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

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September 2018

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This dissertation is submitted for the degree of Doctor of Philosophy

Contents

1 Acknowledgements

I would like to thank David Spring for the opportunity to work on this very interesting project. I would also like to thank Jamie Stokes for helping me get settled into the lab, James Hodgkinson for showing me the biological techniques I will need soon, and Felin Nie and Terrence Kwan for help with the LCMS. Thanks also to Matt Pond, Melvyn Orriss and Nic Davies for help with equipment and glassware and to Jill Vaughan for proof-reading. Most importantly, I would like to thank Yssy Baker for proof-reading, help, support, advice and encouragement. Finally, I would like to thank everyone in the Spring group for their help, advice and delightful company.

2 Declaration

This dissertation describes work carried out in the Department of Chemistry, University of Cambridge under the supervision of Prof. David Spring, and in the Department of Biochemistry, University of Cambridge under the supervision of Dr Martin Welch. This dissertation is the result of my own work and includes nothing that is the outcome of work done in collaboration except as specified in the text. The dissertation does not exceed the word limit specified by the Physics and Chemistry Degree Committee.

Lois Overvoorde September 2018

3 Abstract

Bacterial resistance to antibiotics is becoming a serious global health threat, and the discovery of new, safe and effective antibiotics is required urgently.[1–3] A new class of antibiotic, namely sideophore-antibiotic conjugates, has shown promise in initial studies.[4,5] Siderophores are used by bacteria for iron uptake, and so attaching antibiotics to them allows the antibiotic to be carried across cell membranes. This study investigates conjugates designed using a similar approach, but using bacterial autoinducers[6] instead of siderophores. Autoinducers are required for coordination of bacterial behaviours and are involved in the control of swarming, virulence factor production and biofilm formation.

swarming needs to be mentioned elsewhere

The library was synthesised in two halves which were then coupled together using a copper(I)-catalysed azide-alkyne cycloaddition.[7,8] The autoinducers were functionalised with azide groups and the antibiotics were functionalised with alkynes. The quorum sensing molecules produced by *Pseudomonas aeruginosa* were investigated as it is a significant human pathogen[9] which displays high resistance to many antibiotics[10] and uses quorum sensing to coordinate its group behaviours.[11] Azido analogues of these autoinducers were coupled with alkyne analogues of ciprofloxacin, which was chosen as it is commonly used against *P. aeruginosa*[12] but resistance to it is developing,[13] and trimethoprim. It was hoped that the autoinducers would aid retention of the antibiotic in the cell, thus potentially increasing its potency or even restoring its efficacy against resistant strains.

4 Nomenclature

J	Coupling constant in Hz
m/z	Mass to charge ratio in Daltons
R_f	Retention factor
Ac	Acetate
AIP	Autoinducing peptide
aq.	Aqueous
atm	Atmosphere(s)
BHL	Butyryl homoserine lactone = C_4-HSL **2**
Boc	*tert*-Butyloxycarbonyl
Cip	Ciprofloxacin
conc.	Concentrated
COSY	Correlation spectroscopy
d	Day(s)
Da	Daltons
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMP	Dess-Martin periodinane
DMSO	Dimethylsulfoxide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
eq.	Equivalents
ESI	Electrospray ionization
Et	Ethyl
FT	Fourier transform
h	Hour(s)
HCTL	Homocysteine thiolactone
HHQ	2-Heptylquinolin-4(1H)-one
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HMQC	Heteronuclear multiple-quantum correlation spectroscopy

HOBt 1-Hydroxybenzotriazole

HPLC High-performance liquid chromatography

HRMS High resolution mass spectroscopy

HSL Homoserine lactone

Hz Hertz

IR Infrared

LCMS Liquid chromatography mass spectroscopy

LCT Liquid chromatography time-of-flight

lit. Literature value

M Molar

m.p. Melting point

Me Methyl

MIC Minimum inhibitory concentration

min Minute(s)

mol Mole(s)

Ms Methanesulfonyl

NMR Nuclear magnetic resonance

OdDHL *N*-(3-Oxododecanoyl)-homoserine lactone = 3-oxo-$C_1$2-HSL **3**

P.E. Petroleum ether

PAI-1 *Pseudomonas* autoinducer 1 = 3-oxo-C_{12}-HSL **3**

PAI-2 *Pseudomonas* autoinducer 2 = C_4-HSL **2**

Pd/C Palladium on carbon

PQS Pseudomonas Quinolone Signal

Q-TOF Quadrupole time-of-flight

r.t. Room temperature

s Second(s)

SAM *S*-adenosyl-L-methionine

SAR Structure activity relationship

sat. Saturated

sp. Species

TBAF Tetrabutylammonium fluoride

TBDMS *tert*-Butyldimethylsilyl

TEA Triethylamine

Tf Trifluoromethanesulfonyl

TFA Trifluoroacetic acid

THF Tetrahydrofuran

THPTA Tris(3-hydroxypropyltriazolylmethyl)amine

TLC Thin layer chromatography

TMS Trimethylsilyl

Ts *para*-Toluenesulfonyl

UV Ultraviolet

5 Introduction

5.1 Antibiotic resistance

Antibiotics add, on average, twenty years to a person's life.[2] 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[2] is well known to many scientists. The initial serendipitous discovery of penicillin occurred in 1928 and was reported in 1929,[14] 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.[15,16] 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:[2,3]

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.[17]

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*.[10]

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.[18]

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.[19]

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.[20–25]

5.2 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.'[26] 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 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. 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 since 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.*[6, 27–35] 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.[1]

5.2.1 *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*[27, 34, 35] (see Figure 1). This bacterium receives amino acids[36, 37] from its host in exchange for producing light which the squid uses for counterillumination, to camouflage itself.[38]

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 1: '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 2).

