SYNTHESIS OF P-GLYCO PROTEIN INHIBITOR COMPOUNDS

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SYNTHESIS OF P-GLYCOPEPTIDE INHIBITOR COMPOUNDS

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SYNTHESIS OF P-GLYCOPROTEIN INHIBITOR COMPOUNDS

A Dissertation Presented to the Graduate Faculty of
Dedman College
Southern Methodist University
in
Partial Fulfillment of the Requirements
for the degree of
Doctor of Philosophy
with a
Major in Chemistry
by
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August 4, 2021
SYNTHESIS OF P-GLYCOPROTEIN INHIBITOR COMPOUNDS

Advisor: Professor Alexander R. Lippert

Doctor of Philosophy conferred: August 4, 2021
Dissertation completed: July 14, 2021

A problem with cancer treatment is that many cancers develop resistance to chemotherapeutic agents, causing them to fail to accumulate in resistant cancer cells long enough to have any effect. This is due to the overexpression of a plasma membrane protein called P-glycoprotein (P-gp). Generally, the role of P-gp is to protect the cells from any toxins or foreign substances by pumping these toxins (including chemotherapeutic drugs) out of the cell. I am collaborating with the Wise-Vogel laboratory at Southern Methodist University, who utilize a computer-generated model to predict the structures of P-gp inhibitors that inhibit the action of P-gp. These docking models find drug targets that slow the action of P-gp pumping, as well as help understand the underlying mechanism of how the protein effluxes toxins from the cell. My current research is focused on multiple drug analogues that are predicted to inhibit the P-gp protein based on their docking models. They are tested in cancer cell lines in combination with current chemotherapeutics to determine efficacy and strength of inhibition so that future chemotherapeutic drugs can work effectively in cells.
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ACKNOWLEDGEMENTS

I thank God for giving me the good health and well-being that allowed me to get to where I am. I would like to express my heartfelt appreciation for supervisor Professor Lippert. For all his support during my research, as well as his wisdom, inspiration, and vast expertise. All his advice was invaluable in developing the research questions and methods. All his constructive reviews pushed me to sharpen my thinking and elevate my work. His advice was invaluable in the study and writing of this thesis. I couldn't have asked for a better counselor for my Ph.D. studies.

Besides my adviser, I'd like to thank the rest of my study committee: Prof. John D. Buynak, Prof. Peng Tao and Prof. Isaac Garcia-Bosch for not just their informative comments and inspiration, but also for all questions and comments during the research synopsis and proposal methodology topic that compelled me to broaden my analysis and help me view ideas from several perspectives.

I want to express my gratitude to Professors John Wise and Pia Vogel, whose assistance during our collaboration project was invaluable to me. I feel so fortunate to have worked with someone as wonderful as you. Thank you, really, for you and your group, especially Amila Nanayakkara and Lauren Ammerman. I am honestly so grateful for what we've done and published as two departments, chemistry and biology. I also want to thank my group in Lippert lab, especially Bo and Uroob, for not just your fantastic work but also for all of your support during the project, which made the team stronger. The hard work for us as a team enabled us to complete the project successfully.
Furthermore, I would like to thank my present and past lab colleagues, members of the Lippert group, Dr. Jian Cao, Dr. Weiwei An, Dr. Luke Ryan, Bo Li, Uroob Haris, and Briley Bezner for their support. A big thank you also to all undergraduate students, especially Audrey Reeves, Lyn Mouhaffel, and Andrew Nakatsuka.

I'd like to thank the academic and non-academic members of SMU’s Chemistry Department for their help in a variety of ways.

I am eternally grateful to my wonderful husband Bander and my four children, Sara, Saleh, Tala, and Lara. Their tolerance, compassion, and love have helped me get through the difficult times.

Last but not least, I'd like to thank my family and my husband's family for understanding my goal and supporting me throughout my academic career.

Finally, and probably most importantly, I would want to acknowledge my father, who died before I finished my education. I'm sure he'd be proud, and I'll be always thankful for the knowledge and ideas he instilled in me.
CHAPTER 1
SYNTHESIS OF P-GLYCOPROTEIN INHIBITOR COMPOUNDS

1.1 Introduction

1.1.1 Cancer

The human body is able to control cell growth and death, but when some cells do not respond or grow uncontrollably, they can become unrestrained cancerous cells.¹ Chemotherapy is one of the most common treatments used to kill cancer cells or control their proliferation.² However, cancers will often develop resistance to chemotherapeutic drugs, mainly due to efflux of the drug from the cancer cell.³ Furthermore, chemotherapies used to treat the cancer cells in the body also weaken the patient’s immune system.⁴ These drawbacks show a need for better therapies with less side effects.

When cancer cells become immune to several chemotherapeutic drugs that are chemically and structurally distinct, this is referred to as multi-drug resistance (MDR).⁵ MDR normally results in chemotherapy drug failure and, as a result, a bad patient prognosis. Cancer cells can develop resistance to anticancer drugs by overexpressing one or more ATP-binding cassette (ABC) transporters, which act as drug efflux pumps.⁶ Cancer medications are pumped out of cancer cells before they can do more damage. P-glycoprotein (P-gp, or ABCB1) and Breast Cancer Resistance Protein (BCRP) are two examples of efflux protein pumps.⁷
1.1.2 Chemotherapy and resistance

Chemotherapy is a type of cancer treatment that exploits chemically synthesized drugs to inhibit cancer cells from dividing quickly. Different chemotherapeutic agents are developed and applied depending on cancer type. It is observed that some cancer cells show multidrug resistance due to the presence of a cell surface efflux pump called P-glycoprotein (P-gp), causing various treatment complications. Each drug works to target the cancer through different pathways and mechanisms. Thus, many chemotherapy treatments have been devised based on type of cancer and its pathology. Paclitaxel is one example of chemotherapeutics that has a complex structure from the taxane family isolated from Pacific Yew tree (Taxus brevifolia). This antitumor drug acts by microtubule inhibition, disruption of cell cycle and division as well as induction of apoptosis. Also, pentostatin is being used to treat leukemia and is commercially available under the name Nipen. It acts by inhibiting adenosine deaminase and inhibits cancer cell division by damaging DNA. 5-Fluorouracil (5-FU) is applied as chemotherapy for several types of cancers such as colorectal, skin and stomach cancers. It actually inhibits an enzyme that slows down DNA synthesis. Anthracyclines, too, are used to treat malignancies. They are a type of antibiotic extracted from some types of Streptomyces bacteria. Anthracyclines have the capacity to damage the DNA in cancer cells leading to cell death. These different chemotherapies are usually taken alone or combined with other drugs to improve the result of killing the cancer cells and controlling their proliferation.

Most tumors are quite responsive and highly sensitive to these treatment methods. However, a growing number of patients exhibit multidrug resistance (MDR) to cancer treatment, which has thus become a prevalent concern. From this predicament stemmed the application of
a rotation of drugs with other therapies such as radiation or immunotherapy.\textsuperscript{18} It is apparent that new treatments must be developed that incur less side effects and less lethality in healthy cells.

1.1.3 P-glycoprotein

P-glycoprotein (P-gp) is a plasma membrane protein that is responsible for protecting cells from endogenous toxins.\textsuperscript{19} P-gp is present in many tissues and helps in detoxifying cells by pumping xenobiotics across the plasma membrane. P-gp is able to bind and transport a diverse range of cationic amphipathic molecules ranging in size from 100 to 4000 Da.\textsuperscript{20}

When the cell undergoes distress from an external drug, the P-gp pumps the toxins out of the cell to ensure its survival. While this is a useful function in healthy cells, it can lead to multiple drug resistance in cancer cells. The chemotherapeutic agents are exported out of the tumor before they exhibit their desired effects.\textsuperscript{21}

P-glycoprotein (P-gp), also known as ABCB1, was the first human ABC transporter discovered. It is potentially the most researched ABC transporter, especially in relation to MDR. P-gp, like BCRP, has a diverse substrate set that includes amphipathic, lipid-soluble, and aromatic compounds.\textsuperscript{22} Taxanes, anthracyclines, HIV-protease inhibitors, and antibiotics are among the medications it transports. P-gp is a polypeptide that consists of two nucleotide binding domains. P-gp has a larger role outside of MDR, which is to protect cells from xenobiotics and toxins. The protein is present in the mucosal membrane of the bladder, the kidney epithelia, the liver, and the placenta.\textsuperscript{23}

P-gp is a $\sim$170 kDa protein consisting of two pseudosymmetrical halves, each containing a nucleotide binding domain (NBD) and a transmembrane domain (TMD).\textsuperscript{24} Figure 1.1 shows the crystal structure for P-gp in the “open inward” conformation with NBDs apart and N-terminal half
of the protein (blue) and the C-terminal half (yellow) are linked by a flexible linker (black dashed line).  

![Figure 1.1 P-gp structure in three dimension. Image adapted from ref 25.](image)

It has been hypothesized that ABC-transporter proteins undergo large conformational changes powered by the binding and hydrolysis of ATP and that these changes alter the transmembrane domains from “open to the cytoplasm” (inward-facing) to “open to the extracellular space” (outward-facing) conformations. To understand the mechanism of P-gp, Professor J. Wise at Southern Methodist University used a computational model to study and design synthetic compounds that inhibit P-gp’s efflux pump action by acting as a competitive inhibitor to its nucleotide bindings domains. Through the application of molecular dynamic studies, the development of small molecule P-gp inhibitors has made noteworthy progress. Cells treated with both the P-gp inhibitor and paclitaxel responded with a 2,400-fold increase in cytotoxicity as compared to a positive control treatment with only paclitaxel. These results give conclusive evidence that predicted molecules were effective against the efflux pump and lead to
inhibition of P-gp in prostate cancer cells, reducing drug export and restoring cell sensitivity to chemotherapeutics. Using P-gp inhibitors in combination with chemotherapy could be key in resolving both cost efficiency and efficacy of the treatment, as lower dosages of chemotherapeutics will be needed to achieve their desired effect.

