I. Stereochemical Control of Chiral Assembly and Liquid Crystal Phase Formation of Nonamphiphilic Molecules in Water II. Control Bacterial Quorum Sensing and Quorum Sensing-mediated Activities Using Small Unnatural Molecules

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ABSTRACT

The primary goal of this research is to utilize organic synthesis as a tool to prepare small molecules that find potential application in different areas including colloidal and material science, biological chemistry, and medicinal chemistry.

Chapter 1 describes the interpretation of the conformation of nonamphiphilic mesogen disodium cromoglycate (5´DSCG) when it exists as part of an assembly in water. The study of thermodynamic incompatibility and miscibility suggest that a previously proposed model for the assembly of 5´DSCG may be applicable to nonamphiphilic organic dyes and other mesogens.

Chapter 2 presents a study of stereochemical control on assembly and liquid crystal formation by nonamphiphilic molecules. Three stereoisomers of a disodium chromonyl carboxylate derivative, 5´DSCG-diviol, were designed and synthesized. The chiral stereoisomers formed chiral nematic liquid crystals while the achiral counterpart did not form any kind of liquid crystals. The hydrated assemblies of chiral 5´DSCG-diviol were able to interact with each other across a 6 nm separation in aqueous environment and the chirality information was transmitted through achiral medium.

Chapter 3 describes the synthesis and biological studies of a class of bicyclic brominated furanones. These molecules interacted with quorum sensing in an opportunistic pathogen P. aeruginosa. Some representative compounds in this class inhibited quorum sensing-controlled activities such as biofilm formation and virulence factor production, which were key factors in the pathogenicity of the bacteria. These compounds exhibited significant reduction in the toxicity of human neuroblastoma SK-N-SH and did not inhibit bacterial growth. Furthermore, one compound, 6-BBF, significantly improved P. aeruginosa clearance in the lungs of mice in an immunocompromised pneumonia mouse model in vivo.
Chapter 4 reports the synthesis of a library of squarylated homoserine lactones (SHLs) as analogues to the natural autoinducers N-acyl homoserine lactones (AHL) in Gram-negative bacteria. These SHLs were shown to have no or minimal impact of the growth of *P. aeruginosa* and *V. fischeri*, but maintain the abilities to modulate quorum sensing and inhibit biofilm formation. Primary studies of structural activity relationship revealed that the alkyl chain length was critical to activities of SHLs. These SHLs are promising candidates as modulators of other AHL-mediated QS systems.
I. Stereochemical Control of Chiral Assembly and Liquid Crystal Phase Formation of Nonamphiphilic Molecules in Water

II. Control Bacterial Quorum Sensing and Quorum Sensing-mediated Activities Using Small Unnatural Molecules

by

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Chapter 1

Molecular Conformation and Self-assembly of Nonamphiphilic Liquid Crystals in Water

Summary

NMR spectroscopy was used to interpret the conformation of nonamphiphilic mesogen disodium cromoglycate (5’DSCG) when it exists as part of an assembly in water. In a 1.2 wt% sample, 5’DSCG forms thread assembly, possibly via an isodesmic mechanism. The validity of the thread nature of nonamphiphilic organic dyes was also studied. Polyacrylamide promoted assembly formation by 5’DSCG at concentrations below liquid crystal formation. The same phenomenon was observed for another nonamphiphilic mesogen Sunset Yellow FCF (SSY) but not for 5’MSCG, which does not form liquid crystal at any concentration. While $^1$H NMR alone was not sufficient to show the potential thread nature of the assembly by SSY, a miscibility test further supported this model.

1.1 Background and Significance

1.1.1 Lyotropic, thermotropic, and nonamphiphilic liquid crystals

Liquid crystal (LC) is a state of matter which has properties between the conventional liquid and crystalline solid. Although a liquid crystal may flow like liquids, its molecules do exhibit a certain degree of orientational order (Figure 1.1). The study of liquid crystals began in 1888 when Friedrich Reinitzer noted that cholesteryl benzoate had two distinct melting points. The substance changed from a solid to a hazy liquid at the first melting point and then turned into a clear liquid as the temperature increased further.¹
There are two main types of liquid crystals: thermotropic liquid crystals, the order of which is temperature-dependent, and lyotropic liquid crystals, the order of which is both solvent-dependent and temperature-dependent. Some of the earliest discovered liquid crystals are thermotropic. Within a certain temperature range, cooperative ordering of the mesogens (molecules that form LC) exhibited the LC phase. At too high temperature, the sample becomes isotropic liquid; at too low temperature, the sample turns into crystalline solids. One such example is para-azoxyanisole (Figure 1.2). Lyotropic liquid crystals exhibit liquid-crystalline properties within certain concentration ranges. Typical compounds that form lyotropic liquid crystals are amphiphilic; they consist of two immiscible hydrophobic and hydrophilic parts within the same molecule and form micelles in solution above a critical concentration (critical micelle concentration, or CMC). As the concentration increases further, the assemblies of the micelles become ordered and lyotropic liquid crystalline phases form. There is a minimum temperature for a given lyotropic liquid crystal, termed Krafft emperature, below which micelles
will not form and the liquid crystal phase will not occur. Common examples of such molecules are the salts of fatty acids, many of which are used in soap or detergents (Figure 1.2).

![Chemical structures](https://example.com/structures.png)

**Figure 1.2** Examples of thermotropic and lyotropic mesogens.

Over the past forty years, a new fascinating class of lyotropic liquid crystals has become an active field of research. These molecules structurally differ from the conventional amphiphilic mesogens of lyotropic liquid crystals in that they are usually rigid, disk-like molecules having aromatic cores with polar groups at the periphery, instead of flexible, rod-like molecules having aliphatic chains with ionic groups at one end. They do not possess a critical micelle concentration and the aggregation takes place at all concentrations, the process of which is described as isodesmic, meaning that the energy advantage in adding a molecule to an aggregate is independent of aggregate size. One of the most extensively studied example in this class is disodium cromoglycate (5′DSCG) (Figure 1.3), an anti-asthmatic drug marketed under the trade name INTAL in UK and Chromolyn in US. This special class of liquid crystals extends to other drugs, dyes, and nucleic acids (Figure 1.3), and is also termed “chromonic liquid crystals” (CLCs), not only because of the chromone moiety in 5′DSCG, but also because of the connotations of both color (for dyes) and chromosomes (for nucleic acids). The existence of nonamphiphilic liquid crystals has been known for some time. In 1915, Sandquist described a system of 9-bromo-phenanthrene-3-sulfonic acid in aqueous solution that showed
nonamphiphilic liquid crystal properties.\textsuperscript{16} 5´DSCG was initially studied by Woodard and co-workers in the 1970s\textsuperscript{17,18} and later investigated more extensively by Lydon and co-workers,\textsuperscript{4,19,20} and further by others.\textsuperscript{21-28}

![disodium chromoglycate (5´DSCG)](image1)

![Sunset Yellow FCF (SSY)](image2)

![deoxyguanosine-5´-monophosphate (dGMP)](image3)

Figure 1.3 Examples of nonamphiphilic mesogens.

Nonamphiphilic liquid crystals have potential for a number of new applications, such as preparation of optically anisotropic films,\textsuperscript{29} biosensing,\textsuperscript{30} controlled drug delivery,\textsuperscript{2} microptatterning,\textsuperscript{31} and nanofabrication.\textsuperscript{32} Understanding the assembly formation and the structure-property relationships is important for designing novel nonamphiphilic mesogens, controlling formation of nonamphiphilic LC and optimizing their properties.

1.1.2 Different assembly models of nonamphiphilic liquid crystals

Like in the case of many other nonamphiphilic LCs,\textsuperscript{10,33} there are two mesophases of 5´DSCG.\textsuperscript{18} The one formed at lower concentration shows optical textures typical for thermotropic nematic phases and was termed the N phase. The second mesophases formed at higher concentrations is characteristic of the amphiphile middle (hexagonal) phase and was termed the M phase. The assembly structures of 5´DSCG in the two mesophases have been studied by many and are still not known with certainty. The discoverers Hartshorne and Woodard proposed a simple stacking model -- the planar molecules are arranged with their
planes parallel or approximately parallel for the N phase and the planar discs of molecules aggregate in cylindrical stacks which pack in a hexagonal lattice for the M phase.\textsuperscript{18} Later, a new model was reported without any experimental results, proposing a hollow square columns of 5´DSCG filled with water.\textsuperscript{19} This paper was retracted by the author after a few years,\textsuperscript{15} even though it is still cited in the literature sometimes.

In addition to 5´DSCG, the assembly behavior has been studied for other nonamphiphilic molecules, including organic dyes, such as Sunset Yellow FCF (SSY), Blue 27, Direct Blue 67, and Violet 20.\textsuperscript{10-12,34-37} The original H-stacking model for the nematic phase of these liquid crystals is a continuous subject for discussion.\textsuperscript{15,17,18,38} For instance, a J-stacking model was proposed,\textsuperscript{7,39-41} in which the molecules stack on an offset position by some fixed distance from each other, instead of directly on top of each other (“H” type) (Figure 1.4).

![Stacking models](image)

Figure 1.4 Stacking models for nonamphiphilic mesogens.

Recently, our group reported a novel model for the assembly of nonamphiphilic molecule 5´DSCG in water, which showed that maintaining a nonamphiphilic molecular structure was crucial to form a liquid crystal phase. This model consists of threads, instead of columns, of 5´DSCG molecules. Within each thread, the salt bridges formed between two molecules stacked on another layer of aromatic rings, instead of simply aggregating to form molecular columns via...
π-π interaction of the aromatic rings (Scheme 1.1). Each thread is solvated with a hydration shell of water.\textsuperscript{42} A mesogen mixing experiment was designed to validate the thread model. The addition of mono-charged molecules attenuated or destroyed the liquid crystals phase of 5´DSCG by acting as salt bridge terminators rather than column stackers. In contrast, adding divalent-charged molecules retained the liquid crystal phases of 5´DSCG, suggesting two charged units in the molecule are necessary to maintain a stable assembly and further demonstrating the thread model.

Scheme 1.1 Thread model for the assembly of 5´DSCG in water. The drawing was adapted from literature with modification.\textsuperscript{42}

In another recent study by a previous group member, using self-assembled monolayers of functionalized alkanethiols supported by anisotropic gold films with nanometer-scale topography, it was demonstrated that uniform alignment of liquid crystals formed by hydrated 5´DSCG and Sunset Yellow molecules over a large surface area.\textsuperscript{43} This uniform alignment of liquid crystals cannot be interpreted by the stacking assembly, but rather, by the thread assembly.
While oil-in-water emulsions (hydrophobic-hydrophilic separations) explain many phenomena in conventional colloidal chemistry and novel fabrication methods, entirely water soluble nonamphiphilic molecules can also exhibit phase separations in water. Water-in-water emulsions are usually generated by mixing water-soluble and structurally different polymers, but it was also revealed that some small nonamphiphilic molecules, such as 5′DSCG, can exhibit phase separation with a non-ionic polymer when mixed in water. Later on, our group demonstrated how mixing thermodynamically incompatible molecules, could promote non-covalent polymerization and liquid crystal formation, as long as the assembly formed by nonamphiphilic molecules was isodesmic in nature.

1.2 Results and Discussion

1.2.1 Possible molecular conformation and assembly formation mechanism of 5′DSCG in water

We first used 1D and 2D NMR spectroscopy to investigate the conformation of 5′DSCG in water. We noticed a clear splitting pattern for the protons at the linker region of 5′DSCG for a 1.20 wt% sample at 25 °C, which enabled us to calculate the coupling constants of these protons and therefore the dihedral angles. Protons e and e′ on the methylene group at the linker are both doublet of doublet, with coupling constants of 10.0 and 5.0 Hz; proton f on the methine group is a quintet, with a coupling constant of 5.0 Hz (Figure 1.5A). According to the Karplus relationship of dihedral angles and coupling constant, the dihedral angle of He-C-C-Hf and He′-C-C-Hf are the same (or very similar). In the spectrum of 2D Nuclear Overhauser effect spectroscopy (NOESY), cross peaks between aromatic proton a and methylene protons e/e′ were observed while no cross peak between proton a and proton f was present (Figure 1.5B). Because the cross peaks connect resonances from nuclei that are spatially close to each other, it is very
likely that proton a is further away from proton f than from proton e/e’. Based on the above data, we proposed two possible conformations of 5’DSCG molecules in 1.20 wt% sample in water at 25 °C. If the dihedral angles of both H_e-C-C-H_f and H_e-C-C-H_f are 60 degree (Figure 1.6A), 5’DSCG is in a staggered conformation, meaning proton f is at the maximum distance from the chromone moiety (abbreviated as “OR” in the figure). If the dihedral angles of both H_e-C-C-H_f and H_e-C-C-H_f are 120 degree (Figure 1.6B), 5’DSCG is in an eclipsed conformation, and proton f and the chromone moiety are in closest proximity. Even though the staggered conformation is normally the conformational energy minimum, the repulsion between the lone pairs on the carbonyl groups from the two “wings” of the chromone may disfavor this conformation. The conformation of 5’DSCG under this condition is not known with certainty.

Figure 1.5 NMR spectroscopy of 1.20 wt% (~ 24 mM) 5’DSCG in water at 25 °C. (A) ^1H NMR with the linker region enlarged. (B) Partial NOESY spectrum showing the cross peaks between proton a and proton e/e’.
When 5´DSCG exists as monomer in water, free rotation of the C-C bond in the linker region should enable both protons \( e \) and \( e' \) to experience identical chemical environment and therefore appear as a doublet split by proton \( f \) in the \( ^1H \) NMR. However, this is not the case observed. The different chemical shift of protons \( e \) and \( e' \) that resulted from different chemical environment could only come from a more “locked” conformation, possibly in an assembly. We therefore studied the effect of temperature on the chemical shift of protons in 5´DSCG (1.20 wt\% in water). If assembly exists at this concentration at 25 °C, heating might cause the assembly to dissociate and proton peaks should shift. As shown in Figure 1.7, when the temperature of 1.20 wt\% 5´DSCG sample was increased from 298 K to 363 K, all the proton peaks shifted downfield (Figure 1.7). When the concentration decreased from 1.20 wt\% to 0.45 wt\%, all the peaks also shifted downfield. In addition, the fine splitting pattern of protons \( e/e' \) was lost, suggested a more
“dynamic” conformation. These observations are in accordance with 5´DSCG forming a higher order assembly structure in water. We also note that if 5´DSCG molecules formed assembly in an “H-stacking”, the aromatic protons a-c would have experienced more deshielding due to the aromatic ring current and would have shifted downfield instead of upfield upon assembly formation. The observed upfield shift upon assembly formation (or downfield shift upon assembly dissociation) by 5´DSCG suggested that the aromatic rings stack in an “off-set” fashion, which agrees with the thread model.42

Figure 1.7 Temperature effect on chemical shifts of protons in 1.20 wt% (~24 mM) 5´DSCG (in water). NMR of 0.45 wt% (~ 8.8 mM) 5´DSCG in water at 25 °C is shown for comparison. The
chemical shift of the HOD peak is highly temperature-dependent and the HOD peaks at various temperatures were calibrated to that reported in literature.\textsuperscript{54}

The dependence of the chemical shift of the 5´DSCG aromatic protons on temperature was analyzed in order to investigate the possible mechanism for the assembly formation in water (Figure 1.8). A sudden change in the curve would suggest a cooperative self-assembly mechanism while a smooth sigmoidal curve would suggest an isodesmic mechanism.\textsuperscript{55,56} Within the temperature range tested, no obvious sigmoidal trend was observed for protons a-d; no sudden change suggestive of cooperative mechanism was observed, either. It is very possible that the solvent water limits the temperature range that could be tested and thus the plateau for a sigmoidal curve could not be reached.\textsuperscript{57} However, the gradual, smooth increase of chemical shift values upon heating indicates the assembly formation is more likely isodesmic than cooperative.
Figure 1.8 Effect of temperature on the chemical shifts of protons a-d in 5´DSCG (1.20 wt% in water).

1.2.2 Promote the assembly formation of nonamphiphilic mesogens by thermodynamically incompatible polymers

In a recent study, our group demonstrated LC formation of 5´DSCG molecules promoted by addition of polymers based on thermodynamic incompatibility. A question arises naturally: Does the presence of these polymers promote the assembly of 5´DSCG molecules in water directly into liquid crystal phase (Figure 1.9, Path A) or does the presence of these polymers promote the assembly formation by 5´DSCG before reaching the liquid crystal phase (Figure 1.9, Path B)?
$^1$H NMR experiments were performed to investigate at what stage the polymers promote the liquid crystal formation of 5´DSCG in water. At ambient temperature, 5´DSCG forms LC above ~ 10-11wt%\textsuperscript{17} and SSY forms LC above ~ 25 wt%\textsuperscript{11}. We added 8 wt% polyacrylamide (PAAm, molecular weight 10K) to aqueous 5´DSCG or SSY samples at concentrations far below the LC formation concentrations. Upon concentration increase of SSY, the broadening of proton peaks was a lot more significant in the presence of ~ 8 wt % of PAAm than in the absence of PAAm (Figure 1.10). Similar phenomenon was also observed for 5´DSCG (data not shown). These results indicated that the self-assembly by 5´DSCG or SSY was promoted by PAAm before liquid crystal phase formed. On the contrary, for 5´-monosodium cromoglycate (5´MSCG), which does not form LC at any concentration, the broadening of proton peaks was not much different as the concentration increased, in the absence or presence of ~ 8 wt % of PAAm (10K) (Figure 1.11). The similar self-assembly promoted by polymer suggested that like 5´DSCG, the SSY may also form assembly in LC phases that can be explained by thread model.
Figure 1.10 $^1$H NMR of SSY mixed with ~ 8 wt% of PAAm (10K) in D$_2$O at 25 °C.
Figure 1.11 $^1$H NMR of 5’MSCG mixed with ~ 8 wt% of PAAm (10K) in D$_2$O at 25 °C.

1.2.3 Investigate assembly model of organic dye-based nonamphiphilic molecule by miscibility

The exact assembly structures of organic dye-based nonamphiphilic lyotropic liquid crystals are more difficult to determine than 5’DSCG liquid crystals, because unlike 5’DSCG molecules, the organic dyes, such as SSY, lack a two-fold symmetry. Sunset Yellow dye can exist as two tautomers, either as hydroxyl azo form or NH hydrazone form. NMR and X-ray results indicate that the NH hydrazone form predominantly exists in solution.$^{34,37,58,59}$ Thus, even in an H-stacking assembly, a head-to-tail assembly was possible to facilitate electron-rich rings stacking on electron-deficient rings, as is shown in a recent theoretical study (Figure 1.11).$^{58}$ On the contrary, a simple face-to-face stacking is impossible because of electrostatic repulsion.$^{34}$
Figure 1.12: Asymmetric H-stacking model of Sunset Yellow FCF. Aggregations formed by five SSY molecules are shown.

To further prove that the “thread model”\textsuperscript{42} not only applies to the assembly of 5-DSCG, but also to other nonamphiphilic molecules in general, we chose three commercially available food dyes as the mesogens. Allura Red AC and Acid Red 13 were chosen because of their similar structures to that of SSY. Mono-charged molecule sodium 2-naphthol-6-sulfonate hydrate resembles a portion of SSY and is a potential thread terminator (Figure 1.12). To our best knowledge, the lowest concentration at which Allura Red AC and Acid Red 13 forms a liquid crystal phase has not been reported. We prepared aqueous solutions of Allura Red AC and Acid Red 13 at various concentrations and determined that at or above 22 wt \%, both Allura Red AC and Acid Red 13 displayed birefringence under crossed polarizers at ambient temperature. Below this concentration, isotropic solutions were observed.

Mono-charged molecule sodium 2-naphthol-6-sulfonate was mixed with the three dyes mentioned above at various concentrations (Figure 1.13). With low concentration of the added
mono-charged molecule (0.20 wt %) in SSY (30 wt %), the birefringence was reduced. As the concentration of added mono-charged molecule increased to 0.80 wt%, the birefringence became even less. At 1.51 wt % or higher of the added mono-charged molecule, no birefringence was observed. At 5.19 wt % or higher of the added molecule, precipitate formed. When the mono-charged molecule was added to Allura Red AC (22 wt %) and Acid Red 13 (22 wt %), even very small amount of the addition of the mesogen (0.23 wt % and 0.26 wt %) destroyed the liquid crystal phases completely. The results mentioned above indicated that the “thread model” proposed for 5'DSCG\(^{42}\) could also be true for the LC phase formed by Sunset Yellow, Allura Red AC, and Acid Red 13. Upon addition, sodium 2-naphthol-6-sulfonate acted as a salt bridge terminator and attenuated or even annihilated the liquid crystal phase of these nonamphiphilic dyes.
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Figure 1.13: Optical images of organic dyes mixed with different concentrations of mono-charged molecule sodium 2-naphthol-6-sulfonate hydrate viewed between crossed polarizers.\(^a\) Mixed with SY dye.\(^b\) Mixed with Allura Red AC.\(^c\) Mixed with Acid Red 13. Scale bar = 152 µm.

When di-charged molecules Allura Red AC or Acid Red 12 was added at 5~6 wt% to a 30 wt% SSY sample, birefringence was retained (Figure 1.14 A and B). This phenomenon was expected for the thread model because the di-charged molecules could function as thread connectors. When the concentration of Allura Red AC or Acid Red 13 increased further, precipitated formed due to relatively lower solubility of these molecules in water. When the concentration of SSY was lowered below the nematic liquid crystal concentration, mixing di-

[Chemical Structures]

- Sodium 2-naphthol-6-sulfonate hydrate
- Sunset Yellow
- Acid Red 13
- Allura Red
charged molecule Allura Red AC or Acid Red 13 resulted in similar birefringence observed for pure SSY. For example, when the concentration of SSY was reduced to as low as 14.7 wt %, addition of 15.3 wt % Allura Red AC resulted in birefringence resembled to that formed by 30 wt % SY dye (Figure 1.14 C). Similar birefringence was observed when 14.0 wt % SSY sample was mixed with 14.1 wt % Acid Red 13 (Figure 1.14 D). These results suggest that Allura Red AC and Acid Red 13 not only promoted LC phase of SSY but also were thermodynamically compatible with SSY. Therefore, the presence of Allura Red AC or Acid Red 13 promoted the LC formation of SSY by elongating the salt bridge and further proved the validity of the thread model.

Figure 1.14: Optical images of SY dye mixed with Allura Red AC or Acid Red 13 at different concentrations viewed between cross polarizers. The orientation of the bars shown above the
images indicates the rotation angle of the samples under the cross polarizers. Scale bar = 152 µm.

1.3 Conclusion and Perspectives

In summary, 1D and 2D NMR spectroscopy revealed that 5´DSCG self-assembles into hydrated threads via a possible isodesmic mechanism in a 1.2 wt% sample in water at 25 °C. Similar to that by 5´DSCG, the assembly formation by nonamphiphilic molecule Sunset Yellow FCF can also be promoted by thermodynamically incompatible polymer. In addition, miscibility test showed that mono-charged molecule that is structurally similar to SSY annihilate LC formation by SSY while di-charged molecules retain and promote LC formation. These results suggest that the thread model proposed for 5´DSCG may in general explain the assembly formation by nonamphiphilic dyes and other nonamphiphilic mesogens.

1.4 Experimental Section

Chemicals

Sunset Yellow FCF was purchased from Aldrich (Milwaukee, WI). Acid Red 13 and Allura Red AC were purchased from TCI America. All aqueous solutions were prepared with deionized water with a resistivity of 18.2 MΩ cm (MilliQ system, Millipore, Bedford, MA).

General procedure for preparation of optical cells and birefringence characterization

An aqueous solution of Sunset Yellow FCF (30.08 wt %) and Acid Red 13 (1.97 wt %) was prepared and aged in a vial for 12 h to allow complete dissolution. The sample was assembled in a sandwiched optical cell composed of two glass microscope slides with one sheet of Saran Wrap® (13-15 µm) to afford a spacer. The sheet of Saran Wrap® was punched to create a hole to accommodate the sample to be sandwiched between the glass slides. The sample was loaded between the slides and sealed
with binder clips on each side immediately to prevent the water from evaporating. The sample was viewed and recorded between crossed and parallel polarizers on an Olympus BX51 polarizing microscope.

*General procedure for NMR study*

$^1$H spectra were recorded on a Bruker Advance DPX-300 spectrometer. Chemical shifts are reported in ppm, using tetramethylsilane as the internal standard. NOESY spectra were recorded on a Bruker Advance DRX-500 spectrometer.
Chapter 2

Stereochemical Control of Nonamphiphilic Liquid Crystals: Effect of Chiral Mesogens and Dopants on Assemblies Separated by Six Nanometers of Aqueous Solvents

Summary

Unlike conventional thermotropic and lyotropic liquid crystals, the chromonic liquid crystals consist of hydrated assemblies of nonamphiphilic molecules that are aligned with a separation of about 6 nm between assemblies in an aqueous environment. This separation raises the question of what the assembly structure would be for a chiral nematic (or cholesteric) phase of this class of liquid crystals as the assemblies need to interact with each other that are about 6 nm apart. Here, we report the synthesis of three stereoisomers of disodium chromonyl carboxylate derivative, 5´DSCG-diviol, and the correlation between the molecular structure, bulk assembly and liquid crystal formation. Circular dichroism indicated a chiral conformation with bisignate Cotton effect. Nuclear Overhauser Effect in proton NMR spectroscopy revealed conformations that are responsible for liquid crystal formation. Cryogenic transmission electron microscopy showed that chiral 5´DSCG-diviol form assemblies with crossings. We observed that the chiral isomers formed chiral nematic liquid crystals while the achiral isomer 5´DSCG-meso-diviol did not form any kind of liquid crystals. While generic 5´DSCG in water aligns uniformly on SAMs supported by obliquely deposited gold films, 5´DSCG-(R,R)-diviol 1a exhibited nonuniform alignment of on the same surfaces. Fingerprint texture of 18 wt% 5´DSCG-(R,R)-diviol can be observed with thick cell. Together, these results suggest that the hydrated assemblies of chiral molecule 5´DSCG-(R,R)-diviol 1a interact with each other while being separated by relatively large distance (6 nm) in water, causing a lyotropic chiral nematic liquid crystal phase. These studies suggest that hydrated assemblies of chiral 5´DSCG-diviol can
interact with each other across a 6 nm separation in an aqueous environment, exhibiting a liquid crystal phase with features that may indicate some type of chiral organization.

2.1 Background and Significance

2.1.1 Cholesteric liquid crystal phase

The cholesteric liquid crystal phase is also known as chiral nematic liquid crystal phase. The first materials exhibiting this phase were cholesterol derivatives and hence the name. Nowadays there are many different types of chiral materials that exhibit cholesteric phase and most of them have no resemblance to cholesterol. Cholesteric liquid crystals (LCs) can be visualized as a stack of very thin nematic layers within which molecules have the same orientational orders but no positional order. The directors in each layer twist with respect to those above and below and rotate around a perpendicular direction (helical axis) and form a helix.\(^{60}\) The distance over which the directors complete a full \(360^\circ\) rotation is called a pitch, \(p\) (Figure 2.1). The pitch can vary with temperature, or introduction of other molecules, and the magnitude may range from several hundred of nanometers to micrometers, depending on the chemical compositions. Thermotropic cholesteric phases normally have pitches of a few hundred nanometers.\(^{61-63}\) Lyotropic cholesteric phases have a broader range of pitch, from a few micrometers to a few hundred micrometers.\(^{22,64-67}\) Various materials form cholesteric liquid crystal phase, such as cholesteryl benzoate,\(^{68}\) hydroxypropyl cellulose,\(^{69}\) DNA,\(^{66}\) and even a suspension of viruses.\(^{70}\)

Cholesteric liquid crystal phase can be formed by chiral mesogens alone or can be induced by doping an achiral lyotropic or thermotropic nematic phase with a small amount of chiral molecules.\(^{71-74}\) The molecular asymmetry is amplified in the supramolecular helix structure.
2.1.2 *Chiral chromonic liquid crystals*

There have been many reports on cholesteric liquid crystals formed by either conventional thermotropic\textsuperscript{75-79} or lyotropic\textsuperscript{65,66,72,80-82} mesogens. The mechanism of how a chiral nematic phase arises for lyotropic liquid crystals is still poorly understood.\textsuperscript{60,72,83-85} The most intriguing aspect is that as lyotropic liquid crystals are formed by organized assemblies of molecules in a solvent, the effect of chiral dopants or chiral mesogens must transmit across the solvent between assemblies, and pivot the orientation of the assemblies as a whole continuously throughout the sample.\textsuperscript{72,85}

How the chiral assemblies interact with the solvent molecules surrounding them and influence the neighboring assemblies over distance is unclear. There are few reports on chiral mesogens that form chromonic liquid crystal phase, but several reports examined chromonic liquid crystal phases influenced by chiral dopants that have an interaction over 6 nm between the molecular assemblies.\textsuperscript{22,23,64,86}

To investigate how stereochemistry controls the assembly structure and liquid crystal formation, we designed and synthesized three stereoisomers of derivatives of disodium chromonyl carboxylates (5´DSCG-diviol) which posses a diviol linker (Figure 2.2), and demonstrated a strong correlation between molecular structures and assembly properties, as well
as the ability of the molecules to form liquid crystals. We also present evidence for a liquid crystal phase that requires chemical communication across a distance of 5-6 nm of the aqueous solvent, and is perhaps different from the conventional cholesteric liquid crystal phase.

![Chemical structures](image)

Figure 2.2 The structures of the chiral isomers 5′DSCG-(R,R)-diviol 1a, 5′DSCG-(S,S)-diviol 1b, and achiral isomer 5′DSCG-meso-diviol 1c.

### 2.2 Results and Discussion

#### 2.2.1 Design and synthesis of the stereoisomers of 5′DSCG-diviols

The generic 5′DSCG exhibits a wide range of liquid crystal properties in water that are unmatched by conventional lyotropic liquid crystals that are made of amphiphilic molecules. Several other fused aromatic dye-based molecules also form assemblies and chromonic liquid crystals in water, but 5′DSCG forms liquid crystal phases at lower concentration with higher birefringence compared to other dye-based molecules. To explore the structural requirement for chromonic liquid crystal phase formation, our group recently screened
a series of dichromonyl molecules, and found an optically inactive mixture of stereoisomers that exhibited polymorphism, of which upon rapid cooling resulted in a nematic liquid crystal phase whereas aging at ambient temperature resulted in precipitation. This mixture consists of three stereoisomers: a pair of enantiomers (52%), 5´DSCG-(R,R)-diviol 1a and 5´DSCG-(S,S)-diviol 1b; and an achiral compound (48%), 5´DSCG-meso-diviol 1c.\textsuperscript{42} The precipitates obtained from the above sample after aging overnight were comprised of a mixture of all three stereoisomers, and thus spontaneous resolution\textsuperscript{87,88} was not achieved. To study the effect of stereochemistry on assembly and liquid crystal formation, we synthesized each stereoisomer individually.

The synthesis of the chiral isomers 5´DSCG-(R,R)-diviol and 5´DSCG-(S,S)-diviol was accomplished using the same route (Scheme 2.1), but started with L-(−)-arabitol and D-(+)-arabitol, respectively. For brevity, the synthesis of 5´DSCG-(R,R)-diviol 1a is described below.