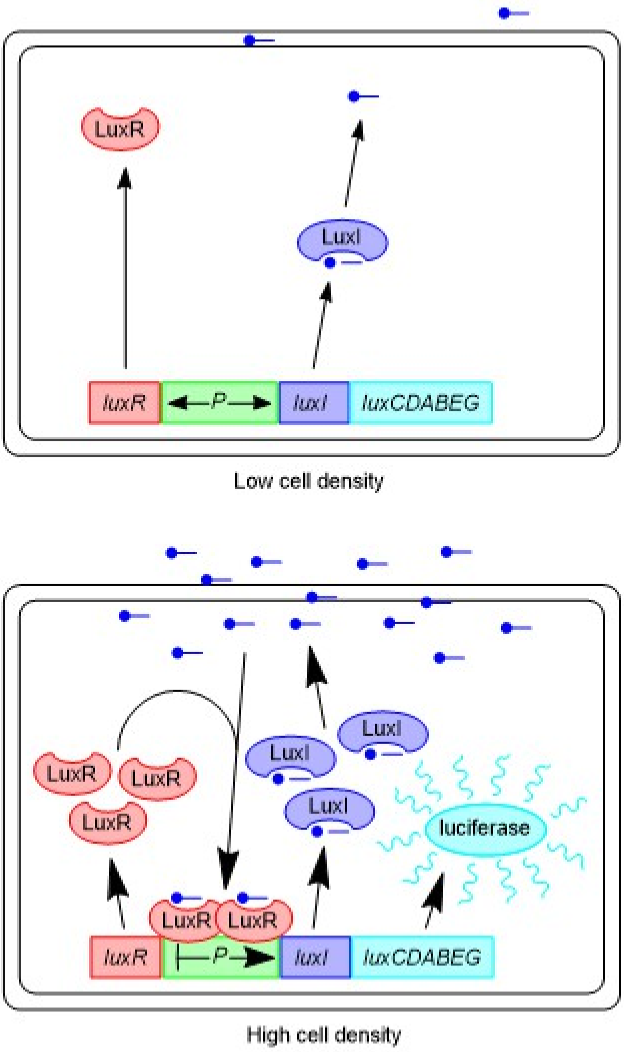


Figure 2: The LuxR-LuxI quorum sensing system in *V. fischeri*.

V. fischeri senses cell concentration by the detection of 3-oxo-C_6-HSL **1**[39] (see Figure 3), a freely diffusible[40] molecule which is synthesised by LuxI[41,42] and secreted by all *V. fischeri* cells[43] at a low basal level.[27] When the bacterial population density, and hence the concentration of 3-oxo-C_6-HSL **1**, reaches a threshold, 3-oxo-C_6-HSL **1** binds to LuxR,[44–46] a receptor which is also synthesised at a low basal level.

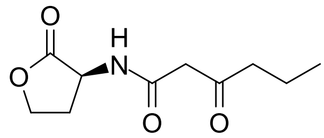


Figure 3: 3-oxo-C_6-HSL **1**.

The LuxR complex binds to the *lux* operator, upregulating production of LuxI and hence 3-oxo-C_6-HSL **1**, and luciferase enzymes and hence blue-green light.[47–49] Production of more 3-oxo-C_6-HSL **1** enables a positive feedback loop, reinforcing the effect of high population density on 3-oxo-C_6-HSL **1** concentration and hence light production. This is the reason that 3-oxo-C_6-HSL **1** is known as an autoinducer.

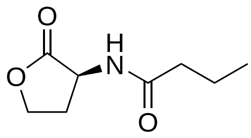
The system also contains a negatively feedback loop to avoid excessive expression of proteins: at high concentrations of 3-oxo-C_6-HSL **1** production of LuxR is inhibited.[50] Such balancing effects, as well as interactions with other quorum sensing and metabolic systems, are very common.

5.2.2 *Pseudomonas aeruginosa*

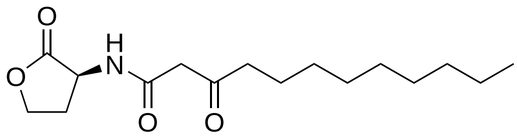
Another well-studied example of quorum sensing is in *P. aeruginosa*.[11,51,52] *P. aeruginosa* is a Gram-negative opportunistic pathogen which typically infects immunocompromised individuals such as those with cystic fibrosis, neutropenia and AIDS. It can infect the pulmonary and urinary tracts as well being the most frequent cause of burn wound infections and the most frequent conloniser of medical devices such as catheters.[9]

P. aeruginosa has a low susceptibility to many antibiotics and readily acquires antibiotic resistance by mutation or horizontal gene transfer.[53] It is difficult for antibiotics to cross into cells due to low cell membrane permeability[54] and biofilm formation,[55] and they are pumped out again by its multiple chromosomally encoded multidrug efflux pumps.[10] *P. aeruginosa* biofilms are more resistant to many drugs including ciprofloxacin **24** and trimethoprim **25** compared with planktonic cells.[55,56] This high level of antibiotic resistance makes *P. aeruginosa* an important target for drug discovery.

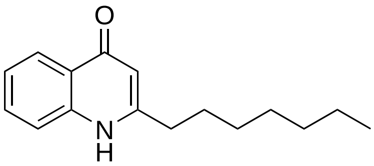
Quorum sensing in *P. aeruginosa* involves a complex interplay of five signalling molecules (see Figure 4) and various proteins (see Figure 5).[11,51,52] These can be broken down into three main, interacting systems: Las, Rhl and Pqs.



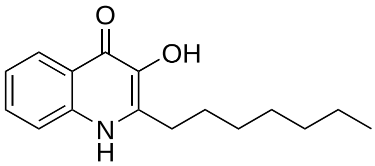
C_4-HSL
2



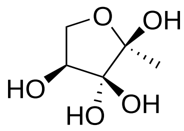
3-oxo-C_{12}-HSL
3



HHQ
4



PQS
5



AI-2
6

Figure 4: *P. aeruginosa* autoinducers.

In the Las system, LasI[57] synthesises the 3-oxo-C_{12}-HSL **3**[58] autoinducer. 3-oxo-C_{12}-HSL **3** binds LasR,[59] and this complex upregulates the production of LasI[60] (thus causing autoinduction) as well as alkaline protease,[61] elastase,[59] exotoxin A,[61] HCN[62] and LasA protease.[63] The LasR complex is also important in late-stage biofilm formation,[31] and upregulates the Rhl[64] and Pqs systems.[65,66]

In the Rhl system, RhlI[67] synthesises the C_4-HSL **2**[68] autoinducer. C_4-HSL **2** binds RhlR,[69] and this complex upregulates the production of RhlI[60] (again causing autoinduction), alkaline protease,[70] elastase,[67] haemolysin,[70] HCN,[62,70] LasA protease,[67] LecA,[71] pyocyanin[67,70] and rhamnolipids.[67] The RhlR complex also downregulates the Pqs system.[66,72] 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[64] and production of both RhlR is upregulated by the PqsR complex.[73]

In the Pqs system, the main autoinducer, PQS **5**,[74] is synthesised by multiple enzymes. PhnAB,[75] PqsA, PqsBC, PqsD[76,77] and PqsE[78,79] produce the precursor HHQ **4**, and PqsH converts HHQ **4** to PQS **5**. PQS **5**[66] or HHQ **4** binds PqsR,[80] and either complex can upregulate the synthesis of HHQ **4** causing autoinduction. The PqsR-PQS complex upregulates the production of chitinase,[81] elastase,[74] HCN,[81] LecA,[82] pyocyanin[65,83] and pyoverdine,[83] as well as increasing biofilm production[82] and vesicle formation.[84] The PqsR-PQS complex also upregulates production of RhlR, so the Pqs system has control over the Rhl system.[73] The Pqs system is controlled by both the Las and Rhl systems, as production of PqsR[66] and PqsH[65] is upregulated by the LasR complex and production of PqsA, PqsBC, PqsD, PqsE[72] and PqsR[66] is downregulated by the RhlR complex.

