyl-1,3-benzoxazol-2(3*H*)-one (AMM43)



Recrystallized from ethanol to yield 41%; FT-IR (KBr), cm$^{-1}$: 1792, 1719 (C=O),$^1$H NMR (DMSO-d6) δ 7.82 (1H, d, 6-methylbenzoxazolone H$^4$), 7.24 (1H, s, 6-methylbenzoxazolone H$^7$), 7.08 (1H, d, 6-methylbenzoxazolone H$^5$), 2.99 (2H, t, –CH$_2$–CO–), 2.35 (3H, s, CH$_3$ ), 1.63 (2H, m, -CH$_2$CH$_2$CO-), 1.33-1.25 (12H, m, CH$_2$), 0.85 (3H, t, CH3). Anal. Calc. for C$_{18}$H$_{25}$NO$_3$: C, 71.26; H, 8.31; N, 4.62. Found: C, 71.05; H, 8.41; N, 4.69%.

### 6-Methyl-3-octanoyl-1,3-benzoxazol-2(3*H*)-one (AMM44)
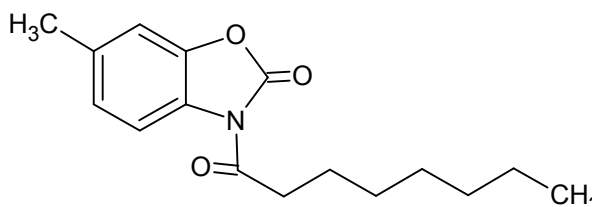


Recrystallized from ethanol to yield 38%; FT-IR (KBr), cm$^{-1}$: 1786, 1718 (C=O),$^1$H NMR (DMSO-d6) δ 7.82 (1H, d, 6-methylbenzoxazolone H$^4$), 7.24 (1H, s, 6-methylbenzoxazolone H$^7$), 7.08 (1H, d, 6-methylbenzoxazolone H$^5$), 3.00 (2H, t, –CH$_2$–CO–), 2.35 (3H,s, CH$_3$ ), 1.64 (2H, m, -CH$_2$CH$_2$CO-), 1.33-1.27 (8H, m, CH$_2$), 0.87 (3H, t, CH$_3$). Anal. Calc. for C$_{16}$H$_{21}$NO$_3$: C, 69.79; H, 7.69; N, 5.09. Found: C, 69.34; H, 7.92; N, 5.14%.

### 3-Heptanoyl-6-methyl-1,3-benzoxazol-2(3*H*)-one (AMM45)
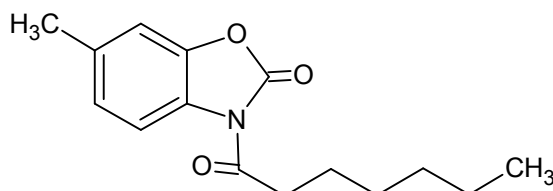


Recrystallized from ethanol to yield 35%; FT-IR (KBr), cm−1: 1785, 1718 (C=O),^{1}H NMR (DMSO-d6) δ 7.82 (1H, d, 6-methylbenzoxazolone H[4]), 7.24 (1H, s, 6-methylbenzoxazolone H[7]), 7.08 (1H, d, 6-methylbenzoxazolone H[5]), 3.00 (2H, t, –$\underline{CH_2}$–CO–), 2.35 (3H,s, CH$_3$), 1.63 (2H, m, -$\underline{CH_2}$CH$_2$CO-), 1.35-1.27 (6H, m, CH$_2$), 0.87 (3H, t, CH3). Anal. Calc. for C$_{15}$H$_{19}$NO$_3$: C, 68.94; H, 7.33; N, 5.36. Found: C, 68.63; H, 7.28; N, 5.40%.
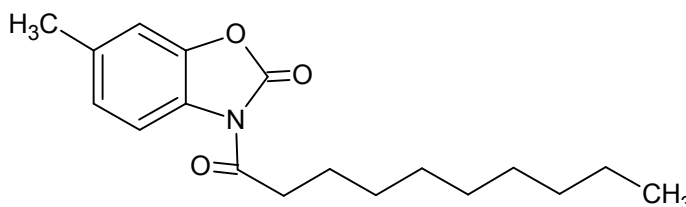
### 3-Heptanoyl-5-methyl-1,3-benzoxazol-2(3*H*)-one (AMM46)
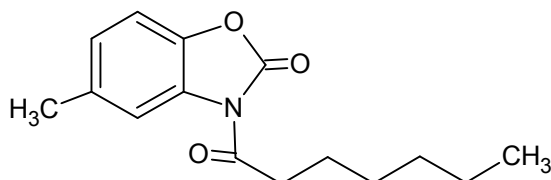


Recrystallized from ethanol to yield 53%; FT-IR (KBr), cm−1: 1793, 1723 (C=O),^{1}H NMR (DMSO-d6) δ 7.78 (1H, s, 5-methylbenzoxazolone H[4]), 7.25 (1H, d, 5-methylbenzoxazolone H[7]), 7.08 (1H, d, 5-methylbenzoxazolone H[6]), 2.98 (2H, t, –$\underline{CH_2}$–CO–), 2.34 (3H,s, CH$_3$), 1.62 (2H, m, -$\underline{CH_2}$CH$_2$CO-), 1.36-1.25 (6H, m, CH$_2$), 0.86 (3H, t, CH3). Anal. Calc. for C$_{15}$H$_{19}$NO$_3$: C, 68.94; H, 7.33; N, 5.36. Found: C, 68.64; H, 7.28; N, 5.43%.

