ROSAMINE AND FLUORESCEIN DERIVATIVES AS DONORS/ACCEPTORS FOR "THROUGH-BOND" ENERGY TRANSFER CASSETTES

A Thesis

by

JUAN CARLOS CASTRO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Chemistry

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Approved by:

Chair of Committee,	Kevin Burgess
Committee Members,	Marcetta Darensbourg
	Coran Watanabe
	Jorge Seminario
Head of Department,	David Russell

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ABSTRACT

Rosamine and Fluorescein Derivatives as Donors/Acceptors for
"Through-bond" Energy Transfer Cassettes. (December 2009)
Juan Carlos Castro, B.S., California State University, San Bernardino
Chair of Advisory Committee: Dr. Kevin Burgess

A series of fluorescein and rosamine derivatives have been prepared and their spectroscopical properties analyzed to determine their usefulness as donor and/or acceptors in "through-bond" energy transfer systems.

Such new systems have been tailored to possess higher quantum yields, increased water solubility and higher pH independence. Some of these compounds have also been designed with handles for bioconjugation for use in intracellular imaging.

The syntheses of most of these compounds have been optimized to afford higher yields in a multigram scale.

Single molecule studies on a fluorescein/rosamine cassette are also reported.

DEDICATION

In memory of Dr. Roger Morgan and Dr. John Hogg

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Kevin Burgess, for his support and advice during my graduate years by providing an environment conducive to independent research and hard work.

I would also like to thank Dr. Marcetta Darensbourg, Dr. Coran Watanabe and Dr. Jorge Seminario for serving on my graduate committee.

Thanks to Dr. Hogstrausser for his single molecule studies.

Thanks to Jill Rutledge for all her help and understanding in my many necessities.

Thanks to everyone in the Burgess group for their time, help and companionship during all these years; may you all enjoy great success.

Finally, thanks to my family for whom all this would not have been possible in many different ways. Thanks to my wonderful wife, Maria, for her patience and support through rough times. Thanks to my beautiful daughters Angel and Elizabeth, for brightening up my life.

NOMENCLATURE

CDCF	carboxydichlorofluorescein
DCF	dichlorofluorescein
DMF	*N*, *N*-dimethylformamide
EtOAc	ethyl acetate
EtOH	ethanol
FRET	fluorescence resonance energy transfer
HCl	hydrochloric acid
MeOH	methanol
TBET	through-bond energy transfer
TEA	triethyl amine
THF	tetrahydrofuran

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CHAPTER I

INTRODUCTION: WHY ENERGY TRANSFER CASSETTES?

The importance of fluorescent compounds for biological applications is enormous. Our research group was first attracted to the idea of "energy-transfer" fluorescent cassette systems primarily for DNA sequencing applications since the dyes utilized mainly for this purpose suffer from several drawbacks. Later on in the course of research, our focus shifted to applications of such fluorescent systems to other biological uses (i.e. pH probes, protein labels), but the background of its significance and the inherited problems are the same as outlined for DNA probes, hence in this introduction the focus is on fluorescent “through-bond” energy transfer cassettes as DNA probes.

The most common dyes used for DNA sequencing were FAM (5-carboxyfluorescein), JOE (2’,7’-dimethoxy-4’,5’-dichloro-6-carboxyfluorescein), TAMRA (*N*,*N*,*N’*,*N’*-tetramethyl-6-carboxyrhodamine), and ROX (6-carboxy-X-rhodamine), these dyes posses four distinct emission maxima of the fluorophores. The drawback of these dyes is that their absorption maxima is different, and they don’t share a common strong absorbance at any wavelength (Figure 1.1).

This thesis follows the style of *Journal of Organic Chemistry*.

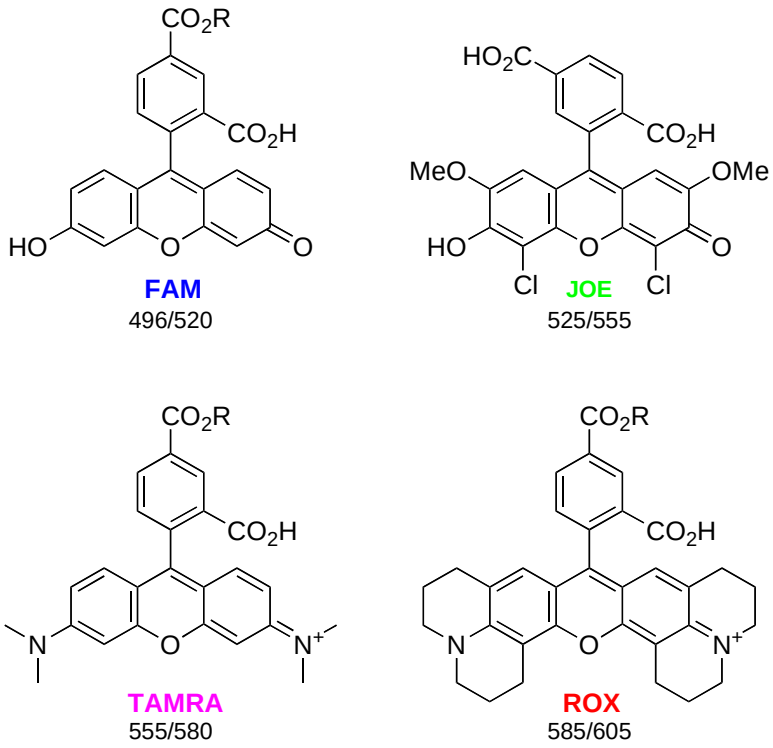


Figure 1.1 Representative fluorescence intensities of JOE, ROX, FAM and TAMRA excited at 488 nm.

It is clearly seen from the representative figure that the fluorescence intensities from the four dyes goes down as the emission wavelength increases. This phenomena occurs as the amount of energy absorbed by the dye decreases due to the change in adsorption maxima of the dye. The ideal set of dyes would have equivalent emission intensities for all four compounds, allowing for easier interpretation by the recording computer (Figure 1.2).

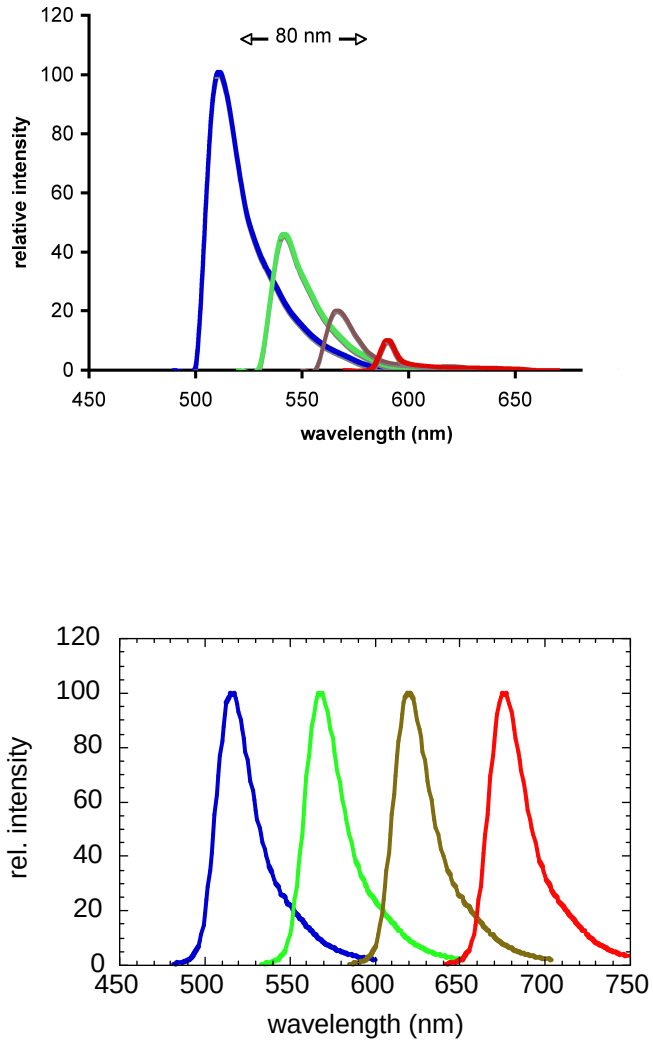


Figure 1.2 Fluorescence intensities of ideal sequencing dyes: resolved and intense.

For the ideal emission of four cassettes, it would be necessary for all of them to have the same absorption maxima, with huge Stoke's shifts for those that emit at longer wavelenghts. This problem could be solved by the use of fluorescence energy transfer (ET) cassettes. The benefit and need of such a system is clear to us. High-throughput DNA sequencing relates to every project for which encoding information is to be obtained on a genomic scale. Obtaining accurate DNA sequencing data is likely to be a

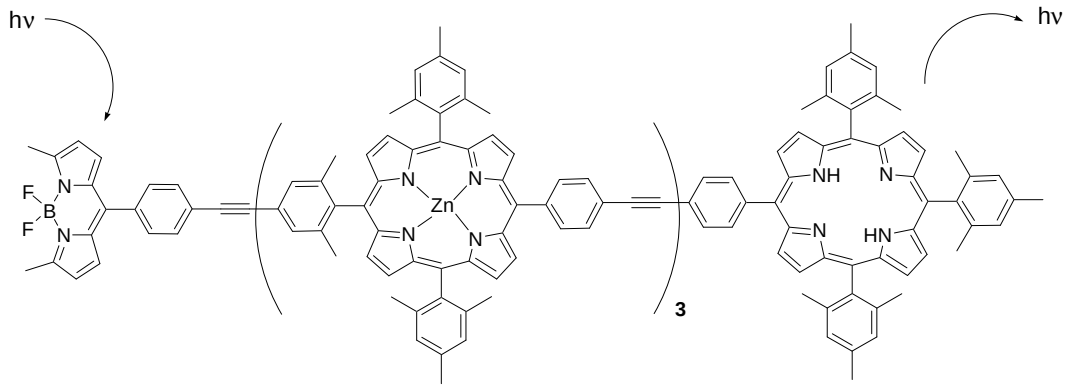
major issue in the biomedical sciences throughout the first part of this century. At present, the efficiency of high-throughput DNA sequencing methods is directly related to the "length of read" that can be obtained from each experiment. This in turn, is dependent upond the levels of fluorescently labeled DNA that can be detected. A small increase in the fluorescence intensity of the label used, translated to longer read lengths and major gains in efficiency for genomic DNA sequencing where thousands of experiments are involved. Scientifically, the chemistry of designing novel dyes that are tailor-made to harvest light from a laser source and emit it with maximal intensity is a challenge. Our through-bond energy transfer approach is novel, hence the project is academically stimulating too. Our systems could be used for intracellular imaging where multiplexing is desired, specifically for protein-protein interactions, and it is this area where our focus have shifted in the past several years.

In 1995, Ju and coworkers from the University of California at Berkeley, utilized the idea of fluorescence energy transfer to construct cassettes that absorb at a common wavelength given off by a laser, and emit at four different ones.[1] The advantage of these cassettes is the relatively close electrophoretic mobility shift of the DNA fragments and distinctive emission wavelengths. By utilizing a fluorescein dye attached to the 5' end of the primer, and other fluorescein and rhodamine derivatives along the primer strand attached to a thymidine residue, the energy absorbed by the fluorescein "donor" is transferred through space to the "acceptor" dyes via a dipole-dipole coupling, which in turn emits at its distinctive wavelength. The use of a common donor dye allows for an equivalent amount of energy absorbance which in turn excites the acceptor dyes more efficiently, obtaining exceptional sensitivity and signal strengths several times greater than those obtained from a single-dye labeled molecule. In this case, the energy transfer occurs via non-conjugated linkers (through-space), but it could also be done with a π-conjugated system

For energy transfer to occur through-space, the emission and adsorption of the donor and acceptor must overlap, this inconvenience does not allow for a big distribution of emission wavelengths, since the Stoke's shift must be relatively close to the emission of the donor. This may be avoided with through-bond energy transfer. In the past recent years, our group has focused on through-bond energy transfer cassettes. Through-bond energy transfer is thought to occur via one of two mechanisms; Dexter energy transfer and superexchange. Dexter energy transfer can be conceptually thought as the effective transfer of two electrons, for this to happen, the orbitals of the excited-state donor and ground-state acceptor must be in physical contact, but it does not necessarily mean that the donor and acceptor should be bonded together, although they usually are. The second mechanism, superexchange, was first suggested by McConnell in 1961.[2] It occurs via energy transfer through the bonds connecting the donor and the acceptor, and so, it occurs over longer distances than Dexter energy transfer. When talking about the bonds connecting the donor and the acceptor, it does not imply a specifically covalent σ-bonded framework, since it can also include solvent molecules,[3] hydrogen bonded bridge groups,[4] or salt bridges.[5] There is no accurate physical description of how the energy transfer in superexchange works, but it may involve mixing of the donor and acceptor orbitals with the appropriate orbitals of the connecting species, but this mixing does not result in the formation of new orbitals that would conjugate the donor, acceptor and connective segment, as one would sugest from simple orbital molecular theory, as explained in recent literature.[6-10]

Our group has focused on dye cassette systems that are in π-conjugation since this affords for a longer distance between the donor dye and the acceptor, and overlap of donor fluorescence with acceptor absorption spectra is not a requirement for through-bond energy transfer. For this reason, it is possible to form a set of through-bond energy transfer cassettes that fluoresce intensely at widely dispersed wavelengths. High throughput DNA sequencing methodology via the dye-primer and dye-terminator approaches could be significantly improved by using fluorescent markers developed to exploit through-bond energy transfer.

The idea of using through-bond energy transfer for linking fluorescent dyes is not a new one. In 1994, Lindsey and coworkers reported the use of a boron-dipyrromethene (BODIPY) dye as an optical input at one end and a free base porphyrin as an optical output at the other end, linked by a linear array of three zinc porphyrins (Figure 1.3).[11]



Observed ET Efficiency = 76 %, Theoretical ET Efficiency = 0.1 to 6%

Figure 1.3 A through-bond ET system by Lindsey *et al.*[11]

Our group has also investigated the use of BODIPY dyes for DNA sequencing cassettes that exploit energy transfer.[12-15] The first generation ET dyes which employed BODIPY dyes as the donor and acceptors enjoyed efficient ET, but were found to have two major setbacks. The two 3,5-diarylBODIPY acceptor dyes suffered from low quantum yield, but more importantly, when attached to oligonucleotides, all four dyes suffered a drastic decrease in their fluorescence emission intensities, which is believed to proceed by aggregation in aqueous solution as a result of their very hydrophobic character, since addition of detergent to the solution restores the fluorescence.

Ideally, these dyes would have very intense fluorescence, with well resolved fluorescence maxima of equal magnitude when excited at 488 nm with an argon laser, which is the most common radiation source in biological applications.

Another set of energy transfer cassettes explored by this group are the uridine-based energy transfer dyes which are useful not only for DNA sequencing but also to generate fluorescent probes for other applications and the nucleotides may be used in fluorescence-based single-molecule sequencing (Figure 1.4). These dyes have fluorescein in conjugation with thymidine via progressively longer, alkyne based linkers and so it was possible to study the dependence of the linker into the incorporation of the nucleoside to DNA by the polymerase.[16]

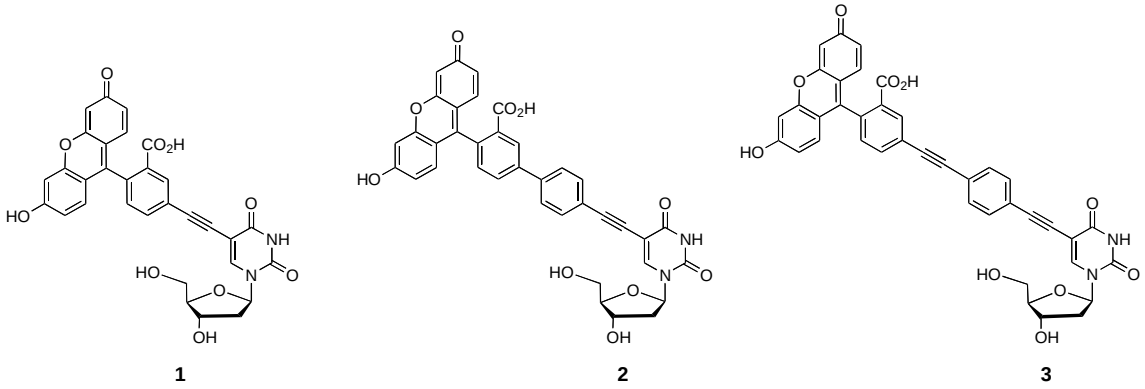


Figure 1.4 2'-Deoxyuridine-fluoresceins with extended linkers.

Spectroscopic analysis of the dyes showed that as the linker gets longer, UV absorption in the 320 – 330 nm range increases. This does not mean that the quantum yield is enhanced, but that the amount of energy being channeled to the dye is increased. The study also showed that the dyes **1** and **2** are too close to the nucleobase, and *TaqFS*

does not incorporates them efficiently, while compound **3** seems to be at the limit of minimum separation which the polymerase requires for incorporation. From this observations, it was concluded that a linker longer than that of compound **3** will not only absorb more strongly, but it will also be more efficiently incorporated into the growing DNA strand.

More recently, our group's efforts have focused on the use of energy transfer to construct fluorescent cassettes in which the dyes are linked in conjugation, allowing for a single donor and four or more different acceptors that would produce a set of resolved and intense emissions when excited at a single wavelength, more desirable in the near-IR range to avoid interference by autofluorescence of the cell.

As part of this masterplan, my research concentrated on the use of xanthene based fluorophores as donors or acceptors for our "through-bond" energy transfer cassettes.

CHAPTER II

OVERVIEW OF XANTHENE DYES

Fluorescein is one the most ubiquitous fluorescent probes and many book chapters and papers have been written,[17-24] one of these in particular being the catalog/handbook "Molecular Probes: Handbook of Fluorescent Probes and Research by Invitrogen®.[25] Fluorescein compounds have found a lot of applications in biotechnology due to their high quantum yield and relatively good water solubility.[26] Some of these uses include covalent attachment to antibodies, lectins and hormone for uses as chemosensors, DNA sequencing beacons, organelle specific labels, laser applications [27] and photosensitizers[17,28]

To include the specifics of these applications is beyond the scope of this chapter, which will only concentrate on the synthesis and derivatizations of fluorescein probes that consist on the basic structure of fluorescein, which is the xanthene core,[17] and how it can be manipulated to form fluorophores such as the fluorone and fluorescein type dyes shown (Figure 2.1).

The fluorophore of fluorescein is the fluorone moiety, which is the tricyclic heterocycle unit (Figure 2.1). The official IUPAC name for fluorescein is 2-(6-hydroxy-3-oxo-xanthen-9-yl)benzoic acid[29] and although some researchers have different nomenclature for the numbering system, the major consensus shows the same pattern as seen in figure 2.1. [30] Almost any substitution around this core alters the properties of the dye such as absorption and emission wavelength, water solubility and quantum yield as seen with CN- instead of H- attachment at the meso position (9').[31] Fluoresceins can also be derivatized by halogenation, alkylation, sulfonation, mercuration and others.

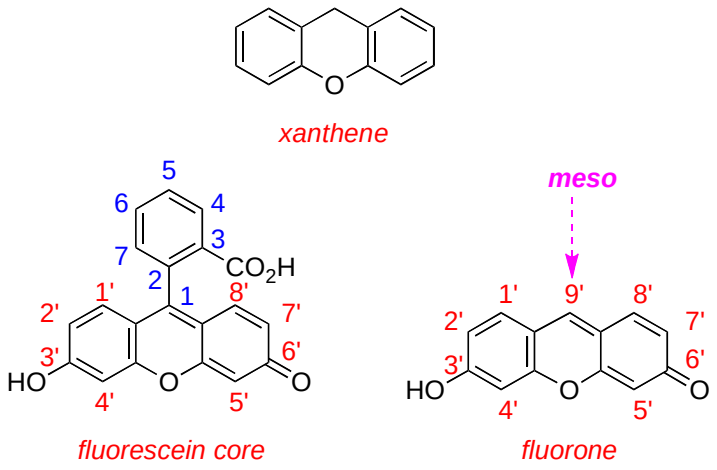


Figure 2.1 Ring numbering system for xanthene dyes.

There is a wide array of fluorescein derivatives and some of them are well known throughout the literature such as Eosin, Rose Bengal, Phloxin and Erythrosin .(Figure 2.2)

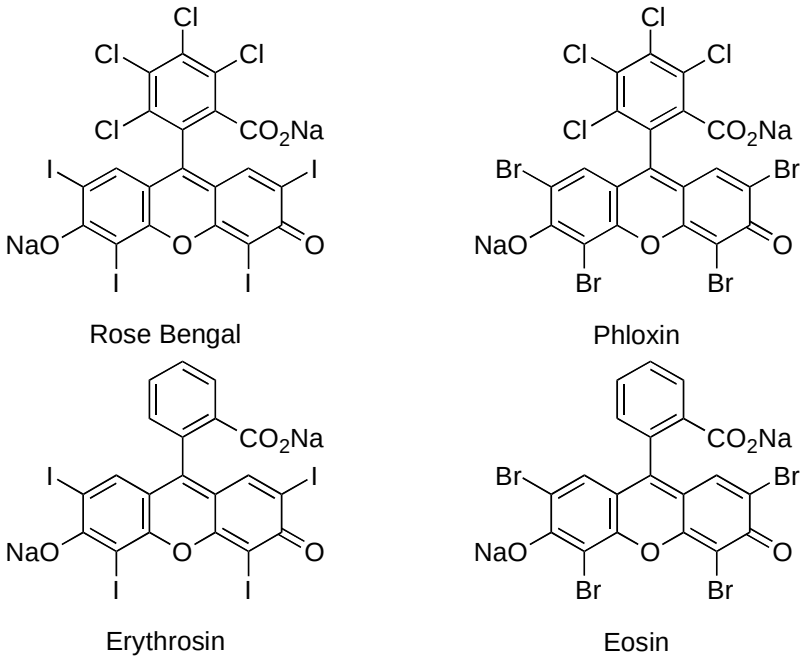
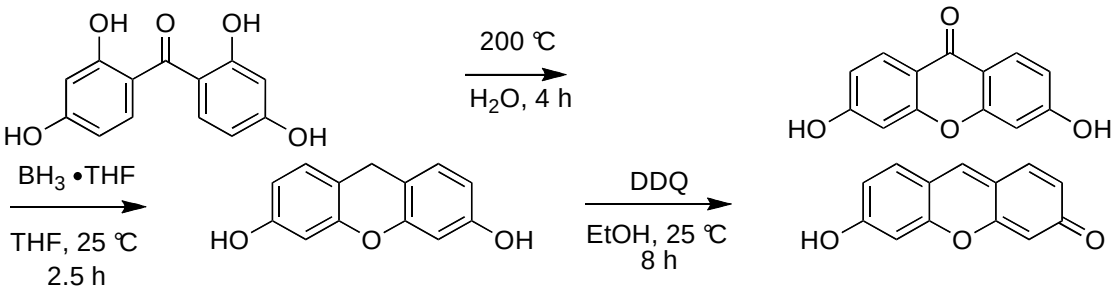


Figure 2.2 Named derivatives of fluorescein.

In the early 1990's, Neckers *et al* developed a new kind of xanthene dye, based on the known structure of fluorescein. Some uses of xanthene dyes require reactions at the C-9' position, but due to crowdedness around this site in fluorone dyes, limits the formation toward intermediates thus slowing down their reactivity.[32] For this reason, a new improved model would consist of a fluorone dye lacking the benzene moiety thus opening up the C-9' site and also removing the possible rotation around that bond, which can theoretically lower the quantum yield of the system as energy is loss through a non-radiative process. This new derivative of fluorone dye was called hydroxyfluorone.[31]

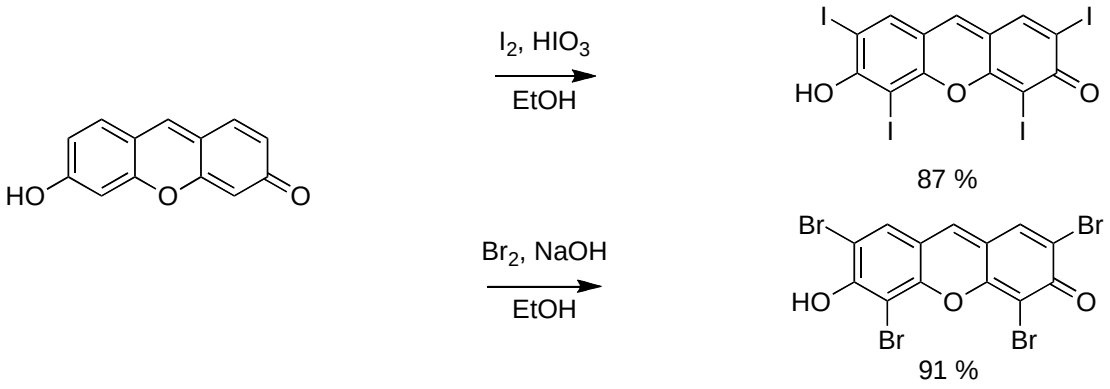
Several derivatives were synthesized and their spectral and photophysical properties investigated, but their synthesis relies on the same scheme, that is, cyclized condensation, reduction and oxidation from 2, 2', 4, 4'-tetrahydroxybenzophenone. (Scheme 2.1)



Scheme 2.1 Synthesis of hydroxyfluorone.

Neckers *et al*. showed that the utility of halogenated dyes could also be used in tailoring the photophysical properties of hydroxyfluorone dyes.[32] It has been explained earlier that the advantage of these dyes rely on the fact that the C-9' position is open for substitution. To establish the effect that a given group at this position may have on the properties of the probe, it was necessary to synthesize halogenated analogs of known halogenated fluorescein dyes, and compare the properties with and without a substituent

at the specified position. The first paper explained the synthesis of these hydroxyfluorone analogues, while in 1993 they reported the actual studies on the substitution behavior of the series.[31] Hydroxyfluorones, as explained earlier in this chapter, are synthesized by the intramolecular condensation of 2,2',4,4'-tetrahydroxybenzophenone followed by reduction to the xanthane and finally oxidation to form the conjugated hydroxyxanthone. Elaboration of this main core by different routes, depending on the product desired, created a series of six hydroxyfluorone derivatives, four of then analogous to a fluorescein counterpart. (Table 2.1) They were named hydroxyfluorone (HF), tetraiodohydroxyfluorone (TIHF), tetrabromohydroxyfluorone (TBHF), diiodomethoxyfluorone (DIMF) and finally diiodofluorone (DIF). (Figure 2.3) Studies on these compounds showed that the photophysical properties of HF, TIHF and TBHF are virtually identical with those of fluorescein, erythrosin and eosin respectively.(Table 2.2) Halogenation of these compounds proceeds by the directed halogenation with either iodine with iodic acid, or bromine under basic conditions. (Scheme 2.2)



Scheme 2.2 Iodination and bromination of hydroxyfluorone.

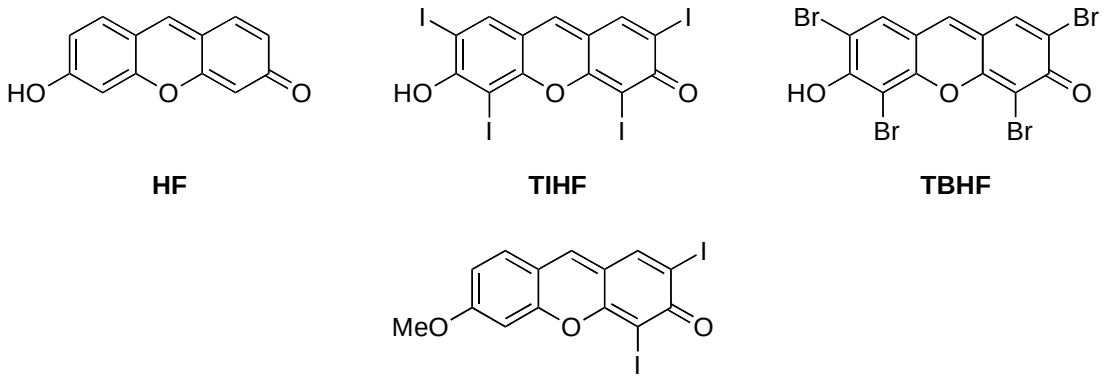


Figure 2.3 Derivatives of hydroxyfluorone dyes.

