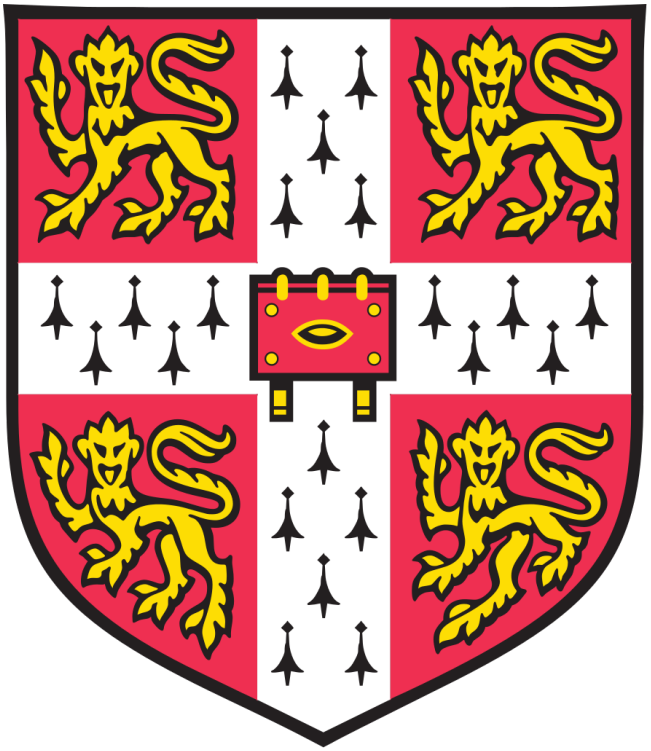
The synthesis and biological evaluation of a library of autoinducer-antibiotic conjugates

Lois Overvoorde



Sidney Sussex College

University of Cambridge

June 2016

Supervised by Prof. David Spring

A dissertation submitted to the University of Cambridge in partial fulfilment of the requirements for the Certificate of Postgraduate Studies

Contents

1 Introduction

1.1 Antibiotic resistance

Antibiotics add, on average, twenty years to a person's life.[1] However, antibiotic resistance is increasing alarmingly and is now recognised as a major threat to global health.[1,2] Antibiotic discovery had its heyday in the 1940s to 60s, which saw the discovery of many new classes of antibiotic. Since then, the rate of discovery of new classes has slowed and resistance to existing treatments has increased.

The story of how Alexander Fleming discovered penicillin by accidentally allowing a Petri dish containing *Staphylococcus aureus* to become contaminated with *Penicillium* mould whilst he was on holiday in Suffolk[1] is well known to many scientists. The initial serendipitous discovery of penicillin occurred in 1928 and was reported in 1929,[3] but it was not until 1943 that the drug was mass produced thanks to the research of Ernst Chain and Howard Florey. However, bacterial resistance to penicillin was being found in hospitals by the late 1940s.[4,5] This alarmingly quick emergence of resistance is a common phenomenon for antibiotics (see Table 1) as bacteria have multiple resistance mechanisms against antibacterial agents. These mechanisms can be broken down into five main categories:

1. The bacterium may inactivate the drug before it can cause damage, for example the hydrolysis of β-lactam antibiotics such as penicillin by β-lactamase enzymes.

2. The bacterium may produce a membrane, cell wall or biofilm which does not allow the drug to pass through, for example biofilm formation may allow bacterial resistance to antibiotics to increase 1000-fold compared with bacteria in suspension culture.[6]

3. The bacterium may pump antibacterial molecules out of its cell membrane using efflux pumps, for example the mexAB and mexXY pumps used by *Pseudomonas aeruginosa*.[7]

4. Mutations may cause the target of the antibacterial molecule to alter such that the molecule no longer effectively binds the target, for example the alteration of penicillin binding proteins which are involved in the final stages of peptidoglycan biosynthesis in the cell walls of MRSA and other penicillin-resistant bacteria.[8]

5. The bacterium may switch to using a metabolic pathway which does not involve the target of the antibacterial molecule, for example sulfonamide resistance may be achieved by taking in folic acid from the environment rather than synthesising it using *p*-aminobenzoic acid - a process which is blocked by sulfonamides.[9]

Antibiotic	Introduction	Resistance
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Trimethoprim	1962	1972
Cephalosporins	1960s	late 1960s
Ciprofloxacin	1987	1988
Linezolid	2000	1997
Daptomycin	2003	2005

Table 1: A timeline of when various antibiotics were first introduced and when resistance to them first appeared.[10–15]

1.2 Quorum sensing

A quorum is defined as 'A fixed minimum number of members of an assembly or society that must be present at any of its meetings to make the proceedings of that meeting valid.'[16] A similar concept is used in bacterial signalling, whereby group behaviour is only triggered when a certain minimum population of bacteria has been reached. Examples of group behaviour include bioluminescence, the production of virulence factors and biofilm formation. It is advantageous for bacteria to coordinate such behaviours as they would be ineffective, and therefore a waste of resources, when carried out by a single bacterium but effective when carried out as a group.

1.2.1 *Vibrio fischeri*

The first example of quorum sensing was discovered in *Vibrio fischeri*, a symbiotic bacterium that produces bioluminescence in the photophore of the Hawaiian bobtail squid, *Euprymna scolopes*[?,17,18] (see Figure 1a). This bacterium receives amino acids[19,20] from its host in exchange for producing light which the squid uses for counterillumination, to camouflage itself[21] (*V. fischeri* also has symbiotic relationships with other species, including the Japanese pinecone fish, *Monocentris japonica*[22])(see Figure 1b).

Figure 1: a) "Euprymna scolopes, South shore of Oahu, Hawaii" by Jamie Foster. Licensed under CC BY-SA 3.0 via Commons. b) "Monocentris japonica.1 - Aquarium Finisterrae" by Drow_male. Licensed under GFDL via Commons.

If a low population of *V. fischeri* is present in the photophore, the light that the bacteria could produce would be insufficient to attract prey. Therefore, the bacteria conserve resources by not producing light. However, if there is a high population of *V. fischeri* it is useful for them all to produce light, as this incentivises the squid to provide them with nutrients.

1.2.1.1 The LuxR-LuxI system

The bacteria sense the population of other *V. fischeri* in their vicinity by the detection of 3-oxo-C_6-HSL **1**[23] (see Figure 2), a freely diffusible[24] molecule which is secreted by all *V. fischeri* cells[25] at a low basal level.[17] When the bacterial population density, and hence the concentration of 3-oxo-C_6-HSL **1**, reaches a threshold, a response is triggered leading to expression of high levels of luciferase, and hence a 10,000-fold[26] increase in the production of (blue-green

The quorum sensing system of *V. fischeri* consists of two operons (see Figure 3). The left operon encodes just one gene, *luxR*, a transcription factor which binds 3-oxo-C_6-HSL **1**. The right operon encodes *luxICDABEG*. *luxI* encodes an enzyme (LuxI) which uses acyl-acyl carrier protein and *S*-adenosyl-L-methionine (SAM) to form 3-oxo-C_6-HSL **1** by lactonisation and acylation.[27,28] *luxCDABEG* encodes luciferase enzymes required for light production. Both operons are continuously expressed at low levels, leading to production of low concentrations of LuxI, 3-oxo-C_6-HSL **1** and LuxR, and low-level light production.[29]

V. fischeri can multiply to very high cell concentrations in the photophore of *E. scolopes* (around 10^9 cells,[30–32] or 10^{11} cells per mL[17] in the organ of a mature squid). As concentrations rise to these levels, the concentration of 3-oxo-C_6-HSL **1** also rises. At a threshold of around 1-10 μg/mL,[23] 3-oxo-C_6-HSL **1** binds to a N-terminal domain of LuxR,[33] leading to unmasking of the C-terminal transcriptional activator domain.[34,35] The LuxR-3-oxo-C_6-HSL complex can then bind to the *lux* operator, which is situated between the left and right operons and, unusually, affects the transcription of both operons in a bidirectional manner, involving both positive and negative regulation.[36] It is thought that the LuxR-3-oxo-C_6-HSL complex forms a homodimer,[37] but this has not been conclusively proven.[38,39]

Binding of LuxR-3-oxo-C_6-HSL complex to the *lux* operator activates transcription of the right operon, leading to production of both 3-oxo-C_6-HSL **1** and light. Production of more 3-oxo-C_6-HSL **1** enables a positive feedback loop, re-inforcing the effect of high population density on 3-oxo-C_6-HSL **1** concentration and hence light production.

Concurrently, transcription of the left operon is also affected by binding of the LuxR-3-oxo-C_6-HSL complex to the *lux* operator, but in a more complex manner. At low concentrations of 3-oxo-C_6-HSL **1** transcription

of the left operon is activated, leading to production of more LuxR. However, at high concentrations of 3-oxo-C_6-HSL **1** production of LuxR is inhibited in an autoinducer-dependent manner.[40] This effect is dependent on DNA sequences found upstream of the left operon, within the right operon, and without them LuxR has a stimulatory effect at all concentrations of LuxR and autoinducer.

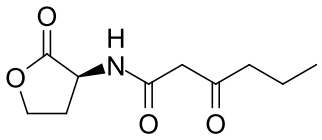


Figure 2: 3-oxo-C_6-HSL **1**.

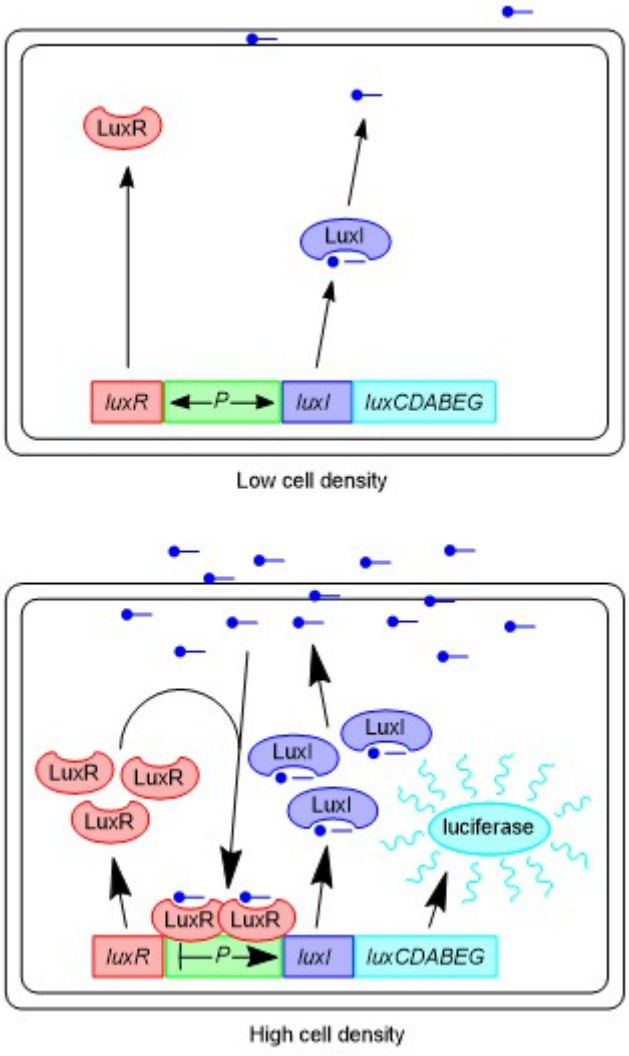


Figure 3: The LuxR-LuxI quorum sensing system in *V. fischeri*.

1.2.1.2 Other quorum sensing systems

Since the discovery in 1970 of the LuxR-LuxI quorum sensing system in *V. fischeri*, several other mechanisms have also been discovered (it should be noted that several of the proteins mentioned in this section were first characterised in *Vibrio harveyi*, and functions may be assigned by analogy to a closely-related *V. harveyi* protein[39]). Systems using two other quorum sensing molecules, *N*-octanoyl-homoserine lactone (C_8-HSL **27**) and a furanosyl borate diester (AI-2 **6**) have been discovered, both of which act via the *luxCDABEG* system and

increase luminescence by increasing luciferase production[39,41] (see Figure 4 and Figure 5). Additional controls in the *lux* promoter region have also been discovered, which respond to O_2 and cAMP[39] (see 1.2.1.3 and 1.2.1.4).

The AinS–AinR system uses C_8-HSL **27**, synthesised by AinS, as its signalling molecule.[39,42,43] C_8-HSL **27** has two main effects on the quorum sensing network. Firstly, it can bind to LuxR,[25] albeit with lower affinity than 3-oxo-C_6-HSL **1**, leading to partial upregulation of lux operon transcription. Secondly, it binds to the histidine kinase AinR, inhibiting its ability to phosphorylate the histidine phosphotransferase LuxU, which links into the LuxR pathway by a less direct route (see later in this section).[44]

The LuxS–LuxP/Q system uses AI-2 **6**, synthesised by LuxS, as its signalling molecule[39,45,46] (this pathway is common to all *Vibrio* species[39]). The receptor of AI-2 **6** is a complex of a two proteins, LuxP and LuxQ. LuxP is a periplasmic protein which binds AI-2 **6**, LuxQ is an inner membrane histidine kinase of the two-component sensor kinase family.[47] It is likely that LuxQ is constitutively dimeric, although this has not yet been demonstrated.[48]

When AI-2 **6** is not bound to LuxP, LuxQ autophosphorylates a histidine residue.[46] This phosphoryl group is then transferred to an aspartic acid residue in LuxQ, and then to a histidine residue in LuxU.

