Contents

1 Introduction

Antibiotic

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. Unfortunately, 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:[1,6]

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.[7]

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*.[8]

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.[9]

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 *para*-aminobenzoic acid - a process which is blocked by sulfonamides.[10]

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.[11–16]

The current pipeline of new antibiotics is widely thought to be worryingly inadequate.[17–19] Significant changes in how we use the antibiotics we already have, as well as investments in the discovery of new ones, are required. Antibiotics currently in late-stage clinical trials nearly all rely on non-novel mechanisms of action,[17] and so it is almost inevitable that resistance to them will develop quickly, as it has done for their predecessors.

There is therefore increasing interest in treatments which would not easily provoke the development of resistance.[20] These treatments often target bacterial virulence rather than killing bacteria outright, hence decreasing selection pressure for resistance.[11] One obvious target is toxin production, for example, an LpxC inhibitor was shown to prevent lethal *Acinetobacter baumannii* infection in mice, despite being inactive against the bacterium *in vitro*.[21] This was due to inhibition of lipopolysaccharide shedding, and hence reduced inflammation in the host. Co-ordination of virulence has also been targeted, for example, analogues of *P. aeruginosa* homoserine lactone autoinducers (see 1.3.1) inhibit the production of virulence factors and increase the survival time of mice in a lethal *P. aeruginosa* lung infection model.[11]

A second strategy in novel antibiotic discovery is to enhance or restore activity of a known antibiotic by lessening or avoiding a resistance mechanism. For example, antibiotics are often excluded from cells due to membrane impermeability or efflux. This may be overcome by attaching the antibiotic 'warhead' to a molecule which the cell imports. The most well known example of this strategy is antibody-drug conjugates[22] in the treatment of cancer, but progress has also made against bacteria. In particular, siderophore-antibiotic conjugates (see 1.2) have been investigated in the hope of hijacking bacterial uptake mechanisms to import antibiotics,[23] and the autoinducer-antibiotic conjugates in this study may gain activity by avoiding efflux pumps (see 1.3). These conjugates may have competing mechanisms of action: either the antibiotic accumulates in the cell to a greater extent and acts by its usual mechanism, or an important bacterial system must be disrupted to avoid accumulation of the antibiotic, hence leading to decreased fitness and/or loss of virulence.

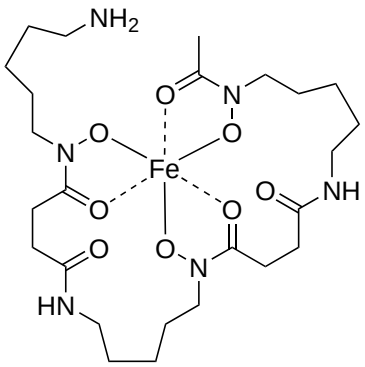
1.2 Siderophore-antibiotic conjugates

Siderophore-antibiotic conjugates have been receiving attention in recent years as a way to enhance the uptake of known antibiotics.[23] This section will discuss the role of siderophores, sideromycins (natural siderophore-antibiotic conjugates), and the synthetic siderophore-antibiotic conjugates inspired by them. Many of the ob-

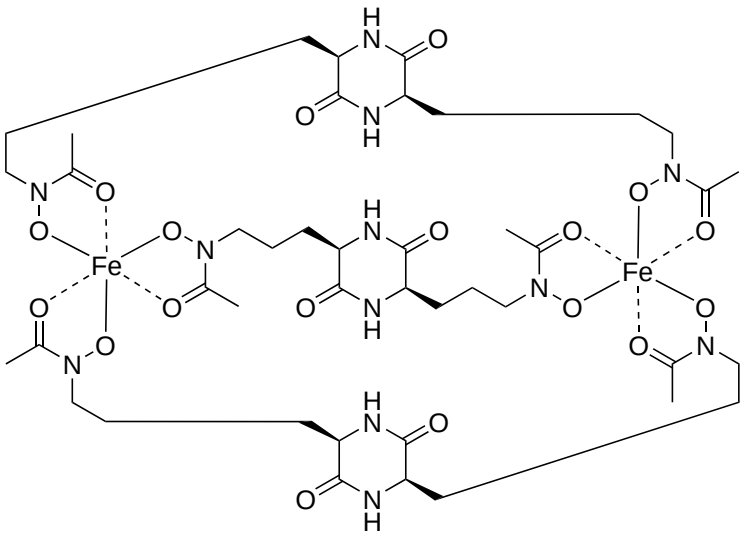
servations made about these molecules could be relevant to the autoinducer-antibiotic conjugates synthesised in this study.

1.2.1 Siderophores

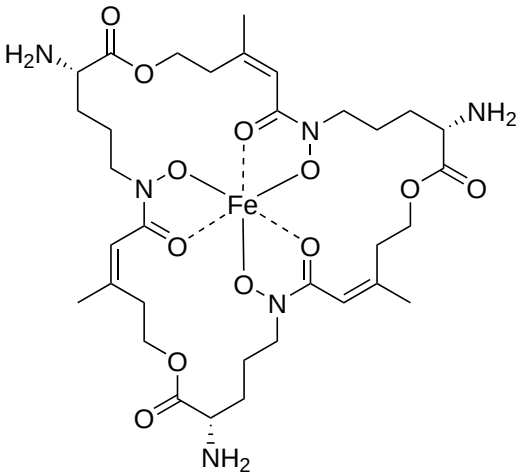
Siderophores are peptides or small molecules used by microorganisms to chelate iron for the purposes of 'iron mining'.[24] Soluble iron is often scarce but it is crucial for many cellular processes including respiration and DNA synthesis. Siderophores are synthesised by the microorganisms and secreted 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 1 and Figure 2), possibly so one species can avoid its siderophores being taken up by another species.[25]



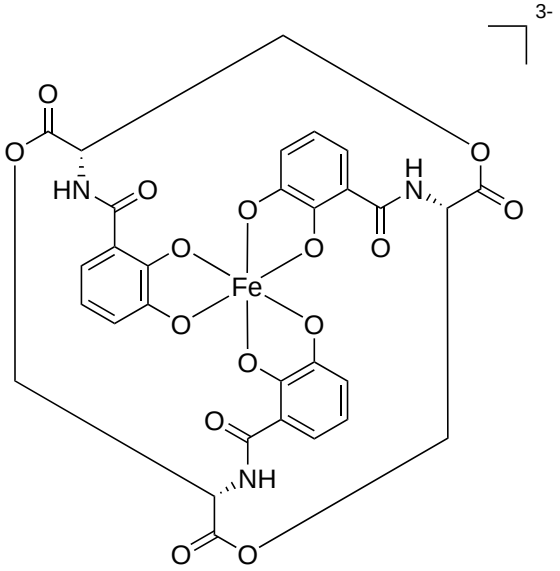
Deferoxamine B
1



Rhodotorulic acid
2



Fusarinine C
3



Enterobactin
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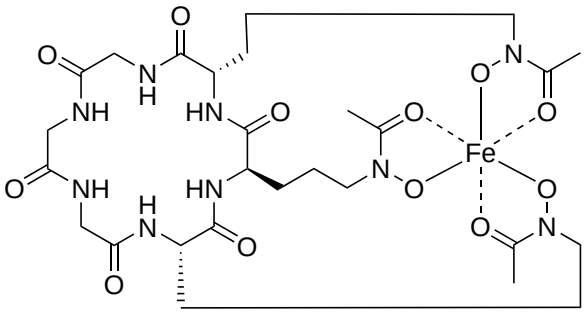
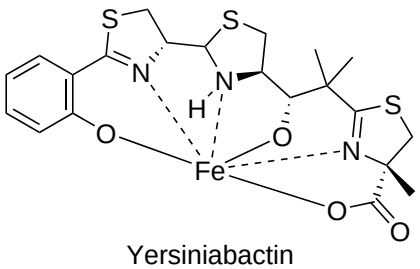
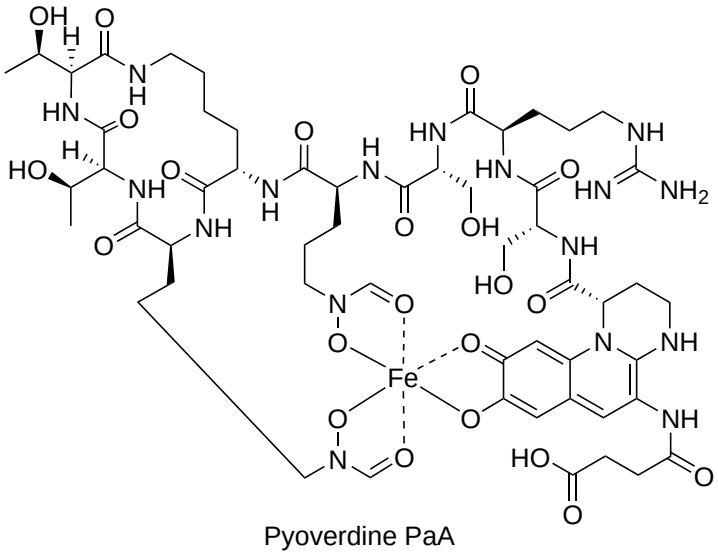


Ferrichrome
5



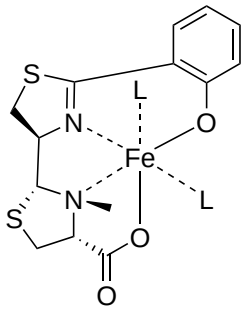
Yersiniabactin
6

Figure 1: Iron-siderophore complexes: Deferoxamine B **1**[26] (*Streptomyces pilosus* and *Streptomyces coelicolor*), rhodotorulic acid **2**[27] (*Rhodotorula pilimanae*), fusarinine C **3**[28] (*Fusarium roseum*), enterobactin **4**[26] (*Escherichia coli* and enteric bacteria), ferrichrome **5**[29] (*Ustilago sphaerogena*, *U. maydis*, *Aspergillus niger*, *A. quadricintus*, *A. duricaulis* and *Penicillium resticolosum*), yersiniabactin **6**[26] (*Yersinia pestis*).



Pyoverdine PaA

7



Pyochelin

8

Figure 2: Iron-siderophore complexes: pyoverdine PaA **7**[26,30] (*P. aeruginosa*, PAO1 strain) and pyochelin **8**[31,32] (*P. aeruginosa*). Note that pyochelin **8** is a tetradentate ligand, hence the iron ion has two sites which can bind other ligands.

1.2.2 Sideromycins

Siderophore-antibiotic conjugates are produced naturally by some bacteria and are known as sideromycins[23] (see Figure 3). Bacteria produce these molecules to attack other bacteria by hijacking their siderophore uptake mechanisms to introduce toxic compounds.

For example, albomycin **9** (see Figure 3) is a sideromycin produced by *Actinomyces subtropicus* and *Streptomyces griseus*[33,34] which has been used to treat infections caused by various bacteria including *Yersinia enterocolitica* and *Streptococcus pneumoniae* in mice and humans.[35,36] Albomycin **9** contains a siderophore coupled to a nuceloside antibiotic via a peptide linker. The siderophore section is structurally similar to ferrichrome **5** (see Figure 1), a siderophore produced by various fungi, but also taken up by bacteria including *Escherichia coli*, *Salmonella typhimurium* and *P. aeruginosa*.[29,37] It has been shown that because of the structural similarity to ferrichrome **5**, *E. coli* will also take up albomycin **9**.[33] 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[35] and other sideromycins such as salmycin A[24,38,39] and ferrimycin A1[40,41] has served as encouragement to many researchers to explore synthetic siderophore-antibiotic conjugates, which will be discussed in the next section.

