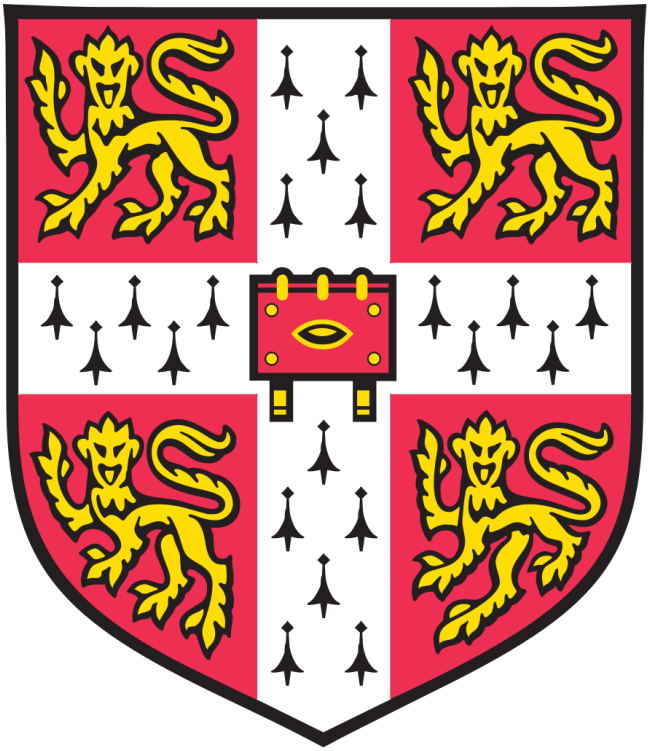
The synthesis and biological evaluation of a library of autoinducer-antibiotic conjugates

Lois Overvoorde



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University of Cambridge

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Supervised by Prof. David Spring

This dissertation is submitted for the degree of Doctor of Philosophy

Contents

1 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

2 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.[7]

The library was synthesised in two halves which were then coupled together using a copper(I)-catalysed azide-alkyne cycloaddition.[8,9] 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[10] which displays high resistance to many antibiotics[11] and uses quorum sensing to coordinate its group behaviours.[12] Azido analogues of these autoinducers were coupled with alkyne analogues of ciprofloxacin, which was chosen as it is commonly used against *P. aeruginosa*[13] but resistance to it is developing,[14] 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.

analogues

3 Acknowledgements

Firstly, I would like to thank David Spring for the opportunity to work on this very interesting topic. I would also like to thank Hannah Sore for guidance and support through the later stages of the project, and Eddy Sotelo and Bin Yu for collaboration and useful discussions. Thank you to Mark Eldridge and Suzie Forrest for help with learning biochemical techniques, to Martin Welch for guidance and advice on data interpretation and especially to Tom O'Brien for stepping in to do some of the testing. Thanks also to Matt Pond, Melvyn Orriss, Nic Davies and Naomi Hobbs for help with equipment and glassware and to Jill Vaughan and Tommy Osberger for proof-reading. Most importantly, I would like to thank Yssy Baker for proof-reading, help, support, advice and encouragement.

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 **19**
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 **20**

P.E. Petroleum ether

PAI-1 *Pseudomonas* autoinducer 1 = 3-oxo-C_{12}-HSL **20**

PAI-2 *Pseudomonas* autoinducer 2 = C_4-HSL **19**

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,[15] 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.[16,17] 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.[18]

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*.[11]

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.[19]

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.[20]

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.[21–26]

The current pipeline of new antibiotics is widely thought to be worryingly inadequate.[27–29] 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,[27] 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.[30] These treatments often target bacterial virulence rather than killing bacteria outright, hence decreasing selection pressure for resistance.[21] 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*.[31] 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 5.3.1) inhibit the production of virulence factors and increase the survival time of mice in a lethal *P. aeruginosa* lung infection model.

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 examples of such conjugates are antibody-drug conjugates[32] in the treatment of cancer, but progress has also made against bacteria. In particular, siderophore-antibiotic conjugates (see 5.2) have been investigated in the hope of hijacking bacterial uptake mechanisms to import antibiotics,[4] and the autoinducer-antibiotic conjugates in this study may gain activity by avoiding efflux pumps (see 5.3). 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.

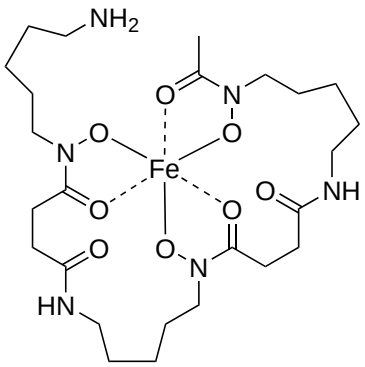
5.2 Siderophore-antibiotic conjugates

Siderophore-antibiotic conjugates have been receiving attention in recent years as a way to enhance the uptake of known antibiotics.[4] 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-

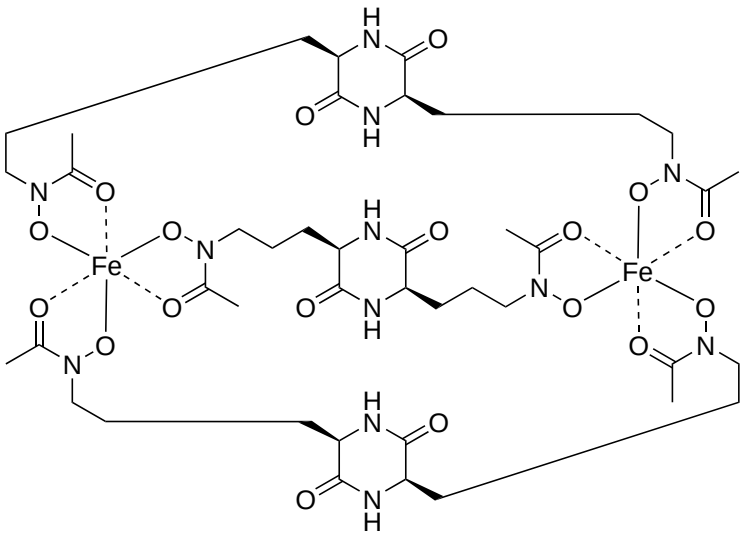
servations made about these molecules could be relevant to the autoinducer-antibiotic conjugates synthesised in this study.

5.2.1 Siderophores

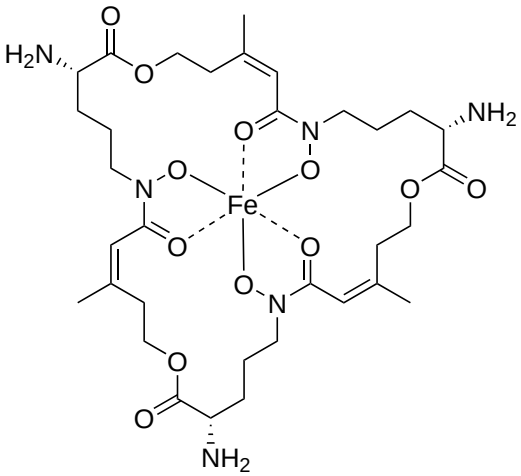
Siderophores are peptides or small molecules used by microorganisms to chelate iron for the purposes of 'iron mining'.[33] Soluble iron is often scarce but it is crucial for many cellular processes including respiration and DNA synthesis. Siderophores are synthesised by the microorganisms and secreted 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.[34]



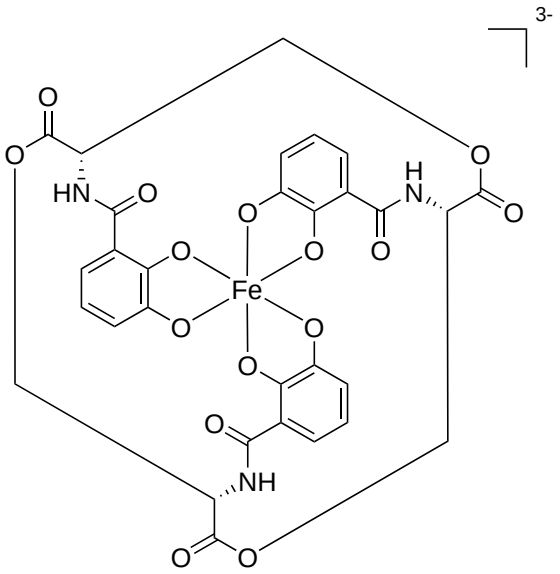
Deferoxamine B
1



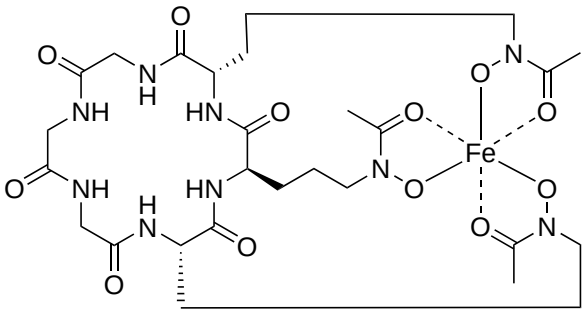
Rhodotorulic acid
2



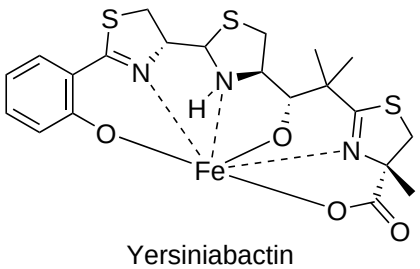
Fusarinine C
3



Enterobactin
4

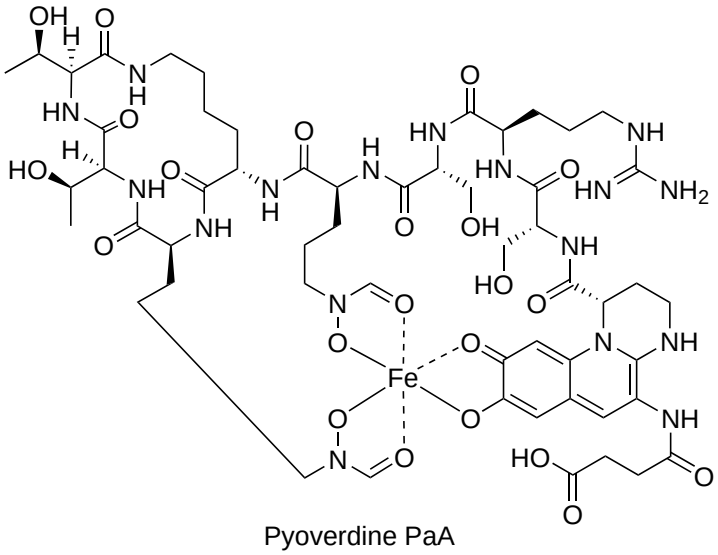


Ferrichrome
5



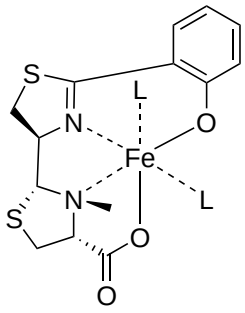
Yersiniabactin
6

Figure 1: Iron-siderophore complexes: Deferoxamine B **1**[35] (*Streptomyces pilosus* and *Streptomyces coelicolor*), rhodotorulic acid **2**[36] (*Rhodotorula pilimanae*), fusarinine C **3**[37] (*Fusarium roseum*), enterobactin **4**[35] (*Escherichia coli* and enteric bacteria), ferrichrome **5**[38] (*Ustilago sphaerogena*, *U. maydis*, *Aspergillus niger*, *A. quadricintus*, *A. duricaulis* and *Penicillium resticolosum*), yersiniabactin **6**[35] (*Yersinia pestis*).



