ABSTRACT


The development of small molecule probes to investigate biological functions is integral to the elucidation of complex biological processes. Achieving a better understanding of biological systems facilitates the discovery of new therapeutics and technologies that can be employed in the treatment of a variety of diseases and disorders. The research described herein addresses the need for novel methodologies for the examination of biological questions via two general approaches. The first approach involves the development of new synthetic methodologies to rapidly access libraries of biologically relevant small molecules. These molecules can then be employed in biological screens to identify new targets for drug discovery. Specifically, the research has developed microwave-assisted [2+2+2] cyclotrimerization reactions towards carbo- and heterocyclic libraries. The second approach of the research involves the development of photochemical technologies to regulate biological processes in a highly specific spatio-temporal fashion using light. This has been applied to the photochemical regulation of DNA hybridization, DNAzyme catalysis, antisense gene regulation, RNA folding, and protein function.
Development of New Tools for Functional Genetics

by

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DEDICATION

This work is dedicated to Edith Malthaner, Susan Young, Ardell Rupert and Dean Rupert.

Without their love and support all of my accomplishments would be meaningless.
Douglas Dean Young was born June 23, 1980 in Longmont, Colorado to Susan and Bruce Young. Against the backdrop of the Rocky Mountains, he spent his formative years there, graduating from Longmont High School in 1998. With some trepidation he moved to Tacoma, Washington to attend college at the University of Puget Sound. While there he worked as a teaching assistant and tutor while majoring in both Biology and Chemistry. It was there that he began performing scientific research, spending four years in the laboratory of Dr. Eric Scharrer, synthesizing and analyzing metal-containing liquid crystals. Additionally, he spent a year in laboratory of Dr. Wayne Rickoll, using transmission electron microscopy techniques to study *Drosophila melanogaster* development. Upon graduation in 2003, Douglas moved to Raleigh, North Carolina to attend graduate school at North Carolina State University. After one year in the laboratory of Dr. Daniel Feldheim, investigating the RNA templated synthesis of nanoparticles, he joined the laboratory of Dr. Alexander Deiters, as the first graduate student. Under the supervision of Dr. Deiters he obtained his Ph.D. and received a variety of prestigious awards, including the ACS Division of Medicinal Chemistry Predoctoral Fellowship, and the CEM M.J. Collins Award. Upon graduation Douglas has accepted a Post-Doctoral Research position in the laboratory of Dr. Peter Schultz at the Scripps Research Institute in La Jolla, California.
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CHAPTER 1: INTRODUCTION

With the sequencing of the human genome and the genomes of several model organisms completed, the foundation has been established to achieve a complete molecular understanding of cells and whole organisms. To achieve this goal, biological research has to proceed beyond the simple genetic information in order to investigate the function and interaction of the discovered genes – a research area termed functional genetics. Gene expression is a complex process that requires a high degree of regulation in order to respond to metabolic and environmental changes. The gene products, which are typically proteins, must be spatially, temporally and quantitatively regulated throughout an organism. Determining the cellular function of a protein generally requires a means to alter that function. The most common way of doing so is indirect, involving the use of inactivating (e.g. deletion or "knock-out") or activating (e.g. oncogenic or “knock in”) mutations in the genes. This is known as the genetic approach, and is commonly and widely employed in biological research.

The genetic approach can be divided into two different methods: forward genetics and reverse genetics. Forward genetics involves the induction of random mutations within the genome followed by a screen for phenotypic results. The phenotype of interest is selected and analyzed to determine the specific gene that was mutated. The second approach is a reverse genetics approach, in which a specific gene of interest is mutated followed by an analysis for phenotypic effects (Figure 1.1). However, these classical methodologies to analyze gene function have several disadvantages.
• Knock-out strategies can lead to lethal phenotypes which make gene function assignments impossible.
• Multi-cellular eukaryotic knock-out organisms are difficult to generate.
• Many of these approaches require a substantial time investment, involving multiple generations of the model system.
• None of these methods can be easily controlled in temporal and spatial manner.
• These approaches are often not “tunable” to achieve variable levels of gene expression.

Figure 1.1. Differentiation between the genetic approach (a) and the chemical genetic approach (b). Image from Stockwell, B.R. *Nature Reviews Genetics, 2000*, 1, 116.
In the last decade a new approach has received increasing attention, in which small molecules are employed as modulating ligands of protein function to facilitate biological studies and to provide an understanding of the function of genes – a research area known as chemical genetics. Chemical genetics can also be subdivided into forward and reverse chemical genetics (Figure 1.1).\(^5\) Forward chemical genetics involves exposing cells or organisms to small molecules followed by a screening for phenotypic variance. The organism exhibiting a phenotypic result is then analyzed to correlate the phenotype to a protein or a gene. In reverse chemical genetics the function of a known protein (or a gene) is modulated with a small organic molecule and the phenotypic result is monitored.\(^6\) The chemical genetics approach is different from established genetic methods in several fundamental aspects:

- The effect of small molecules can be easily controlled in a temporal fashion. It can be initiated at any stage during the development of an organism.

- In most cases the biological effect is reversible (due to depletion of the small molecule), which allows a transient study of protein function.

- The effect is tunable, since varying the small molecule concentration different degrees of phenotype expression can be effected.

- The effect can be studied by anyone who has access to the small molecule probe (simple reproducibility).

A major drawback of the chemical genetic approach is the requirement for chemical libraries with potential biological efficacy. It is not possible to predict what type of molecular structure will lead to the desired phenotypic results, requiring the screening of large arrays of
compounds. As a result, facile synthetic routes must be developed to yield the compound libraries rapidly and efficiently.

Both the genetic and the chemical genetic approaches possess various advantages and disadvantages, and ultimately a combination of both of these methodologies might be employed to de-convolute the complex genetic networks inherent to biological systems. As a result, there is an ever-present need for the development for novel technologies to advance both of these fields.

The research presented here attempts to expand upon the both traditional genetics and chemical genetics approaches via the development of methodologies interfacing chemistry and biology. These unique methods will allow for the elucidation of gene function and afford novel tools for the study of genes. Overall, the research can be divided into two major areas: (1) the development new methodologies for the preparation of diverse compound libraries and subsequent screening for their phenotype inducing ability; and (2) the utilization of photoactivatable molecules to control the regulation of specific biomacromolecules using light. Specifically, the research has the following objectives:

1) Development of solid-supported [2+2+2] cyclotrimerization reactions to yield diverse small molecule libraries.

2) Utilization of microwave irradiation in [2+2+2] cyclotrimerization reactions to develop general reaction conditions applicable to both target and diversity oriented synthesis.

3) Translation of the discovered microwave effects to the activation of enzymatic catalysis and DNA hybridization.
4) Preparation of small molecule libraries and their utilization in whole organism screens for biological activity (forward chemical genetics), followed by the assessment of the active molecules for their biological significance and correlation of phenotype to genotype.

5) Development of directed screens for a reverse chemical genetics approach to library screening, specifically towards the small molecule inhibition of miRNA function.

6) Preparation of photocaged small molecules and biological macromolecules which can be activated via irradiation with non-toxic UV light.

7) Application of these molecules in the spatial and temporal control of various biological functions, especially gene function.

8) Application of these light inducible methodologies towards prominent biological questions.

9) Engineering of a truly light-switchable gene regulation system.

These aims will ultimately afford novel means of regulating and investigating gene function. The developed technologies will be widely applicable to both biologists and chemists, and allow for the improved study of biological systems.
An important requirement for a chemical genetics approach is the preparation of arrays of structurally diverse compounds, in order to maximize the success in subsequent biological assays. As a result, the development of new methodologies for the rapidly assembly of these libraries in high yield and purity is essential for the advancement of the field. We selected the \([2+2+2]\) cyclotrimerization reaction for the preparation of these libraries to its ability to be employed as a multi-component reaction. Moreover, this reaction represents a facile route for the preparation of highly substituted benzene and pyridine derivatives in a single operation (Scheme 2.1).  

Due to its atom economy and convergent nature, the cyclotrimerization approach is advantageous in the construction of highly substituted benzene rings in comparison with conventional strategies (e.g. sequential reactions of aromatic rings in electrophilic aromatic substitutions or ortho-metalations).

\[
\begin{align*}
3 & \quad \text{transition metal} \quad \left[ \begin{array}{c}
\text{metalla}
\end{array} \right] \quad \rightarrow \quad \text{aromatic ring}
\end{align*}
\]

Scheme 2.1. General \([2+2+2]\) cyclotrimerization reaction

The \([2+2+2]\) cyclotrimerization reaction involves three components, each contributing two electrons to the reaction (Scheme 2.1). However, this is not thought to be a concerted reaction, and involves a metallacycle intermediate. Due to the complexity of the
reaction the mechanism is not completely understood; however, key mechanistic studies have afforded some insight towards a proposed mechanism. When employing a catalyst possessing the CpCoL₂ architecture (L = olefin, PPh₃, or CO), the first step of the catalytic cycle is the sequential displacement of the two ligands with alkynes to form complexes 2 and 3, followed by an oxidative coupling to yield a cobaltacyclopentadiene. This intermediate is then thought to coordinate to an additional alkyne to yield complex 4. In a poorly understood step, the third alkyne is incorporated, and the benzene ring (5) is released, regenerating the catalyst in the active form 1 (Scheme 2.2).

Scheme 2.2. Proposed cyclotrimerization mechanism. L = Generic ligand (i.e. CO, PPh₃, alkyne, etc.).

Two possible pathways have been proposed for the incorporation of the third alkyne: The first involves the insertion of the coordinated alkyne into the Co-C σ bond, resulting in the cobaltacycloheptatriene complex (6), which can subsequently undergo a reductive elimination to yield the benzene complex 8. Alternatively, a Diels-Alder cycloaddition can occur between the cobaltacyclopentadiene and the third alkyne, leading to complex 7. This
can then also undergo a reductive elimination to form \( \text{8} \) and regenerate the active catalyst (Scheme 2.3).\(^{13}\)

![Scheme 2.3. Two proposed mechanisms for the incorporation of the final alkyne.](image)

The first reported cycloaddition was performed by Berthelot et al. in 1866 via thermally cyclotrimerizing acetylene at 400 °C.\(^{14}\) The first pyridine formation was demonstrated eleven years later in 1877 when Ramsay et al. passed acetylene and hydrogen cyanide through a red hot glass tube.\(^{8}\) However, the next major advance in this chemistry did not occur until 1949 when Reppe et al. reported the utilization of a nickel catalyst to form both benzene and cyclooctatetraene signifying the first application of a transition metal catalyst in these types of reactions.\(^{15}\) Since the discovery of the first transition-metal mediated cyclotrimerization reactions, further accomplishments have increased the broad utility of this reaction in the assembly of polycyclic aromatic frameworks from simple acyclic precursors.\(^{16}\) The development of several catalyst systems based on Ni, Co, Ru, and Rh have led to mild reaction conditions applicable to organic synthesis.\(^{17-24}\) However, various problems are still associated with these reactions, including chemo- and regioselectivity issues.\(^{25}\)
In order to minimize some of the chemoselectivity issues cyclotrimerization reactions are often conducted with α,ω-diynes to afford a partially intramolecular reaction. The crossed solution-phase reaction of diynes and alkynes often results in only moderate yields and contamination with side-products, due to the participation of the diyne in competing cyclotrimerizations. Scheme 2.4 shows a general [2+2+2] cyclotrimerizations between an unsymmetrically substituted diyne 9 and an unsymmetrical alkyne 10. Besides forming the desired product 11 (as two possible regioisomers 11a and 11b), this reaction can potentially lead to formation of the dimer 12 (as four isomers 12a-12d) and, depending on the catalyst system, the trimer 13 (as three isomers 13a-13c). The formation of products resulting from trimolecular cyclotrimerization reactions of 9 with itself are omitted in this scheme, but represent additional side-products formed in the course of this reaction. To resolve these issues we conducted this reaction on a solid-support by immobilizing the diyne 9 on a polystyrene resin.

2.1 Solid-Supported [2+2+2] Cyclotrimerization Reactions

2.1.1. Introduction

The seminal work by Merrifield which introduced solid phase peptide synthesis, coupled with the advent of combinatorial concepts by Geysen and others, have provided the foundation for the continuously advancing field of combinatorial chemistry.27-30 Embraced by the pharmaceutical industry as an efficient approach to drug discovery, combinatorial chemistry has been extensively employed, resulting in a constant impetus for the development of new combinatorial methodologies. Additionally, a paradigm shift has recently occurred, as the chemical diversity of libraries has become emphasized over the quantity of compounds produced.

To generate a library of small organic molecules, solid-supported combinatorial chemistry is often advantageous. It benefits from easy and fast work-up procedures, pseudo high-dilution conditions, and the advantage of using excesses of reagents to drive reactions to full conversion. In order to efficiently provide access to diverse organic structures, multi-component reactions (MCRs) can be employed as key steps in library syntheses.31-33 Compared to a linear synthesis of organic molecules, MCRs have the advantage of yielding compounds in higher purity with a minimum of time and effort expended. Therefore, structural diversity is generated more quickly and effectively. Most known MCRs (e.g. the Ugi reaction) are based on the chemistry of isocyanates. MCRs employing transition metal-catalyzed chemistry have received much less attention in the past; moreover, transition-metal catalyzed chemistry on solid-support, with the exception of Pd catalyzed reactions, still remains an underdeveloped area.34 We aim to address this problem via conducting transition-
metal catalyzed [2+2+2] cyclotrimerization reactions\(^8, 10, 35\) on the solid-phase. Additionally, we hypothesize that the solid-support may also afford a solution to some of the previously discussed chemo- and regioselectivity issues associated with these reactions. Moreover, solid-phase organic synthesis allows for easy automation, parallelization, and purification - important for the rapid generation of compound libraries.\(^36-40\) Due to the commercial availability of a wide range of alkyne precursors as well as several catalyst systems, [2+2+2] cyclotrimerizations represent ideal tools for the facile assembly of diverse arrays of aromatic compounds. Additionally, the highly convergent and efficient nature of cyclotrimerization reactions makes them ideal candidates for solid-phase combinatorial chemistry.\(^41\)

### 2.1.2 Preparation of Phthalan, Isoindoline, and Indan Libraries

Initially, we selected the partially intramolecular formation of benzenes as a model reaction, to develop the solid-supported technology. Thus, the \(\alpha,\omega\)-diynes 14, 15, and 16 (Figure 2.1a), that possess a functional handle for immobilization, were either purchased or were prepared according to literature procedures (the diyne 15 was prepared by Dr. Senaiar).\(^42, 43\)

![Figure 2.1. Diynes (a) and alkynes (b) employed in solid-supported cyclotrimerization reactions.](image-url)
The diynes were immobilized on polystyrene resins (100-200 mesh, 1% cross-linked) with excellent loadings (1 mmol g\(^{-1}\) on average, as determined by GC/MS analysis). Common trityl (for 14 and 15) and carboxy linkers (for 16) were employed and the couplings occurred under standard conditions (Scheme 2.5).\(^{44}\) The trityl linker was chosen for substrates 14 and 15 since its mild cleavage conditions (1% HCl in CH\(_2\)Cl\(_2\)/MeOH for 1h at room temperature) provided the cyclotrimerization products with highest purity. To our surprise, 16 immobilized via a trityl linker only yielded trace amounts of cyclotrimerization products. Switching to the carboxy resin 31, however, delivered cyclotrimerization products in good yields. This is probably due to the steric constraints imposed by the trityl linker which are lowered by using the sterically less demanding carboxy functionalized resin.

**Scheme 2.5.** Immobilization of diynes 14-16.
Solid-supported cyclotrimerization reactions were conducted with a diverse set of alkynes 17-27 (Figure 2.1b), probing the versatility of this methodology. Acetylene (17) was the most reactive substrate and the cyclotrimerization reaction proceeded smoothly at room temperature with excellent yield. Mono-substituted alkynes appeared to be less reactive and required elevated reaction temperatures. However, the mild reaction conditions tolerated a wide range of functionalities including alkyl chains (in 18 and 26), hydroxy and alkoxy groups (in 20 and 27), aromatic rings (in 19 and 21), carbamates (in 22) cyano groups (in 23), silyl groups (in 24), and chlorines (in 25). The di-substituted alkynes 26 and 27 lead to the formation of up to penta-substituted benzenes 67-68, however, the yields were lower probably due to increased steric repulsion in the metallacycle intermediate of the reaction. Catalyst efficiency appears to be reduced in the presence of hydroxyl functionalities (20); however, via benzyl protection of the alcohol (alkyne 21), activity was restored resulting in excellent yields. A similar trend was observed for amine functionalities as propargylamine failed to undergo cyclotrimerization. Simple installation of a Boc protecting group (in 22) facilitated the cyclotrimerization and conveniently yielded the desired amine product as the protecting group was removed under the acidic conditions utilized for cleavage of 39 and 51 from the resin.

In initial experiments immobilized dipropargylamine 29 was treated with the soluble alkyne reaction partner (10 equiv.) in the presence of 10 mol% Wilkinson’s catalyst (RhCl(PPh₃)₃) at 30 to 60 °C for 12 to 48 hours. Various solvents were employed including toluene, CH₂Cl₂, THF and ethanol; however a 3:1 ratio of CH₂Cl₂ to ethanol at 60 °C was found to be optimal. The cyclotrimerized products 33 were cleaved from the resin by treatment with 1% anhydrous hydrochloric acid in CH₂Cl₂ for 1 hour and analyzed by ¹H
NMR and LC/MS. Under these conditions yields ranged from 30-60% and incomplete conversion of the diyne 14 was observed in most cases. Optimized conditions were found by addition of the catalyst in two portions (10 mol% each, the second aliquot was added after 24 hours). Moreover, it was found that yields could be substantially increased by degassing the solvents prior to catalyst addition. After 48 hours the resins were filtered and washed following standard protocols (alternating rinses with CH₂Cl₂ and methanol). Release from the resin indicated complete consumption of diyne 14, and the isoindolines 34-44 were obtained with 70-95% yield (Scheme 2.6). The compounds were isolated as the HCl salts and purities were determined to be >90% (¹H NMR analysis), obviating the need for further purification.

Scheme 2.6. Solid-supported formation of isoindolines.

Cyclotrimerization reactions with the immobilized diyne 30 were performed under identical conditions; however, to achieve compound purities of >90% (¹H NMR analysis),
the cleavage solutions were filtered through a plug of silica gel to remove unidentified polar impurities. In these cases yields were equally high, and the 2,3-dihydro-indenes 46-56 were obtained in 60-84% yield (Scheme 2.7). The high yields achieved in the cyclotrimerization reactions of 26 and 27 demonstrate the ability of the solid-support to greatly aid in the resolution of chemoselectivity issues. Attempts to conduct this reaction in the absence of a solid support with 18 led to <30% product conversion, most likely due to the incompatibility of the unprotected dipropargylamine with the catalyst. Conveniently, in the case of both resin 29 and 30 the symmetrical nature of the diyne alleviates any regioselectivity issues.

![Scheme 2.7](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R'</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>H</td>
<td>H</td>
<td>78%</td>
</tr>
<tr>
<td>47</td>
<td>(CH$_2$)$_3$CH$_3$</td>
<td>H</td>
<td>81%</td>
</tr>
<tr>
<td>48</td>
<td>Ph</td>
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<td>82%</td>
</tr>
<tr>
<td>51</td>
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<td>CH$_2$CH$_3$</td>
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</tr>
<tr>
<td>56</td>
<td>CH$_2$OCH$_3$</td>
<td>CH$_2$OCH$_3$</td>
<td>61%</td>
</tr>
</tbody>
</table>

Scheme 2.7. Solid-supported formation of indans.

We also investigated the solid-supported formation of phthalans by employing the cyclotrimerization precursor 32 (Scheme 2.8). Due to the presence of an internal triple bond in 32 a higher reaction temperature (80°C in dichloroethane) was required to achieve complete conversion of the starting material. The isolated yields (57-82%) were lower than in case of the other two diynes 29 and 30. Cyclotrimerization products 58-68 were obtained by
Dr. Ramesh Senaiar in excellent purities (>90%). A solution phase control cyclotrimerization reaction of diyne 30 with 18 afforded a 43% yield, with ~30% diyne oligomerization, demonstrating the utility of the solid-support. Cyclotrimerization reactions of the unsymmetrical diyne 32 led to the formation of regioisomers, as determined by $^1$H NMR analysis (Figure 2.2). Low or no regioselectivity was obtained using Wilkinson’s catalyst. However, a slight substrate dependence was observed, which is in accordance with published reports.45 In order to obtain a higher degree of control over the regioisomeric ratio, we examined two Ruthenium catalysts in the cyclotrimerization reaction. In the case of both, Grubbs catalyst (Cl$_2$(Ph$_3$P)$_2$RuCHPh)$^{45, 46}$ and Cp*ClRu(COD)$^{22}$ a high degree of regioselectivity was observed independent of the nature of the alkyne (>90% isomer 59b-66b) (Figure 2.3).

![Figure 2.2. $^1$H NMR of regioisomeric mixture of compound 64 obtained using Wilkinson’s catalyst. Regioisomer ratios were determined via integration of aromatic protons. Aromatic protons assigned to 64a are indicated by a red star, while protons represented by 64b are indicated by a blue star. Overall, the regiomeric ratio of this compound was determined to be 1:3 by integration of the $^1$H NMR signal of aromatic protons.](image-url)
Figure 2.3. $^1$H NMR of compound 64 obtained using Cp*ClRu(COD). Regioisomer ratios were determined via integration of aromatic protons.

Ultimately, the Cp*ClRu(COD) catalyst was utilized due to its favorable reaction conditions (24h, room temperature, addition of 10 mol% catalyst in one aliquot), high yields of the products (69%-95%), and ability to regulate regioselectivity. This is in agreement with the previously reported observation in which the relatively bulky Cp* ligand on the metal center directs the alkyne approach on the metallacycle intermediate to reduce steric interactions.\textsuperscript{22} Additionally, the cyclotrimerization products were obtained in high purities (>90%) without any further purification.
Thus, we have demonstrated the application of solid-supported Rhodium- and Ruthenium-catalyzed [2+2+2] cyclotrimerization reactions in the synthesis of small molecule arrays, enabling their use in the assembly of complex compound libraries. This methodology provides a rapid means for the generation of diverse carbo- and heterocyclic structures, including isoindolines, phthalans, and indans; compound classes which have already been validated as having important biological activities and are found in a variety of natural products. The reaction conditions used for these solid-supported cyclotrimerization reactions are compatible with a variety of functional groups allowing for further diversification of the generated small molecule arrays. This methodology appears to afford a substantial solution to the chemoselectivity issues associated with [2+2+2] cyclotrimerization reactions.

### Scheme 2.8. Solid-supported formation of phthalans.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R'</th>
<th>Yield</th>
<th>a/b</th>
<th>Yield</th>
<th>a/b</th>
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<td>94%</td>
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</tr>
<tr>
<td>59a</td>
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<td>67%</td>
<td>1:3</td>
<td>86%</td>
<td>1:9</td>
</tr>
<tr>
<td>59b</td>
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<td>93%</td>
<td>1:9</td>
</tr>
<tr>
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<td>Ph</td>
<td>H</td>
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<td>1:1</td>
<td>79%</td>
<td>1:9</td>
</tr>
<tr>
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<td>Ph</td>
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<td>1:1</td>
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<td>79%</td>
<td>1:9</td>
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<td>74%</td>
<td>1:1</td>
<td>78%</td>
<td>1:9</td>
</tr>
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<td>73%</td>
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<td>89%</td>
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<td>1:9</td>
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<tr>
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<td>H</td>
<td>SiMe$_3$</td>
<td>68%</td>
<td>2:1</td>
<td>69%</td>
<td>1:9</td>
</tr>
<tr>
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<td>95%</td>
<td>1:9</td>
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<tr>
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<td>CH$_2$OCH$_3$</td>
<td>CH$_2$OCH$_3$</td>
<td>62%</td>
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<td>71%</td>
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</table>
reaction as no dimerization or trimerization of the immobilized diyne occurs. This is apparent through the utilization of di-substituted alkynes, which was not feasible in previous experiments due to the decreased reactivity leading to excessive diyne dimerization.\textsuperscript{45} Gratifyingly, all compounds were obtained in good to excellent yields and with high purities. This approach also demonstrates one means of controlling regioselectivity via alterations in catalyst systems. In this case switching from a Rhodium to a Ruthenium catalyst directed regioisomer formation, affording a less convoluted product mixture and predominantly a single regioisomer.

2.1.3. Application of the Solid-Supported [2+2+2] Cyclotrimerization Reaction to the Synthesis of an Indanone Natural Product

Having successfully developed a new methodology for the rapid preparation of benzene core structures via solid-supported [2+2+2] cyclotrimerization reactions, we set forth to apply this approach towards natural product synthesis. Also, we were interested in investigating additional means of obtaining regioselectivity in conjunction to catalyst tuning. Towards these goals we selected the indanone scaffold as our target. Here we developed a new synthetic route to natural and unnatural indanones via regio- and chemoselective solid-supported [2+2+2] cyclotrimerization reactions. Previous indanone syntheses via cycloadditions either involved completely intramolecular cyclotrimerization reactions,\textsuperscript{51, 52} cyclotrimerization of enones,\textsuperscript{53} or cyclotrimerization reactions with low regioselectivity.\textsuperscript{54} The indanone core structure is widely disseminated among pharmacologically active substances with a wide range of biological activities, hence efficient and selective approaches
to their synthesis are in demand. Additionally, hundreds of indanone natural products are known, most importantly the pterosins (Figure 2.4), including pterosin P (69), mukagolactone (70), and monachosorin A (71).

![Figure 2.4. Selected indanone natural products 69-72.](image)

These molecules, and related structures, display a variety of biological activities including smooth muscle relaxant activity, inhibition of cyclooxygenase 2, and mast cell stabilization. Recently, the indanone natural product 72 has been isolated from a marine cyanobacterium. This compound shows promising biological activity as a regulator of tumor angiogenesis by inhibiting human vascular endothelial growth factor production. However, low *in vivo* activity observed after the initial screening requires additional structural modifications for further improvement, but no total synthesis of 72 has been reported to date. Due to the convergent nature of our synthesis a variety of alkynes can be employed to generate libraries for structure-function relationship studies, and to discover a more potent therapeutic compound.

These studies were performed in collaboration with Dr. Ramesh Senaiar and Jesse Teske to elucidate a chemo- and regioselective approach towards the indanone core structure, and ultimately the natural product 72. Our synthetic route to indanones commenced with the cyclotrimerization precursor 5 which can be rapidly assembled in only 2 steps. Based on our previous results with [2+2+2] cyclotrimerization reactions, we were interested in conducting the reaction via spatial separation of diyne starting materials on a solid support to
alleviate chemoselectivity issues prevalent in solution, such as the di- and trimerization of starting materials.\textsuperscript{22, 25, 65, 66} Performing these reactions on a solid-support, especially with a low loading should prevent these side-reactions. A high level of synthetic flexibility is also achieved by employing an immobilization strategy in which the position of linkage to the solid-phase is not visible in the final product.\textsuperscript{67, 68}

\begin{center}
\textbf{Scheme 2.9.} a) Synthesis of immobilized cyclotrimerization precursors \textsuperscript{76-78}. b) Alkyne reaction partners.
\end{center}

Initial attempts to utilize a similar trityl immobilization strategy as previously employed led to low diyne loadings, sluggish cyclotrimerization reactions, and decomposition upon cleavage. Due to the acid sensitive nature of the benzylic C-O bond in the indanol cyclotrimerization products, we hypothesized the alcohol 73 could best be immobilized via a carboxy linker which can be cleaved under mild basic conditions. Additionally, to solve the decreased reactivity of the immobilized diyne, we selected a NovaSyn\textsuperscript{®} TentaGel carboxy resin, which possesses a polyethyleneglycol (PEG) linker between the polystyrene support and the carboxy functionality, preventing the steric bulk of the resin from interfering with the cyclotrimerization. This resin affords a relatively low loading (0.25 mmol/g); however, due to the increased flexibility affords significantly higher yields and products of higher purity (especially in the case of less reactive internal mono-alkynes). The immobilized 76 was assembled by reacting the resin with 73 (5 equiv.) in
presence of DCC in DCM, and a loading of 0.2 mmol g⁻¹ was obtained (Scheme 2.9a). Resin loadings were determined via the previously described resin cleavage and GC/MS analysis. Due to the synthetic value of the indanone precursors, it is economical that the excess of diyne 73 can be fully recovered and employed in additional immobilization reactions.

The first cyclotrimerization attempts with 76 using the classical Wilkinson’s catalyst (RhCl(Ph₃P)₃)²⁵, ²⁶ led to inconclusive results and cleavage of the diyne from the resin, in spite of the literature prescidence for the utilization of propargyl acetates in Rh-catalyzed cyclotrimerization reactions.²⁵, ⁶⁹, ⁷⁰ Fortunately, the [2+2+2] cyclotrimerization proceeded smoothly at room temperature when Cp*Ru(COD)Cl was used as a catalyst.²² A set of nine alkynes was investigated, revealing that the reaction was compatible with a variety of functionalities, including alkyl chains (in 18), aromatic rings (in 19 and 21), alkoxy groups (in 21, 27, and 79), carbamates (in 22), halides (in 25), and cyano groups (in 23). The cyclotrimerized products were released from the resin by treatment with K₂CO₃ in MeOH/THF. A subsequent solvent change to CH₂Cl₂ followed by oxidation with PDC delivered the indanones 81-89 in good yields (58-78% over three steps) and with excellent purity (>90% by ¹H NMR and GC/MS analysis) (Scheme 2.10). Alternative oxidizing agents (e.g. MnO₂ and Dess-Martin’s periodinane) delivered indanones, albeit in slightly lower yields.
Scheme 2.10. Solid-supported formation of indanones 81-89.

Since the reaction mechanism does not allow differentiation between the two triple bonds in the precursor 8, mixtures of two regioisomers are obtained. The regioisomeric ratio, as determined by $^1$H NMR, ranges from 1:2 to 2:3, with a minimal preference for the formation of the regioisomers 82b-88b. The regioisomeric ratio shown for 82-88 was determined based on the integration of the signal for the aromatic proton at C-7, which occurs farthest downfield in the NMR spectrum. In the case of regioisomers 82a-88a the signal for the proton at C-7 is a singlet, while in the opposite regioisomers 82b-88b this signal occurs as a doublet. This regioisomer assignment was later confirmed by the regioselective preparation of 82b-86b (see Scheme 2.11).

In order to demonstrate the enhancing effect of the solid-support, a solution phase three-step transformation of O-acetylated 73 to 82 under conditions resembling the solid-
phase reaction (20 mM substrate, 10 equiv. 1-hexyne) was conducted. This led to a diminished overall yield of 28% (the solution-phase cyclotrimerization step alone only showed a 37% yield, 1:2 regioselectivity) for 82. This lower yield is a result of competing side reactions in the [2+2+2] cyclotrimerization step (as discussed earlier), which are completely suppressed on the solid-support.

Even though the formation of two regioisomers increases the structural diversity in generated indanone arrays, a selective cyclotrimerization leading to only one product is highly desirable. In order to impose regioselectivity on the solid-supported cyclotrimerization, the precursor 77 was synthesized bearing a removable regio-directing group (TMS).51 Due to the steric bulk of the TMS group, the meta isomer 90 is expected to be the major product (Scheme 2.11). Cyclotrimerization reactions were carried out with terminal alkynes under standard conditions, followed by cleavage and oxidation.22 Investigations into removal of the TMS group were conducted both on the reduced and oxidized indanone. Traditional conditions (TFA, HBr, TBAF, and NH₄F) all yielded little to no desilylation at room temperature or at elevated temperature.71, 72 The TMS group was ultimately removed by treatment with TBAF in 4:1 THF/DMF under microwave irradiation, delivering the indanones 82b-86b in good yields (57-74% over the complete 4 step procedure) and as single regioisomers.
Scheme 2.11. Regioselective solid-supported [2+2+2] cyclotrimerization towards indanones.

The regioisomer shown for 82b-86b was assigned based on the $^1$H NMR spectrum which displays a doublet for the downfield shifted proton H-7 due to its coupling to H-6. The $^1$H NMR spectra of the products correspond to a single regioisomer when compared to the mixtures previously obtained (see Scheme 2.10). Based on the integration of the NMR signal for H-5 the regioisomeric ratio was determined to >95:5. Overall, the [2+2+2] cyclotrimerization reactions of the sterically more hindered 77 lead to comparable product yields to the non-silylated substrate 76, while delivering pure regioisomers.

These studies demonstrate the applicability of solid-supported cyclotrimerization reactions to the chemo- and regioselective formation of natural and unnatural indanones. Since several indanone natural products display a methyl group in the 7-position, the cyclotrimerization precursor 78 was assembled (following a previously employed protocol). Although a highly regioselective [2+2+2] cyclotrimerization as in case of 77 was expected, the regioselectivity inducing effect of a sterically smaller methyl group (compared to a TMS group) needed to be investigated. Dr. Ramesh Senaiar demonstrated that the reaction of 78 with terminal alkynes via the standard cyclotrimerization protocol smoothly lead to the
formation of 91 (Scheme 2.12), followed by the cleavage-oxidation sequence yielding the methyl indanones 92-98 as the only regioisomers. Yields for the three-step process ranged from 63-74% and purities were observed to be >90%. The regioisomer shown for 93-98 was assigned based on singlets in the $^1$H NMR spectrum for the aromatic protons at both C-6 and C-4. In case of the opposite regioisomer, doublets for those protons would be expected. Based on these assignments in the NMR spectrum the regioisomeric ratio was determined to >95:5.

Scheme 2.12. Regioselective solid-supported [2+2+2] cyclotrimerization towards methyl substituted indanones.

These results set the stage for the synthesis of indanone natural products via solid-supported [2+2+2] cyclotrimerization reactions. We selected the marine natural product 4, which was recently isolated from the filamentous marine cyanobacterium *Lyngbya majuscula*, as a target molecule since no total synthesis has been reported to date. Moreover, it exhibits promising anti-angiogenesis activity, which could potentially be improved through the availability of a facile synthetic approach to analogs. The immobilized cyclotrimerization precursor 99 was assembled in 7 steps by Jesse Teske from known material. Again, a carboxy linkage to the polymeric support was chosen due to the acid
lability of the indanol (as discussed above). Since the natural product was reported to be nearly racemic no attempt to generate enantiomerically pure 99 was undertaken. The precursor 99 was cyclotrimerized with propyne to furnish immobilized 100 under the conditions established in previous experiments. After treatment of 100 with K₂CO₃ in THF/MeOH the resulting indanol was oxidized with solid-supported IBX (2-iodoxybenzoic acid polystyrene, Novagen), conveniently leading to an unprecedented acetal cleavage by the solid-supported oxidizing agent. The natural product 72 was obtained in excellent yield (72% over three steps) and as the only regioisomer (Scheme 2.13). All spectroscopic data are in agreement with the literature (Figure 2.5).⁶³

**Scheme 2.13.** Solid-supported synthesis of the indanone natural product 4.
Figure 2.5. $^1$H NMR of natural product 72, demonstrating the compound identity and purity.

In contrast, solution-phase [2+2+2] cyclotrimerization reactions towards 4 were much less successful. A trityl protected precursor of 99 failed to undergo cyclotrimerization, most likely due to strong sterical interactions in the aromatic product between the trityl group on O-1 and the diethylacetal. The solution-phase [2+2+2] cyclotrimerization of an O-1 acetylated precursor resembling the carboxy linker in 99, provided the cyclotrimerization product in only 56% yield. This yield is considerably lower than the 72% yield in case of the solid-phase reaction shown in Scheme 2.13, which also includes the subsequent deprotection and oxidation. Thus, the solid-phase cyclotrimerization approach enables the facile synthesis of analogs of 72 to further improve its anti-angiogenesis activity.

In summary, an efficient and facile approach to natural and unnatural indanones via a solid-supported [2+2+2] cyclotrimerization has been accomplished. An immobilization
strategy was realized which does not show any remains of the linkage site to the solid-support in the final product. Chemo- and regioselectivity issues typically observed in cyclotrimerization reactions have been resolved, and arrays of differently substituted indanones have been constructed. The applicability of the developed approach to total synthesis has been demonstrated through the assembly of a marine natural product. Future work involves the investigation of the biological activities of the synthesized compounds, especially of 72 and analogs.

2.1.3 Solid-phase Synthesis of Pyridines

Based on the successful application of solid-supported cyclotrimerization reaction towards the synthesis of benzenes, we hypothesized that we could adopt a comparable approach towards the synthesis of pyridines. While undergoing a similar catalytic cycle, the decreased reactivity of the nitrile component prevents the incorporation of multiple nitriles when using standard catalysts (Scheme 2.14). Consequently, the final step in the pyridine formation is insertion of the nitrile into the cobaltacycle. Based on this mechanism, it is more feasible to conduct controlled multicomponent reactions towards pyridines relative to benzenes, as we can differentiate all three components: one soluble alkyne, one immobilized alkyne, and one nitrile.
Scheme 2.14. Catalytic cycle for the synthesis of pyridines via the coupling of two alkynes and one nitrile. \( \text{L} = \text{Generic ligand, e.g. CO}_2, \text{PPh}_3, \text{alkyne, etc.} \)

The catalytic solution-phase cyclotrimerization of two alkynes and a nitrile results in the formation of mixtures of products including crossed pyridines (from the incorporation of two different alkynes) and homo pyridines (from the incorporation of two identical alkynes), as well as potential benzene byproducts (Scheme 2.15). The formation of benzene side-products can be disfavored via catalyst selection and by using an excess of the nitrile; however, in a solution phase reaction, there is no means of differentiating between the formation of hetero-pyridines and homo-pyridines.
We envisioned these issues could be resolved via immobilizing one alkyne reaction partner on a polystyrene resin, thereby affording the first catalytic crossed cyclotrimerization reaction of this type. In doing so we can achieve an efficient means of conducting [2+2+2] cyclotrimerization reactions in a true multicomponent fashion. This approach combines the advantages of solid-supported chemistry (easy automatization, parallelization, and purification). The commercial availability of a wide range of alkynes and nitriles makes this reaction an ideal candidate for the rapid assembly of diverse heterocyclic libraries. Additionally, pyridine rings are found in many biologically relevant structures including compounds with antiviral (HIV), antimicrobial, anticaner, and protein kinase inhibition activity.
Our investigation began with the immobilization of a mono-alkyne, and several immobilization strategies were attempted to find the optimal conditions for the cyclotrimerization reaction (Scheme 2.16). Three different resins were investigated, 103, 28, and 31. Resin 103 enabled the cyclotrimerization reaction; however, resin cleavage conditions resulted in a substantial amount of impurities. Resin 31 exhibited high loadings of propargyl alcohol, but exhibited low cyclotrimerization yields, required an additional activation, and entailed a time-consuming cleavage reaction. Ultimately, propargyl alcohol was immobilized on a polystyrene resin (100-200 mesh) 39 using an acid labile trityl linker (28)68, 79 under standard conditions (pyridine, THF, rt, 12 h). The trityl linker was selected due to its mild cleavage conditions, efficiently affording cyclotrimerization products in both high yields and purities. Resin 104 was obtained with a loading of 0.8 mmol g⁻¹ as determined by GC/MS analysis of a sample cleaved with 1% TFA in CH₂Cl₂.

Scheme 2.16. Immobilization strategies for propargyl alcohol.
Scheme 2.17 illustrates the generalized reaction of 104 with an alkyne 105 and a nitrile 106 yielding the immobilized pyridine 107. Only one regioisomer is displayed; however, regioselectivities observed in solution-phase reactions are typically low.\textsuperscript{8, 10, 80} The resin 104 was allowed to swell in degassed toluene in the presence of the alkyne 105, the nitrile 106 (1:10 ratio to favor pyridine formation), and tetramethylammonium oxide (TMAO)\textsuperscript{81} as a catalyst activating additive. The reaction mixture was heated to 80 °C and CpCo(CO)\textsubscript{2} (20 mol\%) was added every 12 h for 48 h.

Due to the pseudo-high dilution conditions on the resin surface, no pyridines resulting from the double incorporation of 104 were observed. Moreover, pyridines that result from the cyclotrimerization of two molecules of 105 and one molecule of 106 were removed in the workup step since they are not immobilized on the resin. The formation of benzenes through the reaction of 104 with two alkynes 105 was suppressed by using the catalyst CpCo(CO)\textsubscript{2} which favors pyridine formation in conjunction with an excess of nitrile 106.\textsuperscript{8} Therefore a highly chemoselective crossed [2+2+2] cyclotrimerization towards 107 was achieved. The pyridine was then cleaved under mild conditions using 1% TFA in DCM yielding clean TFA-
salts of the products 108. These salts were converted into the free bases by passing them through an ion exchange resin (Dowex 50WX8-100). The pyridines 108 were then analyzed by $^1$H NMR, LC/MS, and GC/MS. They are observed in 43-85% yield (typical solution-phase yields are about 65%$^8$) and excellent purity of generally >90%. By using a set of six different alkynes (18-20, 22, 27, 109) and three different nitriles (110-112) an array of 18 pyridines (113-130) was rapidly assembled in collaboration with Dr. Ramesh Senaiar (Figure 2.6).

<table>
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<td>HO</td>
<td>HO</td>
<td>H2N</td>
<td>OMe</td>
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<tr>
<td>114 (85%, 6:3:1)</td>
<td>117 (80%, 13:3:1)</td>
<td>120 (71%, 10:1)</td>
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<tr>
<td>Ph=N</td>
<td>Bu</td>
<td>Ph</td>
<td>HO</td>
<td>HO</td>
<td>H2N</td>
<td>OMe</td>
</tr>
<tr>
<td>115 (75%, 4:1)</td>
<td>118 (66%, 4:3:2)</td>
<td>121 (58%, 2:1)</td>
<td></td>
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Figure 2.6. Alkynes and nitriles (110-112) employed in solid-supported [2+2+2] cyclotrimerization reactions with 62 yielding pyridines 113-130. Yields and ratios of regioisomers are shown in parentheses. The structure of only the 2,4,6-regioisomer is shown, since it generally represents the major product. Compound purities are generally >90% as determined by NMR.

The reaction tolerates a variety of substituents, including alkyl groups (Me, Et, and Bu), aryl groups (Ph), hydroxy groups, alkoxy groups (CH$_2$OMe), and carbamates (BocNH).
amines were not compatible with the reaction conditions; however, the Boc protecting group was conveniently removed from the corresponding carbamate in the acidic cleavage step. Even alkynes which are less reactive due to sterical demand (109) or double substitution (27) underwent cyclotrimerization. Crossed cyclotrimerization reactions lead to the formation of complex mixtures of regioisomers, which can be explained with a generally accepted reaction mechanism (Scheme 2.18).\(^8\) In these cases an assignment of regioisomers was not possible by spectroscopic means and a chromatographic separation was not feasible.

![Catalyst Mechanism Diagram](image-url)

Scheme 2.18. Mechanism leading to eight possible regioisomers in a crossed [2+2+2] cycloaddition yielding pyridines. A, B, and C are different substituents on the alkynes and nitrile, respectively. Ligands on the Co-atom are omitted for clarity and very minor isomers are shown in red.

However, the alkyne reaction partners 20 and 27 greatly simplify the formation of possible regioisomers and allow for an assignment of the major pyridine (the structure shown
in Figure 2.6). In case of 20, a regioselectively less challenging homo cyclotrimerization was performed leading to the predominant formation (67-90%) of the 2,4,6-substituted homopyridines 119-121. This is in agreement with literature observations in the solution phase.\(^8\)

The reaction proceeds through a 2,4-disubstituted cobaltacyclopentadiene as the major reactive species followed by regioselective insertion of the nitrile under carbon-carbon bond formation with the less sterically hindered C-atom attached to the metal. The 2,3,6-trisubstituted pyridine (not shown) is the minor regioisomer (10-33%).

In case of a crossed [2+2+2] cyclotrimerization reaction with the symmetrical alkyne 27, only two regioisomeric pyridines were obtained with the shown 2,3,4,6-pyridines 128-130 (Figure 2.6) being the major isomers and the 2,3,5,6-pyridines (not shown) being the minor isomers. This regioselectivity was established via extensive NMR experiments and can be explained by the mechanism depicted in Scheme 2.19.

\[
\begin{align*}
\text{Co catalyst} & \quad \text{A} \quad + \quad \text{B} \quad \text{B} \\
& \quad \downarrow \\
& \quad \text{C} \quad \equiv \quad \text{N} \\
& \quad \uparrow \\
& \quad \text{131} \quad \text{major} \\
& \quad \downarrow \\
& \quad \text{133} \\
& \quad \downarrow \\
& \quad \text{A} \quad \text{B} \quad \text{A} \quad \text{C} \\
& \quad \downarrow \\
& \quad \text{134} \\
& \quad \downarrow \\
& \quad \text{B} \quad \text{B} \quad \text{A} \quad \text{C} \\
& \quad \downarrow \\
& \quad \text{135} \\
& \quad \downarrow \\
& \quad \text{B} \quad \text{B} \quad \text{B} \quad \text{B} \\
& \quad \downarrow \\
& \quad \text{136} \\
& \quad \downarrow \\
& \quad \text{A} \quad \text{B} \quad \text{A} \quad \text{B} \\
& \quad \downarrow \\
& \quad \text{132} \quad \text{minor} \\
& \quad \downarrow \\
& \quad \text{C} \quad \equiv \quad \text{N} \\
& \quad \uparrow
\end{align*}
\]

Scheme 2.19. Regioselectivity in the cyclotrimerization reaction.
Using a symmetric and an unsymmetric alkyne, two regioisomeric cobaltacyclopentadienes 131 and 132 can be potentially formed. However, the 2,3,4-cyclopentadiene 132 is the minor isomer due to steric interactions between the A and the B substituent. The major 2,3,5-isomer 131 reacts with the nitrile towards the regioisomeric 2,3,4,6- and 2,3,5,6-pyridines, 133 and 134 respectively. The regioisomers 135 and 136 were not observed in the cyclotrimerization reactions described in Scheme 2.19. The ratio of 133/134 was about 2:1 due to similar sterical demand of the two substituents. The slightly higher amount of 133 can potentially be attributed to the higher sterical demand of the trityl group compared to the methyl group.

In summary, we demonstrated the first crossed [2+2+2] cyclotrimerization reaction leading to the formation of highly substituted pyridines. The reaction was conducted on a solid-support facilitating its application in the multi-component synthesis of combinatorial libraries with good yields and excellent purities. While the utilization of the solid support has efficiently solved chemoselectivity issues, further modifications of the reactions (e.g. through catalyst and linker engineering) are required to obtain regioselectivity.

2.2. Microwave Mediated [2+2+2] Cyclotrimerization Reactions

2.2.1 Introduction

Given the success towards the development of solid supported methodologies for [2+2+2] cyclotrimerization reactions, we set forth to improve upon the technology. Standard conditions on the solid-support required long reaction times and often produced only moderate yields. Even without the solid support there are general reactivity issues associated with these reactions, as they often require not only long reaction times, but also high
temperatures, catalyst activating additives, or light irradiation. We hypothesized we may be able to address many of these issues via the application of microwave irradiation.

The development of microwave technologies was greatly advanced during World War II with the application of RADAR devices. It was at this time Percy LeBaron Spencer accidentally discovered the ability of microwaves to cook food as a candy bar melted in his pocket as he experimented with radio waves.\textsuperscript{82} From this point, the application of microwaves to industrial and chemical applications developed rapidly. The first two papers on microwave enhanced organic chemistry were published in 1986,\textsuperscript{83} and the application of microwaves in organic chemistry has exponentially grown in the past two decades. This has been substantially aided by the development of microwave reactors specifically designed for chemical purposes. Due to the enhancing effects of microwave irradiation on many transition-metal mediated reactions (e.g. Suzuki, Heck, and Sonogashira),\textsuperscript{84-86} we suspected the [2+2+2] cyclotrimerization reaction would benefit from microwave irradiation as well.

Microwaves are electromagnetic waves in the 300-300,000 MHz frequency range, and affect molecular rotation. While they possess both an electric and magnetic field, only the electric field contributes to the heating of a sample. This is thought to occur via two mechanisms: dipole alignment and ionic conduction. In both cases, either the dipole or the ion moves to align with the changing electric field, transferring the molecular rotation into thermal energy. Unlike conventional heating, microwaves do not rely upon convection, instead directly interacting with the molecules in the reaction mixture, effectively providing more efficient heating (Figure 2.7). In conjunction to more efficient heating, referred to as a thermal effect, it is postulated that microwaves undergo direct coupling with reaction components leading to other microwave effects.\textsuperscript{87} Most prominent and generally accepted are
“specific” microwave effects, which are defined as accelerations of chemical transformations in a microwave field that cannot be achieved or duplicated by conventional heating.\textsuperscript{88} These effects arise from superheating, increased pressure, or the selective heating of strongly microwave-absorbing heterogeneous catalysts or reagents in a non-polar medium.\textsuperscript{89} Due to these interactions, it may be possible for reactions to occur only under microwave conditions, while no or very little product formation occurs under identical thermal conditions. Perhaps more controversial are “nonthermal” microwave effects which are defined as accelerations of chemical transformations that cannot be explained by either thermal or specific microwave effects.\textsuperscript{87} These effects are thought to arise from the lowering of transition state energies due to the presence of the electromagnetic field and are difficult to conclusively confirm as they may occur in conjunction with other microwave effects.\textsuperscript{87} While the demonstration of these effects is rare, they have the potential to facilitate new chemistries and methodologies that can be synthetically useful.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.7.png}
\caption{Figure 2.7. a) Molecular alignment of dipoles and ions in the microwave electromagnetic field resulting in thermal energy. b) Comparison of microwave versus conventional thermal heating, demonstrating the efficiency of microwave irradiation.}
\end{figure}
Prior to this research there were very few examples of [2+2+2] cyclotrimerization reactions under microwave irradiation. One example of microwave-assisted [2+2+2] cyclotrimerization reactions towards benzene scaffolds was demonstrated in the Ley laboratory, using a completely catalyst free approach; however, the reactions were also completely intramolecular, employing triynes. A second approach by Hrdina and co-workers cyclotrimerized diynes with nitriles to afford atropisomeric pyridines; however, the yields were low and the rate enhancing benefits of the microwave irradiation was not apparent, as the reactions were conducted over 48 hours. In both of these examples, the microwave enhancing effects previously observed with transition metal catalysis were not detected. Consequently, we were interested in investigating this reaction further to make our previously established solid-phase methodologies more efficient and applicable. Concurrently with our work, Zhou et al. were also investigating the same phenomenon and reported the application of microwave irradiation to the synthesis of fused pyridines. Since these initial reports, the methodology has been more widely employed, increasing the applicability of the cyclotrimerization reaction in a variety of fields.

2.2.2. Application of Microwave Irradiation to Solid-Solid Supported Benzene Formation

By conducting [2+2+2] cyclotrimerization reactions on a solid-support we were able to spatially separate the alkyne precursors and thus provided a solution to the chemoselectivity problem. With this problem solved, we next aimed to develop a solution to the reactivity problem by employing microwave irradiation to facilitate formation of otherwise inaccessible ring structures and to greatly reduce the reaction times of solid-supported cyclotrimerization reactions from days to minutes. Moreover, by using
microwave irradiation we can react substrates which previously eluded cyclotrimerization. These developments have lead to unifying conditions for cyclotrimerization reactions of a broad range of alkynes, and can provide excellent reaction conditions for the assembly of combinatorial libraries with aromatic and heteroaromatic core structures. While this initial study was not conducted in a parallel fashion, this methodology can easily be automated to produce libraries of hundreds of compounds in only a few days using commercially available automated microwave synthesizers.

In collaboration with Dr. Lakshminath Sripada five diyne substrates 14-16 and 137-138 (Figure 2.8) used in this study were prepared according to literature conditions, and the mono-alkyne reaction partners were either synthesized (21) or purchased (Sigma-Aldrich). Together, these substrates allow for the probing of the functional group compatibility of the microwave-assisted [2+2+2] cyclotrimerization reaction. The reaction is compatible with a variety of functionalities, including alkoxy groups (in 21), alkyl chains (in 18 and in 26), aromatic rings (in 19, 21 and in 139), chlorine atoms (in 25), cyano groups (in 23), and pyridyl groups (in 139). To investigate the reactivity enhancing effects through microwave irradiation, we were especially interested in employing the otherwise difficult to react diynes 16, 137-138 and the less reactive internal alkyne 26.

![Chemical structures](image1.png)

**Figure 2.8.** Diynes 14-16, 137-138 and mono-alkynes used in this study.
For initial investigations we immobilized the precursor 15 on a standard polystyrene resin (100-200 mesh, 2% crosslinking) using a trityl linker (0.6 mmol/g, Scheme 2.20). The immobilized substrate 30 has previously been cyclotrimerized by us using Wilkinson’s catalyst under thermal conditions (10 mol% Rh(Ph₃P)₃Cl, DCM, 60°C) requiring an extended reaction time of 48 h (Scheme 2.7). However, when we conducted [2+2+2] cyclotrimerization reactions using the Cp*Ru(COD)Cl catalyst (10 mol%) under microwave irradiation in a CEM Discover synthesizer (300 W, 130 °C, toluene), we observed rapid transformation of 30 into 45 (within 10 min) and obtained indanes in excellent yield (75-88%) and high purity (>90%). The shortened reaction time and the higher yields represent a substantial enhancement of the solid-supported [2+2+2] cyclotrimerization reaction and provide an excellent tool for the rapid assembly of small molecule arrays based on fused benzenes (Scheme 2.20). Moreover, it was not necessary to degas the solvent as in the case of previous reactions, thus eventually facilitating the application of this methodology in automated synthesis. As in previous cases, spatial separation on the solid-support completely prevented formation of diyne dimers and trimers, as observed in solution phase reactions (especially with less reactive internal alkynes, R and R’ ≠ H). While these results were promising the thermal control reactions also afforded product, albeit in a reduced yield of ~50%, suggesting that for this diyne substrate microwave irradiation is useful, but not essential.
Scheme 2.20. Microwave assisted [2+2+2] cyclotrimerization reactions towards indanes.

To investigate the facile synthesis of isoindolines and, more importantly, tetrahydroisoquinolines, we immobilized the diynes 14 and 137 on a polystyrene resin as 29 and 141 (0.71 mmol/g and 0.58 mmol/g, respectively) via a trityl linker. As expected, microwave-mediated cyclotrimerization reactions of 29 to 33 proceeded rapidly and almost quantitatively (Scheme 2.21). The isoindolines were isolated in 87-96% yield and with >90% purity, after just a 10 min cyclotrimerization reaction – representing a substantial improvement over previous reaction conditions.41, 66, 105
Scheme 2.21. Microwave assisted [2+2+2] cyclotrimerization reactions towards isoindolines.

Although isoindolines are important pharmacophores found in molecules with a wide range of biological activities, especially antibacterial activity,\textsuperscript{106-108} we were most interested in assembling the homologous tetrahydroisoquinoline skeleton due to its abundance in nature. Gratifyingly, the microwave-mediated cyclotrimerization of 141 smoothly proceeded to the immobilized tetrahydroisoquinoline 143 (Scheme 2.22), and after cleavage form the resin the compounds 144-149 were obtained in good yields (72-89\%) as a 1:1 mixture of regioisomers. Thus, we achieved the facile construction of this important core structure which is found in a wide range of biologically important natural products (for example in protoberine alkaloids, ipecacuanha alkaloids, and benzyltetrahydroisoquinoline alkaloids).\textsuperscript{57}

The transformation 141 $\rightarrow$ 143 showcases the enhancing effects of microwave irradiation in conjunction with a solid-support on the Ru-catalyzed [2+2+2] cyclotrimerization reaction. When the same transformation was carried out in solution-phase, complex compound mixtures were obtained and the product was only isolated in diminished yield.
Scheme 2.22. Microwave-assisted [2+2+2] cyclotrimerization reactions towards tetrahydroisoquinolines.

To further investigate the generality of the microwave-assisted, solid-supported cyclotrimerization reaction, the diyynes \(16-138\) were synthesized and immobilized as \(150-151\) (0.56 mmol/g and 0.45 mmol/g, respectively). The precursor \(151\) was selected to investigate the effects of microwave irradiation on the regioselectivity of cyclotrimerization reaction. Previous solid-phase cyclotrimerization reactions with \(151\) required a sterically less demanding carboxy linkage for immobilization on the polymeric support \((57)\), in contrast, microwave irradiation enables the application of a bulky, but readily cleavable trityl linker. The reactions of \(150\) with the alkynes towards \(152\) proceeded smoothly (Scheme 2.23), delivering up to penta-substituted benzenes in good yields (82-91%) and high purity (>90%). Gratifyingly, with the terminal alkynes, high regioselectivity (9:1) leading to the meta isomer \(152\) (\(R' = \text{H}\)) was observed. These experiments demonstrate that the reactivity enhancing effects of microwave irradiation do not lead to lower regioselectivities. In order to investigate the extent of the reactivity enhancement, we immobilized the diyne \(138\) as \(151\), bearing two internal triple bonds. Interestingly, we had to employ the less sterically demanding carboxy linker to maintain reactivity in this case, emphasizing the importance of a careful linker selection in solid-supported chemistry.\(^{67, 68, 79}\) As expected, the products \(155-161\) were
obtained in slightly lower yields (56-88%), due to two less reactive, internal triple bonds in the precursor 151. The reaction time needed to be increased from 10 to 20 min to achieve full conversion; however, this reaction was especially inefficient under thermal conditions (<10% conversion, data not shown). Additionally, a hexa-substituted benzene 161 could be obtained, which was previously not possible under conventional thermal reaction conditions (data not shown).

Scheme 2.23. Microwave-assisted [2+2+2] cyclotrimerization reactions towards phthalans.

In summary, we demonstrated the reactivity enhancing effects of microwave irradiation combined with the effects of spatial diyne separation on a polymeric support on the ruthenium-catalyzed [2+2+2] cyclotrimerization reaction. The conducted transformations were highly efficient and a high level of chemoselectivity was observed. Moreover, microwave-irradiation did not affect the regioselectivity of the cyclotrimerization reaction when differentially substituted diyne precursors were used. The developed methodology
provides rapid access to a variety of carbo- and heterocyclic structures from simple starting materials. Moreover, it can be directly employed in the synthesis of small molecule arrays of pharmacologically relevant structures (e.g. isoindolines and tetrahydroisoquinolines), due to excellent product yields, extremely short reaction times (minute time scale), and simple reaction conditions (no solvent degassing necessary).

2.2.3. Application of Microwave Irradiation Towards Solid-Supported Heterocyclic Cyclotrimerization Products

Based on the previous results, we were interested in expanding the technology of microwave assisted [2+2+2] cyclotrimerization reactions to other reaction partners. Traditional optimization of transition-metal catalyzed reactions involves the tailoring of substrates, metals, and ligands to achieve the desired transformation with high efficiency, and in order to prevent the incorporation of a third alkyne thus minimizing the formation of benzene side-products. We hypothesized that we could develop a generally applicable and more practical approach to this problem through spatial separation of the substrates combined with microwave irradiation. In order to achieve success, a variety of problems regarding classical cobalt-catalyzed [2+2+2] cyclotrimerization reactions towards the synthesis of heterocycles which still persist must be addressed. These include long reaction times, high dilution conditions, high reaction temperatures, and the necessity of catalyst activation through light irradiation or additives. Moreover, low reactivity with certain substrates, as well as side reactions leading to complex product mixtures have been observed. Recent developments of Co, Ni, Rh, and Ru, catalysts have led to milder reaction conditions and shorter reaction times, but often require specifically designed ligands. Additionally,
with many of the catalysts chemoselectivity (di- and trimerization of starting materials) and regioselectivity issues are still persistent, unless specifically designed substrates are used (e.g. triynes or internal diynes). As a result, no high yielding universal approach to the \([2+2+2]\) cyclotrimerization of a wide range of substrates has been developed to date. Here, we report a different approach to the development of highly efficient \([2+2+2]\) cyclotrimerization reactions, which has the potential to provide unifying conditions for other transition-metal catalyzed cycloadditions as well. It was found that the synergistic application of microwave\(^{88, 98, 112, 113}\) irradiation and a polymeric solid-support makes the \([2+2+2]\) cyclotrimerization highly applicable to a variety of substrates.

Our investigation commenced with a solution phase reaction to elucidate the effect of microwave irradiation on the formation of a fused pyridine. Fortuitously, a literature precedence had been established by Vollhardt et al., employing the cyclotrimerization reaction with benzonitrile and a variety of \(\alpha,\omega\)-diynes (162-163).\(^{16}\) Optimized reaction conditions required a 5 day syringe pump addition of the diyne to the cobalt catalyst (10 mol%) to benzonitrile in refluxing xylene (0.17 M). Even with these optimized conditions, the fused pyridines were only obtained in modest yields. As a starting point, we attempted the identical set of reactions for 10 minutes (300 W) in the microwave and were able to obtain comparable yields (Scheme 2.24). Perhaps the most advantageous aspect of the microwave irradiation is the dramatically reduced reaction time, and the alleviation of the syringe pump requirement. The decreased yield of this cyclotrimerization in the microwave was found to be due to the propensity for dimer and trimerization of the diynes 162-164 leading to 168 and 169, thus indicating that a solid-supported approach could lead to improved yields.
Scheme 2.24. Benchmark formation of fused pyridines, comparing standard thermal conditions with microwave irradiation. Yield are low due to competing dimer (168) and trimerization (169).

An initial solution-phase investigation towards the fused pyridine 171 (Scheme 2.25) was conducted using trityl-protected dipropargylamine and benzonitrile as the starting materials. The cyclotrimerization was performed in non-polar toluene as the solvent at 110°C with 10 mol% CpCo(CO)₂ under microwave irradiation (300W) for 10 min, and after protecting group removal with TFA, 171 was obtained in 46% yield (similar results have been recently observed by others¹⁰⁰, ¹⁰¹). When the same cyclotrimerization was conducted without microwave irradiation, only 9% product formation was observed, even after a prolonged reaction time of 24 hours at 110°C. Previously this has been compensated through light-irradiation, increased reaction temperatures (e.g. 144°C), addition of catalyst activating agents, and extensive reaction times (up to 5 days).¹⁰¹ The modest yield (46%) in the solution-phase cyclotrimerization towards 171 is a result of the formation of benzene byproducts through di- and trimerization of the diyne starting material (as seen in Scheme 2.24), a problem commonly seen in cyclotrimerization reactions of reactive diynes (especially of terminal diynes).²² ²⁵ This problem was solved through spatial separation of the diyne substrates via immobilization on a polystyrene resin.⁶⁷ ⁶⁸ We employed this strategy previously in chemoselective solid-supported cyclotrimerization reactions under
classical conditions.\textsuperscript{65, 66} Preliminary reactions were conducted at various microwave power settings and variable times. Interestingly, addition of TMAO (10\%) to the microwave-mediated reactions led to diminished yields and purities. Excitingly, this previously required catalyst-activating agent could be completely omitted from the reaction, suggesting that the microwave irradiation is effective in catalyst activation without this additive. In conjunction with microwave heating the immobilized diyne 29 delivered the fused pyridines 171-174 in excellent yields (92-95\%) and high purities (>90\%) after cleavage from the resin (Scheme 2.25). The implementation of microwave irradiation in conjunction with the solid-support affords a significant increase in yield, extremely reduced reaction times, and the elimination of catalyst activating additives, excessive heating, or light irradiation. Interestingly, the solid-supported reaction 29 $\rightarrow$ 171 could not be performed under thermal conditions (24 h, 110\(^\circ\)C) and failed to yield significant amounts of product (<5\% yield). The dramatic improvement through microwave irradiation cannot just be attributed to efficient heating (in fact the reaction temperature is lower than under traditional conditions),\textsuperscript{114} but represents one of the most pronounced examples of specific, or still controversially discussed non-thermal microwave effects.\textsuperscript{87, 89, 98} To our surprise electron-poor nitriles could not be reacted towards 170.\textsuperscript{115}
Scheme 2.25. Microwave-mediated solid-supported formation of fused pyridines. Pip = piperidine. \(^5\)CpCo(CO)\(_2\) toluene, 110°C, 24h, MW (300W), no solid-support. \(^6\)CpCo(CO)\(_2\) toluene, 115°C, 24h, no MW irradiation.

We initially employed the symmetrical diyne 29 to investigate the effect of microwave irradiation in conjunction with the solid-support while alleviating the potential for regioisomer formation. However, to explore the ability to impose regioselectivity upon these cyclotrimerization reactions and to investigate potential effects of microwave irradiation on the regioselectivity, we selected 7 (and the structurally related 14) as cyclotrimerization precursors for several reasons: 1) the generated products are chemically stable, 2) the applicability of less reactive internal double bonds will be demonstrated, and 3) the regio-inducing effect of a methylene group can be probed. Employing the previously discovered reaction conditions in the cyclotrimerization of 150 towards 175 using five different nitriles yielded the fused pyridines 176-180 in excellent yields (87-94%) and high purities (>90%) after cleavage from the resin (Scheme 2.26). Most importantly, complete regioselectivity was obtained under microwave irradiation conditions and the obtained regioisomer is in agreement with the generally accepted cyclotrimerization mechanism for the CpCo(CO)\(_2\) catalyst.\(^8\) The regioisomeric identity was confirmed via the \(^1\)H NMR chemical shift of the aromatic proton, as a proton ortho to the nitrogen atom generally affords a signal above 8
ppm, whereas a proton in the meta position often occurs between 7 and 8 ppm, which is what is observed for these compounds.81

Scheme 2.26. Solid-supported regioselective formation of fused pyridines under microwave irradiation. Anth = anthracene.

The synthesis of complementary positional pyridine isomers was achieved through the application of a nitrile tethered to an alkyne.116, 117 Two alkynylnitrile substrates were employed towards the formation of both 5- and 6-membered fused rings. Upon cyclotrimerization of 181 to 182, followed by acid-mediated cleavage from the resin, pyridines 183-190 were obtained in high yields (73-91%), excellent purities (>90%), and as single regioisomers (Scheme 2.27). Regioisomer identity was confirmed by the $^1$H NMR chemical shift of the aromatic protons. Again, the aromatic singlet occurs below 8 ppm, suggesting that only the regioisomer shown in Scheme 2.27 was formed. These yields are significantly higher than in previous solution-phase reactions (especially in the case of reactive terminal alkynes),100, 118 as competing side-reactions are completely suppressed through the spatial separation on the solid-support.
Scheme 2.27. Pyridine formation of fused pyridines from $\alpha,\omega$-alkynenitriles under microwave irradiation.

The cyclotrimerization of alkynes and isocyanates is an effective means of generating pyridones, and catalyst systems based on Ni, Co, Rh, and Ru have been employed.\(^8,115,119-122\) However, these reactions either require specifically tailored substrates (e.g. internal diynes or isocyanate tethered alkynes and alkenes) or specifically tailored ligands. Reported reactions using $\alpha,\omega$-diynes in conjunction with CpCo(CO)$_2$ and CpCo(COD) catalysts produce pyridones only with low efficiency.\(^8,119\) When these cyclotrimerization reactions were performed using our developed reaction conditions, i.e. a solid-support in conjunction with microwave irradiation, highly efficient conversion of 29 into 192-194 (87-96% yield) was observed after cleavage from the resin (Scheme 2.28). Under classical thermal conditions (110°C, 24h) virtually no formation of 192 was detected (<1% yield), thus providing additional evidence for non-thermal microwave effects.
Using carbodiimides in cyclotrimerization reactions under Co and Ni catalysis provides a synthetic entry into 2-iminopyridines. Traditionally, these reactions only show a moderate chemoselectivity with respect to benzene formation and generally low yields, even when conducted with specifically tailored internal akynes. As a result, iminopyridines have not been extensively synthesized via [2+2+2] cyclotrimerization reactions. Moreover, the classical CpCo(CO)$_2$ catalyst has not been successfully employed in these reactions. We solved this problem, as demonstrated by the microwave mediated solid-supported cyclotrimerization of 29 with carbodiimides (Scheme 2.28). The compound 191 ($Y = N$) is formed rapidly and the fused iminopyridines 195-196 were obtained in excellent yield (91-93%) and high purity (>90%). These examples demonstrate that microwave irradiation can activate otherwise inactive Co-catalysts for new cyclotrimerization reactions. Again, under simple thermal heating (110°C, 24h) no iminopyridine 195 could be detected.

In order to validate the enhancing effects of spatial separation on the solid-support, a set of microwave mediated control reactions towards 197-200 (10mol% CpCo(CO)$_2$, toluene, 110°C, MW 300W, 10 min) were conducted in solution-phase (a substrate concentration of

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**Scheme 2.28.** Microwave mediated cyclotrimerization of the diyne 29 with isocyanates, and carbodiimides. CpCo(CO)$_2$ toluene, 110°C, 24h, no MW irradiation.
70 mM was employed, resembling solid-phase conditions). These reactions lead to the formation of complex product mixtures, necessitating chromatographic separation, and greatly diminished yields (16-44%) as a result of undesired side-reactions (Figure 2.9).

![Chemical structures](image)

**Figure 2.9.** Results from solution phase reactions, indicating the necessity of a solid-support.

In conclusion, we have developed microwave-mediated \( [2+2+2] \) cyclotrimerization reactions leading to the formation of pyridines, pyridones, and iminopyridines. The mild and unifying reaction conditions enable the utilization of a wide range of substrates delivering products in high yields, excellent purities, and with complete chemo- and regioselectivity. The universal nature of these mild reaction conditions provide a significant advantage over existing technologies, since the commercially available \( \text{CpCo(CO)}_2 \) catalyst is used for all transformations, and the rapid reaction rates enable the utilization of reaction grade solvents without inert atmosphere. The observed activating effects of microwave irradiation can not be explained by simply more effective heating, but represent pronounced examples of non-thermal microwave effects. Mechanistic explanations of non-thermal microwave effects are still in their infancy\(^{87-89, 98} \) and substantial amounts of future work must be invested to elucidate the nature of this microwave enhancement. A possible explanation could be the lowering of activation barriers in the multi-step cyclotrimerization mechanism\(^{8, 10, 11, 125-127} \) through specific dipole-dipole interactions of the electric field induced by microwave irradiation of polar intermediates (e.g. metallacyclopentadienes) or polar transition states.
Through this methodology, we demonstrate the potential to employ microwave irradiation in the activation of a catalyst system for new synthetic transformations.

2.2.4. Optimization of the Microwave-Mediated Cyclotrimerization Reaction towards Chemo- and Regioselectivity

In order to better understand the optimal conditions and scope of microwave-mediated cyclotrimerization reactions, several studies involving different substrates and reactivity tuning were conducted. Previous reactions were conducted on a trityl functionalized polystyrene resin manufactured by Aldrich; however, the purchase of resin from the Hecheng Corporation (China) is much more cost effective and afforded a wider variety of resins (i.e. higher cross-linking, different mesh sizes). The utilization of this resin for most applications was efficient, except when attempting to cyclotrimerize a less reactive substrate. In this case some dimerization of immobilized alkyne/diyne was observed. This issue became most prominent in the cyclotrimerization reaction of isocyanates to pyridones. We first hypothesized that the undesirable dimerization could be efficiently suppressed by decreasing the loading of the resin. In order to achieve lower resin loadings we examined the effects of utilizing varying equivalents of the diyne 14 in the immobilization step (Scheme 2.29).
As was expected, the loading of the resin decreased in accordance with decreasing amounts of diyne. Three different isocyanates were employed with each of the resins 29 under the previously developed conditions (Scheme 2.30). Reactions conducted on the 1.14 mmol/g resin resulted in a substantial quantity of dimer (202) in the case of all three isocyanates (193, 194, and 201). However, a linear trend was observed for the suppression of dimerization with reduced resin loadings. In fact, at low resin loading (<0.3 mmol/g), very little diyne dimer could be detected by $^1$H NMR, and a 98% yield was obtained when using the butyl isocyanate.
Scheme 2.30. Effects of resin loading on pyridone formation. Complete conversion of starting material was observed in all reactions.

We also varied the crosslinking and mesh size of the resin in order to investigate their effect on the cyclotrimerization reaction. Several resins were obtained from Hecheng Corporation with differing mesh sizes and crosslinking, and dipropargylamine was immobilized on each resin (~0.50 mmol/g). These resins were then cyclotrimerized with cyclohexyl isocyanate (Scheme 2.31).
Reactions with 1% crosslinked resin produced a substantial amount of dimerized diyne independent of mesh size; however, upon increased crosslinking the amount of 194 increased. This decrease in dimerization can be rationalized by considering the rigidity of the resin, as the 2% crosslinked resin is less flexible, prohibiting the potential for the diyne to encounter another immobilized diyne and dimerize. Additionally, it was found with the 2% crosslinked resin that via increasing catalyst loading the amount of dimer was decreased (Scheme 2.31). Ultimately, by employing highly crosslinked resin (2%) and controlling resin loading, the diyne dimerization can be suppressed, even in the case of less reactive substrates.

Another means of preventing dimerization can be achieved via decreasing the diyne reactivity. It has been previously demonstrated that the alkyne reactivity decreases with increasing substitution, thus we prepared bis-trimethylsilyldipropargylamine (203) and immobilized it as the tritylchloride derivatized polystyrene resin (1% crosslinking, 0.56
Cyclotrimerization reactions were conducted based on the previously optimized conditions to obtain the immobilized pyridone product 205, which was subsequently cleaved to yield 206a-209b (Scheme 2.32).

Scheme 2.32. Effect of diyne substitution on pyridone formation. Complete conversion of starting material was observed in all reactions.

This approach towards dimerization suppression was successful as no dimer was detected by LC/MS with any isocyanate substrate. Some desilylation is observed in the microwave, resulting in some impurity of the bis-silylated products 206b-209b; however, it should be possible to completely desilylate the product upon treatment with a fluoride source. While lowering the activity of the diyne 203 does completely suppress the formation of diyne dimer, the preparation of the bis-silylated diyne requires several synthetic operations. This may be prohibitive in the employment of this strategy for more complex molecules.
With the immobilized diproparglyamine 29 in hand, we also explored other potential reaction partners in the cyclotrimerization reaction to access new small molecule scaffolds. Due to the success with isocyanates, we first selected thioisocyanates as reaction partners (Scheme 2.33).\textsuperscript{128}

Overall, thioisocyanates were somewhat problematic when reacted under microwave irradiation. The previously discovered optimal conditions for other substrates could not be employed, as irradiation at 300 W of any isothiocyanate resulted in the spontaneous explosion of the reaction. Additionally, like their isocyanate counterparts, the isothiocyanates were relatively unreactive towards cyclotrimerization. However, it was possible to exclusively obtain 212 in a 67\% yield when employing modified microwave conditions. A 2\% crosslinked resin 29 was employed (loading 0.48 mmol/g) with 200 W of power, resulting in the conversion to 210, which could be readily cleaved from the resin. Product identity and purity was determined by both LC/MS and $^1$H NMR. Interestingly, the product was obtained in >90\% purity and the source for the reduction in yield is unclear. Despite multiple optimization, compound 213 could not be obtained under any of the modified microwave conditions.
We were also interested in investigating the feasibility of cyclotrimerizing the diyne 00 with an aldehyde. First, we conducted a catalyst screen to elucidate the optimal catalyst for this unique cyclotrimerization. Benzyaldehyde was reacted with immobilized dipropargylamine (29), to yield the immobilized product 214, which was readily cleaved with 1% TFA to 215 (Scheme 2.34).

