

DEVELOPMENT OF FLUORESCENT pH INDICATOR, FLUOROGENIC
PROTECTING GROUP, PHOTOACTIVATABLE NUCLEOPHILIC SUBSTITUTION
(S_N1) REACTION AND PHOTOACTIVATABLE FLUOROPHORES FOR
BIOLOGICAL APPLICATIONS.

by

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(Under the Direction of Vladimir V. Popik)

ABSTRACT

In aqueous and alcohol solutions, colorless and non-fluorescent 9-aryl-9*H*-xanthen-9-ols equilibrate with brightly colored and fluorescent 9-arylxanthylium cations. The position of this equilibrium depends only on hydronium ion concentration and is not affected by general acids or other ions. The pH of the equivalence point can be adjusted by substitution in the xanthenol core. 3,6-dialkoxy-9-(*o*-tolyl)-9-xanthenol undergoes dehydroxylation to form corresponding cation at biologically relevant pH = 6.5. 9-Aryl-9*H*-xanthen-9-ol – based pH indicators permit following acidity changes by both fluorescent and absorption spectroscopies.

Non-fluorescent 3,6-*bis*(benzyloxy)-9-aryl-9-xanthenol undergoes photo-dehydroxylation with simultaneous photo-Claisen rearrangement upon irradiation in aqueous methanol to give fluorescent isomeric products. Alcohol substrates are simply caged by refluxing the parent 3,6-*bis*(benzyloxy)-9-aryl-9-xanthenol with the desired alcohol in acetonitrile and catalytic amounts of acetic acid or using the alcohol as neat

solvent for less expensive substrates. Formation of fluorescent fluorescein derivatives can be used to monitor substrate release.

Photolabile protecting groups can also be used to weaken or totally eradicate the fluorescence of a fluorophore. Irradiation of the caged dye removes the protection thereby revealing the fluorophore. We have effectively used 3-(hydroxymethyl)naphthalen-2-ol as a photoremovable protecting group for fluorescein. The caged fluorophore is weakly fluorescent. Photolysis of the caged dye releases the fluorophore with a fluorescent quantum yield $\Phi_F = 0.75 - 0.93$.

Novel light-triggered S_N1 strategy for the formation of glycosidic bond has been developed. The reaction is initiated by the photochemical cleavage of the bond between anomeric carbon and aglycone containing 9-aryl-9-fluorenol. Irradiation of 9-aryl-9-fluorenol derivatives bearing the tetrahydropyranyl moiety in 1,1,1,3,3,3-hexafluoro-2-propanol and tetrahydrofuran in a Royonet reactor at 300 nm presumably resulted in a short-lived 9-aryl-9-fluorenyl cation. The carbocation intramolecularly attacks the ether oxygen. This intramolecular attack of the ether by the initially formed carbocation leads to heterolysis of the C-O bond and the formation of oxonium ion. The oxonium ion thus generated is trapped preferentially with added nucleophile such as 2-phenyl ethanol.

INDEX WORDS: fluorescence, pH probe, fluorogenic, photoactivatable fluorophore, photo- S_N1 , Photo-Claisen rearrangement, photodehydroxylation, photolabile, quinone methide.

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DEDICATION

To my family, especially my father Andrew Nekongo Etum and Uncle George Eyakwe for all they have done.

To my wife Khalilah Nekongo, for her relentless support and encouragement.

And my sister Margaret Yembi of blessed memory, who had always wanted the best of me.

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CHAPTER 1

1.1 INTRODUCTION

The maintenance of specific intracellular acidity is crucial for the proper functioning of cellular machinery. The hydronium ion concentration is kept within very strict limits by a complex system of buffering mechanisms.¹ Normal cytosolic concentration of hydronium ions in mammalian cells is estimated at ca 4×10^{-8} mol/L. A slight variation in this value can impair enzyme activities,² synaptic transmission³ and have been implicated in Alzheimer's,⁴ chronic granulomatous disease,⁵ mucopolipidosis type IV,⁶ as well as cardiopulmonary and neurological conditions.⁷ Furthermore, abnormal alteration in cytoplasmic and vesicular acidity has been associated with cancer.⁸ On the other hand, some acidic organelles require a pH range of about 4.5 – 6.5 to function properly.⁹ Among the various techniques used for the measurement of cellular pH such as NMR, microelectrode, and absorbance spectroscopy, fluorescence spectroscopy is attractive because it is non-invasive, has greater analytical sensitivity and specificity, as well as good spatial and temporal resolution.

Among various pH probes available, fluorescein-based pH probes such as 2',7'-*Bis*-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein **1.1** (BCECF; Figure 1.1) developed by Roger Tsien and co-workers¹⁰ is the most popular. BCECF and its analogue BCPCF **1.5**¹¹ are commercially available as the esters BCECF AM I-III, **1.2** – **1.4** (Figure 1.1) which are easily hydrolyzed by esterases. Halogenated fluoresceins¹² and benzoxanthene dyes with long wavelength emission maxima introduced by Life Technologies¹³ have

also found wide use for pH measurements. In addition, other long wavelength near IR cyanine dyes¹⁴ have also been developed.

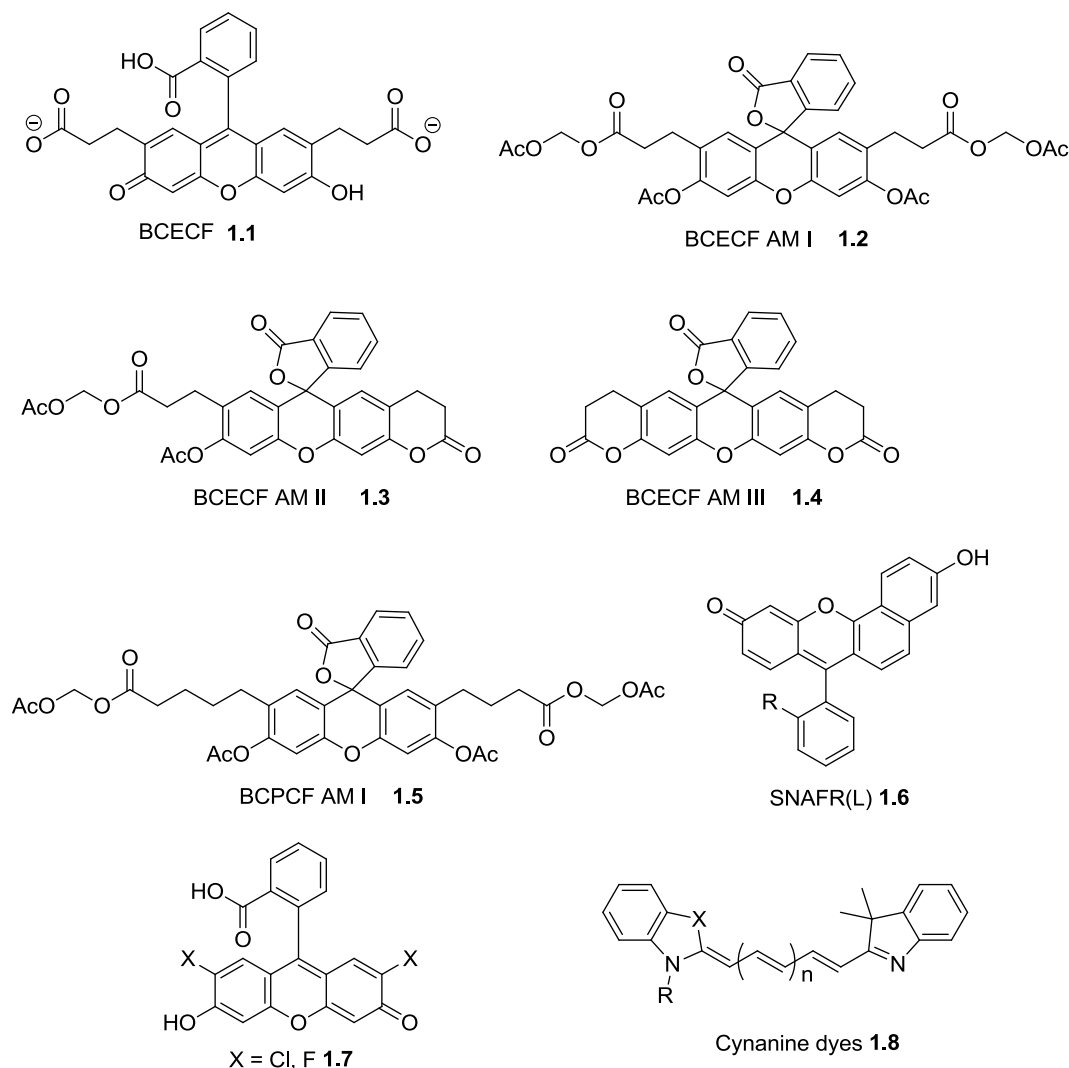


Figure 1.1 Examples of dyes used for pH measurements

There has also been increased interest in the development of fluorescent pH indicators that rely on photoinduced electron transfer (PET) to modulate the emission intensity.¹⁵ In this design, the fluorophore is equipped with an appropriate receptor moiety that possesses high-energy HOMO and a basic site. In the “off-state” of an indicator, fluorescence is quenched by electron transfer from the HOMO to the excited

state of a chromophore. At higher acidities, protonation of the basic moiety reduces the HOMO energy and inhibit PET (Figure 1.2 & 1.3). Unfortunately, stabilization of the receptor HOMO is often not proton-specific.¹⁶ Other general acids and Lewis acids, such as metal cations, can reduce the efficiency of PET.^{17,18} In addition, reactive nitrogen species like the nitric oxide (NO)¹⁹ as well as Lewis bases,²⁰ are known PET quenchers. Solvent polarity²¹ and hydrogen bonding^{20,22} have also been reported to modulate emission intensity.

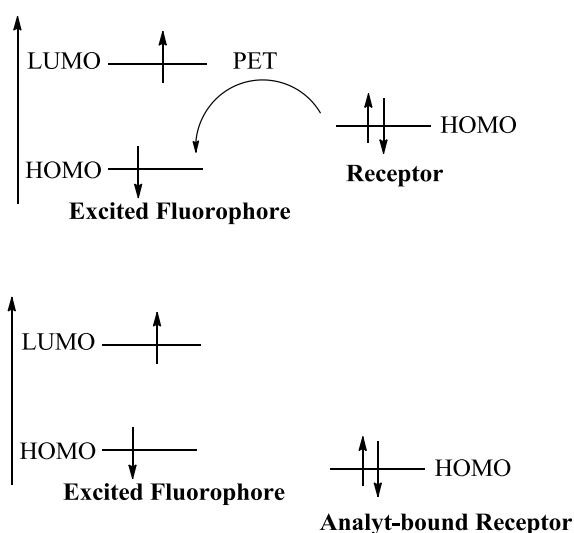


Figure 1.2 Frontier-orbital energy diagram illustrating the thermodynamics of PET .

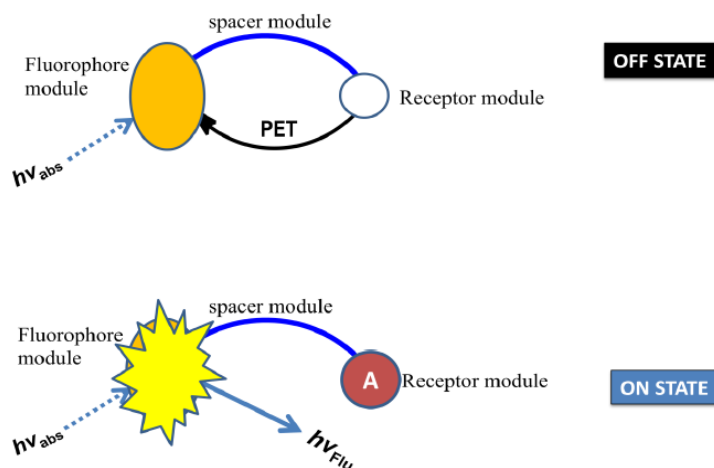
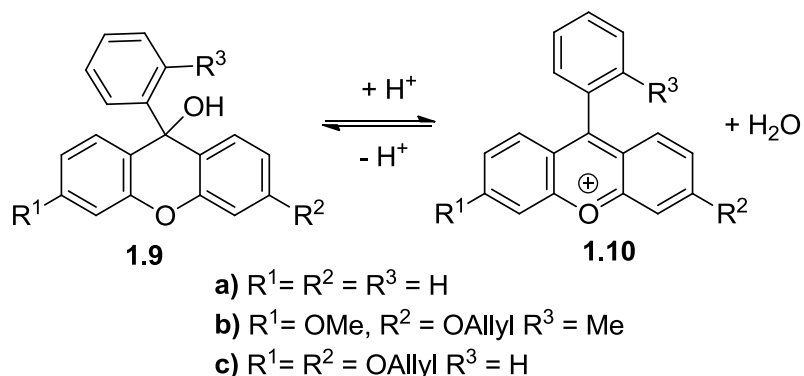


Figure 1.3 Schematic representation of PET

The need for pH sensors has led to the development of several pH probes in recent years.²³ However, many fluorescent pH probes are limited by their sensitivity and negligible stokes shift.²⁴ Therefore, there is a need to develop proton-specific, highly sensitive fluorescent probes with significant bathochromic and stokes shifts for monitoring cellular pH changes.²⁵

We report the design of fluorescent pH indicators, that employ pH-controlled chemical equilibrium to achieve modulation of emission intensity. Such an approach permits the development of more selective or “orthogonal” acidity indicators. In addition, it allows for both fluorescent and colorimetric readout. Our design is based on reversible dehydroxylation of 9-aryl-9-xanthenols (Scheme 1.1). The reversible redox behavior of triphenylcarbenium ions is well documented in early reports by Martin and Smith²⁶ and work by Wada and co-workers²⁷ which indicates that methoxy substituted triarylcarbinols are basic and have varying chemical properties. The parent 9-phenyl-9-xanthenol (**1.9a**) is known to reversibly produce fluorescent 9-phenyl-9-xanthylium cation (**1.10a**) in concentrated acid solutions (25% H₂SO₄). The equivalence point (when [**1.9a**] = [**1.10a**]) was reported at pH = 1.0 ± 0.5.²⁸



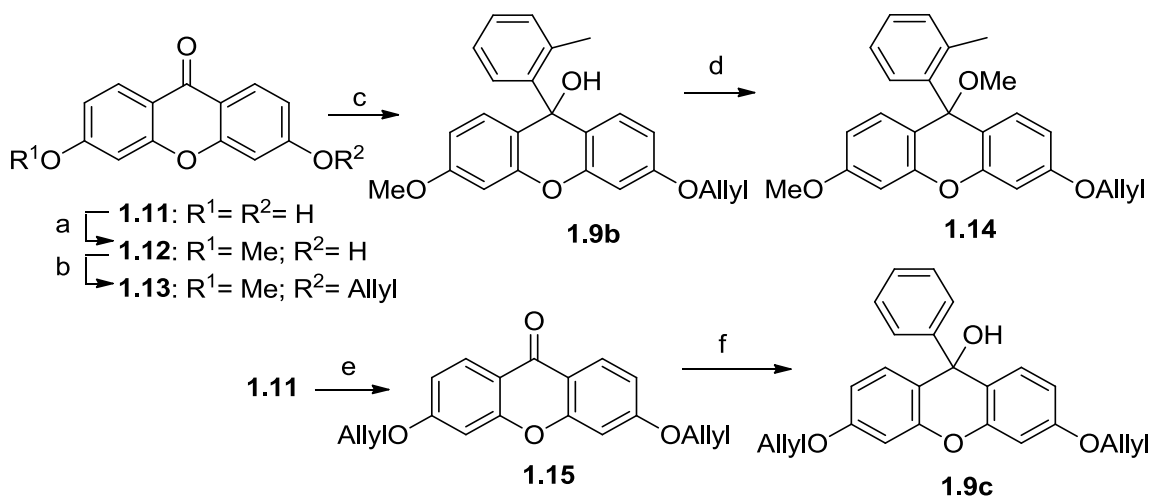
Scheme 1.1 Reversible Dehydroxylation of 9-Aryl-9-Xanthenols

We hypothesized that introduction of electron-donating substituents to the xanthenol structure would shift equivalence point to biologically relevant pH values. To test this hypothesis, we prepared 3,6-*bis*(allyloxy)-9-phenyl-9*H*-xanthen-9-ol (**1.9c**), which showed reversible formation of the cation absorbance (437 nm) at pH < 5. The equivalence point for the **1.9c** – **1.10c** equilibrium is achieved at $pC_{H^+}^{29} = 4.46 \pm 0.02$ (Figure 1.6). *o*-Substituents on the 9-aryl group are known to enhance the fluorescent quantum yield of xanthylium-based fluorophores.³⁰ Following this lead, we replaced the phenyl group in **1.9c** with the *o*-tolyl moiety (Scheme 1, **1.9b**). This substitution resulted in a shift of the indicator equivalence point to higher pH values (*vide infra*). This observation shows that pH response of the indicator can be adjusted not only by using electronic properties of substituent but also by adjusting the structural factors. We believe that increased steric congestion around tertiary alcohol facilitates the dehydroxylation reaction.³¹

1.2 RESULTS AND DISCUSSIONS

Synthesis of 9-aryl-9-xanthenols **1.9b**, **c** and **1.14** started from 3,6-dihydroxy-9*H*-xanthen-9-one (**1.11**), which was prepared by acid-catalyzed cyclization of commercially available 2,2',4,4'-tetra-hydroxybenzophenone.³² Successive alkylation of hydroxy groups in **1.11** produced 9-xanthenone **1.13**, which was treated with *o*-tolylmagnesium bromide to give target compound 3-allyloxy-6-methoxy-9-(2-methylphenyl)-9*H*-9-xanthenol (**1.9b**) in an excellent yield. *Bis*-alkylation of dihydroxyxanthe-9-one (**1.11**) with allyl bromide afforded 3,6-*bis*(allyloxy)-9*H*-xanthen-9-one (**1.15**). The latter was converted to xanthenol **1.9c** by reaction with phenylmagnesium bromide. 9-Aryl-9-

xanthenols can be smoothly etherified with various alcohols. Thus overnight refluxing of **1.9b** in MeOH in the presence of catalytic amount of AcOH quantitatively converts it to methyl ether **1.14**.



^{a)} *Reagents and conditions:* (a) CH_3I , NaOH , BTEAC, toluene, reflux, 6 h, 57%; (b) allyl bromide, K_2CO_3 , acetone, reflux, 1 h, 64%; (c) *o*-tolylmagnesium bromide, RT, THF, 2 h, 80%; THF, (d) AcOH (cat), MeOH, reflux, overnight, 95% ; (e) allyl bromide, Cs_2CO_3 , DMF, overnight, 94%; (f) PhMgBr , RT, 2 h, THF, 77%.

Scheme 1.2. Synthesis of 9-Aryl-9-Xanthenols **1.9b**, c and **1.14**^{a)}

The absorption spectra of **1.9b** in MeOH shows a band at 275 nm ($\log \epsilon = 4.14 \pm 0.016$). In 20% $\text{CH}_3\text{CN}/0.1\text{M HClO}_4$ solution, **1.9b** is completely converted into 9-aryl-9-xanthylium cation **1.10b** as is evident from a strong ($\log \epsilon = 4.77 \pm 0.002$) characteristic band at 437 nm. The formation of cation **1.10b** was also confirmed by HRMS.²⁴ Solutions containing only **1.9b** show no measurable fluorescence, while **1.10b** has intense emission band at 477 nm ($\Phi_{\text{Fl}} = 16\%$). This value is similar to that obtained from other substituted 9-phenylxanthylium ions.³³ The excitation spectrum maximum of **1.9b** corresponds to the major absorbance band at 437 nm (Figure 1.4). It should be noted that reports by Wan,

Yates and Boyd have consistently shown that the emission from parent 9-phenylxanthylium ion is quenched by water;³⁴ emission spectra of **1.10b** were recorded in aqueous solutions.

The dehydroxylation reaction of 9-aryl-9-xanthenols is strongly catalyzed by hydronium ion. At pH<3, equilibration between xanthenol **1.9b** and xanthylium cation **1.10b** occurs virtually instantly, with the lifetime of the leuco-form of the indicator (**1.9b**) being below 100 ms. Rates of the dehydroxylation of xanthenol **1.9b** were therefore studied in acetate, biphosphate ion, TRIS, and bicarbonate ion buffers. The ionic strength of these solutions was kept constant at 0.1 M by adding sodium perchlorate as required. Accurate rate measurements were conducted by stopped-flow kinetic spectroscopy at 25 ± 0.1 °C following the rise of absorbance at 437 nm. Rate measurements in buffered solutions were performed in a series of solutions of varying buffer concentration but constant buffer ratio. No appreciable buffer catalysis was observed. The observed rate constants at various acidities of the solution are shown as the rate profile in Figure 1.5.

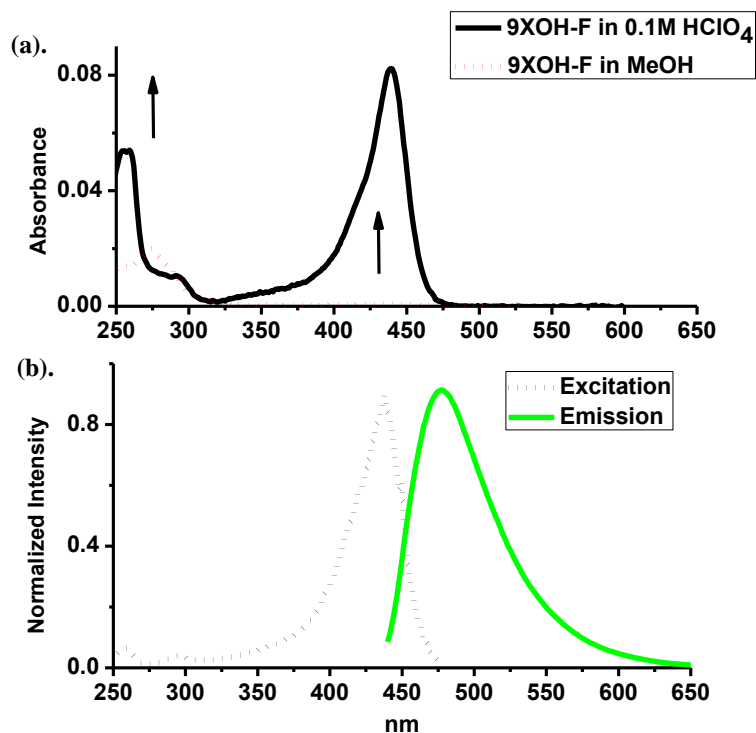


Figure 1.4.(a) Absorption spectra of 1.4 μM solution of **1.9b** in MeOH (dotted red line) and 0.1M HClO₄ (black line) (b) Excitation (dotted black line) and emission spectra (green line) of the latter solution.

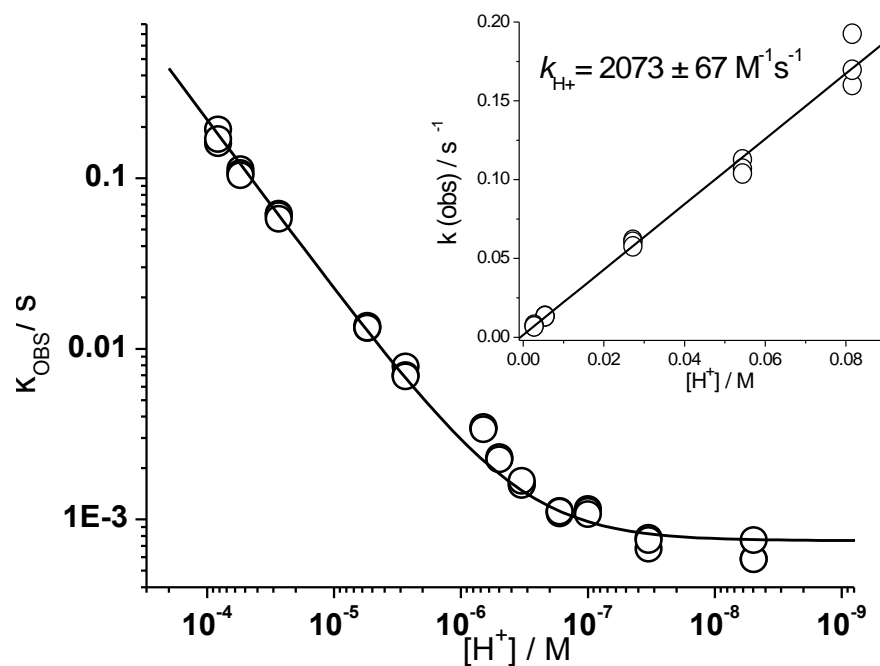


Figure 1.5: Rate profile for the dehydroxylation of xanthanol **1.9b** in aqueous solutions at 25°C. Inset illustrates the linear dependence of the rate on hydronium ion concentration.

The dehydroxylation of **1.9b** exhibits strong hydronium ion catalysis, $k_{H^+} = 2073 \pm 67 \text{ M}^{-1}\text{s}^{-1}$ (inset in Figure 1.5). It is important to note that this reaction is only catalyzed by the specific acid (H_3O^+) but not by general acids, as evidenced by the absence of buffer catalysis. At $\text{pH} > 7$ the rate of **1.9b** – **1.10b** equilibration is virtually independent from the acidity of the solution leveling off at ca. $7 \times 10^{-4} \text{ s}^{-1}$. Therefore accurate rate measurements in this range become progressively difficult due to the very weak signal of the cation **1.10b**. The plateau region has two possible interpretations, the first of which is protonation of the xanthenol **1.9b** by water, followed by the loss of H_2O . This mechanism can be ruled out on the basis of the non-existent general acid catalysis by much stronger acid (AcOH). The more probable mechanism of the uncatalyzed reaction is simple dissociation of **1.9b** to produce cation **1.10b** and hydroxide ion.

Above $\text{pH} 8$, xanthenol **1.9b** has no significant absorbance beyond 350 nm but at higher acidities the intensity of a band at 437 nm grows due to the increased fraction of xanthylium cation **1.10b** in the equilibrium. This growth saturates at $\text{pH} < 5$, indicating complete conversion of the substrate into a cationic form **1.10b**. The equivalence point, where $[\textbf{1.9b}] = [\textbf{1.10b}]$, was determined by spectrophotometric titration to be at $\text{pC}_{H^+} = 6.57 \pm 0.02$ (Figure 1.6).

Similar pH-dependent changes were observed in the emission spectrum (Figure 1.7). Above $\text{pH} = 8$ aqueous solutions of xanthenol **1.9b** show virtually no fluorescence. At lower pH values the emission band at 477 nm appears in the spectrum. The intensity of the band grows in parallel with the increased acidity of the media (Figure 1.7).

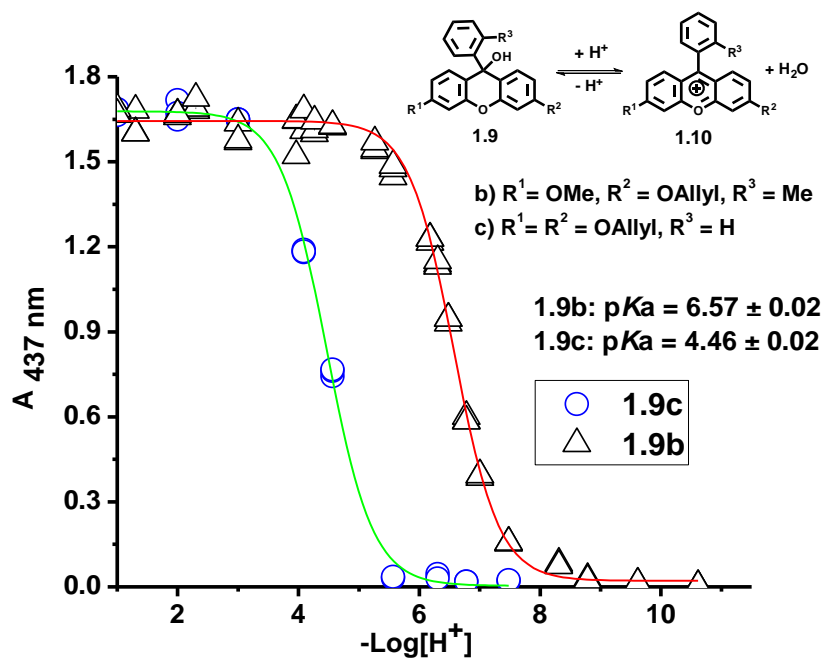


Figure 1.6: Absorbance of 27.5 μM aqueous solutions of xanthenols **1.9b** and **1.9c** at different acidities.

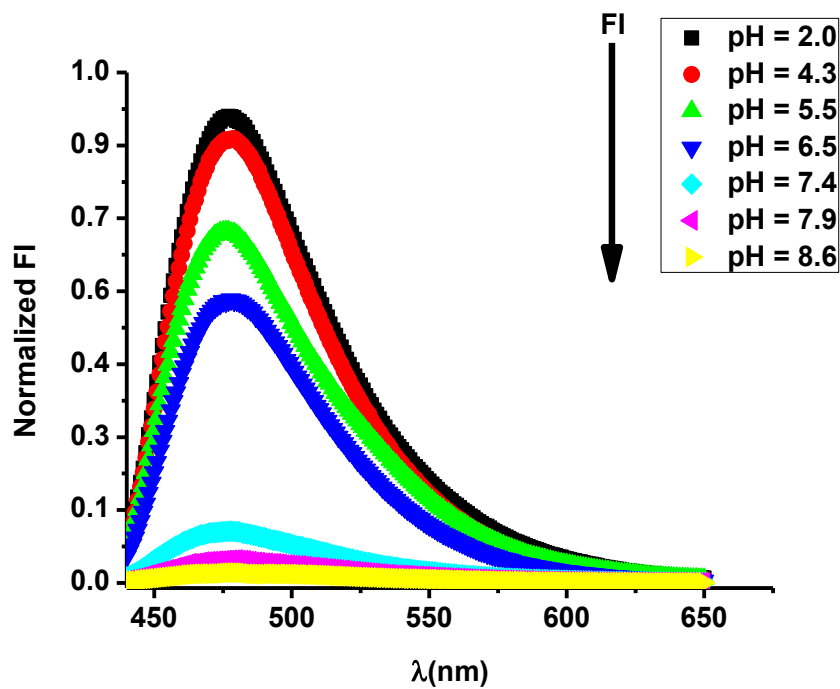


Figure 1.7: Normalized emission spectra of 1.4 μM aqueous solutions of xanthanol **1.9b** at different acidities.

The sensitivity of fluorescence indicators is defined by the difference of the emission intensities between the “on” and “off” states, i.e., the F_{\max}/F_{\min} ratio of the probe.^{25a,35} Xanthenol **1.9b** shows a remarkable 1330 fold increase in fluorescent intensity between acidic ($pC_{H^+}= 3$) solutions and basic ($pC_{H^+}= 10.4$) .

The normalized fluorescent emission intensity of **1.9b** at 477 nm shows a smooth sigmoidal dependence on the pC_{H^+} of the solution with the inflection point corresponding to $pC_{H^+}= 6.52\pm0.05$ (Figure 1.8). This value is in excellent agreement with the equivalence point acidity obtained by spectrophotometric titration. This observation further supports the assignment of photochromic changes in the aqueous solutions of xanthenol **1.9b** to the equilibration with xanthylium cation **1.10b**. It is interesting to note that at $pH<3$, fluorescence emission of xanthylium ion shows an additional small enhancement. This phenomenon is also observed for the parent xanthylium ion **1.10a** but at much higher acidities ($H_0 \geq -3$, ca 39 % H_2SO_4 ^{34a}). It was originally explained by the reduction of effective water concentration in concentrated acids. However, in our case this enhancement is observed in very dilute solutions.

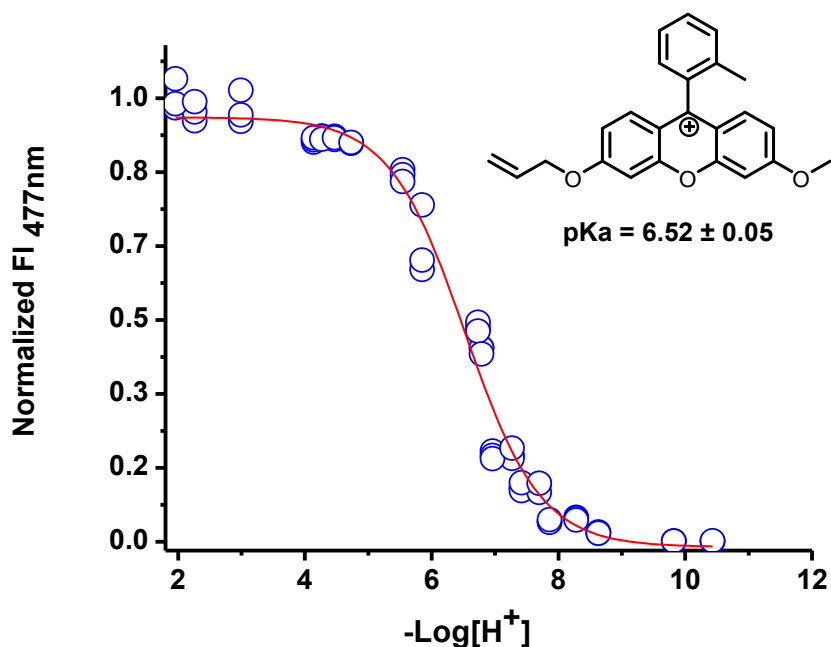


Figure 1.8: Normalized emission ($\lambda_{\text{exc}} = 437$ nm) of 1.4 μ M xanthenol **1.9b** aqueous solutions at various hydronium ion concentrations.

Given the near-neutral pK_a of xanthenol **1.9b**, we next explored the utility of this indicator to visualize acidic compartments in cultured cells.³⁶ To this end, NIH 3T3 fibroblast cells were grown on glass cover slips and incubated with a 5 μ M solution of xanthenol **1.9b** in serum-free Dulbecco's modified Eagle's medium (DMEM) for 1 hour at 37°C. Consistent with the lipophilic character, the dye readily crossed the plasma membrane and localized within cellular compartments, but showed negligible cytosolic fluorescence (Figure 1.9A). To test whether the subcellular staining pattern coincides with acidic compartments, we performed a colocalization experiment in which cells were transfected with a plasmid encoding the red fluorescent protein RFP fused to Lamp1 for lysosomal targeting. Much to our surprise, the compartments stained by xanthenol **1.9b** showed essentially no spatial correlation with lysosomes as illustrated by the red-green

false color overlay in Figure 1.9B (right panel). In contrast, an analogous experiment with mitochondrial targeted red fluorescent protein (mito-dsRed) revealed a high degree of colocalization (Figure 1.9C). At first, the observed mitochondrial localization of **1b** might appear unexpected; however, it is well known that cationic lipophilic compounds are sequestered by mitochondria, driven by the favorable mitochondrial membrane potential.³⁷ As key component of the electron transport chain, protons are extruded from the mitochondrial matrix into the intermembrane space (IMS), resulting in a slightly basic matrix pH of 7.7 and a more acidic IMS pH of 6.8.³⁸ According to Figure 5, such a pH gradient results in a more than 3-fold difference in emission intensity of **1b**, suggesting that the observed cellular staining predominantly originates from dye localized in the IMS.

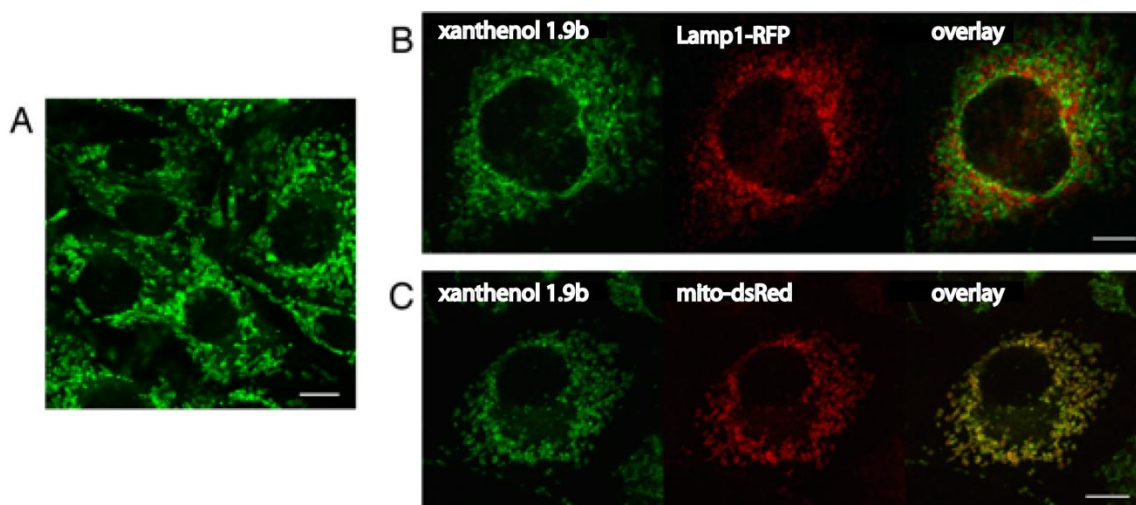


Figure 1.9: Fluorescence micrographs of NIH 3T3 fibroblast cells showing (A) the subcellular distribution of **1.9b** (A) and colocalization with red fluorescent protein markers (red) targeted to lysosomes (B) or mitochondria (C). Cells were transfected either with Lamp1-RFP (lysosomes) or mito-dsRed (mitochondria), followed by incubation with 5 μ M of **1.9b** for 1 h. Areas of colocalization appear yellow in the false color overlays. Scale bar: 10 μ m

1.3 CONCLUSIONS

9-Aryl-9*H*-xanthen-9-ols represent a promising platform for the development of selective fluorescent pH indicators. This platform has several beneficial features: the position of the equilibrium between the leuco- and the colored/fluorescent form of the dye in aqueous solutions depends only on the concentration of hydronium ions; fluorescent xanthylium ions have high absorption coefficient coupled with bright emission; 1300 fold difference in the emission intensity between two forms of the indicator produces high sensitivity; pH changes can be followed by both fluorescent and absorption measurements; the position of equivalence point can be adjusted by introducing appropriate substituents; alkoxy groups and /or 9-aryl moiety can be modified to improve lipophilicity, and binding specificity, or to serve as linkers to the substrates of interest. In addition, 3-Allyloxy-6-methoxy-9-(2-methylphenyl)-9*H*-xanthen-9-ol (**1.9b**) has equivalence point at pH = 6.5, which makes it suitable for numerous biochemical applications. Our current work is focused on exploring cell permeability and intracellular distribution of the dye, derivatization of the dye with substrate-specific binders, and investigation of the substituent effect on the xanthenol-xanthylium cation equilibrium.

1.4 EXPERIMENTAL SECTION

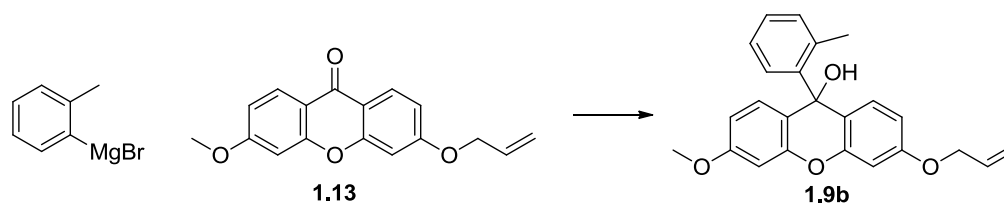
General Methods. All organic solvents were dried and freshly distilled before use. Flash chromatography was performed using 40-75 μ m silica gel. All NMR spectra were recorded in CDCl₃ using 400 MHz instrument (unless otherwise noted). Buffer solutions for kinetic experiments were prepared using literature pK_a values of the buffer acids and

activity coefficient recommended by Bates.³⁹ 2, 2', 4, 4'-Tetrahydroxybenzophenone, phenylmagnesium bromide and o-tolylmagnesium bromide were purchased from TCI America and used as received. 3-Hydroxy-6-methoxy-9*H*-xanthen-9-one (**1.12**)⁴⁰ was synthesized as reported. 3,6-Dihydroxy-9*H*-xanthen-9-one (**1.11**)⁴¹ and 3,6-*bis*(allyloxy)-9*H*-xanthen-9-one (**1.15**)⁴² were prepared using literature procedures.

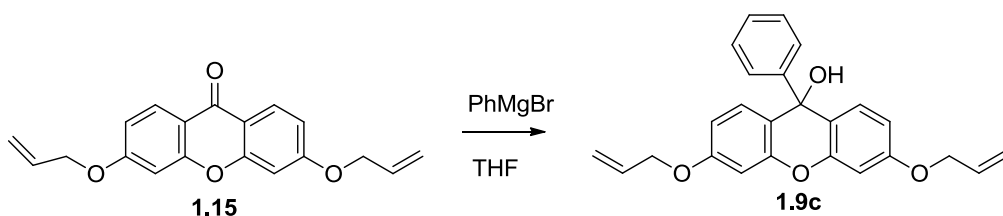
Kinetic experiments. The sample solutions were kept at 25 ± 0.1 ° C. Rate constants for the formation of xanthylium cation **1.10b** were obtained by least-square fitting of experimental data to a single exponential function using Origin 8.1 software. All measurements were done in triplicates. Rate measurements for specific acid catalysis were performed in three series of solutions with varying buffer concentrations but constant buffer ratio in each series.

Fluorescent measurements were conducted in aqueous buffers or acid solutions containing 20% of acetonitrile at 1.4 μ M concentration of **1.9b**. Fluorescent spectra were recorded using 437 nm excitation light source. Fluorimetric titration was conducted following 477 nm emission band. The excitation source and the detector slit were set to 1 nm and 5 nm respectively. The fluorescence quantum yield was determined using fluorescein in 0.1 N NaOH ($\Phi_{\text{Fl}} = 0.95$)⁴³ as the standard reference. Fluorescent spectra were recorded using spectrometer equipped with polarizer. The path length was 1 cm with a cell volume of 3 mL.

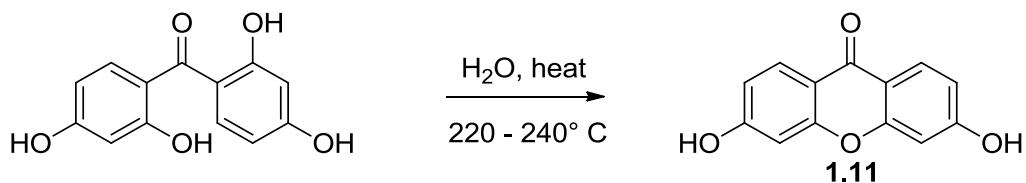
1.5 SYNTHETIC PROCEDURES



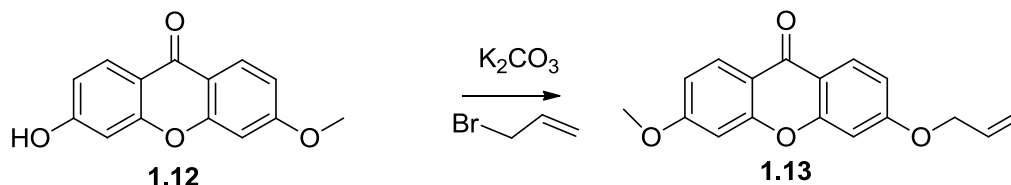
3-Allyloxy-6-methoxy-9-(o-tolyl)-9H-xanthen-9-ol (1.9b). A solution of 3-(allyloxy)-6-methoxy-9H-xanthen-9-one (**1.13**) (0.500 g, 1.771 mmol) in THF (5 mL) was added dropwise to a solution of o-tolylmagnesium bromide (2.125 mL, 2.125 mmol) in THF (4 mL) at 0 °C under nitrogen. The mixture was stirred for 2 h at rt, quenched with a saturated solution of ammonium chloride (2 mL), and the crude product extracted with ethyl acetate (3 x 25 mL). The solvent was evaporated and the product purified by silica gel chromatography eluting with 10 - 30 % EtOAc in hexanes (2% Et₃N) to afford 0.531 g (1.418 mmol, 80%) of 3-(allyloxy)-6-methoxy-9-(o-tolyl)-9H-xanthen-9-ol (**1.9b**) as a pale yellow oil. ¹H NMR: δ 8.30 (d, J = 7.6 Hz, 1H), 7.35 (t, J = 7.6 Hz, 1H), 7.22 (t, J = 8.8, 7.2 Hz, 1H), 6.98 (d, J = 7.2 Hz, 1H), 6.92 (d, J = 8.8 Hz, 2H), 6.64 (dd, J = 7.2, 2.4 Hz, 2H), 6.59 – 6.53 (m, 2H), 6.07 – 5.99 (m, 1H), 5.41 (d, J = 17.2 Hz, 1H-trans), 5.29 (d, J = 10.2 Hz, 2H), 4.51 (d, J = 5.2 Hz, 2H), 3.78 (s, 3H). ¹³C NMR: δ 160.34, 159.33, 151.23, 151.17, 144.19, 135.93, 132.06, 129.79, 127.79, 126.09, 125.44, 119.05, 118.87, 118.09, 111.79, 111.20, 101.40, 100.48, 69.66, 69.19, 55.61, 20.74. HRMS-ESI: calc. for [M - H]⁻; C₂₄H₂₁O₄: 373.1445, found 373.1439



3,6-bis(Allyloxy)-9-phenyl-9H-xanthen-9-ol (1.9c). A solution of 3,6-bis(allyloxy)-9H-xanthen-9-one (**1.15**) 0.279 g, 0.905 mmol) in THF (5 mL) was added dropwise to a solution of phenylmagnesium bromide (1.1 mL, 1.1mmol) in THF (4 mL) at 0 °C under nitrogen. The mixture was stirred for 2 h at rt, quenched with a saturated solution of ammonium chloride (2 mL), and the crude product extracted with ethyl acetate (3 x 25 mL). The solvent was evaporated and the product purified by silica gel chromatography eluting with 10 - 30 % EtOAc in hexanes (2% Et₃N) to afford 0.271 g (0.701 mmol, 77%) of 3,6-bis(allyloxy)-9-phenyl-9H-xanthen-9-ol (**1.9c**) as a pale yellow oil. ¹H NMR: δ 7.39 (d, J = 7.2 Hz, 2 H), 7.27 (t, J = 7.2, 8.4 Hz, 2 H), 7.19 – 7.15 (m, 4 H), 6.66 – 6.62 (m, 4 H), 6.09 – 5.99 (m, 2 H), 5.41 (dd, J = 17.2 , 1.6 Hz, 2 H), 5.29 (d, J = 10.4 Hz, 2 H), 4.53 (d, J = 5.2 Hz, 4 H), 2.58 (br, 1 H). ¹³C NMR: δ 159.24, 150.85, 148.45, 130.37, 128.06, 126.77, 126.65, 118.07, 111.98, 101.44, 70.44, 69.22. EI-MS m/z: 386(M⁺, 4), 370 (11), 369 (M⁺ - OH, 38), 328 (9), 310 (22), 309 (100), 268 (12), 240 (7), 202 (5), 105 (11). HRMS-ESI: calc. for [M - H]⁻; C₂₅H₂₁O₄: 385.1445, found 385.1449.

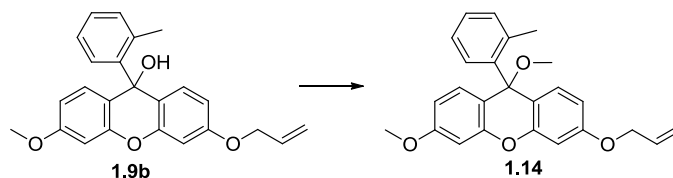


3,6-Dihydroxy-9H-xanthen-9-one (1.11).⁴¹ A suspension of 2,2',4,4'-tetrahydroxybenzophenone (7.0 g, 28.4 mmol) in 30 mL water and 10 mL of acetone was sealed in a pressure vessel and heated at 220 - 230° C in an oven for 4 h. Upon cooling, the resulting solid was filtered out and washed with 100 mL of warm water at about 60° C to afford 6.4 g (28.0 mmol, 99 %) of 3,6-dihydroxy-9H-xanthen-9-one (**1.11**) as colorless needle like solid which did not melt below 350° C. The spectral data are consistent with literature values.⁴¹ ¹H NMR (DMSO-d₆): δ 10.83 (s, 2H -OH), 7.98 (d, J = 8.8 Hz, 2H), 6.86-(dd, J = 8.8, 2.0 Hz, 2H), 6.82 (d, J = 2.0 Hz, 2H). ¹³C NMR (DMSO-d₆): 174.84, 164.29, 158.40, 128.70, 114.93, 114.59, 103.02. DIP-EI/MS: M⁺ 228.

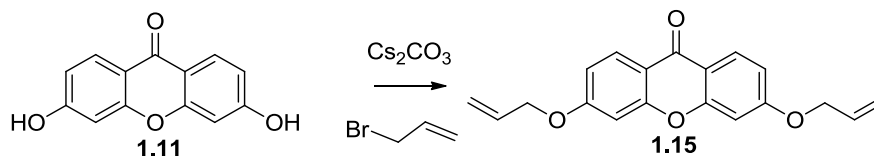


3-(Allyloxy)-6-methoxy-9H-xanthen-9-one (1.13). Powered K₂CO₃ (0.519 g, 3.76 mmol) was added to a solution of 3-hydroxy-6-methoxy-9H-xanthen-9-one (**1.12**) (0.70 g, 2.89 mmol) in acetone (25.0 mL) followed by allyl bromide (0.375 mL, 4.33 mmol). The mixture was refluxed for 1 h and the solvent removed in vacuum. Purification by silica gel chromatography (20% EtOAc in hexanes) afforded 0.520 g (1.842 mmol, 63.7%) of 3-(allyloxy)-6-methoxy-9H-xanthen-9-one(**1.13**) as white fluffy solid. m.p 141-143°C. ¹H NMR: δ 8.24 (d, J = 8.8 Hz, 2H), 6.95 (td, J = 8.8 Hz, 2.4 Hz, 2H), 6.87 (t, J = 2.4 Hz, 2H), 6.08 (m, 1H), 5.47 (d, J = 17.6 Hz, 1H-trans), 5.36 (d, J = 9.6 Hz, 1H-cis), 4.66 (d, J = 5.2 Hz, 2H), 3.93 (s, 3H). ¹³C NMR: δ 175.73, 164.94, 163.87, 158.29, 158.19, 132.45,

128.45, 118.68, 116.14, 113.51, 113.09, 101.44, 100.53, 69.51, 56.02. GC/MS-EI M^+ 282, HRMS-ESI: calc. for $[M+H]^+$ $C_{17}H_{15}O_4$: 283.0965, found 283.0969



3-(Allyloxy)-6,9-dimethoxy-9-(*o*-tolyl)-9*H*-xanthene (1.14). AcOH (5 drops) was added to a solution of 3-allyloxy-6-methoxy-9-(*o*-tolyl)-9*H*-xanthen-9-ol **1.9b** (0.230 g, 0.614 mmol) in MeOH (10 mL) and the mixture refluxed overnight. K_2CO_3 (about 100 mg) was added to a reaction mixture and the solvent evaporated. The resulting residue was adsorbed on silica gel and purified by a short layer of silica gel eluting with 5% EtOAc in hexanes to afford 0.226 g (0.582 mmol, 95%) of 3-(allyloxy)-6,9-dimethoxy-9-(*o*-tolyl)-9*H*-xanthene **1.14** as a pale yellow oil. 1H NMR: δ 8.32 (d, J = 8 Hz, 1.3H), 7.34 (t, J = 7.6 Hz, 1.3H), 7.19 (t, J = 7.2 Hz, 1.3H), 6.96 (d, J = 7.6 Hz, 1.3H), 6.90 (d, J = 8.8 Hz, 2.6 H), 6.66 (m, 2.6 H), 6.60 (t, J = 8.4, 8.0 Hz, 2.6 H), 6.07 (m, 0.8 H), 5.44 (d, J = 17.2 Hz, 0.8 H), 5.31 (d, J = 10.4 Hz, 0.8 H), 4.55 (d, J = 5.2 Hz, 2.0 H), 3.83 (s, 5.50 H), 2.76 (s, 5.5 H). ^{13}C NMR: δ 160.33, 159.32, 153.36, 153.30, 145.23, 130.44, 127.45, 126.02, 125.30, 118.12, 111.30, 100.53, 99.63, 95.66, 74.46, 69.18, 55.60, 49.46, 20.67. HRMS-ESI: calc. for $[M+Na]^+$; $C_{25}H_{24}NaO_4$: 411.1567, found 411.1561.



3,6-Diallyloxy-9H-xanthen-9-one (1.15). Allyl bromide (0.393 mL, 4.54 mmol) was added to a solution of Cs_2CO_3 (2.219 g, 6.81 mmol) and 3,6-dihydroxy-9H-xanthen-9-one **1.11** (0.520g, 2.28 mmol) in DMF (10 mL). The reaction mixture was stirred overnight at rt and quenched with 5% HCl solution. The product was extracted with ether (3 x 50 mL), washed with water (3 x 25 mL), brine (20 mL), and dried over MgSO_4 to afford 0.66 g (2.14 mmol, 94%) of 3,6-bis(allyloxy)-9H-xanthen-9-one **1.15** as colorless crystals. m.p. 137-138 °C. Lit.⁴² 140° C. ^1H NMR: δ 8.23 (d, J = 8.8 Hz, 2H), 6.95 (dd, J = 8.8 Hz, 2.4 Hz, 2H), 6.86 (d, J = 2.0 Hz, 2H), 6.12 -6.04 (m, 2H), 5.47 (dd, J 17.2 Hz, 1.2 Hz, 2H), 5.36 (dd, J = 10.8 Hz, 1.2 Hz, 2H), 4.44 (d, J = 5.2 Hz, 4H). ^{13}C NMR δ 175.75, 163.85, 158.17, 132.43, 128.44, 118.67, 116.21, 113.51, 101.41, 69.49. GC/MS EI-MS m/z : 309, (15), 308 (M^+ , 65), 307 (13), 293 (14), 281 (11), 280 (31), 279 (18), 268 (19), 267 (100), 253 (22), 239 (39), 226 (15), 211 (10), 63 (14). HRMS-EI: calc. for $[\text{M}]^+$; calcd. for $\text{C}_{19}\text{H}_{14}\text{O}_4$ 308.1049, found 308.1046.

