Contents

1 Results and discussion: autoinducer-antibiotic conjugates

1.1 Biological testing

1.1.1 Autoinducer-antibiotic conjugates

The eight triazoles made in **??** (see Figure 1) were tested for antibacterial and anti-biofilm activity in *P. aeruginosa* PAO1[1] and YM64.[2] PAO1 is the *P. aeruginosa* wild-type strain. YM64 is a mutant lacking all of the four major *mex* operons for multidrug efflux pumps: *mexAB-oprM*, *mexXY*, *mexCD-oprJ* and *mexEF-oprN*, making it more sensitive to many antibiotics and hence able to show up moderate effects more clearly.

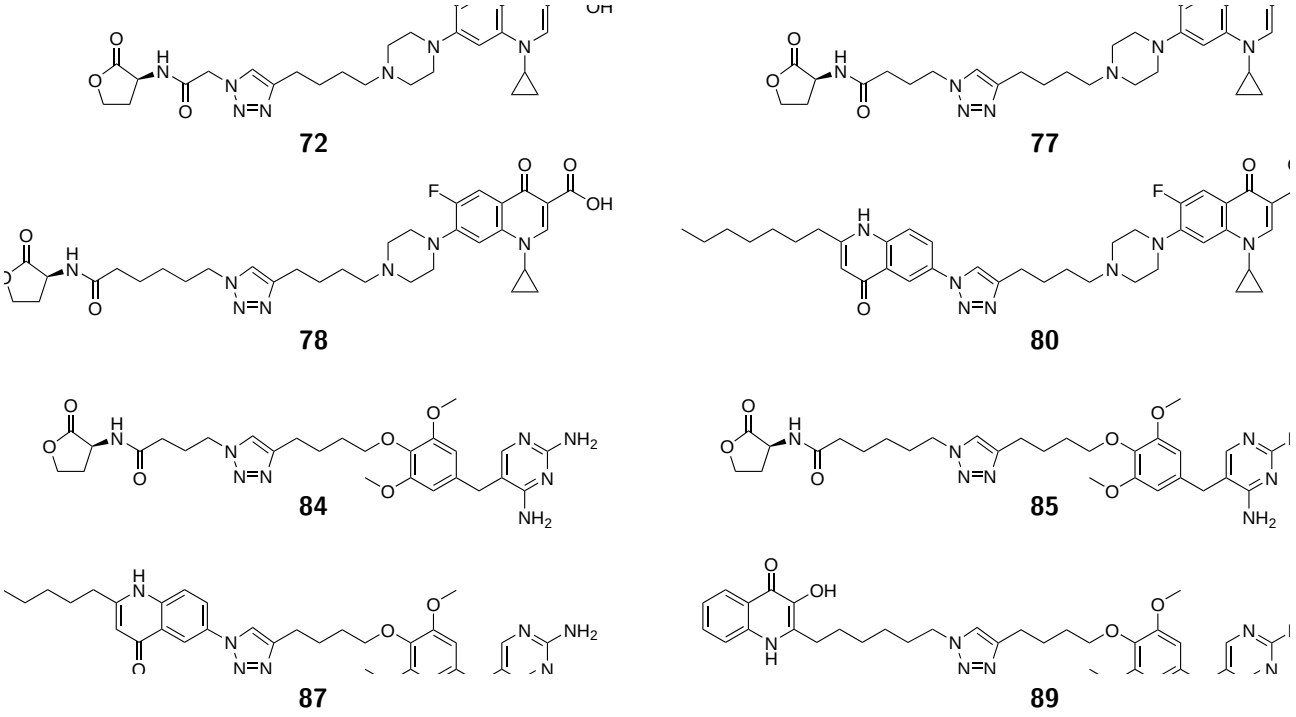

Figure 1: The autoinducer-antibiotic conjugates.

1.1.1.1 YM64

In YM64 at 5 h the HSL-ciprofloxacin conjugates **72**, **77** and **78** showed slight activity at the highest concentration, but not as much as ciprofloxacin **24**. This activity was not visible by 24 h (see Figure 3) and the compounds had no effect on biofilm formation (see Figure 4).

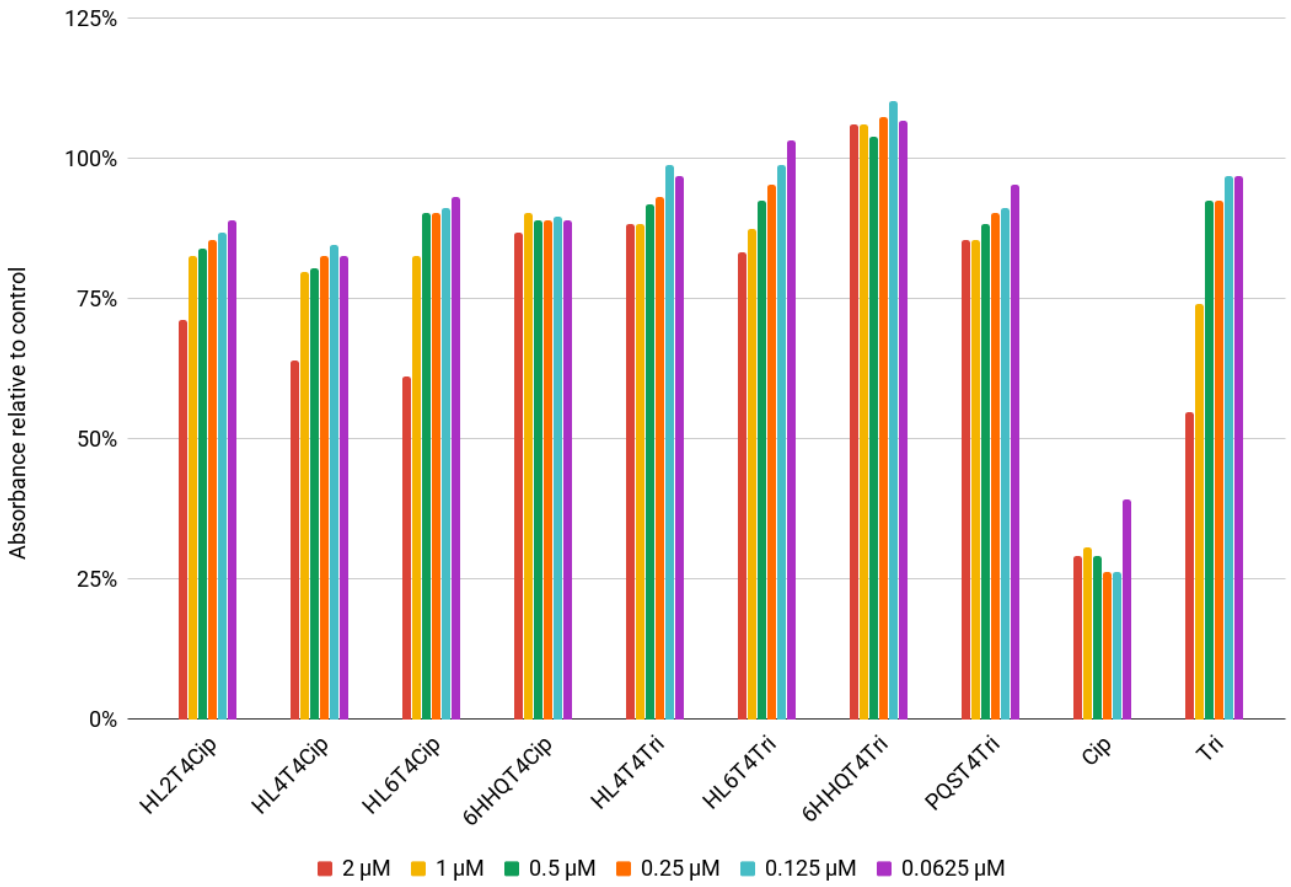

Figure 2: YM64 OD readings at 5 h for the autoinducer-antibiotic conjugates.

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

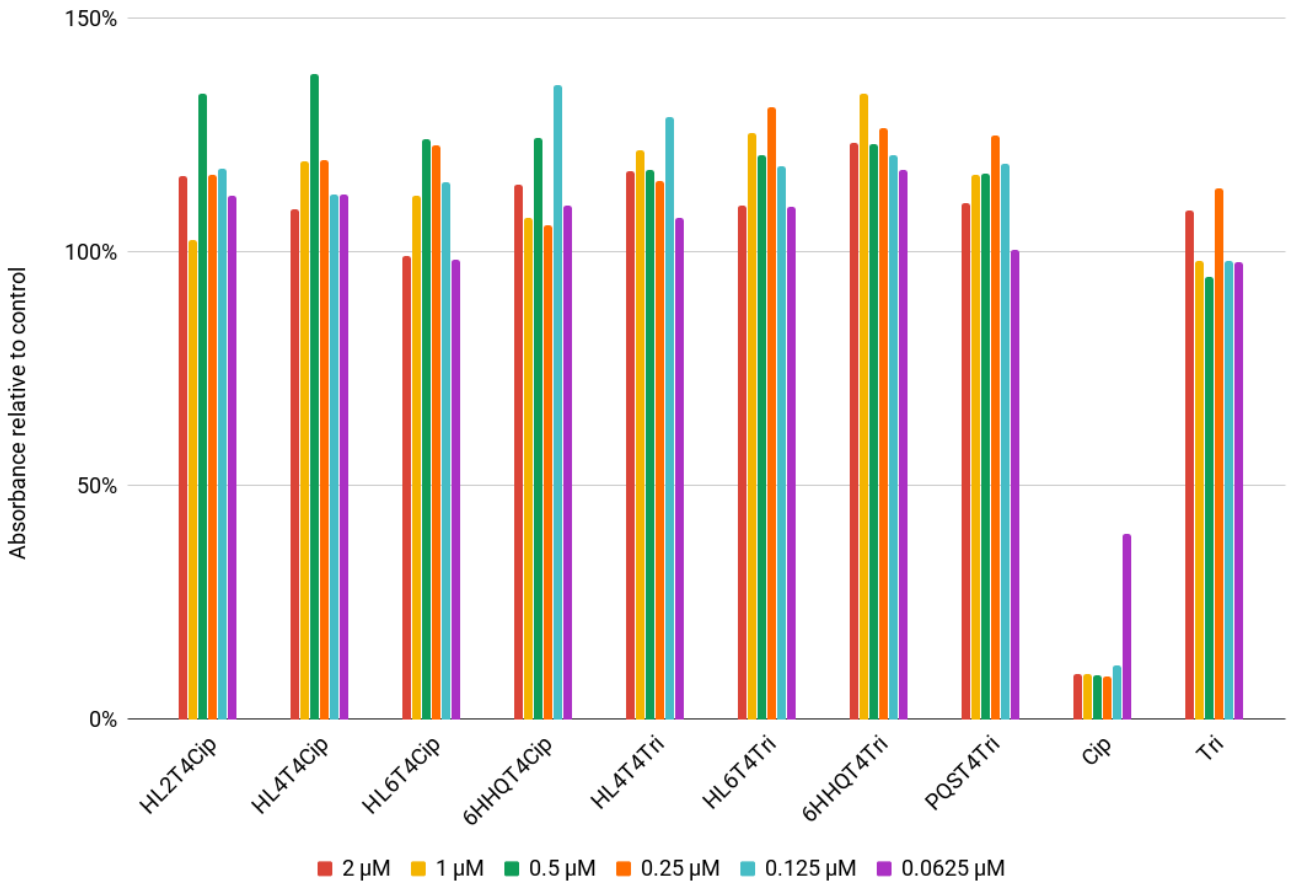

Figure 3: YM64 OD readings at 24 h for the autoinducer-antibiotic conjugates.

