

EFFORTS TOWARD THE SYNTHESIS OF PHENYLALANINE-CONTAINING  
MACROCYCLES DERIVED FROM DIMERIZATION OF TRIAZINE  
MONOMERS TO EXPLORE BRO5 PROPERTIES

by

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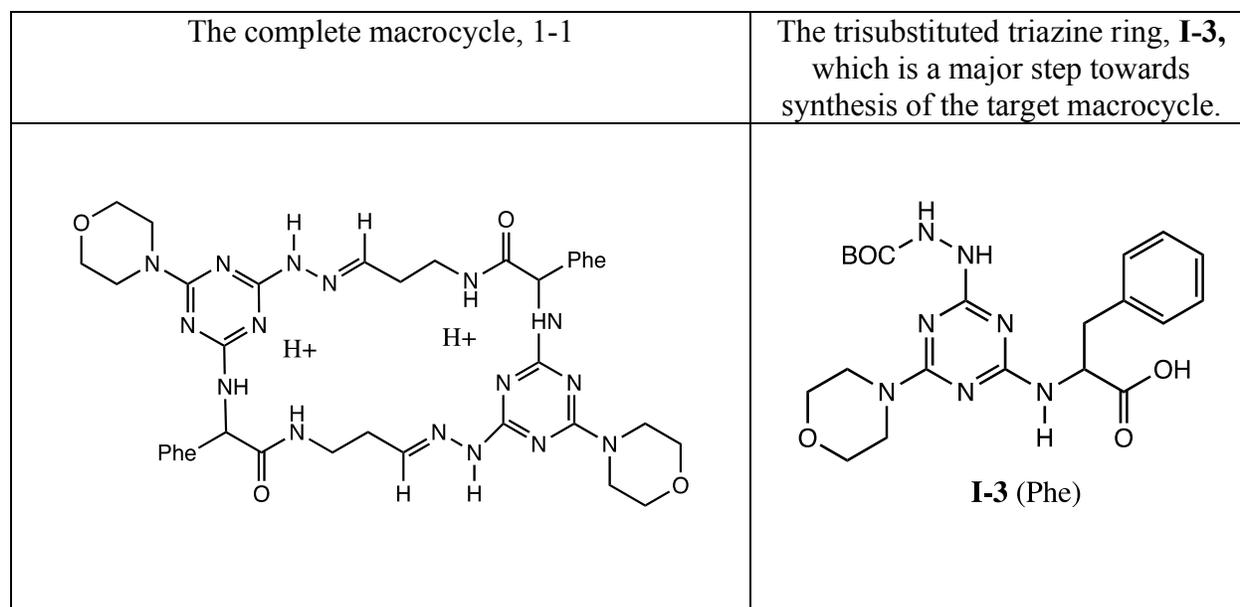
Department of Music

## ABSTRACT

A revival of attention paid to large drugs, such as macrocycles, has been fueled by the idea that there may be therapeutic targets available to them that are unavailable to smaller molecules. The pharmaceutical space that includes large, cyclic molecules has long been ignored by drug developers. This phenomenon is because, orally absorptive drugs shouldn't exist there, based on Lipinski's Rule of 5. Additionally, synthesizing such large molecules is complex, time intensive, and unpredictable. The Simanek group aims to establish a novel and straightforward synthesis of these targets. Establishing this approach allows for investigation into why large drugs, like the immunosuppressant cyclosporin, are so orally bioavailable. Additionally, this research creates opportunities to design large cyclic drugs with intentionality and flexibility. This intentional design includes the addition of specific functional groups and amino acids to maximize therapeutic potential.

The long-term goal of this project is the synthesis of the complete macrocycle, referred to at **1-1** (Figure 1, left). The target molecule has a triazine with a BOC-protected hydrazine group, a morpholine, and phenylalanine. The first step of this experiment is the synthesis of the trisubstituted triazine ring, called intermediate 3 or **I-3** (Figure 1, right). To create the target molecule, I-3, three groups were substituted onto a triazine ring. This synthesis occurred in a stepwise substitution process. The product was purified through silica column chromatography. Upon examination by  $^{13}\text{C}$  and  $^1\text{H}$  NMR, the spectra reveal resonances that are diagnostic for formation of the target intermediate. Thus, it was determined that the trisubstituted molecule was successfully synthesized.

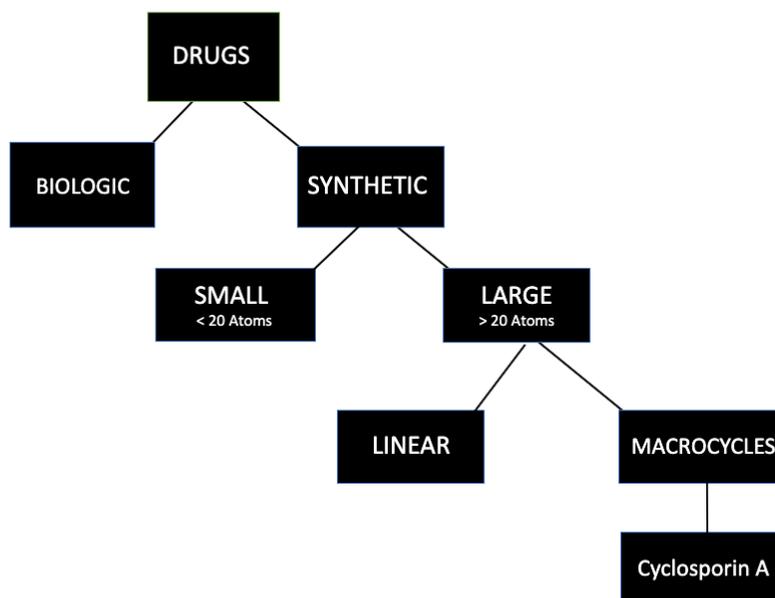




**Figure 1:** The complete macrocycle (left) and the intermediate **I-3** synthesized for this project (right).

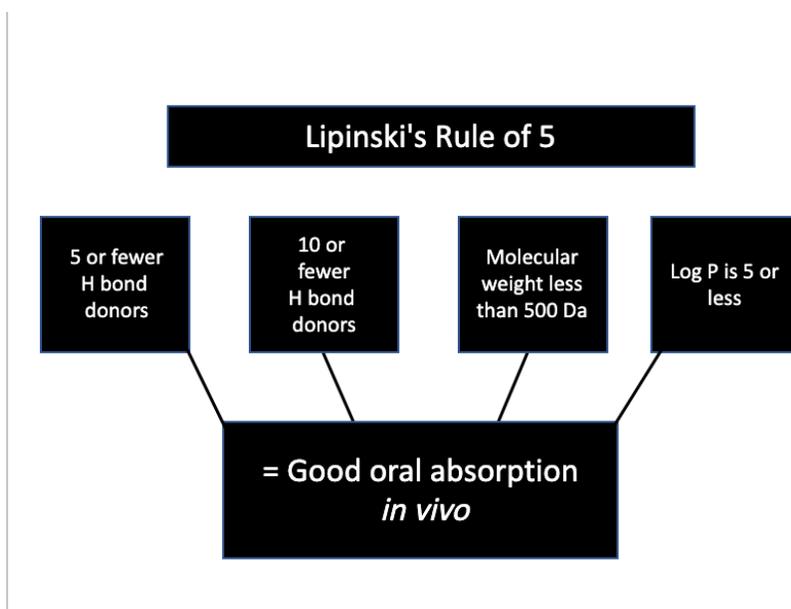
## INTRODUCTION

Drugs can be divided into two main categories: synthetic and biologic. Biologic drugs are made from living sources, whereas synthetic drugs are produced by multistep synthesis chemically, as in a laboratory. Synthetic drugs are classified as small or large, depending on whether they have more or less than 20 atoms. Lipinski's predictive rule of 5 suggest that smaller drugs have a higher likelihood of oral successful oral absorption. As a result, small drugs historically been more popular subjects of drug discovery research. Large drugs include linear molecules and cyclic macrocycles. Cyclosporin A is one such macrocycle.



**Figure 2.** A hierarchal classification of drugs

Pharmaceutical synthesis can be a difficult and time-consuming endeavor. Thus, there is a great deal of value in being able to predict success and failures of these compounds in advance. To evaluate a chemical compound's potential "drug-likeness" in advance of this synthesis, Lipinski's Rule of Five is used. The Rule of Five, or "Ro5" has been a popular general guideline in drug development since 1995. Its name comes not from 5 rules or guidelines, but because each of the four tenets of the rule include a multiple of 5. It is used to evaluate if compounds have the physical and chemical properties that would make them orally active. In creating new molecules, adhering to these guidelines dramatically increases probability of success in terms of drug absorption and permeation in vivo.



**Figure 3.** In order to be considered Ro5 adherent drugs, molecules must break no more than one part of Lipinski's rule.

### Lipinski's Rule of 5

Lipinski's rule of 5 "Ro5" is a general physiochemical guideline for evaluating whether or not a new synthetic drug is likely to be successfully absorbed in the body following oral administration. It is worth noting Lipinski's rule "of five" contains only four parts. It is named the rule "of five" because each part includes a multiple of five as the numerical cutoff. The coefficients for the rules are five, five (x2), five (x100), and five again. The rule suggests that a drug will be orally available if the following four criteria are met.