**1.1.4 Computational model of P-glycoprotein**

Recent advancements have brought upon exceptional techniques which can be used to aid drug discovery and development. Among these are computational methods which have contributed greatly in the discovery of Human Immunodeficiency Virus (HIV) medications such as ritonavir and saquinavir. These methods have also been effective in terms of drug screening to identify and modify particular chemotherapeutics including Luminespib, an experimental anticancer drug. To create highly superior medications, drug screenings often utilize lead optimization in the process. This technique improves the overall performance of the drug while reducing its off-target effects. To elaborate the process, high yield screenings produce small molecules as initial hit molecules. These molecules can then be optimized further into lead molecules, resulting in a drug with better pharmacology properties. Despite the benefits associated with lead optimization, this method requires a considerable amount of cost to be accomplished. To address this problem, computational methods are incorporated into lead optimization as well. Computational lead optimization involves decreasing molecules that require testing and increasing the proportion of produced leads that possess the necessary features, reducing the overall expense of the process.

The diagram below in **Scheme 1.1** is comprised of four components: the orange boxes, the blue boxes, the green boxes and a single yellow box. The orange boxes present the preparation phase wherein the root and alignment atoms of the scaffolds and precursor fragments are marked.
The blue boxes, on the other hand, represent the building phase while the green boxes denote the docking phase. Lastly, the yellow box signifies the final step wherein the dock results are analyzed and the observations are applied into lab configurations.\textsuperscript{31}

\textit{Scheme 1.1}. Step-by-step diagram for \textit{ChemGen} program. Figure adapted from reference 31.
Figure 1.2. SMU-29 structure that successfully inhibited P-gp activity.

P-gp is known as a fundamental membrane efflux pump that acts by transferring compounds such as chemotherapeutic drugs out of the cell. Many studies have been done to develop the multi-drug resistance by inhibiting expression of P-gp. Dr. Wise and his group screened molecules for potential inhibition of P-gp activity by using *in silico* molecular docking assays. One of these molecules, SMU-29 (Figure 1.2), was found to be a successful inhibitor of P-gp activity *in vitro*, resulting in the re-sensitization of cancer cells in culture to chemotherapeutics. Structural variants of SMU-29 were screened *in silico* to create compounds that exhibit higher binding affinity to P-gp's nucleotide binding domain (NBD) by using ChemGen, a novel computational medicinal chemistry software designed by Dr. Wise. An estimated binding affinity was calculated for these compounds when they were docked with multiple models of P-gp. Approximately 500 structural variants of SMU-29 with equivalent or better estimated binding affinity relative to the SMU-29 compound were designed. Three of these compounds target that are shown in Figure 1.3 have been synthesized after specific docking experiments and screened with P-gp *in vitro*. These analogues share a common pyrazole motif and differ with variations in the *N*-chloroacetamide motif. The aim of this project is to synthesize these SMU-29 analogues by conserving the pyrazole and appending various amines to the structure.
1.1.5 Docking simulation of SMU-29 variants to P-gp

The docking studies were used to test P-gp inhibitors in silico. Inhibiting P-gp efflux activity prevents the protein from lowering intracellular concentrations of therapeutics to subtherapeutic levels. P-gp can be responsible for MDR against various cancer chemotherapies. This study aimed to determine binding affinities of the P-gp inhibitors. Using Autodock Vina, and Visual Molecular Dynamics (VMD), docking simulations of SMU-29 variants to P-glycoprotein were made. Through these simulations, it was found that the highest-affinity binding location is near P-gp’s nucleotide binding domain (NBD).
SMU-29-231 is shown in white, docked SMU-29 to P-gp. Figure 1.4 shows the human model of P-gp when it docked with SMU-29-231. SMU-29 in the 2hyd_nbd_1 NBD, showing probable hydrogen-bond interactions.

![Diagram showing SMU-29 binding to amino acids.](image)

*Figure 1.5. SMU-29 binding to amino acids.*

The estimated dissociation constant $K_D$ and binding affinity for SMU-29 variants are listed in Table 1.1. Binding affinities were predicted and compared to original SMU-29. Eight inhibitors with equivalent or better-estimated binding affinity relative to the original SMU-29 except SMU-29-286 and SMU-29-238.
Table 1.1. Estimated value for $K_D$ and binding affinity.

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</tbody>
</table>

![Compound 227](image)

![Compound 231](image)

![Compound 280](image)

![Compound 216](image)
1.2. Results and Discussion

1.2.1 Retrosynthesis

We begin synthesis with the basic concept of using retrosynthesis to provide a roadmap to obtain the SMU-29 variants. We chose to make a disconnection at the carbon-sulfur bond, leaving the thiol on the pyrazole motif as shown in Scheme 1.2 below.

![Scheme 1.2. Retrosynthesis route for SMU-29 analogues.](image)

The methodology behind this synthetic route is that we can form the pyrazole motif as a stable synthetic intermediate, thus allowing for facile screening of all analogues by connecting thiol motif 6 to chloroacetyl motif 7 via nucleophilic substitution. The thiol pyrazole configuration 6 would be formed via hydrolysis of thioacetate 5 after substitution of the chloride in 4 by potassium thioacetate. The N-chloroacetamide 4 is completed by nucleophilic acyl substitution. The amino pyrazole 3 would be produced after phenyl diazoacetonitrile 2 is reacted with phenylhydrazine. The configuration of phenyl diazoacetonitrile can be formed when the aldehyde reacts with diazoacetonitrile 1. The first step in this approach starts with reacting aminoacetonitrile...
hydrochloride with NaNO₂. The second set of configurations N-chloroacetamide derivatives 7 are synthesized by reacting amines through acyl substitution with chloroacetyl chloride.

**Figure 1.6.** Modular synthetic route to access 29 derivatives.

The synthetic route of target inhibitors of P-glycoprotein were devised using this retrosynthetic route.

1.2.2 Synthesis of the pyrazole 6

The synthesis of pyrazole 6 started with the preparation of diazoacetonitrile 1 using aminoacetonitrile hydrochloride and sodium nitrite as shown below in Scheme 1.3.
After that, the diazoacetonitrile 1 was reacted with trimethylbenzaldehyde by using BF₃·OEt₂ as a catalyst to synthesize 3-oxo-3-(2,4,5-trimethylphenyl) propanenitrile 2. Pyrazole is a heterocyclic organic compound that has a five-membered ring and two adjacent nitrogen atoms. This nitrogen made the pyrazole of interest to the chemical industry and pharmaceutical industry. In addition, many previous studies reported anticancer inhibitors that contain pyrazole show promising efficacy in different type of cancer. Synthesis of the five-membered ring was performed under neat conditions; benzoylacetonitrile 2 was reacted with phenylhydrazine to generate 1-phenyl-pyrazole 3 with a good yield. After obtaining the pyrazole, acyl substitution was done by reacting the pyrazole with chloroacetyl chloride using DCM as a solvent and giving a 97% yield. The previous reactions are shown in Scheme 1.3. In addition, potassium thioacetate was used to displace the chloride and give thioacetate 5 in the presence of anhydrous THF as shown below in Scheme 1.4.
Scheme 1.4. Synthetic procedure for compound 5.

1.2.3 Hydrolysis of thioacetate

The last step of the thiol pyrazole intermediate is the hydrolysis of the thioacetate. This step was attempted in both acidic and basic conditions as it shown in Table 1.2.38 The most successful procedure was done through hydrolysis of thioacetate in basic conditions of 2M NaOH and deoxygenated methanol to get the target compound mercapto amino-triphenyl pyrazole 6.

Table 1.2. Overview for acid or base-mediated reactions to hydrolyze thioacetate.

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Acid</th>
<th>Base</th>
<th>Solvent</th>
<th>Product</th>
<th>Yield</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>HCl/MeOH</td>
<td>-</td>
<td>-</td>
<td>SM</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>NaOMe</td>
<td>CH₃OH</td>
<td>6</td>
<td>5-10%</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>NaOH</td>
<td>CH₃OH</td>
<td>6</td>
<td>10-20%</td>
<td>bubbling N₂</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>NaOH</td>
<td>CH₃OH</td>
<td>6</td>
<td>70%</td>
<td>freeze-thaw</td>
</tr>
</tbody>
</table>
Table 1.2 shows the optimization to the best condition to hydrolyze the thioacetate. This compound has two different functional groups, the thioacetate and secondary amide, but hydrolysis using acid failed to give any product. However, when using basic conditions, the reaction gave product. Only a low yield occurred when sodium methoxide was used. Sodium hydroxide in methanol successfully cleaved the thioester groups of compound 5 and gave a high yield up to 70% when degassed with a freeze-thaw method.

1.2.4 Synthesis of the N-chloroacetamide motifs 7-13

The acyl substitution reaction of various amines was done using chloroacetyl chloride to form the intermediate of N-chloroacetamide. The activation of different amines was done by using various amines such as: 2-aminonaphthalene, iminodibenzyl, 3,4,5-trimethoxybenzylamine, 1,3-benzothiazol-6-amine, 1-adamantylamine, benzhydrylamine and 4-fluorobenzylamine. These reactions were done by reacting the various amines with chloroacetyl chloride and Et$_3$N to get the products in high yield as shown in Table 1.3.

Table 1.3. Acylation substitution products for variant amine.
Compound 14 was synthesized by using a Friedel-Crafts acylation with 9-fluorene, chloroacetyl chloride and aluminum chloride as a catalyst. The reaction shown in Scheme 1.5.48

![Scheme 1.5. Friedel-Crafts acylation reaction.](image)

1.2.5 Synthesis of final anticancer drugs

After finishing the previous steps, the last step was done by S2 nucleophilic substitution. The reaction was done by using the thiol pyrazole motif and acetyl motif as shown in Scheme 1.6, in the presence of potassium carbonate and deoxygenated DMF.49 Eight anticancer drugs were successfully synthesized. Characterization was done using proton and carbon NMR.
1.2.6 Synthesis of SMU-544 and SMU-551

Synthesis of SMU-544 was done by reacting isatin with hydrazinecarbothioamide to form aromatic sulfide derivative as is show in Scheme 1.7 below. After that, the sulfide motif 15 was reacted with acetyl chloride 4 in the presence of triethylamine to form final SMU-541 in 58%.
The final analogue SMU-551 was done starting from the sulfide motif 17. First was the formation of 5-bromonicotinoyl chloride 16 by reacting 5-bromonicotinic acid with thionyl chloride. After that, the carbonyl chloride 16 was reacted with 3-aminothiophenol to form the sulfide motif 17, 5-bromo-N-(3-mercaptophenyl) nicotinamide, as it shown below in Scheme 1.7. Finally, SMU-551 was successfully synthesized by reacting the sulfide motif 17 with acetyl chloride 4 by S\texttextsubscript{N}2 reaction.

\begin{center}
\textbf{Scheme 1.8.} Synthesis procedure of sulfide derivative and SMU-551.
\end{center}

1.3. Cell culture methods

1.3.1 Cell lines and cell culture

The cell culture studies have all been performed in the Vogel – Wise Lab, Department of Biological Sciences at SMU by Dr. Amila.