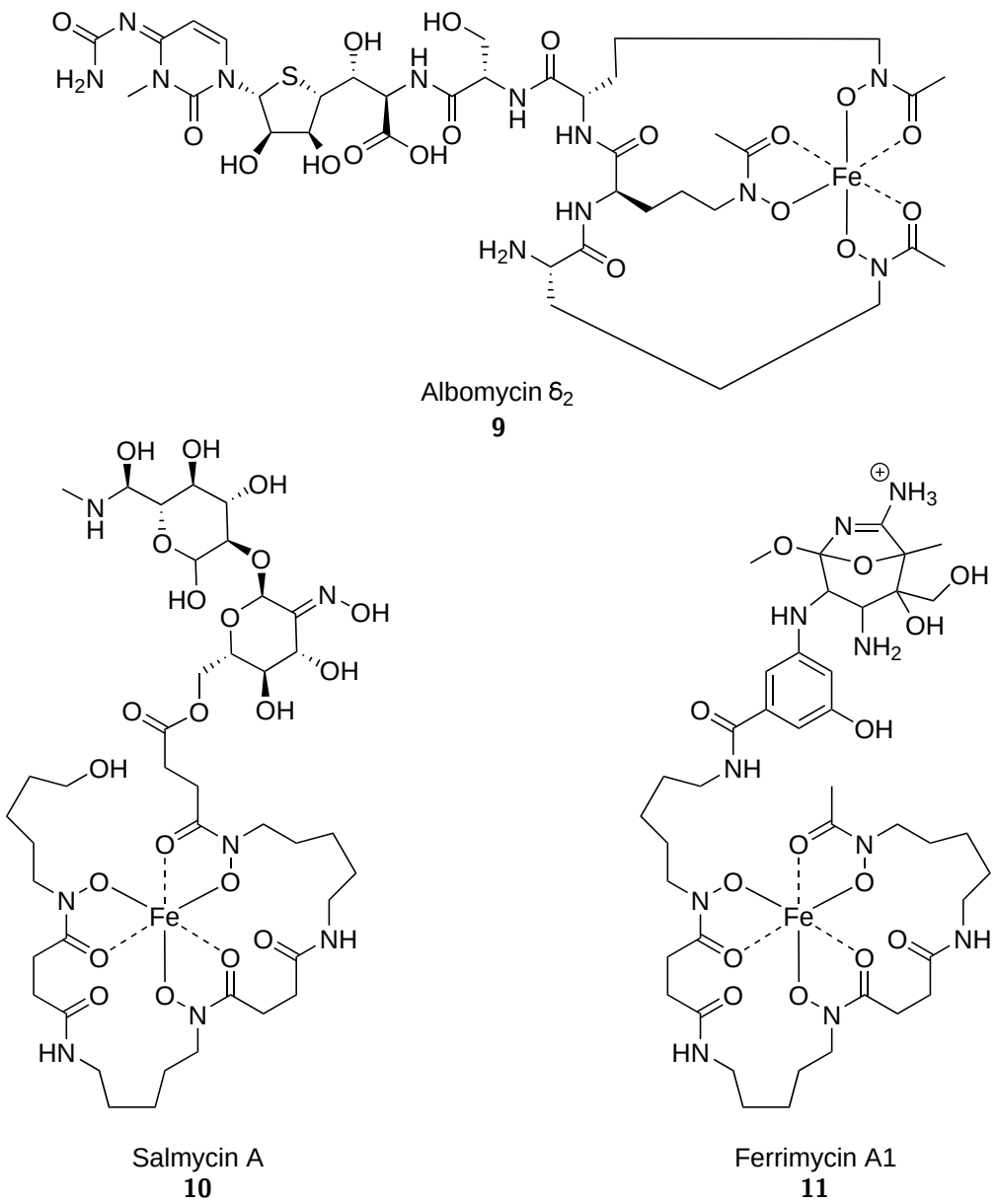


Figure 3: Iron-sideromycin complexes: Albomycin **9**[24,42] (*Actinomyces subtropicus* and *Streptomyces griseus*), salmycin A[24,38,39] (*Streptomyces violaceus*) and ferrimycin[24] (*Streptomyces griseoflavus*).

1.2.3 Synthetic siderophore-antibiotic conjugates

Sideromycins served as inspiration for the design, synthesis and biological evaluation of a wide range of synthetic siderophore-antibiotic conjugates.[23] Antibiotics used include β-lactams,[43–45] nucleosides,[46] glycopeptides[47] and macrolides.[48] Sideromycin-fluoroquinolone conjugates have also been studied by several groups,[49–51] including conjugates with linkers which can be cleaved[50,51] in a similar manner to albomycin.[33] 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.[23] Recently a conjugate (Ent-Amp **12**, see Figure 4) 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.[52] Other

work has focused on monocyclic β-lactams, for example pirazmonam **13** and U-78608 **14**, which show high potency against Gram-negative bacteria including *P. aeruginosa*,[53,54] 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:[55] MC-1 **15**,[56] BAL30072 **16**[23] (see Figure 4) and cefiderocol **17**.[57,58]

MC-1 **15** is reported as being 'in clinical phases of development',[55] but no reports of studies in humans could be found. However, experiments in mice have been promising.[56] BAL30072 **16** 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.[23] However, it is unclear whether it will progress further in trials as it causes liver toxicity.[59] Cefiderocol **17** 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'.[58]

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 **9**-resistant mutants have been shown to be less virulent,[36] 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[60] with an antibiotic can be extended to conjugates of autoinducers and antibiotics in a similar 'Trojan horse' approach.

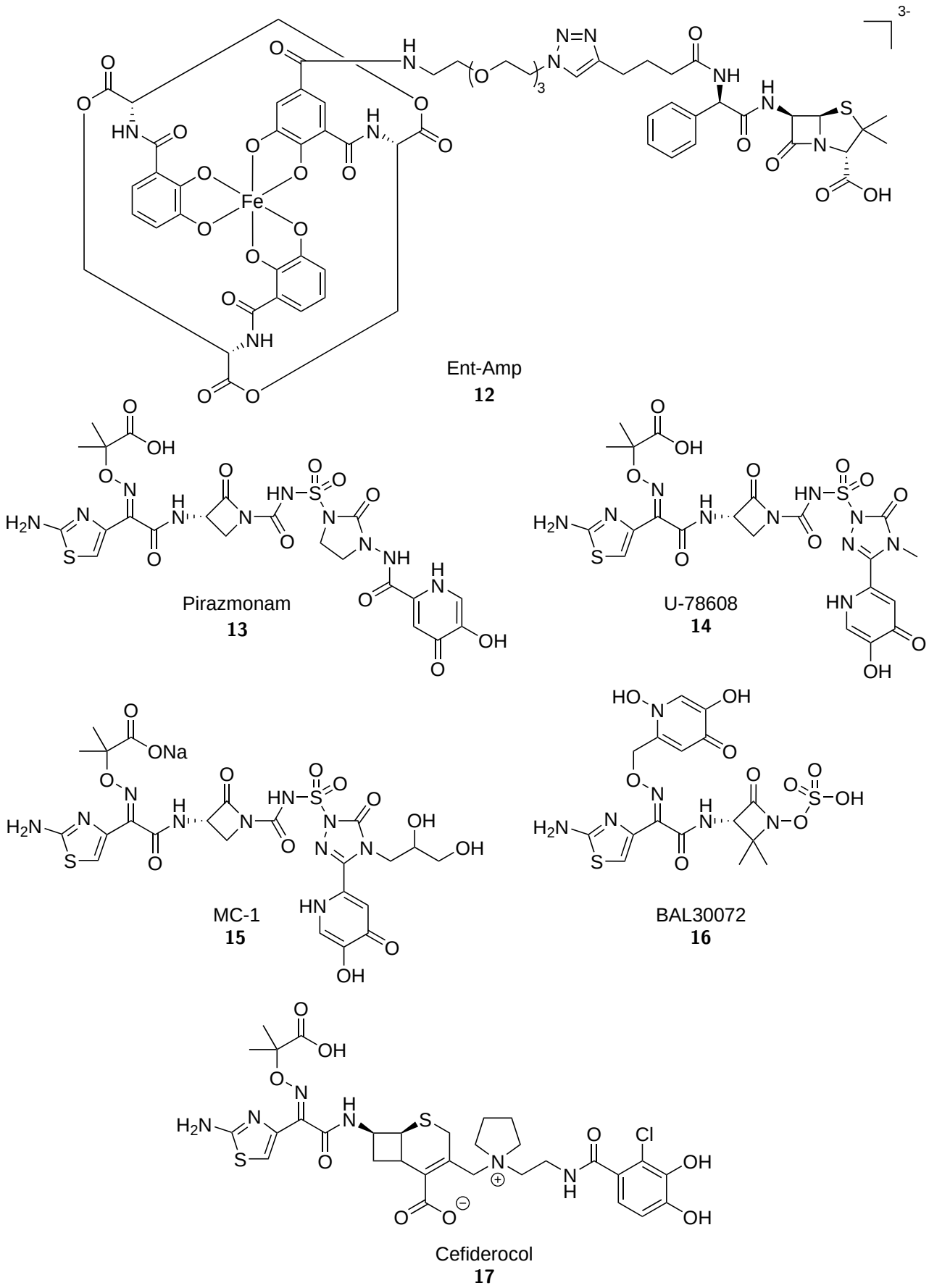


Figure 4: Examples of siderophore-antibiotic conjugates: Ent-Amp **12**,[52] pirazmonam **13**,[53,54] U-78608 **14**,[53,54] MC-1 **15**,[56] BAL30072 **16**[23] and cefiderocol **17**.[57,58]

1.3 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 against resistant strains. This is thought to be the first large study of autoinducer-antibiotic conjugates, with only one such molecule having been reported previously[61] (see 1.3.8). This section begins by introducing the concept of quorum sensing, followed by discussion of the autoinducers and antibiotics used in this study and the mechanisms of their efflux from *P. aeruginosa* cells, and how these mechanisms could be exploited by conjugates.

1.3.1 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.'[62] A similar concept is used in bacterial signalling, whereby group behaviour is only triggered when a certain minimum concentration of bacteria has been reached. Examples of group behaviour include bioluminescence, the production of virulence factors, swarming and biofilm formation.[63] 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. The process by which bacteria determine the concentration of similar bacteria in their vicinity, and act on that information, is known as quorum sensing.

Quorum sensing has been observed in many species of bacteria, including *Vibrio fischeri*, *P. aeruginosa*, *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Staphylococcus aureus*, *Vibrio harveyi*, *Escherichia coli*, *Myxococcus xanthus*, *Salmonella enterica*, *Yersinia enterocolitica*, *Aeromonas sp.* and *Acinetobacter sp.*[63–72] Many of these bacteria are significant causes of disease and death in humans, for example, in a typical year in the U.S. *P. aeruginosa* causes 6,700 multidrug-resistant infections and 440 deaths, methicillin-resistant *S. aureus* causes 80,500 severe infections and 11,300 deaths and non-typhoidal *Salmonella* causes 1.2 million illnesses, 23,000 hospitalisations and 450 deaths.[2]

1.3.1.1 *Vibrio fischeri*

The first example of quorum sensing was discovered in *V. fischeri*, a symbiotic bacterium that produces bioluminescence in the photophore of the Hawaiian bobtail squid, *Euprymna scolopes*[63,71,72] (see Figure 5). This bacterium receives amino acids[73,74] from its host in exchange for producing light which the squid uses for counterillumination, to camouflage itself.[75]

If a low population of *V. fischeri* were present in the photophore, the light that the bacteria could produce would be insufficient to provide counterillumination. 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 incentives the squid to provide them with nutrients.

