SYNTHESIS OF PHOTOCAGED MOLECULES
TOWARDS SPATIAL AND TEMPORAL
CONTROL OF GENE EXPRESSION

by

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Reach for the moon. Even if you miss, you'll land among the stars.
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ABSTRACT

Spatial and temporal control of gene expression can aid in the understanding of gene function and cellular processes. Photo-labile protecting groups are chemical moieties that can be installed on a biologically active compound, blocking its biological function. Upon irradiation of the "caged" compound, the protecting group is removed "releasing" the now biologically active molecule.

In this work, a Tamoxifen aziridine derivative was synthesized and attempts were made to link it to a coumarin-based photolabile protecting group via a variety of synthetic strategies. However, none of the tamoxifen coumarin compounds were successfully synthesized. The failure of linking the tamoxifen core structure to a coumarin derivative can be attributed to two reasons: 1) instability of the coumarin in basic conditions for sufficient deprotonation of the tamoxifen phenol; 2) tendency of the electron-rich tamoxifen olefin to be unstable in acidic conditions.
Chapter 1

SPATIAL AND TEMPORAL CONTROL OF GENE EXPRESSION

1.1 Introduction

Manipulations of gene expression within a cellular environment can help illuminate biological processes. The ability to selectively express a particular set of genes in a temporally- and spatially-controlled manner can give insight to many biological processes and mechanisms. Harnessing the properties of light offers an attractive solution for this goal because light irradiation can be controlled by duration, region, and wavelength.¹

The concept of photocaging refers to modifying a biologically active compound with a photo-labile protecting group such that the modification blocks its biological activity, but can be removed upon irradiation with light (Scheme 1.1). The ideal caged compound possesses fundamental characteristics: because the goal is to perform experiments in the cellular environment, the caged compound must be water soluble; for the same reason, the linkage used to photocage a biologically active compound must be water stable; photodeprotection must occur with high quantum yields to minimize photo-induced cell death; wavelengths used to photodeprotect are within a practical range (300-900 nm); and the byproducts of photodeprotection are nontoxic.
One of the first examples of modifying gene expression using light was reported by Minden and coworkers wherein the authors reported inhibition of DNA binding activity of a transcription factor through the use of a photo-labile protecting group.\(^2\) GAL4-VP16, a potent transcriptional activator,\(^3\) was caged with an \(o\)-nitrobenzyl-4,5-dimethoxy-derived (nitroveratryl) group via a carbonate linkage. Photoactivation enabled expression of GAL4-dependent transgenes and allowed for the marking of activated cells with GFP. This method offered insight to the cellular environment that leads to fate determination of single cells.

In another example, Haselton and coworkers photocaged plasmid DNA to demonstrate the feasibility of targeting spatial and temporal control in vivo of light-dependent gene expression through chemical modification of genetic material.\(^4\) A photocaged plasmid DNA was transfected into the skin of rats and luciferase expression was evaluated. Luciferase expression levels of the caged plasmid were shown to be similar to untransfected skin sites. Upon irradiation at 355 nm, increased expression of luciferase was observed, indicating a release of the activated DNA.

This report was a significant example of the use of a nitroveraryl-derived protecting group for the manipulation of in vivo gene expression, however there were significant limitations. Random modification of the DNA backbone was inefficient and
required multiple caging groups for effective inactivation. Consequently, incomplete restoration of activity was observed.⁴

Tsien and coworkers developed a strategy to chemically modify RNA with a photolabile group to alter translational activity in zebrafish (Scheme 1.2).⁵ The authors introduced bromocoumarin-caged green fluorescent protein (GFP) mRNA at the single-cell stage of zebrafish embryos. Exposure to long wave UV irradiation effected photodeprotection and subsequent synthesis of GFP. Furthermore, embryonic injection of caged eng2a mRNA, crucial for early central nervous system development,⁶ and spot irradiation in the head region during early development led to a significant impairment of eye and brain development of zebrafish. Notably, the authors utilize the properties of a bromocoumarin-based protecting group, which are more sensitive to photo-deprotection than their nitroveratryl counterparts,⁷ to achieve their goals. A discussion on the importance of this feature is forthcoming.

