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A New Class of Antibiotic That Prevents Drug Tolerance, Persistence, and Resistance By Controlling Emergence of Phenotypes.

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Abstract

The fight against bacterial infections and innovations in antibiotic therapy has never halted throughout human history. However, bacteria have evolved smartly and resistance against practically all conventional antibiotics has been developed. Furthermore, bacteria can form biofilms, which are surface-attached multicellular colonies. Biofilms shield bacteria against antibiotics and makes it harder to entirely eradicate infections . Under antibiotics stress, bacteria evolve into different phenotypes like hyper motile, hyper adherent and hyper virulent which are tolerant and persistent to antibiotic treatment. The use of antibiotic therapy to combat such resilient bacterial phenotypes is extremely tough. As a result, the scientific community is always looking for novel ways to defeat these resilient phenotypes. It important to notice that all antibiotic induce tolerant and persistent phenotypes and there is no direct remedy for eradication of these phenotypes. On other hands, resistance to one antibiotic can be tackled by alternative antibiotic usage.

Here, we demonstrate a class of small molecules that inhibits a wide range of phenotypes of *Pseudomonas aeruginosa* and enables the antibiotics to kill tolerant bacteria and to prevent formation of new persistent bacteria. We identified two proteins, type IV pili and lectin LecA as receptors for our molecules by different methods, including a new label-free assay based on bacterial motility sensing the environment, chemical inhibition of bacteriophage adsorption on pili appendages of bacteria, and fluorescence polarization. Structure-activity relationship studies reveal a molecule that inhibits only pili appendage, and a class of chimeric ligands that inhibit

both LecA and pili, with important structural elements of the ligand identified for each protein. This selective control of two proteins makes the correlation between the protein receptor to their controlled phenotypes. Inhibiting LecA results in reducing biofilm formation, eliminating small colony variants, and correlates with killing tolerant bacteria and preventing the development of new persistent bacteria. Inhibiting pili appendages impedes the swarming and twitching motilities, and pyocyanin and elastase production. Because these phenotypes are controlled by a broad range of signaling pathways, this approach simultaneously control the multiple signaling mechanisms, by which bacteria elude antibiotic treatments.

Glycolipid, ganglio-N-tetraosylceramide (asialo-GM1), on the mammalian cells are known to be recognized by type IV pili of *Pseudomonas aeruginosa*. In this work, we show that asialo-GM1 can also be recognized by Lectin A (LecA), another adhesin protein of the *P. aeruginosa*, by a fluorescent polarization assay, a label-free bacterial motility enabled binding assay, and bacterial mutant studies. On hydrated semi-solid gel surfaces, asialo-GM1 enables swarming and twitching motilities, while on solid surfaces facilitates the bacterial adherence of *P. aeruginosa*. These results indicate that asialo-GM1 can modulate bioactivities, adherence, and motilities, that are controlled by opposite signaling pathways. We demonstrate that when a solution of pilin monomers or LecA proteins are spread on hydrated gel surfaces, the asialo-GM1 mediated swarming motility is inhibited. Treatment of artificial liposomes containing asialo-GM1 as a component of lipid bilayer with pilin monomers or LecA proteins caused transient leakage of encapsulated dye from liposomes. These results suggest that pili and LecA proteins not only bind to asialo-GM1 but can also cause asialo-GM1 mediated leakage. We also show that both pili and LecA mutants of *P. aeruginosa* adhere to

asialo-GM1 coated solid surfaces, and that a class of synthetic ligands for pili and LecA inhibits both pili and LecA-mediated adherence of *P. aeruginosa* on asialo-GM1-coated surfaces.

The inspired by adjuvant molecule's control over different *P. aeruginosa* phenotypes, we here designed new class of antibiotics which showed broad spectrum activity against different bacteria. This class of antibiotics inhibits pili and lecA and hence control multiple phenotypes in *P. aeruginosa*. This antibiotic also did not show emergence any tolerant population. We also did not develop any resistance against this class of antibiotics after repeated exposure.

Silver therapy is most commonly used against burn wounds to treat *P. aeruginosa* and *S. aureus* infections. These bacteria develop cooperative resistance against silver with the help of pyocyanin, a virulence factor of *P. aeruginosa*. Here, we showed Farnesol as adjuvant molecule with silver therapy against *P. aeruginosa* and *S. aureus*. Farnesol eradicates resistance against silver by inhibition of pyocyanin. Farnesol also potentiated silver against dual biofilms of P. aeruginosa and *S. aureus*.

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Chapter 1 Introduction: Challenges in antibiotic therapy against bacterial infections

1.1. Pseudomonas aeruginosa and pathogenesis.

Gram-negative bacteria have emerged as a major cause of nosocomial infections and have been associated with higher morbidity and mortality rates.[1-15] Common gram-negative bacteria and the infections they lead to include *Pseudomonas* - lung and urinary tract infections, *Escherichia coli* (*E. coli*)-food poisoning, urinary tract infections, gastroenteritis, *Klebsiella*-meningitis, and pulmonary, urinary tract, and bloodstream infections, *Acinetobacter baumannii*-wound infections, *Neisseria gonorrhoeae*-gonorrhea.[8] *Pseudomonas*, a strictly aerobic, gram-negative bacterium was discovered in 1882 by Gessard. This bacterium is ubiquitous in nature, primarily found in moist environments like soil, water, plants, and animals. *Pseudomonas aeruginosa* (*P. aeruginosa*) is the most common pseudomonal species.[9-15] and reportedly occurs in more than 50% of humans.[14] *Pseudomonas aeruginosa* is a clinically significant and opportunistic pathogen, lead cause of serious and life-threatening diseases in critically ill and immunocompromised patients.[14] The estimated 51,000 nosocomial *Pseudomonal* infections occur in the U.S. each year with 20% - 35% death rates.[9]

1.2 Antibiotic therapy against Pseudomonas aeruginosa are trouble than answer.

The antibiotic therapy used to treat pseudomonal infections includes aminoglycosides (gentamicin, tobramycin, amikacin, netilmicin), carbapenems (imipenem, meropenem), cephalosporins (ceftazidime, cefepime), fluoroquinolones (ciprofloxacin, levofloxacin), penicillin with β-lactamase inhibitors (BLI) (ticarcillin and piperacillin in combination with clavulanic acid or tazobactam), monobactams (aztreonam), Fosfomycin and polymyxins (colistin, polymyxin B).[14]

As *Pseudomonas aeruginosa* quickly acquire resistant to one antibiotic class, infections are usually treated by combinations of antipseudomonal beta-lactam antibiotics and aminoglycoside antibiotics.[14] It has been shown to use combination of carbapenems or quinolones along with aminoglycoside tobramycin in clinical setting.[14]

The copious and misuse of broad-spectrum antibiotics like beta-lactams, quinolones and aminoglycosides contribute to emergence of multi-drug resistant (MDR) pseudomonal species.[15] *Pseudomonas aeruginosa* species showed high propensity to acquire antibiotic resistance through different mechanisms including decreased permeability (porins), expression of efflux systems, production of antibiotic degrading enzymes (beta-lactamases) and target/receptor modifications.[10,16-18] *P. aeruginosa* exhibits these resistance mechanisms through both intrinsic chromosomally encoded or genetically imported resistance determinants affecting the major classes of antibiotics such as β-lactams, aminoglycosides, quinolones and polymyxins.[18-20]

Antibiotic resistance presents an alarming and rising threat over years and technological advances have been trying hard to combat antibiotic resistance.[11] Resistance is the inherited ability of microorganisms to grow in the presence of antibiotics, regardless of the duration of treatment.[11,21] Resistance factors like gene enable resistant bacteria to survive at higher antibiotic concentrations that would usually eradicate susceptible ones.[21] Resistance is measured by the MIC, which can be defined as the minimum inhibitory concentration of an antibiotic that is required to prevent growth of the culture.[21] For resistance, bacteria with a higher MIC will be regarded as more resistant than one with lower MIC.[21]

1.3 Tolerance and persistence paves the way for resistance.

Tolerance, on the other hand, prolongs the duration of antibiotic treatment that bacteria can sustain.[21,22] A tolerant cells do not carry resistance factor like gene and can regrow normally after removal of antibiotic.[21,22] Tolerance enables bacteria to survive longer duration of antibiotic exposure.[21,22] Tolerance can originate from environmental factors like nutrients availability, slow growth, biofilms or genetic.[21,22] As tolerant bacteria may have the same MIC as non-tolerant bacteria, the MIC is not informative as a metric to evaluate tolerance. Recently, the MDK (Minimum duration of Kill) was described as a quantitative measure of tolerance that can be extracted from time–kill curves.[21] The MDK is defined as the typical duration of antibiotic treatment that is required to kill a given proportion of the bacterial population at concentrations higher than the MIC. There have been two main forms of tolerance i.e. 'tolerance by slow growth' and 'tolerance by lag'.[21]

Antibiotic persistence is a phenomenon that comprise of the presence of two subpopulations, consisting of susceptible that are killed fast by the antibiotic and tolerant cells that may survive over longer period.[21,23] By definition, antibiotic persistence is always connected with a heterogeneous population, in which only a part of the population consists of tolerant cells.[21] A persister cell is a tolerant cell originating from a population that displays antibiotic persistence.[21,23-26] Persisters constitute the subpopulation (typically less than 1%) and are not killed by antibiotic treatment. (Fig. 1.1)

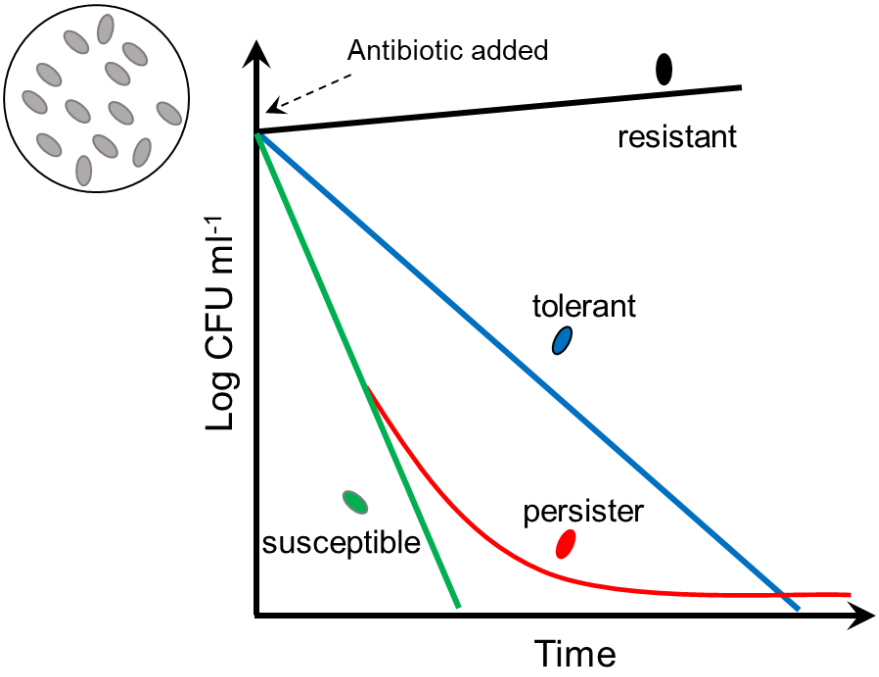
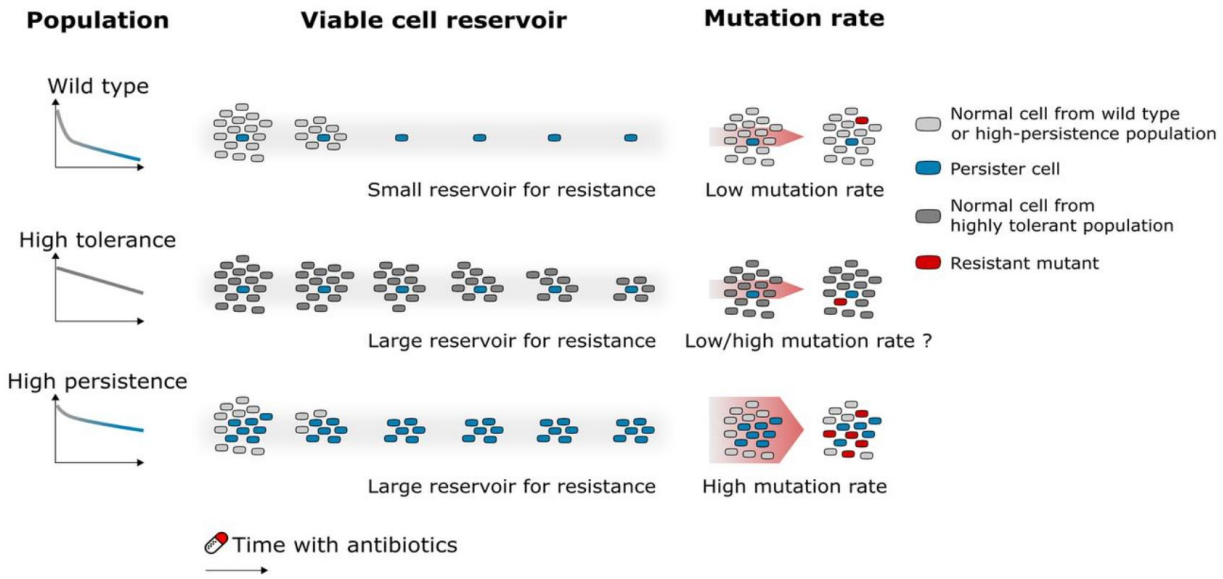


Figure 1.1 Difference between susceptible, tolerance, persistence, and resistance. The plot of bacterial population (log of colony forming units per ml) under antibiotic treatment vs time duration of antibiotic treatment.



Adopted from "E.M. Windels, J.E. Michiels, B. Van den Bergh, M. Fauvart, J. Michiels, Antibiotics: combatting tolerance to stop resistance, MBio, 10 (2019) e02095-02019."

Figure 1.2 Framework for how antibiotic tolerance and persistence accelerate the evolution of genetic resistance. First, in populations displaying high levels of tolerance or persistence, an increased number of viable cells is available for mutation, increasing the likelihood for a resistant mutant to arise. Second, persistence is linked with higher mutation rates, again causing an increased likelihood for the occurrence of resistance-conferring mutations. Future research will reveal if a similar link exists between tolerance and mutation rates.[26]

These resilience by sub-population of bacteria via tolerance or persistence is often overlooked in clinical setting and currently underappreciated role in the recalcitrance and relapse of pseudomonal infections.[15,26] It has been shown that tolerance leads to resistance in long term therapies (Fig.1.2). Unfortunately years of efforts have so far proved inadequate to curb emergence and spread of multidrug resistant species. These direct us to other resilient approaches adopted by bacteria against antibiotic therapy.[14,27-37] It has been shown that *Pseudomonas aeruginosa* exhibit wide variety of responses to different class of antibiotics and leads resilient phenotypes (Table 1).

Table1.1 Different class of antibiotics and emerging resilient phenotypes.

Class	Antibiotics	Emerging phenotypes[27-37]
β-lactams	Carbenicillin, Aztreonam	1. Biofilms 2. Filaments
Polymyxins	Colistin	1. Biofilms 2. Motility
Aminoglycosides	Tobramycin, gentamicin	1. Biofilms 2. Swarming motility 3. Small colony variants
Fluoroquinolones	Ciprofloxacin, Levofloxacin	1. Biofilms 2. Mucoid (Alginates)

Pseudomonas aeruginosa promote biofilms as response to antibiotic treatment. Biofilms are involved in a broad range of infections.[38,39] Indeed, about 80% of the chronic and recurrent microbial infections in humans are caused by biofilms, some of which result in high mortality and morbidity rates.[38-40] In addition, biofilms are highly resistant to the host immune defenses and clearance mechanisms.[40] Other nosocomial infections include those caused by biofilms strongly adhered to implants and catheters or medical devices.[40]

1.4 Pseudomonas respond to antibiotics with emergence of phenotypic resilience.

Biofilms are house of microorganisms attached to each other, or to a surface, and encased in a protective, self-produced matrix.[31,40-42] Biofilm cells are encased within a self-produced matrix comprised of extracellular polymeric substances (EPS) including polysaccharides, extracellular DNA (eDNA), proteins and lipids.[31,41,42] The EPS matrix provides protection for the cells inside biofilms by acting as a shield and is central to biofilm structure and integrity.[31]

The sub-inhibitory concentrations of beta-lactam antibiotics stimulate biofilm formation in *P. aeruginosa* but mechanism of promotion is still unknown. Aminoglycosides antibiotics promote biofilms through aminoglycoside response regulator *(arr)* gene.[33] Interestingly, repeated antibiotic usage has led to emergence of phenotype in clinical setting called as small colony variants (SCVs) which exhibit high resistance to antibiotic treatments.[43-45] SCVs are defined as colonies whose size is 5 or 10 times smaller than normal colony phenotype.[43-45] Compared to wild-type *P. aeruginosa*, SCVs show increased antibiotic resistance, enhanced biofilm formation, reversion to wild-type-like morphotypes, reduced motility, and slow and auto-aggregative growth behavior.[43,44] The clinical patients small colony variants were observed with long term tobramycin treatment.[43]

Antibiotics are shown to behave like intermicrobial signaling molecules in *P. aeruginosa*.[30,46,47] Tobramycin increases bacterial motility, and tetracycline triggers expression of *P. aeruginosa* type III secretion system and consequently bacterial cytotoxicity.[30,47] Interestingly, aminoglycoside antibiotics also promote swarming motility in *P. aeruginosa*.[47] Beta-lactam antibiotics with dose-related affinities for penicillin-binding proteins (PBPs) and at low concentrations, these antibiotics inhibit PBPs, leading to filament formation.[48,49] Filaments are long strands of non-dividing bacteria that contain enhanced quantities of endotoxin molecules. Filamentous bacteria show enhanced resilience to antibiotic treatment.[49]

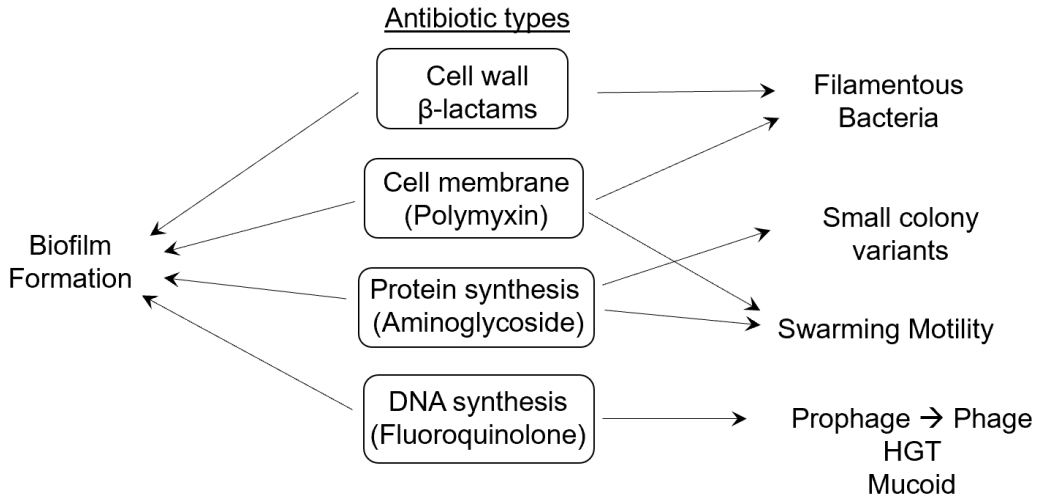


Figure 1.3 Map of antibiotics and promoted bacterial phenotypes that increases their resilience (tolerance, persistence, or resistance). Arrows indicate promotion of the phenotype.

Prophages contribute significantly to resistance to sub-lethal concentrations of fluroquinolones and β-lactam antibiotics primarily through proteins that inhibit cell division. Fluroquinolones induce prophage that mediates horizontal gene transfer.[50-53] Moreover, the prophages play vital role for withstanding osmotic, oxidative, and acid stresses, for increasing growth, and for augmenting biofilm formation Fluroquinolones antibiotics also induce SOS response in *P.aeruginosa* which leads to formation of filamentous bacteria.[51-53] Ciprofloxacin also enhances alginate production in *P.aeruginosa resulting* in mucoid phenotype.[54] Polymyxin antibiotic like colistin induce biofilm formation and these biofilms harbors colistin tolerant cells. These colistin tolerant cells use *P. aeruginosa* type IV pili mediated motility for survival against colistin.[55]

Overall, conventional antibiotics are suffering from either motility mediated tolerance or biofilms mediated tolerance in *P. aeruginosa*. Motilities in *P. aeruginosa* governed by mostly

surface appendages like flagella, pili, and fimbria. There are multiple factors that contribute to biofilm formations but Lectins serve as glue for biofilms and play major role in structure and integrity of *P. aeruginosa* biofilms.[56] Some appendages like pili also play crucial roles in biofilm formation.[57] We believe that antibiotics induce augmented activities of these receptor proteins and lead to enhanced motilities or biofilm formations.

1.5 This dissertation is organized in the following 6 chapters.

In chapter 2, we design and synthesize ligands which targets two different protein receptors in *P. aeruginosa*. We identify two proteins, type IV pili and lectin LecA as receptors for our molecules by different methods, including a new label-free assay based on bacterial motility sensing the environment, chemical inhibition of bacteriophage adsorption on pili appendages of bacteria, and fluorescence polarization. We also discuss structure-activity relationship studies which shades light on how a class of chimeric ligands that inhibit both LecA and pili. Here, we show how chimeric ligands control different phenotypes in *P. aeruginosa*. We also describe how chimeric ligands eradicate antibiotic induced tolerance and prevent formation of new persister populations in *P. aeruginosa* biofilms.

In chapter 3, we expand role type IV pili and Lectin A as adhesin in *P. aeruginosa* infections. We show how pili and Lectin A of *P. aeruginosa* recognize glycosphingolipid present in mammalian cell membrane and initiate infection. We describe how *P. aeruginosa* use pili and Lectin A proteins as adhesins and bind to mammalian cells and mediate motilities or adherence. Here, we show extended application of chimeric ligands from chapter 2 as anti-adhesin molecules. We present chimeric ligands as anti-adhesin molecules which prevent motility and adherence mediated by glycosphingolipid binding to pili or LecA. We also show how binding of

glycosphingolipid to pili or LecA cause transient leakage in artificial liposomes which mimic biological membranes. This provide probable mode of interaction of bacteria and mammalian cells.

In chapter 4, we design and synthesize new class of antibiotic which inhibits functions of penicillin binding proteins of gram-positive and gram-negative bacteria. Here, we show broad spectrum activity of new class of antibiotic and control over resilient phenotypes of *P. aeruginosa*. We also demonstrate that how this antibiotic is chimeric and targets pili and LecA. We also perform multistep resistance studies to determine emergence of antibiotic resistance.

In chapter 5, we illustrate power of naturally occurring terpenoid Farnesol against burn wound microbiome. We also demonstrate how *P. aeruginosa –Staphylococcus aureus* help each other in burn wound environment against silver therapy and develop resistance. We show how farnesol inhibit virulence factors like pyocyanin in *P. aeruginosa* and eradicate collaborative silver resistance in *P. aeruginosa –Staphylococcus aureus* coinfection.

In chapter 6, the conclusions and summary of our findings in this thesis are discussed. We also present the existing open questions and future directions of development of this new class of antibiotic.

Chapter 2 Chimeric Ligands of Pili and Lectin A Inhibit Tolerance, Persistence, and Virulence Factors of *Pseudomonas aeruginosa* over A Wide Range of Phenotypes.

2.1 Background and Significance

Bacteria exhibit adaptive responses to a wide range of stresses from environmental cues to antibiotic treatments leading to many resilient phenotypes,[33,43,55,58-72] including augmented biofilm formation,[33,60] enhanced motilities,[63,64] surface attachment,[66-68] chemical induced virulence,[62,69] and emergence of small colony variants (SCVs).[43,70,71] These diverse phenotypes are often controlled by or correlated to both high and low levels of the global small molecule messenger, bis-(3'-5')-cyclic dimeric guanosine monophosphate (cdG).[73-76] For both in vitro and in vivo conditions, antibiotics treatments or host immune defense can trigger the increase in cdG levels in bacteria, leading to phenotypes that exhibit high drug tolerance and persistence, including biofilms,[33,72] small colony variants (SCVs)[43,70,71] and overexpression of exopolymeric substances (EPS).[77]

Recent studies revealed that low cdG phenotypes can also be highly virulent.[55,69,76] Swarming bacteria, a low cdG phenotype, requires rhamnolipids[78-80] and exhibit antibiotic resistance.[64,65] Dispersing bacteria from biofilms by nitric oxide donor[69] switches bacteria to low cdG phenotypes and causes increased production of virulence factors, including induction of type II and III secretion system, which has been shown to kill even macrophage.[69] Surprisingly, under different conditions, the same antibiotic, tobramycin, can promote P. aeruginosa to form either high or low cdG phenotype to counter the stresses. Sub-lethal dose of tobramycin promotes the high cdG phenotypes including increased biofilms and increased exopolymers

production,[33] and causes formation of SCVs in vivo.[71] In contrast, on hydrated agar surface, tobramycin also promotes the swarming motility of P. aeruginosa.[63] These findings indicate that, although the two types of protein domains, diguanylate cyclases and phosphodiesterases, that regulates the synthesis and hydrolysis of cdG are well-established,[73,75] how they respond to external stresses is complex and still unpredictable. More importantly, these findings suggest that inhibiting high cdG phenotypes alone, such as biofilms formation, would not be sufficient to control drug tolerance and persistence as bacteria can transition readily between high and low cdG phenotypes.

In past study, we showed a broad class of disugar-derivatized hydrocarbons that inhibit both biofilm formation and swarming motility of P. aeruginosa bacteria.[81,82] These dual inhibitors are intriguing as biofilm formation and swarming motility are controlled by the opposite signaling pathways mediated by high and low cdG levels, respectively.[74] Because both high or low cdG phenotypes can be virulent, and bacteria can readily transition between these phenotypes, this class of molecules provide opportunities to control drug tolerance, persistence, and resistance; and also block bacterial signaling that escape drug treatments by transitioning between phenotypes.

In this work, we demonstrate a specific class of molecules that inhibits a wide range of phenotypes that are associated with both high and low cdG levels. These phenotypes include formation of biofilms, small colony variants, pellicles; and swarming and twitching motilities, and production of virulence factors including pyocyanin and elastase. Antibiotics like tobramycin promote these phenotypes[33,70,83] and these phenotypes are shown to be dwelling in chronic and acute infections.[33] We also demonstrate that, when combined with tobramycin, this class of

molecules enables the killing of tolerant bacteria and prevents of the formation of nascent persistent bacteria in biofilms.

To elucidate the mechanism behind this simultaneous control of the wide range of phenotypes associated with opposite signaling pathways (low and high cdG levels), we identify pili appendages and LecA as two protein receptors targeted by the same small molecule (Fig. 2.1).

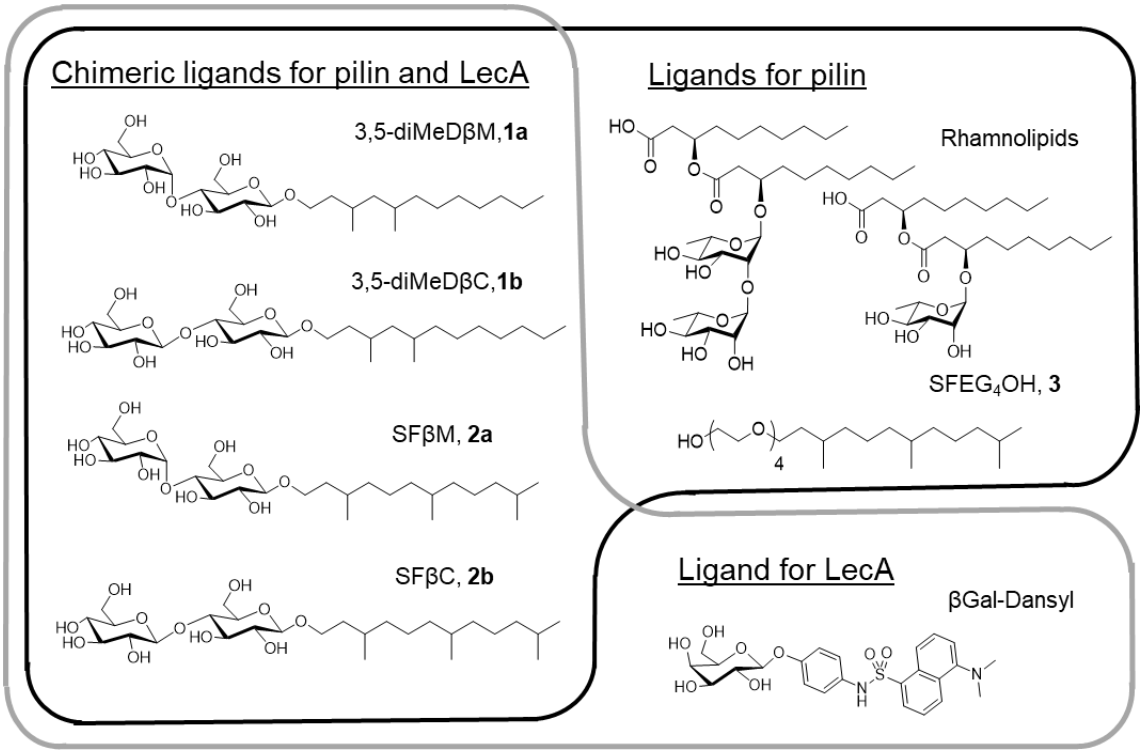


Figure 2.1 Venn diagram of structures of chimeric ligand molecules for type IV pili and Lectin LecA proteins, and ligand molecules for only pilin, and only LecA.

We confirmed the binding of small molecule to pili by multiple methods, including a novel bacterial motility enabled binding assay, which is modified from the swarming experiment without any chemical labeling, and the small molecule inhibition of pili-specific bacteriophage adsorption on of *P. aeruginosa*. The LecA binding was characterized by fluorescence

polarization method and by the bacterial motility enabled binding assay without chemical modification. Structure-activity studies identified the different structural elements of the small molecule ligands that are important for each protein, pilin and LecA, making this class of molecule a structural chimera. The selective ligand-receptor bindings of two proteins reveal the mapping between the proteins and the phenotypes they control.

2.2 Results and discussion

2.2.1 Design and synthesis of ligands

Our past studies reveal that two most active molecules among these dual synthetic inhibitors as saturated farnesol-β-cellobioside or -β-maltoside (SFβC and SFβM, Fig. 2.1.1).[81] Early structural studies indicates that the methyl substitution in the aliphatic chains is important: linear C12-aliphatic chain does not exhibit strong bioactivity, whereas the 3,7,11-trimethyl substituted aliphatic chain does.[81,82] In search for more potent molecules through synthesis of structural variants, we explored new methyl positions on the C12-alphatic chain resulting in new molecules 3,5-diMeDβM and 3,5-diMeDβC (Fig. 2.2 and Fig. S1-S16).

We synthesized alcohol precursor, 3,5-dimethyldodecanol from alkylation of cyclic ketone (3,5-dimethylcyclohexanone) followed by Baeyer-Villiger oxidation. Ring opening was followed by mesylation and one-step reduction of the ester and the mesylated alcohol to generate the desired branched alcohol. Glycosylation with protected maltoside and cellobioside, followed by basic deprotection generated the final products of 3,5-dimethyl-dodecyl maltoside 3,5-diMeDβM, and 3,5-dimethyl dodecyl cellobioside 3,5-diMeDβC (Fig. 2.2).

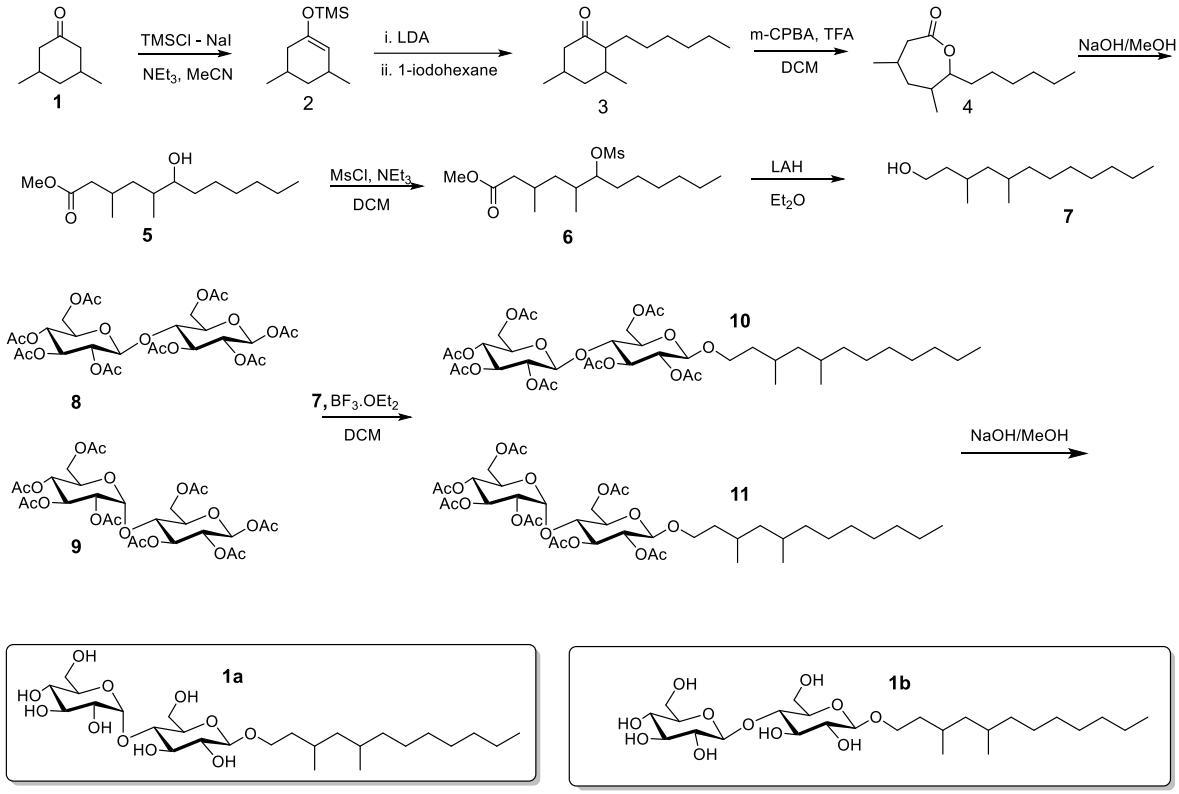


Figure 2.2 Synthetic scheme for 3,5-diMeDβM (**1a**) and 3,5-diMeDβC (**1b**).

We found these disugars tethered with 3,5-dimethyl substituted dodecyl group exhibited stronger biofilm inhibition activities against *P. aeruginosa* with an IC_{50} of 29 µM and 36 µM, respectively, whereas SFβM/βC from past studies showed IC_{50}s of 73 µM and 52 µM, respectively (Table 2.1). Here, we explore these molecules for controlling wide range of bacterial activities induced by sub-MIC of the antibiotic tobramycin.

Table 2.1 3,5-diMeDβM/βC are more potent than SFβM/βC at inhibition and dispersion of wild type *P. aeruginosa* (*wt* PAO1) biofilms.[a]

Molecule	**Biofilm Inhibition IC_{50} ± s.d. [μM]**[b]		**Biofilm Dispersion DC_{50} ± s.d. [μM]**[c]	
	w/o Tob	w/ 0.3 μg/ml Tob	w/o Tob	w/o 0.3 μg/ml Tob
SFβM	73 ±2 μM*	85±4.6 μM	126±5.5 μM*	>150 μM
SFβC	52±2.4 μM*	79±3.3 μM	83±3.3 μM*	>150 μM
3,5-diMeDβM	36±2.2 μM	62±2.2 μM	54±5.5 μM	98±3.3 μM
3,5-diMeDβC	29±3.2 μM	58±2.1 μM	48±4.6 μM	95±2.9 μM

a. The biofilms were characterized by a Crystal violet staining assays on the pegs of MBEC™ microtiter plates b. IC_{50} values were obtained by plotting concentration of the molecule against % biofilm inhibition after 24 h without shaking at 50% of biofilm inhibition. c. DC_{50} values were obtained by plotting concentration of molecule against % biofilm dispersion after 24 h with shaking at 100 rpm shaking, at 50 % biofilm dispersion. Experiments were performed in triplicate and the results are an averaged value; error bars reflect the standard deviation of each point. The curve fitting was performed with GraphPad Prism 6.0 software.*indicated values extracted from literature[81]

2.2.2 Reversing Tobramycin Induced Phenotypes of P. aeruginosa

In general, antibiotics can promote biofilm formation.[33,60] Sub-inhibitory concentration of tobramycin causes two-fold increase in biofilm mass formed by wild type *P. aeruginosa* (wt PAO1) on abiotic surfaces including glass, polystyrene, and stainless steel.[33] The small molecules 3,5-diMeDβM and 3,5-diMeDβC not only inhibit biofilm formation (Table 2.1), but also inhibited the promotion of biofilm formation by 0.3 μg/ml tobramycin (0.3-Tob) and

reduced 0.3-Tob stressed biofilm mass by 80%, revealed by crystal violet staining. For biofilms formed by using wild type *P. aeruginosa* (PAO1) constituently expressing green fluorescent protein (GFP), we validated that these molecules inhibited formation of unstressed biofilms (Fig. 2.3), with a reduction of biofilm mass by ~55 % (from 27.5 $\mu m^3/\mu m^2$ to ~12.3 $\mu m^3/\mu m^2$). This assay demonstrated that 0.3-Tob caused 40% increase in biofilm mass (from 27.5 to 38.6 $\mu m^3/\mu m^2$) on polystyrene surfaces, and combination with 3,5-diMeDβM/βC (85 μM) reduced the biofilm mass by 50% (Fig. 2.4A).

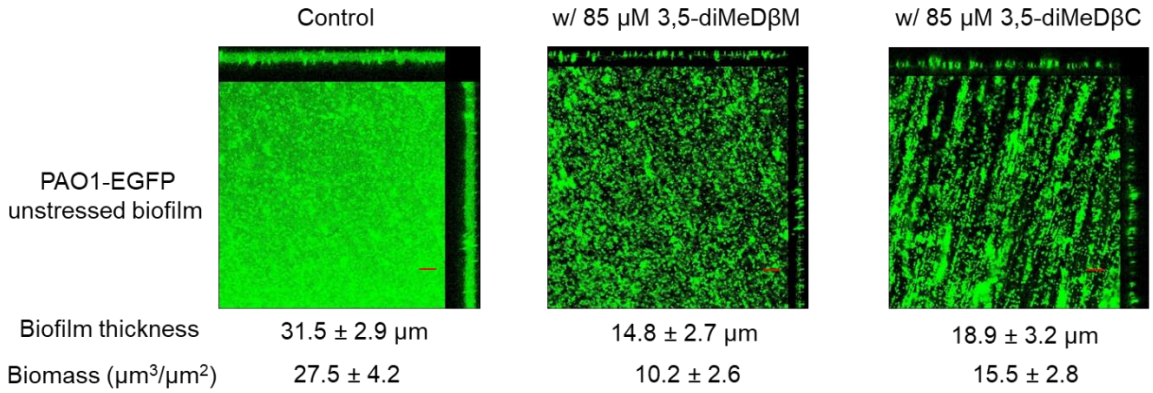


Figure 2.3 3,5-diMeDβM/βC inhibit unstressed biofilm formation in *P. aeruginosa*. 24 h biofilms of GFP-tagged PAO1 were grown in M63 medium with and without 85 μM 3,5-diMeDβM/βC on polystyrene surface. Images were acquired by confocal laser scanning microscope. The central pictures show horizontal optical sections, and the flanking pictures show vertical optical sections. *P. aeruginosa* biomass was quantified with the COMSTAT program. Scale bars, 20 μm. Emission of 488 nm was used to visualize live cells within the biofilms formed by PAO1-EGFP strain. The biomasses fluorescing green reveal the amounts of living bacteria in a biofilm. The COMSTAT program was used to report the percentage of live biomasses in the stacks of images.

Antibiotics of aminoglycosides causes the bacteria to form phenotypes of small colony variants (SCVs) in lungs of cystic fibrosis patients; these SCVs are tolerant in clinical

infections.[70-72] We discovered that, *in vitro*, sub-inhibitory amount of tobramycin also caused SCVs formation (Figure 3B). When *wt* PAO1 were cultured with 0.3-Tob for 6 h followed by growth on Columbia agar gel (24 h) containing 0.3-Tob, 13-fold of SCVs (1 mm in diameter, 4.2×10^7 CFU/ml) was observed over wild type colonies (1 cm in diameter, 3.2×10^6 CFU/ml). Under the same condition, combining 85 μM of 3,5-diMeDβM/βC with 0.3-Tob abolished formation of SCVs entirely (Fig. 2.4B).

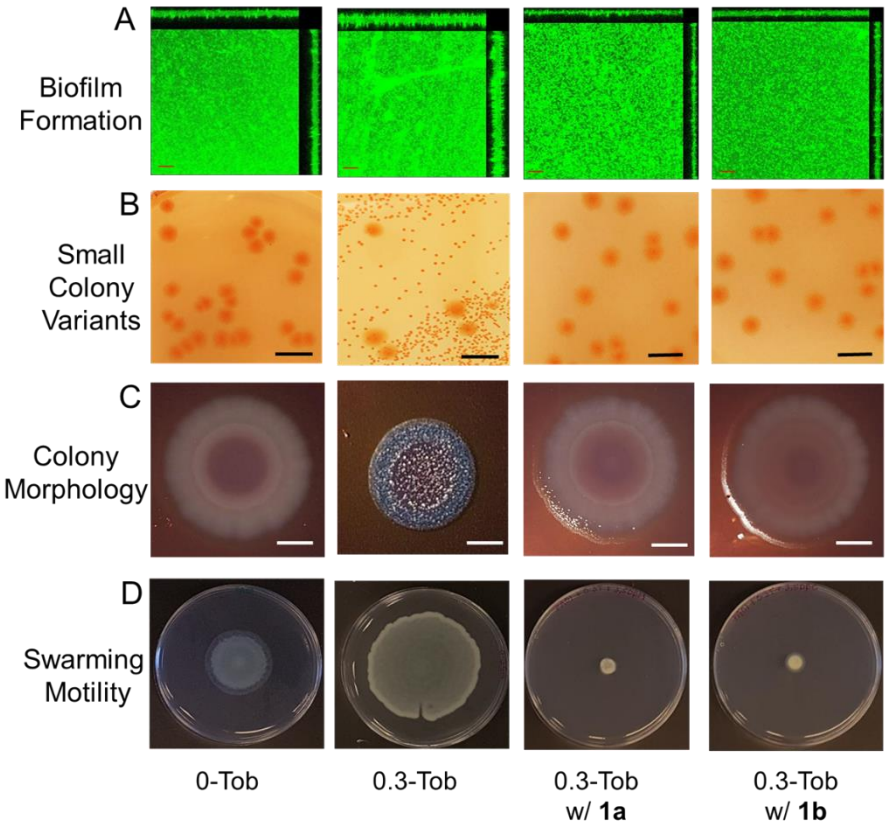


Figure 2.4 (A) Fluorescent images of 24 h unstressed (0-Tob) and 0.3 μg/ml tobramycin (0.3-Tob) stressed biofilms of GFP-tagged *wt* PAO1 grown in M63 medium on polystyrene surface with and without 85 μM of 3,5-diMeDβM/βC. Scale bar = 20 μm. (B) Images of *wt* PAO1 colonies (37 ℃, 3 days) on Columbia blood agar plates. Scale bar = 2 cm. (C) Colony morphology of *wt* PAO1 inoculated on 1% agar plates containing Congo red and Coomassie

brilliant blue dyes. The colonies were grown for 3 days at 37°C. Scale bar =1 cm. (D). Images of swarming patterns of 3 μL of *wt* PAO1 inoculated for 24 h on soft gels (0.5 % agar in LB). The agar plates for SCV, colony morphology, and swarming motility contain 0-Tob, 0.3-Tob, with and without 85 μM of 3,5-diMeDβM/βC.

Antibiotics are also known to increase the production of extracellular polymeric substances (EPS) composed of polysaccharides and proteins.[84] To directly visualize effect of 3,5-diMeDβM/βC on overall EPS, *wt* PAO1 culture (OD_{600}= 0.6) was inoculated on Congo red containing hard agar plates supplemented with 0.3-Tob for 3 days with and without the presence of 3,5-diMeDβM/βC. We found that 0.3-Tob caused rugose/wrinkled colony morphotype (diameter, 2 cm), which is caused by Congo red binding to the exopolysaccharides. In contrast, including 85 μM 3,5-diMeDβM/βC with 0.3-Tob resulted in large colony morphotype that is similar to normal wild type morphotype in size (diameter, 3 cm) and smooth texture (Fig. 2.4C). For pellicle formation,[84] which represent pel polysaccharide among the three types of exopolysaccharides, 0.3-Tob caused increased pellicle (3 days without shaking) at air-liquid interfaces by 60%, whereas including 3,5-diMeDβM/βC (85 μM) completely abolished effect of 0.3-Tob at promoting pellicle formation (Fig. 2.5).

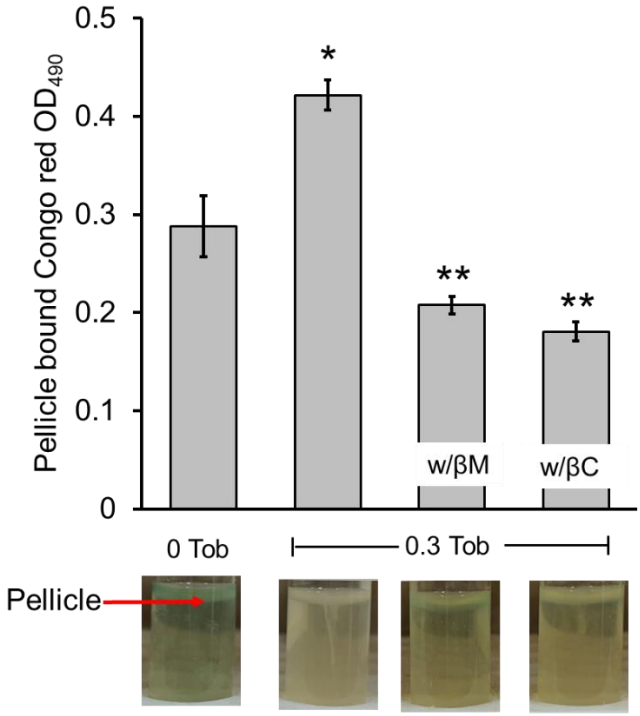


Figure 2.5 3,5-diMeDβM/C inhibit 0.3-Tob induced pellicles in static wild type *P. aeruginosa* cultures. Pellicle formation in *wt* PAO1 were observed and quantified by Congo red (CR) binding assay. Pellicles were formed for 3 days under static conditions. Overnight *wt* PAO1 bacterial cultures were diluted 1:100 in LB medium without NaCl with and without 85 µM 3,5-diMeDβM (βM) or 3,5-diMeDβC (βC) in the presence of 0.3 µg/ml tobramycin (0.3-Tob). The cultures were grown statically (in glass tubes) for 3 days at 37°C. Then Congo red binding by pellicles was calculated as the difference between absorbance for reference solution containing the starting concentration of Congo red (40 µg/ml) and the absorbance of the supernatant. Error bars indicate standard deviations of independent triplicates. The pellicles formed without tobramycin (0-Tob) was considered as control. *P = 0.02 vs control; **P <0.005 vs control as evaluated using two-tailed unpaired Student's t-test.

Subinhibitory concentrations of tobramycin also promote swarming motility in *P. aeruginosa*.[63] This particular response to antibiotic exposure is intriguing because swarming and biofilm formation are inversely regulated in *P. aeruginosa*.[74,75] We show that having 0.3-Tob in

the soft gel (0.5 wt% of agar) caused the swarm area to increase from 24 cm^2 to 30 cm^2 (about 25 %). When the gel supplemented with 85 μM of 3,5-diMeDβM or 3,5-diMeDβC, the swarming motility was inhibited entirely in the presence of 0.3-Tob (Fig. 2.4D). Together, these results showed that 3,5-diMeDβM/βC inhibit both high and low cdG phenotypes that are promoted by tobramycin.

2.2.3 Inhibition of Tobramycin-Induced Persistent Populations in Biofilms

High doses of antibiotics are often used to kill bacteria in biofilms, which inevitably lead to antibiotic tolerance and persistence.[58,60] We first studied the effect of our molecule on persistent and tolerant bacteria in unstressed and 0.3-Tob stressed biofilms. We used a reported isolation method for persistent population,[85] for which all biofilms were sonicated for 15 mins in saline to release the bacteria from the biofilms into a saline solution. The saline solution was treated with 20 μg/ml tobramycin to isolate the persistent population, which was then enumerated on agar plates without antibiotics. This procedure kills the viable biofilm bacteria, including the tolerant population, resulting in isolating the persistent bacteria developed during the drug/agent treatment of biofilm, which will revitalize in the absence of antibiotics.[85] We found that there were about 10 times more persistent bacteria in 0.3-Tob stressed biofilms (1.2×10^5 CFU/ml) than in unstressed biofilms (1.7×10^4 CFU/ml) (Fig. 2.6A).

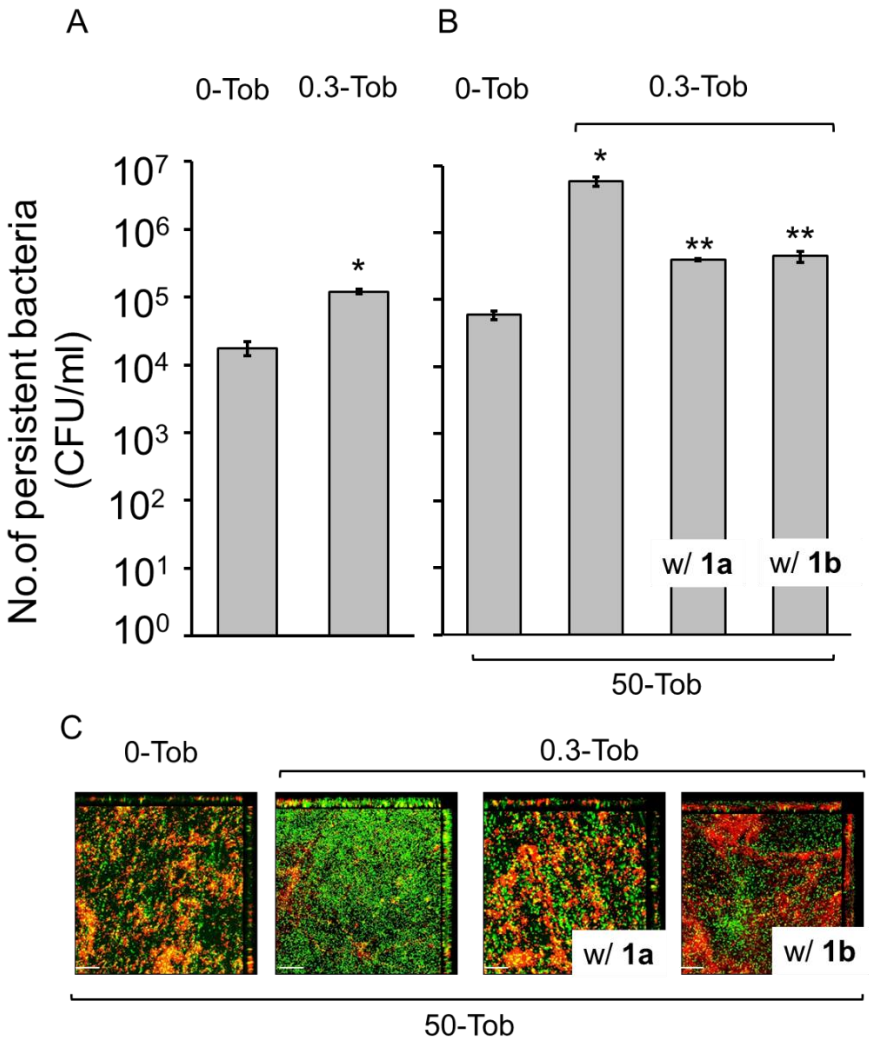


Figure 2.6 Persistent bacterial count in (A) unstressed (0-Tob) and 0.3-Tob stressed biofilms PAO1 biofilm (24-h) without 50-Tob treatment. Error bars indicate the standard deviations of means of triplicates. *$P < 0.001$ vs 0-Tob biofilm. Student's t-test. (B) unstressed and stressed PAO1 biofilm (24 h) that were further treated with 50-Tob in LB, with and without 3,5-diMeDβM (βM) and 3,5-diMeDβC (βC) for another 24 h. *$P < 0.05$; **$P < 0.01$ vs 0-Tob biofilm. Student's t-test. The types of biofilms are shown above the bars, and 50-Tob treatments with and without agents are indicated below. (C) The confocal fluorescent images of unstressed and stressed biofilms of GFP-tagged PAO1 (24 h), further treated with 50-Tob, and with and

without 85 μM 3,5-diMeDβM/βC for another 24 h. The biofilms were stained with propidium iodide dye to show living (green), and dead (yellow/red) bacteria. Scale bar = 20 μm.

To evaluate the effect of high dose of tobramycin on increasing persistent bacteria in a biofilm, 50 μg/ml tobramycin (50-Tob) was applied to already formed biofilm to kill biofilm bacteria.[85,86] When unstressed and 0.3-Tob stressed biofilms were treated with 50-Tob for another 24 h, about 100 times more persistent bacteria were observed in 0.3-Tob stressed biofilm (~5.8×10^6 CFU/ml) than in unstressed biofilm (~5.9×10^4 CFU/ml) (Fig. 2.6B). These results indicate that high dose of tobramycin caused certain precursor bacteria in biofilms to transition to persistent populations. Comparing to without 50-Tob treatment, unstressed biofilm increase the persistent population by about 3 times; but 0.3-Tob stressed biofilms increase about 48 times. Together, stressing biofilms with at sub-MIC tobramycin (0.3-Tob) causes considerable amount of precursor bacteria that can readily transition to persistent populations.

To evaluate the effect of our agents on the persistent population, molecule **1a** or **1b** (85 μM) was combined with 50-Tob treatment. In the presence of 85 of either **1a** or **1b**, 50-Tob did not cause the increase of persistent population seen without the agent (Fig. 2.6B), suggesting that our agents prevents the development of nascent persistent population caused by high applying dosage of tobramycin on biofilms.

To further validate the above results, we grew unstressed and 0.3-Tob stressed biofilms of GFP-tagged PAO1 on polystyrene chips (~1 cm^2) and treated the preformed biofilms with 50-Tob for another 24 h and monitored the development of live and dead subpopulations by green and red fluorescence, respectively, by confocal image acquisition. We found that treatment of 50-Tob caused about 8 times more dead bacteria in unstressed biofilms than 0.3-Tob stressed

biofilms. When combining 85 μM 3,5-diMeDβM/βC with 50-Tob, a substantial decrease in live bacteria, and about 10 times increase in dead bacteria was seen in 0.3-Tob stressed biofilms (Fig. 2.6C).

2.2.4 Killing Tobramycin-Tolerant Bacteria and Preventing Emergence of Nascent Persistent Bacteria in Biofilms

The rate of killing susceptible populations reveal how drug-tolerant the populations are. We believe that the nascent persistent population are transitioned from drug-tolerant bacteria.[61,72,87] To explore this transition, and to examine the source of the nascent persistent bacteria in 0.3-Tob stressed biofilm due to 50-Tob treatment, we performed a time-kill study over 24 h, and used the Minimum Duration for Killing 99.9 % ($MDK_{99.9}$) of total bacterial population as a measure of the tolerance of the bacteria.[72,87] Treating the 24-h old biofilms with 50-Tob over 24 hours showed a rapid decrease of viable bacteria for the first 18 h, followed by plateaus for the rest of the 6 h (Fig. 2.7). This biphasic killing curve is consistent with a mixture of susceptible, tolerant, and persistent population. We found the $MDK_{99.9}$ for unstressed biofilms to be about 12 hours, whereas for 0.3-Tob stressed biofilms, about 18 hours (Fig. 2.7), indicating that there was a higher population of tolerant bacteria in 0.3-Tob stressed biofilms than in unstressed biofilms.

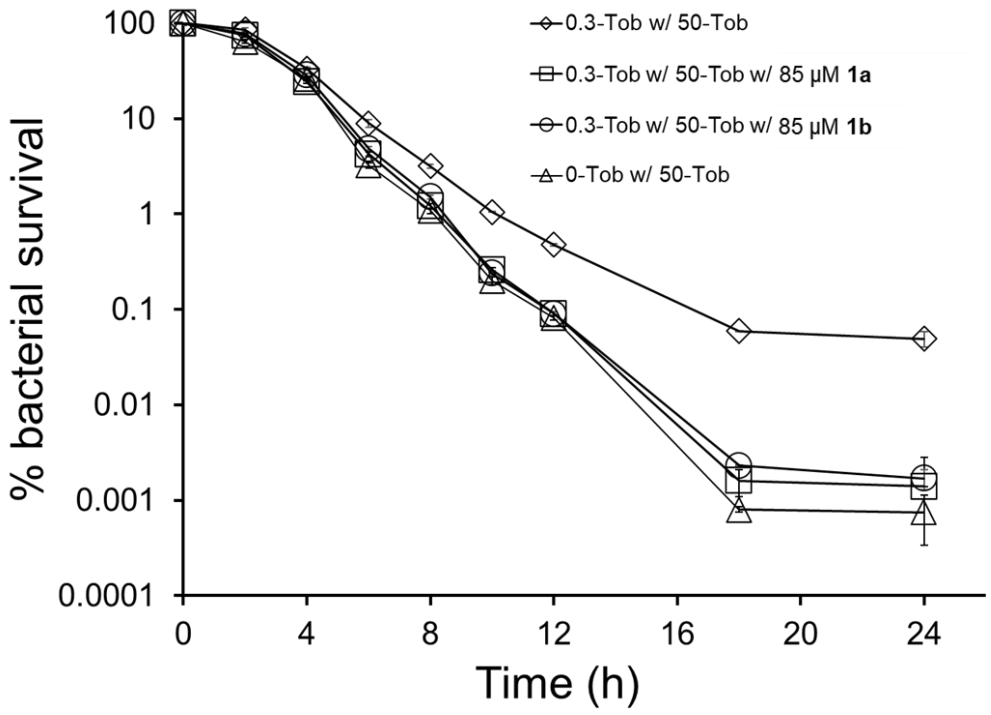


Figure 2.7 Percentage of bacterial survival (CFU during 50-Tob treatment/initial CFU) of 0.3-Tob stressed biofilms and unstressed biofilms of *wt* PAO1 (24 h) further treated with 50 μg/ml tobramycin, and with and without 85 μM of 3,5-diMeDβM or 3,5-diMeDβC, for different time intervals at 37 °C. Error bars indicate the standard deviations of means of triplicates.

These results indicate that 0.3-Tob stressed biofilm contains high level of tolerant populations, and the high dose 50-Tob treatment converts these tolerant bacteria into persistent populations in biofilms. Combining 50-Tob with 85 μM of **1a** or **1b** reduced the $MDK_{99.9}$ from ~ 18 to ~ 12 hours (Fig. 2.7). This killing rate is similar to that for the unstressed biofilm and suggests that the molecules enabled 50-Tob to kill the pre-existed tolerant bacteria in the 0.3-Tob stressed biofilms. Between 18-24 h, the number of viable bacteria started to plateau for all biofilms. For 0.3-Tob stressed biofilms, with the presence of 1a or 1b, the bacterial count

reduced to ~3.9×10^5CFU/ml without our molecules, ~5.8×10^6 CFU/ml; further confirming that our molecules prevented the formation of nascent persistent bacteria. Overall, our molecules enable the high dose tobramycin treatment to reduce 100-fold of persistent bacteria in tobramycin- stressed biofilms.

To understand the mode of action, we first confirmed that these molecules do not affect planktonic growth of *wt* PAO1. We grew the wild type PAO1 in the presence of 85 μM 3,5-diMeDβM for 24 hours. We found that 3,5-diMeDβM does not affect growth of wild type *P. aeruginosa* (Fig. 2.8).

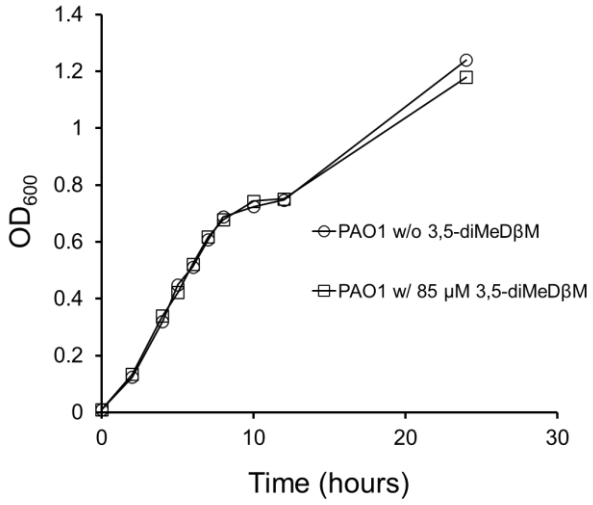


Figure 2.8 3,5-diMeDβM does not affect growth of wild type *P. aeruginosa*. The diluted overnight *wt* PAO1 culture (1:100) in MHB with and without 85 μM 3,5-diMeDβM were incubated for 24 h under 250 rpm shaking conditions and optical density OD_{600} was observed during 24 h .

The glycolipids 3,5-diMeDβM and 3,5-diMeDβC contains both the hydrophilic sugar groups and the lipophilic lipid residues, they likely to behave like amphiphiles. The amphiphiles will self-assemble in aqueous solution beyond critical aggregation concentration.[88] Because of

their amphiphilic character, we characterized the critical aggregation concentrations (CAC) by a Nile red assay. The Nile red dye is minimally fluorescent in water and fluorescence intensity of Nile red increases in hydrophobic micellar environment.[89] We found that 3,5-diMeDβM/βC self-aggregate at 134 and 140 µM, respectively in water (Fig. 2.9). All bioactivities against wild type *P. aeruginosa* and their mutants were observed at concentrations lower than critical aggregation concentration for respective glycolipids molecules.

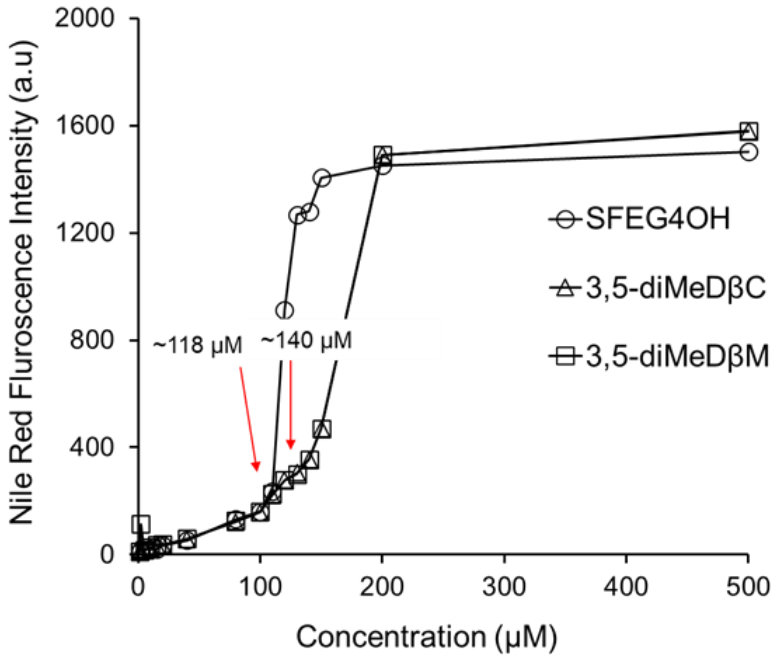


Figure 2.9 The critical aggregation concentrations for 3,5-diMeDβC, 3,5-diMeDβM, and $SFEG_4OH$ was ~140 µM, 138 µM and 118µM. Nile red fluorescence intensity against concentration of 3,5-diMeDβC, 3,5-diMeDβM, and $SFEG_4OH$ in deionized water. The critical aggregation concentrations (CAC) correspond to the concentration at the transition of increase of Nile red fluorescence.

2.2.5 Protein Receptors that Control Opposite Signaling Pathways

To identify potential proteins that are the receptors to our molecule, we consider type IV pili and Lectin A protein on bacteria. We believe that inhibition of these receptors also controls the opposite cdG signaling leading to different phenotypes. From structural point of view, both proteins bind sugar moieties,[90,91] and hydrophobic moieties are reported to be important pili,[90] and hydrophobic spacer between multivalent sugar molecules are vital for LecA inhibition.[91] In addition, type IV pili is necessary for swarming and twitching motility;[78,92,93] and our molecules inhibit both motilities. For signaling, *P. aeruginosa* utilize type IV pili proteins that transduces environment cues to signaling events leading to different phenotypes [66-68,93-97] and facilitate horizontal gene transfer.[98] In contrast, for biofilm formation, pili appendages initiate surface attachment that eventually triggers the transition into high cdG phenotypes, which lead to antibiotic tolerance.[66,96] Interestingly, before colonization and biofilm formation, pili-mediated surface sensing increases the quorum sensing that promote pyocyanin production and induces type II secretion system.[67] These virulent phenotypes are the same low cdG phenotype caused by dispersing bacteria from the biofilms.[69] Small colony variants of *P. aeruginosa*, which exhibit high cdG levels, are also hyperpiliated.[71] Thus, pili appear to mediate transitions between low and high cdG levels and are important for virulence production at both end of signaling. Inadvertently, type IV pili of *P. aeruginosa* have been shown to assemble over wide range of cdG levels.[94]

Another adhesin LecA, is crucial for biofilm formation.[91,99-102] The LecA is positively correlated to biofilm formation.[100] The mutant that overproduces LecA forms more biofilms, whereas the mutant of lacking LecA has low level of cdG and form weak/loose biofilm[100]. Thus, LecA mainly correlates high cdG phenotypes, and has been considered as a therapeutic target.[101] Chemical inhibition of LecA has led to inhibition of biofilm formation.[91,101]

2.2.6 Direct Ligand-Pilin Interaction based on Bacterial Motility Enabled Binding Assay

Type IV pili (TFP) are one of appendages on the surface of bacteria. They are involved in bacterial movement, such as twitching motility, swarming motility in *Pseudomonas* and *Neisseria* species,[96,103] and are essential for microbial adhesion.[57,67] As pili are necessary for swarming and twitching[67,78,96] and bind to different carbohydrate moieties;[90] we propose that 3,5-diMeDβM/βC bind and inhibit pili appendages. We performed four different experiments to test this hypothesis. First we tested effect of 3,5-diMeDβM/βC on *P. aeruginosa* motility. *P. aeruginosa* exhibit different types of motilities like swarming , twitching, and swimming.[96,104-106] We found that 3,5-diMeDβM/βC inhibited swarming motility in wild type *P. aeruginosa*. (Fig. 2.10). At 3,5-diMeDβM/βC concentrations higher than 10 µM, no swarming motility was observed.

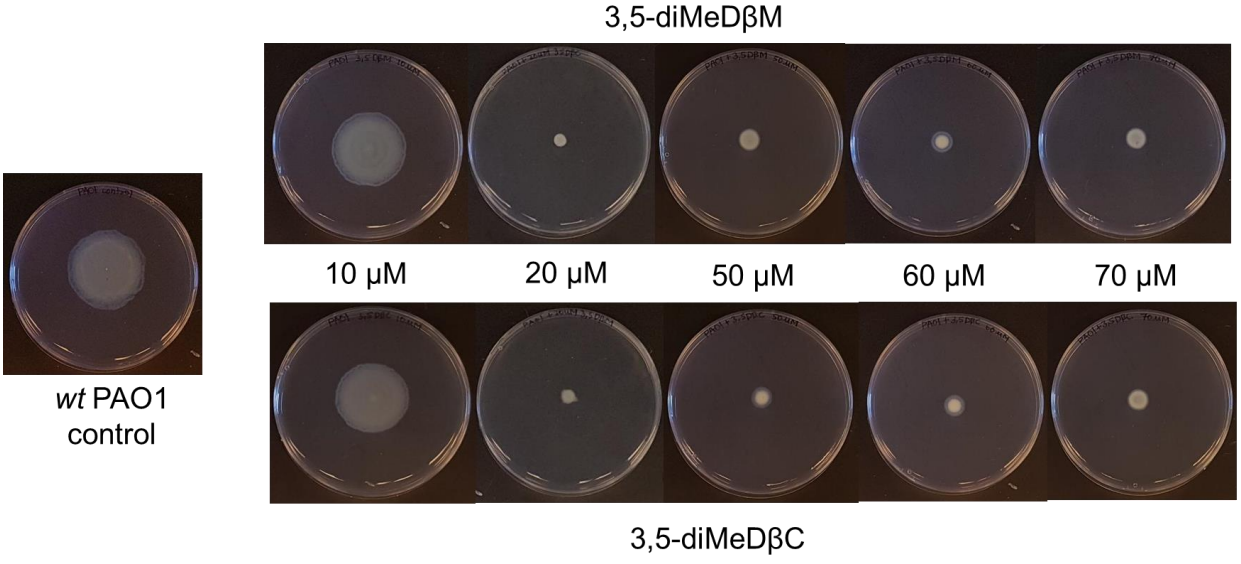


Figure 2.10 3,5-diMeDβM/βC inhibit swarming motility of wild type *P. aeruginosa* Swarming motility assay; The representative images of swarming motilities *wt* PAO1 on semisolid gel (~0.5% agar) with different concentrations of 3,5-DiMeDβM/βC after 24 h. The concentrations are indicated between the images.