2,4-Dihydroxypentane-1,5-diyl bis-(2,4,6-trisopropyl-1-benzenesulfonate) 6a was obtained in 5 steps starting from L-(−)-arabitol.\textsuperscript{89,90} Regioselective acetonization of L-(−)-arabitol afforded diacetal 2a, which reacted with 1,1´-thiocarbonyldiimidazole to give compound 3a. Deoxygenation of 3a lead to compound 4a and deacetonization afforded (2R,4R)-pentane-1,2,4,5-tetraol 5a, which was then converted to bis-sulfonate 6a. Acetonization of bis-sulfonate 6a provided acetal 7a followed by iodination that produced diiodide 8a. Deacetonization of 8a yielded diiodo diol 9a which was treated directly with excess of 2,6-dihydroxyacetophenone to give the substitution adduct 10a. A two-step cyclization sequence from modified conditions\textsuperscript{91} built the chromonyl ring of diester 11a. First, hydroxyl ketone 10a was added to a mixture of sodium methoxide and dimethyl oxalate in a solvent mixture of Et\textsubscript{2}O and MeOH (2:1 by volume) and the reaction mixture was refluxed overnight. Acidification of the reaction mixture gave a yellow precipitate. Second, the yellow precipitate was treated with concentrated hydrochloric
acid under reflux in methanol to give dichromonyl ester 11a. We note that the use of this mixed solvent system was critical for obtaining useful yields. Basic hydrolysis of dichromonyl ester 11a with stoichiometric amount of sodium hydroxide provided the final product 5’DSCG-(R,R)-diviol 1a.

Scheme 2.1 Synthesis of 5’DSCG-(R,R)-diviol 1a and 5’DSCG-(S,S)-diviol 1b. a

Using xylitol as the starting material to attain desired meso stereochemistry, 5’DSCG-meso-diviol was synthesized with a modified synthetic route from that for the chiral stereoisomers (Scheme 2.2) because the same route was not effective to prepare the meso isomer. The preparation of tetraol 5c was essentially the same to that of 5a/5b. However, the reaction of tetraol 5c and TPSCl only provided mono-protection product. There was also a trace amount of byproduct due to the cyclization of the mono-protection product. The different steps included

aTPS = Triisopropylbenzenesulfonyl.
acetyl-bromination of tetraol 5c to give bromoacetate 6c,\textsuperscript{92} epoxidation under basic conditions to generate diepoxide 7c that was purified by bulb-to-bulb distillation,\textsuperscript{93} and iodination of diepoxide 7c to give diiodo diol 8c. Compound 8c was used to obtain the final product 1c under the same reaction conditions as that for the chiral isomers. We note that while the chiral diester 11a or 11b (for the chiral isomers) precipitated out from the reaction mixture, most of the achiral diester 10c remained in the solution (Scheme 2.2).

Scheme 2.2 Synthesis of 5´DSCG-\textit{meso}-diviol 1c.

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\textit{2.2.2 Stereochemistry controls the formation of liquid crystals}

To examine the potential liquid crystal properties, samples of each stereoisomer in water were sandwiched between two microscope glass slides with one sheet of Saran Wrap\textsuperscript{®} (13-15 µm thick) as a spacer. A square hole of ~0.5 x 0.5 cm was cut in the sheet of Saran Wrap\textsuperscript{®} to host the sample. Binder clips were applied on each side to prevent the evaporation of solvent.

Between cross polarizers, 5´DSCG-(\textit{R,}R)-diviol 1a exhibited birefringence at a concentration as
low as 12.0 wt% (with a mole ratio of water to mesogen of 226:1) at ambient temperature, about 21 °C (Figure 2.3 A). At the same concentration (12.0 wt%), 5´DSCG-(S,S)-diviol 1b showed liquid crystals phase at 18 °C (Figure 2.3 B). We believed that this small difference in phase behavior between the (R,R)- and (S,S)- isomers may be caused by the miniscule difference in purity such as small amounts of salts that are beyond the measurement of NMR. Salts are known to change the nematic-isotropic transition temperature of liquid crystals formed by 5´DSCG.28 The achiral stereoisomer 5´DSCG-meso-diviol 1c was a mixture of isotropic solution and a small amount of insoluble solid at the same concentration (12.0 wt%) (Figure 2.3 C). Furthermore, rapid cooling did not induce liquid crystal formation in this sample. This result indicates that 5´DSCG-meso-diviol 1c is either less capable or incapable of forming liquid crystals, which likely contributes to the fact that the mixture of all three stereoisomers requires a lower temperature or a higher concentration than the individual chiral stereoisomers to form a liquid crystal phase. We note that 5´DSCG-meso-diviol 1c has lower water solubility than the chiral isomers 5´DSCG-(R,R)-diviol 1a and 5´DSCG-(S,S)-diviol 1b.

Figure 2.3 Optical images (cross polarizers) of 12.0 wt % 5´DSCG-(R,R)-diviol 1a (A), 5´DSCG-(S,S)-diviol 1b (B), and 5´DSCG-meso-diviol 1c (C) in water sandwiched between two glass slides (spacer: 13-15 µm). Samples were freshly prepared by dissolving solid in water at ambient temperature. Scale bar = 76 µm.
2.2.3 *Stereoisomers of 5´DSCG-diviols exhibit different polymorphism*

To explore liquid crystal formation without any cooling, we prepared 18.0 wt% 5´DSCG-(R,R)-diviol 1a and 18.1 wt% 5´DSCG-(S,S)-diviol 1b (Figure 2.4 A and B). Both of these chiral 5´DSCG-diviols exhibited birefringence over the entire sample prepared at ambient temperature. In contrast, 5´DSCG-*meso*-diviol 1c at this concentration resulted in an isotropic solution with insoluble solids (Figure 2.4 C). Rapid cooling of this sample did not induce liquid crystal formation. After aging overnight at ambient temperature in vials sealed with parafilm to prevent evaporation, we observed that the chiral and meso 5´DSCG-diviol resulted in different assemblies. The sample of 18.0 wt% 5´DSCG-(R,R)-diviol 1a became a mixture of liquid crystals with small precipitates, whereas 18.1 wt% 5´DSCG-(S,S)-diviol 1b remained a mixture of liquid crystals and isotropic solution (Figure 2.4 D and E). Under the same conditions, 18.0 wt% of 5´DSCG-*meso*-diviol 1c in water remained a mixture of isotropic solution and aggregates (Figure 2.4 F). In comparison, the polymorphism of the individual enantiomer was different from that observed in the earlier work of an optically inactive mixture of all three stereoisomers (a mixture of 52% racemic mixture and 48% *meso* isomer): a freshly prepared sample of 18.0 wt% of mesogens transitioned from an isotropic solution to liquid crystal phase by rapid cooling to 15 ºC without observable precipitates, whereas isothermal aging gives precipitates in isotropic solution. Together, these results suggest that, for the optically inactive mixture, the liquid crystal phase represents a local energy minimum, and the precipitation in isotropic solution is of a global minimum in the energy profile.
2.2.4 Conformations of stereoisomers are different

Diastereomers may or may not be of grossly different conformation. In proton NMR spectra, differences in the chemical shifts of the diviol linker in the chiral and meso 5’DSCG-diviol suggest that the conformations between these diastereomers are different from each other. To access the conformations of the diastereomers and their differences, we used two-dimensional NMR spectroscopy to characterize the conformations of the (S,S)-isomer 1b and the meso isomer 1c. Because the meso isomer has a lower solubility than the (S,S)-isomer 1b in water, we believe that even with a concentration at which both molecules appear to dissolve in D$_2$O, the dynamics
of the two molecules in solution may not be the same. For this reason, we focused on using ROESY to characterize the structure to avoid the potential null of NOE signals due to the dynamics of the molecules.94 We observed large differences in the NOE correlations in the ROESY spectra for the diviol bridge region between the two diastereomers in D$_2$O (Figure 2.5). For 0.503 wt% of 5´DSCG-(S,S)-diviol 1b, NOE between protons g and e (and e´), and between g and f were observed with similar peak intensities. This result suggests that these protons (g and e/e´, and g and f) may be in close proximity by similar distances. In contrast, for 5´DSCG-meso-diviol 1c at the same wt%, protons g and g´ on the same methylene group were in different chemical environment, and thus exhibited different chemical shifts. More importantly, one of the “g” protons showed weak NOE correlation with only one of the “e” protons. The other “g” proton exhibited NOE correlation with both of the “e” protons, with strong and weak NOE signal intensities, respectively (Figure 2.6). These NOEs indicate that one of the “g” protons was in close proximity to only one of the “e” protons, whereas the other “g” proton was close in space to both of the “e” protons. These results suggest that the diviol linker region in the meso isomer is twisted through certain preferred bond rotation angle. Together, these spacing of protons in the two diastereomers suggests that 5´DSCG-(S,S)-diviol 1c likely adopts an overall “stretched” conformation while 5´DSCG-meso-diviol 1c is more of a “folded” conformation. These conformational differences also appear to be consistent with the low solubility of the meso isomer 1c and the formation of thread assemblies by the (S,S)-isomer 1b.
Figure 2.5 ROESY of 0.503 wt% of 5´DSCG-(S,S)-diviol and 0.503 wt% of 5´DSCG-meso-diviol in D$_2$O at 25 °C.
2.2.5 **Circular dichroism indicates a chiral conformation**

Circular dichroism (CD) spectra were collected to examine the chirality in chiral 5´DSCG-diviol at feasible concentrations (0.1 to 0.8 mM). The two enantiomers of 5´DSCG-diviol showed CD spectra with mirror signals having minimum and maximum at 274 nm and 339 nm (Figure 2.7). Considering the UV absorption at 256 and 325 nm, bisignate Cotton effects in the
\(\pi-\pi^*\) transition were observed in the CD spectra for 5’DSCG-\(\rightleftharpoons\left(\right)\)-diviol 1a (8 \(\times\) 10^{-4} M) with a positive effect at 339 nm and a negative effect at 274 nm (Figure 2.7). Although bisignate Cotton effect may suggest the presence of ordered chiral assembly, \(^{95,96}\) temperature-dependence study (Figure 2.8) showed insignificant decrease in the intensity of CD signal as the temperature increased from 5 °C to 85 °C. Furthermore, in a concentration-dependent study of the absorbance by 5’DSCG-\(\rightleftharpoons\left(\right)\)-diviol 1a in water, a near constant molar absorptivity was observed at 325 nm in the concentration ranging from 5 µM to 1.6 mM using 1 mm- and 1 cm-path curvettes (data not shown). Together, these results suggest that at this concentration range (<1.6 mM) the molecules are mostly in the monomeric state, and that the chiral conformation is stable at relatively high temperature. However, in a study of concentration-dependent chemical shift in NMR, we observed significant peak broadening and an upfield chemical shift in the proton NMR of 5’DSCG-\(\rightleftharpoons\left(\right)\)-diviol 1a in D\(_2\)O as the concentration was increased from 0.02 wt% (400 µM) to 3.5 wt% (72 mM) (Figure 2.9). The peak broadening resulted from a significant reduction in the molecular mobility due to aggregation and the upfield chemical shift is in accordance with the thread model assembly in which the aromatic rings stack in an “off-set” fashion (also see Section 1.2.1). Considering the upper limit of concentration that could be detected by the CD spectrometer used, it is plausible to suggest that chiral assemblies of 5’DSCG-\(\rightleftharpoons\left(\right)\)-diviol 1a indeed form at relatively high concentrations (at least at above 20 mM).
Figure 2.7 UV spectrum of 5'DSCG-(R,R)-diviol 1a (dot line) and CD spectra of 5'DSCG-(R,R)-diviol 1a (solid line) and 5'DSCG-(S,S)-diviol 1b (dash line) in water (8 × 10⁻⁴ M) at 5 °C. Spectra were taken in a 1 mm path length cuvette.

Figure 2.8 Temperature-dependent CD spectra of 5'DSCG-(R,R)-diviol 1a in water (8 × 10⁻⁴ M). Spectra were taken in a 1 mm path length cuvette.
Figure 2.9 $^1$H NMR of 5’DSCG-(S,S)-diviol 1b from 0.02 wt% (0.4 mM) to 3.5 wt% (72 mM) in water.

2.2.6 *Cryogenic transmission electron microscopy reveals early-staged and crossed assemblies by chiral mesogens*

Cryogenic transmission electron microscopy (cryo-TEM) provides valuable, direct information of the complex nanostructures and microstructures in the solution state. It has been used to characterize the size and shape of peptides, DNA, lipids, virus, as well as the self-
assembly of polymers, surfactants, and liquid crystals.\textsuperscript{28,97} To access the assembly structure of the chiral mesogens, we compared the cryo-TEM images of the 5´DSCG-(R,R)-diviol 1a and the generic 5´DSCG at concentrations below and above which assemblies form (Figure 2.10-2.13).

Figure 2.10 Cryogenic transmission electronic microscopic image of 5.5 wt\% (488) 5´DSCG (inside the curved edge). (Mole ratio) indicates water/5´DSCG.
Figure 2.11 Cryogenic transmission electronic microscopic image of 5.0 wt% (587) 5´DSCG-(R,R)-diviol 1a (inside the curved edge). (Mole ratio) indicates water/5´DSCG-(R,R)-diviol.

Figure 2.12 Cryogenic transmission electronic microscopic image of 8.2 wt% (321) 5´DSCG (inside the curved edge). (Mole ratio) indicates water/5´DSCG.
Figure 2.13 Cryogenic transmission electronic microscopic image of 8.8 wt% (321) 5´DSCG-(R,R)-diviol 1a (inside the curved edge). (Mole ratio) indicates water/5´DSCG-(R,R)-diviol.

A past study of small angle neutron scattering showed that the assembly structure started to appear for 5´DSCG at about 5-6 wt%.[42] Thus, we chose 5´DSCG (5.5 wt%, mole ratio of water molecules to mesogens is 488) and 5´DSCG-(R,R)-diviol 1a (5.0 wt%, mole ratio of water to mesogen is 587) to compare the early stage features of the molecular assembly. We note that the sample of chiral mesogen 5´DSCG-(R,R)-diviol 1a had a lower concentration than the one with achiral mesogen 5´DSCG. Interestingly, assemblies were more visible in the 5´DSCG-(R,R)-diviol 1a samples, whereas mostly blocks of assemblies with about 5-7 nm gap were observed in the 5´DSCG samples (Figure 2.14). These results indicate that the chiral mesogen 5´DSCG-(R,R)-diviol 1a form assemblies at a lower concentration than that by 5´DSCG. At these concentrations, a liquid crystal phase was not observed for either of the samples, and the supramolecular assembly structures appeared to be connected by “blocks” of assemblies.
Although supramolecular assemblies were visible in the 5´DSCG-(R,R)-diviol 1a samples, they were randomly oriented, consistent with a sample of isotropic solution at this concentration.

![Cryo-TEM images of 5.5 wt% of 5´DSCG, and 5.0 wt% of 5´DSCG-(R,R)-diviol 1a. Numbers in the parentheses indicate mole ratio of water and mesogens in the sample. The red dash lines indicate the thread-like assembly. Scale bar = 10 nm.](image)

As the concentration of 5´DSCG-(R,R)-diviol 1a increased to 8.8 wt%, the assemblies were more elongated and more aligned with each other than those observed in samples at lower concentrations (Figure 2.15). This elongation of the assemblies as the concentration increases is consistent with the notion of an isodesmic assembly, \(^{35,56}\) for which there is no critical concentration for assembly but the observed blocks of assemblies continue to grow to form assembly. The cryo-TEM images also showed that the aligned assembly structures were separated by about 6 nm, and the alignment between the assemblies was uniform over at least hundreds of nanometers. Interestingly, there were more crossings between the assemblies formed
by 5´DSCG-(R,R)-diviol 1a than those formed by 5´DSCG (Figure 2.15), and that the chiral mesogen exhibited large curvature in the assemblies.

Figure 2.15 Cryogenic transmission electronic microscopic images of 8.2 wt% of 5´DSCG and 8.8 wt% of 5´DSCG-(R,R)-diviol 1a. Numbers in the parentheses indicate mole ratio of water and mesogens in the sample. Scale bar = 50 nm.

The polymorphism of liquid crystal phases and precipitates under different condition, and the 6 nm-separation between the threads as revealed by cryo-TEM suggest that the assemblies in liquid crystals are likely hydrated by solvent water, which prevent aggregation and precipitation for the chiral isomers. This hydration implies a competition between assemblies getting solvated and aggregating with other assemblies. Whereas aggregation of mesogens leads to insoluble precipitation, hydration of assemblies leads to liquid crystal formation. For the polymorphism shown in the optically inactive mixture, we believe that the achiral mesogen is co-precipitating with the chiral stereoisomers, but rapid cooling can condense the readily available water
molecules around the newly formed assembly to insulate the assemblies from aggregating, and thus facilitate liquid crystal formation.

2.2.7 Nonuniform alignment formed by 5’DSCG-(R,R)-diviol

We note that this chiral mesogen in water at the concentrations studied did not give a conventional fingerprint texture of the classical cholesteric liquid crystal phase.\textsuperscript{98} In addition, without a magnetic field alignment, we did not observe the fingerprint texture of achiral 5’DSCG mixed with small chiral dopants.\textsuperscript{22} To examine the liquid crystal phases comprised of chiral mesogens, we used a surface that can align the generic 5’DSCG liquid crystal uniformly over a large area and explored if a uniform alignment could also be obtained for the liquid crystals formed by the chiral 5’DSCG-(R,R)-diviol \textbf{1a}. Any chiral phase of a liquid crystal will \textit{not} give a uniform single-colored texture over the entire liquid crystal sample because of the continuous change in the molecular orientation in the chiral nematic phase. Thus, a surface that can align a nematic phase uniformly\textsuperscript{99,100} is a useful tool to determine whether a chiral nematic phase exists or not. We have recently reported uniform alignment of 5’DSCG liquid crystal on surfaces presenting self-assembled monolayers (SAMs) of functionalized alkanethiols supported on gold films that were deposited onto glass slides at an incident angle oblique from the surface normal of the glass slides. When sandwiched between two such surfaces, hydrated molecular assemblies of 5’DSCG and Sunset Yellow dye aligned in parallel to the surface of the SAMs, and uniformly in a preferred direction over the entire sample.\textsuperscript{43}

For classical cholesteric liquid crystal samples, the characteristic fingerprint texture can often be observed by chiral mesogens or by achiral mesogens mixed with a chiral dopant. Here, we also compared the liquid crystal formed by chiral 5’DSCG-diviol \textbf{1a} and by achiral 5’DSCG
mixed with a small chiral molecule. In this work, using the same surfaces (two SAMs of HS(CH₂)₁₀(OCH₂CH₂)₃OH on gold films that were deposited with a 45° incident angle), ¹⁴³. ¹⁶.⁸ wt% 5´DSCG in water gave a uniform alignment (Fig 2.16 D), which exhibited a strong modulation in the intensity of transmitted light when the sample is rotated with respect to either one of the crossed polarizers. Uniform alignment was observed as expected for ¹⁴.¹ wt% ⁵´DSCG mixed with ¹⁴.⁵ wt% achiral molecule xylitol (Figure 2.16 E). When ¹³.⁴ wt% ⁵´DSCG was mixed with a racemic mixture of D- and L-mannitol (⁸.¹ wt% of each), uniform alignment of liquid crystal phase was observed (Figure 2.16 F). On the contrary, a nonuniform alignment was observed for a sample containing ¹³.⁴ wt% ⁵´DSCG doped with ¹⁶.² wt% enantiomerically pure D-mannitol (Figure 2.16 G). Two areas of different colors that switched between bright and dark were observed as the sample was rotated between the cross polarizers.

Using the liquid crystal cell with the same monolayers, the ⁵´DSCG-(R,R)-diviol ¹¹ (¹⁸.⁰ wt%) liquid crystal sample also exhibited non-uniform alignments with two domains of different orientations (Figure 2.16 H). We note that all the samples were warmed to above liquid crystal-isotropic transition temperature to remove potential effect of viscosity or flow on the liquid crystal alignment. Because of the ⁶ nm solvent separation between the assemblies, this technique cannot predict if the neighboring assemblies of the chiral molecules can twist relative to each other in the aqueous solution. However, uniform alignment of liquid crystal is not obtained for chiral lyotropic mesogens. This result suggests this liquid crystal phase may have chiral features, perhaps different from those of the classical cholesteric phase.
Figure 2.16 (A). Geometry of gold deposition on glass slides at an oblique angle from the surface normal. (B). Scheme of self-assembly molecules (SAMs) formed by HS(CH$_2$)$_{10}$(OCH$_2$CH$_2$)$_3$OH. (C). Schematic representation of uniform alignment of threads of 5′DSCG liquid crystal on
SAMs supported by gold films deposited at 45° from the surface normal of the glass slides. Optical images (cross polarizers) of (D) 16.8 wt% 5´DSCG, (E) 14.1 wt% 5´DSCG mixed with 14.5 wt% xylitol, (F) 13.4 wt% 5´DSCG mixed with a 16.2 wt% racemic mixture of D- and L-mannitol, (G) 13.4 wt% 5´DSCG doped with 16.2 wt% D-mannitol, and (H) 18.0 wt% 5´DSCG-(R,R)-diviol 1a in water sandwiched between obliquely deposited gold films supporting HS(CH₂)₁₀(O CH₂CH₂)₃OH. Numbers in the parentheses indicate mole ratio of water and mesogens in the sample. Scale bar = 152 µm. The arrows indicate direction of gold deposition projected onto the glass slides, and the orientation of the sample relative to the cross polarizers.

Between two surfaces, cholesteric liquid crystal can align homogeneously or homeotropically. In the homogeneous (planar) alignment, liquid crystals twist as they propagate away from the surface, and all the liquid crystals align parallel relative to the surface. In this case, the pitch is not observable (Figure 2.17 A). In the homeotropic alignment, the surface tends to align the liquid crystal perpendicular to the surface, and the liquid crystals twist as they propagate along the surface. In this case, the pitch can be observed (Figure 2.17 B). For a sample containing 13.5 wt% 5´DSCG doped with 16.5 wt% mannitol, as the thickness of liquid crystal cell (the spacer) increases, the liquid crystal becomes brighter, and more importantly, the modulation of light changing from dark to bright is significantly reduced (Figure 2.18). This result is consistent with a cholesteric phase on a planar-alignment surface (Figure 2.17 A).
Figure 2.17 Illustrations of the molecular assemblies in (A) homogeneous or (B) homeotropic alignment of chiral nematic liquid crystal phase on surface.

Figure 2.18 Optical images (cross polarizers) of 13.5 wt% 5’DSCG doped with 16.5 wt% D-mannitol in water sandwiched between obliquely deposited gold films supporting HS(CH₂)₁₀(OCH₂CH₂)₃OH with different thickness of spacers. Scale bar = 152 µm. The arrows indicate direction of gold deposition projected onto the glass slides, and the orientation of the sample relative to the cross polarizers.

In a conventional cholesteric phase created by doping thermotropic liquid crystals with a small amount of chiral molecules, the chiral effect from the small molecule dopants is transmitted over large distances – up to hundreds of micrometers.¹⁰¹,¹⁰² If one considers the
nematic liquid crystal as the solvent for the chiral dopants, then the dopants are changing the entire organization of the solvent molecules. In our case, the assemblies are separated by water molecules – a medium that comprised of hydrogen bond networks. As the assemblies require strong hydration by water molecules to prevent themselves from coalescing together, at the boundary between the domains, the water molecules are likely organized to facilitate the interaction between the assemblies.

It is important to note that there are precedents of organized water structures.\textsuperscript{103-109} For example, the sucrose density gradients in water that are commonly used for purification of biomolecules are stable over a long period of time.\textsuperscript{103,104} In such a gradient solution, water molecules between the sucrose molecules are dynamically different across the gradient.\textsuperscript{105} In a recent study using molecular dynamics simulation, water dynamics in the solvation shell of proteins have shown to be different from bulk water. The terahertz spectroscopy data suggested an influence on the correlated water network motion beyond two nm between neighboring proteins.\textsuperscript{107} Our recent work also demonstrated that the stereochemistry of polyol diastereomers on surfaces impacted their ability to resist protein adsorption, mammalian cell adhesion and biofilm formation.\textsuperscript{109} As the atomic composition is the same for those surfaces, this resistance to biofouling is likely due to the templated aqueous solvent structure at the interfaces. Surface force studies on bioinert SAMs by Grunze and coworkers measured force effect as far as more than 10 nm from the surfaces.\textsuperscript{110,111} Finally, using chiral lyotropic liquid crystals as solvents, discrimination of enantiomers can be observed in NMR spectra. These studies suggest that the solvent is promoting a preferred orientation of the solutes.\textsuperscript{106,108}

The formation of mesophases for conventional thermotropic liquid crystals (such as 5CB) is primarily a result of anisotropic dispersion forces between molecules.\textsuperscript{60,112} In the present study,
hydrogen bonding appears to be the main factor that governs the interaction between assemblies and the basis for mediating liquid crystal threads to influence each other in an aqueous environment up to 6 nm apart. Together with the anisotropic interactions at meso-scale, liquid crystal materials create complex and interesting assembly structures. With the potential of structuring the organization of water molecules, new assemblies in water can be explored.

2.2.8 Homeotropic alignment (fingerprint texture) of chiral nematic phase of nonamphiphilic liquid crystals

Homeotropic alignment is difficult to obtain for nonamphiphilic chiral nematic liquid crystals. In a nematic liquid crystal, the homeotropic alignment on a surface occurs when the directors of the mesogens are perpendicular to the surface. For a chiral nematic (or cholesteric) phase, an entirely homeotropic alignment for all mesogens is impossible, as the director of each neighboring layer continue to twist with a certain angle. It turns out that homeotropic alignment is very difficult to attain for the achiral nonamphiphilic lyotropic liquid crystal. To date (2013), there is not yet a report on homeotropic alignment of this class of liquid crystal (5'DSCG or other dyes). Homeotropic alignment of conventional thermotropic liquid crystals, however, is easily attainable and is routinely prepared by controlling the surface chemistry and structure. The reason for this “homeotropic” difficulty for nonamphiphilic lyotropic liquid crystal is not clear. From a macroscopic point of view, any long thread-like structure, wire-like or spaghetti-like or else, does not stand vertical to a surface presumably due to weight. At molecular level, the interaction between the long axis of the assembly and the surface is of close to infinitely larger area than between the point of the assembly and the surface. For this reason, the interaction of
the long axes of the assemblies and the surface over a large area outweighs the interaction between the endpoint of the assemblies and the surface.

In a homeotropic alignment of chiral nematic liquid crystals the helical axis is oriented parallel to a surface, with directors in the planes of assembly continuously rotate along the surface. For this surface alignment for chiral nonamphiphilic liquid crystals, some vertical (and thus homeotropic alignment) assembly alignment on a surface is inevitable. Because the surface control strongly favors planar alignment for thread or assembly-based lyotropic liquid crystals, we seek to reduce the surface effect by increasing the cell thickness to achieve fingerprint texture, which provides additional, and likely stronger evidence, for the existence of a lyotropic chiral nematic phase.

*Attaining fingerprint texture of nonamphiphilic chiral lyotropic liquid crystals.* To explore the existence of chiral nematic phase, we study the effect of cell thickness and liquid crystal alignment by preparing a sandwiched sample of 13.4 wt% 5´DSCG mixed with 16.2 wt% D-mannitol in a wedge cell composed of HS(CH$_2$)$_{10}$(OCH$_2$CH$_2$)$_3$OH SAMs supported by gold films. In the wedge cell, the thickness of the cell continuously increases from the thin end (13 µm) to the thick end (1 mm) (Figure 2.19 A). We observe that with thin cell thickness, planar alignment is observed as the color of the optical images does not change much with rotation (Figure 2.19 B and C). However, as the cell thickness increases to 555 um, fingerprint region began to appear (Figure 2.19 D). As the cell thickness increases further, fingerprint region is observed over a larger area (Figure 2.19 E). The helical pitch of the sample of 13.4 wt% 5´DSCG mixed with 16.2 wt% D-mannitol observed in this wedge cell is 25~28 µm. These results are consistent with the notion that with thin spacer, the surface alignment is dominant. As the thickness of liquid crystal cell increases, the liquid crystal is less influenced by the surface, and
the twist-induced (twist from the chiral nematic) vertical or homeotropic alignment becomes possible.

![Diagram of wedge cell](image)

**Figure 2.19 (A)** Schematic representation of a wedge cell composed of HS(CH\(_2\)\(_{10}\))(OCH\(_2\)CH\(_2\))\(_3\)OH SAMs supported by gold films. Optical images (cross polarizers) of 13.4 wt% 5\(^\prime\) DSCG mixed with 16.2 wt% D-mannitol when the thickness of the cell is (B) 13 \(\mu\)m, (C) 296 \(\mu\)m, (D) 555\(\mu\)m, and (E) 778 \(\mu\)m. Scale bar = 76 \(\mu\)m. The arrows indicate direction of gold deposition projected onto the glass slides, and the orientation of the sample relative to the cross polarizers.

With this interpretation, we examined if fingerprint texture for this liquid crystal can be achieved in a uniformly thick cell. Using a cell composed of SAM and spacers of 1 mm to provide a uniform and thick spacing, we also obtained fingerprint texture for sample of 18 wt%
5′DSCG-(R,R)-diviol, which is a strong evidence that the liquid crystal phase formed is chiral nematic (Figure 2.20 A). It is interesting to find that the liquid crystal phase of 13.4 wt% 5′DSCG mixed with 16.2 wt% D-mannitol has a longer helical pitch (17 ~19 µm) than that of the chiral mesogen (10 µm) (Figure 2.20 B). Considering the distance between neighboring thread assembly is ~ 6nm as evidenced in the cryo-TEM, we could calculate the degree of rotation between neighboring assemblies in each case using Equation 2.1. \( P \) represents the helical pitch. In the sample of 18 wt% 5′DSCG-(R,R)-diviol, each thread-like assembly twists ~0.22 degree with respect to the neighboring ones and that angle is 0.13 degree in the case of 13.4 wt% 5′DSCG mixed with 16.2 wt% D-mannitol.

\[
\text{Twist angle between neighboring assemblies} = 360 \div (p/6 \text{ nm}) \quad \text{Eqn 2.1}
\]

Figure 2.20 Optical images (cross polarizers) of (A) 13.4 wt% 5′DSCG mixed with 16.2 wt% D-mannitol and (B) 18.0 wt% 5′DSCG-(R,R)-diviol when the samples were sandwiched between
obliquely deposited gold films supporting HS(CH₂)₁₀(O CH₂CH₂)₃OH in a 1 mm thick cell. Schematic representations of the distance and the twisting angle between two neighboring assemblies are shown below the images.

Optical images with detailed rotations of 13.4 wt% 5´DSCG mixed with 16.2 wt% D-mannitol in a wedge cell (composed of HS(CH₂)₁₀(OCH₂CH₂)₃OH SAMs supported by gold films) with different cell thickness are shown in Figure 2.21 and 2.22.

Figure 2.21 Optical images with detailed rotations of 13.4 wt% 5´DSCG mixed with 16.2 wt% D-mannitol in a wedge cell composed of HS(CH₂)₁₀(OCH₂CH₂)₃OH SAMs supported by gold films when the thickness of the cell ranges from 13 to 222 µm.
Figure 2.22 Optical images with detailed rotations of 13.4 wt% 5′DSCG mixed with 16.2 wt% D-mannitol in a wedge cell composed of HS(CH$_2$)$_{10}$(OCH$_2$CH$_2$)$_3$OH SAMs supported by gold films when the thickness of the cell ranges from 296 to 778 µm.