Scheme 1.2 Photocaged RNA Phosphate Backbone Strategy

More recently, elegant strategies have been developed that harness the unique properties of photocaged biomolecules to control gene expression. Approaches that target transcription and translation are particularly attractive because
of the simplicity of the preparation. In short synthetic sequences, advancements were realized involving antisense mRNA,\textsuperscript{8} siRNA,\textsuperscript{9} negatively charged peptide nucleic acids,\textsuperscript{10} morpholinos,\textsuperscript{11} DNAzymes,\textsuperscript{12} and DNA decoys.\textsuperscript{13}

These methods have a number of drawbacks. One limitation is the challenge of delivering the caged biopolymer into the cell. The caged molecules often required microinjection into the cell. While advantageous in applications studying cellular differentiation during development,\textsuperscript{5} microinjection is not practical in certain applications and model organisms. Additionally, many methods required multiple caging groups for each biologically active molecule for sufficient inactivation. Subsequently, photodeprotection necessitated higher amounts of UV irradiation for complete reactivation.

1.2 Classes of Photo-labile Protecting Groups

There are a number of photolabile protecting groups and an exhaustive look into all of the structures that have been used to cage biologically relevant compounds is beyond the scope of this work. Photolabile protecting groups can be classified by the number of coincidental photons necessary for photolysis. One-photon photolysis involves the absorbance of a single UV photon for initiation of photodeprotection. One-photon photolabile protecting groups include, but are not limited to, o-nitrobenzyls,\textsuperscript{14} benzoins,\textsuperscript{15} and phenacyls.\textsuperscript{16}

Two-photon photolysis requires the excitation of two or more coincidental photons at twice the wavelength.\textsuperscript{7} Thus, two photon irradiation commonly uses infrared light, which is less toxic and has greater tissue penetrance than UV irradiation. Photocaging groups responsive to two-photon deprotection include, but are not limited to, coumarins\textsuperscript{7,17}, quinolines\textsuperscript{18,19}, and thioxanthenes.\textsuperscript{20}
Multi-photon excitation requires the use of very high local light intensities typically through the use of a femtosecond pulsed IR laser. This method enables an extremely precise level of spatial control of photodeprotection because intensities decrease substantially away from the point of focus thereby facilitating a very limited region of photodeprotection.\(^7\) In fact, three-dimensional cross-sections of spatial precision can be achieved through the use of multi-photon excitation.\(^7\)

Quantum yield, described as the ratio of photons absorbed to product formed, provides insight to the efficiency of the photodeprotection. The coumarin class of protecting groups exhibit a higher overall quantum yield, and thus, are more sensitive to deprotection with ultraviolet irradiation than their nitroveratryl counterparts and are also about 30-fold more sensitive to photolysis by two coincident infrared photons.\(^7\) This is significant for two reasons: 1) it enables deprotection of the desired bioactive molecule with less UV irradiation reducing the possibility of phototoxicity to living cells and 2) it allows for the use of two-photon infrared laser photolysis, which permits better three-dimensional localization of photodeprotection.

Multiple factors determine the quantum yield of any given caged molecule. One determinant of quantum yield is the electronic properties of the caging group itself. Additionally, the substituents of the caging group help determine the wavelength required for excitation. A second factor is the mode through which the caging group is linked to the bioactive molecule. Lastly, the electronic nature of the bioactive molecule to be caged affects the overall quantum yield of the photodeprotection.
The effects of the various factors in quantum yield determination can be illustrated in an examination of the reported literature (Figure 1.1). The quantum yields of compounds a-d are representative of the o-nitrobenzyl class of protecting groups. In contrast, compounds e-l, which are part of the coumarin class of protecting groups, exhibit much higher quantum yields overall. Compounds e-h
demonstrate the effect of substituents locating on the protecting group phenyl ring. In particular, the addition of a bromine at the 6-position (compound g) exhibits a higher quantum yield (0.037) as compared to the quantum yield of compound e (0.025), with the bromine omitted at the 6-position. A comparison of the quantum yields for compound i-k demonstrates the effect of methylation of the phenol within the coumarin structure. An increase in quantum yield is observed upon methylation; 0.084 and 0.30 for the free phenol and methylated phenol, respectively.