Figure 5: 'Euprymna scolopes, South shore of Oahu, Hawaii' by Jamie Foster. Licensed under CC BY-SA 3.0 via Commons.

V. fischeri uses the LuxR-LuxI system to sense cell density. This system is seen as a paradigm of quorum sensing, and a simplified explanation of it is presented to show typical features of such a system (see Figure 6).

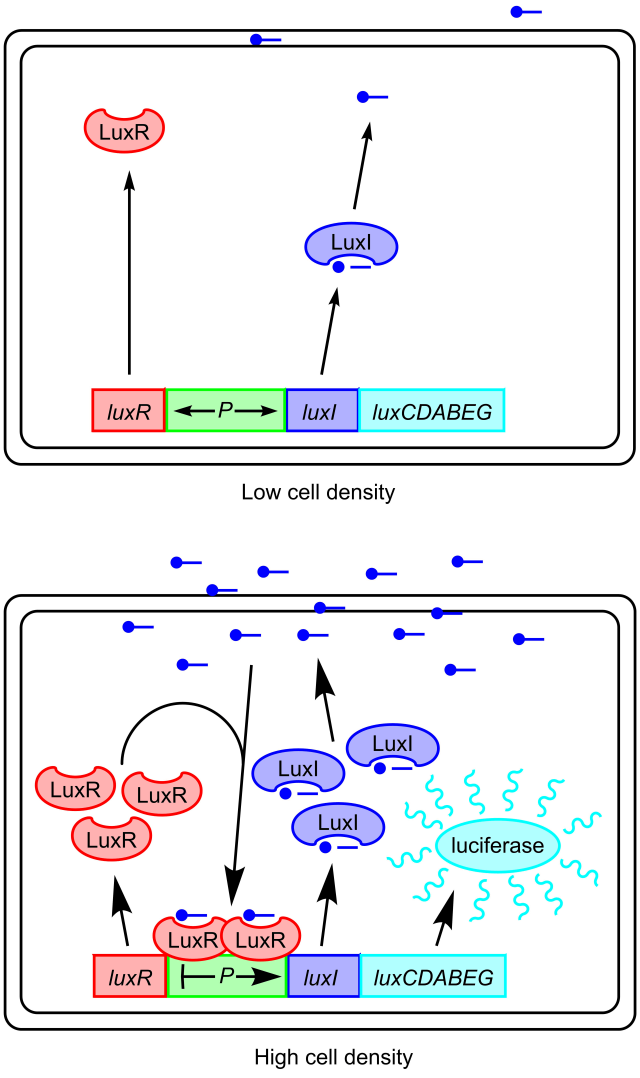


Figure 6: The LuxR-LuxI quorum sensing system in *V. fischeri*.

V. fischeri senses cell concentration by the detection of 3-oxo-C_6-HSL **18**[76] (see Figure 7), a freely diffusible[77] molecule which is synthesised by LuxI[78,79] and secreted by all *V. fischeri* cells[80] at a low basal level.[63] When

the bacterial population density, and hence the concentration of 3-oxo-C_6-HSL **18**, reaches a threshold, 3-oxo-C_6-HSL **18** binds to LuxR,[81–83] a receptor which is also synthesised at a low basal level.

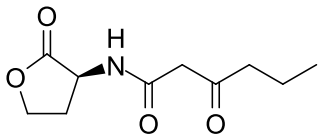


Figure 7: 3-oxo-C_6-HSL **18**.

The LuxR complex binds to the *lux* operator, upregulating production of LuxI and hence 3-oxo-C_6-HSL **18**, and luciferase enzymes and hence blue-green light.[84–86] Production of more 3-oxo-C_6-HSL **18** enables a positive feedback loop, reinforcing the effect of high population density on 3-oxo-C_6-HSL **18** concentration and hence light production. This is the reason that 3-oxo-C_6-HSL **18** is known as an autoinducer.

The system also contains a negatively feedback loop to avoid excessive expression of proteins: at high concentrations of 3-oxo-C_6-HSL **18** production of LuxR is inhibited.[87] Such balancing effects, as well as interactions with other quorum sensing and metabolic systems, are seen in many bacteria.[63,88]

1.3.1.2 *Pseudomonas aeruginosa*

Another well-studied example of quorum sensing is in *P. aeruginosa*.[88–90] *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.[91] Multidrug-resistant *P. aeruginosa* is classified as a 'serious threat' by the United States Centers for Disease Control and Prevention[2] and carbapenem-resistant *P. aeruginosa* is classified as 'priority 1: critical' by the World Health Organisation.[18]

P. aeruginosa has a low susceptibility to many antibiotics and readily acquires antibiotic resistance by mutation or horizontal gene transfer.[92] It is difficult for antibiotics to cross into cells due to low cell membrane permeability[93] and biofilm formation,[94] and they are pumped out again by its multiple chromosomally encoded multidrug efflux pumps.[8] *P. aeruginosa* biofilms are more resistant to many drugs including ciprofloxacin **24** and trimethoprim **25** compared with planktonic cells.[94,95] This high level of antibiotic resistance makes *P. aeruginosa* an important target for drug discovery.

Quorum sensing in *P. aeruginosa* involves a complex interplay of five signalling molecules (see Figure 8) and various proteins (see Figure 9).[88–90] These can be broken down into three main, interacting systems: Las, Rhl and Pqs.

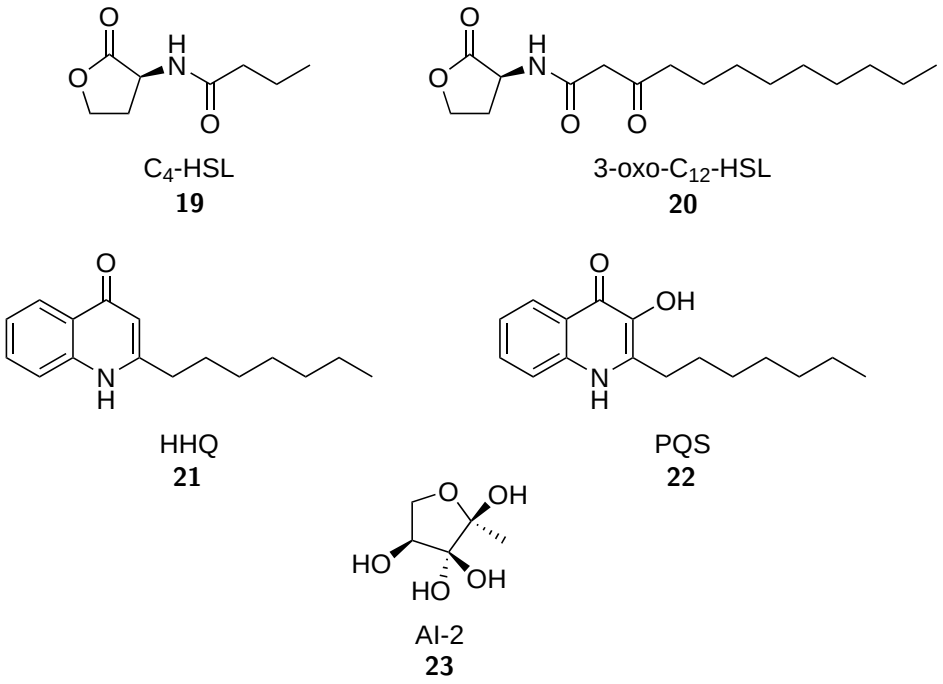


Figure 8: *P. aeruginosa* autoinducers.

In the Las system, LasI[96] synthesises the 3-oxo-C_{12}-HSL **20**[97] autoinducer. 3-oxo-C_{12}-HSL **20** binds LasR,[98] and this complex upregulates the production of LasI[99] (thus causing autoinduction) as well as alkaline protease,[100] elastase,[98] exotoxin A,[100] HCN[101] and LasA protease.[102] The LasR complex is also important in late-stage biofilm formation,[68] and upregulates the Rhl[103] and Pqs systems.[104,105]

In the Rhl system, RhlI[106] synthesises the C_4-HSL **19**[107] autoinducer. C_4-HSL **19** binds RhlR,[108] and this complex upregulates the production of RhlI[99] (again causing autoinduction), alkaline protease,[109] elastase,[106] haemolysin,[109] HCN,[101,109] LasA protease,[106] LecA,[110] pyocyanin[106,109] and rhamnolipids.[106] The RhlR complex also downregulates the Pqs system.[105,111] The Rhl system is controlled by both the Las and Pqs systems, as production of both RhlR and RhlI is upregulated by the LasR complex[103] and production of both RhlR is upregulated by the PqsR complex.[112]

In the Pqs system, the main autoinducer, PQS **22**,[113] is synthesised by multiple enzymes: PhnAB,[114] PqsA, PqsBC, PqsD[115,116] and PqsE[117,118] produce the precursor HHQ **21**, and PqsH converts HHQ **21** to PQS **22**. PQS **22**[105] or HHQ **21** binds PqsR,[119] and either complex can upregulate the synthesis of HHQ **21** causing autoinduction. The PqsR-PQS complex upregulates the production of chitinase,[120] elastase,[113] HCN,[120] LecA,[121] pyocyanin[104,122] and pyoverdine,[122] as well as increasing biofilm production[121] and vesicle formation.[123] The PqsR-PQS complex also upregulates production of RhlR, so the Pqs system has control over the Rhl system.[112] The Pqs system is controlled by both the Las and Rhl systems, as production of PqsR[105] and PqsH[104] is upregulated by the LasR complex and production of PqsA, PqsBC, PqsD, PqsE[111] and PqsR[105] is downregulated by the RhlR complex.