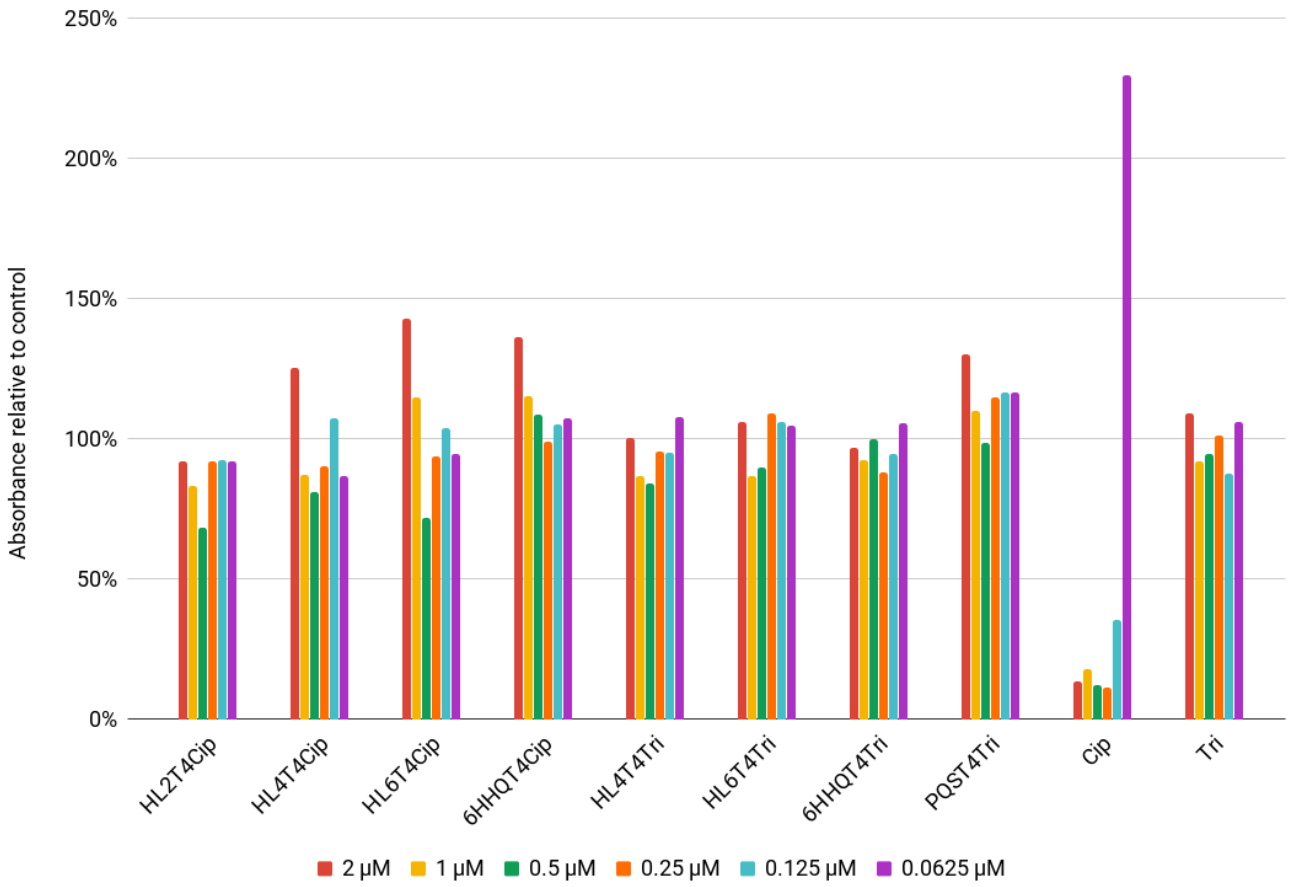

Figure 4: YM64 biofilm quantification at 24 h for the autoinducer-antibiotic conjugates.

1.1.1.2 PAO1

In PAO1 **78** showed similar activity to ciprofloxacin **24** at the highest concentration (see Figure 5), but not at lower concentrations. All other compounds did not show activity, and again there was no activity at 24 h or against biofilms.

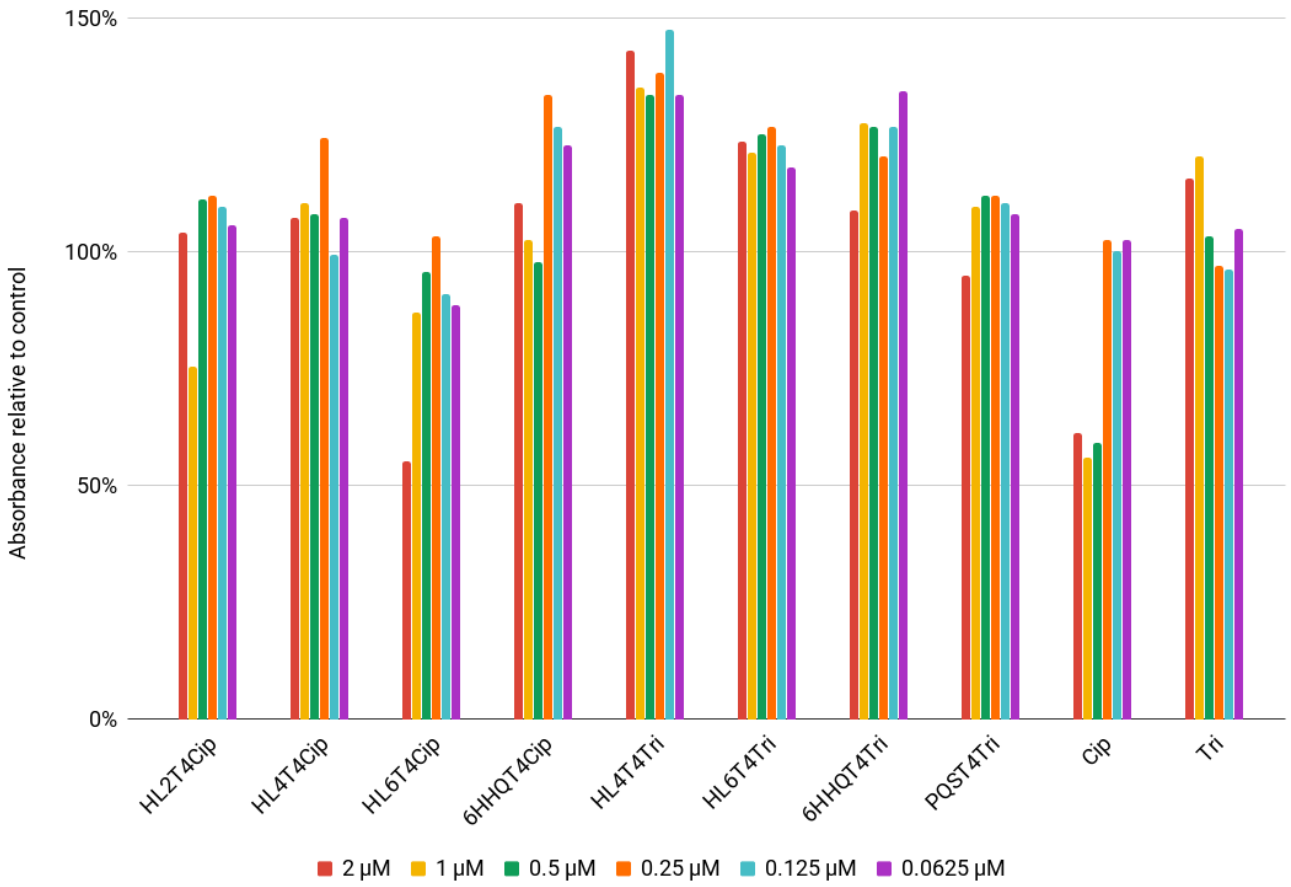

Figure 5: PAO1 OD readings at 5 h for the autoinducer-antibiotic conjugates.