As twitching motility is pili dependent and mutation or defect in pili totally abolish twitching motility in *P. aeruginosa*,[95,96,103,107] we tested effect of 3,5-diMeDβM/βC on twitching motility of wild type *P. aeruginosa*. We discovered that 30 µM of 3,5-diMeDβM/βC inhibited twitching motility (Fig. 2.11). It has been showed that swimming motility is solely mediated by flagella, another surface appendage on *P. aeruginosa* surface.[105,106] We observed no effect on swimming motility of P. aeruginosa in the presence of 3,5-diMeDβM/βC suggesting that these molecules may not inhibit flagella mediated motility. These results are consistent with molecules binding to surface appendage pili and inhibiting its function like swarming and twitching motility.

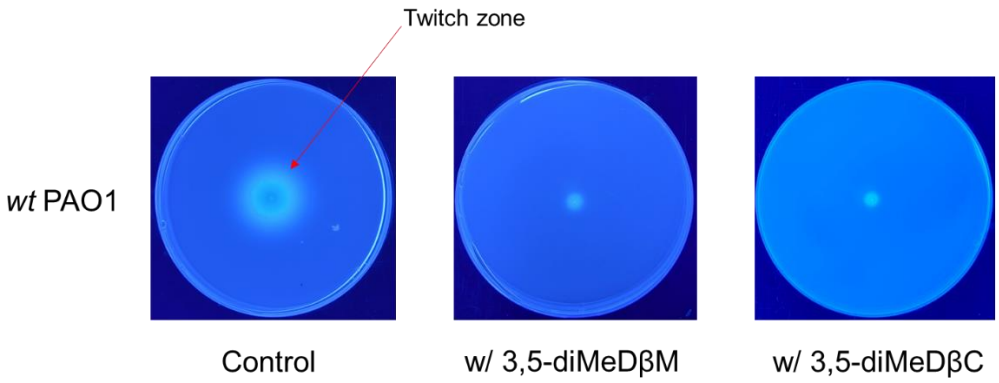


Figure 2.11 3,5-diMeDβM/βC inhibit twitching motility in wild type *P. aeruginosa*. 3,5-DiMeDβM/βC inhibit pili-mediated twitching motility in *wt P. aeruginosa*. LB agar plates (1%) were stab inoculated with a toothpick to the bottom of the plate and incubated for 48 h at 37 ° C with and without 30 µM 3,5-diMeDβM/βC. The light haze of growth, the twitch zone, at the

agar-plate interface is a measure of twitching motility (red arrow). The smaller, denser zone represents surface colony growth. Images were taken under 365/254nm UV irradiation.

Second, as there are no strong synthetic inhibitors reported for type IV pili, and no well-established binding assays for ligand binding to pili, we created a new binding assay based on modifying the swarming motility experiment that studied the direct binding interactions between pilin proteins and proposed ligand candidates.[108] We spread a solution of pilin protein on the swarming gel surface and examined if the ligand candidate in the gel will be sequestered by the added protein, depleting the ligands availability on the gel surface. The experimental scheme is that, as pili appendages on bacteria detect the lack of ligand molecules on the gel surfaces, the swarming motility changes. We demonstrated that spreading the pilin proteins that are expressed and purified from a clinical *P. aeruginosa* strain,[109] PA1244N3-pPAC46 (100 μL of 1 mg/mL, ~616 nmol), on the gel surface (0.5% soft agar, 10 cm in diameter) inhibited the swarming motility of *wt* PAO1 (Fig. 2.12A).

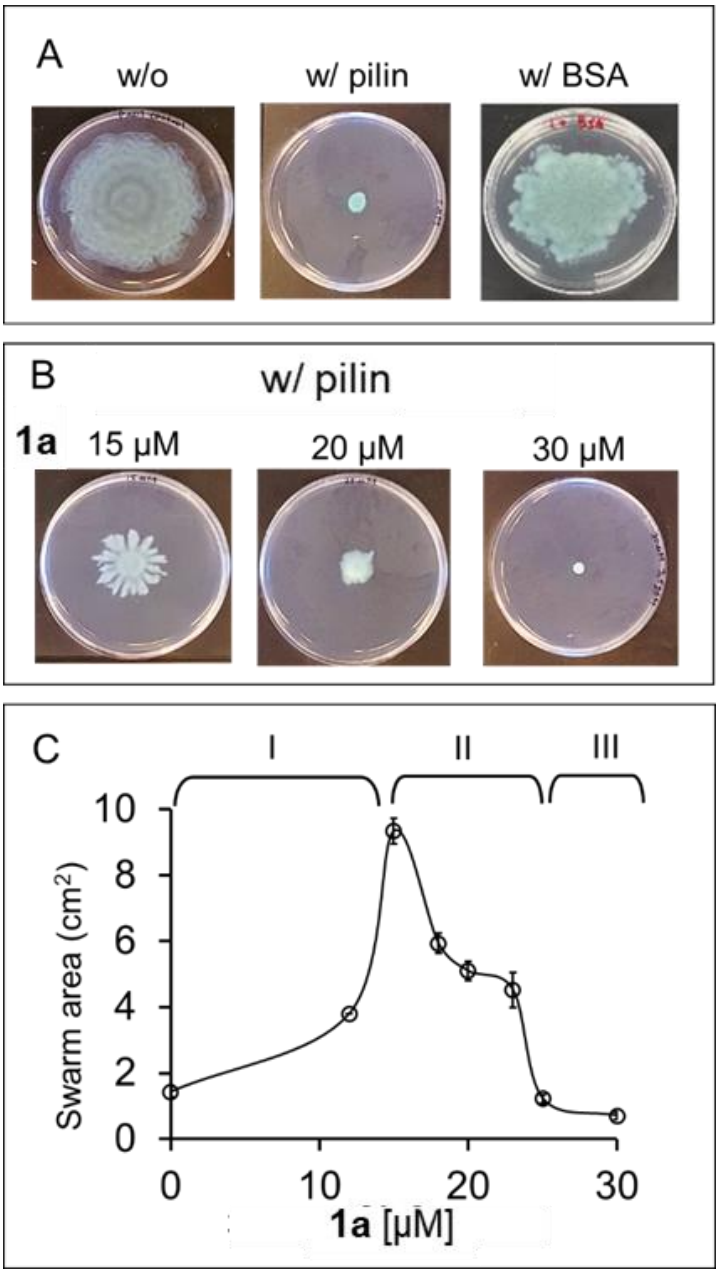


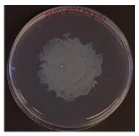
Figure 2.12 Images of swarming patterns of *wt* PAO1 (18 h) on the soft gel (0.5% agar in LB), (A) with and without spreading 100 µl of 1 mg/ml of pilin or Bovine serum albumin (BSA) on gel surface, and (B) with the gel containing 15, 20 and 30 µM of 3,5-diMeDβM (**1a**) and having 100 µl of 1 mg/ml of pilin spread on gel surface. (C) Plot of swarming area of *wt* PAO1 versus

concentration of 3,5-diMeDβM (**1a**) in the gel, with pilin spread on surface. Error bars indicate the standard deviations of means of duplicates.

This result is consistent with externally introduced pilin proteins on gel surfaces sequestered the signaling molecule, likely rhamnolipids, that are necessary for swarming motility,[78,80] making them unavailable to the pili appendage on the bacterial surface and abolished the swarming motility. To test if the expressed pilin proteins bind and sequester our molecules, we spread the pilin protein on swarming gels prepared with different concentrations of 3,5-diMeDβM (0-30 μM) and examined the swarming motility of *wt* PAO1 (Fig. 2.12B, Fig. 2.13). We observed that, while pilin alone or just 3,5-diMeDβM inhibited the swarming motility, having both pilin on gel surface and the molecule in gel caused the reactivation of swarming motility of PAO1 over a small range of concentration of 3,5-diMeDβM. With 626 nmol of pilin protein (100 μL of 1 mg/ml) spread on a gel surface (10 cm in diameter), PAO1 started to swarm when the concentration of 3,5-diMeDβM was increased to 12 μM in gel. The swarming motility reached a maximum (as measured by the area of the swarming pattern) around 15 μM of the 3,5-diMeDβM and decreased as the concentration was increased further (Fig. 2.12C). This reversal of swarming motility as a function of concentration of the molecule is consistent with spread pilin proteins binding and sequestering 3,5-diMeDβM from the gel surface. As the ligand (3,5-diMeDβM) concentration increases in the gel, binding with pilin proteins abolishes each other's ability to inhibit the pili appendages on bacteria, and thus permitting the swarming motility. When the concentration of 3,5-diMeDβM was increased beyond binding capacity of added pilin proteins, the excess ligand molecules inhibit pili appendages on the bacteria, resulting in reducing the swarming motility again. The peak of around 15 μM of 3,5-diMeβM represents the

optimal inhibitory concentration against 7.8 nmol/cm^2 of pilin on gel. The control protein Bovine serum albumin (BSA) has no effect on *wt* PAO1 swarming with and without 3,5-diMeDβM.

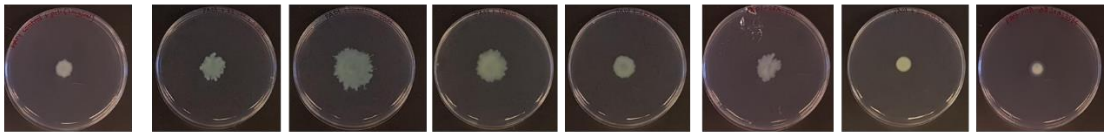
Round I



d 5.6 cm
Area 24.63 cm^2

PAO1 Control

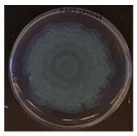
PAO1 + 1 mg/ml Pili



3,5-diMeDβM	0 µM	12 µM	15 µM	18 µM	20 µM	23 µM	25 µM	30 µM
d in cm	1.4	2.2	3.4	2.8	2.6	2.5	1.3	0.9
Area in cm^2	1.54	3.8	9.07	6.15	5.3	4.9	1.32	0.63

100 µL of 1 mg/ml pili protein was spread on agar surface before bacterial inoculation

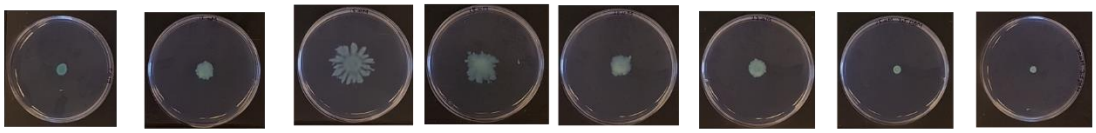
Round II



d 5.8 cm
Area 26.42 cm^2

PAO1 Control

PAO1 + 1 mg/ml Pili



3,5-diMeDβM	0 µM	12 µM	15 µM	18 µM	20 µM	23 µM	25 µM	30 µM
d in cm	1.3	2.2	3.5	2.7	2.5	2.3	1.2	1.0
Area in cm^2	1.32	3.8	9.62	5.72	4.9	4.15	1.13	0.78

100 µL of 1 mg/ml pili protein was spread on agar surface before bacterial inoculation

Figure 2.13 Bacterial Motility Enabled Binding Assay. Externally added pilin protein and 3,5-diMeDβM in gel neutralize each other's swarming inhibition activities. Repetitions of pili-ligand binding assay. 100 µL of pili protein (1mg/ml) expressed from (PA1244N3-pAC46) was spread on the surface of 0.5% agar (10 cm in diameter) containing different concentrations (0-30 µM) of 3,5-diMeDβM, followed by bacterial inoculation (3 µL of culture, OD_{600} = 0.6). Pictures were

taken after the plates were incubated at 37 ℃ for 12 h and then room temperature for another 12 h. The swarming area was estimated by measuring diameter of swarm area on agar gel.

Third, to directly evaluate the effect of this molecule on the pili appendages of PAO1 surfaces, we examined the proteins sheared from the bacterial surfaces (*wt* PAO1) on agar gel (1.5 % agar for growing bacteria),[110] prepared with 85 μM of 3,5-diMeDβM. The amount of pilin and other proteins are characterized by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the same protein mass loaded (See experimental section for details). Without presence of molecule in the hard agar, we observed a band with molecular weight around 16 kD, same as the expressed pilin protein from PA1244N3-pPAC46 (Fig. 2.14). For agar gel containing 85 μM of 3,5-diMeDβM, only a faint band of 16kD was observed, whereas other proteins showed intense bands (Fig. 2.14). This result indicated that the presence of 3,5-diMeDβM reduced the amount of pili on bacterial surfaces. As pili can retract in response to chemotaxis,[66,67] we examined the effect of our molecules on a nonretracting transposon mutant of pilin assembly protein, pilT*(pilT::Tn)*.[111] We observed that the pilin bands were equally intense for bacteria grown with and without 3,5-diMeDβM on agar surfaces (Fig. 2.14) Because our molecules showed no effect on non-retracting pili, we believe that 3,5-diMeDβM binding to pili likely caused pilT-mediated retraction.

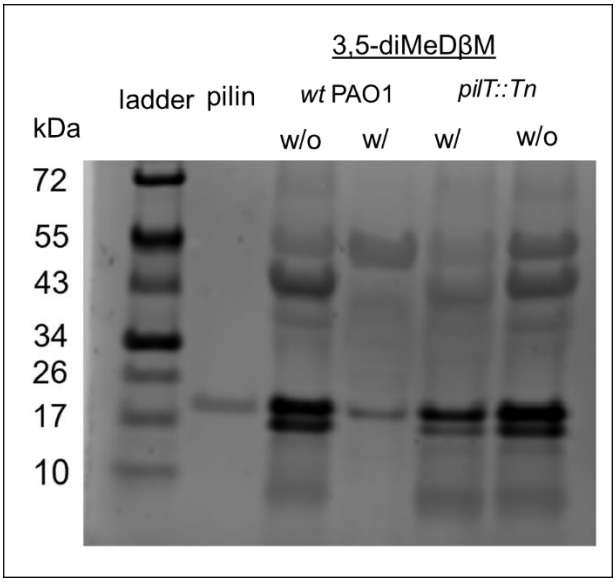


Figure 2.14 Sheared surface proteins assay. *P. aeruginosa* treated with 3,5-diMeDβM have fewer surface pili. Gel electrophoresis (SDS-PAGE) of sheared surface proteins of *wt* PAO1, *pilT::Tn* grown on 1.5% agar with and without 85 μM of 3,5-diMeDβM. From left to right lanes: molecular size markers, standard of expressed pilin from PA1244N3-pAC46, proteins from PAO1 and *pilT::Tn* without and with 3,5-diMeDβM.

2.2.7 Chemical Inhibition of Type IV Pili Mediated Bacteriophage infection on P. aeruginosa

Last, pili appendages are the key receptor proteins for direct binding and adsorption of bacteriophage, which facilitate the injection of DNA into bacteria. Lytic bacteriophage ϕKMV targets specifically type IV pili of *P. aeruginosa* for adsorption on and subsequent lysis of bacteria.[46, 47] Nonpiliated strain Δ*pilA*,[112] or retraction mutant *pilT (pilT::Tn)* are resistant to lysis by bacteriophages.[111] Here, we studied the effect of 3,5-diMeDβM/βC on the bacteriophage ϕKMV adsorption to three *P. aeruginosa* strains: PAO1k, phage sensitive strain *wt* PAO1; pili-deficient mutant Δ*pilA*;[112] and the nonretracting mutant *pilT*.[111] We found that mixing ϕKMV (5.8×10^6 PFU/ml) with excess of PAO1k (2.8×10^7 CFU/ml) for 10 min, the phage titer in supernatant culture was reduced to 6.8×10^5 PFU/ml, indicative of ~86% of the phage was adsorbed on PAO1k (Figure 7A). In contrast, culturing bacteria in the presence of 3,5-diMeDβM

(85 μM), the phage count in solution was 4.2×10^6 PFU/ml, indicating of only ~16 % of phage adsorption on PAO1k. For pili deficient mutant, Δ*pilA*, under the same condition, a low level of adsorption of added phage (~4.5 %) was observed (Fig. 2.15A). The presence of our agent did not exhibit a noticeable effect on phage adsorption on Δ*pilA*. Because of the lack of pili on Δ*pilA*, we consider this ~4.5 % as non-pili mediated phage adsorption. Thus, for PAO1k, the pili mediated phage adsorption is about 86 – 4.5 = 81.5% without the agents, and about 16 – 4.5 = 11.5% with the presence of agents. This result suggests that our agents cause about (81.5 – 11.5)/81.5 = 86 % reduction in bacteriophage adsorption on pili of PAO1. As phage ϕKMV is lytic phage, we also tested effect of 3,5-diMeDβM on the killing kinetics of ϕKMV on phage sensitive *P. aeruginosa* (PAO1k). The kinetics of *P. aeruginosa* PAO1k which is incubated with 3,5-diMeDβM for 6 h (before phage infection) showed slower rate of lysis (measured as drop in optical density per time) by phage ϕKMV than control *P. aeruginosa* (Fig. 2.15B).

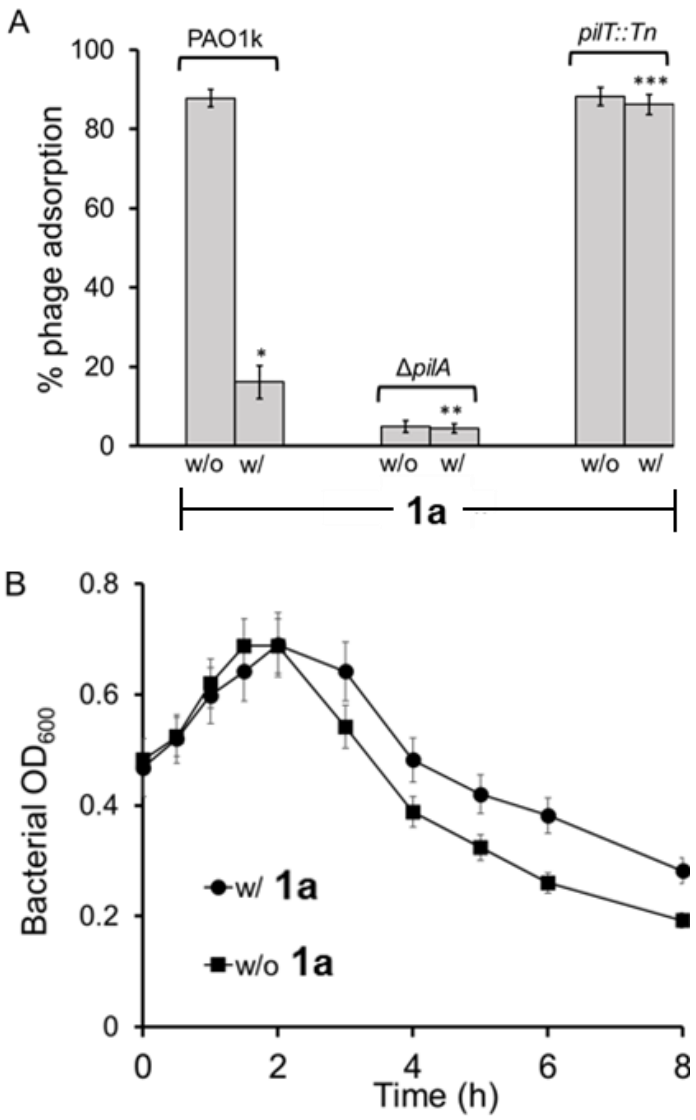


Figure 2.15 (A) Percentage of ϕKMV phage adsorbed on PAO1k (*wt* PAO1 strain that is sensitive to ϕKMV) and Δ*pilA*, *pilT::Tn* mutants over 10 min in LB containing 85 μM of 3,5-diMeDβM (**1a**). Error bars indicate the standard deviations of means of triplicates. *$P < 0.01$; **$P < 0.01$ vs control without 3,5-diMeDβM (**1a**), Student's t-test. (B) ϕKMV phage killing kinetics against PAO1k (OD_{600}=0.6) cultured with and without 85 μM of 3,5-diMeDβM (**1a**) for 8 h. The optical density (OD_{600}) of the bacterial cultures were measured every 30 minutes. Error bars indicate the standard deviations of means of triplicates.

To explore the mechanism of the inhibition of phage adsorption, the study of non-retracting mutant *pilT::Tn* revealed that high levels of phage adsorption (~87%) were observed

(Fig. 2.15A). The presence of 3,5-diMeDβM did not exhibit a noticeable effect on phage adsorption on *pilT::Tn*. These results further support that our molecules bind and retract pili appendages of PAO1 – likely by pilT retraction mechanism.[111] To corroborate these results, we used another synthetic molecule saturated farnesol derivative, $SFEG_4OH$, (Fig. 2.1), which inhibits swarming motility, but not biofilm formation.[81] We found that in the presence of SFEG4OH, phage adsorption on PAO1k pili was also inhibited by ~72% (Fig. 2.16).

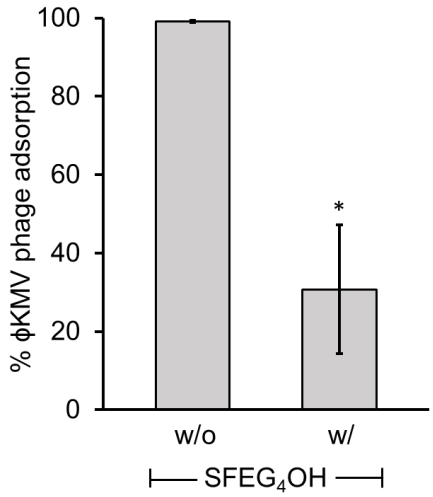


Figure 2.16 $SFEG_4OH$ inhibits bacteriophage adsorption on *P. aeruginosa* PAO1k with functional pili. PAO1k ($6x10^7$ CFU/ml) pretreated with and without 60 μM $SFEG_4OH$ and infected with ~10^6 PFU/ml phage, 10 min, 100 rpm. The phages left in supernatant (not adsorbed on pili) were quantified by plaque formation assay. The % phage adsorption was calculated as 1-(titer in supernatant/ titer of phage added) ×100.[112] Error bars indicate standard deviations of independent triplicates. **P*=0.01 vs control as evaluated using two-tailed unpaired Student's *t*-test.

We note that while pili deficient mutant Δ*pilA* was resistant (Fig. 2.17), PAO1k was killed by phage ϕKMV in 8 h (Fig. 2.15B). The presence of 3,5-diMeDβM, however, caused a delay of killing of PAO1k by 4 h, and an increase in residual live bacteria after 8 h (Fig. 2.15B). This result is consistent with the past finding that anti-pilin antibody can delay the killing of

bacteria by another predator strain through pili binding.[113] The control molecule SDS has no effect on either phage adsorption or phage mediated killing.

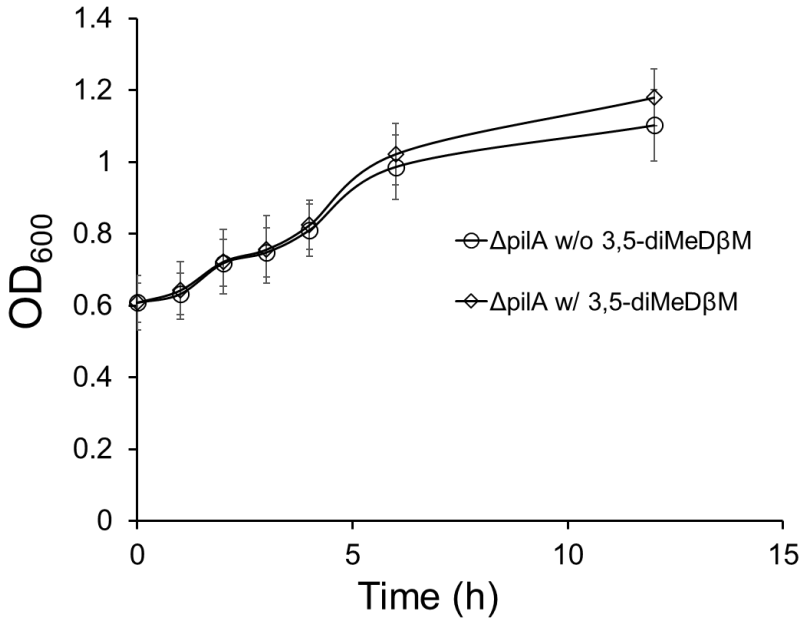


Figure 2.17 ϕKMV phage with and without 3,5-diMeDβM does not kill pili-deficient mutant (Δ*pilA*). Overnight cultures of Δ*pilA* were diluted 100-fold, and subcultured with the presence of 10 mM $MgSO_4$, and with and without 3,5-diMeDβM (85 µM), to an OD_{600} of 0.6, and then infected with ϕKMV phage (PFU/ml ~10^8). The optical density (OD_{600}) of the bacterial cultures were measured after 12 h. Cultures were incubated at 37℃ at 250 rpm.

All in all, pili play different role in motility, receptor for phage adsorption and subsequent lysis. These four different experiments support that our molecules bind to pilin proteins and inhibit function of pili appendages on bacteria. Our results also suggested plausible mechanism for inhibition of pili mediated activities. We suggest that our molecules may target pilT proteins in pili machinery which is responsible for retraction. This proposition is consistent with literature that pilT proteins are necessary for motility and extent of surface piliation.

2.2.8 Ligand binding and inhibition of LecA

We examined the binding of a collection of pili ligands to LecA, including 3,5-diMeDβM/βC, rhamnolipids and $SFEG_4OH$ (Fig. 2.1), by using an established fluorescence polarization-based competitive binding assay.[114] For this assay, we designed and synthesized a Dansyl fluorophore-tagged galactose ligand (βGal-aryl-Dansyl, Figure 1) for LecA protein. The fluorescent polarization of the fluorophore-tagged ligand will increase when bound to a protein receptor because of the reduction in dynamic and will decrease when the fluorophore ligand is displaced from the protein by another ligand. The βGal-aryl-Dansyl (200 nM) showed an increase in its fluorescence polarization with increasing concentrations of LecA (0-100 μM), with a transition indicative of a K_d ~10.7 μM, whereas for the control protein, bovine serum albumin (BSA), no significant increase in fluorescence polarization was observed (Fig. 2.18A).

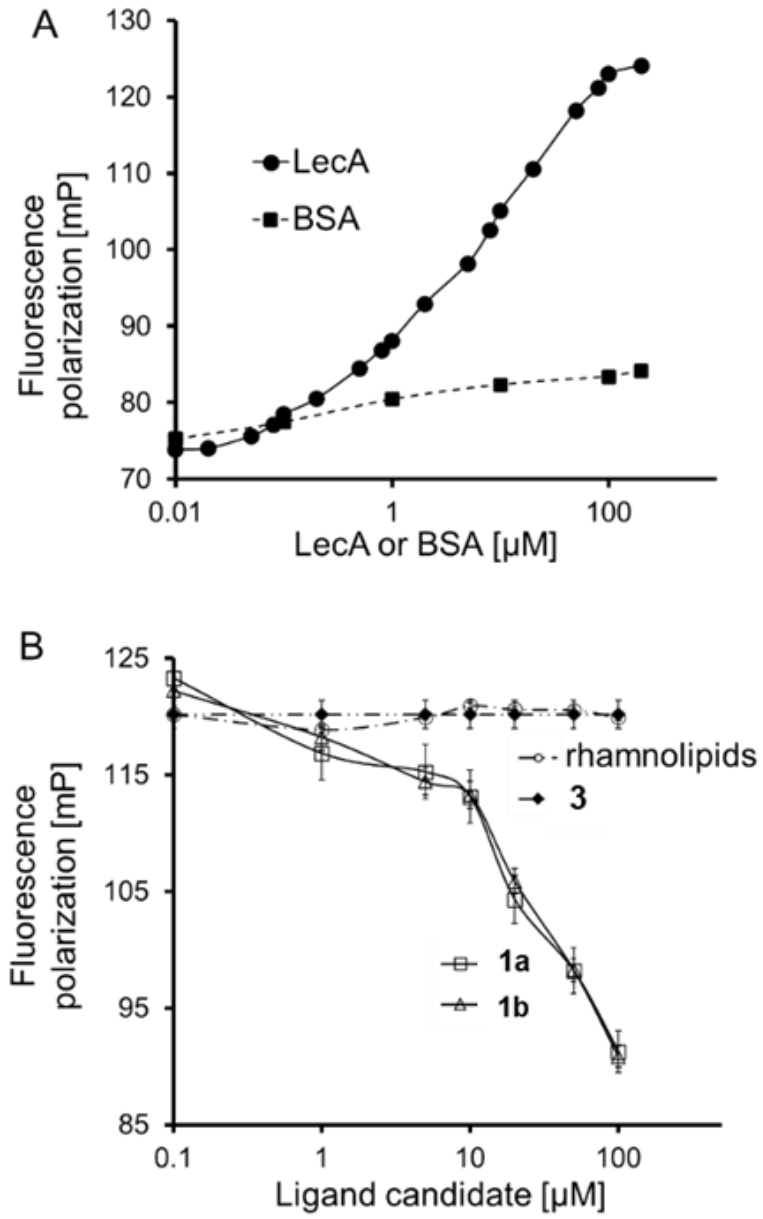


Figure 2.18 Fluorescence polarization of (A) of 200 nM of βGal-Dansyl versus concentrations of LecA and BSA proteins, and (B) of 100 uL of LecA (20 μM) and βGal-Dansyl (200 nM) versus concentration of candidate ligands, 3,5-diMeDβM (βM), 3,5-diMeDβC (βC), $SFEG_4OH$, rhamnolipids mixtures. Error bars indicate the standard deviations of means of triplicates.

To estimate the binding strength of the potential ligands, 3,5-diMeDβM/βC, to LecA protein, the molecules were titrated against complex of βGal-aryl-Dansyl (200 nM) and LecA (20 μM) to displace the βGal-aryl-Dansyl from LecA. We found that 3,5-diMeDβM and 3,5-diMeDβC caused a decrease of fluorescence polarization indicative displacement of βGal-aryl-Dansyl with an IC_{50} of 15 μM and 13 μM, respectively (Fig. 2.18B). Interestingly, rhamnolipids and $SFEG_4OH$ did not cause a decrease of fluorescence polarization. These results are consistent with the knowledge that glucose containing disugars are capable of binding to LecA. [115,116]

While LecA of *Pseudomonas aeruginosa* was initially discovered to have an affinity for galactose, having glucose as part a galactose-glucose disugar can actually increase its binding to LecA protein as compare to other galactose-galactose disuger.[115] More notably, di-glucose linked by oligo(ethylene glycol) without any galactose groups exhibited considerably strong binding to LecA.[116] Varrot and coworkers has demonstrated that crystal structure with a secondary sugar binding site for glucose.[115] It is important to note that binding to LecA is enhanced by multivalent array of galactose,[99,101,117] and glucose[115] The space between the binding sites on LecA likely play a significant role for binding. Evidently, tethering aromatic groups to single sugar galactose greatly enhance its binding to LecA.[101] Binding does not necessarily impact activities of respective protein. Inhibition of biofilm by single galactose derivatives or sugar without attaching groups are often not reported. For our case, β-Gal-Dansyl exhibited a weak biofilm inhibition,IC_{50} 120 uM,. As indicated by Imberty, Romer, Winssinger's work on LecA, the space between the two sugar binding sites has potential to interact with the group attaching the sugar.[101,117]

We believe that the branch hydrocarbon chain tethering the cellobioside or maltoside plays an enhancing role for binding and for controlling LecA's activity. In this case tethering with

a hydrophobic group, glucose has an indispensable advantage of high-water solubility over galactose. This binding effect is an ongoing subject of our research.

To further corroborate for these LecA binding studies, we performed a bacterial motility-enabled binding assay.[108] In this assay, we spread LecA on the soft agar gel containing 3,5-diMeDβM that inhibited swarming motility. We observed that while the swarming motility of PAO1 was inhibited completely on gels containing 20 μM of 3,5-diMeDβM/βC, spreading LecA (100 μL of 1 mg/ml, 780 nmol) on the gel surfaces reactivated the swarming motility (Fig. 2.19). This result is consistent with LecA binding and sequestering 3,5-diMeDβM/C on the gel surface, depleting their availability for pili appendages on bacteria, and thus abolishing their swarming inhibition activities. For pilin binding, in contrast, branched aliphatic chains having different water-soluble group, tetra(ethylene glycol) ($SFEG_4OH$), or disugar of 3,5-diMeDβM/βC, supported binding to pili and inhibition of pili-mediated motilities (Fig. 2.13). These results indicate that 3,5-diMeDβM/βC are chimeric ligands for LecA and pili, whereas $SFEG_4OH$ is a ligand for pili alone.

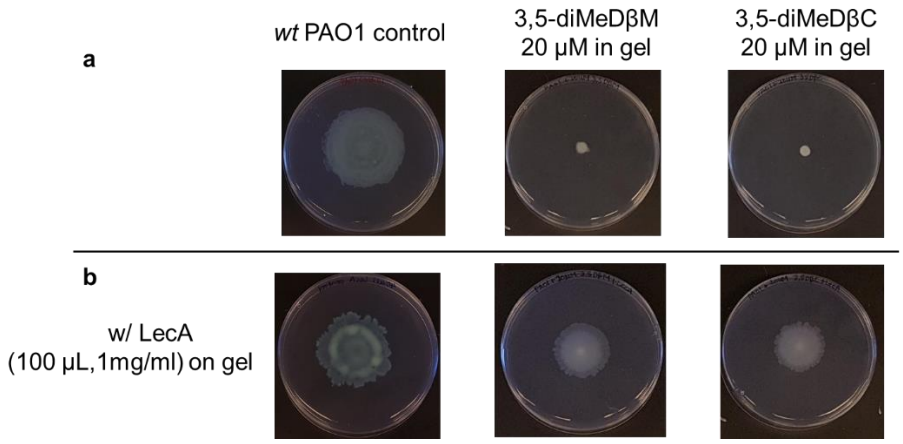


Figure 2.19 LecA remove effect of 3,5-diMeDβM/C on swarming motility. Swarming of *wt* PAO1 on 0.5% agar (10 cm in diameter) containing 20 μM of 3,5-diMeDβM/βC without (a) and with (b) 100 μL of LecA protein (1 mg/mL) was spread on the agar gel surface. Upon

inoculation, the plates were incubated at 37 ℃ for 12 h and then at room temperature for another 12 h, followed by taking the pictures.

2.2.9 Chemical Control and Correlation of Receptor Proteins to Their Phenotypes

The chimeric ligands (3,5-diMeDβM/βC) inhibited biofilms and associated tolerance and persistence, small colony variants, rugose colony, and pellicles formation, as well as swarming and twitching motility for wild type *P. aeruginosa*. Because the two motilities are associated with low cdG levels,[75,94,95] whereas other phenotypes are of high cdG levels. We also evaluated other low cdG controlled virulence factors of *P. aeruginosa*, including the pyocyanin,[76] proteolytic enzyme-elastase. Pyocyanin production is augmented in low cdG mutants.[76] Elastase production increases during pili sensing a surface,[68] We found that pili inhibitor alone, $SFEG_4OH$, reduced type PAO1's pyocyanin and elastase levels by 50% and 49%, respectively (Fig. 2.20). This result makes the correlation of inhibiting pilin proteins to inhibits pili-associated activities that also associated with low cdG levels.[94] The chimeric ligand for both LecA and pili (3,5-diMeDβM/βC) also reduced wild pyocyanin production by ~60%, and elastase activity by ~45%.

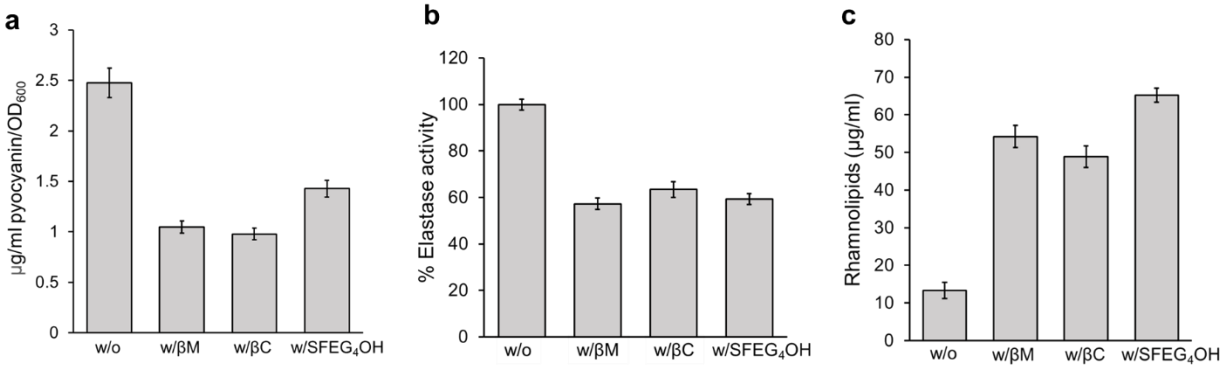


Figure 2.20 *P. aeruginosa* Virulence assay. (a) Pyocyanin levels in supernatant of planktonic cultures of *wt* PAO1 treated with and without 85 μM of 3,5-diMeDβM/βC, $SFEG_4OH$ for 12 h in LB. The pyocyanin levels were measured as μg/ml/OD_{600} of the bacterial culture. (b) Elastase

production in the planktonic culture of *wt* PAO1 treated with and without 85 µM of 3,5-diMeDβM/βC, SFEG$_4$OH for 18 h were determined using the Elastin-Congo Red (ECR) assays. The % of elastase activity was calculated as OD_{495} (with agent) - OD_{495} (without agent). (c) Rhamnolipids productions by planktonic cultures of *wt* PAO1 treated with and without 85 µM of 3,5-diMeDβM/βC, SFEG$_4$OH for 18 h were determined using an established methylene blue complexation assays.[25] The positively charged methylene blue form noncovalent complex preferentially with negatively charged surfactants and does not bind to nonionic surfactants. The methylene blue bound with negatively charged surfactants has an increased partition into the organic chloroform phase bought in contact with the aqueous solution. The methylene blue in the chloroform phase were measured for its UV absorbance, which infers the concentration of rhamnolipids (µg/ml) in the culture.

Overall, the chimeric ligands for LecA and pili proteins inhibited all eight phenotypes (biofilms, SCVs, rugose colony, pellicles, twitching, swarming motilities, elastase, and pyocyanin production). In contrast, pili ligand alone, SFEG$_4$OH, has no effect on biofilm, SCV, rugose and pellicles; but inhibited swarming, twitching motilities, and elastase and pyocyanin production. These results show that the chimeric ligands (for LecA and pili) inhibit phenotypes for both high and low cdG levels, whereas pili ligand inhibits only low cdG levels (Supplementary Table 2). This selective inhibition of LecA and pili enables the correlation between the phenotypes and the receptor proteins. Because inhibiting pili alone by SFEG$_4$OH has no effect on biofilms, SCVs and EPS, pellicle formation, these high cdG phenotypes[99] are primarily controlled by inhibition of LecA. In contrast, the low cdG phenotypes, overproduction of pyocyanin and elastase, as well as swarming and twitching motilities, can be controlled by pili inhibition alone.

Pili retraction resulted from sensing a surface activates virulence factor regulator (*vfr*) gene and type III secretion system, which promotes pyocyanin and elastase, respectively.[66,67]

This important knowledge suggests that pili sensing can feedback to signal to the production of virulence factors correlated with low cdG levels. Our results show that chemical inhibition of pili not only inhibits activities directly controlled by pili (swarming and twitching motility), and but also virulence factors (pyocyanin, elastase) that are signaled by pili sensing the environment.

We also evaluated the production of rhamnolipids in the presence of chimeric ligand for LecA and pili, and pili ligand alone. Rhamnolipids are a special case in the signaling context because they are needed for structured biofilm,[79] which is of high cdG phenotype, but are also necessary for swarming motility,[80] which is of low cdG phenotype.[76,80] Interestingly, both $SFEG_4OH$ and the chimeric ligands cause about 5-fold increase in rhamnolipids production, to ~107 μM (Fig. 2.20c), which is close to rhamnolipid's critical micelle concentration (130 μM).[118] Because pyocyanin and rhamnolipids are inversely regulated in *P. aeruginosa*,[62] and our result shows that pili inhibition leads to pyocyanin reduction, it is consistent that pili inhibition also lead to increase in rhamnolipid production. Furthermore, lowering the cdG level leads to *rhl* quorum sensing that increases rhamnolipid production,[67,69] and lecA inhibition has been shown to correlated to low cdG;[99] thus inhibiting LecA can also promote rhamnolipids production. Together, these results provide a signaling map identifying the phenotypes of pili and LecA proteins, and the effects from chemical inhibition of the two proteins (Fig. 2.21, and Table 2.2). Overall, inhibiting LecA inhibits high cdG phenotypes, whereas inhibit pili suppress low cdG phenotypes. We note that pili appendages are also relates to drug tolerance as increasing swarming motility is also known to lead to antibiotic tolerance.[64,65]

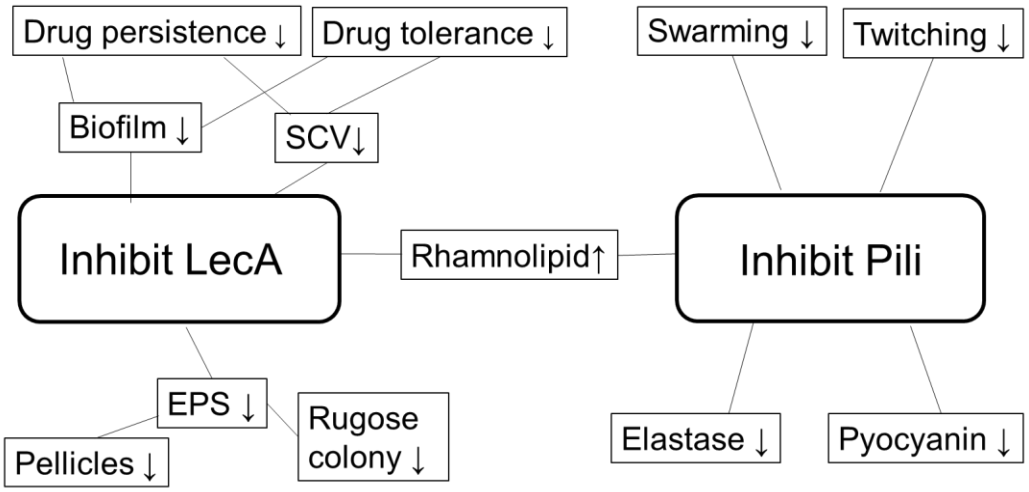


Figure 2.21 Mapping of chemical inhibition of pili and LecA, and the correlation to their controlling phenotypes.

Table 2.2 Ligand correlating phenotypes and corelated receptor.

	Phenotype	Ligand		Inferring Receptor[a]
		Chimeric, 3,5-diMeDβM	Pili only, $SFEG_4OH$	
High cdG phenotypes	Biofilm	Inhibit	No effect	LecA, not pili.
	SCVs	Inhibit	No effect	LecA, not pili.
	Rugose colony EPS	Inhibit	No effect	LecA, not pili.
	Pellicles	Inhibit	No effect	LecA, not pili.
Low cdG phenotypes	Twitching Swarming	Inhibit	Inhibit	Pili only[b]
	Elastase	Decrease	Decrease	Pili only[c]
	Pyocyanin	Decrease	Decrease	Pili only[c,d] Not LecA as low cdG promote pyocyanin.
Both high and low	Rhamnolipids	Increase	Increase	Pili, and may be LecA[e]

[a] Protein receptors (Pili and Lectin A) responsible for controlling phenotypes**.** [b] low cdG promotes motility[69]. [c] Pili surface sensing induce Vfr, which promotes quorum sensing that results in promotion of elastase and rhamnolipids[66,67,119]. [d] low cdG promotes pyocyanin[76]. [e] low cdG promotes rhamnolipids[76].

Comparing to a previous set of molecules, SFβM/βC (Fig. 1) exhibited stronger swarming inhibition, but relatively weaker biofilm inhibition than 3,5-diMeDβM/βC. The

molecules SFβM/βC also inhibited both high and low cdG activities, including biofilm, SCV and pellicle formation, swarming and twitching motilities, pyocyanin and elastase A levels; and promoted rhamnolipids production. These results collectively suggested that small modifications in hydrophobic region of chimeric ligands can give better control over high cdG phenotypes without compromising on control over low cdG phenotypes.

2.3 Conclusions

In this work, we showed a class of molecules that bind and inhibit both Lectin A and pili appendages, and another that inhibit pili alone. We showed the selective chemical inhibition of bacteriophage's adsorption on pili appendages, and a label-free assay that identify direct binding between pilin proteins and chemical ligands. The chemical inhibition of pili appendages likely causes retraction of pili appendages and inhibits phenotypes that are associated with low cdG levels. Chimeric ligands inhibit both LecA protein and pili appendages, and phenotypes associated with both high and low level of cdG. The chimeric ligands also inhibit all the known tobramycin-induced phenotypes, including enabling the killing of tolerant population and the prevention of nascent persistent population in biofilm, and the small colony variants that are specific to tobramycin-stress. Being able to selectively inhibit only pili, and inhibit both pili and LecA, these chemical tools delineate that pili appendages is important for low cdG phenotypes, whereas LecA is important for high cdG phenotypes. Overall, these results suggest an adjuvant approach for existing antibiotics by inhibiting the virulent phenotypes, and a structural consideration for new drug development. The ligands for pili also present opportunities for inhibiting horizontal gene transfer and related drug resistance. Finally, the label-free bacterial

motility-enabled binding assays can be explored for identification and studying new ligand-receptor interactions.

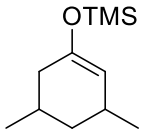
2.4 *Synthesis and Spectral Data*

The synthesis of 3,5-diMeDβM and 3,5-diMeDβC was performed by fellow graduate student Dr. Felicia Burns. The generous amount of pure products were supplied to me for further biological assays.

Materials

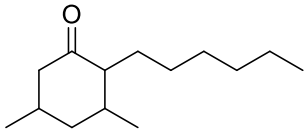
3,5-dimethylcyclohexanone (mixer of isomers) **1** and β-D-Maltose octaacetate **9**, α-D-cellobiose octaacetate **8** were purchased from Sigma Aldrich. Standard solvents and reagents were purchased from commercial sources (Sigma-Aldrich, Fisher, Acros) and used as received. Solvents were removed *in vacuo* using Bűchi rotary evaporator below 40ºC. EMD silica gel 60 F254 pre-coated plates (0.25-mm thickness) were used for TLC. Unless otherwise stated, TLC visualization was done using ceric ammonium molybdate (CAM) stain.

^{1}H-NMR spectra were recorded in deuterated NMR solvents at 400 MHz (^{1}HNMR) and 100 MHz (^{13}CNMR) Bruker instruments, respectively. ^{1}H chemical shifts are reported in ppm relative to $CDCl_3$ δ ppm 7.26, CD_3OD δ ppm 3.31. ^{13}C chemical shifts are reported relative to $CDCl_3$ δ ppm 77.23 and CD_3OD δ ppm 49.0.



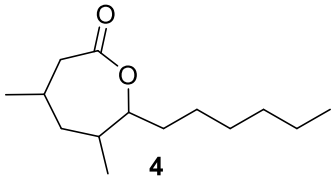
2

To a solution of **1** (2.78 g, 22 mmol) in dry acetonitrile (6 mL) triethylamine (11.5 mL), trimethylchlorosilane (4.1 mL) and sodium iodide (4.63 g, 30.9 mmol) dissolved in acetonitrile (29 mL) were added successively. The reaction mixture was stirred at room temperature. After 4 h, cold water (37 mL) was added and the aqueous solution extracted with cold hexane. The organic layers were combined and evaporated in vacuo to give **2** as a brown oil. Crude product was assessed by NMR and deemed pure enough use crude in next step without further purification (81% yield).



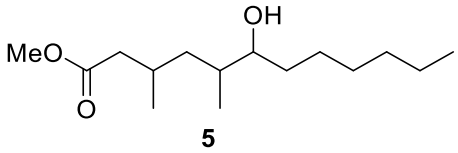
3

A solution of silver trifluoroacetate (2.09 g, 9.45 mmol) suspended in dichloromethane (9 mL) was cooled to -78 ^{o}C and Compound **2** (1.79 g, 9 mmol) was added dropwise. 1-iodohexane (2.0 g, 9.45 mmol) was then added and the reaction was allowed to warm to room temperature then after ten minutes the reaction mixture was filtered and concentrated en vacuo. The crude product was flash chromatographed on silica gel (hexane/AcOEt 95 : 5) to yield **3** as a colorless oil (19% yield). ^{1}H-NMR (400MHz, $CDCl_3$) δ ppm: 0.87 (3H, t), 0.92-0.97 (6H, d), 1.16-1.48 (10H, m), 1.56-1.75 (2H,t), 1.61-1.90 (2H, t), 2.18-2.35 (3H, m)



4

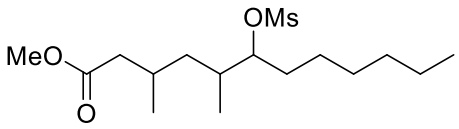
A solution of **3** (160 mg, 0.76 mmol) in dry dichloromethane (2 mL) was cooled to 0ºC and m-chloroperbenzoic acid (350 mg, 2.03 mmol) was added. The suspension and trifluoroacetic acid (0.288 mL, 3.76 mmol) was added dropwise. During this operation, the reaction flask was protected from light. Subsequently, the solution was allowed to warm to room temperature. After 16 h, the mixture was diluted with dichloromethane (2 mL) and the organic layer was washed successively with a 10% aqueous solution of Na_2SO_3 (2 mL), a saturated aqueous solution of potassium carbonate (1 mL) and water (2 mL). Finally, the organic layer was evaporated in vacuo and the product purified by column chromatography (9:1 hexanes: ethyl acetate) to give lactone **4** (46% yield). ^{1}H-NMR (400MHz, $CDCl_3$) δ ppm: δ 0.87 (3H, t), 0.95 (3H, d), 1.00 (3H, d), 1.17-1.59 (12H, m), 1.77-1.92 (2H, d), 2.41-2.54 (2H, dd), 4.29 (1H, td).



5

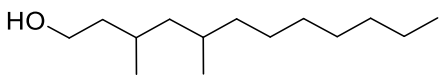
Under nitrogen atmosphere, a solution of **4** (1.608 g, 7.58 mmol) in a 10 mM solution in anhydrous methanol (7.1 mL) was stirred overnight at room temperature. Then, the reaction was neutralized using H^+ amberlite resins. After filtration, the filtrate was evaporated in vacuo to give the methyl ester **5** as a clear, oily liquid (87% yield). ^{1}H-NMR (400MHz, $CDCl_3$) δ ppm: 0.84

(3H, t), 0.87-0.92 (6H, d), 0.96-1.24 (13H, m), 2.14-2.19 (2H, dd), 3.45-3.55 (2H, m), 3.65 (3H, s)



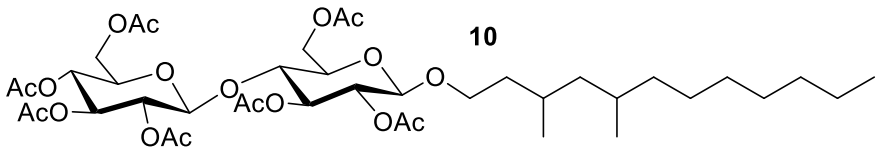
6

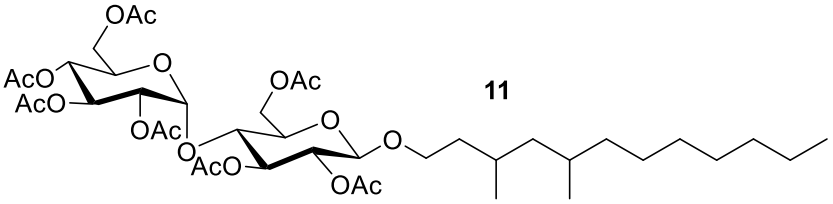
A solution of **5** (1.098 g, 0.25 mmol) in dichloromethane (1.5 mL) was stirred on ice. Triethylamine (0.23 mL, 1.6 mmol) and mesyl chloride (655 mL, 0.51 mmol) were added. The mixture was stirred at 0ºC. After 4 h, aqueous $NaHCO_3$ (1N, 1.2 mL) was added and the resulting solution extracted with dichloromethane. The organic layers were combined and evaporated in vacuo and the product purified by column chromatography (9:1 hexanes: ethyl acetate)to give mesylate **6** as a yellowish oil (91% yield). ^{1}H-NMR (400MHz, $CDCl_3$) δ ppm: δ 0.79-0.91 (9H, m), 1.17-1.34 (13H, m), 2.17-2.39 (2H, d), 2.43 (1H, q) 3.42 (3H, s), 4.27 (1H, td).



7

To a suspension of LAH (63 mg, 1.7 mmol) in anhydrous diethyl ether (1 mL) at 0ºC, a solution of **6** (68 mg, 0.20 mmol) in anhydrous ether (1 mL) was added. The mixture was brought to room temperature then refluxed for 16 h. Then, the solution was cooled to 0ºC before quenching with water. The resulting aqueous layer was extracted with ether. The combined organic fractions were evaporated in vacuo and the oily residue was flash-chromatographed (hexane/AcOEt 9:1) to yield alcohol **7** as an oil (53% yield). ^{1}H-NMR (400MHz, $CDCl_3$) δ ppm: 0.54-0.74 (9H, m), 0.86-1.54 (18H, m), 3.32-3.40 (2H,t).

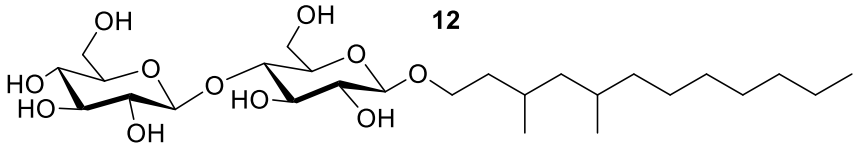


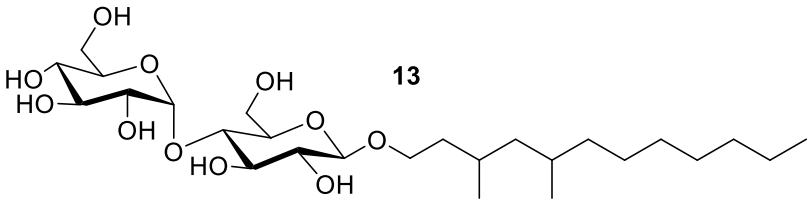


The cellobiose octaacetate **8** or maltose octaacetate **9** was dissolved in dichloromethane (10mL) and **7** (2 equivalents) were added. Under vigorous stirring, BF_3. etherate (2 equivalents as compared to maltose or cellobiose monohydrate) was added to this mixture and the reaction mixture was allowed to stir at rt for 12 h. To the reaction mixture was added aq KBr (10%, 25mL) and PhMe (60mL) under stirring. The organic phase was washed twice with aq. KBr (10%, 2×25mL), once with aq. $NaHCO_3$ (5%, 25mL) and twice with H_2O (2×25mL). Using a gradient elution (100 % hexane to 35 % ethyl acetate in hexane) the crude product was then purified through a silica column to yield white powder.

10 (0.20 mg, 30% yield). ^{1}H (400 MHz, CDCl3) δ 5.16-4.98 (m, 3H), 4.85 (q, *J1-2* = 8.4 Hz, 2H), 4.48-4.29 (m, 4H), 4.06 - 3.97 (m, 2H), 3.82 - 3.69 (m, 2H), 3.66 -3.50 (m, 2H), 3.44 - 3.34 (m, 1H), 2.07-1.93 (s, 7 X 3H), 1.56 -1.32 (m, 2H), 1.20 (br, s, 18 H), 0.83 (m, 9H).

11 (0.18 mg, 12% yield). ^{1}H (400 MHz, CDCl3) 5.43-5.36 (m, 2H), 5.25 (t, 1H), 5.07 (t, 1H), 4.97-4.79 (m, 2H), 4.53-4.42 (m, 2H), 4.29-4.20 (m, 2H), 4.07-3.85 (m, 4H), 3.70-3.65 (m, 1H), 3.53-3.44 (m, 1H), 2.14-2.01 (s, 7 x 3H), 1.66-1.16 (m, 18 H), 0.89 (br, s, 9H).





Zemplen deacetylation was used to deprotect the acetylated glycosides. Briefly, acetylated glycosides were treated with methanolic solution of MeONa (10 mM) followed by neutralization (pH ~ 7) over H+ amberlite resins. The product was filtered and dried under vacuum overnight to get white powder.

12, (9.3 mg, 75%) ^{1}H NMR (300 MHz, MeOD) δ 4.38 (d, *J* = 7.8 Hz, 1), 4.24 (d, *J* = 7.8 Hz, 1H), 3.83 (br, s, 4H), 3.62 (dd, *J1-3* = 12.0 Hz, *J1-2* = 5.1 Hz, 1H), 3.56-3.44 (m, 3H), 3.37-3.27 (m, 5H, overlapping with MeOD), 1.25 (br, s, 18 H), 0.86 (m, 9H).). ^{13}C NMR (75 MHz, CD3OD): δ 102.26, 103.23, 76.72, 76.42, 75.11, 75.04, 73.54, 73.45, 69.97, 67.86, 61.08, 60.54, 37.68, 36.56, 31.67, 29.83, 29.77, 29.65, 29.09, 26.75, 26.65, 22.32, 19.23, 18.48, 13.2.

HRMS (ESI) m/z: Calcd. (C26H50 O11)Na+: 538.68; Found: 537.327526.

13, (6mg, 97%) ^{1}H NMR (300 MHz, MeOD) δ 5.2 (br, s, 1H), 4.27 (d, 1H), 3.95-3.92 (m, 4H), 3.88-3.43 (m, 6H, overlap with CD3OD peak), 3.27-3.20 (m, 4H), 1.79-1.13 (m, 18H), 0.90- 0.87 (m, 9H). ^{13}C NMR (75 MHz, CD3OD): δ 101.54, 101.52, 79.98, 76.50, 75.21, 73.69, 73.35, 72.78, 70.10, 61.36, 60.81, 36.57, 31.67, 29.83, 29.77, 29.65, 29.09, 26.75, 26.65, 22.32, 19.23, 18.48, 13.2.

HRMS (ESI) m/z: Calcd. (C26H50 O11)Na+: 538.58; Found: 537.327637.

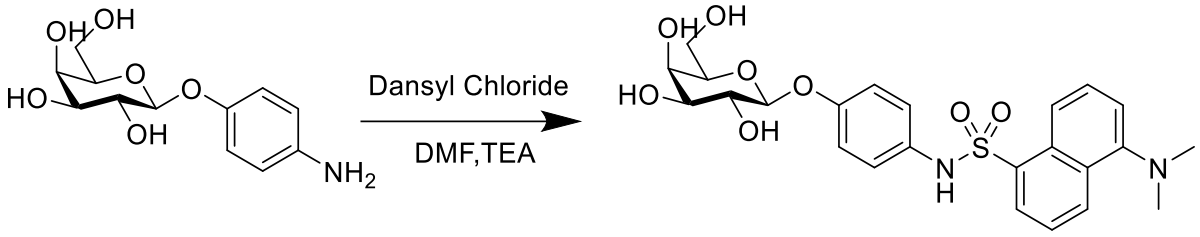


Figure S1: Synthesis scheme for a fluroscent probe, βGal-aryl-Dansyl

4-Aminophenyl β-D-galactopyranoside (0.12 g, 1.2 mmol) in anhydrous DMF (2 mL) was added to Et_3N (0.53 g, 0.53 mmol) in anhydrous DMF (5mL) at 0 ºC. To this solution was added dansyl chloride (0.41 g, 1.5 mmol). After stirring at 0 ºC for 2 hours, the mixture was concentrated, and the residue obtained was subjected to the purification by column chromatography DCM:MeOH 9:1 to yield a yellow solid product (0.44 g, 72 %). ^{1}H NMR (400MHz, $CDCl_3$): δ 8.47 (d, 1 H, dansyl), 8.24 (d, 1 H, dansyl), 8.20 (d, 1 H, dansyl), 7.65-7.69 (m, 2 H, dansyl), 7.39 (d, 1 H, dansyl), , 6.92–6.81 (m, 4H, ArH), 4.75 (d, J =7.7 Hz, 1H), 4.37 (s, 2H, C$\underline{H}_2$NHR), 3.69 (d, J = 3.1 Hz, 1H), 3.58–3.46 (m, 5H), 2.84 (s, 6H,-CH_3).