It should be noted that the ^1H NMR spectrum of this compound as well as many other structurally related compounds are unusual due to the existence of two rotamers that are not equilibrated on the NMR time scale.⁴⁴

Table 1.1: Spectrophotometric titration of **1.9b**

[H₃O⁺]/M	Absorbance at 437 nm		
0.100	1.679	1.687	1.667
0.050	1.682	1.602	1.601
0.010	1.659	1.668	1.667
0.005	1.681	1.689	1.724
0.001	1.571	1.637	1.577
1.09E-04	1.522	1.646	1.648
8.16E-05	1.61	1.66	1.683
5.44E-05	1.599	1.613	1.644
2.72E-05	1.623	1.625	1.628
5.44E-06	1.539	1.545	1.57
2.72E-06	1.447	1.476	1.487
6.64E-07	1.231	1.216	1.23
4.98E-07	1.139	1.132	1.151
3.32E-07	0.948	0.931	0.948
1.66E-07	0.587	0.602	0.585
9.96E-08	0.394	0.386	0.395
3.32E-08	0.157	0.154	0.157
4.92E-09	0.0778	0.0839	0.0747
1.64E-09	0.0293	0.026	0.0234
2.42E-10	0.0102	0.0131	0.0124
2.42E-11	0.00437	0.00424	0.00428

Table 1.2. Observed rates of formation of **1.10b**

$[\text{H}_3\text{O}^+](\text{M})$	$k_{\text{obs}}(\text{s}^{-1})$		
8.16E-05	0.19257	0.16	0.16962
5.44E-05	0.1128	0.10694	0.10385
2.72E-05	0.06177	0.06046	0.05765
5.44E-06	0.01329	0.01319	0.01364
2.72E-06	0.00786	0.00701	0.00688
6.64E-07	0.00342	0.00348	0.00337
4.98E-07	0.00226	0.00231	0.00225
3.32E-07	0.0016	0.00159	0.00168
1.66E-07	0.00108	0.00112	0.00111
9.96E-08	0.00115	0.00112	0.00107
3.32E-08	7.85E-4	6.71E-4	7.54E-4
4.92E-09	1.31E-3	1.41E-3	1.26E-3
1.64E-09	5.01E-4	3.57E-4	4.03E-4
2.42E-10	1.63E-4	2.21E-4	2.02E-4
2.42E-11	7.40E-5	7.75E-5	7.93E-5

Table 1.3.Fluorimetric titration of **1.9b**

[H ₃ O ⁺]/M	Emission Intensity at 477 nm		
0.0110	1.1739E6	1.25239E6	1.18546E6
0.0055	1.13924E6	1.16333E6	1.19046E6
0.0010	1.13727E6	1.22157E6	1.15483E6
7.24E-05	1.08131E6	1.08762E6	1.09299E6
5.37E-05	1.09779E6	1.0959E6	1.08928E6
3.39E-05	1.09699E6	1.08839E6	1.09339E6
1.86E-05	1.0795E6	1.07791E6	1.08016E6
2.88E-06	1.00653E6	992781	974744
1.41E-06	736741	762140	911389
1.86E-07	577759	593638	569816
1.62E-07	524653	523889	507737
1.1E-07	244963	233543	224643
5.37E-08	233068	225600	252962
3.8E-08	145512	138031	159371
2E-08	156907	133025	157652
1.38E-08	54326	52070	59378
5.25E-09	65370	62918	58804
2.34E-09	26141	22469	23233
1.51E-10	1208	1226	1295
3.72E-11	982	443	1112

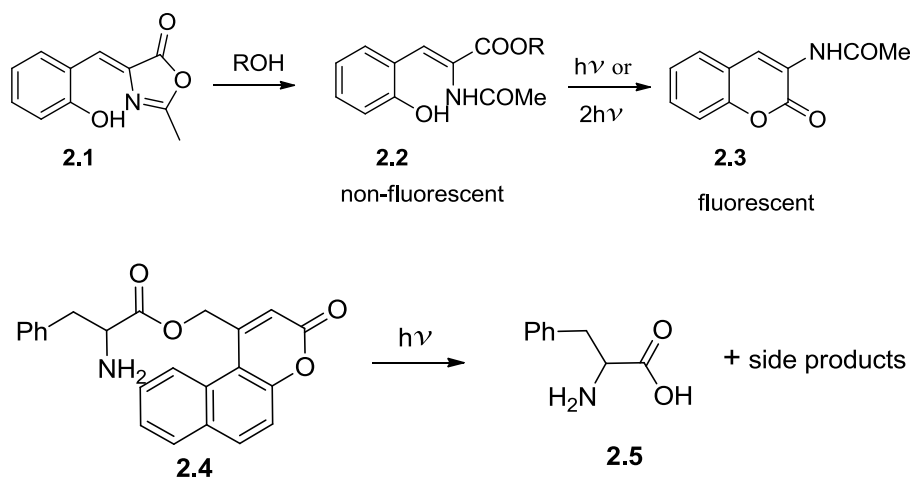
CHAPTER 2

TOWARD THE DEVELOPMENT OF FLUORGENIC PROTECTING GROUP

2.1 INTRODUCTION

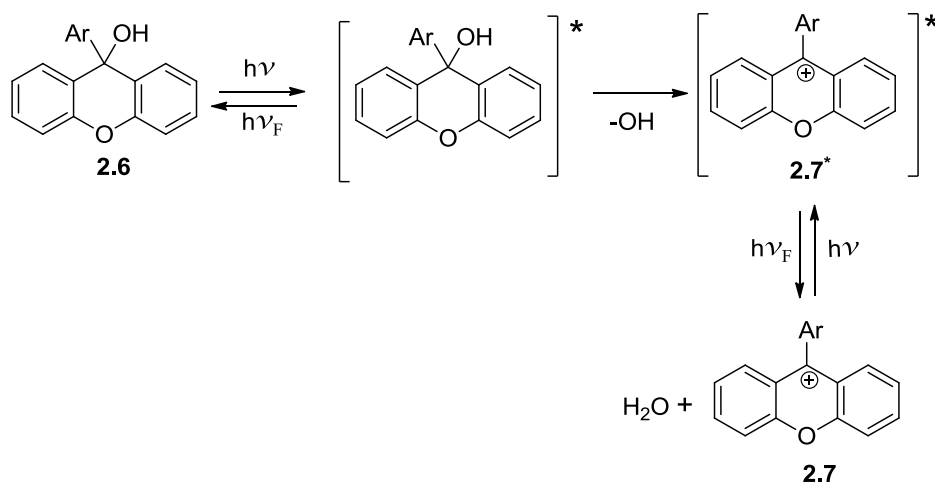
Photo-deprotection is the ideal form of precise delivery as it enables the spatial and temporal control of substrate release, as well as “reagentless” deprotection in a noninvasive manner.⁴⁵ Much attention has been paid to the utility of photolabile protecting groups (PPGs) which have recently found varied and numerous applications including organic synthesis,^{45c,d,46} biochemistry,^{45f,g,47} DNA synthesis, microarray fabrication and photolithography.⁴⁸

Despite the wide range of applications, the utility of PPGs is hampered by the need to characterize uncaging rate in a biological system, where analyte concentration becomes increasingly important.⁴⁹ It is therefore necessary to design methods to quantify sample delivery in biological systems. The use of fluorogenic protecting groups would be a good approach. While there are many reports of fluorescent (fluorescent precursor) photolabile protecting groups,⁵⁰ reports on fluorogenic (non-fluorescent precursor) protecting groups (FPPG) are somewhat limited. The most recent accounts of fluorogenic protecting groups include coumarin based methyloxazolones (**2.1**) by Gagey *et al*⁴⁹ and oxobenzo[f]benzopyrans (**2.4**) by Piloto⁵¹ (Scheme 2.1)



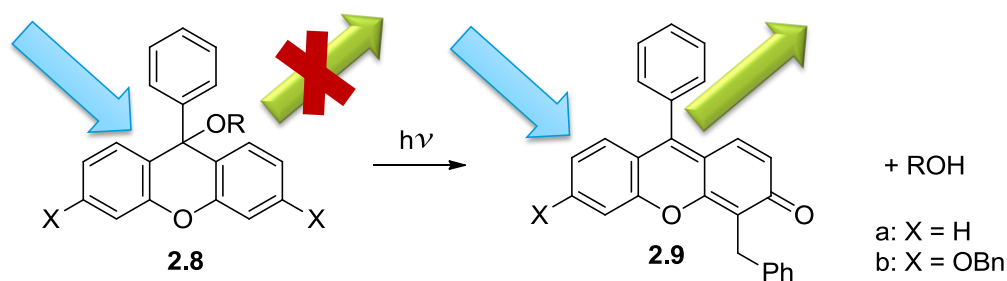
Scheme 2.1 Examples of Fluorogenic Protecting Groups

Our initial approach in the development of FPPG was based on the photodehydroxylation reaction of 9-substituted-9-xanthenols.⁵² Work by Wan *et al*^{52d} shows that in neutral aqueous solutions, 9-aryl-9-xanthenols such as **2.6** (Scheme 2.2) are easily dehydroxylated upon irradiation to give fluorescent 9-aryl-xanthylium cation **2.7**



Scheme 2.2 Photodehydroxylation of 9-aryl-9-xanthenol

Based on this principle, we hypothesized that caged fluorescein derivative such as **2.8** can be developed as fluorogenic protecting group for the protection of hydroxyl functionality (Scheme 2.3). Using non-fluorescent 3,6-*bis*(benzyloxy)-9-phenyl-9-xanthenol **2.8b** as a model, we have shown that caged ethers can be rapidly released after brief irradiation (2 min) with 254 nm or 300 nm lamps to give fluorescent fluorescein derivative **2.9b** with appreciable quantum yields.

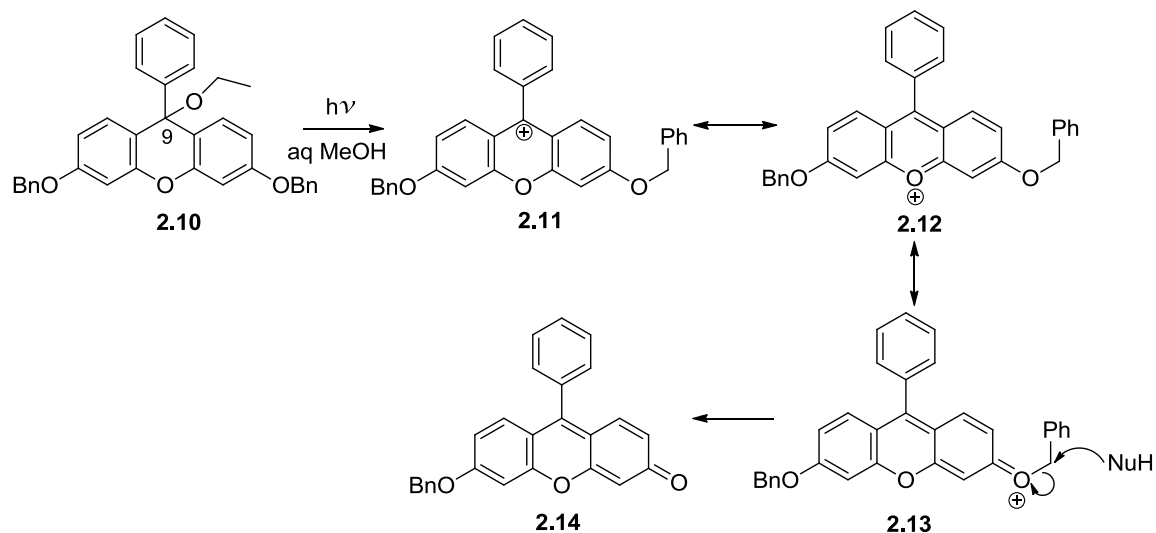


Scheme 2.3 Photodeprotection with fluorescent reporting

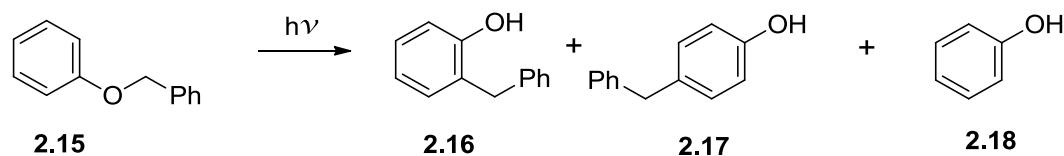
2.2 RESULTS AND DISCUSSION

We had envisioned that irradiation of 3,6-*bis*(benzyloxy)-9-ethoxy-9-phenyl-9*H*-xanthene **2.10** will generate carbocation intermediate **2.11** which can tautomerize to the oxonium ion **2.12** and the quinonoid form **2.13**. Intermediate **2.13** should then undergo nucleophilic attack by the solvent at the activated benzylic position to liberate fluorescent **2.14** (Scheme 2.4). Considering that the ground state reactivity of xanthylium cations (k_{solv}) such as **2.7** is between 11 to 34 s⁻¹, at 25 °C,⁵³ we expected that introduction of electron donating substituents on the xanthene chromophore as in **2.10** would lead to more delocalization and greater stability of cation **2.11**. However, a potential drawback of this approach is the likely competitive classical photo-Claisen rearrangement (Scheme

2.5) typical of allyl and benzyl phenyl ethers.⁵⁴ Upon irradiation of benzyl phenyl ethers mainly 2- and 4-benzilylated products are obtained for ethers lacking *p*- and *o*-substituents.^{54a,55} It is therefore possible that photo-Claisen rearrangement might precede photoheterolysis of the 9-C-O bond in **2.10**.



Scheme 2.4 Envisioned photodehydroxylation of **2.11** to fluorescein derivative **2.14**



Scheme 2.5 Typical photo-Claisen rearrangement of aromatic ether

The synthetic path for caged fluorescein derivative **2.10** is as shown (Scheme 2.6). 3,6-Dihydroxy-9*H*-xanthen-9-one **2.20** was prepared from commercially available 2, 2', 4, 4' -Tetrahydroxybenzophenone **2.19** as previously reported.⁵⁶ Compound **2.20** was protected with benzyl bromide under Williamson ether synthesis conditions to afford

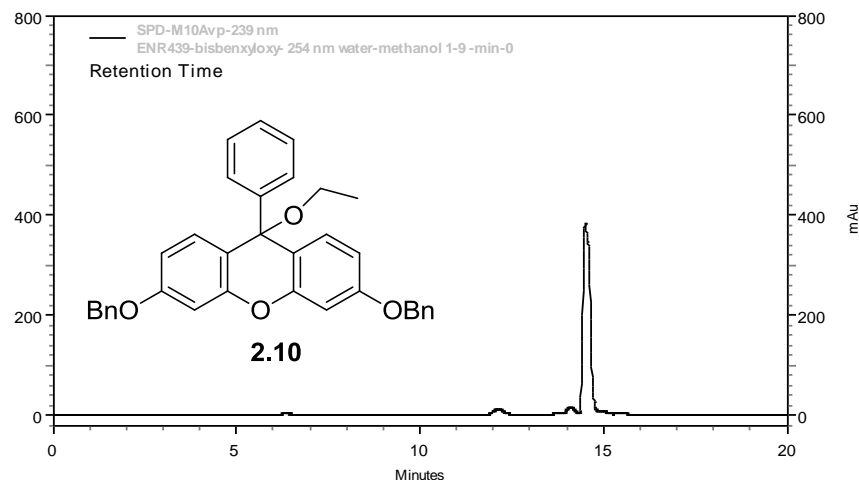


Figure 2.1: HPLC trace of **2.10** before photolysis

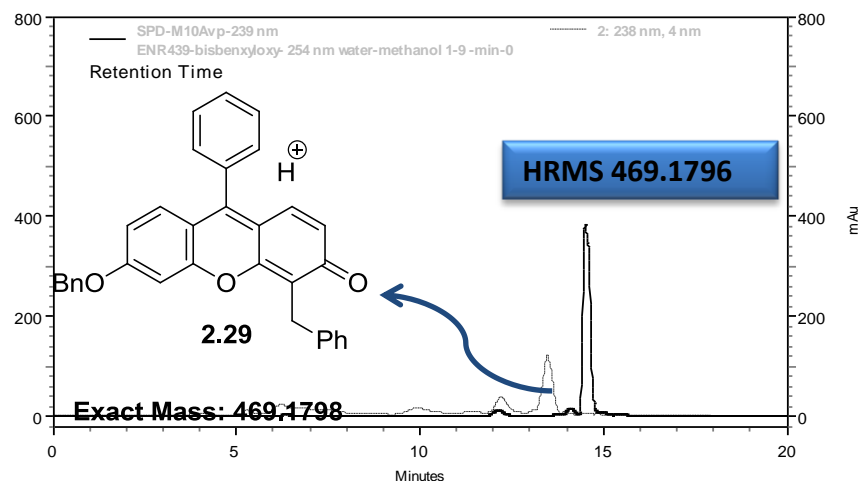
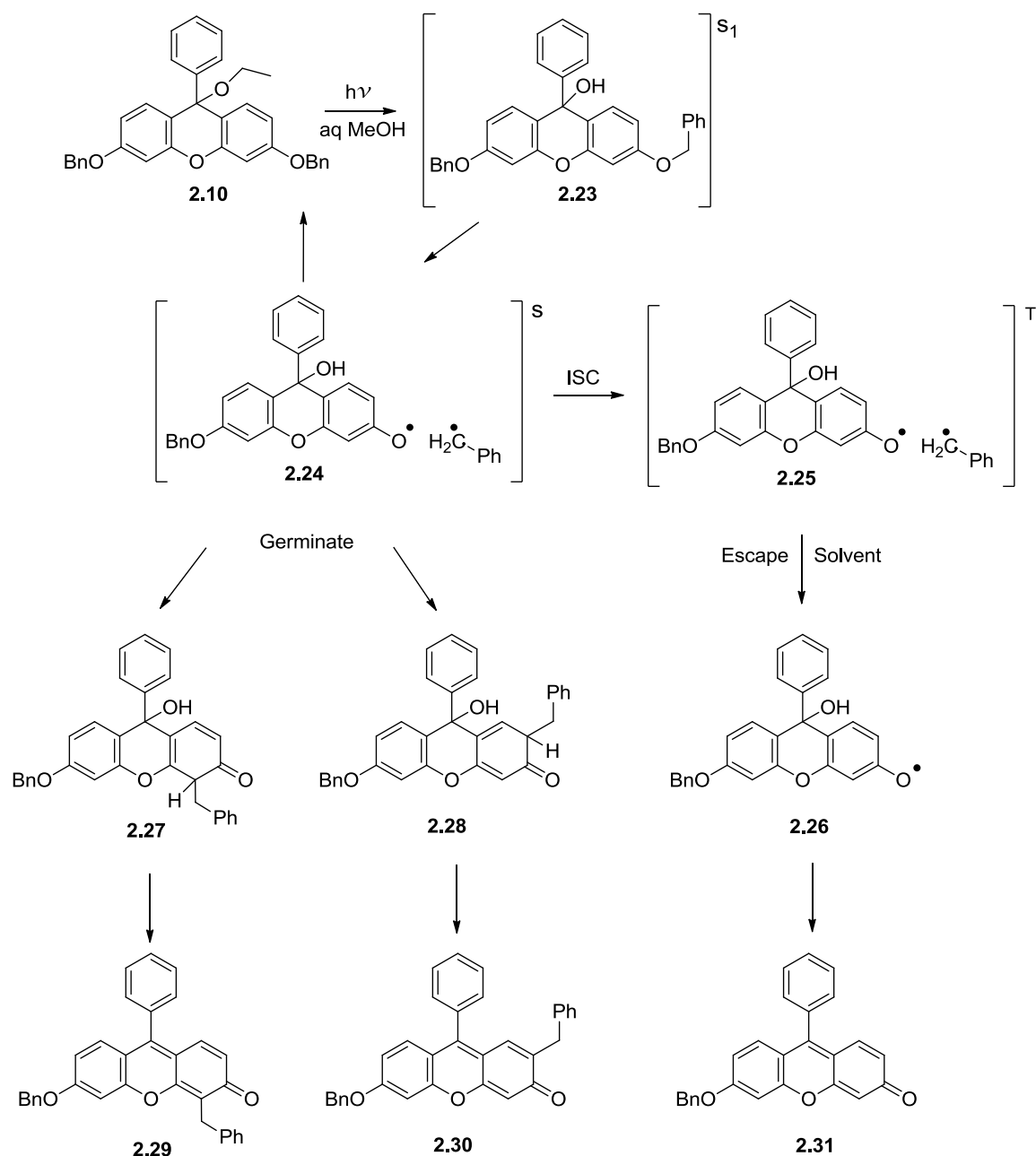


Figure 2.2: HPLC trace of compound **2.10** after 2 min irradiation (254 nm 90% MeOH aq) overlaid with Figure 2.1

Based on the photo-Claisen rearrangement mechanism suggested by Benn and co-workers,^{54g} after excitation, C-O bond homolysis in the singlet excited state results (Scheme 2.7). Recombination of the singlet radical pair would revert to the starting material **2.10** or undergo intersystem crossing to the triplet radical pair **2.25** which eventually escapes from the solvent cage and tautomerizes to give the escape product **2.31**.



Scheme 2.7: proposed mechanism of photodehydroxylation of **2.10** to the fluorescent derivatives

The excited singlet state radical can also react to give in this case, a mixture of *ortho*-substituted products **2.29** and **2.30**. It is possible that the dehydration from **2.24** via intermediate enones **2.27** and **2.28** to give mainly **2.29** and **2.30** may involve a concomitant cleavage of the C-O bond. The out of cage product **2.31** was not observed.

The products obtained after photolysis were analyzed by GC/MS and HRMS and show an m/z of 468 and 469.1796 respectively which corresponds to either of the isomeric structures **2.29** and **2.30**. As expected, uncaging resulted in a drastic change in absorbance with a fluorescent quantum yield ($\Phi_F = 0.02$, Figure 2.4). The highlights of this reaction are that the caged compound is essentially non-fluorescent at the excitation wavelength and the reaction seemed complete in 2 min (Figure 2.2). This could therefore be suitable for use in practical applications requiring fast release after brief photolysis with fluorescent reporting.

From the mechanistic point of view, formation of the aforementioned products may also involve the possibility of intra/intermolecular migration of the benzyl cation in what appears to be an unprecedented electrophilic aromatic substitution (photochemical Friedel–Crafts) reaction or a sigmatropic shift of sort. However, the likelihood of such migrations is questionable, given the planarity of intermediate oxonium ion **2.32** and the distance (d) between the π -electrons of the fluorescein ring and the electrophilic benzyl carbon (Figure 2.3).

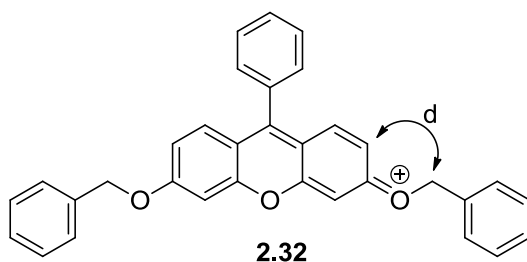


Figure 2.3: Possible Intermediate oxonium ion **2.32**

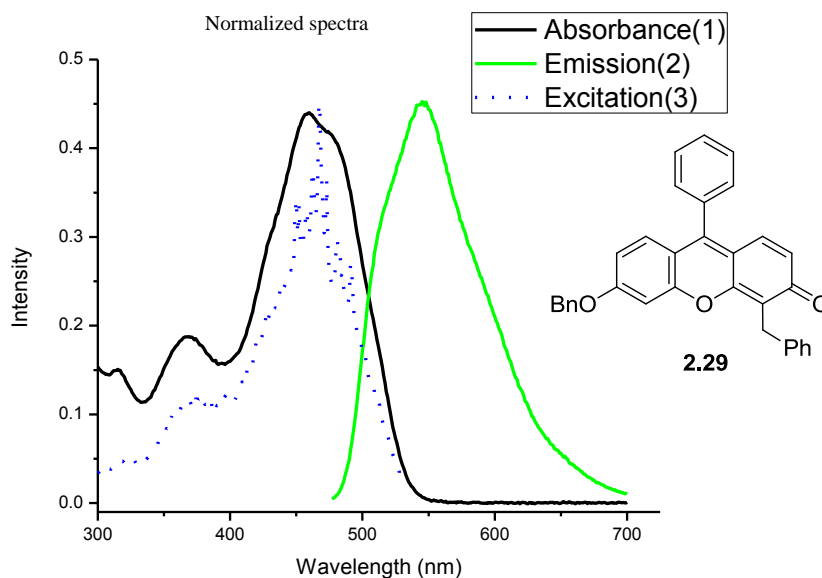
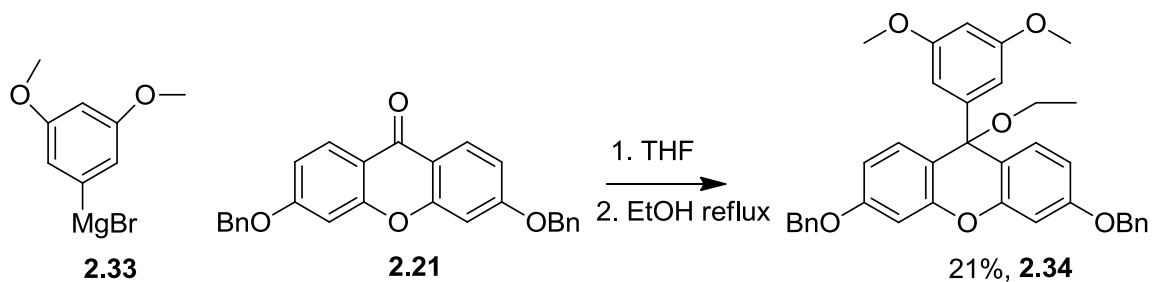


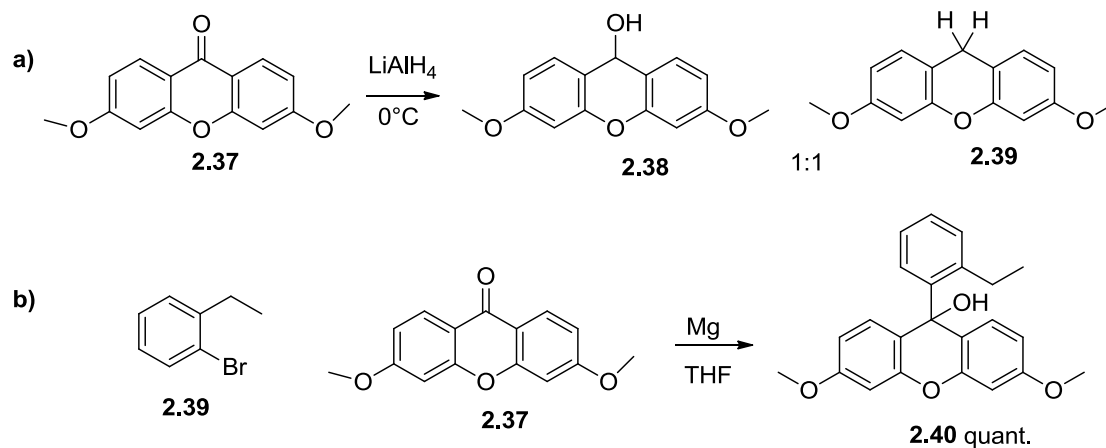
Figure 2.4: Normalized spectra of product mixture **2.29** and **2.30**. Absorbance (3.3×10^{-5} M, solid black line), Emission (8.5×10^{-6} M, green line) and Excitation (dash blue line) in MeOH

To to determine the scope and limitations of this reaction, compound **2.34** an analogue of **2.10** with electron-donating substituents was prepared (Scheme 2.8). Interestingly, photolysis of compound **2.34** in aqueous methanol gave similar results (Scheme 2.9) with a fluorescent quantum yield ($\Phi_{\text{Fl}} = 0.02$).



Scheme 2.8: Synthesis of 3,5-dimethoxyphenyl **2.34**.

for **2.10** and **2.34** involves a photo-Claisen rearrangement which is limited to benzylic and allylic carbons. Although the resulting photochemical products were not analyzed, we believe that **2.40** is slowly dehydroxylated in aqueous methanol to the xanthylium cation. Such dehydroxylation reactions can also be effected photochemically.^{52a,52e}



Scheme 2.10: Synthesis of **2.37** and **2.40**

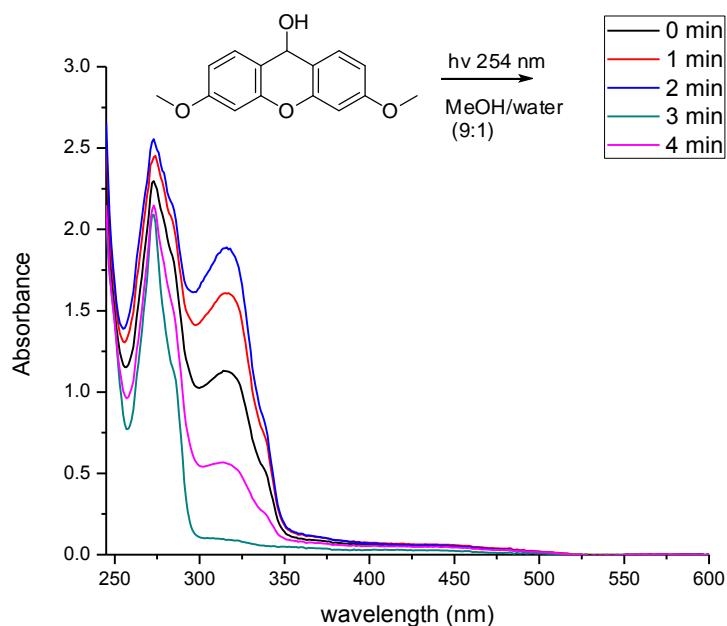


Figure 2.6: Photolysis of 3,6-dimethoxy-9H-xanthen-9-ol **2.38** in aq MeOH

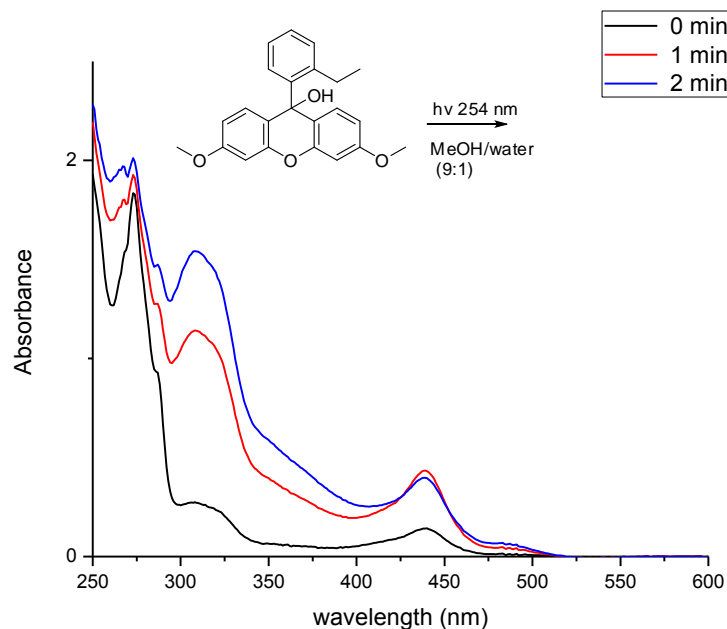
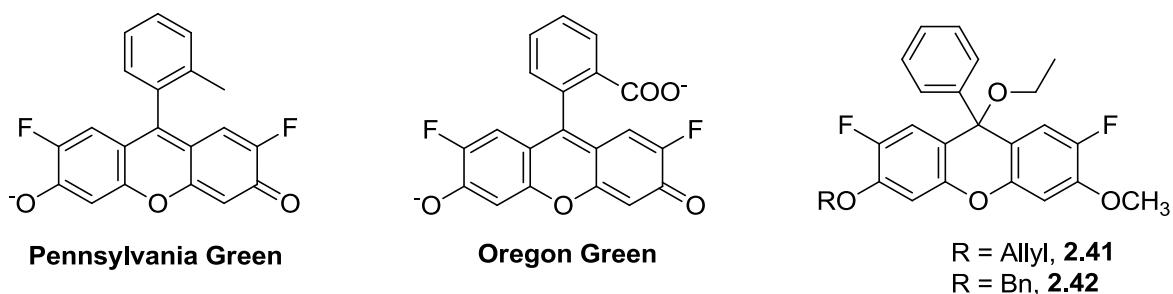


Figure 2.7: Photolysis of **2.40** in aq MeOH

After characterization of the products from irradiation of **2.10** and **2.34** we believed that this reaction gives rise to a mixture of regioisomers based on the NMR of the single band isolated from prep-TLC.

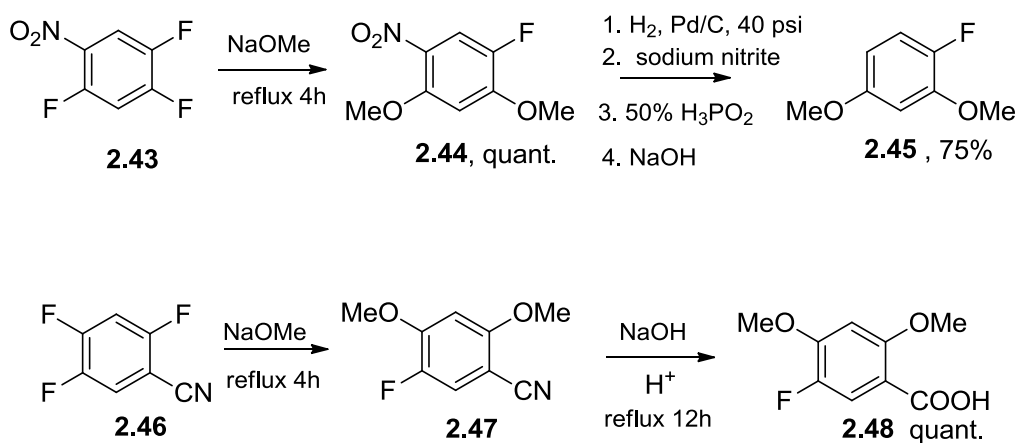
To better understand the nature of this reaction and to simplify product characterization, we envision that incorporating two fluorine atoms at the 2' and 7' positions in **2.41** and **2.42** as in Oregon and Pennsylvania Green⁵⁷ should reduce the number of possible isomeric products. Substitution of fluorine at the 2' and 7' has no effect on absorbance and emission properties⁵⁸ and incorporation of the ethyl group should improve the fluorescence yield.³⁰



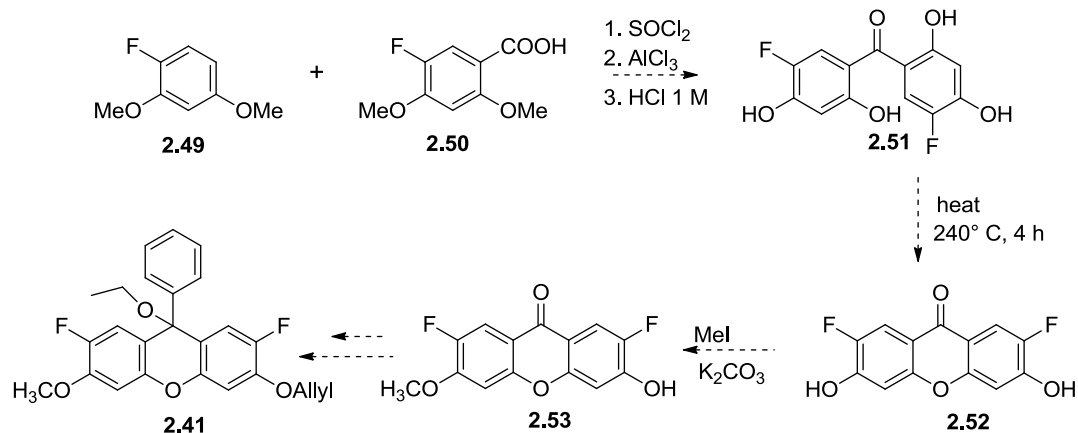
Scheme 2.11: Oregon and Pennsylvania Green, **2.41** and **2.42**

2.3 CONCLUSIONS AND FUTURE DIRECTIONS

The initial steps in the synthesis of **2.41** and **2.42** were carried out (Scheme 2.12). The xanthene moiety of **2.41** and **2.42** can be synthesized from the xanthone precursor **2.51** (Scheme 2.13). Friedel-Crafts acylation of **2.49** with the acid chloride of **2.50** would afford crucial difluoro-tetra-ol **2.51**. Compound **2.51** can be heated in a sealed-tube to furnish 2,7-difluoro-3,6-dihydroxy-9*H*-xanthen-9-one **2.52**. The phenolic moieties of **2.52** can be sequentially protected as allyloxy/methoxy and benzyloxy/methoxy ethers **2.41** and **2.42** respectively.

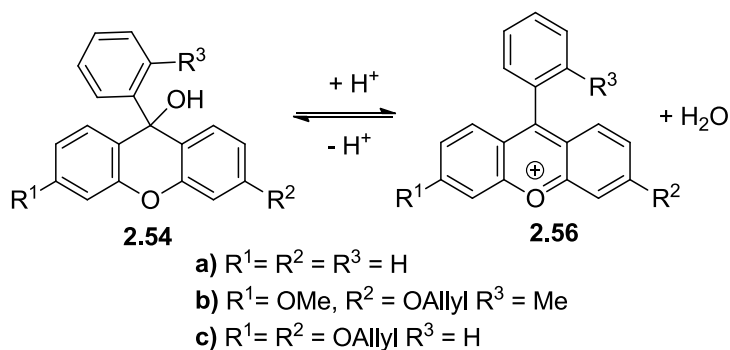


Scheme 2.12: Synthesis of fluorinated precursors



Scheme 2.13: Proposed synthesis of Pennsylvania Green analogue **2.41**.

Unfortunately, during the course of our research, we found out that compounds such as **2.10** and **2.34** are not stable in aqueous solutions and they undergo facile specific acid promoted dehydroxylation as shown below (Scheme 2.14). However, we believe that substitution of electron withdrawing fluorine atoms on the xanthene core as in **2.41** and **2.42** would reduce the pK_a of the corresponding difluorinated xanthenols making them less basic. This approach would hopefully permit the use of **2.41** and **2.42** as fluorogenic protecting group.



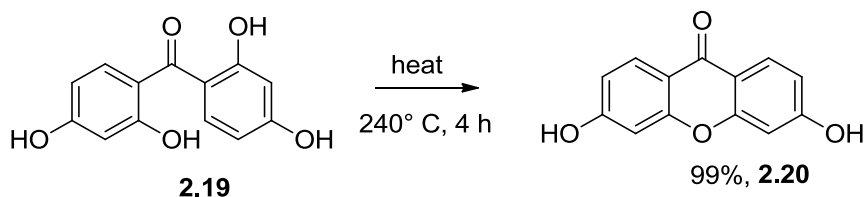
Scheme 2.14 Specific acid promoted dehydroxylation

2.4 EXPERIMENTAL SECTION

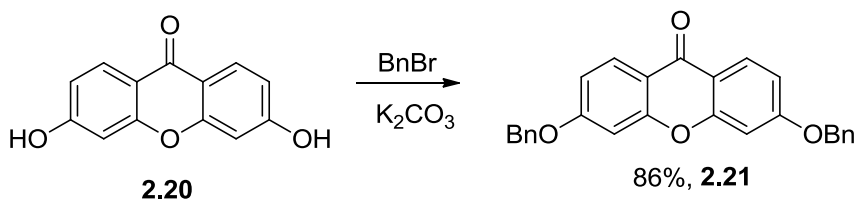
General Methods. All organic solvents were dried and freshly distilled before use. Flash chromatography was performed using 40-75 μm silica gel. All NMR spectra were recorded in CDCl_3 using 400 MHz instrument (unless otherwise noted). Buffer solutions for kinetic experiments were prepared using literature pKa values of the buffer acids and activity coefficient recommended by Bates.³⁹ 2, 2', 4, 4'-Tetrahydroxybenzophenone, phenylmagnesium bromide and *o*-tolylmagnesium bromide were purchased from TCI America and used as received. 2,4,5-trifluorobenzonitrile **2.46**, 1,2,4-trifluoro-5-nitrobenzene **2.43**, 3,6-dihydroxy-9*H*-xanthen-9-one **2.20**,⁴¹ 3,6-dimethoxy-9*H*-xanthen-9-one **2.36**,⁵⁹ 1-fluoro-2,4-dimethoxy-5-nitrobenzene **2.44**,⁵⁸ 1-fluoro-2,4-dimethoxybenzene **2.45**,⁵⁸ and 5-fluoro-2,4-dimethoxybenzoic acid **2.48**⁵⁸ were prepared using literature procedures.

Fluorescent measurements were conducted in aqueous solutions containing 90% methanol. Fluorescent spectra were recorded using 460 nm excitation light. The excitation source and the detector slit were set to 1 nm and 5 nm respectively. The fluorescence quantum yield was determined using fluorescein in 0.1 N NaOH ($\Phi_{\text{Fl}} = 0.95$)⁴³ as the standard reference. Fluorescent spectra were recorded using spectrometer equipped with polarizer. The path length was 1 cm with a cell volume of 3 mL.

2.5 SYNTHETIC PROCEDURES

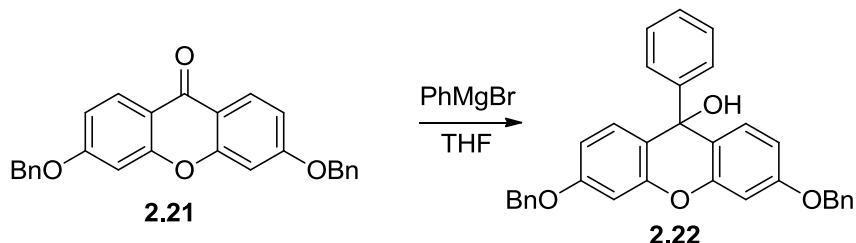


3,6-Dihydroxy-9H-xanthen-9-one (2.20): 2', 4, 4'-Tetrahydroxybenzophenone **2.19** (7.0 g, 28.4 mmol) was heated in water (30 mL) in an oven at 220 - 240° C for 4 h. Upon cooling, the resulting solid was filtered out and washed with 100 mL of water at about 60° C to afford 3,6-dihydroxy-9H-xanthen-9-one⁶⁰ **2.20** (6.4 g, 28.0 mmol, 99 % yield) as colorless needle like solid. ¹H NMR (DMSO) δ 10.80 (s, 2H), 7.95 (d, J = 8 Hz, 2H), 6.84 - 6.82 (dd, J = 8 Hz, 2H), 6.79 (d, 2H). ¹³C NMR (DMSO) δ 174.8, 164.3, 158.4, 128.7, 114.9, 114.6, 103.0. GC/MS (EI) M^{+} 228.

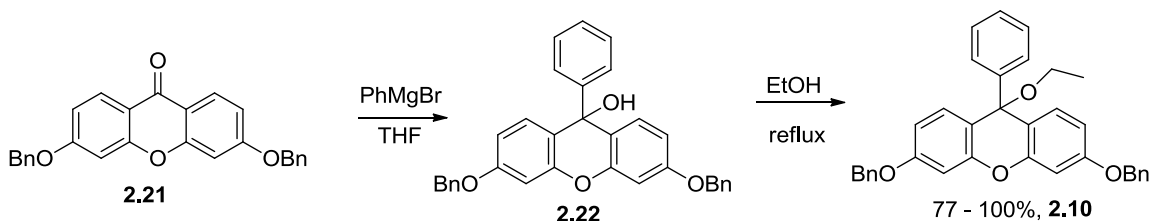


3,6-Bis(benzyloxy)-9H-xanthen-9-one (2.21): Potassium carbonate (4.85 g, 35.1 mmol) and benzyl bromide (2.293 mL, 19.28 mmol) were added to a suspension of 3,6-dihydroxy-9H-xanthen-9-one **2.20** (2.0 g, 8.76 mmol) in acetone (40 mL) at rt was added. The mixture was refluxed overnight and the solvent evaporated. The crude product was then purified through a short plug of silica gel eluting with DCM/hexane (1:1) to afford 3,6-bis(benzyloxy)-9H-xanthen-9-one **2.21**⁶¹ (3.09 g, 7.57 mmol, 86 % yield) as a

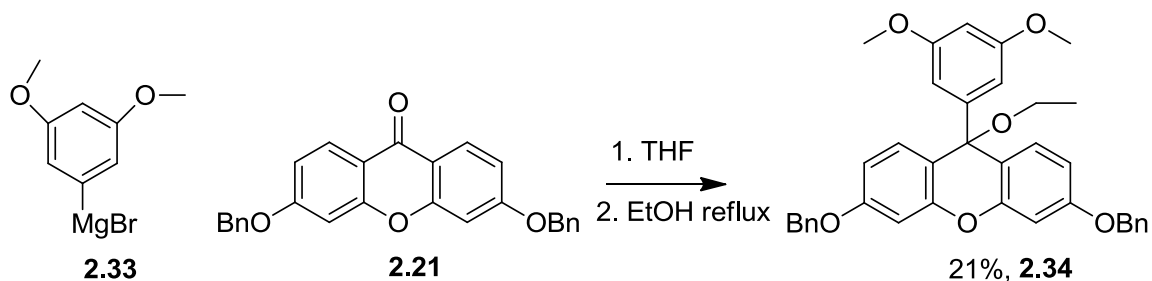
crystalline white solid. ^1H NMR δ 8.25 (d, J = 8.8 Hz, 2H), 7.49 – 7.36 (m, 10H), 7.03 (d, J = 2.3 Hz, 1H), 7.00 (d, J = 2.3 Hz, 1H), 6.93 (d, J = 2.3 Hz, 2H), 5.19 (s, 4H). ^{13}C NMR δ 175.8, 164.1, 158.3, 136.1, 129.1, 128.7, 128.6, 127.9, 116.4, 114.9, 113.8, 101.6, 70.8. GC/MS $M^+ = 408$



3,6-Bis(benzyloxy)-9-phenyl-9H-xanthen-9-ol (2.22): A solution of 3,6-bis(benzyloxy)-9H-xanthen-9-one **2.21** (0.202 g, 0.495 mmol) in THF (5 mL) was added dropwise to a solution of phenylmagnesium bromide (0.297 mL, 0.890 mmol) in ether (4 mL) at 0 °C was added under nitrogen a solution of. The mixture was stirred at rt for 2 h, and then quenched with a saturated solution of ammonium chloride (2 mL). The solvent was evaporated and the residue taken up in DCM (10 mL), washed with water and filtered through a short layer of neutral alumina to afford 3,6-bis(benzyloxy)-9-phenyl-9H-xanthen-9-ol **2.22** (0.154g, 0.317 mmol, 64.0 % yield) as a yellow oil. ^1H NMR δ 8.24 (d, J = 8.9 Hz, 1H), 7.39 – 7.28 (m, 13H), 7.21 – 7.16 (m, 2H), 7.01 (d, J = 8.9 Hz, 1H), 6.93 (s, 1H), 6.77 – 6.67 (m, 2H), 5.18 (s, 2H), 5.07 (s, 2H).

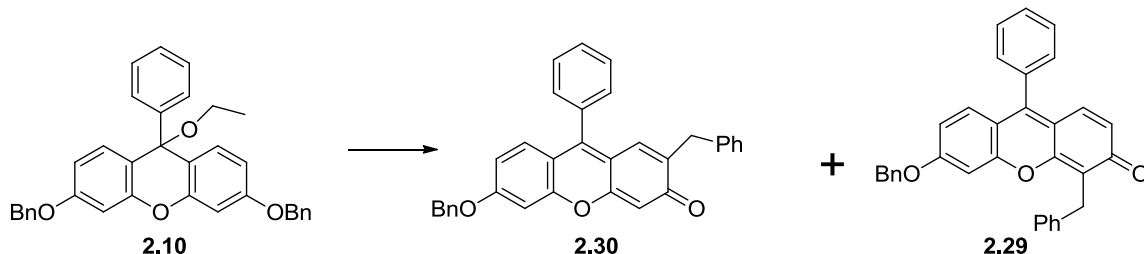


3,6-Bis(benzyloxy)-9-ethoxy-9-phenyl-9H-xanthene (2.10): Solid 3,6-bis(benzyloxy)-9H-xanthen-9-one **2.21** (0.51g, 1.249 mmol) was added to a solution of phenylmagnesium bromide (0.749 mL, 2.248 mmol) in ether (4 mL) at 25°C under nitrogen. The mixture was refluxed for 2 h, and then quenched with a saturated solution of Ammonium chloride. The solvent was evaporated and the crude **2.22** taken up in ethanol (20 mL). AcOH (3 drops) of were added and the reaction mixture refluxed for another 2 h. After cooling to room temperature, excess NaHCO₄ was added and the solvent evaporated. The residue was filtered through a short layer of neutral alumina to afford 3,6-bis(benzyloxy)-9-ethoxy-9-phenyl-9H-xanthene **2.10** (0.65g, 1.263 mmol, 100 % yield) as a thick yellow oil. ¹H NMR δ 7.38 (m, 13H), 7.24 (t, *J* = 7.5 Hz, 2H), 7.14 (d, *J* = 7.3 Hz, 1H), 7.10 (d, *J* = 8.7 Hz, 2H), 6.74 – 6.67 (m, 4H), 5.04 (s, 4H), 2.99 (q, *J* = 7.0 Hz, 2H), 1.11 (t, *J* = 7.0 Hz, 3H). ¹³C NMR δ 159.40, 152.56, 149.75, 137.00, 130.86, 129.06, 128.94, 128.40, 128.01, 127.87, 127.56, 127.47, 126.92, 126.61, 117.02, 112.16, 101.17, 77.66, 77.66, 77.34, 77.34, 77.02, 77.02, 75.35, 70.50, 58.68, 15.76.

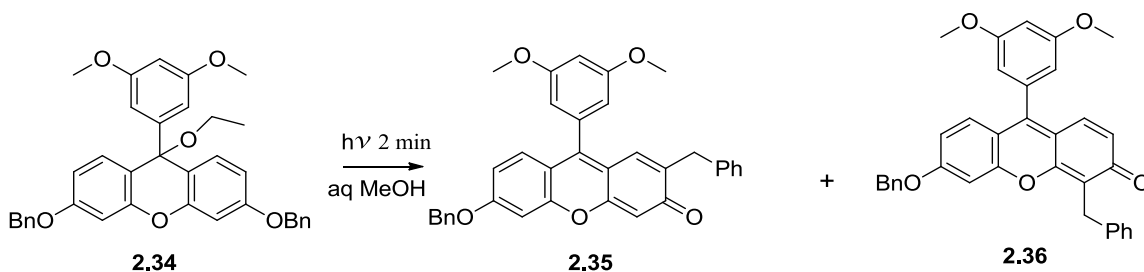


3,6-Bis(benzyloxy)-9-(3,5-dimethoxyphenyl)-9-ethoxy-9H-xanthene (2.34): Solid 3,6-bis(benzyloxy)-9H-xanthen-9-one **2.21** (0.5 g, 1.224 mmol) was added dropwise under nitrogen to a solution of (3,5-dimethoxyphenyl)magnesium bromide **2.33** (1.469 mL, 1.469 mmol) in ether (4 mL) at 0 °C. The mixture was refluxed for 2h, then and quenched with a saturated solution of ammonium chloride (2 mL). The solvent was evaporated and the residue taken up in ethanol (20 mL). 3 drops of AcOH were added and the reaction mixture refluxed for 2 h. After cooling to rt, NaHCO₄ was added and the solvent was evaporated, filtered through a short plug of neutral alumina to afford 3,6-bis(benzyloxy)-9-(3,5-dimethoxyphenyl)-9-ethoxy-9H-xanthene **2.34** (0.15 g, 0.261 mmol, 21.32 % yield) as a colorless oil. GC/MS M⁺ - 45 (EtO) = 529 observed. ¹H NMR δ 7.49 – 7.34 (m, 10H), 7.19 (t, *J* = 8.3 Hz, 3H), 6.73 (d, *J* = 7.6 Hz, 6H), 6.60 (d, *J* = 2.2 Hz, 2H), 5.08 (s, 4H), 3.86 (s, 5H), 3.73 (s, 6H), 2.98 (q, *J* = 7.0 Hz, 2H), 1.11 (t, *J* = 7.0 Hz, 3H). ¹³C NMR δ 161.19, 160.36, 159.33, 152.36, 152.10, 143.69, 136.91, 130.56, 129.25, 128.84, 128.44, 128.29, 127.78, 125.52, 116.65, 112.04, 105.77, 105.67, 101.08, 99.73, 98.01, 77.55, 77.24, 76.92, 75.22, 70.41, 58.65, 55.66, 55.45, 15.64.

