ENHANCING THE ANTIBIOTIC SUSCEPTIBILITY OF PSEUDOMONAS AERUGINOSA BIOFILMS BY QUORUM SENSING INHIBITION

by

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APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

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Caol Philipp Huff

November 27, 2006

To my Dad;

Your exhibition of strength and dignity to adversity is as inspiring as it is aggravating.

ACKNOWLEDGEMENTS

Thanks to Phil Stewart and the CBE. All the encouragement and kind words helped me through what seemed like an unending path of frustration and despair. Phil in particular deserves thanks for his open ear and toleration of my frayed nerves and high blood pressure. Thanks to Tom for teaching me as much as he could about synthetic chemistry and for keeping me on my toes during group meeting with his ever ready barrage of denigrating comments. Your grasp of the organic chemistry literature will always inspire me to spend another hour in the library. Thanks to the Department of Chemistry and Biochemistry for holding me to the higher end of a double standard. I learned that an affable manner, enthusiasm for science and team player attitude are no match for a room full of ivory tower egos.

Lab work was a lot duller once Todd graduated. I'll miss the late night conversations about chemistry, politics and religion. Thanks Magda for your words of encouragement. You were always too willing to lend a helpful hand. Jaemoon, I have no idea how you get so much done in 8 hours and still know what all the famous chemists are up to. Nick and Sol, you've gone to a better place. Enoch, hang in there. Fouchard and Spicka, it was fun barging into your lab with any chemistry question that came to mind.

Lastly, thanks to all my family and friends. I knew I was never alone as long as my cell phone had batteries. Holiday Inn crew, you kept me from going postal. Thanks Johnny, "It only takes a dime to nickel a shoe, it'll do a million dollars worth of good for you." To the Jacks: BOOM!

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ABSTRACT

Biofilm forming bacteria are industrially and medically relevant organisms that are exceptionally resistant to garden variety antimicrobial treatments. This resistance is due in part to a biofilm forming bacteria's ability to sense and communicate with neighboring bacteria. As a result of this intercellular communication, bacteria are able to cooperate as a complex community. This communication system is used to modulate important facets of biofilm behavior and thus is an attractive target for biofilm control and potential antimicrobial agents.

Inhibition of the molecular signaling system used by biofilm forming bacteria could lead to an effective treatment of chronic bacterial infections by interrupting the communication that promotes biofilm formation. Specifically, this will be accomplished be preparing synthetic analogues of signaling molecules possessing the *N*-acyl homoserine lactone structural motif. This structural component is well conserved among the signaling molecules in biofilm forming bacteria and it is hoped that these analogues will inhibit biofilm formation in *Pseudomonas aeruginosa*.

Synthetic analogues of the *N*-acyl-*L*-homoserine lactone structural motif have been prepared that inhibit the signaling in *Pseudomonas aeruginosa*. These synthetic inhibitors have also been shown, using a novel application of the colony biofilm assay, to increase the susceptibility of *Pseudomonas aeruginosa* biofilms to treatment with the antibiotic tobramycin.

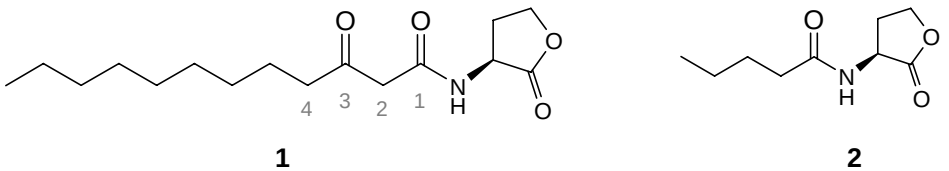
Additional inspiration has been taken from the structure of bacterial communication molecules that has lead to the design and synthesis of a novel class of biocides. These bifunctional molecules incorporate a biocidal property into the *N*-acyl-*L*-homoserine lactone structure. This bifunctionality could potentially enhance the specificity or potency of a biocide over the currently available treatments.

CHAPTER ONE

INTRODUCTION

Background

Biofilm forming bacteria are presently a problem of increasing medical and industrial relevance. Bacteria as biofilms are substantially different organisms (both metabolically and structurally) than their planktonic counterparts, and display an entirely different set of destructive behaviors. Biofilm formation and behavior is known to be modulated via acylhomoserine lactone (ASL) intercellular communication molecules (**1, 2**)[1-3] which are synthesized with a bacterial protein synthase I and activates a transcription factor protein R, which affects biofilm behavior by regulating target genes. This biofilm behavior and the genetic circuitry that supports it are referred to as "quorum sensing" and allow an individual bacterium to sense when the population has reached a critical concentration. The ASL molecules responsible for quorum sensing are constitutively expressed across boundaries of genus with a surprising degree of structural homology. As such, these molecules present themselves as an attractive target for the inhibition of biofilm formation.



1 **2**

Figure 1. Wild type Acyl-homoserine lactones found in *Pseudomonas aeruginosa*

Several naturally occurring compounds have been discovered that instill a certain amount of bacterial immunity in their host organisms by interrupting the molecular mechanism for quorum sensing.[4,5] Application of these isolated compounds to *P. aeruginosa* biofilms increased the bacterial susceptibility to antimicrobial treatment. It has been shown that if one is able to shutdown or inhibit the quorum sensing system, the resulting biofilm bacteria are of a much "softer" variety and are much more readily removed by conventional antimicrobial treatments, be they a host immune response, cleaning with soap, or killing with bleach.[6,7]

Pseudomonas aeruginosa is perhaps the most highly understood of all the biofilm forming bacteria in terms of how ASL molecules regulate the quorum sensing circuitry and for this reason will be the focus of our investigations. ASL molecules **1** and **2** have been shown to be responsible for modulating biofilm through the binding of the *las* and *rhl* receptors, respectively.[8] Once the concentration of these molecules is sufficiently high (as a result of higher bacteria concentration), invading bacteria begin expressing specific virulence factors, surfactants and polysaccharides not associated with its planktonic state.[1]

The aim of this project is the rational design of compounds that will use the homology of the wild type molecules to exploit the quorum sensing system of *Pseudomonas aeruginosa.* At this point in time the project has taken two distinct directions:

1. The preparation of molecular mimics that will act as quorum sensing *inhibitors*, which will attenuate both the virulence and the resilience of the biofilm. It is hoped that effective inhibitors will yield a "softer" biofilm that

is more susceptible to standard treatments like antibiotics or a host immune response.

2. The linkage of known biocides structures to an *N*-acyl-homoserine lactone motif. It is hoped that these novel biocides will be more effective than traditional biocides in that they will incorporate two modes of reactivity into a single conjugate molecule.

Pseudomonas aeruginosa and Quorum Sensing

Pseudomonas aeruginosa is most often described as an opportunistic pathogen. It is responsible for lethal infections in a variety of settings, including Cystic Fibrosis and AIDS. Its existence as a biofilm is noteworthy because once infections have formed biofilms, the organism becomes 10 – 1000 times more resistant to antibiotic treatments.[6,7]

The biofilm phenotype of *Pseudomonas aeruginosa* is regulated by two distinct quorum sensing circuits called Las and Rhl. ASL's **1** and **2** are the respective cognate ligands for these circuits. Planktonic bacteria produce ASL's using a protein synthase I (i.e. LasI). When bacterial cell populations reach a critical density, concentrations of **1** will be sufficiently high to activate LasR, which will activate the transcription of virulence factors and additional biofilm control elements. Although the interaction of Las and Rhl is a complicated control system that has yet to be fully elucidated, it is known that these two systems are responsible not only for sensing population levels, but also for biofilm cell differentiation and response to environmental factors.[9]

Inhibitor Design

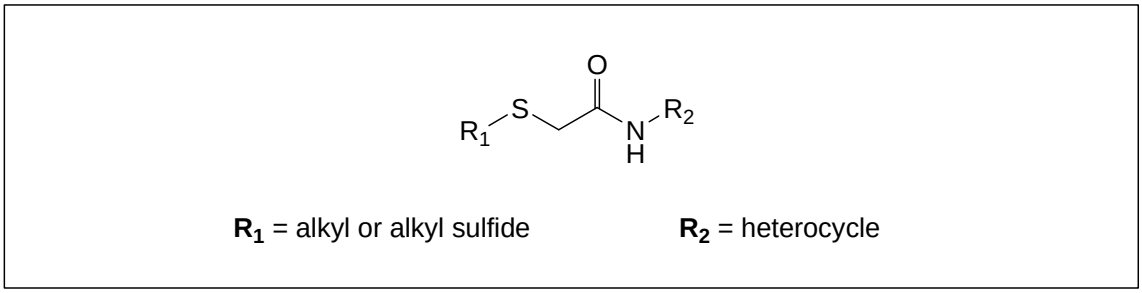


Figure 2. Basic autoinducer design motif

The basic autoinducer design will be based on a heterocyclic core with a sulfide containing acyl side chain (Figure 2). The incorporation of sulfur at the 3 position is based on previous work (unpublished results, T. S. Livinghouse) in which a sulfur in various oxidation states was incorporated into the acyl chain. It was thought that a sulfone or sulfoxide may produce an effective biomimetic of the 3-oxo functionality. However, experimental results have indicated that a sulfide was the most effective in suppressing biofilm activity. This is noteworthy because it completely eliminates oxygen from the 3 position and with it any possible hydrogen bonding capacity.

Additional work[10] has indicated that the sulfide at the 3 position is the most important structural feature of the inhibitor structure. In addition, a saturated carbon at this position failed to produce an effective antagonist (data not shown), indicating that both the bond angle and bond length at this position are crucial in ligand-receptor binding. Accordingly, it may be the hydrogen bond acceptor capacity of the ketone in the wild type autoinducer that activates the conformational change in the receptor protein, LasR. The absence of this hydrogen bonding interaction may allow strong ligand-receptor bonding to occur without activating the transcription factor.

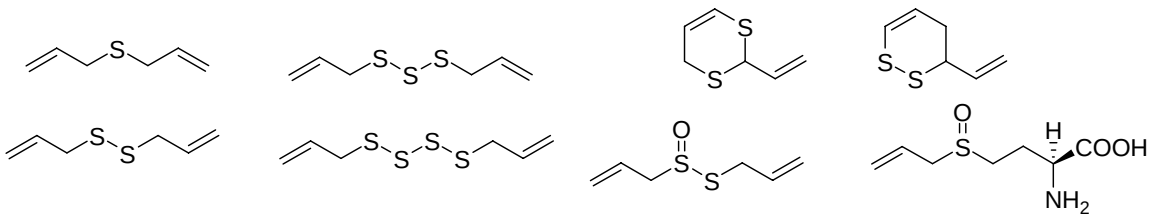
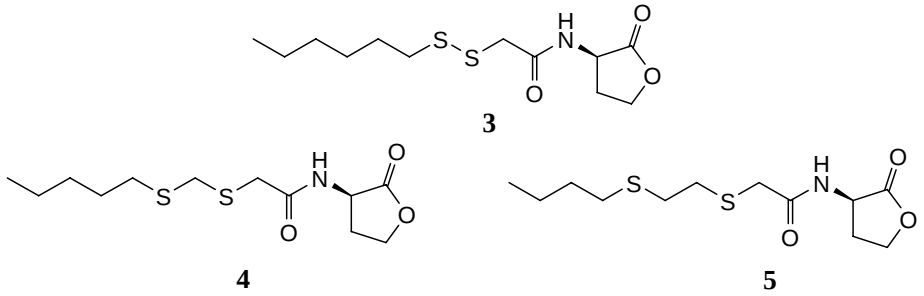


Figure 3. Various garlic components with antibacterial or QSI activity

Another rationale for the incorporation of the sulfide into the inhibitor is the similarity it has with compounds extracted from garlic. Garlic extracts have been shown to have anti-quorum sensing and antimicrobial qualities.[7,11-13] These extracts contain multiple sulfides and polysulfides incorporated into unbranched aliphatic and cyclic hydrocarbons (Figure 3). For this reason the following compounds (Figure 4) were proposed in order to investigate the utility of having multiple sulfurs in the acyl side chain.



3 **4** **5**

Figure 4. Inhibitors incorporating multiple sulfides into the acyl side chain

While considerable work has been done to change the character of R_1,[10,14-17] relatively little has been done to change the nature of the heterocycle. Previous work in which a cyclohexane[10] and phenol[14] substituent replaced the lactone seem to indicate that the presence of an α-amino gamma butyrolactone is not a strict prerequisite for inhibitor binding. Figure 5 indicates 3 novel heterocycles that should prove useful in probing the

structural characteristics that are important in Quorum Sensing Inhibitor (QSI) activity. Chiral components **6**[18,19] and **7**[20] are both available from *L*-glutamine, and achiral **8** is available in one step from 2-hydrazinoethanol and diethyl carbonate.[21,22]

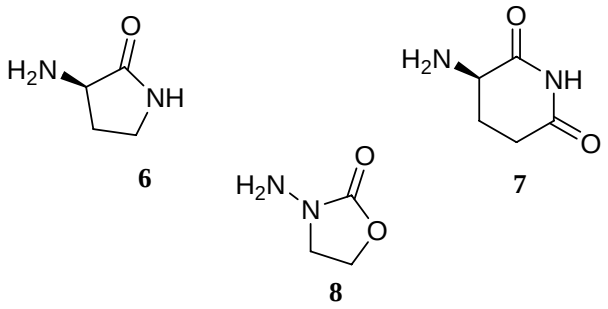


Figure 5. Proposed heterocyclic nuclei for novel autoinducer compounds

Biocide Design

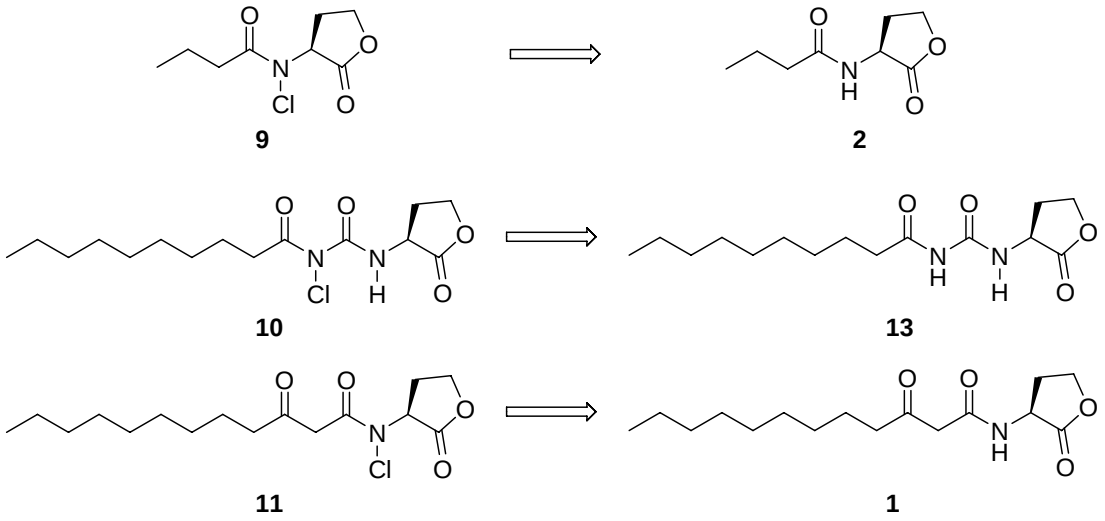
The most recent tangent this project has taken is the design of a novel class of biocides. The rationale for this is that has been a lot of work in academia and industry developing effective quorum sensing inhibitors (QSI's) based on the *N*-acyl-*L*-homoserine lactone structural motif. According to a leader in the quorum sensing field, homoserine lactone type QSI's have not shown the level of activity necessary to produce an effective pharmaceutical. While the rational design of QSI's based on structure activity relationships may be a laudable academic exercise and worth continuing, biocide conjugates could represent an interesting and novel class of antimicrobial agents.

This new class of compounds would still utilize the *N*-acyl-*L*-homoserine lactone structural motif, but instead of simply blocking enzymatic action, they should actively kill bacteria by disrupting cell walls/membranes, destroying cellular machinery, or acting

as a mutagen toward bacterial DNA. It is hoped that the *N*-acyl-*L*-homoserine lactone structure will enhance either the specificity or the potency of the biocide, thereby providing a more effective killing mechanism.

In situ Hypochlorite Generation

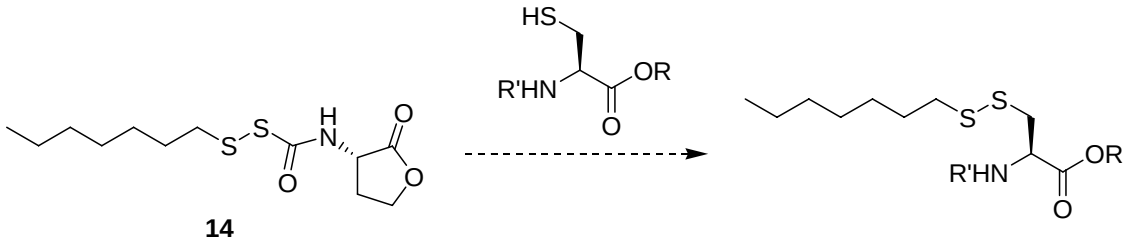
The most familiar biocide to the general public is Clorox, or sodium hypochlorite. While its mechanism of killing is not precisely known, it is thought that it destroys bacterial proteins by oxidizing them. Specifically, the thiol functionalities of cysteine residues are particularly susceptible to irreversible oxidation which could conceivably destroy the structure or function of a protein, especially if the cysteine is directly involved in catalyzing an enzymatic process. Improvements on this theme have been organic molecules that produce hypochlorite *in situ.* This is typically done by chlorination of non-amine nitrogens (i.e. imides, amides and hydantoins). These compounds are often less of an irritant than dilute bleach solutions. It is thought that the action of these compounds is to oxidize thiols thereby killing important protein function. The wild type autoinducer structure could be used to carry the chlorine to the Quorum sensing proteins and then oxidizing them, rendering them nonfunctional. In order to examine the efficacy of this strategy, *N*-chlorination of the butanoyl homoserine lactone **2** will first be carried out using *t*-butyl hypochlorite. Other compounds bearing a hypochlorite-generating moiety are readily available from the corresponding imide and β-ketoamide derivatives (Scheme 1).