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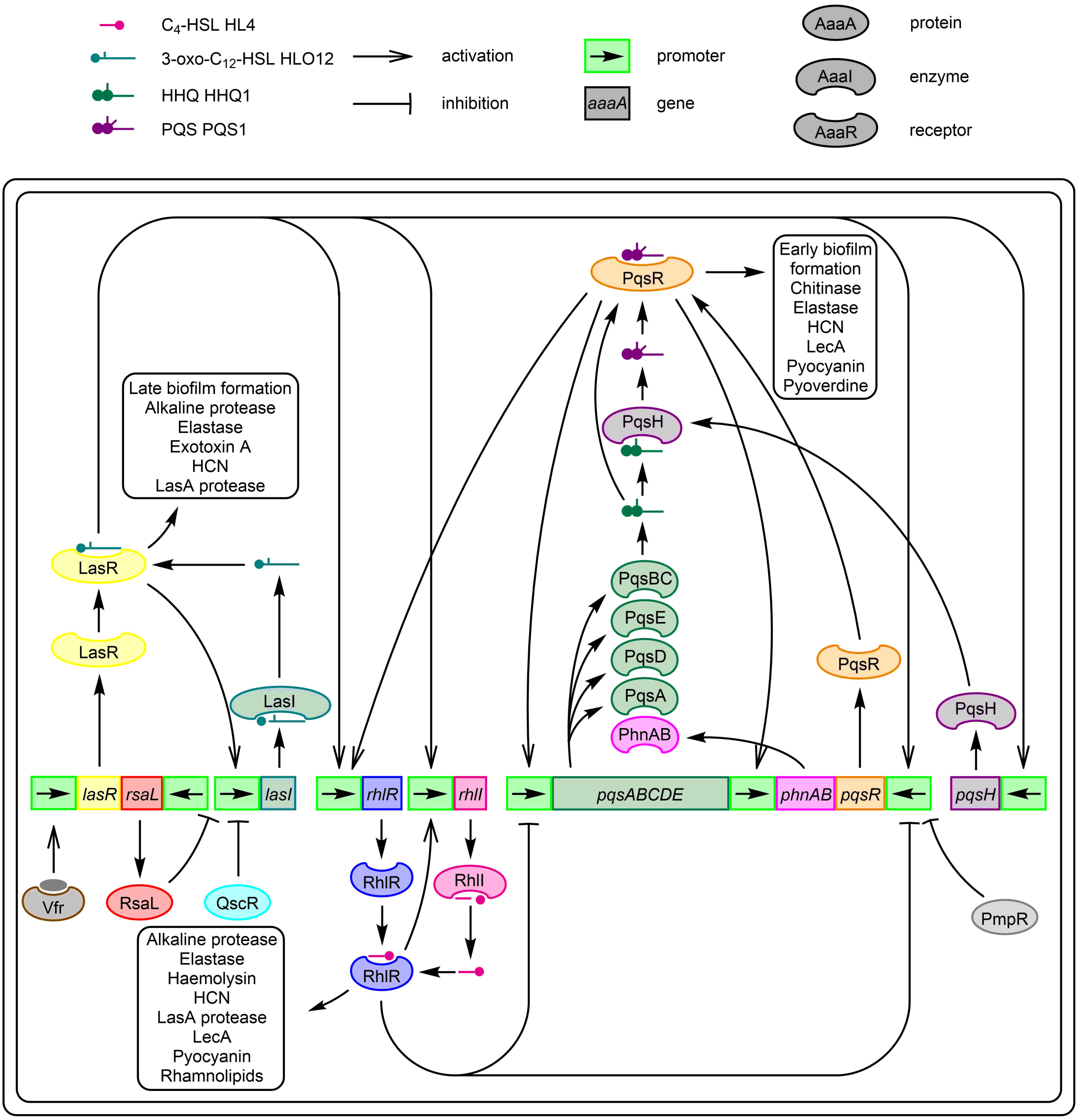


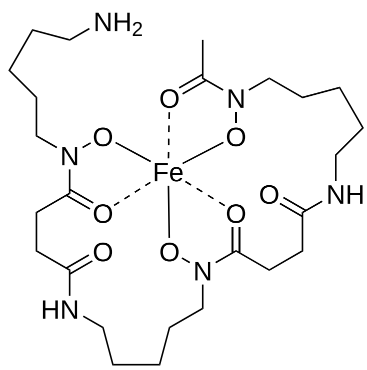
Figure 5: Quorum sensing in *P. aeruginosa*.[11,51,52]

In addition to the above systems, AI-2 (see Figure 4), an interspecies signalling molecule,[85] is known to increase biofilm production and virulence in *P. aeruginosa*.[86,87] This is thought to be achieved by interaction with the Las and Rhl systems, but the exact mechanism is not known.

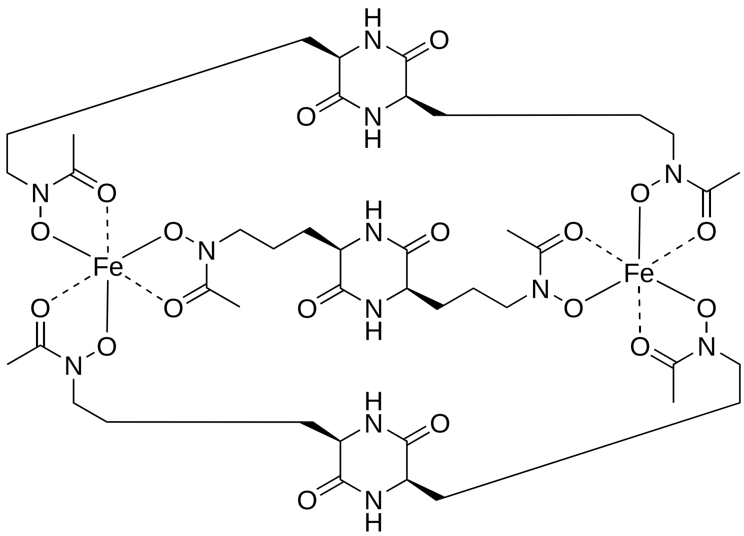
In summary, *P. aeruginosa* uses the autoinducers shown in Figure 4 as part of three interacting quorum sensing systems to coordinate virulence and biofilm production, and this makes these autoinducers interesting therapeutic targets. Quorum sensing has been sucessfully targeted using many different modulators,[51,88] but this study takes a slightly different approach. Inspired by the success of various siderophore-antibiotic conjugates (see 5.5), a library of autoinducer-antibiotic conjugates was synthesised, in the hope that the importance of autoinducers in harmful cellular behaviours would lead to increased activity of the conjugates (see 5.6).