Preparative Photolysis

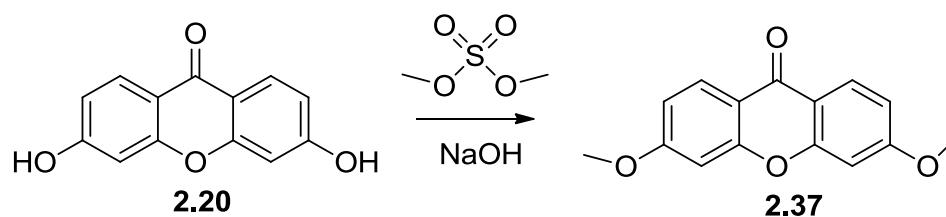


35mg of 3,6-*bis*(benzyloxy)-9-ethoxy-9-phenyl-9*H*-xanthene was dissolved in 400 mL MeOH solution and to give 0.17 mM which was irradiated for 3 min at 254 nm with 8 lamps (4.5W). Solvent was evaporated and the product purified by prep TLC eluting with 5% Methanol/toluene to give two bands (isomeric mixtures) as reddish oil. GC/MS M^+ observed 468. Calculated for $C_{33}H_{25}O_3^+$ 469.1798, HRMS found 469.1796, Fluorescent quantum yield = 0.02



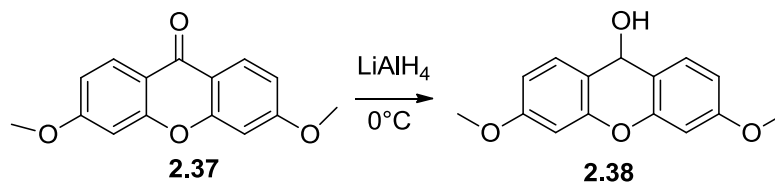
A stock solution of 3,6-*bis*(benzyloxy)-9-(3,5-dimethoxyphenyl)-9-ethoxy-9*H*-xanthene **2.34** in MeCN (2 mL, 10.4 mM) was diluted to 200 mL in MeOH to give ~0.10 mM solution which was irradiated at 254 nm for 3 min in a Rayonet reactor (8 lamps). The solvent was evaporated and the crude mixture taken up in EtOAc. Brine was added and the product extracted with EtOAc. Purification by prep TLC eluting with 5% methanol/toluene gave two bands F1 and F2. (DIP/MS) showed a mass of 528 and the

NMR spectra indicated both bands to be 4-benzyl-6-(benzyloxy)-9-(3,5-dimethoxyphenyl)-3*H*-xanthen-3-one as a mixture of isomeric **2.35** and **2.36**. Fluorescent quantum yield = 0.02. GC/MS $M^+ = 528$ observed. ^1H NMR δ 7.47 - 7.40 (m, 6H), 7.24 - 7.18 (m, 6H), 7.00 - 6.99 (dd, $J = 8.8, 2.2$ Hz, 1H), 6.87 - 6.80 (m, 1H), 6.61 (dd, $J = 25.1, 11.0$ Hz, 1H), 6.48 - 6.34 (m, 2H), 5.18 (d, $J = 10.3$ Hz, 2H), 4.13 (s, 1H), 3.80 (d, $J = 12.6$ Hz, 6H). ^{13}C NMR δ 184.41, 163.19, 161.20, 161.06, 155.00, 154.76, 148.25, 141.39, 135.84, 135.29, 130.08, 129.36, 129.18, 129.07, 128.75, 128.47, 128.43, 127.80, 126.13, 125.91, 118.59, 117.53, 114.80, 113.43, 107.84, 101.70, 77.57, 77.25, 76.94, 70.99, 55.76.

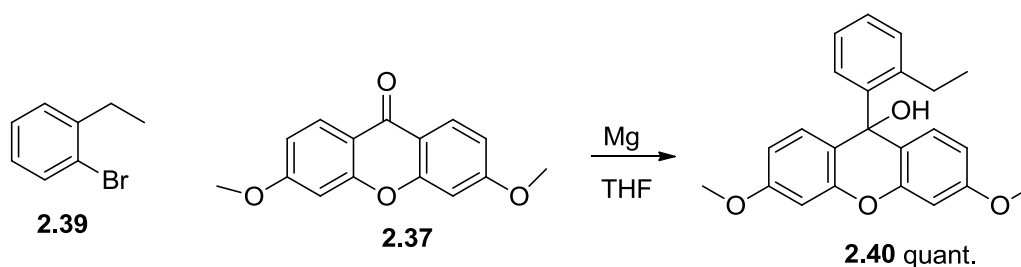


3,6-Dimethoxy-9H-xanthen-9-one (2.37): A solution of 3,6-dihydroxy-9H-xanthen-9-one (**2.20**) (0.600 g, 2.63 mmol) in acetone (30 mL) was treated with sodium hydroxide (10.52 mL, 10.52 mmol) at rt followed by dimethyl sulfate (0.628 mL, 6.57 mmol) and the mixture was stirred overnight. A solution of 5% HCl (3 mL) was added and the product extracted with DCM. The solvent was evaporated and the mixture purified by silica gel chromatography eluting with DCM/ 5% MeOH to afford 3,6-dimethoxy-9H-xanthen-9-one **2.37**⁵⁹ (0.4389 g, 1.713 mmol, 65.1 % yield) as a fluffy white solid. ^1H NMR δ 8.25 (d, 2H, $J = 8$ Hz), 6.96 - 6.93 (dd, $J = 4$ Hz, 8 Hz), 6.78 (d 2H), 3.94 (s, 6H). ^{13}C NMR δ 164.89, 158.25, 128.42, 113.09, 100.46, 77.55, 77.23, 76.91, 56.02.

GC/MS (EI) $M^+ = 256$ observed, calculated 256.07

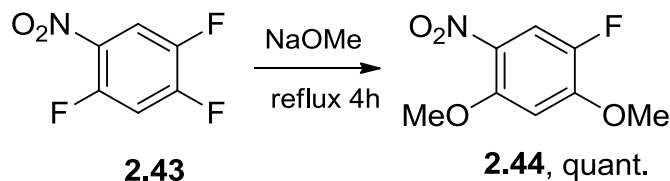


3,6-Dimethoxy-9H-xanthen-9-ol (2.38): Lithium aluminum hydride (0.019 g, 0.496 mmol) was added to a solution of 3,6-dimethoxy-9H-xanthen-9-one (0.106 g, 0.414 mmol) in dry diethyl ether (30 mL) at 0°C. The mixture was stirred for 10 min after which, saturated aqueous sodium sulfate was added until all the excess lithium aluminum hydride had been transformed into a white solid. The resulting mixture was filtered and evaporated to yield the crude 3,6-dimethoxy-9H-xanthen-9-ol **2.37** (0.107 g, 0.414 mmol, 100 % yield) as a creamy solid. ^1H NMR δ 7.49 (d, J = 8.6 Hz, 2H), 6.76 (dd, J = 8.5, 2.5 Hz, 2H), 6.66 (d, J = 2.5 Hz, 2H), 5.74 (d, J = 8.7 Hz, 1H), 3.83 (d, J = 3.6 Hz, 7H), 1.89 (d, J = 8.7 Hz, 1H). ^{13}C NMR δ 160.77, 151.83, 130.84, 115.60, 111.09, 101.03, 77.55, 77.23, 76.91, 63.40, 63.34, 55.71. GC/MS (EI) M^+ 258, FW calculated for M^+ $\text{C}_{15}\text{H}_{14}\text{O}_4$ = 258.27

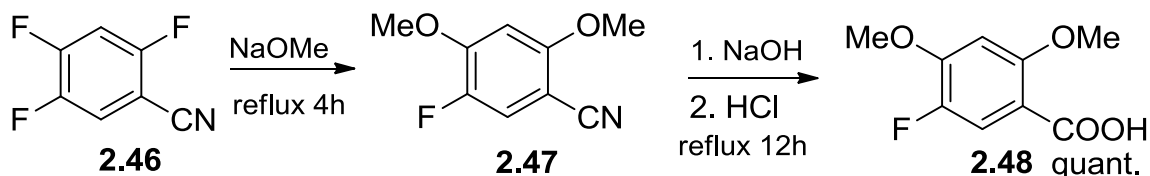


9-(2-Ethylphenyl)-3,6-dimethoxy-9H-xanthen-9-ol (2.40) The Grignard reagent was prepared by the reaction of magnesium (0.023 g, 0.929 mmol) and 1-bromo-2-ethylbenzene **2.39** (0.128 mL, 0.929 mmol) in ether (2 mL) containing a small amount of

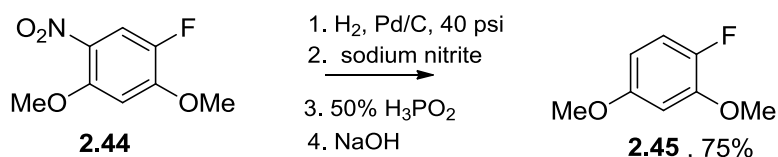
1,2-dibromoethane as the initiator at 25 °C under argon. The mixture was stirred for 30 min. Solid 3,6-dimethoxy-9*H*-xanthen-9-one **2.37** (0.119 g, 0.464 mmol) was added at 0 °C, the mixture was refluxed for 2 h and quenched with a saturated solution of ammonium chloride (2 mL). The mixture was extracted with ether (3 x 20 mL) and the combined ether layers were washed with brine and dried over anhydrous sodium sulfate. The solvent was evaporated and the residue filtered through a short layer of neutral alumina to afford the crude 9-(2-ethylphenyl)-3,6-dimethoxy-9*H*-xanthen-9-ol **2.40** as a yellow syrupy oil which was photolyzed without further purification. GC/MS (EI) M^+ - 17 (OH) found 345, FW calculated for M^+ C₂₃H₂₂O₄ = 362.42



1-Fluoro-2,4-dimethoxy-5-nitrobenzene (2.44) Sodium methoxide (1.342 g, 24.85 mmol) was added to a solution of 1,2,4-trifluoro-5-nitrobenzene **2.43** (2.0 g, 11.29 mmol) (1.0 equiv) in 30 mL MeOH under nitrogen at 4 °C. The resulting reaction mixture was allowed to warm up to rt and refluxed for 4 h. The reaction was then quenched with 10 % HCl (100 mL) acid and extracted with EtOAc (3 x 50 mL), washed brine, dried over anhydrous sodium sulfate and concentrated *in vacuo* to afford crude 1-fluoro-2,4-dimethoxy-5-nitrobenzene **2.44**⁵⁸ (2.3g, 11.43 mmol, 100 % yield) as a pale yellow solid which was used without further purification. ¹H NMR 7.83 (d, 1H, *J* = 8 Hz), 6.59 (d, 1H, *J* = 8 Hz), 3.96 (s, 3H), 3.99 (s, 6H). GC/MS (EI) M^+ found 201, FW calculated for M^+ C₈H₈FNO₄ = 201.15



5-Fluoro-2,4-dimethoxybenzoic acid (2.48): Sodium methoxide (0.984 g, 18.21 mmol) was added to a solution of 2,4,5-trifluorobenzonitrile **2.46** (1.3 g, 8.28 mmol) in MeOH (30 mL) under nitrogen and the mixture refluxed overnight. The reaction was quenched with 10 % HCl and the methanol evaporated. The residue was taken up in EtOAc, washed with brine, dried over anhydrous sodium sulfate and concentrated to give crude 5-fluoro-2,4-dimethoxybenzonitrile **2.47**. The crude **2.47** was then hydrolyzed by refluxing with 6M sodium hydroxide (20 mL, 120 mmol) overnight to give 5-fluoro-2,4-dimethoxybenzoic acid **2.48**⁶² (1.20 g, 6.00 mmol, 72.4 % yield) as a white solid m.p = 166 -167 °C. ¹H NMR (DMSO) δ 12.49 (broad hump, 1H), 7.53 (d, 1H, J = 12 Hz), 6.88 (d, 1H, J = 8 Hz), 3.96 (s, 3H), 3.88b (s, 3H). GC/MS (EI) M^+ observed 200, FW calculated for M^+ C₉H₉FO₄ = 200.16



1-Fluoro-2,4-dimethoxybenzene (2.45): Nitro group was reduced by hydrogenation at 40 psi over 10% Pd/C for 1.5 h in 50 mL EtOAc/EtOH (1:1). Filtration over Celite and evaporation of the solvent gave 5-fluoro-2,4-dimethoxyaniline⁵⁸ (0.315 g, 1.840 mmol,

37.0 % yield) which was used in the next step without further purification. GC/MS (EI) M^+ observed 171, FW calculated for $M^+ C_8H_{10}FNO_2 = 171.17$. A mixture of 5-fluoro-2,4-dimethoxyaniline (315 mg, 1.840 mmol) in water (4.0 mL)/HCl, 37% (2.0 mL) (2:1, 0.3 M) was chilled in an ice-salt bath and treated with a cold solution of sodium nitrite (140 mg, 2.024 mmol) in water (0.5 mL). The resulting solution was stirred for 15 min, and then H_3PO_2 (50%, 20 equiv) was added over 5 min. The mixture was left at 4 °C overnight, stirred at rt for 2 h, and then diluted with water. The reaction mixture was neutralized with aqueous sodium hydroxide and then extracted with EtOAc (2 × 30 mL). The extract was washed with water (5 mL) and brine (5 mL), dried ($MgSO_4$), and concentrated in vacuo. The residue was purified by flash column chromatography, eluting with 10% EtOAc/hexane to afford 1-fluoro-2,4-dimethoxybenzene **2.45**⁵⁸ (214 mg, 1.370 mmol, 74.5 % yield) as a colorless oil. GC/MS (EI) M^+ observed 156, FW calculated for $M C_8H_9FO_2 = 156.15$

CHAPTER 3

3-(HYDROXYMETHYL)NAPHTHALEN-2-OL CAGED FLUORESC EIN DERRIVATIVE AS PHOTOACTIVATABLE FLUOROPHORE

3.1 INTRODUCTION

Fluorescence spectroscopy has become one of the most frequently used analytical techniques in a diverse range of disciplines because it is non-invasive, has greater sensitivity and high spatiotemporal control. As such, a variety of fluorescence probes have found wide ranging applications in biological experiments, particularly in the investigation of cellular dynamics.⁶³ A major drawback in the use of fluorescent molecules in biological assays is that dye conjugates are mostly unstable in regards to the powerful illumination from fluorescence instruments⁶⁴. Some fluorescent molecules like fluorescein can exist as the anion, dianion and neutral species in aqueous solution, thus making its fluorescence and absorption properties very pH dependent.⁶⁵ In addition, protein conjugates of dyes tend to exhibit less fluorescence even when an increasing number of dyes are conjugated to the protein⁶⁶ fluorophore, consequently reducing the assay sensitivity.⁵⁸

To preserve their properties and improve their stability, dyes can be masked with an enzyme-labile substrate⁶⁷ or a photolabile protecting group (PPG), can be removed by brief ultraviolet (UV) irradiation. Photoactivatable fluorescent compounds or

photoactivatable fluorophores (PAFs) also known as caged fluorophores are stable, weakly or non-fluorescent molecules with the potential to become permanently fluorescent. Irradiation of the masked fluorophore at a suitable wavelength removes or ‘opens’ the cage and turns on the fluorescence⁶⁸ thereby allowing for differentiation of otherwise similar molecular species. The light sources used for photodeprotection can be easily controlled to achieve higher precision as well as spatial and temporal control. These unique advantages have made PAFs the probes of choice for biological imaging applications.⁶⁹

Some of the most commonly used dyes in the design of PAFs, include derivatives of caged fluorescein, rhodamine, and coumarin (Figure 3.1). Nagano and co-workers⁷⁰ have reported the synthesis of *o*-nitrobenzyl caged fluorescein derivatives **3.1** and **3.2**. By also incorporating the concept of photoinduced electron transfer (PeT),^{30,71} caged TokyoGreen **3.2** was rendered non-fluorescent. Yuan *et al*⁷² developed *bis*NPE-Fl-Hy **3.3** which upon photodeprotection readily undergoes Cu²⁺ mediated hydrolysis to the fluorescent form. The Li⁷³ group reported a 1-(2-nitrophenyl)ethyl caged coumarin NPE-HCC **3.4** with a remarkable fluorescent enhancement after irradiation. Caged acridinone derivatives like PENB-DDAO **3.5** reported by Goeldner and co-workers⁷⁴ have also been developed and utilized as photoactivatable fluorophore. Photolysis of **3.5** releases 1,3-dichloro-9,9-dimethyl-9*H*-acridin-2(7)-one (DDAO), a far-red emitting fluorophore.

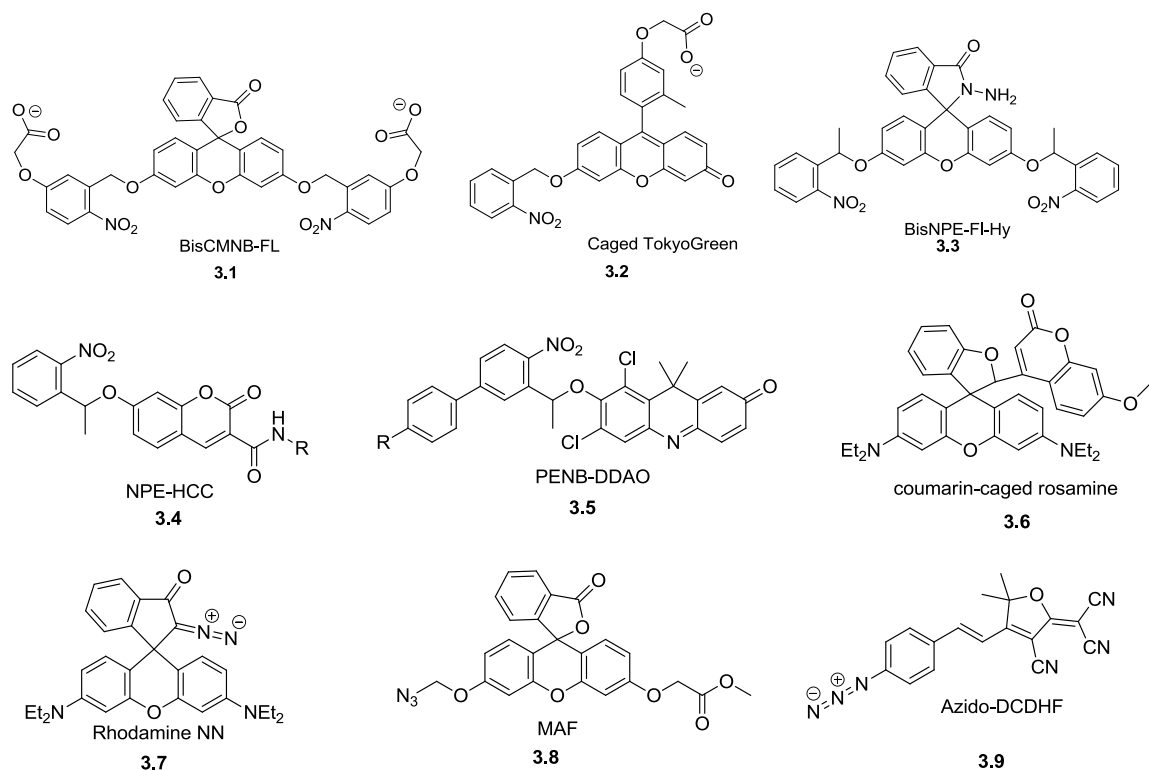


Figure 3.1: New photoactivatable fluorophores

The fluorescence of fluorescein and rhodamine⁷⁵ derivatives rely on their fluorogenic mechanism; the tautomerization between the open and closed lactone forms. Lin and co-workers⁷⁶ employed a remarkable intramolecular carbon–carbon spirocyclization to form a spiro-dihydrobenzofuran caged rosamine **3.6** with a photocleavable coumarin appendage. Diazo and azido moieties have also found use as photocleavable protecting groups. Belov *et al*⁷⁷ utilized a small 2-daizoketone as a PPG for rhodamine NN **3.7**. Upon irradiation, **3.7** extrudes nitrogen to form an intermediate carbene; the carbene undergoes Wolff rearrangement⁷⁸ to provide a very reactive ketene which then reacts with methanol to give uncaged dye. Azido compounds have also been used in caging PAFs. The azidomethyl group was recently employed by Ito and co-workers⁷⁹ to cage a fluorescein derivative **3.8**. Moerner and others⁸⁰ also reported the

Azido-DCDHF **3.9** which converts to fluorescent form when photolyzed via a reactive nitrene intermediate. Excellent reviews on photoactivatable fluorophores were recently published by Li^{69d} and Wysocki.⁸¹

Although a variety of photolabile protecting groups can be used in the caging of PAFs, the most widely used photocleavable protecting group is the *o*-nitrobenzyl⁸² and its derivatives⁸³ (Figure 3.2). Despite its popularity, substrate release from *o*-nitrobenzyl can be slow) and proceeds through several dark steps.⁸⁴ Moreover, the rate of substrate release from other PPGs utilizing C-O bond heterolysis as the mechanism is strongly dependent on the nature of the leaving group.⁸⁵ Therefore, there is a continued need for the development of PPGs for poor leaving groups like alcohols and phenols. As such, reports of other PPGs continue to appear in the literature.

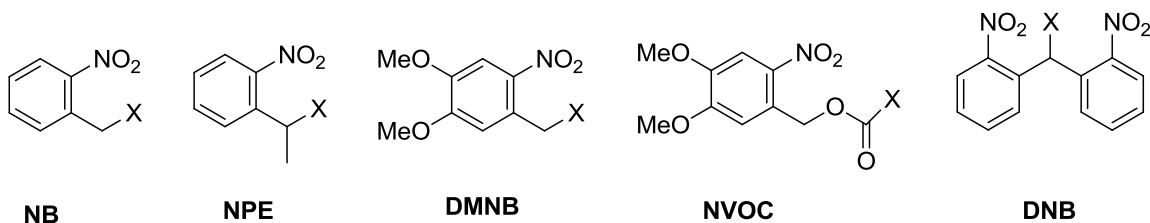
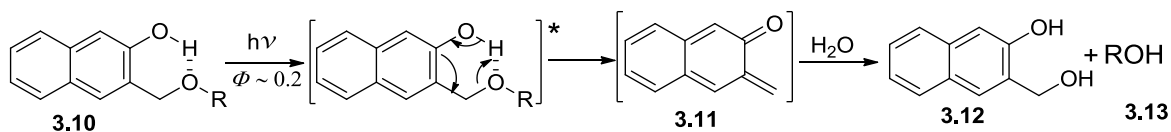


Figure 3.2: Derivatives of the *o*-nitrobenzyl group

Recently, Popik *et al*⁸⁶ reported the 3-(hydroxymethyl)naphthalen-2-ol **3.12** as a photolabile protecting group for alcohols and carboxylic acids. The chemistry and photo physics of **3.12** has been well documented. The Popik lab utilizes the enhanced acidity of phenols and naphthols in the excited state⁸⁷ to photochemically induce proton transfer to weakly basic sites, a concept referred to as excited-state intramolecular proton transfer (ESIPT).⁸⁸ The ESIPT in phenols and naphthols initiates cleavage of the C-O to generate reactive *o*-quinone methide intermediate **3.11**. An *o*-Quinone methide thus formed is

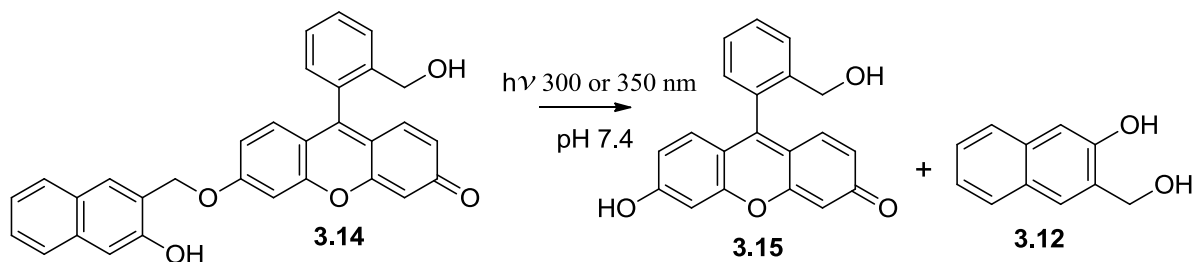
readily trapped by nucleophiles or water to form 3-(hydroxymethyl)naphthalen-2-ol **3.12** accompanied by product **3.13** release (Scheme 3.1). It should be emphasized that the generation of *o*-quinone methide **3.11** is very fast and typically occurs within 12 μ s.



Scheme 3.1: ESIP in 3-hydroxy-2-naphthalenemethanol **3.10**

3.2 RESULTS AND DISCUSSION

We have employed 3-(hydroxymethyl)naphthalen-2-ol **3.12** as a photoremovable protecting group for caged 6-hydroxy-9-(2-(hydroxymethyl)phenyl)-3H-xanthen-3-one **3.15** (Scheme 3.2). We decide to use a fluorescein derivative as our choice of fluorophore due to the high extinction coefficient of fluorescein (at excitation wavelength \sim 488 nm) and remarkable quantum efficiency. In addition, Koide *et al* had recently reported that monoethers of **3.15** such as **3.16**,⁸⁹ **3.17**⁹⁰ and **3.18**⁹¹ (Figure 3.3) are basically



Scheme 3.2: Photoactivation of caged 6-hydroxy-9-(2-(hydroxymethyl)phenyl)-3H-xanthen-3-one **3.14**

non-fluorescent at physiological pH. As a corollary, we assumed that cage fluorescein derivative **3.14** should be equally non-fluorescent. However, in our investigation, we found that photoactivatable fluorophore **3.14** is significantly fluorescent with a quantum yield $\Phi_{\text{Fl}} = 0.29 - 0.32$ at pH 7.4 even before irradiation.

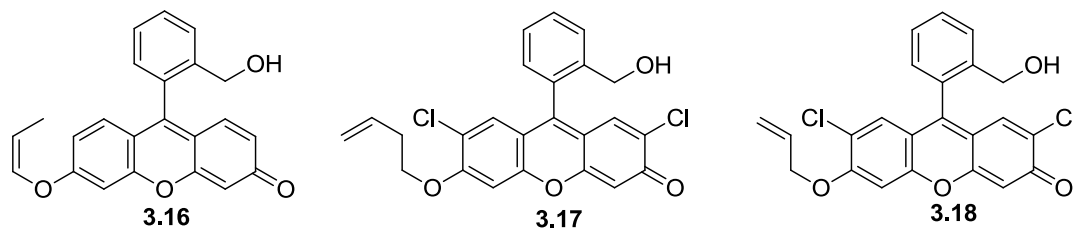
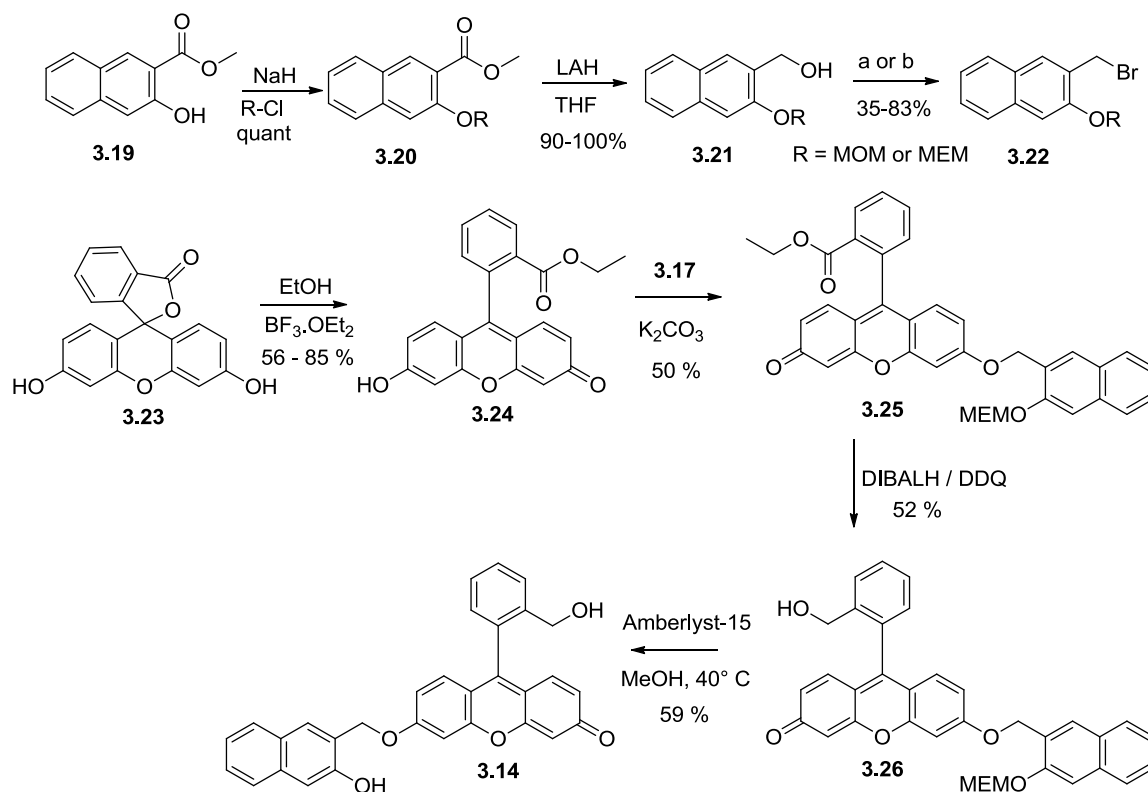


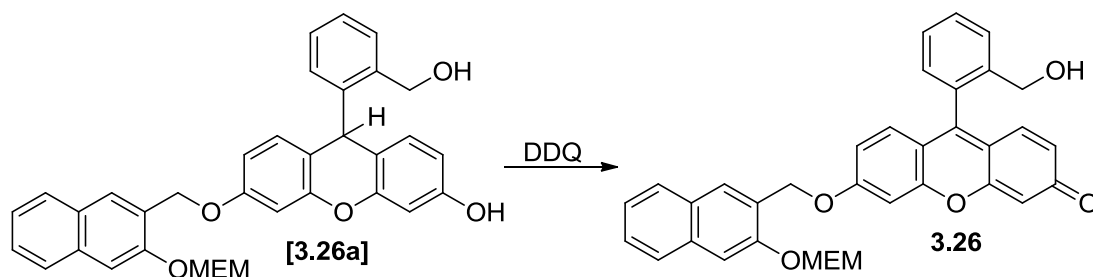
Figure 3.3: non-fluorescent derivatives of Fluorescein



Scheme 3.3: Synthesis of caged 6-hydroxy-9-(2-(hydroxymethyl)phenyl)-3H-xanthen-3-one **3.14**
a). PBr₃, TEA; b). Br₂, PPh₃, imidazole

Photoactivatable fluorophore **3.14** was synthesized as shown in Scheme 3.3. Commercially available methyl 3-hydroxy-2-naphthoate **3.19** was protected as MOM or MEM-ether **3.20**. Lithium aluminum hydride reduction of the MOM-ether afforded the corresponding (3-(methoxymethoxy)naphthalen-2-yl)methanol **3.21** or the MOM analogue quantitatively. Classical use of phosphorous tribromide to prepare 2-(bromomethyl)-3-((2-methoxyethoxy)methoxy)naphthalene **3.22** from **3.21** gave low yields (35 – 50 %), presumably due to the decomposition of the starting material or product **3.22**. However by using a variation of the Appel reaction, the bromide **3.22** was obtained in 85 - 92% yield.

Fisher esterification of commercially available fluorescein **3.23** using 3 equivalents of Lewis acid $\text{BF}_3 \cdot \text{OEt}_2$ afforded fluorescein ethyl ester **3.24**. Fluorescein esters are usually prepared with conventional mineral acids like H_2SO_4 involving complex work-up procedures.^{68f,92} However, we realized that refluxing fluorescein and alcohol with 1-3 molar equivalents of boron trifluoride etherate overnight cleanly affords the corresponding esters. 2-(bromomethyl)-3-(methoxymethoxy)naphthalene **3.22** and **3.24** were subjected to Williamson conditions with potassium carbonate to afford ether **3.25** in 50 % yield. The ethyl ester of **3.25** was reduced to the primary alcohol using DIBALH. Since the quinonoid form of fluorescein can be reduce with other hydride sources like NaBH_4 ,⁹³ we believe that treatment with DIBALH also reduces **3.25** to the corresponding phenol. It was therefore necessary to re-oxidize the crude product mixture by DDQ (Scheme 3.4).⁸⁹ The methoxyethoxy methyl ether on **3.26** was cleaved using amberlyst-15 in methanol⁹⁴ to give the final product **3.14** in 59% yield.



Scheme 3.4: In-situ re-oxidation of reduced **[3.26a]** to quinonoid **3.26**

Photoactivation of our first generation mono-caged-fluorescein derivative **3.14** was carried out using 300 nm or 350 nm lamps. As expected, **3.14** showed an increase in fluorescence (Figures 3.4 & 3.5) upon irradiation in PBS buffer at pH 7.4. HRMS-ESI of the product mixture confirmed the release of 6-hydroxy-9-(2-(hydroxymethyl)phenyl)-3*H*-xanthen-3-one **3.15** (Scheme 3.2).

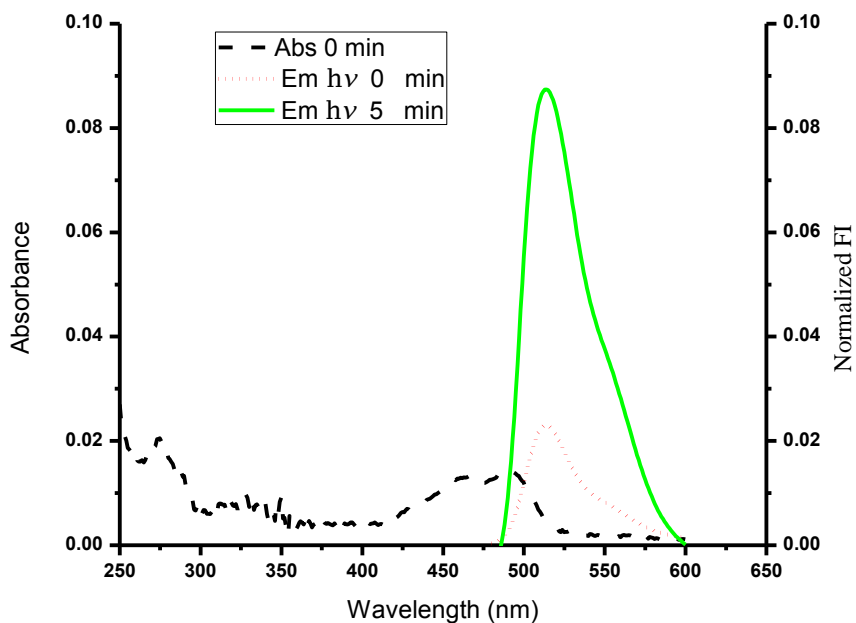


Figure 3.4: Absorbance spectrum (2.8 μM , black dash line) and emission spectra of caged compound **3.14** (3.4 μM) in PBS buffer, pH 7.4 containing 2% DMSO; before (red dotted line) and after (solid green line) 5 min irradiation at 300 nm.

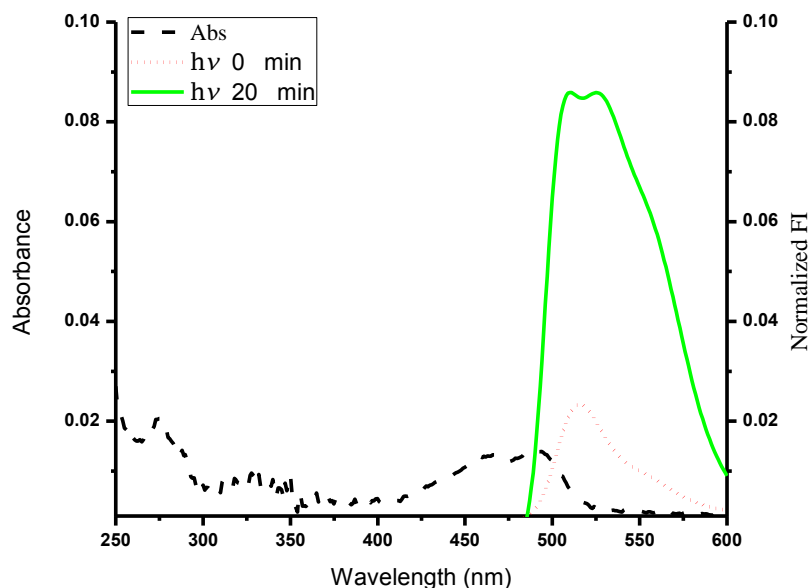


Figure 3.5: Absorbance spectrum (2.8 μM , black dash line) and emission spectra of caged compound **3.14** (3.8 μM) in PBS buffer pH 7.4 containing 8% DMSO; before (red dotted line) and after (solid green line) 20 min irradiation at 350 nm.

Although photoactivatable fluorophore **3.14** is slightly fluorescent, it showed a remarkable fluorescent enhancement after photolysis for 5 min at 300 nm (Figures 3.4) or 20 min at 350 nm in PBS buffer pH 7.4 with a fluorescence quantum efficiency $\Phi_{\text{FI}} = 0.74 - 0.93$. The emission spectrum after 20 min irradiation at 350 nm has two maxima at $\lambda_{\text{max}} = 509 \text{ nm}$ and $\lambda_{\text{max}} = 525 \text{ nm}$ (Figures 3.5). This indicates that prolong photolysis under these conditions gives rise to a secondary species. Fluorescence is due the growth of the band around $\lambda_{\text{max}} = 513 \text{ nm}$ upon photolysis (Figures 3.6 & 3.7). Insert in Figure 3.7 shows the dependence of this band on the irradiation time. Before irradiation, **3.14** has a slight absorbance between 450 -500nm with $\log \epsilon_{460\text{nm}} = 3.65 \pm 0.04$.

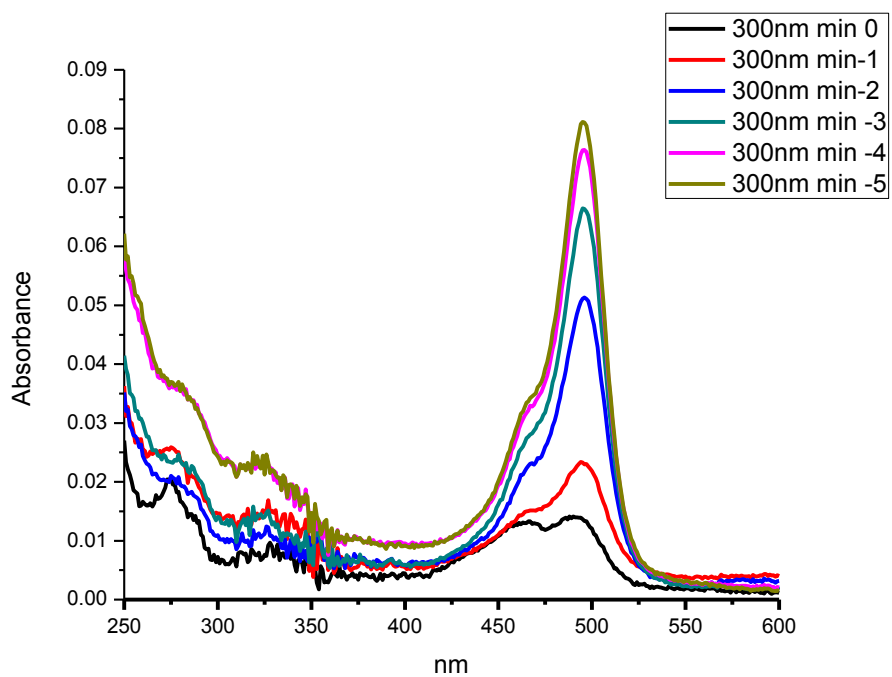


Figure 3.6: Successive 300 nm irradiation of **3.14** 2.8 μM in PBS buffer pH 7.4

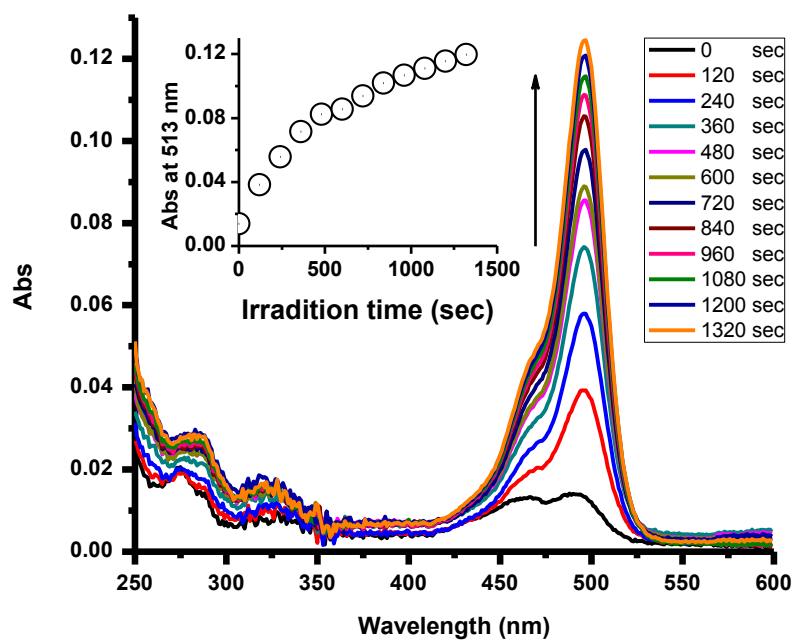


Figure 3.7 Successive 350 nm irradiation of **3.14** 2.8 μM in PBS buffer pH 7.4. Insert shows change in Abs Vs t at $\lambda_{\text{max}} = 513 \text{ nm}$

3.3 CONCLUSIONS AND FUTURE DIRECTIONS.

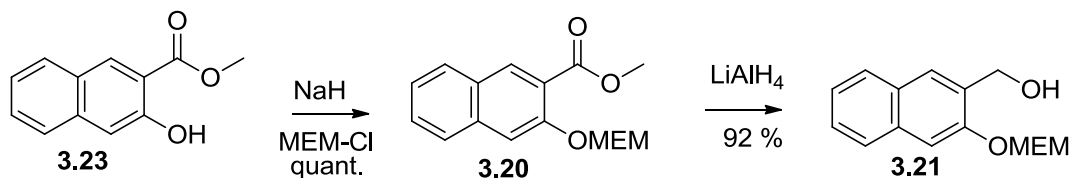
We have designed our first generation photoactivatable fluorophore with a significant fluorescent enhancement upon photolysis at pH 7.4. Photoactivation is achieved by photolysis at 300 nm or 350 nm (for biological samples) to give up to a 6-fold fluorescence enhancement. Having achieved the proof of principle, our next goal is to design a second generation photoactivatable fluorophore that is completely non-fluorescent before irradiation bearing a suitable appendage for bio-conjugation.

3.4 EXPERIMENTAL SECTION

General Methods. All organic solvents were dried and freshly distilled before use. Flash chromatography was performed using 40-75 μm silica gel. All NMR spectra were recorded in CDCl_3 (unless otherwise noted) using 400 MHz instrument. Buffer solutions for kinetic experiments were prepared using literature pKa values of the buffer acids and activity coefficient recommended by Bates.³⁹ Methyl 3-hydroxy-2-naphthoate **3.19** and fluorescein **3.23** were purchased from TCI America and Alfa Aesar respectively. All chemicals were used as received.

Fluorescent measurements were conducted in aqueous buffers containing 4-8% of DMSO as co-solvent at 2.8 or 3.4 μM concentrations of **3.14**. Fluorescent spectra were recorded using 460 nm excitation light. The excitation source and the detector slit were set to 1 nm and 5 nm respectively. The fluorescence quantum yield was determined using fluorescein in 0.1 N NaOH ($\Phi_{\text{Fl}} = 0.95$)⁴³ as the standard reference. Fluorescent spectra were recorded using spectrometer equipped with polarizer. The path length was 1 cm with a cell volume of 3 mL.

3.5 SYNTHETIC PROCEDURES



(3-(methoxymethoxy)naphthalen-2-yl)methanol (3.21) Sodium hydride (1.096 g, 29.7 mmol) to an ice-cold solution of methyl 3-hydroxy-2-naphthoate **3.19** (3.0 g, 14.84 mmol) in DMF (volume: 10 mL). The mixture was stirred for 15 min after which MEM-Cl (2.254 mL, 29.7 mmol) was added. The solution was allowed to warm to room temperature, stirred for 2 h and quenched with 5% HCl (10 mL). Water (50 mL) was added and the crude ether extracted with Ether (4 x 50 mL). The organic layer was washed with saturated aqueous NaHCO₃ (30 mL), NaCl and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and the crude **3.20** was used in the next step without further purification.

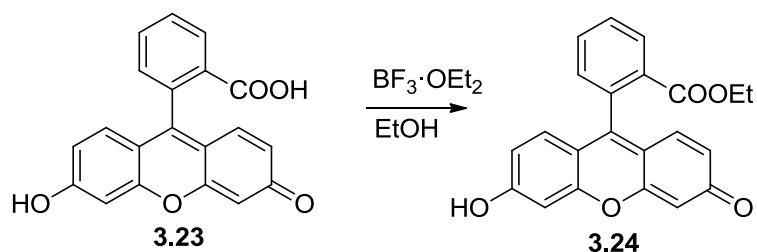
A solution of crude **3.20** in 20 mL of THF was added in portions to a solution of lithium aluminum hydride (1.863 g, 49.1 mmol) in THF (volume: 50 mL) at 0 °C. The mixture was allowed to warm up to rt and stirred for 3 h, quenched with EtOAc and filtered through Celite. Solvent was evaporated and the residue purified thru a short layer of silica gel, eluting with EtOAc/hexane (1:1-7:3) to afford (3-(methoxymethoxy)naphthalen-2-yl)methanol **3.21** (2.995 g, 13.72 mmol, 92 % yield) as a colorless turbid oil. ¹H NMR δ 7.82 (s, 1H), 7.74 (dd, *J* = 8.1, 3.4 Hz, 2H), 7.47 – 7.41 (m, 2H), 7.38 – 7.33 (m, 1H), 5.49 (s, 2H), 4.71 (s, 2H), 3.94 – 3.91 (m, 3H), 3.61 – 3.57

(m, 3H), 3.39 (s, 3H). ^{13}C NMR δ 152.98, 134.94, 130.50, 129.17, 128.10, 127.84, 127.14, 127.09, 124.67, 109.69, 93.57, 77.55, 77.23, 76.91, 71.82, 68.22, 59.22, 29.64.

General procedures for preparation of bromide 3.22.

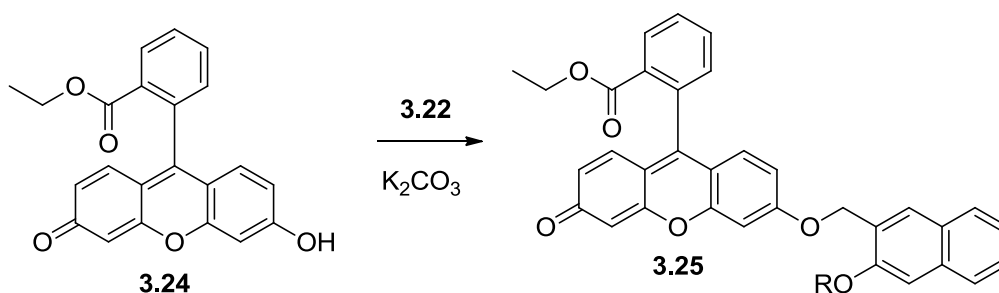
(a). Et_3N (15.10 mmol, 1.2 Equiv.) and PBr_3 (15.10 mmol, 1.2 equiv.) were added to a solution of alcohol **3.21** (12.58 mmol, 1.0 equiv.) in dry DCM at 0°C . The mixture was allowed to warm up to rt and stirred for 2 -12 h. Solvent was evaporated and crude residue purified by silica gel chromatography (eluting with 20% EtOAc/hexane) to give bromide **3.22** as a colorless crystals in 35 -56% yield.

(b). Ph_3P (11 mmol, 1.5 Equiv.) and imidazole (11 mmol, 1.5 equiv.) in DCM (60 mL) was added to an ice-cold solution of Br_2 (11 mmol, 1.5 equiv.). The mixture was stirred for 10 min after which a solution of alcohol **3.21** (7.3 mmol, 1 equiv.) in DCM (40 mL) was added via a cannula. The mixture was stirred at 0°C for 10 – 30 min (TLC) and the precipitate filtered out. The filtered solid was washed with 20% Ether/hexane (2 x 50 mL). The combined filtrate was washed with a saturated solution of sodium dithionite (2 x 50 mL), brine (20 mL) and the solvent evaporated. The crude residue was passed through a short layer of silica gel eluting with hexane to afford bromide **3.22** as colorless crystals in 83 -90% yield.

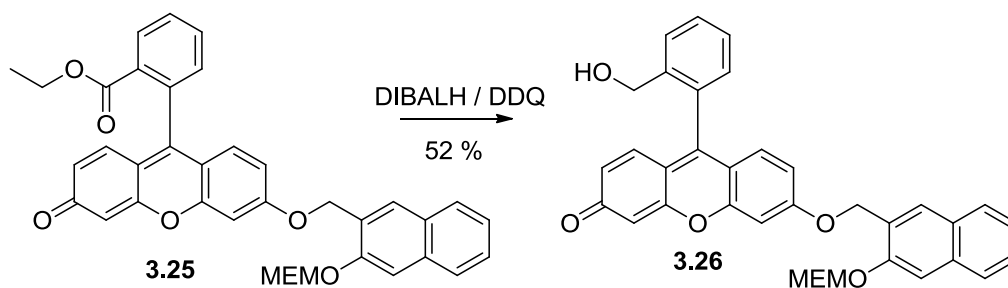


Ethyl 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoate (3.24) $\text{BF}_3 \cdot \text{OEt}_2$ (9.06 mL, 71.5 mmol) was added drop wise to a suspension of fluorescein **3.23** (3.3g, 8.94 mmol) in EtOH (30 mL) and the reaction mixture was refluxed over night with an addition funnel loaded with molecular sieves attached. The mixture was cooled, diluted with EtOAc (100 mL). Water was added and the organic layer extracted with EtOAc (3 x 50 mL). The organic layer was washed with water (2 x 50 mL) and brine (20 mL), dried over sodium sulfate and solvent evaporated to afford crude ethyl 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoate **3.24** (3.2 g, 8.88 mmol, 99 % yield) as a dark red solid which was used without further purification. ^1H NMR (DMSO-d_6) δ 8.17 (d, $J = 7.6$ Hz, 1H), 7.85 (t, $J = 7.3$ Hz, 1H), 7.76 (t, $J = 7.5$ Hz, 1H), 7.48 (d, $J = 7.2$ Hz, 1H), 6.78 (d, $J = 9.8$ Hz, 2H), 6.54 (m, 3H), 3.95 (q, $J = 7.0$ Hz, 2H), 0.85 (t, $J = 7.0$ Hz, 3H). ^{13}C NMR (DMSO-d_6) δ 166.22, 164.95, 157.14, 152.10, 134.97, 134.14, 131.67, 130.97, 130.51, 115.67, 104.27, 53.31, 41.09, 40.88, 40.67, 40.46, 40.25, 40.04, 39.84.

General procedure for Williamson Synthesis of fluorescein naphthyl ethers



Potassium carbonate (2.2 mmol, 1.2 equiv.) was added to a solution of fluorescein ethyl ester **3.24** (1.8 mmol, 1.0 equiv.), and bromide **3.22** (2.8 mmol, 1.6 equiv.) in DMF (5 mL) at room temperature. The mixture was stirred at 60° C for 2 - 12 h and then concentrated in vacuo. The residue was purified by silica gel chromatography eluting with 0-4% EtOH in $CHCl_3$ to afford the corresponding ethers in 50 -89 % yield **3.25** as a yellow solid. **R = MOM**; M.P 72 -74° C. 1H NMR δ 8.26 (d, 2H, $J = 8Hz$), 7.89 (s, 1H), 7.78 - 7.71 (m, 3H), 7.67 (t, 1H, $J = 8Hz$), 7.48 - 7.44 (m, 2H), 7.37(t, 1H, $J = 8Hz$), 7.31 (d,1H, $J = 8Hz$), 7.12 (d, 1H, $J = 2.4Hz$), 6.94 - 6.86 (m,3H), 6.55 (dd, 1H, $J = 8, 2Hz$), 6.45 (d, 1H, $J = 2Hz$), 5.39 (s, 2H), 5.38 (s, 2H), 4.04 (q,2H, Et), 3.55 (s, 3H), 0.95 (t, 3H, Et). ^{13}C NMR δ 185.9, 165.6, 163.5, 159.1, 154.5, 152.8, 150.2, 149.6, 134.5, 132.7, 130.6, 129.8, 129.2, 128.2, 127.9, 127.1, 126.9, 125.9, 124.7, 118.0, 115.4, 109.4, 101.6, 94.8, 66.6, 61.6, 56.5, 13.8.



9-(2-(Hydroxymethyl)phenyl)-6-((3-((2-methoxyethoxy)methoxy)naphthalen-2-

yl)methoxy)-3H-xanthen-3-one (3.26) A solution of DIBAL-H (5.48 mL, 5.48 mmol)

was added dropwise to a solution of ethyl 2-(6-((3-((2-methoxyethoxy)methoxy)naphthalen-2-yl)methoxy)-3-oxo-3H-xanthen-9-yl)benzoate

3.25 (0.663 g, 1.097 mmol) in DCM (15 mL) at -78 °C under argon. The resulting

solution was stirred for 10 min, and then allowed to warm up to rt over 2 h. A saturated solution of ammonium chloride (2 mL) was added and the mixture stirred for 10 min at

room temperature. Ether (50 mL) and DDQ (0.274 g, 1.206 mmol) were added and the

mixture stirred for another 30 min. The reaction mixture was filtered through a pad of

Celite. The solvents were evaporated and the crude mixture purified by silica gel

chromatography eluting with EtOAc/Hexane (1:1) to afford 9-(2-

(hydroxymethyl)phenyl)-6-((3-((2-methoxyethoxy)methoxy)naphthalen-2-yl)methoxy)-

3H-xanthen-3-one **3.26** (0.320 g, 0.569 mmol, 51.9 % yield) as a yellow oil. ¹H NMR δ

7.89 (s, 1H), 7.75 (t, *J* = 6.8 Hz, 3H), 7.49 (s, 1H), 7.46 – 7.31 (m, 4H), 6.95 – 6.71 (m,

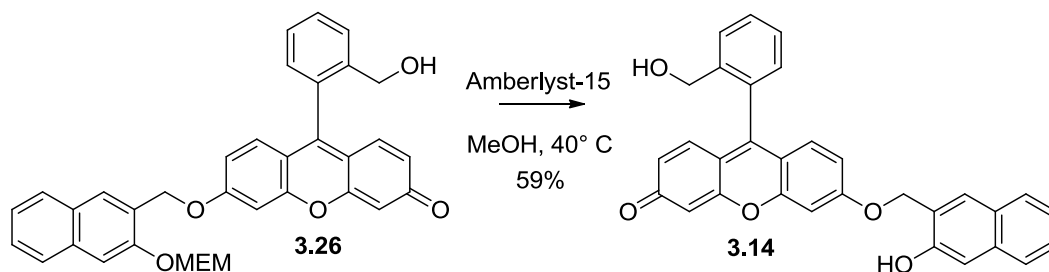
5H), 6.59 (s, 1H), 6.50 (d, *J* = 7.9 Hz, 1H), 5.45 (d, *J* = 2.3 Hz, 2H), 5.25 (s, 2H), 4.85 (s,

1H), 3.88 (t, 2H), 3.59 (t, 2H), 3.38 (m, 3H), 2.09 (s, 3H). ¹³C NMR δ 176.52, 153.47,

152.86, 139.29, 134.28, 134.25, 130.77, 130.05, 129.40, 129.30, 128.52, 128.09, 128.01,

127.92, 127.82, 127.01, 126.99, 126.62, 126.53, 124.54, 124.49, 109.24, 101.70, 93.77, 93.71, 77.55, 77.23, 76.91, 71.89, 71.79, 68.39, 68.10, 66.18, 62.33, 59.18, 20.83.