## 4.2 Biological Activities Results

### 4.2.1 Antibacterial activity results

A list of antibacterial activities of our candidate in comparison with classic test antibiotics activities. (Table 3)

**Table 3 :** List of antibacterial activities of our candidate in comparison with classic test antibiotics

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| AMM33 | 64 | 64 | 64 | 64 | 32 | 32 | 64 |
| AMM34 | 128 | 128 | 128 | 64 | 64 | 64 | 128 |
| AMM37 | 128 | 128 | 128 | 64 | 64 | 64 | 128 |
| AMM38 | 128 | 128 | 128 | 64 | 64 | 64 | 128 |
| AMM39 | 128 | 128 | 128 | 64 | 64 | 64 | 128 |
| AMM40 | 128 | 128 | 128 | 64 | 64 | 64 | 128 |
| AMM43 | 128 | 128 | 128 | 64 | 64 | 64 | 128 |
| AMM44 | 128 | 128 | 128 | 64 | 64 | 64 | 128 |
| AMM45 | 128 | 128 | 128 | 64 | 64 | <4 | 128 |
| AMM46 | 128 | 128 | 128 | 64 | 64 | 128 | 128 |
| Ampicilin | 2 | - | >32 | - | - | 0.5 | >32 |
| Gentamicin | 0.25 | - | >16 | 1 | >8 | 0.5 | 0.25 |
| Ofloxacin | 0.015 | - | >8 | 1 | 2 | 0.25 | 0.5 |
| Tetracycline | 0.5 | - | >8 | 8 | 8 | 0.25 | 4 |
| Ceftriaxone | 0.12 | - | >32 | 64 | 64 | 2 | <0.25* |
| Meropenem | 0.008 | - | <0.25 | 1 | 0.015 | 0.03 | <0.25 |
| Ampicillin sulbactam | - | 16 | - | - | - | - | - |
| Amoxicillin Clavulonic asid | - | 16 | - | - | - | - | - |

**A:** *E. Coli* ATCC 25922,

**B:** *E. Coli* ATCC 35218th

**C:** *E. Coli* isolares,

**D:** *P. aeruginosa* ATCC 27853,

**E:** *P.aeruginosa* isolates,

**F:** *Klebsiella pneumoniae* RSHC 574,

**G:** *K. pneumoniae* isolates

*: ESBL-producing *Klebsiella spp. E. coli* strains in vitro as cephalosporins and sensitive to be shown, but can be clinically resistant to treatment with these drugs.

Candidate codes AMM33, AMM34 and AMM45 due to their strong antibacterial activites eliminated from QSI standard tests. Molecules which have antibiotical activities may cause extensive damage in both membrance and chemistry of QS test bacteria which are selective just for QSI molecules.

## 4.2.2 QSI Activity Results

### 4.2.2.1 Inhibition of violacein production.

The inhibition of QS by synthetic QS analogues was followed by employing the indicator strains *Chromobacterium violaceum* ATCC12472, CV026 and CV VIR07. 10 compounds were tested for their QS inhibitory activity. The culture of *C. violaceum* ATCC12472 was grown overnight and spread onto LB plates. Each compound (75 µl) was loaded into the well as described in Materials and Methods. Lack of purple pigmentation from of *C. violaceum* in the vicinity of the wells indicated the inhibitory effect of the test compounds. The same assays were also performed for CV026 and CV VIR07 by addition of C4-AHL and C12-AHL respectively. But due to strong the antibacterial effect of the test compounds against CV026 and CV VIR07, this assay did not give accurate results. Therefore, we decided to determine first the MIC levels of the compounds then extract the violacein pigment and measure the amount of violacein at 585nm in a UV-spectrophotometer.

The violacein extraction was carried out to quantify the inhibitory activity of test compounds as described in Materials and Methods. The results of these assays were given Table 4.

Results of the 1000 ppm test chemical addition are given in the table below.

**Table 4 :**  Results of compounds caused inhibition

|  | CV 12472 | % Inhibition | CV VIRO7 | % inhibiton | CV 026 | % Inhibition |
|---|---|---|---|---|---|---|
| AMM 37 | 0.910 | 70 | 0.703 | 75 | 0.945 | 39 |
| AMM 38 | 0.395 | 87 | 0.331 | 88 | 1.105 | 29 |
| AMM 39 | 0.670 | 78 | 0.433 | 85 | 0.795 | 49 |
| AMM 40 | 0.485 | 84 | 0.493 | 83 | 0.920 | 41 |
| AMM 43 | - | - | - | - | 0.301 | 81 |
| AMM 44 | 0.915 | 70 | 0.724 | 75 | 0.915 | 41 |
| AMM 46 | - | - | - | - | 0.190 | 88 |
| CONTROL * | 3.065 | | 2.865 | | 1.554 | |

The results shown in the table show that  7 of the compounds caused inhibition.  Two of them (AMM43 and AMM46) caused inhibition only when the CV026 and CV VIR07 de CV12472 did not show inhibition. Indicator strains produced by 1000ppm  added  increased  the violin amount (585 nm)The bottom line of chemicals used has not been added and control wild-type produced by CV12472 in red violin amounts are given.