5.3 Siderophores

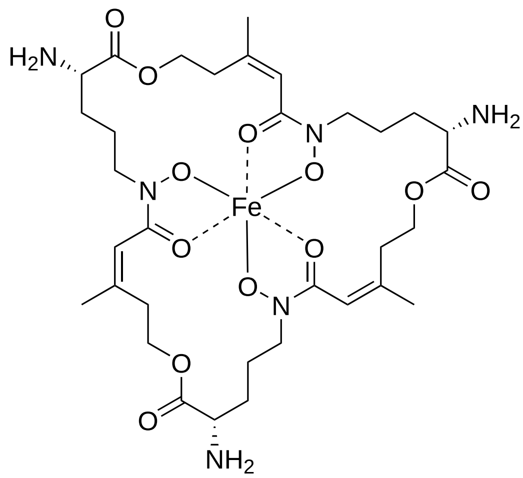
Siderophores are peptides or small molecules used by microorganisms to chelate iron for the purposes of ‘iron mining’.[89] Soluble iron is often scarce but it is crucial for many cellular processes including respiration and DNA synthesis. Siderophores are synthesised by the microorganisms and secreted 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 6 and Figure 7), possibly so one species can avoid its siderophores being taken up by another species.[90]



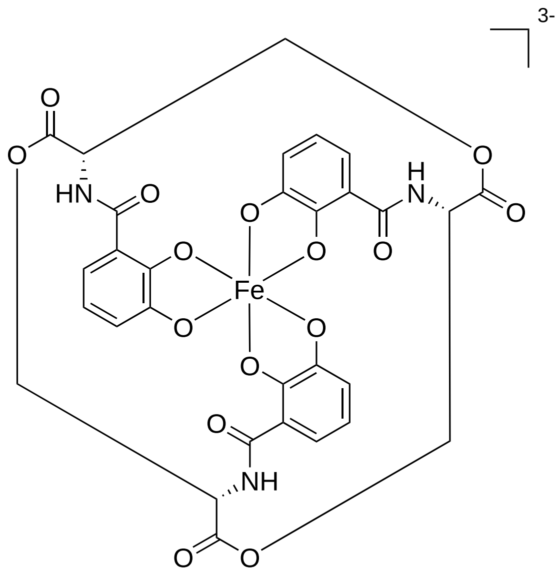
Deferoxamine B
7



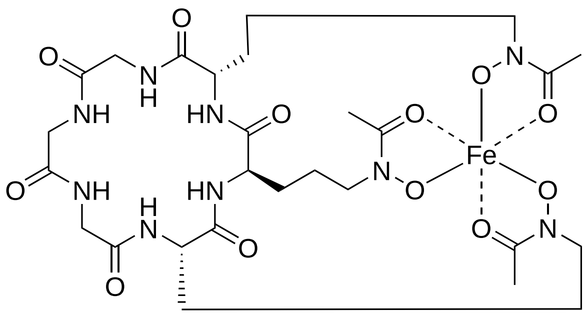
Rhodotorulic acid
8



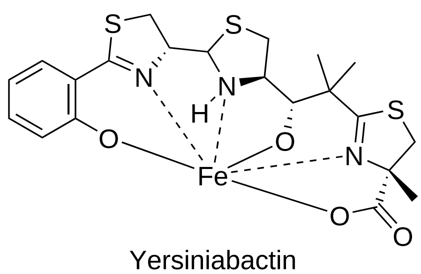
Fusarinine C
9



Enterobactin
10

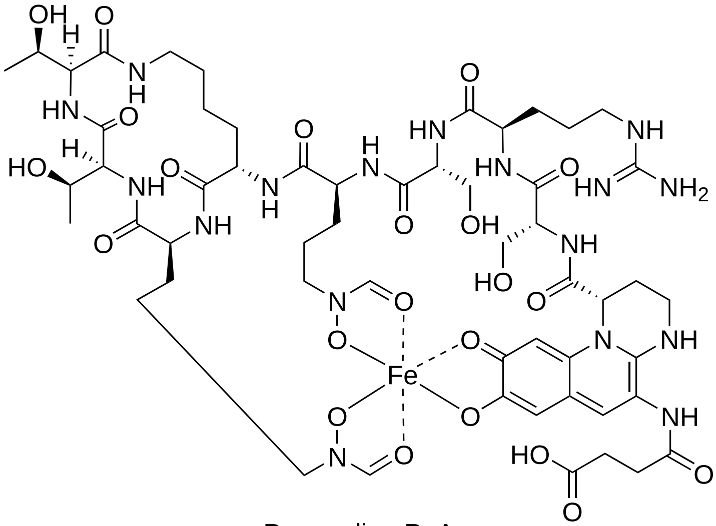


Ferrichrome
11

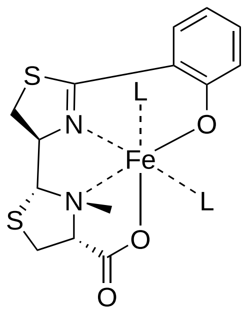


Yersiniabactin
12

Figure 6: Iron-siderophore complexes: Deferoxamine B **7**[91] (*Streptomyces pilosus* and *Streptomyces coelicolor*), rhodotorulic acid **8**[92] (*Rhodotorula pilimanae*), fusarinine C **9**[93] (*Fusarium roseum*), enterobactin **10**[91] (*Escherichia coli* and enteric bacteria), ferrichrome **11**[94] (*Ustilago sphaerogena*, *U. maydis*, *Aspergillus niger*, *A. quadricintus*, *A. duricaulis* and *Penicillium resticolosum*), yersiniabactin **12**[91] (*Yersinia pestis*).