When AI-2 **6** binds to LuxP, this causes a major conformational change in the LuxP/Q complex, replacing one set of contacts between the proteins with another[45,46] and causing the formation of an asymmetric complex of two LuxP/Q dimers. Formation of the asymmetric dimers switches the activity of LuxQ from kinase to phosphatase, which can then dephosphorylate LuxU.

At high cell density, and hence autoinducer concentration, both the AinS–AinR system and the LuxS–LuxP/Q system bring about a decrease in the amount of phosphorylated LuxU. LuxU is a phosphotransferase protein which transfers its phosphate group to an aspartic acid residue in LuxO.[49] Phosphorylated LuxO inhibits quorum sensing responses by via LuxR. Hence, at high cell densities there is a decreased amount of phosphorylated LuxU present, leading to a lack of phosphorylated LuxO, and hence increased quorum sensing responses, e.g. light production. This 'many-to-one' signalling pathway is common in bacterial two-component signalling systems[50] and is found in several other *Vibrionaceae*.[51]

LuxO phosphate inhibits quorum sensing responses via σ_{54}-dependent transcriptional activation of *qrr1*[52,53] (despite the proximity of the *luxOU* and *qrr* promoters, LuxO only affects the activates the production of Qrr1 and not itself[54]). Qrr1 is a small RNA molecule (a quorum regulatory RNA or Qrr) which, with the help of Hfq, can bind to LitR RNA, leading to its degradation.[53] Qrr1 is the only Qrr to regulate LitR expression in *V. fischeri*, and is conserved across all *Vibrionaceae*.[53] In contrast, in other *Vibrionaceae* a family of Qrrs is often used.[55]

Qrr1/Hfq-mediated degradation of LitR mRNA inhibits the production of LitR, an activator of the *lux* operon. LitR binds to a region of the *luxR* promoter, causing increased LuxR production and hence increased bioluminescence.[56]

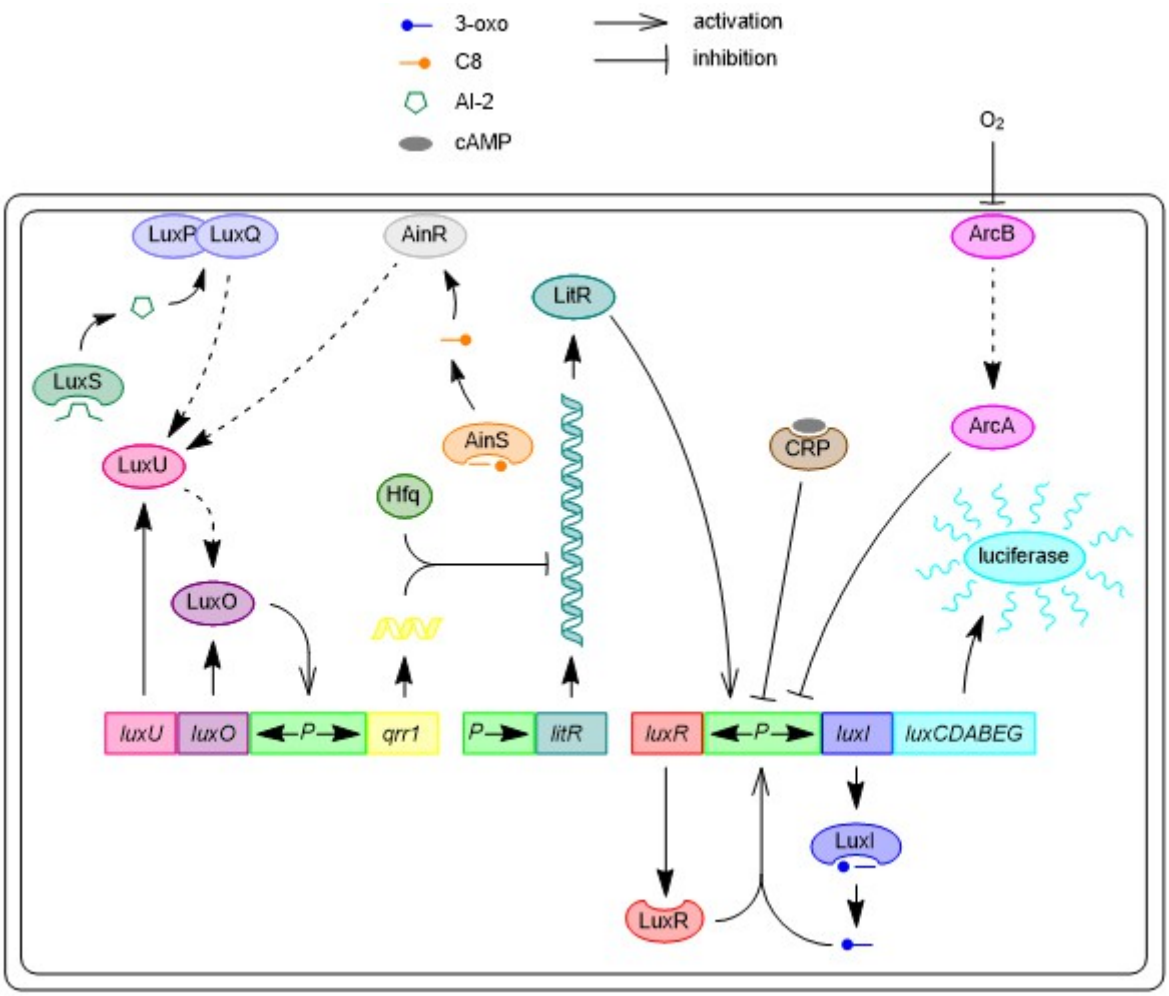


Figure 4: Quorum sensing in *V. fischeri*.

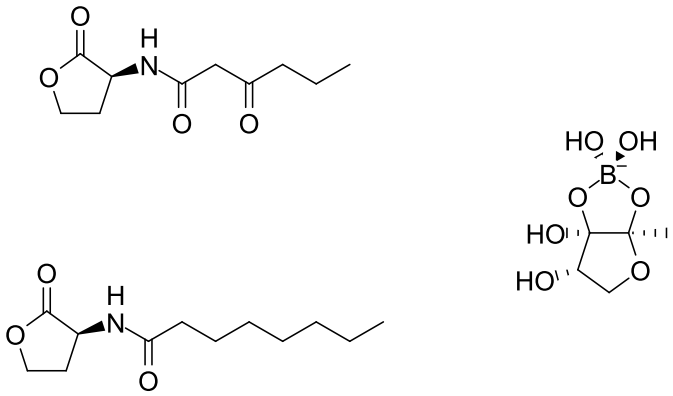


Figure 5: Quorum sensing molecules in *V. fischeri*.

1.2.1.3 The effect of O_2

V. fischeri uses the ArcA-ArcB system to sense how oxygen-rich its environment is, by monitoring the redox state of various quinones produced by the cell. In an oxygen-rich environment, luminescence is stimulated. It is though that luminescence is used by *V. fischeri* make its environment less oxidising, as luminescence consumes oxygen.

ROS are bad and it doesn't want them[57]

ArcB is a histidine kinase which senses the redox state of the quinones. When the environment is low in oxygen or reactive oxygen species, the quinones are reduced and can stimulate ArcB to phosphorylate ArcA. ArcA is a response regulator which represses the transcription of *luxICDABEG* (and to a lesser extent, *luxR*).

1.2.1.4 The effect of cAMP

cAMP

1.2.1.5 The purpose of multiple signalling pathways

It might reasonably be asked: why does *V. fischeri* use three quorum sensing pathways rather than just one? The answer to this question lies in the bacterium's relationship with its squid host. It has been shown that the LuxS-LuxP/Q and AinS-AinR systems are important in the medium cell densities found during early colonisation of the host, whereas the LuxR-LuxI system is important at the higher cell densities found in late colonisation.[42,58]

It has been shown that the LuxS-LuxP/Q system does not have an especially large effect on colonisation of the host squid, although it does have some effect on luminescence.[59] It has therefore been speculated that the LuxS-LuxP/Q system is more important in the colonisation of other marine invertibrates, either in their light organs[22,60] or as part of multi-species colonies in their guts.[59,61]

The AinS-AinR system has a larger effect on colonisation and luminescence, in that *ainS* mutants show only 10-20 % of wild-type luciferase activity at medium cell densities in culture (10_8 to 10_8 cells ml_{-1}).[42] At the higher cell densities in the squid host ($>10_{10}$ cells ml_{-1}), *ainS* mutants show 10-40 % of wild-type luciferase activity, an effect which can be partially attributed to failure of the mutants to colonise the host (bacterial cell numbers are down to 20-80 % compared to the wild type). This failure of *ainS* mutants to colonise the host is due to *ain* regulation of pathways involved in early colonisation. The AinS-AinR system controls around 30 genes via LuxO and LitR.[58] *ain* quorum sensing is thought to repress several motility genes, causing loss of flagella, which are initially required for normal colonisation of the host,[62,63] but are lost as the bacteria colonise the host.[30] *ain* quorum sensing also induces a putative exopolysaccharide, which could be important in biofilm formation inside the host, or evasion of its immune system,[64] as well as two unique. In addition, *ain* quorum sensing affects the transcription several genes involved in metabolism, and new genes of unknown function which could affect colonisation by an as yet unknown pathway.

In contrast to the AinS-AinR, the LuxI-LuxR system is only fully induced at the high cell densities found in the *E. scolopes* light organ. At medium cell densities, C_8-HSL **27** is thought to be the dominant autoinducer, partially activating transcription from the *lux* operon by binding to both AinR and LuxR.[42] At high cell densities, C_8-HSL **27** is displaced from LuxR by 3-oxo-C_6-HSL **1**, leading to full light production.

lux quorum sensing also affects the transcription of five non-*lux* proteins which could potentially act as late colonisation factors.[65,66] Three of these genes, *qsrP*, *acfA*, and *ribB*, are directly activated by LuxR/3-oxo-C_6-HSL[66] and a strain lacking *qsrP* is less effective at colonising *E. scolopes* than the wild type, providing good evidence that it is a late-stage colonisation factor.[65]

ain positive feedback, LuxPQ not[59]

Which do CRP and Arc act on? Add phos. LitR upregulates AinS[59]

Quorum sensing has since been observed in many species of bacteria, including *P. aeruginosa*, *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *S. aureus*, *Vibrio harveyi*, *Escherichia coli*, *Myxococcus xanthus*, *Salmonella enterica*, *Yersinia enterocolitica Aeromonas* sp. and *Acinetobacter* sp.[17,67–74] Many of these bacteria are significant causes of disease and death in humans, for example, it is estimated that in 2005 in the US *S. aureus* caused 477,927 hospitalisations and 11,406 deaths.[75] *S. aureus* uses a peptide autoinducer know as autoinducing peptide (AIP) (see **??** in **??**) which interacts with the *agr* system leading to increased protease and toxin production.[76] *P. aeruginosa* also uses quorum sensing to coordinate biofilm formation, swarming motility and virulence.

1.2.2 *Pseudomonas aeruginosa*

One of the most well-studied examples of QS is in *P. aeruginosa*. *P. aeruginosa* is a Gram-negative opportunistic pathogen which typically infects immunocompromised individuals such as those with cystic fibrosis, neutropenia and AIDS. It can infect the pulmonary and urinary tracts as well being the most frequent cause of burn wound infections and the most frequent conloniser of medical devices such as catheters.[77]

P. aeruginosa uses quorum sensing (QS) to coordinate biofilm formation, swarming motility and virulence. The autoinducers used by *P. aeruginosa* are shown in Figure 7 (HHQ **4** is a precursor to PQS **5** but can bind to its receptor, PQSr, and hence can act as a autoinducer[78]). QS in *P. aeruginosa* involves a complex interplay of the four signalling molecules and various proteins (see Figure 6).[79] QS regulates the production of virulence factors including elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins and superoxide dismutases, as well as regulating biofilm formation.

P. aeruginosa has a low susceptibility to many antibiotics due to its chromosomally encoded multidrug efflux pumps: mexAB and mexXY.[7] It is also difficult for drugs to cross into cells due to low cell wall permeability and biofilm formation. *P. aeruginosa* may also acquire antibiotic resistance by mutation or horizontal gene transfer.[80] This high level of antibiotic resistance makes *P. aeruginosa* an important target for drug discovery.