### 4.3 Molecular Modeling Study Results

On criteria of optimized application of two sample molecules AMM38 and AMM40 in both AUTODUCK 4.1 and VINA, we observed very impressive results. We run computer based drug design software using x-ray of the *p. aeruginosa* LasR ligan binding domin bound to its autoinducer 3-oxo-C(12)-acylhomoserine lactone, PDP ID No 2UV0. [46] Surface presentation of both molecules in very competitive position to ligand, AHL, are shown in (Fig. 14).
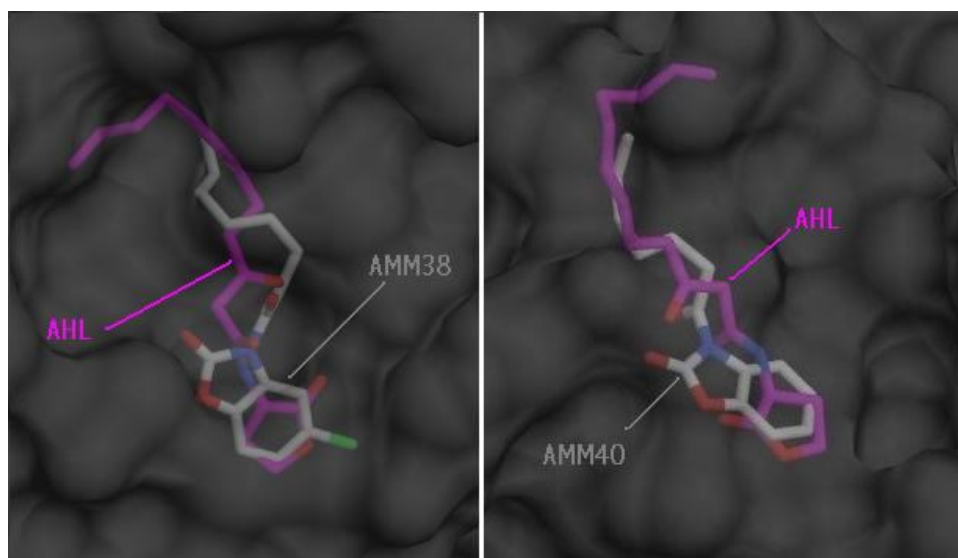
**Figure 14:** Molecular modeling of AMM38 and AMM40 competition with the autoinducer in binding site of quorum sensing protein LasR.

# 5. DISCUSSION

Quorum sensing (QS) is a type of decision-making process used by decentralized groups to coordinate behavior. Many species of bacteria use QS to coordinate their gene expression according to the local density of their population. Similarly, some social insects use QS to make collective decisions about where to nest.

Some of the best-known examples of QS come from studies of bacteria. Bacteria use QS to coordinate certain behaviors based on the local density of the bacterial population. Quorum sensing can occur within a single bacterial species as well as between diverse species and can regulate a host of different processes, essentially serving as a simple communication network. A variety of different molecules can be used as signals. Common classes of signaling molecules are oligopeptides in Gram-positive bacteria, *N*-Acyl Homoserine Lactones (AHL) in Gram-

negative bacteria and a family of autoinducers known as AI-2 in both Gram-negative and Gram-positive bacteria.

The inhibition of QS offers new hope in combatting multi-antibiotic resistant bacteria. Employing the inhibition of bacterial QS systems instead of using bactericidal or bacteriostatic strategies may find application in many different fields such as medicine, agriculture and food technology. In a world where agricultural residues constitute a big problem this approach can pave the way to a very soft antibacterial remedy. Also this approach is highly attractive in terms of medicine because it does not impose harsh selective pressure for the development and emergence of resistance as with antibiotics since QS is not directly involved in processes essential for growth of the bacteria. Furthermore, QSI compounds are not expected to eliminate beneficial bacteria co-existing in the host.

This study aimed to design and synthesize molecules that would be competitive inhibitors for the natural QS process of some certain bacteria.

Among the QS mediators, the best targets may be the ones for which the formula has been given below.



3-oxo-C12-HSL                    3-oxo-C6-HSL
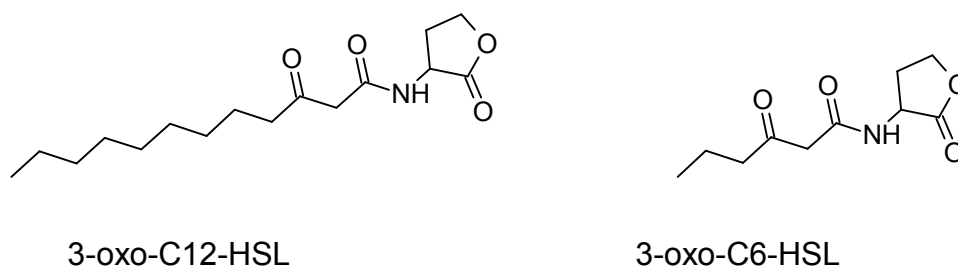
**Figure 15:** *AHL types in P. aeruginosa*