Scheme 1. Chlorinated homoserine lactones

Alkyldithiocarbonyls as Potential Sulfenylating Agents

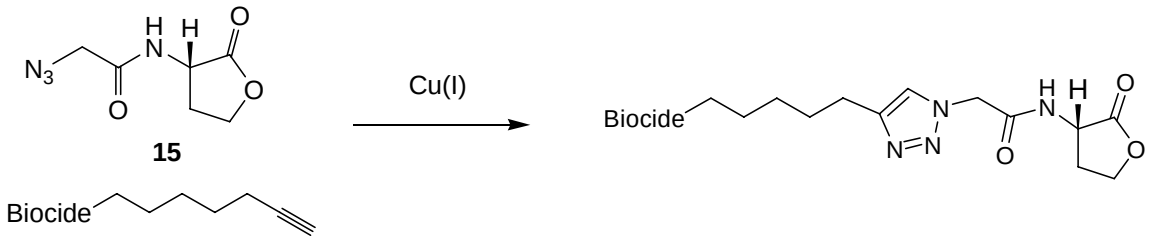
Another strategy currently under consideration would also target protein thiol functionalities. The alkyldithiocarbonyl structure in **14** (Scheme 2) would not be a large structural departure from the current body of QSI's. It may also have the added benefit of being a powerful sulfenylating agent due to its ability to react with thiols to produce disulfides.[23,24]



Scheme 2: Cysteine sulfenylation of alkyldithio carbonyl biocide

Triazole Biocide Conjugates

The most exciting new strategy would employ a covalent triazole linker to attach a known biocide or antimicrobial to the *N*-acyl-*L*-homoserine lactone. A triazole linking an *N*-acyl-*L*-homoserine lactone to a known biocide (Scheme 3) would be easy to assemble from the azido functionalized acyl homoserine lactone and terminal alkyne. This "click" chemistry is a very powerful tool due to its alleged lack of side products, essentially quantitative yields and the stability of the ensuing triazole. Preparation of this azido functionalized acyl homoserine lactone **15** has been completed on the multi gram scale.



Scheme 3: Triazole biocide linker

Although there have been a variety of heterocyclic acyl sidechains prepared as potential QSI's, there have been no reports of a triazole. Therefore, it is important to evaluate the quorum sensing activity of the triazole analogue **16** that is not conjugated to a biocide.

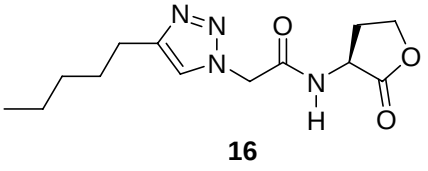


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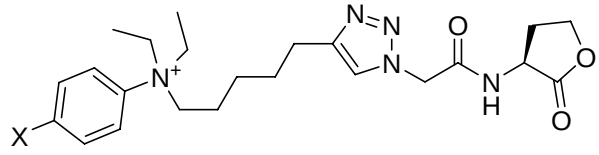
Figure 6. Control triazole which will be used to evaluate the antibacterial and quorum sensing properties of triazole conjugates

In order to demonstrate the utility of this "dual warhead" strategy in the design of *N*-acyl-*L*-homoserine lactone biocides, proof of concept needs to be established. The first compounds to be synthesized will come from 3 different classes: quaternary ammonium salts, mutagens, and aminoglycoside antibiotics. (Figure 7) Theses target molecules will be tested against commercially available molecules of the same class to see if our approach of linking a biocide to *N*-acyl-*L*-homoserine lactone structure gives any enhancement to killing. Once the target molecules are in hand, screening them should be relatively straightforward using a standard microbiology tool called the disc diffusion test. This test consists simply growing a lawn of bacteria on which will be placed a cellulose disk treated with the compound of interest. After overnight incubation, a zone of inhibition can be simply measured with a ruler. Effective biocides will be evaluated on the basis of their ability to create a large zone of inhibition, or killing, at small concentrations.



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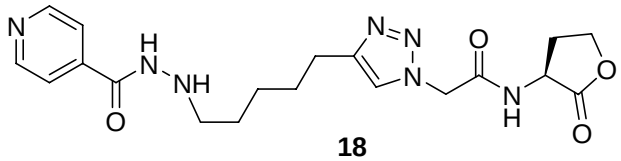

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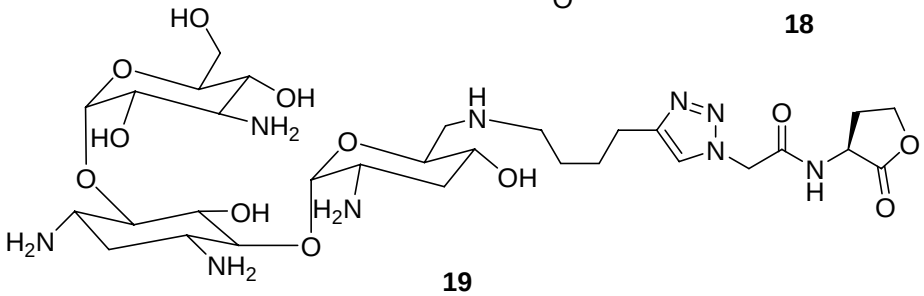

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Figure 7. Quaternary ammonium, mutagen, and antibiotic target biocides

In order to show proof of concept, we must show that these conjugate biocides are more effective than the unlinked biocides by themselves. At this point this effect need only be demonstrated to be statistically significant, unlike the Quorum Sensing Inhibitors (QSI's) which need to show an effect of two orders of magnitude or more.

Summary of Results

Several new synthetic derivatives of the *N*-acylhomoserine lactone autoinducer have been synthesized and demonstrated to be inhibitors of LasR mediated quorum sensing in *Pseudomonas aeruginosa.* In addition, these inhibitors have also been shown enhance the susceptibility of *Pseudomonas aeruginosa* biofilms to the aminoglycoside antibiotic Tobramycin. This enhanced susceptibility was quantitatively determined using a novel modification to the colony biofilm assay.

Three novel biocides which incorporate the bifunctionality of a quorum sensing inhibitor and an antimicrobial moiety in the same molecule have also been synthesized. The antimicrobial moieties utilized were sulfenylating, hypochlorite generating, and mutagenic. These biocides were screened for antimicrobial activity using a disc diffusion zone of inhibition test.

Organization

First, the synthesis and characterization of quorum sensing inhibitors based on the structure of the *N*-acylhomoserine lactone autoinducers (**1, 2**) will be presented.

Secondly, the biological evaluation of these compounds using a flow cytometry and colony biofilm assay will be reported.

The second area of research is the development of new bifunctional biocides. The synthesis and characterization of these compounds will be presented along with zone of inhibition data.

CHAPTER TWO

SYNTHESIS OF INHIBITORS

Synthesis

The following compounds were prepared by members of the Livinghouse group. Synthetic targets prepared by Caol Huff will be discussed in the upcoming paragraphs and schemes. The remaining compounds were synthesized by others in the Livinghouse laboratories. (Figure **8**)

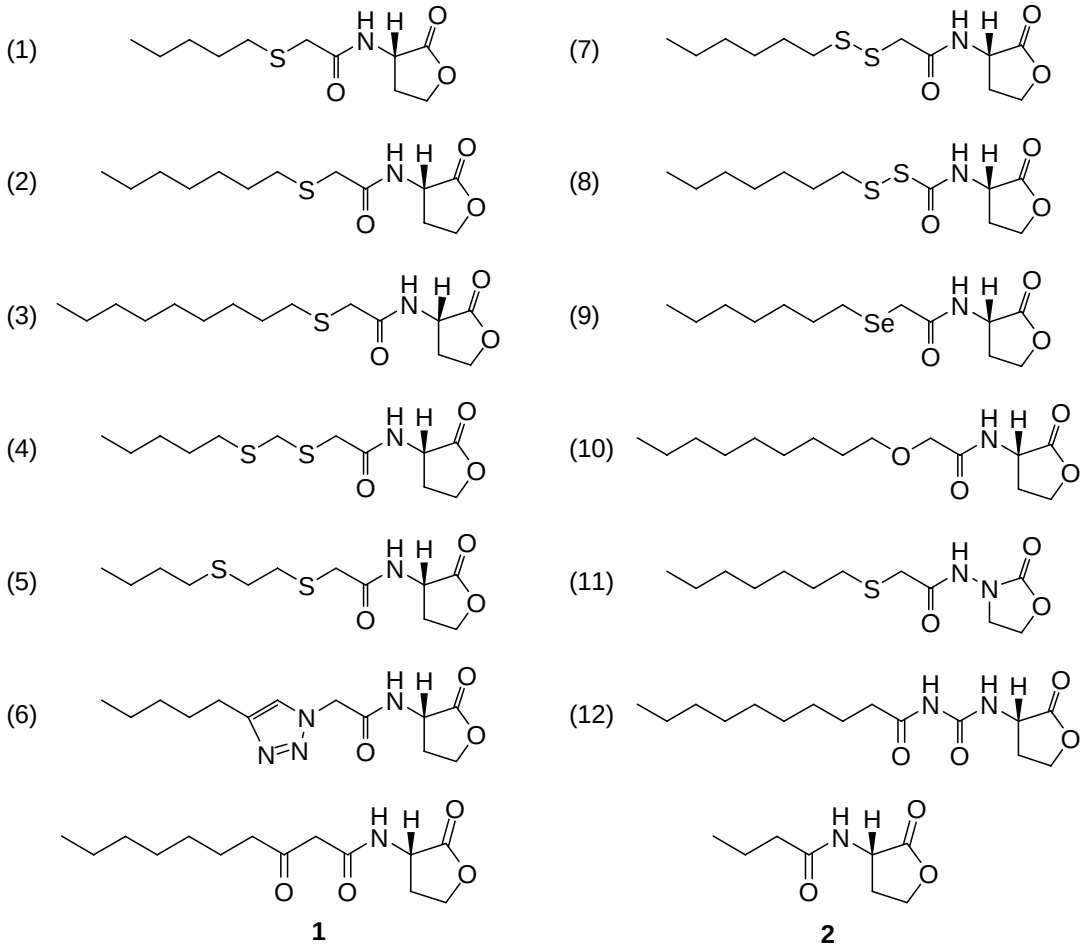


Figure 8. Completed synthetic targets including inhibitors and wild type autoinducers

The selenide (Figure 8, entry 9) was prepared by treatment of the diheptyldiselenide[25] with sodium borohydride to give the sodium heptylselenol [26] which was used as a nucleophile with chloroacetic acid to yield the desired selenide acid side chain.

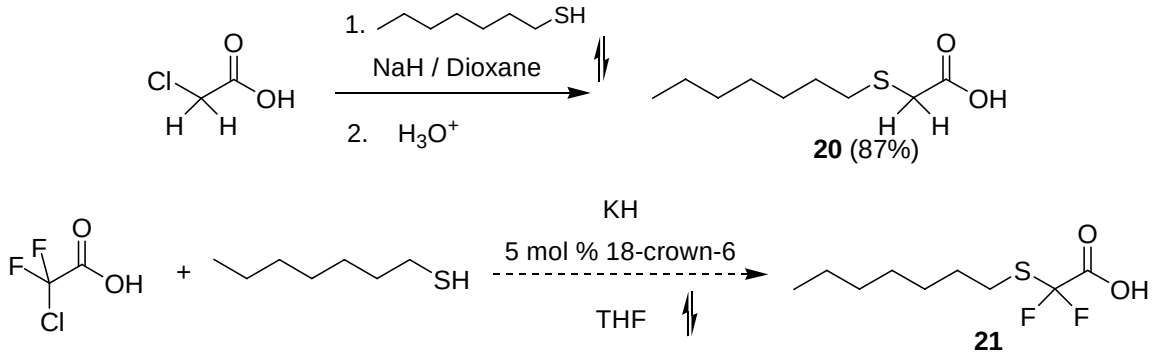
The disulfide acid (Figure 9, entry 7**)** side chain was prepared from the following sequence of reactions: the reaction of sodium sulfide with mesyl chloride yielded sodium methanethiosulfate[27] which was reacted with 1-bromohexane to yield hexyl methanethiosulfate.[28] This intermediate was condensed with mercaptoacetic acid using the same conditions as given in Scheme 9.

Synthesis of Side Chains

The first step in the synthesis of the autoinducer derivatives was the construction of the acyl side chains. The corresponding carboxylic acids would then be coupled with the amine bearing heterocycle of choice to give the desired inhibitor.

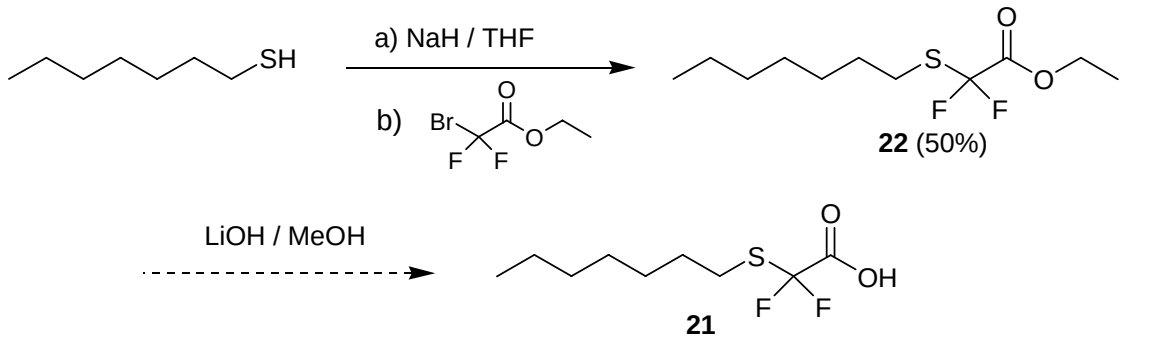
While the synthesis of acid **20** was straightforward, *gem* – difluoro acid **21** was much more problematic (Scheme 4). Using the procedure set forth in Boyle *et al*,[29] acid **20** was isolated in 80% yield. The same procedure was applied to the synthesis of **21**, but similar success did not follow. Many other conditions were attempted, including the use of potassium hydride and crown ethers. Yields came in the 10-20% range, likely owing to the electron withdrawing nature of the *gem*-difluoro carbon, which shortens the carbon-chlorine bond, making the electrophilic carbon sterically less accessible. This electronic effect also gives the chloride leaving group a stronger covalent bond (increasing the

activation energy, making it more reluctant to leave). Crude samples of **21** also quickly decomposed at room temperature making isolation of a pure compound not possible.



Scheme 4. Attempted synthesis of acyl side chains

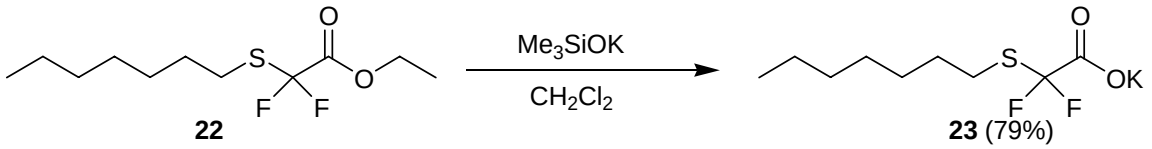
An alternative route to the difluoro acid was taken from ethyl bromodifluoro acetate. (Scheme 5) The sulfide ester was recovered without incident; however, the subsequent saponification encountered obstacles as in the previous route.



Scheme 5. Alternative route to difluoro acid

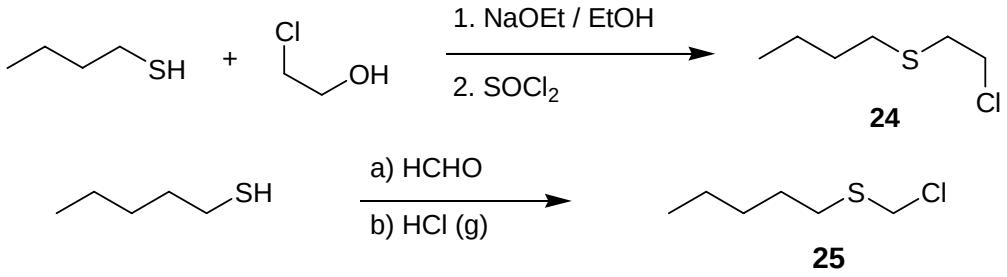
Isolation of this intermediate was not possible because, as was noted previously, the acid readily decomposed at room temperature. In contrast, Boyle *et al* chose to carry the crude intermediate forward to the acid chloride instead of isolating the acid.

In order to avoid the isolation of the free acid, the ester was converted directly to the potassium salt using potassium trimethylsilanoate. (Scheme 6)[30]



Scheme 6. Preparation of Potassium Carboxylate with Potassium Silanoate

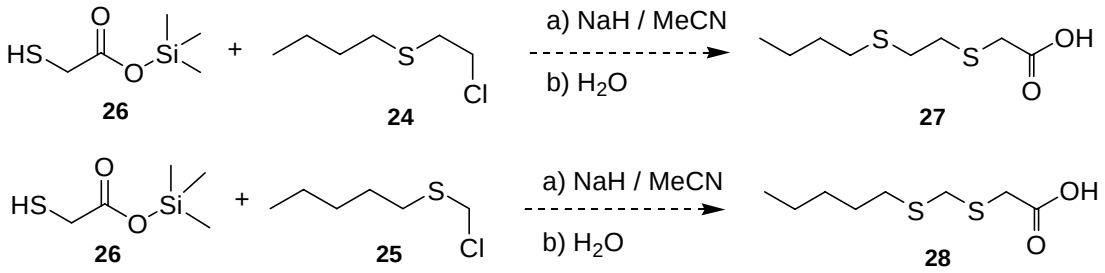
Preparation of the bissulfide side chains was accomplished via condensation of mercaptoacetic acid with the appropriate chloroalkyl sulfide. Chloroalkyl sulfides were prepared from alkylthiols as follows (Scheme 7).[31,32]



Scheme 7. Synthesis of chloroalkyl sulfides

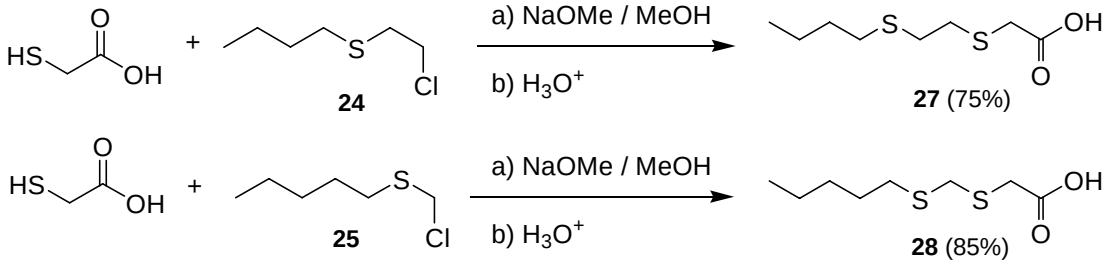
β-chloro sulfides and α–chloro sulfides were prepared in 71% and 89% from their respective alkylthiols.

The condensation with mercaptoacetic acid was first attempted using its silyl ester derivative **26** in order to protect the carboxylic acid. We reasoned that this transformation (Scheme 8) would be facilitated by the neighboring group participation of the sulfur lone pairs with the incipient carbenium center. However, in both cases, after thin layer chromatography indicated the consumption of the sulfide, no substantial amount of product had been formed.



