Pyridyl Bis-Urea Macrocycles as Supramolecular Synthons And the Design and Synthesis of Small Molecule Pharmaceuticals Targeting LY6K

Devan D. Buchanan

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PYRIDYL BIS-UREA MACROCYCLES AS SUPRAMOLECULAR SYNTHONS
AND THE DESIGN AND SYNTHESIS OF SMALL MOLECULE
PHARMACEUTICALS TARGETING LY6K

By

Devan D. Buchanan

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Accepted by:

Linda Shimizu, Director of Thesis

Qian Wang, Reader

Tracy L. Weldon, Vice Provost and Dean of The Graduate School
DEDICATION

To my beloved son, Braxton. Respect is earned by means of honesty, hard work and dedication. When you find your passion, you will know. And when you know, pursue it boundlessly and unapologetically. If you truly love what you do, you will always be successful. Since you were born, you have been--and always will be--my source of motivation. I love you, Brax.
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I would like to thank my beautiful wife, Kendall, for all of her support and unconditional love: You make me a better father, husband, and man. You have never doubted me nor questioned my aspirations. You have taught me so much about myself since I have known you, and you continue to remind me of who I am—who we are. You are my best friend. Without your support and love over these years, this would not have been possible.

To my mother: Without your upbringing, I wouldn’t be who I am today. The most valuable lessons are learned where the worst mistakes are made. You were there to teach me. You showed me the meaning of work ethic and ambition. No one comprehends the amount of respect I have for you until I tell them our story. Thank you for helping me make mine.

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ABSTRACT

Assembled pyridyl bis-urea macrocycles have been utilized as 1D supramolecular synthons to construct hierarchical assemblies. These macrocycles have both urea and pyridyl functional groups and can form non-covalent interactions with hydrogen or halogen bond donors through symmetric, ditopic acceptor motifs. Bis-urea macrocycles offer interesting capabilities as synthons to organize donors into well-defined crystal structures. In part, this is due to the urea motifs’ propensity to assemble through hydrogen bonding. New, asymmetric pyridyl macrocycles were synthesized, crystallized, and experimentally probed to determine how symmetry affects their assembly and utility as supramolecular synthons.

The Uphadhyay group identified small molecules, including NSC11150, from the National Cancer Institute’s (NCI) library to bind lymphocyte antigen 6K (LY6K) protein by the. The LY6K protein has been implicated in the progression of several types of cancer. Docking simulations were performed on NSC11150, as well as several derivatives, to determine the binding mechanism to LY6K. NSC11150 was synthesized on a large scale and purified. Additionally, several derivatives of NSC11150 were synthesized in order to attach functional tags, such as D-biotin and BODIPY, for streptavidin binding assays or fluorescence microscopy, respectively.
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CHAPTER 1

PYRIDYL BIS-UREA MACROCYCLES AS SUPRAMOLECULAR SYNTON\textsuperscript{1, 2}

\textsuperscript{1} Devan D Buchanan, Bozumeh Som, Mark D. Smith, Linda S. Shimizu. \textit{Not submitted.}

1.0 ABSTRACT

Assembled pyridyl bis-urea macrocycles have been utilized as 1D supramolecular synthons to construct hierarchical assemblies. These macrocycles have both urea and pyridyl groups and can form interactions with hydrogen or halogen bond donors through symmetric, ditopic acceptors. Pyridyl bis-urea macrocycles (1-4) were synthesized, crystallized, and characterized as potential supramolecular synthons. Two of the macrocycles in the series (3 and 4) are asymmetric, containing both a pyridine and xylene space group, connected to two urea motifs. These asymmetric macrocycles were synthesized via dynamic covalent chemistry (DCC), developed by Cheng et al, which exploits a condensation reaction between a hindered pyridyl diamine and m-xylene diisocyanate. The DCC method utilizes a thermodynamically driven condensation reaction resulting in efficient macrocyclization. Additionally, macrocycles 1 and 2 were co-crystallized with p-diido-tetrafluorobenzene (p-DITFB), obtained through antisolvent vapor diffusion of water into a DMSO solution and assembled as ditopic 1D pillars. The pillars were indirectly connected by p-DITFB via halogen bonding with a 1:1 host-to-guest ratio for each co-crystal. The co-crystals of 1 and 2 assemble with very different hydrogen bonding networks and connected the guest through either C-Iaryl···Ourea or C-Iaryl···Npyridyl interactions, respectively. The bond lengths and conformational changes of each are quite different and were analyzed individually. All structures were characterized by solid-state frontier transform infrared spectroscopy (FT-IR), powder x-ray diffraction (PXRD) and single-crystal x-ray diffraction (SCXRD). Density functional theory (DFT) was utilized to compare electrostatic potential mapping and optimized host energies free of the crystal field, while Hirshfeld surface analysis and fingerprint plots were generated to analyze the
intermolecular interactions and crystal packing of each macrocycle. The synthetic, structural, and geometric differences of each macrocycle and co-crystals will be discussed and compared in detail. Theoretical computations and spectroscopic changes between each analogue provide evidence for the utility of each host as a supramolecular synthon and probe structural and electronic differences between co-formers. Future work with these macrocycles focuses on their utility as synthons to pre-organize reactive guests through hydrogen or halogen bonding.

1.1 INTRODUCTION

The principle focus in the field of crystal design and engineering is the control of assembly through directional molecular interactions.\textsuperscript{1-3} Predicting supramolecular structures is often challenging and relies on the physical properties and potential non-covalent interactions of individual components that interact to build multicomponent assemblies. The term synthon is often used to define a unique molecular connector which directs the organization of molecules in a crystal structure into a desired supramolecular motif.\textsuperscript{3-4} Having a robust synthon with a predictable manner of assembly, while retaining the ability to direct small molecules into specific motifs, is ideal for supramolecular material engineering. Excellent examples of predictable assembly are \textit{bis}-urea macrocycles, which have demonstrated high-fidelity, columnar self-assembly (Figure 1.1a).\textsuperscript{5-9} These building blocks harness urea’s strong donor and acceptor capabilities, represented by their $\alpha$ and $\beta$ values (3.0 and 8.3, respectively).\textsuperscript{10} The relatively planar C-shaped space groups (Figure 1.1b) attached to each urea motif define the size and functionality while also serving to anchor the ureas perpendicular to the plane of the
macrocycle. This conformation favors columnar self-assembly over other geometries, such as ribbons, tapes, columns, fibers and gels.11-13

Macrocycle 1 displays both hydrogen and halogen bond acceptor abilities through its Lewis basic oxygen lone pairs (C=O⋯X or C=O⋯H) residing along the exterior of its 1D pillars (Figure 1.2b). The characteristic acceptor ability of 1 is owed to the assembled structure having two different hydrogen bonds (N(H)⋯O and N(H)⋯Npyridyl); One urea N-H hydrogen bonds with a carbonyl oxygen (2.90 Å), which is typical of most bis-urea macrocycles, while the other urea N-H forms a hydrogen bond with the pyridyl nitrogen

Figure 1.1. (a) Schematic depiction of bis-urea macrocycles and their directed self-assembly through hydrogen bonding. (b) Examples of C-shaped spacers used to construct bis-urea macrocycles. Adapted with permission from Shimizu Linda S; Hughes Andrew D; Smith Mark D; Davis Matthew J; Zhang B Paul; Zur Loye Hans-Conrad; Shimizu Ken D. Self-assembled nanotubes that reversibly bind acetic acid guests. J. Am. Chem. Soc. 2003, 125, 14972-14973. Copyright 2003. Ref. 45.
(3.08 Å) (Figure 1.2a) which is atypical in bis-urea macrocycles. As a result, two Lewis base oxygen lone pairs align the outside of the columns per macrocycle. This gives rise to electrostatic interactions upon exposure to guests with electrophilic motifs, forming solid state supramolecular complexes. Indeed, 1 has been exploited as a sorbent for alcohols and iodine in solid-to-solid transformations, and as a synthon to organize hydrogen or halogen bond donating guests in co-crystallizations.\textsuperscript{14-16} In this chapter, we will examine the series of symmetric and asymmetric pyridyl macrocycles (1-4, Figure 1.4b) as potential supramolecular synthons in the solid state. Factors contributing to their robustness and acceptor strengths will be probed, both experimentally and computationally. Specifically, the crystal structures of each macrocycle will be compared by differences in geometry, by hydrogen bond strength, and by electrostatic surface potentials. One question of interest is whether the hydrogen bonded assemblies of each macrocycle in the solid state can be used to further organize halogen or hydrogen bond donors in co-crystals.

To answer this question, the crystal structures of each macrocycle were confirmed and evaluated by single crystal X-ray diffraction (SCXRD), solid state frontier transform infrared spectroscopy (FT-IR) and powder X-ray diffraction (PXRD). In each case, the macrocycles self-assembled into strong 1D pillars. Additionally, Hirshfeld surface analysis was computed to compare the intermolecular interactions within each crystal structure, while density functional theory (DFT) computations were performed to compare geometric energies and electrostatic potentials free of the crystal field. Finally, the assemblies of 1-4
were examined as co-crystal formers for ditopic hydrogen and halogen bond donors, including diiodotetrafluorobenzenes, diols and diacids. Urea can directionally assemble through hydrogen bonding in several ways, forming oligomeric and polymeric structures. The most common assemblies include tapes, ribbons and chains. The latter is observed in bis-urea macrocycles, which self-assemble through three-centered bifurcated hydrogen bonds. This is possible due to the hydrogen bonding potential of urea, which is able to form six hydrogen bonds with two acceptor oxygen lone pairs and four electrophilic hydrogens. The hybridization of urea is similar to amino acids, such that the nitrogens are sp2 hybridized and are able to form delocalized bonding structures to the

**Figure 1.2.** Assembly of Macrocycle 1. (a) ChemDraw structure of 1. (b) Self-assembled crystal structure of 1 into 1D pillars, highlighting hydrogen bonds and their respective distances: One between pyridine-N and a urea-H and a second between the carbonyl-O and urea-H. (c) Schematic representation of 1’s columnar assemblies leaving two unsatisfied oxygen lone pairs. Reprinted with permissions from American Chemical Society (ACS), ref. 9, copywrite (2014) Accounts of Chemical Research.
adjacent carbon atom in the event of an electrostatic potential change.\textsuperscript{17} Therefore, kinetics to contribute to urea hydrogen bonding (Figure 1.3), where $K_1$ is the formation of the dimer

\[
\begin{align*}
\text{H}_2\text{N} & \xrightarrow{K_1} \text{H}_2\text{N} - \text{O} - \text{H}_2\text{N} \\
\text{H}_2\text{N} & \xrightarrow{K_2} \text{H}_2\text{N} - \text{O} - \text{H}_2\text{N} - \text{O}
\end{align*}
\]

\textbf{Figure 1.3.} Assembly of urea into a trimer with bifurcated hydrogen bonding through the chain conformation. The value of $K_1$ is 400 M$^{-1}$, and the value of $K_2$ is 900 M$^{-1}$. The difference is due to the initial electrostatic attraction between two molecules which causes polarization and results in a stronger trimer hydrogen bonding system. (Ref. 10)

which in turn polarizes each molecule making $K_2$ (trimer formation) over two-fold larger.\textsuperscript{10} Thus, assembly is initiated by the electrostatic attraction between the oxygen and hydrogens, depleting the electron density around the carbonyl and compensated by donation/delocalization of adjacent nitrogens.

While the same concepts apply to \textit{bis}-urea macrocycles, there is usually less variability in predicting assembly. This is, in part, due to \textit{bis}-urea macrocycles having only two hydrogen bond donors per motif. However, the dominating factor is largely due to the urea adopting a relatively orthogonal geometry to the organic backbone. This results in robust, predictable assemblies—typically through a bifurcated urea-urea motif forming nanotubular system. Depending on whether the space group is large or small, the assemblies form either porous materials\textsuperscript{5,18,19} or 1D pillars,\textsuperscript{6,7,14-16} respectively. Although structurally similar, the assemblies of symmetric hosts 1 and 2 (Figure 1.4b) are significantly different in the solid state. Macrocycle 1 assembled through a hydrogen bonded motif consisting of two different hydrogen bonds: $\text{N(H)}\text{urea} \cdots \text{O}_{\text{urea}}$ and $\text{N(H)}\text{urea} \cdots \text{N}_{\text{pyridyl}}$ (2.90 Å and 3.08 Å, respectively), while 2 displayed typical three-centered urea-urea hydrogen bond motif with two different hydrogen bond distances.
(1N(H)$_{\text{urea}}$···O$_{\text{urea}}$, 2.84 Å and 2N(H)$_{\text{urea}}$···O$_{\text{urea}}$, 2.90 Å), similar to the m-xylene macrocycle 5.$^{19}$ Herein, we probe how symmetry influences both assembly and supramolecular organization. Macrocycles 3 and 4 were synthesized as asymmetric analogues to 1 and 2, respectively (Figure 1.4b). Analogous to 1, macrocycle 3 has one pyridyl nitrogen in close proximity to the urea group. Our first question was whether this asymmetric bis-urea macrocycle, 3, also prefers to assemble through two different hydrogen bonds as macrocycle 1 and what factors associated with assembly influence their differences. Although 2 and 4 are predicted to assemble in a similar fashion, their solid-state geometries may differ, which would subsequently influence their acceptor capabilities from differences in electron distribution and electrostatic potentials. Therefore, our second question was whether these analogs would present different assembly patterns and/or guest absorption capabilities. Using Hirshfeld analysis and density functional theory (DFT) computations, the strength of the acceptors and donors can be ranked for the best donor-
The same computational parameters were performed on each macrocycle to probe the differences in electron distributions and crystal field contacts in the context of symmetry. Finally, we will address how the symmetry of these synthons could influence their co-crystal formation in relation to their assembled structures.

Historically, bis-urea macrocycles have been synthesized under kinetically driven conditions by reacting a bis-bromomethyl space group with triazinanone in base (Figure 1.4b, route B) under high dilution conditions.\textsuperscript{5-7,14-16,18,19} This method leads to reported yields ranging from 3-30%, depending on the organic space group. The low-yielding reaction is mainly due to the possibility of forming linear oligomers and/or ethers (in the presence of water) as well as macrocycles of higher order: trimers, tetramers, etc. Dynamic covalent chemistry (DCC) was recently explored by the Cheng group\textsuperscript{20} as a more efficient
strategy for the synthesis of bis-urea macrocycles. The DCC method is driven mainly by thermodynamics, resulting in a facile condensation reaction between an electrophilic diisocyanate and a bulky secondary amine, forming a hindered urea bond (HUB) (Figure 1.4a, Route A). Deprotection of the bulky group (i.e., tert-butyl) after cyclization is readily achieved by addition of acid at room temperature and is complete within minutes. The efficiency of the DCC method is made possible by the bulky tert-butyl substituent forcing reaction intermediates into a locally minimum energy state, favoring cyclization.

\[ \text{N, N'}-\text{di-substituted ureas, with the chemical structure } R^1\text{N}^1\text{HC(O)N}^2\text{HR}^2, \text{ are typically favored as trans-} R^1/R^2 \text{ (Figure 1.5a). This is attributed to coplanar resonance geometry, which makes this conformation more thermodynamically favored than the cis-} R^1/R^2.\]^{11,21} \text{ Indeed, unhindered polyurea backbones preferentially form a zigzag conformation.}^{21} \text{ However, when the hydrogen of } 2\text{N-}2\text{H is replaced by a group more sterically bulky than } R^1, \text{ the cis- } R^1/R^2 \text{ conformation tends to predominate in effort to}
overcome steric hinderance (Figure 1.5b). In this way, the bulky substituent forces a bond rotation in the urea motif to a more thermodynamically favorable conformation. The cis-$R^1/R^2$ conformation results in a loss of linear geometry and therefore a zigzag polyurea conformation is now energetically unfavorable, lowering the likelihood of polymerization. More importantly, the energetically favored cis-$R^1/R^2$ conformation results in closer proximities of reactive substituents. This favors ring closure over polymerization by increasing the effective molarity of the reactive substituents (Figure 1.5c).
The DCC method can also be used to control the formation of macrocycle products as either dimers or tetramers in two different ways.\textsuperscript{20} First, the \textit{tert}-butyl diamine and diisocyanate should be matched or relatively similar in size. This is true for all macrocyclizations, as one large and one small space group may introduce strain on a dimer system, favoring larger macrocycles or open oligomers. Second, it was observed that diisocyanates lacking a methylene group between the isocyanate and aromatic moiety favored the formation of a tetramer, although only a few examples were synthesized. This theory was tested using a xylene \textit{tert}-butyl amine and ethylene-1,2-diisocyanate. The diisocyanate space group was the smaller of the two, which, in theory, would force the equilibrium to favor the less strained tetramer product. The reaction conditions are outlined in Section 1.5.1 along with the \textsuperscript{1}H NMR.

\textbf{Figure 1.6.} Views from the crystal structure obtained from SCXRD of 1, which assembles into 1D pillars with two different hydrogen bonds. (a) Tetramer assembly highlighting \textit{b}/Å distance of 4.695 Å. (b) Trimer assembly highlighting N\textsubscript{pyr}···(H)N bond length of 3.08 Å. (c) Pillared assembly showing bond length of 2.90 Å for O···(H)N.
Macrocyle 1 was synthesized by a previously reported two-step synthesis,\textsuperscript{5,12-14} reacting triazinanone with commercially available 2,6-\textit{bis}(bromomethyl)pyridine under basic and diluted conditions to give protected macrocycle 1 in 25\% yield.\textsuperscript{18} Deprotection was carried out by refluxing the protected macrocycle in a 1:1 mixture of methanol and 20\% diethanolamine/water solution (pH \textasciitilde 2) for 1 day. Macrocycle 1 was crystallized by dissolution into hot (70 °C) DMSO (\textasciitilde 6 mg/mL) followed by vapor diffusion of methanol as the antisolvent. After 3 days, colorless crystals suitable for x-ray diffraction were obtained in the monoclinic system in the P2\textsubscript{1}/c space group with one-half of 1 on an inversion center making up the asymmetric unit (Figures 1.6 and 1.7). In a dimer circuit, two different hydrogen bonds (\textlt; sum of vdW radii) are present: One hydrogen bond is conventionally formed between one lone pair of the urea oxygen and a urea hydrogen (N(H)\textendash O = 2.90 Å) while the other is extended between the pyridine nitrogen lone pair and a second urea hydrogen (N\textendash (H)N = 3.08 Å) (Figure 1.6c and b, respectively). The urea groups arrange approximately perpendicular to the plane of the pyridine ring systems which are tilted 12.93° (\angle N1-C5-C1) and nearly parallel to the \(c\) axis (Figure 1.7b). The geometries of the urea/methylene moieties were analyzed by measuring the dihedral angles in relation to the plane of the pyridine rings were found to be above and below perpendicular: \(\pm 103.26^\circ\) and \(\pm 77.21^\circ\) (Figure 1.7c, measured reciprocally from \text{Caryl}-\text{CH}_2-\text{NH-C}	ext{carbonyl}). As a result, 1 exhibited parallel-offset stacking of the pyridine rings by 55.70° (\(\angle C4-C6-C6^\circ\) measured within one dimer assembly, Figure 1.7a).
Macrocycle 2 was synthesized through a previously reported literature procedure (Figure 1.4a, Route B).\textsuperscript{22,46} Protected macrocycle 2 was purified by column chromatography, crystallized, and subsequently deprotected by refluxing in a 1:1 mixture of methanol and 20\% diethanolamine/water solution (pH ~2) for 1 day. The precipitate was collected and recrystallized from slow cooling (1 °C/h) from 130 °C to room temperature in a DMSO/acetonitrile solution (1:1.5 \(v/v\)). Colorless crystals suitable for x-ray diffraction were obtained in the monoclinic system with the \(P2_1/n\) space group, similar to the reported structure.\textsuperscript{46} Macrocycle 2 assembled through three-centered, bifurcated hydrogen bonds with lengths of O…N(H) 2.84 and 2.90 Å (Figure 1.8a). The urea groups are closely perpendicular to the plane of the pyridine rings with torsion angles of ±79.89° and ±91.59° (measured from C(H\(_2\))-N(H)-C(O)-N(H)), resulting in parallel-offset stacking of the

**Figure 1.7.** Assembly of 1 exhibiting parallel-offset stacking, backbone tilting and measured dihedral angles. (a) Dimer assembly showing offset stacking parallel to the pyridine plane with a stacking angle of 53.07° with a \(b/\AA\) of 4.695 Å. (b) Pyridine backbone tilted at 14.09°. (c) Torsion angles measured reciprocally from C\(_{aryl}\) to C=O.
pyridine rings at 66.36° (\(\angle \text{C6-C4-C4}'\) dimer assembly) shown in Figure 1.8b. In this structure, 2 adopts a planar geometry compared to 1 with \(\angle \text{N1-C5-C1}\) of 13.46°.