![Scheme 2.34. Cyclotrimerization of dipropargylamine with benzaldehyde.](image)

The cyclotrimerization product 215 was only detected in the case of the CpCo(CO)$_2$ catalyst, with the other catalysts leading to decomposition and remaining starting material. The overall yield of the reaction was low (45%); however, no attempts at reaction optimization were made. Future work will involve both optimization of the reaction and the utilization of other aldehydes to generate products with greater structural diversity.

2.2.5. Microwave-Assisted Multicomponent [2+2+2] Cyclotrimerization Reactions Towards Pyridines

Having successfully developed microwave-mediated intramolecular [2+2+2] cyclotrimerization reactions, we next attempted to translate the reaction into an intermolecular format. In doing so, the methodology becomes more applicable to a wider
range of substrates and increases the potential diversity of the prepared libraries; however, significantly more pronounced chemo- and regioselective issues must be addressed.

Immobilized propargyl alcohol (104) was cyclotrimerized with selected alkynes (10 eq.) and acetonitrile (20 eq.) to examine the rate enhancement of the microwave (Scheme 2.35). Comparing these reaction conditions to the previously developed methodology, a substantial enhancement was discovered both in yield and reaction rate. In the microwave reaction time was reduced from 48 hours to 10 minutes, but more significantly catalyst loading was reduced from 80 mol% to 10 mol%. However, no comparison of the regioselectivity under microwave and non-microwave conditions could be made due to inconsistent NMR and GC analyses.

![Scheme 2.35](image)

**Scheme 2.35.** Comparison of previously established solid-supported cyclotrimerization conditions, with newly established microwave conditions.

Several factors could lead to the more efficient synthesis under microwave irradiation, including: initial catalyst activation in a similar fashion as proposed by light irradiation, direct interaction of the microwave with the catalyst, reaction components, or any...
intermediate in the reaction, and an increase in pressure affording “microwave flash heating.”\textsuperscript{130} We attempted the investigation of several of these factors, considering the obstacle of not being able to spectroscopically analyze reactions while they are being irradiated.

It was first hypothesized that microwave irradiation may change the starting behavior of the catalyst, followed by a traditional thermal reaction at greatly reduced temperatures. This alteration in starting behavior could be attributed to catalyst activation via carbonyl ligand displacement. Previous work by Bonnemann and collaborators, demonstrated the importance of the displaced ligands on the catalyst resting state.\textsuperscript{80} By analyzing pyridine formations at different temperatures and with different catalysts of the general structure \( \text{CpCoL}_2 \), he demonstrated the effect of the ligands \( L \) on the catalytic activity (Figure 2.10). Ligands that are easily displaced (e.g. ethylene) result in active catalyst formation at lower temperatures, suggesting this displacement is one of the reasons for the elevated temperatures required in \( \text{CpCo(CO)}_2 \)-catalyzed reactions. Consequentially, the increased activity in the microwave may be a result of the more facile displacement of the carbonyl ligand in the presence of microwave irradiation. This hypothesis is as also supported by the fact that cyclotrimerization reactions have previously been accelerated by the irradiation with light or the use of catalyst activating agent, both of which are thought to activate the catalyst by the removal of a CO ligand.\textsuperscript{73} Thus, this may explain why the TMAO carbonyl scavenger previously employed in our thermal cyclotrimerization reactions is no longer required.
Figure 2.10. The effect of the ligand L on catalytic activity of CpCoL₂ catalysts. Ligands that are more easily displaced (e.g. ethylene and cyclopentadiene) result in higher catalyst activities at lower temperatures, while more strongly bound neutral ligands (e.g. carbonyl groups) require higher temperatures for catalyst activation. Figure from Bonnemann, H. *Angew. Chem. Int. Ed.* 1985, 24, 248.

If this hypothesis is correct, it should be possible to simply “pre-activate” the catalyst in the microwave to initiate a subsequent thermal cyclotrimerization reaction. In order to examine this possibility, the catalyst was heated in the microwave in the absence of any alkyne or nitrile. This “pre-activated” catalyst was then divided and used in both thermal and microwave-mediated reactions. All thermal reactions at room temperature and 90 °C with the pre-activated catalyst led to no formation of 216, even after 48 hours of reaction time. To ensure that the pre-activation step did not simply lead to catalyst decomposition, an aliquot of the “pre-activated” catalyst was subjected to microwave conditions in the presence of 1-hexyne and acetonitrile. This reaction lead to 79% product formation, confirming the retention of catalyst activity (Scheme 2.36). An additional control experiment was performed to examine if the formation of cobalt precipitates occasionally formed during microwave irradiation are the actual catalytically active species (similar observations have been made in
Thus, a cyclotrimerization was performed and removed from the vial. A second reaction was then attempted in the “dirty” vial in the absence of additional cobalt catalyst; however, this second reaction led to no detectable product. These results indicate that the microwave does not solely activate the catalyst as additional microwave irradiation is required to obtain catalytic turnover and product formation. However, further investigations into the mechanism of microwave acceleration of cyclotrimerization reactions is required. Specifically, it would be intriguing to conduct real-time monitoring of the reaction (via IR or Raman Spectroscopy) to elucidate if the microwave has any direct effect on the catalyst.

We next wanted to investigate if the microwave irradiation was interacting with a specific reaction component leading to the observed microwave effect. The thermal profile of the microwave was examined to compare temperature profiles of heating each component of the reaction individually relative to the combined reaction. This could potentially confirm if the microwaves are specifically interacting with the catalyst, as we initially suspected. We selected the model reaction of hexyne and acetonitrile in xylenes for 10 minutes at 300 W of microwave power. Only slight differences in the thermal profile were observed in the presence of either hexyne or the CpCo(CO)₂ catalyst alone (Figure 2.11). It is important to note that a marked increase in temperature is observed when acetonitrile is a component of
the reaction. This is most likely due to the high dielectric constant of acetonitrile of 37.5. While the thermal profile does not directly implicate a direct interaction with the catalyst, it must be noted that only 10 mol% of catalyst is employed, and we are measuring the bulk temperature. It is still possible that discrete superheating events may be occurring as microscopic hotspots on the surface of the catalyst and further work must be performed to investigate this possibility.

![Figure 2.11. Effects of different reaction components on the bulk reaction temperature measured by an IR pyrometer.](image)

A second possibility is the microwave effect is a result of increased pressure, facilitating the reaction. This has been previously observed, as the increased pressure in the sealed microwave vial affords temperatures unattainable leading to “microwave flash heating.” When separating reaction components, a buildup of pressure was observed to occur with any of the experiments utilizing acetonitrile (data not shown). However, we postulate that this can not be a significant contribution to the microwave effect, as similar
reactions can be conducted under open vessel conditions at atmospheric pressure resulting in similar yields (see Section 2.2.4).

Finally, an interesting observation is that in many of the reactions shown to require microwave irradiation (specifically pyridine, pyridone, and iminopyridine formation), under thermal conditions still afford ~5-10% conversion. This indicates a potential single turnover of the catalyst, suggesting that the microwave irradiation is involved in the perpetuation of the catalytic cycle. We suspect the microwave may be aiding in the disassociation of the final metal complexed intermediate in the reaction, facilitating catalyst turnover. Based on DFT computations by the Vollhardt group for Co-catalyzed alkyne cyclotrimerization reactions towards benzenes, the metal complexed product is at a similar energy level as the final product; however the transition barrier between the two states was not calculated. However, the cobalt complex in the formation of both benzenes and pyridines is often isolable and can be demetallated via further treatment with heat. It certainly is feasible that the microwave is providing this additional energy input to release the cyclotrimerized product from the metal center and regenerate the catalyst for further turnovers. Future work must be invested into elucidating if this is indeed a factor in the microwave acceleration of these reactions. This could potentially be monitored via real-time Raman spectroscopy, or be investigated with stoichiometric amounts of the cobalt complex (both thermally and in the microwave) to examine if the proposed organometallic is formed. If this is indeed the case, it could be isolated and investigated for microwave-mediated demetallation.

Currently, additional experiments are being conducted to further elucidate the role of the microwave in the activation of [2+2+2] cyclotrimerization reactions. Of interest is the addition of a microwave-absorbing agent, such as a silicon carbide plug, which should
rapidly heat the reaction and minimize the actual microwave interaction with the reaction components.\textsuperscript{136, 137} Conversely, it would be intriguing to attempt these reactions under simultaneous cooling conditions to decouple the thermal heating from the microwave interaction with substrates or catalyst. Additionally, significant strides towards understanding this mechanism could be gained via employing real-time monitoring (e.g. via IR or Raman spectroscopy) to monitor changes in the reaction while it is occurring.\textsuperscript{132, 135} Regardless, significant future work is required to better understand the mechanism of microwave activation.

### 2.2.6. Regioselectivity Control of the \([2+2+2]\) Cyclotrimerization Reaction Via Catalyst Modification

While our research has made substantial progress towards the resolution of chemoselectivity and reactivity issues associated with partially intramolecular reactions, the regioselectivity problem has yet not been resolved for intermolecular cyclotrimerization reactions. We have previously established a means of directing regioselectivity through utilization of reaction components possessing a bulky substituent, increasing the propensity of unfavorable steric interactions of the metallacycle intermediate and directing the preferential formation of a specific regioisomer. In the case of benzenes, it has been determined that some regioselective control could be obtained via catalyst selection, as apparent in the regioisomeric ratios between Wilkinson’s catalyst and the Cp*Ru(COD)Cl catalyst (see Scheme 2.8). However, we have yet to explore this catalyst tuning in the case of pyridine formation, although others have employed a variety of different catalysts to examine the effects.\textsuperscript{73} However, it does appear from the literature that catalysts employed in pyridine formations that afford higher regioselectivities typically do so at lower overall yields. Also,
we were interested in investigating to which effect microwave irradiation would shift regioselectivity of intermolecular reactions, potentially affording the production of a single regioisomer. Based on previous results, the steric interactions in the transition state should favor the formation of specific regioisomers over others when conducting a reaction with two identical alkynes (Scheme 2.37).

Scheme 2.37. Mechanism leading to two major regioisomeric pyridines in a homo [2+2+2] cyclotrimerization reaction of two identical alkynes. A and B are different substituents on the alkyne and the nitrile, respectively. Ligands on the Co-atom are omitted for clarity and very minor isomers are shown in red.

The reaction of 1-hexyne (18) and acetonitrile (110) was selected as a model system due to the presence of diagnostic NMR signals in the formed trisubstituted pyridine. This reaction has been found to only yield 2 regioisomers, the 2,4,6- and the 2,3,6-isomer, which can be separated by chromatography. The reaction using 10 equivalents of 110 and 10 mol% CpCo(CO)$_2$ catalyst for 20 minutes at 300W resulted in a 78% yield of 216 with a 2.8 to 1
regioisomeric ratio of 2,4,6- (216a) to 2,3,6-pyridine (216b) (Scheme 2.38). In the literature reported reaction of propyne with propionitrile this catalyst afforded a 1.7 to 1 regioisomeric ratio. The increased regioselectivity in our reaction may be due to the increased steric interactions in the case of the butyl versus the methyl substituent, and thus a matching thermal control reaction was performed to identify if this discrepancy is a result of the microwave or the reactants. This reaction led to a 1.1:1 ratio of 216a to 216b, which was comparable to literature reports of a 1.2:1 ratio of 216a to 216b, which represents an even greater alteration in selectivity. Thus, it does appear that the microwave reactions favor 216a, perhaps due to the rapid rate of the reaction preventing thermal equilibration of the metallacyclopentadiene intermediate.

Scheme 2.38. Microwave cyclotrimerization of 1-hexyne and acetonitrile to yield a regioisomeric mixture of pyridines.

Regioisomer 216a was separated from 216b by flash-column chromatography on silica gel, an was easily distinguishable via the two singlets in the aromatic region (Figure 2.12) of the proton NMR spectrum, while regioisomer 216b gives rise to two doublets in the aromatic region (Figure 2.13). Isomer separation can be assessed via both LC/MS and analysis of the presence of a single CH₃ signal from the incorporated acetonitrile component. Ratios were assessed via both LC/MS integration, and isolated product yield.
As previously noted, an extensive study by Bonnemann investigated a variety of cobalt catalysts and employed thermal reaction conditions cyclotrimerizing propyne and propionitrile (111) at a range of temperatures to investigate the effects on regioselectivity.\textsuperscript{80}
This study varied not only the displaced ligands (as discussed earlier), but also varied the Cp component to best understand the catalyst behavior. It is generally accepted that only changes to the Cp ligand but not the displaced ligand should have an effect on the regioselectivity. As seen in Figure 2.10, the variation of cyclopentadiene ligand also affected the catalyst reactivity (Figure 2.14). This demonstrates that the cyclopentadiene ligand affects the starting behavior of the catalyst as the COD ligand displacement occurs at different temperatures based on substitution affording active catalyst. The relative activity of each catalyst based on the variation of their electronic properties was also elucidated via the utilization of $^{59}$Co NMR experiments. These experiments confirmed previous experiments, finding that the greater the electron donation by the substituent, the greater the electron density on the metal center and the decreased reactivity.

**Figure 2.14.** Effect of cyclopentadiene substitution on the catalyst system in the cyclotrimerization of propyne and propionitrile.

Bonnemann and co-workers also explored the effects of these ligands on regioselectivity. It appears that regioselectivity is inversely proportional to catalyst activity, with more electronrich catalysts yielding higher regioisomeric ratios. However, there does appear to be a steric contribution to the regioselectivity as bulky cyclopentadiene rings were
relatively active, but also demonstrated high levels of regioselectivity favoring $218a$ over $218b$ (Scheme 2.39). The best regioisomeric ratio was observed with the Cp(Me)$_5$ catalyst ligand, leading to a 3.5:1 mixture of $218a/218b$.

![Scheme 2.39. Literature results of the effect of Cp ligand on the reactivity and regioisomeric ratio in the formation of pyridines.](image)

While complete regioselectivity was not achieved, the report is an excellent starting point for further development, and an examination of microwave irradiation on these catalyst systems and the regioselectivity of the reaction. Additionally, we were interested in investigating a more efficient route to the different Co-catalysts. Based on the proposed mechanism for the [2+2+2] cyclotrimerization reaction, it can be hypothesized that by increasing the steric bulk of the ligands on the metal center, the 4,2-cobaltacycle can be favored due to the steric repulsions which may disfavor the 5,2-cobaltacycle. It may also be feasible to tune the electronic nature of the catalyst via Cp ligand substitution to increase reactivity and regioselectivity.

Several routes to modified cyclopentadienyl cobalt catalysts are known; however most require multi-step syntheses of cyclopentadiene precursors followed by laborious formation and purification of the catalyst. Additionally, the preparation of the Cp ligands is not convergent, requiring individual syntheses for each derivative that is employed. Our aim was to start with commercially available or rapidly accessible cyclopentadienyl precursors, and generate active catalysts \textit{in situ} utilizing microwave irradiation. We
suspected that we may be able to simply take substituted cyclopentadienes in the presence of Co$_2$(CO)$_8$ and either pre-generate a catalyst, or generate the active catalyst in the presence of the alkyne and nitrile reaction components. Conveniently, Co$_2$(CO)$_8$ as a Co source does not favor the formation of pyridines under our reaction conditions, but does produce minimal amounts of benzene byproducts (data not shown), thus incomplete Cp catalyst formation should not impact the reaction outcome. Excitingly, it was discovered that under our general microwave reaction conditions, with the addition of 40 mol% CpMe$_5$ and 20 mol% Co$_2$(CO)$_8$ we were able to obtain the pyridine 216 (Scheme 2.40), albeit in moderate yields (59-78%).

We then set forth to determine if the utilization of different cyclopentadiene analogs, under these reaction conditions, could affect the regioselectivity of the reaction. Initial studies employed three commercially available cyclopentadienes with different steric properties (Scheme 2.40).

![Scheme 2.40. One-pot in situ generation of cyclopentadienyl cobalt catalysts in the microwave and their effect on regioselectivity.](image)

Reaction with unsubstituted cyclopentadiene afforded a mixture of regioisomers 216a and 216b in a 78% yield in an approximate 3:1 ratio favoring the 2,4,6-regioisomer 216a. Interestingly, the reaction with CpMe$_5$ resulted in a reversal of regioisomer formation, slightly favoring the 2,3,6–regioisomer 216b. This result contradicts previous reports using

<table>
<thead>
<tr>
<th>&quot;Cp&quot;</th>
<th>Yield</th>
<th>216a</th>
<th>216b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp</td>
<td>78%</td>
<td>2.8</td>
<td>1</td>
</tr>
<tr>
<td>Cp(Me)$_5$</td>
<td>61%</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>Cp(Ph)$_5$</td>
<td>X</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cp(TMS)</td>
<td>59%</td>
<td>1.8</td>
<td>1</td>
</tr>
</tbody>
</table>
thermal conditions (favoring the 2,4,6-regioisomer in a 3.5:1 ratio; see Scheme 2.37), as well as our initial hypothesis; however, the regioisomeric shift is reproducible.\textsuperscript{73} At present, the basis of these results is still under investigation, but will require a more extensive investigation of the active catalyst.

Unfortunately, attempts to perform the penta-phenylcyclopentadiene catalyst formation were unsuccessful under these reaction conditions, and no pyridine formation was observed with this ligand. Fortunately, this catalyst was able to be prepared via a different means (see below), confirming that catalyst formation is possible and does lead to pyridine formation. Reaction with the mono-trimethylsilylcyclopentadiene afforded a 59\% yield of the desired pyridine, and while still favoring regioisomer 216a, doing so in only an 1.8:1 ratio despite the steric bulk of the TMS group. This compares well with to literature results shown in Scheme 2.33. Compared to the unsubstituted cyclopentadiene ligand, the preference of the regioisomer 216a was decreased despite increased steric bulk. Bonneman observed that the steric bulk of the silyl group only began to affect the catalyst regioselectivity upon the addition of multiple TMS groups, which is similar to what we observe.\textsuperscript{80} This result, coupled with the result of the penta-methylcyclopentadiene, suggest that our initial hypothesis of increased steric bulk favoring the 2,4,6 regioisomer was incorrect, and additional factors are involved in the regioselectivity. To better understand this phenomenon additional cyclopentadiene ligands (especially electronpoor ones, e.g. AcCp) need to be prepared to investigate both the steric and electronic effects of ligand substitution on the regioselectivity of the [2+2+2] cyclotrimerization reaction. According to Bonneman, the regioselectivity is inversely proportionate to the activity of the catalyst, except when substantial steric bulk is present.\textsuperscript{80} Thus, to increase the regioselectivity we need to
substantially increase the bulk on the cyclopentadienyl ring, and/or decrease the catalyst activity.

To ensure our results were not limited to this model system we attempted the reaction with propargyl alcohol instead of 1-hexyne. Initial results demonstrated that additional care must be taken to eliminate oxygen from the reaction, as the product is predisposed to oxidize to the aldehyde under the microwave reaction conditions resulting in a mixture of compounds. With thoroughly degassed solutions, it was possible to obtain the desired pyridines in a 49% yield with the Cp*Co(CO)_2 catalyst. The regioisomeric ratio favored the 2,4,6 regioisomer 216a in a 1.5 to 1 ratio, while a 1 to 1.3 ratio was obtained in the case of the reaction with 1-hexyne. A comparable reaction with phenylacetylene resulted in a 1.6 to 1 ratio of 2161a to 216b in a 61% yield, suggesting that the result is consistent for a variety of substrates.

Based on previous reports, we were also interested in the addition of alternate ligands than just the cyclopentadiene. It has been found that addition of phosphine ligands to the reaction have the potential to alter the chemical reactivity of the cobalt catalysts in the [2+2+2] cyclotrimerization reaction. Using our previously employed strategy we added triphenylphosphine (10 to 100 mol%). Unfortunately, when employed with the in situ generated Co_2(CO)_8/Cp catalyst no pyridine formation was detected. When employed with the traditional CpCo(CO)_2 catalyst, pyridine formation was achieved in a reduced 59% yield with no effect on regioselectivity compared to no triphenylphosphine addition.

We next attempted to see if it was possible to pre-form active catalyst, followed by the addition of alkyne and nitrile components. Gratifyingly, this approach was found to be possible, and afforded a route to catalysts, which were not accessible using our previously
described single-pot \textit{in situ} approach. The substituted cyclopentadiene (40 mol\%) and \( \text{Co}_2(\text{CO})_8 \) (20 mol\%) were combined in xylenes and subjected to 10 minutes of microwave irradiation at 300W, followed by the addition of 1-hexyne (1 eq.) and acetonitrile (10 eq.) and an additional irradiation for 20 minutes at 300W (Scheme 2.41).

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\includegraphics[width=\textwidth]{image}};
\end{tikzpicture}
\end{center}

**Scheme 2.41.** Pre-formation of catalyst in the microwave, followed by cyclotrimerization to yield pyridines.

Reaction with penta-methylcyclopentadiene and mono-trimethylsilylcyclopentadiene resulted in comparable regioisomeric ratios as their corresponding \textit{in situ}-generated catalysts (see Scheme 2.40), but reduced yields of 56\% and 48\% respectively. However, using this strategy it was possible to form the penta-phenylcyclopentadiene catalyst and generate the pyridines \(216a\) and \(216b\) in an approximate 5:1 ratio. This corroborates the initial hypothesis that increased steric bulk should favor the 2,4,6 regioisomer, and produced the greatest regioselectivity observed to date.

Additionally, the pre-formation of catalyst afforded the study of phosphine addition on the reaction. It appears that if the catalyst is pre-formed, product formation was obtained after triphenylphosphine addition, suggesting that previously the addition of triphenylphosphine was inhibiting the formation of the active catalyst. While yields were reduced, pyridine products were formed; however, little effect on regioselectivity was noted.
Overall, a variety of methods for catalyst modification were explored towards the optimization of [2+2+2] cyclotrimerization regioselectivity. While these studies represent only the beginning of regioselective control, they suggest that regioselectivity is determined by multiple factors, and catalyst modifications represent a viable method to increase regioselectivities.

Finally, having examined the effects of catalyst modifications on these reactions, we were interested in investigating the role of the substituents in the regioselectivity of the reaction. Thus, we conducted the standard microwave cyclotrimerization with different alkyne reactants and examined the regioisomeric ratio of the products (Scheme 2.42). Interestingly, the bulky alkyens TMS-acetylene and t-butyl acetylene did indeed have a drastic impact on the regioselectivity of the reaction. In fact, in the case of t-butyl acetylene, the regioisomer 219b was barely detectable via $^1$H NMR analysis. The strong regiodirection of the TMS acetylene also suggest that it may be feasible to immobilize alkyens via a silyl linker to the solid-support and conduct regioselective cross pyridine formations. However, the TMS group is unstable, especially when located ortho to the nitrogen atom, leading to a reduced yield. This should be considered when attempting to employ a silyl group to direct regioselectivity.

![Scheme 2.42. Investigation of alkyne component towards the regioselectivity of the pyridine formation.](image_url)
In conclusion, the regioselectivity of the [2+2+2] cyclotrimerization reaction is derived from multiple factors. Ideally, by establishing a better understanding of the individual contributions of each factor we can effectively develop a universal chemo- and regioselective cyclotrimerization. Gratifyingly, we have discovered an effective route to access new catalysts and demonstrated the ability to regulate regioselectivity via both catalyst modification and substituent selection. Future work involves the fusion of these two approaches.

2.2.7 Open-Vessel Microwave-Assisted [2+2+2] Cyclotrimerization Reactions

Although the application of a solid-support in conjunction with microwave irradiation enabled highly selective and efficient reactions, as discussed above, two significant drawbacks remain: 1) due to the low loading of solid-supports, only limited amounts of material can be synthesized, and 2) the starting material needs to be amenable to immobilization through a suitable functional group (typically a carboxy, hydroxy, or amino group).

We hypothesized that we could solve these problems through the application of microwave irradiation under open-vessel conditions. Moving from sealed-vessel conditions to an open-vessel mode enables us to 1) significantly scale-up the microwave mediated [2+2+2] cyclotrimerization reactions, and 2) conduct a slow syringe-pump addition of reactants. The latter minimizes the local concentration of the diyne starting material in the reaction vessel and thus fulfills a similar purpose as the previously employed solid-support.

Initial investigations were conducted by Jesse Teske, and involved the cyclotrimerization of an alkyne with a diyne to afford fused benzenes. Due to its reduced
volatility and other advantageous physical properties dipropargyl diethylmalonate (220) was prepared using previously described literature conditions\(^{143}\) and employed as the model diyne. Additionally, we selected 3-hexyne (26) as the alkyne reaction component for this experiment due to previous reports of the decreased reactivity of internal alkynes leading to a propensity for diyne starting material dimerization.\(^{22, 26}\) Two catalyst systems, Cp*Ru(COD)Cl and Ni(CO)\(_2\)(PPh\(_3\))\(_2\), were employed due to their excellent performance in microwave-mediated [2+2+2] cyclotrimerization reactions towards benzenes.\(^{99, 144}\)

Control closed-vessel conditions under Cp*Ru(COD)Cl catalysis afforded 221 in a poor yield of 28%, which further decreased with reduced equivalents of 3-hexyne due to the extensive self-dimerization and trimerization of 220 to 222 and 223 (Entries 1-3; Scheme 2.43, and Figure 2.15).\(^{22, 145, 146}\) Adaptation to open-vessel conditions generally improved the yield, and the syringe pump addition of 220 to an excess of 26 over 30 minutes provided 221 in a greatly improved yield of 81% (Entry 4; Scheme 2.43). Attempts to minimize the excess of 3-hexyne from 10 to 3-1 equivalents led to lower product yields, even when increasing the reaction time (Entries 2-3 and 5-8; Scheme 2.43). Closed-vessel reactions towards 221 under Ni(CO)\(_2\)(PPh\(_3\))\(_2\) catalysis resulted in a 63% yield, which was diminished with decreasing equivalents of 3-hexyne, as observed for Ru-catalysis (Entries 1-3 and 9-11; Scheme 2.43). When conducted under open-vessel conditions the yield of 221 increased to 82%, and gratifyingly comparable yields could be obtained when reducing the amount of 3-hexyne from 10 to 3 equivalents (Entry 12; Scheme 2.43). Conducting the reaction with only one equivalent of 26 led to decrease yields as starting material remained unreacted (Entries 14; Scheme 2.43).
Overall, these experiments demonstrated that sufficient [2+2+2] cyclotrimerization reactions of difficult to react substrates towards fused benzenes could be obtained using microwave irradiation under open vessel conditions. No need for excessive heating beyond the boiling point of toluene was observed, and the slow syringe-pump addition of the diyne minimized its local concentration thus repressing the formation of side-products.

<table>
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<th>Entry</th>
<th>Catalyst</th>
<th>Equiv.</th>
<th>Syringe</th>
<th>Time/min</th>
<th>Yield</th>
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<tr>
<td>2a</td>
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<td>&quot;</td>
<td>1</td>
<td>-</td>
<td>6%</td>
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</tr>
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<td>4</td>
<td>&quot;</td>
<td>10</td>
<td>30</td>
<td>81%</td>
<td></td>
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<td>8</td>
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<td>1</td>
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<td>30%</td>
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<tr>
<td>9a</td>
<td>Ni(CO)₂(Ph₃P)₂</td>
<td>10</td>
<td>-</td>
<td>63%</td>
<td></td>
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<tr>
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</table>

*aSealed-vessel reaction

Scheme 2.43. Closed- and open-vessel [2+2+2] cyclotrimerization reactions towards benzene formation.

Figure 2.15. Dimerization and trimerization side products.

Having successfully developed open-vessel conditions for the preparation of fused benzenes, we next investigated the synthesis of fused aromatic heterocycles, namely pyridines, and pyridones. We first examined the reaction of benzonitrile (112) with the diyne 220 to generate the fused pyridine 224. We selected benzonitrile as the nitrile component of the reaction based on literature precedence. Using the diyne 220 in a closed-vessel system with 10 equivalents of nitrile 112, we achieved a 72% yield with 16% of starting material.
remaining after a 30-minute reaction time (Entry 1; Scheme 2.44). Attempts to translate these conditions to the open-vessel led to a reduced yield of 34% with substantial amounts of starting material observed, presumably due to a less efficient penetration of the larger reaction vessel by the microwaves. An increase of approximately 10% yield was obtained by extending the reaction time to 60 minutes (Entries 2-3; Scheme 2.44). Finally, with a 120 minute reaction time and slow syringe pump addition of 220, we were able to achieve a 96% yield of 224 with all starting material being consumed (Entry 4; Scheme 2.44).

In an attempt to avoid the high excess of nitrile previously employed in solid-supported cyclotrimerization reactions, we attempted to develop conditions for the use of stoichiometric quantities of the nitrile. When performed under closed-vessel conditions a 74% yield of 224 was attained using only one equivalent of 112; however, instead of unreacted diyne 220, we observed some dimer 222 and trimer 223 formation (Entry 5; Scheme 2.44). Comparable results were found with either 30 or 120-minute reaction times (data not shown). In open-vessel mode, the same reaction with a 120 minute reaction time afforded moderately higher yields (80-86%); however, syringe pump addition was not required (Entries 6-7; Scheme 2.44). Interestingly, no diyne dimer 222 or trimer 223 was observed, and only small quantities of starting material remained. Thus, the open-vessel application of this reaction towards fused pyridines is advantageous, facilitating larger reaction scales and a reduction of the equivalents of the nitrile component was possible.
Next, we investigated the utilization of isocyanates as reaction partners to afford fused pyridones. Hexyl isocyanate (225) was selected as the model isocyanate for this study due to its commercial availability. Microwave-mediated cyclotrimerization reactions of isocyanates have significant chemoselectivity issues due to dimerization and trimerization of the diyne starting material. As mentioned before, these chemoselectivity issues have previously been addressed using a solid-support, here we demonstrate an alternative solution through the combination of open-vessel conditions and slow substrate addition. An initial reaction of 220 and 225 yielded 226 in 56% under open-vessel conditions (Entry 1; Scheme 2.45). Due to the propensity for the diyne to dimerize and trimerize to 222 and 223 in the presence of the less reactive isocyanate reaction partner, we speculated that it may be possible to increase the yield via a more slowly addition of the diyne 220 thus keeping its local concentration low. Gratifyingly, we were able to achieve an 82% yield in the formation of the pyridone 226 with no detectable dimer or trimer formation by decreasing the isocyanate equivalents to 5, and by increasing the syringe pump addition time to 2 hours (Entry 3; Scheme 2.45). Closed-vessel conditions under similar conditions, but without syringe pump addition, yielded substantial amounts of the trimer 223 and afforded 226 in a
much lower yield of 47\% (Entry 4; Scheme 2.45). Attempts to lower the amount of isocyanate were unsuccessful as the competing diyne self-trimerization to 223 started to dominate (Entries 2 and 5; Scheme 2.45). It is interesting to note that while the isocyanate can react under these conditions, its reactivity is substantially different than that observed with the nitrile 112. As a result, optimization of reaction conditions must be performed for different reaction partners; however, the generality of the approach is maintained as the same catalyst system can readily be employed for both substrates.

Scheme 2.45. Closed- and Open-Vessel Cyclotrimerization Reactions Towards Pyridone Formation

In order to demonstrate the applicability of the developed methodology we set forth to scale up the reaction. As a model reaction we chose the intermolecular pyridine formation of two 1-hexyne molecules (18) with acetonitrile (110) to form a pyridine 216. A sealed vessel reaction using 100 mg (1.2 mmol) of 1-hexyne yielded 78\% of the pyridine 216 as an expected ~3:1 mixture of regioisomers favoring the 2,4,6-isomer, as determined by $^1$H NMR (Entry 1; Scheme 2.47). Initial attempts to scale the reaction to 1.0 g (12 mmol) of 1-hexyne involved mixing all components, followed by open-vessel microwave irradiation; however, these conditions provided 216 in only a 52\% yield (Entry 2; Scheme 2.46). Due to the large amount of microwave receptive 110 ($p = 3.92$ D; $\varepsilon = 36.6$) the reaction was vigorously refluxing, potentially leading to some evaporation of the 1-hexyne (b.p. = 71 °C). To
overcome this problem, the 1-hexyne (18) was slowly added over 120 minutes using a syringe pump leading to an increased yield of 68%, but extremely vigorous refluxing due to the extended reaction time was still problematic (Entry 3; Scheme 2.46). To avoid the rapid heating and vigorous reflux, the reaction was attempted by slowly adding both the 1-hexyne (18) and acetonitrile (110) via syringe pump. Gratifyingly, this afforded a 74% yield, which is similar to the optimized sealed-vessel approach (Entry 4; Scheme 2.46). The performance of this reaction in an open vessel mode using a slow syringe pump addition of substrates was also advantageous as the amount of acetonitrile could be dropped from 10 equivalents to 1.5 equivalents, and catalyst loading could be decreased from 10 mol% of CpCo(CO)$_2$ down to 1 mol% while maintaining an good yield of 216 (Scheme 2.41).

Overall, we developed new reaction conditions to address chemoselectivity issues and scale-up issues associated with the microwave-mediated [2+2+2] cyclotrimerization reaction towards a variety of products. While conditions must be slightly optimized for different substrates, utilizing the open-vessel reaction conditions with or without syringe pump addition of reagents is advantageous for large scale application of this methodology. This was demonstrated via the chemoselective preparation of benzenes, pyridines, and pyridones.

Moreover, the opportunity to perform a substantial reaction scale up was shown by the
synthesis of gram quantities of pyridines. We believe that similar open-vessel conditions enable the addressing of similar selectivity and scale issues of other microwave-assisted transition-metal catalyzed cycloaddition reactions.
2.3 Experimental

General. Solvents and reagents were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification unless noted. Tritylchloride and Carboxy resins, 100-200 mesh, 1% crosslinking, were purchased from Sigma-Aldrich and Novabiochem. Diynes 15 and 16 were prepared according to literature procedures. Reactions were conducted under N\textsubscript{2} atmosphere using dry solvents distilled from appropriate drying agents prior to use. NMR data was acquired on a Varian Gemini 300MHz NMR, GC/MS data was obtained on an HP 5890 Series II G1800A, LC/MS data was obtained on an HP 1100MSD system with a ZorbaxSB C-18 3.5 µM pore size 4.5X100mm column, and HRMS was conducted on a JEOL HX110HF mass spectrometer with a resolving power of 10,000 and an accelerating voltage of 10 keV. Compound purity was assessed via gradient runs of 9:1 to 1:9 H\textsubscript{2}O/Acetonitrile at a flow rate of 0.75ml/min for 15 minutes.

2,2-Di(2-propynyl)propane-1,3-diol (15). In a flame dried flask, diethylmalonate (5.0 mL, 33 mmol) was added dropwise over 15 minutes to a suspension of NaH (1.74g, 72 mmol, 2.2 eq) in dry THF (30 mL), and the reaction mixtures was stirred for 15 additional minutes at room temperature. The mixture was then cooled to 0 °C and propargyl bromide (7.1 mL, 66 mmol, 2 eq.) was added dropwise over a 15 minutes. The reaction was then heated to 60 °C for 16 hours, cooled to room temperature and extracted with ethyl acetate (3 × 15 mL). The organic layer was dried (MgSO\textsubscript{4}), concentrated and purified by silica gel chromatography (4:1 Hexanes/Ethyl Acetate) to yield dipropargyl diethyl malonate as a yellow oil, 4.9 g (64% yield). Dipropargyl diethylmalonate (1.0 g, 4.2 mmol) was dissolved in dry THF (20 mL) and
added dropwise to a slurry of lithium aluminum hydride (1.60 g, 4 eq.) in dry THF (20 mL) at 0 °C. The reaction mixture was stirred overnight and was transferred to a large beaker. Diethylether (40 mL) was added followed by water (in a dropwise manner until all unreacted lithium aluminum hydride was quenched). The solution was then filtered through a celite plug and rinsed with ether. The flow-through was dried with MgSO₄, filtered and concentrated in vacuo to yield pure 15 as a white solid (0.62 g, 3.4 mmol, 82%).¹H NMR (300 MHz, CDCl₃) δ 3.74 (s, 4 H), 2.37 (d, J = 2.7 Hz, 4 H), 2.05 (t, J = 2.7 Hz, 2 H); ¹³C NMR (75 MHz, CDCl₃) δ 80.5, 71.5, 66.8, 42.4, 22.0.

4-(Prop-2’-ynyloxy)but-2-yn-1-ol (16).⁴³ In a flame dried flask, 1,4-butyne-diol (1.00 g, 11.6 mmol) was dissolved in THF (15 mL). The solution was cooled to 0 °C and tosylated propargyl alcohol (1.22 g, 5.8 mmol, 0.5 eq) was added dropwise, followed by KOH (0.65 g, 11.6 mmol, 1 eq.). The reaction mixture was stirred at room temperature for approximately 1 hour followed by heating to 60 °C for 16 hours. The reaction was quenched with water (30 mL), and extracted with DCM (3 x 15 mL). The organic layer was dried with MgSO₄, filtered, concentrated, and purified via silica gel chromatography (1:1 Hexane/Ethyl Acetate) to yield 16 as a yellow oil (0.43 g, 3.4 mmol, 61%).¹H NMR (300 MHz, CDCl₃) δ 4.32-4.30 (m, 4 H), 4.26-4.25 (m, 2 H), 2.46 (t, J = 2.1 Hz, 2 H); ¹³C NMR (75 MHz, CDCl₃) δ 85.4, 81.2.5, 79.1, 75.3, 57.1, 56.9, 51.4.

**Immobilization of diproparglyamine (29).** Resin 28 (990 mg, 1.9 mmol) was allowed to swell for 15 minutes in CH₂Cl₂ (10 mL). Dipropargylamine (0.59 mL, 5.7 mmol, 3 eq.) was added, followed by triethylamine (2.65 mL, 19.0 mmol, 10 eq.). The reaction was shaken at
room temperature for 12 hours. The resin was transferred to a syringe filter and washed with alternating rinses of CH$_2$Cl$_2$ and MeOH (4 × 7 mL). The resin was then dried under vacuum and 15 mg were removed and cleaved (1% HCl in CH$_2$Cl$_2$/MeOH, 1h) to determine the loading via GC analysis.

**Immobilization of 4,4-bis(hydroxymethyl)-1,6-heptadiyne (30).** Resin 28 (500 mg, 0.95 mmol) was allowed to swell for 15 minutes in THF (5 mL). Diyne 15 (370 mg, 2.43 mmol, 3 eq.) was added, followed by pyridine (0.77 mL, 9.5 mmol, 10 eq.) and the reaction was stirred at room temperature for 12 hours. The resin was transferred to a syringe filter and washed with alternating rinses of CH$_2$Cl$_2$ and MeOH (4 × 5 mL). The resin was then dried under vacuum and 15 mg were removed and cleaved (1% HCl in CH$_2$Cl$_2$/MeOH, 1 h) to determine the loading via GC analysis.

**Immobilization of 4-(prop-2-ynyloxy)but-2-yn-1-ol (32).** Resin 31 (1.0 g, 1.2 mmol) was allowed to swell for 15 minutes in CH$_2$Cl$_2$ (6 mL). Diyne 16 (750 mg, 6 mmol, 5 eq.) was added, followed by DMAP (29 mg, 0.24 mmol, 0.2 eq.), and diisopropylcarbodiimide (0.93 mL, 6 mmol, 5 eq.) and the reaction was shaken at room temperature for 12 hours. The resin was transferred to a syringe filter and washed with alternating rinses of CH$_2$Cl$_2$ and MeOH (4 × 5 mL). The resin was dried under vacuum and 15 mg was cleaved (25 mg K$_2$CO$_3$, 4:1 THF/MeOH, 12 h) to determine the loading via GC analysis.

**General procedure for the determination of resin loading.** Standard solutions of the immobilized compound were prepared by two serial dilutions from an initial 0.1 M solution.
of the compound in DCM leading to a 0.05 M and a 0.025 M solution. The immobilized resin (15 mg) is then cleaved in 1% TFA in DCM (500 µL) for 1 hour, and the solution is carefully removed from the resin for GC analysis. The standards and resin cleavage are then injected onto the HP 5890 Series II G1800A GC/MS. The peak integration for the standard solution was plotted versus the concentration to establish a standard curve which is used to elucidate the concentration of the compound in the cleavage solution. This concentration can then be correlated to the amount of resin cleaved to generate a resin loading.

**Cyclotrimerization to isoindolines (34-44).** Derivatized resin 29 (50 mg, 0.07 mmol) was placed in a flame dried vial with 3:1 CH₂Cl₂/EtOH (2 mL). The soluble alkyne (0.70 mmol, 10 eq.) was added and the solution was degassed with three freeze-pump-thaw cycles. Wilkinson’s Catalyst (6 mg, 0.07 mmol, 0.1 eq) was added and the reaction was heated to 60°C. After 24 hours additional catalyst was added (6 mg, 0.07 mmol, 0.1 eq.) and the reaction was allowed to progress for an additional 24 hours. The resin was then transferred to a syringe filter and washed with alternating rinses of CH₂Cl₂ and MeOH (4 × 5 mL). The resin was then treated with 1% HCl in CH₂Cl₂/MeOH for 1 hour. The filtrate was concentrated to yield the cyclotrimerized product. Yields ranged from 69-95% and products were obtained in quantities of 7.2-3.4 mg.

**Cyclotrimerization to indans (46-56).** Derivatized resin 22 (50 mg, 0.05 mmol) was placed in a flame dried vial with 3:1 CH₂Cl₂/EtOH (2 mL). The soluble alkyne (0.49 mmol, 10 eq.) was added and the solution was degassed with three freeze-pump-thaw cycles. Wilkinson’s catalyst (5 mg, 0.05 mmol 0.1eq) was added and the reaction was heated to 60°C. After 24
hours additional catalyst was added (5 mg, 0.05 mmol 0.1 eq) and the reaction was allowed to progress for another 24 hours. The resin was then transferred to a syringe filter and washed with alternating rinses of CH$_2$Cl$_2$ and MeOH (4 × 5 mL). The resin was then treated with 1% HCl in CH$_2$Cl$_2$/MeOH for 1 hour. The filtrate was removed, concentrated, dissolved in ether and filtered through a plug of silica gel to yield the cyclotrimerized product. Yields ranged from 60-84% and products were obtained in quantities of 6.4-2.8 mg.

**Cyclotrimerization to phthalans using Wilkinson's catalyst (58-68).** Derivatized resin 32 (50 mg, 0.05 mmol) was placed in a flame dried vial with dichloroethane (2 mL). The soluble alkyne (0.50 mmol, 10 eq.) was added and the solution was degassed with three freeze-pump-thaw cycles. Wilkinson’s catalyst (4 mg, 0.005 mmol, 0.1 eq.) was added and the reaction was heated to 80°C. After 24 hours a additional catalyst was added (4mg, 0.005mmol, 0.1eq) and the reaction was allowed to progress for another 24 hours. The resin was then transferred to a syringe filter and washed with alternating rinses of CH$_2$Cl$_2$ and MeOH (4 × 3mL). The resin was then treated with K$_2$CO$_3$ in 4:1 THF/MeOH for 12 hours. The filtrate was removed, concentrated, dissolved in ether and filtered through a plug of silica gel to yield the cyclotrimerized product. Yields ranged from 52-87% and products were obtained in quantities of 6.4-2.2 mg.

**Cyclotrimerization to phthalans using Cp*ClRu(COD) as the catalyst (58-68).** Derivatized resin 32 (50mg, 0.05mmol) was placed in a flame dried vial with dichloroethane (2mL). The soluble alkyne (0.50mmol, 10equiv.) was added and the solution was degassed with three freeze-pump-thaw cycles. Cp*ClRu(cod) catalyst (2mg, 0.01mmol, 0.1equiv.) was
added and the reaction was shaken at room temperature for 24 hours. The resin was then transferred to a syringe filter and washed with alternating rinses of CH$_2$Cl$_2$ and MeOH (4 × 3mL). The resin was then treated with K$_2$CO$_3$ in 4:1 THF/MeOH for 12 hours. The filtrate was removed and concentrated to yield the cyclotrimerized product. Yields ranged from 69-95% and products were obtained in quantities of 8.1-3.9 mg.

1,6-Heptadiyn-3-ol (73). A flame dried flask charged with oxalyl chloride (166 mg, 1.3 mmol) in dry DCM (2.5 mL) was cooled to −78 °C, and dry DMSO (206 mg, 2.6 mmol) in DCM (500 µL) was added dropwise. The reaction mixture was stirred for 30 minutes at reduced temperature and pentyn-1-ol (100 mg, 1.19 mmol) in DCM (1 mL) was added dropwise while maintaining the temperature at −78 °C. The reaction mixture was stirred for an additional hour and triethylamine (541 mg, 5.35 mmol) was added, followed by an additional hour of stirring at −78 °C, then the reaction mixture was warmed to room temperature for 2.5 hours. The reaction mixture was cooled in an ice bath and quenched with water (5 mL), washed with brine (2 × 5mL) and extracted with DCM (2 × 5 mL). The organic layer was dried with MgSO$_4$, filtered and concentrated by rotatory evaporation, with caution taken due to the volatility of the product. The pent-4-ynal product was then taken on to the next step without further purification.

A solution of pent-4-ynal (50 mg, 0.61 mmol) was added dropwise to a solution of ethynylmagnesium bromide (1.46 mL of 0.5M, 0.73 mmol) in THF (10 mL) at 0 °C and stirred at that temperature for 2 hours. The reaction was then quenched with water (10 mL) and extracted with ether (3 x 15 mL). The combined organic layers were dried with MgSO$_4$, filtered and concentrated by rotatory evaporation. The residue was purified by silica gel
chromatography (5:1 Hexane/EtOAc) to yield **73** as a clear oil (47 mg, 0.44 mmol, 72%). Analytical data is in accordance to the literature reports.

**1-(Trimethylsilyl)-1,6-heptadiyn-3-ol (74).** In a flame dried flask n-butyllithium (460 µL of a 1.59M solution, 0.73 mmol) was added to a solution of trimethylsilyl acetylene (78 mg, 0.79 mmol) in THF (10 mL) at 0 °C. The mixture was stirred for 30 minutes, cooled to –78 °C, and pent-4-ynal (50 mg, 0.61 mmol, prepared according to the previous method) dissolved in THF (2 mL) was added dropwise. The reaction temperature was then slowly raised to 0 °C and stirring was continued for 2 hours before water (15 mL) was added. The reaction mixture was extracted with ether (3 x 10 mL), the combined organic layers were dried with MgSO₄, filtered and concentrated by rotatory evaporation. The residue was purified via silica gel chromatography (5:1 Hexanes/EtOAc) yielding **74** as a white solid (85 mg, 0.47 mmol, 78%). Analytical data is in accordance to the literature reports.

**Immobilization of 73, 74, and 75.** Carboxylic acid derivatized resin (1.0 g, 1.2 mmol) was allowed to swell for 15 minutes in DCM (6 mL). The diyne (73-75) (6 mmol) was added, followed by DMAP (29 mg, 0.24 mmol), and diisopropylcarbodiimide (0.93 mL, 6 mmol), and the reaction was shaken at room temperature for 12 hours. The resin was transferred to a syringe filter and washed with alternating rinses of DCM and MeOH (4 × 5 mL). The resin was dried under vacuum and an aliquot of 15 mg was cleaved (25 mg K₂CO₃, 500 µL 4:1 THF/MeOH, 12h). The loading was determined via GC/MS analysis by generating a standard curve of the diyne starting material within the range of expected loading (100 mM, 50 mM, and 25 mM).
**Indanone cyclotrimerization procedure.** Diyne derivatized resin (76-78) (50 mg, 0.05 mmol) was placed into a flame-dried vial and was suspended in dichloroethane (2 mL) under a nitrogen atmosphere. The soluble alkyne (17-19, 21-23, 25, 27, or 79, 0.50 mmol.) was added and the solution was degassed with three freeze-pump-thaw cycles. The Cp*Ru(COD)Cl catalyst (2 mg, 0.01 mmol) was added and the reaction was shaken at room temperature for 24 hours. The resin was then transferred to a syringe filter, washed with alternating rinses of DCM and MeOH (4 × 3mL), and subsequently dried under vacuum.

**Indanone cleavage and oxidation procedure.** Dried resin (80, 90, or 91) carrying the cyclotrimerized product was transferred to a vial, THF and MeOH (4:1, 500 µL) were added, followed by K$_2$CO$_3$ (20 mg, 0.14 mmol), and the suspension was shaken for 16 hours at room temperature. The filtrate was removed, concentrated, and dissolved in DCM (1 mL). PCC (15 mg, 0.07 mmol) or PDC (26 mg, 0.07 mmol) was added and the solution was stirred overnight at room temperature. The reaction mixture was filtered through a silica plug followed by a rinse with DCM (2 mL), and concentrated to yield pure indanone products (81-89, and 92-98). All yields were determined by measuring the mass balance of pure indanones and correlating the amount of material to the loading of the resin.

**Indanone desilylation procedure.** Oxidized TMS-indanones were dissolved in THF (400 µL), transferred to a microwave vial, and treated with DMF (100 µL) followed by 1.0 M TBAF in THF (50 µL, 0.05 mmol), and the reaction was placed in a CEM Discover Microwave Synthesizer for 2 min (300 W, ~200 °C). The reaction was then passed through a
silica plug which was subsequently rinsed with hexanes (2 mL), and the filtrate was concentrated to yield pure desilylated indanone products (82b-86b). Overall indanone yields ranged from 57-78% and products were obtained in quantities of 2.6-7.2 mg.

**Immobilization of propargyl alcohol (104).** Resin 28 (500 mg, 0.95 mmol) was allowed to swell for 15 minutes in THF (5 mL). Propargyl alcohol (530 mg, 9.5 mmol, 10 eq.) was added, followed by pyridine (0.77 mL, 9.5 mmol, 10 eq.) and the reaction was stirred at room temperature for 12 hours. The resin was transferred to a syringe filter and washed with alternating rinses of CH₂Cl₂ and MeOH (4 × 5 mL). The resin was then dried under vacuum and 15 mg were removed and cleaved (1% HCl in CH₂Cl₂/MeOH, 1 h) to determine the loading via GC analysis.

**Pyridine cyclotrimerization protocol.** Trimethylamine oxide (0.1 eq.) was added to a flame dried vial containing the propargyl alcohol derivatized trityl resin (50 mg), the nitrile (100 eq.), the soluble alkyne (10 eq.), and toluene (2 mL). The solution was degassed, the Co catalyst was added (0.25 eq.), and the reaction was heated to 80 °C. The catalyst addition was repeated every 12 h for 48 h. The resin was transferred to a syringe filter and washed with DCM and MeOH (4 alternating rinses with 4 mL each). The product was then cleaved from the resin by treatment with 1% TFA (DCM, 1 h), filtered and concentrated. The residue was then dissolved in diethyl ether (500 µL) and neutralized with 2 drops of K₂CO₃ (sat. aq.) for 1 hour at room temperature, and the diethylether layer was transferred into a short Dowex WX8-100 column (200 mg) and eluted with ammonia in methanol (2 M, 2 mL) to yield the cyclotrimerization product.
Microwave assisted benzene cyclotrimerization protocol. Diyne derivatized resin (29, 30, 32, 141, 150, or 151) (50 mg, 0.05 mmol) was placed into a flame-dried microwave vial and was suspended in toluene (0.5 mL) under a nitrogen atmosphere. The resin was allowed to swell for approximately 10 minutes, followed by the addition of the soluble alkyne (18, 19, 21, 23, 25, 26, or 139, 0.50 mmol) and the Cp*Ru(COD)Cl catalyst (2 mg, 0.01 mmol). The reaction was then irradiated at 300 W in a CEM Discover microwave synthesizer for 10 minutes (20 minutes in case of internal diynes (26-27). The resin was then transferred to a syringe filter, washed with alternating rinses of DCM and MeOH (4 × 3 mL), and subsequently dried under vacuum. The dried resin was then either cleaved with anhydrous 1% HCl in 0.5 mL 3:2 DCM/MeOH for 1 hour at room temperature (trityl resin), or K$_2$CO$_3$ in 4:1 THF/MeOH for 12 hr at room temperature (carboxy resin). The solution was then filtered, passed through a silica plug and concentrated to yield the cyclotrimerization products. These compounds were analyzed by $^1$H NMR and LC/MS to assess identity and purity.

Microwave assisted pyridine cyclotrimerization in solution. To validate the application of microwave irradiation on the [2+2+2] cyclotrimerization reactions, solution phase cyclotrimerizations were performed and compared with the literature precedence established by the Vollhardt laboratory.$^{21}$ Three $\alpha,\omega$-diynes (162-164) were selected for comparison to the literature. The diyne (25 mg) was placed in a flame dried microwave vessel with benzonitrile (10 eq.) and toluene (4 mL) and degassed. The CpCo(CO)$_2$ catalyst (10 mol%) was then added and the reaction was irradiated at 300W for 10 minutes, reaching a maximum
temperature of 135 °C. The reaction was then transferred to a vial and concentrated by rotatory evaporation. The residue was purified by silica gel chromatography to afford the fused pyridine (47-64%).

**Microwave assisted cyclotrimerization protocol.** Derivatized resin (29, 150, or 181) (40 mg, 0.5-1.2 mmol/g substrate loading) was placed in a microwave reaction vessel and swelled in anhydrous toluene (500 µL) for 10 min. The soluble reaction partner (10 eq.) was added followed by CpCo(CO)$_2$ (0.1 eq.) and the reaction was irradiated in a CEM Discover microwave synthesizer for 10 min at 300 W. The vessel was removed and the resin was washed in a syringe filter with four alternating cycles of DCM and MeOH (2 mL ea.) The resin was dried in vacuo, transferred into a vial, and cleaved for one hour with 500 µL of either a 1% TFA in DCM solution or 1% anhydrous HCl in 3:2 DCM/MeOH. The solution was filtered through a celite plug, concentrated, and then analyzed by $^1$H NMR and LC/MS.

**Microwave Pressure/Temperature Curves of Representative Solid-Phase Reactions.** The microwave synthesizer employed (CEM Discover) allows for the simultaneous measurement of microwave power, temperature, and pressure over the course of the reaction. Do the use of toluene, a microwave ‘transparent’ solvent, only a modest rise in temperature (and pressure) was observed.
Formation of Pyridine 171

Formation of Pyridine 183
Solution phase control reactions. The α,ω-diyne (either trityl protected dipropargylamine or 1,6-heptadiyne, 25 mg) was placed in a flame dried microwave vessel with the appropriate reaction partner (10 eq.) and toluene (to bring the reaction to 70mM of diyne). The CpCo(CO)$_2$ catalyst (10 mol%) was then added and the reaction was irradiated at 300W for
10 minutes, reaching a maximum temperature of 135 °C. The reaction mixture was concentrated by rotatory evaporation and the residue was purified by silica gel chromatography to afford the product (197-200) (16-46%).

**Catalyst pre-activation experiments.** A flame dried microwave vial was charged with xylenes (1 mL) and CpCo(CO)$_2$ (4 µL, 0.035 mmol) and subjected to microwave irradiation for 10 minutes at 300 W. The irradiated catalyst was then divided and added to two separate vials containing 1-hexyne (100 µL, 71 mg, 0.87 mmol) and acetonitrile (300 µL, 236 mg, 5.75 mmol) in xylenes (1 mL). One vial was subjected to microwave irradiation for 20 minutes at 300 W, while the other vial was first stirred at room temperature for 2 hours then heated to 90 ºC for 2 days. The reaction was concentrated by rotatory evaporation and purified via silica gel chromatography (3:1 Hexanes/EtOAc) to yield the pyridine product 216 as a yellow oil (0 % for thermal reactions, 79% for microwave reaction).

**In situ formation of CpCo catalysts for cyclotrimerization reactions.** A flame dried microwave vial was charged with xylenes (2 mL), 1-hexyne (100 µL, 71 mg, 0.87 mmol) and acetonitrile (300 µL, 236 mg, 5.75 mmol). The cyclopentadiene derivative (0.12 mmol, 40 mol%) and Co$_2$(CO)$_8$ (8 mg, 0.03 mmol, 10 mol%) were added, and the reaction was irradiated in the microwave for 20 minutes at 300W. The reaction was concentrated by rotatory evaporation and purified via silica gel chromatography (3:1 Hexanes/EtOAc) to yield the pyridine product 216 as a yellow oil and as a mixture of regioisomers (0-78%; 1:1.3 – 2.8:1).
**Pre-formation of CpCo catalysts for cyclotrimerization reactions.** A flame dried microwave vial was charged with the cyclopentadiene derivative (0.12 mmol, 40 mol%) and xylenes (2 mL), followed by the addition of Co₂(CO)₈ (8 mg, 0.03 mmol, 10 mol%). The reaction mixture was irradiated for 10 minutes at 300 W in the microwave. After the reaction was cooled to room temperature, 1-hexyne (100 µL, 71 mg, 0.87 mmol) and acetonitrile (300 µL, 236 mg, 5.75 mmol) were added under a nitrogen atmosphere and the reaction mixture was subjected to microwave irradiation at 300 W for 10 minutes. The reaction mixture was concentrated by rotatory evaporation and purified via silica gel chromatography (3:1 Hexanes/EtOAc) to yield the pyridine product **216** as a yellow oil and as a mixture of regioisomers (48-59%, 1:1 – 4.8:1). The analytical data was identical to that reported in the literature.

**3-Phenyl-5,7-dihydro[2]pyrindine-6,6-dicarboxylic acid diethyl ester (224):** The diyne **220** (100 mg, 0.42 mmol) was dissolved in xylenes (10 mL) and added over 120 minutes to a solution of benzonitrile (220 µL, 4.2 mmol) and CpCo(CO)₂ (4.9 µL, 0.042 mmol) in xylenes (5 mL) while being irradiated in a CEM Discover microwave synthesizer at 300 W. After addition the reaction was irradiated for a further 10 min, cooled to room temperature, concentrated *in vacuo*, and purified by flash chromatography on silica gel, eluting with hexanes/EtOAc (5:1 to 3:1) to give 136 mg (96%) of **224** as an off-white solid. The analytical data for 7 was identical with data reported in the literature.¹⁴⁵

**2-Hexyl-2,3-dihydro-3-oxocyclopentapyridine-6,6-dicarboxylic acid diethyl ester (226):**

The diyne **220** (100 mg, 0.42 mmol) was dissolved in xylenes (10 mL) and added over 120
minutes to a solution of hexylisocyanate (305 µL, 2.1 mmol) and CpCo(CO)₂ (4.9 µL, 0.042 mmol) in xylenes (5 mL) while being irradiated in a CEM Discover microwave synthesizer at 300 W. After addition the reaction was irradiated for a further 10 min, cooled to room temperature, concentrated in vacuo, and the residue purified by flash chromatography on silica gel, eluting with hexanes/EtOAc (2:1 to 1:3) to give 125 mg (82%) of 226 as a tan solid. ¹H NMR (300 MHz, CDCl₃) δ 7.07 (br s, 1 H), 6.35 (br s, 1 H), 4.20 (q, J = 6.9 Hz, 4 H), 3.82 (t, J = 7.5 Hz, 2 H), 3.63 (s, 2 H), 3.30 (s, 2 H), 1.67-1.64 (m, 2 H), 1.28-1.20 (m, 12H), 0.84 (t, J = 6.6, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 170.9, 155.2, 131.5, 119.3, 114.9, 62.2, 61.0, 50.2, 39.9, 36.7, 31.6, 29.6, 26.6, 22.7, 14.2. HRMS calcd for [M + H]+ C₂₀H₃₀N₂O₅ 364.2046, found 364.2030.

2,4-Dibutyl-6-methylpyridine (216a) and 3,6-Dibutyl-2-methylpyridine (216b): 1-Hexyne (1 g, 12.0 mmol) and acetonitrile (500 µL, 9 mmol) were dissolved in xylenes (10 mL) and added over 120 minutes to a solution of CpCo(CO)₂ (14 µL, 0.12 mmol) in xylenes (20 mL) while being irradiated in a CEM Discover microwave synthesizer at 300 W. After addition the reaction was irradiated for a further 10 min. cooled to room temperature, concentrated in vacuo, and purified by flash chromatography on silica gel, eluting with hexanes/EtOAc (3:1 to 1:1) to give 0.91 g (74%) of a 3:1 ratio of 216a/216b as a yellow oil. The analytical data for 216a and 216b was identical with data reported in the literature.

Analytical Data

Isoindoline (34): ¹H NMR (300 MHz; CD₂OD) δ 10.46 (s, 2H), 7.28-7.24 (m, 4H), 4.50 ppm (s, 4H); LC/MS m/z 119.1 [M⁺+H], Rᵣ 4.04 min.
5-Butylisoindoline (35): $^1$H NMR (300 MHz; CDCl$_3$) δ 10.40 (s, 2H), 7.12 (d, $^3$J(H,H)=8.1 Hz, 1H), 7.14 (d, $^3$J(H,H)=8.1 Hz, 1H), 7.08 (s, 1H) 4.62 (s, 4H), 2.61 (t, $^3$J(H,H)=7.5 Hz, 2H), 1.58 (p, $^3$J(H,H)=6.9 Hz, 2H), 1.35 (m, 2H), 0.93 ppm (t, $^3$J(H,H)=7.2 Hz, 3H); LC/MS $m/\zeta$ 176.1 [M$^+$+H], $R_t$ 5.93 min.

5-Phenylisoindoline (36): $^1$H NMR (300 MHz; CDCl$_3$) δ 10.49 (s, 2H), 7.56-7.33 (m, 8H), 4.73 ppm (s, 4H); $^{13}$C NMR (75 MHz; CDCl$_3$) δ 142.6, 140.2, 134.8, 132.9, 129.1, 128.3, 128.0, 127.4, 123.4, 121.7, 50.9, 50.7; HRMS (FAB): $m/\zeta$ calculated for C$_{14}$H$_{14}$N [M$^+$+H]: 196.1126, found: 196.1114; LC/MS $m/\zeta$ 196.1 [M$^+$+H], $R_t$ 5.75 min.

5-Hydroxymethylisoindoline (37): $^1$H NMR (300 MHz; CD$_3$OD) δ 7.39 (d, $^3$J(H,H)=7.6 Hz, 2H), 7.38 (s, 1H), 4.63 (s, 4H), 3.09 ppm (s, 2H); LC/MS $m/\zeta$ 150.1 [M$^+$+H], $R_t$ 1.97 min.

5-Benzylxoxymethylisoindoline (38): $^1$H NMR (300 MHz; CDCl$_3$) δ 10.42 (s, 2H), 7.35-7.22 (m, 8H), 4.64 (s, 4H), 4.55 (s, 2H), 4.53 ppm(s, 2H); LC/MS $m/\zeta$ 240.1 [M$^+$+H], $R_t$ 5.81 min.

5-Aminomethylisoindoline (39): $^1$H NMR (300 MHz; CD$_3$OD) δ 7.52 (d, $^3$J(H,H)=8.1 Hz, 2H), 7.50 (s, 1H), 4.67 (s, 4H), 4.17 ppm (s, 2H); LC/MS $m/\zeta$ 149.1 [M$^+$+H], $R_t$ 1.34 min.

5-(1-Cyanopropyl)isoindoline 40: $^1$H NMR (300 MHz; CDCl$_3$) δ 10.52 (s, 2H), 7.23-7.12 (m, 3H), 4.67 (s, 4H), 2.35 (t, $^3$J(H,H)=7.2 Hz, 2H) 2.18 (t, $^3$J(H,H)=7.2 Hz, 2H), 2.00 ppm
(quin, $^3J(H,H)$=7.2 Hz, 2H); $^{13}$C NMR (75 MHz; CDCl$_3$) δ 141.2, 134.7, 132.3, 129.5, 123.4, 123.1, 119.3, 50.7, 50.6, 34.4, 27.1, 16.8; HRMS (FAB): $m/z$ calculated for C$_{12}$H$_{15}$N$_2$ [M$^+$+H]: 187.1235, found: 187.1231; LC/MS $m/z$ 187.1 [M$^+$+H], $R_t$ 4.41 min.

5-Trimethylsilylisoindoline (41): $^1$H NMR (300 MHz; CDCl$_3$) δ 10.44 (s, 2H), 7.47 (d, $^3J(H,H)$=7.5 Hz, 1H), 7.43 (s, 1H), 7.29 (s, 1H), 4.68 (s, 4H), 0.28 ppm (s, 9H); LC/MS $m/z$ 192.0 [M$^+$+H], $R_t$ 6.37 min.

5-(1-Chlorobutyl)isoindoline (42): $^1$H NMR (300 MHz; CDCl$_3$) δ 10.41 (s, 2H), 7.25-7.09 (m, 3H), 4.63 (s, 4H), 3.55 (t, $^3J(H,H)$=6.3 Hz, 2H), 2.65 (t, $^3J(H,H)$=6.6 Hz, 2H), 1.78-1.76 ppm (m, 4H); LC/MS $m/z$ 210.0 [M$^+$+H], $R_t$ 6.03 min.

5,6-Diethylisoindoline (43): $^1$H NMR (300 MHz; CDCl$_3$) δ 10.31 (s, 2H), 7.06 (s, 2H), 4.61 (s, 4H), 2.64 (q, $^3J(H,H)$=7.2 Hz, 4H), 1.21 (t, $^3J(H,H)$=7.2 Hz, 6H); LC/MS $m/z$ 176.1 [M$^+$+H], $R_t$ 5.93 min.

5,6-Dimethoxymethylisoindoline (44): $^1$H NMR (300 MHz; CDCl$_3$) δ 10.34 (s, 2H), 7.33 (s, 2H), 4.64 (s, 4H), 4.49 (s, 4H), 3.40 ppm (s, 6H); LC/MS $m/z$ 208.1 [M$^+$+H], $R_t$ 4.20 min.

2,2-Bishydroxymethyl-Indan (46): $^{14}$H NMR (300 MHz; CDCl$_3$) δ 7.18-7.13 (m, 4H), 3.78 (s, 4H), 2.86 (s, 4H), 2.39 ppm (s, 2H); LC/MS $m/z$ 179.0 [M$^+$+Na], $R_t$ 5.01 min.
2,2-Bishydroxymethyl-5-butyl-indan (47): $^1$H NMR (300 MHz; CDCl$_3$) δ 7.08-6.95 (m, 3H), 3.77 (s, 4H), 2.81 (s, 4H), 2.57 (t, $^3J(H,H)$=7.8 Hz, 2H), 2.12 (s, 2H), 1.61-1.55 (m, 2H), 1.40-1.32 (m, 2H), 0.93 ppm (t, $^3J(H,H)$=7.5 Hz, 3H); LC/MS $m/z$ 257.1 [M$^+$+Na], $R_t$ 6.73 min.

2,2-Bishydroxymethyl-5-phenyl-indan (48): $^1$H NMR (300 MHz; CDCl$_3$) δ 7.55 (d, $^3J(H,H)$=6.9 Hz, 2H), 7.43-7.23 (m, 6H), 3.82 (s, 4H), 2.91 (s, 2H), 2.90 (s, 2H), 2.34 ppm (s, 2H); $^{13}$C NMR (75 MHz; CDCl$_3$) δ 142.4, 141.6, 140.9, 140.1, 128.9, 127.3, 127.2, 125.9, 125.5, 124.0, 69.9, 49.5, 38.8, 38.6; HRMS (FAB): $m/z$ calculated for C$_{17}$H$_{19}$O$_2$ [M$^+$+H]: 255.1385, found: 255.1381; LC/MS $m/z$ 277.0 [M$^+$+Na], $R_t$ 6.16 min.

2,2-Bishydroxymethyl-5-hydroxymethyl-indan (49): $^1$H NMR (300 MHz; CD$_3$OD) δ 7.14-7.09 (m, 3H), 4.53 (s, 2H), 3.78 (s, 4H), 2.76 ppm (s, 4H); LC/MS $m/z$ 231.0 [M$^+$+Na], $R_t$ 3.89 min.

2,2-Bishydroxymethyl-5-benzyloxymethyl-indan (50): $^1$H NMR (300 MHz; CDCl$_3$) δ 7.37-7.15 (m, 8H), 4.56 (s, 2H), 4.52 (s, 2H), 3.75 (s, 4H), 2.83 (s, 4H), 2.23 ppm (s, 2H); LC/MS $m/z$ 321.1 [M$^+$+Na], $R_t$ 6.07 min.

2,2-Bishydroxymethyl-5-aminomethyl-indan (51): $^1$H NMR (300 MHz; CD$_3$OD) δ 7.25-7.20 (m, 3H), 4.04 (s, 2H), 3.55 (s, 4H), 2.81 ppm (s, 4H); LC/MS $m/z$ 230.0 [M$^+$+Na], $R_t$ 1.97 min.
2,2-Bishydroxymethyl-5-(1-cyanopropyl)-indan (52): $^1$H NMR (300 MHz; CDCl$_3$) δ 7.12-6.94 (m, 3H), 3.76 (s, 4H), 2.83 (s, 2H), 2.81 (s, 2H), 2.74 (t, $^3$J(H,H)=7.5 Hz, 2H), 2.35-2.30 (m, 2H), 2.29 (s, 2H), 1.97 ppm (t, $^3$J(H,H)=7.2 Hz, 2H); $^{13}$C NMR (75 MHz; CDCl$_3$) δ 142.5, 140.1, 138.3, 127.0, 125.4, 125.3, 119.9, 69.7, 49.3, 38.6, 38.4, 34.5, 27.3, 16.6; HRMS (EI): m/z calculated for C$_{15}$H$_{20}$NO$_2$ [M$^+$+H]: 246.1494, found: 246.1488; LC/MS m/z 268.0 [M$^+$+Na], Rt 5.12 min.

2,2-Bishydroxymethyl-5-trisilylmethyl-indan (53): $^1$H NMR (300 MHz; CDCl$_3$) δ 7.35-7.15 (m, 3H), 3.77 (s, 4H), 2.85 (s, 4H), 2.43 (s, 2H), 0.25 ppm (s, 9H); LC/MS m/z 273.0 [M$^+$+Na], Rt 6.77 min.

2,2-Bishydroxymethyl-5-(1-chlorobutyl)-indan (54): $^1$H NMR (300 MHz; CDCl$_3$) δ 7.10-6.94 (m, 3H), 3.77 (s, 4H), 3.55 (t, $^3$J(H,H)=6.3 Hz, 2H), 2.82 (s, 4H), 2.61 (t, $^3$J(H,H)=7.5 Hz, 2H), 2.32-2.10 (bs, 2H), 1.84-1.75 ppm (m, 4H); LC/MS m/z 291.0 [M$^+$+Na], Rt 6.36 min.

2,2-Bishydroxymethyl-5,6-diethyl-indan (55): $^1$H NMR (300 MHz; CDCl$_3$) δ 6.99 (s, 2H), 3.78 (s, 4H), 2.81 (s, 4H), 2.62 (q, $^3$J(H,H)=7.2 Hz, 4H), 1.91 (s, 2H), 1.21 ppm (t, $^3$J(H,H)=7.2 Hz, 6H); LC/MS m/z 257.1 [M$^+$+Na], Rt 6.48 min.

2,2-Bishydroxymethyl-5,6-methylmethoxy-indan (56): $^1$H NMR (300 MHz; CDCl$_3$) δ 7.20 (s, 2H), 4.74 (s, 4H), 3.74 (s, 4H), 3.93 (s, 6H), 2.82 (s, 4H), 2.34-2.22 ppm (bs, 2 H); LC/MS m/z 289.1 [M$^+$+Na], Rt 4.72 min.
4-Hydroxymethyl-phthalan (58): $^1$H NMR (300 MHz; CDCl$_3$) $\delta$ 7.28-7.18 (m, 3H), 5.19 (s, 2H), 5.12 (s, 2H), 4.67 ppm (d, $^3$J(H,H)=5.7 Hz, 2H); LC/MS $m/z$ 151.0 (M+H), $R_t$ 5.37 min.

4-Hydroxymethyl-5-butyl-phthalan and 4-hydroxymethyl-6-butyl-phthalan (59): $a/b = 1:3$, $^1$H NMR (300 MHz; CDCl$_3$) $\delta$ 7.11 (s, 0.50H), 7.06 (s, 0.75H), 7.00 (s, 0.75H), 5.24 (s, 1H), 5.15 (s, 1.50H), 5.09 (1.50H), 4.87 (s, 0.5H), 4.64 (s, 1.50H), 2.71 (t, $^3$J(H,H)=7.8 Hz, 0.50H), 2.63 (t, $^3$J(H,H)=7.8 Hz, 1.50H), 1.43-1.30 (m, 4H), 0.92 ppm (t, $^3$J=7.2 Hz, 3H); LC/MS $m/z$ 207.1 [M$^+$+H], $R_t$ 8.25 min.

4-Hydroxymethyl-5-phenyl-phthalan and 4-hydroxymethyl-6-phenyl-phthalan (60): $a/b = 3:1$, $^1$H NMR (300 MHz; CDCl$_3$) $\delta$ 7.57 (d, $^3$J(H,H)=6.9 Hz, 0.50H, 7.44-7.35 (m, 5H), 7.22 (s, 1.50H), 5.32 (s, 1.50H), 5.22 (s, 1H), 5.17 (s, 1.50H), 4.73 (d, $^3$J(H,H)=5.4 Hz, 0.50H), 4.58 ppm (d, $^3$J(H,H)=5.4 Hz, 1.50H); $^{13}$C NMR (75 MHz; CDCl$_3$) $\delta$ 141.6, 141.1, 140.8, 136.7, 134.8, 129.0, 127.7, 127.4, 125.2, 119.2, 73.7, 72.7, 63.9; HRMS (FAB): $m/z$ calculated for C$_{15}$H$_{15}$NO$_2$ [M$^+$+H]: 227.1072, found: 227.1069; LC/MS $m/z$ 227.0 [M$^+$+H], $R_t$ 7.64 min.

4,5-Dihydroxymethyl-phthalan and 4,6-dihydroxymethyl-phthalan (61): $a/b = 1:1$, $^1$H NMR (300 MHz; CDCl$_3$) $\delta$ 7.67-7.44 (m, 1H), 7.17 (s, 0.5H), 7.14 (s, 0.5H), 5.22-5.10 (m, 4H), 4.77-4.66 ppm (m, 4H); LC/MS $m/z$ 203.0 [M$^+$+Na], $R_t$ 4.50 min.
4-Hydroxymethyl-5-benzyloxymethyl-phthalan and 4-hydroxymethyl-6-benzyloxymethyl-phthalan (62): \(\text{a/b} = 1:1\), \(^1\text{H NMR (300 MHz; CDCl}_3\) \(\delta 7.37-7.16\) (m, 7H), 5.24 (s, 1H), 5.17 (s, 1H), 5.12 (d, \(3^J_{(H,H)}=4.5\) Hz, 2H), 4.67 (s, 2H), 4.60-4.56 ppm (m, 4H); LC/MS \(m/z\) 293.0 [M\(^++Na\)], \(R_t\) 7.83 min.

4-Hydroxymethyl-5-\(t\)-butylmethylcabamate-phthalan and 4-hydroxymethyl-6-\(t\)-butylmethylcabamate-phthalan (63): \(\text{a/b} = 1:1\), \(^1\text{H NMR (300 MHz; CDCl}_3\) \(\delta 7.24-7.09\) (m, 3H), 5.23-5.02 (m, 4H) 4.65 (d, \(3^J_{(H,H)}=5.4\) Hz, 2H), 4.38 (d, \(3^J_{(H,H)}=6.0\) Hz, 2H), 4.31 (d, \(3^J_{(H,H)}=5.4\) Hz, 2H), 1.46 (s, 4.5H), 1.43 ppm (s, 4.5H); LC/MS \(m/z\) 302.1 [M\(^++Na\)], \(R_t\) 5.37 min.