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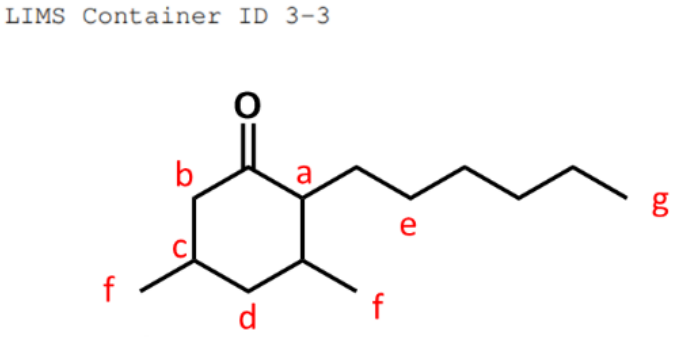

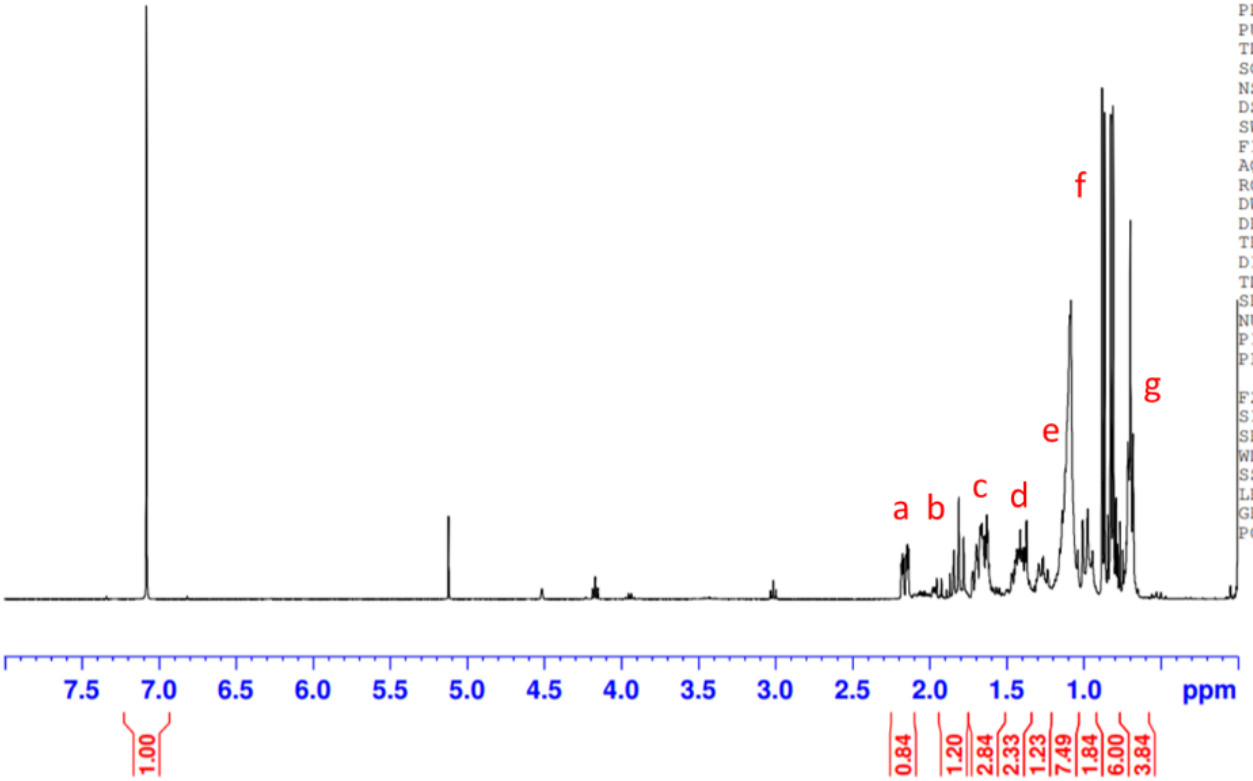


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Figure S2: ^{1}H NMR spectrum of **3** in $CDCl_3$

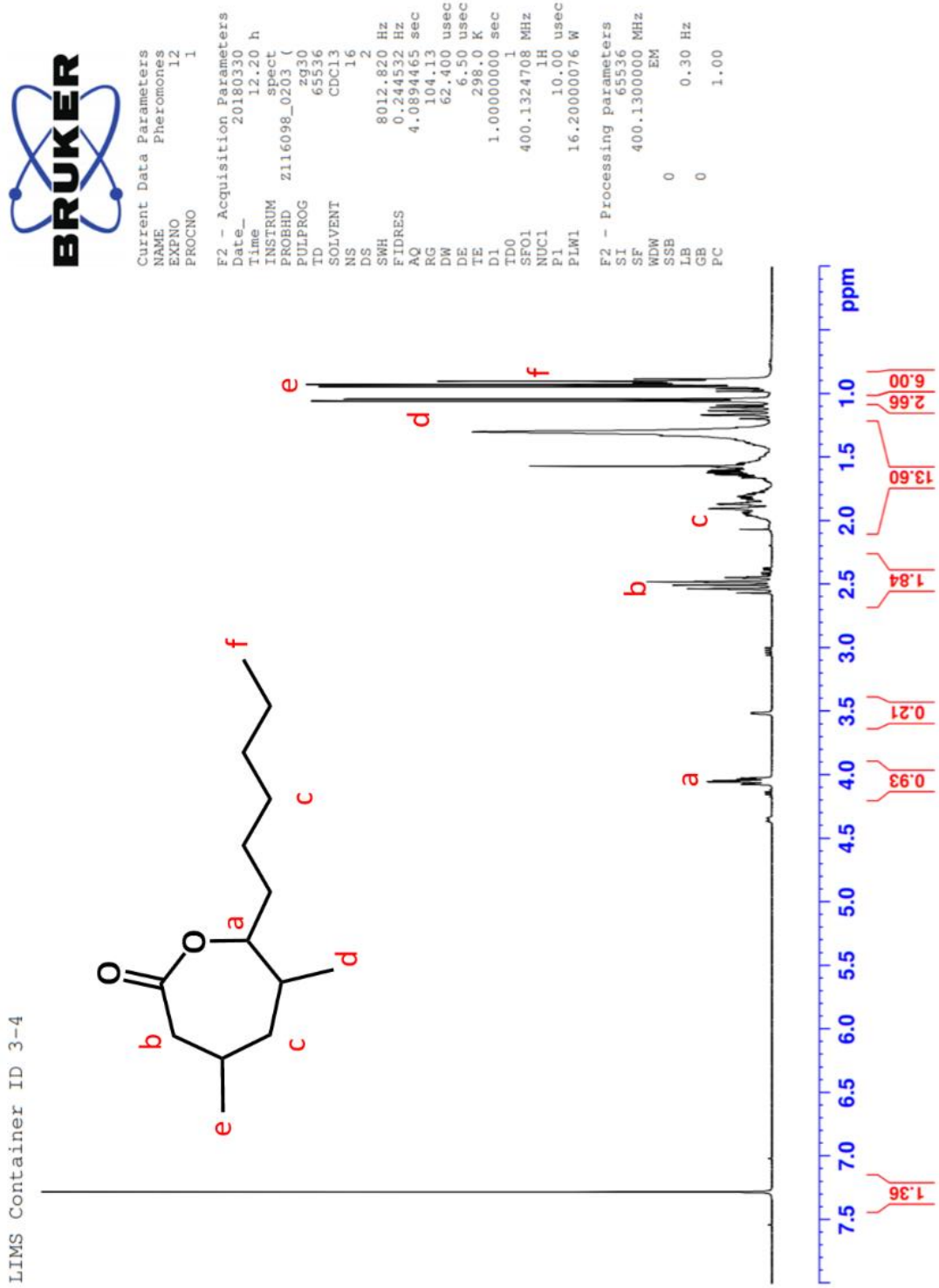

Figure S3: ^{1}H NMR spectrum of **4** in $CDCl_3$

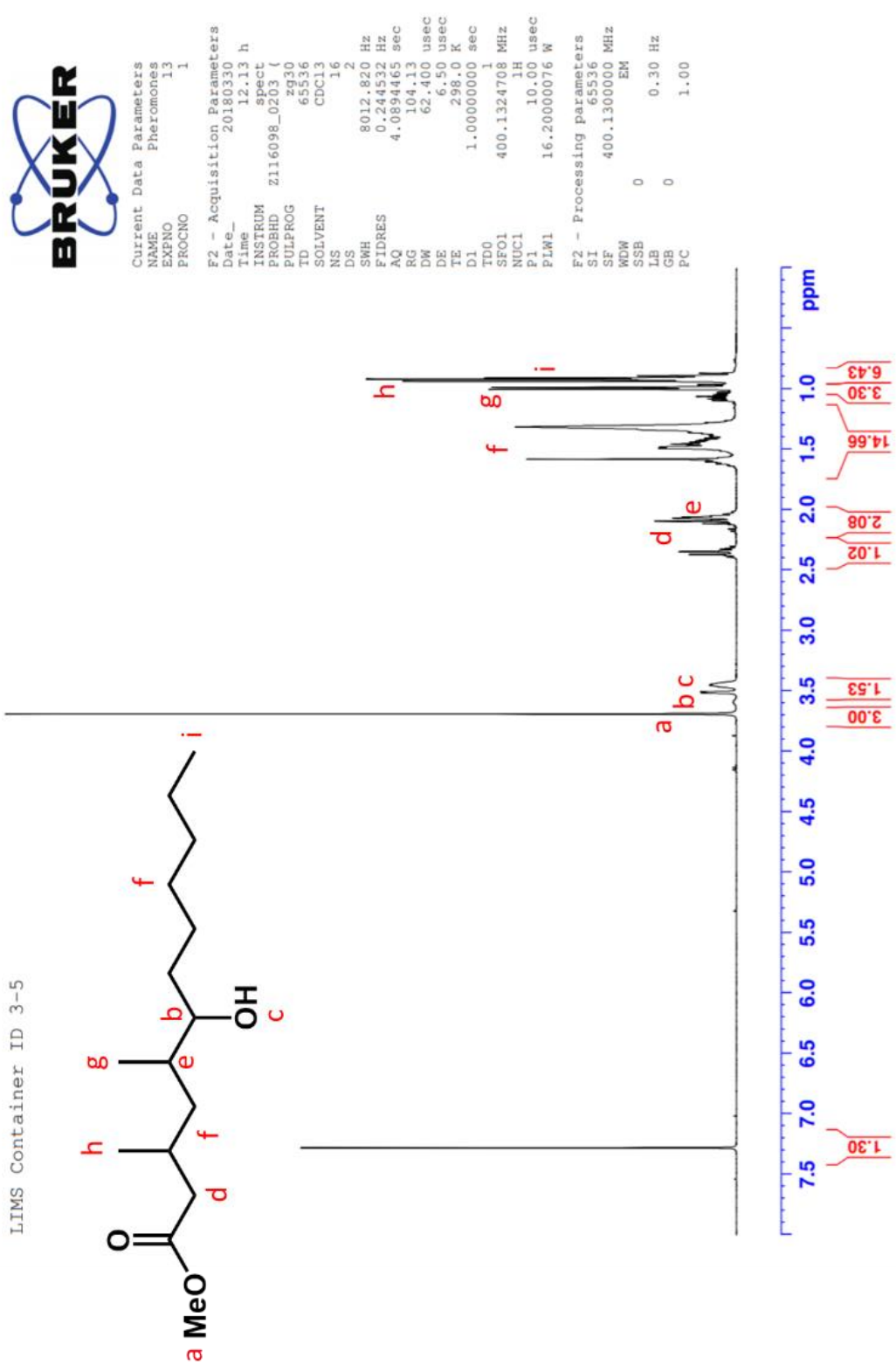

Figure S4: ^{1}H NMR spectrum of **5** in $CDCl_3$

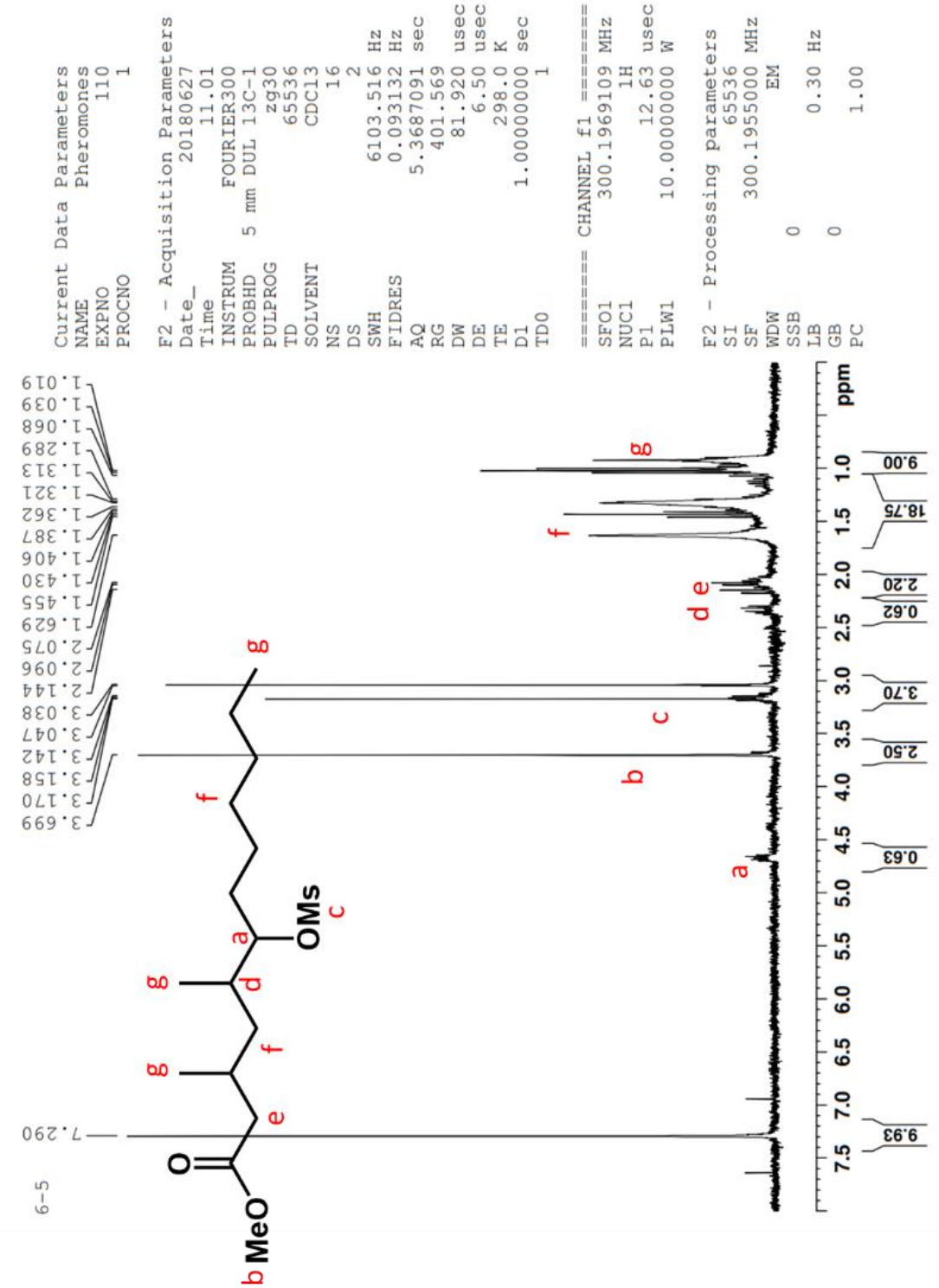

Figure S5: ^{1}H NMR spectrum of **6** in $CDCl_3$

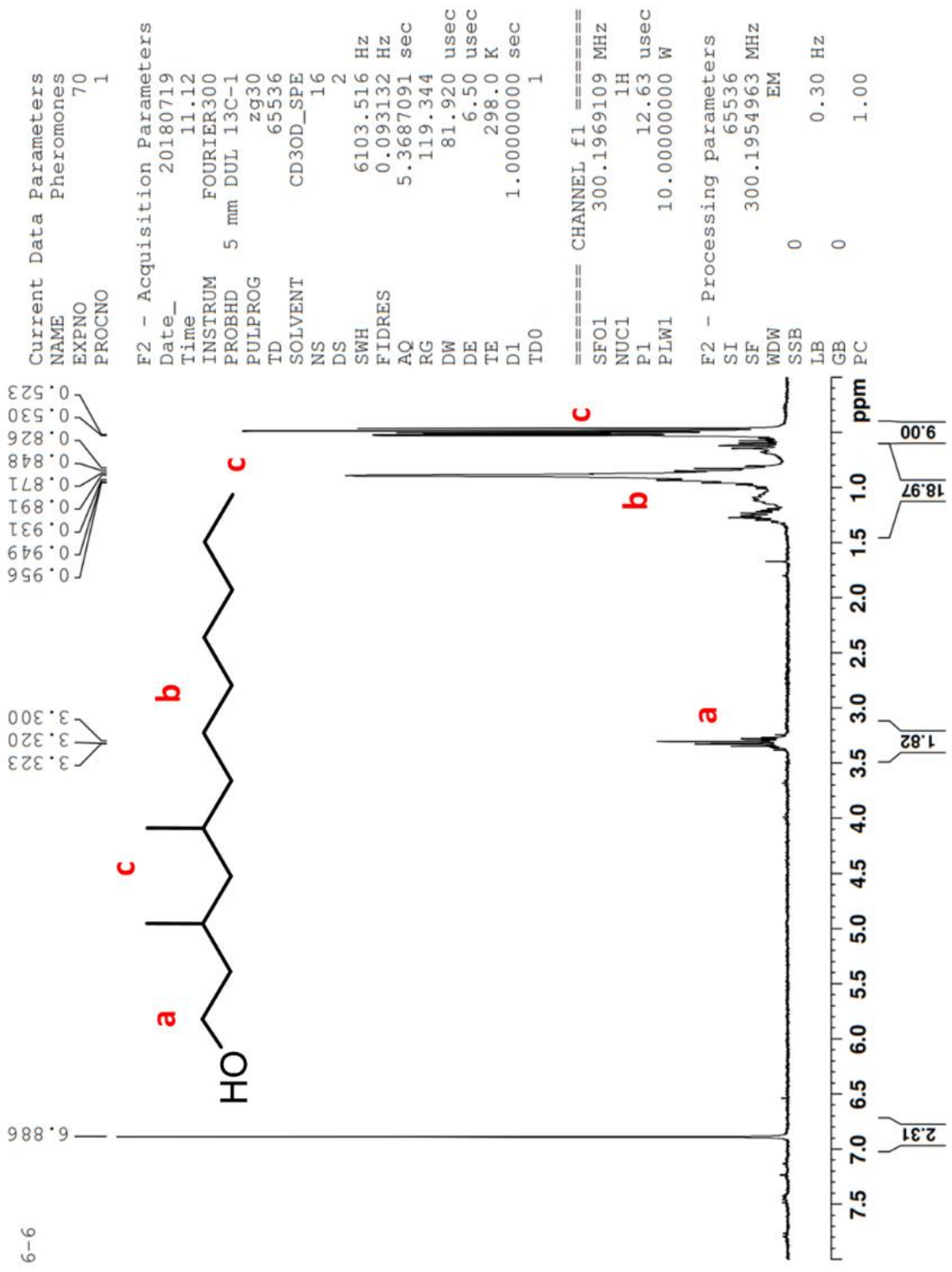

Figure S6: ^{1}H NMR spectrum of **7** in $CDCl_3$

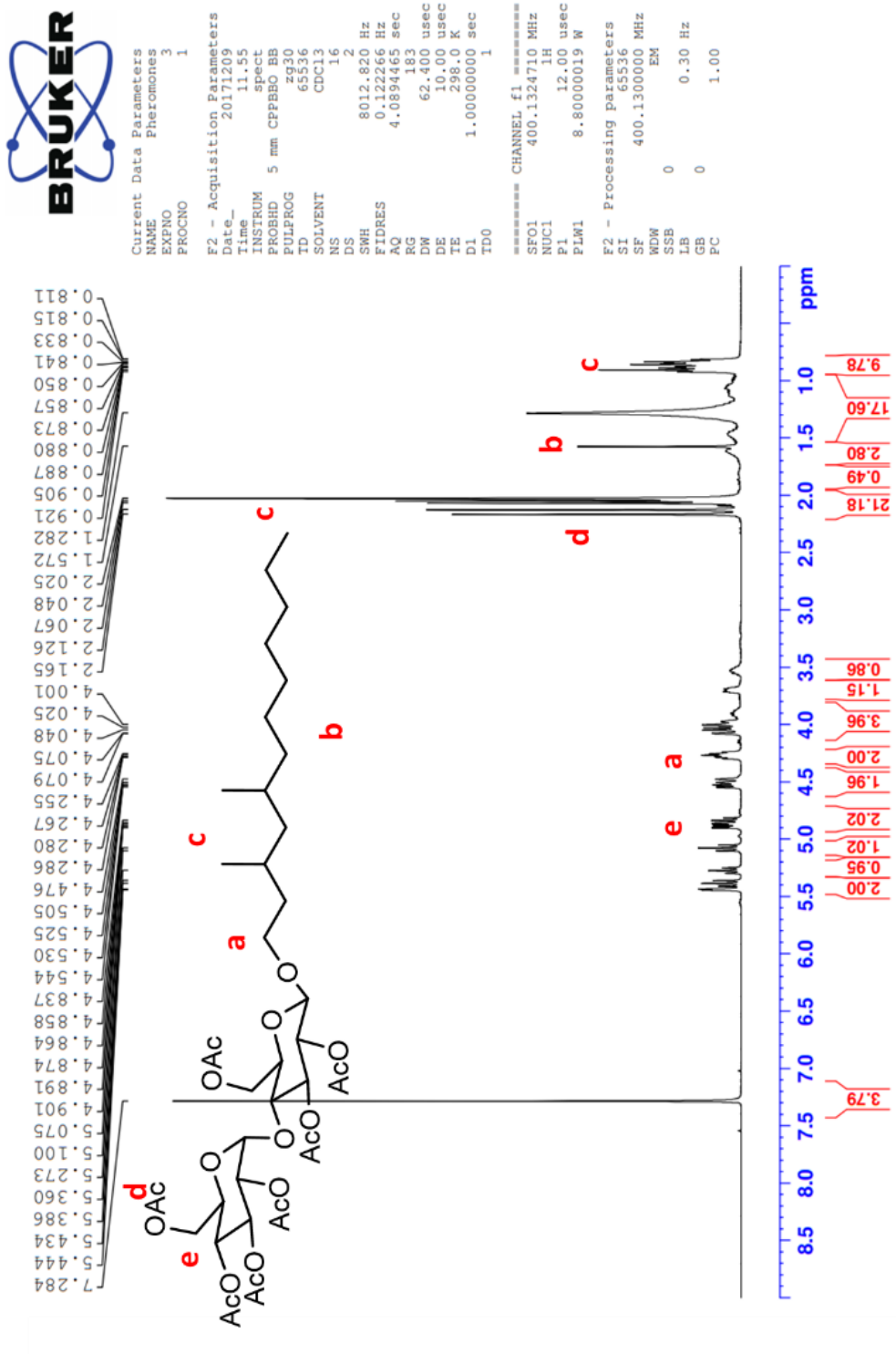

Figure S7: ^{1}H NMR spectrum of **11** in $CDCl_3$

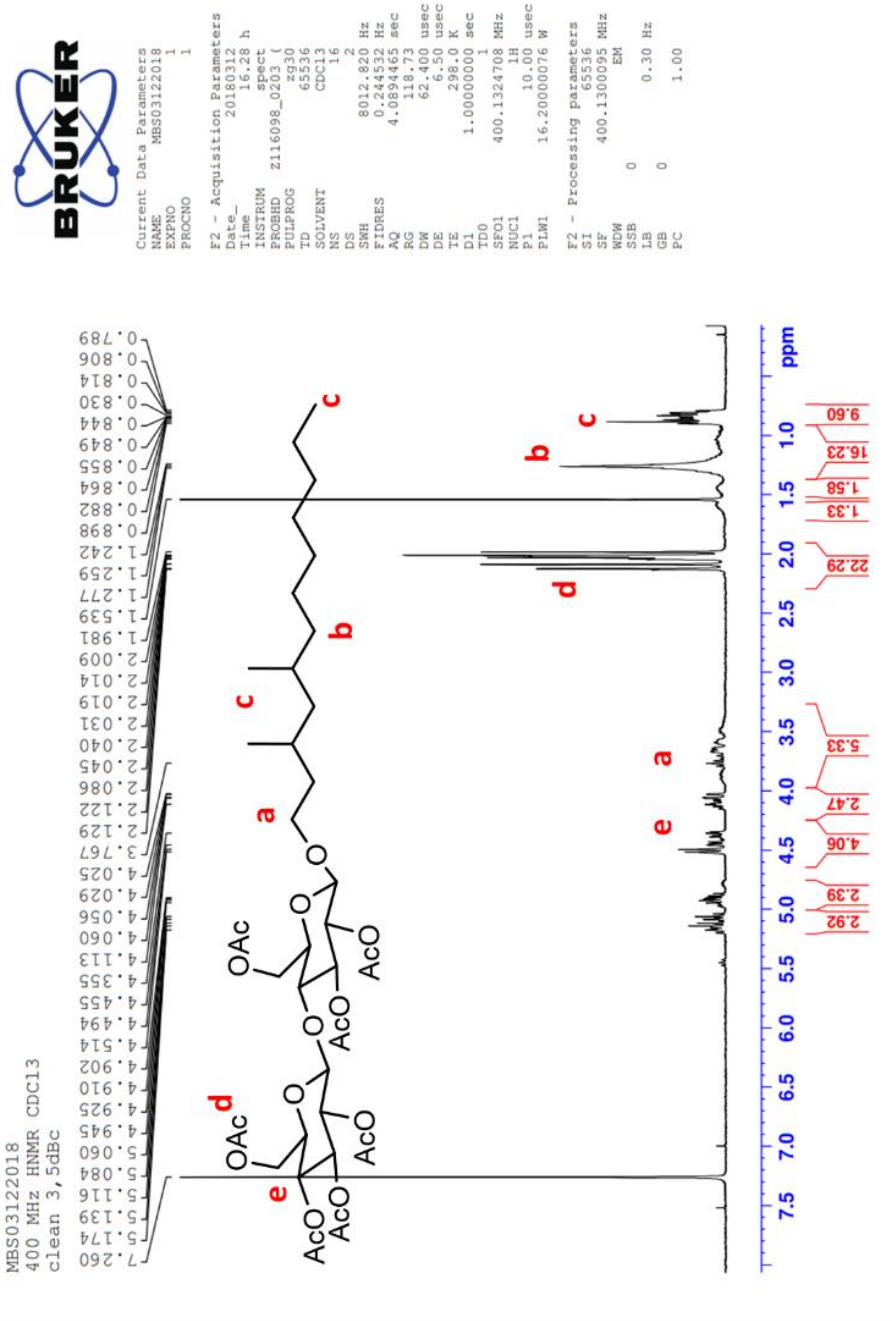

Figure S8: ^{1}H NMR spectrum of **10** in $CDCl_3$

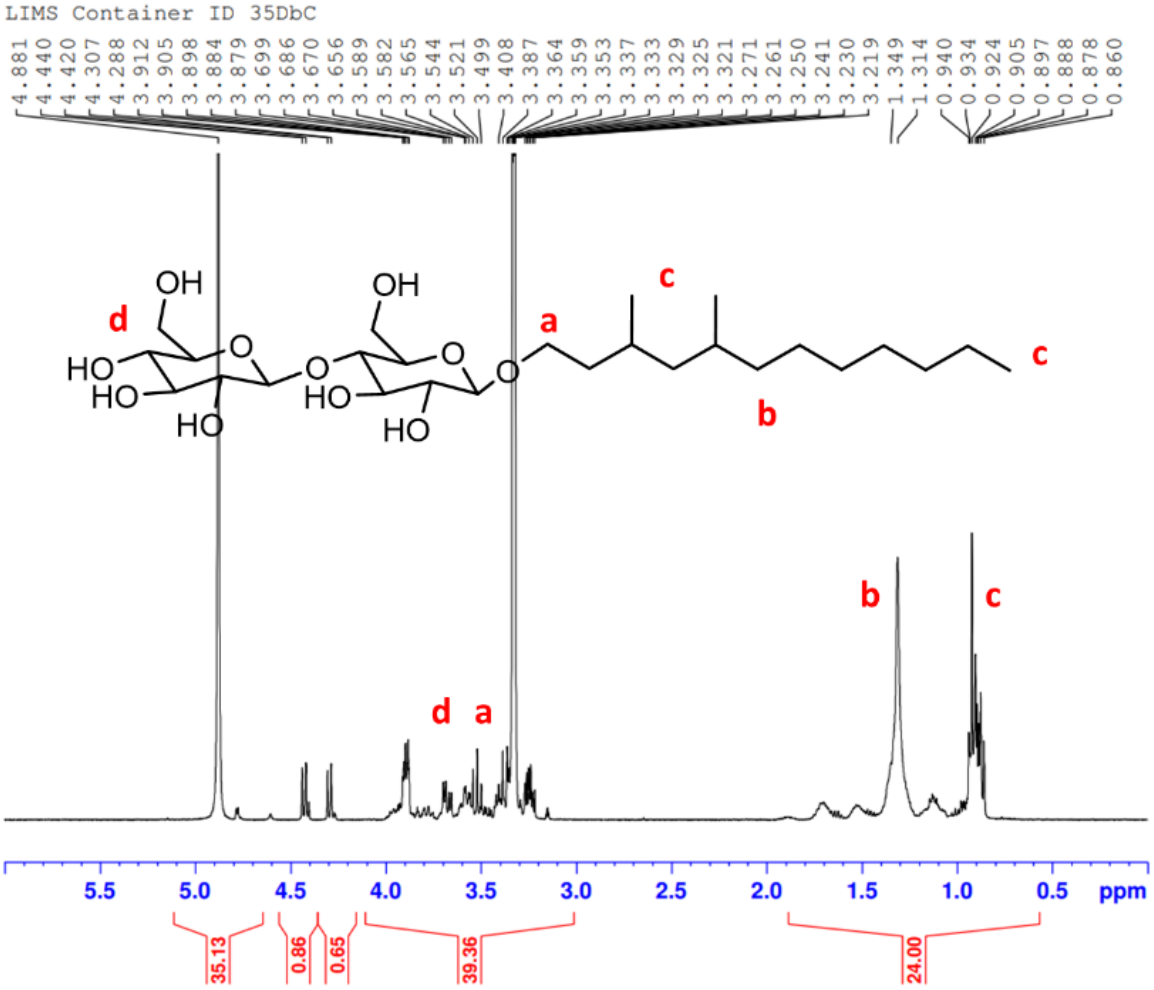


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Figure S9: ^{1}H NMR spectrum of **12** in MeOD

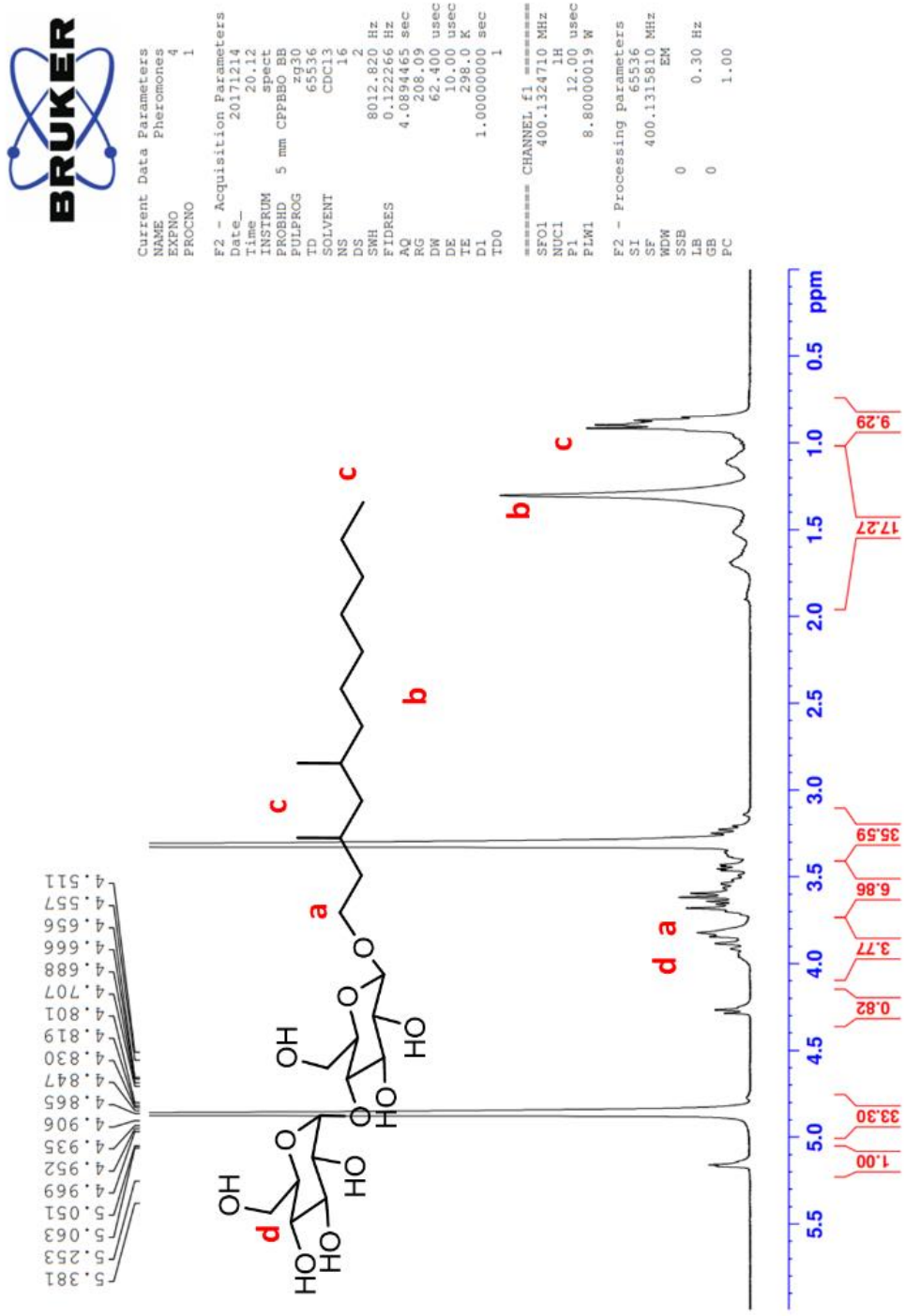

Figure S11: ^{1}H NMR spectrum of **13** in MeOD

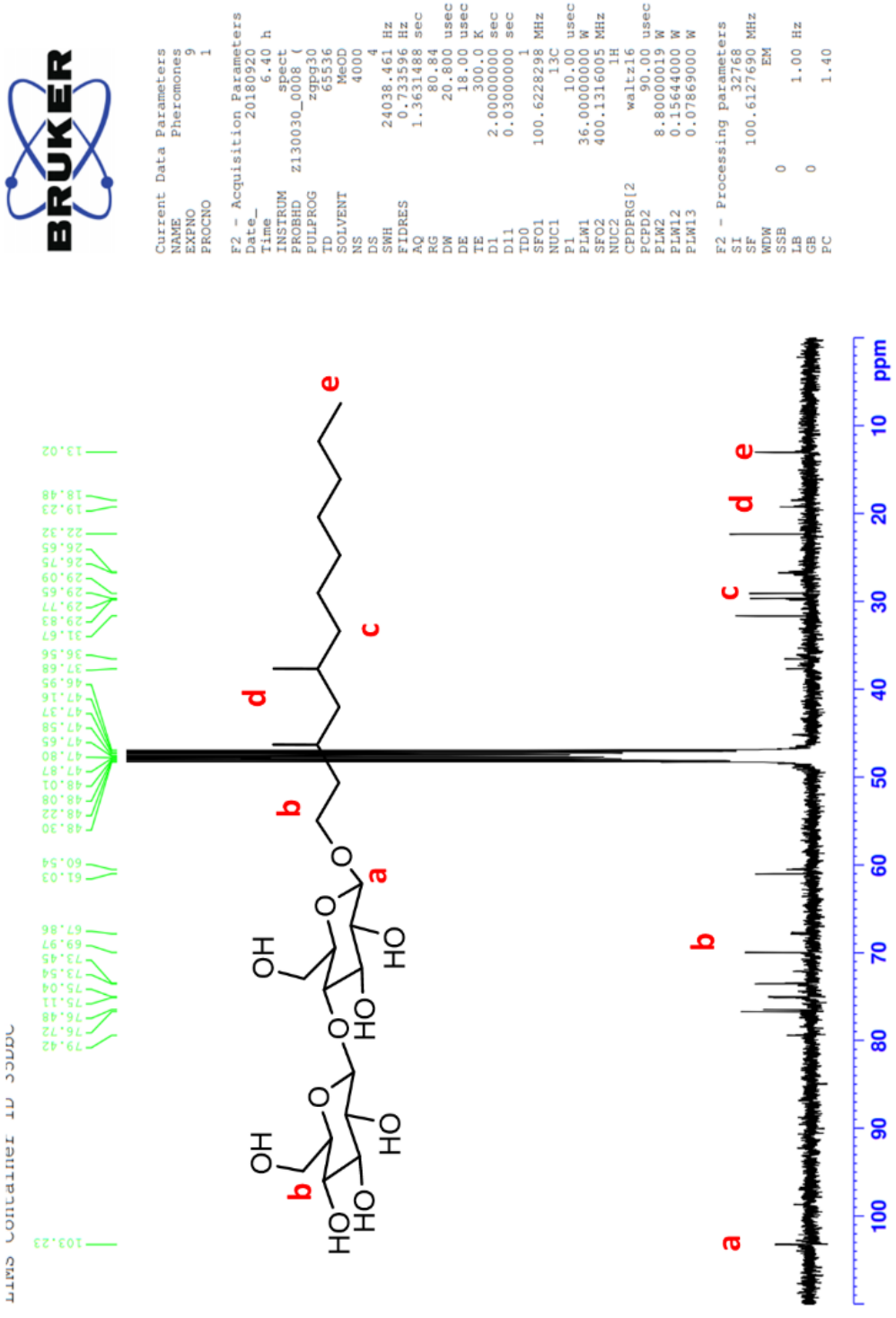

Figure S12: ^{13}C NMR spectrum of **12** in MeOD

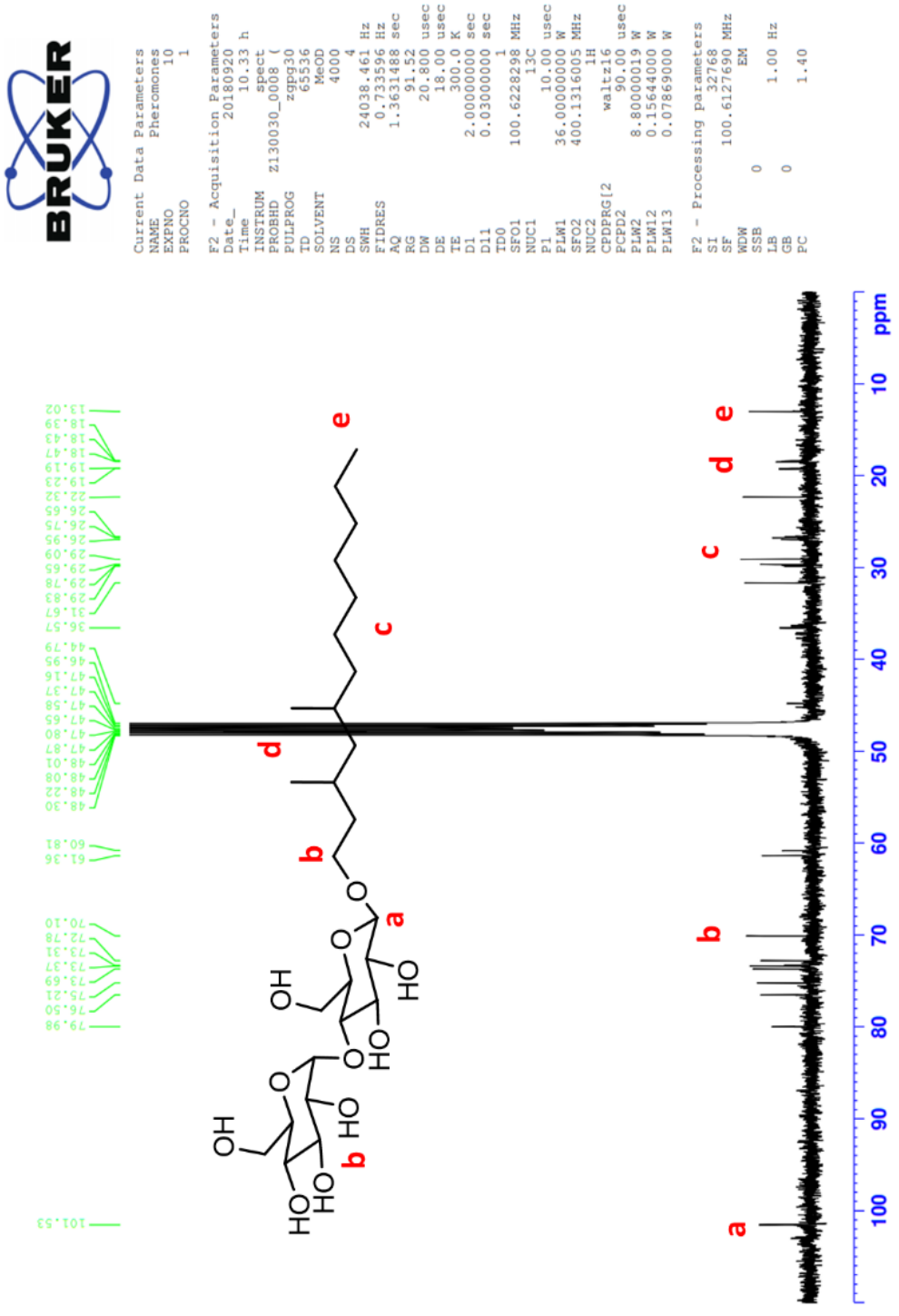

Figure S13: ^{13}C NMR spectrum of **13** in MeOD

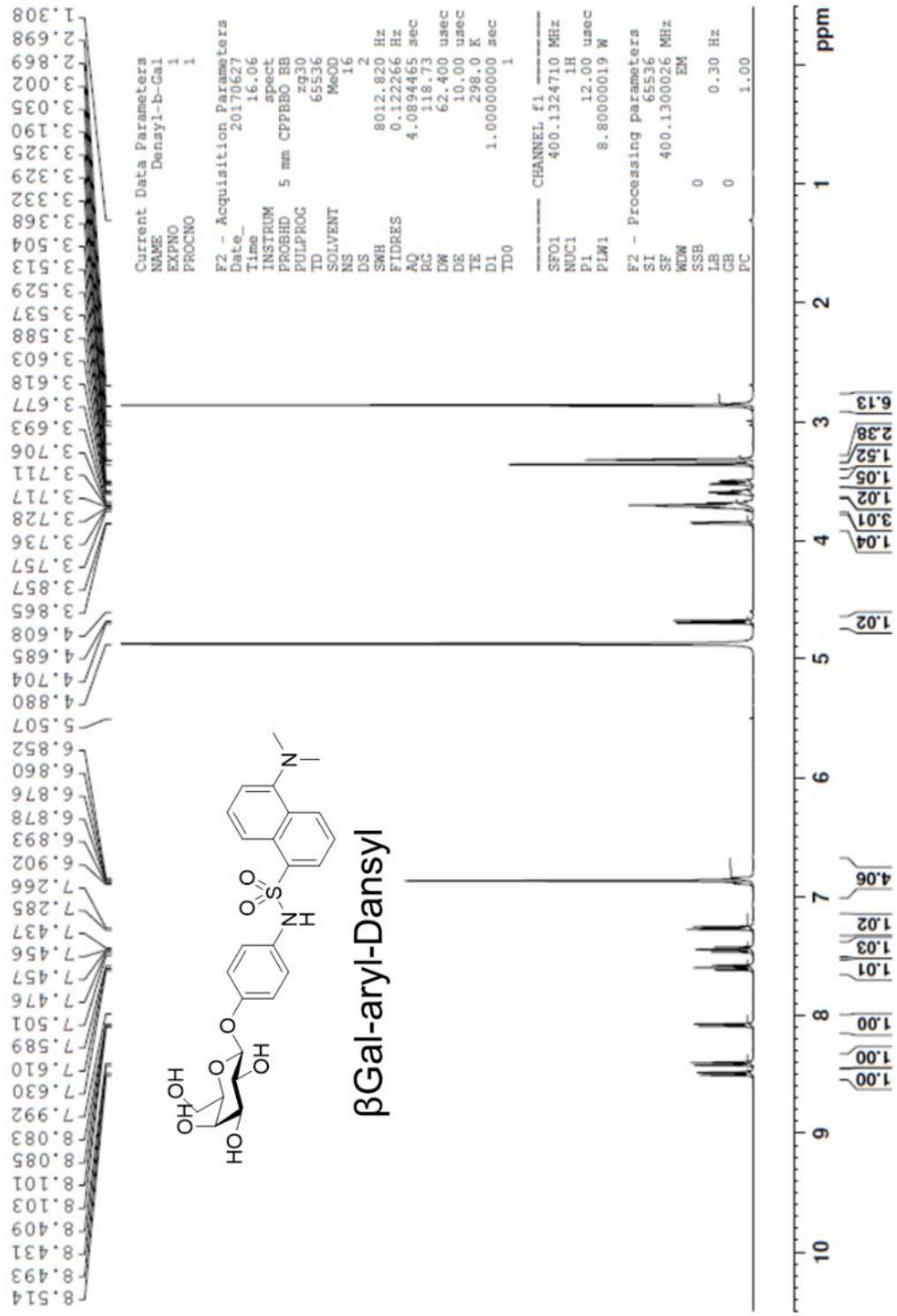

Figure S14: ^{1}H NMR spectrum of βGal-aryl-Dansyl in $CDCl_3$

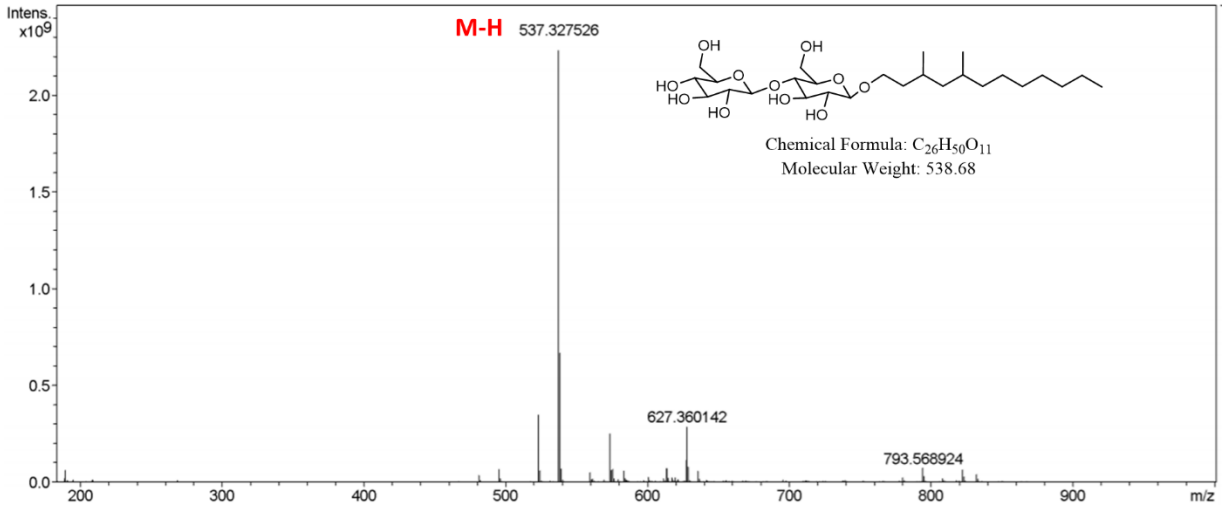

Figure S15: Mass spectroscopy by - negative-ion mode electrospray ionization of **12**

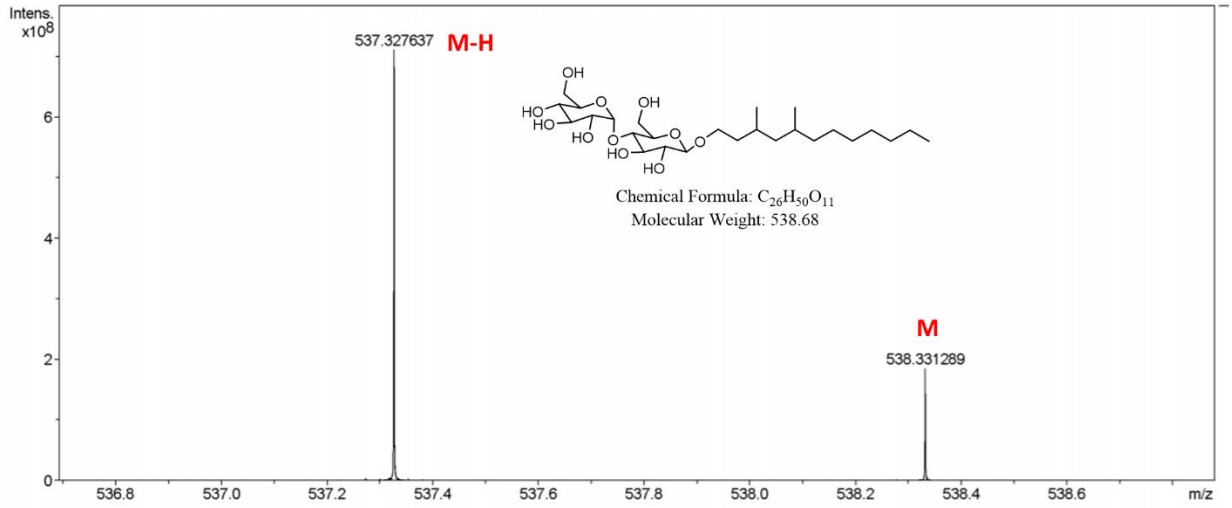

Figure S16: Mass spectroscopy by - negative-ion mode electrospray ionization of **13**

2.5. *Experimental section*

Freezer stocks of all strains (Supplementary for Bacterial strains) were stored at -80 ^{o}C in lysogeny broth (LB) with ~20% glycerol. All strains were grown in LB (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl) or Mueller-Hinton Broth MHB (beef infusion solids, 2.0 g/l. casein hydrolysate, 17.5 g/l. starch, 1.5 g/l) at 37 ^{o}C with shaking at 250 rpm. All biofilm inhibition, dispersion assays were performed in LB or MHB medium, and plates were incubated at 37 ^{o}C under static conditions. Stock solutions of 10 mM of 3,5-diMeDβM, 3,5-diMeDβC, SFEG$_4$OH, and 10 mg/ml of tobramycin sulfate were prepared in sterile (autoclaved) water. Stock solutions of agent (3,5-diMeDβM, and 3,5-diMeDβC), and non-autoclavable reagents were further filtered through cellulose acetate syringe filter (0.2 µm pore, Millipore). All overnight cultures of bacteria were grown in Lysogeny Broth, Miller (LB-Miller, 10 g/l tryptone, 5 g/l yeast extract, 10 g/L NaCl). All biofilm assays were performed by immersing the pegs of a modified polystyrene microtiter lid of MBEC™ plates (Innovotech, Alberta, Canada) in M63 medium. All the optical density/absorbance measurements were carried out on Biotek ELx800™ absorbance microplate reader (BioTek Instruments, Inc. Winooski, VT).

Blood agar for revealing small colony variants: To prepare the blood agar gel containing our agents for visualizing small colony variants (SCVs), 1.75 g of premixed powder (Thermo Scientific™ Oxoid™ Columbia Blood Agar Base) containing peptone mixture 25.1 g/l, soluble starch 1.0 g/l, sodium chloride 5.0 g/l, agar 12.0 g/l was mixed and autoclaved with 100 mL of sterile water. The solution was cooled briefly without solidifying and followed by adding 5 mL of sheep blood (Hemostat) and mixed gently by swirling. Portions of 20 mL of this solution was

poured into a 50 mL falcon tube, followed by adding 170 µL of a stock solution of agents 3,5-diMeDβM/βC (10 mM) to obtain gel solutions containing 85 µM of 3,5-diMeDβM/βC. The 20 mL gel solutions were poured into petri dishes (10-cm diameter) and solidified for inoculation and visualizing colonies of SCVs.

Strain	**Number**	**Source**
P. aeruginosa PAO1 wild type		Dr. Guirong Wang (Upstate Medical University)
PAO1-EGFP		Dr. Guirong Wang (Upstate Medical University)
Δ*pilA* knock out mutant		Dr. Rob Lavigne (Katholieke Universiteit Leuven)
pilT transposon mutant	PA0395	**PAO1 transposon mutant library (Manoil lab)**
LecA transposon mutant	PA2570	**PAO1 transposon mutant library (Manoil lab)**
P. aeruginosa pili source strain	PA1244N3 (pPAC46)	Dr. Castric **(Duquesne University) &** Dr. Horzempa (Liberty University)
PAO1k, phage sensitive strain *wt* PAO1		Dr. Rob Lavigne (Katholieke Universiteit Leuven)
Lytic Phage ϕKMV specific for PAO1k		Dr. Rob Lavigne (Katholieke Universiteit Leuven)

2.5.1 Bacterial strains and bacteriophage used in studies

Table 2.3 Bacterial strains and bacteriophage used in these studies.

Here, we describe experiments in this work that are new or modified from literatures.

2.5.2 *In vitro Small colony variants assay*[71]

Overnight cultures (100 µL) of wt PAO1 was diluted 10 mL of LB containing 0.3 µg/ml tobramycin with and without 85 µM 3,5-diMeDβM/βC and incubated at 37°C without shaking for 6 h. These cultures were serially diluted (10^5-10^7) in LB medium and spread on Columbia blood agar plates (Thermo Scientific™ Oxoid™ Columbia Blood Agar Base (Dehydrated) supplemented with 5% sheep blood) containing 85 µM 3,5-diMeDβM or 3,5-diMeDβC agar plates were incubated at 37°C for 1 day.

2.5.3 *Bacterial Swarming assay*[80]

The soft gels for swarming motility were prepared by autoclaving 0.5 wt% Bacto Agar in M8 medium (0.6 % Na_2HPO_4, 0.3 % KH_2PO4 and 0.05 % NaCl). The gel solution was cooled to ~60 ºC, supplemented with filtered 0.2 % glucose, 0.5 % casamino acid, and 1 mM $MgSO_4$ (0.22 µ filter). The gel solutions were poured into a Falcon tube for 20 mL portions, followed by adding aliquots of (1-20 µL) of 3,5-diMeDβM or 3,5-diMeDβC stock solutions to achieve the desired concentrations. The falcon tubes were closed, the agar solution was mixed by gently rocking, and then poured into polystyrene petri dishes (10 cm-diameter). The agar solution was solidified by cooling and air-drying in a laminar hood for 1 h. Bacterial culture of wild-type PAO1 (3 µL) with an OD_{600} between ~0.4–0.6 was inoculated on the center of the surface of the soft agar gel. These "swarm plates" were incubated at 37 ºC for 12 h and then incubated for additional 12 h at room temperature. After a total of 24 h, pictures of the swarming plates were taken.

2.5.4 *Bacterial motility enabled binding assay*

For soft gel prepared for swarming motility (see above), 100 µL of 1 mg/ml pilin, LecA, or BSA protein was introduced onto the center of the agar gel and immediately spread over the surface of the ger using a sterile cell spreader. The proteins were prepared in NaPB (4 mM, pH 7.2), Tris-HCl (0.1 M Tris-HCl, pH 7.5 and 6 µM $CaCl_2$) and Tris buffer (2 mM Tris, 7 mM NaCl, pH 7.5). The agar gel spread with protein solution was dried for an additional 30 min. A bacterial subculture (3 µL, 0.6 OD_{600}) was inoculated on the center of the soft gel (10-cm diameter plate). The bacteria on the soft gel were incubated 12 h at 37ºC and an additional 12 h at room temperature.

2.5.5 Persistent population isolation assay[85,86]

An overnight culture (100 µL) of bacteria in LB was diluted in 10 mL of M63 and incubated to reach an OD_{600} value of ~0.1. Aliquots (150 µL) of the subculture were added into the wells of 96-well MBEC™ microtiter plate with and without 0.3 µg/ml tobramycin, 85 µM 3,5-diMeDβM/βC. MBEC™ pegs were immersed in the bacterial culture and further incubated at 37 °C without shaking for 24 h, to form biofilms. The MBEC™ peg lid was then removed, and pegs were washed with 0.9% saline to remove unattached or loosely attached bacteria and antibiotic. The biofilm mass was quantified by crystal violet (CV) dye assay for 6 pegs. The 10 unstained pegs were cut with sterile plier and suspended in saline water. The saline solution with pegs were sonicated 15 minutes at 30 kHz (Symphony VWR Internationals Ltd.). Pegs were removed, and the solutions were centrifuged at 6000 rpm for 10 minutes, to collect the bacteria. The collected pellets were resuspended and supplemented with 20 µg/ml tobramycin (20×MIC) in MHB

medium and incubated at 37 ℃ with shaking at 250 rpm for 8 h, to isolate persistent population. The sample was then centrifuged at 8000 rpm for 10 min at room temperature, to collect the persister pellet. The supernatant was discarded, the bacterial pellet was washed twice with 0.9% saline. The number of persistent bacteria w then quantified by counting CFU on MHB agar plate.

2.5.6 Chemical inhibition of bacteriophage adsorption ϕKMV on P. aeruginosa strains[111,112]

Bacteriophage adsorption assay were adopted as described previously,[112] with addition of our agents. An overnight culture (100 µL) of wt P. aeruginosa strain PAO1k that is ϕKMV sensitive, knockout mutant ΔpilA, and transposon mutant pilT mutant (pilT::Tn) were diluted with 10 mL of LB supplemented with 10 mM $MgSO_4$ (LB-Mg^{2+}) and subcultured to OD_{600} around 0.6 at 37°C with shaking at 250 rpm with and without 85 µM 3,5-diMeDβM/βC, or 60 uM $SFEG_4OH$. The bacteria subculture (100µL) was mixed with 900 µL of LB-Mg^{2+}/ml ϕKMV phage. The titer of added phage was individually determined for every experiment from the phage stock solution. Following incubation for 10 min at 37 °C with shaking at 100 rpm, bacteria were removed by centrifugation (10,000 g, 5 min at 4°C), and 900 µL of the supernatant was transferred to an Eppendorf tube. The plaque-forming units (PFU) in the supernatant with and without the added agents was determined by the top agar overlay method with PAO1k. The percentage of phage bound to bacteria was calculated as [(titer of added phage − titer in supernatant)/(titer of added phage)] × 100.

2.5.7 Crystal violet dye-based biofilm assay[120]

An overnight culture (100 µL) of bacteria in LB was diluted in 10 mL of M63 and incubated to reach an OD_{600} value of ~0.1. The bacterial culture (150 µL) was added to the wells of MBEC™ microtiter plate, followed by predetermined volumes of 3,5-DiMeDβM/βC stock solution for targeted concentrations (6 wells/concentration). The MBEC™ plates were incubated under

stationary conditions at 37 ℃ for 24 h. After incubation, the pegs were transferred and immersed into 96 wells containing sterile water (200 µL) twice to briefly rinse the biofilms to remove unattached or loosely attached bacteria and were dried at 37 ℃ for 30 min. The peg-attached biofilms were immersed into 96 wells containing crystal violet (CV) dye solution (150 µL, 0.1%) at ambient temperature for 30 min, to stain the biofilms. The CV-stained pegs were then washed twice with sterile water (200 µL). To solubilize the CR stains, 150 µL of 30% acetic acid solution was added to wells, and the pegs was immersed in the wells, the plates were shake on a microplate mixer (Scilogex MX-M) at 100 rpm for 15 min. The amount of biofilms was inferred and quantified by measuring the OD_{600} of the 150 µL acetic acid solution on plate reader. The absorption from stained pegs containing just M63 medium was subtracted from pegs treated with agents.

2.5.8 Confocal microscopy of biofilms and image acquisition[55,81]

In a 24-well microtiter plate, biofilms were grown by placing 100 µL of *wt* PAO1 culture with and without tobramycin, and with and without 85µM 3,5-diMeDβM/βC, on polystyrene coupons (roughly 3/8 in. × 3/8 in.) that were cut from a polystyrene petri dish. The Saran-wrapped plate was then incubated at 37 ^{o}C without shaking. Each polystyrene coupon was then washed gently by immerging into saline twice and then placed on a microscope cover glass (50 x 24 mm, No. 2, Fisher Scientific, Pittsburgh, PA). All microscopy images were acquired using a Zeiss LSM confocal laser scanning microscope (Carl Zeiss, Germany) for monitoring Green Fluorescent protein (GFP) and propidium iodide (PI) fluorescence.

2.5.9 Colony morphology assay[99]

Overnight cultures of wt PAO1 (100 µL) was diluted with 10 mL MHB medium containing 0.3 ug/ml tobramycin with and without 85 µM of 3,5-diMeDβM or 3,5-diMeDβC and cultured further for 6 h. These cultures (10 µL) were inoculated on a 1 wt% LBNS agar gels containing 0.3 µg/ml tobramycin with and without 85 µM of 3,5-diMeDβM or 3,5-diMeDβC. The agar plates were incubated at 37°C for 3 days, and images of the colonies were taken. The agar gels were prepared by autoclaving 1 wt% bacto agar in LBNS medium. The gel solution was cooled to about 60 ºC, and poured into Falcon tubes for 20 mL, and supplemented with filtered solutions of Congo red (40 µg/ml), Coomassie brilliant blue dye (20 µg/ml), and tobramycin (0.3 ug/ml), 3,5-diMeDβM or 3,5-diMeDβC (85 µM). The falcon tubes were closed, gently mixed, and then the agar solution was poured into polystyrene petri dishes (10 cm-diameter, Fisherbrand™), and solidified at room temperature.

2.5.10 Characterization of rugose and smooth colony morphology of PAO1 caused by 0.3 µg/ml tobramycin with and without 85 µM 3,5-diMeDβM/βC[121,122]

The overnight culture (100 µL) of *wt* PAO1 was diluted in 10 mL LB medium and treated with and without 0.3 µg/ ml tobramycin with and without 85 µM 3,5-diMeDβM/βC 37 ºC with shaking at 250 rpm for 6 h. The 10 µL of these cultures were spotted on 1 % bacto agar plates containing 20 mL colony morphology assay medium (1% tryptone; Bacto), with 40 µg/ml Congo red (CR) and 20 µg/ml Coomassie brilliant blue dyes (EMB). Colonies were incubated at 37°C for 3 days.

2.5.11 Pellicle formation and quantification[122]

Pellicle formation assay was adopted as previously described. Briefly, overnight culture (100 µL) of *wt* PAO1 were inoculated in borosilicate glass tubes (18 mm by 150 mm) containing 10

mL of MHB supplemented with and without 0.3 µg/ml tobramycin, 85 µM 3,5-diMeDβM/βC. Tubes were incubated without shaking at 37°C for 3 days. Pellicle formed at the air-liquid interface was resuspended in 1.5 mL PBS buffer. Congo red stock solution (10 mg/ml) was added to a final concentration of 40 µg/ml and incubated with shaking for 2 h at 37°C. Following this incubation, the bacteria were pelleted via centrifugation (6000 rpm, 15 min); and the absorbance (490 nm) of 200 uL of the supernatant was measured on plate reader. The PBS with 40 µg/ml Congo red was used as the reference to determine the amount of Congo red bound to pellicles.

2.5.12 Quantification of biofilm bacteria and mass[55,81]

We quantified the killing of bacteria in biofilms under different chemical treatment (tobramycin and 3,5-diMeDβM/βC) by CFU counting, and measured biofilm mass by CV dye assay. An overnight culture (100 µL) of bacteria in LB was diluted in 10 mL of M63 and subcultured to reach an OD_{600} value of ~0.1. The bacterial culture (150 µL) was added to the wells of MBEC™ microtiter plate, followed by predetermined volumes of 3,5-diMeDβM/βC stock solution for targeted concentrations (6 wells/concentration). The 24 h-old biofilm on pegs were transferred to wells of saline briefly, and then transferred to wells containing 150 µL of fresh M63 medium containing of tobramycin, 3,5-diMeDβM or 3,5-diMeDβC with targeted concentrations and were incubated for an additional 0-24 h at 37 ºC. The pegs were washed with saline and clipped with sterile plier. Each condition consisting of 6 pegs was sonicated in 5 ml of saline and serially diluted in saline followed by plating on LB agar plates for 24 hours for counting colony forming units. The amount of the biofilms on pegs were measured by using the crystal violet staining

method described in biofilm inhibition assay. The bacterial count (CFU/ml) was normalized by amount of biofilm.

2.5.13 Hemolysis assay[123]

Single donor Human Red Blood Cells (Innovotech) suspension (300 µL) was mixed with different volumes of 3,5-diMeDβM/C to reach final concentrations of 0-500 µM in PBS buffer, respectively. mix 300 µL of RBC suspensions with of 30 µL of Triton X (1%) and PBS, respectively as Positive and negative controls After incubation at 37℃ with 100 rpm shaking for 1 hthe samples were then centrifuged for 2 min at 2000 rpm. The absorbance of 200 µL supernatant was recorded at 540 nm. % Hemolysis was calculated as $(OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control}) \times 100\%$. Experiments were performed in triplicate.

2.5.14 Critical Aggregation Concentration (CAC) assay[124]

A 3,5-diMeDβM/βC stock solution (10 mM) was diluted in PBS buffer at various concentrations (0-200 µM). An ethanol solution (1 µL) of Nile red (2.5 mM) was added to 1 mL assay solution, and the fluorescence emission was measured on a using Synergy 2 multimode microplate reader an excitation wavelength of 550 nm after 20 minutes of incubation at 37℃. Fluorescence intensity was recorded at 635 nm. Experiments were performed in triplicate.

2.5.15 Bacterial Growth as 3,5-diMeDβM as the sole carbon source[125]

Wild type *P. aeruginosa* strain was grown on 1.5 % agar gel containing modified M9 minimal salts, 3.5 g of $NaNH_4HPO_4 \cdot 4\ H_2O$, 7.5 g of $K_2HPO_4 \cdot 3H_2O$, 3.7 g/liter KH_2PO_4, and 0.1% (vol/vol) trace salts solution. One liter of trace salts solution contained 2.86 g of $FeCl_3 \cdot 6H_2O$, 1.98 g of $MnCl_2 \cdot 4H_2O$, 3.31 g of $CoCl_2 \cdot 6H_2O$, 1.47 g of $CaCl_2 \cdot 2H_2O$, 0.17 g of $CuCl_2 \cdot 2H_2O$, and 0.61 g of $ZnCl_2$. Combinations of either 85μM of 3,5-diMeDβM or 2% glucose were provided as the sole carbon source. Growth of bacterial colonies was monitored at 37°C for 1-3 days.

2.5.16 Twitching assays[126,127]

Subsurface twitching assays were modified from previously reported procedures. Briefly, 100 μL of overnight culture of PAO1-EGFP was diluted in 900 μL of Luria Bertani broth without NaCl (LBNS). Sterile pipette tips (10 μL) were dipped in this LBNS suspension and were then stabbed through a one-day-old 1% LBNS agar, with and without 85 μM of 3,5-diMeDβM or 3,5-diMeDβC, to inoculate bacteria at bottom of the agar gel. Plates were incubated upright (not inverted) in an incubator at 37 ℃ for 5 days, and images of the plates were taken under ultraviolet light (Accuris™ E3000 UV Transilluminator - 302nm) in dark room.

2.5.17 Gel electrophoresis of sheared surface proteins from wild type P. aeruginosa[110]

The levels of pili on bacterial surface were analyzed by gel electrophoresis as described previously with slight modifications[110]. The *wt* PAO1 and *transposon mutant pilT::Tn* were streaked on LB agar plates with and without 85μM 3,5-diMeDβM/βC and incubated at 37 °C for ~ 16 h. The bacterial cells were scraped from the agar gels with glass coverslips and resuspended in 5 mL of phosphate buffered saline, pH 7.0, with and without 85μM 3,5-diMeDβM/βC, respectively. The solutions were centrifuged at 6000 rpm for 15 min to collect bacterial cells. The collected bacterial pellet was resuspended in 1 ml PBS buffer, and vortexed for 30 s to shear

surface proteins from suspended bacterial cells. The suspensions were transferred to three separate 1.5 ml Eppendorf tubes and centrifuged at 11,688 x *g* for 5 min to pellet the bacterial cells. The supernatant was transferred to fresh tubes and centrifuged at 11,688 x *g* for 20 min to further pellet remaining cells. The supernatants were collected in new tubes, mixed with 1:10 volume of 5 M NaCl and 30% (w/v) polyethylene glycol (PEG 8000; Sigma-Aldrich), and incubated on ice for 90 min, to precipitate the surface proteins. The solutions were centrifuged at 11,688 x *g* for 20 min, to collect the aggregated proteins. The protein pellets were resuspended in 100 µL PBS buffer and the amount of total surface sheared protein was determined by Bradford assay that resulted for 120 µg/ml protein mass for *wt* PAO1 without 3,5-diMeDβM/βC treatment, and 136 µg/ml for *wt* PAO1 with 3,5-diMeDβM/C treatment, which were diluted to 120 µg/ml; and 470 µg/ml for *transposon mutant pilT::Tn* without 3,5-diMeDβM/βC treatment, which was diluted to 321 µg/ml, and 321 µg/ml for *transposon mutant pilT* with 3,5-diMeDβM/βC treatment. The protein samples (80 µL) were mixed with 150 µL of 1X sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris, pH 6.8, 2% β-mercaptoethanol, 20% glycerol, 4% SDS and 0.001% bromophenol blue), and boiled for 10 min, cooled to room temperature; 20 µL of the sample were loaded to for separation on 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels. Proteins were visualized by staining with Coomassie brilliant blue.

2.5.18 Quantification of phage titer, plaque forming unit (PFU), by top overlay method[112]

All phage counts were quantified by phage titer using top overlay method to report the plaque forming units.[112] *Top agar preparation.* Mix 3 mL of warm top agar solution (7 g/l agar, 0.5 g/l sodium chloride, 10 g/l tryptone, 5 g/l yeast extract in sterile water) and 200 µL of LB culture of

PAO1k (OD 0.6); spread this mixture solution on 1.5% LB agar plate (LB-Miller, 10 g/l tryptone, 5 g/l yeast extract, 10 g/L NaCl ,15 g/l agar, 10-cm in diameter). Cool for 15 min in biosafety level 2 hood to solidify the gel. Serial dilutions of supernatant of containing phage were prepared in LB-Mg^{2+}. Nine or six drops of 10 µL of the diluted supernatant were placed on the top agar plates to observe plaques formation on bacterial lawn after 12 h. The number of plaques were counted and reported as plaque forming units per ml of sample.

2.5.19 Lysis of bacterial cells by bacteriophage ϕKMV[112,128,129]

The overnight culture (100 µL) of (PAO1k, Δ*pilA, pilT::Tn*) was diluted in 10 mL LB medium with $MgSO_4$ (10 mM) and was grown to mid-log phase (OD=0.5) with and without 85 µM 3,5-diMeDβM or 3,5-diMeDβC at 37℃ at 250 rpm. These cultures (OD_{600} = 0.5) were infected with φKMV phage (PFU/ml = 3.4×10^9). The optical density (OD_{600}) of bacterial culture was monitored over 12 h. The clear bacterial lysate (OD < 0.1) is considered as the end point for lysis experiment.

2.5.20 Direct binding of fluorescent ligands to LecA[114]

The fluorescent ligand (βGal-aryl-Dansyl) were dissolved in water to a final concentration of 3 mg/ml. 2 mg LecA (Sigma) was dissolved in 1 mL of 0.1M Tris-HCl pH 7.5 supplemented with $CaCl_2$ (6 µM). The solution of fluorescent ligand (βGal-aryl-Dansyl, 200 nM) is mixed with different concentrations of LecA solution (0-100 µM). After incubation for 1 h at room temperature in dark, fluorescence polarization was measured by using Edinburgh FLS9801 Spectrometer, with the samples being illuminated with vertically polarized light at 330 nm (for

βGal-aryl-Dansyl). The vertical and horizontal fluorescence components of emission 570 nm were recorded, and the fluorescence polarization was calculated and data were analyzed using a four-parameter fit model.

2.5.21 Competitive binding of ligands to LecA[114]

To a solution of LecA (final concentration: 20 μM) and fluorescent ligand (final concentrations of βGal-aryl-Dansyl: 200 nM) in 0.1M Tris-HCl pH 7.5 supplemented with $CaCl_2$ (6 μM), serial dilutions (0.1 μM to 140 μM) of test compounds (SFβM/C, rhamnolipids, $SFEG_4OH$ and 3,5-diMeDβM/βC) were done. The mixture samples were incubated for 6 hours at room temperature. The fluorescence polarization was measured using Edinburgh FLS9801 Spectrometer. During measurement, the samples were illuminated with vertically polarized light at 330 nm (for βGal-aryl-Dansyl) and vertical and horizontal fluorescence components were measured, and the fluorescence polarization was calculated and data were fitted with four parameter variable slope model.

2.5.22 Elastase assay[126,130]

Elastase activity in *P. aeruginosa* culture was determined by an elastin Congo red (ECR) assay with modifications[126,130]. Briefly, 100 μL of bacteria from overnight cultures in MHB were diluted in fresh MHB to an OD_{600} of 0.01 with and without 3,5-diMeDβM or 3,5-diMeDβC, $SFEG_4OH$ (final concentration: 85 μM). After culturing for 18 h at 37°C with shaking, culture supernatants were filtered (0.45-μm pore-size filter). Triplicates of culture filtrates (100 μL) were added to 1 mL of Tris buffer (0.1 M Tris, pH 7.2, 1 mM $CaCl_2$) containing 20 mg of Elastin-Congo red (Sigma Aldrich). The glass tubes were incubated for 6 h at 37°C with shaking

at 100 rpm and were added with 0.1 ml of 0.12 M EDTA and placed on ice. Insoluble ECR was removed by centrifugation at 16,000 rpm and the OD_{495} of the supernatant was measured. The elastase activity was reported as OD_{495} (with agent) - OD_{495} (without agent).

2.5.23 Rhamnolipid assay[118]

Quantification of rhamnolipids in planktonic cultures of *P. aeruginosa* was determined by methylene blue-rhamnolipid complex assays by an established assay.[118] In this assay, the positively charged methylene blue form noncovalent complex preferentially with negatively charged surfactants, and does not bind to nonionic surfactants. The methylene blue bound with negatively charged surfactants has an increased partition into the chloroform phase bought in contact with the aqueous solution. The methylene blue in the chloroform phase were measured for its UV absorbance, which infers the amount of negatively surfactants. Briefly, 100 µL bacteria from overnight cultures in MHB were diluted in fresh MHB to an OD_{600} of 0.01 with and without 3,5-diMeDβM or 3,5-diMeDβC, $SFEG_4OH$ (final concentration: 85 µM). After 18 h at 37°C with shaking, culture supernatants were filtered with 0.45-µm pore-size filter (SCBT, USA). The filtrate pH was first adjusted to 2.3 ± 0.2 using 1 N HCl. The acidified sample was then extracted with five-fold volume of chloroform. The chloroform extract (4 mL) was transferred to put in contact with 5 mL of a freshly prepared methylene blue aqueous solution (40 µg/ml, pH pre-adjusted to 8.6 ± 0.2 by adding the 50 mM borax buffer). The pH of this aqueous solution of methylene blue had been pre-adjusted to 8.6 ± 0.2 by adding the 50 mM borax buffer (~15 µL). After being vigorously mixed for 4 min, the samples were left to stand for 15 min. The chloroform phase was transferred into a cuvette and the absorbance was measured at 638 nm

with a UV/Vis spectrophotometer with a reference of blank chloroform. The absorbance values were converted to rhamnolipid concentrations using a calibration curve.

2.5.24 Pyocyanin assay[131]

An overnight culture of PAO1 (100 µL) was added to 10 mL fresh LB medium, 5 mL of which were grown with and without 85 µM 3,5-diMeDβM, or 3,5-diMeDβC, $SFEG_4OH$ for 12 h. The absorbance of bacterial culture was measured at 600 nm (A_{600}), and then centrifuged to remove bacterial pellet. Supernatant (5 mL) was extracted with 3 mL chloroform. The pyocyanin-containing chloroform layer was acidified with 2 mL of 0.1 N HCl to give a pink solution, the absorbance at 520 nm of 200 µL of the solution was measured. The absorbance reading at 520 nm was converted into µg/ml of supernatant with standard curve reported in literature. Pyocyanin levels were expressed as µg/ml of supernatant/A_{600} of culture where A_{600} is absorbance of bacterial culture.

Chapter 3 Chemical control over Asialo-GM1: A dual ligand for Pili and Lectin A that activates swarming motility and facilitates adherence of *Pseudomonas aeruginosa*.

3.1 Background and Significance

The bacterium *Pseudomonas aeruginosa* is one major opportunistic pathogen that contribute to a wide range of infection conditions, including burnt wounds, nosocomial bacterial infections [132] cystic fibrosis lung infection [133], and other immune compromised illnesses [134]. The initial contact and adherence to either a host cell surface or any abiotic surfaces is facilitated by surface bound proteins, and appendages [100,135-144]. For *P. aeruginosa*, type IV pili and lectin proteins are one of the commonly employed adhesins proteins [137,142]. This bacterium interacts with different small molecules on human epithelial cells, including glycosphingolipids, including globotriaosylceramide (Gb3) and ganglio-N-tetraosylceramide (asialo-GM1), as well as receptors protein, such as transmembrane conductance regulator of cystic fibrosis lungs [145-152].

