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Synthesis and Characterization of pH-Sensitive Rhodamine 6G Spirolactam Structures

Grace H. Taumoefolau

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Synthesis and Characterization of pH-Sensitive Rhodamine 6G Spirolactam Structures

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelors of Arts / Science in Chemistry from The College of William and Mary

by

Grace Hikari Taumoefolau

Accepted for ______________

Dr. Elizabeth J. Harbron, Director

Dr. Robert J. Hinkle

Dr. Deborah C. Bebout

Dr. Gexin Yu
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Abstract

As an extension of the RB sterics project, which studies the tunability of fluorescence turn-on pKₐ as a function of substituent A-value, similar efforts with R6G are presented. In light of previous failed attempts at di-ortho R6G derivative synthesis, this study sought to optimize synthetic procedures as well as explore alternative synthetic pathways. Optimization efforts, summarized by maintaining a cold and anhydrous reaction environment, were ultimately fruitful, significantly raising the chances at isolating product although purification of these products still poses some questions. Relatively pure products were then characterized with fluorescence studies, NMR spectroscopy and computational methods. R6G derivatives generally fluoresced at higher pKₐ as compared to their RB counterparts, albeit to a lesser extent than expected. Computation of R6G derivatives’ optimized geometries aided in understanding the NMR spectra of a few compounds. Methods for observing RB and R6G FRET were also explored, namely by investigating dye-embedded pHEMA films and attempting to covalently bind the two dyes together using azide-alkyne click chemistry.
Acknowledgements

My sincerest thanks goes to Dr. Elizabeth Harbron for letting me join her research group and allowing me to work with this project until the end. The details of my project can be grueling to say the least, but our shared excitement and frustrations over research fueled my motivation to keep going. Furthermore, words cannot express my gratitude for her patience and compassion when real life issues outside academics hit home. My four years at William & Mary would not have been the same if I did not take Organic Chemistry I with her in the spring of my freshman year, and my time as her advisee will ground my scientific career for many years to come.

I would also like to express my thanks to my committee members, Dr. Robert Hinkle, Dr. Deborah Bebout and Dr. Gexin Yu along with the Chemistry department. You all taught and helped me so much at different stages of my undergraduate years. I relish the opportunity to share the culmination of my time here through my honors thesis. I also want to specifically thank Professor Jeffrey Molloy for the countless times I bombarded him with questions regarding the NMR, especially as I often asked them in an untimely fashion.

Thank you to current and former Harbron lab members for coloring my research experience with fond memories. I want to mention Christian Chamberlayne, for engaging with my half-baked thoughts on confusing observations and Grace Purnell, for mentoring me both inside and outside lab. All the shenanigans from the summers and semesters will stay with me and I will miss spending time in lab with you all.

My gratitude extends to my friends and family for supporting me through this journey. I especially want to thank my parents, Nofomuli Taumoefolau and Lily Hsu, for expressing genuine interest and pride whenever I discussed a subject matter from research they were not quite familiar with. Without your support, I would not be standing where I am today.
My final thanks goes to the Charles Center and William & Mary’s HHMI program for funding my project over two years. The resources and activities they provided during my senior year alleviated the honors process, for which I am grateful.
Introduction

Chemical investigations in the Harbron lab often examine the chemical and physical properties of fluorescent compounds. The process spans techniques in organic photochemistry in the most literal sense – we synthesize and characterize these desired compounds before carrying out fluorescence experiments on the compound of interest. These turn-on or turn-off fluorescent sensors synthesized in our lab are then interrogated via incident light, metal ion concentrations or variable pH. The independent variable in my project is pH change within a biologically relevant range.

pH variance plays a regulatory role in a variety of cellular processes, such as proliferation, apoptosis and multidrug resistance\(^1\). In particular, the acidic window (pH 4.5-6) characterizes the pH values of acidic organelles and common cell dysfunctions, including cancer and Alzheimer’s. Compared to other methods of measuring pH, fluorescent turn-on probes report pH concentrations with great precision owing to their superior sensitivity and are ideal for measuring intracellular pH concentrations that tend to be heterogeneous with respect to time and space.

Although fluorescent pH probes are proven tools in biological imaging, few fluorescent indicators in literature to date probe for the acidic region\(^2\); even fewer can demonstrate dynamic color change over a narrow pH range. With this realization, our lab started a series of investigations with the long-term goal of engineering a fluorescent probe that can report for this important region with high accuracy.

Before delving into the specifics of my project, I will briefly explain the principles of fluorescence. Fluorescence is a physical phenomenon that describes the reemission of absorbed light and it is often observed in conjugated molecules.
Upon absorption, the molecule reaches a higher energy state and promptly undergoes energy loss by non-radiative vibrational relaxation. This manifests as a shift in band maxima between the absorption and emission spectra (see Figure 2), also known as the Stokes shift. Following this relaxation process, the molecule emits a photon of longer wavelength than the absorbed photon to return to its ground state. Figure 1 is a simplified representation of this phenomenon, where $S_0$ is the molecule’s singlet ground state and $S_1$ the singlet excited state. We often manipulate this phenomenon to observe color in solutions that are otherwise colorless by treatment with UV-light, as the emitted light would be visible to the human eye. Conjugation and structural rigidity allows fluorescence in molecules: $\pi$-bonds excite at longer wavelengths than $\sigma$-bonds and rigid structures prohibit purely non-radiative processes back to the ground state.

Figure 1: Jablonski Diagram of Fluorescence

Figure 2: (blue) Fluorescence Excitation (Absorption) and (red) Emission Spectra
Figure 3: (left) Rhodamine B, (right) Rhodamine 6G

The dyes that we deployed for this investigation were Rhodamine B and Rhodamine 6G (hereafter RB and R6G). Both are of a family of dyes categorized by a xanthene moiety with two amines attached in positions shown in Figure 3. RB has tertiary amines attached to the xanthene, with a quantum yield of 0.65 in absolute ethanol at the $\lambda_{\text{max}}$ (wavelength of maximum fluorescence) of 610nm$^3$. On the contrary, R6G has secondary amines with two extra methyl groups adjacent to them and a quantum yield of 0.95 at the $\lambda_{\text{max}}$ of 566nm.

The Harbron lab will eventually have to test the efficacy of our fluorescent probes in vitro. Good sensors for biological systems entail the following properties: (1) sensitivity, (2) affordability, (3) reversibility from fluorescent to non-fluorescent form, (4) stability in a wide pH range and (5) solubility in aqueous media. Although toxic at high concentrations, both RB and R6G are prominent fluorescence markers in the realm of biological research and roughly adhere to the aforementioned requirements. The abundance of research for related xanthene dyes also make them ideal candidates for the target pH sensor. I mostly work with the R6G in my project, but RB, due to being more synthetically tractable, is better understood in our lab. Often, I will refer to RB to frame my argument for the R6G case.
**Scheme 1** describes the synthesis of rhodamine spirolactam structures (hereafter RSL) that allow the RB and R6G to have a non-fluorescent leuco form\(^4\). The additional C-N bond induced by RSL formation prevents the conjugation of the xanthene core. Upon exposure to an acidic medium, the spirolactam ring undergoes a two-step process where the heterocyclic nitrogen gets protonated and the closed ring opens back up to its fluorescent form. Until a few years ago, little was understood on how to systematically tune the pKa at which these rhodamine spirolactam structures open and fluoresce. Anecdotal evidence from a paper published by Yuan and coworkers pointed to the possible connection between pK\(_a\) of ring opening and substituent on the upper ring system\(^5\). A notable example is the adamantyl substituted R6G-based RSL that sees a stark increase in pK\(_a\) of ring opening at 6.5 as opposed to its ethanol substituted analogue that possesses a pK\(_a\) of 2.8.

**Figure 4**: Yuan’s RSL compounds (left) ethanol, (right) adamantyl substituted R6G
To unravel the relationship between turn-on pKₐ and the physical properties of substituent groups, we decided to derivatize RB and R6G into RSL structures as shown in Scheme 1, where the R and R’ groups are the only variable parts of the series of compounds. The R and R’ groups were each characterized by their electronic and steric properties, quantified by their Hammett constants and A-values. My predecessor, Will Czaplyski, synthesized a series of RB-based RSLs with para substituted R-groups: -OCH₃, -t-C₄H₉, -CH₃, -H, -Cl, -CF₃, -CN and -NO₂. Surprisingly, the average pKₐ of ring opening for this series was 4.14, with a very narrow distribution of values (±0.04); in other words, electronic properties of substituents have no impact on the pKₐ of ring opening. Instead, the kinetics of ring opening changed. In the case of RB at least, the substituent effects of steric bulk and electronic properties seem to compartmentalize, a fortuitous finding with regards to the tunability of RSLs.

Naturally, the extension of this project was to test for the effects of steric bulk as defined by A-values, positioning the substituents ortho with respect to the rest of the RSL (R’-groups). A substituent’s A-value is a numerical value based on the energy difference between the axial and equatorial orientation of a mono-substituted cyclohexane ring. Grace Purnell and Sarah Stratton’s work on di-ortho substituted RB compounds (with substituents -H, -F, -Cl, -i-Pr, -CH₃, -OC₂H₅) demonstrated that increased steric bulk of the R’-groups generally raise the pKₐ of ring opening. We hypothesize that substituent size, or steric strain, has a destabilizing effect that accelerates the breakdown of the spirolactam ring after the initial protonation.