Two photon uncaging cross sections, $\delta_u$ (expressed as units of GM, or Göppert-Mayer) can be measured to indicate efficiency of photolysis and intrinsic three-dimensional resolution.\textsuperscript{23} Values of $\delta_u$ greater than 0.01 GM are considered biologically favorable,\textsuperscript{17} a criterion o-nitrobenzyl-derived groups often fail to meet or are unable to be measured (Figure 1.1, Compound a).\textsuperscript{7,17,18,22} In contrast, coumarin-based photolabile protecting groups exhibit excellent two-photon cross-sections (Compounds e-i, l), enabling precise and efficient deprotection.

1.3 Previous Work on Photoactivatable Gene Expression by the Koh Group

The Koh group has successfully demonstrated that a nitroveratryl group can be used to photocage a tamoxifen aziridine as part of a strategy to control recombination of DNA with estrogen receptor (ER) dependent Cre recombinase.\textsuperscript{24} Tamoxifen aziridine (Taz) is an antagonist of the estrogen receptor (ER) that forms a covalent bond with a cysteine residue within the ER active site (Scheme 1.3).\textsuperscript{25} Phenolic modification of Taz with a nitroveratryl caging group (nvTaz) prevents entrance into the ER active site.
Irradiation of HEK293T cells treated with nitroveratryl tamoxifen aziridine (NvTaz) at 345 nm led to exposure-dependent expression of CRE recombinase, an enzyme responsible for the intra- (or inter) molecular splicing of DNA at loxP sites. Recombination can be measured using a reporter gene that is flanked by loxP sites, "floxed", which after recombination, places the β-galactosidase gene LacZ in front of the promoter. LacZ expression can be measured by x-gal staining (Scheme 1.4).26

The aforementioned report is an attractive strategy for gene photo-patterning cells through spatial and temporal control of gene expression because it addresses key limitations of existing methods. In general, transcription response of a
ligand to its target is limited two-fold: 1) rate of diffusion of the uncaged ligand out of the cell, and 2) stability of the ligand receptor complex.\textsuperscript{27} The Koh group addressed these key limitations through NvTaz covalent modification of ER. As such, the ER/Taz complex has an exceptionally long half-life ($t_{1/2} \approx 32$ hr),\textsuperscript{26} which allows gene expression to be sustained for a longer duration. Additionally, recombination places the gene of interest downstream of the promoter, permanently turning it on in the photo-induced cells. The reported method, however, required up to 180 seconds of UV irradiation. It is well-known that extended exposure of cells to UV irradiation can be detrimental and may lead to UV-induced apoptosis.\textsuperscript{28,29} Attempts to increase recombination efficiency by increasing UV irradiation cycles lead to significant cytotoxicity from excessive UV exposure.

Herein, the goal of this research was to modify Taz to incorporate a 6-bromo-7-methoxycoumarin photo-labile protecting group so as to take advantage of the higher quantum yields exhibited by this class of compounds.
1.4 References


Chapter 2

DEVELOPMENT OF BROMOCOUMARIN-CAGED TAMOXIFEN AZIRIDINE

2.1 Introduction

The design and synthetic route of a bromocoumarin-caged tamoxifen aziridine (2) was based on the previously synthesized nitroveratryl-caged amoxifen aziridine (Figure 2.1).<sup>1</sup> It has been shown that a bulky caging group through a phenolic ether linkage blocks entrance into the estrogen receptor active site. An aziridine moiety on the adjacent phenol was known to form a covalent bond within the active site of ER to prevent dissociation of ligand from complex.<sup>1</sup>

![Figure 2.1 Structure of Nitroveratryl-caged Tamoxifen Aziridine and Proposed Bromocoumarin-caged Tamoxifen Aziridine](image-url)
The synthesis of 1 involved McMurry coupling to construct the tamoxifen core, followed by installation of the nitroveratryl caging group. Mitsunobu reaction of aziridine ethanol and the free phenol of the tamoxifen core completed the synthesis.¹

The proposed synthetic route to 2 remained congruent, however, the nitroveratryl caging group was substituted for a bromocoumarin derivative to take advantage of their high quantum yields and ultimately utilize less cytotoxic UV or high-flux IR irradiation. Since the bromocoumarin subunit was not commercially available, synthesis of the bromocoumarin group bearing an electrophilic carbon for installation at the tamoxifen phenol was necessitated.

The initial synthetic route of the bromocoumarin component was based on a previously reported synthesis wherein a nucleotide was caged via a diazomethyl derivative.² It was envisaged that the bromocoumarin caging group could be installed onto the phenol of the tamoxifen core by utilizing the inherent reactivity of a diazomethyl derivative.

