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

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Contents

1 Declaration

This dissertation describes work carried out in the Department of Chemistry, University of Cambridge under the supervision of Professor 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. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution, except those parts which were included in my CPGS dissertation. The dissertation does not exceed the word limit specified by the Physics and Chemistry Degree Committee.

Lois Overvoorde
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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 investigated 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 initial library was synthesised in two halves which were coupled together using a copper(I)-catalysed azide-alkyne cycloaddition.[8,9] The autoinducers (specifically three produced by *Pseudomonas aeruginosa*) were functionalised with azide groups and the antibiotics (specifically ciprofloxacin and trimethoprim) were functionalised with alkynes. Two cleavable alkynyl ciprofloxacin derivatives were also included.

The quorum sensing molecules produced by *P. 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] Ciprofloxacin was chosen as it is commonly used against *P. aeruginosa*[13] but resistance to it is developing.[14] *P. aeruginosa* is resistant to trimethoprim, but it was hoped that conjugation to an autoinducer could restore its potency. It was hypothesised that the autoinducers would aid retention of the antibiotic in the cell, hence increasing activity.

A second set of compounds, namely homoserine lactone analogue-ciprofloxacin conjugates were then synthesised, building on the one known report of a conjugate of a quorum sensing modulator and an antibiotic.[15]

2.1 Conclusions

2.1.1 Library synthesis

In this section, a range of 1,2,3-triazole-linked autoinducer-antibiotic conjugates was successfully synthesised and tested for antibiotic and anti-biofilm activity. Reliable routes to the azido autoinducers and alkynyl antibiotics were found, but the copper(I)-catalyzed alkyne-azide cycloaddition reactions used to link them proved rather capricious. The main reasons for this were insolubility of the starting materials and air-sensitivity. Air-sensitivity is not expected in a click reaction, but can be explained by many of the reactions being too dilute.[16] This led to ascorbate being used up by the oxygen dissolved in the reaction solvent and present in the air above the reaction mixture. Even when the solvent was degassed and the reaction performed under argon, a small amount of air leaking in through a perished septum was enough to cause the reaction to stall. Low concentrations were used because of the insolubility of the starting materials, but this would have been better addressed by more thorough screening of solvents. In addition, it was later shown that THPTA may not be necessary for a sufficiently concentrated reaction to take place,[17] so this expensive reagent could be omitted.

Assuming the click reaction could be further optimised, this library could be easily expanded by the addition of more azido autoinducers and alkynyl antibiotics (see 2.2). In particular, autoinducers which are actively transported into cells, such as AI-2, are attractive targets.

2.1.2 Biology

2.2 Future work

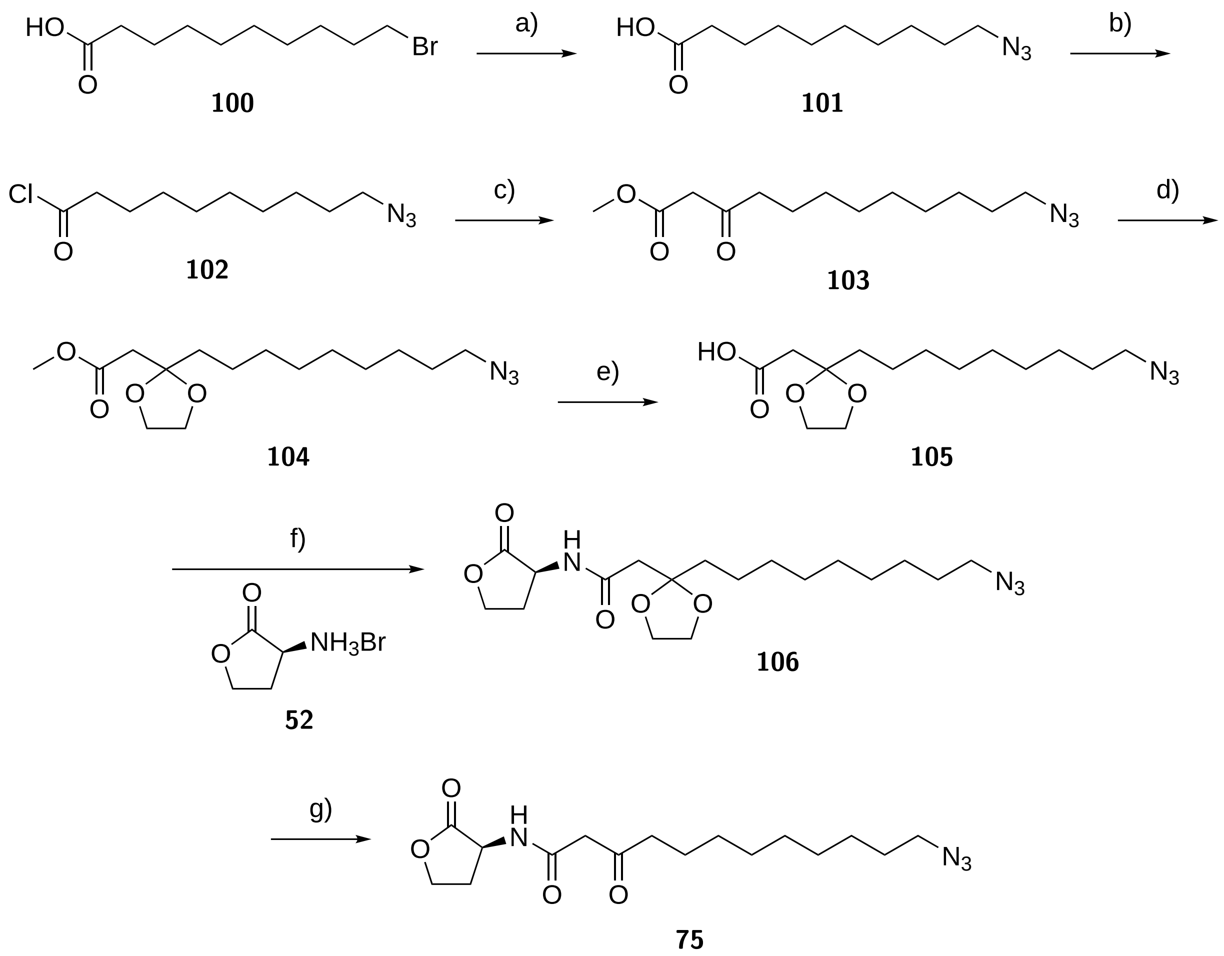
This section begins with discussion of further autoinducers and antibiotics which could be used in future conjugates. Some have have already been partially or fully synthesised by myself or other members of the Spring group. Plans for further biological testing of the conjugates synthesised in this study are then presented.