The compounds that have been synthesized by microwave assisted methods in this study, have the main chemical structure as follows.(Table 5)

**Table 5:** Main chemical structure and list of substitutions
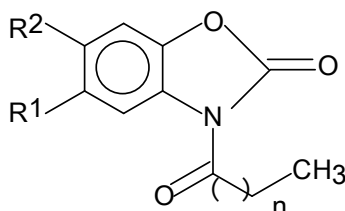


| Code Name | n | R$_1$ | R$_2$ |
|---|---|---|---|
| AMM33 | 8 | -H | -H |
| AMM34 | 6 | -H | -H |
| AMM40 | 5 | -H | -H |
| AMM37 | 8 | -Cl | -H |
| AMM38 | 6 | -Cl | -H |
| AMM39 | 5 | -Cl | -H |
| AMM43 | 8 | -H | -CH$_3$ |
| AMM44 | 6 | -H | -CH$_3$ |
| AMM45 | 5 | -H | -CH$_3$ |
| AMM46 | 5 | -CH$_3$ | -H |

The synthetic routes for the synthesized compounds are outlined in (Fig.16). The starting compound, 1,3-benzoxazol-2(3H)-one was readily prepared by the reaction of urea and o-aminophenol under MWI and the procedures were in accordance with a previously published method. [40] MWI had a dramatically reduced reaction time for the synthesis of 1,3-benzoxazol-2(3H)-one, from 24 h to 10 min. Similarly, other starting rings, 5-methyl-1,3-benzoxazol-2(3H)-one and 6-methyl-1,3-benzoxazol-2(3H)-one, were prepared by the reaction of urea and 4-methyl-2-aminophenol / 5-methyl-2-aminophenol under MWI. The acylation of core rings with acyl chloride derivatives was carried out in tetrahydrofurane (THF) by using microwave assisted method. The reaction time for the synthesis of the 3-acyl-1,3-benzoxazol-2(3H)-one derivatives by the

conventional method was up to 4h longer in comparison to the microwave conditions (20 min).

The structures of the compounds were elucidated by IR, ^{1}H-NMR, and microanalyses. Crystallization solvents, melting points, % yields, and the spectral data of the compounds are given in the Results Part. (Fig. 16)
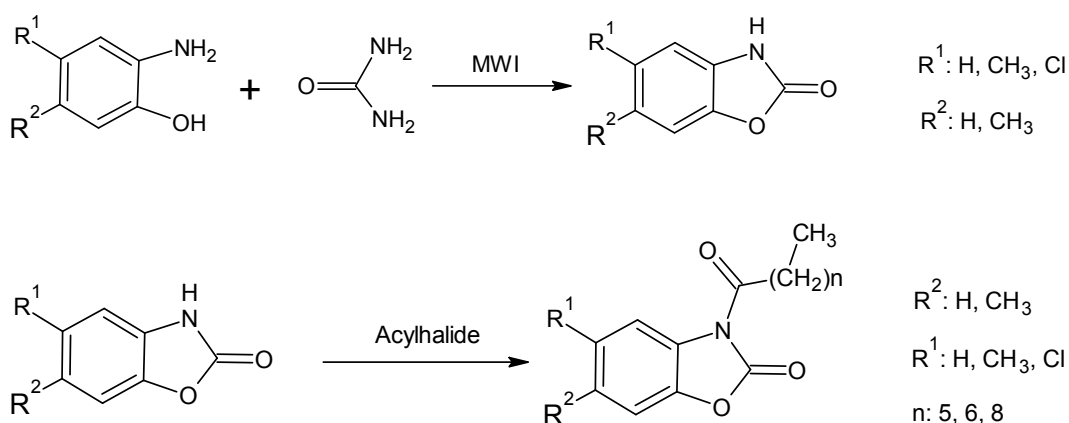


**Figure 16:** Synthetic route of the title compounds.

The results shown in the table indicate that 7 of the compounds caused quorum sensing inhibition on 3 standard QS test bacteria.

Candidate codes AMM33, AMM34 and AMM45 due to their strong antibacterial activities were eliminated from QSI standard tests. Molecules which have antibiotic activities may cause extensive damage in both the membrane and chemistry of the QS test bacteria which were selected just for their QSI molecules.

**Table 6:** QSI results

| | CV 12472 | % Inhibition | CV VIRO7 | % inhibiton | CV 026 | % Inhibition |
|---|---|---|---|---|---|---|
| AMM 37 | 0.910 | 70 | 0.703 | 75 | 0.945 | 39 |
| AMM 38 | 0.395 | 87 | 0.331 | 88 | 1.105 | 29 |
| AMM 39 | 0.670 | 78 | 0.433 | 85 | 0.795 | 49 |
| AMM 40 | 0.485 | 84 | 0.493 | 83 | 0.920 | 41 |
| AMM 43 | - | - | - | - | 0.301 | 81 |
| AMM 44 | 0.915 | 70 | 0.724 | 75 | 0.915 | 41 |
| AMM 46 | - | - | - | - | 0.190 | 88 |
| **CONTROL** | **3.065** | | **2.865** | | **1.554** | |