2.2 Results and Discussion

The tamoxifen core structure was synthesized by McMurry coupling³ of propriophenone and 4,4'-dihydroxybenzophenone in the presence of titanium tetrachloride and zinc dust in THF to afford compound 3 in 84% yield (Scheme 2.1).⁴⁵ A Mitsunobu reaction of aziridine ethanol and 3 effected the installation of the aziridine moiety. Care was required to minimize exposure of the aziridine ethanol starting material to triphenyl phosphine in the absence of the phenol 3 since reactivity between the phosphine and the aziridine was observed.
Scheme 2.1 Construction of Tamoxifen Core

With the tamoxifen component in hand, attention was then turned to the synthesis of the diazomethylcoumarin, which commenced with acid-catalyzed condensation of 4-bromoresorcinol and ethyl acetoacetate (Scheme 2.2). This step noted an improvement on a previously reported transformation and used benzene as azeotropic solvent in a Dean-Stark apparatus. Treatment with methyl iodide in the presence of potassium carbonate masked the phenol group. Benzylic oxidation of compound 6 with selenium dioxide under reflux in chlorobenzene afforded aldehyde 7. Conversion to tosyl hydrazone 8 was effected by treatment of p-toluenesulfonyl hydrazine in ethanol at 40 °C. Compound 8 was transformed into diazo derivative 9 upon exposure to triethyl amine in methanol.
Scheme 2.2 Synthesis of 6-bromo-7-methoxy-4-diazo
coumarin

The stage was set for the coupling of the tamoxifen and
diazomethylcoumarin subunits. However, all efforts to construct the phenolic ether
link between the tamoxifen phenol and the diazomethylcoumarin were unfruitful.
Attempts were made to form the diazo functionality \textit{in situ} from the hydrazone,
though it did not form the desired product (\textbf{Scheme 2.3}). To avoid complications with
the coupling chemistry and the 3-membered aziridinyl ring, the coupling was also tried
with compound 3 having the aziridine moiety omitted.
Scheme 2.3 **Attempts to Couple Tamoxifen and Diazomethylcoumarin**

An updated synthetic route involved installation of a leaving group at the hydroxyl position was undertaken (**Scheme 2.4**). The synthesis of the bromocoumarin subunit was modified to present a more streamlined approach that avoided the use of selenium dioxide for hydroxy functionalization at 4-C. The acid catalyzed condensation of 4-bromoresorcinol and ethyl 4-chloroacetoacetate proceeded in good yields to form chloride 11. Methylation of the phenol with methyl iodide in the presence of potassium carbonate gave compound 12 in 60% yield. Reflux of compound 13 in water overnight effected the conversion of the chloride to a hydroxyl group, which was subsequently treated with mesyl chloride and cesium carbonate to form mesylate 14.
Attempts to displace the mesylate leaving group with the tamoxifen phenolate, generated upon reaction of tamoxifen and a strong base, proved unsuccessful. Other hydroxy leaving groups were surveyed, however the coupled product was not able to be isolated. In most cases, consumption of the bromocoumarin starting material was observed by TLC, however this was attributed to coumarin decomposition.

Concurrently, attempts were undertaken to displace the chloride of compound 12 through a silver-assisted S_N2. The rationale was that silver complexation with the chloride would increase the electrophilic character of the chloride-bearing carbon, thereby facilitating the ease of nucleophilic substitution of the electron-deficient tamoxifen phenolate (Scheme 2.5).
Discouragingly, attempts to couple the tamoxifen phenolate (3) and 4-chloro bromocoumarin (12) via silver-assisted substitution were unsuccessful. This outcome can be attributed to unwanted side reactions of the silver salt and the aryl bromide of the coumarin.\(^7\)

A scan of the literature uncovered other modes in which the coumarin and tamoxifen subunits could be linked. An appealing method emerged that involved formation of a reactive chloroformate and subsequent reaction with a phenol to form a carbonate linkage.\(^8,9\) An attractive feature of this method was that a common intermediate from a previous synthetic route could be utilized. Thus, attention was directed towards formation of the chloroformate of compound 13 followed by reaction with the tamoxifen phenol (Scheme 2.6).
Ungratifyingly, all attempts to isolate the chloroformate from alcohol 13 were unsuccessful (Scheme 2.7). It was believed that the chloroformate moiety was considerably labile and therefore may undergo hydrolysis to the original alcohol. Therefore, additional attempts were conducted to couple the crude chloroformate directly, however these trials were unsuccessful as well.