The first criterion is that there are no more than 5 hydrogen or "H" bond donors. This number is calculated as the sum of the OH's and NH's present on the molecule. If the number is less than or equal to five, the criterion is met.

The second criterion is that there are no more than 10 hydrogen or "H" bond acceptors. This number is calculated as the sum of the O's and N's present on the molecule. If the number is less than or equal to 10, the criterion is met.

The third criterion is that molecular weight is less than 500 Da. The molecular weight is a sum of the atomic mass units of the atoms in the molecule. If the number is less than or equal to 500 dalton, the criterion is met.

The fourth and final criterion is that the logP is no more than 5. The logP is a measure of lipophilicity. This is described as the log of the partition coefficient, P between n-octanol and water. If the logP is less than or equal to 5, the criterion is met.<sup>1</sup>

If these guidelines are not adhered to, however, common belief is that there is poor chance of oral absorption and, thus, a poorer chance of treating disease. As a result, there has historically been little interest and research going into areas of drug development that contradict, or “go beyond” the Ro5.

The most notable exception to the Ro5 is the success of the BRo5 drug Cyclosporin. Despite existing outside the Ro5 space at every opportunity, it is still orally available (Table 1). Not only this, but it is so successful that it has become the predominant immunosuppressant drug prescribed for heart, liver, and kidney transplant patients, saving innumerable lives in the process. Table 1 provides a rule-by-rule breakdown of the similarities between the successful Cyclosporin and the target molecule, **1-1**.

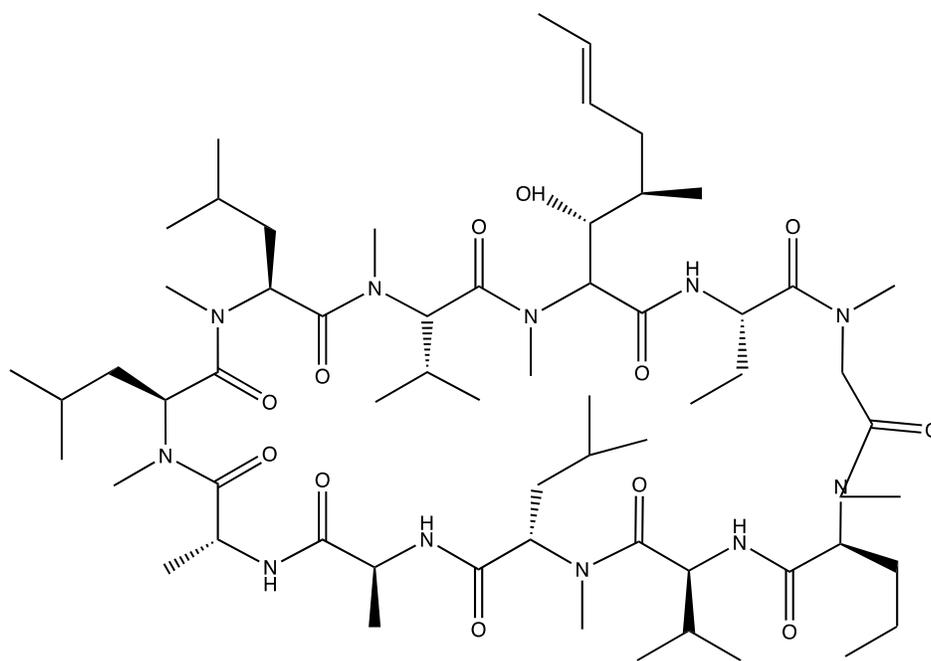
<b>Lipinski's Rule</b>	<b>Cyclosporin</b>	<b>Target Macrocycle 1-1</b>
	$C_{62}H_{111}N_{11}O_{12}$	$C_{42}H_{55}N_{18}O_6^{2+}$
1. No more than 5 H bond donors	5	6
2. No more than 10 H- bond acceptors (N's and O's)	23	24
3. Small molecular weight, less than or equal to 500 DA.	Mw 1202.6 g·mol <sup>-1</sup>	Mw 908.2 g·mol <sup>-1</sup>

4. Log of P is less than or equal to 5	23	Unknown.
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**Table 1.** A comparison of the BRo5 drugs Cyclosporin and the target macrocycle.<sup>2</sup>

### Cyclosporin

Cyclosporin is an immunosuppressant that was approved by the FDA in 1983 for preventing organ rejection. Since its original FDA approval rate in 1983, it has been prescribed over 25 million times from 2007-2017. It is frequently one of the 300 most prescribed medicines in the world.<sup>3</sup> Prescribed 1,834,555 times in 2017 alone, Cyclosporin is a shining example that demonstrates how Ro5 defying drugs can save tens of millions of lives and are worth immediate investigation.<sup>4</sup>



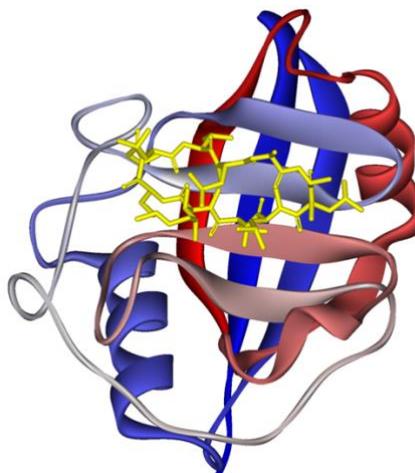
**Figure 5.** Cyclosporin is large macrocycle that breaks all guidelines in Lipinski's rule

Cyclosporin is able to permeate the phospholipid bilayer of the cell membrane. Normally, this penetration would require adhering to the "lipophilicity" guidelines of Lipinski's fourth rule.

Cyclosporin is able to bypass this because of its remarkable conformational changes.<sup>5</sup> Acting as a chameleon, it has the flexibility to display solubility in an aqueous environment by displaying its polar backbone, or to adopt a different conformation in apolar solvents. In these apolar solvents, its lipophilic groups cover its external surface and the polar groups are tucked away internally, engaging in intramolecular hydrogen bonds.

Cyclosporin is a cyclic peptide that binds to cyclophilin.<sup>6</sup> Cyclophilins are proteins associated with isomerase activity that are involved in mediating protein-protein interactions. They contribute to activation of immune responses. Cyclosporin binds to these cyclophilins, which suppresses activation of the immune system. This allows the cessation of immune responses to newly transplanted organs that might otherwise lead to harmful inflammation and organ rejection.<sup>6</sup>

The X-ray structure of this interaction between cyclophilin and cyclosporin is pictured below in (Figure 4).<sup>7</sup> Notably, the BRo5 drug cyclosporin does not tuck into a small pocket within cyclophilin. Cyclosporin forms a complex with cyclophilin and takes up a great deal of space on cyclophilin. This complex is able to suppress immune responses that would lead to organ rejection. Despite its space-occupying approach that differs from the mechanism of most Ro5 drugs, cyclosporin works. The bioavailability of cyclosporin is 29%, which is “remarkably high.”<sup>8</sup> Based on this model, successful drugs exist in the space Bro5.

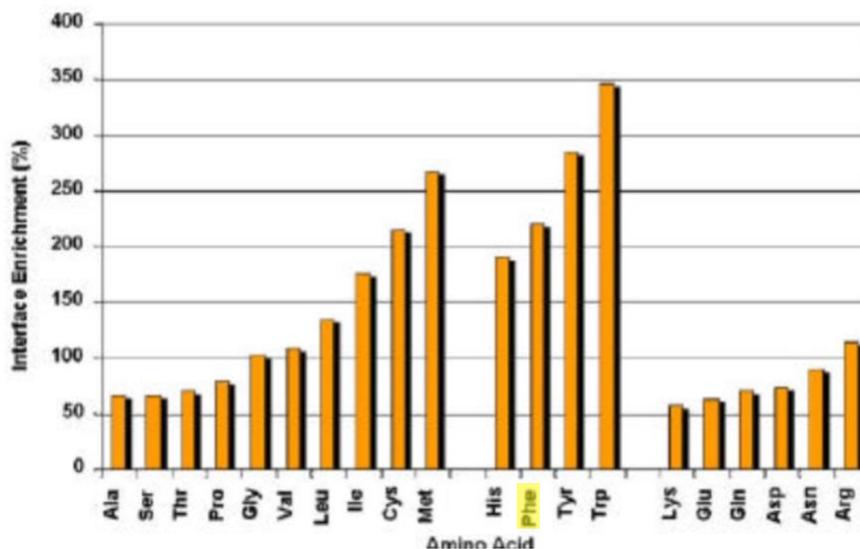


**Figure 4.** The macrocycle cyclosporin (yellow) binds the protein cyclophilin (red). Cyclosporin does not bind within a small pocket.<sup>9</sup>

When the Ro5 is taken as a cardinal rule, instead of a general guideline, a massive space is ignored in the drug development scene. Within this space lies cyclosporin, macrocycles such as those synthesized by the Simanek group, and innumerable others awaiting discovery. This space is historically under-researched and has unknown potential for lifesaving medicines.