2.2.1 Autoinducer derivatives

2.2.1.1 3-oxo-C_{12}-HSL derivative 75

N_3-3-oxo-C_{12}-HSL **75** (see Scheme 1) was synthesised by Ryan Howard, a master's student under my supervision. The synthesis was based on a synthesis of 3-oxo-C_{12}-HSL **20** reported by Hodgkinson *et al.*[18] Conjugates of this compound was not included in the library as it degraded during the click reaction. However, reaction conditions could be further optimised, or the acetal protected azide **106** could be used in the click reaction, followed by deprotection.

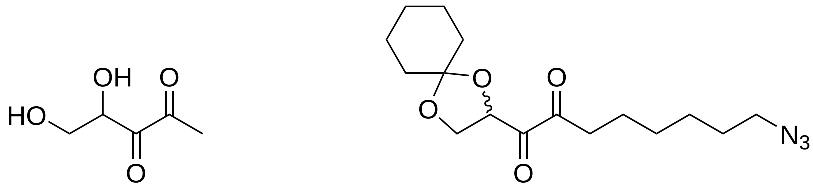
This compound would be a useful addition to the library as it would demonstrate whether the 3-oxo group and/or longer alkyl chain are required for activity. As the head group is added fairly late in the synthesis it would also be easy to swap it for the other head groups described in **??**, thus expanding the library further.



Scheme 1: The synthesis of N_3-3-oxo-C_{12}-HSL **75** carried out by Ryan Howard. a) NaN_3, DMF, 60 °C, 6 h, 93%. b) Oxalyl chloride, DMF, CH_2Cl_2, 3 h, r.t.. c) MeOAc, *N*-methyl imidazole, $TiCl_4$, DIPEA, toluene, r.t., 2 h, 43% over two steps. d) $HO(CH_2)_2OH$, TsOH, $CH(OMe)_3$, r.t., 5 h, 78%. e) NaOH, water, r.t., 6 h, 85%. f) EDC, DMAP, CH_2Cl_2, r.t., 16 h. g) TFA, r.t., 5 h, 29% over two steps.

2.2.1.2 AI-2 derivatives

AI-2 **23** is perhaps a more attractive choice of autoinducer for inclusion in conjugates than the others used in this study as it is actively transported into cells[19] and used by a wide range of bacterial species.[20] The synthesis of conjugates of AI-2 **23** with ciprofloxacin **24** and trimethoprim **25** has been attempted in the Spring group by Dr Jamie Stokes. However, the protected azido AI-2 derivative **107** synthesised was found to be unstable, and the click reactions attempted were unsuccessful.[17] AI-2 **23** is known to interconvert between multiple forms (including forming a furanosyl borate diester)[21] so it is to be expected that syntheses involving it might be challenging. If a more stable azido AI-2**23** derivative cannot be developed, another approach would be to use an azido AI-2 **23** analogue which is capable of being taken up by the same active transport mechanism.



AI-2
23

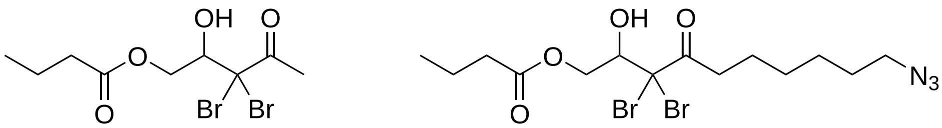
107

Figure 1: AI-2 **23** in its DPD form and the protected azido AI-2 derivative **107** synthesised by Dr Jamie Stokes.

Two types of AI-2 **23** receptors have been identified: LuxP, present in *Vibrio sp.*,[22] and LsrB, first discovered in *Salmonella enterica* serovar Typhimurium.[23] LuxP is a periplasmic binding protein that relays the signal, but not the actual AI-2 **23** molecule into the cell, and hence is not a useful target.[24] LsrB is the ligand binding protein of a system that transports AI-2 **23** into the cell,[19] and hence can be targeted. LsrB orthologs are found in a wide range of bacterial families including *Enterobacteriaceae*, *Rhizobiaceae*, and *Bacillaceae*.[25] In addition, several bacterial species, including *P. aeruginosa*, are known to respond to AI-2 **23** but do not have either of these two known types of receptors, and thus the discovery of new receptor types is expected.[25] Any postulated receptor would need to internalise the AI-2 analogue in order for conjugates to be effective against the bacterium.