I am conducting the R6G analogue of this study. R6G-based RSLs tend to have a slightly higher pKₐ as compared to their RB counterparts. For instance, Lin’s adamantyl substituted R6G shows a pKₐ of 6.5 whereas its RB equivalent is down at 5.6. The rationale behind testing both R6G and RB is to build a generalized knowledge base on how to systematically tune both dyes.
I will often use Will and Grace’s findings to interpret the data acquired during my time at William & Mary. This honors thesis will heavily feature preliminary results as well as unrealized ideas for further investigation.
I. Optimization of R6G derivatives Reaction Conditions

The recurring problem that arises with synthesizing R6G-based RSLs (hereafter, R6G derivatives) is low yield; often, the experimental yield is so low that it makes acquiring a good NMR spectra challenging. The cause of low yield has been attributed to the secondary amine on the R6G xanthene core. While the tertiary amine on the RB’s xanthene core is also a site for derivatization, the replacement of the ethyl group by hydrogen makes R6G more reactive, forming multiple by-products alongside the desired product in the reaction mixture. Such low yield is still tolerable when the target aniline is para-substituted: percent yield falls between 10-20%. However, synthesis with di-ortho substituted anilines presents more challenges due to steric hindrance about the reaction site. Simply doubling the amount of the starting material proved to be unhelpful and warranted a systematic investigation to improve overall yield.

In order to establish appropriate reaction conditions for making R6G derivatives, I revisited the Harbron Lab’s standard synthetic procedure (that Will arranged) by consulting two other synthetic pathways. The first is from Best’s paper, a two-step synthetic procedure that closely resembles Lin’s synthesis of RB and R6G aniline derivatives\(^8\). The second alternative is a one-step procedure, a reaction scheme that harkens back to the original procedures Grace Purnell tested before making any of her RB aniline derivatives\(^9\). I unknowingly backtracked in our history of RB aniline synthesis, replacing the contestant with R6G.

The first chapter of this thesis focuses on my investigation for the optimal reaction conditions, based off of Scheme 1 as introduced earlier. While describing the methodologies I used to achieve higher yield, I will also list the many unsuccessful attempts that helped provide insight.
Identification of By-product Formed Upon Addition of POCl$_3$

Unlike RB, the esterified R6G does not effectively react with phosphorus oxychloride or POCl$_3$, so R6G must undergo nucleophilic acyl substitution under basic conditions to give R6G-acid, an analogue of RB. The polarity (and therefore conveniently low $R_f$) of R6G-acid also helps later in the purification steps because it traps the R6G-acid up top the stationary phase of the column.

![Chemical Structure](image)

**Figure 5:** Rhodamine 6G with the functional group at the upper ring system altered from an ethyl acetate to a carboxylic acid (shorthand, R6G-acid)

Compared to RB, R6G-acid has an overwhelming tendency to precipitate from solution with the addition POCl$_3$. The precipitate has a maroon waxy appearance and does not dissolve back into the organic solvent, usually chloroform or 1,2-dichloroethane. For a while, this phenomenon was attributed to the dimerization of R6G-acids by the reaction of the R6G acid chloride derivative with the secondary amine of another R6G-acid instead of the primary amine on the target aniline. Several observations compelled me to challenge this hypothesis; for instance, the precipitate and supposed dimer of R6G-acids preferred to dissolved in protic solvents such as water and ethanol. The dimer of R6G-acids makes a large organic molecule – it should not dissolve in water more readily than a lone R6G-acid. To better understand the activity of POCl$_3$ in R6G reaction mixtures, I devised a systematic approach to uncover the identity of this mysterious precipitate.
Initially, I chose from three variables to change in the standard procedure (as with RB-aniline derivative synthesis): temperature, dryness and solvent. If the identity of the waxy precipitate were indeed the product of the dimerization of R6G-acids, a cold reaction mixture should slow down its formation. The idea for testing anhydrous conditions stemmed from the fact that dry reaction conditions commonly yielded better results for synthesis of R6G aniline derivatives for reasons that were not understood. I changed the solvent (to ethanol) simply because I knew it dissolved R6G-acid better than our standard solvent for both RB and R6G aniline syntheses, 1,2-dichloroethane. Each trial had reaction mixtures comprised of 0.05g of R6G-acid and 10mL of solvent in a 25mL round bottom flask. The time for the formation of product were recorded as shown in the table below.

<table>
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<th>Reagent</th>
<th>Cold?</th>
<th>Dry?</th>
<th>Solvent</th>
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<td>N</td>
<td>N</td>
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<td>~3</td>
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<td>DCE</td>
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<td>H₂SO₄</td>
<td>N</td>
<td>N</td>
<td>DCE</td>
<td>Y</td>
<td>28</td>
</tr>
</tbody>
</table>

**Table 1**: Chart of reaction conditions and the formation of maroon and waxy precipitate in a transparent and yellowish solvent

The first four trials provided strong evidence that POCl₃’s interaction with water directly related to the formation of the waxy precipitate. According to literature, POCl₃ reacts with water to form HCl in the gaseous form, which can get reabsorbed by the reaction mixture to make a formerly neutral solvent environment acidic.

\[
O=PCl₃ + 3 H₂O \rightarrow O=P(OH)₃ + 3 HCl
\]

To confirm this, I designed a fifth trial where I add a few drops of 2M HCl to the mixture, leaving out the POCl₃. Almost immediately after HCl was added, the waxy precipitate formed. Addition of H₂SO₄ had similar effects, albeit requiring sonication or additional encouragement to precipitate. The precipitate formed with H₂SO₄ also had a less waxy and more oil-like
appearance, suggesting that HCl was indeed responsible for the troublesome formation of the waxy precipitate. Thus, I concluded that the identity of the precipitate was an R6G-acid salt, protonated at the secondary amine on the xanthene ring, the expected reactive site.

My deduction is consistent with the behavior of the precipitate when exposed to protic solvents and base. When I added a few squirts of NaOH to the fifth trial, an aqueous layer and an organic layer formed in the reaction vessel, of which the former had a dark red color and the latter a brick red (as R6G-acids should appear.) This implies that I was able to retrieve some R6G-acids by adding NaOH, which apparently deprotonated the secondary amine. After isolating the organic layer and letting the mixture stir overnight, the waxy precipitate made another reappearance. Perhaps adding molecular sieves could have prevented its formation, but the point still stands: the similarities of this experiment to a back extraction validate the hypothesis that the waxy precipitate is an R6G-acid salt. The formation of the R6G-acid salt should be prevented altogether, as both R6G-acid and the target aniline are also soluble in water, rendering back extraction ineffectual.

![Figure 6: R6G-Acid salt, with the quaternary ammonium cation at the xanthene core](image)

These results explain why previous syntheses that underwent acidic work up with cold sulfuric acid gave a higher yield; with 2M HCl, the mixture along with the desired product was protonated and then extracted into the aqueous layer, leaving little to purify from in the crude organic layer. TLC of both the organic layer and aqueous layer after acid work-up confirmed that
the desired product was present in both solutions. From then on, the acid work-up was skipped in favor of base, brine and DI water washes.

It should be noted that this process is not mutually exclusive with the first hypothesis regarding dimerization of R6G – regardless of precipitation, dimerized R6G-acid by-products have been previously observed in post-reflux reaction mixtures, suggestive of the countless variations of extraneous products that can be synthesized in **Scheme 1**. In later experiments, it is revealed that certain reaction conditions dramatically increase the number of by-products, making the extraction of the desired product an impossible endeavor.
Tracking Experiment: Attempted Synthesis of Rhodamine 6G – dimethylaniline

After establishing that an anhydrous reaction mixture was crucial to successfully synthesizing the desired products, I decided to test two different synthetic schemes for higher yield. In both cases, I repeatedly sampled the reaction mixture during reflux, monitoring the formation of the desired product as well as extraneous UV-active by-products on TLC plates. In this investigation, 2,6-dimethylaniline was deployed as the target aniline for its more or less average A-value and predictable electronic behavior. An example of bad anilines to generalize reaction procedures with would be 2,6-dinitroaniline. Due to the nitro group’s extreme electron-withdrawing tendencies, 2,6-dinitroaniline regularly makes for RSL derivatives that express peculiar photochemical or physical behavior, such as maintaining the fluorescent open-form in a basic environment or precipitating out mid-purification.

The first synthetic scheme that I tracked was the classic two-way method utilized by Lin and Best’s groups – it requires the acid chloride intermediate to enable the desired aniline to form the closed ring spirolactam structure. The first reflux drives the reaction of R6G-acid and POCl₃ (and thus the formation R6G acid chloride) to completion. The second reflux encourages the primary aniline on the desired aniline to attack the previously synthesized acid chloride intermediate.
Scheme 2: An example of the two-step (three-step, including the synthesis of R6G-acid) synthesis of RSLs

After roughly 19 hours of the first reflux, without aniline, 6 distinct UV-active spots were observed on the TLC plates. As no other starting material other than R6G-acid is UV-active and the spots appeared at a higher R_f than the R6G-acid, this implies the dimerization of R6G-acid under high temperature. The addition of aniline and forty more hours of reflux saw an increase in by-products by 9 (giving a total of 15). Both steps of the synthesis generated plenty of competing reactions along with that of Scheme 1.