### **Design**

The Simanek group aims to ameliorate this situation by designing large macrocycles similar in shape and size to cyclosporin. The triazine ring allows for the attachment of different functional groups, such as amino acids. In this experiment, phenylalanine was chosen as the amino acid because of the desire to target protein-protein interaction sites. Figure 5 shows the uneven distribution of various amino acids present at protein-protein interaction sites within the body.<sup>10</sup> Amino acids containing aromatic groups are the most abundant at these sites. Phenylalanine has an aromatic group and had near 225% interface enrichment, which reflects its high frequency of appearance at the desired sites.<sup>10</sup> Therefore, it was the candidate of choice.

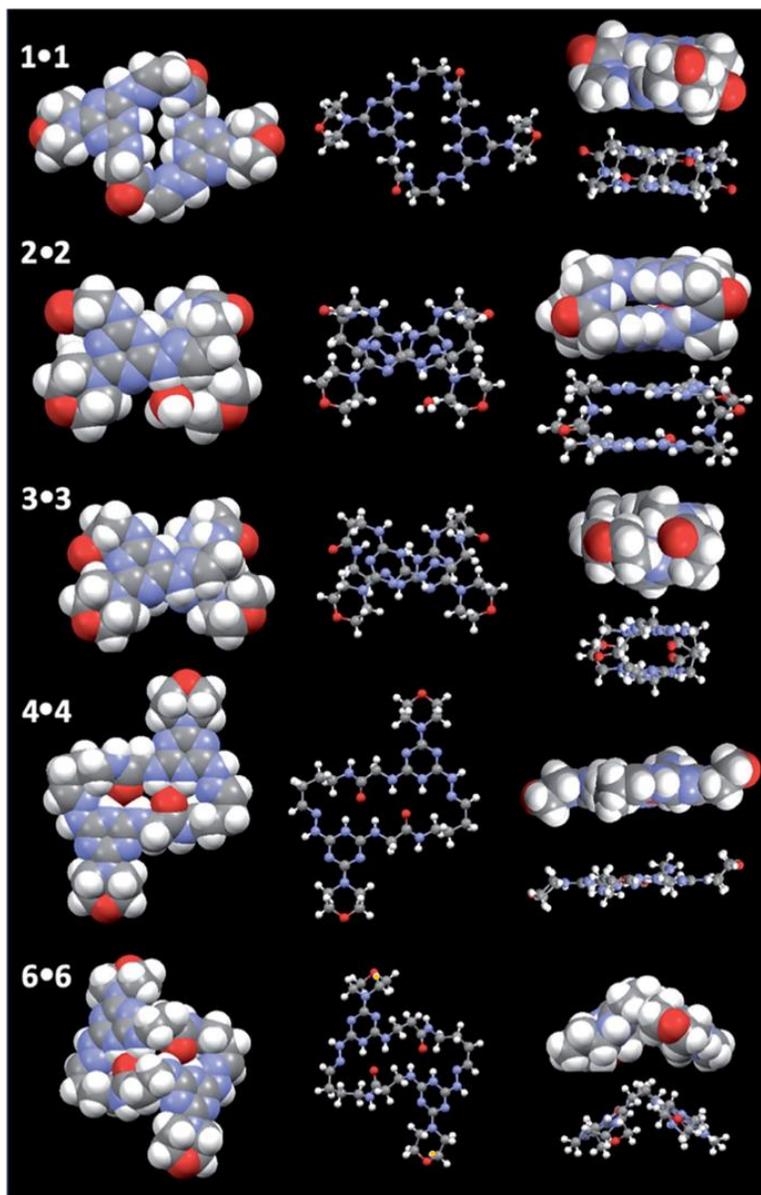


**Figure 5.** Amino Acid distributions at protein-protein interaction sites show that aromatic groups dominate.<sup>10</sup>

With one additional procedural step, the intermediate, **I-3**, spontaneously dimerizes into **1-1**. This dimer may have conformational variety like cyclosporin. While some portions of the molecule are rigid and unchanging, it is hypothesized that alterations at a few small points can contribute to folding that contributes to overall flexibility. One such point is the presence of a protonated nitrogen on the triazine ring. This cation has the ability to engage in bonding in two different locations, folding the macrocycle into several different conformations.

Such bonding can be better understood through ball and stick and space-filling modeling. X-ray crystallography has allowed for the modeling of these macrocycles, including those shown in Figure 5. In Figure 6, The (x,y) notation corresponds to a variable length of the macrocycle. They are labeled in order from smallest, **1.1**, to largest, **6.6**. These figures specifically demonstrate a visually apparent protonated nitrogen on the triazine ring, which is located opposite the morpholine ring. This proton has the ability to engage in hydrogen bonding with several different inner-molecular targets. In **1.1**, **2.2**, and **3.3**, the hydrogen is engaged in

hydrogen bonding with trifluoroacetate counter ion. In **4.4** and **6.6**, however, the hydrogen is engaged in hydrogen bonding with the carbonyl group of the monomer opposing it.<sup>11</sup> These differences in bonding lead to a dramatic difference in conformation.



**Figure 6.** Crystal structures of **1.1**, **2.2**, **3.3**, **4.4** and **6.6**.<sup>11</sup>

Differences in hydrogen bonding determine conformation: which parts of the macrocycle are folded to the interior or exposed externally. Based on this, the macrocycle will behave differently and exhibit different properties in vivo. It is hypothesized that these conformational changes will allow for a chameleon-like effect in the body, where the molecule is able to alter its properties such as solubility and lipophilicity. In this way, it is hypothesized the macrocycle could also would mimic the flexibility of cyclosporin. If so, it is possible that it could eventually also mirror its great utility.

### **Lipophilicity**

One key characteristic of Ro5 drugs is their lipophilicity, or solubility in nonpolar solutions such as lipids. In Ro5 adhering drugs, this solubility is low. The goal is to ensure that these small molecules are able to permeate the phospholipid bilayer of the cell membrane, and thus able to affect change within the cell. In large macrocycles such as cyclosporin, conformational changes allow for this solubility to change depending on which part of the molecule is exposed to the environment.<sup>12</sup> This eliminates the need for this constant nonpolar solubility, or lipophilicity. The lipophilicity of the Simanek group's target **1-1** macrocycle is yet to be calculated. However, based on an informed understanding of how cyclosporine's changing conformations subvert the need for constant lipophilicity and the computational models, it is believed that target macrocycle will be able to do the same.

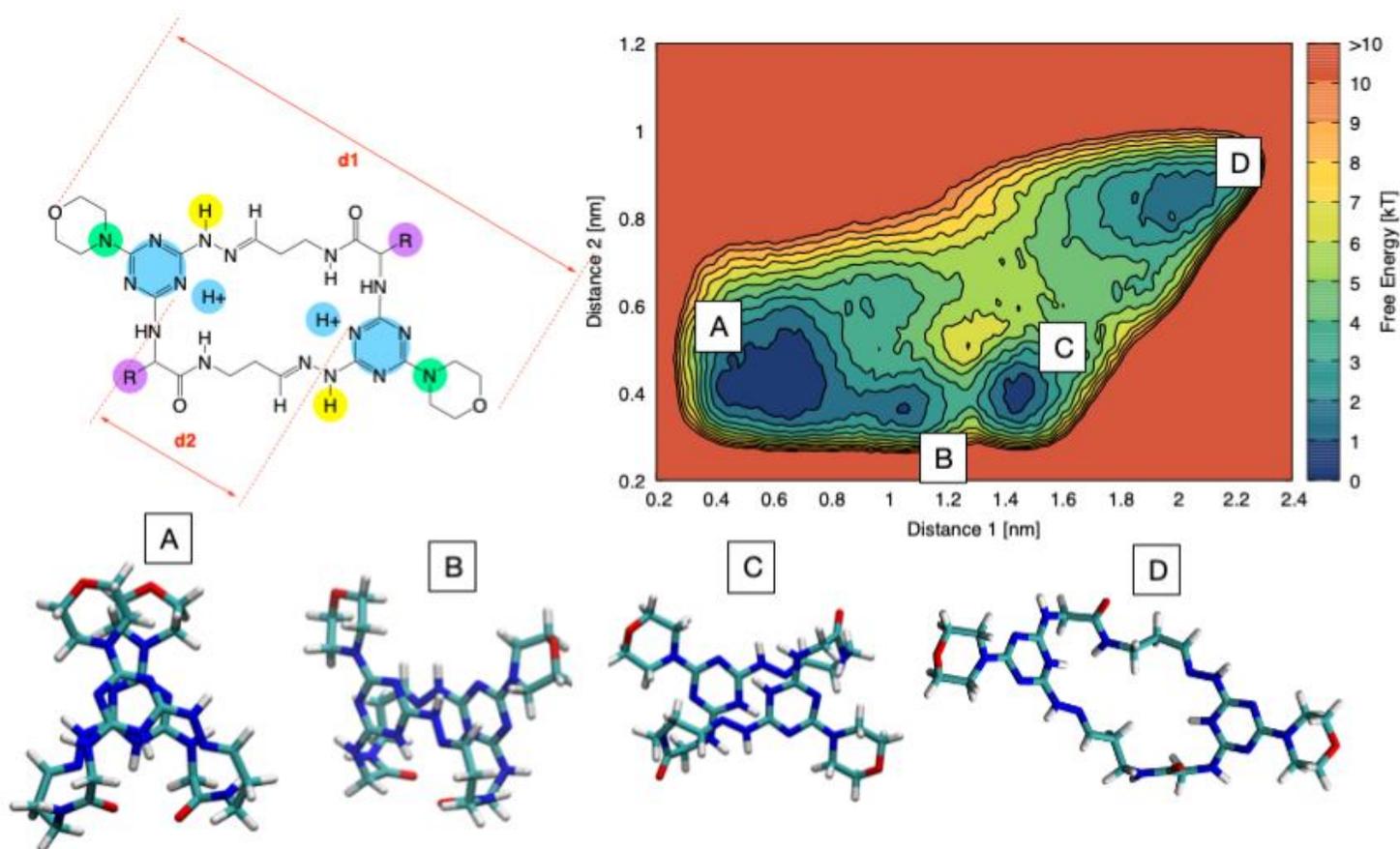
### **Conformations**

Due to its large size, the proposed macrocycle in the BRo5 space will be unable to accomplish this protein inhibition in a mechanism similar to that of small Ro5 adhering molecules. Instead of inhibiting protein-protein interaction by fitting into small cave-like