Type IV Pili of *P. aeruginosa* are often involved initial attachment to surfaces [136,139,140] and facilitate transition between motility and irreversible attachment [136]. For host infection, pili recognizes asialo-GM1, asialo-GM2 and related gangliosides present in epithelial cell membrane and initiate subsequent virulence factors [145-147,153]. Surprisingly, *P. aeruginosa* mutants lacking pili still adhere to epithelial cells after extended exposure [154] suggesting that additional non-pilus adhesins on bacterial surfaces are involved in stabilizing host-pathogen interactions, and that lack of pili may only prevent initial short-term interaction with host cells [141,154].

Another adhesin protein Lectin (LecA) exhibits preferential binding affinity for α-D-galactosylated glycans and has been shown to interact with wide range of ganglioside molecules [149-151,155]. The interactions between LecA and ganglioside molecules like globotriaosylceramide (Gb3) leads to the bending of mammalian cell membrane and the entry of bacteria into the cells are crucial for initiating bacterial infection [149]. These findings suggest that LecA and pili may complement each other for the attachment to and the entry into mammalian cells. As LecA has binding affinity towards various galactose and glucose terminated lipids [100,155,156], we believe that LecA can also interact aGM1 and function as a non-pilus adhesin. The adherence of bacteria caused by pili, are known to lead to a wide range of phenotypes, including motility of twitching and swarming, as well as biofilm formation [66,136]. Furthermore, both pili and LecA have been shown to act as invasion factor damaging host epithelial cells [138,149]. Pili lead to epithelial cell injury followed by bacterial internalization [157] while LecA induce permeability and growth defect in epithelial cells [149,158]. Considering that bacteria can use more than one adhesin protein for adherence, controlling multiple adhesin's binding is important for both understanding the chemical signaling and phenotypes, and for therapeutic developments.

Our past studies show that disaccharides tethered with different aliphatic chain structures can control both swarming motility and biofilm formation of the bacterium *Pseudomonas aeruginosa*[81,82] [81,82]. Interestingly, the control of these two bioactivities is extremely sensitive to the structural changes in the aliphatic chains, and tolerate changes in disaccharide structures (maltoside or cellobioside) [81]. Furthermore, for a multivalent glycopeptide dendrimer binding to LecA, hydrophobic spacer adjacent to anomeric center of saccharide enhanced binding of the molecule to LecA, suggesting that hydrophobic regions adjacent to the galactose are also important for binding [102,117,151]. To that end, the artificial liposomes offer a model for studying ligand-receptor binding on a lipid bilayer that resembles the basic structure of host cell membrane [159-161]. In this model, the contribution of the aliphatic chains to ligand-receptor binding may be revealed by the change of liposomal membrane structural integrity. Because of their ability to compartmentalize dye molecules in the internal aqueous environment, liposomal systems are used to study integrity of membrane [160,161].

In this work, we propose and demonstrate that lectin LecA is also a receptor protein for ganglio-N-tetraosylceramide (aGM1). We show that aGM1 displaces a synthetic fluorescence ligand molecule from LecA to solution. Surprisingly, we find that on hydrated gel surface, aGM1 activates swarming motility of a non-swarming *rhlA* transposon mutant, of *Pseudomonas aeruginosa*. In contrast, on solid surfaces, aGM1 facilitates *P. aeruginosa*'s adherence that is mediated by binding to LecA or pili protein. A novel bacterial motility enabled binding assay shows direct binding between unlabeled LecA and pilin to aGM1, for which externally added proteins (LecA and pilin) inhibit the swarming motility activated by aGM1. We further demonstrate a class of synthetic dual ligands comprised of saturated farnesol derivatized with disaccharide (Fig. 1) for LecA and pili appendages inhibits the adherence of wild type *P.*

aeruginosa, and mutant strains with no LecA protein (but still has pili), and with no pili protein (but still has LecA), on aGM1-coated surfaces. We also explored the potential contribution of the lipid aliphatic chains, by incorporating a small percentage of aGM1 into artificial liposomes of 1,2-dioleoyl-sn-glycero-3-phosphocholine: cholesterol (DOPC:Chol 65:30 w/w) and studied the effect of LecA and pili's binding to aGM1 on the leakage of fluorescent dye, Sulforhodamine B encapsulated in liposomes.

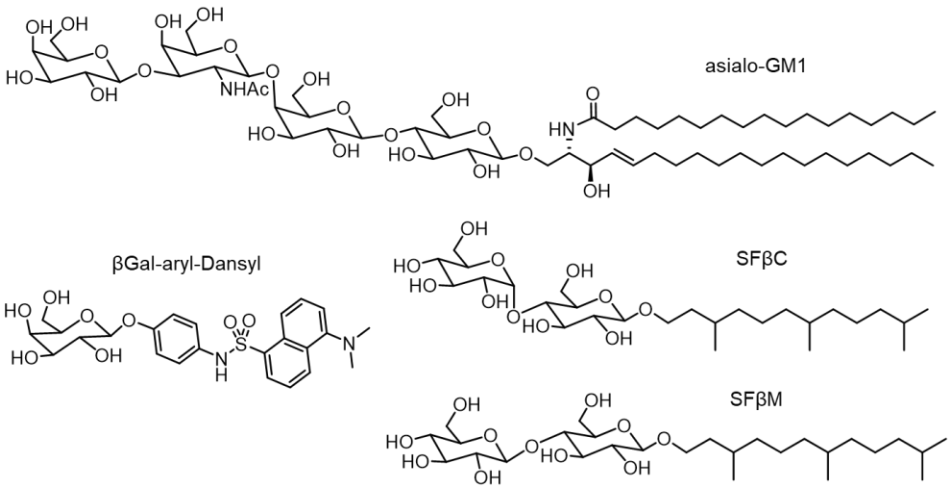

Figure 3.1 Chemical structures of dual ligands targeting both lectin LecA and pilin of *P. aeruginosa*: Asialo-ganglioside-monosialic acid (asialo-GM1), saturated farnesol-β-cellobioside (SFβC), saturated farnesol-β-maltoside (SFβM) and synthetic fluorescent lectin LecA ligand βGal-aryl-Dansyl.

3.2 Results and Discussion

Gangliosides, and asialo gangliosides, including asialo-GM1 in general consist of four sugar moieties, with three galactose at the terminus [151,162]. Because LecA has a binding affinity for galactose, we explore the binding of aGM1 by LecA by five different methods, including a fluorescence polarization assay that measure the displacement of a fluorophore from LecA by

aGM1, a label-free bacterial motility enabled binding assay that examine the effect of direct binding between a protein spread on the surface of hydrated gel, and aGM1 molecules prepared in the gel, a liposome leakage assay that characterize the effect of LecA on aGM1-loaded liposome; and the bacterial adhesion and inhibition of a *lecA* transposon mutant *lecA::Tn* and a pili mutant (Δ*pilA*). Asialo-GM1 on a host mammalian cell is known to bind to pili appendages and facilitate bacterial adherence [157,163]. However, the bacteria's motility on the surface caused by the aGM1-mediated adherence is not addressed. Here, by including the aGM1 in hydrated gels, we examine that effect of aGM1 on the swarming motility of the bacteria. By incorporating the aGM1 in an artificial liposome, we also examine the effect on pili binding on the leakage of liposome.

3.2.1 Asialo-GM1 is a specific ligand for Lectin A.

We have reported molecules of disugar tethered with methyl-substituted aliphatic chains that inhibit swarming and biofilm formation [81]. We propose that these molecules bind to both LecA and pili appendages, as a class of synthetic dual ligands for both proteins. Here, we use fluorescence polarization to demonstrate a specific binding between LecA and aGM1, SFβM and SFβC. An efficient one-step synthesis resulted in a fluorescently labeled ligand βGal-aryl-Dansyl that binds to LecA. Direct titration with increasing amounts of LecA to 200 nM βGal-aryl-Dansyl, caused an increase in the fluorescence polarization of the dye with a transition to plateau of signals indicative of a binding constant of 10.7 μM between the fluorescent ligand and LecA (Fig. 3.2). A half maximal inhibitory concentration (IC_{50}) of our synthetic molecules against βGal-aryl-Dansyl were determined by competitive binding assay, in which small molecule ligand

candidates displace the fluorescent ligand, resulting in increasing the tumbling motion of the dissociated fluorescent ligands, and a decrease in fluorescent polarization signal.

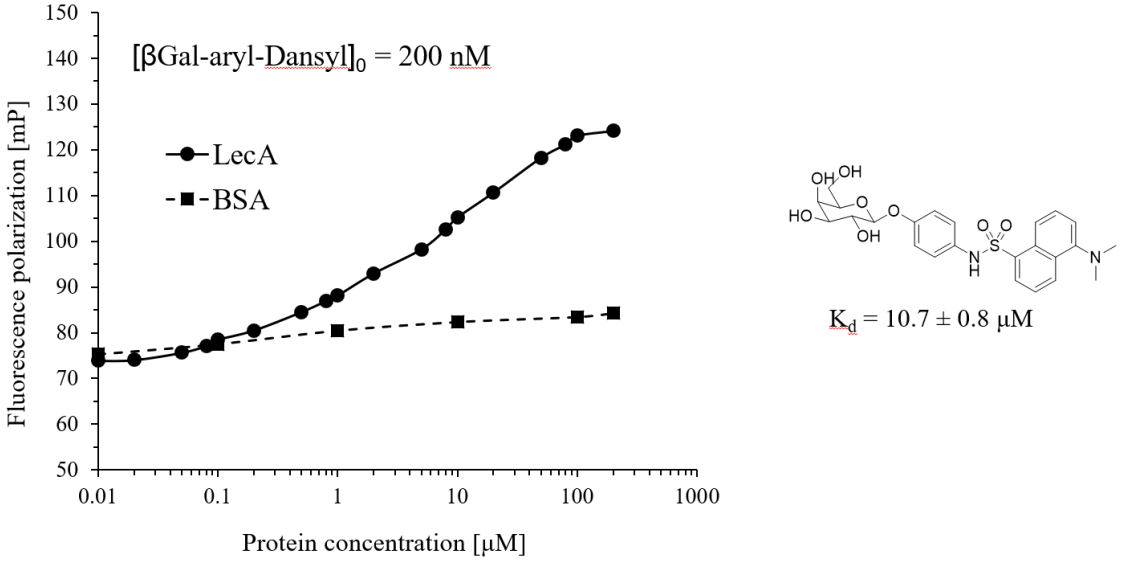

Figure 3.2 βGal-aryl-Dansyl binds to Lectin A. Titration of βGal-aryl-Dansyl with increasing Lectin (LecA) and bovine serum albumin (BSA) proteins. Dissociation constants were obtained from four parameter fitting procedure to the dose-dependent increase in fluorescence polarization. βGal-aryl-Dansyl showed binding to LecA (K_d = 10.7 ± 0.8 μM) while showed no binding to bovine serum albumin (BSA) protein.

The ligand candidates, aGM1, SFβM and βC, were titrated into a mixture of βGal-aryl-Dansyl (200 nM) and LecA (20 μM). We found that SFβM and SFβC caused a decrease of fluorescence polarization indicative of an apparent IC_{50} of 17 μM and 16 μM, respectively, whereas aGM1 showed apparent IC_{50} of 42 μM, against β-Gal-aryl-Dansyl-LecA complex (Fig. 3.3). Detailed studies of the fluorescence polarization for LecA protein revealed that the IC_{50} values for galactoside ligands varied between 10 -100 μM and are highly sensitive to both the ligand structures of galactose derivatives, and to the fluorescent probe structures tethered on the galactose [114,155]. Thus, comparing two IC_{50}s reflect a relative binding strength of two ligand

molecules to LecA, but does not necessarily reflect the impact on the LecA folding structure and thus on its bioactivities.

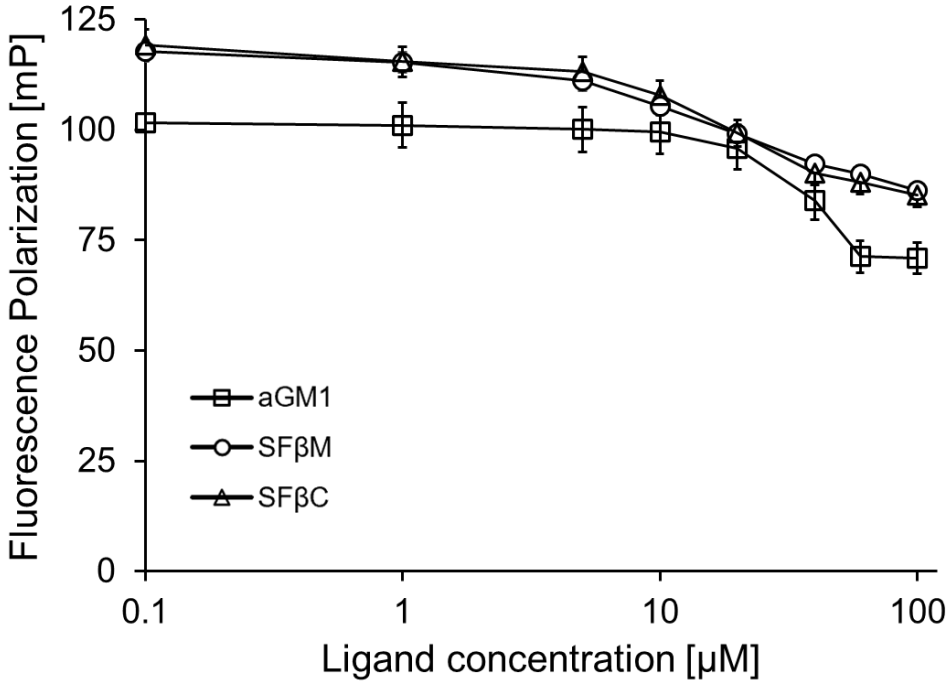

Figure 3.3 Binding of asialo-GM1, SFβM and SFβC to Lectin A protein. The fluorescent probe β-Gal-Dansyl-LecA displacement assay with asialo-GM1, SFβM and SFβC. 100 μL of LecA (20 μM) and β-Gal-Dansyl (200 nM) versus different concentrations of ligand asialo-GM1, SFβM and SFβC. Error bars indicate the standard deviations of means of triplicates.

3.2.2 aGM1 activates swarming motility, which is inhibited by LecA and Pilin spread on gel surfaces

The type IV pili is responsible for two types of motilities in *P. aeruginosa* viz. twitching and swarming. These pili mediated motilities were important for *P. aeruginosa* cytotoxicity against epithelial cells and for virulence *in vivo* [164] . Rhamnolipids, a class of self-secreted small molecule by *P. aeruginosa* [79,80], is necessary for swarming motility; mutant with impaired rhamnolipid production does not swarm [79,80]. When exogenous rhamnolipids are added to

hydrated gel, the swarming motility of nonswarming mutant *rhlA* is reactivated [79,80,82]. Here, we evaluate the effect of aGM1 on swarming motility of this bacterium. We discovered that aGM1 activates and modulates the swarming motility of *rhlA* mutant as a function of concentration in the hydrated agar gel (Fig.3.4 and Fig. 3.5A). At 10 µM, aGM1 started to activate swarming motility and swarm area was increased from 1.5 to 5.3 cm^2. As concentration increased to 20-60 µM, aGM1 caused more tendrils formation with an increase in swarm area from 8.12 to 34.22 cm^2 (Fig. 3 and Fig.S2). At 80 µM, aGM1 resulted in defined and dense tendrils with a similar swarming area of 34.42 cm^2 (Fig. 3.4).

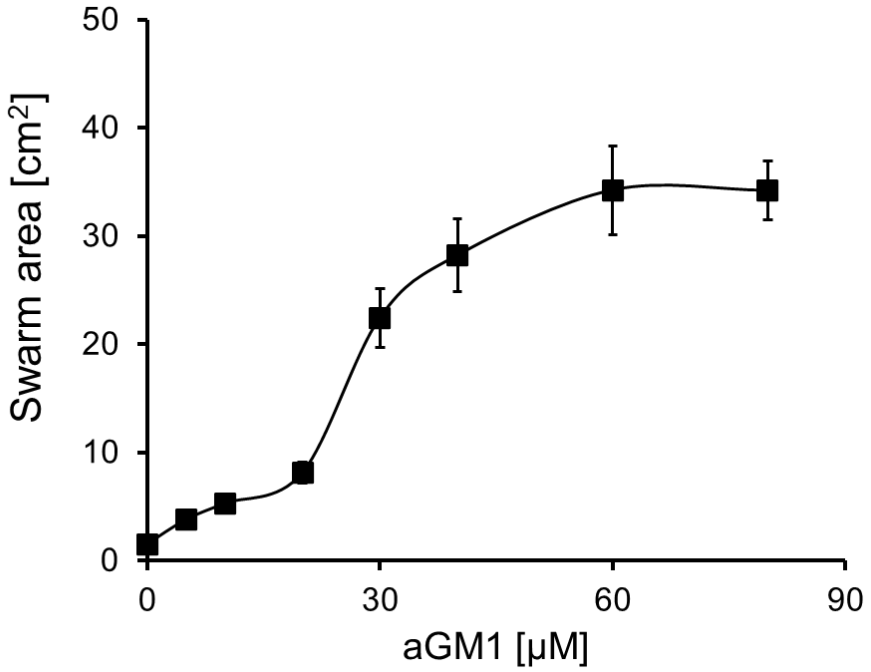

Figure 3.4 Asialo-GM1 activates and modulates swarming of *P. aeruginosa* rhamnolipid deficient *rhlA* mutant. Swarming motilities *rhlA* mutant on semisolid gel (~0.5% agar) with different concentrations of aGM1 in DMSO was observed after 24 h. The swarming area was estimated by measuring diameter of swarm area on agar gel and plotted against respective concentration of aGM1 used in swarm gel. The average of two independent experiments was plotted and error bars representing standard error from the mean.

This result is consistent with possibility that both aGM1 and rhamnolipids are specific ligands for pili appendages, and binding of both small molecules activate swarming motilities, but with different characteristics. Rhamnolipids prepared in the hydrated gel start to activate swarming motilities at 5 μM and inhibit swarming motilities as the concentration increases to about 30 μM. The patterns of the swarming bacteria activated by rhamnolipids are radial and without formation of tendrils. Asialo-GM1 activates swarming motility around 10 μM and causes formation of increasingly large tendrils as concentration increases. We believe that the tendrils are caused by the formation of two different phenotypes, the hyper swarming, and lazy swarming phenotypes, while the original normal swarming phenotype is abolished. This subject is of our current research. To corroborate the binding hypothesis, we apply a label-free bacterial motility-enabled binding assay to examine the effect of direct binding interaction between aGM1 prepared in gel and protein (LecA or pilin) spread on the gel surface on the swarming motility. We propose that when a protein (spread on the gel surfaces) selectively recognizes the ligand molecules from the gel surfaces, their binding may sequester the ligands molecule, and deplete their presence on and near the gel surfaces, because, in a gel, the diffusion is slow, and replenish the molecule from the bulk to surface is ineffective. In the absence of ligand molecules, the swarming motility is abolished.

We observed that while the swarming motility of *rhlA* mutant was activated on gels containing 10 μM of aGM1, spreading Pili or LecA (100 μL of 1 mg ml^{-1}), about 626 and 780 nmol, respectively, on the gel surfaces (10-cm in diameter) abolished the swarming motility (Fig. 3.5B). This result is consistent with pilin and LecA bind and sequester aGM1 on the gel surface, depleting their availability for pili appendages on bacteria, and thus abolishing the swarming

motility of *rhlA* mutant. Spreading a solution of bovine serum albumin (BSA) protein has no effect on swarming motility of mutant in the presence of aGM1. This set of results indicates a direct interaction between the lecA or pilin protein with aGM1, for which the swarming motility activated by aGM1 is inhibited.

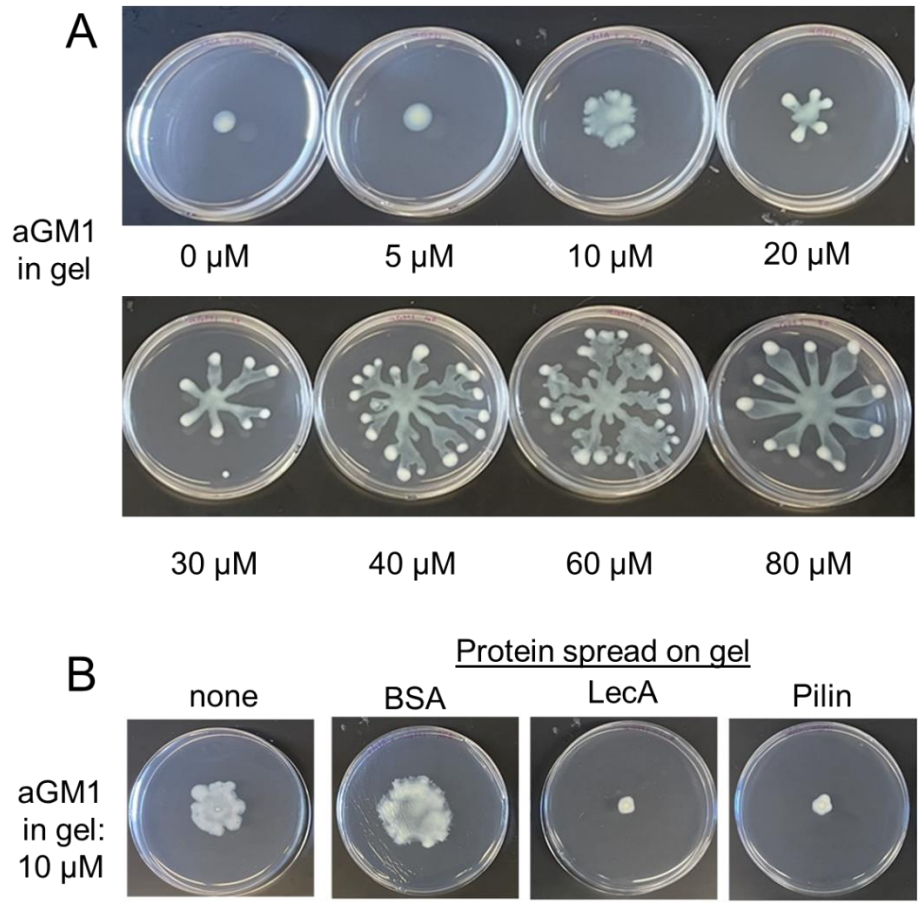

Figure 3.5 Swarming motility assays. Asialo-GM1 activates and modulates swarming of *P. aeruginosa* rhamnolipid deficient *rhlA* mutant.(A) Swarming motility assay; The representative images of swarming motilities *rhlA* mutant on semisolid gel (~0.5% agar) with different concentrations of asialo-GM1 in DMSO after 24 h. The concentrations are indicated between the images. (B) Asialo-GM1 binds to Lectin A protein and Pilin. Images of swarming patterns of *rhlA* mutant (18 h) on the soft gel (0.5% agar in LB) with the gel containing 10 μM of aGM1 and having 100 μl of 1 mg $ml^{-1}$of pilin, LecA or BSA spread on gel surface.

As pili are necessary for twitching motility in *P. aeruginosa* [92,164,165], we also examined the effect of aGM1 on twitching motility. Asialo-GM1 (10 µM) promoted twitching motility by about 14 % based on the sub-surface twitch zone area (Fig. 3.6). This result is consistent with aGM1 binds to pili of the bacterium, and likely promote the extension-retraction dynamics of the pili appendages on the bacterium. In *P. aeruginosa*, twitching motility is required for the initial attachment and pathogenesis [165]. This result showed that pili binding to aGM1 does not just facilitate adherence, but can also initiate twitching or swarming motility, and lead to virulence signaling related to motilities [66,166,167].

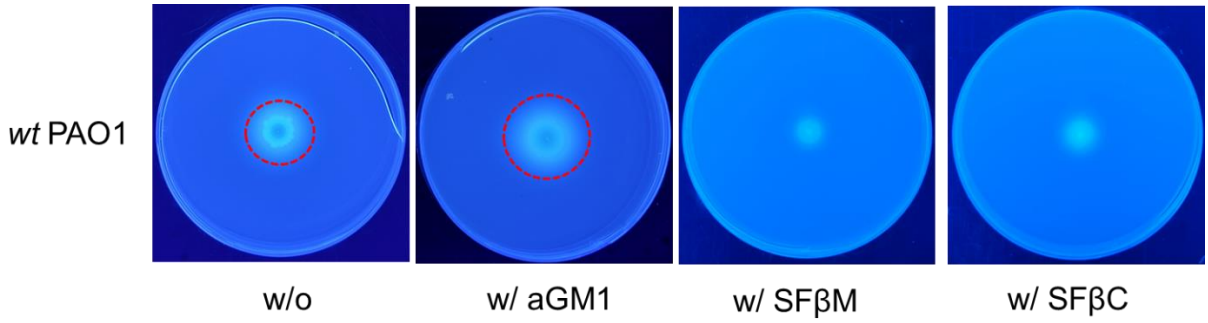

Figure 3.6. Asialo-GM1 and SFβM/βC affects pili-mediated twitching motility. aGM1 promotes twitching motility while SFβM and βC inhibit twitching motility. LB agar plates (1%) were stab inoculated with a toothpick to the bottom of the plate and incubated for 48 h at 37 ° C with and without 10µM aGM1 or 20 µM SFβM or βC. The light haze of growth, the twitch zone, at the agar-plate interface is a measure of twitching motility (red dashed circle). Twitch zone is measured as area of red circle. The smaller, denser zone represents colony growth. Images were taken under UV lights.

3.2.3 Synthetic ligand and aGM1 reduces phage adsorption on pili

Pili appendages are the key receptor protein for the adsorption of bacteriophage and lysis of bacteria [112]. Lytic bacteriophage ϕKMV requires specifically type IV pili of *P. aeruginosa* for

adsorption [112]. Here, we studied the effect of aGM1 and SFβM and βC on the adsorption of bacteriophage ϕKMV to a known phage sensitive *P. aeruginosa* strain, PAO1k [112]. We found that mixing bacteriophage ϕKMV (1.6×10^6 PFU ml^{-1}) with excess of PAO1k (2.0×10^7 CFU ml^{-1}) for 8 min and removing the bacteria and adsorbed phage from the culture, the remaining phage titer in supernatant was reduced to 1.2×10^5 PFU ml^{-1}, indicating that ~93% of the phage was adsorbed on PAO1k (Fig. 3.7). In contrast, culturing bacteria in the presence of 20 μM of SFβM or βC, the phage count in solution was 1.2×10^6 PFU ml^{-1}, indicating that only ~25 % of phage was adsorbed on pili of PAO1k. This result indicated that SFβM or βC (20 μM) inhibited phage adsorption on pili appendages by 73%. Presence of aGM1 (10 μM) showed weaker impact on phage adsorption (82% of phage was adsorbed on PAO1k). While SFβM or βC showed a strong inhibition of phage adsorption (and twitching, swarming motility), aGM1 had a weak impact on phage adsorption, and promoted twitching motility, modulated swarming motility. These results are consistent with the notion that both molecules bind to pili appendages but have an opposite effect on the pili-mediated activities.

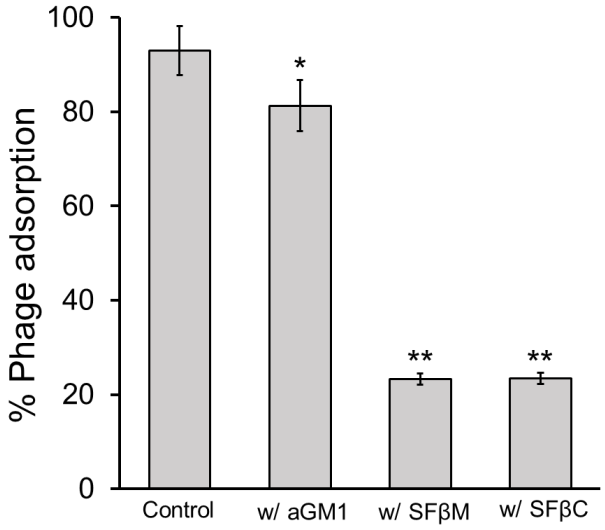

Figure 3.7 Phage adsorption assay. aGM1 and SFβM or βC binds to pili aGM1. aGM1 has no effect on phage adsorption while SFβM or βC inhibit phage adsorption. Percentage of ϕKMV (~10^6 PFU ml^{-1}) phage adsorbed on PAO1k (*wt* PAO1 strain that is sensitive to ϕKMV) over 10 min in LB containing 10 μM aGM1, or 20 μM of SFβM or βC. Error bars indicate the standard deviations of means of triplicates. *$P < 0.05$; **$P < 0.01$ vs control, Student's t-test.

3.2.4 LecA and pilin monomer cause leakage of aGM1-loaded liposomes.

Both the binding strength study of galactose derivatives by fluorescence polarization[114] and the development of multivalent ligands to LecA [156] indicated that nonpolar groups attached to the galactose have a substantial impact at increasing the binding to LecA protein. To explore if aGM1's lipid aliphatic chains also contribute to binding to Pili and LecA proteins, we conducted the binding by incorporating aGM1 into artificial liposomes [159]. Substantial interaction between fatty hydrocarbons and proteins such as lipoproteins can results in compromise membrane and leakage of the liposome causing the encapsulated molecules to escape from the interior aqueous compartment of the liposome [168]. We prepared liposomes consist of 1,2-dioleoyl-sn-glycero-3-phosphocholine:cholesterol (DOPC:Chol 65:30 w/w) with and without 5% asialo-GM1 as membrane component [161]. The soluble dye Sulforhodamine B (50 mM) was encapsulated in the liposomes, in which the fluorescence was inhibited by self-quenching [161]. When the liposomes containing 5 % of aGM1 were treated with LecA and pili proteins (1 mg ml^{-1}) for 1 hour, 70% and 55% leakage of Sulforhodamine B, respectively, was observed (Fig. 5). Without aGM1 in the liposome, the same treatment with pili and LecA, only 10% of leakage of Sulforhodamine B was observed (Fig. 5). Here, we used 0.1% triton X-100, a nonionic surfactant, to lyse the liposome, and treat this lysis-caused leakage as 100 % as the basis for comparing the effect by protein binding to aGM1 on liposomes [159,160]. These results indicate that pili and LecA recognize

and bind asialo-GM1 component from lipid membrane of liposomes and cause a leakage in the membrane. Gangliosides like GM1,GM3 exhibit a non-uniform distribution and forms clusters in artificial liposomes [169-171], and receptor protein binding are known to interact with gangliosides and cause changes in membrane curvature which may lead to transient leakage [159,172-174]. Thus, the leakage of membrane system can be the result of receptor protein binding induced restructuring of lipid bilayer, or clustering of ligand, or partial extraction of the ligand molecules from lipid membrane. Interestingly, antibodies against aGM1 and BSA only resulted in minimal leakage (12-15%) (Fig. 5). This result implies that binding does not necessarily lead to disruption of lipid membrane and adhesin proteins binding to aGM1 possibly involves changes in membrane curvature.

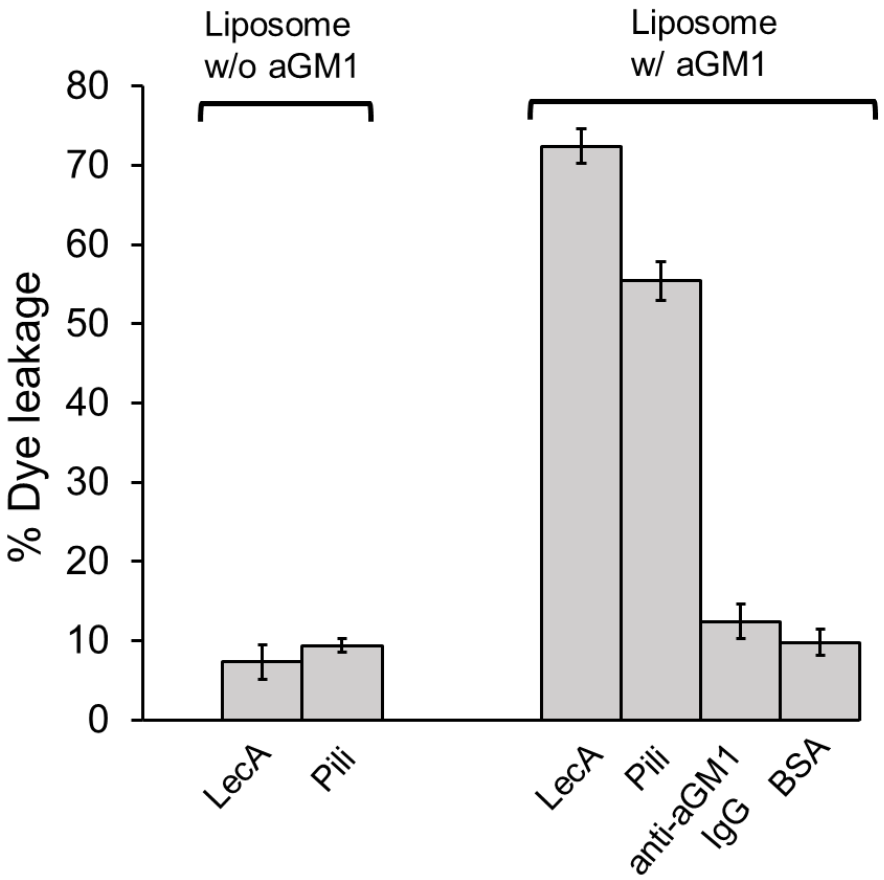

Figure 3.8 Pili and LecA proteins bind to asialo-GM1 and interact with asialo-GM1 from liposomes membrane. 100 µL saline solution of liposomes (w/ and w/o aGM1 as part of the lipid bilayer) encapsulating 100 mM Sulforhodamine B, was mixed with a. 100 µl of 0.1 mg ml^{-1} (pilin or LecA) and 0.5 mg ml^{-1} BSA and anti-asialo-GM1 antibody for 1 h at room temperature. The mixture was incubated in dark at ambient temperature for 1 h. The fluorescence measurement was made at λ_{ex} 565nm; λ_{em} 585 nm. As a control for 100% of liposome lysis and leakage, 0.1% Triton-X was used. Percentage of (%) of liposome leakage was calculated as (fluorescence after incubation – fluorescence before incubation) / fluorescence after incubation with 0.1% Triton-X ×100.

3.2.5 Synthetic inhibitors for LecA and pili reduce bacteria adherence on aGM1-coated surfaces

With aGM1 binding to LecA and pili proteins, we examined the adherence of wild type *P. aeruginosa* (*wt* PAO1), *lecA* transposon mutant, and knockout *pilA* mutant on aGM1-coated surfaces. These two mutants provide means to study the impact of each adhesin protein on aGM1 mediated adherence. as the *pilA* mutant still has LecA protein; and the *lecA* mutant still has surface pili. Here, we also study the inhibition of the adherence of these strains on aGM1-coated surfaces by small molecule ligands, SFβM and SFβC. We cultured ligand-pretreated bacterial cultures in aGM1-coated wells for 1 h followed by rinsing to remove unadhered bacteria. The residual adhered bacteria were resuspended in fresh LB media by sonication and then enumerated by Colonies Forming Units (CFU) counting on LB agar plates. We found that about 6.2×10^5 CFU ml^{-1} *wt* PAO1 adhered on aGM1-coated wells, whereas for *lecA* and *pilA* mutants substantially lesser adherent bacteria were observed, about 3.4×10^4 and 5.2×10^3 CFU ml^{-1}, respectively. These results indicate that both LecA and pili protein contribute the bacterial adherence on aGM1-coated surfaces.

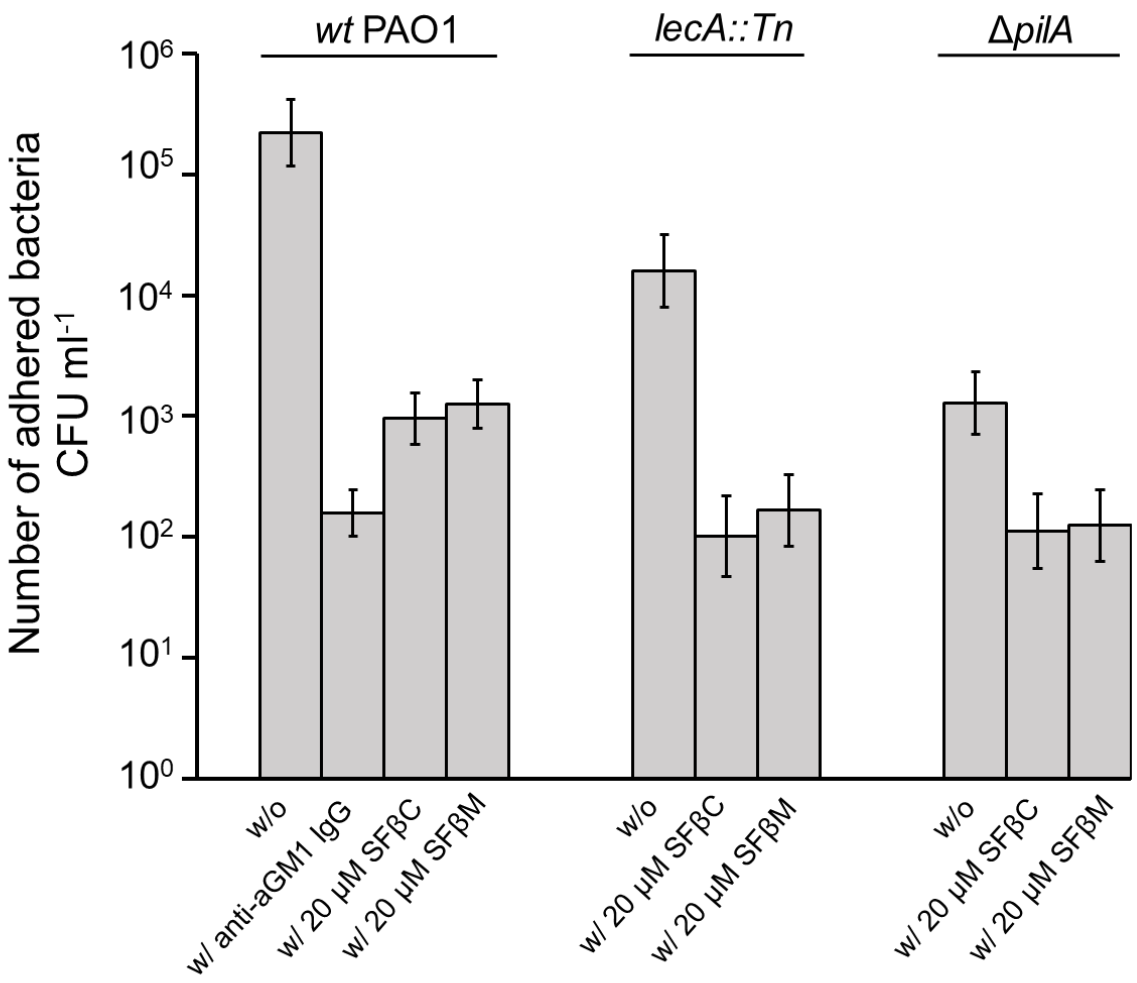

Figure 3.9 Bacterial adherence assay. Number of bacteria (CFU ml^{-1}) adhered on 2 μg ml^{-1} (100 μL) asialo-GM1 coated 96 well plate surface. The bacterial strains *wt* PAO1, pili, and LecA mutants (CFU $ml^{-1} = 10^7$) were cultured with and without 20 μM SFβM or βC for 1 h, and the cultures were incubated in wells coated with aGM1 for 1h. The adhered bacteria were counted by colony forming units on LB agar plates. For antibody adherence assay, the aGM1-coated wells are incubated with a PBS buffer containing 0.5 mg ml^{-1} anti-asialo-GM1 IgG antibody for 15 min. The wells were rinsed and added with bacterial culture (*wt* PAO1, pili, and LecA mutant CFU $ml^{-1} = 10^7$) for 1 h.

To study the chemical inhibition of bacterial adherence, we first examined the effect of blocking the aGM1-coated well surface with the anti-asialo-GM1 antibody (100 μL, 0.5 mg ml^{-}

[1]). We found that the adherence of *wt* PAO1 was reduced to about 2.2×10^2 CFU ml^{-1} (from 6.2 x 10^5 CFU ml^{-1}). In comparison, when the *wt* PAO1 was pretreated with SFβM or βC (20 μM), the bacterial adherence was reduced nearly to 8.8×10^2 and 1.2×10^3 CFU ml^{-1}, respectively (Fig. 3.9). These results indicated blocking the aGM1 by its antibody reduced the bacterial adherence by 1000 times, whereas bacterial pretreatment with dual ligand of SFβM or βC, reduced the adherence by about 100 times. For *lecA* and *pilA* mutants, pretreatment with SFβM (or βC) further reduced the bacterial adherence by 100 and 10 times, respectively (Fig. 3.9). These results indicated that *lecA* and *pilA*, each mutant exhibits considerable adherence to aGM1-coated surface, and these adherences can be further inhibited by the dual synthetic ligand SFβM and SFβC.

3.2.6. Binding, and inhibition, of LecA and pilin proteins to aGM1-coated surface

We studied the direct binding between LecA and pilin proteins, and aGM1 coated on microplate surface [175], and the chemical inhibition of this binding by the synthetic dual ligands, SFβM and βC. The commercially obtained LecA and expressed pilin monomer protein were modified by PEGylated biotin [150,175]. First, to validate direct binding of LecA protein and pilin protein to aGM1, we incubated aGM1-coated surfaces (100 μl, 2 μg ml^{-1} in chloroform, overnight) with solutions of biotin-tagged LecA or biotin-tagged pilin (100 μL, different concentrations from 0.1 to 5 μg ml^{-1} in PBS buffer) for 1 h. The wells were rinsed with PBS to remove unbound proteins and treated with 4'-hydroxyazobenzene-2-carboxylic acid (HABA)-bound avidin, which exhibit a strong absorbance in solution. When HABA-bound avidin bind to biotinylated proteins on surface, the chromophore HABA is displaced into solution by biotin-avidin binding. Upon dissociating from the avidin, the free HABA exhibit a low absorbance. We

found that saturation of binding curves were obtained when the concentration of both LecA and pilin monomer reached ~ 1.0 μg ml^{-1} (Fig. 3.10). The binding curves also suggested that aGM1 may have a stronger binding constant for LecA (K_d of 0.44 μg ml^{-1}) than that for pilin monomers (K_d of 0.82 μg ml^{-1}). These results are consistent with LecA being more effectively interacts with aGM1 than pilin monomers from aGM1-loaded liposomes.

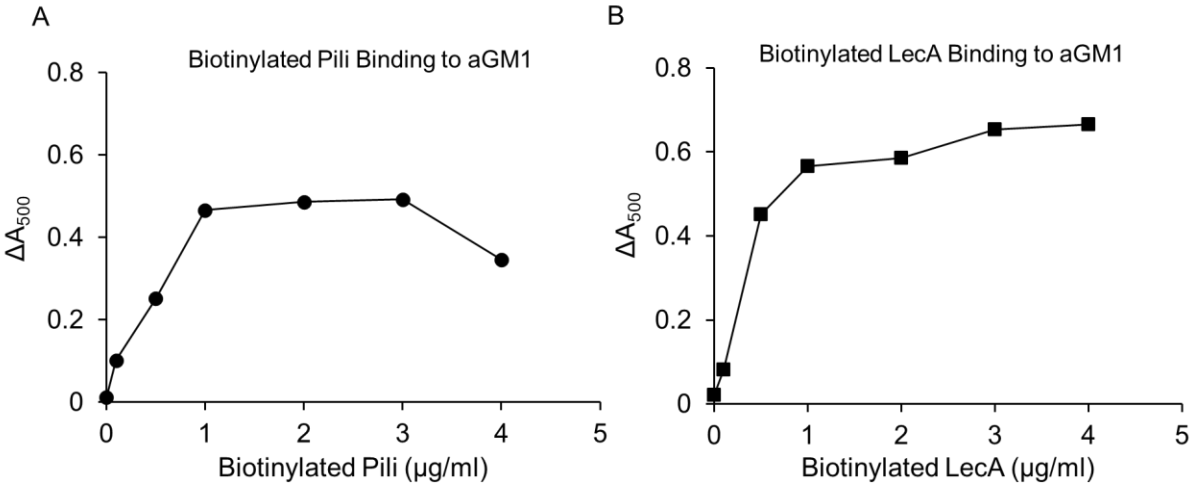

Figure 3.10 Asialo-GM1 is ligand for Pili and LecA. Aliquots of (A) biotinylated pili or (B) LecA were added to the aGM1-coated wells (100 μL per well) to reach different concentrations (0.1 to 5 μg ml^{-1}) and were incubated for 1 h at 37ºC. The amount of adhered biotinylated proteins was measured by HABA-Biotin assay (Pierce™ Biotin Quantitation Kit).

To evaluate the binding of synthetic ligands SFβM or βC to LecA and pilin monomer, we incubated biotinylated LecA or biotinylated pilin with different concentration of SFβM or βC for 1 h. The aGM1-coated wells were then treated with these protein-ligand commixture for another 1 h. The amount of bound biotinylated proteins was estimated by HABA-avidin solution as described previously. We found that SFβM (or βC) inhibited the binding of biotinylated LecA and biotinylated pili binding to aGM1-coated surfaces by IC_{50s} around 15 (17) μM and 24 (26) μM, respectively (Fig. 3.11). These results demonstrated that SFβM and βC are synthetic dual

ligands for pili and LecA and are consistent with their excellent ability to inhibit aGM1 mediated bacterial adherence.

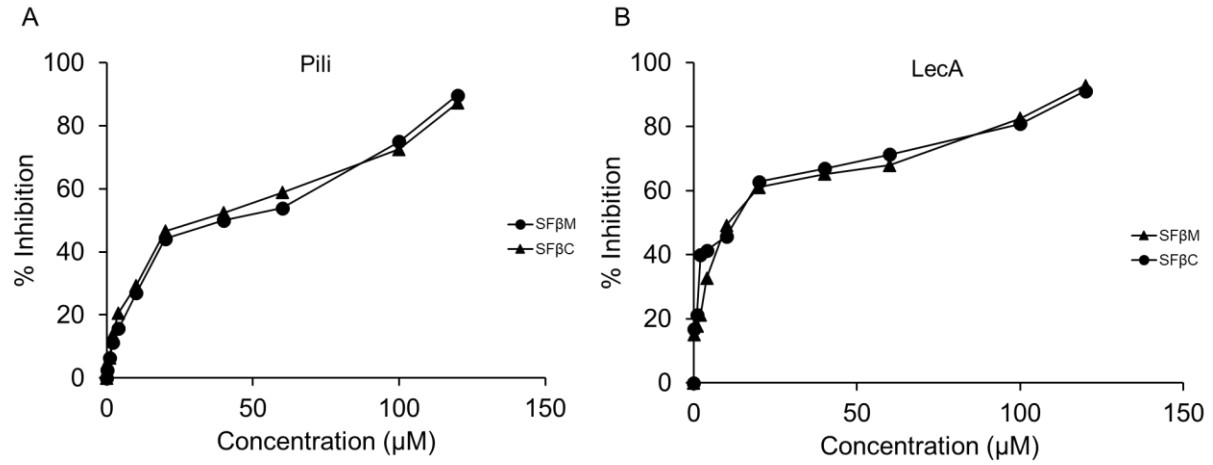

Figure 3.11 SFβM and SFβC inhibit pilin monomer and LecA binding to aGM1-coated black 96 wells. % inhibition of binding of biotinylated pilin (A) and biotinylated LecA (B) against concentrations of ligands SFβM and βC. The two proteins were incubated with the ligands for 1 h, and the mixture of ligand-proteins were introduced to the aGM1-coated wells and incubated for another 1 h (see experimental for details).

In this work, we note four findings and their implications. First, aGM1 activate the swarming motility and promote twitching motility of *P. aeruginosa*. Bacteria respond and counter to external stimuli by emergence of phenotypes that are associated with either high or low levels of a global messenger small molecule 3',5'-cyclic diguanylic acid (cdG) [176]. Both swarming and twitching motilities are governed by low cdG levels and associated signaling, whereas adherence of bacteria often leads to biofilms formation which is governed by high cdG signaling. Thus, aGM1 can demonstrate dual functions: facilitating motilities or supporting adherence. Second, aGM1 also bind to LecA protein. LecA mediates adhesion of bacterial cells to the epithelial tissue surface resulting in colonization, which may subsequently lead to biofilm

formation [100]. LecA protein appears to associate only with high cdG levels and related signaling pathways [99]. The LecA is positively correlated to biofilm formation [99,100]. The mutant that overproduces LecA forms more biofilms, whereas the mutant of lacking LecA has low level of cdG and form weak/loose biofilm [100]. Thus, bacteria likely can utilize the expression levels of LecA and pili to tune and determine the outcome of adherence or motility. Third, besides adherence and motilities, bacteria are known to enter the host cells. Such entry is often initiated by adhesins binding to specific host cell receptors followed by internalization, or by bacteria secreting and injecting proteins that mimic or hijack specific host cell factors to trigger the bacterial uptake process [177]. Our results here demonstrate that both LecA and pili can selectively bind aGM1 from the lipid bilayers in an artificial liposome system. This binding may serve as an important step for either of the two mechanisms of bacteria's entry into the host cells. At the molecular level, we believe that aliphatic chains of aGM1 likely contribute to its binding to LecA and pilin. Finally, as aGM1 is dual ligand for LecA and pili, the binding sites of the proteins to ligands share certain common features. Here, we demonstrate that a class of strong synthetic ligands for LecA and pili can inhibit the function of both proteins and provide an effective inhibition for bacterial activities over a broad level of cdG signaling.

3.3 Conclusions

While aGM1 is known to bind pili, here we showed that aGM1 also bind to LecA. We demonstrated that aGM1 activates and modulates the swarming motility of non-swarming *rhlA* mutant, promotes sub-surface twitching motility of wild type *P. aeruginosa*, and has a weak effect on the adsorption of pili-specific bacteriophage on PAO1k. These results suggested that aGM1 binding to pili can cause different bioactivities. A label-free bacterial motility-enabled

binding assay revealed that both pilin and LecA proteins can sequester aGM1 from hydrated gel surface and abolish aGM1 activated swarming motility. Both pilin and LecA proteins caused leakage of artificial liposomes containing aGM1 as a lipid component, whereas BSA and anti-asialo-GM1 antibody did not show a similar effect. These results showed that both pilin and LecA bind to aGM1, and likely also recognize the aliphatic chains of aGM1, and cause leakage of aGM1-loaded liposomal system. Synthetic dual ligands, SFβM or βC for pili and LecA inhibited phage adsorption on pili appendages. These synthetic molecules inhibit swarming motility, also inhibit the surface adherence of *P. aeruginosa* that is mediated by either LecA or pili appendages, providing an opportunity for therapeutic development.

3.4 Experimental section

All overnight cultures of bacteria were grown in Lysogeny Broth-Miller (LB-Miller, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl). All the optical density/absorbance measurements were carried out on Biotek ELx800™ absorbance microplate reader (BioTek Instruments, Inc. Winooski, VT). The asialo-GM1 (98%), Sulforhodamine B dye was purchased from Sigma Aldrich and dissolved in chloroform: methanol (1:1) mixture and PBS buffer respectively for microtiter plate and liposomal assays. FITC tagged polyclonal rabbit anti-asialo-GM1 IgG was purchased from Wako Pure Chemicals (Osaka, Japan). Cholesterol and DOPC lipids were purchased from Avanti Polar Inc. For swarming assay and fluorescence polarization assay, aGM1 was dissolved in dimethyl sulfoxide filtered through cellulose acetate syringe filter (0.2 μm pore, Millipore).

P. aeruginosa LecA protein was purchased from Sigma Aldrich. Pili protein was expressed and purified according to literature.[178,179] EZ-Link NHS-PEG4-Biotin and Slide-A-Lyzer™ G1 Dialysis Cassettes, 1K MWCO were purchased from ThermoFisher Scientific. The

fluorescent ligand βGal-aryl-Dansyl and SFβM/SFβC were synthesized as per literature with slight modifications [81]. Stock solutions of 10 mM of SFβM, SFβC and βGal-aryl-Dansyl were prepared in sterile (autoclaved) water. Stock solutions of agents and non-autoclavable reagents were further filtered through cellulose acetate syringe filter (0.2 µm pore, Millipore).

3.4.1 Competitive binding of ligands to LecA by fluorescent polarization

To a solution of LecA (final concentration: 20 µM) and fluorescent ligand (final concentrations of βGal-aryl-Dansyl: 200 nM) in 0.1M Tris-HCl pH 7.5 supplemented with $CaCl_2$ (6 µM), serial dilutions (0.1 µM to 140 µM) of test compounds (SFβM and SFβC) were done. The mixture samples were incubated for 6 h at room temperature. The fluorescence polarization was measured using Edinburgh FLS9801 Spectrometer. During measurement, the samples were illuminated with vertically polarized light at 330 nm (for βGal-aryl-Dansyl) and vertical and horizontal fluorescence components were measured, and the fluorescence polarization were fitted with four parameter variable slope model [114].

3.4.2 Bacterial Swarming assay

The soft gels for swarming motility were prepared by autoclaving 0.5 wt% Bacto Agar in M8 medium (0.6 % Na_2HPO_4, 0.3 % KH_2PO4 and 0.05 % NaCl). The gel solution was cooled to ~60 ºC, supplemented with filtered 0.2 % glucose, 0.5 % casamino acid, and 1 mM $MgSO_4$ (0.22 µ filter). The gel solutions were poured into a Falcon tube for 20 mL portions, followed by adding aliquots of (1-20 µL) of SFβM or βC stock solutions to achieve the desired concentrations. The falcon tubes were closed, the agar solution was mixed by gently rocking, and then poured into polystyrene petri dishes (10 cm-diameter). The agar solution was solidified by cooling and air-drying in a laminar hood for 1 h. Bacterial culture of *rhlA* mutant strain (3 µL) with an OD_{600} between ~0.4–0.6 was inoculated on the center of the surface of the soft agar gel. These "swarm

plates" were incubated at 37 ^{o}C for 12 h and then incubated for additional 12 h at room temperature. After a total of 24 h, pictures of the swarming plates were taken.

3.4.3 Bacterial motility enabled binding assay

For soft gel prepared for swarming motility (see above), 100 µL of 1 mg ml^{-1} pilin, LecA, or BSA protein was introduced onto the center of the agar gel and immediately spread over the surface of the ger using a sterile cell spreader. The proteins were prepared in NaPB (4 mM, pH 7.2), Tris-HCl (0.1 M Tris-HCl, pH 7.5 and 6 µM $CaCl_2$) and Tris buffer (2 mM Tris, 7 mM NaCl, pH 7.5). The agar gel spread with protein solution was dried for an additional 30 min. A bacterial subculture (3 µL, 0.6 OD_{600}) was inoculated on the center of the soft gel (10-cm diameter plate). The bacteria on the soft gel were incubated 12 h at 37^{o}C and an additional 12 h at room temperature to develop the swarming pattern.

3.4.4 Quantification of phage titer, plaque forming unit (PFU), by top overlay method

All phage counts were quantified by phage titer using top overlay method and reported as the plaque forming units (PFU). *Top agar preparation.* Mix 3 mL of warm top agar solution (7 g L^{-1} agar, 0.5 g L^{-1} sodium chloride, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract in sterile water) and 200 µL of LB culture of PAO1k (OD 0.6); spread this mixture solution on 1.5% LB agar plate (LB-Miller, 10 g L^{-1}tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl ,15 g L^{-1} agar, 10-cm in diameter). Cool for 15 min in biosafety level-2 hood to solidify the gel. Serial dilutions of supernatant of containing phage were prepared in LB-Mg^{2+}. Nine or six drops of 10 µL of the diluted supernatant were placed on the top agar plates to observe plaques formation on bacterial lawn after 12 h. The number of plaques were counted and reported as plaque forming units per ml of sample.

3.4.5 Adsorption of bacteriophage ϕKMV on P. aeruginosa strains

Bacteriophage adsorption assay were adopted as described previously [112], with addition of our ligands. An overnight culture (100 µL) of *wt P. aeruginosa* strain PAO1k that is ϕKMV sensitive was diluted with 10 mL of LB supplemented with 10 mM $MgSO_4$ (LB-Mg^{2+}) and subcultured to OD_{600} around 0.6 at 37°C with shaking at 250 rpm with and without 20 µM SFβM and βC or aGM1. The bacteria subculture (100µL) was mixed with 900 µL of LB-Mg^{2+} ml^{-1} ϕKMV phage. The titer of added phage was individually determined for every experiment from the phage stock solution. Following incubation for 10 min at 37 °C with shaking at 100 rpm, bacteria were removed by centrifugation (10,000 g, 5 min at 4°C), and 900 µL of the supernatant was transferred to an Eppendorf tube. The plaque-forming units (PFU) in the supernatant with and without the added agents was determined by the top agar overlay method with PAO1k. The percentage of phage bound to bacteria was calculated as [(titer of added phage − titer in supernatant)/(titer of added phage)] × 100.

3.4.6 Liposomes preparation

Large liposomes were prepared using reported methods with slight modification [161]. The liposomes were prepared with 30 mol% cholesterol, 65 mol% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids) and 5 mol% asialo-GM1. A mixture of lipids consists of 30 mol% cholesterol, 65 mol% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPE) and 5 mol% asialo-GM1 were dissolved in chloroform: methanol (2:1) and evaporated on rotary evaporator under high vacuum at room temperature. Thin films so formed were dried overnight under high vacuum. Dried lipid thin films were rehydrated with 100 mM Sulforhodamine B in

saline (0.9w/v% NaCl in water). The resulting liposome solution was dialyzed with 5K Dalton dialysis tube to remove free Sulforhodamine B that was not entrapped in liposomes.

3.4.7 Liposome Leakage assay

The liposome encasing the Sulforhodamine B dye in saline (10 µL) were resuspended in PBS buffer (1 mL), followed by adding 100 µL of pili (1 mg ml^{-1}), LecA (1 mg ml^{-1}), anti-asialo-GM1 antibody (0.5 mg ml^{-1}), BSA (0.5 mg ml^{-1}), or Triton X-100 (1 mg ml^{-1}). The liposome and proteins solution were incubated in dark for 6 h. The fluorescence of leaked Sulforhodamine B into the solution containing liposomes was measured at excitation wavelength λ_{ex} 565nm and emission was measured at wavelength λ_{em} 585 nm over a period of 0-6 h. The Triton X-100 was used as 100% lysed/leakage control.

3.4.8 Bacterial adhesion assay

The wells of the black microtiter plate were coated with 100 µL of 2 µg ml^{-1} solution of asialo-GM1 dissolved in chloroform: methanol (1:1). The plate was kept in BSL-2 hood for overnight to evaporate solvent and form asialo-GM1 coating. The wells were rinsed with 200 µL PBS buffer once and each well was added with 150 µL of 50 µg ml^{-1} of BSA solution and incubated for 1 h in at 37 ℃ to passivate the surfaces. The wells were washed with PBS containing 0.05% (w/v) BSA (Buffer A). Overnight cultures (100 µL) of *wt* PAO1, Δ*pilA*, and *lecA::Tn* - in LB medium were diluted in 10 mL of LB. When the OD_{600} of the sub-cultures reached ~0.6, an aliquot of the sub-culture (200 µL) was transferred to the asialo-GM1-coated wells in black microtiter plate, The black microtiter plate was wrapped in plastic cling wrap and incubated at 37ºC for 1 h. The bacterial cultures were then discarded by pipetting, and each well was washed once with saline water (0.85 w/v% aqueous NaCl). An aliquot of fresh LB (200 µL) was then

added to the wells and sonicated for 5 min (10 L water bath cooled with ice, 40 kHz). The suspended bacteria were enumerated by colony forming units (CFU ml^{-1}) on LB agar plates. For antibody assay, aGM1-coated surfaces were treated with anti-asialo-GM1 antibody (100 μL, 0.5 mg ml^{-1}) for 15 min prior to addition of bacteria. For competitive inhibition assay, bacteria were cultured with SFβM or βC.

3.4.9 Synthesis of fluorescent probe, βGal-aryl-Dansyl

4-Aminophenyl β-D-galactopyranoside (0.12 g, 1.2 mmol) in anhydrous DMF (2 mL) was added to Et_3N (0.53 g, 0.53 mmol) in anhydrous DMF (5mL) at 0 ºC. To this solution was added dansyl chloride (0.41 g, 1.5 mmol). After stirring at 0 ºC for 2 hours, the mixture was concentrated, and the residue obtained was subjected to the purification by column chromatography DCM:MeOH 9:1 to yield a yellow solid product (0.44 g, 72 %). ^1H NMR (400MHz, $CDCl_3$): δ 8.47 (d, 1 H, dansyl), 8.24 (d, 1 H, dansyl), 8.20 (d, 1 H, dansyl), 7.65-7.69 (m, 2 H, dansyl), 7.39 (d, 1 H, dansyl), , 6.92–6.81 (m, 4H, ArH), 4.75 (d, J =7.7 Hz, 1H), 4.37 (s, 2H, C<u>H</u>2NHR), 3.69 (d, J = 3.1 Hz, 1H), 3.58–3.46 (m, 5H), 2.84 (s, 6H,-CH3).

3.4.10 Direct binding of fluorescent ligand to LecA[114]

The fluorescent ligand (βGal-aryl-Dansyl) were dissolved in water to a final concentration of 3 mg ml^{-1} . 2 mg ml^{-1} LecA (Sigma) and bovine serum albumin (Sigma) was dissolved in 1 mL of 0.1M Tris-HCl pH 7.5 supplemented with $CaCl_2$ (6 μM). The solution of fluorescent ligand (βGal-aryl-Dansyl, 200 nM) is mixed with different concentrations of LecA solution (0-100 μM). After incubation for 1 h at room temperature in dark, fluorescence polarization was measured by using Edinburgh FLS9801 Spectrometer, with the samples being illuminated with

vertically polarized light at 330 nm (for βGal-aryl-Dansyl). The vertical and horizontal fluorescence components of emission 570 nm were recorded, and the fluorescence polarization was calculated and data were analyzed using a four-parameter fit model.

3.4.11 Twitching motility assay[180]

Subsurface twitching assays were modified from previously reported procedures. Briefly, 100 µL of overnight culture of PAO1-EGFP was diluted in 900 µL of Luria Bertani broth without NaCl (LBNS). Sterile pipette tips (10 µL) were dipped in this LBNS suspension and were then stabbed through a one-day-old 1% LBNS agar, with and without 10 µM of aGM1 or 20 µM SFβM or βC, to inoculate bacteria at bottom of the agar gel. Plates were incubated upright (not inverted) in an incubator at 37 ℃ for 2 days, and images of the plates were taken under ultraviolet light (Accuris™ E3000 UV Transilluminator - 302nm) in dark room.

3.4.12 Synthesis of biotinylated Pili and LecA proteins [175,181]

The 500 µL of pili (0.5 mg ml^{-1}) or LecA (0.5 mg ml^{-1}) were biotinylated with 1 ml solution of EZ-Link NHS-PEG4-Biotin (1 mg ml^{-1}) in 1 ml carbonate-bicarbonate buffer (pH 9.2). The reaction was carried out at 37 ℃ for 4 h under 100 rpm shaking condition. The resultant mixture was pipetted into Slide-A-Lyzer™ G1 Dialysis Cassettes, 1K MWCO and stir for 24 h in distilled water. The dialyzed proteins were lyophilized to obtain biotinylated pili or LecA. The extent of biotinylating reaction was accessed by Pierce™ Biotin Quantitation Kit (ThermoFisher Scientific).

3.4.13 Protein binding, and inhibition, to aGM1 on surfaces [175,182]

The wells of the microtiter plate were coated with 100 µL of 2 µg ml^{-1} solution of asialo-GM1 dissolved in chloroform: methanol for overnight in biosafety level-2 hood. The excess sites in the wells were blocked with a 5% (w/v) BSA solution (150 µL per well) for 1 h in a 37 ℃ incubator for drying. The wells were washed with PBS containing 0.05% (w/v) BSA (Buffer A). Aliquots of biotinylated pili or biotinylated LecA were added to the wells (100 µL per well) to reach different concentrations (0.1 to 5 µg ml^{-1}) and incubated for 1 h at 37℃. The plate was washed three times with Buffer A. The amount of biotinylated pili and biotinylated LecA bound on aGM1-coated wells were characterized by the 4'-hydroxyazobenzene-2-carboxylic acid (HABA)-Biotin assay (Pierce™ Biotin Quantitation Kit, ThermoFisher Scientific). The absorbance of HABA at 500 nm was measured and plotted against concentration of pili or LecA proteins. The hyperbolic curve model was fitted by using non-linear regression program in Mathcad and binding constant K_d was extrapolated from model.

For inhibition assay, we used 1 µg ml^{-1} of biotinylated pili and biotinylated LecA as 1 µg ml^{-1} concentration provides a near full binding of aGM1 on surfaces as depicted in previous protein binding studies. The 1 µg ml^{-1} protein solutions are mixed with different concentrations of SFβM or βC (final concentrations 0.1 to 120 µM) for 1 h, and the mixture were added to aGM1-coated wells for 1 h. The binding of biotinylated pili/LecA was measured same as above. The inhibition of binding of biotinylated pili/LecA was plotted against concentrations of ligands SFβM or βC. The half-maximal inhibition concentration (IC_{50}) was calculated from standard dose-response curves using Dr-Fit software.

Chapter 4 Antibacterial agents that inhibit tolerance, persistence, and resistance: receptor identification and mechanism

4.1 Background and significance

The chemical action of killing bacteria by traditional antibiotics always cause the bacteria to develop certain resilient phenotypes that survive. All these resilient phenotypes have the characteristic of either drug tolerance, drug persistence, or drug resistance, and are manifested in the form of increased biofilm formation[183] and motility,[30,46] over production exopolymers,[183] formation of small colony variants.[36,184] All clinically approved antibiotics promote biofilm formation, by a sub-lethal concentration in laboratory setting, and by less well-defined conditions on an infection site. These biofilms host high populations of drug tolerant, drug persistent, or drug resistant population, which overall requires thousands fold of concentration of antibiotics to kill in comparison to bacteria in a planktonic form.[185-188] More intriguing cases are where, bacteria respond to the same antibiotic treatment with different resilient phenotypes under different conditions.

4.1.1 Antibiotic leads to tolerance, persistence, and resistance.

Use of antibiotics on bacteria readily causes the bacteria to be tolerant to the antibiotics in a matter of minutes to hours.[21] This tolerance causes the requirement of a high dosage or a longer treatment time to kill bacteria.[21] In addition, this tolerance transition to persistence for some small population of the bacteria under antibiotic treatment. These bacteria do not grow and do

not get killed during the antibiotic treatment; but once the antibiotics are removed, these bacteria revitalize to a population of bacteria that are mostly susceptible, with some small percentage of persistent bacteria. These small percentage of persistent bacteria, however, is larger than that before the antibiotic treatment. When drug resistance is fully formed, these population can grow slowly in the presence of antibiotics . In the absence of antibiotics, the gene mutations for drug resistance are usually stable.

4.1.2 Antibiotic tolerance and persistence facilitate resistance.

While the development of drug resistance is due to spontaneous gene mutation, which is random in nature; under the stress of antibiotic treatment, the mutation that cause resistance is under the natural selection for survival by the antibiotic treatment.[22,23,189,190] Thus, the repeated applications of a conventional antibiotic on a bacterial population promotes both drug tolerance, and drug resistance. In a batch culture, both developments occur. A recent study by Balaban and coworkers reveals the relation between the two develop.[23,189-192] In a batch culture, the tolerance mutation and resistance mutation does not occur independent of each other. Rather, the resistant population carry both mutation responsible for drug resistance and drug tolerance, whereas the tolerant population carry only mutated gene relevant to tolerance. This important result illustrates an important genetic dependence (epistasis) of the development of tolerance for the development of resistance. The resistance mutations establish significantly faster for drug tolerant and persistent bacteria. Theoretical analysis suggests that, for example, *ampC* mutations for resistance are often lost during antibiotic treatment. With a tolerance background, the probability of lost or reversal of resistance mutation is lowered. One plausible explanation is that tolerance

arises from many mutations, and stable resistance typically requires several important mutations. Thus, mutations for tolerance facilitates mutations for stable resistance (Fig. 4.1).

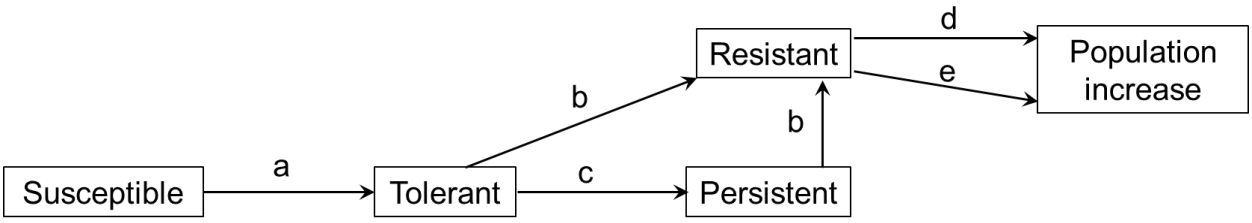

Figure 4.1 Scheme of development of resistance under antibiotic stress (steps a to e). a. Antibiotic treatment caused tolerance mutation.[189-191] b. Tolerance and persistence facilitate resistance mutation.[25,191] c. Tolerance transitions to persistence.[21,23] d. Generational gene transfer.[193,194] e. Horizontal gene transfer.[24,195,196]

Interestingly, it is also mentioned that at low concentrations of antibiotics, fully resistant single step mutants can occur without a priori developed tolerance.