Pyoverdine PaA

7



Pyochelin

8

Figure 2: Iron-siderophore complexes: pyoverdine PaA **7**[35,39] (*P. aeruginosa*, PAO1 strain) and pyochelin **8**[40,41] (*P. aeruginosa*). Note that pyochelin **8** is a tetradentate ligand, hence the iron ion has two sites which can bind other ligands.

5.2.2 Sideromycins

Siderophore-antibiotic conjugates are produced naturally by some bacteria and are known as sideromycins[4] (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*[42,43] which has been used to treat infections caused by various bacteria including *Yersinia enterocolitica* and *Streptococcus pneumoniae* in mice and humans.[44,45] 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*.[38,46] It has been shown that because of the structural similarity to ferrichrome **5**, *E. coli* will also take up albomycin **9**.[42] 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[44] and other sideromycins such as salmycin A[33,47,48] and ferrimycin A1[49,50] has served as encouragement to many researchers to explore synthetic siderophore-antibiotic conjugates, which will be discussed in the next section.

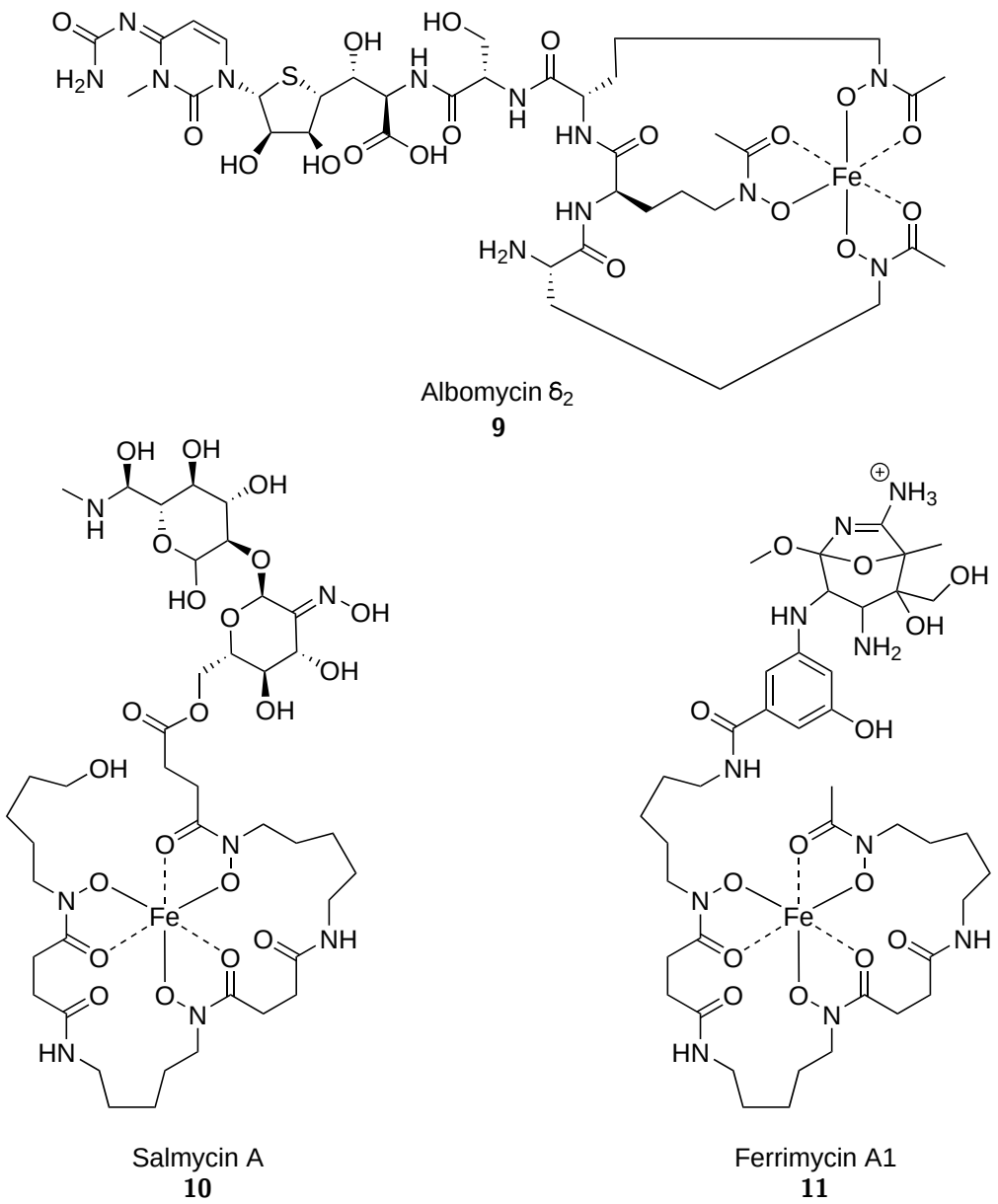


Figure 3: Iron-sideromycin complexes: Albomycin **9**[33,51] (*Actinomyces subtropicus* and *Streptomyces griseus*), salmycin A[33,47,48] (*Streptomyces violaceus*) and ferrimycin[33] (*Streptomyces griseoflavus*).

5.2.3 Synthetic siderophore-antibiotic conjugates

Sideromycins served as inspiration for the design, synthesis and biological evaluation of a wide range of synthetic siderophore-antibiotic conjugates.[4] Antibiotics used include β-lactams,[52–54] nucleosides,[55] glycopeptides[56] and macrolides.[57] Sideromycin-fluoroquinolone conjugates have also been studied by several groups,[58–60] including conjugates with linkers which can be cleaved[59,60] in a similar manner to albomycin.[42] 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 **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.[61] 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*,[62,63] 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 **15**,[64] BAL30072 **16**[4] (see Figure 4) and cefiderocol **17**[65,66].

pre-release paper

MC-1 **15** 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.[64] 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.[4] However, it is unclear whether it will progress further in trials as it causes liver toxicity.[67] 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'.[66]

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,[45] 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[68] with an antibiotic can be extended to conjugates of autoinducers and antibiotics in a similar 'Trojan horse' approach.

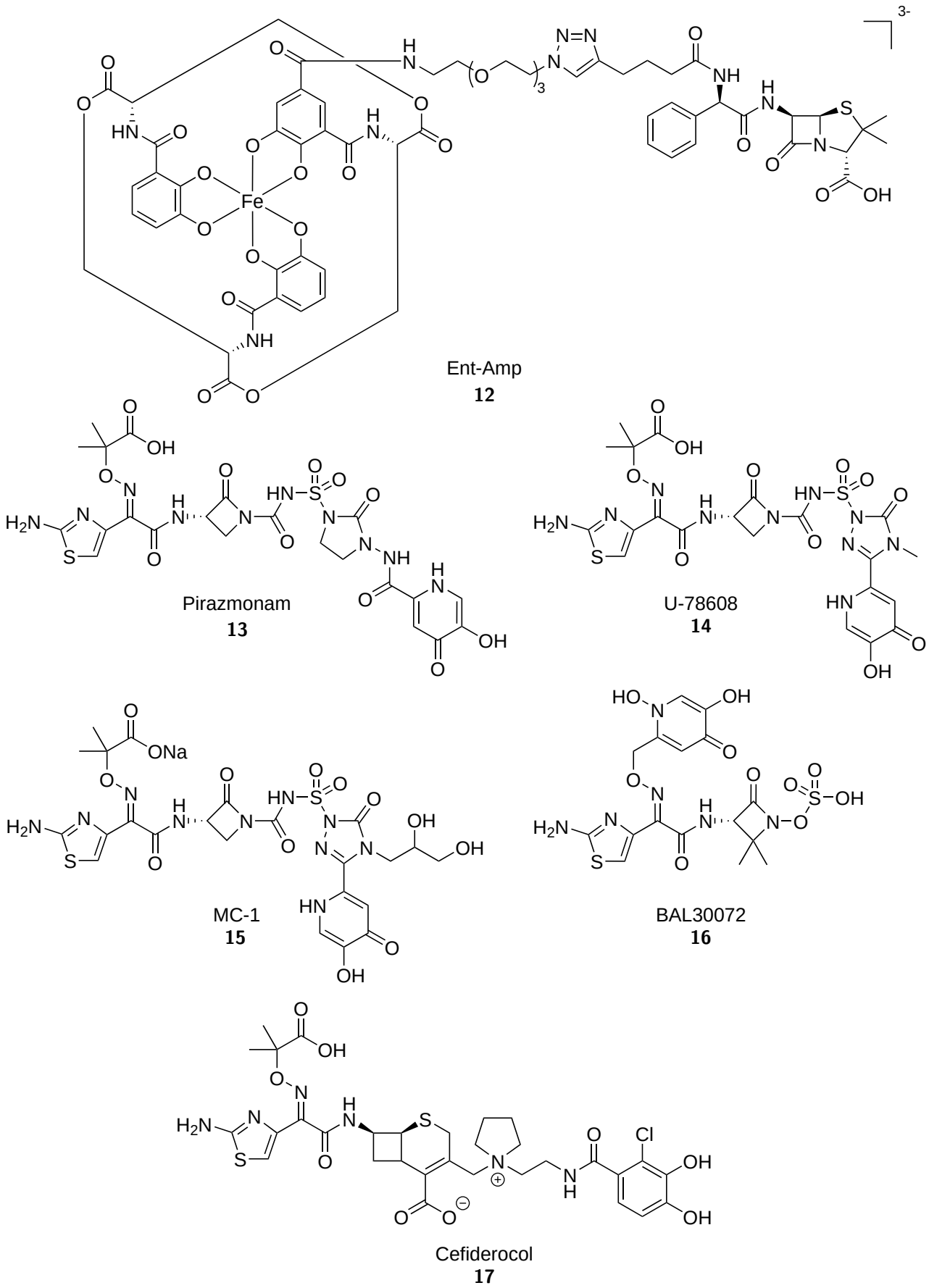


Figure 4: Examples of siderophore-antibiotic conjugates: Ent-Amp **12**,[61] pirazmonam **13**,[62,63] U-78608 **14**,[62,63] MC-1 **15**,[64] BAL30072 **16**[4] and cefiderocol **17**.[65,66]

5.3 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 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.

5.3.1 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.'[69] 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.[7] 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.*[6,7,70–77] 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]

5.3.1.1 *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*[7,76,77] (see Figure 5). This bacterium receives amino acids[78,79] from its host in exchange for producing light which the squid uses for counterillumination, to camouflage itself.[80]

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).

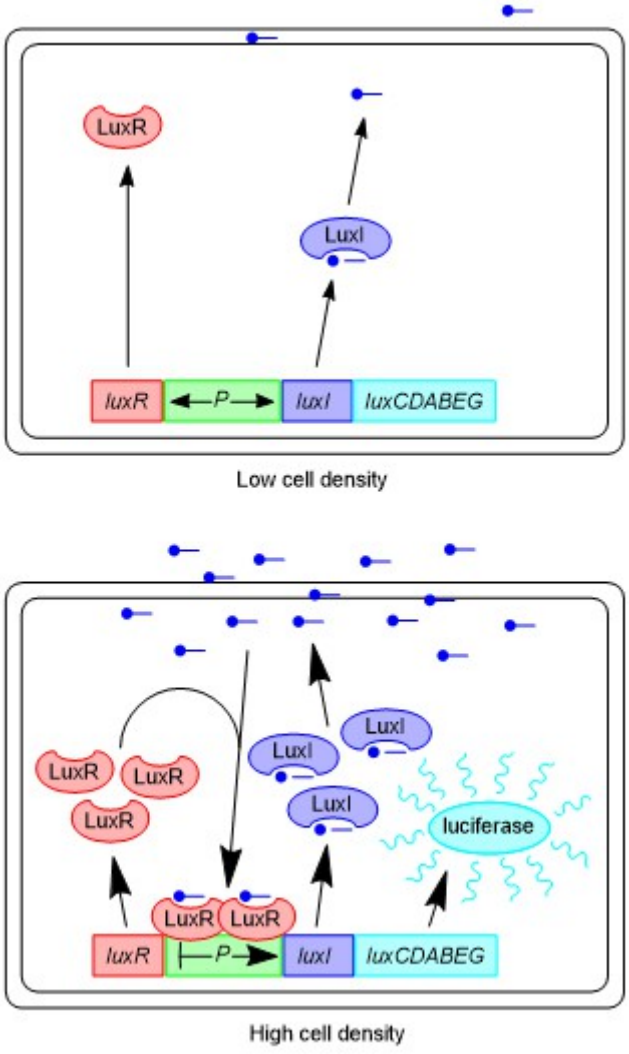


Figure 6: The LuxR-LuxI quorum sensing system in *V. fischeri*.