openings on the target, it has to work indirectly. The large size of these macrocycles allows for breadth of surface coverage on the target.

It is hypothesized that the target macrocycle **1-1** has the flexibility to adhere to different conformations, as cyclosporin does. These four different conformations are key for flexibility and adaptability to a variety of diverse biologic environments. This macrocycle has been modeled in four major conformations, which are shown below in Figure 7 as “A, B, C, and D.” The A and B conformations are labeled colloquially as the folded “taco” conformations. Each of the four conformations vary in amount of free energy.

Free energy allows for the conformational flexibility, but not without challenges. In the 2-D figure at the top left of Figure 7, the red line d1 represents the distance between oxygens. D2 represents the distance between nitrogen. Each pixel represents the energy associated with a specific d1 and d2 combination. Additionally, the colors are used to identify pairs within the homodimer. D1 and d2 values are combined in the top left figure to explain the relationship between macrocycle conformation and macrocycle free energy. A is the most compact, then B, then C, then D.<sup>13</sup> Moving between various conformations requires meeting necessary energy requirements. Several conformations exist, and energy barriers suggest that the molecule is flexible to adopt different shapes. Free energy (kT) is a product of the Boltzmann constant and temperature. It is worth noting that all four of these conformations are possible at room temperature.



**Figure 7.** Four conformations of the Simanek group's 1-1 macrocycle and their respective free energies <sup>13</sup>

## MATERIALS AND METHODS

### **Experimental Procedures: “D” Synthesis of 2,4-dichloro-6-BOC-Hidrazinyl Triazone**

*Preparation of Intermediate 1 (I-1).* Cyanuric chloride (368mg, 2mmol) was added as a solid to a 100 mL round bottom flask with stir bar. THF (20mL) was added to bring the concentration to 0.1 M. This flask was chilled in an ice bath with water. In a separate vial, 1 equivalent of BOC-Hydrazide (265mg, 2 mmoles) was dissolved in ~20mL water to a concentration of 0.5 M. The solution of BOC-hydrazide was added dropwise over 7 minutes to the chilled solution of

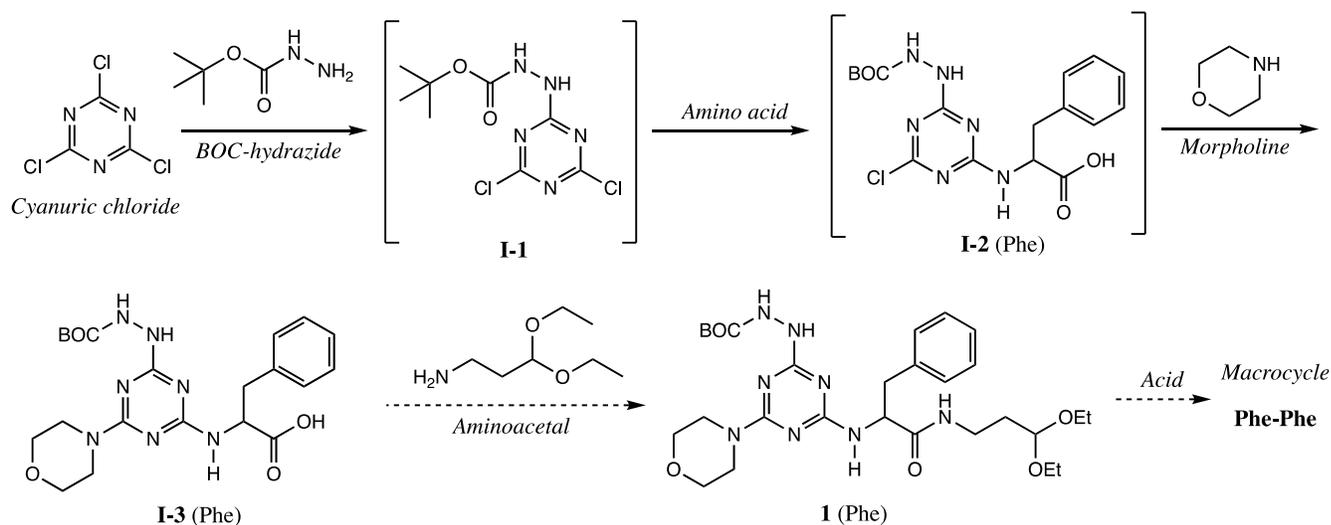
cyanuric chloride still on ice. One half equivalent of sodium bicarbonate solid (168mg, 2 mmoles)  $\text{NaHCO}_3$ , which was added directly to the stirring mixture. The solution became more basic as the sodium bicarbonate dissolved; this was confirmed by darkening litmus paper strips showing an increase in pH value. A small aliquot was removed for TLC and the solution was sealed. Solid phenylalanine was dissolved in water with ~20 drops THF. After 24h, slight head was added. Two equivalents (332mg, 2 mmoles) of powdered amino acid L-Phenylalanine were added directly to the solution. One equivalent (167mg, 1 mmoles) of solid sodium bicarbonate were added directly to the solution to decrease the likelihood of side reactions. After 24 hours, 8 equivalents of morpholine (1mL) were directly added dropwise. The solution stirred overnight. TLC plate was run, to confirm BOC-Hydrazine attachment and morpholine placement. The solution in the round bottom flask was placed on the rotary evaporator for 1.5 hours to remove as much morpholine as possible. The solution was put under airflow for 48 hours. A TLC plate was run in 19/1 MeCl/MeOH, with 1%  $\text{NH}_4\text{OH}$ . The  $\text{NH}_4\text{OH}$  was burnt off before staining with Ninhydrin. The product was isolated by silica gel chromatography using 9/1 DCM/MeOH as an eluent.

## RESULTS AND DISCUSSION

### **Synthesis procedures**

Scheme 1 shows the route for stepwise organic synthesis. The solid arrows reflect the path to the intermediate **I-3** that was synthesized in this experiment. The dotted arrows reflect the synthetic steps necessary to synthesize the macrocycle **1-1** from the intermediate **1-3**. The brackets around the intermediates designate that each of these reactions was done in the same round bottom reaction flask. This simplicity is a significant value to this synthetic approach.

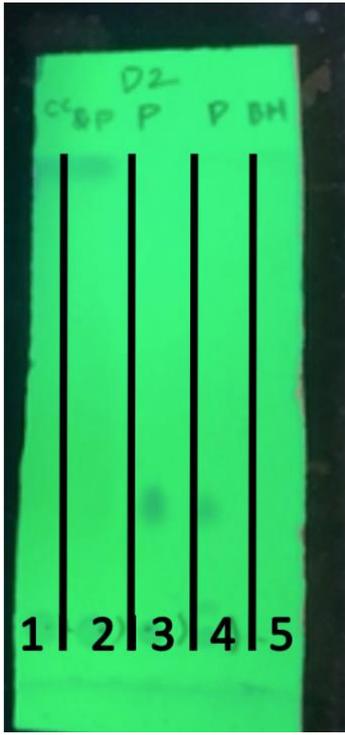
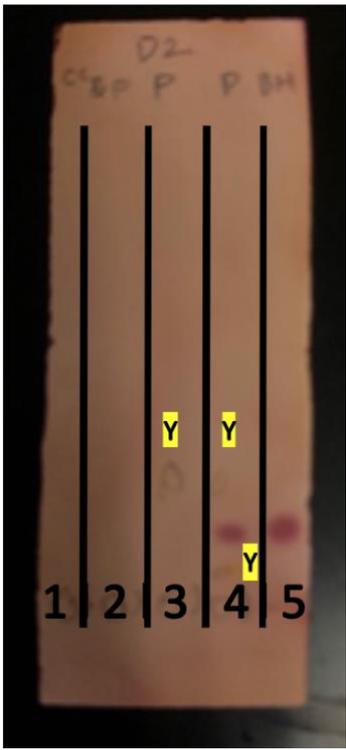
In this experiment, the three stepwise additions were made to the cyanuric acid-based triazine ring. In summary, BOC-hydrazide was added to cyanuric chloride, which was then treated with the amino acid Phenylalanine. Then, morpholine was added and the product was purified through chromatography. After each addition, the product was analysed by thin layer chromatography (TLC) in order to confirm desired attachment onto the triazine ring. This was based on examination under UV light, where the synthesized target **I-3** was compared with a comparison solution of **I-3**, which is usually labelled “FA.” In these comparisons, spots closely resemble the comparison solution in size, shape, and location on the TLC plate. TLC plates were then tested with a Ninhydrin stain for primary amines. Red-tinted plates reveal similar results; that the synthesized target **I-3** closely resembles the comparison solution of **I-3**. This is further evidenced by the presence of yellow spots, often found slightly above the maroon spots. These yellow spots reflect the attachment of BOC-Hydrazine onto the triazine ring, which is a vital component of **I-3**'s synthesis. Upon analysis of the TLC plates of trial “D,” it was predicted with confidence that the target intermediate, **I-3** had been successfully synthesized. Both  $^1\text{H}$  and  $^{13}\text{C}$  NMR confirm that **I-3** was prepared. The following paragraphs provide additional details for each step.