4-Hydroxymethyl-5-(1cyanopropyl)-phthalan and 4-hydroxymethyl-6-(1-cyanopropyl)-phthalan (64): \(\text{a/b} = 1:3\), \(^1\text{H NMR (300 MHz; CDCl}_3\) \(\delta 7.13\) (s, 0.50H), 7.07 (s, 0.75H), 7.00 (s, 0.75H), 5.23-5.09 (m, 4H), 4.69-4.64 (m, 2H), 2.90 (t, \(3^J_{(H,H)}=7.8\) Hz, 0.50H), 2.81 (t, \(3^J_{(H,H)}=7.8\) Hz, 1.50H), 2.42-2.32 (m, 2H), 2.04-1.95 ppm (m, 2H); \(^13\text{C NMR (75 MHz; CDCl}_3\) \(\delta 140.6, 139.8, 135.9, 134.6, 126.1, 120.4, 119.6, 73.6, 72.7, 63.7, 34.5, 27.4, 16.8\); HRMS (EI): \(m/z\) calculated for C\(_{13}\)H\(_{15}\)NO\(_2\)Na [M\(^++Na\)]: 240.1000, found: 240.0995; LC/MS \(m/z\) 40.0 [M\(^++Na\)], \(R_t\) 6.02 min.

4-Hydroxymethyl-5-trimethylsilyl-phthalan and 4-hydroxymethyl-6-trimethylsilyl-phthalan (65): \(\text{a/b} = 2:1\), \(^1\text{H NMR (300 MHz; CDCl}_3\) \(\delta 7.43\) (d, \(3^J_{(H,H)}=7.5\) Hz, 0.33H), 7.34 (d, \(3^J_{(H,H)}=7.5\) Hz, 1.33H), 7.17 (d, \(3^J_{(H,H)}=7.5\) Hz, 0.33H), 5.26 (s, 1.33H), 5.19 (s,
1.33H), 5.13 (s, 1.33H), 4.75 (d, $^3J(H,H)=5.1$ Hz, 0.33H), 4.68 (d, $^3J(H,H)=5.1$ Hz, 1.67H), 0.37 (s, 3H), 0.29 ppm (s, 6H); LC/MS m/z 223.0 [M$^+$$+$$H$], R$\text{t}$ 8.37 min.

4-Hydroxymethyl-5-(1-chlorobutyl)-phthalan and 4-hydroxymethyl-6-(1-chlorobutyl)-phthalan 66: a/b = 2:1, $^1$H NMR (300 MHz; CDCl$_3$) $\delta$ 7.25 (s, 0.67H), 7.06 (s, 0.33H), 6.99 (s, 0.33H), 5.23 (s, 1.33H), 5.15 (s, 1.33H), 5.09 (s, 1.33H), 4.68 (d, $^3J(H,H)=4.2$ Hz, 1.33H), 4.64 (d, $^3J(H,H)=4.2$ Hz, 0.67H), 3.56 (q, $^3J(H,H)=6.3$ Hz, 2H), 2.76 (t, $^3J(H,H)=7.5$ Hz, 1.33H), 2.67 (t, $^3J(H,H)=7.5$ Hz, 0.67H), 1.90-1.75 ppm (m, 4H); LC/MS m/z 241.0 [M$^+$$+$$H$], R$\text{t}$ 7.92 min.

4-Hydroxymethyl-5,6-diethyl-phthalan (67): $^1$H NMR (300 MHz; CDCl$_3$) $\delta$ 7.04 (s, 1H), 5.24 (s, 2H), 5.22 (s, 2H), 5.08 (s, 2H), 4.69 (d, $^3J(H,H)=5.4$ Hz, 2H), 2.77 (q, $^3J(H,H)=7.6$ Hz, 2H), 2.69 (q, $^3J(H,H)=7.6$ Hz, 2H), 1.26-1.21 ppm (m, 6H); LC/MS m/z 207.0 [M$^+$$+$$H$], R$\text{t}$ 8.21 min.

4-Hydroxymethyl-5,6-dimethyloxymethyl-phthalan (68): $^1$H NMR (300 MHz; CDCl$_3$) $\delta$ 7.19 (s, 1H), 5.24 (s, 2H), 5.12 (s, 2H), 4.65 (s, 2H), 4.57 (d, $^3J(H,H)=6.0$ Hz, 2H), 4.53 (s, 2H), 3.47 (s, 3H), 3.40 ppm (s, 3H); LC/MS m/z 261.0 [M$^+$$+$$Na$], R$\text{t}$ 5.50 min.

Indanone (81).$^{149}$ $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.77 (d, $J = 7.8$ Hz, 1H), 7.61 (t, $J = 7.8$ Hz, 1H), 7.46 (d, $J = 7.8$ Hz, 1H), 7.34 (d, $J = 7.8$ Hz, 1H), 3.18 (t, $J = 5.5$ Hz, 2H), 2.65 (t, $J = 5.5$ Hz, 2H); HRMS calcd for C$_9$H$_8$O 133.0653, found 133.0649.
5-Butylindanone (82b).\textsuperscript{150} \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ 7.67 (d, $J = 7.8$ Hz, 1H), 7.19 (d, $J = 7.8$ Hz, 1H), 3.11 (t, $J = 5.4$ Hz, 2H), 2.70-2.67 (m, 4H), 1.62 (p, $J = 7.8$ Hz, 2H), 1.41-1.34 (m, 2H), 0.94 (t, $J = 7.5$ Hz, 3H); HRMS calcd for C\textsubscript{13}H\textsubscript{16}O 189.1279, found 189.1267.

5-Phenylindanone (83b).\textsuperscript{151} \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ 7.82 (d, $J = 7.8$ Hz, 1H), 7.64-7.53 (m, 4H), 7.49-7.39 (m, 3H), 3.21 (t, $J = 5.4$ Hz, 2H), 2.76 (t, $J = 5.5$ Hz, 2H); HRMS calcd for C\textsubscript{15}H\textsubscript{12}O 209.0966, found 209.0956.

5-(4-Chlorobutyl)indanone (84b). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ 7.67 (d, $J = 8.1$ Hz, 1H), 7.28 (s, 1H), 7.18 (d, $J = 8.1$ Hz, 1H), 4.39 (t, $J = 5.4$ Hz, 2H), 3.12 (t, $J = 5.4$ Hz, 2H), 2.75-2.67 (m, 4H), 1.81-1.71 (m, 4H); HRMS calcd for C\textsubscript{13}H\textsubscript{15}ClO 223.0890, found 223.0871.

5-(3-Cyanopropyl)indanone (85b). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ 7.69 (d, $J = 7.6$ Hz, 1H), 7.30 (s, 1H), 7.19 (d, $J = 7.6$ Hz, 1H), 3.13 (t, $J = 5.4$ Hz, 2H), 2.89 (t, $J = 7.5$ Hz, 2H), 2.71 (t, $J = 5.4$ Hz, 2H), 2.37 (t, $J = 7.4$ Hz, 2H), 2.03 (p, $J = 7.5$ Hz, 2H); HRMS calcd for C\textsubscript{13}H\textsubscript{13}NO 200.1075, found 200.1072.

5-(Benzyloxymethyl)indanone (86b). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) $\delta$ 7.73 (d, $J = 7.8$ Hz, 1H), 7.49 (s, 1H), 7.38-7.33 (m, 6H), 4.63 (s, 2H), 4.61 (s, 2H) 3.14 (t, $J = 5.4$ Hz, 2H), 2.71 (t, $J = 7.5$ Hz, 2H); HRMS calcd for C\textsubscript{16}H\textsubscript{15}O\textsubscript{2} 239.1072, found 239.1076.
5-((Tetrahydro-2H-pyran-2-yloxy)methyl)indanone and 6-((tetrahydro-2H-pyran-2-yloxy)methyl)indanone (87). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.76 (s, 0.3H), 7.72 (d, \(J = 8.1\) Hz, 0.7H), 7.60 (d, \(J = 7.8\) Hz, 0.3H), 7.49 (s, 0.7H), 7.45 (d, \(J = 8.1\), 0.3H), 7.35 (d, \(J = 7.8\), 0.7H), 4.89-4.85 (m, 1H), 4.79-4.71 (m, 1H), 4.60-4.52 (m, 1H) 3.94-3.89 (m, 1H), 3.59-3.54 (m, 1H), 3.14 (t, \(J = 5.4\) Hz, 2H), 2.71 (t, \(J = 6.3\) Hz, 2H) 1.93-1.57 (m, 6H); HRMS calcd for C\(_{15}\)H\(_{18}\)O\(_3\) 247.1334, found 247.1337.

tert-Butyl(1-oxo-indan-5-yl)methylcarbamate and tert-Butyl(1-oxo-indan-6-yl)methylcarbamate (88). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.71 (d, \(J = 7.8\) Hz, 0.7H), 7.65 (s, 0.3H), 7.53 (d, \(J = 7.8\) Hz, 0.3H), 7.43 (d, \(J = 7.8\), 0.3H), 7.42 (s, 0.7H), 7.27 (d, \(J = 7.8\), 0.7H), 4.93 (bs, 1H), 4.41-4.37 (m, 2H), 3.13 (t, \(J = 5.4\) Hz, 2H), 2.73-2.68 (m, 2H) 1.47 (m, 9H); HRMS calcd for C\(_{15}\)H\(_{19}\)NO\(_3\) 262.1443, found 262.1443.

5,6-Bis(methoxymethyl)indanone (89). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.72 (s, 1H), 7.59 (s, 1H), 4.60 (s, 1H), 4.51 (s, 2H), 3.48 (s, 3H), 3.37 (s, 3H), 3.14 (t, \(J = 5.4\) Hz, 2H), 2.71 (t, \(J = 5.4\) Hz, 2H); HRMS calcd for C\(_{13}\)H\(_{16}\)O\(_3\) 221.1178, found 221.1167.

7-Methylindanone (92). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.42 (d, \(J = 7.5\) Hz, 1H), 7.27 (d, \(J = 7.5\) Hz, 1H), 7.09 (d, \(J = 7.5\) Hz, 1H), 3.10 (t, \(J = 5.7\) Hz, 2H), 2.67 (t, \(J = 5.7\) Hz, 2H), 2.65 (s, 3H); HRMS calcd for C\(_{10}\)H\(_{10}\)O 147.0810, found 147.0807.
5-Butyl-7-methylindanone (93). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.07 (s, 1H), 6.91 (s, 1H), 3.05 (t, $J = 5.7$ Hz, 2H), 2.67-2.63 (m, 4H), 2.61 (s, 3H) 1.60 (p, $J = 7.2$ Hz, 2H), 1.42-1.36 (m, 2H), 0.95 (t, $J = 7.2$ Hz, 3H); HRMS calcd for C$_{14}$H$_{18}$O 203.1436, found 203.1434.

7-Methyl-5-phenylindanone (94). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.61 (d, $J = 7.2$, 2H), 7.48-7.40 (m, 4H), 7.32 (s, 1H), 3.14 (t, $J = 5.7$ Hz, 2H), 2.72 (t, $J = 5.7$ Hz, 2H), 2.70 (s, 3H); HRMS calcd for C$_{16}$H$_{14}$O 223.1123, found 223.1112.

5-(4-Chlorobutyl)-7-methylindanone (95). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.08 (s, 1H), 6.91 (s, 1H), 3.56 (t, $J = 5.2$, 2H), 3.05 (t, $J = 5.7$ Hz, 2H), 2.69-2.63 (m, 4H), 2.62 (s, 3H) 1.81 (m, 4H); HRMS calcd for C$_{14}$H$_{17}$ClO 237.1046, found 237.1038.

5-(3-Cyanopropyl)-7-methylindanone (96). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.10 (s, 1H), 6.91 (s, 1H), 3.06 (t, $J = 5.7$ Hz, 2H), 2.82 (t, $J = 7.5$ Hz, 2H), 2.65 (t, $J = 5.7$ Hz, 2H), 2.62 (s, 3H), 2.35 (t, $J = 7.5$ Hz, 2H) 2.02 (p, $J = 7.5$, 2H); HRMS calcd for C$_{14}$H$_{15}$NO 214.1232, found 214.1223.

7-Methyl-5-((tetrahydro-2H-pyran-2-yloxy)methyl)indanone (97). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.28 (s, 1H), 7.07 (s, 1H), 4.82 (d, $J = 12.6$ Hz, 1H), 4.73 (t, $J = 3.3$ Hz, 1H), 4.52 (d, $J = 12.6$ Hz, 1H), 3.96-3.88 (m, 1H), 3.60-3.54 (m, 1H), 3.08 (t, $J = 5.7$ Hz, 2H), 2.67 (t, $J = 5.7$ Hz, 2H), 2.63 (s, 3H), 1.92-1.56 (m, 6H); HRMS calcd for C$_{16}$H$_{20}$O$_3$ 261.1491, found 261.1476.
tert-Butyl(7-methyl-1-oxo-indan-5-yl)methylcarbamate (98). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.19 (s, 1H), 6.99 (s, 1H), 4.82 (bs, 1H), 3.34 (d, $J = 6.1$ Hz, 2H), 3.06 (t, $J = 5.7$ Hz, 2H), 2.67 (t, $J = 5.7$ Hz, 2H), 2.62 (s, 3H), 1.48 (s, 9H); HRMS calcd for C$_{16}$H$_{21}$NO$_3$ 276.1600, found 276.1582.

2,3-Dihydro-6-phenyl-1H-pyrrolo[3,4-c]pyridine (171). $^1$H NMR (300 MHz, CD$_3$OD) δ 8.66 (s, 1H), 7.97-7.90 (m, 3H), 7.42-7.45 (m, 3H), 4.73 (s, 4H); HRMS calcd for C$_{13}$H$_{13}$N$_2$ 197.2478, found 197.2480.

2,3-Dihydro-6-vinyl-1H-pyrrolo[3,4-c]pyridine (173), $^1$H NMR (300 MHz, CD$_3$OD) δ 8.61 (s, 1H), 7.80 (s, 1H), 6.95 (dd, $J_1 = 17.4$ Hz, $J_2 = 11.1$ Hz, 1H), 6.29 (d, $J = 17.4$ Hz, 1H), 5.71 (d, $J = 11.1$ Hz, 1H), 4.74 (s, 4H) ; HRMS calcd for C$_9$H$_{11}$N$_2$ 147.1891, found 147.1886.

2,3-Dihydro-6-((piperazin-1-yl)methyl)-1H-pyrrolo[3,4-c]pyridine (174). $^1$H NMR (300 MHz, CD$_3$OD) δ 8.48 (s, 1H), 7.41 (s, 1H), 4.61 (s, 4H), 3.43-3.00 (m, 8H) ; HRMS calcd for C$_{12}$H$_{20}$N$_4$ 219.2981, found 219.2986.

1,3-Dihydro-6-methyl-furo[3,4-c]pyridine-4-methanol (176). $^1$H NMR (300 MHz, CDCl$_3$) δ 6.97 (s, 1H), 5.14 (s, 4H), 4.62 (s, 2H), 2.57 (s, 3H) ; HRMS calcd for C$_9$H$_{10}$NO$_2$ 165.1891, found 165.1883.
1,3-Dihydro-6-phenyl-furo[3,4-c]pyridine-4-methanol (177). $^1$H NMR (300 MHz, CDCl$_3$) δ 8.08 (d, $J$=8.1, 1H), 7.63-7.31 (m, 5H), 5.25-5.14 (m, 4H), 4.67 (s, 2H); HRMS calcd for C$_{14}$H$_{14}$NO$_2$ 228.2585, found 228.2588.

1,3-Dihydro-6-vinyl-furo[3,4-c]pyridine-4-methanol (178). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.97 (s, 1H), 7.01 (dd, $J_1$=17.4 Hz, $J_2$=11.4 Hz, 1H), 6.43 (d, $J$=17.4 Hz, 1H), 5.90 (d, $J$=11.4 Hz, 1H), 5.25 (s, 2H), 5.18 (s, 2H), 4.88 (s, 2H); HRMS calcd for C$_{10}$H$_{12}$NO$_2$ 178.1998, found 178.1998.

1,3-Dihydro-6-ethyl-furo[3,4-c]pyridine-4-methanol (179). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.00 (s, 1H), 5.18-5.06 (m, 4H), 4.63 (s, 2H), 2.87 (q, $J$=6.7 Hz, 2H), 1.31 (t, 3H); HRMS calcd for C$_{10}$H$_{14}$NO$_2$ 179.2157, found 179.2167.

1,3-Dihydro-6-anthacenyl-furo[3,4-c]pyridine-4-methanol (180). $^1$H NMR (300 MHz, CDCl$_3$) δ 8.68 (s, 1H), 8.57 (s, 1H), 8.49 (s, 1H), 8.19-8.01 (m, 4H), 7.76 (s, 1H), 7.53-7.50 (m, 2H), 5.23 (s, 2H), 5.18 (s, 2H), 4.79 (s, 2H); HRMS calcd for C$_{22}$H$_{18}$NO$_2$ 328.3759, found 328.3763.

2-Butyl-5,7-dihydro-furo[3,4-b]pyridine-4-methanol (183). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.00 (s, 1H), 5.17 (s, 2H), 5.03 (s, 2H), 4.68 (s, 2H), 2.79 (t, $J$=7.8 Hz, 2H), 1.72-1.68 (m, 2H), 1.42-1.25 (m, 2H), 0.94 (t, $J$=7.8 Hz, 3H); HRMS calcd for C$_{12}$H$_{18}$NO$_2$ 208.2689, found 208.2680.
2-Phenyl-5,7-dihydro-furo[3,4-b]pyridine-4-methanol (184). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.97-7.92 (m, 2H), 7.56 (s, 1H), 7.45-7.43 (m, 3H), 5.23 (s, 2H), 5.12 (s, 2H), 4.72 (s, 2H); HRMS calcd for C$_{14}$H$_{14}$NO$_2$ 228.2585, found 228.2591.

2-t-Butyl-5,7-dihydro-furo[3,4-b]pyridine-4-methanol (185). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.17 (s, 1H), 5.18 (s, 2H), 5.05 (s, 2H), 4.69 (s, 2H), 1.36 (s, 9H); HRMS calcd for C$_{12}$H$_{18}$NO$_2$ 208.2689, found 208.2700.

2,3-Diphenyl-5,7-dihydro-furo[3,4-b]pyridine-4-methanol (186). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.27-7.23 (m, 4H), 7.15-7.13 (m, 4H), 7.07-7.04 (m, 2H), 5.43 (s, 2H), 5.15 (s, 2H), 4.54 (s, 2H); HRMS calcd for C$_{20}$H$_{18}$NO$_2$ 304.3545, found 304.3551.

(2-Butyl-7,8-dihydro-5H-pyrano[4,3-b]pyridin-4-yl)methanol (187). $^1$H NMR (300 MHz, CDCl$_3$) δ 7.08 (s, 1H), 4.73 (s, 2H), 4.58 (s, 2H), 4.04 (t, $J$=5.7 Hz, 2H), 3.00 (t, $J$=5.7 Hz, 2H), 2.75 (t, $J$=7.5 Hz, 2H) 1.71-1.63 (m, 2H), 1.49-1.36 (m, 2H), 0.93 (t, $J$=7.5 Hz, 3H); HRMS calcd for C$_{13}$H$_{20}$NO$_2$ 222.2955, found 222.2949.

(2-Phenyl-7,8-dihydro-5H-pyrano[4,3-b]pyridin-4-yl)methanol (188). $^1$H NMR (300 MHz, CDCl$_3$) δ 8.11 (d, $J$=8.2 Hz, 2H), 7.69 (s, 1H), 7.45-7.83 (m, 3H), 4.81 (s, 2H), 4.63 (s, 2H), 4.15 (t, $J$=5.8 Hz, 2H) 3.18 (t, $J$=5.8 Hz, 2H); HRMS calcd for C$_{15}$H$_{16}$NO$_2$ 242.2851, found 242.2850.
(2-t-Butyl-7,8-dihydro-5H-pyrano[4,3-b]pyridin-4-yl)methanol (189). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.23 (s, 1H), 4.78 (s, 2H), 4.60 (s, 2H), 4.04 (t, $J=5.7$ Hz, 2H), 3.77 (t, $J=5.7$ Hz, 2H), 4.15 (t, $J=5.8$, 2H) 1.38 (s, 9H); HRMS calcd for C$_{13}$H$_{20}$NO$_2$ 222.2955, found 222.2957.

(2,3-Diphenyl-7,8-dihydro-5H-pyrano[4,3-b]pyridin-4-yl)methanol (190). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.38-7.06 (m, 10H), 5.03 (s, 2H), 4.25 (s, 2H), 4.72 (t, $J=5.7$ Hz, 2H), 2.68 (t, $J=5.7$ Hz, 2H); HRMS calcd for C$_{21}$H$_{20}$NO$_2$ 318.3811, found 318.3807.

2,3-Dihydro-5-phenyl-1H-pyrrolo[3,4-c]pyridin-6(5H)-one (192). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 7.83 (s, 1H), 7.56-7.42 (m, 5H), 6.74 (s, 1H), 4.68 (s, 2H), 4.56 (s, 2H); HRMS calcd for C$_{13}$H$_{13}$N$_2$O$_2$ 213.2472, found 213.2470.

2,3-Dihydro-5-butyl-1H-pyrrolo[3,4-c]pyridin-6(5H)-one (193). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 7.74 (s, 1H), 6.50 (s, 1H), 4.47 (s, 2H), 4.43 (s, 2H) 3.99 (t, $J=7.8$ Hz, 2H), 1.71-1.65 (m, 2H), 1.38-1.27 (m, 2H) 0.93 (t, $J=7.8$ Hz, 3H); HRMS calcd for C$_{11}$H$_{17}$N$_2$O$_2$ 193.2575, found 193.2581.

2,3-Dihydro-5-cyclohexyl-1H-pyrrolo[3,4-c]pyridin-6(5H)-one (194). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 7.84 (s, 1H), 6.50 (s, 1H), 4.45 (s, 2H), 4.40 (s, 2H) 4.29 (m, 1H), 1.98-1.19 (m, 9H), 1.38-1.27 (m, 2H) 0.93 (t, $J=7.8$ Hz, 3H); HRMS calcd for C$_{13}$H$_{19}$N$_2$O$_2$ 219.2948, found 219.2951.
N-(2,3-Dihydro-5-isopropyl-1H-pyrrolo[3,4-c]pyridin-6(5H)-ylidene)propan-2-amine (195). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 8.24 (s, 1H), 7.31 (s, 1H), 4.70 (s, 2H), 4.68 (s, 2H) 4.09-3.98 (m, 2H), 1.43 (d, $J$=6.3 Hz, 6H), 1.29 (d, $J$=6.3 Hz, 6H); HRMS calcd for C$_{13}$H$_{21}$N$_3$ 220.3259, found 220.3259.

N-(2,3-Dihydro-5-cyclohexyl-1H-pyrrolo[3,4-c]pyridin-6(5H)-ylidene)propan-2-amine (196). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 8.30 (s, 1H), 7.34 (s, 1H), 4.69 (s, 2H), 4.59 (s, 2H) 3.75-3.59 (m, 2H), 2.00-1.26 (m, 20H), 1.29 (d, $J$=6.3 Hz, 6H); HRMS calcd for C$_{19}$H$_{30}$N$_3$ 300.4357, found 300.4350.

2,3-Dihydro-5-phenyl-1H-pyrrolo[3,4-c]pyridine-6(5H)-thione (212). $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 7.79-7.23 (m, 7H), 3.99 (s, 2H), 3.97 (s, 2H); HRMS calcd for C$_{13}$H$_{13}$N$_2$S 229.3128, found 229.3133.

Additional Compounds. Compounds 140, 142, 144-149, and 154-161 were prepared in collaboration with Dr. Lakshminath Sripada. Compounds 206-209 were obtained as mixtures and characterized by $^1$H NMR and LC/MS. Analytical data for compounds 216-219 was identical to literature reported values. $^{81}$
CHAPTER 3: DEVELOPMENT OF BIOLOGICAL SCREENS FOR SMALL MOLECULE LIBRARIES

Having successfully developed a methodology to rapidly access various biologically relevant core structures and having assembled collections of these compounds, we became interested in assessing their activity in a biologically relevant context. The library approach to drug discovery is fundamental to the development of new therapeutics for a plethora of disease states. Within the pharmaceutical industry, high throughput screens are the most viable technology for the elucidation of new drugs from compound libraries. While the compounds in the initial library are important, the success of the screens is also determined via the inception of meaningful assays. Thus, the development of novel biological screens coupled with the preparation of new compounds is imperative to the discovery of new therapeutic agents.

It is estimated that virtually all drugs on the market target less than 500 biomolecules, which only a fraction of the overall components of the cell. However, many of the current screening assays skew drug discovery towards receptor proteins (Figure 3.1). As a result, we became interested in the development of screens to target biological questions not typically investigated in conventional screening processes.
Specifically, we were interested in the discovery of small-molecule regulators of microRNA pathways, as well as modifiers of developmental pathways. Both of these targets are interesting in the context of drug discovery; however have yet to be extensively explored due to the scarcity of viable screening protocols. MicroRNAs represent increasingly interesting drug targets, as their misregulation has been correlated to a variety of diseases;\textsuperscript{154} however, due to their relatively recent discovery and the bias of screens assays towards proteins, no small molecule regulators have been discovered. Additionally, due to the lack of knowledge of specific targets in the field of developmental disorders, it is difficult to devise adequate screening methodologies. As a result, we strive to develop new assays to address these biological problems, and ideally develop a better understanding of these important biological pathways.

As previously discussed, we developed a novel methodology for the preparation of carbo- and heterocyclic core structures. The biological relevance of these types of structures is apparent via a simple search of the Beilstein Database which contains 9,862,953 organic structures of which the vast majority are heterocyclic (5,480,018 structures, 56%) and
homocyclic/isocyclic (3,545,766 structures, 36%). Only 8% of the indexed structures are acyclic. Additionally, a substantial number of therapeutic drugs on the market possess these core structures, and thus it is our hope that our compound collection will yield several hit compounds in biological screens. Through the integration of our novel screening targets with our biologically relevant compound collection, our goal is the discovery of novel drug targets that arise from our synthesized compound libraries. Towards this goal, we have taken both a forward and reverse chemical genetics approach; developing whole-organism phenotypic screens as well as directed assays targeting specific components of biological systems. Herein we report the establishment and employment of these screens with both our compounds as well as commercially available libraries.

3.1 Forward Chemical Genetics: Establishment of a Phenotypic Screen in Xenopus Embryos

3.1.1. Introduction

As previously discussed, forward chemical genetics screens involve the incubation of compounds with an organism, followed by detection for phenotypic variance. These screens are useful in obtaining small molecule inducers, and ideally protein targets, for a variety of disease states and phenotypes; however, since compounds are simply incubated with the organism, it is extremely difficult to bias the screen towards a desired phenotype. The difficulty in screen biasing can primarily be attributed to the lack of knowledge of the protein target. As a result, they are most commonly employed as chemical rescue screens, involving the ability of the small molecule to restore wild type function to an organism possessing a disease phenotype. One major disadvantage of this approach is the often difficult nature of
correlating a phenotypic hit to its biomolecular target. While not as common as reverse chemical genetics screens, when successfully employed, forward chemical genetics screens can provide access to a small molecule that can be employed at a specific time-point and at a tunable concentration for the precise study of the observed phenotype.

Existing libraries have been sifted for bioactive compounds via a multitude of high throughput screening platforms, including assays for morphological phenotypes or biochemical activity in cell culture. These approaches have already uncovered numerous small molecules that have been used as tools for the study of cell biological processes, to uncover new signaling pathways, or to serve as lead compounds for drug development. In fact, this approach has been employed in the discovery of cholchicine as a microtubule inhibitor, the Hsp90 inhibitor geldanamycin, and the golgi disruptor Brefeldin A. Assays with cultured cells are limited in their ability to provide insight into complex biological processes that occur in the context of intact three-dimensionally organized tissues, such as organogenesis. As a result, a transition to small molecule screens in whole organisms has recently begun to be employed for this purpose. Promising studies have demonstrated that the phenotypes identified by chemical genetic screening strategies in can phenocopy known genetic disorders, suggesting that embryo screening may be a powerful alternative to conventional genetic investigations of developmental mechanisms.

Based on an active collaboration with Dr. Nanette Nascone-Yoder, we became interested in employing our compounds in forward chemical genetic screens using *Xenopus laevis* embryos. These types of screens for compounds with unknown bioactivity have been conducted previously in zebrafish embryos, but the transition to *Xenopus laevis* frog embryos has not been extensively studied. However, the *X. laevis* model system offers several
advantages over the zebrafish in that the embryos are relatively large (~1.3 mm) easily obtained in large numbers by in vitro fertilization techniques, can be grown in a single well of a multi-well plate, and will complete development to a tadpole with fully functional organs within a week, thus facilitating the screening of many compounds in a short period of time. The transparency of the tadpoles provides a means visualization of organ looping and rotation events in the developing organism at multiple stages of development. Convenienly, this process, and many others, is highly analogous to human development. Additionally, the *Xenopus* model system has served as a fundamental tool in the understanding of the mechanisms of early vertebrate development, including gastrulation, organogenesis, and nervous system development. Thus, perhaps the most unique advantage of the *Xenopus* system with respect to other aquatic vertebrate embryos, is the facility of physical manipulation at all stages of embryogenesis. For decades, classical experimental embryologists have performed explantations, tissue recombinations and organ cultures with *Xenopus* tissues. Such studies have afforded unparalleled insight into key morphogenetic processes involved in both *Xenopus* and human development. Despite the overwhelming advantages of using Xenopus embryos in small molecule screening efforts, the possibilities have been largely unexplored by the *Xenopus* community. In fact, to date only one study has been conducted using *Xenopus* for chemical genetic screens, employing previously selected compounds from a zebrafish screen. Of the seven compounds assayed, five yielded comparable pheonotypes, demonstrating that a chemical genetics screen in *X. laevis* embryos is possible. However, a true library screen with compounds of unknown biological activity has yet to be attempted. We suspect this is primarily due to the size of the embryos inhibiting high-throughput assays, limiting the number of compounds which can be screened.
Additionally due to the pseudotetraploid genome of the *X. laevis* embryos, genetic manipulations are difficult to perform to elucidate the genetic mechanism of action of hit compounds.\textsuperscript{166}

### 3.1.2. Assay Development and Compound Screening

Based on the previously described [2+2+2] cyclotrimerization methodology development, an array of compounds has been prepared and was subjected to a forward chemical genetics screen. A general screening protocol was developed in the Nascone-Yoder laboratory, and involved arraying compounds in a multi-well plate and exposing *Xenopus* embryos at different stages of development for different periods of time. After compound exposure the embryos were evaluated using a dissecting microscope for abnormalities in organ morphogenesis and other unique phenotypes. Compounds that produce interesting phenotypes were then further evaluated to examine their mechanism of action (Figure 3.2).

![Diagram of assay development and compound screening](image)

**Figure 3.2.** Novel compounds were screened for unique organ phenotypes in *Xenopus laevis* embryos.
The original screen was based on both the pyridine and benzene libraries previously synthesized on the solid support. Compounds were arrayed at 20 mM concentrations in DMSO, and then further diluted to 100 µM for the screen. In the case of pyridines formed by a multi-component reaction, mixtures of regioisomers were employed (Figure 3.3). Embryos were screened by the Nascone-Yoder laboratory in quadruplet to ensure statistical significance of the phenotypic scoring.

Figure 3.3. Initial 48 compounds screened in *Xenopus laevis* embryos. The heterotaxia inducing compound is highlighted in the blue box, and compounds affecting pigmentation are indicated by the yellow boxes.
The initial phenotypic screen afforded several promising compounds, including phenotypic alterations of pigmentation, tail elongation and eye shape (indicated by a yellow box in Figure 3.3). This result validates the biological relevance of our compound library, as indanes and isoindolines have previously been demonstrated to inhibit protein kinase activity, TNF-\(\alpha\) production, and smooth muscle relaxant activity.\(^{49, 50}\) However, the most interesting phenotype was elicited from compound \(114\) (blue box, Figure 3.3). This compound induces abnormal left-right asymmetric organ development with high penetrance, phenocopying a human birth defect known as heterotaxia.

Heterotaxia is a group of congenital disorders identifiable by the malrotation, malformation, or misplacement of vital organs relative to the left-right axis of the organism.\(^{167}\) All vertebrates normally develop morphological left-right asymmetries in their vital organs. In individuals with perturbed left-right asymmetry, the relative spatial orientations of asymmetric structures, such as the position of the heart or the liver, can either develop in a mirror image of their normal left-right configuration (\textit{situs inversus}), or be completely randomized, resulting in an uncoordinated arrangement of internal organs with “mixed up” left-right orientations (\textit{situs ambiguous} or heterotaxia), and other deformities.\(^{167}\) Interestingly, several deformities that are part of the heterotaxia syndrome, such as cardiac valvuloseptal defects and intestinal malrotations, are also commonly found in individuals with otherwise normal asymmetry at the rate of 1 in 100 to 1 in 500 births.\(^{168}\) This disorder often leads to the necessity for multiple surgeries, and potentially death in newborns.\(^{169}\) As a result, the discovery of a small molecule inducer of heterotaxia is advantageous as it provides a means to better understand this phenomenon by elucidating the biomolecular mechanism leading to this disorder. Given the better understanding of the biochemical causes of
heterotaxia, efficient diagnostic tests and therapeutics can be developed to alleviate this disabling phenotype.

To investigate the underlying mechanisms of abnormal left-right asymmetric organ development, we have begun to further characterize the biological effects of 114, which we have named heterotaxin. At a concentration of 200 µM of heterotaxin, a range of left-right heart deformities are developed, including both reversals of looping and isomeric hearts (Figure 3.4). Likewise, the gut morphogenesis phenotypes induced by heterotaxin include reversals of foregut looping and intestinal malrotations, as well as straight, unlooped isomeric gut tubes (Figure 3.6). Heterotaxin’s effects are highly penetrant, since 100% of embryos exhibited left-right defects in at least one organ at both 100 and 200 µM concentrations. Although some embryos with heart isomerisms induced by the higher concentrations of heterotaxin exhibit mild edema (the abnormal accumulation of fluid perhaps due to poor cardiac function), overall, the specificity of this chemically induced phenotype is quite high, as it does not seem to perturb axial elongation, or other aspects of development, even though embryos are exposed to the compound during gastrulation.

**Figure 3.4.** Left-right asymmetric heart defects induced by exposure to heterotaxin. The asymmetry of heart looping is either reversed (“reversal”) or lost (“isomerism”) in embryos treated with 114. Two examples of each phenotype are shown with arrows indicating the looping direction.
Due to the well studied development of *Xenopus*, their embryos can be treated at specific stages to elucidate the temporal effect of the active compound. Post-fertilization the embryo undergoes a series of cellular divisions to lead to the blastula at stage 8. This then undergoes gastrulation, or the migration of cells to establish the three primary germ layers and establish primary body axes, from stages 10-12. These stages are followed by neurulation in stages 13-15, establishing the central nervous system. Finally, stages 16-45 are characterized by organogenesis leading to the tadpole stage. This is followed by metamorphosis to the adult frog at stage 56 (Figure 3.5). To determine what stages of left-right asymmetric development are perturbed to cause these phenotypes, we exposed embryos to heterotaxin at successively later stages of development.

![Figure 3.5. Developmental stages of *Xenopus laevis* embryos.](image)

The Nascone-Yoder laboratory found that embryos exposed to 100 µM heterotaxin at stage 10-11 developed organ reversals and malrotations, while embryos of the same age exposed to a higher concentration (200 µM) of heterotaxin also developed isomerisms (Figure 3.6, Trial 1 and 2). Interestingly, when treatments were initiated at stage 18-19,
heterotaxin had no effect at 100 μM, but induced reversals and isomerisms at the higher concentration. Not surprisingly, embryos treated at stage 31-32 were insensitive at 100 μM (results of higher concentrations were not determined). Due to the establishment of symmetry axes during gastrulation (stages 10-12), this effect correlates to the activity of heterotaxin in the disruption of left-right differentiation (Figure 3.6). Although, these results reveal both stage- and concentration-dependence, these effects need to be further investigated at a finer scale to fully take advantage of the empirical control enabled by chemical genetics.

**Figure 3.6.** Left-right asymmetric gut defects induced by exposure to heterotaxin. Asymmetric gut morphology is perturbed in embryos treated with 100-200 μM heterotaxin at the indicated stages, while DMSO controls exhibit normal gut development. The results of two independent trials are shown for the stage 10-11 treatments. The “200 μM derivative” trial was performed with the indicated concentration of heterotaxin added at stage 11.

3.1.3. Optimization and Purification of Heterotaxin

While this chemical genetics screen was successful, several major issues towards the application of heterotaxin must be addressed. Perhaps most troubling is the fact that the initial screen employed a mixture of regioisomers of compound 114, and it is unclear if there is a single active regioisomer, or if the combination of isomers is required for biological activity. Additionally, the preparation of the compound on a solid-support was initially
achieved thermally, and required long reaction times to access very small quantities of compound. In order to further understand and explain the biological activity of this molecule, these problems needed to be addressed.

We first attempted to address the problems associated with the initial synthesis, as compound preparation required almost 3 days due to long reaction times. This can readily be solved via the application of our previously established microwave assisted cyclotrimerization conditions; however, we needed to confirm that the microwave irradiation does not affect regioisomeric ratios. Therefore, we conducted the experiment both thermally and in the microwave (300W, 10 min) and verified the regioisomeric ratio via $^1$H NMR spectroscopy. Gratifyingly, no substantial difference in the reaction regioselectivity was observed in the $^1$H NMR spectrum, affording the utilization of microwave irradiation in the synthesis, and subsequently reducing reaction times (Figure 3.7). It does appear that some new aromatic products form; however these have been determined to be benzenes, which can be easily separated from the mixture.

![Figure 3.7. Determination of regioisomeric ratios between synthetic conditions. a) $^1$H NMR of a mixture of 114 regioisomers prepared under thermal conditions. b) $^1$H NMR of a mixture of 114 regioisomers prepared under microwave conditions. No alteration in the regioisomeric ratio is observed.](image-url)
With the application of microwave irradiation greatly enhancing the reaction rate, we next focused on solving the issues associated with reaction scale. This issue was addressed by conducting multiple microwave preparations on 200 mg of resin. Following the reaction the resins were pooled for cleavage and neutralization with sodium bicarbonate. This afforded enough product to be separated via silica gel chromatography to attain an adequate separation of regioisomers. The individual compounds were analyzed by GC/MS and $^1$H NMR to identify regioisomers and confirm purity (Figures 3.8 and 3.9). Structures were assigned to regioisomers via both the multiplicity and chemical shift of the aromatic protons in conjunction with the known regioselectivity of the reaction. Compounds 114a and 114b both produce two doublets in the aromatic region, however, the shift of the propargylic methylene is shifted further downfield in 114b. In the case of 114c the aromatic region is distinctive with two singlets.
Figure 3.8. $^1$H NMR spectra of the three isolable regioisomers.

Figure 3.9. GC/MS analysis of the formation of heterotaxin. a) GC/MS of crude product mixture, indicating the formation of several regioisomers (retention times 16.03-17.57 min) and some benzene formation (retention time 12.12 min). b) Sample GC/MS after column chromatography of the most prevalent regioisomer (114c) demonstrating the purity of the compound.
The isolated regioisomers were then subjected to the previously described embryo assay to identify the active isomer. The regioisomer 114c was able to elicit the phenotypic effect that was observed with the mixture, whereas the other three regioisomers were inactive.

3.1.4. Structure Activity Relationship Study of Heterotaxin

With the discovery of heterotaxin as a compound capable of phenocopying heterotaxia, we were next interested in elucidating its mechanism of action, and ascertain if we could find compound analogs that may display greater activities. We first conducted a preliminary structure activity relationship study by varying the C-4 substituents, as well as the C-2 hydroxymethylene (Figure 3.10). Replacement of the C-4 butyl group with a hexyl substituent (232) led to embryonic toxicity at similar concentrations. Introduction of a pentyl chain (228) or an alkene (229) resulted in some toxicity, but some retention of activity. Shortening of the C-4 substituent to an ethyl group (230) or a hydroxymethylene (231) resulted in complete loss of activity.
Interestingly, we found that methylation of the hydroxy group (227) resulted in a retention of heterotaxin activity. This result is especially promising, as it suggests we may have found a functional handle for immobilization of the molecule on an affinity matrix. The immobilized compound can be incubated with a cellular lysate prepared from *Xenopus* embryos isolated at the developmental stage most susceptible to the effects of heterotaxin. Ideally, the target protein will bind the immobilized heterotaxin, affording the identification of the mechanism of action of the small molecule (Scheme 3.1). This process has been employed previously for the isolation of small molecule-protein interactions, and it is our hope that it is applicable to this interaction. With a protein target in hand, we will be better able to understand the biological mechanism of this compound, and ultimately the genetic basis of heterotaxia.
Scheme 3.1. Protocol for the immobilization of heterotaxin (114c) via pegylation to 233 and reaction with commercially available Affi-gel (Bio-Rad) to the immobilized analog 234. This is followed by incubation with Xenopus cellular lysate to afford a small molecule-protein complex 235, followed by resin washing and elution of protein for analysis.

3.1.5. Investigations Into the Biological Mechanism of Heterotaxin

Due to the technical difficulties associated with the isolation and identification of protein targets via incubation with cellular lysate, Dr. Nanette Nascone-Yoder is also investigating the biological mechanism of heterotaxin via a cellular approach. Due to the disruption in left-right differentiation, the Nascone-Yoder laboratory first chose to investigate several genes thought to be involved in asymmetric development.

The two sides of the embryo are first distinguished as “left” and “right” by the rotational beating of cilia at the embryonic node during gastrulation, which generates an asymmetric “nodal flow” to break bilateral symmetry. The small initial asymmetry becomes amplified via a reaction-diffusion based mechanism involving the left-side specific
expression of the TGF-β family members, *nodal* and *lefty*.\textsuperscript{171-173} The asymmetric expression of these molecules ultimately leads to the left-side specific expression of the transcription factor *Pitx2* on the left side of the developing organs that will actually become morphologically asymmetric structures, including the heart and gut.\textsuperscript{174}

Our collaborators employed *in situ* RNA hybridization experiments to investigate the expression of these important genes associated with left-right development. First, gastrulating embryos were exposed to heterotaxin or a DMSO control and subject to *in situ* hybridization with an RNA probe specific for *Xenopus Nodal (Xnr1)*. The Heterotaxin appeared to have a dramatic effect on the localization of *nodal* expression. The DMSO control treated embryos resulted in normal gene expression of *nodal*, localized on the left side of the embryo. Conversely, embryos treated with 114c, exhibited bilateral, left, right, and no expression of this gene. This lack of localization of *nodal* expression may substantially contribute to the heterotaxia phenotype observed when treated with 114c (Figure 3.11).
Figure 3.11. Demonstration of misregulation of the *nodal* gene in embryos treated with 114c. A) In control embryos treated with DMSO the normal expression of the *nodal* gene occurs on the left side of the embryo. B) No *nodal* expression is apparent on the right half of the control embryos. C) Some normal expression of *nodal* occurs on the left side of embryos treated with 114c; however some embryos do not possess the RNA transcript for the gene. D) Misregulation of heterotaxin embryos is clear on the right side of treated embryos, as bilateral expression and right-side expression is clear. E) The results demonstrate the mislocalization of this gene.

Similar results were observed with the *lefty* and *Pitx2* genes, which are also known regulators of left-right development. The misregulation of all three genes demonstrates the role of heterotaxin in the asymmetric development of the embryos. Via the delocalization of these key developmental genes, the correct right-left differentiation of the embryo is lost. Unfortunately, the signaling cascade for the regulation of these proteins is not completely elucidated, and thus the true molecular target of the small molecule is still evasive. It is suspected that the heterotaxin is ultimately interfering with nodal function and expression, affording ectopic distribution of the protein. This results in activation of the nodal signaling cascade on both the left and right side of the embryo, and thus disrupts the proper left-right patterning (Figure 3.12). The Nascone-Yoder laboratory is currently investigating several key kinases thought to be involved in these pathways to determine if heterotaxin is altering their function.
In conclusion, we have successfully developed a forward chemical genetics screen in *Xenopus laevis*, and discovered a new compound capable of phenocopying a human birth defect. Future work must be conducted to better understand the mechanism of action of this compound *in vivo*, as well as elucidate its actual protein target. Additionally, other hit compounds from the screen must be explored for potential therapeutic properties.

### 3.2. Reverse Chemical Genetics: Development of Small Molecule miRNA Inhibitors

#### 3.2.1 Introduction

MicroRNAs (miRNAs) have recently emerged as an important class of gene regulators, and their misregulation has been linked to a variety of cancers. Small molecule inhibitors of miRNAs would be excellent tools to elucidate the detailed mechanisms of...
miRNA function and provide lead structures for the development of new therapeutics. Thus, we aim to develop a cellular screen for miRNA pathway inhibitors and identify the first small molecule modifiers of miRNA function.

miRNAs are single-stranded noncoding RNAs of 21-23 nucleotides. They are a novel class of gene regulators that function by binding to the 3’ untranslated region of target messenger RNAs leading to either suppression of their translation or acceleration of their degradation.\textsuperscript{176-178} The majority of miRNAs are initially transcribed by RNA polymerase II as primary transcripts (pri-miRNAs) that require subsequent processing to yield a functional mature miRNA (Figure 3.13).\textsuperscript{179} Pri-miRNAs are processed in the nucleus by the enzyme Drosha, partnering with DGCR8 (in vertebrates) or Pasha (in invertebrates), thus transforming pri-miRNAs into shorter stem-loop-structured, double-stranded RNAs called precursor miRNAs (pre-miRNAs).\textsuperscript{180-182} Pre-miRNAs are then transported from the nucleus to the cytoplasm and are processed by Dicer into mature miRNAs.\textsuperscript{183-187} Mature miRNAs enter the effector complex, called the RNA-induced silencing complex (RISC), to then target single-stranded complementary mRNAs.\textsuperscript{188-190} It is estimated that miRNAs are involved in the regulation of about 30% of all genes and almost every genetic pathway.\textsuperscript{191} Moreover, recent evidence suggests that they can function as oncogenes and tumor suppressors.\textsuperscript{192-195} Due to these characteristics, small molecule inhibitors would be important tools to further elucidate the details of miRNA biogenesis and function. Moreover, small molecule regulation of deregulated miRNAs has the potential to provide a novel area of therapeutics. So far, specific miRNA inhibition has been only achieved by antisense nucleic acid approaches.\textsuperscript{196}
Figure 3.13. The endogenous miRNA pathway contrasted to the exogenous siRNA pathway.

Brain tumors are the leading cause of cancer-related death among children and the second leading cause of cancer-related death among males aged 20-39. Glioblastoma is the most aggressive and most common type of brain cancer, affecting approximately 40,000 people each year in the US. Only 10% of all patients diagnosed with glioblastoma survive more than two years after the initial diagnosis, while the majority of patients have a much shorter survival rate. Although surgery is difficult to perform on these cancers due to their
diffuse nature, it remains the first treatment option due to a lack of highly efficient chemotherapeutic agents for brain cancers.

Interestingly, the expression levels of several miRNAs differ considerably between normal tissue and malignant tissue from brain tumor patients, thus linking brain tumor development to miRNA function. The expression of miR-212, 026a, 150, 152, 191, and 192 are upregulated in pituitary adenomas compared with normal pituitary, while miR-15 and 16 are downregulated.\textsuperscript{199, 200} The expression pattern of miRNAs can also be used to accurately predict the nature of clinical samples.\textsuperscript{201} Most importantly, suppression of miR-150, 152, 191, and 192 using antisense oligonucleotides inhibits cell growth in culture.\textsuperscript{202} In clinical glioblastoma samples, miR-21, 25, 9-2, 10, 130, 221, 125, and 123 are highly overexpressed, while miR-128 and 181 are downregulated.\textsuperscript{203} It has been demonstrated that knockdown of miR-21 induces cell death in glioblastoma cells by triggering the activation of caspases 3 and 7,\textsuperscript{201} making miR-21 a highly promising and novel target for the development of brain cancer therapy. Glioblastoma cells that are transduced with an miR-21 oligonucleotide suppressor do not grow or grow very slowly compared with their respective control cells in an ectopic mouse model, demonstrating that inhibition of miR-21 has high potential for the development of efficient chemotherapies. In this context, small organic molecules are highly desirable therapeutic agents over, for example, antisense oligonucleotides, peptides (e.g. aptamers), or proteins (e.g. antibodies), since small molecules are much more likely to have favorable pharmacokinetic properties and are more likely to pass the blood brain barrier.\textsuperscript{204}
3.2.2. Development and Validation of miRNA Screen

Our collaborator, Dr. Qihong Huang, developed an efficient screen with the potential to identify potential miRNA inhibitors. Based on the previously discussed mechanism of miRNA activity, mature miRNAs bind to their complementary sequences on mRNAs and either suppress the translation of the mRNAs or induce the degradation of the mRNAs (Figure 3.14). This assay exploits the miRNA pathway by cloning an miRNA binding sequence in the 3’ untranslated region of a luciferase reporter gene. Thus, in this assay, active miRNAs should bind sequence and downregulate the expression of the luciferase reporter.

![Diagram of miRNA binding and luciferase signal](image)

**Figure 3.14.** General assay for the discovery of small molecule miRNA inhibitors. Based on the assay, if a small molecule is capable of inhibiting miRNA function, and increased luciferase signal should be observed.

Hence, the luciferase-complementary sequence plasmids can serve as sensors to detect the presence of specific mature miRNAs (here, miR-21 and miR-30A). Any interference of the miRNA processing pathway, for example by small molecules, which results in the deficiency of mature miRNA processing can be detected using via quantitating the luciferase signal. The Huang laboratory generated HeLa cells that stably express Luc-miR-21, Luc-miR-30A, or a luciferase control (Luc-linker) reporter by infecting these cells with lentiviruses containing the reporter plasmids. HeLa cells express high levels of miR-21, but only low levels of miR-30A, as both others and we have previously demonstrated. As a result, they cloned miR-30 into a lentiviral expression vector and infected HeLa cells that already contained the reporter.
In order to validate the specific detection of one miRNA, Luc-miR-21, Luc-miR-30A, and Luc-linker (control) were introduced into HeLa cells by viral infection. The specificity of the reporter system was tested by assaying cells that contained both the Luc-miR-30A reporter and a construct expressing exogenous miR-30. These cells displayed a much lower luciferase signal than cells with a mismatched Luc-miR-30A reporter/miR-21 combination (Figure 3.15). This demonstrates that the Luc-miR-21 and Luc-miR-30A reporters are highly specific and only react to miR-21 and miR-30, respectively.

The reporter systems were then analyzed for their ability to detect endogenous miRNAs. The Luc-miR-21 reporter, stably introduced into HeLa cells, displayed a 90% decreased luciferase signal in comparison to the control luciferase-linker construct, visualizing the high level of matching endogenous miR-21 expression in HeLa cells (Figure 3.15); Additionally, in the case of the miR-30A reporter, only a modest decrease is detected, since HeLa cell express only low levels of endogenous miR-30A (Figure 3.16). These experiments demonstrate that the reporter systems can be used for the detection of endogenous miRNAs and are specific enough to differentiate between different miRNAs.
With the assay constructed and validated, we were able to begin to screen for miRNA inhibiting compounds.

![Graph showing luminescence CPS for different constructs.]

**Figure 3.16.** Assessment of the developed screen to detect endogenous miRNA. Due to the endogenous expression of miR-21 in HeLa cells, the construct possessing the miR-21 binding sequences displayed a significantly lower signal than the other constructs.

3.2.3. Screening of Compound Libraries to Discover a Specific miRNA21 Inhibitor

The compound screen was initiated with a primary screen of >1000 compounds from our own compound collection, consisting of the previously described 96 well plate (Figure 3.3) and the Library of Pharmacologically Active Compounds (Sigma-Aldrich). All compounds were stored at a 10mM concentration in DMSO to keep the DMSO concentration in the actual screen at 0.1%, which has minimal effects on the cells.

Compounds were screened at a 10 µM concentration and an initial hit compound 236, which restored luciferase activity, was discovered (Figure 3.17). This diazobenzene led to an increase of the luciferase signal by 251% compared to untreated cells (the DMSO control had no effect on the luciferase signal).
Figure 3.17. Identification of a compound by the developed miRNA screening assay that perturbs the miRNA pathway, resulting in an increased luciferase signal relative to the control.

In order to exclude the possibility that this compound may increase the luciferase signal through a non-specific mechanism, the compound was tested in HeLa cells expressing the Luc-linker, a control luciferase reporter. The diazobenzene 236 does not represent a false-positive hit, since it does not affect the luciferase signal in HeLa cells expressing the Luc-linker control, thus demonstrating that this compound is specific to the miRNA pathway (Figure 3.18).

Figure 3.18. Control assay demonstrating 236 does not affect the luciferase signal in HeLa cells expressing the control reporter.
Through several rounds of screening and structural modification a preliminary structure-activity relationship was developed (Figure 3.19). Chemical modifications of the amino group in 236 through acylation and alkylation led to diminished activities. Unfortunately, no compounds demonstrating a higher level of activity were determined, requiring further screening.

![Chemical structure](image)

**Figure 3.19.** Preliminary SAR study of the initial hit in the miRNA screen.

Fortuitously, this hit compound contained a diazobenzene core structure, a scaffold that has been thoroughly explored in our laboratory. This allowed us to rapidly conduct a secondary screen, to find a more active miRNA inhibitor. Again, compounds were treated at 10 µM concentrations for 48 hours, followed by luminescence measurements with a Bright-Glo Luciferase Kit (Promega). Compound activities are reported in fold activation relative to the DMSO control (Figure 3.20).
Figure 3.20. Compounds assayed to elucidate an improved miR21 inhibitor. The blue numbering indicates the fold increase in activity relative to a DMSO control. The most active compounds are indicated by blue and yellow shading.
This subsequent screen of structurally modified molecules (synthesized in collaboration with Dr. Dennis Xiong) related to the azobenzene core structure delivered the highly active compound 237 (5-fold increase of the luciferase signal at 10 µM.). Additionally, two other compounds 238 and 239 were found to have substantial activity within the established screen. This led to a more intensive structure-activity relationship study. Other molecules derived from 237 through introduction of an amino or nitro group in the 4’ position led to 12% or 64% reduced activity, respectively (Figure 3.21). Additionally, investigated amide substituents displayed a substantial loss of activity (24-53%), with the exception of allyl and propyl groups, which showed 11% and 16% lower activity, respectively. An exchange of the amide group for a sulfonamide group delivered compounds with no activity and, interestingly, the styrene analog of 237 had a 40% lower activity (Figure 3.21).
Figure 3.21. Structure activity relationship studies for the most active core molecules found in the secondary screen.

Thus far, 237 is the most effective small molecule inhibitor of microRNA miR-21 function inducing a 485% increase in the luciferase reporter signal at 10 µM. The diazobenzene 237 does not display any cytotoxic effects at this concentration as determined by an MTT assay. Dose response studies from 0-10 µM revealed a concentration dependence of the luciferase signal with an EC$_{50}$ of 2 µM (Figure 3.22).
Several experiments were conducted in order to investigate the mode of action of the inhibitor 237. The compound does not affect the luciferase signal in HeLa cells expressing the Luc-linker control with a random miRNA target sequence (Figure 3.23a), thus indicating that it does not increase the luciferase signal through means other than inhibiting the miRNA pathway. Furthermore, HeLa cells stably expressing both the miR-30 luciferase reporter construct and miR-30 were treated with 237. In this case, no increase of the luciferase signal was detected (Figure 3.23a), demonstrating that 237 is somewhat specific towards miR-21 and does not have a general effect on the miRNA pathway.
Figure 3.23. Determination of the specificity and mode of action of the active compound 237. A) Performing the assay with the different miR-luciferase constructs demonstrate that the increase in luciferase signal is specific to the knockdown of miR-21. B) RT-PCR quantitation of the miRNA levels for miR-21, primary miR-21 and two other miRNAs.

The specificity of 237 for the inhibition of miR-21 function was further validated by measuring the intracellular miRNA levels via quantitative RT-PCR (Figure 3.23b). The Huang laboratory found that levels of the stably expressed, exogenous mature miR-30 and the endogenous mature miR-93 are not reduced by treatment with 237 (relative to DMSO) (Figure 3.23b). Gratifyingly, miR-21 expression is reduced by 78% compared to the DMSO control in HeLa cells. Quantitative RT-PCR experiments with primers selective for the primary miR-21 (pri-miR-21) sequence but not mature or precursor miR-21 revealed that the pri-miR-21 levels in cells treated with 237 were reduced by 87% (Figure 3.23b). Similar observations were also made in three other cell lines, MCF-7, A172, and MDA-MB-231, which endogenously express miR-21 (Figure 3.24). These results strongly suggest that compound 237 is an inhibitor that targets the transcription of the miR-21 gene into pri-miR-21, but not downstream processes of the common miRNA pathway.
3.2.4 Induction of Apoptosis in Glioblastoma Cells Via miRNA Inhibitory Compounds

We were next interested in the therapeutic properties of 237, specifically its ability to induce apoptosis in the A172 glioblastoma cell line. Unfortunately, compound 237 was unable to stimulate cell death at a 10 µM concentration. As a result, we obtained the NIH/NCI COMBO screening plate, which contains 77 compounds, 23 of which are FDA-approved anti-cancer drugs. Many of these compounds have known mechanisms of action, including tubulin binding, DNA damage, Hsp90 binding, topoisomerase inhibition, angiogenesis, and kinase inhibition. Based on previous studies, which indicate the potential to induce apoptosis in cancer cells using an miR-21 antisense agent in combination with similar anti-cancer therapeutics,205 we hypothesized that it may be feasible to induce apoptosis via combining 237 with a low dose of one of these compounds.
The A172 cell line was screened with 10 µM compound 237 or a DMSO control in the presence of 10-fold lower concentration than the EC50 of each compound in the COMBO plate. Initial confluence of cells was recorded, as well as final confluence after 48 hours of incubation, to ensure a precise measurement of cell death. After 48 hours, the cells were subjected to a Cell-Titer Blue assay (Promega) to assess cellular viability. The cells were incubated for 3 hours with the resazurin compound from the assay kit, leading to its reduction (and corresponding shift in wavelength) in vivo from cellular processes occurring in viable cells. The cellular incubations were then measured for absorbance at 570 nm to elucidate the viability. From this initial screen, three compounds were found to be able to induce cell death at concentrations below their EC50 in the presence of 237 (Figure 3.25). While other compounds in the plate also induced a decrease in cellular viability, the reduced viability also occurred in control incubations in the absence of 237. The most promising compound was curcumin (240), which when treated with 237 reduced cellular viability 10-fold relative to its treatment with the DMSO control. Both fluorouracil (241) and doxorubicin (242) afforded an ~ 4-fold reduction in cellular viability; however, the doxorubicin in the absence of 237 generated a rounded cell morphology and led to some cytotoxicity relative to the other two compounds. As a result, only the curcumin and fluorouracil were taken forward in further studies.
Figure 3.25. Relative cell viability in the presence and absence of 237. Structures of the most active compounds are displayed above their activities. Compounds treated with doxorubicin (242), fluorouracil (241), or curcumin (240) displayed a substantial decrease in cellular viability in the presence of 222. Cell viability was assessed using a Cell Titer Blue assay with a 3 hour incubation, followed by a measurement of the absorbance at 570 nm.

We were next interested in assessing the functional range of the sensitization of the cancer cells to curcumin and fluorouracil by our miR-21 inhibitor 237. In order to achieve this, we treated the glioblastoma cells with variable concentrations of both 237 and the anti-cancer agent. The most effective results were observed when using 10 µM 237, and very effective cell death was observed at concentrations 1000-fold lower than the EC50 of fluorouracil (241). Ultimately, the largest degree of cellular death occurred at either 10-fold lower
concentrations (10 µM) than the EC50 of curcumin, or 100-fold lower concentration than the EC50 of flurouracil (0.5 µM) (Figure 3.26).

Figure 3.26. Survey of concentrations of 237 and anti-cancer agents 240 and 241 required to elicit cell death in the glioblastoma A172 cell line. Results from each well was conducted in triplicate.

While the Cell-Titer Blue assay is a useful measurement of cellular viability, the effects of the compounds can also be observed visually. The A172 glioblastoma cell line has a distinct morphology when adhered to the surface of the well (Figure 3.27A). When cells were treated with DMSO and curcumin (0.1 X, 10 µM), the cellular confuency and morphology remained normal (Figure 3.26A). However, upon the treatement of 237 (10 µM) with the same concentration of curcumin (0.1 X, 10 µM), a substantial alteration in cellular confuency and morphology was observed (Figure 3.27B). The rounding of cells and lack of
adherence correlates to the decreased activity in the Cell-Titer Blue assay, and also the expected cellular death.

Figure 3.27. Visual effects of cellular viability upon co-treatment of A172 cells with 237 and 240. A) Cells treated with DMSO and 240. B) Cells treated with 10 µM 237 and 240. Similar results were observed with a combination of 237 and 241.

Finally, we were interested in confirming that the induced cellular toxicity was specific to only the cancer cell lines overexpressing miRNA 21. Thus, standard human embryonic kidney (HEK 293T) cells were treated with 237 at 10 µM and different concentrations of the anti-cancer agents. Additionally, the A172 cell line was treated under similar conditions. After 48 hours of treatment, both cell lines were assayed for viability and visually inspected for confluency. Gratifyingly, under all treatment conditions, no distinguishable decrease in cellular viability was noted for the HEK 293T cell line, while the A172 cell line demonstrated comparable decreases in viability as previously observed (Figure 3.28). Additionally, no changes in cellular confluency could be noted in the 293T line between any of the treatment conditions. This suggests that the sensitization of the cells to anti-cancer agents by 237 is specific to only cell lines overexpressing microRNA-21.
Figure 3.28. Determination of cellular viability in different cell lines treated with combinations of 237 and anti-cancer agents 240 and 241 (240: 0.01X = 1 µM, 0.1X = 10 µM; 241: 0.001X = 0.05 µM, 0.01X = 0.5 µM). The A172 glioblastoma line displayed a decreased viability in response to compound treatments, while the non-cancerous HEK 293T cell line lost no significant cellular viability upon compound treatment.

In summary, a method to identify inhibitors of the miRNA pathway in live cells, specifically of miR-21, an important anti-apoptotic factor in several cancers, was developed. Screening of >1000 small organic molecules followed by a structure activity relationship analysis of an initial hit delivered the azobenzene 237 as a somewhat specific and efficient inhibitor of miR-21 expression. Research on miRNAs is still in its infancy and the biogenesis of many miRNAs (including miR-21) is not fully understood, thus specific inhibitors of the miRNA pathway (like 237) will be unique tools for the investigation of miRNAs and their involvement in the manifestation of various types of cancers in greater detail. Additionally, the therapeutic relevance of this miR-21 inhibitor was evaluated, and while the compound alone is unable to induce apoptosis in a glioblastoma cell line, it can be employed in
combination with known low dose of anti-cancer agents to decrease cellular viability. This effect is theoretically a result of knockdown of miR-21 expression leading to an increased sensitization of the cells to the cancer therapeutic, and is only active in the cancer cell lines, while in non-cancerous cell lines no effect is observed upon treatment with the compound.

3.2.5 Development of a miRNA-122 Small Molecule Assay

Given the success of our original screen, we became interested in developing a general approach for discovering small molecule inhibitors of any miRNA. Moreover, the screen should possess a transfection control and not require stable transfection. In order to achieve this we started with the psiCHECK-2 (Promega) expression plasmid which is commonly employed in the screening of siRNA. This plasmid expresses both Renilla luciferase as well as Firefly luciferase, and contains a multi-cloning site at the 3’ terminus of the Renilla gene. Typically, the gene of interest is cloned into this site, and active siRNA agents for the gene of interest lead to the degradation of transcript and silencing of the luciferase gene. We were interesting in exploiting this construct to insert the desired miRNA binding sequence into this 3’ UTR and prepare a dual-reporter system for miRNAs and their small molecule inhibitors (Figure 3.29).
Figure 3.29. The psiCHECK 2 dual reporter vector to be employed in the assay development. Ideally, by cloning in the miRNA binding sequence of interest into the 3’ UTR, the miRNA to be investigated will bind, silencing the Renilla luciferase gene. In the presence of a small molecule inhibitor, the knock-down of the Renilla luciferase signal should be inhibited, identifying active compounds. Images are adapted from www.promega.com

We were initially interested in generating an assay for small molecule miR-122 inhibitors using this approach. This miRNA is a liver specific miRNA, and is an interesting drug target as its inhibition by PS DNA antigomers has been found to be an effective treatment for high cholesterol. This antisense agent reduced miR-122 levels 90% and led to reduced cholesterol levels, a reduced rate of cholesterol synthesis, and no detectable off-target effects in a mouse model. Additionally, this miRNA has been found to play a role in Hepatitis C growth and proliferation, with inhibition of miRNA-122 leading to decreased viral replication. Therefore, this miRNA is extremely relevant in a therapeutic
context, and a small molecule inhibitor could be a useful agent in the management of these disorders.

We began by cloning either the miR-21 binding sequence (as a control) or the miR-122 binding sequence into the MCR of the psiCHECK-2 vector using the Pmel/SgfI restriction sites. Conveniently, due to the size of the insert, the DNA could be synthetically obtained, accounting for restriction sites, and hybridized which obviated the necessity for enzymatic digestion of insert. The presence of insert was confirmed via PCR screening and vector sequencing, the use of phosphorylated DNA was not necessary.

With the constructs in hand, we needed to assess the ability of endogenous miRNAs to inhibit the expression of the Renilla luciferase reporter gene. In order to do so, we transfected the psiCHECK-2/miRNA constructs into Huh7, HEK-293T, and HeLa cell lines. Huh7 cells are isolated from the liver, and have previously been demonstrated to over-express miRNA122, serving as an excellent system for investigations involving these miRNA. Additionally, it has been previously demonstrated that this specific miRNA is not expressed at all in HEK-293T cells, providing a negative control for the assay. As previously noted, HeLa cells overexpress miR-21, but should not possess elevated levels of miR-122. After a 24 hour incubation, the cells were assayed using a Dual-Luciferase Assay Kit (Promega), which affords the simultaneous assay of both the Renilla luciferase and control Firefly luciferase as a transfection control (Figure 3.30).
Figure 3.30. Dual Luciferase Assay of psiCHECK-miRNA constructs. A) Huh7 cell line B) 293T cell line C) HeLa cell line
The psiCHECK-miRNA constructs appeared to be functional in all three cell lines. Since Huh7 cells express miR-122, an expected drop in fluorescence was observed, correlating to the miRNA knocking down luciferase expression. Conversely, the 293T and HeLa cells do not possess this miRNA, and no silencing relative to the psiCHECK empty vector control is observed. Additionally, miR-21 is present in all three cell lines, and effectively reduces Renilla luciferase expression at a level that corresponds to its literature reported expression in the different cell lines.\textsuperscript{209,210}

Having established a miR-21 assay system, we were interested in comparing this to the miRNA screen established via the stable transfection of an alternate construct. Fortuitously, we already had \textsuperscript{237}, which was validated using the previously described assay, and could readily be employed with the psiCHECK-miR-21 vector. The HeLa cells were initially grown to 70\% confluence, followed by transfection with the psiCHECK-miR-21 vector. After a 4 hour incubation the transfection media was removed and replaced with standard growth media, supplemented with 10 μM \textsuperscript{237}. Cells were then incubated for 48 hours, and then quantitated using the Dual-luciferase assay (Figure 3.31).
Figure 3.31. Small molecule inhibition of miR-21 by 237 using the psiCHECK-miR-21 construct in HeLa cells.

The effect of 237 is not as prominent in this assay system relative to the previously describe assay, affording only a 163% increase in the luciferase system. Although repeated under several conditions, no substantial further increase in luciferase signal was obtained. We suspected this may be a result of the amount of luciferase transcript relative to the endogenous miR-21 levels, and attempted a similar experiment in the HEK 293T cell line which expresses less miR21, but observed a similar result. In order to increase the functional range of this assay, it may be necessary to alter the promoter strength in the plasmid in order to obtain a better ratio of transcript to miR-21. Additionally, the functional range of the assay also needs to be elucidated via the employment of an antisense agent to investigate maximum restoration of luciferase signal that can be obtained. Thus, future work must be done to characterize and optimize the assay output for the psiCHECK vector systems.

Once this has been accomplished it will be possible to screen a library of small molecules to identify inhibitors of miR-122 in Huh7 cells. Also, this assay could be employed towards the discovery of inhibitors for virtually any known miRNA. Overall,
future work will involve elucidating the specific targets of previously discovered inhibitors as well as assaying compounds in the miR-122 screen.

3.3 Application of [2+2+2] Cyclotrimerization Reaction to the Preparation of Biologically Relevant Fluorophores

3.3.1 Introduction

While we have had substantial success in the employment of our libraries in the development of novel therapeutics and developmental probes, we are also interested in the application of our [2+2+2] cyclotrimerization methodology towards the development of new biological diagnostic tools. Specifically, due to the convergence of our cyclotrimerization approach towards benzene and pyridine rings it can be envisioned that libraries of fluorescent molecules can be rapidly accessed. A fluorophore library can then be screened for specific cellular interactions, e.g. DNA binding, to afford environmentally sensitive cellular sensors and imaging agents. The combinatorial approach to fluorescent dyes is not a novel approach, and has employed in a variety of applications. Due to the difficulty in predicting fluorescent properties and biological interactions of small molecules, the screening of combinatorial libraries is one of the most efficient means to develop fluorescent stains. Significant work in this field has been performed by the Chang laboratory in both the preparation of fluorescent dye libraries, and their screens for biological relevance. To date this approach has been applied towards the staining of myotubes, amyloid fibrils, and the detection of cellular Hg(II) ions, to name a few; however, the preparation of novel libraries and cellular screens is still in high demand. One previously unexplored, yet biologically relevant fluorescent core structure is the anthracene moiety. Anthracenes are found in a variety of practical
applications, including potential therapeutics, optical devices, and polymeric materials. Most importantly, anthracenes have interesting fluorescent properties and have received attention as imaging agents for cellular processes.

As previously noted, we have developed a microwave-assisted [2+2+2] cyclotrimerization assay to rapidly access carbo- and heterocyclic structures. Utilizing this technology it is possible to rapidly assemble libraries of fluorophores based on an anthracene and an azaanthracene scaffold. Although several synthetic routes to anthracenes have been reported, the synthesis of 2-azaanthracenes is an undeveloped field. Moreover, access to these compounds are even more important as we discovered that the synthesized 2-azaanthracenes have very unique fluorescent properties in contrast to regular anthracenes.

3.3.2 Anthracene Methodology Development and Library Preparation

The preparation of anthracene and azaanthracene dyes was discovered during the preparation of anthraquinone libraries. The precursor was immobilized on a trityl chloride resin and cyclotrimerized to yield the reduced anthraquinone precursor; however, upon the acidic cleavage from the resin, instead of the desired diol, a product resulting from the elimination of one alcohol was obtained, which could then be reduced to the azaanthracene. The environmentally sensitive fluorescence properties of these compounds was immediately realized, and thus we initiated our investigation into these core structures. Unfortunately, the generality of this approach to both anthracenes and azaanthracenes was limited, and despite attempts at optimization, yields remained relatively low (Scheme 3.2), especially in the attempted synthesis of anthracenes.
In order to address the issues associated with the previously developed synthesis, we decided to explore the preparation of these anthracenes and azanthracenes beginning with the known diyne 249, which can be prepared in three steps from commercially available o-xylene dibromide with an overall yield of 72%. Due to the lack of immobilization handles of this substrate, we were required to employ solution phase cyclotrimerizations; however, based on previous results in our laboratory with similar diynes, we postulated that we could minimize chemoselectivity issues often associated in reactions conducted without a solid support by using high dilution conditions. The precursor 249 was cyclotrimerized with a set of six alkynes containing alkyl chains, benzenes, hydroxy groups, nitriles, and imides. These reactions were conducted with 10 mol% \((\text{PPh}_3)_2\text{Ni(CO)}_2\) in toluene at 120 °C under microwave irradiation (300W) in 10 min, delivering the tricyclic compounds 250-255 in 66-86% yield (Scheme 3.3). After the initial optimization of the reaction conditions, the anthracene derivatives were prepared by Yan Zou. Reactions conducted thermally in the absence of microwave irradiation displayed diminished yields (e.g. 35% in case of compound 252 under otherwise identical conditions). Byproducts observed in some reactions resulted from the dimerization of 249, as well as cyclotrimerization of 249 with two mono-alkyne molecules.
The anthracences 256-261 were generated in yields of 70-79% through a rapid microwave-assisted oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (Scheme 3.3). This two-step procedure provides a flexible and facile approach to the introduction of an anthracene moiety into a wide range of alkynes.

![Scheme 3.3. Anthracene synthesis via [2+2+2] cyclotrimerization](image)

We subsequently investigated the feasibility of this route towards the synthesis of 2-azaanthracenes, a mostly unexplored class of compounds. Here, the diyne 249 was reacted with nitriles bearing a variety of functional groups, including alkyl and alkyne chains, hydroxy groups, benzene, and pyridine rings. The reactions were conducted under CpCo(CO)_2 catalysis in toluene using microwave irradiation (300W) delivering the cyclotrimerization products 262-267 in 80-94%. The change in catalyst system was necessary in order to achieve cyclotrimerization reactions towards pyridines.\(^7e-k\) In the case of thermal heating, but in absence of microwave irradiation, very low product yields were obtained under otherwise identical conditions (e.g. 8% in case of compound 266). Moreover, the subsequent DDQ oxidation step proceeded smoothly and yielded the azaanthracences 245-247, and 268-270 in 53-85% yield. In order to investigate the effects of a permanently positively charged nitrogen center on the fluorescent properties of azaanthracenenes, and in
In order to increase their solubility in an aqueous environment, Yan Zou methylated the azaanthracenes 245-247, and 268-270 in neat methyl iodide at 60 °C to obtain the salts 271-276 in quantitative yields (Scheme 3.4).


3.3.3 Characterization and Biological Screening of Anthracene Based Library

A prominent feature of anthracenes is their intrinsic fluorescence;230, 231 a property which is largely unexplored in case of the corresponding 2-azaanthracenes. The developed array approach to both compound classes prompted us to investigate the fluorescent properties of the synthesized molecules, since combinatorial approaches to fluorophores have been successfully applied to biological imaging problems.214, 232, 233 We were especially interested in their application as environmentally sensitive probes, and therefore investigated the dependence of their fluorescent spectra on a) the polarity of the solvent, b) the pH of the solvent, and c) the presence of different metal cations. These experiments were conducted in a 96-well format (Figure 3.32).
While the anthracenes 256-261 did not show any significant changes in fluorescence under different conditions, many of the azaanthracenes 245-247, and 268-270 exhibited environmental sensitivity (Figure 3.32). This can be explained by the ability of the nitrogen center to undergo coordination to the solvent, to protons, and to metal ions. Moreover, the azaanthracenes 245-247, and 268-270 exhibited generally higher fluorescence levels than the anthracenes 256-261. A significant change in fluorescence intensity was observed for most of the azaanthracenes 245-247, and 268-270 between protic (H$_2$O at pH 4-10) and aprotic (DMSO) solvents (Figure 3.32). More drastic changes in fluorescence emission were visually observed in other non-polar solvents such as CH$_2$Cl$_2$ and toluene; however, these solvents were incompatible with the 96-well microtiter plates employed in the fluorescence measurements. Initial fluorescence experiments involved the irradiation of different compounds on a UVB Transilluminator (25 W) at 365 nm in the presence of different
organic solvents. While little solvent dependence was observed in the case of anthracenes, a drastic change in emission was observed for both the azaanthracenes and their quaternized analogs (Figure 3.33).