One example of an AI-2 analogue which could be derivatised is a geminal dibromo compound **108** synthesised by Guo *et al.*[21] (see Figure 2). It is as potent as AI-2 at dissociating the LsrR repressor from the promotor region in a reporter strain, and may be more stable. It is also esterified, making it less volatile and thus easily purified using column chromatography. The esters are presumably cleaved by cellular esterases as the compounds can me used in QS assays without deprotection.[26]

A possible azido derivative **109** of this analogue is shown in Figure 2. If a route to it could be found, it appears to be a promising partner in future conjugates given the known properties of AI-2 **23**.



108

109

Figure 2: An AI-2 analogue **108** synthesised by Guo *et al.* and the proposed azido AI-2 analogue derivative **109**.

2.2.2 Antibiotic derivatives

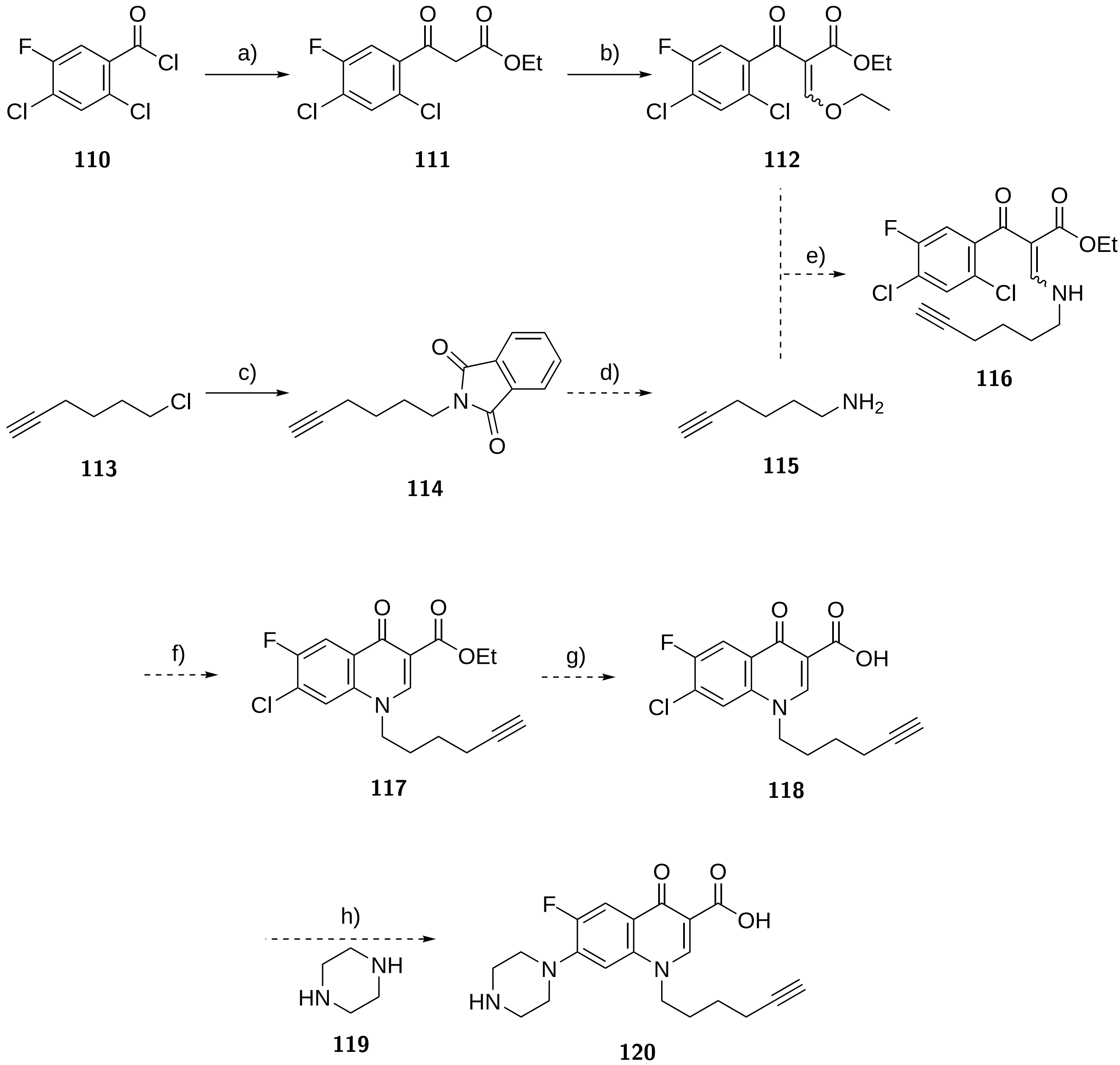
2.2.2.1 Ciprofloxacin derivative 120

A second alkynyl ciprofloxacin derivative **120** was planned and partially synthesised during this project, and finishing this synthesis would provide a useful intermediate for future conjugates.

The derivative **120** has an alkyne tail attached in place of the cyclopropane ring as it has been shown that bulkier groups in this position can be tolerated.[27,28] This synthesis follows a conventional route to ciprofloxacin similar to that reported by Mitscher *et al.*[27] but using hex-5-yn-1-amine **115** instead of cyclopropylamine.