The above results are all on surfaces that provide uniform planar alignment of liquid crystals (both thermotropic and this class of nonamphiphilic lyotropic liquid crystals). The observed homeotropic alignment is caused by the splay strain and facilitated by the large thickness (see below). To show that the chiral nematic phase forms on any surface for this class of liquid crystal, we used a plane glass slides and wedge cells. Optical images with detailed rotations of
13.4 wt% 5′DSCG mixed with 16.2 wt% D-mannitol in a wedge cell (composed of plain glass slides) with different cell thickness are shown in Figure 2.23

<table>
<thead>
<tr>
<th>Distance from the thin end</th>
<th>Thickness of the spacer</th>
<th>Optical Images</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mm</td>
<td>41 ± 15 µm</td>
<td></td>
</tr>
<tr>
<td>4 mm</td>
<td>178 ± 78 µm</td>
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<tr>
<td>8 mm</td>
<td>298 ± 99 µm</td>
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<tr>
<td>11 mm</td>
<td>407 ± 107 µm</td>
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<tr>
<td>16 mm</td>
<td>595 ± 195 µm</td>
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<tr>
<td>21 mm</td>
<td>778 ± 278 µm</td>
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<tr>
<td>No polarizer</td>
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</table>

Figure 2.23 Optical images with detailed rotations of 13.4 wt% 5′DSCG mixed with 16.2 wt% D-mannitol in a wedge cell composed of plain glass slides when the thickness of the cell ranges from 13 to 778 µm.
For the planar alignment on the thin end, there are more domains on the plain glass slides than on the SAMs supported by obliquely deposited gold films. This result is consistent with uniform alignment on SAMs and random alignment on plane glass. As the samples are rotated between the cross polarizers, the color did not change in either samples. These results are consistent with a homogeneous planar alignment and random planar alignment of the chiral nematic liquid crystal phase on oblique gold-supported SAMs and on plane glasses, respectively.

The helical pitch of the sample of 13.4 wt% 5´DSCG mixed with 16.2 wt% D-mannitol is not uniform. For example, when cell thickness is 555, 593, and 778 µm, the helical pitch is 25, 22, and 28 µm, respectively (Figure 2.24). The correlation between the size of the pitch and cell thickness is not yet clear.

Figure 2.24 Optical images of 13.4 wt% 5´DSCG mixed with 16.2 wt% D-mannitol in a wedge cell composed of HS(CH$_2$)$_{10}$(OCH$_2$CH$_2$)$_3$OH SAMs supported by gold films when the cell thickness is (A) 555 µm, (B) 593 µm, and (C) 778 µm.

_Splay strain-induced homeotropic alignment._ It is interesting that the “critical thickness” of the wedge cell at which fingerprint region begins to show and the size of the helical pitch vary with the shape of the wedge cell. For example, when a sample of 13.4 wt% 5´DSCG mixed with 16.2 wt% D-mannitol was viewed in a 27 mm wedge cell with an angle of 2.12° composed of HS(CH$_2$)$_{10}$(OCH$_2$CH$_2$)$_3$OH SAMs supported on gold films, the critical thickness that the
fingerprint region begins to show is ~555 µm and the helical pitch observed is ~25 µm (Figure 2.25 A). When the same sample was viewed in a 17 mm long wedge cell with an angle of 3.18°, the critical thickness is ~222 µm and the helical pitch observed is ~18 µm (Figure 2.25 B).

Figure 2.25 Optical images (cross polarizers) of 13.4 wt% 5΄DSCG mixed with 16.2 wt% D-mannitol viewed in a wedge cell with (A) 27 mm and (B) 17 mm cell length. Both of the wedge cells are composed of HS(CH₂)₁₀(OCH₂CH₂)₃OH SAMs supported by gold films and the spacer is 13 µm on one end and 1 mm on the other. We note that the wedge cell with 1 mm thickness spacer provide an asymmetry triangle rather than isosceles triangle.

Examining the fingerprint texture of the liquid crystal in Figure 2.21 A, the longest undisrupted lines (by defects) spanned a distance of > 849 µm, which is larger than the critical thickness 555 µm at which the fingerprint texture started to appear. This result indicates that the threads aligned perpendicular to the surface are shorter than the threads parallel to the surface in
the fingerprint domains of the liquid crystals. Interestingly, a more wedged cell (wedge angle 3.18°) caused the fingerprint texture (and thus homeotropic alignment) to occur at a relatively small thickness (222 µm) whereas a less wedged cell (wedge angle 2.12°) caused the fingerprint to occur at 555 µm thickness. As a larger wedge angle causes more splay strain in liquid crystals, this result suggests that although the thickness of the cell is important, the strong splay strain can also induce a forced homeotropic alignment of chiral nematic (i.e. fingerprint texture). As two neighboring assemblies are splayed, the energy for a homeotropic alignment to fill up the void space between the splayed assemblies is lowered (Figure 2.26 B).

![Figure 2.26 Schematic representation of splay deformation of liquid crystals in a wedge cell of (A) small angle and (B) large angle.](image)

*Significance of chiral nematic phase created by assemblies of chiral mesogens in water versus achiral mesogens mixed with chiral molecules in water.* In comparison with conventional chiral nematic for thermotropic liquid crystals, the key question for a chiral nematic version of lyotropic liquid crystal is how the chiral information is being transmitted between the assemblies that are separated by a rather large volume of water molecules, more than 80% by weight. In conventional thermotropic liquid crystal, the mesogens are in van der Waals contact; the chiral information is transmitted through dispersion interaction between molecules and manifested by
molecules twisting relative to each other continuously throughout each domain in the liquid

crystal sample. In lyotropic liquid crystals, the assemblies are not in direct van der Waals contact

with each other. There are two scenarios. One is chiral mesogen in water, the other is achiral

mesogen mixed with a chiral molecule in water. We discuss each separately and first, the case of

chiral mesogen in water.

We believe that there are three possible modes for chemical information transmission

between the assemblies of chiral mesogens in water. First, the assemblies are separated by pure

water, and there is no or negligible amount of chiral mesogens. For both isodesmic and

cooperative assemblies, this case is probable when the self-association constant is high. In this

case chiral information is transmitted through an achiral solvent medium (Figure 2.27). For a

rough estimate in our case, the two neighboring assemblies are separated by at least 14 to 18

water molecules (Figure 2.27 and 2.28). This number is estimated by assuming a linear hydrogen

bonding arrangement between the water molecules.
Figure 2.27 Schematic representation of chiral information transmitted through water molecules if the molecular assemblies (thread model) are separated by pure water. The number of water molecules between neighboring thread-like assemblies is estimated.
Figure 2.28 Schematic representation of chiral information transmitted through water molecules if the molecular assemblies (H-stacking model) are separated by pure water. The number of water molecules between neighboring assemblies is estimated.

Second, there may be some amount of chiral mesogens solvated as individual free molecules in the aqueous solvent between the threads (Figure 2.29 and 2.30). These molecules may function as a chiral dopant to twist the molecular assemblies. This scenario is possible, but the transmission of chiral information is still mediated by the solvent molecules because the free molecules are still solvated.
Figure 2.29 Schematic representation of chiral information transmitted through water molecules with free solvated chiral mesogens between the molecular assemblies (thread model). The number of water molecules between neighboring thread-like assemblies is estimated.
Figure 2.30 Schematic representation of chiral information transmitted through water molecules with free solvated chiral mesogens between the molecular assemblies (H-stacking model). The number of water molecules between neighboring assemblies is estimated.

Third, the chiral mesogens branch out (or grow out) of the assemblies and connect to the neighboring ones (Figure 2.31 and 2.32). Through this connection, the chiral information is somehow transmitted and manifested as twisting neighboring assemblies. This scenario is unlikely because such a cross-linking network will cause gel formation instead of a fluid phase.
In the thread model

Figure 2.31 Schematic representation of chiral information transmitted through chiral mesogens between the molecular assemblies (thread model).

In the H-stacking model

Figure 2.32 Schematic representation of chiral information transmitted through chiral mesogens between the molecular assemblies (H-stacking model).

In the case of achiral mesogens mixed with chiral molecules in water, the transmission of chemical information is not likely through pure water as the concentration of the chiral molecules are high. However, because the chiral molecules are small molecules, the transmission
of the chemical information must still go through many molecular interactions between the chiral molecules and the solvent water molecules, and eventually the neighboring assemblies. In an aqueous solution, hydrogen bonds are ubiquitous. It is important to note that one does not know the contribution from the dispersion force which is present regardless the types of functional groups. What types of the molecular interactions are contributing and enabling the chiral information transmission is a still an open question.

2.3 Conclusions and perspectives

In conclusion, enantiomers of 5′DSCG-\((R,R)\)-diviol 1a and 5′DSCG-\((S,S)\)-diviol 1b, and achiral 5′DSCG-\(meso\)-diviol 1c were individually synthesized. Nuclear Overhauser Effect in proton NMR spectroscopy revealed that the 5′DSCG-\(meso\)-diviol 1c adopted a more “folded” conformation while the chiral 5′DSCG-diviol had a more “stretched” conformation. Circular dichroism suggested a stable chiral molecular conformation by 5′DSCG-\((R,R)\)-diviol 1a. Proton NMR revealed significant upfield chemical shift and peak broadening around 72 mM. Cryo-TEM showed visible assembly at 5 wt% (~103 mM), and that chiral stereoisomer 5′DSCG-\((R,R)\)-diviol 1a forms more elongated assembly at low concentration in water, and exhibited more crossings between the assemblies at high concentration than those shown by generic 5′DSCG in water. Chiral stereoisomers formed chiral nematic liquid crystal phases while the \(meso\) isomer did not any liquid crystal phase. While generic 5′DSCG in water aligns uniformly on SAMs supported by obliquely deposited gold films, 5′DSCG-\((R,R)\)-diviol 1a exhibited nonuniform alignment of on the same surfaces. Splay strain-induced homeotropic alignment of liquid crystals formed by 13.4 wt% 5′DSCG mixed with 16.2 wt% D-mannitol. Fingerprint texture of 18 wt% 5′DSCG-\((R,R)\)-diviol can be observed with thick cell. Together, these results suggest that the hydrated assemblies of chiral molecule 5′DSCG-\((R,R)\)-diviol 1a interact with
each other while being separated by relatively large distance (6 nm) in water, causing a lyotropic chiral nematic liquid crystal phase. Further works include investigate the assembly structure of chiral 5’DSCG-diviol at higher concentration than already studied using cuvette with shorter path length, and further investigate the mechanism and chiral features of the assembly thus formed.

2.4 Experimental Section

Chemicals.

All reagent grade starting materials were obtained from commercial supplies (Sigma-Aldrich, TCI, Alfa Aesar, and Acros) and used as received. Anhydrous solvents were purchased from Sigma-Aldrich. Water used to prepare all buffers and solutions had resistivity of 18 MΩ cm (Millipore, Billerica, MA).

General procedure.

All air sensitive reactions were performed in oven dried glassware under an atmosphere of argon unless otherwise notified. Analytical thin layer chromatography was performed on EM silica gel 60 F254 glass plates (0.25 mm). Visualization of analytical thin layer chromatography was achieved using UV absorbance (254 nm), KMnO₄, and ceric ammonium molybdate stains. Flash column chromatography was performed using SiliaFlash P60 silica gel (40-60 Å) from SiliCycle, Inc (Quebec City, Quebec, Canada). 1D ¹H and ¹³C NMR spectra were recorded on a Bruker Advance DPX-300 spectrometer. Chemical shifts are reported in ppm, using tetramethylsilane as the internal standard. ROESY spectra were acquired at a temperature of 300 K recorded on a Bruker AVANCE-II NMR spectrometer equipped with a 5 mm TCI CryoProbe with z-gradient operating at a frequency of 600.13 MHz for ¹H. For 2D ROESY experiments, a total of 320 t₁ increments of 2048 complex points with 16 scans each were collected. The ROESY spin lock
was set to 250 ms for all experiments. NMR data were processed using Bruker TOPSPIN 3.0 software by setting forward linear prediction to 640 points and zero filled to 2048 points for F1 dimension. Mass spectra were measured using a MAT 95 XP mass spectrometer, carried out by the Mass Spectroscopy Facility at Indiana University.

Circular dichroism.

Wavelength scan and thermal study of chiral 5′DSCG-diviols were performed on an AVIV 420 CD spectrometer (AVIV Biomedical, Inc., Lakewood, NJ) using a 1 mm path length cuvette. For thermal study, the temperature was increased at a rate of 2 °C/min and samples were equilibrated for 60 s prior to each data collection.

UV.

UV measurements were performed on an Agilent 8453 spectrometer (Agilent Technologies, Santa Clara, CA) using a 1mm or 1cm path length cuvette at ambient temperature.

Cleaning of glass substrates.109

Substrates used for gold films were Fisher’s Finest premium microscope slides purchased from Fisher Scientific (Pittsburgh, PA). Prior to gold deposition, the glass slides were cleaned with Piranha solution. The slides were soaked in Piranha solution (7 parts of 35% aqueous hydrogen peroxide solution and 3 parts of concentrated sulfuric acid) for 45 min at 70 °C. Warning!

Piranha solution is extremely corrosive and can potentially detonate when mixed with significant amounts of oxidizable materials. It is advised to neutralize the Piranha solution with sodium hydroxide before disposal. After cooling to ambient temperature, the Piranha solution was poured off and the glass slides rinsed 20 times sequentially with deionized water, followed by 10 times with ethanol, and then 10 times with methanol. The cleaned slides were dried individually with a stream of nitrogen gas and kept in an 80 °C oven overnight.
Deposition of Gold Films.

Deposition of gold films was done following literature procedure. Semitransparent gold films were deposited onto the glass substrate using an electron beam evaporation system (Thermionics, Port Townsend, WA). A layer of titanium (~70 Å) was deposited first to enhance the adhesion of the gold. A layer of gold (~280 Å) was then deposited at an oblique angle of 45° to the surface normal of the substrate. The rate of deposition was kept at 0.2 Å/s for both gold and titanium and the pressure was maintained at no higher than 2 × 10^{-6} Torr throughout the deposition.

Preparation of SAMs on obliquely deposited gold films.

Gold films deposited on microscope glass slides were cut into strips (8 mm × 2.5 mm) along the direction of gold deposition. The strips were rinsed with 200 proof ethanol (3 ×), dried with a stream of nitrogen gas and then immersed in ethanolic solutions containing 2 mM of thiols overnight (~15 h). Each strip was rinsed with 200 proof ethanol (3 ×), dried with a stream of nitrogen gas and used immediately.

Assembly of optical cells and characterization of birefringence.

Aqueous solutions of 5´DSCG and 5´DSCG-diviols were prepared by dissolution in deionized water in vials. The vials were subjected to vortex mixing for at least 1 min before use. The liquid crystal samples were assembled in a sandwiched cell composed of two microscope glass slides or gold films supporting SAMs with one sheet of Saran Wrap® (13 – 15 µm) inserted in between as a spacer. A square hole was cut on the sheet of Saran Wrap® to accommodate the liquid crystal sample. The liquid crystal samples were loaded between the slides in the square hole and sealed with binder clips on each side to prevent the evaporation of solvent. The samples were viewed under crossed polarizers on an Olympus BX51 polarizing microscope and were rotated from an arbitrary starting point to record the birefringence.
Sample preparation for cryo-TEM.

The samples were prepared by applying ~5 µL droplet of the sample on the microperforated cryo-TEM grid, followed by blotting the grid with a filter paper to produce a thin layer. The grid was then rapidly plunged into liquid ethane that had been cooled to near liquid nitrogen temperature to freeze the sample.

General information of synthesis.

All air sensitive reactions were performed in oven dried glassware under an atmosphere of argon unless otherwise notified. Analytical thin layer chromatography was performed on EM silica gel 60 F254 glass plates (0.25 mm). Visualization of analytical thin layer chromatography was achieved using UV absorbance (254 nm), KMnO₄, and ceric ammonium molybdate stains. Flash column chromatography was performed using SiliaFlash P60 silica gel (40-60 Å) from SiliCycle, Inc (Quebec City, Quebec, Canada). 1D ¹H and ¹³C NMR spectra were recorded on a Bruker Advance DPX-300 spectrometer. ¹H chemical shifts are reported in ppm, downfield from tetramethylsilane using residual CHCl₃ as the internal standard (δ 7.26 ppm). ¹³C chemical shifts are reported in ppm, downfield from tetramethylsilane relative to CDCl₃ (δ 77.0 ppm), DMSO-d₆ (δ 39.5 ppm) and CD₃CN (δ 1.94 ppm). ROESY spectra were acquired at a temperature of 300 K recorded on a Bruker AVANCE-II NMR spectrometer equipped with a 5 mm TCI CryoProbe with z-gradient operating at a frequency of 600.13 MHz for ¹H. For ROESY experiments, a total of 320 t₁ increments of 2048 complex points with 16 scans each were collected. The ROESY spin lock was set to 250 ms for all experiments. NMR data were processed using Bruker TOPSPIN 3.0 software by setting forward linear prediction to 640 points and zero filled to 2048 points for F1 dimension. Mass spectra were measured using a MAT 95 XP mass spectrometer, carried out by the Mass Spectroscopy Facility at Indiana University.
Synthetic procedures and spectral data.

1,2:4,5-bis-acetal 2a: To a stirred suspension of L-arabitol (3.008 g, 19.37 mmol) in anhydrous THF (48 mL) was added 2,2-dimethoxypropane (5.1 mL, 41 mmol) at ambient temperature. The reaction mixture was refluxed for 15 min and then L-(−)-camphorsulfonic acid (461.2 mg, 1.946 mmol) was added at refluxing temperature. After refluxing for another 5 minutes, the reaction was quenched with 2 M NaOH (11 mL) at refluxing temperature. The solvent was removed under reduced pressure and residue was extracted with diethyl ether (11 mL × 3). The combined organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. The colorless oil residue was dissolved in anhydrous methylene chloride (48 mL) and treated with Et₃N (2.9 mL). The reaction mixture was then brought to reflux and succinic anhydride (488.9 mg, 4.837 mmol) was added. After refluxing for 5 h, the reaction was quenched with saturated aqueous NaHCO₃ (7.0 mL) at refluxing temperature. The mixture was cooled to ambient temperature and the organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate, 5:1 to 3:1) provided 1,2:4,5-bis-acetal 2a: (3.0925 g, 69 %) as a pale yellow oil. TLC Rₜ = 0.48 (hexane:ethyl acetate, 1:1). ¹H NMR (300 MHz, CDCl₃): δ 4.26 (ddd, 1H, J = 6.6, 6.6, 4.2 Hz), 4.13 (m, 2H), 4.04 (dd, 1H, J = 10.8, 5.4 Hz), 3.98 (m, 1H), 3.92 (dd, 1H, J = 8.3, 6.6 Hz), 3.43(m, 1H), 2.29 (d, 1H, J = 6.3 Hz), 1.45 (s, 3H), 1.41 (s, 3H), 1.39 (s, 3H), 1.36 (s, 3H).
**Imidazolyl thiocarbonyl derivative 3a:** To a stirred solution of 1,2:4,5-bis-acetal 2a (2.241 g, 9.657 mmol) in anhydrous 1,2-dichloroethane (32 mL) was added 1,1’-thiocarbonyl diimidazole (2.249 g, 11.36 mmol) at ambient temperature. The resulting brown solution was refluxed for 7 h and then cooled to ambient temperature. The solvent was removed under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate 3:1) provided imidazolyl thiocarbonyl derivative 3a (3.17 g, 96 %) as a yellow oil. TLC Rₖ = 0.36 (hexane:ethyl acetate, 1:1). ¹H NMR (300 MHz, CDCl₃): δ 8.34 (m, 1H), 7.63 (m, 1H), 7.03 (m, 1H), 5.88 (dd, 1H, J = 5.7, 3.3 Hz), 4.45(m, 2H), 4.08 (m, 3H), 3.83 (dd, 1H, J = 9.0, 6.0 Hz), 1.39 (s, 3H), 1.33 (s, 9H); ¹³C NMR (75 MHz, CDCl₃): δ 184.5, 137.0, 131.0, 118.0, 109.7, 109.6, 80.9, 74.8, 74.5, 65.8, 65.2, 26.3, 26.1, 25.0, 24.9. HRMS: Cacl. for (M + H)⁺: 343.1328, found: 343.1314.

![Diagram of 3a]

**1,2:4,5-bis-acetal 4a:** Benzoyl peroxide (202.9 mg, 0.821 mmol) was added to a stirred solution of imidazolyl thiocarbonyl derivative 3a (1.444 g, 4.223 mmol) in triethylsilane (25 mL) at refluxing temperature. The reaction mixture was refluxed for 2 h during which similar amount of benzoyl peroxide was added after 30 min, 60 min, and 90 min, respectively. The solvent was then removed under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate, 3:1) provided 1,2:4,5-bis-acetal 4a as a light yellow oil which contained some aromatic impurities and triethyl silane but was good enough to be carried on. TLC Rₖ = 0.65 (hexane:ethyl acetate, 1:1).

![Diagram of 4a]

**[(2R,4R)-pentane-1,2,4,5-tetraol 5a]**: 0.5 M H₂SO₄ (5.4 mL, 2.7 mmol) was added to a stirred solution of 1,2:4,5-bis-acetal 4a (1.508 g, impure) in ethanol (5.4 mL) at ambient temperature.
The reaction mixture was refluxed for 4.5 h and then BaCO$_3$ was added to adjust the pH to 7. The resulting milky mixture was filtered and the white solid thus collected was heated with MeOH (5 mL) at 50 °C for 10 min. The combined filtrate was concentrated under reduced pressure. Flash column chromatography (SiO$_2$: DCM:MeOH, 7:3) provided (2$R$,4$R$)-pentane-1,2,4,5-tetraol 5a (546.7 mg, 95 % over 2 steps) as a white solid. TLC R$_f$ = 0.25 (DCM:MeOH, 7:3). $^1$H NMR (300 MHz, MeOH-d$_4$): δ 3.85 (m, 2H), 3.47 (m, 4H), 1.52 (dd, 2H, $J$ = 7.1, 5.6 Hz); $^{13}$C NMR (75 MHz, MeOH-d$_4$): δ 70.1, 67.9, 38.1. HRMS: Cacl. for (M + Na)$^+$: 159.0633, found: 159.0631.

(2$R$,4$R$)-2,4-Dihydroxypentane-1,5-diyl Bis-(2,4,6-triisopropyl-1-benzenesulfonate) 6a: To a stirred solution of (2$R$,4$R$)-Pentane-1,2,4,5-tetraol 5a (92.2 mg, 0.678 mmol) in anhydrous pyridine (0.55 mL) was added 2,4,6-triisopropyl benzenesulfonyl chloride (529.2 mg, 1.695 mmol) at 0 °C. The pale yellow cloudy mixture was stirred at ambient temperature for 13 h and then the solvent removed under reduced pressure. Flash column chromatography (SiO$_2$: hexane:acetone, 3:1) provided (2$R$,4$R$)-2,4-Dihydroxypentane-1,5-diyl bis-(2,4,6-triisopropyl-1-benzenesulfonate) 6a (325.5 mg, 72 %) as a white fluffy solid. TLC R$_f$ = 0.70 (hexane:ethyl acetate, 1:1). $^1$H NMR (300 MHz, CDCl$_3$): δ 7.20 (s, 4H), 4.23 (m, 2H), 4.10 (m, 4H), 3.98 (dd, 2H, $J$ = 10.2, 6.9 Hz), 2.92 (septet, 2H, $J$ = 6.9 Hz), 2.87 (br, 2H), 1.64 (t, 2H, $J$ = 5.7 Hz), 1.27 (d, 24H, $J$ = 6.9 Hz), 1.26 (d, 12H, $J$ = 6.9 Hz).

Acetonide 7a: To a stirred solution of (2$R$,4$R$)-2,4-Dihydroxypentane-1,5-diyl bis-(2,4,6-triisopropyl-1-benzenesulfonate) 6a (472.8 mg, 0.708 mmol) in acetone (7.1 mL) was added 2,2-
dimethoxypropane (0.89 mL, 7.1 mmol) and \( p \)-toluenesulfonic acid (18.2 mg, 0.094 mmol) at ambient temperature. The resulting colorless solution was stirred at ambient temperature overnight (23 h). The reaction mixture was quenched with aqueous saturated NaHCO\(_3\) (8 mL) and the solvent removed under reduced pressure. The residue was extracted with methylene chloride (4 mL \( \times \) 3) and the combined organic layer dried over Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. Flash column chromatography (Si\(_2\)O\(_2\); hexane:ethyl acetate, 3:1) provided acetonide 7a (473.4 mg, 94 %) as a pale yellow solid. TLC \( R_f \) = 0.64 (hexane:ethyl acetate, 3:1). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta \) 7.19 (s, 4H), 4.13 (septet, 4H, \( J = 6.9 \) Hz), 4.03 (m, 6H), 2.92 (septet, 2H, \( J = 6.9 \) Hz), 1.66 (t, 2H, \( J = 7.7 \) Hz), 1.26 (d, 36H, \( J = 6.9 \) Hz), 1.22 (s, 6H); HRMS: Cacl. for (M + Na)\(^+\): 731.3627, found: 731.3651.

\[ \text{Diiodide 8a:} \] To a stirred solution of (2\( R \),4\( R \))-2,4-Dihydroxypentane-1,5-diyl bis-(2,4,6-triisopropyl-1-benzenesulfonate) 7a (938.1 mg, 1.325 mmol) in 2-butanone (13 mL) was added sodium iodide (1.985 g, 13.24 mmol) followed by anhydrous pyridine (0.54 mL, 6.7 mmol) at ambient temperature. The resulting light yellow suspension was refluxed overnight (25 h). The reaction mixture was cooled to ambient temperature and the solvent was removed under reduced pressure. The residue was treated with water (10 mL), extracted with ethyl acetate (10 mL \( \times \) 4), dried over MgSO\(_4\), filtered and concentrated under reduced pressure. Flash column chromatography (Si\(_2\)O\(_2\); hexane to hexane:ethyl acetate, 15:1) provided diiodide 8a (484.7 mg, 92 %) as a colorless oil. TLC \( R_f \) = 0.55 (hexane:ethyl acetate, 10:1). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \( \delta \) 3.85 (m, 2H), 3.18 (m, 4H), 1.78 (t, 2H, \( J = 3.3 \) Hz), 1.39 (s, 6H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)): \( \delta \) 101.6 (C), 66.9 (CH), 38.6 (CH\(_2\)), 24.6 (CH\(_3\)), 8.9 (CH\(_2\)).
**Diiodo diol 9a:** To a stirred solution of diiodide 8a (100.9 mg, 0.255 mmol) in THF (2.5 mL) was added 1N HCl (2.6 mL) at ambient temperature. The reaction mixture was stirred at ambient temperature for 45 min. NaHCO₃ (powder) was added to adjust pH to 7 and then the organic solvent was removed under reduced pressure. The residue was extracted with ethyl acetate (3 mL × 4), dried over MgSO₄, filtered and concentrated under reduced pressure. Diiodo diol 9a was obtained as a white solid which was in good enough quality to be carried on without further purification. TLC Rf = 0.14 (hexane:ethyl acetate, 3:1). ¹H NMR (300 MHz, CDCl₃): δ 3.94 (m, 2H), 3.39 (dd, 2H, J = 10.2, 4.5 Hz), 3.28 (dd, 2H, J = 10.2, 6.9 Hz), 2.54 (d, 2H, J = 4.5 Hz), 1.86 (dd, 2H, J = 6.3, 5.4 Hz); ¹³C NMR (75 MHz, CDCl₃): δ 68.4, 41.6, 15.0.

**Hydroxyacetophenone 10a:** To a stirred solution of diiodo diol 9a (90.7 mg, 0.255 mmol, assuming quantitative yield from previous step) in 2-propanol (5.1 mL) was added 2,6-dihydroxyacetophenone (240.3 mg, 1.532 mmol) at ambient temperature. The resulting yellow solution was heated to reflux and a mixture of KOH (118.5 mg, 1.901 mmol) in water (0.20 mL) and 2-propanol (0.26 mL) was added. The reaction mixture was refluxed for 24 h, cooled to ambient temperature, and then concentrated under reduced pressure. The dark brown residue was treated with ethyl acetate (5 mL) and filtered. The filtrate was concentrated under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate, 5:1 to 3:1 to 1:1 to 1:2) provided hydroxyacetophenone 10a (87.9 mg, 85 %) as a light yellow solid. TLC Rf = 0.24 (hexane:ethyl acetate, 1:2). ¹H NMR (300 MHz, DMSO-d6): δ 12.21 (br, 2H), 7.34 (t, 2H, J =
8.3 Hz), 6.56 (d, 2H, J = 8.4 Hz), 6.49 (d, 2H, J = 8.1 Hz), 4.97 (d, 2H, J = 5.4 Hz), 4.08 (m, 2H), 3.99 (m, 4H), 2.62 (s, 6H), 1.64 (t, 2H, J = 6.3 Hz); HRMS: Cacld. for (M + Na)^+: 427.1369, found: 427.1354.

Diester 11a: To a freshly prepared NaOMe solution (206.8 mg of sodium cube in 18 mL of anhydrous MeOH) was added anhydrous diethyl ether (36 mL) followed by dimethyl oxalate (1.065 g, 8.931 mmol). After the solid disappeared, hydroxylacetophenone 10b (360.3 mg, 0.892 mmol) was added to the reaction mixture. The resulting yellow solution was refluxed for 24 h, cooled to ambient temperature, and then concentrated under reduced pressure. The yellow solid residue was treated with water (45 mL) and acidified with concentrated HCl (0.20 mL) until pH ~ 2. The yellow precipitated was filtered off, dissolved in anhydrous MeOH, and treated with concentrated HCl (2.7 mL). The reaction mixture was refluxed for 40 min and then cooled to ambient temperature. The pale brown precipitate was filter off and found to be pure to be carried on (191.7 mg, 40 %). TLC R_f = 0.10 (ethyl acetate:ethanol, 9:1). $^1$H NMR (300 MHz, DMSO-d6): δ 7.73 (t, 2H, J = 8.4 Hz), 7.22 (d, 2H, J = 8.4 Hz), 7.09 (d, 2H, J = 8.4 Hz), 6.75 (s, 2H), 4.07 (m, 6H), 3.92 (s, 6H), 3.50 (br, 2H), 1.78 (t, 2H, J = 6.0 Hz); $^{13}$C NMR (75 MHz, DMSO-d6): δ 176.6, 160.6, 158.6, 157.1, 150.1, 135.4, 115.4, 114.8, 110.4, 109.7, 74.4, 65.2, 53.4, 37.2. HRMS: Cacld. for (M + Na)^+: 563.1165, found: 563.1149.

5´DSCG-(R,R)-diviol 1a: To a stirred suspension of diester 11a (101.5 mg, 0.188 mmol) in ethanol (9.4 mL) was added aqueous NaOH solution (2.5 M, 0.15 mL, 0.38 mmol) at ambient
temperature. The reaction mixture was refluxed for 10 min, cooled to ambient temperature, and then at 0 °C for 20 min. After filtration and drying under high vacuum for 24 h, 1a was obtained as brown solid (91.2 mg, 89%). 1H NMR (300 MHz, D₂O): δ 7.60 (t, 2H, J = 8.4 Hz), 7.08 (d, 2H, J = 8.4 Hz), 6.89 (d, 2H, J = 8.4 Hz), 6.64 (s, 2H), 4.37 (m, 2H), 4.13 (m, 4H), 1.97 (t, 2H, J = 6.2 Hz); 13C NMR (75 MHz, D₂O): δ 181.4, 165.1, 157.2, 157.2, 156.2, 135.2, 113.0, 112.0, 110.6, 108.2, 73.1, 65.5, 35.5. HRMS: Cacld. for (M + Na)+: 579.0483, found: 579.0491.