Pyoverdine PaA
13



Pyochelin
14

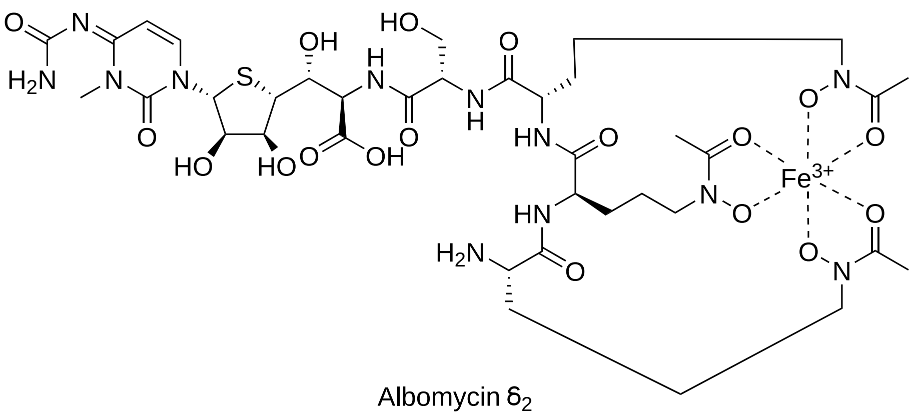
Figure 7: Iron-siderophore complexes: pyoverdine PaA **13**[91,95] (*P. aeruginosa*, PAO1 strain) and pyochelin **14**[96,97] (*P. aeruginosa*). Note that pyochelin **14** is a tetradentate ligand, hence the iron ion has two sites which can bind other ligands.

5.4 Sideromycins

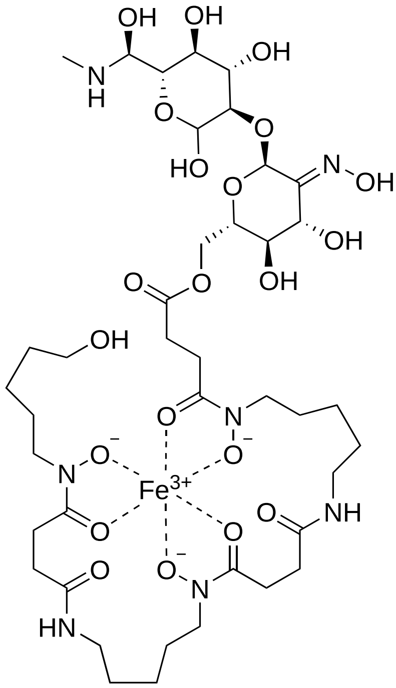
Siderophore-antibiotic conjugates are produced naturally by some bacteria and are known as sideromycins[4] (see Figure 8). Bacteria produce these molecules to attack other bacteria by hijacking their siderophore uptake mechanisms to introduce toxic compounds.

For example, albomycin **15** (see Figure 8) is a sideromycin produced by *Actinomyces subtropicus* and *Streptomyces griseus*[98,99] which has been used to treat infections caused by various bacteria including *Yersinia enterocolitica* and *Streptococcus pneumoniae* in mice and humans.[100,101] 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 6), a siderophore produced by various fungi, but also taken up by bacteria including *Escherichia coli*, *Salmonella typhimurium* and *P. aeruginosa*.[94,102] It has been shown that because of the structural similarity to ferrichrome **11**, *E. coli* will also take up albomycin **15**.[98] 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.

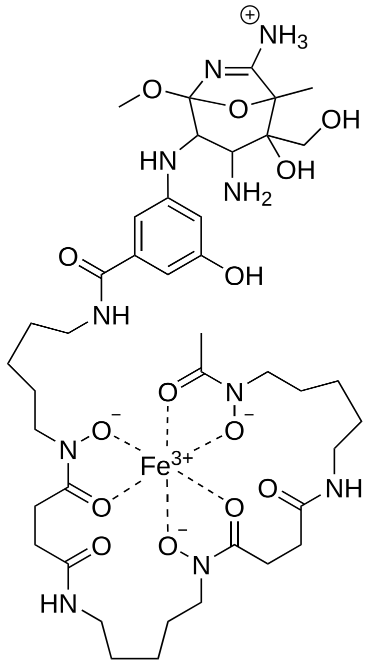
The success of albomycin[100] and other sideromycins such as salmycin A[89,103,104] and ferrimycin A1[105,106] has served as encouragement to many researchers to explore synthetic siderophore-antibiotic conjugates, which will be discussed in the next section.



Albomycin δ$_2$
15



Salmycin A
16



Ferrimycin A1
17

Figure 8: Iron-sideromycin complexes: Albomycin **15**[89,107] (*Actinomyces subtropicus* and *Streptomyces griseus*), salmycin A[89,103,104] (*Streptomyces violaceus*) and ferrimycin[89] (*Streptomyces griseoflavus*).

5.5 Synthetic siderophore-antibiotic conjugates

Sideromycins served as inspiration for the design, synthesis and biological evaluation of a wide range of synthetic siderophore-antibiotic conjugates.[4] Antibiotics used include β-lactams,[108–110] nucleosides,[111] glycopeptides[112] and macrolides.[113] Sideromycin-fluoroquinolone conjugates have also been studied by several groups,[114–116] including conjugates with linkers which can be cleaved[115,116] in a similar manner to albomycin.[98] 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.