We indicated that our agonist-based QSI design block QS by enering the AHL-binding pocket. Conversely, the mutant LuxR was still senstive to inhibition by benzoxazol ring, indicating that they bind at a different location on the Lux-box protein. [47] As it is shown in (Fig.11) antagonistic analogues of cognate AHLs compete for binding to QSP but do not result in the activation of the protein.This pair with *lux-Box inhibits* gene translation and thus protein production. This step is important from the standpoint of the inhibition of virulence factor production caused by biofilm formation in pathogenic microorganisms such as *P. aeruginosa.*

## 6. CONCLUSION

The results showed that 7 of the 10 compounds which were successfully synthesized in this study, caused quorum sensing inhibition on 3 standard QS test bacteria. QSI activity for CV 12472 test bacteria was AMM38 %87, AMM40 %84, AMM39 %78, AMM37 %70, AMM44 %70, consequently in 1mg/ml concentration.

QSI activity for CV VIR07 test bacteria was AMM38 %88, AMM39 %85, AMM40%83, AMM44 %75, AMM37 %75, consequently in 1mg/ml concentration.

QSI activity for CV 026 test bacteria was AMM46 %88 and AMM43 %81, consequently in 1mg/mL concentration.

Our biological research revealed that the amount of violin pigment extracted was again determined by the more sensitive HPLC method.

This is an important human pathogen which is made  of chemical substances by *P. aeruginosa* produced under the control of QS piyosiyanin mechanisms, such as elastase and ramnolipit to determine the effects virulence factors.

The tests are still being conducted to follow up on the above results thus expanding our research further. The design and evalution of new molecules based on these primary data will lead us to more novel active and selective molecules.

# 7. SUMMARY

## DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY STUDIES ON SOME NOVEL ACYL HOMOSERINE LACTONE ANALOGUES

The inhibition of QS (Quorum Sensing) offers new hope in combatting multi-antibiotic resistant bacteria. Inhibiting bacterial QS systems instead of employing bactericidal or bacteriostatic strategies may find application in many different fields, such as medicine, agriculture and food technology. In a world where agricultural residues constitute a big problem, this approach can pave the way to a very soft antibacterial remedy. Also, this approach is highly attractive in terms of medicine because it does not impose harsh selective pressure on the development and emergence of resistance, from antibiotics, since QS is not directly involved in the processes essential for the growth of bacteria. Furthermore, QSI compounds are not expected to eliminate beneficial bacteria co-existing in the host. [48]

This study aimed to design and synthesize molecules that would be competitive inhibitors for the natural QS process of some certain bacteria.

3-Acyl-2(3H)-benzoxazolone derivatives that have been synthesized by microwave assisted methods in this study, have major chemical structures such as AHL inhibitors. The structures of the compounds have been elucidated by IR, $^1$H-NMR, and microanalyses.

The results showed that 7 of the 10 compounds which were successfully synthesized in this study, caused quorum sensing inhibition on 3 standard QS test bacteria. QSI activity for CV 12472 test bacteria was AMM38 %87, AMM40 %84, AMM39 %78, AMM37 %70, AMM44 %70, consequently in 1mg/ml concentration. QSI activity for CV VIR07 test bacteria was AMM38 %88, AMM39 %85, AMM40%83, AMM44 %75,

AMM37 %75, consequently in 1mg/ml concentration. QSI activity for CV 026 test bacteria was AMM46 %88 and AMM43 %81, consequently in 1mg/ml concentration. Candidate codes AMM33, AMM34 and AMM45 due to their strong antibacterial activities were eliminated from QSI standard tests. Molecules which have antibiotic activities may cause wide damages in both membrane and chemistry of QS test bacteria which are selective just for QSI molecules.

Promising results of molecular Modeling for AMM38 and AMM40 in competition with autoinducer in binding site were impressive.
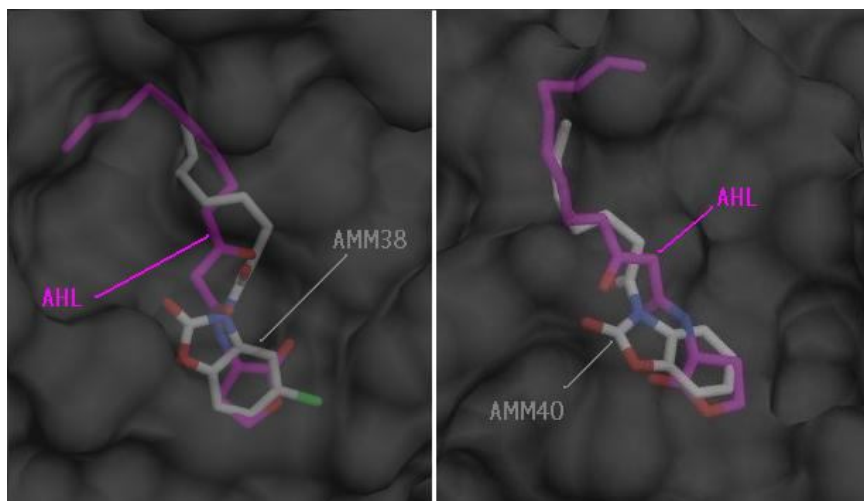


**Figure 14:** Molecular modeling of AMM38 and AMM40 competition with the autoinducer in binding site of quorum sensing protein LasR.