4.1.3 Horizontal or lateral gene transfer for propagating the resistant genes.

The above describes diverse types of drug resistance mechanisms.[24,193-196] Once the resistant mutant forms, their genes can be transferred by the different ways. First, generational gene transfer causes the daughter bacterium to carry the gene from the parent gene. Second, a common mechanism of gene transfer for bacteria is the direct physical contact between two bacteria causing the transfer of DNA from one to the other: or the release of DNA materials into the environment by one bacterium and be taken up by another. These gene transfers does not involve a bacterium's replication and multiplying, but horizontally between bacteria of the same species or between different species, and thus are called horizontal gene transfer (HGT).

Horizontal gene transfers (HGT) result in genetic variation in bacteria and are a major contribution to the spread of antibiotic resistance genes.

There are three mechanism of horizontal gene transfers[196-198]. 1. Conjugation: Two bacteria can pair up and connect through structures in the cell membranes and then transfer DNA from one bacterial cell to another. 2. Transformation: Some bacteria can take up pieces of DNA directly from the environment around the cell. 3. Transduction: There are viruses called bacteriophages that can infect bacteria. These viruses sometimes bring along genes that they picked up during infection of another bacterium. These genes may then be incorporated into the DNA of the new bacterial host.

Any gene has the potential to be transferred between bacteria by HGT, including antibiotic resistance genes.[196] The transferred genes may be integrated into the DNA genome of a recipient bacterium or may exist as a circular plasmid DNA. Foreign DNA can be harmful to a bacterium, and there are machineries in place that degrades incoming DNA. However, these systems are not always 100% efficient. If the incoming DNA is incorporated into the recipient genome and provides a benefit for the bacterium, the integrated DNA is more likely to be maintained. For example, if a bacterium picks up an antibiotic resistance gene and is subsequently exposed to that antibiotic, this bacterium can proliferate.[196]

4.1.4 Our advances in inhibition of resilient phenotypes and design of antibacterial agents that inhibit resistance.

We believe that the fundamental issue we face for development of antibiotics is that primary or only goal in the tradition approach to develop antibiotics is to kill the bacteria with high potency. Here, we first develop agents to inhibit broadly all the resilient phenotypes, and

then modify the structure so that the molecules kill the bacteria while retaining the ability to inhibit the formation of resilient phenotypes.

We showed a broad class of disugar-derivatized hydrocarbons that inhibit both biofilm formation and swarming motility of *P. aeruginosa* bacteria. Our past studies revealed that two most active molecules among these dual synthetic inhibitors are saturated farnesol-β-cellobioside or -β-maltoside (SFβC and SFβM).[81,199] Early structural studies indicated that the methyl substitution in the aliphatic chains is important: linear C12-aliphatic chain does not exhibit strong bioactivity, whereas the 3,7,11-trimethyl substituted aliphatic chain does. In search for more potent molecules through synthesis of structural variants, we explored new methyl positions on the C12-alphatic chain resulting in new molecules 3,5-diMeDβM and 3,5-diMeDβC. These molecules inhibits tolerance and prevents formation of new persistent populations in *P. aeruginosa*. To introduce bactericidal or bacteriostatic activities into these chimeric agents, we note a recent development of dodecyl triazole cellobioside (C_{12}TC, Fig. 4.2) that has exhibited antibacterial activities. This molecule showed mild bacteriostatic activity against wild type *P. aeruginosa.*

To understand structure activity relationship between antibacterial activity and structural elements of dodecyl triazole cellobioside (C_{12}TC), we designed following variants of Dodecyl triazole cellobioside (C_{12}TC). We have previously showed that saturated farnesol derivatized control bioactivities in *P. aeruginosa* and hence we designed farnesol triazole cellobioside (FTC) (Fig. 4.2) and saturated farnesol triazole cellobioside (SFTC). To understand importance of sugar moiety, we designed farnesol derivatized tetra ethylene glycol with click chemistry. The fatty acids/long chained alkyl groups play role in biological system hence to understand role of

hydrophobic alkyl group in antibacterial activity we designed short chained trimethyl hexyl triazole cellobioside (triMeHTC).

We found that SFTC and FTC (Fig. 4.2) showed bactericidal activities against *P. aeruginosa* while other molecules showed bioactivities like inhibition of swarming motility, biofilms against *P. aeruginosa* but lacked antibacterial activity. These results hinted us towards significance of triazole and sugar moieties in antibacterial activities against *P. aeruginosa*. Hence to test this hypothesis we designed Farnesol oxadiazole cellobioside (FOC) which contains farnesol and sugar (cellobioside) but lack triazole moiety. We introduced oxadiazole as heterocycle linker in place of triazole.

4.1.5 Journey from adjuvant agents to design of antibacterial agents with no resistance.

We showed a broad class of disugar-derivatized hydrocarbons that inhibit both biofilm formation and swarming motility of *P. aeruginosa* bacteria.[81,199] Our past studies revealed that two most active molecules among these dual synthetic inhibitors are saturated farnesol-β-cellobioside or -β-maltoside (SFβC and SFβM).[81,199] Early structural studies indicated that the methyl substitution in the aliphatic chains is important: linear C12-aliphatic chain does not exhibit strong bioactivity, whereas the 3,7,11-trimethyl substituted aliphatic chain does. In search for more potent molecules through synthesis of structural variants, we explored new methyl positions on the C12-alphatic chain resulting in new molecules 3,5-diMeDβM and 3,5-diMeDβC. These molecules inhibits tolerance and prevents formation of new persistent populations in *P. aeruginosa*. To introduce bactericidal or bacteriostatic activities into these chimeric agents, we note a recent development of Dodecyl triazole cellobioside (C_{12}TC). that has

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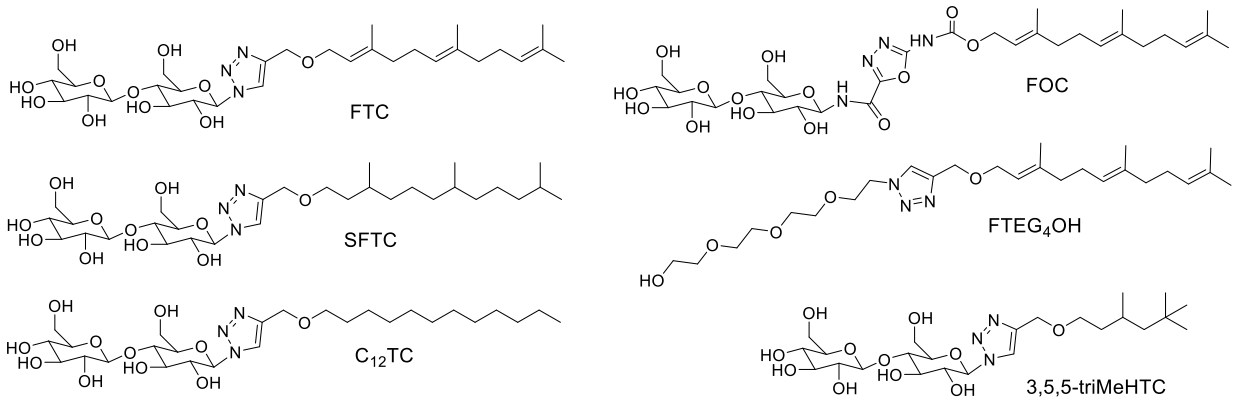


Figure 4.2 Structures of Farnesol triazole cellobioside (FTC), Saturated farnesol triazole cellobioside (SFTC), Farnesol oxadiazole cellobioside (FOC), Dodecyl triazole cellobioside (C_{12}TC) and farnesol triazole tetraethylene glycol ($FTEG_4OH$), 3,5,5-trimethylhexyl triazole cellobioside (3,5,5-triMeHTC)

4.2 Farnesol Triazole Cellobioside is active against both planktonic and biofilm bacteria

The failure of existing antibiotics because of the development of drug tolerance and drug resistance is increasingly severe worldwide. While bacteria are usually sensitive to antibiotic in planktonic cultures, bacteria form biofilms under almost all conditions. In biofilms, it has become clear, bacteria is more difficult to kill by antibiotics, resulting in relapse and recurrence of infections[190,191,200]. Bacteria evade antibiotics treatments by three different phenotypes, drug tolerant, drug persistent, and drug resistant populations[22,26,189-192,200-202]. The resistance is often defined as intrinsic or acquired genetic process that leads bacterial survival under antibiotic treatment. Biofilms show enhanced tolerance to antibiotics and harbor persistent populations which are precursor to resistance under prolonged antibiotic exposure[189,190]. In fact, all current antibiotics are known to promote biofilm formation by bacteria[33,34]. As higher dose of antibiotics are necessary to eradicate biofilms,[34,38] vicious circles are created that increase the overuse of antibiotic and the drug tolerance, and persistence. In general, this overuse of antibiotics often leads to resistance[202].

In our past studies, we have identified a class of adjuvant molecules that are specific ligands to two proteins Lectin A and pili. When combined with an existing antibiotic tobramycin, this class of chimeric ligands enables tobramycin to kill drug tolerant population and prevents the

formation of nascent persistent populations. Based on the structures of the chimeric ligands, we have developed a class of antibacterial molecules with no resistance that is able to kill a wide range of bacteria strains, include both gram-positive and gram-negative (see previous section 4.1). Here we present the effectiveness of this class of molecules at killing the planktonic bacteria by evaluating the minimum inhibitory concentrations (MIC), and at killing biofilm bacteria by minimum bactericidal concentration (MBC). In the next section, we will characterize the effectiveness of this class of molecules at inhibiting the inhibiting and killing tolerant and persistent populations

4.2.1 FTC is active against both gram-negative and gram-positive pathogens in planktonic cultures.

First, we established the growth curves of wild type *Pseudomonas. aeruginosa* (*wt* PAO1) and *Staphylococcus aureus* (*S. aureus*) in the presence of FTC (1 - 100 µg/ml). The *P. aeruginosa* and *S. aureus* are common pathogens for many infection conditions, such as in the lung of cystic fibrosis patients and the sites of burnt wounds. For both *wt* PAO1, and *S. aureus*, we found that FTC significantly reduce the growth of both strains at around 10 µg/ml (Fig. 4.3).

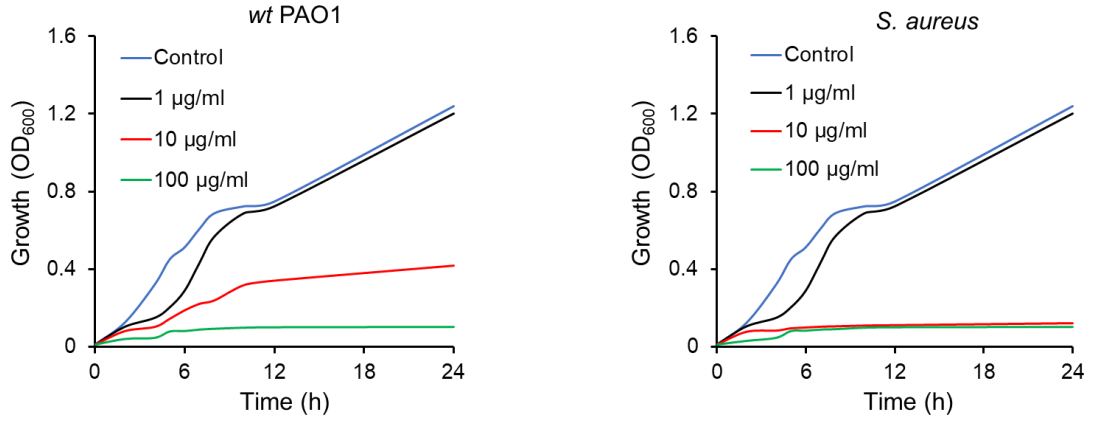


Figure 4.3 Representative growth curves of wild type *P. aeruginosa* (*wt* PAO1) and *Staphylococcus aureus* (*S. aureus*) in the presence of different concentrations of FTC (1 – 100 µg/ml). *P. aeruginosa* PAO1 in Luria–Bertani broth media. *Staphylococcus aureus* (*S. aureus*) in Tryptic soy broth (TSB) OD_{600} = 0.01 were grown with different concentrations of FTC ((1 – 100 µg/ml) or without FTC (control) at 37°C for 24 hours under 250 rpm shaking.

4.2.2 Inhibiting growth of planktonic bacteria : Minimum inhibitory concentration (MIC)

To characterize the killing of the bacteria in planktonic culture, minimum inhibitory concentration (MIC) – the lowest concentration of FTC that prevents visible growth of bacteria (cloudiness of the culture) is determined by microtiter broth dilution method as described by Eloff according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[203,204]. The FTC molecule was diluted serially on 96-well plates, in cultures with an initial optical density (OD_{600}) of ~0.01 of gram-negative strains, including *Pseudomonas aeruginosa* PAO1 and PA14, *E. coli*; and gram-positive strains, including *Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis, Nontuberculous mycobacteria* (NTM) of *Mycobacterium avium* and *Mycobacteroides abscessus*. The MIC values are displayed in Table 4.1.

4.2.3 Killing planktonic bacteria: Minimum bactericidal concentration (MBC)

The MBC is identified as the lowest concentration of antibacterial agent that reduces the viability of the initial bacterial inoculum by a pre-determined reduction of ≥99.9%. MIC test demonstrates the lowest level of antimicrobial agent that greatly inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent resulting in microbial death.[204]

FTC showed most promising activity against gram positive bacteria *Staphylococcus aureus, Bacillus subtilis* and *Staphylococcus epidermidis* and *Mycobacterium avium*. FTC was bactericidal against *Staphylococcus aureus* with MIC of 2 µg/ml and MBC of 8 µg/ml. FTC showed bactericidal activity against *Bacillus subtilis* with MIC of 1 µg/ml and MBC of 4 µg/ml. FTC was more potent against *Staphylococcus epidermidis* with MIC of 1.5 µg/ml and MBC of 4 µg/ml. FTC was active against *Mycobacterium avium* with MIC of 8 µg/ml. FTC showed very mild activity against multidrug resistant *Mycobacteroides abscessus* with MIC of 64 µg/ml (Table 4.1).

FTC exhibited mild bactericidal activity against all the gram-negative strains (Table 4.1). FTC was bactericidal against *P. aeruginosa* strains PAO1 and PA14 with MIC of 4 µg/ml/MBC of 48 µg/ml and MIC of 12 µg/ml and MBC of 64 µg/ml. FTC was active against *E. coli* with MIC of 8 µg/ml and MBC of 72 µg/ml.

Table 4.1 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of FTC against different gram-negative and gram-positive bacteria.

Bacterium strain	**MIC (µg/ml)**	**MBC (µg/ml)**
Gram-negative		
Pseudomonas aeruginosa (PAO1)	4-8	48
Pseudomonas aeruginosa (PA14)	12	64
E. coli	8	72
Gram-positive		
Staphylococcus aureus	2	8
Bacillus subtilis	1	4
Staphylococcus epidermidis	0.5	4
*Mycobacterium avium****	8	-
*Mycobacteroides abscessus****	64	-

Two different strains are used for *P. aeruginosa* *wild-type Pseudomonas aeruginosa reference strain PAO1. **PAO1 isolated form lungs of cystic fibrosis patients. *** NTM strains are evaluated by Seattle Children's Hospital.

Table 4.2 The MIC values of commercially available antibiotics on PAO1 and *S. aureus.*

Antibiotic	**PAO1 (MIC µg/ml)**	**SA (MIC µg/ml)**
Tobramycin	1	0.125-1
Aztreonam	3[205-207]	Inactive
Colistin	1[205-207]	>100[205-207]
Imipenem	2[205-207]	0.05
Ceftazidime	2[205-207]	Inactive
Ciprofloxacin	0.25[205-207]	0.2
Moenomycin A	32	0.5
*FTC	4-8	2

* relisted from Table 4.1

4.2.4 Antibacterial activities of FTC against P. aeruginosa clinical strains from cystic fibrosis patients.

Bacteria extracted from patients, clinical isolates, or strains, always exhibit high tolerance, sometimes, resistance to treatments of conventional antibiotics. During early infections, *P. aeruginosa* is typically in a planktonic, non-mucoid form and can be easily killed by repeated antibiotic exposure. However, during chronic infections *P. aeruginosa* forms biofilms and becomes more tolerant to antibiotics. The repeated exposure to higher antibiotic concentrations leads tolerant and persistent clinical isolates in cystic fibrosis patients.[208-211] The clinical isolates are known to exhibit persistent phenotypes like mucoid production, hyper adherent, and small colony variants. We used three clinical isolates of *P. aeruginosa* characterized as non-mucoid (SMC1587, SMC1595 and SMC1596) and three

characterized as mucoid (SMC5450, SMC5451 and SMC1585) obtained from Dr. George O'Toole laboratory.[208-213] Clinical *P. aeruginosa* isolates SMC1585, SMC1587, SMC1595, SMC1596, SMC5450 and SMC5451 were collected from the sputa of CF patients by the clinical microbiology laboratory at the Dartmouth-Hitchcock Medical Center (DHMC). Preliminary biofilms study with these phenotypes revealed that these strains form more biofilms than wild type PAO1. SMC1587 and SMC1585 cause small colony variants under tobramycin and aztreonam treatments, and the other four strains did not. Mucoid strains overproduce exopolymer alginate, and often cause cultures with slims. This phenotype, when populate in patients' lung, causes patients' difficulty in beathing. We also studied a clinical, hyper-mucoid *P. aeruginosa* strain PA2192. This strain overproduces mucoid and is considered highly virulent because patients show poor clinical outcome despite having a heightened immune response.[54,214-216] Mucoid PA 2192 was obtained from Dr. Joanna B. Goldberg (Emory University, Atlanta GA).[214,217,218]

Here, we examined planktonic minimum inhibition concentration (MIC) of FTC on three nonmucoid strains and four mucoid strains. We found that FTC is effective against all clinical *P. aeruginosa* strains. FTC showed bactericidal activity against non-mucoid SMC1587, SMC1595 and SMC1596 with MIC <16 μg/ml. FTC also showed excellent activity against resilient mucoid strains (Table 4.3). For SMC1585, SMC5450 and SMC5451, the MICs are 8 μg/ml. The clinical cystic fibrosis isolate of *P. aeruginosa* PA2192, produces visibly slimier (alginate) than other mucoid strains, which cause the significant increase of viscosity of culture. The MIC for this strain is lower than 16 μg/ml.

Table 4.3 MIC of Farnesol Triazole Cellobioside against clinical isolates of *P. aeruginosa*.

Clinical strain		MIC (µg/ml)	Note
Nonmucoid	SMC1587	8<MIC <16	Colistin resistant
	SMC1595	8<MIC <16	
	SMC1596	16<MIC <32	
Mucoid	SMC1585	4<MIC <8	
	SMC5450	4<MIC <8	
	SMC5451	4<MIC <8	β-lactam resistant
	Mucoid PA2192	8<MIC <16	

During MIC assay, we made a surprising discovery that SMC1587 appeared to be resistant to colistin (10 µg/ml, MIC for *wt* PAO1 : 1 µg/ml) (Fig. 4.4). We further validated this colistin resistance by subculturing SMC1587 in the presence of 100 µg/ml colistin and observed that the growth if this strain was as fast as if there was no colistin in the culture. Interestingly, FTC showed activity against colistin resistant SMC1587 with MIC = 16 µg/ml.

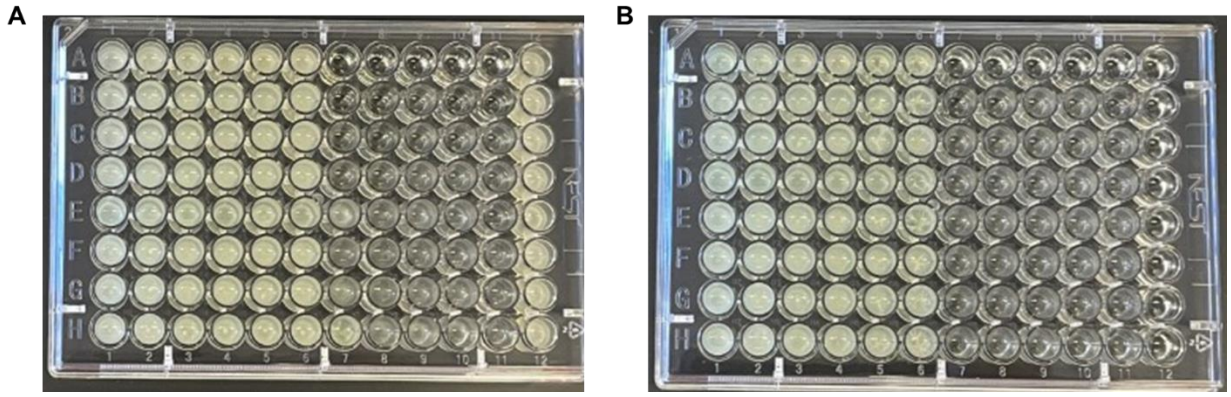


Figure 4.4 Typical 96-well plates for determination of MIC values. Specific examples here for examining FTC against clinical strain SMC1587 (A) and SMC1595 (B) Column 1 contains no agent Column 11 to 2 contain, 256, 128, 64, 32, 16, 8, 4,2, 1, 0.5 µg/ml of FTC. Column 12 contains 10 mg/ml of colistin. Strain SMC1587 showed resistance to colistin, while strain SMC1595 was susceptible to colistin.

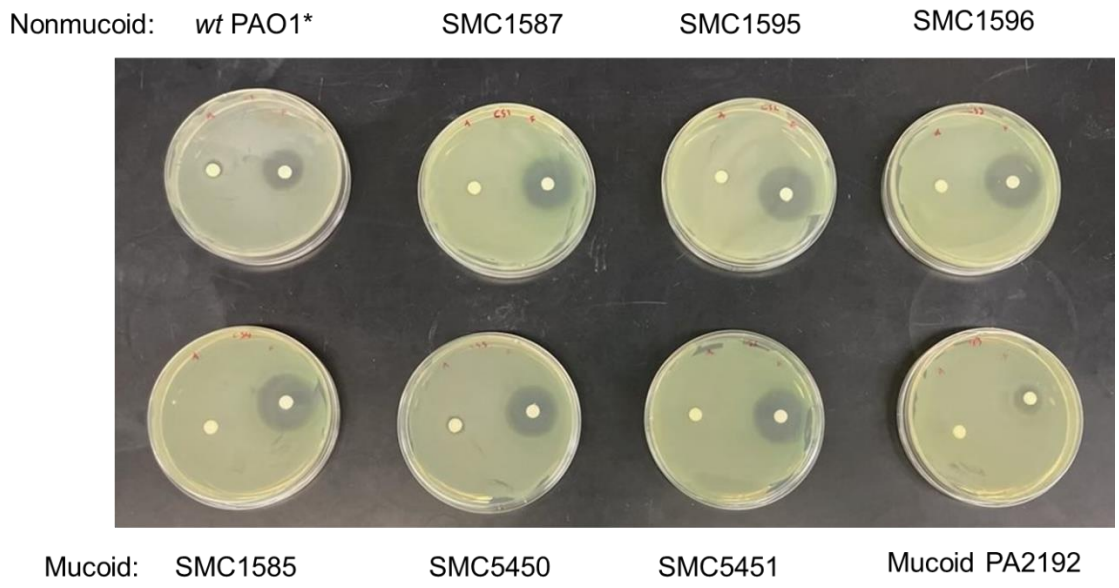


Figure 4.5 Antibiotic susceptibility tests of non-mucoid and mucoid *P. aeruginosa* strains by disc diffusion method showing zone of inhibition. 10 µL of 3 µg/ml AZT and 8 µg/ml FTC was dropped on discs , incubated for 24 h at 37°C. **wt* PAO1 laboratory strain has been obtained from multiple antibiotic abuse.

We compared antibacterial efficacy of FTC with aztreonam antibiotic by disc diffusion assay. As clinical strains are more resistant to aztreonam, we increased concentrations of aztreonam from 3 µg/ml (the reported MIC against *wt* type for AZT is 3 µg/ml[219]) to 6 µg/ml and to 8 µg/ml. At these two concentrations (6 µg/ml and 8 µg/ml, 10 µL on the disks), inhibition zones were observed for clinical strains, except for SMC 5451. This result suggests that SMC 5451 is resistant to aztreonam, which is consistent with O'Toole and coworkers reported finding that that SMC5451 is tolerant to aztreonam in planktonic culture with a MIC >256 µg/ml[208].

Overall, with MIC values, we believe that these MIC values suggest that FTC is potent enough for an immediate exploration for therapeutic applications. The most significant finding here is that FTC is active against aztreonam- and colistin-resistant clinical strains.

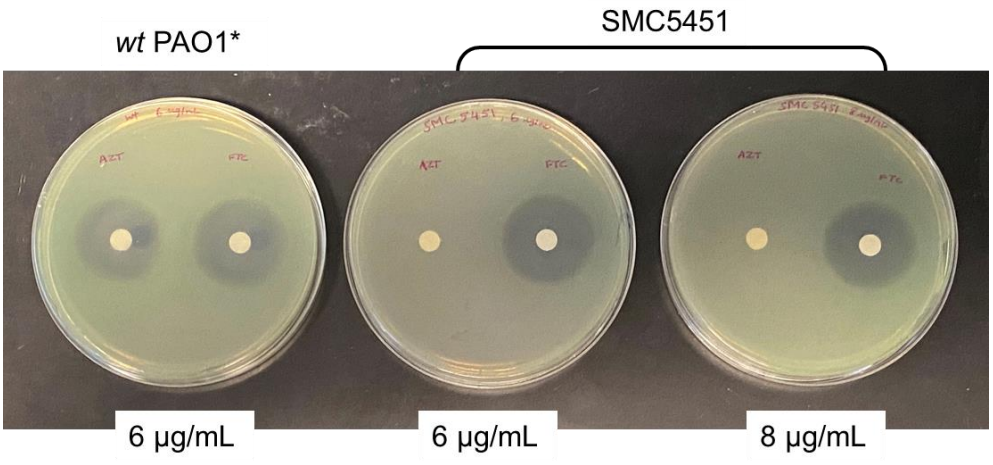


Figure 4.6 FTC is active against aztreonam resistant strain of *P. aeruginosa* SMC5451. Antibiotic susceptibility tests of wild type PAO1 and SMC5451 by disc diffusion method showing zone of inhibition. 10 µL of 6 µg/ml, 8 µg/ml AZT and 8 µg/ml FTC was dropped on discs; incubated for 24 h at 37ºC.

Table 4.4 Minimum Biocidal Concentration (MBC) of antibiotics on wild type *P. aeruginosa*

Antibiotic	**MBC µg/ml** [220,221]
Tobramycin	2
Aztreonam	8
Colistin	1
Imipenem	4
Ceftazidime	8-12
Ciprofloxacin	0.5-1
FTC	**48***

*FTC is done by us. All other values are from literature.

4.2.5 FTC against Biofilms: Minimal biofilm-eradication concentration (MBEC)

The MBEC is defined as the lowest concentration of antibiotic required to eradicate the biofilm or, the lowest concentration of antimicrobial agent that prevents visible growth in the recovery medium used to collect biofilm cells.[203,204,222,223] We tested efficacy of FTC against 1- day old biofilms of wild type *P. aeruginosa* PAO1 and *S. aureus* grown on peg surface by Calgary devices (MBEC Assay® Biofilm Inoculator). FTC reduced viability of biofilm bacteria in both PAO1 and *S. aureus*. FTC was active against PAO1 with MBEC of 48 µg/ml and MBEC of 16 µg/ml against *S. aureus* (Table 4.4).

Table 4.4 Minimal biofilm-eradication concentration (MBEC) and Biofilm bactericidal concentration (BBC) of FTC against biofilms of gram-negative and gram-positive bacteria.

Bacteria	**MBEC (µg/ml)**	**BBC (µg/ml)**
Gram-negative		
Pseudomonas aeruginosa (PAO1)	48	64
Gram-positive		
Staphylococcus aureus	16	32

Table 4.5 Minimal biofilm-eradication concentration (MBEC) and Biofilm bactericidal concentration (BBC) of different antibiotics against biofilms of wild type PAO1.

Antibiotic	**Type/receptor**	**MBEC µg/ml** [220,221]	**BBC µg/ml** [220,221,224]
Aztreonam	β-lactam/PBP	1028	-
Imipenem	β-lactam/PBP	256	256
Ceftazidime	β-lactam/PBP	64	1024
Colistin	Polymyxin/outer membrane LPS	32	64

Tobramycin	Amino glycoside/ribosome	4	64
Ciprofloxacin	Fluoroquinolone/topoisomerase	4	64
FTC		48*	64*

4.2.6 FTC against Biofilms: Biofilm bactericidal concentration (BBC)

The BBC is defined as lowest concentration of an antibiotic producing a 99.9% reduction of the CFUs recovered from a biofilm culture as compared to the growth control[203,204,222,223]. We tested efficacy of FTC against bacteria in 1- day old biofilms of wild type *P. aeruginosa* PAO1 and *S. aureus* grown on peg surface by Calgary devices (MBEC Assay® Biofilm Inoculator). FTC reduced viability of biofilm bacteria in both PAO1 and *S. aureus*. FTC was active against PAO1 with BBC of 64 µg/ml and BBC of 32 µg/ml against *S. aureus* (Table 4.5).

We demonstrated killing of both planktonic and biofilm bacteria; for a wide range of bacterium species, including both gram-positive and gram-negative strains. FTC is indeed broad-spectrum antibiotic. The activity against nontuberculous mycobacteria (NTM) is promising as recently there has been a considerable rise in pulmonary infections caused by NTM. These mycobacteria species have developed resistance to conventional antibiotics leading to treatment failure. FTC has showed better anti-biofilm activity than existing antibiotic used in clinical settings[220]. β-lactam antibiotics like aztreonam, Imipenem and ceftazidime showed MBEC of 512 µg/ml, 1028 µg/ml, and 64 µg/ml, respectively against wild type PAO1 biofilms while FTC showed MBEC of 48 µg/ml.

We showed that FTC is bactericidal against and gram-negative and positive bacteria. The MIC values of FTC range from 2 µg/ml to 4 ug/ml for gram positive, and from 4 ug/ml-16ug/ml

for gram negative. These MIC values suggest that FTC is more potent for planktonic gram-positive than gram negative bacteria. The clinical isolates of P. aeruginosa are showed to be tolerant to antibiotic treatments.[208] FTC showed activity towards to 7 clinical isolates of *P. aeruginosa*, both mucoid and non-mucoid phenotypes. The bactericidal concentration for FTC was higher than other antibiotics. However, all current antibiotics are reported to promote biofilms in both laboratory settings and in patients. We showed that even though FTC exhibit higher MIC or MBC than conventional antibiotics, FTC do not promote biofilm formation. It is important to note that, at least for aztreonam, the planktonic MIC does not correlate with tolerance in biofilms. In fact, SMC5451 biofilm is reported to be susceptible to aztreonam, while SMC5451's planktonic MIC is reported to >256 μg/ml.[208]

However, in a collaboration with O'Toole, results indicate that FTC is more potent than aztreonam for *wt* PAO1 biofilms formed on airway cells conducted an *in vitro* experiment.

Many conventional antibiotics exhibit strong activity for planktonic bacteria, as illustrated by the relatively low minimum inhibitory concentration (MIC) and minimum bactericidal concentration(MBC), see Table 4.2 and Table 4.3. However, these antibiotics exhibit disappointingly low activities against biofilm bacterial, as shown in minimum biofilm eradication concentration (MBEC) that measure the inhibition biomass, and biofilm bactericidal concentration (BBC) that measures the killing of biofilm bacteria. These values against biofilms are 100 to 1000 times less potent than the values against planktonic values. While these measurements are in vitro data, the clinical treatment of infections related to biofilms are even worse, requiring dosage of antibiotics 1000 times or more than that for planktonic bacteria.[34,187,225]

In contrast, for FTC the activities against planktonic bacteria (MIC: 4-12 μg/ml) and activities biofilms (MBEC: 48 μg/ml) are not so many magnitudes different. This sharp contrast between FTC and all conventional antibiotics is interesting and likely therapeutically valuable. We believe the reason for the activity of FTC is rooted in the fact that FTC inhibit biofilm formation, and disperse already formed biofilms, whereas all conventional antibiotics promote biofilm formation.

The mechanisms for FTC against biofilm and killing bacteria will be studied in the following sections with following sequence. First, we will explore FTC's effect at causing, in fact not causing, drug tolerance and drug resistance; in comparison with other conventional antibiotics that are all known to rapidly cause drug tolerance, and over repeated exposure, drug resistance. Second, we will identify the protein receptors for FTC that are related to bacterial signaling that inhibit the formation of biofilms and the emergence of drug tolerance, as well as the spread of drug resistance.

Table 4.6 Structural Activity correlation between different molecules.

	Bacteriostatic	**Biofilm inhibition**	**Swarming inhibition**
FTC	Yes	Yes	Yes
SFTC	Yes	Yes	Yes
FOC	No	-- not done	-- not done
C_{12}TC	Yes	Yes	--not done
FTEG4OH	No	No	Yes
3,5,5-triMeHTC	No	No	No ??

C_{12}TC showed mild antibacterial activity against wild type *P. aeruginosa* with MIC of 32 μg/ml. The MBC for C_{12}TC was 256 μg/ml. According to CLSI, antibacterials with MBC/MIC >

4 are defined as bacteriostatic, hence C_{12}TC is bacteriostatic. Though C_{12}TC showed biofilm inhibition with MBEC = 128 µg/ml, C_{12}TC could not eradicate 24-h PAO1 biofilms.

Summary

We found that MIC value for FTC against *P. aeruginosa* against planktonic culture is higher than antibiotics used to treat pseudomonal infections. But FTC has excellent activity against *P. aeruginosa* biofilms. When existing antibiotics fail against (higher MBEC and BBC) biofilms, we found that FTC is better than existing antibiotics.

Clinical strain SMC1587 isolated from CF patients showed resistance to colistin which is last resort antibiotic for treatment of pseudomonal infections. FTC showed bactericidal activity against this clinical strain. In cystic fibrosis patients, antibiotic therapy includes both a beta-lactam (Aztreonam) and an aminoglycoside antibiotic (tobramycin, gentamicin). This combination therapy help prevent or delay the emergence of resistance which is often observed with single-drug therapy.[226] But it has been shown that clinical isolates of *P. aeruginosa* are resistant to aztreonam like SMC5451. [210]

FTC is active against aztreonam resistant strain of *P. aeruginosa* SMC5451. FTC can be used in combination with tobramycin against P. aeruginosa and can be better alternative to aztreonam.

Experimental section

4.2.7 MIC Determination by Broth Microdilution Method

On a sterile 96-well plate, 100 µL of Cation-adjusted Mueller-Hinton broth (MHB-2) was introduced to 8 wells of 12 columns. The 100 µL of stock solution FTC (1.024 mg/ml) was introduced to column 11 to generate a concentration of 1/2 of the stock conc., then 100 µL from column 11 was introduced to column 10 to generate ¼ of stock conc., repeated pass of 100 µL

down the columns generated 1/8, 1/16, 1/32, 1/64, 1/128, 1/512, 1/1024, 1/2048, of the stock conc. For the 12^{th} column, 10 µL of colistin stock solution (10 mg/mL) was introduced to the eight wells as positive control. All bacteria including clinical strains, nonmucoid, mucoid were cultured overnight in MHB-2, 200 µL of the overnight culture was mixed 20 mL of MHB-2. Then, 100 µL of this mixture was introduced to all 12 columns. For 12 column, 10 µL*10 µg/uL *(1/210) = 10/21 µg/uL = ~0.5 mg/mL colistin (MIC =1 µg/mL). For 11^{th} to 2^{nd} column, ¼, 1/8, 1/16, 1/32, 1/64, 1/128, 1/512, 1/1024, 1/2048, 1/4096 of the stock conc (1024 µg/mL) of FTC, corresponding to concentrations (µg/mL) of 256, 128,64, 32, 16, 8, 4,2, 1, 0.5, respectively. The 1^{st} column contains no agents or antibiotics and serves as negative (growth) control. The plate was incubated at 37^{o}C without shaking for 24 hours. The MIC was determined by the minimum concentration for which there was no visible bacterial growth.

4.2.8 Antibiotic susceptibility by Disc diffusion test

A suspension of the desired P. aeruginosa strains following overnight growth was made to equal a 0.5 MacFarland Standard, or approximately 10^8 CFU/ml. Using a sterile swab, a lawn of the inoculum was made on Mueller Hinton agar by covering the entire surface of the plate. The Mueller Hinton agar was stamped with antibiotic discs within 15 minutes of the inoculated preparation and incubated in a 37^{o}C incubator for 24 hours. Using calipers, the diameters of the zone of clearing were measured and the zone sizes recorded and interpreted according to CLSI procedures.

4.3 Inhibition of tolerant and persistent phenotypes by Farnesol Triazole Cellobioside

Dr. Alexander Fleming, who discover the first antibiotic penicillin in 1928, described rather clearly in his Nobel lecture in 1945 on development of drug resistance by microbes: *"It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."*

This development of antibiotic resistance by bacteria has become increasingly more severe over time since their discoveries, as predicted by Dr. Fleming. Since then, three different phenotypes that all seem to be resistant are better defined, drug tolerant bacteria require a high dose or longer drug treatment time to kill; drug persistent bacteria are not killed during drug treatment periods but revitalize to be a population mixture of majorly susceptible bacteria and a small percentage of persistent bacteria; and drug resistant bacteria grow slowly in the presence of antibiotic.[192,227-229] In addition, biofilms are discovered to be a major lifestyle besides planktonic form.[192,225] In general, biofilms host bacteria with low metabolic rates, and are the host all three types of resilient phenotypes, drug tolerant, persistent, and resistant population.[40,230,231] We not that there always exist a small percentage of persisters in a bacterial population, which does not get killed and does not grow during drug treatments and revitalize to become a population of mainly susceptible and with a small percentage of persisters when drug treatment is removed.[227]

At sub-lethal concentration (such as <MIC in lab), almost all commercially used antibiotics actually promote biofilm formation.[30,231] Treatment of a biofilms with antibiotics

causes increase in drug tolerant population in minutes and increase in persistent population in perhaps hours to days. There are two types of drug resistant population of a strain of bacterium, the acquired drug resistance and intrinsic drug resistance.[21,186,190,232] Natural resistance may be intrinsic (always expressed in the species), or induced (the genes are naturally occurring in the bacteria, but are only expressed to resistance levels after exposure to an antibiotic).[190] Acquisition of genetic material that confers resistance is possible through all of the main routes by which bacteria acquire any genetic material: transformation, transposition, and conjugation (all termed horizontal gene transfer—HGT); plus, the bacteria may experience mutations to its own chromosomal DNA. The acquisition may be temporary or permanent.[186,232] Intrinsic resistance may be defined as a trait that is shared universally within a bacterial species, is independent of previous antibiotic exposure, and not related to horizontal gene transfer.[186,232] We will address resistance in the next section.

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP, cdG) plays an important role in biofilm formation and motility of gram-negative bacteria.[233-236] Bacterial intracellular cdG levels controlled by diguanylate cyclases (DGCs) that catalyze the formation of cdG and phosphodiesterases (PDEs), which degrade c-di-GMP.[234] When intracellular cdG levels are high, bacteria halt motility and switch to biofilm formation. In contrast, biofilm cells when needed increase their motility and disperse from biofilms by reducing cdG levels.[234,236] C-di-GMP signaling can be triggered by stress conditions immune attack or antimicrobial exposure.[236] The impact of cdG on mediating stress response by microbial communities during both planktonic and biofilm modes of growth remains unclear. Elevated levels of cdG contribute to antimicrobial tolerance in *Pseudomonas aeruginosa*.[237] It is well known that higher cdG contribute to antibiotic tolerance in P. aeruginosa by enhancing biofilm formation, high cdG can

also leads to biofilm independent increased antibiotic tolerance.[238] The reasons for the biofilm tolerance are multiple, including slow growth or the presence of an extracellular matrix.[239]

Over the years, different anti-biofilm strategies are employed as means to eradicate biofilms and hence biofilm-related antibiotic tolerance. [228,230,240-244] 2-Aminoimidazole/triazole-conjugated compounds can induce the dispersal of bacterial biofilms.[243,244] 2-Aminoimidazole/triazole showed to inhibit bacterial signaling (quorum sensing).[244] Most of the anti-biofilm compounds act as anti-virulence agents and has no effect on bacterial growth. Antimicrobial peptides (AMPs), specifically lytic peptides are used against bacterial biofilms.[242] Combinatorial therapy of anti-biofilm agents with antibiotics look promising but suffer potential rise of motile lifestyle for bacteria. Motility especially swarming motility leads to enhanced antibiotic tolerance in *P. aeruginosa*.[245-247] Swarming bacteria move in multicellular groups and exhibit adaptive tolerance to multiple antibiotics.[246] Swarming bacteria also have increased virulence which can add to antibiotic tolerance.[245] To counter swarming motility, researchers have developed swarming inhibitor compounds but detailed mechanisms underlying swarming inhibition remain unknown. *P. aeruginosa* swarming motility is inhibited by several compounds, including branched-chain fatty acid anteiso-$C_{15:0}$, cranberry proanthocyanidins and other tannins, and several amino acids, such as arginine.[248-250]

Another important resilient phenotype, small colony variants phenotype (SCV) on semi-solid surfaces are linked to elevated cdG levels.[27,35,37,251] SCVs are hyperpiliated, showed increased twitching motility and capacity for biofilm formation. SCVs were found to be multi-drug tolerant.[37]

Tolerance unlike resistance, that's a growth of the drug concentration for bacteria can grow, tolerance is an extension of the time frame that bacteria can live under high concentrations of an antibiotic (regularly way greater than MIC).

We divide tolerance into two major types: biofilm dependent tolerance and biofilm independent tolerance. Biofilm dependent tolerance can be easily evaluated as reduction in biofilms and biofilm related phenotypes. Although research into defining planktonic tolerance is ongoing, direct measurement techniques are also lacking and limited. There are numerous mechanisms probably underlying planktonic tolerance recently for example, tolerance to β-lactams can be done via way of means of a reduced cell wall synthesis, and tolerance to fluoroquinolone consequences from a reduced DNA replication. The standard method used to evaluate tolerance is by performing time-kill measurements. In this method, bacteria are exposed to a higher antibiotic concentration, and viable counts are plotted against time. Recently, Balaban group have termed this parameter the minimum duration for killing a certain percentile of the population (MDK), with the percentile added as an index—thus, MDK_{99} is the minimum duration for killing 99% of the population. A comparatively high MDK suggests that bacterial killing requires more time, i.e., corresponds to high tolerance.

4.3.1 Inhibition and dispersion of P. aeruginosa biofilms.

Biofilms are strongly correlated to antibiotic tolerance.[34,252] *P. aeruginosa* forms biofilms which consists of three main polysaccharides*:* Psl, pel and alginates.[31,32] The Pel plays protective role in the biofilm matrix of *Pseudomonas aeruginosa* and pel overproduction leads aminoglycoside antibiotic tolerance.[31,32] Alginate production leads mucoid biofilms which exhibit antibiotic tolerance. *P. aeruginosa* biofilms harbor tolerant and persister cells which can survive transient

antibiotic treatments and revive after in the absence of antibiotic treatment.[31,32] Interestingly, sub-lethal antibiotic causes biofilm formation in *P. aeruginosa.*[33,34] Antibiotics can have two opposing effect on biofilms: enhancing biofilm formation or disrupting existing biofilms.[33,34]

We first tested effect of different concentrations of FTC on biofilm formation of *P. aeruginosa* wild type PAO1 and PA14. We found that FTC inhibits biofilm formation in PAO1 and PA14 with half maximal inhibition (IC_{50}) of 0.5 µg/ml and 0.8 µg/ml, respectively. At 1µg/ml, FTC completely (>95% inhibition) inhibits biofilm formation in both PAO1 and PA14. We then evaluated effect of sub-MIC of FTC on dispersion of 24-h old biofilms. We found that FTC was capable of dispersing 24-h old PAO1 and PA14 biofilms with half maximal dispersion (DC_{50}) values of 1 µg/ml and 1.5 µg/ml, respectively (Table 4.7).

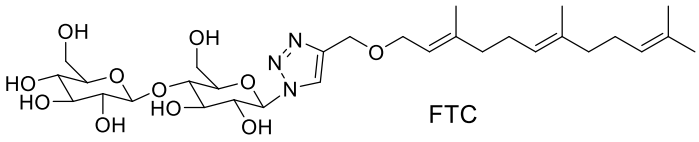


Table 4.7 Inhibition and dispersion of *P. aeruginosa* biofilms by farnesol triazole cellobioside (FTC).

Strain	**Biofilm Inhibition (IC_{50}) µg/ml**	**Biofilm Dispersion (DC_{50}) µg/ml**
PAO1	0.5	1
PA14	0.8	1.5

We compared biofilm inhibition and dispersion efficacy to previous generation anti-biofilm adjuvants SFβM/ βC and 3,5-diMeDβM/βC. SFβM/βC showed biofilm inhibition with IC_{50} = 15 µg/ml and biofilm dispersion with DC_{50} = 54 µg/ml. The 3,5-diMeDβM/βC showed better anti-

biofilm activity than SFβM/βC. 3,5-diMeDβM/βC showed biofilm inhibition with IC_{50} = 9 µg/ml and biofilm dispersion with DC_{50} = 32 µg/ml. We believe antibacterial effect of FTC synergistically increases biofilm inhibition and biofilm dispersion.

4.3.2 Inhibition of the formation of small colony variants.

P. aeruginosa forms small colony variants (SCVs) as adaptive response to antibiotic treatments.[35-37] SCV are a phenotypic subset of the bacterial population surviving in biofilms.[37] SCVs are persistent phenotype, which is dormant, hyper adherent, exhibit enhanced biofilm formation and slow, auto-aggregative growth.[35-37] Antibiotics like aminoglycosides leads SCVs formation.

We tested effect of sub-lethal concentrations of FTC on small colony formations in *P. aeruginosa.* We grew 24-h PAO1 biofilms in the presence of sub-MIC of FTC and cultivated, visualized biofilms bacteria on Columbia blood agar. We found that sub-MIC concentrations of FTC does not promote SCVs formations in PAO1 biofilms. We previously showed that tobramycin induced SCVs in P. aeruginosa. Here, we tested effect of combination of tobramycin and FTC on SCVs formation in biofilms. We also tested effect of aztreonam, well known combination antibiotic used with tobramycin in cystic fibrosis on SCVs formation in biofilms. We found that sub-MIC of tobramycin induces SCVs in *P. aeruginosa* biofilms. Aztreonam does not prevent induction of SCVs by sub-MIC of tobramycin. Tobramycin -FTC combination prevented formation of SCVs in 24-h biofilms (Fig. 4.7).

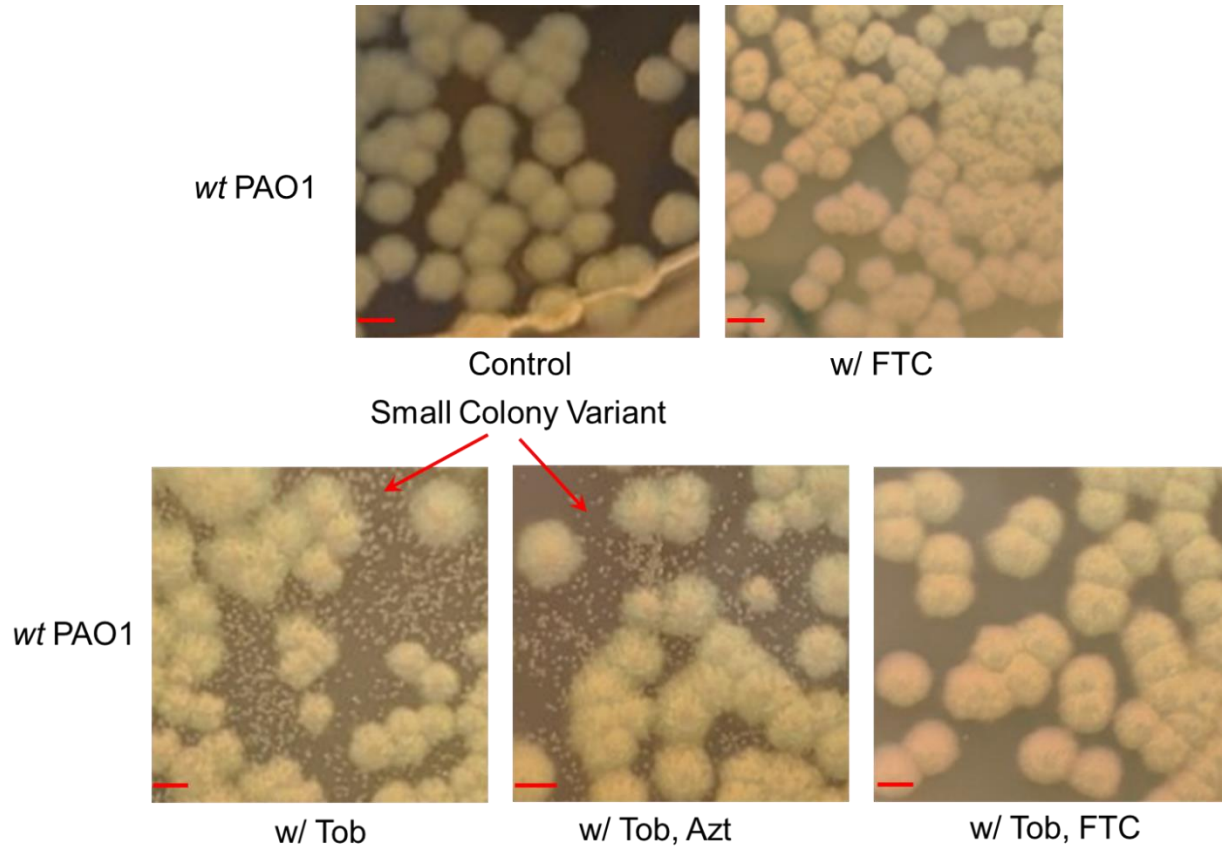


Figure 4.7 Colony morphology assay. Images of biofilm bacterial colonies (37 ℃, 24 h) on MHB agar plates. Scale bar = 2 cm. *wt* PAO1 was treated with sub-MIC concentrations of FTC or Tobramycin or aztreonam or combination and inoculated in MBEC peg plates to form biofilms for 24 h and then biofilm bacteria were obtained and plated on MHB agar plates for 24 h. TOB- tobramycin, AZT-aztreonam, FTC-farnesol triazole cellobioside; For assay concentrations of antibiotics used are, 0.3 ×MIC for TOB, 0.5 × MIC for AZT, 0.5 × MIC for FTC.

4.3.3 Tolerance Detection test (TDtest).

We used a simple modification of the standard Kirby-Bauer disk-diffusion assay (**T**olerance **D**etection/TDtest) which allows the semi-quantitative evaluation of tolerance to

antibiotic.[253-256] TDtest enables the detection of tolerant and persistent bacteria by promoting the growth of the surviving bacteria in the inhibition zone once the antibiotic has diffused away. Using the TDtest, we can detect different levels of antibiotic tolerance in different bacteria. In typical Kirby–Bauer disk diffusion method, gradient of antibiotic prevents the growth of bacteria creating an "inhibition zone" around the disk. The TDtest enables the detection of tolerant strains by overcoming the nutrient depletion that occurs in the Kirby–Bauer disk diffusion method. The surviving bacteria in the inhibition zone are then recovered by replacing the antibiotic disk with a new disk impregnated with nutrients like glucose. As nutrients diffuse away from the new disk, they now promote the growth of tolerant bacteria that can form detectable colonies in the inhibition zone. We found that aztreonam showed tolerant bacteria while FTC had almost no tolerant bacteria in zone of inhibition. Furthermore, a qualitative evaluation of tolerance can be done by the number of colonies inside a typical inhibition zone. Makarova et.al has used TD test to identify cross tolerance of *Salmonella enterica* to herbicide.[256] Kotkova et.al has successfully used TD test to detect persistent or tolerant bacterial cells in clinical isolates of *Staphylococcus aureus.*[255]

We found that wild type PAO1 showed medium tolerance to aztreonam (medium tolerance: 10 to 100 colonies in inhibition zone) while low or no tolerance to FTC (Fig. 4.8). We also tested if colonies grown inside zone of inhibition are all tolerant or mixture of tolerant and resistant. We picked single colony from inside zone of inhibition and regrew in fresh medium in the presence of antibiotic. We found that none of the colonies survive antibiotic treatment indicating that all those colonies were tolerant (Fig. 4.9).

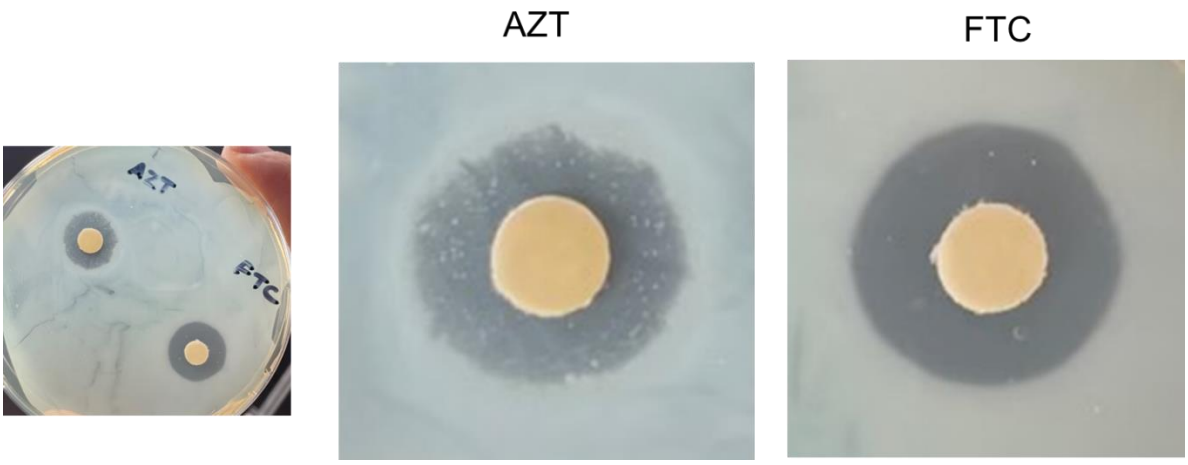


Figure 4.8 TDtest for direct visualization of tolerance/persistence strains of wild type PAO1. FTC showed low tolerance while Aztreonam showed high tolerance/persistence. We first exposed *wt* type PAO1 to antibiotic (8×MIC for AZT and 10×MIC for FTC) for 24 h and then followed by 24 h exposure to glucose disk. Colonies inside the inhibition zone after the second step (please see experimental section) of the TDtest shows tolerant/persistent strains. Disk diameter: 6 mm.

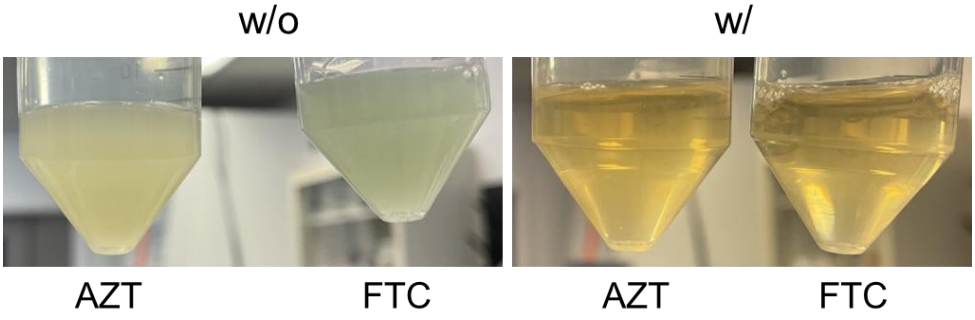


Figure 4.9 Bacterial growth assay. The large majority of colonies growing inside the inhibition zone after the second step of TDtest are not resistant. The single colony inside zone of inhibition was regrew with and without antibiotic AZT (8×MIC) and FTC (10×MIC) for 24 h to observe visible bacterial growth.

4.3.4 FTC causes low or no antibiotic-tolerance in planktonic culture.

In this method, bacteria are cultured with a higher concentration (higher than MIC, 10-15×MIC) of antibiotic, and number of viable bacteria (CFU/ml) are plotted against time. When the killing curve is exponential (not biphasic) the killing rate can be used to quantify tolerance. Balaban group used parameter the minimum duration for killing (MDK) a certain % (e.g. 99%) of the population; MDK99.9 is the minimum duration for killing 99.9% of the population. A comparatively high MDK suggests that bacterial killing requires more time, i.e., corresponds to high tolerance[257,258].

Planktonic *P. aeruginosa*, *wt* PAO1, was cultured with and without higher concentrations of aztreonam or FTC for 8 h. The concentration of aztreonam was 45 µg/ml (15xMIC), and that of FTC was 80 µg/ml (10xMIC); at these concentrations, the frequency of mutants was less than 10^{-11}.. The residual live bacteria were isolated from the entire culture volume by centrifugation and rinse with fresh LB medium to remove the antibiotics. The bacteria were then cultured overnight again to obtain an ancestral (without antibiotic) and evolved (with antibiotic) bacterial population.

We first validated if these ancestral and evolved strains are not resistant strains because resistance is described as a shift in the MIC to higher drug concentration. We determined MIC of aztreonam and FTC against ancestral and evolved strains by disk diffusion test. We found that under aztreonam treatment, evolved strain showed same zone of inhibition (11±1.5 mm) as ancestral strain (12±0.5 mm). For FTC, evolved strain showed same zone of inhibition (9±1 mm) as ancestral strain (9±0.5 mm) (Fig. 4.10).

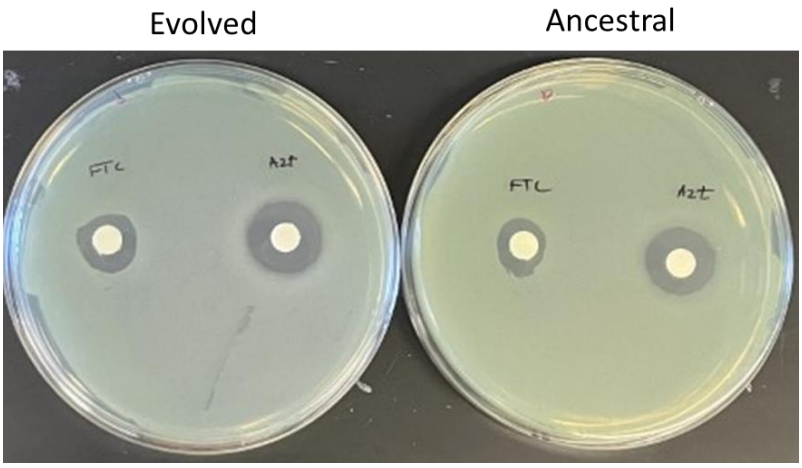


Figure 4.10 Kirby-Bauer disk diffusion susceptibility test on ancestral and evolved wild type PAO1 grown on Mueller-Hinton agar plates with 4 µg/ml Aztreonam and FTC. The zones of clearing (zone of inhibition) are measured to determine susceptibility to aztreonam and FTC.

The ancestral, and AZT/FTC-evolved population (10^6 CFU/ml) were cultured again with FTC (10xMIC) and AZT (15xMIC); and the survival population were monitored (by colony forming units) over 0,3,6, 8, 12, 24 h; and the MDK_{99} was obtained (Fig. 4.11).

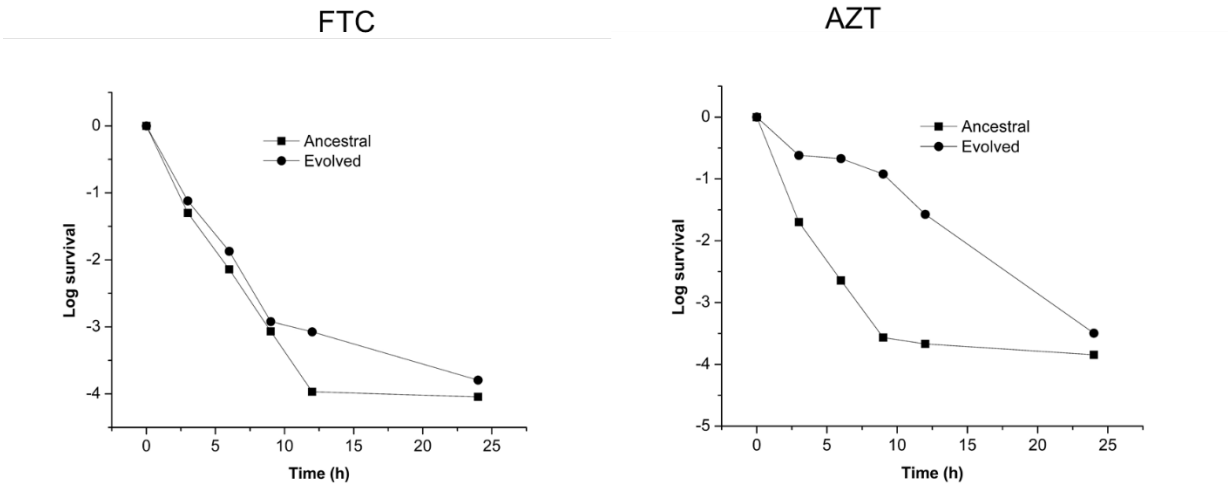


Figure 4.11 Time-kill curves under FTC and aztreonam treatment of two population ancestral and evolved with different tolerance levels. The tolerant population (evolved) was obtained from the ancestral strain under intermittent antibiotic exposure (8h). The ancestral, and AZT/FTC-evolved population (10^6 CFU/ml) were cultured again with FTC (80 µg/ml) and AZT (45

μg/ml); and the survival population were monitored (by colony forming units) over time. From killing curve, tolerance is reflected in the terms of MDK_{99}, i.e., the time to kill 99% of the population, or Survival = 10^{-2}.

Table 4.8 The minimum duration for killing (MDK_{99}) of ancestral and evolved population of wild type PAO1 under the treatment of FTC and aztreonam.[a]

Antibiotic	**Ancestral**	**Evolved**
FTC[b]	~6 h	~6 h
Aztreonam[c]	~5 h	~15 h

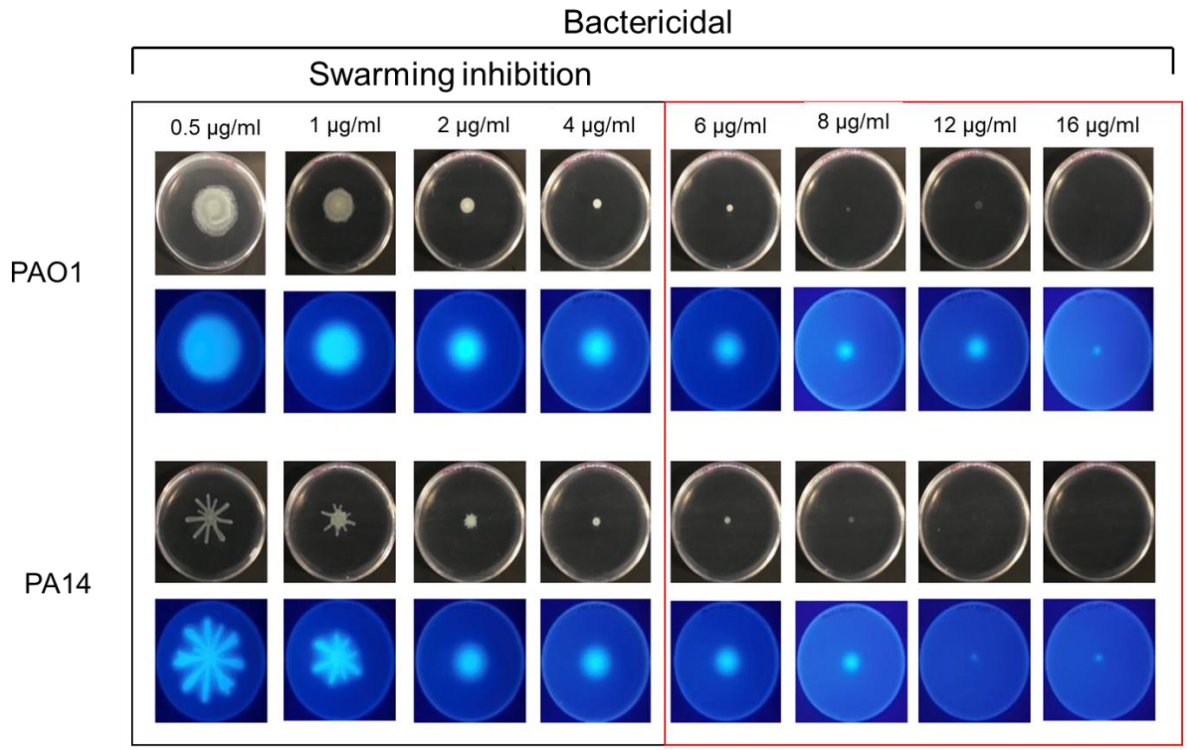
[a]8 h culture with high concentrations of antibiotics. [b]FTC conc. 80 μg/ml (10xMIC). [c]Aztreonam conc. 45 μg/ml (15xMIC).

We found that MDK_{99} for ancestral and evolved population of PAO1 after FTC treatments were both about 6 h (Table 4.8). We found that MDK_{99} for ancestral and evolved PAO1 after FTC treatments were 6 h. The similar MDK_{99}s between the ancestral and evolved PAO1 suggest that high concentrations of antibiotic FTC (10xMIC) did not cause a tolerant phenotypes after an 8 h treatment of FTC. For aztreonam, one exposure cycle caused an increase in MDK_{99} from 5 h to 15 h, suggesting that the tolerant bacteria population has evolved.

4.3.5 Inhibition swarming motility while killing P. aeruginosa.

Bacteria exhibit activities with complex high cell density in swarming motility, which is regarded to protect bacteria against antibiotic exposure.[30,245-247,259] Swarming bacteria are believed to be more tolerant to antibiotics.[246,259] Interestingly, antibiotic of aminoglycoside (tobramycin) also promotes the swarming motility in *P. aeruginosa*.[30]

We evaluated effect of FTC on swarming motility of wild type *P. aeruginosa* strains PAO1 and PA14. We found that FTC inhibits swarming of both PAO1 and PA14 below minimum inhibitory concentration (12 µg/ml). We found that concentrations range (0.5 – 4 µg/ml) FTC could inhibit swarming motility in PAO1 and PA14 without affecting viability (clearly visible under UV light). At concentrations higher than 4 µg/ml, FTC cause both swarming inhibition and killing in PAO1 and PA14



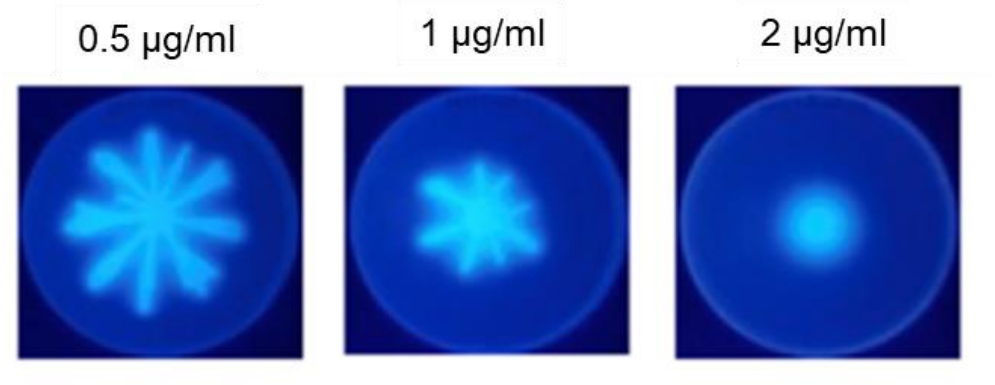


Figure 4.12 (A) FTC inhibits swarming motility in *P. aeruginosa* strains. Swarming motility assay; The representative images of swarming motilities *wt* PAO1 and PA14 on semisolid gel (~0.5% agar) with different concentrations of FTC after 24 h. Images were with and without 365/254nm UV irradiation. The concentrations are indicated over the images. (B) Detail examination of the swarming pattern of PA14 in the presence of FTC (0.5, 1.0, 2.0 ug/ml).

Because FTC kills bacteria, whether FTC also inhibits swarming motility need a close examination. We studied effect of different concentrations of FTC prepared in the hydrated gel for swarming motility for PAO1 and PA14 (Fig. 4.12). At higher concentrations, 6-16 ug/ml the inoculation spot of the bacteria fade out, indicating that the bacteria was killed. For low concentrations, 0.5-4 ug/ml, the area of swarming bacteria reduces, which can be attributed to either killing of the bacteria or inhibiting the swarming motility of the bacteria, or both. Close examination of swarming patterns of PA14 suggest that the tendrils of the swarming pattern reduced more than the area in the center. This result suggests that swarming motility is likely also inhibited in addition to killing of bacteria. If only killing is occurring, then the swarming tendril pattern likely will be maintained, and both the tendril and the center of the swarming pattern should fade to a similar extend (Fig. 4.12).

To validate FTC does inhibit swarming at low concentrations, we evaluated effect of $FTEG_4OH$ on swarming motility of wild type PAO1. $FTEG_4OH$ is structural variant of FTC which has no bactericidal activity. $FTEG_4OH$ and FTC both share structural features needed for pili inhibition.