Table 2.1 Selected photophysical properties of hydroxyxanthone derivatives

	HF	**TBHF**	**TIHF**	**DIMF**
λ_{max} **(MeOH)**	500	526	532	470
λ_{fl} **(MeOH)**	506	537	544	542
ϕ_{fl}	0.95	0.52	0.13	0.03
pK_a	5.97	3.29	4.08	-
E_{ox}	1.04	1.09	1.34	-
E_{red}	-0.95	-0.95	-.099	-

Table 2.2 Most common halogenated fluoresceins, absorbance and quantum yield

Solvent	**HF (nm)**	**TBHF (nm)**	**TIHF (nm)**	**DIMF (nm)**
MeOH - 10% H_2O	490	516	526	-
MeOH	500 / 506 [a]	526 / 537 [a]	532 / 544 [a]	470 / 542 [a]
EtOH	504 / 513 [a]	530 / 539 [a]	536 / 548 [a]	470 / 544 - 468 [a]
i-PrOH	510 /516 [a]	532 / 543 [a]	538 / 550 [a]	472
t-BuOH	514	534	538	-
BzOH	508	536	542	-
CH_3CN	518	534	538	466
acetone	520	536	542	466
THF	520	536 / 544 [a]	540 / 553 [a]	468 / 543 - 573 [a]
DMF	524	536 / 543 [a]	542 / 552 [a]	468
toluene	-	-	-	474 / 546 - 584 [a]

[a] emission

Neckers *et al.* also showed that substitution at the C-9' position from a proton to a cyano group, dramatically red-shifted the absorbance of the fluorophore. [33] Substituting this main core with halogen atoms at the X and Y positions (Table 2.3)

Table 2.3 Absorbance properties of substituted hydroxyfluorones

No.	R	X	Y	Abs. λ_{max} (EtOH)	ε (x 10^3)
1	H	H	H	504	24.7
2	CN	H	H	548, 594	24.7, 50.3
3	H	Br	Br	530	39.3
4	CN	Br	Br	576, 626	24.5, 51.4
5	H	I	I	536	91.2
6	CN	I	I	586, 638	35.0, 80.0
7	H	H	I	520	86.0
8	CN	H	I	570, 618	16.6, 30.5
9	H	t-Bu	H	518	101.0
10	CN	t-Bu	H	564, 614	23.2, 47.4
11	H	t-Bu	I	532	90.8
12	CN	t-Bu	I	582, 636	33.6, 68.3

In 1871, Adolph Von Baeyer synthesized fluorescein. It was created by the condensation of two equivalents of resorcinol with one equivalent of phthalic anhydride in the presence of a strong acid, usually concentrated sulfuric acid, and zinc chloride as a catalyst.[34] The formed dye has been proposed to consist of a directly linked donor-acceptor system since both parts are not conjugated due to an orthogonal twist along the axis.[35] These parts have been referred to as the benzene and the xanthene moieties where a back to back photoinduced electron transfer (PeT) occurs.[36] (Figure 2.4)

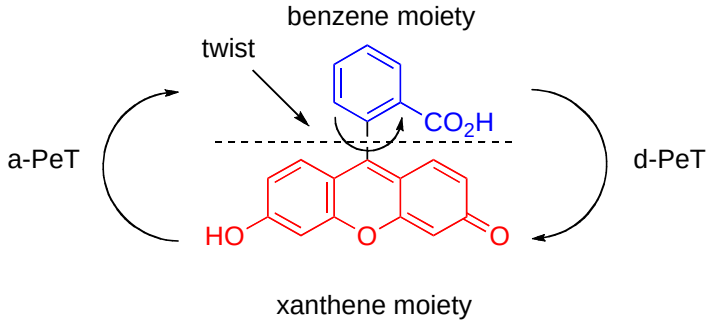
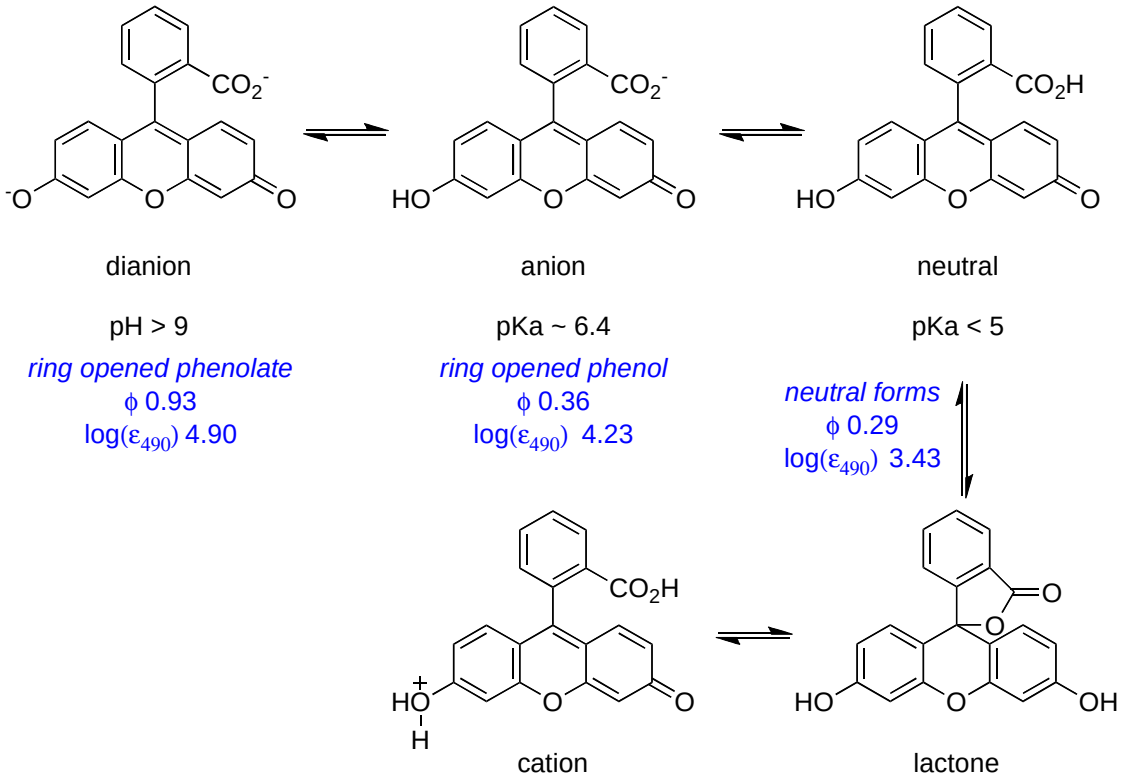


Figure 2.4 Implied donor-acceptor system.

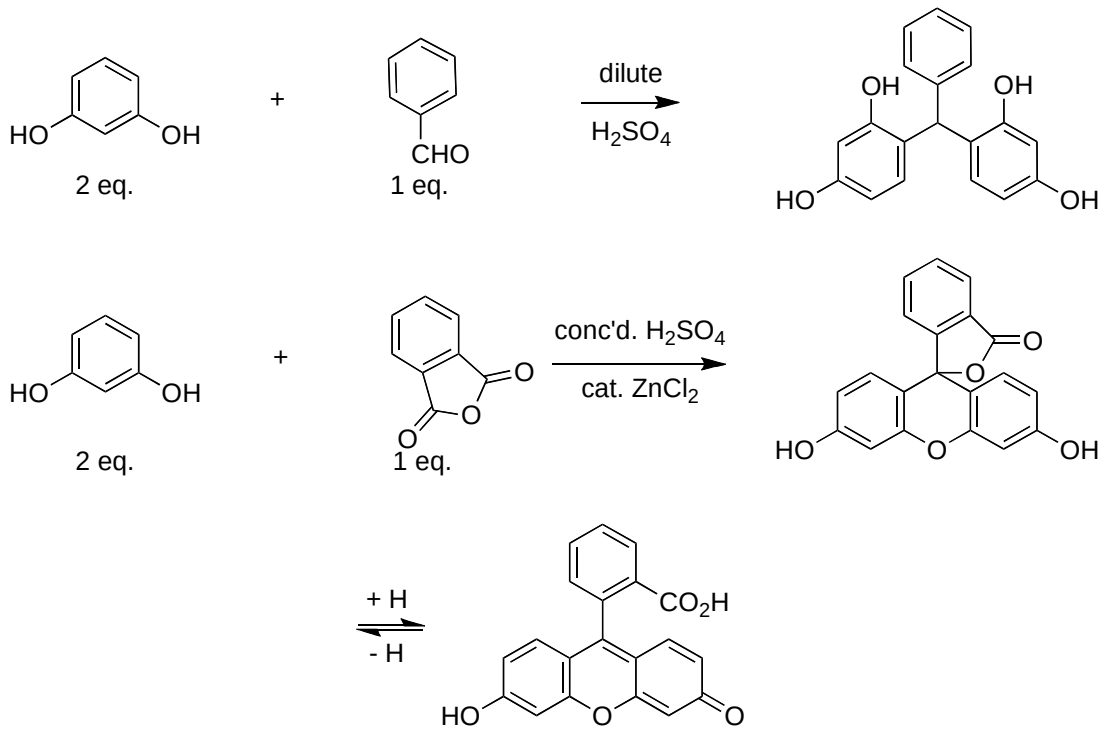
Fluorescein has a high molar absorptivity and good quantum yield in aqueous environments (0.92 at pH 9)[37,38] making this probe ideal for biological applications, although it is dependent on pH, having a lactone-locked formed under acidic conditions thus effectively inhibiting its fluorescent properties. Fluorescein does not transform from an open form to a lactone form directly. Instead, it consists of five different structural forms that shift according to pH changes. This alterations in the structure causes fluorescein to undergo consecutively a large, a small and then another large bathochromic shift (red shift) as the pH increases, going from the neutral form, to the anion and then to the dianion respectively (Figure 2.5).[39-41]



Species	Quantum Yield	Lifetime (ns)
dianion	0.93	4.06 ±0.02
anion	0.36	3.37 ±0.02
neutral	0.29	2.97 ±0.02
cation	0.9-1.0	3.5 - 4.4

Figure 2.5 pKa dependence of fluorescein.

Originally fluorescein was synthesized by the high temperature condensation of resorcinol and phthalic anhydride in the presence of a strong acid, such as H_2SO_4 (Scheme 2.3), but variations to this procedure have included the use of a catalytic amount of $ZnCl_2$, solventless media and more recently microwave-assisted heating.



Scheme 2.3 Classical synthesis of fluorescein.

During the years, the improvement of fluorescent probes, specially those of fluorescein, has brought a lot of attention, mainly the way of efficiently synthesizing them under milder conditions with relatively high yields. Usually, synthesis of these probes involves the use of zinc chloride as a catalyst, or strongly acidic conditions at high temperatures which result in the formation of undesirable byproducts which are difficult to separate from the desired product. In recent years, the utility of microwave

radiations have shown a remarkable improvement in yields and reaction times. [42,43] Also, different alternative reaction methodologies which allow accelerated coupling of both starting materials in a more efficient way, such as Grignard reactions, have been used, although they mostly consist of the improved synthesis of fluorones.[44] New, tailored probes with required functional groups at the core of the probe has also required the need of milder conditions which would not affect the added sites.

It has also been shown that the use of microwave radiation under solvent free conditions efficiently enhances the yield and reaction time for the formation of sulfonated fluoresceins.[42] In 2005, Lippard *et al*. reported the formation of isomerically pure sulfonate-substituted fluoresceins.[45] In this case, the presence of the anhydride is not necessary for the condensation with resorcinol, which occurs at 90 °C in the presence of methane sulfonic acid. A mixture of isomers is obtained, but further protection with pivaloyl anhydride allows the separation of each isomer by crystallization from a dichloromethane/diethyl ether mixture, obtaining them as the diisopropylethylammonium salt, which could then be hydrolyzed to the free fluorescein form.

I have already mentioned two of the four most common halogenated fluorescein derivatives, Eosin and Phloxin, but there is also Rose Bengal, and Erythrosine.[33] (Table) Another very useful halogenated fluorescein, which has been exploited greatly by recent research groups such as Necker's and Lippard's, is dichlorofluorescein (DCF) (Figure 2.6). The synthesis of this important dye consists on the condensation of 4-chlororesorcinol with phthalic anhydride under the same typical conditions for the formation of fluorescein. This dye form the basic skeleton to which other fluoresceins are derived, such as the Mannich produced chemosensors already presented.

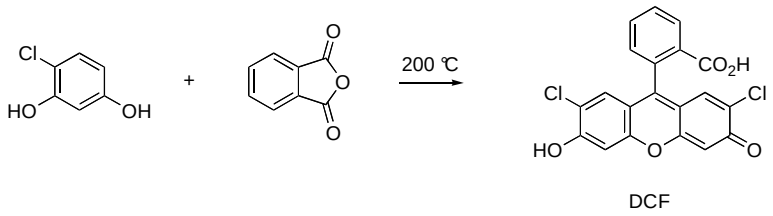


Figure 2.6 Classical synthesis of dichlorofluorescein.

A difluorofluorescein, analogous to dichlorofluorescein has been synthesized, although the synthesis of this compound calls for the prior formation of the 4-fluororescorcinol. Even though this compound is commercially available, it was not at the time the given reference was published.[46] The formation of 4-fluororesorcinol, they claim, was the key in developing a series of fluorinated fluoresceins, and by following the same strategy, they were able to synthesize 2-fluororesorcinol, 2,4-difluororesorcinol and 2,4,5-trifluororesorcinol, which allowed them to create a series of polyfluorinated fluoresceins also called "Oregon Green" dyes. (figure 2.7)

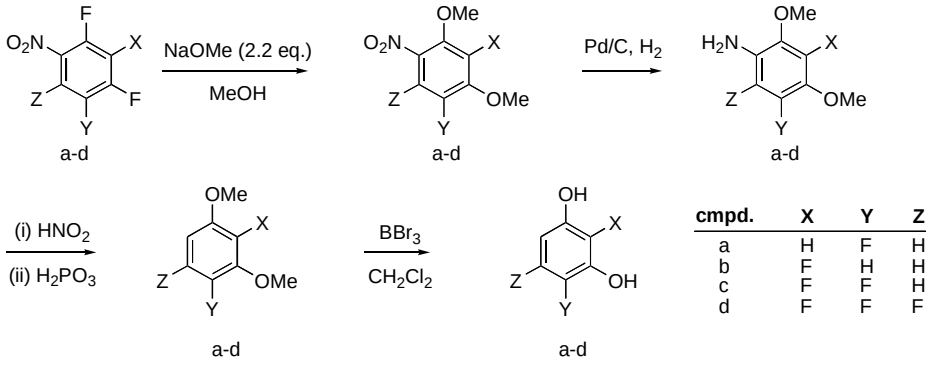
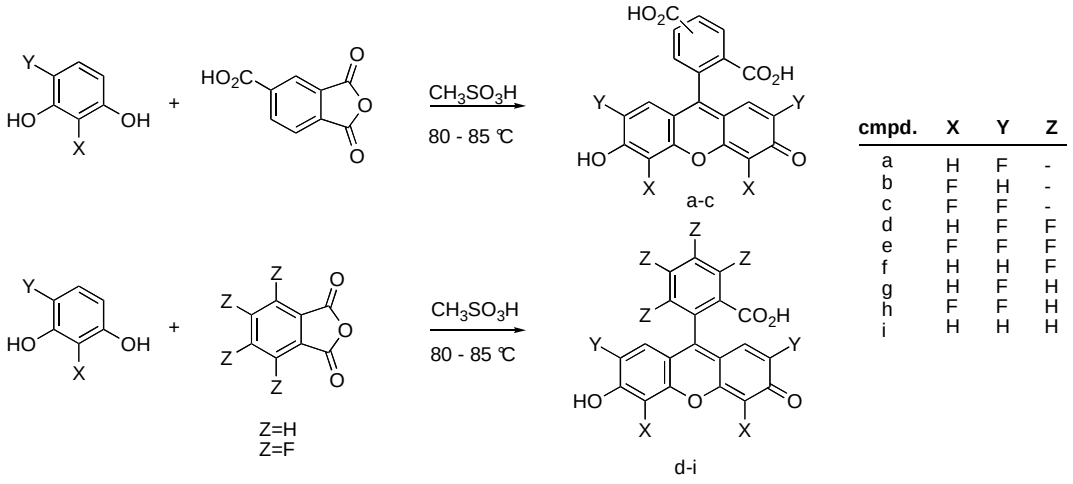


Figure 2.7 Synthesis of fluorinated resorcinols.

The actual synthesis of the fluorescein dyes required minor modifications since classical synthesis using zinc chloride at high temperatures caused a large amount of the

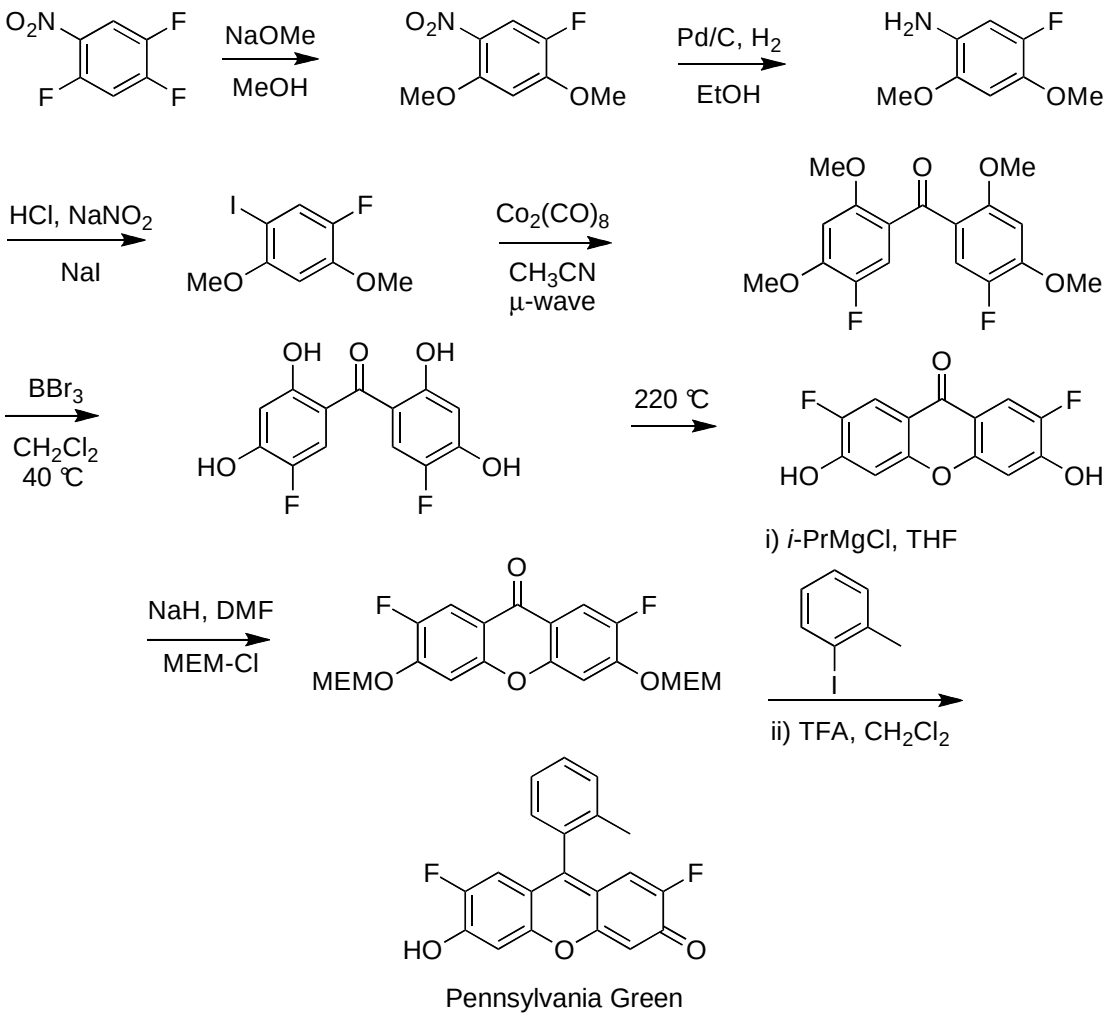
fluorinated resorcinol to sublime. For this reason, the use of methanesulfonic acid (CH_3SO_3H) as both a solvent and Lewis acid catalyst gave an improved procedure with higher yields under lower temperatures although the formation of the fluorescein dye using 2,4,5-trifluororesorcinol or 5-fluororesorcinol failed to give the desired product. Purification of these dyes was accomplished by diacetylation, which allowed for easier recrystallization or chromatography, followed by hydrolyzation to obtain the free dye. (Scheme2.4)



cmpd.	X	Y	Z
a	H	F	-
b	F	H	-
c	F	F	-
d	H	F	F
e	F	F	F
f	H	H	F
g	H	F	H
h	F	F	H
i	H	H	H

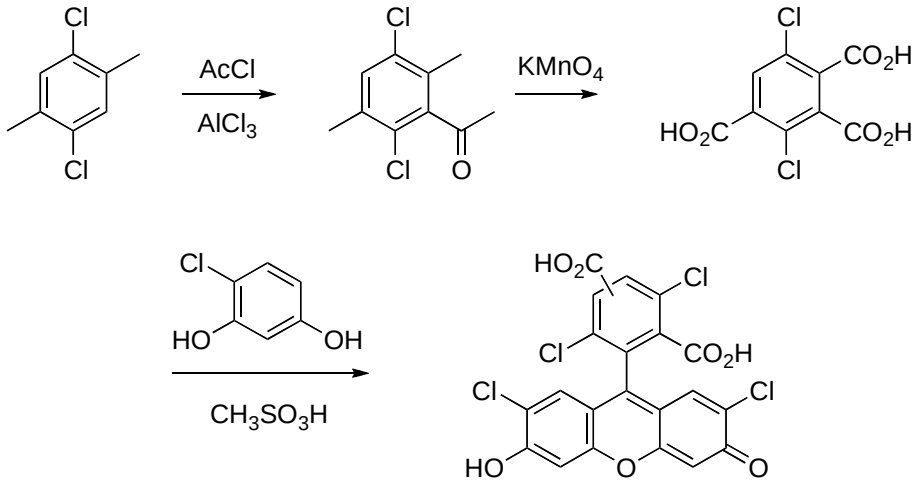
Scheme 2.4 Synthesis of Oregon Green Dyes.

More recently, a new derivative of Oregon Green was synthesized by Peterson *et al.* [37] This new dye, called "Pennsylvania Green" by the authors, is a hybrid of Oregon Green and Tokyo Green. By combining the properties of each dye, that is, the incorporation of the fluorine atom at the 2'- and 7'- position, and replacing the benzoic acid by a methyl group in the benzene moiety respectively, it was possible to form a monoanionic form of fluorescein, which combined with the ability of the attached fluorine atoms to lower the pK_a of the system to 4.8, allows the fluorescence intensity of the dye to remain high even under much acidic conditions than regular fluorescein. This dye was synthesize via a route that utilizes a fluorone as an intermediate developed in a novel approach.

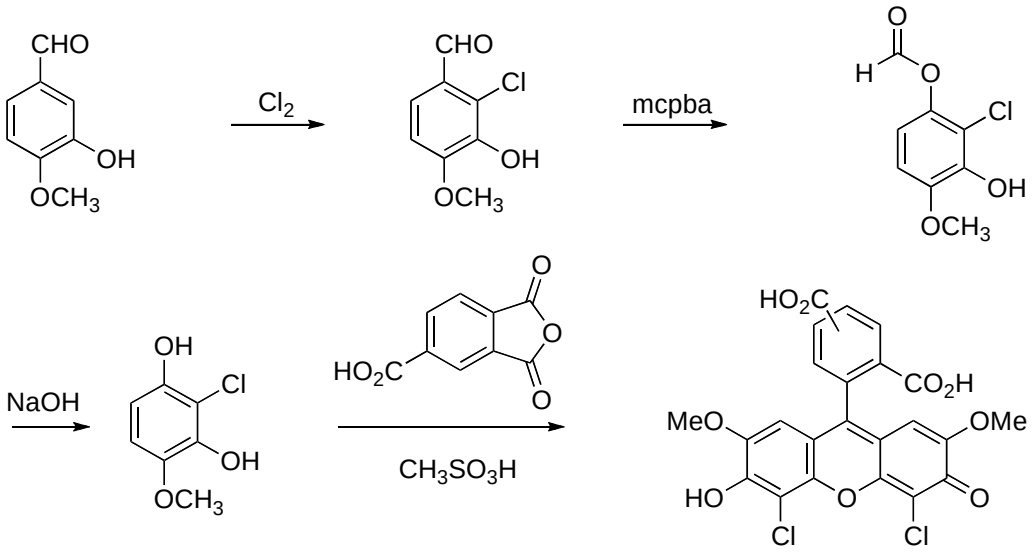


Scheme 2.5 Synthesis of Pennsylvania Green.

Other synthesis of chlorinated fluoresceins include 4,7,2'7'-tetrachloro- and 4'5'-dichloro-2'7'-dimethoxy-5(and 6)-carboxyfluoresceins. Even though these same compounds have been reported previously in patents,[47-50] Lyttle *et al.* reported in 2001 an improved procedure for these same compounds, which could be prepared in large preparative amounts.[51]



Scheme 2.6 Synthesis of 4,7,2',7'-tetrachloro-(5 and 6)-carboxyfluorescein.

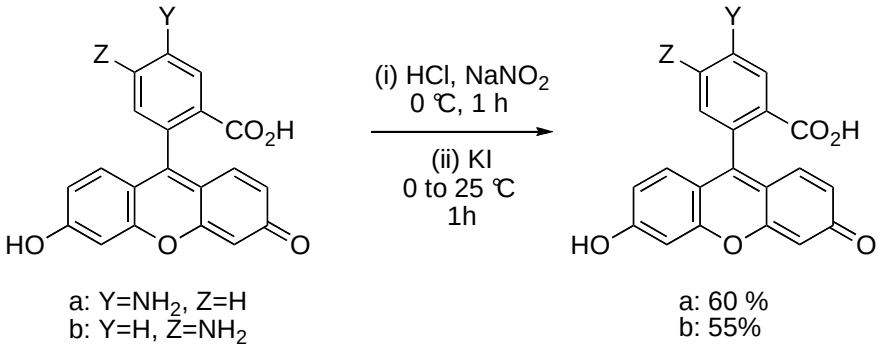


Scheme 2.7 Synthesis of 4',5'-dichloro-2',7'-dimethoxy-(5 and 6)-carboxyfluorescein.

Although the synthesis of both compounds have been improved, no procedure for the separation of the 5- and 6- isomers is given. A procedure for the separation of such isomers was published in by Rossi and Kao,[52] in which pivaloyl protection of the

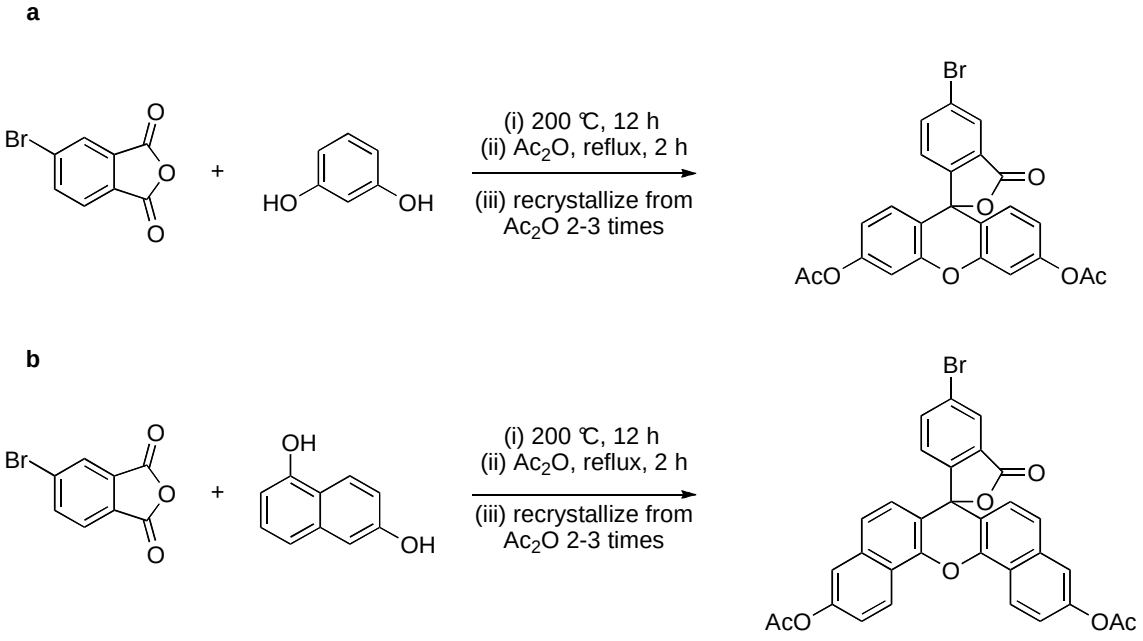
carboxy fluorescein isomeric mixture, followed by the formation of the diisopropylamine salt, afford the 6- isomer in pure form as a precipitates. This procedure has been claimed to be not easily reproduced,[53] and instead opted for the reduction of the acid functionality, thus allowing for easier separation of the isomers. An improved procedure for the separation of the mixture by recrystallization from methanol- or ethanol-hexane solvent systems was later reported.[54]

In the course of our research, it was necessary to find efficient procedures for the separation of such 5- and 6- isomers, specifically, those of halogenated fluorescein, which are essential to the formation of our "Through-bond" energy transfer cassette scheme.[55] These 5- or 6-halofluorescein are valuable substrates for various metal-catalyzed coupling reactions such as Sonogashira or Suzuki-Miyaura, specially for biological applications, since a mixture of isomers would affect anisotropy measurements, cause possible differences in quantum yields, or interact differently with the attached biomolecule. It is possible to obtain the desired pure regioisomer from the commercially 5- or 6-aminoflourescein, but this compound, in its pure regioisomerically pure form is extremely expensive and therefore not desirable for preparative work.(Scheme 2.8)



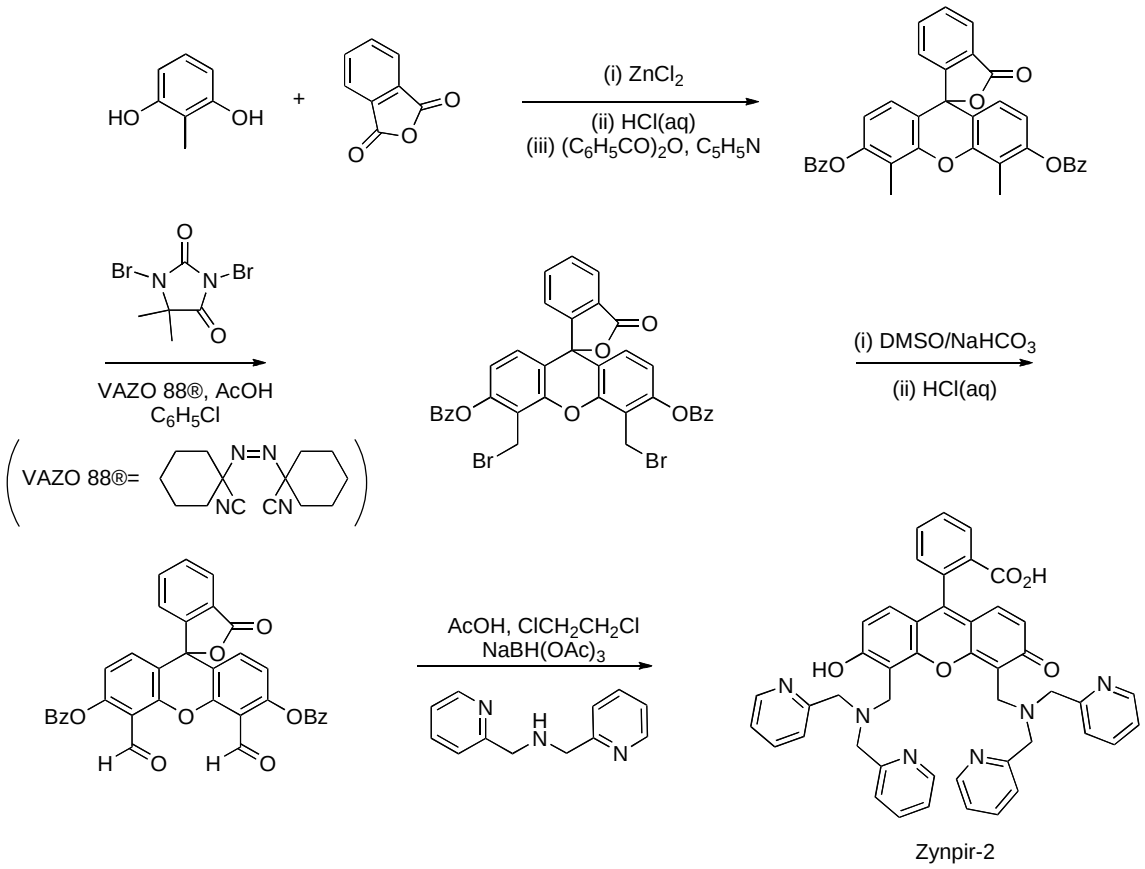
Scheme 2.8 Synthesis of regioisomerically pure 5- and 6-iodofluorescein.