HRMS calc. for $[M+H]^+$; $C_{35}H_{31}O_7^+$ 563.2064, found 563.2071



9-(2-(Hydroxymethyl)phenyl)-6-((3-hydroxynaphthalen-2-yl)methoxy)-3H-xanthen-3-one (3.14) Amberlyst-15 resin (100 mg) was added to compound **3.26** (0.100 g, 0.178 mmol) in 15 mL of aqueous methanol (95/5, v/v). The mixture was stirred at 50 °C and monitored by TLC. After 2 h, the reaction mixture was cooled and filtered. Evaporation of the solvents under reduced pressure provided the unprotected **3.14** (0.050 g, 0.105 mmol, 59.3 % yield). 1H NMR (DMSO- d_6) δ 10.16 (s, 1H), 9.83 (s, 1H), 7.88 (s, 1H), 7.78 (d, J = 8.4 Hz, 1H) 7.69 (d, J = 8.4 Hz, 1H), 7.47 – 7.34 (m, 4H), 7.30 – 7.18 (m, 3H), 6.89 (d, J = 2.4 Hz, 2H), 6.84 (d, J = 8.7 Hz, 1H), 6.76 (dd, J = 14.9, 8.4 Hz, 3H), 6.58 (d, J = 2.3 Hz, 1H), 6.51 (dd, J = 8.6, 2.5 Hz, 1H), 5.41 (s, 1H), 5.23 (s, 4H). HRMS calc. for $[M+H]^+$; $C_{31}H_{23}O_5^+$ 475.1540, found 475.1544

CHAPTER 4

SYNTHESIS OF 2nd GENERATION PHOTOACTIVATABLE FLUOROPHORE WITH EASILY FUNCTIONALIZABLE APPENDAGE FOR BIO-CONJUGATION

4.1 INTRODUCTION

In chapter 3, we reported the synthesis of our first generation PAF, a mono caged derivative of fluorescein **3.14**. However, photoactivatable fluorophore **3.14** is significantly fluorescent with a quantum yield $\Phi_{\text{Fl}} = 0.29 - 0.32$ at pH 7.4 before photoactivation. **3.14** also show some detectable absorbance in the excitation region (between 450 – 500 nm), which might inevitably give rise to significant background fluorescence. This feature might therefore limit or preclude its use as an effective caged fluorophore.

To reduce the fluorescence quantum yield before photoactivation, fluorescein derivatives are usually transformed into the *bis*-caged lactones. Some examples include the *bis*(5-carboxymethoxy-2-nitrobenzyl)-fluorescein (*Bis*CMNB-FL, **4.1**, Figure 4.1), which is commercially available and the *bis*-caged carboxyfluorescein (C2CF, **4.2**, Figure 4.1) developed by Mitchison.⁹⁵ However, etherifying both phenolic groups of fluorescein with a PPG requires the removal of two protecting groups which often renders the uncaging process less efficient.

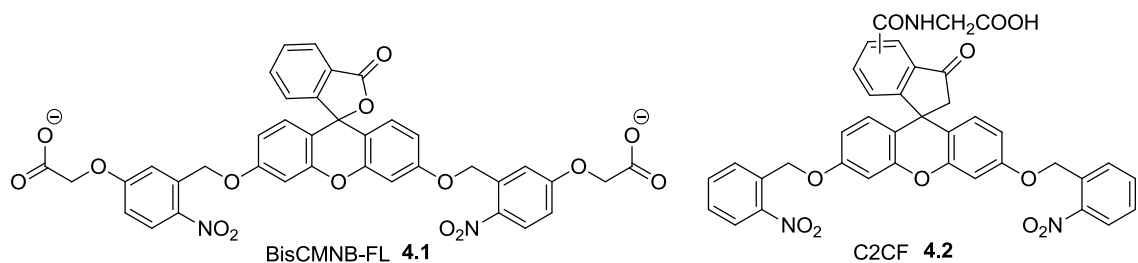


Figure 4.1 Examples of non-fluorescent *bis*-caged fluorescein derivatives.

Another approach used to reduce the fluorescence before irradiation is to unsymmetrically etherify the phenolic groups such that only one PPG is used while the other phenol is functionalized as desired. Krafft and co-workers^{68f} were the first to employ this approach utilizing the *o*-nitrobenzyl (NB) protecting group. The NB group was also used by Trentham *et al*⁹⁶ to synthesize mono-caged fluorescein derivatives with various functionalities. To improve on the properties of our 1st generation PAF **3.14**, we have synthesized 3-(hydroxymethyl)naphthalen-2-ol caged fluorescein (NQMP-FL-A, **4.3**, Figure 4.2) with a suitable appendage that can be conveniently used for bio-conjugation.

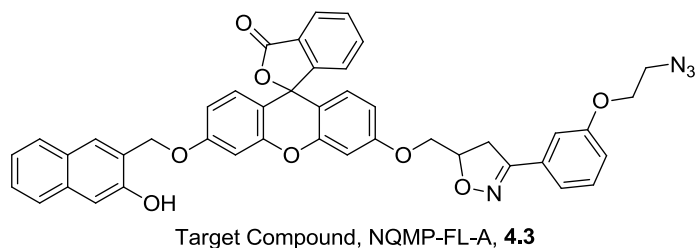
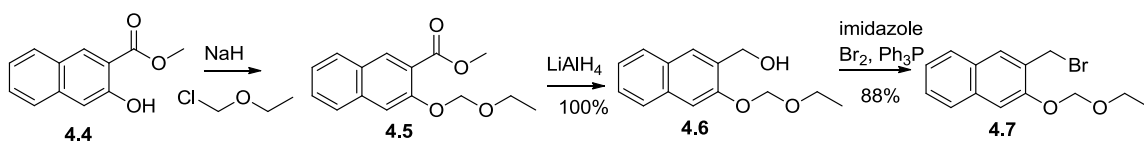


Figure 4.2 2nd Generation PAF 3-(hydroxymethyl)naphthalen-2-ol caged fluorescein (NQMP-FL-A) **4.3**

4.2 RESULTS AND DISCUSSION

While the synthesis of caged fluorescein with similar substitutions on both phenolic moieties can be very straight forward, the preparation of our unsymmetrical caged fluorescein turns out to be a bit tricky due to the inherent fluoregenic mechanism of the dye. Kraft and co-workers⁹⁷ had noted similar challenges in their work with *o*-nitrobenzyl derivative. However, they utilized a step wise silver(I)oxide (Ag₂O) mediated alkylation in a non-polar solvent (benzene) to achieve high overall yield.

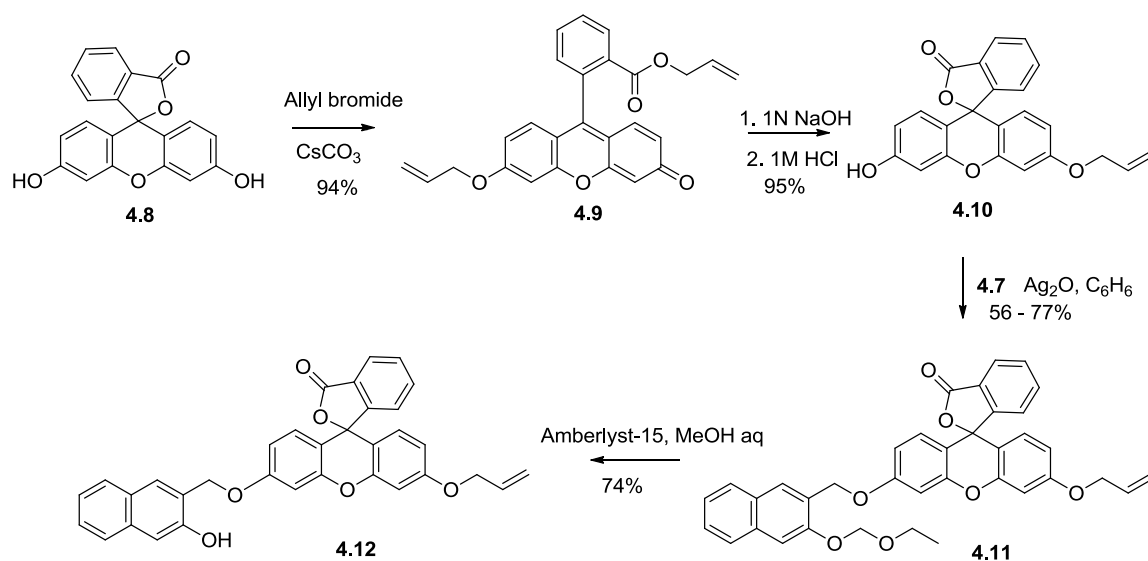
To prepare NQMP-FL-A **4.3**, the phenolic hydroxyl group of commercially available methyl 3-hydroxy-2-naphthoate **4.4** was protected with (chloromethoxy)ethane to afford **4.5**. This conversion proceeded smoothly and cleanly to afford the corresponding methyl naphthanoate ether in high yields. Subsequent reduction of the crude ether **4.5** using LiAlH₄ gave alcohol **4.6** in excellent overall yields. (3-(ethoxymethoxy)naphthalen-2-yl)methanol **4.6** was converted to the corresponding bromide **4.7** using a variation of the Appel reaction (Scheme 4.1).



Scheme 4.1 Synthesis of 2-(bromomethyl)-3-(ethoxymethoxy)naphthalene **4.7**

Allyl fluorescein **4.9** was obtained from commercially available fluorescein **4.8** by treatment with cesium carbonate and allyl bromide. Saponification of allyl fluorescein **4.9** with 1.0 N NaOH at rt followed by acidification afforded fluorescein lactone **4.10**.

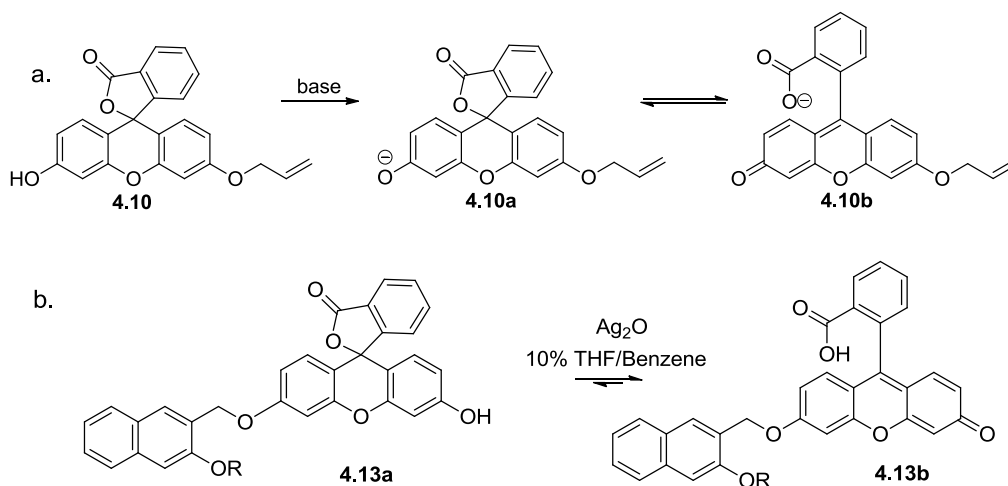
Compound **4.7** was then coupled to fluorescein lactone **4.10** using Ag_2O mediated alkylation of the free phenolic oxygen in non-polar solvent benzene to give caged fluorescein **4.11**. The use of standard protocols involving strong acids like TFA and HCl were not successful for the removal of MOM, MEM or the 2-(ethoxymethoxy) protecting group on **4.11**. These conditions led to the formation of intense yellow solutions, presumably from cleavage of the naphthyl group. However, we found the method employed by Michelot *et al*⁹⁴ for the deprotection of MOM ethers to be very effective for our substrate. The removal of the 2-(ethoxymethoxy) protecting group was therefore achieved using amberlyst-15 resin in 5% aqueous methanol at 50° C to give **4.12** in 74% yield (Scheme 4.2).



Scheme 4.2 Synthesis of cage fluorescein

It is noteworthy that the use of classical Williamson ether synthesis conditions as well as Mitsunobu reaction with alcohol **4.6** to prepare caged fluorescein **4.11** are less efficient as the lactone **4.10** tautomerizes to the carboxylate under basic conditions. Under these

conditions, esterification of the carboxylate **4.10b** becomes predominant affording the undesirable fluorescent carboxylate ester (Scheme 4.3a). In addition, fluorescein lactones bearing a naphthyl group such as **4.13a** do not easily undergo the silver oxide mediated alkylation or rather do so sluggishly. We observed that a benzene solution of **4.13a** containing silver oxide instantly becomes yellow and fluorescent upon the addition of THF as a co-solvent, this is characteristic of the quinonoid form **4.13b**. We believe this could be due to electronics rather than steric factors (Scheme 4.3b). However no such color change is observed for the corresponding fluorescein lactone **4.10** bearing an allylic moiety. Therefore, the order of substituent introduction on the fluorescein ring is equally important. Corrie and co-workers⁹⁶ also reported similar concerns with their use of o-nitrobenzyl bromide for the preparation of mono substituted fluorescein lactone.

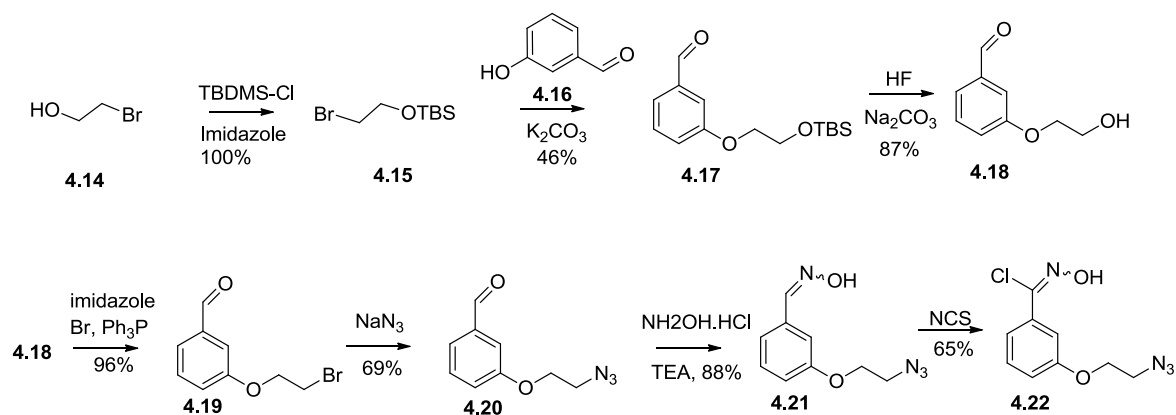


Scheme 4.3 Fluorescein lactones and tautomers in (a) basic (b) neutral conditions.

The caged fluorescein **4.12** is equipped with an alkene handle for easy functionalization. Among the various derivatizations that could be carried out on the alkene, we decided to append the versatile azido moiety which can either be clicked with

alkynes or subjected to the Staudinger reaction to give the corresponding amine. By using the alkene as a flexible handle, **4.12** offers convenient derivatization to functional group(s) of choice.

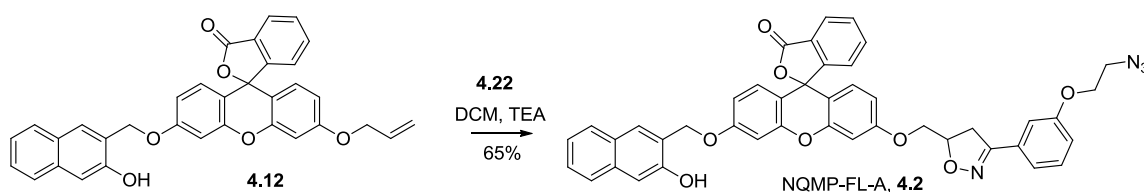
Alkenes are excellent dipolarophiles for the Huisgen cycloaddition. We decided to exploit the utility of 1,3-dipolar cycloaddition between a dipolarophile such as **4.12** and nitrile oxide to incorporate the azido moiety on to the caged fluorophore via an isoxazoline ring. Nitrile oxides can be generated in-situ from hydroximoyl chlorides^{95,98} such as 3-(2-azidoethoxy)-*N*-hydroxybenzimidoyl chloride **4.22** (Scheme 4.4).



Scheme 4.4 Synthesis of chloro oxime **4.21**

3-(2-Azidoethoxy)-*N*-hydroxybenzimidoyl chloride **4.22** was prepared in 7 steps (Scheme 4.4) from commercially available 2-bromoethanol **4.14** which was protected as silyl ether **4.15**. The TBDMS ether was then coupled to 3-hydroxy benzaldehyde **4.16** to afford aldehyde **4.17**. Removal of the TBDMS group under classical conditions gave alcohol **4.18** in 87% yield. Bromination of the alcohol followed by substitution with an azide afforded azido aldehyde **4.20** in excellent yield. Treatment of aldehyde **4.20** with

hydroxylamine hydrochloride gave the corresponding aldoxime **4.21** in 88% yield. The key intermediate 3-(2-azidoethoxy)-*N*-hydroxybenzimidoyl chloride **4.22** was obtained in 65% yield from aldoxime **4.21** using *N*-chlorosuccinimide. Dehydrochlorination of hydroximic acid chloride **4.22** generated the reactive nitrile oxide intermediate in-situ which was trapped by olefin **4.12** to give the target compound NQMP-FL-A **4.2** in 65% yield (Scheme 4.5).



Scheme 4.5 Synthesis of target compound NQMP-FL-A **4.2**

A solution of caged fluorophore NQMP-FL-A **4.2** in a PBS buffer at pH = 7.4 has a slight absorbance with $\log \epsilon_{460\text{nm}} = 2.86 \pm 0.04$ compared with $\log \epsilon_{460\text{nm}} = 3.65 \pm 0.04$ for our first generation **3.14**. Irradiation of a 2.33 μM PBS solution of NQMP-FL-A **4.2** at 350nm for 20 min, shows a significant rise in absorbance between 400 – 500nm (Figure 4.3) due to fluorophore release. A 460 nm excitation of the solution of released fluorophore gives an emission band with $\lambda_{\text{max}} = 515 \text{ nm}$ (Figure 4.4).

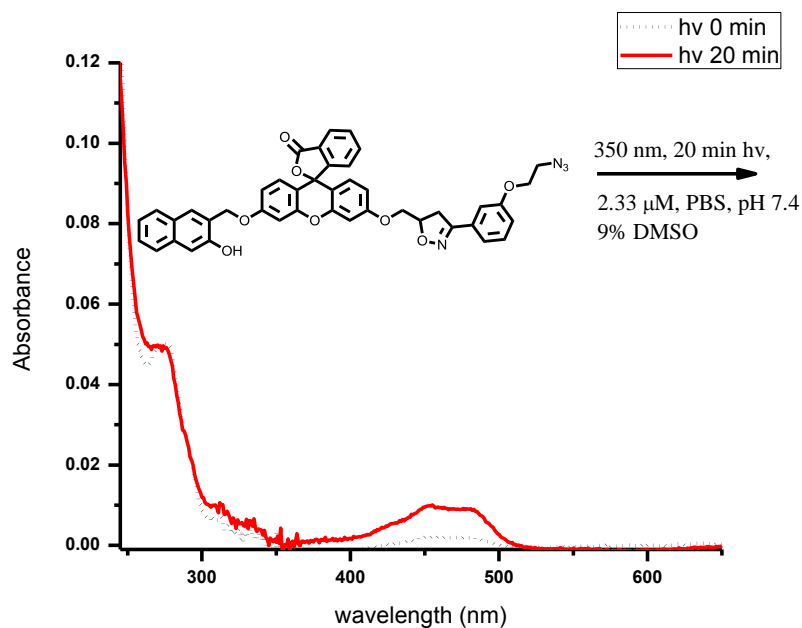


Figure 4.3 Absorption spectra of 2.33 μ M NQMP-FL-A **4.2** in 0.1M PBS buffer pH 7.4 containing 9% DMSO co-solvent; dotted dark line before and solid red line after photolysis

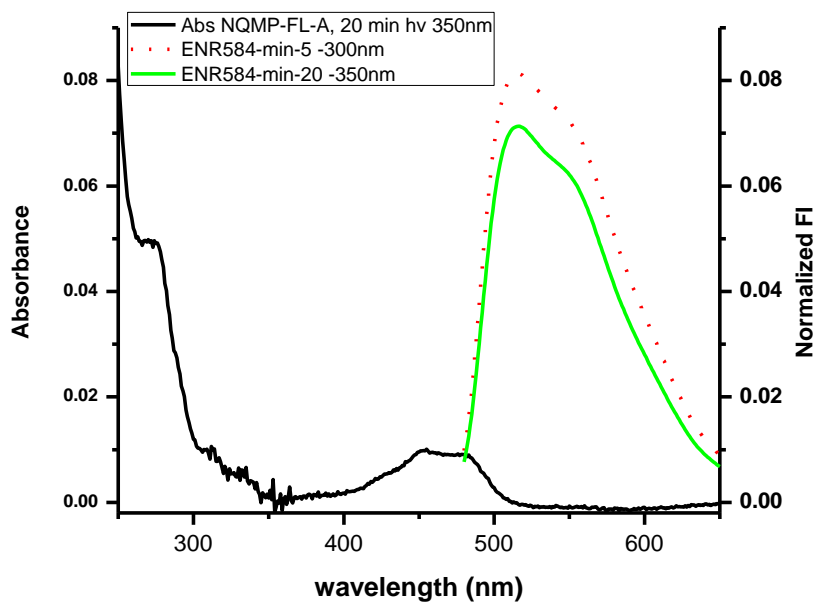


Figure 4.4 Absorption and emission spectra of 2.33 μ M NQMP-FL-A **4.2** in 0.1M PBS buffer pH 7.4 containing 9% DMSO co-solvent; (solid dark line) absorbance, (dotted red line) emission after 20 min photolysis at 350 nm, solid green line after hv 5 min at 300nm.

In 90% aqueous methanol solution, NQMP-FL-A **4.2** does not show any absorbance between 400 -500 nm. However, in PBS buffer at pH 7.4 containing 9% of DMSO as co-solvent, a small band could be observed within this region. It becomes necessary to determine the stability of NQMP-FL-A **4.2** in wholly aqueous solutions.

4.3 CONCLUSIONS AND FUTURE DIRECTIONS

We have developed a second generation caged fluorophore with an easily functionalizable handle for further derivatization. We are currently working on the fluorescence quantum determination and the investigation of the dark stability of NQMP-FL-A **4.2** in PBS buffer at pH 7.4.

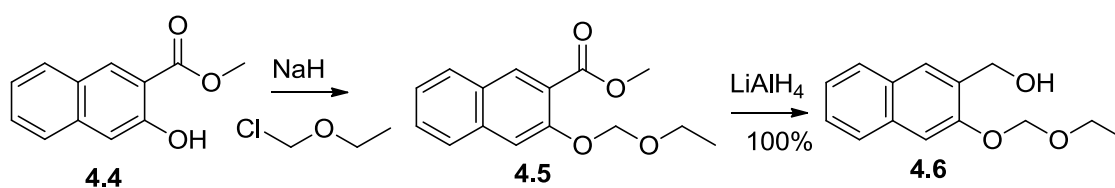
4.4 EXPERIMENTAL SECTION

General Methods. All organic solvents were dried and freshly distilled before use. Flash chromatography was performed using 40-75 μm silica gel. All NMR spectra were recorded in CDCl_3 using 400 MHz instrument (unless otherwise noted). Buffer solutions for kinetic experiments were prepared using literature pKa values of the buffer acids and activity coefficient recommended by Bates.³⁹ Methyl 3-hydroxy-2-naphthoate **4.4**, 2-bromoethanol **4.14** and chloromethyl ethyl ether were purchased from TCI America. 3-hydroxybenzaldehyde **4.16**, allyl bromide and fluorescein **4.8** were purchased from Alfa Aesar. All chemicals were used as received.

Fluorescent measurements were conducted in aqueous buffers containing 4-8% of DMSO as co-solvent at 2.8 or 3.4 μM concentrations of **3.14**. Fluorescent spectra were recorded using 460 nm excitation light. The excitation source and the detector slit were

set to 1 nm and 5 nm respectively. The fluorescence quantum yield was determined using fluorescein in 0.1 N NaOH ($\Phi_{\text{Fl}} = 0.95$)⁴³ as the standard reference. Fluorescent spectra were recorded using spectrometer equipped with polarizer. The path length was 1 cm with a cell volume of 3 mL.

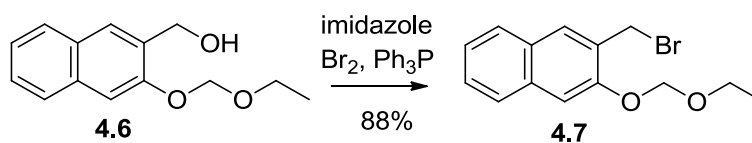
4.5 SYNTHETIC PROCEDURES



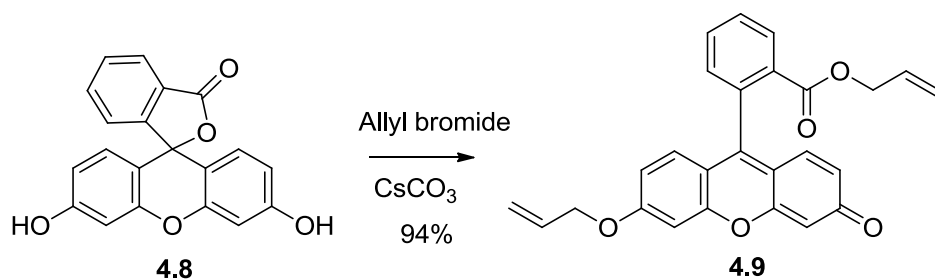
(3-(Ethoxymethoxy)naphthalen-2-yl)methanol (4.6) Sodium hydride (0.833 g, 22.55 mmol) was added to an ice-cold solution of methyl 3-hydroxy-2-naphthoate (1.52 g, 7.52 mmol) in THF (80 mL). The mixture was stirred for 20 min and chloromethyl ethyl ether (1.395 mL, 15.03 mmol) was added dropwise. The solution was allowed to warm to rt, stirred overnight and quenched with 5% HCl (20 mL). The crude ether was extracted with ether (3 x 50 mL). The organic layer was washed with saturated aqueous NaCl and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*. The crude product was used in the next step without further purification.

Powered lithium aluminum hydride (0.571 g, 15.03 mmol) was added in portions to a solution of crude naphthyl ether **4.5** in THF (50 mL) at 0 °C. The mixture was allowed to warm up to rt, stirred overnight, quenched with EtOAc (10 mL) and filtered through a layer of Celite. 5% HCl (20 mL) was then passed through the Celite, followed by EtOAc (40 mL). The organic layer was extracted with ether (3 x 50 mL) and washed with brine

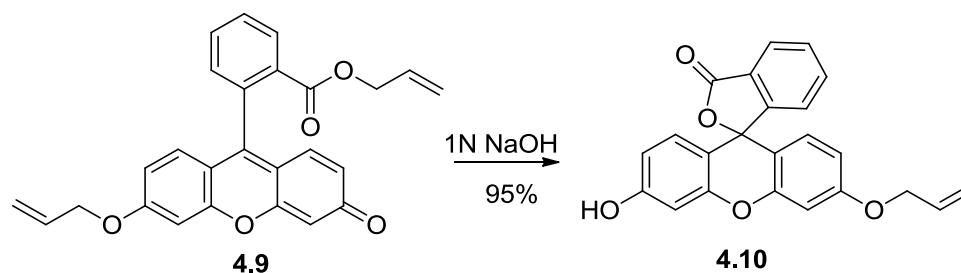
(20 mL). Solvents were evaporated and the crude product purified by silica gel chromatography eluting with 20-50% EtOAc/hexane containing 2%TEA to afford (3-(ethoxymethoxy)naphthalen-2-yl)methanol (1.74 g, 7.49 mmol, 100 % yield) as a colorless oil. ^1H NMR δ 7.79 – 7.73 (m, 3H), 7.48 – 7.41 (m, 2H), 7.40 – 7.35 (m, 1H), 5.41 (s, 2H), 4.85 (s, 2H), 3.78 (q, J = 7.1 Hz, 2H), 1.26 (t, J = 7.1 Hz, 3H). ^{13}C NMR δ 153.61, 134.18, 131.09, 129.37, 127.79, 127.64, 126.97, 126.43, 124.45, 109.20, 109.18, 93.53, 77.56, 77.24, 76.92, 64.90, 62.33, 15.33, 15.31.



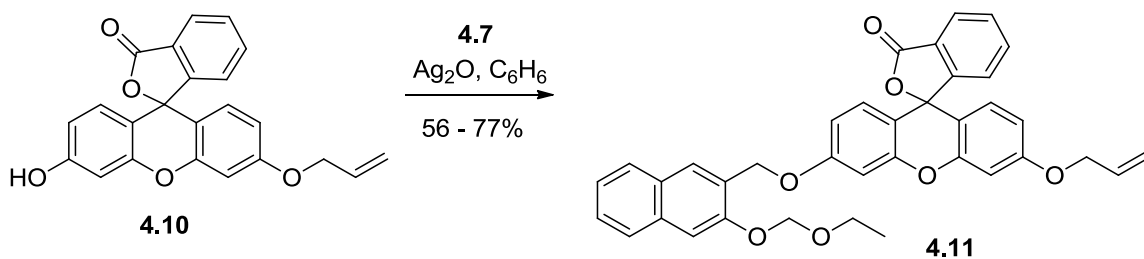
2-(Bromomethyl)-3-(ethoxymethoxy)naphthalene (4.7) Br_2 (0.566 mL, 10.98 mmol) was added to an ice-cold solution of Ph_3P (2.88 g, 10.98 mmol) and imidazole (0.747 g, 10.98 mmol) in DCM (60 mL) and the mixture stirred for 10 min after which a solution of (3-(ethoxymethoxy)naphthalen-2-yl)methanol **4.6** (1.7 g, 7.32 mmol) in DCM (40 mL) was added via a cannula. The mixture was stirred at 0°C for 1h and the precipitate filtered out. The filtered solid was rinsed with 20% Ether/hexane (2 x 50 mL). The combined filtrate was washed with a saturated solution of sodium dithionite (2 x 50 mL), brine (20 mL) and the solvent evaporated. The crude residue was passed through a short plug of silica gel eluting with hexane to afford 2-(bromomethyl)-3-(ethoxymethoxy)naphthalene **4.7** (1.9 g, 6.44 mmol, 88 % yield) as colorless crystals. M.p = 108 - 110 $^\circ\text{C}$



Bis-allyl-fluorescein (4.9) Allyl bromide (2.60 mL, 30.1 mmol) was added to a solution of fluorescein **4.8** (2.5 g, 7.52 mmol) and cesium carbonate (6.13 g, 18.81 mmol) in DMF (75 mL). The mixture was stirred at 60 °C overnight, then poured into cold water (150 mL). The orange precipitate was filtered off, washed with cold water (3 x 50 mL) and dried to afford *bis*-allyl-fluorescein **4.9** (2.82 g, 6.84 mmol, 91 % yield) as a yellow solid which was recrystallized from CCl₄. m.p = 150 -152 °C. Lit.⁸⁹ 153 -155° C. ¹H NMR δ 8.86 (d, 1H, *J* = 7.6 Hz), 7.74 (t, 1H, *J* = 7.6, 6.8 Hz), 7.68 (t, 1H, *J* = 7.6, 6.8 Hz), 7.31 (d, 1H, *J* = 7.2 Hz), 6.95 (d, 1H, *J* = 2 Hz), 6.89 (m, 2H), 6.76 (dd, 1H, *J* = 8.8, 2.4 Hz), 6.54 (dd, 1H, *J* = 9.6, 1.6 Hz), 6.45 (d, 1H, *J* = 1.6 Hz), 6.11 -6.01 (m, 1H), 5.64 - 5.54 (m, 1H), 5.46 (d, 1H, *J* = 17.2 Hz), 5.36 (d, 1H, *J* = 10.4 Hz), 5.12 (d, 1H, *J* = 4.4 Hz), 5.08 (s, 1H), 4.65 (d, 2H, *J* = 5.2 Hz), 4.52 - 4.42 (m, 2H). ¹³C NMR δ 185.9, 165.2, 163.2, 159.1, 154.4, 149.6, 134.6, 132.8, 132.1, 131.4, 131.2, 130.7, 130.2, 129.9, 129.1, 119.4, 118.9, 118.0, 115.2, 113.9, 106.0, 101.4, 69.7, 66.2

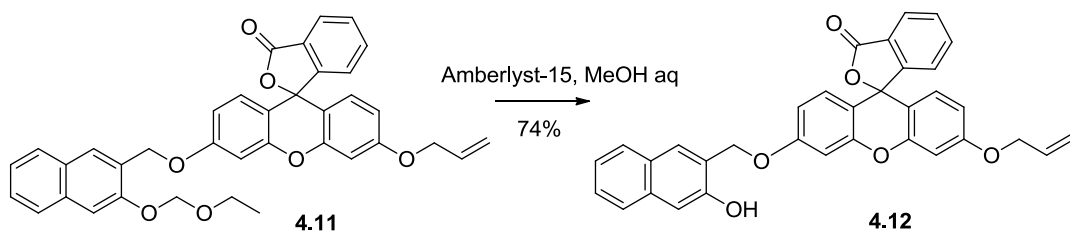


3'-Hydroxy-6'-allyloxy-fluorescein (4.10) A solution of sodium hydroxide (25.5 mL, 25.5 mmol) was added to a solution of *bis*-allyl-fluorescein **4.9** (0.750 g, 1.818 mmol) in MeOH(40 mL). The mixture was stirred at rt for 1 h and MeOH was evaporated. The reaction was neutralized with 25 mL of 1.0 M HCl and extracted with DCM (3 x 20 mL).The organic layer was concentrated in vacuo and the crude purified by silica gel chromatography eluting with 40% EtOAc/hexane to afford 3'-hydroxy-6'-allyloxy-fluorescein **4.10** (0.644 g, 1.729 mmol, 95 % yield) as a yellow solid. ^1H NMR (DMSO- d_6) δ 10.16 (s, 1H), 8.00 (d, J = 7.5 Hz, 1H), 7.79 (t, J = 7.4 Hz, 1H), 7.72 (t, J = 7.4 Hz, 1H), 7.28 (d, J = 7.6 Hz, 1H), 6.94 (d, J = 2.4 Hz, 1H), 6.75 – 6.67 (m, 2H), 6.64 (d, J = 8.8 Hz, 1H), 6.57 (s, 2H), 6.10 – 5.96 (m, 1H), 5.41 (dd, J = 17.3, 1.5 Hz, 1H), 5.28 (d, J = 10.6 Hz, 1H), 4.64 (d, J = 5.2 Hz, 2H). ^{13}C NMR (DMSO- d_6) δ 168.54, 163.83, 159.82, 159.47, 152.37, 151.74, 151.71, 135.56, 133.15, 130.06, 128.98, 128.85, 125.99, 124.57, 123.93, 117.72, 112.72, 112.30, 111.12, 109.40, 104.24, 102.15, 101.55, 82.61, 68.54, 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89. M.p 189 -190 °C. Lit ⁹⁶ 206 -207 °C.

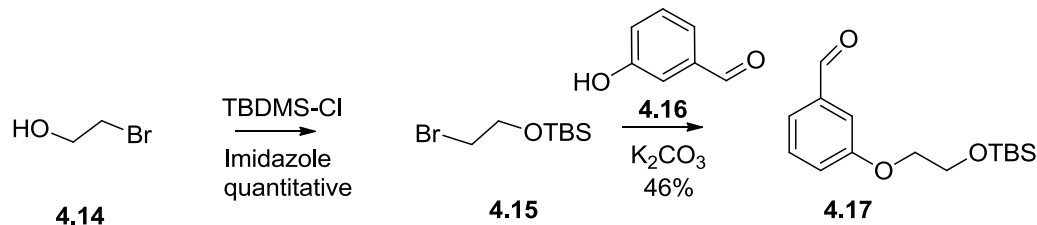


3'-(Allyloxy)-6'-((3-(ethoxymethoxy)naphthalen-2-yl)methoxy)-3H-

spiro[isobenzofuran-1,9'-xanthen]-3-one (4.11) Silver(I)oxide (0.317 g, 1.370 mmol) was added to a solution of 3'-(allyloxy)-6'-hydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one **4.10** (0.300 g, 0.806 mmol) and 2-(bromomethyl)-3-(ethoxymethoxy)naphthalene **4.7** (0.380 g, 1.289 mmol) in dry benzene (10 mL) and THF (2 mL) and the mixture refluxed for 16 h. The mixture was allowed to cool and filtered through a thin layer of Celite and the Celite rinsed with EtOAc (80 mL). The solvents were evaporated and the residue purified by silica gel chromatography eluting with 25% acetone/hexane to afford 3'-(allyloxy)-6'-((3-(ethoxymethoxy)naphthalen-2-yl)methoxy)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one **4.11** (0.363 g, 0.619 mmol, 77% yield) as yellowish oil. ^1H NMR δ 8.03 (d, J = 7.6 Hz, 1H), 7.90 (s, 1H), 7.78 (t, J = 7.6 Hz, 2H), 7.70 - 7.63 (m, 2H), 7.50 - 7.42 (m, 2H), 7.37(t, J = 7.6 Hz, 1H), 7.19(d, J = 7.5 Hz, 1H), 6.92(d, J = 2.2 Hz, 1H), 6.79 - 6.68 (m, 4H), 6.66 - 6.61 (m, 1H), 6.11 - 5.98 (m, 1H), 5.45 - 5.41 (m, 3H), 5.34 - 5.26 (m, 3H), 4.57(d, J = 5.3 Hz, 2H), 3.80 (q, J = 7.1 Hz, 2H), 1.30 (t, 3H). ^{13}C NMR δ 169.58, 160.87, 160.52, 153.38, 153.02, 152.75, 152.69, 135.13, 134.35, 132.85, 129.86, 129.32, 129.24, 127.99, 127.95, 127.14, 127.02, 126.69, 126.68, 125.22, 124.49, 124.18, 118.32, 112.59, 112.35, 111.75, 111.72, 109.17, 102.14, 102.01, 93.47, 83.42, 77.55, 77.23, 76.91, 69.30, 66.28, 64.87, 15.41. HRMS-ESI $[\text{M}+\text{H}]^+$ calc for $\text{C}_{37}\text{H}_{31}\text{O}_7^+$ 587.2064 found 587.2068.

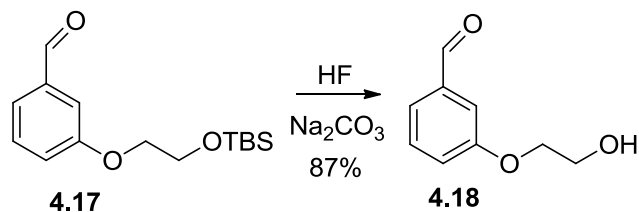


3'-((Allyloxy)-6'-((3-hydroxynaphthalen-2-yl)methoxy))-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (4.12) Amberlyst-15 (300 mg) and water (1 mL) were added To a solution of 3'-((allyloxy)-6'-((3-(ethoxymethoxy)naphthalen-2-yl)methoxy))-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one **4.11** (0.300 g, 0.511 mmol) dissolved in MeOH (20 mL) at 50 °C. The mixture was stirred at 50 °C overnight. Amberlyst-15 was filtered off and the solvents were evaporated to afford crude 3'-((allyloxy)-6'-((3-hydroxynaphthalen-2-yl)methoxy))-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one **4.12** (0.201 g, 0.380 mmol, 74.4 % yield) as pale yellow oil which was used without further purification. m.p = 92 - 95 °C ^1H NMR (Acetone- d_6) δ 9.11 (s, 1H), 8.00 (d, J = 7.6 Hz, 1H), 7.95 (s, 1H), 7.82 – 7.77 (m, 2H), 7.75 – 7.66 (m, 2H), 7.45 – 7.35 (m, 2H), 1.15 – 1.09 (m, 1H), 7.31 – 7.25 (m, 3H), 6.86 (dd, J = 8.1, 1.8 Hz, 2H), 2.99 – 2.95 (m, 1H), 7.00 (d, J = 2.5 Hz, 1H), 6.77 (d, J = 8.8 Hz, 1H), 6.73 (d, J = 2.0 Hz, 2H), 6.13 – 6.03 (m, 1H), 5.43 (dd, J = 17.3, 1.7 Hz, 1H), 5.37 (s, 2H), 5.27 (dd, J = 10.6, 1.4 Hz, 2H), 4.67 – 4.61 (m, 2H). ^{13}C NMR (Acetone- d_6) δ 169.48, 161.78, 161.42, 154.16, 153.97, 153.36, 153.32, 136.18, 135.64, 134.23, 130.91, 130.10, 130.02, 129.39, 129.23, 128.67, 127.81, 127.46, 127.23, 126.77, 125.53, 124.98, 124.21, 117.96, 113.39, 113.25, 112.78, 112.72, 110.10, 102.72, 102.68, 102.59, 102.54, 83.35, 69.77, 66.81. HRMS-ESI $[\text{M}+\text{H}]^+$ calc for $\text{C}_{34}\text{H}_{25}\text{O}_6^+$ 529.1646, found 529.1656.

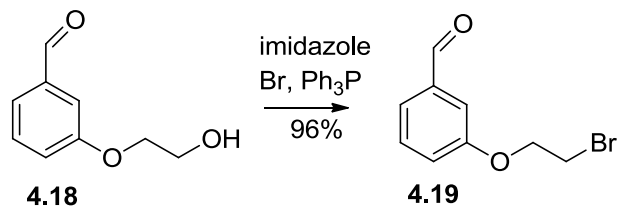


3-(2-((tert-Butyldimethylsilyl)oxy)ethoxy)benzaldehyde (4.17) Imidazole (6.27 g, 92 mmol), TBDMS-Cl (12.81 g, 85 mmol) and catalytic amount of DMAP were added to a round bottom flask containing 2-bromoethanol **4.14** (5 mL, 70.8 mmol) in DCM (25 mL). The mixture was stirred at rt for 12 h, water was added and the mixture was extracted with ether (3 x 20 mL). Solvents were evaporated and the crude mixture purified by silica gel chromatography eluting with 5% ether/hexane to afford silyl ether **4.15** (17 g, 71.1 mmol, 100 % yield) as a colorless oil.

K_2CO_3 (4.53 g, 32.8 mmol) was added to a solution of 3-hydroxybenzaldehyde **4.16** (2.0 g, 16.38 mmol) in DMF (20 mL) at rt and the mixture stirred for 20 min after which silyl ether **4.15** (4.70 g, 19.65 mmol) was added and the mixture stirred overnight. Work up and purification through a short layer of silica gel afforded 3-(2-((tert-butyldimethylsilyl)oxy)ethoxy)benzaldehyde **4.17** (4.201 g, 7.49 mmol, 45.7 % yield) as a colorless oil with 50% purity (NMR). 1H NMR δ 9.95 (s, 1H), 7.45 – 7.36 (m, 3H), 7.18 (dt, J = 6.8, 2.5 Hz, 1H), 3.87 (t, J = 6.5 Hz, 2H), 3.38 (t, J = 6.5 Hz, 2H), 0.89 (d, 15H).

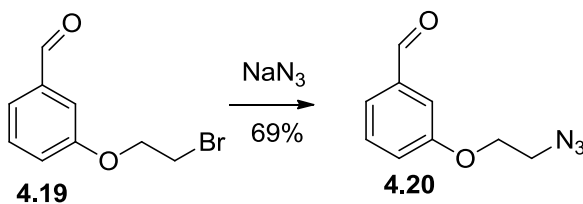


3-(2-Hydroxyethoxy)benzaldehyde (4.18) Aqueous HF (0.473 mL, 14.26 mmol) was added to a solution of 3-(2-((tert-butyldimethylsilyl)oxy)ethoxy)benzaldehyde **4.17** (2.0 g, 7.13 mmol) in Acetonitrile (40 mL) at rt. The reaction was stirred and monitored by TLC. After 15 min, solid NaHCO₃ (1.512 g, 14.26 mmol) was added and the product filtered. The solid NaHCO₃ was washed with ether (3 x 5 mL). Evaporation of the solvents and purification by flash chromatography on silica gel eluting with 40% EtOAc/hexane afforded 3-(2-hydroxyethoxy)benzaldehyde **4.18** (1.036 g, 6.23 mmol, 87 % yield) as a colorless oil. ¹H NMR δ 9.93 (s, 1H), 7.46 – 7.41 (m, 2H), 7.38 – 7.36 (m, 1H), 7.18 (dt, J = 7.3, 2.3 Hz, 1H), 4.12 (t, 2H), 3.98 (s-broad, 2H), 2.65 (s-broad, 1H). ¹³C NMR δ 192.22, 159.40, 137.93, 130.28, 123.95, 122.03, 113.08, 77.55, 77.23, 76.91, 69.70, 61.35.



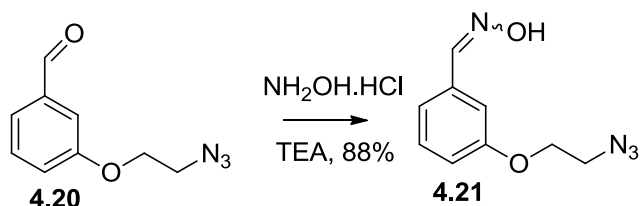
3-(2-Bromoethoxy)benzaldehyde (4.19) Br₂ (0.316 mL, 6.14 mmol) was added to an ice-cold solution of Ph₃P (1.503 g, 5.73 mmol) and imidazole (0.418 g, 6.14 mmol) in DCM (10 mL). The mixture was stirred for 10 min after which a solution of 3-(2-

hydroxyethoxy)benzaldehyde **4.18** (0.680 g, 4.09 mmol) in DCM (10 mL) was added in portions. The reaction mixture was stirred at 0° C for 1h and the precipitate filtered out. The filtered solid was washed with Ether (2 x 50 mL). The combined filtrate was then washed with a saturated solution of sodium dithionite (2 x 30 mL), brine (20 mL) and dried over sodium sulfate. The solvent was then evaporated and the crude residue purified by silica gel chromatography eluting with 20% EtOAc/hexane (R_f = 0.34) to afford 3-(2-bromoethoxy)benzaldehyde **4.19** (0.900 g, 3.93 mmol, 96 % yield) as colorless oil. ^1H NMR δ 9.97 (s, 1H), 7.50 – 7.43 (m, 2H), 7.40 – 7.35 (m, 2H), 7.22 – 7.18 (m, 1H), 4.35 (t, J = 6.1 Hz, 2H), 3.66 (t, J = 6.1 Hz, 2H). ^{13}C NMR δ 192.03, 162.88, 158.89, 138.05, 130.43, 124.31, 122.22, 113.16, 77.55, 77.23, 76.91, 68.25, 60.58, 53.59, 34.83, 31.74, 28.95, 22.81, 14.25.

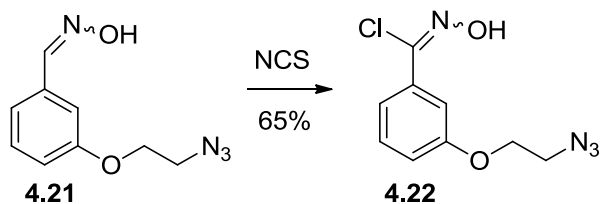


3-(2-Azidoethoxy)benzaldehyde (4.20) Sodium azide (0.461 g, 7.09 mmol) was added to a solution of 3-(2-bromoethoxy)benzaldehyde **4.19** (0.650 g, 2.84 mmol) in DMF (10 mL) and the mixture stirred at 100° C overnight. The reaction mixture was allowed to cool to rt, poured into a saturated solution of ammonium chloride (50mL) and extracted with DCM (3 x 50 mL). The organic phase was washed with water (3 x 30 mL), brine (20 mL), dried over sodium sulfate and the solvent evaporated. The crude residue was passed through a short layer of silica gel eluting with hexane to afford 3-(2-

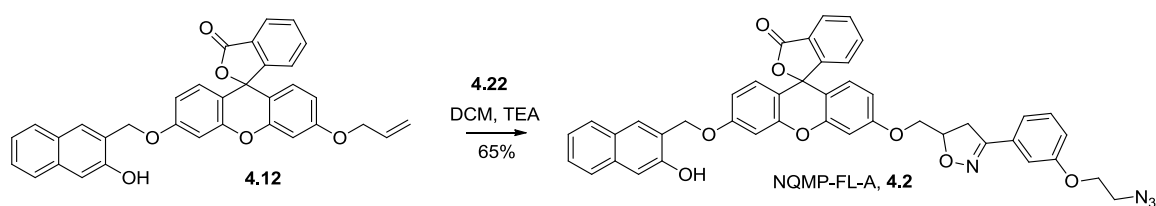
azidoethoxy)benzaldehyde **4.20** (0.372 g, 1.946 mmol, 68.6 % yield) as colorless oil (R_f = 0.29, 20% EtOAc/Hexane). ^1H NMR δ 9.97 (s, 1H), 7.52 – 7.43 (m, 2H), 7.42 – 7.37 (m, 2H), 7.24 – 7.19 (m, 1H), 4.23 – 4.19 (m, 2H), 3.63 (t, J = 4.9 Hz, 2H). ^{13}C NMR δ 192.01, 159.00, 138.06, 130.41, 124.32, 122.18, 112.85, 77.55, 77.23, 76.91, 67.40, 50.24.



3-(2-Azidoethoxy)benzaldehydeoxime (4.21) Hydroxylamine hydrochloride (0.191 g, 2.75 mmol) was added to a solution of 3-(2-azidoethoxy)benzaldehyde **4.20** (0.350 g, 1.831 mmol) and Et_3N (0.383 mL, 2.75 mmol) in DCM (10 mL). The mixture was stirred at rt for 2 h, then diluted with water (10 mL). The aqueous layer was extracted with DCM (3 x 15 mL). The combined organic layer was washed with saturated NaHCO_3 (10 mL) and brine (5 mL). The organic layer was dried with sodium sulfate, filtered and concentrated. Purification by silica gel chromatography eluting with 20% EtOAc/hexane gave (*E*)-3-(2-azidoethoxy)benzaldehydeoxime **4.21** (0.334 g, 1.620 mmol, 88 % yield) as a colorless oil. ^1H NMR δ 8.71 – 8.68 (s, 1H), 8.17 – 8.11 (s, 1H), 7.36 – 7.30 (t, J = 7.8 Hz, 1H), 7.20 – 7.15 (m, 3H), 7.01 – 6.94 (dd, J = 8.2, 1.6 Hz, 1H), 4.20 – 4.15 (t, J = 5.0 Hz, 2H), 3.63 – 3.58 (t, J = 4.9 Hz, 2H). ^{13}C NMR δ 158.72, 150.38, 133.59, 130.15, 121.01, 117.23, 112.03, 77.55, 77.23, 76.91, 67.23, 50.32.



3-(2-Azidoethoxy)-N-hydroxybenzimidoyl chloride (4.22) NCS (0.194 g, 1.455 mmol) was added in portions to a solution of oxime **4.21** (0.300 g, 1.455 mmol) in DMF (2 mL). A small portion of HCl gas (0.1 mL) from the headspace above a solution of conc. HCl was bubbled into the solution to initiate the reaction. After 4 min, the temperature rose to 31° C. After about 20 min, the temperature dropped to 25°C, and then ether (20 mL), water (5 mL) and saturated ammonium chloride (10 mL) were added. The organic phase was extracted with ether (3 x 10 mL), dried over sodium sulfate and the solvent removed under reduced pressure to give crude (Z)-3-(2-azidoethoxy)-N-hydroxybenzimidoyl chloride **4.22** (0.227 g, 0.943 mmol, 64.8 % yield) as a pale yellow oil. ¹H NMR δ 10.39 – 10.37 (s, 1H), 7.50 – 7.47 (m, 1H), 7.40 (s, 1H), 7.34 – 7.27 (m, 1H), 7.02 – 6.95 (m, 1H), 4.20 – 4.15 (t, *J* = 5.0 Hz, 2H), 3.64 – 3.57 (t, *J* = 5.0 Hz, 2H). ¹³C NMR δ 158.34, 138.45, 134.52, 129.69, 120.60, 117.23, 113.11, 67.69, 67.31, 50.33, 50.21, 81.