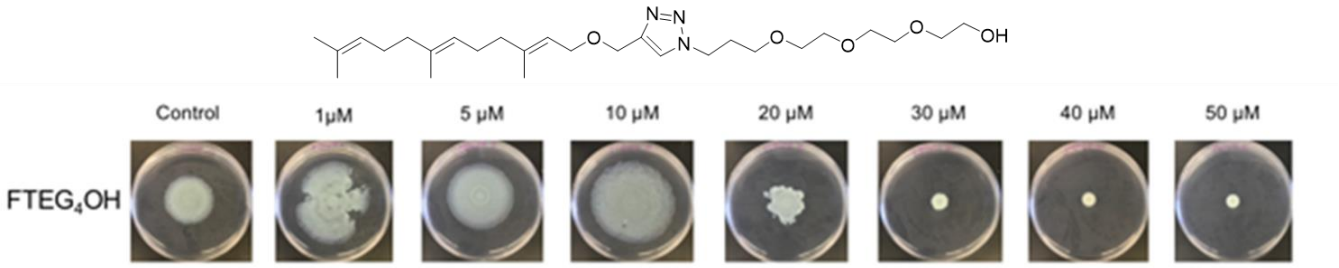


Figure 4.13 Farnesol triazole tetraethylene glycol ($FTEG_4OH$) inhibits swarming motility in *P. aeruginosa*. Swarming motility assay; The representative images of swarming motilities *wt* PAO1 on semisolid gel (~0.5% agar) with different concentrations of $FTEG_4OH$ after 24 h. The concentrations are indicated over the images.

We found that FTEG4OH inhibits swarming motility of wild type PAO1 at concentrations higher than 30 μM (Fig. 4.13).

Discussion

These results suggest that FTC inhibits biofilm formation in *P. aeruginosa*. Previously, we showed that sub-lethal concentrations of antibiotics promote biofilm formations but sub-lethal concentrations of FTC inhibits biofilms in *P. aeruginosa*. FTC does not induce small colony variants in biofilms unlike other antibiotics like tobramycin, gentamicin. Collectively, we showed that FTC inhibit biofilm dependent tolerance in *P. aeruginosa*.

FTC and $FTEG_4OH$ inhibit swarming motility in *P. aeruginosa*. Antibiotics have been shown to promote swarming motility in *P. aeruginosa*[30] and swarming bacteria are more tolerant to

antibiotics[245-247]. FTEG4OH and FTC both have C_{12} branched hydrocarbon as structural moiety. These results are consistent with previous finding that branched chain hydrocarbons are important for swarming inhibition.[81,199,249] We believe that branched chain hydrocarbon inhibit pili, appendage needed for swarming motility and hence inhibit swarming motility in *P. aeruginosa*. The branched chain hydrocarbon structural feature of FTC gives antibiotic advantage at inhibiting swarming motility.

Summary

We showed that FTC inhibits swarming motility and biofilm formation in PAO1 and PA14. FTC does not promote swarming motility or biofilm formation unlike other antibiotics. We believe that FTC eradicates swarming and biofilm-induced antibiotic tolerance. FTC also does not induce SCVs formation in *P. aeruginosa*. FTC inhibits tobramycin induced SCV formation in *P. aeruginosa*. The mechanism of inhibition is still not clear. Inhibition of biofilm, swarming, and SCV are inhibiting tolerance, and inhibition of swarming is likely due to inhibition of pili, which we will elaborate in the receptor identification section.

Experimental section

Freezer stocks of all strains were stored at -80 ^{o}C in lysogeny broth (LB) with ~20% glycerol. All strains were grown in LB (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl) or Mueller-Hinton Broth MHB (beef infusion solids, 2.0 g/l. casein hydrolysate, 17.5 g/l. starch, 1.5 g/l) at 37 ^{o}C with shaking at 250 rpm. All biofilm assays were performed by immersing the pegs of a modified polystyrene microtiter lid of MBEC™ plates (Innovotech, Alberta, Canada) in M63 medium. All biofilm inhibition, dispersion assays were performed in M69 medium, and MBEC peg plates were incubated at 37 ^{o}C under static conditions.

4.3.6 Crystal violet dye-based biofilm assay[260]

An overnight culture (100 µL) of bacteria in LB was diluted in 10 mL of M63 medium and incubated to reach an OD_{600} value of ~0.1. The bacterial culture (150 µL) was added to the wells of MBEC™ microtiter plate, followed by predetermined volumes of FTC stock solution for targeted concentrations (6 wells/concentration). The MBEC™ plates were incubated under stationary conditions at 37 ℃ for 24 h. After incubation, the pegs were transferred and immersed into 96 wells containing sterile water (200 µL) twice to briefly rinse the biofilms to remove unattached or loosely attached bacteria and were dried at 37 ℃ for 30 min. The peg-attached biofilms were immersed into 96 wells containing crystal violet (CV) dye solution (150 µL, 0.1%) at ambient temperature for 30 min, to stain the biofilms. The CV-stained pegs were then washed twice with sterile water (200 µL). To solubilize the CR stains, 150 µL of 30% acetic acid solution was added to wells, and the pegs was immersed in the wells, the plates were shake on a microplate mixer (Scilogex MX-M) at 100 rpm for 15 min. The amount of biofilms was inferred and quantified by measuring the OD_{600} of the 150 µL acetic acid solution on plate reader. The absorption from stained pegs containing just M63 medium was subtracted from pegs treated with agents. For dispersion assay, biofilms were grown for 24 hours and then treated with different concentrations of FTC for 24 hours. The amount of biofilm mass was determined by same CV dye method described previously.

4.3.7 Disk diffusion test[261,262]

The 100 µL bacterial strain of interest (0.6 OD, ~10^6–10^7 CFU/ml) was spread onto a Mueller–Hinton agar plate. The antibiotic disks (Whatman® Antibiotic Assay Discs , diam 6 mm, Sigma Aldrich) were used. The 5 µL of antibiotics were dropped on disks and incubated for 24 h at

37°C. After 24-h incubation, zone of inhibition was measured and efficacy of antibiotic was expressed as diameter of zone of inhibition.

4.3.8 TDtest for visualization of tolerance/persistence[253]

The TDtest consists of two steps: Step I: similar to reference disk diffusion assay, with the following exceptions: 100 µL of *wt* PAO1 bacteria (0.6 OD, ~10^6–10^7 CFU/ml) were plated on LB agar plate, with diffusion disks containing 5 µL of AZT and FTC with concentrations 24 µg/ml (8×MIC) and 80 µg/ml (10×MIC), respectively. The disk/agar was incubated for 24 h at 37°C. The amount of antibiotics in the disks was adjusted low enough (still multiple of MICs) so that the glucose diffusion ring will cover the abx/agents ring, and not generate spontaneous mutation. Multiple trials indicate that the optimal amount of antibiotic for this TDtest is 24 µg/ml (8×MIC) for AZT and 80 µg/ml (10×MIC) for FTC.

Step II: The antibiotic disks were replaced with a fresh blank disk, and applied 5 µl of 40% sterile glucose solution, and were incubated for an additional 24 h. The tolerant strains can grow in the inhibition zoom in this experiment because glucose gradients is introduced to replace the 24-h old antibiotics disks.

4.3.9 Planktonic tolerance assay[258]

The overnight cultures of *wt* PAO1 was diluted to OD=0.01 in MHB medium and then was cultured to OD=0.6. We treated *wt* PAO1 (10 mL) with a high concentration of FTC (80µg/ml;10×MIC) and aztreonam (45µg/ml;15×MIC). These two values 10xMIC and 15xMIC for FTC and AZT, respectively, are determined by the single step resistance assay, for which the frequency of mutants that were resistant to these concentrations of antibiotics was less than 10^{-11}. After 8 h of exposure to these antibiotics, the cultures were washed to remove the drug,

resuspended in fresh MHB medium, and grown for total 24 h. After 24 h, we got four populations, two for each of ancestral and evolved by FTC/AZT. We measured MDK_{99} (the minimum duration for killing 99% of cells) may be defined to quantify tolerance. (See procedure below). We will also find out susceptibility of these strains to AZT and FTC by disk diffusion assay to show validate these strains are not resistant.

4.3.10 MDK_{99} assay[258]

We cultured *wt* PAO1 ancestral and evolved strains in MHB medium and adjusted to ~10^6 CFU/ml. We supplemented 10^6 CFU/ml bacterial cultures with high concentrations of FTC (80µg/ml ,10×MIC) and aztreonam (45µg/ml, 15×MIC) for 24 h. The bacterial viability was determined by colony forming units on LB agar plate every 3, 6, 9, 12 and 24 hours. We plotted % survival bacterial population vs time and calculate MDK_{99} as the time to kill 99% of the population, or survival = 10^{-2}.

4.3.11 Bacterial Swarming assay[263]

The soft gels for swarming motility were prepared by autoclaving 0.5 wt% Bacto Agar in M8 medium (0.6 % Na_2HPO_4, 0.3 % KH_2PO4 and 0.05 % NaCl). The gel solution was cooled to ~60 ºC, supplemented with filtered 0.2 % glucose, 0.5 % casamino acid, and 1 mM $MgSO_4$ (0.22 µ filter). The gel solutions were poured into a Falcon tube for 20 mL portions, followed by adding aliquots of (1-20 µL) of FTC, $FTEG_4OH$ stock solutions to achieve the desired concentrations. The falcon tubes were closed, the agar solution was mixed by gently rocking, and then poured into polystyrene petri dishes (10 cm-diameter). The agar solution was solidified by cooling and air-drying in a laminar hood for 1 h. Bacterial culture of *wt* PAO1/PA14 (3 µL) with an OD_{600} between ~0.4–0.6 was inoculated on the center of the surface of the soft agar gel. These “swarm

plates" were incubated at 37 ºC for 12 h and then incubated for additional 12 h at room temperature. After a total of 24 h, pictures of the swarming plates were taken.

4.3.12 Examination of SCV formation and inhibition[251]

An overnight culture (100 µL) of *wt* PAO1 bacteria in LB was diluted in 10 mL of M63 medium, and sub-cultured to reach an OD_{600} value of ~0.1. The sub-culture (150 µL) was added to the wells of MBEC™ microtiter peg plate and supplemented with (and without) tobramycin (0.3 ug/ml), and aztreonam (0.5 ug/ml) or FTC (0.5 ug/ml). Each condition is duplicated in 6 wells and incubated under at 37 ℃ without shaking for 24 h. The pegs were then transferred and immersed into 96 wells containing sterile water (200 µL) twice to briefly rinse the biofilms to remove unattached or loosely attached bacteria. The 6 pegs were cut with sterile plier and put in saline in a test tube. The saline solution with pegs were bath sonicated 15 minutes at 30 kHz (Symphony VWR Internationals Ltd.). Pegs were removed, and the solutions were centrifuged at 6000 rpm for 10 minutes, to collect the bacteria. The collected pellets were resuspended in 1 mL saline, which were serially diluted (10^5-10^7) in LB medium, spread on Mueller Hinton Agar plates, and incubated at 37℃ for 1 day.

4.4 Inhibition of drug resistance development

Antibiotics disrupt the essential structures or processes in bacteria, which either kills the bacteria or stops them from multiplying.[185-188,225,264-266] In response, bacteria have evolved to develop many types of resistance to withstand the actions of antibiotics.[185-188,225,264-266] Generally, the major mechanisms of bacteria used to counter antibiotic attack can be classified into intrinsic, acquired and adaptive resistance. The intrinsic resistance of bacteria includes low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic inactivating enzymes. The acquired resistance of *P. aeruginosa* can be achieved by either horizontal transfer of resistance genes or mutational changes.

The primary mechanism for drug resistance development is spontaneous mutation, which is random and rare. Under stress of a drug treatment, however, the natural selection of cause the selected mutant that survived the mutation to multiply and populate. Such a natural selection is not random. There are ways by which the drug resistant mutants pass on their genes. First, by generational gene transfer that the resistant gene is passed on to the daughter bacteria when the bacteria replicate and multiple and proliferate. Second, by horizontal gene transfer, for which the bacteria can release their genes to the environment and be picked up another; or the bacterium can directly make direct physical contact with another bacterium and transfer the genes to the other bacterium.

The gene mutations that resist the effects of an antibiotic cause one of the two effects;[188,264-266] one, to reduce antibiotic concentration by pumping the drug out or by chemically destroying or modifying the drugs; or two, to modify or bypass the drug target, which are often enzymes in bacteria, that the antibiotic acts on.[188,266] Bacteria produces protein pumps

that sit in their membrane or cell wall. These so-called efflux pumps are very common in bacteria and can transport a variety of compounds such as signal molecules and toxins. Gene mutation can cause bacteria produce more of a certain pump or make certain pumps more effective at pumping the antibiotics out of the bacterium lowering the antibiotic concentration inside the bacterial cell, resulting in drug resistance, for which the bacteria grow slowly in the presence of drugs.[188,264-266] Gene mutation can also decrease the permeability of the bacterial membrane. This change decreases the amount of antibiotics getting into the bacteria, resulted in drug resistance and growth. Gene mutation can also make enzymes that are more effective at destroying or modifying certain antibiotics, making the antibiotics not being able to bind to the protein targets. One example is β-lactamase that destroys the β-lactam ring of penicillin, the first and most used antibiotics for treating human infections. Over the years, bacteria evolve to produce many different β-lactamases. These bacteria form a class called extended spectrum beta-lactamases (ESBL)-producing bacteria, has become a major health issue. These bacteria can degrade a wide spectrum of β-lactam antibiotics, sometimes also the last resort drugs like aztreonam for infections with these bacteria.[185,187] Gene mutation can also change the composition or the structure of the drug target (often enzymes) in the bacterium. The modification render the antibiotics missing the target, and the biology and livelihood unchanged. Gene mutation can also cause the making of alternative proteins to replace the drug targets. For example, the bacterium *Staphylococcus aureus* can acquire the resistance gene *mecA* and produce a new penicillin-binding protein. The new penicillin-binding protein has low affinity to β-lactam antibiotics and is thus resistant to β-lactam treatment. This type of resistance is the basis for methicillin-resistant Staphylococcus aureus (MRSA). Gene mutation can also reprogram or modify the structures of the target molecules (nonprotein). For example,

Vancomycin-resistant bacteria make a different cell wall lipid structures compared to susceptible bacteria. The antibiotic is not able to interact as well with this type of cell wall[185,187].Some bacteria are naturally resistant to certain antibiotics. Imagine for example an antibiotic that destroys the cell wall of the bacteria. If a bacterium does not have a cell wall, the antibiotic will have no effect. This phenomenon is called intrinsic resistance. When a bacterium that was previously susceptible to an antibiotic evolves resistance, it is called acquired resistance[264,265].

Both serial passage assay and single step mutation assay are used to evaluate the "resistance" development by bacteria under antibiotic treatment in a laboratory settings. Often both assays are studied together[267,268].[269] Serial passage assay are believed to allow cumulation of multiple mutations, each with incremental survival benefit; and single step mutation assay causes drug resistance in a single step.[18,267,269]

Inhibition of acquired drug resistance, and mechanism:

4.4.1 Serial multi-passage assay:

Serial Passage: This assay develops resistance in bacteria by continuously passaging bacteria grown in sub-MIC (sub-inhibitory) concentrations of antimicrobial agent. For serial passage assay, the bacterial culture were treated with a range of doubling MIC concentrations of the wild type, such as 0.5x MIC, 1x MIC, 2x MIC,…,etc., for 12h. The lowest concentration that causes no visible bacterial growth are regarded at the new MIC. The culture with high concentration that shows visible bacterial growth is diluted and cultured with a new series of doubling concentration of the NEW MIC for the next passage. It is important to note that in between each passage, the bacteria are not culture without antibiotics. Thus, a series of questions are not

answered. Is the resistance mutation in the new "resistant" population stable? In other words, if the bacteria population is isolated and cultured again, is the entire population of the bacteria resistant to drugs, or some of new generation will be susceptible? More importantly, will some of the initial "resistant" population, cultured without antibiotic, revert back to susceptible? If some of the initial "resistant" population revert back to susceptible, then these bacteria are really tolerant rather than resistant. For the latter case, it is not clear if the increased MIC due to multiple passages will or will not revert back to a lower value if the bacteria are cultured again without antibiotics.

Illustration of serial passage assay was done in Fig.4.14 A parent culture is prepared and used to inoculate a serially diluted series of antimicrobial compound.

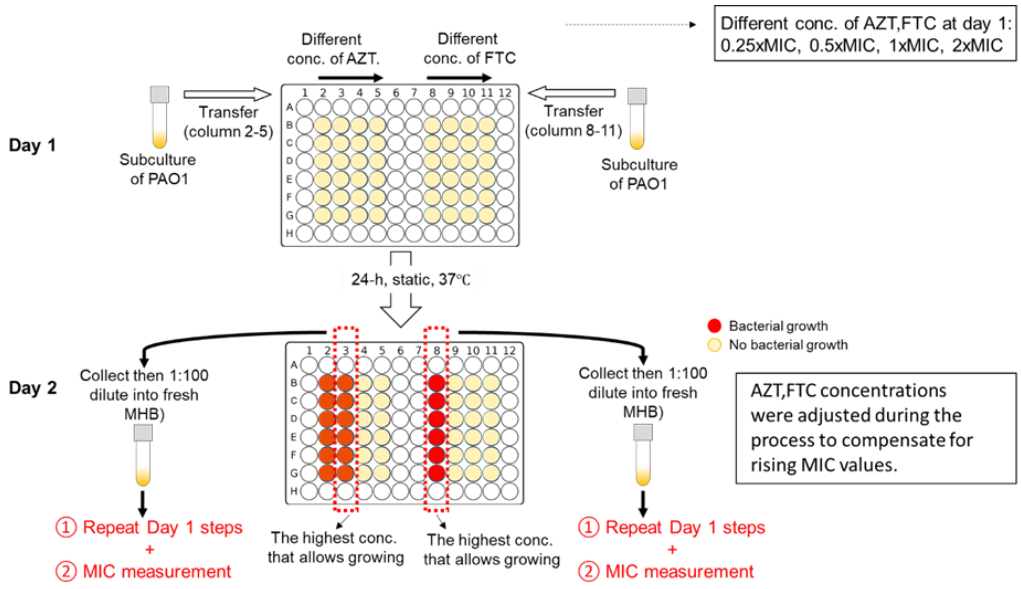


Figure 4.14 Schematic representation of multi-passage assay.

The culture is incubated and on day 1 the MIC concentration is determined to be the lowest concentration in which no growth is observed. The sub-MIC concentration culture is used to inoculate a second serially diluted series of antimicrobial compound and incubated overnight.

The next day the MIC (which may remain the same or increase) is determined and the sub-MIC concentration culture from the latest passage is used to inoculate a new series of diluted antimicrobial compound. The process continues for up to 21 days or until a predetermined end point concentration is reached.

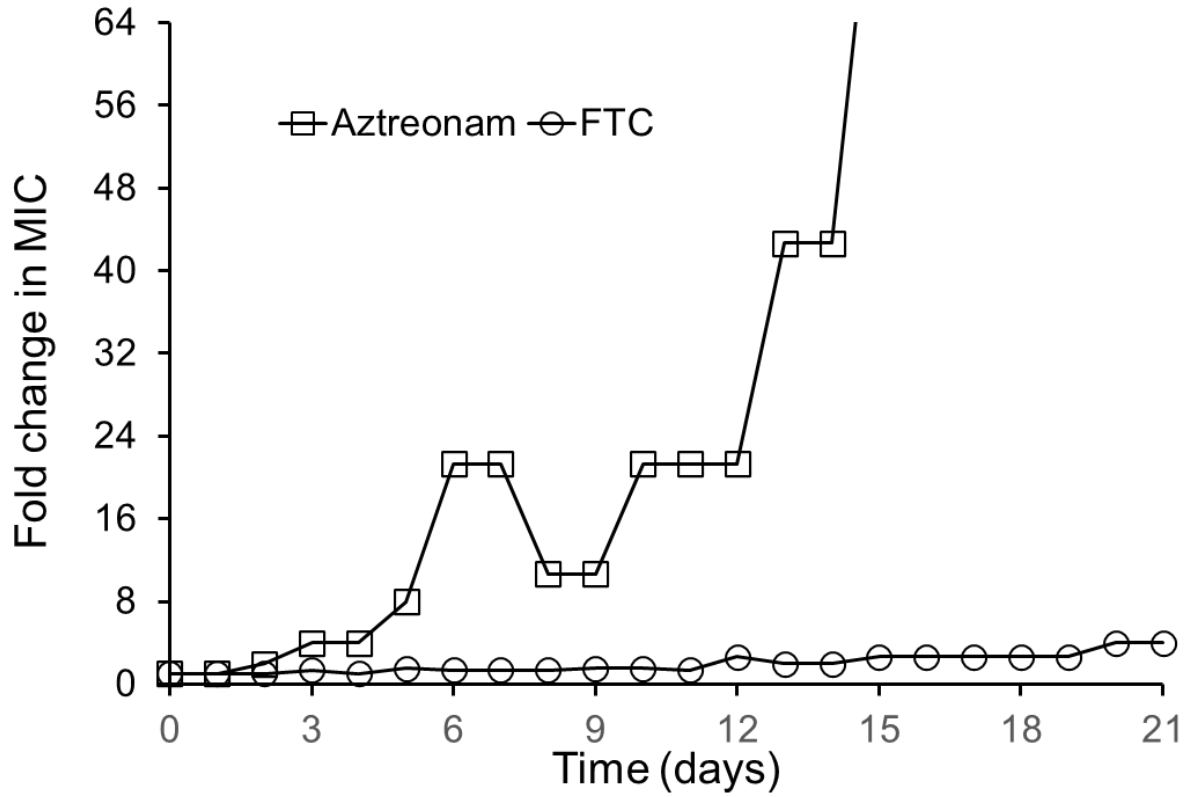


Figure 4.15 Multi-passage resistance assay for *P. aeruginosa*. Bacteria were serially passaged over a 21-day period and the broth microdilution assay was used to determine the minimum inhibitory concentration of each compound against wild type PAO1 after each successive passage.

Examining the details for the data (Table 4.9), we note increase of MIC in the serial passage assay is exponential over time, whereas the increase of MIC in the same assay but with

the presence of pili inhibitor appears to be linear. This substantial suppression of antibiotic resistance development by a non-killing agent was not known before this work and is a surprise when pondering on the possible mechanisms.

Table 4.9 MIC values for aztreonam and FTC after each passage.

Day	**AZT (µg/ml)**	**FTC (µg/ml)**
0	3	12
1	3	12
2	6	12
3	12	16
4	12	12
5	24	18
6	64	16
7	32	16
8	32	16
9	32	18
10	64	18
11	64	16
12	64	32
13	128	24
14	128	24
15	256	32
16	512	32
17	512	32
18	>512	32

19	-	42
20	-	42
21	-	42

Horizontal gene transfers promote drug resistance by increasing drug tolerant population.

To understand the mechanism for reducing drug resistance in a serial passage assay by inhibiting horizontal gene transfer using pili inhibitors, we first discuss the establish background knowledge, and then state the key question, and describe the logical answer that elucidate a proposed mechanism. First, we note that in the presence of antibiotics, bacteria develop drug tolerance over short period of time, in 24 h.[257] Such drug tolerance is still presence even after the antibiotic is removed, and the bacteria is cultured for another 24 h.[257] Thus, the gene mutation for tolerance is stable. Also, the MIC are the same for the ancestral and the evolved/tolerant (one passage) bacteria.[257] Second, the MIC of a bacterial population is primarily the property of individual bacterium, meaning that, on average, each bacterium needs a higher concentration of antibiotic to inhibit its growth for a high MIC strain than that for a low MIC. Furthermore, measuring the MIC of a culture grown to an OD 0.8 versus the same culture diluted with LB to an OD of 0.01 should not be greatly different. Third, for stable gene mutation, horizontal gene transfer are readily possible, and HGTs merely increase the population of bacteria to carry the mutated genes.

These establish background knowledge naturally leads to the question of how does inhibiting horizontal gene transfers reduce or subpress the development of drug resistance as we have seen in our results? In a series of recent seminal work, tolerance mutation is found to be the genetic background or foundation that greatly facilitate the development of stable resistance, and

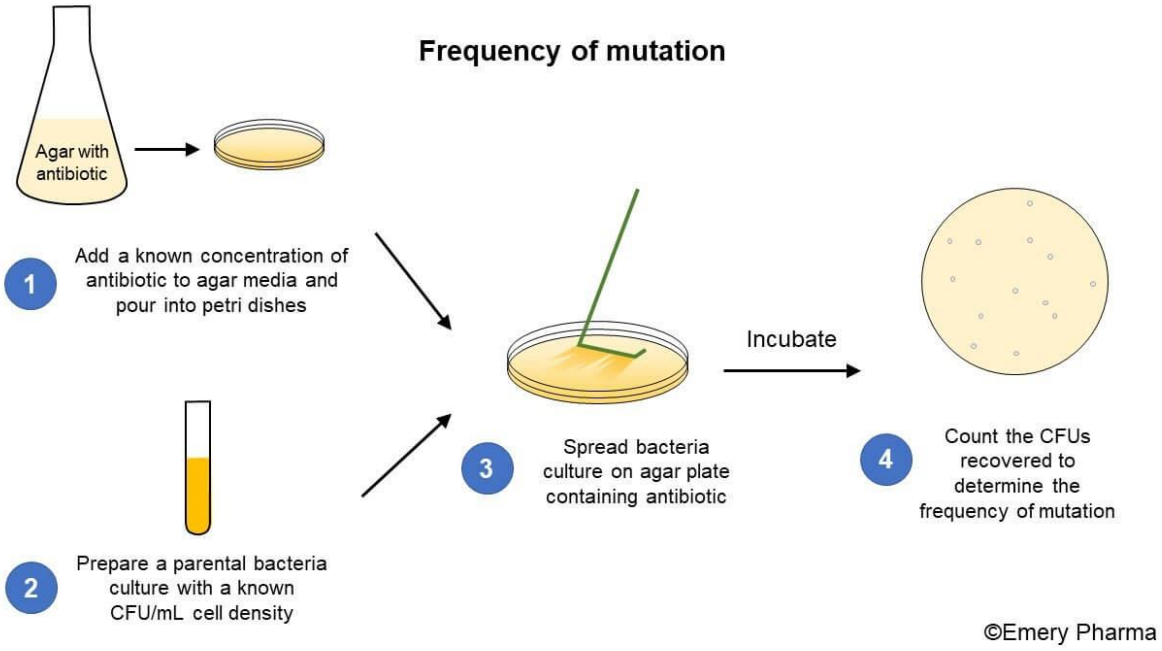
thus tolerance is regarded as the epigenetics for resistance.[23,189,191,257] These findings together with our results deduce that horizontal gene transfers increase MIC values in the serial passage assay by greatly increase the tolerant population, which by itself does not increase the MIC values, but such tolerant population readily transitions into resistant one in each bacterium, which requires a higher MIC for inhibiting their growth.

In summary, the effect of horizontal gene transfers on resistance development was not clear before. Here, we found that the effect is significant: inhibiting HGTs not only inhibit the drug resistance development for at least two weeks, but also change the drug resistance development from exponential over time to apparently linear over time. Furthermore, the mechanism suggests that, while HGTs transfer mutated genes between bacteria, HGTs suppress the development of drug resistance by inhibiting the spread of tolerance mutation in a population, which would have facilitated the population to be resistant that requires a high MIC of the antibiotic.

4.4.2 Single step resistance development in P. aeruginosa and S. aureus, mechanism: unknown

Single step resistance assay causes a set of mutations that permits surviving and growing bacteria under a single treatment (often 12 h) of a high concentration (multiples of planktonic MIC) of an antibiotic. The mutation rate in single step resistance assay is defined as the *in vitro* frequency at which detectable mutants arise in a bacterial population in the presence of a high antibiotic concentration.[268,270] It is important to note that, in this single step resistance assay, the number of mutant bacteria are recorded as colonies, and that the number of mutation events are not yet

characterized.[270] Therefore, only the favorable mutations are recorded for the bacteria that lead to a visible antibiotic resistance phenotype on agar plates (Fig. 4.16).



Adopted from Emery Pharma Ref: Antimicrobial Agents and Chemotherapy, Apr. 2006, p. 1228–1237 Vol. 50, No. 4, Mani et al, In Vitro Characterization of the Antibacterial Spectrum of Novel Bacterial Type II Topoisomerase Inhibitors of the Aminobenzimidazole Class.

Figure 4.16. Single step mutation assay: This figure illustrates the general workflow for conducting a single-step, spontaneous mutation frequency assay. (1) Liquid agar containing a known concentration of antibiotics (typically 4-fold or 8-fold or greater the MIC value of the bacteria being assayed) is prepared, poured into petri dishes, and allowed to solidify. (2) A parental bacteria culture is prepared and the bacterial density in CFU/ml is determined. (3) The parental bacteria culture is spread onto the agar plate containing antibiotic and incubated for up to 2 days. (4) The number of CFUs recovered is enumerated and this value is compared to the parental bacterial density to generate a frequency of mutation.

We tested mutational frequencies of wild type PAO1 under high concentrations of aztreonam and FTC. We found that aztreonam had resistant colonies in the presence of 4×MIC,

8×MIC and 16×MIC with a mutation frequency of 2.8×10^{-9} at 4×MIC. FTC did not show any resistant colonies at 8×MIC and 16×MIC, but 2 resistant colonies in the presence of 4×MIC with a mutation frequency of 2×10^{-10} (Fig. 4.17) We also tested if the resistance of bacteria (by aztreonam and FTC) in these resistant colonies is "stable". We picked single colony from agar plates containing 4 × MIC of aztreonam or FTC and cultured them in the presence of 10 × MIC of aztreonam or FTC at 37 C with shaking (250 rpm). For both cases, the bacteria did group to cloudiness under such high concentration of antibiotics (10x MIC of aztreonam and FTC) These results indicated that bacteria survived in the single-step assay are stable resistant mutants.

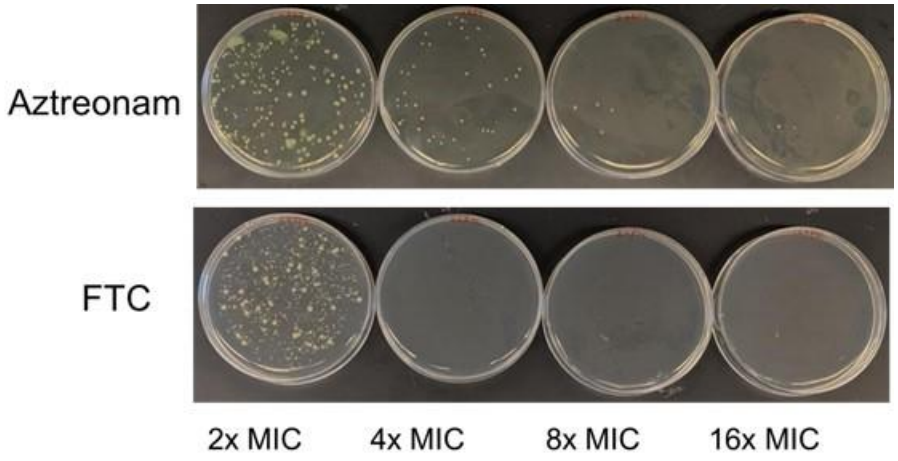


Figure 4.17 Single step resistance assay for *P. aeruginosa*. For single step resistance, *wt* PAO1 (10^{10} CFU/ml) was plated on MHB containing 2×, 4×, 8× and 10 × MIC of aztreonam and FTC. After 48 h of incubation at 37 °C, no resistant colonies were detected for FTC. Aztreonam showed resistant colonies for high concentration (16 × MIC). mutational frequency = no. of resistant colonies / CFU of bacteria added (10^{10}); FTC = 2×10^{-10}; aztreonam = 2.8×10^{-9}

We tested mutational frequencies of methicillin susceptible *S. aureus* (MSSA) under high concentrations of vancomycin and FTC. We found that vancomycin had resistant colonies in the presence of 4×MIC, 8×MIC and 16×MIC. Vancomycin showed mutational frequency of $12 \times 10^{-}$

^{8} in the presence of 4×MIC vancomycin. Interestingly, FTC did not show any resistant colonies at 2×MIC, 4×MIC 8×MIC and 16×MIC (Fig.18).

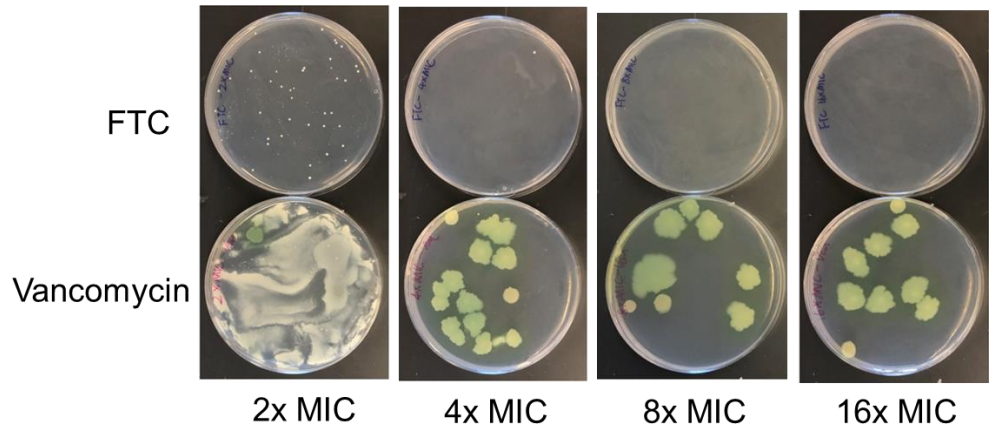


Figure 4.18 FTC Does Not Cause Resistance by Staphylococcus aureus (SA) while vancomycin does after 48 h. After 48 h of incubation at 37 °C, no resistant colonies were detected for FTC. Vancomycin showed resistant colonies for high concentration (16 × MIC). mutational frequency = no. of resistant colonies / CFU of bacteria added (10^{10}) Vancomycin = 12×10^{-8}

Discussion

Single-step mutation assay and serial passage assay are used to study bacterial survival under antibiotic treatments, and to characterize the development of resistance by the survival colonies and increasing MIC, respectively. Typically, single step mutation assays only consider situations in which bacteria acquire genetic mutations by a single high dose antibiotic treatment that renders them practically immune to therapy.[267,268,271] However, this is not the case in nature. Bacteria undergo multiple antibiotic treatments or abuse in clinical settings. Serial passage assay considers multi-step resistance, where the accumulation of several mutations of low individual benefit is necessary for high-level resistance.

For single-step mutation assay, our results showed that at 2x MIC aztreonam (at higher concentrations than MIC) developed single-step mutants, while FTC did not show any colonies for *P. aeruginosa*. The possible explanation for occurrence of aztreonam-resistant mutants are the change in porin structures or increased efflux pump activity.[272,273] The development of resistance against FTC has yet to be elucidated, and is likely different than that of beta-lactam,[274] particularly because the molecular weight cut off for hydrophilic drugs passing through different porins is shown to be around 600 Daltons.[274-276] The molecular weight of aztreonam is 435.4 Daltons while that of FTC is 627.23 Daltons, thus FTC will likely not pass through the porins to get into the bacteria, and the resistance via porin permeability is likely irrelevant.

For *S. aureus*, higher number of resistant colonies were observed under one-time high concentrations of vancomycin treatment. These results are consistent with literature that vancomycin has high propensity to develop single-step mutations in the presence of high antibiotics concentrations.[277-279] Numerous changes related to *S. aureus* cell wall structure or cell wall metabolism are shown to lead to an increased number of false binding sites for vancomycin.[277] S aureus did not show any resistant mutants in the presence of FTC.

For serial passage assay, from day 5, aztreonam MIC showed two-fold increase, and started to increase exponentially. Multiple genes are reported to recurrently mutate in *P. aeruginosa* when repeatedly passaged with aztreonam, these multiple gene mutations occurred cumulatively, including those related efflux pumps, porins and beta lactamase, all appears to be specific to aztreonam.[273,280] FTC did not show exponential increase in MIC over days during serial passage. We believe that exponential increase in MIC is a result of the resistant mutation has become stable, and the spread resistance is facilitated by both generational passing

compounded by horizontal gene transfer. Whereas the linear slow increase in MIC is a result of unstable resistant mutation, and thus ineffective generation passing, and inactive horizontal gene transfer. In such a case, the bacteria may be in a state of drug tolerant and drug persistent. Dr.Luk group is working on identification of population diversity under serial passage assay under aztreonam and FTC treatment.

Most of the antibiotics exhibit rapid drug resistance development by serial passage assay, except the newly developed ones, such as the quaternary positively charged molecules, cationic steroid antibiotics (CSA) and Teixobactin and a particularly peculiar case, Moenomycin A. Moenomycin is family of antibiotics that inhibits the peptidoglycan glycosyltransferases (PGTs) in cell wall synthesis of gram-positive bacteria and showed activity against methicillin sensitiveS. aureus with MIC of 0.5 μg/ml, [281-284] This class of antibiotics is not active against gram-negative bacteria. The lead antibiotic in this class, Moenomycin A failed as a drug for human due to suboptimal pharmacokinetics. Instead, Moenomycin A is used as supplements livestock feeds for animals to promote animal growth as well as an antibiotic.[283] Surprisingly, after decades of higher dose feed of Moenomycin A to animals, no moenomycin-resistant bacteria have been reported to date.[281,282] Single-step mutation assay showed very low mutational frequency for moenomycin in vitro (2.2×10^{-10}).[281] Moenomycin also showed no transferable resistance or cross resistance.[283,284]

Comparing the structures, FTC and Moenomycin share two key common structural elements, both bear sugars and aliphatic chains. However, FTC is overall smaller, having two sugars instead of 4, and the aliphatic chain is shorter. One key functional advantage of FTC is that FTC is active against both gram-negative and positive bacteria, whereas Moenomycin is

only active against gram positive. FTC also does not seem to develop drug resistance, just like Moenomycin.

Experimental section

4.4.3 Multi-passage resistance assay

Multi-passage assay was adopted as described previously.[269,285] To prepare the initial bacterial culture, an overnight culture of PAO1 wild type was prepared in MHB in falcon tube at 37°C with 250 rpm shaking. Subculture of PAO1 was prepared by diluting 100 µL of overnight culture into 10 mL fresh MHB (1:100). For the serial passage experiment, the prepared subculture of PAO1 was transferred into a 96-well plate with adding antibiotics (Aztreonam, FTC) stock solution to get different final concentration (0.25 x MIC, 0.5 x MIC, 1 x MIC, 2 x MIC) in totally 200 µL culture into each well (6 replicates of each concentration were performed in the same column). After a 24-h incubation in a static incubator at 37°C, the column of wells with the highest concentration of antibiotics that still showed bacteria growth was sub-cultured again: Culture from six wells of this column were taken out and combined, and 100 µL of the combined culture was mixed with 10 mL of fresh MHB (1:100). This freshly diluted culture was transferred into another column of 6 wells with addition of a new set of concentrations of antibiotics. The new concentrations were adjusted such that the lowest concentration of antibiotic that showed non-growth in column was the new MIC value, and thus 0.25 x new MIC, 0.5 x new MIC, 1 x new MIC, 2 x new MIC, were applied.

To quantify the MIC change for PAO1, this lowest concentration of antibiotics that showed non-growth in column was taken as the new MIC for PAO1 for each passage. The added antibiotics

concentrations were increased for the rising MIC values. This process was repeated for 21 times (passages).

4.4.4 Single step resistance assay

We tested single step resistance assay as described in literature.[269,285] For single step resistance, *P. aeruginosa* at 10^{10} CFU/ml was plated onto MHB containing 2×, 4×, 8× and 16 × MIC of Aztreonam and FTC. For single step resistance, *S. aureus* at 10^{10} CFU/ml was plated onto MHB containing 2×, 4×, 8× and 10 × MIC of vancomycin and FTC. After 48 h of incubation at 37 °C, no resistant colonies were detected for FTC in both strains. We obtained no mutants of *P. aeruginosa* or *S. aureus* to FTC with a low dose (4 × MIC) of the compound.

4.5 Farnesol triazole cellobioside exhibits synergy with conventional antibiotics

4.5.1 FTC showed synergy with β-lactam antibiotics in gram-negative bacteria.

In previous section, we have shown that FTC inhibits NagZ enzyme activity in *P. aeruginosa.* NagZ promotes AmpC β-lactamase expression through activating AmpR and enhances resistance to β-lactam antibiotics in *P. aeruginosa*[286-289] and *E. coli*[290].

These results prompted us to evaluate the efficacy of combining different class of antibiotics with FTC for killing gram-negative bacteria. We determined Fractional Inhibitory Concentration (FIC) index values by microdilution checkerboard analysis[291]. These assays determined minimum inhibitory concentration (MIC) of different antibiotics and FTC. We determined the impact on potency of the combination of antibiotics in comparison to their individual activities against both bacteria.

Table.4.10 Synergistic effect of FTC with different antibiotics.

Antibiotic	**Type/receptor**	**FIC Index*** **(*E. coli*)**	**FIC Index*** **(PAO1 *wt*)**
Aztreonam	β-lactam/PBP	0.44	0.58
Imipenem	β-lactam/PBP	0.28	0.45
Ceftazidime	β-lactam/PBP	0.36	0.44
Cefoxitin	β-lactam/PBP	0.48	0.58
Tobramycin	Aminoglycoside/Ribosome	0.78	0.80
Ciprofloxacin	fluroquinolone /DNA gyrase	1.0	1.0
Colistin	Polymyxin/outer membrane/LPS	1.6	1.4

*$FIC_{antibiotic} = MIC_{antibiotic+FTC}/MIC_{antibiotic}$, $FIC_{FTC} = MIC_{FTC+antibiotic}/MIC_{FTC}$ The FIC index is the sum of $FIC_{antibiotic}$ and FIC_{FTC}.A FIC index of < 0.5 indicates synergism, > 0.5–1 indicates additive effects, > 1 to < 2 indifference, and ≥ 2 is considered to be antagonism.

We found that FTC showed synergy with β-lactam antibiotics aztreonam, imipenem, ceftazidime and cefoxitin against *E. coli* and *P. aeruginosa*. FTC was additive with aminoglycoside antibiotic tobramycin and fluroquinolone antibiotic ciprofloxacin against *E. coli aureus* and *P. aeruginosa* (Fig. 4.19)

Overall, FTC showed enhanced activities of β-lactam class of antibiotics against gram-negative bacteria like *E. coli* and *P. aeruginosa*. FTC showed additive effect with other antibiotics like aminoglycoside tobramycin, fluroquinolone ciprofloxacin. These results showed that FTC may have synergy with β-lactam antibiotics via NagZ inhibition which targets peptidoglycan (PG) synthesis pathway.

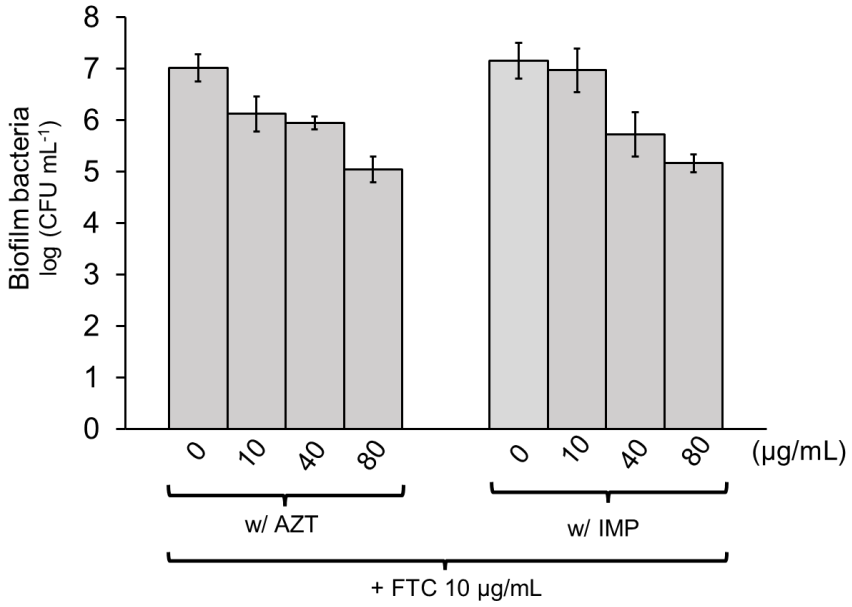


Figure 4.19 Biofilm eradication by aztreonam and Imipenem in the presence of FTC. The 24-h PAO1 biofilms were subjected to different concentrations of Aztreonam and Imipenem in the presence of 10 µg/ml for 24 h. The number viable biofilm bacteria were obtained by sonication and enumerated by plating on LB agar plate and expressed as colony forming units.

We found that FTC (10 µg/ml) potentiate aztreonam and imipenem (10, 40 and 80 µg/ml) against 24-h PAO1 biofilms.

Even though extended β-lactam antibiotics are active against planktonic *P. aeruginosa*, they fail against *P. aeruginosa* biofilms[29,220,292]. In *P. aeruginosa* , β-lactamases was associated with strong biofilms and β-lactamases results in enhanced resistance in biofilms[220].Aztreonam and Imipenem showed higher tolerance towards *P. aeruginosa* biofilms than planktonic bacteria. The MBEC for both aztreonam and imipenem against *P. aeruginosa* biofilms are greater than 512 µg/ml (MIC_{AZT} = 3 µg/ml; MIC_{IMP} = 2 µg/ml).

4.5.2 FTC is active against Clinical Isolate of P. aeruginosa (Non-mucoid strain 1587) in Biofilm

P. aeruginosa non mucoid isolates were shown to be tolerant to aztreonam.[208] we tested efficacy of aztreonam and FTC, combination against Clinical Isolate of *P. aeruginosa* (Non-mucoid strain 1587)

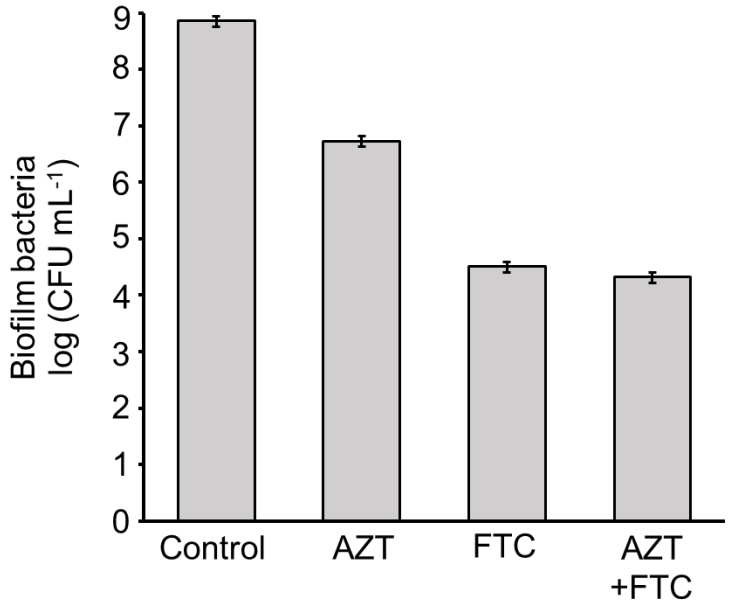


Figure 4.20 Biofilm eradication of Clinical *P. aeruginosa* strain 1587 by aztreonam and FTC. The 24-h PAO1 biofilms were subjected to different concentrations of 10 µg/ml Aztreonam in the presence of 10 µg/ml for 24 h. The number viable biofilm bacteria were obtained by sonication and enumerated by plating on LB agar plate and expressed as colony forming units.

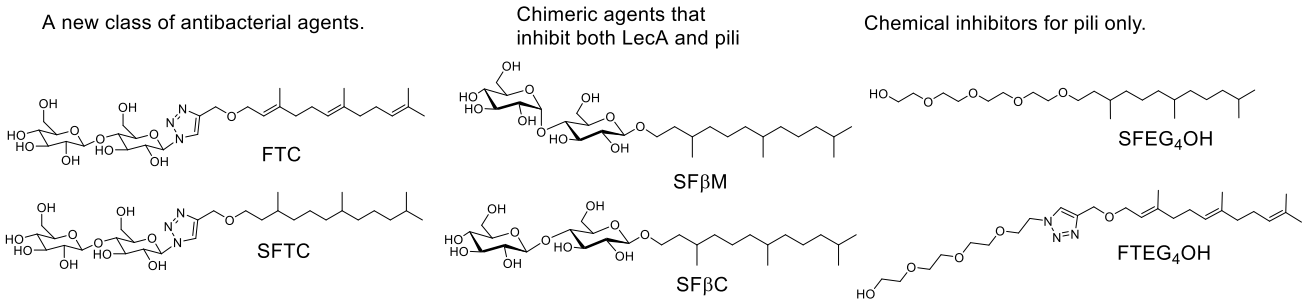
We found that aztreonam (AZT) was moderately active against 24-h biofilms of strain 1587 and reduced biofilm bacteria by 2 log. FTC alone showed good activity against strain 1587 and reduced biofilm bacteria by 4 log. Aztreonam and FTC combination did not show any significant reduction in biofilm bacterial count than FTC alone though FTC prevented formation (Fig. 4.20).

4.6 Receptor identification and mode of action of FTC

4.6.1 Identification of Receptors and Mode of action of FTC

We showed that FTC inhibited swarming motilities in *P. aeruginosa* strains (PAO1 and PA14) at concentrations lower than MIC. Pili are necessary for swarming motility.[293-295] Previously Luk group has shown group of molecules which targets pili and inhibit pili-mediated motilities like swarming.[81,199] Previous generation of adjuvant agents like SFβC, 3,5-diMeDβC and SFEG$_4$OH, FTEG$_4$OH inhibit swarming motility in *P. aeruginosa*. Luk group has shown that SFβC/3,5-diMeDβC and SFEG$_4$OH/ FTEG$_4$OH inhibits pili mediated activities by the small molecules direct binding to pili and causing retraction of pili (See Chapter 2). LecA play major role in biofilm formation and inhibition of LecA leads to inhibition of biofilms in *P. aeruginosa*.[296-298] Luk group has shown that SFβC/3,5-diMeDβC binds to LecA. Because of this strong similarity between the activities of the molecules that kill bacteria and the chimeric molecule, we hypothesize that that FTC and SFTC also binds to pili and LecA, but also bind to a new receptor protein that causes killing of the bacteria. . Binding and inhibiting pili and LecA are responsible inhibiting a wide range of phenotypes that is controlled by both high and low level of cdG, controlling these phenotypes inhibits the development of drug tolerance and drug resistance.

Table 4.11 Structure and phenotypes relation of small molecules provide receptor hypothesis.



		Small molecules		
		FTC, SFTC	SFβC[81], 3,5-diMeDβC[a]	$SFEG_4OH$[81], $FTEG_4OH$[b]
Phenotypes	Biofilm	Inhibit	Inhibit	No effect, or slightly promote
	Swarming	Inhibit	Inhibit	Inhibit
	Killing	Yes	No	No
Protein receptor	LecA	??	Yes	No
	Pili	??	Yes	Yes
	(for killing)	??	NA	NA

a.derived from chapter 2 b.Another Graduate student Yuchen Jin's work

Bacterial populations susceptible to antibiotics become resistant either through genetic mutation (spontaneous) or through horizontal transfer and expression of resistance genes from other strains.[193,195,196,299] Horizontal gene transfer (HGT) is one of major cause of acquired antibiotic resistance[193,195,300]. All resistance genes from the super bugs can be mobilized by HGT mechanisms to other pathogenic and non-pathogenic bacteria. Pili also play important role in horizontal gene transfer in *P. aeruginosa*[299,301,302]. A gene cluster encoding type IV pilus is crucial for horizontal gene transfer.[193,195] We believe that FTC prevents horizontal gene transfer and hence prevents resistance development in *P. aeruginosa*.

4.6.2 Killing mechanism of FTC

The farnesol triazole cellobioside (FTC) is an amphiphilic glycolipid. The amphiphiles are known to damage bacterial cell membrane and cause leakage causing cell leakage and lysis, and bacterial death.[303,304] To test effect of FTC on bacterial cell membrane permeability, we used propidium iodide (PI), a fluorescent probe which exhibit fluorescence after binding to DNA.[305] PI dye is not capable to cross an intact cell membrane, but only crossed compromised cell membrane that results in DNA binding and fluorescence increases. Based on this PI dye assay, we found that FTC has no effect on bacterial cell membrane permeability of either B. *subtilis* or PAO1, suggesting that cell membrane damage is not a mechanism of killing by FTC.

To identify the protein receptor for FTC that resulted in killing of the bacteria, we first found out that sub-inhibitory concentration of FTC (0.5×MIC) caused filament formation for both gram-negative wild type *P. aeruginosa* and gram-positive *B. subtilis* (Fig. 4.21). This result provides a strong entry point for identifying the protein receptors that are responsible for killing

the bacteria. There are a few conditions that would cause bacteria to form filaments, including inhibition of penicillin binding proteins (PBPs), undecaprenyl diphosphate synthase (UppS), reactive oxygen species (ROS) formation under anaerobic conditions.[48,49,306-309] Among these conditions, we believe the potential protein receptor that is responsible for FTC binding and killing of the bacteria is either penicillin binding proteins or undecaprenyl diphosphate synthase (UppS). The reactive oxygen species (ROS) formation under antibiotic stress requires strictly anaerobic conditions hence we ruled out ROS formation as potential mode of action. Penicillin binding proteins (PBPs) is a critical enzyme that catalyze the cross-links in the cell walls, and inhibition of PBP by β-lactam kill bacteria by impairing the cell wall synthesis. Undecaprenyl diphosphate synthase (UppS) is another important enzyme in the biosynthesis of bacterial cell wall, it catalyzes the condensation of farnesyl pyrophosphate (FPP) with eight consecutive isopentenyl pyrophosphate units (IPP), to generate undecaprenyl pyrophosphate. UppS also serves as a lipid carrier for peptidoglycan synthesis.[309-311] Small molecule inhibitors of UppS inhibits bacterial the growth of a variety of bacteria.[309-311] Both β-lactam and UppS inhibitor at sub-MIC concentrations cause filamentous bacteria formation. FTC showed activity against both gram-positive and gram-negative bacteria, hence we believe that FTC kill bacteria by either inhibition of undecaprenyl diphosphate synthase (UppS) or glycosyltransferase (GT) and transpeptidase (TP) activities of penicillin-binding proteins (PBPs).

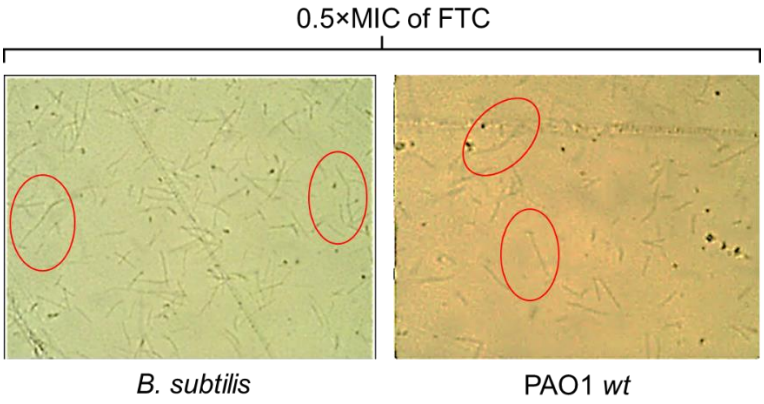


Figure 4.21 Morphology of *B. subtilis* and *P. aeruginosa* under treatment of Farnesol triazole cellobioside (FTC) at 0.5×MIC for 1 h in planktonic culture and visualized by light microscopy.

We first evaluated effect of FTC on undecaprenyl diphosphate synthase (UppS) for gram-positive bacteria by using a screening platform described in literature.[312-314] For Gram-positive bacteria like *S. aureus*, the cell wall is made up of peptidoglycan and teichoic acid (WTA), both are synthesized from the lipid carrier undecaprenyl phosphate (Und-P).[310,312] UppS is an enzyme responsible for synthesizing undecaprenyl phosphate (Und-P), which is the precursor WTA.[312] Both Und-P and WTA are required for cell wall synthesis. Targocil is a drug that inhibit enzyme tarG which acts on WTA, resulting in the inhibition of bacterial growth.[315] Past studies by Eric Brown group has shown that as sub-inhibitory concentration of UppS inhibitors reduces WTA levels.[312] Targocil becomes less effective as a bacteriostatic agent due to unavailability of WTA, indicated by the increase of MIC of Targocil at inhibiting bacterial growth. This increase in MIC of Targocil has been used as an indicator for the presence of UppS inhibitors. Our results show that the known UppS inhibitor, Clomiphene increased Targocil's MIC from 1.5 to 8 µg/ml (Table 4.11). Our results showed that FTC did not change MIC of Targocil (1.5 to 2 µg/ml), suggesting that FTC may not inhibit UppS enzyme (Table 4.12).

For a separate experiment, we study another effect that is known for UppS inhibitors. Presence of UppS inhibitors, such as Clomiphene, at sub-MIC concentrations increases the level of Staphyloxanthin pigment – a yellow-to-orange pigment produced by *S. aureus*.[312] At sub-MIC concentration of FTC, the levels of Staphyloxanthin pigment did not change. These results together indicated that FTC is unlikely targeting UppS.

Table 4.12 Susceptibility of *S. aureus* against different cell wall synthesis inhibitors.

	MIC (µg/ml)		
Antibiotic/Agent	by itself	with Clomiphene	with FTC
Targocil	1.5	8	2
Clomiphene	8		
FTC	2		

We next moved to explore another potential receptor, penicillin binding proteins (PBPs).[316-319] PBPs are members of a subgroup of enzymes called transpeptidases.[316-319] The beta lactam antibiotics inhibits penicillin binding proteins, and also causes bacteria to form filaments at sub-MIC concentrations.[316-319] We tested effect of FTC on inhibition of transpeptidase activity of the PBP1a from *E. coli*. PBPs are found in every bacterial species. *E. coli*, for example, has eight well-known PBPs, named 1A, 1B, and 2-7. PBPs have molecular masses ranging from 20 to 120 kDa and are split into two categories: high molecular mass (HMM) and low molecular mass (LMM) PBPs.[16,320,321] HMM PBPs are required for bacterial survival and are –lactam antibiotics' fatal targets, but LMM PBPs are not required for cell viability.[320,321] Moreover, PB1a is correlated to filament formation in bacteria.[49] Hence we chose commercially available PBP1a as representative protein for small molecule studies. FTC is also active against *E. coli* with MIC of 8 ug/ml while $FTEG_4OH$ showed no antibacterial activity against *E. coli*. We used microtiter

plate-based assay for inhibitor binding to the PBPs based on competition with biotin-ampicillin (BIO-AMP) conjugate binding. Ampicillin is a beta-lactam that has a high affinity for PBPs.[316-318] In this assay, purified PBP1awere immobilized onto microtiter plate wells by physical adsorption and labeled with BIO-AMP. The amounts of the BIO-AMP labeled PBP are determined by avidin based biotin quantification kit.[322] The binding/labelling of the BIO-AMP to PBP1a can be inhibited with different concentrations of unlabeled PBP inhibitors, in our case FTC. We used moenomycin A antibiotic as positive control. Moenomycin A is active against gram-positive bacteria and binds to PBP, inhibit transpeptidase activity of PBPs.[281,283] We used $FTEG_4OH$ as negative control; $FTEG_4OH$ is not bactericidal against gram-positive or gram-negative bacteria.

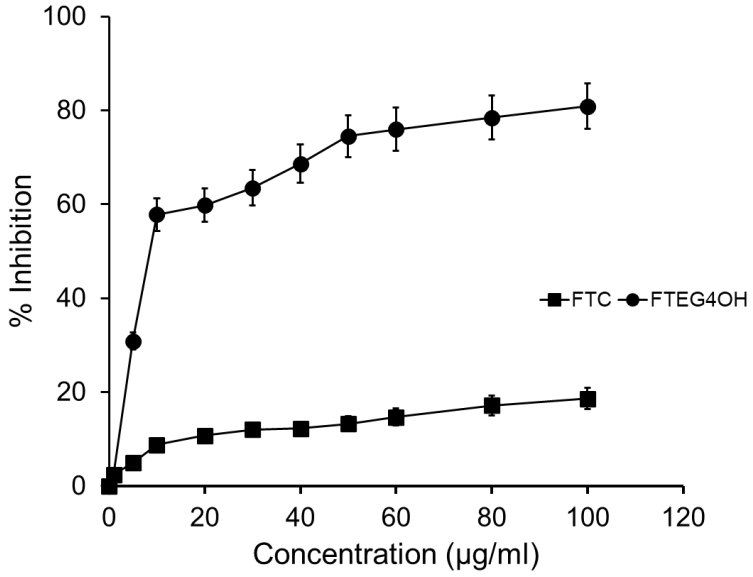


Fig. 4.22 Inhibition curves of unlabeled PBP inhibitor against BIO-AMP labeled *E. Coli* PBP1a The different concentration of FTC, FTEG4OH were added to 2 µg *E. Coli* PBP1a and then 100 µM BIO-AMP was added to mixture. After 10 min, amount of Bound biotin-Amp-PBP was quantified by Pierce™ Biotin Quantitation Kit. % inhibition was calculated as % inhibition = 100 x [1 - (amount of BIO-AMP bound at given concentration – amount of BIO-AMP bound with Moenomycin A)/(amount of BIO-AMP bound with no inhibitor - amount bound with Moenomycin A)].

We found that FTC inhibited transpeptidase activity of *E. Coli* PBP1a enzyme while FTEG4OH did not inhibit transpeptidase activity of *E. Coli* PBP1a (Fig.4.22). These results are consistent with the bactericidal activity of FTC and FTEG4OH against *E.coli*. In this assay, moenomycin A showed highest inhibition of *E. Coli* PBP1a which is consistent with literature.[281,283] Together , these results suggest that FTC inhibits transpeptidase activity of *E. Coli* PBP1a and shows bactericidal activity against *E. coli*. We believe that FTC may inhibits different PBPs proteins in gram-positive and gram-negative bacteria and showed antibacterial activity against these bacteria.

4.6.3 Inhibition of HGTs, conjugation, transformation, and transduction by FTEG$_4$OH

Drug resistance can be developed by acquiring gene materials that encode for proteins that causes drug resistance from environment or from another strain. Classically, this type of gene transfer was thought to occur by three different mechanisms: Conjugation: the use a surface appendages called a pilus to transfer genes between two bacteria. Transformation: naked DNA is taken up by a bacteria. Transduction: genes are transferred using a phage particles. We previously showed that FTEG$_4$OH which inhibits pili and pili-mediated swarming motilities in wild type *P. aeruginosa*. Here, we tested efficacy of FTEG$_4$OH at inhibition of three mechanisms of gene transfer.

First, we used well established ***P****seudomonas* ***A****eruginosa* **P**athogenicity **I**sland I (*PAPI-I*) gene transfer assay between *P. aeruginosa* species via conjugation mode of transfer.[301,302] In this conjugation assay, we used two *P. aeruginosa* strains PA14-140495 (*PAPI-I* donor) and PAO1Cb^R*lacZ* (*PAPI-I* acceptor). The *P. aeruginosa* PA14 carries *PAPI-I* gene which is absent in wild type PAO1.[301,302] *PAPI-1* gene is horizontally transferable into recipient strains lacking *PAPI-I* gene via conjugation mechanism by the type IV pilus.[301] We found that *PAPI-I* gene transferred from PA14-PAPI-I to PAO1 with transfer frequency of 1.1×10^{-5} (Fig. 4.23). In the presence of pili inhibitor FTEG$_4$OH, gene transfer frequency reduced to 1.6×10^{-6}. This results suggested that FTEG$_4$OH inhibits pili mediated conjugative gene transfer.

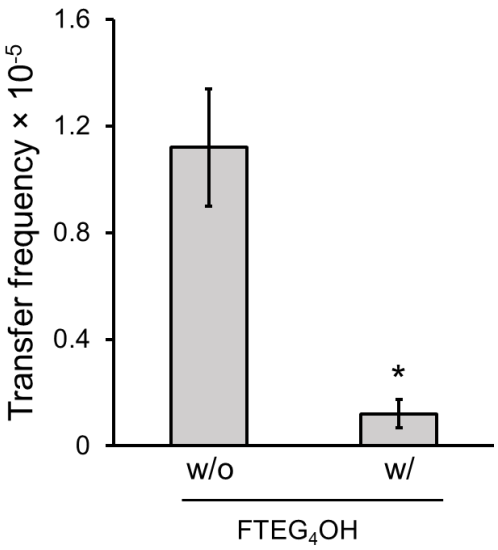


Figure 4.23 $FTEG_4OH$ reduces *PAPI-I* gene transfer from PA14-PAPI-I to PAO1 by inhibition of conjugation mechanism. PA14-140495 (*PAPI-I* donor) and $PAO1Cb^R$*lacZ* were co-incubated for at 37°C for 48 h statically with and without 100 µM $FTEG_4OH$. The number of transconjugants (blue colonies) was counted on selective agar containing 75 µg/mL gentamicin, 150 µg/mL carbenicillin and 40 µg/mL X-Gal. The transfer frequency was calculated by the total number of transconjugants (blue colonies) divided by the total recipients (CFU after 48 h culture at 37°C).

Bacterial transformation is a process of horizontal gene transfer by which *P. aeruginosa* take up foreign genetic material (naked DNA, plasmid) from the environment.[323,324] To test if pili inhibitors have effect on gene transfer mediated by transformation mechanism, we used *mCherry* (Tc^R) plasmid as genetic material and wild type *P. aeruginosa* as acceptor strain. We found that in the absence of inhibitor wild type PAO1 pick up *mCherry* (Tc^R) plasmid from cultures with transformation frequency (expressed as number of colonies of transformants per nanogram of plasmid per 10^7 CFU/ml of recipient bacteria) of 2.4. In the presence of 100 µM $FTEG_4OH$, transformation frequency reduced to 1.1 (Fig. 4.24). We also performed effect of different

concentrations of pili inhibitor ($FTEG_4OH$) on transformation frequency of wild type *P. aeruginosa*. We found that transformation frequency decreased as concentration of pili inhibitor $FTEG_4OH$ (Fig. 4.25). These results collectively suggested that $FTEG_4OH$ inhibits pili and pili mediated transformation mechanism of gene transfer.

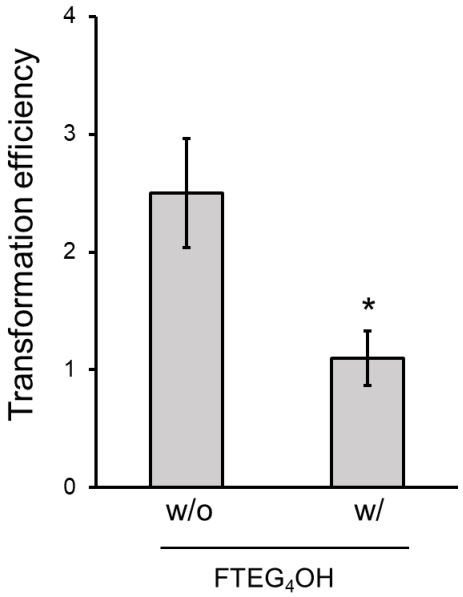


Figure 4.24 $FTEG_4OH$ inhibits *mCherry* (Tc^R) plasmid transfer by transformation mechanism. *wt* PAO1 (10^7 CFU/ml) was cultured with *mCherry* (Tc^R) plasmid for 24 h under 37°C with shaking (250 rpm). To quantify the plasmid transfer, colony forming units (CFU) of the transformants was counted on agar plates containing 250 µg/mL tetracycline. Transformation efficiency was expressed in number of colonies of transformants per nanogram of plasmid per 10^7 CFU/ml of recipient bacteria. Error bars indicate the standard deviations of means of duplicates. *$P < 0.05$; vs control without $FTEG_4OH$, Student's t-test.

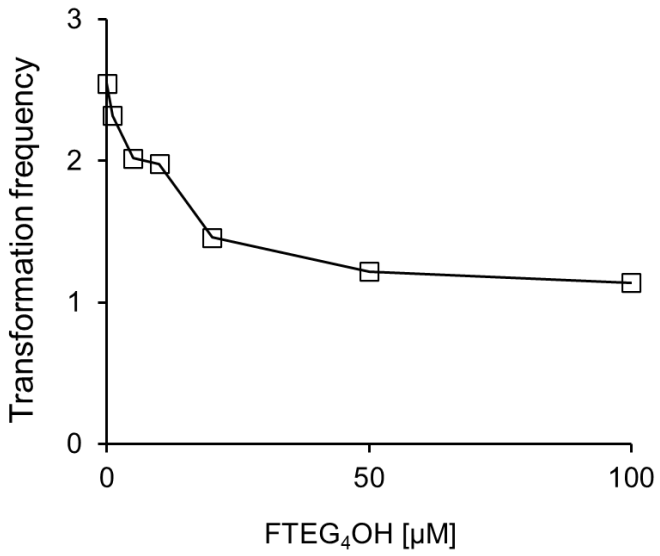


Figure 4.25 Transformation efficiency of $FTEG_4OH$. *wt* PAO1 (10^7 CFU/ml) was cultured with *mCherry* (Tc^R) plasmid for 24 h under 37°C with shaking (250 rpm). To quantify the plasmid transfer, colony forming units (CFU) of the transformants was counted on agar plates containing 250 μg/mL tetracycline. Transformation efficiency was expressed in number of colonies of transformants per nanogram of plasmid per 10^7 CFU/ml of recipient bacteria and plotted vs corresponding concentration of $FTEG_4OH$.