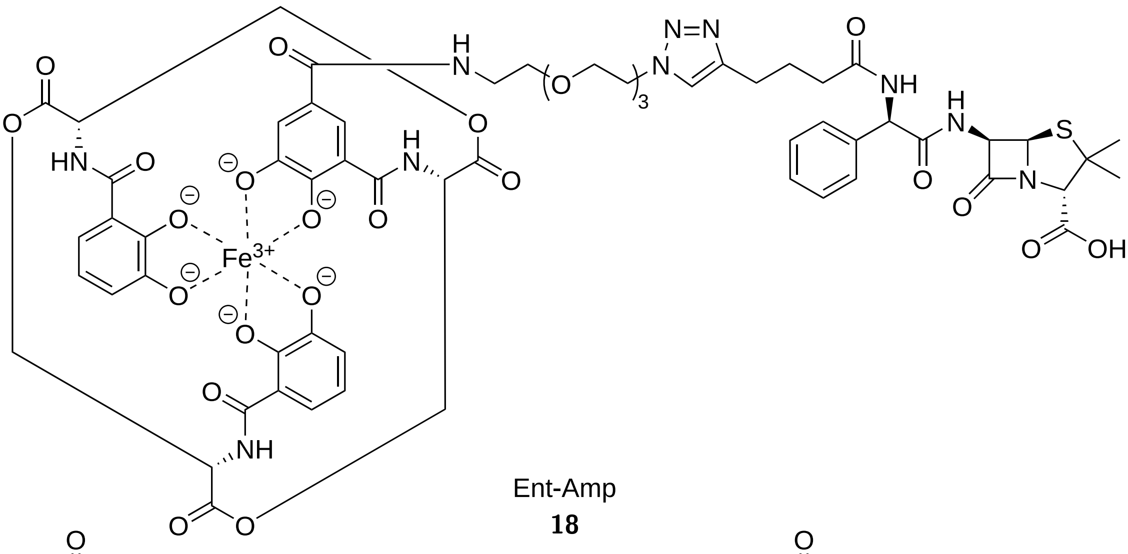
β-lactam-sideromycin conjugates have been more widely investigated and show good activity *in vitro*, however, resistance can evolve by loss of the TonB transporter or of the relevant siderophore receptor, e.g. Cir and Fiu for catecholate siderophores or FhuA for hydroxamate siderophores.[4] Recently a conjugate (Ent-Amp **18**, see Figure 9) 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.[117] Other work has focused on monocyclic β-lactams, for example pirazmonam **19** and U-78608 **20**, which show high potency against Gram-negative bacteria including *P. aeruginosa*,[118,119] 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:[5] MC-1 **21**,[120] BAL30072 **22**[4] (see Figure 9) and cefiderocol **23**[121,122]. pre-release paper

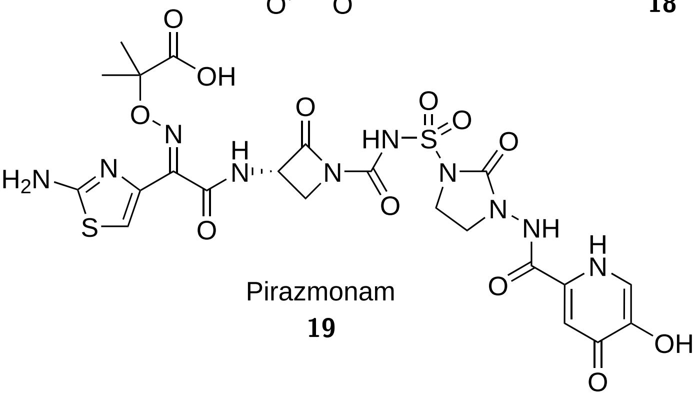
MC-1 **21** is reported as being "in clinical phases of development",[5] but no reports of studies in humans could be found. However, experiments in mice have been promising.[120] BAL30072 **22** is a siderophore-β-lactam conjugate which showed initial promise as it is a poor substrate for β-lactamases, and resistance due to loss of transport proteins is infrequent.[4] However, it is unclear whether it will progress further in trials as it causes liver toxicity.[123] 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'.[122]

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,[101] indicating that bacteria may lose out either by susceptibility to the antibiotic or by loss of fitness due to decreased iron transport.

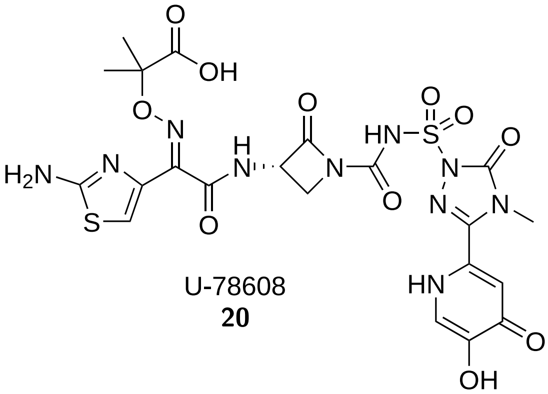
Building on these positive examples, it is hoped that the strategy of conjugating a molecule which is important for virulence[124] with an antibiotic can be extended to conjugates of autoinducers and antibiotics in a similar 'Trojan horse' approach.



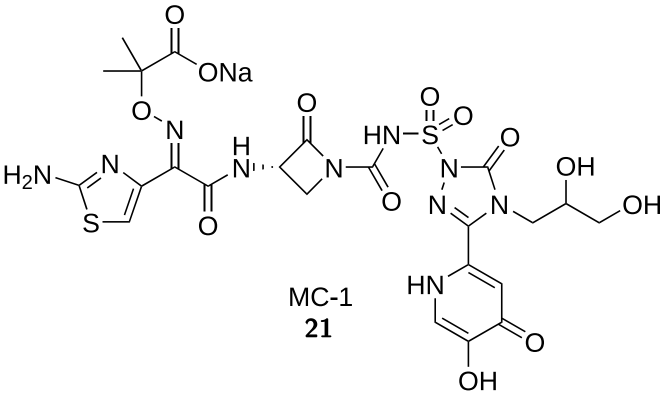
Ent-Amp
18



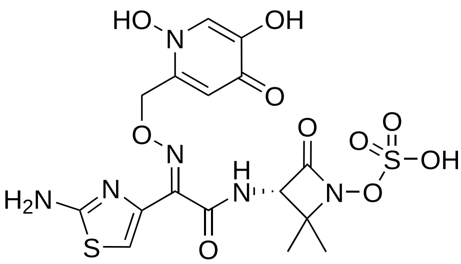
Pirazmonam
19



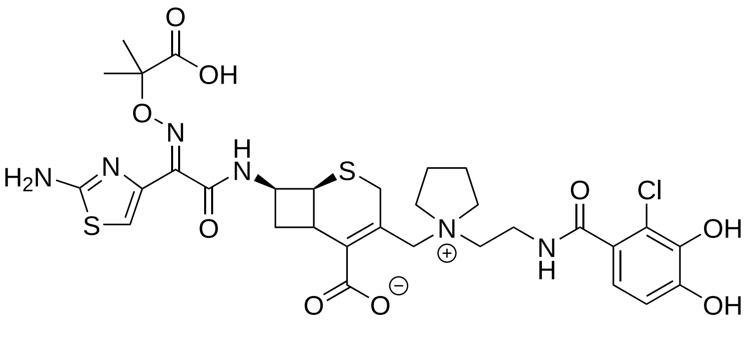
U-78608
20



MC-1
21



BAL30072
22



Cefiderocol
23

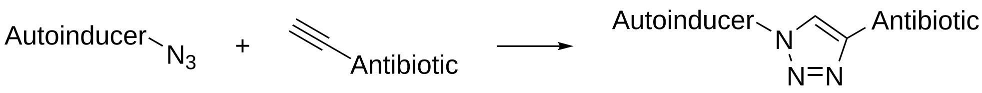
Figure 9: Examples of siderophore-antibiotic conjugates: Ent-Amp **18**,[117] pirazmonam **19**,[118,119] U-78608 **20**,[118,119] MC-1 **21**,[120] BAL30072 **22**[4] and cefiderocol **23**.[121,122]

5.6 Autoinducer-antibiotic conjugates

This study has extended 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. The work is divided into two main sections. The first section focuses on conjugates of three *P. aeruginosa* autoinducers (see Figure 4) with ciprofloxacin and trimethoprim (see Figure 10). The second section focuses on conjugates of homoserine lactone analogues with ciprofloxacin (see 5.7).