NQMP-FL-A (4.2) A solution of TEA (0.036 mL, 0.255 mmol) in DCM (2 mL) was added dropwise to a stirred solution of 3'-(allyloxy)-6'-((3-hydroxynaphthalen-2-yl)methoxy)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one **4.12** (90 mg, 0.170 mmol) and 3-(2-azidoethoxy)-*N*-hydroxybenzimidoyl chloride **4.22** (45.1 mg, 0.187 mmol) in DCM (3.5 mL) at rt and the mixture stirred overnight. Solvent was evaporated and the residue purified by silica gel chromatography eluting with DCM/EtOAc/hexane (2:3:7) to afford 3'-((3-(3-(2-azidoethoxy)phenyl)-4,5-dihydroisoxazol-5-yl)methoxy)-6'-((3-hydroxynaphthalen-2-yl)methoxy)-3*H*-spiro[isobenzofuran-1,9'-xanthen]-3-one NQMP-FL-A **4.2** (66 mg, 0.090 mmol, 52.9 % yield) as a pale yellow fluffy solid, m.p (softens) 92 - 93°C. IR (neat, cm⁻¹) 3345, 3058, 2926, 2873, 2108, 1762, 1612, 1501, 1425, 1345, 1284, 1248, 1188, 1110. ¹H NMR (Acetone-d₆) δ 9.09 (s, 1H), 8.00 (d, *J* = 7.6 Hz, 1H), 7.95 (s, 1H), 7.82 – 7.67 (m, 4H), 7.48 – 7.27 (m, 7H), 7.07 – 7.05 (m, 1H), 7.00 (s, 1H), 6.91 (s, 1H), 6.88 – 6.86 (m, 1H), 6.85 – 6.74 (m, 3H), 5.37 (s, 2H), 5.20 – 5.08 (m, 1H), 4.31 – 4.23 (d, *J* = 4.1 Hz, 4H), 3.71 – 3.65 (t, *J* = 4.8 Hz, 2H), 3.64 – 3.36 (m, 2H). HRMS-ESI [M+H]⁺ calc. for C₄₃H₃₃N₄O₈ 733.2293, found 733.2292

CHAPTER 5

PHOTOACTIVATABLE NUCLEOPHILIC SUBSTITUTION (S_N1)

5.1 INTRODUCTION

Destabilized carbocations have been of interest as transients observable by laser flash photolysis in neutral solutions. The 9-fluorenol system was chosen for the present investigation because of the known ease of formation of cationic 9-fluorenyl intermediates⁹⁹ through photodehydroxylation. The 9-fluorenyl cation is thermodynamically unstable, largely due to its antiaromatic ($4n\pi$) character, and attempts by Olah and co-workers¹⁰⁰ to generate the parent ion even in cold strong acid solution were not successful. However, following the initial detection of transient carbocations by laser flash photolysis (LFP) in 1986,¹⁰¹ the 9-fluorenyl cation has been observed directly and found to have a life time of less than 20 ps in aqueous solution.¹⁰² Thereafter, the existence and characterization of transient 9-fluorenyl cation and a number of its derivatives have been reported.¹⁰²⁻¹⁰³

Early studies by Wan *et al*¹⁰⁴ showed that photolysis of 9-fluorenol and some of its derivatives in aqueous protic solvents initiates photohomolysis and photoheterolysis involving the C-O bond. Similar results were also obtained by Mecklenburg and Hilinski¹⁰² as well as Steenken and coworkers.¹⁰⁵ These reactions were extraordinary in that the hydroxide ion which is normally a poor leaving group in the ground state surprisingly becomes a better leaving group in the excited state, given the appropriate solvent and chromophore.^{104b} The primary pathway for photodehydroxylation depends on

the solvent used in the reaction. In polar protic solvents, such as water, or the weakly nucleophilic 2,2,2-trifluoroethanol (TFE) and 2,2,2,2',2',2'-hexafluoroisopropanol (HFIP), the heterolytic pathway is favored. The homolytic pathway is predominant in less polar solvents like alcohols.^{103b,105}

The relative ease of the cation formation even with the poor leaving group (OH⁻) has been attributed to the enhanced reactivity of excited states leading to a system with internal cyclic array (ICA) containing $4n\pi$ systems^{104a,b,106} (Figure 5.1, **5.2**). Conversely, these types of cations are somewhat destabilized in the ground state as evident by solvolysis reactions proceeding via 9-fluorenyl cations that occur far more slowly than their corresponding analogues lacking the central ring.¹⁰⁷ Alcohols such as diphenylmethanol do not undergo this remarkable photo-reaction or do so considerably less efficiently.^{104b}

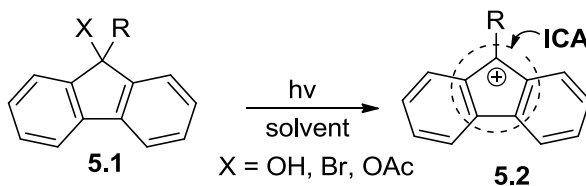
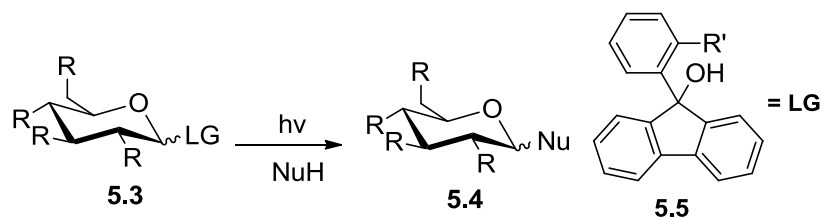


Figure 5.1 Photolysis of 9-fluorenyl derivatives

5.2 RESULTS AND DISCUSSIONS

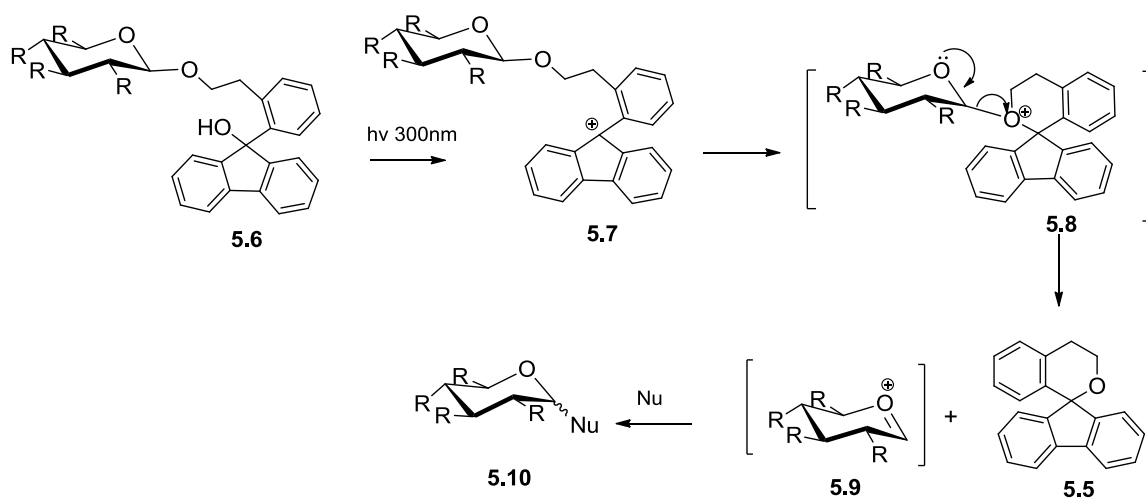
Herein, we report the photoactivation of S_N1 -type reaction at anomeric carbon atoms by photochemical generation of the oxonium ion and the subsequent nucleophilic trapping. Irradiation of a pyranose such as **5.3** bearing a suitably designed leaving group (LG = 9-aryl-9-fluorenyl derivatives) at 300 nm in HFIP/THF mixture affords the corresponding glycosylated product **5.4** (Scheme 5.1). The proposed mechanism for this

reaction is shown in Scheme 5.2. Irradiation of suitably designed 9-fluorenyl derivative **5.6** leads to C-O bond heterolysis and the formation of 9-fluorenyl cation **5.7**.



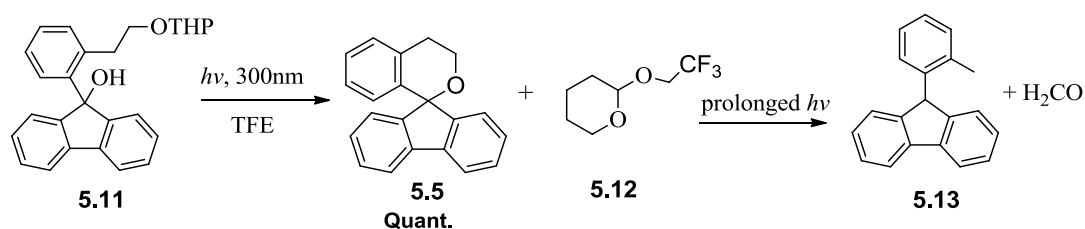
Scheme 5.1 Photo-activation of S_N1 -type reaction.

Cation **5.7** undergoes intramolecular attack by the ether oxygen atom to form intermediate **5.8**. The intermediate **5.8** releases the spiro ether **5.5** as leaving group with the help of the anomeric oxygen. The resulting oxonium ion **5.9** is then trapped by nucleophile to give the addition product **5.10**.



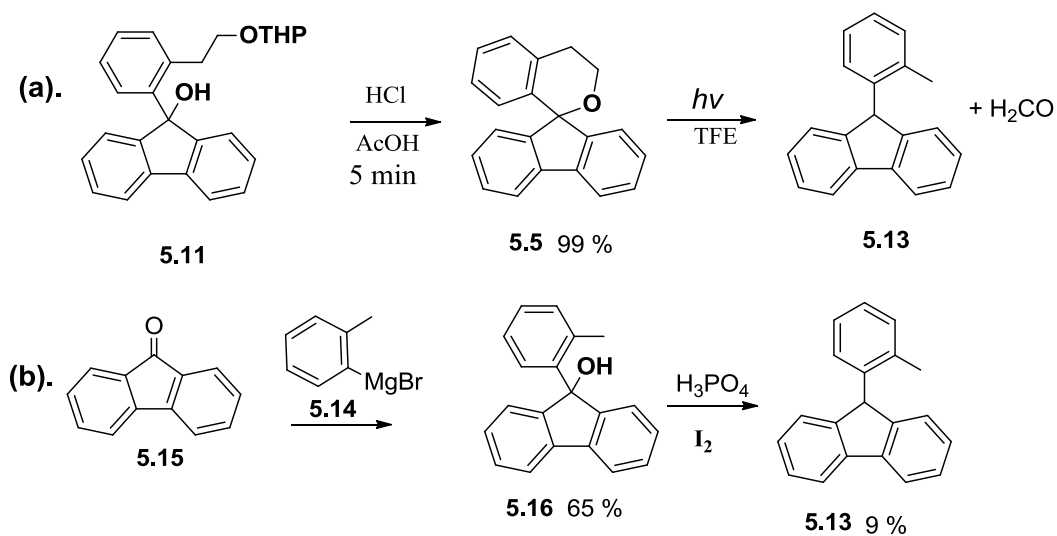
Scheme 5.2 Proposed mechanism for photoactivation of S_N1 -type reaction.

To test our hypothesis, 9-(2-(2-(tetrahydro-2H-pyran-2-yloxy)ethyl)phenyl)-9*H*-fluoren-9-ol **5.11** was used as a model for sugars. Photolysis of 1.03 mM solution of **5.11** in 1.0 mL of polar weakly nucleophilic solvent 2,2,2-trifluoroethanol (TFE) affords spiro compound **5.5** and 2-substituted tetrahydropyranyl ether **5.12** as expected in quantitative yields at up to 40 % conversion (Scheme 5.3). Prolonged irradiation (> 5min) gives rise to secondary photoproduct **5.13** and presumably formaldehyde, which was confirmed by the photolysis of an authentic sample of 3'H-spiro[fluorene-9,1'-isobenzofuran] **5.5** (Scheme 5.4a).



Scheme 5.3 Irradiation of **5.5** in TFE gives 2-substituted tetrahydropyranyl ether **5.12** and secondary photoproduct **5.13**.

We supposed that **5.13** resulted from the photolysis of **5.5** rather than **5.11**. To ascertain this assumption, the spiro ether **5.5** was synthesized from tertiary alcohol **5.11**. Intramolecular cyclization of **5.11** is rapidly accomplished in strong acid to afford spiro ether **5.5**. Photolysis of **5.5** under similar conditions gave 9-(*o*-tolyl)-9*H*-fluorene **5.13** as expected (Scheme 5.4a). The identity of secondary photoproduct **5.13** was further established by reference to an authentic sample prepared through an alternative pathway. Addition of *o*-tolylmagnesium bromide **5.14** to 9-fluorenone **5.15** gave alcohol **5.16**. Acid catalyzed reduction of tertiary alcohol **5.16** afforded 9-(*o*-tolyl)-9*H*-fluorene **5.13** (Scheme 5.4b).



Scheme 5.4 Preparation and irradiation of **5.5** (a) and synthesis of 9-*o*-tolyl-9*H*-fluorene **5.13** (b).

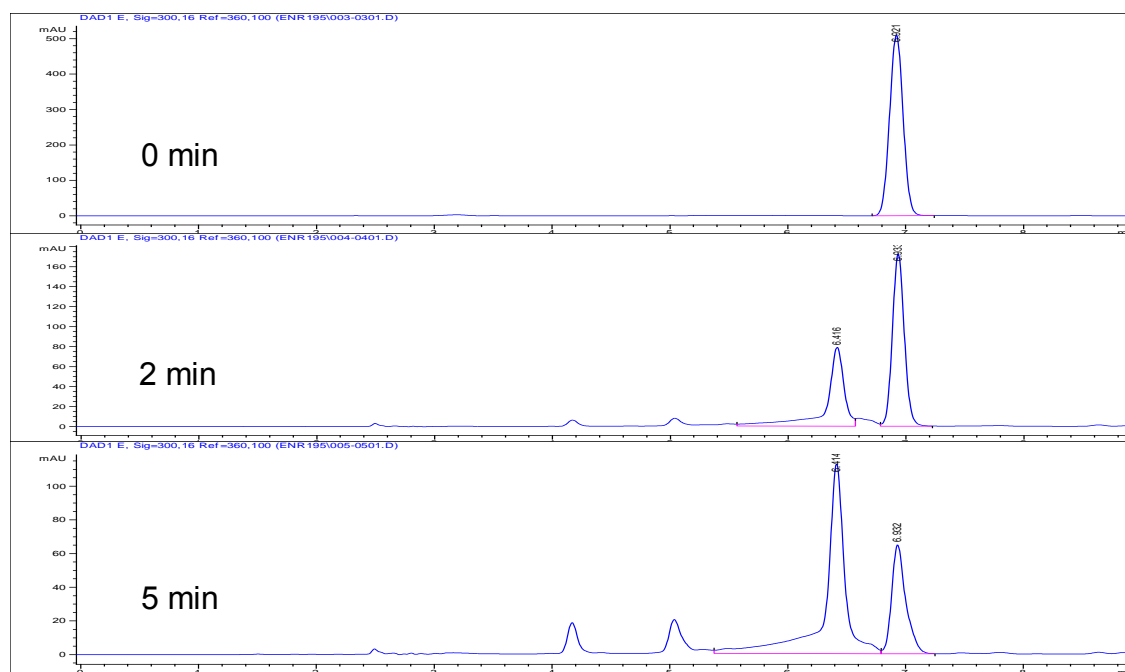
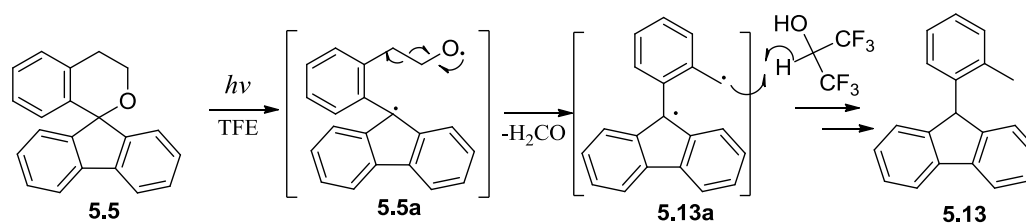


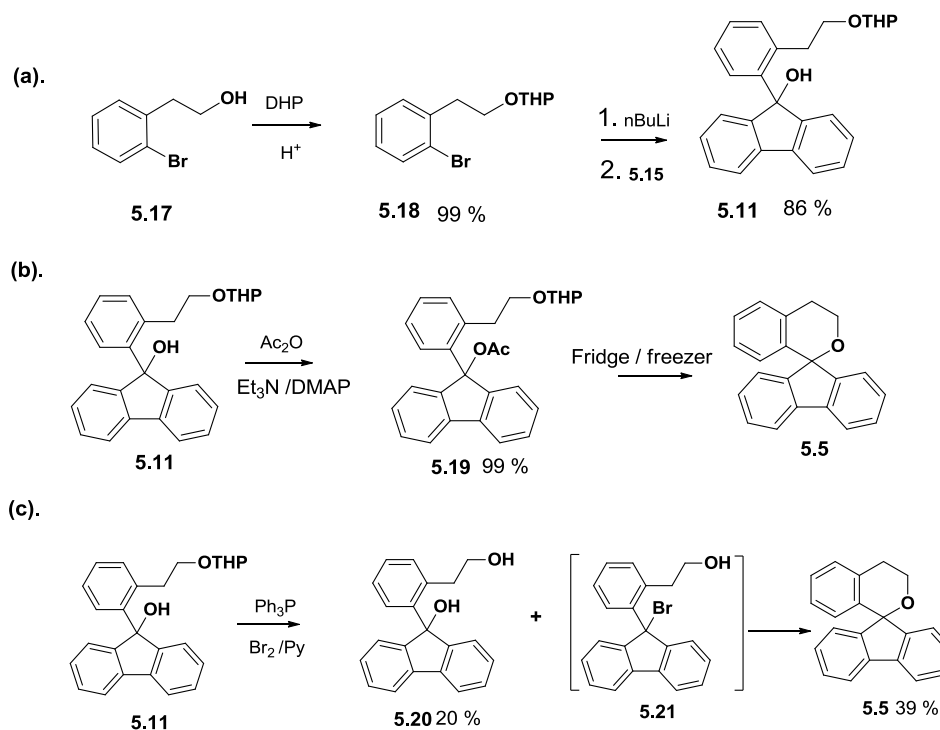
Figure 5.2 HPLC trace of 3.5 mM solution of **5.5** before (0 min) and after irradiation in TFE at 300nm.

The loss formaldehyde in photolysis of **5.5** is rather unusual and unprecedented. We believe that irradiation of spiro ether **5.5** leads to homolysis of the C-O bond to form diradical intermediate **5.5a**. Diradical **5.5a** may recombine to give the starting material or undergo further hemolytic cleavage of the benzylic C-C to form a more stable benzylic diradical **5.13a**. Proton abstraction from solvent then gives the observed product **5.13** (Scheme 5.5).



Scheme 5.5 Proposed mechanism for the formation of **5.13**

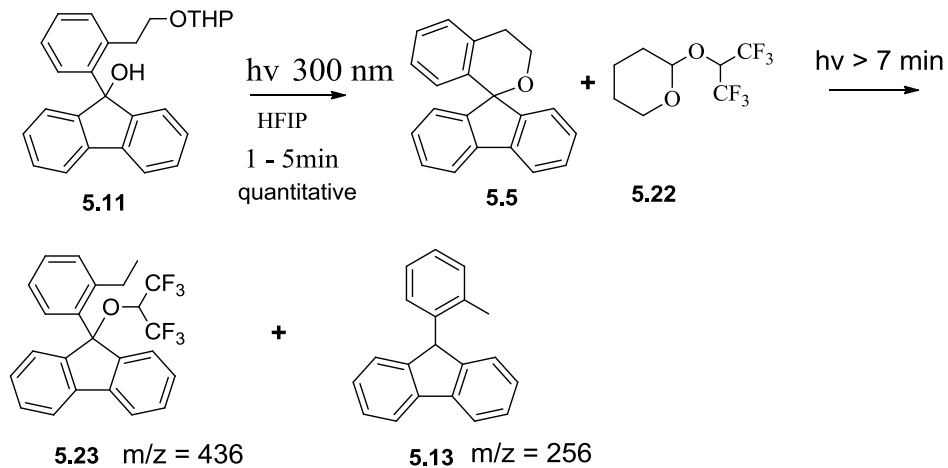
Target compound **5.11** was synthesized as depicted in (Scheme 5.6a) below. 2-Bromophenylethanol **5.17** was protected as the tetrahydropyranyl ether **5.18** in 99% yield. Nucleophilic addition of the anion of **5.18** to 9-fluorenone **5.15** gave the target compound **5.11** in 86% yield. We had also prepared the corresponding acetate **5.19** and attempted the synthesis of the bromide **5.21** to investigate their reactivity. Unfortunately, acetate **5.19** cyclizes to the spiro compound **5.5** upon storage in the dark (Scheme 5.6b). Bromide **5.21** could not be isolated but a substantial amount of the cyclized spiro compound **5.5** was obtained (Scheme 5.6c). We believe that bromide **5.21** cyclizes spontaneously to spiro compound **5.5**.



Scheme 5.6 Synthesis of **5.11** (a), **5.19** (b) and attempted synthesis of 2-(2-(9-bromo-9H-fluoren-9-yl)phenoxy)tetrahydro-2H-pyran **5.21**.

Considering that hexafluoro isopropanol (HFIP) is less nucleophilic than trifluoro ethanol (TFE), we decided to irradiate **5.11** in HFIP as well. Photolysis of **5.11** for up to 5 min at 300 nm (84% conversion) gave the expected hexafluoropropan-2-yl)oxy)tetrahydro-2H-pyran **5.22** in quantitative yield (Scheme 5.7). In these experiments, formation of the spiro product **5.5** was used to determine the extent of the reaction (Figure 5.3).

To achieve complete conversion, it was essential to irradiate the mixture for up to 15 min. As earlier observed with TFE, extended irradiation gave rise to secondary photoproducts **5.23** and **5.13** which arises from photo-decomposition of **5.5** (Scheme 5.7). To reduce the formation of secondary photoproducts, it was therefore necessary to minimize the reaction time to less than 5 min (Table 5.1).



Scheme 5.7 Prolonged irradiation in HFIP gave presumably ether **5.23** with $m/z = 436$ and **5.13**

Time	% conversion 5.11 TFE	% conversion 5.11 HFIP	Chemical yield (cpd 5.5)
1 min	23	27	quantitative
2 min	40	41	quantitative
3 min	69	66	quantitative
5min	N/A	84	quantitative
7min	N/A	90	95%
10min	N/A	96	81%
15min	N/A	98	54%

Table 5.1 Irradiation of 0.67 mM solution of **5.11** in TFE and HFIP at 300 nm

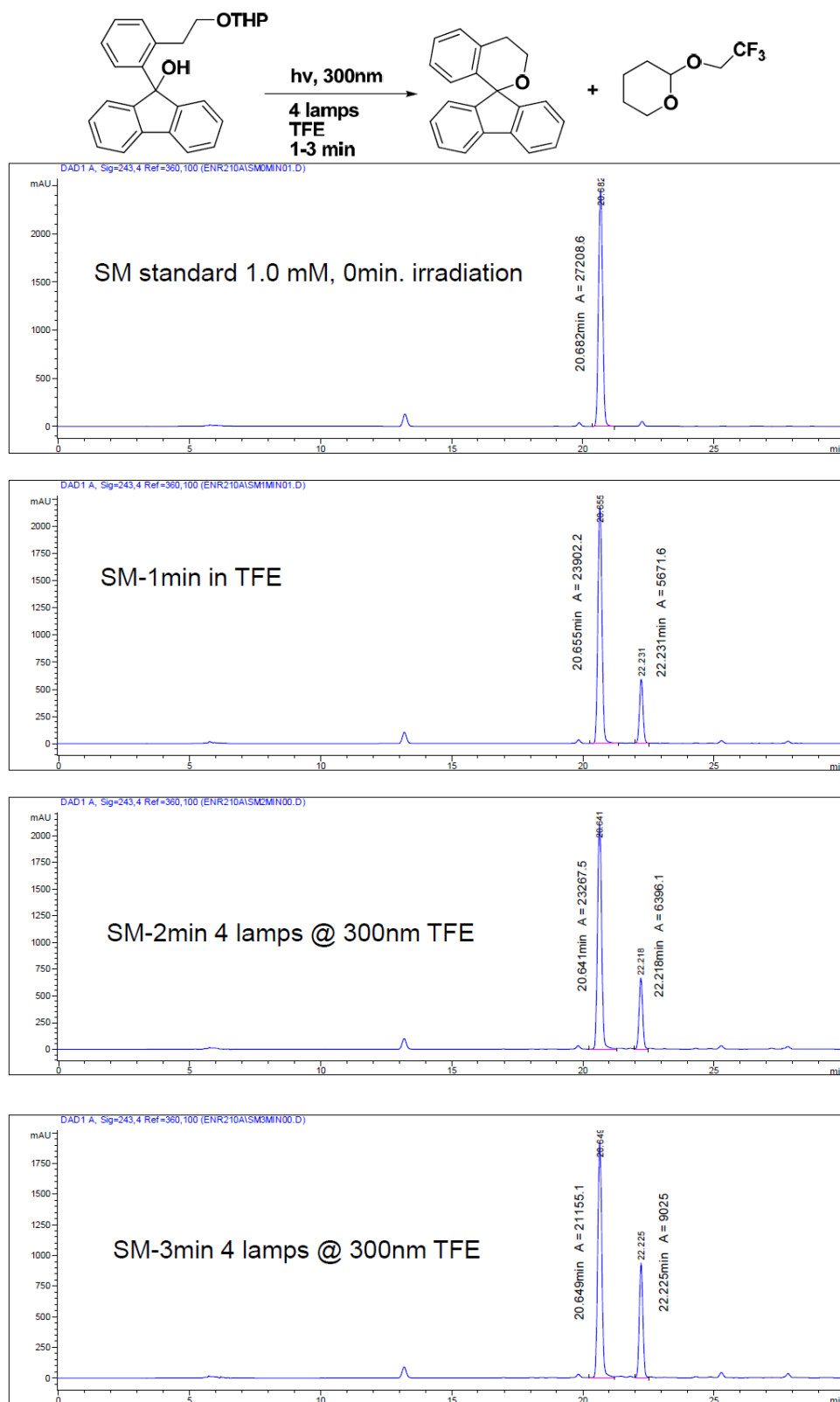
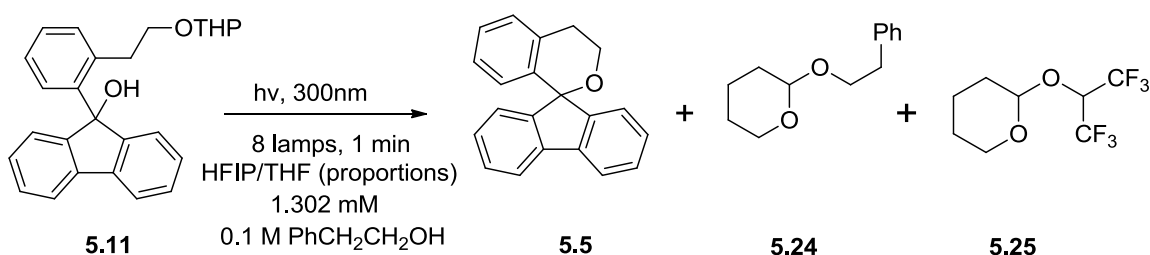


Figure 5.3 HPLC traces **5.11** before and after irradiation

Having determined the reaction conditions we decided to irradiate **5.11** in the presence of a nucleophile; 2-phenyl ethanol. However, in order to achieve this, we needed to use a minimum amount of the required HFIP so as to reduce the formation of side product **5.22**. We found out that a 7:3 HFIP/THF mixture, gave the yield of **5.24**. Under these conditions, the formation of side product **5.25** was negligible and therefore not quantified (Scheme 5.8, Table 5.2)



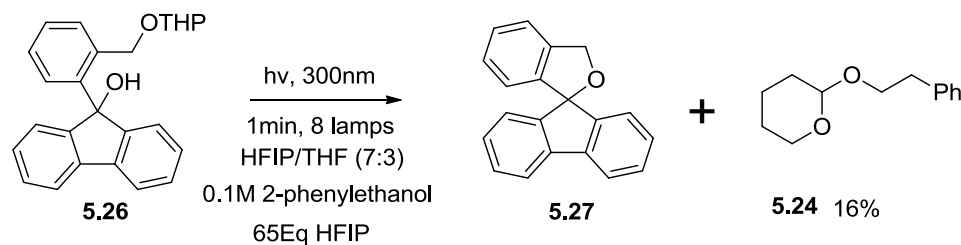
Scheme 5.8 Photolysis of **5.11** in HFIP/THF mixture

HFIP: THF	% Conversion	% Yield of 5.24 (GC/MS)	Yield 5.5 (HPLC)
3:1	28	60	quantitative
4:1	34	63	quantitative
7:3	49	44	80 %

Table 5.2 Photolysis of **5.11** in HFIP/THF mixture

Next we decided to investigate the reactivity of 9-(2-(((tetrahydro-2H-pyran-2-yl)oxy)methyl)phenyl)-9H-fluoren-9-ol **5.26**. We had hoped that cyclization of **5.26** to spiro benzofuran **5.27** would be thermodynamically more favorable than that of **5.11**. On the other hand, photolysis of **5.26** under similar conditions afforded 16% of the desired spiro benzofuran **5.27**. The yield was only increased to 22% after 2 min. Unfortunately,

relatively higher yields of hexafluoroisopropoxy ether **5.25** were observed by GC/MS which may account for the reduce yields of the desired compound **5.24** (Scheme 5.9, Table 5.3).

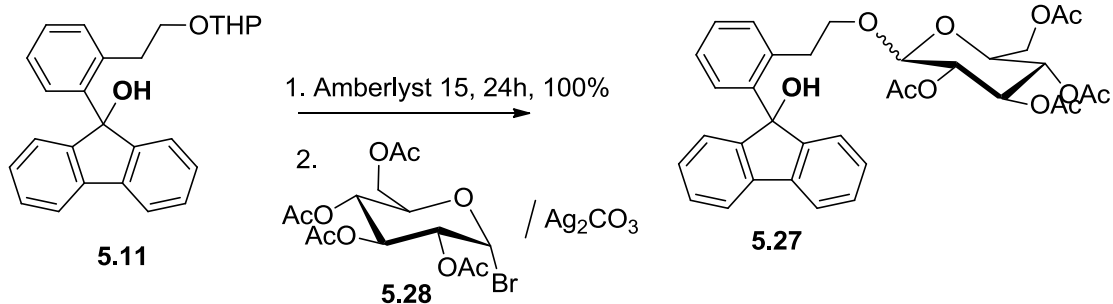


Scheme 5.9 Photolysis of **5.26** in HFIP/THF mixture

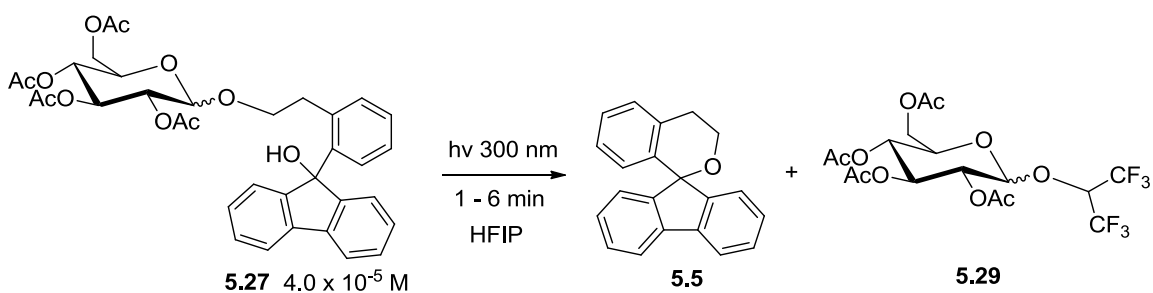
Time (min)	% Conversion of 5.26	% Yield of 5.24 (GC/MS)
1	35	16
2	57	22

Table 5.3 Photolysis of **5.26** in HFIP/THF mixture

Using these conditions, we decided to explore the reaction of per acetylated glycoside **5.27** from **5.11**. 2,3,4,6-Tetra-*O*-acetyl-B-D-glucoside was synthesized from acetobromo glucose **5.28** by classical Koenig Knorr conditions. Removal of the tetrahydropyranyl moiety from **5.11** was accomplished using Amberlyst-15 resin in methanol. The resulting diol was then glycosylated with acetobromo glucose to give the desired product **5.27** in 28% yield (Scheme 5.10). Compound **5.27** irradiated in HFIP using the Rayonet reactor and the reaction analyzed by HPLC (Figure 5.4) and monitored by UV-vis spectroscopy (Figure 5.5). As expected, the formation of spiro compound **5.5** was observed and the UV-vis spectra showed neat isosbestic points.



Scheme 5.10 Synthesis of peracetylated glycoside **5.27**



Scheme 5.11 Photolysis of **5.27** in HFIP

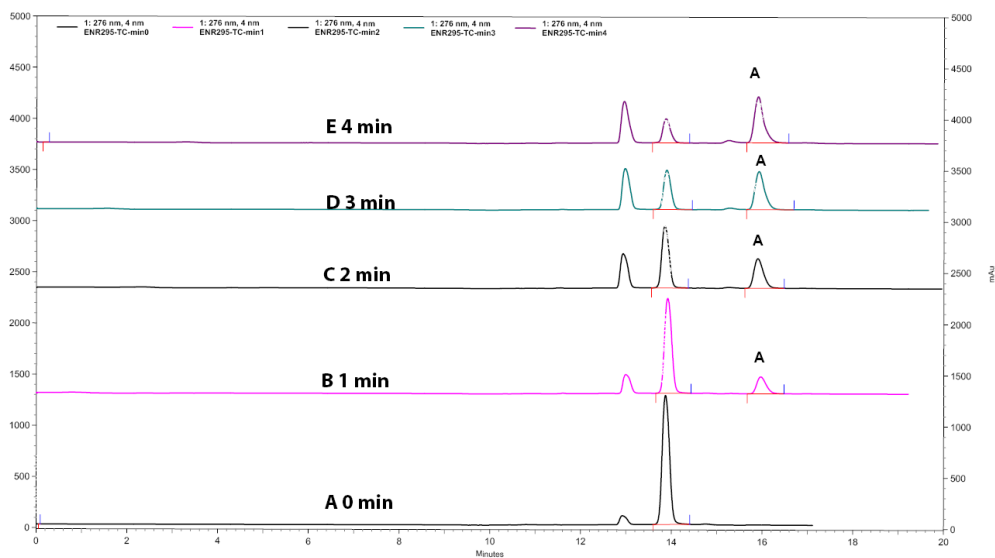


Figure 5.4 HPLC traces **5.27** before and after irradiation at 300 nm in HFIP; (A = **5.5**)

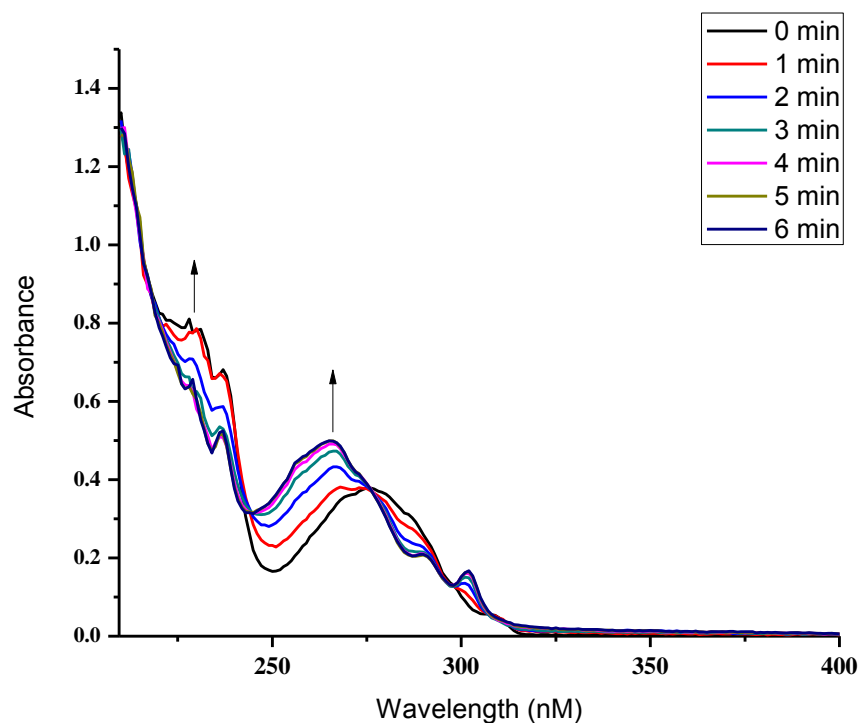
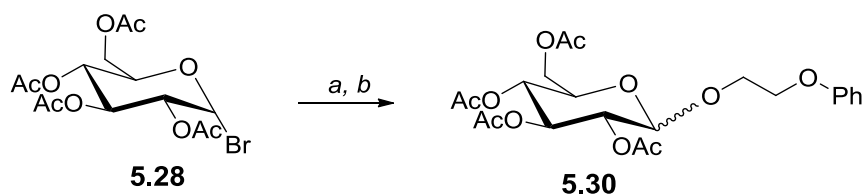


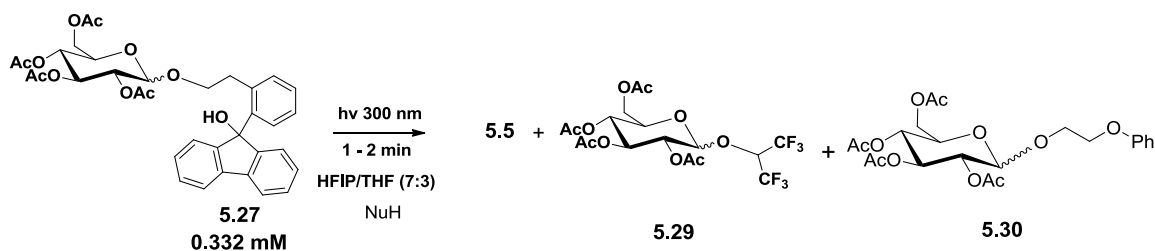
Figure 5.5 Photolysis of **5.27** in HFIP monitored by UV-vis

To analyze the reaction products with the presence of a nucleophile, it is essential for the nucleophilic species to have a considerable absorbance well above 220 nm. In view of the fact that expected photoproduct **5.24** with 2-phenylethanol has very little absorbance ($\log \epsilon_{250\text{nm}} = 2.47 \pm 0.02$) above 220 nm, we envisioned that incorporation of a hetero atom like oxygen next to the aromatic ring as in 2-phenoxyethanoxyl chromophore as **5.30** (Scheme 5.12) would lead to a secondary absorption band that might enable the expected photo-product **5.30** to be monitored at wavelengths greater than 220 nm. As expected, 2-phenoxyethyl 2,3,4,6-tetra-O-acetyl- β -D-glucoside synthesized from acetobromo glucose **5.28** by classical Koenig Knorr conditions has an absorbance at about 270 nm with $\log \epsilon_{270\text{nm}} = 2.95 \pm 0.02$.



Scheme 5.12 Reagents and conditions: a. Ag_2CO_3 , $\text{PhOCH}_2\text{CH}_2\text{OH}$; b. Ac_2O , pyridine, (20 % over two steps)

Having obtained the phenoxyethoxy glycoside **5.30**, the previously prepared target compound **5.27** was irradiated successively for 2 min in a HFIP/THF (7:3) mixture at 300nm in a Rayonet reactor. The formation of the spiro compound **5.5** was observed by HPLC as well as a small amount of what we think to be glycoside **5.30** (Figure 5.6 B).



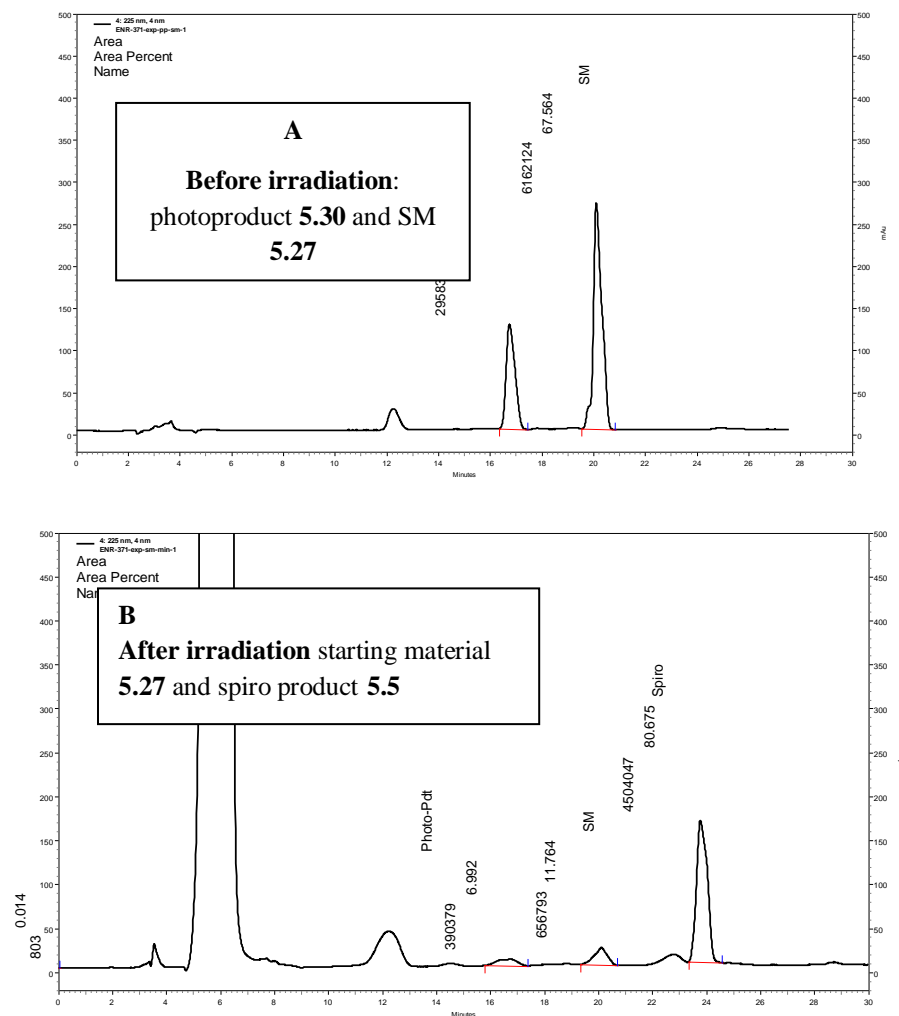


Figure 5.6 Irradiation of target compound in HFIP/THF in the presence of nucleophile

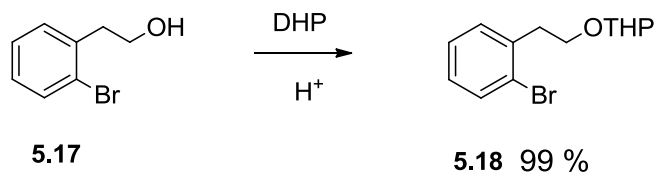
5.3 CONCLUSIONS AND FUTURE DIRECTIONS

It would be desirable to fine-tuning the present reaction conditions so as to minimize the formation of byproduct **5.29** and improve the yield of photoproduct **5.30**. Nucleophiles with good extinction coefficients would also make product analysis by HPLC easier and convenient.

5.4 EXPERIMENTAL SECTION

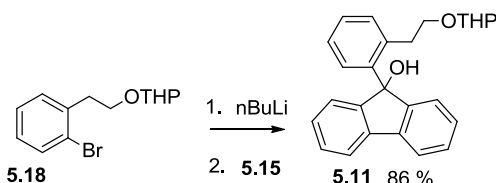
General Methods. All organic solvents were dried and freshly distilled before use. Flash chromatography was performed using 40-75 μm silica gel. All NMR spectra were recorded in CDCl_3 using 400 MHz instrument (unless otherwise noted). HPLC solvent grade were used for analysis. 9-Fluorenone **5.15**, 2,3,4,6-tetra-O-acetyl-B—D-glycoside **5.28**, 2-phenylethanol, 2-bromophenethyl alcohol **5.17** and trifluoroethanol were purchased from Aldrich, *o*-tolyl bromide was purchased from TCI America. 1,1,1,3,3,3-Hexafluoropropan-2-ol was purchased from Oakwood Products Inc. and distilled before use. 2-bromobenzyl bromide was bought from Alfa Aesar. All chemicals were used as received.

5.5 SYNTHETIC PROCEDURES



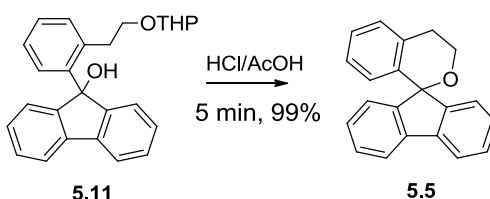
2-(2-Bromophenethoxy)tetrahydro-2H-pyran (5.18). Dihydropyran (0.669 mL, 7.38 mmol) **5.17** and catalytic amount of PTSA were added to a 25 mL round-bottomed flask containing 2-bromophenethyl alcohol (0.50 mL, 3.69 mmol) in 10 mL of THF at rt. The resulting solution was stirred at this temperature for 2 h and the reaction monitored by TLC (one spot). Purification through a short layer of silica gel eluting with DCM with 1% Et_3N ($R_f = 0.6$) afforded 2-(2-bromophenethoxy)tetrahydro-2H-pyran **5.18** (1.00 g, 3.51 mmol, 95 % yield) as a colorless liquid. ^1H NMR δ 7.48-7.51(d, 1H), 7.26-7.28 (d,

1H), 7.18-7.22(t, 1H), 7.01-7.05(t, 1H), 4.59(s, 1H), 3.43-3.95(m, 4H), 3.04(t, 2), 1.46-1.82(m, 6H). ¹³C NMR 138.4, 132.7, 131.2, 128.0, 127.3, 124.7, 98.6, 66.4, 62.1, 36.5, 30.7, 25.5, 19.5. GC/MS m/z: (M⁺, 286)

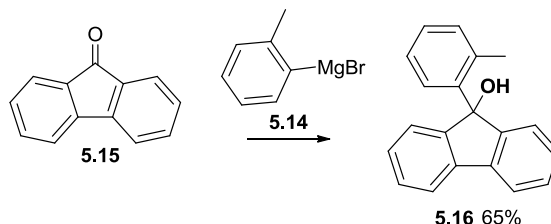


9-(2-(2-(Tetrahydro-2H-pyran-2-yloxy) ethyl) phenyl)-fluoren-9-ol (5.11). 2-(2-Bromophenethoxy) tetrahydro-2H-pyran **5.18** (0.800 g, 2.81 mmol) was added to a dry 100 mL round-bottom flask containing THF (15 mL) under nitrogen to give a colorless solution. The solution was cooled to -78 °C and 2.2mL solution of butyl lithium (0.216 g, 3.37 mmol) in hexane (2.5M) was added using a syringe pump. After the addition was complete, the solution was stirred for 45 min at -78 °C, after which a light greenish color is observed. A solution of 9-fluorenone (0.506 g, 2.81 mmol) in THF (5 mL) was added under nitrogen. The ice bath was removed and the mixture allowed to reach rt and stirred overnight. Ammonium chloride (10 mL) was added and the layers separated, the organic layer was extracted with ethyl acetate (3 x 20 mL). The combined organic layers was evaporated to give a yellow solid which was recrystallized in hexane to afford 9-(2-(2-(tetrahydro-2H-pyran-2-yloxy) ethyl) phenyl)-fluoren-9-ol **5.11** as whitish needles. m.p 167-168 °C. The ¹H NMR spectrum of this compound as well as many other structurally related compounds are unusualⁱ and was obtained at -23°C. ¹H NMR δ 1.24 -1.61 (m, 6H), 3.20- 3.41 (m, 2H), 3.89- 4.15 (broad s, 1H), 6.11(s,1H), 7.06 (broad s, 3H), 7.20-

7.40 (m, 7H), 7.83- 7.85 (d, 2H). ^{13}C NMR δ 19.00, 24.90, 30.08, 60.98, 97.57, 120.34, 124.24, 124.36, 125.37, 126.91, 127.00, 128.06, 128.11, 128.51, 139.63, 141.55, 151.09, 151.19. GC/MS-DIP: m/z [$M^+ = 386(2)$], 302(14), 286(15), 285(26), 284 (100), 283(27), 272(15), 270(7), 269(19), 268(53), 267(27), 266(50), 265(25), 258(14), 255(25), 254(86), 253(74), 252(47), 250(12), 241(8), 239(15), 181(31), 180(11), 165(12), 152(13), 126(8), 85(78), 67(13), 57(14), 55(13). HRMS-EI M^+ calc for $\text{C}_{26}\text{H}_{26}\text{O}_3$ 386.1882 found 386.1887



^{13}C NMR δ 29.3, 62.2, 85.3, 120.2, 125.4, 126.6, 126.9, 127.3, 128.4, 128.9, 129.3, 134.5, 136.9, 140.4, 150.6 GC/MS m/z ; 285(22), 284(100), 283(53), 266(10), 256(15), 255(11), 254(29), 253(79), 252(42), 250(13), 239(13), 228(10), 126(13), 119(11), 113(12), HRMS –EI M^+ calc. for $\text{C}_{21}\text{H}_{16}\text{O}$ 284.1201, found 284.1196

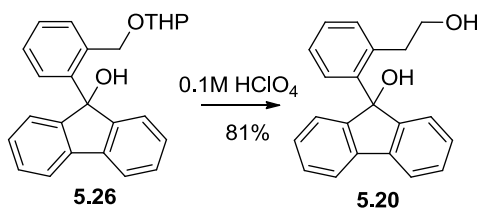


9-o-Tolyl-9H-fluoren-9-ol (5.16). A solution of 9-fluorenone (3.28 g, 18.20 mmol) **5.15** in 20 mL of dry ether was added over 20 min to a refluxing solution of *o*-tolylmagnesium bromide **5.14** (3.55 g, 18.18 mmol) and the reaction heated at reflux for 1h. The mixture was quenched with 10 % H_2SO_4 solution (20 mL), diluted with water (20 mL) and extracted with ethyl acetate (20 mL x 3). The combined extract was dried (MgSO_4) and concentrated using the rotary evaporator to give yellowish slurry which was recrystallized in hexane to afford 9-*o*-tolyl-9H-fluoren-9-ol **5.16** (3.23 g, 11.86 mmol, 65.2 % yield) as yellowish crystals. (m.p. 115 - 116 ° C: Lit¹. 119 - 120 C). ^1H NMR δ 1.30 (broad s, 3H), 2.32 (s, 1H), 6.91-6.92 (d, 1H), 7.13- 7.22 (m, 5H), 7.31- 7.35 (m, 3H), 7.64- 7.66 (d, 2H), 8.28-8.29 (broad d, 1H).

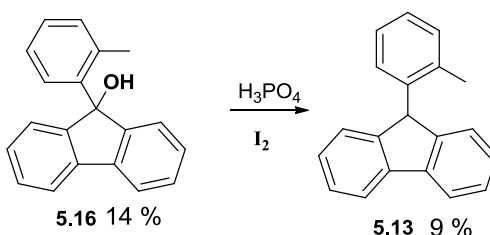
¹ C. Y. Meyers, P. D. R. a. A. W. M. *Acta Cryst.* **2003**, C59, 0712-0714

Preparation of Grignard reagent

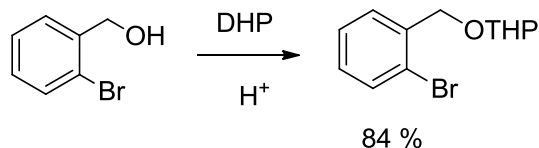
A solution of 2-Bromotoluene (2 mL, 18.18 mmol) in 5 mL of dry ether was added to a stirred mixture of magnesium turnings (0.486 g, 20.00 mmol) in 10 mL of dry ether. A few drops of bromine were added to initiate the reaction. The reaction mixture was stirred at rt and the initially clear solution turned cloudy, then brown and boiled spontaneously. After the boiling had subsided, the reaction mixture was refluxed for 2 h at 55 °C until most of the magnesium had been consumed. The Grignard reagent was used as described above.



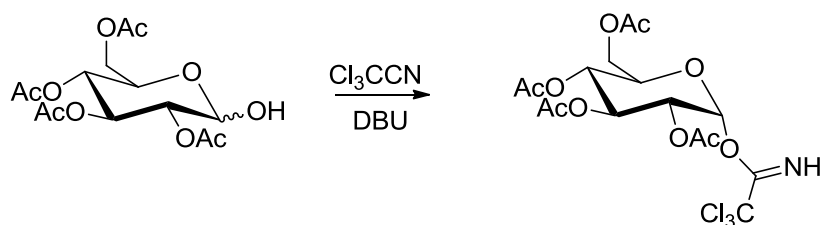
9-(2-(2-hydroxyethyl)phenyl)-9H-fluoren-9-ol (5.20). Mild hydrolysis of 9-(2-(2-(tetrahydro-2H-pyran-2-yloxy)ethyl)phenyl)-9H-fluoren-9-ol (71.00 mg, 0.184 mmol) with 0.1 M perchloric acid (11.09 μ l, 0.184 mmol) was carried out by stirring a 5 mL THF/H₂O (4:1) solution overnight. The resulting mixture was extracted with DCM (5 mL x 3), dried with MgSO₄ and filtered through a short plug of silica gel eluting with 3% MeOH in toluene to afford (45 mg, 0.149 mmol, 81 % yield) as a pale yellow oil. Like that of its precursor, the NMR of this compound is unusual¹. HRMS-EI M⁺ calc. for C₂₁H₁₈O₂; 302.1307, found 302.1297



9-o-Tolyl-9H-fluorene (5.13). A solution of iodine (932 mg, 7.34 mmol), 85% orthophosphoric acid (854 μ l, 14.69 mmol) and glacial acetic acid (10 mL) was heated at reflux for 2 h under inert atmosphere after which a solution of 9-o-tolyl-9H-fluoren-9-ol (200mg, 0.734 mmol) in 2 mL of acetic acid was then added. The mixture was refluxed under inert atmosphere for 3 days and monitored by GCMS (64 % yield of product). After cooling, water (10 mL) was added and the mixture extracted with ethyl acetate 10 mL x 3. The combined extracts was washed with NaHCO_3 (3 x 20 mL), brine (20 mL), dried over MgSO_4 and concentrated in *vacuo*. The mixture was then filtered through a short plug of silica gel eluting with petroleum ether to afford 9-o-tolyl-9H-fluorene **5.13** (16 mg, 0.062 mmol, 8.50 % yield) isolated as pure product. m.p. 84 C, lit. 90 C. GC/MS $m/z = 256$. ^1H NMR δ 1.14 (s, *ap*, 1.2H), 2.78 (s, *sp*, 1.8H, CH_3 , two rotamers), 5.02 (s, *ap*, 0.3H), 5.42 (s, *sp*, 0.6H, 9-H, two rotamers), 6.91-6.98 (m, 1H), 7.24-7.30 (m, 6H), 7.36-7.39 (m, 2H), 7.80-7.82 (d, 2H).