1,2:4,5-bis-acetal 2b: To a stirred suspension of D-arabitol (3.004 g, 19.55 mmol) in anhydrous THF (49 mL) was added 2,2-dimethoxypropane (5.1 mL, 41 mmol) at ambient temperature. The reaction mixture was refluxed for 15 min and then L-(-)-camphorsulfonic acid (463.7 mg, 1.956 mmol) was added at refluxing temperature. After refluxing for another 3 minutes, the reaction was quenched with 2 M NaOH (11 mL) at refluxing temperature. The solvent was removed under reduced pressure and residue was extracted with diethyl ether (11 mL × 3). The combined organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. The colorless oil residue was dissolved in anhydrous methylene chloride (49 mL) and treated with Et₃N (3.0 mL). The reaction mixture was then brought to reflux and succinic anhydride (504.6 mg, 4.891 mmol) was added. After refluxing for 6.5 h, the reaction was quenched with saturated aqueous NaHCO₃ (8.0 mL) at refluxing temperature. The mixture was cooled to ambient temperature and the organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate, 5:1 to 3:1) provided 1,2:4,5-bis-acetal 2b: (2.7085 g, 60 %) as a pale yellow oil. TLC Rf = 0.49 (hexane:ethyl acetate, 1:1). 1H NMR (300 MHz, CDCl₃): δ 4.26 (ddd, 1H, J = 6.6, 6.6, 4.2 Hz),
4.13 (m, 2H), 4.04 (dd, 1H, $J = 10.8, 5.4$ Hz), 3.98 (m, 1H), 3.92 (dd, 1H, $J = 8.3, 6.6$ Hz),
3.43(m, 1H), 2.29 (d, 1H, $J = 6.3$ Hz), 1.45 (s, 3H), 1.41 (s, 3H), 1.39 (s, 3H), 1.36 (s, 3H).

**Imidazolyl thiocarbonyl derivative 3b:** To a stirred solution of 1,2:4,5-bis-acetal 2b (2.678 g, 11.54 mmol) in anhydrous 1,2-dichloroethane (38 mL) was added 1,1′-thiocarbonyl diimidazole (2.8955g, 14.62 mmol) at ambient temperature. The resulting brown solution was refluxed for 7 h and then cooled to ambient temperature. The solvent was removed under reduced pressure. Flash column chromatography (SiO$_2$; hexane:ethyl acetate 3:1 to 1:1) provided imidazolyl thiocarbonyl derivative 3b (3.8679 g, 98%) as a yellow oil. TLC $R_f$ = 0.25 (hexane:ethyl acetate, 1:1). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 8.34 (m, 1H), 7.63 (m, 1H), 7.03 (m, 1H), 5.88 (dd, 1H, $J = 5.7, 3.3$ Hz), 4.45(m, 2H), 4.08 (m, 3H), 3.83 (dd, 1H, $J = 9.0, 6.0$ Hz), 1.39 (s, 3H), 1.33 (s, 9H);
$^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 184.5, 137.0, 131.0, 118.0, 109.7, 109.6, 80.9, 74.8, 74.5, 65.8, 65.2, 26.3, 26.1, 25.0, 24.9. HRMS: Calcd. for (M + H)$^+$: 343.1328, found: 343.1313.

**1,2:4,5-bis-acetal 4b:** Benzoyl peroxide (560.0 mg, 2.266 mmol) was added to a stirred solution of imidazolyl thiocarbonyl derivative 3b (3.8679 g, 11.31 mmol) in triethylsilane (56 mL) at refluxing temperature. The reaction mixture was refluxed for 2 h during which similar amount of benzoyl peroxide was added after 30 min, 60 min, and 90 min, respectively. The solvent was then removed under reduced pressure. Flash column chromatography (SiO$_2$, hexane to hexane:ethyl acetate, 30:1 to 15:1) provided 1,2:4,5-bis-acetal 4b (~2.42 g) as a light yellow oil
which contained some aromatic impurities and triethyl silane but was good enough to be carried on. TLC R\textsubscript{f} = 0.65 (hexane:ethyl acetate, 1:1).

\[
\begin{align*}
\text{HO} & \quad \text{HO} \\
\text{OH} & \quad \text{OH} \\
\text{5b}
\end{align*}
\]

\textbf{(2S,4S)-pentane-1,2,4,5-tetraol 5b:}\textsuperscript{117} 0.5 M H\textsubscript{2}SO\textsubscript{4} (14 mL, 7.0 mmol) was added to a stirred solution of 1,2:4,5-bis-acetal 4b (~2.42 g, 11.1 mmol, impure) in ethanol (14 mL) at ambient temperature. The reaction mixture was refluxed for 4 h and then BaCO\textsubscript{3} was added to adjust the pH to 7. The resulting milky mixture was filtered and the white solid thus collected was heated with MeOH (15 mL) at 50 °C for 10 min. The combined filtrate was concentrated under reduced pressure. Flash column chromatography (SiO\textsubscript{2}; DCM:MeOH, 7:3) provided (2S,4S)-pentane-1,2,4,5-tetraol 5b (960.5 mg, 62 % over 2 steps) as a white solid. TLC R\textsubscript{f} = 0.25 (DCM:MeOH, 7:3). \textsuperscript{1}H NMR (300 MHz, MeOH-d4): δ 3.85 (m, 2H), 3.47 (m, 4H), 1.52 (dd, 2H, J = 7.1, 5.6 Hz).

\[
\begin{align*}
\text{TPSO} & \quad \text{OH} \\
\text{OTPS} & \quad \text{OH} \\
\text{6b}
\end{align*}
\]

\textbf{(2S,4S)-2,4-Dihydroxypentane-1,5-diyl Bis-(2,4,6-triisopropyl-1-benzenesulfonate) 6b:}\textsuperscript{117} To a stirred solution of (2S,4S)-pentane-1,2,4,5-tetraol 5b (85.3 mg, 0.627 mmol) in anhydrous pyridine (0.63 mL) was added 2,4,6-triisopropyl benzenesulfonyl chloride (490.5 mg, 1.571 mmol) at 0 °C. The pale yellow cloudy mixture was stirred at ambient temperature for 10 h and then the solvent removed under reduced pressure. Flash column chromatography (SiO\textsubscript{2}; hexane:acetone, 3:1) provided (2S,4S)-2,4-dihydroxypentane-1,5-diyl bis-(2,4,6-triisopropyl-1-benzenesulfonate) 6a (293.3 mg, 70 %) as a white fluffy solid. TLC R\textsubscript{f} = 0.77 (hexane:ethyl acetate, 1:1). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}): δ 7.20 (s, 4H), 4.23 (m, 2H), 4.10 (m, 6H), 3.98 (dd,
2H, \( J = 10.2, 6.9 \text{ Hz} \), 2.92 (septet, 2H, \( J = 6.9 \text{ Hz} \)), 2.87 (br, 2H), 1.64 (t, 2H, \( J = 5.7 \text{ Hz} \)), 1.27 (d, 24H, \( J = 6.9 \text{ Hz} \)), 1.26 (d, 12H, \( J = 6.9 \text{ Hz} \)).

**Acetonide 7b:** To a stirred solution of (2S,4S)-2,4-Dihydroxypentane-1,5-diyl bis-(2,4,6-triisopropyl-1-benzenesulfonate) 6b (1.115 g, 1.670 mmol) in acetone (17 mL) was added 2,2-dimethoxypropane 2.1 mL, 17 mmol) and p-toluenesulfonic acid (36.5 mg, 0.189 mmol) at ambient temperature. The resulting colorless solution was stirred at ambient temperature overnight (25 h). The reaction mixture was quenched with aqueous saturated NaHCO₃ (17 mL) and the solvent removed under reduced pressure. The residue was extracted with methylene chloride (17 mL \( \times 3 \)) and the combined organic layer dried over Na₂SO₄, filtered, and concentrated under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate, 3:1) provided acetonide 7b (2.099 g, 80 %) as a white solid. TLC \( R_f = 0.73 \) (hexane:ethyl acetate, 3:1). \(^1\text{H NMR (300 MHz, CDCl}_3\)): \( \delta \) 7.19 (s, 4H), 4.08 (m, 10H), 2.92 (septet, 2H, \( J = 6.9 \text{ Hz} \)), 1.65 (t, 2H, \( J = 7.8 \text{ Hz} \)), 1.27 (d, 12H, \( J = 7.5 \text{ Hz} \)), 1.26 (d, 24H, \( J = 6.9 \text{ Hz} \)), 1.23 (s, 6H); \(^{13}\text{C NMR (75 MHz, CDCl}_3\)): \( \delta \) 153.7, 150.8, 129.3, 123.8, 101.0, 70.1, 64.5, 34.2, 30.0, 29.6, 24.7, 24.3, 23.5. Caclđ. for (M+Na)^+: 731.3627, found: 731.3610.

**Diiodide 8b:** To a stirred solution of (2S,4S)-2,4-Dihydroxypentane-1,5-diyl bis-(2,4,6-triisopropyl-1-benzenesulfonate) 7b (1.343 g, 1.897 mmol) in 2-butanone (19 mL) was added sodium iodide (2.815 g, 18.78 mmol) followed by anhydrous pyridine (0.77 mL, 9.5 mmol) at ambient temperature. The resulting light yellow suspension was refluxed overnight (22 h). The
reaction mixture was cooled to ambient temperature and the solvent was removed under reduced pressure. The residue was treated with water (30 mL), extracted with ethyl acetate (30 mL × 3), dried over MgSO$_4$, filtered and concentrated under reduced pressure. Flash column chromatography (SiO$_2$; hexane to hexane:ethyl acetate, 15:1) provided diiodide 8b (1.632 g, 85%) as a light yellow oil. TLC $R_f$ = 0.53 (hexane:ethyl acetate, 10:1). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 3.85 (m, 2H), 3.18 (m, 4H), 1.78 (t, 2H, $J = 3.3$ Hz), 1.39 (s, 6H); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 101.6 (C), 66.9 (CH), 38.6 (CH$_2$), 24.6 (CH$_3$), 8.9 (CH$_2$).

Diiodo diol 9b: To a stirred solution of diiodide 8b (972.0 mg 2.455 mmol) in THF (25 mL) was added 1N HCl (25 mL) at ambient temperature. The reaction mixture was stirred at ambient temperature for 2 h. NaHCO$_3$ (powder) was added to adjust pH to 7 and then the organic solvent was removed under reduced pressure. The residue was extracted with ethyl acetate (20 mL × 3), dried over MgSO$_4$, filtered and concentrated under reduced pressure. Diiodo diol 9b (1.339 g, 92%) was obtained as a white solid which was in good enough quality to be carried on without further purification. TLC $R_f$ = 0.14 (hexane:ethyl acetate, 3:1). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 3.94 (m, 2H), 3.39 (dd, 2H, $J = 10.2$, 4.5 Hz), 3.28 (dd, 2H, $J = 10.2$, 6.9 Hz), 2.53 (d, 2H, $J = 5.1$ Hz), 1.86 (dd, 2H, $J = 6.3$, 5.4 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 68.4, 41.6, 15.0. HRMS: Caclld. for M$^+$: 355.8765, found: 355.8768.

Hydroxyacetophenone 10b: To a stirred solution of diiodo diol 9b (651.1 mg, 1.829 mmol) in 2-propanol (37 mL) was added 2,6-dihydroxyacetophenone (1.7231 g, 10.99 mmol) at ambient temperature.
temperature. The resulting yellow solution was heated to reflux and a mixture of KOH (843.8 mg, 13.53 mmol) in water (1.0 mL) and 2-propanol (1.9 mL) was added. The reaction mixture was refluxed for 22 h, cooled to ambient temperature, and then concentrated under reduced pressure. The dark brown residue was treated with ethyl acetate (50 mL) and filtered. The collected solid was washed with ethyl acetate and the combined filtrate was concentrated under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate, 1:1 to 1:2 to 1:5, and then ethyl acetate) provided hydroxyacetophenone 10b (g, 35 %) as a light yellow solid. TLC Rf = 0.18 (hexane:ethyl acetate, 1:2). ¹H NMR (300 MHz, DMSO-d6): δ 12.21 (s, 2H), 7.34 (t, 2H, J = 7.8 Hz), 6.56 (d, 2H, J = 7.8 Hz), 6.50 (d, 2H, J = 7.8 Hz), 4.97 (br, 2H), 4.11 (m, 2H), 3.99 (m, 4H), 2.62 (s, 6H), 1.64 (m, 2H); ¹³C NMR (75 MHz, DMSO-d6): δ 204.4, 161.1, 159.5, 134.9, 113.2, 109.5, 103.0, 73.7, 65.2, 38.1, 33.5. HRMS: Cacl’d for (M + Na)⁺: 427.1369, found: 427.1354.

Diester 11b: To a freshly prepared NaOMe solution (206.8 mg of sodium cube in 18 mL of anhydrous MeOH) was added anhydrous diethyl ether (36 mL) followed by dimethyl oxalate (1.0653 g, 8.931 mmol). After the solid disappeared, hydroxylacetophenone 10b (360.3 mg, 0.892 mmol) was added to the reaction mixture. The resulting yellow solution was refluxed for 24 h, cooled to ambient temperature, and then concentrated under reduced pressure. The yellow solid residue was treated with water (45 mL) and acidified with concentrated HCl (0.20 mL) until pH ~ 2. The yellow precipitated was filtered off, dissolved in anhydrous MeOH, and treated with concentrated HCl (2.7 mL). The reaction mixture was refluxed for 40 min and then cooled to ambient temperature. The pale brown precipitate was filter off and found to be pure to be
carried on (191.7 mg, 40 %). TLC $R_f = 0.10$ (ethyl acetate:ethanol, 9:1). $^1$H NMR (300 MHz, DMSO-d6): $\delta$ 7.73 (t, 2H, $J = 8.4$ Hz), 7.22 (d, 2H, $J = 8.4$ Hz), 7.09 (d, 2H, $J = 8.4$ Hz), 6.75 (s, 2H), 4.85 (d, 2H $J = 4.8$ Hz), 4.07 (m, 6H), 3.92 (s, 6H), 1.78 (t, 2H, $J = 6.0$ Hz); $^{13}$C NMR (75 MHz, DMSO-d6): $\delta$ 176.6, 160.6, 158.6, 157.1, 150.1, 135.4, 115.4, 114.8, 110.4, 109.7, 74.4, 65.2, 53.4, 37.2. MS: Cacld. for (M + Na)$^+$: 563.1165, found: 563.1178.

5´DSCG-(S,S)-diviol 1b: To a stirred suspension of diester 11b (120.5 mg, 0.223 mmol) in ethanol (11 mL) was added aqueous NaOH solution (2.5 M, 0.18 mL, 0.45 mmol) at ambient temperature. The reaction mixture was refluxed for 10 min, cooled to ambient temperature, and then at 0 °C for 20 min. After filtration and drying under high vacuum for 24 h, 1b was obtained as brown solid (108.1 mg, 87%). $^1$H NMR (300 MHz, D$_2$O): $\delta$ 7.59 (t, 2H, $J = 8.4$ Hz), 7.06 (d, 2H, $J = 8.7$ Hz), 6.87 (d, 2H, $J = 8.4$ Hz), 6.62 (s, 2H), 4.38 (m, 2H), 4.11 (m, 4H), 1.97 (t, 2H, $J = 6.2$ Hz); $^{13}$C NMR (75 MHz, D$_2$O): $\delta$ 181.4, 165.1, 157.1, 157.1, 156.2, 135.2, 113.0, 112.0, 110.5, 108.1, 73.1, 65.5, 35.4. HRMS: Cacld. for (M + Na)$^+$: 579.0483, found: 579.0491.

1,2:4,5-bis-acetal 2c: To a stirred suspension of xylitol (2.590 g, 16.85 mmol) in anhydrous THF (48 mL) was added 2,2-dimethoxypropane (4.6 mL, 37 mmol) at ambient temperature. The reaction mixture was refluxed for 15 min and then L-(-)-camphorsulfonic acid (398.7 mg, 1.682 mmol) was added at refluxing temperature. After refluxing for another 5 minutes, the reaction was quenched with 2 M NaOH (10 mL) at refluxing temperature. After removing the majority of the solvent under reduced pressure, the aqueous layer was extracted with diethyl ether (10 mL ×
The combined organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. The colorless oil residue was dissolved in anhydrous DCM (50 mL) and Et₃N (2.5 mL) was added. The reaction mixture was then brought to reflux and succinic anhydride (427.6 mg, 4.203 mmol) was added. After refluxing for another hour, the reaction was quenched with saturated aqueous NaHCO₃ (5 mL) at refluxing temperature. The mixture was cooled to ambient temperature and the organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. Flash column chromatography (SiO₂; diethyl ether:hexane) provided 1,2:4,5-bis-acetal 5: (1.720 g, 44 %) as a pale yellow oil. TLC Rf = 0.23 (diethyl ether:hexane, 1:1). ¹H NMR (300 MHz, CDCl₃): δ 4.18 (ddd, 2H, J = 6.6, 6.6, 5.1 Hz), 4.07 (dd, 2H, J = 8.1, 6.6 Hz), 3.89 (dd, 2H, J = 8.3, 6.8 Hz), 3.60 (dt, 1H, J = 6.0, 5.1 Hz), 2.44 (d, 1H, J = 6.0 Hz), 1.45 (s, 6H), 1.38 (s, 6H).

Imidazolyl thiocarbonyl derivative 3c: To a stirred solution of 1,2:4,5-bis-acetal 5 (265.2 mg, 1.143 mmol) in anhydrous 1,2-dichloroethane (4.1 mL) was added N,N´-thiocarbonyldiimidazole (396.1 mg, 2.000 mmol) at ambient temperature. The resulting brown solution was refluxed for 4 h and then cooled to ambient temperature. The solvent was removed under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate) provided imidazolyl thiocarbonyl derivative 6 (371.8 mg, 95 %) as a yellow oil. TLC Rf = 0.30 (diethyl ether:hexane, 3:2). ¹H NMR (300 MHz, CDCl₃): δ 8.38 (t, 1H, J = 0.9 Hz), 7.68 (t, 1H, J = 1.5 Hz), 7.06 (dd, 1H, J = 1.8, 0.9 Hz), 5.84 (t, 1H, J = 5.4 Hz), 4.13 (m, 4H), 3.92 (dd, 2H, J = 8.9, 5.9 Hz), 1.43 (s, 6H), 1.35 (s, 6H). HRMS: Cacl. for (M + H): 343.1328, found: 343.1312.
**1,2:4,5-bis-acetal 4c:** Benzoyl peroxide (53.4 mg, 0.216 mmol) was added to a stirred solution of imidazolyl thiocarbonyl derivative 6 (371.8 mg, 1.087 mmol) in triethylsilane (8.0 mL) at refluxing temperature. The reaction mixture was refluxed for 2 h during which similar amount of benzoyl peroxide was added after 30 min, 60 min, and 90 min, respectively. The solvent was then removed under reduced pressure. Flash column chromatography (SiO$_2$; hexane:ethyl acetate) provided 1,2:4,5-bis-acetal 7 (199.7 mg, 85%) as a yellow oil. TLC $R_f$ = 0.71 (diethyl ether:hexane, 1:1). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 4.19 (m, 2H), 4.09 (dd, 2H, $J = 8.1, 6.0$ Hz), 3.62 (dd, 2H, $J = 7.8, 7.4$ Hz), 2.01 (m, 1H), 1.79 (dt, 1H, $J = 13.8, 6.0$ Hz), 1.42 (s, 6H), 1.36 (s, 6H).

**5c**

(2S,4R)-pentane-1,2,4,5-tetraol 5c: 0.5 M H$_2$SO$_4$ (1.0 mL) was added to a stirred solution of 1,2:4,5-bis-acetal 7 (170.0 mg, 0.787 mmol) in ethanol (1 mL) at ambient temperature. The reaction mixture was refluxed for 3.5 h and then BaCO$_3$ was added to adjust the pH to 7. The resulting milky mixture was refluxed for another 10 min, cooled to ambient temperature, and then filtered. The solid was treated with MeOH (5 mL) and the mixture was stirred at 50°C for 10 min. Filtered again and washed the solid with another 5 mL of MeOH. The combined filtrated was concentrated under reduced pressure. Flash column chromatography (SiO$_2$, DCM:MeOH) provided (2S,4R)-pentane-1,2,4,5-tetraol 5c (119.4 mg, 97%) as a white solid. TLC $R_f$ = 0.01 (ethyl acetate). $^1$H NMR (300 MHz, MeOH-d$_4$): $\delta$ 3.82 (m, 4H), 3.49 (m, 2H), 1.73 (dt, 1H, $J = 14.1, 4.8$ Hz), 1.53 (dt, 1H, $J = 14.1, 8.1$ Hz); $^{13}$C NMR (75 MHz, MeOH-d$_4$): $\delta$ 71.7 (CH), 67.4 (CH$_2$), 37.7 (CH$_2$).
**Bromoacetate 6c**: To a stirred suspension of (2S,4R)-pentane-1,2,4,5-tetraol 5c (597.7 mg, 4.395 mmol) in anhydrous 1,4-dioxane (11 mL) was added acetyl bromide (0.80 mL, 10 mmol) dropwise at 0 °C. The reaction mixture was stirred at ambient temperature overnight (22 h) and then the solvent was removed under reduced pressure. The residue was taken up in diethyl ether (20 mL) and washed with saturated aqueous NH₄Cl. The aqueous layer was extracted with diethyl ether (20 mL × 3), dried over MgSO₄, filtered, and concentrated under reduced pressure. ¹H NMR of the crude indicated the presence of the desired product bromoacetate 6c, which was unstable and carried on immediately without further purification.

**Diepoxide 7c**: The crude bromoacetate 6c obtained from previous step was dissolved in anhydrous diethyl ether (44 mL) and then cooled to 0 °C. Freshly ground KOH (904.7 mg, 14.51 mmol) was added to the reaction mixture in one portion. The reaction mixture was then warmed to ambient temperature while stirred vigorously. Another portion of freshly ground KOH (905.8 mg, 14.53 mmol) was added to the reaction mixture after 1 h at 0 °C. After stirring at ambient temperature for 5 h, the reaction mixture was filter through a pad of MgSO₄. The filtrate was carefully distilled from an ice-water bath under reduce pressure with the receiving flask immersed in a bath at -78 °C. The pale yellow oil left in the distilling flask was in good enough purity to be carried on. TLC Rₜ = 0.40 (hexane:ethyl acetate, 3:1).
**Diiodo diol 8c:** The crude diepoxide 7c obtained from previous step was dissolved in anhydrous acetonitrile (11 mL). To the reaction mixture was added sodium iodide (1.352 g, 9.022 mmol) followed by cerium (III) chloride heptahydrate (1.643 g, 4.409 mmol) at ambient temperature. The resulting yellow suspension was refluxed for 2 h and then cooled to ambient temperature. The reaction mixture was washed with water (15 mL), extracted with methylene chloride (15 mL × 3), dried over MgSO₄, filtered, and then concentrated. The crude yellow oil was carried on without further purification. TLC Rᵣ = 0.15 (hexane:ethyl acetate, 3:1).

![Image of diiodo diol 8c](image_url)

**Hydroxyacetophenone 9c:** To a stirred solution of the crude diiodo diol 8c in 2-propanol (88 mL) was added 2,6-dihydroxyacetophenone (3.616 g, 23.05 mmol) at ambient temperature. The resulting yellow solution was heated to reflux and a mixture of KOH (1.7024 g, 27.31 mmol) in water (1.5 mL) and 2-propanol (4.4 mL) was added. The reaction mixture was refluxed for 15 h, cooled to ambient temperature, and then concentrated under reduced pressure. Flash column chromatography (SiO₂; hexane:ethyl acetate, 1:1 to 1:2 to 1:5 to 1:8, and then ethyl acetate) provided hydroxyacetophenone 9c (438.5 mg, 25 % over 4 steps) as a light yellow solid. TLC Rᵣ = 0.26 (hexane:ethyl acetate, 1:2). ¹H NMR (300 MHz, CDCl₃): δ 12.2 (s, 2H), 7.34 (t, 2H, J = 8.3 Hz), 6.56 (d, 2H, J = 8.4 Hz), 6.49 (d, 2H, J = 8.1 Hz), 5.02 (d, 2H, J = 4.8 Hz), 4.05 (m, 6H), 2.61 (s, 6H), 1.83 (m, 1H), 1.71 (m, 1H); ¹³C NMR (75 MHz, DMSO-d₆): δ 204.4, 161.0, 159.5, 134.9, 113.2, 109.5, 103.0, 73.0, 66.0, 37.8, 33.4. HRMS: Cacld. for M⁺: 404.1466, found: 404.1458.
**Diester 10c:** To a freshly prepared NaOMe solution (202.3 mg of sodium cube in 17.6 mL of anhydrous MeOH) was added anhydrous diethyl ether (35.2 mL) followed by dimethyl oxalate (1.060 g, 8.886 mmol). After the solid disappeared, hydroxylacetophenone 9c (355.7 mg, 0.880 mmol) was added to the reaction mixture. The resulting yellow solution was refluxed for 24 h, cooled to ambient temperature, and then concentrated under reduced pressure. The yellow solid residue was treated with water (45 mL) and acidified with concentrated HCl (0.22 mL) until pH ~ 2. The yellow precipitated was filtered off, dissolved in anhydrous MeOH, and treated with concentrated HCl (2.7 mL). The reaction mixture was refluxed for 1.5 h, cooled to ambient temperature and then 0 °C for 1 h. The brown precipitate was filter off and found to be the desired product diester 10c (8.6 mg, 2 %). However, the majority of the diester 10c remained in the filtrate as oily substance. The filtrate was concentrated to about half the volume and carried on without further purification. $^1$H NMR (300 MHz, DMSO-d6): $\delta$ 7.71 (t, 2H, $J = 8.4$ Hz), 7.20 (d, 2H, $J = 8.4$ Hz), 7.08 (d, 2H, $J = 8.4$ Hz), 6.73 (s, 2H), 4.91 (br, 2H), 4.08 (m, 6H), 3.92 (s, 6H), 2.03 (m, 1H), 1.81 (m, 1H). HRMS: Cacld. for (M + Na)$^+$: 563.1165, found: 563.1167.

![Structure of Diester 10c](image)

**5´DSCG-meso-diviol 1c:** To the filtrate from the previous step was added aqueous NaOH solution (2.5 M, 5.0 mL, 13 mmol) at ambient temperature. Some pale brown precipitate formed and the pH of the liquid was approximately 1. The reaction mixture was warmed to reflux and treated with NaOH pellet in 10 portions. The resulting suspension was refluxed for 1.5 h, cooled to ambient temperature, and then 0 °C for 30 min. The pale brown precipitate was collected by centrifugation and dried under vacuum overnight. 5´DSCG-meso-diviol 1c was obtained as pale brown powder (332.3 mg, 73 % over 2 steps). $^1$H NMR (300 MHz, D$_2$O): $\delta$ 7.58 (t, 2H, $J = 8.4$ Hz), 7.38 (d, 2H, $J = 8.4$ Hz), 7.18 (d, 2H, $J = 8.4$ Hz), 7.06 (d, 2H, $J = 8.4$ Hz), 4.95 (br, 2H), 4.15 (m, 6H), 4.05 (s, 6H), 2.05 (m, 1H), 1.83 (m, 1H). HRMS: Cacld. for (M + Na)$^+$: 563.1165, found: 563.1167.
Hz), 7.06 (d, 2H, $J = 8.4$ Hz), 6.91 (d, 2H, $J = 8.4$ Hz), 6.60 (s, 2H), 4.25 (m, 4H), 4.09 (m, 2H),
2.36 (m, 1H), 1.92 (m, 1H); $^{13}$C NMR (75 MHz, D$_2$O): 181.1, 165.1, 157.0, 156.9, 156.2, 135.2,
113.1, 112.1, 110.7, 108.4, 72.5, 65.9, 37.6. HRMS Cacl'd. for (M + Na)$^+$: 579.0491, found:
579.0471.
1.964
1.927
2.006
1.211
6.68
5.516
1.953

OH
OH
CO₂Me 11a
CO₂Me

7.757
7.729
7.720
7.701
7.233
7.205
7.099
7.071
6.746
6.709

4.176
4.139
4.114
4.099
4.082
4.067
4.037
4.011
4.005
3.984
3.917

2.505
2.500
2.494
2.488
1.003
1.783
1.763
1.230
Chapter 3

Bicyclic Brominated Furanones: A new Class of Quorum Sensing Modulators that Inhibit Biofilm Formation with Reduced Toxicity, and Improved Bacterial Clearance in vivo

Summary

Both natural and synthetic brominated furanones are known to inhibit biofilm formation by bacteria, but their toxicity to mammalian or human cells is often not reported. Here, we designed and synthesized a new class of bicyclic brominated furanones (BBFs) that contained only one bromide group with controlled regiochemistry. This class of molecules exhibited significant reduction in the toxicity to human neuroblastoma SK-N-SH cells and did not inhibit bacterial (E. coli and P. aeruginosa) growth, but retained the inhibitory activity towards biofilm formation and virulence factor expression of bacteria. A representative compound 6-BBF also increased the susceptibility of P. aeruginosa in biofilms to the antibiotic tobramycin. All BBFs exhibited antagonistic activities in the lasI quorum sensing circuit, while only 5-BBF showed agonistic activity for the rhlI quorum sensing circuit. Furthermore, 6-BBF significantly improved P. aeruginosa clearance in the lung of mice in an immunocompromised pneumonia mouse model in vivo. This study suggests that structural variation of brominated furanones can be designed for targeted biological activities.