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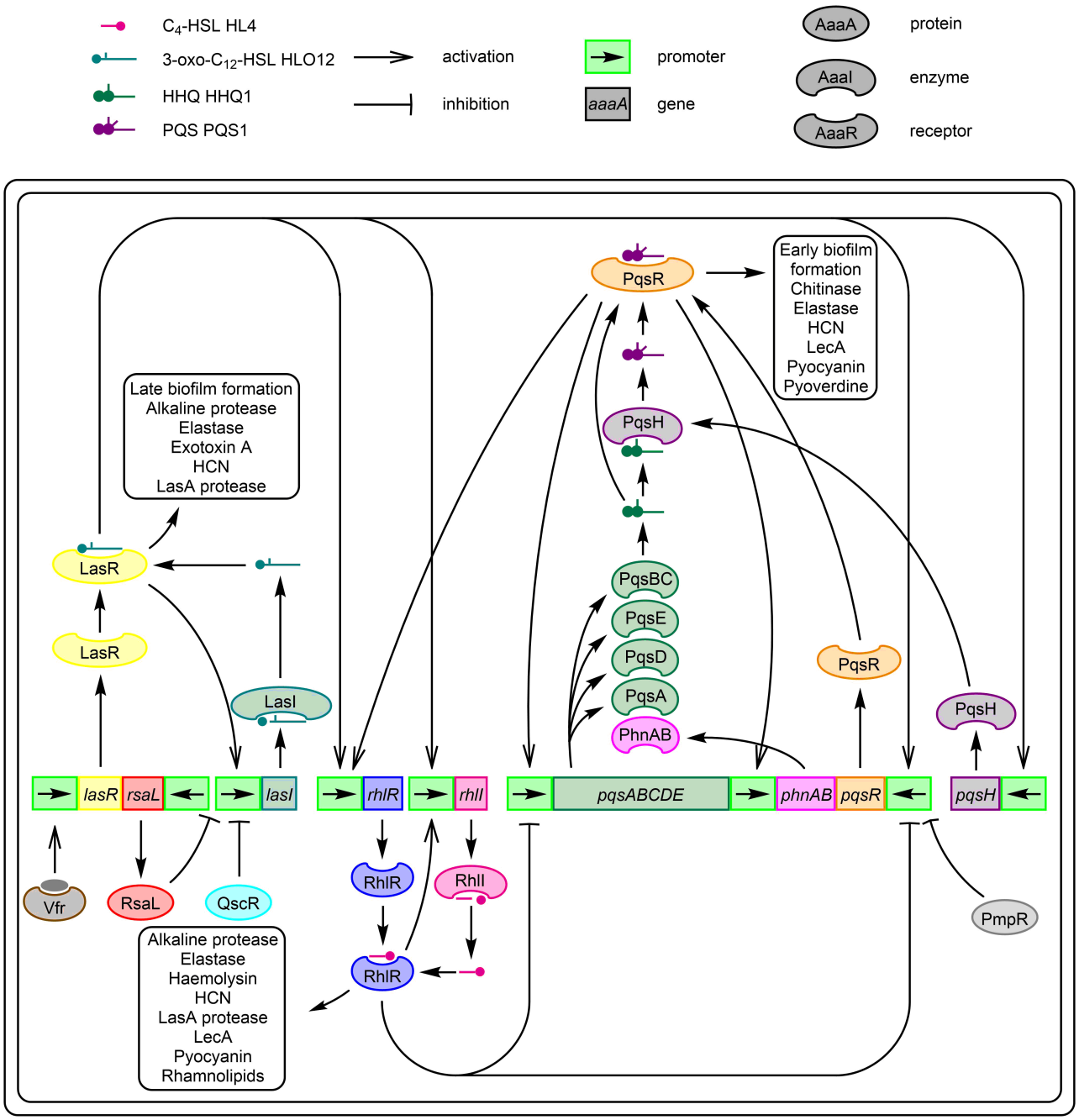


Figure 9: Quorum sensing in *P. aeruginosa*.[88–90]

In addition to the above systems, AI-2 (see Figure 8), an interspecies signalling molecule,[124] is known to increase biofilm production and virulence in *P. aeruginosa*.[125,126] This is thought to be achieved by interaction with the Las and Rhl systems, but the exact mechanism is not known.

In summary, *P. aeruginosa* uses the autoinducers shown in Figure 8 as part of three interacting quorum sensing systems to coordinate virulence and biofilm production, and this makes these autoinducers interesting therapeutic targets.

1.3.2 Autoinducers

Quorum sensing has been sucessfully targeted using many different modulators,[89,127] but this study takes a slightly different approach. Inspired by the success of various siderophore-antibiotic conjugates (see 1.2.3),

a library of autoinducer-antibiotic conjugates was synthesised to test the hypothesis that the importance of autoinducers in harmful cellular behaviours could lead to increased activity of the conjugates (see 1.3).

The *P. aeruginosa* autoinducers (see Figure 8) were chosen for use in this study as *P. aeruginosa* is a significant human pathogen which shows high antibiotic resistance and utilises quorum sensing to coordinate pathogenic behaviours (see 1.3.1.2). Specifically, C_4-HSL **19**, HHQ **21** and PQS **22** derivatives were chosen as they were considered to be the most synthetically tractable.

1.3.3 Autoinducer efflux

Autoinducers must be exported from the cell in order to be used for intercellular communication, and the five known *P. aeruginosa* autoinducers are exported by various different transport mechanisms. The mechanism is not well known for HHQ **21** or AI-2 **23**, but it is know that PQS **22** is exported in vesicles,[128] C_4-HSL **19** passively diffuses in and out of cells,[129] and 3-oxo-C_{12}-HSL **20** 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 **19** and 3-oxo-C_{12}-HSL **20** 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 **18** has been shown to be freely diffusable through *V. fischeri* membranes.[77]

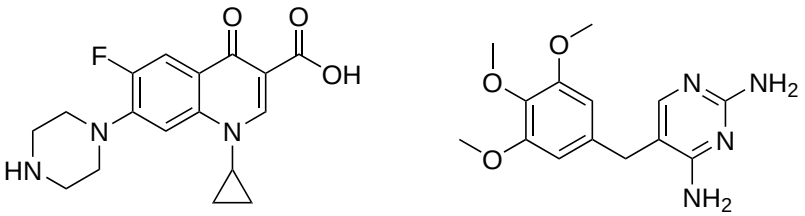
3-oxo-C_{12}-HSL **20** is exported primarily via the MexAB-OprM efflux system.[8,130] The increased removal of 3-oxo-C_{12}-HSL **20** from the cell by upregulation of the MexAB-OprM system leads to decreased production of additional 3-oxo-C_{12}-HSL **20** (as the positive feedback loop is disrupted, see 1.3.1.2), 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_{12}-HSL **20** levels would disrupt Las-mediated quorum sensing,[131] but no direct studies of this could be found.

1.3.4 Antibiotics

Ciprofloxacin **24** and trimethoprim **25** (see Figure 10) were chosen as the antibiotic sides of the conjugates.

Ciprofloxacin **24** is second-generation fluoroquinolone antibiotic used to treat both Gram-positive and Gram-negative bacterial infections including *P. aeruginosa*.[132,133] Ciprofloxacin **24** inhibits DNA replication by binding to DNA gyrase and topoisomerase IV.[134]

Trimethoprim (see Figure 10) is a dihydrofolate reductase inhibitor used primarily to treat bladder infections.[135] It is active against several significant human pathogens including *Streptococcus pneumoniae* and *Haemophilus influenzae*, but not against *P. aeruginosa*. It was primarily chosen in this study as it was considered easy to functionalise, but also to test the feasibility of creating antibiotic activity against *P. aeruginosa*.



Ciprofloxacin **24**

Trimethoprim **25**

Figure 10: The antibiotics used in this section.

1.3.5 Antibiotic efflux

Ciprofloxacin **24** enters *P. aeruginosa* by diffusion,[136] but is pumped out by efflux pumps.[137] In the planktonic state several efflux pumps are known to pump out ciprofloxacin **24**, including MexAB–OprM, MexCD–OprJ, MexEF–OprN, MexXY–OprM, MexJK–OprM and MexVW–OprM.[8] However, in biofilms only MexEF-OprN has an effect.[138]

Trimethoprim **25** is mainly exported by the MexAB–OprM,[139] MexCD–OprJ[140] and MexEF–OprN[141] multidrug efflux systems[8,142] in the planktonic state. It is not known which pumps are used to export trimethoprim **25** from biofilms, but biofilms do show increased resistance to it.[95]

1.3.6 Conjugate efflux and antibiotic action

There are two ways in which the conjugates could disrupt *P. aeruginosa* growth:

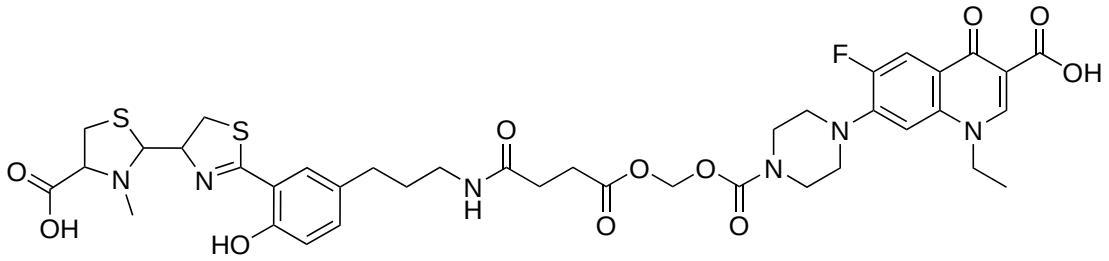
1. *P. aeruginosa* could develop resistance to an autoinducer-antibiotic conjugate by upregulation of its export mechanism, but this would also lead to increased export of the native autoinducer, thus disrupting the quorum sensing system and hence biofilm formation and virulence.[88,130,131] For HSL conjugates this would mean upregulation of the MexAB-OprM pump, as this is the pump used for export of 3-oxo-C_{12}-HSL **20**.[8,130] For PQS conjugates this would mean upregulation of vesicle formation.[128]

2. The autoinducer section could make the conjugate a poor substrate for the antibiotic section's usual efflux mechanism, leading to accumulation of the conjugate within cells and hence increased antibacterial activity. For autoinducer-ciprofloxacin conjugates acting on planktonic *P. aeruginosa* this would mean the conjugate being a poor substrate of the various efflux pumps listed in the previous section. For autoinducer-ciprofloxacin conjugates acting on biofilms this would mean the conjugate being a poor substrate of MexEF–OprN (the sole exporter of ciprofloxacin **24** in biofilms[138] and not an exporter of HSLs **19** or **20**, or PQS **22**[8]). This mechanism could in principal work for trimethoprim **25** as well, but it is not known which pumps are active against this antibiotic in biofilms.

These synergistic mechanisms of action made autoinducer-antibiotic conjugates a promising target. An initial library was designed using a copper(I)-catalysed azide-alkyne cycloaddition,[143,144] commonly referred to as a click reaction (although this is a more general term), to join each combination of autoinducer and antibiotic together.

1.3.7 Cleavable linkers

As part of the library, a set of cleavable HSL-ciprofloxacin triazole conjugates was synthesised in collaboration with Professor Eddy Sotelo. These were based on the cleavable pyochelin–norfloxacin conjugates synthesised by Rivault *et al.*[50] (see Figure 11). It was envisaged that the linker would be stable under the extracellular assay conditions, but would be cleaved upon entry into the cell by intracellular esterases. It was hoped that the attached HSLs would improve retention of the conjugate in cells.



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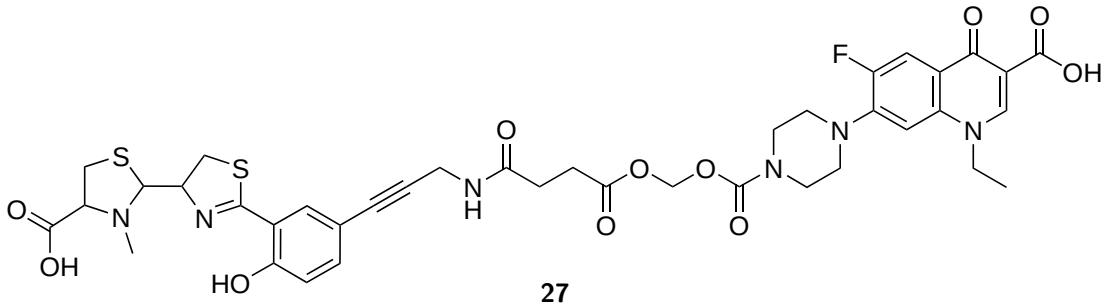


27

Figure 11: The cleavable pyochelin–norfloxacin conjugates synthesised by Rivault *et al.*[50]

The properties of similar linkers (see Figure 12, R = Me) were studied by Gogate *et al.*, who found that they were stable for more than 3 years under optimal conditions.[145] The hydrolysis of a secondary amine prodrug is dependent on ester hydrolysis rate, therefore the cleavage rate can be tuned by changing the R group between the ester and amide.[146] The *N*-(acetoxyethoxycarbonyl) (R = Me) linkers have been shown to be cleaved by esterases at an enhanced rate compared to buffer, and thus show promise in prodrugs.[147] It was therefore hoped that they will allow intracellular release of the ciprofloxacin **24** payload from the conjugates in this study. Both the *N*-(acetoxymethoxycarbonyl) (R = H) and *N*-(acetoxyethoxycarbonyl) (R = Me) were used, to investigate whether differences in cleavage rate could tune activity.