It is also possible to obtain the desired 5- or 6-halogenated fluorescein by the sequential recrystallization of the crude product of the condensation of 4-halophthalic anhydride and the desired resorcinol derivative, such as 4-chlororesorcinol or 1,6-naphthalenediol.[56]



Scheme 2.9 Synthesis of Regioisomerically pure halofluorescein derivatives.

Similarly, the same complexing product could be obtained using fluorescein. In this case, a new synthetic strategy had to be developed to position both DPA units at the desired positions, since the 2'- and 7'- sites are also open for substitution and a mixture of products could be obtained by using typical Mannich conditions. The synthesis call for the substitution of resorcinol with 2-methylresorcinol in the standard fluorescein condensation procedure, followed by protection of the phenolic groups in the lactone form of the dye. The next step involves radical bromination of both methyl groups in the core, which could then be oxidized to the dialdehyde. This intermediate not only can be aminated under reducing conditions as shown, allowing substitution with DPA, but leaving a very important precursor for other reactions as mentioned by Lippard.[57]



Scheme 2.10 Synthesis of Zynpir-2.

Halogenated fluorescein dyes have been prepared and studied. The insertion of halogenated groups into the fluorescein main core affects its electronic configuration in a way that its spectroscopical properties differ from one another. The substitution in the benzene moiety does not directly affects the fluorophore as much as the substitution in the xanthene part, as it is the actual fluorophore of the molecule as can be seen in table 2.4

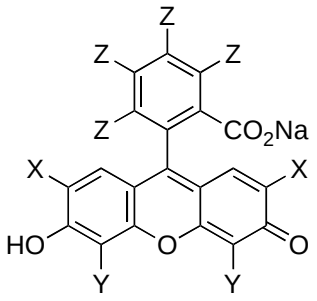


Table 2.4 Structure and comparison of halogenated hydroxyfluorone derivatives

Xanthene	X	Y	Z	Abs. λ_{max} (EtOH)
Rose Bengal	I	I	Cl	557
Erythrosin	I	I	H	532
Phloxin	Br	Br	Cl	548
Eosin	Br	Br	H	523

Fluorescein has been used extensively as a covalently bound cellular probe for different targets, but due to its properties such as high quantum yield, it has drawn attention toward its use as a solid-state fluorophore for various applications. Strangely as it may seem, fluorescein in a solid state by itself has not been studied deeply, but rather when covalently bound to a solid matrix. These solid-state compounds show different absorption and emission wavelengths differently than those in aqueous solutions (λ_{exc}=490, λ_{em}=515 nm) when covalently bound to starch or other polymers (λ_1=490 nm, λ_2=530 nm absorption; λ=570 nm emission)(Figure 2.8). Some argue that this shift in wavelengths may be due to a normal and an charge transfer state (CT) species present in the solid state while citing findings by the Ueno group,[58] difference in polarization energy of the S_0 and S_1 state, or wavelength bands identified as the monomer or excimer emission.(PAT, 2008, 19, 385-392)

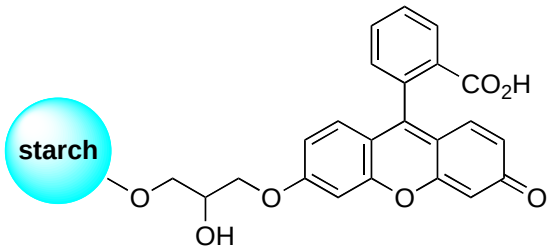


Figure 2.8 Covalently bound fluorescein with starch.

Other solid supports used have been polymers and copolymers formed by the oxidative coupling of fluorescein-containing bis-acetylenes.[59] In this case, two approaches were used; direct polycondensation of fluorescein as a monomer or oxidative coupling of fluorescein bis-acetylene to form the fluorescein polymer (figure 2.9).

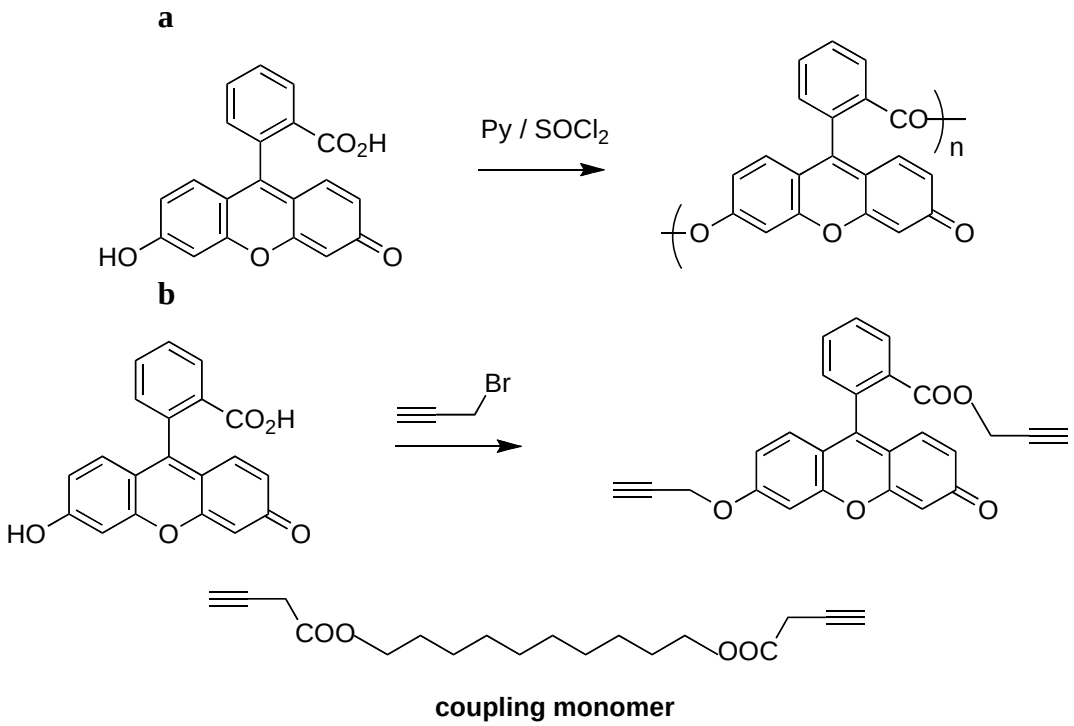
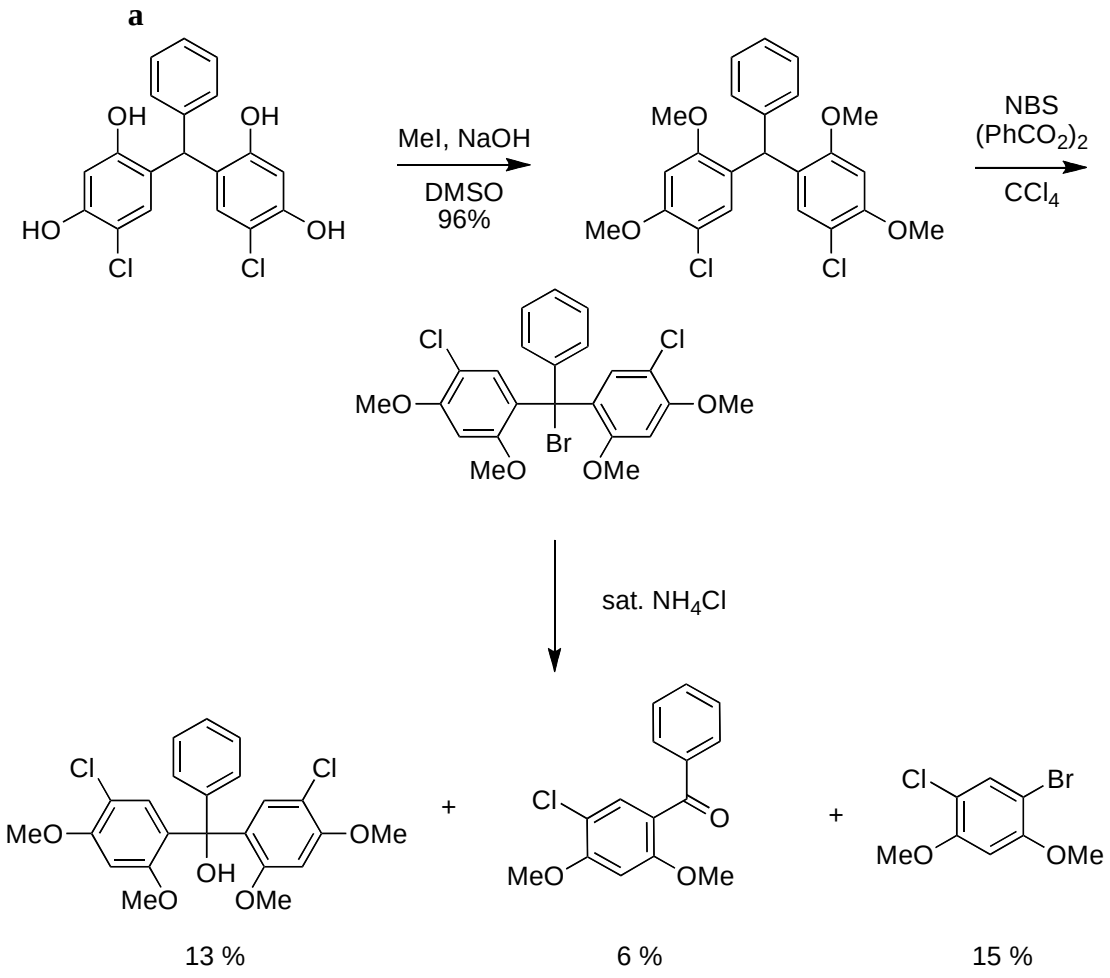


Figure 2.9 Synthetic routes to form polymeric fluorescein. **a.** Direct polymerization of fluorescein as a monomer and, **b.** formation of fluorescein bis-acetylene.

In both of the previous cases, a film was cast from a pyridine solution and remained relatively brittle when removed from the support. The polymers produced by these routes show absorption band identical to those of fluorescein, while emissions ranged from and single maximum at 544 nm, to longer wavelength emissions at 560, 568 and 588 nm.

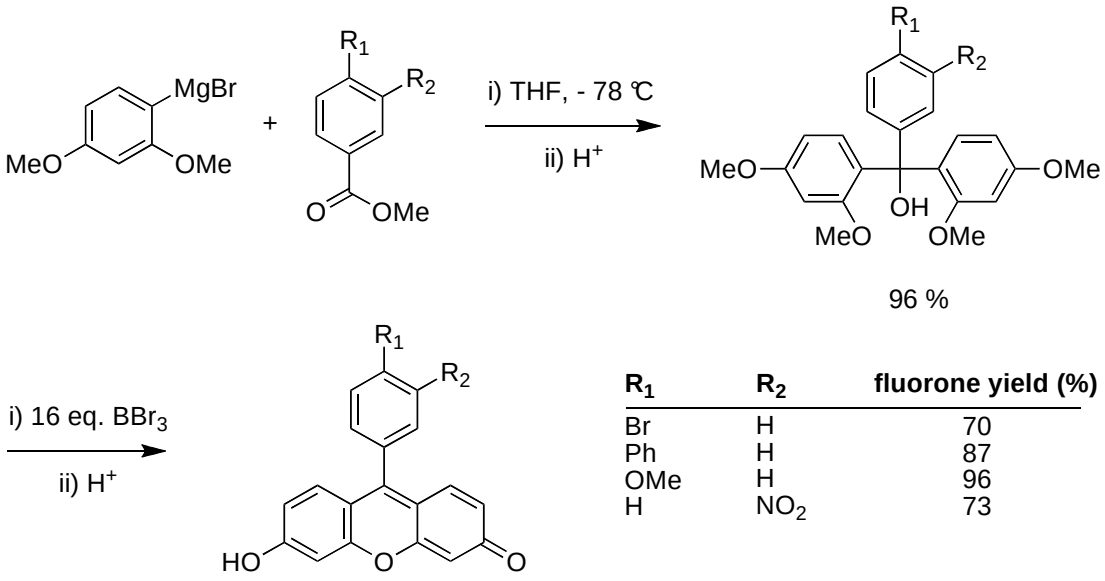
During the years, the improvement of fluorescent probes, especially those of fluorescein, has brought a lot of attention, specially the way of efficiently synthesizing them under milder conditions with relatively high yields. Usually, synthesis of these probes involves the use of zinc chloride as a catalyst, or strongly acidic conditions at high temperatures that result in the formation of undesirable byproducts that are difficult to separate from the desired product. In recent years, the utility of microwave radiations has shown a remarkable improvement in yields and reaction times.[42,43] Also, different

alternative reaction methodologies which allow accelerated coupling of both starting materials in a more efficient way, such as Grignard reactions, have been used, although they mostly consist of the improved synthesis of fluorones.[44] New, tailored probes with required functional groups at the core of the probe has also required the need of milder conditions which would not affect the added sites (Scheme 2.5).



Scheme 2.11 Synthesis of fluorescein derivatives by alternate route.

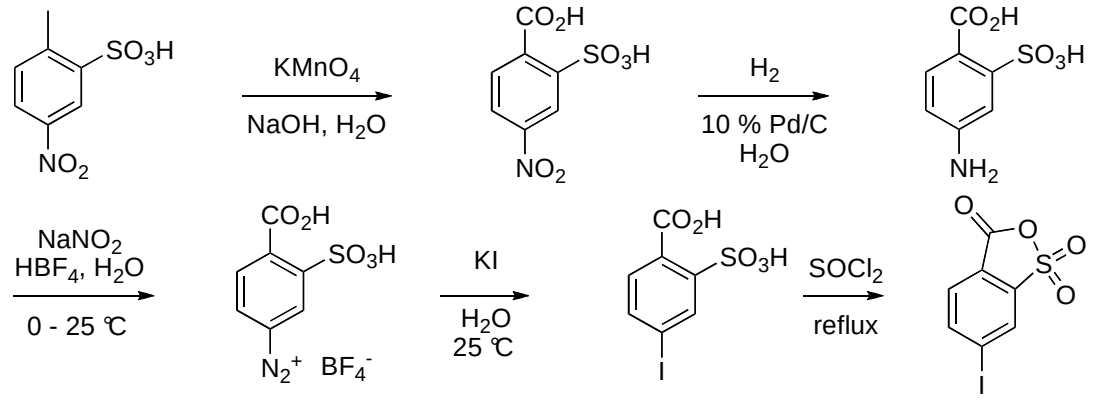
b



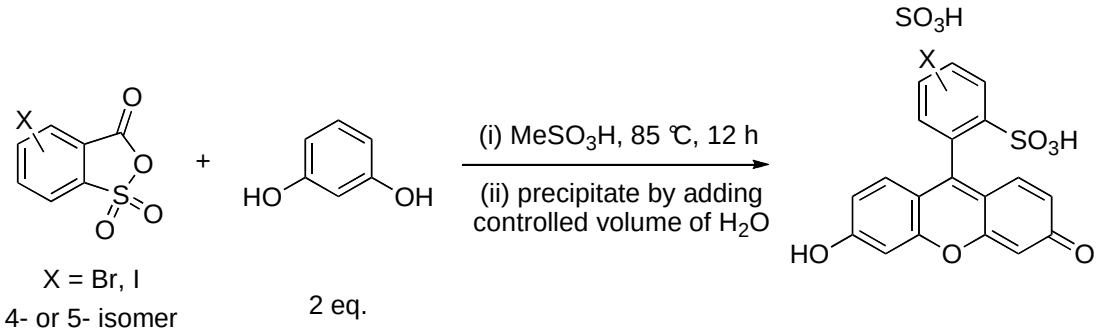
R_1	R_2	fluorone yield (%)
Br	H	70
Ph	H	87
OMe	H	96
H	NO_2	73

Scheme 2.11 Continued.

The use of ortho-sulpho-benzoic acid in the condensation with phenols, or resorcinol, for this matter, was first suggested in 1884 by Remsen.[60] This kind of reaction relies on the use 2-sulfobenzoic acid anhydride and condensing it with resorcinol to form the desired sulfonated fluorescein. A practical protocol for the synthesis of *o*-sulfobenzoic anhydride is known since the early 1940's,[61,62] and the utility of this compound, which provides the same benefits as the use of phthalic anhydride, is also enhanced by the addition of a water soluble group, which also eliminates the formation of regiosiomers.(Scheme 2.6)



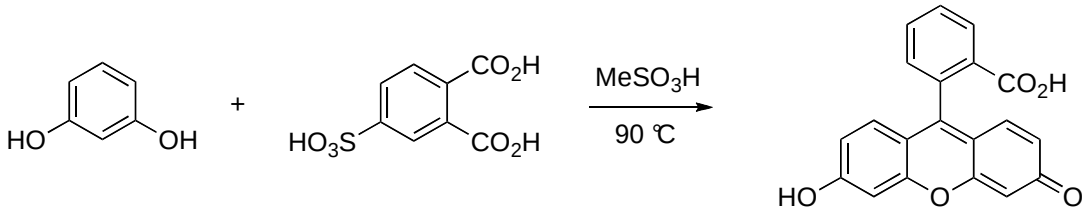
Scheme 2.12 Synthesis of 3-iodo-*o*-sulfobenzoic anhydride.



Scheme 2.13 Synthesis of 5- or 6-halogenated fluoresceins.

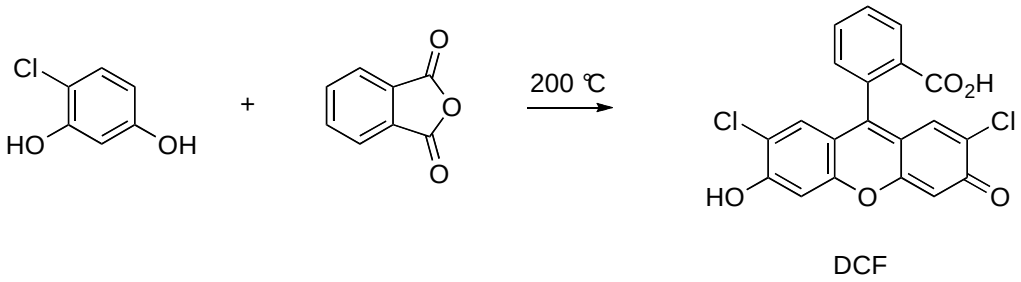
It has also been shown that the use of microwave radiation under solvent free conditions efficiently enhances the yield and reaction time for the formation of sulfonated fluoresceins.[42] In 2005, Lippard *et al*. reported the formation of isomerically pure sulfonate-substituted fluoresceins.[45] In this case, the presence of the anhydride is not necessary for the condensation with resorcinol, which occurs at 90 °C in the presence of methane sulfonic acid. A mixture of isomers is obtained, but further protection with pivaloyl anhydride allows the separation of each isomer by crystallization from a dichloromethane/diethyl ether mixture, obtaining them as the diisopropylethylammonium salt, which could then be hydrolyzed to the free fluorescein form.

It is interesting to know, however, that the condensation of sulfonated resorcinol with phthalic anhydride provided unsubstituted fluorescein as the major product. Likewise, direct sulfonation of fluorescein with fuming sulfuric acid provides only unreacted starting material.[45]



Scheme 2.14 Synthesis of regioisomerically pure 5- and 6-sulfofluorescein.

We have already mentioned two of the four most common halogenated fluorescein derivatives, Eosin and Phloxin, but there is also Rose Bengal, and Erythrosine.[31] (Table) Another very useful halogenated fluorescein, which has been exploited greatly by recent research groups such as Neckers and Lippard, is dichlorofluorescein (DCF). The synthesis of this important dye consists on the condensation of 4-chlororesorcinol with phthalic anhydride under the same typical conditions for the formation of fluorescein. This dye forms the basic skeleton to which other fluoresceins are derived, such as the Mannich produced chemosensors already presented.



Scheme 2.15 Classical synthesis of dichlorofluorescein.

This derivative of fluorescein can be manipulated in many different ways, and due to its interesting spectroscopical properties, became the focus of my research at the end of my graduate education.

CHAPTER III

MICROWAVE-ASSISTED SYNTHESES OF REGIOISOMERICALLY PURE BROMORHODAMINE DERIVATIVES

Regioisomerically pure, bromo-substituted rhodamine derivatives would be desirable starting materials for elaboration of fluorescent dyes via organometallic coupling reactions. We required them for syntheses of superior fluorescent dyes for multiplexing in applications like high-throughput DNA sequencing.[63,64] However, the well-established condensation route to rhodamines is not ideal to make brominated derivatives because it can give two regioisomeric products that can be hard to separate (Figure 3.1a). The corresponding compounds without carboxylic acid functionalities, that have been called "rosamines",[65] had not previously been reported with one halogen substituent but we found them interesting because, unlike bromorhodamine syntheses, condensation reactions to form them should give only one regioisomer (Figure 3.1b). Literature routes to rosamines involve extended high temperature reactions that tend to give complicated mixtures.[65]

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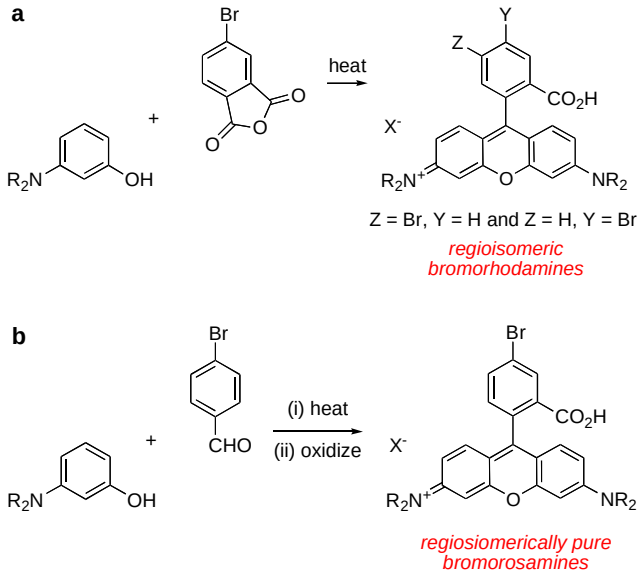


Figure 3.1 Regioisomerically pure bromorhodamines are hard to obtain via condensations **a**, but the corresponding rosamine derivatives should be more accessible, **b**.

Rosamines **1** – **4** were made via the reactions of 4-bromobenzaldehyde with the phenolic amines **5** – **8**. Preparation of **3** was chosen as a model system. Attempts to optimize reaction conditions using NMR were complicated by the extent of impurities formed at the high temperatures involved, and by insolubilities of the products in some solvent systems. Consequently, a UV-based method was developed. Standardized concentrations of the reagents were reacted under various conditions, an aliquot of the reaction mixture was then diluted in DMF, allowed to oxidize in the air until the UV showed no change, then analyzed via UV. Figure 3.2a illustrates that the starting materials do not absorb significantly above 350 nm, whereas the desired products do. Thus the extent of conversion was obtained via a calibrated UV plot.

Figure 3.2b shows how this method was used to determine that a good yield of product **3** could be obtained at 150 °C using 5 min microwave irradiation time. These reactions were performed using 60 % sulfuric acid as the medium. Similar experiments using methanesulfonic acid as solvent gave inferior results. Shorter periods of irradiation gave diminished yields, whereas longer ones gave no advantage.

Consequently, 10 min microwave irradiation at 150 °C was set as a standard to compare with typical thermal syntheses of this system (the reaction time was increased from 5 to 10 min because the scale of the reactions was also increased). Preparations of the other dyes were similarly optimized.

a

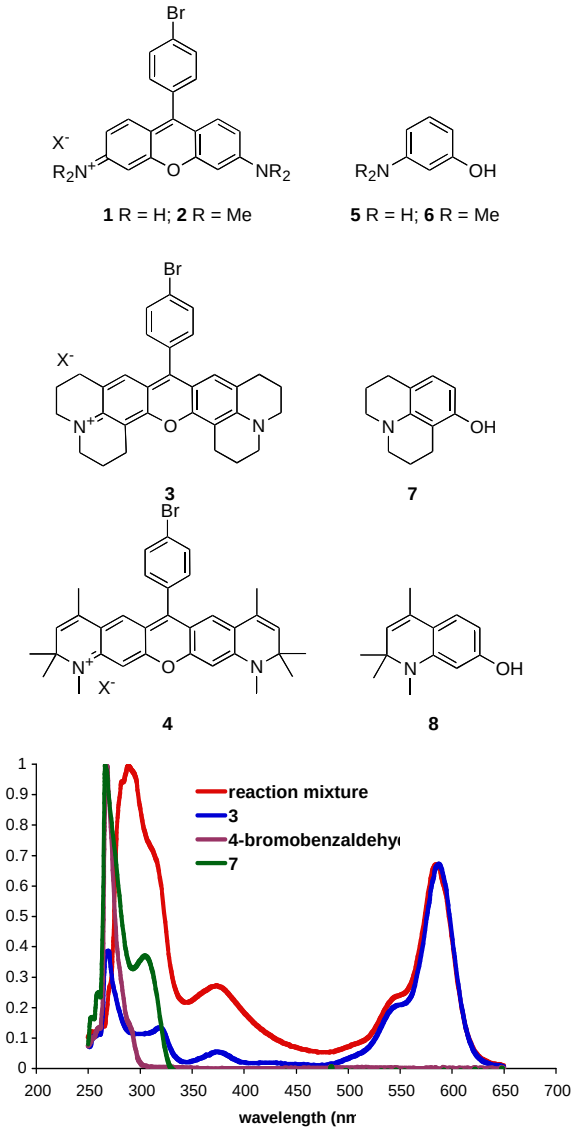


Figure 3.2 a UV spectra of reactants, product and a typical reaction mixture in the microwave synthesis. **b** Temperature and time optimization for the microwave synthesis of **3**.

b

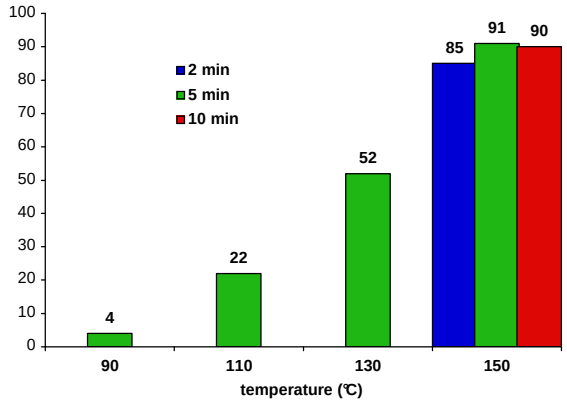


Figure 3.2 Continued.

Table 3.1 compares isolated yields of compounds **1 - 4** under various microwave and thermal conditions. Reaction times in the microwave reactions were significantly shorter; thus the transformations could be performed more conveniently in the microwave apparatus, and, perhaps more importantly, the process of optimizing the reaction conditions was more facile. In each case, chloranil was added after the reaction period, then the mixtures were stirred for at least 10 min at 25 °C to ensure oxidation of the intermediate condensation product.

Table 3.1 Isolated yields of the dyes under microwave and thermal conditions[a]

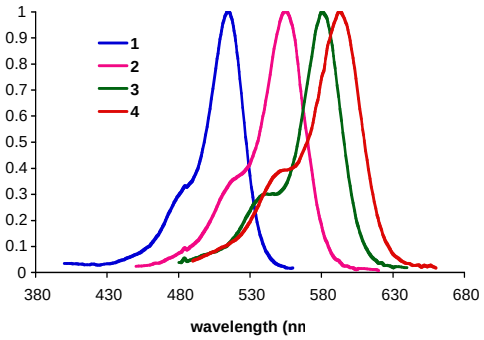
dye	microwave[b]		thermal	
	temp./time (°C, min)	yield (%)	temp./time (°C, h)	yield (%)
1	150, 20	27	160, 24	8
2	90, 30	41	160, 22	12
3	90, 30	38	90, 18	35
	150, 10	73		
4	150, 10[c]	53	160, 24[c]	5

[a] In 60 % H_2SO_4 unless otherwise indicated. [b] After microwave, 2 equiv. chloranil was added to the reaction mixtures to ensure complete oxidation, then the products were isolated via flash chromatography. [c] Neat: no solvent or acid.

Compounds **1** – **4** are highly colored in solution (dilute EtOH solutions are yellow, pink, magenta, and purple respectively). In fact, they are so strongly colored that it is difficult to recrystallize them, simply because it is almost impossible to see when the solid dissolves and when crystals form. Consequently, there is some ambiguity about the counter ion for these salts, but we believe they are obtained from the syntheses as hydroxide salts.

Figure 3.3 shows the UV absorption and fluorescence emission spectra of **1** – **4**. Their fluorescence maxima span the range 532 nm to 616 nm in EtOH. Consequently, these molecular fragments can be used as acceptors for energy transfer cassettes to enable fluorescence detection in multiplexing via well-resolved emission maxima.

a



b

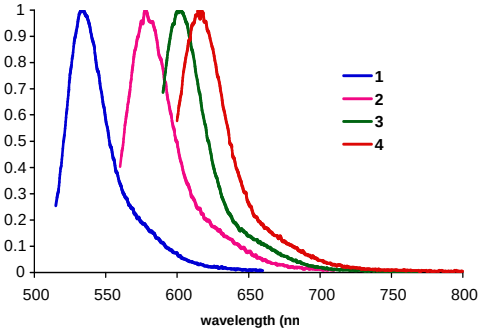


Figure 3.3 a UV spectra, and **b** fluorescence emission spectra of dyes **1** – **4** in EtOH.

In conclusion, the high temperatures required for formation of rhodamine derivatives are easily obtained via microwave heating. Microwave-assisted syntheses of compounds **1** – **4** were efficiently optimized and rapidly repeated as a direct result. There is a high probability that microwave-heating also could be applied to make similar systems like fluoresceins and other xanthene-based dyes. In this work, use of 4-bromobenzaldehyde to give the rosamine compounds rather than 3-bromophthalic anhydride to give rhodamines circumvents the issue of regioisomer formation.