This phenomenon has previously been investigated for 2-methyl-azaanthracene (245), finding that the addition of a polar solvent causes a broadening of the $\pi\rightarrow\pi_1^*$ energy levels resulting in a bathachromic shift.\textsuperscript{234} The characterization of all dyes was performed under all conditions; however, only the general trends are presented herein (Figure 3.34). A loss of solvent sensitivity was observed upon quaternization of the nitrogen center in 271-276, and a moderate broadening of the emission spectrum occurred when compared to the non-quaternized analogs (Figure 3.34A). Additionally, a visible bathochromic shift in emission
could be detected upon quaternization from blue to green (see compounds 245-247, and 268-270 versus 271-276 in Figure 3.33). At low pH (<4), protonation of the azaanthracenes 245-247, and 268-270 lead to a general bathochromic shift (~480 to 520 nm) of their fluorescence emission spectra, similar to their quaternized analogs 271-276 (Figure 3.34B). No changes in fluorescence were observed between pH 7-10. As expected, the azaanthracene 270 demonstrated a fluorescence sensitivity towards metal ions, as a result of the chelating ability of the bipyridyl motif. An increase in the fluorescence intensity of 270 was observed in the presence of different divalent metal ions, suggesting a trend in binding affinity Cu$^{2+} <$ Mg$^{2+} \approx$ Zn$^{2+}$ (Figure 3.34C).

**Figure 3.34.** Fluorescence spectra (350 nm excitation wavelength) of A) representative azaanthracenes 245 and 256 demonstrating the effect of quaternization on the fluorescence properties in DMSO and H$_2$O; B) the representative azaanthracene 269 in the presence of different solvents and different pH; and C) the pyridylazaanthracene 270 in the presence of different divalent metal cations.

In addition to general environmental conditions, the assembled fluorophore array was also investigated for potential DNA intercalation. The azaanthracene structure is reminiscent
of several known DNA stains, possessing a planar heteroaromatic core. In structures such as ethidium bromide, the nitrogen center is quaternized to solubilize the dye and to increase its affinity for polyanionic DNA. This is evident as ethidium bromide displays a substantial increase in fluorescence emission upon DNA intercalation. To assess the alterations in fluorescent properties upon DNA binding all compounds were incubated with varying concentrations of commercially available salmon sperm DNA. The fluorescence spectrum was then measured (with a set excitation wavelength of 350 nm). Sample fluorescence spectra are displayed in Figure 3.35. In the case of compound 245, and other azaanthracenes a shift in fluorescence was observed in the presence of DNA from 515 nm to 450 nm (Figure 3.35A). Interestingly, in the case of most quaternized analogs a fluorescence quenching was observed, with no shift of wavelength (Figure 3.35B).

**Figure 3.35.** Effects of DNA intercalation on the fluorescence spectrum of selected fluorophores. A) Compound 245 in the presence of different concentrations of DNA. B) Compound 271 under identical conditions.

We also examined the application of the synthesized compounds for the fluorescent labeling of mammalian cells. Compounds 245-247, and 268-276 (20 μM) were incubated with human embryonic kidney (HEK-293T) cells overnight in standard DMEM growth media. To our surprise, compound 246, selectively forms ~200 nm sized particles within the
cells but not outside the cells, as confirmed by confocal microscopy using a Leica TSC SP1 laser scanning confocal microscope (Figure 3.36). By imaging the cells on varying planes of focus, the upper and lower membranes are apparent with the aggregates inside of the cell, not on the surface. No observable effect on the cell phenotype and the cell viability was observed (Promega Cell-Titer Blue assay).\textsuperscript{236} To the best of our knowledge, this is the first example of a small organic molecule that selectively forms a fluorescent crystal inside live cells.

**Figure 3.36.** A) HEK-293T cells incubated with azaanthracene \textit{246} (20 \textmu M) for 12 h were imaged at 365 nm UV irradiation (100\times magnification). B) Confocal microscopy image of azaanthracene \textit{246} crystallized inside a single human embryonic kidney cell (400\times magnification)

In summary, we have developed a rapid route to anthracenes and azaanthracenes via microwave assisted [2+2+2] cyclotrimerization reactions. These compounds have unique photochemical and biological properties and can act as environmentally sensitive dyes, metal sensors, pH sensors, and cellular stains. Future work will reveal further interesting phenomena, and lead to the application of these fluorophores in a biological context.
3.4 Experimental.

Cyclotrimerization towards Heterotaxin (114). Propargyl alcohol derivatized resin 104 (200 mg, 0.9-1.2 mmol/g substrate loading) was placed in a microwave reaction vessel and swelled in anhydrous toluene (3 mL) for 10 min. The 1-hexyne (10 eq.) and propionitrile (20 eq.) was added followed by CpCo(CO)$_2$ (0.1 eq.) and the reaction was irradiated in a CEM Discover microwave synthesizer for 10 min at 300 W. The vessel was removed and the resin was washed in a syringe filter with four alternating cycles of DCM and MeOH (2 mL ea.). The resin was dried in vacuo, transferred into a vial, and cleaved for one hour with 500 µL of 1% anhydrous HCl in 3:2 DCM/MeOH. The solution was filtered through a celite plug, concentrated, and neutralized with NaH$_2$CO$_3$ (aq.)/ether for 1 hour. The aqueous layer was extracted (3 X Et$_2$O), dried, concentrated and purified by silica gel chromatography (3:1 Hexanes/Ethyl Acetate to 1:1 Hexanes/Ethyl Acetate) to separate regioisomers. The fractions were then analyzed by $^1$H NMR and LC/MS.

Preparation of Heterotaxin Analogs. Prior to the elucidation of the active regioisomer, twelve analogs were prepared (Scheme 2.A.1) and screened for biological activity as the mixture of regioisomers. Derivatized resin (200 mg, 0.9-1.2 mmol/g substrate loading) was placed in a microwave reaction vessel and swelled in anhydrous toluene (3 mL) for 10 min. The soluble alkyne (10 eq.) and nitrile (50 eq.) was added followed by CpCo(CO)$_2$ (0.1 eq.) and the reaction was irradiated in a CEM Discover microwave synthesizer for 10 min at 300 W. The vessel was removed and the resin was washed in a syringe filter with four alternating cycles of DCM and MeOH (2 mL ea.) The resin was dried in vacuo, transferred into a vial,
and cleaved for one hour with 500 µL of 1% anhydrous HCl in 3:2 DCM/MeOH. The solution was filtered through a celite plug, concentrated, and neutralized with K₂CO₃ (aq.)/DCM for 6 hours. The organic layer was washed with water (2 x 1 mL), dried and incubated with DOWEX 50WX8-100 resin (~100 mg; pre-washed with 10 mL of DCM) for 2 hours at room temperature. The resin was transferred to a filter syringe and washed with DCM (5 x 3 mL), the collection tube was changed and the pyridine was eluted with 2 M NH₃/MeOH (5 x 3 mL). The flow through was then concentrated and the neutralized pyridines were analyzed by ^1H NMR for purity (32-76%).

Azobenzene-4-propargylamide (237). 4-Phenylazobenoic acid (30mg, 0.133mmol) was dissolved in DCM (1mL), followed by the addition of 1-ethyl-3-(3’-dimethylaminopropyl)carbodiimide (42mg, 0.22mmol) and hydroxybenzotriazole (21mg, 0.15mmol). Propargylamine (15mg, 0.27mmol) was added and the reaction was stirred at room temperature for 12 hours. The reaction was quenched with water (5mL) and extracted
with DCM (3 X 5mL). The organic layer was dried with sodium sulfate, concentrated and purified by silica gel chromatography (2:1 hexane/ethyl acetate) to yield an orange solid (29mg, 0.11mmol, 86%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.98-7.92 (m, 6H), 7.57-7.49 (m, 3H), 6.46 (bs, 1H), 4.29 (dd, $J_1$ = 2.4 Hz, $J_2$ = 4.8 Hz, 2H), 2.13 (t, $J$ = 2.4, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 166.6, 154.6, 152.7, 135.6, 131.9, 129.4, 128.3, 123.4, 123.2, 79.5, 72.4, 30.2. HRMS Calcd for C$_{16}$H$_{14}$N$_3$O (MH$^+$): 264.1131, Found: 264.1135.

$^1$H NMR spectrum:
Cell Culture Protocol. Human Breast cancer cell lines MDA-MB-231 and MCF-7, Human glioblastoma cell line A172, and Human cervical cancer cell line HeLa (obtained from American Type Cell Collection, Manassas, VA) were grown in DMEM media (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml, Invitrogen). All cells were incubated at 37°C in a humidified chamber supplemented with 5% CO₂.

Screening protocol. HeLa cells were cultured in DMEM and 10% FBS. HeLa cells containing the Luc-miR-21 construct were passaged into a 96-well plate and incubated overnight at 37°C with 5% CO₂. After reaching ~70% confluency, media was removed and replaced with media (198 µL) supplemented with the test compound (20 µM; 2 µM of a 2

$^{13}$C NMR spectrum:
mM DMSO stock) and incubated for 48 hours at 37 °C with 5% CO₂. Luciferase signals were determined 48 hours after compound treatment using a Bright-Glo Luciferase Assay Kit (Promega). All incubations were performed in triplicate and normalized to a DMSO control.

**Determination of Cell Viability.** The Glioblastoma A172 and Human Embryonic Kidney 293T cell lines was cultured in standard Dulbecco’s modified Eagle’s media (DMEM; Hyclone), supplemented with 10% Fetal Bovine Serum (FBS; Hyclone) and 10% streptomycin/ampicillin (MP Biomedicals). Cells were passaged into 96 well plates (200 μL per well; ~1×10⁴ cells) and grown to ~80% confluency. The media was then removed and replaced with fresh media supplemented with the miRNA inhibitor compound (0 to 10 μM) and the NCI COMBO compound (0.001 to 0.1X of the EC₅₀ concentration, Figure 2.A.1) and incubated for 48 hours at 37 °C with 5% CO₂. Cell viability was then monitored via either the Cell-Titer Glo or the Cell-Titer Blue assay (Promega) in conjunction with visual inspection on an inverted compound microscope.
Construction and Screening of psiCHECK-miRNA Reporter Systems. The psiCHECK plasmid (1 µg; Promega) was doubly digested with Sgfl (Promega, 1 unit) and PmeI (New England Biolabs, 1 unit) in Promega Buffer C for 2 hours at 37 ºC, followed by enzymatic deactivation at 70 ºC for 20 minutes. The vector was then gel purified (1% Agarose), and extracted with a Qiagen Gel Extraction Kit. The miRNA constructs (Table 2.A.1) were hybridized via an initial heating step of 95 ºC for 3 minutes, followed by a gradual cooling to 4 ºC. The inserts were then ligated with the digested vector in a 10:1 ratio with T4 DNA Ligase (New England Biolabs, 1 unit) at 4 ºC for 16 hours. Ligated vectors were transformed into Top 10 cells and plated on Ampicillin plates. Colonies were selected and screened via PCR and sequencing (Sequencing Primer: 5’ GCTAAGAAGTTCCT 3’). Appropriate constructs (1 µg) were then transfected into the appropriate cell line (Huh7, HeLa, or 293T) at ~70% confluency using FugeneHD transfection reagent. Transfection media was removed.
after 4 hours and replaced with standard growth media (DMEM; 10% FBS; 1% Streptomycin/Ampicillin). Cells were then incubated at 37 °C, 5% CO₂ for 24 hours then assayed using a Dual Luciferase Kit (Promega), and quantitated on a Wallac luminometer.

In order to examine the effect of 237 with this assay system, HeLa cells were passaged into a 96 well plate, and grown to 60% confluence. The cells were then transfected with the psiCHECK-miR21 construct (1 µg) or the empty psiCHECK construct (1 µg) using the FugeneHD transfection reagent and OPTI-MEM media. The transfection media was removed after 4 hours and replaced with standard growth media supplemented with 237 (20 µM; 1% DMSO final concentration). Cells were incubated at 37 °C with 5% CO₂ for 48 hours, then analyzed with a Dual Luciferase Kit (Promega), and quantitated on a Wallac luminometer.

Table 2.A.1. Sequences for Synthetic miR target DNA

<table>
<thead>
<tr>
<th>DNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR122 Forward</td>
<td>5' CGCAGTAGAGCTCTAGTACAAACACCATTGTCA CACTCCAGTTT 3'</td>
</tr>
<tr>
<td>miR122 Reverse</td>
<td>5' AAACTGGAGTGTGACAATGGTGTTTGTACTAGAGCTCTACTGCGAT 3'</td>
</tr>
<tr>
<td>miR21 Forward</td>
<td>5' CGCAGTAGAGCTCTAGTTCAACATCAGTCTGATA AGCTAGTTT 3'</td>
</tr>
<tr>
<td>miR21 Reverse</td>
<td>5' AAACTAGTTATCAGACTGATGTTGAACTAGCTCTACTGCGAT 3'</td>
</tr>
</tbody>
</table>

The following protocol for the cyclotrimerization reactions toward 250-255 was used:

The diyne 249 (20 mg, 0.13 mmol), alkyne (1.3 mmol), dry toluene (4 ml) and (Ph₃P)₂Ni(CO)₂ (8.3 mg, 0.013 mmol) were added to a flame dried microwave vial equipped with a stir bar. The vial was flushed with nitrogen, capped with a microwave vial septum and irradiated for 10 min in a CEM Discover microwave synthesizer at 300W. After cooling, the volatiles were evaporated, and the crude mixture was purified by silica gel chromatography,
eluting with hexanes/EtOAc. Due to compound instability, the product was only characterized via NMR and directly subjected to the next reaction without HRMS measurement.

2-Butyl-9,10-dihydroanthracene (250): 23 mg, 75%; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.28 (m, 2H), 7.16 (m, 3H), 7.11 (s, 1H), 7.00 (d, \(J = 7.2\) Hz, 1H), 3.90 (s, 4H), 2.58 (t, \(J = 8.0\) Hz, 2H), 1.58 (m, 2H), 1.34 (m, 2H), 0.91 (q, \(J = 8.0\) Hz, 3H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 140.7, 136.9, 136.8, 136.5, 133.8, 127.4, 127.4, 127.2, 126.1, 126.0, 36.2, 35.8, 35.3, 33.9, 22.4, 14.0.

9,10-Dihydro-2,3-diphenylanthracene (251): 36 mg, 86%; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.37 (s, 2H), 7.32 (m, 2H), 7.21 (m, 9H), 7.12 (m, 5H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 141.7, 138.7, 136.7, 136.2, 130.1, 129.8, 128.0, 127.7, 126.5, 126.4, 36.0.

9,10-Dihydro-2-(hydroxymethyl)anthracene (252): 18 mg, 66%; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.29 (m, 4H), 7.17 (m, 3H), 4.66 (s, 2H), 3.93 (s, 3H), 1.60 (s, 1H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 142.8, 139.0, 137.3, 136.8, 136.5, 127.8, 127.6, 127.2, 126.9, 126.4, 125.1, 65.6, 36.4, 36.1.

4-(9,10-Dihydroanthracen-6-yl)butanenitrile (253): 24 mg, 74%; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.28 (m, 2H), 7.24 (t, \(J = 1.2\) Hz, 1H), 7.19 (m, 2H), 7.11 (bs, 1H), 7.00 (m, 1H), 2.75 (t, \(J = 7.2\) Hz, 2H), 2.29 (t, \(J = 7.2\) Hz, 2H), 1.96 (p, \(J = 7.2\) Hz, 2H); \(^{13}\)C NMR (75
MHz, CDCl3) δ 151.6, 137.7, 137.3, 136.9, 136.2, 127.9, 127.7, 127.6, 126.4, 126.4, 119.8, 36.4, 36.0, 34.2, 27.3, 16.6.

3-(9,10-Dihydroanthracen-6-yl)propan-1-ol (254): 24 mg, 78%; 1H NMR (300 MHz, CDCl3) δ 7.27 (m, 2H), 7.17 (m, 4H), 7.01 (dd, $J_1=7.6$ Hz, $J_2=1.4$ Hz, 1H), 3.90 (s, 4H), 3.65 (b, 2H), 2.68 (t, $J=7.8$ Hz, 2H), 1.80 (m, 2H), 1.26 (s, 1H); 13C NMR (75 MHz, CDCl3) δ 161.7, 160.7, 146.8, 146.0, 139.8, 137.1, 136.9, 134.4, 127.7, 127.6, 126.4, 126.3, 62.5, 36.4, 36.0, 34.6, 31.9.

2-(2-(9,10-Dihydroanthracen-6-yl)ethyl)isoindoline-1,3-dione (255): 39 mg, 86%; 1H NMR (300 MHz, CDCl3) δ 7.82 (d, $J=3.3$ Hz, 1H), 7.81 (d, $J=3.3$ Hz, 1H), 7.69 (d, $J=3.0$ Hz, 1H), 7.67 (d, $J=3.0$ Hz, 1H), 7.26 (m, 2H), 7.17 (m, 4H), 7.09 (dd, $J_1=7.8$ Hz, $J_2=1.5$ Hz, 1H), 3.89 (m, 6H), 2.96 (t, $J=6.9$ Hz, 2H); 13C NMR (75 MHz, CDCl3) δ 203.0, 168.4, 137.1, 136.9, 136.8, 136.0, 135.2, 134.1, 132.3, 128.1, 127.8, 127.6, 127.6, 126.8, 126.3, 123.4, 39.6, 36.4, 36.0, 34.5.

The following protocol for the cyclotrimerization reactions toward 262-267 was used:
The diyne 249 (20 mg, 0.13 mmol), nitrile (1.3 mmol), dry toluene (4 ml) and CpCo(CO)2 (1.56 µl, 0.013 mmol) were added to a flame dried microwave vial equipped with a stir bar. The vial was flushed with nitrogen, capped with a microwave vial septum and irradiated for 20 min in a CEM Discover microwave synthesizer at 300W. After cooling, the reaction was purified by silica gel chromatography, eluting with hexanes/EtOAc. Due to compound
instability, the products were only characterized via NMR and directly subjected to the next reaction without HRMS measurement.

5,10-Dihydro-3-methylbenzo[g]isoquinoline (262): 94%; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.57 (s, 1H), 7.42 (m, 4H), 7.24 (s, 1H), 4.05 (s, 4H), 2.69 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 155.8, 147.4, 146.0, 135.7, 134.8, 129.3, 127.7, 127.6, 126.5, 126.4, 121.8, 35.3, 32.3, 23.9.

5,10-Dihydro-3-propylbenzo[g]isoquinoline (263): 87%; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.42 (s, 1H), 7.27 (m, 2H), 7.19(m, 2H), 7.06(s, 1H), 3.89 (s, 4H), 2.72 (t, $J$ = 7.5 Hz, 2H), 1.73 (m, 2H), 0.95 (t, $J$ = 7.5 Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 160.1, 147.8, 146.0, 136.0, 135.2, 129.7, 128.0, 126.7, 126.6, 121.5, 40.2, 35.6, 32.6, 23.5, 14.1.

2-(5,10-Dihydrobenzo[g]isoquinolin-3-yl)ethanol (264): 83%; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.38 (s, 1H), 7.36 (m, 4H), 7.08 (s, 1H), 4.00 (t, $J$ = 5.4 Hz, 2H), 3.89 (s, 4H), 2.97 (t, $J$ = 5.7 Hz, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 158.3, 147.3, 146.7, 135.6, 134.9, 130.5, 127.9, 127.9, 126.8, 126.7, 122.1, 62.2, 38.8, 35.6, 32.6.

5,10-Dihydro-3-vinylbenzo[g]isoquinoline (265): 90%; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.42 (s, 1H), 7.22 (m, 5H), 6.77 (dd, $J_1$ = 10.8 Hz and $J_2$ = 17.2 Hz, 1H), 6.13 (d, $J$ = 17.2 Hz, 1H), 5.40 (d, $J$ = 10.8 Hz, 1H), 3.89 (s, 4H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 153.6, 147.9, 136.8, 135.3, 134.6, 131.3, 127.7, 127.6, 126.6, 126.5, 119.7, 117.3, 35.4, 32.5.
5,10-Dihydro-3-phenylbenzo[g]isoquinoline (266): 87%; $^1$H NMR (400 MHz, CDCl$_3$) δ 8.59 (s, 1H), 7.98 (s, 1H), 7.96 (s, 1H), 7.63 (s, 1H), 7.46 (t, $J = 7.6$ Hz, 2H), 7.39 (d, $J = 6.4$ Hz, 1H), 7.30 (m, 2H), 7.23 (m, 2H), 3.97 (m, 4H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 155.6, 148.4, 146.5, 139.7, 135.6, 135.0, 131.1, 128.9, 128.8, 128.0, 127.9, 127.0, 126.8, 126.7, 119.4, 35.8, 32.7.

5,10-Dihydro-3-(pyridin-2-yl)benzo[g]isoquinoline (267): 80%; $^1$H NMR (400 MHz, CDCl$_3$) δ 8.66 (d, $J = 4.4$ Hz, 1H), 8.58 (s, 1H), 8.36 (d, $J = 8.0$ Hz, 1H), 8.32 (s, 1H), 7.79 (dt, $J_1 = 7.6$ Hz, $J_2 = 1.6$ Hz, 1H), 7.30 (m, 3H), 7.22 (m, 2H), 4.04 (s, 2H), 4.02 (s, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 156.3, 154.0, 149.0, 147.8, 146.5, 136.9, 135.2, 134.8, 132.6, 127.8, 126.6, 123.5, 121.0, 119.6, 35.5, 32.6.

The following protocol for the oxidation reactions toward 256-261, 245-247 and 268-270 was used: The precursors 250-255, and 262-267 (0.10 mmol), DDQ (27 mg, 0.12 mmol), and dry toluene (2 ml) were added to a flame dried microwave vial equipped with a stir bar. The vial was flushed with nitrogen, capped with a microwave vial septum and irradiated for 5 min in a CEM Discover microwave synthesizer at 300W. After cooling, the crude mixture was purified by silica gel chromatography, eluting with hexanes/EtOAc to yield the anthracene products 256-261. The azaanthracenes 245-247 and 268-270 were obtained by adding EtOAc (10 mL) to the reaction mixture which was subsequently washed with NaHCO$_3$ (3 × 5 mL), dried with MgSO$_4$, and concentrated under reduced pressure to yield
the products in high purity. The analytical data for 256-258 was identical with literature reports.237, 238

4-(Anthracen-6-yl)butanenitrile (259): 74%; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.38 (s, 1H), 8.35 (s, 1H), 7.98 (m, 3H), 7.46 (s, 1H), 7.45 (m, 2H), 7.27 (dd, $J_1$ = 8.9 Hz, $J_2$ = 1.5 Hz, 1H), 2.96 (t, $J$ = 7.2 Hz, 2H), 2.36 (t, $J$ = 7.1 Hz, 2H), 2.12 (p, $J$ = 7.2 Hz, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 136.7, 132.2, 131.9, 131.7, 130.8, 129.0, 128.4, 128.3, 126.8, 126.3, 125.9, 125.7, 125.5, 119.9, 34.9, 26.6, 16.7; HRMS calcd for [M + H]$^+$ C$_{18}$H$_{16}$N$_2$ 246.1283, found 246.1281.

3-(Anthracen-6-yl)propan-1-ol (260): 70%; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.37 (s, 1H), 8.33 (s, 1H), 7.96 (m, 3H), 7.76 (s, 1H), 7.43 (m, 2H), 7.32 (d, $J$ = 8.8 Hz, 1H), 3.73 (t, $J$ = 6.4 Hz, 2H), 2.90 (t, $J$ = 7.6 Hz, 2H), 2.01 (m, 2H), 1.33 (s, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 138.9, 132.1, 131.6, 130.8, 128.5, 128.4, 128.3, 127.5, 126.2, 125.7, 125.5, 125.2, 62.5, 33.9, 32.6; HRMS calcd for [M + H]$^+$ C$_{17}$H$_{17}$O 237.12799, found 237.1279.

2-(2-(Anthracen-6-yl)ethyl)isoindoline-1,3-dione (261): 76%; $^1$H NMR (300 MHz, CDCl$_3$) δ 8.36 (s, 1H), 8.31 (s, 1H), 7.95 (d, $J$ = 3.0 Hz, 2H), 7.93 (s, 1H), 7.81 (m, 3H), 7.66 (m, 2H), 7.42 (m, 3H), 4.05 (t, $J$ = 7.7 Hz, 2H), 3.18 (t, $J$ = 7.7 Hz, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 203.1, 168.5, 135.1, 134.1, 132.3, 132.0, 131.7, 130.9, 128.8, 128.4, 128.3, 127.3, 127.3, 126.3, 125.9, 125.6, 125.4, 123.5, 39.1, 35.2; HRMS calcd for [M + H]$^+$ C$_{24}$H$_{18}$NO$_2$ 352.1338, found 352.1340.
3-Methylbenzo[g]isoquinoline (245): 75%; $^1$H NMR (400 MHz, CDCl$_3$) δ 9.41 (s, 1H), 8.53 (s, 1H), 8.24 (s, 1H), 8.03 (d, $J$ = 8.4 Hz, 1H), 7.97 (d, $J$ = 8.4 Hz, 1H), 7.51 (m, 3H), 2.71 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 154.2, 149.4, 134.6, 133.0, 132.0, 129.1, 128.2, 127.8, 127.5, 125.9, 125.5, 124.1, 117.5, 24.4; HRMS calcd for [M + H]$^+$ C$_{14}$H$_{12}$N 194.0970, found 194.0961.

3-Propylbenzo[g]isoquinoline (268): 85%; $^1$H NMR (300 MHz, CDCl$_3$) δ 9.43 (s, 1H), 8.53 (s, 1H), 8.27 (s, 1H), 8.03 (d, $J$ = 8.4, 1H), 7.97 (d, $J$ = 8.7 Hz, 1H), 7.56 (s, 1H), 7.49 (m, 2H), 2.93 (t, $J$ = 7.5 Hz, 2H), 1.87 (m, 2H), 1.02(t, $J$ = 7.5 Hz, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 154.3, 153.3, 134.6, 133.0, 132.0, 129.1, 128.2, 127.7, 127.5, 125.9, 125.8, 124.3, 117.1, 40.3, 23.1, 14.1; HRMS calcd for [M + H]$^+$ C$_{16}$H$_{16}$N 222.1283, found 222.1281.

2-(Benzo[g]isoquinolin-3-yl)ethanol (269): 53%; $^1$H NMR (400 MHz, CD$_3$OD) δ 9.41 (s, 1H), 8.72 (s, 1H), 8.42 (s, 1H), 8.12 (d, $J$ = 8.4 Hz, 1H), 8.06 (d, $J$ = 8.4 Hz, 1H), 7.78 (s, 1H), 7.57 (m, 2H); 4.00 (t, $J$ = 6.8 Hz, 2H), 3.15 (t, $J$ = 6.4 Hz, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 155.4, 150.3, 136.5, 135.6, 134.1, 133.8, 130.2, 129.4, 129.3, 129.1, 127.4, 125.7, 120.4, 62.7, 47.9, 41.7; HRMS calcd for [M + H]$^+$ C$_{15}$H$_{14}$NO 224.1075, found 224.1072.

3-Vinylbenzo[g]isoquinoline (247): 80%; $^1$H NMR (300 MHz, CDCl$_3$) δ 9.45 (s, 1H), 8.56 (s, 1H), 8.32 (s, 1H), 8.05 (d, $J$ = 8.4 Hz, 1H), 7.99 (d, $J$ = 8.4 Hz, 1H), 7.62 (s, 1H), 7.56 (m, 2H), 6.95 (dd, $J$ = 17.3 Hz and J=9.9, 1H), 6.44 (dd, $J$=17.3 and J=1.8, 1H), 5.49 (dd, J=9.9 and J=1.8, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 154.77, 147.34, 136.76, 134.75, 132.72,
132.36, 129.18, 128.31, 128.06, 127.75, 126.51, 126.29, 125.31, 118.03, 117.29; HRMS calcd for [M + H]^+ C_{15}H_{12}N 206.0970, found 206.0960.

**3-Phenylbenzo[g]isoquinoline (246):** 84%; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 9.56 (s, 1H), 8.59 (s, 1H), 8.40 (s, 1H), 8.17 (s, 1H), 8.16 (d, \(J = 5.6\) Hz, 2H), 8.06 (d, \(J = 8.4\) Hz, 1H), 8.02 (d, \(J = 8.4\) Hz, 1H), 7.55 (m, 4H), 7.43 (t, \(J = 7.2\) Hz, 1H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 154.6, 149.1, 139.8, 134.8, 133.0, 132.5, 129.2, 129.0, 128.6, 127.8, 127.7, 127.1, 126.3, 126.2, 125.5, 115.9; HRMS calcd for [M + H]^+ C_{19}H_{14}N 256.1126, found 256.1124.

**3-(Pyridin-2-yl)benzo[g]isoquinoline (270):** 81%; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 9.56 (s, 1H), 8.90 (s, 1H), 8.73 (d, \(J = 4.2\) Hz, 1H), 8.62 (s, 1H), 8.56 (m, 2H), 8.06 (t, \(J = 8.7\) Hz, 2H), 7.85 (t, \(J = 7.7\) Hz, 1H), 7.55 (m, 2H), 7.32 (t, \(J = 4.8\) Hz, 1H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 156.7, 154.4, 149.7, 147.8, 147.3, 137.3, 135.1, 134.8, 132.9, 129.2, 128.6, 127.9, 127.8, 127.5, 126.6, 125.2, 123.4, 121.5, 117.4; HRMS calcd for [M + H]^+ C_{18}H_{13}N\(_2\) 257.1079, found 257.1067.

**General methylation procedure to 271-276.** The azaanthracenes 245-247 and 268-270 (0.05 mmol) and MeI (32 \(\mu\)l, 0.5 mmol) were added to a flame-dried vial under nitrogen atmosphere. The reaction mixture was heated at 60 °C overnight. After cooling to room temperature, the solvent was removed under reduced pressure to yield the desired products 271-276 in quantitative yield.
2-Methyl-3-methylbenzo[g]isoquinolinum iodide (271): quant.; $^1$H NMR (400 MHz, DMSO-d6) $\delta$ 10.31 (s, 1H), 9.24 (s, 1H), 8.83 (s, 1H), 8.49 (s, 1H), 8.42 (d, $J = 8.4$ Hz, 1H), 8.32 (d, $J = 8.8$ Hz, 1H), 7.89 (t, $J = 7.4$ Hz, 1H), 7.80 (t, $J = 7.4$ Hz, 1H), 4.43 (s, 3H), 2.84 (s, 3H); $^{13}$C NMR (75 MHz, DMSO-d6) $\delta$ 154.6, 141.2, 137.0, 132.6, 132.4, 130.9, 129.6, 128.4, 128.1, 125.1, 124.5, 123.0, 45.8, 18.8; HRMS calcd for [M] $^+$ C$_{15}$H$_{14}$N 208.1126, found 208.1117.

2-Methyl-3-propylbenzo[g]isoquinolinum iodide (272): quant.; $^1$H NMR (400 MHz, DMSO-d6) $\delta$ 10.36 (s, 1H), 9.24 (s, 1H), 8.88 (s, 1H), 8.46 (s, 1H), 8.43 (d, $J = 8.8$ Hz, 1H), 8.32 (d, $J = 9.2$ Hz, 1H), 7.89 (t, $J = 7.8$ Hz, 1H), 7.80 (t, $J = 7.4$ Hz, 1H), 4.46 (s, 3H), 3.13 (t, $J = 7.8$ Hz, 2H), 1.88 (q, $J = 7.6$ Hz, 2H), 1.11 (t, $J = 7.6$ Hz, 3H); $^{13}$C NMR (75 MHz, DMSO-d6) $\delta$ 155.1, 144.1, 137.0, 132.7, 132.4, 131.5, 130.9, 129.6, 128.5, 128.1, 125.4, 123.4, 122.9, 45.5, 32.6, 20.5, 13.5; HRMS calcd for [M] $^+$ C$_{17}$H$_{18}$N 236.1439, found 236.1429.

2-(2-Methyl(benzo[g]isoquinolinum)-3-yl)ethanol iodide (273): quant.; $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 10.06 (s, 1H), 9.12 (s, 1H), 8.75 (s, 1H), 8.43 (s, 1H), 8.27 (d, $J = 8.4$ Hz, 1H), 8.22 (d, $J = 8.4$ Hz, 1H), 7.81 (td, $J_1 = 6.6$ Hz, $J_2 = 0.9$ Hz, 1H), 7.72 (t, $J = 6.6$ Hz, 1H), 4.52 (s, 3H), 4.05 (t, $J = 6.0$ Hz, 2H), 3.37 (t, $J = 6.0$ Hz, 2H); $^{13}$C NMR (75 MHz, CD$_3$OD) $\delta$ 154.8, 138.2, 134.6, 133.8, 132.5, 131.1, 129.5, 128.5, 128.4, 126.8, 125.7, 123.7, 120.6, 60.2, 45.8, 34.3; HRMS calcd for [M] $^+$ C$_{16}$H$_{16}$NO 238.1232, found 238.1230.
2-Methyl-3-vinylbenzo[g]isoquinolinum iodide (274): quant.; $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 10.12 (s, 1H), 9.19 (s, 1H), 8.86 (s, 1H), 8.71 (s, 1H), 8.32 (d, $J = 9.0$ Hz, 1H), 8.27 (d, $J = 8.4$ Hz, 1H), 7.89 (td, $J_1 = 6.6$ Hz, $J_2 = 1.2$ Hz, 1H), 7.78 (td, $J_1 = 6.6$ Hz, $J_2 = 1.2$ Hz, 1H), 7.27 (dd, $J_1 = 11.0$ Hz, $J_2 = 17.0$ Hz, 1H), 6.31 (dd, $J_1 = 17.0$ Hz, $J_2 = 0.6$ Hz, 1H), 5.96 (dd, $J_1 = 11.0$ and $J_2 = 0.6$, 1H), 4.52 (s, 3H); $^{13}$C NMR (75 MHz, DMSO-d$_6$) $\delta$ 155.6, 141.2, 137.6, 133.6, 133.4, 131.9, 131.8, 130.4, 129.3, 129.2, 128.7, 127.0, 125.6, 123.8, 123.1, 47.1; HRMS calcd for [M]$^+$ C$_{16}$H$_{14}$N 220.1126, found 208.1119.

2-Methyl-3-phenylbenzo[g]isoquinolinum iodide (275): quant.; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 11.65 (s, 1H), 9.59 (s, 1H), 8.57 (s, 1H), 8.14 (m, 3H), 7.76 (t, $J = 8.4$ Hz, 2H), 7.68 (t, $J = 8.4$ Hz, 2H), 7.59 (m, 3H), 4.50 (s, 3H); $^{13}$C NMR (75 MHz, DMSO-d$_6$) $\delta$ 155.7, 142.9, 138.3, 134.7, 134.1, 131.9, 131.3, 130.5, 129.9, 129.8, 128.9, 128.5, 126.4, 125.9, 123.8, 47.5; HRMS calcd for [M]$^+$ C$_{20}$H$_{16}$N 270.1277, found 270.1275.

2-Methyl-3-(pyridin-2-yl)benzo[g]isoquinolinum iodide (276): quant.; $^1$H NMR and $^{13}$C NMR not obtained due to solubility issues. HRMS calcd for [M]$^+$ C$_{19}$H$_{15}$N$_2$$^+$271.1230, found 271.1226.

**Fluorescence Measurements.** In order to quantitatively measure the fluorescence of the compounds in different environments, a 96 well plate was assembled at 20 µM concentrations of compound (diluted from a 1 mM stock in DMSO), and analyzed on a Molecular Devices Gemini EM microplate spectrofluorimeter. The solutions at different pH were prepared as 10 mM PBS solutions, and all salt solutions were prepared at 1 mM (pH
7.0, 10mM PBS Buffer). All solutions were added to the 96 well plate (180 µL) followed by addition of the prepared compounds. We selected 350 nm as our excitation wavelength as it provided reproducible fluorescence data within the detection limits of the instrument, and it represents a common excitation wavelength in laboratory instruments (e.g. transilluminators and hand-held UV lamps). Similar experimental setups were employed for the DNA intercalation studies, with DNA solutions prepared via serial dilutions (0 ng to 100 µg) in 10 mM PBS buffer (pH 7.0). Upon addition of the test compound the plates were incubated at room temperature for 2 hours prior to analysis.
**Cellular Assays.** Human Embryonic Kidney (HEK-293T) cells were passaged into two 96-well culture plate, and grown to 60% confluence in the presence of standard growth media (Dulbecco’s modified Eagle’s media (Hyclone) with 10% Fetal Bovine serum (Hyclone) and 10% streptomycin/ampicillin (MP Biomedicals). Cells were then treated in triplicate with 20 µM of compound (245-247, and 268-276; 1% DMSO final concentration) or 1% DMSO. Cells were then incubated at 37 °C (5% CO₂) for 24-48 hours and imaged on a Leica DM5000B compound microscope. Compounds demonstrating interesting phenotypes were then incubated in chamber slides and examined on a Leica TCS SP1 laser scanning confocal microscope to more accurately characterize their cellular location and interactions. Finally, to test cell viability, similar incubations were performed in a 96 well plate and then assayed with a Cell Titer Blue Kit (Promega; 48 hours after initial incubation), to confirm the lack of compound toxicity.
CHAPTER 4: APPLICATION OF MICROWAVE IRRADITATION TO THE ACTIVATION OF BIOLOGICAL PROCESSES

The value and relevance of microwave irradiation in organic chemistry has been extensively demonstrated by research in our laboratory, as well as in the laboratories of numerous others. However, the employment of microwaves towards biological applications is still an underdeveloped field. This extension to a more biological context is logical when considering the fundamental basis of microwave chemistry. Due to the electromagnetic field, dipoles orient with the oscillating electric field, creating molecular motion and generating thermal energy. While the dipoles of small organic molecules are significant enough to accelerate reactions, they are minimal compared to the dipoles which exist in biomacromolecules (Table 4.1). This suggests the potential to exploit microwave irradiation for the activation of enzymes (and potentially DNA) due to their propensity to align with the electric field.

**Table 4.1.** Dipole moments of biologically important systems.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Dipole Moment µ (Debye)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.8</td>
</tr>
<tr>
<td>Peptide Bond</td>
<td>3.7</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>170</td>
</tr>
<tr>
<td>Horse Serum Albumin</td>
<td>380</td>
</tr>
<tr>
<td>Horse Carboxy Hemoglobin</td>
<td>480</td>
</tr>
</tbody>
</table>

The helical structures found in both proteins and DNA result in substantial dipole moments, which can be oriented with the microwave field, potentially activating or deactivating their function (Figure 4.1). Microwaves have recently been exploited
extensively in the field of proteomics. They have been found to expedite protein digestion (trypsin), \(^{241}\) more effectively deglycosylate antibodies (PNGase F), increase the yield in N-terminal digests for Edman’s sequencing (PGAP), \(^{242}\) and increase reaction rates (lipases and polymerases). However, the extent of the microwave activation has yet to be fully established as almost all of these examples are conducted in aqueous solutions at standard enzyme temperature of approximately 37 °C, which limit the microwave power input to 2-5 W. As a result more comprehensive studies must be conducted to investigate the effects of substantial microwave input into enzymatic reactions.

![Figure 4.1](image)

**Figure 4.1.** Alignment of important biological motifs in a microwave field. It is hypothesized that certain biological motifs (e.g. α-helices) possess substantial dipole moments and thus are specifically activated under microwave irradiation. In this case the α-helix flips directionality to align with the electric field.

In terms of the actual characterization of microwaves on enzymatic catalysis, examples are few and far between. Perhaps the most extensive study was conducted by the Leadbeater laboratory on a *C. antartica* lipase. \(^{243}\) Lipases have previously been studied under microwave irradiation, primarily because they are commercially available immobilized on resins. This feature of the enzymes affords their application in organic solvents. While the
activity of enzymes in organic solvents is often muted, the ability to employ microwave transparent solvents (unlike aqueous buffers) enables the application of high microwave powers. This enzyme is capable of performing a transesterification between methyl acetoacetate and primary alcohols, and was examined under both microwave and thermal conditions. Ultimately, despite attempting multiple microwave conditions including simultaneous cooling and cryogenic cooling, no difference between microwave irradiation and thermal heating was observed (Figure 4.2).

![Figure 4.2. Progression of lipase reaction in the presence and absence of microwave irradiation. From Leadbeater, N.E. et. al. Org. Biomol Chem. 2007, 5, 1052.](image)

These results are in contrast to findings in other research groups with similar immobilized lipases, which report an increase product conversion of up to 20% when irradiated in the microwave. Conversely, enzymatic deactivation has been observed as well. The conflicting results represent the complexity involved in subjecting complex molecules to microwave irradiation, and the need for further investigations into the behavior of biomolecules in a microwave field.
Another example of the implementation of microwaves in biotechnological applications has been explored in the polymerase chain reaction. Preliminary studies have found that cycling times can be reduced by half due to the more efficient heating of the reactions allowing the optimal temperatures to be reached more rapidly (Figure 4.3).\textsuperscript{246} Additionally, it was feasible to scale up the PCR, which was previously unattainable due to challenges associated with temperature maintenance in the conventional heating of large volumes of aqueous media. The reaction was able to be cycled 33 times without any detectable enzymatic degradation.

![Microwave-Heated PCR, 15 mL](image)

**Figure 4.3.** Temperature profile of large scale PCR reactions which were only made possible by microwave irradiation. From Orrrling, K. *Chem. Commun.* 2004, 790.

Based on these intriguing results we were interested in participating in this rapidly advancing field on several levels. Substantial work must be done to understand the role of large biomacromolecules such as DNA and proteins in the field of microwave chemistry. Due to their unique properties, including, high dipole moments, substrate specificity and catalytic efficiency, they are ideal candidates for microwave activation of their function, yet
provide a challenge due to their relatively fragile nature and evolved nacent catalytic efficiency (making further optimization difficult).

4.1. Microwave Activation of Enzymatic Catalysis

4.1.1. Introduction

Microwave induction of enzymatic reactions has been considered;\textsuperscript{241, 246-249} however, whether non-thermal microwave effects are important in biocatalysis is not clear.\textsuperscript{243} These effects are difficult to measure because rapid heating of aqueous solutions under high-power microwave irradiation can result in protein denaturation and inactivation.\textsuperscript{250, 251} Moreover, the intrinsic catalytic ability of enzymes at a certain temperature can obscure the potential rate enhancement through microwave irradiation. There have been attempts to conduct enzymatic reactions under microwave irradiation in non-aqueous solvents but this has yielded inconclusive results because of greatly reduced enzymatic activity.\textsuperscript{243, 244, 252-254}

4.1.2. Application of Hyperthermophilic Enzymes in Microwave Catalysis

Non-thermal microwave effects in enzymatic catalysis could be observed if, at high levels of microwave irradiation, minimal catalytic activity arises from thermal heating. This can be done in an aqueous environment, if care is taken to: 1) effectively cool the reaction mixture during irradiation using a jacketed reaction vessel and cryogenic cooling, while precisely measuring the temperature using a fiber optics probe (CEM Coolmate), and 2) use hyperthermophilic enzymes which have minimal catalytic activity at temperatures below 40°C and denature at much higher temperatures than their mesophilic counterparts. Here, a \(\beta\)-glucosidase (CelB) from the hyperthermophilic archaeon, \textit{Pyrococcus furiosus}, was
examined for biocatalytic function under microwave irradiation; this enzyme is optimally active at ~110 °C. *P. furiosus* CelB (Pfu CelB) cleaves exo-glycosidic linkages in both natural (e.g., cellulbiose) and synthetic substrates (e.g., 277, Scheme 4.1). 255, 256

Scheme 4.1. Colorimetric CelB assay. The enzymatic hydrolysis of 277 produces glucose (278) and nitrophenol (279).

4.1.3. Enzymatic Assay in the Microwave Reactor

Enzymatic assays were conducted by cooling the nitrophenolate substrate 277 in reaction buffer (50 mM NaOAc pH 5.5, 10% DMSO) to –20 °C, followed by the addition of heat-pretreated *E. coli* cell extract containing recombinant CelB. Microwave irradiation (300 W) was applied until the reaction temperature reached 40 °C (all temperatures were measured using a fiber optics temperature sensor); coolant at –60 °C was simultaneously circulated through the jacketed reaction vessel. The reaction was then quenched with a 1 M Na2CO3 solution, and the absorbance of the alkoxide of 279 was measured at 405 nm. No significant substrate hydrolysis was observed under microwave irradiation without Pfu CelB addition. Initial investigations were conducted to determine the effect of microwave power on the enzymatic catalysis and elucidate the optimum conditions for microwave irradiation. Thus, we conducted the enzymatic reaction with 300, 200, 100, and 50 W of microwave power (Figure 4.4). Interestingly we found a strong correlation between enzymatic activity and microwave power when the reactions were conducted from –20 to 40 °C. The maximum
power input allowed by the CEM Coolmate is 300 W, thus higher power settings could not be accessed to further probe this effect.

![Graph](image)

**Figure 4.4.** Pfu CelB enzymatic activity dependence on microwave power. All experiments were conducted in triplicate, 5 mM substrate concentration. In all cases, the temperature increased from –20 to 40°C.

Furthermore, under identical thermal conditions (–20 to 40 °C) (Figure 4.4), no significant enzymatic activity (<10^{-11} mol min^{-1} µg^{-1}) was detected in the absence of microwave irradiation (0 W). However, a more than four orders of magnitude increase in enzymatic activity of Pfu CelB was achieved with 300 W of microwave irradiation (2.3×10^{-6} mol min^{-1} µg^{-1}). Thus, a substantial microwave effect on enzymatic catalysis was observed.\textsuperscript{257,258} This illustrates for the first time that microwaves can trigger high biocatalytic rates of a hyperthermophilic enzyme at bulk solution temperatures far below (70 °C or more) its thermal optimum. In order to confirm that the microwave reaction at 300W and the corresponding control reaction were conducted under similar thermal profiles, we employed the fiber optics probe in both conditions to allow us to best mimic the microwave temperature profile in the thermal control (Figure 4.5).
4.1.4. Determination of Kinetic Parameters for the Enzymatic Activation

In order to determine the kinetic parameters of the enzyme, we first obtained purified protein from Inci Ozdemir (Kelly lab). The optimized microwave conditions (300 W, –20 to 40 °C) were compared to the optimal thermal conditions (90 °C, 2 min) to establish the different kinetic parameters. Conveniently, previous work in the Kelly lab had established many of these parameters for the CelB enzyme for comparison. We varied substrate concentration and analyzed enzymatic activity to generate a Michalis-Menten kinetics model (Figure 4.6). Based on the data, the $K_m$ of the thermal reaction was found to be 0.21 mM, which was comparable to the literature value of 0.20 mM. The microwave $K_m$ was found to be 0.036 mM, suggesting a more efficient processing of the substrate. Further analysis must be conducted to determine $k_{cat}$ values for the reaction, and these calculations are currently underway. Additionally, we are also examining the enzymatic specificity in the microwave by employing other p-nitrophenolic substrates, ideally comparison with previously published thermal data will indicate if the mechanism of catalysis alters in the microwave, and if the promiscuity of the enzyme increases.
4.1.5. Investigation of Other Hyperthermophilic Enzymes

To further probe the generality of the discovered effect, two other hyperthermophilic enzymes, an α-galactosidase from *Thermotoga maritima* (TmGalA),\(^\text{259}\) and a carboxylesterase from *Sulfolobus solfataricus* P1 (SsoP1 CE),\(^\text{260}\) were investigated (Figure 4.7, note the logarithmic scale). A similar colorimetric assay was used based on α-galactopyranoside or hexanoic acid analogs of 277 (Scheme 4.1). In both cases, for the same thermal heating, microwave irradiation significantly stimulated enzyme activity. Note that both TmGalA and SsoP1 CE have lower T\(_{\text{opt}}\) than Pfu CelB (~90-95 °C\(^\text{259, 260}\) vs. 110 °C for CelB), such that some thermally-induced activity was observed in the absence of microwave irradiation.
Figure 4.7. Effect on microwave irradiation on Pfu CelB, Pdu CelB, Tm GalA, and SsoP1 CE activity. All experiments were conducted in triplicate.

Note that no microwave biocatalytic activation was observed in the case of a mesophilic homolog of CelB from *Prunus dulcis* (Pdu CelB) (Figure 4.7). We suspect this is a result of thermal stimulation during heating between –20 to 40 °C leading to a mixing of microwave and thermal catalysis. Therefore, the microwave effect is much more prominent in hyperthermophilic enzymes, perhaps due to their decreased activity at low temperatures allowing for the separation of the microwave activation from the thermal activation.

4.1.6. Circular Dichroism Analysis of the Effects of Microwave Irradiation

Hyperthermophilic enzymes have highly compact structures and limited conformational flexibility at temperatures far below their normal functional range. We hypothesize the observed specific microwave effect (enzyme activity at unusually low temperatures) most likely derives from molecular motion induced by a rapid dipole alignment of the peptide bonds with the oscillating electric field.240,243
The strong induction of molecular motion in the enzyme through microwave irradiation is corroborated by the observation that high microwave power (300 W) at moderate temperatures (75 °C) led to a denaturation of Pfu CelB, confirmed by a loss of activity and a loss of tertiary structure as measured by circular dichroism (Figure 4.8).

Figure 4.8. Circular dichroism results for the Pfu CelB enzyme after heating with and without microwave irradiation.

Figure 4.8 indicates that secondary structure is observed in the enzyme subjected to thermal heating at 75 °C; however, this structural organization is lost when irradiated at 300W at the same temperature for the same amount of time. The ellipticity between 200-230 nm in the thermal sample is characteristic in shape and magnitude of the presence of α-helices (which have been observed in crystal structures of homologs of CelB as ~40% of the structure); however in the irradiated sample this ellipticity is absent, correlating to protein denaturation. This loss of structure is coupled with a loss of activity in the pNP assay (5.9x10⁹ mol min⁻¹ µg⁻¹), and can be correlated to the denaturation of the protein under microwave irradiation.
To further investigate this phenomenon, CD experiments were performed using the optimal microwave conditions (–20 to 40 °C, 300W) as well as a control thermal heating (40 °C, 0W). CD spectra were taken ~5 minutes post irradiation. This data is displayed in Figure 4.9, and indicates that no significant change in final enzyme folding occurs between the two conditions, and no appreciable denaturation of the enzyme occurs under our assay conditions.

Figure 4.9. Circular dichroism results for the Pf CelB enzyme after heating with and without microwave irradiation at –20 to 40 °C and 300W (black) and 40 °C at 0W (red).

To examine if the microwave affected the stability of a mesophilic analog of the enzyme from *P. dulcis*, CD experiments were also conducted at –20 to 40 °C with 300W of power and at 40 °C without microwave irradiation. Under these conditions, no denaturation of the enzyme was observed (Figure 4.10). When compared to the results of the enzymatic assay, it appears that the high thermal activity of the mesophilic enzyme obscures the detection of potential microwave activation, making it difficult to truly investigate microwave effects in this system.
The results reported here illustrate for the first time the intrinsic effect of microwave irradiation on biocatalysis. Furthermore, they indicate that hyperthermophilic enzymes can be activated at temperatures far below their optimum, presumably by microwave-induced conformational flexibility. This finding offers the prospect of using hyperthermophilic enzymes at ambient temperatures to catalyze reactions with thermally labile substrates and products. Furthermore, microwaves could be used to regulate biocatalytic rates at very low temperatures for enzymes from less thermophilic sources. Both of these possibilities are being considered.

**4.1.7. Investigation into Microwave Activation of Proteases**

Having successfully employed hyperthermophilic enzymes we were interested in attempting to expand the technology to more accessible mesophilic proteins. While these enzymes may not exhibit as prominent of microwave effects, it may be possible to extend their catalytic properties via microwave irradiation. We initiated our investigation with proteases due to their intensive application for *in vitro* protein digestions. Specifically, we
were interested in the trypsin mesophilic protease. This enzyme is a serine protease, which cleaves proteins on the carboxyl side of lysine and arginine residues. Trypsin is naturally found in the digestive tract; however, is employed in a variety of biotechnical applications. These uses include resuspension of adherent cells in tissue culture, dissolution of blood clots, pre-digestion of baby food, and extensively in the field of proteomics to digest protein samples for mass spectrometry.

The application of microwave irradiation has previously been applied to the trypsin enzyme, and is currently employed in industrial applications towards the more rapid digest of protein samples. These findings have reduced the time of enzymatic digest from days to hours, increasing the efficiency of sample processing; however, the digestions are conducted in the microwave at a standard temperature of 37 °C, with minimal microwave power (1 to 5 W). To the best of our knowledge, no studies have been conducted under high microwave power or with simultaneous cooling. We hypothesized that by increasing the microwave power of the enzymatic digest we may be able to increase the catalytic rate of these reactions even further, as well as process proteins samples that are more difficult to digest and provide more efficient and complete digestion of known samples.

Due to the optimal activity of trypsin being 37 °C, as opposed to the hyperthermophilic enzymes, which approach or exceed 100 °C, we decided to explore various microwave conditions to determine the maximal activity for the enzyme. The enzymatic assay employed was very similar to that utilized for CelB, except we used a Tris Buffer (pH 8), and N-\(\alpha\)-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as the enzymatic substrate. The enzymatic activity was then quantified based on the concentration of p-nitroaniline liberated by the enzyme (405 nm). Reactions were conducted without microwave
irradiation (2 min, 37 °C), in a CEM Discover microwave without simultaneous cooling (2 min, 45-110 °C depending on power input), in a CEM Discover microwave with simultaneous cooling using compressed air (2 min, 35-72 °C depending on power input), and in a CEM Coolmate (5 min, –20 to 40 °C). Control reactions in the absence of enzyme were also conducted in triplicate to correct for background hydrolysis, and all reactions were monitored using a fiber-optic probe inserted in the reaction vial (Figure 4.11).

![Figure 4.11](image-url) Investigation into optimum conditions for microwave activation of trypsin.

In contrast to the hyperthermophilic enzymes investigated earlier, the Coolmate system was not the most effective means of activating enzymatic digestion of the substrate; however, simultaneous cooling with 100-200 W of power appears to provide an increased level of substrate cleavage. It appears that 300W of microwave power lowers the enzymatic activity, which is most likely due to denaturation of the mesophilic enzyme as a combination
of both high microwave irradiation, and in the case of the Discover microwave, elevated temperatures. Excitingly, increased activities of enzymatic catalysis were observed in the microwave reaction relative to the thermal control at similar temperature profiles.

These results motivated us to explore the kinetic parameters of microwave activation on trypsin. We thus conducted a similar experiment as with CelB where the substrate concentration was varied and the reactions were repeated under thermal conditions at 42 °C, or under microwave irradiation with simultaneous cooling (200 W, 42 °C) (Figure 4.12). In the case of trypsin, it appears that the microwave is able to promote much higher levels of catalysis, with a higher \( V_{\text{max}} \). The microwave enzyme possesses a \( K_m \) of 1.20 mM, while the \( K_m \) of the thermal conditions is 2.06 mM, almost twice as high. This increased activity obtained by microwave irradiation may have interesting consequences on the digestion of actual proteins.

![Figure 4.12](image_url)

**Figure 4.12.** Kinetic analysis for the microwave activation of trypsin. A substantial increase in activity can be noted for the conditions employing microwave irradiation with simultaneous cooling.
Based on the promising differential activity observed between microwave and thermal conditions, we were interested in attempting actual protein digests under these conditions. We thus obtained a melittin protein sample (a standard for protein digests) from the Muddiman laboratory, and conducted protein microwave irradiations (2 min, 200 W, 42 °C) in the presence and absence of trypsin. Additionally, the Muddiman lab performed a standard control digestion (37 °C, 4 h) and then conducted a standard mass spectroscopy analysis of the various digests. The two microwave samples (with and without trypsin) afforded similar digest patterns to the thermal control. Perhaps the most surprising result is the presence of digested protein in the absence of trypsin for the irradiated sample. This suggests that for this particular protein, microwave input affords enough energy to promote peptide bond cleavage, a result that requires further investigation. Between the trypsinized samples, similar digestion patterns were observed, but a substantial time saving occurred (2 minutes versus 4 hours). Future work needs to be conducted to attempt similar digestions in the Coolmate to examine potential differences in cleavage patterns. Additionally, as melittin is a relatively easy protein to digest, it would be interesting to investigate more challenging proteins under microwave conditions, as microwave irradiation may facilitate proteolysis that can not occur under thermal conditions.

4.2. Microwave Disruption of DNA Hybridization

4.2.1. Introduction

Despite its biological relevance, DNA has virtually been unexplored in relation to microwave irradiation. Most studies conducted in the microwave are more in the context of cellular effects of the irradiation leading to DNA damage and cellular death, and are
typically done in household microwave ovens. The overall application of these approaches is the sterilization of surfaces and solutions from bacterial films. Very few investigations examine the \textit{in vitro} effect of microwaves on DNA stability and controlled irradiation.\textsuperscript{264}

Specifically, we are interested in examining the hybridization of DNA oligomers subjected to microwave irradiation. Hybridization is an extremely valuable property of DNA, and the ability to modulate it with microwave irradiation possesses several intriguing applications, including studies involving DNA hybridization probes, interactions with proteins, and experiments utilizing hybridization for materials science purposes. Additionally, if it can be demonstrated that DNA is sensitive to microwave effects, then this also may have implications in DNA and RNA synthesis, similar to the effects observed in the synthesis of peptides, as well as enzymatic reactions processing DNA and RNA, e.g. PCR.

\textit{4.2.2. Assay Development}

In order to probe the effects of microwave irradiation, we first needed to establish a viable assay to detect DNA hybridization. While many real-time protocols exist for the detection of double-stranded DNA (e.g. real-time PCR using an intercalating fluorescent probe), we do not have the capabilities of monitoring microwave reactions in a real-time. Thus, we need an offline assay to determine if the DNA is melting or annealing while being subjected to microwave irradiation. As a result, we designed an end-point assay that relies upon fluorescence quenching to detect a prior melting and annealing of DNA in the microwave synthesizer.

Fundamentally, the assay uses two sets of double-stranded DNA. One of the pairs possesses a 5’ fluorescein modification (5’ Fluor-CGCACCCAGGCTTAGCTACAA 3’).
which hybridizes in close proximity to a 3’ dabcy1 modification (5’ TTGTAGCTAAGCCTGGGTGCG-Dabcy1 3’), and the other pair consists of the identical sequence without the modifications (Calculated $T_m = 60 \, ^\circ\text{C}$; Experimental $T_m = 74.2 \pm 1.2 \, ^\circ\text{C}$).

Due to the proximity of the fluorophore and quencher on the hybridized modified oligomers, very little fluorescence should be present initially. Upon irradiation of the system and melting of the dsDNA, the ssDNA will subsequently anneal in a statistical fashion to either its previous partner, or to the non-modified complement. If the fluorescein modified strand anneals to the non-modified strand, the quencher is removed leading to an increase in fluorescence signal. A similar result is observed if the DNA melts, but does not re-hybridize. If the perturbation is not capable of inducing DNA melting, then no change in fluorescence should be observed (Figure 4.13). Ideally, this experimental setup will allow us to detect if it is possible to induce DNA melting via microwave irradiation.

**Figure 4.13.** Experimental assay to determine if microwave irradiation is capable of melting dsDNA. Open circle = fluorophore (fluorescein); closed circle = quencher (dabcy1).
4.2.3. *Effects of Microwave Irradiation on DNA Hybridization*

In order to probe the assay viability, we designed a DNA sequence with a calculated $T_m$ of 60 °C, and obtained the required modified and unmodified oligomers (Alpha DNA Technologies). Initial hybridizations were conducted thermally to generate the two sets of double stranded DNA, and an aliquot was kept to obtain an initial fluorescence reading. Various stoichiometric iterations were employed in these initial hybridizations to minimize the fluorescence signal. Ultimately, a 10-fold excess of dabcyl-modified oligomer to the fluorescein-modified oligomer was employed to completely drive the equilibrium to maximum hybridization, and effectively suppress the fluorescence signal.

In order to differentiate microwave effects on the melting of dsDNA from thermal effects we elected to conduct these experiments in the CEM Coolmate, in an analogous fashion to our work with hyperthermophilic enzymes. By attempting the DNA melting at temperatures well below its calculated melting point (e.g. –20 to 40 °C) we believe it is possible to demonstrate if the melting is a direct effect of the microwave irradiation, as opposed to the combination of some thermal influence convoluting the results. Thus, we mixed our pre-hybridized oligomers and subjected them to various conditions to probe hybridization. Based on initial results we employed a 10-fold excess of the non-labeled oligomers to the fluorescein monomer (Final ratio 10:10:10:1 Dabcyl/Non-labeled/Non-Labeled/Fluorescein). The mixture of dsDNA was either irradiated in the Coolmate (2 min, –20 to 40 to –20 °C, 300W), heated to 70 °C for 5 minutes, or subjected to thermal conditions mimicking the microwave temperature profile (2 min, –20 to 40 to –20 °C). All reactions were subsequently cooled to 4 °C to ensure hybridization. A positive control involving the
hybridization of the fluorescein modified oligomer with its unmodified complement to determine the maximum attainable fluorescence signal was also conducted (Figure 4.14).

![Figure 4.14](image)

**Figure 4.14.** Analysis of DNA hybridization under microwave conditions.

Gratifyingly, it appears that microwave irradiation is capable of inducing DNA melting, even at temperatures well below the calculated $T_m$ of the DNA duplex. Both perturbations of the system with either microwave irradiation (300W), or thermal stimulation ($70 \, ^\circ\text{C}$) were able to restore some fluorescence signal, signifying the melting of the initial DNA sequences and re-hybridization with non-quencher modified complements. A similar restoration of fluorescence was not observed for the thermal controls, which mimicked the temperature profile of the Coolmate. All reactions were conducted in triplicate to ensure statistical significance. Based on the dabcy1-free control, the maximum fluorescence was not restored; however, this is not expected, as fluorescein sequences can re-anneal to their complementary dabcy1 quenching oligomers providing 50% of the signal. While we do not
observe a complete restoration to 50% of the signal, we do observe a statistically relevant increase in fluorescent signal. Further optimization must be performed to demonstrate the generality of this approach, to establish a microwave power-melting relationship, and elucidate the optimal conditions for DNA melting in the microwave. Ultimately, the sequence may be altered to utilize DNA hybridization as an internal temperature sensor for some microwave reactions, which may be a more accurate measure than current temperature sensing methods.

In summary, these results demonstrate the interactions of DNA oligomers with a microwave field, suggesting the microwave can be responsible for DNA melting. This result corresponds well to the previous observations we have made when attempting restriction enzyme digests in the microwave, as no cleavage was observed. Based on the discoveries discussed here, this was presumably due to the absence of double-stranded DNA. Additionally, this opens the possibility for a variety of applications employing DNA in the microwave. However, these results are still preliminary and substantial work must still be conducted to verify the universal nature of this interaction.
4.3 Experimental

**CelB Activity Assay.** Recombinant hyperthermophilic enzymes were expressed and purified according to previously reported protocols.\(^1\)\(^2\) An enzyme working solution was prepared by diluting the enzyme (0.1ng CelB, 1ng TmGalA, or 1ng SsoP1) to 100µL in dIH\(_2\)O. A microwave reaction vial was charged with a solution of the nitrophenoate substrate (5mM; pNp-β-glucopyranoside for CelB; pNp-α-galctopyranoside for TmGalA; SsoP1 pNp-hexanoate) in a sodium acetate buffer solution (50mM, pH 5.5, 10% DMSO, 600µL). The vial was placed in a CEM Discover instrument and cooled to −40 °C using the CEM Coolmate system, the reaction was irradiated at 300W, and upon reaching −20 °C the enzyme solution was quickly added. The reaction was allowed to proceed until the temperature reached 40 °C, and then stopped by the addition of 1M Na\(_2\)CO\(_3\) (500µL). All temperatures were measured using a fiberoptics probe. The absorbance of the solution at 405nm was recorded on a Nanodrop spectrophotometer. Enzymatic activity was calculated by using the extinction coefficient of the nitrophenolate (ε = 17,700 M\(^{-1}\) cm\(^{-1}\)) to calculate its concentration. The enzymatic activity was calculated using the following formula:

\[
[pNP] = \frac{\text{Absorbance}}{(\varepsilon \times l)}
\]

\[
\text{Activity} = \frac{[pNP] \times \text{Reaction Volume}}{\text{Reaction Time} \times \text{Amount of Enzyme Used}}
\]
All reactions were conducted in triplicate. Also, they were repeated in triplicate under all conditions in the absence of enzyme, to control for potential background hydrolysis. This background absorbance was then subtracted from the enzymatic reactions to afford a true measure of enzyme catalysis.

Control reactions were performed under identical conditions in a microwave reaction vessel. The reaction mixture was cooled to –40 °C in an isopropanol/CO$_2$ bath followed by the transfer to a 90 °C water bath. The enzymes were added at –20 °C and all temperatures were recorded using the same fiberoptics probe.

**Dependence of Enzymatic Activity of Microwave Power.** The activity assays described above were also conducted to establish a power/activity relationship. In these reactions, all concentrations, buffers, and the temperature range (−20 °C to 40 °C) were identical with the conditions above, only the microwave power was varied (50 W, 100 W, 200 W, and 300 W). Due to the different power settings, the reaction times varied from 3.00 min (50 W) to 13 sec (300 W, see above); however, control reactions at 0 W were performed to mimic the temperature profile at each different power setting. Since the reaction time is factored into the activity calculation (see above), the varying times are negligible when examining the overall activity.

**Determination of Kinetic Parameters for CelB.** Assays were conducted as previously described at both 300 W (−20 °C to 40 °C) and 0 W (−20 °C to 40 °C), but the substrate concentration was varied from 0.2 mM to 20 mM. Again, reactions were conducted in triplicate in conjunction to control reactions to eliminate contributions from non-enzymatic
substrate hydrolysis. Enzymatic activity was plotted versus substrate concentration to determine enzymatic parameters.

**Circular Dichroism.** A CelB assay (1ng) in a sodium acetate buffer solution (50mM, pH 5.5, 10% DMSO, 600µL) was heated to 75 °C at both 300W (in a CEM Discover microwave reactor) and without microwave irradiation (water bath at 75 °C) for 1 min. The sample was then transferred into a quartz cuvette and a CD spectrum was recorded at 25 °C using a Jasco J-600 Circular Dichroism Spectrophotometer.

**Trypsin Activity Assay.** Trypsin was obtained from MP Biomedicals and a 5 mg/mL stock solution was made in Tris Buffer (0.1 M Tris, 1 mM MgCl₂, 1 M glycerol). For each assay the enzyme was diluted to 50 µg/mL (200 µL final volume, 10 µL of trypsin stock in 990 µL of Tris Buffer). The assay was performed thermally at 37 °C for 1 hour by mixing 200 µL of N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) substrate (Aldrich; 0.8 mM in a Tris/EDTA Buffer (50 mM Tris and 1 mM EDTA, pH 7.2)) with the 200 µL of enzyme working solution. The reaction was then measured at 410 nm on a Nanodrop spectrophotometer. Activity was then calculated in an analogous manner as previously described with CelB.

\[[pNP] = \text{Absorbance} / (\varepsilon \times l)\]

Activity = ([pNP] × Reaction Volume) / Reaction Time × Amount of Enzyme Used
Identical reactions were conducted in triplicate in the Coolmate (−20 °C to 40 °C) as well as in the CEM Discover (with and without simultaneous cooling with air) instrument at various microwave power settings to determine the optimal conditions.

Kinetic parameters were examined by taking the optimal microwave conditions (200 W, with air, 2 min), as well as the thermal conditions (37 °C, 2 min), and applying them in the reaction with different BAPNA substrate concentrations (0.2 mM to 20 mM). Again, reactions were conducted in triplicate in conjunction to control reactions to eliminate contributions from non-enzymatic substrate hydrolysis. Enzymatic activity was plotted versus substrate concentration to determine enzymatic parameters.

**DNA Hybridization Assay.** Fluorescein modified DNA (5’ Fluro-CGCACCAGGCTTAGCTACAA 3’; 150 pmol; Alpha DNA) and Dabcyl modified DNA (5’ TTGTAGCTAAGCCTGGGTGCG-Dab 3’; 150 pmol; Alpha DNA) were combined with 10X Taq DNA Polymerase Buffer (New England Biolabs) and water to a final volume of 200 µL. A complementary reaction was set up using the non-modified DNA (IDT DNA Technologies) and the Fluorescein modified DNA. Both hybridizations were heated to 70 °C for 5 minutes, and then cooled to 4 °C. The fluorescein/dabcyl modified hybridization was then split into three separate reactions (37.5 pmol of hybridized DNA each) and incubated with the non-modified DNA sequences (300 pmol each) with to afford 1X buffered solutions (Taq DNA Polymerase Buffer) at a final volume of 200 µL. One reaction was subjected to microwave irradiation (300W, −20 to 40 to −20 °C) in a CEM Coolmate microwave reactor (simultaneously cooled with −60 °C coolant) for 5 minutes. Another reaction was subjected
to a similar temperature profile, employing a dry ice/acetone bath followed by boiling water before returning the reaction to the dry ice/acetone bath. The final reaction was heated to 70 °C for 2 minutes, followed by cooling to 4 °C. All reactions were then measured for fluorescence using a Molecular Devices Gemini EM microplate spectrofluorimeter.
In order to elucidate biological processes on a molecular level, precise external control over these processes is required.\textsuperscript{265-268} Light represents an ideal external control element as it possesses several advantages over traditional modulators of gene function. Most importantly, light irradiation can be easily controlled in a spatial and a temporal fashion, conveying spatio-temporal control of biological activity to the system under study. Light irradiation is a non-invasive technique that results in minimal secondary perturbations of cellular processes, and the potential to regulate its amplitude enables the ability to tune the desired biological effect. Hence, the photochemical regulation of gene function is a rapidly advancing research field in the functional genomics area.

Light-induced activation of biological processes is most commonly achieved through the initial deactivation of a particular molecule through installation of a photoprotecting group at a critical position. This renders the molecule inactive, in a practice known as “caging”.\textsuperscript{269} The photoprotecting group is removed upon irradiation with UV light, thus restoring the biological activity, in a practice known as “decaging” (Scheme 5.1). Several very effective caging groups are known, including derivatives of the 2-nitrobenzyl group \textsuperscript{280} and the coumarin moiety \textsuperscript{281}. Nitrobenzyl groups \textsuperscript{280} are by far the most common caging groups due to their facile synthesis and easy installation on the molecule under study. They are typically decorated with electron donating groups (e.g. OCH\textsubscript{3}) to shift the absorption maximum to a longer wavelength, allowing efficient decaging with non-photodamaging UV light of 365nm (Scheme 5.1). The caging
technology has been employed extensively in biochemical reactions to elicit an additional degree of control over biological function.

While the concept of caging has been around since 1978, only relatively recently have scientists investigated the caging of DNA and other oligonucleosides. The photoregulation of DNA is especially intriguing as it possess a multitude of functions, and the spatial and temporal regulation of these functions can have innumerable advantages. Over the years, several different approaches have been taken to achieve efficient caging strategies, with each possessing different advantages and disadvantages.

A successful caging approach must address several stringent criteria. Perhaps the most important is that the installation of the caging group must abrogate the nascent function of the DNA oligomer, and afford the rapid restoration of activity upon a brief period of irradiation. Additionally, the caging group installation must be stable to both synthetic and physiological conditions, as loss of the group under these conditions would nullify the value
of the caging experiment. While not required, it is advantageous if the caging can occur in a controlled fashion, providing a high level of control over the number of installed caging groups and their exact position. Finally, a high yielding synthesis of the caging group and the caged DNA molecule is favorable.

Within the literature three major approaches to DNA caging have been attempted. The first involves the statistical caging of the DNA backbone. The first example of this approach was taken by Haselton et al. via the reaction of a standard 2-nitrobenzyl derivative under benzylating conditions with plasmid DNA encoding GFP. The plasmid was then introduced to an \textit{in vivo} model system, rendering the plasmid transcriptionally inactive until light irradiation. While this approach inhibited function prior to light irradiation, the complete restoration of transcriptional activity could not be attained. The statistical caging approach is not limited to DNA, but has found more extensive application in RNA caging; however, as illustrated in this example faces numerous difficulties in finding widespread application. Primarily, due to the non-specific nature of this approach, it is difficult to control the number of caging groups installed, and there is no sequence specificity as to where they are located on the phosphodiester backbone. Due to the incorporation of multiple caging groups, it is difficult to ensure the removal of every group on every molecule through light irradiation, and thus restore full activity to the system. Additionally, since much of the function of the DNA molecule occurs as a result of base paring interactions, the positioning of caging groups on the backbone is a less efficient means of disrupting function. However, the most significant advantage to this approach is the limited synthetic requirement, as complex DNA monomers and complicated caging groups do not need to be synthesized.
A second approach, which has found a substantial amount of utility of late, is the installation of a photocleavable linker into the backbone of the DNA oligomer. The fundamental basis of this approach was established by Taylor and Ordoukhanian by the simple incorporation of a 2-nitrobenzyl group between two DNA bases. Irradiation led to DNA scission, disrupting its hybridization.277 More notably, the Dmochowski group and the Chen group have developed photocleavable linkers, which link complementary oligomers.278, 279 Upon exposure to UV irradiation, the linker is removed, leading to dehybridization, and the utilization of the single stranded oligomers in antisense technologies. This has been demonstrated to be a valid approach to gene regulation in multicellular organisms (Figure 5.1). While a useful technology, there is significant synthetic preparation of caged linkers required, and due to the indirect caging of DNA, complete abrogation, followed by complete restoration of function requires substantial experimental design.

The final approach to DNA caging involves the direct installation of a caging group on the nucleoside base. This approach is the most straightforward and has found substantial success in the disruption of nascent function of the oligomer due to the direct perturbation of hydrogen bonding and thus DNA hybridization. While this approach requires substantial synthetic preparation of DNA monomers for oligomer synthesis on a DNA synthesizer, the specificity of incorporation tends to outweigh this disadvantage. Prior to and concurrent with our research several other groups have prepared caged DNA monomers and applied them towards various biological questions (Figure 5.2). The application of each will be discussed and addressed later, in relation to our approach. However, each group has advantageous and disadvantages which we hope to improve upon in our research.
Based on all of these considerations, we became interested in the installation of a photolabile protecting group directly on the base of DNA. Conveniently, a DNA monomer could synthetically be prepared and incorporated in the DNA sequence via standard DNA synthesis. This could then be exploited towards the spatio-temporal regulation of DNA function both in vivo and in vitro.
5.1. Development of DNA Caging Technologies and Application to DNAzyme Catalysis

5.1.1. Introduction

Many recent discoveries have revealed the multifactorial roles oligonucleotides play in vitro and in vivo. It has been demonstrated, that they can act as catalysts (ribozymes and DNAzymes),\textsuperscript{283, 284} sensors (aptamers),\textsuperscript{285} gene expression platforms (riboswitches and antiswitches),\textsuperscript{2, 286, 287} and gene regulatory elements (antisense DNA, siRNA, and miRNA).\textsuperscript{286, 288, 289}

As previously discussed, approaches to the site-specific caging of DNA have been reported. The introduction of a O-4 caged thymidine has been successfully applied to the photochemical activation of transcription and aptamer binding.\textsuperscript{280, 290} However, due to the lability of the caging group special DNA synthesis conditions were necessary. An adenosine modified with a sterically demanding, photo-removable imidazolylethylthio group has been used to photochemically activate an 8-17E DNAzyme.\textsuperscript{281} After irradiation for 8-10 min with short-wavelength UV light (254-310 nm) only 30% of RNA cleavage was observed after a 60 min reaction time. Since the caging group was installed at C-8, no hydrogen bonding of the adenosine was disrupted. Reversible switching of DNAzyme activity was previously achieved through incorporation of diazobenzene motifs, however, only a 5- to 9-fold rate modulation upon irradiation was obtained.\textsuperscript{291, 292}

Our goal was to develop a caging approach which fulfills all of the following requirements: a) allows for specific probing of hydrogen bonding of oligonucleotide bases, b) enables introduction of the caged monomer under standard DNA synthesis conditions, c) provides a caged oligomer which is stable to a wide range of chemical and
physiological conditions, and d) allows for excellent restoration of DNA activity upon brief irradiation with non-photodamaging UV light.