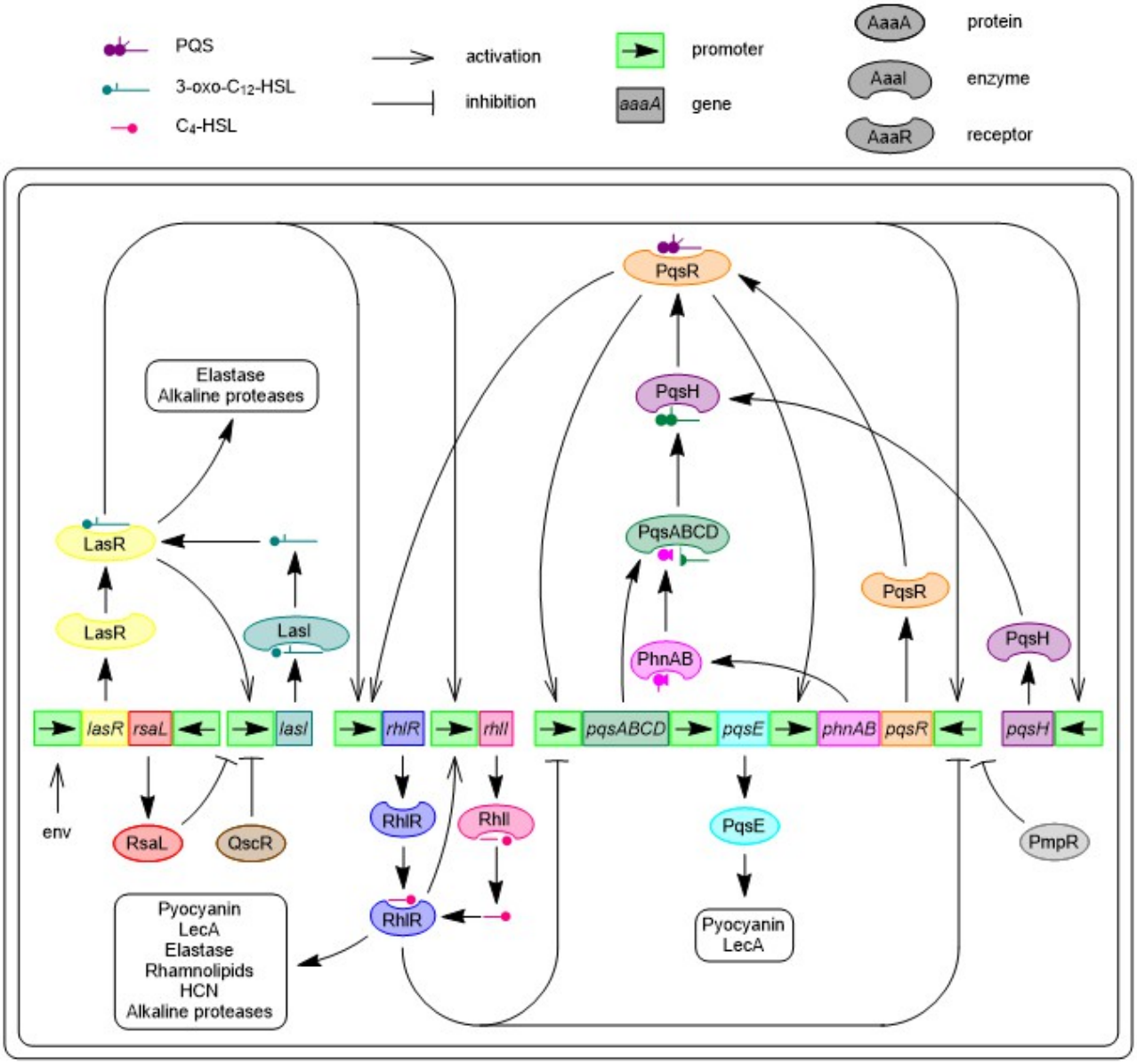


Figure 6: Quorum sensing in *P. aeruginosa*.[79]

add biofilm to diagram, late and early development Sauer2002

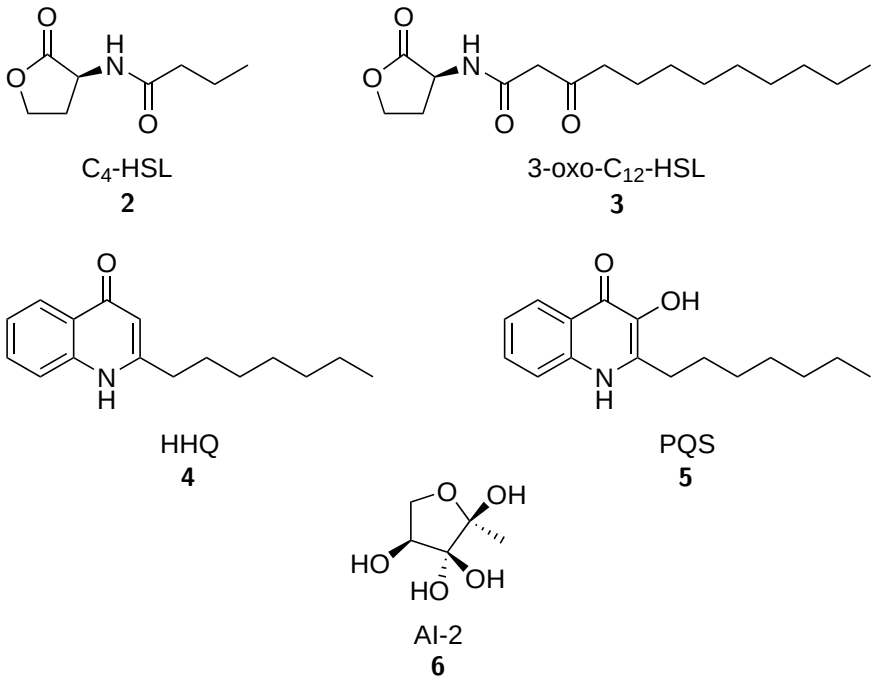
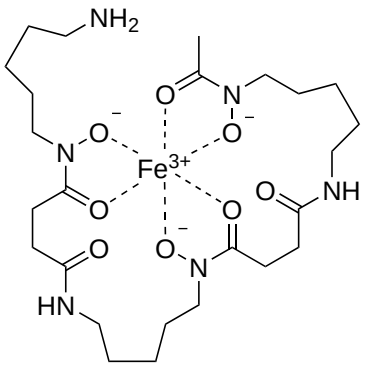


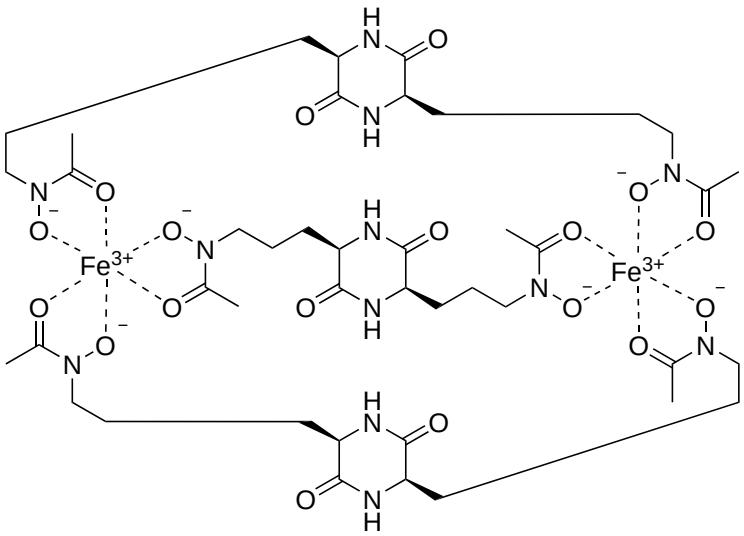
Figure 7: *P. aeruginosa* quorum sensing molecules.

1.3 Siderophores

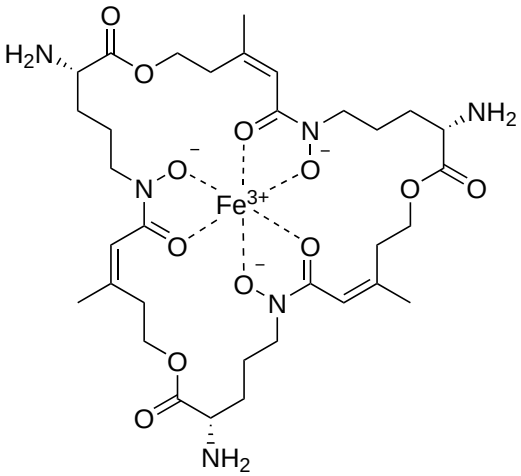
Siderophores are peptides or small molecules used by microorganisms to chelate iron for the purposes of 'iron mining'.[81] Soluble iron is often scarce but it is crucial for many cellular processes including respiration and DNA synthesis. Siderophores are synthesised by the microorganisms and secreted into the extracellular environment where they bind to Fe^{3+}, often with exceptionally high affinities. The iron-bound siderophores are then brought back into the cell by active transport and the iron is released, either by reduction of the Fe^{3+} to Fe^{2+} or by enzymatic degradation of the siderophore. Siderophores have a wide range of structures (see Figure 8 and Figure 9), possibly so one species can avoid its siderophores being taken up by another species.[82]



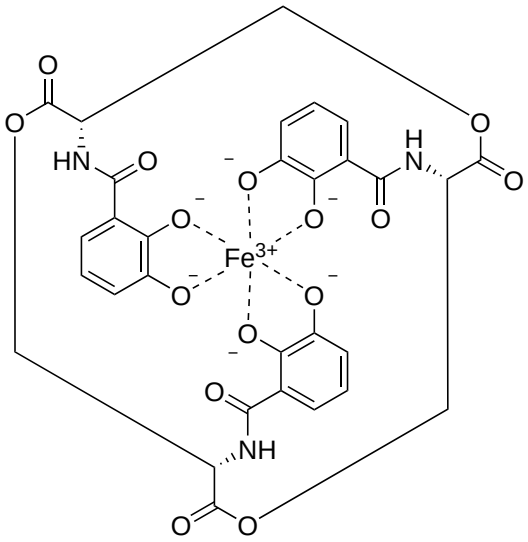
Deferoxamine B
7



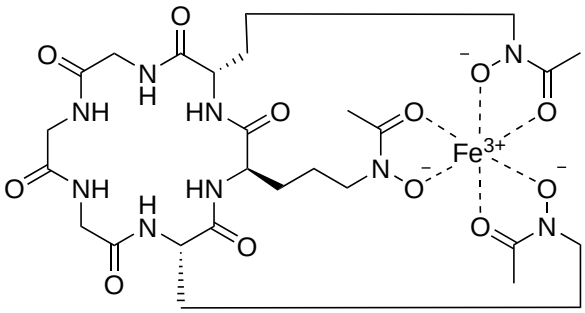
Rhodotorulic acid
8



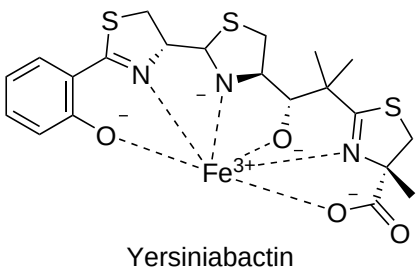
Fusarinine C
9



Enterobactin
10

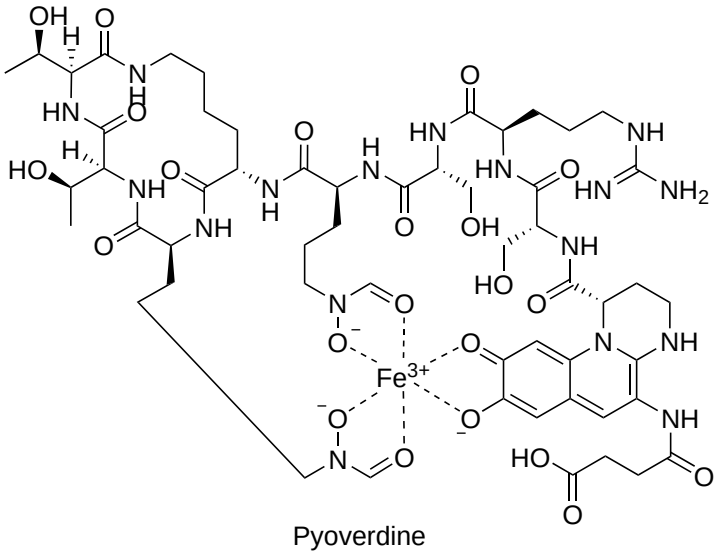


Ferrichrome
11



Yersiniabactin
12

Figure 8: Iron-siderophore complexes: Deferoxamine **7**[83] (*Streptomyces pilosus* and *Streptomyces coelicolor*), rhodotorulic acid **8**[84] (*Rhodotorula pilimanae*), fusarinine C **9**[85] (*Fusarium roseum*), enterobactin **10**[83] (*Escherichia coli* and enteric bacteria), ferrichrome **11**[86] (*Ustilago sphaerogena*, *U. maydis*, *Aspergillus niger*, *A. quadricintus*, *A. duricaulis* and *Penicillium resticolosum*), yersiniabactin **12**[83] (*Yersinia pestis*).