**Scheme 1.** The macrocycle, **1-1** is synthesized by a series of stepwise reactions, where I-3 is an intermediate.

*Preparation of Intermediate 1 (I-1).* Briefly, cyanuric chloride was dissolved in tetrahydrofuran and stirred while chilled on ice. A solution containing one equivalent of BOC-hydrazide was added dropwise over a period of 7 minutes. To this iced solution, one equivalent of sodium bicarbonate was added to increase basicity. The reaction was then allowed to progress in an ice bath for 50 minutes. TLC analysis was run to confirm BOC-hydrazine addition onto the triazine ring. The solution remained a clear, homogenous liquid without any notable color change.

Figure 8 shows the TLC plate D2, on which P represents a comparison reaction mixture. *Product is I-1*, a mixture of cyanuric chloride and BOC-Hydrazide. Under short wave UV light, baseline spots can be seen in all lanes that contain the product. The ninhydrin stain for primary amines reveals spots in both lanes where BOC Hydrazine was placed. Additionally, it contains yellow-staining spots. These spots reflect the attachment of BOC-Hydrazine onto the triazine ring. In these plates, P is a mixture of cyanuric chloride and BOC-Hydrazine.

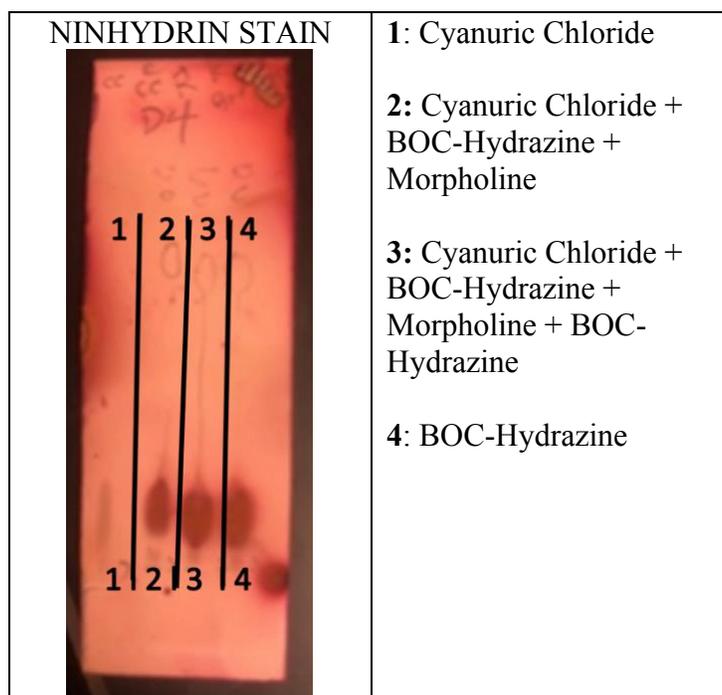
SHORT WAVE UV LIGHT		NINHYDRIN STAIN	
	<p><b>1:</b> Cyanuric Chloride + product</p> <p><b>2:</b> Cyanuric Chloride + product</p> <p><b>3:</b> Product</p> <p><b>4:</b> Product + BOC-Hydrazine</p> <p><b>5:</b> BOC-Hydrazine</p>		<p><b>1:</b> Cyanuric Chloride + product</p> <p><b>2:</b> Cyanuric Chloride + product</p> <p><b>3:</b> Product</p> <p><b>4:</b> Product + BOC-Hydrazine</p> <p><b>5:</b> BOC-Hydrazine</p> <p>*Y denotes yellow spot indicating BOC-Hydrazine attachment</p>

**Figure 8.** Short wave UV light and ninhydrin-stained TLC plate “D2.”

*Preparation of Intermediate 2 (I-2).* At room temperature, one equivalent of solid phenylalanine was added directly to the reaction mixture including intermediate **I-1**. This was in an effort to retain the simplicity of the one-pot synthesis.

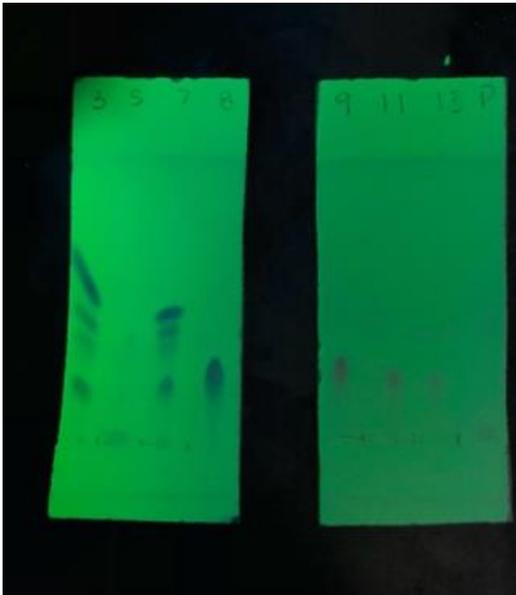
*Preparation of Intermediate 3 (I-3).* One equivalent of liquid morpholine was added dropwise directly to the reaction mixture. The reaction mixture was measured by TLC D4 to confirm morpholine addition onto the triazine ring. When this morpholine addition was confirmed, the reaction mixture was placed under a rotary evaporator for one hour, then under direct airflow at room temperature for 48 h.

TLC D4 was stained with Ninhydrin. Dark spots at the baseline reflect a great deal of morpholine, which appears heavy and maroon in Figure 9. This informed the decision to evaporate some excess morpholine by using the rotary evaporator and holding the product under constant airflow for 48 hours.



**Figure 9.** TLC plate D4 shows dark morpholine spots.

Purification of this target, **I-3**, was accomplished by silica gel chromatography. The solvent used was 19/1/ DCM/MeOH with 1% NH<sub>4</sub>OH. Approximately 23 fractions were obtained and allowed to evaporate for 48 hours at room temperature under a fume hood. TLC tests were continually run on fractions to identify when product exited the column. They were compared against a control of a purified intermediate **I-3**. This comparison was a provided sample. Fractions 8 and 9 resembled this target intermediate most closely.

<p style="text-align: center;">SHORT WAVE UV LIGHT</p> 	<p><b>Left plate:</b> Fractions 3, 5, 7, and 8</p> <p><b>Right plate:</b> Fractions 9, 11, 13, control/comparison F Phenylalanine acid product</p>
<p style="text-align: center;">NINHYDRIN STAIN</p> 	<p><b>Left plate:</b> Fractions 3, 5, 7, and 8</p> <p><b>Right plate:</b> Fractions 9, 11, 13, control/comparison F Phenylalanine acid product</p> <p>*No yellow Ninhydrin-stained plate indicating successful attachment onto triazine.</p>

**Figure 10.** TLC results from a variety of fractions collected from column chromatography.

Upon visual examination of the fractions, a cloudy white film appeared above the solution line on fractions 8 and 9. These fractions were tested by TLC. TLC plate analysis via short wave UV light and ninhydrin-staining demonstrated patterned spots that were remarkably similar in shape, size, and location to the patterned spots of the target intermediate **I-3** (right

lane). Dark ninhydrin stains at the baseline indicate morpholine is present. Additionally, after ninhydrin staining, yellow spots were still visually apparent, which indicated BOC-hydrazine was still attached. Based on this confirmation, NMR was run.

<p style="text-align: center;">SHORT WAVE UV LIGHT</p> 	<p><b>Left:</b> Fraction 8</p> <p><b>Center:</b> Fraction 9</p> <p><b>Right:</b> Phenylalanine acid product</p>
<p style="text-align: center;">NINHYDRIN STAIN</p> 	<p><b>Left:</b> Fraction 8</p> <p><b>Center:</b> Fraction 9</p> <p><b>Right:</b> Phenylalanine acid product</p>

**Figure 11.** TLC plates from the most promising fractions, 8 and 9, compared with a solution of phenylalanine acid product.