5.6.1 Synthesis of the conjugates

A copper(I)-catalysed azide-alkyne cycloaddition,[7,8] 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). The autoinducers were functionalised with azide groups, and the antibiotics with alkynes. This modular approach would allow the library to be expanded easily 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.

5.6.2 Autoinducers

The *P. aeruginosa* autoinducers (see Figure 4) were chosen as *P. aeruginosa* is a significant human pathogen which shows high antibiotic resistance and utilises quorum sensing to coordinate pathogenic behaviours (see 5.2.2). Specifically, C_4-HSL **2**, HHQ **4** and PQS **5** derivatives were chosen as they were considered to be the most synthetically tractable.

5.6.3 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 **4** or AI-2 **6**, but it is know that PQS **5** is exported in vesicles,[125] C_4-HSL **2** passively diffuses in and out of cells,[126] and 3-oxo-C_{12}-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_{12}-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.[40]

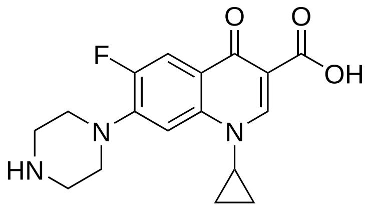
3-oxo-C_{12}-HSL **3** is exported primarily via the MexAB-OprM efflux system.[10,127] The increased removal of 3-oxo-C_{12}-HSL **3** from the cell by upregulation of the MexAB-OprM system leads to decreased production of additional 3-oxo-C_{12}-HSL **3** (as the positive feedback loop is disrupted, see 5.2.2), 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_{12}-HSL **3** levels would disrupt Las-mediated quorum sensing,[128] but no direct studies of this could be found.

5.6.4 Antibiotics

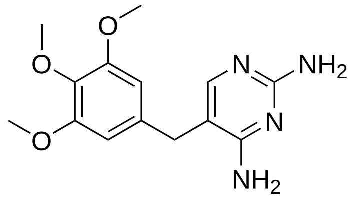
Ciprofloxacin **24** and trimethoprim **25** (see Figure 10) were chosen as the antibiotic sides of the conjugates.

Ciprofloxacin **24** is second-generation fluoroquinolone antibiotic used to treat both Gram-positive and Gram-negative bacterial infections including *P. aeruginosa*.[12,129]

Trimethoprim (see Figure 10) is a dihydrofolate reductase inhibitor used primarily to treat bladder infections.[130] 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.

5.6.5 Antibiotic efflux

Ciprofloxacin **24** enters *P. aeruginosa* by diffusion,[131] but is pumped out by efflux pumps.[132] 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.[10] However, in biofilms only MexEF-OprN has an effect.[133]

Trimethoprim **25** is mainly exported by the MexAB–OprM,[134] MexCD–OprJ[135] and MexEF–OprN[136] multidrug efflux systems[10,137] 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.[56]

5.6.6 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.[11,127,128] 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 **3**.[10,127] For PQS conjugates this would mean upregulation of vesicle formation.[125]

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[133] and not an exporter of HSLs **2** or **3**, or PQS **5**[10]). 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.

5.7 Autoinducer analogue-ciprofloxacin conjugates

Following on from the library of compounds based on *P. aeruginosa* autoinducers, a series of conjugates based on *analogues* of HSL were planned. This strategy was inspired by a paper[138] and patent[139] by Ganguly *et al.*, who synthesised and characterised a conjugate **111** of methyl ciprofloxacin with homocysteine thiolactone (see Figure 11). 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.[43,140–146]

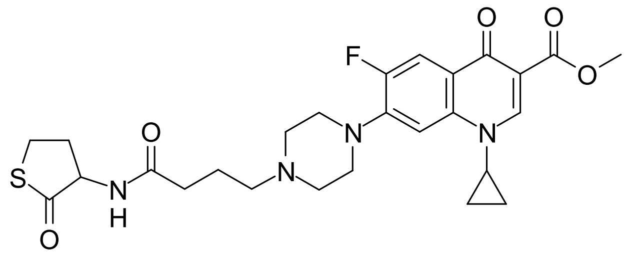


Figure 11: The HCTL-CipMe conjugate **111** studied by Ganguly *et al.*[138,139]

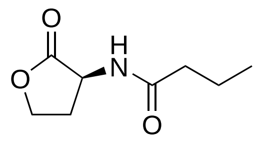
As part of their characterisation of the HCTL-CipMe conjugate **111**, Ganguly *et al.* found the minimum inhibitory concentration (MIC) of the conjugate in *P. aeruginosa* under standard planktonic conditions. The MIC was found to be ten times higher for the conjugate vs. ciprofloxacin (50 vs. 5 μm), 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'.

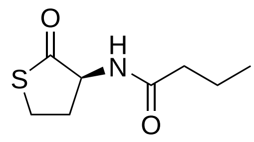
These results are exciting as they hint that an autoinducer conjugate might be able to combat an established *P. aeruginosa* infection more effectively than the unmodified antibiotic. Ganguly *et al.* suggest that their conjugate is more effective than ciprofloxacin in penetrating biofilms, and/or better 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 **26** (see Figure 12) has 'either enhanced uptake or functional activity' when compared with C_4-HSL **2**.

It is possible that the conjugate **111** has higher activity against biofilms when compared with ciprofloxacin **24** because conjugate **111** avoids being pumped out by multidrug efflux pumps, or selects for the survival of mutants with upregulated efflux pumps, and hence disrupted quorum sensing systems (see 5.6.6).