P. aeruginosa species like PA14 which carries PAPI-I gene use transduction via bacteriophage,[325,326] and all clinical isolates have prophages in their genomes.[326-330] Transfer of phage DNA (transduction of phage followed by lysogeny) between *P. aeruginosa* populations has been reported in patients with cystic fibrosis.[326,330] Generalized transduction mechanism occurs when induced phage particles by either oxidative stress or fluroquinolone antibiotics, package plasmid DNA that deliver host DNA to new recipient *P. aeruginosa* strain. [325,330] To test effect of pili inhibitor on phage mediated gene transfer, we induced phage particles in *P. aeruginosa* PA14 via exposure to fluroquinolone antibiotic ciprofloxacin as reported in literature.[326,330] We used these phage particles for gene transfer into recipient *P. aeruginosa*

strain (PAO1Cb^{R}*lacZ*). We treated PAO1Cb^{R}*lacZ* with supernatant containing phage particles and selected new transductant bacteria on selective agar plates. We found that in the absence of pili inhibitor FTEG$_4$OH, 98 colonies of transductant. In the presence of 100 µM FTEG$_4$OH, we found almost no or ~3 transductant colonies. This results suggested that FTEG$_4$OH has strong effect on phage mediated gene transfer.

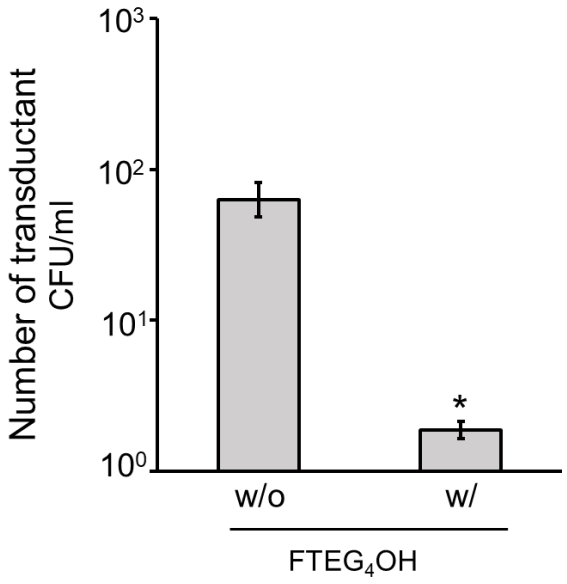


Figure 4.26 FTEG$_4$OH Inhibits ciprofloxacin induced phage mediated PAPI-I gene transfer by transduction mechanism. PAO1Cb^{R}*lacZ* (with and without 100µM) were treated with bacteria free supernatant of PAPI-1 donor PA14-140495 (Gm^{R}) which contains ciprofloxacin induced phage for 48 h without shaking. The transductant were counted as blue colonies on selective agar (75 µg/ml gentamicin, 150 µg/ml carbenicillin, and 40 µg/ml X-Gal). Error bars indicate the standard deviations of means of duplicates. *$P < 0.01$; vs control without FTEG$_4$OH, Student's t-test.

As pili inhibitor exhibited strong inhibition of transduction mechanism of gene transfer, we explored effect of pili inhibitor on phage adsorption and killing. We used P. aeruginosa specific lytic phage (ϕKMV) for adsorption and killing assay. *Pseudomonas aeruginosa* bacteriophage ϕKMV requires type IV pili for adsorption and subsequent killing.[331] We tested effect of pili inhibitor FTEG4OH on phage adsorption of P. aeruginosa sensitive to ϕKMV (PAO1k). We found that mixing ϕKMV (5.8×10^6 PFU/ml) with excess of PAO1k (2.8×10^7 CFU/ml) for 10 min, the phage titer in supernatant culture was reduced to 6.1×10^5 PFU/ml, indicative of ~81% of the phage was adsorbed on PAO1k (Fig. 4.27). In contrast, culturing bacteria in the presence of $FTEG_4OH$ (100 µM), the phage count in solution was 4.8×10^6 PFU/ml, indicating of only ~20 % of phage adsorption on PAO1k.

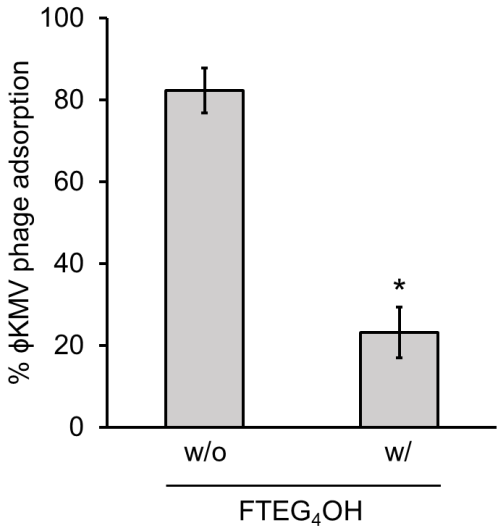


Figure 4.27 $FTEG_4OH$ inhibits ϕKMV Phage adsorption on *P. aeruginosa*. Percentage of ϕKMV phage adsorbed on PAO1k (*wt* PAO1 strain that is sensitive to ϕKMV) mutants over 10 min in LB containing 100 µM of $FTEG_4OH$. Error bars indicate the standard deviations of means of duplicates. *$P < 0.02$; vs control without $FTEG_4OH$, Student's t-test.

FTEG$_4$OH (100 µM) Delays Cip induced Phage mediated PAO1k killing.

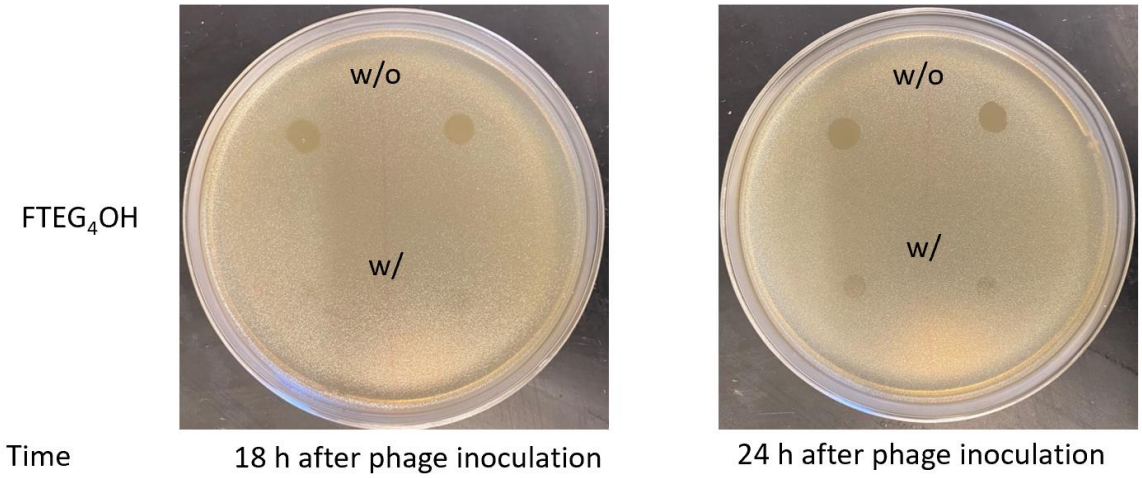


Figure 4.28 Phage plaque formation assay. FTEG$_4$OH (100 µM) delays Cip-induced phage mediated PAO1k killing.

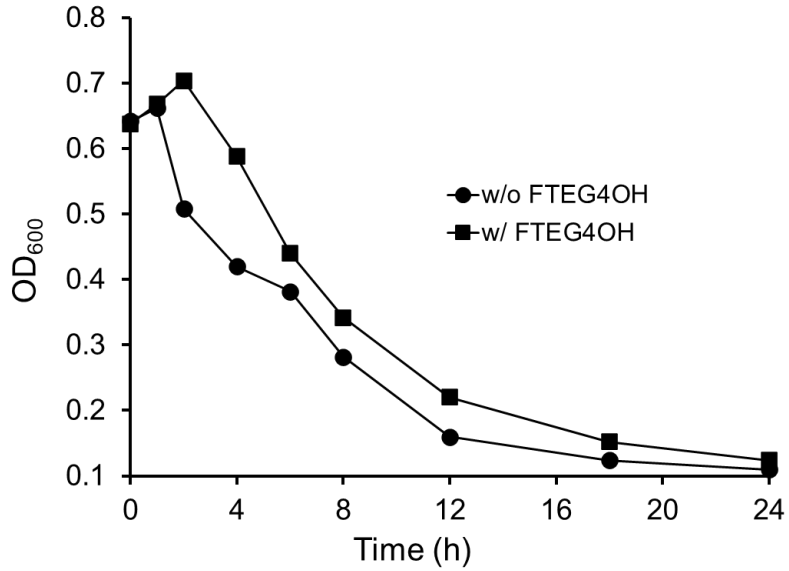


Figure 4.29 Planktonic Killing assay. FTEG$_4$OH (100 µM) delays Cip-induced phage mediated PAO1Cb^R*lacZ* killing.

We also tested if bacteria survived Cip-induced phage exposure with and without $FTEG_4OH$ after 24 hours. We found that in both cases, there are survival bacteria and we selected them on selective agar containing 75 µg/ml gentamicin, 150 µg/ml carbenicillin . In the presence of $FTEG_4OH$, we found that 10 times less bacteria survived.

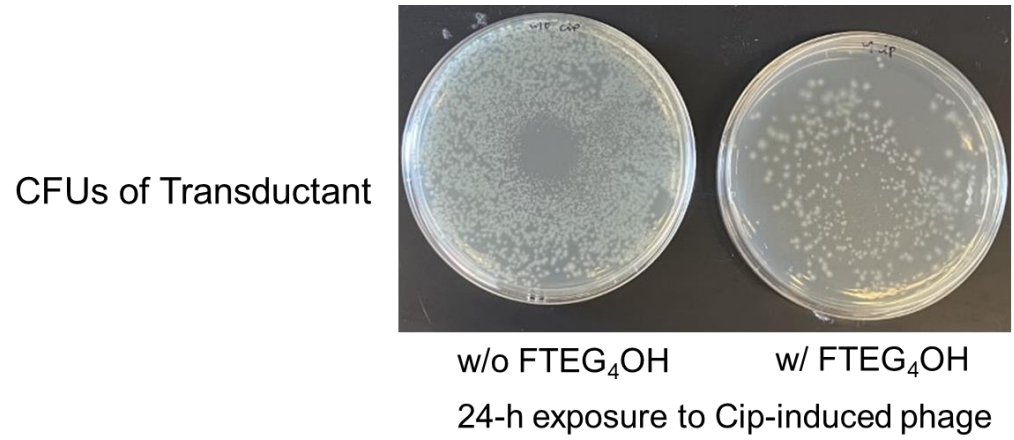


Figure 4.30 Colony forming unit assay. 100 µL of survived bacteria after 24 h cip-induced phage exposure was counted on selective agar containing 75 µg/ml gentamicin, 150 µg/ml carbenicillin.

To test if Cip induced phage caused transductional gene exchange with recipient PAO1Cb^R*lacZ,* we isolated bacteria survived after 24 h phage exposure in planktonic culture , and prepared a bacterial lawn using these bacteria, and then introduced drops of cip-induced phage supernatant to lawn to perform the plaque formation assay. We discovered that new transductant bacteria did not show plaques. in the presence of Cip-induced phages. This result suggests that this phage particle are lysogenic to the survived bacteria that was infected with cip-induced phages.

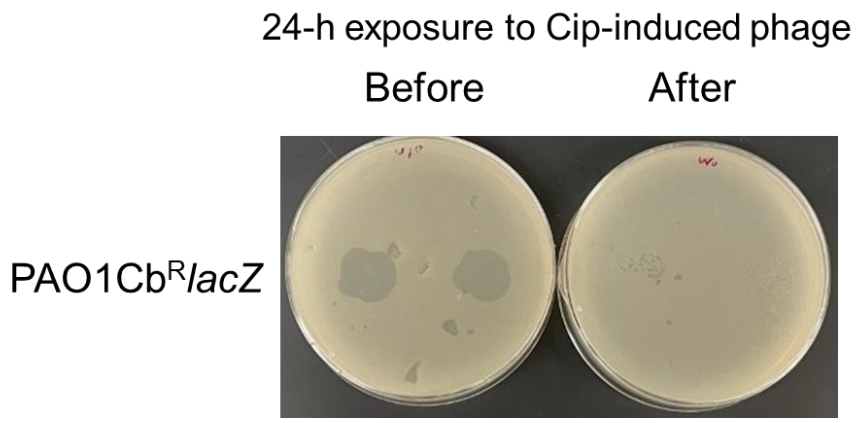


Figure 4.31 Plaque formation assay. 100 µL of PAO1Cb^{R}*lacZ* (before exposure and after 24-h exposure of cip-induced phages in planktonic culture was mixed in 1 mL of top agar and spread on 1.5% LB agar plates for 1 h, and 10 µL of supernatant cip-induced phage from PA14-PAPI was spotted on top agar and images were taken after 18 h.

4.6.4 Inhibition of intrinsic drug resistance to β-lactam, mechanism: FTC inhibits NagZ protein reducing the β-lactamase production.

β-lactam antibiotics like penicillin, cephalosporins, carbapenems showed activity against gram-negative bacteria like *E. coli* and *P. aeruginosa*.[220,288,289] Even though gram-negative bacteria acquire resistance to β-lactam antibiotics by overexpression of different β-lactamase enzymes which degrades antibiotic, extended spectrum β-antibiotics like aztreonam, imipenem, cefoxitin and ceftazidime tolerate some chromosomally encoded β-lactamase enzymes[288,289]. These extended spectrum β-antibiotics still are not totally resistant to broad spectrum β-lactamase enzymes found in clinical strains. The chromosomally encoded AmpC β-lactamase play crucial role in increased tolerance to extended spectrum β-antibiotics in gram-negative bacteria.

Previously, triazolyl glycolipid derivatives increase the susceptibility of a drug-resistant *S. aureus* (MRSA) to β-lactam antibiotics.[332] These molecules supposedly suppress penicillin binding protein 2a (PBP2a) which is responsible for β-antibiotic resistance in drug-resistant *S. aureus* (MRSA).[333,334]. These triazolyl glycolipid derivatives also showed to increase cell membrane permeability for β-antibiotics [332].

We tested effect of FTC on β-lactamase activity in gram-negative bacteria *P. aeruginosa*. We found that FTC reduces expression of basal β-lactamase activity in wild type PAO1. Usually β-lactam antibiotics induce expression of β-lactamase enzyme in bacteria resulting in increased resistance to β-lactam antibiotics. We tested effect of FTC on induction of β-lactamase activity in the presence of β-lactam antibiotic, Cefoxitin. Cefoxitin is potent inducer of β-lactamase and can antagonize the activity of other β-lactams against bacteria. We found that FTC reduced β-lactamase activity in the presence of Cefoxitin and increased efficacy against wild type PAO1 by 2-folds. (MIC changed from 1028 µg/ml to 64 µg/ml).

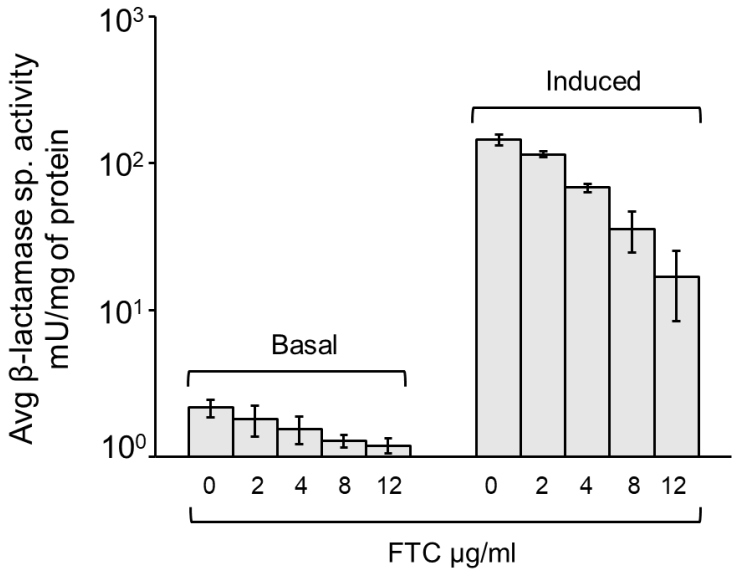


Figure 4.32 AmpC β-lactamase activity (measured in nanomoles per minute per milligram nitrocefin hydrolyzed) were measured by nitrocefin hydrolysis assay. Specific activity is in units of nanomoles of nitrocefin hydrolyzed per minute (mU) per milligram of protein. Induction was carried out by culturing PAO1 in the presence of 50 μg/ml cefoxitin for 3 h at 37°C.

β-N-Acetylglucosaminidase (NagZ), encoded by the *nagZ* gene, is a critical enzyme for *ampC* expression in gram-negative bacteria.[286,287,335]Activation of AmpC is needed for b-lactamase production in several pathogenic Gram-negative bacteria,[336,337]and *ampC* gene expression is regulated by cell wall peptidoglycan recycling. Hence, blocking NagZ activity with small-molecule inhibitors has garnered interest as a therapeutic strategy to suppress AmpC mediated b-lactam resistance. These inhibitors like O-(2-acetamido-2deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate (PUGNAc) and derivatives inhibits NagZ non-selectively.[336,337] NagZ offers conformational flexibility in binding pocket for inhibitor molecules.[336,337] Analogous to PUGNAc, addition of bulky substituents to the acetamido moiety of this inhibitor also makes it selective for NagZ.[337] This knowledge suggest that saccharide with hydrophobic moiety can potentially inhibit NagZ.

We hypothesized that FTC may have inhibitory effect on NagZ enzyme activity resulting in downregulation of β-lactamase expression[286,287,335]. We tested commercially available potent non-selective NagZ inhibitor PUGNAc and FTC against NagZ enzyme activity. We found that FTC reduced NagZ enzyme activity in wild type PAO1 by 65%.

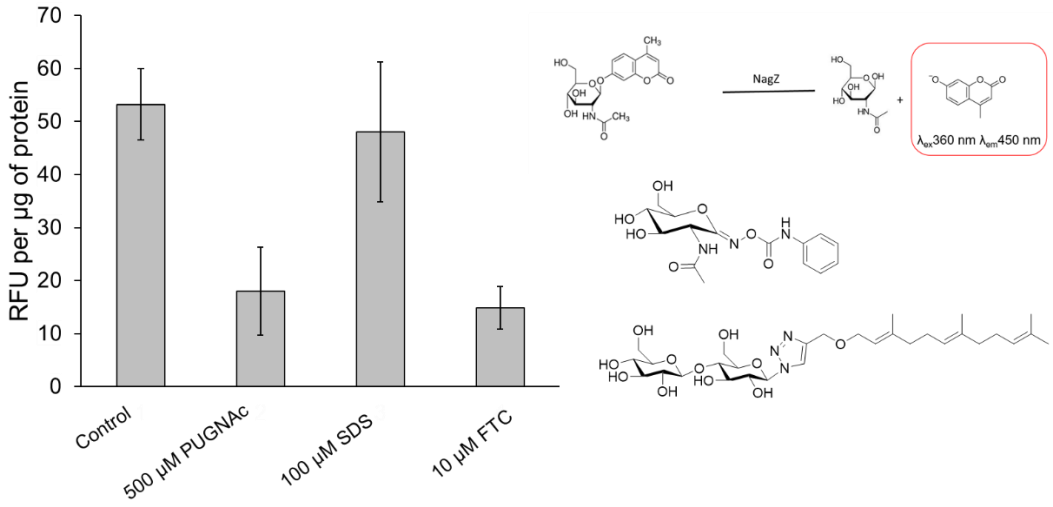


Figure 4.33 NagZ activity assay of wild-type *P. aeruginosa* in the presence of non-selective NagZ inhibitor PUGNAc (500µM) and FTC (10µM). NagZ activity was determined from sonicated cultures by monitoring fluorescence of 4-methylumbelliferone (4-MU) liberation at an excitation wavelength of 360 nm and monitoring of the emission at 450 nm.

The wild-type *P. aeruginosa* (PAO1) were treated with and without inhibitor PUGNAc (500µM) and FTC (10µM) for 12 h and lysate of cells of wild-type *P. aeruginosa* (PAO1) was assayed for N-acetyl-β-glucosaminidase activity by using 4-methylumbelliferyl N-acetyl-β-d-glucosaminide (4-MUGlcNAc) as the substrate. Liberated 4-methylumbelliferone (4-MU) was detected by measurement of the fluorescence by using an excitation wavelength of 360 nm and monitoring of the emission at 450 nm. We found that PUGNAc reduced NagZ activity by 52% while FTC reduced NagZ activity by 65%.

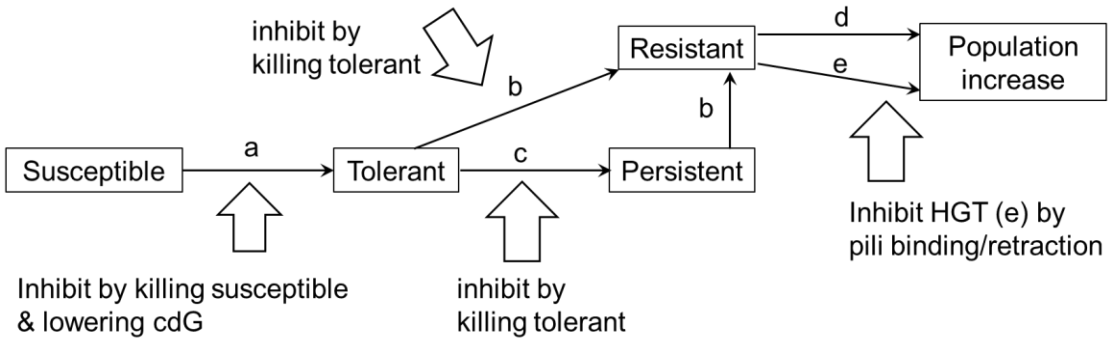


Figure 4.34 Possible schematic representation of emergence of resistance via tolerance and persistence route. a. Antibiotic treatment caused tolerance mutation. b. Tolerance and persistence facilitate resistance mutation. c. Tolerance transition to persistence. d. Generational gene transfer. e. Horizontal gene transfer.

4.6.5 FTC binds to Lectin A

The galactophilic protein Lectin A (LecA) of *P. aeruginosa* has a significant role in controlling biofilm formation and is positively correlated to the levels of cdG in bacteria.[297,298] FTC inhibited biofilm formation in *P. aeruginosa* PAO1 and PA14. We believe that FTC inhibits biofilm formation via LecA inhibition. We examined the binding of FTC to LecA (Fig. 1), by using an established fluorescence polarization-based competitive binding assay[296]. For this assay, we designed and synthesized a Dansyl fluorophore-tagged galactose ligand (βGal-aryl-Dansyl, Fig. X) for LecA protein. As a fluorophore-tagged ligand bind to a receptor protein, its fluorescent polarization increases as the motion and dynamics reduce in the solution. The βGal-aryl-Dansyl (200 nM) showed an increase in its fluorescence polarization with increasing concentrations of LecA (0-100 μM), with a transition indicative of a K_d ~10.7 μM, whereas for the control protein, bovine serum albumin (BSA), no significant increase in fluorescence polarization was observed. To estimate the binding strength of FTC to LecA protein, the molecules were titrated into a mixture of βGal-aryl-Dansyl (200 nM) and LecA (20 μM) to

displace the βGal-aryl-Dansyl from LecA. We found that FTC caused a decrease of fluorescence polarization indicative of an IC_{50} of 20 µg/ml.

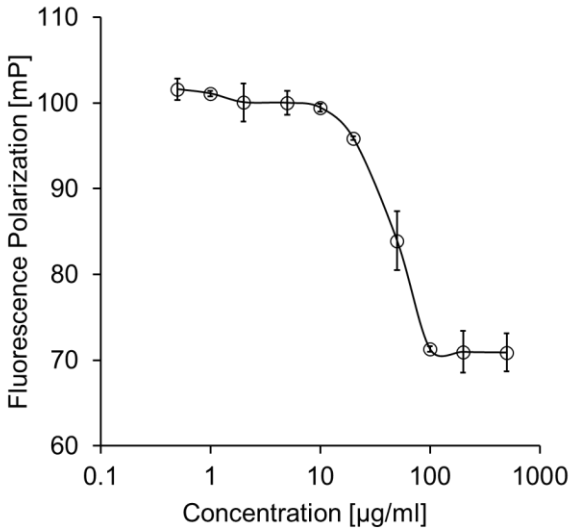


Figure 4.35 FTC binds to Lectin A. 100 uL of LecA (20 µM) and βGal-Dansyl (200 nM) versus concentration of FTC (1-500 µg/ml). Error bars indicate the standard deviations of means of triplicates.

Summary

The primary mechanism is spontaneous mutation, which is random and rare. Under stress, natural selection is not random, and cause the selected mutant to populate. There are two possible cases of "spontaneous mutations". A. Unprovoked, natural mutations. B. Under killing or growth inhibition stress, the resistant strains develop from tolerant and persistent one. For natural mutations, FTC does not show any resistant mutant. The mechanism of action is unclear. From initial high dose of antibiotics, there are two scenarios that the resistant mutant can emerge and populate. 1. vertical gene transfer. 2. Horizontal gene transfer. Vertical gene transfer is the transfer of genetic information, including any genetic mutations, from a parent to its offspring. The vertical gene transfer is hard to inhibit. The FTC and $FTEG_4OH$ inhibit pili and pili mediated activities which are necessary for horizontal gene transfer and different mechanisms of gene transfer. FTC also inhibits another receptor proteins Lectin A which is closely related to global messenger c-di-GMP. The levels of c-di-GMP dictate tolerance and persistence in *P. aeruginosa* and different bacteria. As tolerant and persistent bacteria are precursor to resistance bacteria, we believe that FTC can prevent emergence of resistant bacteria over the time.

All in all, we believe that FTC can be future of antibiotics which exhibit broad spectrum activity against different bacteria and prevent resistance development.

Experimental section

4.6.6 Strains and Plasmids.

PAO1-mCherry (Tc^R) was given by George A. O'Toole's lab (Dartmouth College). PA14 140495 (PAPI-1) and PAO1Cb^R*lacZ* were given by Stephen Lory's lab (Harvard Medical School). All strains were grown in LB medium supplemented with the appropriate antibiotics. PAO1-mCherry (Tc^R) was served as plasmid donors, while PAO1 wild type was served as plasmid recipient in transformation assays. PA14 140495 (Gm^R) and PAO1*lacZ* (Cb^R) were served as donor and recipient in conjugation assays respectively.

4.6.7 Inhibition of transpeptidase activity of Penicillin binding proteins (PBPs)

An ELISA-like protocol was used in black-walled Costar microtiter plates (Costar #3631), with gentle rocking at room temperature used for all steps. For PBP1a attachment, wells were treated with 2 μg of PBP in 50 μL of PBS/20% glycerol at 25 °C for 30 min, followed by treatment (3x) with 150 μL/well of blocking buffer (PBS/0.2% Tween-20), and then washed (3x) with 200 μL/well of washing buffer (PBS/0.05% Tween-20). To label PBP1a in initial proof-of-principle experiments, 50 μL of 100 μM BIO-AMP in PBS was added to the wells. After 10 min the PBP1a was denatured heating at 80 °C for 3 min followed by quick cooling on ice. The plates were then washed (3x) with washing buffer. Bound biotin-Amp-PBP was quantified by Pierce™ Biotin Quantitation Kit.

With inhibitor: For PBP attachment, wells were treated with 2 μg of PBP1a in 50 μL of PBS/20% glycerol at 25 °C for 30 min, followed by treatment (3x) with 150 μL/well of blocking buffer (PBS/0.2% Tween-20), and then washed (3x) with 200 μL/well of washing buffer (PBS/0.05% Tween-20). 50 μL of different concentration of inhibitor FTC in PBS was added to the wells. After 1 h, 50 μL of 100 μM BIO-AMP in PBS was added to the wells. After 10 min

the PBP1a was denatured heating at 80 °C for 3 min followed by quick cooling on ice. The plates were then washed (3x) with washing buffer. Bound biotin-Amp-PBP was quantified by Pierce™ Biotin Quantitation Kit.

4.6.8 Inhibition of conjugation

We adopted the method of mating in static liquid culture[338] with the modification that inhibitor molecule, $FTEG_4OH$ was cultured with recipient strain ($PAO1Cb^R$*lacZ*), and included in the mating mixture culture. To prepare the culture of donor strain, PA14-140495 was grown with 75 µg/ml gentamicin in LB medium at 37°C overnight with shaking (250 rpm). The donor culture (50 µL, OD_{600} 1.0) was further diluted into 1 mL of LB and incubated at 37°C for 2-h with shaking (250 rpm). To prepare the culture of recipient strain, $PAO1Cb^R$*lacZ* was grown with 75 µg/mL carbenicillin in LB medium at 37°C overnight with shaking (250 rpm). $FTEG_4OH$ (stock 10 mM) was added to a 50 µL of recipient cultures (OD ~1.0) for a final concentration of 100 µM, and further cultured for 3 h at 37 °C. For gene transfer by conjugation, the prepared recipient culture (50 µL) was mixed with the prepared donor subculture (1.05 mL) to incubate at 37°C for 48 h statically. The resulting mating culture generated observable slimy substances, and was mixed with 1 mL of fresh LB, and then sonicated in bath for 10 min. The diluted culture in 15 mL falcon tube was immersed a water bath in the sonicator [SRA TruPower, UC-45D-PRO] and sonicated for 10 min with 50% power. To quantify the PAPI-1 transfer, 100 µL of the final mating mixture was poured and spread on a selective agar containing 75 µg/mL gentamicin, 150 µg/mL carbenicillin and 40 µg/mL X-Gal. The number of transconjugants (blue colonies) was counted after 48 h incubation. The transfer frequency was calculated by the total number of transconjugants (blue colonies) divided by the total recipients (CFU after 48 h culture at 37°C)

4.6.9 Inhibition of transformation

For transformation, the plasmid donor strain, PAO1-mCherry (Tc^R) was cultured with or without the pili inhibitor, $FTEG_4OH$. The donor culture was then sonicated by a tip sonicated to release the DNA plasmids from the bacteria, the bacteria and its debris was removed by centrifugation and filtration. The supernatant containing mCherry (Tc^R) plasmid was mixed with the recipient strain, *wt* PAO1 culture with the presence of $FTEG_4OH$ under stress of sub-lethal amount of tetracycline (20 µg/ml). Sub-lethal concentrations of antibiotic promote the intake of plasmid.[50] The number of transformed bacteria was quantified by colony forming units on LB agar plate containing 250 µg/ml tetracycline. To prepare the recipient culture, the inhibitor molecule, $FTEG_4OH$ (10 mM stock), was added into 1 mL of PAO1 recipient subculture (OD = 0.8) to obtain final concentration of 100 µM, and further cultured for 3 h at 37°C with shaking (250 rpm). To prepare the supernatant of the donor strain, an overnight culture of donor strain (PAO1-mCherry) was centrifuged to obtain pellet. Pellet was resuspended in PBS buffer and cold sonicated using a probe sonicator at power level 1, ON for 5 sec., and OFF for 5 sec., and repeat 6 times; the culture was then centrifuged (4185 g, 15 min) to pellet the bacterial debris. This supernatant of donor strain was filtered with 0.22 µm syringe filter to further remove residual bacterial cells. Plasmid DNA concentration was estimated by measuring the absorbance at 260nm, adjusting the A260 measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that an A260 of 1.0 = 50 µg/ml pure plasmid DNA. Concentration (µg/ml) = (A260 reading – A320 reading) × dilution factor × 50µg/ml. The Plasmid DNA yield was calculated as plasmid DNA yield (µg) = DNA concentration × total sample volume (ml).[339]

For the transfer experiment, the supernatant (5 mL) of donor culture (PAO1-mCherry) was mixed with 1 mL of recipient culture that contained 100 μM of $FTEG_4OH$. To this mixture culture, 50 μL of 10 mM stock of $FTEG_4OH$ was added to maintain 100 μM in the total 6 mL mixture. Tetracycline was also added to the mixture to get a concentration of 20 μg/mL. The same mixed culture was also prepared without adding tetracycline as a control. The mixtures were cultured for 24 h at 37℃ with shaking (250 rpm). To quantify the plasmid transfer, colony forming units (CFU) of the recipient strains was counted on agar plates containing 250 μg/mL tetracycline.

4.6.10 Chemical inhibition of bacteriophage adsorption ϕKMV on P. aeruginosa.

Bacteriophage adsorption assay were adopted as described previously,[331,340] with addition of our agents. An overnight culture (100 μL) of *wt P. aeruginosa* strain PAO1k that is ϕKMV sensitive, was diluted with 10 mL of LB supplemented with 10 mM $MgSO_4$ (LB-Mg^{2+}) and subcultured to OD_{600} around 0.6 at 37°C with shaking at 250 rpm with and without 100 μM of $FTEG_4OH$. The bacteria subculture (100 μL) was mixed with 900 μL of ϕKMV phage. The titer of added phage was determined for every experiment from the phage stock solution. Following incubation for 10 min at 37 °C with shaking at 100 rpm, bacteria were removed by centrifugation (10,000 g, 5 min at 4°C), and 900 μL of the supernatant was transferred to an Eppendorf tube. The plaque-forming units (PFU) in the supernatant with and without the added agents was determined by the top agar overlay method with PAO1k. The percentage of phage bound to bacteria was calculated as [(titer of added phage − titer in supernatant)/(titer of added phage)] × 100.

4.6.11 Chemical inhibition of transduction on bacterial lawns and in planktonic culture.

To prepare phage-loaded supernatant from the donor strain, an overnight culture (150 µL) of PAPI-1 donor PA14-140495 (Gm^R) was diluted with 15 mL of LB and cultured at 37°C with shaking at 250 rpm to reach an OD_{600} of 0.6. Ciprofloxacin (stock 1 µg/mL) was added into this subculture to reach 0.05 µg/ml and cultured for additional 3 h at 37℃ without shaking; to promote the production of phages. The culture was centrifugated at 4185 g for 10 min to pellet the bacterial cells. The supernatant (10 mL) was filtered through a 0.22 µm filter to further remove bacterial cells and treated with 0.5 µL of Universal Nuclease (10 kU stock) and incubated for 1 h at ambient temperature; to degrade the DNA molecules in the supernatant.

To prepare the agar plate (0.7% top agar and 1.5% LB agar), 3 mL of warm top agar solution (7 g/l agar, 0.5 g/L sodium chloride, 10 g/l tryptone, 5 g/l yeast extract in sterile water) was mixed with 200 µL of PAO1k culture (OD 0.6), spread on 1.5% "hard" agar plate (LB-Miller, 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl ,15 g/l agar, 10-cm in diameter), and cooled for 15 min in biosafety level-2 hood to solidify the top gel at ambient temperature. The agar plates were incubated at 37 °C for 1 h statically. 10 µL of ciprofloxacin treated supernatant from PA14-PAPI-more details culture was added and incubated for 24 h.

To prepare the recipient culture, an overnight culture (100 µL) of the recipient strain PAO1Cb^R*lacZ* was sub-cultured in 10 mL LB supplemented with and without 100 µM of inhibitor molecule, $FTEG_4OH$ (from a stock of 10 mM) at 37°C with shaking (250 rpm) to reach an OD_{600} of 0.6.

On bacterial lawn. 100 µM of $FTEG_4OH$ was added onto solidified top agar layer and incubated for 15 min in biosafety level-2 hood at ambient temperature. The agar plates were incubated at

37 °C for 1 h statically. 10 µL of ciprofloxacin treated supernatant from PA14-PAPI-more details culture was added onto same spot as $FTEG_4OH$ and incubated for 24 h.

In planktonic culture. Overnight culture of wild type PAO1 was diluted 100x in LB, and subcultured (10 mL) with and without FTEG4OH (100 µM) to an OD of 0.6. Phage loaded supernatant (1 mL, see above) was added to both subculture, and the optical density (OD_{600}) was monitored over 24 h.

4.7. Synthesis and Characterization data

4.7.1 Synthesis of Farnesol Triazole tetraethylene glycol (FTEG$_4$OH)

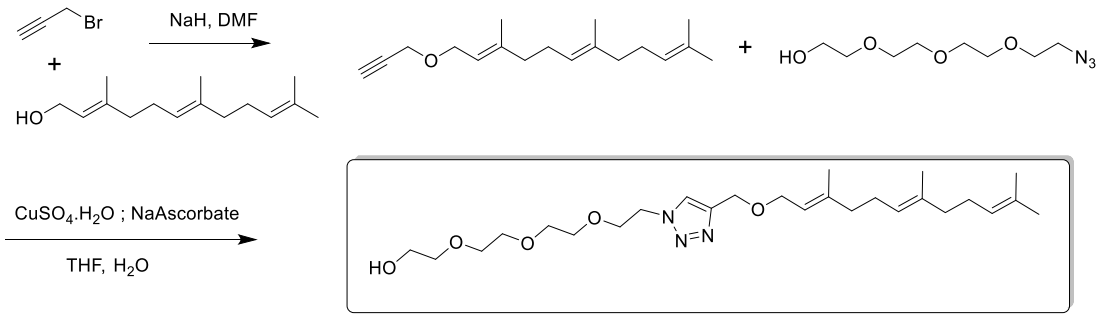


Figure 4.36 Synthesis scheme for Farnesol Triazole Tetraethylene glycol (FTEG$_4$OH)

4.7.2 Synthesis of Farnesol Triazole Cellobioside (FTC)

Synthesis of Farnesol triazole cellobioside(FTC) is based on convergent synthesis that couples an alkyne-derivatized farnesol and a protected disugar derivatized with an azide by Click chemistry, followed by the deprotection of the hydroxyl groups of the disugar under basic conditions (Scheme X).

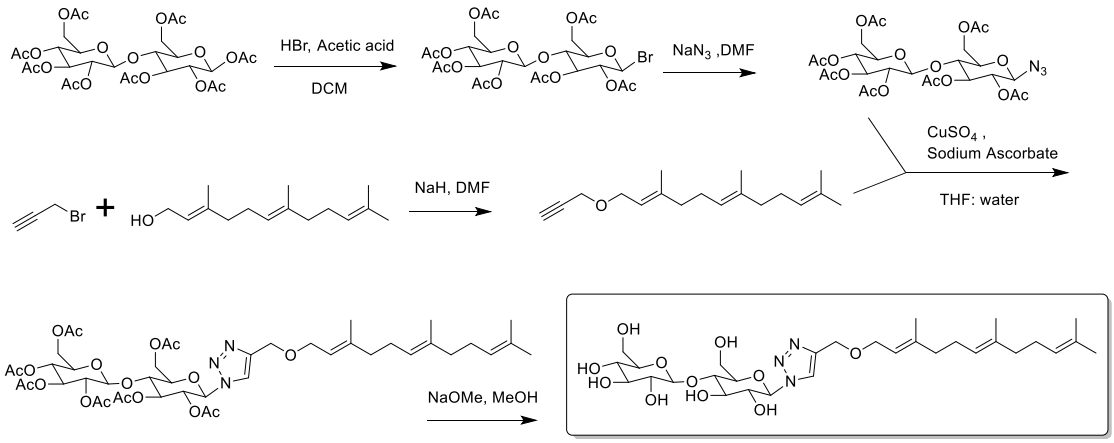


Figure 4.37 The synthesis scheme for Farnesol triazole cellobioside (FTC).

4.7.3 Synthesis of Farnesol oxadiazole cellobioside (FOC)

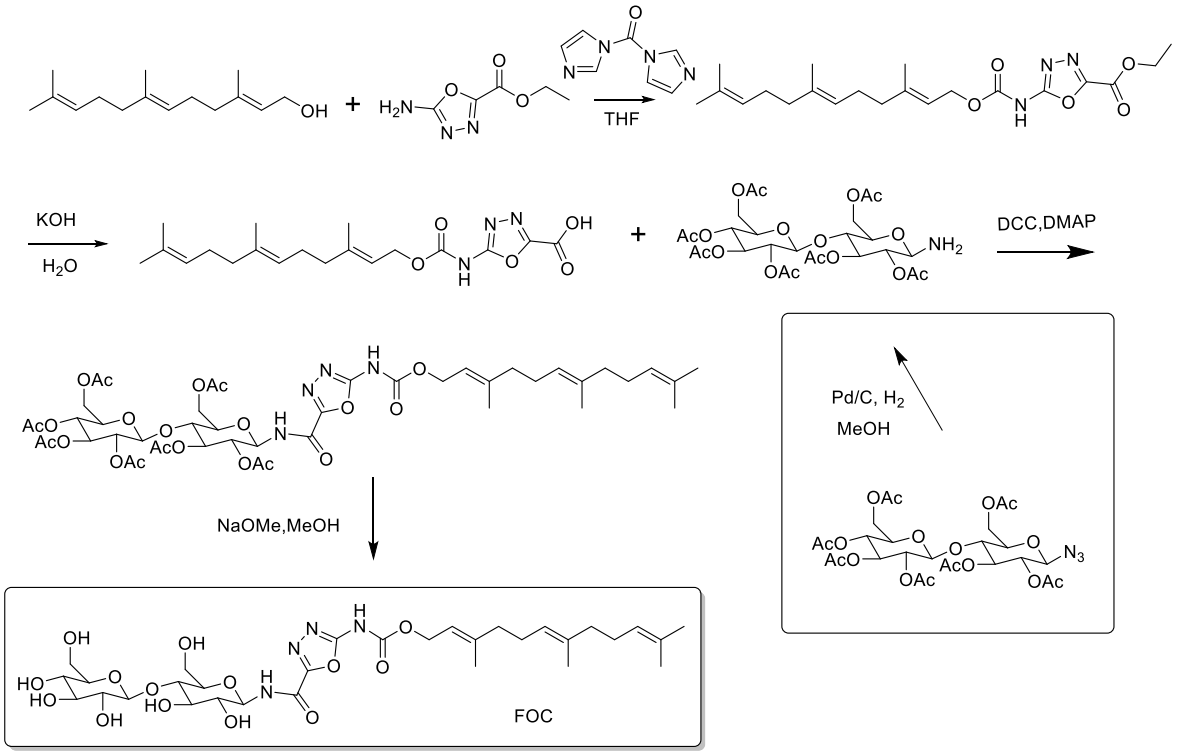


Figure 4.38 Synthesis scheme for Farnesol oxadiazole cellobioside (FOC).

7.4 Synthesis and characterization of intermediates for FTC,FOC and FTEG$_4$OH

First step of the synthesis involved conversion of acetocellobioside into acetobromocellobioside in the presence of hydrobromic acid in acetic acid and conversion of farnesol to propargyl farnesol ether.

Figure 4.39 Synthesis of acetobromocellobioside

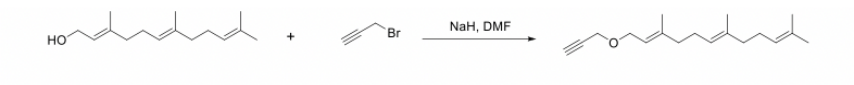


Figure 4.40 Synthesis of propargyl farnesol ether.

The ^{1}H NMR and ^{13}C NMR both showed the compound to be pure for acetobromocellobioside.

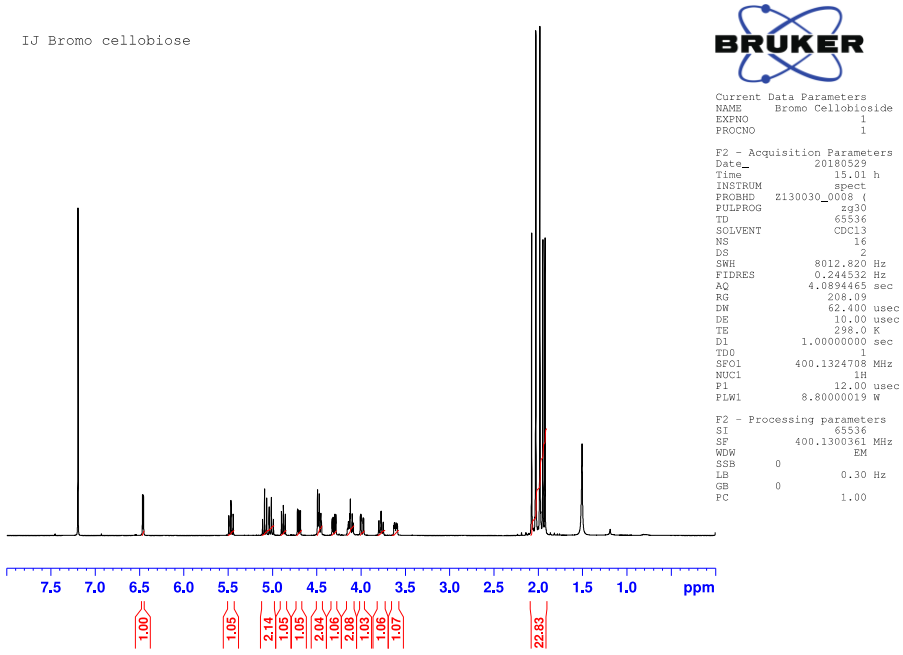


Figure 4.41 ^{1}H NMR of acetobromocellobioside.

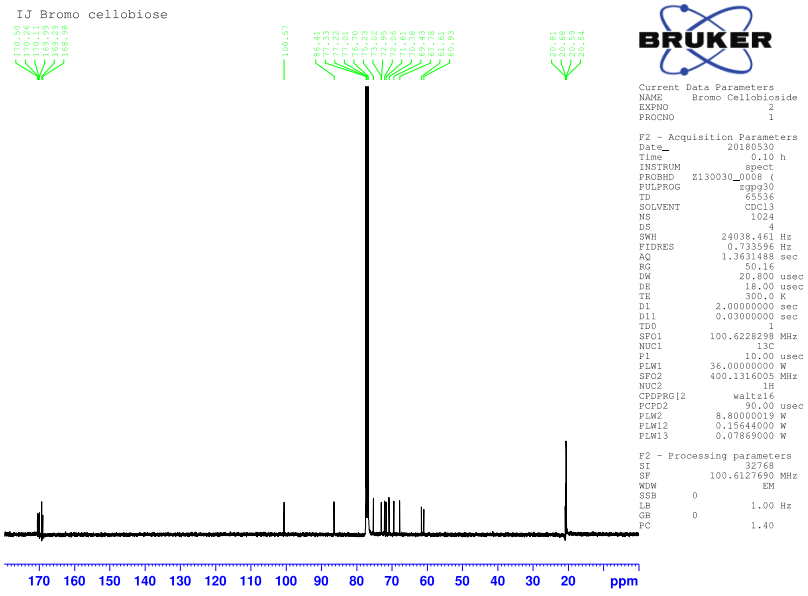


Figure 4.42 ^{13}C NMR of acetobromocellobioside

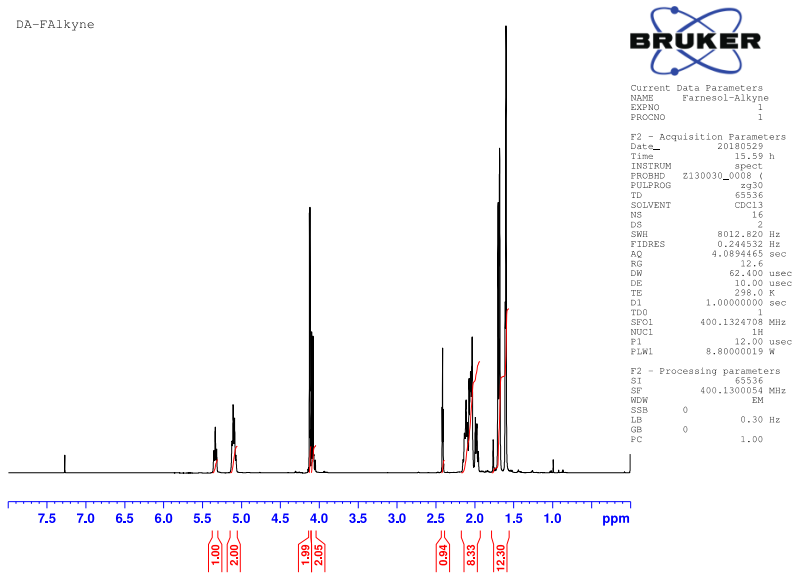


Figure 4.43 ^{1}H NMR of propargyl farnesol ether.

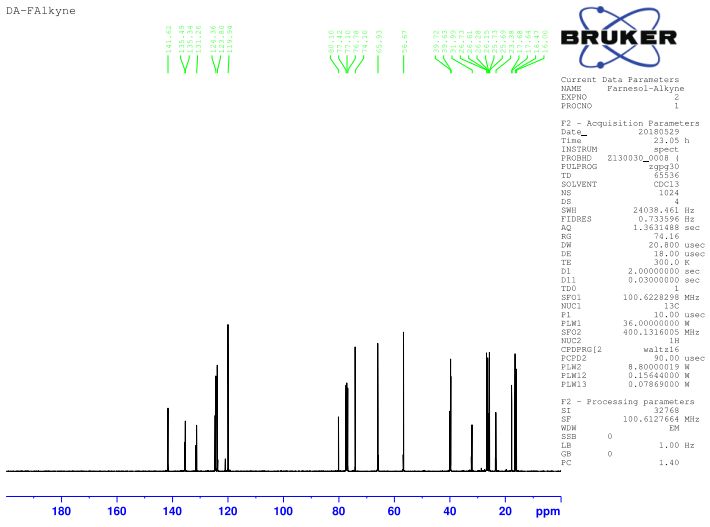


Figure 4.44 ^{13}C NMR of propargyl farnesol ether.

The bromoacetocellobioside was converted to azidoacetocellobioside by simple nucleophilic substitution reaction with sodium azide.

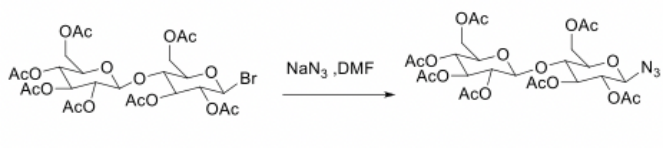


Figure 4.45 Synthesis of azidoacetocellobioside

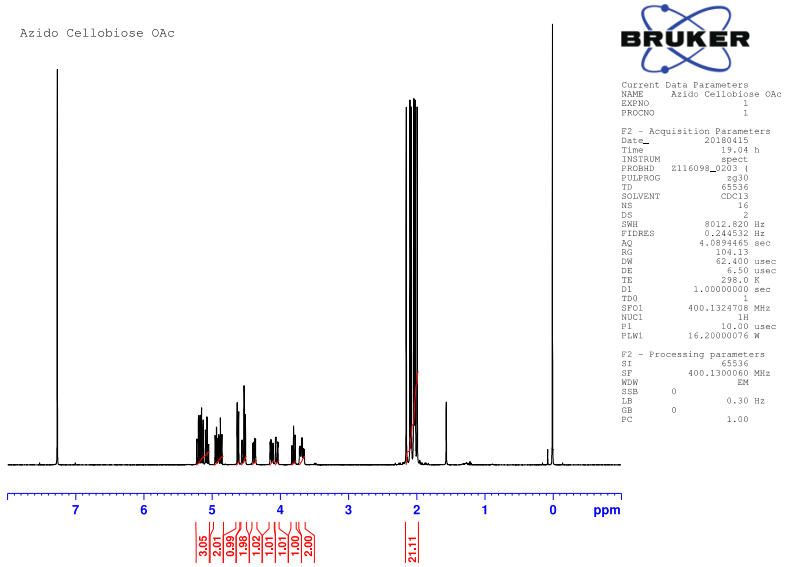


Figure 4.46 ^{1}H NMR of azidoacetocellobioside

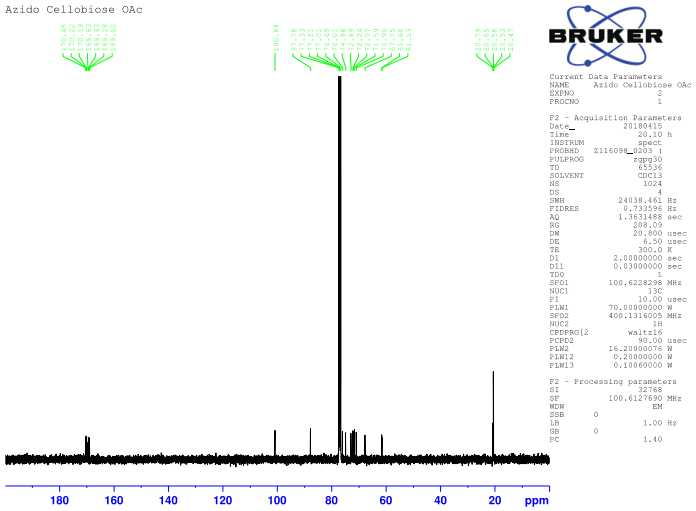


Figure 4.47 ^{13}C NMR of azidoacetocellobioside

After synthesis of azidoacetocellobioside, click chemistry was performed on farnesol propargyl ether and azidoacetocellobioside.

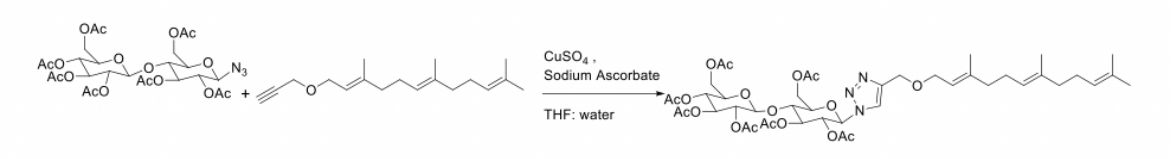


Figure 4.48 Synthesis of acetylated farnesol triazole cellobioside (AcFTC).

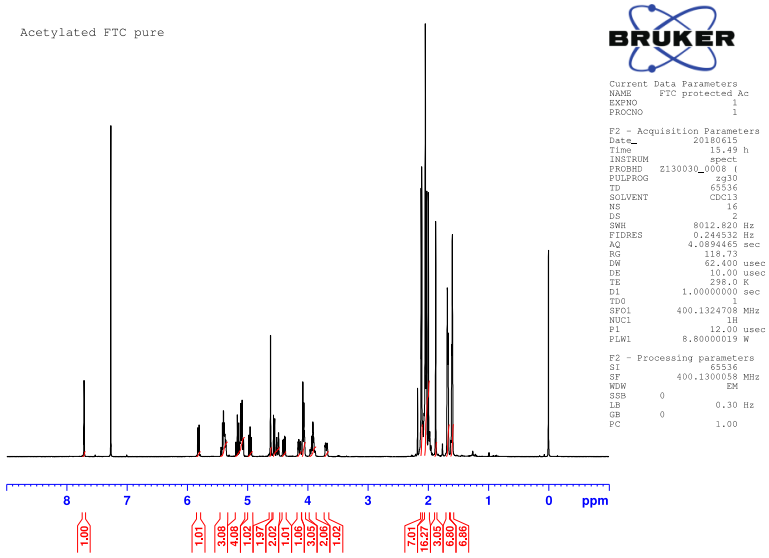


Figure 4.49 ^{1}H NMR of acetylated FTC

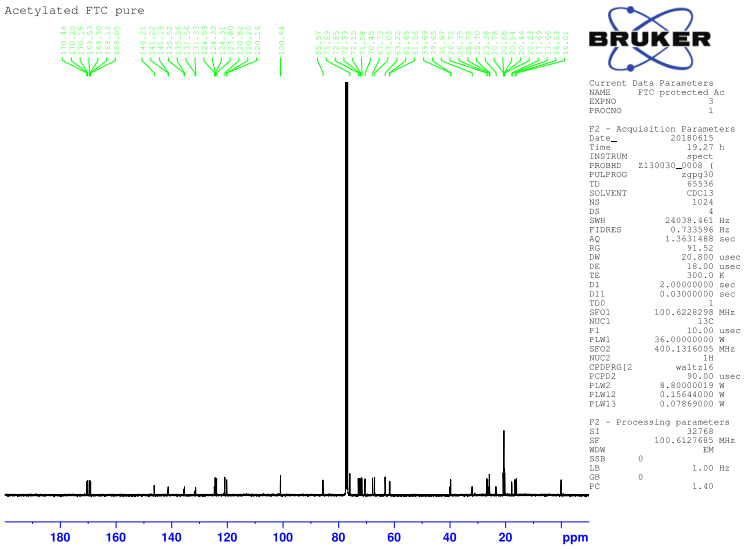


Figure 4.50 ^{13}C NMR of acetylated FTC.

The final step of the reaction was the deacetylation of the acetylated FTC by Zemplén deacetylation using sodium methoxide solution (MeONa/MeOH).

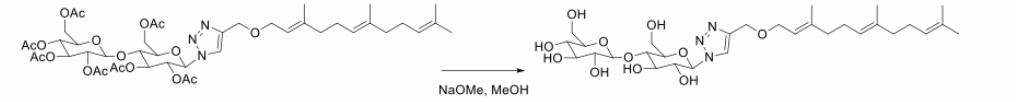


Figure 4.51 Deacetylation of acetylated FTC.

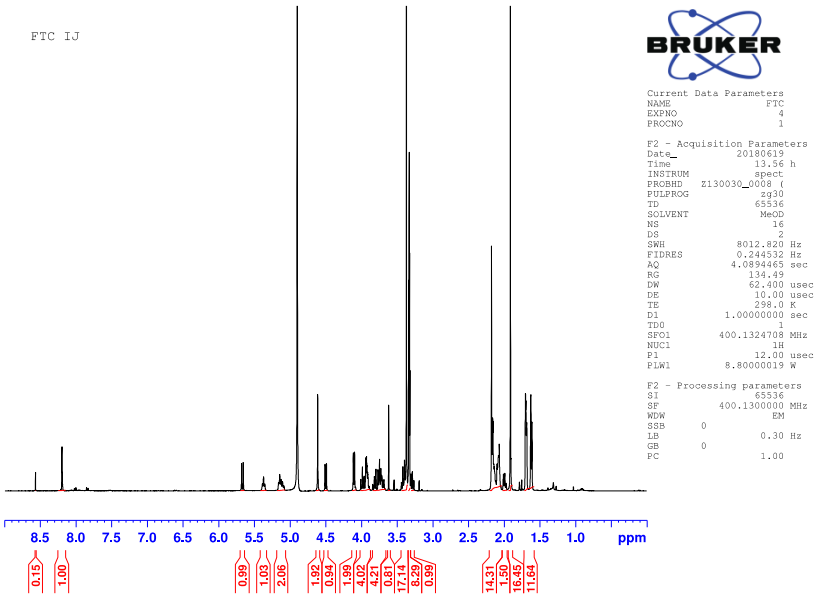


Figure 4.52 ^{1}H NMR of FTC

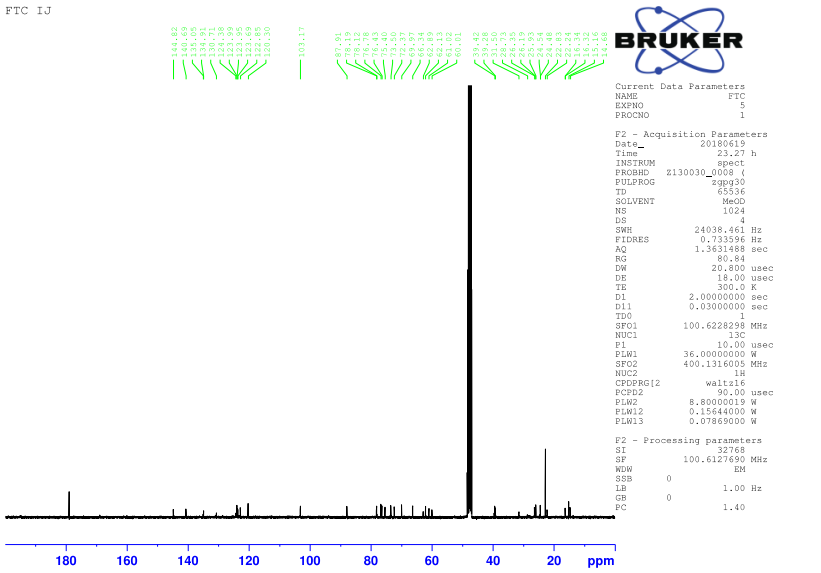


Figure 4.53 ^{13}C NMR of FTC.

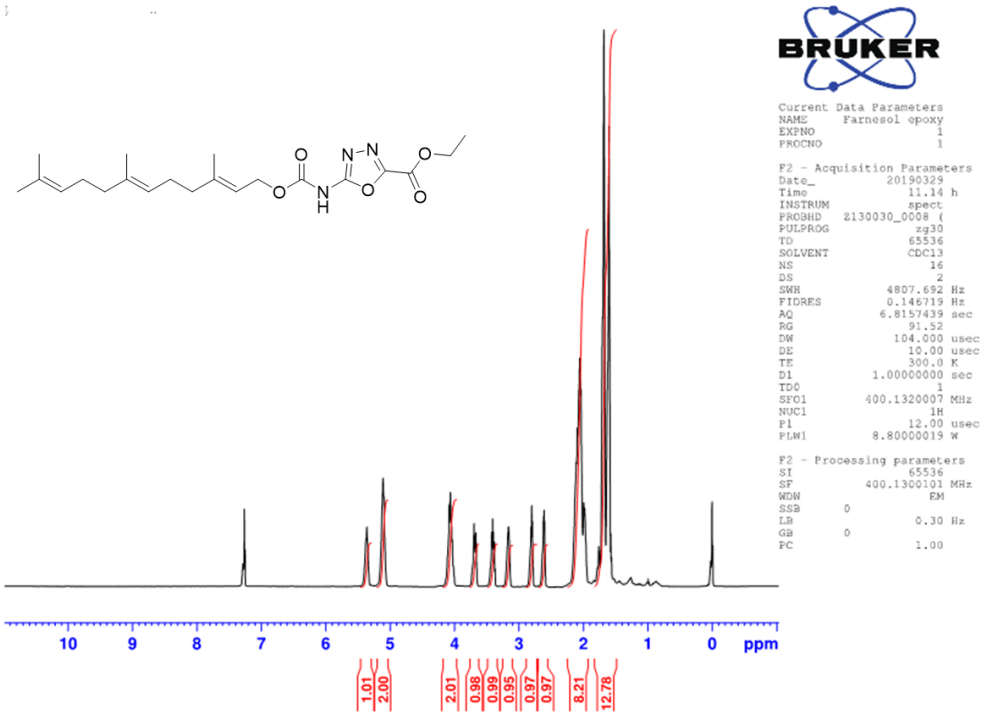


Figure 4.54 ^{1}H NMR of farnesol oxadiazole ethyl ester.

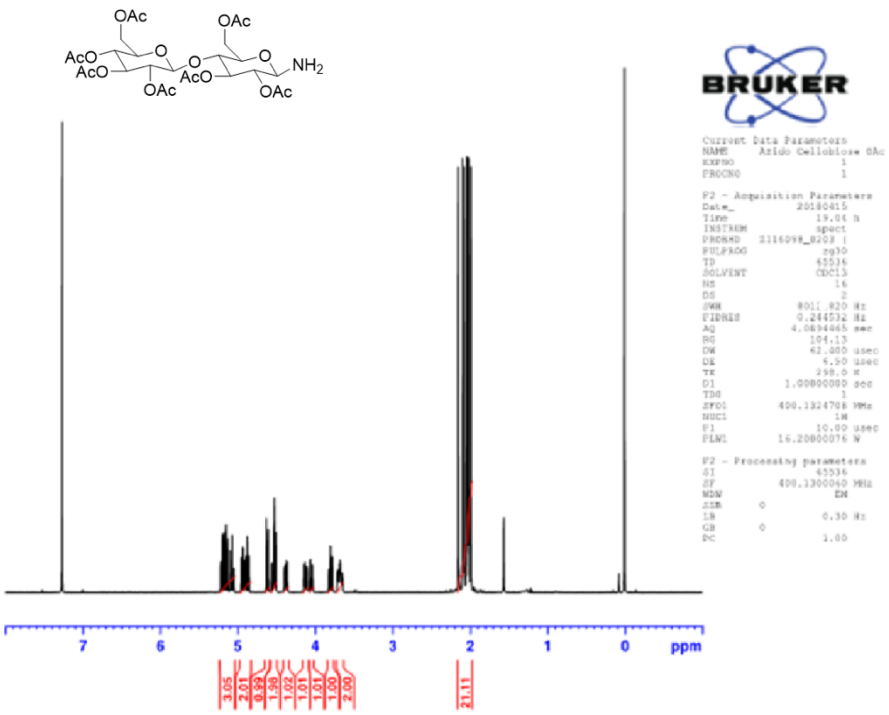


Figure 4.55 ^{1}H NMR of aminoheptaacetocellobioside.

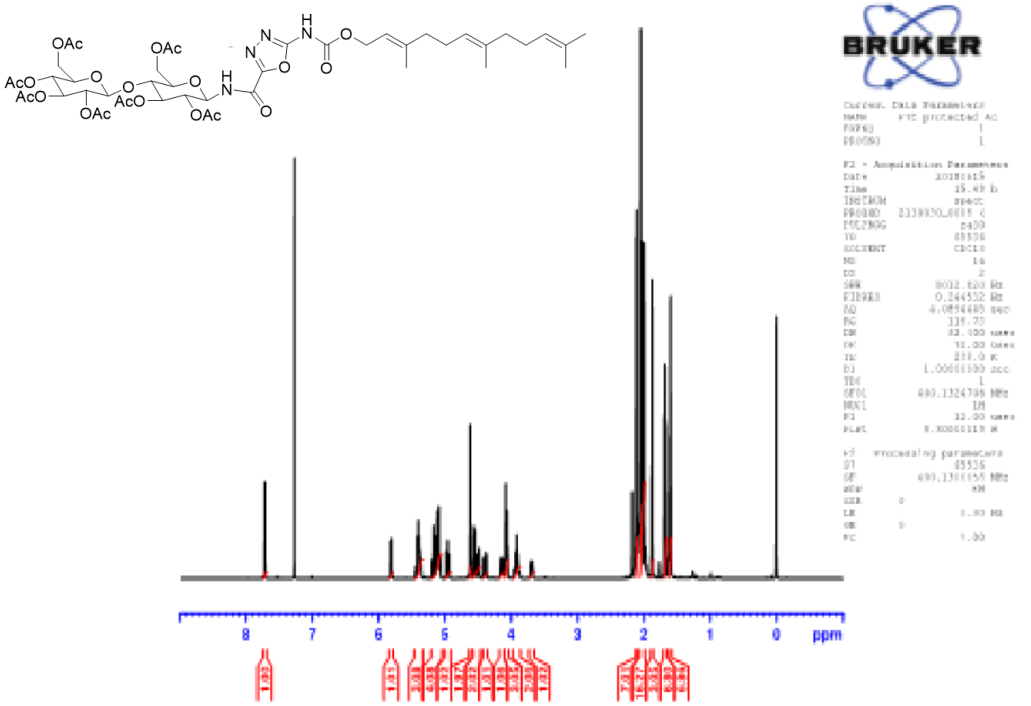


Figure 4.56 ^{1}H NMR of protected farnesol oxadiazole cellobioside.

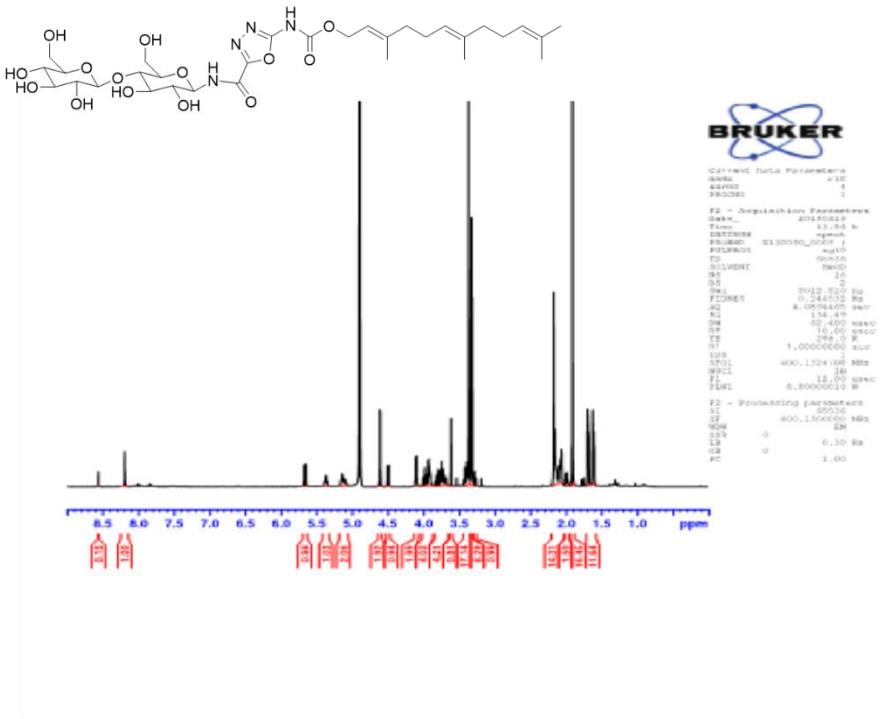


Figure 4.57 ^{1}H NMR of deprotected farnesol oxadiazole cellobioside.

Chapter 5. Farnesol Potentiates Silver against Burn Wound Microbiome by Inhibiting Pyocyanin and Promoting Rhamnolipids Production.