5.1.2. Caging Group Design and Incorporation into DNA

Recently, the Deiters lab developed a new caging group (NPOM = 6-nitropiperonyloxyomethyl) which proved to be highly efficient in the caging of nitrogen heterocycles. This group was specifically designed to solve previous problems associated with chemical stability or slow decaging rates of photo-protecting groups on nitrogen atoms. Consequently, we selected this group for application to the caging of the thymidine N-3, thus disrupting an essential hydrogen bond. The phosphoramidite (Scheme 5.2) was synthesized in 5 steps from thymidine starting with the preparation of the known acetylated thymidine (Ac₂O, DMAP, 98%). Caging with 6-nitropiperonyloxyomethyl chloride (NPOM-Cl) was achieved in 82% (Cs₂CO₃, DMF, rt) yielding 287. Removal of the acetate groups (K₂CO₃, MeOH, 78%) towards 288 followed by selective tritylation of the primary hydroxy group (DMTCl, DMAP, pyridine) delivered 289 in 91% yield. Installation of the phosphoramidite (2-cyanoethyl-diisopropyl-chloro phosphoramidite, DCM DIPEA) was achieved in 80% under classical conditions completing the synthesis of 290 (Harry Lusic).
Scheme 5.2. Synthesis of the caged phosphoramidite 290, followed by incorporation can lead to photocaged DNA until light irradiation.

The stability of 289 to DNA synthesis conditions and its rapid decaging through irradiation with UV light of 356 nm ($\varepsilon_{365} = 6887 \text{ cm}^{-1} \text{ M}^{-1}$) was demonstrated (see supporting information). The quantum yield ($\phi = 0.094$) for the photochemical removal of the NPOM group was determined by 3,4-dimethoxynitrobenzene actinometry.$^{295, 296}$

5.1.3. Application of the Caged Thymidine to Photoregulation of DNAzyme Activity

Using standard DNA synthesis conditions 290 has been incorporated at all thymidine positions of the 10-23 DNAzyme D1 providing the mutants D2-D7 (Figure 5.3). The 10-23 DNAzyme is a highly active and sequence specific RNA cleaving deoxyoligonucleotide.$^{284, 297}$ It has been successfully applied to the suppression of genes in vitro and in model organisms.$^{298}$
To probe the necessity of free 3-NH groups in these thymidine residues for the maintenance of DNAzyme activity, the RNA substrate 5'-GGAGAGAGAUGGG-UGCG-3' was radioactively 5'-labeled using $^{32}$P-ATP and exposed to the seven DNAzymes D1-D7 in a standard reaction buffer (100 mM MgCl$_2$, pH 8.2, 15 mM Tris buffer) for 30 min at 37°C (Figure 5.4). As expected, the original 10-23 DNAzyme D1 led to almost complete RNA cleavage. DNAzyme D2 exhibited completely inhibited activity due to the installation of a single caging group on T$_{12}$. This was expected, since a previous mutagenesis study of the catalytic core revealed this to be an essential residue.$^{299}$ These experiments also demonstrated that the least essential thymidine residue is located at position 16. This was confirmed through the incorporation of 290 at this position leading to still catalytically active D3, even in presence of the sterically demanding caging group. We then probed the tolerance of base pair mismatches in the substrate recognition domains by caging the thymidine residues T$_{25}$, T$_{27}$, and T$_{29}$. The resulting DNAzymes D4-D6 displayed lower activity but still induced substantial RNA cleavage. Previously, single mismatches between the RNA substrate and the flanking regions have led to reduced cleavage activity as well.$^{297}$ However, selective
installation of three caging groups on T₂₅, T₂₇, and T₂₉ lead to complete inhibition of RNA cleavage activity in D₇, presumably due to the disruption of multiple Watson-Crick base paring interactions with the substrate.

![RNA D1 D2 D3 D4 D5 D6 D7](image)

**Figure 5.4.** Cleavage of the RNA substrate for 30 min with the 10-23 DNAzymes D₁-D₇ without prior UV irradiation. 100 mM MgCl₂, pH 8.2, 15 mM Tris buffer, 37 °C, 40 nM substrate, 400 nM enzyme.

We then became interested in determining the optimal decaging conditions for the restoration of DNAzyme activity. In order to do so, we employed various irradiation sources for variable time intervals with DNAzyme D₂, and monitored the amount of RNA cleavage over time. From this initial study it was determined that a 1 minute irradiation at 365 nm on a transilluminator was the most efficient for DNAzyme activation (Figure 5.5).
Figure 5.5. Differential activation of DNAzyme D2 with differing irradiation times and sources.

We conducted HPLC experiments on the caged DNA oligomers to ensure the photolabile protecting groups were completely removed. Samples were irradiated under the previously established optimal conditions of 1 minute with the transilluminator at 10 μM concentrations and then analyzed via HPLC. We also analyzed the non-caged oligomer as well as the pre-irradiated caged oligomer to examine differences in retention times. Based on HPLC results, it appears that all caging groups are removed via this brief irradiation, as the retention time of the irradiated sample was identical to the non-caged oligomer (Figure 5.6).
Figure 5.6. Decaging of the caged DNAzyme followed by HPLC analysis. After irradiation, the peak corresponding to the caged oligomer disappeared while a new peak was observed with a similar retention time as the wild-type oligomer.

Subsequently, a more detailed time-course investigation of the light-activation of D2 was conducted (Figure 5.7). A control experiment of just the RNA substrate exposed to UV light did not result in any cleaved product. Complete cleavage of the RNA substrate was achieved within 30 min using the unmodified D1, whereas no cleavage was observed with caged D2 under identical conditions. However, brief irradiation with non-photodamaging UV light of 365 nm (25 W) for 1 min initiated decaging and activation of D2. Figure 5.7 displays the resulting RNA cleavage with complete consumption of the substrate by 30 min.

![DNAzyme HPLC analysis](image)

Figure 5.7. Progressing cleavage of the RNA substrate with the 10-23 DNAzyme D2 after a 1 min UV irradiation (365 nm). Complete RNA cleavage is observed after 30 min. 100 mM MgCl₂, pH 8.2, 15 mM Tris buffer, 37 °C, 40 nM substrate, 400 nM enzyme.
In order to determine the cleavage rates $k$ of the DNAzymes D1, D2, and D7, the amount of cleaved RNA was quantified at nine different time points under single-turnover conditions through integration (using Molecular Dynamics ImageQuant 5.2TM) of the corresponding radioactive bands in 15% denaturing TBE polyacrylamide gels using a PhosphorImager (Figure 5.8).

![Figure 5.8](image)

**Figure 5.8.** Cleavage of the RNA substrate with the wild-type DNAzyme D1 and the caged DNAzymes D2 and D7 (with and without UV irradiation). 10 mM MgCl$_2$, pH 7.4, 15 mM Tris buffer, 37 °C, 40 nM substrate, 400 nM enzyme. The cleaved RNA has been normalized and the experiments were conducted in triplicate.

The data was fitted (using Microcal Origin 5.0TM) with an exponential decay curve $\sim -e^{kt}$, and, as previously observed, the wild-type DNAzyme D1 showed a high cleavage rate ($k_{D1} = 0.242\pm0.013 \text{ min}^{-1}$) under the assay conditions. As expected from the results shown in Figure 5.4, the caged DNAzymes D2 and D7 displayed no cleavage activity ($k_{D2} = \text{ND}$ and $k_{D7} = \text{ND}$), demonstrating that caging group installation on thymidine can completely abrogate both catalytic activity and substrate binding. Gratifyingly, brief irradiation for 1 min (365 nm, 25 W) of the caged DNAzymes led to restoration of catalytic activity of D2 ($k_{D2,UV} = 0.131\pm0.007 \text{ min}^{-1}$) and D7 ($k_{D7,UV} = 0.129\pm0.011 \text{ min}^{-1}$) to 54%
and 53% of the original D1 activity, respectively. After a 30 min incubation time 80% of the RNA substrate was cleaved by the non-caged D1, 73% by irradiated D2, and 55% by irradiated D7. Thus DNAzymes with an excellent light-triggered switch have been developed.

5.1.4. Photodeactivation of DNAzyme Catalysis

Having successfully demonstrated the photochemical activation of DNAzyme function, we also wanted to devise a means of deactivating DNAzymes. This could be particularly useful in the spatio-temporal activation of gene expression through a light-deactivatable DNAzyme as an antisense agent. If the DNAzyme is constitutively active, gene expression will be suppressed via mRNA cleavage; however, upon light irradiation, deactivation of DNAzyme activity should lead to intact mRNA and thus expression of the gene of interest. We proposed several approaches towards this photochemical deactivation.

We first hypothesized that by incubating the DNAzyme with a DNA strand complementary to the DNAzyme binding arms should lead to a competition between the DNA and RNA substrate. If used in excess, DNA/DNA hybridization should be most prevalent, efficiently prohibiting RNA cleavage. Additionally, the DNAzyme should be inactive towards DNA cleavage and the substrate should not be degraded. If the DNA inhibitor possesses a caging group, it should be incapable of hybridization until the photolabile protecting group is removed with light. In the absence of DNA/DNA hybridization the DNAzyme catalysis should function normally, cleaving the RNA substrate (Figure 5.9).
Figure 5.9. Assay design for the inhibition of DNAzyme function with complementary DNA. DNAzyme (black line) catalysis should cleave the RNA substrate (red line); however, in the presence of a complementary DNA inhibitor (blue line), the cleavage of the RNA should be suppressed.

To validate this approach, we first attempted an experiment with a non-caged inhibitor to determine optimal conditions for DNAzyme deactivation. As with previous experiments, the RNA substrate was labeled with $\gamma^{32}$P ATP and employed in the experiments along with the 10-23 DNAzyme and the DNAzyme DNA complement (5’-GGAGAGAGATGGGTGCG-3’). Several experimental parameters were varied to optimize DNAzyme deactivation including: Mg$^{2+}$ concentrations, DNA substrate to RNA substrate ratio, and temperature (Figure 5.10).

<table>
<thead>
<tr>
<th>DNAzyme [Mg$^{2+}$]</th>
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<th>10</th>
<th>10</th>
<th>100</th>
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<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Temperature</td>
<td>37</td>
<td>37</td>
<td>25</td>
<td>25</td>
<td>37</td>
<td>25</td>
<td>37</td>
<td>37</td>
<td>25</td>
<td>37</td>
</tr>
</tbody>
</table>

Figure 5.10. Polyacrylamide gel electrophoresis analysis of the inhibition of DNAzyme cleavage by a complementary DNA inhibitor. Different conditions were assessed, including magnesium concentration, temperature and DNA inhibitor/RNA substrate ratio, in order to determine the optimal conditions for DNAzyme inhibition.
Ultimately, it was determined that a 10:1 ratio of DNA inhibitor to RNA substrate, at 10 mM Mg\(^{2+}\) for 30 minutes at 10 °C, was optimal for suppressing DNAzyme activity. Based on these results we were next interested in regulating this process in a photochemical fashion. In order to do so the DNA inhibitor was synthesized with both thymidine residues caged (5'-GGAGAGAGAT*GGGT*T*GCG 3', T* denotes a caged thymidine). Employing the optimized conditions, we evaluated the photoregulation of the caged inhibitor, either not irradiating the reaction, or irradiating for 10 minutes at 365 nm prior to RNA substrate addition (Figure 5.11).

![Figure 5.11. Photochemical inhibition of DNAzyme function using a caged complementary inhibitor. WT = non-caged complementary inhibitor.](image)

Gratifyingly, the approach worked very well, as the irradiated DNA inhibitor efficiently prevented RNA cleavage. The non-irradiated sample remained inactive towards DNAzyme inactivation; as comparable levels of cleavage were observed as when no competing DNA inhibitor was added. The irradiated sample was also comparable in deactivation as the non-caged substrate.
Similarly, we were interested if designing a DNA inhibitor complementary to the catalytic core of the DNAzyme would also be as effective. This approach would be advantageous as it eliminates competition with the RNA target. To examine this possibility, we first conducted the reactions with a non-caged DNA complement (5’ TCGTTCTAGCTAGCC 3’). Again, several variables were altered to ascertain the ideal reaction conditions for DNAzyme inactivation. Based on the previous experiment, we only altered magnesium concentrations and DNA inhibitor to RNA substrate ratios (Figure 5.12).

![Figure 5.12. Inhibition of DNAzyme function via implementation of DNA inhibitor complementary to the catalytic core of the DNAzyme.](image)

Interestingly, this DNAzyme silencing strategy was much more applicable and efficient than employing a DNA substrate complementary to the binding arms of the DNAzyme. In this case, even at high magnesium concentrations where the 10-23 DNAzyme has been demonstrated to be highly active, its catalytic ability is suppressed at low DNA/RNA substrate ratios. This is not necessarily surprising, as the two sequences are now
not competing for binding, but rather have different sites for hybridization. However, once the DNA inhibitor hybridizes with the catalytic portion of the DNAzyme, catalytic ability is lost. In fact, virtually no DNAzyme cleavage of RNA substrate is observed under any condition using the complementary DNA oligomer. Based on the success of these results, we prepared a caged DNA substrate (5’ T*CGT*TGTAGCT*TAGCC 3’, T* denotes the caged thymidine), which was complementary to the DNAzyme catalytic core. In order to mimic the previously utilized conditions, we continued to use 10 mM Mg$^{2+}$; however dropped our caged DNA/RNA ratio to 5:1. We either irradiated the reaction mixture for 5 minutes at 365 nm, or prevented its exposure to UV irradiation, then incubated the reaction with the substrate at 25 °C for 30 minutes (Figure 5.13). This approach of DNAzyme deactivation was also successful, as the DNAzyme was active in the presence of the non-irradiated caged complement, cleaving RNA substrate at a comparable fashion as in the absence of any complementary substrate. However, irradiation of the caged substrate completely deactivated DNAzyme activity as it was capable of hybridizing to the catalytic core effectively shutting down catalysis (Figure 5.13).
We next hypothesized that we could develop a similar approach using intramolecular DNAzyme inhibition. Since the intramolecular hybridization should be significantly more efficient than the intermolecular hybridization previously employed, this should be the ideal solution to deactivation of DNAzyme activity. This can be achieved via the synthesis of a longer DNAzyme with a self-complementary binding arm. When caged, the DNAzyme should remain active; however, upon decaging the intramolecular hybridization event should dominate, forming a hairpin and suppressing RNA hybridization and cleavage. This design may lead to more reliable deactivation, as the intramolecular hybridization should be faster and more effective than relying on the DNAzyme substrate out-competing the RNA substrate in an intermolecular fashion. To test this we first prepared two hairpin DNAzymes with different degrees of complementarity to ascertain how much hybridization was required to inhibit catalysis (HP1: 5’ CATGCCCTGGTGGCTTTTTCGCCACCCAGGCTAGCTACAACG ACTCTCTCCG 3’ and HP2: CCTGGTGGCTTTTCGCACCCAGGCTAGCTACAACGA

Figure 5.13. Photochemical deactivation of DNAzyme catalysis using a caged DNA inhibitor complementary to the catalytic core.
CTCTCTCCG 3’, self-complementary sequence is underlined). We conducted these reactions at 10 mM and 100 mM concentrations of Mg$^{2+}$, and employed the previously described radioactivity labeled RNA substrate (Figure 5.14).

![Figure 5.14. Inhibition of DNAzyme activity via complementary hairpin formation.](image)

From these exploratory experiments, it appears this approach is not as versatile as utilizing a DNA substrate complementary to the catalytic core. Only the HP1 DNAzyme was capable of suppressing RNA cleavage, and only at 10 mM Mg$^{2+}$, whereas the HP2 DNAzyme was constitutively active. Additional optimization could be performed via the designing of sequences with a greater degree of hairpin hybridization; however, this approach is limited by the synthesis of relatively long sequences of caged DNA. Nonetheless, we attempted to see if this could be performed in a photochemical fashion. We installed
photolabile groups on the hairpin of HP1 DNAzyme (5’
CAT*GCC*T*GGT*GCCTTTCGCACCCAGGCTAGCTACAACGACTCTCTCCG 3’,
T* denotes the caged thymidine, complementary sequence is underlined), and analyzed its
sensitivity to light activation under the optimal conditions of 10 mM Mg$^{2+}$ at 25 °C for 30
minutes (Figure 5.15).

![Diagram of DNAzyme deactivation](image)

Figure 5.15. Photodeactivation of DNAzyme function using a caged hairpin approach.

Gratifyingly, the caged hairpin approach to DNAzyme deactivation was also
successful. However, the caged hairpin DNAzyme in the absence of UV irradiation
maintained some catalytic activity. If the caged hairpin was decaged for 5 minutes with 365
nm light, the DNAzyme was deactivated towards the catalytic cleavage of RNA. Thus, we
have demonstrate three different approaches to DNAzyme deactivation, which complement
our previous studies of DNAzyme activation.

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5.1.5. Utilization of DNAzymes in vivo

Having successfully developed methodologies for the photochemical activation and deactivation of DNAzymes we hoped to employ this technology in vivo to silence a gene of interest. This application of DNAzymes has substantial therapeutic value as they can target genes for degradation in a site specific fashion. However, single-stranded DNA is still intrinsically unstable to intracellular conditions, but a recent report demonstrated that via creating double-stranded hairpins on the ends of a DNAzyme, the system could be used in vivo to knock-down reporter genes, as the agent was more stable against exonuclease degradation.  

It was our hope to utilize a caged analog of this DNAzyme to achieve spatio-temporal control of gene silencing in mammalian cells. In order to test the feasibility of the approach we first reproduced the literature results, and thus obtained the non-caged sequence for a DNAzyme that silences the DsRed reporter gene. First, to determine the optimal conditions for gene silencing the concentration of DNAzyme was varied. The DNAzyme (0, 250, and 500 pmol) was co-transfected into HEK-293T cells along with a DsRed expressing plasmid (pIRES-DSRed-Express; Clontech).

Figure 5.16. Silencing of DsRed expression in 293T cells with a hairpin DNAzyme. A) Control transfection without DNAzyme. B) Transfection with 250 pmol DNAzyme. C) Transfection with 500 pmol DNAzyme.
Based on the transfection results, it appears that 250 pmol DNAzyme transfection is unable to completely knock-down DSRed expression; however, 500 pmol effectively silences the gene (Figure 5.16). This experiment also demonstrated the need for a transfection control as it is unclear whether or not the cells are not fluorescent because the silencing is effective or if the cells are no longer viable, or if the co-transfection was ineffective. Additionally, it is unclear if the silencing is due to actual DNAzyme activity leading to transcript cleavage and degradation, or if the DNAzyme was simply functioning as an antisense agent, suppressing gene expression. To fully investigate the mechanism of silencing we obtained two additional oligomers; one which had the essential thymidine in the catalytic core mutated to an adenosine, and one where we removed the entire catalytic core from the sequence. If silencing is observed in either of these cases it will confirm the DNAzyme is not functioning via site-specific DNAzyme cleavage, but rather by an antisense mechanism. Thus, we co-transfected the DNAzyme constructs (500 pmol) with the pIRES-DSRed-Express plasmid (1 µg) and a GFP control plasmid (1 µg; Clontech) and examined fluorescent protein expression via fluorescence microscopy (Figure 5.17).

![Figure 5.17](image_url)

**Figure 5.17.** Probing the mechanism of “DNAzyme” silencing. A) Co-transfection of DsRed plasmid and GFP plasmid with no DNAzyme construct. B) Transfection with the literature reported DNAzyme. C) Transfection with DNAzyme with an essential base mutated. D) Transfection with a DNAzyme with the catalytic core removed.

Interestingly, the DSRed gene is silenced with all of the DNAzyme constructs. This suggests that DNAzyme activity is not necessary for efficient gene silencing, which most
likely occurs via a traditional antisense mechanism, in contrast to the literature report. Thus, we have demonstrated that this construct can be employed to silence genes in vivo, and has applications towards the photoregulation of gene expression. The next steps in the research require the synthesis of caged constructs to achieve spatio-temporal control over gene regulation. Caging in the DNA recognition arms should prohibit hybridization, and thus silencing until an irradiation event. Additionally, the catalytic core can be caged at the essential thymidine residue to further confirm the silencing mechanism.

In summary a new photocaged nucleoside (synthesized by Harry Lusic), which was incorporated into DNA using standard synthesis conditions (by Dr. Mark O. Liveley at WFU). This caging approach was then used to probe the necessity of specific hydrogen bonds for activity of a DNAzyme, and we found that disruption of a single H-bond can be sufficient to completely inhibit the enzyme. Surprisingly, installation of the bulky caging group was tolerated at several positions within the DNAzyme and the caging of three thymidine residues was necessary to abrogate binding to the RNA substrate. Restoration of DNAzyme activity was achieved through decaging with a brief irradiation of 365 nm UV light (UVA light of this wavelength is far less toxic to cells than UVB light of shorter wavelength and is typically considered to be non-photodamaging\textsuperscript{269, 271, 302-305c,19}), providing an excellent on/off switch for oligonucleotide activity.

5.2 Photochemical Regulation of the Polymerase Chain Reaction

5.2.1. Introduction

Based on the results from the DNAzyme studies, we expected that the developed technology was generally effective in regulating the hybridization of DNA. Since
DNA/DNA and DNA/RNA hybridization are essential for many of the processes in which DNA is involved we were interested in studying this result more extensively. Perhaps one of the best examples of the use of DNA/DNA hybridization in vitro is the polymerase chain reaction (PCR). Ideally we could exploit the technology to photochemically regulate this important reaction.

PCR was developed in 1983, and is employed in the in vitro isolation and exponential amplification of specific DNA sequences. By utilizing thermophilic DNA polymerases with specifically designed DNA primers, extremely small amounts of DNA can be rapidly enriched to substantial quantities (Figure 5.18). In the few years since its discovery, PCR has revolutionized the field of molecular biology, facilitating genome sequencing, genetic disease diagnosis, and genetic fingerprinting.

![PCR Reaction Diagram](image)

**Figure 5.18.** Representative PCR reaction leading to the exponential amplification of a DNA template.

5.2.2. Initial Hybridization Studies Utilizing Caged Oligomers

As previously discussed, we developed a novel caging group for N-heterocyclic molecules and applied it to the specific caging of a thymidine nucleotide on its
heterocyclic base. The corresponding caged phosphoramidite was incorporated into DNA oligomers using standard DNA synthesis equipment and protocols. Installation of the sterically demanding caging group in conjunction with the disruption of an NH bond critical for Watson-Crick base pairing allowed for the attenuation of catalytic activity of a DNAzyme. Brief irradiation with UV light of 365 nm (25 W, handheld UV lamp) removes the caging group and generates the regular DNA oligomer (Figure 5.19). In order to apply this approach to the photochemical regulation of PCR, we first investigated the effect of one or multiple caging groups on the pairing to a complementary DNA strand. The DNA oligomers \( \text{P1-P7} \), consisting of 19 nucleotides, a typical length for PCR primers, and containing 0-4 caged thymidines have been synthesized. These primers were than analyzed for their annealing and melting properties in the presence of a complementary oligonucleotide.

**Figure 5.19.** Analysis of the hybridization of caged DNA oligomers via the installation of caging groups (blue squares) at different positions on the DNA sequence.

Melting curves were measured on a BioRad MyiQ RT-PCR thermocycler by conducting a sequence of 3 heating and cooling cycles (1\( \mu \)M of both primer and complementary DNA with 12.5 \( \mu \)L iQ SYBR Green Supermix to a total volume of 25\( \mu \)L; 40 \( ^\circ \)C to 80 \( ^\circ \)C with a 0.5 \( ^\circ \)C/min ramp). The melting temperatures were determined to be 65.3 \( ^\circ \)C (P1), 62.1 \( ^\circ \)C (P2), 54.3 \( ^\circ \)C (P3), 55.5 \( ^\circ \)C (P4), and 50.0 \( ^\circ \)C (P6). No melting temperatures
could be measured for P5 and P7, leading to the assumption that no hybridization occurs (Table 5.1).

**Table 5.1** Melting temperatures of non-caged (P1) and caged oligonucleotides (P2-P7) before and after UV irradiation (365 nm).

<table>
<thead>
<tr>
<th>DNA Sequence</th>
<th>Mp / °C –UV</th>
<th>Mp / °C +UV</th>
</tr>
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<tbody>
<tr>
<td>P1 5’ CTGATTTCGACCAGGTTGC 3’</td>
<td>65.3 ± 0.3</td>
<td>65.0 ± 0.8</td>
</tr>
<tr>
<td>P2 5’ CTGATTTTCGACCAGGTTGC 3’</td>
<td>62.1 ± 0.7</td>
<td>64.1 ± 0.2</td>
</tr>
<tr>
<td>P3 5’ CTGATTTCGACCAGGTTGC 3’</td>
<td>54.3 ± 1.1</td>
<td>64.1 ± 0.8</td>
</tr>
<tr>
<td>P4 5’ CTGATTTCGACCAGGTTGC 3’</td>
<td>55.5 ± 0.5</td>
<td>63.8 ± 0.3</td>
</tr>
<tr>
<td>P5 5’ CTGATTTCGACCAGGTTGC 3’</td>
<td>ND</td>
<td>64.2 ± 0.2</td>
</tr>
<tr>
<td>P6 5’ CTGATTTCGACCAGGTTGC 3’</td>
<td>50.0 ± 1.0</td>
<td>64.5 ± 0.5</td>
</tr>
<tr>
<td>P7 5’ CTGATTTCGACCAGGTTGC 3’</td>
<td>ND</td>
<td>64.2 ± 0.3</td>
</tr>
</tbody>
</table>

aT denotes the caged thymidine. bMelting temperatures (Mp) determined with the non-caged complement (5’ CGAACCTGGTCGAAATCAG 3’). ND = not detectable.

These results indicate that both the number of caging groups and the position of the caged thymidine residues affect DNA hybridization. Installation of a single caging group results in a melting temperature depression of 3.2 °C and 11.0 °C as seen in P2 and P3, respectively. This effect is less pronounced in P2 perhaps due to the caged T’s close proximity to the 5’ terminus, leading to a lower level of interference with the hybridization of neighboring nucleotides. Very similar melting point depressions and positional variations have previously been observed in T-mismatches.\(^{311, 312}\) With the incorporation of additional caging groups in P4-P7, melting temperatures decrease further. However, addition of a single caged thymidine close to the 5’ terminus of P3 had no effect in P4. A positional effect was also observed with three caging groups, as seen in P5 and P6. The primer P6 contains a cluster of three caged thymidines and displays a higher melting temperature than P5 containing three caging groups distributed throughout the DNA oligomer, thus leading to a more effective disruption of hybridization. In order to ensure a complete removal of the
photolabile group, each primer was irradiated for 8 minutes at 365 nm (Figure 5.20), and then analyzed in the same melting temperature assay. As expected, irradiation led to full restoration of DNA hybridization, as each primer displayed a comparable melting temperature to the non-caged analog P1.

5.2.3. Application of the Caging Technology to the PCR Reaction

The hybridization experiments revealed that the presence of three caging groups distributed evenly throughout a 19 nucleotide oligomer is sufficient to disrupt hybridization and thus will prevent annealing of a PCR primer to its cognate DNA template, at the typical annealing temperature range of 50-65°C. The stability of the caging group to PCR conditions was examined on the monomeric caged thymidine, and found to be unaffected by the elevated temperatures required for PCR (data not shown). These results set the stage for the application of P5 in a light-activated PCR experiment (Scheme 2).

![Figure 5.20. PCR activation by light. Black line: PCR template, blue line: primer, red circles: caged thymidines.](image)

The DNA template (1.5 ng/µL of plasmid DNA) was incubated in the presence of P5 and a reverse primer (5’AGAGAGCTCGAGATCGCCATCTTCCAG)
CAGGCGCACCATTGCCCCTGT 3’, 1 µM each; the same reverse primer was used in all PCRs) with nucleotide triphosphates (dNTPs, 0.3 mM each), in Taq Reaction Buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, pH 8.3) and water. Taq DNA Polymerase (3 units/µL) was added to initiate the reaction. Prior to placement in a PCR thermocycler (Eppendorf Mastercycler) the reaction was either irradiated for 8 minutes at 365 nm, or maintained in the dark. An initial denaturation at 95 °C was performed, followed by 40 cycles consisting of 95 °C (30 sec), 50 °C (30 sec), and 72 °C (1 min), with a final extension at 72 °C (2 min). The reactions were conducted in triplicate, visualized on a 1% agarose gel, and quantitated (all DNA was quantified by band integration in ethidium bromide stained agarose gels using Image Quant 5.2). A regular PCR reaction with non-caged P₁ produced approximately 140 ng of PCR product (Figure 5.21, lane 5); however when caged P₅ was employed in the absence of UV irradiation, only trace amounts of product were detected (Figure 5.21, lane 6). After irradiation, the function of P₅ was restored (Figure 5.21, lane 7), leading to comparable amounts of PCR product as found in the reaction with P₁ (Figure 5.21, lane 5).

![Figure 5.21. Agarose gel of dsDNA amplified by light regulated PCR](image-url)
This experiment represents the first example of a light-activated PCR. While we initially employed irradiation prior to the thermal cycling, temporal control can be achieved by initiating the reaction through irradiation at a specific timepoint. Here, an identical reaction with UV irradiating at cycle 15 was conducted, as shown in Figure 5.22. Prior to irradiation, no PCR product can be detected in reactions conducted with the caged primer \( P_5 \). In contrast, the non-caged primer \( P_1 \) leads to the expected exponential amplification. Upon UV irradiation of the reaction with \( P_5 \) at cycle 15 the amount of DNA increases exponentially, while no amplification occurs in the corresponding non-irradiated reaction.

![Figure 5.22. Time course of a PCR reaction light-activated at cycle 15. All experiments were performed in triplicate.](image)

After achieving photochemical control of the activation of PCR at a specific time point, the possibility to switch-off PCR activity via light irradiation was examined. This can be accomplished by designing a self-complementary primer which is predisposed to form a hairpin, rather than act as a PCR primer. By installing caging groups on the complementary portion of this sequence it is possible to block self-hybridization, thus enabling the polymerization reaction. The PCR is then stopped by removing the caging groups, leading to
hairpin formation and primer deactivation (Figure 5.23). In order to achieve photochemical deactivation, an appropriate hairpin primer \textbf{P8} was designed (5’ GGTCAGTAAATTGTTTTTCAATTCTACTGACCG 3’), and a photocaged analog \textbf{P9} was synthesized (5’ GGT*CAG*T*AAAT*T*-GTCTTTCAATTCTACTGACCG 3’, T* denotes the caged thymidine). PCR using a typical primer which only possesses half of the hairpin and not its complement led to DNA amplification after 25 cycles (Figure 5.23 and Figure 5.21, lane 1). Conversely, the non-caged hairpin primer \textbf{P8} failed to amplify the DNA leading to very little PCR product (Figure 5.21, lane 2). However, in the absence of light irradiation the caged hairpin primer \textbf{P9} was successful in acting as a primer, yielding 137 ng of PCR product (Figure 5.21, lane 3). This suggests that the 3 installed caging groups prevented hairpin formation, and allowed the complementary sequence to act as a PCR primer. If \textbf{P9} was irradiated for 8 minutes at 365 nm, the caging groups were removed, leading to hairpin formation and suppression of DNA amplification (Figure 5.21, lane 4).

These light-regulatory mechanisms could also be employed in a temporally controlled fashion by irradiating the caged primer after 10 cycles of PCR (Figure 5.24).
The non-caged primer P8 was used as a control, as it formed a hairpin immediately, blocking Taq DNA polymerase and inhibiting amplification. Because the caged primer P9 was able to bind to the DNA template, amplification occurred. At cycle 10 the reaction containing P9 was irradiated leading to removal of the caging groups, hairpin formation, and effective inhibition of DNA amplification. At the same point, amplification continued in the non-irradiated reaction with P9.

![Graph](image)

**Figure 5.24.** Time course of a PCR reaction light-deactivated at cycle 10. All experiments were performed in triplicate.

With the two caged primers P5 and P9 possessing opposing effects on the PCR reaction upon light irradiation, it was possible to utilize both primers simultaneously to stop the production of one PCR product, while also triggering the amplification of a different PCR product via irradiation with UV light (Figure 5.25). Thus, P5, P9, and the reverse primer were included in the PCR reaction mixture. A product band of ~1.0 kb was detected in the non-irradiated reaction after 20 cycles, which is attributed to the caged and inactive P5 and the caged and active P9 (Figure 5.21, lane 9). Alternatively, a product band of ~0.6 kb was observed in the irradiated reaction, as a result of the
decaging and activation of $P5$ and the deactivation of $P9$ (Figure 5.21, lane 10). As expected, both bands were observed in the control reaction using non-caged and non-hairpin primers (Figure 5.21, lane 8).

![Diagram](image)

**Figure 5.25.** Light-switching between two different PCR reactions through simultaneous activation and deactivation of primers.

In summary, a photochemical activation and deactivation of the polymerase chain reaction has been developed. This was achieved through the incorporation of caged thymidine phosphoramidites into oligonucleotide primers using standard DNA synthesis protocols. By effectively disrupting DNA hybridization through the site-specific installation of caging groups and restoring it with light irradiation it was possible to control activation and deactivation of PCR in a temporal fashion. Moreover, by conducting a simultaneous activation and deactivation, light switching from one DNA amplification product to another was accomplished. We believe that real-time PCR thermocyclers could be easily equipped with UV light sources enabling primer decaging at any time of the experiment. Caged PCR primers could prevent non-specific amplification until the reaction mixture has been heated to the annealing temperature followed by irradiation. This allows for caged primers to be employed in hot-start PCR to
simply the process and prevent polymerization until the mixture has been heated, eliminating non-specific amplification.

### 5.3. Investigation of DNA Polymerase Interactions with Photolabile Protecting Groups

#### 5.3.1. Introduction

Our application of caging groups towards the light-deactivation of the Polymerase Chain Reaction reaction employed primers which created an internal hairpin upon the removal of the caging group; however, in the absence of light irradiation, the caging group remained on the primer and a PCR product was formed. These results evoked an investigation into the interaction between the caged primer and the DNA polymerase. It was unclear if the polymerase was capable of reading through the caging group, or rather if it stopped upon encountering the non-natural base. If the polymerase was incapable of reading through the caging group, one could envision a variety of molecular biology applications for this technology, several of which we sought to exploit.

DNA polymerases are essential enzymes for both DNA synthesis applications in vitro and for the propagation of genetic information in vivo. These enzymes are responsible for the polymerization and replication of DNA using a template strand of DNA and deoxynucleotide triphosphates as monomeric building blocks, effectively catalyzing the formation of phosphodiester bonds. However, DNA polymerases are not capable of the de novo synthesis of DNA and thus require a “primer” possessing a free 3’ hydroxyl group to initiate the polymerization. In vivo these enzymes are necessary for the replication of genomic DNA and thus are essential for cell division. In vitro they are employed in several
molecular biology techniques, most importantly the polymerase chain reaction. DNA polymerases represent a diverse set of enzymes with numerous homologs in various species (5 prokaryotic and over 15 eukaryotic DNA polymerases are known) and their various functions have been extensively studied. Depending on their function, polymerases have different polymerization rates, proof-reading capacities, and fidelities. Due to the importance of these enzymes we are interested in obtaining a greater degree of external control over their enzymatic activity. In order to achieve this we explored the response of these polymerases to the presence of a photo-labile protecting group (caging group) installed on either the DNA template being amplified or the primers utilized to amplify the DNA.

In previous studies different modifications both in the DNA phosphodiester backbone of the DNA template, as well as on the nucleobases themselves have been incorporated. Very recently, by both us and others, a photo-caged base has been employed in PCR primers. Depending on the specific polymerase employed, varied results were obtained ranging from termination of polymerization to enzymatic proof-reading of the abnormal base to yield a correct, full length product.

5.3.2. Primer Extension Reactions

Our experiments commenced with investigations into the effects of caged nucleotides on DNA polymerase catalyzed primer extensions. In order to achieve this, we incorporated a single caged thymidine residue into a short oligonucleotide (32 bases) at two different positions, and examined the reaction of various polymerases to the presence of the caging group. Depending on the position of the caging group, different length products of polymerase extensions should be obtained if the polymerase is incapable of reading beyond
the caging group, allowing us to investigate if the polymerase could read through the NPOM caged thymidine nucleotide (Figure 5.26). Previous research by Dmochowski et al using the Klenlow fragment of DNA Polymerase I suggests that the polymerase is capable of reading through a caging group depending on the distance from the caging group. However, if the 5’ end of the primer is aligned directly with the caged nucleotide in the template, prohibiting the enzyme from generating any velocity, polymerization does not occur until the caging group is removed through light irradiation.

![Figure 5.26. DNA polymerization through extension of a primer using a 32 nucleotide template with a caged thymidine 12 or 16 nucleotides from the 3’ end of the oligomer. A single caging group blocked polymerization by T4 and T7 DNA polymerase.](image)

Three different polymerases were examined: DNA Polymerase I (polymerase family A), T7 DNA Polymerase (polymerase family A), and T4 DNA Polymerases (polymerase family B) were selected due to their different fidelities (ability to exactly copy templates) and exonuclease activities. Family A polymerases share similar sequence homologies and are known for their replicative and repair capabilities, whereas family B polymerases are known mostly for their replicative properties. T4 DNA polymerase has the highest degree of 3’ → 5’ proof-reading capacity, while T7 DNA polymerase has a high fidelity and rapid extension rate. DNA Polymerase I, on the other hand, possesses low proof-reading ability and has a 5’ → 3’ exonuclease function.

After radioactively labeling a DNA primer for the reaction, each polymerase was examined in the presence of a non-caged DNA template, and the two caged templates, D1 and D2. Extension reactions employed the labeled primer (1 pmol), template (1 pmol), and
one of the polymerases (New England Biolabs, 3 units) and were allowed to proceed for 30 minutes at 37 °C. Extension products were then analyzed via polyacrylamide gel electrophoresis and imaged on a STORM phosphorimager (Figure 5.27).

A)

5' AATGGGGCGGAGAGAG

D1 GCCCTCTCAGCAACATCGATCGGACCACGC 5'

5' AATGGGCGGAGAGAG

D2 GCCCTCTCAGCAACATCGATCGGACCACGC 5'

B) T4 WT D1 D2 C) T7 WT D1 D2 D) DNA Poly I WT D1 D2

Figure 5.27. Effects of a caged thymidine nucleobase on the polymerization of mesophilic DNA polymerases. A) Experimental setup using a caged template and a radiolabeled primer to achieve DNA Polymerase promoted primer extension. Blue = Distance from primer to caging group, T = caging group. B) Polymerase extension reaction with T4 DNA Polymerase, when using a non-caged template full length product is obtained; however, using either D1 or D2 containing differently positioned caging groups, polymerization is halted, leading to truncated product. C) Polymerase extension reaction with T7 DNA Polymerase, similar truncation is observed. D) Polymerase extension reaction with DNA Polymerase I, demonstrating a polymerase read through to yield full length product in the case of both caged templates.

The first lane of each gel contains the polymerase product of non-caged DNA template, yielding a full length product of 38 bases. The extension reaction was then conducted in the presence of a caged template with photolabile thymidines at different positions. The template D1 affords 11 base pairs prior to the caging group, while the template D2 only provides 7 bases prior to the caging group. Ideally, this will afford insight into the behavior of the polymerase before encountering the caged thymidine residue. The reaction utilizing D1 with both T4 and T7 Polymerases yields a 12 base pair shorter product than the
full length template (Figure 5.27A-B; Lane 2). Additionally, reaction with D2 and both T4 and T7 affords a 16 base pair shorter product than the full length product (Figure 5.27A-B; Lane 3). This data suggests that T4 and T7 DNA polymerase are unable to continue DNA polymerization past an NPOM caged thymidine nucleotide. Conversely, DNA Polymerase I appears to read through the caged nucleotide, producing full length product in all three cases (Figure 5.27C; lanes 1, 2 and 3). This is likely due to its lower degree of fidelity and proof-reading ability, resulting in the read through of the caged nucleotide. This corroborates the findings of Dmochowski et al., as the Klenow fragment used by them is simply DNA Polymerase I without the 5’→3’ exonuclease domain. This enzyme’s proof-reading capabilities thus are comparable to DNA Polymerase I, and the polymerase is able to read through the caging group.

5.3.3. Caging Groups in the PCR

Based on the results of the primer extension reaction, we next investigated the effects of caged nucleotides on the polymerization of DNA with thermophilic DNA polymerases, thus enabling a polymerase chain reaction. Thus, we employed caged primers with either a single or three consecutive caged thymidine nucleosides. In order to visualize truncated products we amplified a 45mer with either Taq DNA polymerase or Phusion DNA polymerase (Figure 5.28).
Figure 5.28. DNA polymerization by polymerase chain reaction using a caged primer (17 nucleotides) containing the first caged thymidine 10 nucleotides from the 5' end. PCR generates a caged template which results in a stop of Taq and Pfu polymerase due to the presence of a single caged thymidine.

Each PCR was conducted with one of the forward primers (WT, P1, P2, or T1; Table 5.3), a radioactively labeled reverse primer (Integrated DNA Technologies; 50 pmol), DNA template (1 ng), dNTPs, and either Taq DNA polymerase (New England Biolabs, 5 units) or Phusion DNA polymerase (New England Biolabs, 2 units) for 30 cycles. The PCR was then run on a polyacrylamide gel to identify PCR termination by the caged nucleotide. The gel was imaged on a STORM phosphorimager via detection of the $^{32}$P labeled PCR product. Primer T1 was designed to generate a PCR product of the same length as a product resulting from polymerase termination (Figure 5.29).

![Figure 5.29](image)

Figure 5.29. Effects of a caged thymidine nucleobase on the polymerase chain reaction. A) PCR product using Phusion DNA Polymerase. Polymerization is halted in the presence of a caging group, which is confirmed using a truncated primer (T1) that affords the same length product. B) PCR product using Taq DNA Polymerase. Polymerization is halted in the presence of a caging group, which is confirmed using a truncated primer (T1) that affords the same length product.
The non-caged primer afforded full length PCR product for both enzymes (Figure 5.29; lane 1), while, as expected, reactions conducted with primer T1 yielded products which were 7 bases shorter (Figure 5.29; lane 2). Interestingly, PCR reactions with both the singly caged primer P1 and the triply caged primer P2 afforded truncated products the same size as the T1 product (Figure 5.29; lanes 3 and 4). These results demonstrate that both Taq DNA polymerase and Phusion DNA polymerase are stopped by a single caged thymidine.

5.3.4. Light Mediated Mutagenesis and Cloning

The ability of caging groups to stop DNA polymerization by polymerases enables the enzymatic synthesis of double-stranded DNA with a 5’ single-stranded DNA overhang of virtually any size. When applied to the construction of plasmid DNA and molecular cloning, it provides the opportunity to easily and rapidly introduce single or multiple base mutations and to insert synthetic DNA of any length into plasmids. Moreover, it enables the deletion of any sequence of DNA from any plasmid. Importantly, all these processes can be conducted without the need of endonucleases and the use of restriction sites. Site-directed mutagenesis and related modifications of plasmid DNA are important tools for the introduction of stop codons, gene knock-outs, and alterations in the codon set to exchange amino acids.\textsuperscript{322,323} The most widely used commercially available mutagenesis kits (e.g. Stratgene’s QuikChange and Invitrogen’s GeneTailor) require overlapping primers which are used to replicate plasmid DNA. Due to the complementarity of these primers, exponential amplification of plasmid DNA does not occur, resulting in the need to digest parental plasmid. Additionally, because these primers rely upon hybridization of the mutagenized region, typically only one
or two nucleic acids can be mutated for each reaction, and the number of nucleotides that can
be inserted or deleted is very limited. Other strategies currently exist to modify plasmids;
however they possess several disadvantages. Most notably, they require the use of a ligation
reaction or other enzymatic processing, which decreases the overall efficiency of the process
(examples include Clontech’s Transformer, and Promega’s Altered Sites). Additionally,
restriction enzyme free cloning protocols have been developed, but rely upon the use several
primers (up to 8) and thus require complex experimental design.\textsuperscript{324,325}

Previously, we demonstrated that multiple caging groups inhibit hybridization, thus
large, single-stranded regions can be complementary but remain unhybridized until the
caging group is removed, enabling efficient amplification by PCR. Initially, a set of caged
primers with a specified caged base (blue square) was employed in a polymerase chain
reaction, yielding a linearized plasmid with sticky ends (red). Upon light irradiation the
caging groups are removed, enabling hybridization, and affording transformation into \textit{E.
coli}. Intracellular repair of the remaining nicks provide a mutagenized plasmid (Figure
5.30). Additionally, due to the non-complementary nature of the primers to the plasmid,
exponential amplification is achieved in the PCR, affording an excess of mutagenized DNA
over template DNA.
Figure 5.30. The blocking of PCR using a caged thymidine can be used for the insertion of plasmid DNA as well as for the site-directed mutagenesis or deletion of DNA. The installation of a caged thymidine enables the generation of double stranded DNA with long single stranded overhangs. Removal of the caging group through UV irradiation (8 min, 365 nm, transilluminator 25 W) generates a nicked plasmid which can be transformed into \textit{E. coli} where it is propagated after nick repair.

To demonstrate the feasibility of this approach, we synthesized primer sets which either inhibit GFP expression in a pGFPuv plasmid via introduction of a stop codon upstream of the GFP gene, or activate GFP via removing a stop codon generated from the prior mutation (Table 5.4). To obtain optimized conditions for this mutagenesis, we designed 3 sets of knock-out primers with different numbers of caging groups, and thus different lengths of single-stranded overhangs produced. Conveniently, due to the nature of the desired mutation, preliminary assessment of the successful mutagenesis was visually observed by the lack of GFP expression in transformed bacterial cells. Multiple conditions were employed to determine the optimal protocol for a fast and efficient mutagenesis. These included DpnI digestion of template DNA, different polymerase enzymes, phosphorylation of primers, ligation, different irradiation sources, gel purification of PCR products, iterative PCR
reactions, different annealing protocols for the preparation of the nicked plasmids, and different competent cells.

Two substantial problems arose when optimizing this technology; first, the removal/dilution of original template must be achieved as the circular plasmid is transformed much more efficiently than the nicked mutated vector. Secondly, excess of primers must be removed to prevent their undesired hybridization to the PCR product upon irradiation. To overcome the first problem, the best results were achieved by either gel purifying the PCR reaction, or by conducting two iterative PCR reactions to dilute the template relative to the PCR product. An added convenience of the gel purification is the subsequent decaging within the gel on a transilluminator; however, complete removal of template was difficult, as the circular DNA often migrated a similar distance as the linearized PCR product. Unfortunately, this often required an additional DpnI digestion depending on the efficiency of the separation. In order to remove the excess primers, gel purification was sufficient, or in the case of iterative PCR reactions, a PCR cleanup kit (Wizard SV; Promega) could simply be employed.

The optimal light-mediated mutagenesis protocol involved a PCR using caged primers (50 pmol), pGFPuv template (0.1 ng), deoxynucleotide triphosphates (50 pmol), and Phusion DNA polymerase (New England Biolabs, 2 units). An identical PCR was then repeated, using 5 µL of the previous reaction as the template, followed by purification with a PCR cleanup kit. The purified product was then irradiated for 8 minutes on a transilluminator (25 W), and subjected to a hybridization protocol (95 ºC for 5 minutes followed by three cycles of 65 ºC for 5 minutes and 30 ºC for 15 minutes). The nicked plasmid was then transformed and plated on ampicillin LB plates. Colonies were first assessed visually for a
GFP phenotype, and several clones were sequenced verifying that the site-directed mutation and no other mutation occurred (Figure 5.31).

![Figure 5.31. Site-directed mutagenesis of pGFPuv to introduce a stop codon. Primers aligned with the pGFPuv DNA sequence demonstrating the mutation, and the sequencing results confirming the mutation of the DNA, introducing a thymidine residue in place of the adenosine residue effectively introducing a stop codon. Caged thymidines are shown in bold.](image)

Overall, with relatively short overhangs, efficient mutagenesis can progress using a single caging group to stop polymerization reactions; however, with longer overhangs, PCR efficiency can be increased by employing multiple caging groups to prevent undesired hybridization. Conveniently this protocol does not require any restriction sites, endonucleases or additional enzymes, lengthy incubations, or highly competent or specialized cells.

Based on the initial success we set forth to examine the scope of the methodology by designing primer sets to not only introduce DNA and make point mutations, but to remove DNA from the plasmid template. Specifically, we designed a primer set to completely remove the GFPuv gene from the plasmid (Figure 5.32). Primers could readily be designed by selecting DNA sequences on both sides of the GFP gene, coupled by a region that would promote hybridization once the caging groups were removed affording circularization of the plasmid. Two sets of primers were synthesized, a set with multiple caging groups in the complementary sequence (P11/P12), and a set with a single caging group prior to the
complementary sequence (P13/P14). Based on the initial PCR, it was quickly evident that the primer set (P13/14) possessing a single caging group was not suited for this application. While some PCR product could be obtained, the reaction was low yielding, and side products were observed via gel electrophoresis. This is most likely due to the potential of these primers to participate in non-productive hybridization based on their 15-base long complementary sequence. However, PCR of the primer set with multiple caging groups facilitated a clean PCR product, as competing hybridization is prevented by the multiple caging groups. Based on the protocol developed with the site-specific mutagenesis primers, a similar approach was taken for the deletion primers. Thus, a second PCR reaction was performed to enrich the amount of PCR product relative to the initial template. The PCR product was purified (Wizard SV; Promega), followed by irradiation at 365 nm (8 min, transilluminator, 25 W), and hybridization of the generated “sticky ends” (95 °C for 5 minutes followed by three cycles of 65 °C for 5 minutes and 30 °C for 15 minutes). The nicked plasmid was then transformed into Top 10 cells and plated on ampicillin supplemented LB agar. Conveniently, the removal of the GFP gene affords a visible assessment of the success of the mutagenesis, as successful mutations yield colonies capable of growth on ampicillin media, but which lack fluorescence. Non-mutated plasmids also yield colonies; however these produce GFP and are fluorescence upon irradiation with UV light. The light-directed deletion mutagenesis approach was deemed to be successful, affording only a single colony which was not mutated (Table 5.2). The deletion was confirmed via the sequencing of non-fluorescent colonies (Figure 5.32).
Figure 5.32. Strategy to delete entire sequences from a plasmid using the developed methodology, and sequencing data from the successful deletion of the GFPuv gene.

Table 5.2. Light-Mediated Mutagenesis Results.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Positive Colonies</th>
<th>Negative Colonies</th>
<th>Total Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAA Stop Introduction</td>
<td>26</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>AAA Stop Removal</td>
<td>68</td>
<td>6</td>
<td>74</td>
</tr>
<tr>
<td>GFP Deletion</td>
<td>9</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

In summary, we effectively investigated the effects of photocaged nucleosides on the DNA polymerizaton reaction, finding that most polymerases are unable to recognize and read-through the presence of a single caging group on the DNA template. However,
exceptions were observed for DNA Polymerase I, which possess a low fidelity, and thus a high mutation rate. This feature of caged DNA enables its utilization for a variety of molecular biology applications. We developed a new method of introducing mutations into plasmid DNA via a light-mediated mutagenesis protocol. This methodology is advantageous over several common approaches in that it requires the use of only two PCR primers, a PCR reaction, and a single transformation. Additionally, this approach enables not only site-directed mutations, but also insertion of long strands of DNA and the deletion of entire genes.

5.4. Photoregulation of Phosphorothioate Antisense Activity

5.4.1. Introduction

The demonstrated ability of our caged DNA oligomers to regulate both hybridization and catalysis led us to examine the potential application of caged DNA towards gene regulation. One of the most widely used approaches for the down-regulation of specific genes is the application of antisense agents. Antisense agents are oligomers which have the ability to hybridize to specific mRNAs, inhibiting translation and potentially leading to mRNA degradation through RNAse H recruitment (Figure 5.33). As previously discussed, other researchers have previously accomplished the phoregulation of antisense activity; however have done so using a caged linker strategy. Thus, it was our hope to cage the antisense in a more direct fashion via the application of our previously devised caged oligomer synthesis.
For the investigation of gene function, antisense agents can be transfected into cells in cell culture experiments or injected into embryos at an early time-point in development, e.g. into zebrafish embryos at the one- to four-cell stage. Moreover, antisense oligomers are being investigated as therapeutics and one reagent, Vitravene (Isis Pharmaceuticals) is available for the treatment of cytomegalovirus retinitis. However, a substantial drawback of current antisense technologies is the inability to regulate their activity with spatial and temporal control. When injected into early embryos, antisense agents can be distributed to all cells in the developing organism and are active directly after injection. This inhibits the investigation of the spatial and/or temporal regulation of genes. Moreover, genes that are essential in early development cannot be targeted with antisense agents, since they are silenced directly after injection inducing death in the organism under study. These problems can be solved using light-activated antisense agents enabling the spatio-temporal regulation of gene silencing.

Figure 5.33. General schematic of the *in vivo* gene regulation with antisense agents.
We envisioned the photochemical regulation of antisense activity through the incorporation of a caged base unit into the oligomer (Figure 5.34). This strategy is more predictable, synthetically less complex, and less prone to the generation of undesired side products as previous solutions involving statistical caging of the phosphate backbone or the application of photocleavable inhibitors.\textsuperscript{278, 279, 337, 338}

5.4.2. Synthesis of Caged Phosphorothioate Oligomers and Decaging Studies

One of the most commonly employed antisense agents is based on a DNA phosphorothioate (PS DNA) backbone.\textsuperscript{339, 340} PS DNA antisense agents have been used in mammalian cell culture, murine models, and have been FDA approved as therapeutic agents in humans. They have been employed in the study and potential therapy of Crohn’s disease, Hepatitis C, and various cancers.\textsuperscript{341-343} As in the case of locked nucleic acids (LNA),\textsuperscript{333} peptide nucleic acids (PNA),\textsuperscript{332} and morpholinos (MO),\textsuperscript{327} the phosphorothioate modification
conveys intracellular stability to the oligomer. The light-regulation of LNA, PNA, MO, and PS DNA antisense agent can be advantageous over siRNAs\textsuperscript{338, 344} as they do not induce a catalytic cascade of RNA degradation as seen in the case of RNA interference,\textsuperscript{288, 331} thus providing a more tunable control over gene silencing. An advantage of PS DNA over other antisense agents is their ease of synthesis using commercially available monomers and conventional DNA synthesizers. We hypothesized that the hybridization of a PS DNA antisense agent to its corresponding mRNA target can be disrupted through the installation of NPOM-caged thymidine residues on the PS DNA (Figure 5.34), as previously shown for DNA/DNA hybridization.\textsuperscript{318} We discovered that in order to effectively inhibit DNA duplex formation of a caged DNA 19-mer, we need at least three NPOM caging groups evenly distributed throughout the oligomer.\textsuperscript{318} Moreover, brief UV light-irradiation has previously been shown to quantitatively restore hybridization.

Thus, we synthesized a non-caged PS DNA antisense agent targeting the \textit{Renilla} luciferase reporter gene (Table 5.3).\textsuperscript{345} Moreover, PS DNA antisense agents carrying 3 or 4 NPOM-caged thymidine residues where synthesized using standard DNA synthesis conditions in conjunction with Beaucage’s reagent\textsuperscript{346} for the introduction of the phosphorothioate backbone. We selected 3 and 4 caging groups evenly distributed throughout the PS DNA 19-mer based on our previous experiments of the PCR light-regulation.\textsuperscript{318} Finally, we prepared a control PS DNA sequence which should not induce silencing of the \textit{Renilla} luciferase reporter gene.
Table 5.3. Synthesized caged and non-caged phosphorothioate DNA oligomers\(^{[a]}\).

<table>
<thead>
<tr>
<th>PS DNA</th>
<th>Sequence</th>
<th>(T_m/\degree C) –UV</th>
<th>(T_m/\degree C) +UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5’ TCCAGAACAAAGGAAACG 3’</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Non-</td>
<td>5’ CGTTTCCTTGTCTGGA 3’</td>
<td>39.5 ±0.5</td>
<td>39.3 ±0.8</td>
</tr>
<tr>
<td>caged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Caged</td>
<td>5’ CGTTT(^{\ast})CCTT(^{\ast})GTTCT(^{\ast})GGA 3’</td>
<td>ND</td>
<td>39.2 ±0.6</td>
</tr>
<tr>
<td>4-Caged</td>
<td>5’ CGTT(^{\ast})TCCT(^{\ast})TTGT(^{\ast})TCT(^{\ast})GGA 3’</td>
<td>ND</td>
<td>38.9 ±0.8</td>
</tr>
</tbody>
</table>

\(^{[a]}\)Melting temperature of PS DNA/RNA hybrids before and after irradiation (5min, 365nm, 23W). \(\text{T}^{\ast}\) denotes the caged thymidine. ND = non-detectable.

In order to assess the effective decaging of the synthesized PS DNA oligomers we first performed decaging experiments which were monitored by HPLC (Figure 5.35).
For both the 3-caged and 4-caged oligomers, 5 minutes of irradiation with a hand-held UV lamp (23W) lead to complete disappearance of the caged oligomer and the exclusive detection of non-caged PS DNA. We then examined the capacity of the three and four caging groups to inhibit hybridization of the caged PS DNA oligomer to the complementary RNA sequence (5’ UCCAGAACAAAGGAAACG 3’). Hybridization was monitored on a BioRad MyiQ RT-PCR thermocycler by conducting a sequence of 3 heating and cooling cycles (10 µM of both PS DNA and RNA with 12.5 µL iQ SYBR Green Supermix to a total volume of 25µL; 30 °C to 80 °C with a 0.5 °C/min ramp). No hybridization was detected in this
temperature range for the control PS DNA or the non-irradiated caged PS DNA oligomers. The non-caged PS DNA/RNA hybrid exhibited a melting temperature of approximately 39 °C, which is in agreement with similar PS DNAs. Complete hybridization was restored upon a 5-minute irradiation at 365 nm for both caged analogs (Table 5.4).

5.4.3. Phosphorothioate Activity in Mammalian Cell Culture

These antisense oligomers were transfected into mouse fibroblast cells (NIH 3T3) together with a dual reporter system encoding Renilla luciferase and firefly luciferase as a transfection control. As previously described, the non-caged PS DNA induced a 70% down-regulation of the Renilla luciferase signal, and Figure 5.36 displays all luciferase readouts normalized to that signal. The control PS DNA has no effect on the luciferase signal as observed by comparison to cells, which have not been transfected with PS DNA. Moreover, the UV irradiation (365nm, 25W hand-held UV lamp, 5min) of 3T3 cells has no effect on the luciferase signal (within the error margin of the experiment) as can be seen in case of transfection of the non-caged and the control PS DNA followed by irradiation. Gratifyingly, the installation of 3 caging groups completely inhibits the antisense activity of the PS DNA oligomer, as expected from the hybridization experiments. Moreover, a brief irradiation with UV light quantitatively restores antisense activity to the level of the non-caged antisense agent. The same result was achieved with the PS DNA oligomer containing 4 caging groups (Figure 5.36). This clearly demonstrates the ability to regulate gene silencing activity using light through the incorporation of caged monomeric building blocks into phosphorothioate antisense oligomers. The brief UV irradiations did not elicit any toxic effects on 3T3 cells as demonstrated by a cell viability assay.
Figure 5.36. *Renilla* luciferase signal after PS DNA transfection ±UV light irradiation (365nm, 25W hand-held UV lamp, 5 min), normalized to the transfection of the non-caged PS DNA antisense agent. The error bars represent the standard deviation of three independent experiments.

5.4.4. Spatial Control of Antisense Activity

In order to demonstrate spatial regulation of gene expression using caged PS DNA antisense agents, we transfected cells with the *Renilla* luciferase plasmid and with or without PS DNA in a six-well format. After a four hour incubation, the media was removed and the cells were irradiated at 365 nm (5min, 23W) in a specific location using a mask. After a further 24h incubation for luciferase expression to occur, the plate was imaged on a Xenogen Lumina system (Figure 5.37). A high level of spatial control of antisense activity was achieved, as only irradiated areas of the cell monolayer transfected with caged PS DNA agents display little to no luciferase expression. In contrast, when wells containing no caged PS DNA were irradiated the same way, no luciferase silencing was observed.
In summary, we developed an effective light-regulated gene silencing methodology through the incorporation of a caged thymidine phosphoramidite into phosphorothioate antisense agents using standard DNA synthesis conditions. The disruption of antisense activity and its restoration using UV light irradiation was demonstrated through hybridization studies and in mammalian cell culture using a luciferase reporter gene. Moreover, precise spatial control of gene expression was obtained. Due to its easy synthesis and its excellent light-activation properties, we believe that the developed antisense technology will find widespread application in the investigation and regulation of gene function.
5.5. Photoregulation of CpG Response

5.5.1. Introduction

The CpG motif refers to DNA sequences in which a cytosine nucleotide is followed by a guanosine, and thus the “p” represents the phosphate linkage. Interestingly, the occurrence of this dinucleotide within vertebrate genomes is surprisingly rare. When considering the statistical probability of a CG dinucleotide sequence in the human genome (with a 42% GC content), the likelihood of finding this sequence is approximately 4.4%; however, the frequency of this dinucleotide sequence is actually less than 1%. It is suspected that this decreased frequency is a direct result of an increased propensity for cytosine methylation of these sequences, which leads to transitional mutations, decreasing the evolutionary viability of these CpG sequences. However, the CpG motif is much more common in bacteria, where the unfavorable methylation event is less common. From an evolutionary standpoint, the increased frequency of this sequence has been advantageous to eukaryotic systems as they have evolved an immune response to CpG motifs as they are indicative of a bacterial infection.

The mammalian immune system combats infection via two mechanisms, the innate immune response and the adaptive immune response. Upon initial exposure to an antigen, the innate immune response is activated and is non-specific; reacting only to pathogen associated molecular patterns (PAMPs). PAMPs are highly conserved, but limited molecular structures, which are present in pathogen, but are rarely present in the host organism. Thus, CpG oligonucleotides are ideal, as they are common within bacteria, but relatively rare in mammalian systems. The activation of the innate immune response leads to the production of stimulatory cytokines and polyreactive IgM antibodies, which afford a preliminary non-
specific response to the infection. This response results in inflammation and recruits immune cells to mount a more specific adaptive immune response.\textsuperscript{351} This initial immune response is meant to limit the proliferation and spread of the infectious agent. After the initial innate response the adaptive response involves specific antigen recognition by immune cells and the production of high-affinity antibodies and cytotoxic T cells, which specifically combat the infection and afford long-lasting protection.

The ability of CpG motifs to trigger the innate immune response was discovered in 1995 by Krieg \textit{et al.},\textsuperscript{352} however, the mechanism of activation eluded scientists until recently when Hemmi and co-workers firmly established the interaction of Toll-like Receptor 9 (TLR9) with CpGs to induce an immune response.\textsuperscript{353} Toll-like receptors are membrane receptor proteins, distinguished via an amino-terminal extracellular leucine rich domain, followed by a single transmembrane domain, and a globular cytoplasmic domain called the Toll/Interleuken 1 receptor domain (TIR). There are currently 14 known TLRs in mammalian systems each of which recognizes a specific PAMP and homodimerizes initiating a signaling cascade which activates the innate immune response.

The commonly accepted mechanism of immune response activation via CpG is through the internalization of the oligomers into endocytotic vesicles containing TLR9.\textsuperscript{351} This interaction leads to a swelling and acidification of the vesicles, coupled with the generation of reactive oxygen species. In conjunction with these processes, the activated TLR9 proceeds via a similar catalytic cascade as other toll-like receptors, recruiting myeloid differentiation response gene 88 (MYD88), interleukin-1 (IL-1) receptor-activated kinase (IRAK) and tumor-necrosis factor receptor (TNFR)-associated factor 6 (TRAF-6), leading to the activation of various transcription factors (nuclear factor-\(\kappa\)B, activating protein 1,
CCAAT-enhancer binding protein, and cAMP-responsive element binding protein). This signaling cascade culminates in the upregulation of cytokine gene expression and the recruitment of immune cells (Figure 5.38).

Based on the ability of CpG oligonucleotides to elicit an immune response, they have found a variety of uses in therapeutic applications. One major application of the CpG motif is the induction an innate immune response in immunosuppressed individuals, such as pregnant and newborn individuals, to afford a degree of protective immunity. Additionally, CpGs have found application in affording an increased response to vaccines as they efficiently activate the immune system facilitating the adaptive immune system to more effectively
produce antibodies. In fact, co-treatment of vaccines with CpGs has been demonstrated to generate specific IgG responses that were 10-10,000 fold higher than in the absence of the CpG. Finally, the CpG oligonucleotides have potential utility in cancer therapy as they activate natural killer cells and cytotoxic T lymphocytes that are typically employed in some cancer treatments. Based on the plethora of therapeutic applications of CpG motifs they have more recently been employed in human clinical trials.

There are currently three generally accepted structural classes of synthetic CpG oligonucleotides which are capable of activating TLR9. The CpG-B class possess multiple CpG sites and consist of a purely phosphorothioate backbone, while the CpG-A class contain a mixed phosphodiester-phosphorothioate backbone and contain a hexameric CG sequence, flanked by complementary bases thought to create stem-loop structures, and possess a poly-G tail. The C class of CpGs has a pure phosphorothioate backbone, with multiple palindromic CpG motifs, and a 5’ TCG dimer. Each class of CpGs has slightly different modes of immunoactivation, and is employed based on the desired application. However, all undergo the activation of the toll-like receptor 9.

Based on our previous results with the direct caging of phosphorothioate DNA, we hypothesized that we could employ a similar methodology to regulate this DNA/protein interaction, and effectively control a protein-signaling cascade in a photochemical fashion. Conveniently, all synthetic CpGs possess a substantial degree of thymidine content especially surrounding the CpG sequence. Ideally, by caging some of these residues it will disrupt the CpG interaction with the protein receptor until activated by UV irradiation. This would ultimately afford a high degree of spatio-temporal control over an immune response.
5.5.2. Development of an Assay for Caged CpGs

In order to develop a photochemical mechanism for regulation of the immune response, we first needed to find a viable assay, which is amenable to the detection of CpG activity. We selected a commercially available B-type CpG sequence from Invivogen and synthesized phosphorothioate oligomers with either no caging groups, or 4 caging groups located throughout the sequence. Additionally, we synthesized a control oligomer, which should not be recognized by TLR9, and thus should not elicit an immune response (Figure 5.39).

Non-Caged: \[\text{5'} \text{TCGTCGTTTTGTTCGTTTTGTTCGTT 3'}\]
4A: \[\text{5'} \text{TCG} \text{T} \text{CGTTTTGTTCGTTTTGTTCGTT 3'}\]
4B: \[\text{5'} \text{TCTCGTTTTGTGCCTTTTGTTCGTT 3'}\]
Control: \[\text{5'} \text{TCCAGAACAAGGAAACG 3'}\]

![Caging strategy for CpG sequences](image)

Figure 5.39. Caging strategy for CpG sequences. All sequences possess a complete phosphorothioate backbone, and caged thymidine residues are represented by T.

With the caged oligomer prepared, we next obtained from the Dean lab at NCSU, a HEK-293T cell line stably transfected with a plasmid expressing the TLR9 protein.\(^{354}\) This provided the means of activating TLRs in mammalian cell culture; however, alone does not provide a viable assay, as there is no measure for TLR activation.\(^{354}\) Consequently, we obtained a pNiFty-SEAP plasmid (Invitrogen) which can be introduced into the cell line via co-transfection with the CpG oligomer. This plasmid possesses the human secreted embryonic alkaline phosphatase (SEAP) gene whose expression is controlled by an NF-κB inducible ELAM-1 composite promoter. As TLR9 activation by CpGs leads to an
upregulation of NF-κB transcription factors, this event will increase the amount of SEAP expression within the cells, providing a quantitative measure of TLR9 activation.

We first examined CpG 4A by co-transfecting it (or a control CpG) with the pNiFty-SEAP vector using X-tremeGENE transfection reagent. One set of cells was irradiated at 365 nm for 5 minutes, while the other set remained non-irradiated. The cells were incubated for 48 hours, followed by supernatant removal for a SEAP (SEcreted Alkaline Phosphatase) assay. Using the Phospha-Light Kit (Applied Biosystems), cleavage of the supplied SEAP substrate led to a luminescent signal, which was quantitated on a luminometer.

The control sequence does not elicit a significant amount of TLR9 activation, with relatively low levels of secreted phosphatase (Figure 5.40). Additionally, irradiation does not appear to affect the levels of expression within the control. In the case of the non-caged CpG an approximate 4-fold increase in SEAP signal was observed, and similar levels were detected with or without UV irradiation. This activation tracts well with previous literature reports of 4-5 fold activation when using this system. Gratifyingly, the caged CpG 4A, in the absence of light irradiation possessed similar SEAP levels as the control sequence; however, upon irradiation at 365 nm, the expression levels increased to comparable levels as the non-caged control. This data suggests that it is indeed feasible to regulate the immune response with light using caged CpG motifs (Figure 5.40A).
In order to examine if the position of the caging groups is important, we conducted a similar assay using CpG 4B. In this case, the secreted phosphatase levels were substantially higher, even in the control incubation. However, a similar ~4-fold increase in signal was observed between the control and non-caged CpG experiment. The activity of the caged CpG 4B is slightly higher than the control sequence, indicating some activation of TLR9; however, a significant increase in expression is observed upon decaging (Figure 5.40B).

Overall, the experiments suggest that the photoregulation of the immune response is feasible. It appears that the caged CpG possessing a more even distribution of caging groups is more effective at inhibiting CpG function than when the caging groups are concentrated at the 5’ and 3’ terminus of the sequence. However, both caged oligomers induce the signaling cascade to similar degrees as the non-caged analog. The next step in expanding this technology is the implementation of the caged CpG in more complex biological systems (e.g. multicellular organisms) to trigger an actual immune response. Based on the phosphorothioate backbone, it is our hope that these caged oligomers will be substantially more stable under physiological conditions than standard bacterial CpG, and will remain...
innocuous until irradiated with light. The ability to spatially induce an immune response, affording inflammation and recruiting immune cells has potential therapeutic applications.

5.6. Photoactivation of Restriction Enzyme Activity

5.6.1. Introduction

As previous chapters have demonstrated we have been able to photoregulate a variety of biological processes via the utilization of photocaged DNA oligomers. The majority of these applications involved the disruption of hybridization via the installation of several caging groups on the DNA oligomer. However, we demonstrated a single caging group still enabled DNA/DNA and DNA/RNA hybridization, but could disrupt processing of the oligomer by polymerases and inactivate the catalytic ability of DNAzymes. As a result, we became interested in exploring other biologically relevant processes involving DNA that did not involve caging group perturbation of hybridization. Due to the prevalence of DNA/protein interactions both in vivo and in vitro, we postulated it may be feasible to disrupt processes which relied upon these interactions via our caged thymidine nucleotide.

Restriction endonucleases are enzymes which are capable of the site-specific recognition and cleavage of double stranded DNA. These enzymes were discovered in the 1960’s by Daniel Nathans, Werner Arber, and Hamilton Smith, leading to their receipt of the Nobel Prize in 1978. These enzymes are found within bacteria, and are thought to have evolved as a defense mechanism in response to invading viruses.\(^{359}\) Based on their unique activity, they have been employed extensively in molecular biology and have facilitated the development of recombinant DNA technology and cloning. To date over 3000 restriction enzymes are known, and over 600 are commercially available.\(^{359}\) There are three major
classes of restriction enzymes, which differ in their utilization of cofactors, their target sequence, and the location of their cleavage relative to their target sequence. The most commonly employed restriction endonucleases are from the Type II family, which typically only require Mg$^{2+}$ as a cofactor, recognize a 4-8 base sequence, and cleave directly within that sequence (Figure 5.41). Based on their extensive use in the manipulation of DNA and the site specific mechanism of action, we hypothesized that these enzymes were ideal targets for photochemical regulation using our developed nucleobase caging technology.

![Figure 5.41. Restriction endonuclease cleavage of double-stranded DNA.](image)

5.6.2. Initial Investigations into Photoregulation of Restriction Enzyme Function

We examined our previously synthesized oligonucleotides containing single caged thymidines and found a NheI restriction site at the same location as a caged thymidine residue in the DNAzyme D2. As a proof-of-concept experiment, we were interested in probing the effect of this caging group on NheI activity. In order to do so, we end-labeled the complementary sequence with $^{32}$P and hybridized it with both the caged and wild type DNA oligomer. Gratifyingly, due to the fact that there is only a single caging group on the sequence, hybridization proceeded efficiently, and the double-stranded DNA could be probed for restriction digest. A standard restriction digest was conducted with NheI (1 unit), and the
double-stranded DNA, which was either not irradiated, or exposed to 365 nm light for 5 minutes prior to enzymatic incubation. The reaction was allowed to proceed for 1 hour at 37°C, and was then analyzed by polyacrylamide gel electrophoresis (Figure 5.42).

\[
\begin{align*}
D1 & \quad 5' \text{CGCACCCAGGCTAGC} \quad TACAACGACTCTCTCCG \; 3' \\
D2 & \quad 5' \text{CGCACCCAGGCTAGC} \; \text{TACAACGACTCTCTCCG} \; 3'
\end{align*}
\]

\[
\begin{array}{cccc}
D1 & D1 & D2 & D2 \\
\text{NheI} & - & + & + & + \\
\text{UV} & + & + & - & + \\
\end{array}
\]

*Figure 5.42. Photoregulation of NheI restriction enzyme cleavage.*

Based on the results of the gel, it is apparent that restriction enzyme activity can indeed be regulated in a photochemical fashion. Labeled DNA incubated without the restriction enzyme resulted in no DNA cleavage; however upon the addition of *NheI*, substantial DNA cleavage was observed. Similarly, in the presence of the enzyme, the non-irradiated caged DNA led to virtually no cleavage. Incubation with caged double-stranded DNA which was irradiated, enzymatic activity was restored leading to the cleaved product.
5.6.3. Extended Application of the Photoregulation of Restriction Endonuclease Activity

Based on our initial success, we assessed the generality of this approach. In order to achieve this goal we designed a DNA construct, which possessed multiple restriction sites and thymidine residues at various positions relative to the site of cleavage. Ideally, this should facilitate a better understanding of the mechanism of enzyme inhibition through the presence of the caging group. The EcoRI, BglII, and BamHI, sites were selected for the study as they are some of the most common restriction enzymes employed, and have thymidine residues at variable positions relative to their cleavage site. The non-caged DNA and its complement were obtained and an initial study was performed to demonstrate the efficient cleavage of the substrate by the three enzymes (Figure 5.43).
**Figure 5.43.** Caged restriction enzyme substrates. This DNA sequence possesses three unique restriction sites, and has been systematically modified with caged thymidine residues to probe the response of the restriction enzyme. The EcoRI site is highlighted in yellow, the BglII site is highlighted in blue, and the BamHI site is highlighted green in the non-caged (WT) sequence. The caging groups are indicated by T. The non-caged substrate WT is readily cleaved by all three enzymes.