While one might expect the conjugate **111** to behave like C_4-HSL **2**, 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 **3**. 3-oxo-C_{12}-HSL **3**'s accumulation in membranes and interaction with efflux pumps is thought to be based primarily on tail chain length (see 5.6.3), and the ciprofloxacin half of the conjugate **111** could be seen as a long tail, especially as the carboxylic acid is methylated and hence less polar.



C_4-HSL
2



C_4-HCTL
26

Figure 12: C_4-HSL **2** and C_4-HCTL **26**. Note that Ganguly *et al.* tested the *S* enantiomer of C_4-HCTL **26**, 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 known as part of quorum sensing modulators.[88, 147]

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 **??**.

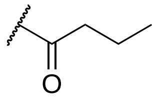
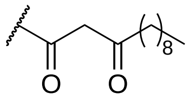
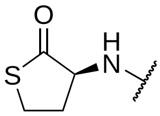
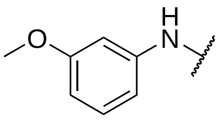
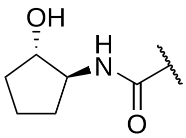
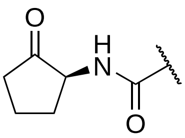
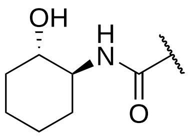
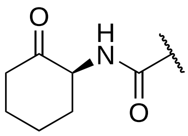
Head group		
	Partial agonist and antagonist against LasR.[144] Shown to increase biofilm formation in *P. aeruginosa*.[138]	Strong agonist against LasR, with comparable activity to the native ligand.[141, 142, 144, 148]
	Partial agonist against LasR.[147]	Strong antagonist against LasR.[147]
	Poor agonist and antagonist against RhlR.[149, 150]	Strong antagonist against LasR.[149]
	Strong agonist against RhlR.[149] *SS* enantiomer is more potent.[150]	Partial agonist against LasR.[149]
	Strong agonist against RhlR.[149] *SS* enantiomer is more potent, with comparable activity to the native ligand.[150]	Strong agonist against LasR.[142, 149] *SS* enantiomer is more potent, with comparable activity to the native ligand.[150]
	Strong agonist against RhlR.[149] *SS* enantiomer is more potent.[150]	Partial antagonist against LasR.[149] Shown to reduce biofilm formation in *P. aeruginosa*.[149]

Table 2: Activities of autoinducers containing the chosen head groups when coupled with C_4 or 3-oxo-C_{12} tails.

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6 Autoinducer-antibiotic conjugates

6.1 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 Figure 4). Azido derivatives of these compounds were coupled to alkynyl dervitatives of antibiotics, specifically ciprofloxacin **24** and trimethoprim **25** (see Figure 10), using a copper(I)-catalysed azide-alkyne cycloaddition.[7,8]

6.1.1 Azido autoinducer derivatives

The structure-activity relationships in HHQ **4** and PQS **5** have been previously studied,[151–153] and it was shown various substitutions on the benzene ring could be made without significantly decreasing activity. The 6-azido derivatives (see Figure 13) were chosen for this study as routes to them have previously been found.[154]

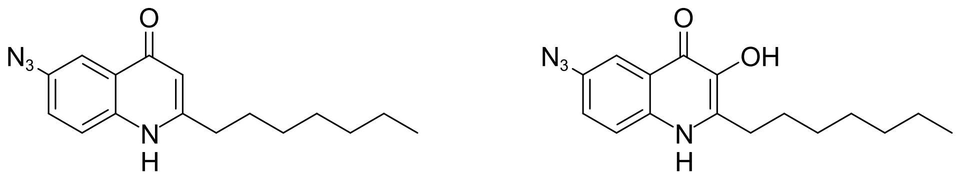


Figure 13: The azido derivatives of HHQ **4** and PQS **5**: **46** and **57**.

Alteration of the lactone group of HSL derivatives is known to significantly decrease activity, especially where the number of H-bond donors or acceptors is altered.[88] Hence, the azide group was included on the tail.[155] Acyl tail length is known to play an important role in affinity,[88] 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 14).

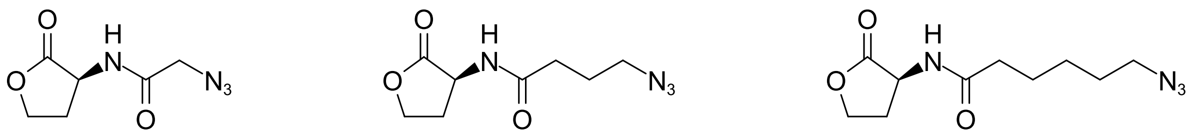
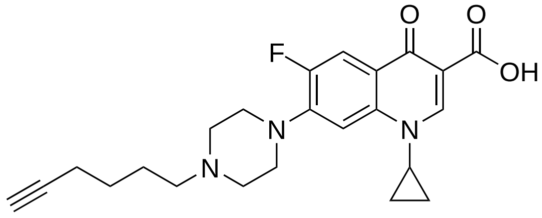


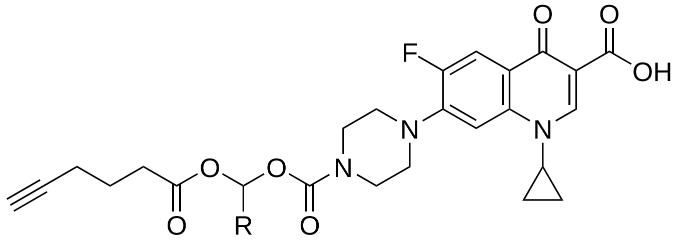
Figure 14: The azido derivatives of C_4-HSL **2**: **63**, **66** and **69**.

6.1.2 Alkynyl antibiotic derivatives

The structure-activity relationships for ciprofloxacin have been investigated[156] 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 15) 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 **??**).



76



R = H **98**
R = Me **99**

Figure 15: The alkynyl ciprofloxacin derivatives **76**, **98** and **99**.

The choice to of alkyne tail attachment point on trimethoprim **25** (see Figure 16) is based on the use of that same point in a fluorogenic trimethoprim tag synthesised by Jing *et al.*[157]

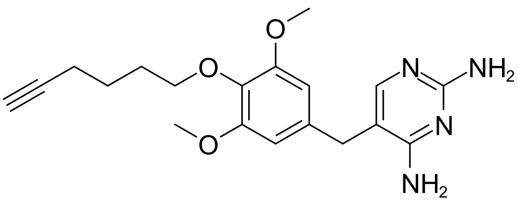


Figure 16: The alkynyl trimethoprim derivative **79**.

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