CHAPTER IV

MICROWAVE-ASSISTED FUNCTIONALIZATION OF BROMO-FLUORESCEIN AND BROMO-RHODAMINE DERIVATIVES*

Methods to form fluorescein and rhodamine dyes typically feature high temperature condensation reactions that are not readily adapted to form small libraries of derivatives.[32,65-67] A project in these laboratories to prepare fluorescent tags for labeling and observation of several biomolecules in one system[64] led us to consider ways of linking fluorescein and rhodamine fragments. More specifically, it was necessary to link them to form twisted systems that would be conjugated if they became planar. Consequently, we decided to investigate organometallic couplings of starting materials that could be used to prepare a diverse set of dyes. For this purpose we compared conventional and microwave heating[68] of palladium-catalyzed reactions featuring the regioisomerically pure brominated starting materials **1** and **2**.[56]

The most obvious way to couple fragments **1** and **2** is to form an organometallic species from one of them, then couple this with the other. Suzuki couplings[69] might be preferred because they are high yielding and experimentally convenient. Use of this method would require that one component be converted to a boronic acid, hence it was necessary to decide which. Molecules like **2** (rhodamines lacking a carboxylic acid functionality) have been called *rosamines*.[65] Experimentally, they tend to be more difficult to manipulate than the fluorescein derivatives **1**, because of their charge. We hypothesized that their positive charge also makes the aryl bromide more electron

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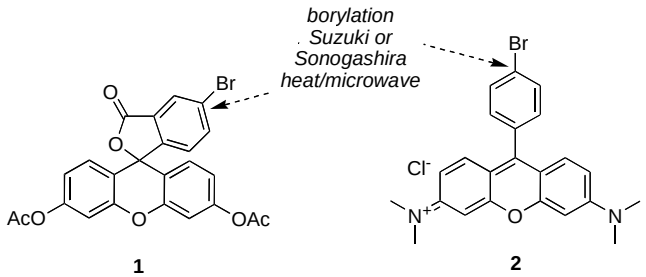
deficient than in compound **1**. Electron deficient aryl bromides tend to give more efficient coupling reactions in catalytic cycles involving oxidative addition, while the transmetallation component is less sensitive to electronic factors.[69] Consequently, it was decided to focus on formation of the boron-containing fragment from the fluorescein derivative **1**.



1 **2**

Initially, attempts were made to borylate **1** using pinacolborane,[70,71] since that reagent is cheaper than the corresponding diboron reagents. Conventional heating was investigated first. The 1,1'-diphenylphosphinoferrocenyl-based catalyst, $PdCl_2$(dppf) gave poor conversion and much of the material that was formed was the unwanted reduction product **4** (Table 4.1, entry 1; similar results were obtained using 100 °C reaction temperature, data not shown).

Table 4.1 Borylation of 5-bromofluorescein diacetate **1**

entry[a]	borylating agent	base	catalyst	solvent	heating method (temp °C, time)	reactant/product ratio[c] **1**:**3**:**4**
1	$H\text{-}BO_2C_6H_{12}$	NEt_3	$PdCl_2(dppf)$	dioxane	conventional (80, 20 h)	90: 0.0 :10
2	$H\text{-}BO_2C_6H_{12}$	NEt_3	$PdCl_2(P^tBu_2OH)_2$	dioxane	conventional (80, 20 h)	0.0 :15:85
3	$H_{12}C_6O_2B\text{-}BO_2C_6H_{12}$	NEt_3	$PdCl_2(P^tBu_2OH)_2$	dioxane	conventional (80, 20 h)	94:3.0:3.0
4	$H_{12}C_6O_2B\text{-}BO_2C_6H_{12}$	KOAc	$PdCl_2(P^tBu_2OH)_2$	dioxane	conventional (80, 20 h)	35:50:15
5	$H_{12}C_6O_2B\text{-}BO_2C_6H_{12}$	KOAc	$PdCl_2(P^tBu_2OH)_2$	toluene	conventional (100, 20 h)	0.0:80:20
6	$H_{12}C_6O_2B\text{-}BO_2C_6H_{12}$	KOAc	$PdCl_2(dppf)$	toluene	microwave, 50 W[a] (25 to 147, 5 min)	85:15: 0.0
7	$H_{12}C_6O_2B\text{-}BO_2C_6H_{12}$	KOAc	$PdCl_2(dppf)$	toluene	microwave, 100 W[a] (25 to 157, 5 min)	61:39: 0.0
8	$H_{12}C_6O_2B\text{-}BO_2C_6H_{12}$	KOAc	$PdCl_2(dppf)$	toluene	microwave, 200 W[a] (25 to 238, 15 min)	0.0 :>98: 0.0
9	$H_{12}C_6O_2B\text{-}BO_2C_6H_{12}$	KOAc	$PdCl_2(dppf)$	toluene	microwave[b] (150, 5 min)	0.0 :>98: 0.0

[a] Constant power, temperature allowed to vary.

[b] Variable power, constant temperature.

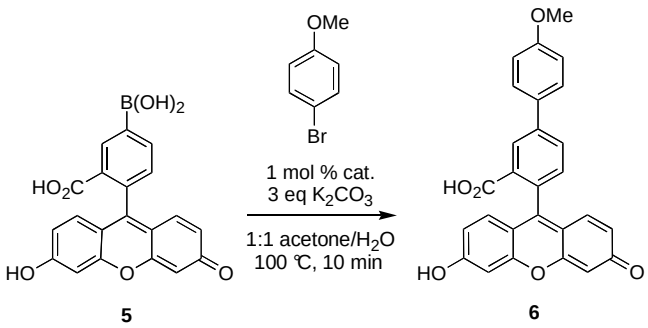
[c] Determined by ^1H NMR; errors estimated as $\pm$ 5 %

One of Li's phosphinite catalysts,[72] that has proved useful for other coupling reactions, was then investigated; it gave some of the desired product, but reduction was still a complication (entry 2). It seemed likely that the hydrogen that gave the reduction product was derived from the pinacolborane, hence the borylating agent was switched to dipinacolatodiboron.[73,74] Entries 4 and 5 demonstrate that when the base was also switched to KOAc then an appreciable conversion to the desired product was obtained. Still, however, reduction products were formed. It may be that the adventitious hydrogen that causes formation of these reduction products is solvent-derived.

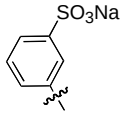
At this stage, it became evident that the optimization process was too slow due to the reaction times involved, hence subsequent studies focused on microwave acceleration of the reactions.[75,76] These reactions were performed using sealed tubes in a CEM Discover instrument that allows either the microwave power or the reaction temperature to be held constant, and the temperature in the reaction vessel to be monitored. Control of the reaction temperature would not be an option if a domestic microwave instrument was used. Entries 6 – 8 illustrate that if the power is modulated then the reaction temperature rises abruptly. These erratic experiments are scientifically unsatisfying because they would be hard to reproduce, especially on a different instrument or for different reaction scales. However, they did illustrate that high temperatures were tolerated. Finally, entry 9 shows the most favorable conditions identified. The diboron reagent with KOAc as base, microwave heated at a relatively high temperature for a short time gave approximately 98 % selectivity for the desired product. A 93 % yield of the desired product was isolated when this reaction was repeated on a 1 mmol scale (microwave, 150 °C, 10 min) and the crude material was purified via crystallization.[77]

It was convenient to store compound **3** because when it is hydrolyzed to the corresponding deacylated boronic acid **5** cyclotrimerization to the corresponding boroxine ensues. Consequently, the boronate **3** was hydrolyzed (K_2CO_3, 1:1 THF/H_2O, 3 h; 79 %) to **5** then isolated immediately prior to use in Suzuki coupling reactions.

Scheme 4.1 shows three model reactions that were performed to evaluate Suzuki couplings to compound **5**. A water-soluble palladium catalyst gave a superior result to $Pd(PPh_3)_4$ under these conditions, and the best isolated yield of the product **6** was obtained from the microwave-accelerated transformation in a sealed vessel.

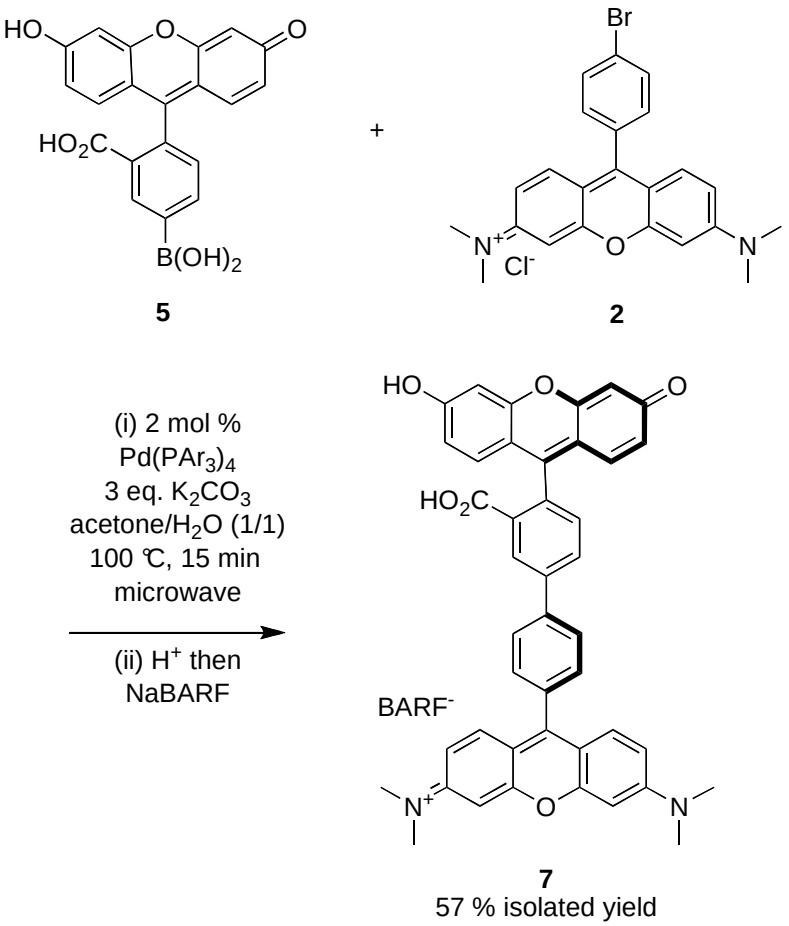


catalyst/heating	isolated yield (%)
$Pd(PPh_3)_4$/microwave	52
$Pd(PAr_3)_4$/microwave	81
$Pd(PAr_3)_4$/conventional	60

Ar = 

Scheme 4.1 Model reactions.

Scheme 4.2 illustrates how these findings were then applied to the coupling of the rosamine **2** with compound **5**.[78] The water soluble catalyst was used to couple the rosamine fragments, the product was converted to a protic form, then the counterion was metathesized to the *tetra*(3,5-trifluoromethylbenzene)boronate (BArF) anion; this allows convenient chromatography isolation of the product.



Scheme 4.2 Formation of cassette via Suzuki reaction.

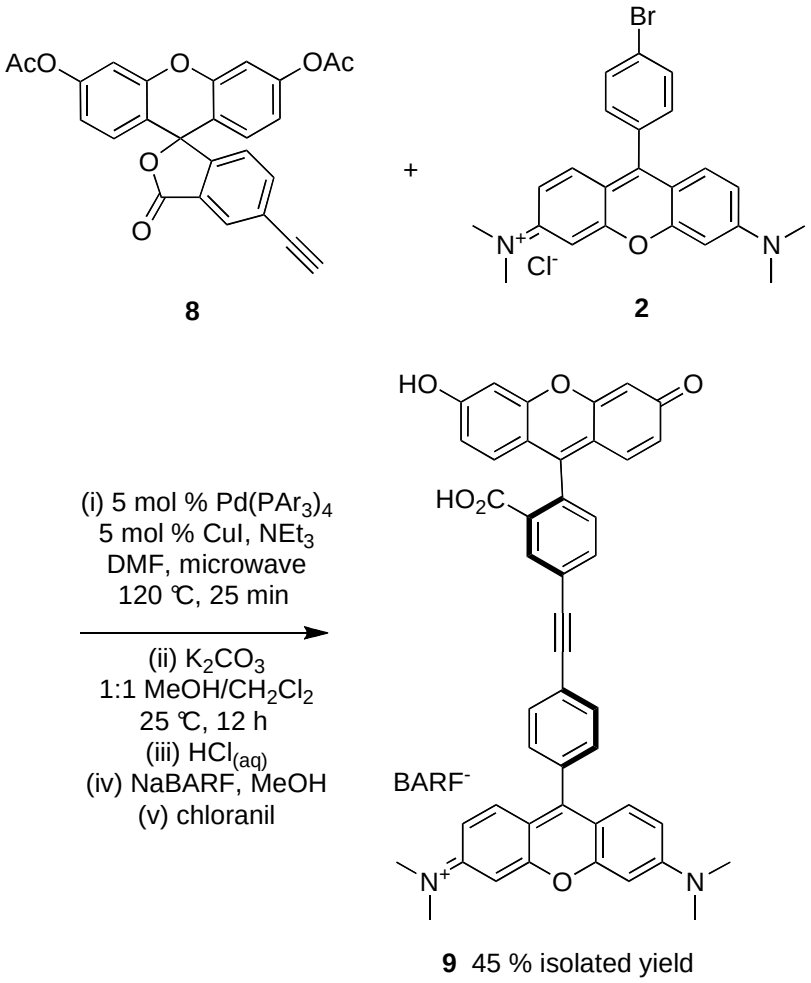
System **7** would be totally conjugated were it not for the twists imposed by the adjacent aromatic rings. For comparison, analogs of this structure with alkyne units inserted between the rings were desired, hence microwave-assisted Sonogashira couplings[79] of the dyes were investigated.

Alkyne **8** was formed in a two step process that involved first coupling trimethylsilylethyne with the protected fluorescein **1** under microwave conditions (5 mol % $Pd(PPh_3)_4$, CuI, NEt_3, DMF, 120 °C, 25 min; 84 % isolated). Like the other microwave reactions reported in this paper, this coupling was performed in a sealed tube. The procedure for sealing tubes for use in the microwave instrument by crimping on an

aluminum ring is very easy. Use of such sealed tubes for reactions such as this where volatile reagents are used is clearly an advantage. Deprotection of the alkyne was performed using TBAF (2 equiv., CH_2Cl_2, -78 °C, 5 min; 90 %).

Scheme 4.3 illustrates how the alkyne **8** was coupled with the bromorosamine **2** to give the desired alkyne-containing product **9** via a microwave accelerated coupling using the water-soluble catalyst. The product from this step was hydrolyzed directly to remove the acetate groups, acidified, then the counterion was exchanged with $BArF^-$ to assist chromatographic isolation.

However, Figure 4.1 shows the UV absorption spectra and fluorescence emission spectra of these molecules. The most significant features are that for the molecules **7** and **9** but not **6**, emission in the 520 nm region, that would be attributed to the fluorescein component of these systems, is greatly suppressed; most of the fluorescence of **7** and **9** occurs at a longer wavelength characteristic of the rosamine fragment.



Scheme 4.3 Formation of cassette via Sonogashira reaction.

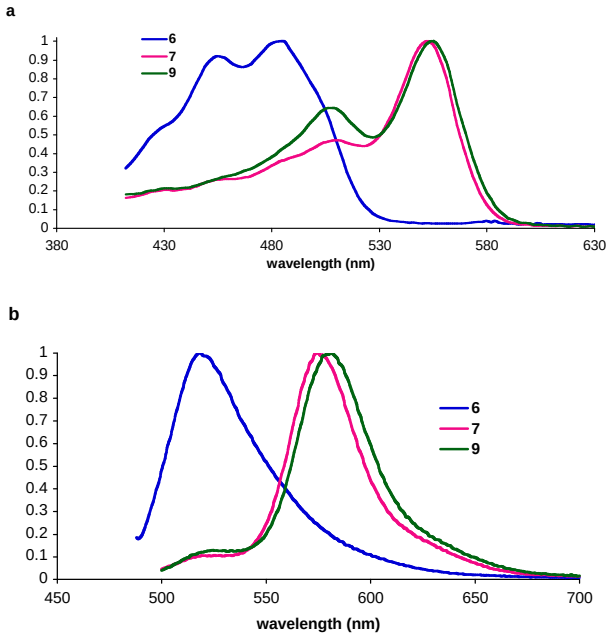


Figure 4.1 **a** UV absorption, and **b** fluorescence emission spectra of compounds **6**, **7** and **9**.

Organometallic coupling reactions are rarely used to functionalize fluorescein- and rhodamime-based systems. Here, halogenated dyes were transformed into organoboron compounds, biaryls, and alkynes. Fluorescein and rhodamine derivatives tend to be stable to high temperatures, so they might be expected to be amenable to microwave assisted steps. Indeed, the couplings described here worked well using bursts of microwave irradiation applied to give high temperatures for relatively short times.

CHAPTER V

SYNTHESIS OF REGIOISOMERICALLY PURE 5-FUNCTIONALIZED 2',7'-DICHLOROFLUORESCEINS*

Fluorescein undergoes transitions between various ionization states at pH levels around physiological (Figure 5.1 a). Quantum yields and extinction coefficients of fluorescein therefore may change significantly as the pH of the solvent varies. Consequently, fluorescein is non-ideal, or at least, difficult to use, for many quantitative biochemical experiments that rely on intensity of fluorescence.

The phenolic-OH groups of 2',7'-dichlorofluorescein are more acidic than those of the parent compound because of inductive stabilization of the phenolate form. 2',7'-Dichlorofluorescein therefore is completely ionized at pH levels near physiological and even slightly below.[82] Figure 5.1b shows data recorded for this paper wherein the pH of a 2',7'-dichlorofluorescein solution was varied around physiological levels; the fluorescence of the solution remained near stable until the pH of the medium fell to 5 and below. This dye therefore has some attributes that fluorescein does not. To be widely useful, however, probes such as these must have functional groups that allow them to be attached to biomolecules. This usually involves preparation of derivatives as regioisomeric mixtures then separation via recrystallization.[46,51,56]

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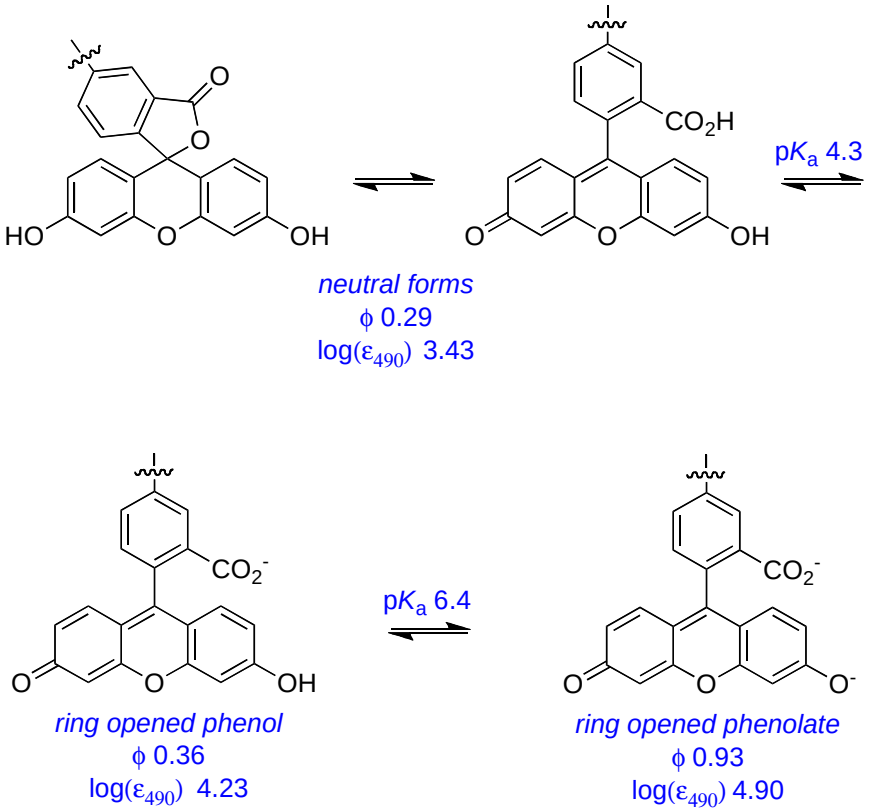
a



b

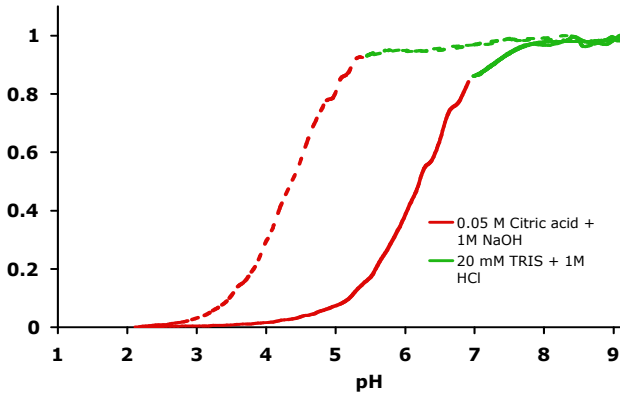
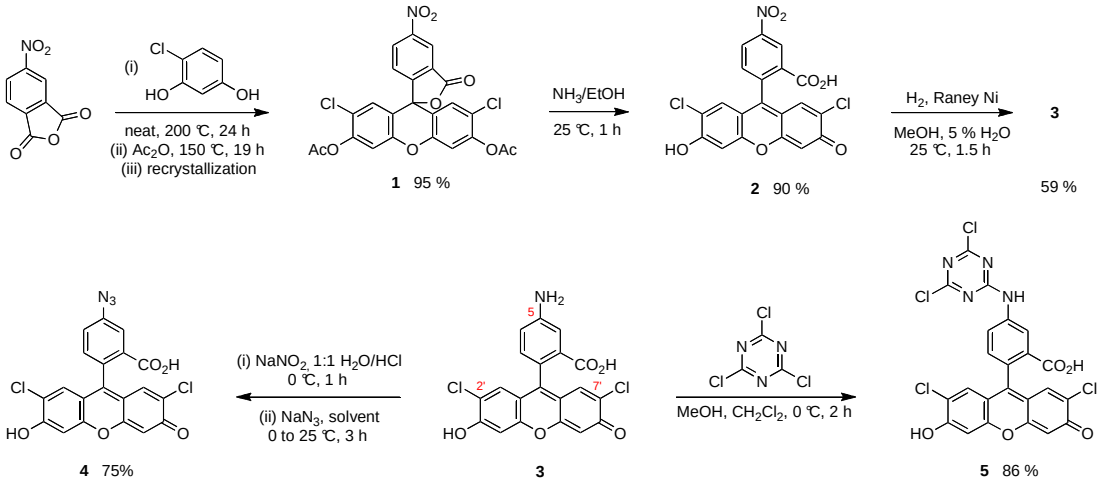


Figure 5.1. **a**; Ionization states of fluorescein at various pH levels; and **b**; pH dependence of fluorescence intensity for fluorescein vs. 2',7'-dichlorofluorescein.

Separation of the isomers of 5-nitro-2',7'-dichlorofluorescein was pivotal to the success of this project. Fortunately, 3-nitrophthalic anhydride could be condensed with chloro-2,4-dihydroxybenzene on a large scale to give a mixture of the 5- and 6-nitro isomers. Treatment of this mixture with acetic anhydride gave the diacetate **1**. This material was recrystallized six times from acetic anhydride to give the pure 5-nitro compound. This procedure gave 46 % of the target material and could conveniently be performed to give 35 g of the desired product.

An ammonia solution in ethanol (or methanol) was used for removal of the acetate groups from the lactone **1** which gave the free, ring-opened form **2**. This intermediate is then subjected directly to reduction of the nitro group via treatment with hydrogen and Raney Ni to give the free amine **3**.[83]

Amine **3** could be functionalized in several ways to enable it to be conjugated to biomolecules. Two approaches were chosen to illustrate this: formation of an azide **4** and conversion to the dichlorotriazine derivative **5**. The azide **4** is suitable for copper-mediated azide-to-alkyne coupling reactions.[84,85] Compound **5** is of particular interest to us for its ability to add nucleophilic groups sequentially.[86] The fluorescein analog of this compound[87] has been used extensively by us as a linker to form bivalent peptidomimetics.[88,89] However, we have found that direct binding assays based on measurement of fluorescence for these mimics requires very careful control of pH.



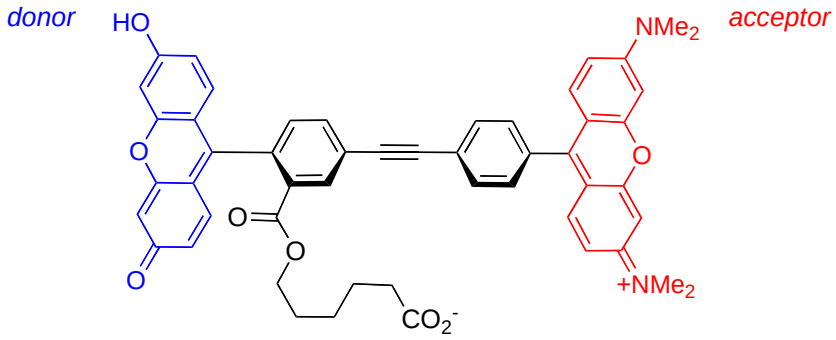
Scheme 5.1 Derivatization of 5-nitro-2', 7'-dichlorofluorescein.

The procedures outlined in this report are scalable to preparative quantities. Syntheses of the 5-nitro and (5 amino-) 2', 7'-dichlorofluorescein are easily obtained in multi-gram scale (ca. 35 g or above). The subsequent derivatization into the azido dichlorofluorescein is also scalable.

CHAPTER VI

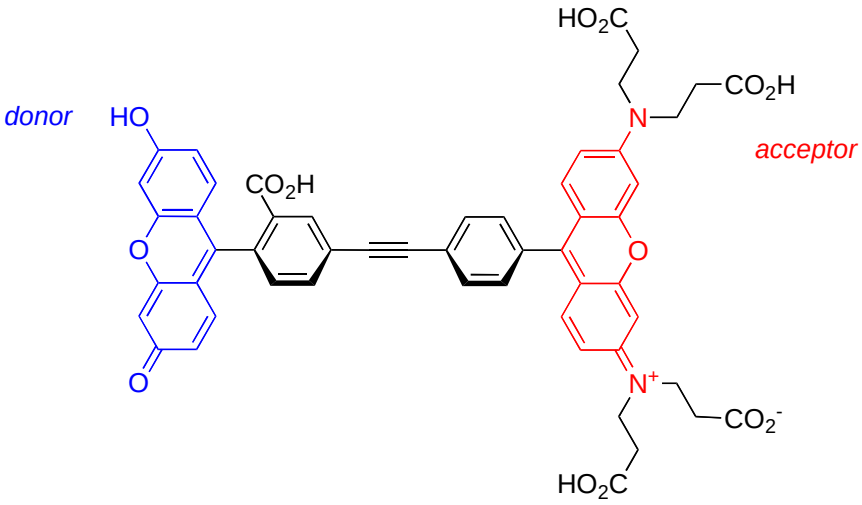
CONCLUSION: USEFUL FUTURE POTENTIAL OF 2',7'-DICHLOROFLUORESCEIN

Earlier research from these laboratories has produced cassettes based on conjugation of fluorescein donors with rhodamine acceptors (Figure 6.1).[80,81] The first generation of these probes, *eg* **A**, had very poor water solubility, but later designs, like **B**, are soluble in aqueous media. However, several improvements are necessary, and the most important of these are: (i) improved energy transfer efficiencies between the donor and the acceptor in aqueous media; and, (ii) greater dispersion of the fluorescence outputs.



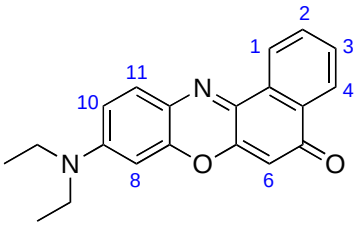
A
first generation cassette

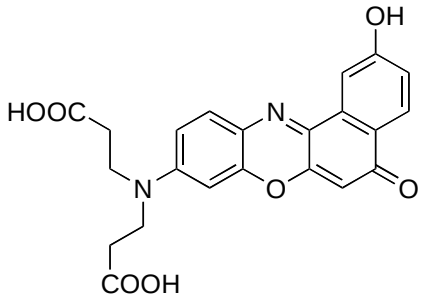
Figure 6.1 Nile red fluorophores.



B

second generation cassette

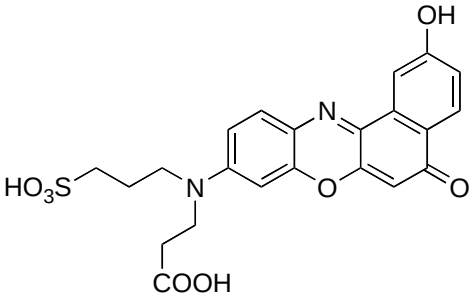




C

Nile Red

D



E

Figure 6.1 Continued.

Nile Red, **C**, has a very poor water solubility but otherwise desirable properties as a potential acceptor in cassettes, notably emissions in the region of 648 nm (phosphate buffer, pH 7.4). Recent developments from these laboratories demonstrated methods to prepare water soluble, functionalized, 2-hydroxy Nile Red derivatives like **D** and **E**. In this paper, we demonstrate how such compounds can be integrated into through bond energy cassettes, and report on the spectroscopic properties of the first fluorescein/Nile Red cassettes of this kind. Specifically, the syntheses and spectroscopic properties of cassettes **1** – **3** are reported (Figure 6.2). Cassette **1** is a model compound with no functional group to allow convenient attachment to biomolecules, while **2** and **3** have such a group. The dichlorofluorescein functionality of cassettes **3** and **4** was investigated as a likely design upgrade since that particular donor functionality is less sensitive to pH changes (Figure 6.3) Finally, cassette **4** has two desirable features that are not inherent in structures **1** – **3**: a handle on the donor part, and lack of an alkyne linker. It is beneficial to include the handle for bioconjugation on the donor part because this is intended to be constant in sets of cassettes for multiplexing. To include the handle on the acceptor part tends to be less practical because then every acceptor synthesis has to accommodate this, and that is extra work. Deletion of the alkyne linker removes brings the donor and acceptor fragments closer together; this may have increase the efficiency of donor-to-acceptor energy transfer, but it also perturbs the molecular geometry and that may have unknown effects.