I performed flash chromatography to separate R6G-dMA from the resulting crude oil. In the 155 fractions retrieved from the 40:60 EtOAc/Hexane column, fractions 99-144 gave a deep red product; this is half the R_f of the desired product, which eluted at fractions 44-60. The NMR spectra of this unknown by-product was beyond recognition, but due to color-wise similarity to R6G-acid, it’s likely the massive amount of deep red by-products are variations of R6G-acid dimers. The bottom line is that these by-products form only in the occasion that extended reflux takes place and an ideal reaction procedure should avoid producing these as much as possible.
In the second tracking experiment, less time (4 hours) was devoted to heating the initial R6G-acid and POCl₃ mixture but the reaction still produced four UV-active by-products on the TLC. Both trials yielded product, albeit in negligible amounts (<5%). In light of the cold temperature trial from the precipitation experiment, I concluded that heat generally encourages other competing reactions over the desired reaction pathway, significantly lowering the experimental yield. A chilled reaction tempers the formation of by-products.

The other reaction scheme that I tested was one that avoided using POCl₃. Scheme 3 describes a process where reflux eventually forces the R6G’s ethyl acetate to be replaced by the aniline through nucleophilic acyl substitution and locks into its closed spirolactam by interacting with triethylamine (hereafter Et₃N), an organic base, in the reaction mixture.

Scheme 3: An example of the one-step synthesis of RSLs where (iii) includes CH₃OH, Et₃N, reflux, 12hrs⁹

Without POCl₃ to form the acid chloride intermediate that readily reacts with other rhodamine species, Scheme 3 could potentially allow a clean reaction, provided that the desired reaction occurs. It also circumvents the synthesis of R6G-acid, which is another possible source of UV-active by-products. Unfortunately, no product was observed. Tracking with TLC plates over 72 hours showed four different UV-active compounds, of which none of them turned pink (note that open-form R6G derivatives fluoresce yellow-green but appears pink) upon pipetting a half-drop of 2M HCl.
Of the two synthetic pathways, Scheme 2 (which is a more elaborate version of Scheme 1) seems to be the only pathway that ensures the desired product, however miniscule. Although little product came out of this endeavor, the tracking experiments revealed that the synthesis of R6G derivatives cannot tolerate heat. Successful synthesis of the desired products comes down to tempering all the other extraneous reactions. Following Yuan and Best’s procedures that consistently incorporates a reflux step somehow lowers the overall yield of the reaction.
**Solubility Test of R6G-acid**

In effectively carrying out the synthesis of R6G derivatives, knowing the solubility of the starting material is important. Unlike RB, which readily dissolves in chlorinated solvents like 1,2-dichloroethane (DCE), R6G-acid requires sonication and larger volumes of solvent. In order to understand the R6G-acid’s behavior in solvents, I conducted a simple solubility test with solvents involved in the synthetic and purification steps. In 50mL of solvent, I poured a pre-weighed amount of R6G-acid, sonicated the mixture and subtracted from the pre-weighed amount the weight of the solute that remained undissolved. In the interest of conserving R6G-acid, the exact solubility of R6G-acid in the polar protic solvents, water and ethanol, is left without exact solubility measures. These numbers suggest that a good amount of R6G-acid can be removed during the acid-base work-up into the aqueous layer. It also calls for more DCE to saturate R6G-acid.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Solubility (g/100mL solvent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>soluble</td>
</tr>
<tr>
<td>DCE</td>
<td>$2.96 \times 10^{-3}$</td>
</tr>
<tr>
<td>chloroform</td>
<td>similar to DCE</td>
</tr>
<tr>
<td>diethylether</td>
<td>$1.03 \times 10^{-3}$</td>
</tr>
<tr>
<td>ethanol</td>
<td>very soluble</td>
</tr>
</tbody>
</table>

**Table 2: Solubility (g/100mL) Test of R6G-acid**
**Synthesis of R6G-diisopropylaniline (R6G-dIA)**

Informed by experiments mentioned in previous sections and seeing that the more successful syntheses in the past involved ice baths and molecular sieves, I tested Scheme 1 under a different condition: cold and anhydrous. Not only was the reaction mixture maintained dry, but the work-up was also performed with chilled 2M NaOH, brine and DI water (they prevented emulsions and precipitates).

The percent yield was 11.8%, a significant improvement from the reactions that underwent reflux. However, these procedures still produce one by-product with roughly the same R_f as the desired product. The two cannot be separated even after three consecutive ethyl acetate/hexane columns. Further discussion on the identity of this by-product will be brought up in the next section.

<table>
<thead>
<tr>
<th>Trial</th>
<th>POCl₃</th>
<th>DCE (mL)</th>
<th>Reflux Time (hrs)</th>
<th>Work-up</th>
<th>Eluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Excess</td>
<td>8</td>
<td>~60</td>
<td>N/A</td>
<td>CHCl₃ &amp; MeOH</td>
</tr>
<tr>
<td>2*</td>
<td>1eq.</td>
<td>20</td>
<td>27</td>
<td>1M 3×50mL H₂SO₄, 2M 3×50mL NaOH, brine</td>
<td>Hexane &amp; EtOAc</td>
</tr>
<tr>
<td>3</td>
<td>1eq.</td>
<td>50</td>
<td>N/A</td>
<td>2M 3×50mL NaOH, DI H₂O, brine</td>
<td>Hexane &amp; EtOAc</td>
</tr>
</tbody>
</table>

*Table 3: A set of different conditions that gave progressively more desired product (*trials 1 and 2 are of Scheme 2 with R6G-dMA) Note that my approach goes from attempting to optimize formation of the R6G-acid chloride intermediate to tempering unwanted side-reaction.*
Finding the Optimal Column Chromatography for Purification Procedure

In our lab, we commonly utilize silica columns to purify our crude products – the mobile phase is most often a combination of hexanes and ethyl acetate with some Et₃N, with the occasional exception chloroform columns when the crude product does not properly dissolve in the aforementioned solvents. A few experimental measures were taken to ensure that the column does not take away from higher yield, as R6G derivatives once again behaved differently from their RB counterparts in terms of solubility.

Ethyl acetate and hexane columns exhibited superior separation of the crude products, although chloroform columns (with 1% methanol) observed more concentrated bands and less smearing all over the stationary phase due to higher solubility.

Of the identified compounds, R6G-dIA, R6G-dMA, R6G-dCA (di-chloro) and R6G-dFA (di-fluoro), and failed purification of R6G-dNA, the more electron-withdrawing substituents generally gave lower yield, with the exception of R6G-dCA. Apart from the electronic effects the substituents have on their respective aniline before forming the spirolactam structure (electron-donating groups are bound to attack the acid chloride intermediate more effectively), interactions with the weakly acidic silica column should be noted. Particularly, R6G-dNA, with low Rₚ owing to the negative character on the nitro group, had the tendency to saturate the column. Other R6G-diortho compounds also saw wide bands of the desired product, so the secondary amine also likely interacts with silica, a weakly acidic oxide.

In an attempt to remedy this, I tested a different stationary phase to see if compounds can be extracted from more concentrated fractions. The alumina column was washed with methanol before saturating with the eluent (hexane and ethyl acetate). Unfortunately, unlike the silica
column, observing colored bands on the alumina column proved to be rather difficult. Despite the difficulty, a certain variation of R6G-dMA was successfully isolated.

Figure 7: Aromatic region in the NMR of R6G-dMA before the alumina column. The two small peaks near 6.5ppm is likely trace amounts of open-form R6G-dMA, judging from the integral

Figure 8: Aromatic region in the NMR of R6G-dMA byproduct. The distinct hydrogen atoms on the xanthene core are the four rightmost peaks.

Although the supposed R6G-dMA in the fractions showed a very pure product, some alterations in the structure must have happened during the flash chromatography. The NMR spectra before and after the alumina column showed marked change in the aromatic region, exhibiting four distinct peaks for the four hydrogen atoms on the xanthene ring.

One significant observation regarding this isolated R6G-dMA by-product is that the compound retains its fluorescence turn-on properties. The compound still fluoresces with exposure to acid and reverts to colorless form after treatment with Et₃N. Again, observations suggest the involvement of the secondary amine on the xanthene core. Two possible scenarios explain this phenomenon.
The first hypothesis attributes the unique hydrogen atoms to the protonation of one of the secondary amines. As a result, the electronic configuration of the xanthene core alters. Although this scenario is certainly possible, several piece of evidence argue against it. Alumina is a basic oxide and the fractions were treated with Et$_3$N afterwards. Protonation at the secondary amine should not occur in such an explicitly basic environment. Even if it did happen, the spirolactam nitrogen should be protonated first. The NMR spectra also showed one product, not an equilibrium between two species. For these reasons, I hesitate to conclude that another acid-base process is responsible for this complication.

Figure 9: An example of an oxidized amine due to alumina$^{12}$

Although I have my reservations for the second hypothesis, I find it more plausible than the first one. The second scenario describes a situation where the alumina oxidizes one of the amines. Again, this changes the electronic configuration of the xanthene core and quite possibly prevents a second oxidation process from taking place. One can confirm this process by taking the IR spectra of the pure by-product and comparing the peaks in between 1400-2000cm$^{-1}$ with that of the actual R6G-dMA. Since N-O peaks are fairly distinct, identification of an oxidized R6G-dMA in comparison to the desired R6G-dMA should be possible.