For these experiments, multidrug-resistant DU145TXR cells were used that derived from the human prostate cancer cells DU145. Dr. Evan Keller (University of Michigan, Ann Arbor, MI)
generously provided both the chemotherapeutic sensitive DU145 human prostate cancer cells specimens, together with the multidrug resistant sub-line, DU145TXR. The DU145TXR cell lines were maintained under positive selection pressure by supplementing the complete medium with 10 nM of paclitaxel from Acros Organics, NJ. Both DU145 and DU145TXR cell lines were then maintained in complete media composed of RPMI1640 with L-glutamine, 10% fetal bovine serum (FBS; BioWest, Logan, UT), 100 U/mL penicillin, and 100 µg/mL streptomycin and were kept in a humidified incubator at 37 degrees Celsius with 5% CO₂. Dr. Robert Harrod (Southern Methodist University, Dallas, TX) provided the noncancerous human fetal lung cell line, HFL1. This was maintained in complete media as well which is composed of F12K with L-glutamine, 10% FBS (BioWest, Logan, UT), 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified incubator at 37 degrees Celsius and 5% CO₂ as well. In addition, the HFL1 cells’ growth surfaces were treated with 0.1 mg/mL rat tail collagen (BD Biosciences, Palo Alto, CA) in 0.02 N acetic acid for 10 minutes and rinsed with PBS after use to enhance the cells’ attachment properties. All cell culture materials were retrieved from Corning Inc. (Corning, NY) unless otherwise stated.

1.3.2 Calcein AM assay

Calcein AM, DU145TXR cells were seeded in 96 wells plates and permitted to grow completely until they reach confluency. This was done to assess the inhibition of P-gp-catalyzed transport of the P-gp pump substrate. Afterwards, the medium was removed and the cells were treated without the presence of 2 μM P-gp inhibitory compounds as well as 1 μg/mL calcein AM. This was then diluted into phenol red free RPMI 1640 media. The effect of pre-incubation of compound cells were investigated by treating the cells with P-gp inhibitory compounds, incubating the specimens by 37 degrees Celsius for 6 hours, and adding calcein AM afterwards. Measurements were done with the following parameters: fluorescence excitation at 485 nm with
a 20 nm gate emission at 535 nm with a 20 nm gate. The BioTek Cytation 5 imaging multi-mode reader was used over 60 minutes in 20-minute intervals. The results of the investigations were plotted as the mean with the standard deviation (SD) of six replicates per concentration. This serves as representatives of at least two independent experiments as well.

1.3.3 MTT cell viability assay

The cells were initially trypsinized from monolayers and were seeded with 3000 cells in 150 µL of complete medium in a 96 well plate afterwards. After 24 hours, the cells were treated with paclitaxel retrieved from Acros Organics, NJ for 48 hours. This process is done with or without the presence of P-gp inhibitor compounds dissolved in DMSO or DMSO controls. 48 hours after the treatment, MTT assays were executed using 5 mg/mL of MTT (Acros Organics, NJ) solution prepared in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, and pH 7.4). The media was then removed and the formazan crystals were mixed and dissolved in 100 µL of DMSO 4 hours after incubation with MTT. Using a BioTek Cytation 5 imaging multi-mode reader (Bio-Tek, Winooski, VT), the absorbance at 570 nm was measured. The DMSO treated cells were assigned as representatives for 100% viability. With this, percent viability is calculated using the following formula:

\[
\text{% Viability} = \frac{\text{Absorbance at 570 nm of test well}}{\text{Absorbance at 570 nm of DMSO treated cells}}
\]

Using MTT and complete medium without cells, background absorbance was quantified. This was then subtracted from all the test values. Lastly, a four-parameter variable non-linear slope were utilized in generating the graphical representations and in identifying IC50 values.
1.4 Cell culture results

![Graph showing cell viability results for different derivatives.

Figure 1.7. Optimization of the 29 variants that inhibit the P-gp by reduce the cell viability of MDR prostate cancer cells in the presence of chemotherapeutic more efficiently compared to original SMU-29.

The viability of the DU145TXR cells treated with derivatives 238, 255, 278, 280, and 286 with or without 100 nM of paclitaxel is shown in Figure 1.7. Similar to the previous group, the inhibitory action of these variants can be observed at 3 µM concentrations and higher. Performance-wise, it can be observed that the Group 2 variations incorporated with paclitaxel were superior to the original 29-compound. This is the case for all derivates except for 286. This variant, however, showed high toxicity levels at a concentration of 15 µM. Similarly, variant 280 displayed signs of toxicity to cancer cells at 3 µM concentrations without the presence of paclitaxel. Another test was performed to determine the IC50 values necessary for reversing MDR with these