Based on this confidence,  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  tests were run to assess the content of the final purified product.  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  tests reveal the target molecule was synthesized via trisubstitution with BOC-hydrazine, phenylalanine, and morpholine. An excess of morpholine appears to be present via NMR analysis. This step's successful completion

provides a foundation for further steps to synthesize the complete macrocycle, as evidenced by nuclear magnetic resonance spectroscopy.

### **Characterization by $^1\text{H}$ NMR.**

Three signal sets are diagnostic for successful triazine ring substitution. These sets of signals derive from each of the three substituted groups: morpholine, the BOC group, and the amino acid. The NMR spectra in Figure 12 shows these three groups.

The appearance of diagnostic resonance around 7.0 ppm corresponds to the hydrogens on the aromatic ring of the amino acid phenylalanine. Impurity around  $\sim 5.0$  ppm is unclear. Due to quantity, this is most likely water. The upfield region of the spectra presents a poorly-resolved alpha proton around  $\sim 4.8$  ppm on the chiral alpha carbon of phenylalanine. Slightly upfield at  $\sim 3.7$  ppm are two peaks reflecting the protons on the morpholine ring. The BOC group is identified far upfield near  $\sim 1.4$  ppm.

The integrated intensity of a signal in a  $^1\text{H}$  NMR spectrum is based on the ratio between the number of hydrogens that give rise to each signal. This allows for the calculation of the total number of hydrogens in the sample. In this spectrum, the integration value is 1.0; this reflects a 1:1 ratio. The three methyl groups on BOC represent a known value of nine hydrogens. The peak "E" gives an integration value of exactly nine, reinforcing this ratio. Based on this, it also becomes apparent that there are excess hydrogens due to impurities. These impurities are most likely excess morpholine and acetone. The amount of any specific impurity could be quantified by dividing the integration value of the impurity over the integration value of the whole, then multiplying this value by 100 to get a percentage.

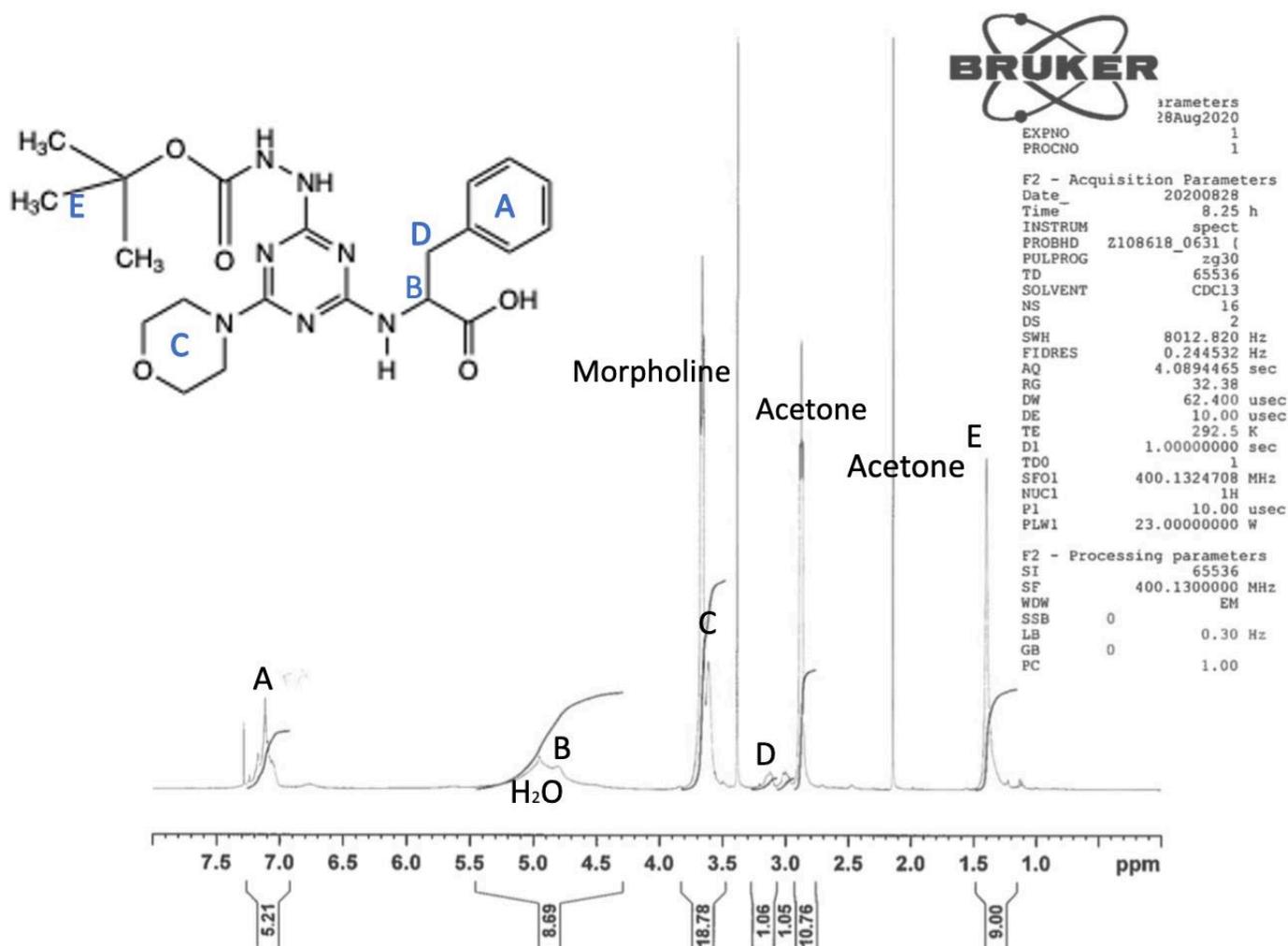
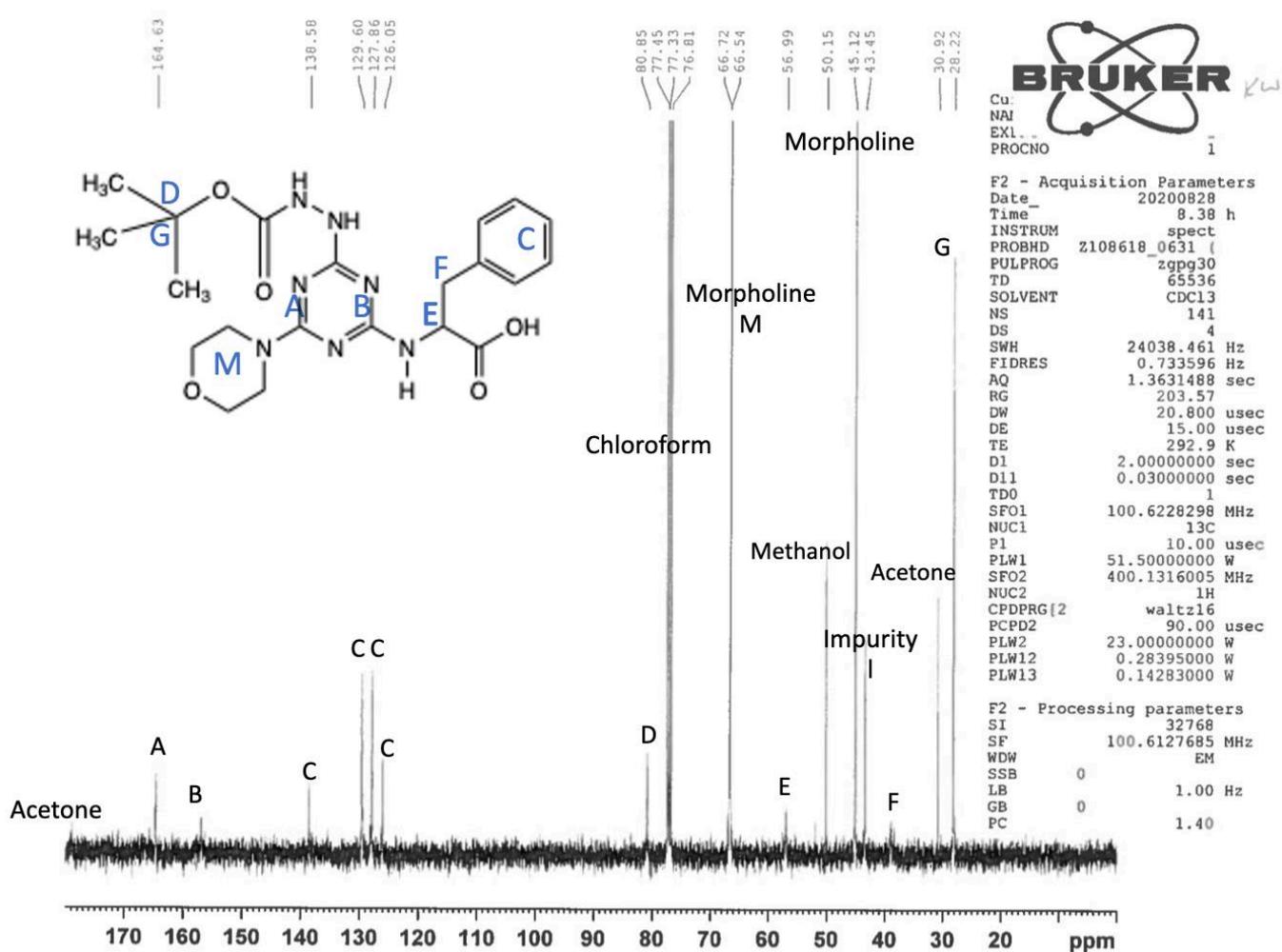


Figure 12.  $^1\text{H}$  NMR spectrum with corresponding macrocycle locations.