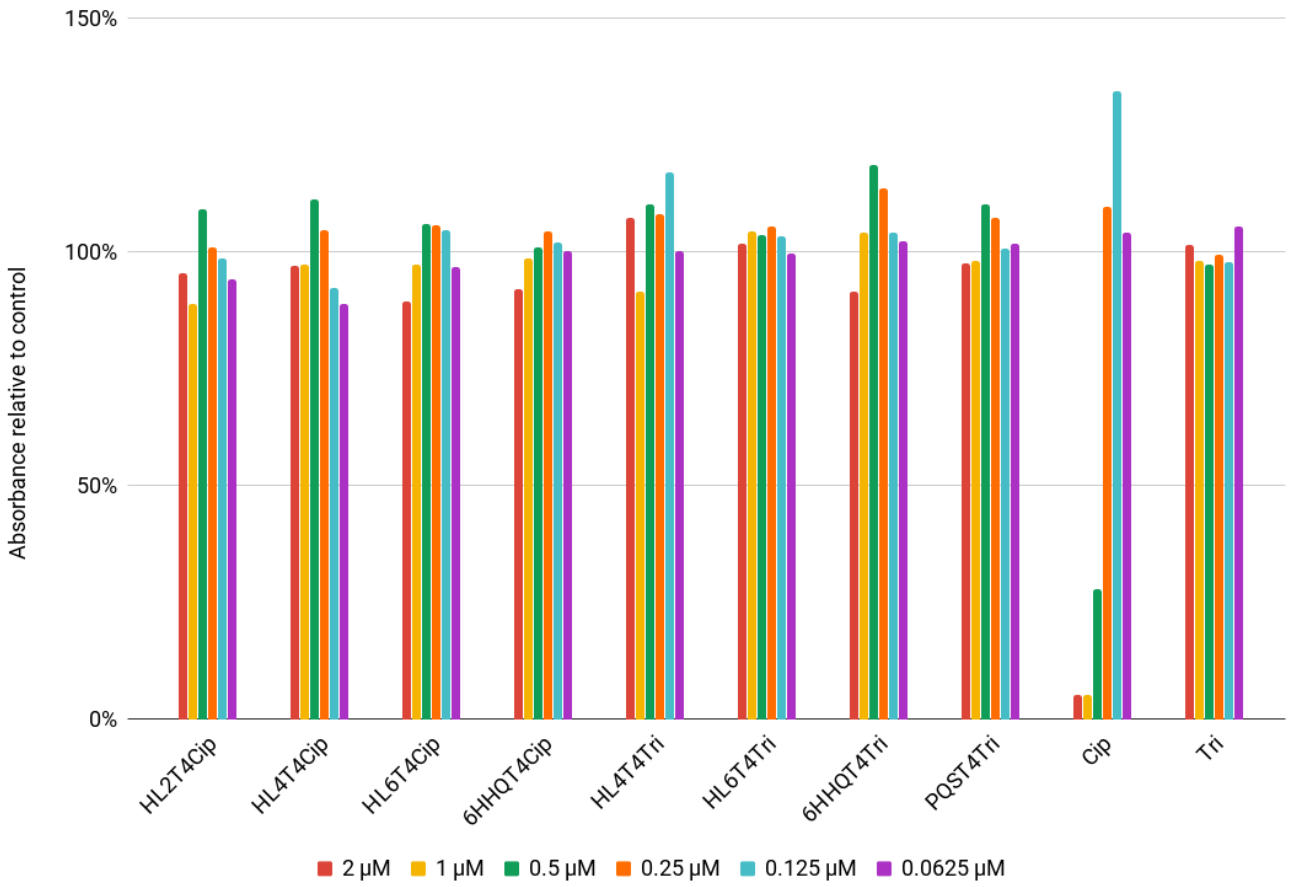

Figure 6: PAO1 OD readings at 24 h for the autoinducer-antibiotic conjugates.

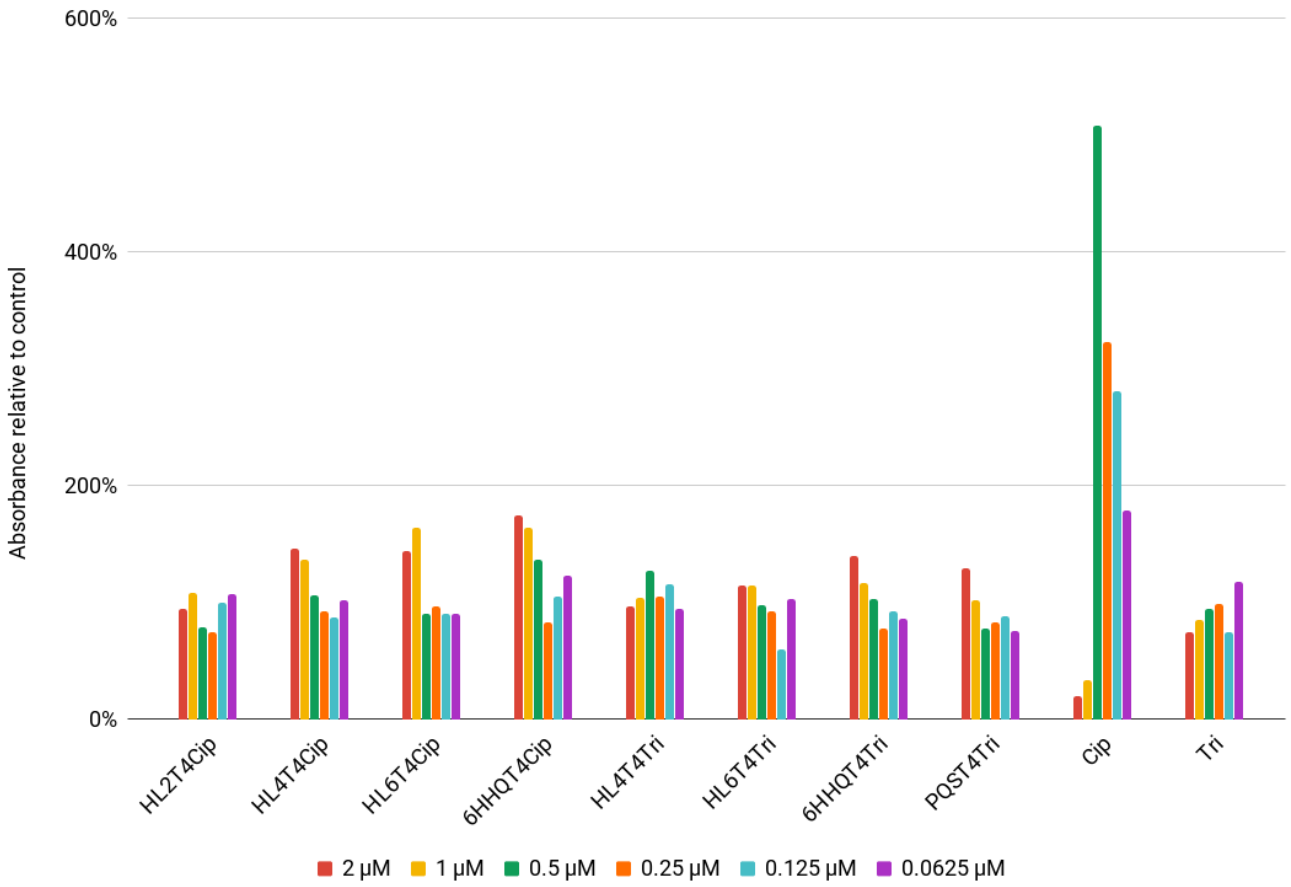

Figure 7: PAO1 biofilm quantification at 24 h for the autoinducer-antibiotic conjugates.

1.1.2 Cleavable HSL-ciprofloxacin conjugates

The eight cleavable HSL-ciprofloxacin conjugates, two controls and two alkynes described in **??** (see Figure 8) were tested for antibacterial and anti-biofilm activity in *P. aeruginosa* YM64.

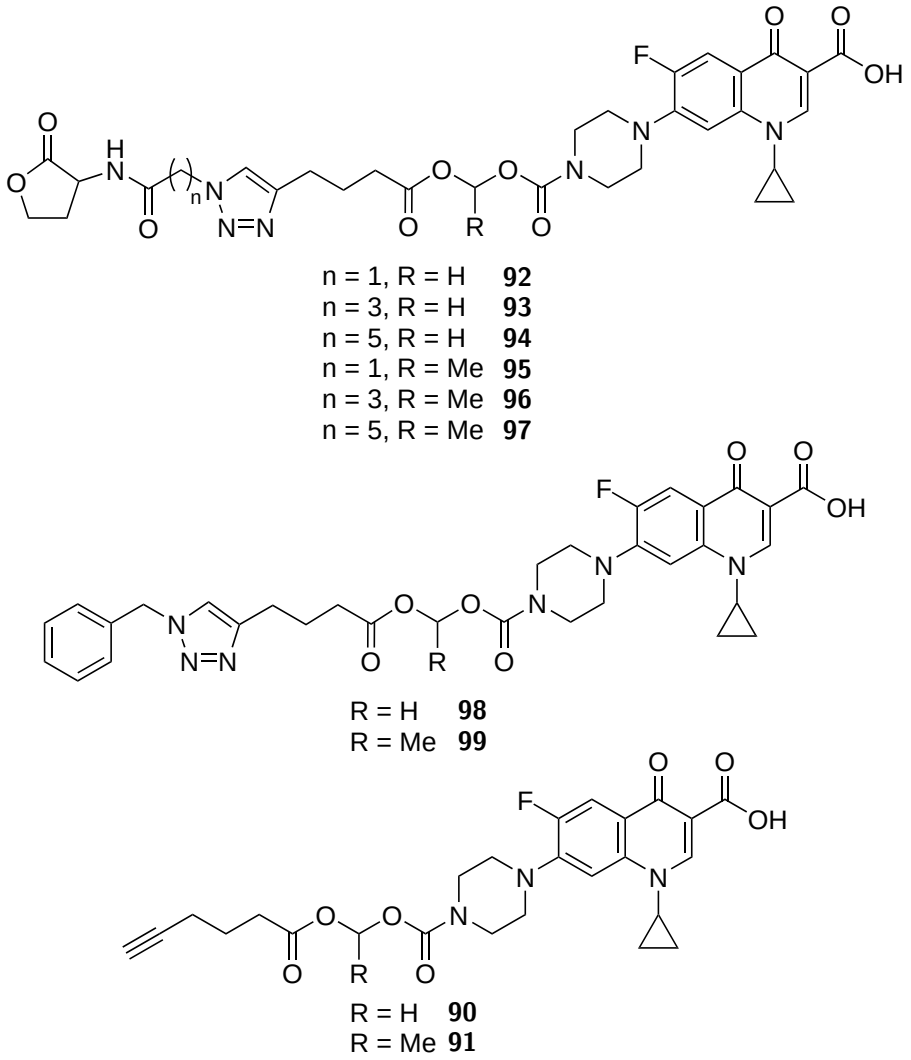

Figure 8: The cleavable HSL-ciprofloxacin conjugates.