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```
22
```
inhibitors. In this test, the DU145TXR cells were treated with fixed concentrations of inhibitors while adding varying doses of paclitaxel as seen in Figure 1.8.

![Figure 1.8. Compound 29 reverses paclitaxel resistance by rationally designed versions of the Pgp inhibitor.](image)

### 1.4.1 Calcein accumulation

The P-gp substrate real-time accumulation in the presence or absence of P-gp inhibitors was previously evaluated through calcein AM accumulation assays. The process involved the incubation of P-gp overexpressing DU145TXR cells with the corresponding inhibitors in the presence of the P-gp substrate. This led to cellular accumulation of calcein-AM and the cleavage of its acetoxymethyl ester groups, generating the highly fluorescent compound, calcein. The calcein remains in the cells and its relative fluorescence is observed and measured over time. The results of these assays are presented in the left panel of Figure 1.9.
From these records, it can be seen that the cellular accumulation of fluorescent calcein in cells treated with any of the three Group 1 \(29\) variants were lower than that of the cells treated with the parental compound. Several variables are presumed to affect the previous results. The lower accumulation of calcein may be associated with the retention of the compounds in the cellular membrane due to their increased \(\log P\) values relative to \(29\) values. These observations were further tested using similar calcein accumulation assays performed after a 6-hour pre-incubation with the \(29\)-variants and the parental compound \(29\). To clarify, the preferential partitioning variants in the hydrophobic part of the cell membrane would keep them more distant from the putative allosteric site on P-gp located adjacent to the membrane in the cytoplasm. Once again, the results of these tests are shown in Figure 1.8. The data indicates that the 6-hour preincubation resulted in a slight improvement in the calcein accumulation in the presence of variants for all Group 1 compounds. The same cannot be said for the specimens that did not undergo preincubation. Nevertheless, the performance of these variants in these assays was still inferior compared to the parental compound.

Figure 1.9. Cellular accumulation of the fluorescent dye calcein.
Summarizing, the prostate cancer cell line, DU145TXR, overexpressing P-gp was treated with calcein AM and 2 µM of the indicated P-gp inhibitors. For comparison, two setups were done, one with a 6-hour preincubation and the other without.

1.4.2 Comparison between predicted $K_D$ and IC50

The correlation between the predicted $K_D$ dissociation constant and IC50 value is shown in Table 1.4.

Table 1.4. Estimated value for predicted $K_D$ and measured IC50.

<table>
<thead>
<tr>
<th>SMU-29 variants</th>
<th>$K_D$ (M)</th>
<th>IC50 (M)</th>
<th>SMU-29 variants</th>
<th>$K_D$ (M)</th>
<th>IC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.54E-09</td>
<td>1.86E-06</td>
<td></td>
<td>1.53E-08</td>
<td>2.54E-06</td>
<td></td>
</tr>
<tr>
<td>1.30E-08</td>
<td>2.4E-06</td>
<td></td>
<td>1.24E-07</td>
<td>2.85E-06</td>
<td></td>
</tr>
</tbody>
</table>
1.5. Conclusion

Our results give conclusive evidence that the ten analogues we have synthesized against the efflux pump all effectively lead to inhibition of P-gp in prostate cancer cells. Reducing drug export and restoring cell sensitivity to the chemotherapeutic drugs. Four P-gp inhibitors, namely 216, 227, 231, and 278, that reversed multidrug resistance in *in vitro* were produced in this study. From the four inhibitors, three variants were capable of reversing MDR without being substrates for P-gp. These inhibitors were variants generated from the original P-gp inhibitor, 29, and all exhibited greater efficacy in reversing MDR compared to the parental compound. Further research is recommended to evaluate the inhibitors’ effectiveness in *in vivo* as this approach shows potential of saving overall cost and time in identifying drugs both in *in vitro* and *in vivo* conditions.

ChemGen was the main platform used in the synthesis involved in this study. A docking routine was implemented and using MTT assays, it was observed that three Group 1 derivatives of 29, namely 216, 227, and 231, enabled the P-gp overexpressing prostate cancer cell line,
DU145TXR, to re-sensitize to paclitaxel, a chemotherapeutic. Given this, the active range of the concentration of the variants of 29 was analyzed.

Our research could be key to solving issues of cost and side effects since less drug will be needed to achieve maximum effect. This project shows promising results and that our synthetic work in the lab has validated the computational model. In addition, the project will be continued by selecting the top compounds predicted to have high binding affinity, synthesizing it as shown below in Scheme 1.9, and test it \textit{in vitro}.

\begin{center}
\includegraphics[width=\textwidth]{image.png}
\end{center}

\textit{Scheme 1.9.} Retrosynthesis future anticancer drug that are predicted by computer.
1.6 Experimental section

1.6.1 Synthetic procedures

General materials and methods.

The reactions were performed under nitrogen and dried glassware. Reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), Oakwood Chemical (West Columbia, SC), and Cayman Chemical (Ann Arbor, MI). Silica gel P60 (SiliCycle) was used for column chromatography and Analytical Chromatography TLC Silica gel 60 F₂₅₄ (Merck Millipore, Darmstadt, Germany) was used for analytical thin layer chromatography. \(^1\)H NMR and \(^{13}\)C NMR spectra were used for analyzed the compounds by using CDCl₃ (Cambridge Isotope Laboratories, Cambridge, MA) on a JEOL 500 MHz and

BRUKER 400 MHz spectrometer in the Department of Chemistry at Southern Methodist University. Chemical abbreviations are used as follows: CH₂Cl₂, dichloromethane; EtOAc, ethyl acetate; THF, tetrahydrofuran; DMF, dimethylformamide; H₂O, water; HBTU, ObenzotriazoleN,N',N',N'-tetramethyl-uronium-hexafluoro-phosphate; DIPEA, N,N-diisopropylethylamine; KOH, potassium hydroxide; DMSO, dimethylsulfoxide N₂, nitrogen. High resolution mass spectroscopy was performed on a Shimadzu IT-TOF (ESI source) and low resolution mass spectroscopy was performed on a Shimadzu LCMS-8050 Triple Quadrupole LCMS (ESI source) or a Shimadzu Matrix Assisted Laser Desorption/Ionization MS (MALDI) at the Shimadzu Center for Advanced Analytical Chemistry at the University of Texas, Arlington.
**Diazodacetonitrile (2)**\(^{11}\) Aminoacetonitrile hydrochloride (0.575 g, 6.21 mmol, 1.0 equiv) was dissolved in water (10 mL) and CH\(_2\)Cl\(_2\), and then placed in an ice bath. The mixture was stirred and then sodium nitrite (0.43 g, 6.21 mmol, 1.0 equiv) was added over 5 min. After 15 min, the reaction mixture was extracted with CH\(_2\)Cl\(_2\), washed with brine, and dried with Na\(_2\)SO\(_4\). The solution was filtered and used directly in the next step using an estimated yield of 30%.

![3](image)

**3-oxo-3-(2,4,5-trimethylphenyl)propanenitrile (3)**\(^{11}\) 2,4,5-trimethylbenzaldehyde 1 (183 mg, 1.24 mmol, 1.5 equiv) was dissolved in a minimum amount of CH\(_2\)Cl\(_2\) and added to a solution of diazodacetonitrile 2 (1.9 mmol, 1.0 equiv) in dichloromethane. BF\(_3\)-OEt\(_2\) (0.4 mmol, 0.004 mL, 0.2 equiv) was added dropwise to the reaction mixture until gas stopped evolving and the color was changed from light green to dark red. After 20 min, the reaction mixture was concentrated and purification done by silica column chromatography using (1:5 Ethyl Acetate: Hexane) to give a tan solid (162 mg, 0.86 mmol, 70% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.36 (s, 1H), 7.06 (s, 1H), 4.02 (s, 2H), 2.50 (s, 3H), 2.28 (s, 6H); \(^13\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 19.4, 20.0, 21.6, 31.3, 114.4, 130.9, 131.3, 134.3, 134.5, 138.4, 143.2, 188.7.

![4](image)

**1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5-amine (4)** Compound 4 was prepared by adaptation of a literature procedure.\(^{12}\) Benzoylacetonitrile 3 (250 mg, 1.33 mmol, 1.0 equiv) and
phenylhydrazine (0.31 mL, 1.3 mmol, 1.0 equiv) were added to pressure tube and heated to 165 °C for six hours. The mixture was purified by silica column chromatography using CH$_2$Cl$_2$ to give a yellow solid (258 mg, 0.931 mmol, 70% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.66 (m, 2H), 7.48 (m, 2H), 7.42 (m, 1H), 7.32 (m, 1H), 7.00 (s, 1H), 5.81 (s, 1H), 3.82 (s, 2H), 2.46 (s, 3H), 2.24 (s, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 18.7, 19.5, 20.4, 91.4, 123.6, 126.9, 129.5, 130.2, 130.5, 132.2, 133.2, 133.5, 135.9, 139.1, 144.6, 152.4; HRMS calcd for C$_{18}$H$_{19}$N$_3$ (M+H)$^+$ 278.1652, found 278.1650.

2-chloro-N-[1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5-yl]acetamide (5) Pyrazole 4 (530 mg, 1.93 mmol, 1.0 equiv) was dissolved in CH$_2$Cl$_2$ and placed in an ice bath. Chloroacetyl chloride (260 mg, 2.30 mmol, and 0.22 mL) was added dropwise. The reaction mixture was stirred overnight at room temperature. The reaction washed with H$_2$O and brine, dried over Na$_2$SO$_4$, filtered and concentrated. The product was obtained as a brown crystal (660 mg, 1.87 mmol, 97% yield) and used without any further purification, $^1$H NMR (500 MHz, CDCl$_3$) δ 8.77 (s, 1H), 7.53 (m, 4 H), 7.43 (s, 2H), 7.03 (s, 1H) 6.90 (s, 1H), 4.15 (s, 2H), 2.48 (s, 3H), 2.25 (s, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 19.3, 19.5, 20.7, 42.7, 98.7, 124.6, 128.7, 129.6, 129.8, 130.2, 132.2, 133.4, 133.2, 134.0, 134.5, 136.6, 137.4, 152.8, 162.6, 169.2; HRMS calcd for C$_{20}$H$_{20}$N$_3$OCl(M+H)$^+$ 354.1368, found 354.1371.