Trichloroacetimidates, common motifs found in glycoside synthesis, were evaluated as an alternative method that avoids the use of a strong base to effect the coupling. They are of particular interest due to the conditions under which they react.
with nucleophiles. Instead of requiring the use of a strong base for deprotonation of
the phenol, catalytic amounts of Lewis acids can be used to facilitate the attack of the
nucleophile at the electrophilic carbon of the acetimidate.\textsuperscript{10-12} It has been demonstrated
that trichloroacetimidates readily couple to phenols in the presence of boron trifluoride
etherate in good yield.\textsuperscript{13,14}

It was envisaged that the trichloroacetimidate of the coumarin, formed
from reaction of the 4-hydroxycoumarin (13) and trichloroacetonitrile with catalytic
amounts of DBU, can be displaced with the tamoxifen phenol (3). Unfortunately,
 attempts to make the desired trichloroacetimidate intermediate were unsuccessful
(Scheme 2.8). After extensive study of the crude reaction mixture, the identity of the
trichloroacetimidate could not be confirmed.

![Scheme 2.8 Attempts to Generate Acetimidate](image)

Scheme 2.8 Attempts to Generate Acetimidate

It is interesting to note that the proposed trichloroacetimidate coumarin
structure (15) in this context is allylic. Presumably, a [3,3] sigmatropic arrangement
could be suggested, which would account for the observed lack of desired reactivity, however, analogous transformations require catalysts, such as cobalt.\textsuperscript{15} Additionally, this pathway could not be confirmed from crude reaction mixture analysis.

2.3 Conclusion

Synthesis of a aziridinyl tamoxifen derivative was described. A number of bromocoumarin derivatives were synthesized, and their ability to couple \textit{via} a phenolic ether and carbonate linkage to tamoxifen was evaluated. Unfortunately, all attempts to form the desired coupling product were unsuccessful. These unfavorable observations are attributable to a variety of factors. In the reaction conditions necessary to effect sufficient tamoxifen phenolate generation, decomposition of the coumarin subunit was observed. Additionally, in the conditions required for coupling of the trichloroacetimidate and phenol, decomposition of both the coumarin structure and the electron rich olefin of the tamoxifen core structure was observed. This observed dichotomy presented an insurmountable impediment for progress toward the project's goal.
2.4 Experimental

General Considerations
All reactions were carried out in glassware that was flame-dried under vacuum and cooled under nitrogen. Chromatography was performed on silica gel (Silicycle 40-63D, 60Å) and deactivated silica gel (Silicycle 40-63D, 60Å), which was prepared by treating silica gel with EtSiCl$_3$. Reagents were used from commercial sources without further purification. The purity of the compounds was determined by 1H NMR. All compounds were measured to be greater than 95% pure unless otherwise noted otherwise.

1,1-Bis(4-hydroxyphenyl)-2-phenylbut-1-ene (3)

Zinc powder (4.8 g, 74 mmol) and dry THF (84 mL, 0.1 M) was brought to 0 °C to which TiCl$_4$ (4.2 mL, 37.0 mmol) was added dropwise. The solution was warmed to room temperature and refluxed for 4 hr. A solution of 4,4'-dihydroxybenzophenone (2.0 g, 9.3 mmol), propiophenone (3.86 mL, 28 mmol), in dry THF (124 mL, 0.05 M), was added to the reaction mixture and refluxed for 12 hours. After 12 hours, the reaction mixture was allowed to cool, quenched with 10% K$_2$CO$_3$ (100 mL), extracted with ethyl acetate (3x, 150 mL), dried over MgSO$_4$,
filtered, and concentrated by rotary evaporation. The crude residue was purified by silica gel column chromatography using a gradient of (0-10%) ethyl acetate in dichloromethane to afford 2.5 g of 3 (7.8 mmol, 84%), having identical 1H and 13C NMR spectrum to that previous reported.\textsuperscript{16}