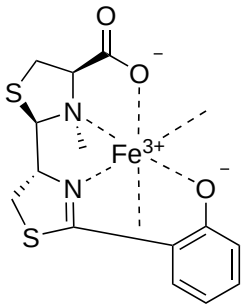


Figure 9: Iron-siderophore complexes: Pyochelin **13**[87] (*P. aeruginosa*), Pyoverdine **14**[83] (*P. aeruginosa*). Note that pyoverdine **14** is a tetradentate ligand, hence the iron ion has two sites which can bind other ligands.

1.4 Sideromycins

Siderophore-antibiotic conjugates are produced naturally by some bacteria and are known as sideromycins[88] (see Figure 10). Bacteria produce these molecules to attack other bacteria by hijacking their siderophore uptake mechanisms to introduce toxic compounds.

For example, albomycin **15** (see Figure 10) is a sideromycin produced by *Actinomyces subtropicus* and *Streptomyces griseus*[89,90] which has been used to treat infections caused by various bacteria including *Yersinia enterocolitica* and *Streptococcus pneumoniae* in mice and humans.[91,92] Albomycin **15** contains a siderophore coupled to a nuceloside antibiotic via a peptide linker. The siderophore section is structurally similar to ferrichrome **11** (see Figure 8), a siderophore produced by various fungi, but also taken up by bacteria including *Escherichia coli*, *Salmonella typhimurium* and *P. aeruginosa*.[86,93] It has been shown that because of the structural similarity to ferrichrome **11**, *E. coli* will also take up albomycin **15**.[89] The linker is hydrolysed in the cytoplasm of the *E. coli*, releasing the active nuceloside antibiotic. This leads to 500-fold concentration of the antibiotic within the *E. coli* cells, enough to have significant effect on growth.

The success of albomycin[91] and other sideromycins such as salmycin A[94,95] and ferrimycin A1[96,97] has served as encouragement to many researchers to explore synthetic siderophore-antibiotic conjugates, which will be discussed in the next section.

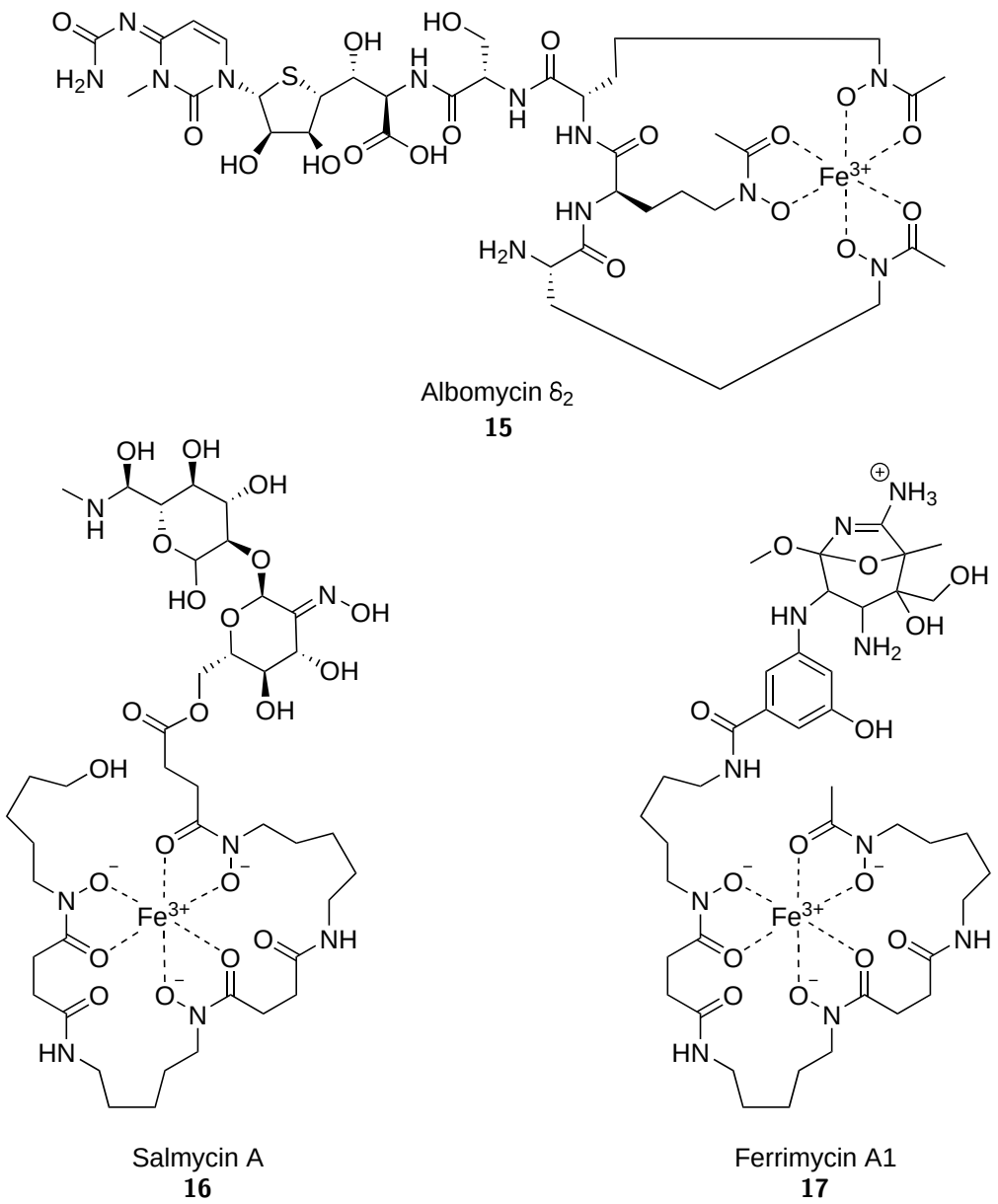


Figure 10: Iron-sideromycin complexes: Albomycin **15**[94,98] (*Actinomyces subtropicus* and *Streptomyces griseus*), salmycin A[94,95] (*Streptomyces violaceus*) and ferrimycin[94] (*Streptomyces griseoflavus*).

1.5 Synthetic siderophore-antibiotic conjugates

Sideromycins served as inspiration for the design, synthesis and biological evaluation of a wide range of synthetic siderophore-antibiotic conjugates.[88] Antibiotics used include β-lactams,[99–101] nucleosides,[102] glycopeptides[103] and macrolides.[104] Sideromycin-fluoroquinolone conjugates have also been studied by several groups,[105–107] including conjugates with linkers which can be cleaved[106,107] in a similar manner to albomycin.[89] Some of these showed comparable activity to the parent antibiotic, but it is not clear whether attachment of the siderophore improved uptake or whether the conjugates acted as classical prodrugs.

β-lactam-sideromycin conjugates have been more widely investigated and show good activity *in vitro*, however, resistance can evolve by loss of the TonB transporter or of the relevant siderophore receptor, e.g. Cir and Fiu for catecholate siderophores or FhuA for hydroxamate siderophores.[88] Recently a conjugate (Ent-Amp **18**, see Figure 11) of enterobactin and ampicillin joined using a copper(I)-catalyzed azide-alkyne cycloaddition has been shown to have increased activity against pathogenic *E. coli* when compared to native ampicillin.[108] Other work has focused on monocyclic β-lactams, for example pirazmonam **19** and U-78608 **20**, which show high po-

tency against Gram-negative bacteria including *P. aeruginosa*,[109,110] Monocyclic β-lactams are generally fairly stable to β-lactamase activity, which is an advantage compared with many bicyclic β-lactams.

Three siderophore-antibiotic conjugates are reported as being in clinical trials:[111] MC-1 **21**,[112] BAL30072 **22**[88] (see Figure 11) and cefiderocol **23**[113,114].

pre-release paper

MC-1 **21** is reported as being "in clinical phases of development",[111] but no reports of studies in humans could be found. However, experiments in mice have been promising.[112] BAL30072 **22** is a siderophore-β-lactam conjugate which showed initial promise as it is a poor substrate for β-lactamases, and resistance due to loss of transport proteins is infrequent.[88] However, it is unclear whether it will progress further in trials as it causes liver toxicity.[115] Cefiderocol **23** is a cephalosporin-catechol conjugate in phase 1 trials. Recent results indicate that 'single and 35 multiple intravenous doses of cefiderocol at up to 2000 mg were well tolerated in healthy 36 subjects'.[114]

These examples show that siderophore-antibiotic conjugates are a promising strategy to deliver antibiotics across bacterial membranes, but it is worth noting that conjugation to a siderophore may lead to loss of activity, or resistance may be acquired by loss of transport proteins. Encouragingly though, albomycin **15**-resistant mutants have been shown to be less virulent,[92] indicating that bacteria may lose out either by susceptibility to the antibiotic or by loss of fitness due to decreased iron transport.

Building on these positive examples, it is hoped that the strategy of conjugating a molecule which is important for virulence with an antibiotic can be extended to conjugates of autoinducers and antibiotics in a similar 'Trojan horse' approach.

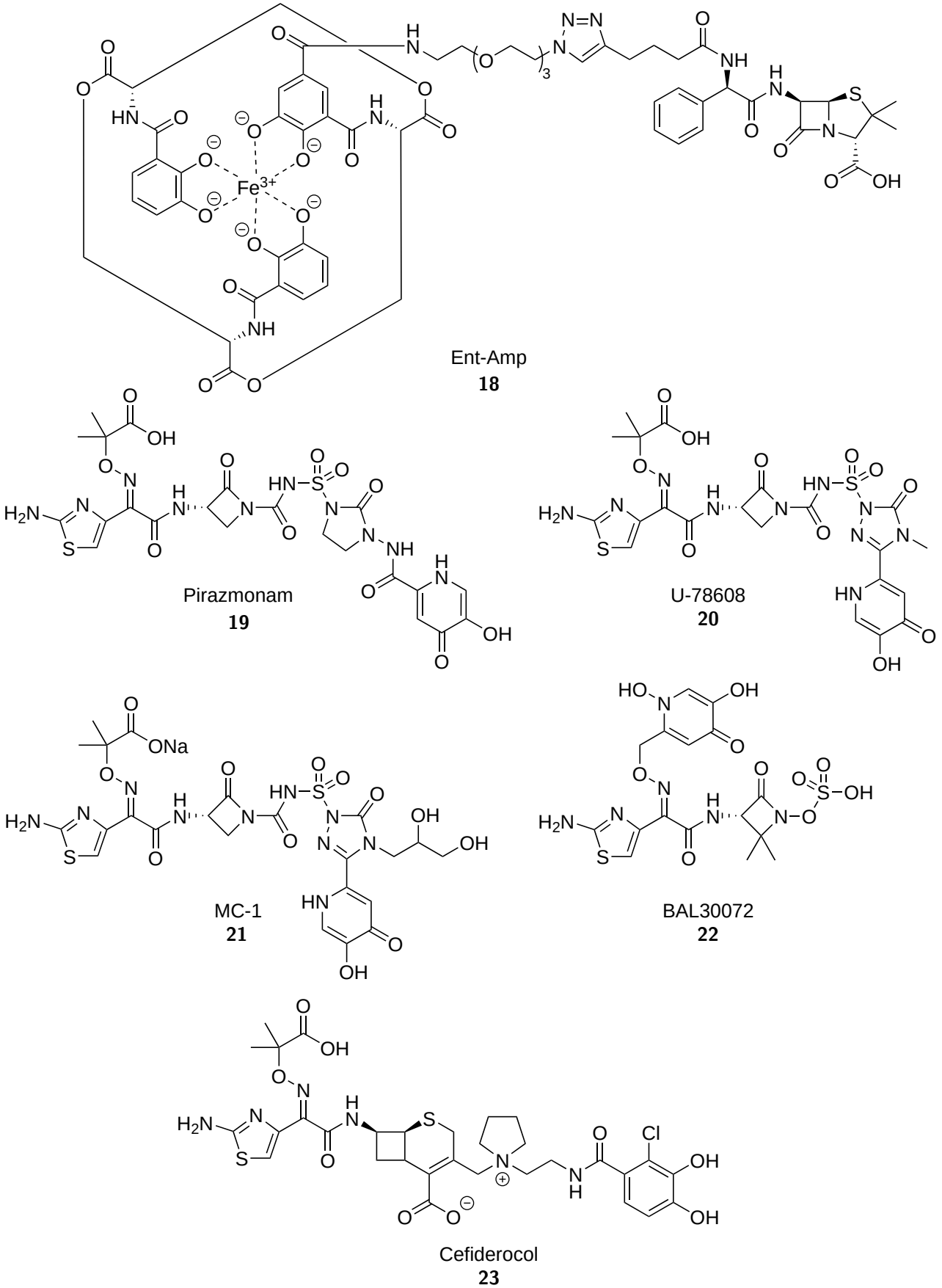


Figure 11: Ent-Amp **18**,[108] pirazmonam **19**,[109,110] U-78608 **20**,,[109,110] MC-1 **21**,[112] BAL30072 **22**[88] and cefiderocol **23**.[113,114]

1.6 Autoinducer-antibiotic conjugates

This study extends the conjugation strategy discussed above by creating autoinducer-antibiotic conjugates. It was hypothesised that attaching an autoinducer to a known antibiotic could lead to increased cellular retention of the antibiotic, and could potentially restore function in resistant strains. The work is divided into two main sections. The first section focuses on conjugates of three *P. aeruginosa* autoinducers (see **??**) with ciprofloxacin and trimethoprim (see Figure 12). The second section focuses on conjugates of homoserine lactone analogues with ciprofloxacin (see 1.7).