5.1 Background and Significance

5.1.1 Silver exhibit broad-spectrum antibacterial activity.

The antibacterial activity of silver has been known for known for thousands of years dated back around 400 B.C.[341,342] Since ancient Greeks utilized silver for stomach aches and wound healing, silver was likely the most important antibacterial compound before the introduction of antibiotics.[343] It is currently utilized on hospital surfaces to minimize nosocomial illness.[344,345] It's also found in water purification systems including hospital hot water circuits, swimming pools, and potable water distribution systems.[345] Silver can also be found in Japanese Jintan tablets, which are used to treat nausea, vomiting, hangovers, foul breath, and sunstroke, among other things.[341,342] Similarly, silver ions have been recognized as a highly effective potentiator of different classes of antibiotics.[346,347] Finally, silver nanoparticles are one of the most commonly employed nanomaterials in medicinal, antibacterial, and electrical applications.[345]

5.1.2 Silver targets multiple bacterial macromolecules.

Despite their long and widespread use, the exact mechanism of silver as antibacterial agent remains unknown. Interestingly, metal silver has no biological function while only ionic state (Ag+) is highly toxic to microbes. Silver ions are strongly bind to nucleic acids and form tight complexes with DNA or RNA.[348] They interact preferentially with bases rather than the

negatively charged backbone of DNA. Ag^{+} toxicity arises from nonspecific binding to DNA.[348] Silver ions also target thiol containing cytoplasmic proteins like enzymes and leads to inactivation of enzymes. membrane proteins, including those associated with electron transport.[349,350] Silver ions also showed to alter cell envelope and shrinking of cell membrane.[350] There is also debatable mode of action of silver ions like ROS production and perturbation of iron homeostasis.[351]

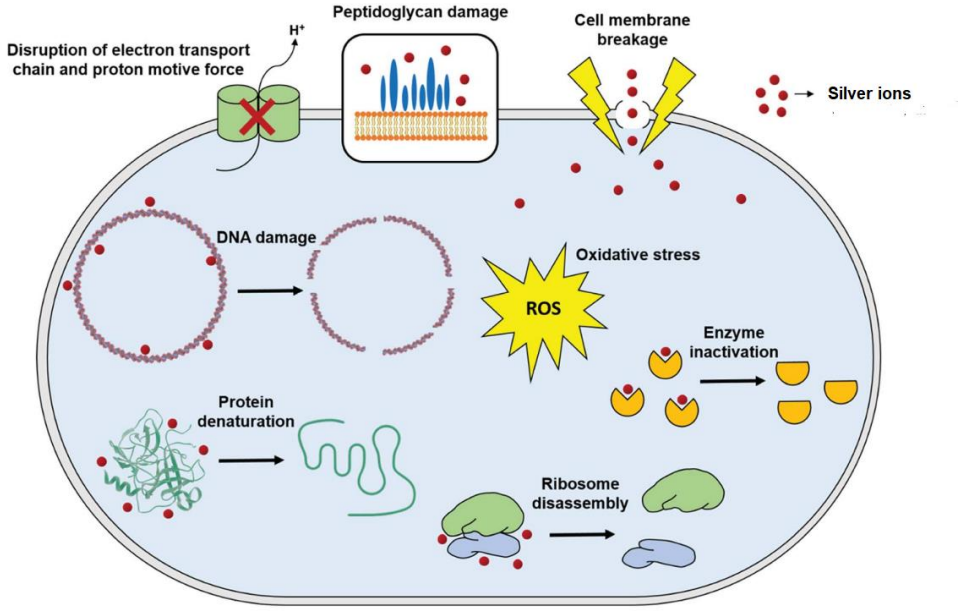


Figure 5.1 General mechanisms for antimicrobial mode of action of silver ions against bacteria. (This figure is adapted and modified from "A. Roy, O. Bulut, S. Some, A.K. Mandal, M.D. Yilmaz, Green synthesis of silver nanoparticles: biomolecule-nanoparticle organizations targeting antimicrobial activity, RSC advances, 9 (2019) 2673-2702.")

5.1.3 Emergence of silver resistance.

While antibacterial targeting multiple cellular targets as mode of action for killing are believed to combat development of drug resistance, [343,349,352] silver resistance has occurred over the years.[353-356] Bacterial develop resistance to silver typically by gene mutation and requires changes in

silver-binding proteins, associated efflux pumps which export the bound silver ion from the bacterial cell.[353,354] Horizontal gene transfer also showed to rise silver resistance in many bacterial species.[357,358] Recently, a novel mechanism of silver resistance was reported for the well-known opportunistic gram-negative bacterium *Pseudomonas aeruginosa*. *P. aeruginosa* produces pyocyanin pigment which reduces active silver ions to inactivate silver ion leading to resistance by reducing Ag+ to Ag metal. Pyocyanin is a blue-green redox-active secondary metabolite (Fig.5.2) that is produced by *Pseudomonas aeruginosa*.[359-361]Pyocyanin acts as an extracellular electron carrier and participate in redox reactions.[361,362] Due to its redox activity, pyocyanin increases the production of reactive oxygen species and also depletes the pools of antioxidant molecules like glutathione.[360,361,363] Pyocyanin is a zwitterion that can easily penetrate biological membranes and is readily recovered in large quantities from the wound exudates.[360] Pyocyanin can also act as interspecies communication signal between *P. aeruginosa* and *S. aureus*.[364] These two common bacterial species are highly sensitive to Ag^{+} individually, but when they are dwelling together, they develop resistance to silver ions.[364]

5.1.4 Silver therapy is widely used against burn wound infections.

Burn wounds are ideal site for harvest of bacteria and cause persistent infection than surgical wounds, mainly because of the larger area involved and longer duration of patient stay in the hospital.[365-367] Bacterial infection is a major cause of morbidity and mortality in hospitalized burn patients. About 75% of the mortality from burn injuries is estimated to be related to bacterial infections rather than osmotic shock.[365] Burn wound harbors microorganisms like *Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus pyogenes*.[365,368] Burn wound impetigo and folliculitis are usually caused by bacterial colonization (high concentrations ($>10^5$

CFU bacteria/g of tissue) in the burn wound and scab. However, no evidence exists that the infection is invasive.[365,368] Burn wound infections usually harbors *P. aeruginosa* and large amounts of pyocyanin have been identified in burn wound exudates of infected patients.[369,370] Silver ion has been used as first line antibacterial to treat burn wound infections. Silver ion-containing creams are favored topical ointments for large burns and marketed as Flamazine (10 mg/ml silver sulfadiazine) and Silvazine (10 mg/ml silver sulfadiazine plus 2 mg/ml chlorhexidine digluconate).[371] The silver-coated polyurethane negative-pressure wound therapy (NPWT) sponge are newer silver products, which has the advantages of NPWT with a sustained release of 20 to 40 ppm of silver ion.[372,373] These products are also helpful against biofilm-causing organisms, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, including methicillin resistant staphylococcus aureus(MRSA) more than other silver formulations, leading to faster healing in infected diabetic foot ulcers.[372-374]

5.1.5 Silver therapy face serious challenges in treatments of burn wounds.

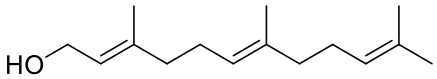
Silver formulations are widely used for the treatment of second-degree burns. However, sometimes silver therapy can have some of the worst outcomes in burn treatment, in terms of infection and wound healing. Silver ion treatments suffer major concerns like silver ion is effective only for the first few days/weeks, after which non-silver dressings should be used instead. For clean wounds and closed surgical incisions, silver confers no benefit and can lead to risk of resistance.[356]Burn wound infections have shown resistance to silver treatments.[356,370,375] Silver ions formulation like silver sulfadiazine impairs wound healing.[376,377]

The *Pseudomonas aeruginosa* and *Staphylococcus aureus* are two notorious bacteria that contribute to major burn wound infections and acquired resistance to silver therapy. The

dominant flora of burn wounds during hospitalization changes from Gram-positive bacteria such as *Staphylococcus* to Gram-negative bacteria like *Pseudomonas aeruginosa*.[378] The pattern of antimicrobial susceptibility of *S. aureus* and *P. aeruginosa* is a concern as these two species can cross communicate and develop adaptive resistance to antimicrobial treatment.[378-380] The coexistence of *S. aureus* and *P. aeruginosa* have been extensively studied in cystic fibrosis disease and coinfections leads to poor clinical outcomes[381-383].

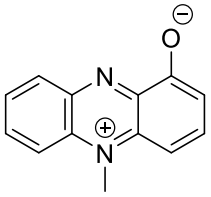
Even though *S. aureus* and *P. aeruginosa* frequently co-colonize chronic wounds,[384-386] the connection between them in the chronically infected wound is not well understood. Biopsies of chronically infected wounds have revealed that *S. aureus* and *P. aeruginosa* coexist,[379] with *P. aeruginosa* located in the deeper tissues and *S. aureus* at the wound's surface. These two pathogens are believed to initially co-exist and interact in a beneficial manner to develop resistance to antimicrobial therapy.[385,386] *P. aeruginosa* and *S. aureus* coinfections are more virulent than single infections.[387,388] Thus, coexistence of these two organisms is believed to create cooperative adaptive silver ion resistance.[364]

Farnesol is a sesquiterpene alcohol (Fig. 5.2) that exists widely in fruits such as peaches, herbs such as lemon grass, chamomile, and in the essential oils of ambrette seeds and citronella.[389,390] Farnesol is nontoxic to humans and found to alleviate massive inflammation, oxidative stress, and lung injury.[389-391]



Farnesol

(2E,6E)-3,7,11-Trimethyldodeca-2,6,10-trien-1-ol.



Pyocyanin

5-methylphenazin-1-one

Figure 5.2 Structure and IUPAC names of Farnesol and Pyocyanin.

Here we report that how Farnesol, the naturally occurring sesquiterpenoid reduce production of pyocyanin by *P. aeruginosa* and eradicate cooperative adaptive silver ion resistance when both *P. aeruginosa* and *S. aureus* are present.

5.2 Results

5.2.1 Farnesol is not toxic to burn wound pathogens.

Farnesol has been shown to inhibit growth of different bacteria like *Paracoccidioides brasiliensis, Staphylococcus epidermidis*.[392-394] To test effect of Farnesol on growth of burn wound bacteria, we established the growth curve of *P. aeruginosa* (wild type PA14) and *Staphylococcus aureus* (SA) in the presence of different concentrations of farnesol (10 – 500 µM). We found that farnesol does not affect growth of wild type PA14 or *S. aureus* (Fig.5.3).

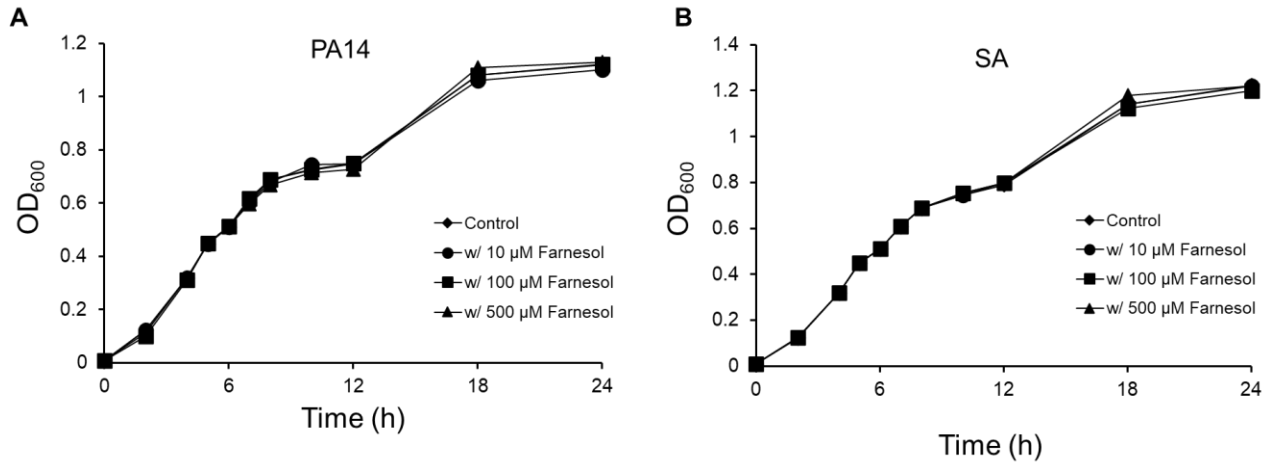


Figure 5.3 Growth curves of *P. aeruginosa* wild type PA14 and *S. aureus* in the presence of different concentrations of farnesol. Overnight cultures of A. wild type PA14 and B. *S. aureus* were diluted in MHB to optical density (OD_{600}) ~0.01 and incubated with different concentrations of Farnesol (10, 100, 500 µM) for 24 hours at 37 ℃ under 250 rpm shaking. The OD_{600} was measured at different time intervals.

5.2.2 Farnesol reduce pyocyanin production in P. aeruginosa PA14.

Pyocyanin has been shown to reduce toxic Ag^+ ion to inactive Ag metal.[395] We used mutant of P. aeruginosa which has deletion of pyocyanin synthesis gene *phzM*.[396]The pyocyanin deficient mutant strain (Δ*phzM*) showed increased susceptibility to silver ions (MIC = 5 ppm) in contrast to wild type PA14 (MIC = 15 ppm).[395] We tested minimum inhibitory concentration (MIC) of silver ions against wild type PA14, Δ*phzM* with and without Farnesol. We found that 100 µM Farnesol increased efficacy of silver ions against wild type PA14 by ~2 folds (MIC dropped from 15 ppm to 8 ppm). Interestingly, for pyocyanin deficient mutant (Δ*phzM*), MIC of silver ions was unaffected by presence of 100 µM Farnesol (Table 5.1). These results suggest that

Farnesol enhances the silver ion efficacy against *P. aeruginosa* PA14, and this enhancement is related to silver ion's effect on pyocyanin production.

Table 5.1 Effect of farnesol on the efficacy of silver ions against *P. aeruginosa* PA14, and pyocyanin deficient strains.

	MIC* (ppm)	
Strain	w/o Farnesol	w/ Farnesol
wild type PA14	15	8
Δ*phzM*	5	5

* Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of silver ions that completely inhibit visible growth of bacteria.

To evaluate effect of farnesol on pyocyanin production, we cultured PA14 with and without 100 μM Farnesol for 12 hours and quantified pyocyanin secreted in supernatant. We found that farnesol reduces pyocyanin production in PA14 by 80% (from ~ 5.1 μg/ml to 0.84 μg/ml) (Fig.5.4A). As pyocyanin is known to destroy silver ions by converting Ag^{+} to $Ag^{0}$and reduce its biological activity,[395] we test the hypothesis Farnesol increase silver ions efficacy by reducing levels of pyocyanin. We examined the level of silver ions in PA14 cultures (12 hours) with and without 100 μM Farnesol. Pyocyanin converts molecular oxygen to the superoxide free radical by oxidizing reduced nicotinamide adenine dinucleotide phosphate (NADPH) to Nicotinamide adenine dinucleotide phosphate ($NADP^{+}$).[360] We incubated silver ions (100 μM) in PA14 supernatant and NADPH (100 μM), and determined Ag^{+} concentration by sensitive dimethylaminobenzylidene rhodamine method. Without farnesol, PA14 supernatant reduced 80

% of silver ions (Ag^{+}) to silver metal (Ag^{0}). The PA14 supernatant treated with farnesol only reduced Ag+ by 15% (Fig.5.4 B).

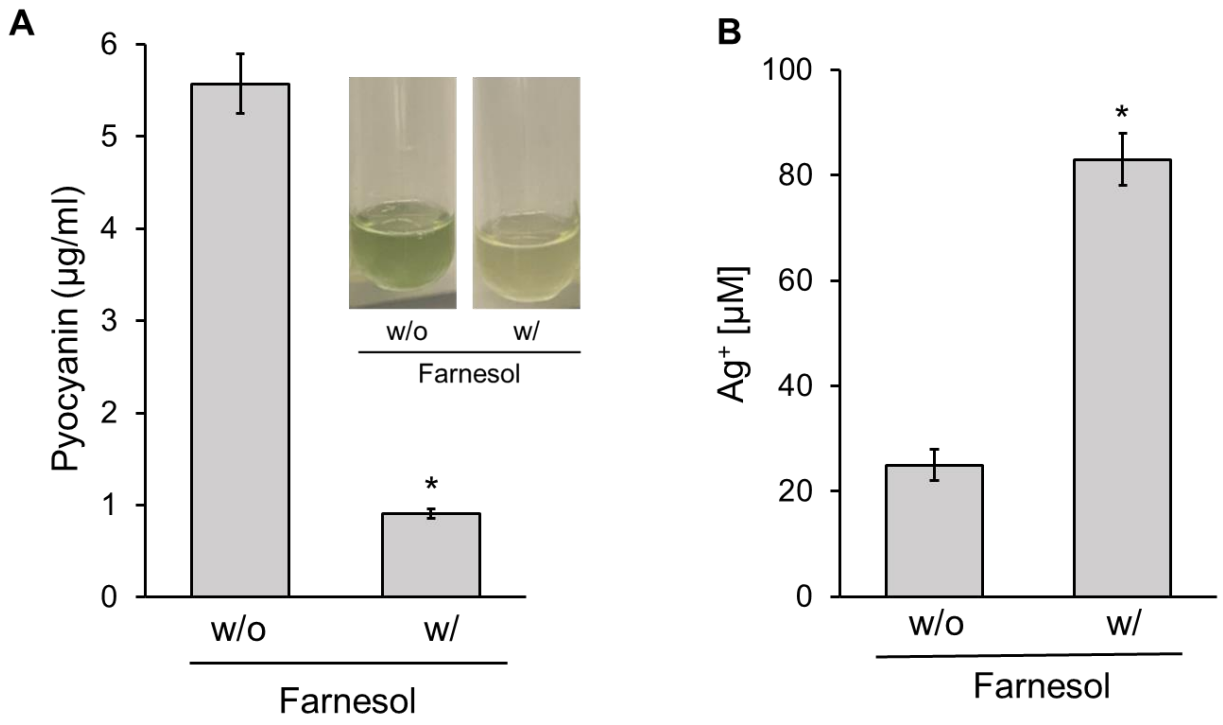


Figure 5.4 Effect of Farnesol on Pyocyanin production and silver degradation by *P. aeruginosa*. A. Pyocyanin levels in supernatant of planktonic cultures of wild type PA14 treated with and without 100 µM of Farnesol for 12 h in fresh King's A medium. The pyocyanin levels were measured as µg/ml of supernatant. Images represent green pigment pyocyanin production with and without Farnesol. More green color represents more pyocyanin production. Error bars are standard error of the mean from three replicates. The rhamnolipids produced without Farnesol was considered as control. *P = 0.01 vs control as evaluated using two-tailed unpaired Student's t-test. B. Silver ion concentration after treatment of 100 µM silver ions with supernatant of PA14 treated with and without Farnesol for 12 hours. A solution of Ag^{+} (100 µM) was reduced in the presence of PA14 supernatant and NADPH (100 µM) in water (pH 7.0; 32°C) and remaining

silver ions concentrations were determined by dimethylaminobenzylidene rhodamine dye method. Error bars are standard error of the mean from three replicates. The silver ions concentration without Farnesol was considered as control. *P = 0.05 vs control as evaluated using two-tailed unpaired Student's t-test.

5.2.3 Farnesol Increases rhamnolipid production in P. aeruginosa PA14.

P. aeruginosa uses quorum sensing (QS) to sense the presence of small molecules they secreted, and thus their population, and response by forming different phenotypes. This QS is regulated by the interlinked *las*, *rhl*, *pqs* gene systems. Interestingly, modulation of *rhl* and *pqs* activities induces inverse regulation of pyocyanin and rhamnolipid production in *P. aeruginosa*.[397,398] *P. aeruginosa* produces biosurfactant named rhamnolipids which modulate motility and biofilms.[399,400] Our previous results showed that Farnesol reduces pyocyanin production in PA14, here we also evaluated effect of Farnesol on rhamnolipids production. We quantified rhamnolipids production in the presence of Farnesol by methylene blue complexation method.[401] We found that Farnesol increased rhamnolipids production by 50% (94 µg/ml to 144 µg/ml) (Fig.5.5).

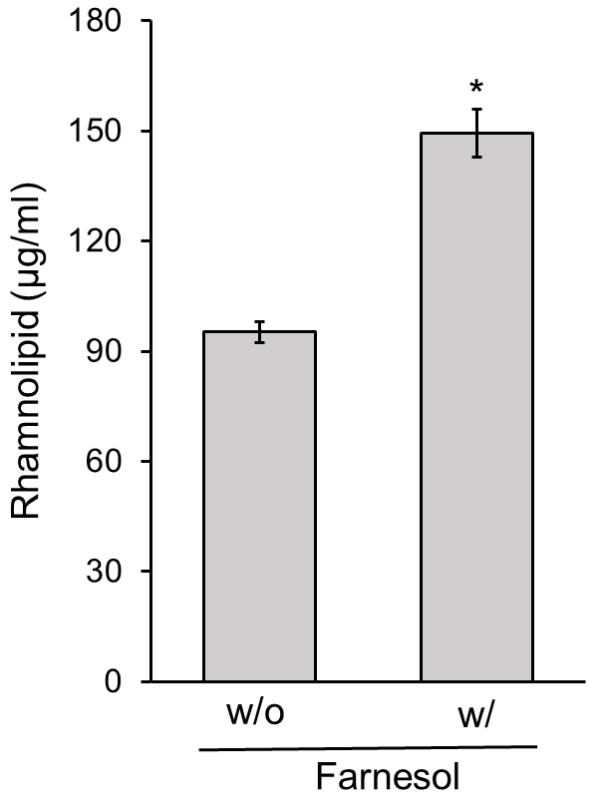


Figure 5.5 Effect of Farnesol on rhamnolipids production by *P. aeruginosa* PA14. Rhamnolipids productions by planktonic cultures of wild type PA14 treated with and without 100 µM of Farnesol for 18 hours were determined using an established methylene blue complexation assays. The positively charged methylene blue form noncovalent complex preferentially with negatively charged surfactants and does not bind to nonionic surfactants. The methylene blue bound with negatively charged surfactants has an increased partition into the organic chloroform phase bought in contact with the aqueous solution. The methylene blue in the chloroform phase were measured for its UV absorbance, which infers the concentration of rhamnolipids (µg/ml) in the culture. Error bars are standard error of the mean from three replicates. The rhamnolipids produced without Farnesol was considered as control. **P* = 0.02 vs control as evaluated using two-tailed unpaired Student's t-test.

5.2.4 Farnesol Increased Dispersion in Dual Species Biofilms.

Considering that both *P. aeruginosa* and *S. aureus* may co-exist in wound infections, the impact of these bacterial interaction on biofilm formation is inevitable. The mono and dual species biofilms by these both bacteria are highly tolerant to silver ions.[402,403] Pyocyanin in *P. aeruginosa* biofilms contribute positively to silver tolerance.[403,404] Moreover, dual species biofilms are always hard to eradicate due to pyocyanin mediated cross signaling.[364] *S. aureus* showed enhanced biofilm dependent and biofilm independent tolerance during coexistence of *P. aeruginosa* species.[379,405] *P. aeruginosa* rhamnolipids enable and maintain the formation of biofilm structure with channels and pores. For mature biofilms, rhamnolipids are also needed for dispersing bacteria from biofilms.[399,400] Thus, rhamnolipids have been used as dispersal agents against *P. aeruginosa* biofilms (strain and concentration dependent)[397,399,400,406,407] and *S. aureus*, *S. epidermidis.*[408,409] Because farnesol also increase the production of rhamnolipids, we hypothesize that Farnesol can eradicate silver tolerance in mono and dual species biofilms. We formed mono (*S. aureus* or PA14) and dual (*S. aureus* and PA14) species biofilms and treated respective biofilms with Farnesol. Farnesol caused dispersion of *S. aureus* biofilms by 40%, consistent with literature[410] while moderately dispersed (16%) PA14 biofilms (Fig.5.6). Interestingly, Farnesol showed enhanced biofilm dispersion (82%) in dual species (SA+PA14) biofilms. These results together suggest that while Farnesol might not cause strong biofilm dispersion for mono species, Farnesol can be more effective at dispersing biofilms of PA-SA dual species biofilms.

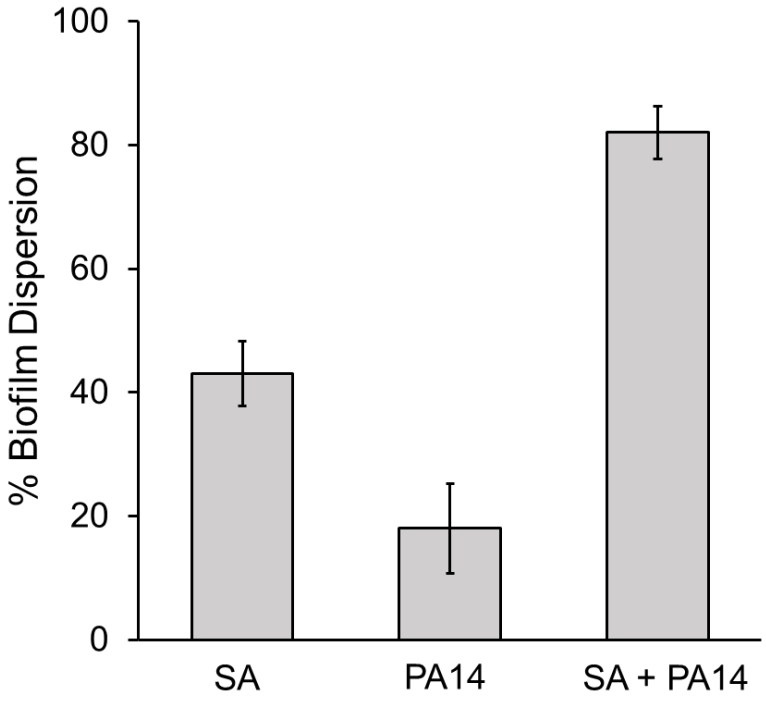


Figure 5.6 *S. aureus* and *P. aeruginosa* biofilms dispersion in the presence of Farnesol. Percent biofilms remaining after treating 24-h biofilms grown on pegs with and without 100 µM farnesol (100 rpm shaking) for another 24 h. The biofilms treated without Farnesol were considered as 100 % control. Error bars are standard error of the mean from six replicates.

5.2.5 Sub-lethal concentrations of silver ions promote swarming motility in P. aeruginosa PA14.

P. aeruginosa show increased resistance to antimicrobials by increased motilities like swarming, surfing.[246,247,411] Silver nanoparticles especially at sub-lethal concentrations showed to enhanced motilities in *E. coli, P. aeruginosa* and contribute to silver resistance.[412,413] We tested effect of sub-lethal concentrations of silver ions on *P.aeruginosa* wild type PA14. We found that sub-lethal concentration of silver ions (0.5 ppm, MIC = 15ppm) promoted swarming motility in wild type PA14 by 43% (swarming diameter increased from 58 mm $\pm$ 3 mm to 83 mm $\pm$ 4 mm) (Fig. 5.7A)

5.2.6 Farnesol inhibits Ag^+ promoted swarming motility in P. aeruginosa PA14.

PA14 always exhibit tendril formation when they swarm on a soft agar gel, whereas PAO1 swarm evenly outward radially, resulting in a circle pattern. There are currently no reported chemicals that control the swarming of PA14. We previously showed that saturated farnesol derivatives inhibit swarming motility in wild type PAO1, but not PA14.[81] We tested effect of farnesol on swarming motility of wild type PA14. We discovered that, when prepared in the hydrated gel with a concentration as low as 10 µM, farnesol inhibits swarming motility of PA14 (Fig. 5.7B). The swarming motility of PA14 was completely abolished at farnesol concentration higher than 10 µM with disappearance of tendrils. We also evaluated effect of farnesol on swarming motility promoted by sub-lethal silver ions. In the presence of farnesol (10 µM), sub-lethal Ag^+ ions did not promote swarming motility in PA14 (Fig. 5.7A).

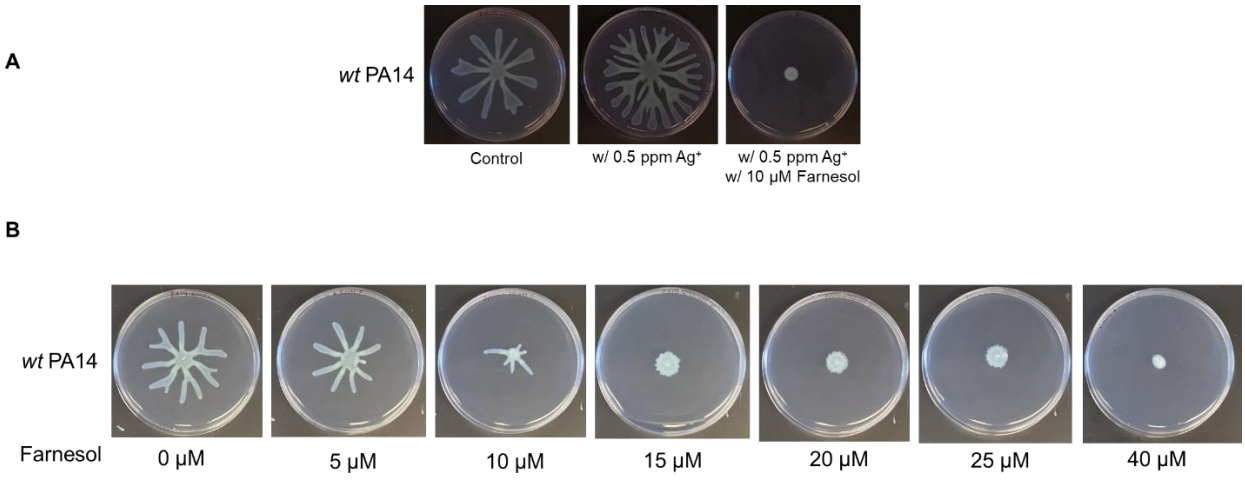


Figure 5.7 Swarming motility assay. Effect of sub-lethal silver ions and Farnesol on swarming motility of *P. aeruginosa* PA14. A. The representative images of swarming motilities PA14 on semisolid gel (~0.5% agar) with and without sub-lethal silver ions (0.5 ppm) in the presence of 10 µM farnesol. B. The representative images of swarming motilities PA14 on semisolid gel (~0.5% agar) with different concentrations of Farnesol. The concentrations are indicated below

the images. Pictures were taken after the plates were incubated at 37 ℃ for 12 h and then room temperature for another 12 h.

5.2.7 Farnesol potentiate silver ions against P. aeruginosa PA14.

We previously tested efficacy of silver ions (Ag^{+}) against *P. aeruginosa* wild type PA14. The minimum inhibitory concentration (MIC) against PA14 was 15 ppm (15 mg/l). When we added 100 µM Farnesol to PA14 cultures, MIC was reduced to 8 ppm (Table 5.1). This 2-fold reduction in MIC against PA14 suggests a synergistic effect between farnesol and silver ions. To further investigate effect of farnesol on efficacy of silver ion treatments, we treated PA14 culture (10^{8} CFU/ml) with Ag+ (50 ppm), and with and without 100 µM of farnesol. Silver ions (50 ppm) reduced viability after 24 hours where ~1% bacteria survived (Fig.5.8) without farnesol. With farnesol, only ~0.02% bacteria survived silver treatment.

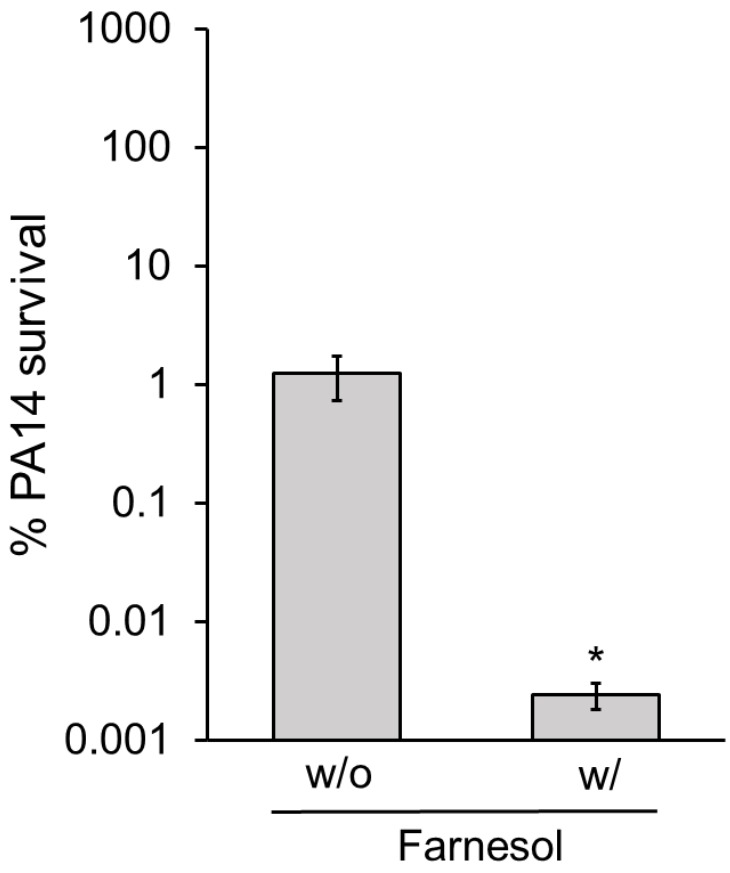


Figure 5.8 Efficacy of silver ions against *P. aeruginosa* in the presence of 100 μM of Farnesol. 10^8 CFU/ml PA14 was treated with 50 ppm silver ions for 6 hours under 250 rpm shaking and viability was measured by colony forming units on MHA plates. Error bars are standard error of the mean from three replicates. The PA14 survival without Farnesol was considered as control. **P* = 0.02 vs control as evaluated using two-tailed unpaired Student's t-test.

5.2.8 Farnesol eliminates P. aeruginosa - S. aureus cooperative silver resistance.

S. aureus alone is susceptible to silver ions at low concentrations but pyocyanin produced by *P. aeruginosa* renders redox advantage to *S. aureus* against silver ions[364,414]. To investigate effect of farnesol on *S. aureus* survival against silver ions in the presence of PA14, we exposed *S. aureus*

to silver ions in the presence of bacteria free PA14 supernatant (obtained from PA14 treated with and without farnesol). We could not use direct PA14 bacteria with *S. aureus* for this assay as PA14 causes changes in viability of *S. aureus*.[415] We found that supernatant from farnesol-treated PA14 culture caused more *S. aureus* killing (100 times) than that without farnesol (Fig.5.9). We found that ~ 0.1% *S. aureus* bacteria survived in the absence of farnesol. In the presence of 100 uM farnesol, less than ~ 0.001% bacteria survived silver ions treatment.

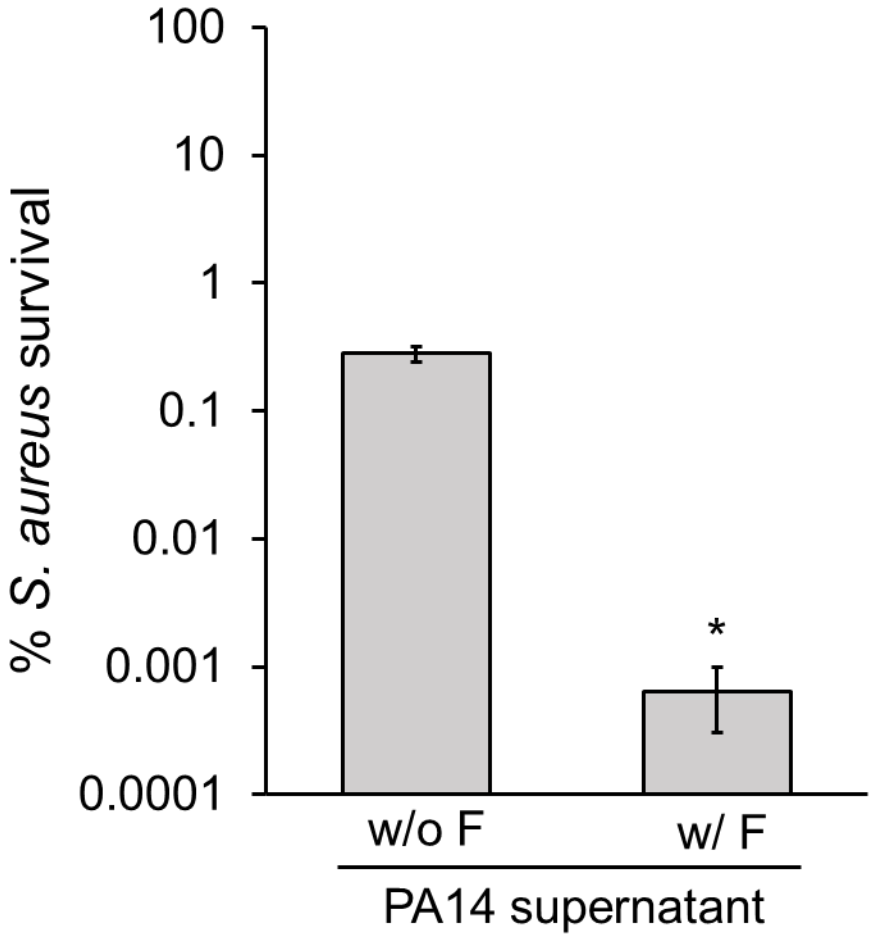


Figure 5.9 Survival of *S. aureus* under silver ions in the presence of *P. aeruginosa* (PA14) supernatant. 10^8 CFU/ml *S. aureus* was treated with 50 ppm silver ions in the presence of PA14 supernatant (supernatant from PA14 treated with and without 100 µM Farnesol) for 6 hours

under 250 rpm shaking and viability was measured by colony forming units on MHA plates. Error bars are standard error of the mean from three replicates. The *S. aureus* survival in the presence of PA14 supernatant without Farnesol treatment was considered as control. *P = 0.05 vs control as evaluated using two-tailed unpaired Student's t-test.

5.2.9 Farnesol prevents formation of small colony variants (SCVs) in S. aureus in vitro.

S. aureus forms small colony variants in the presence of *P. aeruginosa* as a defensive mechanism[27,416,417] possibly through pyocyanin mediated stress[417]. Our previous results showed that farnesol reduced cooperative resistance between *S. aureus* and *P. aeruginosa*, and farnesol reduced pyocyanin production in PA14, we tested effect of Farnesol on PA14 supernatant treated *S. aureus* culture. *S. aureus* shows SCVs when cultured with PA14 supernatant, consistent with that reported in literature.[416] For supernatant from a PA14 culture having 100 uM of farnesol, 10 times less SCVs were observed as compared to supernatant from culture without farnesol (Fig.5.10A). These results suggest that Farnesol prevents formation of small colony variants (SCV) by SA that are induced by PA14 supernatant. To test if pyocyanin secreted by *P. aeruginosa* play role in SCV formation of *S. aureus*, we used supernatant of pyocyanin deficient mutant of *P. aeruginosa* (Δ*phzM*)[396] for *S. aureus* culture. We found that *S. aureus* treated with Δ*phzM* supernatant showed no SCVs (Fig.5.10B). These results collectively suggest that Farnesol inhibits SCV formation in S. aureus-*P. aeruginosa* coculture by inhibiting PA14's pyocyanin production.

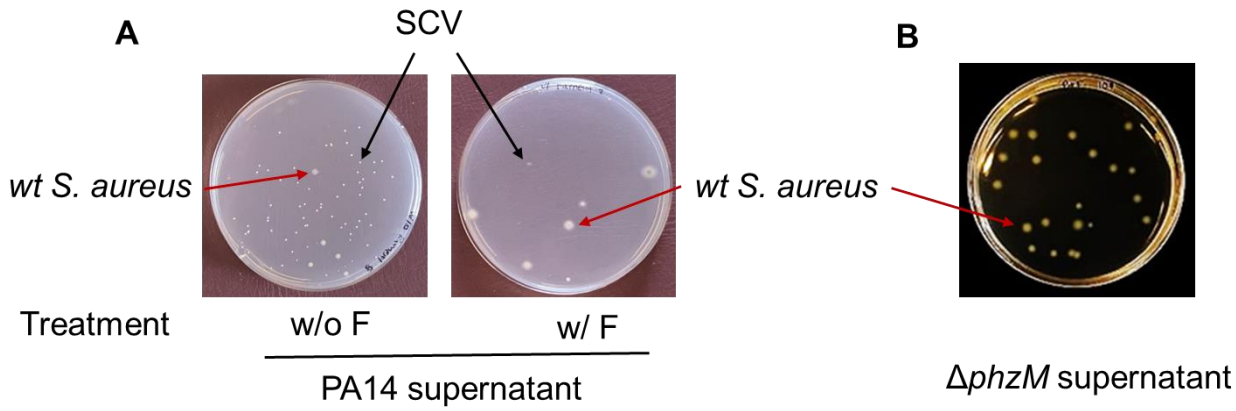


Figure 5.10 Visualization of Small colony variants of *S. aureus* A. Effect of Farnesol on small colony variants SCVs formation in *S. aureus*. B. Effect of Δ*phzM* supernatant on *S. aureus* SCVs were identified by their appearance on Luria-Bertani agar (LBA) generally appearing to be <1/10th the size of wild-type colonies.

5.3 Discussion

The *sil* genes are responsible for bacterial silver resistance and served as predictors of resistance in the wound environment.[353,418] Muller have shown that pyocyanin-caused resistance against silver ions is independent of the *sil* genes and the silver-binding proteins and efflux pumps.[395] Pyocyanin-caused silver resistance is likely due to pyocyanin caused reduction of Ag+ to Ag metal, inactivating the Ag+ ions.[395]

Our results showed that Farnesol reduced pyocyanin levels in *P. aeruginosa* and pyocyanin is known to reduce silver ions to inactive silver metal, hence silver ions showed increased efficacy in the presence of Farnesol. Pyocyanin and rhamnolipids are inversely correlated in *P. aeruginosa* PA14. As farnesol negatively regulated pyocyanin we hypothesize that farnesol might have effect on rhamnolipids production. Our results showed that Farnesol increased production of rhamnolipids in *P. aeruginosa*. Rhamnolipids not only disperse multi species

biofilms,[407,419,420] but also increase efficacy of antibiotics against different bacteria.[414,421-423] Our results indicates that Farnesol disperse mono and dual species biofilms of wound pathogens *P. aeruginosa* and *S. aureus*, likely by increasing production of rhamnolipids as one of the contributing factors. Rhamnolipids also promote wound healing and used as anti-scar therapy in wound therapy.[424-426]

We demonstrated that Farnesol inhibits swarming motility In *P. aeruginosa* PA14. We previously showed that rhamnolipids inhibit swarming of P. aeruginosa PA14 at concentration higher than 60 μM.[427] As Farnesol increased rhamnolipids production in *P. aeruginosa* PA14, Farnesol caused inhibition of swarming motility via increased rhamnolipids production. Swarming motility has been positively correlated to increased antibiotic resistance.[247,411] Because sub-lethal concentrations of antibiotics are known to promote swarming motility in *P. aeruginosa*,[30] antibiotic and swarming motility becomes vicious cycle. We showed that sub-lethal silver ions promote swarming motility of *P. aeruginosa* PA14, and Farnesol abolished promotion of swarming motility by silver ions in PA14.

Under clinical settings, higher pyocyanin concentrations have been detected in burn wound fluids.[370] *P. aeruginosa* is shown to reside in the immediate vicinity of pyocyanin-deficient species like *S. aureus*, and provide *S. aureus* protection from silver ions.[364] The absence of pyocyanin can lead to increased efficacy of silver ions. Farnesol cuts down pyocyanin supply to *S. aureus* and increased efficacy of silver ions against *S. aureus*.

Farnesol inhibited small colony variants of *S. aureus* in the presence of *P. aeruginosa* supernatant. *P. aeruginosa* and *S. aureus* frequently are coisolated in burn wound patients and prolonged growth of *S. aureus* in the presence of *P. aeruginosa* leads small colony variants of *S.*

aureus.[416] Externally added pyocyanin or oxidative stress are shown to be responsible for small colony variants in *S. aureus*.[416] Farnesol may be inhibiting small colony variants by inhibiting pyocyanin production.

5.4 Conclusion

We showed that Farnesol inhibits pyocyanin production in *P. aeruginosa*. Farnesol also increased production of rhamnolipids which results in not only dual species biofilm dispersion but also contribute to inhibiting swarming motility of *P. aeruginosa*. We also showed that Farnesol increased efficacy of silver ions against wound pathogens *P. aeruginosa* and *S. aureus*. Farnesol inhibited small colony variant formation in dual species cultures, which may also reduce small colony variants mediated antibiotic persistence. We believe that Farnesol can be great adjuvant to silver ion treatments because of its synergy with silver ions, which not only increase the potency of silver ions, but also prevents tolerance and resistance development against silver ions.

5.5 Experimental Section

5.5.1 Materials

Farnesol was dissolved in sterile (autoclaved) water. Farnesol has solubility of 0.059 g/l in water ~ 265 µM. All assays were performed at concentration less than 265 µM.[428] For concentration, higher than 265 µM , Farnesol was dissolved in sterile dimethyl sulfoxide. Silver nitrate (10 mg/ml) was prepared in sterile (autoclaved) water. Stock solutions of agents were further filtered through cellulose acetate syringe filter (0.2 µm pore, Millipore). All overnight cultures of bacteria were grown in Mueller Hinton II Broth (MHB-Cation-Adjusted, Beef extract 3 g/l, Acid Hydrolysate of Casein 17.5 g/l, Starch 1.5 g/l). All biofilm assays were performed by immersing

the pegs of a modified polystyrene microtiter lid of MBEC™ plates (Innovotech, Alberta, Canada) in M63 medium. For pyocyanin extraction, King's A medium; 1.5 g/l dipotassium hydrogen phosphate, 1.5 g/l magnesium sulfate, 20 g/l mixed peptone (Himedia, Mumbai, India) was used. All the optical density/absorbance measurements were carried out on Biotek ELx800™ absorbance microplate reader (BioTek Instruments, Inc. Winooski, VT). *P. aeruginosa* wild type PA14 strain was obtained from Dr. George O'Toole, Geisel School of Medicine at Dartmouth. *Staphylococcus aureus* 23235 was obtained from Dr. Olga Makhlynets from Department of Chemistry, Syracuse University. Δ*phzM* was obtained from Manoil lab, University of Washington.

5.5.2 Bacterial Growth Curve analysis

An overnight culture (150 µL) of bacteria (wild type PA14, *S. aureus*) in MHB was diluted in 15 mL of fresh MHB and incubated with and without different concentrations of Farnesol at 37°C under 250 rpm shaking. The 200 µL of bacterial cultures were removed and the optical density was measured at 600 nm at different time intervals.

5.5.3 Preparation of pyocyanin rich bacterial supernatant

An overnight culture of wild type PA14 (100 µL) was added to 10 mL fresh King's A medium[429] with and without 100 µM Farnesol and incubated with 160 rpm shaking for 12 h. After 12 h at 37°C with shaking, cultures were centrifuged at 6000 rpm for 15 min. Supernatants so obtained were filtered with 0.45-µm pore-size filter (SCBT, USA). The filtered supernatant was used as source of pyocyanin for biological assays.

5.5.4 Pyocyanin assay[131]

An overnight culture of wild type PA14 (100 µL) was added to 10 mL fresh King's A medium , 5 mL of which were grown with and without 100 µM Farnesol for 12 h. The absorbance of

bacterial culture was measured at 600 nm (A_{600}), and then centrifuged to remove bacterial pellet. Bacterial supernatant (5 mL) was extracted with 3 mL chloroform. The pyocyanin-containing chloroform layer was acidified with 2 mL of 0.1 N HCl to give a pink solution, the absorbance at 520 nm of 200 µL of the solution was measured. The absorbance reading at 520 nm was converted into µg/ml of supernatant with standard curve reported in literature. Pyocyanin levels were expressed as µg/ml of supernatant.

5.5.5 Assay for free silver ions (Ag^+).

The concentration of free Ag^+ was determined using the sensitive dimethylaminobenzylidene rhodanine method, as described previously.[430] Silver nitrate (100 µM, 500 µL) was incubated with 500 µL wild type PA14 supernatant (obtained by method described previously) and NADPH (100 µM) in water (pH 7.0; 32°C) for 10 mins. Ag^0 nanoparticles were removed prior to assay of Ag^+ by centrifugation at 5,000 rpm for 5 min using an Eppendorf microcentrifuge. 1000 µL of 25 µM 5-(4-dimethylaminobenzylidene) rhodanine (Sigma Aldrich) in acetone, mix, and incubate 2-5 min at room temperature. Centrifuge for 2 min at 15,000 rpm to remove the silver-dye chelate and measure the absorbance of the supernatant at 463 nm. The standard curve obtained from literature[430] for correlation between silver ions concentration and absorbance was used to calculate free silver ions concentration.

5.5.6 Crystal violet dye-based biofilm dispersion assay[120]

An overnight culture (100 µL) of bacteria (wild type PA14, *S. aureus*) in MHB was diluted in 10 mL of M63 and incubated to reach an OD_{600} value of ~0.1. The bacterial culture (150 µL) was added to the wells of MBEC™ microtiter plate. The MBEC™ plates were incubated under stationary conditions at 37 ℃ for 24 h. After incubation, the pegs were transferred and immersed into 96 wells containing sterile water (200 µL) twice to briefly rinse the biofilms to remove

unattached or loosely attached bacteria and resuspended in wells containing 100 µM Farnesol in M63 media and incubated at 37 ℃ for 24 hours. After incubation, the pegs were transferred and immersed into 96 wells containing sterile water (200 µL) twice to briefly rinse the biofilms to remove unattached or loosely attached bacteria and air dried for 30 min in BSL-2 hood. The peg-attached biofilms were immersed into 96 wells containing crystal violet (CV) dye solution (150 µL, 0.1%) at ambient temperature for 30 min, to stain the biofilms. The CV-stained pegs were then washed twice with sterile water (200 µL). To solubilize the CR stains, 150 µL of 30% acetic acid solution was added to wells, and the pegs was immersed in the wells, the plates were shake on a microplate mixer (Scilogex MX-M) at 100 rpm for 15 min. The amount of biofilms was inferred and quantified by measuring the OD_{600} of the 150 µL acetic acid solution on plate reader. The absorption from stained pegs containing just M63 medium was subtracted from pegs treated with agents.

5.5.7 Rhamnolipid assay[118]

Quantification of rhamnolipids in planktonic cultures of *P. aeruginosa* was determined by methylene blue-rhamnolipid complex assays by an established assay.[118] In this assay, the positively charged methylene blue form noncovalent complex preferentially with negatively charged surfactants, and does not bind to nonionic surfactants. The methylene blue bound with negatively charged surfactants has an increased partition into the chloroform phase bought in contact with the aqueous solution. The methylene blue in the chloroform phase were measured for its UV absorbance, which infers the amount of negatively surfactants. Briefly, 100 µL wild type PA14 bacteria from overnight cultures in MHB were diluted in fresh MHB to an OD_{600} of 0.01 with and without 100 µM Farnesol. After 18 h at 37°C with shaking, culture supernatants

were filtered with 0.45-µm pore-size filter (SCBT, USA). The filtrate pH was first adjusted to 2.3 ± 0.2 using 1 N HCl. The acidified sample was then extracted with five-fold volume of chloroform. The chloroform extract (4 mL) was transferred to put in contact with 5 mL of a freshly prepared methylene blue aqueous solution (40 µg/ml, pH pre-adjusted to 8.6 ± 0.2 by adding the 15 µL 50 mM borax buffer). After being vigorously mixed for 4 min, the samples were left to stand for 15 min. The bottom chloroform phase (1 mL) was transferred into a cuvette and the absorbance was measured at 638 nm with a UV/Vis spectrophotometer with a reference of blank chloroform. The absorbance values were converted to rhamnolipid concentrations using a calibration curve.

5.5.8 Bacterial Swarming assay[80]

The soft gels for swarming motility were prepared by autoclaving 0.5 wt% Bacto Agar in M8 medium (0.6 % Na_2HPO_4, 0.3 % KH_2PO4 and 0.05 % NaCl). The gel solution was cooled to ~60 ºC, supplemented with filtered 0.2 % glucose, 0.5 % casamino acid, and 1 mM $MgSO_4$ (0.22 µ filter). The gel solutions were poured into a Falcon tube for 20 mL portions, followed by adding aliquots of (1-20 µL) of Farnesol, silver nitrate stock solutions to achieve the desired concentrations. The falcon tubes were closed, the agar solution was mixed by gently rocking, and then poured into polystyrene petri dishes (10 cm-diameter). The agar solution was solidified by cooling and air-drying in a laminar hood for 1 h. Bacterial culture of wild type PA14 (3 µL) with an OD_{600} between ~0.4–0.6 was inoculated on the center of the surface of the soft agar gel. These "swarm plates" were incubated at 37 ºC for 12 h and then incubated for additional 12 h at room temperature. After a total of 24 h, pictures of the swarming plates were taken.

5.5.9 Bacterial killing by silver ions

To determine the efficacy of silver ions against killing of the wild type PA14 and *S. aureus* in the presence of Farnesol, the bacterial culture (10^8 CFU/ml) was treated 50 ppm silver nitrate for 6 h. For PA14, overnight culture (100 µL) of bacteria in MHB was diluted in 10 mL of MHB and incubated to reach 10^8 CFU/ml with and without 100 µM Farnesol. The bacterial culture (10^8 CFU/ml) was treated with 50 ppm silver nitrate for 6 h. For *S. aureus*, supernatant obtained from PA14 treated with and without Farnesol was added to bacterial culture (10^8 CFU/ml) and then treated with 50 ppm silver nitrate for 6 h. 100 µL aliquots were removed from cultures and the number of CFU was determined by serial dilution on MHA plates.

5.5.10 In vitro Small colony variants assay[71]

Overnight cultures (100 µL) of S. aureus was diluted 9 mL of MHB containing 1 mL of supernatant of wild type PA14 or ΔphzM (prepared as described above) and incubated at 37°C with 250 rpm shaking for 6 h. These cultures were diluted (10^5-10^7) in MHB medium and spread on Mueller Hinton Agar (MHA) plates and incubated at 37°C for 1 day.

Chapter 6 Conclusions and Future Perspective

6.1 Conclusions from this thesis

In this thesis, we described class of synthetic adjuvant molecules which targets two receptor proteins in *P. aeruginosa*. We identified type IV pili and Lectin LecA which serve as adhesin proteins *P. aeruginosa* as a target for these synthetic adjuvant molecules. We demonstrated how inhibition of pili and LecA controls different phenotypes in *P. aeruginosa*. These phenotypes are antibiotic tolerant and persistent and eventually lead to resistance in bacteria. We also explored anti-adhesin abilities of synthetic adjuvant molecules against pili and LecA. We showed that pili and LecA recognized asialo-GM1 glycosphingolipid in mammalian cells and promote motilities and adherence. We demonstrated how chimeric ligands inhibits pili and LecA binding to asialo-GM1 and thwart asialo-GM1 promoted motilities, adherence.

Inspired from designed of adjuvant molecules which inhibit pili and LecA, we developed new class of antibiotic, Farnesol triazole cellobioside (FTC) which inhibits penicillin binding proteins and showed broad spectrum antibacterial activity. We also showed that this new class of antibiotic did not develop resistance in *P. aeruginosa* after repeated exposures. Farnesol triazole cellobioside also did not promote phenotypes like enhanced motility, biofilms, and small colony variants unlike other conventional antibiotics. We also showed how Farnesol based antibiotics can prevent horizontal gene transfer via pili inhibition. This discovery by Luk group can be utilized in the future to design more antibiotics with inhibitory control over horizontal gene

transfer. Burn wounds microbiome show enhanced resistance to silver therapy. We demonstrated use of farnesol as adjuvant molecule in silver therapy. Farnesol inhibits pyocyanin, one of the virulence factor in *P. aeruginosa* which is responsible for silver resistance.

6.2 Future work and thoughts

Other strains. Antibiotic resistance in *P. aeruginosa* is still a major issue in infections. As a result, new techniques of eradicating diseases caused by drug-resistant bacteria strains are constantly being pursued. The Luk lab's synthetic ligands bind to adhesin proteins, pili and LecA and inhibit emergence of phenotypes associated with these proteins. In this thesis, we primarily focused on *P. aeruginosa* pathogen, but pili and LecA proteins are not limited to just *P. aeruginosa*. In future, these molecules have potential to be broad spectrum and show efficacy at controlling phenotypes of other microbes.

Do bacteria develop resistance towards non-killing bacterial drugs? Bacteria develop resistance as response to the antibiotic "attack". "Survival of the fittest" is a consequence of an immense genetic plasticity of bacteria that trigger variety of responses that result in mutational adaptations, acquisition of genetic material or alteration of gene expression. This resistance development comes with a fitness cost. The ability of the bacteria to genetically compensate for such fitness costs determines development of resistance. Do bacteria incur such fitness costs only under severe stress and "need for survival"? Researchers have shown that antibiotic adjuvants molecules do not develop resistance after repetitive usage. In this thesis, we show that inhibiting LecA lead to reduction of biofilm formation, which does not kill bacteria, but increase the susceptibility of bacteria to antibiotics. We ask the question if bacteria are repeatedly exposed to LecA inhibitor, will bacteria mutate to regain the ability to form biofilms?

Likewise, inhibiting pili does not kill bacteria but inhibits swarming motility and horizontal gene transfer, which increase the susceptibility of a bacteria population to antibiotics. We ask the similar question if a bacteria population is repeatedly exposed to a pili inhibitor in both planktonic culture and on hydrated soft gel, will bacteria ever mutate to regain the ability to swarm, and conduct other surface appendage functions to facilitate horizontal gene transfer? In other words, killing stresses cause bacteria to mutate to increase survival chances, does inhibition of certain phenotypes cause bacteria to mutate regain that phenotype? Specifically for phenotypes associated with LecA and or pili, answer to this question leads to another important question. If, in the presence of such inhibitors, bacteria do not regain the ability to form biofilm and conduct horizontal gene transfer, does that mean the path to drug tolerance is permanently blocked, and thus drug resistance to agents such as FTC will never be developed?

Correlation or causation for signaling by LecA inhibition and low cdG levels?

Tolerance "cultivates" resistance development. Our results showed that inhibition of Lectin A and pili proteins controlled different tolerant phenotypes. These tolerant phenotypes are precursor of resistant bacteria. Lectin A is correlated to high cdG phenotypes but direct correlation between Lectin A and cdG is still missing. It is unclear if Lectin A transduce signals to control levels of cdG or cdG levels control Lectin A activities. We need to do more experiments to map correlation between LecA and cdG to get better control over tolerant phenotype. Pili functions at low and high levels of cdG which makes control of cdG levels tricky. Again, direct correlation between pili and cdG is not clear in literature. We can perform few basic experiments to establish Lectin A and Pili receptor correlation to cdG. We can use high and low cdG mutants and determine levels of Lectin A and pili gene expression and

proteins in these mutants. We can also use commercially available cdG modulator like nitric oxide donors to see changes in levels of Lectin A and pili genes as function of cdG levels.

Covalent conjugate between antibiotics and inhibitor of horizontal gene transfer?

Horizontal gene transfer is a major cause of resistance development in bacteria. Pili play an important role in horizontal gene transfer and pili inhibitor can prevent potential horizontal gene transfer. Luk group has demonstrated that farnesol (saturated and unsaturated) derivatives inhibit horizontal gene transfer. The use of farnesol derivatives as adjuvant could be one strategy to develop antibiotic therapy without emergence of resistance. Antibiotic conjugates are one of new approach to prevent antibiotic resistance. The farnesol conjugated conventional antibiotics can be very promising in future. From another perspective, FTC is a covalent integration between chimeric ligands for LecA and pili, and a killing agent targeting cell wall enzymes.

Effect of inhibiting horizontal gene transfer in the bacteria world. The significance of horizontal gene transfer on the bacteria's gene composition is still of debate to this date. To the gene composition, the contribution of horizontal gene transfer is difficult to be clearly separated from the veritical inheritance. Here, we have created a unique opportunity of isolating out the horizontal gene communication between bacteria, and ask the open question of what will happen in the long run. Bacteria communicate with its environment and each other by protein appendages, when two major ones, LecA and pili, are shut down, what happen to their mutation in general over time? What happen also between bacterial strains killing each other? Furthermore, for a population of bacteria, when spontaneous mutation occurs, it is unlikely that the same mutation occur at the same time for the entire population, and thus, horizontal gene transfer likely play role for contributing to spreading the dominating mutation in the population.

In the presence of an inhibitor for HGT, what happen to the frequency and stability of spontaneous mutation is another open question for exploration when HGT is inhibited.

Other protein targets by chimeric ligands? In this thesis, we described that chimeric ligands inhibit pili activities in *P. aeruginosa*. We also suggested pilT protein mediated inhibition of retraction as possible mode of action of chimeric ligands. Whether chimeric ligands also bind to pilT protein to inhibit subsequent signal transduction is not clear. We believe that methylated hydrocarbon shows inhibitory effect on *P. aeruginosa* motilities and results as chemorepellent. To explore if methylated hydrocarbons inhibit motility through inhibition of pilT mediated retraction, additional experiments must be conducted. We suggest direct observation of extension and retraction of type IV pili in *P. aeruginosa*.

Nature of low resistance observed in serial passage assay. For multi-step resistance assay for farnesol triazole cellobioside (FTC), we observed slight increase in minimum inhibitory concentration against *P. aeruginosa*. It is very interesting to see if bacteria survived at end of 21 passages are truly resistant genotype or tolerant/persistent phenotype mutations. If bacteria are resistant genotype, the total gene sequencing of bacteria at end of 21 passages should be done to understand site of mutation in bacteria.

Effect on persisters. The conventional antibiotic induces persister formation and leads to recurrent infections. The efficacy of antibiotic farnesol triazole cellobioside (FTC) against persister formation and preexisted persisters should be tested.

develop antibiotic therapy without emergence of resistance. Antibiotic conjugates is one of new approach to prevent antibiotic resistance. The farnesol conjugated conventional antibiotics can be very promising in future.

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Educational Background

- **Ph.D (Chemistry)** Syracuse University, USA (2016-April 2022,expected)
  (Under the guidance of Dr.Yan Yeung Luk)
  Research highlights: Synthesis and evaluation of adjunct molecules to prevent antibiotic drug tolerance and resistance in Cystic fibrosis. Design and synthesis of preventive medicine for SARS-CoV-2 viral infection.
- **Senior Research Fellow(Biomedical engineering)** (DST project) IIT, Mumbai (2015-2016)
  (Under the guidance of Prof. Rohit Srivastava)
  Research highlights: Hemostatic wound healing product for trauma care.
- **M.tech (Pharmaceutical Sciences and Technology)** ICT/IIT, Mumbai (2013-2015)
  (Under the guidance of Prof.K.G.Akamanchi)
  Thesis title: Design and synthesis novel dendritic heterolipids for pharmaceutical applications.
  GPA: 9.23/10
  Class Rank: 1st Rank in M.tech (Pharma) and 2nd Rank in Department of Pharmacy,Mumbai.
- **B.Tech (Pharmaceutical Sciences and Technology)** ICT, Mumbai (2009-2013)
  GPA: 7.45/10

Research Experience

- **Research Project 1:** Working on preventive medicine for SARS-CoV-2 infections(2020-ongoing)
- **Research Project 2:** Working on design and synthesis of molecules for controlling bacterial behaviour in cystic fibrosis patients (2017-ongoing).
- **Research Project 3:** Worked on design and synthesis of dendritic heterolipids for solubility enhancement of drugs (2014)
  - ✓ Synthesis of novel heterolipid as solubility enhancer for Amphotericin B and Camptothecin.
- **Research Project 4:** Worked on polymeric formulation 'Wound Healing Wafers'(2014)
  - ✓ Wound Healing Wafers with dendritic surfactant as advanced penetration enhancer for transdermal drug delivery.
- **Research Project 5:** Designed and formulated lipidic formulation LipiSol(2014)
  - ✓ Use of heterolipid as oil based system to improve solubility and permeability of drugs.
- **Research Project 6:** Designed flow reactor for Sandmeyer Reaction and IBX Synthesis.
  - ✓ Use of flow reactor in order to get pure and high yeild of various reactions.(2013)
- **Research Project 7:** Synthesized nanocatalyst for hydrogenation of nitriles(2012)
  - ✓ Study of Chemical kinetics of nanocatalyst for hydrogenation reaction.

Publications/Patents

1. **Pankaj D. Patil**, Hewen Zheng, Yuchen Jin, Yan-Yeung Luk. Synthetic chimeric ligands targeting both Pili and Lectin A inhibit binding of *Pseudomonas aeruginosa* to a natural chimeric ligand asialo-GM1. Colloids and Surfaces B: Biointerfaces p.112478.
2. **Pankaj D. Patil**, Hewen Zheng, Felicia N. Burns, Arizza C. S. Ibanez, Yuchen Jin, Yan-Yeung Luk. Chimeric Ligands for Pili and Lectin A inhibit antibiotic-induced tolerance and persistence, and virulence of *Pseudomonas aeruginosa* over a broad range of second messenger signaling. (Under preparation for ACS Infectious Diseases).
3. Burns, F.N., Alila MA, Zheng H, **Patil PD**, Ibanez A., Exploration of Ligand-Receptor Binding and Mechanisms for Alginate Reduction and Phenotype Reversion by Mucoid Pseudomonas aeruginosa. ChemMedChem, 2021.
4. Yuchen Jin **,Pankaj D. Patil**, Yan-Yeung Luk. Synthetic pili inhibitors prevent horizontal gene transfer mediated antibiotic resistance in *Pseudomonas aeruginosa.* (Manuscript under preparation).
5. Dhumal DM, **Patil PD**, Kulkarni RV, Akamanchi KG. Experimentally Validated QSAR Model for Surface pK_a Prediction of Heterolipids Having Potential as Delivery Materials for Nucleic Acid Therapeutics. ACS Omega. 2020 Nov 30;5(49):32023-32031.
6. Yuchen Jin; Hewen Zheng; Arizza Chiara Ibanez; **Pankaj Dinkar Patil**; Suqi Lv; Minrui Luo; Thomas M. Duncan; Yan-Yeung Luk, Cell Wall-Related Antibiotics Cause Slow-Growing Bacteria to Form Surface-Mediated Filaments Leading to Promoted Biofilms and Suspended Aggregates, *ChemBioChem, 2020, 21, 825.*
7. S.Mapari, **P.Patil**. Nanocarriers for the Treatment of HIV and Fungal Infections, Int J Pharma Bio Sci. 2015; 6(1); 149-169.
8. **P.Patil**, R.Tayade, A potent Target for Cancer treatment–Tumor Hypoxia Necrosis:An overview PharmaTutor,2014;2(7);8-28.
9. **P.Patil**, S.Rojekar, Self-Assembled Cyclodextrin Nanoparticles as Drug Carrier,Int J Pharma Bio Sci.,2014;5(4);569-588.
10. "Neutralization of severe acute respiratory syndrome coronaviruses by chemical inhibition of spike proteins" U.S. Patent Application No. 63/141,018
11. "Adjuvant chemicals that prevent drug tolerance and persister formation by bacteria" U.S. Patent Application No. 16/240,527.
12. "Silver ions and a pyocyanin inhibitor combination", U.S. Patent Application No. 62/733,102.

Industrial Training

1. Internship at LifeUnit INC. to develop synthetic molecules to eradicate drug tolerance in Cystic fibrosis.
2. Underwent a four week In-Plant Training (Internship) at the Formulation plant of **Rubicon Private Ltd**.,Mumabai, India. And successfully completed the assigned project work.
3. Internship at **Arch Ltd** ., an Active Pharmaceutical Ingredient (API) manufacturing unit. I worked with the Technology Transfer Department on an assignment on Process and Scale-Up Design.My suggestions were well appreciated and implemented in the production batches

Presentations and Seminars

1. Delivered a presentationon "**Carbon Sequestration Techinques** : **A Concise Review**"at a seminar organized by the Department of Pharmaceutical Sciences and Technology at ICT under the guidance of Prof. K.G. Akamanchi (2013).
2. Presented a Technical Paper solution to a problem statement (Industrial Defined Problem based on **Post Operative Wound Healing** Technolgy) posed by VBP Research Group.
   Awarded the 2nd prize (2013).
3. Delivered a seminar on "**Oxide Supported Thin Film Catalyst"** as a part of the curriculum under guidance of Dr.V.N.Telvekar (2012)**.**

Conferences and Workshops

1. Participated as Delegate at the **ISDNDD -2014** (2nd International Symposium On Dendrimer in Nano Drug Delievry) CSIR-CDRI, Lucknow and delivered oral presentation entitled "**Oleodendrimer:A chemical Penetration Enhancer for Transdermal Drug Delievry**".
2. Participated in the International Workshop On "**Research Writing Skills – Publish or Perish**" held on Nov 2013.
3. Participated in the International Workshop by **American Chemical Society ACS** On "How to get Published,a lecture on "Excitement in Chemical Sciences and Engineering" held on Nov 2014.

Achievements

1. Featured in Graduate student spotlight-2020 Syracuse Chemistry department.
2. Qualified and ranked AIR 10 in GATE 2013(Graduate Apptitude Test in Engineering) in Chemical Engineering.
3. Merit certificate from the State Board of Maharashtra awarded to TOP 0.1% of over 1 million students appearing for the SSC Exams.

Research skills

1. Competent with the multistep organic synthesis, HPLC, spectroscopy, and PCR, ELISA.
2. Worked on high Pressure autoclave hydrogenation apparatus, flow reactors.
3. Worked with Biosafety level II microbes(bacteria, viruses).

Declaration

I hereby declare that the information furnished above is true to the best of knowledge and I bear the responsibility for the correctness of the above-mentioned particulars.

PANKAJ D PATIL