Synthesis of macrocycle 3 was achieved through a three-step synthesis using the DCC method for cyclization. The first step involved the reaction of commercially available 2,6-\textit{bis}(dibromomethyl)pyridine and \textit{tert}-butylamine, resulting in the respective pyridyl \textit{tert}-butyl diamine (85%). The pyridyl diamine was subsequently treated with \textit{m}-xylene diisocyanate in a precise 1:1 ratio with a final concentration of 32 mM. The reaction was

**Figure 1.8.** Assembly of 2 adopting a parallel-offset stacking motif, backbone tilting and unique urea torsion angles. (a) Dimer assembly showing offset stacking parallel to the pyridine plane with a stacking angle of 53.07° with a \(b/\text{Å}\) of 4.595 Å. Hydrogen bonding indicated by black dashes. (b) Parallel-offset stacking measured at 66.36°, angle indicated in green. (c) Pyridine backbone tilted at 13.46° (green). (d) Torsion angles (green) measured reciprocally from C(H\(_2\)) to N(H).
heated at 55 °C for 1 day to obtain the crude tert-butyl-protected macrocycle 3 (89%). The intermediate was readily purified by column chromatography. The tert-butyl groups were removed by addition of trifluoroacetic acid and 10 minutes of sonication. The desired bis-urea 3 was collected as a precipitate after neutralization with 1M NaOH (99%). The solubility of 3 was screened in three polar solvents (acetonitrile, DMF, and DMSO) under heat (50-120 °C) and ambient conditions; however, 3 was only soluble when gently heated in DMSO (6.4 mg/mL). After cooling to room temperature, the macrocycle stayed in solution and was crystallized by antisolvent vapor diffusion of H₂O. After six days, colorless crystals suitable for x-ray diffraction were obtained in the monoclinic system with

![Figure 1.9. Geometric analysis of 3 assembly. (a) Parallel-offset stacking of a dimer circuit at 56.30° measured from \( \angle \) C6-C4-C4\(^\alpha \). Hydrogen bonds were omitted for clarity. (b) Off-plane geometry of a pyridine/xylene backbone with symmetric tilting at 12.93°, measured from \( \angle \) N1-C5-C1. (c) Torsion angles measured reciprocally from methylene to the urea motif. Viewed down the b axis.](image)
the P2₁/c space group as a centrosymmetric arrangement. The asymmetric unit consisted of one unique six-membered ring per unit, implying disorder and scrambling over the C/N crystallographic site. Refinements of the site as C(8)H(8)/N3 group showed occupancy values of 0.5, giving good support for the disordered C/N model. The pyridine/xylene backbone of macrocycle 3 adopts a near-planar structure, with a tilt angle of 12.93° (N1-C5-C1), and assembles through bifurcated, three-centered hydrogen bonding with bond lengths of O···N(H) 2.89 and 2.98 Å (Figure 1.9a and b). Surprisingly, 3 does not display the pyridine-urea hydrogen bond assembly observed in 1, despite having a similarly placed pyridine nitrogen. The urea groups orient approximately perpendicular to the mixed m-xylene/pyridine backbone with torsion angles of ± 85.98° and ± 96.66°, measured from Caryl-CH₂-N(H)-C(O) (Figure 1.9c). Assembled 3 also exhibits parallel-offset stacking of the pyridine/xylene moieties at 56.03° (measured within a dimer circuit, C6-C4-C4°).

Macrocycle 4 was synthesized through a six-step synthesis using the DCC method for macrocyclization. The first three steps yielded 3,5-bis(bromomethyl)pyridine using a
previously reported literature procedure,\textsuperscript{15} identical to the macrocycle 2 intermediate (dibromo-pyridine). The intermediate was aminated by reaction of 3,5-\textit{bis-}(bromomethyl)pyridine with \textit{tert}-butylamine (84\%), and subsequently cyclized by addition of \textit{m}-xylene diisocyanate diluted to 32 mM with dichloromethane (91\%). Deprotection of 4 was achieved by addition of trifluoroacetic acid and 10 minutes of alternating sonication and manual stirring to remove the \textit{tert}-butyl groups (99\%). The solution was neutralized with NaOH (1 M) and the precipitate was collected as 4. The product was collected, and its solubility was screened in three polar solvents including DMF, acetonitrile and DMSO under a range of temperatures (r.t.-120 °C). However, 4 was only partially soluble in DMSO at room temperature, while gentle heating and sonication provided complete dissolution (5.5 mg/mL). The crystallization conditions were optimized, and 4 was crystallized by vapor diffusion of H\textsubscript{2}O into DMSO-4. After 5-6 days, colorless needle-like crystals were collected, which were suitable for x-ray diffraction. Crystalline 4 was obtained in the monoclinic system with the P2\textsubscript{1}/n space group centrosymmetrically. The asymmetric unit consisted of once unique six-membered ring per unit. However, refinements of the site as a C(5)H(5) or N3 showed site occupancy values of 0.50(2), and is consistent with C/N disordered scrambling over the same crystallographic site. Macrocycle 4 adopts a near-planar geometry with respect to the pyridine/xylene moieties at an angle of 13.18° (\angle N1-C5-C1, Figure 1.10a). Individual macrocycles assemble into columns (Figure 1.10a) through bifurcated urea-urea hydrogen bonding typically seen
Table 1.1. Comparison of SCXRD geometric differences and melting points between macrocycles.

<table>
<thead>
<tr>
<th>Geometric Property</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Offset Stacking angle</td>
<td>53.70</td>
<td>66.36</td>
<td>56.03</td>
<td>62.98</td>
</tr>
<tr>
<td>(°)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spacer angle (°)</td>
<td>14.09</td>
<td>13.46</td>
<td>12.93</td>
<td>13.18</td>
</tr>
<tr>
<td>Torsion angles&lt;sup&gt;b&lt;/sup&gt;</td>
<td>±77.21, ±79.89, ±85.98, ±96.86, ±103.26, ±91.59, ±96.66, ±80.67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b/Å</td>
<td>4.69(5)</td>
<td>4.59(5)</td>
<td>4.56(5)</td>
<td>4.59(8)</td>
</tr>
<tr>
<td>Melting point (°C)</td>
<td>291-293</td>
<td>283-286</td>
<td>287-289</td>
<td>273-274</td>
</tr>
</tbody>
</table>

in symmetric bis-urea macrocycles (O···N(H), 2.82 Å and 1.96 Å).<sup>14-16</sup> The two urea motifs orient approximately perpendicular to the pyridine/xylene backbone (Figure 1.10). Displaced-parallel stacking of 62.98° is observed in a dimer circuit (∠ C6-C4-C4<sup>α</sup>) between the pyridine/xylene space groups. The dihedral angles measured along reciprocal methylene to urea motifs (±96.86° and ±80.67°, Figure 1.10c). The stacking angle is smaller than in macrocycle 2, indicating a slightly more pronounced offset geometry, as their b/Å values are nearly identical (2 = 4.59 Å, 4 = 4.59 Å Figure 1.10a, table 1.1.).

Investigating the local chemical environment of symmetric versus asymmetric analogues within the crystal field was an important step in determining the preorganization abilities of potential of each supramolecular synthon. Mentioned previously, 1 formed an
assembly with two distinct hydrogen bonds and includes the assistance of the pyridine nitrogen as a hydrogen bond acceptor. The self-assembly of macrocycle 2, on the other hand, was guided by conventional bifurcated urea hydrogen bonding. We sought to investigate how molecular symmetry contributes to self-assembly by comparing the local crystal environments of symmetric versus asymmetric co-formers using Hirshfeld surface analysis.

Important solid-state interactions are typically characterized by identifying distances between two atoms and comparing this distance to the van der waals radii of the two atoms. However, the complex interplay between close crystal packing and intermolecular interactions cannot be determined without consideration of the crystal environment, molecular shape and electron densities. Hirshfeld surface analysis was developed with this in mind by generating a method encompassing shape-defining criteria within the crystal lattice using electron distributions and the weight function \( w(r) \):

\[
    w(r) = \frac{\rho_{\text{promolecule}}(r)}{\rho_{\text{procrystal}}(r)} = \frac{\sum_{A \in \text{molecule}} \rho_A(r)}{\sum_{A \in \text{crystal}} \rho_A(r)} \approx \frac{\rho_{\text{molecule}}(r)}{\rho_{\text{crystal}}(r)}
\]  

(1)

where \( \rho_A(r) \) is the averaged electron density centered on nucleus A. The ‘promolecule’ is spherically summed atoms belonging to the molecule, while the ‘procrystal’ is the sum of atoms belonging to the crystal. Hirshfeld analysis is an isosurface where \( w(r) = 0.5 \). The efficiency of this approximation is observed by comparison to conventional models, where surface volumes are much larger and fill the crystal space greater than 95% (compared to 60-80% in conventional models). This is because the ratio of electron densities in the ‘promolecule’ and ‘procrystal’ are reasonably accurate in reflecting the actual molecule and actual crystal, respectively. The \( d_i \) (interior) and \( d_e \) (exterior) characterizes the
distances relative to the Hirshfeld surface from the nuclei, with respect to their given vDW radii.\textsuperscript{25} The Hirshfeld surfaces were generated using CrystalExplorer 21.3 using B3LYP basis set and G-31G (d, p) level of theory.\textsuperscript{26,27} The Hirshfeld surfaces are mapped over $d_{\text{norm}}$ and depicts areas of white (= sum vDW), blue (> sum vDW) and red (< sum vDW). While red areas indicate the strongest, shortest interactions. Additionally, 2D fingerprint plots were generated under the same basis set and level of theory.\textsuperscript{26-28} The fingerprint plots are used as a quantification tool to calculate the distance of an atom from the interior or exterior of the crystal surface ($d_i$ and $d_e$, respectively).\textsuperscript{24}
Although 1 and 3 both crystallized in the same crystal system and space group, 3 displayed a typical bifurcated, three-centered hydrogen bond assembly, according to the SCXRD data. This was further investigated by Hirshfeld surface analysis and subsequent fingerprint plotting by comparison of their respective Hirshfeld profiles, shown in Figures 1.11 and 1.12, respectfully (summarized in Table 1.1). In Figure 1.11a, there are three contacts in the total Hirshfeld surface, indicated as red circles, for macrocycle 1. The O···H/H···O contacts within the fingerprint plot (Figure 1.11b) displays a spike at \( de = di \) \( \sim 1.3 \) Å ending at \( \sim 2.3 \) Å and is visualized in the O···H/H···O surface in red. Considering

**Figure 1.11.** Hirshfeld surface analysis and fingerprint plots of 1. (a) crystal structure and Hirshfeld surface analysis of macrocycle 1. (b) O···H/H···O fingerprint plot. (c) N···H/H···N fingerprint plot. (d) Caryl···Caryl stacking fingerprint plot.
the SCXRD hydrogen bond lengths, 1 showed a N(H)···O of 2.90 Å, which is a reasonable assessment from the Hirshfeld data. The O···H/H···O urea hydrogen bonds contributed a total of 16.1% to the overall surface contacts. As expected, the N···H/H···N contacts of 1 are a significant contributor to the overall crystal structure at 15.1% of the total contacts. In Figure 1.11c, spikes in the N···H/H···N fingerprint plot show a $de = di \sim 1.18$ Å and spans to roughly 2.25 Å. The hydrogen bond length from SCXRD were evaluated and confirmed the N(H)···N of 1 = 2.235 Å. C$_{aryl}$···C$_{aryl}$ is shown in Figure 1.11d, revealing only a 0.4% contribution to the overall surface contacts.

Figure 1.12. Hirshfeld surface analysis and fingerprint plots of 3. (a) Crystal structure and Hirshfeld surface analysis of macrocycle 3. (b) O···H/H···O fingerprint plot. (c) N···H/H···N fingerprint plot. (d) C$_{aryl}$···C$_{aryl}$ stacking fingerprint plot.
In figure 1.12a, the Hirshfeld surface for 3 is shown. Analysis of the individual surface contact values is much different compared to 1. The crystallographic data showed no appreciable hydrogen bonding from the urea hydrogen to a proximal pyridyl nitrogen, as N(H)$\cdots$N$_{pyridyl}$ seen in 1, but the Hirshfeld surface analysis showed three contacts shorter than the sum of the vdW radii. A closer look into the single crystal data showed a short contact between the pyridine N3 and urea H2 with a bond length of 3.23 Å. However, this bond is only slightly less than the sum of the vdW radii (3.29 Å) and is therefore a weak candidate for a hydrogen bond interaction. Consideration of the O$\cdots$H/H$\cdots$O fingerprint plot for 3 (Figure 1.12c) revealed contacts peaking at $de = di \sim 1.1$ Å and ending around 2.0 Å while contributing 13.6% to the overall surface contacts. As depicted in Figure 1.12b,
there were four contacts (red, two per asymmetric unit) displayed under these parameters, exhibiting bond lengths of 2.79 and 2.91 Å and in agreement with a three-centered urea-urea assembly complex. CrystaExplorer was unable to decipher between the pyridyl nitrogen versus a phenyl carbon (position C8/N3) in 3 due to disorder over this specific crystallographic site in the crystallographic information file’s (CIF) data. The reciprocal C···H/H···C fingerprint was generated to investigate this site (Figure 1.13). In Figure 1.12a, the full Hirshfeld surface is shown with hydrogen bonds between the urea N2-H2 and a

**Figure 1.13.** Comparison of the total computed Hirshfeld surface and C···H/H···C reciprocal contacts of Host 3. (a) Total Hirshfeld surface showing six contacts (red) less than the sum of the vdW radii indicated by green arrows. Zoomed orientation of a dimer assembly showing hydrogen bonding from the position of the pyridine to urea N-H. (b) Hirshfeld surface under reciprocal C···H/H···C filter, showing two contacts (red) oriented over the urea N-H and pyridine positions indicating a close contact and potential hydrogen bonding. (c) Fingerprint plot of host 3 under reciprocal C···H/H···C filtering.
proximal pyridyl N3(C8). Figure 1.13b shows the C···H/H···C Hirshfeld surface and fingerprint plot which suggests two contacts: one over the N3/C8 and N2-H2 positions, analogous to macrocycle 1. Although there are short contacts indicated in the fingerprint, we can only look at this data qualitatively and suggest a possible weak interaction. Next, analysis of N···H/H···N contacts (Figure 1.13c) showed no significant contribution to the total surface contacts for 3, which totaled to < 4%. This confirmed that the crystallographic disorder at site N3/C8 inhibits analysis of the contact surface. Finally, C<sub>aryl</sub>···C<sub>aryl</sub> stacking interactions were analyzed (Figure 1.13d). The total surface contact equaled 2.3% due to the offset stacking of the pyridyl/xylene rings, suggesting minimal π-π interactions.

To summarize, macrocycle 1 exhibited a total hydrogen bond surface contact contribution of 31.2% (N···(H)N + N(H)···O) compared to ~14% for macrocycle 3. With

![Figure 1.14](image)

**Figure 1.14.** Comparison between crystal structures of macrocycles 2 and 4. (a) Crystal structure of 2 showing bifurcated hydrogen bonding between O<sub>urea</sub>···(H)N<sub>urea</sub>. (b) View of 2 on the crystallographic b axis. (c) Crystal structure of 4 showing bifurcated hydrogen bonding between O<sub>urea</sub>···(H)N<sub>urea</sub>. (d) View of 4 along the crystallographic b axis.

this comparison, we can suggest that 1 assembles into a more stable structure than 3, despite having a free oxygen lone pair unsatisfied within the assembly. This is also consistent when comparing the melting points of 1 and 3 (291-293 °C vs. 287-289 °C). Macrocycle 3 does, however, possess more aryl stacking interactions at 2.3% (compared to 0.4% in 1) and is
correlated with \( b/\text{Å} \) unit cell distances of 4.694 Å for 1 and 4.565 Å for 3. The pillared assembly of 1 is therefore dominated by hydrogen bonding, having more than double the percent hydrogen bond contacts compared to 3. The close contact in 3 between the urea N2(H2) and N3pyridine (\( \sim2.71 \) Å) is only slightly less than the sum of the vdW radii (2.75 Å) and can only be viewed in the fingerprint plot under C···H/H···C. This suggests the proximal pyridine nitrogen 3 cannot be considered a significant contributor to the overall assembled structure under the present data at this time. The fingerprint plots suggest aryl stacking as a seemingly insignificant contributor to the overall crystal contacts for both 1 and 3. This sheds light on the differences in stacking patterns observed for each analogue. While 1 has an infinite pyridine-pyridine-pyridine stacking pattern, 3 assembles in an infinitely alternating xylene-pyridine-xylene stacking pattern. The differences in \( b/\text{Å} \) could be an artifact of this, (Table 1.1) but, according to Hohenstein and Sherrill’s computational approach,\(^4\) pyridine aryl stacking induces a dipole, reduces polarizability and the spatial extent of the electron density which leads to reduced exchange-repulsion compared to benzene dimers. The extent of the energetic favorability, in either pyridine-benzene or pyridine-pyridine dimers, depends on the stacking conformation and spatial orientations of the pyridine nitrogen. Comparing the conformations of 1 and 3 to Hohenstein and Sherrill’s data, the aryl interactions observed in 3 should be more favorable. This could explain the significant difference in Caryl···Caryl contacts between these analogues in the Hirshfeld data. This led us to further investigate the electrostatic potentials (below) between each analogue as possible evidence to explain the differences in assembly.
The Hirshfeld surfaces and fingerprint plots were also compared for macrocycles 2 and 4. Figure 1.14 compares the crystal structures of each macrocycle. In comparison, macrocycle 2 crystallized in the monoclinic system in the P2\textsubscript{1}/c space group, while 4 crystallized in the monoclinic system in the P2\textsubscript{1}/n space group. In macrocycle 4, the disorder within the asymmetric unit inhibited clarity in defining the pyridine vs. phenyl ring, but further refinement showed a 0.50(3) probability of N\textsubscript{pyridine}/C\textsubscript{phenyl} occupancy per unit cell. The Hirshfeld surface analysis and fingerprint plots are characterized in Figures 1.15 and 1.16 for 2 and 4, respectively. Analyzing the total surface contacts for macrocycle 2 (Figure 1.15a), two obvious contacts less than the sum of the vdW radii (red) are located
over the urea oxygen and N-H groups indicating bifurcated hydrogen bonding. Subsequent fingerprint plotting with the O···H/H···O surface filter shows spikes starting at $de = di \sim 1.1$ Å and ending at $\sim 2.4$ Å and accounts for 14.4% of total contacts (Figure 1.15b). The crystal structure was analyzed as confirmation and is in good agreement with the fingerprint plot of 2, showing two urea hydrogen bonds $N(H)$···$O = 2.84$ and $2.90$ Å. The $N$···$H/H$···$N$ fingerprint plot (Figure 1.15c) was generated to assess intercolumn contacts and serve as a control for macrocycles 1 and 3. The $N$···$H/H$···$N$ contacts equaled 17.1%, which corresponds to interactions between aryl hydrogens on neighboring macrocycles and the pyridyl nitrogen. This can be visualized when looking down the $a$ axis (Figure 1.17) showing a bond distance between $N3$···$H4$ of $2.642$ Å which is less than the sum of the vDW radii ($2.98$ Å). Finally, $C_{aryl}$···$C_{aryl}$ stacking is shown in Figure 1.15d and has a total surface contribution of 1.7%. 


The Hirshfeld surface and fingerprint plots of macrocycle 4 are shown in Figure 1.16. Two contacts (red, Figure 1.15a) are observed in the total surface computation, indicating bifurcated hydrogen bonding. The O···H/H···O fingerprint plot revealed a contribution of 13.2% of the total contacts (Figure 1.16b), with a $de = di$ range starting at ~1.1 Å and ending at ~2.1 Å. Crystallographic analysis revealed N(H)···O = 2.96 and 2.82 Å, which agrees with the fingerprint data. N···H/H···N contacts were analyzed as a second control and to assess the presence of intercolumn contacts (Figure 1.16c). Under the N···H/H···N parameters, the data were insignificant, accounting for 2.8% of the total surface contacts exhibiting much less intercolumn contacts compared to 2. The fingerprint
There were minor differences between macrocycles 2 and 4. Macrocycle 2 exhibits shorter hydrogen bond lengths than 4, on average, at 2.87 and 2.94 Å, respectfully. Macrocycle 2 showed an additional short contact at 2.64 Å (vdW radii ~3.1 Å) between N3 and H4 (Figure 1.17). Although both 2 and 4 had similar \( b/\text{Å} \) values and offset stacking angles (table 1.1), 4 showed a larger \( C_{\text{aryl}} \cdots C_{\text{aryl}} \) contribution at 2.4% (compared to 1.7% for 2). This could be explained as the same phenomena discussed for 1 and 3, in which the xylene-pyridine-xylene stacking pattern is suggested as more favorable for these macrocycles and correlates well to the theoretical models of benzene-pyridine under their
Table 1.2 Comparison if hydrogen bond distances and Hirshfeld surface interactions between macrocycles 1-4.