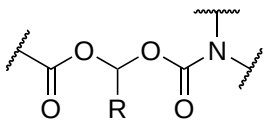


Figure 12: The cleavable linkers investigated in this study.

1.3.8 HSL analogue-ciprofloxacin conjugates

Following on from the library of compounds based on *P. aeruginosa* autoinducers, a series of conjugates based on analogues of HSL were planned. This strategy was inspired by a paper[61] and patent[148] by Ganguly *et al.*, who synthesised and characterised a conjugate **154** 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.[80,149–155]

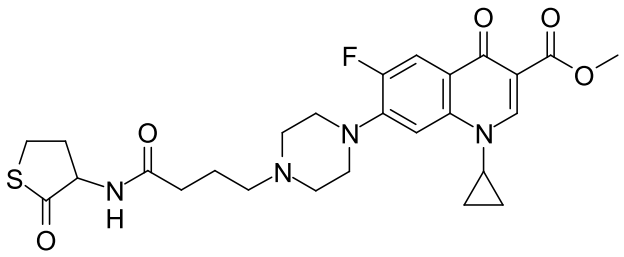


Figure 13: The HCTL-CipMe conjugate **154** studied by Ganguly *et al.*[61,148]

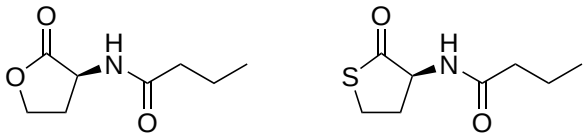
As part of their characterisation of the HCTL-CipMe conjugate **154**, Ganguly *et al.* found the minimum inhibitory concentration (MIC) of the conjugate in *P. aeruginosa* under standard planktonic conditions. The MIC was found to be ten times higher for the conjugate vs. ciprofloxacin (50 vs. 5 μm), 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 **154**. They then incubated cultures for 24 h, to allow biofilms to grow, before adding the compounds. In contrast, they found that the conjugate **154** 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 **28** (see Figure 14) has 'either enhanced uptake or functional activity' when compared with C_4-HSL **19**.

It is possible that the conjugate **154** has higher activity against biofilms when compared with ciprofloxacin **24** because the conjugate **154** avoids being pumped out by multidrug efflux pumps, or selects for the survival of mutants with upregulated efflux pumps, and hence disrupts quorum sensing systems (see 1.3.6).

While one might expect the conjugate **154** to behave like C_4-HSL **19**, and hence passively diffuse in and out of cells, it is possible that its transport more closely resembles that of 3-oxo-C_{12}-HSL **20**. 3-oxo-C_{12}-HSL **20**'s accumulation in membranes and interaction with efflux pumps is thought to be based primarily on tail chain length (see 1.3.3), and the ciprofloxacin half of the conjugate **154** could be seen as a long tail, especially as the carboxylic acid is methylated and hence less polar.



C_4-HSL
19

C_4-HCTL
28

Figure 14: C_4-HSL **19** and C_4-HCTL **28**. Note that Ganguly *et al.* tested the *S* enantiomer of C_4-HCTL **28**, but used a racemic mixture in their HCTL-CipMe conjugate.

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 taken from known quorum sensing modulators,[127,156] a selection of which are described in Table 2.

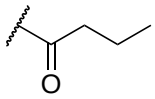
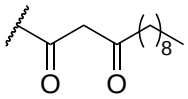
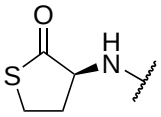
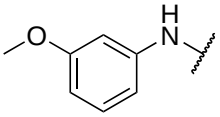
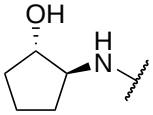
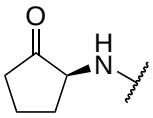
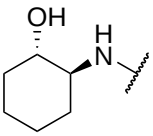
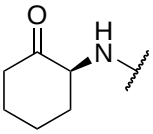
Head group		
	Partial agonist and antagonist against LasR.[153] Shown to increase biofilm formation in *P. aeruginosa*.[61]	Strong agonist against LasR, with comparable activity to the native ligand.[150,151,153,157]
	Partial agonist against LasR.[156]	Strong antagonist against LasR.[156]
	Poor agonist and antagonist against RhlR.[158,159]	Strong antagonist against LasR.[158]
	Strong agonist against RhlR.[158] *SS* enantiomer is more potent.[159]	Partial agonist against LasR.[158]
	Strong agonist against RhlR.[158] *SS* enantiomer is more potent, with comparable activity to the native ligand.[159]	Strong agonist against LasR.[151,158] *SS* enantiomer is more potent, with comparable activity to the native ligand.[159]
	Strong agonist against RhlR.[158] *SS* enantiomer is more potent.[159]	Partial antagonist against LasR.[158] Shown to reduce biofilm formation in *P. aeruginosa*.[158]

Table 2: Activities of quorum sensing modulators containing the head groups used in this study.

2 Project aims and summary

The aim of this project is to produce and test a library of autoinducer-antibiotic conjugates with the goal of producing conjugates with greater potency than the parent antibiotics. The work is divided into two main sections. Section 3 focuses on conjugates of three *P. aeruginosa* autoinducers (see Figure 8) with ciprofloxacin and trimethoprim (see Figure 10) joined using a copper(I)-catalyzed azide-alkyne cycloaddition. Section **??** focuses on conjugates of homoserine lactone analogues with ciprofloxacin (see 1.3.8) joined either using a copper(I)-catalyzed azide-alkyne cycloaddition or an S_N2 reaction or peptide coupling.

3 Results and discussion: autoinducer-antibiotic conjugates

3.1 Biological testing

3.1.1 Autoinducer-antibiotic conjugates

The eight triazoles made in **??** (see Figure 15) were tested for antibacterial and anti-biofilm activity in *P. aeruginosa* PAO1[160] and YM64.[161] PAO1 is the *P. aeruginosa* wild-type strain. YM64 is a mutant lacking all of the four major *mex* operons for multidrug efflux pumps: *mexAB-oprM*, *mexXY*, *mexCD-oprJ* and *mexEF-oprN*, making it more sensitive to many antibiotics and hence able to show up moderate effects more clearly.

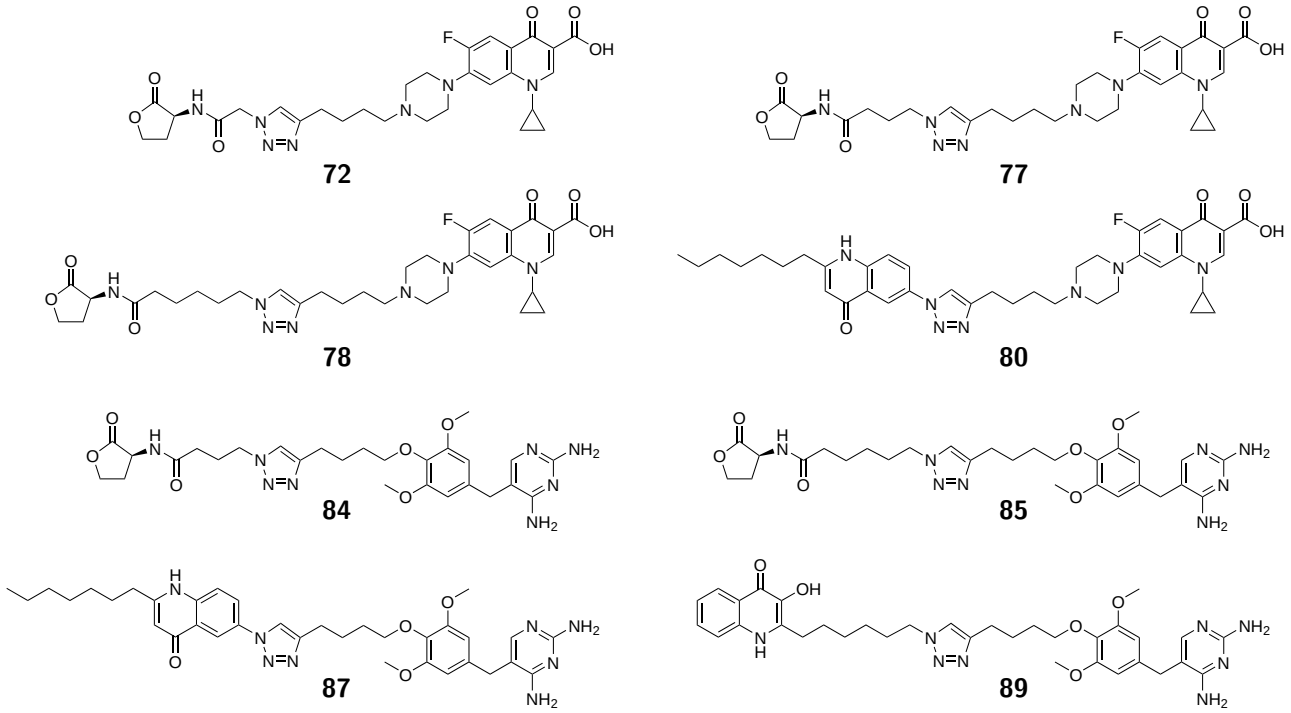


Figure 15: The autoinducer-antibiotic conjugates

In YM64 at 5 h the HSL-ciprofloxacin conjugates **72**, **77** and **78** showed slight activity at the highest concentration, but not as much as ciprofloxacin **24**. This activity was not visible by 24 h (see Figure 17) and the compounds had no effect on biofilm formation (see Figure 18).