3.1 Background and Significance

3.2.1 Quorum sensing and autoinducers

A number of bacteria species use small molecule signals to coordinate gene expression in a cell-density dependent manner. This mechanism of sophisticated intercellular communication is
termed quorum sensing (QS).\textsuperscript{119,120} Quorum-sensing bacteria intracellularly synthesize small diffusible molecules (autoinducers or AIs) that are released into the surroundings. The extracellular concentration of AIs increases as the number of cells in a bacterial colony increases. Upon reaching a threshold concentration, AIs bind to cognate receptors within the bacterial cells, triggering a signal transduction cascade that results in a population-wide alteration in gene expression. Therefore, the response of bacterial population of AIs facilitates multicellular activities, such as bioluminescence, virulence factors expression, swarming, biofilm formation, production of antibiotics, conjugation and sporulation.\textsuperscript{121} The first QS system studied was that in the marine bacterium \textit{Vibrio fischeri} which produces luminescence at high but not low cellular densities.\textsuperscript{122} Although originally thought to be limited to a small number of marine bacterial species, it is now widely recognized that QS is a common part of regulatory machinery utilized by many bacterial species.\textsuperscript{120,123} A number of AIs have been identified, among which \textit{N}-acyl homoserine lactones (AHLs) are the most common signals used by Gram-negative bacteria and oligopeptides by Gram-positive bacteria. A furanosyl borate diester (autoinducer-2 or AI-2) is produced and used by both Gram-negative and Gram-positive bacteria and is proposed to enable interspecies communication.\textsuperscript{124} Other small molecule signals have also been associated with QS in Gram-negative bacteria, such as 2-heptyl-3-hydroxy-4-quinolone (PQS) used by \textit{P. aeruginosa}.\textsuperscript{125,126}

One of the best-understood bacterial QS systems to date is that mediated by AHLs, which are composed of a homoserine lactone ring (HSL) and an acyl chain. The types of AHLs produced vary greatly among bacterial species (Table 3.1). Some species such as \textit{Agrobacterium tumefaciens}\textsuperscript{127} and \textit{Erwinia carotovara}\textsuperscript{128} produce mainly a single type of AHL, \textit{N}-(3-oxo-octanoyl)-\textit{L}-homoserine lactone (3-oxo-C8-HSL) for \textit{Agrobacterium tumefaciens} and \textit{N}-(\textbeta-
ketocaproyl)-L-homoserine lactone (3-oxo-C6-HSL) for *Erwinia carotovara*, respectively. Other species such as *Pseudomonas aeruginosa* has two AHL synthases, LasI and RhlI, which produce *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12-HSL)\(^\text{129}\) and *N*-butanoyl-L-homoserine lactone (C4-HSL)\(^\text{130}\), respectively. In addition, some of the AHLs are produced by multiple species. For example, 3-oxo-C6-HSL is produced by both *Erwinia carotovara* and *Vibrio fischeri*.\(^\text{122}\)
Table 3.1 Representative AHL systems and corresponding bacteria. (Adapted and modified from the review by Spring and co-workers.\textsuperscript{131})

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>AHL</th>
<th>Nomenclature\textsuperscript{a}</th>
<th>LuxI/R homologs</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Agrobacterium tumaei}</td>
<td></td>
<td>3-oxo-C8-HSL</td>
<td>Tral/R</td>
</tr>
<tr>
<td>\textit{Burkholderia cenocepacia}</td>
<td></td>
<td>C8-HSL</td>
<td>CepI/R</td>
</tr>
<tr>
<td>\textit{Chromobacterium violaceum}</td>
<td></td>
<td>C6-HSL</td>
<td>CviI/R</td>
</tr>
<tr>
<td>\textit{Erwinia carotovar}</td>
<td></td>
<td>3-oxo-C6-HSL</td>
<td>ExpI/R; Carl/R</td>
</tr>
<tr>
<td>\textit{Pseudomonas aeruginosa}</td>
<td></td>
<td>3-oxo-C12-HSL</td>
<td>LasI/R; Qsc\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C4-HSL</td>
<td>RhII/R</td>
</tr>
<tr>
<td>\textit{Pseudomonas putida}</td>
<td></td>
<td>3-oxo-C12-HSL</td>
<td>PpuI/R</td>
</tr>
<tr>
<td>\textit{Serratia marcescens AS-I}</td>
<td></td>
<td>3-oxo-C6-HSL</td>
<td>SpnI/SpnR</td>
</tr>
<tr>
<td>\textit{Vibrio fischeri}</td>
<td></td>
<td>3-oxo-C6-HSL</td>
<td>LuxI/R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C8-HSL</td>
<td>AinS/R</td>
</tr>
<tr>
<td>\textit{Vibrio harvey}\textsuperscript{c}</td>
<td></td>
<td>3-hydroxy-C4-HSL</td>
<td>Generated by LuxM, detected by LuxN\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}AHL abbreviation used in this chapter. \textsuperscript{b}QscR is an orphan receptor that responds to 3-oxo-C12-HSL produced by LasI. \textsuperscript{c}This system is distinct from the typical \textit{V. fischeri} as the genes coding for LuxM and LuxN are not homologs with LuxI and LuxR.
3.2.2 Biofilm formation and consequences

Biofilms are surface-attached sessile communities of microorganisms enclosed in extracellular polymeric substance (EPS). Approximately 65-80% of microbial infections occurring in human body are biofilm-related according to the National Institute of Health (NIH) estimation.\textsuperscript{132} Biofilms are associated with many diseases including tooth decay, catheter infections, endocarditis, otitis media, chronic prostatitis, periodontal disease, chronic urinary tract infections, and osteomyelitis,\textsuperscript{133-135} and cause enormous burden and detrimental effects in medical and industrial settings.\textsuperscript{136,137} For example, the nosocomial infections caused and perpetuated by the persistent bacterial biofilm colonization of hospital facilities place a $10 billion burden on the U.S. healthcare system annually.\textsuperscript{138}

The formation of biofilm is regulated by multiple genes in the quorum sensing systems, which results in highly complex biofilm structures on the surface of microbes.\textsuperscript{123,139,140} There are several stages of biofilm formation (Figure 3.1).\textsuperscript{141} After the initial reversible attachment of planktonic cells to a surface and irreversible attachment of the cells by secreting extracellular polymeric matrix, quorum sensing triggers further development of biofilm and maturation. Finally, the dispersion allows the detachment of cells from the biofilm and colonization of new surfaces. The bacteria reside in the complex structure of biofilm are often up to 1000-fold more tolerant to antibiotics and biocides than planktonic bacteria.\textsuperscript{133,142-144}
There are several mechanisms of biofilm resistance to antimicrobial compounds, which vary with the species of bacteria present in the biofilm and the antimicrobials used.\textsuperscript{133,144} One of the mechanisms is the failure of an agent to penetrate the biofilm. It has been suggested that the exopolysaccharide matrix, one of the characteristics of biofilms, can be an initial barrier and
limit the transportation of the agents to the cells within the biofilm by either reacting with or sorption to the compound. For example, Hoyle et al. formed *P. aeruginosa* biofilms on one side of a dialysis membrane and found that the biofilms was a diffusion barrier to piperacillin.\(^{146}\)

Bacteria cells experience different nutrient and oxygen availability at different locations in the biofilms, both due to consumption by cells on the periphery of biofilm and by reduced diffusion of nutrients and oxygen deep into the biofilm.\(^{147}\) At the surface of the biofilm, sufficient nutrients and oxygen allow bacteria to grow actively. However, nutrients and oxygen are more limited deeper in the biofilms, and therefore bacteria grow more slowly or not at all. Brown et al. have suggested that other environmental factors can also cause a general stress response by bacteria reside in the biofilm that results in slow growth. These factors include heat shock, cold shock, pH changes and many chemical agents.\(^{148}\) The consequent heterogeneous metabolic activity of cells accounts for an increase in resistance to antibiotics.\(^{149-151}\) Quorum sensing (QS) has also been found to play a role in the resistance of biofilms to antimicrobials. Davies et al. demonstrated that a lasI mutant of *P. aeruginosa* formed flat, undifferentiated biofilms which were sensitive to the biocide sodium dodecyl sulfate (SDS) while the wild-type biofilms were not. In addition, the mutant biofilms appeared normal when grown in the presence of the autoinducer 3-oxo-C12-HSL (naturally produced by LasI synthase in wild-type).\(^{139}\) Similarly, a double mutant (lasR and rhlR) of *P. aeruginosa* forms biofilms that are more susceptible to killing by hydrogen peroxide and antibiotic tobramycin than the wild-type biofilms.\(^{152}\)

Biofilms have also shown increased resistance to host defense systems. In bronchopulmonary *P. aeruginosa* infections in cystic fibrosis (CF) patients, bacteria mainly exist in the biofilm mode and persist despite an intact host immune defense and frequent antibiotic treatment. Jensen
et al. showed a reduced activation of a complex of blood serum proteins of the immune system in biofilm-grown *P. aeruginosa* compared with planktonic cells.\textsuperscript{153}

3.2.3 *Use quorum sensing modulators to control biofilm formation*

Controlling the formation of biofilm has been challenging because inhibition of biofilm formation and dispersion of already formed biofilm are difficult.\textsuperscript{154} One rational approach to control biofilm formation is to interfere with the chemical communication that results in a quorum sensing between bacteria, which is one of the key events leading to the biofilm formation.\textsuperscript{121,139} Existing antibiotics generally inhibit bacterial cellular processes that are essential for survival and thus exerts an evolutionary selective pressure for drug-resistant mutations.\textsuperscript{155} Although quorum sensing is essential for bacterial group behaviors such as biofilm formation and production of virulence factors, it is not essential for survival.\textsuperscript{156} Thus inhibiting quorum sensing may inhibit biofilm formation and attenuate pathogenicity without leading to the development of drug-resistance.

The QS circuit in *V. fischeri*, the first discovered AHL-mediated system of its kind, is composed of the autoinducer synthase LuxI, which is responsible for the synthesis of AHL autoinducer, and the receptor LuxR, which is a transcriptional activator and upon binding to the cognate autoinducer, promotes transcription of the luciferase structural operon *luxCDABE* and the production of more AHL autoinducer.\textsuperscript{157-159} This circuit is the basic paradigm for AHL-based QS systems in Gram-negative bacteria. The majority of autoinducer synthases and receptors subsequently discovered in other Gram-negative bacteria are termed LuxI-type and LuxR-type proteins, respectively. Even though several bacteria species have been demonstrated to have a more complicated system for cell-to-cell signaling which involves more than one AHL signaling
molecules, and even other types of AIs, the general principles of AHL-based QS apply to the majority of Gram-negative bacteria.

The strategies applied to inhibit QS can be generally separated into three categories. The first is to interfere with the AHL production by targeting LuxI-type synthase protein. The second is to interfere with the AHL signaling molecules themselves. And the majority of work focuses on the third category that is to discover agents which can interact with the LuxR-type receptors. In this chapter, I will mainly discuss existing examples and our efforts to synthesize and identify small molecules as quorum sensing inhibitors (QSIs).

One of the most significant efforts lies in the design and synthesis of mimics to natural AHL autoinducers. Several synthetic autoinducer analogs have been reported to induce or inhibit quorum sensing of Gram-negative bacteria. Suga and coworkers reported autoinducer analogues that reduced the production of virulence factors and biofilm formation by inhibiting quorum sensing in *P. aeruginosa*.\(^\text{160,161}\) They also found that the agonist/antagonist activity of these molecules could be tuned by structural modifications. Blackwell and coworkers reported a series of systematic and comprehensive studies of synthesis of range of non-natural AHLs which were shown to inhibit quorum sensing.\(^\text{162-167}\) Some of these AHLs were found to selectively modulate one, two, or three LuxR-type proteins, namely LuxR in *V. fischeri*, TraR in *A. tumefaciens*, and LasR in *P. aeruginosa*. Moreover, a few synthetic AHLs show moderate to high inhibition of biofilm formation by the opportunistic pathogen *P. aeruginosa*.\(^\text{162,167}\)

### 3.2.4 Other biofilm inhibitors

By dissecting the natural products instead of mimicking natural AHL autoinducers, Melander and coworkers reported a library of 2-aminoimidazole derivatives that can inhibit and disperse...
biofilm formation by one or multiple species, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Bordetella bronchiseptica*, *Enterococcus faecium*, *Streptococcus mutans*, and methicillin-resistant *Staphylococcus aureus* (MRSA). Davies *et al.* discovered that a fatty acid produced by *P. aeruginosa* is able to induce dispersion of biofilms formed by a number of other bacterial species. Chemical library screening has also been utilized to discover biofilm formation inhibitors.

3.2.5 *Quorum sensing and biofilm inhibitors with furanone moiety*

Brominated furanones (BFs) were first isolated in 1970s from a red marine alga *Delisea pulchra* largely unfouled in nature, found on the south-eastern coast of Australia. They are released on the surface of the alga and inhibit the colonization by both prokaryotes and eukaryotes, and are proposed to be an evolutionary response to the adverse effects of AHL-driven colonization. Brominated furanones differ on the extent of bromination, both on the furanone ring and on the exocyclic alkene, as well as on the substitution of the alkyl chain with hydroxy or acetoxy functionality (Figure 3.2). There are apparent structural similarities between brominated furanones and AHLs: both groups of compounds consist of a relatively polar “head” and a non-polar aliphatic carbon “tail”. Studies have shown that some of the natural brominated furanones are capable of inhibiting biofilm formation and swarming of both Gram-positive and Gram-negative bacteria. These findings have led to the efforts to chemically modify the furanone scaffold to identify the key components in the structure for biological activity and to discover potent modulators of biological activities.
A few brominated furanones have thus been synthesized and studied along with natural brominated furanones for their activity to modulate quorum sensing and biofilm formation (Figure 3.3). Both natural and synthetic brominated furanones were reported to control the expression of AHL-dependent phenotypes.\textsuperscript{185} As a result, many brominated furanones have been shown to inhibit a wide range of quorum sensing-controlled activities, including bioluminescence,\textsuperscript{186-189} swarming,\textsuperscript{186,190-194} biofilm formation,\textsuperscript{183,192,194-201} production of prodigiosin,\textsuperscript{193,202} and production of virulence factor.\textsuperscript{195,203-206} Brominated furanones are also reported to restore the antibiotic tolerance of persister cells.\textsuperscript{207} To give a few examples, Hentzer \textit{et al.} used \textit{in vitro} studies to demonstrate that brominated furanone 1 inhibit QS-controlled reporter genes and virulence factor expression. Although this compound did not inhibit the formation of biofilms, it did affect biofilm architecture.\textsuperscript{195} In a subsequent study, brominated furanone 2 was shown to not only inhibit virulence factor expression of \textit{P. aeruginosa} both \textit{in vitro} and \textit{in vivo} but also increased the susceptibility of \textit{P. aeruginosa} biofilms to SDS and antibiotic tobramycin. Moreover, in a mouse pulmonary infection model, compound 2 accelerated clearances of infecting bacteria from the lungs by the host.\textsuperscript{208} Wu \textit{et al.} found that both brominated furanones 1 and 2 were able to accelerate bacterial clearance in the lungs of infected mice and reduced the severity of lung pathology. In mice treated with lethal \textit{P. aeruginosa} infections, treatment of compound 1 significantly prolonged the survival time.\textsuperscript{209} In an effort to investigate the important structural elements responsible for biological activities of
brominated furanones, Han et al. discovered potent inhibitors 3-5 for *E.coli* biofilms, together with inactive compounds 6 and 7, as well as compounds 8 and 9, which are toxic to the growth of strain *E. coli* RP437. Janssens et al. reported that brominated furanone 10 inhibit flagella biosynthesis and biofilm formation by *Salmonella enterica* and increased the susceptibility of *Salmonella* biofilms to antibiotics without affecting currently known QS systems in *Salmonella*. A couple of adamantine tethered brominated furanones 13 and 14, together with some of the synthesis intermediate 11 and 12, were tested for their cytotoxicity and inhibitory activity of biofilm formation of the strain *E. coli* RP437. All the molecules tested in this work showed moderate inhibition of biofilm formation without having significant impact on the growth of bacteria under the conditions tested.

![Figure 3.3 Examples of synthetic brominated furanones.](image)

The mechanism of how brominated furanones interfere with quorum sensing is still unclear. Manefield et al. demonstrated that brominated furanones may inhibit QS by binding to and inducing degradation of the LuxR-type protein. Defoirdt et al. indicated that a natural brominated furanone from *Delisea pulchra* blocks QS in *V. harveyi* by disabling the LuxR...
protein to bind to the promoter sequence of QS-regulated genes.\textsuperscript{212} It is also proposed that brominated furanones may disrupt AHL-mediated QS by competitively binding to the receptor site.\textsuperscript{186,191,195,213-215} A natural brominated furanone from \textit{Delisea pulchra} was suggested to inhibit QS of \textit{E. coli} via AI-2.\textsuperscript{184,216}

In this work, we aim to develop new structures of inhibitors of biofilm formation that are nonmicrobicidal to bacteria and nontoxic to mammalian cells which should have potent use in clinics. Han \textit{et al.} designed and studied a library of structurally closely related synthetic brominated furanones and suggested the most important structural element of this class of molecules to be a conjugated exocyclic vinyl bromide on the furanone ring as in compounds 3-5. Supporting this hypothesis, brominated furanones 6 and 7 lack the conjugated exocyclic vinyl bromide and were inactive. Brominated furanones 8 and 9 which bear monosubstituted bromides on an exocyclic methyl group were found to be toxic to the growth of \textit{E. coli} cells.\textsuperscript{198} Based on these findings, we speculated that a bicyclic version of brominated furanones which retain the conjugated exocyclic vinyl bromide in the furanone moiety could potentially reduce their toxicity while retaining the biofilm inhibitory activities. Therefore, we designed a new class of bicyclic brominated furanones (BBFs), 5-BBF 15, 6-BBF 16, and 7-BBF 17, with [3,3,0], [4,3,0], and [5,3,0]-fused ring structures, respectively (Figure 3.4). Compared to the known brominated furanones (will use “BF” from now on for brevity), such as 2\textsuperscript{208} and 3,\textsuperscript{198} the fused bicyclic systems bear only one bromo-substitution, and introduce bulkier but semi-rigid cyclic hydrocarbon skeletons into the molecules that can potentially increase the binding and selectivity to the receptor proteins.
3.2 Results and Discussion

3.2.1 Synthesis of BBFs

Bicyclic brominated furanones, 5-BBF 15, 6-BBF 16, and 7-BBF 17, were synthesized via a similar route starting with keto acids. We describe the longest synthesis sequence that was used to make 7-BBF in Scheme 1. Under basic conditions, coupling of cycloheptanone and dimethyl carbonate provided methyl 2-oxo-1-cycloheptanecarboxylate 19 followed by acetoacetic ester synthesis to give 2-oxocycloheptanecacetic acid 20. This intermediate was then subjected to bromination and dehydration to build the fused ring framework, followed by elimination to obtain the conjugated final product without isolating the intermediates (Scheme 3.1). Brominated furanones 2 and 3 were also synthesized to compare their toxicities and biofilm inhibition activities to BBFs. Compound 2 was synthesized by Dr. Debjoyoti Bandyopadhyay in my research group.
Scheme 3.1 Synthesis of BBFs

```
Reagents and conditions: (a) Br₂, CH₂Cl₂, 0 °C to rt; (b) P₂O₅, DCM, 0 °C to reflux; (c) Et₃N, DCM, 0 °C to reflux; (d) LiOH, THF/H₂O (9:4), 23 h, 1M HCl (aq.), rt; (e) NaH, benzene, rt to 85 °C; (f) ethyl bromoacetate, K₂CO₃, acetone, rt to reflux, 16 h; (g) 6 M HCl (aq.), AcOH, rt to reflux, 2 d.
```

3.2.2 Inhibition of E.coli biofilm formation by BBFs

We first evaluated the inhibitory activity of BBFs on biofilm formation by strain E.coli RP437 (pRSH103), which constantly expresses red fluorescence and enables easy visualization of the biofilm using confocal laser scanning microscopy (CLSM). BF 3 was a known biofilm inhibitor to this strain at ~200 μM and was used as a positive control. Biofilms were grown on sterile steel coupons in the absence or presence of 200 μM brominated furanones for 24 h before visualization. Appropriate amount of DMSO was added to the non-BF treated control to eliminate solvent effect. E. coli RP437 (pRSH103) biofilms grown in the presence of the brominated furanones all displayed less fluorescence than the DMSO control. The formation of
biofilm was quantified from fluorescence images using COMSTAT software. Biomass, mean thickness, and surface area values were calculated from 4 random locations on the steel coupons as 4 replicates and were normalized by that of the DMSO control. Biomass represents the overall volume of the biofilm. Mean thickness is the average thickness of the biofilm. Surface area is the area summation of all biomass voxel surfaces exposed to the background. It is noteworthy that 6-BBF 16 results in the least amount of biofilm formation (based on quantified biomass) of this strain (31.3 ± 2.5%) \((P < 0.01)\) compared with 5-BBF 15 (56.3 ± 5.0%) \((P < 0.05)\) and 7-BBF 17 (49.7 ± 2.4%) \((P < 0.01)\) and even less than that treated with BF 3 (49.1 ± 8.4%) \((P < 0.05)\).

Figure 3.5 The effect of brominated furanones on biofilm formation by *E.coli* RP437 (pRSH103). Representative confocal laser scanning microscopy (CLSM) images of biofilm formed by *E.coli* RP437 (pRSH103) in the (A) absence and presence of 200 µM (B) 3, (C) 5-
BBF 15, (D) 6-BBF 16 and (E) 7-BBF 17. The control is supplemented with the same amount (0.4%) of DMSO as present in the brominated furanone-treated conditions. Scale bar = 100 µm.

Figure 3.6 Quantification of biofilm formation by *E. coli* RP437 (pRSH103) in the absence and presence of 200 µM brominated furanones. Biomass, mean thickness, and surface area were quantified from fluorescence image using COMSTAT software. Z-Stack images from four different locations were used. Values are normalized by that of the brominated furanone-free control and represent the means ± standard deviation from 4 replicates. Significant differences in the biofilm formation from the BF-free control are indicated by asterisks: *, \( P < 0.05 \); **, \( P < 0.01 \).

3.2.3 *Cytotoxicity study of BBFs on the growth of E. coli*

We conducted cytotoxicity study of BBFs on the growth of *E. coli* to investigate if the observed biofilm inhibition was due to cytotoxicity. Bacteria cells were allowed to grow in the
absence or presence of 200 µM BBFs and the OD<sub>600</sub> values were measured every 2 h for the first 12 h and then after 24 h. BF 3 was used as a positive control (Figure 3.7). None of the BBFs exhibited obvious impact on the growth of <i>E. coli</i> RP437. The growth curve of bacteria in the presence of BF 3 deviated from that of the control for up to 8 h and then the OD<sub>600</sub> values were essentially the same to the control after then. Therefore, BBFs all showed moderate to good biofilm inhibition of <i>E. coli</i> RP437 at concentrations that do not inhibit the growth of bacteria cells.

![Graph](image)

Figure 3.7 Growth curve of <i>E. coli</i> RP437 in the absence and presence of 200 µM brominated furanones. Values are normalized by that of the BF-free control and represent the means ± standard deviation from 6 replicates.

3.2.4 Inhibition of <i>P. aeruginosa</i> biofilm formation by BBFs

<i>P. aeruginosa</i> is an opportunistic pathogen and the most common Gram-negative bacterium found in nosocomial and life-threatening infections of immunocompromised patients, including
cystic fibrosis (CF), cancer, and AIDS patients. The airways of CF children are colonized by *P. aeruginosa* soon after birth; an infection is initiated followed by massive immune response from the host that causes severe damage to the lung tissues. This infection is reoccurring and develops into a serious chronic infection that resists antibiotic therapies by 10 years of age. The presence of biofilm and the resistance to antimicrobial therapy appear to be the main reasons for the persistence of *P. aeruginosa* infections. Controlling and eradicating *P. aeruginosa* biofilm is therefore a significantly clinically related issue.

We used the wild type PAO1-GFP that constitutively expresses green fluorescent proteins (GFP) to grow biofilm on steel coupons with and without BBFs, and monitored the biofilm formation directly by using confocal fluorescent microscopy. BF 2 has been reported to inhibit biofilm formation by *P. aeruginosa* and was thus used as a positive control. All three BBFs showed moderate to good inhibitory activity against biofilms formed by *P. aeruginosa* PAO1 at 400 µM, with 6-BBF 16 provided more inhibition than that by 5-BBF 15 and 7-BBF 17. BF 2 reduced biofilm almost completely (Figure 3.8). To evaluate the biofilm inhibition more quantitatively, we also calculated the biomass, mean thickness and surface area of *P. aeruginosa* biofilms formed with and without agents by using COMSTAT software (Figure 3.9). The results were normalized by the BF-free control. Compound 6-BBF 16 exhibited the strongest inhibition as it reduced the biofilm formation by 71% (*P* < 0.01), followed by 5-BBF 15 at 53% (*P* < 0.01) and 7-BBF 17 at 50% (*P* < 0.05). The mean thickness of biofilm was reduced by 6-BBF 16 to 60% (*P* < 0.01) and by 7-BBF 17 to 74% (*P* < 0.05). We note that the mean thickness of biofilm treated by 5-BBF 15 did not seem to reduce. One possibility is that the biofilm becomes more “fluffy” in the presence of 400 µM 5-BBF 15. The surface areas of biofilm in the presence of all three BBFs were about 60% of that formed in the absence of BF (*P* < 0.05).
Figure 3.8 The effect of brominated furanones on biofilm formation by *P. aeruginosa*.

Representative confocal laser scan microscopy (CLSM) images of biofilm formed by PAO1-GFP (expresses green fluorescence on plasmid pSMC2) (A) in the absence of agents, and in the presence of 400 μM (B) 2, (C) 5-BBF 15, (D) 6-BBF 16, and (E) 7-BBF 17. The control is supplemented with the same amount (0.8%) of DMSO as present in the brominated furanone-treated conditions. Scale bar = 50 μm.
Figure 3.9 Quantification of biofilm formation by PAO1-GFP in the absence and presence of 400 µM brominated furanones. Biomass, mean thickness, and surface area were quantified from fluorescence image using COMSTAT software. Z-Stack images from four different locations were used. Values are normalized by that of the BF-free control and represent the means ± standard deviation from 4 replicates. Significant differences in biofilm formation from BF-free control are indicated by asterisks: *, P < 0.05; **, P < 0.01.

We followed up with a dose-dependence study on inhibitory activity of BBFs on *P. aeruginosa* biofilm using a colorimetric assay in 96-well plates employing crystal violet (CV) (Figure 3.10). IC$_{50}$ values obtained from the dose-response curves were more than 400 µM for 5-BBF 15, and 145.8 µM and 139.7 µM for 6-BBF 16 and 7-BBF 17, respectively. These results suggest that 6-BBF 16 and 7-BBF 17 are stronger biofilm inhibitors than 5-BBF 15 for *P. aeruginosa*. We note that the percentage of relative biofilm formation in the presence of 400 µM BBFs obtained from confocal laser scan microscopy and that from the CV assay did not match perfectly. This discrepancy most likely comes from the different experimental conditions and
procedures used in the two different assays. However, the general trend that 6-BBF and 7-BBF showed more inhibitory activity against *Pseudomonas* biofilm formation is similar in both assays.
Figure 3.10 *P. aeruginosa* biofilm formation dose-response curves with IC<sub>50</sub> values for (A) 5-BBF 15, (B) 6-BBF 16, and (C) 7-BBF 17. Values are normalized by that of the BF-free control.
and represent the means ± standard deviation from 4 replicates. Data shown is a representative of at least three separate experiments.

3.2.5 Cytotoxicity study of BBFs on the growth of P. aeruginosa

The toxicity of brominated furanones to the growth of bacteria is difficult to predict based on structures. For example, BF 3 does not inhibit the growth of E. coli, whereas similar structures do. Here, we compared the toxicity of BBFs and BF 2 to the growth of P. aeruginosa. At 400 µM, none of the three BBFs showed dramatic inhibition to the growth of P. aeruginosa PA01 (Figure 3.11), and bacteria grown in the presence of BBFs reached the same optical density (OD 600) as those in the absence of BBFs (control) after 24 h. At the same concentration and under the same conditions, BF 2 completely inhibited bacterial growth (P < 0.01). Therefore, BBFs all inhibited P. aeruginosa biofilm at concentrations that did not affect the bacterial growth. Together with other reports,198,210,229 these results suggest that small structural modifications of brominated furanones can have substantial impact on their activities.230 In particular, the toxicity and biological activity of brominated furanones is highly sensitive to small variation in the structure whereas the biofilm inhibition activity may be quite general for most of the structures. We note that all three BBFs appear to be more stable than BF 2 and 3. Bacteria culture containing BF 2 and 3 became pinkish after incubating for ~3 h and the coloration deepens over time, which may indicate decomposition. However, BBFs did not show any color change even after 72 h of incubation under the same condition.
Figure 3.11 Growth curves of *P. aeruginosa* PAO1 in the absence (control) and presence of 400 µM brominated furanones. Values represent the means ± standard deviation from six replicates. Significant differences in the optical density from BF-free control are indicated by asterisks: **, $P < 0.01$.

### 3.2.6 Study of synergistic effect between BBFs and tobramycin

As our results indicated that bicyclic brominated furanones were effective at inhibiting biofilm formation with very low toxicity, we studied the effect of combined use of antibiotics and these biofilm inhibitors on killing *P. aeruginosa* within the biofilm. We choose tobramycin because of its clinical relevance, superior effectiveness against *Pseudomonas* than other antibiotics, and wide use in treating *Pseudomonas*-related diseases.\(^{231}\) We applied 6-BBF 16 at 200 µM, a relatively low concentration at which the inhibition of biofilm formation is still effective without killing bacteria cells. Tobramycin was initially applied at 2.0 µg/mL as commonly used in clinics to one-day-old biofilm. However, tobramycin alone at this
concentration was highly effective at killing bacteria (> 95%) in an *in vitro* setting. After concentration screening, we used 0.25 µg/mL of tobramycin and one-day-old biofilm to study the synergistic effect in the presence of 6-BBF 16. Either 200 µM 6-BBF 16 or 0.25 µg/mL tobramycin alone resulted in only a mild decrease in the colony forming units (CFUs) of *P. aeruginosa* PAO1 biofilm cells (14.6 ± 7.4% and 24.1 ± 6.8%, respectively) (*P* < 0.05). The combination of consecutive treatment of 6-BBF 16 and tobramycin resulted in significant decrease of viable cell counts in the PAO1 biofilm (70.5 ± 1.6%) (*P* < 0.05) (Figure 3.12). Only less than half log drop was observed for the viable counts in our case whereas a drop of more than one magnitude would be more useful in practice. This result suggests that treatment with 6-BBF 16 slightly increased the susceptibility of PAO1 biofilm to tobramycin *in vitro*. A past study has used a LIVE/DEAD BacLight Bacterial Viability staining kit to show that BF 2 (10 µM) significantly increased the susceptibility of bacteria in *P. aeruginosa* biofilms to tobramycin (100 µg/mL). However, biofilms used in this study was grown under different conditions (ABtrace minimal medium in flow chamber compared to LB broth in 24-well plate in our case). It has been shown that the susceptibility of the *P. aeruginosa* biofilms toward tobramycin can be model and strain dependent. We note that the dosage used in our study is lower than that normally used in clinical studies, about 1.7-2.0 µg/mL. 
Figure 3.12 Viability of cells within the biofilm treated with and without 6-BBF 16 after exposure to tobramycin. Preformed 24 h-old biofilms grown on steel coupons were treated with 200 µM 6-BBF 16 for 24 h and then with and without 0.25 µg/mL tobramycin for another 24 h. Two independent experiments were performed, with four replicates per experiment. Values represent the means ± standard error of a total of eight replicates. Significant differences in the CFU from the control are indicated by asterisks: *, \( P < 0.05 \).

The mechanism of how the presence of 6-BBF 16 increases the effectiveness of antibiotics in a synergistic way is not yet clear. One possible explanation is that 6-BBF 16 inhibits quorum sensing, leading to a reduction of biofilm formation, which reduces the number of biofilm-associated bacteria that bear high resistance to antibiotics. Changes in the biofilm structure may also facilitate the transport of antibiotics to bacteria in the biofilm. In addition, quorum sensing inhibitors may also lead to a reduction in persister formation, as a recent study have shown that quorum sensing molecules (not inhibitors) can increase the persister formation by \( P \).
Alternatively, these bicyclic brominated furanones may directly target the proteins that are required to maintain the persisters, and subsequently increase the susceptibility of the persister bacteria to antibiotics.