<table>
<thead>
<tr>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td><strong>Hydrogen Bonds</strong> (Å)⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(H)···O</td>
<td>2.90(4)</td>
<td>2.84(7),</td>
<td>2.89(7),</td>
<td>2.82(7),</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.90(1)</td>
<td>2.98(8)</td>
<td>2.96(2)</td>
</tr>
<tr>
<td>N&lt;sub&gt;pyr&lt;/sub&gt;···(H)N</td>
<td>3.08(2)</td>
<td></td>
<td>3.43(3)</td>
<td></td>
</tr>
</tbody>
</table>

| **Hirshfeld Contact (%)⁵** |         |         |         |         |
| O···H/H···O              | 16.1    | 14.4    | 13.6    | 13.2    |
| N···H/H···N              | 15.1    | 17.1    | 3.8     | 2.8     |
| C<sub>aryl</sub>···C<sub>aryl</sub> | 0.4     | 1.7     | 2.3     | 2.4     |

| **Total Contact (%)⁴** |         |         |         |         |
|                       | 31.6    | 33.2    | 19.7    | 18.4    |

⁶ measured using SCXRD

⁵ measured using d<sub>norm</sub> under the given parameters

⁴ computed by summing the three d<sub>norm</sub> parameters
adopted conformations.

Assessing the electron distribution of a supramolecular host is important in determining the individual pre-organization abilities as synthons.\textsuperscript{29} Halogen bonds are electrostatic attractions,\textsuperscript{30} and therefore the strongest donor/acceptor complexes can be theoretically quantified from density functional theory (DFT) computations while the results of which can be qualitatively compared between subjects.\textsuperscript{31} The presence of multiple donor/acceptor sites and varying geometric constraints can alter each analogue’s supramolecular assemblies, giving rise to materials with highly different properties for applications in a variety of different subfields in material chemistry.\textsuperscript{29} However, multiple donor and acceptor sites present allow for competition between sites for hydrogen-bonded assembly. An electrostatic potential difference greater than 75 kJ mol\textsuperscript{-1} between two competing sites is predicted to assemble, whereas a difference less than 30 kJ mol\textsuperscript{-1} is inadequate for assembly.\textsuperscript{47} Therefore, we sought to explore structure-function commonalities (and/or differences) between each asymmetric host, 3 and 4, in comparison to their symmetric counterparts which are known co-crystal formers. Macrocycle 1 has been reported as a robust supramolecular synthon in the preorganization of activated halogen and hydrogen bond donors and as a sorbent for alcohols and iodine in solid-state transformations.\textsuperscript{14-16} Macrocycle 2 has shown strong preliminary results as a robust synthon in the co-crystallization with an activated halogen bond donor, 1,4-diiodotetrafluorobenzene (Figures 1.21b and 1.23). To compare each macrocycle’s electron distribution, DFT computations were used to generate fully optimized equilibrium geometries and electrostatic potential maps using standard B3LYP function and 6-311++G** basis set. The negative values indicate areas of high electron density (acceptor
potential, red), and large positive values indicate electron deficient regions (donor potential, blue).

**Figure 1.18.** Computed surface electrostatic potential maps of hosts 1-4, comparing potentials of each donor (blue) and acceptor (red) site in each host using Density functional theory and standard B3LYP function and 6-311++G** basis set. (a) macrocycle 1. (b) Macrocycle 2. (c) Macrocycle 3. (d) Macrocycle 4.

The electrostatic potentials of each macrocycle’s donor and acceptor sites and computed energies are summarized in Table 1.3. Typically, one expects the best donor to pair selectively with the best acceptor, leaving the second-best donor to pair with the second-best acceptor, etc. This assumes that the best acceptor is at least 30 kJ mol\(^{-1}\) better than the second-best acceptor.\(^{47}\) Both macrocycles 1 and 3 have the urea N-Hs as their best donors, although 1 showed lower donating potential (187.2 kJ mol\(^{-1}\)) than 3 (222.9 kJ mol\(^{-1}\)). According to Figure 1.18, the best acceptors in both 1 and 3 are the urea oxygens by between 77.5-77.6 kJ mol\(^{-1}\), well above the typical 30 kJ mol\(^{-1}\) which is needed for selective
assembly. These data suggest there should be no competition between the two acceptors in either 1 or 3, and we expect the urea oxygen to act as the best acceptor and form the shorter, stronger hydrogen bond with the best donor. Once that occurs, there is currently not a good method for estimating the ability of the second lone pair on the urea oxygen to act as an acceptor. Ideally, one would like to know if this oxygen lone pair is a better or worse acceptor than the pyridyl nitrogen. Experimentally, we observed that 1 formed two different hydrogen bonds, which suggested that the pyridyl nitrogen is a better than acceptor versus the urea oxygen that is already involved in one hydrogen bond. Surprisingly, analysis of 3 suggests that it forms bifurcated urea-urea hydrogen bonds or two C=O⋯(H)N instead. In this case, perhaps hydrogen bonding propensity is based more on geometric constraints/orientation, instead of electron distribution and donor/acceptor potentials.

In figure 1.18c and d, the electron distributions of 2 and 4 were much different. Macrocycle 2 showed less electron density (-184.8 kJ mol\(^{-1}\)) at the pyridine nitrogen compared to 4 (-197.8 kJ mol\(^{-1}\)). The same trend was observed at the carbonyl oxygen site of 2 having a potential of -180.1 kJ mol\(^{-1}\) and 4 having a potential of -208.6 kJ mol\(^{-1}\). The donating potential in 2 at the urea N-H site was higher compared to 4 (249.9 and 234.7 kJ mol\(^{-1}\), respectively). The differences in electrostatic potentials between acceptor sites in both 2 and 4 suggest low fidelity assembly (less than 30 kJ mol\(^{-1}\)). These data would suggest 2 to exhibit more competition between donor sites, on average, compared to 4. Indeed, each macrocycle still assemble into similar structures, suggesting conformational/stability constrains despite competing acceptor sites.
Table 1.4. Comparison of hydrogen bond lengths of N(H)⋯O, N\textsubscript{pyr}⋯(H)N and indicative solid-state FT-IR stretches for hosts 1-4 and co-crystals of 1 and 2.

\begin{tabular}{|c|ccc|ccc|}
\hline
 & \textbf{Bond Length\textsuperscript{a}} & & & \textbf{IR stretch (cm\textsuperscript{-1})} & & \\
 & \(d(\text{N(H)}\cdots\text{O})/\text{Å}\) & \(d(\text{N(H)}\cdots\text{N}\text{pyr})/\text{Å}\) & \(d(\text{C=N})/\text{Å}\) & \(\nu(\text{C=O})\) & \(\nu(\text{N-H})\) & \(\nu(\text{C=N})\) \\
\hline
1 & 2.90(4) & 3.08(2) & 1.34(5) & 1650 & 3263, 3320 & 1563 \\
2 & 2.84(7), 2.90(1) & & 1.33(8) & 1594 & 3325 & 1462 \\
3 & 2.89(7), 2.98(8) & 3.42(8) & 1.36(5) & 1602 & 3329 & 1576 \\
4 & 2.96(2), 2.82(7) & & 1.36(4) & 1601 & 3306, 3321 & 1584 \\
1\textsubscript{p-DITFB} & 2.154 & 3.12(2) & 1.34(6) & 1626 & 3304 & 1556 \\
2\textsubscript{p-DITFB} & 2.83(8), 2.91(9) & & 1.347) & 1597 & 3328 & 1435 \\
\hline
\end{tabular}

\textsuperscript{a} Obtained from SCXRD data
Supramolecular assemblies of complementary molecular recognition sites are often utilized to modulate the solid-state properties of materials.\textsuperscript{32} This has been applied in areas of organic electronics,\textsuperscript{33} photo- and thermo-responsive materials\textsuperscript{34} and pharmaceuticals.\textsuperscript{35} Co-crystallizations can be used to control product distributions of photoreactive guests in the solid-state.\textsuperscript{36} Supramolecular synthons paired with complementary photoreactive co-formers can restrict molecular orientation and/or accentuate electron densities, resulting in enhanced yields or product distributions which otherwise aren’t observed in solution.\textsuperscript{32} In this respect, it is essential to understand supramolecular assembly to manufacture predictable synthons. In practice, crystallization conditions influence the physiochemical properties and crystal quality. Likewise, optimization and control over the crystallization conditions can enhance the final crystal properties.

Selecting the mode of crystallization is generally the first step in determining the conditions for co-crystallization. Choosing the crystallization method is normally limited to the least-soluble component in a multicomponent system. Therefore, each co-former’s

<table>
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<tr>
<th>Electrostatic potential (kJ mol(^{-1}))</th>
<th>(N_{\text{pyr}})</th>
<th>N-H</th>
<th>C=O</th>
<th>Energy (a.u.)</th>
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<td>1</td>
<td>-135.5</td>
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<td>249.9</td>
<td>-180.1</td>
<td>-1099.84</td>
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<tr>
<td>4</td>
<td>-197.8</td>
<td>234.7</td>
<td>-208.6</td>
<td>-1083.80</td>
</tr>
</tbody>
</table>

Table 1.3. Comparison of electrostatic potentials at donor/acceptor sites and total ennergy (Hartrees).
equilibrium data should be determined. This can be achieved by generating a solubility curve, or Miers’ plot, similar to the example in Figure 1.19. Each co-former’s solubilities is first screened in common organic solvents (or combination of solvents) under ambient and low temperatures and controlled heated conditions. Bis-urea macrocycles are only partially soluble in polar organic solvents at room temperature. Therefore, solubility is typically best in highly polar solvents (DMSO, DMF, acetic acid or ethylene diamine) and full dissolution is aided by controlled heat (80-160 °C), vigorous stirring and sonication. The solubility of each guest should also be evaluated under the same conditions, as multicomponent nucleation requires similar solubilities between co-formers in the given solvent system.
In Figure 1.19, the Miers’ plot depicts three lines. The solubility line is located in the center of figure 1.19 and is the equilibrium between temperature and concentration. The curve below this line is an undersaturated region. The ‘metastable limit’ is the supersaturation point where crystallization takes place. The metastable limit largely encompasses the kinetics associated with nucleation, and is the supersaturation limit up to which the solution is stable (or metastable), with respect to nucleation. This is helpful for crystallization because the morphology of the solubility curve can be evaluated as a starting point for determining which crystallization method is best suited for a particular system. Thus, if the equilibrium curve is relatively flat, evaporation (or vacuum) should be utilized at constant temperature, as this will increase each co-former’s concentration over time and force supersaturation and subsequent nucleation/crystallization. If the solubility curve is
highly temperature-dependent, slow-cooling or antisolvent vapor diffusion are most suitable. These methods are indicated because they take advantage of the co-former’s low solubility and forces nucleation by slowly decreasing its solubility, with temperature or solvent(s), respectively. Bis-urea macrocycle crystallization has been achieved by each of the above methods, while slow-cooling (1 °C/h) is the most common since these macrocycles are essentially insoluble at ambient temperatures. Co-crystallization also depends on the relative solubility of the guest, which is (usually) more soluble in polar solvents compared to the bis-urea macrocycle. As a result, the host and guest could reach supersaturation at different rates. This can be overcome by increasing the concentration of the guest or using the ‘drowning-out’ method. The process of ‘drowning-out’ (or ‘watering-out’) is an effective strategy involving the addition of a second solvent (antisolvent) to reduce the effectiveness of the first, while simultaneously increasing the effective concentration of each component. A common co-crystallization method using this technique is antisolvent vapor diffusion. This involves the dissolution of each co-former into a solvent (or solvent system) and placing the open solution into an environment where the antisolvent can slowly diffuse into the solution via vapor phase exchange (Figure 1.20). The gradual addition of the antisolvent vapors slowly allows the solution to reach supersaturation, due to both the host and guest being mostly insoluble in the antisolvent and allowing nucleation to take place. Additionally, the ‘drowning- or watering-out’ technique is commonly combined with slow cooling in a solvent:antisolvent system. To do this, both co-formers are dissolved in a minimum amount of a single solvent and the antisolvent is slowly added. For bis-urea macrocycles, addition of the antisolvent is typically attempted under heated conditions (dissolution temperature) with the macrocycle
solution and antisolvent incubated at the same temperature. As in the previous example, the antisolvent reduces the effectiveness of the original solvent while slow cooling induces supersaturation and nucleation. Choosing the antisolvent is crucial for these two techniques, as the macrocycle and guest should be mostly insoluble in the antisolvent.

The metastable limit width has proven useful for analyzing nucleation kinetics and has been widely studied in the context of slow cooling crystallizations. Many mathematical models have been generated for predicting optimal supercooling conditions while accounting for volume and cooling rate as influential variables.\textsuperscript{38-40} Several other factors influence the width of the metastable limit and can only be controlled experimentally, such as foreign particles, reactor geometry, agitation rate and the thermal history of solution.\textsuperscript{39} As the width of the supersaturation limit increases, the likelihood of nucleation decreases; the opposite is true for a narrow metastable limit width. In the event of the former, seeding is strongly indicated for crystallization. Seed-crystals added to a supersaturated solution increase the rate of nucleation and are typically made from crystals of the target structure by grinding and then adding to the solution. For co-crystallization, seeds can be generated by mechanochemical interactions and are utilized for primary nucleation. Manual grinding with a mortar and pestle is the most common practice but other, more efficient, methods have been developed as well such as ball, oscillating and vibratory mills. Mechanical grinding using a mortar and pestle is a sufficient and cost-effective tool to achieve seed crystals and can be done through neat or liquid-assisted grinding. For neat grinding, both co-formers are placed in a mortar dish and ground with a pestle, while liquid-assisted grinding uses a small, sub-stoichiometric amount of solvent to facilitate interactions. Drying is an additional step required after liquid-assisted grinding, as this is usually done
with a semi-volatile solvent and is left to evaporate. Once the components are sufficiently ground, the powder is dissolved in the chosen solvent for crystallization. The process of mechanically grinding induces particle size reduction and facilitates solid state interactions by conversion of mechanical work into heat. As a result, mechanochemical transformations take place, noncovalent interactions are achieved, and crystal seeds are usually generated.

Both 1-p-DITFB and 2-p-DITFB were obtained from similar crystallization conditions. Host 1 or 2 (3 mg, 0.009 mmol) was dissolved in a minimum amount of DMSO (6 mg/mL, 0.018 mM) with the aid of heat (~120 °C) and sonication. A 1:1 stoichiometric equivalent of p-DITFB was added to the mixture. After filtering in to a 5 mL vial, water was allowed to vapor diffuse into the solution by placing the open vial into a larger vial (20 mL) containing ~2-3 mL of water, closed to the atmosphere (Figure 1.20). Crystals suitable for x-ray diffraction were obtained after 3-5 days. Once the structures were

**Figure 1.20.** General overview of the vapor diffusion method used for co-crystallization (and crystallization). From left to right: Host and guest co-formers are dissolved in a primary (1°) solvent. The open vial is placed in a larger vessel containing an antisolvent and sealed from the atmosphere (red arrow). Over time, antisolvent vapors diffuse into the smaller vial via vapor phase exchange (black arrow) and induces supersaturation of the solutes in the primary solvent. Subsequently, nucleation is induced as the solution becomes more dilute, preceding co-crystallization/crystallization.
confirmed by SCXRD, the crystals were individually subject to PXRD to determine if the bulk structure was single phase. In addition, solid-state FT-IR was used to probe and compare the noncovalent interactions of these assemblies. The hydrogen bond lengths and FT-IR wavenumber stretches of each supramolecular assembly are listed in Table 1.3.

Single crystals of 1·p-DITFB were obtained in the triclinic system with the P-1 space group with a 1:1 host to guest ratio. The distinct hydrogen bonding pattern of host 1 results in the urea oxygens pointing in opposite directions (up and down), which is commonly observed to minimize dipoles. The orientation of assembly and the 𝑁pyridyl⋯(H)𝑁 hydrogen bond leaves one oxygen lone pair unsatisfied. As a result, this generates two unsatisfied lone pairs per macrocycle, which are situated approximately linear to one another. Crystallization with the ditopic halogen bond donor p-DITFB forms a short halogen bond between one oxygen lone pair and an aryl iodine (C=O⋯I). In the structure, p-DITFB is interpenetrated between columns of 1, connecting two adjacent 1D pillars (Figure 1.21a). Observed along the 𝑏-𝑐 plane is a Host-Guest pattern of 𝐻⋯𝐺⋯𝐻⋯𝐺, forming infinite 1D linear chains. The hydrogen bonded pillars display structural similarities to 1. Two different hydrogen bonds are observed: One hydrogen bond extends from a urea hydrogen N-H to an adjacent urea C=O (N(H)⋯O), while the other is formed between a proximal pyridine nitrogen and a urea hydrogen (N(H)⋯N) with bond lengths of 2.83 and 3.12 Å, respectively. These bond lengths are slightly longer compared to the assembled ‘guest free’ structure of 1, which has distances of N(H)⋯O = 2.90 Å and N(H)⋯N = 3.08 Å. In figure 1.22a, the iodine is shown donating to the carbonyl oxygen (C=O) at a 135.70° angle, indicating the location of the previously unsatisfied lone pair.
This bond features high directionality with the angle of the halogen bond from C-I⋯O is measured nearly orthogonal at 178.68° and a bond length of 2.825 Å. The centroid distance between each guest is measured at 4.561 Å (Figure 1.22b), while a I⋯π interaction is observed at I1⋯C13 between each guest was measured at 3.665 Å (vdW radii = 3.945 Å, Figure 1.22c). The tilted displaced tilt angle was measured at 63.10° for p-DITFB.

Solid-state FT-IR was used to probe hydrogen and halogen bond interactions in these crystals (Table 1.4). The two N-H peaks in 1 change into a single stretch in 1-p-

![Figure 1.21](image_url)  
**Figure 1.21.** Crystal structures of 1-p-DITFB and 2-p-DITFB, highlighting interpenetrated halogen bonding connecting 1D host pillars. a) A trimer assembly of 1-p-DITFB showing one halogen bond per asymmetric unit and a zoomed look at the halogen bond interaction at 2.825 Å. b) A trimer assembly of 2-p-DITFB viewed along the a axis, displaying interpenetrated guest molecules between each pillar connected by halogen bonds at 2.838 Å.
DITFB. In host 1, the N-H stretches at 3263 and 3320 cm\(^{-1}\) correspond to the hydrogen bonds of N(H)···O and N(H)···N, respectively. In 1\(p\)-DITFB, the N-H bonds are characterized as a single peak at 3304 cm\(^{-1}\). The increased wavenumber from 3263 to 3304 cm\(^{-1}\) is indicative of a typical blueshift in which the vibrational bond frequency is increased due to decreased bond polarity from an increase in electron density.\(^{38}\) The band shifts are therefore expected due to decreased electron density on the carbonyl oxygen as it interacts with iodine. Both N-H bond length lengths in 1\(p\)-DITFB decreased compared to host 1. The N1-H1 bond distance decreased by 0.060 Å and N2-H2 decreased by 0.086 Å. However, the convergence of the N(H)···N bond stretch is difficult to interpret since there is conflicting evidence based on typical trends. A redshift and decreased intensity are observed at the C=O stretch in 1\(p\)-DITFB with a frequency of 1626 cm\(^{-1}\) (compared to 1650 cm\(^{-1}\) in 1) is a result of a weakened C=O bond due to the interaction with the iodine donor. This is indicative of n→\(\sigma^*\) donation from the oxygen to iodine (C=O···I).\(^{22}\) This was confirmed by measuring the C=O distance using the SCXRD data showing an increased bond length by 0.009 Å in 1\(p\)-DITFB. The C=O···I halogen bonding interaction was also examined by X-ray photoelectron spectroscopy (XPS) comparing several co-crystals of 1. The data showed that the XPS binding energy of the carbonyl C(1s) was exceptionally sensitive to halogen bond formation.\(^{14}\)
Single crystals of $2\times p$-DITFB were obtained in the Triclinic system with the $P-1$ space group (No. 2). The asymmetric unit consisted of half of 2 and half of $p$-DITFB, both located on crystallographic inversion centers. Macrocycle 2 in the co-crystal structure assembled through a three-centered hydrogen bond network, similar to the ‘empty’ host 2 assembly. As expected, 2 crystallized as a dipotic acceptor when exposed to a ditopic halogen bond donor (Figure 1.23). 1D hydrogen bonded pillars of 2 are seen approximately parallel to the $a$ axis, while layers of $p$-DITFB are interpenetrated between each pillar spanning the $b$-$c$ plane. Along the $b$ plane, the Host-Guest pattern is $H\cdots G\cdots H\cdots G$ forming...
infinite 1D linear chains. The halogen bond connecting pillars of 2, by C-I⋯N, measures at 2.838 Å with a bond angle of 171.10°. The assembly of 2 versus the co-crystal 2·p-DITFB adopted similar hydrogen bonding patters. However, the hydrogen bond distances in 2·p-DITFB were only slightly longer than in (empty) host 2 (2.83(8) and 2.91(9) Å; 2.84(7) and 2.90(1) Å, respectively). The increase in hydrogen bond length between 2 and 2·p-DITFB is less than 0.005 Å, indicating a high fidelity assembly. The guest centroid distance is measured at 4.574 Å in 2·p-DITFB (Figure 1.23b), with minimal electrostatic interactions less than the sum of the vdW radii. Interestingly, the displaced stacking angle of the pyridine backbone in 2·p-DITFB was smaller compared to the empty host at 61.41° (66.36° in 2). This suggests a small conformational change leading to less aryl overlap within the pyridine backbone. Despite marginal differences in the hydrogen bonds in the assembly, the urea N-H bond lengths were much shorter on average in by 0.093 Å. The carbonyl bond distance (C=O) was nearly identical in both structures. This was expected given only slight changes in hydrogen bond distances. A slight increase of the pyridine C=N bond (Δ d(C-N) ~ 0.01 Å) suggests a weaker bond due to the iodine donor.
The solid-state FT-IR spectra were compared between structures 2 and 2·p-DITFB to probe changes in assembly interactions. There were essentially no changes in the vibrational frequencies at the urea N-H and carbonyl bonds (Table 1.4) between the two structures, suggesting the urea hydrogen bonding strength was not modulated by cocrystal formation. Broadening of the N-H peak in the 2·p-DITFB compared to 2 alone is quite evident and could be explained by the presence of water on the crystal surface. The C=N vibrational frequency displayed a redshift and decreased in intensity from 1462 cm\(^{-1}\) in 2 to 1435 cm\(^{-1}\) in 2·p-DITFB. This is explained by a decreased dipole moment at the pyridine nitrogen.