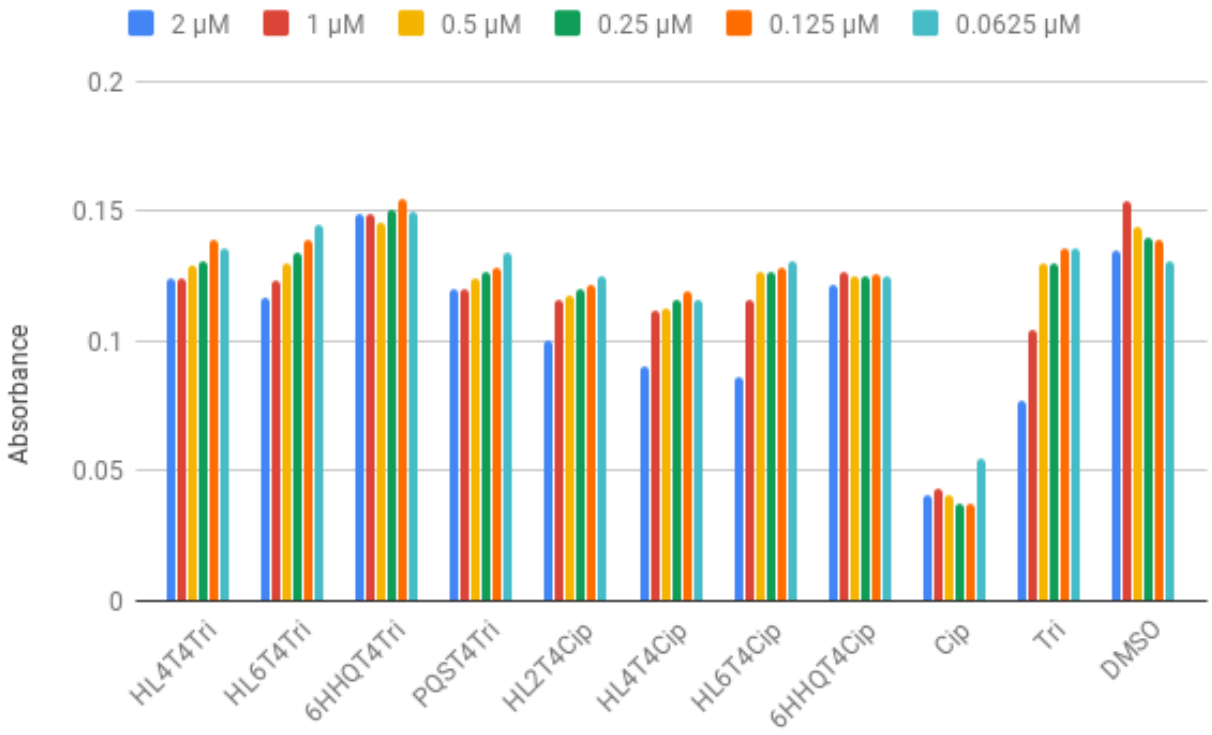


Figure 16: YM64 OD readings at 5 h for the autoinducer-antibiotic conjugates.

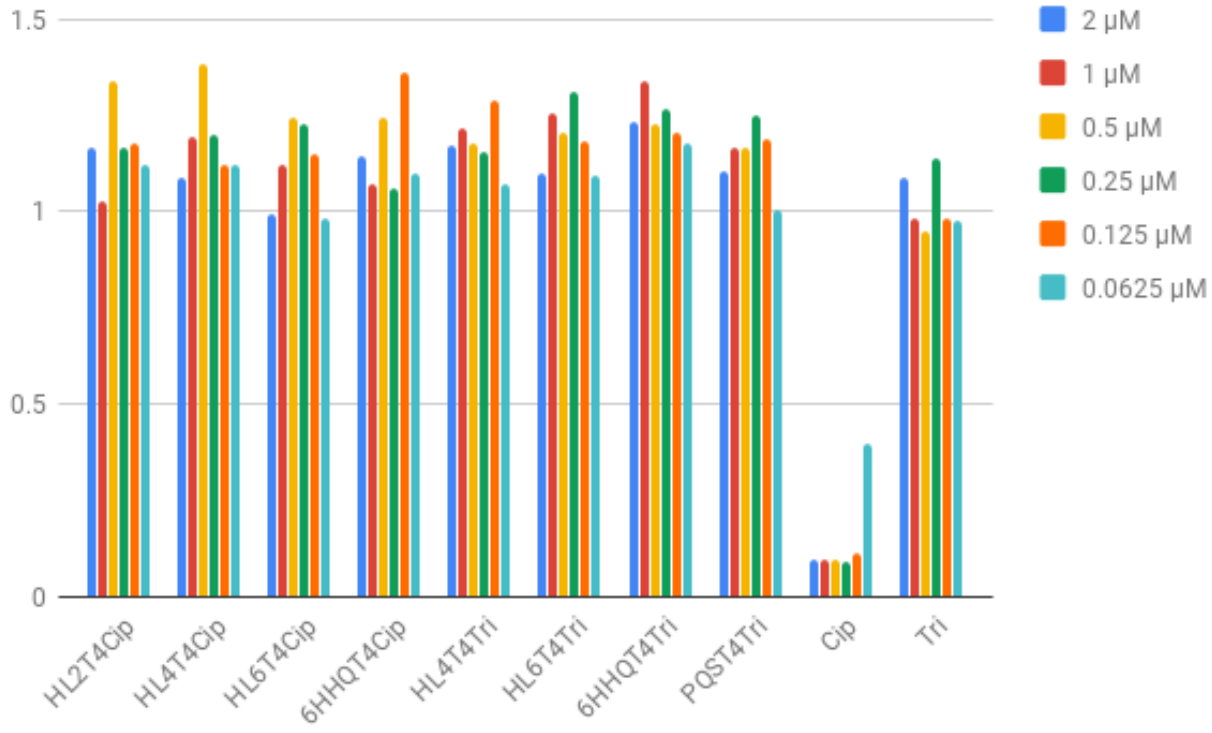


Figure 17: YM64 OD readings at 24 h for the autoinducer-antibiotic conjugates.

please ignore the wandering legends and confusing compound codes, these graphs will be updated when I've settled on the ordering of compounds in the thesis

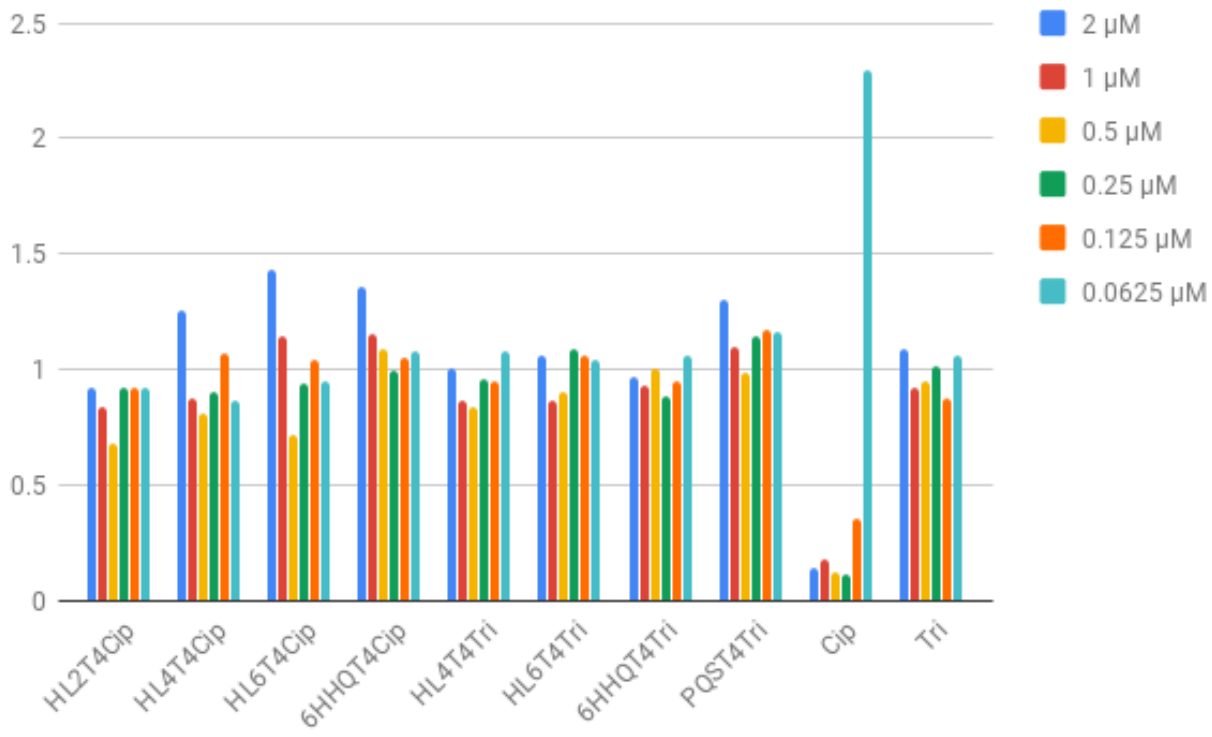


Figure 18: YM64 biofilm quantification at 24 h for the autoinducer-antibiotic conjugates.

In PAO1 **78** showed similar activity to ciprofloxacin **24** at the highest concentration (see Figure 20), but not at lower concentrations. All other compounds did not show activity, and again there was no activity at 24 h or against biofilms.

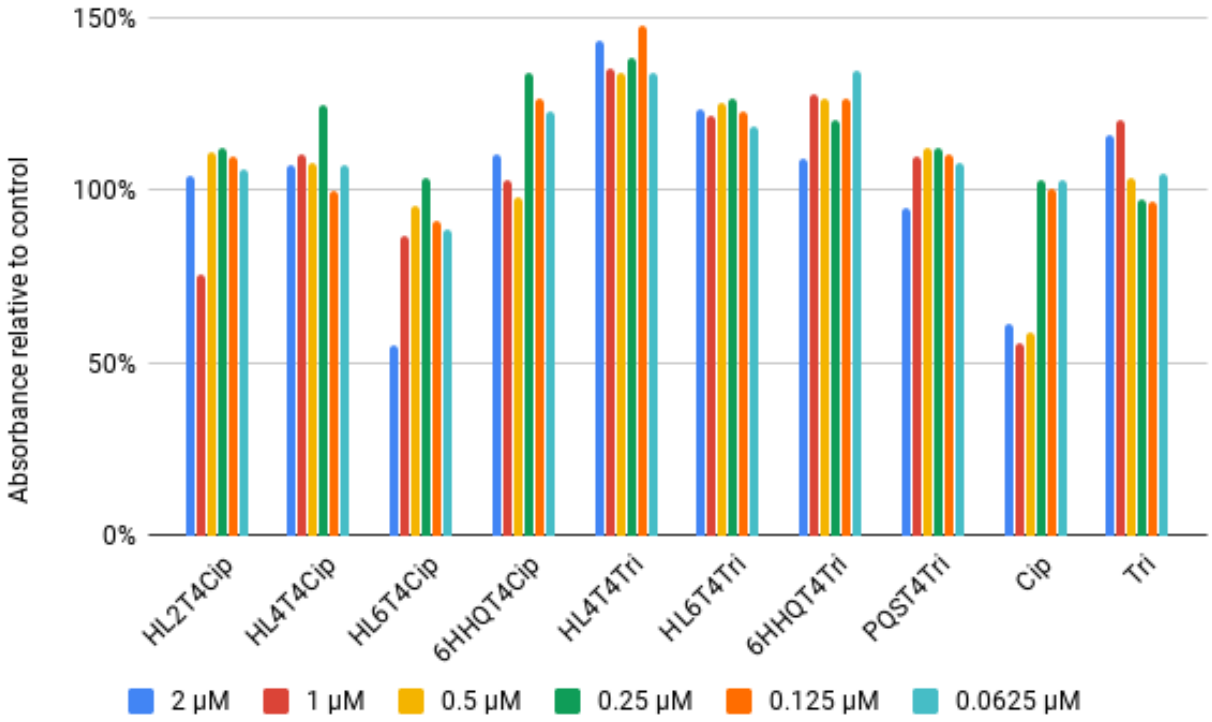


Figure 19: PAO1 OD readings at 5 h for the autoinducer-antibiotic conjugates.