### 3.2.7 Modulation of the quorum sensing in P. aeruginosa by BBFs

To investigate whether BBFs inhibit biofilm formation via interference with QS, we studied the agonistic and antagonistic effects of these BBFs on the two quorum sensing systems in *P. aeruginosa*. There are two identified AHL-mediated quorum sensing circuits in *P. aeruginosa*. The *las* circuit involves autoinducer 3-oxo-C12-HSL and the *rhl* circuit utilizes autoinducer C4-HSL.

A double-knockout reporter strain PAO-JP2 (ΔlasIΔrhlI) harboring the plasmid *plasl*-LVAgfp was used to evaluate the *las* quorum sensing system. When bacteria are growing in the presence of exogenously introduced autoinducer 3-oxo-C12-HSL or analogues, LasR receptor binds to the AI and activates transcription of the *lasI* promoter which controls green fluorescence protein (GFP) expression. The GFP production is quantified by correcting the measured fluorescence signal for cell density as shown in Equation 3.1. “RFU<sub>sample</sub>” is the relative fluorescence unit measured by the plate reader. “No AI” represents bacteria culture grown in the presence of no AI analogue, supplemented with the same amount of DMSO present in the SHL stocks.

\[
\text{Relative fluorescence} = \frac{\text{RFU}_{\text{sample}} - \text{RFU}_{\text{no AI}}}{\text{OD}_{600 \text{sample}}} \quad (3.1)
\]

The antagonism assay was conducted with competition of BBFs against 1 µM natural AI 3-oxo-C12-HSL (a concentration within the range produced by wild type *P. aeruginosa*). All BBFs decreased fluorescence signals as the concentration was increased from 50 to 150 to
300 µM, indicating inhibition of GFP expression (Figure 3.13). Of all the BBFs, 5-BBF 15 appeared to be the strongest inhibitor at all concentrations tested. In the presence of 50 µM agents, the relative fluorescence was 68.5 ± 4.4% (P <0.001), 85.0 ± 14.2%, and 72.9 ± 1.5% (P <0.01) for bacteria treated with 5-BBF 15, 6-BBF 16, and 7-BBF 17, respectively. At 300 µM, the GFP expression in the presence of 5-BBF 15 was reduced to about 27.6 ± 4.7% (P <0.05) as compared to control with only 1 µM of 3-oxo-C12-HSL, while 6-BBF 16 and 7-BBF 17 reduced the GFP expression to 55.2 ± 17.8% (P < 0.01) and 39.8 ± 6.2% (P <0.05), respectively. BF 3 showed similar inhibitory activity to 5-BBF 15.

Figure 3.13 GFP expression by PAO-JP2 (plasI-LVAgfp) in the presence of 1 µM 3-oxo-C12-HSL alone (control) or 1 µM 3-oxo-C12-HSL plus various concentration of 5-BBF 15, 6-BBF 16, 7-BBF 17 or BF 3. Fluorescence signals were corrected for cell density as shown in Equation 3.1 and the results were normalized to the BF-free control. Values represent the means ± standard deviation from four replicates. Data shown is a representative of duplicate experiments. Significant differences in the GFP expression from the control are indicated by asterisks: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Similar results were obtained using the wild type strain PAO1 harboring plasmid \textit{plasI-LVAgfp} (Figure 3.14). Both 6-BBF 16 and 7-BBF 17 decreased fluorescent signals as the concentration was increased from 50 to 150 to 300 \(\mu\text{M}\) (Figure 3.15). At 300 \(\mu\text{M}\), the GFP production in the presence of 6-BBF 16 or 7-BBF 17 was \(78 \pm 1.1\%\) \((P < 0.05)\) and \(69 \pm 4.0\%\) \((P < 0.001)\), respectively, compared to that of the BF-free control. BF 3 showed similar inhibitory activity to 5-BBF 15 in this strain, too, with \(46 \pm 4.8\%\) \((P < 0.001)\) GFP expression at 300 \(\mu\text{M}\), compared to that of the BF-free control \((P < 0.001)\). However, BF 3 is in general more toxic than 6-BBF 16 and 7-BBF 17. Compound 5-BBF inhibited the GFP production slightly at 50 \(\mu\text{M}\) and 150 \(\mu\text{M}\), and reduced the GFP expression by approximately half at 300 \(\mu\text{M}\). These results suggest that the BBFs are weak to moderate antagonists of LasR protein within the concentration range of 50 to 300 \(\mu\text{M}\).

![Figure 3.14](image-url)

Figure 3.14 GFP expression by PAO1 (\textit{plasI-LVAgfp}) in the absence or presence of 5-BBF 15, 6-BBF 16, 7-BBF 17 or BF 3 at 50, 150, and 300 \(\mu\text{M}\). Fluorescence signals were corrected for cell density as shown in Equation 3.1 and the results were normalized to the BF-free control.
Values represent the means ± standard deviation from four replicates. Data shown is a representative of at least three separate experiments. Significant differences in the GFP expression from the control are indicated by asterisks: *, $P < 0.05$; ***, $P < 0.001$.

We also tested the effects of BBFs on the quorum sensing system of $rhlI$ using PAO-JP2 ($prhlI$-LVAgfp) in the presence of 1 µM 3-oxo-C12-HSL and 10 µM C4-HSL (Figure 3.15).

Surprisingly, 5-BBF 15 promoted, instead of suppressed, the GFP expression significantly as its concentration was increased. At 50 µM and 150 µM, 5-BBF 15 increased the GFP expression by 16.4 ± 11.8% and 62.2 ± 14.2% ($P < 0.001$), respectively. When the concentration was increased to 300 µM, the fluorescence was increased by 154 ± 18.6% ($P < 0.001$) compared to the control.

On the contrary, 6-BBF 16, 7-BBF 17 or BF 3 did not show observable increase in green fluorescence. Similar results were also obtained when reporter strain PAO1 ($prhlI$-LVAgfp) was used (Figure 3.16). At 150 µM and 300 µM, 5-BBF 15 increased the GFP expression by 82.6 ± 4.4% and 165 ± 17.6%, respectively ($P < 0.01$). On the contrary, 6-BBF 16, 7-BBF 17 and BF 3 had no effect on the GFP expression at all concentrations tested. These results suggest that 5-BBF is a strong agonist of RhlR, while 6-BBF 16 and 7-BBF 17 are not.
Figure 3.15 GFP expression by PAO-JP2 (prhlI-LVAgfp) in the presence of 1 µM 3-oxo-C12-HSL and 10 µM C4-HSL alone (control) or 1 µM 3-oxo-C12-HSL and 10 µM C4-HSL plus various concentration of 5-BBF 15, 6-BBF 16, 7-BBF 17, or BF 3. Fluorescence signals were corrected for cell density as shown in Equation 3.1 and the results were normalized to the BF-free control. Values represent the means ± standard deviation from four replicates. Data shown is a representative of at least three separate experiments. Significant differences in the GFP expression from the control are indicated by asterisks: ***, $P < 0.001$. 
Figure 3.16 GFP expression by PAO1 (prhl-LVAgfp) in the absence or presence of 5-BBF 15, 6-BBF 16, 7-BBF 17, or BF 3 at 50, 150, and 300 µM. Fluorescence signals were corrected for cell density as shown in Equation 3.1 and the results were normalized to the BF-free control. Values represent the means ± standard deviation from four replicates. Data shown is a representative of at least three separate experiments. Significant differences in the GFP expression from the control are indicated by asterisks: ***, \( P < 0.001 \).

3.2.8 Effect on elastase B production in P. aeruginosa by BBFs

Another bioactivity of P. aeruginosa that is also controlled by quorum sensing (las pathway)\(^ {238} \) is the production of the virulence factor metalloprotease elastase B. This highly toxic virulence factor facilitates the invasion and destruction of host tissues,\(^ {239} \) induces inflammatory responses from the host\(^ {240} \) and also signals the biofilm formation.\(^ {241} \) Thus elastase B is a potential target for developing antimicrobial agents. Molecules that inhibit virulence factor production without bactericidal action would place little selection pressure on the bacteria strains.
for the emergence of resistance and therefore have been proposed as a class of second-generation antibiotics. BF 2, for example, was reported to inhibit the production of virulence factors and to increase the bacterial susceptibility to tobramycin. Another synthetic BF 1 was also shown to reduce the production of virulence factors, including protease and chitinase. Because BBFs appeared to be potent antagonists of LasR, we also tested their ability to inhibit elastase B production. A colormetric assay that utilized elastin-congo red substrate was conducted to measure the elastase B activity produced by *P. aeruginosa* PAO-JP2. Natural autoinducers 3-oxo-C12-HSL and C4-HSL were added at 5 µM and 10 µM, respectively, for consistent induction of elastase B activity in this strain. At 300 µM, all BBFs significantly reduced the production of elastase B by ~80% (*P* < 0.05) (Figure 3.1). In a dose-dependent study, the elastase B activity in the presence of 50 µM 6-BBF 16 was not significantly different from the non-treated control. But the inhibitory activity of 6-BBF 16 increased as the concentration increased up to 300 µM (Figure 3.17, insert). Because the elastase B production is controlled by *las* quorum sensing pathway, these results are consistent with BBFs being antagonists of LasR in *P. aeruginosa*. 
Figure 3.17 Elastase B activity produced by *P. aeruginosa* PAO-JP2 in the presence of 5 µM 3-oxo-C12-HSL and 10 µM C4-HSL alone (control) or 5 µM 3-oxo-C12-HSL and 10 µM C4-HSL plus 300 µM BBFs or 5 µM PAI1 and 10 µM PAI2 plus various concentration of 6-BBF 16 (insert). Values were normalized to that of the brominated furanone-free control and represent the means ± standard deviation from four replicates. Significant differences in the elastase B activity from the control are indicated by asterisks: *, $P < 0.05$.

3.2.9 *Improved bacterial clearance by 6-BBF in vivo*

A more clinically-related method to evaluate the *in vivo* function of a QSI compound is by means of a pulmonary infection model. To further explore the potential utility of BBFs *in vivo*, we used a SP-A/D KO mouse model that lacked the expression of two host defense proteins SP-A and SP-D in the lung and thus increased the mouse’s susceptibility to *P. aeruginosa* infection. This model mimics microenvironments of the lung in the pulmonary immunocompromised patients, who have a high susceptibility to lung infections. Four
independent experiments with a total of 68 age and gender matched mice were performed. The results showed that the average CFU in mice treated with 6-BBF at 200 µM was significantly lower ($P < 0.05$) than that in mice of the control group. The average CFU in the 6-BBF-treated group and control group were $(4.1 \pm 0.29) \times 10^4$ CFU per lung and $(18.6 \pm 5.9) \times 10^4$ CFU per lung, respectively, 48 hours after infection (Figure 3.18). Our results from this in vivo model demonstrated that 6-BBF 16 can improve bacterial clearance compared to the controls (without 6-BBF treatment). The mechanisms underlying this increase clearance may be through the inhibition of biofilm formation of bacteria by 6-BBF and/or increase of phagocytic efficiency of bacteria by alveolar macrophages and neutrophils in the lung.

![Figure 3.18 Effect of 6-BBF on the bacterial clearance of mouse lung in vivo.](image)

Figure 3.18 Effect of 6-BBF on the bacterial clearance of mouse lung in vivo. Each SP-A/D KO mouse was infected with 50 µl (containing $1 \times 10^7$ CFU of wilt-type $P. aeruginosa$) intratracheally. Bacterial suspension contained either saline as the control group or 200 µM 6-BBF 16. The mice were sacrificed 48 hours after infection and CFUs of each lung were determined. The data showed one representative experiment (n= 8 mice for each group) of four independent experiments and values are means ± standard deviation. Significant differences in the bacterial clearance from the control are indicated by asterisk: *, $P < 0.05$. This experiment of
mouse model was performed by Dr. Osama A. Abdel-Razek from Dr. Guirong Wang’s research group at Upstate Medical University (SUNY).

3.2.10 Cytotoxicity study of BBFs on human cells

To avoid invoking bacterial resistance, nonmicrobicidal agents that do not kill bacteria or influence bacterial growth are being sought. However, for any potential therapeutic development, the toxicity issue of the molecules towards mammalian or human cells must be addressed. Although many natural and non-natural brominated furanones inhibit biofilm formation by a wide range of bacteria, few studies have addressed their toxicity to mammalian or human cells. Here, we compare the toxicity of BBFs to a nonmicrobicidal brominated furanone, BF 3, against human neuroblastoma SK-N-SH. Cells were allowed to attach to and grow in 96-well tissue culture-treated microtiter plates for 24 h, after which they were treated with at 100 µM of each agent (5-BBF 15, 6-BBF 16, 7-BBF 17 and BF 3) for 1 h, and then the agent-containing medium was replaced with fresh medium. After the 1-h treatment of agents, the cells were then allowed to recover for 0, 24, and 48 h (recovery time), at which time the number of live cells was determined by the CCK 8 assay. Survival (%) was calculated based on cells without any agent treatment as shown in Equation 3.2. The sample absorbance values were obtained from the wells containing cells and brominated furanones, and the control absorbance values were obtained from BF-free wells containing cells and DMSO.

$$\text{Survival} \ (%) = \frac{A_{450\text{sample}} - A_{450\text{medium}}}{A_{450\text{control}} - A_{450\text{medium}}} \times 100 \quad (3.2)$$

At 100 µM, BF 3 exhibited moderate toxic effect toward human neuroblastoma SK-N-SH cells after 0 h of recovery; about 50.3 ± 9.3% of cells were alive compared to those not treated
with agents ($P < 0.001$). None of the three BBFs showed any obvious toxicity at this time (Figure 3.19). As the culture time prolonged after agent-treatment, survival (%) of cells treated with 5-BBF 15 and BF 3 both decreased significantly, to $21.4 \pm 3.6\%$ ($P < 0.001$) and less than 5% ($1.9 \pm 0.3\%, P < 0.01$), respectively, after 48 h. In contrast, cells treated with 6-BBF 16 and 7-BBF 17 did not show any decrease in the number of live cells, but rather a slight increase of cells treated with 6-BBF 16 after 48 h of agent-treatment. These results suggest that 6-BBF 16 and 7-BBF 17 are not particularly toxic to this cell line under the conditions studied, whereas 5-BBF 15 and BF 3 are.

Figure 3.19 Survival (%) of human neuroblastoma SK-N-SH cells at 0, 24, and 48 h after 1 h treatment of 100 µM 5-BBF 15, 6-BBF 16, 7-BBF 17 and BF 3, respectively. Values are normalized by that of the SHL-free control as shown in Equation 3.2 and represent the means ± standard deviation from six replicates. Significant differences in the survival% from BF-free control are indicated by asterisks: **, $P < 0.01$; ***, $P < 0.001$. 
To further explore the toxic effect of 6-BBF and 7-BBF, we increased the exposure time to 2, 4, 6 h to the human cells. The $A_{450}$ was measured immediately after incubation with the agents. The results showed that the survival (%) decreased as the exposure time prolonged for cells treated with 5-BBF 15 and BF 3, and was lower than that for 1h agent treatment. This result further confirms that these two compounds are toxic to this cell line. The survival (%) for cells treated with 6-BBF 16 remained the same after 2, 4, and 6 h treatment, and the survival (%) decreased by ~20% for cells treated with 7-BBF 17 (Figure 3.20). These results suggest that 6-BBF 16 and 7-BBF 17 are not as toxic as BF 3 to the cells line studied.

Figure 3.20 Survival (%) of SK-N-SH when treated with 100 µM 5-BBF 15, 6-BBF 16, 7-BBF 17 and BF 3 for 2, 4, and 6 h. Values are normalized by that of the SHL-free control as shown in Equation 3.2 and represent the means ± standard deviation from six replicates. Significant differences in the survival (%) with 2 h exposure time are indicated by asterisks: *, $P < 0.05$. 
Cytotoxicity of BBFs against this human neuroblastoma cell line SK-N-SH was also evaluated at the highest concentration tested for biofilm inhibition (400 µM) following the same protocols. After the 1 h treatment of agents and 0 h recovery time, BF 3 exhibited the strongest toxic effect toward human neuroblastoma SK-N-SH cells; 29.0 ± 14.1 % of cells were alive compare to those not treated with agents (P < 0.001). 5-BBF 15 and 6-BBF 16 showed mild toxicity (~ 76% cell survival for both), and 7-BBF 17 resulted in ~ 44% of cell survival (Figure 3.21). At 24 h and 48 h of recovery time, survival (%) for cells treated with 5-BBF 15 dropped to ~ 21% and ~ 6%, respectively. For cells treated with BF 3, almost no live cell remained (< 2%) after 24 and 48 h. For 7-BBF 17, cell survival dropped to ~ 7 % after 24 h of recovery time, but appears to increase to 13 % after 48 h. In contrast, 6-BBF 16 exhibited the least toxic effect to this human cell line. After 24 h and 48 h of recovery time, about 50 % survival was observed for cells treated with 6-BBF 16.

Figure 3.21 Survival (%) of human neuroblastoma SK-N-SH cells at 0, 24, and 48 h after 1 h treatment of 400 µM 5-BBF 15, 6-BBF 16, 7-BBF 17 and BF 3, respectively. Values represent
the means ± standard deviation from six replicates. Significant differences between BF-treated conditions in the survival% and BF-free control at each time point are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

### 3.3 Conclusion and Perspectives

The bicyclic brominated furanones are a new class of synthetic brominated furanones with increased chemical stability and significant reduction in toxicity to human cancer cells compared to the known compound 3. These BBFs inhibit biofilm formation by *E. coli* and *P. aeruginosa* and inhibit production of virulence factor elastase B in *P. aeruginosa* at nonmicrobicidal concentrations. At 200 µM, the representative molecule 6-BBF 16 moderately increased the susceptibility of *Pseudomonas* biofilm to tobramycin. These biological activities are probably realized through interfering with quorum sensing in bacteria. The relative high IC$_{50}$ of biofilm inhibition of these brominated furanones plague their use in pharmaceutical area. However, they may still be useful in treatment of external wound infections. This work indicates a complex and sensitive structure-bioactivity relationship for brominated furanones. Future work would include studying the ability of BBFs to modulate QS in other species such as *V. fischeri* and *A. tumefaciens*. Using X-ray crystal structures of a few LuxR-type proteins bound to their cognate AHL ligands as a guidance, one could design and tune the structure of brominated furanones and other potential quorum sensing modulators. Computer simulated binding of candidates to the active site of LuxR-type receptors would help to predict and understand the activity of these molecules.
3.4 Experimental Section

3.4.1 Biological studies

Chemicals.

Minimum essential medium with Eagle’s salts and L-glutamine (EMEM) was obtained from Mediatech (Herndon, VA). Trypan blue stain was purchased from Sigma-Aldrich (Milwaukee, WI). Cell counting kit 8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD). Water used for preparing all buffers and solutions had resistivity of 18 MΩ cm (Millipore, Billerica, MA).

Bacteria strains and growth media.

Double-knockout mutants of *Pseudomonas aeruginosa*, PAO-JP2 (plasI-LVAgfp) and PAO-JP2 (prhlI-LVAgfp) were kindly provided by Dr. Helen E. Blackwell (University of Wisconsin-Madison) with permission by Dr. Babara H. Iglewski (University of Rochester Medical Center).\(^{236,249}\) Plasmids plasI-LVAgfp and prhlI-LVAgfp were kindly provided by Dr. Hiroaki Suga (The University of Tokyo). *Escherichia coli* RP437\(^{250}\) and *E. coli* RP437 with pRSH103 plasmid (constitutively expresses red fluorescence proteins)\(^{251}\) were kindly provided by Dr. Dacheng Ren (Syracuse University). *E. coli* RP437 and *E. coli* RP437 (pRSH103) were grown in Luria-Bertani (LB) broth (containing 10 µg/mL of tetracycline for *E. coli* RP437 (pRSH103)) at 37 °C. *P. aeruginosa* PAO1-BAA-47 (wild type) from ATCC and PAO1-GFP (constitutively expresses green fluorescence proteins on plasmid pSMC2)\(^{228}\) were grown in LB broth (containing 300 µg/mL of carbenicillin for PAO1-GFP) at 37 °C. All the other *P. aeruginosa* strains were grown in M9 minimal medium containing 300 µg/mL of carbenicillin.

Stock solutions of brominated furanones.
Stock solutions of all brominated furanones (BFs) (50 mM) were prepared in DMSO, sterilized by filtering through a 0.2 µm syringe filter, and stored at -20 °C in sealed vials. Appropriate amount of DMSO was added to controls in all assays to eliminate solvent effect. The amount of DMSO in all cases was no more than 0.8%.

**Confocal laser scanning microscopy (CLSM) analysis of biofilms.**

Biofilms were grown on 316 stainless steel coupons (ca. 3/8 in. × 3/8 in., McMaster-Carr, Elmhurst, IL) with or without BBFs in a 24-well microplate. The plate was wrapped in a Saran wrap and incubated at 37 °C for 24 h without shaking. Each steel coupon was then washed gently by dipping into 0.9% NaCl saline buffer 3 times (fresh saline buffer was used for each dipping) and then placed upside down on a microscope cover glass (50 x 24mm, No. 2, Fisher Scientific, Pittsburgh, PA). The biofilms were visualized using a Zeiss LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss, Jena, Germany). The biofilms formed by strain *E. coli* RP437 (pRSH103) were visualized by excitation with a HeNe laser at 543 nm and fluorescent emission was detected with a LP 560 nm emission filter. A 488 nm laser line was used to visualize biofilms formed by PAO1-GFP. Z-stacks from four randomly picked spots were taken for each steel coupon. Quantification analysis of biomass, mean thickness, and surface area of the biofilms formed in the absence and presence of brominated furanones were obtained from fluorescence image using COMSTAT software. Values are normalized by that of the BF-free control.

**Crystal violet biofilm assay in 96-well plates.**

An overnight bacterial culture was subcultured at an optical density (OD$_{600}$) of 0.1 in LB broth. Appropriate amount of stock solutions of BBFs in DMSO were added to each well. The outermost wells of the 96-well polystyrene microtiter plates (Costar 3370) were filled with 100
µL of sterile autoclaved water to prevent uneven evaporation. The plates were wrapped in Saran wrap and incubated under stationary conditions at 37 °C for 24 h. The media was then discarded and the plates were washed with deionized water (125 µL × 1) and dried for 30 min. Biofilms were stained with 100 µL of 0.1% aqueous solution of crystal violet for 30 min at room temperature. The crystal violet was discarded and then the plates were washed with deionized water (125 µL × 3) and dried again for 30 min. The remaining stained cells were solubilized with 200 µL of 95% EtOH and 125 µL of the solubilized CV stain was transferred to a new polystyrene microtiter dish. The amount of biofilm formation was determined by measure the absorbance at 540 nm. The relative biofilm formation was determined using the following equation: relative biofilm formation (%) = (A$_{540}$ sample – A$_{540}$ medium)/(A$_{540}$ control – A$_{540}$ medium) × 100. The sample absorbance values were obtained from the wells containing bacteria and brominated furanones, and the control absorbance values were obtained from BF-free wells containing bacteria and DMSO.

**Growth curve of planktonic bacteria.**

An overnight bacterial culture was diluted and grown to an OD$_{600}$ of 0.05. The subculture was aliquoted into 96-well plates at 200 µL per well. Appropriate amount of stock solutions of brominated furanones in DMSO was added to each well. The outermost wells of the 96-well plates were filled with 200 µL of sterile autoclaved water to prevent uneven evaporation. The plates were incubated at 37 °C with shaking (250 rpm). The OD$_{600}$ readings were acquired aseptically at 0, 2, 4, 6, 8, 10, 12, and 24 h using Biotek ELx800™ absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT) using Gen5™ data analysis software. The growth curves were plotted as OD$_{600}$ values (mean ± SD) versus time from six replicates.

**Reporter gene assay for P. aeruginosa.**

$^{162,167,195}$
An overnight culture of PAO1 (\textit{plasI-LVA}_gf\textit{p}) and PAO1 (\textit{prhlI-LVA}_gf\textit{p}) in M9 medium (300 µg/mL carbenicillin) was grown from a single colony picked from an LB agar plate supplemented with 300 µg/mL carbenicillin. The cell culture was diluted and grew to an OD$_{600}$ of 0.1 in M9 medium containing 300 µg/mL of carbenicillin. Bacteria culture (200 µL) was added to each well of a polystyrene 96-well microplate (Costar 3370, Corning Incorporated, Corning, NY) containing appropriate amount of natural autoinducers with and without brominated furanones. The plate was incubated at 37 °C for 24 h in a rotary shaking incubator (250 rpm). The culture from each well was then transferred to a 96-well plate with black wall (μClear, 655096, Greiner Bio-One North America, Inc., Monroe, NC). The fluorescence (an excitation wavelength of 500 nm and an emission wavelength of 540 nm) and OD absorbance (OD$_{600}$) in each well was measured by Synergy H1 multi-mode microplate reader with a Gen5 data analysis software. Background signals from M9 medium were deducted from all samples.

\textit{Determination of combined effect of BBFs and tobramycin.}

An overnight culture of \textit{P. aeruginosa} PAO1 in LB broth was diluted 1:100 and grew to OD$_{600}$ of 0.1. The cell culture was inoculated on to 316 stainless steel coupons (ca. 3/8 in. x 3/8 in.) in a 24-well plate. After 24 h of biofilm formation at 37 °C, the old medium was then replaced by fresh LB broth that contained 200 µM BBF and then the biofilms were allowed to grow for another 24 h. Subsequently, the medium was replaced by fresh LB broth that contained 0.25 µg/mL tobramycin and then the biofilms were allowed to grow for another 24 h. Each steel coupon was then washed gently by dipping into saline buffer (0.9% NaCl) 3 times and placed in 2 mL of saline buffer. The coupons were sonicated for 1 min and vortexed for 1 min; this process was repeated twice to release biofilm cells. The resulting cell suspensions were serially
diluted in saline buffer, spread on LB agar plates, and incubated at 37 °C for 24 h to count the number of CFU.

*Evaluating the production of virulence factor in the presence of BBFs.*

The production of virulence factor elastase B by *P. aeruginosa* was measured as described previously.\(^{160,253}\) Bacteria were grown overnight in PTSB media (5% Peptone, 0.1% Tryptic Soy Broth) at 37 °C, diluted and grown to midlog phase, and subcultured to an OD\(_{600}\) of 0.05. The culture was then added to test tubes containing BBFs at the desired final concentrations. The tubes were incubated for 24 h at 37 °C with shaking (250 rpm). Culture supernatants were recovered by centrifugation at 3000 rpm (Galaxy 5D centrifuge, VWR, Radnor, PA) for 10 min at room temperature and then passed through a 0.45 µm PVDF syringe filter (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A 100 µL aliquot of the supernatant was added to 900 µL of Elastin-Congo red (ECR) buffer (100 mM Tris-HCl, 1 mM CaCl\(_2\), pH 7.2) containing 4.5 mg of Elastin-Congo red and incubated for 24 h at 37 °C with shaking (250 rpm). After incubation, 0.2 mL of 0.12 M EDTA was added to stop the reaction. Insoluble ECR was removed by centrifugation and the absorbance of the supernatant (OD\(_{490}\)) was measured. Elastase B activity was represented by OD\(_{490}\) of the samples treated with BBFs minus the OD\(_{490}\) of the bacteria-free samples.

*Mice* (The protocols for SP-A/D KO mouse model, lung infection, tissue collection, lung bacteriology and bronchoalveolar lavage were performed and described by Dr. Osama A. Abdel-Razek from Dr. Guirong Wang’s research group at Upstate Medical University (SUNY)).

SP-A and SP-D double knockout (SP-A/D KO) mice with C57BL/6 background were used for this study. The original SP-A/D KO mice were kindly provided by Dr. Samuel. Hawgood (The University of Califonia San Francisco) and these mice had been backcrossed at least 10
generations against a C57BL/6 background. Mice used in this study were bred in the animal core facility at SUNY Upstate Medical University under pathogen-free conditions. All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee guidelines of SUNY Upstate Medical University and the National Institutes of Health guidelines on the use of laboratory animals.

**Lung infection model.**

*P. aeruginosa* strain PAO1- BAA-47 (wild type) from frozen stocks were streaked onto LB agar plates and incubated at 37°C for 24 hours, and a single colony was picked up and transferred to a flask contains 20 mL of LB Broth and incubated at 37°C for 13 hours with shaking at 250 rpm. The optical density at 600 nm (OD₆₀₀) was measured and it is usually about 2.1 by this time. The bacterial cells were recovered by centrifugation, resuspended in saline and diluted to an optical density of 0.6 at OD₆₀₀. One mL of this solution was estimated to contain 2 × 10⁹ CFU. The solution was diluted 10 times with saline for use. Based on our preliminary data, a 50 µl (containing 1 × 10⁷ CFU) of the diluted bacterial solution was used to inject each mouse intratracheally. The tracheal delivery of [50 µl (1 × 10⁷ CFU)/ mouse] was accomplished by anesthetizing mice with 30 µl of the mixture of ketamine: xylazine (100 mg/kg:10 mg/kg). In 6-BBF-treated group bacterial suspension was mixed with 6-BBF (final concentration to 200 µM of 6-BBF). In control group bacterial suspension was added with same volume of saline. Forty eight hours after lung infection the mice were sacrificed.

**Tissue Collection.**

After anesthetizing mice with 30 µl of the mixture of ketamine: xylazine (100 mg/kg:10 mg/kg), a large abdominal incision was made and the intestine was turned to the left side of the
inferior vena cava and aorta were cut using iris scissors and the animal was bleed and then lung tissue was harvested or was lavaged.

**Lung Bacteriology.**

Mice from both 6-BBF-treated group and controls were studied for quantitative bacteriology. The left half of the lung of each mouse was removed aseptically and homogenized in 1 ml of sterile saline and 100 µl of appropriately serial diluted lung homogenates were plated on LB agar, and incubated at 37°C for 24 hours after which bacterial colonies were counted. CFUs were determined using the Quantity One colony-counting software (Bio-Rad, Hercules, CA).

**Bronchoalveolar Lavage (BAL).**

Mice from both 6-BBF-treated group and controls were prepared for BAL. After anesthetizing mice with 30 µl of the mixture of ketamine/xylazine (90mg/kg/10mg/kg, respectively), the animal was bleed and then the lung was lavaged 3 times each with 0.5 ml saline. Then 100 µl of diluted BAL Fluid with saline was plated on LB agar plate, and incubated at 37°C for 16 hours. Bacterial CFUs were determined using the Quantity One colony-counting software (Bio-Rad, Hercules, CA).

**Cytotoxicity Assay for human cells.**

All cell cultures were maintained in a humidified incubator, at 37 °C, with 5% CO₂ atmosphere. Human neuroblastoma SK-N-SH cells (a donation from Bonnie B. Toms at SUNY Upstate Medical University) were cultured in 96-well plates with 100 µL of culture medium (EMEM + 10% FBS, 100 µg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM L-glutamine). Each well contained 100 µL of cell suspension with a concentration of 5×10⁴ cells/mL. The viability of SK-N-SH was determined with a hemacytometer using standard trypan blue protocol. For cytotoxicity assay, the colorimetric cell counting assay (CCK 8 assay) was used as described
previously. In brief, after being plated, the SK-N-SH cells were allowed to adhere to the bottom of the wells for 24 h. The medium was then replaced with fresh ones (supplemented with DMSO to eliminate solvent effect for the negative control) with or without brominated furanones. After 1 h, the BF-containing media were removed and the cells were washed twice with fresh culture medium without DMSO. The cells were then allowed to recover for 0, 24, and 48 h (recovery time), at which time the number of live cells was determined by the CCK 8 assay. The survival (%) was calculated using the equation: survival (%) = (A_{450 \text{ sample}} – A_{450 \text{ medium}})/(A_{450 \text{ control}} – A_{450 \text{ medium}}) × 100. The sample absorbance values were obtained from the wells containing cells and brominated furanones, and the control absorbance were obtained from BF-free wells containing cells and DMSO.