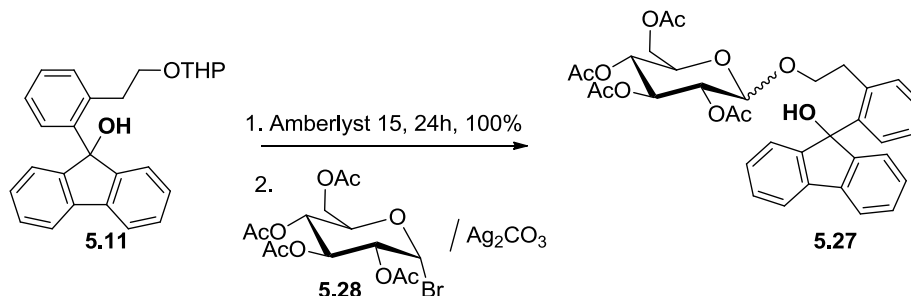


2-(2-Bromobenzoyloxy)tetrahydro-2H-pyran. (2-bromophenyl)methanol (5 g, 26.7 mmol) was added to a 25 mL round-bottomed flask containing 3,4-dihydro-2H-pyran (4.85 mL, 53.5 mmol) and catalytic amount of PTSA in 10 mL of THF at rt. The resulting solution was stirred at this temperature for 2 h and the reaction monitored by TLC. Purification through a short plug of silica gel eluting with DCM afforded 2-(2-bromobenzoyloxy)tetrahydro-2H-pyran (6.117 g, 22.56 mmol, 84 % yield) as a colorless liquid. ^1H NMR δ 7.50-7.54 (m, 2H), 7.28-7.32 (t, 1H), 7.11-7.15 (t, 1H), 4.81-4.84 and 4.55-4.59 (2H, AB(roof effect), $J = 17$ Hz, CH_2O), 4.77(t, 1H, CH), 3.89 -3.95(m, 1H), 3.54-3.59 (m, 1), 1.59-1.95 (m, 6H) GC/MS m/z : ($M^+ = 272$)



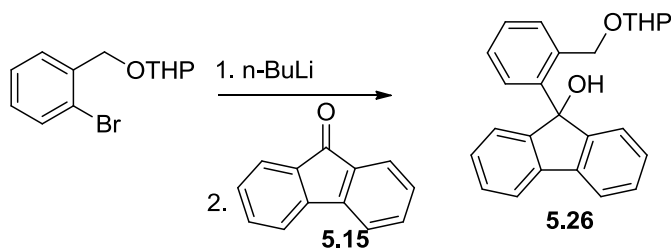
Glycosyl trichloroacetimidate. Trichloroacetonitrile (2.56 mL, 25.5 mmol) and 1,8-diaza bicyclo[5.4.0]undec-7ene, DBU (0.12 mL, 0.85 mmol) were added drop wise to a solution of 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose (1.48 g, 4.25 mmol) in 2 mL of dry DCM at 0 °C under nitrogen and stirred for 2.5 h at rt. After the reaction was complete, the mixture was concentrated in vacuo without aqueous work up. The crude mixture was

purified through a short plug of silica gel eluting with hexane/ethyl acetate (2:1) to afford glycosyl trichloroacetimidate (1.53 g, 3.10 mmol, 72.9 % yield) as colorless oil. ^1H NMR δ 8.70 (s, 1 H, NH), 6.56-6.57 (d, 1H, J = 4.0 Hz, H-1), 5.57 (t, 1H), 5.51-5.21 (m, 3H), 4.21-4.30 (m, 3H), 2.09, 2.06, 2.04, 2.03 (4s, 12H, 4 CH_3).



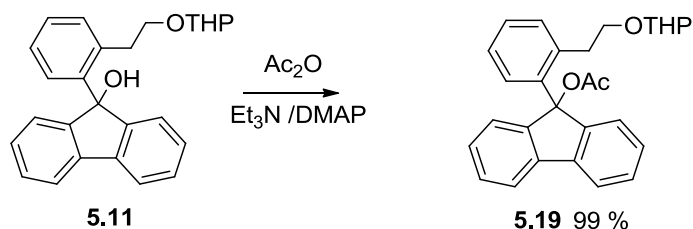
Per acetylated 9-fluorenol-9-aryl glucoside (2.27). Amberlyst deprotection (24 h, rt) of 9-(2-(2-(tetrahydro-2H-pyran-2-yloxy)ethyl)phenyl)-9H-fluoren-9-ol **5.11** gave the diol **5.20** in quantitative yield. "Drierite" (0.80 g) was added to a solution of the resulting 9-(2-(2-hydroxyethyl)phenyl)-9H-fluoren-9-ol (116 mg, 0.384 mmol) and glycosyl bromide **5.28** (174 mg, 0.422 mmol) in dry DCM and the mixture stirred under nitrogen for 30 min. Silver carbonate (127 mg, 0.460 mmol) was then added and the reaction stirred in the dark for 18 h at rt. The reaction mixture was diluted with DCM (50 mL), filtered through wet Celite, washed consecutively with sat. NaHCO_3 (25 mL x 2) and brine (30 mL). The organic layer was dried over MgSO_4 , filtered, concentrated and purified (30 % EtOAc/Hexane) to afford the per acetylated 9-fluorenol-9-aryl glucoside **2.27** (67 mg, pure 28 %) as a white fluffy solid. m.p 64-65. The corresponding spiro compound was also isolated (23mg). ^1H NMR (CD_3CN): δ 7.79-7.77 (dd, 2H, J = 4 Hz), 7.42-7.24 (m, 7H, aromatic), 7.12-7.08 (m, 3H, aromatic), 5.33 (d, 1H, H -1, J = 4Hz), 4.98 (s, 1H),

4.78 (d, 1H, $J = 8\text{Hz}$), 4.15-3.97 (m, 4H), 3.79-3.76 (m, 2H), 2.66 (m, 2H, hump), 2.14 (m, 1H), 2.09 – 2.00 (3s, 9H). ^{13}C NMR (CD_3CN): δ 169.63, 168.91, 168.33, 150.16, 140.38, 139.22, 139.14, 130.97, 128.30, 127.72, 126.51, 126.37, 125.36, 123.68, 123.56, 120.04, 119.72, 95.77, 71.53, 68.73, 67.17, 65.96, 62.34, 19.35, 19.32, 19.18, 19.16. ESI: calc. for ($\text{M}^+ + \text{H}$) = 633.0



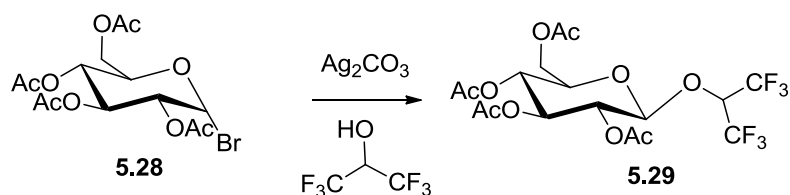
9-(2-((Tetrahydro-2H-pyran-2-yloxy)methyl)phenyl)-9H-fluoren-9-ol (5.26**)**. A solution of 2-(2-bromobenzyl) tetrahydro-2H-pyran (1.3 g, 4.79 mmol) in ether (20 mL) was added to a 100 mL round-bottomed flask under nitrogen to give a colorless solution. The solution was cooled to $-78\text{ }^\circ\text{C}$ and a 2.0 mL solution of n-butyllithium (0.307 g, 4.79 mmol) in hexane (2.9 M) was added using a syringe pump to give an orange colored solution. After the addition was complete, the solution was allowed to warm up to rt (pink colored) and stirred for 45 min. A solution of 9-fluorenone **5.15** (0.864 g, 4.79 mmol) in ether (20 mL) was added under nitrogen and the mixture was refluxed for 3 h and then stirred at rt overnight. Ammonium chloride (10 mL) was added and the layers separated. The organic layer was extracted with ethyl acetate (3 x 20 mL). The combined organic layers were evaporated to give a yellow solid which was recrystallized in hexane to afford 9-(2-((tetrahydro-2H-pyran-2-yloxy)methyl)phenyl)-9H-fluoren-9-ol **5.26** (1.56 g, 4.19 mmol, 87 % yield) as white solid. m.p = 106-108. GC/MS -DIP, m/z: $\text{M}^+ = 372$

HRMS-EI M^+ calc for $C_{25}H_{24}O_3$ 372.1725, found 372.1704. 1H NMR aromatic ~12H.



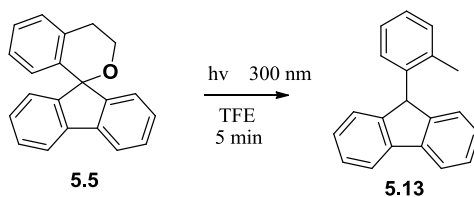
9-(2-(2-(Tetrahydro-2H-pyran-2-yloxy)ethyl)phenyl)-9H-fluoren-9-yl acetate (5.19).

Acetic anhydride (1106 mL, 11.70 mol) was added to a solution of 9-(2-(2-(tetrahydro-2H-pyran-2-yloxy)ethyl)phenyl)-9H-fluoren-9-ol **5.11** (0.540 g, 1.40 mmol) in Et_3N (7 mL) and DCM (2 mL) followed by DMAP (catalytic) at rt. After stirring overnight, the reaction mixture was diluted with MeOH (10 mL) and concentrated under reduced pressure. The residue was quenched with water, extracted with EtOAc, washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was chromatographed on silica gel, eluting with 20% AcOEt/hexane and 1% Et_3N to give a 9-(2-(2-(tetrahydro-2H-pyran-2-yloxy)ethyl)phenyl)-9H-fluoren-9-yl acetate **5.19** (0.60 g, 1.40 mmol, 100 % yield) as colorless viscous liquid which solidified to a white solid on cooling (mixture of diastereoisomers). As previously mentioned, The 1H NMR spectrum of this compound as well as many other structurally related compounds are unusual due to the existence of two rotamers that are not equilibrated on the NMR time scale.^{44a} 1H NMR: aromatic = 12H GC/MS-DIP, m/z M^+ = 428.



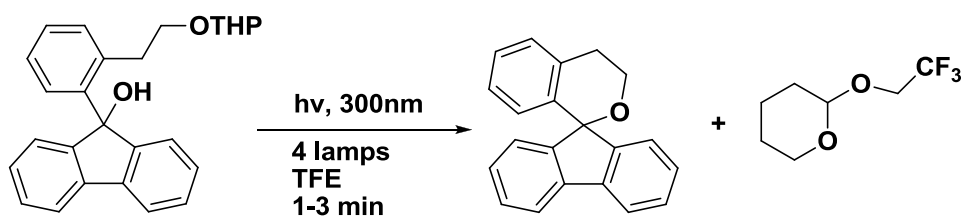
2,3,4,6-Tetra-O-acetyl-B-D-glucoside Hexafluoro-2-propanol (5.29). Hexafluoro-2-propanol (768 μL , 7.30 mmol) and aceto bromoglucose **5.28** (300 mg, 0.730 mmol) were added to a solution of previously stirred DCM (10 mL) and "Drierite" (800 mg) under nitrogen. The mixture was stirred for 30 additional min. then silver carbonate (221 mg, 0.803 mmol) was added and the reaction stirred for 18 h at rt under darkness. The reaction mixture was diluted with DCM (50 mL), filtered through wet Celite, washed consecutively with saturated NaHCO_3 (25 mL x 2) and brine. The organic layer was dried over MgSO_4 , filtered, concentrated and purified (30 % EtOAc/Hexane) to afford 2-(1,1,1,3,3,3-hexafluoro-2-propoxy) 2,3,4,6-tetra-O-acetyl-B-D-glucoside **5.29** (193 mg, 0.387 mmol, 53.1 % yield) as white solid recrystallized in MeOH/Hexane. m.p = 74 – 76 $^\circ\text{C}$. ^1H NMR δ 5.74 (d, 1H, *H*-1, *J* = 8Hz), 5.20 (t, 1H, *H*-5, *J* = 4 Hz), 4.92-4.89 (dd, 1H, *J* = 4Hz), 4.50 (m, 1H), 4.40 (dd, 1H, *J* = 4Hz), 4.23-4.16 (m, 2H), 3.88 (m, 1H), 2.13-2.10 (2s, 9 H), 1.78 (s, 3H). ^{13}C NMR δ 170.77, 169.69, 169.19, 122.46, 97.75, 73.56, 70.28, 69.95, 69.67, 69.62, 68.09, 67.72, 63.14, 21.52, 20.91, 20.89.

Preparative photolysis:



9-o-Tolyl-9H-fluorene (5.13). A solution of spiro [fluorene-9, 1'-isochroman] (92 mg, 0.324 mmol) in TFE (100 mL) was irradiated for 5 min in a Rayonet reactor at 300 nm using 8 lamps (4.5W) and the product mixture analyzed by GC/MS and HPLC. The GC/MS analysis indicated that 9-o-tolyl-9H-fluorene **5.13** is the major product. The product mixtures were separated by preparative TLC to give 9-o-tolyl-9H-fluorene (9 mg, 0.035 mmol, 10.85 % isolated yield) and 1 mg of a minor product with an $m/z = 270$ which would corresponds to 9-(2-ethylphenyl)-9H-fluorene. A sample of 9-o-tolyl-9H-fluorene **5.13** prepared separately had the same retention time (HPLC and GC/MS) as the product from irradiation. The ^1H NMR is consistent with that reported² for this compound. Spiking of HPLC peak (RT = 9.8 min) with authentic sample of 9-o-tolyl-9H-fluorene **5.13** also confirms this. ^1H NMR 1.14 (s, *ap*, 1.1H), 2.77 (s, *sp*, 1.9H, CH₃, two rotamers), 5.02 (s, *ap*, 0.3H), 5.41 (s, *sp*, 0.6H, 9-H, two rotamers), 6.89-6.99 (m, 1H), 7.11-7.13 (t, 1H), 7.24-7.31 (m, 6H), 7.36-7.39 (m, 2H), 7.80-7.82 (d, 2H).

² C. Y. Meyers, P. D. R. a. A. W. M. *Acta Crystallogr. Sect. C: Cryst. Struct. Commun.* **2004**, 60, O156 - O157.



General procedure for photolysis

A 1.04mM solution of 9-(2-(2-(tetrahydro-2H-pyran-2-yloxy)ethyl)phenyl)-9H-fluoren-9-ol in TFE was irradiated for 1, 2, and 5 min in a Rayonet reactor at 300 nm using 4 lamps and the chemical yield calculated by HPLC analysis at isosbestic point (243nm).

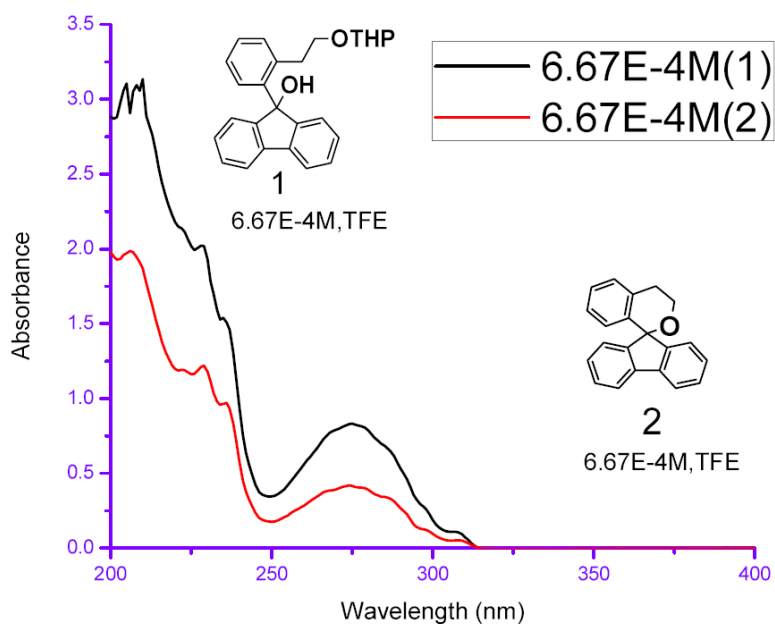


Figure 5.7 UV-vis spectra of product and starting material

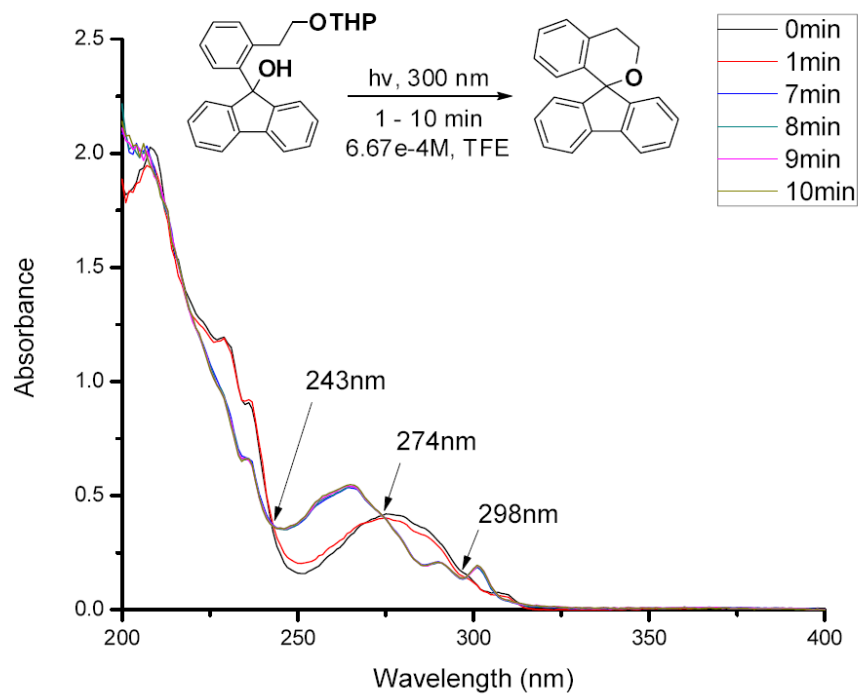


Figure 5.8 UV-vis spectra of reaction mixture showing isosbestic points.

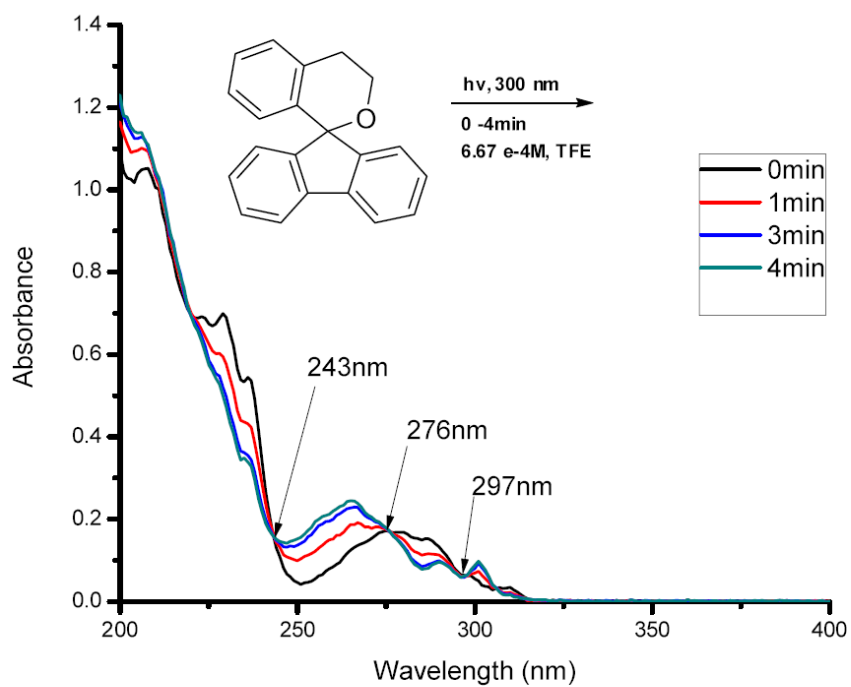


Figure 5.9 UV-vis spectra of reaction mixture for photolysis of **5.5**

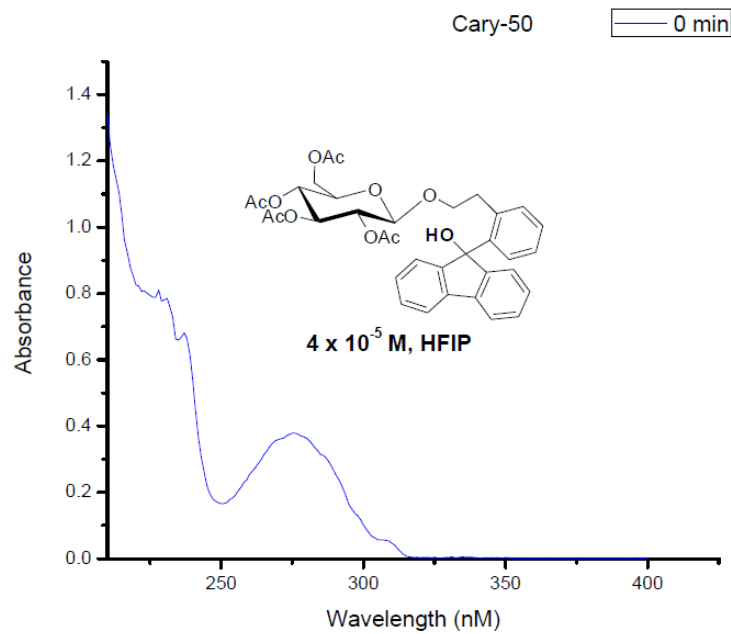


Figure 5.10 UV-vis spectrum of **5.27** in HFIP

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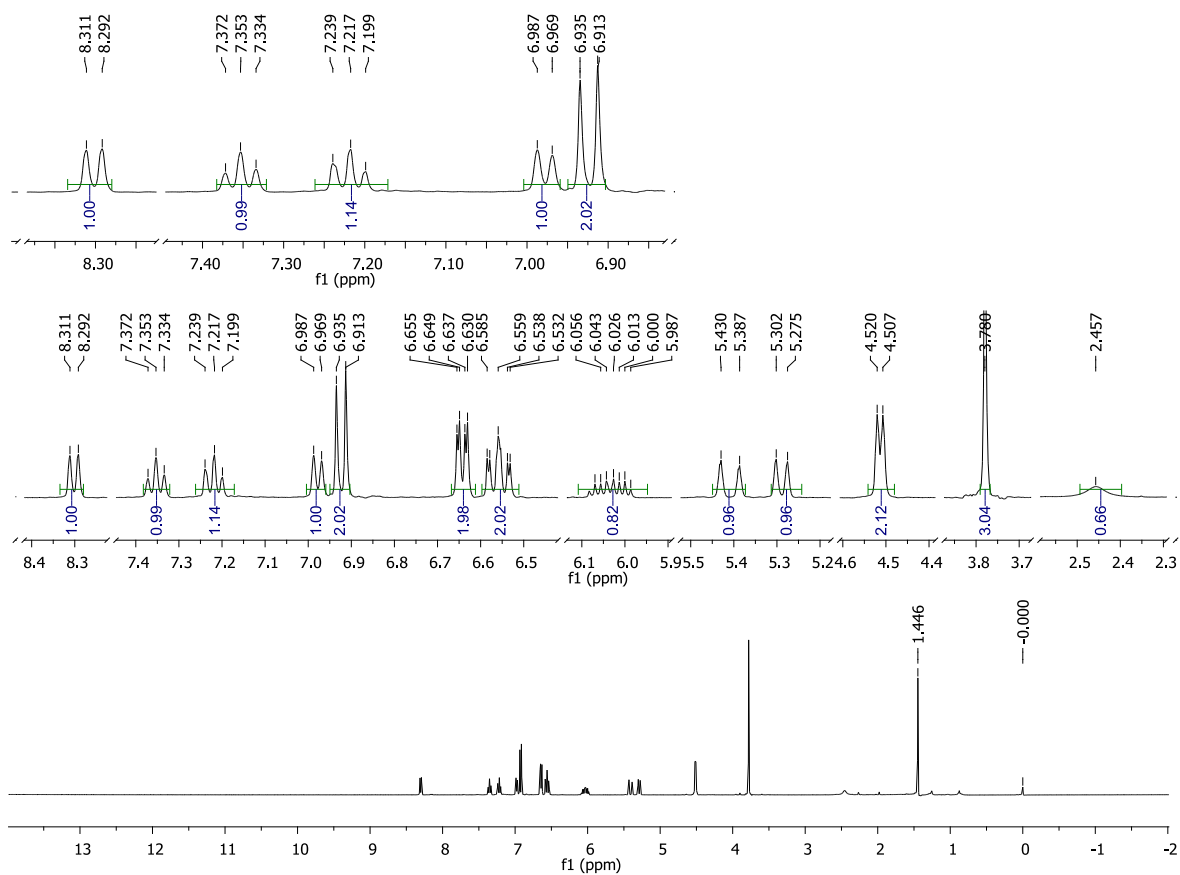
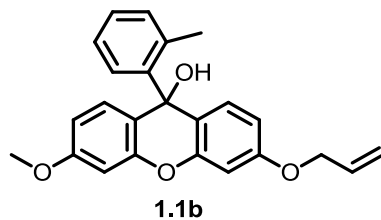
APPENDIX A
LIST OF ACRONYMS

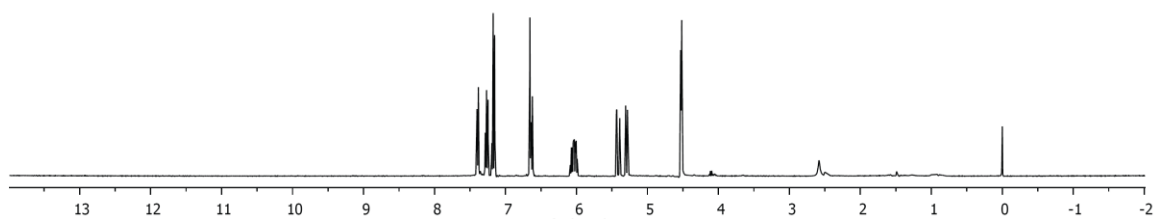
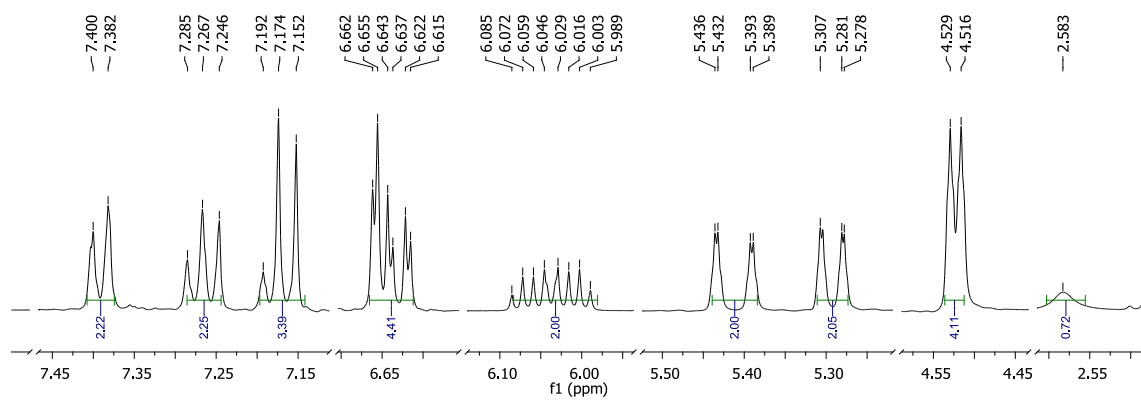
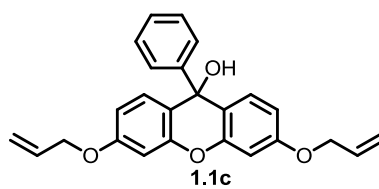
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
PET	Photoinduced Electron Transfer
DIBAL	Diisobutylaluminium Hydride
DIP-MS	Direct Inlet Probe/Mass Spectrometry
DMSO	Dimethyl Sulfoxide
GC/MS	Gas Chromatography/Mass Spectrometry
HPLC	High-performance Liquid Chromatography
TFE	2,2,2-Trifluoroethanol
HFIP	1,1,1,3,3,3- Hexafluoropropan-2-ol
NMR	Nuclear Magnetic Resonance
PPG	Photoremovable Protecting Groups
UV	Ultraviolet
EI	Electron Impact
HRMS	High Resolution Mass Spectrometry
NCS	<i>N</i> -Chlorosuccinimide
DMF	<i>N,N</i> -Dimethylformamide
TEA	Triethylamine
DCM	Dichloromethane

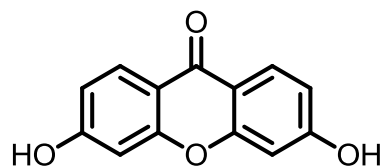
DDQ	2,3-Dichloro-5,6-Dicyanobenzoquinone
rt	Room Temperature
TLC	Thin Layer Chromatography
MOM	Methoxymethyl ether
MEM	Methoxyethoxymethyl ether
TFA	Trifluoroacetic Acid
TBDMS	Tert-butyldimethylsilyl (TBS)
THF	Tetrahydrofuran

APPENDIX B

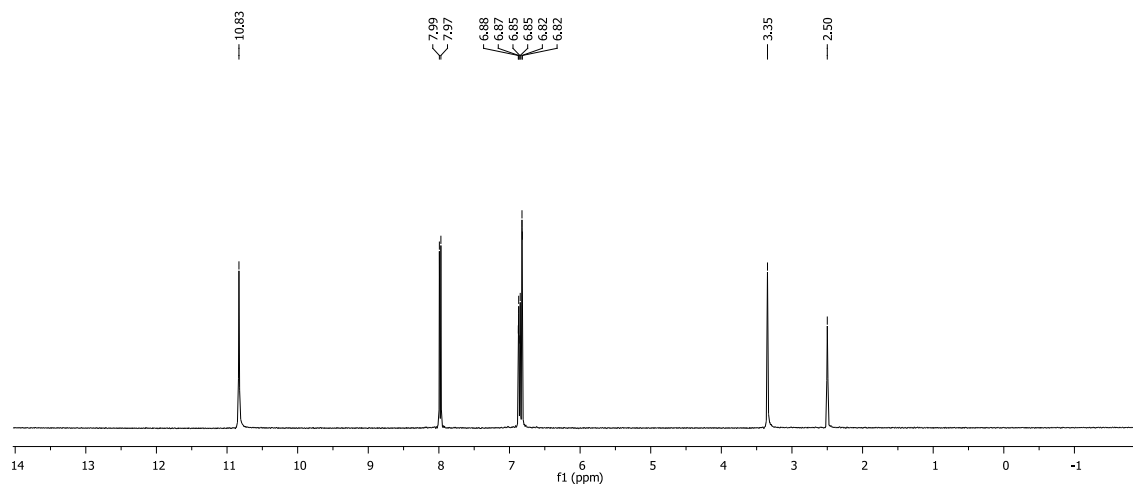
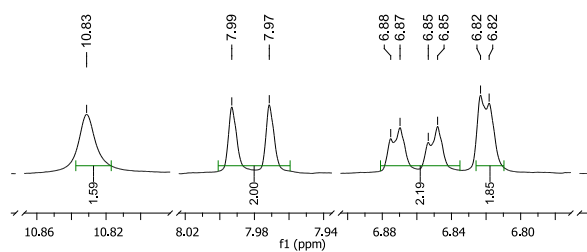
^1H NMR, ^{13}C NMR

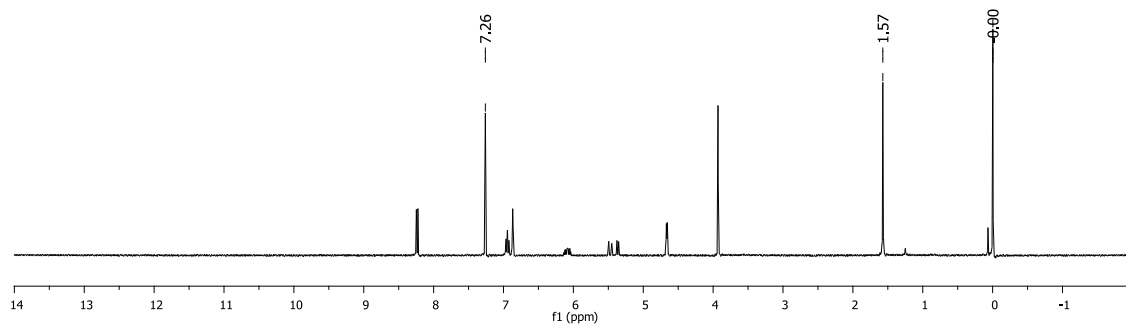
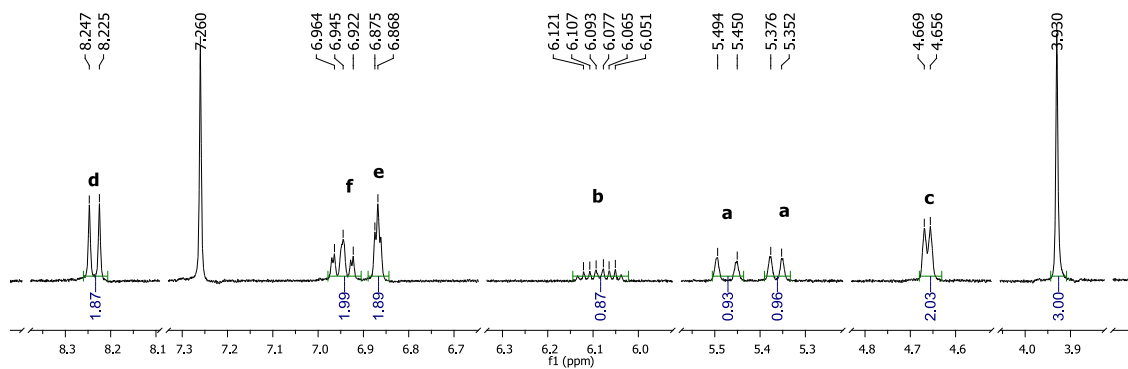
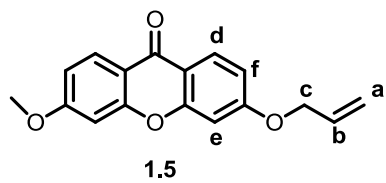


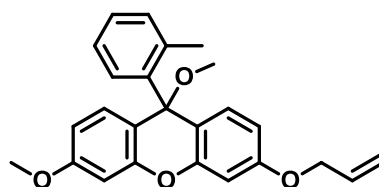




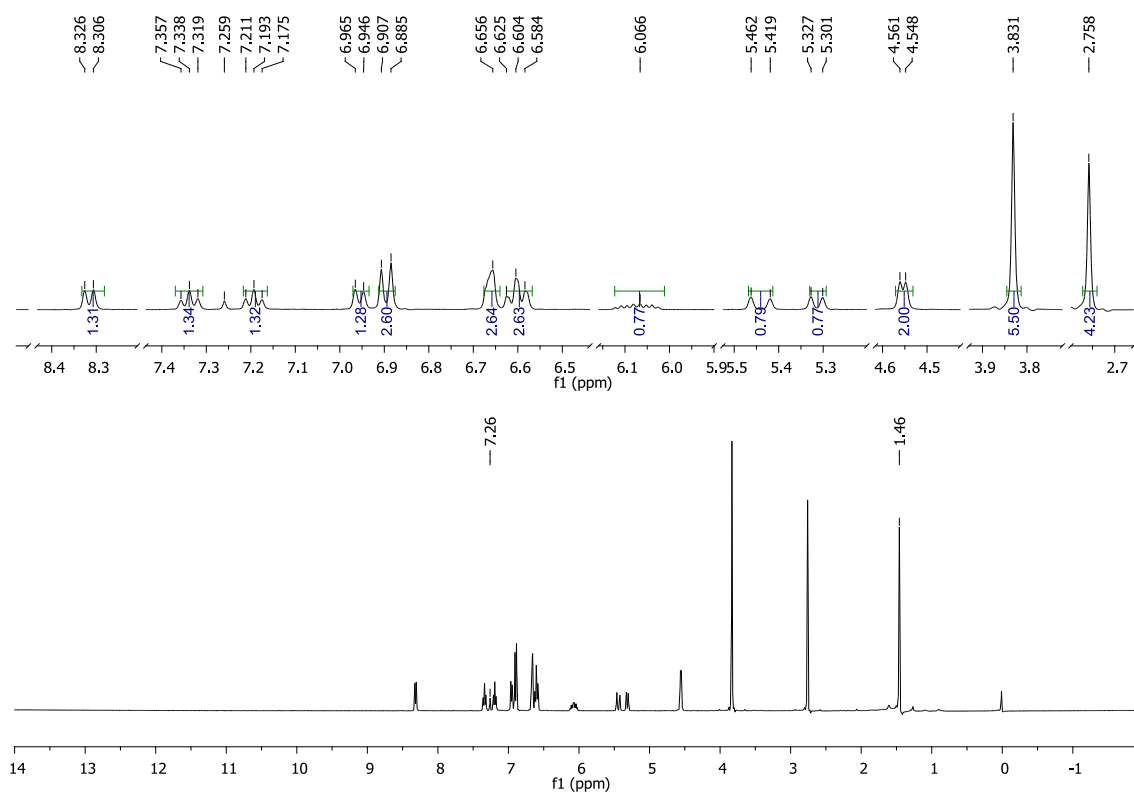
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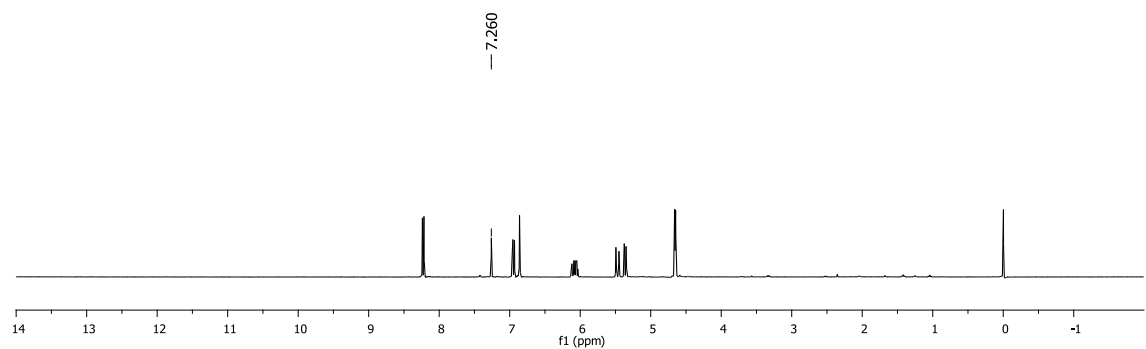
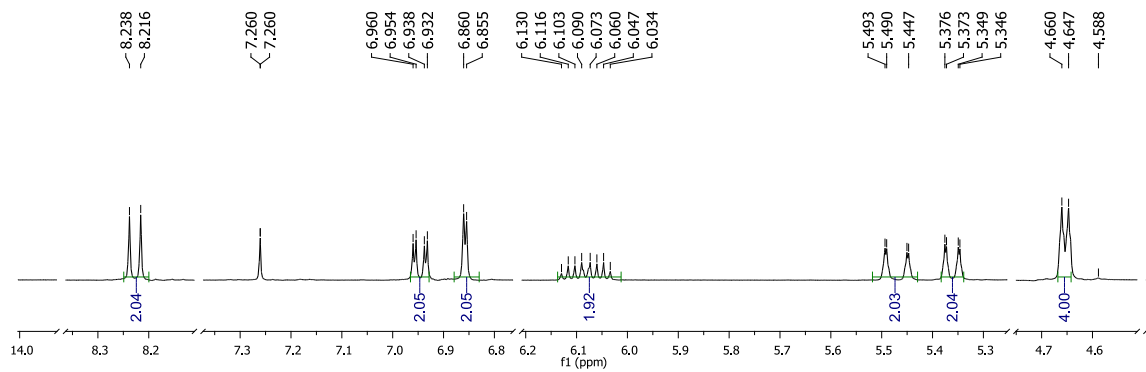
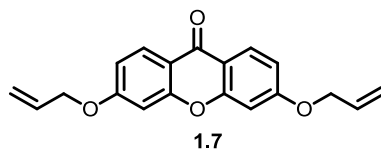




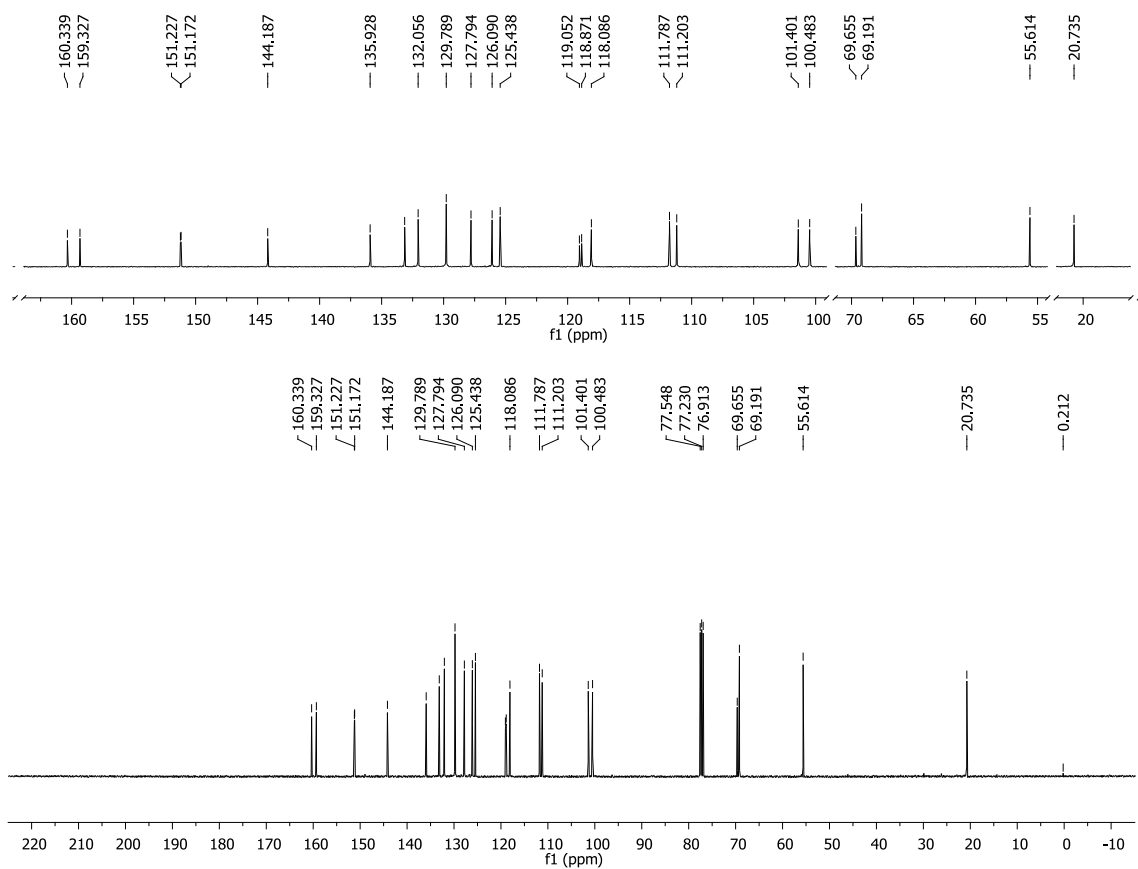
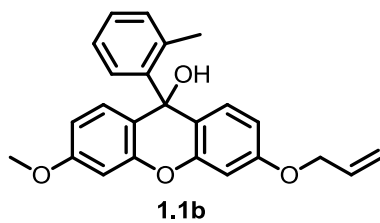


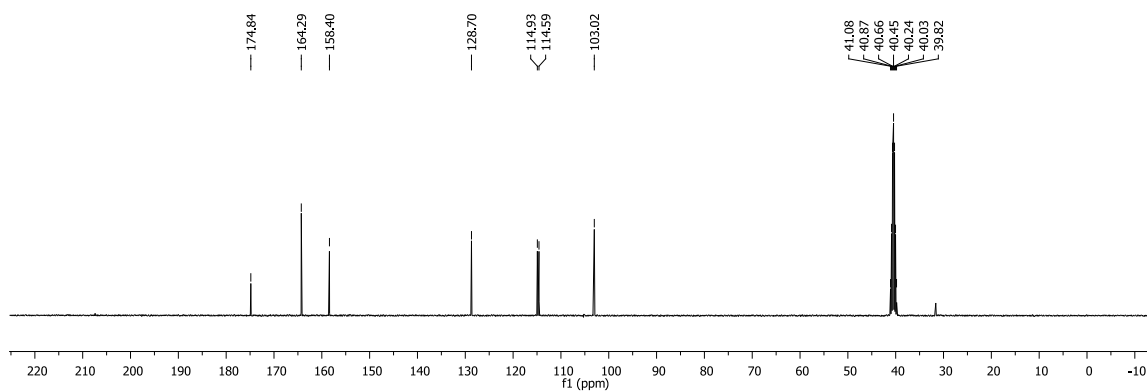
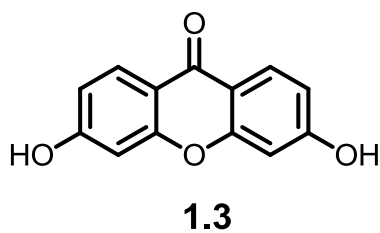
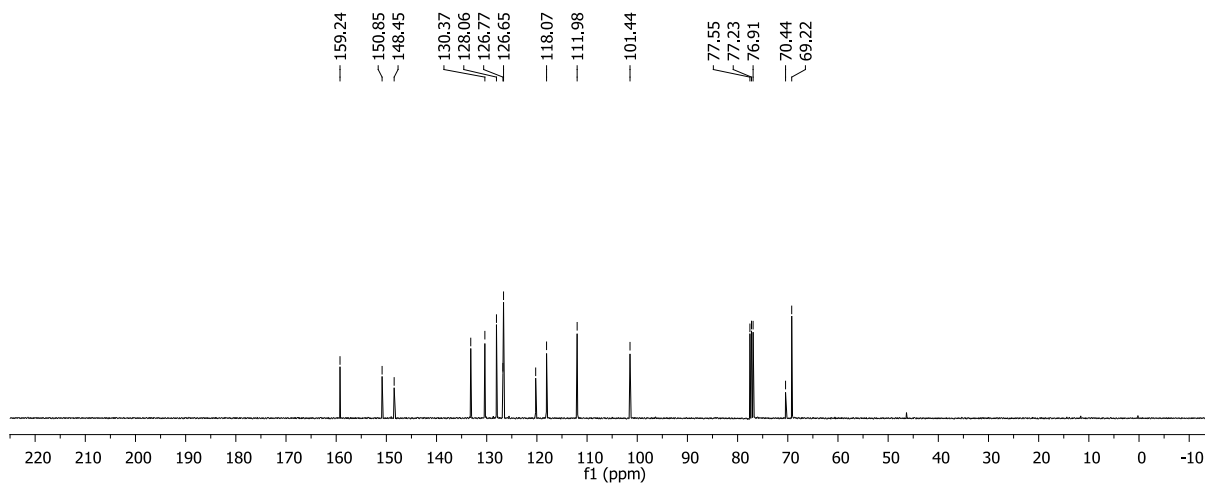
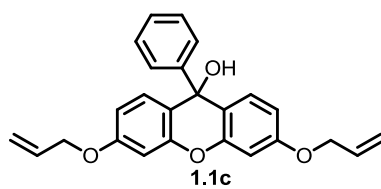
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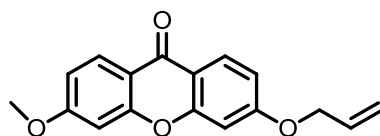