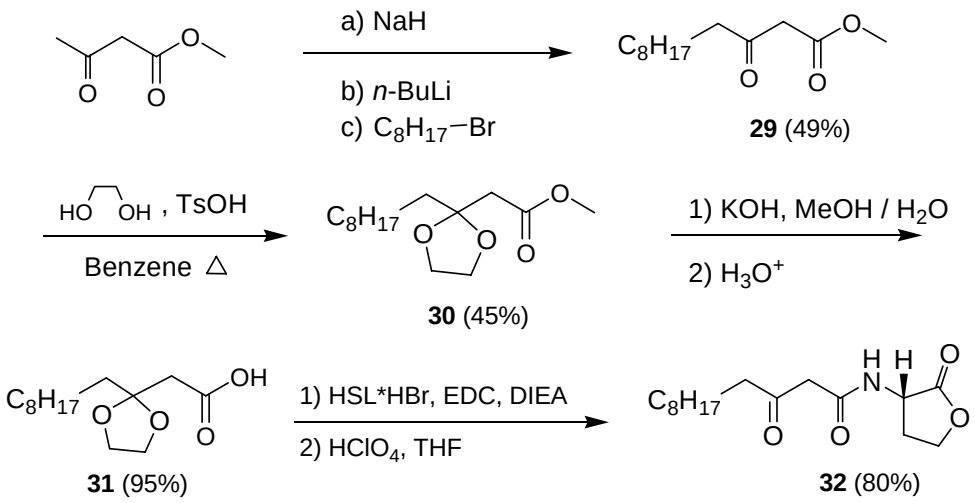
Scheme 8. Attempted preparation of bissulfides from protected mercaptoacetic acid

Interestingly, this transformation was accomplished in good yield using the unprotected acid in sodium methoxide (Scheme 9).



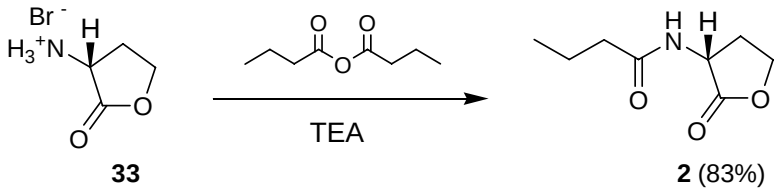
Scheme 9. Successful preparation of bissulfide acids

The acid **31** corresponding to the ketal protected side chain of the wild type C(12)-3-oxo-*N*-acyl homoserine lactone[15,33] was prepared via the dianion of methyl acetoacetate (Scheme 10) according to literature methods[34,35]. It was necessary to protect the β-ketoester as the ketal in order to ensure a smooth coupling with the homoserine lactone. The ethylene glycol acetal was easily removed after the peptide coupling step with aqueous perchloric acid in tetrahydrofuran.



Scheme 10. Preparation of ketal protected C(12) wild type sidechain

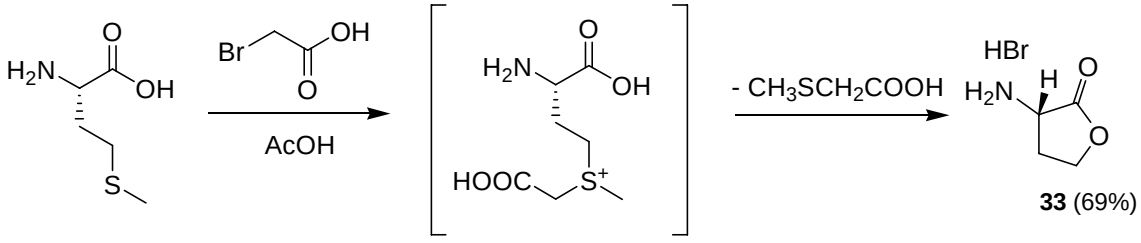
The C(4) wild type autoinducer **2** which corresponds to the RhlR ligand was prepared in 92% yield by treating HSL **33** with butyric anhydride and 2 equivalents of triethylamine.[36]



Scheme 11. Synthesis of *N*-butanoyl Homoserinelactone

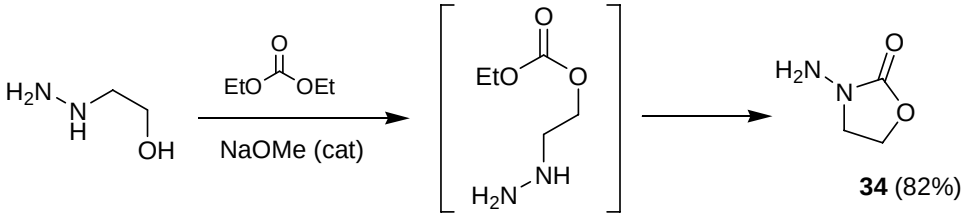
Synthesis of Heterocycles

Starting with *L*-methionine and using the approach of Persson,[10] S_N2 attack on bromoacetic acid will yield the sulfonium ion shown, which activates the β carbon of the amino acid side chain to nucleophilic attack by the carboxyl[10] which forms the resulting amino lactone (Scheme 12). There was little problem with this transformation and **33** was isolated in 69% yield.



Scheme 12. Construction of Homoserine Lactone from *L*-methionine

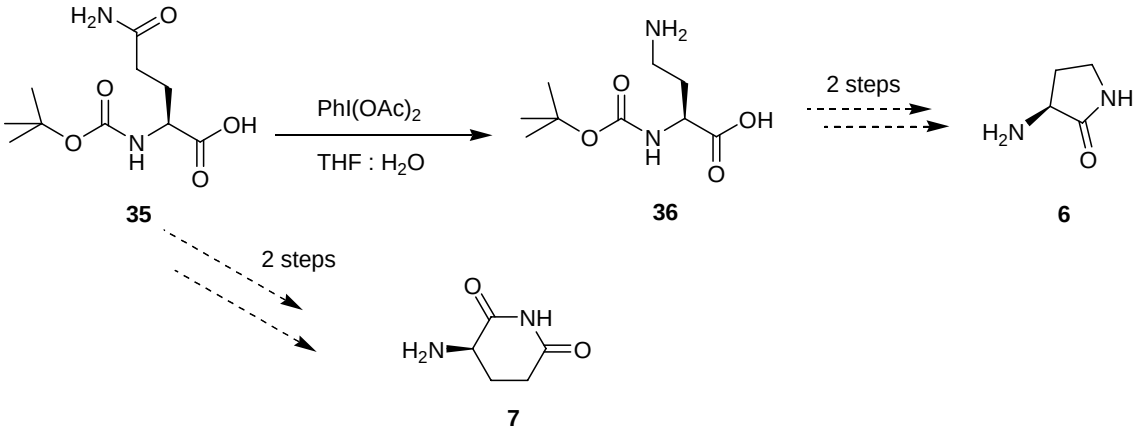
3-amino-oxazolidone was prepared by treatment of 2-hydrazinoethanol with diethyl carbonate and a catalytic amount of sodium methoxide.[21,22] Upon heating (Scheme 13) the primary alcohol is deprotonated and performs a nucleophilic attack on the carbonate center. This releases an ethoxide anion which can continue the catalytic cycle by deprotonating another alcohol. The oxazolidone ring closing is completed by attack by the more nucleophilic secondary nitrogen on the carbonate center, which releases another ethoxide ion. This reaction is driven to completion because the five membered ring is a more entropically favored thermodynamic well.



Scheme 13. Preparation of 3-amino-oxazolidone from hydrazinoethanol

Some work was also made toward the preparation of the lactam and succinate heterocycles from the chiral amino acid *L*-glutamine.(Scheme 14) Boc protection of the primary amine was accomplished using di-*tert*-butyldicarbonate using a variation of a published procedure[37] which furnished carbamate **35** in 50% yield. This cumbersome

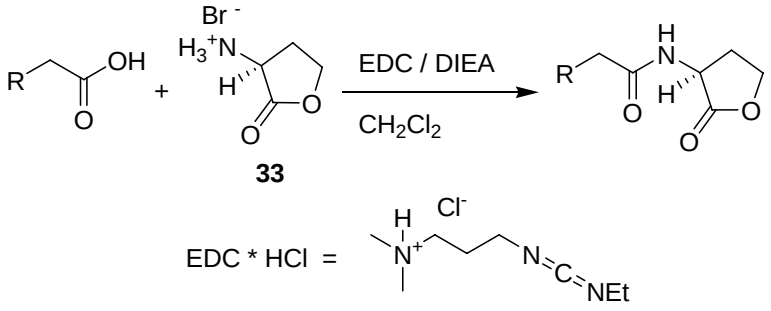
hygroscopic foam was subjected to modified Hoffman rearrangement conditions[18] which proceeded smoothly to the primary amine. Intermediates **35** and **36** require only a cyclization and deprotection to give the desired lactam **6**[18,19] and piperdinedione **7**[20]**,** respectively.



Scheme 14. Preparation of amino γ-butryolactone and 3-amino-piperdinedione

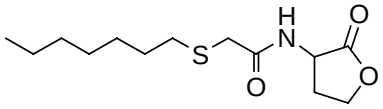
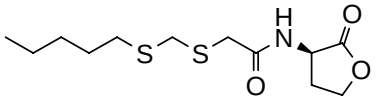
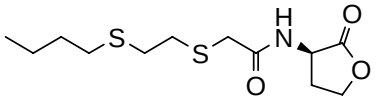
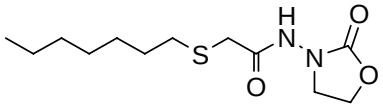
Amide Coupling

The peptide bond in the inhibitors was prepared using the coupling agent 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC).[38,39] This reagent is much more convenient to use than Dicyclohexylcarboimide (DCC) because its water solubility lends itself to easy removal in the work up (Scheme 15). In my hands the coupling gave modest to good yields (Table 1) and most of the time did not require any chromatography other than filtration through a plug of fluorosil to give the pure inhibitor.



Scheme 15. Generalized coupling reaction

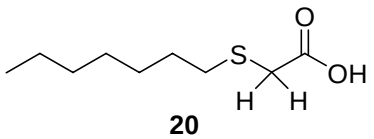
Table 1: Completed inhibitors and yields of amide coupling

Entry	Structure	Coupling Yield
1		79
2		60
3		72
4		68

Experimental Procedures

General. All ^{1}H NMR spectra were measured at 500 MHz on a Bruker Avance 500 Digital NMR. Chemical shifts are reported in δ units to 0.01 ppm with coupling constants reported in Hertz to 0.1 Hz. Residual chloroform (δ = 7.27 ppm) was used as an internal reference for all spectra recorded in $CDCl_3$. Residual DMSO (δ = 2.46 ppm) was used as an internal reference for all spectra recorded in DMSO(d_6). All ^{13}C NMR spectra were

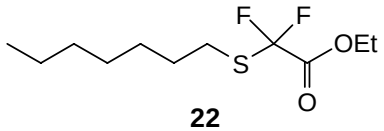
recorded at 500 MHz on a Bruker Avance 500 Digital NMR. Residual chloroform (δ = 77.23 ppm) was used as an internal reference for all spectra recorded in $CDCl_3$. Residual DMSO (δ = 40.51 ppm) was used as an internal reference for all spectra recorded in DMSO(d_6). Infrared spectra were recorded on a ThermoNicolet IR200 Spectrometer. Melting Points were measured using a Mel-TempII melting point apparatus and are uncorrected. Analytical Gas Chromatography was performed on a Varian Model 3700 Gas Chromatograph equipped with an Alltech Econo-Cap EC-5 column.



20

Heptylthioethanoic acid (20): The title compound was prepared according to a variation of the literature procedure.[29] Sodium hydride (400 mg, 10.1 mmol, 60% dispersion in oil) was washed by stirring with hexanes and removing the hexanes via cannula. This was repeated three times and the remaining hexanes were removed *in vacuo*. The sodium hydride was then placed under an atmosphere of argon and cooled to 0 ^{o}C. Freshly distilled dioxane (8 mL) was then added to the reaction flask followed by chloroacetic acid (0.60 g, 4.6 mmol) over 10 min. After the evolution of hydrogen gas had ceased, heptanethiol (0.64 g, 4.8 mmol) was added over 15 min. The reaction was allowed to return to room temperature and was then heated to reflux for 4 h. The solvent was then removed *in vacuo* and the residue was dissolved in H_2O. This mixture was extracted three times with pentane and the remaining water layer was acidified using concentrated HCl to pH 1. The acidified aqueous layer was extracted three times with diethyl ether. The

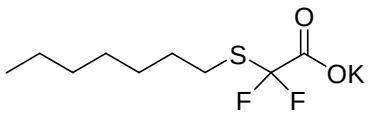
combined ether extracts were washed with brine and dried over magnesium sulfate. After the solvent was removed, 0.73 g (87%) of a white crystalline solid remained: m.p. 35-40 ºC; IR 2800 – 3300, 2922, 2860, 1710, 1457, 1293, 1143 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 3.23 (s, 2H), 2.63 (t, 2H, *J* = 7.0), 1.58 (quintet, 2H, *J* = 7.5), 1.35 (quintet, 2H, *J* = 8.0), 1.25-1.28 (m, 6H), 0.85 (t, 3H, *J* = 7.0); ^{13}C NMR (500 MHz, $CDCl_3$) δ 176.82, 67.31, 33.72, 33.10, 31.91, 29.15, 29.05, 28.92, 22.81, 14.27.



22

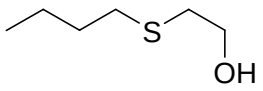
(2,2) *gem*-Difluoro-heptylsulfanylacetic acid ethyl ester (22): The title compound was prepared according to a variation of the literature procedure.[29] Sodium hydride (370 mg, 9.3 mmol, 60% dispersion in oil) was washed by stirring with hexanes and removing the hexanes via cannula. This was repeated three times and the remaining hexanes were removed by *in vacuo*. The sodium hydride was then placed under argon and cooled to 0 ^oC. Freshly distilled DMF (10mL) was then added to the reaction vessel. Heptanethiol was then added dropwise over 5 min. After the evolution of H_2 ceased, ethyl bromodifluoroacetate was added dropwise over 15 min and the reaction was allowed to come to room temperature. The reaction was allowed to stir for 4 h and then quenched with water. The mixture was extracted 3 times with ether. The combined ether extracts were washed with saturated sodium bicarbonate and brine and dried over magnesium sulfate. After the solvent was removed, 1.3 g of a golden oil remained yielding 50%. IR: 2929, 2857, 1769, 1298, 1101 cm^{-1}; ^1H NMR(500 MHz, $CDCl_3$) δ 4.368 (q, 2H, *J* = 7),

2.878 (t, 2H, J = 7.5), 1.678 (quint, 2H, J = 7.5), 1.378 (t, 3H, J = 7), 1.2-1.4 (m, 8H), 0.888 (t, 3H, J = 7.5); ^{13}C NMR (500 MHz, $CDCl_3$) δ 162.2, 121, 63.7, 31.8, 29.8, 28.98, 28.88, 28.84, 22.77, 14.23, 14.10.



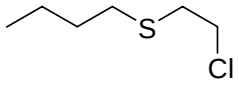
23

(2,2) *gem*-Difluoro-heptylsulfanyl acetic acid potassium salt (23): The title compound was prepared according to a variation of the literature procedure.[30] Ethyl ester **(22)** (200 mg, 0.79 mmol) was added in one portion to a stirring slurry of potassium trimethylsilanoate (100 mg, 0.8 mmol) in 5ml CH_2Cl_2. The reaction mixture was stirred for 4 h at which point the resulting solid was filtered and washed with cold ether yielding 166 mg (79%) of the white salt: mp 220-230 ºC; IR: 2922, 2852, 1640, 1015 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ 2.712 (t, 2H, J = 7.5), 1.518 (quint, 2H, J = 7.5), 1.1-1.3 (m, 8H), 0.717 (t, 3H, J = 7.25); ^{13}C NMR (500 MHz, D_2O) δ 123.4, 30.94, 29.17, 28.39, 27.906, 27.826, 21.94, 13.37.



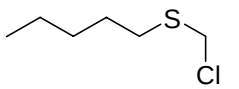
(2-Hydroxyethyl)butyl sulfide: The title compound was prepared according to a variation of the literature procedure.[32] Sodium metal (180 mg, 8.0 mmol) was added in two portions to 24 mL absolute ethanol at 5 ºC. Butanethiol (720 mg, 8.0 mmol) was added over 5 min. This was followed by addition of 2-chloroethanol (1.2 mL, 8.0 mL) over 15 min at room temperature. The reaction became cloudy as NaCl was formed. The

reaction was quenched after 6 h with water and extracted 3 times with ether, washed with saturated sodium bicarbonate and brine and dried over magnesium sulfate. After the solvent was removed 850 mg (65%) of a golden oil remained. This material was used without purification for the next step. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.717 (t, 2H, J = 6), 2.729 (t, 2H, J = 6), 2.525 (t, 2H, J = 7.35), 2.246 (s, 1H), 1.577 (quint, 2H, J = 7.5), 1.406 (sextet, 2H, J = 7.2), 0.92 (t, 3H, J = 7.2); ^{13}C NMR (300 MHz, $CDCl_3$) δ 60.4, 35.5, 32, 31.5, 22.1, 13.8.



24

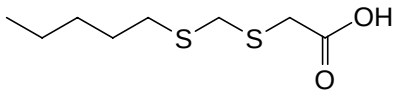
(2-Chloroethyl)butyl sulfide (24): The title compound was prepared according to a variation of the literature procedure.[32] Thionyl chloride (4.3 mL, 43.3 mmol) was added slowly to 20 mL CH_2Cl_2 at -5ºC followed by (2-hydroxyethyl)propyl sulfide (4.47 g, 33.3 mmol). The reaction mixture was stirred at 5 ºC for 3 h and at room temperature overnight. Solvent and thionyl chloride were then removed *in vacuo* and product was purified by fractional distillation (35 ºC @ .05 mm Hg). 3.6 g (70.9%) of sulfide was obtained as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.758 (s, 2H), 2.758 (t, 2H, J = 7.35), 1.672 (quint, 2H, J = 7.2), 1.399 (m, 4H), 0.922 (t, 3H, J = 7.2).



25

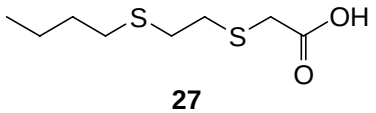
(Chloromethyl)pentyl sulfide (25): The title compound was prepared according to a variation of the literature procedure.[31] A slurry of pentanethiol and paraformaldehyde was

cooled to below 0 ºC with an ice-salt bath. A slow stream of gaseous HCl was then bubbled through the reaction mixture until the formaldehyde had dissolved. Excess $CaCl_2$ was then and the reaction mixture was stirred at 0 ºC for 3 h. Argon was then bubbled through the mixture for 20 min to remove any excess HCl. The product was isolated by distillation (23 ºC @ 0.07mm Hg) to yield 2.73 g (89%) of a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.748 (s, 2H), 2.748 (t, 2H, *J* = 7.35), 1.662 (quint, 2H, *J* = 7.2), 1.391 (m, 4H), 0.912 (t, 3H, *J* = 7.5); ^{13}C NMR (300 MHz, $CDCl_3$), b.p. 23 ºC/ 0.07mm Hg.