The tests are still being conducted to follow up on the above results and will lead us to do more extensive research. The design and evaluation of the new molecules based on these primary data will lead us to more novel, active and selective molecules.

**Key words:** Acyl Homoserine Lactone, Quorum Sensing, 2(3H)-Benzoxazolone

## 8. ÖZET

## BAZI YENI AÇILHOMOSERINLAKTON ANALOGLARININ TASARIMI, SENTEZI VE BIYOLOJIK AKTIVITELERI ÜZERINDE ÇALIŞMALAR

Bakterilerin izole varlıklar olarak yaşamadıkları, değişen ortam koşullarına uyumlarını kolaylaştırmak için karmaşık hücrelerarası haberleşme sistemleri kullanan topluluklar halinde bulundukları giderek artan bir yaygınlıkla kabul edilmektedir. Bu tip hücreler arası haberleşmenin iyi karakterize edilmiş bir örneği, çevreyi algılamadır. Çevreyi algılama, bir bakteriye kendi hücre populasyon yoğunluğunu izlemesine olanak veren autoinducer veya feromon olarak adlandırılan sinyal moleküllerinin üretimine bağımlıdır. Değişik bakteri türlerinde, çeşitli fizyolojik işlemler, çevreyi algılama ile regüle edilir. Bu işlemlere örnek olarak, biyolüminesens, antibiyotik biyosentezi, biyofilm oluşumu, konjugasyon ve hayvan, bitki ve balık patojenleri tarafından oluşturulan virülens etkenlerinin üretimi verilebilir. Gram-negatif bakterilerde en yaygın olarak bulunan sinyal molekülleri N-acyl homoserine lakton türevleridir (acyl HSLs).

Yeter Çoğul Algılama (YÇA)(Quorum Sensing) toplanmamış gruplar tarafından karar mekanizmaları için kullanılan bir süreçtir. Bir çok bakteri türü YÇA'yı nüfusun yoğunluğuna göre kendi gen ifadeleri için kullanırlar. Benzer şekilde bazı sosyal böcekler yuvalarını nerede yapsınlar diye YÇA'yı kullanırlar.

YÇA çalışlarında en iyi örnekler bakteriler üzerinde yapılan çalışmalardır. Bakteriler, YÇA'yı kullanarak bakteriyel nüfusun yerel yoğunluğuna bağlı bazı davranışları düzenler. YÇA basit bir iletişim mekanizması olarak hizmet vermekte, bir tür içinde iletişimi sağlamakta, türler arası iletişimi sağlamakta veya farklı süreçleri düzenlemede

kullanılmaktadır. Çeşitli moleküller ileti için kullanılabilir. Genellikle gram pozitif bakterileride oligopeptitler kullanılırken, gram negatiflerde N-Açil Homoserin Lakton (AHL) ve Kendini Uyarıcı (KU) ( autoinducer) adlı AI-2 ler her iki tür bakteri kullanmaktadır.

YÇA'yı durdurmak antibiotiklere dirençli bakteriler ile mücadele için umut vadetmektedir. Bakteri üremesi veya ölümünden daha ziyade YÇA durdurmak, ilaç, tarım ve gıda teknolojisi gibi yeni alanlar vadetmektedir. Tarımsal kimyasal atıkların ciddi bir sorun olduğu bir dünyada, bu yöntem daha yumuşak antibakteriyal eylemlerin önünü açabilir. Ayrıca YÇA ilaç araştırmalarında çok önemli bir alandır zira çeştili yöntemlerle bakterilerin direncini artıran meknizmalardan uzak, bakterinin temel işlevlerini doğrudan engellememektedir. En önemlisi, YÇA durduran bileşikler bakterilerin evsahibine verecekleri yararlı katkıları da engellememektedir.

Bu çalışma bazı özgün bakteriside kullanılan YÇA sürecini durdurmak için çeşitli kompetetif antagonist moleküllerin tasarımı ve sentezini hedeflemiştir.

En iyi hedef olan YÇA arabulucuların formülleri aşağıda verilmiştir.
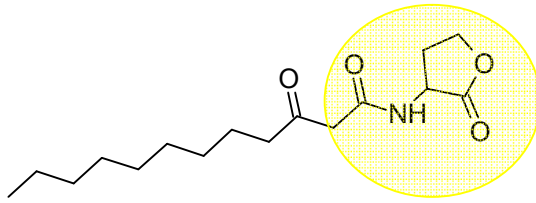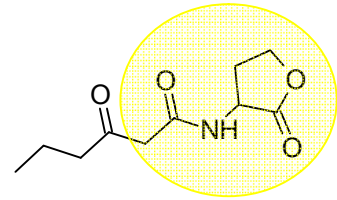


3-okso-C12-HSL                3-okso-C6-HSL

Bu çalışmada mikro dalga destekli sentez yöntemleri ile sentezlenen bileşiklerin ana kimyasal yapısı aşağıda verilmiştir:
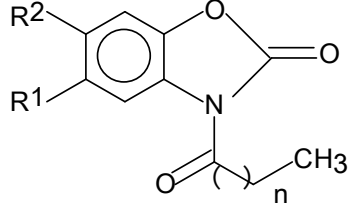


| Code Name | n | $R_1$ | $R_2$ |
|-----------|---|-------|-------|
| AMM33 | 8 | -H | -H |
| AMM34 | 6 | -H | -H |
| AMM40 | 5 | -H | -H |
| AMM37 | 8 | -Cl | -H |
| AMM38 | 6 | -Cl | -H |
| AMM39 | 5 | -Cl | -H |
| AMM43 | 8 | -H | $-CH_3$ |
| AMM44 | 6 | -H | $-CH_3$ |
| AMM45 | 5 | -H | $-CH_3$ |
| AMM46 | 5 | $-CH_3$ | -H |

Tablodaki 10 madde için gösterilen sonuçlarda, 7 tanesi 3 farklı standart YÇA test bakterisi üzerinde YÇA inhibitör etki göstermiştir.