**Figure 1.23.** Crystal structure of 2·p-DITFB, highlighting geometric configurations. (a) Viewing 2·p-DITFB along the \(a\) axis are two halogen bonds extending from a single guest molecule, connecting each pillar at an angle of 171.1°. (b) Side view of 2·p-DITFB column, featuring a centroid distance of 4.574 Å between each guest and a tilted stacking angle of 40.76°.
To summarize the differences between each co-crystal, the halogen bond is slightly shorter in 1·p-DITFB, suggesting a stronger interaction. In 2·p-DITFB, an increase in bond distance at the pyridine N-C bond (Δ d(C-N) ~ 0.01 Å) suggests weakening of the C-N bond, compared to 1 and 1·p-DITFB as controls, which showed minimal change (Δ d(C-N) ~ 0.001 Å). The stacking orientation of p-DITFB is also different between co-crystals. Co-crystal 2·p-DITFB showed more overlapped stacking between guests by 1.242 Å while the tilt angle is significantly less in 2·p-DITFB at 40.76°. In 1·p-DITFB, the guest featured an additional C-I···π interaction between C13-I1 at 80.40°. The parallel stacking angle in 2·p-DITFB was smaller at 61.41° than the empty host (66.36°). This could be conformational artifact of a decreased dipole moment in the co-crystal, as the centroid distance between pyridines is also smaller (4.574 Å) despite longer hydrogen bonds than the host assembly. As a control, there was virtually no change in the parallel stacking angles between 1 and 1·p-DITFB at 53.07° and 53.89°, respectively.

1.3. FUTURE DIRECTIONS

Our preliminary results exhibited exciting potential directions that can be utilized to compare each host’s ability to pre-organize guests as a supramolecular synthon. Hosts 1 and 2 were able to organize an activated halogen bond donor, p-DITFB, without disrupting assembly. This displays their ability to accommodate other complementary guests through molecular recognition. Asymmetric hosts 3 and 4 could also be utilized this way but attempts to co-crystallize these hosts with activated halogen bond donors have failed. Thus, conditions for co-crystallization using these hosts need to be optimized. Given centrosymmetric assemblies, larger guests may be indicated for asymmetric hosts, as the
intercolumnar space between pyridine nitrogens or carbonyl oxygens is essentially twice as long as the symmetric hosts. Additionally, host 3 should be further investigated for use in this capacity. Although 3 is structurally similar to the supramolecular synthon 1, little evidence was uncovered in our analysis for its use as a synthon in this paper. However, co-crystallization with appropriate guest(s) could result in enhancement of the pyridine nitrogen as a result of a conformational change, as 1 did show conformational differences when co-crystallized with p-DITFB.

![Chemical structures](image)

**Figure 1.24.** Potential ditopic and monotoptic guests for future co-crystallizations. Left Column: activated halogen and hydrogen bond donors. Middle column: Ditopic and monotoptic stilbenes containing hydrogen bond donating moieties. Right column: Azobenzene guests with hydrogen bond donating group(s).

Figure 1.24 highlights potential guests for co-crystallization using the current series of macrocycles. In the first column, additional activated halogen and hydrogen bond donors are depicted. The DITFB series would be an interesting study on the induced conformational differences each host displays when co-crystallized with the different structural isomers. Potential charge-transfer complexes could be synthesized with the
activated hydroquinone (and other derivatives) as well. The second column in Figure 1.24 features carboxylic acid and phenol stilbenes as possible ditopic and monotopic guests. Stilbenes can undergo [2+2] dimerizations and photoisomerizations when exposed to UV light. Co-crystallization with these guests could accentuate their reactivities and therefore employ each host as a solid state nanoreactor. Similarly, in the last column in Figure 1.24, azobenzenes undergo an induced photoisomerization when exposed to UV light. Solid state yields of the trans-cis isomerization could be enhanced when these guests are pre-organized within the host series.

The ¹H NMR of hosts 3 and 4 display very broad methylene peaks around ~4.2-4.3 ppm (Figure 1.25). This is indicative of multiple conformations assumed in solution. 2D NMR studies, coupled with temperature-dependent NMR, could identify these conformations, and determine what temperatures or solvents influence each macrocycle’s conformation equilibrium. This could facilitate a better understanding of the crystallization

**Figure 1.25.** ¹H NMR of hosts 3 and 4, highlighting broad methylene peaks. (a) ¹H NMR of 3 with the methylene peak shown from 4.03 – 4.51 ppm. (b) ¹H NMR of 4 showing the methylene peak at 3.71 – 4.91 ppm. Peaks are scaled larger for visual clarity of the methylene peak.
conditions most conducive to isolating a specific conformer in the solid state, and thus the synthesis of new co-crystalline materials.

Lastly, macrocycle 3 displayed interesting changes in its $^1$H NMR after treatment with 1 equivalent of acid (Figure 1.26). The coupling constants of the two methylene peaks in $3 + H_3O^+$ could be indicative of cis-trans conformations in equilibrium. Additionally, two amide protons experienced a significant downfield shift, while many aromatic protons experienced convergence. The amide protons recovered after addition of D$_2$O (1.2 eq) along almost all of the aromatic protons. This could be a good starting place for probing conditions which induce cis-trans conformations. 2D COSY was also performed on $3 + H_3O^+$ (Figure 1.48). Further investigation was prematurely halted.

Collectively, these are just a few avenues which could be pursued for future publications.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.26.png}
\caption{$^1$H NMR of macrocycle 3 treated with acid water and highlighting the differences in chemical shifts between the methylene peaks and amide (urea) proton peaks. (a) $^1$H NMR of 3 in DMSO-\textit{d}$_6$ after treatment with 0.5 eq. of acidic water. Zoomed view of aromatic region and methylene peaks highlighted. $^1$H NMR (300 MHz, DMSO-\textit{d}$_3$): $\delta = 7.68$ (t, $J = 5.76$ Hz, 2H), 7.25 (d, $J = 7.92$ Hz, 2H), 7.13 (m, 5H), 6.42 (d, $J = 5.85$ Hz, 2H), 4.17 (d, $J = 6.00$ Hz, 4H), 4.21 (d, $J = 16.23$ Hz, 4H). (b) Comparison of $^1$H NMR of 3: Original $^1$H NMR (blue), after treatment with 0.5 eq. acidic water (red) and quenched with 0.8 eq. of D$_2$O.}
\end{figure}
1.4. CONCLUSIONS

In this chapter, symmetric and asymmetric bis-urea macrocycles 1-4 were examined and compared as analogues of each other and as potential supramolecular synthons for absorbing hydrogen or halogen bond-donating guests in the solid state. Each analogue formed robust 1D pillars with slightly different geometric conformations, while macrocycles 1 and 2 were successfully co-crystallized with an activated p-diiodobenzene. Macrocycle 1 self-assembled through two different hydrogen bonds: N(H)···O (2.90 Å) and N(H)···N\textsubscript{pyridyl} (3.08 Å), compared to the asymmetric analogue, 3, which assembled through a typical urea-urea bifurcated hydrogen bond network of N(H)···O (2.89, 2.98 Å). Hirshfeld surface analysis was used to confirm the differences in hydrogen bonding based on the crystal packing parameters and revealed a stark difference between the total contacts of 1 and 3 (33.1% and 19.7%, respectively). In macrocycle 3, since there was disorder over the C8/N3 position from the SCXRD data, the \textit{d}_{norm} for N···H/···N did not show an interaction between the proximal pyridine N and urea N-H (N···(H)N), as seen in host 1. However, the Hirshfeld surface did show an interaction less than the sum of the vdW radii between N3 and H2 when the \textit{d}_{norm} parameters were changed to C···H/···C. This was due to the program recognizing the C8/N3 position as a carbon, by default, due to the disorder in the CIF data. The SCXRD did reveal a hydrogen bond contact between N3···H2 at a bond angle of 144.88° and distance of 2.711 Å, which is only slightly less than the sum of the vdW radii (≈2.75 Å). Given the length of the hydrogen bond and the angle of the interaction, there is weak evidence for 3 to act as an asymmetric synthon through the same supramolecular contacts as 1. DFT calculations revealed similar electrostatic potentials at
the pyridine N for 1 and 3 (135.5 and 132.0 kJ mol\(^{-1}\), respectively), indicating analogous acceptor potentials, while the donating potential of 1 was lower (187.2 kJ mol\(^{-1}\)) than 3 (222.8 kJ mol\(^{-1}\)). This suggests that the contribution of the pyridine N to assembly in 1 and not 3 is due to conformational differences rather than donor/acceptor capacities. This is also observed in the Hirshfeld surface, revealing a C\(_{aryl}\)···C\(_{aryl}\) stacking contribution of 0.4% which is ~58% less than 3. Despite the weak evidence for pre-organization capabilities, co-crystallization of 3 with a certain guest(s) could induce enhancement of the proximal nitrogen and leave an oxygen lone pair unsatisfied, analogous to 1, to act as a ditopic synthon. Macrocycles 2 and 4, on the other hand, displayed very similar assembly patterns. Each macrocycle exhibited conventional bifurcated hydrogen bonding from N(H)···O, forming strong 1D pillars. Hirshfeld surface analysis confirmed this, showing four surface contacts per macrocycle. Macrocycle 2 showed slightly shorter hydrogen bond lengths than 4, on average (2.874 and 2.878 Å, respectfully). Macrocycle 2 showed an additional short contact at 2.64 Å (vdW radii ~3.1 Å) between N3 and H4 and displayed more N···H/H···N on the periphery of each column at 17.1%, compared to 2.8% in 4. DFT calculations uncovered very different electrostatic potentials at the hydrogen bond donating and accepting sites. Macrocycle 2 had higher electrostatic potentials at both the pyridine N and urea O (-184.8 and -180.1 kJ mol\(^{-1}\), respectfully), compared to 4 (-197.8 and 208.6 kJ mol\(^{-1}\), respectfully), suggesting inferior hydrogen bond accepting potential compared to 4 despite having shorter hydrogen bonds on average. However, this could be explained at the urea motif, as 2 showed significantly higher donating ability (249.9 kJ mol\(^{-1}\)) compared to 4 (234.7 kJ mol\(^{-1}\)). Additionally, 2 was successfully co-crystallized as a ditopic synthon and compared with co-crystals of 1.\(^{14}\) Both systems formed 1D pillars interpenetrated by
the guest $p$-DITFB through strong halogen bonds. While $1\cdot p$-DITFB pre-organized the guest via an unpaired oxygen lone pair (O···I), $2\cdot p$-DITFB preorganized the same guest through a N···I interaction (2.825 and 2.838 Å, respectfully). The halogen bonding angle for $1\cdot p$-DITFB was nearly linear directional at 178.7°, compared to $2\cdot p$-DITFB displaying a halogen bond angle of 171.1°. Co-crystals $1\cdot p$-DITFB and $2\cdot p$-DITFB displayed similar guest centroid distances, but $1\cdot p$-DITFB exhibited two additional I-π interactions between I1···C13 per molecule measured at 3.665 Å (vdW radii = 3.945 Å). This interaction was not observed in $2\cdot p$-DITFB. In conclusion, host 1 and 2 were exemplified as robust, predictable assemblies, which were not affected by the introduction of a strong halogen bond donor. Thus, giving precedence for the asymmetric analogues, 3 and 4, to act as hosts for organizing reactive guests and potentially modulating their chemical properties and reactivities.

1.5 EXPERIMENTAL

1.5.1 Materials and Methods

All reagents, solvents and guests (1,4-diiodotetrafluorobenzene) were purchased from either VWR, Sigma Aldrich, or Fisher Scientific and used as received. 1H NMR spectra were collected from a Bruker Avance III HD 300 or a Varian Mercury/VX 400 NMR. FT-IR were obtained with a Perkin Elmer Spectrum 100 FT-IR spectrometer over the range of 4000-650 cm⁻¹ with 2 cm⁻¹ resolution and 64 scans per sample.

1.5.2 Synthesis and Characterization of Macrocycles 1-4 and Co-crystals
**Dimethyl pyridine-3,5-dicarboxylate.** 3,5-pyridinedicarboxylic acid (5 g, 29.9 mmol) was suspended in 100 mL of dry methanol and cooled to ~0°C in an ice bath with stirring under N\textsubscript{2}. To the cooled suspension, thionyl chloride was added in excess (299 mmol, 21.5 mL) dropwise over 30 minutes with vigorous stirring. The mixture was refluxed for 1-2 days and followed by TLC (90:10 DCM/MeOH) with an N\textsubscript{2} inlet. The reaction mixture was cooled over an ice bath and slowly basified with saturated NaHCO\textsubscript{3} until pH ~8. A white precipitate formed around pH ~6. The white precipitate was suction filtered, washed with saturated NaHCO\textsubscript{3} (50 mL) and water (100 mL) to remove unreacted

**Figure 1.27.** \textsuperscript{1}H NMR of dimethyl pyridine-3,5-dicarboxylate. \textsuperscript{1}H NMR (300 MHz, MeOD): \(\delta = 9.36 \text{ (d, } J = 2.06 \text{ Hz, 2H)}\), 8.56 (t, \(J = 2.11 \text{ Hz, 1H})\), 4.0 (s, 6H).
diacid and salts, respectively. The white powder was dried under vacuum (~1 mbar) for 12-24 hours to give dimethyl pyridine-3,5-dicarboxylate (5.25 g, 90%) as a white solid. The compound was used in the next step without purification. \(^1\)H NMR (300 MHz, MeOD): \(\delta = 9.36 (d, J = 2.06 \text{ Hz}, 2\text{H}), 8.56 (t, J = 2.11 \text{ Hz}, 1\text{H}), 4.0 (s, 6\text{H}).\)

**Pyridine-3,5-dimethanol.** To an oven-dried 250 mL Schlenk tube, LiAlH\(_4\) (0.87 g, 23.0 mmol) was suspended in 40 mL of cold, dry THF, cooled to ~0 °C under \(\text{N}_2\). The suspension was stirred vigorously while a solution of dimethyl pyridine-3,5-dicarboxylate (1.00 g, 5.12 mmol) in 17 mL of dry THF was added dropwise over 30 minutes with continuous stirring. The reaction mixture was stirred at room temperature for 24 h with an \(\text{N}_2\) inlet. The reaction was cooled to 0°C, quenched with saturated \(\text{Na}_2\text{SO}_4\) solution (2.3 mL), diluted with cold water (8 mL) and neutralized with concentrated HCl, guided by litmus paper. The mixture was removed from the ice bath, allowed to return to room temperature, and stirred for an additional hour with intermittent sonication to break up emulsions. The white precipitate was filtered and washed thoroughly with hot methanol (80 mL). The filtrate was collected and concentrated under reduced pressure to a yellow oil (0.463 g, 65%) and was used in the following step without further purification. The next step produced the best results when this product was allowed to air-dry for 24-48 h after thoroughly removing solvents.
3,5-bis(bromomethyl)pyridine. To an oven-dried 250 mL round bottom flask, pyridine-3,5-dimethanol (0.45 g, 3.2 mmol) was added and dissolved in 20 mL HBr (48%) at 0°C with stirring. Once fully dissolved, the mixture was refluxed at 125°C for 6-12 hours, followed by TLC (DCM). The mixture was cooled to 0°C and basified to pH ~8 with saturated NaHCO₃ and kept at 0°C for 30 minutes after a brown-ish white precipitate formed. The brown precipitate was suction filtered, dried, and washed with cold diethyl ether (100 mL) into a clean filtrate flask. The filtrate was concentrated under reduced pressure to give 3,5-bis(bromomethyl)pyridine (0.300 g, 35%) as a crystalline, white solid.

¹H NMR (300 MHz, CDCl₃):  δ = 8.56 (d, J = 2.0 Hz, 2H), 7.77 (t, J = 2.0 Hz, 1H), 4.47 (s, 4H).
N,N'-(pyridine-3,5-diylbis(methylene))bis(2-methylpropan-2-amine). To a clean 250 mL Schlenk flask 3,5-bis(bromomethyl)pyridine (0.300 g, 1.13 mmol) was dissolved in 35 mL of dry THF. While stirring, tert-butylamine (1.2 mL, 11.3 mmol) and K₂CO₃ (0.16 g, 1.13 mmol) were added to the mixture. The reaction was stirred at room temperature and monitored by TLC (Ethyl acetate:MeOH:NH₃OH, 58:40:2). After 24 h, the solution
was filtered, and the filtrate was concentrated under reduced pressure to a clear, yellow oil. The product was purified by column chromatography using DCM/EtOAc/MeOH/NH₄OH (60:30:8:2) as the eluant to obtain N,N'-(pyridine-3,5-diylbis(methylene))bis(2-methylpropan-2-amine) (0.190 g, 71%) as a brownish/yellow oil, dried under vacuum to a solid. \(^1\)H NMR (300 MHz, CDCl₃): \(\delta = 8.45\) (s, 2H), 7.86 (s, 1H), 3.78 (s, 4H), 1.23 (s, 18H).

**Protected macrocycle 4.** To an oven-dried Schlenk flask (100 mL), N,N'-(pyridine-3,5-diylbis(methylene))bis(2-methylpropan-2-amine) (60 mg, 0.240 mmol) was added and dissolved in dry CH₂Cl₂ (20 mL) and stirred at room temperature with a N₂ inlet. A solution of m-xylene diisocyanate (37.7 mL, 0.240 mmol) in 5 mL of dry CH₂Cl₂ was added dropwise over 10 minutes. For each subsequent cyclization, the concentration was carefully set at 30 to 32 mM. The reaction was heated at 45°C for 1-2 days, or until the TLC of the reaction mixture showed no starting material and a single spot (10% DCM:MeOH). The solution was cooled to room temperature and concentrated under reduced pressure. The residue was recrystallized by dissolving the off-white solid in CHCl₃ and acetonitrile (2:1) and allowed to partially evaporate for 24 h at room temperature, then stored at 0°C for 3 days to obtain the product (90%, 0.10 g) as needle-like crystals. \(^1\)H
NMR (300 MHz, CDCl$_3$): $\delta = 8.37$ (s, 1H), 7.86 (s, 1H), 7.20 (t, $J = 7.85$, 1H), 7.04 (d, $J = 7.44$, 2H), 6.87 (s, 1H), 4.62 (s, 4H), 4.41 (d, $J = 4.43$, 4H), 1.51 (s, $J = 1.14$, 18H)

**Figure 1.29.** $^1$H NMR of protected macrocycle 4 (300 MHz, CDCl$_3$): $\delta = 8.37$ (s, 1H), 7.86 (s, 1H), 7.20 (t, $J = 7.85$, 1H), 7.04 (d, $J = 7.44$, 2H), 6.87 (s, 1H), 4.62 (s, 4H), 4.41 (d, $J = 4.43$, 4H), 1.51 (s, $J = 1.14$, 18H)

**Macrocycle 4.** To an unused 30 mL vial, protected macrocycle 4 (0.10 g, 0.22 mmol) was added and dissolved in ~10 mL of trifluoroacetic acid. The mixture was sonicated and manually stirred for roughly ~10 minutes. The solution was cooled to ~0 °C in an ice bath and quenched with cold H$_2$O (5 mL). The solution was basified to pH ~9
with NaOH (1M) and a white precipitate formed. The new suspension was stored at ~3 °C for 1 hour. The precipitate was collected by suction filtration and washed with water, methanol and acetone. The white precipitate was dried at ~1 mbar to obtain the product (74 mg, 99%) as a white solid. $^1$H NMR (300 MHz, DMSO-$d_3$): δ = 8.27 (s, 2H), 7.64 (s, 1H), 7.28 (s, 1H), 7.17 (t, $J= 7.21$ Hz, 1H), 7.03 (d, $J= 7.36$ Hz, 2H), 6.56 (s, 4H) 4.21 (broad s, 8H).