The $TiCl_4$-catalysed crossed Claisen condensation of the acid chloride **110** and ethyl acetate described by Hashimoto *et al.*[29] was used to produce the β-ketoester. The ethoxymethylene group in **112** was installed by the reaction of β-ketoester **111** and triethyl orthoformate to give a mixture of the E and Z isomers.[27,30] Hex-5-yn-

1-amine **115** was prepared using a Gabriel synthesis[31] described by Rożkiewicz *et al.*[32] Unfortunately the amine was surprisingly volatile and was lost on evaporation of the reaction solvent. If a better purification method could be found the rest of the synthesis could be performed and the resulting alkynyl ciprofloxacin derivative **120** could be used to form more triazole-linked conjugates.

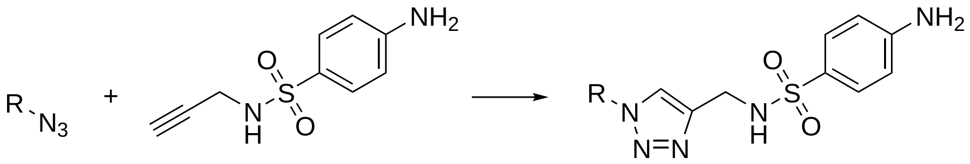


Scheme 2: The synthesis of **120**. a) EtOAc, $TiCl_4$, DIPEA, *N*-methyl imidazole, toluene, r.t., 30 min. b) Triethyl orthoformate, Ac_2O, reflux, 2 h. c) Potassium phthalimide, potassium iodide, DMF, 80 °C, 18 h. d) $N_2H_2 \cdot H_2O$, EtOH, reflux, 18 h. e) EtOH. f) NaH, dioxane. g) KOH, THF. h) DMSO.

2.2.2.2 Sulfanilamide derivatives

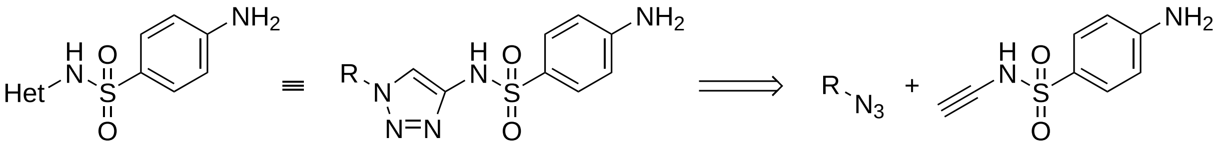
Sulfanilamide antibiotics were the first class of antibiotics to be widely used.[33,34] They are all derivatives of 4-aminobenzenesulfonamide, very commonly with the sulfonamide nitrogen linking to a heterocycle. Sulfanilamide antibiotics function by inhibiting bacterial synthesis of folic acid.

Derivatives of 4-aminobenzenesulfonamide **208** have previously been synthesised using copper(I)-catalyzed alkyne-azide cycloaddition reactions to append various groups[35] (see Scheme 3). However, if one considers sulfonamide antibiotics already in use, nearly all have a heterocycle linked directly to the sulfur atom, rather than with a methylene group in between.



Scheme 3: The sulfanilamide derivatives synthesised using click chemistry by Wang et al.[35]

Therefore, it was postulated that a 1,2,3-triazole could be introduced in the position occupied by a heterocycle in other known sulfonamide antibiotics by attachment of an alkyne directly to the sulfonamide nitrogen to form an alkynyl sulfanilamide derivative **121** or a protected version of it (see Scheme 4).