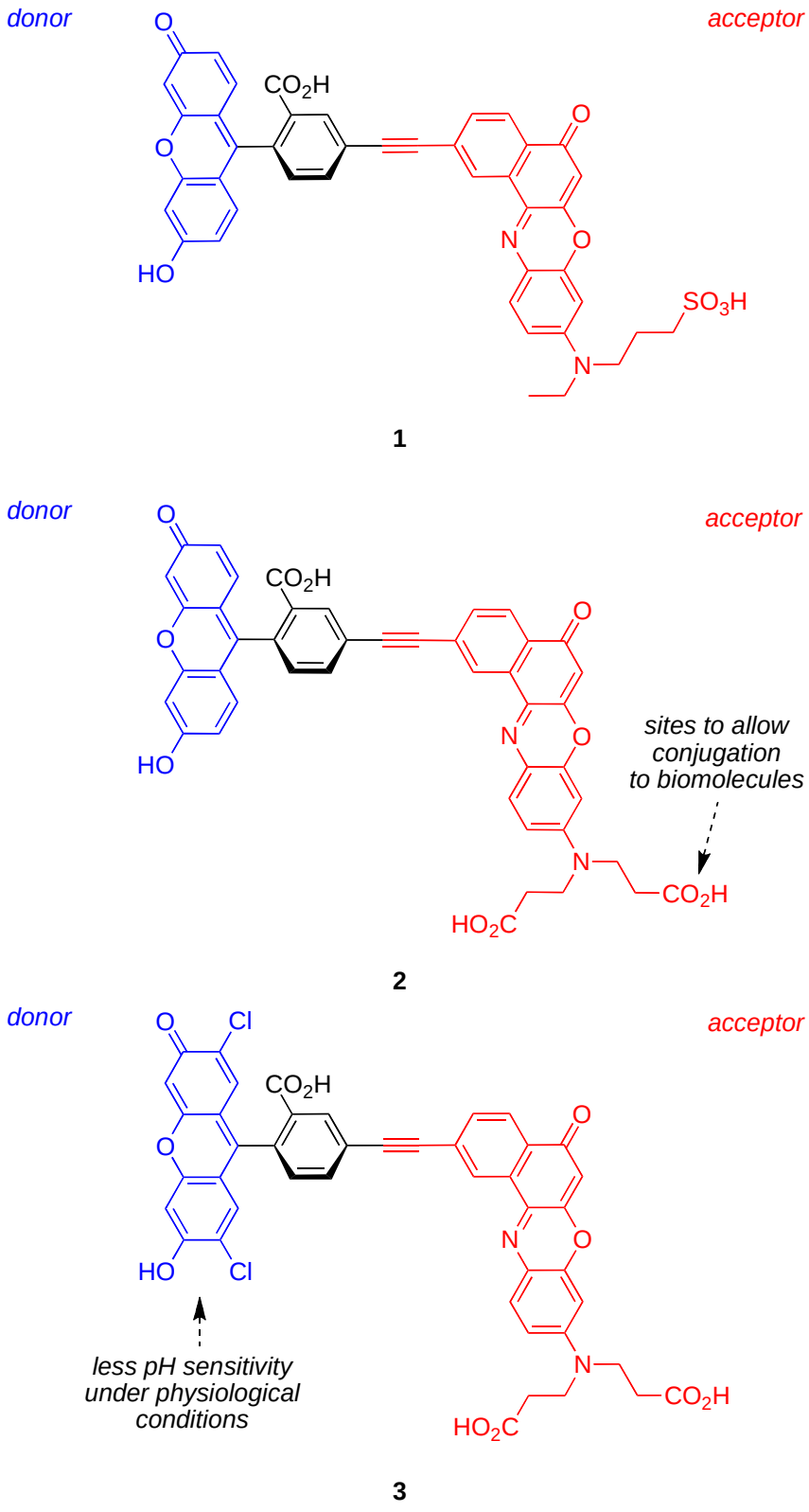


Figure 6.2 Synthesized cassettes.

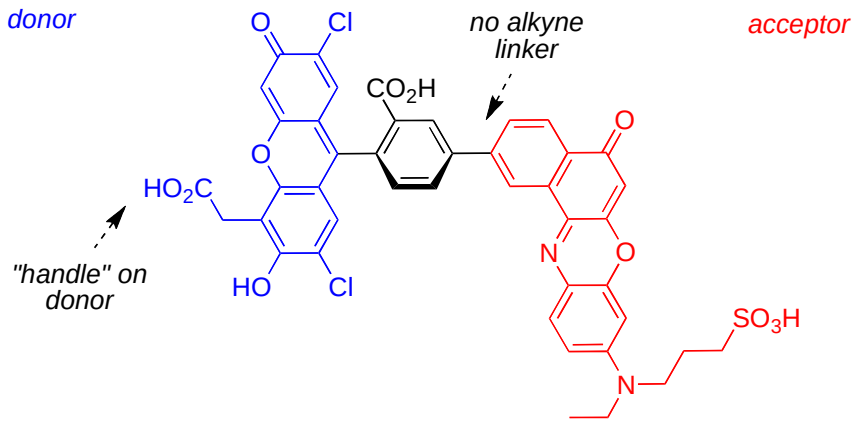


Figure 6.2 Continued.

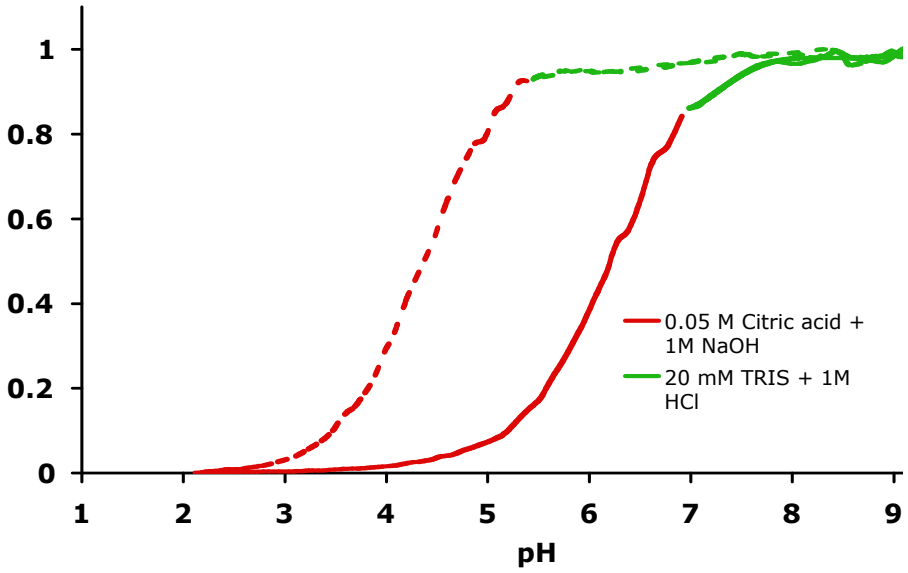
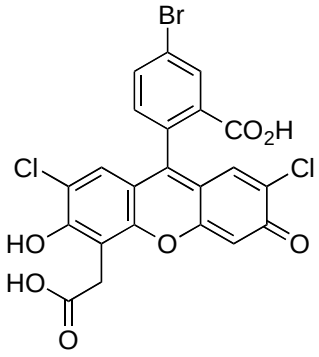


Figure 6.3. Intensity pH dependency of DCF and fluorescein.

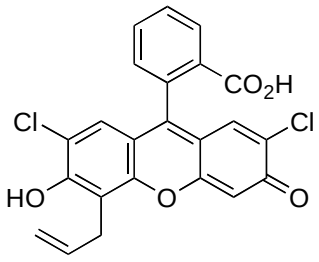
It was therefore required to develop a synthetic methodology toward the desired fluorescein "donor". In our laboratory, we have already developed a strategy toward the multiscale synthesis of 5-bromo-2', 7'-dichlofluorescein. This is achieved by the high temperature condensation of 4-chlororesorcinol with 4-bromophthalic anhydride, followed by sequential recrystallization from acetic anhydride to yield the 5-bromo

isomer in high yield and purity. By following Koide's lead into the synthesis of 1'-alyllflourescein (figure 6.3), we were able to modify our approach to form the allyl analog of 2', 7'-dichlorofluorescein required to obtain our desired compound (Figure 6.4) in a multistep approach which led us to obtain the diacid target in relatively good yield. (Scheme 6.1)



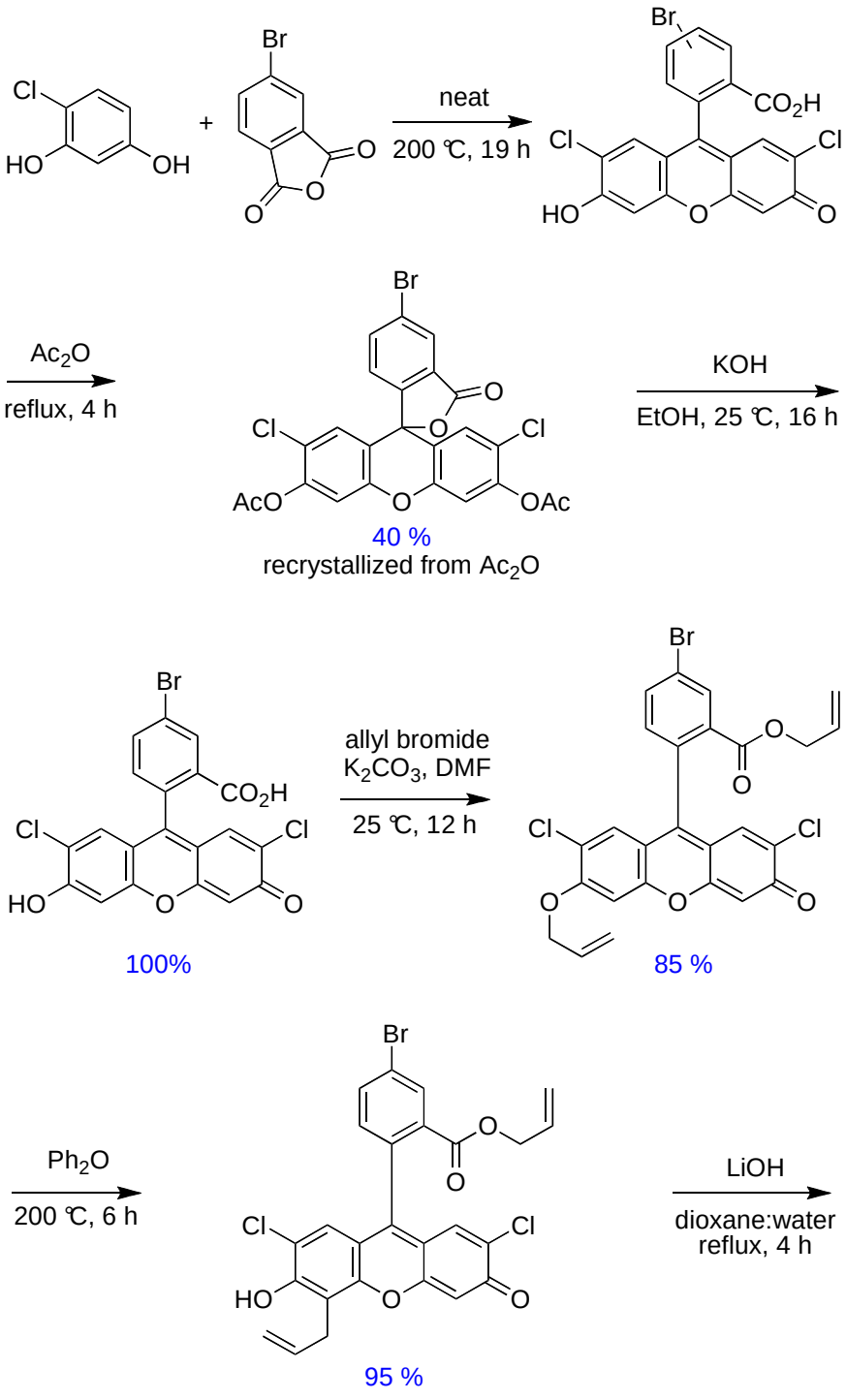
3

Obtained product

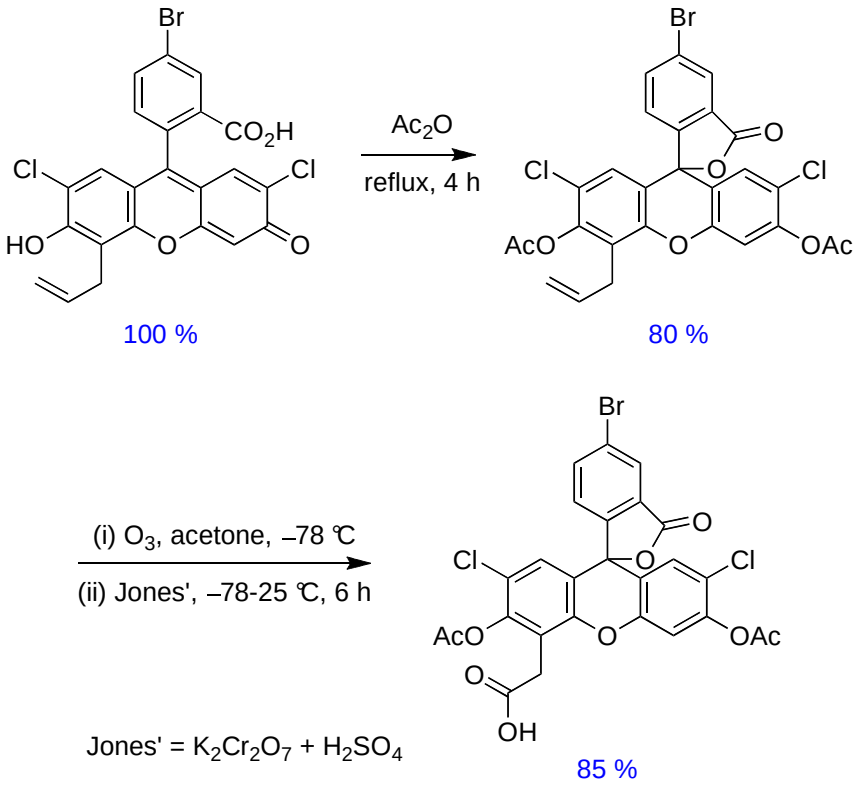


4

Figure 6.4 Allyl dichlorofluorescein prepared by Koide.



Scheme 6.1 Stepwise synthesis of desired product.



Scheme 6.1 Continued.

The attempts to incorporate this compound into cassettes with Nile Red as an acceptor resulted in very poor yields, unable to fully characterized the obtained compound. Preliminary data suggested the presence of the cassette, but further attempts to obtain higher amounts were unsuccessful probably due to the solubility differences between both molecules.

Future studies in this system should be attempted.

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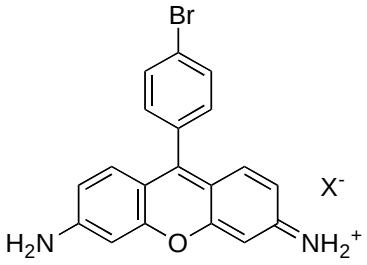
APPENDIX: EXPERIMENTAL

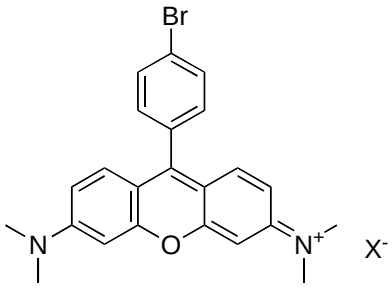
General Procedures. Melting points are uncorrected. High field NMR spectra were recorded on Varian Unity Plus (^{1}H at 300 MHz, ^{13}C at 75 MHz) or Inova (^{1}H at 500 MHz, ^{13}C at 125 MHz) NMR spectrometers. Chemical shifts are reported in units of ppm relative to solvent ($CDCl_3$: 7.27 ppm for ^{1}H and 77.0 ppm for ^{13}C; CD_3OD: 3.30 ppm for ^{1}H and 49.0 ppm for ^{13}C). Mass spectra were obtained from the Mass Spectrometry Applications Laboratory at Texas A&M University. Thin layer chromatography was performed using silica gel 60 F254 plates. Flash chromatography was performed using silica gel (230-600 mesh). 7-Hydroxy-*N*-methyl-2,2,4-trimethyl-l,2-dihydroquinoline[1] was prepared following the literature procedure. Other chemicals were purchased from commercial suppliers and used as received. All the experiments involving microwave irradiation were performed on a CEM Discover instrument.

CHAPTER III



6-amino-9-(4-bromophenyl)-3*H*-xanthen-3-iminium.

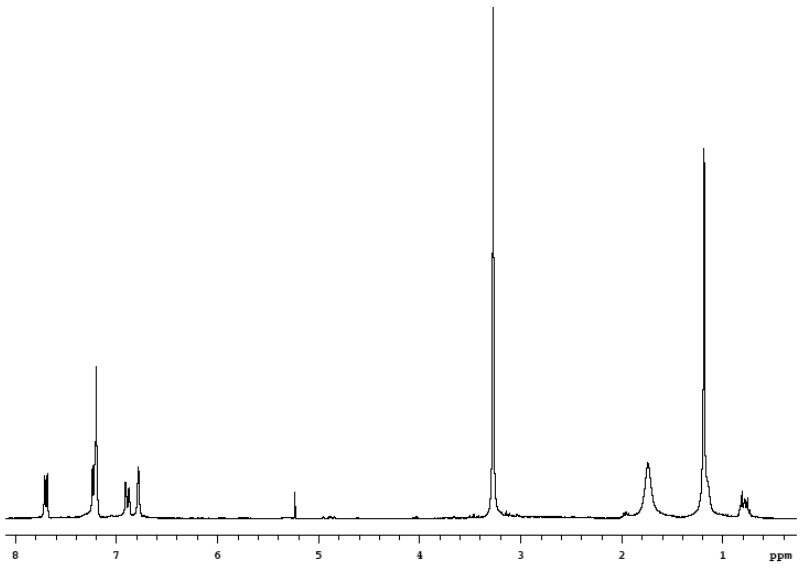
3-Aminophenol (1.00 g, 9.17 mmol) and 4-bromobenzaldehyde (0.85 g, 4.59 mmol) were dissolved in 20 mL $CHCl_3$. The solvent was then removed *in vacuo*. The mixture was suspended in 20 mL of ice-cold 60% H_2SO_4. A condenser was fitted, and the flask was placed in the microwave reactor and heated to 150 ^oC for 20 min (constant temperature mode). The nitrogen cooling flow was applied in order to maximize the power . Then tetrachloro-1,4-benzoquinone (1.69 g, 6.89 mmol) was added to the solution, and allowed to stir at room temperature for 10 min. The dark brown mixture was neutralized with 45 mL 10 M potassium hydroxide to pH ≈ 7. The mixture was then extracted with 5 % *i*-PrOH/CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with brine (200 mL) and water (200 mL), dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was further purified by flash chromatography (5 to 15% MeOH/CH_2Cl_2) to give the title compound as a red-orange solid (0.47 g, 27 % yield). R_f 0.10 (10 % MeOH/CH_2Cl_2); ^1H NMR (1:1 $CD_3OD/CDCl_3$, 500 MHz): δ = 6.79 (d, J = 2.2 Hz, 2H), 6.82 (dd, J = 9.3 Hz, 2.2 Hz, 2H), 7.21 (d, J = 9.3 Hz, 2H), 7.27 (d, J = 8.5 Hz, 2H), 7.76 (d, J = 8.5 Hz, 2H); ^{13}C NMR (1:1 $CD_3OD/CDCl_3$, 125 MHz): δ = 98.4, 113.8, 117.7, 125.3, 131.5, 131.6, 132.6, 132.7, 157.5, 159.0, 160.3; MS (ESI) m/z 365/367 (M^+).



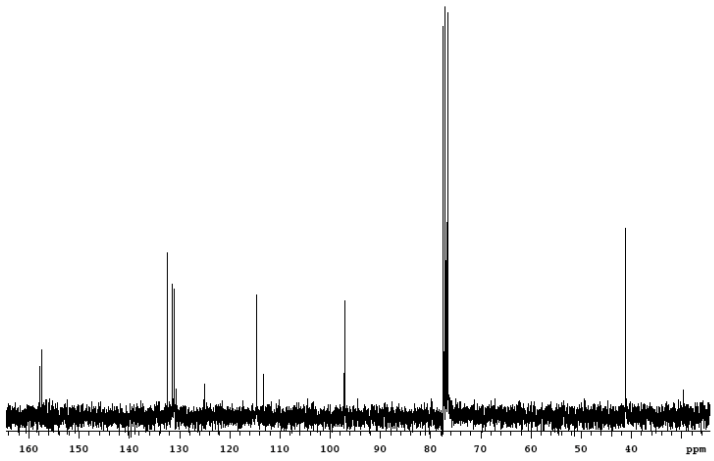
***N*-(9-(4-bromophenyl)-6-(dimethylamino)-3*H*-xanthen-3-ylidene)-*N*-methylmethanaminium.**

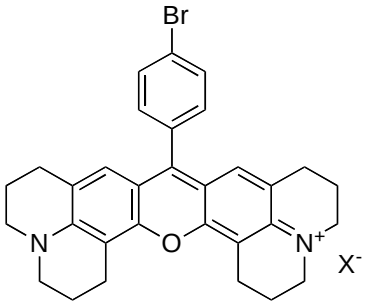
3-Dimethylaminophenol (5.00 g, 36.0 mmol) and 4-bromobenzaldehyde (3.37 g, 18.0 mmol) were dissolved in 50 mL dichloromethane and the solvent was removed *in vacuo* to make a homogenous mixture. The mixture was then suspended in 70 mL ice-cold 60% H_2SO_4. A condenser was fitted, and the flask was heated in the microwave reactor for 30 min at 90 ^oC (constant temperature mode) with vigorous stirring. A nitrogen cooling flow was applied throughout to maximize the power. Tetrachloro-1,4-benzoquinone ("chloranil", 8.85 g, 36.00 mmol) was added to the solution, which was then stirred at room temperature for 15 min. The dark violet mixture was neutralized with *ca.* 150 mL of 10 M potassium hydroxide to pH $\approx$ 7. The mixture was then extracted with 5 % *i*-PrOH/CH_2Cl_2 (3 x 200 mL). The combined organic layers were washed with brine (200 mL) and water (300 mL), then dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was further purified by flash chromatography (5 to 10% MeOH/CH_2Cl_2) to give the title compound as a dark violet solid (2.69 g, 41 %). Mp = 227 ^oC (dec); R_f 0.27 (10 % MeOH/CH_2Cl_2); ^1H NMR ($CDCl_3$, 300 MHz): δ = 3.38 (s, 12H), 6.91 (d, J = 2.4 Hz, 2H), 7.02 (dd, J = 9.5 Hz, 2.4 Hz, 2H), 7.29 (d, J = 8.3 Hz, 2H), 7.33 (d, J = 9.5 Hz, 2H), 7.78 (d, J = 8.3 Hz, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 41.5, 97.4, 113.5, 114.9, 125.3, 130.9, 131.3, 131.6, 132.6, 156.6, 157.6, 157.9 MS (ESI) m/z 421/423 (M^+).

H^1-NMR



C^{13}-NMR

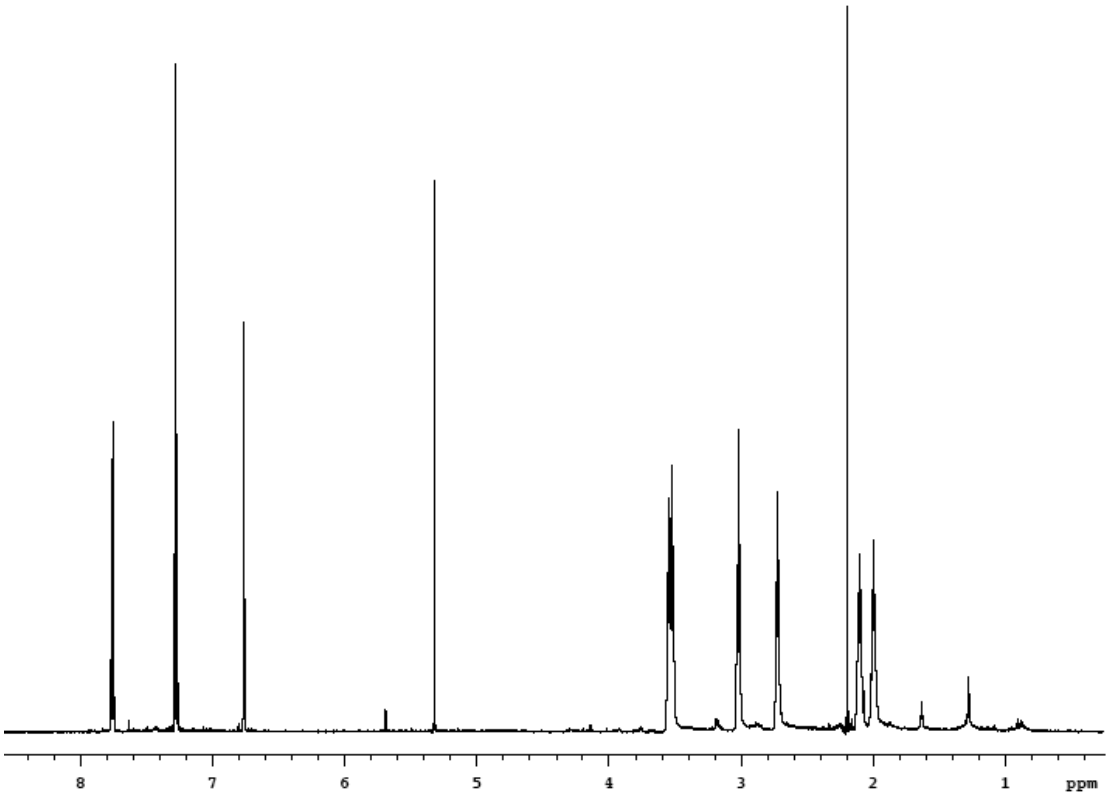


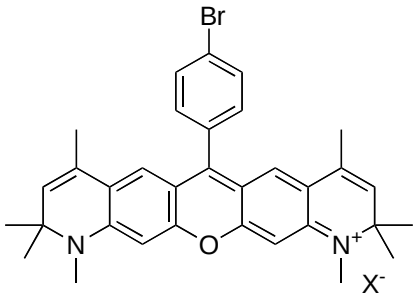


5-bromo-julolidinorosamine

8-Hydroxyjulolidine (1.00 g, 5.29 mmol) and 4-bromobenzaldehyde (0.49 g, 2.65 mmol) were dissolved in 20 mL $CHCl_3$. The solvent was then removed *in vacuo*. The mixture was suspended in 20 mL of ice-cold 60% H_2SO_4. A condenser was fitted, and the flask was heated in the microwave reactor with vigorous stirring for 10 min at 150 ^oC (constant temperature mode). Throughout, the nitrogen cooling flow was applied in order to maximize the power. Tetrachloro-1,4-benzoquinone (0.98 g, 3.98 mmol) was added to the solution, which was then stirred at room temperature for 10 min. The dark blue mixture was neutralized with *ca.* 45 mL 10 M potassium hydroxide to pH ≈ 7. The mixture was then extracted with 5 % *i*-PrOH/CH_2Cl_2 (3 x 100 mL). The combined organic layers were washed with brine (200 mL) and water (200 mL), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The residue was further purified by flash chromatography (5 to 10% MeOH/CH_2Cl_2) to give the title compound as a dark violet solid (1.05 g, 73 % yield). Mp = 140 ^{o} C (dec); R_f 0.18 (10 % MeOH/ CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 1.99 (p, *J* = 5.5 Hz, 4H), 2.10 (p, *J* = 5.5 Hz, 4H), 2.70 (t, *J* = 6.0 Hz, 4H), 3.02 (t, *J* = 6.0 Hz, 4H), 3.53 (t, *J* = 5.5 Hz, 4H), 3.56 (t, *J* = 5.5 Hz, 4H), 6.73 (s, 2H), 7.20 (d, *J* = 8.5 Hz, 2H), 7.87 (d, *J* = 8.5 Hz, 2H); ^{13}CNMR ($CDCl_3$, 125 MHz): δ = 19.5, 19.8, 20.5, 27.5, 50.4, 50.8, 105.4, 112.4, 123.8, 124.0, 126.1, 130.9, 131.5, 132.0, 151.1, 152.0, 152.6 MS (ESI): m/z 525/527 (M^+).

H^1-NMR

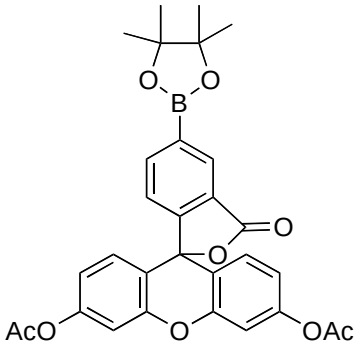




Extended 5-bromo rosamine

7-Hydroxy-*N*-methyl-2,2,4-trimethy-l,2-dihydroquinoline[1] (1.50 g, 7.39 mmol) and 4-bromobenzaldehyde (0.68 g, 3.69 mmol) were dissolved in 20 mL $CHCl_3$. The solvent was then removed *in vacuo*. A condenser was fitted, and the flask was heated in the microwave reactor with vigorous stirring for 10 min at 150 ^oC (constant temperature mode). A nitrogen cooling flow was applied throughout to maximize the power . The reaction mixture was dissolved in 20 mL 5 % $MeOH/CH_2Cl_2$ and tetrachloro-1,4-benzoquinone (1.36 g, 5.54 mmol) was added to the solution, which was then stirred at room temperature for 10 min. The solvent was removed under reduced pressure. The residue was further purified by flash chromatography (5 to 10% $MeOH/CH_2Cl_2$) to give the title compound as a dark blue solid (1.13 g, 53 % yield). R_f 0.20 (10 % $MeOH/CH_2Cl_2$); ^1H NMR ($CDCl_3$, 500 MHz): δ = 1.46 (s, 12H), 1.79 (d, J = 1.2 Hz, 6H), 3.17 (s, 6H), 5.49 (d, J = 1.2 Hz, 2H), 6.1 (s, 2H), 6.85 (s, 2H), 7.27 (d, J = 8.3 Hz, 2H), 7.76 (d, J = 8.3 Hz, 2H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 18.4, 29.3, 33.3, 59.8, 96.2, 113.5, 121.4, 123.6, 124.8, 125.4, 130.9, 131.5, 132.3, 132.7, 152.8, 153.3, 158.3; MS (ESI) m/z 553/555 (M^+).