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

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

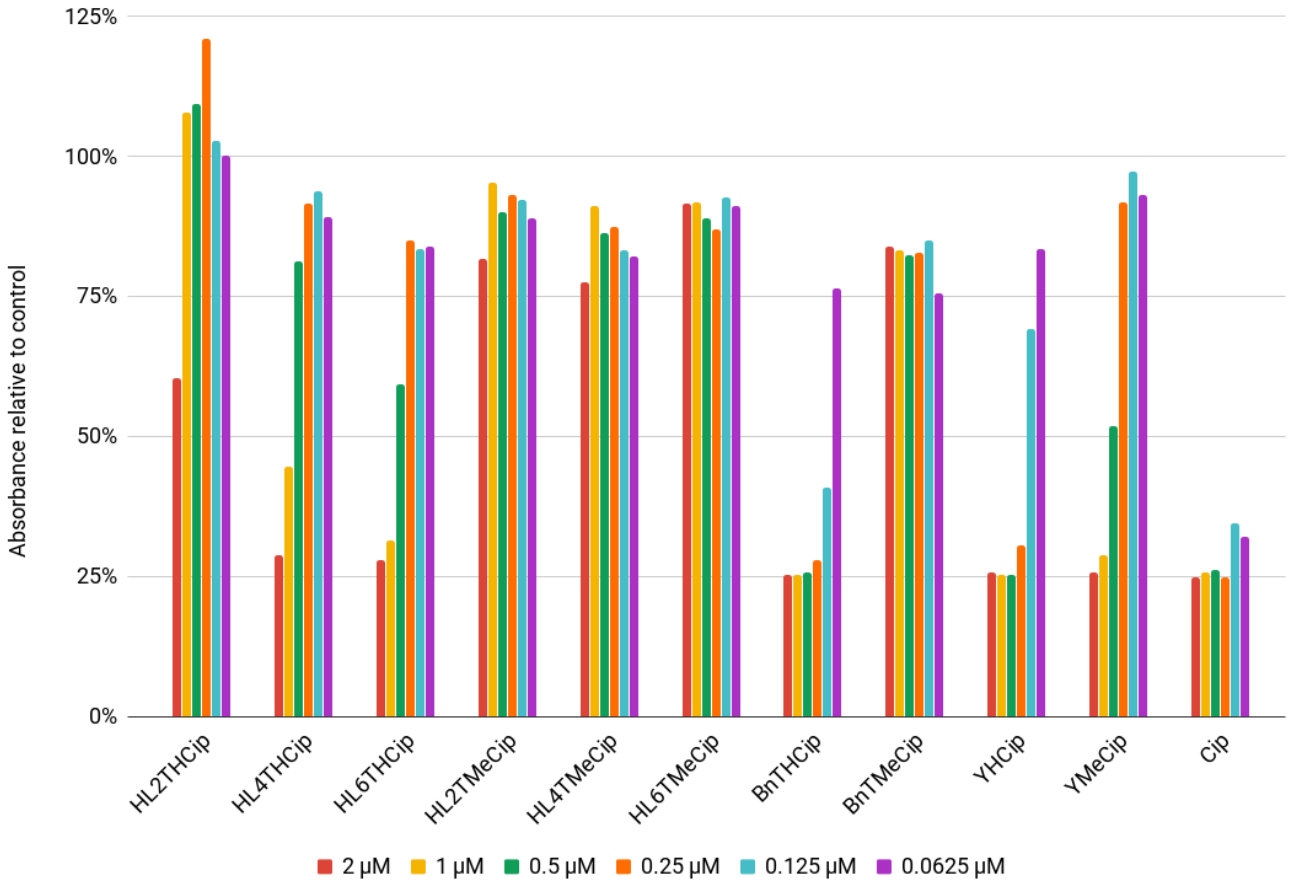

Figure 9: YM64 OD readings at 5 h for the cleavable HSL-ciprofloxacin conjugates.

1.2 Conclusions

1.2.1 Library synthesis

In this section, a range of 1,2,3-triazole-linked autoinducer-antibiotic conjugates was successfully synthesised and tested for antibiotic and anti-biofilm activity. Reliable routes to the azido autoinducers and alkynyl antibiotics were found, but the copper(I)-catalyzed alkyne-azide cycloaddition reactions used to link them proved rather capricious. The main reasons for this were insolubility of the starting materials and air-sensitivity. Air-sensitivity is not expected in a click reaction, but can be explained by many of the reactions being too dilute.[3] 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,[4] 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 1.3). In particular, autoinducers which are actively transported into cells, such as AI-2, are attractive targets.

1.2.2 Biology

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

1. Restriction of the binding of ciprofloxacin to DNA gyrase and topoisomerase IV[5] or trimethoprim to dihydrofolate reductase.[6]
2. Failure of the autoinducers to mask the antibiotics from recognition by efflux pumps.
3. Failure to penetrate the cell wall/biofilm.
4. Non-specific binding to the cell wall.

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

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

1.3 Future work

1.3.1 Biology

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

1. 24 h OD readings and biofilm quantifications for the cleavable HSL-ciprofloxacin conjugates.
2. Biofilm dispersal assays on all compounds (see **??** for a discussion of biofilm dispersal using a HSL analogue-ciprofloxacin conjugate and 3.1.4 for the methodology to be used).

2 Results and discussion: HSL analogue-ciprofloxacin conjugates

2.1 Biological testing

The testing presented in this section was planned by me but carried out by Tom O'Brien, a PhD student in the Department of Biochemistry.

The HSL analogue-ciprofloxacin conjugates (see Figure 10), as well as C_4-HSL **19**, ciprofloxacin **24**, methyl ciprofloxacin **151**, the alkynyl ciprofloxacin derivative **68**, the *tert*-butyl ester methyl ciprofloxacin derivative **198** and the carboxylic acid methyl ciprofloxacin derivative **199** were tested for antibacterial and anti-biofilm activity in *P. aeruginosa* PAO1[1] and YM64.[2]

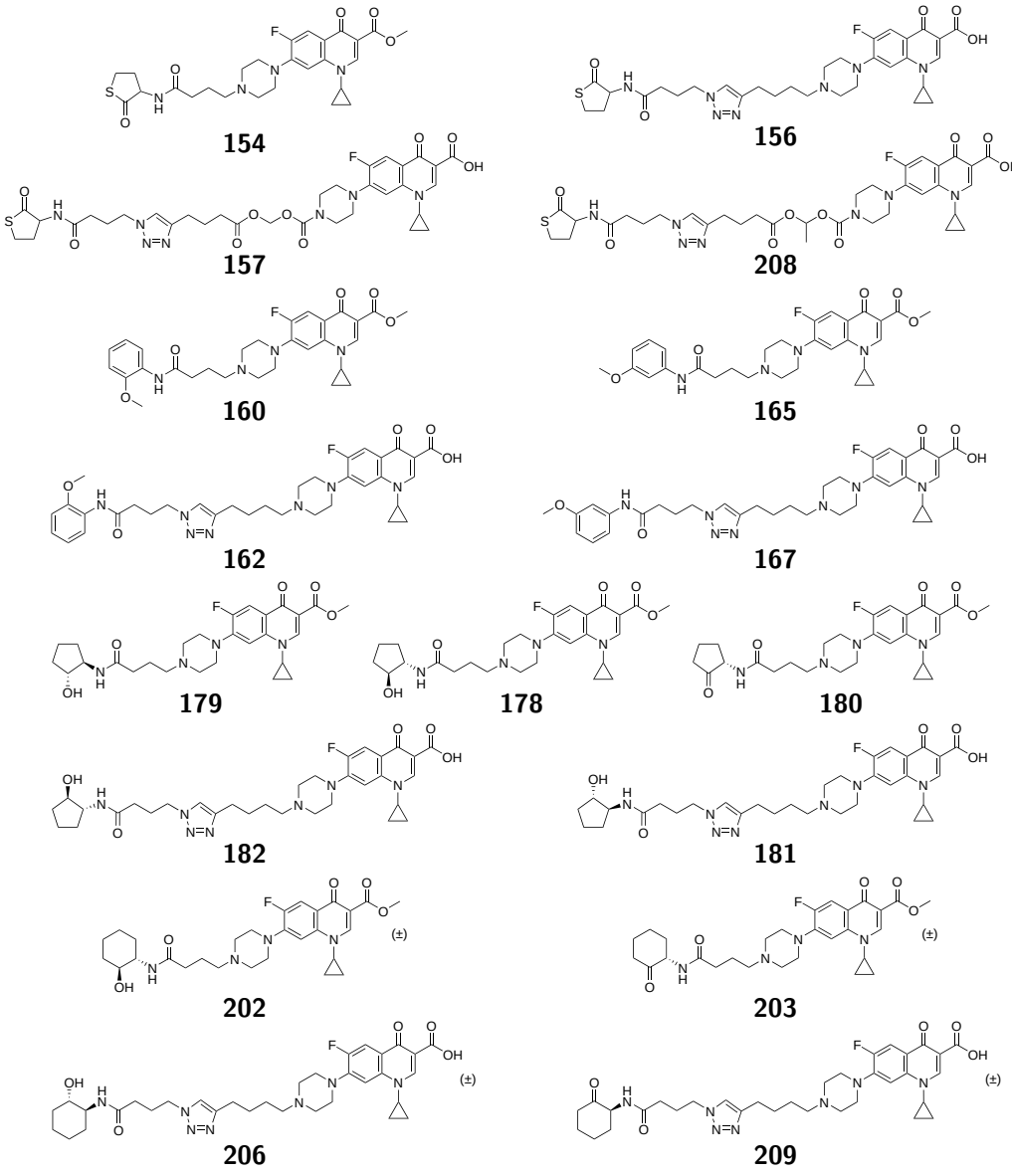

Figure 10: The HSL analogue-ciprofloxacin conjugates.

2.1.1 Antibacterial activity

2.1.1.1 YM64

In YM64 at 5 h several of the HSL analogue-ciprofloxacin conjugates showed activity at the highest concentration (see Figure 11 and Figure 12). Conjugates **162** and **167** showed similar activity to ciprofloxacin **24** and the cleavable conjugate **157** showed better activity (see Figure 11). The activity of the cleavable conjugate **157** was even more pronounced at 24 h (see Figure 13).

It should be noted that the highest concentration tested was 25 μM in this set of assays as opposed to 2 μM in the previous set (see 1.1), but oddly all compounds including ciprofloxacin **24** showed less activity. This is thought to be due to a change in the plate seals used (see 3.1.1).