2-(acetylsulfanyl)-N-[1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5-yl]acetamide (6)

Chloride 5 (224 mg, 0.640 mmol, 1.0 equiv) was dissolved in 3 mL of anhydrous THF and placed in an ice bath. KSAc (143 mg, 1.28 mmol) was then added. The reaction was stirred overnight at room temperature. An orange solid was obtained (243 mg, 0.62 mmol, 97% yield). H NMR (500 MHz, CDCl$_3$) $\delta$ 8.38 (s, 1H), 7.51 (m, 4H), 7.42 (m, 2H), 7.08 (s, 1H), 6.85 (s, 1H), 3.59 (s, 2H), 2.48 (s, 3H), 2.32 (s, 6H), 2.25 (s, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 19.3, 19.4, 20.6, 30.2, 33.2, 98.9, 129.8, 133.4, 133.9, 135.4, 136.4, 137.9, 152.4, 165.2, 196.3; HRMS calcd for C$_{22}$H$_{23}$N$_3$O$_2$S (M+H)$^+$ 394.1584, found 394.1576.

2-mercapto-N-(1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5-yl)acetamide (7) In a dry Schlenk flask, the thioester 6 (80 mg, 0.2 mmol, 1.0 equiv) was dissolved in 3 mL anhydrous CH$_3$OH and then 0.8 mL of 2 M NaOH (16 mg, 1.6 mmol, 8.0 equiv) was added. The reaction was degassed using a freeze-pump-thaw procedure. The solvent and reactants in the Schlenk flask were frozen by submerging in liquid nitrogen. Then, the flask was opened to the vacuum for one minute. After that, the flask was sealed and allowed to warm up until the solvent has completely become liquid again. This procedure was repeated two times. After the last cycle was complete, the flask
was brought to room temperature and filled with N₂. After stirring one hour, the reaction mixture was concentrated and diluted with ethyl acetate, and then acidified to pH = 1 by using HCl. The extraction was done by using ethyl acetate /water and dried over Na₂SO₄. The purification was done by silica column chromatography using 1:2 (Ethyl Acetate:Hexane) to obtain white crystals (49 mg, 70% yield). ¹H NMR (500 MHz, CDCl₃) δ 8.94 (s, 1H), 7.52 (m, 4H), 7.40 (m, 2H), 7.00 (s, 1H), 6.90 (s, 1H), 3.36 (s, 2H), 2.47 (s, 6H), 2.24 (s, 3H), 1.8 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 19.2, 19.7, 21.1, 28.6, 98.1, 124.7, 128.6, 130.0, 130.3, 132.3, 133.3, 133.9, 135.2, 136.4, 138.0, 148.5, 152.7, 165.5; HRMS calcd for C₂₀H₂₁N₃OS (M+H)⁺ 352.1478, found 352.1481.

General procedure for the synthesis of 2-chloro-acetamide derivatives

Each substituted amine (1.0 equiv) was dissolved in 3 mL of anhydrous THF, followed by addition of one equivalent of Et₃N. After placing the reaction mixture in an ice bath, 2-chloroacetyl chloride (1.2 equiv) was added dropwise for one hour and the reaction was stirred overnight at room temperature. After being concentrated, CH₂Cl₂ and water were added and the organic compounds were extracted three times with CH₂Cl₂. The organic layers were washed with brine, dried over Na₂SO₄, filtered, and concentrated.
2-chloro-N-(naphthalen-2-yl) acetamide (8)\(^\text{13}\) Light orange solid (112 mg, 72% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 8.44\) (s, 1H), 8.25 (s, 1H), 7.80 (m, 3H), 7.50 (m, 3H), 4.24 (s, 2H).

![Structure 9](image)

2-chloro-1-(10,11-dihydro-5\textit{H}-dibenzo[\textit{bf}]azepin-5-yl)ethan-1-one (9).\(^\text{14}\) White solid (199 mg, 72% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 7.45–7.25\) (m, 4H), 7.08 (m, 2H), 6.79 (m, 2H), 4.13 (t, 2H, \(J = 12.6\) Hz), 4.02 (t, 2H, \(J = 12.6\) Hz), 3.50 (m, 2H), 3.35 (m, 2H), 3.10 (s, 2H).

![Structure 10](image)

2-chloro-N-(3,4,5-trimethoxyphenyl)acetamide (10).\(^\text{15}\) White solid (212 mg, 75% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 8.24\) (s, 1H), 6.81 (s, 2H), 4.14 (s, 2H), 3.79 (s, 9H).

![Structure 11](image)
N-(benzo[d]thiazol-6-yl)-2-chloroacetamide (11). White solid (56 mg, 75% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.94 (s, 1H), 8.54 (s, 1H), 8.45 (br s, 1H), 8.11 (d, 1H, $J = 9.1$ Hz), 7.42 (d, 1H, $J = 9.1$ Hz), 4.23 (s, 1H).

![Image](N-(benzo[d]thiazol-6-yl)-2-chloroacetamide.png)

N-((3s,5s,7s)-adamantan-1-yl)-2-chloroacetamide (12). White solid (0.97 g, 75% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 6.22 (s, 1H), 3.90 (s, 2H), 1.99 (m, 9H), 1.66 (m, 6H).

![Image](N-((3s,5s,7s)-adamantan-1-yl)-2-chloroacetamide.png)

N-benzhydryl-2-chloroacetamide (13). The mixture was purified by silica column chromatography using 1:4 Ethyl Acetate:Hexane to give a white solid (0.81 g, 56% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.34 (m, 6H), 7.30 (d, 1H, $J = 12.6$ Hz), 7.24 (m, 4H), 4.12 (s, 2H), 4.06 (s, 2H).

![Image](N-benzhydryl-2-chloroacetamide.png)

2-chloro-N-(4-fluorobenzyl)acetamide (14). White solid (241 mg, 75% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 7.24 (m, 2H), 6.98 (m, 2H), 6.88 (s, 1H), 4.40 (s, 2H), 4.06 (s, 2H).
2-chloro-1-(9H-fluoren-2-yl)ethan-1-one (15).\textsuperscript{20} 9-fluorene (100 mg, 0.6 mmol, 1.0 equiv) was dissolved in 100 mL of methylene chloride. After the reaction mixture was cooled to 0 °C, anhydrous aluminum chloride (120 mg, 0.9 mmol, 1.5 equiv) was added. The reaction was stirred for 15 min. Chloroacetyl chloride (102 mg, 0.9 mmol, 1.5 equiv) was added in dropwise. After 15 min at 0 °C and 45 min of stirring at room temperature, the reaction mixture was poured into a mixture of 500 mL of ice and 100 mL of hydrochloric acid. The organic phase was extracted and washed with brine. The product was dried over sodium sulfate collected after concentrated. The product was obtained as white solid (140 mg, 96% yield).\textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) δ 8.13 (m, 1H), 7.96 (m, 1H), 7.82 (m, 1H), 7.56 (m, 1H), 7.40 (m, 3H), 4.73 (s, 2H), 3.93 (s, 2H).

General synthesis for S\textsubscript{2}2 coupling of alkyl thiols

The reaction was performed by dissolving the thiol (1.0 equiv) in 3 mL of DMF (deoxygenated by bubbling N\textsubscript{2}) and adding K\textsubscript{2}CO\textsubscript{3} (2.0 equiv). The chloroacetamide (1.2 equiv) was then added to the reaction mixture and stirred overnight at room temperature. The reaction was diluted in EtOAc and washed with water. The water layer was extracted three times with EtOAc, washed with brine, dried over Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated to give the crude product, which was purified as indicated.
2-[(2-(9H-fluoren-2-yl)-2-oxoethyl)]-N-[1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5-yl]acetamide (216) The mixture was purified by silica column chromatography using (1:3 Ethyl Acetate: Hexane) to give a white solid (0.017 g, 50% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.94 (s, 1H), 8.55 (s, 1H), 8.09 (s, 1H), 7.73 (m, 3H), 7.52–7.44 (m, 8H), 7.00 (s, 1H), 6.82 (s, 1H), 3.4 (s, 2H), 3.30 (s, 2H), 2.42 (s, 3H), 2.24 (s, 6H); \(^13\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 193.4, 165.2, 152.3, 147.4, 144.5, 143.4, 140.0, 137.8, 136.1, 135.3, 133.6, 133.1, 132.7, 132.1, 130.17, 129.9, 128.3, 128.0, 127.9, 127.1, 125.2, 125.1, 121.0, 119.8, 98.7, 60.3, 53.3, 37.9, 36.7, 36.2, 20.6, 19.3; HRMS calculated for C\(_{35}\)H\(_{31}\)N\(_3\)O\(_2\)S (M+H)\(^{+}\) 558.2210, found 558.2201.

2-[(2-[2-azatricyclo[9.4.0.0]pentadeca-1(11),3(8),4,6,12,14-hexaen-2-yl]-2-oxoethyl)sulfanyl] -N-[1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5-yl]acetamide (227) The mixture was purified by silica column chromatography using (1:2 Ethyl Acetate:Hexane) to give a white solid (0.013 g, 60% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.57 (s, 1H), 7.52 (m, 2H), 7.42 (m, 5H), 7.23–7.25 (m, 5H), 7.01 (s, 2H), 6.85 (s, 2H), 3.40 (s, 2H), 3.30 (s, 2H), 2.88 (m, 4H), 2.45 (s, 3H), 2.22 (s, 6H); \(^13\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 168.8, 166.5, 152.3, 141.0, 139.2, 138.2, 137.7, 136.2, 135.9, 134.6, 133.3, 132.2, 130.9, 129.4, 128.1, 127.7, 127.2, 126.6, 125.5, 124.6, 99.7, 35.62, 32.1, 29.7, 28.4, 20.7, 20.5, 18.9; HRMS calculated for C\(_{36}\)H\(_{34}\)N\(_4\)O\(_2\)S (M+H)\(^{+}\) 587.2475, found 587.2477.
2-(((naphthalen-2-yl)carbamoyl)methyl)sulfanyl)-N-[1-phenyl-3-(2,4,5 trimethylphenyl)-1H-pyrazol-5-yl]acetamide (231) The mixture was purified by silica column chromatography using (1:3 Ethyl Acetate: Hexane) to give a white solid (0.019 g, 50% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 9.12 (s, 1H), 8.03 (s, 1H), 7.88 (m, 1H), 7.84 (m, 1H), 7.81 (m, 1H), 7.59 (m, 1H), 7.50 (m, 2H), 7.42 (m, 6H), 7.35 (m, 1H), 7.03 (s, 1H), 6.91 (s, 1H), 3.93 (s, 2H), 3.41 (s, 2H), 2.49 (s, 3H), 2.25 (s, 6H); \(^1^3\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 166.7, 166.2, 152.4, 137.9, 136.3, 135.1, 134.6, 133.6, 133.1, 132.2, 130.8, 130.2, 129.8, 129.7, 128.9, 128.7, 128.3, 127.7, 127.6, 127.1, 126.6, 125.2, 125.1, 121.0, 119.8, 117.0, 99.6, 36.6, 36.0, 20.6, 19.3, 19.0; HRMS calculated for C\(_{32}\)H\(_{30}\)N\(_4\)O\(_2\)S (M+H)\(^+\) 535.2162, found 535.2157.

N-[1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5-yl]-2-(((3,4,5 trimethoxyphenyl)carbamoyl)methyl)sulfanyl)acetamide (238) The mixture was purified by silica column chromatography using (2:1 Ethyl Acetate: Hexane) to give a white solid (0.016 g, 62% yield). \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.67 (s, 1H), 8.35 (s, 1H), 7.53 (m, 2H), 7.50 (m, 2H), 7.40 (m, 2H), 7.04 (s, 1H), 6.85 (s, 1H), 6.80 (s, 2H), 3.78 (s, 9H), 3.41 (s, 2H), 3.27 (s, 2H), 2.46 (s, 3H), 2.25 (s, 6H); \(^1^3\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 166.6, 153.2, 152.6, 138.0, 134.9, 133.7, 133.3, 128.3, 127.8, 127.7, 126.6, 125.2, 125.1, 121.0, 119.8, 117.0, 99.6, 36.6, 36.0, 20.6, 19.3, 19.0.
124.9, 100.0, 97.6, 61.0, 56.1, 36.6, 36.0, 20.6, 19.2; HRMS calculated for C\textsubscript{31}H\textsubscript{34}N\textsubscript{4}O\textsubscript{5}S (M+H)