V. fischeri senses cell concentration by the detection of 3-oxo-C_6-HSL **18**[81] (see Figure 7), a freely diffusible[82]

better quality diagrams

molecule which is synthesised by LuxI[83,84] and secreted by all *V. fischeri* cells[85] at a low basal level.[7] 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,[86–88] a receptor which is also synthesised at a low basal level.

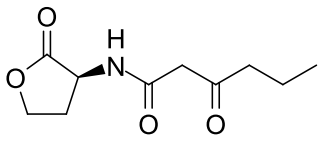


Figure 7: 3-oxo-C_6-HSL **18**.

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.[89–91] 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.[92] Such balancing effects, as well as interactions with other quorum sensing and metabolic systems, are very common.

5.3.1.2 *Pseudomonas aeruginosa*

Another well-studied example of quorum sensing is in *P. aeruginosa*.[12,93,94] *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.[10] Multidrug-resistant *P. aeruginosa* is classified as a 'serious threat' by the United States Centers for Disease Control and Prevention[1] and carbapenem-resistant *P. aeruginosa* is classified as 'priority 1: critical' by the World Health Organisation.[28]

P. aeruginosa has a low susceptibility to many antibiotics and readily acquires antibiotic resistance by mutation or horizontal gene transfer.[95] It is difficult for antibiotics to cross into cells due to low cell membrane permeability[96] and biofilm formation,[97] and they are pumped out again by its multiple chromosomally encoded multidrug efflux pumps.[11] *P. aeruginosa* biofilms are more resistant to many drugs including ciprofloxacin **24** and trimethoprim **25** compared with planktonic cells.[97,98] 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).[12,93,94] These can be broken down into three main, interacting systems: Las, Rhl and Pqs.

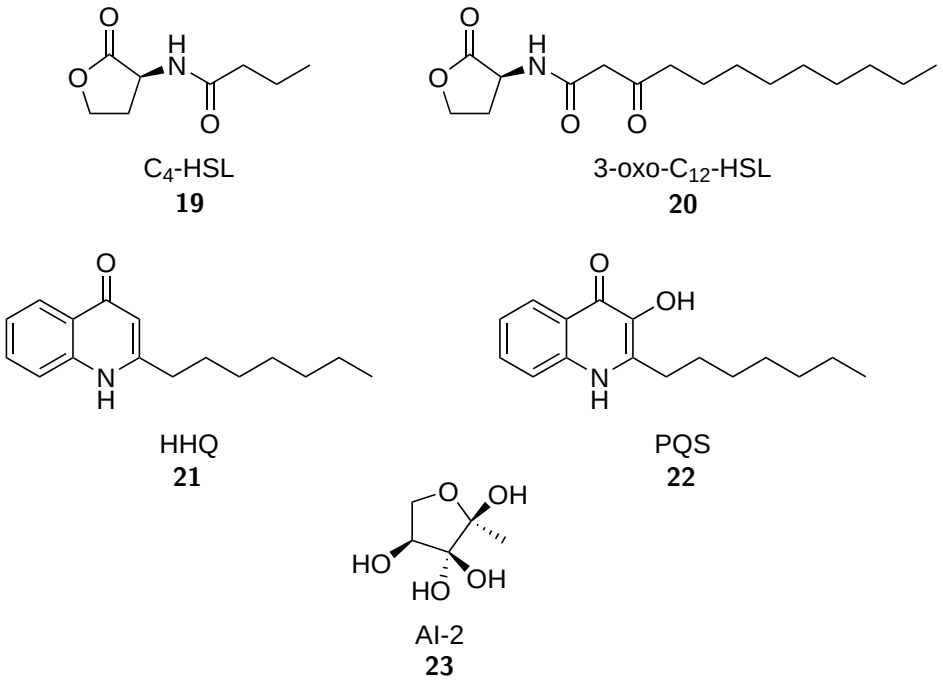


Figure 8: *P. aeruginosa* autoinducers.

In the Las system, LasI[99] synthesises the 3-oxo-C_{12}-HSL **20**[100] autoinducer. 3-oxo-C_{12}-HSL **20** binds LasR,[101] and this complex upregulates the production of LasI[102] (thus causing autoinduction) as well as alkaline protease,[103] elastase,[101] exotoxin A,[103] HCN[104] and LasA protease.[105] The LasR complex is also important in late-stage biofilm formation,[73] and upregulates the Rhl[106] and Pqs systems.[107,108]

In the Rhl system, RhlI[109] synthesises the C_4-HSL **19**[110] autoinducer. C_4-HSL **19** binds RhlR,[111] and this complex upregulates the production of RhlI[102] (again causing autoinduction), alkaline protease,[112] elastase,[109] haemolysin,[112] HCN,[104,112] LasA protease,[109] LecA,[113] pyocyanin[109,112] and rhamnolipids.[109] The RhlR complex also downregulates the Pqs system.[108,114] 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[106] and production of both RhlR is upregulated by the PqsR complex.[115]

In the Pqs system, the main autoinducer, PQS **22**,[116] is synthesised by multiple enzymes. PhnAB,[117] PqsA, PqsBC, PqsD[118,119] and PqsE[120,121] produce the precursor HHQ **21**, and PqsH converts HHQ **21** to PQS **22**. PQS **22**[108] or HHQ **21** binds PqsR,[122] and either complex can upregulate the synthesis of HHQ **21** causing autoinduction. The PqsR-PQS complex upregulates the production of chitinase,[123] elastase,[116] HCN,[123] LecA,[124] pyocyanin[107,125] and pyoverdine,[125] as well as increasing biofilm production[124] and vesicle formation.[126] The PqsR-PQS complex also upregulates production of RhlR, so the Pqs system has control over the Rhl system.[115] The Pqs system is controlled by both the Las and Rhl systems, as production of PqsR[108] and PqsH[107] is upregulated by the LasR complex and production of PqsA, PqsBC, PqsD, PqsE[114] and PqsR[108] is downregulated by the RhlR complex.

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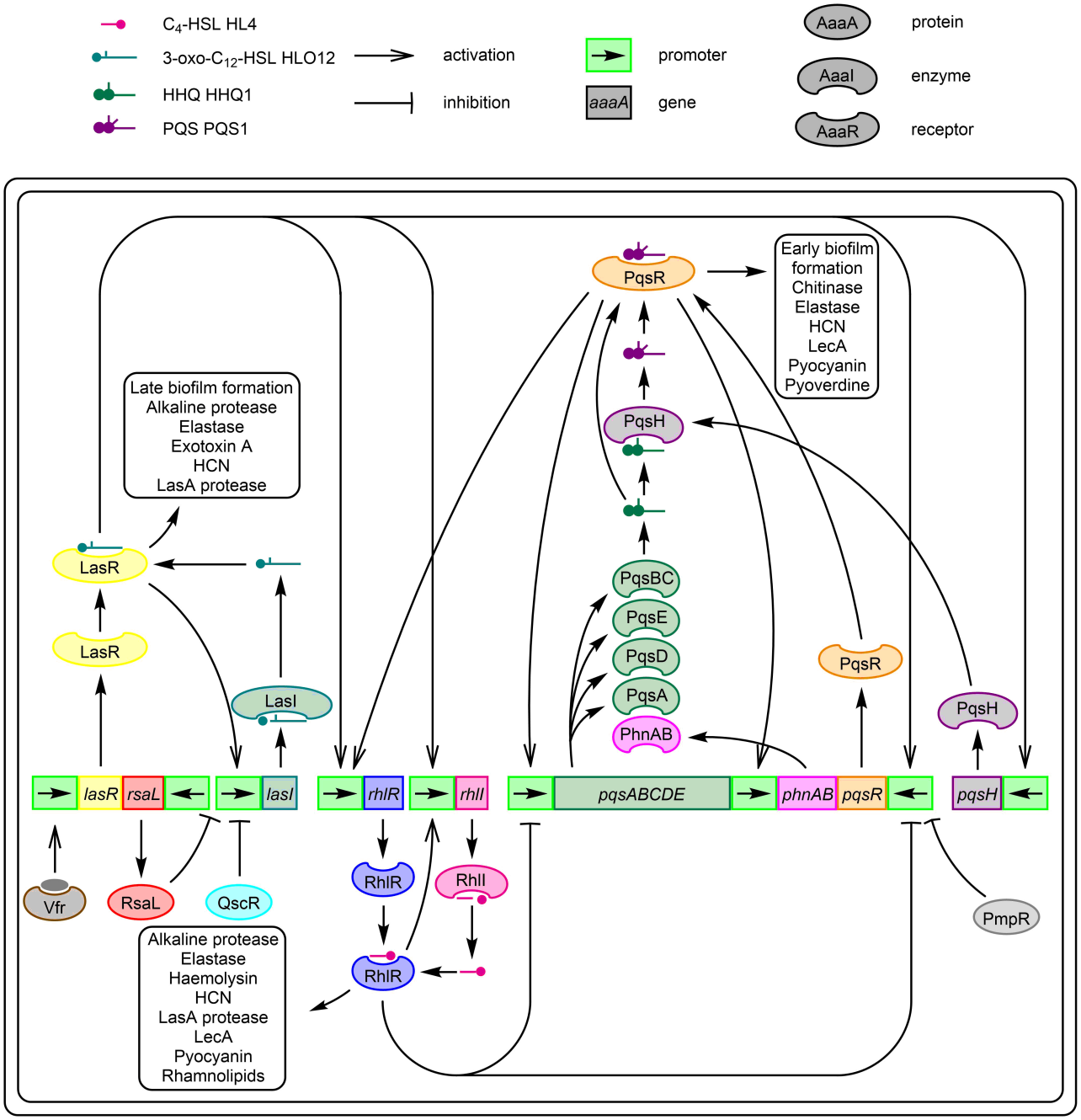


Figure 9: Quorum sensing in *P. aeruginosa*.[12,93,94]

In addition to the above systems, AI-2 (see Figure 8), an interspecies signalling molecule,[127] is known to increase biofilm production and virulence in *P. aeruginosa*.[128,129] 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.

5.3.2 Autoinducers

Quorum sensing has been sucessfully targeted using many different modulators,[93,130] but this study takes a slightly different approach. Inspired by the success of various siderophore-antibiotic conjugates (see 5.2.3), 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 5.3).

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 5.3.1.2). Specifically, C_4-HSL **19**, HHQ **21** and PQS **22** derivatives were chosen as they were considered to be the most synthetically tractable.