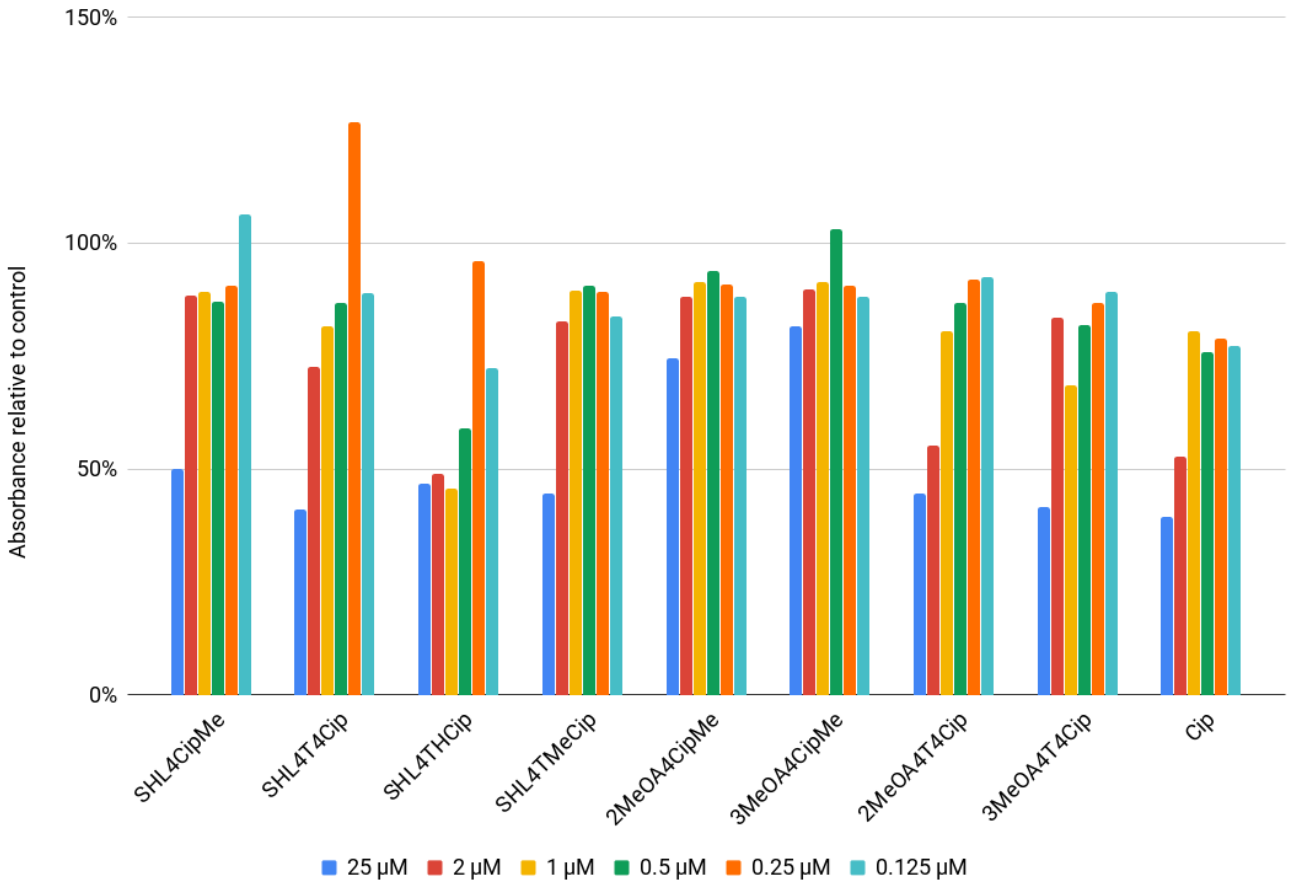

Figure 11: YM64 OD readings at 5 h for the HCTL, 2-methoxybenzene and 3-methoxybenzene HSL analogue-ciprofloxacin conjugates.

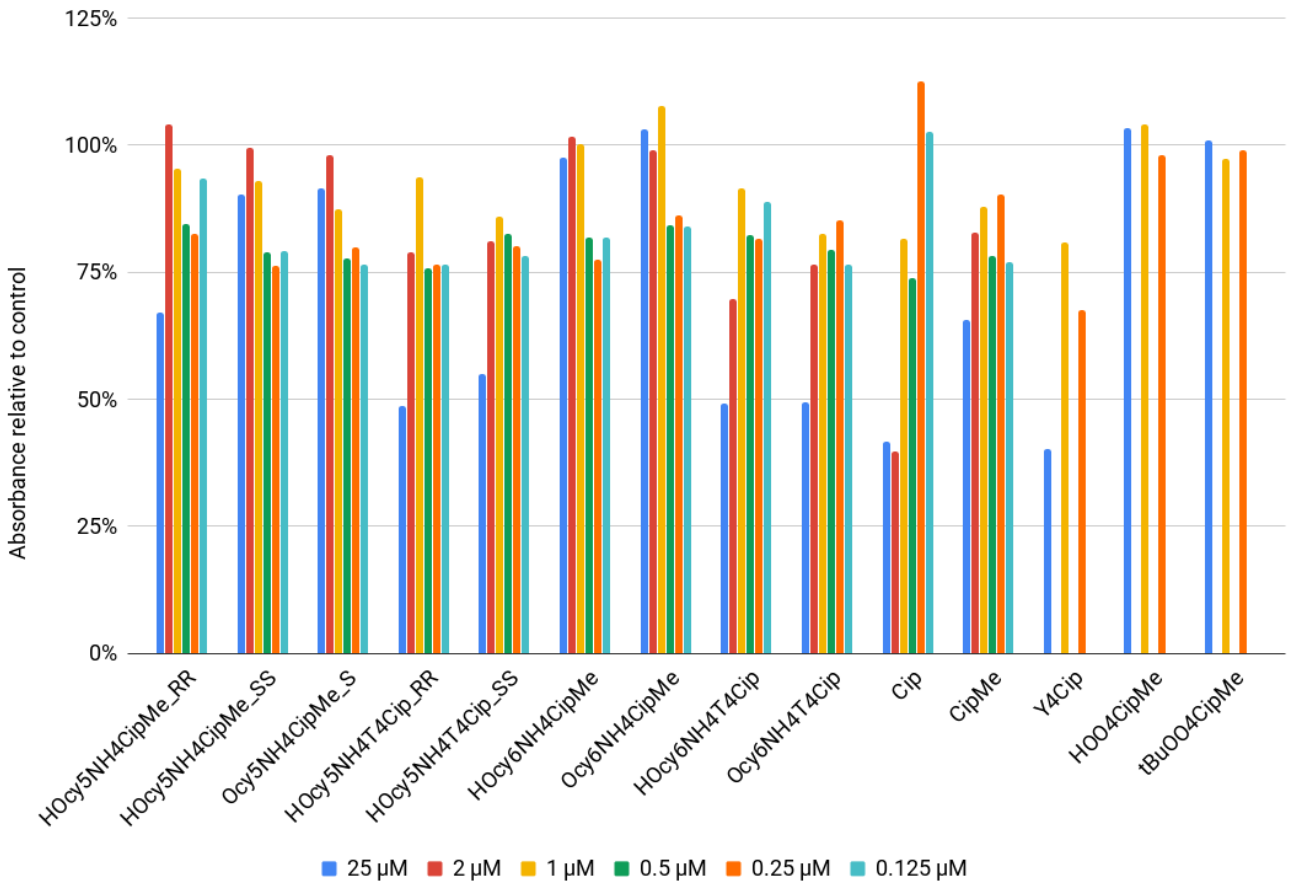

Figure 12: YM64 OD readings at 5 h for the alcohol and ketone HSL analogue-ciprofloxacin conjugates.

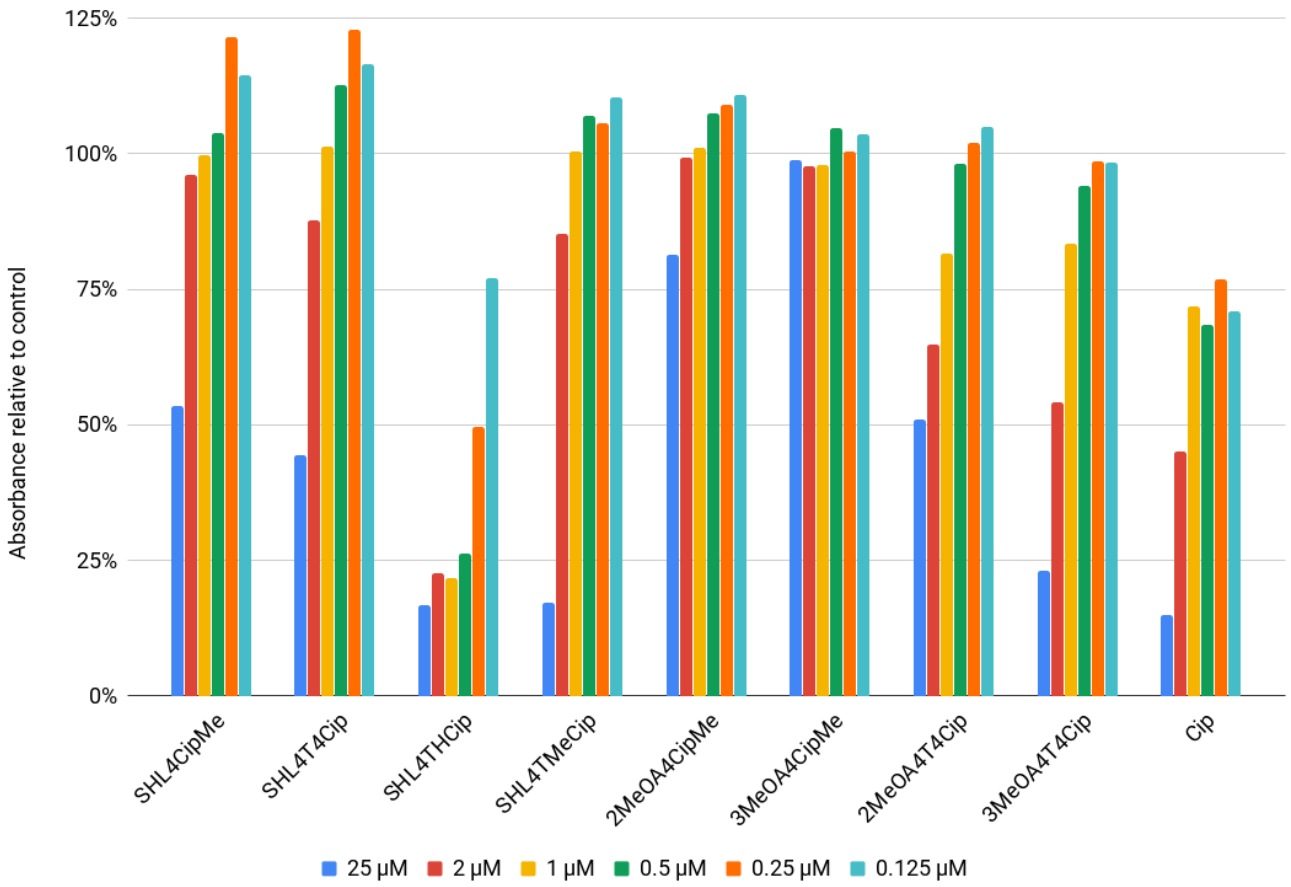

Figure 13: YM64 OD readings at 24 h for the HCTL, 2-methoxybenzene and 3-methoxybenzene HSL analogue-ciprofloxacin conjugates.