Initially we explored the propensity for a caging group to inhibit EcoRI based on position of the caged thymidine residue to the site of cleavage. To do so, we selected oligomers T0-T2, T4, and T5, as well as the wild type oligomer. These sequences, both non-irradiated and irradiated for 5 minutes at 365 nm, were digested with the restriction enzyme for 1 hour at 37 °C. The cleavage was then analyzed via polyacrylamide gel electrophoresis (Figure 5.44).
**EcoRI**

wt: 5’ GGGTGAATTCAAGATCTGGATCCAAAAG 3’
T0: 5’ GGGTGAATTCAAGATCTGGATCCAAAAG 3’
T1: 5’ GGGTGAATTTCAGATCTGGATCCAAAAG 3’
T2: 5’ GGGTGAATTTCAGATCTGGATCCAAAAG 3’
T4: 5’ GGGTGAATTTCAGATCTGGATCCAAAAG 3’
T5: 5’ GGGTGAATTTCAGATCTGGATCCAAAAG 3’

<table>
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<th>WT</th>
<th>WT</th>
<th>T0</th>
<th>T0</th>
<th>T1</th>
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<tbody>
<tr>
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<td>-RE</td>
<td>+RE</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
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*Figure 5.44.* Photochemical activation of *EcoRI*. The enzyme was incubated with caged sequences that were either not irradiated, or irradiated at 365 nm for 5 minutes. The cleavage was then assessed by polyacrylamide gel electrophoresis.

The digest provides some interesting perspectives on the ability of the caging group to modulate enzymatic activity. The control reaction in the absence of the restriction enzyme leads to no degradation of the double-stranded DNA (Lane 1, Figure 5.44), while the enzyme in the absence of any caged oligonucleotides results in complete cleavage of the substrate within the 1 hour digest (Lane 2, Figure 5.44). The **T0** construct contains a caged thymidine outside of the recognition site of the enzyme; however, it is only two bases away from the cleavage site. Interestingly, the digest of the non-irradiated substrate leads to a mixture of cleaved and uncleaved substrate (Lane 3, Figure 5.44), which suggests that there is some inhibition of the reaction by the caging group. Additionally, since this is outside of the recognition site of the enzyme, the caging group is not directly affecting the recognition of the substrate, leading to the incomplete cleavage of the substrate, possibly by steric blocking of enzyme recognition. Upon irradiation of **T0**, complete enzymatic cleavage is observed.
(Lane 4, Figure 5.44). Substrates T1, T2, and T5 possess either one or two caging groups within the recognition site of the enzyme, and are either 3 or 4 residues away from the cleavage site. The non-irradiated double-stranded oligonucleotides are not cleaved by EcoRI, however, complete cleavage is observed after a brief UV irradiation (Lanes 5-10, Figure 5.44). Substrate T4 possesses a caging group well outside the enzymatic recognition and cleavage site, and the caging group has no effect on DNA cleavage (Lanes 11-12, Figure 5.44). The amount of cleavage was quantified using ImageQuant software to provide an adequate comparison between the different substrates (Figure 5.45).

Figure 5.45. Analysis of the extent of EcoRI cleavage with different caged substrates. A) Amount of DNA uncleaved after the enzymatic digestion for samples that were either not irradiated, or irradiated. B) Amount of DNA cleaved after the enzymatic digestion for samples that were either not irradiated, or irradiated.

We next investigated the cleavage reaction of BglII. Here, we employed oligomers T2-T4 and T6 to probe the effect of the caging group on BglII activity. Oligomer T3 possesses a caging group within the enzyme recognition site that is 3 nucleotides away from the cleavage site. The T4 oligomer is also within the enzyme recognition site and is 5 bases away from the cleavage site; however is located directly in the cleavage site of the opposite
strand. Finally, both residues caged in T3 and T4 are caged in the T6 oligomer. As a control oligomer T2 was also used, which contains a caging group well outside the recognition site of this enzyme. As with the EcoRI digest, each double-stranded DNA was digested for 1 hour at 37 °C, then analyzed by polyacrylamide gel electrophoresis (Figure 5.46).

![Diagram](image)

Figure 5.46. Photochemical activation of BglII. The enzyme was incubated with caged sequences that were either not irradiated, or irradiated at 365 nm for 5 minutes. The cleavage was then assessed by polyacrylamide gel electrophoresis.

As observed with EcoRI, the digestion of the substrate with BglIII can be regulated in a photochemical fashion via the installation of caged bases. A similar degree of enzymatic digestion was observed using the wild-type DNA (WT) in the presence and absence of restriction enzyme (Lanes 1-2, Figure 5.46). A single caged thymidine completely inhibits enzymatic cleavage if it is located within the enzyme recognition site, as observed with the T3 and T4 double-stranded DNA (Lanes 3 and 5, Figure 5.46). This principle logically extends to the installation of two caging groups, in the case of T6, in which enzymatic activity is abrogated completely prior to irradiation (Lane 7, Figure 5.46). Additionally, the deactivation of substrate towards cleavage is only achievable if the caged residue is within
the enzymatic recognition site as observed by the similar rates of cleavage in T2 (Lanes 9-10, Figure 5.46). In all cases, enzymatic activity is restored upon brief irradiation with UV light at 365 nm (Lanes 4, 6, and 8, Figure 5.46). The degree of cleavage was again analyzed by quantification using ImageQuant software (Figure 5.47).

A

![Graph A](image1)

B

![Graph B](image2)

**Figure 5.47.** Analysis of the extent of BglII cleavage with different caged substrates. A) Amount of DNA uncleaved after the enzymatic digestion for samples that were either not irradiated, or irradiated. B) Amount of DNA cleaved after the enzymatic digestion for samples that were either not irradiated, or irradiated.

Finally we examined the application of this methodology towards the regulation of BamHI on the same substrate. In this investigation we employed the T3-T5 oligomers. In this case only the T5 oligomer possessed a caging group within the recognition site of the enzyme, 3 bases from the cleavage site. Oligomer T4 contained a caging group outside of the recognition site, but only 2 bases away from the cleavage site. The T3 oligomer was used as a control caged sequence, in which the caging group was spaced substantially away from the enzyme recognition and cleavage site. Again, the enzymatic digestions were incubated for 1 hour at 37 °C, prior to analysis by gel electrophoresis (Figure 5.48).
Figure 5.48. Photochemical activation of BamHI. The enzyme was incubated with caged sequences that were either not irradiated, or irradiated at 365 nm for 5 minutes. The cleavage was then assessed by polyacrylamide gel electrophoresis.

The photochemical regulation of restriction endonuclease activity appears to be general, as digestion was again regulated via light irradiation. When employing the non-caged primer, standard results in the presence and absence of the restriction enzyme were observed (Lanes 1-2, Figure 5.48). The BamHI enzyme was incapable of enzymatic cleavage when the caging group was centered in the enzyme recognition site (T5, Lane 3, Figure 5.48). A similar effect was observed when the caging group was outside the enzyme recognition site; however, inhibition was slightly less efficient (T4, Lane 5, Figure 5.48). Again, no effect on enzymatic cleavage was observed when the caging group was further separated from the enzyme cleavage and recognition site in T3 (Lanes 7-8, Figure 5.48). Irradiation by UV light appears to restore the degradation of the DNA (Lanes 4 and 6, Figure 5.48). Again, the extent of BamHI cleavage was determined by quantification using ImageQuant software (Figure 5.49).
5.6.4. Investigation of the Effects of Caging Groups on Hyperthermophilic Restriction Enzymes

Finally, we prepared a substrate for digestion that possessed a restriction site for TaqαI, a hyperthermophilic restriction endonuclease with an optimal activity at 65 °C. We were interesting in exploring the scope of the technology by using this enzyme, which is active at high temperatures where DNA hybridization is weaker leading to faster DNA cleavage. While we examined enzymatic activity at the optimal temperature, we surreptitiously also probed enzymatic activity at 37 °C. The caged substrate was again hybridized with its radioactively labeled complement, then subjected to a 1 hour digestion at the appropriate temperature (Figure 5.50).
Interestingly, in the case of TaqαI, the presence of a caging group (P3) was found to actually activate enzymatic cleavage of the substrate at 37 °C. At this temperature the non-caged substrate (P1) remains uncleaved as the enzyme is not appreciably active (Lane 1, Figure 5.50); however, the caged substrate without irradiation affords a substantial amount of substrate degradation (Lane 3, Figure 5.50). This activation is abrogated upon irradiation, as very little substrate cleavage is observed in the absence of a caging group (Lane 2, Figure 5.50). At the optimal temperature, the caging group has no effect on the enzymatic cleavage as virtually all substrate is cleaved irrespective of the presence of a caging group (Lanes 4-6, Figure 5.50). To ascertain if this effect is specific to the enzyme, or if this substrate is simply prone to degradation, we conducted the enzymatic digest with the caged substrate and a variety of restriction endonucleases (Figure 5.51). However, this effect was only observed in the presence of the TaqαI enzyme, as no other enzyme was capable of cleaving the substrate.

<table>
<thead>
<tr>
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<td>P1</td>
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</table>

Figure 5.50. Investigation of the effects of caging group on inhibition of TaqαI activity.
In summary, we have effectively demonstrated both the activation and deactivation of restriction endonucleases via the installation of a photolabile protecting group on the DNA substrate of these enzymes. The results suggest that an interplay of enzyme recognition and cleavage inhibition give rise to this phenomenon; however future work must be conducted to elucidate the details of the restriction site protection with caging groups. This can potentially be accomplished via fluorescence polarization assays, detecting whether the enzyme can no longer bind to the substrate, or if it is capable of recognizing the substrate, but incapable of cleaving it. Based on the current results, coupled to the site-specificity of these enzymes, we hypothesize that enzyme recognition plays a larger role in restriction enzyme regulation by caging groups, than inhibition of catalysis, as catalysis is blocked even when caging groups are distanced from the site of cleavage. Additionally, significant work must be performed to better understand the activation of the hyperthermophilic Taqα restriction endonuclease. Specifically, a crystal structure of the hybridized caged substrate could provide deeper

Figure 5.51. Analysis of the room temperature cleavage of P3 by various restriction enzymes.
insight into the perturbation of the DNA double helix in presence of the caging group, thus facilitating cleavage. Gratifyingly, in all cases, normal endonuclease activity is restored upon the photochemical removal of the caging group, confirming that it is the agent of regulation.
5.7 Experimental

**DNA synthesis protocol.** DNA synthesis was performed using an Applied Biosystems (Foster City, CA) Model 394 automated DNA/RNA Synthesizer using standard β-cyanoethyl phosphoramidite chemistry at the Wake Forest University Nucleotide Core Facility. All caged DNAzymes were synthesized using 40 nmole scale, low volume solid phase supports obtained from Glen Research (Sterling, VA). Reagents for automated DNA synthesis were also obtained from Glen Research. Standard synthesis cycles provided by Applied Biosystems were used for all normal bases using 2 minute coupling times. The coupling time was increased to 10 minutes for the positions at which the caged-T modified phosphoramidites were incorporated. Each synthesis cycle was monitored by following the release of dimethoxy trityl (DMT) cations after each deprotection step. No significant loss of DMT was noted following the addition of the caged-T for any of the DNAzymes D1-D7, so 10 minutes was sufficient to allow maximal coupling of the caged-T. Yields of D1-D7 were close to theoretical values routinely obtained.

**Decaging and DNAzyme activation through light irradiation.** RNA substrate (5’ GGAGAGAGAGUGGGUGCG 3’) was purchased from IDT and end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P] ATP (37°C, 30min). The labeled substrate was purified by Microcon 3 centrifugation and resuspended in DEPC water. DNAzyme assays were performed under single turnover conditions with the DNAzyme (40nM) and the RNA substrate (4nM) in standard reaction buffer (100 mM MgCl2, pH 8.2, 15 mM Tris base). The DNAzyme was equilibrated at 37°C in the reaction buffer for approximately 15 minutes, followed by the addition of RNA substrate to initiate the reaction.
In the case of photochemical activation of caged DNAzyme, the substrate was irradiated in a disposable cuvette with either a handheld Spectroline UV lamp (23W) or a UVP Transilluminator (25W) at a distance of approximately 5 cm prior to equilibration at 37 °C and RNA substrate addition. Aliquots of the reaction were removed at 0.5, 1, 3, 5, 7, 10, 15, and 30 min and the reaction was stopped via addition of 6X stop/loading dye. The samples were analyzed by 15% denaturing PAGE (160V, 40min). Acrylamide gels were visualized using a Storm phosphorimaging system, and radioactive band intensities were quantified using Image Quant 5.2 and correlated to RNA concentrations (Figure S1). To obtain the rate constants under single-turnover conditions, exponential decay curves (cleaved RNA is proportional to $-e^{kt}$, with k being the first order rate constant) were fitted through the data points using Origin 5.0. Initial studies with D2 were performed to elucidate the optimal irradiation conditions to achieve the greatest DNAzyme activation. Ultimately, the best results ($k = 0.054\pm0.013$) were found for a 1 min irradiation (365nm) using a transilluminator (25W).

**HPLC Assessment of Decaging.** To ensure effective decaging, samples were analyzed on a Hamilton reverse phase preparatory column (10 µM, 250 x 4.1 cm, PRP-1) via HPLC on a Waters 2796 HPLC coupled with a Waters 2996 photodiode array. A 10 µM sample of non-caged DNAzyme was initially analyzed to establish optimal conditions (90% H₂O/ 10% acetonitrile isocratic 10 min, ramp to 90% acetonitrile 10 min; each solvent with 0.1% TFA).

**Polymerase Extension Reactions.** All DNA polymerases were obtained from New England Biolabs and used with the supplied buffers. Non-caged DNA controls were obtained from
Integrated DNA Technologies (IDT), and pGFPuv was obtained from Clontech. The caged Thymidine monomer was prepared according to the previously reported route. Control mutagenesis reactions were performed using a Stratagene QuikChange Site-Directed Mutagenesis Kit, following standard protocols. Oligonucleotides were end labeled using $\gamma^{32}$P-ATP (MP Biomedicals) and T4 Kinase (New England Biolabs) at 37 °C for 1 hour, and then purified using TE Midi Select-D, G25 microcentrifuge spin columns (Shelton Scientific).

### Primer Extension Reactions.

Three templates D1 (5’ CGCACCCAGGCT*AGCTACAACGACTCTCTCCG 3’), D2 (5’ CGCACCCAGGCTAGC*ACAACGACTCTCTCCG 3’), and WT (5’ CGCACCCAGGCTAGCTACAACGACTCTCTCCG 3’) were resuspended in sterile water (10 µM). The template (20 pmol) was then incubated with end-labeled $^{32}$P primer (5’ AATGGGCGGAGAGAG 3’) with either DNA Polymerase I, T7 DNA Polymerase, or T4 DNA Polymerase (5 units) in the appropriate buffer for 1 hour at 37 °C. The DNA Polymerase was then deactivated at 70 °C for 10 minutes, and the reaction was separated on a 25% polyacrylamide gel (400V, 40 min).

### Caged Primers in the Polymerase Chain Reaction.

Standard PCR reactions were conducted using either WT, P1, P2, or P3 forward primers (50 pmol) with an end-labeled $^{32}$P reverse primer (5’ AGCGATCGCTATTTTCCATG 3’; 50 pmol). Additionally plasmid template (1 ng), dNTPs (1 mM each), and appropriate buffer were added and diluted to 50 µL with dH$_2$O, followed by the addition of either Taq Polymerase (5 units), or Phusion...
Polymerase (2 units). An initial denaturation at 95 °C (2 min), followed by 30 cycles consisting of 95 °C (30 sec), 50 °C (30 sec), and 72 °C (1 min). The reactions were then separated on a 20% polyacrylamide gel (400V, 30 min) and imaged on a Storm Phosphorimager.

**Table 1.** Primer set employed in the analysis of PCR read-through

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5' CTGATTTCGACCAGGTT 3'</td>
<td>Forward PCR Primer</td>
</tr>
<tr>
<td>P1</td>
<td>5' CTGATTTCGACCAGGTT 3'</td>
<td>Forward PCR Primer</td>
</tr>
<tr>
<td>P2</td>
<td>5' CTGATTTCGACCAGGTT 3'</td>
<td>Forward PCR Primer</td>
</tr>
<tr>
<td>P3</td>
<td>5' CGACCAGGTT 3'</td>
<td>Truncated PCR Primer</td>
</tr>
</tbody>
</table>

**Optimized Mutagenesis Protocol.** Primers to accomplish point mutations (P4-P10), insertions (P11-P12), and deletions (P13-P14) with varying numbers of caging groups and base pair overlaps were designed and synthesized (Table 1). Both the forward and reverse primers were used (50 pmol each) with a pGFPuv template (0.1 ng) in a PCR reaction with Phusion DNA Polymerase (2 units). An initial denaturation at 95 °C was performed, followed by 40 cycles consisting of 95 °C (30 sec), 40 °C (60 sec), and 72 °C (3.3 min), with a final extension at 72 °C (2 min). An identical PCR reaction was then repeated, using 5 µL of the previous reaction as the template, followed by purification with a PCR cleanup kit (Promega). The purified product was then irradiated 8 minutes on a transilluminator (25 W), and subjected to a hybridization protocol (95 °C for 5 minutes followed by three cycles of 65 °C for 5 minutes and 30 °C for 15 minutes). The hybridized DNA was then transformed (2 ng, 30 min 4 °C, 45 sec at 42 °C, 2 min at 4 °C, followed by a 1 hr recovery at 37 °C) into chemically competent Top 10 cells (4 × 10^7 cfu) and 100 µL was plated on Ampicillin LB.
(Luria-Bertani) plates. Colonies were assessed for mutations via visual observation of GFP, by PCR screens, and through DNA sequencing.

**Table 2. Primers for light controlled mutagenesis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>5' AATAATGAGTAAAGGAGAAGAAC 3'</td>
<td>GFP Stop Codon Foward</td>
</tr>
<tr>
<td>P5</td>
<td>5' ATTTATTCTACGGGTACCCGGG 3'</td>
<td>GFP Stop Codon Reverse</td>
</tr>
<tr>
<td>P6</td>
<td>5' AATAATGAGTAAAGGAGAAGAAC 3'</td>
<td>GFP Stop Codon Foward</td>
</tr>
<tr>
<td>P7</td>
<td>5' ACTCATATTCTACGGGTACCCGGG 3'</td>
<td>GFP Stop Codon Reverse</td>
</tr>
<tr>
<td>P8</td>
<td>5' ACCGGTAGAATGAAAGGAGAAGAAC 3'</td>
<td>GFP Stop Codon Foward</td>
</tr>
<tr>
<td>P9</td>
<td>5' ACCGGTAGAATGAAAGGAGAAGAAC 3'</td>
<td>GFP Start Codon Foward</td>
</tr>
<tr>
<td>P10</td>
<td>5' ATTTTTTCTACGGGTACCCGGG 3'</td>
<td>GFP Start Codon Reverse</td>
</tr>
<tr>
<td>P11</td>
<td>5' ACCGGTAGAATGAAAGGAGAAGAAC 3'</td>
<td>GFP Deletion Forward</td>
</tr>
<tr>
<td>P12</td>
<td>5' ATTTTTTCTACGGGTACCCGGG 3'</td>
<td>GFP Start Codon Reverse</td>
</tr>
<tr>
<td>P13</td>
<td>5' ACCGGTAGAATGAAAGGAGAAGAAC 3'</td>
<td>GFP Deletion Reverse</td>
</tr>
<tr>
<td>P14</td>
<td>5' ATTTTTTCTACGGGTACCCGGG 3'</td>
<td>GFP Deletion Forward</td>
</tr>
</tbody>
</table>

**Phosphorothioate DNA Synthesis Protocol.** DNA synthesis was performed using an Applied Biosystems (Foster City, CA) Model 394 automated DNA/RNA Synthesizer using standard β-cyanoethyl phosphoramidite chemistry. All caged DNAzymes were synthesized using 1 umole scale, low volume solid phase supports obtained from Glen Research (Sterling, VA). Reagents for automated DNA synthesis were also obtained from Glen Research. Standard synthesis cycles provided by Applied Biosystems were used for all normal bases using 2 minute coupling times. The coupling time was increased to 10 minutes for the positions at which the caged-T modified phosphoramidites were incorporated. Each synthesis cycle was monitored by following the release of dimethoxy trityl (DMT) cations after each deprotection step. No significant loss of DMT was noted following the addition of
the caged-T for any of the phosphorothioates, so 10 minutes was sufficient to allow maximal coupling of the caged-T. Sulfurization of each base position was performed following each coupling step using 3H-1,2-benzodithiol-3-one 1,1-dioxide, known as Beaucage Reagent, obtained from Glen Research. The reagent was dissolved in acetonitrile at a concentration of 1 g / 100 mL. Following the final coupling step and sulfurization, the DMT-protecting group was left on the completed oligonucleotide. After deprotection and cleavage from the resin, the DMT-containing phosphorothioates were isolated using a C-18 reverse phase solid phase extraction column (Waters Sep-Pak Classic C18 cartridge WAT051910). Removal of the last DMT group was accomplished by treatment of the oligonucleotide bound to the Sep-Pak column with 2% (v/v) trifluoroacetic acid in water and the fully deprotected oligo was eluted with 40% (v/v) MeOH in water.

**Hybridization Assay.** Complementary RNA was purchased from IDT DNA. Melt curves were measured on a BioRad MyiQ RT-PCR thermocycler by conducting a sequence of 3 heating and cooling cycles (10 µM of both PS DNA and complementary RNA with 12.5 µL iQ SYBR Green Supermix to a total volume of 25µL; 30 °C to 80 °C with a 0.5 °C/min ramp). The non-caged PS DNA, 3-caged PS DNA, 4-caged PS DNA, and control PS DNA were analyzed in triplicate, and samples were either irradiated for (365nm, 5min, 23W), or were not irradiated prior to measurement of the melting temperature.

**HPLC Assessment of Decaging.** To ensure effective decaging, samples were analyzed on a Hamilton reverse phase preparatory column (10 µM, 250 x 4.1 cm, PRP-1) via HPLC on a Waters 2796 HPLC coupled with a Waters 2996 photodiode array. A 10 µM sample of non-
caged PS DNA was initially analyzed to establish optimal conditions (90% H₂O/10% acetonitrile isocratic 10 min, ramp to 90% acetonitrile 10 min; each solvent with 0.1% TFA). The 3-caged PS DNA was then run (10 µM) and the same sample was then irradiated with a hand-held UV lamp (365nm, 5min, 23W) and injected again. The original peak at 3.3 min completely disappeared and the chromatogram was similar to the wild type chromatogram with a peak at approximately 2.3 min.

**Luciferase Assay.** Mouse fibroblast cells (NIH/3T3) were grown at 37 °C and 5% CO₂ in Dulbecco’s modified Eagle’s media (DMEM; Hyclone); supplemented with 10% Fetal Bovine serum (FBS; Hyclone) and 10% streptomycin/ampicillin (MP Biomedicals). Cells were passaged into two 96 well plates (200 µL per well; ~1×10⁴ cells) and grown to ~80% confluence within 24 hours. The media was changed to OPTIMEM (Invitrogen), and the cells were co-transfected with pGL3 (0.43 µg, Promega), pRL-TK (0.043µg, Promega), and the phosphorothioate DNA (250 pmol) using X-Treme GENE (3:2 reagent/DNA ratio; Invitrogen). The following conditions were used: no phosphorothioate oligomer, a sense-strand control phosphorothioate oligomer, the non-caged *Renilla* luciferase targeting phosphorothioate, and the phosphorothioate with either 3 or 4 caging groups. All transfections were performed in triplicate. Cells were incubated at 37 °C for 6 hours and the transfection media was removed. One of the 96 well plates was briefly irradiated with a hand-held UV lamp (365 nm, 25W) for 5 min. The media was then replaced with standard growth media and the cells were incubated for an additional 24 hours. After the 24-hour incubation, the cells were observed and no changes in growth or morphology were visible when comparing the irradiated cells with the non-irradiated cells. Following the visible
inspection, the media was removed and the cells were assayed with the Dual-Luciferase Reporter Assay system (Promega) using a Wallac VICTOR\textsuperscript{3} luminometer with a measurement time of 1 s and a delay time of 2 s. The ratio of \textit{Renilla} to Firefly luciferase expression was calculated for each of the triplicates, the data was averaged, and standard deviations were calculated using Microsoft Excel.

**Spatial Control of PS DNA Activity.** Mouse fibroblast cells (NIH/3T3) were grown at 37 °C and 5% CO\textsubscript{2} in Dulbecco’s modified Eagle’s media (DMEM; Hyclone); supplemented with 10% Fetal Bovine serum (FBS; Hyclone) and 10% streptomycin/ampicillin (MP Biomedicals). Cells were passaged into a 6-well plate (2 mL per well; ~1×10\textsuperscript{4} cells) and grown to ~80% confluence within 24 hours. The media was changed to OPTIMEM (Invitrogen), and the cells were transfected with pRL-TK (0.5µg, Promega), and the phosphorothioate DNA (250 pmol) using X-Treme GENE (3:2 reagent/DNA ratio; Invitrogen). The following conditions were used: no phosphorothioate oligomer, a sense-strand control phosphorothioate oligomer, and the phosphorothioate with either 3 or 4 caging groups. All transfections were performed in triplicate. Cells were incubated at 37 °C for 6 hours and the transfection media was removed. A mask was made using aluminum foil, and cells were irradiated from underneath for 3 minutes with a hand-held UV lamp (365 nm, 25W). The media was then replaced with standard growth media and the cells were incubated for an additional 24 hours. After the 24 hour incubation, the cells were observed and no changes in growth or morphology were visible when comparing the irradiated areas with the non-irradiated areas. Following the visible inspection, the media was removed and replaced with 1 mL of fresh media and 4 µg/mL of coelenterazine (Gold Biotechnologies) and
incubated at room temperature for 10 min. The 6 well plate was then imaged on a Xenogen Lumina in vivo imaging system with a 60 s exposure time.

**Cell Viability Assay.** In order to assess the cellular toxicity of UV irradiation, 3T3 cells were either irradiated (hand held UV lamp, 365 nm, 5 min, 25W) or not irradiated (3 wells each in a 96-well plate). Cultures were allowed to grow for 24 hours (a comparable time to the luciferase experiment), and then assayed using Cell Titer Blue Assay (Promega). A control experiment with dead cells was achieved via dessication of three wells. To each well 20uL of Cell Titer Blue reagent was added and incubated with the cells for 4 hours. Fluorescence measurements (λ<sub>ex</sub> = 530 nm/λ<sub>em</sub> = 590 nm) were taken on a Labsystems Fluoroskan Ascent FL plate reader. Non irradiated cells: 240 ± 34 RFU; irradiated cells: 233 ± 34 RFU; dead cells: 13 ± 1 RFU. Thus, no measurable cytotoxicity was observed as a direct effect of UV irradiation.

**Photochemical CpG Activation.** HEK-293T Cells stably expressing TLR-9 were grown (DMEM, 10% FBS, 0.5mg/mL G418-Sulfate, 10 µg/mL Ciprofloxacin), and passaged into two 96 well plates. Cells were transfected at 60% confluence with pNIFTY (1 µg; Invivogen) and CpG (250 pmol) using X-TREME Gene transfection reagent (Roche). After 4 hours, transfection media was removed and one well plate was irradiated 5 minutes with a hand-held UV lamp (23W). All transfections were conducted in triplicate. After irradiation, growth media was replaced, and cells were incubated for 48 hrs at 37 °C (5% CO₂). After incubation the media was removed and used in the Phospha-Light SEAP assay (Applied
Biosystems). Luminescence measurements were taken using a Wallac VICTOR3V luminometer with a measurement time of 1 s and a delay time of 2 s.

**Photochemical Restriction Enzyme Digests.** Synthetic DNA constructs were purchased from IDT DNA, and caged oligomers were prepared by our collaborator Dr. Mark O. Lively as previously discussed. Oligonucleotides were end labeled using $\gamma^{32}$P-ATP (MP Biomedicals) and T4 Kinase (New England Biolabs) at 37 °C for 1 hour, and then purified using TE Midi Select-D, G25 microcentrifuge spin columns (Shelton Scientific). The $\gamma^{32}$P-end labeled substrate (10 µL; 1 nmol) was incubated with its complementary strand (10 µL; 1 nmol) at 90 °C for 1 min, and then gradually cooled to 4 °C over 2 hours. The dsDNA construct (2 µL; 0.1 nmol) was then subjected to an 50 µL enzymatic digest according to manufacturer’s protocols with the appropriate buffer (New England Biolabs). Upon completion, the enzyme was deactivated (70 °C, 20 min), and digests were analyzed on a 20% polyacrylamide gel (400V, 40 min). Acrylamide gels were visualized using a Storm phosphorimaging system, and radioactive band intensities were quantified using Image Quant 5.2.
CHAPTER 6: PHOTOREGULATION OF RNA FUNCTION

RNA has proven to be a versatile biomolecule capable of not only its traditional biological role, the transient storage of genetic information, but also of catalysis and small molecule recognition. RNA has been demonstrated to catalyze a number of important organic reactions.\(^{361}\) Specifically, there are catalytically active RNA sequences known as ribozymes. These molecules are naturally occurring and are capable of promoting sequence-specific phosphodiester bond scission of nucleic acids.\(^{286}\) Additionally, recent studies have proven that RNA may also be involved in the modulation of gene expression via binding to naturally occurring metabolites.\(^{362}\) In addition to ribozymes, RNA has been found to have the ability to adopt complex secondary structures which are capable of binding both small molecules as well as other biomacromolecules. These three-dimensional sequence specific RNA structures are referred to as aptamers, and are pertinent to both therapeutic and sensing applications.\(^{363}\)

Although, traditionally proteins have been considered the most important biological catalysts and regulators, RNA possesses several features that more easily facilitate enzymatic design and gene control, making it ideal for bioengineering. Perhaps the greatest advantage of RNA over proteins is the development of \textit{in vitro} molecular techniques that afford the ability to enzymatically replicate, amplify, and sequence these molecules. These properties can be exploited to select RNA sequences with unique properties. The process of \textit{in vitro} selection of RNA involves the fusion of molecular biology techniques with chemistry, becoming a valuable tool in the design of RNA sequences. Through the SELEX process (\textit{Systematic Evolution of Ligands by EXponential enrichment}) RNA aptamers have been engineered against numerous targets including a variety of small organic molecules, e.g.
Traditionally, in vitro selections have been performed via the immobilization of the molecule of interest on a resin, followed by incubation with RNA molecules possessing a randomized region. However, a new means of selecting aptamers has recently been developed involving allosteric ribozymes. This type of selection is advantageous as no functional handles are required on the small molecule for resin immobilization, and the evolved aptamer is evolved in the context of a functional ribozyme. This allosteric ribozyme can then more directly be applied to gene regulation, whereas aptamers isolated from resin selections must be functionalized for biological activity (e.g. incorporated into a ribozyme, riboswitch, or sensor).

The overarching goal of our research is the development of novel technologies for the light-induced in vivo regulation of RNA. RNA is substantially more challenging to photoregulate than DNA and proteins due to its widely accepted instability in a cellular environment and lack of transcriptional systems which afford its modification. The first examples of in vivo photocontrol of RNA function involve mRNA. The groups of Okamoto and Tsien reported light-activated gene expression in zebrafish embryos by employing chemically modified, caged mRNA. Specifically, in vitro generated mRNA encoding GFP or β-galactosidase reporter genes was treated with diazo-modified hydroxycoumarin, resulting in the formation of statistically caged mRNA on the phosphate backbone. This RNA was injected into the cytoplasm of one-cell-stage zebrafish embryos which were subsequently incubated at 28 °C in the dark. After irradiation with UV light, 2/3 of the embryos showed expression of GFP, whereas non-irradiated zebrafish displayed little to no fluorescence (Figure 6.1). Quantification of β-galactosidase activity indicated a 4.5-fold increased expression level after irradiation.
Moreover, spatially restricted activation of caged mRNA was achieved by using a microscope equipped with a special illumination system. Although an interesting approach, the level of activation achieved is modest and the necessity to transcribe mRNA \textit{in vitro} followed by caging with varying efficiency hampers a widespread application of this technology.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6_1.png}
\caption{Photochemical regulation of gene function in zebrafish through injection of caged mRNA. a) Wild type GFP mRNA; b) caged GFP mRNA; c) caged GFP mRNA followed by irradiation with UV light (365 nm, 10 sec).}
\end{figure}

While we are interested in the direct caging of mRNA, we hope to accomplish this in a more controlled fashion via the site-specific incorporation of caged nucleosides. Our previous experience involving the application of caging groups on DNA (see Chapter 5) can be readily transferred to the context of RNA. However, as previously noted, the implementation of these methodologies is challenging on the RNA level due to the chemical and enzymatic instability of RNA. Additionally, while the site specific caging of RNA is scientifically interesting and applicable to addressing biological questions, we are also interested in harnessing the other properties of RNA. Specifically, the photochemical control of RNA catalysis and its recognition properties is a technique that has substantial application to the regulation of genes. Due to the inherent versatility of RNA function, we have developed several divergent approaches to the photochemical regulation of this biomolecule. The ultimate goal is then to translate these \textit{in vitro} techniques to an \textit{in vivo} setting.
Theoretically, this will lead to the successful \textit{in vivo} regulation of RNA, providing a spatio-temporal means of studying and investigating gene function.

6.1. Photoactivation of Theophylline Allosteric Ribozyme Function

6.1.1. Introduction

The hammerhead ribozyme is a small RNA motif consisting of three loops that intersect at a conserved catalytic core. It is able to promote the sequence specific cleavage of RNA phosphodiester bonds with a rate of about 1 min$^{-1}$ at 25$^\circ$C and neutral pH. This represents a 10$^6$-fold rate enhancement compared to the non-catalyzed hydrolysis of RNA.$^{283}$ Using Watson-Crick base pairing, the hammerhead ribozyme has been engineered to cleave a variety of different RNA substrates.$^{286}$ Numerous studies have shown that \textit{trans}-acting ribozymes can be successfully employed in living cells to knock-down expression of specific genes.$^{371, 372}$ Advantages of utilizing ribozymes in Functional Genomics studies include, 1) unlike siRNA, ribozymes do not rely on the cellular machinery to inactivate the target mRNA, 2) cleavage by a ribozyme is a catalytic process, and 3) ribozymes can be easily expressed \textit{in vivo}. This technology has found wide application in basic and applied research, and several ribozymes are currently being investigated in clinical trials.$^{373}$

Recently, the catalytic capabilities of these RNAzyme molecules have been further tuned via the integration of a small-molecule binding aptamer sequence to the ribozyme yielding an allosteric ribozyme. For example, an allosteric hammerhead ribozyme composed of a catalytic domain connected to a theophylline binding domain via a communication module has been created (other small organic ligands for allosteric ribozymes include e.g. ATP, and cAMP).$^{285, 286, 374}$ Upon binding of the ligand, base pairing at the communication
module linking the aptamer and the catalytic domain is stabilized. This activates the ribozyme leading to subsequent cleavage of the RNA substrate (Figure 6.2). This technology has been applied to the generation of RNA based sensors for small organic molecules and to the evolution of new aptamers using an *in vitro* selection.\textsuperscript{285, 374} However, its utilization in the small molecule mediated regulation of gene expression has not yet been exploited.

![Figure 6.2. General structure and activation of allosteric ribozymes.](Image)

### 6.1.2. Development of a Theophylline Caging Strategy

Our initial attempts at photoregulation at the RNA level involve the utilization of a hammerhead ribozyme. A photo-activatable ribozyme represents a potential photochemical gene control element.\textsuperscript{375} This is a convenient approach because it involves minimal handling of RNA, as a small molecule regulator can be chemically modified instead of the RNA directly. Additionally, ribozymes are well studied in terms of their catalysis, providing a wealth of literature resources. Our initially proposed system for photochemical activation was comprised of 1) a photo-caged small organic ligand\textsuperscript{304, 376, 377} and 2) an allosteric ribozyme which is activated by this ligand.

For 1), we selected theophylline (291) as a small organic ligand, due to its absence in bacterial, yeast, and mammalian cells, its high solubility in an aqueous environment, its low
toxicity, and its ability to bind selectively to certain RNA sequences.\textsuperscript{378, 379} Since 291 is not present in animal cells it can be used as an exogenous regulator of gene control in model organisms.\textsuperscript{380, 381} Hence, we synthesized the photocaged compounds 292-295, blocking the 9-NH group as previous studies have demonstrated that this group is crucial for efficient binding to a known RNA aptamer (Scheme 6.1).\textsuperscript{378, 379}

\[
\text{Scheme 6.1. Caged theophyllines 292-295, and their half-life times under irradiation conditions (365 nm, 23 W, hand-held UV lamp).}
\]

In 292-295 we caged this position with a photo-removable nitrobenzyl group. Irradiation with non-photodamaging UV light (365 nm, 23 W, hand-held UV lamp) irreversibly removes the photo-protecting group and delivers free theophylline (276) (Scheme 6.1). Our studies commenced with 292 (Figure 6.3), which is easily synthesized and decages readily. However, it produces a potentially toxic benzaldehyde as a byproduct in the decaging reaction and displays low solubility in aqueous environments. This problem was solved by synthesizing the carboxy modified molecule 293. It displayed high aqueous solubility but unfortunately slow decaging properties. In 294 we employed a carbamate protecting group which has been proven to provide rapid decaging. However, 294 was highly
unstable in an aqueous environment. Finally, with 295 we introduced the (2-nitropiperonyl)oxymethyl (NPOM) group as a new amino photo-protecting group, and investigated its decaging properties (Scheme 6.1). 382

![Graph](image)

**Figure 6.3.** Decaging timecourse of compound 292.

The caged theophylline (295) was prepared via the reaction of the activation of (α-Methyl-6-nitropiperonyloxy)methylthiomethylether (298) into a chloromethylether, followed by reaction with theophylline. Precursor 297 was prepared via methylation of nitropiperonal (296) with trimethylaluminum, and subsequent installation of the methylthiomethylether functionality (Scheme 6.2).

This caging group has several advantages over other amino caging groups: it decages rapidly via irradiation with non-photodamaging UV light, it produces a less toxic acetophenone byproduct in the photo-decaging, the caged molecule is highly stable in an aqueous environment at various pH, and the caging step proceeds in high yield. Moreover, the caged theophylline 295 is non-toxic (Figure 6.4).
Figure 6.4. Growth curves of *E. coli* incubated with no small molecule, theophylline (291) and NPOM caged theophylline 295 (1 mM). No significant alteration in growth is observed as a result of incubation with either small molecule.

For this approach to be viable in an *in vivo* context, effective uptake of the small molecule must occur, and thus we set forth to elucidate if the caging group dramatically affected the physiological uptake of theophylline. Cellular uptake of the caged molecule was assessed in zebrafish embryos. These embryos (~20 animals in each experiment) were treated with both theophylline and NPOM caged theophylline 295 directly after fertilization and observed 24 hours later (Table 6.1). Overall, no phenotypic differences were visually observable after incubation with either compound and in all experiments a survival rate of 75-89% was observed (84% in case of the H2O control). The embryos were then snap-frozen, lysed, and analyzed by LC/MS.
Table 6.1. Zebrafish embryo incubations with theophylline and NPOM caged theophylline

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time Treated</th>
<th>Time Observed</th>
<th>Incubation Conc.</th>
<th>Alive Embryos</th>
<th>Cellular Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O control</td>
<td>0-24hpf</td>
<td>24hpf</td>
<td>-</td>
<td>84%</td>
<td>-</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0-24hpf</td>
<td>24hpf</td>
<td>1.0 mM</td>
<td>74%</td>
<td>0.17mM</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0-24hpf</td>
<td>24hpf</td>
<td>0.5mM</td>
<td>79%</td>
<td>0.11mM</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0-24hpf</td>
<td>24hpf</td>
<td>0.25mM</td>
<td>85%</td>
<td>0.11mM</td>
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<td>Theophylline</td>
<td>0-24hpf</td>
<td>24hpf</td>
<td>0.1mM</td>
<td>89%</td>
<td>0.10mM</td>
</tr>
<tr>
<td>295</td>
<td>0-24hpf</td>
<td>24hpf</td>
<td>0.25mM</td>
<td>75%</td>
<td>0.14mM</td>
</tr>
<tr>
<td>295</td>
<td>0-24hpf</td>
<td>24hpf</td>
<td>0.1mM</td>
<td>75%</td>
<td>0.10mM</td>
</tr>
</tbody>
</table>

6.1.3. Application of Caged Theophylline to Allosteric Ribozyme Activation

The allosteric ribozymes we chose for this study are comprised of the catalytic domain of a hammerhead ribozyme coupled with a theophylline aptamer via a communication module shown in Figure 6.5. Ribozyme A cleaves itself in an intramolecular fashion and is used as a model system. Ribozyme B can be utilized to cleave virtually any RNA by tailoring its flanking sequences.

![Diagram of ribozymes A and B](image)

**Figure 6.5.** Theophylline responsive ribozymes that cleave RNA in a *cis* (intramolecular) (A) and a *trans* (intermolecular) (B) fashion. B is shown with its RNA substrate bound. They consist of an aptamer domain (I), a communication module (II), and a catalytic domain (III). The flanking regions (IV) define the target RNA.

In the absence of the small organic molecule the allosteric ribozyme A is inactive, however in the presence of its effector 291, binding to the aptamer occurs. This event is
transmitted to the catalytic domain via the communication module, activating the ribozyme to induce intramolecular cleavage. The RNA degradation was detected by polyacrylamide gel electrophoresis of radiolabeled oligonucleotides. The gel shown in Scheme 6.3 displays the photoactivation event: In absence of the small organic ligand the ribozyme is inactive and remains uncleaved even after 90 min of incubation (lane 1). Presence of theophylline (291) induces complete cleavage after 90 min (lane 2). In contrast, the photocaged theophylline 295 does not activate the ribozyme as lane 3 shows, verifying the importance of the 9-NH group. However, lanes 4-6 display the progressing cleavage of the photo-activated ribozyme by irradiation with a hand-held UV lamp (365 nm, 23 W) for just 5 minutes. Complete cleavage was observed after 90 min.
We were also interested in investigating the photoregulation of the ribozyme cleavage in a trans fashion. This has been demonstrated for non-allosteric ribozymes directed against the mRNA expression product of targeted gene.\textsuperscript{384-389} As it is possible to introduce modified sequences in the flanking regions of the ribozyme, this method can be specifically tailored to knockdown virtually any gene of interest. The sequence of the ribozyme B and the target RNA are depicted in Figure 6.4. Rapid decaging of 295, via irradiation with a hand-held UV lamp (365 nm, 23 W) for 5 minutes, leads to activation of the allosteric ribozyme B, followed by complete RNA degradation within 480 minutes. This photo-activated RNA cleavage is documented by an SDS PAGE gel (Scheme 6.4). Only the 5' $^{32}$P-endlabeled RNA substrate
(13 nucleotides) is visible in lane 1. In presence of a non-allosterically regulated hammerhead ribozyme (5’- GGGCGACCCUGAUAGGCUCUUCGGCCGAACC GGU-3’) complete cleavage of the substrate is achieved in 240 min (lane 2). In presence of ribozyme B but absence of theophylline (291) (lane 3), or in presence of caged theophylline (295) but in absence of UV light (lane 4), no ribozyme activity is detected and the RNA substrate remains intact even after 240 min of incubation. As expected, presence of 295 and brief UV irradiation (5 minutes) induces complete RNA degradation over the course of 480 min (lane 5-8).

Scheme 6.4. Light-activation of the trans-acting allosteric hammerhead ribozyme B followed by cleavage of a single-stranded RNA oligomer. Lane 4 shows uncleaved RNA in presence of 295 but absence of UV light. Lane 8 shows the same reaction after 5 min of UV irradiation followed by incubation for 480 min, indicating complete cleavage. The other lanes are described in more detail in the text.

In summary, we have demonstrated the photochemical activation of cis and trans
acting allosteric ribozymes by irradiation of a photocaged exogenous ligand. This enables photo-activation of ribozyme function in a precise temporal and spatial control. Future developments will demonstrate the in vivo activity of this system.

6.2. Photochemical Regulation of Ribozymes in vivo

6.2.1. Introduction

While we have extensively explored photochemical ribozyme activation in vitro, the developed systems are not applicable to in vivo settings. This is primarily due to their dependence on high magnesium concentrations to provide correct RNA folding, and optimal activity at elevated pH increasing their propensity for phosphodiester cleavage. Despite the inactivity of the minimal hammerhead ribozyme in vivo, the involvement of RNA in the regulation of gene function is becoming increasingly prominent. However, in vivo applications of the minimal hammerhead ribozyme have been hampered by the necessity of high salt concentrations for sufficient catalytic activity.\textsuperscript{286, 386} Recently, the Mulligan lab reported a mutated version of the full-length hammerhead ribozyme, which is approximately 50% larger than the minimized construct and >1,000 times more active. X-ray crystal analysis confirmed the interactions from the previously eliminated stem loops contribute to this activity, making the full length sequence catalytic under physiological conditions.\textsuperscript{390} Implementation of this ribozyme led to transgene inactivation in mammalian cell culture and mice when inserted into the 5’ UTR of a gene.\textsuperscript{391} The active ribozyme removes the 5’ cap from the mRNA through self-cleavage leading to an inhibition of translation, mRNA degradation, and thus gene silencing (Scheme 6.5).
Subsequent screening of compound collections for inhibitors of ribozyme activity delivered the natural product toyocamycin (299). This antibiotic efficiently inhibits ribozyme function in vivo at a micromolar concentration and thus induces gene expression.

Scheme 6.5. A self-cleaving hammerhead ribozyme located in the 5′ untranslated region of a transgene leads to gene silencing. Toyocamycin (299) inhibits ribozyme function and induces expression of an open reading frame (ORF). Using a photocaged toyocamycin 300, this process can be controlled with light.

6.2.2. Caging of Toyocamycin

Due to the modular nature and its simplicity of this gene expression system (in comparison to transcription factors, operators, promoters, etc.), and its function in mammalian cell culture and mice, we adapted it to achieve photochemical gene regulation.

325
All previously reported eukaryotic light-inducible gene expression systems are based on transcriptional activation.\textsuperscript{393-395} In order to achieve photochemical control over translation we decided to install a light-removable photocaging group on \textbf{299}.

The precise mechanism of action of how \textbf{299} inhibits hammerhead ribozyme function is still unknown, however, preliminary evidence suggests the incorporation of \textbf{299} into the ribozyme by RNA polymerase II.\textsuperscript{392} Thus, we hypothesized that an inactive analog of \textbf{299} can be generated by blocking either the 3’ or the 5’ position with a caging group. Initial experiments of synthesizing a 2-nitrobenzyl or 6-nitropiperonyl ether at the 5’ hydroxyl group of \textbf{299} were unsuccessful. A synthesized \textit{ortho}-nitroveratryl carbonate at the 5’ position (as a mixture together with 3’ and 2’ carbonates) was not stable under physiological conditions. Finally, we synthesized the dioxolane caged \textbf{300} in one step from \textbf{299} through treatment with zinc bromide\textsuperscript{396} in neat \textit{ortho}-nitrobenzaldehyde at 60 °C for 24 h (67% yield, Scheme 6.6).\textsuperscript{397} All attempts to conduct the same reaction with 6-nitropiperonal in order to obtain a favorable bathochromic shift in the absorption maximum of the caged compound failed. The identity and purity of the caged toyocamycin \textbf{300} was confirmed by both NMR and HRMS.
Scheme 6.6. Conversion of toyocamycin (299) into caged toyocamycin 300. UV light irradiation generates the esters 301 and 302 which are hydrolyzed intracellularly to generate toyocamycin (299).

6.2.3. Application of Caged Toyocamycin in vivo

We speculated that 300 is inactive as a repressor of ribozyme function, which was verified by a luciferase assay in 293T cells (Figure 6.7). Transcripts encoded by the N117-Luc plasmid contain the self-cleaving ribozyme upstream of the firefly luciferase open reading frame (Scheme 6.5, ORF = luciferase).\(^{391}\) Here, the caged toyocamycin 300 (10 µM) leads to a low luciferase signal which is within the error margin of the signal obtained when the cells harboring the reporter construct are not exposed to any small molecule, demonstrating the inactivity and cellular stability of 300 (Figure 6.7). In initial experiment of irradiating 300 in the absence of cells with UV light of 365 nm for 10 min (25 W handheld UV lamp) revealed a complete photochemical conversion into the benzoic esters 301 and 302 in a ratio of 1:1 as determined by \(^1\)H NMR and GC (Scheme 6.6 and Figure 6.6).
Figure 6.6 ¹H NMR analysis of the photochemical conversion to benzoic esters of toyocamycin. A) Initial spectrum prior to irradiation. B) Irradiated spectrum yielding the mixture of benzoic esters in a 1:1 ratio. Proton assignments were made based on literature reports for adenosine benzyl esters.

We hypothesized that the esters 301 and 302 will be enzymatically hydrolyzed to active toyocamycin (299) intracellularly. Thus, 293T cells transfected with the N117-Luc construct were exposed to 300 (10 µM) for 48 hours followed by a change to media not containing 300 and a brief UV irradiation (365 nm, 5 min, 25 W hand-held UV lamp). A 20-fold activation of luciferase activity was detected providing expression levels virtually identical with the induction using regular toyocamycin. Within the error margin, UV irradiation itself had no effect on luciferase activity in any of these experiments (Figure 6.7).
Figure 6.7. Luciferase assay demonstrating the induction of gene expression with toyocamycin (299), the inactivity of caged toyocamycin (300) in the absence of UV light, and the restoration of gene activity through UV irradiation (365 nm, 25 W, 5 min). In the case of 300, the cells were incubated with the caged compound for 48 h, the supernatant was removed, the cells were washed with PBS, irradiated, and assayed after 24 h. All experiments were conducted in triplicate and the average ratio of luciferase light units (firefly/Renilla) is reported.

6.2.4. Spatial Control of Ribozyme Activity in Mammalian Cell Culture

In order to apply the developed photochemical deactivation of ribozyme function in the spatial control of gene activity, we generated the N117-GFP plasmid encoding a green fluorescent protein (GFP) transcript with the self-cleaving ribozyme located in the 5' UTR. A monolayer of 293T cells was transfected with N117-GFP (and a DsRed transfection control) and incubated with growth media containing 300 (10 µM) for 48 hours. The media containing 300 was removed, the cells were washed with PBS buffer, and subsequently irradiated for 30 sec through an inverted compound microscope equipped with a 100 W Xe/Hg lamp using a DAPI fluorescence filter (340-380 nm excitation). The cells were imaged after 24 hours (to provide GFP expression and maturation) with a Leica DM5000B compound microscope revealing precise spatial control of gene expression since GFP activity was only observed in the irradiated area (Figure 6.8). No diffusion and gene activation by the
decaged toyocamycin in neighboring cells was observed. Thus, a highly stringent, spatially and temporally regulated gene expression system was discovered.

**Figure 6.8.** Spatial control of gene expression in 293T cells harboring N117-GFP. DsRed expression was used as a transfection control. The cells were incubated with 300 for 48 h, the supernatant was removed, the cells were washed with PBS buffer, and the area within the white circle was irradiated for 1 min with UV light. A) Montage of the GFP and DsRed images after a further 24 h incubation. B) GFP expression is only visible in the irradiated area. C) DsRed transfection control. D) Brightfield image of the cell monolayer.

In summary, we developed a light-activated gene expression system for mammalian cells based on a self-cleaving hammerhead ribozyme and a photocaged small molecule inhibitor of ribozyme function. Excellent induction of gene expression after UV irradiation was observed, and spatial control of gene expression was achieved through UV irradiation of a cell monolayer through the optics of a regular fluorescent microscope. We expect that this light-activation methodology will find widespread application in the spatial-temporal investigation of gene function, especially since this ribozyme gene regulation system is structurally and conceptually much simpler than previously
described photochemical gene regulation systems, and since it has shown to be active in vertebrate species.\cite{391}

6.3. Caging of Uridine for the Application in Light-Activation of RNA

6.3.1. Introduction

Having effectively caged small molecule activators of allosteric ribozymes, we next directed our efforts towards directly caging a ribozyme, and thus directly caging RNA. Based on our success in the caging of a thymidine DNA base, we were interested in extending the technology to the caging of a uridine RNA base. We speculate that the direct ribozyme caging could have multiple mechanism for the inhibition of function. First, as witnessed with the DNAzymes, the caging group could directly prevent catalysis via the caging of an essential uridine. Secondly, the caging group could inhibit proper folding due to the steric bulk of the caging group, abrogating its function. Finally, multiple caging groups can be installed, inhibiting hybridization in a analogous fashion to the caged thymidine. This technology could ultimately lead to an alternate means of photoregulating gene function via the utilization of a caged trans-acting ribozyme.

The direct caging of RNA has previously been conducted by various laboratories. This includes the previously noted site-specific caging of the mRNA transcript by Tsien and Okamoto to modulate GFP expression.\cite{274, 275} However, more recently RNA has been caged site specifically by both the Heckel and Silverman groups. The Silverman laboratory prepared all four caged nucleotide phosphoramidites and incorporated them into an RNA sequence to investigate the effects of caging groups on RNA folding. They found that while the caging group sufficiently disrupts tertiary RNA structure prior to irradiation, the
perturbation can be compensated for by increased levels of magnesium ions. Additionally, the Heckel group has employed their caged deoxynucleotides towards the caging of siRNA. They found that the incorporation of a single deoxynucleotide base in the siRNA does not affect the function of the siRNA, and the installation of a caged deoxynucleotide provides light-activated RNA interference. While this approach is useful, it employs non-natural bases (DNA) in the RNA sequence and in the best case does not completely inactivate the siRNA and does not completely restore the activity.

6.3.2. Synthesis of Caged Uridine

The synthesis conditions and sensitivity of RNA are somewhat different than those observed in DNA, and thus we were interested in examining multiple caging strategies. In order to elucidate the optimal caging group for uridine we prepared a variety of derivative utilizing different caging groups. By first acetylation of the uridine we were able to protect the reactive hydroxyls and easily access the 3-N position. The acetylated uridine 304 is then treated with either the bromomethylpiperonyl caging group 307 to yield 308, or the NPOM caging group 298 to yield 306 (Scheme 6.7). Alternatively, uridine (303) can be protected in situ then caged with a chloroformate caging group to afford caged uridine 310, which can then be acylated (310) for comparison to the other caged uridines (Scheme 6.8). A final caged uridine was prepared from acylated uridine 304, to install the caging group on the 4-O position to yield caged uridine 309, which is comparable to the caged thymidine prepared by the Heckel laboratory (Scheme 6.7).
Scheme 6.7. Synthetic route to caged uridine derivatives 305, 307, and 308.
Scheme 6.8. Synthetic route to chloroformate caged uridine (310)

6.3.3. Assessment of Caging Group Properties

Having successfully prepared four alternatively caged uridines, we were next interested in exploring their photochemical properties and physiological stabilities to select the best caging group for uridine. All of the acylated derivatives (10 mM) were irradiated for 10 minutes (handheld UV lamp, 365 nm, 23 W) and their decaging properties were measured by LC/MS for disappearance of starting material and appearance of uridine (Figure 6.9).

A

B

Figure 6.9. LC/MS trace of decaging of 310. A) Chlorofomate caged uridine 310 prior to irradiation. Two diasteriomers are visible. B) The same sample after a 10 minute irradiation at 365 nm. More than 80% of the starting material has been converted to acylated uridine 304.
Figure 6.10. Decaging timecourses of the caged uridine derivatives. Decagings were performed with 10 mM of each acylated monomer in MeOH with a 10 min irradiation (365 nm, 23 W, handheld UV lamp).

$\tau_{1/2}(307) = 60$ min; $\tau_{1/2}(308) = 26$ min; $\tau_{1/2}(310) = 52$ min; $\tau_{1/2}(305) = 2$ min.
All four caged uridines were capable of being decaged at 10 mM concentrations; however, the rates of decaging were dramatically different (Figure 6.10). The chloroformate caged uridine 310, possessed a half-life of approximately 1 hour, and was not stable towards RNA synthesis conditions. Caged uridine 307 had a similar 1 hour half life, but was found to be stable to both RNA synthesis and physiological conditions. The 4-O caged uridine 308 was decaged slightly more rapidly with a half-life of approximately 30 minutes; however, demonstrated some decomposition under some of the acidic RNA synthesis conditions. Finally, the NPOM caged uridine 305, was found to be the optimal caging group as it demonstrated excellent decaging properties \( t_{1/2} = 2 \text{ min} \), and a good stability when subjected to physiological conditions (pH 7.3), as determined by LC/MS analysis at various timepoints for compound degradation.

6.3.4. Incorporation of a Caged Uridine into a Hammerhead Ribozyme

With the development of a caged uridine possessing rapid decaging properties and high stability to both RNA synthesis and physiological conditions, we set forth to demonstrate its utility in a practical application. The acylated NPOM caged uridine 305 was prepared on a large scale according to the previously described synthesis. The acetyl protection groups were removed with standard \( \text{K}_2\text{CO}_3/\text{MeOH} \) conditions to yield the unprotected caged uridine. This was then sent to Dharmacon for conversion into a phosphoramidite that is compatible with their 5’-silyl-2’-orthoester chemistry for the preparation of RNA oligomers.

400
To initiate the study we were interested in preparing both the intramolecular and intermolecular hammerhead ribozyme that does not possess the allosteric core. Conveniently, this ribozyme possesses a highly conserved uridine residue within the catalytic core, as replacement of this uridine with any other nucleoside has resulted in a complete loss of activity. Due to this Dharmacon synthesized RNA oligomers with only one caging group installed on the uridine on the catalytic core for both inter- (5’ GGGCGACCCUGAUAGGC CUUCGGGCGACCCUGAUAGGC 3’) and intramolecular (5’ GGGCGACCCU*GAUGAGGCCU U*CGGGCGACCCUGAUAGGCU*CGGGCGACCCUGAUAGGC 3’) hammerhead ribozymes (Figure 6.11).

![Diagram](image)

**Figure 6.11.** Nucleotide sequence of caged intermolecular ribozyme. The RNA substrate is highlighted yellow, and the caged uridine in the catalytic core is indicated by a red circle.

The RNA oligomers were deprotected according to standard Dharmacon protocols. Attempts to perform light activation of the oligomers led to inconclusive results, and thus the oligomers were purified via HPLC prior to utilization in ribozyme assays. Purification of the intramolecular ribozyme led to two major RNA oligomer fractions, potentially as a result of inadvertent ribozyme activation via decaging.
The intramolecular ribozyme assay was performed with both RNA fractions. The RNA was first end labeled using $\gamma^{32}$P [ATP], and purified on microcon filters. The ribozyme was either not irradiated, or irradiated for 5 minutes at 365 nm, then incubated in ribozyme cleavage buffer (50 mM Tris-HCl, 20 mM MgCl$_2$, pH 7.5), and aliquots were removed at both 30 and 120 minutes then analyzed via polyacrylamide gel electrophoresis. A non-caged control ribozyme was also subjected to the same conditions to monitor the normal behavior of the ribozyme construct. Unfortunately, no light-induced activation of ribozyme activity was observed. The non-caged ribozyme undergoes complete self-cleavage after only 30 minutes. The first RNA fraction obtained from the purification appears to be the cleaved ribozyme. This suggests that at some point during the purification or labeling of the RNA the caging group was removed, facilitating ribozyme activation. Specifically this may have occurred during the HPLC purification as the RNA elution is monitored by UV, which at such low concentrations may be substantial enough to lead to the photodecomposition. The second fraction of purified RNA appears to be the intact ribozyme. Some photoactivation appears to occur as a minimal band is observed at the same location as the cleaved product, only in the irradiated sample. However, the majority of the RNA is not cleaved, even after 120 minutes. Attempts to increase the rate of cleavage via increased irradiation time or increased magnesium time; however, not more than ~20% cleavage was observed (Figure 6.12).
We next investigated the activity of the intermolecular ribozyme. As in the case of the intermolecular allosteric ribozyme, we end labeled the RNA substrate with $^{32}$P to monitor its cleavage. Again, in the case of this ribozyme, no activity was observed upon irradiation with light at 365 nm for 10 minutes. Due to the slower cleavage rate of the intermolecular ribozyme, reactions were allowed to progress for 12 hours and monitored by gel electrophoresis. The substrate was incubated in the absence of any ribozyme to afford a means of detecting uncleaved product. Also, the substrate was incubated in the presence of non-caged ribozyme to confirm ribozyme activity. In the absence of UV irradiation, no cleavage was observed in the case of the caged ribozyme; however, activity was not restored upon irradiation (Figure 6.13).
In summary, we effectively developed a means of caging a uridine base with the NPOM caging group which was subsequently incorporated into RNA oligomers; however, we failed to successfully employ the caged uridine to activate either an intra- and intermolecular hammerhead ribozyme. Future work must be applied towards the preparation of the RNA phosphoramidite for standard RNA synthesis conditions, due to the previously demonstrated stability of the NPOM caging group towards these synthesis conditions. Additionally, due to the instability of RNA, additional care must be given towards the handling and purification of the prepared oligomers.
6.4. Ribozyme Selection for Diazobenzene Allosteric Ribozymes

6.4.1. Introduction

Allosteric ribozyme selections represent a useful strategy for the discovery of specific RNA aptamers by employing the functional activity of ribozymes to gage aptamer binding. These selections have been employed in the selection of flavin mononucleotide (FNM), theophylline, cAMP as well as photochemically switchable molecules. Conveniently, these types of selections result in a functional aptamer, which regulates ribozyme activity and could potentially be directly employed in gene regulation. Additionally, this type of selection could be employed to generate allosteric ribozymes that are more active under physiological conditions, based on the selection conditions.

Thus far, irreversible caging technologies have been employed towards photochemical RNA regulation; however, the reversible regulation of RNA function would be advantageous. Many important processes, e.g. information storage, signal transduction, signal processing, and gene expression all need to be regulated in a reversibly fashion. An important example is the regulation of hox genes which have been found to be turned on, off, and on again in model organisms during development. The reversible regulation of these genes facilitates proliferation, differentiation, and morphogenesis of bone tissue and other tissue involved in digitation. Possessing a means of externally regulating these (or similar) events with Nature’s precision has the potential to aid in the understanding of fundamental developmental processes.

Obviously, Nature has evolved her own highly sophisticated mechanism to reversibly sense light using the photochemical cis→trans isomerisation of retinal. This configurational change is detected by opsin proteins, which then trigger a signal transduction cascade.
Synthetic molecules which are isomerizable in a reversible fashion using light irradiation of different wavelengths have been developed as well, including diazobenzenes, dihydropyrenes, spirooxazines, anthracenes, fulgides, and spiropyrans.\textsuperscript{405-409} These molecules have been used in the investigation of transport channels, CAP binding affinity, papain activity, and DNAzyme cleavage, as well as various other applications.\textsuperscript{291, 410-413} However, no synthetic reversible photochemical genetic switch has been demonstrated to date. We envisioned the design of such a switch by mimicking Nature, however, substituting the small molecule-protein interaction with a small molecule-RNA interaction. Thus, we hoped to employ an allosteric ribozyme selection to develop aptamers for photoisomerizable diazobenzene molecules (Scheme 6.9).\textsuperscript{414, 415} With these allosteric \textit{trans}-ribozymes gene expression will be turned “off” in the presence of one geometrical isomer; however, upon irradiation with light photoisomerization occurs, preventing aptamer recognition, halting mRNA degradation and turning “on” gene expression.

\textbf{Scheme 6.9.} Photoisomerization of a diazobenzene from \textit{trans} to \textit{cis}.

\textit{6.4.2. The Allsosteric Ribozyme Selection Cycle}

The ribozyme selection cycle commences with synthetic DNA possessing the hammerhead ribozyme machinery fused to a 25 nucleotide long randomized sequence
encoding the aptamer domain (Figure 6.14). The library (~$10^{14}$ RNA molecules) is reverse transcribed to yield the double-stranded DNA required for transcription. Initially, the photochemical component of the selection was omitted in an attempt to enrich diazobenzene binding aptamers. The switching from $trans$ (311a) to $cis$ (312b) could then be employed in later cycles to apply increased stringency to the selection. The active ribozyme library is generated with T7 polymerase, as well as $\alpha$-$^{32}$P ATP to label the molecules, and subjected to a negative selection to remove any sequences, which are naturally active. After excising the uncut ribozyme band from an acrylamide gel, a positive selection is performed with the $trans$ diazobenzene (311a) (0.1mM). After gel electrophoresis, the band corresponding to the cleaved ribozyme is excised. Due to the negative selection, the active ribozymes selected should only be active due to the presence of the small molecule effector. These sequences are reverse transcribed and amplified by PCR to obtain the double stranded DNA. At this point the library can then be subjected to another selection cycle or cloned and sequenced to elucidate the active ribozymes. Typically selections require 6-12 rounds in order to see enrichment of an active species.
6.4.3. Selection Results

The ribozyme selection cycle was conducted for 8 rounds of selection; however, it became difficult to assess library enrichment, as the band representing the cleaved ribozyme
in the positive selection became increasingly difficult to isolate. At this point, the selection was re-initiated with a fresh pool of RNA. In this second selection attempt, incubation times were increased to 1 hour for the first three rounds, then reduced to 30 minutes; however, similar problems were encountered in detecting enrichment of active sequences. Ultimately, the selections were discontinued due to the inability to reliably separate active and inactive sequences and detect enrichment. Perhaps the greatest shortcoming of the selection strategy was the extraction of RNA from the acrylamide gels. Due to the sensitive nature of RNA coupled with the low extraction efficiency, a substantial amount of RNA was lost in the process, limiting the number of viable sequences carried through the selection. These selections need to be repeated to isolate a viable allosteric ribozyme; however, in the future, the RNA should be run on a higher percentage acrylamide gel (18-20%), and more importantly should be isolated via electroelution. Taking these measures should increase the efficiency of the selection cycle, affording a highly active evolved pool of aptamers.

6.5. Selection of a Photoswitchable RNA Aptamer

6.5.1 Introduction

Given the failure of the ribozyme selections, we attempted selecting an aptamer via the classical SELEX process. Using the SELEX (systematic evolution of ligands by exponential enrichment) process RNA aptamers have been engineered to selectively bind to a variety of different small molecules. In order to afford a greater difference in the geometrical isomers, as well as provide more functionality for the RNA to interact with, we decided to alter our photoisomerizable small molecule. A spiropyran was been selected as the light-responsive molecule since it displays unique chemical properties. Spiropyrans are
both light and pH sensitive, exhibit distinctive chromophores in different forms (colorless, purple, and yellow) enabling a visual detection of the switching event (Scheme 1), and undergo rapid isomerization between the spiropyran (312) and the merocyanine form (313a and 313b) upon irradiation with UV light (or pH changes).\textsuperscript{407, 418-420} These molecules have been extensively employed in the construction of nanoscale switches and photoisomerizable polymers for materials applications.\textsuperscript{421-423} Conveniently, the spiropyran 312 is photochemical switching is virtually complete (>95% of 313a after UV irradiation at 365nm) due to distinctively different absorption maxima of 312 (350nm) and 313a (563 nm).\textsuperscript{424} This is in contrast to diazobenzenes which reach a photostationary state of 70-90% cis when exposed to UV light of 365nm.\textsuperscript{425}

Scheme 6.10. Spiropyran 312 (colorless) and its merocyanine forms 313a (purple) and 313b (yellow).

6.5.2. Application of the SELEX Process to Select an RNA Aptamer for a Spiropyran Isomer

We applied the in vitro selection process in the evolution of an RNA aptamer capable of selectively recognizing only one photochemical isomer of a spiropyran molecule. We first needed to prepare a molecule capable of being immobilized on a resin, while still maintaining the ability to be reversibly switched. After several iterations, the optimal handle
was found to be an β-alanine linker. Compound 317 was prepared by the coupling of the commercially available spiropyran 314 to N-Boc protected β-alanine 315. Following the carbodiimide coupling, the protecting group was removed, and the amine was neutralized to provide a basic functionality for immobilization (Scheme 6.11).

Scheme 6.11. Synthesis and immobilization of the spiropyran for SELEX process.

The spiropyran 316 was immobilized on a sepharose resin (Sepharose 6B; Amersham Biosciences), and subjected to ten rounds of affinity selection (Figure 6.15). The selection was designed to employ the photoisomerization as part of the partitioning process. A \(^{32}\)P-labeled RNA library (~ \(10^{14}\) sequences) was incubated with resin carrying the spiropyran in its closed form 317, and nonbinding members were removed through subsequent washing of the resin. The resin was then subjected to irradiation with UV light at 365 nm for 1 h (25 W, hand-held UV lamp), resulting in a visible isomerization to the purple merocyanine 313a.
(such prolonged irradiation is not necessary with the non-immobilized spiropyran as complete switching is observed within 2 min). The RNA aptamers that selectively bind only the closed isomer 295 dissociate from the resin and are collected, whereas nonspecific binders remain bound to the resin and are discarded. This feature of the selection obviates the need for the negative selection step traditionally required in \textit{in vitro} evolution strategies.

\textbf{Figure 6.15} Photochemical \textit{in vitro} selection. An RNA library is incubated with a resin containing the spiropyran 312 (red square). RNA aptamers incapable of binding are washed away and the resin is switched to the merocyanine form 313a (open circle) with UV light of 365nm. RNAs which are non-specifically bound, or recognize both isomers are retained, while specific binders are eluted and collected. This enriched pool of RNA aptamers is then reverse transcribed, PCR amplified, and transcribed back to RNA to continue the cycle. The selection cycle was performed 10 times until significant enrichment of the RNA pool was detected.

The collected aptamers were then reverse transcribed, amplified by PCR, and transcribed into RNA again to be taken through another round of selection. After ten rounds,
an enrichment of 13% of sequences carried through the selection was noted (as detected by radioactive quantitation by liquid scintillation counting, Figure 6.16), and the resulting aptamer pool was cloned and sequenced (Figure 6.17). A similar selection was performed on an immobilized diazobenzene; however after 11 rounds of selection, no sequence convergence was observed.

**Figure 6.16.** Spiropyran selection RNA recovery. After 10 rounds of selection ~13% enrichment was achieved. A decrease in recovery was observed for round 7 as the stringency of the selection was increased through a shorter incubation time (30 min).

**Figure 6.17.** Conserved sequences within clones of the enriched pool of aptamers after 10 rounds of selections.
6.5.3. Characterization of Selected RNA Sequences

Sequencing results revealed five aptamer families with a certain degree of sequence homology, thus suggesting convergence of the selection. Several RNA aptamers were analyzed for their binding affinity and selectivity for the desired spiropyran isomer 312. Initial titration experiments were performed to assess the binding of individual sequences to the spiropyran. Whereas several of the isolated aptamers exhibited micromolar binding to the resin, to our surprise very few exhibited preferential binding to 312 over 313a (Figure 6.18).

![Figure 6.18](image)

**Figure 6.18.** Assessment of the specificity for spiropyran regioisomers of several isolates from the selection. Aptamers were bound on the resin, and after washing, the supernatant was assessed for radioactive RNA molecules (dark). The resin was then switched (5 min, 365 nm, 25 W) and aptamers capable of only binding the closed form were eluted from the resin allowing for an assessment of the radioactivity present in the supernatant (light).

Radioactively labeled RNA aptamers were incubated with resin carrying 316, which was then washed until no radioactivity was detected in the eluent. The resin was then suspended in binding buffer, and, after 1 hour, the amount of RNA aptamer present in solution was determined on a scintillation counter. The suspended resin was then switched to
by irradiation at 365 nm (25 W, hand-held UV lamp), and after 1 hour the solution was assayed again for radioactivity. A randomly selected control sequence (CNTL) was determined to have no binding to the spiropyran (in any form), thus showing comparable levels of RNA present before and after irradiation. The other aptamers (SP3, SP4, SP5, and SP18) were found to bind immobilized 312. Of all the aptamers assayed, SP3 exhibited binding with the highest level of selectivity, as a significant portion of the RNA was released from the resin into solution upon switching from 312 to 313a. SP5 was capable of binding both photochemical states, 312 and 313a, of the spiropyran, but did not exhibit specificity. SP4 and SP18 exhibited some specificity for 312, however it was minimal compared to SP3.

These experiments indicate that the SP3 aptamer (5’-GGAUUAACUCCUAUCCCGAUUGAAAGCAGUACCUAUUCCA-3’) is a selective binder for the closed spiropyran form 312 and does not bind to the merocyanine form 313a, as indicated by a marked 14-fold increase of radioactivity after resin switching (Figure 6.18). As SP3 demonstrated the best selectivity, its binding constant was determined to be 19 µM by both radioactive titration assays and equilibrium dialysis (Figure 6.19). We are aware that 312 is present as a racemate; this results in a binding constant of 10 µM in the case of enantioselective aptamer recognition.426
6.5.4. Demonstration of Reversible Aptamer Binding Via Surface-Plasmon Resonance Imaging

Following the identification of an aptamer sequence, the reversibility of the developed photochemical switch was demonstrated by surface plasmon resonance (SPR) studies. The spiropyran molecule 319 was modified with a poly(ethylene glycol) spacer and a thiol group and immobilized on a gold slide along with a mercaptohexanol control. The commercially available spiropyran 314 was first converted to the bromide 318 in an Appel reaction. The bromide was displaced with sodium azide then reduced in a Staudinger reduction to the amine 319. The amine was next conjugated to a protected thiol linker (Quanta BioDesigns) to yield compound 320 in an 81% yield. Finally, the thiol protecting group was removed to 321, followed by immediate immobilization on a gold surface (Scheme 6.12).

The surface was then incubated with the SP3 aptamer (90 µM); this resulted in an increase of the SPR signal (the mercaptohexanol control did not display any signal change; data not shown). Subsequent irradiation with UV light of 365 nm for 10 min by using fiberoptics connected to a UV LED system (Prizmatix BLCC-02) led to a decrease in the SPR signal indicating a dissociation of the aptamer from the surface (Figure 6.20). This correlates to the isomerization event as the spiropyran is switched from closed form \( 312 \) to the open form \( 313a \). After irradiation, the spiropyran moiety thermally reverts back to the closed form \( 312 \) in a gradual process, and rebinding of the aptamer is observed (Figure 6.21). This process was then repeated with a second 20 min irradiation event. Thermal, instead of photochemical switching of \( 313a \) to \( 312 \) was conducted because of an interference of white light irradiation and the SPR camera.
Figure 6.20. SPR apparatus utilized in the experiment. A standard chamber was modified to incorporate a UV window for irradiation during the experiment. The Prizmatix LED fiberoptics was then inserted into the quartz window. Control experiments demonstrated that the UV irradiation on a gold surface, a mercaptohexanol, or a spiropyran coated surface without aptamer lead to no detectable change in the SPR signal.