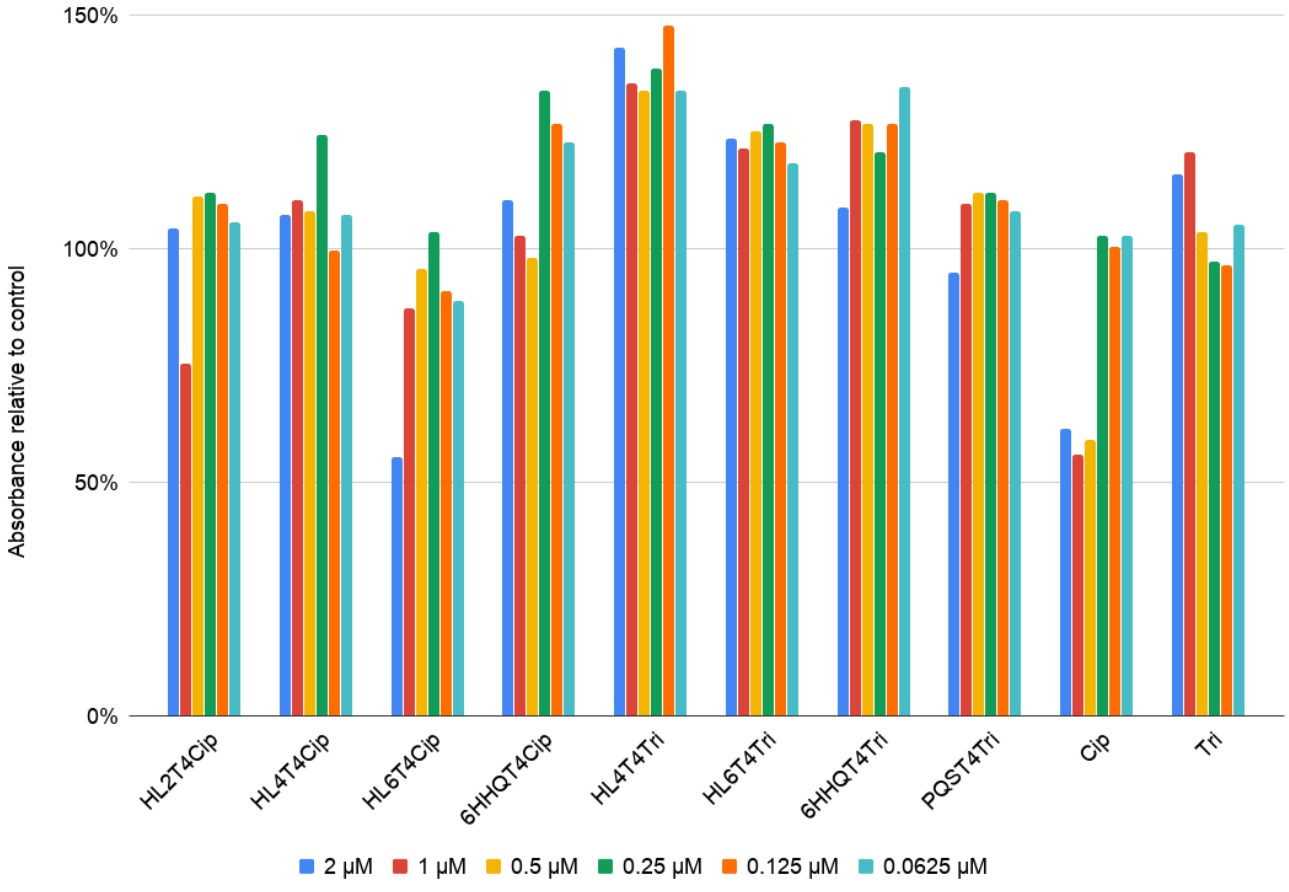


Figure 20: PAO1 OD readings at 5 h for the autoinducer-antibiotic conjugates.

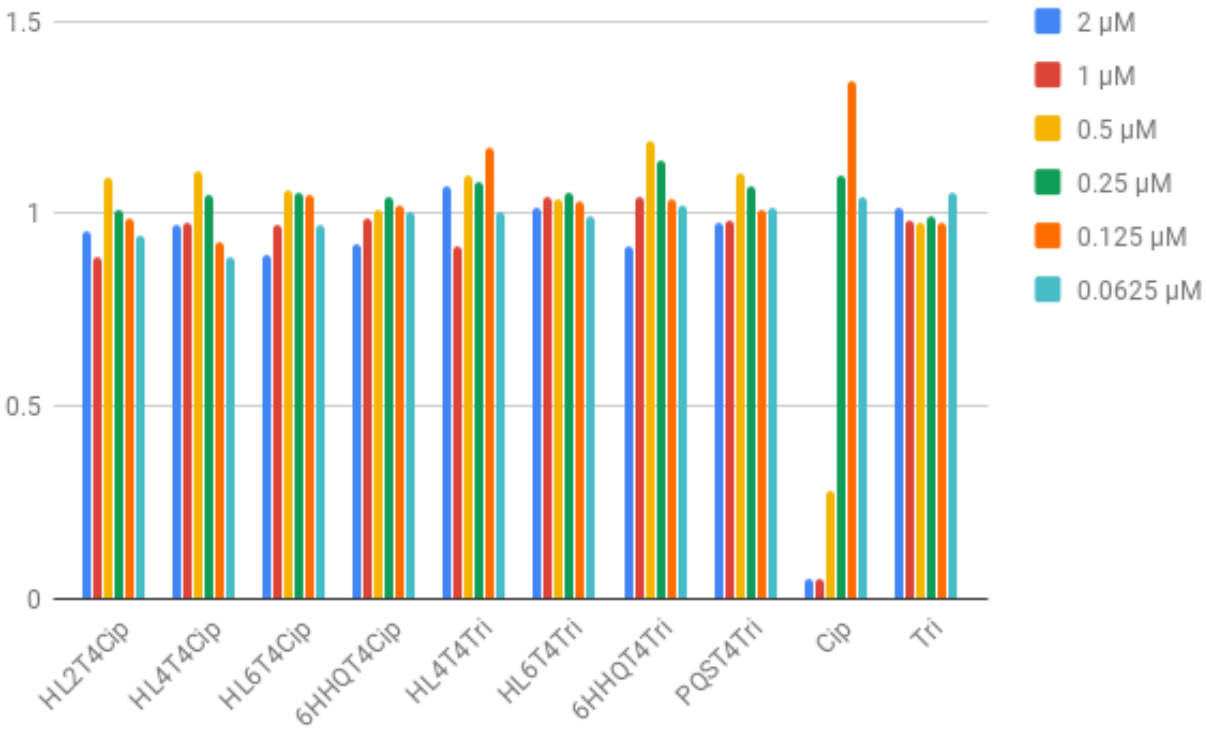


Figure 21: PAO1 OD readings at 24 h for the autoinducer-antibiotic conjugates.

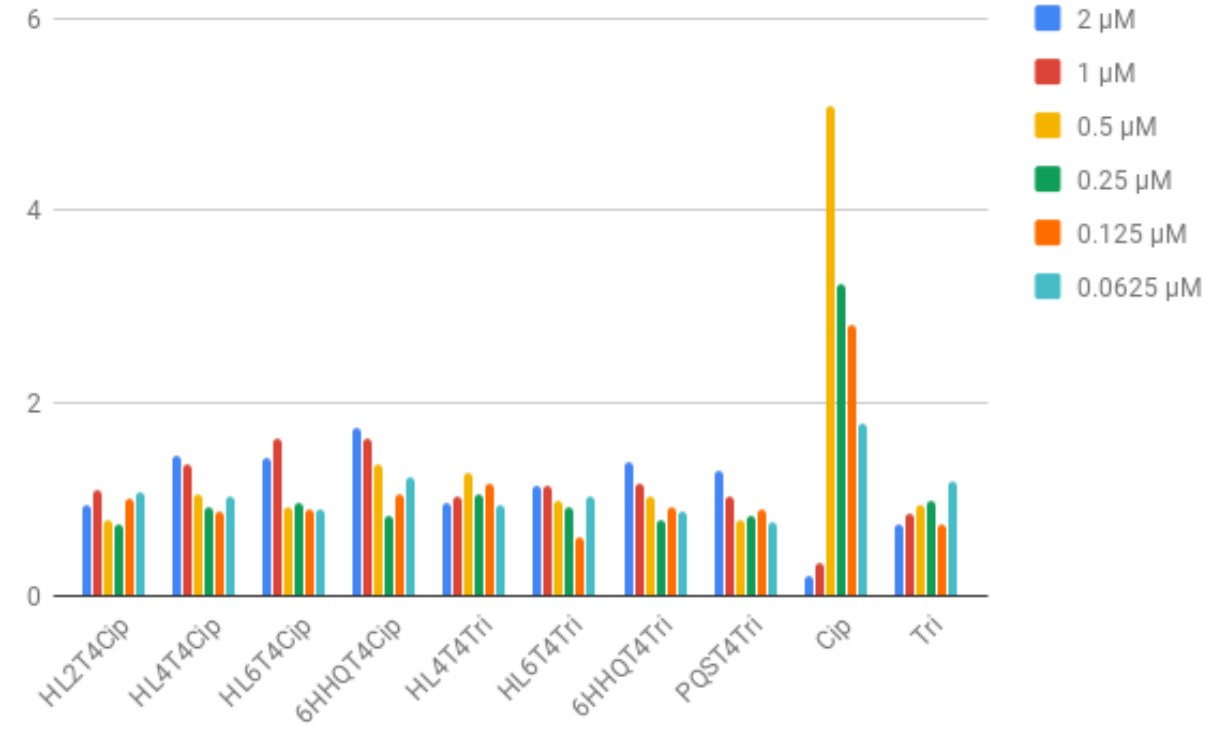


Figure 22: PAO1 biofilm quantification at 24 h for the autoinducer-antibiotic conjugates.

3.1.2 Cleavable HSL-ciprofloxacin conjugates

The eight cleavable HSL-ciprofloxacin conjugates, two controls and two alkynes described in **??** (see Figure 23) were tested for antibacterial and anti-biofilm activity in *P. aeruginosa* YM64.