**(E/Z)-1-(4-(2-N-aziridinyl)ethoxy)phenyl)-1-(4-hydroxyphenyl)-2-phenylbut-1-ene (4)**

Diethyl azodicarboxylate (1.3 mL, 0.78 mmol) was added dropwise to a solution of aziridine ethanol (76 µL, 0.95 mmol) and THF (1 mL, 0.8 M) at 0 °C. A solution of 3 (250.0 mg, 0.79 mmol), triphenyl phosphine (289.0 mg, 1.10 mmol) and THF (3.9 mL, 0.2 M) was then added dropwise to the aziridine/DIAD/THF solution at 0 °C. The reaction mixture was allowed to gradually warm to room temperature and stirred overnight. The crude reaction mixture was concentrated onto deactivated silica gel\textsuperscript{17} by rotary evaporation and purified on deactivated silica gel using a gradient (0-100%) ethyl acetate in hexanes to afford 40 mg of 4 (0.10 mmol, 14%) as a mixture of E/Z isomers. 1H NMR (400 MHz, (CD\textsubscript{3})\textsubscript{2}CO, δ): 0.92 (t, J = 6 Hz, 6H), 1.28 (t, J = 3 Hz, 3H), 1.39 (t, J = 3 Hz, 3H), 2.48 (q, J = 6 Hz, 4H), 6.39 (d, J = 6 Hz, 2H), 6.54 (d, J = 6 Hz, 2H), 6.64 (d, J = 6 Hz, 2H), 6.77 (d, J = 6 Hz, 2H), 6.88 (d, J = 6 Hz, 2H), 7.0-7.2 (m, 7H); 13C NMR (400 MHz, (CD\textsubscript{3})\textsubscript{2}CO, δ): 13.9, 15.0, 27.4, 29.8,
6-bromo-7-hydroxy-4-methylcoumarin (5)

To dry benzene (55 mL, ) in a round bottom flask of a Dean-Stark apparatus was added \( p \)-toluenesulfonic acid hydrate (503.1 mg, 2.645 mmol) and brought to reflux for 1 hour. The reaction mixture was cooled and 4-bromoresorcinol (5.0 g, 26.45 mmol) and ethyl acetoacetate (5.1 mL, 39.7 mmol) was added and the solution brought to reflux overnight. The reaction mixture was cooled, poured over ice water, and stirred for 30 min. The precipitate was collected and dried over \( \text{P}_2\text{O}_5 \) under vacuum to yield 4.9 g of compound 5 (19.5 mmol, 74%), estimated to be about 85% pure, and was carried on to the next step without further purification.\(^2\) \( ^1\text{H} \text{NMR} \) (400 MHz, \( \text{CD}_3\text{OD} \), \( \delta \)): 2.40 (s, 3H), 6.15 (s, 1H), 6.83 (s, 1H), 7.88 (s, 1H); \( ^{13}\text{C} \text{NMR} \) (400 MHz, \( \text{CD}_3\text{OD} \), \( \delta \)): 19.0, 104.2, 111.8, 114.2, 129.8, 153.5, 154.2, 158.0, 160.3; LRMS (ES pos) calculated for \( \text{C}_{10}\text{H}_7\text{BrO}_3 \) 253.95, found \( \text{M}^+ \) 254.8.
6-bromo-7-methoxy-4-methylcoumarin (6)

Compound 5 (2.5 g, 9.8 mmol) was dissolved in dry acetone (50 mL, 0.2 M) to which was added K₂CO₃ (1.4 g, 10.3 mmol) followed by iodomethane (2.9 mL, 47.0 mmol). The reaction mixture was stirred at reflux overnight. The reaction mixture was allowed to cool to room temperature and the solvent was removed by rotary evaporation. The residue was diluted in chloroform and washed with 1M HCl (3x, 10 mL) and the organic layer was dried over MgSO₄ and concentrated. The residue was purified by column chromatography using a gradient (0-100%) ethyl acetate in hexanes to afford 1.79 g of 6 (5.9 mmol, 60%).⁹ ¹H NMR (400 MHz, CDCl₃, δ): 2.40 (s, 3H), 3.95 (s, 3H), 6.38 (s, 1H), 6.84 (s, 1H), 7.82 (s, 1H); ¹³C NMR (400 MHz, CDCl₃, δ): 19.0, 56.7, 100.3, 107.4, 112.8, 114.5, 128.6, 151.5, 154.3, 158.4, 161.4.