5.3.3 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,[131] C_4-HSL **19** passively diffuses in and out of cells,[132] 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.[82]

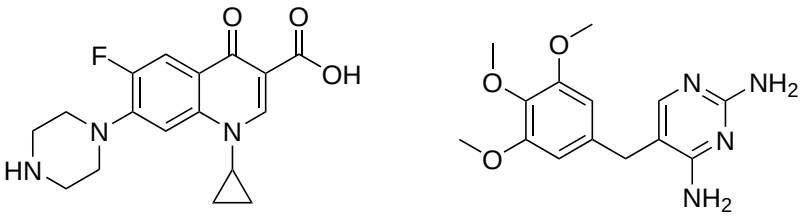
3-oxo-C_{12}-HSL **20** is exported primarily via the MexAB-OprM efflux system.[11,133] The increased removal of 3-oxo-C_{12}-HSL **20** from the cell by upregulation of the MexAB-OprM system leads to decreased production of additional 3-oxo-C_{12}-HSL **20** (as the positive feedback loop is disrupted, see 5.3.1.2), 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,[134] but no direct studies of this could be found.

5.3.4 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*.[13,135] Ciprofloxacin **24** inhibits DNA replication by binding to DNA gyrase and topoisomerase IV.[136]

Trimethoprim (see Figure 10) is a dihydrofolate reductase inhibitor used primarily to treat bladder infections.[137] 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.

5.3.5 Antibiotic efflux

Ciprofloxacin **24** enters *P. aeruginosa* by diffusion,[138] but is pumped out by efflux pumps.[139] 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.[11] However, in biofilms only MexEF-OprN has an effect.[140]

Trimethoprim **25** is mainly exported by the MexAB–OprM,[141] MexCD–OprJ[142] and MexEF–OprN[143] multidrug efflux systems[11,144] 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.[98]

5.3.6 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.[12,133,134] 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**.[11,133] For PQS conjugates this would mean upregulation of vesicle formation.[131]

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[140] and not an exporter of HSLs **19** or **20**, or PQS **22**[11]). 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.

5.3.7 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[145] and patent[146] by Ganguly *et al.*, who synthesised and characterised a conjugate **101** 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.[85,147–153]

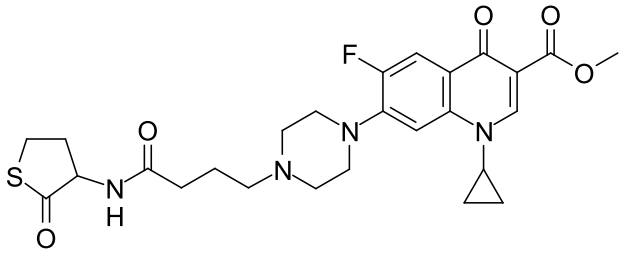


Figure 11: The HCTL-CipMe conjugate **101** studied by Ganguly *et al.*[145,146]

As part of their characterisation of the HCTL-CipMe conjugate **101**, Ganguly *et al.* found the minimum inhibitory concentration (MIC) of the conjugate in *P. aeruginosa* under standard planktonic conditions. The

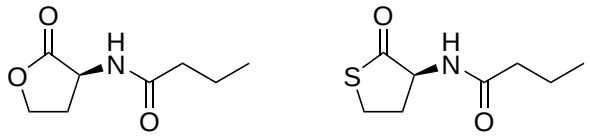
MIC was found to be ten times higher for the conjugate vs. ciprofloxacin (50 vs. 5 μm), indicating that the conjgate was less effective than ciprofloxacin under planktonic conditions.

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 **101**. They then incubated cultures for 24 h, to allow biofilms to grow, before adding the compounds. In contrast, they found that the conjugate **101** 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 **19**.

It is possible that the conjugate **101** has higher activity against biofilms when compared with ciprofloxacin **24** because conjugate **101** 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 5.3.6).

While one might expect the conjugate **101** 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 5.3.3), and the ciprofloxacin half of the conjugate **101** 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 **26**

Figure 12: C_4-HSL **19** 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.[130,154]

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 **??**.

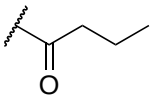
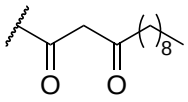
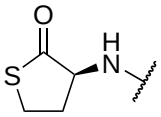
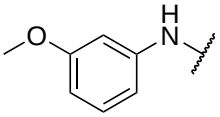
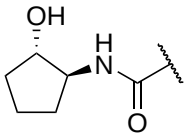
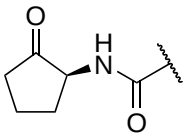
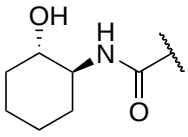
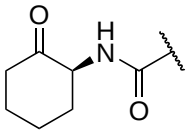
Head group		
	Partial agonist and antagonist against LasR.[151] Shown to increase biofilm formation in *P. aeruginosa*.[145]	Strong agonist against LasR, with comparable activity to the native ligand.[148,149,151,155]
	Partial agonist against LasR.[154]	Strong antagonist against LasR.[154]
	Poor agonist and antagonist against RhlR.[156,157]	Strong antagonist against LasR.[156]
	Strong agonist against RhlR.[156] *SS* enantiomer is more potent.[157]	Partial agonist against LasR.[156]
	Strong agonist against RhlR.[156] *SS* enantiomer is more potent, with comparable activity to the native ligand.[157]	Strong agonist against LasR.[149,156] *SS* enantiomer is more potent, with comparable activity to the native ligand.[157]
	Strong agonist against RhlR.[156] *SS* enantiomer is more potent.[157]	Partial antagonist against LasR.[156] Shown to reduce biofilm formation in *P. aeruginosa*.[156]

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

6 Project aims and summary

The aim of this project is to produce and test a library of autoinducer-antibiotic conjugates with the hope of producing conjugates with greater potency than the parent antibiotics. The work is divided into two main sections. Section 7 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 5.3.7) joined either using a copper(I)-catalyzed azide-alkyne cycloaddition or an S_N2 reaction or peptide coupling.

7 Results and discussion: autoinducer-antibiotic conjugates

7.1 Overview

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 **19**, HHQ **21** and PQS **22** (see Figure 8). 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.[8,9]

7.1.1 Azido autoinducer derivatives

The structure-activity relationships in HHQ **21** and PQS **22** have been previously studied,[158–160] 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.[161]

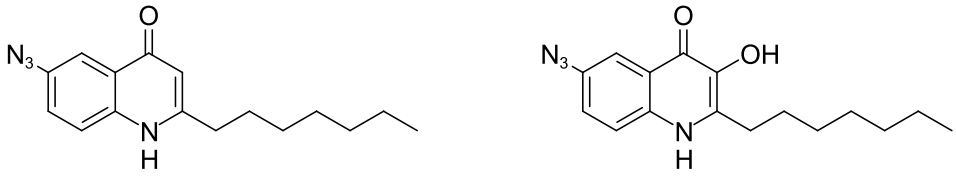


Figure 13: The azido derivatives of HHQ **21** and PQS **22**: **36** and **47**.

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.[130] Hence, the azide group was included on the tail.[162] Acyl tail length is known to play an important role in affinity,[130] so three derivatives of C_4-HSL **19** were synthesised: N_3-C_2-HSL **53**, N_3-C_4-HSL **56** and N_3-C_6-HSL **59** (see Figure 14).

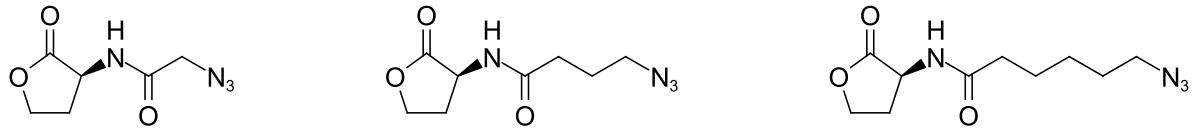


Figure 14: The azido derivatives of C_4-HSL **19**: **53**, **56** and **59**.

7.1.2 Alkynyl antibiotic derivatives

The structure-activity relationships for ciprofloxacin have been investigated[163] 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 **66** (see Figure 15) was synthesised in this study (see 7.3.1), and two cleavable alkynyl ciprofloxacin derivatives **88** and **89** were synthesised by Dr Eddy Sotelo and combined with some of the azido HSL derivatives made in this study (see 7.2.3 and 7.4.3).

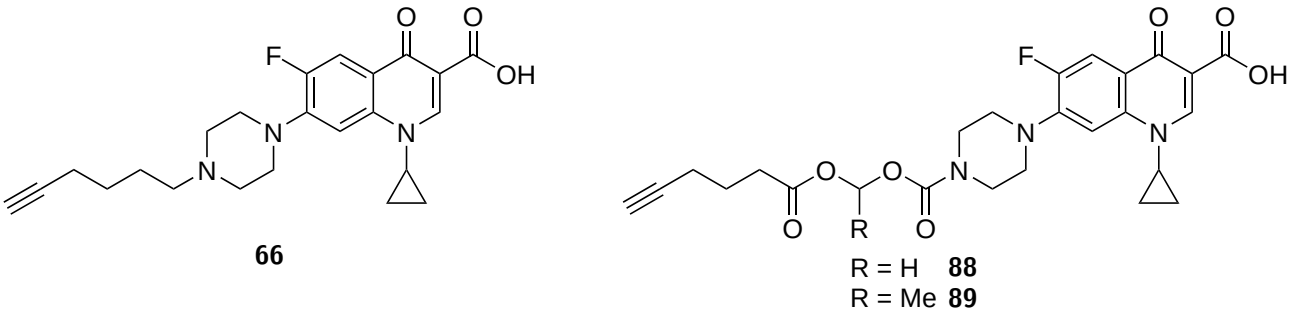


Figure 15: The alkynyl ciprofloxacin derivatives **66**, **88** and **89**.

The choice to of alkyne tail attachment point on trimethoprin **25** (see Figure 16) is based on the use of that same point in a fluorogenic trimethoprim tag synthesised by Jing *et al.*[164]

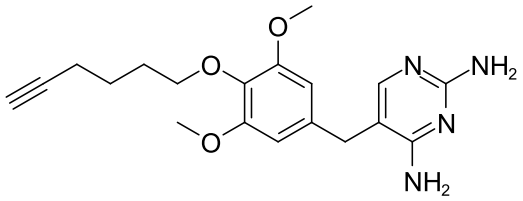
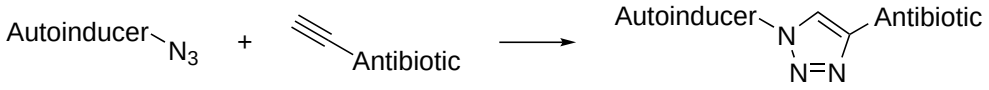


Figure 16: The alkynyl trimethoprim derivative **69**.

7.1.3 Synthesis of the conjugates

A copper(I)-catalysed azide-alkyne cycloaddition,[8,9] 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).