The four unique hydrogen atoms on the asymmetric xanthene were also been observed in R6G-dIA. The plain R6G-dIA was virtually inseparable from the by-product –after three silica columns, the four aromatic peaks still appeared in small amounts alongside the two singlets that indicate symmetric xanthene.
Seeing that the alumina column dramatically changed the structure of the desired product, continued usage of silica columns is highly recommended.
Future Directions for Optimization of R6G derivatives Synthesis

Although I tested different methods and analyzed many more, the “optimized” reaction procedure still room for improvement. Below are several extra measures that may lead to improved yield.

- Initially, our lab used 1,2-dichloroethane as the reaction medium because the solvent boiled at higher temperature. Since the synthesis R6G derivative requires cold and anhydrous reaction conditions, we can go back to synthesizing compounds in chloroform. Reagents may dissolve better in chloroform.
- Neither Yuan nor Best purifies R6G-acid prior to Scheme 1 but it would not hurt to eliminate extraneous material, especially since the synthesis of R6G-acid involves strong base, strong acid and overnight reflux. Chloroform and 1,2-dichloroethane are both appropriate solvents for recrystallization.
- Dry-packing before flash chromatography may help, as the dominant eluents, ethyl acetate and hexane, do not necessarily dissolve the crude mixture effectively. Since the desired products have a tendency to linger in the stationary phase, minimal silica powder should be packed in columns.

Besides micro-managing every step of the synthesis, we need to determine the pKₐ of the secondary amine on the xanthene. Its sensitivity has been this Chapter’s mantra and we may need to decide if the two sites ultimately interfere with the R6G derivative’s ability to function as a pH sensor. I am also curious to see if the experimental yield for para-substituted R6G derivatives improves with cold, anhydrous and neutral conditions. If yes, clickable rhodamine probes should become easier to make, a topic I will delve into in detail in Chapter 3.
II. Characterization of di-ortho R6G Derivatives

Before the laborious optimization experiments from Chapter 1, the nature of di-ortho R6G derivatives was unknown. Once they were finally made, comparing and contrasting R6G derivatives with their RB counterparts lead to vital insights that informed the sterics project as a whole. This section delves into characterization of R6G derivatives via NMR and fluorescence titrations. Molecular modeling of the compounds from our lab as well as other relevant compounds from literature are meant to complement the spectroscopic investigations.

**Figure 10:** NMR spectra of R6G-aniline, the most generic of R6G derivatives, synthesized by Grace Purnell in the summer of 2012 (left) aromatic region, (right) aliphatic region
**R6G-dMA after alumina column and R6G-dIA after DCl**

The $^1$H NMR spectrum’s aromatic region for R6G derivatives should show two peaks in 6-6.5 ppm, each representative of two hydrogen atoms on the xanthene core. **Figure 12** (left) is the second attempt at synthesizing of R6G-dMA after a single attempt at purification.

![Figure 11: R6G-dMA](image)

**Figure 12**: Partial aromatic region showing the hydrogen atoms on the aniline and the xanthene core. (left) actual R6G-dMA with the two xanthene peaks and two benzene peaks, (right) R6G-dMA by-product

To reiterate, the first batch of R6G-dMA that I attempted to purify via an alumina column transformed into a different compound mid-purification. The four peaks (refer to **Figure 12**, right) that appear in place of the two desired peaks suggest an alteration on a single secondary amine. The peak at 6.8 ppm for the R6G-dMA by-product also exhibits peculiarities, as it deviates from the supposed doublet of the two hydrogen atoms adjacent to the benzene’s substituent. To formally test if this alteration is merely due to protonation on one side of the xanthene core, I mixed one drop of DCl with R6G-dIA’s NMR sample (because I had relatively
larger portions of it, as oppose to the precious amounts of R6G-dMA that I had) and scanned for its aromatic region. Figure 14 shows an open ring R6G-dIA and we see here that the symmetry of the xanthene core is not be lost with the presence of acid.

Figure 13: (left) Closed-from R6G-dIA, (right) Open-form R6G-dIA

Figure 14: Aromatic Region in the NMR of R6G-dIA with one drop of DCl (open-form) Note the two xanthene peaks that represent two sets of symmetric hydrogen atoms are still present.

The acid does not impact the doublet near 6.9ppm either. Here is proper spectroscopic evidence that refutes the first hypothesis for the formation of pure R6G-dMA by-product presented in Chapter 1.
Chemical non-equivalence of R6G-dIA’s isopropyls

Figure 15 shows the $^1$H NMR spectrum of R6G-dIA after three consecutive ethyl acetate/ hexane columns, with trace amounts of aforementioned asymmetric by-product. Curiously, the isopropyl peaks that appear from 0.3-1.0 ppm appear as two separate peaks rather than one. Not only were they separate but also the difference in chemical shift is 0.52 ppm – a huge number for what should be equivalent peaks.

Figure 15: Aliphatic region in the NMR of R6G-dIA
The same peaks at 0.36 and 0.88ppm can be found in RB-dIA. On top of that, the 0.88ppm peak disappears when one of the isopropyl is removed (the compound is RB-2-isopropylaniline or RB-2IA).

Figure 16: Aliphatic region in the NMR of RB-dIA, synthesized by Grace Purnell. Note the two isopropyl peaks are present at nearly identical chemical shifts.

Figure 17: Aliphatic region in the NMR of RB-2IA, also synthesized by Grace Purnell. The doublet that represents six isopropyl hydrogen atoms is gone.

At first, I suspected that the apparent chemical topicity rooted from conformational differences. To support that hypothesis, I ran the compound through a quick-and-dirty variable temperature NMR experiment. The two isopropyls rotates fast enough by raising the temperature
that the two peaks will eventually coalesce. However, the two peaks did show the slightest signs of coalescence upon raising the temperature by 15K. Perhaps the temperature needs to be raised even higher, which can only be afforded with higher boiling deuterated solvent.

If temperature did not matter and the difference did not come from conformational differences, the two isopropyls must be experiencing a different chemical environment, through-space or otherwise. To understand what is happening, I ran Gaussian optimizations at the HF/3-21 level to visualize the geometry of the molecule. The specifics will be discussed in a different section.
**R6G derivative’s pK\textsubscript{a} values of ring opening and similarity to RB counterparts**

To find the pK\textsubscript{a} at which the synthesized R6G derivatives fluoresce (or more precisely, when the open form and closed form is in equilibrium) our lab does fluorescence titrations, utilizing the pH meter and the fluorometer.

As the quantum yield of R6G compounds far exceeds that of RB compounds, I added a tenfold dilution step to the ordinary procedures of RB-derivatives’ serial dilution. In an ordinary procedure, 10-15mg of the target compound is first dissolved in 25mL of ethanol. 10mL of that was diluted to volume in a 100mL volumetric flask with 40mL ethanol and 50mL water to give a 1:1 ethanol to water solvent. For R6G derivatives, 10mL of that was diluted to volume again in a 1:1 ethanol water mixture, giving an approximate molarity of 0.001 M. Evidently, not a lot of product is needed to derive pK\textsubscript{a} values – purifying enough product for a decent NMR spectra is the biggest problem. Keeping the bottles of absolute ethanol fresh becomes important because deviation from the 1:1 ratio of ethanol and water causes solvatochromic effects as well as alter the amount of 2M HCl needed to change the pH of the solution.

By adding increasingly larger increments of 2M HCl, the stirring solution’s pH value goes down by 0.2-0.3 with every scan. **Figure 18** shows a generic fluorescence titration data. By using the Henderson-Hasselbach-type mass action equation, we extrapolate the pK\textsubscript{a}.
Contrary to what we expected, the R6G derivative’s $pK_a$ of ring opening are not dramatically higher than their RB counterparts. Preliminary titrations for R6G-dCA gave a $pK_a$ of 5.6, which is 0.2 above that of RB-dCA, as did R6G-dIA’s 5.8 which was also higher in value than RB-dIA by 0.2. These results suggest that the substituent’s $A$-value and R6G derivatives’ methyl group on the xanthene core does not have additive effect on the $pK_a$ of fluorescence turn-on, calling for structural and geometric studies of the di-ortho compounds.
**Computational Study of RB and R6G Derivatives with Sterically Unique Groups**

Shown below are the calculated structures for R6G-A, R6G-dFA, R6G-dCA and R6G-dIA in order. The following were optimized to the HF/3-21G level with the exception of R6G-A to examine the geometric placement and alignment of separate parts of the molecule such as the xanthene ring, spirolactam and the di-ortho benzenes. Calculations often took more than 5 hours so instead of computing in one sitting, computations were continued and discontinued from the checkpoint file by typing in the command: Opt=Restart. Calculations also proceeded faster if the structure was optimized at the semi-empirical PM3 level beforehand.

![Figure 19: Bird eye view of R6G-A (optimized at semi-empirical PM3)](image)

**Figure 19:** Bird eye view of R6G-A (optimized at semi-empirical PM3)

![Figure 20: Optimized structure of R6G-dFA (left) birds eye view, (right) side/behind view](image)

**Figure 20:** Optimized structure of R6G-dFA (left) birds eye view, (right) side/behind view
According to these optimized calculations, the geometry of the xanthene ring undergoes a deplanarization with sterically bulkier substituents. As shown in Figures 19-22, R6G-dIA and R6G-dCA have non-planar xanthene cores whereas R6G-dFA and R6G-A exhibits a relatively planar geometry. At first glance, it may seem as if the angle strain on the xanthene ring can potentially explain some compounds’ spirolactam ring opening at a higher pKₐ. If the angle strain destabilizes the spirolactam nitrogen, that explains the higher pKₐ of fluorescence turn-on.