+ 575.2323, found 575.2329.

\[
\text{2-}((1,3\text{-benzothiazol-6-yl})\text{carbamoyl}\text{methyl})\text{sulfanyl})\text{-}N\text{-}[1\text{-phenyl-3-(2,4,5 trimethylphenyl)1H-pyrazol-5-yl}]\text{acetamide (255)}\]

The mixture was purified by silica column chromatography using (2:1 Ethyl Acetate:Hexane) to give a white solid (0.005 g, 49% yield). ¹H NMR (500 MHz, CDCl\textsubscript{3}) δ 8.91 (s, 1H), 8.57 (m, 2H), 8.45 (s, 1H), 8.04 (s, 1H), 7.55 (m, 5H), 7.39 (m, 2H), 7.01 (s, 1H), 6.88 (s, 1H), 3.47 (s, 2H), 3.36 (s, 2H), 2.47 (s, 3H), 2.25 (s, 6H); ¹³C NMR (125 MHz, CDCl\textsubscript{3}) δ 167.2, 166.1, 153.9, 152.5, 150.2, 138.0, 136.4, 135.2, 133.9, 133.2, 132.5, 130.5, 129.5, 128.3, 125.2, 123.7, 119.2, 112.8, 99.6, 36.7, 36.1, 20.7, 19.2; HRMS calculated for C\textsubscript{29}H\textsubscript{27}N\textsubscript{5}O\textsubscript{2}S\textsubscript{2} (M+H)
+ 542.1679, found 542.1661.

\[
\text{2-}(((\text{adamantan-1-yl})\text{carbamoyl}\text{methyl})\text{sulfanyl})\text{-}N\text{-}[1\text{-phenyl-3-(2,4,5 trimethylphenyl)1H-pyrazol-5-yl}]\text{acetamide (278)}\]

The mixture was purified by silica
column chromatography using (1:2 Ethyl Acetate: Hexane) to give a white solid (0.017 g, 57% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 9.44 (s, 1H), 7.53 (m, 2H), 7.42 (m, 2H), 7.35 (m, 2H), 7.00 (s, 1H), 6.79 (s, 1H), 3.29 (s, 2H), 2.93 (s, 2H), 2.40 (s, 3H), 2.20 (s, 6H), 1.98 (s, 3H), 1.83 (m, 6H), 1.57 (m, 6H); $^{13}$C NMR (125MHz, CDCl$_3$) δ 167.6, 166.2, 152.4, 138.3, 136.3, 135.7, 133.9, 133.3, 132.2, 130.2, 128.0, 99.2, 41.4, 36.4, 29.5, 20.5, 19.2; HRMS calculated for C$_{32}$H$_{38}$N$_4$O$_2$S (M+H)$^+$ 543.2788, found 543.2789.

![Chemical Structure](image)

N-(diphenylmethyl)-2-[[[1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5-yl]carbamoyl]methyl)sulfanyl]acetamide (280) The mixture was purified by silica column chromatography using (1:4 Ethyl Acetate: Hexane) to give a white solid (0.017 g, 52% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 9.11 (s, 1H), 7.48 (m, 2H), 7.40 (m, 3H), 7.29 (m, 8H), 7.18 (m, 3H), 7.02 (m, 1H), 6.80 (s, 1H), 6.08 (d, 1H, $J = 8.5$ Hz), 3.26 (s, 2H), 3.13 (s, 2H), 2.48 (s, 3H), 2.25 (s, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 168.0, 166.2, 152.3, 140.7, 138.0, 136.4, 135.6, 129.5, 128.7, 127.2, 125.3, 124.8, 99.6, 57.5, 35.7, 35.1, 20.7, 19.1; HRMS calculated for C$_{35}$H$_{34}$N$_4$O$_2$S (M+H)$^+$ 575.2475, found 575.2472.
N-[(4-fluorophenyl)methyl]-2-[[[1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol5yl]carbamoyl]methyl]sulfanyl]acetamide (286) The mixture was purified by silica column chromatography using (1:3 Ethyl Acetate: Hexane) to give a white solid (0.014 g, 54% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 9.05 (s, 1H), 7.55 (m, 2H), 7.47 (m, 2H), 7.38 (m, 2H), 7.19 (m, 2H), 6.99 (m, 3H), 6.83 (m, 1H), 6.47 (s, 1H), 4.30 (m, 1H), 3.38 (s, 2H), 3.13 (s, 2H), 2.49 (s, 3H), 2.23 (s, 6H); $^{13}$C NMR (125MHz, CDCl$_3$) δ 168.4, 166.1, 152.5, 138.0, 136.5, 135.4, 132.4, 130.4, 129.7, 128.4, 125.1, 115.8, 99.5, 43.2, 36.2, 35.6, 20.7, 19.5, 19.2; HRMS calculated for C$_{29}$H$_{29}$N$_{4}$O$_{2}$FS (M+H)$^+$ 517.2068, found 517.2068.

**Synthetic procedures for aromatic sulfide derivatives**

5H-[1,2,4]triazino[5,6-b]indole-3-thiol (15)$^{27}$ In a round-bottom flask, isatin (200 mg, 1.36 mmol, 1.0 equiv) and potassium carbonate were dissolved 5 mL of water. (124 mg, 1.36 mmol, 1.0 equiv) of thiosemicarbazide was added to a solution. The reaction was reflux for 16 h. The reaction mixture was acidified by using 0.5 mL acetic acid. The yellow precipitate was afforded and washed with water and acetic acid (12:1). The yellow solid was tritutrated with hot DMF. The
product was filtered and dried under high vacuum to give a yellow solid (119 mg, 0.59 mmol, 43% yield). $^1$H NMR (500 MHz, DMSO-D$_6$) δ 7.99 (d, 1H, $J = 8.0$ Hz), 7.94 (s, 1H), 7.61 (t, 1H, $J = 8.0$ Hz), 7.42 (d, 1H, $J = 8.9$ Hz), 7.33 (t, 1H, $J = 8.0$ Hz); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 113.4, 118.4, 122.3, 123.5, 132.3, 135.9, 143.6, 149.6, 179.6; HRMS calculated for C$_9$H$_6$N$_4$S (M+H)$^+$ 203.0389, found 203.0386.

2-((5H-[1,2,4]triazino[5,6-b]indol-3-yl)thio)-N-(1-phenyl-3-(2,4,5-trimethylphenyl)1Hpyrazol-5-yl)acetamide (541) The reaction was performed by dissolving the thiol 15 (10 mg, 0.049 mmol, 1.0 equiv) in 3 mL of methanol and adding triethyl amine (1.5 equiv). The chloroacetamide (1.0 equiv) was added to the reaction mixture and stirred overnight at room temperature. The reaction mixture was filtered and washed with methanol. The product was collected after high vacuum as a yellow powder (15 mg, 0.028 mmol, 58% yield). $^1$H NMR (500 MHz, CDCl$_3$) δ 10.37 (s, 1H), 8.29 (d, 1H, $J = 10.8$), 7.66 (m, 1H), 7.56 (m, 3H), 7.41 (m, 1H), 7.32 (m, 4H), 7.19 (m, 1H), 6.96 (s, 1H), 6.64 (s, 1H), 4.19 (s, 2H), 2.39 (s, 3H), 2.15 (s, 6H); $^{13}$C NMR (500 MHz, DMSO-D$_6$) δ 18.6, 20.3, 39.5, 102.0, 112.5, 116.7, 121.1, 122.7, 123.9, 126.8, 128.7, 129.5, 130.8, 140.3, 146.4, 150.9, 166.0, 166.9; HRMS calculated for C$_{29}$H$_{25}$N$_7$OS (M+H)$^+$ 520.1919, found 520.1914.
5-bromonicotinoyl chloride (16) 5-Bromonicotinic acid (100 mg, 0.5 mmol, 1.0 equiv) was dissolved in 3 mL of 1,2-dichloroethane. Thionyl chloride (0.11 mL, 1.5 mmol, 3.0 equiv) was added to the reaction mixture followed by one drop of DMF. The reaction was heated and reflux for overnight. The reaction was stopped and cooled to room temperature. The excess thionyl chloride was removed under reduced pressure to give a carbonyl chloride that was used for the next step without further purification.

5-bromo-N-(3-mercaptophenyl)nicotinamide (17) 3-Aminothiophenol (46 mg, 0.37 mmol, 1.0 equiv) was dissolved in 5 mL of dichloromethane. The carbonyl chloride 16 (82 mg, 0.37 mmol, 1.0 equiv) and pyridine (0.044 mL, 0.55 mmol, 1.5 equiv) were added to the solution at –10 ºC and the reaction was stirred overnight at room temperature. The reaction mixture was washed with 10 mL of 1M HCl and the solvent was removed under reduced pressure. The solid was dissolved in 2:1 methanol and water. Potassium carbonate (51 mg, 0.37 mmol, 1.0 equiv) was added and the reaction mixture was stirred for one hour at room temperature. The crude mixture was acidified to pH = 1 by using 1M HCl. The methanol was removed under reduced pressure and the aqueous layer was extracted by dichloromethane. The organic layer was washed with brine, dried over Na₂SO₄, filtered, and concentrated. The mixture was purified by silica column chromatography using (2:1 Ethyl Acetate: Hexane) to give a white solid (40 mg, 0.129 mmol, 35%
yield. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.96 (s, 1H), 8.83 (s, 1H), 8.31 (s, 1H), 7.99 (s, 1H), 7.65 (s, 1H), 7.31 (d, 1H, $J = 8.6$), 7.26 (m, 1H), 7.07 (d, 1H, $J = 8.6$), 3.53 (s, 1H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 117.2, 120.3, 124.8, 129.6, 132.4, 137.8. 139.3, 147.5, 153.2, 162.5.

5-bromo-N-(3-((2-oxo-2-((1-phenyl-3-(2,4,5-trimethylphenyl)-1H-pyrazol-5yl)amino)ethyl(thio)phenyl)nicotinamide (551). The thiol 17 (22 mg, 0.071 mmol, 1.0 equiv) was dissolved in 3 mL of DMF (deoxygenated by bubbling N$_2$) and K$_2$CO$_3$ (22 mg, 0.163 mmol, 2.3 equiv) was added. The chloroacetamide (25 mg, 0.071 mmol, 1.0 equiv) was added to the reaction mixture and stirred overnight at 110 °C. The reaction was diluted in EtOAc and washed with water. The organic layer was washed with brine, dried over Na$_2$SO$_4$, filtered and concentrated. The purification was done by using (2:1 Ethyl acetate: Hexane) to give (23 mg, 0.037 mmol, 51% yield). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.98 (s, 1H), 8.90 (s, 1H), 8.74 (s, 1H), 8.26 (s, 2H), 7.46 (m, 2H), 7.32–7.23 (m, 5H), 6.93 (m, 2H), 6.88 (s, 1H), 6.80 (s, 1H), 3.73 (s, 2H), 2.37 (s, 3H), 2.13 (s, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 19.2, 19.5, 20.7, 37.2, 98.4, 118.7, 118.9, 124.6, 128.4, 130.0, 130.3, 133.2, 137.8, 138.1, 146.0, 152.6, 153.7, 162.6, 164.7; LRMS calculated for C$_{32}$H$_{29}$BrN$_5$O$_2$S (M+2H)$^{2+}$ 313.6, found 313.9.
1.7. Chapter 1 References


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CHAPTER 2
SYNTHESIS AND CHARACTERIZATION OF GREEN LIGHT EMITTING SPIROLACTAM RHODAMINE BASED PHOTOSWITCH FOR USE IN A 3D LIGHT PAD

Fluorescence emission from small organic photoswitch molecules can be used to generate images and animations in a volumetric three-dimensional light photoactivatable dye display (3D Light PAD). These high-resolution 3D light structures can be formed by projecting two light beams with UV and visible wavelengths simultaneously through a solution of fluorescent photoswitches. Our group has successfully used photoactivatable red-emitting N-phenyl spirolactam rhodamine B derivatives in a first-generation 3D Light PAD. To move towards our goal of developing a true-color 3D volumetric display, we have synthesized fluorescent photoswitches with green emission. This chapter will highlight the design and synthesis of a green emitting spirolactam rhodamines, as well as the characterization of their photochemical properties.

2.1. Introduction

2.1.1 Volumetric 3D Displays

Volumetric 3D displays may well be used for medical imaging, engineering, education, and alternative areas due to explicit attractive properties. Compared with traditional 3D displays like stereoscopic displays, volumetric 3D displays fully incorporate physical depth cues that account for some physical factors, like the eyes’ activities upon focusing at completely different distances and angles, motion optical phenomenon distinction, and binocular inequality that is