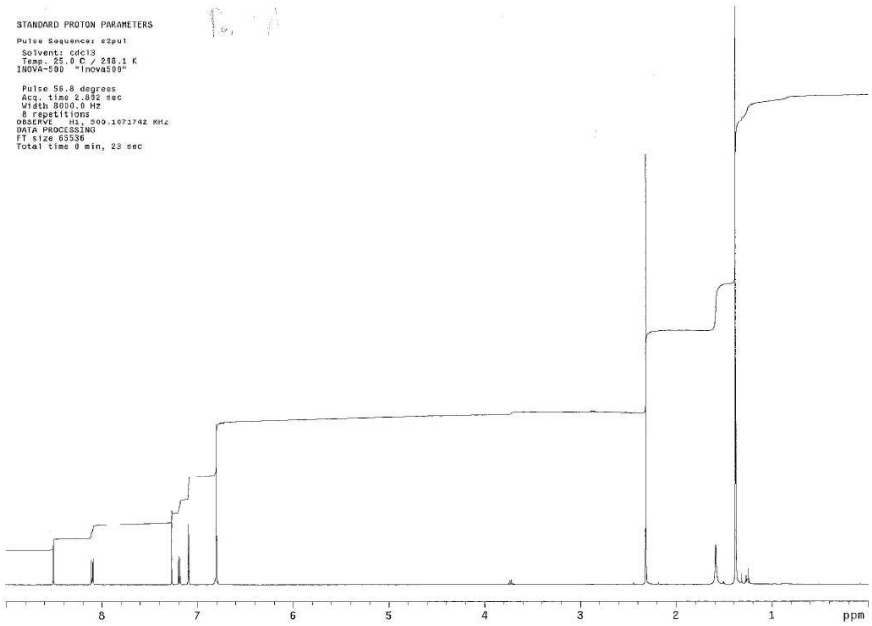
CHAPTER IV



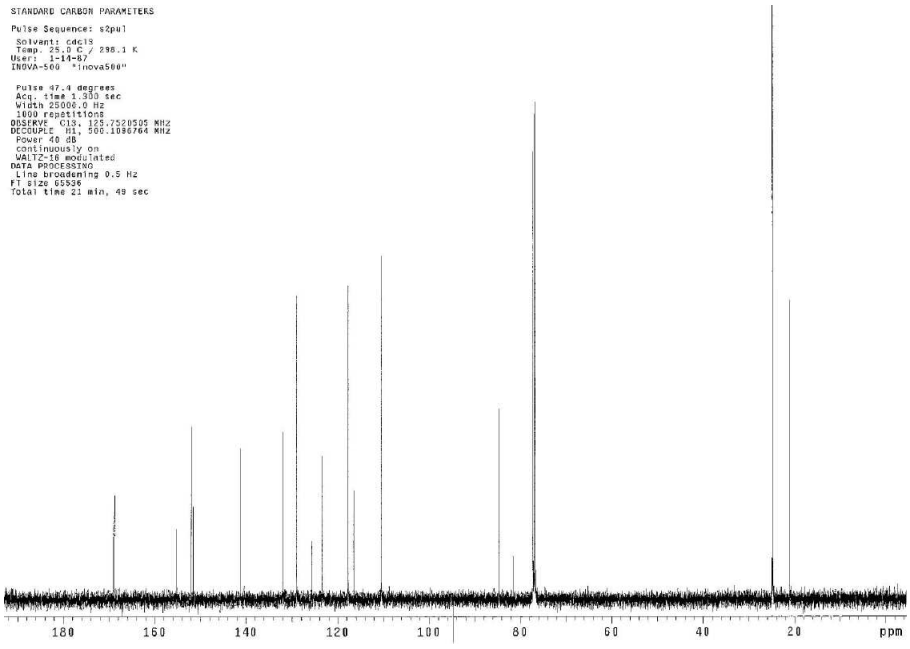
3-oxo-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3*H*-spirow-3',6'-diyl diacetate.

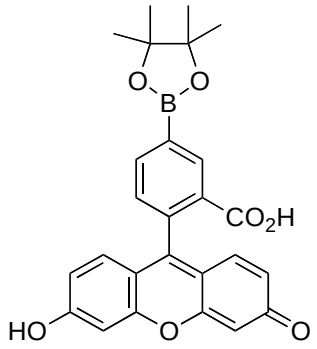
A typical procedure is given for entry 9. A mixture of 5-bromofluorescein diacetate (495 mg, 1.0 mmol), dipinacolatodiboron (279 mg, 1.1 mmol), $PdCl_2$(dppf) (7.3 mg, 0.01 mmol), and KOAc (294 mg, 3.0 mmol) in 4 mL of toluene was sealed in a microwave tube. The reaction tube was microwave heated at 150 °C for 10 min. After cooling, the reaction mixture was poured into aqueous NH_4Cl solution. The mixture was extracted with benzene and the organic extract was washed with aqueous $NaHCO_3$ solution and aqueous NaCl solution successively. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by solidification from benzene/EtOH to give 506 mg (93% yield) of **3** as a white solid. Characterization of **3**: mp 241 °C (dec). ^{1}H NMR ($CDCl_3$): δ = 8.51 (s, 1H), 8.10 (d, *J* = 7.5 Hz, 1H), 7.19 (d, *J* = 7.5 Hz, 1H), 7.10 (m, 2H), 6.80 (m, 4H), 2.32 (s, 6H), 1.38 (s, 12H); ^{13}C NMR ($CDCl_3$): δ = 169.0, 168.8, 155.2, 152.0, 151.5, 141.3, 131.9, 129.0, 125.7, 123.4, 117.7, 116.4, 110.4, 84.5, 81.6, 24.9, 21.1. HRMS (ESI) *m/z* (M^+ + H) calcd. for $C_{30}H_{28}BO_9$: 543.1824. Found: 543.1815.

H^1 NMR



C^{13} NMR

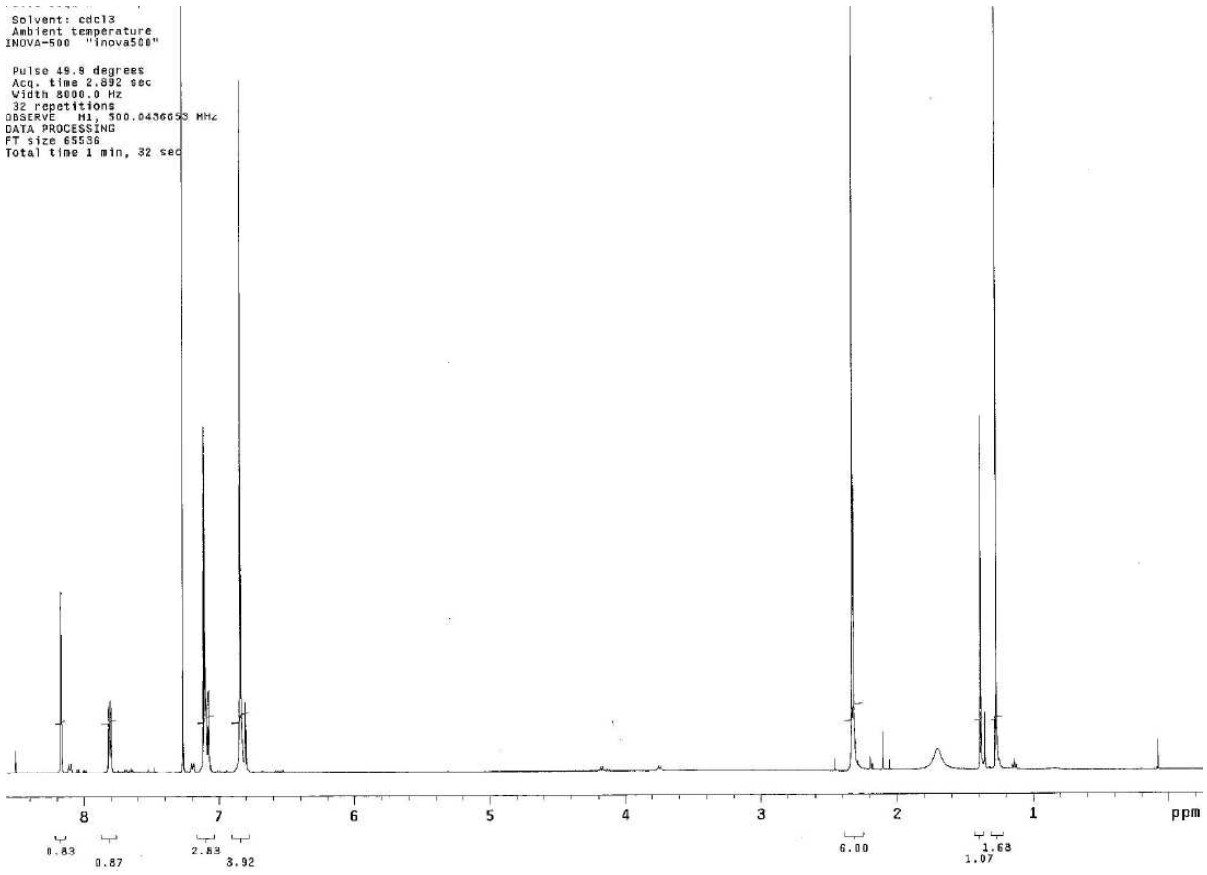




2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoic acid.

A mixture of 3-oxo-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate (300 mg, 0.55 mmol) and K_2CO_3 (382 mg, 2.76 mmol) in 10 mL of THF/H_2O (1/1) was stirred at room temperature for 3 h. The reaction mixture was acidified with concentrated HCl. The resulting solid was filtered and washed with H_2O and diethyl ether successively. The solid was dried under reduced pressure to give 164 mg (79% yield) of 5-borono-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid as a yellow solid. Mp 392 °C (dec). ^{1}H NMR (DMSO-d_6): δ = 10.09 (s, 2H), 8.42 (s, 2H), 8.39 (s, 1H), 8.14 (d, J = 7.5 Hz, 1H), 7.21 (d, J = 7.5 Hz, 1H), 6.66 (m, 2H), 6.53 (m, 4H); ^{13}C NMR (DMSO-d_6): δ = 169.0, 159.4, 154.2, 151.8, 141.0, 130.2, 129.1, 125.5, 123.0, 112.6, 109.6, 102.2, 82.9. HRMS (ESI) *m/z* (M^+ + H) calcd. for $C_{20}H_{14}BO_7$: 377.0833. Found: 377.0817.

H^1 NMR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32 repetitions
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DATA PROCESSING
FT size 65536
Total time 1 min, 32 sec
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7
6
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4
3
2
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ppm
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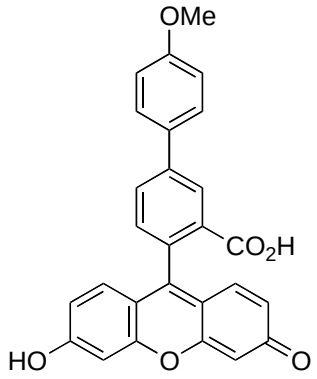
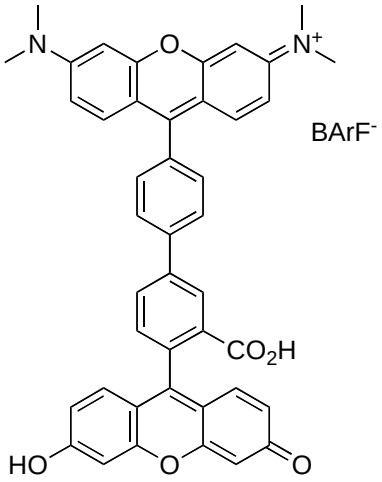
6.00
1.07
1.68



4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-4'-methoxybiphenyl-3-carboxylic acid.

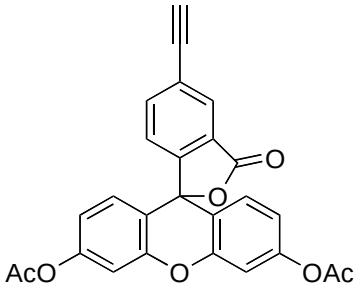
A mixture of **5** (50 mg, 0.13 mmol), 4-bromoanisole (0.050 mL, 0.40 mmol), $Pd(P(3\text{-}C_6H_4SO_3Na)_3)_4$, (3.2 mg, 0.0013 mmol) and K_2CO_3 (55 mg, 0.40 mmol) in 2 mL of acetone/H_2O (1/1) was microwave heated in a sealed tube at 100 °C for 10 min. After cooling, the reaction mixture was poured into 6 N HCl solution. The mixture was extracted with 25% *i*PrOH/$CHCl_3$ and the organic extract was washed with aqueous NaCl solution. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by flash column chromatography (CH_2Cl_2/MeOH = 15/1) to give 47 mg (81% yield) of **6** as an orange solid. Characterization of **6**: mp 174 °C (dec). ^{1}H NMR (DMSO-d_6): δ = 10.11 (s, 2H), 8.12 (s, 1H), 8.02 (d, *J* = 8.0, 1.5 Hz, 1H), 7.77 (d, *J* = 7.0 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.07 (d, *J* = 7.0 Hz, 2H), 6.68 (d, *J* = 2.0 Hz, 2H), 6.63 (d, *J* = 9.0 Hz, 2H), 6.56 (dd, *J* = 8.5, 2.5 Hz, 2H), 3.82 (s, 3H); ^{13}C NMR (DMSO-d_6): δ = 168.6, 159.5, 159.5, 151.8, 150.7, 141.8, 133.7, 130.7, 129.1, 128.3, 127.1, 124.4, 121.4, 114.6, 112.6, 109.6, 102.2, 83.1, 55.2. HRMS (ESI) *m/z* (M^+ - H) calcd. for $C_{27}H_{17}O_6$: 437.1025. Found: 437.1009.



***N*-(9-(3'-carboxy-4'-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)biphenyl-4-yl)-6-(dimethylamino)-3*H*-xanthen-3-ylidene)-*N*-methylmethanaminium.**

A mixture of **5**(38 mg, 0.10 mmol), **2** (45 mg, 0.10 mmol), $Pd(P(3\text{-}C_6H_4SO_3Na)_3)_4$ (4.8 mg, 0.0020 mmol), and K_2CO_3 (41 mg, 0.30 mmol) in 3 mL of acetone/H_2O (1/1) was microwave heated in a sealed tube at 100 °C for 15 min. After cooling, the reaction mixture was poured into 6 N HCl solution. The mixture was extracted with 25% *i*PrOH/$CHCl_3$ and the organic extract was washed with H_2O. The organic layer was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. To a solution of crude product in 10 mL of CH_2Cl_2/H_2O (1/1) was added 133 mg (0.15 mmol) of sodium tetra(3,5-trifluoromethylbenzene)boronate (NaBARF). The mixture was stirred at room temperature for 12 h. The mixture was extracted with 25% *i*PrOH/$CHCl_3$ and the organic extract was washed with H_2O. The organic layer was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (CH_2Cl_2/MeOH = 15/1) to give 87 mg (57% yield) of **7** as a purple solid. Characterization of **7**: mp 120 °C (dec). ^{1}H NMR (DMSO-d_6): δ = 10.16 (s, 2H), 8.37 (s, 1H), 8.24 (d, *J* = 8.0 Hz, 1H), 8.17 (d, *J* = 8.5 Hz, 2H), 7.67 (s, 4H), 7.68 (d, *J* = 8.0 Hz, 2H), 7.60 (s, 8H), 7.43 (d, *J* = 7.5 Hz, 1H), 7.37 (d, *J* = 9.5 Hz, 2H), 7.18 (dd, *J* = 9.5, 2.5 Hz, 2H), 6.99 (d, *J* = 2.5 Hz, 2H), 6.70 (d, *J* = 2.5 Hz, 2H), 6.67 (d, *J* = 9.0 Hz, 2H), 6.58 (dd, *J* = 9.0, 2.5 Hz, 2H), 3.28 (s, 12H); ^{13}C NMR

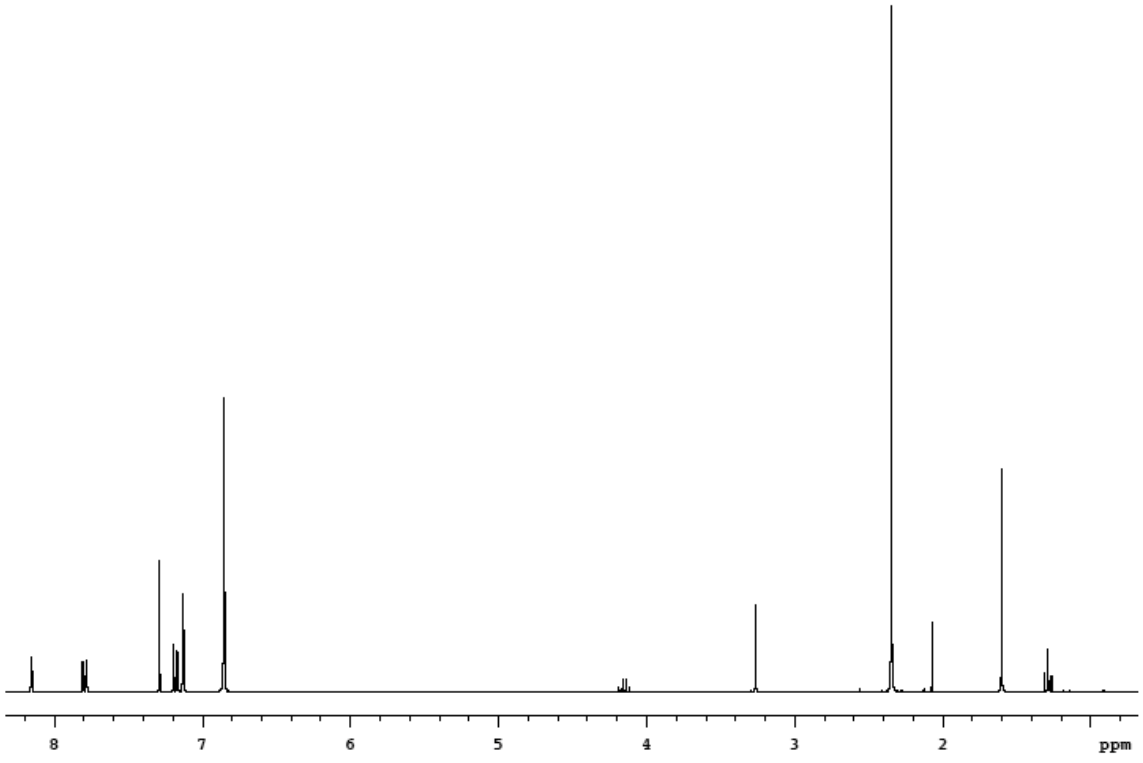
(DMSO-d_6): δ = 168.5, 160.9 (q, J = 49.4 Hz), 159.6, 157.1, 156.9, 156.5, 152.1, 151.9, 141.0, 140.1, 134.4, 134.0, 131.7, 131.2, 130.5, 129.1, 128.5 (m), 127.5, 127.3, 124.8, 124.0 (q, J = 271 Hz), 122.6, 117.6, 114.7, 112.7, 112.7, 109.4, 102.3, 96.3, 83.1, 40.5. Anal. calcd for $C_{75}H_{45}BF_{24}N_2O_6$: C, 58.61; H, 2.95; found: C, 58.58; H, 3.02.

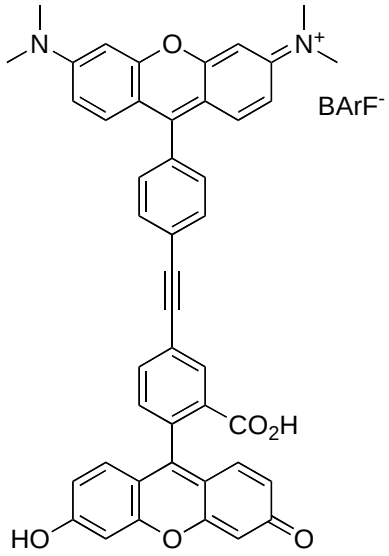


5-ethynyl-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diacetate.

A mixture of 5-bromofluorescein diacetate (0.248 g, 0.5 mmol), trimethylsilylacetylene (54.0 mg, 0.55 mmol), $Pd(P(3\text{-}C_6H_4SO_3Na)_3)_4$ (59.5.0 mg, 25.0 mmol), copper(I) iodide (5.0 mg, 25.0 mmol), and triethylamine (1.014 g, 10.0 mmol) in 0.5 mL DMF was microwave heated in a sealed tube at 120 ^oC for 25 min. in constant temperature mode. After cooling, 10 mL of diethyl ether were added to the solution and filtered over celite. The filtrate was then poured in 10.0 mL 0.1 M HCl. The product was extracted with 2 x 20 mL diethyl ether and dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. The solid was further purified by flash chromatography (5% MeOH/CH_2Cl_2) to give 0.215 g (84% yield) of **8** as a pale yellow solid. Characterization of **8.** R*f* 0.55 (35 % EtOAc/hexanes); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 0.29 (s, 9H), 2.33 (s, 6H), 6.82 (m, 4H), 7.10 (m, 2H), 7.13 (d, *J* = 8.1 Hz, 1H), 7.75 (dd, *J* = 8.1 Hz, 1.2 Hz, 1H), 8.11 (s, 1H); ^{13}C-NMR ($CDCl_3$, 75 MHz): δ = -0.3, 21.1, 81.8, 102.6, 110.5, 116.0, 117.8, 124.1, 125.6, 126.5, 128.5, 128.9, 135.5, 151.5, 152.1, 186.8; MS (ESI): *m/z* 513 $(M+H)^+$.

H^1-NMR



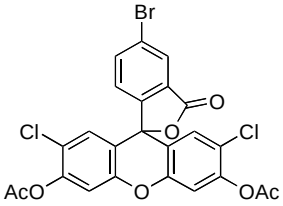


***N*-(9-(4-((3-carboxy-4-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)phenyl)ethynyl)phenyl)-6-(dimethylamino)-3*H*-xanthen-3-ylidene)-*N*-methylmethanaminium**.

A mixture of 8 (88 mg, 0.2 mmol), 2 (92 mg, 0.2 mmol), tetrakis(triphenylphosphine) palladium (11 mg, 5 % mol), copper(I) iodide (2 mg ,5 % mol), and triethylamine (304 mg, 3.0 mmol) in 0.5 mL DMF was microwave heated in a sealed tube at 120 ^{o}C for 15 min. in constant temperature mode. After cooling, 166 mg of potassium carbonate (1.2 mmol) were added to the solution and stirred at 25 ^{o}C for 6 h. The solution was then poured into 20 mL of methanol, and acidified to pH » 2 with concentrated HCl. To this solution, 60 mL of 25 % *i*PrOH / $CHCl_3$ were added and washed with 3 x 100 mL water, the organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. To the dark purple residue, 20 mL of a 1:1 CH_2Cl_2/H_2O solution were added, and 106 mg (0.12 mmol) of sodium tetra(3,5-trifluoromethylbenzene)boronate (NaBARF) were added and the mixture stirred at 25 ^{o}C for 12 h. The mixture was once again extracted with 25 % *i*PrOH/$CHCl_3$ and the organic extract was washed with H_2O. The organic layer was dried over anhydrous Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography (10 % MeOH/CH_2Cl_2) to give 62 mg (38 % yield) of 9 as a purple solid. ^{1}H NMR (DMSO-d_6 500 Mhz): δ = 10.16 (s, 2H), 8.19 (s, 1H), 7.99 (d, J = 8.0 Hz, 1H), 7.93 (d, J = 8.5 Hz,

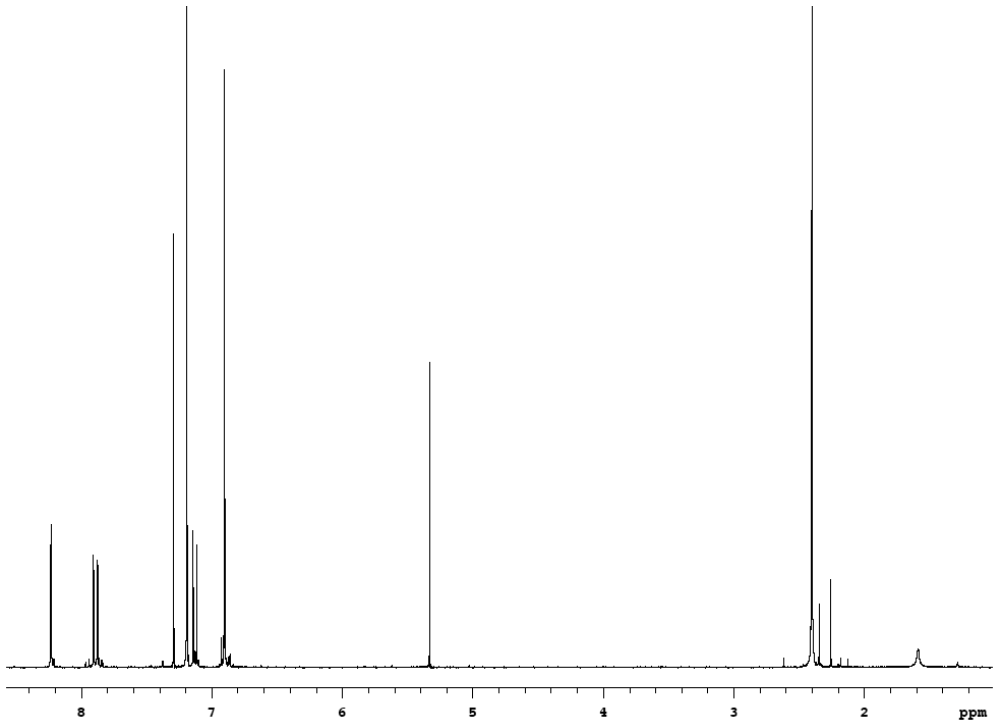
2H), 7.72 (s, 3H), 7.62 (s, 6H), 7.38 (d, *J* = 8.0, 1H), 7.32 (d, *J* = 9.5 Hz, 2H), 7.17 (dd, *J* = 9.5, 2.5 Hz, 2H), 6.99 (d, *J* = 2.5 Hz, 2H), 6.70 (d, *J* = 2.5 Hz, 2H), 6.65 (d, *J* = 9.0 Hz, 2H), 6.58 (dd, *J* = 9.0, 2.5 Hz, 2H), 3.30 (s, 12H); ^{13}C NMR (DMSO-d_6, 125 Mhz): δ = HRMS (ESI) *m/z* (M^+) calcd for $C_{45}H_{33}N_2O_6^+$ 697.2339 found 697.2363.