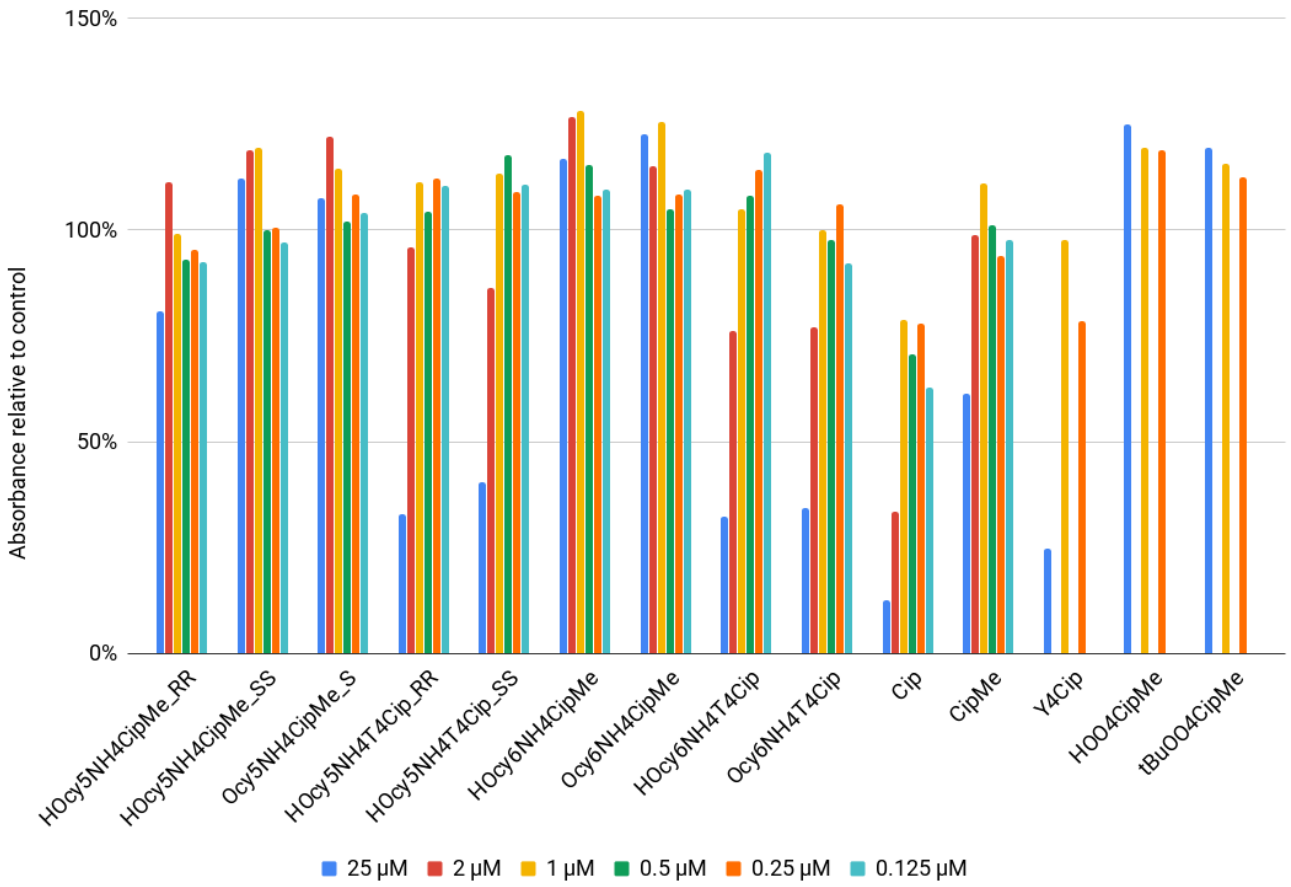

Figure 14: YM64 OD readings at 24 h for the alcohol and ketone HSL analogue-ciprofloxacin conjugates.

2.1.1.2 PAO1

In PAO1 at 5 h conjugates **157**, **162** and **167** showed activity at the highest concentration (see Figure 15). The cleavable conjugate **157** showed similar activity to ciprofloxacin **24**. At 24 h conjugate **167** still showed some activity, and cleavable conjugate **157** showed similar activity to ciprofloxacin **24** (see Figure 17).

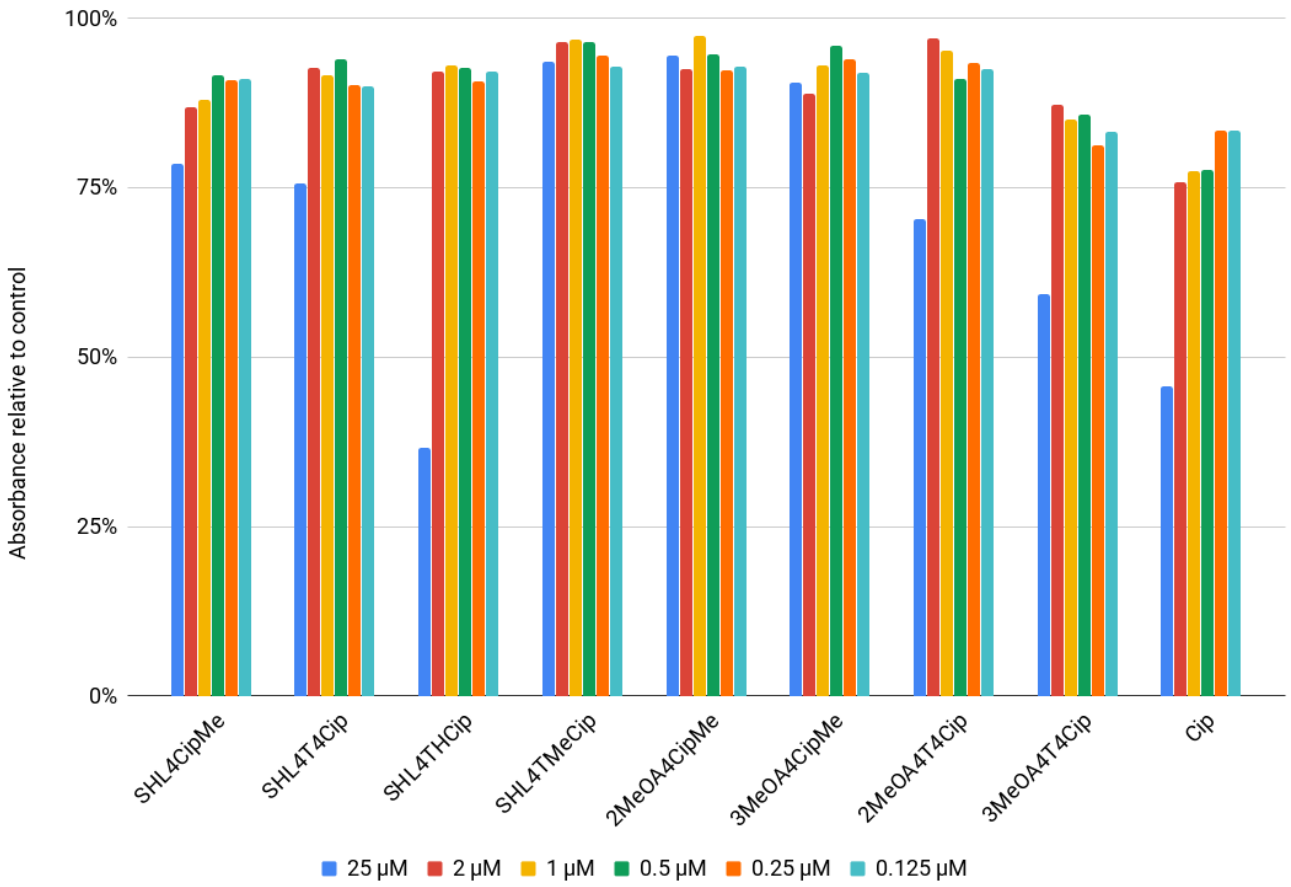

Figure 15: PAO1 OD readings at 5 h for the HCTL, 2-methoxybenzene and 3-methoxybenzene HSL analogue-ciprofloxacin conjugates.

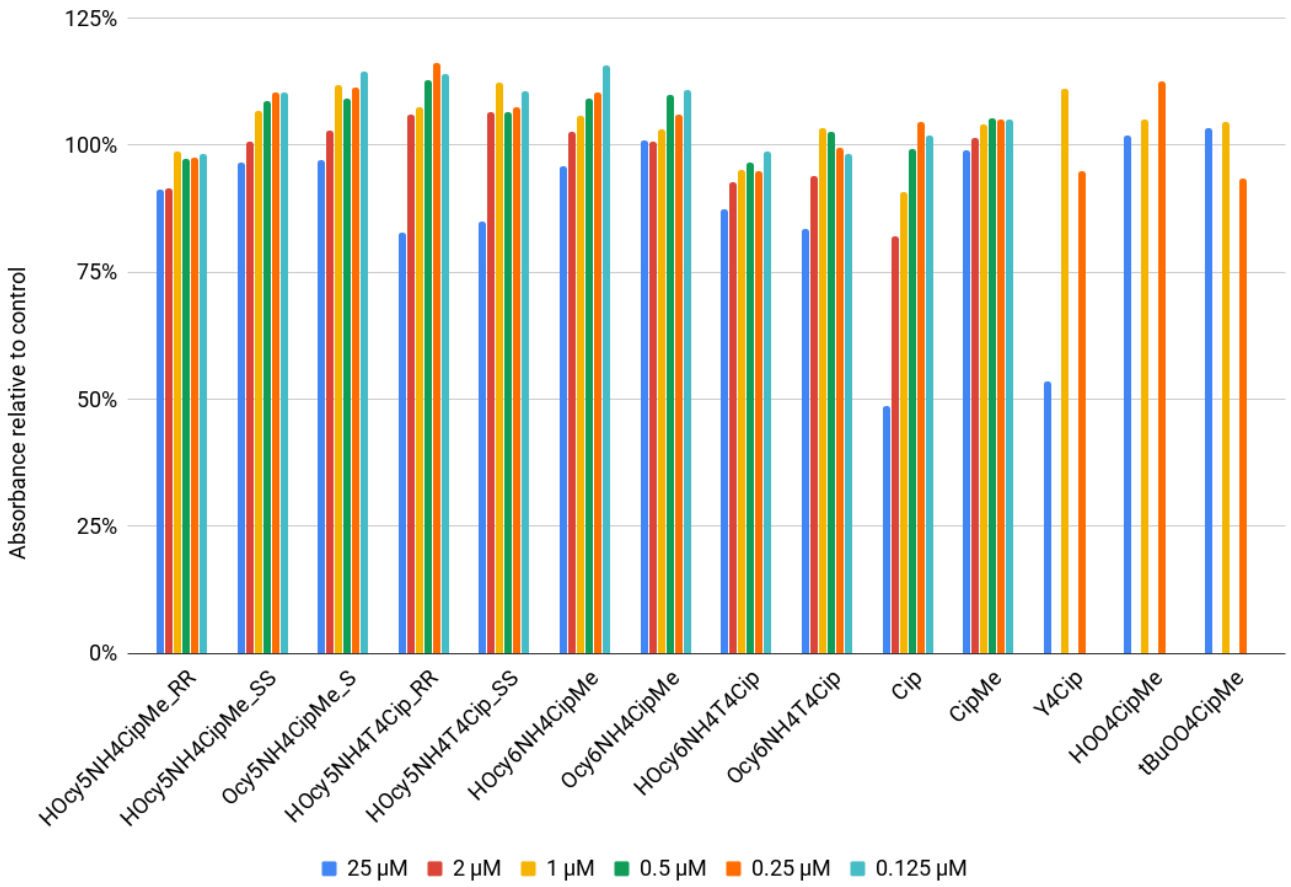

Figure 16: PAO1 OD readings at 5 h for the alcohol and ketone HSL analogue-ciprofloxacin conjugates.