AMM33, AMM 34 ve AMM 45 güçlü bakterisit etkilerinden dolayı, standart YÇA testlerinden çıkartılmıştır. Bu tür güçlü antibakteriyal etkisi olan bileşikler, YÇA'lamaya duryarlı olan test bakterilerin çeperi ve kimyasında yaygın hasarlara yol açabilir.

**Tablo 6:** YÇA durdurucularının sonuçları

| | CV 12472 | % Inhibisyon | CV VIRO7 | % Inhibisyon | CV 026 | % Inhibisyon |
|---|---|---|---|---|---|---|
| AMM 37 | 0.910 | 70 | 0.703 | 75 | 0.945 | 39 |
| AMM 38 | 0.395 | 87 | 0.331 | 88 | 1.105 | 29 |
| AMM 39 | 0.670 | 78 | 0.433 | 85 | 0.795 | 49 |
| AMM 40 | 0.485 | 84 | 0.493 | 83 | 0.920 | 41 |
| AMM 43 | - | - | - | - | 0.301 | 81 |
| AMM 44 | 0.915 | 70 | 0.724 | 75 | 0.915 | 41 |
| AMM 46 | - | - | - | - | 0.190 | 88 |
| **KONTROL GRUBU** | **3.065** | | **2.865** | | **1.554** | |

Tasrladığımız möleküllerin YÇA proteinine bağlanarak yapısal değişiklik yapıp, sonra Lux-Kutusunu bağlanma noktasında işlevi durduracak steriyokimyasal düzensizliğe yol açtığını öngürlmektedir. Resimde görüldüğü gibi YÇA durducuları AHL ile yarışarak YÇA proteininie bağlandığı halde ödevin oluşmasını engellemektedirler. Lux-Kutusu ile oluşan bu yapı gen çevirisi ve protein sentezini durdurmaktadır. Bu *P. aeruginosa* gibi hastalık yapan mikroorganizmalarda biofilm oluşumu sonucu ortaya çıkan hasta edici faktörleri durdurmak açısında önemlidir.
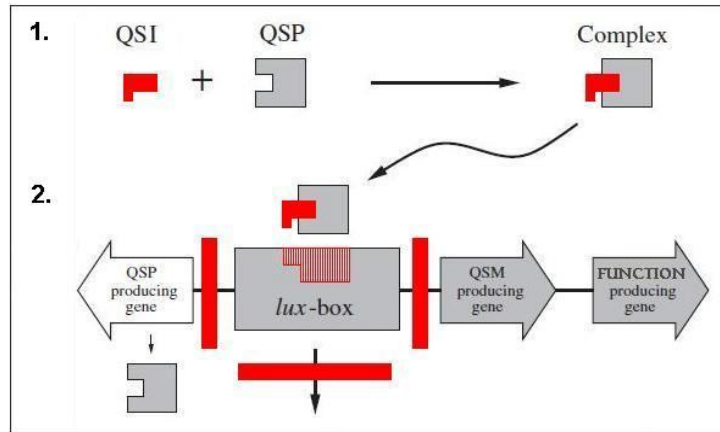


**Figure 11:** Bakteri Hücresinde YÇA durducuların şematik çizimi.
**(1)** YÇA durducunun YÇA proteinine bağlanması ( LuxR) ve anormal yapının oluşumu **(2)** bu ikililin *lux*-box bağlanarak işlev durdurulmasına yol açması.

AMM 38 ve AMM40 in bilgisayar modellemesi ile elde edilen ve AHL ile bağlanma yöresinde yarışmayı gösteren etkileyici sonuçlar, gelecek vaadetmektedir.
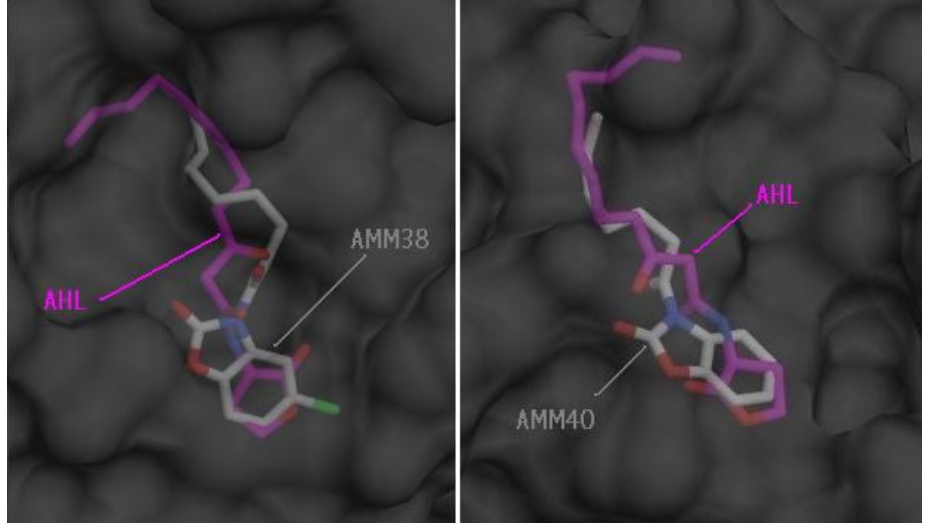