1.6.1 Autoinducers

The *P. aeruginosa* autoinducers (see Figure 7) were chosen as *P. aeruginosa* is a significant human pathogen which shows high antibiotic resistance and utilises quorum sensing to coordinate pathogenic behaviours (see 1.2.2).

The five known *P. aeruginosa* autoinducers have different transport mechanisms in and out of cells: The mechanism is not well known for HHQ **4** or AI-2 **6**, PQS is exported in vesicles,[116] C_4-HSL **2** passively diffuses in and out of cells,[117] and 3-oxo-$C_1$2-HSL **3** is taken up passively, accumulates in the cell membrane and is actively pumped out by efflux pumps.

The difference in transport mechanism for C_4-HSL **2** and 3-oxo-$C_1$2-HSL **3** is thought to be largely due to chain length rather than the 3-oxo modification, as a shorter-chain version, 3-oxo-C_6-HSL **1** has been shown to be freely diffusable through V. fischeri membranes.[24]

Upregulation of the MexAB-OprM efflux system leads to increased efflux of 3-oxo-$C_1$2-HSL **3**.[?] The removal of 3-oxo-$C_1$2-HSL **3** from the cell leads to decreased production of additional 3-oxo-$C_1$2-HSL **3** (as the positive feedback loop is disrupted, see 1.2.2), and hence decreased production of pyocyanin, elastase and casein protease. It is expected that MexAB-OprM upregulation would also disrupt biofilm formation as a decrease in 3-oxo-$C_1$2-HSL **3** levels would disrupt both Las- and Rhl-mediated quorum sensing,[?] but no direct studies of this could be found.

HHQ **4**, PQS **5** and C_4-HSL **2** (see Figure 7) derivatives were chosen as they were deemed most synthetically tractable.

1.6.2 Antibiotics

Ciprofloxacin **34** and trimethoprim **36** (see Figure 12) were chosen as the antibiotic sides of the conjugates.

Ciprofloxacin **34** is second-generation fluoroquinolone antibiotic used to treat both Gram-positive and Gram-negative bacterial infections including *P. aeruginosa*.[118,119] *P. aeruginosa* biofilms are more resistant to ciprofloxacin **34** than planktonic cells.[120]

Ciprofloxacin **34** enters *P. aeruginosa* by diffusion,[121] but is pumped out by efflux pumps.[122] In the planktonic state several efflux pumps are known to pump out ciprofloxacin **34**, including MexAB–OprM, MexCD–OprJ, MexEF–OprN, MexXY–OprM, MexJK–OprM and MexVW–OprM.[7] However, in biofilms only MexEF-OprN has an effect.[123]

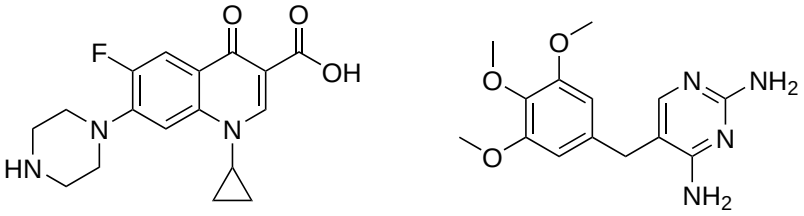
It is hoped that *P. aeruginosa* would develop resistance to a conjugate of HSL and ciprofloxacin **34** by upregulation of the MexAB-OprM pump. This would also lead to increased export of 3-oxo-$C_1$2-HSL **3**, thus disrupting the quorum-sensing system and hence biofilm formation and virulence.

It is hoped that the HSL group would make the conjugate a poor substrate of MexEF–OprN (the sole exporter of ciprofloxacin **34** in biofilms[123] and not an exporter of HSLs[?]) as this would lead to accumulation of the conjugate within cells and hence cell death.

Trimethoprim (see Figure 12) is a dihydrofolate reductase inhibitor used primarily to treat bladder in-

fections.[124] It is active against several significant human pathogens including *Streptococcus pneumoniae* and *Haemophilus influenzae*. It was primarily chosen in this study as it was easy to functionalise.

These results demonstrate that the mexABoprM multidrug efflux system is mainly responsible for the intrinsic resistance of P. aeruginosa to TMP[125]



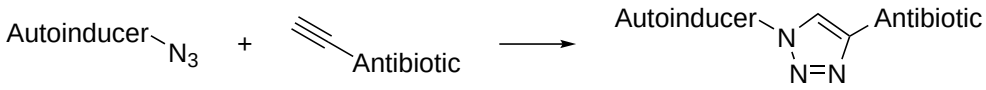
Ciprofloxacin **34** Trimethoprim **36**

Figure 12: The antibiotics used in this section.

consisten
on figure cap-
tions/labe
of molecules

1.6.3 Synthesis of the conjugates

A copper(I)-catalysed azide-alkyne cycloaddition,[126,127] commonly referred to as a click reaction (although this is a more general term), was used to join each combination of autoinducer and antibiotic together (see Scheme 1). This modular approach would allow the library to be easily expanded by adding more autoinducers or antibiotics, or indeed other groups such as siderophores, fluorescent or affinity tags, or resin beads.



Scheme 1: The construction of the triazole-linked autoinducer-antibiotic conjugate library using a copper(I)-catalysed azide-alkyne cycloaddition.

1.7 Autoinducer analogue-ciprofloxacin conjugates

Following on from the library of compounds based on *P. aeruginosa* autoinducers, a series of conjugates based on *analogues* of C_4-HSL were planned. This strategy was inspired by a paper[128] and patent[129] by Ganguly *et al.*, who synthesised and characterised a conjugate **111** of methyl ciprofloxacin with homocysteine thiolactone (see Figure 13). Homocysteine thiolactone is an analogue of homoserine lactone with the ring oxygen replaced by sulfur, and has been used as the head group in several other known quorum sensing modulators.[25,130–136]

show com-
pounds
it's in
and de-
scribe?

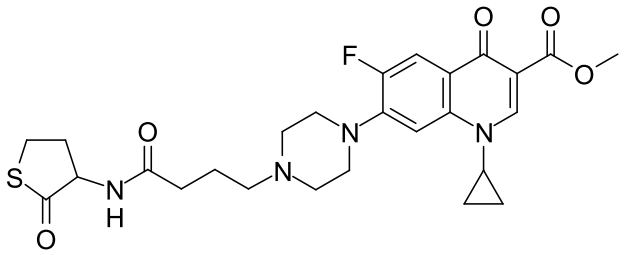


Figure 13: The HCTL-CipMe conjugate **111** studied by Ganguly *et al.*[128,129]

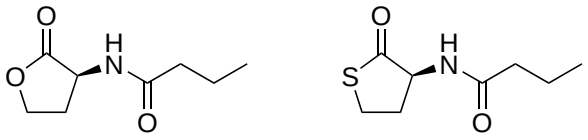
(good
it has
Me so
it ac-
cumu-
lates in
mem-
branes
like 3

As part of their characterisation of the HCTL-CipMe conjugate **111**, Ganguly *et al.* found the minimum

inhibitory concentration (MIC) of the conjugate in *P. aeruginosa* under standard planktonic conditions. The MIC was found to be ten times higher for the conjugate vs. ciprofloxacin (50 vs. 5 μm), indicating that the conjgate was less effective than ciprofloxacin under planktonic conditions.

Ganguly *et al.* then investigated the effect of the conjugate on biofilms. The conjugate and ciprofloxacin were first added to dilute *P. aeruginosa* liquid culture at 25 μm. As expected, the culture failed to grow and form biofilm in the presence of ciprofloxacin, but did grow in the presence of the conjugate **111**. They then incubated cultures for 24 h, to allow biofilms to grow, before adding the compounds. In contrast, they found that the conjugate **111** disrupted the biofilm more effectively than ciprofloxacin. When the biofilm was grown for 48 or 72 hours the conjugate had similarly disruptive effects, whereas ciprofloxacin 'did not show any significant antibacterial activity'.

These results are exciting as they hint that an autoinducer conjugate might be able to combat an established *P. aeruginosa* infection more effectively than the unmodified antibiotic. Ganguly *et al.* suggest that their conjugate is more effective than ciprofloxacin in penetrating biofilms, and/or better at avoiding being pumped out by multidrug efflux pumps. They posit that this could be due to the thiolactone head, as they also showed that unconjugated C_4-HCTL **24** (see Figure 14) has 'either enhanced uptake or functional activity' when compared with C_4-HSL **2**.



2 **24**

Figure 14: C_4-HSL **2** and C_4-HCTL **24**. Note that Ganguly *et al.* tested the *S* enantiomer of C_4-HCTL **24**, but used a racemic mixture in their HCTL-CipMe conjugate.

is it racemic?

While the results found by Ganguly *et al.* show promise, they only test one conjugate, and do not include controls to show that the HCTL group specifically is necessary for the enhanced effect. It was therefore decided to build on this work by synthesising a series of ciprofloxacin conjugates with head groups known as part of quorum sensing modulators.[137,138]

read these again, put ones I didn't do in further work

The activity of the chosen head groups against *P. aeruginosa* receptors when coupled with the native C_4 and 3-oxo-C_12 tails is summarised in Table 2. It is hoped that high activity of these molecules should correlate with high activity of their ciprofloxacin conjugates. This is not a comprehensive list of active head groups, and other possible choices are covered in **??**.

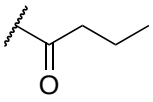
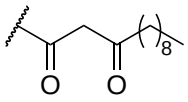
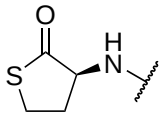
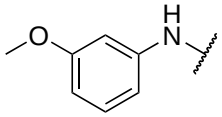
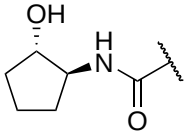
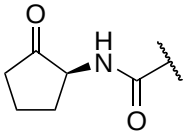
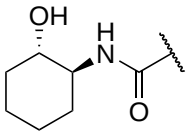
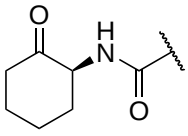
Head group		
	Partial agonist and antagonist against LasR.[134] Shown to increase biofilm formation in *P. aeruginosa*.[128]	Strong agonist against LasR, with comparable activity to the native ligand.[131,132,134,139]
	Partial agonist against LasR.[138]	Strong antagonist against LasR.[138]
	Poor agonist and antagonist against RhlR.[140,141]	Strong antagonist against LasR.[140]
	Strong agonist against RhlR.[140] *SS* enantiomer is more potent.[141]	Partial agonist against LasR.[140]
	Strong agonist against RhlR.[140] *SS* enantiomer is more potent, with comparable activity to the native ligand.[141]	Strong agonist against LasR.[132,140] *SS* enantiomer is more potent, with comparable activity to the native ligand.[141]
	Strong agonist against RhlR.[140] *SS* enantiomer is more potent.[141]	Partial antagonist against LasR.[140] Shown to reduce biofilm formation in *P. aeruginosa*.[140]

Table 2: Activities of autoinducers containing the chosen head groups when coupled with C_4 or 3-oxo-C_12 tails.

2 Autoinducer-antibiotic conjugates

2.1 Introduction

The first part of this project was focused on producing a library of autoinducer-antibiotic conjugates. *P. aeruginosa* autoinducers were used, in particular C_4-HSL **2**, HHQ **4** and PQS **5** (see **??**). Azido derivatives of these compounds were coupled to alkynyl dervitatives of antibiotics, specifically ciprofloxacin **34** and trimethoprim **36** (see Figure 12), using a copper(I)-catalysed azide-alkyne cycloaddition.[126,127]

2.1.1 Azido autoinducer derivatives

The structure-activity relationships in HHQ **4** and PQS **5** have been previously studied,[142–144] and it was shown various substitutions on the benzene ring could be made without significantly decreasing activity. The 6-azido derivatives (see Figure 15) were chosen for this study as routes to them have previously been found.[145]

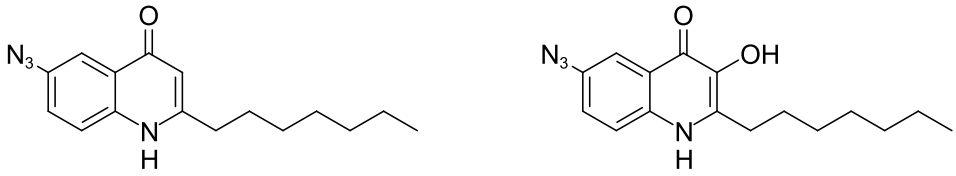


Figure 15: The azido derivatives of HHQ **4** and PQS **5**: **46** and **57**.