### Characterization by $^{13}\text{C}$ NMR

As with  $^1\text{H}$  NMR, three signal sets are diagnostic for successful trisubstitution in this experiment. The three sets derive from the three substituted groups: morpholine, the BOC group, and the amino acid. The NMR spectra in Figure 13 shows these three groups. In this spectrum, broad and poorly resolved triazine signals are common of rotational isomers that are derived

from restricted rotation around the triazine bonds. One triazine ring resonance is visible near  $\sim 157$  ppm, while the other two are found downfield near  $\sim 165$  ppm. This is characteristic for triazine resonances, which usually appear between 166-162 ppm. In this spectra, the amino acid's aromatic rings are identified  $\sim 125$ -140 ppm. The BOC, or tert-butyloxycarbonyl protecting group, is identified near  $\sim 81$  ppm. It is fragmented and also present near  $\sim 27$  ppm. Also apparent in this spectrum is the asymmetry of the morpholine ring, a large peak near  $\sim 66$  ppm. Impurities such as acetone are present around 45, 43, and 31 ppm.



**Figure 13.**  $^{13}\text{C}$  NMR spectrum with corresponding macrocycle locations.

**Barriers to completing organic synthesis.**

The threefold difficulties with chemical substitution all have to do with its characterization as a sometimes time consuming and unpredictable process. The synthesis described in this experiment was comprised of several steps, which necessitated wait times of 1-48 hours between each step. This reaction was simplified significantly by the “one-pot” reaction approach. The reaction was complicated by experimental error. Frequent problems arise with synthesis, which necessitate starting over. One such problem that arose in this experiment was an excess of morpholine that would not dissipate despite forced air flow stationary evaporation, rotary evaporation, and test-tube extraction with HCl. While the hope would be to purify this product and remove the excess morpholine, if this was unsuccessful, it is possible that starting over would have been a better approach. Major problems arose with synthesis attempts A-C. In order to increase the yield and clarity of results, all reactant values were doubled for trial “D” in order to synthesize a “double batch.” Eventually, the synthesis of the proposed intermediate is confirmed through NMR.

CONCLUSIONS AND FUTURE DIRECTIONS

It would be a mistake to ignore the pharmaceutical space that contains large molecules that do not adhere to Lipinski’s rule of 5. In this space exists large, cyclic macrocycles of particular interest to the Simanek group. One model for an orally absorptive molecule in this space is Cyclosporin, which has proven to be tremendously successful and vital to lifesaving practices for millions of organ transplant recipients, arthritis patients, and others. Establishing the presence of orally absorptive molecules beyond the rule of 5 could dramatically redirect the research focus of the pharmaceutical industry. In a broader sense, this could pave the way for a tremendous amount of research in a previously unexplored place. This place could be rich with

macromolecules that, in time, prove to be as valuable as cyclosporin, but further exploration is necessary to determine this.

Efforts in this experiment center around the design and synthesis of an intermediate, **I-3**, of a target macrocycle, **1-1**. This macrocycle is one of many examples being targeted to demonstrate the value in drugs that exist BRo5. Part of this utility is due to **1-1**'s ability to make conformational changes, such as folding, at room temperature. This folding can be attributed to hydrogen bonding. It is hypothesized that this "dance" between conformations could contribute to **1-1**'s successful membrane permeation and oral absorption in a manner similar to cyclosporin.

Adaptability is supported by the addition of a variety of different side groups, which could include a variety of different any amino acids. In this experiment, the amino acid was phenylalanine. This is because the macrocycle's intended target is protein-protein interaction sites, and phenylalanine is prevalent at such protein-protein interaction sites. This prevalence, along with favorable energetics, made it an ideal candidate.

In this experiment, the three stepwise additions of were made the cyanuric acid-based triazine ring. These each occurred in a series of days and were verified at each addition step and intermediate step by thin layer chromatography (TLC) in order to confirm desired attachment onto the triazine ring. Upon analysis of the TLC plates of trial "D," it was predicted with confidence that the target intermediate, "I-3" had been successfully synthesized. The product was then purified through silica gel chromatography and analyzed via H-NMR and C-NMR. Upon analysis of this data, despite messy contributions from excess morpholine, it was determined that the product did indeed contain target molecule, which was successfully synthesized.

The process of bringing these drugs to market is extremely long and highly selective as well. Because most marketable drugs are orally absorptive and adhere to the Ro5, most research exists in this space. It is difficult to garner support for BRo5 drugs who don't have the same broad history of success. Further research into the BRo5 space is thus slightly dependent upon the marketable success of existing drugs in the BRo5 space. Researchers need to prove successful drugs exist in this space if they hope to ever garner additional breadth and depth of interest. Thus, barriers to finish research in the BRo5 space involve the lack of support for BRo5 drugs as compared to Ro5 adhering drugs. This support comes in forms of money and researchers, it and creates quite an initial hurdle to overcome in order to begin.

### **Future directions**

A familiar adage in the synthetic drug development world is "Methyl-ethyl-butyl-futile." This refers to the exhaustive search through compositional space in order to try and find a real drug. This inevitably leads to a significant amount of "futile" dead ends. In essence, medicinal chemists often experience such lack of success because drug design is incredibly challenging. The Simanek group aims to eliminate some of these challenges by providing a straightforward path to a synthetic molecule that can be easily modified with additions and substitutions of amino acids. This will allow for specific biologic targeting of certain proteins. This targeting allows for the selective inhibition of certain protein-protein interactions. Inhibiting such interactions is a common mechanism for many successful pharmaceutical drugs and could elicit reactions such as the genesis or cessation of any number of biological functions. The discovery and development of a macrocycle ceasing the function of biologically harmful cells, such as cancer cells, could be life-saving technology.

### ACKNOWLEDGEMENTS

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### ABBREVIATIONS

BOC- tert-butyloxycarbonyl.

BRO5- Beyond the rule of 5 (contradicts criteria in Lipinski's rule).

C- Carbon.

H- Hydrogen

DCM- Dichloromethane.

MeOH-Methanol.

NH<sub>4</sub>OH – Ammonia hydroxide

NMR- Nuclear magnetic resonance spectroscopy.

Phe- Phenylalanine.

Ro5- Rule of 5 (characterized as aligning with criteria in Lipinski's rule)

THF- Tetrahydrofuran.

TLC- Thin Layer Chromatography

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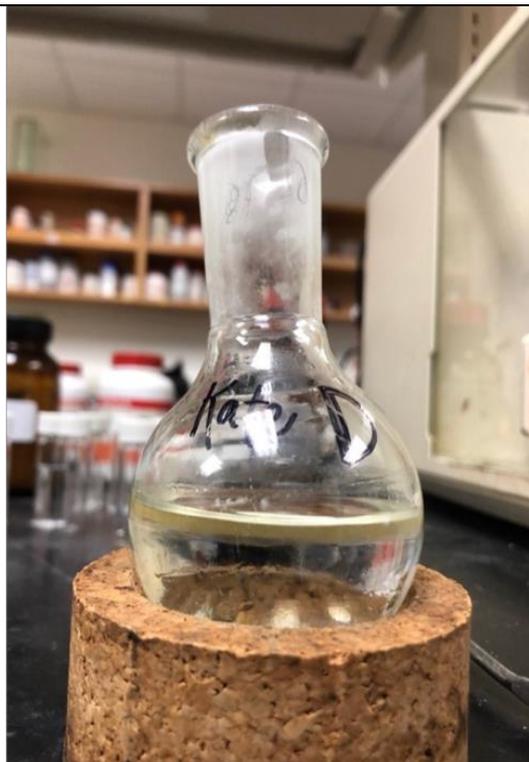
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[https://meps.ahrq.gov/mepsweb/data\\_stats/download\\_data\\_files-\\_results.jsp?cboDataYear=All](https://meps.ahrq.gov/mepsweb/data_stats/download_data_files-_results.jsp?cboDataYear=All) (accessed Nov 1, 2020).