Statistical analysis.

Experimental data were analyzed by SigmaStat 3.5 software (Systat Software, Inc., San Jose, CA) and presented as means ± standard error. Two-group comparisons were performed using Student’s t test. A P value of <0.05 was considered to be statistically significant.

3.4.2 Chemical synthesis and characterization

\[
\begin{align*}
\text{HO} & \xrightarrow{\text{Br}_2, \text{DCM}, 0 \degree \text{C to RT}} \text{HO} \\
\text{O} & \xrightarrow{\text{P}_2\text{O}_5, \text{DCM}, 0 \degree \text{C to reflux}} \text{O} \\
\text{O} & \xrightarrow{\text{Et}_3\text{N, DCM, 0 \degree \text{C to reflux}}} \text{O}
\end{align*}
\]

\( \text{n = 1: 5-BBF} \)
\( \text{n = 2: 6-BBF} \)
\( \text{n = 3: 7-BBF} \)

General procedure for synthesis.

All air sensitive reactions were performed in oven dried glassware under an atmosphere of argon unless otherwise notified. All reagent grade starting materials were obtained from commercial supplies and used as received. Anhydrous solvents were purchased from Sigma-
Aldrich. Analytical thin layer chromatography was performed on EM silica gel 60 F254 glass plates (0.25 mm). Visualization of analytical thin layer chromatography was achieved using UV absorbance (254 mm), KMnO₄, and ceric ammonium molybdate stains. Flash column chromatography was performed using SiliaFlash P60 silica gel (40-60 Å) from SiliCycle, Inc. 1D ¹H and ¹³C NMR spectra were recorded on a Bruker Advance DPX-300 spectrometer. ¹H chemical shifts are reported in ppm, downfield from tetramethylsilane using residual CHCl₃ as the internal standard (δ 7.26 ppm). ¹³C chemical shifts are reported in ppm, downfield from tetramethylsilane relative to CDCl₃ (δ 77.0 ppm). Mass spectra were measured using a MAT 95 XP mass spectrometer, carried out by the Mass Spectroscopy Facility at Indiana University.

**5-BBF 15**: Bromine (0.79 mL, 15 mmol) was added dropwise to a solution of 2-oxocyclopentaneacetic acid (1.13 g, 7.69 mmol) in anhydrous methylene chloride (7.7 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 40 min and then ambient temperature for 100 min. The resulting solution was washed with water (10 mL) followed by aqueous 1M Na₂S₂O₃ solution (10 mL) and then extracted with methylene chloride (10 mL × 3). The combined organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. The crude oil was taken up in anhydrous methylene chloride (30 mL) and cooled to 0 °C, followed by addition of P₂O₅ (2.65 g, 18.6 mmol) in portions. The reaction mixture was stirred at 0 °C for 30 min, allowed to warm to ambient temperature and then stirred under reflux for 2 h. The solid thus formed was filtered off and the filtrate concentrated under reduced pressure. The crude oil was dissolved in anhydrous methylene chloride (15 mL), cooled to 0 °C and treated with anhydrous
Et₃N (1.12 mL, 8.0 mmol). The reaction mixture was stirred at ambient temperature for 30 min and then under reflux for 2 h. After cooled to ambient temperature, the reaction mixture was washed with aqueous saturated NH₄Cl and extracted with methylene chloride (10 mL × 3). The combined organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. Flash chromatography (SiO₂, hexane:ethyl acetate, gradient) provided 5-BBF 15 (54.3 mg, 3% over 3 steps) as off white solid. TLC Rf = 0.29 (hexane:ethyl acetate, 4:1). ¹H NMR (300 MHz, CDCl₃): δ 5.67 (t, 1H, J = 0.9 Hz), 3.12 (t, 2H, J = 4.2 Hz), 2.95 (td, 2H, J = 3.9, 1.5 Hz). HRMS: Cacl. for M⁺: 199.9467, found: 199.9466 (96.3%), 201.9442 (100.0%).

6-BBF 16: To a mixture of ethyl 2-cyclohexaneacetate (2.00 g, 10.6 mmol) in THF (72 mL) and water (32 mL) was added lithium hydroxide (688.0 mg, 34.4 mmol). The reaction mixture was stirred vigorously at ambient temperature overnight. The reaction mixture was concentrated to remove THF and acidified with hydrochloric acid until pH was one. The mixture was extracted with ethyl acetate (30 mL × 3), washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. 2-Oxocyclohexaneacetic acid 18 was obtained as a light yellow crude oil that was carried on to the next step without further purification. Bromine (1.14 mL, 22.3 mmol) was added dropwise to a solution of crude 2-oxocyclohexaneacetic acid 18 (1.65 g, 10.6 mmol) in anhydrous methylene chloride (10.6 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then ambient temperature for 2 h. The resulting solution was washed with water (6 mL) followed by aqueous 1M Na₂S₂O₃ solution (6 mL) and then extracted with methylene chloride (6 mL × 3). The combined organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. The crude oil was taken up in anhydrous methylene
chloride (42 mL) and cooled to 0 °C, followed by addition of P₂O₅ (3.67 g, 25.9 mmol) in portions. The reaction mixture was stirred at 0 °C for 30 min, allowed to warm to ambient temperature and then stirred under reflux for 2 h. The solid thus formed was filtered off and the filtrate concentrated under reduced pressure. The crude oil was dissolved in anhydrous methylene chloride (26 mL), cooled to 0 °C and treated with anhydrous Et₂N (1.50 mL, 10.8 mmol). The reaction mixture was stirred at ambient temperature for 30 min and then under reflux for 1 h. After cooled to ambient temperature, the reaction mixture was washed with aqueous saturated NH₄Cl and extracted with methylene chloride (18 mL × 3). The combined organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. Flash chromatography (SiO₂, hexane:ethyl acetate, gradient) provided 6-BBF (350.5 mg, 16% over 4 steps) as off white solid. TLC Rf = 0.66 (hexane:ethyl acetate, 1:1). ¹H NMR (300 MHz, CDCl₃): δ 5.83 (s, 1H), 2.79 (t, 2H, J = 6.0 Hz), 2.73 (td, 2H, J = 6.0, 1.2 Hz), 2.00 (m, 2H); ¹³C NMR (75 MHz, CDCl₃): δ 168.5, 154.9, 148.3, 111.0, 107.4, 34.0, 24.0, 23.6. HRMS: Cacld. for M⁺: 213.9624, found: 213.9626 (100.0%), 215.9596 (99.6%).

7-BBF 17: To a stirred suspension of NaH (5.10 g, 60% suspension in mineral oil, 128 mmol, 15 mL × 2 hexane washed) in anhydrous benzene (80 mL) was added dimethyl carbonate (7.6 mL, 90 mmol) via syringe. The reaction was stirred under reflux for 1 h. A solution of cycloheptanone (4.3 mL, 36 mmol) in anhydrous benzene (6.0 mL) was then added via cannula and the reaction mixture was stirred under reflux for 3 h. The heterogeneous mixture was cooled to ambient temperature and then quenched with acetic acid (8 mL). The mixture was diluted with
water (200 mL), extracted with ethyl acetate (50 mL × 4), washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. Methyl 2-oxo-1-cloheptanecarboxylate 19 was obtained as yellow oil that was carried on to the next step without further purification. To a solution of crude methyl 2-oxo-1-cloheptanecarboxylate 19 in acetone (100 mL) was added potassium carbonate (23.2 g, 168 mmol) followed by ethyl bromoacetate (3.8 mL, 34 mmol). The reaction mixture was stirred under reflux overnight (~17 h). The suspension was cooled to ambient temperature and concentrated under reduced pressure to remove ~ half of the acetone. The residue was diluted with water (50 mL), extracted with diethyl ether (50 mL × 3), washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. The resulting yellow oil was treated with a solution of hydrochloric acid (27 mL, 6M) and acetic acid (27 mL). The reaction mixture was stirred under reflux for 2 days and cooled to ambient temperature. The mixture was diluted with water (50 mL), extracted with methylene chloride (30 mL × 5), washed with brine, dried over MgSO₄, filtered and concentrated under reduced pressure. Flash chromatography (SiO₂, methylene chloride:ethyl acetate, gradient) provided 2-oxocycloheptaneacetic acid 20 (5.35 g, 87% over 3 steps) as a light yellow oil. TLC Rf = 0.31 (methylene chloride:ethyl acetate, 1:1). The identity of this compound was confirmed by comparing ¹H NMR data with that previously reported. Bromine (0.93 mL, 18 mmol) was added dropwise to a solution of 2-oxocycloheptaneacetic acid 20 (1.47 g, 8.66 mmol) in anhydrous methylene chloride (8.7 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min and then ambient temperature for 2 h. The resulting solution was washed with water (5 mL) followed by aqueous 1M Na₂S₂O₃ solution (5 mL) and then extracted with methylene chloride (5 mL × 3). The combined organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. The crude oil was taken up in anhydrous methylene chloride (35 mL) and
cooled to 0 °C, followed by addition of P₂O₅ (3.00 g, 21.1 mmol) in portions. The reaction mixture was stirred at 0 °C for 40 min, allowed to warm to ambient temperature and then stirred under reflux for 2 h. The solid thus formed was filtered off and the filtrate concentrated under reduced pressure. The crude oil was dissolved in anhydrous methylene chloride (22 mL), cooled to 0 °C and treated with anhydrous Et₃N (1.50 mL, 8.93 mmol). The reaction mixture was stirred at ambient temperature for 30 min and then under reflux for 1 h. After cooled to ambient temperature, the reaction mixture was washed with aqueous saturated NH₄Cl and extracted with methylene chloride (15 mL × 3). The combined organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. Flash chromatography (SiO₂, hexane:ethyl acetate, gradient) provided 7-BBF (194.8 mg, 10% over 3 steps) as off white solid. TLC Rf = 0.62 (hexane:ethyl acetate, 1:1). ¹H NMR (300 MHz, CDCl₃): δ 6.01 (s, 1H), 2.98 (t, 2H, J = 6.0 Hz), 2.82 (td, 2H, J = 6.0, 1.5 Hz), 1.91 (m, 2H), 1.81 (m, 2H); ¹³C NMR (75 MHz, CDCl₃): δ 168.0, 157.0, 148.2, 117.2, 115.4, 39.5, 29.3, 27.8, 23.9. HRMS: Cacl. for M⁺: 227.9780, found: 227.9785 (97.3%), 229.9766 (100.0%).
Chapter 4

Modulation of Quorum Sensing in Gram-negative Bacteria by Squarylated Homoserine Lactones

Summary

An expanded library of squarylated homoserine lactones (SHLs) were designed and synthesized as analogues to the natural AHLs. Together with some of the existing SHLs, these compounds were evaluated for their activity to modulate the quorum sensing (QS) systems in two Gram-negative bacteria, an opportunistic pathogen \textit{P. aeruginosa} and a symbiont \textit{V. fischeri}. None of the SHLs were identified as agonists to LasR in \textit{P. aeruginosa} or LuxR in \textit{V. fischeri} at the concentrations tested, except for octyl-SHL, which showed weak agonistic activity to LasR at 150 \( \mu \text{M} \). The SHLs were weak to moderate antagonist to LasR in at 150 and 300 \( \mu \text{M} \). All except one SHL inhibited LuxR, with the alkyl-SHLs showing more inhibition in general than the aryl-SHLs. The cytotoxicity study suggested that the SHLs have no or minimal inhibition to the growth of these two bacteria species and the inhibition of QS or biofilm formation by \textit{P. aeruginosa} were not due to bactericidal effect. Primary investigation of structural activity relationship indicated that alkyl chain length is critical to activity of SHLs. These SHLs are promising candidates as modulators of other AHL-mediated QS systems.

4.1 Background and Significance

4.1.1 AHL-based quorum sensing in Gram-negative bacteria

\textit{N}-Acyl homoserine lactones (AHLs) are the most common class of signaling molecules used by Gram-negative bacteria in their intercellular communication called quorum sensing (QS).\textsuperscript{123} This signaling process allows the bacteria to coordinate gene expression in response to
population density. Once the concentration of the signaling molecules reaches a threshold, the bacteria undergo a lifestyle switch from that of a signal cell to that of a multicellular group, initiating the gene expression that benefit the colony as a whole. These signaling molecules are called autoinducers (AIs). There have been a number of AHLs identified in various bacteria species (Table 3.1) but they are all composed of a homoserine lactone ring (HSL) with an acyl chain (Figure 4.1). The AHLs may vary in acyl chain length (generally 4 to 18 carbons) and the degree of oxidation at the AHL C3 position, and may have different degree of unsaturation on the acyl chain. Some species produced mainly a single type of AHL while others produce multiple AHLs.

![General chemical structure of N-acyl homoserine lactones.](image)

The first QS system studied was that in the marine bacterium *Vibrio fischeri*. This QS system is composed of the autoinducer synthase LuxI, which is responsible for the synthesis of autoinducer (AI) 3-oxo-C6-HSL, and the receptor protein LuxR, which is a transcriptional activator and upon binding to the autoinducer, promotes transcription of the luciferase structural operon *luxCDABE* and the production of more AHL autoinducer. This principle of AHL-based QS applies to the majority of Gram-negative bacteria. The autoinducer synthases and receptors in other Gram-negative bacteria are termed LuxI-type and LuxR-type proteins, respectively.
4.1.2 Modulation of LuxI/LuxR-type quorum sensing system

As discussed in Chapter 3, one of the most commonly used strategies to control QS is to interfere with the binding of AIs to the LuxR-type receptors. There have been a number of attempts to discover small molecules that can potentially interact with the LuxR-type receptors and therefore modulate QS and QS-controlled bioactivities in bacteria. One powerful method is the high-throughput screen which has been utilized to identify small molecules as inhibitors of quorum sensing or biofilm formation.\textsuperscript{175-177} For example, ursolic acid was identified from 13000 compounds purified from plants as a biofilm inhibitor that inhibits biofilm formation by \textit{E. coli}, \textit{P. aeruginosa}, and \textit{V. harveyi} without being toxic.\textsuperscript{175} A rapid screening of 4509 compounds revealed that ferric ammonium citrate inhibited biofilm formation by \textit{P. aeruginosa} PA14 in a dose-dependent manner.\textsuperscript{176}

The human kind has long been utilizing plants and animals and fungus as traditional medicines and we are still learning from Mother Nature as new technology allows us to identify the active components in the therapy. We are therefore able to design and synthesize mimics of the identified natural products which do not exist in nature, with similar or even higher biological activities. One of the most successful approaches is based on the synthesis of mimics of naturally occurring bacterial signaling molecules (e.g. AHLs).

The first a few AHL analogue studies were published by groups led by Eberhard,\textsuperscript{256} Greenberg,\textsuperscript{257} and Iglewski.\textsuperscript{258} They explored the agonistic and antagonistic activities of synthetic AHL mimics against LuxR in \textit{V. fischeri} or LasR in \textit{P. aeruginosa}. These researchers demonstrated that: (1). In general, the acyl chain length is critical for the activity of AHL analogues. (2). For species whose natural AHLs possess a 3-oxo group, the presence of this group in synthetic AHL analogues is important for activity, but not essential. The removal of the
3-oxo group most often results in reduced agonistic activity or results in antagonistic activity rather than agonistic activity. (3). Replacement of the lactone ring in AHL analogues with γ-thiolactone normally retains the same overall activity but switching to a γ-lactam is not tolerant.

In a series of studies reported by Doutheau and co-workers on AHL analogues and their activities against LuxR, the authors found that medium-sized, branched acyl derivatives were agonists of LuxR while phenyl derivatives were antagonists of LuxR. Moreover, they made a group of AHLS in which the carbonyl group of the amide bond was replaced with a sulfonyle group and determined that the sulfonyl AHLS with acyl chains close in length to 3-oxo-C6-HSL and shorter were the strongest inhibitors of LuxR. None of the sulfonyl AHLS showed agonistic activity against LuxR. In another report by the same group, it was shown that replacing the amide in AHL with a urea resulted in compounds that displayed antagonistic but not agonistic activities against LuxR.

Suga and co-workers prepared a library of 96 AHL mimics, in which the lactone ring was replaced with various heterocycles and carbocycles that could hydrogen-bond at the same position as the carbonyl in the parent γ-lactone. They concluded that a five- or six-membered ring with a hydrogen bond acceptor was necessary to activate LasR in P. aeruginosa by AHL analogues. AHL mimics that contain an aromatic ring in place of the γ-lactone with a hydrogen bond acceptor present could inhibit LasR. The authors speculate that compounds with aromaticity at the AHL lactone ring position cause a conformational change of LasR upon binding which then presumably reduces the ability of LasR to bind DNA, and therefore inhibits transcription.

The most comprehensive and systematic work to investigate the structure activity relationship (SAR) for synthetic AHL analogues to date was done by Blackwell and co-
workers. Using a microwave-assisted, solid-phase route, these researchers synthesized more than 100 AHL analogues and evaluated their activity to modulate QS across three Gram-negative bacteria species: the symbiont *V. fischeri* and the pathogens *P. aeruginosa* and *A. tumefaciens*. Their studies revealed that several of the AHL analogues exhibited antagonistic activity in all three species examined, while other analogues were only active in one or two species. Broad-spectrum agonists with high activity were not identified (Figure 4.2). Several of the AHL analogues appeared to be agonist in one strain but antagonist in another. Very interestingly, a few of these ligands were antagonists at lower concentrations but were able to activate the transcriptional regulators at higher concentrations. From these studies, Blackwell and co-workers summarized several SAR trends, a few of which are congruent with those found by other groups previous reports as discussed above. In addition, the authors noted that:

1. The TraR protein was the most sensitive to the length of the AHL acyl chains. Assuming the synthetic AHLs target the same ligand-binding site on TraR compared to the natural AHL, this finding suggests that the TraR may have a sterically crowded ligand-binding site, which is in accordance with the X-ray crystal structure of TraR.

2. On the contrary, the LasR protein was the most tolerant of varying AHL acyl chain length, functionality on the acyl chain, and the stereochemistry of the homoserine lactone ring. This finding implies that LasR has a larger ligand-binding site than TraR, which is in accordance with the X-ray crystal structure of LasR.

3. The LuxR protein was most strongly activated by AHLs containing a phenylacetanoyl group in the acyl chain with electron-withdrawing group in the 3-position, while was most strongly inhibited by AHLs with acyl chain length of 6 to 14 carbons that had a 3-oxo group. These works not only supply a highly efficient chemical tool to develop and expand new libraries of QS.
modulators but also provide broad insights into the structural basis for QS mediated by AHL signals in Gram-negative bacteria.

Figure 4.2 Venn diagrams showing the structures of selective most potent LuxR-type protein antagonists and agonists identified and their selectivities for different LuxR-type proteins from *A. tumefaciens* (TraR), *V. fischeri* (LuxR), and *P. aeruginosa* (LasR). Ligands in the intersections of the circles have significant selectivity for two or more proteins. NA: No applicable ligands identified.
4.1.3 **Biofilm inhibitors containing squarate moiety**

AHLs are prone to hydrolysis either at the lactone ring due to elevated pH or the activity of acyl-HSL lactonases produced by strains of *Agrobacterium, Arthrobacter, Bacillus*, and *Klebsiella*, or at the acyl-amide linkage catalyzed by acyl-HSL acylases produced by strains of *Pseudomonas, Ralstonia*, and *Variovorax*. In addition, the flexible structures of the acyl chains provide numerous possible conformations which may reduce the affinity of these molecules to the receptor proteins. Taking these facts into consideration, Narasimhan *et al.* designed and synthesized a group of squarylated homoserine lactones (SHLs) in which the homoserine lactone is attached to a squarate moiety, which bears saturated or unsaturated aliphatic chains. Most SHLs were prepared in a racemic form except for vinyl-SHL, which was prepared in both enantiomerically pure forms (4 and 5) and the racemic form (3). Together with four squarylated esters and two squarylated amides, these squarate-based AHL analogues were tested for their inhibitory activity against biofilm formation by *E.coli* RP437 (Figure 4.3). Vinyl-SHL 3 and butyl-SHL 6 were the most potent biofilm inhibitors among the molecules tested, with ~50% inhibition at 200 µM. No different biofilm inhibitory activity was observed for the L- and D- vinyl-SHLs and both resulted in only half biofilm inhibition compared to the racemic form.
Figure 4.3 Chemical structures of squarate-based AHL analogues examined by Narasimhan et al. on inhibitory activities against biofilm formation by *E. coli* RP437. Figure adapted and modified from Ph. D. thesis by Sri Kamesh Narasimhan.266

Bandyopadhyay *et al.* added three alkyl SHLs and three aryl SHLs to the library and evaluated the ability of the new compounds to inhibit biofilm formation by *E. coli* RP437 (Figure 4.4).211 Butyl-SHL 6 was used as a control. In general, the aromatic SHLs appeared to exhibit stronger biofilm inhibition, with the most potent compound tolyl-SHL 18 resulting in ~55% inhibition while the least potent compound ethyl-SHL 14 showing ~34% inhibition.
Although *E. coli* is not capable of producing AHL molecules because it lacks an AHL synthase encoding gene, it does possess a LuxR-type receptor protein, called SdiA, and can respond to AHL signals produced by other bacteria.\textsuperscript{267,268} The two publications described above did not directly study the ability of SHLs to modulate QS in *E. coli*. However, since biofilm formation is known to be regulated by AHL-based QS,\textsuperscript{139} it is possible that the SHLs inhibit *E. coli* biofilm formation by binding to the LuxR-type receptor (e.g. SdiA) in *E. coli*.\textsuperscript{211}

The most common Gram-negative bacterium found in nosocomial and life-threatening infections of immunocompromised patients is the opportunistic pathogen *P. aeruginosa*. These infections are very persistent because of the presence of *Pseudomonal* biofilm and the resistance to antimicrobial therapy. Controlling the QS in *P. aeruginosa* may lead to inhibition or eradication of *P. aeruginosa* biofilm and therefore is a significantly clinically related issue.

There are two identified AHL-mediated QS circuits in *P. aeruginosa*. In the las circuit, autoinducer synthase LasI is responsible for the production of the AHL signal 3-oxo-C12-HSL while in the rhl circuit, the RhlII protein is responsible for the production of a second AHL signal,
C4-HSL. In the effort of evaluating the ability of synthetic AHLs to stimulate the expression of the gene for elastase, one of many biological activities controlled by AHL-mediated QS in *P. aeruginosa*, Passador *et al.* demonstrated that the length of the acyl side chain of the AHL analogues is the most critical factor of activity.\(^{258}\) Therefore we designed the synthesis of SHLs with chain length similar to that in the natural AI 3-oxo-C12-HSL (8 and 12 carbons for SHLs 19 and 20, respectively) (Scheme 4.1). Introduction of aromatic substituent to the structure of AHL analogues leads to agonists and antagonists with increased activities.\(^{162,163,264}\) In addition to the aryl-substituted SHLs 16-18 in hand, we also designed *m*-xylol-SHL 21, which has more substituents on the benzene ring. SHL 6, 13, and 15-21 constitute a library of candidates as modulators for QS in *P. aeruginosa* (Figure 4.5).

![Figure 4.5 The library of SHLs used in this study.](image)

### 4.2 Results and Discussion

#### 4.2.1 Synthesis of SHLs

The synthesis route used to prepare SHLs was adapted and modified from that previously reported by Narasimhan\(^{266}\) and Bandyopadhyay (Scheme 4.1).\(^{211}\) Briefly, 1,2-addition of various alkylation reagents at one of the carbonyl groups of 3,4-dibutoxy-3-cyclobutene-1,2-dione 9

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Note: Methyl-SHL 13, butyl-SHL 6, and tolyl-SHL 17 were synthesized by Debjyoti Bandyopadhyay. Phenyl-SHL 16 and the precursor for tolyl-SHL 17 were synthesized by Nisha Varghese. All the other SHLs were synthesized by Sijie Yang. Department of Chemistry, Syracuse University, Syracuse NY.
gave cyclobutenols 22-25, which yielded cyclobutenediones 26-30 upon treatment with concentrated hydrochloric acid. The alkylating reagents were Grignard reagents or organolithium reagent made from corresponding commercially available bromides. The cyclobutenediones 26-30 then reacted with α-amino-γ-butyrolactone hydrobromide in the presence of triethylamine and provided the desired SHLs, namely hexyl-SHL 15, thienyl-SHL 18, octyl-SHL 19, dodecyl-SHL 20, and xylyl-SHL 21. All the SHLs precipitated out from the reaction solvent EtOH, and did not need further purification by column chromatography.

Scheme 4.1 Synthesis of SHLs

\[
\begin{align*}
\text{R = n-hexyl, 22 (44\%)} & \quad \text{R = n-hexyl, 26 (65\%)} & \quad \text{R = n-hexyl, 15 (53\%)} \\
n-octyl, 23 (48\%) & \quad n-octyl, 27 (80\%) & \quad n-octyl, 19 (65\%) \\
n-dodecyl, 24 (45\%) & \quad n-dodecyl, 28 (21\%) & \quad n-dodecyl, 20 (30\%) \\
thieryl, 25 (21\%) & \quad \text{thieryl, 29 (59\%)} & \quad \text{thieryl, 18 (60\%)} \\
m-xylyl, (N/A) & \quad m-xylyl, 30 (27\% \text{ over 2 steps}) & \quad m-xylyl, 21 (65\%)
\end{align*}
\]

\(^{\text{a}}\text{N/A indicates the shown compound was not obtained in purified form and a yield was not available.}\)

4.2.2 Cytotoxicity study of SHLs on the growth of P. aeruginosa

The increasing occurrence of drug-resistant bacteria strains and lack of new antibiotics pose a significant challenge in treating diseases. It is essential to develop quorum sensing modulators that do not exert evolutionary selective pressure on bacteria by inhibiting bacterial survival. We first evaluate the cytotoxicity of SHLs on the growth of P. aeruginosa. Optical density values of bacteria culture grown in the presence of 300 μM SHLs were measured at 600 nm (OD\text{600}) every 2 h for the first 12 h and then after 24 h of incubation. Bacteria grown in the absence of SHLs
were used as the control (Figure 4.5). The same amount of DMSO introduced from the SHL stocks was added to the control to eliminate solvent effect. None of the SHLs tested resulted in any deviation of OD values from that of the SHL-free control and therefore these SHLs do not inhibit the growth of *P. aeruginosa* PAO1 under the conditions used.

**Figure 4.5** Growth curves of *P. aeruginosa* PAO1 in the absence (control) and presence of 300 µM SHLs. Values represent the means ± standard deviation from four replicates.

### 4.2.3 Modulation of quorum sensing in *P. aeruginosa* by SHLs

A double-knockout reporter strain PAO-JP2 (ΔlasIΔrhlII)\(^{236}\) harboring the plasmid *plasI-LVAgfp*\(^{237}\) was used to evaluate the effect of SHLs on the *las* QS system in *P. aeruginosa*. When bacteria are growing in the presence of exogenously introduce autoinducer 3-oxo-C12-HSL or functional analogues, LasR receptor protein binds to the AI, activating transcription of the *lasI* promoter and thus the expression of green fluorescence protein (GFP). The GFP production is quantified by correcting the measured fluorescence signal for cell density as shown in Equation...
4.1. “RFU<sub>sample</sub>” is the relative fluorescence unit measured by the plate reader. “No AI” represents bacteria culture grown in the presence of no AI analogue, supplemented with the same amount of DMSO present in the SHL stocks.

\[
\text{Relative fluorescence} = \frac{\text{RFU}_{\text{sample}} - \text{RFU}_{\text{No AI}}}{\text{OD}_{600\text{sample}}} \tag{4.1}
\]

In the agonism assay, bacteria cultures grown in the presence of 150 µM SHLs were compared to that grown in the presence of 1 µM natural AI 3-oxo-C12-HSL (a concentration within the range produced by wild type P. aeruginosa).\textsuperscript{130,160-162} Only octyl-SHL 19 was able to activate GFP production by a measurable amount, ~20% that of the control (1 µM 3-oxo-C12-HSL). None of the other SHLs showed any activation at this concentration (Figure 4.6).

The antagonism assay was conducted with competition of 150 or 300 µM SHLs against 1 µM 3-oxo-C12-HSL (Figure 4.7). As the concentration increased from 150 to 300 µM, almost all of the SHLs resulted in reduced GFP expression. The SHLs appeared to be weak to moderate antagonists to LasR at the concentrations tested. The most potent antagonists were octyl-SHL 19 and tolyl-SHL 17, which showed ~35% and 42% inhibition, respectively.
Figure 4.6 GFP expression by PAO-JP2 (\textit{plasl}-LVA\textit{gfp}) in the presence of 1 µM 3-oxo-C12-HSL alone (control) or 150 µM SHLs. Fluorescence signals were corrected for cell density as shown in Equation 4.1 and the results were normalized to the control. Values represent the means ± standard deviation from four replicates. Data shown is a representative of at least three separate experiments.

Figure 4.7 GFP expression by PAO-JP2 (\textit{plasl}-LVA\textit{gfp}) in the presence of 1 µM 3-oxo-C12-HSL alone (control) or 1 µM 3-oxo-C12-HSL plus different concentrations of SHLs.
Fluorescence signals were corrected for cell density as shown in Equation 4.1 and the results were normalized to the control. Values represent the means ± standard deviation from four replicates. Data shown is a representative of at least three separate experiments.

4.2.4 Inhibition of *P. aeruginosa* biofilm formation by SHLs

Biofilm formation by *P. aeruginosa* in the presence of 300 µM SHLs was quantified using a colorimetric assay in 96-well plates employing crystal violet (CV) (Figure 4.8). The amount of biofilm formation was determined indirectly by measuring the absorbance of CV at 540 nm after workup (see experimental section for details) and normalized by that of the SHL-free control as shown in Equation 4.2. The SHL-free control was supplemented with the same amount of DMSO present in the SHL stocks to eliminate solvent effect.

\[
\text{Relative biofilm formation (\%)} = \frac{OD_{540\text{sample}} - OD_{540\text{medium}}}{OD_{540\text{control}} - OD_{540\text{medium}}} \tag{4.2}
\]

In contrast to the trend that aryl-SHLs showed more biofilm inhibition than alkyl-SHLs for *E. coli* as observed by Bandyopadhyay,\(^{211}\) alkyl-SHLs seemed to have stronger inhibitory activities than aryl-SHLs against biofilm formation by *P. aeruginosa*. This difference may be due to different structure at the binding site of receptors SdiA (in *E. coli*) and LasR (in *P. aeruginosa*). Moreover, a clear structure activity relationship was observed for the alkyl-SHLs. Within the range of one to eight carbons in the alkyl side chain, the biofilm inhibitory activity increased as the chain length increased. Octyl-SHL 19, which possess the chain length most similar to the natural AI 3-oxo-C12-HSL, resulted in the most inhibition, ~76% compared to the control. We note that dodecyl-SHL 20 did not seem to be completely soluble in the bacterial
culture at the concentration tested. All of the aryl-SHLs were moderate biofilm inhibitors, with ~50% inhibition in all cases.