Alteration of the lactone group of C_4-HSL **2** and other HSL derivatives is known to significantly decrease activity, especially where the number of H-bond donors or acceptors is altered.[137] Hence, the azide group was included on the tail of C_4-HSL **2**. Acyl tail length is known to play an important role in affinity, so three derivatives of C_4-HSL **2** were synthesised: N_3-C_2-HSL **63**, N_3-C_4-HSL **66** and N_3-C_6-HSL **69** (see Figure 16).

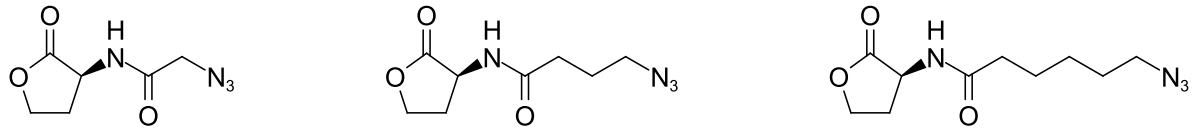


Figure 16: The azido derivatives of C_4-HSL **2**: **63**, **66** and **69**.

2.1.2 Alkynyl antibiotic derivatives

The structure-activity relationships for ciprofloxacin have been investigated[146] and modifications at the cyclopropane and piperazine groups were found not to cause loss of activity. It was decided an alkyne tail would be added onto the free NH of the piperazine ring, as this position is more synthetically accessible. Alkynyl ciprofloxacin derivative **76** (see Figure 17) was synthesised in this study (see **??**), and two cleavable alkynyl ciprofloxacin derivatives **98** and **99** were synthesised by Dr Eddy Sotelo and combined with some of the azido HSL derivatives made in this study (see **??** and **??**).

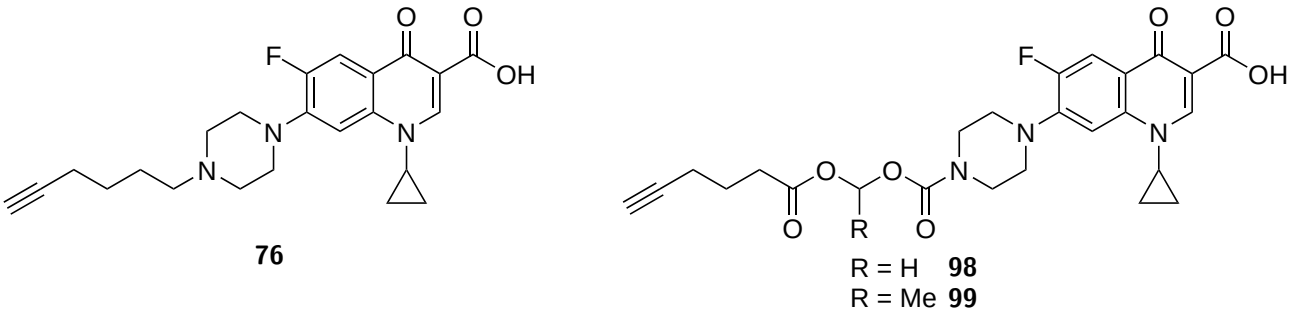


Figure 17: The alkynyl ciprofloxacin derivatives **76**, **98** and **99**.

The choice to of alkyne tail attachment point on trimethoprin **36** (see Figure 18) is based on the use of that same point in a fluorogenic trimethoprim tag synthesised by Jing *et al.*[147]

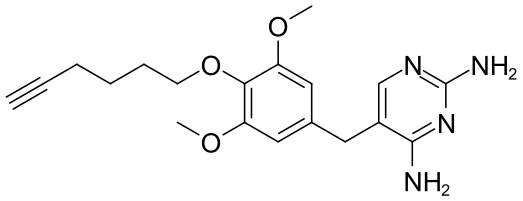


Figure 18: The alkynyl trimethoprim derivative **79**.

3 References

[1] S. C. Davies, *The Drugs Don't Work: A Global Threat*, Penguin Books Limited. 2013.

[2] *Antibiotic Resistance Threats in the United States*. 2013.

[3] A. Fleming. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. *The British Journal of Experimental Pathology*, 10(3):226–236. 1929.

[4] M. Barber. Staphylococcal infection due to penicillin-resistant strains. *British Medical Journal*, 2(4534):863–865. 1947.

[5] P. M. Rountree and E. F. Thomson. Incidence of penicillin-resistant and streptomycin-resistant staphylococci in a hospital. *The Lancet*, 254(6577):501–504. 1949.

[6] P. S. Stewart and J. W. Costerton. Antibiotic resistance of bacteria in biofilms. *The Lancet*, 358(9276):135–138. 2001.

[7] K. Poole. Efflux-mediated multiresistance in Gram-negative bacteria. *Clinical Microbiology and Infection*, 10(1):12–26. 2004.

[8] C. Fuda, M. Suvorov, S. B. Vakulenko and S. Mobashery. The basis for resistance to β-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *The Journal of Biological Chemistry*, 279(39):40802–40806. 2004.

[9] O. Sköld. Sulfonamide resistance: mechanisms and trends. *Drug Resistance Updates*, 3(3):155–160. 2000.

[10] A. E. Clatworthy, E. Pierson and D. T. Hung. Targeting virulence: a new paradigm for antimicrobial therapy. *Nature Chemical Biology*, 3(9):541–548. 2007.

[11] S. R. Palumbi. Humans as the World's Greatest Evolutionary Force. *Science*, 293(5536):1786–1790. 2001.

[12] J. W. Ogle, L. B. Reller and M. L. Vasil. Development of resistance in Pseudomonas aeruginosa to imipenem, norfloxacin, and ciprofloxacin during therapy: proof provided by typing with a DNA probe. *The Journal of Infectious Diseases*, 157(4):743–748. 1988.

[13] P. Huovinen. Resistance to Trimethoprim-Sulfamethoxazole. *Antimicrobial Resistance*, 32(11):1608–1614. 2001.

[14] M. C. Birmingham, C. R. Rayner, A. K. Meagher, S. M. Flavin, D. H. Batts and J. J. Schentag. Linezolid for the treatment of multidrug-resistant , Gram-positive infections: experience from a compassionate-use program. *Clinical Infectious Diseases*, 36(2):159–168. 2003.

[15] D. K. Lee, Y. Kim, K. S. Park, J. W. Yang, K. Kim and N. J. Ha. Antimicrobial activity of mupirocin, daptomycin, linezolid, quinupristin/dalfopristin and tigecycline against vancomycin-resistant enterococci (VRE) from clinical isolates in Korea (1998 and 2005). *Journal of Biochemistry and Molecular Biology*, 40(6):881–887. 2007.

[16] *Oxford English Dictionary*, Oxford University Press. 2014.

[17] M. B. Miller and B. L. Bassler. Quorum sensing in bacteria. *Annual Review of Microbiology*, 55:165–199. 2001.

[18] K. L. Visick and E. G. Ruby. Vibrio fischeri and its host: it takes two to tango. *Current Opinion in Microbiology*, 9(6):632–638. 2006.

[19] J. Graf and E. G. Ruby. Host-derived amino acids support the proliferation of symbiotic bacteria. *Proceedings of the National Academy of Sciences*, 95(4):1818–1822. 1998.

[20] J. D. Lemus and M. J. McFall-Ngai. Alterations in the proteome of the *Euprymna scolopes* light organ in response to symbiotic *Vibrio fischeri*. *Applied and Environmental Microbiology*, 66(9):4091–4097. 2000.

[21] B. W. Jones and M. K. Nishiguchi. Counterillumination in the Hawaiian bobtail squid, *Euprymna scolopes* Berry (Mollusca: Cephalopoda). *Marine Biology*, 144(6):1151–1155. 2004.

[22] E. G. Ruby and K. H. Nealson. Symbiotic association of *Photobacterium fischeri* with the marine luminous fish *Monocentris japonica*: a model of symbiosis based on bacterial studies. *The Biological Bulletin*, 151(3):574–586. 1976.

[23] A. Eberhard, A. L. Burlingame, C. Eberhard, G. L. Kenyon, K. H. Nealson and N. J. Oppenheimer. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry*, 20(9):2444–2449. 1981.

[24] H. B. Kaplan and E. P. Greenberg. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. *Journal of Bacteriology*, 163(3):1210–1214. 1985.

[25] A. L. Schaefer, B. L. Hanzelka, A. Eberhard and E. P. Greenberg. Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interactions with autoinducer analogs. *Journal of Bacteriology*, 178(10):2897–2901. 1996.

[26] J. H. Devine, G. S. Shadel and T. O. Baldwin. Identification of the operator of the lux regulon from the *Vibrio fischeri* strain ATCC7744. *Proceedings of the National Academy of Sciences*, 86(15):5688–5692. 1989.

[27] M. R. Parsek, D. L. Val, B. L. Hanzelka, J. E. Cronan and E. P. Greenberg. Acyl homoserine-lactone quorum-sensing signal generation. *Proceedings of the National Academy of Sciences*, 96(8):4360–4365. 1999.

[28] W. T. Watson, T. D. Minogue, D. L. Val, S. B. von Bodman and M. E. A. Churchill. Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. *Molecular Cell*, 9(3):685–694. 2002.

[29] J. Engebrecht, K. Nealson and M. Silverman. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell*, 32(3):773–781. 1983.

[30] E. G. Ruby and L. M. Asato. Growth and flagellation of *Vibrio fischeri* during initiation of the sepiolid squid light organ symbiosis. *Archives of Microbiology*, 159(2):160–167. 1993.

[31] E. G. Ruby and K. H. H. Lee. The *Vibrio fischeri-Euprymna scolopes* light organ association: current ecological paradigms. *Applied and Environmental Microbiology*, 64(3):805–812. 1998.

[32] S. V. Nyholm and M. J. McFall-Ngai. Sampling the light-organ microenvironment of *Euprymna scolopes*: description of a population of host cells in association with the bacterial symbiont *Vibrio fischeri*. *The Biological Bulletin*, 195(2):89–97. 1998.

[33] B. L. Hanzelka and E. P. Greenberg. Evidence that the N-terminal region of the *Vibrio Fischeri* LuxR protein constitutes an autoinducer binding domain. *Journal of Bacteriology*, 177(3):815–817. 1995.

[34] S. H. Choi and E. P. Greenberg. The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain. *Proceedings of the National Academy of Sciences of the United States of America*, 88(24):11115–11119. 1991.

[35] S. H. Choi and E. P. Greenberg. Genetic dissection of DNA binding and luminescence gene activation by the *Vibrio fischeri* LuxR protein. *Journal of Bacteriology*, 174(12):4064–4069. 1992.

[36] G. S. Shadel and T. O. Baldwin. The *Vibrio fischeri* LuxR protein is capable of bidirectional stimulation of transcription and both positive and negative regulation of the *luxR* gene. *Journal of Bacteriology*, 173(2):568–74. 1991.

[37] S. Choi and E. Greenberg. Genetic evidence for multimerization of LuxR, the transcriptional activator of *Vibrio fischeri* luminescence. *Molecular Marine Biology and Biotechnology*, 1(6):408–413. 1992.

[38] L. C. M. Antunes, R. B. R. Ferreira, C. P. Lostroh and E. P. Greenberg. A mutational analysis defines *Vibrio fischeri* LuxR binding sites. *Journal of Bacteriology*, 190(13):4392–4397. 2008.

[39] T. Miyashiro and E. G. Ruby. Shedding light on bioluminescence regulation in *Vibrio fischeri*. *Molecular Microbiology*, 84(5):795–806. 2012.

[40] P. V. Dunlap and J. M. Ray. Requirement for autoinducer in transcriptional negative autoregulation of the *Vibrio fischeri luxR* gene in *Escherichia coli*. *Journal of Bacteriology*, 171(6):3549–3552. 1989.

[41] S. Verma and T. Miyashiro. Quorum sensing in the squid-*Vibrio* symbiosis. *International Journal of Molecular Sciences*, 14(8):16386–16401. 2013.

[42] C. Lupp, M. Urbanowski, E. P. Greenberg and E. G. Ruby. The *Vibrio fischeri* quorum-sensing systems *ain* and *lux* sequentially induce luminescence gene expression and are important for persistence in the squid host. *Molecular Microbiology*, 50(1):319–331. 2003.

[43] L. Gilson, A. Kuo and P. V. Dunlap. AinS and a new family of autoinducer synthesis proteins. *Journal of Bacteriology*, 177(23):6946–51. 1995.

[44] M. Timmen, B. L. Bassler and K. Jung. AI-1 influences the kinase activity but not the phosphatase activity of LuxN of *Vibrio harveyi*. *Journal of Biological Chemistry*, 281(34):24398–24404. 2006.

[45] M. B. Neiditch, M. J. Federle, S. T. Miller, B. L. Bassler and F. M. Hughson. Regulation of LuxPQ receptor activity by the quorum-sensing signal autoinducer-2. *Molecular Cell*, 18(5):507–518. 2005.