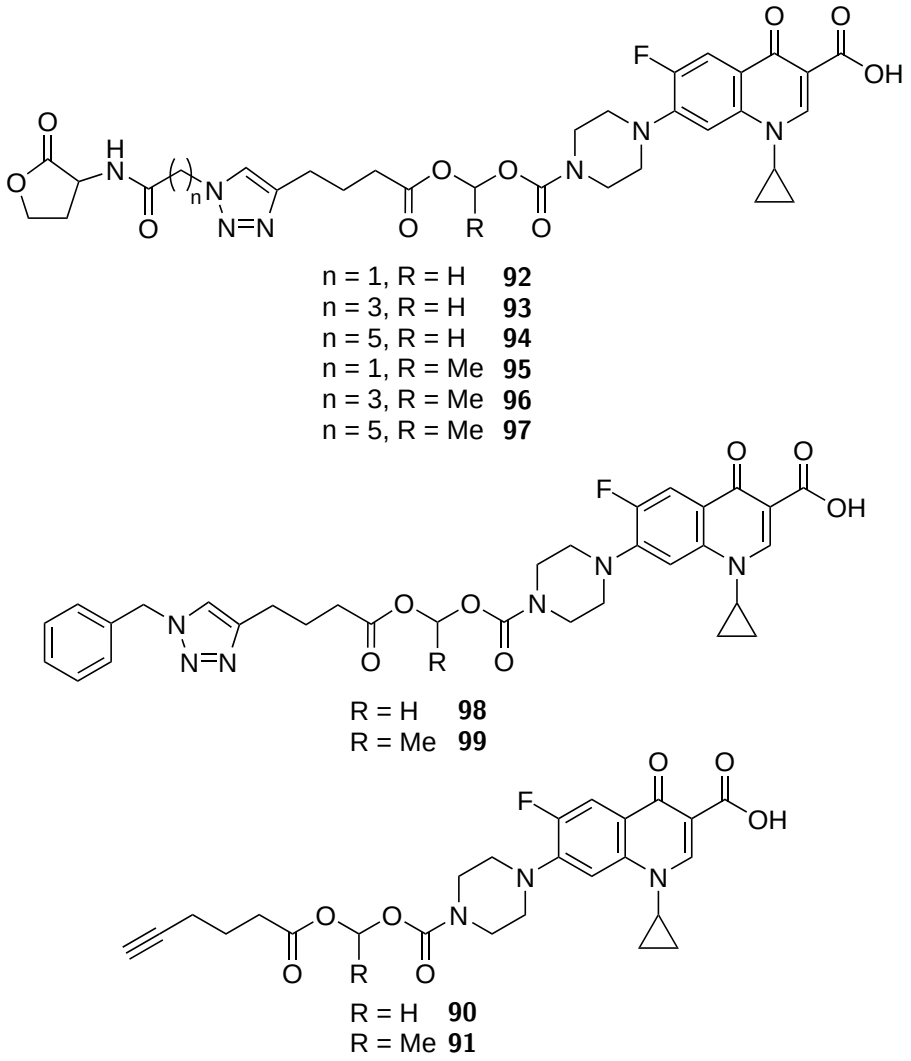


Figure 23: The cleavable HSL-ciprofloxacin conjugates.

Here there was more success, although the activity was still not as high as for ciprofloxacin **24**. The HSL-ciprofloxacin conjugates with *N*-(acetoxymethoxycarbonyl) linkers (R = H) showed activity at high concentrations. A longer linker seems to give higher activity; **93** and **94** showed activity comparable with ciprofloxacin **24** at high concentrations. Unfortunately the control **98** and alkyne **90** with *N*-(acetoxymethoxycarbonyl) linkers (R = H) showed higher activity than the conjugates, indicating that the HSL head wasn't contributing to the activity of the conjugates.

The conjugates with an *N*-(acetoxyethoxycarbonyl) linker (R = Me) did not show any activity. This suggests that they either didn't enter cells or weren't suitable substrates for esterases. The *N*-(acetoxyethoxycarbonyl) linked alkyne (R = Me) did show some activity, indicating that maybe it could penetrate cells more easily than the conjugates due to its lower molecular weight and/or lower polarity.

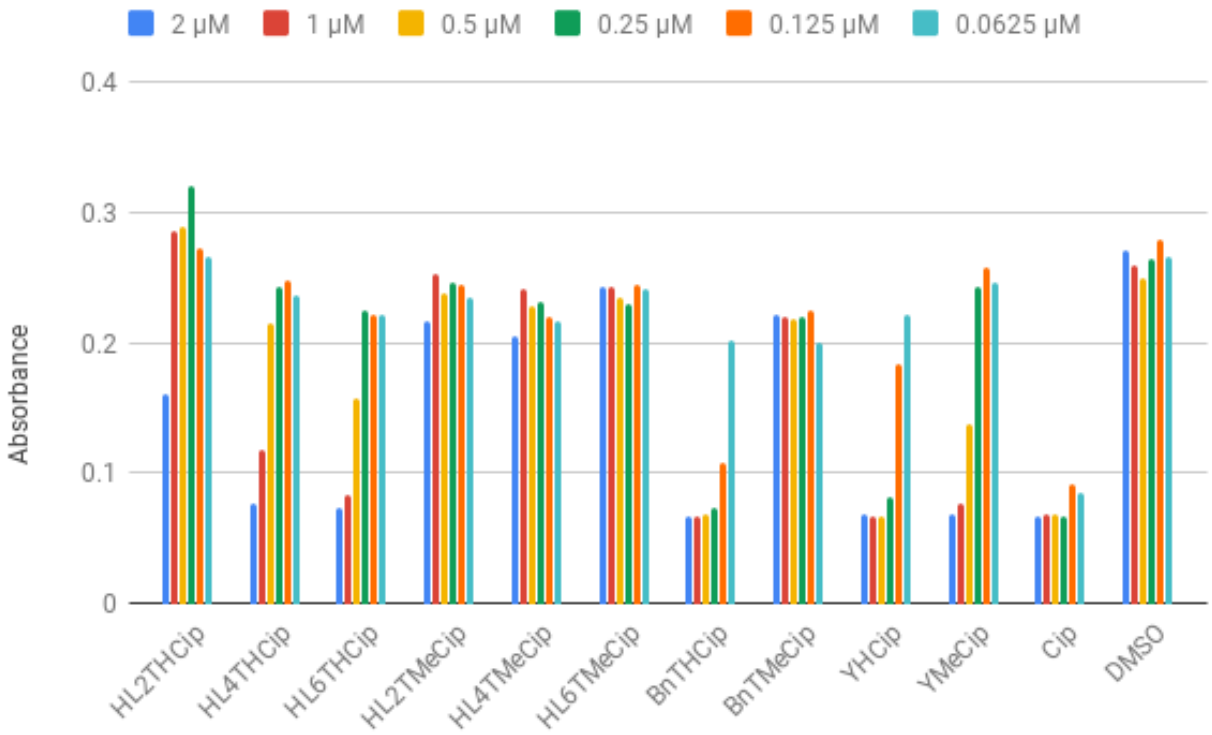


Figure 24: YM64 OD readings at 5 h for the cleavable HSL-ciprofloxacin conjugates.

3.2 Conclusions

3.2.1 Library synthesis

In this section, a range of 1,2,3-triazole-linked autoinducer-antibiotic conjugates was successfully synthesised and tested for antibiotic and anti-biofilm activity. Reliable routes to the azido autoinducers and alkynyl antibiotics were found, but the copper(I)-catalyzed alkyne-azide cycloaddition reactions used to link them proved rather capricious. The main reasons for this were insolubility of the starting materials and air-sensitivity. Air-sensitivity is not expected in a click reaction, but can be explained by many of the reactions being too dilute.[162] This led to ascorbate being used up by the oxygen dissolved in the reaction solvent and present in the air above the reaction mixture. Even when the solvent was degassed and the reaction performed under argon, a small amount of air leaking in through a perished septum was enough to cause the reaction to stall. Low concentrations were used because of the insolubility of the starting materials, but this would have been better addressed by more thorough screening of solvents. In addition, it was later shown that THPTA may not be necessary for a sufficiently concentrated reaction to take place,[163] so this expensive reagent could be omitted.

Assuming the click reaction could be further optimised, this library could be easily expanded by the addition of more azido autoinducers and alkynyl antibiotics (see 3.3). In particular, autoinducers which are actively transported into cells, such as AI-2, are attractive targets.

3.2.2 Biology

Little biological activity was seen in the non-cleavable autoinducer-antibiotic conjugates. This could be due to a number of factors, for example:

1. Restriction of the binding of ciprofloxacin to DNA gyrase and topoisomerase IV[134] or trimethoprim to

dihydrofolate reductase.[135]

2. Failure of the autoinducers to mask the antibiotics from recognition by efflux pumps.
3. Failure to penetrate the cell wall/biofilm.
4. Non-specific binding to the cell wall.

If binding of the antibiotics to target proteins is indeed restricted by the attachment of the autoinducer, this could be affected by the size and polarity of the linker and autoinducer. With this in mind, the next set of compounds synthesised contain HSL analogues, which are smaller than HHQ **21** and PQS **22**, and some omit the triazole in the linker, hence affecting polarity.

The cleavable HSL-ciprofloxacin conjugates showed a little more activity, but unfortunately this did not require the HSL, and probably was mostly affected by the polarity and size of the attached group and the ease of hydrolysis of the linker.

3.3 Future work

3.3.1 Biology

The further biological testing required for these compounds is as follows:

1. 24 h OD readings and biofilm quantifications for the cleavable HSL-ciprofloxacin conjugates.
2. Biofilm dispersal assays on all compounds (see 1.3.8 for a discussion of biofilm dispersal using a HSL analogue-ciprofloxacin conjugate and 4.1.4 for the methodology to be used).

4 Experimental

4.1 Biological testing

Compounds were tested against *P. aeruginosa* PAO1[160] and YM64.[161] C_4-HSL **19**, HHQ **21**, PQS **22**, ciprofloxacin **24**, trimethoprim **25** and DMSO were included as controls, along with LB to check for contamination of the plates. All absorbances are shown relative to DMSO. The first set of autoinducer-antibiotic conjugates (see 3.1) were tested at 2, 1, 0.5, 0.25, 0.125 and 0.0625 μM. OD readings were taken at 5 and 24 h, and biofilm quantification was carried out soon after the 24 h OD reading. Only a 5 h OD reading was obtained for the cleavable HSL-ciprofloxacin conjugates. The HSL analogue-ciprofloxacin conjugates (see **??**) were tested at 25, 2, 1, 0.5, 0.25 and 0.125 μM in triplicate. OD readings were taken at 0, 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h. Biofilm inhibition testing was carried out on plates grown for 24 and 48 h. Biofilm dispersal testing was carried out by growing plates for 24 h, followed by addition of the compounds, incubation for a further 24 h and quantification of the biofilms.

4.1.1 Antibiotic susceptibility

Antibiotic susceptibility was determined using spectrophotometry measurements. Colonies of the desired strains were grown at 37 °C overnight on LB agar. The colonies were used to inoculate LB (10 ml) and these cultures were grown at 37 °C overnight. The cultures were diluted 1/100 with LB, and 99 μl diluted culture per well was added to flat-bottomed clear 96-well plates. 1 μl of compound solution in DMSO was then added from master plates. The plates were covered with adhesive aeration filters and placed in a open box containing tissue paper wetted with distilled water in order to control evaporation. The box was shaken at 37 ° and 100 rpm. The optical density was recorded periodically using a Biochrom EZ Read 400 microplate reader at 595 nm.

4.1.2 Quantification of biofilms

Biofilms were quantified using a method described previously.[125,164] After the bacteria had grown for the desired amount of time, the culture was aspirated out of the wells using a pipette tip attached to a vacuum pump, making sure not to touch the sides of the wells. Water (120 μl) was then added and aspirated out again. This process was repeated twice more to thoroughly wash out planktonic cells. Crystal violet (120 μl, 0.1% m/v) was added and left for 15 min, then aspirated out. The wells were washed again with water (3 × 120 μl). Acetic acid (120 μl, 30% v/v aq.) was added and left for 15 min then the plate was vortexed and read using a Biochrom EZ Read 400 microplate reader at 595 nm.

4.1.3 Biofilm inhibition

The plates were prepared as in 4.1.1. The box of plates was shaken at 37 ° and 100 rpm for 24 h followed by quantification of biofilm growth as shown in 4.1.2.

4.1.4 Biofilm dispersal

The plates were prepared as in 4.1.1, initially without the addition of compound solutions. The box of plates was shaken at 37 ° and 100 rpm for 24. 1 μl of compound solution in DMSO was then added to each well from master plates and the plates were shaken as above for a further 24 h followed by measurement of OD and quantification of biofilm growth as shown in 4.1.2.

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