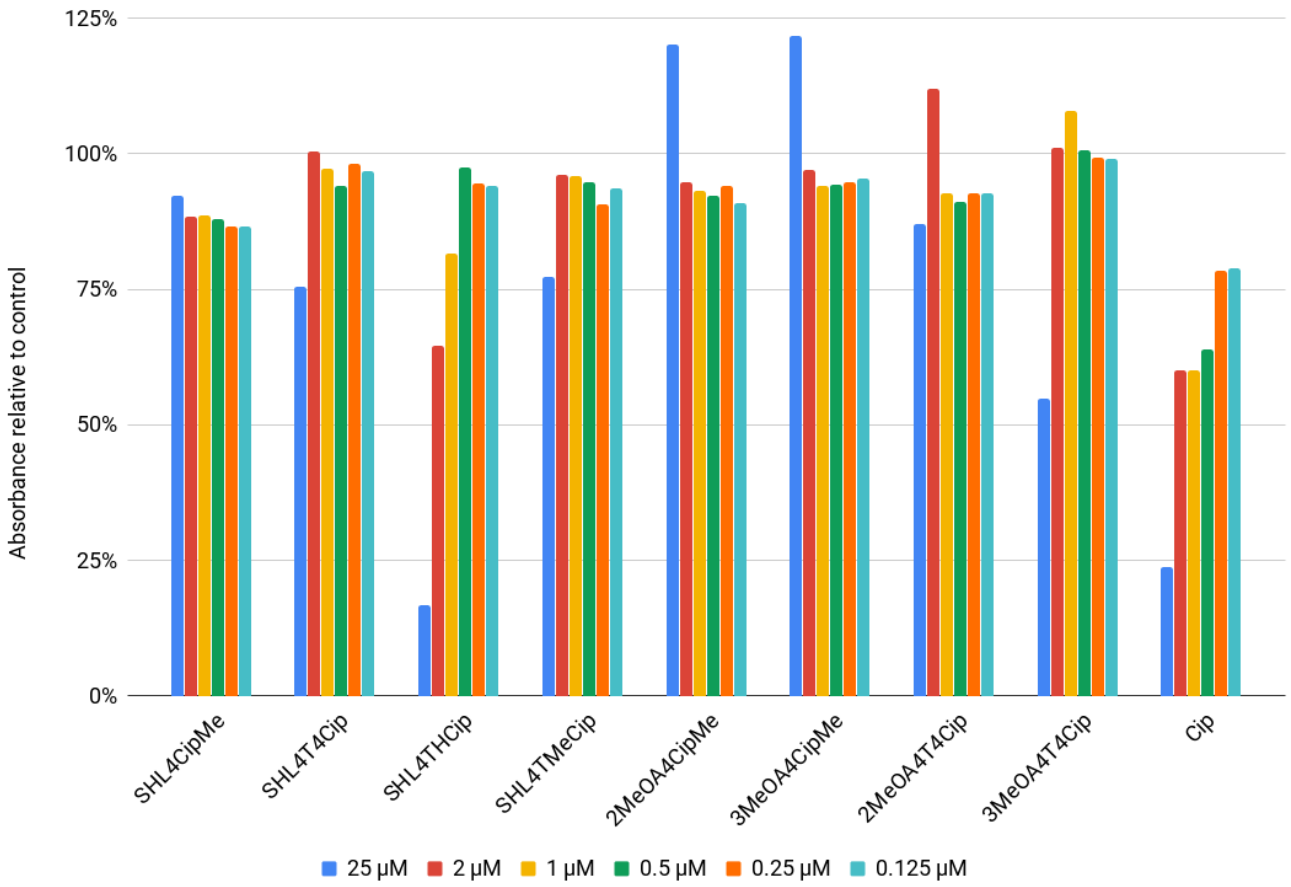

Figure 17: PAO1 OD readings at 24 h for the HCTL, 2-methoxybenzene and 3-methoxybenzene HSL analogue-ciprofloxacin conjugates.

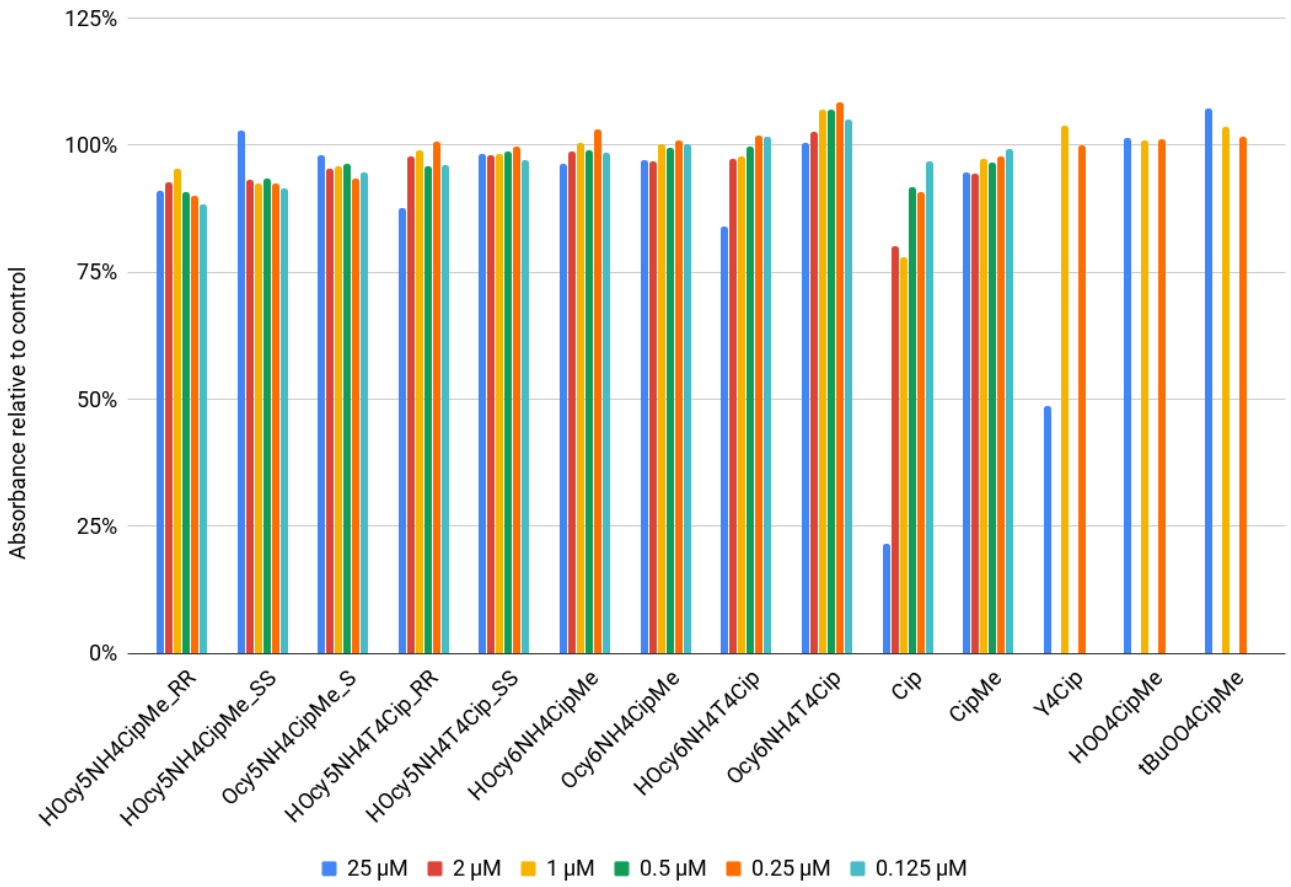

Figure 18: PAO1 OD readings at 24 h for the alcohol and ketone HSL analogue-ciprofloxacin conjugates.

Approximate MICs for the more active compounds are shown in **??**

Compound	**YM64 - 5 h**	**YM64 - 24 h**	**PAO1 - 5 h**	**PAO1 - 24 h**
SHL4THCip **157**				0.0455 ±
2MeOA4T4Cip **162**	0.0406 ±	0.0391 ±		
3MeOA4T4Cip **167**		0.0364 ±		
Cip **24**				

Table 1: .

In addition to its promising antibacterial activity, the cleavable conjugate **157** has an interesting growth curve (see Figure 19). Whereas *P. aeruginosa* treated with ciprofloxacin **24** continues to grow over the course of a 48 h assay, the growth curve for compound **157** remains flat. It seems that **157** kills/inhibits the growth all of the cells, whereas ciprofloxacin **24** either allows slow growth, or resistant mutants emerge and start to replicate.