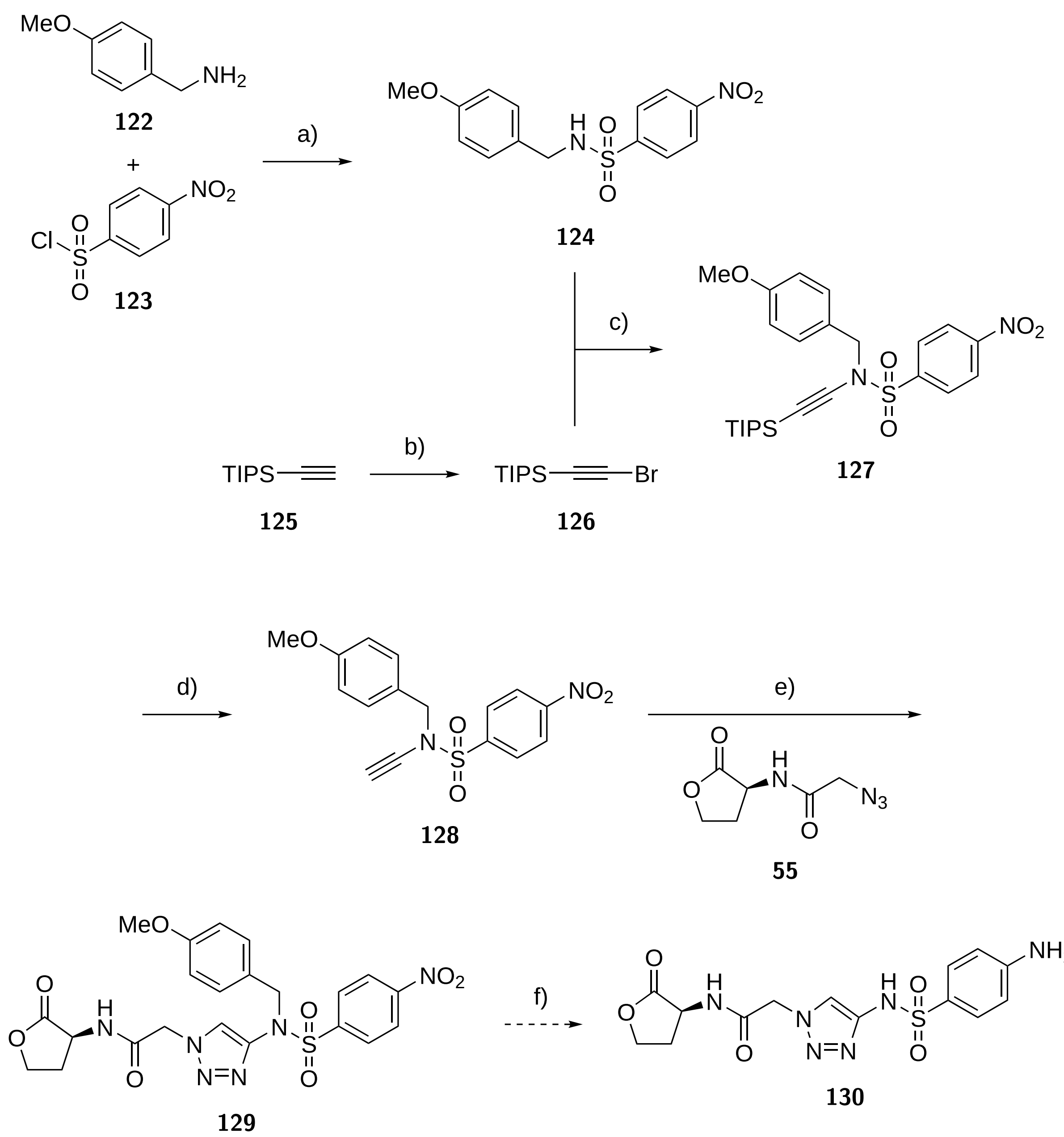
121

Scheme 4: Retrosynthesis of a 1,2,3-triazole-containing autoinducer-sulfonamide conjugate. R = autoinducer.

It was hoped that sulfanilamide derivative **121** could be synthesised and reacted with the azido autoinducer derivatives directly. However, it appears that no secondary ynamides have been synthesised to date. Conversely, the synthesis of tertiary ynamines has been studied more widely.[36] In particular, tertiary ynamides have been shown to be relatively stable and easy to work with in a variety of reactions including copper(I)-catalyzed alkyne-azide cycloadditions.[37,38]

The study of copper(I)-catalyzed alkyne-azide cycloadditions of ynamides by IJsselstijn et al.[37] includes terminal ynamides protected using a benzyl and a tosyl group. Although their click reactions proceed with high yield, they fail to present the deprotection of their final compounds. However, these reactions provided a promising suggestion that click reactions between a protected alkynyl sulfanilamide derivative and the azido autoinducer derivatives were feasible. The tosyl group used by IJsselstijn et al.[37] to protect their ynamide is very similar to the *p*-aminobenzenesulfonyl group needed in the alkynyl-sulfanilamide derivative. However, because installation of the alkyne could be problematic in the presence of a second amine, the NH_2 group was installed as a NO_2 group and reduced after the click reaction.

The synthesis proceeded as shown in **??**.[37,39,40] It was hoped that the methoxybenzyl group could be removed and the nitro group converted to an amine simultaneously by reduction in the last step, but unfortunately the methoxybenzyl group proved difficult to remove. On reflection, methoxybenzene was a poor choice of protecting group, and a more reduction-labile group such as benzyl or diphenylmethyl should have been chosen.[41] This reaction could be repeated with a different choice of protecting group to provide another set of autoinducer-antibiotic conjugates.