Curiously, the computation of two of Lin’s RSL derivatives with high pKₐ’s of ring opening did no exhibit a huge deviation from the planar xanthene geometry. The two derivatives were RB and R6G analogues of each other, with an adamantane in place of di-ortho anilines. As indicated in the images produced below, there seems to be no direct correlation between the

Figure 21: Optimized structure of R6G-dCA (left) birds eye view, (right) side/behind view

Figure 22: Optimized structure of R6G-dIA (left) birds eye view, (right) side/behind view

angle strain on the xanthene and the pK$_a$ of ring-opening, because R6G-dIA clearly shows more dramatic strain than does the sterically bulkier RSL of adamantylaniline.

**Figure 23**: Optimized structure of RB-Adamantane (left) birds eye view, (right) side/behind view

**Figure 24**: Bird eye view of R6G-Adamantane

I organized a table with relevant angles and level of theory to understand the differences between these structures more quantitatively.
As mentioned earlier, the angle of the xanthene core becomes increasingly acute with the bulkier substituents. Angle 2 is provided for future reference, as the slight tilt of the RSL attached group is peculiar in what is supposed to be a symmetrical ground state molecule.

Although the variation in planarity may not explain the pK$_a$ of ring opening, it may provide valuable insight on the apparent topicity of the isopropyls on R6G-dIA. Perhaps, the strain on the xanthene core raises the energy barrier to rotate the bond that connects the spirolactam ring and the aniline. Recall that in the NMR of the mono-isopropyl RB (RB-2IA), one of the peaks in the aliphatic region that is present in RB-dIA disappeared. This may suggest that the chemical nonequivalence is between the two isopropyl groups rather than the two enantiotopic methyl on each isopropyl; otherwise, RB-2IA should show the two peaks present in RB-dIA, but with halved integral numbers. To gain further confirmation on this, a second set of calculations should be made, examining the rotational barrier of the bond between the spirolactam and di-ortho isopropyl benzene. Running higher temperature VT NMRs with this information in mind could also lead to the eventual rotation of the bond in question.

Table 4: Angles derived from the optimized structures of RSL derivatives. The asterisk signifies an incomplete job, although both had computed for at least three hours.

<table>
<thead>
<tr>
<th>RSL</th>
<th>Turn-on pK$_a$</th>
<th>Level of Theory</th>
<th>Angle 1</th>
<th>Angle 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6G-A</td>
<td>N/A</td>
<td>Semi-empirical/P3</td>
<td>111.857</td>
<td>119.947</td>
</tr>
<tr>
<td>R6G-dFA</td>
<td>N/A</td>
<td>HF/3-21G*</td>
<td>111.064</td>
<td>123.990</td>
</tr>
<tr>
<td>R6G-dCA</td>
<td>5.6</td>
<td>HF/3-21G*</td>
<td>109.697</td>
<td>124.534</td>
</tr>
<tr>
<td>R6G-dIA</td>
<td>5.8</td>
<td>HF/3-21G</td>
<td>109.002</td>
<td>124.483</td>
</tr>
<tr>
<td>RB-adamantane</td>
<td>5.6</td>
<td>HF/3-21G</td>
<td>111.840</td>
<td>126.332</td>
</tr>
<tr>
<td>R6G-adamantane</td>
<td>6.5</td>
<td>Semi-empirical/P3</td>
<td>111.835</td>
<td>124.844</td>
</tr>
</tbody>
</table>

Figure 25: Angle 1 and Angle 2
In processing computations on the school computers, there are several tips that can minimize the interruption of jobs. The school computers automatically log out in 20 minutes; by playing a DVD on the same system, one can avoid auto-log outs accidentally killing jobs. The school computers also undergo Windows updates without warning near 4am in the morning. Simply avoiding overnight computation should help. With these little details in mind, computation should resume smoothly – although, installing GaussView on a personal computer should circumvent all these complications (note that the school does not provide Gaussian for Mac books.)
Future Directions for Characterization of R6G derivatives

The observations I make in Chapter 2 more often than not leave more questions than answers. This open-endedness is partly due to the general lack of clean NMR spectra, both H-NMR and C-NMR; R6G-dIA is still not pure enough after three runs of flash chromatography. The other part of the problem was that I did not know what to look for in more advanced NMR methods until I ran Gaussian calculations. Now that the calculations show at least two variables – planarity of the xanthene core and tilt of the di-ortho substituted benzene – here are my immediate goals:

- I should run optimization calculations for more compounds, including RB derivatives. It would be interesting to see if the planarity of the xanthene core breaks down in the same way it does with R6G. For compounds that will undergo calculations about the energy barrier of rotation, the geometry should be optimized at a higher level of theory.

- Our lab has only run mono-ortho compounds through variable temperature NMRs. R6G-dIA, R6G-dMA and its RB counterparts should also be subject to VT NMR, to see if the substituent peaks change with enough heat.

- After NMR experiments are done, the R6G derivatives should be titrated. If the pKₐ values are still barely above that of their RB counterparts, we should reevaluate why Lin’s adamantyl RB and R6G showed such stark difference in pKₐ.
III. Attempt at achieving RB and R6G FRET

While fluorescent probes possess many desirable properties as chemosensors, they are not immune to photochemical degradation processes. For instance, fluorescence probes often undergo photobleaching (another term for fading): a process described by the deactivation of fluorophores under non-specific chemical processes.

One of the final goals of this project is to make a tunable ratiometric FRET probe to circumvent these complications. Covalently binding a RB and R6G FRET pair is the first step to actualizing it. R6G has higher pKₐ than RB to permit FRET, a photophysical phenomenon where the donor fluorophore (R6G) transfers energy to a nearby acceptor chromophore (RB), resulting in sensitized emission of red light. Energy transfer between these two dyes happen because of the significant overlap of R6G’s emission spectra and RB’s absorption spectra. This interrelated dual emission as underpinned by FRET enables ratiometric pH measurement because the ratio of yellow-green or red-orange light (as oppose to intensity) should depend solely on the solution’s pH level. Calibrating to the pH-independent isoemissive point (see Figure 26), a necessary consequence of ratiometric probes, eliminates ambiguity in fluorescence intensity signals.

![Emission spectra of ratiometric metal sensor from the Harbron Lab (isoemissive point at ~565nm)](image)

**Figure 26:** Emission spectra of ratiometric metal sensor from the Harbron Lab (isoemissive point at ~565nm)
Several attempts were made to achieve the response seen in Figure 26. The following section will give an overall methods description for the fluorescence titration of dye-embedded polymer films and document synthetic attempts at covalently bonding the two dyes.
Making the dye-embedded pHEMA films

\[
\text{Figure 27: Poly (2-hydroxyethyl methacrylate)}
\]

pHEMA is a polymer that can behave as a hydrogel, absorbing 38 wt\%\textsuperscript{15} upon complete immersion in water. The same polymer is utilized for contact lenses, which are known for drying out in a matter of seconds and finally rehydrating after hours. By doping dyes into hydrogel, the dyes can respond to the pH change of an aqueous environment. However, before undertaking titration studies, the optimal combination of polymer to dyes needs to be established as well as the proper procedure to make reasonably even polymer films. I tested several combinations with different RB compounds, the first of which was RB-hydrazide. I quickly discovered that RB-hydrazide had much too low a quantum yield for the fluorescence titration to work.

\[
\text{Figure 28: RB-hydrazide}
\]

Although the RB-hydrazide films produced little results, the process helped determine the procedure to make spin coated pHEMA films. First, 5 wt\% of pHEMA to ethanol made a film that was too thin – 10 wt\% to 15 wt\% allowed noticeable intensity for fluorescence scans. I discovered while cleaning pHEMA off the glass films that methanol dissolves pHEMA more
effectively than ethanol. Improved solubility of pHEMA meant fewer air bubbles in the polymer film. pHEMA in methanol had to be gently heated (near 40°C) in a 10mL round bottom flask for at least 2 hours to dissolve and mix uniformly. It was crucial to cover the mouth of the round bottom flask to prevent methanol from evaporating out. By the end of heating and stirring, the mixture was usually bright pink. Because the mixture dried quickly without heat, I spin coated the polymer onto thin glass films immediately after taking the mixture off the heat. In most cases, I set the spin coating at 10,000 rpm (the highest setting) throughout the two-stage spin to spread the viscous polymer solution as evenly as possible. The amount of hydrogel dropped onto the glass film varied, but most of the time, it was one or two drops, straight from the round bottom flask (as opposed to the tip of the pipette.)

Figure 29: (left) RB-nitroaniline (RB-NA) and (right) RB-chloroaniline (RB-CA), substituents are para-substituted

In place of RB-hydrazide, I utilized RB-NA because our lab tended to get higher yield of RB-NA. This decision proves to be a bad one, as the negative character on the nitro group makes the RB-NA behave differently than other RB aniline compounds – more will be discussed in the next section. At first, 1mg/1mL (10mg) and then eventually 0.5mg/1mL (5mg) of RB-NA was added to each batch of polymer solution. After seeing that the RB-NA was not the best candidate to acquire consistent data, I changed the dye to RB-CA. Since R6G-CA had already been synthesized in our lab, pairing RB-CA with R6G-CA seemed appropriate, with minimal side effects from substituent interactions.
Annealing films was one of the things that I did not explore in this project. The $T_g$ of pHEMA is 84.8°C°C. Perhaps evening the surface area of the film will help fluorescence titrations, as varied thickness and surface on the polymer film introduced inconsistency in the overlaid fluorescence intensities. The problem is roughly avoidable as long as one keeps track of the slide’s positioning on the stand.
Fluorescence Titration for various compounds

Deciphering the appropriate conditions for titrating the polymer films involved several steps: understanding how long the polymer film took to absorb water and become a hydrogel, assembling the titration apparatus that mimics the liquid phase titrations we usually undertake and finding optimal excitation and emission wavelengths.