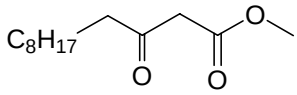
28

(Pentylsulfanyl)methylsulfanylacetic acid (27): Sodium metal (230 mg, 10 mmol) was added to 10 mL of HPLC grade MeOH at 0 ºC. Thioglycolic acid (0.35 mL, 5.0 mmol) was then added to the reaction mixture followed by (chloromethyl) pentyl sulfide **25**. The reaction mixture was then warmed to room temperature and stirred overnight. The solvent was then removed *in vacuo* and the residue was dissolved in water and extracted with ether. The aqueous layer was then acidified to pH 2 and extracted 2 times with ether. The combined organic layers were combined and dried with $MgSO_4$ and placed under high vacuum for several hours yielding 850 mg (85%) of a colorless oil. IR: 3093, 2926, 2869, 2672, 2566, 1708, 1421, 1296, 1199 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.82 (s, 2H), 3.46 (s, 2H), 2.63 (t, 2H, *J* = 6.5), 1.6 (q, 2H, *J* = 7.5), 1.35 (m, 4H), 0.0911 (t, 3H, *J* = 7); ^{13}C NMR (500 MHz, $CDCl_3$) δ 176.8, 35.9, 31.3, 30.98, 30.8, 28.6, 22.2, 13.96.



27

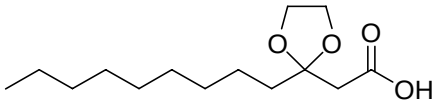
(Propylsulfanyl)ethylsulfanylacetic acid (27): Sodium metal (230 mg, 10 mmol) was added to 10 mL of HPLC grade MeOH at 0 ºC. Thioglycolic acid (0.35 mL, 5.0 mmol) was added to the reaction mixture followed by (1-chloroethyl) propyl sulfide **24**. The reaction mixture was then warmed to room temperature and stirred overnight. The solvent was then removed in vacuo and the residue was dissolved in water and extracted with ether. The aqueous layer was then acidified to pH 2 and extracted 2 times with ether. The combined organic phases were dried as before yielding 770 mg (74%) of a pale yellow oil. IR: 3106, 2957, 2874, 2666, 2567, 1708, 1424, 1297, 1201 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.8-11.7 (s, 1H), 3.307 (s, 2H), 2.905 (t, 2H, *J* = 8.7), 2.772 (t, 2H, *J* = 8.7), 2.57 (t, 2H, *J* = 7.2), 1.583 (quint, 2H, *J* = 7.5), 1.428 (quint, 2H, *J* = 7.5), 0.925 (t, 3H, *J* = 7); ^{13}C NMR (500 MHz, $CDCl_3$) δ 176.96, 33.64, 32.73, 32.11, 31.94, 31.7, 22.26, 13.87.



29

Methyl 3-oxododecanoate (29): The title compound was prepared according to a variation of the literature procedure.[34] A reaction flask was equipped with a magnetic stirring bar and was charged with sodium hydride – 60% dispersion in oil (1.2 g, 49.9 mmol) under an atmosphere of argon. The sodium hydride was deoiled by washing three times with hexanes. 110 mL of dry tetrahydrofuran was added to the reaction vessel at 0

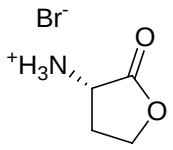
ºC followed by dropwise addition of methyl acetoacetate (4.49 mL, 41.6 mmol). A pale yellow color developed as the ester was deprotonated. The reaction mixture was allowed to stir at 0 ºC for 1 h whereupon *n*-butyl lithium – 2.68 M in hexanes (16.3 mL, 43.7 mmol) was added dropwise over 15 min. A solution of 1-bromooctane (7.9 mL, 45.8 mmol) in 10 mL tetrahydrofuran was added from an addition funnel over 30 min. The orange color of the dianion faded somewhat and the reaction was allowed to stir at 10 ºC overnight. The reaction mixture was quenched with 8 mL of concentrated HCl in 20 mL of water. The resultant mixture was extracted with diethyl ether (3 x 50 mL). The combined ether extracts were washed with water until neutral, and dried over anhydrous magnesium sulfate, filtered and concentrated *in vacuo*. Flash chromatography (10% ethyl acetate in hexanes) yielded 4.65 g (49%) of a pale yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.744 (s, 3H), 3.452 (s, 2H), 2.533 (t, 2H, J = 7.2), 1.595 (quint, 2H, J = 7.2), 1.27 (m, 12H), 0.884 (t, 3H, J = 6.6).



31

2-Ethanoic acid 2-nonyl-1,3-dioxolane (31): The title compound was prepared according to a variation of the literature procedure.[35] β-ketoester **29** was added to a mixture of 50 mL benzene and ethylene glycol (1.25 mL, 22.4 mmol) in a reaction vessel fitted with a Dean Stark trap and reflux condenser. *p*-Toluenesulfonic acid (100 mg, 0.5 mmol) was added in one portion and the mixture was heated to reflux for 24 h. The reaction mixture was then quenched with 400 mL 10% K_2CO_3. The organic layer was

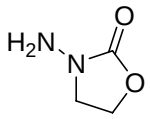
separated and washed twice with 150 mL of 10% K_2CO_3 followed by a 9:1 mix of saturated sodium chloride (aq) and 10% K_2CO_3. The organic layer was then dried with $MgSO_4$, filtered and concentrated *in vacuo*. The crude ester was dissolved in 3.5 mL methanol and was then added to a solution of KOH (672 mg, 12 mmol) in 12 mL anhydrous methanol at 0 ºC over 30 min. The mixture was then allowed to come to room temperature and stir for 24 h. After a few hours the solution lost its yellow/gold color and developed a red-brown color that was maintained throughout the reaction. The solvent was then removed *in vacuo* and the residue was dissolved in 30 mL H_2O. This solution was extracted once with ether and the ether layer was discarded. To the aqueous layer was added with 15 mL EtOAc, 4.5 mL concentrated HCl was added dropwise and the biphasic mixture was stirred for 30 min. The EtOAc layer was removed, dried with brine and Na_2SO_4 and filtered through a pad of silica (EtOAc for elution) to give the desired acid (2.58 g, 45 %). IR: 3100, 2925, 2852, 1713 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 9.8-10 (s, 1H), 4.05 (m, 4H), 2.73 (s, 2H), 1.777 (m, 2H), 1.39 (quint, 2H, J = 7.5), 1.26 (m, 12H), 0.886 (t, 3H, J = 7); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.6, 109.54, 65.31, 42.46, 37.77, 32.08, 29.84, 29.74, 29.71, 29.5, 23.72, 22.89, 14.32.



33

(*L*)-Homoserine lactone hydrobromide (33): The title compound was prepared according to the literature procedure.[10] To a 14.4 mL mixture of water, isopropanol, and

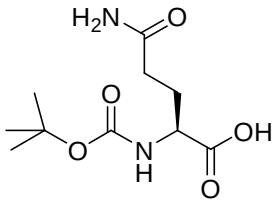
acetic acid (1 **:** 1 **:** 0.6) was added *L*-methionine (1.52 g, 10 mmol) and bromoacetic acid (1.54 g, 11.0 mmol). The mixture was refluxed overnight (12 h) and the solvent was removed first using a rotary evaporator followed by high vacuum overnight. The resulting opaque brown semi-solid was then dissolved in 10 mL of a 4:1 mixture of 2-propanol – HBr in 30% AcOH and stirred for 5-10 min until a homogeneous precipitate had formed. The precipitate was filtered and the filtrate was concentrated *in vacuo*. The residue was then redissolved and refiltered. The combined filtrants were then washed with isopropanol until the orange color was largely removed. After drying, 1.254 g of white solid was recovered: mp = 218-221°C; IR 2400-4000, 1774 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.54 (b s, 3H), 4.42 (t, 1H, J = 9.0), 4.33 (dd, 1H, J = 9.0, J = 11.5), 4.27-4.22 (m, 1H), 2.51 (m, 1H), 2.21 (quintet, 1H, J = 11.5); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.82, 66.66, 48.28, 27.50.



34

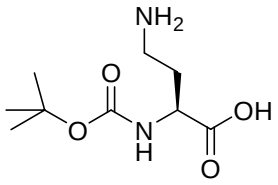
3-Amino-1-oxazolidone (34): The title compound was prepared according to the literature procedure.[21] A mixture of sodium metal (31 mg, 1.3 mmol) in 4.2 mL anhydrous methanol was added to a solution of 2-hydrazinoethanol (760 mg, 10 mmol) and diethyl carbonate (1.42 g, 12 mmol) at room temperature. The reaction mixture was refluxed for 3 h and cooled to room temperature. The solution was evaporated to dryness and filtered through a pad of silica (CH_2Cl_2 elution). The solvent was removed *in vacuo* yielding 835 mg (82%) of pale yellow crystals: mp 72-74 ºC; IR: 3327, 3195, 2986,

2910, 1748, 1033 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.317 (t, 2H, *J* = 8), 3.703 (t, 2H, *J* = 8), 3.4-4.1 (s, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 159.9, 61.4, 48.8.



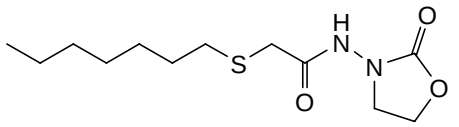
35

***N-tert*-Butoxycarbonyl-(*S*)-glutamine (35):** The title compound was prepared according to a variation of the literature procedure.[37] A 50 mL round bottom flask was charged with *L*-glutamine (1.5 g, 10.26 mmol) followed by 7.5 mL of water. This was followed by addition of 1.5 mL Et_3N and 7.5 mL n-PrOH followed by di-*tert*-butyl dicarbonate (1.8 g, 8.25mmol). After stirring for 24 h, the solution was evaporated to 5 mL, diluted with water (50 mL), washed with ether (20 mL), acidified to pH 1 with 10% HCl, and extracted with 25% i-PrOH in $CHCl_3$ (3 x 30 mL). The combined extracts were dried and evaporated to give 1.10 g (50%) as a white foam. IR: 3407, 3364, 3209, 2983, 1714, 1684, 1666, 1529 cm^{-1}; ^{1}H NMR (500 MHz, DMSO d_6) δ 11.5 (s, 1H), 7.268 (s, 2H), 7.037-7.052 (d, 1H, J = 4), 6.736 (d, 1H, J = 4), 2.128 (t, 2H, J = 7), 1.91 (m, 1H), 1.707 (m, 1H), 1.38 (s, 9H); ^{13}C NMR (500 MHz, DMSO d_6) δ 174.01, 173.7, 155.63, 78.08, 53.19, 31.482, 28.26, 26.58, 25.5.



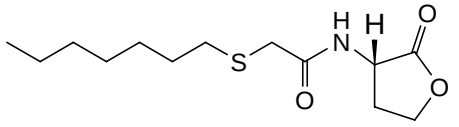
36

***N-tert*-Butoxycarbonyl-(*S*)-2,4-diaminobutanoic acid (36):** The title compound was prepared according to the literature procedure.[18] To a solution of *N-tert*-butoxycarbonylglutamine **35**[37] (295 mg, 1.2 mmol) in 2.88 mL tetrahydrofuran and 0.72 mL H_2O was added phenyliodosodiacetate (463 mg, 1.44 mmol) while stirring at 4 ºC. The reaction mixture was stirred for 6 h in the cold room (~3 ºC) after which the reaction mixture was evaporated to dryness and the crude solid was washed several times with cold chloroform and dried *in vacuo* to afford 166 mg (63%) of the pure acid. mp 209-211 ºC; BP Not measured. IR: 3417, 2922, 1702, 1460, 1373 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 11.5 (s, 1H), 7.268 (s, 2H), 7.037-7.052 (d, 1H, J = 4), 6.736 (d, 1H, J = 4), 2.128 (t, 2H, J = 7), 1.91 (m, 1H), 1.707 (m, 1H), 1.38 (s, 9H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 172.95, 155, 78.07, 54.46, 37.54, 30.85, 28.32.



General Coupling procedure using EDC as a coupling agent: (3-thioether) *N*-heptanoyl-3-aminooxazolidone:[38] A solution of 3-amino-1-oxazolidone (204 mg, 2.0 mmol) in 6 mL CH_2Cl_2 was cooled to 0 ºC and diisopropylethylamine (696 µl, 4.0 mmol) was added dropwise over 3 min. Acid **20**[40] (399 mg, 2.1 mmol) was added in one portion followed by EDC (403 mg, 2.1 mmol). The reaction mixture was stirred at 0 ºC for one h

and then at room temperature overnight. The reaction was then quenched with 6 mL of half saturated brine. The organic layer was removed and washed twice with 3 mL 0.5 M HCl, 3 mL half saturated brine, 3 mL half saturated $NaHCO_3$ and 6 mL brine. The organic layer was dried with Na_2SO_4 and filtered through a plug of fluorosil. The solvent was removed *in vacuo* yielding 395 mg (68%) of pure white crystals: mp 72-74 ºC; IR: 3278, 2969, 1735, 1690, 1465 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 8.527 (s, 1H), 4.453 (t, 2H, *J* = 7.75), 3.853 (t, 2H, *J* = 7.75), 3.298 (s, 2H), 2.663 (t, 2H, *J* = 7.75), 1.617 (quint, 2H, *J* = 7.25), 1.381 (quint, 2H, *J* = 7.5), 1.378 (m, 6H), 0.918 (t, 3H, *J* = 7.5); ^{13}C NMR (500 MHz, $CDCl_3$) δ 168.4, 157.3, 62.1, 46.3, 34.5, 33.2, 31.9, 29.2, 29.03, 28.9, 22.8, 14.27.



***N*-Heptylsulfanylacetyl-*L*-homoserine lactone:** The title compound was prepared according to the above peptide coupling procedure. mp 90-92 ºC; IR: 3305, 2950, 2922, 2855, 1773, 1648, 1549, 1175, 1014 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.37 (d, 1H, *J* = 5), 4.53-4.62 (ddd, 1H, *J* = 11.5, 9.5, 6), 4.468 (, 1H, *J* = 9), 4.23-4.32 (ddd, 1H, *J* = 11.5, 9, 6.5), 3.25 (App. d, 2H, *J* = 1), 3.75-2.84 (ddd, 1H, *J* = 10.5, 7.5, 6), 2.549 (t, 2H, *J* = 7.5), 2.10-2.24 (ddd, 1H, *J* = 23.7, 11.4, 8.7), 1.578 (quint, 2H, *J* = 7.5), 1.16-1.38 (m, 8H), 0.877 (t, 3H, *J* = 7); ^{13}C NMR (300 MHz, $CDCl_3$) δ 175.00, 170.00, 66.10, 49.50, 36.16, 33.40, 31.92, 30.42, 29.36, 29.06, 28.95, 22.82, 14.30.

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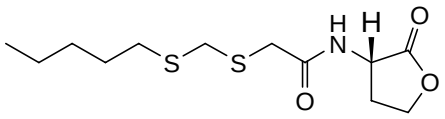
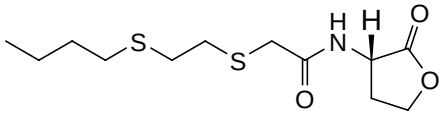
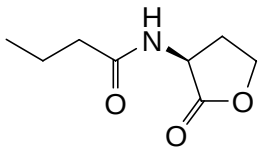


***N*-(Pentylsulfanyl)methylsulfanylacetyl -*L*-homoserine lactone:** The title compound was prepared according to the above peptide coupling procedure. mp 103-105 ºC IR: 3312, 2957, 2922, 1777, 1648, 1554, 1170 cm^{-1}; ^{1}H NMR (500 MHz, CDCl3) δ 7.35 (d, 1H, J = 4.5), 4.584-4.638 (ddd, 1H, J = 11.5, 8.5, 7), 4.487-4.523 (t, 2H, J = 9), 4.283-4.336 (ddd, 1H, J = 11, 9.5, 6), 3.289-3.363 (ABq, 2H, J = 16.5), 2.745-2.845 (m, 5H), 2.554 (t, 2H, J = 7), 1.538-1.582 (App. Quint, 2H), 1.372-1.430 (App. Sextet, 2H), 0.92 (t, 3H, J = 7); ^{13}C NMR (500 MHz, CDCl3) δ 174.99, 169.70, 66.06, 49.42, 35.95, 33.25, 32.05, 31.85, 31.72, 29.99, 22.11, 13.83; Mass: Calculated - 291.1036, Found - 291.0997.



***N*-(Propylsulfanyl)ethylsulfanylacetyl -*L*-homoserine lactone:** The title compound was prepared according to the above peptide coupling procedure. mp 103-105 ºC; IR: 3312, 2957, 2922, 1777, 1648, 1554, 1170 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 (d, 1H, J = 4.5), 4.584-4.638 (ddd, 1H, J = 11.5, 8.5, 7), 4.487-4.523 (t, 2H, J = 9), 4.283-4.336 (ddd, 1H, J = 11, 9.5, 6), 3.289-3.363 (ABq, 2H, J = 16.5), 2.745-2.845 (m, 5H), 2.554 (t, 2H, J = 7), 1.538-1.582 (App. Quint, 2H), 1.372-1.430 (App. Sextet, 2H), 0.92 (t, 3H, J = 7); ^{13}C NMR (500 MHz, $CDCl_3$) δ 174.99, 169.70, 66.06, 49.42, 35.95, 33.25, 32.05, 31.85, 31.72, 29.99, 22.11, 13.83; Mass: Calculated - 291.1036, Found - 291.0992.



2

***N*-Butanoyl homoserinelactone (2):** The title compound was prepared according to a variation of the literature procedure.[36] A suspension of *L*-homoserine lactone (611.6 mg, 3.36 mmol) in 12 mL CH_2Cl_2 was cooled to 0 ºC and diisopropylethylamine (1.81 mL, 10.4 mmol) was added dropwise while vigorous stirring was maintained. The resulting homogeneous solution was stirred for 15 min and butyric anhydride (822 µL, 5.04 mmol) was added dropwise. The reaction was stirred for 2 h and poured into brine (25 mL). The biphasic mixture was stirred at 25 ºC for 15 min and the organic layer was removed and the aqueous layer was treated with excess brine and extracted with EtOAc (2 x 20 mL). The CH_2Cl_2 and EtOAc layers were combined, dried over $MgSO_4$, filtered and concentrated. The residual white solid was redissolved in EtOAc and filtered through a short column of fluorosil. This yielded 865 mg (83%) of the title compound: m.p. 124-127 ºC; IR: 3312, 2955, 1755, 1645, 1546, 1170 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.939 (s, 1H), 4.51 (m, 1H), 4.484 (t, 1H, J = 7.2), 4.301 (m, 1H), 2.892 (m, 1H), 2.247 (t, 2H, J = 7.2), 2.137 (m, 1H), 1.698 (sext, 2H, J = 7.5), 0.987 (t, 3H, J = 7.5); ^{13}C NMR (500 MHz, $CDCl_3$) δ 175.45, 173.54, 66.1, 49.29, 38.037, 30.73, 18.845, 13.64.