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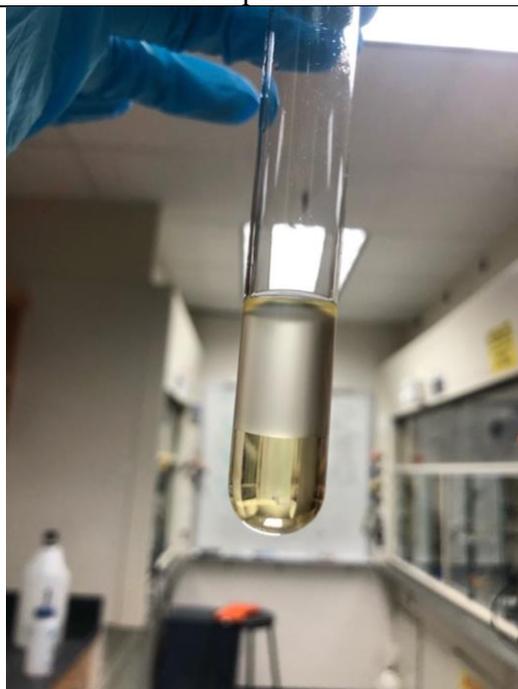
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APPENDIX

Product was placed on a rotary evaporator to evaporate excess morpholine



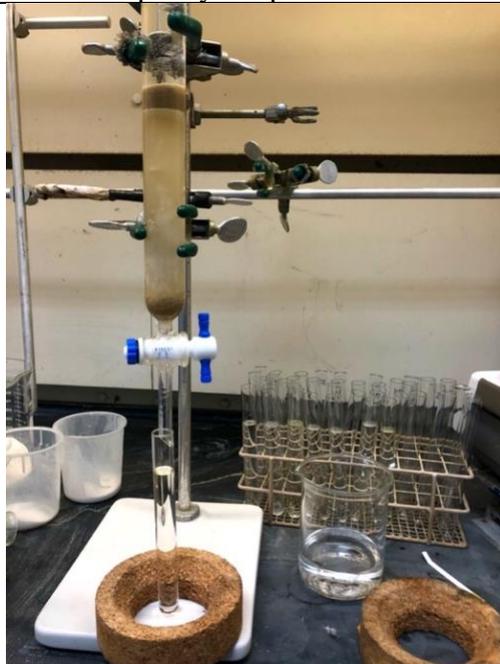
The round bottom flask reveals a heterogeneous solution that required extraction.



A test tube extraction shows the organic

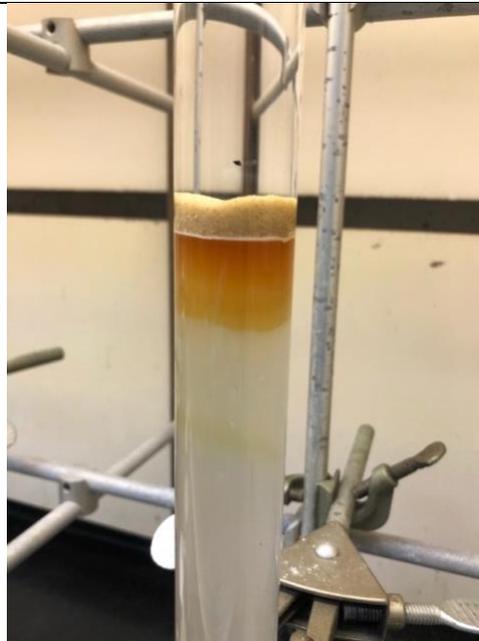


(cloudy) and aqueous (yellow) layers. Dilute HCl drops were added to reach a pH of 4. This was to protonate morpholine, making it water soluble and purify the product.

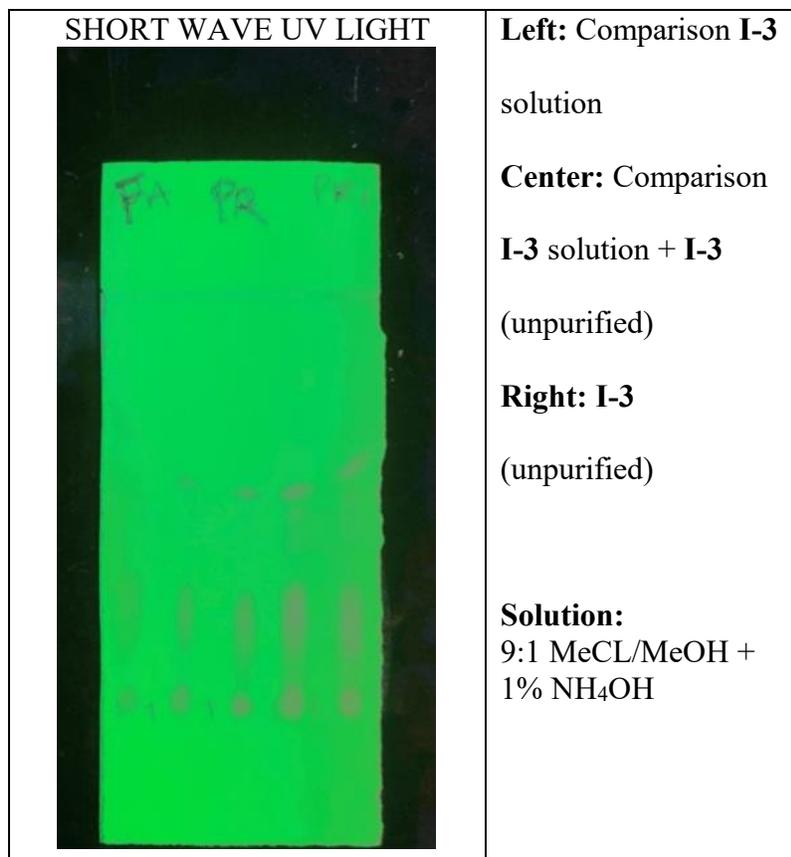


Silica column chromatography. Fraction 8 is pictured, which contained the final product.

Thick yellow oil is still present after the first "Rotovap" procedure to remove morpholine.



Column after product addition. Separation of orange product becomes visible with the downward movement of faint orange bands.



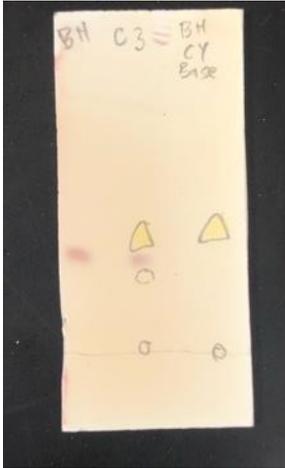
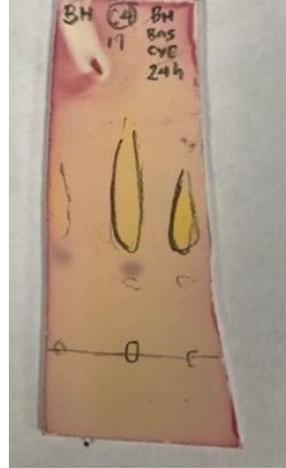
### Previous Trials: Synthesis: A-C.

#### Methods:

Cyanuric chloride (186mg, 1 mmoles) was added in as a solid into a 100 mL round bottom flask with stir bar. THF (10mL) was added to bring the concentration to 0.1 M. This flask was chilled in an ice bath with water. In a separate vial, 1 equivalent of BOC hydrazide (133mg, 1 mmole) was dissolved in water to reach a concentration of 0.5 M. The solution of BOC- Hydrazide was added dropwise over 3 minutes to the chilled solution of cyanuric chloride still on ice. Sodium bicarbonate solid (65mg, 0.06 mmoles) in the amount of a ½ equivalence was added to the stirring mixture. Litmus paper tests were completed until pH became more basic as the sodium carbonate dissolved. As small aliquot was removed for TLC. A stopper was placed on the solution. After 24 hours, 5 drops of THF were added to

dissolve one equivalence of phenylalanine (4 mmoles). At room temperature, phenylalanine was added dropwise into the solution over 7 minutes. After 24 hours, morpholine (0.35mL, 4 mmoles) was dissolved in water (7.5mL) and added dropwise into the solution. The solution was placed on the rotary evaporator for 1.5h and then analyzed via column chromatography in 19/1 MeCl/MeOH. 72 fractions were collected, fractions 70-72 were combined and tested via H-NMR and C-NMR. Calculated yield, including impurities, was near 385mg (4 mmoles).

### Trial C – Thin Layer Chromatography (TLC) ninhydrin stained plates

	<p>C3 In 19/1 DCM/MeOH</p> <p><b>Left:</b> BOC Hydrazine</p> <p><b>Center:</b> BOC Hydrazine + Cyanuric Chloride + NaHCO3</p> <p><b>Right:</b> BOC Hydrazine + Cyanuric Chloride + NaHCO3</p>		<p>C4 In 19/1 DCM/MeOH</p> <p><b>Left:</b> BOC Hydrazine</p> <p><b>Center:</b> BOC Hydrazine + NaHCO3 + Cyanuric Chloride</p> <p><b>Right:</b> BOC Hydrazine + NaHCO3 + Cyanuric Chloride (after 24h)</p>
	<p>C5 In 19/1 DCM/MeOH</p> <p><b>Left:</b> BOC Hydrazine + Cyanuric Chloride + NaHCO3 + F</p> <p><b>Center:</b> BOC Hydrazine + Cyanuric Chloride + NaHCO3 + F</p> <p><b>Right:</b> BOC Hydrazine</p>		<p>C6 In 19/1 DCM/MeOH</p> <p><b>Left:</b> F + Morpholine</p> <p><b>Center:</b> BOC Hydrazine + F + Morpholine</p> <p><b>Right:</b> BOC Hydrazine</p>