At first, the polymer film was placed in stirring water with descending pH values, much like a normal titration procedure but without ethanol. This yielded poor results, as the water failed to penetrate the film and the dye’s fluorescent turn-on was less likely to happen without proper exposure to water. Soaking the film for at least 90 minutes prior to titration studies proved to be effective, showing faster response from the dyes doped in the film. Polymer films with thicker layers (as with 15 wt% pHEMA films) required 15 minutes of soaking in the acidic solution between titration scans just to guarantee that the change of pH permeated the film.

In regards to preparing an adequate apparatus, I used tweezers to secure the film while dipping in the stirring solution. I also attached a piece of tape on one of the polymer film’s vertices to indicate which side the tweezers should hold. Marking the edge also helped with aligning the pHEMA film in a consistent manner when setting it up on the fluorometer. For each scan, the stand was taken apart from the fluorometer so that I can safely insert the film while applying as little force as possible. The pHEMA films are very delicate.

The question of the appropriate excitation and emission wavelength was slightly more complicated, especially with the RB-NA case. Both the excitation and emission spectra gradually redshifted with each subsequent drop in pH, producing the spectra in Figure 30.
The final $\lambda_{\text{max}}$ of RB-NA doped in pHEMA film is closer to that of RB derivatives (580-590nm). In other words, the initial $\lambda_{\text{max}}$ in a weakly acidic environment is already significant blueshifted and redshifts back to its expected $\lambda_{\text{max}}$ at very low pH. This irregular behavior is likely due to the explicitly negative character that the attached nitro group possesses. The degree of red shifting varied widely with each film (in Figure 29, the final $\lambda_{\text{max}}$ is 574nm, but another film red-shifted all the way to 590nm), suggesting that trial-wise compositions of RB-NA ensembles leads to this photochemical behavior rather than a phenomenon that assumes uniformity in the film, such as protonation at the nitro group. If the latter was the case, the spectra should redshift to the same wavelength at a given pH value.

So far, pH-dependent redshift of the excitation and emission spectra has not been observed when RB-NA is immersed in solution; the phenomenon is unique to RB-NA doped in polymer films. Such discrepancy may not be surprising after all considering that the proximity of the dyes between the two mediums is incomparable. Further experimentation of RB-NA with
raised concentrations in solution environment and single molecule experiments in the pHEMA film should help clarify if the red shifting is indeed an ensemble behavior or an interaction unique to pHEMA films.

![Emission spectra of RB-NA and R6G-CA doped in pHEMA film](image)

**Figure 31**: Emission spectra of RB-NA and R6G-CA doped in pHEMA film (starting pH= 5.55, end pH= 0.10) Note that the wavelength at which RB-NA ends and R6G-CA begins is highly ambiguous.

Replacing RB-NA with RB-CA proved to be an immediate solution to the redshifting spectra and pHEMA film titrations came to closely resemble our lab’s solution based fluorescence titration experiments thereafter. I fixed RB-CA and R6G-CA as the standard dyes to used in film titrations. **Figure 32** is an example of a pHEMA film with both RB-CA and R6G-CA doped in it.
Some inherent concerns with the goals of this investigation rose with continual trials. Distance between the two dyes is crucial to observe an isoemissive point, and the chances that the dyes successfully FRET rises with increased concentrations of both dyes. However, this meant the data would represent two competing processes: the overall fluorescence intensity rising with increasing acidity and occasional FRET (and enhanced intensity of RB-CA) from dye pairs that happened to be in near enough proximity. The combination of these two processes results in Figure 32, which is far from an ideal emission spectra of a FRET pair.

The solution lies in another set of optimizations unique to this established experimental set-up (RB-CA and R6G-CA in pHEMA). A few suggestions are: bringing down the wt% of pHEMA to prevent a pH gradient within the hydrogel, further increasing the concentration of dyes to increase the ratio of paired dyes to non-paired dyes and annealing the films to create an even pHEMA coating.
Ultimately, the film experiments were foregone in favor of purifying R6G derivatives. However, many results derived from pHEMA film experiments left open-ended questions, especially with regard to the unique properties of RB-NA. Continuing this line of investigation will only help broaden our understanding for RB and R6G derivatives.
Click Chemistry – Covalently binding RB and R6G via the upper ring system

Before delving into synthesizing di-ortho R6G derivatives, I synthesized a couple of R6G and RB derivatives to used as reagents for a dual-colored fluorescent pH sensor. This is another project that was sidelined in favor of the sterics project, but since picking up where the project left of should not be too difficult, I will list a few insights that may come to use.

The one synthetic pathway we considered towards covalently binding RB and R6G was the Azide-alkyne Huisen cycloaddition\textsuperscript{17}, with the alkyne and azide functional groups attached as substituents of para-substituted RSL derivatives. Scheme 4 was prioritized over a few other alternative schemes (see next section) for its quantitative yield. Taking in consideration the reactivity of alkyne, I decided to attach it to the relatively well-behaved RB instead of R6G. This gives the overall synthetic scheme as shown below.

![Scheme 4](image)

**Scheme 4**: Azide-alkyne click chemistry between RB-alkylaniline and R6G-azidoaniline affords a dual-colored fluorescent pH probe

R6G-azidoaniline could be synthesized with standard Harbron lab procedures and possibly my optimized procedure as described in Chapter 1. Synthesis of RB-ethynylaniline underwent the biggest procedural changes because the terminal alkyne frequently reduced to an alkene through alkyne halogenation (Figure 33). Figure 38 shows the NMR spectrum of RB-ethynylaniline with its alkene by-product.
Figure 33: Alkene by-product from RB-ethynylaniline synthesis. Since alkyne halogenation is a room temperature process, both POCl₃ and acid work-up can generate this compound.

The by-product as shown in Figure 38 overlaps with the desired product in the fractions retrieved from flash chromatography and the difficulty to separate the two reduces the final yield (<5%). The majority of the addition reaction occurred when washing the post-reflux crude product with 2M HCL, but TLC analysis indicated that the by-product can formed before reflux. For reasons stated in Chapter 1, POCl₃ is likely responsible for the HCl formation in the pre-reflux reaction mixture. Drying the apparatus thoroughly and setting the reaction under anhydrous conditions solved this issue.

The optimum reaction condition for the Azide-alkyne Huisen cycloaddition (Scheme 4) requires high concentration of rhodamines and a solvent system that effectively dissolves both reagents, copper (II) sulfate and sodium ascorbate. The biggest problem I encountered was the incompatibility of these three reagents in terms of their preferred solvents; both RB-alkylaniline and R6G-azidoaniline are soluble in organic halogenated solvents whereas copper (II) sulfate and sodium ascorbate dissolve in water. The fact that the latter two undergo a reduction reaction to produce Cu(I), a transition metal that is unstable in water without stabilizing ligands, only complicates the picture. Separately dissolving the rhodamine derivatives in ethanol and two other reagents in water with Et₃N or THF, and then combining the two mixtures may finally afford the desired bis-rhodamine probe.
It should be noted that we considered two alternative synthetic pathways to covalently binding RB and R6G: attaching RB and R6G to a dianiline compound or attaching the RB and R6G derivatives via Suzuki coupling. The first option was not attempted. The endeavor will likely be a synthetic nightmare, with its potential for many similar by-products. In the event that the dianiline synthetic route is attempted, the RB derivative of the chosen dianiline should be isolated first. Only the R6G-CA was successfully synthesized in preparation for the Suzuki coupling and RB-boronic acid is yet to be isolated. There is no glaring incentive behind pursuing this reaction pathway; synthesis based on this scheme requires more procedures for what would probably be lower yield.
<table>
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**Table 5**: Structures and Abbreviations of Rhodamine Derivatives
**Synthesis of R6G-Acid.** 1.604 g NaOH (40.1 mmol, excess) was dissolved in 20 mL DI H₂O and 10 mL absolute ethanol. The mixture was combined with 1.04 g of R6G (2.17 mmol, 1 eq) and a stir bar. Since the reaction makes the boiling flask and condenser stuck by the end of reflux, vacuum grease was applied around the mouth of the round bottom flask. The solution was refluxed at 90°C for 24 hours with an oil bath. After 24 hours, the solution was taken off the heat. 2M HCl was added to the stirring solution, until the pH meter indicated a pH of 6.25. Buchner filtered the deep red mixture and washed the resulting brick red powder with cold DI H₂O. After collecting the powder into a sample vial, it was left on the high vacuum for more than five hours to dry. The product was used without further purification, as in Lin and Best’s papers.