CHAPTER 3

QUORUM SENSING INHIBITION AND ANTIBIOTIC SUSCEPTIBILITY ENHANCEMENT OF BIOFILMS

Biological Results

Until now, most quorum sensing inhibitors have been evaluated using a GFP or a β-Galactosidase fluorescence assay. In these systems, a fluorescent reporter is spliced into the *las* genetic circuitry so that when the LasR transcription factor was activated, the cell culture glows.[10,14-17] When an effective inhibitor of LasR binding is present, a decrease in fluorescence is seen. We have developed two **new** assays for evaluating QSI effectiveness, one based on measuring an inhibition of binding to LasR, and another that evaluates an inhibitors ability to enhance a biofilm's susceptibility to killing by antibiotics. The first assay uses a flow cytometer to measure fluorescence of a GFP reporter on a *per cell* basis in contrast to the conventional reporter assays which will measure the *total* fluorescence in a sample cuvette. The second assay grows biofilms under various growth conditions evaluating the biofilm's ability to resist an antimicrobial challenge.

Flow Cytometry

A flow cytometer is able to accomplish more than a conventional fluorimeter because it utilizes an advanced fluidics system that allows individual cells to be passed single file past a set of optics at a high rate of speed. As a result, a flow cytometer can

look at a whole population of bacteria one at a time and use that set of data to perform population analyses not possible by taking a single reading with a fluorimeter.

Flow cytometry using microbial samples is much more challenging than corresponding experiments using eukaryotic cells due to differences in size. Bacteria in general have a volume 1000 times smaller than a eukaryotic cell. At this size, the cells of interest are approaching the size of extraneous particles that may slough off the tubing. As a result, the signal to noise ratio is drastically decreased.

The first experiment undertaken to demonstrate the usefulness of the flow cytometer was to grow 3 separate samples: one in the presence of an effective QSI, One in the presence of the wild type autoinducer, and one control with no additive. As shown below in the overlay graphs in Figure 9, as time passes and bacterial growth passes through the exponential phase, there is a marked change in fluorescence between the samples. As one would expect, the C(7) sulfide QSI was effective in lowering the fluorescence and the wild type autoinducer enhanced the fluorescence. The difference was most pronounced at the end of the exponential phase of bacterial growth, so that was chosen as the best time point to evaluate samples grown in the presence of the novel QSI's. Figure 10 shows preliminary evaluation of these compounds according to the aforementioned conditions. At this point in time there is a very wide distribution of fluorescence populations. It was originally hoped that this experiment would show a much sharper population of fluorescence. This is most likely due to inadequate sample preparation, but up to this point all attempts to clean up the signal have not offered any improvements. The first problems that came to mind were the refractive index matching of the sample fluid to the sheath fluid and cellular aggregation. The refractive index

matching problem was fixed by spinning down planktonic cells and then re-dissolving them in sterile filtered PBS (sheath fluid is also PBS). Attempts to disaggregate cells consisted of only extended vortexing and light sonication. Neither of these changes to the procedure improved the quality of the signal.

Normally, bacterial flow cytometry techniques make use of gating multiple thresholds in order to improve the quality of the signal. In the experiments that have been preformed to date, only side scatter has been used to separate the signal of the bacteria from the signal of the noise. The side scatter signal has a very loose correlation with particle size, so the threshold is set that all particles falling below a certain signal intensity will not be included in the final analysis. In eukaryotic cell systems, side scatter is used to differentiate different levels of cellular granularity (for example, this is good for separating signals of leukocytes from lymphocytes), not particle/cellular size. In eukaryotic systems the cells are sufficiently large that forward scatter provides a much better discrimination of particle size. To make up for the inherent inability of forward scatter and side scatter to distinguish particle size at the micron range where rod shaped *Pseudomonas aeruginosa* grow, organic fluorescent dyes specific for cell membranes or DNA are used so that a fluorescence signal may also be used to make effective thresholding conditions. When a fluorescence signal is used in conjunction with a side scatter signal the cytometer is much more able to separate the bacterial signals from junk. Specifically, these dyes can also be used to target viable, or living, cells. During normal culture growth and sample manipulation, some cells are certain to be killed. Since we are only interested in cells that are metabolically active, it would be nice to have a reagent that would allow specific examination of only viable cells. Unfortunately, *Pseudomonas*

aeruginosa has very effective efflux pumps that not only make it especially resistant to antimicrobials, but also make it especially troublesome for staining. For this reason, the standard stains employed for flow cytometry will not be effective. There are stains available that will accomplish this end but they have not been acquired as of yet.

In Figure 10 the fluorescence data is normalized against a negative control (no additive) so that any compound inhibiting the action of LasR would have a number less than one while a molecule enhancing the activation of LasR should have a number greater than one.

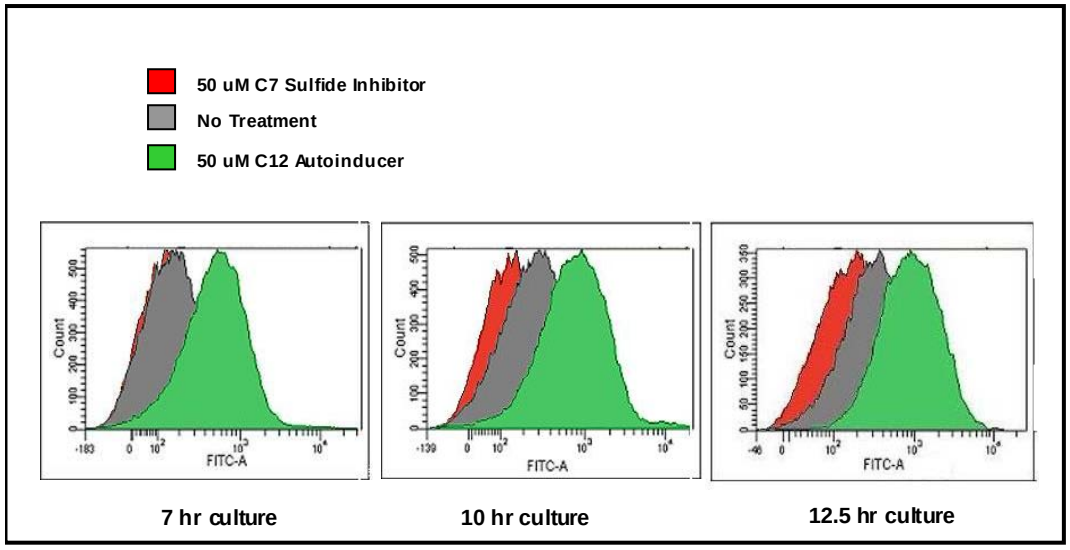


Figure 9. Fluorescence Response to the C(7) Sulfide Inhibitor as Measured by Flow Cytometry

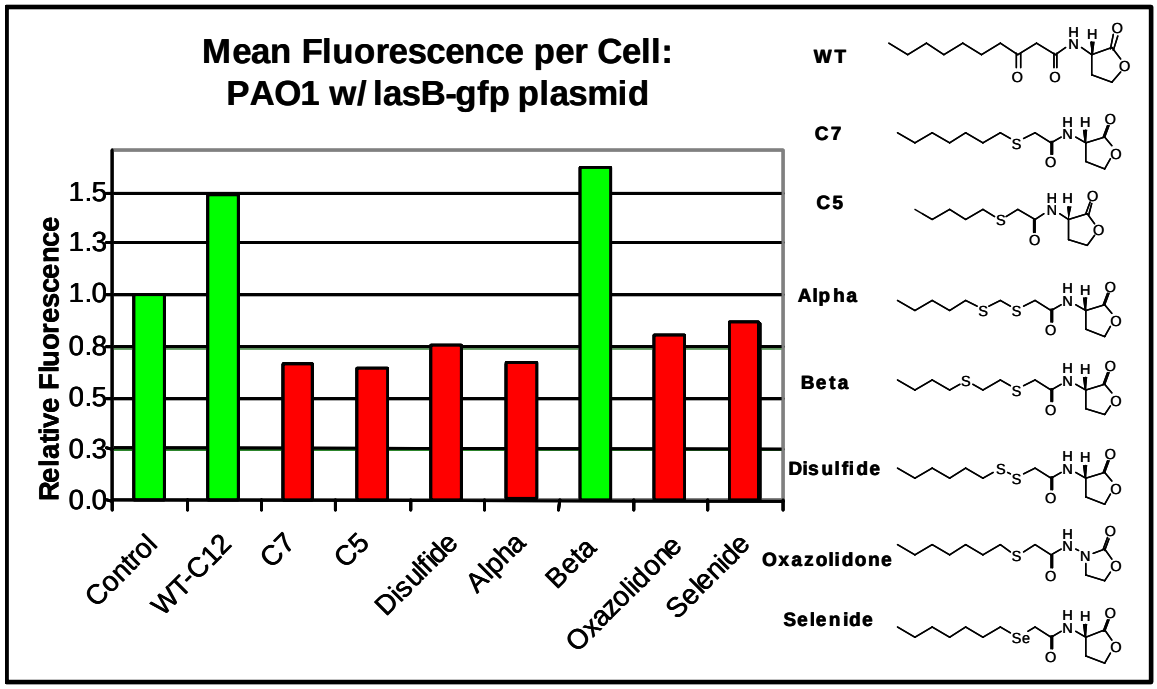


Figure 10. Fluorescence inhibition in Quorum Sensing linked *Pseudomonas aeruginosa* in response to treatment with various Quorum Sensing Inhibitors

As one would expect, the wild type C(12) autoinducer enhanced the level of fluorescence while the inhibitors disrupted fluorescence anywhere from 10-40%. Of particular interest is the Beta bissulfide which seems to act as an autoinducer instead of an inhibitor, which of course is contrary to what would have been expected. This data also shows that the rest of the novel compounds have some effectiveness as Quorum Sensing Inhibitors.

At this point it is too early to say quantitatively exactly *how* effective these new compounds are in antagonizing ligand-receptor binding of LasR. These compounds first need to be evaluated at varying concentrations in order to more effectively quantify their efficacy.

Colony Biofilm

The colony biofilm assay is the first use of a stationary biofilm to evaluate the ability of a QSI to enhance the susceptibility of a biofilm to an antimicrobial (antibiotic in our case) challenge. This *quantitative* test for biofilm susceptibility stands in stark contrast to the other biofilm tests in the literature.[6,7,10,41] In these studies the effectiveness of a quorum sensing inhibitor was only evaluated qualitatively using confocal microscopy. In addition, it is worth mentioning that most evaluations of QSI's is done using a fluorescent reporter construct that is linked to LasR, the quorum sensing receptor protein. While it is important to quantify the level of quorum sensing inhibition, it is crucial that this inhibition of quorum sensing be correlated to the increased susceptibility of biofilms, preferably in a quantitative manner. The colony biofilm method is an effective way of quantifying this susceptibility effect.

This test is carried out by growing a biofilm on a permeable polycarbonate membrane that is placed on an agar plate (Figure 11). The cells in the biofilm remain stationary because they are too big to pass through the pores in the membrane. Instead, they are allowed to grow in place because nutrients are still allowed to pass up through the membrane.

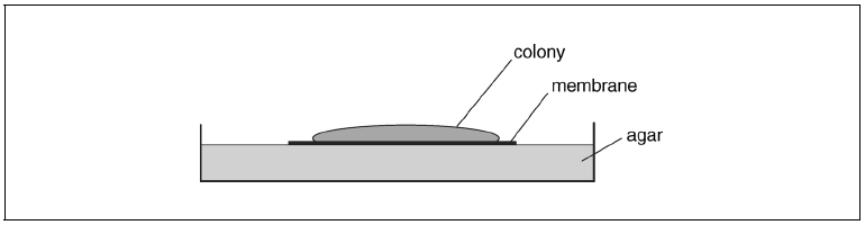


Figure 11. Side view of a colony biofilm assay

Specifically, the Colony Biofilm is inoculated onto the membrane and allowed to grow for 48 hours. At this point the membrane (with biofilm attached) is transferred to a treatment plate where it is subjected to some sort of antimicrobial treatment. In our case, the treatment period consisted of three different sample groups: Negative control containing no additives, Positive control containing tobramycin, and an inhibitor sample that also contained tobramycin. Some tests were also done to show that the inhibitors were not killing the biofilms when there was no antibiotic present. (data not shown) After a predetermined treatment period the membranes are harvested separately so that a plate count of Colony Forming Units (CFU) (or viable bacteria) can be performed.

This test was developed to quantify the increased tolerance to antibiotics that biofilm bacteria exhibit. While this is a labor intensive setup, it involves less work than similar apparatuses that involve flowing liquid media to feed the growing biofilms. Initially, a treatment period of 12 hours was chosen to evaluate the ability of the QSI's to lower the tolerance of the colony biofilms to antibiotics. (Figure 12) This data showed only a nominal reduction in the number of viable bacteria after treatment. In the case of the negative control (-) the agar contained only normal growth media. In these conditions there was a 0.19 log reduction by having tobramycin in the treatment media and an additional 0.19 log reduction with the presence of the C(7) sulfide QSI.

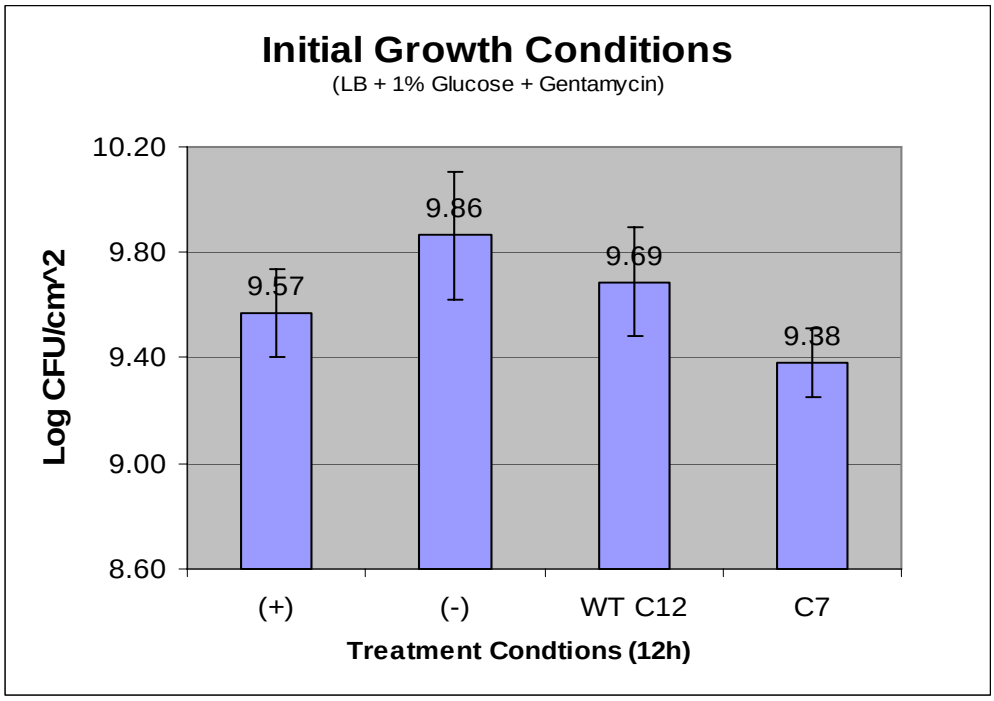


Figure 12. Evaluation of Tobramycin tolerance of *Pseudomonas aeruginosa* biofilms in the presence of an Autoinducer and QSI.

These results were improved when sampling was done after a 24 hour treatment instead of a 12 hour treatment. (Figure 13) Unfortunately, these results were still not statistically significant, as shown by the size of the error bars which were in some cases as large as the measured effect. However, the measured effect was still improved from the previous conditions. With the 24 hour treatment period, there was a 0.20 log reduction from the tobramycin negative to the tobramycin positive controls and an additional 0.50 log reduction with the addition of the C(7) QSI. In order to further improve the resolution of this assay, it was decided that a more dramatic effect would be shown if the biofilms were grown in the presence of inhibitor for the entire 48 hour growth period instead of just the 24 hour treatment period. Before this change, it was originally thought that in order to mimic the conditions that exist in the cystic fibrosis

lung, bacteria should be allowed to grow into mature biofilms without Quorum Sensing interference. This way the effectiveness of the novel QSI's with respect to their potential use as an adjuvant therapy (with classical antibiotics) for cystic fibrosis could be more directly evaluated. As a result of the poor repeatability and resolution of these conditions, the presence of inhibitors in the growth media was evaluated. (data not shown) These conditions improved the antibiotic susceptibility of the *Pseudomonas aeruginosa* biofilms, but it was still not the drastic effect that had been originally envisioned for an inhibitor that had been shown to be so much more effective in pseudo-*in vitro* QSI assays. [10,14]

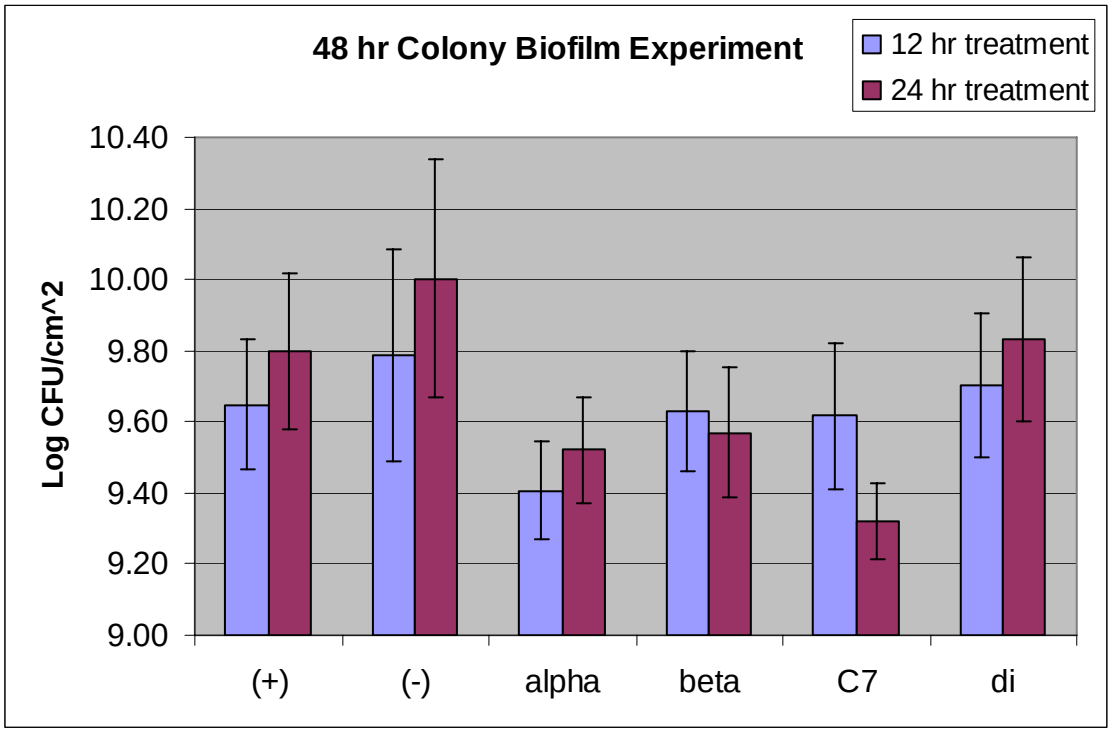


Figure 13. Comparison of 12 and 24 hour sample points.