[46] M. B. Neiditch, M. J. Federle, A. J. Pompeani, R. C. Kelly, D. L. Swem, P. D. Jeffrey, B. L. Bassler and F. M. Hughson. Ligand-induced asymmetry in histidine sensor kinase complex regulates quorum sensing. *Cell*, 126(6):1095–1108. 2006.

[47] B. L. Bassler, M. Wright and M. R. Silverman. Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Molecular Microbiology*, 13(2):273–286. 1994.

[48] A. M. Stock, V. L. Robinson and P. N. Goudreau. Two-component signal transduction. *Annual Review of Biochemistry*, 69(1):183–215. 2000.

[49] J. A. Freeman and B. L. Bassler. Sequence and function of LuxU: a two-component phosphorelay protein that regulates quorum sensing in *Vibrio harveyi*. *Journal of Bacteriology*, 181(3):899–906. 1999.

[50] M. T. Laub and M. Goulian. Specificity in two-component signal transduction pathways. *Annual Review of Genetics*, 41:121–145. 2007.

[51] D. L. Milton. Quorum sensing in vibrios: Complexity for diversification. *International Journal of Medical Microbiology*, 296(2-3):61–71. 2006.

[52] C. M. Miyamoto, Y. H. Lin and E. A. Meighen. Control of bioluminescence in Vibrio fischeri by the LuxO signal response regulator. *Molecular Microbiology*, 36(3):594–607. 2000.

[53] T. Miyashiro, M. S. Wollenberg, X. Cao, D. Oehlert and E. G. Ruby. A single qrr gene is necessary and sufficient for LuxO-mediated regulation in Vibrio fischeri. *Molecular microbiology*, 77(6):1556–67. 2010.

[54] B. N. Lilley and B. L. Bassler. Regulation of quorum sensing in Vibrio harveyi by LuxO and Sigma-54. *Molecular Microbiology*, 36(4):940–954. 2000.

[55] D. H. Lenz, K. C. Mok, B. N. Lilley, R. V. Kulkarni, N. S. Wingreen and B. L. Bassler. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in Vibrio harveyi and Vibrio cholerae. *Cell*, 118(1):69–82. 2004.

[56] P. M. Fidopiastis, C. M. Miyamoto, M. G. Jobling, E. a. Meighen and E. G. Ruby. LitR, a new transcriptional activator in Vibrio fischeri, regulates luminescence and symbiotic light organ colonization. *Molecular Microbiology*, 45(1):131–143. 2002.

[57] K. L. Visick, J. Foster, J. Doino, M. McFall-Ngai and E. G. Ruby. Vibrio fischeri lux genes play an important role in colonization and development of the host light organ. *Journal of Bacteriology*, 182(16):4578–4586. 2000.

[58] C. Lupp and E. G. Ruby. Vibrio fischeri Uses Two Quorum-Sensing Systems for the Regulation of Early and Late Colonization Factors Vibrio fischeri Uses Two Quorum-Sensing Systems for the Regulation of Early and Late Colonization Factors. 187(11):3620–3629. 2005.

[59] C. Lupp and E. G. Ruby. Vibrio fischeri LuxS and AinS: Comparative Study of Two Signal Synthases. *Journal of Bacteriology*, 186(12):3873–3881. 2004.

[60] J. L. REICHELT, K. NEALSON and J. W. HASTINGS. The Specificity of Symbiosis: Pony Fish and Luminescent Bacteria. *Arch. Microbiol.*, 112:157–161. 1977.

[61] A. Ramesh, R. Nandakumar and V. K. Venugopalan. Enteric Luminous Microflora of the Pond-Cultured Milk Fish Chanos chanos (Forskal). pages 231–235. 1986.

[62] D. S. Millikan and E. G. Ruby. Alterations in Vibrio fischeri motility correlate with a delay in symbiosis initiation and are associated with additional symbiotic colonization defects. *Applied and Environmental Microbiology*, 68(5):2519–2528. 2002.

[63] J. Graf, P. V. Dunlap and E. G. Ruby. Effect of transposon-induced motility mutations on colonization of the host light organ by Vibrio fischeri. *Journal of Bacteriology*, 176(22):6986–6991. 1994.

[64] I. S. Roberts. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual Review of Microbiology*, 50(1):285–315. 1996.

[65] S. M. Callahan and P. V. Dunlap. lux Genes Define a Quorum-Sensing Regulon in Vibrio fischeri LuxR- and Acyl-Homoserine-Lactone-Controlled Non- lux Genes Define a Quorum-Sensing Regulon in Vibrio fischeri. *Journal of bacteriology*, 182(10):2811–2822. 2000.

[66] N. Qin, S. M. Callahan, P. V. Dunlap and A. M. Stevens. Analysis of LuxR regulon gene expression during quorum sensing in Vibrio fischeri. *Journal of Bacteriology*, 189(11):4127–4134. 2007.

[67] W. C. Fuqua, S. C. Winans and E. P. Greenberg. MINIREVIEW Quorum Sensing in Bacteria : the LuxR-LuxI Family of Cell Density-Responsive Transcriptional Regulatorst. *Journal of Bacteriology*, 176(2):269–275. 1994.

[68] C. M. Waters and B. L. Bassler. Quorum sensing: cell-to-cell communication in bacteria. *Annual Review of Cell and Developmental Biology*, 21:319–346. 2005.

[69] S. Atkinson, C.-Y. Chang, R. E. Sockett, M. Cámara and P. Williams. Quorum Sensing in Yersinia enterocolitica Controls Swimming and Swarming Motility. *Journal of Bacteriology*, 188(4):1451–1461. 2006.

[70] K.-G. Chan, S. D. Puthucheary, X.-Y. Chan, W.-F. Yin, C.-S. Wong, W.-S. S. Too and K.-H. Chua. Quorum sensing in Aeromonas species isolated from patients in Malaysia. *Current Microbiology*, 62(1):167–72. 2011.

[71] K. Sauer, A. K. Camper, G. D. Ehrlich, J. W. Costerton and D. G. Davies. Pseudomonas aeruginosa Displays Multiple Phenotypes during Development as a Biofilm. *Journal of Bacteriology*, 184(4):1140–1154. 2002.

[72] B. Michael, J. N. Smith, S. Swift and F. Heffron. SdiA of Salmonella enterica Is a LuxR Homolog That Detects Mixed Microbial Communities. *Journal of Bacteriology*, 183(19):5733–5742. 2001.

[73] B. M. M. Ahmer. Cell-to-cell signalling in Escherichia coli and Salmonella enterica. *Molecular Microbiology*, 52(4):933–945. 2004.

[74] K. H. Nealson, T. Platt and J. W. Hastings. Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System. *Journal of bacteriology*, 104(1):313–322. 1970.

[75] E. Klein, D. L. Smith and R. Laxminarayan, *Hospitalizations and Deaths Caused by Methicillin-Resistant Staphylococcus aureus, United States, 1999-2005*. 2007.

[76] L. C. M. Antunes, R. B. R. Ferreira, M. M. C. Buckner and B. B. Finlay. Quorum sensing in bacterial virulence. *Microbiology*, 156:2271–2282. 2010.

[77] G. P. Bodey, R. Bolivar, V. Fainstein and L. Jadeja. Infections caused by *Pseudomonas aeruginosa*. *Reviews of Infectious Diseases*, 5(2):279–313. 1983.

[78] G. Xiao, E. Déziel, J. He, F. Lépine, B. Lesic, M.-H. Castonguay, S. Milot, A. P. Tampakaki, S. E. Stachel and L. G. Rahme. MvfR, a key Pseudomonas aeruginosa pathogenicity LTTR-class regulatory protein, has dual ligands. *Molecular Microbiology*, 62(6):1689–99. 2006.

[79] J.-F. Dubern and S. P. Diggle. Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. *Molecular BioSystems*, 4(9):882–888. 2008.

[80] P. Cornelis, *Pseudomonas: Genomics and Molecular Biology*, Caister Academic Press. 2008.

[81] R. C. Hider and X. Kong. Chemistry and biology of siderophores. *Natural Product Reports*, 27(5):637–657. 2010.

[82] M. R. Seyedsayamdost, S. Cleto, G. Carr, H. Vlamakis, M. João Vieira, R. Kolter and J. Clardy. Mixing and matching siderophore clusters: structure and biosynthesis of serratiochelins from Serratia sp. V4. *Journal of the American Chemical Society*, 134(33):13550–135503. 2012.

[83] T. Zheng and E. M. Nolan. Siderophore-based detection of Fe(III) and microbial pathogens. *Metallomics*, 4:866–880. 2012.

[84] C. J. Carrano and K. N. Raymond. Synthesis and characterization of iron complexes of rhodotorulic acid: a novel dihydroxamate siderophore and potential chelating drug. *Journal of the Chemical Society, Chemical Communications*, (12):501. 1978.

[85] M. B. Hossain, D. L. Eng-Wilmot, R. A. Loghry and D. van der Helm. Circular Dichroism , Crystal Structure , and Absolute Configuration of the Siderophore Ferric N,N',N"-Triacetylfusarinine, FeC39H57N60. *Journal of the American Chemical Society*, 102:5766–5773. 1980.

[86] D. van der Helm, J. R. Baker, D. L. Eng-Wilmot, M. B. Hossain and R. A. Loghry. Crystal Structure of Ferrichrome and a Comparison with the Structure of Ferrichrome A. *Journal of the American Chemical Society*, 102(12):4224–4231. 1980.

[87] K. Schlegel, J. Lex, K. Taraz and H. Budzikiewicz. The X-ray structure of the pyochelin Fe3+ complex. *Zeitschrift für Naturforschung*, 61c:263–266. 2006.

[88] M. G. P. Page. Siderophore conjugates. *Annals of the New York Academy of Sciences*, 1277:115–126. 2013.

[89] A. Hartmann, H.-P. Fiedler and V. Braun. Uptake and conversion of the antibiotic albomycin by Escherichia coli K-12. *European Journal of Biochemistry*, 99(3):517–24. 1979.

[90] H. Fiedler, F. Walz, A. Diihle and H. Ziihner. Albomycin : Studies on fermentation , isolation and quantitative determination. pages 341–347. 1985.

[91] G. F. Gause. Recent studies on albomycin, a new antibiotic. *British Medical Journal*, 2(4949):1177–1179. 1955.

[92] A. Pramanik, U. H. Stroeher, J. Krejci, A. J. Standish, E. Bohn, J. C. Paton, I. B. Autenrieth and V. Braun. Albomycin is an effective antibiotic , as exemplified with Yersinia enterocolitica and Streptococcus pneumoniae. *International Journal of Medical Microbiology*, 297(6):459–469. 2007.

[93] M. Hannauer, Y. Barda, G. L. A. Mislin, A. Shanzer and I. J. Schalk. The Ferrichrome Uptake Pathway in Pseudomonas aeruginosa Involves an Iron Release Mechanism with Acylation of the Siderophore and Recycling of the Modified Desferrichrome. *Journal of Bacteriology*, 192(5):1212–1220. 2010.

[94] R. C. Hider and X. L. Kong. Chemistry and Biology of Siderophores. (January). 2015.

[95] V. Braun, A. Pramanik, T. Gwinner, M. Köberle and E. Bohn. Sideromycins : tools and antibiotics. pages 3–13. 2009.

[96] W. SACKMANN, P. REUSSER, L. NEIPP, F. KRADOLFER and F. GROSS. Ferrimycin A, a new iron-containing antibiotic. *Antibiotics & Chemotherapy*, 12:34–45. 1962.

[97] D. Gottlieb and P. D. Shaw, *Mechanism of Action*, Springer Berlin Heidelberg. 2012.

[98] G. Benz, T. Schröder, J. Kurz, C. Wünsche, W. Karl, G. Steffens, J. Pfitzner and D. Schmidt. Constitution of the Deferriform of the Albomycins $\delta 1$, $\delta 2$ and ϵ. *Angewandte Chemie International Edition in English*, 21(7):527–528. 1982.

[99] U. Möllmann, L. Heinisch, A. Bauernfeind, T. Köhler and D. Ankel-Fuchs. Siderophores as drug delivery agents: application of the "Trojan Horse" strategy. *Biometals*, 22(4):615–624. 2009.

[100] C. Dini and J. Aszodi. Synthesis of a dihydroxythiophene analogue of catechosporines. *Bioorganic & Medicinal Chemistry Letters*, 10(4):349–352. 2000.

[101] T. Kline, M. Fromhold, T. E. Mckennon, S. Cai, J. Treiberg, N. Ihle, D. Sherman, W. Schwan, M. J. Hickey, P. Warrener, P. R. Witte, L. L. Brody, L. Goltry, L. M. Barker, S. U. Anderson, S. K. Tanaka, R. M. Shawar, L. Y. Nguyen, M. Langhorne, A. Bigelow, L. Embuscado and E. Naeemi. Antimicrobial Effects of Novel Siderophores Linked to -Lactam Antibiotics. *Bioorganic & Medicinal Chemistry*, 8(2000):73–93. 1999.