Figure 1.30. $^1$H NMR of 4 (300 MHz, CDCl$_3$): δ = 8.27 (s, 2H), 7.64 (s, 1H), 7.28 (s, 1H), 7.17 (t, $J= 7.21$ Hz, 1H), 7.03 (d, $J= 7.36$ Hz, 2H), 6.56 (s, 4H) 4.21 (broad s, 8H).
N,N'-((pyridine-2,6-diylbis(methylene))bis(2-methylpropan-2-amine). To an oven-dried Schlenk flask, 2,6-bis(bromomethyl)pyridine (0.50 g, 1.88 mmol) was added and dissolved in THF (25 mL). While the solution stirred, tert-butylamine (2 mL, 18.8 mmol) and K$_2$CO$_3$ (0.260 g, 1.88 mmol) were added to the flask. The mixture was stirred at room temperature for 24 h and monitored by TLC (ethyl acetate/DCM/MeOH, 40:50:10). Once the TLC showed no starting material and only one spot, the mixture was suction filtered to remove the salty precipitate. The filtrate was collected separately and concentrated under reduced pressure to give a clear-yellow oil. The residue was purified by column chromatography using CH$_2$Cl$_2$:CH$_3$OH (85:15) as the eluant to obtain N,N'-((pyridine-2,6-diylbis(methylene))bis(2-methylpropan-2-amine) (450 mg, 96%) as a white, crystalline
solid. $^1$H NMR (300 MHz, CD$_3$OD): $\delta = 7.95$ (t, $J = 7.76$ Hz, 1H), 7.55 (d, $J = 7.77$ Hz, 2H), 4.42 (s, 4H) 1.56 (s, 18H).

Figure 1.31. $^1$H NMR of N,N'-(pyridine-2,6-diylbis(methylene))bis(2-methylpropan-2-amine) (300 MHz, CDCl$_3$): $\delta = 7.95$ (t, $J = 7.76$ Hz, 1H), 7.55 (d, $J = 7.77$ Hz, 2H), 4.42 (s, 4H) 1.56 (s, 18H).

Protected Macrocycle 3. To an oven-dried Schlenk flask (100 mL), N,N'-(pyridine-2,6-diylbis(methylene))bis(2-methylpropan-2-amine) (60 mg, 0.240 mmol) was added and dissolved in dry CHCl$_3$ (20 mL) and stirred at room temperature with a N$_2$ inlet.
A solution of m-xylene diisocyanate (37.7 mL, 0.240 mmol) in 5 mL of dry CHCl₃ was added dropwise over 10 minutes. For each subsequent cyclization, the concentration was carefully set to 32 mM. The reaction was heated at 45°C for 1 day, or until the TLC of the reaction mixture showed no starting material and a single spot (Eluent: 10% DCM:MeOH). The solution was cooled to room temperature and concentrated under reduced pressure to a solid. The residue was recrystallized by dissolving the off-white solid in a minimal amount of CHCl₃ (room temperature) and acetonitrile (2:1) and allowed to partially evaporate for 24 h at room temperature, then stored at 0°C for 3 days to obtain the product (91%, 0.10 g) as needle-like crystals. ¹H NMR (300 MHz, DCM-d₂): δ = 7.78 (t, J = 7.74, 1H), 7.35 (d, J = 7.71, 2H), 7.20 (d, J = 4.43, 1H), 7.09 (s, 2H), 6.64 (s, 1H), 4.72 (s, 2H), 4.58 (s, 4H) 4.26 (d, J = 4.86, 4H), 1.56 (s, 18H)

**Figure 1.32.** ¹H NMR of Protected Macrocyle 3 (300 MHz, DCM-d₂): δ = 7.78 (t, J = 7.74, 1H), 7.35 (d, J = 7.71, 2H), 7.20 (d, J = 4.43, 1H), 7.09 (s, 2H), 6.64 (s, 1H), 4.72 (s, 2H), 4.58 (s, 4H) 4.26 (d, J = 4.86, 4H), 1.56 (s, 18H)
1H), 7.35 (d, J = 7.71, 2H), 7.20 (d, J = 4.43, 1H), 7.09 (s, 2H), 6.64 (s, 1H), 4.72 (s, 2H), 4.58 (s, 4H) 4.26 (d, J = 4.86, 4H), 1.56 (s, 18H).

**Macrocycle 3.** To an unused 30 mL vial, protected macrocycle 4 (0.10 g, 0.22 mmol) was added and dissolved in ~10 mL of trifluoroacetic acid. The mixture was sonicated and manually stirred for roughly ~10 minutes. The solution was cooled to ~0 °C in an ice bath and quenched with cold H2O (5 mL). The solution was basified to pH ~9 with NaOH (1M) and a white precipitate formed. The new suspension was stored at ~3 °C for 1 hour. The precipitate was collected by suction filtration and washed with water, methanol and acetone. The white precipitate was dried at ~1 mbar to obtain the product (74 mg, 99%) as a white solid. (0.74 g, 99%). 1H NMR (300 MHz, DMSO-d3): δ = 7.63 (t, J =
7.61 Hz, 1H), 7.25 (s, 1H), 7.15 (d, J = 7.65 Hz, 3H), 7.00 (d, J = 7.43 Hz, 2H), 6.46 (s, 2H), 6.35 (s, 2H), 4.31 (broad s, 8H).

**Protected Macrocycle 2.** The synthesis of 3,5-**bis**(bromomethyl)pyridine is outlined above. To an oven-dried 500 mL round bottom flask triazinanone (0.30 g, 1.88

![Chemical structure](attachment:chemical_structure.png)

Figure 1.33. $^1$H NMR of Macrocycle 3 (300 MHz, DMSO-$d_6$): $\delta = 7.78$ (t, $J = 7.74$, 1H), 7.30 (s, $J = 7.71$, 1H), 7.19 (d, $J = 4.43$, 3H), 7.09 (d, 2H), 6.49 (s, 1H), 6.42 (s, 1H), 4.26 (s, 8H).
mmol) and NaH (0.39 g, 9.76 mmol) were suspended in 100 mL of dry THF and refluxed for 2 hours under nitrogen. The mixture was cooled to room temperature and a solution of 3,5-bis(bromomethyl)pyridine (0.65 g, 1.88 mmol) in dry THF (50 mL) was added. The reaction stirred at reflux for ~48 h (monitored by TLC), cooled to room temperature and quenched with water (100 mL). The solvent volume was reduced to a minimum using rotary evaporation and the aqueous layer was subsequently extracted with DCM (3 x 100 mL). The organic layers were combined, washed with brine, dried with anhydrous MgSO₄ and the solvent was removed under reduced pressure to give a yellow solid. The product was purified by column chromatography using CHCl₃/CH₃OH (9:1) and yielded macrocycle 1 (0.15 g, 30.6%). The product was recrystallized by slow evaporation of toluene (3 mg/1 mL). ¹H NMR (400 MHz, TCE) δ = 8.40 (s, 2H), 7.98 (s, 1H), 4.64 (s, 4H), 4.26 (s, 4H), 1.14 (s, 9H); ¹³C NMR (101 MHz, CD₃CN) δ = 156.58, 148.67, 135.86, 62.92, 54.78, 46.16, 28.50: Mp: 268-271 °C.

**Macrocycle 2.** The triazinanone protected pyridyl bis-urea macrocycle (0.12 g, 0.23 mmol) was heated to reflux in 20 mL of a 1:1 mixture of 20% [diethanolamine/water solution adjusted to pH ~ 2 with conc. HCl]: MeOH for 24 h. The methanol was removed in vacuum and cooled in an ice-bath to afford white solid precipitate. The solid was collected by suction filtration, washed with 10 mL H₂O and dried in vacuo to obtain the product as white powder (68 mg, 90 %). ¹H NMR (400 MHz, DMSO-d6) δ = 8.27 (s, 2H), 7.66 (s, 1H), 6.41 (t, J = 5.8 Hz, 2H), 4.32 (d, J = 6.0 Hz, 4H). ¹³C-NMR (101 MHz, DMSO-d6); δ = 158.51, 146.78, 137.36, 132.04, 40.42. Material turns brown (decomposes) in the temperature range of 283-286 °C.
**Synthesis of Macrocycle 1.** Triazinanone protected bis-urea macrocycle 1 (0.260 g, 4.99 x 10^-1 mmol) was heated to reflux in 50 mL of a 1:1 mixture of 20% (diethanolamine/water solution adjusted to pH ~ 2 with conc. HCl): MeOH for 24 h. The methanol was removed in vacuo resulting a light brown aqueous solution. A white solid precipitated out of solution upon cooling in an ice-bath. The solid was collected by suction filtration, washed with 45 mL H2O and dried in vacuo to obtain the product as white powder (0.163 g, 93%). ^1H NMR of 1 matched that of the reported.

**Crystallization of Macrocycle 1:** To a new, 10 mL vial, 1 (10 mg, 0.031 mmol) was added and dissolved in DMSO to make a 6 mg/mL solution. Dissolution was achieved by heating to 100 °C and sonicating (1-2 mins) intermittently for 30 minutes. The solution was allowed to cool to room temperature to ensure the compound stayed in solution. The solution was then filtered through glass wool into a new, 10 mL vial. The new vial’s cap was removed and was placed in a new, 20 mL vial containing 2-3 mL of methanol closed to the atmosphere for vapor diffusion. After 3-5 days, colorless crystals of 1 were obtained.

**Crystallization of Macrocycle 2:** To an oven dried 100 mL flask, macrocycle 2 (10 mg, 0.031 mmol) was suspended in 4.5 mL of a DMSO and acetonitrile solution (1:1.5 v/v). Dissolution was achieved by heating to 130 °C with stirring. The solution was hot
filtered through glass wool into an oven dried pressure tube. The solution was allowed to slow cool from 130 °C to room temperature (1 °C/h) which yielded colorless crystals of 2.

**Figure 1.34.** Solid-state FT-IR of macrocycle 2 crystals

**Crystallization of Macrocycle 3:** To a new, 10 mL vial, 3 (5 mg, 0.015 mmol) was added and dissolved in DMSO to make a 6.4 mg/mL solution. Dissolution was achieved by heating to 120 °C and sonicating (1-2 mins) intermittently for 30 minutes. The solution was allowed to cool to room temperature to ensure the compound stayed in solution and subsequently filtered through glass wool into a new 10 mL vial. The new vial’s cap was removed and was placed in a new, 20 mL vial containing 2-3 mL of water closed to the atmosphere for vapor diffusion. After 5 days, colorless crystals of 3 were obtained.
**Figure 1.35.** Solid-state FT-IR of macrocycle 3 crystals from 500 to 4000 cm\(^{-1}\), 64 scans

**Figure 1.34.** Observed powder diffractogram of Macrocycle 3, 2theta (~1.54 Å)
Figure 1.37. Simulated powder diffractogram of Macrocycle 3, 2theta (~1.54 Å)

**Crystallization of Macrocycle 4:** To a new, 10 mL vial, 4 (5 mg, 0.015 mmol) was added and dissolved in DMSO to make a 5.5 mg/mL solution. Dissolution was achieved by heating to 120 °C and sonicating (1-2 mins) intermittently for 30 minutes. The solution was allowed to cool to room temperature to ensure the compound stayed in solution and subsequently filtered through glass wool into a new 10 mL vial. The new Macrocycle 4

Figure 1.38. Solid-state FT-IR of macrocycle 4 crystals from 500 to 4000 cm⁻¹, 64 scans
vial’s cap was removed and was placed in a new, 20 mL vial containing 2-3 mL of water closed to the atmosphere for vapor diffusion. After 5 days, colorless crystals of 4 were obtained.

**Co-crystallization of 1-p-DITFB:** In a new 10 mL vial, 1 (3 mg, 0.0091 mmol) was dissolved in DMSO to make a 6 mg/mL solution. Dissolution was aided by heating to 120 °C for 30 minutes. A 1:1 stoichiometric amount of p-DITFB was added to the solution and remained heated. After 20 minutes, the solution was allowed to cool to room temperature and subsequently filtered through glass wool into a clean vial. Vapor diffusion with water yielded 1-p-DITFB as colorless block crystals.

**Co-crystallization of 2-p-DITFB:** In a new 10 mL vial, 2 (3 mg, 0.0091 mmol) was dissolved in DMSO to make a 6 mg/mL solution. Dissolution was aided by heating to 120 °C for 30 minutes. A 1:1 stoichiometric amount of p-DITFB was added to the solution and

![2p-DITFB Experimental](image)

**Figure 1.39.** Observed powder diffractogram of 2-p-DITFB, 2theta (~1.57Å)
remained heated. After 20 minutes, the solution was allowed to cool to room temperature and subsequently filtered through glass wool into a clean vial. Vapor diffusion with water yielded 2-p-DITFB as colorless block crystals.

![Graph of 2-DITFB simulated](image)

**Figure 1.40.** Observed powder diffractogram of 2-p-DITFB, 2theta (~1.57 Å)

### 1.5.3 X-ray Structure Determination

![Macrocycle 3 (C₁₇H₁₉N₅O₂)](image)

X-ray intensity data from a colorless needle were collected at 100(2) K using a Bruker D8 QUEST diffractometer equipped with a PHOTON-II area detector and an Incoatec microfocus source (Mo Kα radiation, λ = 0.71073 Å). The raw area detector data
frames were reduced, scaled and corrected for absorption effects using the Bruker APEX3, SAINT+ and SADABS programs. The structure was solved with SHELXT. Subsequent difference Fourier calculations and full-matrix least-squares refinement against $F^2$ were performed with SHELXL-2018 using OLEX2.

The compound crystallizes in the monoclinic system. The pattern of systematic absences in the intensity data was consistent with the space group $P2_1/c$, which was confirmed by structure solution. The asymmetric unit consists of half of one molecule located on a crystallographic inversion center. There is one unique six-membered ring per asymmetric unit. In accordance with the synthesis, this ring must therefore be a disordered pyridyl / phenyl ring, with the pyridyl nitrogen (N3) and phenyl carbon atom (C8) disordered on one crystallographic site. Trial refinements of this site as a carbon atom resulted in a site occupancy value greater than 100%, while refinement as a nitrogen atom gave an occupancy value less than 100%. Trial refinements of this site as a mixed C(8)H(8) group / N3 atom resulted in site occupancy values near 0.5 for both components, giving good support for the disordered C/N model. For the final cycles, the C(8)/H(8)/N3 site occupancies were fixed at 0.5. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms bonded to carbon were located in difference Fourier maps before being placed in geometrically idealized positions and included as riding atoms with $d$(C-H) = 0.95 Å and $U_{iso}$(H) = 1.2$U_{eq}$(C) for arene hydrogen atoms and $d$(C-H) = 0.99 Å and $U_{iso}$(H) = 1.2$U_{eq}$(C) for methylene hydrogen atoms. Hydrogen atoms bonded to nitrogen were located and refined freely. The largest residual electron density peak in the final difference map is 0.24 e$^-$/Å$^3$, located 1.33 Å from H2A.
Table 1.5. Crystal data and structure refinement for Macrocycle 3.

<table>
<thead>
<tr>
<th>Identification code</th>
<th>Macrocycle 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Empirical formula</strong></td>
<td>C_{17}H_{19}N_{5}O_{2}</td>
</tr>
<tr>
<td><strong>Formula weight</strong></td>
<td>325.37</td>
</tr>
<tr>
<td><strong>Temperature/K</strong></td>
<td>100(2)</td>
</tr>
<tr>
<td><strong>Crystal system</strong></td>
<td>monoclinic</td>
</tr>
<tr>
<td><strong>Space group</strong></td>
<td>P2_{1}/c</td>
</tr>
<tr>
<td><strong>a/Å</strong></td>
<td>7.9999(7)</td>
</tr>
<tr>
<td><strong>b/Å</strong></td>
<td>4.5647(4)</td>
</tr>
<tr>
<td><strong>c/Å</strong></td>
<td>20.9453(19)</td>
</tr>
<tr>
<td><strong>α/°</strong></td>
<td>90</td>
</tr>
<tr>
<td><strong>β/°</strong></td>
<td>93.181(3)</td>
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<td><strong>γ/°</strong></td>
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<td><strong>Volume/Å³</strong></td>
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<td><strong>Z</strong></td>
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<td><strong>ρ_{calc}/g/cm³</strong></td>
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<td>0.097</td>
</tr>
<tr>
<td><strong>F(000)</strong></td>
<td>344.0</td>
</tr>
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<td><strong>Crystal size/mm³</strong></td>
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<tr>
<td><strong>Radiation</strong></td>
<td>MoKα (λ = 0.71073)</td>
</tr>
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<td><strong>2Θ range for data collection/°</strong></td>
<td>6.244 to 50.124</td>
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<td><strong>Reflections collected</strong></td>
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<tr>
<td><strong>Independent reflections</strong></td>
<td>1352 [R_{int} = 0.0594, R_{sigma} = 0.0335]</td>
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<tr>
<td><strong>Data/restraints/parameters</strong></td>
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</tr>
<tr>
<td><strong>Goodness-of-fit on F²</strong></td>
<td>1.195</td>
</tr>
<tr>
<td><strong>Final R indexes [I&gt;2σ(I)]</strong></td>
<td>R₁ = 0.0613, wR₂ = 0.1304</td>
</tr>
</tbody>
</table>
Final R indexes [all data] | $R_1 = 0.0717$, $wR_2 = 0.1347$
---|---
Largest diff. peak/hole / e Å$^{-3}$ | 0.24/-0.21

Table 1.6. Atomic occupancy for macrocycle 3.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Occupancy</th>
<th>Atom</th>
<th>Occupancy</th>
<th>Atom</th>
<th>Occupancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8</td>
<td>0.5</td>
<td>H8</td>
<td>0.5</td>
<td>N3</td>
<td>0.5</td>
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</table>

Macrocycle 4 ($C_{17}H_{19}N_5O_2$)

X-ray intensity data from a colorless needle were collected at 100(2) K using a Bruker D8 QUEST diffractometer equipped with a PHOTON-II area detector and an Incoatec microfocus source (Mo Kα radiation, $\lambda = 0.71073$ Å). The raw area detector data frames were reduced, scaled and corrected for absorption effects using the Bruker APEX3, SAINT+ and SADABS programs.$^{41,42}$ The structure was solved with SHELXT.$^{43}$ Subsequent difference Fourier calculations and full-matrix least-squares refinement against $F^2$ were performed with SHELXL-2018$^{43}$ using OLEX2.$^{44}$ The compound crystallizes in the monoclinic system. The pattern of systematic
absences in the intensity data was consistent with the space group $P2_1/n$, which was confirmed by structure solution. The asymmetric unit consists of half of one molecule located on a crystallographic inversion center. There is one unique six-membered ring per asymmetric unit. In accordance with the synthesis, this ring must therefore be a disordered pyridyl / phenyl ring, with the pyridyl nitrogen (N3) and phenyl carbon atom (C5) scrambled over one crystallographic site. Trial refinements of this site as a C(5)H(5) group / N3 atom resulted in site occupancy values of 0.50(2) for both components, giving good support for the disordered C/N model. For the final cycles, the C(5)/H(5)/N3 site occupancies were fixed at 0.5. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms bonded to carbon were located in difference Fourier maps before being placed in geometrically idealized positions and included as riding atoms with $d$(C-H) = 0.95 Å and $U_{iso}$(H) = 1.2$U_{eq}$(C) for arene hydrogen atoms and $d$(C-H) = 0.99 Å and $U_{iso}$(H) = 1.2$U_{eq}$(C) for methylene hydrogen atoms. Hydrogen atoms bonded to nitrogen were located and refined freely. The largest residual electron density peak in the final difference map is 0.28 e$^-$/Å$^3$, located 0.73 Å from C7.

Table 1.7. Crystal data and structure refinement of Macrocycle 4.