CHAPTER V

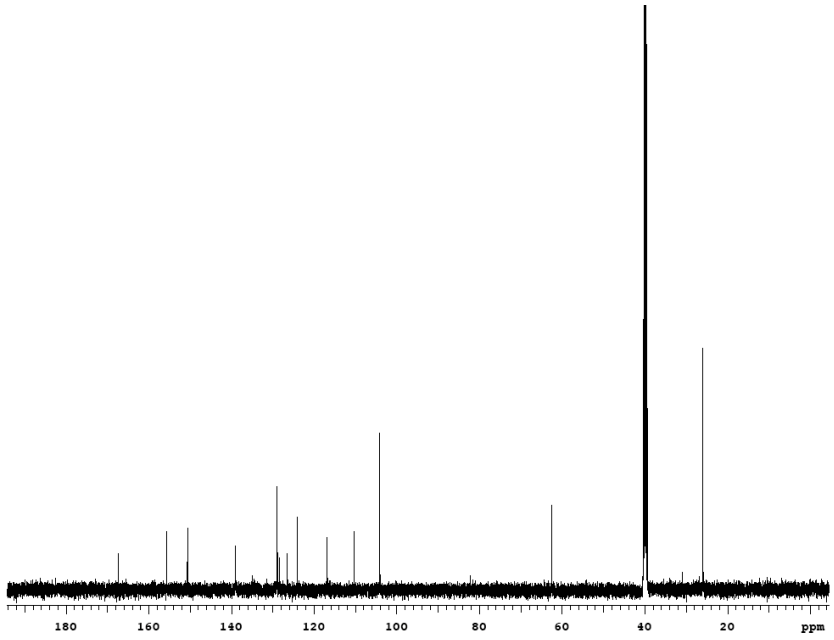


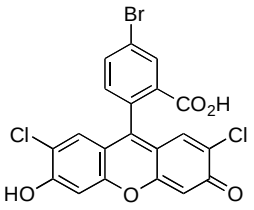
5-bromo-2',7'-dichlorofluorescein-3',6'-diacetate. 50.0 g of 4-chlororesorcinol (346 mmol) and 35.7 g of 4-bromophthalic anhydride (157.3 mmol) were heated to 200 °C for 24 h. It was then allowed to cool to room temperature before venting the flask. A dark orange/brown solid was obtained and dissolved in sufficient amount of 1 N NaOH (ca. 1.5 L). This solution was filtered and the resulting filtrate carefully acidified to pH 2.5 using concentrated HCl in an ice bath. An orange precipitate was obtained, this mixture was allowed to stand in an ice bath for about 1 h before filtrating. The orange solid was collected by filtration, and dissolved in 25 % ^{i}PrOH/$CHCl_3$, dried over Na_2SO_4, filtered and concentrated in vacuo. The orange solid obtained was dried under vacuum and 300 mL of acetic anhydride was then added, and the solution refluxed at 150 °C for 4 h. It was then concentrated in vacuo, and ca. 150 ml fresh acetic anhydride added. The solution was heated then allowed to crystallize. This recrystallization was repeated at least six more times to increase the purity of the desired isomer obtaining 35.5 g (40 %) of the desired product as a white crystalline solid. mp = 235 - 237 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.21 (dd, *J* = 1.8, 0.7 Hz, 1H), 7.88 (dd, *J* = 7.9, 1.9 Hz, 1H), 7.17 (s, 2H), 7.12 (dd, *J* = 8.2, 0.7 Hz, 1H), 6.80 (2, 2H), 2.38 (s, 6H); ^{13}C NMR (125 MHz, DMSO-*d*6): δ = 168.0, 166.6, 150.0, 149.4, 148.4, 138.8, 129.0, 128.3, 128.0, 126.2, 124.0, 122.0, 117.2, 113.2, 80.0, 20.4; ES HRMS calcd for $C_{24}H_{13}BrCl_2O_7$ $(M+Li)^+$:568.9382; found: 568.9344. TLC (60% EtOAc/Hexanes) R_f = 0.80

H^1-NMR



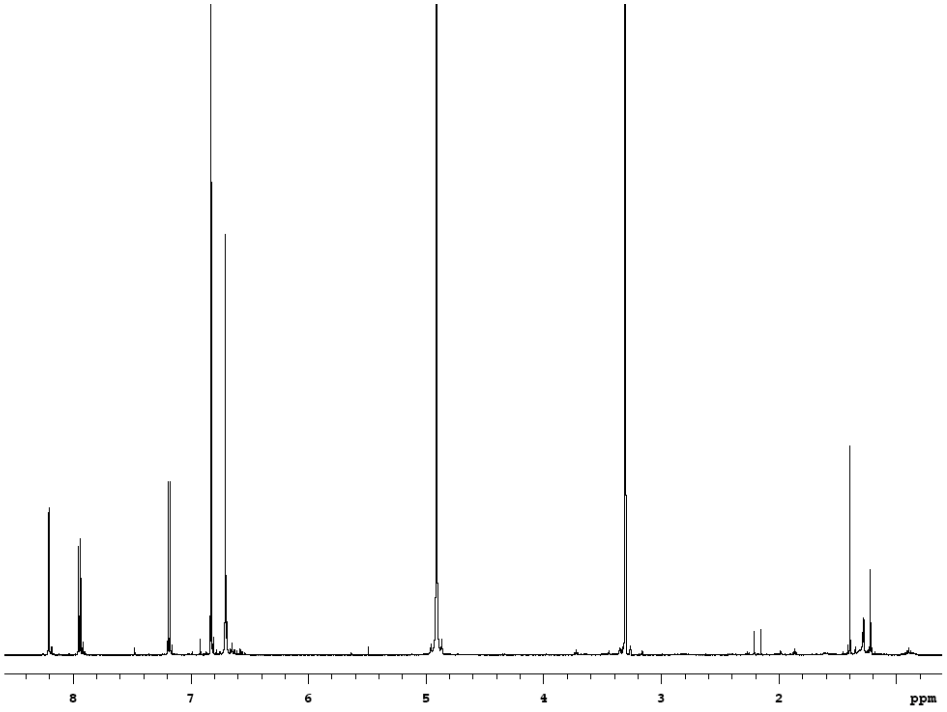
C^{13}-NMR



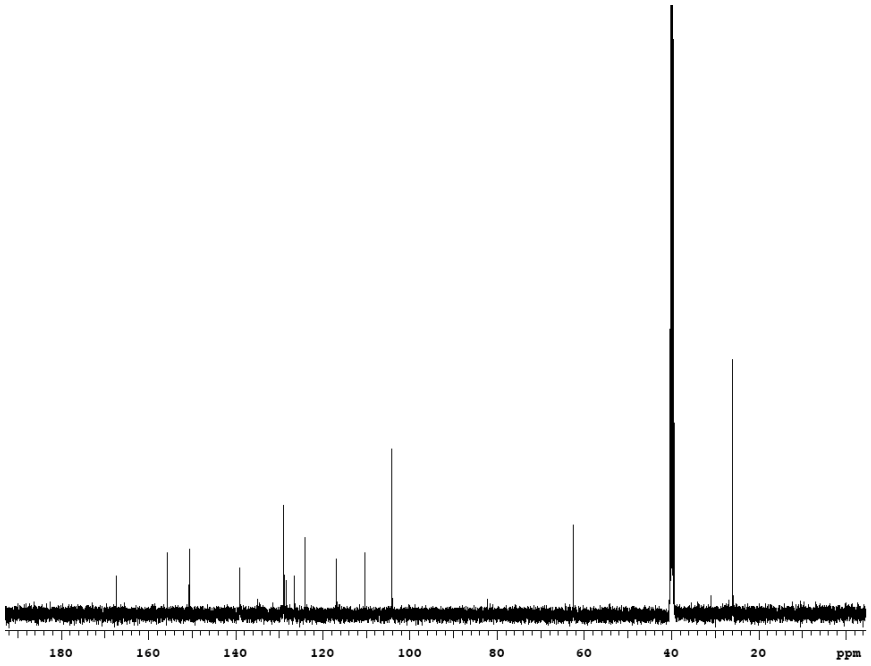


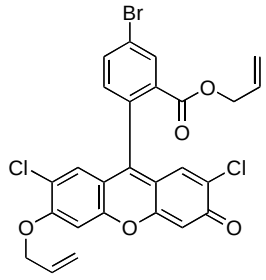
5-bromo-2',7'-dichlorofluorescein. 10 g of 5-bromo-2',7'-dichlorofluorescein diacetate (17.7 mmol) and 9.8 g of potassium carbonate (70.9 mmol) were combined and stirred in a 1:1 mixture of methanol/THF at 25 °C for 4 h. The dark brown/orange solution obtained was filtered and concentrated in vacuo. The obtained solid was dissolved in water and carefully acidified to pH 2.5 using 1 N HCl. The precipitate obtained was filtered, dissolved in 25 % ^{i}PrOH/$CHCl_3$, washed with 2 x 200 mL brine, 2 x 200 mL H_2O, dried over Na_2SO_4, filtered and concentrated in vacuo obtaining 8.5 g (>99%) of the desired product as a brick red color solid. ^{1}H NMR (500 MHz, CD_3OD): δ = 8.21 (d, *J* = 1.9 Hz, 1H), 7.95 (dd, *J* = 8.0, 1.9 Hz, 1H), 7.19 (d, *J* = 8.0 Hz, 1H), 6.83 (s, 2H), 6.71 (s, 2H); ^{13}C NMR (125 MHz, DMSO-*d*6): δ = 166.9, 155.3, 150.4, 150.1, 138.6, 128.5, 128.4, 127.9, 126.0, 123.5, 116.4, 109.9, 103.6, 62.1; ES HRMS calcd for $C_{20}H_9BrCl_2O_5$ $(M-H)^-$: 476.8932; found: 476.8940. TLC (10 % MeOH/CH_2Cl_2) R_f = 0.46

H^1 NMR



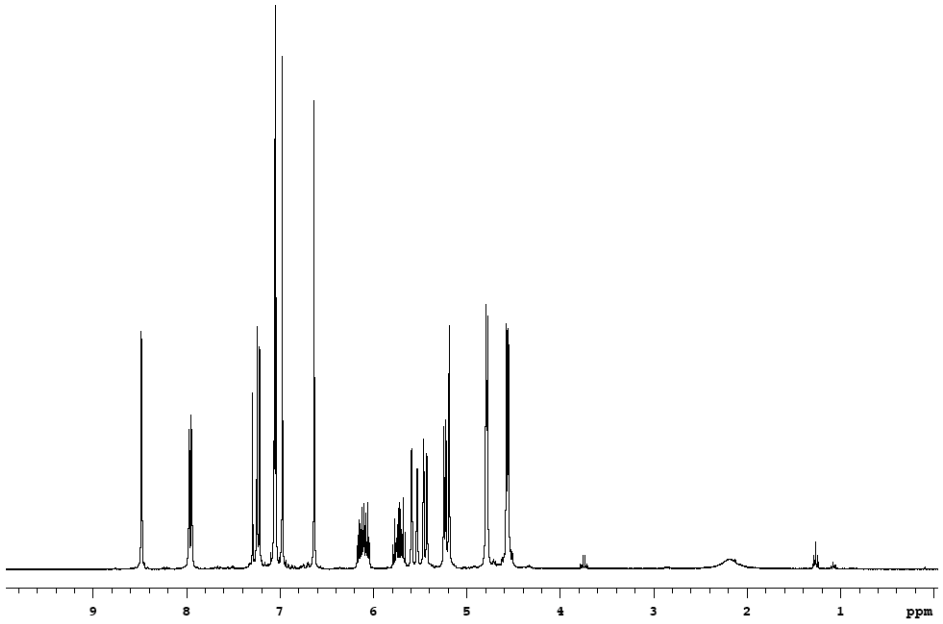
C^{13}-NMR



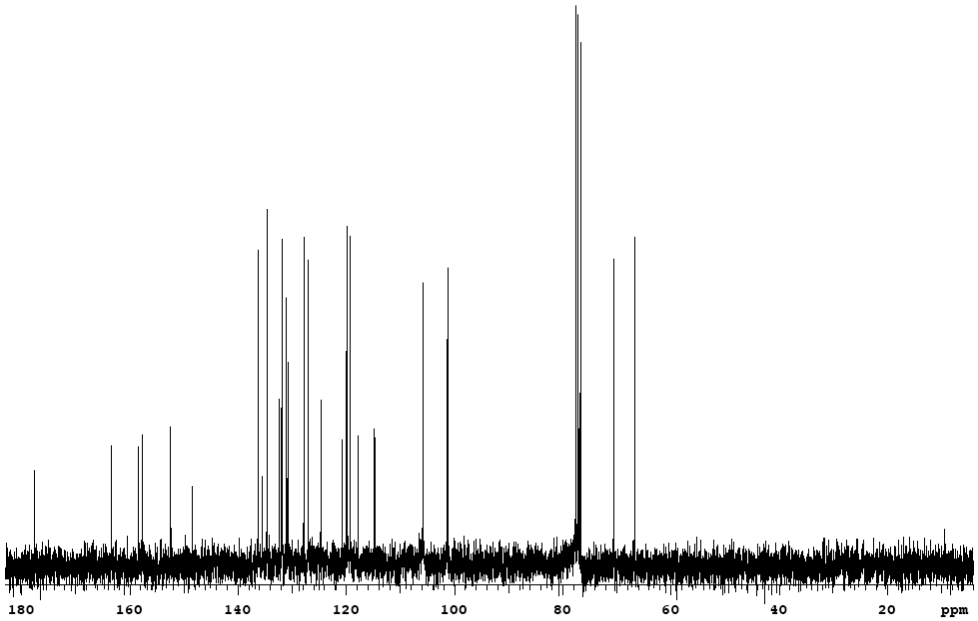


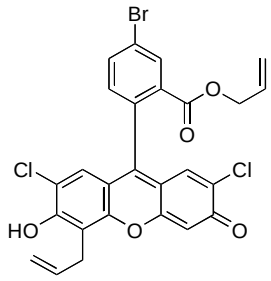
5-bromodichlorofluorescein diallyl. 10.5 g (21.9 mmol) of 5-bromo-2',7'-dichlorofluorescein and 12.09 g (87.5 mmol) potassium carbonate were combined in 150 ml DMF and stirred at 25 °C for 10 min. To this solution, 7.6 ml (87.5 mmol) allyl bromide was added dropwise and stirred for 24 h. The reaction mixture was cooled to 0 °C in an ice bath and added to 500 ml ice cold water. A bright orange precipitate was obtained. It was filtered and dried under vacuum. The product was further purified by recrystallization from EtOH obtaining 10.2 g (85 %) of a bright orange solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.46 (d, *J* = 2.0 Hz, 1H), 7.95 (dd, *J* = 8.2, 2.0 Hz, 1H), 7.22 (d, *J* = 8.0 Hz, 1H), 7.04 (s, 1H), 7.02 (s, 1H), 6.95 (s, 1H), 6.61 (s, 1H), 6.15 (m, 1H), 5.77 (m, 1H), 5.57 (dd, *J* = 17.3, 1.3 Hz, 1H), 5.44 (dd, *J* = 10.5, 1.2 Hz, 1H), 5.22 (dd, *J* = 7.1 Hz, 1.1 Hz, 1H), 5.16 (s, 1H), 4.77 (d, *J* = 5.4 Hz, 2H), 4.6 (d, *J* = 6.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 177.8, 163.6, 158.7, 157.9, 152.7, 148.7, 136.6, 135.8, 134.9, 132.6, 132.2, 132.1, 131.3, 131.0, 128.1, 127.3, 124.9, 121.0, 120.2, 119.5, 118.1, 115.0, 106.1, 101.5; ES HRMS calcd for $C_{26}H_{17}BrCl_2O_5$ $(M+Li)^+$: 564.9796; found: 564.9791; TLC (10 % MeOH/CH_2Cl_2) R_f = 0.66

H^1-NMR



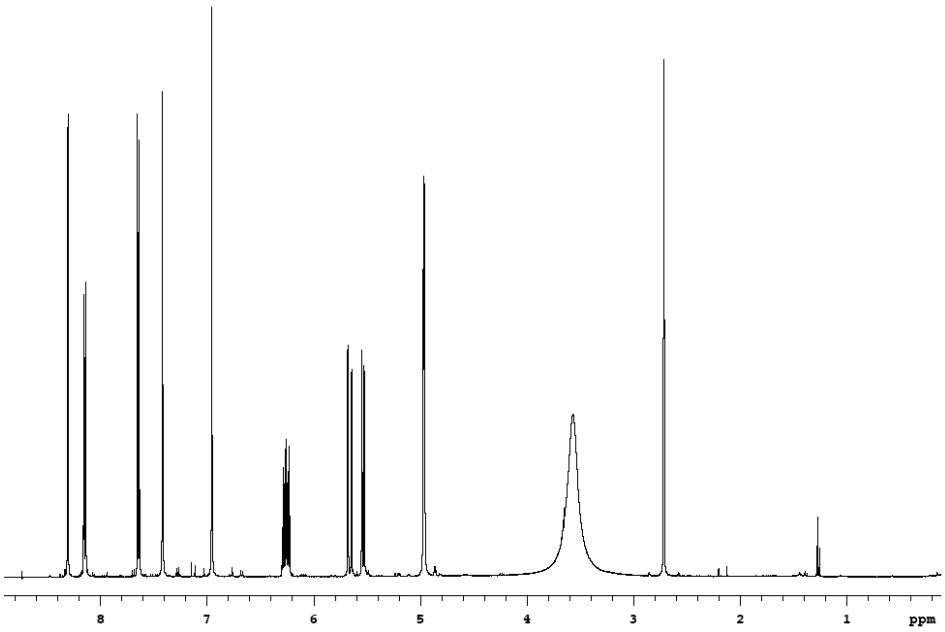
C^{13}-NMR



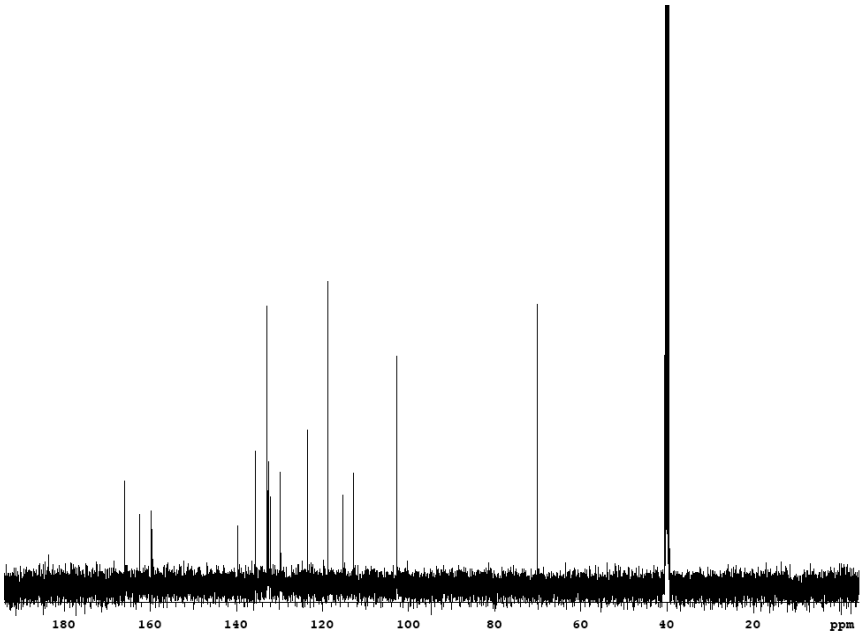


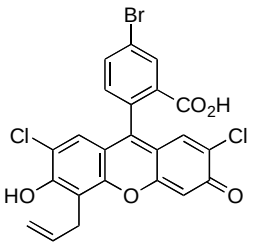
5-bromo-4'-allyl-2',7'dichlorofluorescein-3-allyl ester. 8.2 g (15.1 mmol) of 5-bromodichlorofluorescein diallyl was heated to 200 °C in diphenyl ether and stirred for 7 h. It was then allowed to cool to room temperature. The resulting solution was passed through a silica gel column eluting with 100 % CH_2Cl_2 to remove diphenyl ether. The desired product was eluted with 10 % MeOH/CH_2Cl_2 obtaining 7.8 g (95 %) of desired product as a red/orange solid. ^{1}H NMR (300 MHz, CD_3OD): δ = 8.46 (s 1H), 8.07 (dd, *J* =, 1H), 7.42 (d, *J* =, 1H), 7.10 (s, 1H) 7.04 (s, 1H), 7.03 (s, 1H), 5.99 (m, 1H), 5.59 (m, 1H), ^{13}C NMR (125 MHz, DMSO-d_6): δ = 163.3, 148.6, 136.2, 135.1, 133.5, 132.7, 131.4, 128.0, 126.5, 123.6, 118.6, 115.4, 114.5, 103.2, 65.8, 27.2; ES HRMS calcd for $C_{26}H_{17}BrCl_2O_5$ $(M-H)^-$: 556.9558; found: 556.9547; TLC (10 % MeOH/CH_2Cl_2) R_f = 0.65

H^1-NMR



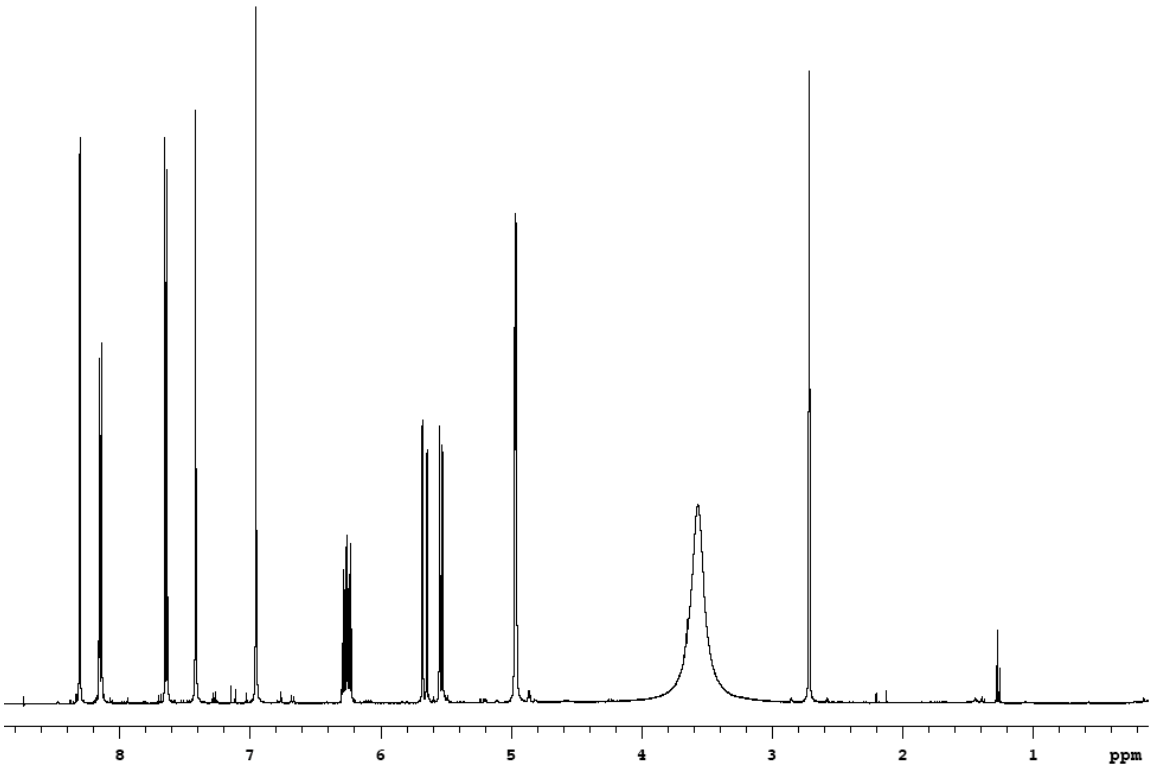
C^{13}-NMR

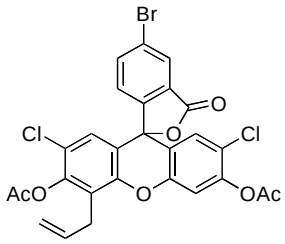




5-bromo-4'-allyl-2',7'-dichlorofluorescein. 8.2 g (15.1 mmol) of 5-bromo-4'-allyl-2',7'dichlorofluorescein-3-allyl ester and 3.2 g (75.5 mmol) of lithium hydroxide monohydrate in 300 ml dioxane/water (10:3) was refluxed at 100 °C for 3 h. The solution was allowed to cool for 12 h. It was then concentrated in vacuo to almost dryness. dH_2O (400 ml) was added and stirred, acidifed with 2N HCl until a precipitate formed and stirred for another 10 min. It was filtered, and the solid dissolved in 200 ml 25% $^{i}PrOH/CHCl_3$, washed with 2 x 200 ml 0.1 N HCl and 2 x 100 ml dH_2O. The combined aqueous layers were extracted with 1 x 200 ml 25% $^{i}PrOH/CHCl_3$ and added to previous organic. Dried over Na_2SO_4, filtered and concentrated in vacuo. Obtained 7.8 g (>99%) of the desired product as a red solid. ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.09 (d, *J* = 1.9 Hz, 1H), 7.93 (dd, *J* = 8.2, 2.1 Hz, 1H), 7.42 (d, *J* = 8.2 Hz, 1H), 7.20 (s, 1H), 6.74 (s, 1H), 6.04 (m, 1H), 5.44 (dd, *J* = 17.2, 1.8 Hz, 1H), 5.32 (dd, *J* = 10.7, 1.6 Hz, 1H), 4.75 (d, *J* = 5, 2H); ^{13}C NMR (125 MHz, DMSO-d_6): δ = 176.7, 163.8, 161.3, 157.5, 155.55=, 154.31=, 135.2, 132.9, 131.8, 131.1, 130.9, 130.0, 124.8, 123.5, 121.1, 120.1, 119.2, 116.6, 116.3, 116.0, 114.5, 103.3, 29.2; ES HRMS calcd for $C_{23}H_{13}BrCl_2O_5$ $(M-H)^-$: 516.9245; found: 516.9240; TLC (10 % MeOH/CH_2Cl_2) R_f = 0.60.

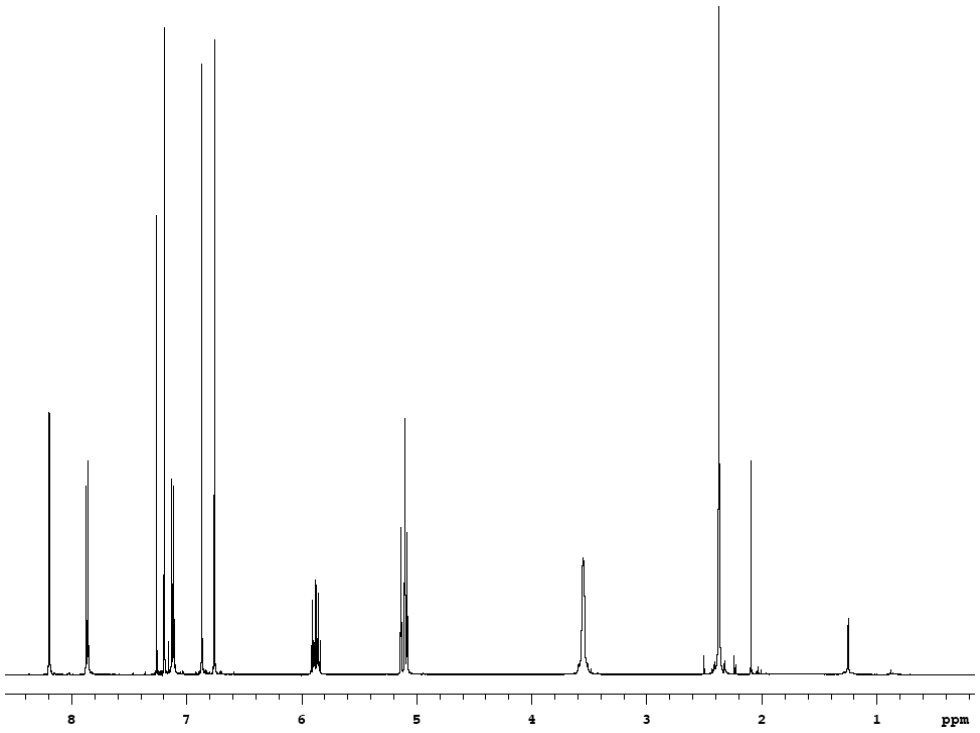
H^1-NMR



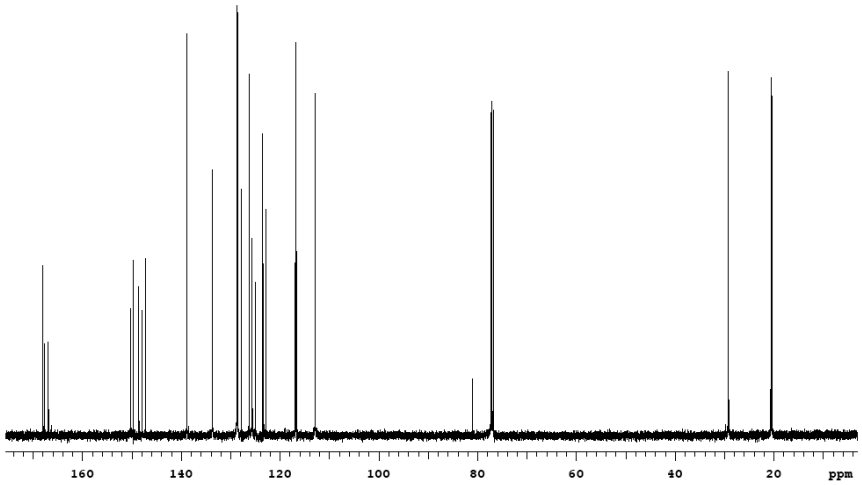


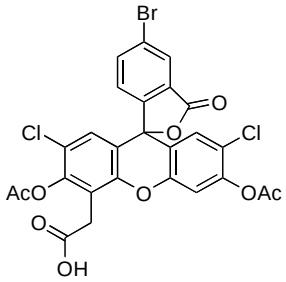
5-bromo-4'-allyl-2',7'-dichlorofluorescein-3',6'-diacetate. 7.8 g (15.1 mmol) of 5-bromodichlorofluorescein alllyl was refluxed in acetic anhydride at 150 °C for 4 h. It was then concentrated in vacuo. Recrystallized from acetic anhydride to afford 7.5 g (80 %) of the desired product as a crystalline white solid. mp: 200.1 - 2002.4 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.21 (s, 1H), 7.87 (dd, *J* = 8.3, 1.7 Hz, 1H), 7.21 (s, 1H), 7.13 (d, *J* = 8.3, 1H), 6.88 (s, 1H), 6.77 (s, 1H), 5.89 (m, 1H), 5.15 (d, *J* = 1.6 Hz, 1H), 5.12 (d, *J* = 1.6 Hz, 1H), 3.56 (s, 1H), 2.38 (s, 6H); ^{13} C NMR (125 MHz, $CDCl_3$): δ = 168.3, 168.0, 167.2, 150.6, 150.0, 148.9, 148.3, 147.6, 139.2, 134.0, 129.1, 128.9, 128.1, 126.6, 126.0, 125.2, 123.9, 123.6, 123.2, 117.3, 117.1, 117.0, 113.2, 81.3, 29.5, 20.9, 20.8; ES HRMS calcd for $C_{27}H_{17}BrCl_2O_7$ $(M+H)^+$: 602.9613; found: 602.9603; TLC (60 % EtOAc/Hexanes) R_f = 0.78

H^1-NMR



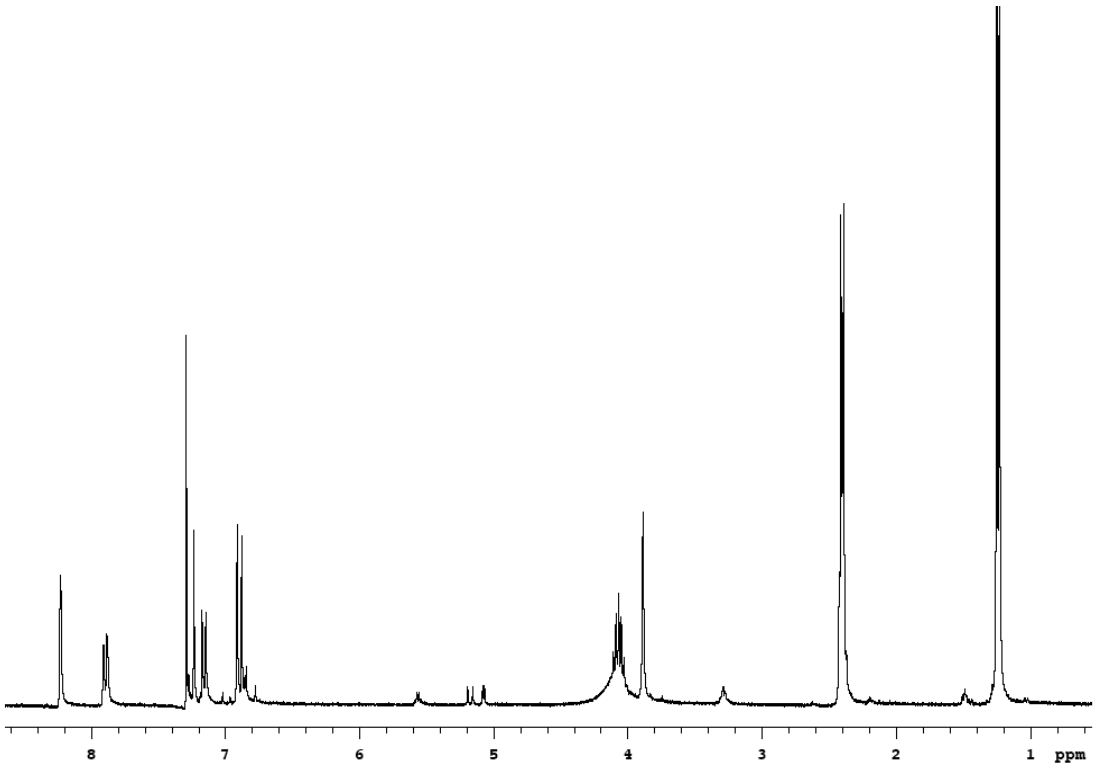
C^{13}-NMR

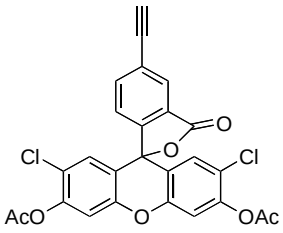




5-bromo-2',7'-dichloro-4'-carboxyfluorescein-3',6'-diacetate. 0.5 g (0.8 mmol) of 5-bromodichlorofluorescein diacetate allyl dissolved in 50 ml acetone was cooled to -78 °C. The solution was flushed with oxygen for 10 min. then ozonolysed for 45 min. The solution was quenched with 5 eq. of Jone's reagent ($K_2Cr_2O_6$; H_2SO_4 in H_2O) and allowed to slowly warm to 25 °C for 12 h. The solution was then filtered, and the resulting solution concentrated in vacuo until most of the acetone had evaporated. A yellow precipitate was formed which was collected by filtration. Washed with 2 x 200 ml H_2O and dried under high vacuum. ^{1}H NMR (500 MHz, CD_3OD): δ = 8.22 (s, 1H), 7.93 (dd, *J* = 2.9, 8.3, 1H), 7.21 (d, *J* = 8.3, 1H), 6.87 (s, 1H), 6.75 (s, 2H), 3.73 (s, 2H);

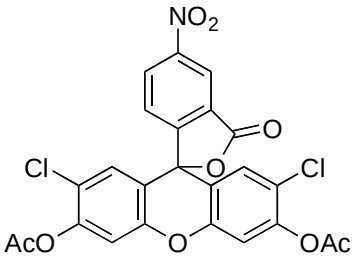
H^1-NMR





2',7'-dichloro-5-ethynyl-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diethanoate 2',7'-dichloro-3-oxo-5-((trimethylsilyl)ethynyl)-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl diethanoate (290 mg, 0.499 mmol) was dissolved in 10 mL CH_2Cl_2. Then the mixture was cooled to -78 ^oC for 15 min. To the solution was added tetrabutyl ammonium fluoride (TBAF, 1.50mL, 1.0M) dropwise. Then the solution was slowly warmed up to room temperature. The reaction solvent was removed under reduced pressure and the crude product was purified by flash column eluting with 66% hexane:ethyl acetate to give the desired product as a white yellow solid (125 mg, 49%). ^1H NMR (500 MHz, $CDCl_3$): δ = 8.15 (m, 1H), 7.81 (dd, *J* = 8.0, 1.5 Hz, 2H), 7.17(dd, *J* = 8.0, 1.0 Hz, 1H), 7.15 (s, 2H), 6.86 (s, 2H), 3.27 (s, 1H), 2.36 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 167.9, 167.5, 151.4, 149.6, 148.5, 139.2, 129.1, 128.8, 126.0, 125.2, 124.1, 122.7, 117.0, 112.8, 81.2, 80.5, 80.4, 20.5; MALDI MS calcd for $C_{26}H_{15}Cl_2O_7^+$ $(M+H)^+$: 509.0117; found: 509.0167; TLC (50 % EtOAc-Hexane): Rf = 0.50.