[102] Y. Lu and M. J. Miller. Syntheses and Studies of Multiwarhead Siderophore-5-fluorouridine Conjugates. *Bioorganic & Medicinal Chemistry*, 7(1999):3025–3038. 1999.

[103] M. Ghosh and M. J. Miller. Synthesis and in vitro antibacterial activity of spermidine-based mixed catechol- and hydroxamate-containing siderophore–vancomycin conjugates. *Bioorganic & Medicinal Chemistry*, 4(1):43–48. 1996.

[104] M. Ghosh and M. J. Miller. Design, synthesis, and biological evaluation of isocyanurate-based antifungal and macrolide antibiotic conjugates: iron transport-mediated drug delivery. *Bioorganic & Medicinal Chemistry*, 3(11):1519–1525. 1995.

[105] S. R. Md-Saleh, E. C. Chilvers, K. G. Kerr, S. J. Milner, A. M. Snelling, J. P. Weber, G. H. Thomas, A.-K. Duhme-Klair and A. Routledge. Synthesis of citrate-ciprofloxacin conjugates. *Bioorganic & Medicinal Chemistry Letters*, 19(5):1496–1498. 2009.

[106] F. Rivault, C. Liébert, A. Burger, F. Hoegy, M. A. Abdallah, I. J. Schalk and G. L. A. Mislin. Synthesis of pyochelin-norfloxacin conjugates. *Bioorganic & Medicinal Chemistry Letters*, 17(3):640–644. 2007.

[107] C. Ji and M. J. Miller. Chemical syntheses and in vitro antibacterial activity of two desferrioxamine B-ciprofloxacin conjugates with potential esterase and phosphatase triggered drug release linkers. *Bioorganic & Medicinal Chemistry*, 20(12):3828–3836. 2012.

[108] T. Zheng and E. M. Nolan. Enterobactin-Mediated Delivery of β-Lactam Antibiotics Enhances Antibacterial Activity Against Pathogenic Escherichia coli. *Journal of the American Chemical Society*. 2014.

[109] G. E. Zurenko, S. E. Truesdell, B. H. Yagi, R. J. Mourey and A. L. Laborde. In vitro antibacterial activity and interactions with beta-lactamases and penicillin-binding proteins of the new monocarbam antibiotic U-78608. *Antimicrobial Agents and Chemotherapy*, 34(5):884–8. 1990.

[110] J. M. Harrington, T. Gootz, M. Flanagan, M. Lall, J. O'Donnell, J. Winton, J. Mueller and A. L. Crumbliss. Characterization of the aqueous iron(III) chelation chemistry of a potential Trojan Horse antimicrobial agent: Chelate structure, stability and pH dependent speciation. *BioMetals*, 25(5):1023–1036. 2012.

[111] I. J. Schalk and G. L. A. Mislin. Bacterial Iron Uptake Pathways: Gates for the Import of Bactericide Compounds. 2017.

[112] C. J. McPherson, L. M. Aschenbrenner, B. M. Lacey, K. C. Fahnoe, M. M. Lemmon, S. M. Finegan, B. Tadakamalla, J. P. O. Donnell, J. P. Mueller and A. P. Tomaras. Clinically Relevant Gram-Negative Resistance Mechanisms Have No Effect on the Efficacy of MC-1 , a Novel Siderophore-Conjugated. 56(12):6334–6342. 2012.

[113] A. Ito, T. Sato, M. Ota, M. Takemura, T. Nishikawa, S. Toba, N. Kohira, S. Miyagawa, N. Ishibashi, S. Matsumoto, R. Nakamura, M. Tsuji and Y. Yamanoa. In Vitro Antibacterial Properties of Cefiderocol, a Novel Siderophore Cephalosporin, against Gram-Negative Bacteria. *Antimicrobial Agents and Chemotherapy*, 62(1):1–11. 2018.

[114] J. S. Yutaka Saisho, Takayuki Katsube, Scott White, Hiroyuki Fukase. Pharmacokinetics, Safety, and Tolerability of Cefiderocol, a Novel Siderophore Cephalosporin for Gram-Negative Bacteria, in Healthy Subjects. *Antimicrobial Agents and Chemotherapy*, (January). 2018.

[115] F. Paech, S. Messner, J. Spickermann, M. Wind, A. Hortense, S. Hoffmann, A. Therese, W. Brett, A. H. Rachel, J. C. Jeff, W. Marc, S. Krähenbühl and M. Maurer. Mechanisms of hepatotoxicity associated with the monocyclic β - lactam antibiotic BAL30072. *Archives of Toxicology*, 91(11):3647–3662. 2017.

[116] C. Florez, J. E. Raab, A. C. Cooke and J. W. Schertzer. Membrane Distribution of the Pseudomonas Quinolone Signal Modulates Outer Membrane Vesicle Production in Pseudomonas aeruginosa. pages 1–13. 2017.

[117] J. P. Pearson, C. Van Delden and B. H. Iglewski. Active Efflux and Diffusion Are Involved in Transport of Pseudomonas aeruginosa Cell-to-Cell Signals. *J Bacteriol*, 181(4):1203–1210. 1999.

[118] C. M. Oliphant and G. M. Green. Quinolones: a comprehensive review. *American Family Physician*, 65(3):455–464. 2002.

[119] A. P. Macgowan, M. Wootton and H. A. Holt. The antibacterial efficacy of levofloxacin and ciprofloxacin against *Pseudomonas aeruginosa* assessed by combining antibiotic exposure and bacterial susceptibility. *Journal of Antimicrobial Chemotherapy*, 43:345–349. 1999.

[120] D. J. Evans, D. G. Allison, M. R. W. Brown and P. Gilbert. Susceptibility of Pseudomonas aeruginosa and Escherichia coli biofilms towards ciprofloxacin : effect of specific growth rate. (August):177–184. 1991.

[121] R. A. Celesk and N. J. Robillard. Factors Influencing the Accumulation of Ciprofloxacin in Pseudomonas aeruginosa. 33(11):1921–1926. 1989.

[122] K. Poole. MINIREVIEW Efflux-Mediated Resistance to Fluoroquinolones in Gram-Negative Bacteria. 44(9):2233–2241. 2000.

[123] T. R. De Kievit, M. D. Parkins, R. J. Gillis, R. Srikumar, H. Ceri, K. Poole, B. H. Iglewski and D. G. Storey. Multidrug Efflux Pumps : Expression Patterns and Contribution to Antibiotic Resistance in Pseudomonas aeruginosa Biofilms. 45(6):1761–1770. 2001.

[124] R. N. Brogden, A. A. Carmine, R. C. Heel, T. M. Speight and G. S. Avery. Trimethoprim: A Review of its Antibacterial Activity, Pharmacokinetics and Therapeutic Use in Urinary Tract Infections. *Drugs*, 23(6):405–430. 1982.

[125] T. Köhler, M. Kok, M. Michea-hamzehpour, P. Plesiat, N. Gotoh, T. Nishino, L. K. Curty and J.-c. Pechere. Multidrug Efflux in Intrinsic Resistance to Trimethoprim and Sulfamethoxazole in Pseudomonas aeruginosa. 40(10):2288–2290. 1996.

[126] C. W. Tornøe, C. Christensen and M. Meldal. Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *The Journal of Organic Chemistry*, 67(9):3057–3064. 2002.

[127] V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless. A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angewandte Chemie International Edition*, 41(14):2596–2599. 2002.

[128] K. Ganguly, R. Wu, M. Ollivault-Shiflett, P. M. Goodwin, L. A. Silks and R. Iyer. Design, synthesis, and a novel application of quorum-sensing agonists as potential drug-delivery vehicles. *Journal of Drug Targeting*, 19(7):528–539. 2011.

[129] R. Iyer, K. Ganguly and L. A. Silks. Synthetic analogs of bacterial quorum sensors. Los Alamos National Laboratory. 2012.

[130] A. Eberhard, C. A. Widrig, P. Mcbath and J. B. Schineller. Analogs of the autoinducer of bioluminescence in Vibrio fischeri. *Archives of Microbiology*, 146(1):35–40. 1986.

[131] L. Passador, K. D. Tucker, K. R. Guertin, M. P. Journet, A. S. Kende and B. H. Iglewski. Functional analysis of the Pseudomonas aeruginosa autoinducer PAI. *Journal of Bacteriology*, 178(20):5995–6000. 1996.

[132] K. M. Smith, Y. Bu and H. Suga. Library Screening for Synthetic Agonists and Antagonists of a Pseudomonas aeruginosa Autoinducer. *Chemistry & Biology*, 10(6):563–571. 2003.

[133] S. R. Chhabra, P. Stead, N. J. Bainton, G. P. Salmond, G. S. Stewart, P. Williams and B. W. Bycroft. Autoregulation of carbapenem biosynthesis in Erwinia carotovora by analogues of N-(3-oxohexanoyl)-L-homoserine lactone. *The Journal of Antibiotics*, 46(3):441–454. 1993.

[134] C. E. McInnis and H. E. Blackwell. Thiolactone modulators of quorum sensing revealed through library design and screening. *Bioorganic & Medicinal Chemistry*, 19(16):4820–4828. 2011.

[135] G. D. Geske, J. C. O. Neill, D. M. Miller, M. E. Mattmann and H. E. Blackwell. Modulation of Bacterial Quorum Sensing with Synthetic Ligands : Systematic Evaluation of N-Acylated Homoserine Lactones in Multiple Species and New Insights into Their Mechanisms of Action. *Journal of the American Chemical Society*, 129(44):13613–13625. 2007.

[136] J. C. A. Janssens, K. Metzger, R. Daniels, D. Ptacek, T. Verhoeven, L. W. Habel, J. Vanderleyden, D. E. De Vos and S. C. J. De Keersmaecker. Synthesis of N -Acyl Homoserine Lactone Analogues Reveals Strong Activators of SdiA , the Salmonella enterica Serovar. *Applied and Environmental Microbiology*, 73(2):535–544. 2007.

[137] W. R. J. D. Galloway, J. T. Hodgkinson, S. D. Bowden, M. Welch and D. R. Spring. Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chemical Reviews*, 111(1):28–67. 2011.

[138] J. T. Hodgkinson, W. R. J. D. Galloway, M. Wright, I. K. Mati, R. L. Nicholson, M. Welch and D. R. Spring. Design, synthesis and biological evaluation of non-natural modulators of quorum sensing in Pseudomonas aeruginosa. *Organic & Biomolecular Chemistry*, 10(30):6032. 2012.

[139] M. E. Boursier, D. E. Manson, J. B. Combs, E. Helen and H. E. Blackwell. A comparative study of non-native N-acyl L-homoserine lactone analogs in two Pseudomonas aeruginosa quorum sensing receptors that share a common native ligand yet inversely regulate virulence. *Bioorganic & Medicinal Chemistry*, pages 1–17. 2018.

[140] K. M. Smith, Y. Bu and H. Suga. Induction and Inhibition of Pseudomonas aeruginosa Quorum Sensing by Synthetic Autoinducer Analogs. *Chemistry & Biology*, 10(1):81–89. 2003.

[141] G. J. Jog, J. Igarashi and H. Suga. Stereoisomers of P. aeruginosa Autoinducer Analog to Probe the Regulator Binding Site. *Chemistry and Biology*. 2006.

[142] C. Lu, B. Kirsch, C. Zimmer, J. C. De Jong, C. Henn, C. K. Maurer, M. Müsken, S. Häussler, A. Steinbach and R. W. Hartmann. Discovery of antagonists of PqsR, a key player in 2-alkyl-4-quinolone- dependent quorum sensing in Pseudomonas aeruginosa. *Chemistry and Biology*, 19(3):381–390. 2012.

[143] C. Lu, C. K. Maurer, B. Kirsch, A. Steinbach and R. W. Hartmann. Overcoming the unexpected functional inversion of a PqsR antagonist in Pseudomonas aeruginosa: An in vivo potent antivirulence agent targeting pqs quorum sensing. *Angewandte Chemie - International Edition*, 53(4):1109–1112. 2014.

[144] J. Hodgkinson, S. D. Bowden, W. R. J. D. Galloway, D. R. Spring and M. Welch. Structure-activity analysis of the Pseudomonas quinolone signal molecule. *Journal of Bacteriology*, 192(14):3833–3837. 2010.

[145] Y. R. Baker. Investigating quinolone based quorum sensing in Pseudomonas aeruginosa using a chemical proteomics approach. PhD thesis, University of Cambridge. 2015.

[146] T. E. Renau, J. P. Sanchez, J. W. Gage, J. A. Dever, M. A. Shapiro, S. J. Gracheck and J. M. Domagala. Structure-activity relationships of the quinolone antibacterials against mycobacteria: effect of structural changes at N-1 and C-7. *Journal of Medicinal Chemistry*, 39(3):729–735. 1996.

[147] C. Jing and V. W. Cornish. A fluorogenic TMP-tag for high signal-to-background intracellular live cell imaging. *ACS Chemical Biology*, 8(8):1704–12. 2013.

Todo list