<table>
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<th>Identification code</th>
<th>Macrocycle 4</th>
</tr>
</thead>
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<td>Empirical formula</td>
<td>C$<em>{17}$H$</em>{19}$N$<em>{5}$O$</em>{2}$</td>
</tr>
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<td>Formula weight</td>
<td>325.37</td>
</tr>
<tr>
<td>Temperature/K</td>
<td>100(2)</td>
</tr>
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<td>Crystal system</td>
<td>monoclinic</td>
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<tr>
<td>Space group</td>
<td>$P2_1/n$</td>
</tr>
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<td>a/Å</td>
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<tr>
<td>b/Å</td>
<td>4.5982(3)</td>
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c/Å  13.7044(8)  
α/°  90  
β/°  103.605(2)  
γ/°  90  
Volume/Å³  769.10(8)  
Z  2  
ρcalc/cm³  1.405  
μ/mm⁻¹  0.096  
F(000)  344.0  
Crystal size/mm³  0.52 × 0.05 × 0.02  
Radiation  MoKα (λ = 0.71073)  
2Θ range for data collection/°  6.118 to 54.208  
Index ranges  -15 ≤ h ≤ 16, -5 ≤ k ≤ 5, -17 ≤ l ≤ 17  
Reflections collected  12882  
Independent reflections  1686 [Rint = 0.0411, Rsigma = 0.0243]  
Data/restraints/parameters  1686/0/118  
Goodness-of-fit on F²  1.174  
Final R indexes [I>=2σ (I)]  R₁ = 0.0493, wR₂ = 0.0969  
Final R indexes [all data]  R₁ = 0.0556, wR₂ = 0.0994  
Largest diff. peak/hole / e Å⁻³  0.28/-0.18  

Table 1.8. Atomic occupancy for Macrocycle 4.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Occupancy</th>
<th>Atom</th>
<th>Occupancy</th>
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<th>Occupancy</th>
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<tbody>
<tr>
<td>C5</td>
<td>0.5</td>
<td>H5</td>
<td>0.5</td>
<td>N3</td>
<td>0.5</td>
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</table>
Co-crystal 2-DITFB ($C_{16}H_{18}N_6O_2$($p$-$C_6F_4$I$_2$)

X-ray intensity data from a colorless needle were collected at 100(2) K using a Bruker D8 QUEST diffractometer equipped with a PHOTON-II area detector and an Incoatec microfocus source (Mo Kα radiation, $\lambda = 0.71073$ Å). The raw area detector data frames were reduced, scaled and corrected for absorption effects using the Bruker APEX3, SAINT+ and SADABS programs.$^{41,42}$ The structure was solved with SHELXT.$^{43}$ Subsequent difference Fourier calculations and full-matrix least-squares refinement against $F^2$ were performed with SHELXL-2018$^{43}$ using OLEX2.$^{44}$

The compound crystallizes in the triclinic system. The space group $P$-1 (No. 2) was confirmed by structure solution. The asymmetric unit consists of half each of one $C_{16}H_{18}N_6O_2$ and one $p$-$C_6F_4$I$_2$ molecule, both located on crystallographic inversion centers. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms bonded to carbon were located in difference Fourier maps before being placed in geometrically idealized positions and included as riding atoms with $d$(C-H) = 0.95 Å and $U_{iso}$(H) = 1.2$U_{eq}$(C) for arene hydrogen atoms and $d$(C-H) = 0.99 Å and $U_{iso}$(H) = 1.2$U_{eq}$(C) for methylene hydrogen atoms. The two unique hydrogen atoms bonded to nitrogen were located and refined freely. The largest residual electron density peak in the final difference map is 0.56 e$^-$/Å$^3$, located 0.69 Å from C4.
Table 1.9. Crystal data and structure refinement for $2\rho$-DITFB.

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<tr>
<th>Identification code</th>
<th>$2\rho$-DITFB</th>
</tr>
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<td><strong>Temperature/K</strong></td>
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<td><strong>Crystal system</strong></td>
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<td><strong>Space group</strong></td>
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<td>$a$/Å</td>
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<td>$\gamma$/°</td>
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<td><strong>ρcalc/cm$^3$</strong></td>
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<td><strong>F(000)</strong></td>
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<td><strong>Crystal size/mm$^3$</strong></td>
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<td><strong>Radiation</strong></td>
<td>MoKα ($\lambda = 0.71073$)</td>
</tr>
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<td><strong>2θ range for data collection/°</strong></td>
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<td><strong>Index ranges</strong></td>
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<td><strong>Reflections collected</strong></td>
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<td><strong>Independent reflections</strong></td>
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<td><strong>Goodness-of-fit on F$^2$</strong></td>
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<td><strong>Final R indexes [I≥2σ(I)]</strong></td>
<td>R$_1 = 0.0189$, wR$_2 = 0.0362$</td>
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</tbody>
</table>
1.5.4 Computational Details

Electrostatic potentials were computed with the Spartan 20’ software package. The crystal structure files (CIFs) of macrocycles 1-4 were imported into the program. The host were fully optimized using density functional theory (DFT) and energies were directly calculated using B3YLP level of theory and 6-311++G** basis set under vacuum. The electrostatic potentials were obtained from the electrostatic potential map (0.002 e a.u. isovalue) and were automatically distinguished by the software. The electrostatic potentials were determined by clicking on the region of interest on the molecule until the highest (or lowest) value was obtained for each binding-site.
1.6 REFERENCES


18. Kittikhunnatham, Preecha; Som, Bozumeh; Rassolov, Vitaly; Stolte, Matthias; Würthner, Frank; Shimizu, Linda S.; Greytak, Andrew B. Fluorescence Polarization Measurements to Probe Alignment of a Bithiophene Dye in One-Dimensional Channels of Self-Assembled Phenylethynylene Bis-Urea Macrocyle Crystals. J. phys. Chem. C. 2017, 33, 18102-18109.


22. Bozumeh Som, Mark D. Smith, Linda Shimizu. Multidimensional Copper (I) and Silver (I) coordination polymers assembled with a pyridyl bis-urea macrocyclic ligand. Polyhedron. 2021, 201, 115170.


CHAPTER 2
SYNTHESIS OF SMALL MOLECULE PHARMACEUTICALS
TARGETING LY6K
2.0 ABSTRACT

The Upadhyay group identified small molecules, including NSC11150, from the National Cancer Institute’s (NCI) library to bind lymphocyte antigen 6K (LY6K) protein by the Upadhyay group. The LY6K protein has been implicated in the progression of several types of cancer. Recent efforts uncovered correlations in disrupted triple negative breast cancer cell growth after suppressing the expression of LY6K. The two small molecule inhibitors, unfortunately, are not commercially available and required strategic synthesis and careful purification prior to biological testing. In this chapter, methods for synthesis of NSC11150, purification and strategies for scale-up synthesis are discussed. Additionally, the binding mechanism of NSC11150 to LY6K was evaluated by two different approaches. First, functionalized derivatives of NSC11150 for attaching either biotin or a fluorophore were synthesized for streptavidin pulldown assays or visualization of drug occupancy within tumor cells, respectively. Second, an in silico approach was implemented by performing molecular docking studies on each derivative and comparing these data to positive, in vitro controls performed by the Upadhyay group. Advances in small molecule binding specificity and cellular localization of the potential small molecule inhibitor, and its derivatives, should aid in the development of new therapeutics for triple negative breast cancer or other cancers expressing LY6K.
2.1 INTRODUCTION

The Lymphocyte antigen 6K (LY6K) protein is a glycosylated transmembrane protein endogenously expressed in testicular germ cells.\textsuperscript{1} The LY6 clusters (30 clusters) are located on chromosomes 6, 8, 11, and 19 and perform a variety of functions including spermatogenesis, complement-mediated lysis, macrophage communication and neutrophil activation.\textsuperscript{1} Overexpression of LY6K mRNA in cancer cells has been associated with poor survival in several cancers such as head, neck, esophageal, bladder, breast, cervical and colorectal.\textsuperscript{3-7} The presence of LY6 clusters on chromosome 8q24 has been implicated in association with Myc overexpression, a commonly overexpressed gene in many cancers located at gene loci 8q24.\textsuperscript{8-10} Additionally, overexpression of LY6K was observed to increase TGF\(\beta\) signaling, a growth factor associated with cancer progression and metastasis.\textsuperscript{11} shRNA-mediated LY6K knockdown cancer cells showed reduced tumor growth \textit{in vivo}.\textsuperscript{11} Interestingly, due to the location of LY6K normal expression, male LY6K knockout mice exhibited loss of fertility while female knockout mice showed normal fertility function under the same knockdown expression.\textsuperscript{12} Therefore, LY6K was identified

\textbf{Figure 2.1.} Overview of NSC11150 derivative synthesis. From left to right: site of attachment, attachment chemistry showing either amide bond (top) or ester bond (bottom), linker type of either polyethylene glycol (top, \(n = 2, 3, \text{ or } 4\)) or boronophenyl propionic acid (bottom); tag type of either D-biotin or BODIPY.
as a potential target for women’s cancers with poor outcomes, such as triple negative breast cancer (TNBC), or others expressing LY6K.

These preliminary results prompted the Upadhyay group to explore small molecule inhibition of LY6K and test the implicative role of LY6K in cancer progression. The National Cancer Institute (NCI) library of small molecules was used to screen ~2,600 small molecules to identify potential inhibitors of LY6K. Only strong candidates were selected
for subsequent surface plasmon resonance (SPR) assay. LY6K was immobilized and coated on a Biacore chip while ~2,600 small molecules were injected over the target at the same concentration. Roughly 180 small molecules were chosen by having a 50% or higher binding capacity. A cell-titer-blue (CTB) assay was performed with the 180 small molecules to determine cell viability in LY6K knockdown cells versus control HeLa cells. A heat map was used to depict the live cells in red and green cells as dead. These data were
replotted to determine which drug candidate showed the most cell death due to loss of LY6K in controls compared to LY6K knockdown cells. Cell viability index was plotted in knockdown cells vs. control cells and identified NSC11150 (Figure 2.1) as a potential inhibitor.

The binding mechanism of NSC11150 to LY6K was not well understood, initially. Therefore, the Shimizu group not only focused on the initial synthesis of NSC11150 in high purity (>98%) on a gram-scale, but also targeted the synthesis of functionalized derivatives of NSC11150 by attaching small fluorophores (i.e. BODIPY, Pyrene) (Section 2.3.1, Figure 2.1) or a D-biotin tag. In addition, docking simulations were used as an in silico approach to elucidate the binding mechanism, drug-target interactions and potential binding pockets. Retrosynthetic analysis and synthesis schemes for NSC11150 (and derivatives thereof) are outlined in Section 2.3.2 and were first carried out by Dr. Dustin Goodlett, a graduate of the Shimizu group (Figure 2.1, 2.13). The synthesis of functional derivatives for the attachment of D-biotin and a small fluorophore were hopeful routes to determine the binding mechanism/location of NSC11150 to LY6K by subsequent streptavidin pulldown assays for binding affinities or cellular/tissue localization and migration using fluorescent microscopy, respectively. Synthesis of the functional derivatives required careful consideration, as the goal was to retain the drug’s ability to bind to LY6K while also avoiding significant decreases in solubility. Therefore, an in silico approach offered a two-fold solution. First, theoretical docking computations allowed us to identify potential binding pocket(s) of LY6K and determine which functional groups of NSC11150 were most essential for binding. This forced us to carefully consider the location of attachment moiety for the synthesis of functional derivatives. Secondly,
docking computations were compared to the preliminary, Surface Plasmon resonance (SPR) assay results and were used as a guide in interpreting our theoretical data. Collectively, the *in silico* approach provided a swift, strategic prediction of each small molecule’s binding propensities prior to synthesis. The above approach was implemented for the synthesis of three additional small molecule derivatives of NSC11150 (derivatives 11-13).

After analyzing the theoretical binding affinity of NSC11150 *in silico*, it was evident the phenol groups, located at positions 1 and 3 on NSC11150, were essential for hydrogen bonding interactions with LY6K. To test this hypothesis, three small molecules were synthesized with different hydrogen bond donating and accepting groups at the functionalized positions of NSC11150 at positions 1, 3 and 4’ (Table 2.1, Compounds 11-13). First, docking computations were performed for each analogue: 11, 12 and 13 (Figure 2.9). With good results, compared to previous computations, the commercially available benzophenone derivatives of these molecules were synthesized and purified to ~98-99% in

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**Figure 2.3.** Minimized protein structure of LY6K generated via iTasser; viewed and minimized in UCSF Chimera after simulations were performed on the SwissDock web server. (a) Ribbon structure of LY6K. Black arrow indicating binding pocket exhibited by 1 (NSC11150). (b) Hydrophobicity surface of LY6K generated through UCSF Chimera. Red indicates most hydrophobic regions; white areas are neutral; blue indicates hydrophilic regions.
exceptional yields. Once the purity was confirmed, the compounds were sent for SRP binding assays. The Biacore testing results of the three compounds were simultaneously tested against NSC11150 (positive control) and 2 (negative control) and compared to our simulated results.

2.2 RESULTS AND DISCUSSION

2.2.1 Docking Simulations for NSC11150 and its Derivatives

Since the x-ray structure of LY6K has not been determined, an accurate model of LY6K needed to be generated. Therefore, a portion of the protein from NCBI was submitted to three different web servers, each applying a different algorithm to the sequence: Swiss-Model, i-Tasser and Phyre2. The Phyre2 model showed the best results after assessing the RMSD between each model (0.833 Å). However, our results showed no differences in binding affinities between drug analogues, which was speculated to be due to the incomplete NCBI model. Dr. Tomasz Borowski was able to provide a more complete model using the i-Tasser webserver. Moving forward, this model’s minimized structure was used for all subsequent docking simulations. The molecular docking studies were performed on the SwissDock web server (https://swissdock.ch) and visualized in UCSF Chimera\textsuperscript{13-15} (https://www.cgl.ucsf.edu/chimera/). Once a docking model is completed, SwissDock provides an output of multiple binding parameters generated from the ligand-protein simulation. Although many of the quantities computed by SwissDock are helpful, the two important quantification measures were determined to be Full Fitness (FF, kcal mol\textsuperscript{-1}) and $\Delta G$ (kcal mol\textsuperscript{-1}).\textsuperscript{15,16} The more negative values for these two quantities identify a more favorable binding interaction between the ligand and LY6K. We used these data in
a relativistic context, comparing the docking simulations to the positive and negative controls from in vitro experiments performed by the Upadhyay group. The in vitro positive

![Figure 2.4. Docking simulations of NSC11150 (1) and 2, the in vitro positive and negative controls, respectively, viewed in UCSF Chimera. Hydrogen bonds indicated in cyan. (a) Chemdraw structure of 1. (b) Best docking frame of 1 binding to LY6K viewed in a hydrophobicity surface. (c) Best docking frame of 1 binding to LY6K (depicted as ribbon structure. (d) Chemdraw structure of 2. (e) Best docking simulation of 2 and LY6K (viewed as hydrophobicity surface. (f) Best docking frame of 2 and LY6K in ribbon form.](image)

and the negative control (compound 2) was replicated in SwissDock under the same parameters as other derivatives for fair consideration (Figure 2.4d). Figure 2.4a depicts NSC11150 binding to LY6K. One of the two phenol groups is shown participating in a hydrogen bond (shown in cyan) within the potential binding pocket. Figure 2.4b shows 2, a synthetic intermediate and negative control, binding to the same location but without the involvement of hydrogen bonds. The FF and ΔG for NSC11150 are more favorable (more negative) than compound 2.
The results provided insight into the drug’s binding location(s), which functional
groups participated most in binding to LY6K and which amino acid residues contribute to
binding/sterics. This enabled computational biochemist collaborators in Dr. Tomasz
Borowski’s group to perform molecular dynamic (MD) simulations on target ligands
exhibiting favorable binding quantifications at specific binding sites identified by our
simulations. The crystal structure of the LY6K protein is still not known, leaving some
uncertainty associated with the docking results and models used. Table 1 summarizes the
results of the docking computations and their respective $\Delta G$ and FF values.

**Table 2.1.** Comparison of docking computations between derivatives. Compounds 1 and 2 served as positive and negative controls, respectively. Derivative name, structure and binding computations (FF and $\Delta G$) calculated from the SwissDock web server, analyzed in Chimera.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>FF (kcal mol$^{-1}$)</th>
<th>$\Delta G$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>-613.16</td>
<td>-6.69</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>-497.62</td>
<td>-5.73</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>-609.38</td>
<td>-7.26</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>-629.18</td>
<td>-6.83</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>-656.29</td>
<td>-8.53</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>-662.91</td>
<td>-8.51</td>
</tr>
</tbody>
</table>
Analysis of the docking results considered the binding location, $\Delta G$ and FF. Docking compounds 1 and 2 served as internal positive and negative controls, respectively, based on past \textit{in vitro} and Biacore assay results. Depicted in Table 1, compounds 1-10 are inherently different from each other with respect to the type of tag, location of attachment or functional group. An amide group links each derivative to its functional group (D-biotin,
pyrene or aryl acetamide) in either the 3’ or 4’-positions (ortho or para, with respect to the methylene group). Compounds 11-13 vary in the substituents at positions 2, 4 and 4’ on the two aryl rings. At these positions, either a hydroxyl group, methoxy group or a hydrogen (only at 4’-position) are found. These were tested to probe the protein/ligand interactions by varying each positions’ hydrogen bond donating and acceptor capabilities.

Unfortunately, Swissdock was unable to process the structure of BODIPY, and we were unable to compute the simulations for those derivatives. Compounds 3 and 4 were synthesized (Section 2.2) after showing favorable ΔG and FF and similar binding locations to 1 in their docking simulations with LY6K. In Table 1.1, the ΔG and FF of 3 was -609.38 and -7.26 kcal mol⁻¹, respectfully while 4 had ΔG = -629.18 kcal mol⁻¹ and FF = -6.83 kcal mol⁻¹. These results are closely related to the simulations for 1. Although 3 and 4 showed

![Figure 2.5](image_url)
favorable binding $\Delta G$ and FF to same binding location as the parent compound, 1, the Biacore assay results for 3 showed no binding (Table 2) to LY6K-WT but bound weakly to LY6K-P79A. Compound 4 was synthesized successfully, but unfortunately was not available for Biacore testing due to degradation. Compounds 5 and 6 exhibited more favorable $\Delta G$ and FF than 1. However, their docking orientations showed LY6K preferentially binding to the biotin moiety, rather than the drug portion, but in the same location as 1 (Figure 2.6). The location of D-biotin within the binding pocket is unfortunately not conducive to accurate SRP assays. The same phenomena were observed for compounds 7 and 8: Their $\Delta G$ and FF indicated very favorable interactions with the target, but their docking results showed preferential interactions with either D-biotin or the

Figure 2.6. Docking of compounds 5 and 6 viewed in UCSF Chimera. (a) Best docking frame of 5 with target shown as hydrophobicity surface. (b) Best docking frame of 5 with target viewed in ribbon form. (c) Best docking frame of 6 with target shown as hydrophobicity surface. (d) Best docking frame of 6 with target viewed in ribbon form.
polyethylene glycol chain (Figure 2.7). The polyethylene glycol chain was initially hypothesized to increase the separation of the D-biotin tag from the binding motif of the compounds while also enhancing solubility. Future docking studies could be carried out with constraint parameters applied to the linker in an extended position to further test the binding capacities of D-biotin versus the drug. Compounds 9 and 10 feature pyrene as the
fluorophore in either the 3’ or 4’ position. These derivatives showed the least favorable quantities with respect to FF but slightly enhanced \( \Delta G \) values compared to 1: Compound 9 \( \Delta G = -8.60 \text{ kcal mol}^{-1} \), FF = -506.14 \text{ kcal mol}^{-1} and compound 10 \( \Delta G = -8.09 \text{ kcal mol}^{-1} \), FF = 503.22 \text{ kcal mol}^{-1}. Collectively, the \( \Delta G \) and FF of these derivatives were most similar to the negative control, 2. Additionally, the binding orientation of 9 and 10 were different from the parent compound 1 and could be explained by the hydrophobicity of pyrene and its alkyl chain preferentially interacting with more nonpolar amino acid side chain residues. The binding locations identified were similar to the positive control, however (Figure 2.8).

Compounds 11-13 in Table 2.1 were used to probe the role of each substituent on the drug. We wished to identify the influence of hydrogen bond donors versus acceptors as either enhancing or disrupting binding, as well as determine if the absence or replacement of chlorine influences binding. Derivative 11 is predicted to bind in a similar orientation and location as 1, the positive control. Interestingly, it also exhibited similar \( \Delta G \) (-7.35 \text{ kcal mol}^{-1}).

![Docking frames of compounds 11-13](image-url)
mol\(^{-1}\)) and FF (-528.73 kcal mol\(^{-1}\)) as compound 1. Compound 12 showed the least favorable \(\Delta G\) and FF out of the three derivatives at -6.85 and -516.53 kcal mol\(^{-1}\), respectively. Derivative 13 fell in between the two other derivatives with a \(\Delta G = -7.33\) kcal mol\(^{-1}\) and FF = -528.77 kcal mol\(^{-1}\) but more closely related to 11. Each showed similar orientations and binding locations to LY6K in the docking simulations (Figure 2.9).

A Biacore assay was subsequently performed on compounds 3, 11, 12 and 13 (and repeated on compound 1 as a positive control). The results are summarized in Table 2.1. The experimental details of the Biacore assays are outlined in Section 2.5, and the synthesis of each compound is detailed in Section 2.2. Each compound was tested against wild type LY6K (LY6K-WT) and a LY6K variant with a substitution of Proline to Alanine at position P79 (LY6K-P79A). A relative dose response was assessed at two different concentrations (1 and 5 \(\mu\)M) for each compound as a function of time. As expected, compound 1 bound to both LY6K variants but showed a greater response to LY6K-WT. In Figure 2.10b, the binding response peaked around \(s = 70\) with a relative response of \(~2000\)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>hLY6K-WT (FC:2-1)</th>
<th>hLY6K-P79A (FC:4-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>3</td>
<td>NO</td>
<td>YES (Weak)</td>
</tr>
<tr>
<td>11</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>12</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>13</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>

Table 2.2. Results of a Biacore assay (SPR assay) of compounds 1, 3 and 11-13 (Analyte). The two ligands tested are hLY6K-WT and variant hLY6K-P79A.
at 5 µM. At concentration 1 µM, compound 1 exhibited approximately 30% of the response at 5 µM (~600). Compound 3 showed no binding to LY6K-WT at either concentration (Figure 2.10e). However, a very weak binding response in the presence of LY6K-P79A was indicated at both concentrations; the binding for this assay peaked around a response of 2 at 5 µM (Figure 2.10f).