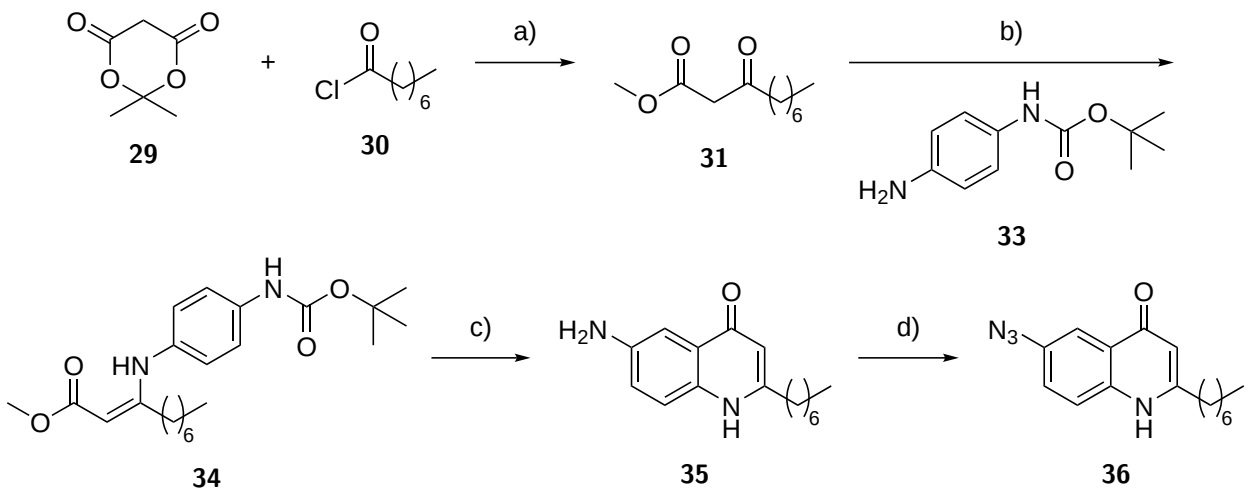
Scheme 1: The construction of the triazole-linked autoinducer-antibiotic conjugate library using a copper(I)-catalysed azide-alkyne cycloaddition.

7.2 Azido autoinducer derivatives

7.2.1 Synthesis of 6-N_3-HHQ 36

The synthesis of 6-N_3-HHQ **36** is shown in Scheme 2 and follows a route devised by Baker.[161] Octanoyl chloride **30** was converted to β-ketoester **31** via a Meldrum's acid adduct.[165,166] The β-ketoester **31** was condensed with *N*-Boc-*para*-phenylenediamine **33** to form enamine **34**. The disappointing yield of this step was in part due to the reaction proceeding to an equilibrium state rather than to completion, and hence not all of the starting material being consumed; starting materials can be recycled to improve the yield. Alternatively, Baker later found a higher-yielding reaction using a $ZrCl_4$ catalyst.

The enamine **34** was cyclised with polyphosphoric acid to form amino-HHQ **35** in good yield. The amine group of amino-HHQ **35** was converted to a diazo group by reaction with $NaNO_2$ and HCl, followed by displacement with NaN_3 to form the final azido-HHQ product **36**.[167]



Scheme 2: The synthesis of **36**. a) i) Pyridine, CH_2Cl_2, 0 °C. ii) MeOH, reflux, 66 % over two steps. b) MeOH, reflux, 19 %. c) Polyphosphoric acid, 120 °C, 72 %. d) i) $NaNO_2$, HCl, H_2O, 0 °C. ii) NaN_3, H_2O, r.t., 46.5 %.

7.2.2 Synthesis of 6-N_3-PQS 47

The synthesis of 6-N_3-PQS **47** is shown in Scheme 3, and also follows a route devised by Baker.[161] The Weinreb amide **41**[93] was prepared from chloroacetyl chloride, followed by attack with heptyl magnesium bromide **38** to form 1-chlorononan-2-one **42** following a procedure described by Hodgkinson *et al.*[168]

The synthesis of PQS **22** described by Hodgkinson *et al.*[168] used a microwave reaction of 1-chlorononan-2-one **42** with anthranilic acid. It was hoped that the azide group could be installed by using 5-nitroanthranilic acid **43** in the place of anthranilic acid in this microwave reaction, so that the nitro group could then be converted to an azide group via an amine. However, the microwave-catalysed reaction failed when 5-nitroanthranilic acid **43** was used.[161] Therefore, a two step process was employed instead.

5-Nitroanthranilic acid **43** was heated with K_2CO_3 to deprotonate the carboxylic acid, followed by addition of 1-chlorononan-2-one **42** to form the ester **44** by S_N2 displacement of the chlorine atom in a procedure adapted from Hlaváč *et al.*[169] Cyclisation with polyphosphoric acid produced nitro-PQS **45** cleanly.[169,170]

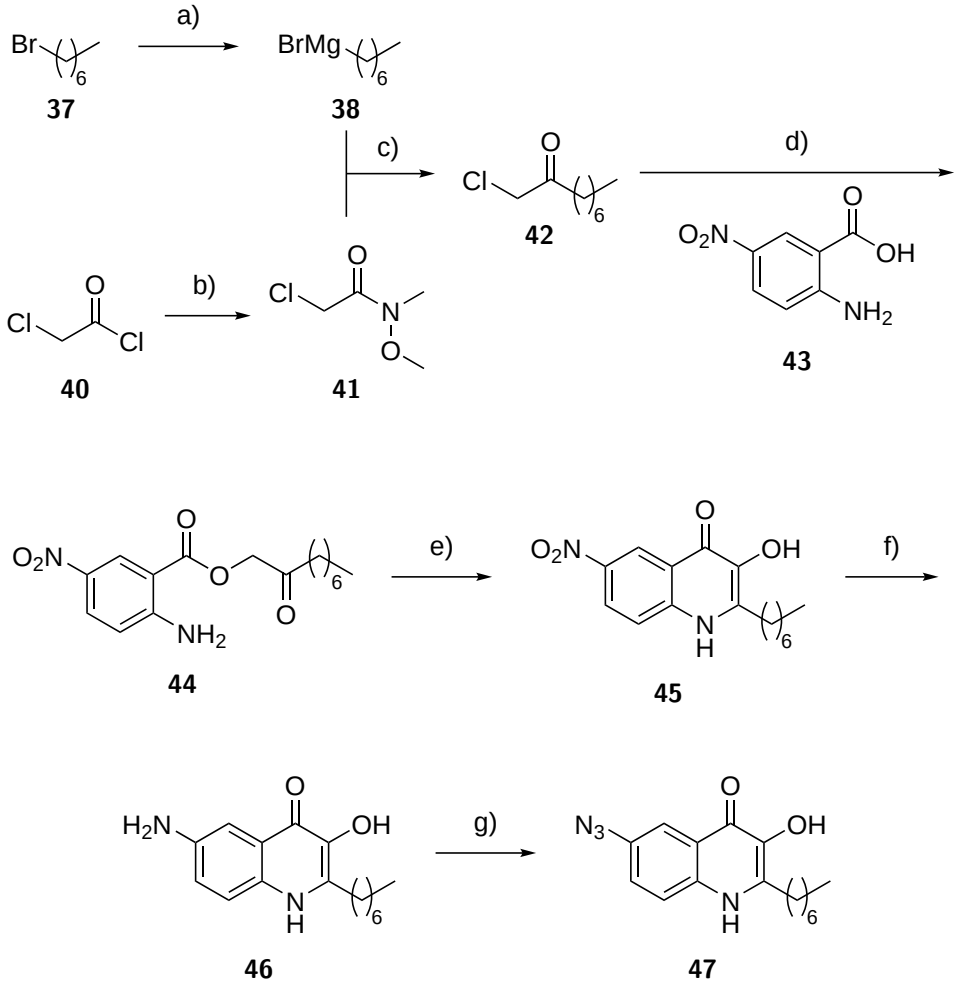
Conditions for the reduction of the nitro group were then compared (see Table 3). Baker initially used Zn and HCl, however this gave a yield over 100 % suggesting coordination of Zn to the amino-PQS **46**[161] (this product was taken through and purified after the next step). She also attempted reduction with Pd/C and H_2 or ammonium formate, but no reaction was observed.

Further conditions were tested in *this* work in order to obtain a clean sample of amino-PQS **46**. An initial test of reduction with $SnCl_2$ produced no detectable product by LCMS. Catalytic hydrogenation using harsher conditions was then attempted, and it was determined that increasing the pressure to 3 atm using a Paar hydrogenator causes full conversion in 4 h using Pd/C and H_2. Good yields (80 %) were also achieved using PtO_2 as a catalyst, with the advantage that the reaction proceeds more quickly, and at atmospheric pressure and temperature.[171]

Finally, amino-PQS **46** was converted to azido-PQS **47** by reaction with $NaNO_2$ and HCl to form diazo-PQS, followed by displacement of the diazo group using NaN_3 to give the azido-PQS **47**.[167] The yield of this reaction was rather disappointing (28 %), and is probably due to loss of product in the supernatant following precipitation.[161]

Conditions	**Outcome**
H_2, Pd/C, 1 atm, r.t., 18 h	No reaction
NH_4HCO_2, Pd/C, 1 atm, r.t., 18 h	No reaction
Zn, HCl (aq), r.t., 5 min h	Product **46** + Zn, assumed quantitative yield
$SnCl_2.2H_2O$, MeOH, r.t., 18 h	No reaction
H_2, Pd/C, MeOH, 3 atm, r.t., 4 h.	Product **46**, 100 % yield
H_2, PtO_2, MeOH, 1 atm, r.t., 45 min	Product **46**, 80 % yield

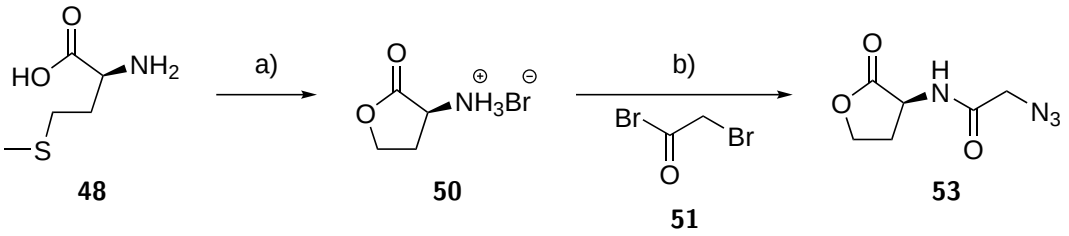
Table 3: Conditions attempted for the synthesis of **46**. Rows 1-3 were carried out by Baker,[161] rows 4-6 were carried out as part of this study.



Scheme 3: The synthesis of **47**. a) Mg turnings, THF, r.t., 2 h then reflux, 2 h. b) *N,O*-dimethylhydroxyl amine hydrochloride, K_2CO_3, toluene, H_2O, - 5 °C to r.t., 30 min, 71 %. c) THF, 0 °C to r.t., 15 h, 96 %. d) **43**, K_2CO_3, DMF, 90 °C, 1 h, then **42**, r.t., 18 h, 100 %. e) Polyphosphoric acid, 90 °C, 5.5 h, 40 %. f) H_2, PtO_2, MeOH, 1 atm, r.t., 45 min, 80 %. g) i) $NaNO_2$, HCl, H_2O, 0 °C, 50 min. ii) NaN_3, H_2O, r.t., 4 h, 28 % over two steps.

7.2.3 Synthesis of the azido C_4-HSL derivatives 53, 56 and 59

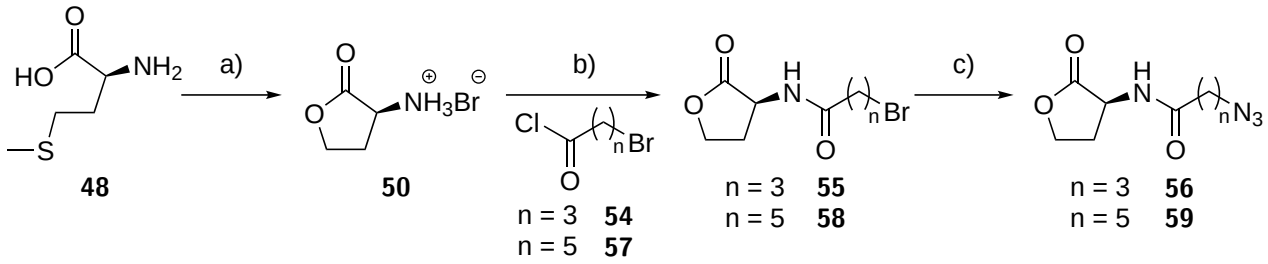
N_3-C_2-HSL **53** (the azido derivative of C_4-HSL with a C_2 chain, see Scheme 4) has previously been prepared by Stacy *et al.*[162] Their synthesis was followed, starting with the cyclisation of L-methionine **48** using bromoacetic acid to form the homoserine lactone HBr salt **50**. The disappointing yield can be attributed to difficulties in precipitating the final product. The homoserine lactone HBr salt **50** was then converted by a biphasic one-pot process to N_3-C_2-HSL **53** using bromoacetyl bromide **51** and NaN_3.