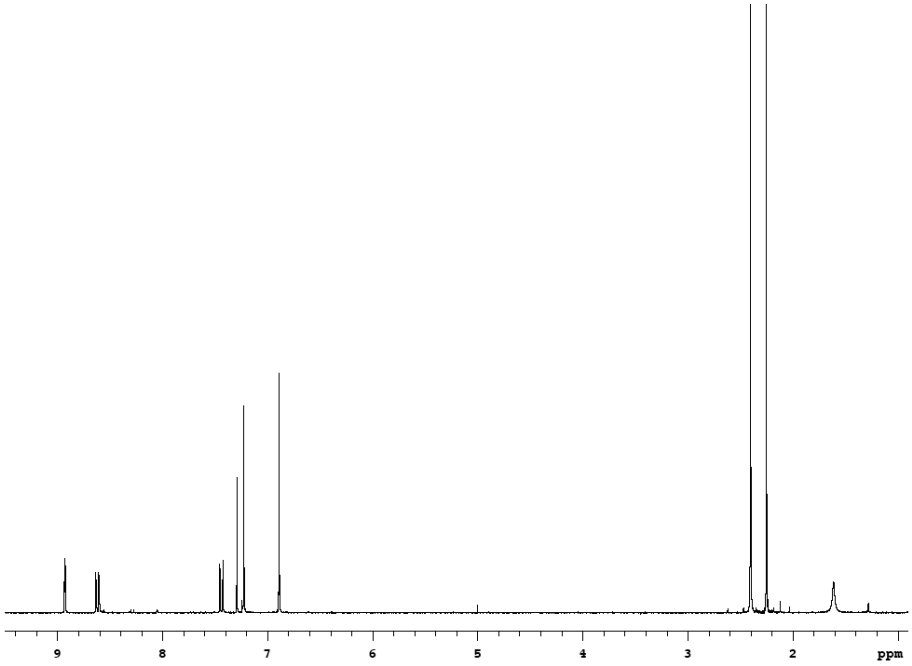
CHAPTER VI



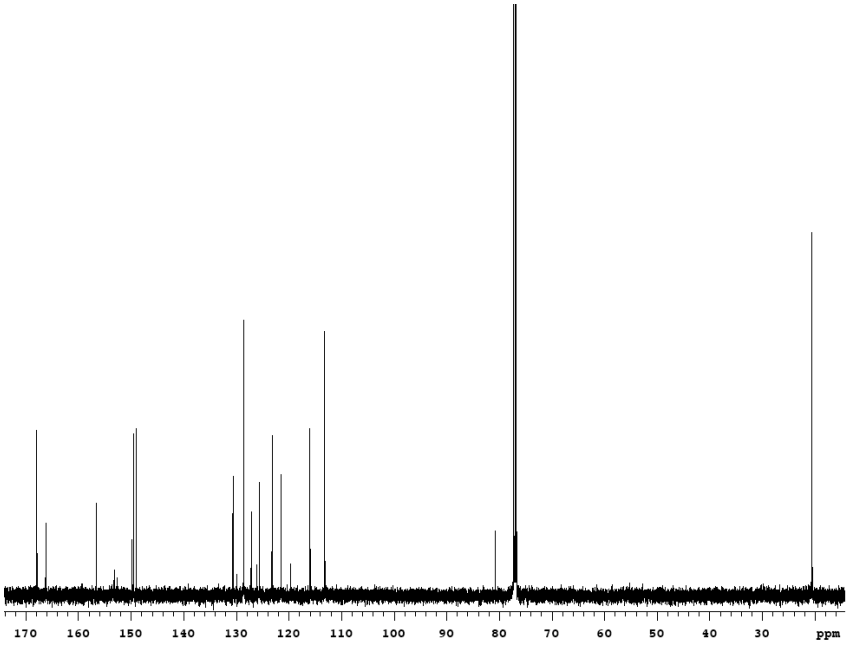
5-nitro-2',7'-dichlorofluorescein-3',6'-diacetate.

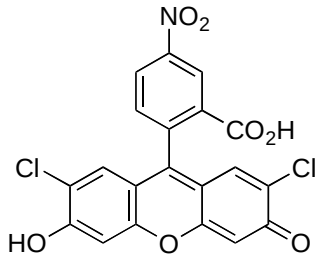
11.6 g (60.4 mmol) of 4-nitrophthalic anhydride and 17.46 g (120.8 mmol) of 4-chlororesorcinol were heated to 200 °C for 24 h. It was then allowed to cool to room temperature before venting the flask. A dark orange/brown solid was obtained and dissolved in sufficient amount of 1 N NaOH (ca. 1.0 L). This solution was filtered and the resulting filtrate carefully acidified to pH 2.5 using concentrated HCl in an ice bath. An orange precipitate was obtained, this mixture was allowed to stand in an ice bath for about 1 h before filtrating. The orange solid was collected by filtration, and dissolved in 25 % ^{i}PrOH/$CHCl_3$, dried over Na_2SO_4, filtered and concentrated in vacuo. The orange solid obtained was dried under vacuum and 300 mL of acetic anhydride was then added, and the solution refluxed at 150 °C for 4 h. It was then concentrated in vacuo, and ca. 150 ml fresh acetic anhydride added. The solution was heated then allowed to crystallize. This recrystallization was repeated at least six more times to increase the purity of the desired isomer obtaining 16.1 g (50 %) of the desired product as a white crystalline solid. M.p. = compound decomposes at about 210 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.91 (dd, *J* = 2.2, 0.6 Hz, 1H), 8.6 (dd, *J* = 8.3, 2.2 Hz, 1H), 7.42 (d, *J* = 9.1 Hz, 1H), 7.21 (s, 2H), 6.87 (s, 2H), 2.23 (s, 6H); ^{13}C NMR (126 MH, $CDCl_3$): δ = 168.0, 166.2, 156.8, 149.6, 149.1, 130.8, 128.7, 128.7, 127.3, 125.7, 123.3, 121.6, 116.1, 113.3, 80.9, 20.8. ES HRMS calcd for $C_{24}H_{13}Cl_2NO_9$ $(M + Li)^+$: 536.0127; found: 536.0121. TLC (60 % EtOAc/Hexanes): R_f = 0.73; Anal. Calcd for $C_{24}H_{13}Cl_2NO_9$: C, 54.36; H, 2.47; O, 27.16. Found C, 54.29; H, 2.53; O, 27.41.

H^1-NMR



C^{13}-NMR

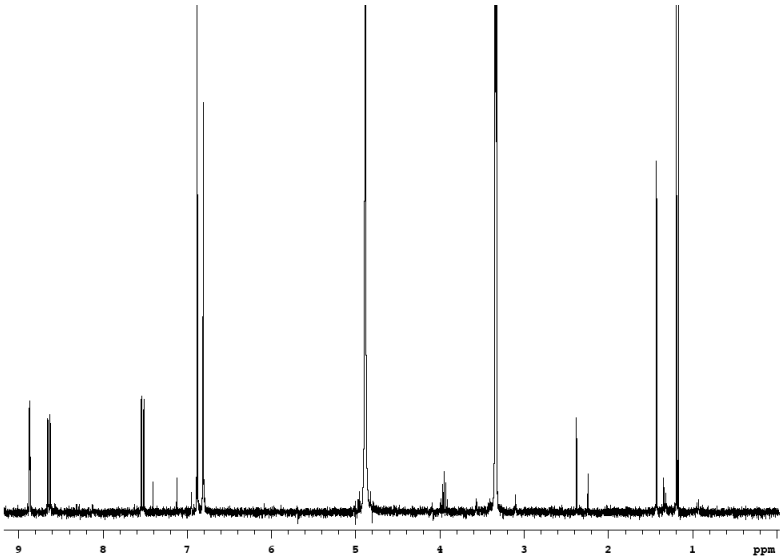




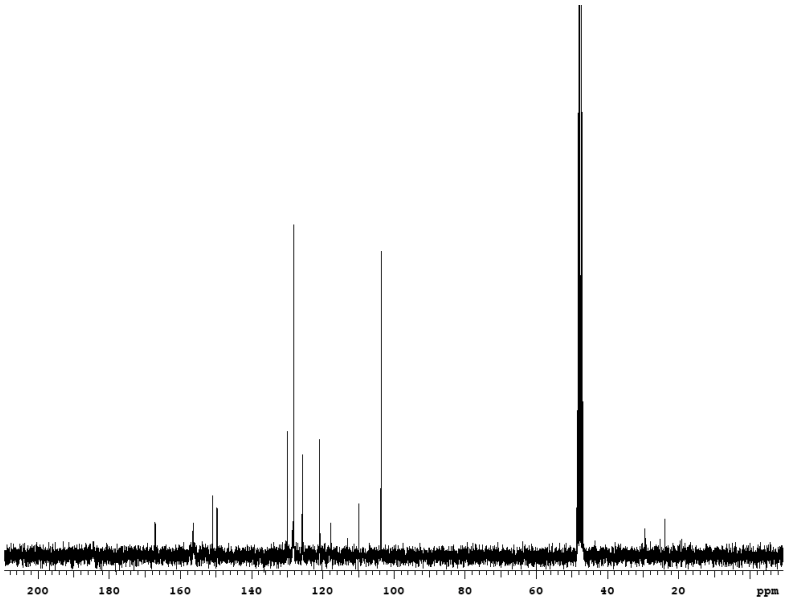
5-nitro-2',7'-dichlorofluorescein.

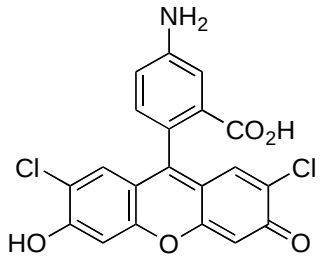
2.4 g (4.52 mmol) of 5-nitro-2',7'-dichlorofluorescein diacetate and 2.5 g (18.1 mmol) of potassium carbonate were combined and stirred in a 1:1 mixture of methanol/THF at 25 °C for 4 h. The dark brown/orange solution obtained was filtered and concentrated in vacuo. The obtained solid was dissolved in water and carefully acidified to pH 2.5 using 1 N HCl. The precipitate obtained was filtered, dissolved in 25 % ^{i}PrOH/$CHCl_3$, washed with 2 x 200 mL brine, 2 x 200 mL H_2O, dried over Na_2SO_4, filtered and concentrated in vacuo obtaining 1.84 g (90 %) of the desired product as a brick red color solid. M.p. = compound decomposes; ^{1}H NMR (500 MHz, CD_3OD): δ = 8.85 (d, *J* = 2.2 Hz, 1H), 8.62 (dd, *J* = 8.4, 2.3 Hz, 1H), 7.51 (d, *J* = 8.4 Hz, 1H), 6.86 (s, 2H), 6.78 (s, 2H); ^{13}C NMR (75 MHz, CD_3OD): δ = 168.6, 157.9, 152.5, 151.2, 131.4, 129.7, 127.2, 122.3, 119.2, 114.5, 111.3, 105.1; ES HRMS calcd for $C_{20}H_9Cl_2NO_7$ $(M - H)^-$: 443.9756; found: 443.9837; TLC (10 % MeOH/CH_2Cl_2): R_f = 0.59; Anal. Calcd for $C_{20}H_{11}Cl_2NO_7$: C, 53.59; H, 2.47; N, 3.13. Found: C, 53.58; H, 2.74; N, 2.87.

H^1-NMR



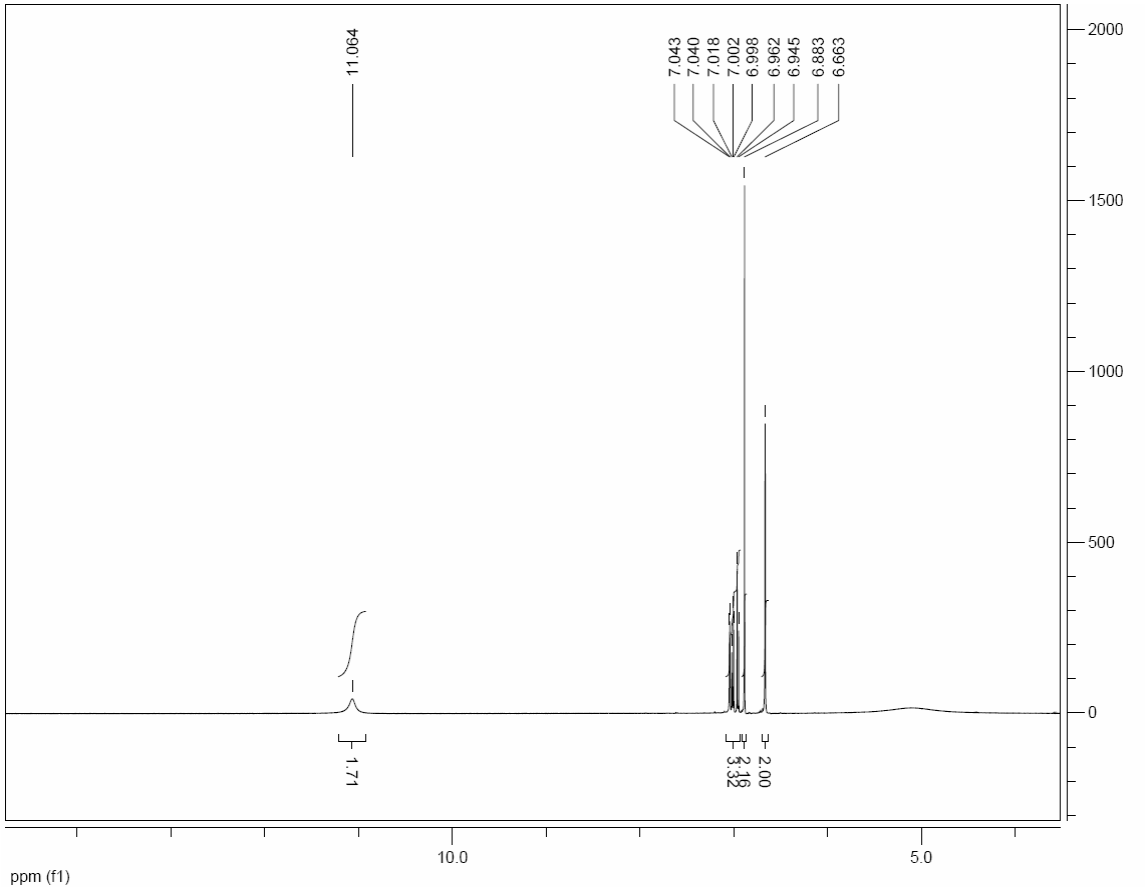
C^{13}-NMR





5-amino-2',7'-dichlorofluorescein.

1.0 g (1.88 mmol) of 5-nitro-2',7'-dichlorofluorescein diacetate was dissolved in 20 ml of MeOH:THF (5:1) mixture. To this solution, 5 mL of 2N NH_3 in MeOH was added and allowed to stir at 25 °C for 2 h, after which time TLC showed completion of the reaction. It was then concentrated under vacuum. The obtained residue was dissolved in 15 mL of 5 % H_2O/MeOH. To this solution, 110 mg (1.88 mmol) Raney Nickel was added and stirred under hydrogen (1 atm) for 1.5 h. (It is important to stop the reaction immediately after starting material has disappeared). The reaction mixture was filtered through celite and concentrated under vacuum. It was further purified by silica gel column chromatography (1:1 MeOH/CH_2Cl_2), and the obtained dark red solid recrystallized from approximate 1:1 mixture of EtOH/^{i}PrOH obtaining 460 mg (59 %) of fine red crystals. M.p.= decomposes at about 125 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 11.06 (s, 1H), 7.04 (d, J = 1.8 Hz, 1H), 7.00 (dd, J = 2.1 Hz; 8.2 Hz, 1H), 6.95 (d, J = 8.2 Hz, 1H), 6.88 (m, 2H), 6.66 (m, 2H), 5.19 (br, 2H); ^{13}C NMR (126 MHz, DMSO-d_6): δ = 169.6, 155.6, 155.6, 151.0, 150.9, 139.6, 139.5, 128.8, 127.9, 124.9, 124.9, 122.9, 116.7, 112.2, 107.5, 104.3, 95.0, 82.0, 56.7, 19.3; TOF MS calcd for $C_{20}H_{11}Cl_2NO_5$: 416.00; found: 416.99 (M+H); TLC (30 % MeOH/CH_2Cl_2): R_f = 0.63. Anal. Calcd for $C_{20}H_{11}Cl_2NO_5$: C, 57.71; H, 2.66; N, 3.37. Found: C, 57.78; H, 3.11; N, 3.24.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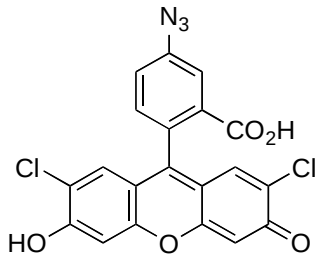
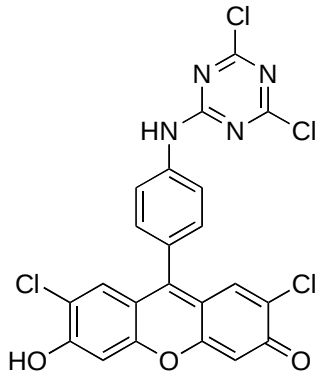
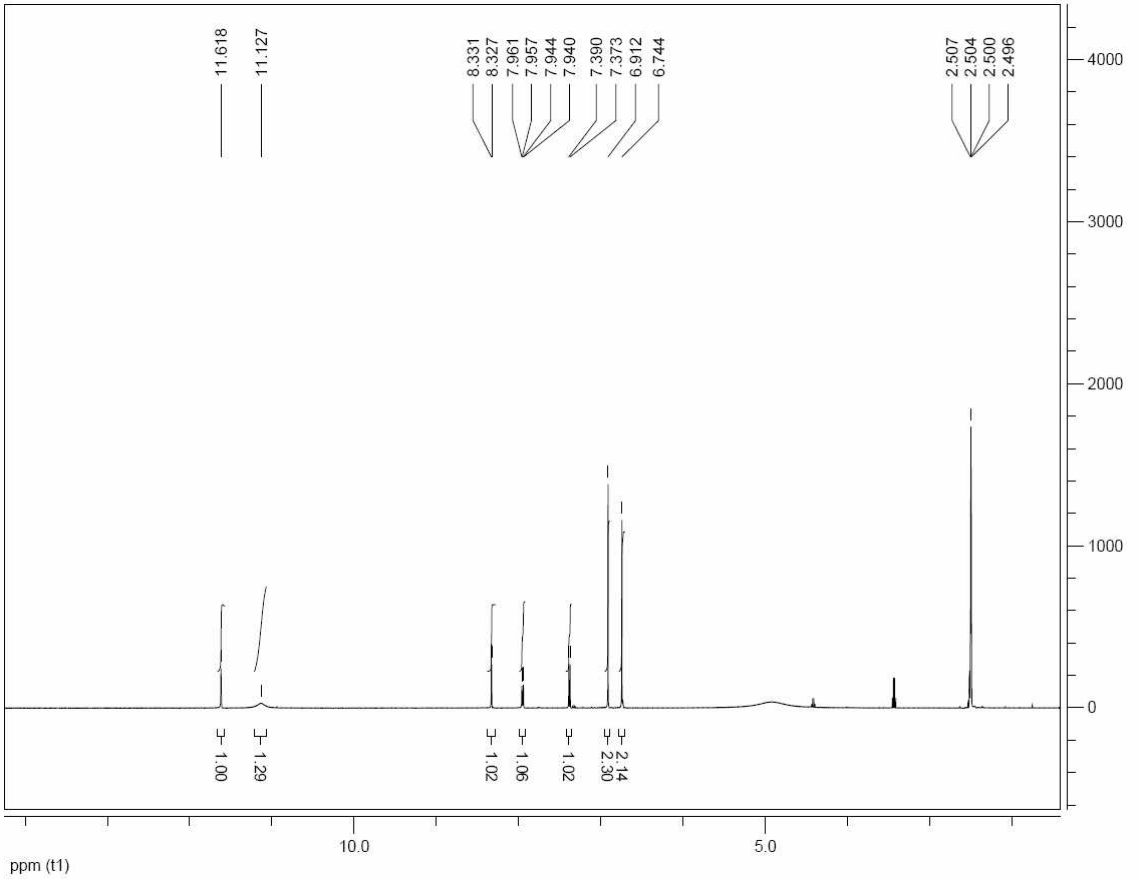


5-azido-2', 7'-dichlorofluorescein (4). 15 mg (0.036 mmol) of 5-amino-2', 7'-dichlorofluorescein was dissolved in 2 mL of H_2O:HCl (1:1) and 1 mL absolute ethanol, sonicated until dissolved and cooled to 0 °C in an ice bath. To this solution, 5 mg (0.072 mmol) of $NaNO_2$ dissolved in 0.5 mL of H_2O was added dropwise to the previous solution. The reaction was placed under nitrogen and allowed to stir for 1 h until TLC indicated the reaction was complete. At this point, 3 mg (0.043 mmol) of NaN_3 dissolved in 0.1 mL H_2O was added dropwise and the solution was allowed to warm slowly to 25 °C while stirring for 6 h. It was then washed with 2 X 50 mL brine solution, dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. It was further purified by a silica gel column chromatography (10 % MeOH/CH_2Cl_2) obtaining 8 mg (47 %) of an orange solid. R_f = 0.59 (10 % MeOH/CH_2Cl_2); M.p: compound decomposes ca. 159 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.69 (d, *J* = 1.9 Hz, 1H), 7.33 (dd, *J* = 8.3, 2.2 Hz, 1H), 7.14 (d, *J* = 8.3 Hz, 1H), 6.94 (s, 2H), 6.72 (s, 2H); ^{13}C NMR (126 MH, 10 % CD_3OD/ $CDCl_3$): δ= 168.5, 151.3, 143.1, 128.7, 128.5, 126.5, 126.1, 115.3, 110.8, 104.0, 90.9; HRMS-ES: *m/z* $(M - H)^-$ calcd for $C_{20}H_9Cl_2N_3O_5$: 439.9846; found: 439.9841; Anal. Calcd for $C_{20}H_9Cl_2N_3O_5$: C, 54.32; H, 2.05; N, 9.50. Found C, 51.50; H, 3.33; N, 7.08



5-(4,6-dichloro-1,3,5-triazin-2-ylamino)-2-(2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid.

A solution of 5-amino-2',7'-dichlorofluorescein 900 mg (2.16 mmol) in methanol (25 mL) was added dropwise into a solution of cyanuric chloride (518 mg; 2.81 mmol) in CH_2Cl_2 at 0 °C. The reaction mixture was stirred at 0 °C for 2 h. After that conc. HCl (0.7 mL) was added and reaction stirred for 1h at 0 °C. The reaction mixture was concentrated under vacuum. It was further purified by silica gel column chromatography (d = 2 cm, h = 15 cm, 1:9 MeOH/CH_2Cl_2 + 0.1% AcOH), the product was obtained as dark yellow solid. Yield 1.05 g (86 %). M.p. = compounds decomposes at about 320 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 11.61 (s, 1H), 11.12 (s, 1H), 8.33 (d, *J* = 1.9 Hz, 1H), 7.95 (dd, *J* = 1.9Hz; 8.3 Hz, 1H), 7.39 (d, *J* = 8.3, 1H), 6.91 (s, 2H)), 6.74 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6): δ = 171.2, 170.2, 169.7, 169.7, 168.6, 164.7, 155.9, 155.8, 150.8, 139.9, 129.1, 129.1, 129.0, 127.6, 125.3, 117.0, 111.0, 104.3, 56.7, 19.3. TOF MS calcd for $C_{23}H_{10}Cl_4N_4O_5$ 564.16; found: 562. (M-H); TLC (10 % MeOH/CH_2Cl_2 +0.1% AcOH): TLC (10 % MeOH/CH_2Cl_2 +0.1% AcOH): R_f = 0.61. Anal. Calcd for $C_{23}H_{10}Cl_4N_4O_5$: C, 48.97; H, 1.79; N, 9.93. Found: C, 49.05; H, 2.20; N, 9.92.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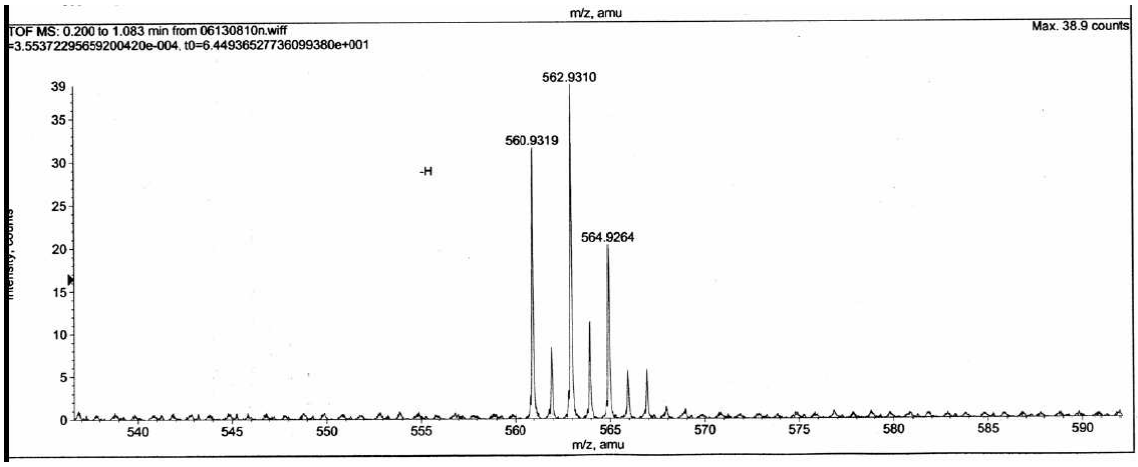
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560.9319
564.9264
-H
Intensity, cps
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35
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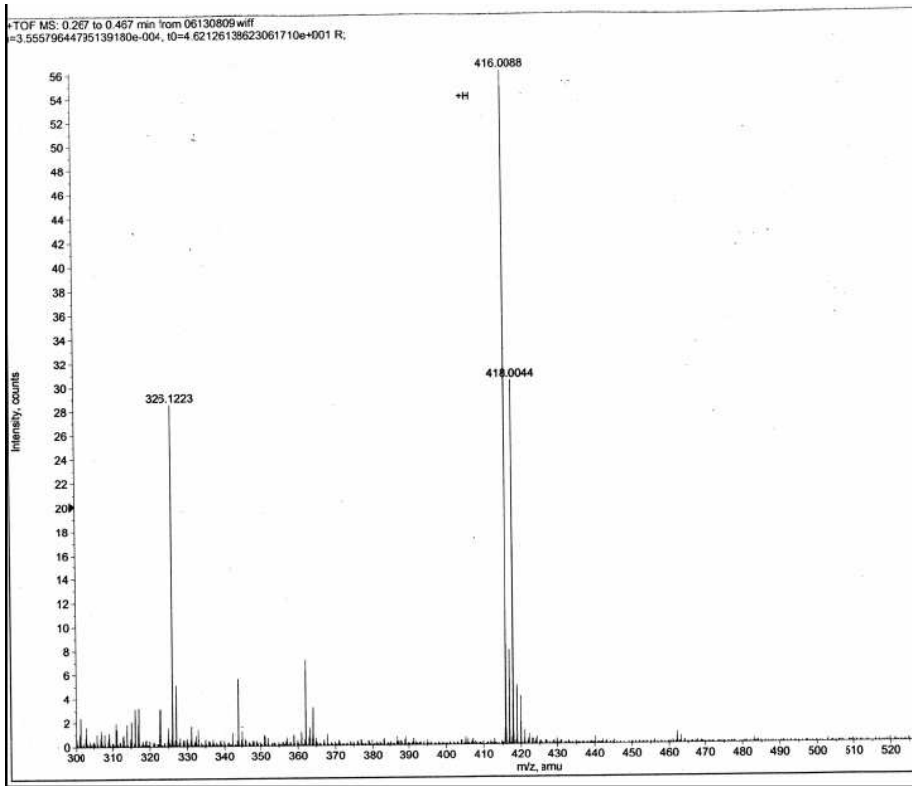
m/z, amu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+H
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Intensity, counts
m/z, amu

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