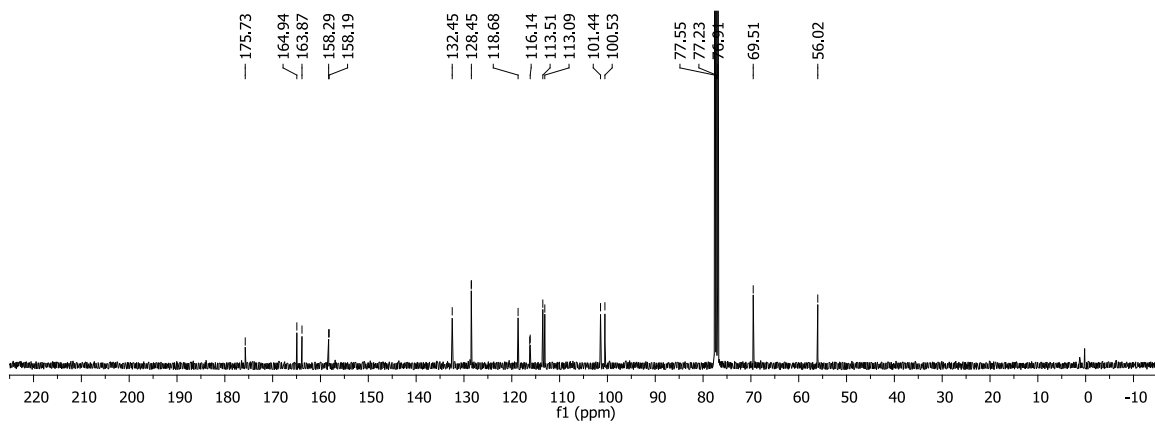
¹³C NMR

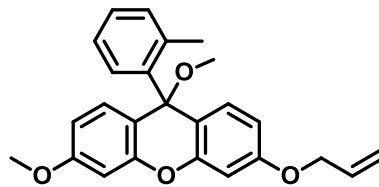




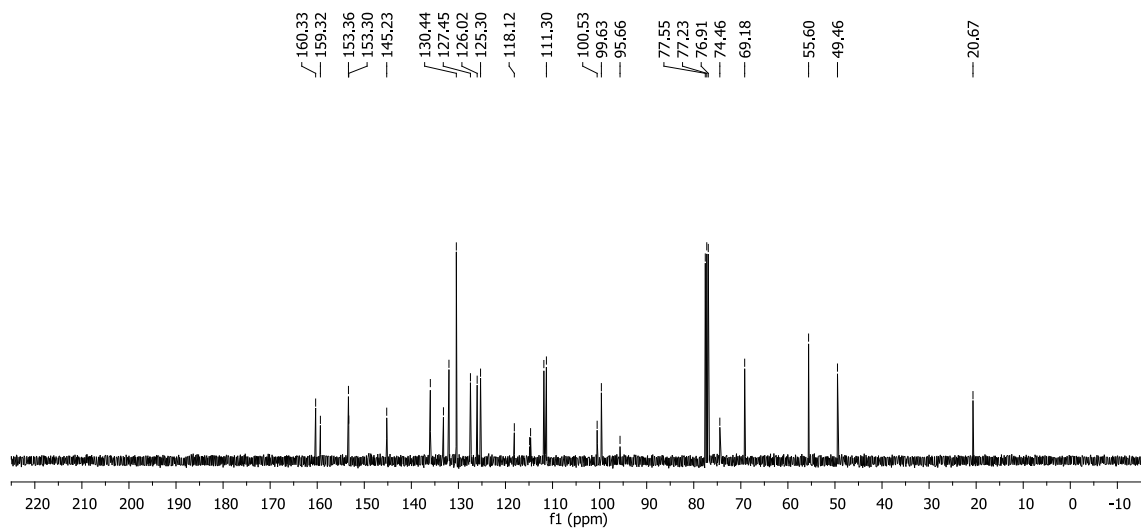


1.5





1.6



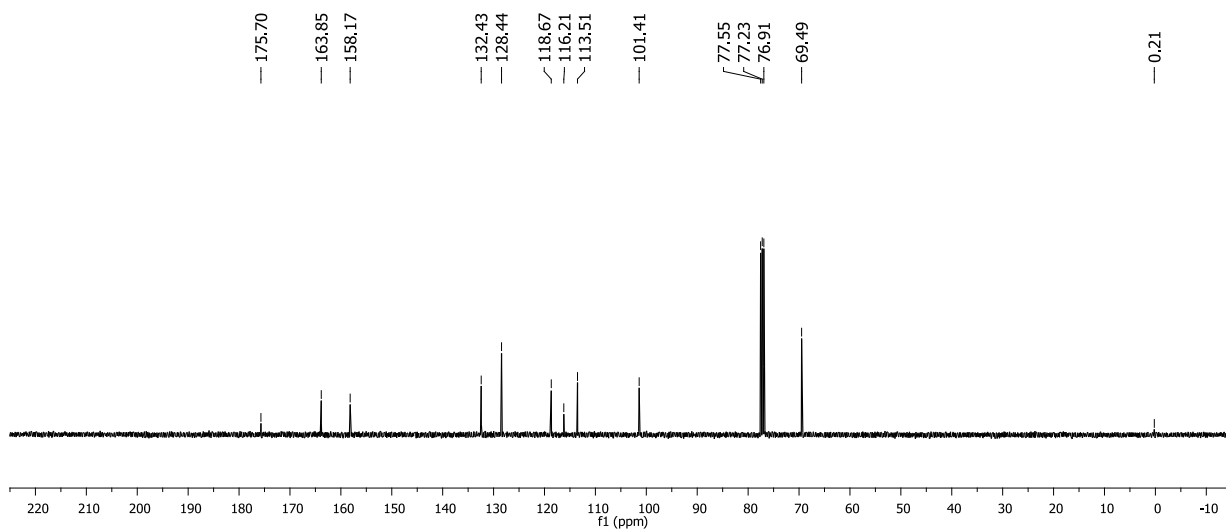
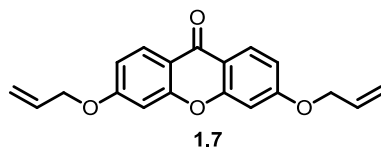
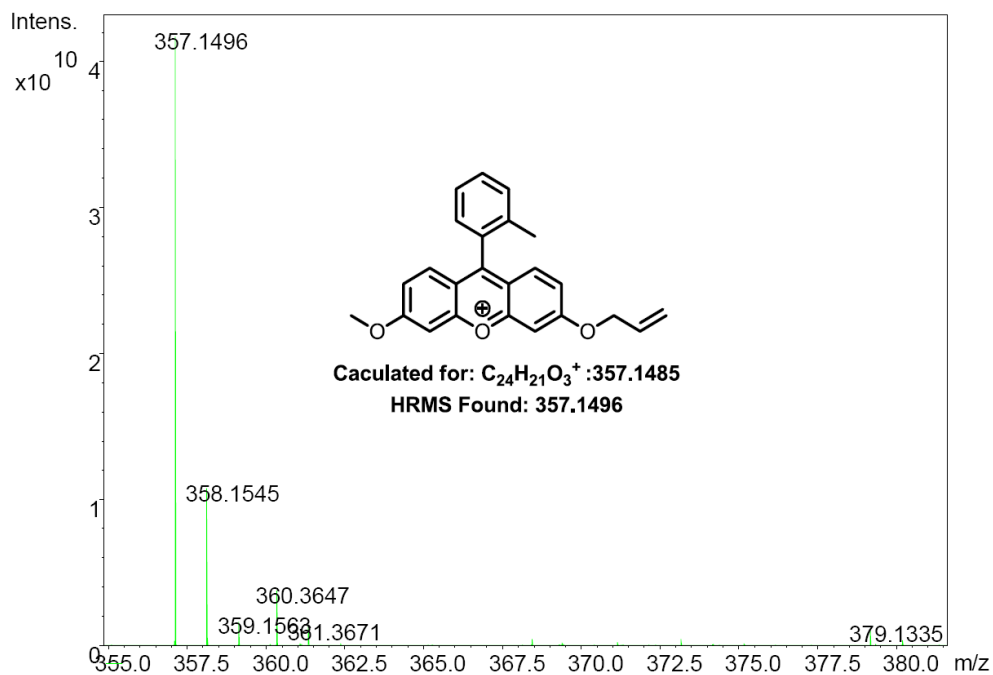
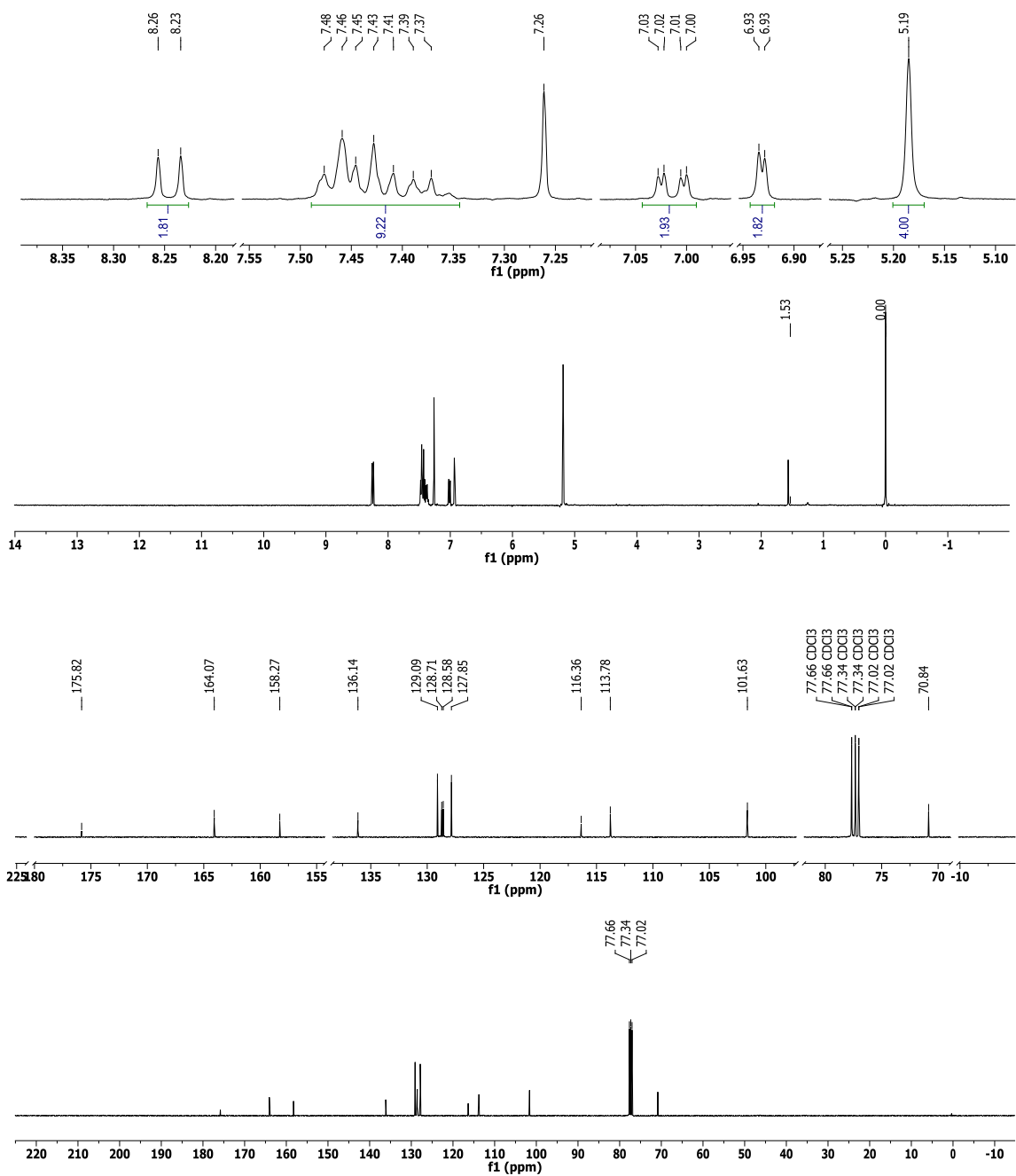
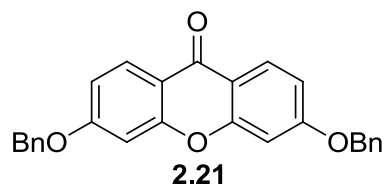
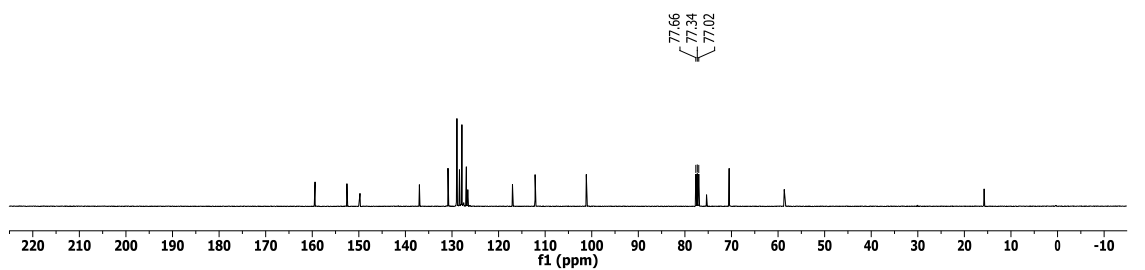
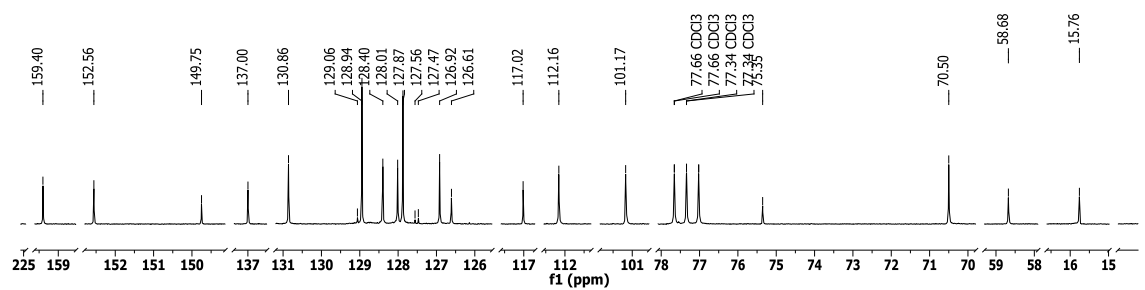
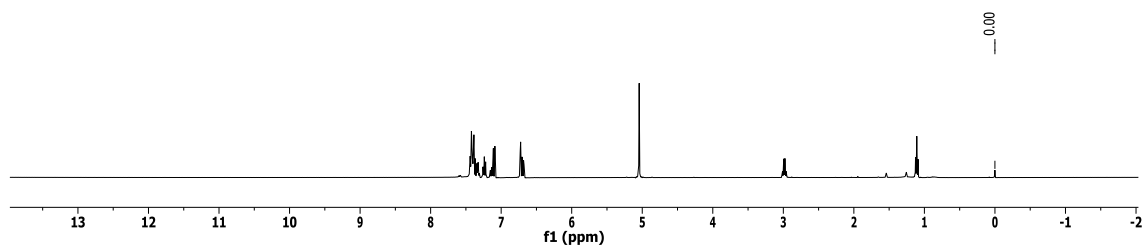
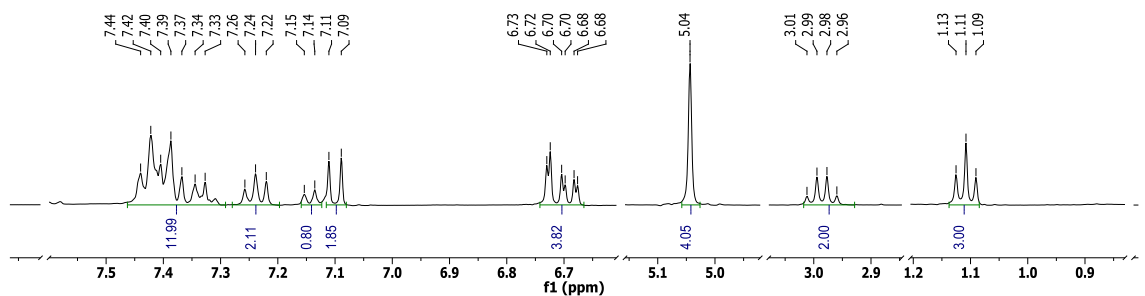
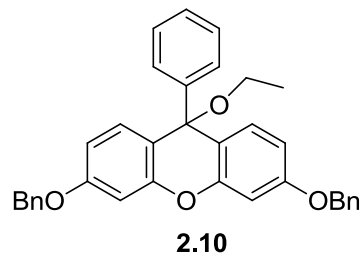
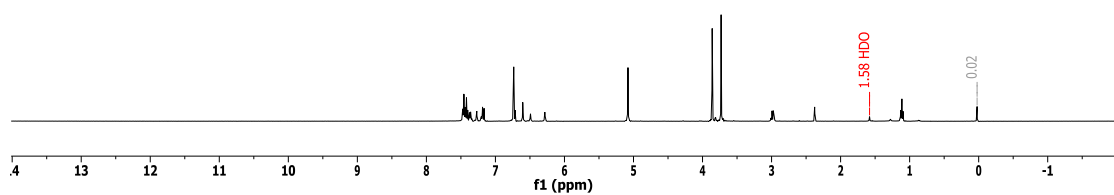
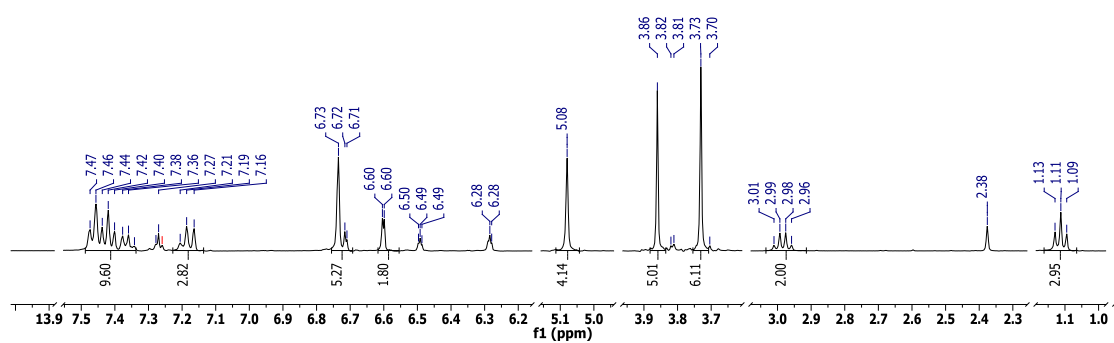
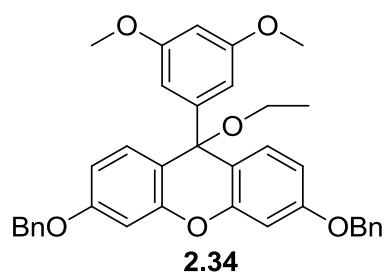


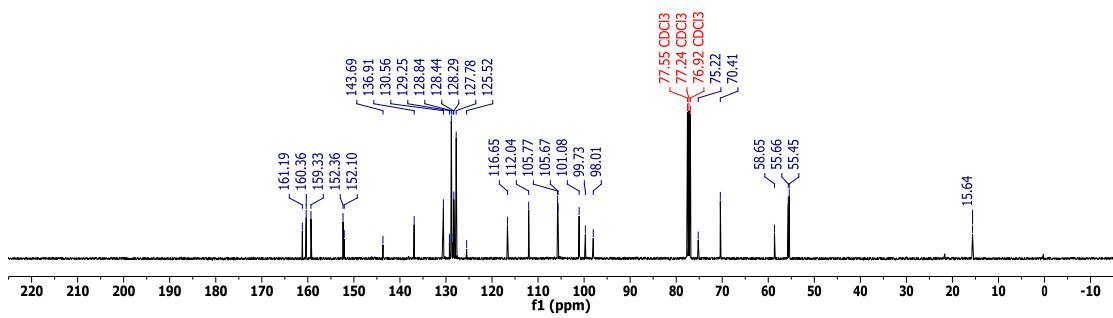
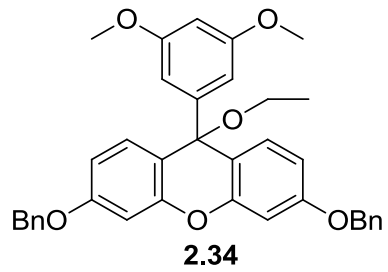
Figure S1. HRMS of cation 1.2b in Methanolic TFA

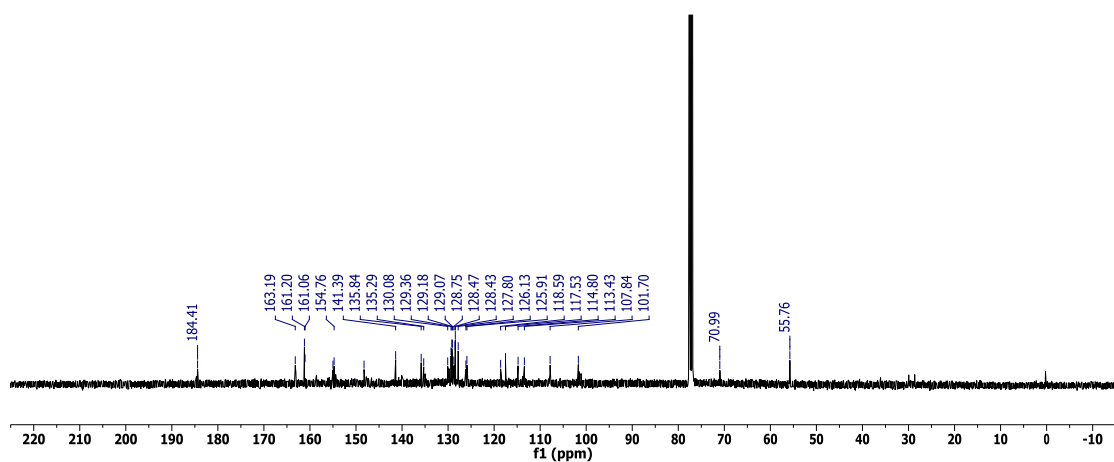
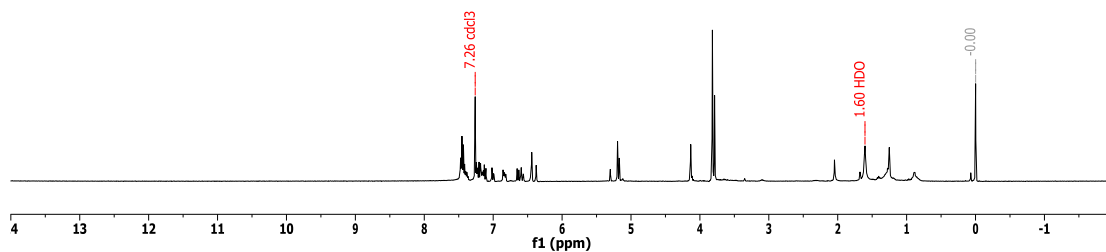
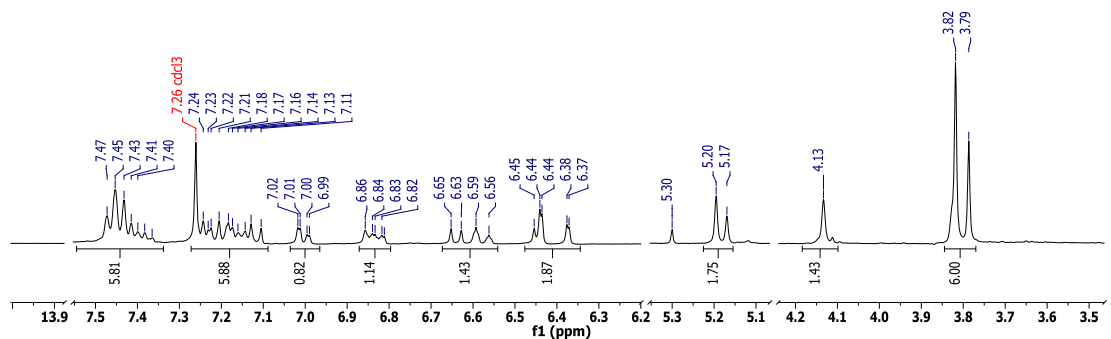
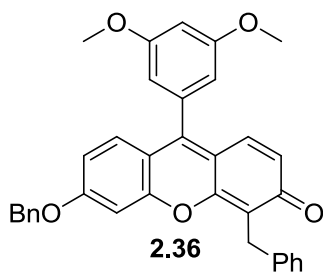


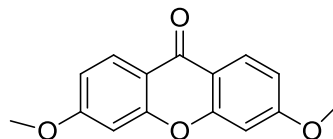




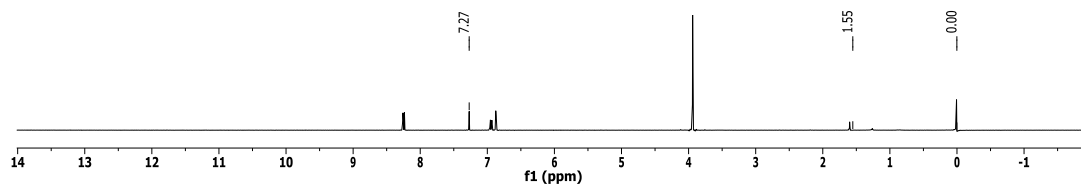
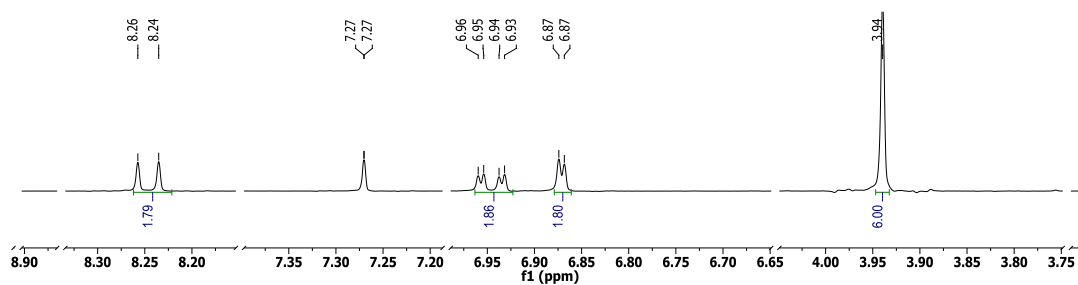
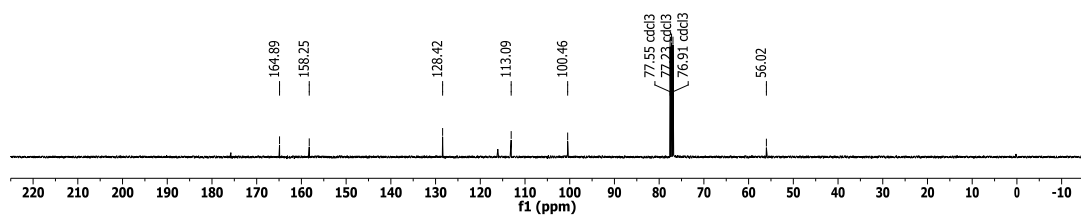


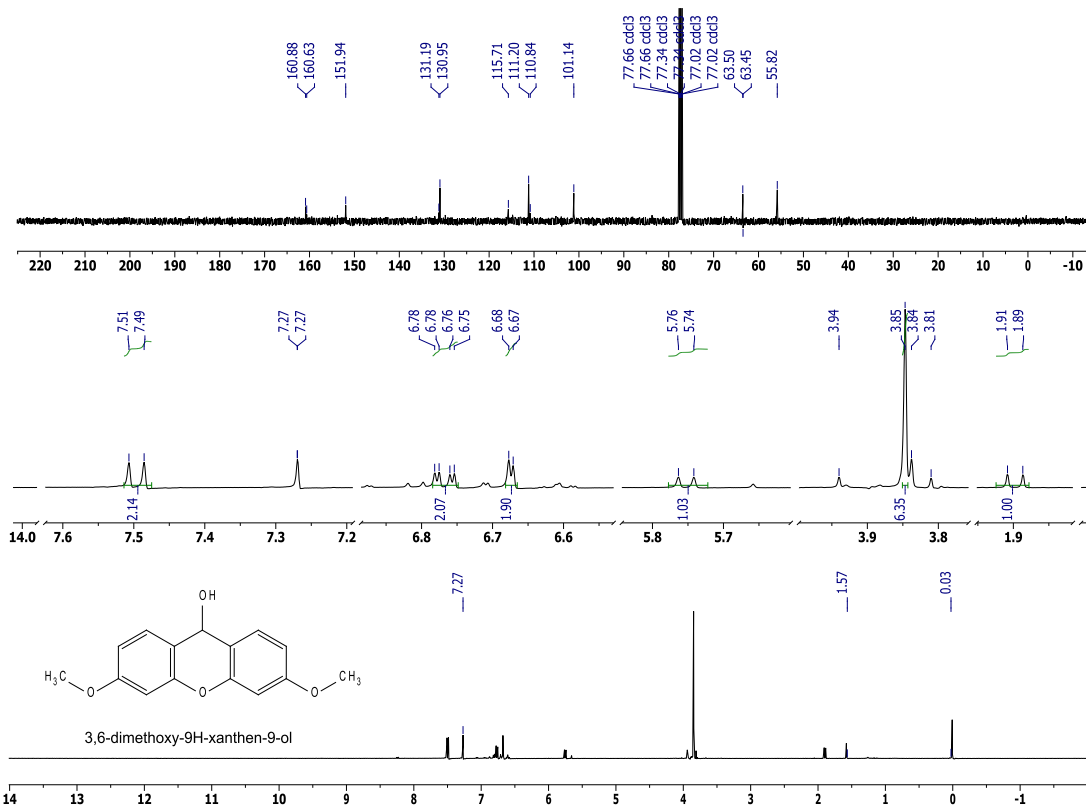
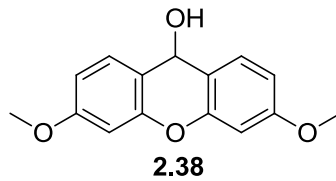


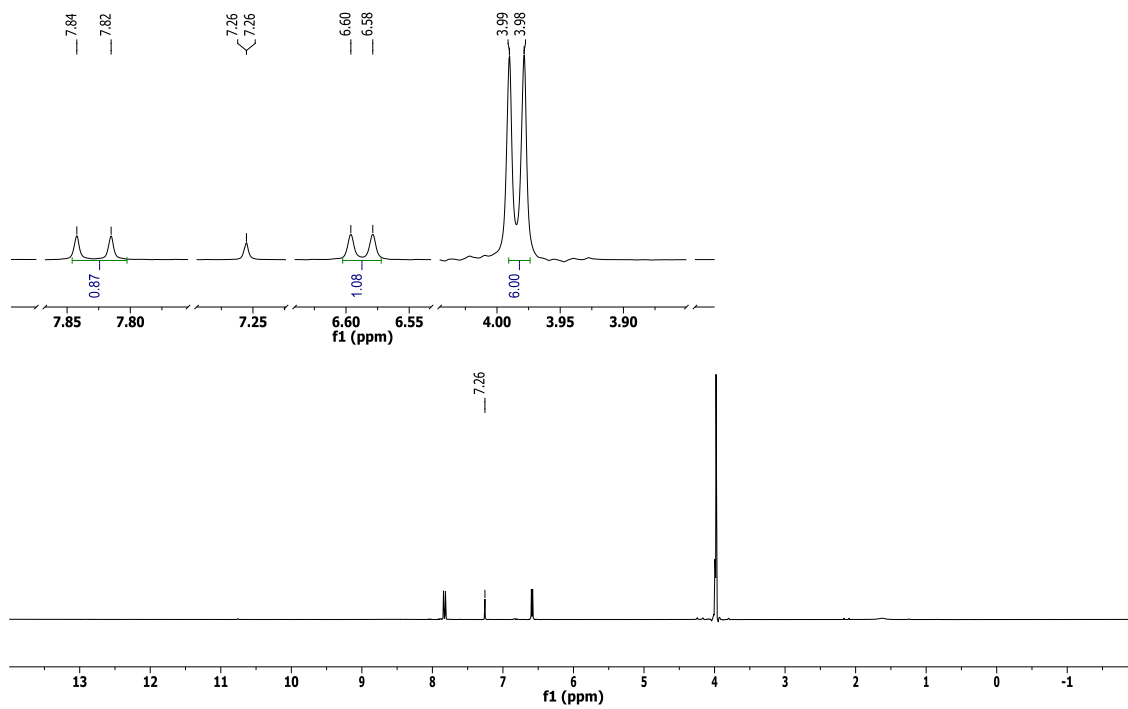
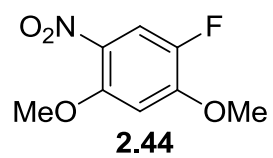


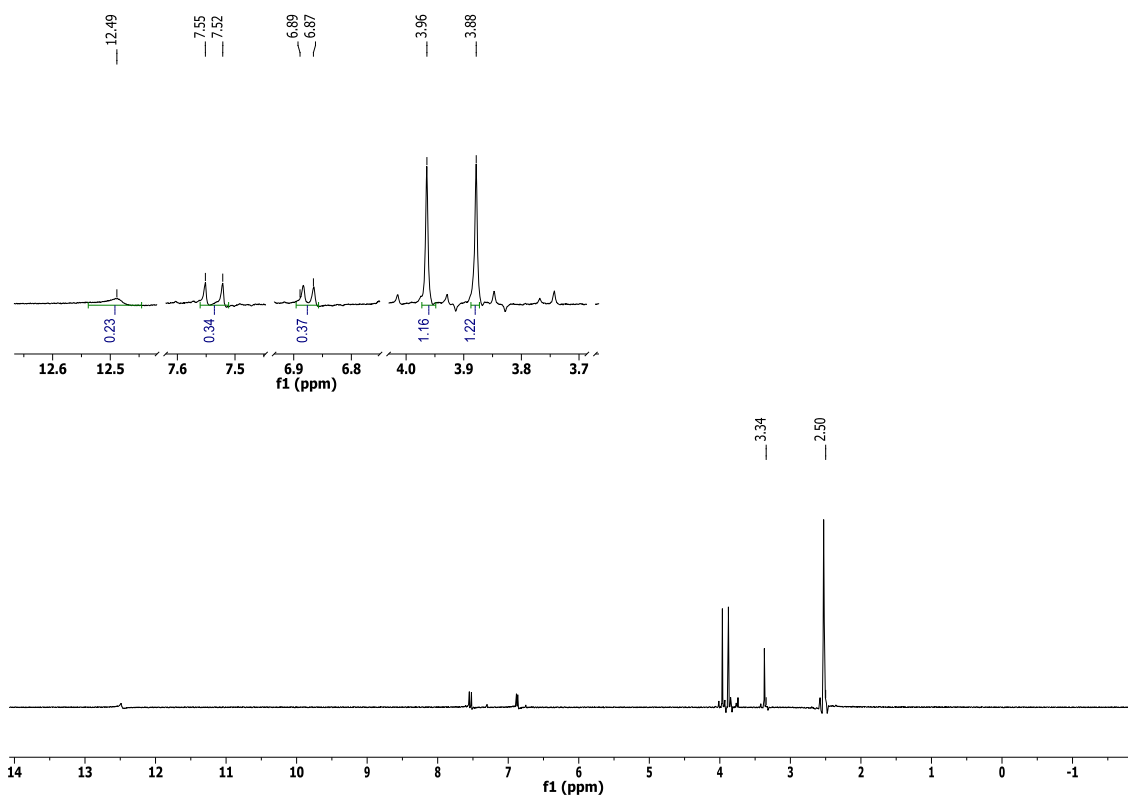
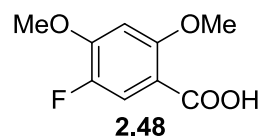


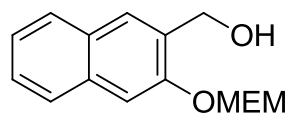
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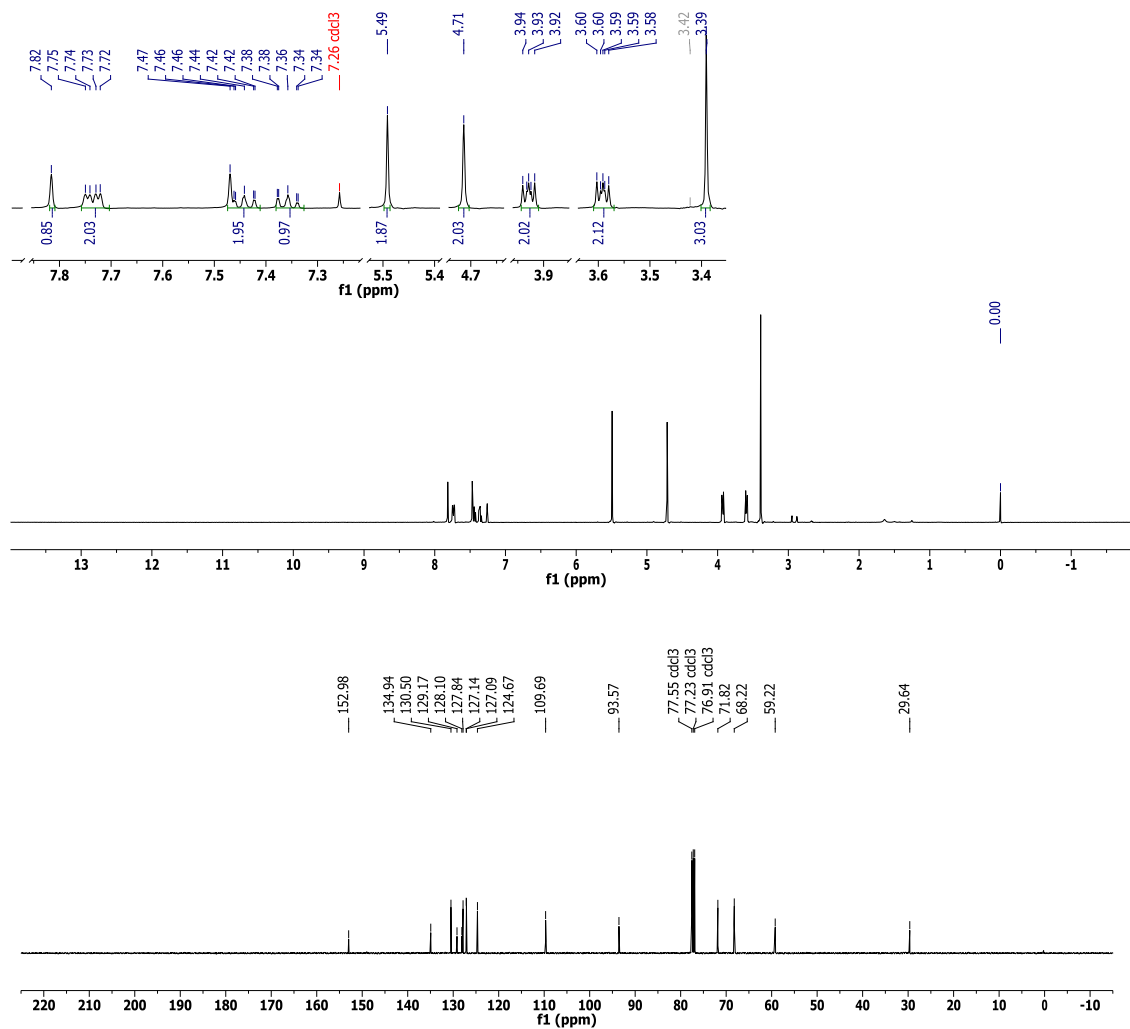


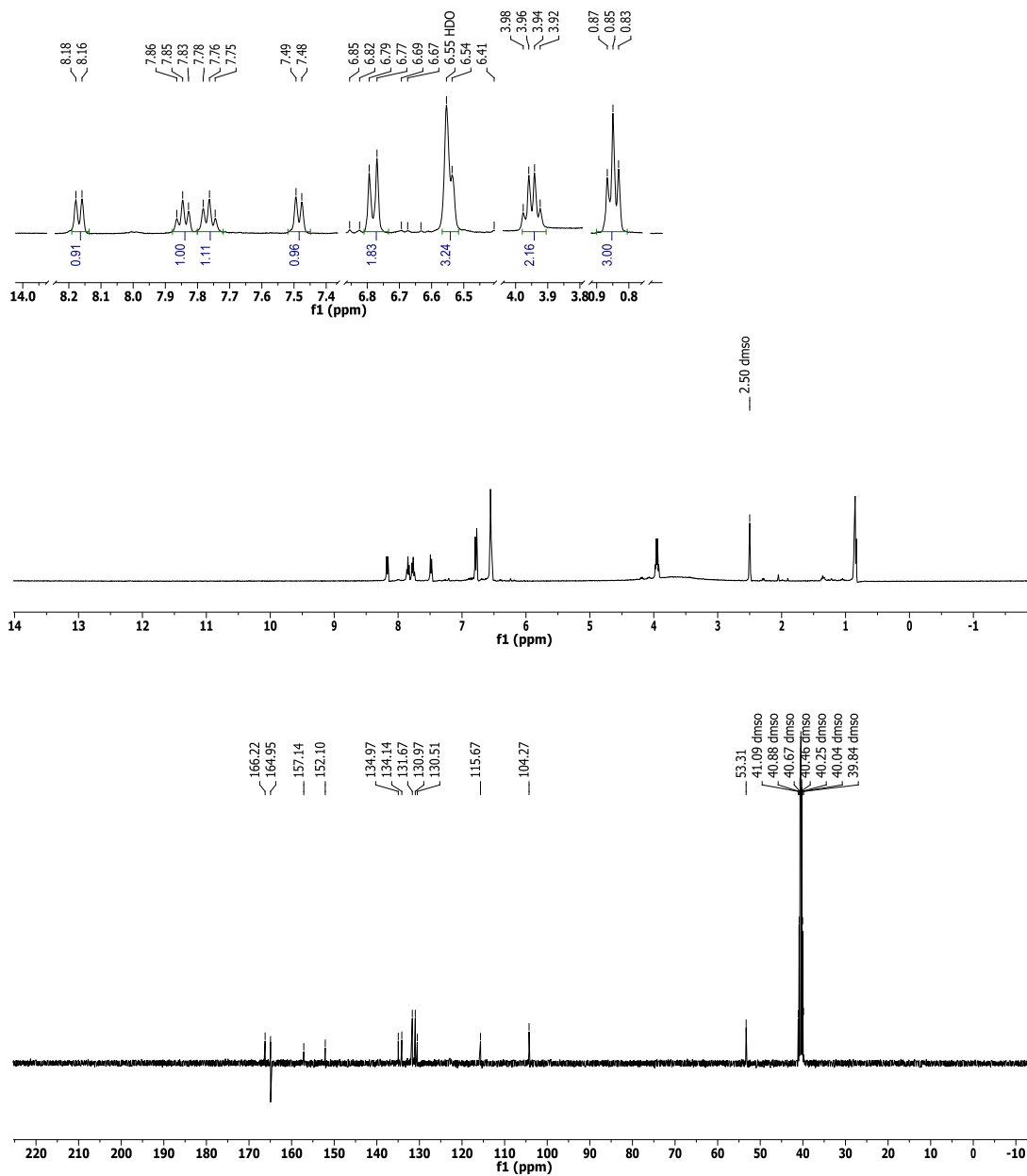
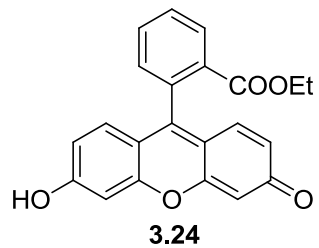


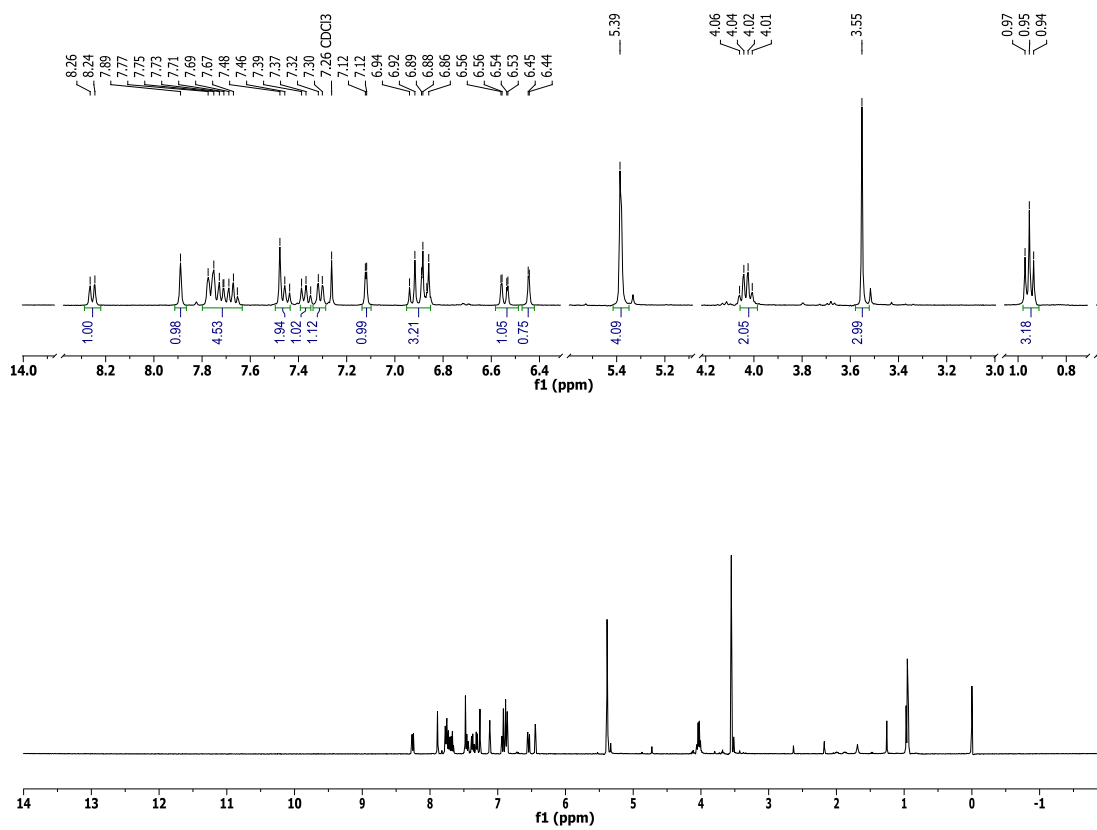
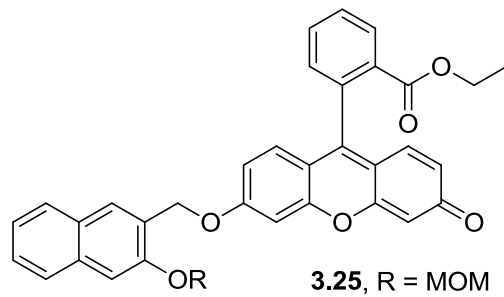


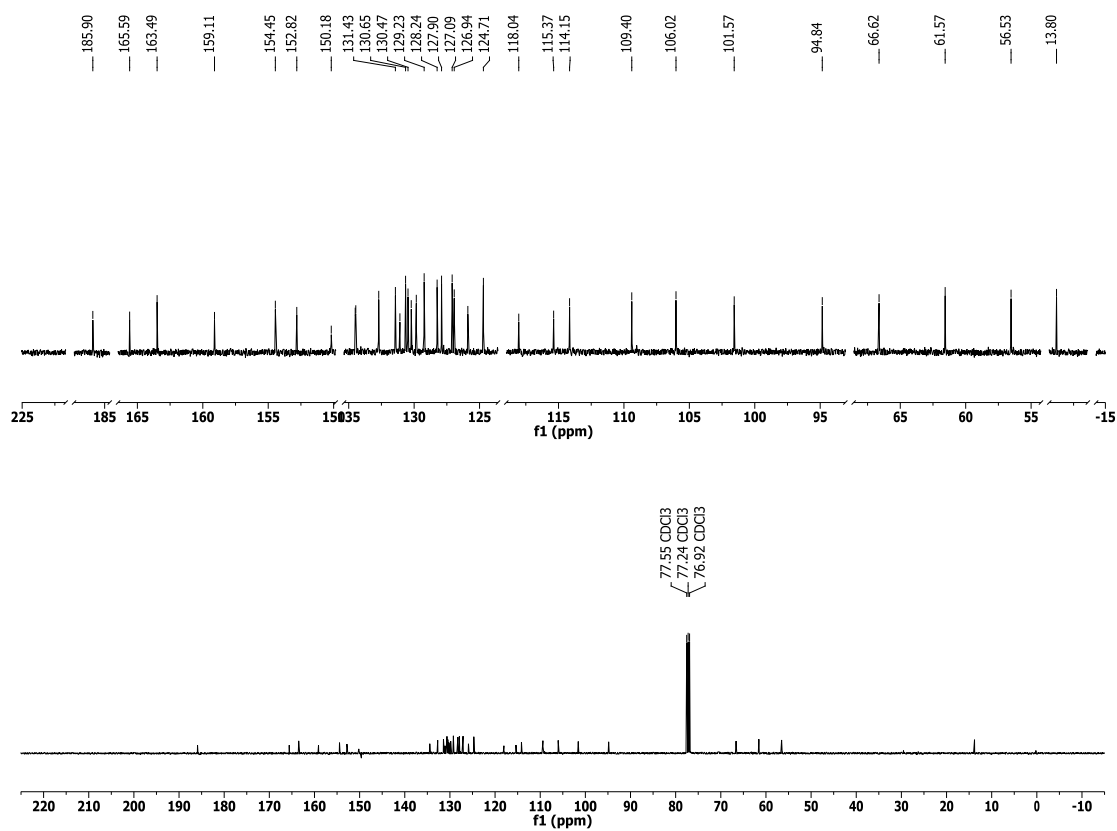
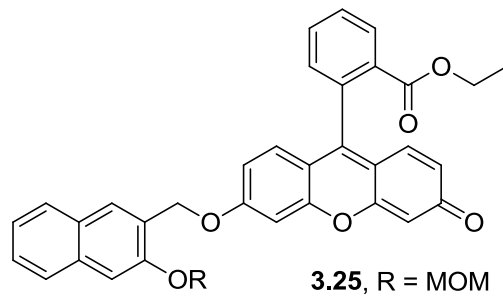


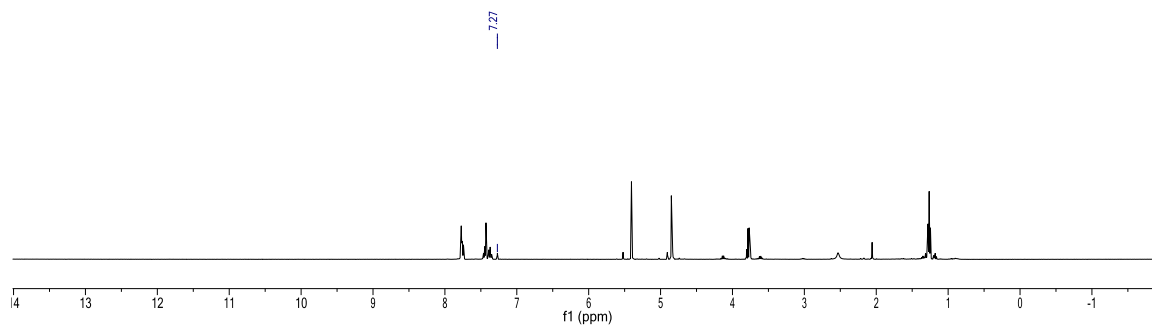
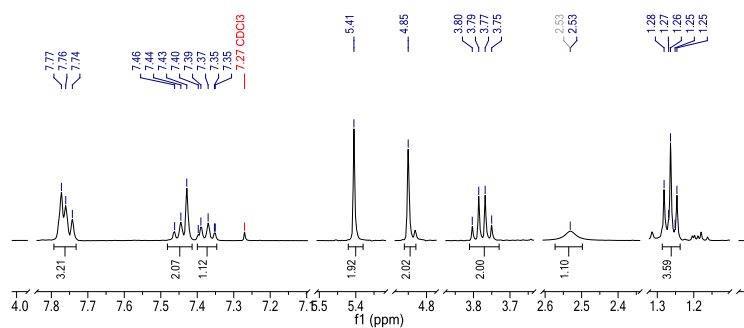
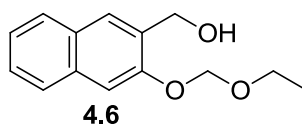
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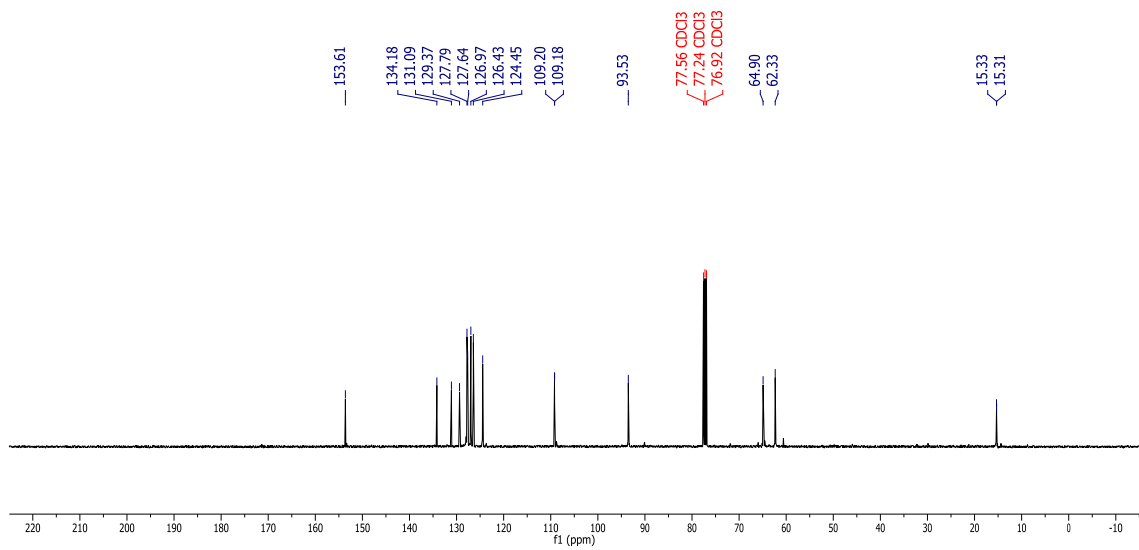
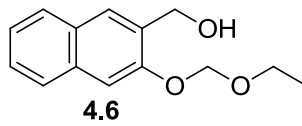


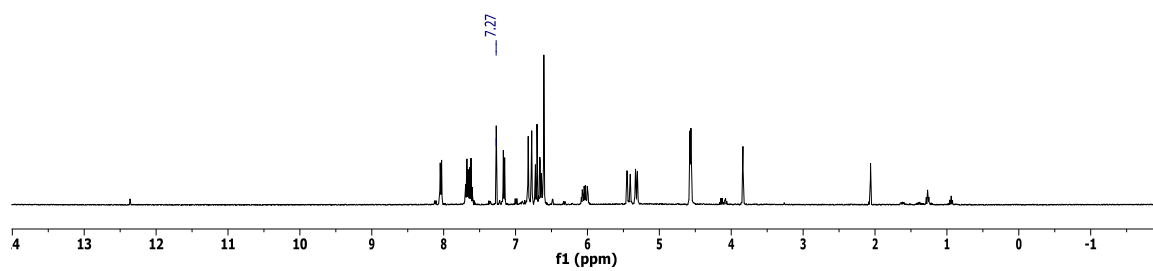
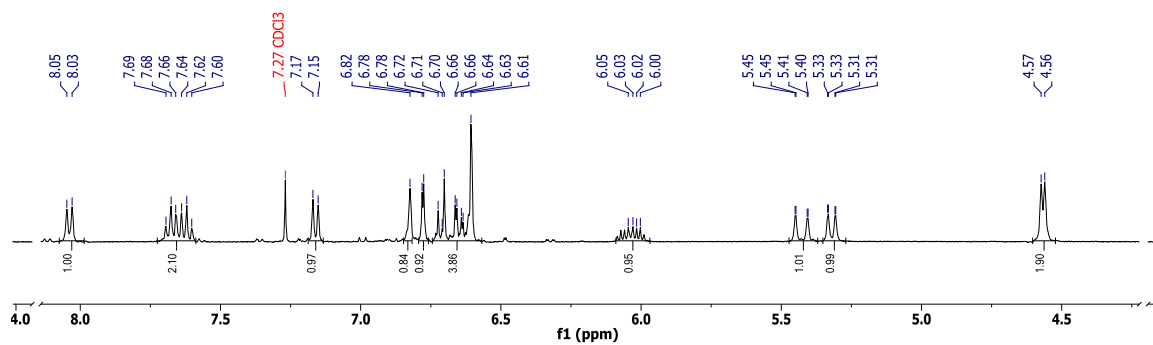
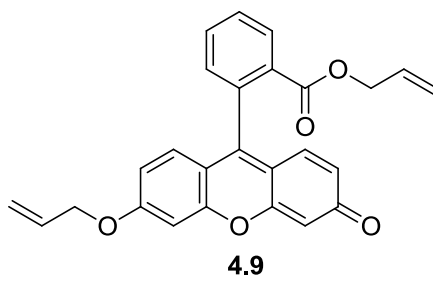


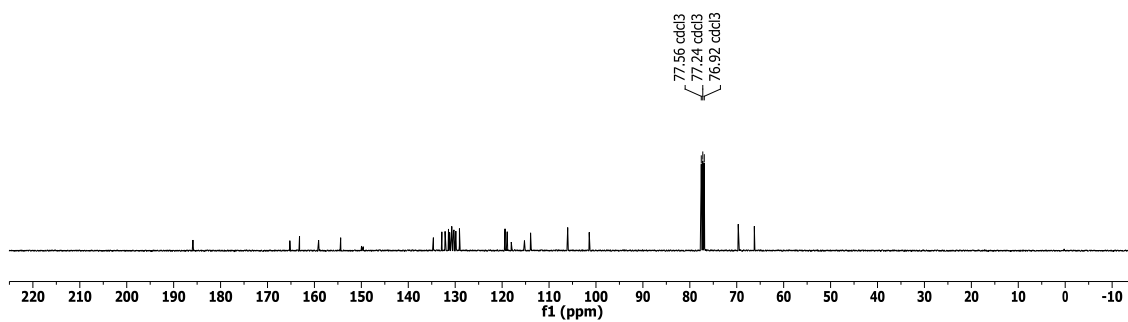
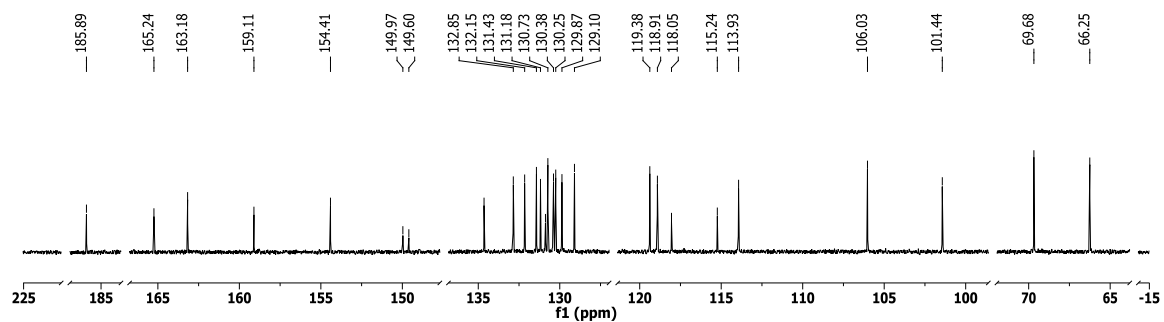
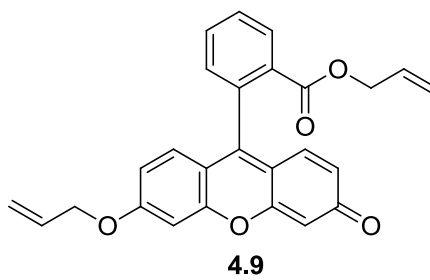