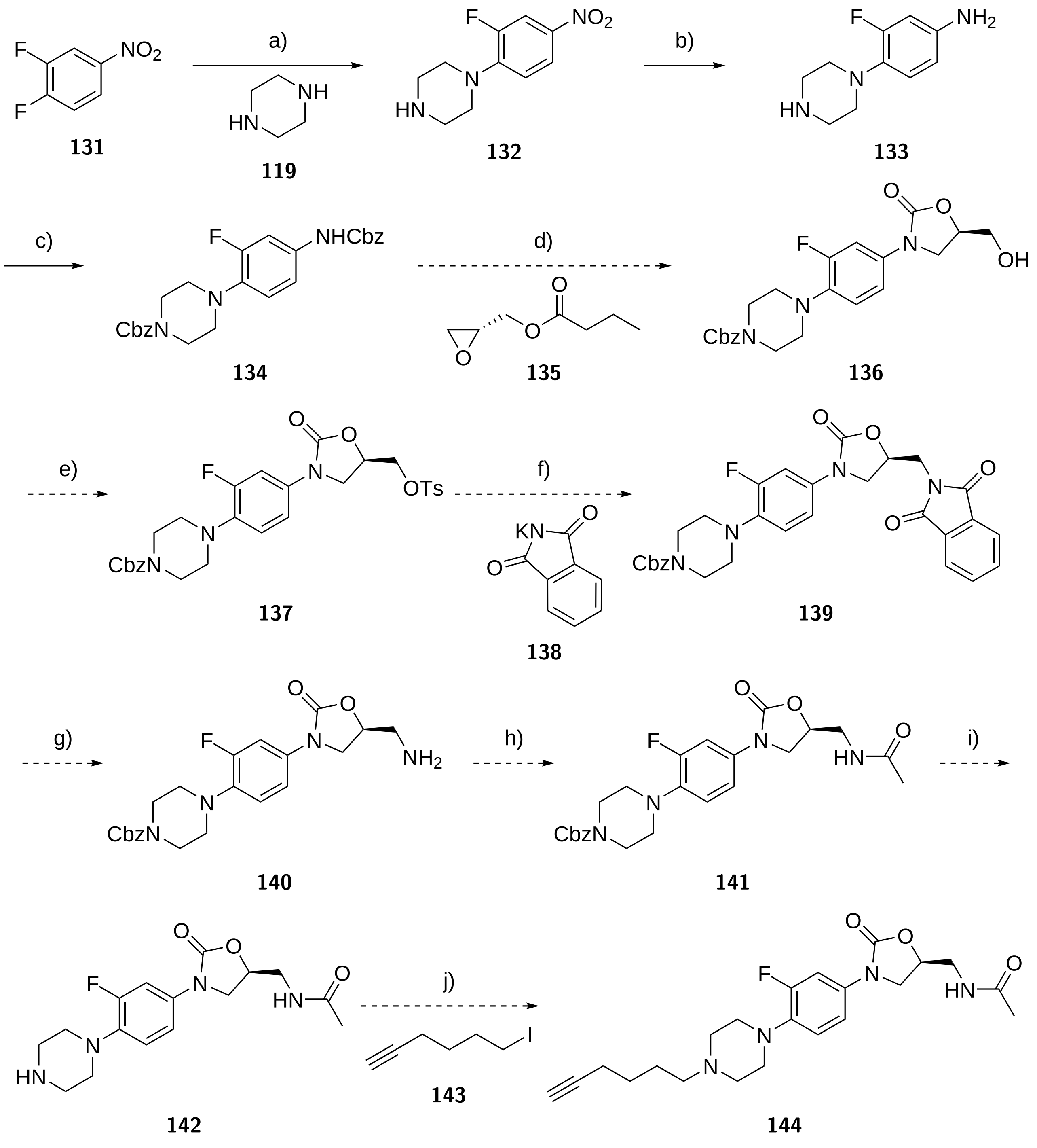
Scheme 5: Synthesis of a 1,2,3-triazole-containing sulfonamide antibiotic-autoinducer hybrid. a) CH_2Cl_2, r.t., 24 h. b) $AgNO_3$, acetone, r.t., 3 h. c) $CuSO_4 \cdot 5\,H_2O$, 1,10-phenanthroline, K_2CO_3, toluene, 80 °C, 48 h. d) TBAF, THF, −78 °C, 3 h. e) $Cu(OAc)_2$, sodium ascorbate, CH_2Cl_2, *t*-BuOH, water, r.t., 16 h. f) H_2, PtO_2, MeOH, 1 atm, r.t., 3 h.

2.2.2.3 Linezolid derivative 144

Linezolid is a monoamine oxidase inhibitor used for the treatment of infections caused by Gram-positive bacteria. Gram-negative bacteria, including *P. aeruginosa* are resistant to linezolid due to the activity of efflux pumps, and hence it might be possible to increase its activity in such organisms by increasing its uptake and/or retention by conjugation to an autoinducer.

An alkynyl linezolid derivative **209** was partially synthesised by Ryan Howard (see Scheme 6). The route follows a literature procedure described by Phetsang *et al*[42] where the morpholine ring of linezolid is replaced by piperazine, allowing an alkynyl tail to be attached to the molecule.

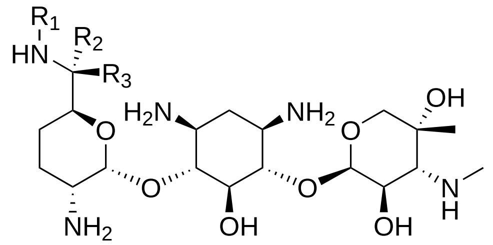
The first three steps were carried out on a large scale, producing 55.7 g of **134**. As all steps except the final one are reported in the literature[42, 43] it is hoped that the alkynyl linezolid derivative **210** could be synthesised fairly straightforwardly.



Scheme 6: Proposed and partially completed synthesis of linezolid derivative **210**.[42] a) MeCN, reflux, 3 h, 91%. b) H_2, 10% Pd/C, THF, 40 psi, <50 °C, 1.5 h, 95%. c) CbzCl, Na_2CO_3, acetone, water, 5 °C, 1 h then r.t., 16 h, 56%. d) *n*-BuLi, THF, -78 °C, 1 h then add epoxide then -78 °C to r.t., 5 h. e) TsCl, TEA, CH_2Cl_2, 0 °C to r.t. 4.5 h. f) Acetonitrile, water, reflux, 48 h. g) $MeNH_2$, EtOH, water, reflux, 5.5 h. h) Ac_2O, pyridine, 0 °C to r.t., 16 h. i) H_2, 10% Pd/C, MeOH/CH_2Cl_2, 1 atm, r.t., 16 h. j) NEt_3, EtOH, reflux.