Scheme 4: The synthesis of **53**. a) Bromoacetic acid, *i*-PrOH:H_2O:AcOH (5:5:2), r.t., 18 h, 41 %. b) NaN_3, $NaHCO_3$, H_2O/CH_2Cl_2, r.t., 18 h, 41 %.

It was hoped that this procedure could also be used to produce the C_4 and C_6 derivatives, however, attempts to convert homoserine lactone **48** to N_3-C_4-HSL **56** using 4-bromobutyryl chloride **54** produced a complex mixture of products. This is likely to be because the S_N2 reaction in which the azide anion displaces bromine is slower for the C_4 derivative as the bromine atom being displaced is no longer adjacent to a carbonyl group. In addition, the longer chain length allows intramolecular cyclisation of the bromide with the secondary amide. The conversion was therefore carried out as a two-step process, where a bromoacyl chain was initially installed, followed by the S_N2 reaction with NaN_3 (see Scheme 5).

Reaction of the homoserine lactone HBr salt **50** with 4-bromobutyryl chloride **54** or 6-bromohexanoyl chloride **57** produced Br-C_4-HSL **55** or Br-C_6-HSL **58** respectively, in good yields. Heating with NaN_3 in DMF converted Br-C_6-HSL **58** to N_3-C_6-HSL **59**. Similar conditions were used by Dr. Bin Yu, a visiting PhD student in the Spring group, to convert the bromo-C_4 derivative **55** to the azido-C_4 derivative **56**, and this compound was kindly donated to complete the set. Yields for the S_N2 reaction could probably be improved by decreasing the temperature (see **??**, for example).



Scheme 5: The synthesis of **56** and **59**. a) Bromoacetic acid, *i*-PrOH:H_2O:AcOH (5:5:2), r.t, 18 h, 41 %. b) $NaHCO_3$, H_2O/CH_2Cl_2, r.t., 18 h, **55**: 80 %, **58**: 66 %. c) NaN_3, DMF, 100 °C, 5 h, **59**: 27 % (donated by Dr. Bin Yu), **59**: 56 %.

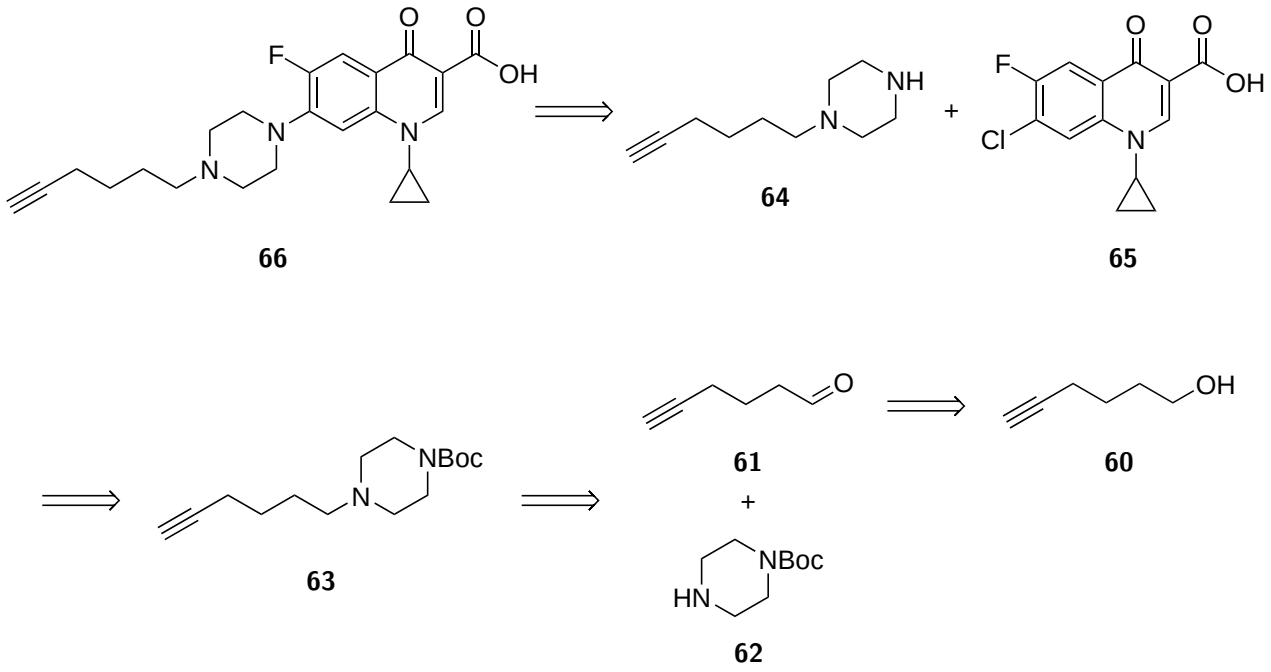
7.3 Alkynyl antibiotic derivatives

7.3.1 Synthesis of the alkynyl ciprofloxacin derivative 66

The retrosynthesis of ciprofloxacin derivative **66** is shown in Scheme 6. The disconnection to an alkynyl piperazine **66** and a commercially available ciprofloxacin precursor **65** was chosen based on a study by Renau *et al.*, who found this route to be "...superior to previous reports which involved alkylation of piperazine with an appropriate alkyl halide.".[163,172]

It was envisaged that the alkynyl piperazine **66** could be prepared from mono-Boc-protected piperazine **62** and hex-5-ynal **61** using conditions similar to those used by Renau *et al.*[163]

Unlike the aldehydes and ketones used by Renau *et al.*,[163] hex-5-ynal **61** is not commercially available and so it was hoped that this could be prepared by oxidation of hex-5-ynol **60**.



Scheme 6: The retrosynthesis of **66**.

The synthesis of ciprofloxacin derivative **66** is shown in Scheme 7. Hex-5-ynal **61** was prepared by PCC oxidation of hex-5-ynol **60** in good yield according to the procedure described by Kocsis *et al.*[173]

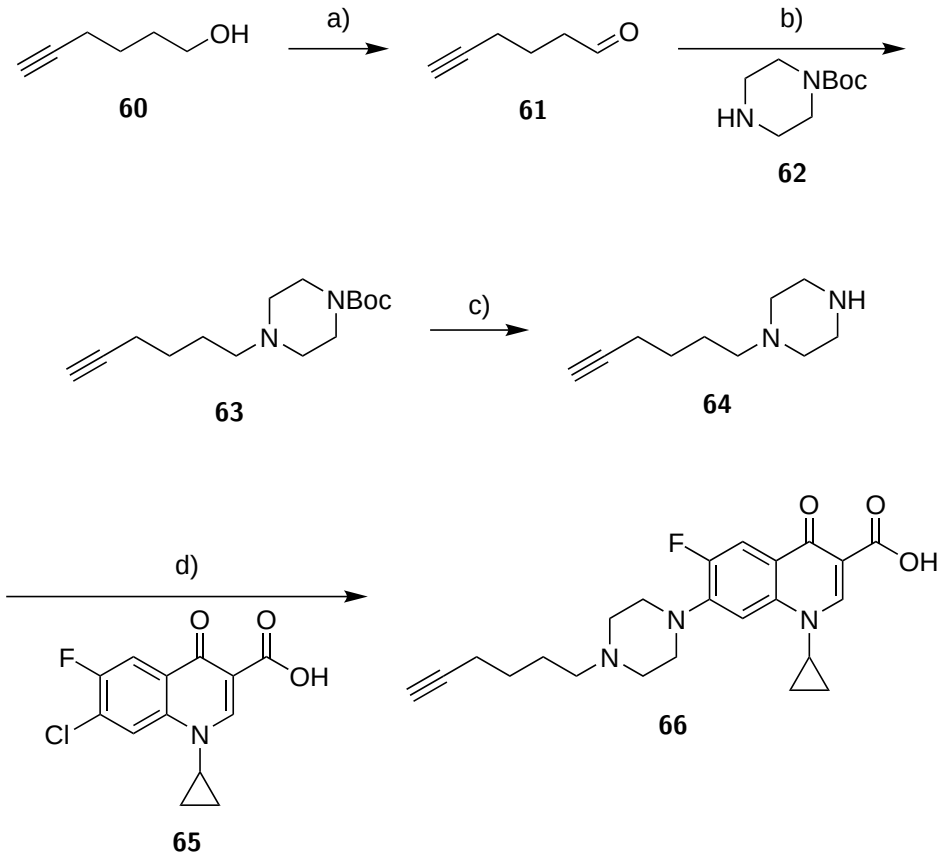
Renau *et al.*[163] used sodium cyanoborohydride to facilitate the reductive amination of hex-5-ynal **61** and 1-Boc-piperazine **62**. However, it was decided to attempt this transformation using the less toxic sodium triacetoxyborohydride following a procedure reported by Abdel-Magid *et al.*[174] This reaction yielded compound **63** in excellent yield, which was deprotected using TFA using the procedure described by Renau *et al.*[163] to give the alkynyl piperazine **64** quantitatively.

The alkynyl piperazine **64** was refluxed in MeCN with the ciprofloxacin precursor **65** according to the procedure described by Renau *et al.*,[163] however the reaction did not proceed. Addition of 2 eq. of NEt_3 did not lead to reaction, however it was found that refluxing in neat NEt_3 led to conversion to the final ciprofloxacin derivative **66**.

With a small sample of the final product in hand, less harsh conditions were sought for a larger-scale version of the final reaction. Mircowave irradiation at 115 °C was used, following a procedure by Reddy *et al.*[175] DMSO and NMP were tested as solvents, with or without the addition of TEA. The reactions were monitored using LCMS, and NMP without TEA was found to give the highest conversion.

Work-up of this reaction proved difficult, with an unknown dark brown viscous liquid being formed which was difficult to separate from the white solid product. A pure sample was obtained by recrystalisation from EtOAc, but the yield was rather poor (11.8 %). The reaction was observed to stall after a certain point, while still having some of the ciprofloxacin precursor **65** present. The alkynyl piperazine **64** was not observed by TLC despite having been added in two-fold excess, suggesting that it degraded to a by-product before having chance to react.

Further attempts to refine this reaction might involve lower temperatures, higher ratios of the alkynyl piperazine **64** or improvement of the purification, e.g. by finding better precipitation conditions or by using reverse-phase chromatography. A Buchwald-Hartwig coupling or Ullmann reaction could also be attempted, but, as seen later, coordination of ciprofloxacin to Cu can hinder catalysis.