At this point it was convincing that these inhibitors (both the novel inhibitors and the C(7) sulfide that was being used as a benchmark) were producing a statistically

significant effect, but it seemed that the biofilms being grown were *too* resilient to show an increased antibiotic susceptibility effect with good resolution and repeatability. By lowering the concentration of the media the resulting biofilms were smaller and more susceptible to the treatment systems (Figure 14).

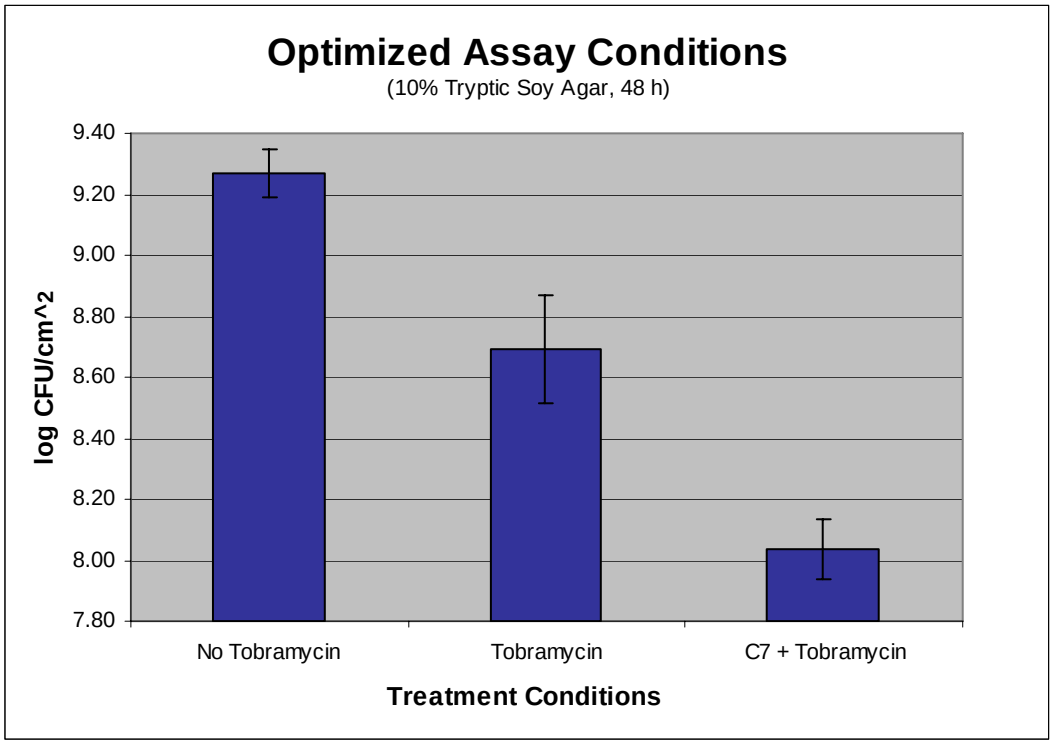


Figure 14. Antibiotic susceptibility enhancement by C7 sulfide QSI

At this point in time, assay conditions have been worked out and repeatability of this assay has been demonstrated on the C7 sulfide inhibitor. When the biofilms were grown in the presence of the inhibitor, the treatment showed a much more noticeable effect. We have also shown with this assay that the inhibitors themselves are not killing the biofilm, but that they are "softening" the biofilm so that they are more easily killed by the antibiotic treatment.

Experimental Procedures

Planktonic Culture Preparation

An overnight culture of PAO1 pMH509 inoculated from a frozen stock was diluted to an OD 600 of 0.05 in LB. The culture was grown and diluted using LB media supplemented with 1% glucose and 100 μg/mL Gentamycin. 50 μl of this diluted culture was used to inoculate 15 mL culture tubes containing 4 mL of LB media supplemented with 1% glucose, 100 μg/mL Gentamycin and the indicated concentration of inhibitor. These cultures were grown for 12 hours at 37 °C in a rotary shaker. The fluorescence of these cultures were then evaluated using flow cytometry. A 1/1000 dilution of the 12 h culture in sterile PBS was filtered through a 100 μm membrane and vortexed for 30 sec.

Flow Cytometry

These samples were evaluated with a Becton Dickinson FACSAria™ using a side scatter threshold of 200. The mean fluorescence (average of 30,000 events) of each sample was normalized to the control sample which contained no exogenous inhibitor or inducer.

Colony Biofilm Preparation

Colony biofilms[42-44] were grown on polycarbonate membranes resting on agar (10% TSA) plates. Starter cultures were inoculated from a frozen culture. Planktonic starter cultures of *P. aeruginosa* (PAO1 pMH509) were grown overnight in a 37 ºC shaker in LB and diluted to an optical density (at 600 nm, with a 1-cm path length) of 0.10 in LB. Gentamycin (100 μg/mL) was added to the broth used to grow the inoculum

for the preparation of PAO1. The antibiotic was also included in the agar medium used to grow colony biofilms. One 5-µl drop of diluted planktonic culture was used to inoculate individual sterile, grey, polycarbonate membrane filters (25-mm diameter, 0.2-µm pore size; Poretics Corp., Livermore, Calif.) resting on 10% TSA supplemented with the appropriate amount of inhibitor. The control samples were grown on agar media without any inhibitor. The membranes were sterilized by UV exposure (15 min per side) prior to inoculation. The plates were inverted and incubated at 37 °C for 48 h, and the membrane-supported biofilms were transferred to fresh culture medium once after 24 h.

Biofilm Susceptibility

The 48 h biofilms were transferred to antibiotic and inhibitor containing agar, and the agar plates were incubated at 37 °C. The biofilms were sampled after 24 h. When sampled, each membrane-supported biofilm was placed in 9.0 mL of PBW, and the mixture was vortexed at high speed for 1.0 min with a Maxi Mix II Vortex mixer (Barnstead/Thermolyne, Dubuque, Iowa) and then serially diluted in PBW. The viable bacteria were enumerated as described below.

Viable Bacteria Enumeration and Comparison

Serially diluted samples were plated onto Tryptic Soy Agar (Difco Laboratories) by the drop plating method,[45,46] which consists of plating 10 µl drops (x 5) of each serial dilution (0-8) on TSA agar plates. Each plating was done in duplicate, changing the pipet tip each time. The plates were then incubated at 37 °C for 18 h. After incubation, the numbers of colonies were counted from the serial dilution where 3-30 individual colonies were discernable inside each drop made 18 hours before. The numbers inside each drop

were averaged and this number was used to calculate the number of Colony Forming Units (CFU). The change in the number of CFU was normalized for each experiment by converting the measurements of the number of viable bacteria to the log reduction of the number of CFU. The log reduction of the number of CFU, or simply log reduction, at the 24 h sampling time was defined as the negative log10 of the quotient of the number of CFU at that time and the number of CFU present in the control samples. A positive log reduction represents a decrease in the number of CFU. The log reduction values at the 24 h time point for identical experiments were averaged, and the standard error of the mean was calculated.

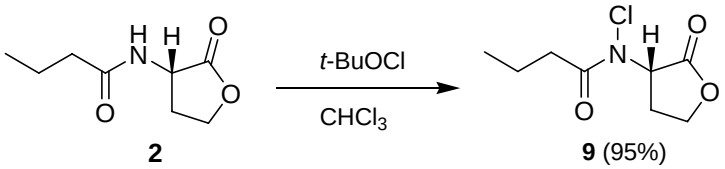
CHAPTER 4

SYNTHESIS OF BIOCIDES

Synthesis

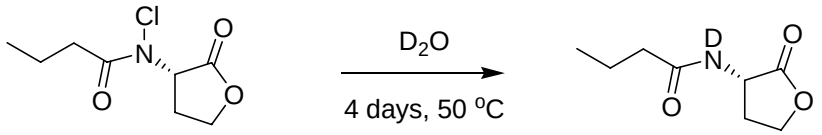
Synthesis of an *N*-chlorinated analogue of the C(4) wild type autoinducer **2** would result in a molecule that could produce hypochlorite upon hydrolysis. If this were to occur inside of a biofilm, it would result in a compound that was able to selectively deliver a biocide to a biofilm cell or community.

Chlorination of the amide nitrogen was accomplished using *t*-butyl hypochlorite which was prepared from *t*-butanol and Clorox.[47] Initially, methanol was used as a solvent, but since the mechanism of chlorination involves nucleophilic attack onto the hypochlorite chlorine, methanol would compete with the amide nitrogen for the *t*-butyl hypochlorite reagent.[48] Using this method, the *N*-chlorinated analogue could be separated chromatographically from the amide starting material with yields around 25%. When the solvent was changed to the non-nucleophilic chloroform, the reaction proceeded much more smoothly, yielding 95%.



Scheme 16. *N*-chlorination of *N*-butanoyl homoserinelactone

Since it is thought that the most probable mechanism for bacterial killing will involve production of hypochlorite, a simple experiment was performed in order to determine the relative stability of the amide chlorine to hydrolysis. Using deuterium oxide as a solvent, the hydrolysis was monitored using ^{1}H NMR (Scheme 17). The reaction was held at 50 ºC for 4 days while disappearance of the lactone alpha proton was monitored. These experiments indicated that the rate of hydrolysis of the lactone[49] was faster than the hydrolysis of the nitrogen chlorine bond. After 18 h neither hydrolysis had occurred to any substantial degree. This information was encouraging because it indicated that the *N*-chloro-*N*-butanoyl homoserine lactone would not be so reactive that it would immediately produce hypochlorite when exposed to water before it would have the opportunity to penetrate a cell or biofilm.



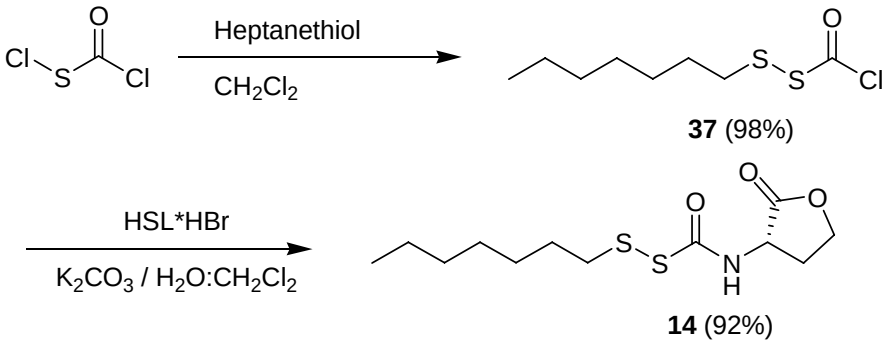
9 **2**

Scheme 17. Hydrolysis of *N*-chloro-*N*-butanoyl homoserinelactone

Similar to the *N*-chlorinated analogues, the alkyldithiocarbonyl analogue **14** could conceivably gain selective entry to the interior of a bacterial cell and oxidize thiols to their corresponding disulfide (Scheme 2), thus selectively destroying the function of bacterial enzymes.

Alkyldithiocarbonyl **14** was synthesized via dithiocarbonylchloride **37**.[50] This material was readily available from treatment of the sulfanyl chloride with heptanethiol.[51] The thiol preferentially condensed with the sulfur in quantitative yield (Scheme 18) after

removal of HCl *in vacuo*. Amidation with HSL*HBr was accomplished using a biphasic medium of aqueous potassium carbonate and dichloromethane.[49] This method was a substantial improvement over using only dichloromethane and diisopropylethylamine in a purely organic medium.



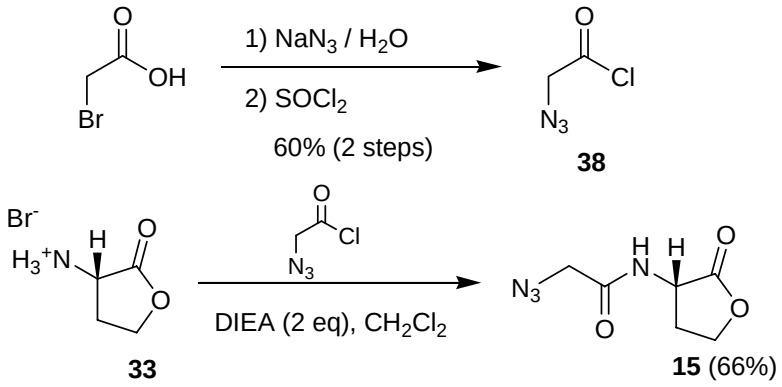
37 (98%)

14 (92%)

Scheme 18. Synthesis of alkyldithiocarbonyl HSL

Azido acetylchloride **38** was prepared from bromoacetic acid and sodium azide (Scheme 19) using a modification of the procedures used by Hennig and Dyke.[40,52,53] The workup of this acid was cumbersome due to the high water solubility of azidoacetic acid. It was necessary to extract the reaction mixture many times with a large amount of diethyl ether in order so secure the desired acid. The acid was isolated by removal of the majority of the solvent *in vacuo* and treated with thionyl chloride without further purification. This reaction mixture was distilled directly to give the desired acid chloride in 70% yield.

The acid chloride was then coupled to the HSL*HBr using 2 equivalents of Hunig's base in dichloromethane producing the amide in 66% yield. With this azide in hand, known biocides could now be functionalized with terminal alkynes and linked to the *N*-acyl azido homoserine lactone by copper catalyzed dipolar Huisgen cycloaddition.



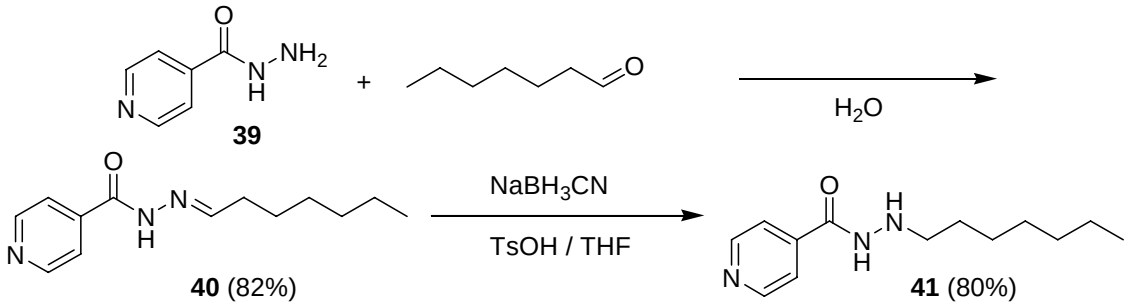
Scheme 19. Synthesis of acyl azido HSL linker

In order to construct the homoserine lactone conjugate with isonicotinic acid hydrazide **39** it was first necessary to alkylate the hydrazide using a reductive amination. Since the omega alkyne aldehyde was not commercially available and the precursor was expensive (~$30 / gram) the reductive amination was first optimized using the fully saturated heptanal (Scheme 20). Possession of alkyl hydrazide **41** would also allow a control biological experiment to determine what the effect of alkylation of the terminal nitrogen has on biocidal activity.

Preparation of hydrazone **40** was accomplished by a simple condensation of stoichiometric amounts of 6-heptanal and isonicotinic acid hydrazide in water.[54] After 2 hours at room temperature the hydrazone crystallized out of solution and filtration and recrystallization yielded 83%.

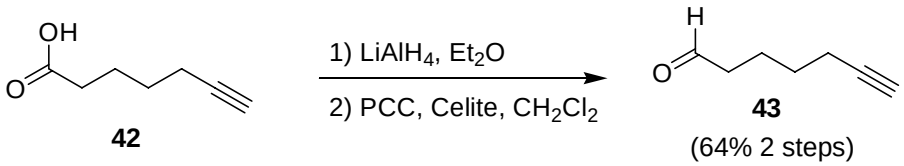
Reduction to the corresponding alkyl hydrazide was not so straightforward. Although a variety of methods exist to accomplish this transformation, various conditions using sodium borohydride and triethylsilane as hydride donors resulted mostly decomposition with only very small amounts of reduction product. Successful reduction was accomplished using sodium cyanoborohydride with *p*-toluenesulfonic acid.

Bromocresol green was also used as a pH indicator so that a solution of acid in THF could be added to keep the reaction at a pH of 3-5. Although chromatography was required to isolate the pure hydrazide, the final yield was a reasonable 80%.



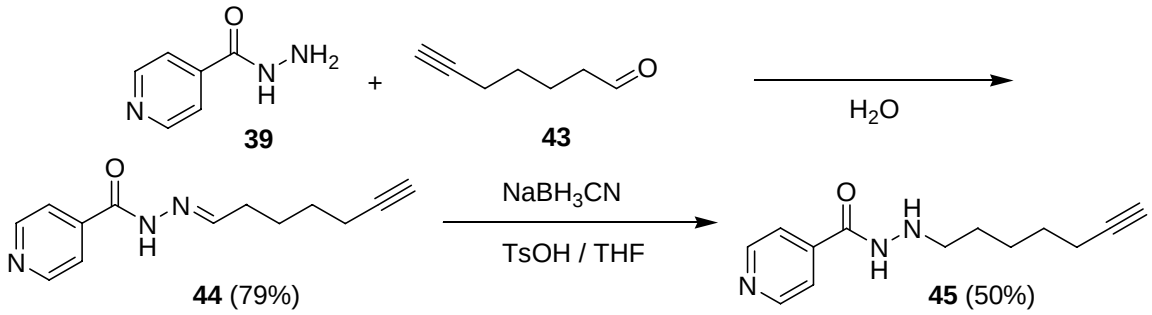
Scheme 20. Alkylation of isonicotinic acid hydrazide

Preparation of 6-heptyn-1-al[55] was accomplished by reduction of the corresponding acid to the alcohol using lithium aluminum hydride followed by oxidation to the aldehyde using Magtrieve™ or $Cr(IV)O_2$.[56] This oxidation procedure produced slightly better yields than the Swern oxidation[57], and has a much less cumbersome work up. The Magtrieve™ reagent requires only filtration to remove the chromium oxide followed by removal of the solvent *in vacuo.* Both the Swern and Magtrieve oxidation produced a variety of products and required chromatography to separate to secure the pure aldehyde. Problems with oxidation of primary alcohols with terminal alkynes are not unprecedented as Swern reported having similar difficulties in his original publication disclosing the dimethylchlorosulphonium oxidation of alcohols.[58] Efficient oxidation to the aldehyde was ultimately accomplished using pyridinium chlorochromate in a preparation with Celite and dichloromethane.[59]



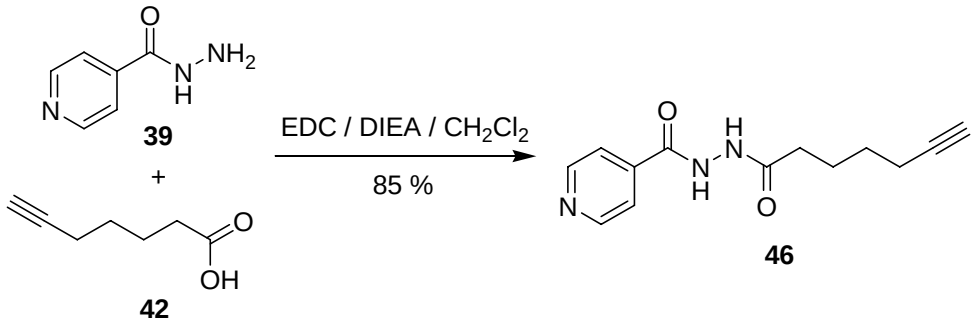
Scheme 21. Preparation of 6-heptyn-1-al

Alkylation of isonicotinic hydrazide was accomplished as before with isolation of the hydrazone intermediate followed by reduction to hydrazide **45**.