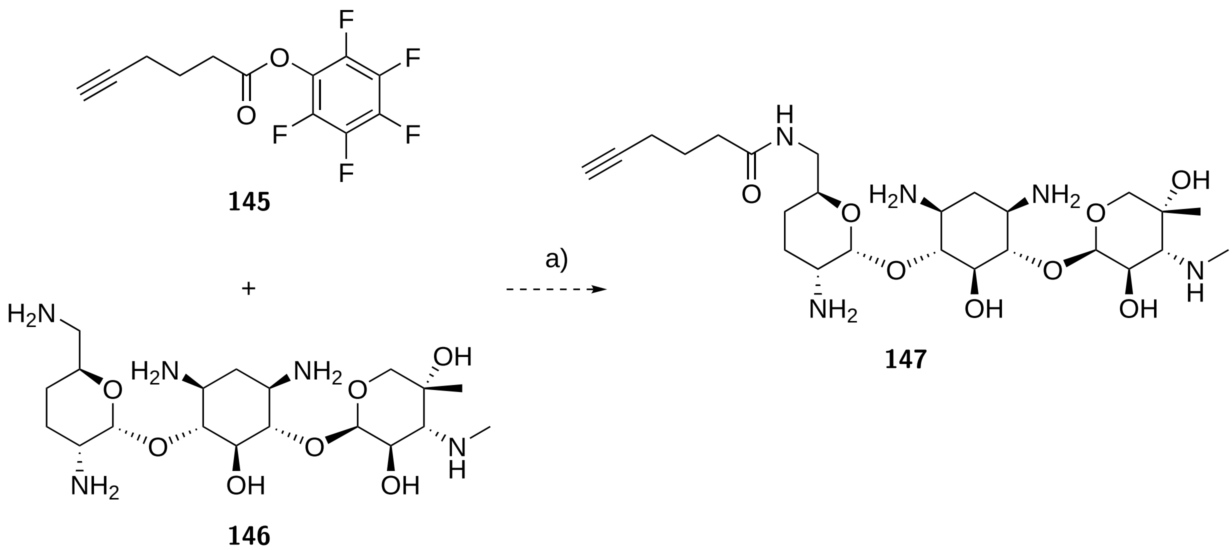
2.2.2.4 Gentamicin derivative 147

Gentamicin is an aminoglycoside antibiotic used to treat many bacterial infections, particularly those caused by Gram-negative organisms, by binding to the bacterial ribosome. Gentamicin is actually a mixture of components (see Figure 3) synthesised by *Micromonospora sp.*, a genus of Gram-positive bacteria. Separation of the gentamicin components has been achieved by Grote *et al.*[44] by reaction with benzyl chloroformate followed by HPLC and hydrogenolysis of the protecting groups. Gentamicin C1a **146** was isolated pure, and is particularly useful because it the only component which contains a CH_2NH_2 group. This group is less hindered than all other amine groups in gentamicin C1a **146** and hence it is possible to selectively derivatise the molecule at this position. Grote *et al.* attached a tag needed for an immunoassay using a pentafluorophenyl ester.[45] Hence, it may be possible to achieve selective reaction of this site with the pentafluorophenyl ester of 5-hexynoic acid **145** (see Scheme 7). It may even be possible to react the original gentamicin mixture with the pentafluorophenyl ester **145** and then separate out the desired component.



Gentamicin	R_1	R_2	R_3
C1	Me	Me	H
C1a	H	H	H
C2	H	Me	H
C2a	H	H	Me
C2b	Me	H	H

Figure 3: Gentamicin components.

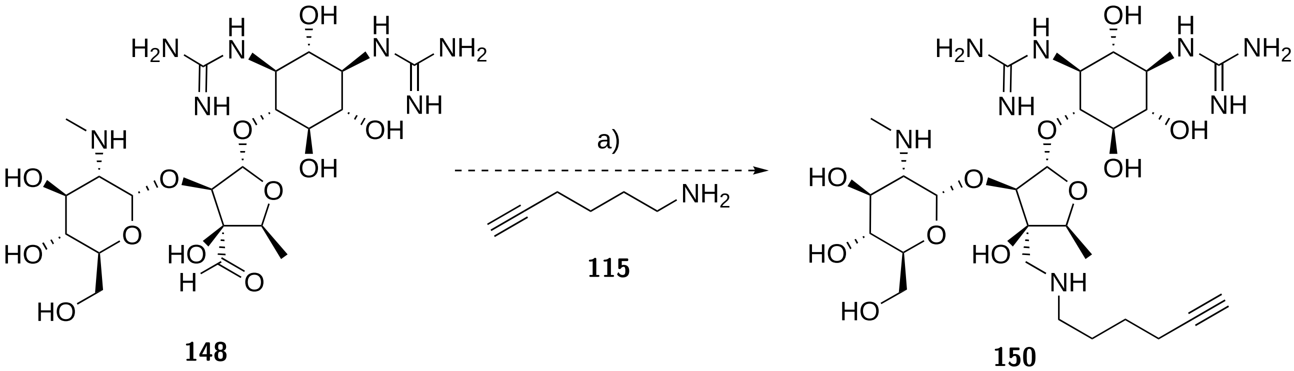


Scheme 7: Proposed synthesis of gentamicin C1a derivative **147**. a) DIPEA, DMF, - 55 °C.