6-Bromo-4-formyl-7-methoxycoumarin (7)

Compound 6 (200 mg, 0.67 mmol) was placed in a flame dried flask under nitrogen. Chlorobenzene (6 mL, 0.1 M) was then added followed by SeO₂ (89.2 mg, 0.80 mmol). The reaction was brough to reflux and stirred for 6 days. The reaction
mixture was cooled, filtered, and concentrated. The residue was recrystallized from toluene and the crystals were collected by vacuum filtration to afford 39.6 mg of aldehyde 7 (0.13 mmol, 20%).\(^{9}\) 1H NMR (400 MHz, CDCl\(_3\), \(\delta\)): 3.95 (s, 3H), 6.83 (s, 1H), 7.10 (s, 1H), 8.86 (s, 1H), 10.04 (s, 1H); 13C NMR (400 MHz, CDCl\(_3\), \(\delta\)): 60.8, 100.3, 107.4, 112.8, 114.5, 127.7, 130.2, 142.2, 151.5, 154.3, 159.2, 167.7, 190.7

### 6-Bromo-4-formyl-7-methoxycoumarin p-tosylhydrazone (8)

![Chemical structure of 8](image)

To a stirring solution of ethanol (1.0 mL, 0.6 M) and aldehyde 7 (200 mg, 0.64 mmol) was added p-toluenesulfonyl hydrazine (131.66 mg, 0.71 mmol). An additional 0.17 mL ethanol was added to the reaction mixture and was allowed to stir for 1 hour at 40 °C. The reaction mixture was cooled, filtered, and the resulting precipitate was washed with cold ethanol and dried under vacuum. The product was purified by column chromatography using a gradient (0-100%) ethyl acetate in dichloromethane to afford 232.1 mg (0.48 mmol, 74%). 1H NMR (400 MHz, DMSO-d\(_6\), \(\delta\)): 2.50 (s, 3H), 3.5 (s, 3H), 6.74 (s, 1H), 7.45 (d, J = 7.6 Hz, 2H), 7.53 (s, 1H), 7.82 (d, J = 7.6 Hz, 2H), 8.17 (s, 1H), 8.91 (s, 1H); 13C NMR (400 MHz, DMSO-d\(_6\), \(\delta\)): 20.5, 21.1, 111.0, 112.8, 115.6, 119.3, 127.2, 131.3, 136.0, 141.8, 143.8, 144.3, 149.8, 153.9, 160.1, 168.0.
6-Bromo-4-diazomethyl-7-methoxycoumarin (9)

![Chemical structure of 6-Bromo-4-diazomethyl-7-methoxycoumarin (9)]

To a stirring solution of tosyl hydrazone 8 (21.0 mg, 0.045 mmol) and methanol (210 µL, 0.2M) was added triethyl amine (6.7 µL, 0.47mmol). The reaction mixture was stirred at room temperature for 1 hour. A yellow precipitate was filtered, washed with cold methanol, and dried under vacuum to afford 5.5 mg of 9 (0.02 mmol, 40%). The residue was taken on without further purification. 1H NMR (400 MHz, DMSO-d$_6$, δ): 3.95 (s, 3H), 6.63 (s, 1H), δ;13C NMR (400 MHz, DMSO-d$_6$, δ): 19.9, 46.2, 99.4, 111.2, 112.9, 115.4, 128.5, 145.9, 150.0, 151.9, 152.7, 159.1, 168.2.

6-Bromo-7-methoxycoumarin-4-carbaldehyde Hydrazone (10)

![Chemical structure of 6-Bromo-7-methoxycoumarin-4-carbaldehyde Hydrazone (10)]

To a stirring suspension of aldehyde 7 (10 mg, 0.03 mmol) and ethanol (1 mL, 0.03 M) was added hydrazine hydrate (75.0 mg, 1.5 mmol) and allowed to stir at room temperature until a yellow/orange precipitate formed. The reaction mixture was cooled in an icebath and the precipitate was collected by vacuum filtration to afford 0.4 mg of hydrazine 10 (0.001 mmol, 4%). The precipitate was taken on without further purification. 1H NMR (400 MHz, CDCl$_3$, δ): 3.95 (s, 1H), 6.63 (s, 1H), 6.96 (s, 1H),
8.40 (s, 1H), 9.06 (s, 1H); 13C NMR (400 MHz, CDCl₃, δ): 55.7, 100.8, 110.3, 111.8, 130.9, 146.7, 155.6, 160.7, 162.0.