Figure 2.10. Biacore dose response experiment for compounds 1 and 3 at analyte concentrations of 1 µM (red) and 5 µM (green). Flow cell (FC) 1 was used as a reference for FC2; FC3 was used as a reference for FC4. (a) Chemdraw structure of 1 (analyte). (b) Biacore dose response binding assay of 1 with human wild type LY6K (hLY6K-WT). Peak binding response of 2000 seen at ~60-70 s. (c) Biacore assay of 1 with human LY6K variant P79A. Peak response of ~260 seen at s = 60-70. (d) Chemdraw structure of 3 (analyte). (e) Biacore binding assay of 3 with human wild type LY6K (hLY6K-WT). No binding was indicated. (f) Biacore dose response binding assay of 3 with human LY6K variant P79A. Weak binding response of ~2-2.5 seen at s = 60-70.

This was unanticipated given superior ΔG and FF in the docking simulations, compared to 1 (Table 1.2). Compound 11 also did not bind to either LY6K target in the Biacore assay (Figure 2.11b and c). Similarly, this was unexpected since 11 displayed the most favorable ΔG and FF values out of compounds 11-13 from the docking results. These values were also superior to the simulated ΔG and FF of 1.
Compound 12 displayed an analogous dose response to LY6K-WT compared to compound 1 (Figure 2.10e and 2.11b, respectively). In Figure 2.11e, the peak response is displayed around s = 21 at an analyte concentration of 5 µM. At 1 µM, 12 responded 75% less than

![Chemdraw structure of 11 (analyte).](image1)
![Biacore assay of 11 with human wild type LY6K (hLY6K-WT) at ligand concentrations of 1 µM (red) and 5 µM (green). No response occurred.](image2)
![Chemdraw structure of 12 (analyte).](image3)
![Biacore assay of 12 with human wild type LY6K (hLY6K-WT) at ligand concentrations of 1 µM (red) and 5 µM (green). Peak response of ~1300 observed at s = 21.](image4)
![Chemdraw structure of 13 (analyte).](image5)
![Biacore assay of 13 with human wild type LY6K (hLY6K-WT). Peak response of ~900 observed at s = 15, 30 and 70.](image6)

**Figure 2.11.** Biacore dose response binding assays for compounds 11-13 at analyte concentrations of 1 µM (red) and 5 µM (green). Flow cell (FC) 1 was used as a reference for FC2; FC3 was used as a reference for FC4. (a) Chemdraw structure of 11 (analyte). (b) Biacore assay of 11 with human wild type LY6K (hLY6K-WT) at ligand concentrations of 1 µM (red) and 5 µM (green). No response occurred. (c) Biacore assay of 11 with human LY6K variant P79A at ligand concentrations of 1 µM (red) and 5 µM (green). No response occurred. (d) Chemdraw structure of 12 (analyte). (e) Biacore assay of 12 with human wild type LY6K (hLY6K-WT) at ligand concentrations of 1 µM (red) and 5 µM (green). Peak response of ~1300 observed at s = 21. (f) Biacore assay of 12 with human LY6K variant P79A. (g) Chemdraw structure of 13 (analyte). (h) Biacore assay of 13 with human wild type LY6K (hLY6K-WT). Peak response of ~900 observed at s = 15, 30 and 70. (i) Biacore assay of 13 with human LY6K variant P79A. No response observed.
at 5 μM. Figure 2.11f shows the negative binding response of 12 to LY6K-P79A. Compound 13 bound to LY6K-WT but with a response 30 to 50% less (~900) than compounds 12 and 1 at the same concentration, respectively. In Figure 2.11h, the peak binding response for 13 is observed approximately s = 15, 30 and 70. Additionally, 13 showed a ~55% decrease in binding at 1 μM (Figure 2.11h) and did not bind to the LY6K-P79A variant (Figure 2.11i).

These data suggest a 50% success rate of in silico to in vitro; two of the four compounds tested in vitro were predicted to bind in similar capacities to 1 based on the simulated docking results. Indeed, more precise in silico predictions will be possible once the crystal structure of LY6K is determined.

2.2.2 Synthesis of NSC11150 and its Derivatives

Evaluating the synthesis of NSC11150, our goal was to develop a synthesis requiring the least amount of steps/purification and resulting in the highest yields. The target compound resembles commercially available 4-benzylresorcinol but lacks a chlorine in the para-position of the 4-benzyl ring. Electrophilic aromatic substitution of 4-benzylresorcinol could result in the product, but a reaction of this nature would inevitably chlorinate the more electronically activated ring before the position required for the target compound. Therefore, a coupling reaction of two aryl derivatives, one containing the 1,3-positioned phenols and one containing the p-chlorine, was investigated. As an added step, however, the product of the coupling reaction would require subsequent reduction. The type of coupling reaction was considered regarding the functional groups present to prevent cross reactions and unwanted side products. Three transformations were considered: Friedel Craft Acylation, a Grignard reaction, or a Palladium cross-coupling reaction. A
Friedel Craft or Grignard reaction would require additional steps to protect/deprotect the phenol groups to avoid cross reactions under their respective conditions. A reported procedure identified a cross-coupling reaction using an aryl aldehyde and an aryl boronic acid, similarly derivatized to our starting materials. While the starting materials were commercially available, the yields were low (~25%) and speculated to be due to the presence of the phenol groups. Finally, the first parent intermediate was synthesized through a reported aldehyde cross-coupling reaction, catalyzed by palladium, between an iodobenzene derivative and benzaldehyde. Under these conditions, the reaction between p-chloroiodobenzene and 2,4-dihydroxybenzaldehyde gave the desired benzophenone intermediate in moderate (avg. ~50%) yields (Scheme 2.1). The reduction of the ketone to methylene was first tested using a simple hydrogenation at room temperature using H₂ and Pd/C at ~50 psi. However, many products with similar polarities resulted, and the product was difficult to isolate in a pure form. The final target compound was acquired under the reducing conditions of sodium borohydride (NaBH₄) and trifluoroacetic acid (TFA) in

**Figure 2.12.** Retrosynthetic analysis of 1 (NSC11150).

**Scheme 2.1.** Synthesis of 1 (NSC11150)
moderate (~60%) yields. The final product was subject to silica column chromatography for purification. HPLC analysis was used to confirm the purity before sending for biologic testing (Section 2.5), as >97% purity was required.

Scheme 2.2. Synthesis of meta- and para-NH$_2$ derivatives of NSC11150. (a) Synthesis of meta-NH$_2$ derivative with a one-pot double reduction under Clemmensen conditions. (b) Synthesis of para-NH$_2$ derivative using a Boc-protected amine.

A pathway for developing drug conjugates with either biotin or a small fluorophore was also developed. Derivatives of NSC11150 were based on the binding orientation to LY6K in preliminary docking results performed by our collaborators using molecular dynamic (MD) simulations. This revealed the ligand:protein interaction primarily relied on the contacts between the phenol groups located at the 1,3-positions of NSC11150. This led us to develop derivatives with an aryl amine at the 3’- and 4’-positions, serving as an attachment point for a fluorophore or biotinylation via amide bonds. The synthesis of the amine derivatives was achieved using similar conditions to NSC11150, but with different starting materials. The meta-NH$_2$ synthesis starts with a Pd-catalyzed coupling between 3-nitroiodobenzene and 2,4-dihydroxybenzaldehyde (Scheme 2.2a). The resulting dihydroxy-nitro-benzophenone intermediate’s carbonyl and nitro group were originally
reduced in a two-step process: The ketone was reduced to a methylene using NaBH₄ and TFA, and subsequently the nitro group was reduced to an amine by a room temperature hydrogenation (H₂ + Pd/C). However, these two steps were later optimized by implementing a Clemmensen reduction using activated zinc powder and concentrated hydrochloric acid. Shown in Scheme 2.2a, the benzophenone intermediate was reduced at both the ketone and nitro locations simultaneously in good (avg.~85%) yields under these conditions. The para-NH₂ synthesis required a protected aniline derivative (Scheme 2.2b) because the Pd-catalyzed coupling reaction of p-iodonitrobenzene with 2,4-dihydroxybenzaldehyde failed. Thus, the Boc-protected p-iodoaniline in scheme 2.2b was purchased and resulted in moderate yields (avg. ~55%) of the benzophenone intermediate.

After purification, a test reaction to reduce the ketone to methylene was carried out using a hydrogenation at room temperature. Strangely, this reduction was only successful on a small scale (~50 mg). Attempts of scaling up the synthesis rendered this step unsuccessful, even at H₂ pressures of 100-200 psi (versus 50-55 psi normally). Regardless, the para-NH₂
synthesis was completed after a simple Boc-deprotection using trifluoroacetic acid (TFA) in good yields (> 90%).

Synthesizing the two amine derivatives allowed us to utilize a common condensation reaction between an amine and carboxylic acid (Figure 2.13) using 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) for the formation of an amide bond. The reaction was carried out under basic conditions using Hunig’s base in DMF. Each successful synthesis required column chromatography. Compound 5, containing D-biotin in the meta-position, was successfully synthesized, and required automated column chromatography for purification. The coupling reaction between acetic acid and the meta-NH$_2$ and para-NH$_2$ derivatives were also successfully carried out. However, attempts of coupling either BODIPY and pyrene to the NH$_2$-derivatives resulted in many products. These derivatives were, unfortunately, never isolated. Compound 6 (D-biotin in the para-position) was not attempted after a negative SRP assay of 5.

Additionally, three small molecule derivatives of NSC11150 were synthesized using commercially available benzophenone derivatives (Figure 2.14). These derivatives were synthesized after a simple Boc-deprotection using trifluoroacetic acid (TFA) in good yields (> 90%).

![Figure 2.14. Synthesis of compounds 11, 12, and 13 via reduction of commercially available benzophenone derivatives in excellent yields. (a) Benzophenone reduction conditions for derivatives. (b) Final structures of compounds 11-13.](image)
were used to grasp a more complete understanding of the binding mechanism of NSC11150 to LY6K by correlating our theoretical docking simulations to SPR assay results (Section 2.3). The conditions for synthesis of each derivative were first tested with a Clemmensen reduction, analogous to the conditions described previously, using different acids (TFA, HCl, and Acetic acid). Each condition, however, resulted in many products with similar polarities, making isolation and purification difficult. The reduction was successfully carried out by a Pd/C catalyzed hydrogenation in the presence of H2 (50-55 psi) and provided us with essentially quantitative yields of each product. Purification of each derivative was simply achieved by recrystallization from hot (80-110 °C) hexanes. The docking simulations and in vitro testing results of these compounds were discussed in Section 2.3.

2.3 FUTURE DIRECTIONS

Future work will be aided by x-ray structure determination of LY6K. Other proteins in the LY6-family are also being explored as additional targets contributing to cancer progression and poor prognosis. Determining the role and mechanism of LY6K (and other LY6-variants) in relation to cancer is the principle focus of the Upadhyay group. Future work within the Shimizu group will focus on the synthesis of fluorophore-appended derivatives of NSC11150 for fluorescent microscopy studies which will facilitate the Upadhyay group in biologic aspects. Attaching D-biotin for streptadivin pull-down assays is another focus which will also aid the Upadhyay group in measuring binding constants and drug affinities. However, D-biotin inherently increases the solubility of the drug—especially with a PEG linker present—which yields issues regarding purification. Once the
crystal structure and a definitive binding pocket of LY6K are identified, a more strategic synthetic approach should be made for attaching D-biotin.

During Dr. Uphadhyay’s initial investigation into small molecule inhibitors,

\[
\text{Scheme 2.3. Synthesis of NSC243928.}
\]

another molecule was discovered to bind LY6K, NSC243928 (Scheme 2.3). Biologic testing has since revealed NSC243928 having significant affinity for LY6K and anticarcinogenic activity. The synthesis of NSC243928 is shown in Scheme 2.X. An amine derivative of this compound was synthesized for the attachment BODIPY and/or D-biotin, analogous to NSC11150. However, the amide coupling reaction failed under several reaction conditions. We have speculated this to be due to the amine having low nucleophilicity, secondary to the presence of the acridine ring system. Efforts to attach the other fluorophores via reductive amination were also explored. However, commercially available aldehydes were not found for BODIPY or D-biotin.
2.4 CONCLUSIONS

LY6K has been implicated in the progression and poor outcome of several types of cancer. Small molecule NSC11150 (1) was discovered to bind LY6K as a potential pharmaceutical inhibitor. We successfully synthesized NSC11150 on a gram scale via a two-step synthesis in high purity (>97%). Derivatives of NSC11150 contained either D-biotin, a small fluorophore, an aryl acetamide, or substitutions at the 1-, 3-, or 4’-positions with hydrogen bond donors or acceptors (Table 2.1). Two amino derivatives were successfully synthesized, differing in the placement of the aryl amine at either meta- or para-positions. These were used to as attachment points for coupling different functional groups described above. Our in silico approach was utilized as a synthetic guide prior to synthesis, giving results suggesting 50% accuracy from in silico to in vitro. The docking simulations aided our understanding of the binding location(s) and interactions leading to a facile binding to LY6K. Additionally, our docking results allowed Dr. Tomasz Borowski’s group to perform molecular dynamic computations with promising ligands at potential binding pockets identified by our simulations. Compounds 3, 11, 12, and 13 were

Scheme 2.4. Synthesis of the amine derivative of NSC243928. (a) Synthesis of the chloroacridine ring. (b) Synthesis of the amine derivative using product from ‘a’.
assessed by surface plasmon resonance assays, along with 1 as a positive control. Compound 3 bound to LY6K-P79A weakly but did not bind LY6K-WT. Compound 11 did not bind either LY6K variant protein. However, compounds 12 and 13 bound LY6K-WT with similar capacities to the positive control. Once the x-ray structure of LY6K is uncovered, docking simulations will offer a more accurate approach for developing potential inhibitors of LY6K. This work, in collaboration with the Chruszcz, Upadhyay and Borowski groups, has facilitated the development of new pharmaceuticals targeting LY6K to combat cancer—especially in the context of untreatable breast cancer in women.

2.5 EXPERIMENTAL DETAILS

2.6.1 Materials and methods

Unless otherwise specified, all chemicals were purchased from either Sigma-Aldrich or VWR and used without further purification (with the exception of zinc powder, below). Synthesis of intermediates and/or starting materials are listed in synthesis schemes and figures above. $^1$H NMR and $^{13}$C NMR spectra were collected from a Bruker Avance III DH 300 or a Varian Mercury/VX 400 NMR.

2.6.2 Synthesis and Characterization of Compounds and Intermediates

Activated Zinc. To a 250-mL flask equipped with a magnetic stir bar, zinc powder (3.3 g) and 1M HCl (20 mL) was added. The mixture was stirred at room temperature for 30 minutes. The zinc precipitate was vacuum filtered and washed with water (5 mL),
ethanol (5 mL), acetone (5 mL) and ether (5 mL), then dried at 300°C for 30 minutes. Yield: 3.10 g, 94%.

Scheme 2.6.1. Synthesis of 4'-chloro-2,4-dihydroxybenzophenone

4'-chloro-2,4-dihydroxybenzophenone. 2,4-Dihydroxybenzaldehyde (1 g, 7.24 mmol), p-chloriodobenzene (3.6 g, 14.5 mmol, 2 eq), PdCl₂ (64 mg, 0.36 mmol, 0.05 eq), Na₂CO₃ (1.53 g, 14.5 mmol, 2 eq.), and LiCl (123 mg, 2.90 mmol, 0.4 eq) were suspended in 20 mL of dry dimethylformamide (DMF) in a 250 mL oven dried Schlenk flask. The vessel was subsequently subject to four cycles of freeze-pump-thaw under nitrogen. After the mixture was fully thawed, the contents were stirred at 110 °C under nitrogen for 12 h. The reaction was removed from heat, filtered and adsorbed to silica and dried under reduced pressure. The solid was purified using column chromatography (eluted with a 70:30 hexane:ethyl acetate mixture). Fractions were combined and evaporated under reduced pressure, which yielded a white solid (1.29g, 72%). ¹H NMR (300 MHz, MeOD) δ = 7.62 (d, J = 8.3 Hz 2H), 7.54 (d, J = 8.4 Hz, 2H), 7.45 - 7.38 (m, 1H), 6.35 (dd, J = 6.3, 2.2 Hz, 2H). ¹³C NMR (100 MHz, MeOD) δ = 199.87, 167.34, 167.06, 138.43, 136.74, 131.52, 129.64, 113.42, 109.25, 103.98. HRMS (DIP) m/z: [M+H]⁺ calculated for [C₁₃H₁₉ClO₃]⁺, 248.0240; found 248.0242.
Figure 2.15. $^1$H NMR of 4’-chloro-2,4-dihydroxybenzophenone.
Figure 2.16. $^{13}$C NMR of 4’-chloro-2,4-dihydroxybenzophenone.

Scheme 2.6.2. Synthesis of NSC 11150, compound 1

4-(4-chlorobenzyl)benzene-1,3-diol (NSC11150, Compound 1). NaBH$_4$ (259 mg, 6.85 mmol) was added to an oven dried Schlenk tube and sealed with a rubber septum. The flask was placed in an ice bath and connected to a N$_2$ inlet which was connected to a gas bubbler. Trifluoroacetic acid (TFA, 4.31 mL, 55.65 mmol) was cooled to ~0 °C and added to the reaction vessel in 0.05 mL additions via syringe. After each addition of TFA, the flask was vented and the gas bubbler was monitored until the flow rate returned to
normal N₂ flow. 4’-chloro-2,4-dihydroxybenzophenone (213 mg, 0.856 mmol) was dissolved separately in tetrahydrofuran (THF, ~5 mL) and added to the reaction flask dropwise by syringe. The vessel was periodically vented until N₂ flow rate returned to normal. The reaction was removed from ice and allowed to warm to room temperature and stirred for 2 hours. The reaction was quenched with 4 mL of H₂O and neutralized with 3 mL NaOH (1 M). The crude product was extracted with dichloromethane (3 x 10 mL) and the organic layers were combined and dried over MgSO₄. The solvents were removed under reduced pressure and the crude product was subject to silica gel column chromatography (95:5 Dichloromethane/ethyl acetate) to give the product as a white solid (110 mg, 55%). ¹H NMR (300 Hz, MeOD) δ = 7.20 (d, J = 8.5 Hz 2H), 7.15 (d, J = 8.5 Hz 2H), 6.79 (d, J = 8.2 Hz 1H), 6.29 (d, J = 2.3 Hz, 1H), 6.21 (dd, J = 8.2, 2.4 Hz, 1H), 3.80 (s, 2H). ¹³C NMR (100 MHz, MeOD) δ = 157.93, 157.08, 142.37, 132.17, 131.93, 131.25,
129.03, 119.75, 107.47, 103.47, 35.47. HRMS (DIP) m/z: [M+H]^+ calculated for [C_{13}H_{11}ClO_2]^+, 234.0448; found 234.0454.

Figure 2.17. ^1H NMR of NSC11150 (compound 1)
2,4-dihydroxy-3'-nitrobenzophenone (2). 2,4-Dihydroxybenzaldehyde (1.00 g, 7.24 mmol), m-nitriodobenzene (3.61 g, 14.5 mmol, 2 eq), PdCl₂ (64 mg, 0.362 mmol, 0.05 eq), Na₂CO₃ (1.53 g, 14.5 mmol, 2 eq.), and LiCl (123 mg, 2.90 mmol, 0.4 eq) were suspended in dry DMF (50 mL) in an oven dried Schlenk flask (250 mL). The vessel was subsequently subject to four cycles of freeze-pump-thaw under nitrogen. After the mixture was fully thawed, the contents were stirred at 110 °C under nitrogen for 12 h. The reaction...
was removed from heat, filtered, and adsorbed to silica and dried under reduced pressure. The solid was purified using column chromatography (eluted with a 70:30 hexanes:ethyl acetate). Pure fractions were combined and evaporated under reduced pressure, which yielded a white solid (1.46 g, 50%). $^1$H NMR (300 MHz, MeOD) $\delta = 8.45$ (d, $J = 5.6$ Hz, 2H), 8.02 (d, $J = 7.6$ Hz, 1H), 7.83 - 7.75 (m, 1H), 7.39 (d, $J = 9.5$ Hz, 1H), 6.38 (dd, $J = 6.3$, 2.2 Hz, 2H). HRMS (DIP) m/z: [M+H]$^+$ calculated for [C$_{13}$H$_{19}$ClO$_3$]$^+$, 259.0481; found 259.0482.