**Synthesis of RB-ethynylaniline (RB-EA, see Figures 34-39).** Dissolved 0.500 g Rhodamine B (1.04 mmol, 1 eq.) and 0.4890 g 4-ethynylaniline (4.16 mmol, 4 eq.) in 50 mL of dried 1,2-dichloroethane and sonicated the solution briefly to dissolve the reagents. Flame dried a 100 mL round bottom flask containing stir bar and molecular sieves. Utilized a 15 mL syringe to put the solution in the dried round bottom flask and chilled the flask to 0°C with an ice bath. Let the solution stir for 30 minutes while adding 0.05 mL of POCl₃ (0.55 mmol, 1.2 eq.) dropwise with 1 mL syringe and stirred for 30 more minutes. The solution was set up with an argon condenser and oil bath, and refluxed at 85°C for 4 hours. After taking off the heat, the solution was Buchner filtered to remove the molecular sieves and stir bar. The solution was washed with cold 1M H₂SO₄ (3×50 mL), 2M NaOH (3×50 mL) and finally, brine (2×50 mL) in a separatory funnel. Dried the organic layer with sodium sulfate and Buchner filtered out the drying agent 1,2-dichloroethane was evaporated under reduced pressure in the rotary evaporator. The crude product was loaded in a silica column with ethyl acetate and hexane (30:70 by volume) eluent. During flash chromatography, the desired product appeared to be a pink band. Evaporating the
fractions under reduced pressure (both rotary evaporator and high vacuum) gave 0.116g of white powdery product (20.5% yield). Residual ethyl acetate is present in the NMR spectra. The $R_f$ of RB-EA in 40:60 ethyl acetate and hexane is 0.40. RB-EA: $^1$H NMR (400 MHz, CDCl3) $\delta$ 7.98 (m, 1H), 7.48 (m, 2H), 7.12 (m, 1H), 6.88 (d, 2H), 6.59 (d, 2H), 6.29 (m, 4H), 6.27 (s, 2H), 3.31 (q, 8H), 2.98 (s, 1H), 1.15 (t, 12H). RB-EA by-product: $^1$H NMR (400 MHz, CDCl3) $\delta$ 7.98 (m, 1H), 7.48 (m, 2H), 7.12 (m, 1H), 6.95 (d, 2H), 6.62 (d, 2H), 6.29 (m, 4H), 6.27 (s, 2H), 5.65 (d, 1H), 5.41 (d, 1H), 3.31 (q, 8H), 2.98 (s, 1H), 1.15 (t, 12H).

**Synthesis of R6G-azidoaniline (R6G-AA, see Figures 40-42).** Dissolved 0.500g R6G-acid (1.11mmol, 1eq.) and 0.5670g 4-azidoaniline (3.33mmol, 3 eq.) in 50mL of dried 1,2-dichloroethane and sonicated the solution for an hour to dissolve the reagents. Flame dried a 100mL round bottom flask containing stir bar and molecular sieves. Utilized a 15mL syringe to put the solution in the dried round bottom flask and chilled the flask to 0°C with an ice bath. Let the solution stir for an hour while adding 0.122mL of POCl$_3$ (1.33mmol, 1.2eq.) dropwise with 1mL syringe. The solution was set up with a argon condenser and oil bath, and refluxed at 85°C for 5 hours. The solution was Buchner filtered to remove the molecular sieves and stir bar. The solution was washed with 2M HCl (3×50mL), 2M NaOH (3×50mL) and finally, 50mL of brine in a separatory funnel. Dried the organic layer with sodium sulfate and Buchner filtered out the drying agent. 1,2-dichloroethane was evaporated under reduced pressure in the rotary evaporator to give a reddish oil. TLC showed product formation. The crude product was loaded in a silica column with chloroform and methanol (2%) eluent. During flash chromatography, the desired product appeared to be an orange band and eluted in two fractions. Evaporating the fractions gave 0.1088g of pink-orange powder, the isolated product. The $R_f$ of R6G-AA in 40:60 ethyl acetate and hexane is 0.57. R6G-AA: $^1$H NMR (400 MHz, CDCl3) $\delta$ 8.11 (m, 1H), 7.51 (m, 2H),
7.12 (m, 1H), 6.73 (m, 4H), 6.40 (s, 2H), 6.23 (s, 2H), 3.49 (br, 2H?), 3.16 (q, 4H), 1.93 (s, 6H),
1.31 (t, 6H).

**Synthesis of R6G-CA (see Figures 43-46).** Dissolved 0.200g R6G-acid (0.44mmol, 1eq.) and 0.2263g 4-chloroaniline (1.77mmol, 4 eq.) in 50mL of dried 1,2-dichloroethane and sonicated the solution for an hour to dissolve the reagents. Flame dried a 100mL round bottom flask containing stir bar and molecular sieves. Utilized a 15mL syringe to put the solution in the dried round bottom flask and chilled the flask to 0°C with an ice bath. Let the solution stir for 30 minutes while adding 0.05mL of POCl₃ (0.55mmol, 1.2eq.) dropwise with 1mL syringe. The solution was set up with an argon condenser and oil bath, and refluxed at 85°C for 4.5 hours. The solution was Buchner filtered to remove the molecular sieves and stir bar. The solution was washed with 2M HCl (3×50mL), 1M NaOH (4×50mL) and finally, 50mL of brine in a separatory funnel. Dried the organic layer with sodium sulfate. Buchner filtered out the drying agent. 1,2-dichloroethane was evaporated under reduced pressure in the rotary evaporator. The crude product was loaded in a silica column with chloroform and methanol (3%) eluent. During flash chromatography, the desired product appeared to be an orange band. Evaporating the fractions under reduced pressure gave 0.0436g of product (18.8% yield). The Rₜ of R6G-CA in 40:60 ethyl acetate and hexane is 0.63. R6G-CA: ¹H NMR (400 MHz, CDCl₃) δ 8.19 (m, 1H), 7.50 (m, 2H), 7.11 (m, 1H), 7.05 (d, 2H), 6.71 (d, 2H), 6.39 (s, 2H), 6.23 (s, 2H), 3.49 (br, 2H), 3.16 (q, 4H), 1.93 (s, 6H), 1.30 (t, 6H). ¹³C NMR (400 MHz, CDCl₃) δ 167.99, 153.48, 151.71, 147.61, 135.50, 133.34, 132.38, 130.85, 128.99, 128.60, 128.49, 128.38, 124.24, 123.64, 118.20, 106.70, 96.80, 38.57, 31.19, 17.03, 14.94, 1.26.

**Attempted Synthesis of RB-R6G clicked probe.** Attempted to dissolve 0.010g of R6G-AA (17.8µmol, 20eq.) and 0.010g of RB-EA (18.5µmol, 20eq.) in 1:1 water and ethanol. Added
0.355mg of CuSO₄ (0.89µmol, 1eq.) and 0.881mg of C₆H₇NaO₆ (17.8µmol, 2eq.) Left the solution to stir in room temperature overnight. Buchner filtered the solution and observed green-brown precipitate that was not product. Both TLC and NMR did not show product formation.

**Synthesis of R6G-dCA.** Dissolved 0.200g R6G-acid (0.44mmol, 1eq.) and 0.2874g 4-chloroaniline (1.77mmol, 4 eq.) in 50mL of dried 1,2-dichloroethane and sonicated the solution for an hour to dissolve the reagents. Flame dried a 100mL round bottom flask containing stir bar and molecular sieves. Utilized a 15mL syringe to put the solution in the dried round bottom flask and chilled the flask to 0°C with an ice bath. Let the solution stir for 30 minutes while adding 0.05mL of POCl₃ (0.55mmol, 1.2eq.) dropwise with 1mL syringe. The solution was set up with a argon condenser and oil bath, and refluxed at 85°C for approximately 5 hours. The solution was Buchner filtered to remove the molecular sieves and stir bar. The solution was washed with 2M HCl (3×50mL), 2M NaOH (3×50mL) and finally, 50mL of brine in a separatory funnel. Dried the organic layer with sodium sulfate and Buchner filtered out the drying agent. 1,2-dichloroethane was evaporated under reduced pressure in the high vacuum apparatus to give a reddish oil. The crude product was loaded in a silica column with chloroform and methanol (2.5%) eluent. During flash chromatography, the desired product appeared to be an orange band. Evaporating the fractions in the rotary evaporator gave ~0.040g of product (10-15% yield).

**Synthesis of R6G-dFA (see Figures 47-49).** Dissolved 0.200g R6G-acid (0.44mmol, 1eq.) in 50mL of dried 1,2-dichloroethane and sonicated the solution for 20 minutes to dissolve the reagents. Flame dried a 100mL round bottom flask containing stir bar and molecular sieves. Brought the flask to 0°C in an ice water bath for 2 hours. Added 0.05mL of POCl₃ (0.55mmol, 1.2eq.) dropwise with 1mL syringe over 90 minutes while the solution stirred in ice water bath. After adding POCl₃, 0.25mL 2,6-Difluoroaniline (1.33mmol, 3 eq.) was added all at once. TLC
showed product formation. Allowed the solution to stir at 0°C for 6 hours. The solution was Buchner filtered to remove the molecular sieves and stir bar and washed with cold 2M NaOH (3×50mL), DI H₂O (3×50mL) and 50mL brine in a separatory funnel. The organic layer was immediately evaporated under reduced pressure using the rotary evaporator. The crude product was loaded in a silica column with ethyl acetate and hexane eluent (from 40% to 80% hexane). During flash chromatography, the desired product appeared colorless. Fractions 7-11 were collected and evaporated under reduced pressure using the rotary evaporator to give a sticky tannish solid which seemed to amount to 5% yield. The R_f of R6G-dFA in 40:60 ethyl acetate and hexane is 0.38. R6G-dFA: ^1^H NMR (600 MHz, CDCl3) δ 8.06 (m, 1H), 7.58 (m, 2H), 7.22 (m, 1H), 7.10 (m, 1H), 6.67 (t, 2H), 6.45 (s, 2H), 6.19 (s, 2H), 3.49 (br, 2H?), 3.16 (q, 4H), 1.95 (s, 6H), 1.30 (m, 6H).