caused by the space of left eye and right eye. Additionally, volumetric 3D displays are often characterized into swept-volume displays, which implies they will be used for high-speed image generation and rotating screen-based image generation, and static volume displays that embody active-matrix displays and beam-addressable displays. However, there are several challenges for final image generation, together with advanced fabrication, moving components, high-speed projectors, and appropriate optical device powers, which will limit the wide use of this technology.

Our group has used innovative chemistry to enable a volumetric 3D digital light photoactivatable dye display (3D Light PAD) and has shown that it is a good methodology for 3D image generation. There are two necessary factors for this system, one could be a special category of photoactivatable molecules, and the other is digital light processing (DLP) technology to get structured light in three dimensions.

Photoactivatable molecules with a specific set of optical properties: a non-fluorescence state, a visible fluorescent state and simple photochemical change from off-state to on-state. Our group used spirolactam rhodamine photoswitches as the chemical agent in the 3D Light PAD. Rhodamine dyes and fluorogenic compounds are often used as laser dyes, tracer agents, and biological probes.

N-aryl spirolactam rhodamine derivatives with fluorinated aminoalkyl groups (SRCF3) that have green (518 nm) emission wavelengths were prepared so that we can better understand the design principles of spirolactam rhodamine photoswitches and ultimately generate photoswitches of different colors that would be suitable for use in a multi-color 3D Light PAD. Our group reported the synthesis of these derivatives. This includes a new procedure of preparing fluorinated spirolactam rhodamine derivatives and a method to measure the kinetics and quantum efficiency.
yields of the photoswitching process. The information gathered was used to provide a preliminary demonstration of 3D voxel formation using a green-emitting spirolactam rhodamine photoswitch in a 3D Light PAD display for a multi-color volumetric 3D display. Also, the fluorinated spirolactam rhodamines containing trifluoromethyl amino groups as a photoswitch with green fluorescence emission at 518 nm were identified.

The research team under Professor Lippert’s direction collected exciting preliminary results that display the use of innovative chemistry for 3D image generation. The 3D light structures can be formed by projecting the dual-wavelength beams into a solution of a fluorescent photoswitch. They synthesized and measured the photophysical properties of a range of N-aryl spirolactam rhodamine B (SRB) photoswitches with red (585 nm) emission wavelengths. The 3D light structures can be formed by projecting the dual-wavelength beams into a solution of a fluorescent photoswitch. Following this initial report, we successfully synthesized a significant number of new fluorescent photoswitches and measured their photoswitching properties.

A 3D Light PAD emits light at specific points in 3D space only at the intersection of ultraviolet (UV) and visible patterned light provided by commercially available Digital Light Processing (DLP) projectors using fluorescent photoswitches dissolved within a solvent as it shown in Figure 2.1A. The development of a scalable and true-color 3D monitor requires a greater understanding of the fundamental molecular information of photoswitch chemistry. Spirolactam rhodamines are photoswitches with properties suitable for use in a 3D Light PAD, such as (1) balance between photoactivation and thermal relaxation rates, (2) a fluorescent on-state, (3) photoswitching caused by non-visible ultraviolet radiation. Under normal conditions, these photoswitches show a colorless and non-fluorescent "closed" conformation (1a). When exposed
to UV light, the spirolactam ring opens, resulting in a completely conjugated "open" shape (2b) that fluoresces when excited with visible light (Figure 2.1 B).

Figure 2.1. (A) Diagram and function of the fluorescent photoswitch in a 3D Light PAD. (B) 3D images generated by a 3D Light PAD.

The photochromic properties of N-phenyl spirolactam rhodamines, and subsequent experiments on the process showed significant effects from solvent and pressure. This was first identified by Knauer and Gleiter. When it was discovered that the photoswitching properties of spirolactam rhodamines made them suitable for superresolution microscopy, derivatives suitable for this technique were prepared and tested. This research yielded a variety of N-substituted spirolactam rhodamines with emission ranging from green to far-red and water solubility.

2.2 Results and Discussion:

Our group (Bo Li, Uroob Haris, Andrew Nakatsuka, and I) worked on three aims:

1. Develop rationally designed red fluorescent photoswitch derivatives with better water solubility and photoswitching kinetics.

2. Synthesize a green fluorescent photoswitch and characterize it.

3. Measure the kinetics of the photoswitching process.
Green emitting photoswitches with trifluoromethyl substituents on the rhodamine nitrogens are established, but their turn-on rates are slow and turn-off rates are fast, respectively.\textsuperscript{60} Green-emitting derivatives were prepared as shown in Scheme 2.1 below, after formation of fluorescein ditriflates from fluorescein, followed by a palladium cross-coupling between fluorescein triflates and 2,2,2-trifluoroethylamine.\textsuperscript{61} The reaction was done by using tris(dibenzylideneacetone)dipalladium(0) (Pd\textsubscript{2}dba\textsubscript{3}) as a catalyst with biaryl ligand 2-Dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl XPhos in dioxane. POCl\textsubscript{3}-mediated acid chloride formation was accompanied by acyl substitution with aniline or alkyl amine to produce diverse amination substrates as it show in Table 2.1.\textsuperscript{62}

\textbf{Scheme 2.1.} Synthesis of Green Spirolactam Rhodamine (SRCF3).
Figure 2.3 shows the green spirolactam rhodamine with various amines. Starting with aniline 23 as the parent compound, analogues were prepared by adding electron-withdrawing groups to various amines such as (21, 22, 24, 26, 27, 29, 31 and 32). On other hand, we also synthesized the green spirolactam rhodamine with sterically hindered amines groups such as (25 and 30). Finally, the large conjugated system in the coumarinic ring was successfully synthesized as compound 28.

As a preliminary demonstration of 3D voxel formation, we used a 254 nm excitation source and the 2-fluoro SRF3 in dichloromethane. In a 2.5 cm x 2.5 cm x 5 cm cuvette, the photoswitch was dissolved at 5 M in dichloromethane with 7.2 M triethylamine. A mask was made to fit over a 254 nm TLC lamp, allowing a line of UV light to project up from the TLC lamp into the cuvette (Figure 2.3A). A LightCrafter 4500 projector was used to project a square of blue light from the side. A voxel of green light can be seen using this configuration (Figure 2.3 B), demonstrating the feasibility of using green SRF3 photoswitches in a 3D Light PAD. Unfortunately,
photoactivation was only effective at 254 nm, so patterning could not be accomplished using the commercially available WinTech 4500 UV projector (as used in our previous work), which has a 385 nm LED.

Figure 2.4. (A) Schematic and (B) photograph of the 3D voxel formation design, which includes a 254 nm TLC lamp, the blue LED of a LightCrafter 4500 projector, and a cuvette loaded with 5 mM 22 and 7.2 M triethylamine.

2.3 Conclusion

The synthesis of rhodamine derivatives was done successfully with fluorinated aminoalkyl groups (SRCF3) that have green (518 nm) emission wavelengths. This includes a new procedure of preparing fluorinated spirolactam rhodamine derivatives so that we can better understand the design principles of spirolactam rhodamine photoswitches and ultimately generate photoswitches of different colors that would be suitable for use in a 3D Light PAD.
2.4 Experimental Section

3-oxo-3H-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl bis(trifluoromethanesulfonate) (18)

Fluorescein (1020 mg, 3.1 mmol, 1 equiv) was dissolved in 30 mL of dichloromethane. Trifluoromethanesulfonic Anhydride (2215 mg, 6.2 mmol, 2 equiv) and N,N-Diisopropylethylamine (1.6 mL, 9.3 mmol, 3 equiv) added to the reaction mixture and run for overnight at room temperature. The reaction mixture washed with 15 mL of 1M HCl and 15 mL of NaHCO₃. The mixture extracted with CH₂Cl₂, dried with Na₂SO₄, filtered, and concentrated. Purification done by using silica column chromatography (20:1 DCM/CH₃OH) affording the product as a light yellow solid (3.56 g, 71% yield). ¹H NMR (500 MHz, CDCl₃) δ 8.05 (d, J = 7.0 Hz, 1H), 7.73–7.69 (m, 2H), 7.29 (s, 2H), 7.19 (d, J = 6.6 Hz, 1H), 7.01–6.97 (m, 4H); ¹³C NMR (125 MHz, CDCl₃) δ 168.7, 152.1, 151.4, 150.2, 131.0, 130.0, 125.8, 123.7, 119.3, 117.7, 110.7, 80.1.

3',6'-bis((2,2,2-trifluoroethyl)amino)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (19)

Fluorescein ditriflate (1 g, 1.68 mmol, 1 equiv), tris (dibenzylideneacetone) dipalladium (155 mg,