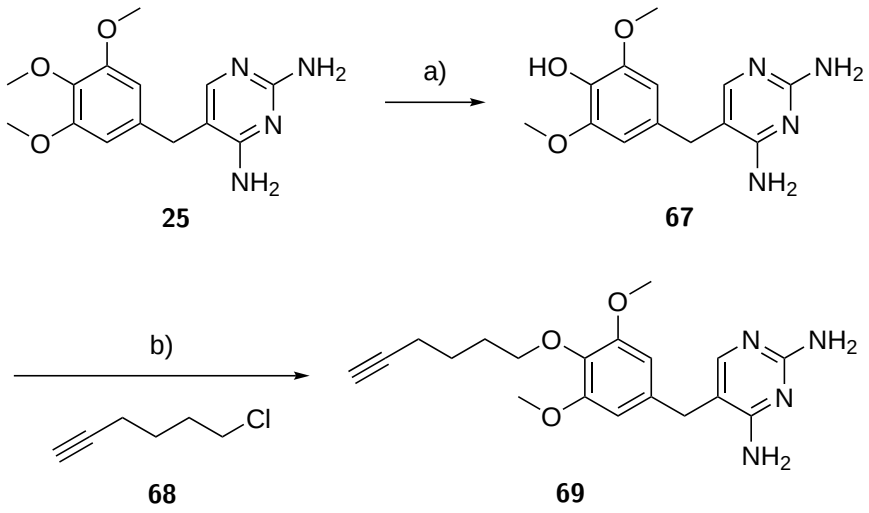
Scheme 7: The synthesis of **66**. a) Pyridinium chlorochromate, CH_2Cl_2, r.t., 5 h, 72 %. b) $NaBH(AcO)_3$, 1,2-dichloroethane, r.t., 10.5 h, 99 %. c) TFA, r.t., 1 h, 100 %. d) NMP, microwave, 115 °C 24 h, 11.8 %.

7.3.2 Synthesis of the alkynyl trimethoprim derivative 69

The synthesis of trimethoprim derivative **69** is shown in Scheme 8. Trimethoprim was selectively deprotected using HBr (aq.) using a procedure described by Jing *et al.*[164] to form **67**. A slightly longer reaction time (40 min vs 20 min) probably led to the yield being slightly somewhat lower than that obtained by Jing *et al.*. The main impurity was asymmetrically di-demethylated trimethoprim, which could be identified by the presence of two aryl peaks at 6.41 (d, J=2.0 Hz, 1 H) and 6.34 (d, J=2.0 Hz, 1 H) and a corresponding methyl peak at 3.82 (s, 3 H) in the crude NMR.

The alkynyl trimethoprim derivative **69** was synthesised from the demethylated trimethoprim **67** and 6-chloro-1-hexyne **68** using a Cs_2CO_3-catalysed S_N2 reaction similar to that used by Jing *et al.*.

weigh Y4Tri then discuss



Scheme 8: The synthesis of **69**. a) HBr (aq.), 100 °C, 40 min, 43.4 %. b) Cs_2CO_3, DMF, 70 °C, 7 h, 19.6 %.

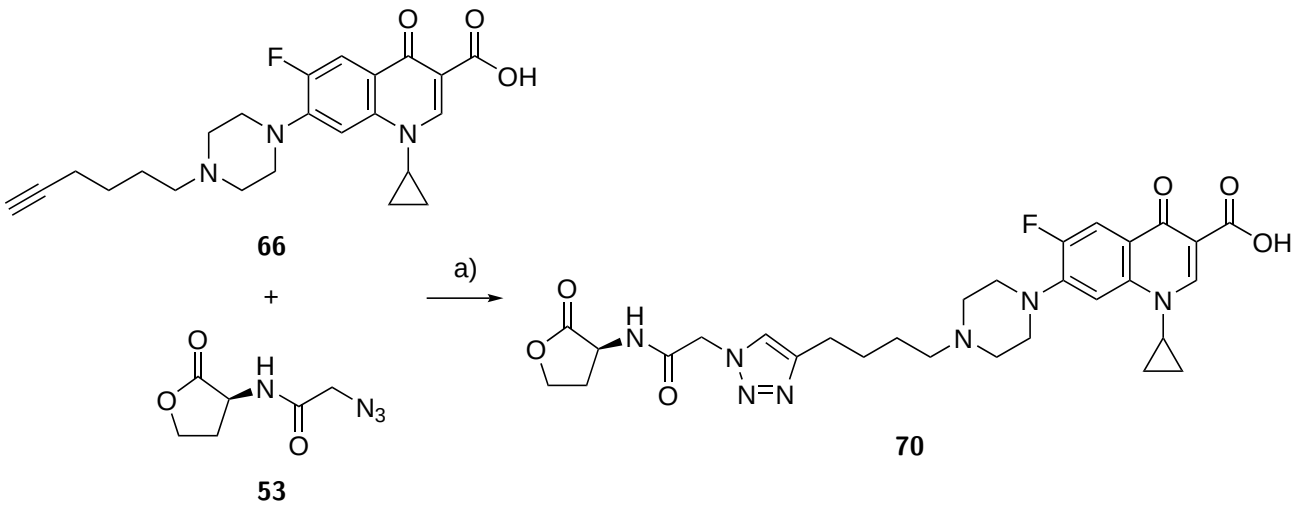
7.4 Triazole-linked autoinducer-antibiotic conjugates

7.4.1 Optimisation of the click reaction

Test reactions using N_3-C_2-HSL **53** and the alkynyl ciprofloxacin derivative **66** were performed to find conditions for the click reactions between the azido autoinducers and the alkynyl antibiotics (see Table 4 and Scheme 9). Stirring at r.t. had no effect even with an extended reaction time. Heating to 50 °C did lead to slow formation of the product, but a mixture of the 1,4 **70** and 1,5 **71** isomers was observed in an approximately 4:1 ratio by LCMS (see Figure 18). Use of the ligand tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) **72** (see Figure 17) led to some conversion at room temperature, however the reaction stopped before completion, probably due to oxidation of the Cu(I) catalytic species. When degassed solvent and an argon atmosphere were used the reaction proceeded to completion at room temperature in around 3 h.

Conditions	**Outcome**
$CuSO_4 \cdot H_2O$, sodium ascorbate, H_2O, *t*-BuOH, air, r.t., 7 d.	No reaction
$CuSO_4 \cdot H_2O$, sodium ascorbate, H_2O, *t*-BuOH, air, 50 °C, 5 d.	1,3-Triazole product **70** and 1,5 triazole impurity **71** 4:1
$CuSO_4 \cdot H_2O$, sodium ascorbate, THPTA **72**, H_2O, *t*-BuOH, air, r.t., 3 h.	1,3-Triazole product **70** and starting materials **53** and **66**
$CuSO_4 \cdot H_2O$, sodium ascorbate, THPTA **72**, H_2O, *t*-BuOH, Ar, r.t., 3 h.	1,3-Triazole product **70**

Table 4: Conditions attempted for the synthesis of **70** (see Scheme 9).



Scheme 9: Synthesis of **70**. For conditions see Table 4.

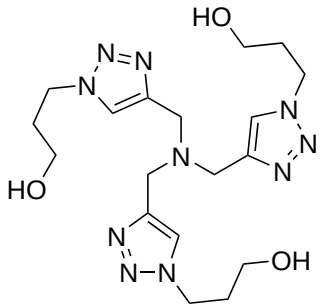


Figure 17: Tris[(1-benzyl-*1H*-1,2,3-triazol-4-yl)methyl]amine (THPTA) **72** .

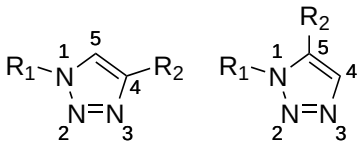
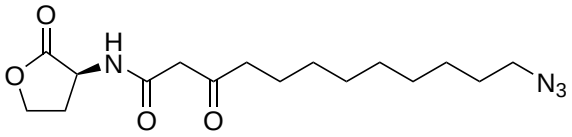


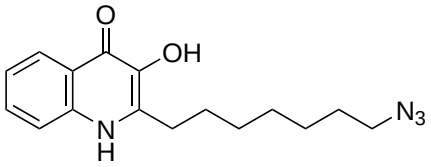
Figure 18: 1,4 (left) and 1,5 (right) triazoles .

7.4.2 Synthesis of the autoinducer-ciprofloxacin and autoinducer-trimethoprim triazole conjugates

Once conditions had been found for the click reaction, the synthesis of other conjugates was attempted. Two additional azides were kindly donated by members of the Spring group: the azido derivative of 3-oxo-C_{12}-HSL **73** was synthesised by Ryan Howard, a master's student under my supervision[176] and the tail azide derivative of PQS **74** was synthesised by Ysobel Baker[161] (see Figure 19).



73

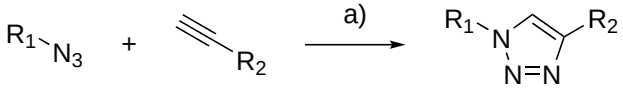


74

Figure 19: Further azido autoinducer derivatives synthesised by Howard[176] **73** and Baker[161] **74**.

Synthesis of the conjugates proved more difficult than expected, for several reasons. Firstly some compounds did not dissolve in the reaction solvent (50 % water/*t*-BuOH) requiring addition of co-solvents such as CH_2Cl_2. Secondly, some compounds were unstable: HSL derivatives hydrolysed upon attempted preparative HPLC purification and the 3-oxo-C_{12}-HSL conjugates degraded during the reaction. Finally, the reaction was highly air-sensitive which led to stalling. The most reliable procedure was determined over the course of several reactions, and is shown in **??**.

Nonetheless, several conjugates were produced for testing. The results of the reactions are shown in Table 5, Table 6, Table 7 and Table 8. It was intended that the failed reactions would be repeated, but as preliminary biological testing proved unpromising it was decided that attention should be focused elsewhere. ref



Scheme 10: General scheme for the click reaction, where R_1-N_3 is an azido autoinducer derivative and R_2-≡ is an alkynyl antibiotic derivative a)$CuSO_4$, sodium ascorbate, THPTA, H_2O, *t*-BuOH.

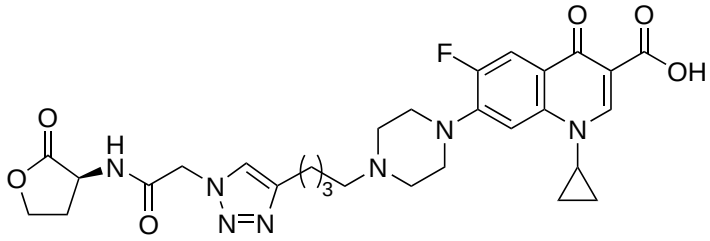
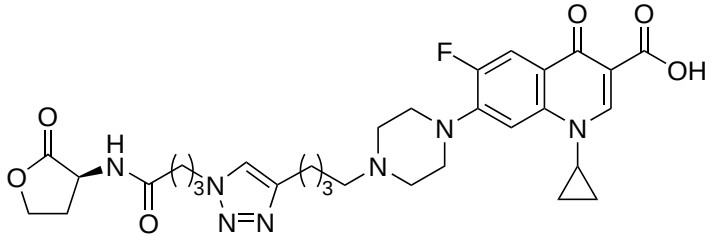
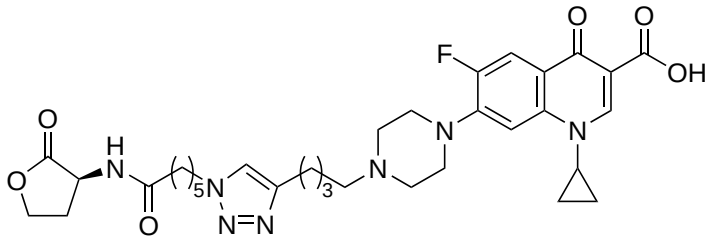
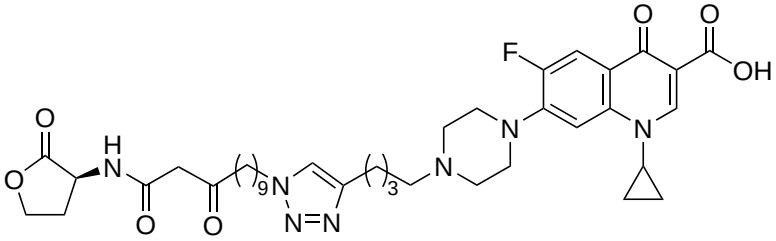
Starting materials	Product	Outcome	Yield
53 and **66**	 **70**	✓ Reaction complete by LCMS in 3 h. Purified by column chromatography (SiO_2, 0 - 20 % $MeOH/CH_2Cl_2$).	29.6 %
56 and **66**	 **75**	✓ Reaction complete by LCMS in 3 h. Purified by column chromatography (SiO_2, 0 - 20 % $MeOH/CH_2Cl_2$).	46.8 %
59 and **66**	 **76**	✓ Reaction complete by LCMS in 3 h. Purified by column chromatography (SiO_2, 0 - 20 % $MeOH/CH_2Cl_2$).	38.0 %
73 and **66**	 **77**	✗ Reaction complete by LCMS in 3.5 h, but product degraded when subjected to column chromatography (SiO_2, 20 % $MeOH/CH_2Cl_2$).	