**Synthesis of R6G-dMA (see Figures 50-56).** Dissolved 0.200g R6G-acid (0.44mmol, 1eq.) in 50mL of dried 1,2-dichloroethane and sonicated the solution for 20 minutes to dissolve the reagents. Flame dried a 100mL round bottom flask containing stir bar and molecular sieves. Brought the flask to 0°C in an ice water bath for 2 hours. Added 0.05mL of POCl₃ (0.55mmol, 1.2eq.) dropwise with 1mL syringe over 90 minutes while the solution stirred in ice water bath. After adding POCl₃, 0.25mL 2,6-Dimethylaniline (1.33mmol, 3 eq.) was added all at once. TLC showed product formation. Allowed the solution to stir at 0°C for 6 hours. The solution was Buchner filtered to remove the molecular sieves and stir bar and washed with cold 2M NaOH (3×50mL), DI H₂O (3×50mL) and 50mL brine in a separatory funnel. The organic layer was immediately evaporated under reduced pressure using the rotary evaporator. TLC showed at least three by-products. The crude product was loaded in a silica column with ethyl acetate and hexane eluent (from 40% to 80% hexane). During flash chromatography, the desired product appeared to
be a pinkish band. Fractions were collected and evaporated under reduced pressure using the rotary evaporator to give a light yellow powder that seemed to amount to 5% yield. The compound needs to go through another flash chromatography. The R of R6G-dMA in 40:60 ethyl acetate and hexane is 0.34. R6G-dMA: $^1$H NMR (600 MHz, CDCl3) δ 8.08 (m, 1H), 7.67 (m, 2H), 7.38 (m, 1H), 7.00 (t, 1H), 6.80 (d, 2H), 6.35 (s, 2H), 6.19 (s, 2H), 3.12 (m, 4H), 1.94 (s, 6H), 1.32 (s, 6H), 1.29 (m, 6H). R6G-dMA by-product: $^1$H NMR (600 MHz, CDCl3) δ 8.10 (m, 1H), 7.69 (m, 2H), 7.38 (m, 1H), 7.00 (t, 1H), 6.80 (t, 2H), 6.35 (s, 2H), 6.45 (s, 1H), 6.44 (s, 1H), 6.35 (s, 1H), 6.19 (s, 1H), 3.37 (br, 1H), 3.15 (m, 2H), 2.05 (s, 3H), 1.94 (s, 3H), 1.35 (t, 3H), 1.30 (t, 6H), 1.28 (t, 3H). $^{13}$C NMR (600 MHz, CDCl3) δ 166.05, 155.09, 154.24, 153.66, 148.70, 147.64, 138.88, 138.69, 133.37, 132.38, 132.26, 130.40, 129.59, 128.87, 127.98, 127.90, 127.86, 124.85, 123.92, 119.03, 117.08, 113.69, 108.82, 102.50, 96.83, 50.91, 38.37, 18.27, 18.23, 16.78, 15.243, 14.63, 8.353.

**Synthesis of R6G-dIA (see Figures 57-59).** Dissolved 0.200g R6G-acid (0.44mmol, 1eq.) in 50mL of dried 1,2-dichloroethane and sonicated the solution for 20 minutes to dissolve the reagents. Flame dried a 100mL round bottom flask containing stir bar and molecular sieves. Stirred the mixture under argon gas for 6 hours to dry the reaction mixture. Brought the flask to 0°C in an ice water bath for another 2 hours. Added 0.05mL of POCl$_3$ (0.55mmol, 1.2eq.) dropwise with 1mL syringe over 40 minutes while the solution stirred in ice water bath. After adding POCl$_3$, 0.2mL 2,6-Diisopropylaniline (1.06mmol, 2.4 eq.) was added dropwise over roughly 30 minutes. TLC showed product formation. Allowed the solution to stir in room temperature for 10 hours. The solution was Buchner filtered to remove the molecular sieves and stir bar and washed with 1M NaOH (2×50mL), DI H$_2$O (2×50mL) and brine (2×50mL) in a separatory funnel. Dried the organic layer with sodium sulfate by leaving the mixture to sit for
90 minutes. Buchner filtered out the drying agent and evaporated 1,2-dichloroethane under reduced pressure using the high vacuum. The crude product was loaded in a silica column with ethyl acetate and hexane eluent (from 40% to 80% hexane). During flash chromatography, the desired product appeared to be a tannish yellow band. Fractions 5-32 were collected and evaporated under reduced pressure using the rotary evaporator to give 0.0301g of whitish pink powder. Some product was lost due to bumping during the rotary evaporator stage. Flash chromatography with small scale ethyl acetate and hexane columns were repeated two more times in an attempt to purify the product and produced very white powder. The compound needs to go through another flash chromatography. The R_f of R6G-dIA in 40:60 ethyl acetate and hexane is 0.69. 1H NMR (600 MHz, CDCl3) δ 8.08 (m, 1H), 7.63 (m, 2H), 7.24 (m, 1H), 7.20 (t, 1H), 6.93 (d, 2H), 6.33 (s, 2H), 6.17 (s, 2H), 3.48 (br, 2H), 3.12 (quint, 4H), 2.38 (sept, 2H), 1.93 (s, 6H), 1.25 (m, 6H), 0.88 (d, 6H), 0.36 (d, 6H).

**Attempted Synthesis of R6G-dNA.** Dissolved 0.200g R6G-acid (0.44mmol, 1eq.) in 50mL of dried 1,2-dichloroethane and sonicated the solution for 20 minutes to dissolve the reagents. Flame dried a 100mL round bottom flask containing stir bar and molecular sieves. Brought the flask to 0°C in an ice water bath for 2 hours. Added 0.05mL of POCl₃ (0.55mmol, 1.2eq.) dropwise with 1mL syringe over 90 minutes while the solution stirred in ice water bath. After adding POCl₃, 0.25mL 2,6-Dinitroaniline (1.33mmol, 3 eq.) was added all at once. TLC showed product formation. Allowed the solution to stir at 0°C for 6 hours and subsequently stored in the freezer for two nights. The solution was then Buchner filtered to remove the molecular sieves and stir bar and washed with cold 2M NaOH (3×50mL), DI H₂O (3×50mL) and 50mL brine in a separatory funnel. The organic layer was immediately evaporated under reduced pressure using the rotary evaporator to give a red-orange mixture. The crude product was loaded
in a silica column with ethyl acetate and hexane eluent (from 40% to 80% hexane). During flash chromatography, the desired product appeared a faint pink and extremely broad band. Fractions 25-65 were collected and evaporated under reduced pressure using the rotary evaporator. The product was put through another small-scale ethyl acetate and hexane column but due to R6G-dNA’s tendency to saturate the stationary phase and the eluted fractions showed little product, the procedure was aborted. The $R_f$ of R6G-dNA in 40:60 ethyl acetate and hexane is 0.51.
References


Appendix

Figure 34: $^1$H NMR Spectrum of RB-EA
Figure 35: $^1$H NMR Spectrum of RB-EA (6.0-8.5ppm)
Figure 36: $^1$H NMR Spectrum of RB-EA (0-4.5ppm)
Figure 37: $^1$H NMR Spectrum of RB-EA with by-product
Figure 38: $^1$H NMR Spectrum of RB-EA with by-product (5.0-8.0ppm)
Figure 39: $^1$H NMR Spectrum of RB-EA with by-product (0-4.0ppm)
Figure 40: $^1$H NMR Spectrum of R6G-AA
Figure 41: $^1$H NMR Spectrum of R6G-AA (6.0-8.5ppm)
Figure 42: $^1$H NMR Spectrum of R6G-AA (aliphatic)
Figure 43: $^1$H NMR Spectrum of R6G-CA
Figure 44: $^1$H NMR Spectrum of R6G-CA (6.0-8.0ppm)
Figure 45: $^1$H NMR Spectrum of R6G-CA (0-4.0ppm)
Figure 46: $^{13}$C NMR Spectrum of R6G-CA
Figure 47: $^1$H NMR Spectrum of R6G-dFA
Figure 48: $^1$H NMR Spectrum of R6G-dFA (6.0-8.5 ppm)
Figure 49: $^1$H NMR Spectrum of R6G-dFA (0-4.0ppm)
Figure 50: $^1$H NMR Spectrum of R6G-dMA
Figure 51: $^1$H NMR Spectrum of R6G-dMA (6.0-8.5ppm)
Figure 52: $^1$H NMR Spectrum of R6G-dMA (0-4.0ppm)
Figure 53: $^1$H NMR Spectrum of R6G-dMA by-product
Figure 54: $^1$H NMR Spectrum of R6G-dMA by-product (6.0-9.0ppm)
Figure 55: $^1$H NMR Spectrum of R6G-dMA by-product (1.0-4.0ppm)
Figure 56: $^{13}$C NMR Spectrum of R6G-dMA by-product
Figure 57: $^1$H NMR Spectrum of R6G-dIA
Figure 58: $^1$H NMR Spectrum of R6G-dIA (6.0-9.0ppm)
Figure 59: $^1$H NMR Spectrum of R6G-dIA (0.3-3.6 ppm)