Figure 6.21. Surface plasmon resonance experiment demonstrating the reversibility of the light-regulated SP3 aptamer binding. The spiropyran 312 was immobilized on a gold surface, and was incubated with the SP3 aptamer and irradiated at 365 nm (yellow). SPR = change in percent reflectivity.

The engineered RNA aptamer SP3 is specific for one geometrical state of the spiropyran 312, and the binding event can be reversibly switched using light irradiation thus representing a unique tool in the development of biological switches. Over the course of our studies, two other photoswitchable molecule-aptamer pairs using a diazobenzene427 and a dihydropyrene403 have been reported, and effectively complement our switch. The spiropyran 312 is advantageous over other reversible systems in that its photochemical switching is virtually complete (>95% of 313a after UV irradiation at 365nm) due to distinctively different absorption maxima of 312 (350nm) and 313a (563 nm),424 unlike diazobenzenes.
which reach a photostationary state of 70-90% cis when exposed to UV light of 365nm. The developed light-switchable small molecule aptamer pair has the potential to be developed into a photochemical riboswitch, which then can be used to spatially and temporally regulate gene function in a reversible fashion. This switch also has potential in materials applications including nanodevices (similar to the thrombin DNA aptamer amperometric biomolecular device) and data processing circuits and storage devices.

6.5.5. Assessment of Toxicity and Cellular Uptake of Spiropyrans

In order for the evolved aptamer to find broader application, it must be employed in an in vivo setting. In order to discern if this is viable, we initially examined the toxicity and uptake of the molecule in zebrafish embryos. Our collaborators in the Yoder laboratory treated embryos with 316 at different concentrations by incubating the embryo in egg water supplemented with the organic compound for a 24 hour period from 0 to 24 hpf or 24 to 48 hpf (Table 6.2). At 48 hrs of growth the embryos were washed with system water and visually examined under a dissecting microscope. When simply treated with water, normal embryos were observed, and 72% of the embryos were viable. When treated with 1 mM of DMSO, embryo viability remained similar; however, a phenotypic curvature of the tail was observed and some embryos were not capable of swimming. At the same concentration (1 mM) of the spiropyran 316, the embryos were not viable, and actually disintegrated. At higher concentrations some loss in viability was observed; however at 0.1 and 0.05 mM concentrations most embryos were normally developed and viable.
Table 6.2. Percent viability of zebrafish embryos treated with 316 at different developmental stages.¹²

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0.50 mM</th>
<th>0.25 mM</th>
<th>0.10 mM</th>
<th>0.05 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24 hpf</td>
<td>83% (n=23)³</td>
<td>63% (n=19)³</td>
<td>89% (n=19)</td>
<td>75% (n=24)</td>
</tr>
<tr>
<td>24-48 hpf</td>
<td>100% (n=23)³</td>
<td>88% (n=16)³</td>
<td>88% (n=17)³</td>
<td>86% (n=21)</td>
</tr>
</tbody>
</table>

[¹] Unpublished results, Yoder Lab. [²] Percent viability of control embryos was 72% (n=25). [³] These embryos appeared to be developmentally delayed.

The viable embryos were then subjected to a brief irradiation with UV light at 365 nm to afford the switching of the spiropyran (colorless) to the merocyanine (purple) form. This switching confirmed the uptake of 316, as a definite purple color was observed in the embryos. Interestingly, this compound appears to be concentrated in the yolk sacks of the developing embryos, but some coloration was observed in other regions of the zebrafish (Figure 6.22). After visual observations were performed, the embryos were snap-frozen, centrifuged, lysed, and analyzed by LC/MS to ascertain the exact intracellular concentration of 316. LC/MS analysis of the lysate indicated complete compound uptake with intracellular concentrations of 0.25 mM. We believe these concentrations are sufficient to achieve strong small molecule-aptamer interactions in an in vivo environment.

![Figure 6.22. Zebrafish embryos 24 h after fertilization and 24 h treatment with 0.1 M spiropyran 316. A) Untreated control embryo. B) Treated embryos before irradiation at 365 nm. C) Treated embryos after irradiation (5 min) at 365 nm. D) Irradiated embryo 2 hr after irradiation, demonstrating the in vivo switching of the spiropyran.](image-url)
Thus, it appears that the spiropyran is mildly toxic to zebrafish embryos; however, at lower concentrations (< 0.5 mM) the compound is taken up by the organism and no toxicity is observed. This leads us to believe that reasonable concentrations for aptamer binding can be achieved in an in vivo environment, allowing for the application of the evolved aptamer. As a result, the next step involves the integration of the aptamer into a functional expression system, e.g. a riboswitch, and its incorporation into a zebrafish system to reversibly regulate gene expression.

6.6. Caging of siRNA

6.6.1. Introduction

RNA interference (RNAi) refers to the ability of dsRNA to elicit a gene-silencing event, and has been demonstrated in a variety of model systems. Thought to have evolutionarily evolved as a defence mechanism against double-stranded RNA viruses, RNAi was first demonstrated in plants in the 1980s, but not formally recognized and characterized until a decade later. In an attempt to alter the color of petunias, scientists over-expressed the gene responsible for plant pigmentation; however, instead of increased purple coloration, substantial lack of pigmentation was observed (Figure 6.23). Several other documented cases of this phenomenon, referred to as quelling, were observed leading up to the formal discovery of the mechanisms behind gene silencing were elucidated. The formal discovery of RNAi in C. elegans was accomplished in 1998 as the unc22 and gfp genes were silenced by injection of dsRNA into the nematode. The discovery of RNAi in C. elegans ultimately led to the awarding of the Nobel Prize in 2006 to Fire and Mello. Later investigations discovered other methods of introduction of the dsRNA into the organism, which could be applied to
achieve the RNAi response. *C. elegans* itself is an extremely useful model organism for the study of RNA interference as it possesses a rapid life cycle, exhibits fast reproductive growth, possesses a sequenced genome, and is employable under a multitude of laboratory conditions. In addition to *C. elegans*, RNAi has also been extensively employed in *Drosophila melanogaster* and in human tissue culture, as well as a variety of other model organism.\textsuperscript{430,431}

![Figure 6.23. Demonstration of RNA interference in petunias. Introduction of pigmentation RNA led to a decrease in pigmentation relative to wild type petals.](image)

The RNA interference pathway begins with the introduction of dsRNA into the biological system. The can be achieved via multiple approaches including; viral introduction, feeding/injection of plasmids which possess expression cassettes which either yield hairpin dsRNA or long dsRNA strands by using dual promoters, or via transfecting or soaking the organism with synthetically prepared hairpin dsRNA or siRNA. The dsRNA is then processed by the dicer enzyme into siRNA duplexes containing 20-25 nucleotides, including a 2-3 base overhang. The duplex is then loaded into the RNA-induced silencing complex (RISC) leading to sequence specific silencing of complementary mRNA transcripts via transcript cleavage and degradation (Figure 6.24).
The photoregulation of RNAi has previously been investigated by both the Heckel and Friedman laboratories; however, the controlled photoactivation still has issues which must be addressed in order to make the technology applicable to broader studies. Friedman and co-workers demonstrated the caging of a siRNA molecule targeting the GFP gene. They conducted a non-specific caging of double stranded RNA using the diazo caging group 322 (Scheme 6.13).

Figure 6.24. RNA interference pathway.
This reactive intermediate is readily generated from a commercially available kit (Invitrogen/Molecular Probes), and can be used to photoprotect a wide range of weak acids with a pKₐ of 3-7, including carboxylic acids, phenols, and phosphates (Scheme 6.13). They determined a caging efficiency of 3% with approximately 1.4 caging groups per RNA molecule. A significant difference in GFP expression was observed between the caged and irradiated siRNAs in mammalian cell culture; however, some GFP down-regulation was still observed with the caged siRNA. This was resolved by increasing the caging efficiency to 15.2%, but resulted in decreased photoinduced activation, potentially due to the lack of removal of all caging groups.

The disadvantage of this strategy is the lack of control over caging, resulting in either incomplete light activation or incomplete initial deactivation. Friedman later addressed this issue via the installation of a caging group on the 5' phosphate group, inhibiting its loading into RISC. This approach employed only a single caging group; however did not lead to complete deactivation of the siRNA pathway. More recently, Heckel and co-workers introduced site-specifically caged DNA monomers into the RNA sequence to elicit photochemical regulation of the gene silencing of GFP. This approach was significantly
more successful than the caging of the phosphate backbone (10% active when caged; 80% active upon decaging); however, required the use of DNA bases within the RNA sequence. Ultimately, we envision the application of our previously developed caged uridine monomer into the siRNA sequence at specific bases to afford light regulated gene silencing. This approach uses RNA monomers, as opposed to DNA, and allows for the site-specific incorporation of caging groups. This is also advantageous as the synthetically prepared siRNAs can be used to better understand the mechanism of RNAi, as based on the location of the caged RNA base, we can study both the loading into RISC, but also the mRNA recognition and cleavage.

6.6.2. Development of siRNA Assay

In order to investigate the photoregulation of the RNAi pathway, we first need to establish a viable assay. We were first interested in employing a reporter gene in cell culture to measure the degree of silencing by siRNA constructs, and thus selected GFP, due to the commercial availability of known siRNA sequences that effectively silence this gene. The Silencer® GFP (eGFP) double-stranded siRNA was obtained from Ambion, and has previously been demonstrated to effective in the silencing of the gfp gene in mammalian tissue culture (Figure 6.25). In addition to the siRNA targeting GFP, a negative control construct with no sequence homology was obtained.

\[
\text{Sense} \quad 5'\text{-CAA GCU GAC CCU GAA GUU Ctt-3'}  \\
\text{Anti-Sense} \quad 5'\text{-GAA CUU CAG GGU CAG CUU Gtt-3'}
\]

\textbf{Figure 6.25.} Sequence of GFP siRNA

We initiated our studies with a stably transfected GFP expressing HEK-293T cell line (obtained from the NCSU Biotchnology Department), to probe the ability of the non-caged
siRNA to decrease GFP expression, and optimize silencing parameters. The GFP siRNA and control siRNA were transfected at different concentrations (3 or 6 pmol total RNA) using X-tremeGENE siRNA transfection reagent, and the cells were incubated for 24 to 48 hours prior to analysis on a fluorescence plate reader. Due to the constitutive expression of GFP in the stably transfected line, no significant change in GFP expression was observed after 24 hours; however, after 48 hours approximately a 50% reduction in GFP expression was observed in the cells incubated with the siRNA GFP construct (Figure 6.26). Interestingly, no substantial change was observed between the cells transfected with 3 pmol versus 6 pmol, suggesting that 3 pmol is sufficient for induction of the RNAi cascade.

![Figure 6.26. Silencing of GFP in stably transfected HEK-293T, 48 hours post transfection.](image)

While the initial results were promising, a 50% reduction in gene expression is not especially useful in the study of genes. However, we suspected that due to the stability of the GFP protein, and the constitutive expression a more substantial silencing was actually occurring, but was difficult to detect due to the regular expression of GFP for almost 24
hours prior to introduction of the siRNA. In order to better assess the amount of gene silencing occurring with this construct, we co-transfected a CMV promoter driven GFP plasmid with the siRNA using the same X-tremeGENE transfection reagent. This alleviates the background expression observed from the constitutively expressed GFP, as the silencing agent and the gene are introduced simultaneously. This approach appears to work extremely well, as almost a complete reduction of the GFP signal was observed using only 3 pmol of the GFP siRNA construct. Conversely, the negative control exhibited a similar level of GFP expression as the cells transfected with only the GFP plasmid (Figure 6.27). The gene silencing could also be visually observed using a fluorescent confocal microscope (Figure 6.27).

![Figure 6.27. Silencing of gfp by a siRNA construct. Quantitative measurement of the relative fluorescence of each condition. A) No transfection control. B) Transfection with pGFP plasmid only. C) Transfection with pGFP plasmid and GFP siRNA. D) Transfection with pGFP plasmid and negative control siRNA.]

Using a co-transfected GFP construct with the siRNA, a successful assay for gene knockdown by RNAi has been established, leading to >90% gene silencing. Given the success of this initial assay it is feasible to apply this assay to the evaluation of caged siRNAs. In order to achieve this we need to prepare the caged siRNA construct.
Additionally, we are interested in extending this application towards a *C. elegans* model system; however, this requires the adjustment of the siRNA construct to account for the change in codon preference in *C. elegans* versus the mammalian system. Additionally, the mechanism for delivery must be examined, as traditionally plasmids expressing dsRNA precursors are delivered (either by feeding or soaking) for *in vivo* expression and processing. In the case of caged siRNA, the constructs must be microinjected, requiring optimization of injection time-points and concentrations. All of these issues will be addressed upon the successful preparation of caged siRNA constructs.

6.6.3. Synthesis of a Caged Uridine Phosphoramidite

In order to provide a universal approach to caged RNA synthesis, we adopted an alternative synthesis of caged RNA than previously described in Chapter 6.3.2, which is compatible with standard DNA/RNA synthesizers and synthesis conditions. To afford a highly selective synthesis, differentiating between the 2’ and 3’ hydroxyl groups, we employed a silyl protecting group that selectively protects the 5’ and 3’ hydroxyl groups, affording the selective protection of the 2’ hydroxyl group with a bulky TBDMS protecting group that is retained throughout the preparation of the RNA oligomer and cleaved after hydrolysis from the solid-support. With the uridine fully protected, it was reacted with the previously employed NPOM chloride, followed by the selective deprotection of the 5’ and 3’ hydroxyl protecting group. The 5’ hydroxyl group was then readily converted to the DMT ether, and the 3’ hydroxyl group was reacted with the chloro-phosphoramidite to yield the RNA synthesis precursor 324 (Scheme 6.14). This monomer can readily be utilized on a standard DNA/RNA synthesizer to produce the desired caged oligomers.
Scheme 6.14. New synthetic route to a caged uridine phosphoramidite (324).
6.6.4. Future Work

With the prepared monomer in hand, the next step involves the incorporation of the monomer into the desired siRNA sequence. Initial investigations will involve the incorporation of variable caging groups within the GFP siRNA sequence. Additionally, due to the presence of two thymidine residues on the ends of the RNA, which afford increased cellular stability from exonuclease activity, our caged thymidine monomer can be employed. This may aid in the investigation and feasibility of preventing siRNA loading into RISC. After establishing the induction of RNAi in mammalian culture, we hope to extend the photoregulation to *C. elegans*, affording a technology for spatio-temporal regulation in a model organism.
6.7 Experimental

Materials. Solvents and reagents were obtained from either Sigma-Aldrich or Fisher Scientific and used without further purification unless noted. Reactions were conducted under N\textsubscript{2} atmosphere using dry solvents distilled from appropriate drying agents prior to use. NMR data was acquired on a Varian Gemini 300MHz NMR and LC/MS data was obtained on an HP 1100MSD system with a ZorbaxSB C-18 3.5 μM pore size 4.5X100mm column. Irradiation for decaging was performed with a handheld Spectroline ENF-280C 23W lamp at 365nm.

\(\alpha\)-Methyl-6-nitropiperonyl alcohol (297). A solution of nitropiperonal (296) (1.0g, 5.1mmol) in DCM (40mL) was stirred at room temperature and trimethylaluminum (0.81g, 11.3mmol, 2.2eq) was added over the course of 2 hours. The solution was stirred for 3 additional hours, quenched with methanol (20mL) and aqueous HCl (20mL), and extracted with DCM (3\times40mL). The organic layer was dried (Mg\textsubscript{2}SO\textsubscript{4}) and concentrated under vacuum. The product was purified via column chromatography on silica gel (2:1 hexanes/EtOAc) to yield 297 as an orange solid (1.01g, 4.7mmol, 93%). The analytical data were identical with literature reports.\(^{434}\)

(\(\alpha\)-Methyl-6-nitropiperonyloxy)methylthiomethylether (298). A solution of 297 (0.59g, 2.8mmol) in DMSO (5mL) and acetic anhydride (3.5mL) was stirred at room temperature.
Acetic anhydride (2.5mL) was added and the reaction was allowed to progress at room temperature for 48 hours followed by the addition of aq. NaHCO₃ (sat.). The reaction was stirred an additional 24 hours then quenched with 2:1 EtOAc/Hexanes (30mL) and washed with aqueous NaHCO₃ (3×30mL). The organic layer was dried (Mg₂SO₄) and concentrated under vacuum. The product was purified via column chromatography on silica gel (2:1 Hexanes/EtOAc) to yield 298 as a brown oil (0.51g, 1.9mmol, 67%). ¹H NMR (300MHz; CDCl₃) δ 7.64 (s, 1H), 7.16 (s, 1H), 6.11 (d, ³J(H,H)=5.6 Hz, 2H), 5.44 (m, 1H), 4.59 (d, ³J(H,H)=11.2 Hz, 1H), 4.28 (d, ³J(H,H)=11.2 Hz, 1H), 2.13 (s, 3H), 1.50 ppm (d, ³J(H,H)=6.4 Hz, 3H). ¹³C NMR (300MHz; CDCl₃) δ 152.64, 147.18, 142.36, 137.18, 106.63, 105.32, 103.19, 73.35, 70.65, 23.71, 14.51 ppm. LC/MS m/z 294.1 (M+Na⁺), Rᵣ 10.37 min, Rᵣ 0.73 (hexanes/EtOAc = 4:1).

NPOM Caged Theophylline 295. To an ice-cold solution of 298 (50mg, 0.18mmol) in DCM (1mL) was added sulfuryl chloride (37mg, 0.28mmol, 1.5eq.). The reaction was stirred for 4 hours and the solvent was removed under vacuum. In a separate flask theophylline (49mg, 0.28mmol) was stirred in DMF (1mL) containing cesium carbonate (176mg, 0.54mmol, 3eq.) for 1 hour at room temperature. The caging group was then dissolved in DMF (1mL) and added dropwise to the theophylline solution. The reaction was allowed to progress for 12 hours and quenched with water (1mL). The reaction was extracted with DCM (3×3mL), and the organic layer was dried (Mg₂SO₄), and the solvents were evaporated. The crude product was purified via column chromatography (3:1 EtOAc/Hexanes) to yield 295 as a yellow solid (88mg, 0.21mmol, 82%). ¹H NMR (300MHz; CDCl₃) δ 7.65 (s, 1H), 7.41 (s,
$^1$H NMR (300 MHz; CDCl$_3$) $\delta$ 205.75, 205.71, 151.61, 150.82, 146.52, 143.45, 140.80, 136.65, 105.64, 104.06, 103.42, 91.55, 74.49, 73.25, 29.44, 27.54, 23.51 ppm. LC/MS $m/z$ 404.5 (M+H$^+$), $R_t$ 6.70 min, $R_f$ 0.21 (hexanes/EtOAc = 1:3).

**Synthesis of Caged Toyocamycin (300).** A flame dried vial under a nitrogen atmosphere was charged with toyocamycin (299)(14 mg, 0.05 mmol), 2-nitrobenzaldehyde (151 mg, 1.00 mmol) and ZnBr$_2$ (45 mg, 0.20 mmol). The reaction mixture was heated to 60 °C for 12 hours and directly purified via flash chromatography (100% EtOAc) on SiO$_2$ to yield an off-white solid (14 mg, 0.03 mmol, 68%). $^1$H NMR (300 MHz; CD$_3$OD) $\delta$ 8.24 (s, 1H), 8.22 (s, 1H), 8.11 (dd, $J_1 = 7.8$ Hz, $J_2 = 1.5$ Hz, 1H), 7.98 (dd, $J_1 = 7.8$ Hz, $J_2 = 1.5$ Hz, 1H), 7.80 (dt, $J_1 = 7.8$ Hz, $J_2 = 1.5$ Hz, 1H), 7.66 (dt, $J_1 = 7.8$ Hz, $J_2 = 1.5$ Hz, 1H), 6.60 (s, 1H), 6.33 (d, $J = 3.3$ Hz, 1H), 5.35 (dd, $J_1 = 6.3$ Hz, $J_2 = 3.3$ Hz, 1H), 5.16 (dd, $J_1 = 6.3$ Hz, $J_2 = 2.1$ Hz, 1H), 4.42 (dd, $J_1 = 3.9$ Hz, $J_2 = 2.1$ Hz, 1H), 3.77 (m, 2H); $^{13}$C NMR (75 MHz; CD$_3$OD) $\delta$ 157.5, 153.5, 149.9, 133.3, 132.5, 131.0, 130.5, 127.9, 124.3, 114.7, 102.9, 91.2, 86.4, 85.3, 84.3, 83.1, 62.1; HRMS (MALDI TOF): $m/z$ calculated for C$_{19}$H$_{17}$N$_6$O$_6$: 425.1137, found: 425.1235; UV-VIS $\lambda_{\text{max}}$ 290nm ($\varepsilon = 931$ cm$^{-1}$M$^{-1}$); $\lambda$ 365nm ($\varepsilon = 174$ cm$^{-1}$M$^{-1}$).
**UV Irradiation of Caged Toyocamycin.** A solution of 300 (0.1 mM in MeOH) was irradiated for 5 minutes at 365 nm with a transilluminator (25 W). After irradiation the product was concentrated and analyzed by NMR and LC/MS. Based on the results of these two experiments the ratio of 301/302 was determined to be 1:1.

**E. coli viability studies.** An *E. coli* culture was grown to log phase, then diluted and split into 3 experimental conditions: no small molecule, 0.1mM theophylline, and 0.1mM NPOM caged theophylline. OD<sub>600</sub> measurements were taken every 30 minutes, and no significant change in culture growth was observed under any of the conditions. The cultures were then centrifuged, lysed and analyzed by LC/MS for the presence of the small molecule. Both
theophylline and NPOM caged theophylline were found in approximately 0.06mM intracellular concentrations.

**Zebrafish embryo toxicity.** Zebrafish embryos (~20 animals in each experiment) were treated with both theophylline and NPOM caged theophylline 295 directly after fertilization and observed 24 hours later (Table 6.1). Overall, no phenotypic differences were visually observable after incubation with either compound and in all experiments a survival rate of 75-89% was observed (84% in case of the H2O control). The embryos were then snap-frozen, lysed, and analyzed on an LC/MS instrument. Intracellular concentration appears to be independent of the incubation concentration and reaches its maximum at approximately 0.17mM.

**Photochemical Ribozyme Activation.** DNA templates for the ribozyme depicted in Figure 1 and the oligonucleotides used for RT-PCR were purchased from Integrated DNA Technologies, Inc. The DNA template encoding the allostERIC cis acting ribozyme A (5’GGGCGACCCUGAUGAGCCAGGAUACCAGCCGAAAGGCCCUUGGCAGAUUGACGAAACGGUGAAAGCCGUAGGUUGCCC 3’) or trans acting ribozyme B (5’GGGCGACCCUGAUGAGCCAGGAUACCAGCCGAAAGGCCCUUGGCAGAUUGACGCGAAACCGGU 3’) was converted into double-stranded DNA in the presence of the primer 5’TAATACGACTCTATAGGGCGACCTGTGATGAG, which introduces the promoter for T7 RNA polymerase (New England Biolabs). The DNA extension reaction was carried out using Super Script II Reverse Transcriptase (Invitrogen) according to the manufacturer’s
directions. The resulting double-stranded DNAs were suspended in the transcription mixture containing 50 mM Tris-HCl (pH 7.5 at 23 °C), 75 mM MgCl₂, 3 mM KCl, 10 mM dithiothreitol and 0.5 mM of each of the four NTPs, 30 µCi [α-3²P] ATP, and T7 RNA polymerase (200 U). The transcription mixture was incubated at 37 °C for 6 h and purified on a Microcon 10 filtration column. The transcript was re-suspended in water and quantified on a liquid scintillation counter.

Ribozyme substrate (5’ GCCGUAGGUUGCC 3’) for Ribozyme B was purchased from Integrated DNA Technologies, Inc., and 5’ labeled with T4 Polynucleotide Kinase (New England Biolabs) and 10 µCi [γ-3²P]ATP according to the manufacturers instructions. The substrate was purified via a chloroform/isoamyl alcohol/phenol extraction followed by an ethanol precipitation and quantified on a liquid scintillation counter.

Ribozyme A (100 nM) was incubated at 23 ºC for 90 minutes in a buffer containing 50 mM Tris-HCl (pH 7.5) and 20 mM MgCl₂ in the presence and absence of the natural and caged theophylline (100 µM) and with and without UV irradiation (5 min, hand-held UV lamp, 365 nm, 23 W). Aliquots were removed at 30, 60, and 90 minutes and placed in stop dye (87 mM Tris base, 89 mM boric acid, 20% glycerol, 0.05% bromophenyl blue, 0.05% xylene cyanol, 0.1% SDS, 7.3 M urea, 40 mM EDTA). Samples were run on a 10% denaturing polyacrylamide gel and visualized on a phosphorimager (Scheme 2).

Ribozyme B (100nM) was incubated at 23 ºC for 10 minutes in a buffer containing 50 mM Tris-HCl (pH 7.5) and 20 mM MgCl₂ in the presence and absence of the natural and caged theophylline (100 µM) and with and without UV irradiation (5 min, hand-held UV lamp, 365 nm, 23 W). The initial incubation was followed by the addition of 5’ labeled RNA
substrate (1 nM), and the reaction was subjected to the appropriate experimental conditions for 8 hours. Aliquots were removed at 1, 2, 4, and 8 hours and placed in stop dye (87 mM Tris base, 89 mM boric acid, 20% glycerol, 0.05% bromophenyl blue, 0.05% xylene cyanol, 0.1% SDS, 7.3 M urea, 40 mM EDTA). Samples were run on a 10% denaturing polyacrylamide gel and visualized on a phosphorimager.

**Luciferase Assay in Mammalian Cell Culture.** Human Embryonic Kidney (HEK-293T) cells were passaged into two 96-well culture plate, and grown to 60% confluence. The cells were then transfected with the N117-luc construct (0.5 µg) and a phRL-CMV (Promega) transfection control construct (0.5 µg) using Fugene HD (3:2 Fugene/DNA; Roche Biomedicals) in OptiMEM media (Invitrogen). The transfection was incubated at 37 °C for 4 hours, followed by replacement of transfection media with standard growth media (Dulbecco’s modified Eagle’s media (Hyclone) with 10% Fetal Bovine serum (Hyclone) and 10% streptomycin/ampicillin (MP Biomedicals). The cell media was then supplemented with either 1% DMSO, 10 µM Toyocamycin (1% DMSO), or 10 µM caged toyocamycin (1% DMSO) and incubated 48 hours at 37 °C (5% CO₂) to afford compound diffusion into cells. All incubation conditions were repeated in triplicate on each plate. After 48 hours the media was removed and one plate was irradiated for 5 minutes with a hand-held UV Lamp (25W). Standard growth media was replaced and the cells were incubated for another 24h at 37 °C to afford luciferase expression. Following a visual inspection, the media was removed and the cells were assayed with the Dual-Luciferase Reporter Assay system (Promega) using a Wallac VICTOR³V luminometer with a measurement time of 1 s and a delay time of 2 s. The
ratio of Renilla to Firefly luciferase expression was calculated for each of the triplicates, the data was averaged, and standard deviations were calculated using Microsoft Excel.

**GFP Reporter System Construction.** In order to construct a N117-EGFP plasmid, the luciferase open reading frame (ORF) was removed from N117-luc (NotI/BamHI) and replaced with a PCR amplified ORF of EGFP (derived from EGFP-N1; Clontech). In brief, the ORF of EGFP was amplified with primers that introduced a 5' NotI site and a 3' BglII site and ligated into the NotI/BamHI sites of N117-luc. PCR employed Pfu DNA polymerase (Stratagene), a forward primer 5'-GATCGCGGCCGCGGTACCCCGGGCCCGGGATCCCA CC-3' and reverse primer 5'-GATCAGATCTTTACTTTGTACAGCTCGTCCATGCC-3' (restriction sites are underlined).

**Spatial Control of GFP Expression in Mammalian Cell Culture.** Human Embryonic Kidney (HEK-293T) cells were passaged into a 6-well culture plate, and grown to 40% confluence. The cells were then transfected with the N117-GFP construct (1 µg) and a pIRES2-DsRed Express transfection control construct (1 µg; Clontech) using Fugene HD (3:2 Fugene/DNA; Roche Biomedicals) in OptiMEM media (Invitrogen). The transfection was incubated at 37 °C for 4 hours, followed by replacement of transfection media with standard growth media (Dulbecco’s modified Eagle’s media (Hyclone) with 10% Fetal Bovine serum (Hyclone) and 10% streptomycin/ampicillin (MP Biomedicals) supplemented with caged toyocamycin (10 µM final concentration; 1% DMSO). The cells were then incubated at 37 °C (5% CO₂) for 48 hours to afford compound diffusion into cells, followed by the removal of the media and spot irradiation with a Jenco Epi-fluorescence inverted
microscope equipped with a 100W mercury lamp and a DANSA filter cube (330-400 nm excitation) for 30 seconds. The media was replaced and the cells were incubated for another 24 hours at 37 °C to allow for protein expression and folding. Cells were then imaged on a Leica DM5000B compound microscope to assess the spatial control of GFP expression.

**Synthesis of Caged Uridines.**

**Synthesis of Compound 304.** A flame dried round bottom flask was charged with nitrogen and a stir bar. Uridine (1.94g, 7.94mmol) and catalytic amounts of DMAP were added. Acetic anhydride (5mL) was added and the reaction was stirred at room temperature. The reaction was monitored by TLC (eluent: EtOAc) until disappearance of starting material was achieved (approximately 60 minutes). Excess acetic anhydride was removed *in vacuo*. The residual oil was dissolved in DCM (30mL), and washed with H₂O (2 x 30mL), followed by 2 washes with saturated NaHCO₃ (15mL). The organic layer was dried with MgSO₄, filtered and evaporated to dryness, yielding a foamy white solid (2.85 g, 7.70mmol, 96% yield). ¹H NMR (300MHz, CDCl₃) δ 8.66 (s, 1H), 7.38 (d, 1H, J=8.4), 6.02 (d, 2H, J= 5.1), 5.78 (dd, 1H, J₁=7.8, J₂=2.1), 5.30 (m, 2H), 4.35 (s, 2H), 2.13 (m, 9H).

**Synthesis of Compound 305.** A flame dried flask was charged with acylated uridine (0.36 g, 0.96 mmol 1.5 eq.) and dissolved in DMF (5 mL), Cs₂CO₃ (0.63g, 1.94 mmol, 3 eq.) was added and the solution was cooled to 0 °C. The NPOM chloride (0.17 g, 0.65 mmol) was dissolved in DMF (3 mL) and added dropwise to the reaction mixture over 30 minutes. The reaction was stirred and allowed to warm to room temperature over 16 hours. The reaction was quenched with H₂O (15 mL) and extracted with DCM (3 x 15 mL). The organic layer
was dried with MgSO$_4$, filtered and concentrated by rotatory evaporation. The residue was then subjected to purification via silica gel chromatography (1:3 Hexanes/ EtOAc) to yield a slightly yellow solid (0.44 g, 0.75 mmol, 78%). $^1$H NMR (400MHz, CDCl$_3$) $\delta$ 7.43 (s, 1H), 7.30 (s, 1H), 7.28 (d, 0.5 H, $J$=2.4), 7.26 (d, 0.5 H, $J$=2.0), 7.20 (s, 0.5 H), 7.16 (s, 0.5 H), 6.09-6.06 (m, 2H), 5.94 (d, 0.5 H, $J$=2.0), 5.90 (d, 0.5 H, $J$=4.4), 5.72 (d, 1H, $J$=8.0), 5.33-5.09 (m, 5H), 4.35-4.32 (m, 3H), 4.14 (q, 1H, $J$=7.2), 2.12-2.11 (m, 9H), 1.48 (d, 3H, $J$=6.4).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 170.4, 170.0, 169.8, 162.2, 162.1, 152.5, 151.0, 151.0, 147.2, 138.2, 137.7, 137.6, 106.6, 106.5, 105.2, 103.1, 103.1, 103.0, 102.9, 89.0, 88.9, 79.9, 79.8, 73.6, 73.3, 73.2, 73.0, 70.0, 69.7, 69.3, 63.2, 63.1, 23.9, 23.8, 21.0, 20.7, 20.6.

**Synthesis of Compound 307.** Sodium hydride (27 mg, 0.68 mmol, 1.6 eq.) was added to a flame dried flask under nitrogen. A slurry was made with DMF (6mL), and the acetylated uridine (81 mg, 0.22 mmol, 1 eq.) was added, and the reaction was stirred at room temperature for 1 hour. In a vial, 1-methyl nitropiperonyl bromide (67 mg, 0.25 mmol, 1.2 eq) was dissolved in DMF (4 mL) and added dropwise to the reaction, and then stirred overnight at room temperature. The reaction was monitored by TLC (100% EtOAc) for the disappearance of acylated uridine (approximately 12 hours). The reaction was quenched with H$_2$O (20 mL) and extracted with DCM (3 x 15 mL). The combined organic layer were then back extracted with brine (2 x 15mL), dried, filtered and evaporated *in vacuo*. The crude product was purified via flash chromatography (1:3 EtOAc/Hexanes) to yield a yellow glassy solid (18 mg, 0.05 mmol, 6% yield). $^1$H NMR (300MHz, CDCl$_3$) $\delta$ 7.33-7.24 (m, 4 H), 6.38 (t, 1 H, $J$=7.2), 6.10-6.08 (m, 2 H), 6.01 (d, 1 H, $J$=5.1), 5.71-5.69 (m, 1 H), 5.28-5.26 (m, 2 H), 4.36-4.32 (m, 3 H), 2.14-2.05 (m, 9 H), 1.84-1.81 (m, 3 H).
Synthesis of Compound 308. A flame dried flask was charged with the acylated uridine (0.10 g, 0.27 mmol) and acetonitrile (5 mL) was added. Reagents were then added in the following order: DMAP (cat.), Et₃N (0.14 g, 0.18 mL, 1.35 mmol, 5 eq.), followed by iPr₅C₆H₂SO₂Cl (0.25 g, 0.81 mmol, 3 eq.). The reaction turned yellow then quickly darkened and was stirred at room temperature for 3 hours. The 1-methyl nitropiperonyl alcohol (0.11 g, 0.54 mmol, 2 eq.) was dissolved in acetonitrile (1 mL) and added dropwise to the reaction, which was then allowed to stir at room temperature for 12 hours. The reaction was quenched with H₂O (10 mL) and extracted with DCM (3 x 10 mL). The organic layer was dried with MgSO₄, filtered, and concentrated by rotatory evaporation. The residue was purified via silica gel chromatography (1:1 Hexanes/EtOAc) to yield a yellow solid (0.13 g, 0.23 mmol, 86%).¹H NMR (300MHz, CDCl₃) δ 7.69-7.63 (m, 1 H), 7.47 (d, 1 H, J=3.6), 7.01 (d, 1 H, 6.9), 6.75-6.72 (m, 1 H), 6.10-6.07 (m, 2 H), 6.00-5.92 (m, 2 H), 5.33-5.26 (m, 2 H), 4.34 (s, 4 H), 4.09 (q, 1 H, J=7.2), 2.13-2.04 (m, 9 H), 1.68 (d, 3 H, J=6.6).

Synthesis of Compound 309. A flame dried flask was charged with uridine (0.12 g, 0.48 mmol) and dissolved in DMF (10 mL). Triethylamine (0.29 g, 2.85 mmol, 6 eq.) was added and the solution was cooled to 0 °C in an ice bath. Trimethylsilyl chloride (0.17 g, 1.57 mmol, 3.3 eq.) was added dropwise over 15 minutes and the reaction was stirred at 0 °C for 2 hours. The reaction was then gravity filtered into a second flame dried flask to remove the white precipitate and pyridine (0.04 g, 0.48 mmol, 1 eq.) was added. The solution was cooled to 0 °C in an ice bath and the chloroformate caging group (0.13 g, 0.48 mmol, 1 eq., in 3 mL DMF) was added dropwise. The reaction was allowed to progress 12 hours at room
temperature, then HF (200 µL) was added and the solution was stirred for 1 hour at room temperature. The reaction was quenched with H₂O (20 mL) and extracted with DCM (3 x 20 mL). The organic layer was dried with MgSO₄, filtered and concentrated by rotatory evaporation. The residue was purified via silica gel chromatography to yield a yellow solid (0.16 g, 0.34 mmol, 70%). ¹H NMR (300MHz, CDCN) δ 7.98 (d, 2 H, J=8.1), 7.94 (s, 1 H), 7.55 (s, 1 H), 7.25 (s, 1 H), 6.45 (q, 1 H, J=6.3), 6.19 (d, 2 H, J=6.0), 5.79-5.75 (m, 2 H) 4.16 (bs, 2 H), 3.99-3.97 (m, 1 H), 3.81-3.30 (m, 2 H), 3.50 (bs, 1 H), 3.30 (bs, 1 H), 1.71 (d, 3 H, J=6.3).

**Allosteric Ribozyme Selection Cycle.** The DNA template 5' GGCAACCTACGGCTTTCA CCGTTTCGACGT(N25)AAGGCTCATCAGGGTCGCC (purchased from IDT) encoding the randomized allosteric ribozyme was converted into double-stranded DNA in the presence of the primer 5'-TAATACGACTCACTATAGGGCGACCCTGATGAG, which introduces the promoter for T7 RNA polymerase (New England Biolabs). The DNA extension reaction was carried out using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s directions (2 µM of both template and primer (200 pmol); 0.5 mM each dNTP, 10 mM DTT, 200 units SSII RT, 20 µL total volume; 65 °C for 5 min, 4 °C for 2 min, 42 °C for 55 min, and 70 °C for 15 min). The resulting double-stranded DNAs were isolated by ethanol precipitation where 3M NaOAc, pH 5.0 was added (2 µL; 1/10 of the initial volume), followed by 100% EtOH (60 µL; 3X the initial volume). The sample was then incubated at -70 °C for 4 hours then centrifuged at the maximum speed for 25 minutes. The supernatant was removed and 70% isopropanol was added (100 µL), followed by a second 25 minute centrifugation to yield a DNA pellet. The isopropanol was removed and the pellet
was air dried and suspended in the transcription buffer containing the four NTPs, 200 µCi [α-\[^{32}\text{P}\]]\text{ATP}, and T7 RNA polymerase (20 units/µL; New England Biolabs) in a final volume of 200 µL. The transcription mixture was incubated at 37 °C for 1 h and the resulting uncleaved precursor RNAs (internally \[^{32}\text{P}\]-labeled) were isolated by denaturing 10% PAGE (95 V, 40 min). This PAGE purification eliminates ribozymes that undergo self-cleavage, thereby introducing an additional negative selection step that disfavors the isolation of ribozymes that function without activation by an external small organic ligand. The gel was imaged on a Storm Phosphorimager and the image was used to excise the appropriate band from the gel. These bands were then incubated 12 hours at 4 °C in 200 µL Soak Buffer (0.2M NaCl, 0.01M Tris, 1 mM EDTA) then centrifuged, and the supernatant was removed and ethanol precipitated to afford the purified RNA.

The \textit{in vitro} selection for allosteric ribozymes was carried out using repeated rounds of negative and positive selection. For the first round of negative selection, an initial pool of RNA precursors (approx. 10 nmol representing \(\sim10^{15}\) RNA molecules) was incubated at 23 °C for 5 h in a reaction mixture containing 50 mM Tris-HCl (pH 7.5) and 20 mM MgCl\(_2\). Ribozymes that resist cleavage during this incubation were isolated by denaturing 10% PAGE. Purified allosteric ribozymes were then subjected to the first round of positive selection at 23 °C for 30 min in the same reaction buffer in the presence of diazobenzene \textbf{311a} (1 mM). At this stage, cleaved products are purified by denaturing 10% PAGE and the 5' cleavage fragments are recovered from the gel by crush-soak elution and amplified by RT-PCR. Reverse transcription was conducted on the RNA using SuperScript II RT according to the manufacturer’s directions (0.5 mM each dNTP, 10 mM DTT, 200 units SSII RT, 20 µL total volume) using the primer 5’-GGGCAACCTACGGCTTCCACCGTTTTCG (10 µM).
Subsequent PCR amplification of the resulting cDNA using both primers mentioned above was performed in a reaction mixture containing 10 mM Tris-HCl (pH 8.3 at 23 ºC), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, and 50 U Taq polymerase (Promega). The reaction was cycled at 94 ºC for 30s, 55 ºC for 30s and 72 ºC for 60s for the appropriate number of cycles as determined by PCR pilot reactions (6, 8, 10, 13, 16, and 19 cycles run with 2 µL of reaction removed at each time point). The PCR pilots were run on a 10% polyacrylamide gel, and stained with ethidium bromide to ascertain the correct number of cycles required to see the correct MW band without any primer dimer or non-specific amplification. Additional rounds of selective amplification were repeated in a similar fashion using shorter positive selection reactions (15min) until caged theophylline activated ribozyme function is detected.

**Synthesis of 315.** 1’-(2-Hydroxyethyl)-3’,3’-dimethyl-6-nitrospiro[1(2H)-benzopyran-2,2’-indoline] (30 mg, 0.09 mmol) was dissolved in DCM (2 mL), and hydroxybenzotriazole (11 mg, 0.09 mmol) and 1-ethyl-3-(3’-dimethylaminopropyl) carbodiimide hydrochloride (16 mg, 0.09 mmol) were added, followed by N-Boc-β alanine (32 mg, 0.17 mmol). The reaction was stirred at room temperature 16 hours then concentrated and purified via chromatography (6:1 hexanes/ethyl acetate) to yield a glassy yellow solid (23 mg, 0.04 mmol, 52%); HRMS (MALDI TOF) m/z calcd for C₂₈H₃₄N₃O₇: 524.2319; found: 524.2298.

**Synthesis of 317.** Spiropyran 315 (10 mg, 0.019 mmol) was dissolved in anhydrous HCl (500 µL) and stirred for 5 hours at room temperature. The reaction was concentrated under vacuum, and neutralized with 1M NaOH (100 µL) for 2 hours at room temperature, then
dried with sodium sulfate and concentrated to yield a purple solid (7 mg, 0.016 mmol, 87%). The deprotected spiropyran 316 was directly dissolved in DMF (166 µL, 100 mM), and diluted to 10 mM in a 2:1 H<sub>2</sub>O/DMF mixture (1 mL). This solution was incubated with epoxy-activated Sepharose 6B resin (500 mg, GE Healthcare) for 24 hours at 40 °C. The resin was then washed with 2:1 H<sub>2</sub>O/DMF (5 x 3 mL), H<sub>2</sub>O (5 x 5 mL), alternating washes of 0.1M sodium acetate (0.5 M NaCl, pH 4.0, 3 x 3 mL) and 0.1 M Tris-HCl (0.5 M NaCl, pH 8.0, 3 x 3 mL), and finally DEPC water (5 x 5 mL).

**Synthesis of 318.** 1'-(2-Hydroxyethyl)-3',3'-dimethyl-6-nitrospirol[1(2H)-benzopyran-2,2'-indoline] (30 mg, 0.09 mmol) was dissolved in THF (2 mL) at 0 °C, and carbon tetrabromide (33 mg, 0.1 mmol) followed by triphenylphosphine (27 mg, 0.1 mmol) were added. The reaction was stirred 12 hours at room temperature then concentrated and purified via chromatography (6:1 hexanes/ethyl acetate) to yield a purple solid (17 mg, 0.04 mmol, 49%).

**Synthesis of 319.** Spiropyran 318 (17 mg, 0.04 mmol) was dissolved in DMF (1 mL) and sodium azide (5 mg, 0.08 mmol) was added. The reaction was allowed to progress 12 hours at room temperature, concentrated under vacuum and re-dissolved in THF (2 mL). The reaction was then filtered to remove excess sodium azide, and treated with triphenylphosphine (22 mg, 0.08 mmol) for 24 hours at room temperature. The reaction was then concentrated and purified by chromatography (4:1 hexanes/ethyl acetate) to yield a glassy yellow solid (9 mg, 0.03 mmol, 63%); HRMS (MALDI TOF) m/z calcd for C<sub>20</sub>H<sub>22</sub>N<sub>3</sub>O<sub>3</sub>: 352.1583; found: 352.1719.
Synthesis of 321. Spiropyran 319 (5 mg, 0.014 mmol) was dissolved in methanol (500 µL) and reacted with S-acetyl-dPEG₈ NHS ester (10 mg, 0.016 mmol, Quanta BioDesign, Ltd.) for 6 hours at room temperature. The reaction was concentrated to dryness and purified by chromatography (10:1 hexane/ethyl acetate) to yield a viscous orange liquid (9 mg, 0.011 mmol, 81%); HRMS (MALDI TOF) m/z calcd for C₄₁H₄₀N₃O₁₃S: 834.3769; found: 834.3912. Spiropyran 320 was then dissolved in methanol (1.2 mL, 100mM) and 10 µL was added to 990 µL of a NH₂OH (0.5 M, 2:1 H₂O/MeOH, 0.1 M NaH₂PO₄, 10 mM EDTA, pH 7.2) for 2 hours at room temperature to yield the free thiol 321. Acetal cleavage was determined via LC/MS analysis (100% conversion). HRMS (MALDI TOF) m/z calcd for C₃₉H₅₈N₃O₁₂S: 792.3663; found: 792.3699.

SELEX Protocol. A library of DNA containing a 40mer randomized sequence was purchased from IDT (5’ GCCTGTGTGAGCCTCTTGTCGAAN(N)₄₀TTGGAGCGTTTA TTCTTTGCTCCCC 3’) and 250 pmol was amplified via PCR (Forward primer: 5’ CCGAATTCTAATACGACTCACTATAGGGAGACAAGAAATAAACGCTCCAA 3’, Reverse Primer: 5’ CCGGATCCGCTGTGTGAGCCTCTTGTCGAA 3’; dNTPs (1 µM each final concentration), Taq Buffer (10X from NEB), Taq polymerase (240 units, NEB, 5 units/µL), 100 µL total volume) for 8 cycles (initially 95 °C for 2 min, 95 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min) and purified by ethanol precipitation (see previous allosteric selection cycle for protocol). The dsDNA (1 nmol) was then transcribed with T7 RNA polymerase (New England Biolabs) and [α-³²P] ATP (2.8 pmol; MP Biomedicals) for 6 hours at 37 °C, and purified on Microcon 10 columns. The RNA was quantitated on a liquid scintillation counter and used for the partitioning step. Approximately 100 mg of
immobilized spiropyran resin was washed 3 times with 200 µL binding buffer (250 mM NaCl, 50 mM Tris, 5 mM MgCl₂, pH 7.5), followed by a 2 hour incubation with 100 pmol of the RNA randomer. The resin was then washed 6-8 times with 400 µL binding buffer until supernatant was free of radioactivity as detected by a Geiger counter, additional binding buffer was added (400 µL) and the resin was irradiated for 1 hour at 365 nm using a 500 W Hg lamp (Newport Model 66902). Spiropyran switching was observed as the color of the resin changed from white to a deep purple. Post irradiation, the supernatant was removed and the resin was washed 2 additional times. The supernatant and washes were combined, quantitated and concentrated under vacuum. The RNA was resuspended in DEPC water (20µL) and reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen). The reverse transcript was then amplified by PCR (as above), ethanol precipitated and quantitated. The DNA was then transcribed again and the selection cycle was repeated. After 10 cycles of selection, library enrichment was detected. The enriched DNA library was then cloned with a StrataClone PCR Cloning Kit (Stratagene) into a pSC-A plasmid. Individual colonies were selected, grown in liquid culture, mini-prepped (Qiagen Spin Mini-Prep Kit), and sequenced (University of Michigan DNA Sequencing Core). Sequences were processed to reveal several conserved motifs within the enriched pool.

**Binding Specificity Assay.** Selected clones were amplified from the plasmid via PCR (Taq Polymerase, 20 cycles), ethanol precipitated, and transcribed with [α-³²P] ATP using T7 RNA polymerase. After purification on a Microcon 10 column, and quantitation, the RNA aptamer was incubated with 50 mg of immobilized spiropyran in 200 µL of binding buffer (250 mM NaCl, 50 mM Tris, 5 mM MgCl₂, pH 7.5) for 2 hours. The resin was then washed 6
times with 400 µL binding buffer to remove unbound RNA, and incubated an additional hour in 200 µL binding buffer. The binding buffer was then removed and quantitated on a scintillation counter (3 µL of sample in 3 mL scintillation cocktail), and 200µL of fresh buffer was added followed by irradiation at 365 nm with the 300W Hg lamp for 1 hour. The buffer was removed and quantitated to determine the amount of RNA released upon resin switching.

**Binding Constant Determination.** Selected clones were amplified from the plasmid via PCR (50 µL reaction, in accordance to the previously employed conditions for the selection, Taq Polymerase, 20 cycles), ethanol precipitated and transcribed with [α-32P] ATP using T7 RNA polymerase. After centricon purification (previously described) and quantitation, two separate assays were used to determine the binding constant of each aptamer. First, the washed spiropyran resin was incubated with the RNA (50 pmol) in the presence of different concentrations of free spiropyran (0 mM, 0.010mM, 0.05mM, 0.1mM, and 10 mM). After 1 hour aliquots were removed and quantitated on a scintillation counter, yielding a titration curve (plotting actual concentration versus incubation concentration) with a binding constant at the inflection point. With a rough binding constant determined, equilibrium dialysis was performed with DispoEquilibrium Dialyzers (Harvard Apparatus) with 40 µM RNA aptamer in one chamber and 0, 10, 50 or 100 µM spiropyran in the alternate chamber. After 4 hours of equilibration, the solution in the spiropyran chamber was removed and analyzed by LC/MS to determine the final concentration. This data was then plotted versus initial concentration to afford a binding constant for the aptamer at the inflection point of the curve.
**SPR Protocol.** The spiropyran 321 (1 µM, 3 x 1.5 µL) and a mercaptohexanol control (1 µM, 3 x 1.5 µL) were incubated on a gold surface (SPRchip; GWC Technologies) for 2 hours to afford efficient immobilization. The surface was then washed with water (3 x 1 mL), and placed into in the SPR imager (SPRimager II, GWC Technologies). The RNA aptamer was transcribed, purified, resuspended in binding buffer, and quantitated on a Nanodrop Spectrophotometer. Approximately 100 µL of the aptamer solution (30-96 µM) was injected into the SPR chamber and allowed to bind to the immobilized aptamer for 30 minutes. After binding was detected the gold slide was irradiated with UV light of 365 nm for approximately 10 minutes, dissociation and adsorption were monitored in real time, and the switching was repeated to demonstrate reversibility of the aptamer binding.

**Synthesis of Caged Uridine Phosphoramidite (324).** The NPOM caging group was prepared according to the previously described synthesis. Uridine (2.0 g, 8.2 mmol) was co-evaporated with dry pyridine (2 x 10 ml), then dissolved in dry DMF (20 mL), and cooled to 0 ºC. The (t-Bu)2(OTf)2Si (2.85 mL, 8.8 mmol, 1.1 eq.), was added via syringe pump addition over 45 min and the reaction mixture was stirred for another 45 min at 0 ºC. Then, imidazole (2.72 g, 40 mmol, 5 eq.) was added, and the reaction was brought to room temperature, followed by the slow addition of tert-butyldimethylchlorosilane (1.578 g, 10.4 mmol). The reaction mixture was then heated to 60 ºC for 4 h, cooled to room temperature, and all the solvent was removed by rotatory evaporation. Water (60 mL) and chloroform (120 mL) were added, the layers were separated and the organic layer was washed with brine (2 x 30 mL) and dried with Na2SO4, gravity filtered, and evaporated by rotatory evaporation. Thee oily residue was purified by column chromatography (CHCl3:MeOH = 100:1),
delivering the product as a white solid (3.9 g, 97%). $^1$H NMR (300 MHz, CDCl$_3$): δ 0.18-0.21 (d, 6 H), 0.19 (s, 9 H), 1.05-1.10 (d, 18 H), 3.82-3.87 (m, 1 H), 3.90-3.99 (m, 1 H), 4.12-4.19 (m, 1 H), 4.26-4.27 (d, 1 H), 4.47-4.50 (m, 1 H), 5.65 (s, 1 H), 5.70-5.74 (m, 1 H), 7.22 (s, 1 H), 8.14 (s, 1 H).

The 2’-$O$-(tert-butyldimethylsilyl)-3’,5’-$O$-(di-tert-butylsilanediyl)uridine (1.0 g, 2.62 mmol) was dissolved in dry DMF (15 ml) and cooled to 0 ºC, and Cs$_2$CO$_3$ (2.0 g, 6.06 mmol) was added. The NPOM chloride (0.62 g, 2.38 mmol) was dissolved in DMF (15 mL), cooled to 0 ºC, and the solution was slowly added into the protected uridine over 10 minutes and then allowed to warm to room temperature. After 16 hours, the solvent was removed by rotary evaporation and the residue was dissolved in EtOAc (100 mL) and water (50 mL), the organic phase was separated, washed with brine (3 x 15 mL), dried over Na$_2$SO$_4$, filtered and evaporated. The oily residue was purified by column chromatography on silica gel (Hexanes/EtOAc 6:1 to 3:1), delivering the product as an off-white solid (1.12 g, 77%). $^1$H NMR (400 MHz, CDCl$_3$): δ 0.12-0.18 (d, 6 H), 0.85-1.04 (m, 27 H), 1.48-1.49 (d, 3 H), 3.75-3.84 (m, 1 H), 3.92-3.99 (m, 1 H), 4.11-4.23 (m, 2 H), 4.45-4.51 (m, 1 H), 5.13-5.21 (m, 1 H), 5.27-5.35 (m, 1 H), 5.55-5.59 (m, 1 H), 5.65-5.67 (m, 1 H), 6.05-6.10 (m, 2 H), 7.10-7.13 (m, 1 H), 7.18-7.21 (m, 1 H), 7.24-7.25 (s, 1 H), 7.42 (s, 1 H).

The silyl protected caged uridine (0.50 g, 0.82 mmol) was dissolved in DCM (10 mL) and cooled to 0 ºC. A chilled solution of hydrogen fluoride-pyridine (0.5 mL, 8.33 M) in 2 ml pyridine was added and the reaction was stirred at 0 ºC for 2 h. The solution was washed with water (3 x 10 mL) and with an aqueous sodium hydrogen carbonate-saturated solution (2 x 10 mL). The organic layer was dried with Na$_2$SO$_4$, filtered and concentrated via rotatory evaporation. The residue was purified by column chromatography on silica gel.
(CHCl₃:MeOH 100:1 to 25:1), furnishing the product as a colorless oil (0.34 g, 72%). ¹H NMR (400 MHz, CDCl₃): δ 0.07-0.09 (m, 6 H), 0.88-0.89 (s, 9 H), 1.47-1.48 (m, 3 H), 2.56-2.70 (m, 1 H), 3.79-3.80 (m, 1 H), 3.80-3.82 (m, 1 H), 3.93-3.98 (m, 1 H), 4.14-4.18 (m, 1 H), 4.49-4.55 (m, 1 H), 5.12-5.20 (m, 1 H), 5.29-5.33 (m, 2 H), 5.44-5.45 (m, 1 H), 5.53-5.55 (m, 1 H), 5.66-5.71 (m, 1 H), 6.06-6.09 (m, 2 H), 7.17-7.20 (d, 1 H), 7.44-7.45 (m, 1 H), 7.46-7.50 (m, 1 H).

The uridine analog (0.20 g, 0.34 mmol) was dissolved in pyridine (5 mL), and 4,4′-dimethoxytrityl chloride (0.17 g, 0.51 mmol) was added. The reaction was then stirred at room temperature for 12 h. Methanol (2 mL) was added and the reaction was concentrated by rotatory evaporation. The residue was purified by column chromatography on silica gel (Hexanes/EtOAc/Et₃N 6:1:0.25 then CHCl₃:MeOH:Et₃N 100:1:5) delivering the product as a yellow solid. (0.24 g, 80%). ¹H NMR (400 MHz, CDCl₃): δ 0.04-0.22 (m, 6 H), 0.82-0.93 (m, 9 H), 1.47-1.55 (m, 3 H), 2.69 (s, 1 H), 3.46-3.48 (m, 1 H), 3.78-3.79, (s, 6 H), 4.09-4.18 (m, 2 H), 4.28-4.34 (m, 1 H), 5.12-5.24 (m, 2 H), 5.26-5.38 (m, 3H), 5.66-5.71 (m, 1 H), 5.84-5.89 (m, 1 H), 6.03-6.12 (m, 2 H), 6.79-6.84 (m, 4 H), 7.14-7.19 (m, 2 H), 7.21-7.36 (m, 8 H), 7.42-7.50 (m, 1 H).

The DMT protected caged uridine (0.24 g, 0.27 mmol) was dissolved in DCM (5 ml) and chilled to 0 °C and flushed with N₂ gas. DIPEA (0.14 g, 1.08 mmol, 4eq.) was added and the reaction was stirred at 0 °C for 10 min. NCCH₂CH₂OP[N(i-C₃H₇)₂]Cl (0.13 g, 0.54 mmol, 2 eq.) was added and the reaction was gradually allowed to warm to room temperature over 12 hours. MeOH (0.5 mL) was added and the reaction was concentrated by rotatory evaporation. The residue was purified by column chromatography on silica gel (Hexanes/CHCl₃/Et₃N 100:20:1), delivering the product as a foamy yellow solid (0.26 g,
91%). $^1$H NMR: (400 MHz, CD$_3$CN): $\delta$ 0.08-0.09 (m, 6 H), 0.81-0.82 (m, 3 H), 0.87-0.88 (m, 6 H), 1.02-1.04 (m, 2 H), 1.09-1.23 (m, 12 H), 1.43-1.45 (d, 2 H), 2.57-2.60 (m, 1 H), 2.64-2.69 (m, 1 H), 3.27-3.31 (m, 1 H), 3.38-3.54 (m, 2 H), 5.56-3.64 (m, 3 H), 3.77-3.83 (s, 6 H), 4.06-4.10 (m, 1 H), 4.18-4.23 (m, 1 H), 4.26-4.33 (m, 1 H), 5.03-5.07 (m, 1 H), 5.13-5.27 (m, 3 H), 5.34-5.40 (m, 1 H), 6.05-6.11 (m, 2 H), 6.87-6.91 (m, 4 H), 7.09-7.13 (m, 2 H), 7.28-7.35 (m, 6 H), 7.39-7.45 (m, 3 H).
CHAPTER 7: LIGHT REGULATION OF PROTEIN FUNCTION

With the successful regulation of both DNA and RNA function using photolabile or photoswitchable light receptors, we were interested in expanding the technology to the final tier of the central dogma, that of protein function. As proteins are the biological workhorses of the cell, their direct spatial and temporal regulation can be advantageous in the regulation of a plethora of cellular processes. Additionally, the ability to regulate proteins using an external stimulus can lead to a better understanding of their regulation and function. Finally, due to the increased stability of proteins relative to RNA and ssDNA, their propensity for photoregulation is significantly higher and more applicable to \textit{in vivo} applications. In order to achieve photoregulation of protein function, either a small molecule effector of proteins can be converted into a photosensitive moiety, or the protein itself can be directly modified to become photoresponsive. Most importantly, when a light activated gene expression system is employed, it takes time for the protein to be made; however, via direct regulation of the protein a much more rapid response is achieved. We are interested in investigating the feasibility of both approaches towards the light regulation of protein function.

Many mechanisms of gene regulation \textit{in vivo} rely on small molecule inducers of gene expression. Due to their facile chemical manipulation, these organic ligands have become prime targets for the photochemical regulation of genes. In many of these cases, gene transcription is initiated or inhibited by a small molecule-protein interaction. Most commonly the small molecule binds to a protein, the activator, which subsequently binds to a promoter sequence on the DNA, thus turning on gene expression (Figure 7.1). Examples of small molecule modulators of gene expression include
doxycycline/tetracycline, lactose, β-estradiol, and ecdysone. Several of these molecules have been caged to achieve photoregulation of expression systems.

**Figure 7.1** General mechanism of many small molecule inducers of gene activation. Corresponding activator/promoter pairs discussed in this report are the estrogen receptor/estrogen response element and the reverse tetracycline-controlled transactivator/tetracycline responsive promoter element (Tet-ON system).

The first example of employing a caged small molecule in an inducible gene control system was based on the estrogen receptor, and was reported by the Koh group. The estrogen receptor (ER) is a nuclear hormone receptor that acts as a ligand-dependent transcriptional activator in eukaryotes. Upon binding of estradiol (326, Figure 7.2), the ER undergoes a conformational change that leads to release from a binding protein. It then binds to a specific eukaryotic promoter, the estrogen response element, activating transcription (Figure 7.1). Koh *et al.* synthesized the 3-OH photocaged estradiol 327 and exposed HEK293 cells harboring a luciferase reporter system under control of the ER to this compound. Cells irradiated with UV light showed 86% of maximum luciferase activity, in contrast to non-irradiated cells, which only showed minimal background activity.

**Figure 7.2.** Photocaged estradiol 326-328 and ecdysone 325.
Subsequently, Lawrence et al. achieved photochemical control of an ecdysone-inducible gene expression system employing the same strategy. Caging of ecdysone was accomplished on the most reactive 2-hydroxy position leading to 325. A luciferase reporter under control of the ecdysone inducible system was transfected into mammalian cells, and probed for luciferase expression with and without light irradiation. This was achieved in a spatial fashion via spot irradiation with a 100W Hg lamp through a light microscope. Luciferase expression was detected at approximately 60% of the level induced by the natural molecule, and spatial control was achieved in tissue culture. Advantages of both systems include low basal expression and high inducibility upon light irradiation. Moreover, the ecdysteroids are exogenous to mammalian systems and appear to have no toxic effects.

A problem associated with both systems is the limited duration of transcriptional activation through diffusion of estradiol and ecdysone out of the cell. Recently, Koh et al. solved this problem by creating a permanent light-induced switching event through covalent bond formation between the activated ligand and the estrogen receptor (Figure 7.3). Here, they employed a tamoxifen selective mutant of the ligand binding domain of the ER fused to Cre recombinase. In combination with the photocaged tamoxifen aziridine 330, which is decaged to 329 and then reacts with a cysteine residue in the binding pocket of the ER, recombination at a level of 26% of maximal activity (β-galactosidase reporter gene) was observed after 3 UV irradiations at 24 h intervals. Although the low level of gene activity, the necessity for multiple irradiations, and the potential diffusion of decaged 329 into neighboring cells prevents this from being a
system with broad applicability, it represents a step further towards a general light-induced gene activation.

![Chemical structure]

**Figure 7.3.** Caged tamoxifen aziridine 329 for irreversible estrogen receptor activation. After decaging to 330, a cysteine in the binding pocket of the receptor undergoes covalent bond formation.

As an application to showcase the utility of photocaged nuclear hormones in a model organism, Hayashi and co-workers recently synthesized the estradiol 329, which is caged on the 17-OH group, and employed it towards gene regulation in transgenic *Arabidopsis* plants. An estrogen receptor based transactivator system was employed to strongly express GFP in the presence of the small molecule activator. As a means of comparison the caged estradiol 5 was also employed, and in both cases GFP expression was regulated via irradiation with light; surprisingly, only compound 329 was capable of providing discrete spatial resolution (Figure 7.4). The researchers employed a similar tactic to regulate a gene responsible for the development of the lateral root and root hairs. While limited by small molecule delivery and diffusion, the ability to use light to control developmental processes within complex organisms was demonstrated.
Figure 7.4. Spatial control over GFP expression in Arabidopsis roots using 329. a) No light irradiation; b) whole root irradiation; c) spot irradiation.

The Tet system is a commonly employed conditional gene control system in eukaryotic cells and is functional in a wide range of model organisms. Cambridge and co-workers recently developed a caged doxycycline molecule 331 (through the reaction of doxycycline with the commercially available diazo DMNPE 322 in 16% yield), which was used to regulate GFP expression under control of the Tet-ON system in cell culture (Figure 7.5).395 In the absence of light the doxycycline remained inactive, resulting in no GFP expression. However, after a brief irradiation, the caging group was removed leading to the production of the transgenic GFP in cell culture. To demonstrate the ability to achieve spatial control in a higher organism, the system was employed in the regulation of β-glucuronidase (GUS) in transgenic tobacco leaves.395 The GUS transgene was under the control of a Tet-ON regulated CMV promoter. After incubation of the plant tissue with the caged doxycycline followed by partial irradiation, areas exposed to UV light expressed the GUS protein and thus displayed a blue pigmentation (Figure 7.5). While successful, the Tet-ON system does suffer intrinsically from a delayed induction, with maximal protein levels being detected after 12-14 hours, and potential diffusion problems of decaged doxycycline. Nevertheless, this example
displays photo-triggering of a significant gene expression system and demonstrates the feasibility of obtaining spatiotemporal control within cells and tissues.

![Figure 7.5. Caged doxycycline 331 and its application in spatial control of gene expression in plant tissue using the Tet-ON system.](image)

Recently, Dore and co-workers employed light-activation of a small molecule towards the opposite aim: the inhibition of protein expression. Although the caging of inhibitors of biological processes is not new, inhibitory regulation in a spatial fashion was reported for the first time. The ribosome inhibitor anisomycin (333) was caged by installing a BHC caging group on the pyrrolidine ring via a carbamate linker (332). This caging group was superior to other, more commonly used caging groups (DMNB and p-nitrobenzyl), since it has a 10-fold greater quantum efficiency and a large absorption cross-section, making it amenable to two-photon decaging at 740nm. Complete decaging of 332 was observed after a brief 2s irradiation at 365nm (Scheme 7.1). Its potential to control protein expression in vivo was successfully demonstrated in cell culture using GFP as a reporter gene. Irradiation of a subset of cells on a plate showcased the spatial control of the developed methodology; only irradiated cells exhibited decreased fluorescence, whereas surrounding non-irradiated cells showed constant or increased fluorescence. Future experiments will reveal if this approach is amenable to the study of biological processes within a multi-cellular model organism. It
is surprising that no two-photon excitation\textsuperscript{441} studies have been reported, since the BHC group is an excellent caging group for this purpose.

![Scheme 7.1. Light-activation of protein synthesis inhibitor 333 through decaging of 332.](image)

In addition to the photochemical regulation of gene function, several reports describe the caging of low-molecular weight regulators of other biological processes, including Ca\textsuperscript{2+},\textsuperscript{442} phosphatidic acid,\textsuperscript{443} and nitric oxide.\textsuperscript{444}

Proteins have traditionally been caged in a non-specific fashion (mostly on lysine residues) through isolation, installation of a reactive caging group \textit{in vitro}, and subsequent purification. This approach affords little control over photochemical regulation as multiple caging groups are installed, and caging of essential residues is not guaranteed. Additionally, this is limited to accessible amino acid residues, and often to only those exposed at the surface of the protein. An alternative approach is the chemical synthesis of caged peptides and proteins and their subsequent introduction into the biological system.\textsuperscript{445-447} The stepwise synthesis of proteins limits both their size and their quantity. These problems have been addressed through the native chemical ligation\textsuperscript{448} allowing for the semi-synthesis of caged proteins.\textsuperscript{449}

An alternative approach involves site-specific caging using unnatural amino acid mutagenesis with chemically synthesized misacylated tRNAs, as developed by Schultz and co-workers.\textsuperscript{450} Although, this technology provides site-specific incorporation of caged amino acids into proteins and has been demonstrated to regulate the activity of ion channels in
Xenopus oocytes by the groups of Dougherty and Lester, a limiting factor is the laborious chemical synthesis of the misacylated tRNA. Recently, the Schultz group modified the translational machinery of yeast and bacterial cells by introducing a completely orthogonal tRNA/tRNA synthetase pair. Two tRNA synthetases were engineered to only accept a caged tyrosine and a caged cysteine as the substrate (Figure 7.6), and to charge the corresponding tRNA with these amino acids. This tRNA then allows for site-specific in vivo incorporation of the caged amino acid into proteins by the ribosome, in response to the amber stop codon, TAG. The in vivo incorporation of was employed in the caging of a β-galactosidase reporter gene at the essential residue Tyr503. Cells incorporating the caged tyrosine displayed greatly reduced β-galactosidase activity, which was subsequently restored through UV irradiation at 365 nm (67% of wild-type activity, as demonstrated by a Miller assay). This technology removes limitations associated with the previous misacylation approach and enables the production of substantial quantities of site-specifically caged proteins in vivo. However, it is currently limited to tyrosine and cysteine amino acids and the employed ONB caging group is not optimal for irradiation with non-photodamaging UV light.

![Figure 7.6. Caged tyrosine (334) and cysteine (335).](image)

Dougherty and co-workers demonstrated the photolytic cleavage of 2-nitrophenylglycine (Npg) incorporated into proteins in vivo (Scheme 7.2). Specifically,
this approach was employed to probe the structure-function relationships of ion channel domains. The Npg residue was site-specifically incorporated into intracellular, extracellular, and transmembrane positions of both a potassium ion channel and the nicotinic acetylcholine receptor by employing the tRNA misacylation approach. In all cases protein cleavage was observed after UV irradiation. While the cleavage efficiency was only approximately 50%, these studies were still effective in the assignment of vital structural motifs for ion channel function. This methodology provides an alternative means to photocaging when probing protein function with light; however, prolonged irradiation times of 4h might not be compatible with most biological systems and prevent a high temporal resolution.

![Scheme 7.2. Photolytic cleavage of the peptide backbone.](image)

Although the vast majority of light-regulated biological processes involve the application of caging groups, the potential to photoregulate biological macromolecules through the incorporation of photoswitchable small organic molecules has also been realized. Typically, this is achieved by incorporating a diazobenzene moiety, whose configuration can be reversibly switched from trans to cis through light irradiation (Scheme 7.3), into a polypeptide, a protein, double-stranded DNA, or single-stranded RNA.
Recently, Loudwig and Bayley generated a protein pore which was modified with a single diazobenzene residue.\(^{460}\) This was achieved by introducing a single cysteine residue into one subunit of the \(\alpha\)-hemolysin heptamer, and subsequent bioconjugation of a thiol reactive diazobenzene molecule. The pore was assembled within a lipid bilayer by combining this subunit with wild-type subunits. The photochemical switching event could be detected on the single molecule level by applying an electric potential and measuring the current across the bilayer. Isacoff, Trauner, and co-workers successfully applied the same approach \textit{in vivo} using GluR6, a glutamate responsive ion channel.\(^{461}\) Here, the diazobenzene was not only covalently linked to a cysteine residue which was engineered into the pore, but was also connected to a glutamate allosteric activator. Photochemical switching to the \textit{cis} form allowed binding of the glutamate to the binding pocket of GluR6, thus opening the ion channel and allowing the flow of \(\text{Na}^+\), \(\text{K}^+\), and \(\text{Ca}^{2+}\). This was conducted in transfected HEK293 cells and the cation flow into the cell was measured by \(\text{Ca}^{2+}\) imaging and whole-cell patch clamping. These experiments revealed that the engineered channel does not open completely upon irradiation with UV light of 380nm, but it is fully closed when exposed to 500nm light.
A fundamentally different way to obtain regulation of gene expression on a transcriptional level has been achieved via the engineering of natural proteins which respond to light. This has been recently demonstrated by the groups of Quial, Ellington, and Voigt in the development of photoresponsive cells.\textsuperscript{462,463}

Quail and co-workers reported a truly light-switchable cellular system which is based on the classical yeast two-hybrid technology, allowing the regulation of gene expression in eukaryotic cells.\textsuperscript{463} Here, a phytochrome chromophore domain (Phy) is fused to the DNA binding domain of a GAL4 transcriptional activator. Through absorption of a red photon, the phytochrome is converted from its inactive form (Pr) into an active conformer (Pfr). This active conformer Phy(Pfr) then undergoes a specific interaction with a basic helix-loop-helix protein (PIF3), which itself is fused to the activating domain of the GAL4 transcriptional activator. Hence, irradiation with red light recruits the transcriptional machinery towards expression of a reporter gene (\textit{lacZ} or \textit{HIS3}) downstream of the GAL4 promoter sequence. Detectable increase of the \textit{lacZ} reporter was present five minutes after a 60sec irradiation and reached a maximal 1000-fold increase after three hours. Furthermore, photochemical control of the \textit{HIS3} reporter gene conveyed growth to a histidine auxotroph strain in the presence of light (Figure 7.7). Upon irradiation with light of a different wavelength (far-red), it was possible to turn off gene expression instantly. Most likely, this represents the only gene-expression system to date which can be reversibly switched with light.
Yeast two-hybrid assay employing a HIS3 reporter gene on selective media. a) Cell growth is only observed in case of irradiation with red light for three days. b) No growth was observed in the dark.

In a related approach, Ellington et al. created photoresponsive bacteria by fusing a membrane bound cyanobacterial photoreceptor (PCB) to a histidine kinase domain (EnvZ). The kinase domain is responsible for phosphorylation of a transcriptional activator (OmpR), which then binds to a corresponding promoter region (ompC promoter), thus effectively turning on gene expression (Scheme 7.4). In the absence of light the kinase domain is active, promoting gene expression. However, upon light exposure a conformational change inhibiting kinase activity is induced, thus turning both phosphorylation of OmpR and gene expression off.

Scheme 7.4. Photoresponsive bacterial system.
This method was employed in bacterial lithography using a β-galactosidase reporter system encoded by the lacZ gene, demonstrating the feasibility of obtaining excellent spatial control of gene expression using light (Figure 7.8). An intrinsic limitation of both systems is the need for the complete exclusion of ambient light, which interferes with the selective activation. Moreover, the phycocyanobilin cofactor is not naturally produced in yeast and bacterial cells, and needs to be added to the cell media. Ellington et al. elegantly solved this problem through the addition of the phycocyanobilin biosynthetic pathway to E. coli.462

Figure 7.8. Bacterial lithography utilizing the PCB photoresponsive expression system.

More recently, the Benkovic laboratory investigated the intrinsic light sensitivity of naturally evolved proteins as a mechanism to control protein function.464 Due to the ubiquitous nature of light, many species have developed mechanisms to respond to this external stimulus (as previously discussed in the PCB protein). Light, oxygen, or voltage (LOV) domains are common functional domains of proteins found across all kingdoms of life, and are involved in the light sensitivety of phototropins, chloroplast movement, and stomatal opening.465 The light sensitivity of this domain is derived from the covalent adduct formed between a cysteine residue with an excited flavin mononucleotide (FMN).466
Benkovic et al. were interested in engineering allosteric control into proteins using the LOV domain to convey a degree of photosensitivity to standard proteins. Protein allostery is a common mechanism of signaling for proteins, as interactions which occur on distinct protein surfaces are communicated to distant regions on the protein (Figure 7.9). For this study dihydrofolate reductase (DHFR) was selected as a target protein which would be engineered with the LOV domain to engineer a non-natural allosteric signaling construct. After identification of the optimal site in DHFR for LOV incorporation, light-dependent allosteric control was realized (Figure 7.9). However, the effect is relatively small (~2 fold), this interaction was not optimized, and represents a novel mechanism for the introduction of photosensitivity to any protein of interest.

Figure 7.9. Regulation of protein function through allosteric signaling. A) Demonstration of the concept of protein allostery, where distant active sites on the protein (yellow dots) communicate signal inputs through the protein (red dots and blue arrows). B) The photosensitivity of an LOV domain integrated into a DHFR protein. While different insertion positions were tested one exhibited photosensitivity (dark = black bars, light = white bars), increasing DHFR catalysis in the presence of light irradiation. Adapted from Lee. J. et al. Science, 2008, 322, 438.
Ultimately, we would like to expand the photoregulation of protein function in an *in vivo* context via the application of both photoregulated small molecule effectors and direct protein photoregulation. While we plan to initiate our studies with reporter proteins, it is our hope to quickly expand to more biologically relevant systems, which can benefit from photochemical control. We also hypothesize that we can utilize our caging technology to introduce near-natural amino acids into proteins that cannot be incorporated by any other means. All of these applications should provide useful tools to better harness protein activity using a non-invasive external stimulus.