2.2.2.5 Streptomycin derivative 150

Streptomycin **148** is an aminoglycoside antibiotic used to treat *Mycobacterium tuberculosis* and *S. aureus* which works by binding to the bacterial ribosome. There is limited SAR data on streptomycin but it is known that conversion of the aldehyde to a carboxylic acid destroys activity, whereas conversion an alcohol retains it.[46]

Reductive amination can be used to install an alkyne group by reaction of the aldehyde with an amine such as hex-5-yn-1-amine **115** (see Scheme 8). This approach has been used by Zhang *et al.*[47] to form a conjugate of streptomycin **148** and chitosan which was active against biofilms. Reductive amination replaces the aldehyde O with NH; it is known that an OH is tolerated at this position so it makes sense that NH is as well.



Scheme 8: Proposed synthesis of streptomycin derivative **150**. a) $NaBH_3CN$, water, r.t..

2.2.3 Biology

2.3 Conclusions

In this section, a library of HSL analogue-ciprofloxacin conjugates was successfully synthesised and tested for antibiotic activity. A range of 7 head groups (see **??**) and two linking strategies were used. Unfortunately the branching route that was initially proposed (see **??**) was not feasible for the alcohol-containing head groups and was low yielding for others, probably due to internal cyclisation (this side reaction could with hindsight be avoided by changing the linker length).

Given the difficulties in the branching synthesis, routes to the differently-linked compounds were optimised separately: the alkyl-linked conjugates were best formed using peptide coupling and the triazole-linked conjugates via a chloride intermediate. Direct comparisons of routes are not possible without repeating syntheses, but if it is assumed that peptide coupling of homocysteine thiolactone hydrochloride **152** to carboxylic acid **199** would have a similar yield to the coupling with (1*R*,2*R*)-2-aminocyclopentan-1-ol **173**, approximate comparisons can be made. The synthesis of the HCTL-CipMe conjugate **154** described in **??** has an overall yield of 11%, whereas the route to the cyclopentanol-CipMe conjugate **178** shown in **??** has an overall yield of 26%. Moreover, if the yield starting from the head group is considered, the yield is 55% vs. 11%. Therefore, the peptide coupling route is recommended for further investigation if the alkyl-linked library is to be expanded.

Synthesis of the azido autoinducer analogues via the chloride is also recommended as the bromide is thought to cyclise readily (this could explain the poor yields of the 2- and 3-methoxybenzene derivatives).

Preparative HPLC was identified as the best purification method for these conjugates (note that the standard acidic method used hydrolyses the lactone of native HSL and so cannot be used in that case).

2.4 Future work

2.4.1 HSL analogue derivatives

A selection head groups which could be used in future conjugates are shown in Figure 4. These have all been shown to modulate HSL-mediated quorum sensing as part of acyl-HSLs.[48–54] The most obvious targets are the cyclopentanone derivatives, as this could be synthesised from the alcohols above. The aniline, pyridine, quinoline and cyclopentyl amine head groups are commercially available and hence derivatives of these could be easily obtained. The 3- and 4-substituted HSL analogues require synthesis, but a convenient route has been devised.[51]

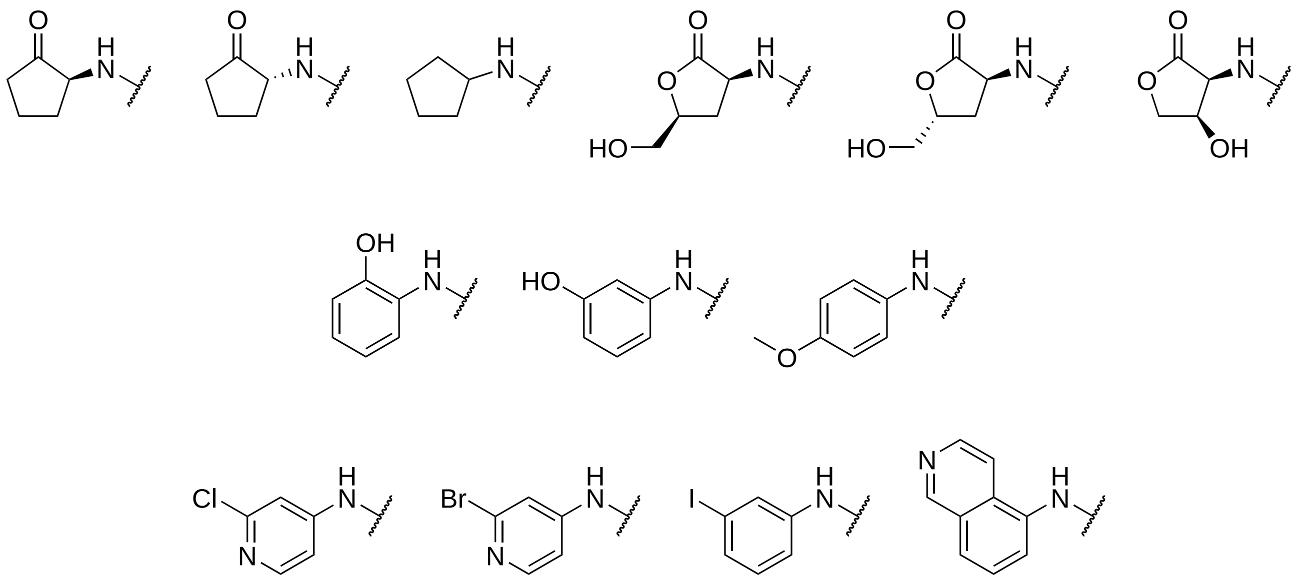


Figure 4: HSL analogue head groups for use in future conjugates.

2.4.2 Biology

3 References

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