Table 5: Click reactions attempted.

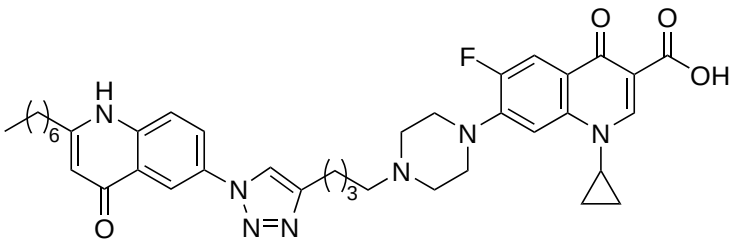
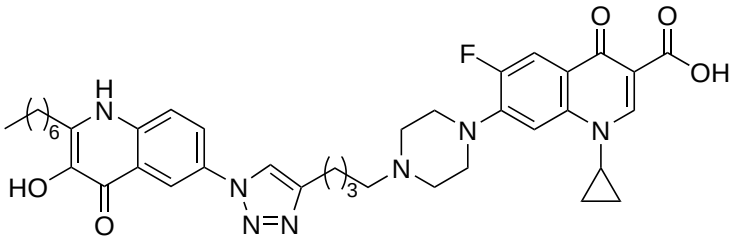
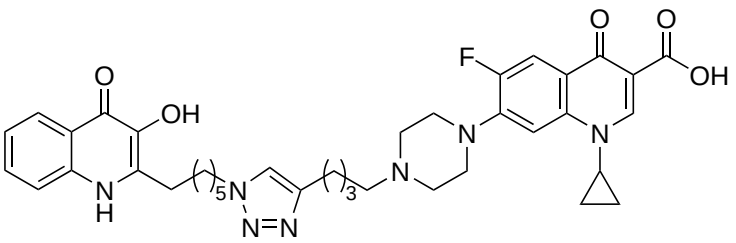
Starting materials	Product	Outcome	Yield
36 and **66**	 **78**	✓ Reaction complete by LCMS in 1.5 h. Purified by prep. HPLC.	27.0 %
47 and **66**	 **79**	✗ Reaction did not go to completion by LCMS. Attempted purification by prep. HPLC but unsuccessful.	
74 and **66**	 **80**	✗ No reaction seen by LCMS.	

Table 6: Click reactions attempted.

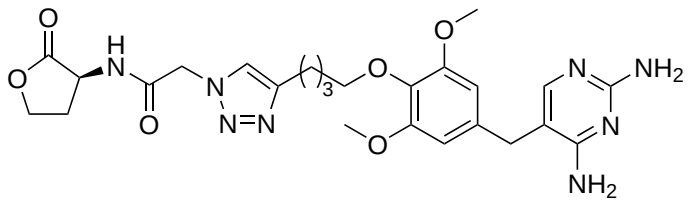
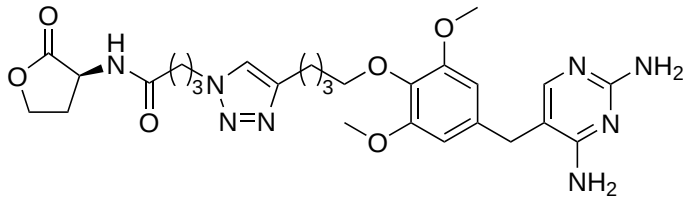
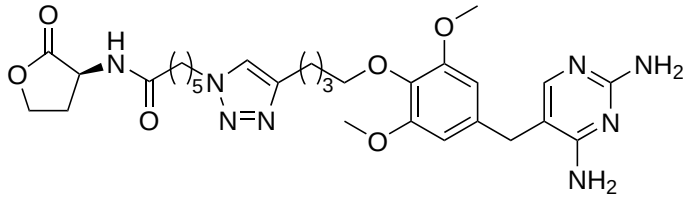
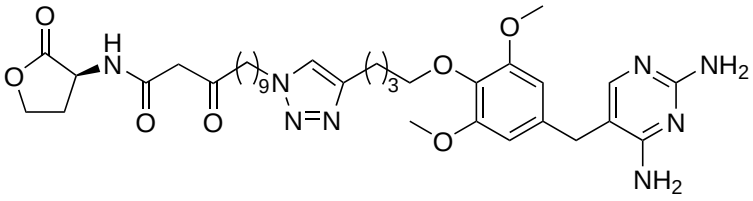
Starting materials	Product	Outcome	Yield
53 and **69**	 **81**	✗ Reaction complete by LCMS in 2 h, but lactone hydrolysed on prep. HPLC column.	
56 and **69**	 **82**	✓ Reaction complete by LCMS in 2 weeks (stalled). Purified by column chromatography (SiO_2, 20 % $MeOH/CH_2Cl_2$).	16.8 %
59 and **69**	 **83**	✓ Reaction complete by LCMS in 2 weeks (stalled). Purified by column chromatography (SiO_2, 20 % $MeOH/CH_2Cl_2$).	26.8 %
73 and **69**	 **84**	✗ Degraded during reaction.	

Table 7: Click reactions attempted.

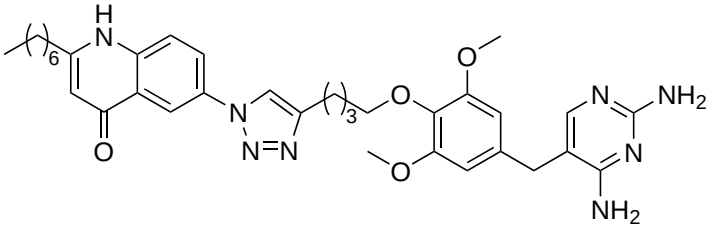
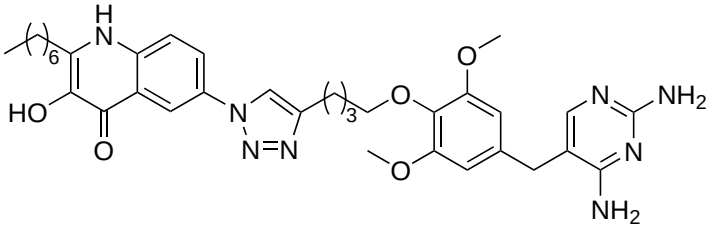
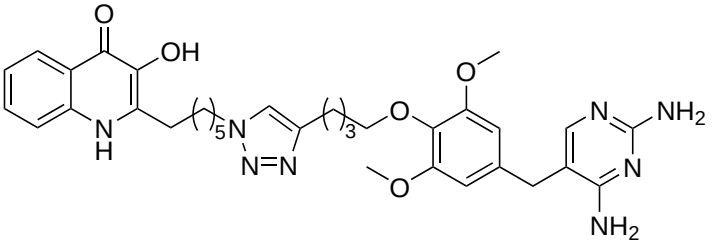
Starting materials	Product	Outcome	Yield
36 and **69**	 **85**	✓ Reaction complete by LCMS in 1.5 h. Purified by prep. HPLC.	41.0 %
47 and **69**	 **86**	✗ Reaction did not go to completion by LCMS. Attempted purification by prep. HPLC but unsuccessful.	
74 and **69**	 **87**	✓ Reaction complete by LCMS in 3 h. Purified by column chromatography (SiO_2, 20 % $MeOH/CH_2Cl_2$).	18.3 %

Table 8: Click reactions attempted.

7.4.3 Synthesis of homoserine lactone-ciprofloxacin triazole conjugates with cleavable linkers

In addition to the conjugates shown in the previous section, a further collection was synthesised in collaboration with Prof. Eddy Sotelo, a visiting researcher in the Spring group. Prof. Sotelo synthesised two alkyne-linked ciprofloxacin derivatives **88** and **89** (see Figure 20), both with cleavable linkers (see **??**).

link this up

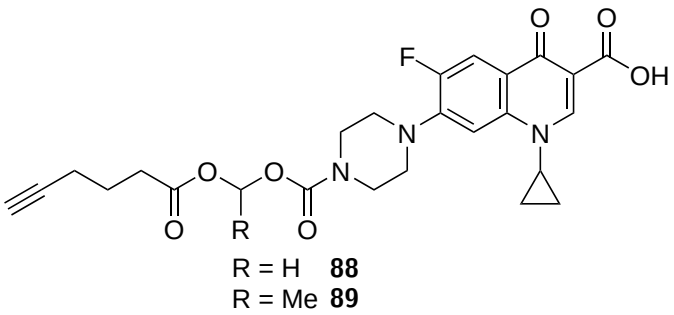
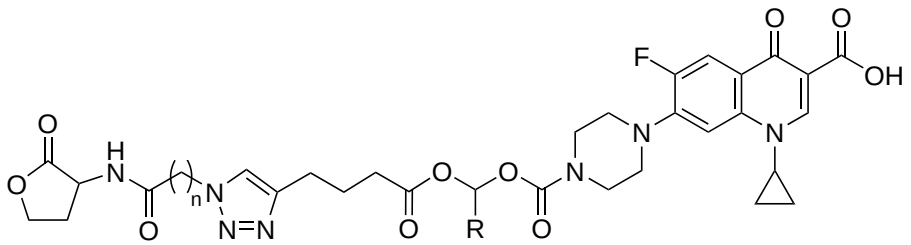


Figure 20: The cleavable alkyne-Cip derivatives synthesised by Prof. Sotelo.

Prof. Sotelo then performed click reactions using the AHL azide derivatives **53**, **56** and **59** shown in 7.2.3 to form a library of conjugates (see Figure 21). It was hoped that these conjugates would enter the cell and then be cleaved by esterases to release ciprofloxcin (see **??**).

link this up

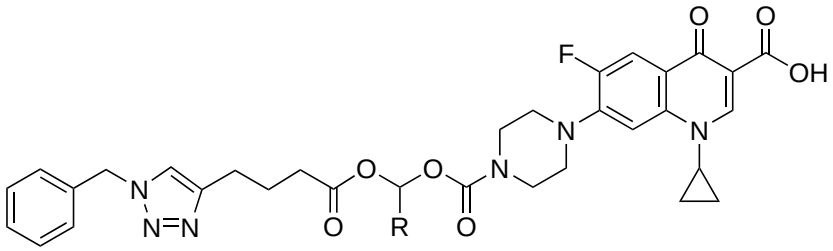
should I show the synthesis?



n = 1, R = H **90**
n = 3, R = H **91**
n = 5, R = H **92**
n = 1, R = Me **93**
n = 3, R = Me **94**
n = 5, R = Me **95**

Figure 21: The cleavable HSL-Cip triazole conjugates synthesised by Prof. Sotelo.

In addition, two control compounds **96** and **97** with benzyl head groups were produced by Prof. Sotelo (see Figure 22). It was hoped that these would show whether the AHL head group is required for activity.



R = H **96**
R = Me **97**

Figure 22: The cleavable Bn-Cip triazole conjugates **96** and **97** synthesised by Prof. Sotelo.

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Todo list