### 7.1. Light Regulation of the Lac Operon

#### 7.1.1. Introduction

Small-molecule inducible gene expression systems are available in a variety of cell types including bacterial cells, yeast cells, and mammalian cells.\(^{265, 266}\) They have been used for the conditional protein expression in higher organisms ranging from plants to mice.\(^{267, 268}\)\(^{467}\) These systems are typically comprised of a natural receptor taken from one organism and transferred into a second organism, in conjunction with a natural or unnatural small-molecule ligand, which is orthogonal to all endogenous molecules of the organism of interest. Exposure of the organism to the small-molecule typically activates gene expression, which has found application in the production of recombinant proteins, the programming of biological processes, and the study of gene function. Although this technique enables temporal control over gene function on a minute to hour timescale, it does not permit spatial control.
We hypothesize that spatial control can be accessed utilizing the previously discussed caging strategy. As noted earlier, this approach has been applied to the light-activation of small-molecule inducers of protein expression. Both reported systems, the doxycycline and the nuclear hormone (e.g. estradiol) conditional gene expression system, are restricted to eukaryotic cells. As a result, we were interested in developing a light-inducible gene expression system which can be used in bacterial cells. We based this system on the lactose (lac) repressor which binds to the lac operator (lacO) thereby inhibiting RNA polymerase to perform gene transcription. In presence of the small-molecule effector isopropyl-β-D-thio-galactoside (IPTG) the repressor is released from the DNA through an allosteric binding event resulting in a conformational change and leading to gene expression (Scheme 7.5).

**Scheme 7.5.** IPTG induced expression of an open reading frame (ORF) through formation of the IPTG/lac repressor complex.

### 7.1.2. Design of a Caged Small Molecule Inducer

The crystal structure of the LacI/IPTG complex (PDB 1LBH) reveals interactions between the small-molecule and the protein in a tight binding pocket, as well as four essential hydrogen bonds between 4-OH and Arg197, 3-OH and Arg197, as well as 2-OH and Asp274 and Asn246 (Figure 7.10). We speculated that disruption of the hydrogen bond network and installation of a sterically demanding caging group would inhibit formation of the LacI/IPTG complex and thereby inhibit gene expression. If the installed group is removable
through irradiation with UV light, IPTG can be generated in a spatio-temporal manner, thereby enabling spatio-temporal control over protein expression.

Figure 7.10. Protein crystal structure of the LacI/IPTG complex. The intrinsic hydrogen bonding in the binding pocket suggests a target for disruption via the installation of a photolabile protecting group.

In order to achieve this, the caged IPTG 337 was synthesized in a single step (78% yield) by the reaction of IPTG with 6-nitropiperonal, furnishing selective dioxolan formation at the 4- and 6-hydroxyl groups. This is in accordance with NMR experiments and previous observations in similar reactions (Scheme 7.6).
7.1.3. Photochemical Assessment of Caged IPTG

With the successful preparation of a caged small molecule inducer, we first needed to characterize its photochemical properties. Irradiation of an aqueous solution of 337 (≤0.5 mM, $\varepsilon_{365} = 4533 \text{ cm}^{-1} \text{ M}^{-1}$) with non-photodamaging UV light (hand-held UV lamp, 365 nm, 0.5 W/cm$^2$) for 5 min leads to quantitative formation of the ester 2, as a 1:1 mixture of regioisomers (4-OH/6-OH, Scheme 7.6), as determined via $^1$H NMR.

Half-lives for the conversion $337 \rightarrow 338$ after irradiation depend on the concentration of 337 and amount to 11 sec (0.1 mM), 5.1 min (0.5 mM), and 11.8 min (1.0 mM, Figure 7.11A). This facile photolytic conversion of a 1,3-dioxane is in remarkable contrast to previous observations with bromohydroxycoumarin caged 1,3 diols.$^{394}$ The quantum yield ($\phi = 0.131$) for the photochemical conversion of 337 to 338 has been determined by 3,4-dimethoxynitrobenzene actinometry.$^{295, 296}$
Figure 7.11. Photochemical IPTG ester formation and physiological degradation. A) Decaging of 337 demonstrates a rapid formation of the ester at low concentrations. B) Intracellular concentration of the IPTG ester (338) decreases over the course of 12 hours signifying its intracellular hydrolysis to IPTG.

While the caged IPTG 337 is capable of rapidly being converted into the ester 338, it is our hope that this will undergo further hydrolysis to yield the IPTG (336). The ester 338 has been found to be stable in an aqueous solution; however, as observed for other carbohydrate esters, 338 is hydrolyzed to IPTG through esterases found in a cellular environment \( (t_{1/2} = 63 \text{ min} \pm 2 \text{ min}) \). These results suggest that it should be feasible to decage 337 \textit{in vivo} to afford IPTG.

In order to be useful in a biological environment, we needed to assess if the caged IPTG or any of its photodecomposition products are toxic to cells. Hence, we selected \textit{E. coli} for our toxicity studied, as this is the initial model for our photoregulation of gene expression. Growth of bacterial cells exposed to 0.5 mM concentrations of 337 and 338 revealed that both compounds do not reduce growth rates and are non-toxic, but are easily taken up by the cells (Figure 7.12). Thus, we are primed to employ this compound in \textit{in vivo} studies of small-molecule/protein interactions to achieve spatio-temporal control of gene expression.
Figure 7.12. *E. coli* growth curve demonstrating the lack of cytotoxicity of both the caged IPTG (337) and the IPTG ester (338).

7.1.4 Assessment of in vivo Activation of Caged IPTG

Photochemical induction of a liquid culture expression of a β-galactosidase reporter showed a strong dependence of expression on UV irradiation. It is of importance to note that this photochemical induction is only possible in cells (BL-21(DE3)), which express the lac repressor. A pUC19 (Novagen) plasmid containing the *lacZ* gene under the control of the lac operator was utilized to perform a Miller assay, thus quantifying the regulation of gene expression through caged IPTG. Cells were grown to an OD<sub>600</sub> of 0.6 and then induced with either IPTG or caged IPTG (to a final concentration of 0.5 mM) and either irradiated or kept in the dark. After 6 hours, cells were lysed, treated with o-nitrophenyl galactopyranoside, and the absorbance of the lysates was measured at 420 and 550 nm (Figure 7.13). Both the non-induced control and cells growing in presence of caged IPTG 337 displayed no β-galactosidase activity, demonstrating that installation of a caging group on the 4- and 6-OH...
completely disrupts binding of IPTG to the lac repressor. Additionally, irradiation of a bacterial culture with UV light in the absence of any small molecule also failed to induce β-galactosidase expression. Brief irradiation with non-photodamaging UV light (365 nm, 15 min, 25 W, handheld UV-lamp) of cells grown in presence of 337 (0.5 mM) lead to rapid decaging and levels of protein expression comparable to a standard IPTG induction (Figure 7.13).

![Figure 7.13](image.png)

**Figure 7.13.** β-Galactosidase (Miller) assay showing that in presence of 337 lacZ expression is only observed in cells, which have been irradiated with light of 365 nm (15 min, 25 W) or were incubated with regular IPTG. In absence of the small molecule inducer minimal protein expression is observed. In all cases the concentration of the small molecule was 0.5 mM.

### 7.1.5. Application of Caged IPTG Towards Bacterial Lithography

An application of the developed light-inducible gene expression system was demonstrated by bacterial lithography. Here, the synthetic small molecule 337 functions as a light sensor transforming a lawn of bacteria into a biological film. The resolution of such a film has been estimated to be 15 megapixels cm$^{-2}$, however, this theoretical limit might be diminished through IPTG diffusion.$^{462}$ For bacterial lithography the lacZ reporter gene was used to convert colorless X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) into a
blue dye. Hence, a negative image is obtained where irradiated regions are dark and non-irradiated regions remain white. The same pUC19 plasmid in BL21(DE3) cells was employed as before. Cells were seeded on Agar plates surface pre-treated with caged IPTG and X-gal, followed by immediate irradiation for only 30 sec at 365 nm (25 W). A mask blocking one half of the plate was used to demonstrate spatial control over gene expression. After 12 hours of incubation at 37 °C, only cells on the irradiated half of the plate showed a blue color and hence β-galactosidase activity (Figure 7.14). To obtain a positive image, an identical experiment was performed using green fluorescent protein (GFP) as a reporter. Here, *E. coli* cells transformed with a pGFPuv plasmid were seeded on agar plates pre-treated with caged IPTG, irradiated as before, and incubated at 37 °C for 12 hours. Plates were analyzed for fluorescence on a Storm 480 PhosphorImaging system, indicating a spatially restricted expression of GFP (Figure 7.14) and revealing a positive image. In both cases spatial control of gene expression is observed, with minimal diffusion of irradiated IPTG. The short exposure time is advantageous over previously reported bacterial lithography methods which required 12-15 hours of light exposure.\textsuperscript{462}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{image.png}
\caption{Bacterial lithography using UV irradiation of 365 nm for 30 sec while blocking the left half of a Petri dish (Ø 10 cm). Two different reporter genes were employed; lacZ (left) and GFP (right) delivering a negative and a positive image, respectively.}
\end{figure}
In summary we achieved light-controlled activation of gene expression in bacterial cells in a spatio-temporal fashion. Due to the widespread application of the lac repressor in biotechnology, a broad utilization of the developed methodology is expected. Moreover, since the lac repressor is also functional in plant cells, mammalian cells, and higher organisms, this approach has the potential to provide a general solution to temporal and spatial control over gene function in more complex biological environments. Importantly, it could allow for a photochemical regulation of bacterial quorum sensing, providing detailed information on the mechanism of biofilm formation through cell-to-cell communication. Additional applications range from the creation of biological materials with certain spatial patterns to the studies of genetic circuits in developmental model organisms.

7.2. Photocaging of Rapamycin

7.2.1. Introduction

Rapamycin (339), also known as Sirolimus, is a triene macrolide natural product produced from a polyketide synthetase that has been demonstrated to have substantial biological relevance. First isolated in 1975 from the soil bacterium Streptomyces hygroscopicus, on Easter Island (Rapa Nui), rapamycin was first examined as an antifungal agent. As research progressed, rapamycin was found to be a highly specific inhibitor of the mammalian target of rapamycin (mTOR) protein, which is involved in the regulation of cell growth, proliferation, motility, survival, as well as protein synthesis and transcription. The value of rapamycin has also been demonstrated by the numerous total syntheses towards this
natural product. As a result, rapamycin has become a potent therapeutic agent, and recently has been marketed under the name of Rapamune by Wyeth as an immunosuppressant for transplant patients. More recently, due to its antiproliferative effects, rapamycin is being investigated for a variety of purposes, including applicability as an anti-cancer agent, a treatment for autism, and for prevention of restenosis in arterial stents.

Figure 7.15. Structure of rapamycin.

The mTOR protein (also known as FRAP and RAFT) is a 289 kDa serine/threonine kinase that exists in two heterodimeric complexes mTORC1 and mTORC2, and is highly conserved between a variety of organisms (greater than 95% of sequence). Interestingly, mTORC1 is the only known target of rapamycin, and mTORC2 displays no sensitivity to rapamycin. Prior to the discovery of rapamycin, little was known about its function, as there were no known antagonists. The integral nature of this protein, mTOR knock-outs and mutants have led to embryonic lethality, increases the difficulty of studying this protein. The mTOR protein appears to be activated by a variety of upstream signals, and is known to phosphorylate a variety of targets, leading to multiple signaling cascades (Figure 7.16).
The mechanism of rapamycin interaction with the mTOR protein appears to occur via initial binding of rapamycin with the FK506 (also known as FKBP12 or FKBP) protein, this protein-small molecule complex is then capable of binding mTOR in a highly specific fashion, leading to its inactivation. The FK506 protein alone is known to inhibit rotamase activity; however, its best-known function is as a part of the FK506-rapamycin-mTOR complex (Figure 7.17). Fortunately, the binding sites on the rapamycin to both the mTOR and
FKBP proteins have been well characterized, leading to an excellent understanding of this complex formation.\textsuperscript{486}

Due to the high binding affinity and specificity of the FKBP-rapamycin complex to mTOR, this interaction has been utilized as a chemical dimerization tool to engineer novel function into biological systems. The two proteins are expressed as fusion proteins to secondary proteins, and upon introduction of rapamycin, or a rapamycin analog, the mTOR-rapamycin-FKBP complex forms, bringing the two secondary proteins together to induce a biological effect. This chemical induction of dimerization has been employed in a variety of applications, including transcriptional activation, protein localization, protein stabilization, protein degradation, and protein splicing (Figure 7.18). Due to these results, significant work has been performed by the Crabtree laboratory to engineer orthogonal systems using rapamycin analogs, referred to as “rapalogs”, and mutated Frb domains of the mTOR protein.\textsuperscript{488} Ideally, these mutated mTOR and rapalogs can be used in the same system to selectively perform functions based on what their corresponding protein fusion (Figure 7.19).\textsuperscript{265, 489}
Figure 7.18. Application of mTOR-rapamycin-FKBP interaction to control biological processes. These include protein stabilization, protein degradation, and protein splicing. From Banaszynski, L.A. *Chemistry & Biology*, 2006, 13, 11.

Figure 7.19. Application of orthogonal rapalog systems. Using a combination of mTOR analogs and rapalogs different proteins can be exported utilizing the mTOR-rapamycin-FKBP interaction. From Bayle, J.H. *Chemistry & Biology*, 2006, 13, 99.

Based on the plethora of *in vivo* and *in vitro* applications of rapamycin, we can envision several scenarios where a photoregulated rapamycin would be advantageous. Due to
the specific and tight protein-small molecule interactions of rapamycin with its two protein binders, we hypothesized the installation of a photolabile protecting group on the rapamycin should inactivate this chemical dimerization until activated via light irradiation.

7.2.2. Caging of Rapamycin

A variety of modified rapamycin derivatives have been synthesized in the literature, and based on these chemical modifications, we attempted to synthesize several caged rapamycin derivatives (Figure 7.20). Due to the complexity of the molecule, only mild conditions can be employed in synthetic protocols to modify rapamycin. Dr. Xin Xiong installed a methyl-2-nitropiperonyl group on the C40 hydroxyl group via displacement of the 4-nitrobenzylchlorofomate with the Methyl-2-nitropiperonyl alcohol to yield caged rapamycin 340. Additionally, he prepared caged rapamycin 341 in a 74% yield via treatment with TFA to generate the carbocation, followed by quenching with 2-nitrobenzyl alcohol. Modifications at this C7 position are somewhat common and the source of modification for various rapalogs.

Interestingly, one of the more orthogonal rapalog/mutant mTOR systems involves the installation of 3-methylindole on this position. This derivative, known as the iRap analog now possesses an indole functionality, which may be caged via previously developed protocols. Thus, Yan Zou prepared the iRap derivative 342, and attempted its caging with a chlorofomate caging group to yield 343. To date, the caging has remained elusive; however, a variety of other strategies are being explored to synthesize caged iRap 343.
7.2.3. Rapamycin Assays

With several caged rapamycin analogs in hand, we next needed to assay their activity towards binding both FKBP and mTOR. As previously discussed, the FKBP-rapamycin association is necessary to bind mTOR and inhibit its function as a kinase, thus we suspected an assay could be conducted measuring the phosphorylation ability of mTOR. Conveniently, there are several mTOR kinase assays commercially available, and we selected the K-LISA mTOR activity kit (Calbiochem) to determine the activity and photoactivation of the caged rapamycins.

The K-LISA mTOR activity kit is an ELISA based assay beginning with a glutathione coated plate which allows for the immobilization of an mTOR substrate. In the absence of rapamycin, the mTOR should be an active kinase and phosphorylate its substrate.
on the surface, the phosphorylation event can be detected via a primary antibody to the phosphorylated substrate. This primary antibody is then recognized by a secondary antibody that is conjugated to the horseradish peroxidase (HRP) enzyme, which after incubation with an appropriate substrate will afford a color change correlated to the amount of HRP present. This colorometric assay can be used to quantitatively measure the amount of mTOR activity.

In the presence of rapamycin and the FKBP protein (R&D Systems), the mTOR activity should be diminished, leading to less phosphorylated substrate, and ultimately a decreased absorbance at 450 nm. Ideally, the caged rapamycin prior to irradiation should afford a comparable activity as that measured in the absence of rapamycin, but upon irradiation should reduce the signal to the same level as measured in the presence of rapamycin.

An assay of caged rapamycin demonstrated no loss of activity in the non-irradiated sample, which suggests that modifications on C40 can be tolerated and do not appear to inhibit the formation of the terinary complex (data not shown). This is not necessarily surprising, as many rapamycin analogs used in clinical treatments possess modifications on this cyclohexyl ring in order to aid in drug formulation without inhibiting function.

Performing the K-LISA assay on caged rapamycin led to some interesting results. The non-irradiated compound restored approximately 20% of the activity relative to the non-caged rapamycin; however, it does not restore activity to 100% (Figure 7.21). This indicates that this modification is inhibiting some binding, but is still be accommodated in the binding pocket. Gratifyingly, irradiation of provides the comparable amount of mTOR inhibition as the non-caged rapamycin. We hypothesize that an increased level of activity may be
achieved in the caged analog via the installation of a more bulky caging group, decreasing the propensity of the rapamycin to bind its targets.

In order to investigate the effects of the iRap analog \textbf{342} in the non-mutated mTOR system, we utilized the K-LISA assay. The 3-methylindole modification leads to a similar loss in mTOR activity as the caged rapamycin \textbf{341} (Figure 7.22). These groups are comparable in size, and thus suggest that the installation of a larger group may further inhibit function. As a result, the synthesis of the caged iRap \textbf{342} may further abrogate activity. Additionally, it would be interesting to employ \textbf{341}, \textbf{342} and \textbf{343} in an assay with the appropriate mutated mTOR due to the orthogonal nature of the system.
In summary, we have initiated a program to photochemically regulate the important mTOR pathway. Additionally, the photoregulation of rapamycin activity has substantial application in chemical dimerization experiments in the regulation and transport of proteins. However, further work is still required to develop an optimal system, in which, the caging group completely abrogates function. Due to the high degree of functionality on the rapamycin core, potential chemical modifications are challenging, but ideally the installation of a sterically bulkier caging group at the C7 position may be the best option. Additionally, the caging of iRap 342 should also provide a large enough of a perturbation to disrupt enzyme recognition; however, when using the standard mTOR protein, some of the inhibitory activity relative to rapamycin is lost. Finally, the analysis of new chemistries to modify other regions of rapamycin should be considered. As with the caged IPTG, it may be possible to form a caged dioxolane rapamycin on one of the keto-functionalities located within the enzymatic
recognition sites. Ultimately, the successful caging of rapamycin possesses a plethora of therapeutic and technological possibilities, which can facilitate the study and treatment of diseases such as cancer.

7.3. Photoregulation of Cre Recombinase

7.3.1. Introduction

While the previous research has involved the caging of biologically active small molecules that target proteins, leading to a disruption of their interactions with other biomacromolecules, we are also interested in directly installing the photocaging group on the protein of interest. This may be a more efficient mechanism for photoregulation, as the photosensitive group can be specifically placed in the enzymatic active site to prohibit catalysis, or prevent proper folding and function of the protein. Conveniently, various research groups have previously explored this approach towards protein photoactivation. Specifically, the Schultz group has installed a photocaged tyrosine and cysteine within a protein, as well as a photoswitchable diazobenzene moiety directly into proteins.\textsuperscript{453, 490, 491}

Within the genetic code, there are only 20 naturally encoded amino acids utilized in the biosynthesis of all proteins. This is remarkable when considering the diversity of all protein function is relegate to only 20 monomers; however, one can envision the expansion of the genetic code to additional amino acids to facilitate novel protein activity. This can be accomplished via several routes including: 1) the \textit{in vitro} solid-phase synthesis of proteins containing non-natural amino acids; 2) the \textit{in vitro} chemical modification of amino acid residues; and 3) the \textit{in vivo} incorporation of non-natural amino acids via modification of the translational machinery. While all three methodologies have been successfully employed in
the introduction of new functionalities to proteins, they each possess different advantages and disadvantages. The solid-phase incorporation of unnatural amino acids is limited to the constraints imposed by peptide synthesis, limiting the number of amino acids coupled before synthetic impurities dominate the synthesis. The in vitro chemical modification requires reaction conditions that are amenable to proteins (e.g., typically aqueous solutions), and is often non-specific, reacting with any accessible residue bearing the modification site. The in vivo incorporation of amino acids requires substantial effort to generate an expression system; however, once accomplished provides the site-specific incorporation of the desired amino acid. Due to the ability to generate large proteins (>40 amino acids) with a site-specific modification, we selected the in vivo approach to investigate the ability to introduce a means for the photochemical activation of proteins.

Protein synthesis is derived from translation of mRNA on the ribosome, and this process relies upon tRNAs to recognize the RNA sequence in a triplet code (known as codons). Since there are only 4 nucleotide bases, the triplet nature of the genetic code results in 64 codons that encode the 20 naturally occurring amino acids, resulting in some degeneracy in the system. The Schultz laboratory has exploited this degeneracy to develop a mechanism to expand the genetic code for the in vivo incorporation of a 21st amino acid (Figure 7.23). The technique requires an initial selection to develop an orthogonal tRNA/tRNA synthetase pair which recognizes the unnatural amino acid and incorporates it in response to a degenerate codon. The degenerate codon of choice is typically the amber stop codon (AUG) as it does not encode the insertion of any natural amino acid, and is the least frequent in occurrence of all stop codons.
To begin, a suppressor tRNA must be generated that recognizes the amber stop codon (AUG) and inserts an unnatural amino acid at this position; because of the inefficient aminoacylation between the tRNAs of different species, a suppressor tRNA\textsuperscript{Tyr} library can be constructed from the tyrosyl-tRNA (tRNA\textsuperscript{Tyr}) of the archaeabacterium \textit{Methanococcus jannaschii} for use in an \textit{E. coli} expression system. The most important feature of this exogenous \textit{M. jannaschii} tRNA\textsuperscript{Tyr} is that it must efficiently suppress the stop codon so that protein biosynthesis can occur, while remaining uncharged by any endogenous \textit{E. coli} aminoacyl tyrosyl-tRNA synthetases (TyrRS).\textsuperscript{53} In order to optimize this orthogonality, 11 nucleotides of the tRNA that do not directly interact with the TyrRS are randomly mutated to generate a suppressor tRNA library for screening. The suppressor library is then subjected to a negative selection to eliminate library members that are aminoacylated by \textit{E. coli}.

\textbf{Figure 7.23.} A general approach for the site-specific incorporation of unnatural amino acids into proteins \textit{in vivo}.
synthetases. This is followed by a positive selection to identify those tRNAs^{Tyr} able to incorporate tyrosine in the presence of their cognate \( M. \textit{jannaschii} \) TyrRS.\(^{492}\)

Next, the \( M. \textit{jannaschii} \) TyrRS must be evolved to recognize an unnatural amino acid and use it to charge the previously evolved cognate tRNA^{Tyr}. A TyrRS library is established via the generation of a library of mutants, which vary at five or six residues selected based on the crystal structure of the \( M. \textit{jannaschii} \) TyrRS active site (Figure 7.24). The pool of synthetases is then subjected to iterative selection cycles of positive and negative selections to evolve a mutant TyrRS capable of charging a suppressor tRNA^{Tyr} with almost any unnatural amino acid (Figure 7.25). As previously noted, this process has been applied towards the successful incorporation of photocaged amino acids, to yield light-regulated proteins.\(^{54-56}\)

**Figure 7.24.** Active site of \( M. \textit{jannaschii} \) tyrosine tRNA synthetase and the amino acids varied for the generation of a mutant library.
The tRNA/tRNA synthetase pair for the incorporation of 2-nitrobenzyl tyrosine (ONBY) was procured from the Schultz laboratory, as the tRNA/tRNA synthetase pairs have already been subjected to the intense selection cycle and have been found to readily incorporate this unnatural amino acid in a variety of reporter proteins. We hoped to exploit this technology to harness the power of the photoactivation event in more biologically relevant proteins.

7.3.2. Cre Recombinase

Site-specific recombinase proteins catalyze strand exchange between defined target sequences on each of two DNA segments. The well-characterized and versatile Cre recombinase of bacteriophage P1 recognizes two palindromic loxP sites consisting of two 13 base pair repeats flanking an eight base pair asymmetric core; orientation of the loxP sites
results in deletion or insertion (direct repeats), or inversion (inverted repeats) of intervening DNA (Figure 7.26).\(^4\)

![Figure 7.26](image)

**Figure 7.26.** Cre recombinase catalyzes the inversion or excision/integration of DNA between *loxP* sites depending on their directionality.

Cre has an extensive range of applications in a variety of organisms (e.g. in mice, zebrafish, drosophila, and plants),\(^4\) from basic vector construction to silencing or inducing gene expression, and has been an invaluable tool for engineering knockouts and conditional alleles.\(^2\) Importantly, Cre is active both *in vitro* and *in vivo*, as successful recombination occurs without an external energy source or accessory proteins, and with no constraint on substrate conformation.\(^5\)

While wide-spread use of the Cre/*loxP* system is due to its simplicity and effectiveness, limitations exist in achieving tight control over Cre activity, and thus the recombination event, as organisms harboring a Cre-encoding plasmid or transgene typically express the enzyme constitutively. Attempts to regulate Cre activity temporally have involved the use of inducible systems,\(^5\) and spatial control has been achieved with tissue-specific promoters to control Cre expression. However, those systems have a variety of
limitations, including the toxicity of small molecule inducers of gene expression, leakiness of the gene expression system leading to background Cre activity, incomplete tissue specificity of the regulatory elements, and the unavailability of promoters for every tissue. Importantly, spatial control of the enzyme cannot be achieved on the level of a single cell. Alternatively, Cre has been fused to various ligand binding domains that are regulated by small molecules, such as tamoxifen. While small molecules provide a means of external control, they are nonetheless susceptible to problems with cellular uptake, diffusion into and out of the cell, and subsequent off-target recombination. To limit this unwanted recombination, the activity of tamoxifen was previously modulated by the installation of a light-responsive protecting group, termed caging group. While photocaged tamoxifen provided the first attempt at photo-control over recombination using a photoinducible system, multiple light irradiations were required to decage the small molecule to activate Cre, and recombination activity was low, presumably due to the low uptake of tamoxifen. To obviate the need for small molecule induction, bypass difficulties with cellular uptake, and increase recombination efficiency, we sought to photoregulate Cre by installing a caging group directly on the enzyme. Previous studies have shown that wild type Cre is membrane-permeable and readily translocates to the nucleus.

Cre utilizes a nucleophilic tyrosine at position 324 to catalyze sequential strand exchange amongst its cognate loxP sites. Initially, a covalent DNA-protein intermediate is formed that is essential to recombination-site base pairing and strand exchange (Figure 7.27). The tyrosine hydroxyl attacks a specific phosphodiester bond within the loxP site, cleaving the first strand; ligation of the remaining DNA strand and resolution of the nucleoprotein intermediate complete the recombination event. We reasoned that the presence
of a caging group on the nucleophilic hydroxyl group of Tyr324 would render Cre inactive, and thus completely inhibit recombination. Light irradiation would then remove the caging group and restore Cre activity.

![Figure 7.27](image)

**Figure 7.27.** Cre recombinase cleaves DNA strands by the nucleophilic attack of Tyr324 onto the DNA phosphodiester backbone

### 7.3.3. Synthesis of Caged Tyrosine

In order to prepare caged tyrosine on a large scale several synthetic routes were explored; however, it was ultimately determined that the most efficient synthetic route involved the formation of the dityrosine copper complex, followed by reaction with 2-nitrobenzyl bromide and copper deprotection (Scheme 7.7). This afforded gram quantities of caged tyrosine 334 in 54-72% yield.
Using the Schultz technology, Wesleigh Edwards expressed and purified Cre recombinase containing the caged tyrosine 317 in position Tyr324. Moreover, Wesleigh confirmed in an *in vitro* assay that photocaged Cre is completely inactive prior to removal of the caging group through light irradiation (Figure 7.28).

**Figure 7.28.** Expression of photocaged Cre protein.
7.3.4. Application of the Expressed Caged Cre Recombinase to in vivo Systems

To assess the function of this protein in mammalian cell culture, we obtained a previously reported Cre Stoplight plasmid from the Hughes lab (Figure 7.29).515

![Figure 7.29](image)

**Figure 7.29.** Cre stoplight vector. Before recombination, the translational stop prevents the expression of GFP; however upon recombination by Cre, the loxed DsRed gene with the corresponding stop codon is removed, facilitating GFP expression.

HEK-293T cells were transfected with the Cre Stoplight plasmid,515 which encodes DsRed and a transcription termination region, both flanked by *loxp* sites and located upstream of the eGFP gene. Prior to Cre-mediated recombination, cells should exclusively express DsRed under control of the CMV promoter; after recombination, which results in the excision of DsRed and its terminator, cells should express GFP. To demonstrate the efficacy of this system, cells were first transfected with the Cre Stoplight; leading to the cells producing only the DsRed reporter protein, as expected (Figure 7.30A). To eliminate *in vitro* recombination, cells were then sequentially transfected with the Cre Stoplight, followed six
hours later by wild type Cre to ensure that the observed recombination occurred *in vivo*. Gratifyingly, in this case some GFP expression was observed, demonstrating that the wild type Cre protein was active in an *in vivo* setting (Figure 7.30B). Having previously demonstrated that the caged Cre is completely inactive, sequential transfections of the caged analog were unnecessary, and thus cells were therefore co-transfected with both Cre-ONBY and the Cre Stoplight using a DOTAP transfection reagent known to tranfect both proteins and DNA. Non-irradiated cells resulted in only DsRed expression after 48 hours (Figure 7.30C); however, a brief irradiation at 365nm for 5 minutes yielded some GFP expression after 48 hours (Figure 7.30D). This confirmed that DNA recombination in HEK293T cells can be effectively triggered with light using photocaged Cre recombinase.

**Figure 7.30.** Photochemical control of Cre-catalyzed DNA recombination in 293T cells transfected with the Cre-Stoplight plasmid.

To further confirm the observed recombination activity of Cre-ONBY, the transfections were repeated and incubated for 48 hours to afford adequate gene expression. After a brief visual inspection, the cells were trypsinized to remove them from the surface of the plate and transferred into eppendorf tubes to be FACS counted. Each reaction condition was individually counted on a Becton-Dickinson FACSCalibur instrument for the number of cells expressing GFP and DsRed. Non-transfected 293T cells did not display any fluorescence as no GFP or DsRed was expressed, and thus all cells appear in the lower left
quadrant of Figure 7.31A. Cells transfected with the Stoplight plasmid and wild type Cre protein contained highly fluorescent cells with a number of them possessing both GFP and DsRed, as a result of the reversibility of the recombination reaction (Figure 7.31B). The 293T cells transfected with only the Cre stoplight resulted in cells predominantly expressing the DsRed protein (Figure 7.31C; upper left quadrant). A similar result was observed in cells co-transfected with the Stoplight plasmid and caged Cre that were not irradiated (Figure 7.31D); however, in the irradiated cells, a statistical increase in GFP was observed, indicating activation of the Cre protein and subsequent recombination (Figure 7.31E; left-hand quadrants).

**Figure 7.31.** Results of FACS counting of transfected cells. The number of cells expressing DsRed and GFP were counted and a significant amount of recombination was observed in the irradiated cells containing caged Cre.
7.3.5. Spatial Control of the Cre Recombinase Protein

Having effectively demonstrated the photoregulation of the caged Cre protein in mammalian tissue culture, we were next interested in investigating the feasibility of obtaining spatial control over Cre activation. In order to achieve this goal, HEK-293T cells were co-transfected with the Cre Stoplight plasmid and the caged Cre protein and incubated for 24 hours. The transfection media was then removed and a small area of cells was irradiated for 20 seconds using a Jenco Epi-fluorescence inverted microscope equipped with a 100W mercury lamp and a DANSA filter cube (330-400 nm excitation) for 30 seconds, followed by replacement of media with standard growth media and incubation for an additional 48 hours. The cells were then imaged on a Leica DM5000B microscope to determine if GFP expression could be observed in the irradiated area. Gratifyingly, spatial control was achieved, as the majority of the cultured cells only expressed DsRed; however, only in the irradiated cells was the GFP protein expressed (Figure 7.34).
In summary, we have accomplished the photochemical activation of DNA recombination in a spatial and temporal fashion through the incorporation of a photocaged tyrosine into Cre recombinase at an essential site. Ultimately, we aim to utilize this technology in multi-cellular organisms, specifically zebrafish and *C. elegans*; however, we must first optimize conditions for the introduction of the caged protein into the organism, and establish a *loxP* system for a reporter gene of interest, which could optimally be stably incorporated into the genome.
7.4. Microwave Assisted Synthesis of Unnatural Amino Acids

7.4.1. Introduction

The synthesis of unnatural amino acids is interesting due to advancing technologies for their incorporation into proteins both in vivo and in vitro. Since unnatural amino acids can have a repertoire of functional groups that vastly extends beyond the common set of endogenous amino acids, they can be used as probes to obtain a better understanding of biological processes or in an applied fashion to generate proteins with novel functions. While several routes to amino acid synthesis currently exist, microwave irradiation has rarely been employed. To the best of our knowledge, only one synthetic route to unnatural amino acids using microwave irradiation has been attempted via the Michael addition of methyl N-(diphenylmethylene)-2,3-didehydroalaninate and nitroalkanes. Thus we set forth to explore a more general microwave-assisted alkylation approach to afford a wide variety of unnatural amino acid derivatives in a facile fashion.

7.4.2. Microwave-Assisted Alkylation Optimization

Initial investigations commenced with the optimization of the alkylation of diethyl acetamidomalonate (346) with benzyl bromide (347a) in the presence of base to yield the diester 348a (Table 7.1). Several bases were examined including NaH, NaOEt, K₂CO₃ and Cs₂CO₃. Ultimately Cs₂CO₃ was found to be optimal as it afforded high yields with a minimum number of side products compared to the other bases. We next investigated the role of the solvent and the microwave power on the alkylation reaction (Table 7.1). Based on our previous experience in microwave-mediated reactions, toluene was employed as a microwave transparent solvent, thus enabling a high level of microwave power input to the substrates. However, even at 300W of microwave irradiation overall conversion was low,
presumably due to the poor solubility of Cs$_2$CO$_3$. When the alkylation was conducted in DMF, a polar, strongly microwave absorbing solvent, a temperature of 170 °C was reached very quickly; however, the yield of 348a was still relatively low at 45%. Conducting reactions in microwave absorbing solvents limits the ability to use high microwave power inputs due to the rapid heating of the solvents beyond their boiling point, leading to a substantial pressure formation in the sealed reaction vessel. Based on these observations, we next performed the reaction in microwave transparent THF, resulting in decreased temperatures but also significant amounts of the starting material 346. However, these conditions afforded slightly higher yields of 348a at high microwave powers: 47% yield at 200W and a 59% yield at 300W. We then conducted the reaction in acetonitrile, which is a common solvent employed in similar alkylations. Acetonitrile was found to be the optimal solvent under standard microwave irradiation at ~130 °C for 10 minutes affording 348a in 82% yield. To investigate the rate enhancement effects of the microwave, similar conditions were attempted thermally for the same reaction time and temperature, yielding only 43% of 348a with significant amounts of 346 remaining.
Table 7.1. Optimization of the microwave-assisted alkylation reaction.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>MW Power (W)/ Temperature (°C)</th>
<th>Yield 3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PhMe, 10 min</td>
<td>300 / 120°F</td>
<td>31%</td>
</tr>
<tr>
<td>2</td>
<td>DMF, 10 min</td>
<td>0-200 / 170°F</td>
<td>45%</td>
</tr>
<tr>
<td>3</td>
<td>THF, 10 min</td>
<td>300 / 85°F</td>
<td>59%</td>
</tr>
<tr>
<td>4</td>
<td>THF, 10 min</td>
<td>200 / 80°F</td>
<td>47%</td>
</tr>
<tr>
<td>5</td>
<td>ACN, 10 min</td>
<td>0-300 / 130°F</td>
<td>82%</td>
</tr>
<tr>
<td>6</td>
<td>ACN, 10 min</td>
<td>0 / 130°F</td>
<td>43%</td>
</tr>
</tbody>
</table>

*Fixed microwave power input and variable temperature.
*Fixed temperature and variable microwave power input.

7.4.3. Generation of a Library of Nonnatural Amino Acid Precursors

With optimized conditions in hand (Cs$_2$CO$_3$, CH$_3$CN, 130 °C, microwave irradiation), the reaction scope was investigated with several bromides (347a-347g) to yield the alkylated products 347a-347g (Table 7.2). Overall the amino acid precursors 347a-347g could typically be prepared in moderate to high yields within a short 10 minute reaction time. The methodology tolerates a wide range of functionalities including alkyl (347g), aryl (347a, 347d, and 347e-347f), alkene (347b and 347d), alkyne (347c), fluorine (347e), and ketone (347f) functional groups. However, aliphatic chains (347g) lead to reduced yields despite further optimization attempts, presumably due to their intrinsic lower reactivity and their propensity to undergo eliminations. In order to increase the yield in case of certain bromides (347b, 347e, and 347g), sodium iodide (20 mol%) was added to the reaction mixture to facilitate substitution via an *in situ* leaving group exchange.
Table 7.2. Preparation of unnatural amino acid precursors 348.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R Group</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Image" /></td>
<td>348a</td>
<td>82%</td>
</tr>
<tr>
<td>2</td>
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<td>348b</td>
<td>78%a</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Image" /></td>
<td>348c</td>
<td>68%</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Image" /></td>
<td>348d</td>
<td>76%</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Image" /></td>
<td>348e</td>
<td>73%a</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Image" /></td>
<td>348f</td>
<td>92%</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Image" /></td>
<td>348g</td>
<td>33%a</td>
</tr>
</tbody>
</table>

*a Sodium iodide (20 mol%) was added.

7.4.4. Microwave-Assisted Hydrolysis of Amino Acid Precursors

After generating a small array of the amino acid precursors 348a-348g, a global deprotection of the carboxy and the amino groups followed by an instant decarboxylation was performed to yield the amino acids 349a-349g in a single step. Typically, this reaction is conducted in refluxing aqueous 6N HCl for approximately 12 hours. However, we discovered that this transformation also benefits from microwave irradiation. After just 10 min of microwave irradiation of 348a-348g in 6N HCl, the amino acids 349a-349g were obtained in excellent yields of 85-99% and purities of >95%, as determined by 1H NMR.
(Table 7.3). The analytical data of 349a-349g was in agreement with literature reports.\textsuperscript{526-531} Thermal reactions mimicking the microwave reaction conditions afforded minimal product conversion <20\%, and significant amounts of the starting materials 349a-349g were retained. Overall, the preparation of unnatural amino acids was achieved in a combined 20 minutes of reaction time with a single chromatography purification between reactions.

**Table 7.3. Conversion of precursor 348 to amino acid 349.**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Diester</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>349a</td>
<td>98%</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>6</td>
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<td>349f</td>
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</tr>
<tr>
<td>7</td>
<td>348g</td>
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<td>92%</td>
</tr>
</tbody>
</table>

In conclusion, a new microwave-assisted two-step methodology to rapidly prepare unnatural amino acids in high yield and high purity was developed. Due to the wide range of functional groups tolerated via this approach, several important unnatural amino acids could be attained. Upon incorporation into proteins and peptides these amino acids can potentially be employed in bioconjugation reactions (349c and 349f),\textsuperscript{456, 491, 532-535} as NMR labels (349e),\textsuperscript{536} and in photochemical transformations (349b).\textsuperscript{537, 538} Due to the high convergence of this approach and the wide range of inexpensive commercially available bromides, the methodology will find widespread application in the rapid assembly of unnatural amino acids
7.5. Application of Caging Technologies for Introduction of Near-Natural Amino Acids

7.5.1. Introduction

Elucidating the molecular details of enzymatic activity is a pertinent and constantly changing field in biochemistry. Historically, enzyme active sites are first identified and studied using site-directed mutagenesis or chemical modification to predict residues (and the corresponding functional groups) that are directly involved in catalysis.\textsuperscript{539-541} While such approaches can implicate an amino acid, they cannot definitively support a proposed chemical mechanism. A mutation that results in total or even partial loss of activity only implicates the residue as being important for catalysis, and provides very little detailed molecular or mechanistic information. Other traditional experiments that address this information, such as pH rate profiles, inhibitor studies, and substrate analogues, are classic tools but far from perfect when dissecting the complex and intricate details of protein function. One of the more recent approaches protein scientists have turned to in order to more effectively investigate enzymatic mechanisms is the use of unnatural amino acids that display slightly modified functional groups. Amino acids that contain these subtle perturbations are often termed near-natural amino acids, and can alter functional group placement or electronic properties to reveal a precise structure-function relationship for protein active sites and provide valuable mechanistic detail that is not attainable through any other method.

Currently no generally applicable method for the site-specific incorporation of near-natural amino acids into proteins exists. Often, the near-natural amino acid analogues are
incorporated into synthetic peptides created by solid-phase peptide synthesis.\textsuperscript{542} This approach is necessary because researchers are typically interested in mutating a single site within the protein followed by studying the effects of this single mutation on protein catalysis. While this approach offers a high level of flexibility when working on smaller peptide substrates (<50 residues), it is not ideal in the context of large proteins due to problems with synthesis efficiency and post-synthesis purification. With larger proteins, site-specific incorporation of a near-natural amino acids requires the peptide synthesis to be coupled with expressed protein ligation.\textsuperscript{543} Therefore this method is only compatible if the position of the mutation is within 50 residues of the N- or C-termini of the protein. In addition the peptide ligation step is normally conducted under denaturing conditions followed by refolding and purification of the final product. Thus, depending on the stability of the target protein, the position and sequence context of the desired mutation, a synthesis-peptide ligation approach may or may not be practical.

Ideally, one would want to be able to perform site-directed, near-natural mutagenesis with the ease of the previously described Schultz mutagenesis experiment in the lab; produce large amounts of protein in \textit{E. coli} or yeast, purify, and perform the experiment of interest. Unfortunately many of the near-natural amino acids that could be useful tools to enzymologists infiltrate normal protein biosynthesis.\textsuperscript{490} Because they are similar in structure, they serve as substrates for many of the endogenous aminoacyl-tRNA synthetases and end up globally incorporated throughout the proteome. In many cases,\textsuperscript{490} this results in severe toxicity. For instance the antibiotic canavanine is a close analogue of arginine that accumulates in many seeds to serve as a defense mechanism towards herbivores.\textsuperscript{544} This issue of toxicity can sometimes be overcome through the use of auxotrophic strains of \textit{E. coli}
or by coordinating protein expression with amino acid addition. Essentially, the auxotrophic cells are grown to full density in rich media to achieve a large biomass that is used as a “production factory”. Then the cells are washed, purged of the natural amino acid (for example histidine, tyrosine, or cysteine) and then protein expression is induced in new media containing the near-natural amino acid (such as fluorohistidine, fluorotyrosine, or selenocysteine). However, an additional severe problem of such a general in vivo incorporation is the complete lack of specificity. The fact that these amino acids are recognized by endogenous aminoacyl-tRNA synthetases limits their use as site-specific probes because they are globally incorporated. Also, even with the use of auxotrophic strains, it is impossible to avoid some natural amino acid incorporation. In these cases, cells do not grow in the presence of these amino acids, they only produce protein with established machinery. Based on these toxicity issues and selectivity limitations, it is currently impossible to use natural genetic methods to site-specifically incorporate near-natural amino acids in vivo with high efficiency.

We hypothesize that we can employ the previously described non-natural amino acid technology combined with caged near-natural amino acids to effectively solve the various issues associated with the use of near-natural amino acids. Due to the ability of the Schultz tRNA/tRNA synthetase technology the site-specific incorporation of a variety of non-natural amino acids is possible; however, as previously noted, near-natural amino acids are not structurally different enough to not be recognized by endogenous synthetases. In order to solve this selectivity issues we suspect that it is feasible to synthesize caged analogs of the near-natural amino acids, which due to their similarities to the evolved non-natural synthetases, can be incorporated utilizing this system. This approach should protect near-
natural amino acids from leaking into the natural protein biosynthesis and thus causing toxic effects or leading to non-specific incorporation (Figure 7.35). The caging group dramatically changes the structure of the unnatural amino acid, in essence “hiding” it from endogenous protein biosynthesis. After biosynthetic incorporation and purification of the protein, the photocaging group can be removed by light, generating the desired mutation, affording the means to study the effects of this perturbation on enzymatic activity. This methodology will represent a general approach for introducing near-natural amino acids into site-specific positions in proteins, in vivo. Ideally, this strategy will bring the powerful tool of near-natural amino acid mutagenesis to any lab capable of normal protein expression and purification.

Figure 7.35. Near-natural amino acids are often incorporated into all proteins at all sites of the corresponding natural amino acid whereas the caged equivalents are not.

7.5.2. Synthesis and Incorporation of Caged Selenocysteine

An important analog of the canonical 20 amino acids is in fact Nature’s own 21st amino acid, selenocysteine (Sec, U). While structurally analogous to cysteine, the selenol side chain has a dramatically reduced pKₐ of 5.3. This makes the side chain of selenocysteine anionic at physiological pH and thus much more nucleophilic than that of cysteine (which with a pKₐ of 8.3 is rarely ionized). The ability to create proteins with site-specific selenocysteine mutations would allow enzymologists to determine the implications of this residue in selenoprotein catalysis. In general, proteins with active sites containing
selenocysteines are much more efficient than the same enzyme containing cysteine. As a result, it is possible to use the properties of selenocysteine towards the evolution of new enzyme function via directed evolution strategies. Furthermore, the high nucleophilicity of selenocysteine could undoubtedly be adapted for the site-selective bioconjugation with highly selective electrophiles.

Unfortunately, mutagenesis employing selenocysteine is technically too challenging to find widespread application. Selenocysteine is unique in that it is encoded in few select examples in nature by a UGA codon, normally a universal stop codon. In eubacteria this in-frame UGA codon is always followed by a segment of structured mRNA called a Selenocysteine Insertion Sequence (SECIS), which recruits the specialized tRNA and other elements involved in Sec decoding. This sequence must be present or the codon will be read as a normal stop codon, resulting in premature termination of protein translation. Based on this limitation, selenocysteine mutagenesis is not general because any mutation must be accompanied by the SECIS that by default will also be translated. Thus, in order to perform a Sec mutation, other amino acids must also be inserted.

In an alternative approach, Hilvert and co-workers were the first to create a synthetic selenoprotein using chemical conversion. The active site serine was first activated by phenylmethanesulfonyl fluoride (PMSF) and then converted to selenocysteine by reaction with a large excess of sodium hydrogenselenide. The resulting protein had a dramatically altered function, since it was converted from a protease to an acyl-transferase, potentially capable of joining proteins. The enzyme also displayed secondary glutathione peroxidase activity (which is itself a natural selenoprotein). Unfortunately, because the serine must be activated by PMSF, this semi-synthetic approach requires an extremely nucleophilic serine.
residue like the one found in subtilisin, which is activated by a catalytic triad. Furthermore, the yield of the artificial selenoprotein was only ~40% and had to be purified from the precursor protein by thiol affinity chromatography. Based on these requirements, this approach of synthetic modification could most likely not be used to produce other selenoproteins.

Other methods to introduce selenocysteine into proteins include peptide synthesis. The limitations of this approach are the laborious assembly of large peptides, the associated cost, and the size limitations. The latter has previously been overcome through clever native chemical ligation approaches by Kent, Muir, and Dawson, but is still very technically challenging. As a result, this near-natural amino acid is an ideal candidate for the proposed methodology.

Ultimately, due to the presence of a previously evolved caged cysteine tRNA/tRNA synthetase system, we hypothesized that the selenocysteine near-natural amino acid was an ideal starting point for the investigation into the feasibility of this approach. In order to be a viable approach we need to demonstrate the facile synthesis of a caged selenocysteine, its efficient incorporation into a protein, and its effective decaging leading to generation of the near-natural residue in the protein of interest. Gratifyingly, a high yielding synthesis was designed to yield the caged selenocysteine 353 from the commercially available seleno-L-cysteine 350. The first step of the synthetic route is a Boc-protection of the amino groups to furnish the compound 351 in 92% yield. The caging group was then installed through a reduction of the diselenide bond, followed by reaction with the 2-nitrobenzyl bromide 345 (R = H) under basic conditions delivering 352 in 86% yield. The synthesis of 353 was
completed through deprotection of the amino group in a quantitative fashion using 3% TFA in DCM (Scheme 7.8).

**Scheme 7.8. Synthesis of caged seleocysteine (353)**

Unfortunately, initial decaging experiments with 353 using a hand-held UV lamp (23W, 365nm, 1-10min) failed to deliver decaged selenocysteine. Based on LC/MS analysis the caged derivative decreased in concentration; however, a mass corresponding to the near-natural derivative was not detected. It is suspected that the lack of this mass is due to the high reactivity of the selenide, and thus decagings were attempted at different pH, as well as in the presence of selenide trapping or reducing agents (3 eq. MeI, 5 eq. semicarbazide, or 2 eq. ascorbic acid). Ultimately, a substantial amount of the diselenide was detected by LC/MS at low or neutral pH in the absence of trapping reagents, confirming the decaging was in fact occurring (365 nm, 5 min, transilluminator, 25W, 1mM or 0.1mM).

Based on these preliminary results the *in vivo* incorporation of this near-natural amino acid was attempted by our collaborator Dr. Ashton Cropp. It was found that the caged selenocysteine is somewhat toxic to cells, and that care must be taken in the final deprotection steps, as the amino acid decomposes over time when subjected to acidic
conditions. An aminoacyl-tRNA synthetase/tRNACUA pair specific for caged cysteine has already been developed. As an initial test of the flexibility of this synthetase, the Cropp laboratory transformed this plasmid into a strain of yeast which expresses a Trp33TAG human superoxide dismutase (hSOD)\textsuperscript{47}. As can be seen in Figure 7.36, this strain of yeast only produces full-length SOD in the presence of caged selenocysteine\textsuperscript{335}. This data supports our hypothesis that this amino acid is of approximate size and shape as the caged cysteine.

![Image](image_url)

**Figure 7.36.** Incorporation of caged selenocysteine into the hSOD protein.

Further application of this amino acid has proven limited due to decaging issues. The protein does not appear to be functional, even after light irradiation. This may be a result of either inefficient decaging, or decaging followed by increased reactivity/nucleophilicity of the selenocysteine functionality leading to covalent bond formation with neighboring electrophilic on lysine residues abrogating catalytic function. Further investigations must be conducted to identify which mechanism is at work. Initially, the caged selenocysteine protein can be irradiated, followed by MS analysis, which should confirm the presence or absence of the caging group based on the mass. Future research should be driven based on the results of these experiments. If the decaging is inefficient, than selection of an alternate caging group in conjunction with tRNA/tRNA synthetase selection can be performed. If heightened reactivity
is the issue, alternative proteins can be employed to probe the function of the near-natural amino acid.

### 7.5.3. Synthesis and Introduction of Caged Fluorotyrosine

One notable utilization of near-natural amino acids is the application of fluorinated amino acids. These analogues have seen extensive use as chemical probes in enzymology with mono-fluorinated residues being near perfect isosteric replacements for natural amino acids.\(^{554}\) Substitution of hydrogen with fluorine represents a fairly small change in structural size (~0.15 Å van der Waals radius difference) and therefore minimizes steric effects. Once introduced into proteins, however, these analogues can be used as probes of protein folding and dynamics using \(^{19}\text{F}\) NMR.\(^{555-559}\) Fluorinated amino acids also have altered electronic properties due to the high electronegativity of fluorine, resulting in lower pK\(_a\)s of heteroatoms located in the adjacent side chain and higher oxidation potentials.\(^{560-562}\)

As an example, the natural amino acid tyrosine has a phenolic proton with a pK\(_a\) of ~10 whereas the tetrafluoro derivative has a dramatically lowered pK\(_a\) of 5.2 (Figure 7.38), approaching that of a carboxylic acid!\(^{563,564}\) This difference in side chain acidity has been used to investigate the protonation state of tyrosines that are implicated in protein function. Recently, Cole and co-workers have employed fluorinated tyrosines to probe the mechanism of protein tyrosine kinase domains.\(^{565}\) In these experiments, these near-natural amino acids were incorporated by chemical peptide synthesis and the peptides were then used as a surrogate for the natural kinase substrate. By controlling the tyrosine pK\(_a\) (through the use of mutant tyrosines) and buffer pH they were able to ascertain that the natural substrate of the insulin receptor kinase is indeed a neutral tyrosine, and that the de-protonated phenoxide anion is actually a
competitive inhibitor of the enzyme. This unequivocal evidence would not be obtainable by just traditional pH rate profiles or mutagenesis to one of the 19 other natural amino acids.

![Figure 7.38](image)

**Figure 7.38.** Structures of fluorinated tyrosine analogues 354-359 showing the effects of substitution on side chain acidity. Coupled with buffer pH, the protonation state in enzyme active sites can be precisely controlled.

Using a combination of synthetic peptide synthesis and expressed protein ligation, Stubbe and co-workers have recently expanded the use of fluorotyrosine analogues to the study of electron transfer processes within ribonucleotide reductase.\(^{564, 566, 567}\) While tryptophan and cysteine have oxidation potentials similar to tyrosine, mutations to those amino acids are much more likely to disrupt the protein structure, thus complicating the interpretation of results. However, by maintaining the size and shape of tyrosine and only altering its redox properties they were able to achieve very accurate comparisons to the wild-type protein. Specifically, the fluorinated tyrosines provided a spectrum of oxidation potentials that were used to clearly implicate an active site tyrosyl radical in the reaction mechanism of ribonucleotide reductase.

The synthesis of caged fluorinated tyrosine amino acids commences with the enzymatic production of the non-caged amino acids 354-359 in one step from the corresponding fluoro-phenols, catalyzed by tyrosine phenol lyase (TPL). An over-expression plasmid for this enzyme was provided by Prof. Robert Phillips of the University of Georgia, and the synthesis of fluorinated tyrosine derivatives has been optimized in the Cropp laboratory.\(^{564, 568}\) These reactions are compatible for multi-gram scale synthesis of all
fluorinated tyrosine analogues 354-359 using a 1 L reaction. Installation of the caging group on 354-359 can be conducted in one step via the previously described formation of the corresponding copper complex and a subsequent reaction with the benzyl bromide 32 (Scheme 7.9).

![Scheme 7.9. Synthesis of caged fluoro-tyrosines.]

With the caged 2-fluorotyrosine 360a and caged 3-fluorotyrosine 360b prepared, we next investigated the ability to incorporate them into a protein using the pSup-ONBY expression system previously employed in the caging of Cre recombinase. In this case we selected the lacZ protein, due to the integral tyrosine residue implicated in its hydrolysis of glycosidic bonds. Via the incorporation of the fluorinated residues into this position, we cannot only demonstrate the applicability of this methodology, but also probe the catalytic importance of this tyrosine residue by altering the acidity of the phenol.

7.5.4. Assay of Caged β-Galactosidase

In order to identify the activity of analogs of the lacZ protein we conducted standard Miller assays to quantitate their catalytic potential. Having performed an expression with the non-fluorinated caged tyrosine 334, we first wanted to establish a benchmark for wild-type lacZ activity, and optimal decaging conditions. As a result, we initially performed the Miller assay using the cellular lysate rather than the purified protein. Both the lacZ proteins
containing 334 and 360b were irradiated for different times and with different irradiation sources. Ultimately, it was determined that the maximum enzymatic activity was restored with a 20-minute irradiation at 365 nm using a transilluminator (25W). Interestingly, the non-fluorinated protein demonstrated a significantly higher activity upon photoactivation than the 3-fluoro analog. This suggest that either the expression of the caged fluorinated analog was unsuccessful, or that the incorporation of a fluorinated tyrosine dramatically effects the catalytic activity (Figure 7.39).

![Bar chart showing Miller Assay results](image)

**Figure 7.39.** Miller Assay to determine optimal decaging conditions. A substantial photoactivation occurs with the ONBY expression lysate; however, little to no photoactivation appears to occur with the 3F-ONBY lysate.

The masking of near-natural amino acids with photolabile protecting groups for site specific incorporation into proteins remains elusive, and requires further optimization to be generally applicable. Significant work must still be accomplished to fully exploit this novel means of installing near-natural amino acid residues. Specifically, the expression of lacZ
proteins with the various fluorinated tyrosine analogs must confirmed and optimized, and the purified proteins must be assayed at various pH to elucidate the effect of the near natural amino acid on catalysis. This should remedy abnormalities observed when using the cellular lysate and provide kinetic parameters for enzymatic catalysis. Additionally, the incorporation of near naturals must be expanded to more biologically relevant systems that require the specific substitution of a near natural amino acid as a means of understanding enzymatic catalysis.
7.6 Experimental

Synthesis of Caged IPTG (337). Isopropyl-β-D-thio-galactoside (50 mg, 0.18 mmol) was dissolved in DMSO (0.5 mL) at 0 °C. 6-Nitropiperonal (103 mg, 0.53 mmol) and concentrated H₂SO₄ (0.2 mL) was added, and the reaction was allowed to warm to room temperature. After 24 hours the reaction was quenched with water (0.5 mL), the layers were separated, and the organic layer was extracted with ethyl acetate (3 × 2 mL). The combined organic layers were dried, concentrated, and the residue was subjected to column chromatography on SiO₂ (5:1 EtOAc/hexanes) to yield a yellow solid (56 mg, 0.13 mmol, 78%). ¹H NMR (300 MHz; CDCl₃) δ 7.40 (s, 1H), 7.33 (s, 1H), 6.16 (s, 1H), 6.10 (s, 2H), 4.40 (d, ³J(H,H)=9.3 Hz, 1H), 4.29 (m, 2H), 4.08 (m, 2H), 3.70 (m, 2H), 3.50 (m, 1H), 3.25 (sep, ³J(H,H)=6.9, 1H), 1.38 ppm (6H, ³J(H,H)=6.9); ¹³C NMR (75 MHz; CDCl₃) δ 151.79, 148.30, 128.94, 107.77, 105.43, 103.32, 96.89, 85.81, 76.25, 74.04, 70.39, 70.20, 69.91, 35.64, 24.53, 24.34; HRMS (FAB): m/z calculated for C₁₇H₂₁NO₉S: 416.1015, found: 415.0984.
Irradiation Analysis of 337. A solution of 337 (0.2 mM in CH₂Cl₂) was irradiated for 5 minutes at 365 nm with a handheld UV lamp (25 W). After irradiation the product was concentrated and analyzed by COSY NMR. Based on the COSY assignment of 337, the methylene protons on carbon 6 were integrated to elucidate the regioisomeric ratio. The ratio of 338a/338b was determined as 1:1. An irradiation timecourse study was performed to analyze the rates of the formation of the ester 338. Irradiation reactions were performed in water (1% DMSO), at concentrations of 0.1, 0.5, and 1.0 mM with 5 minutes of irradiation at 365 nm (Hand-held UV lamp, 23 W). Aliquots were analyzed by LC/MS to determine the concentration of 338. Half-lives of 337 after irradiation are calculated via fitting of an
exponential decay curve (Microcal Origin 5.0™), and are as follows: 11 sec (0.1 mM), 5.1 min (0.5 mM), and 11.8 min (1.0 mM).

**Cellular Uptake and Toxicity Analysis of 337.** *E. coli* BL21 cells harboring the pUC19 plasmid were grown for 12 h to reach log phase and were used to inoculate cultures under various conditions. Each culture consisted of 2 mL LB medium, 2 µL ampicillin (50 µg/mL), and either 0.5 mM IPTG, 337, 338, or no small molecule addition (control). Cultures were grown at 37 °C and monitored every 60 min by UV-VIS to determine OD<sub>600</sub> values. No difference in growth was observed for any of the conditions, demonstrating no toxicity of the compounds 337 and 338. After 8 hours, cells were pelleted (6000 rpm; 10 min), lysed with 0.1% SDS/lysozyme, and intracellular small molecule concentrations were quantified by LC/MS. The caged IPTG 337 was detected at an intracellular concentration of 0.5 mM.

**Miller Assay with Caged IPTG (337).** Five liquid cultures (2 mL) of BL21(DE) cells possessing the pUC19 plasmid were grown in LB media with Ampicillin (50 µg/mL). At an OD<sub>600</sub> of 0.6, either 0.5 mM (final concentration) IPTG (2 samples), caged IPTG (2 samples), or no compound (1 sample) was added to the samples, and one of the samples was irradiated for 15 minutes (365 nm, hand-held lamp, 23W). Cells were then incubated for 6 hours and the OD<sub>600</sub> was recorded. Then 100 µL of each sample was added to 900 µL Z-Buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 50 mM β-mercaptoethanol, pH 7.0) and 25 µL 0.1% SDS and vortexed for 2 minutes to promote cellular lysis. The ONPG substrate (200 µL, 4 µg/µL) was added and the samples were heated to 30 °C for 10 minutes (or until a distinct yellow color appeared). The reaction was then quenched with 500 µL of 1
M Na₂CO₃ and the absorbance at 550 nm and 420 nm were recorded. Miller units were calculated from the following formula:

\[
\text{Miller Units} = 1000 \times \left( \frac{\text{OD}_{420} - 1.75 \times \text{OD}_{550}}{\text{T} \times \text{V} \times \text{OD}_{600}} \right)
\]

\[T = \text{reaction time (typically 10 min)}\]

\[\text{V} = \text{Final volume}\]

**Intracellular Hydrolysis of 338.** LB media containing compound 338 (0.5 mM) was inoculated with BL21(DE) cells to an OD₆₀₀ of 0.4. The culture was grown at 37 °C, and 1mL aliquots were removed at 1, 2, 3, 4, 5, 6, 8, 10, and 12 hours, and the cells were pelleted. The cells were lysed with 0.1% SDS/lysozyme for 1h at room temperature, and the lysates were analyzed by LC/MS to determine the concentration of 338 (corrected for the number of cells). The half-life of the hydrolysis was calculated to \(t_{1/2} = 63\) min ± 2 min via fitting of an exponential decay curve (Microcal Origin 5.0™).

**Spatial Control of Protein Expression.** Ampicillin resistant constructs pUC19 or pGFPuv with the lacZ or the gfp gene, respectively, under the control of the lac repressor in BL21(DE) cells were grown to log phase for 12 hours at 37 °C in LB broth containing ampicillin (20 µg/mL). Bacteria (100 µL, OD₆₀₀ = 1.1) were then spread as lawns on LB/Amp agar plates (Ø 10 cm) which were pre-treated with caged IPTG (10 mM, 40 µL) and X-gal (2% in DMF, 40 µL) in the case of lacZ expression. One half of the plate was covered with a mask, and the plate was irradiated for 30 seconds at 365 nm. Following the brief irradiation, the bacteria were grown for 12 hours at 37 °C, and the plates were imaged.
**Rapamycin Assay.** The assay protocol was adapted from that provided in the K-LISA mTOR Activity Kit (Calbiochem). The glutathione-coated strips were first treated with a 1:400 dilution of the provided mTOR substrate in TBS (100 µL/well) for 1 hour at room temperature. During this incubation a separate pre-incubation of the provided mTOR standard (50 µL) with FKBP (1.8 µg, 1 µL; R&D Systems), and either DMSO (1 µL), rapamycin (1.8 µg, 1 µL), or modified rapamycin (1.8 µg, 1 µL) for 20 minutes at 4 °C. In the case of irradiated samples, a 5-minute irradiation at 365 nm (23 W) was performed prior to the incubation on ice. The modified glutathione wells were then washed with TBS (4 x 200 µL) and incubated with the mTOR/FKBP/rapamycin incubations (50 µL) followed by 2X Kinase Assay Buffer (50 µL) for 30 minutes at 30 °C. The phosphorylation reaction was quenched with 1X Kinase Stop Solution (10 µL) and washed with 1X Plate Wash (3 x 200 µL). The assay was then incubated with a 1:1000 dilution of the Anti-p70S6K-T389 primary antibody (100 µL) for 1 hour at room temperature, followed by washing with 1X Plate Wash (4 X 200 µL). Finally, the wells were incubated with a 1:400 dilution of the HRP-Antibody Conjugate (100 µL) for an additional hour at room temperature, and then washed with 1X Plate Wash (4 X 200 µL). The TMB Substrate (100 µL) was added to each well and incubated 20 minutes at room temperature followed by the addition of ELISA Stop Solution (100 µL) and the measurement of absorbance at 450 nm, 540 nm, and 595 nm on a SpectraMax 384 plus plate reader (Molecular Dynamics). The absorbance at 450 nm was subtracted from the absorbance of 595 nm, and the data was normalized to the no rapamycin control.
Synthesis of Caged Tyrosine 334. Tyrosine (0.84 g, 4.63 mmol) was dissolved in 2 N NaOH (5.5 mL) and a solution of CuSO₄ (0.58 g, 2.32 mmol) in H₂O (2 mL) was added and heated to 60 °C for 10 minutes. The reaction was then cooled to room temperature and neutralized with acetic acid to pH 7.0, and gravity filtered (washed with water 3 x 10 mL). The product is transferred to a new flask and resuspended in 3:1 DMF/H₂O (10 mL), and 2-nitrobenzyl bromide (1.0 g, 4.63 mmol, 1 eq.) is then added and the reaction is stirred in the dark at room temperature for 48 hours. The mixture is then filtered and washed with 3:1 DMF/H₂O (3 x 10 mL), H₂O (3 x 10 mL), and cold acetone (3 x 5 mL). The solid is then transferred to a new flask and stirred at room temperature in 1 M HCl for 2 hours, then filtered and washed with 1 M HCl (3 x 10 mL), H₂O (3 x 10 mL) and cold acetone (3 x 5 mL) to yield a slightly yellow powder (0.98 g, 3.10 mmol, 67%). All analytical data correspond to reported values.453

Photochemical Regulation in Mammalian Cell Culture Using Caged Cre Recombinase. Human Embryonic Kidney (HEK-293T) cells were passaged into two 4-well chamber slides, and grown to 40% confluence. The cells were then transfected with the Cre stoplight construct (1 µg) alone (2 wells per slide), or with either wild-type Cre recombinase (3 µg, 1 well per slide) or caged Cre recombinase (10 µg, 1 well per slide) using DOTAP transfection reagent (5:2 DOTAP/DNA; Roche Biomedicals) in OptiMEM media (Invitrogen). The transfection was incubated at 37 °C for 4 hours, followed by replacement of tranfection media in one of the non-protein treated Cre stoplight wells, followed by a subsequent transfection with wild type Cre recombinase (3 µg) using the DOTAP transfection reagent. This and the remaining wells were then incubated for 24 hours, followed by the removal of
the transfection media, irradiation of one slide (2 min, 365 nm, 23 W handheld UV lamp), and replacement of media with standard growth media (Dulbecco’s modified Eagle’s media (Hyclone) with 10% Fetal Bovine serum (Hyclone) and 10% streptomycin/ampicillin (MP Biomedicals). The cells were then incubated at 37 °C (5% CO₂) for 24 hours then imaged on a Leica DM5000B to assess GFP and DsRed expression.

**Flow Cytometry.** Human Embryonic Kidney (HEK-293T) cells were passaged into 2 6-well culture plate, and grown to 40% confluence. The cells were then transfected with a no transfection control well, a Cre stoplight plasmid control well, a wild-type Cre and Cre stoplight plasmid well, and a caged Cre and Cre stoplight well using the previously described protocol. After 24 hours the transfection media was removed and one 6-well plate was irradiated for 2 minutes with a hand-held UV lamp (365 nm, 23 W). The media was then replaced with standard growth media and the cells were incubated at 37 °C for 48 hours. The growth media was then removed, and the cells were trypsinized (500 µL, HyClone Trypsin, VWR International), and transferred to culture tubes. Cells from each well were then counted on a Becton-Dickinson FACSCalibur cell counter for 20,0000 events monitoring GFP (525 nm) and DsRed (600 nm) emission wavelengths.
Spatial Regulation of Cre Recombinase Activity. Human Embryonic Kidney (HEK-293T) cells were passaged into a 4-well chamber slide, and grown to 40% confluence. The cells were then transfected with the Cre stoplight construct (1 µg) and caged Cre recombinase (10 µg, 1 well per slide) using DOTAP transfection reagent (5:2 DOTAP/DNA; Roche Biomedical) in OptiMEM media (Invitrogen). The transfection was incubated at 37 °C, 5% CO₂ for 24 hours, followed by the removal of tranfection media and spot irradiation using a Jenco Epi-fluorescence inverted microscope equipped with a 100W mercury lamp and a DANSA filter cube (330-400 nm excitation) for 30 seconds. The media was then replaced with standard growth media, and the cells were incubated 48 hours then analyzed on a Leica DM5000B compound microscope for expression of GFP and DsRed reporter proteins.
Synthesis of Unnatural Amino Acids. Diethyl acetamidomalonate (50 mg, 0.23 mmol), Cs₂CO₃ (150 mg, 0.46 mmol, 2 eq.) and bromide 347 (0.46 mmol), and acetonitrile (3 mL) were added to a flame dried microwave vial. The vial was placed in a CEM Discover microwave reactor and irradiated in standard mode (130 °C) for 10 min. The reaction mixture was cooled to room temperature, filtered to remove Cs₂CO₃, and concentrated under reduced pressure. Purification via column chromatography (Hexane/EtOAc) yielded pure alkylation products 348a-348g. These compounds were dissolved in 6N HCl (aq.) and irradiated for 10 min in a CEM Discover microwave reactor (standard mode, 90 °C). Removal of the volatiles under reduced pressure yielded pure 349a-349g without further purification.

Synthesis of Caged Selenocysteine 353. Seleno-L-cystine (0.10 g, 0.30 mmol) was dissolved in 2:1 Dioxane/H₂O (1.2 mL), and K₂CO₃ (0.12 g, 0.9 mmol, 3 eq.) was added and the reaction was cooled to 0 °C, followed by di-tert-butyl-dicarbonate (0.20 g, 0.9 mmol, 3 eq.). The reaction was allowed to gradually warm to room temperature over 4 hours then evaporated to remove the dioxane. An additional 5 mL of H₂O was added and washed with EtOAc (3 x 10 mL). The aqueous layer was then cooled to 0 °C and neutralized with 1 N HCl, forming a white precipitate. The solution was then extracted with EtOAc (3 x 10 mL) and the organic layer was dried with MgSO₄, filtered and concentrated via rotary evaporation to yield a yellow solid, 351 (0.156 g, 0.29 mmol, 98%). All analytical data was consistent with published reports.⁵⁶⁹

The Boc-seleno-L-cysteine (341) (43 mg, 0.08 mmol) was dissolved in methanol (2 mL) and cooled to 0 °C and NaBH₄ (12 mg, 0.32 mmol, 4 eq.) was slowly added. The reaction was stirred at 0 °C for 30 minutes until the disappearance of the yellow color was
observed, and 2-nitrobenzyl bromide (86 mg, 0.40 mmol, 5 eq.) was added followed by K$_2$CO$_3$ (55 mg, 0.40 mmol, 5 eq.) and the reaction was allowed to warm to room temperature over the course of 6 hours. The reaction was quenched with H$_2$O (5 mL) and extracted with EtOAc (3 x 10 mL). The organic layer was then dried with MgSO$_4$, filtered and concentrated by rotary evaporation. The product was then purified via silica gel chromatography (1:1 Hexanes/EtOAc to 1:3 Hexanes/EtOAc) to yield the product as an off-white solid, 352 (43 mg, 0.11 mmol, 68%). ONB-Boc-seleno-L-cystine (352) (20 mg, 0.05 mmol) was resuspended in 3% TFA/DCM (2 mL) and stirred at room temperature for 2 hours. The solvent was then removed by rotary evaporation to quantitatively yield the ONB-seleno-L-cystine as an slightly tan solid, 353 (15 mg). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.89 (d, 1 H, J=8.4), 7.48 (t, 1 H, J=8.0), 7.33-7.30 (m, 2 H), 4.09 (t, 1 H, J=5.2), 4.01-3.99 (m, 2 H), 3.01-2.85 (m, 2 H). HRMS (FAB): m/z calculated for C$_{10}$H$_{14}$N$_2$O$_4$Se: 305.1812, found: 305.1824.

**Synthesis of Caged Fluorinated Tyrosines (360a and 360b).** Fluorinated tyrosine (3-Fluoro, or 2-Fluoro, 0.25 g, 1.25 mmol) was dissolved in 2 N NaOH (3 mL) and a solution of CuSO$_4$ (0.16 g, 0.63 mmol) in H$_2$O (2 mL) was added and heated to 60 °C for 10 minutes. The reaction was then cooled to room temperature and neutralized with acetic acid to pH 7.0, and gravity filtered (washed with water 3 x 5 mL). The product is transferred to a new flask and resuspended in 3:1 DMF/H$_2$O (10 mL), and 2-nitrobenzyl bromide (0.54 g, 2.5 mmol, 2 eq.) is then added and the reaction is stirred in the dark at room temperature for 48 hours. The mixture is then filtered and washed with 3:1 DMF/H$_2$O (3 x 5 mL), H$_2$O (3 x 5 mL), and cold acetone (3 x 2 mL). The solid is then transferred to a new flask and stirred at room temperature in 1 M HCl for 2 hours, then filtered and washed with 1 M HCl (3 x 5 mL), H$_2$O
(3 x 5 mL) and cold acetone (3 x 2 mL) to yield a slightly yellow powder (43-68%).$^1$H NMR (400 MHz, CD$_3$OD) δ 8.06 (s, 1H), 7.88-7.82 (m, 1 H), 7.65-7.62 (m, 1 H), 6.91-6.80 (m, 4 H), 5.15-5.09 (m, 2 H), 4.61-3.58 (m, 1 H), 3.01-2.85 (m, 2 H). HRMS m/z calculated for C$_9$H$_{11}$FNO$_3$: 200.1790, found: 200.1799.

**Miller Assay with Tyrosine Caged β-Galactosidases.** Liquid cultures (2 mL) of Top10 cells possessing a pBAD-lacZ-TAG plasmid and the pSup-ONBY plasmid were grown in LB media with Ampicillin (50 µg/mL), chloramphenicol (50 µg/mL), the appropriate tyrosine analog (2 mM), and 0.2% arabinose for constitutive expression overnight at 37 °C. The OD$_{600}$ of each expression was recorded, and 100 µL of each sample was added to 900 µL Z-Buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 50 mM β-mercaptoethanol, pH 7.0) and 25 µL 0.1% SDS and vortexed for 2 minutes to promote cellular lysis. One sample for each expression condition was then irradiated 20 minutes on the transilumminator (365 nm, 25W), while the other was not exposed to UV irradiation. The ONPG substrate (200 µL, 4 µg/µL) was added and the samples were heated to 30 °C for 10 minutes (or until a distinct yellow color appeared). The reaction was then quenched with 500 µL of 1 M Na$_2$CO$_3$ and the absorbance at 550 nm and 420 nm were recorded. Miller units were calculated from the following formula:

$$\text{Miller Units} = 1000 \times \frac{(\text{OD}_{420} - 1.75 \times \text{OD}_{550})}{(T \times V \times \text{OD}_{600})}$$

$T =$ reaction time (typically 10 min)

$V =$ Final volume
REFERENCES


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