Figure 4.8 *P. aeruginosa* biofilm formation in the presence of 300 µM SHLs. Values are normalized by that of the SHL-free control as shown in Equation 4.2 and represent the means ± standard deviation from 4 replicates. Data shown is a representative of at least three separate experiments. Significant differences in the biofilm formation from the SHL-free control are indicated by asterisks: ***, *P* < 0.001.

4.2.5 Cytotoxicity study of SHLs on the growth of *V. fischeri*

Blackwell and co-workers discovered a few AHL analogues that exhibited antagonistic activities against LuxR-type proteins in more than one bacteria species.\textsuperscript{163,165,166} We were also interested in if any of our SHLs was capable of activating or inhibiting LuxR-type proteins in more than one species. We selected the *V. fischeri* for this purpose. The QS system in *V. fischeri* is representative of all AHL-mediated QS systems in Gram-negative bacteria and the natural AI
3-oxo-C6-HSL is structurally similar to the 3-oxo-C12-HSL produced by *P. aeruginosa*. We expected at least one or more of the SHLs with shorter alkyl side chain should show some activity for the LuxR protein.

The cytotoxicity of the SHLs to the *V. fischeri* was first studied. The strain VCW2G7 we used lacks the *luxI* gene that encodes the AI synthase LuxI and does not produce the natural AI 3-oxo-C6-HSL, but possesses the native *lux* operon that expresses luminescence. The EC$_{50}$ of 3-oxo-C6-HSL was determined to be 3 µM. We used this concentration as the control and compared the growth curves of bacteria culture in the presence of 3 µM 3-oxo-C6-HSL plus 10 µM SHLs to the control (Figure 4.9). At 10 µM, none of the SHLs except methyl-SHL 13 had negative impact on the growth of bacteria. Methyl-SHL 13 caused about ~20% reduction in OD$_{600}$ values than the control throughout the experiment. Therefore, the SHLs had no or very little effect on the growth of *V. fischeri*.

Figure 4.9 Growth curves of *V. fischeri* VCW2G7 in the presence of 3 µM 3-oxo-C6-HSL alone (control) or 3 µM 3-oxo-C6-HSL plus 10 µM SHLs. Values represent the means ± standard deviation from four replicates.
4.2.6 Modulation of quorum sensing in *V. fischeri* by SHLs

The ability of SHLs to modulate the QS in *V. fischeri* was evaluated similarly to that in *P. aeruginosa* except luminescence was measured instead of fluorescence. The luminescence expression is quantified as shown in Equation 4.3. “RLU\textsubscript{sample}” is the relative luminescence unit measured by the plate reader. “No AI” represents bacteria culture grown in the presence of no AI analogue, supplemented with the same amount of DMSO present in the SHL stocks.

\[
\text{Relative luminescence} = \frac{\text{RLU}_{\text{sample}} - \text{RLU}_{\text{no AI}}}{\text{OD600}_{\text{sample}}} \quad (4.3)
\]

In the agonism assay, 10 µM SHLs were compared to 3 µM 3-oxo-C6-HSL and none of the SHLs showed measurable agonistic activity (data not shown). When 10 µM SHLs competed with 3 µM 3-oxo-C6-HSL in the antagonism assay (Figure 4.10), hexyl-SHL 15 and octyl-SHL 19 were the most potent antagonists identified, as expected, with ~90% and >99% reduction in luminescence expression, respectively. Moreover, the alkyl-SHLs in general resulted in less luminescence expression than aryl-SHLs. We note that while m-xylyl-SHL 21 (two methyl substitution on the benzene ring) appeared to be a strong antagonist (23% relative fluorescence signal of the control), tolyl-SHL 17 (one methyl substitution on the aromatic ring) appeared to be a weak agonist (112% relative luminescence expression of the control). This difference in activity is likely caused by structural difference and the specific mechanism is under investigation.
Figure 4.10 Luminescence expression by *V. fischeri* VCW2G7 in the presence of 3 µM 3-oxo-C6-HSL alone (control) or 3 µM 3-oxo-C6-HSL plus 10 µM SHLs. Luminescence signals were corrected for cell density as shown in Equation 4.3 and the results were normalized to the control. Values represent the means ± standard deviation from four replicates. Data shown is a representative of at least three separate experiments. Significant differences in the luminescence expression from the SHL-free control are indicated by asterisks: ***, $P < 0.001$.

4.3 Conclusion and Perspectives

An expanded library of squarylated homoserine lactones were designed and synthesized as analogues to the natural AHLs. The SHLs were weak to moderate antagonist to LasR in *P. aeruginosa* at 150 and 300 µM while only the octyl-SHL 19 was a weak agonist to LasR at 150 µM. Although none of the SHLs were capable of activating LuxR in *V. fischeri* at 10 µM, all except one SHL inhibited LuxR, with the alkyl-SHLs showing more inhibition in general than the aryl-SHLs. Octyl-SHL 19 was the most potent inhibitor of biofilm formation by *P.
*Pseudomonas aeruginosa* among all SHLs tested. Primary investigation of structural activity relationship indicated that alkyl chain length is critical to the activity of SHLs. Future works include study of the ligand-receptor binding between SHLs to LuxR-type receptor proteins by computationally docking to elucidate the mechanism of observed structural activity relationship. This computational analysis should provide important guidance in the *in silico* design of new QS modulators of LuxI/LuxR-type QS systems in Gram-negative bacteria.

4.4 Experimental Section

**Bacteria strains and growth media.**

Double-knockout mutants of *Pseudomonas aeruginosa*, PAO-JP2 (*plasI*-LVAgfp) was kindly provided by Dr. Helen E. Blackwell (University of Wisconsin-Madison) with permission by Dr. Babara H. Igliewski (University of Rochester Medical Center). P. aeruginosa PAO1-BAA-47 (wild type) from ATCC and PAO-JP2 (*plasI*-LVAgfp) were grown in Luria-Bertani (LB) broth (containing 300 µg/mL of carbenicillin for PAO-JP2 (*plasI*-LVAgfp)) at 37 °C. *V. fischeri* VCW2G7 was grown in LB salt media (LBS) at ambient temperature. LBS media contained 2% NaCl, 1% tryptone, 0.5% yeast extract, 0.3% (v/v) glycerol, and 50 mM Tris-HCl buffer (pH = 7.5).

**Stock solutions of SHLs.**

Stock solutions of all SHLs (200 µM to 5 mM) were prepared in DMSO, sterilized by filtering through a 0.2 µm syringe filter, and stored at -20 °C in sealed vials. Appropriate amount of DMSO was added to controls in all assays to eliminate solvent effect. The amount of DMSO in all cases was no more than 0.2%.

**Growth curve of planktonic bacteria, reporter gene assay for P. aeruginosa, and crystal violet biofilm assay in 96-well plates.**
These experiments were performed as described in Chapter 3.

*Reporter gene assay for V. fischeri.*

An overnight culture of *V. fischeri* VCW2G7 in LBS broth was grown from a single colony picked from an LBS agar. The cell culture was diluted by 10 times and aliquoted (200 µL per well) to a polystyrene 96-well microplate (Costar 3370, Corning Incorporated, Corning, NY) containing appropriate amount of natural autoinducers with and without SHLs. The plate was incubated at ambient temperature (~21 °C) for 5~6 h in a rotary shaking incubator. The culture from each well was then transferred to a 96-well plate with white wall (µClear, 655095, Greiner Bio-One North America, Inc., Monroe, NC). The luminescence and OD absorbance (OD\(_{600}\)) in each well was measured by Synergy H1 multi-mode microplate reader with a Gen5 data analysis software.

*General procedure for synthesis.*

All air sensitive reactions were performed in oven dried glassware under an atmosphere of argon unless otherwise notified. All reagent grade starting materials were obtained from commercial supplies and used as received. Anhydrous solvents were purchased from Sigma-Aldrich. Analytical thin layer chromatography was performed on EM silica gel 60 F254 glass plates (0.25 mm). Visualization of analytical thin layer chromatography was achieved using UV absorbance (254 mm), KMnO\(_4\), and ceric ammonium molybdate stains. Flash column chromatography was performed using SiliaFlash P60 silica gel (40-60 Å) from SiliCycle, Inc. \(^1\)H and \(^{13}\)C NMR spectra were recorded on a Bruker Advance DPX-300 spectrometer. \(^1\)H chemical shifts are reported in ppm, downfield from tetramethylsilane using residual CHCl\(_3\) as the internal standard (δ 7.26 ppm). \(^{13}\)C chemical shifts are reported in ppm, downfield from tetramethylsilane relative to CDCl\(_3\) (δ 77.0 ppm), (δ 39.5 ppm) and CD\(_3\)CN (δ 1.94 ppm). Mass spectra were measured...
using a MAT 95 XP mass spectrometer, carried out by the Mass Spectroscopy Facility at Indiana University.

To a stirred solution of 3,4-butoxy-3-cyclobutene-1,2-dione (269.0 mg, 1.165 mmol) in anhydrous THF (12 mL) was added hexylmagnesium bromide (2.0 M solution in diethyl ether, 0.61 mL, 1.2 mmol) dropwise at -78 °C. The reaction mixture was stirred at -78 °C for 4 h and another portion of hexylmagnesium bromide (2.0 M solution in diethyl ether, 0.30 mL, 0.60 mmol) was added. After stirring at -78 °C for 1 h further, the reaction mixture was poured into a separatory funnel containing 5% NH₄Cl (14 mL) and diethyl ether (14 mL). The aqueous layer was extracted with diethyl ether (14 mL × 3) and the combined organic layer was washed with brine, dried over MgSO₄, filtered, and concentrated under reduced pressure. Column chromatography (SiO₂; hexane:ethyl acetate, 50:1 to 3:1, gradient) provided alcohol 22 (161.5 mg, 44%) as a colorless oil. TLC Rf = 0.20 (hexane:ethyl acetate, 5:1). ¹H NMR (300 MHz, CDCl₃) δ 4.35 (m, 2H), 4.19 (td, 2H, J = 6.6, 1.8 Hz), 3.68 (br, 1H), 1.76 (m, 4H), 1.59 (m, 2H), 1.38 (m, 4H), 1.25 (m, 8H), 0.93 (t, 3H, J = 7.5 Hz), 0.89 (t, 3H, J = 7.4 Hz), 0.83 (t, 3H, J = 6.5 Hz); ¹³C NMR (75 MHz, CDCl₃): δ 187.6, 167.9, 132.6, 86.4, 72.8, 70.5, 32.6, 31.7, 31.5, 31.4, 29.3, 24.9, 22.4, 18.7, 18.6, 13.9, 13.6, 13.5.

To a stirred solution of alcohol 22 (138.5 mg, 0.444 mmol) in anhydrous methylene chloride (3.0 mL) was added one drop of concentrated hydrochloric acid (12 M) at ambient temperature. The reaction mixture was stirred at ambient temperature for 20 min and concentrated under reduced pressure. Column chromatography (SiO₂; hexane:ethyl acetate, 50:1 to 15:1, gradient) provided
3-butoxy-4-hexyl-3-cyclobutene-1,2-dione 26 (68.7 mg, 65%) as a colorless oil. TLC Rf = 0.71 (hexane:ethyl acetate, 3:1). $^1$H NMR (300 MHz, CDCl$_3$): δ 4.73 (t, 2H, J = 6.6 Hz), 2.60 (t, 2H, J = 7.5 Hz), 1.80 (quintet, 2H, J = 7.0 Hz), 1.67 (m, 2H), 1.45 (m, 2H), 1.31 (m, 6H), 0.98 (t, 3H, J = 7.4 Hz), 0.88 (t, 3H, J = 6.6 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 198.6, 195.5, 194.3, 184.5, 74.3, 31.8, 31.3, 29.2, 25.7, 25.0, 22.4, 18.5, 14.0, 13.5.

α-Amino-γ-butyrolactone hydrobromide (55.6 mg, 0.299 mmol) was added to a stirred solution of 3-butoxy-4-hexyl-3-cyclobutene-1,2-dione 26 (58.7 mg, 0.247 mmol) in ethanol (2.5 mL). The resulting suspension was then treated with triethylamine (41 µL, 0.30 mmol). After stirring at ambient temperature for 2.5 h, the solvent was removed under reduced pressure. The crude was washed with diethyl ether (3 mL), dissolved in a mixture of water (3 mL) and ethyl acetate (3 mL), and extracted with ethyl acetate (3 mL x 2). The combined organic layer was dried over MgSO$_4$, filtered, and concentrated under reduced pressure. Hexyl-SHL 15 was obtained as a pale yellow solid (34.5 mg, 53%). TLC Rf = 0.20 (hexane:ethyl acetate, 1:2).$^1$H NMR (300 MHz, CD$_3$CN): δ 7.11 (br, 1H), 5.00 (b, dd, J = 20.4, 7.8 Hz, 1H), 4.42 (t, 1H, J = 7.8 Hz), 4.28 (m, 1H), 2.68 (m, 1H), 2.54 (t, 2H, J = 7.5 Hz), 2.34 (m, 1H), 1.62 (m, 2H), 1.32 (m, 6H), 0.91 (t, 3H, J = 7.2 Hz); $^{13}$C NMR (75 MHz, CD$_3$CN): δ 195.2, 193.0, 186.0, 175.9, 175.8, 66.8, 53.9, 32.5, 30.9, 30.3, 27.0, 25.9, 23.6, 14.7; HRMS: Calcd. for M$: 265.2309$, found: 265.1310.

A solution of 1-bromooctane (1.2 mL, 7.0 mmol) in anhydrous THF (4.5 mL) was added to a stirred suspension of freshly ground magnesium turnings (201.3 mg, 8.39 mmol) in anhydrous THF (1.0 mL) dropwise at ambient temperature. The mixture was stirred at ambient temperature
for 5 h and then added to a solution of 3,4-dibutoxy-3-cyclobutene-1,2-dione (0.77 mL, 3.5 mmol) in anhydrous THF (70 mL) via cannula at -78 °C. The reaction mixture was stirred at this temperature for 4 h and then quenched with saturated aqueous NH₄Cl. The aqueous layer was extracted with diethyl ether (25 mL × 3), dried over MgSO₄, filtered, and concentrated under reduced pressure. Column chromatography (SiO₂; hexane:ethyl acetate, 20:1 to 5:1, gradient) provided alcohol 23 (569.6 mg, 48%) as a colorless oil. TLC Rf = 0.23 (hexane:ethyl acetate, 5:1). ¹H NMR (300 MHz, CDCl₃): δ 4.31 (m, 2H), 4.14 (m, 2H), 1.71 (m, 4H), 1.55 (m, 2H), 1.35 (m, 4H), 1.18 (m, 13H), 0.88 (t, 3H, J = 7.5 Hz), 0.85 (t, 3H, J = 7.5 Hz), 0.79 (t, 3H, J = 6.6 Hz); ¹³C NMR (75 MHz, CDCl₃): δ 187.8, 168.0, 132.4, 86.2, 72.7, 70.3, 32.5, 31.6 (2 C), 31.3, 29.5, 29.2, 29.0, 24.9, 22.4, 18.6, 18.5, 13.8, 13.5, 13.4.

To a stirred solution of alcohol 23 (333.8 mg, 0.982 mmol) in anhydrous methylene chloride (6.5 mL) was added two drops of concentrated hydrochloric acid (12 M) at ambient temperature. The reaction mixture was stirred at ambient temperature for 40 min and concentrated under reduced pressure. Column chromatography (SiO₂; hexane:ethyl acetate, 20:1 to 10:1, gradient) provided 3-butoxy-4-octyl-3-cyclobutene-1,2-dione 27 (209.9 mg, 80%) as a colorless oil. TLC Rf = 0.54 (hexane:ethyl acetate, 5:1). ¹H NMR (300 MHz, CDCl₃): δ 4.73 (t, 2H, J = 6.6 Hz), 2.59 (t, 2H, J = 7.5 Hz), 1.79 (m, 2H), 1.68 (m, 2H), 1.45 (m, 2H), 1.26 (m, 10H), 0.97 (t, 3H, J = 7.5 Hz), 0.89 (t, 3H, J = 6.6 Hz); ¹³C NMR (75 MHz, CDCl₃): δ 198.6, 195.6, 194.3, 184.5, 74.3, 31.8, 31.7, 29.5, 29.0 (2 C), 25.7, 25.0, 22.6, 18.5, 14.0, 13.5. HRMS: Cacl. for M⁺: 266.1882, found: 266.1868.
α-Amino-γ-butylrolactone hydrobromide (179.3 mg, 0.965 mmol) was added to a stirred solution of 3-butoxy-4-octyl-3-cyclobutene-1,2-dione (209.9 mg, 0.789 mmol) in ethanol (7.9 mL). The resulting suspension was then treated with triethylamine (0.13 mL, 0.94 mmol). After stirring at ambient temperature for 1.5 h, the solvent was removed under reduced pressure. The crude was washed with diethyl ether (10 mL), dissolved in a mixture of water (10 mL) and ethyl acetate (10 mL), and extracted with ethyl acetate (10 mL × 2). The combined organic layer was dried over MgSO₄, filtered, and concentrated under reduced pressure. Octyl-SHL 19 was obtained as a white solid (150.3 mg, 65%). TLC Rf = 0.41 (ethyl acetate). ¹H NMR (300 MHz, CD₃CN): δ 6.99 (br, 1H), 5.00 (td, 1H, J = 11.7, 9.0 Hz), 4.42 (t, 1H, J = 9.0 Hz), 4.28 (m, 1H), 2.69 (m, 1H), 2.54 (t, 2H, J = 7.5 Hz), 2.32 (m, 1H), 1.63 (m, 2H), 1.28 (m, 10H), 0.88 (t, 3H, J = 6.6 Hz) ¹³C NMR (75 MHz, CD₃CN): δ 195.2, 193.0, 186.0, 176.0, 175.8, 67.0, 66.9, 54.0, 32.9, 31.0, 30.7, 30.3, 27.1, 26.0, 23.7, 14.8. HRMS: Cacl. for M⁺: 293.1627, found: 293.1624.

A solution of 1-bromododecane (1.23 mL, 4.97 mmol) in anhydrous THF (4.0 mL) was added to a stirred suspension of freshly ground magnesium turnings (148.3 mg, 6.18 mmol) in anhydrous THF (0.5 mL) dropwise at ambient temperature. The mixture was stirred at ambient temperature for 5 h and then added to a solution of 3,4-dibutoxy-3-cyclobutene-1,2-dione (0.55 mL, 2.5 mmol) in anhydrous THF (45 mL) via cannula at -78 °C. The reaction mixture was stirred at this temperature for 2 h and then quenched with saturated aqueous NH₄Cl. The aqueous layer was extracted with diethyl ether (10 mL × 3), dried over MgSO₄, filtered, and concentrated under reduced pressure. Column chromatography (SiO₂; hexane:ethyl acetate, 20:1 to 5:1, gradient) provided alcohol 24 (407.5 mg, 45%) as a pale yellow oil. TLC Rf = 0.27 (hexane:ethyl acetate, 5:1). ¹H NMR (300 MHz, CDCl₃): 4.27 (m, 2H), 4.10 (td, 2H, J = 6.6, 1.8 Hz), 3.88 (br, 1H),
To a stirred solution of alcohol 24 (407.5 mg, 1.03 mmol) in anhydrous methylene chloride (7.0 mL) was added three drops of concentrated hydrochloric acid (12 M) at ambient temperature. The reaction mixture was stirred at ambient temperature for 20 min and concentrated under reduced pressure. Column chromatography (SiO$_2$; hexane:ethyl acetate, 20:1 to 10:1, gradient) provided 3-butoxy-4-dodecyl-3-cyclobutene-1,2-dione 28 (69.5 mg, 21%) as a pale yellow oil. TLC R$_f$ = 0.62 (hexane:ethyl acetate, 5:1). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 4.73 (t, 2H, $J = 6.6$ Hz), 2.60 (t, 2H, $J = 7.5$ Hz), 1.80 (m, 2H), 1.67 (m, 2H), 1.46 (m, 2H), 1.25 (m, 18H), 0.98 (t, 3H, $J = 7.5$ Hz), 0.88 (t, 3H, $J = 6.6$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 198.8, 195.5, 194.3, 184.5, 74.3, 31.9, 31.8, 29.6 (3C), 29.5, 29.4, 29.3, 29.1, 25.7, 25.0, 22.7, 18.5, 14.1, 13.5. HRMS: Cacl. for M$^+$: 322.2508, found: 322.2505.

$\alpha$-Amino-$\gamma$-butylrolactone hydrobromide (48.6 mg, 0.262 mmol) was added to a stirred solution of 3-butoxy-4-dodecyl-3-cyclobutene-1,2-dione 28 (69.5 mg, 0.216 mmol) in ethanol (2.2 mL). The resulting suspension was then treated with triethylamine (0.37 mL, 0.27 mmol). After stirring at ambient temperature for 1 h, the solvent was removed under reduced pressure. The crude was washed with diethyl ether (5 mL), dissolved in a mixture of water (2 mL) and ethyl acetate (2 mL), and extracted with ethyl acetate (2 mL $\times$ 2). The combined organic layer was dried over MgSO$_4$, filtered, and concentrated under reduced pressure. Octyl-SHL 20 was obtained as a white solid (22.3 mg, 30%). TLC R$_f$ = 0.77 (ethyl acetate). $^1$H NMR (300 MHz,
CD$_3$CN): $\delta$ 7.06 (br, 1H), 5.00 (td, 1H, $J = 11.7, 9.0$ Hz), 4.42 (t, 1H, $J = 6.0$ Hz), 4.28 (m, 1H), 2.68 (m, 1H), 2.54 (t, 2H, $J = 7.5$ Hz), 2.33 (m, 1H), 1.63 (m, 2H), 1.27 (m, 18H), 0.88 (t, 3H, $J = 6.6$ Hz); $^{13}$C NMR (75 MHz, CD$_3$CN): $\delta$ 195.0, 192.6, 185.6, 175.7, 175.5, 118.4, 66.5, 53.6, 32.7, 30.7, 30.4 (3C), 30.3, 30.1, 30.0, 26.8, 25.7, 23.5, 14.5. HRMS: Caclld. for M$^+$: 349.2253, found: 349.2251.

To a stirred solution of 2-bromothiophene (52 µL, 0.53 mmol) in anhydrous THF (7.0 mL) was added $n$-butyllithium (2.5 M solution in hexane, 0.21 mL, 0.53 mmol) dropwise at -78 °C. The reaction mixture was stirred at -78 °C for 20 min and then added to a stirred solution of 3,4-dibutoxy-3-cyclobutene-1,2-dione (111.8 mg, 0.484 mmol) in anhydrous THF (17 mL) via cannula at -78 °C. The reaction mixture was stirred at -78 °C for 20 min and then poured into a separatory funnel containing 5% NH$_4$Cl (15 mL) and diethyl ether (5 mL). The aqueous layer was extracted with diethyl ether (5mL × 3) and the combined organic layer was washed with brine, dried over MgSO$_4$, filtered, and concentrated under reduced pressure. Column chromatography (SiO$_2$; hexane:ethyl acetate, 20:1 to 5:1, gradient) provided alcohol 25 (31.5 mg, 21%) as a colorless oil. TLC $R_f$ = 0.38 (hexane:ethyl acetate, 5:1). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.30 (dd, 1H, $J = 5.1, 1.2$ Hz), 7.10 (dd, 1H, $J = 3.6, 1.2$ Hz), 7.01 (dd, 1H, $J = 5.1, 3.6$ Hz), 4.39 (m, 2H), 4.30 (t, 2H, $J = 6.6$ Hz), 3.53 (br, 1H), 1.70 (m, 4H), 1.43 (m, 4H), 0.95 (t, 3H, $J = 7.2$ Hz), 0.93 (t, 3H, $J = 7.2$ Hz).

To a stirred solution of alcohol 25 (31.5 mg, 0.102 mmol) in anhydrous methylene chloride (2.0 mL) was added one drop of concentrated hydrochloric acid (12 M) at ambient temperature. The
reaction mixture was stirred at ambient temperature for 10 min and concentrated under reduced pressure. Column chromatography (SiO$_2$; hexane:ethyl acetate, 20:1 to 10:1, gradient) provided 3-butoxy-4-thienyl-3-cyclobutene-1,2-dione 29 (14.1 mg, 59%) as needle-like yellow crystals.

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.91 (dd, 1H, $J = 3.6, 1.2$ Hz), 7.81 (dd, 1H, $J = 5.1, 1.2$ Hz), 7.28 (dd, 1H, $J = 5.1, 3.6$ Hz), 4.92 (t, 2H, $J = 6.6$ Hz), 1.90 (m, 2H), 1.53 (m, 2H), 1.02 (t, 3H, $J = 7.2$ Hz).

α-Amino-γ-butylrolactone hydrobromide (14.8 mg, 0.080 mmol) was added to a stirred solution of 3-butoxy-4-thienyl-3-cyclobutene-1,2-dione 29 (14.1 mg, 0.060 mmol) in ethanol (2.0 mL). The resulting suspension was then treated with triethylamine (11 µL, 0.079 mmol). After stirring at ambient temperature for 2 h, the precipitate was collected by filtration. Diethyl ether (8.0 mL) was added to the filtrate and precipitate thus formed was collected by filtration. The combined solid was washed with pentane (5 mL) and dried under vacuum. Thieryl-SHL 18 was obtained as a pale yellow solid (9.4 mg, 60%). TLC $R_f = 0.35$ (hexane:ethyl acetate, 1:3).$^1$H NMR (300 MHz, DMSO-d$_6$): $\delta$ 9.35 (d, 1H, $J = 4.5$ Hz), 8.04 (d, 1H, $J = 4.8$ Hz), 7.87 (d, 1H, $J = 3.6$ Hz), 7.39 (t, 1H, $J = 4.2$ Hz), 5.27 (m, 1H), 4.48 (t, 1H, $J = 8.9$ Hz), 4.32 (m, 1H), 2.29 (m, 1H), 2.41 (m, 1H); $^{13}$C NMR (75 MHz, DMSO-d$_6$): $\delta$ 190.3, 186.6, 176.8, 174.6, 157.7, 132.2, 128.8, 128.8, 128.7, 65.6, 52.9, 29.3. HRMS: Cacld. for M$^+$: 263.0247, found: 263.0240.

To a stirred solution of 5-bromo-m-xylene (0.45 mL, 3.2 mmol) in anhydrous THF (15 mL) was added n-butyllithium (2.5 M solution in hexane, 1.2 mL, 3.0 mmol) dropwise at -78 ºC. The
reaction mixture was stirred at -78 °C for 20 min and then added to a stirred solution of 3,4-
dibutoxy-3-cyclobutene-1,2-dione (0.24 mL, 1.1 mmol) in anhydrous THF (35 mL) via cannula
at -78 °C. The reaction mixture was stirred at -78 °C for 45 min and then poured into a
separatory funnel containing 5% NH₄Cl (15 mL) and diethyl ether (15 mL). The aqueous layer
was extracted with diethyl ether (15 mL × 3) and the combined organic layer was washed with
brine, dried over MgSO₄, filtered, and concentrated under reduced pressure. Column
chromatography (SiO₂; hexane:ethyl acetate, 10:1 to 3:1, gradient) provided a mixture of mono-
and di-substituted product. The mixture was dissolved in DCM (5 mL) and treated with 2 drops
of concentration hydrochloric acid (12 M). The reaction mixture was concentrated under reduced
pressure after stirred at ambient temperature for 45 min. Column chromatography (SiO₂;
hexane:ethyl acetate, 5:1) provided 3-butoxy-4-(3,5-dimethyl-phenyl)-3-cyclobutene-1,2-dione
30 (41.5 mg, 27% over 2 steps) as a light yellow oil. TLC Rf = 0.52 (hexane:ethyl acetate, 5:1).
¹H NMR (300 MHz, CDCl₃): δ 7.67 (s, 2H), 7.19 (s, 1H), 4.93 (t, 2H, J = 6.6 Hz), 2.39 (s, 6H),
1.92 (quintet, 2H, J = 7.2 Hz), 1.53 (m, 2H), 1.03 (t, 3H, J = 7.5 Hz); ¹³C NMR (75 MHz,
CDCl₃): δ 194.5, 193.1, 192.6, 174.2, 138.8, 134.6, 127.6, 125.3, 75.1, 32.0, 21.2, 18.6, 13.6.
HRMS: Caclld. for M⁺: 258.1256, found: 258.1252.

α-Amino-γ-butyrolactone hydrobromide (35.6 mg, 0.192 mmol) was added to a stirred solution
of 3-butoxy-4-(3,5-dimethyl-phenyl)-3-cyclobutene-1,2-dione 30 (41.5 mg, 0.161 mmol) in
ethanol (1.6 mL). The resulting suspension was then treated with triethylamine (27 µL, 0.20
mmol). After stirring at ambient temperature for 1 h, the precipitate was collected by filtration.
Diethyl ether (10 mL) was added to the filtrate and precipitate thus formed was collected by
filtration. The combined solid was washed with pentane (5 mL) and dried under vacuum. Xylyl-SHL 21 was obtained as a pale yellow solid (29.8 mg, 65%). TLC Rf = 0.75 (ethyl acetate).\textsuperscript{1}H NMR (300 MHz, DMSO-d6): \(\delta\) 9.26 (br, 1H), 7.62 (s, 1H), 7.18 (s, 1H), 5.32 (m, 1H), 4.48 (t, 1H, \(J = 8.7\) Hz), 4.37 (m, 1H), 2.70 (m, 1H), 2.43 (m, 1H), 2.34 (s, 6H); \textsuperscript{13}C NMR (75 MHz, DMSO-d6): \(\delta\) 192.6, 188.9, 179.1, 174.7, 163.0, 138.4, 132.6, 128.7, 123.9, 65.4, 52.9, 29.3, 20.8, for M\(^{+}\): 285.1001, found: 285.0955.

To a stirred solution of 3,4-dibutoxy-3-cyclobutene-1,2-dione (0.22 mL, 1.0 mmol) and 1-phenyl-1-(trimethylsilyloxy)ethylene (0.42 mL, 2.0 mmol) in anhydrous DCM (4.0 mL) was added titanium tetrachloride (0.11 mL, 1.0 mmol) at -15 °C. After stirring at this temperature for 20 min, another portion of 1-phenyl-1-(trimethylsilyloxy)ethylene (0.21 mL, 1.0 mmol) and titanium tetrachloride (0.05 mL, 0.5 mmol) was added. The reaction mixture was stirred for another 20 min and quenched by pouring into cold water. The aqueous layer was extracted with DCM (5 mL x 3), dried over MgSO\(_4\), filtered, and concentrated under reduced pressure.

\(\alpha\)-Amino-\(\gamma\)-butylrolactone hydrobromide (123.2 mg, 0.677 mmol) was added to a stirred solution of crude (124.8 mg, 0.552 mmol) in ethanol (5.5 mL). The resulting suspension was then treated with triethylamine (77 \(\mu\)L, 0.56 mmol). After stirring at ambient temperature for 1 h, the precipitate was collected by filtration. Diethyl ether (10 mL) was added to the filtrate and precipitate thus formed was collected by filtration. The combined solid was washed with pentane (5 mL) and dried under vacuum. Phenylethyl-SHL was obtained as a pale yellow solid (55 mg,
33%). TLC $R_f = 0.18$ (hexane:ethyl acetate, 1:1). $^1$H NMR or $^{13}$C NMR either indicate a mixture or rotamers and/or decomposed products during the time of NMR experiment. This batch of compound showed no activity in preliminary biofilm or reporter gene assay.
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**Publications**


**Presentations**