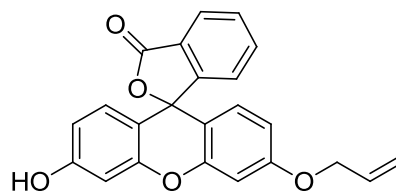




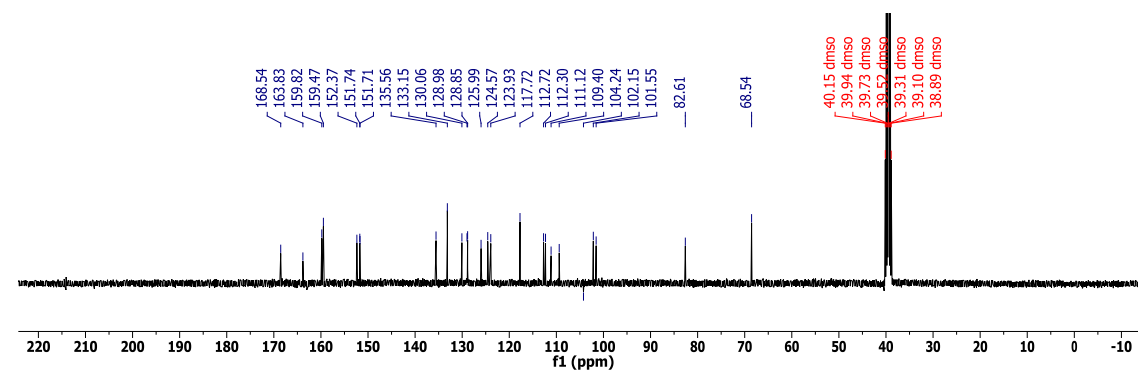
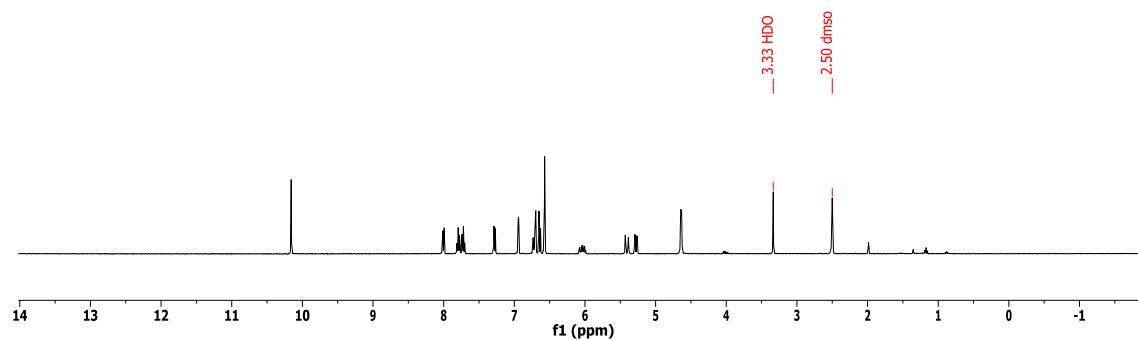
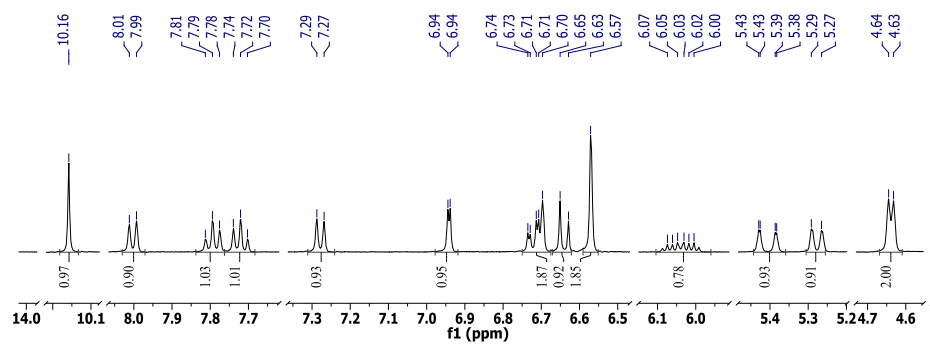


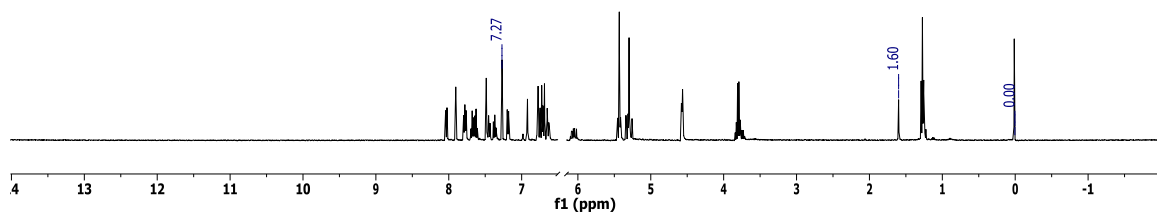
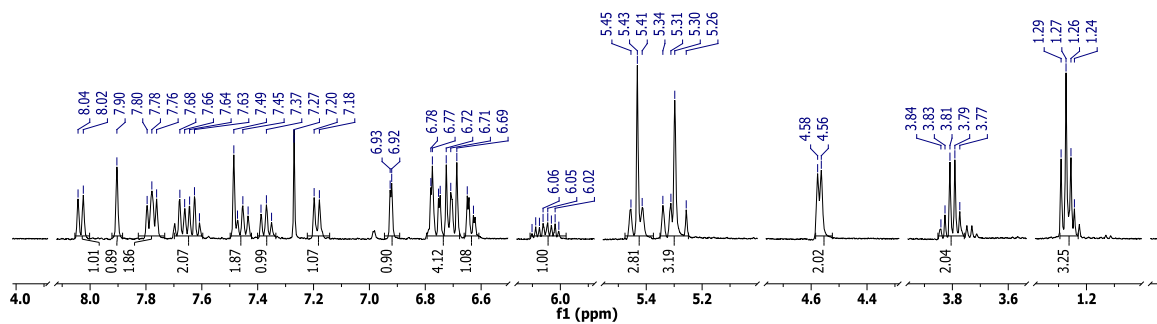
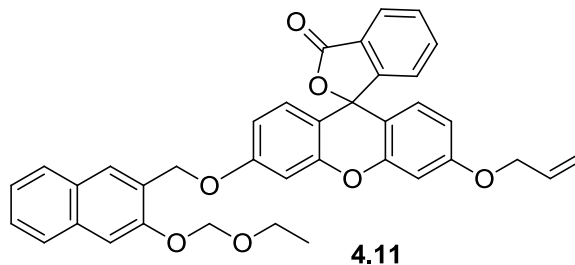


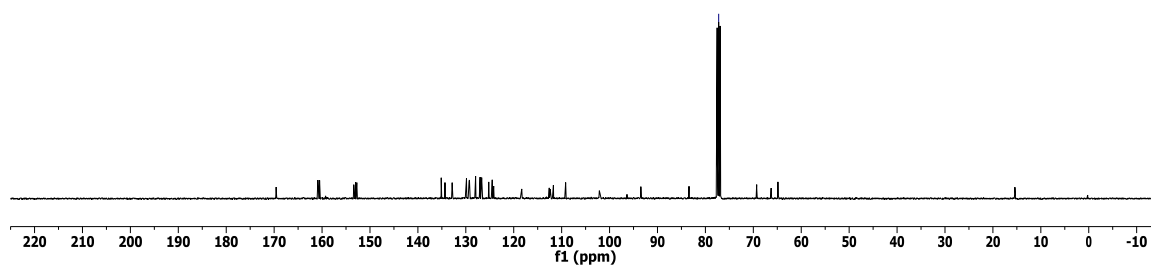
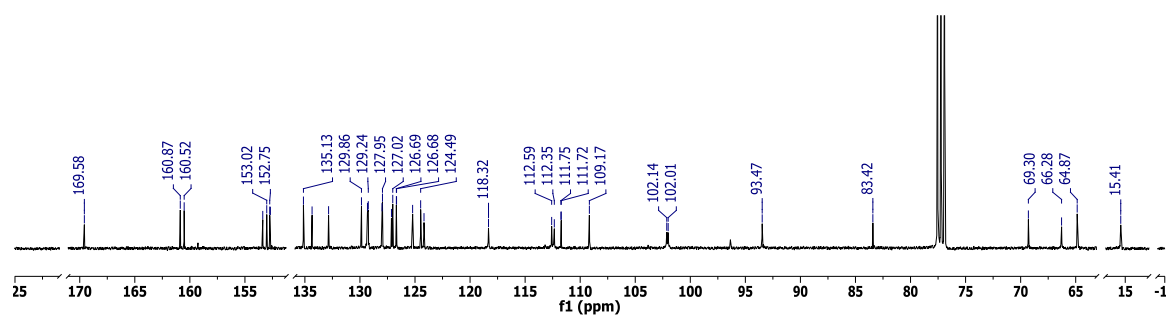
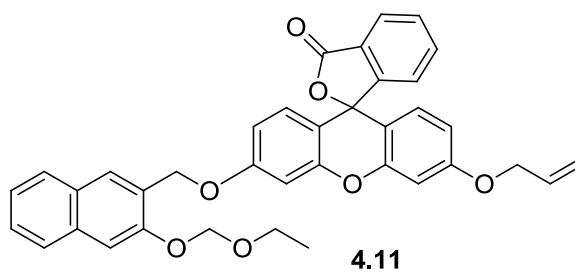


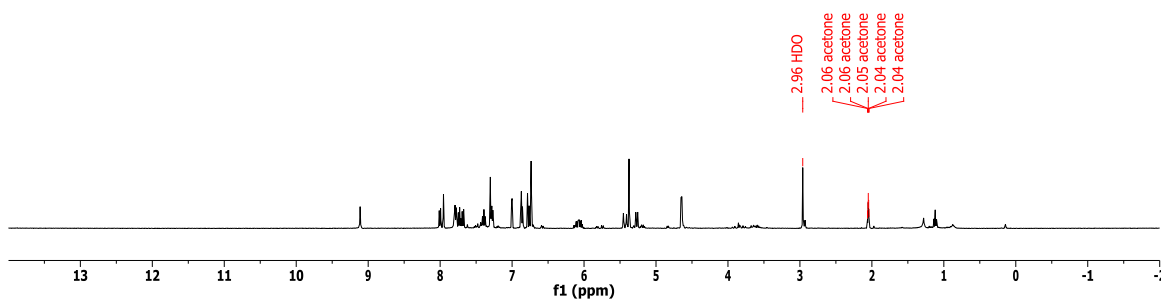
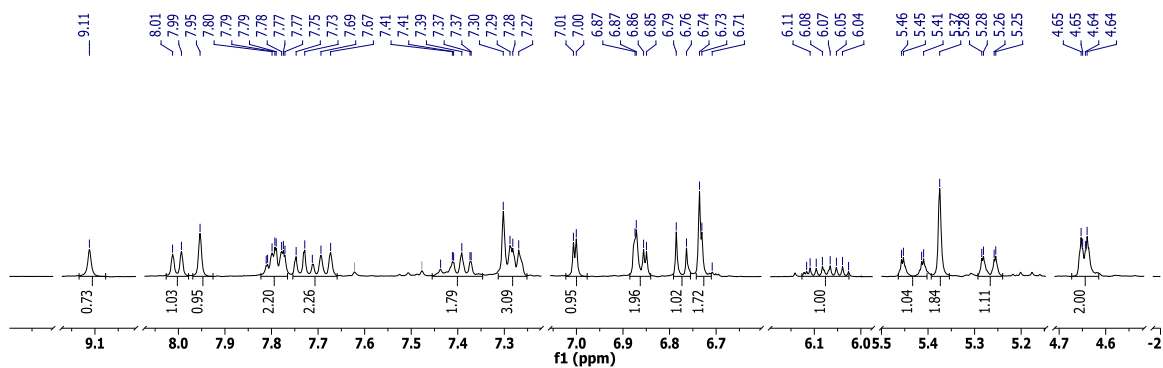
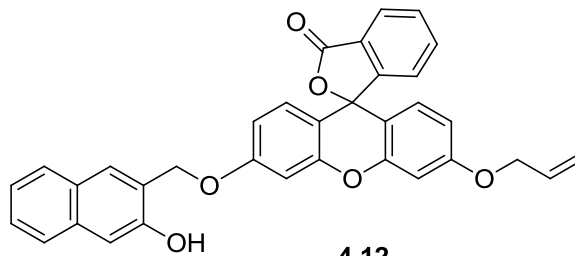


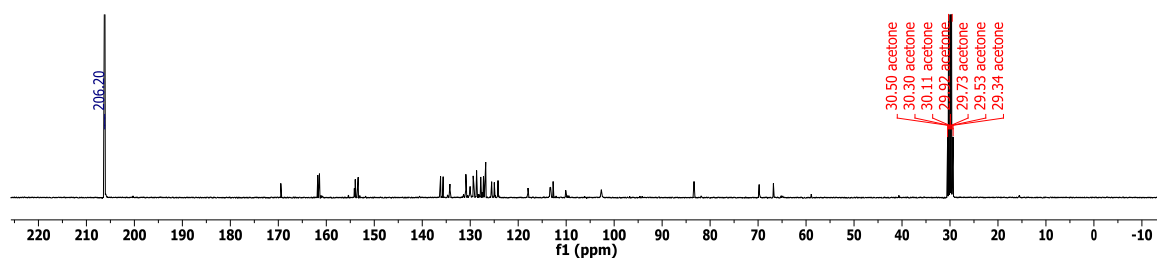
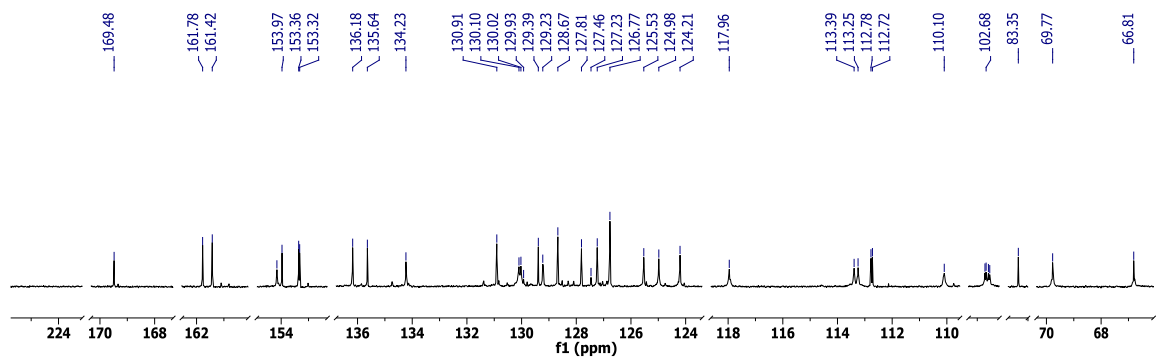
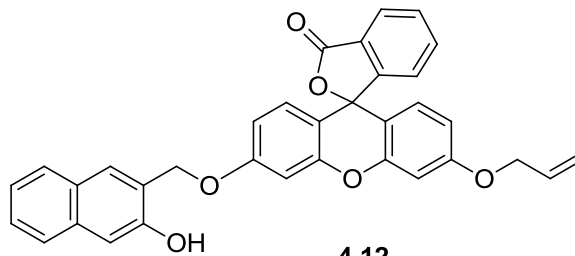
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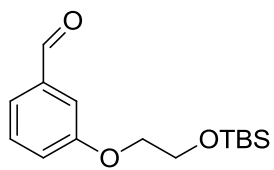




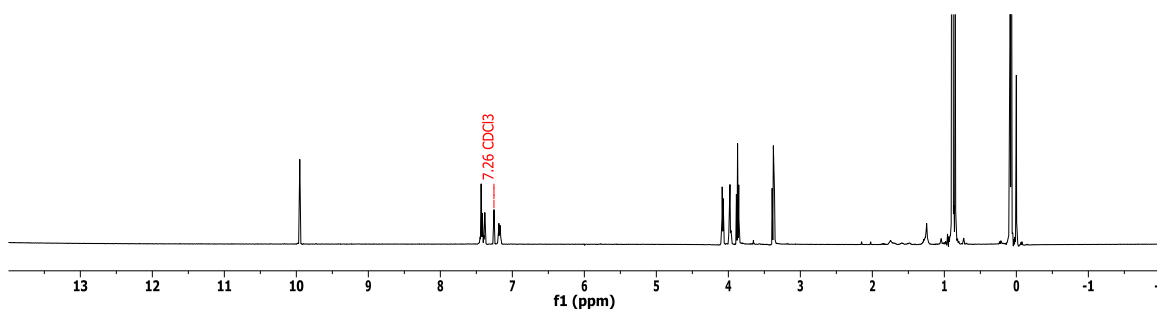


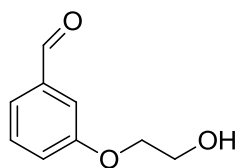




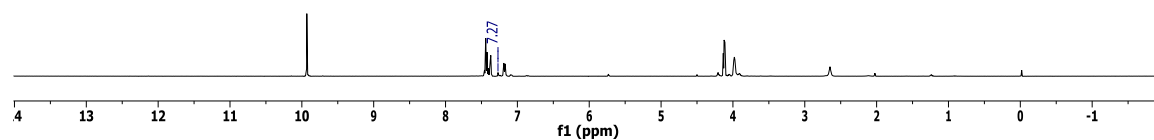
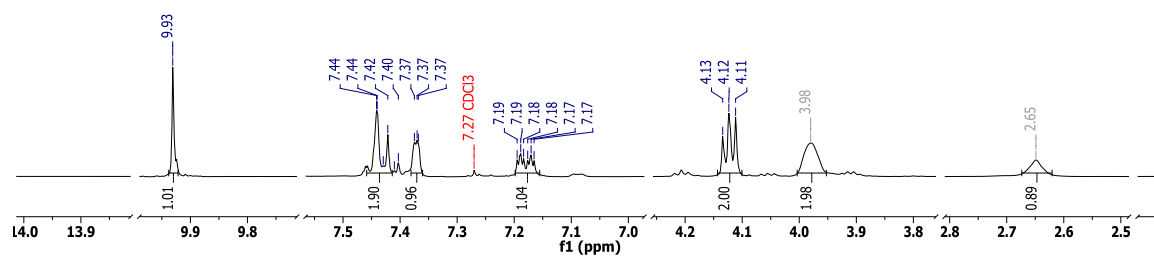
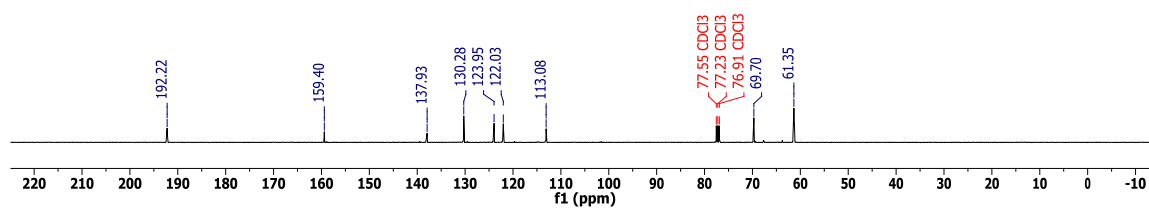


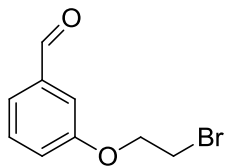
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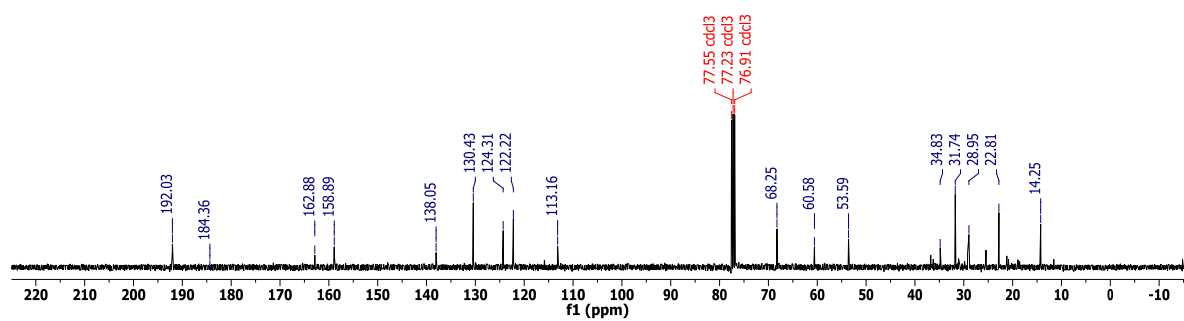
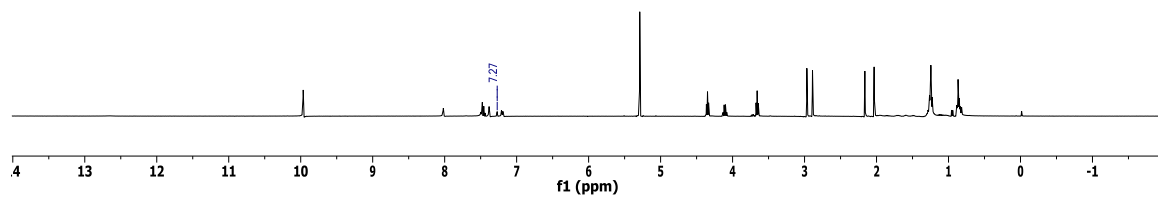
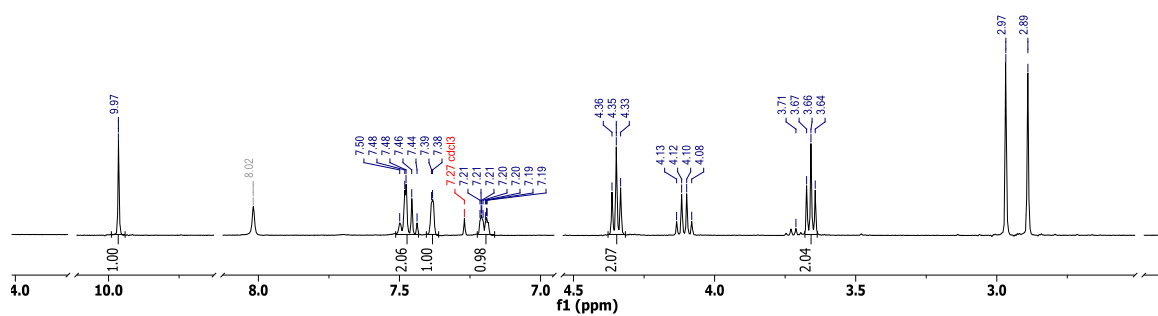


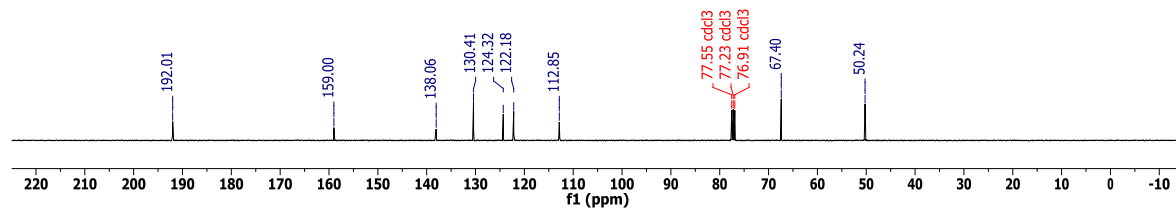
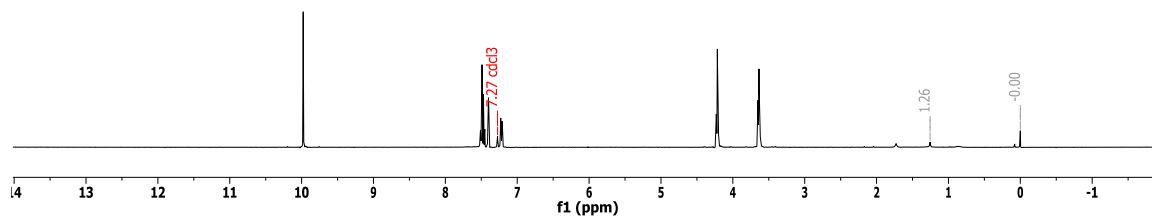
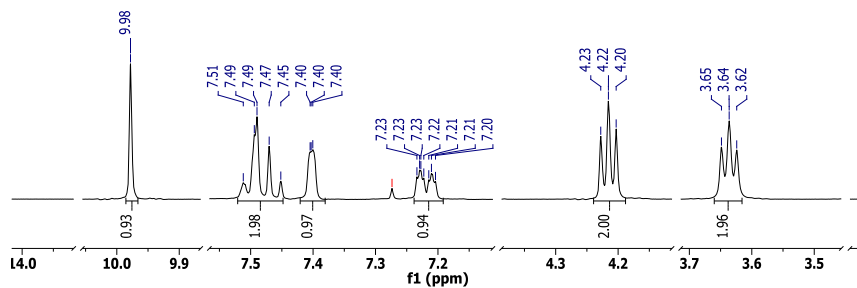
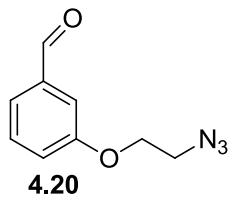
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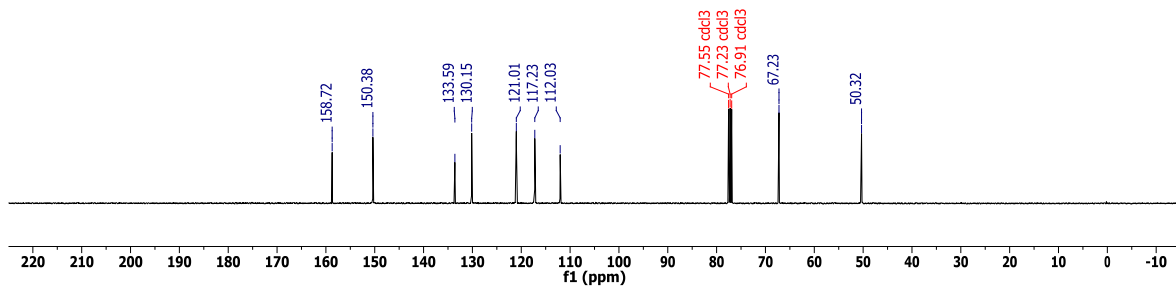
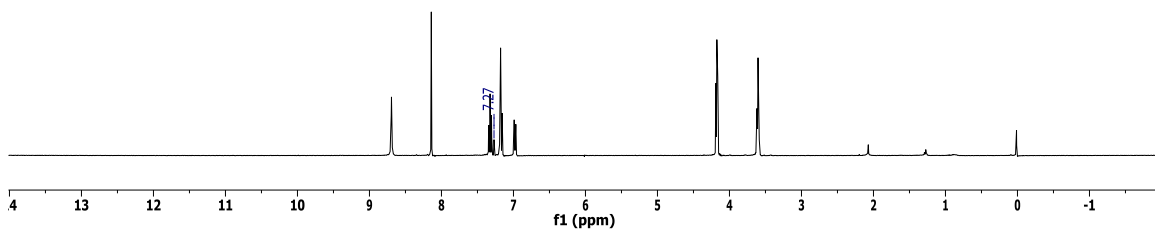
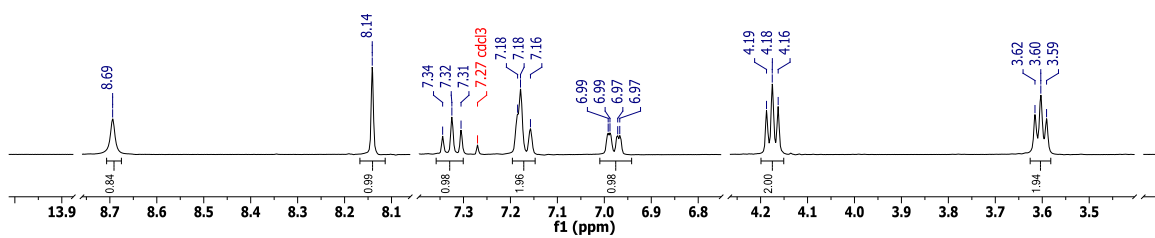
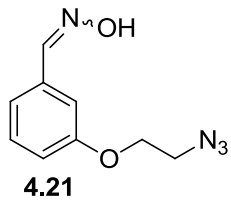


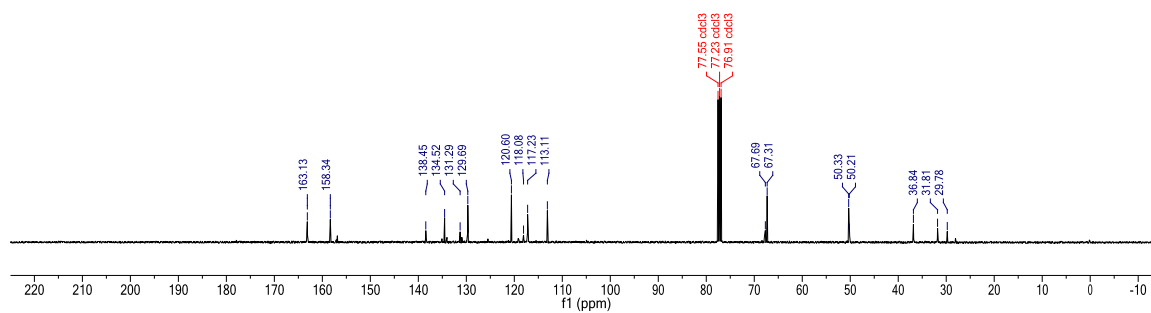
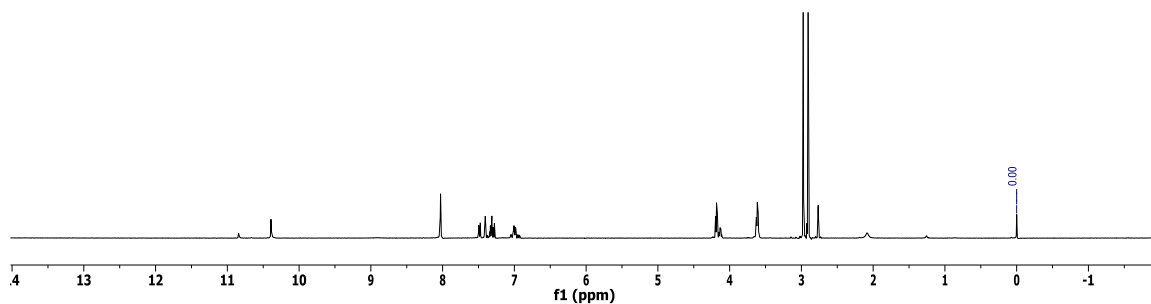
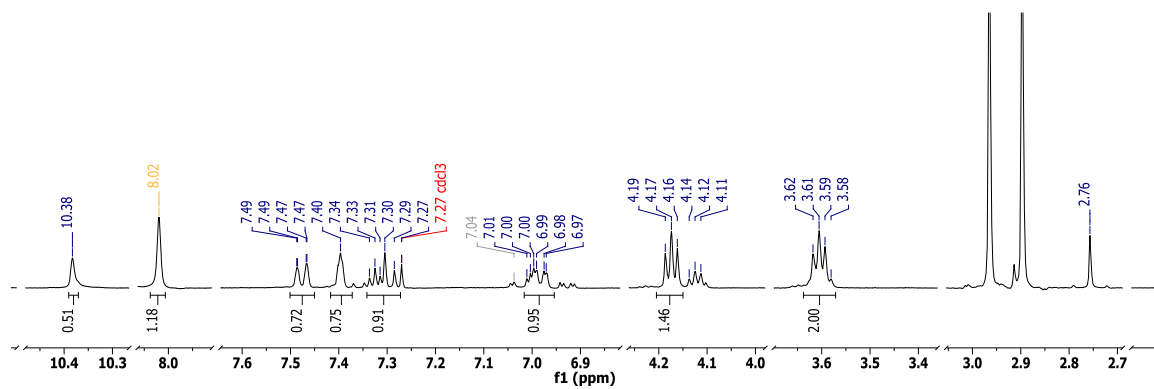
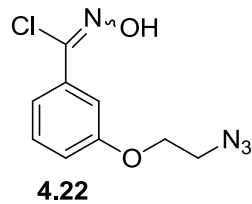


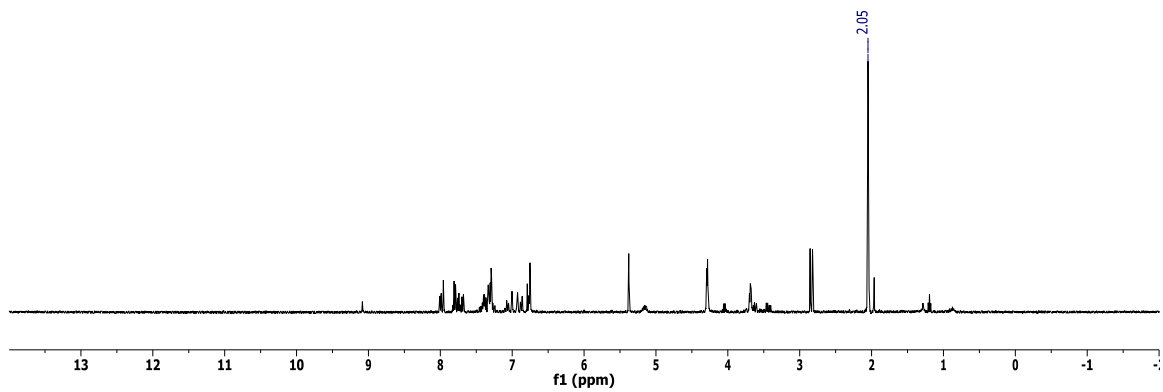
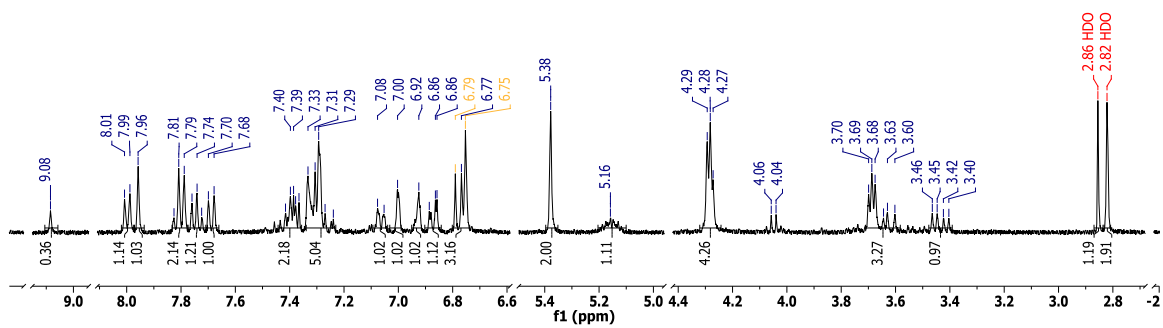
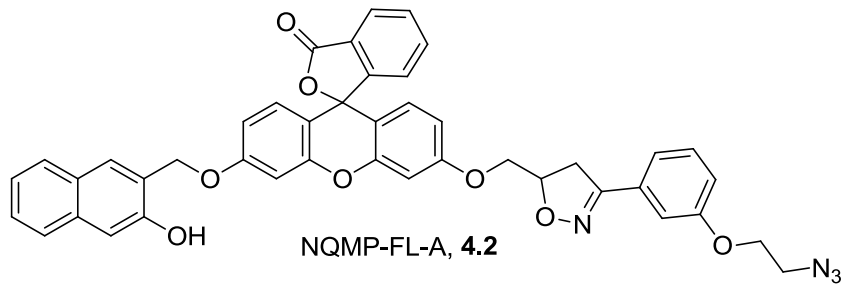
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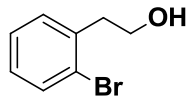




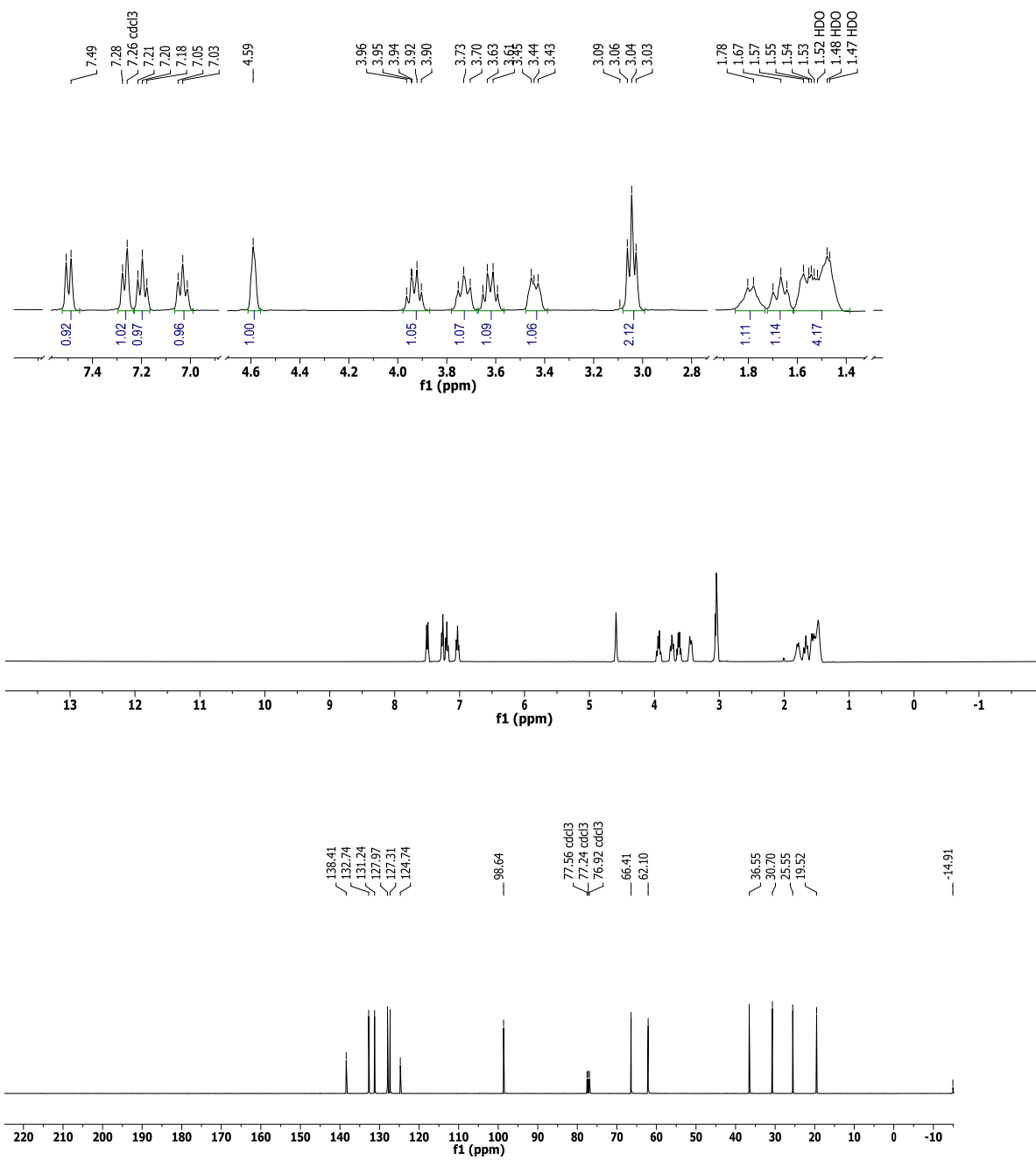


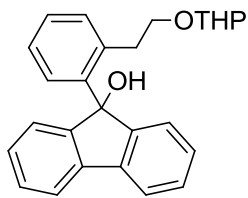




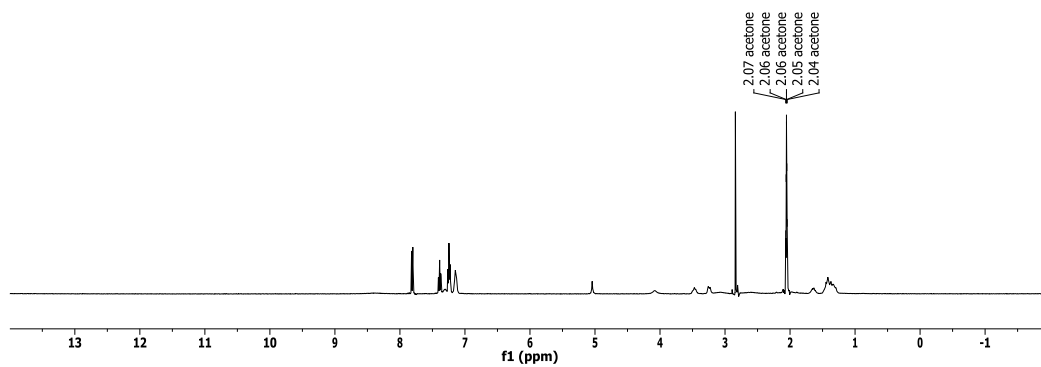
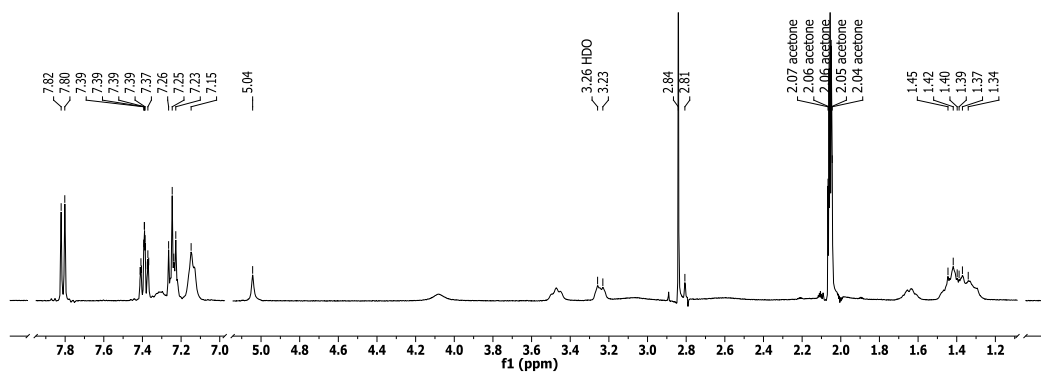
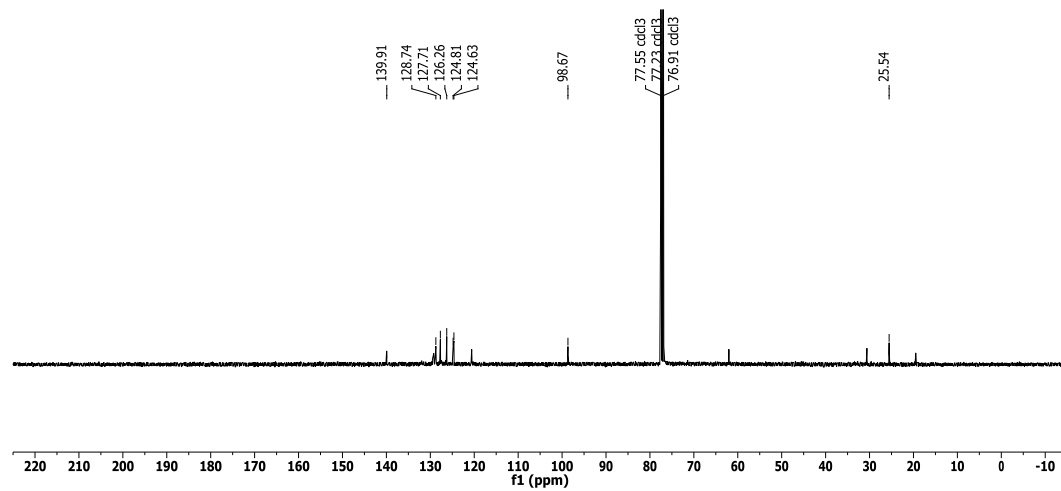


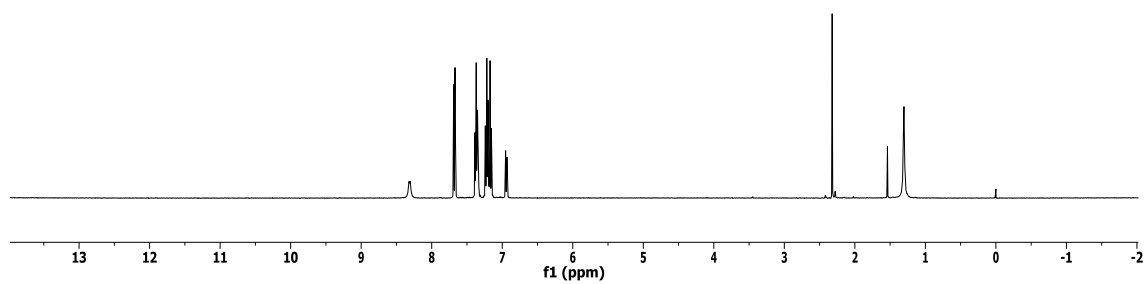
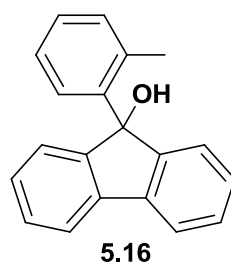
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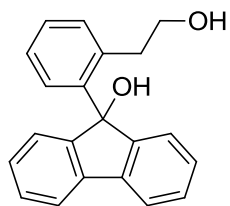




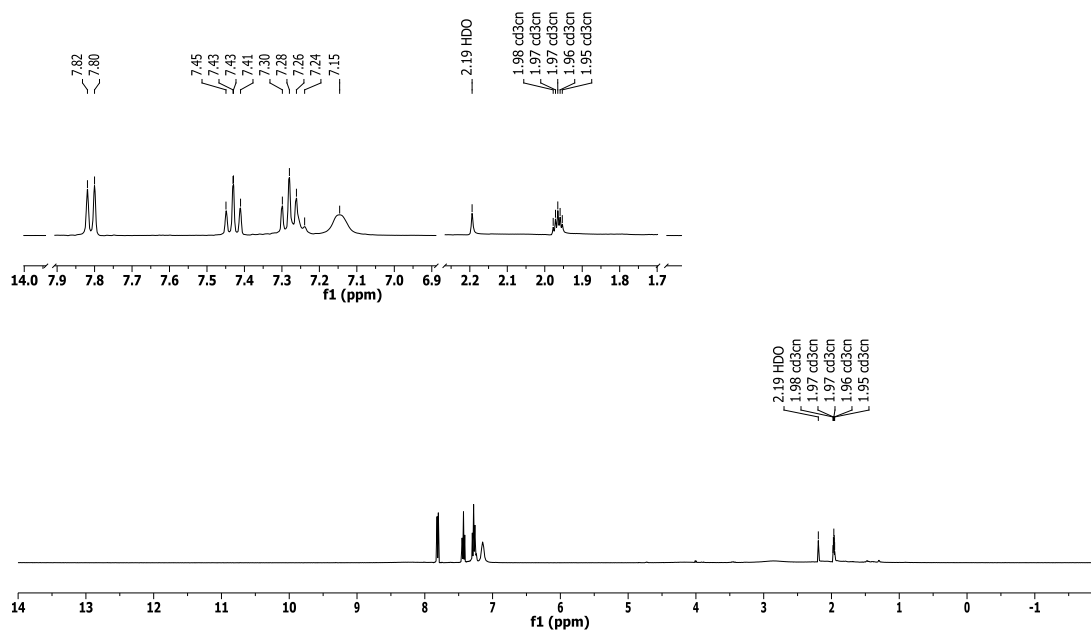
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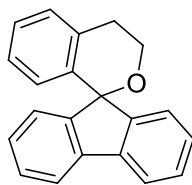




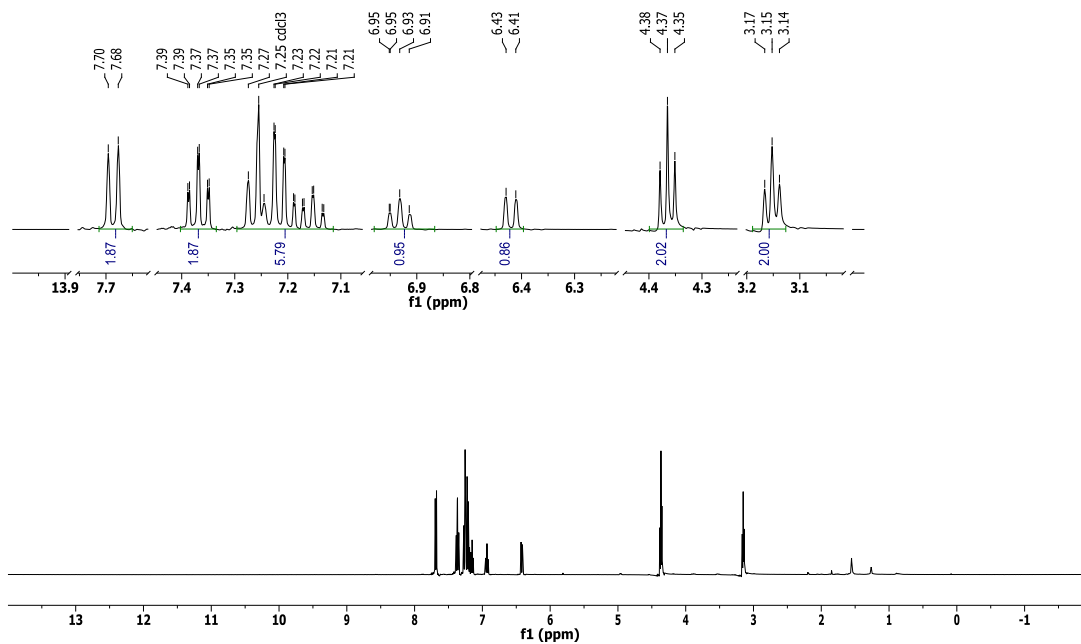


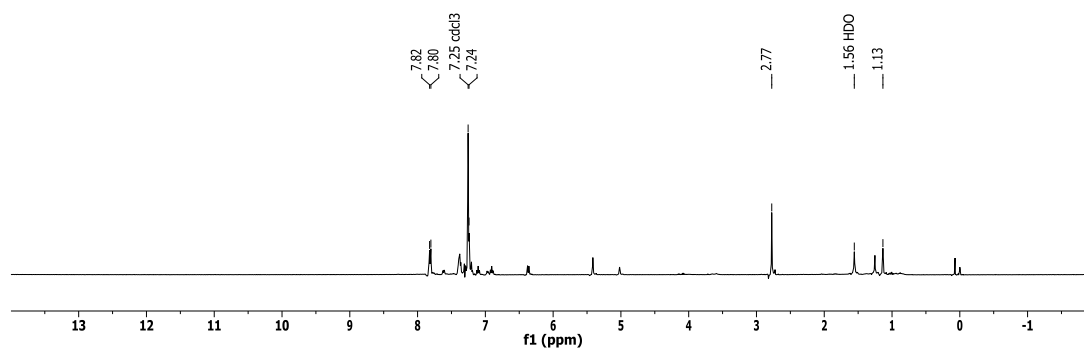
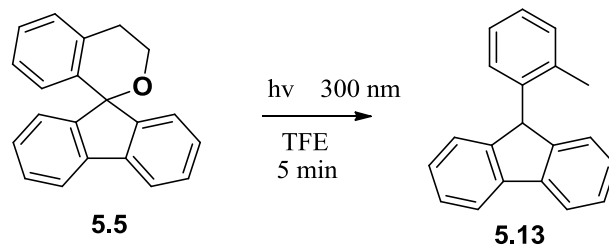
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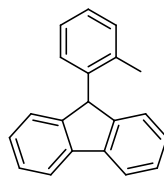




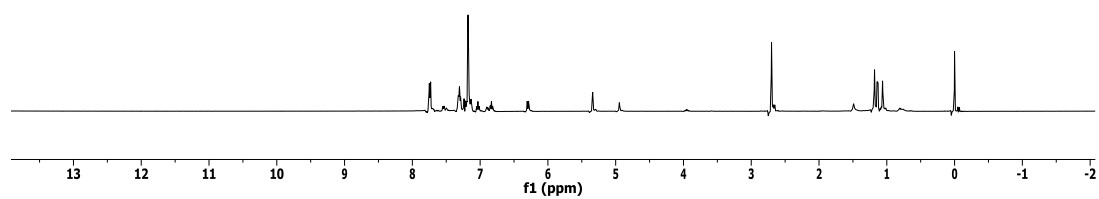
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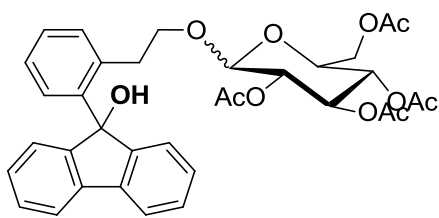




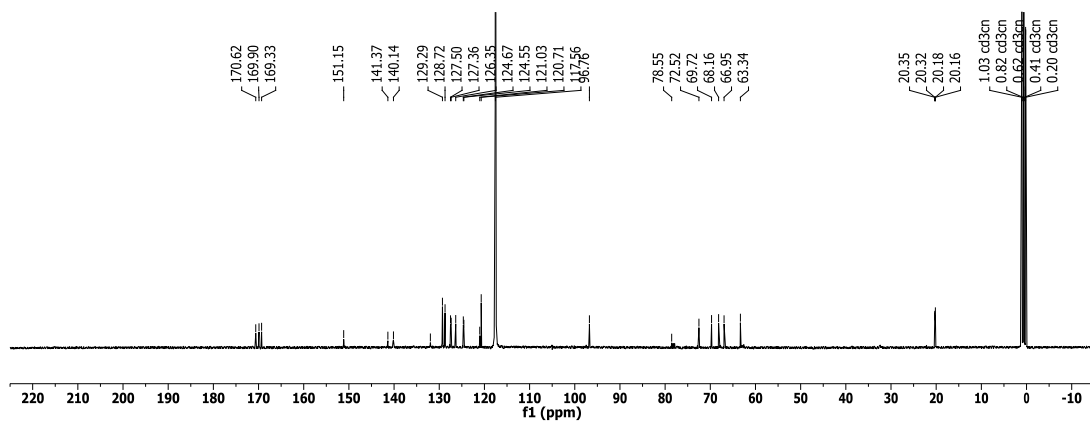
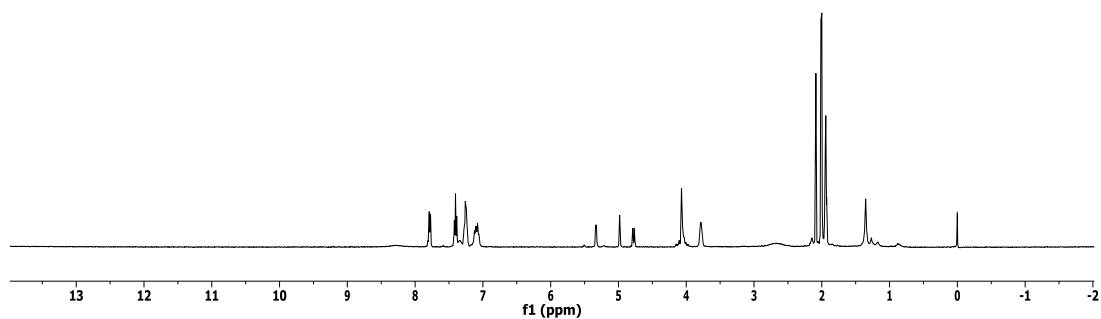


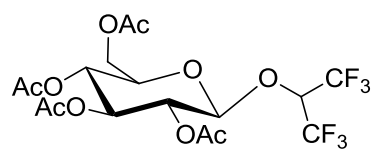
5.13





5.29





5.29