0.17 mmol, 0.1 equiv), 2-Dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (238 mg, 2.5 mmol, 0.3 equiv) and cesium carbonate (1.5 g, 4.6 mmol, 2.8 equiv), were added to a pressure flask. The flask was evacuated and backfilled with N\textsubscript{2} three times. Then 8 mL of anhydrous dioxane was added to the flask and let it run for 20 minute at room temperature. 2,2,2-Trifluoroethylamine (1.3 mL, 4 mmol, 2.4 equiv) was added to the pressure flask and sealed immediately. The mixture was stirred for 20 minute at room temperature. Then the mixture was heated to 100 °C and stirred for 16 h. The reaction was concentrated and the crude was purified by the silica column chromatography (4:1 DCM/isopropanol) to provide dark red crystal (600 mg, 72%). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 7.99 (m, 1H), 7.65 (m, 1H), 7.59 (m, 1H), 7.17 (d, 1H), 6.58 (s, 2H), 6.51 (m, 2H), 6.36 (t, \(J = 8.3\) Hz, 2H), 4.23 (s, 2H), 3.78 (m, 4H); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \(\delta\) 167.7, 154.8, 154.0, 133.3, 131.9, 130.6, 128.7, 123.9, 108.7, 98.1, 69.3, 44.6, 37.9, 30.0, 14.9, 12.6; HRMS calculated for C\textsubscript{24}H\textsubscript{16}N\textsubscript{2}O\textsubscript{3}F\textsubscript{6} \[M+H\]+ 495.1121, found 495.1119.

\textbf{CF\textsubscript{3} aniline (20). Rhodamine-CF\textsubscript{3} (50 mg, 0.1 mmol, 1 equiv) was dissolved with 8 mL of DCM. Aniline (0.028 mL, 0.3 mmol, 3 equiv) was added to the reaction mixture. The reaction was cooled in ice bath before adding phosphoryl chloride (0.012 mL, 0.12 mmol, 1.2 equiv). The reaction was run for 20 min at 0 °C and reflux for overnight. The reaction mixture was washed with 10 mL of 1 M HCl and 10 mL of 1M of NaOH. The extraction was done with DCM, washed with brine, dried with Na\textsubscript{2}SO\textsubscript{4}, filtered, and concentrated. Purification done by using silica column chromatography (1:1 ACOEt/ Hexane) affording the product 20 as a red solid (1.59 g, 64% yield).}
$^1$H NMR (500 MHz, CDCl$_3$)  δ 7.99 (m, 1H), 7.65 (m, 1H), 7.59 (m, 1H), 7.17 (d, 1H), 6.58 (s, 2H), 6.51 (m, 2H), 6.36 (t, J = 8.3 Hz, 2H), 4.23 (s, 2H), 3.78 (m, 4H); $^{13}$C NMR (125 MHz, CDCl$_3$)  δ 167.7, 154.8, 154.0, 133.3, 131.9, 130.6, 128.7, 123.9, 108.7, 98.1, 69.3, 44.6, 37.9, 30.0, 14.9, 12.6; HRMS calculated for C$_{24}$H$_{16}$N$_2$O$_3$F$_6$ [M+H]$^+$ 495.1121, found 495.1119.

![Chemical Structure](image)

2-phenyl-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one  (23)

**Rhodamine-CF$_3$** (305 mg, 0.607 mmol, 1.0 equiv) and aniline (0.17 mL, 1.82 mmol, 3.0 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl$_3$ (0.07 mL, 0.73 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate:hexane (1:4) as the eluent, affording the product 23 as a white solid (141.2 mg, 40% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). $^1$H NMR (500 MHz, CDCl$_3$)  δ 8.01 (d, J = 7.1 Hz, 1H), 7.54–7.50 (m, 2H), 7.14–7.08 (m, 4H), 6.54 (d, J = 8.0 Hz, 4H), 6.30 (d, J = 8.6 Hz, 4H), 3.73 (q, J = 6.6 Hz, 4H). $^{13}$C NMR (125 MHz, CDCl$_3$)  δ 167.6, 152.7, 147.0, 135.9, 132.9, 131.3, 129.1, 127.3, 126.2, 123.7, 110.3, 99.3, 67.1, 45.9, 29.7; HRMS calculated for C$_{30}$H$_{21}$N$_3$O$_2$F$_6$ [M+H]$^+$ 570.1533, found 570.1531.
2-(2-fluorophenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-one

(22) Rhodamine-CF₃ (311 mg, 0.607 mmol, 1.0 equiv) and 2-fluro aniline (0.18 mL, 1.83 mmol, 2.9 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl₃ (0.07 mL, 0.73 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate:hexane (1:2) as the eluent, affording the product as a white solid (221.4 mg, 62% yield). The compound 22 can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). ¹H NMR (500 MHz, CDCl₃) δ 8.01–8.05 (m, 1H), 7.54 (t, J = 4.9 Hz, 2H), 7.12–7.16 (m, 2H), 6.77 (t, J = 10.3 Hz, 1H), 6.61 (d, J = 8.6 Hz, 2H), 6.57 (m, 2H), 6.31 (dd, J = 2.3, 8.6 Hz, 4H), 4.12 (t, J = 8.8 Hz, 2H), 3.73 (p, J = 13.3 Hz, 4H); ¹³C NMR (125 MHz, CDCl₃) δ 166.8, 159.6, 152.9, 152.6, 147.6, 133.1, 130.9, 129.8, 129.5, 129.0, 128.7, 125.9, 124.0, 123.9, 123.7, 116.4, 116.2, 110.2, 109.6, 99.0, 66.9, 53.4, 45.9, 45.6, 29.7; HRMS calculated for C₃₀H₂₀N₃O₂F₇ [M+H]^+ 588.15, found 588.15.
2-(2,6-diisopropylphenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9']xanthen-3-one (30) Rhodamine-CF3 (152 mg, 0.307 mmol, 1.0 equiv) and 2,6-diisopropyl aniline (0.16 g, 0.906 mmol, 2.9 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl₃ (0.04 mL, 0.36 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate:hexane (1:3) as the eluent, affording the product 30 as a white solid (120.1 mg, 62% yield). The compound can be additionally purified by recrystallization from ethyl acetate:hexanes (1:2).

¹H NMR (500 MHz, CDCl₃) δ 8.06 (d, J = 6.9 Hz, 1H), 7.63 (m, 1H), 7.03 (d, J = 7.6 Hz, 2H), 6.97 (m, 2H), 6.82 (m, 2H), 6.59–6.57 (m, 1H), 6.33 (d, J = 6.8 Hz, 2H), 6.26 (d, J = 5.4 Hz, 2H), 3.72 (m, 4H), 2.30 (m, 2H), 1.16 (s, 2H), 0.88 (d, J = 6.9 Hz, 6H), 0.41 (d, J = 6.3 Hz, 6H); ¹³C NMR (125 MHz, CDCl₃) δ 167.6, 157.0, 150.3, 133.8, 130.3, 129.7, 127.4, 123.6, 123.2, 112.0, 110.3, 100.2, 70.0, 45.4, 28.6, 21.4; HRMS calculated for C₃₆H₃₃N₃O₂F₆ [M+H]+ 654.2550, found 654.2548.
2-(3-chlorophenyl)-3',6'-bis((2,2,2-trifluoroethyl)amino)spiro[isoindoline-1,9'-xanthen]-3-one (17). Rhodamine-CF3 (172 mg, 0.347 mmol, 1.0 equiv) and 3-chloro aniline (0.10 mL, 0.86 mmol, 2.5 equiv) were dissolved in dichloromethane (10 mL). The mixture was cooled to 0 °C, then POCl3 (0.04 mL, 0.34 mmol, 1.2 equiv) was added dropwise. The reaction mixture was allowed to stir at 0 °C for 15 min and then heated to 45 °C for 20 h. The mixture was diluted with 10 mL dichloromethane and then washed with 1 M HCl (3 x 10 mL), 1 M NaOH (3 x 10 mL) and brine. The organic layer was collected, dried over Na2SO4, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel chromatography with ethyl acetate: hexane (1:2) as the eluent, affording the product 31 as a white solid (117 mg, 68% yield). The compound can be additionally purified by recrystallization from ethyl acetate: hexanes (1:2). 1H NMR (500 MHz, CDCl3) 8.00 (d, J = 6.8 Hz, 1H), 7.54–7.50 (m, 2H), 7.11–7.02 (m, 3H), 6.81 (s, 1H), 6.60 (q, J = 8.3 Hz, 3H), 6.33 (d, J = 8.2 Hz, 4H), 4.1 (m, 2H), 3.75 (q, J = 6.6 Hz, 4H). 13C NMR (125 MHz, CDCl3) 167.3, 147.6, 137.6, 134.1, 133.2, 130.6, 130.1, 129.7, 128.9, 126.7, 125.8, 124.8, 110.6, 109.7, 99.0, 67.2, 45.9. HRMS calculated for C30H20N3O2F6Cl [M+H]+ 604.1221, found 604.1221.
2.5. Chapter 2 References


APPENDIX I

$^1$H AND $^{13}$C NMR SPECTRA
Figure A-1. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 2.
Figure A-2. $^{13}$C NMR spectrum (100 MHz, CDCl$_3$) of 2.
Figure A-3. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 3.
Figure A-4. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 3.
Figure A-5. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 4.
Figure A-6. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 4.
Figure A-7. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 5.
Figure A-8. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 5.
Figure A-9. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 6.
Figure A-10. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 6.
Figure A-11. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 7.
Figure A-12. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 8.
Figure A-13. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 9.
Figure A-14. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 10.
Figure A-15. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 11.
Figure A-16. $^1$H NMR spectrum (400 MHz, CDCl₃) of 12.
Figure A-17. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 13.
Figure A-18. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 14.
Figure A-19. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 216.
Figure A-20. $^{13}\text{C}$ NMR spectrum (125 MHz, CDCl$_3$) of 216.
Figure A-21. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 227.
Figure A-22. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 227.
Figure A-23. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 231.
Figure A-24. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 231.
Figure A-25. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 238.
Figure A-26. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 238.
Figure A-27. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 255.
Figure A-28. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 255.
Figure A-29. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 278.
Figure A-30. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 278.
Figure A-31. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 280.
Figure A-32. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 280.
Figure A-33. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 286.
Figure A-34. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 286.
Figure A-35. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 15.
Figure A-36. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 15.
Figure A-37. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 541.
Figure A-38. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 541.
Figure A-39. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 17.
Figure A-40. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 17.
Figure A-41. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 551.
Figure A-42. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 551.
Figure A-43. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 19.
Figure A-44. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 19.
Figure A-45. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 23.
Figure A-46. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 23.
Figure A-47. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 22.
Figure A-48. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 23.
Figure A-49. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 30.
Figure A-50. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 30.
Figure A-51. $^1$H NMR spectrum (400 MHz, CDCl$_3$) of 31.
Figure A-52. $^{13}$C NMR spectrum (125 MHz, CDCl$_3$) of 31.