**Figure 14:** YÇA proteini LasR'e bağlanmak için KU ile yarışan AMM38 ve AMM40'ın bilgisayar modellemeleri.


**Anahtar Kelimeler:** Açilhomoserinlakton, Yeterçoğul algılama, 2(3H)-Benzoksazolon

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**CURRICULUM VITAE**

**Anooshirvan M. Miandji**
Tehran. Iran 7th January 1971

**EDUCATION**

2005       BSc. Pharmacy Gazi University

**LANGUAGES**

- **Persian** Native
- **Azeri** Native
- **Turkish** Fluent *(academic)*
- **English** Advanced *(CBT 217, 2006)*
- **Arabic** Advanced reading

**PUBLICATIONS**

2010       **A Standard Dicitonary of Pharmacy -**A first illustartated guide to the most common terminology in the field of Pharmacy, classifications and molecular structures of chemicals, abbreviations, generic lists in the world market,8000 entries, European-Books, London, UK

2010       **Dicitonary of Pharmacy ( Turkish) -** First online guide to the most common terminology in Pharmacy field, classifications and molecular structures of chemicals, abbreviations, generic lists in Turkish market,5000 entries, European Business Matching Center, London, UK, Turkey Branch

2010       **Magistral Formulary (Turkish),** a unique and up-to-date resource for extemporaneous preparations which is essential for all pharmacists to know in daily pharmacy services. More than 800 formulas with directions and all essentials on compunding methods, 650 p., EGAŞ, Ankara, Turkey

2010       **Farsi-English/ English-Farsi Standart Dictionary** - An Up-to-date dictionary, 1200 p., 2007, [Eng.- Pers./Pers.- Eng.], 40000 entries, Hippocrene Books, NY, USA

| 2010 | **Atlas of Medicinal Plants-** A practical pictorial reference for 170 common diseases and pharmaceutical dosed herbal remedies for them, Bilgi Yayınevi, Ankara, Turkey |
| --- | --- |
| 2003 | **Farsi-English/ English-Farsi Concise Dictionary-** An Up-to-date dictionary, 450 p., [Eng.- Pers./Pers.- Eng.], ISBN 078180860X, Hippocrene Books, NY, USA |
| 1997 | **Beginner's Persian-** A Beginners guide to the Persian language, 315 p., [Pers.- Eng.], ISBN 0781805678, Hippocrene Books, NY, USA |
| 1996 | **Digital Conversation Dictionary** A Common Phrases Dictionary, 168 p., Eng.-Tur.], Palme Publications, Ankara, Turkey |
| 1994 | **Modern Farsi** - An Intensive guide to the Farsi Phonological System - Tourist Guide of Iran, 304 p., 1995, [Pers.-Eng.], Mehran Publications, Tabriz, Iran |

## PAPERS, LECTURES AND PRESENTATIONS

| July 2008 | Reportage "**Inventor Pharmacist and A Medication Reminder System**", Turkish Pharmacist's Association Bulletin |
| --- | --- |
| Oct 2006 | **Seminar,** "**Bacterial Esperanto**" Quorum Sensing and New Antibiotics, Department of Medicinal Chemistry, Faculty of Pharmacy, Gazi University |
| July 2001 | Report of **3rd EPSA Summer University**, Turkish Pharmacist's Association Bulletin |
| Aug 2000 | Report of **23rd EPSA Congress**, Turkish Pharmacist's Association Bulletin |
| April 2000 | **Poster Presentation, "Biotechnology in Pharmacy" - 23rd EPSA Congress**, Uppsala, Sweden, Topic Biotechnology, Official delegate |

**INVENTION**

| | |
|---|---|
| 2007 | **1st Place Winner** of 6. Region of Channel D TV competition "Our Inventors" |
| 1999 | **INVENTION,** inventor of an electronic medication reminder system "Pharminder", patent pending since 2007, www.pharminder.com |

**GRANTS**

| | |
|---|---|
| 2001 | TEB (Turkish Pharmacist's Association) Travel Grant-3rd EPSA Summer University, Istanbul, Turkey |
| 2000 | TEB (Turkish Pharmacist's Association) Travel Grant- 23rd EPSA Congress, Uppsala, Sweden |
| 2000 | Gazi University Travel Grant- 23rd EPSA Congress, Uppsala, Sweden |
| 1999-2000 | Gazi University, Faculty of Pharmacy Alumni Education Grants |
| 1998-1999 | YÖK (Turkish Higher Education Consul ) Educational Grants |