Scheme 22. Synthesis of Isonicotinic acid, 2-(6-heptynyl)hydrazide

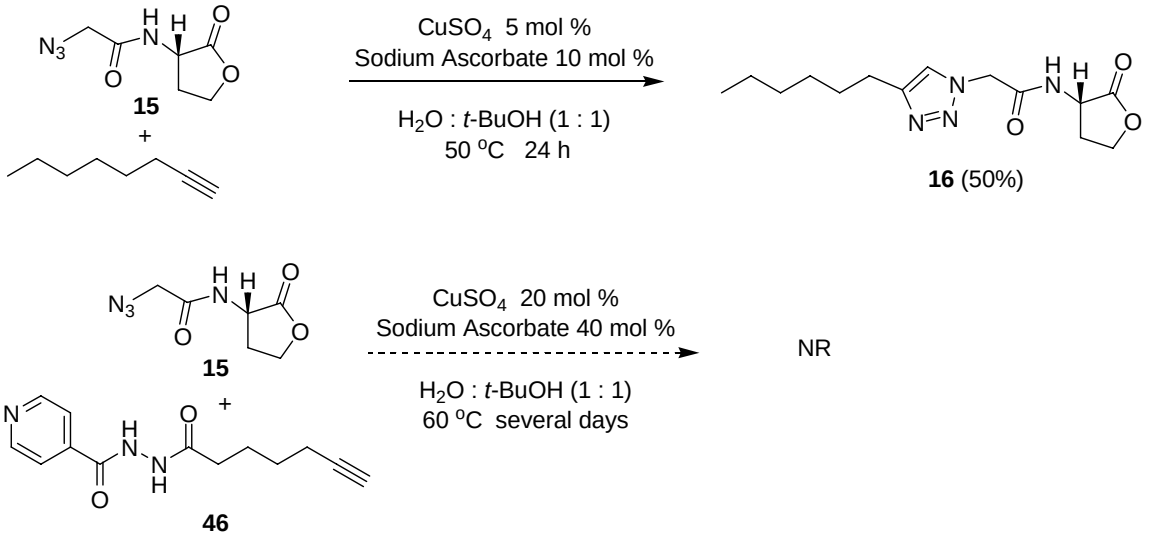
The acyl hydrazide analogue of the terminal alkyne was also synthesized because it seemed like a good idea. Although acylation completely changes the electronics of the hydrazide functionality, it functionalized isonicotinic acid hydrazide with a terminal alkyne in one step. Using the EDC coupling described previously (Scheme 15), **46** was isolated in good yield on the one gram scale (Scheme 23).



Scheme 23. Acylation of isonicotinic acid hydrazide

The final step to synthesize the novel bifunctional mutagenic biocide was to ligate the alkyne functionalized isonicotinic acid hydrazide analogues with azido homoserine lactone **15**.

To first probe cyclization conditions 1-octyne was used with azido homoserine lactone **15** to give triazole **16** using conditions outlined by Sharpless (Scheme 24).[60] The advantage of this method is that the sensitive and insoluble Cu(I) catalyst is generated *in situ* as Cu(II) is reduced by sodium ascorbate. These conditions yielded the desired triazole in 50% yield (Scheme 24). Due to the leftover starting material recovered using chromatography, it is reasonable to assume that a longer reaction time or high temperatures may have produced the near quantitative yields normally reported with this type of cycloaddition.



Scheme 24. Copper catalyzed azide alkyne ligation

Much to our dismay, the above conditions were not conducive to triazole formation in alkyne functionalized isonicotinic acid hydrazide analogues. After several days at 50 ºC only starting material was recovered. It has been reported that some

problematic and insoluble substrates required high catalyst loadings to efficiently complete the copper catalyzed cycloaddition.[61] Unfortunately for my graduation aspirations, this change also failed to yield any triazole. These results contrasted sharply with the reported robustness and functional group tolerance of this transformation.

Although there have been countless reports of copper catalyzed triazole formation since its initial independent reporting by Meldal[62] and Sharpless[60], most do not involve substrates that contain basic and/or sterically unhindered nitrogens. It is possible that the pyridine of the isonicotinic acid hydrazide is coordinating to the Cu(I) in such a fashion that causes a problem with catalyst turnover. Because the majority of the conditions employed in the body of literature (especially those involving more exotic substrates) did not use Cu(II) sulfate, it was decided to seek out an alternative Cu(I) source. Using the more popular copper iodide allowed direct introduction of Cu(I) instead of *in situ* reduction of Cu(II). The drawback to using CuI (in addition to its sensitivity to oxidation) was the idiosyncratic nature of the many conditions under which successful cycloaddition had been accomplished.

Inspection of the [1,2,3]-triazole literature revealed that a large variety of conditions exist to overcome some inherent problems with the generality of the alkyne azide cycloaddition. Issues such as electronic differentiation of the cycloaddition partners, solvent choice, presence of base as well as stability of Cu(I) ion to the reaction conditions all contribute to the efficiency of the reaction.

Although it is not widely reported, the efficiency of triazole formation is greatly improved when electronically differentiated alkynes and azides are used.[63] For instance, electron deficient internal alkynes lead smoothly to 1,4,5-substituted triazoles even in the

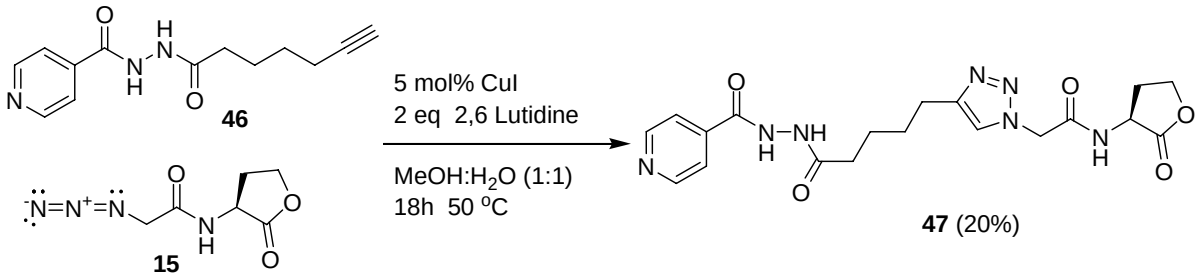
absence of a catalyst, a transformation that is typically very sluggish and requires high temperatures.[64]

Additionally, Cu(I) oxidation and disproportionation are much more prevalent when CuI is used. For this reason, dry organic solvents and an inert atmosphere are required in order to avoid Cu(I) degradation. This presents an additional problem of alkyne deprotonation. In aqueous conditions, deprotonation of the terminal alkyne in the presence of Cu(I) is very favorable and can take place without any base other than water. Using organic solvents increases the energy required for this deprotonation and subsequent copper acetylide formation.[65] Accordingly, high temperatures and or addition of base is required.

Wong *et al* has reported the extreme sensitivity of triazole formation to the reaction conditions.[66] Simply changing the base from triethylamine to DIEA increased the yield from 0% to 38%. Interestingly, he was able to obtain 1,4-triazoles in high yield on very small scales by using a large excess of CuI (5 eq) in acetonitrile and elevated temperatures. In our case, a variety of conditions in dry acetonitrile resulted in no conversion even when an inert atmosphere was used. Particularly notable was the formation of a bright yellow precipitate immediately upon addition of CuI to a slurry of alkyne **46** and azide **15** in acetonitrile[67]. Subsequent addition of a base resulted in change of precipitate color from yellow to green, but with or without base, room temperature or reflux, all resulted in 0% conversion.

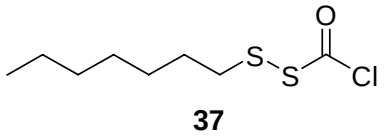
By abandoning the amine bases and changing the solvent back to an alcohol/water mixture the desired triazole was formed in 20% yield after purification using column chromatography (Scheme 25). This formation of the desired product may have been the

result of ligand effects on the Cu(I) ion. The decreased σ-basicity of lutidine as compared to acetonitrile may have resulted in a more labile copper complex that would allow more facile formation of the copper-acetylide and faster catalyst turnover. It is thought that ideal conditions exist when ligands bind the copper sufficiently to not compromise the redox stability of the Cu(I) ion, but labile enough to allow π complexation of the alkyne and subsequent deprotonation and formation of the copper-acetylide complex. [68]



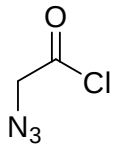
Scheme 25. Successful cycloaddition conditions for alkenylacylhydrazide 46

Experimental Procedures



Heptyldithiocarbonyl chloride (37):[50] The title compound was prepared according to a variation of the literature procedure[51]. A solution of heptanethiol (591 mg, 4.5 mmol) in 1 mL CH_2Cl_2 was added dropwise to a solution of chlorocarbonylsulfenyl chloride (650 mg, 5 mmol) in 1.5 mL CH_2Cl_2 under an atmosphere of argon. Stirring was continued overnight to ensure complete consumption of the starting thiol. The solvent and excess chlorocarbonylsulfenyl chloride were removed *in vacuo* to yield the desired alkyldithiocarbonyl chloride in 95% yield. 100% purity was determined by ^{1}H NMR.

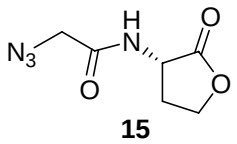
IR: 2956, 2928, 2856, 1780, 792 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.882 (t, 2H), 1.69 (quint, 2H), 1.291 (m, 8H), 0.895 (t, 3H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 167.1, 39.71, 31.84, 29.1, 28.96, 28.46, 22.77, 14.25; Mass: Calculated - 291.1036, Found - 292.1161.



38

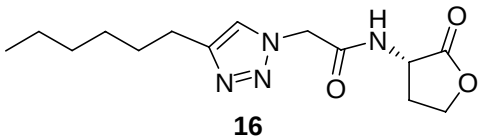
Azidoacetyl chloride (38): The title compound was prepared according to a variation of the literature procedures.[52,53] A solution of sodium azide (16.0 g, 246 mmol) in 24 mL H_2O (to make ~40% solution) was stirred at 50 ºC for 30 min, as to dissolve as much of the NaN_3 as possible. The solution was then cooled to 0 ºC and bromoacetic acid (20 g, 143 mmol) was added portionwise while maintaining vigorous stirring. The solution stirred at 0 ºC and developed a red color as all the undissolved material was solvated. The reaction mixture was then allowed to stir for 24 h at which point some of the color of the solution had faded. The solution was then cooled to 0 ºC and acidified to pH 4 using ice cold H_2SO_4:H_2O (1:1). The solution was then extracted ten times with a total of 800 mL of diethyl ether. The organic layer was dried with Na_2SO_4, filtered and concentrated *in vacuo* until most of the ether was removed. This afforded a yield of about 8 g (56%) of crude azidoacetic acid. This was carried forward without further purification. The reaction flask was then cooled to 0 ºC and thionyl chloride (15 mL, 205 mmol) was added over a period of 10 min. This reaction mixture was stirred at 45 ºC for 3 h and at room temperature overnight. The reaction mixture was then directly fractionally distilled using a water aspirator affording 8.0 g (47% over 2 steps) of the title compound: bp 42 ºC/

20mm Hg; IR: 2989, 2912, 2111, 1795, 1407, 1272, 905 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.28 (s, 2H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 169.8, 59.5.



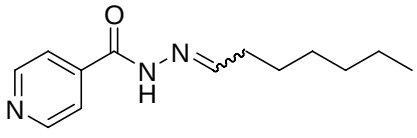
15

Acetamide, 2-azido-*N*-[(3*S*)-tetrahydro-2-oxo-3-furanyl] (15): The title compound was prepared according to variation of the published amide coupling protocol.[38] A solution of HSL (650 mg, 3.6 mmol) in 5 mL DCM was cooled to 0 ^{o}C and stirred while 1.28 mL of diisopropylethylamine was added dropwise. The mixture was stirred for 15 min or until HSL was completely solvated. The solution was cooled to – 20 °C and azidoacetylchloride (350 µl, 3.7 mmol) was then added dropwise over 10 min. The solution was stirred for one hour at – 20 °C and was then allowed to come to room temperature. The stirring continued for about two hours or until HSL was consumed by TLC. The reaction was then quenched with 5 mL brine and allowed to stir for 5 min. The organic layer was separated and the solvent was removed *in vacuo*. This residue was dissolved in a small amount of DCM and filtered through a plug of silica (DCM elution). The solvent was removed *in vacuo* yielding 435 mg (66 %) of a white solid. mp 92.6-93.6 ºC; IR: 3293, 2920, 2109, 1777, 1666, 1534, 1178 cm^{-1}; ^{1}H NMR (500 MHz, CDCl3) δ 6.858 (s, 2H, J =), 4.584-4.637 (ddd, 1H, J = 11.5, 8.5, 7), 4.5 (t, 1H, J = 9), 4.282-4.335 (ddd, 1H, J = 11.5, 9.5, 5.75), 2.804-2.856 (m, 1H, J =), 2.174-2.263 (m, 1H, J =); ^{13}C NMR (500 MHz, CDCl3) δ 175.03, 167.66, 66.2, 52.5, 49.17, 30.



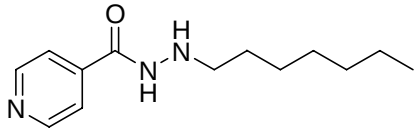
16

1H-1,2,3-Triazole-1-acetamide, 4 pentyl-*N*-[(3*S*)-tetrahydro-2-oxo-3-furanyl] (16): The title compound was prepared according to a variation of the literature procedure.[60] A solution of 1-octyne (443 μl, 3.0 mmol), acylazido HSL (552 mg, 3.0 mmol), *t*-butanol (6 mL) and water (6 mL) was stirred at room temperature while 0.6 mL of a freshly prepared solution of 0.5 M sodium ascorbate was added followed by copper(II) sulfate (22 mg, 0.1 mmol). The reaction was stirred at 50 °C for 24 h at which point the reaction mixture was evaporated to dryness. The residue was dissolved in a minimal amount of *N*,*N*-dimethylformamide and the product was precipitated by adding cold water to the solution. The solid material was filtered off and washed with small amounts of cold water to yield 440 mg (50%) of the pure triazole. MP 164.8 – 165.6 °C. IR: 3300, 3076, 2923, 2854, 1777, 1672, 1557, 1459, 1376 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.515 (s, 1H), 6.969 (s, 1H), 5.138 (s, 2H), 4.547-4.601 (ddd, 1H, J = 11.5, 9, 7), 4.482 (t, 1H, J = 9), 4.255-4.308 (ddd, 1H, J = 11.5, 9.5, 6), 2.698-2.763 (m, 3H), 2.25-2.33 (m, 1H), 1.662-1.722 (q, 2H, J = 7.5), 1.297-1.389 (m, 6H), 0.893 (t, 3H, J = 7); ^{13}C NMR (500 MHz, $CDCl_3$) δ 174.7, 166.21, 149.3, 122.9, 66.1, 52.73, 49.4, 31.7, 29.4, 29.3, 29.1, 25.7, 22.7, 14.2; Mass: Calculated - 294.1765, Found - 294.1771.



40

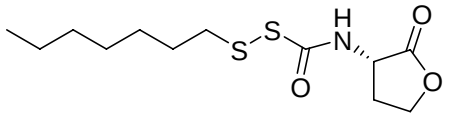
1-Isonicotinyl-2-heptylidene hydrazine (40): Isonicotinyl hydrazide (750 mg, 5.5 mmol) was added to 10 mL deionized water followed by heptanal (783 µl, 5.6 mmol). A small amount of precipitate formed almost immediately and the reaction mixture continued to stir at room temperature for 2 h at which point a thick white mixture had developed. The mixture was filtered and washed with small portions of cold water. This solid was dried and recrystallized from ~25 mL xylenes. The crystals were filtered and dried yielding 1.05 g (82%). mp 100.4-101.6 ºC; IR: 3438, 3215, 3047, 2927, 2857, 1658, 1552, 1296 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.15 (s, 1H), 8.76 (m, 2H), 7.64-7.73 (m, 3H), 2.28-2.45 (m, 2H), 1.51-1.58 (m, 2H), 1.3-1.37 (m, 6H), 0.89 (t, 3H, J = 7); ^{13}C NMR (500 MHz, $CDCl_3$) δ 162.5, 155, 150.7, 149.8, 140.7, 123.8, 121.4, 32.7, 31.7, 29.1, 26.7, 22.7, 14.2.



41

Isonicotinic acid, 2-heptylhydrazide (41): A RBF was charged with sodium cyanoborohydride (230 mg, 4 mmol) and c.a. 0.5 mg of bromocresol green. 13 mL of THF was then added via syringe and the reaction flask was cooled to 0 °C. Isonicotinic heptylidene hydrazine (230 mg, 1 mmol) was then added in one portion while efficient stirring was maintained. A solution of *p*-toluenesulfonic acid was added dropwise as a solution in 4 mL THF. The solution was added to maintain the reaction mixture at pH 3 – 5 as indicated by a light tan color of the indicator. The solution was then allowed to warm to room temperature and stirred for 5 hours. The reaction was subsequently quenched

with water and the solvent was removed under reduced pressure. The residue was then dissolved in ethyl acetate and the organic layer was washed with brine, which was followed by water. The organic layer was then dried with sodium sulfate and the majority of the solvent was removed *in vacuo*. The crude product was purified by flash chromatography (50% ethyl acetate in hexanes) yielding 193 mg (82%) of yellow crystals. mp 96.5-97.8 ºC; ^{13}C NMR (500 MHz, $CDCl_3$) δ 161.7, 147.8, 145.5, 124.8, 52.3, 31.9, 29.3, 28.2, 27.1, 22.8, 14.3.