6-Bromo-4-chloromethyl-7-hydroxycoumarin (11)

A solution of 4-bromoresorcinol (15 g, 79.2 mmol), H₂SO₄ (70 mL), and ethyl 4-chloroacetoacetate (16 mL, 119 mmol) was stirred at room temperature for 6 days. The reaction mixture was poured into ice water and stirred for two hours. The precipitate was filtered, washed with cold distilled water and dried over P₂O₅ overnight under vacuum to afford 18 g of 11 (62.4 mmol, 79%).⁶ ¹H NMR (400 MHz, CD₃OD, δ): 4.82 (s, 2H), 6.42 (s, 1H), 6.85 (s, 1H), 7.94 (s, 1H); ¹³C NMR (400 MHz, CD₃OD, δ): 42.1, 104.2, 108.1, 111.8, 114.2, 127.3, 129.8, 152.3, 154.2, 158.0, 159.32, 162.3.
6-Bromo-4-chloromethyl-7-methoxycoumarin (12)

![Chemical Structure](image)

Compound 11 (500 mg, 1.7 mmol) was dissolved in dry acetone (8 mL, 0.21 M) to which was added K₂CO₃ (281.9 mg, 2.04 mmol) followed by iodomethane (0.54 mL, 8.4 mmol). The reaction mixture was stirred at reflux overnight. The reaction mixture was allowed to cool to room temperature and the solvent was removed by rotary evaporation. The residue was diluted in chloroform and washed with 1M HCl. The organic layer was dried over MgSO₄, filtered, and concentrated. The product was purified by column chromatography using a gradient (0-50%) ethyl acetate in hexanes to afford 92.1 mg of 12 (1.02 mmol, 60%). 1H NMR (400 MHz, CDCl₃, δ): 3.95 (s, 3H), 4.34 (s, 2H), 6.38 (s, 1H), 6.84 (s, 1H), 7.82 (s, 1H); 13C NMR (400 MHz, CDCl₃, δ): 54.3, 59.0, 111.2, 112.6, 117.1, 128.3, 149.8, 152.7, 155.3, 159.5, 168.0.
6-Bromo-4-hydroxy-7-methoxycoumarin (13)

A suspension of 12 (10 g, 35.0 mmol) in distilled water (50 mL, 0.7 M) was heated at reflux overnight. The reaction mixture was cooled and evaporated under vacuum. The residue was purified by column chromatography using a gradient (0-80%) ethyl acetate in hexanes to afford 4.3 g of 13 (15.8 mmol, 45%).

$^1$H NMR (400 MHz, CD$_3$OD, $\delta$): 3.97 (s, 3H), 4.78 (s, 2H), 6.43 (s, 1H), 7.04 (s, 1H), 7.88 (s, 1H); $^{13}$C NMR (400 MHz, CD$_3$OD, $\delta$): 57.2, 60.9, 101.5, 106.6, 108.7, 111.8, 129.0, 157.1, 160.3.

6-Bromo-7-methoxycoumarin-4-yl Methanesulfonate (14)

To a stirring solution of 13 (50 mg, 0.18 mmol) dissolved in anhydrous DMF (1 mL, 0.2 M) at 0 °C was added Cs$_2$CO$_3$ (58.8 mg, 0.18 mmol), followed by mesyl chloride (22.9 mg, 0.2 mmol). The reaction mixture was warmed to room temperature and stirred overnight. The reaction mixture was diluted with ether, washed with distilled water (10 mL), extracted with ether (3x, 10 mL), dried over MgSO$_4$ to afford 25 mg of crude 14 (0.07 mmol, 40% crude yield). The residue was
taken on without further purification. 1H NMR (400 MHz, CDCl₃, δ): 3.21 (s, 3H), 3.97 (s, 3H), 4.34 (s, 2H), 6.42 (s, 1H), 7.04 (s, 1H), 7.71 (s, 1H); 13C NMR (400 MHz, CDCl₃, δ): 39.2, 57.2, 68.7, 108.7, 109.7, 111.8, 129.0, 157.1, 160.3.
2.5 References


7. Li, P.; Wang, L. *Synlett* **2006**, 14, 2261