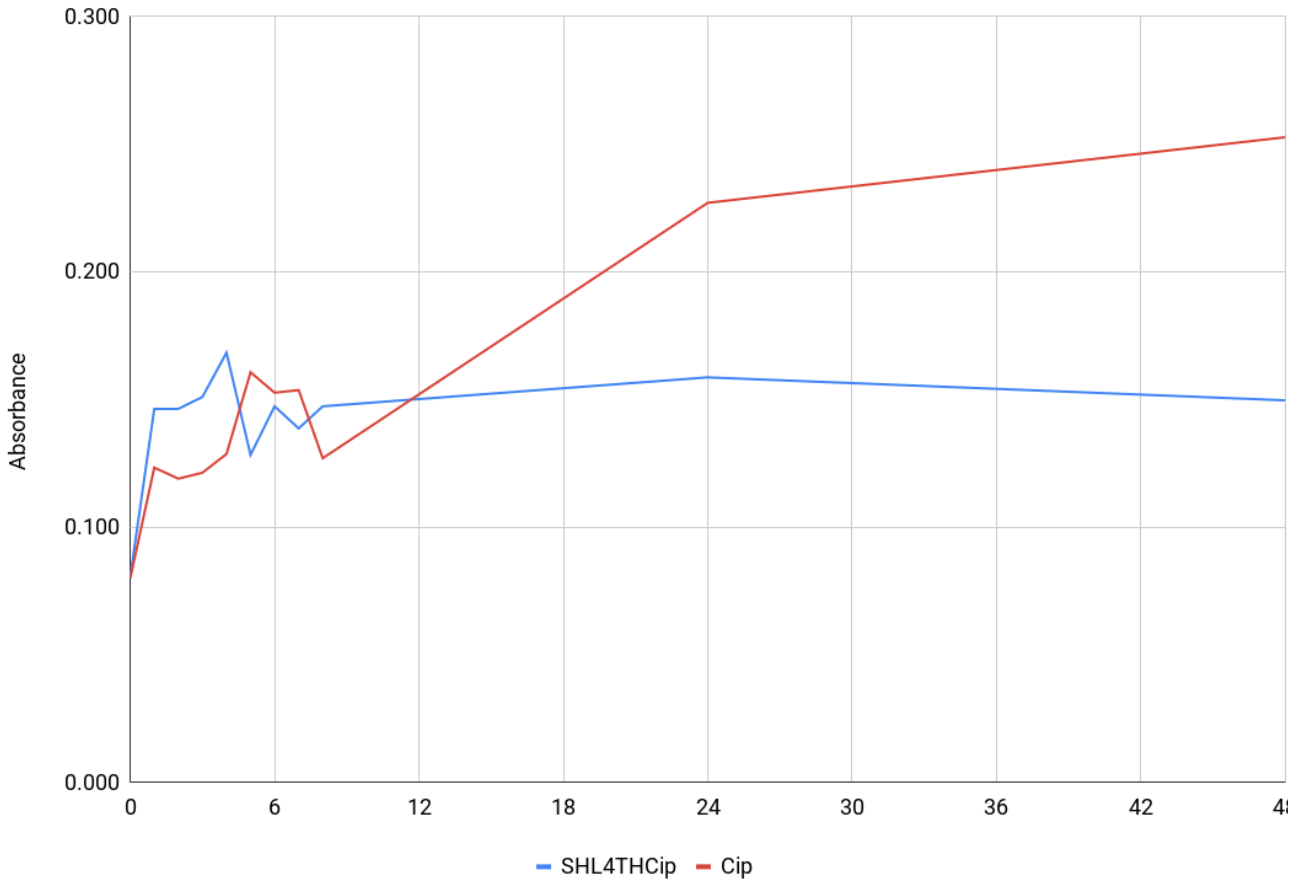

Figure 19: PAO1 25 μM.

2.1.2 Determination of anti-biofilm activity

Biofilm inhibition and dispersal were measured using crystal violet staining (see 3.1). Unfortunately the results were largely unreliable as it was obvious that staining was higher in the wells at the edges of the plates, and that this was overwhelming any other trends. This effect was probably due to increased evaporation from the outer wells. It is likely that this effect was seen in these results, but not those in 1.1, due to a change in the conditions that the plates were incubated in. Specifically, a different type of plate seal was used and a humid environment was maintained in the incubator (see 3.1) in the previous experiments.

2.2 Conclusions

2.2.1 Library synthesis

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).

2.2.2 Biology

2.2.2.1 Controls

2.2.2.2 Cip vs CipMe

The Cip triazole conjugates had higher activity than the CipMe conjugates. This was mirrored in the controls: CipMe**151** showed very little activity compared to ciprofloxacin **24**. It was assumed that methyl ciprofloacin would act as a prodrug, and was used in the Ganguly conjugate, but this was apparently not the case. The methylated controls also didn't show any activity, but Y4Cip did.

2.2.2.3 Best ones

The most promising compounds were **157**, **162** and **167**.

PA doesn't develop resistance to **157** over the course of a 48 h incubation whereas Cip does.[7]

2.2.2.4 biofilm fail

Biofilm inhibition and dispersal were measured using crystal violet staining (see 3.1). Unfortunately the results were largely unreliable as it was obvious that biofilm was growing more in the wells at the edges of the plates, and that this was overwhelming any other trends. This effect was probably due to increased evaporation from the outer wells. It is likely that this effect was seen in these results, but not those in 1.1, due to a change in the conditions that the plates were incubated in. Specifically, a different type of plate seal was used and a humid environment was maintained in the incubator (see 3.1) in the previous experiments.

As biofilm formation is induced by hypoxia[8] (which might occur in the centre of the plate when a plastic lid was used in addition to the adhesive plate seal) this cannot account for the increased biofilm growth at the edges of the plate. Neither can the increased concentration of NaCl that would occur due to evaporation of water from the edges of the plate, as this too decreases biofilm formation.[9] A reasonable explanation could be that evaporation would leave a residue of dried planktonic cells the edges of the wells which would be stained by the crystal violet. To avoid this problem in the future the plates should be incubated in a humid atmosphere without a plastic plate lid.

culture conc?

2.3 Future work

2.3.1 HSL analogue derivatives

HL4CipM

A selection head groups which could be used in future conjugates are shown in Figure 20. These have all been shown to modulate HSL-mediated quorum sensing as part of acyl-HSLs.[10–16] The most obvious targets are the cyclopentanone derivatives, as this could be synthesised from the alcohols above. The aniline, pyridine,

HSL analogue cip conjugates with no

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.[13]

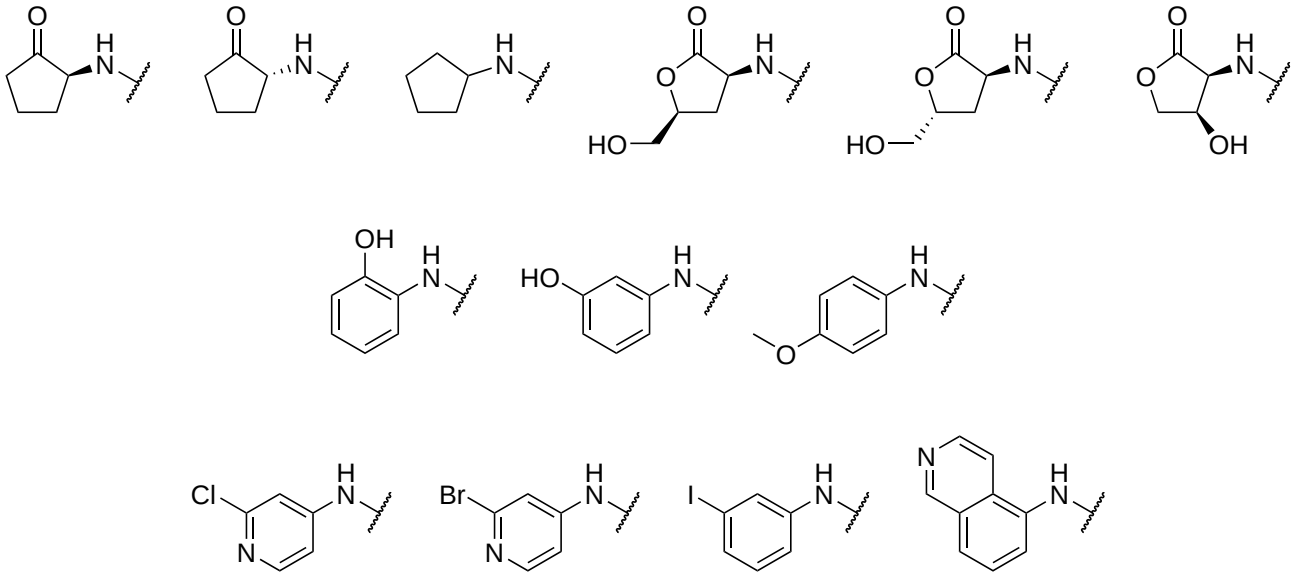

Figure 20: HSL analogue head groups for use in future conjugates.

2.3.2 Biology

Repeat biofilm stuff

Ganguly *et al.* used Bac-Light Live/Dead staining and confocal microscopy to image the biofilms, whereas so far I have used crystal violet staining. Crystal violet does not differentiate between live or dead cells, and so might not pick up on the antibacterial effects of compounds. However, their confocal microscopy results show a quantifiable decrease in biofilm thickness, and it may be possible to detect this using crystal violet.

XTT stain

3 Experimental

3.1 Biological testing

Compounds were tested against *P. aeruginosa* PAO1[1] and YM64.[2] C_4-HSL **19**, HHQ **21**, PQS **22**, ciprofloxacin **24**, trimethoprim **25** and DMSO were included as controls, along with LB to check for contamination of the plates. All absorbances are shown relative to DMSO. The first set of autoinducer-antibiotic conjugates (see 1.1) were tested at 2, 1, 0.5, 0.25, 0.125 and 0.0625 μM. OD readings were taken at 5 and 24 h, and biofilm quantification was carried out soon after the 24 h OD reading. Only a 5 h OD reading was obtained for the cleavable HSL-ciprofloxacin conjugates. The HSL analogue-ciprofloxacin conjugates (see 2.1) were tested at 25, 2, 1, 0.5, 0.25 and 0.125 μM in triplicate. OD readings were taken at 0, 1, 2, 3, 4, 5, 6, 7, 8, 24 and 48 h. Biofilm inhibition testing was carried out on plates grown for 24 and 48 h. Biofilm dispersal testing was carried out by growing plates for 24 h, followed by addition of the compounds, incubation for a further 24 h and quantification of the biofilms.

3.1.1 Antibiotic susceptibility

Antibiotic susceptibility was determined using spectrophotometry measurements. Colonies of the desired strains were grown at 37 °C overnight on LB agar. The colonies were used to inoculate LB (10 ml) and these cultures were grown at 37 °C overnight. The cultures were diluted 1/100 with LB, and 99 μl diluted culture per well was added to flat-bottomed clear 96-well plates. 1 μl of compound solution in DMSO was then added from master plates and the plates were covered with adhesive aeration filters.

In the first round of testing, Breathe-Easy® sealing membranes from Diversified Biotech were used and the plates were placed in a open box containing tissue paper wetted with distilled water in order to control evaporation. In the second round of testing, AeraSeal™ films from Excel Scientific were used, and the humidified box was not.

The plates were shaken at 37 °C and 100 rpm and optical density was recorded periodically using a Biochrom EZ Read 400 microplate reader at 595 nm.

3.1.2 Quantification of biofilms

Biofilms were quantified using a method described previously.[17,18] After the bacteria had grown for the desired amount of time, the culture was aspirated out of the wells using a pipette tip attached to a vacuum pump, making sure not to touch the sides of the wells. Water (120 μl) was then added and aspirated out again. This process was repeated twice more to thoroughly wash out planktonic cells. Crystal violet (120 μl, 0.1% m/v) was added and left for 15 min, then aspirated out. The wells were washed again with water (3 × 120 μl). Acetic acid (120 μl, 30% v/v aq.) was added and left for 15 min then the plate was vortexed and read using a Biochrom EZ Read 400 microplate reader at 595 nm.

3.1.3 Biofilm inhibition

The plates were prepared as in 3.1.1. The plates were shaken at 37 ° and 100 rpm for 24 h followed by quantification of biofilm growth as shown in 3.1.2.

3.1.4 Biofilm dispersal

The plates were prepared as in 3.1.1, initially without the addition of compound solutions. The box of plates was shaken at 37 ° and 100 rpm for 24. 1 μl of compound solution in DMSO was then added to each well from master plates and the plates were shaken as above for a further 24 h followed by measurement of OD and quantification of biofilm growth as shown in 3.1.2.

4 References

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