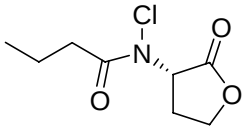


14

***N*-[(3*S*)-Tetrahydro-2-oxo-3-furanyl]-carbamo(dithioperoxoic) acid, heptyl ester (14):** Homoserine Lactone * HBr (100 mg, 0.55 mmol) was dissolved in a solution of sodium carbonate (106 mg, 1 mmol) and 1.5 mL deionized water. 1.5 mL CH_2Cl_2 was added to form a biphasic mixture that was subsequently cooled to -10 °C with an ice/salt bath. Heptyldithiocarbonyl chloride (113 mg, 0.5 mmol) was then added dropwise over 15 min while maintaining the reaction temperature below 0 °C. Vigorous stirring was maintained while the reaction warmed to ambient temperature. The reaction was allowed to proceed for 1.5 hours until the starting material was no longer visible by TLC. The organic layer of the reaction mixture was then separated and the aqueous layer was extracted twice with CH_2Cl_2. The combined organic layers were washed with brine and dried over magnesium sulfate. The solvent was removed *in vacuo* yielding 125 mg of a white solid: mp 73.5 – 74.4 ºC; IR: 3303, 2951, 2924, 2853, 1777, 1691, 1511, 1382,

1011 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.559-7.568 (d, 1H, J = 4.5), 4.550-4.602 (ddd, 1H, J = 11.75, 8.5, 5.75), 4.486-4.522 (t, 1H, J = 9), 4.286-4.339 (ddd, 1H, J = 5.5, 8.5, 11.5), 2.859-2.918 (m, 1H), 2.815-2.848 (ABq, 2H, J = 15, 1.5), 2.177-2.266 (m, 1H), 1.688-1.751 (dq, 2H, J = 7, 1.5), 1.366 (q, 2H, J = 7), 1.258-1.338 (m, 6H), 0.889 (t, 3H, J = 7); ^{13}C NMR (500 MHz, $CDCl_3$) δ 174.49, 166, 66.14, 50.79, 40.08, 31.82, 30.55, 29.07, 29, 28.55, 22.75, 14.24; Mass: Calculated - 291.1036, Found - 292.1161.

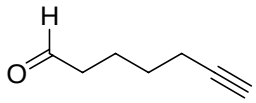
***tert*-Butylhypochlorite:** (prepared in the dark) A 4% aqueous solution of sodium hypochlorite was prepared by diluting sodium hypochlorite (100 mL, 12% available chlorine, supplied by Albertson's) in deionized water (150 mL) and the resulting solution was cooled to 0°C. To this was added a solution of glacial acetic acid (12 mL) and *tert*-butanol (18 mL) in one portion and the resulting mixture was stirred for 3 min. The organic layer was separated and washed successively with sodium carbonate (10% solution, 25 mL) and water (25 mL). The product was dried over calcium chloride and filtered to yield the title compound as a yellow liquid. The product was stored below 4 °C in the dark over calcium chloride.



9

***N*-chloro-*N*-butanoyl-*L*-homoserinelactone (9):** This reaction was performed in the dark. *N*-butanoyl homoserinelactone (178 mg, 1.04 mmol) was dissolved in 1.5 mL $CHCl_3$ and cooled to 0 °C. Using gas tight syringe, *t*-butyl hypochlorite (0.226 mg, 2.08

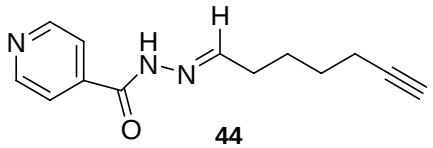
mmol) was added dropwise over 5 min. The syringe was then rinsed with 500 µL $CHCl_3$ and added to the reaction mixture. The reaction was then allowed to stir for 3 hours, or until the starting amide was no longer visible by TLC (R_f = 0.25, 100% ethyl acetate). The reaction mixture was then filtered through a short plug of silica and eluted with CH_2Cl_2. The solvent was then removed in vacuo using a water aspirator and rotary evaporator with a cold water bath (~ 10 °C) yielding 200 mg (95%) of the title compound. IR: 2965, 2933, 2874, 1783, 1684, 1375, 1216, 1019 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.65 (t, 1H, J = 10), 4.53-4.67 (td, 1H, J = 1. 9.5), 4.37-4.42 (m, 1H), 2.60-2.65 (m, 1H), 2.26-2.38 (m, 3H), 1.58-1.66 (sext, 2H, J = 7.5), 0.916 (t, 3`H, J = 7.25); ^{13}C NMR (500 MHz, $CDCl_3$) δ 175.46, 171.91, 65.45, 57.47, 35.35, 25.36, 18.28, 13.77; Mass: Calculated - 206.0578, Found - 206.0505.



43

6-Heptyn-1-al (43): A solution of lithium aluminum hydride (160 mg, 4.2 mmol) in 4 mL ethyl ether was cooled to 0 °C and 6-heptynoic acid (260 mg, 2.1 mmol) was added dropwise over ten min as H_2 gas was seen to evolve vigorously. The syringe was then washed with an additional 200 µL ethyl ether which was added in one portion to the reaction mixture. The reaction was allowed to stir at 0 °C for forty min and then at room temperature for 5 h or until complete conversion of starting material by TLC. The reaction was then cooled to 0 °C and quenched slowly with 260 µg H_2O, 260 µg 15% NaOH, then 480 µg H_2O. This mixture was stirred for 15 min, filtered and the organic

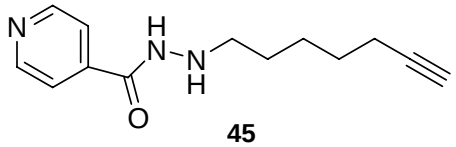
layer was separated. The aqueous layer was extracted twice with ethyl ether. The combined organic layers were then washed twice with brine and dried over Na_2SO_4. The solvent was then removed *in vacuo* yielding 197 mg (83 %) of the desired alcohol as a clear oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.656 (t, 2H, J = 6.9), 2.184-2.237 (td, 2H, J = 6.75, 2.4), 1.949 (t, 1H, J = 2.7), 1.45-1.64 (m, 7H). The resulting alcohol was carried on without further purification to the next step. A 100 mL round bottom flask was charged with 6-heptyn-1-ol (450 mg, 4.0 mmol), pyridinium chlorochromate (6.49 g, 30.1 mmol) and 40 mL $CHCl_3$. The reaction was stirred for 4 h. The mixture was then diluted with 50 mL diethyl ether and filtered through a plug of silica. The solvent was then removed using a rotary evaporator in an ice bath yielding 350 mg (79%) of a pale yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.79 (s, 1H), 2.45-2.50 (td, 2H, J = 1.3, 7), 2.21-2.26 (td, 4, H, J = 2.4, 7), 1.97 (t , 1H, J = 2.4), 1.73-1.83 (quint, 2H, J = 7), 1.53-1.63 (quint, 2H, J = 7).



44

1-Isonicotinyl-2-(6-heptynylidene) (44): Isonicotinyl hydrazide (75 mg, 0.55 mmol) was added to 2 mL deionized water followed by 6-heptyn-1-al (61.6 mg, 0.56 mmol). A small amount of precipitate formed almost immediately and the reaction mixture continued to stir at room temperature for 2 h at which point a thick white mixture had developed. The mixture was filtered and washed with small portions of cold water. This solid was dried and recrystallized from ~25 mL xylenes. The crystals were filtered and

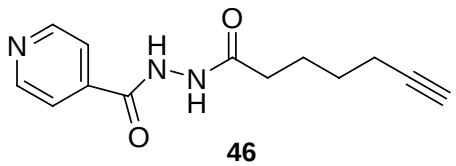
dried yielding 90.8 mg (72%). IR: 3278, 3228, 3046, 2934, 1659, 1556, 1298 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.122-9.49 (s, 1H), 8.76 (d, 2H, J = 4.5), 7.77 (App d, 3H, J = 4.5), 2.24-2.47 (m, 4H), 1.96 (t, 1H, J = 2), 1.61-1.71 (m, 4H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 162.1, 154.4, 150.5, 149.7, 140.1, 121.6, 68.9, 32.2, 28.1, 25.7, 18.3; Mass: Calculated - 229.1288, Found - 229.1286.



45

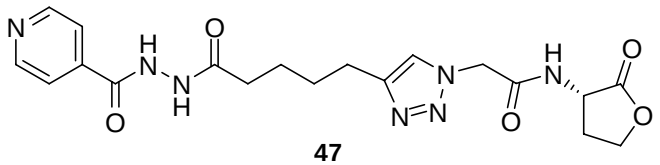
Isonicotinic acid, 2-(6-heptynyl)hydrazide (45): A RBF was charged with sodium cyanoborohydride (234 mg, 4 mmol) and c.a. 0.5 mg of bromocresol green. 13 mL of THF was then added via syringe and the reaction flask was cooled to 0 °C. Isonicotinic acid 6-heptynylidene was then added in one portion while efficient stirring was maintained. A solution of *p*-toluenesulfonic acid was added dropwise as a solution in 4 mL THF. The solution was added to maintain the reaction mixture at pH 3 – 5 as indicated by a light tan color of the indicator. The solution was allowed to warm to room temperature and stirred for 5 hours. The reaction was quenched with water and solvent was removed under reduced pressure. The residue was then dissolved in ethyl acetate and the organic layer was washed with brine, which was followed by water. The organic layer was then dried with sodium sulfate and the majority of the solvent was removed *in vacuo*. The crude product was purified by flash chromatography (90% ethyl acetate in hexanes) yielding 115 mg (49%) of yellow crystals. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.78 (s, 2H), 7.64 (s, 2H), 2.96 (t, 2H, J = 7), 2.19-2.23 (td, 2H, J = 2.5, 7), 1.95 (t, 1H, J = 2.25), 1.48-

1.59 (m, 6H); ^{13}C NMR (500 MHz, $CDCl_3$) δ 165.4, 150.7, 140.3, 121.1, 84.5, 68.7, 52.2, 28.4, 27.6, 26.2, 18.5; Mass: Calculated - 231.1372, Found - 232.1459.



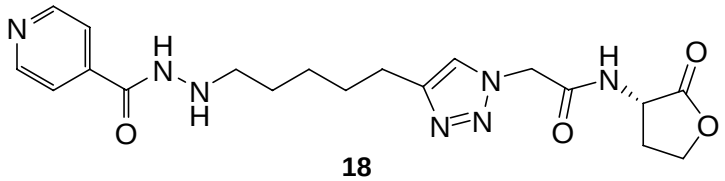
46

Isonicotinic acid, 2-(6-heptynyl)acylhydrazide (46): A solution of isonicotinic acid hydrazide (750 mg, 5.5 mmol) in 18 mL CH_2Cl_2 was cooled to 0 ºC and diisopropylethylamine (956 µl, 5.5 mmol) was added dropwise over 3 min. 6-Heptynoic acid (730 mg, 5.8 mmol) was added in one portion followed by EDC (1.11 g, 5.8 mmol). The reaction mixture was stirred at 0 ºC for one h and then at room temperature overnight. The reaction was then quenched with 6 mL of half saturated brine. The organic layer was removed and washed twice with 6 mL 0.5 M HCl, 6 mL half saturated brine, 6 mL half saturated $NaHCO_3$ and 18 mL brine. The organic layer was dried with Na_2SO_4 and filtered through a plug of fluorosil. The solvent was removed *in vacuo* yielding 1.10 g (86%) of pure white crystals. mp 172.7-175.7 ºC; IR cm^{-1} 3194, 3031, 2923, 2854, 1605, 1551, 1497, 1464, 1414, 1210; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.71-8.72 (d, 2H, J = 6), 7.81-7.83 (App q, 2H), 2.35 (t, 2H, J = 7.5), 2.21-2.25 (m, 3H), 1.78-1.84 (quint, 2H, J = 7.5), 1.59-1.64 (quint, 2H, J = 7); ^{13}C NMR (500 MHz, $CDCl_3$) δ 175.1, 166.9, 151.2, 142.1, 123.3, 84.7, 69.9, 34.4, 29.2, 25.8, 18.9; Mass: Calculated - 245.1056, Found - 245.1044.



47

1H-1,2,3-Triazole-1-acetamide, 4-(4'-isonicotinic acid pentylacylhydrazide)-*N*-[(3*S*)-tetrahydro-2-oxo-3-furanyl] (47): A scintillation vial was charged with azido homoserine lactone **15** (30 mg, 0.16 mmol) and acyl hydrazide **46** (40 mg, 0.16 mmol) followed by 1 mL deionized water and 1 mL methanol. 2,6 Lutidine (38 μL, 0.32 mmol) was then added slowly and the reaction mixture was heated to 50 ºC. Copper iodide (1.6 mg, 0.008 mmol) was added in one portion and the mixture stirred at 50 ºC for 18h. The reaction mixture was filtered and the solvent and base were removed in *vacuo*. The crude solid was purified using flash chromatography (10% Methanol in CH_2Cl_2) and recrystallized from ethanol yielding 14 mg (20%) white crystals.



18

1H-1,2,3-Triazole-1-acetamide, 4-(4'-isonicotinic acid pentylhydrazide)-*N*-[(3*S*)-tetrahydro-2-oxo-3-furanyl] (18): A scintillation vial was charged with azido homoserine lactone **15** (30 mg, 0.16 mmol) and hydrazide **45** (40 mg, 0.16 mmol) followed by 1 mL deionized water and 1 mL methanol. 2.6 Lutidine (38 μL, 0.32 mmol) was then added slowly and the reaction mixture was heated to 50 ºC. Copper iodide (1.6 mg, 0.008 mmol) was added in one portion and the mixture stirred at 50 ºC for 18h. The reaction mixture was filtered and the solvent and base were removed in *vacuo*. The crude

solid was purified using flash chromatography (15% Methanol in CH_2Cl_2) and recrystallized from ethanol yielding 14 mg (20%) white crystals.

CHAPTER 5

BIOLOGICAL SCREEN FOR ANTIMICROBIAL ACTIVITY IN NOVEL BIFUNCTIONAL BIOCIDES

Zone of Inhibition

In order to quickly determine the effectiveness of the novel biocides that include antibiological activity and quorum sensing inhibition into the same molecule, a screen for antimicrobial activity was needed. To this end, a modification of a common clinical practice was devised.[42]

Clinical microbiologists use what is called a "disc diffusion test" to determine the susceptibility of certain bacterial strains to known antimicrobials. In the clinical setting, small (~ 6 mm) filter paper discs are preloaded with a known mass of antimicrobial and placed onto a freshly inoculated agar plate bacterial lawn. At this point, the antimicrobial is able to diffuse freely into the agar surrounding the disc. This provides a crude gradient of antimicrobial around the disc that will inhibit growth where a suitably high concentration of antimicrobial is present. At the end of an incubation period, a bacterial lawn will be visible around a "zone of inhibition". The diameter of this zone can be measured and qualitatively used to determine the susceptibility of the bacteria to the antimicrobial present on the disc.

In our version of this test, a Tryptic Soy Agar plate was inoculated with a specific turbidity of *Pseudomonas aeruginosa* cultures. Filter paper discs were when pressed into

the agar plate and loaded with a solution of antimicrobial in DMF. After an 18 hour incubation the size of inhibition was measured with a ruler (Tables 2 and 3).

Table 2. Disc diffusion zones of inhibition measured on a PAO1 bacterial lawn

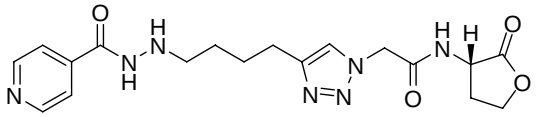
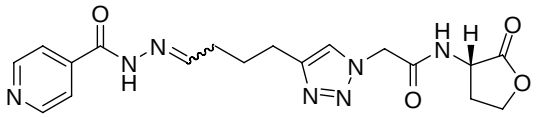
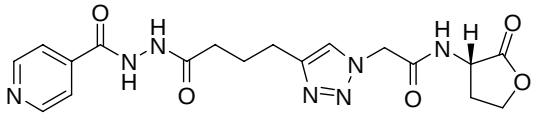
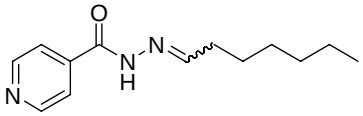
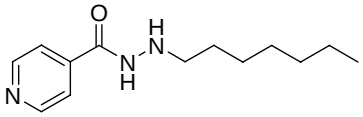
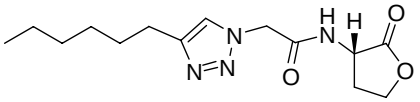
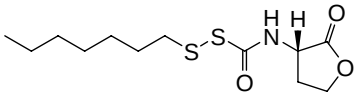
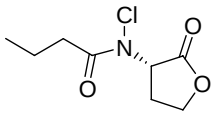
Entry	Biocide	Zone Diameter (mm)
1		not performed
2		not performed
3		not performed
4		9
5		9.5
6		6
7		8
8		8

Table 3. Zone of inhibition with known biocides

Biocide	Zone Diameter (mm)
Ceftazidime	13.5
Tobramycin	15.5
Chloramphenicol	0
Tetracycline	6
Isonicotinic acid hydrazide (Isoniazid)	14.5

Experimental Procedures

A frozen stock of PAO1 pMH509 was used to inoculate a Tryptic Soy Agar plate using a sterile streaking loop. The plate was then incubated at 37 ºC for at least 18 h at which point mature colonies were visible on the plate. The plates were then stored at ~4 ºC. Colonies were picked directly from this plate directly into a sterile solution of 0.85% NaCl. The number of colonies placed into the solution was adjusted until its turbidity roughly matched that of a 0.5 McFarland standard. 200 µL of the turbid NaCl solution was then pipetted directly onto a Tryptic Soy Agar plate. The inoculum was immediately spread across the plate using a sterile cotton swab or sterile glass applicator stick in order to obtain a uniform inoculum across the entire of the plate. Three 6 mm filter discs were then pressed gently onto the surface of the agar in an evenly spaced pattern using sterile tweezers. The discs were then loaded with 20 µL of a 10,000 µg/mL solution of the appropriate biocide. These agar plates were allowed to dry for about 3 min and then

inverted and placed in a 37 ºC incubator for 16-20 h. After this incubation period the plates were removed and the zones of inhibition were measured using a ruler.

CHAPTER 6

SUMMARY AND CONCLUSIONS

In this project effective Quorum Sensing Inhibitors have been synthesized based on the structure of naturally occurring autoinducers containing acylated homoserine lactones. The efficacy of these inhibitors has been screened using two novel assays. One of these assays used a Flow Cytometer to quantify reduction in fluorescence on a per cell basis. The second assay developed was based on the colony biofilm system. This assay is particularly noteworthy because it quantifies the ability of an inhibitor to enhance the susceptibility of a biofilm to antibiotic treatment. To date, only qualitative measurements (using microscopy) of biofilm killing in the presence of inhibitors has been performed.

Novel biocides that incorporate the acylhomoserine lactone structural motif have also been synthesized. These biocides are hypothesized to be a selective oxidizing agent, sulfenylating agent, and a targeted mutagen.

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