**Figure 2.19.** $^1$H NMR of 2,4-dihydroxy-3'-nitrobenezophenone (2).
4-(3-aminobenzyl)benzene-1,3-diol. 2,4-dihydroxy-3'-nitrobenzophenone (1.67 g, 6.44 mmol) was added to a 150-mL two-neck round bottom flask and dissolved in 4:1 ether:ethanol (10 mL:2 mL). Activated zinc powder (1.9 g, 30.2 mmol) was suspended in the reaction vessel (see activated zinc procedure), and the heterogeneous mixture was stirred for 30 minutes at 0°C. Concentrated HCl (4.3 mL, 50.25 mmol) was added dropwise over 15 minutes at 0°C. The reaction was removed from the ice bath and stirred at room temperature for 3 h. The reaction was diluted with 8 mL of deionized water, neutralized with 1M NaOH and filtered to remove salt precipitate. The filtrate was extracted with ethyl acetate (3 x 15 mL). The organic layers were combined and washed with water (3 x 5 mL) and dried over MgSO₄. The solvents were evaporated under reduced pressure to give the crude product as a brown oil. The oil was adsorbed to silica and subject to column chromatography (Eluted with: 80:20 DCM:ethyl acetate to 50:50 DCM:ethyl acetate.). Combined fractions were evaporated to a white solid (1.26 g, 92%). (300 Hz, MeOD) \( \delta = \)
6.97 (t, J = 7.7 Hz, 1H), 6.76 (d, J = 8.2 Hz, 1H), 6.62 – 6.49 (m, 3H), 6.31 – 6.26 (m, 1H),
6.19 (dd, J = 8.2, 2.4 Hz, 1H), 3.72 (s, 2H).

Figure 2.20. $^1$H NMR of 4-(3-aminobenzyl)benzene-1,3-diol.

Scheme 2.6.5. Synthesis of Compound 3.

$N$-(3-(2,4-dihydroxybenzyl)phenyl)acetamide (3). To a 150-mL Schlenk flask, a
solution of acetic acid (0.159 mL, 2.80 mmol, 1.5 eq.) in dry DMF (1 mL) and a solution
of DIPEA (0.580 mL, 3.30 mmol, 1.8 eq) in dry DMF (1 mL) was added and stirred for 10
minutes under nitrogen at room temperature. A solution of HATU (544 mg, 2.20 mmol,
1.2 eq) in dry DMF (2 mL) was added and stirred for 30 minutes under nitrogen, or until
the mixture became light brown. A solution of 4-(3-aminobenzyl)benzene-1,3-diol (NIHA-3d) (400 mg, 1.85 mmol) in dry DMF (5 mL) was added dropwise to the mixture over 5 minutes. The reaction was stirred under nitrogen at room temperature for 24-24 h, followed by TLC. The mixture purified using silica gel chromatography (50:50 DCM:Ethyl acetate). Fractions were combined and evaporated under reduced pressure to a yellow-brown oil, which was re-crystallized by dissolving in DCM and storing at 0°C for 2 days yielding white, crystalline material (322 mg, 68%). (300 Hz, MeOD) δ = 7.14 (d, J = 7.99 Hz, 1H), 7.28 (s, 1H), 7.17 (t, J = 7.83 Hz, 1H), 6.95 (d, J = 7.62 Hz, 1H), 6.80 (d, J = 8.19 Hz, 1H), 6.31 (s, J = 7.31 Hz, 1H), 3.83 (s, 2H), 2.09 (s, 3H)

Figure 2.21. 1H NMR of N-(3-(2,4-dihydroxybenzyl)phenyl)acetamide (Compound 3).
Figure 2.22. LC/MS TOF MS ES+ TIC of \(N\)-(3-(2,4-dihydroxybenzyl)phenyl)acetamide (Compound 3).

Figure 2.23. LC/MS chromatogram of \(N\)-(3-(2,4-dihydroxybenzyl)phenyl)acetamide (Compound 3) looking at peak A, [M+H]+
Figure 2.24. LC/UV (at 254 nm) chromatogram of N-(3-(2,4-dihydroxybenzyl)phenyl)acetamide (Compound 3) suggesting purity of 99.42%
Figure 2.25. LC/CAD chromatogram of N-(3-(2,4-dihydroxybenzyl)phenyl)acetamide (Compound 3) suggesting purity of 98.1%.

Scheme 2.6.6. Synthesis of BODIPY.
5,5-difluoro-1,3,7,9,10-pentamethyl-5H-4λ,4,5λ4-dipyrrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine (BODIPY). To a 3-neck round bottom flask equipped with a reflux condenser, 2,4-dimethylpyrrole (3.25 mL, 31.5 mmol) was dissolved in 18 mL of dry DCM under N₂. Acetyl chloride (15.5 mL, 72.5 mmol) was added dropwise over 30 minutes. The mixture was stirred for 1 h at 40 °C. The reaction was removed from heat and hexanes (90 mL) was added. The solvents were then removed by rotary evaporation and the dried reactants were redissolved in dry DCM (200 mL). Triethylamine (TEA, 12.74 mL, 91.43 mmol) was added dropwise at room temperature and stirred for 10 minutes. BF₃·OEt₂ (17.03 mL, 135.6 mmol) was added to the reaction mixture and stirred for 1 h at room temperature. The mixture was transferred to a separatory funnel where the mixture was washed with saturated NaHCO₃ (3 x 150 mL) to remove HF and acetyl chloride. The organic layer was collected and dried over MgSO₄. The organic solvents were removed by rotary evaporation. The product was obtained by dissolving the crude solid in a minimal amount of DCM and dropwise adding MeOH until a precipitate formed. The precipitate was suction filtered and collected to give the product as a red solid (2.6 g, 63%). ¹H NMR (300 MHz, CDCl₃) δ = 6.05 (s, 2H), 2.58 (s, 3H), 2.52 (s, 6H), 2.41 (s, 6H).

Scheme 2.6.7. Synthesis of BODIPY-Mono-I.
BODIPY-mono-I. BODIPY (200 mg, 0.76 mmol) was added to an oven dried flask and dissolved in dry DCM (20 mL). NIS (170 mg, 0.76 mmol) was added to the reaction and the mixture was stirred for two days at room temperature. DCM was removed by rotary evaporation and the crude solid was adsorbed onto silica for column chromatography (isocratic, 1:1 Hexanes/DCM). Pure fractions were combined and evaporated under

Figure 2.26. $^1$H NMR of BODIPY
reduced pressure to give the product as an orange solid (130 mg, 44%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta = 6.12$ (s, 1H), 2.61 (s, 6H), 2.53 (s, 3H), 2.44 (d, $J = 7.6$ Hz, 6H).

Figure 2.27. $^1$H NMR of BODIPY-Mono-I

Scheme 2.6.8. Synthesis of BODIPY-COOH.

3-(4-(5,5-difluoro-1,3,7,9,10-pentamethyl-5H-5$\lambda^4,6\lambda^4$-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-2-yl)phenyl)propanoic acid (BODIPY-COOH). To an oven dried
Schlenk flask, BODIPY-mono-I (38 mg, 0.098 mmol), 3-(4-boronophenyl)propanoic acid (38 mg, 0.196 mmol), Pd(dppf)Cl$_2$ (7.2 mg, 0.01 mmol), and K$_2$CO$_3$ (68 mg, 0.49 mmol) were added and suspended in 1:1 dry MeOH/Toluene (4 mL). The mixture was subject to three cycles of freeze-pump-thaw and allowed to warm to room temperature. The reaction was stirred overnight at 55 °C. The solvents were removed by rotary evaporation and adsorbed to silica for column chromatography. The silica mixture was dry loaded and eluted using 100% DCM to 90:10 DCM/MeOH gradient. Fractions were collected and the solvents were removed under reduced pressure to obtain the product as a red solid. (38 mg, 94%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ = 7.33 (d, $J$ = 8.0 Hz, 2H), 7.18 (d, $J$ = 7.9 Hz, 2H).

Figure 2.28. $^1$H NMR of BODIPY-COOH
2H), 6.27 (s, 1H), 2.86 (dd, J = 16.0, 8.0 Hz, 2H), 2.69 (d, J = 9.3 Hz, 3H) 2.63 – 2.55 (m, 2H), 2.41 (dd, J = 12.9, 2.3 Hz, 6H), 2.32 (t, J = 9.5 Hz, 6H).

Scheme 2.6.9. Synthesis of Compound 5.

**N-(3-(2,4-dihydroxybenzyl)phenyl)-5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (5).** To an oven dried Schlenk flask, 4-(3-Aminobenzyl)resorcinol (194 mg, 0.901 mmol), D-biotin (264 mg, 1.08 mmol) HATU (411 mg, 1.08 mmol) and N, N-diisopropylethylamine (DIEA) (0.245 mL, 1.406 mmol) were dissolved in 15 mL of dry dimethylformamide. The flask was shrouded in aluminum foil and stirred at room temperature for 2 days under nitrogen. The reaction mixture was concentrated under reduced pressure and injected into a Biotage Isolera automated column, equipped with a silica column for purification. A dichloromethane/methanol gradient was used to elute the product as a white solid (234 mg, 61%) 1H NMR (400 MHz, MeOD) δ = 7.41 (d, J = 8.3 Hz, 1H), 7.29 (s, 1H), 7.17 (t, J = 7.8 Hz, 1H), 6.95 (d, J = 7.5 Hz, 1H), 6.79 (d, J = 8.2 Hz, 1H), 6.29 (d, J = 2.3 Hz, 1H), 6.21 (dd, J = 8.2, 2.3 Hz, 1H), 4.48 (dd, J = 7.7, 4.9 Hz, 1H), 4.29 (dd, J = 7.8, 4.4 Hz, 1H), 3.81 (s, 1H), 3.24 – 3.17 (m, 1H), 2.92 (dd, J = 12.7, 5.0 Hz, 1H), 2.70 (d, J = 12.7 Hz, 1H), 2.36 (t, J = 7.4 Hz, 2H), 1.82 -1.40 (m, 7H). 13C NMR (100 MHz, MeOD) δ = 174.44, 103.44, 63.33, 61.63, 56.93, 49.00, 41.04, 37.61, 35.99, 29.75, 29.46, 26.74. HRMS (ESI) m/z: [M+H]+ calculated for [C23H27N3O4S], 442.1795; found 442.1791.
Figure 2.29. $^{13}$C NMR of compound 5.

Figure 2.30. $^1$H NMR of compound 5.
2-benzyl-5-methoxyphenol (12). To a pressure reaction vessel, 2-hydroxy-4-methoxybenzophenone (1 g, 4.38 mmol) was added and dissolved in methanol (30 mL). A spatula tip of Pd/C was added to the vessel and sealed with a rubber stopper connected to the hydrogenator apparatus and secured appropriately. The flask was purged with H\(_2\) (3 x 20 psi). Immediately after, the H\(_2\) pressure was raised to 50 psi and shaken for 12 h. The reaction vessel was removed from the hydrogenator apparatus, the flask purged with N\(_2\), and the mixture was filtered and washed with methanol. The filtrate was evaporated under reduced pressure to a brown oil. The oil was dissolved in diethyl ether (20 mL) and washed with deionized water (3 x 10 mL) in a separatory funnel. The organic layer was dried over MgSO\(_4\), filtered, and evaporated under reduced pressure to obtain a brown solid. The solid was heated to reflux in hexanes, hot filtered, and stored at 0°C for 24 h. A white, crystalline solid resulted (928 mg, 99%). (300 Hz, DMSO-\(d_6\)) \(\delta = 9.41\) (s, 1H), 7.22 (d, \(J = 7.12\) Hz, 1H), 7.21 (d, 1H), 7.18 (d, 1H), 7.16 (d, 1H), 7.13 (d, 1H), 6.92 (d, \(J = 8.27\) Hz, 1H), 6.38 (d, \(J = 2.3\) Hz, 1H), 6.31 (dd, \(J = 8.31, 2.22\) Hz, 1H), 3.78 (s, 2H), 3.65 (s, 3H)
Figure 2.31. $^1$H NMR of compound 12.

Figure 2.32. LC/MS chromatogram of compound 12.
Figure 2.33. LC/MS chromatogram showing peak analysis of compound 12. Correct peak is A [M+H]+.

Figure 2.34. LC/UV chromatogram of compound 12 suggesting 97.97% purity.
Figure 2.35. LC/CAD chromatogram of compound 12 suggesting 76.15% purity. Background (blank).

Figure 2.36. LC/CAD ‘blank’ chromatogram after initial assessment of compound 12 suggesting 76.15% purity. Significant background was detected.
Scheme 2.6.11. Synthesis of Compound 11.

4-benzylbenzene-1,3-diol (11). To a pressure reaction vessel, 2,4 dihydroxybenzophenone (1 g, 4.66 mmol) was added and dissolved in methanol (30 mL). A spatula tip of Pd/C was added to the vessel and sealed with a rubber stopper connected to the hydrogenator apparatus and secured appropriately. The flask was purged with H\textsubscript{2} (3 x 20 psi). Immediately after, the H\textsubscript{2} pressure was raised to 50 psi and shaken for 12 h. The reaction vessel was removed from the hydrogenator apparatus, the flask was purged with N\textsubscript{2} and the mixture was filtered. Methanol was evaporated under reduced pressure to a brown oil. The oil was dissolved in diethyl ether (20 mL) and washed with deionized water (3 x 10 mL). The organic layer was dried with MgSO\textsubscript{4} and evaporated under reduced pressure to obtain a brown solid. The solid was heated in hexanes to reflux, hot filtered, and stored at 0°C for 24 h. A white solid resulted (924 mg, 99%). (300 Hz, DMSO-\textit{d}_6) \(\delta = 9.20 (s, 1H), 9.03 (s, 1H), 7.22 (d, J = 7.12 \text{ Hz}, 1H), 7.20 (d, 2H), 7.17 (d, 2H), 6.78 (d, 1H), 6.27 (s, 1H), 6.15 (d, 1H), 3.72 (s, 2H).\)
Figure 2.37. $^1$H NMR of compound 11.
Figure 2.38. LC/MS TOF MS ES+ TIC chromatogram of compound 11.

Figure 2.39. LC/MS chromatogram of compound 11 showing peak analysis of peak A [M+H]⁺.
Figure 2.40. LC/UV chromatogram of compound 11 suggesting purity of 99.42%.

Figure 2.41. LC/CAD chromatogram of compound 11 suggesting purity of 98.73%.

4-(2,4-dimethoxybenzyl)phenol (13). To a pressure reaction vessel, 2,4-dimethoxy-4'-hydroxybenzophenone (1 g, 3.87 mmol) was added and dissolved in methanol (30 mL). A spatula tip of Pd/C was added to the vessel and sealed with a rubber stopper connected to the hydrogenator apparatus and secured appropriately. The flask was purged with H\textsubscript{2} (3 x 20 psi). Immediately after, the H\textsubscript{2} pressure was raised to 50 psi and shaken for 12 h. The reaction vessel was removed from the hydrogenator apparatus, the flask was purged with N\textsubscript{2} and the mixture was filtered. Methanol was evaporated under reduced pressure to a brown oil. The oil was dissolved in diethyl ether (20 mL) and washed with deionized water (3 x 10 mL). The organic layer was dried with MgSO\textsubscript{4} and evaporated under reduced pressure to obtain a brown solid. The solid was heated in hexanes to reflux, hot filtered, and stored at 0°C for 24 h. A white solid resulted (943 mg, 99%). (300 Hz,
CD$_2$Cl$_2$ $\delta$ = 7.08 (d, J = 8.37 Hz, 2H), 6.98 (d, J = 8.21 Hz, 1H), 6.75 (d, J = 8.43 Hz, 2H), 6.50 (d, J = 2.3 Hz, 1H), 6.45 (d, J = 8.22 Hz, 1H), 3.85 (s, 2H), 3.81 (d, J = 2.71 Hz, 6 H).

Figure 2.42. $^1$H NMR of compound 13.
Figure 2.43. LC/MS TOF MS ES+ TIC chromatogram of compound 13.

Figure 2.44. LC/MS chromatogram of compound 13 showing peak analysis of three different peaks present
2.6.3 Computations/Docking Studies

Each compound was submitted to the SwissDock online service engine for docking. First, the compounds were generated in ChemDraw and converted to SMILES format. Each SMILES formula was converted to a 3D ‘mol2’ document in USF Chimera. The mol2 document was uploaded to SwissDock as the ‘ligand’. Our LY6K target was generated using the iTasser online web server and the structure was fully minimized. Once generated, the model was uploaded to SwissDock as the ‘target protein’. SwissDock gives quantifications of ΔG and FullFitness in units of kJ mol⁻¹. Each compound was evaluated.
based on the magnitude of the absolute values of ΔG and Fullfitness from Swissdock, as well as the binding location compared to the *in vitro* positive control (compound 1) simulations. The results are summarized in Table 2.1 and discussed in Section 2.2).

2.6.4 Biacore (Surface Plasmon Resonance) Assay

Biacore T200 was used. All experiments were carried out at 25 °C. hLy6E (~11234.7 Da, 50 ng/µL stock concentration), hLy6D (~11196.4 Da, 70 ng/µL stock concentration), and hLy6K (~16936.2 Da, 117 ng/µL stock concentration) were used as ligands to immobilize onto the CM5 sensor surface. NIHB-3A (502.8 Da, 100 mM stock concentration in DMSO), NIHB-3B (452.5 Da, 10 mM stock concentration in DMSO), NIHB-5B (648.8 Da, 10 mM stock concentration in DMSO), NIHA-1C (259.2 Da, 10 mM stock concentration in DMSO), and NIHA-4D (441.6 Da, 10 mM stock concentration in DMSO) were used as analytes to inject over the ligand immobilized surface. Flow cell (FC) 1 was used as reference for FC2, FC3, and FC4. hLy6E was diluted in 10 mM acetate buffer at pH 4.5 (1:50 dilution) and immobilized onto FC2 to a level of ~1400 RU. hLy6D was diluted in 10 mM acetate buffer at pH 5.5 (1:25 dilution) and immobilized onto FC3 to a level of ~2200 RU. hLy6K was diluted in 10 mM acetate buffer at pH 4.0 (1:25 dilution) and immobilized onto FC4 to a level of ~2500 RU. Standard amine coupling chemistry was utilized to immobilize the ligands. PBS-P (10 mM Phosphate buffer pH 7.4, 0.27 mM KCl, 137 mM NaCl, 0.05% surfactant P20) was used as the immobilization running buffer.

Based on the immobilized response values, theoretical $R_{max}$ values were calculated and are presented in slide 4. The $R_{max}$ values assume 1:1 interaction mechanism. Overnight
kinetics experiments were performed for all analytes binding to the immobilized ligands, separately, in the presence of PBS-P+5%DMSO. The flow rate of all the solutions was maintained at 50 μL/min. The contact and dissociation times were 60s and 180 s, respectively. No regeneration was used. Analyte concentrations were 0 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM. The sensorgrams obtained from overnight binding experiments were evaluated using steady state affinity analysis.

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NB: No binding
NA: KD values could not be determined from fitting, poor data quality

**Figure 2.46.** Biacore assay of compounds 1 and 5 (latter two rows) against Human LY6E, D and K. No binding was observed for either compound.
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**Figure 2.47.** Theoretical Rmax of compounds 2 and 5. NIHA-A1C = Compound 2. NIHA-4D = Compound 5.

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<th>Ligand Binding (RU)</th>
<th>MWL (Da)</th>
<th>MWA(Da)</th>
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**Figure 2.48.** Theoretical Rmax of compounds 1, 3, and 11-13. NIHA-parent = Compound 1. NIHA-2,4 OMe4-OH = Compound 13. NIHA-2-OH4-OMe = Compound 12. NIHA-2,4-OH = Compound 11.
**Figure 2.49.** Biacore assay results of compound 2 with human LY6E. Graph depicts response versus time at varying concentrations of analyte.
Figure 2.50. Biacore assay results of compound 2 with human LY6E. Graph depicts response versus time at varying concentrations of analyte.
Figure 2.51. Biacore assay results of compound 2 with human LY6K. Graph depicts response versus time at varying concentrations of analyte.
Figure 2.52. Biacore assay results of compound 5 with human LY6D. Graph depicts response versus time at varying concentrations of analyte.
Figure 2.53. Biacore assay results of compound 5 with human LY6E. Graph depicts response versus time at varying concentrations of analyte.
Figure 2.54. Biacore assay results of compound 5 with human LY6K. Graph depicts response versus time at varying concentrations of analyte.
2.6 REFERENCES


7. Luo, L.; McGarvey, P.; Madhavan, S.; Kumar, R.; Gusev, Y.; Upadhyay, G. Distinct lymphocyte antigens 6 (Ly6) family members Ly6D, Ly6E, Ly6K and


10. Liao, Minru; Qin, Rui; Huang, Wei; Zhu, Hong-ping; Peng, Fu; Han, Bo; Liu, Bo. *J. Hematol. Oncol.* **2022**, 15, 1260.


APPENDIX A

PERMISSION TO REPRINT: CHAPTER 1
APPENDIX B

PERMISSION TO REPRINT: CHAPTER 1

Self-Assembled Nanotubes that Reversibly Bind Acetic Acid Guests
Author: Linda S. Shimizu, Andrew D. Hughes, Mark D. Smith, et al
Publication: Journal of the American Chemical Society
Publisher: American Chemical Society
Date: Dec 1, 2003
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