WATER-SOLUBLE BENZOPHENOXAZINE DYES: SYNTHESES, DERIVATIZATION AND PHOTOPHYSICAL STUDIES

A Thesis

by

JINEY JOSE

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 2006

Major Subject: Chemistry

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Approved by:

Chair of Committee,	Kevin Burgess
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ABSTRACT

Water-soluble Benzophenoxazine Dyes: Syntheses, Derivatization and Photophysical Studies.

(December 2006)

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Chair of Advisory Committee: Dr. Kevin Burgess

A set of three benzophenoxazine dyes, two completely soluble and one partially soluble in aqueous media, has been prepared and their spectroscopic properties examined. These dyes can be used as either donor or acceptor in synthesis of through-bond energy transfer cassettes. Structural modifications prevented aggregation in water and improved their fluorescence properties in water. Their absorption and emission were studied in both organic and aqueous media. Two of the three dyes have superior quantum yields in aqueous media as compared to other reported dyes. Improved quantum yield makes these dyes attractive candidates for biological studies in aqueous media.

We have also prepared alkynes and iodo derivatives of benzophenoxazines, which can be used for synthesis of water-soluble, through-bond, energy transfer cassettes. Alkynes were prepared via Sonogashira coupling.

ACKNOWLEDGMENTS

I would like to thank Professor Kevin Burgess for his support and advice through out this project. I am very thankful to him for all the help and for providing a conducive environment for carrying out independent research. Thanks to Professors Brian Connell, Marcetta Darensbourg and Robert C Burghardt for serving on my graduate advisory committee. Thanks to Yuichiro Ueno and Dr. Rakeshwar Bandichhor for their valuable help in various projects and all others in the Burgess group for their friendship. Thanks to Jill Rutledge for assistance with plenty of office related work. Thanks to Lauren Kulpa for help with Endnote and reference collection.

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LIST OF ABBREVIATIONS

DBU	1,8-diazabicyclo[5,4,0]undec-7-ene
CH_2Cl_2	dichloromethane
DMF	*N*, *N*-dimethylformamide
EtOAc	ethyl acetate
EtOH	ethanol
FRET	fluorescence resonance energy transfer
HCl	hydrochloric acid
LAH	lithium aluminum hydride
MeOH	methanol
TBAF	tetrabutylammonium fluoride
TEA	triethylamine
THF	tetrahydrofuran

CHAPTER I
INTRODUCTION

1.1 Cell Imaging

Cell imaging is defined as visualizing particular groups of cells, single cells and intracellular entities. Various imaging techniques are available and analysis of the data can afford reproducible results.[1] A plethora of detection systems are available to perform such imaging, each with its own degree of sensitivity and applicable to either quantitative or qualitative measurements. In most case a combination of different techniques rather than a single detection tool is used depending upon the complexity of the biomolecules being studied and the level of information to be obtained. Cellular imaging provides a means for measuring different parameters in a single experiment, thereby reducing time and increasing the efficiency of analysis. This is extremely important in treating any disease where in early detection of affected cell lines can result in therapeutic intervention at an early stage. Automated and user friendly image analysis software has helped in gaining valuable information not only regarding visible characteristics, but also subcellular changes from cells. Laser-scanning microscopy, fluorescence resonance energy transfer (FRET), multiphoton microscopy, epifluorescence microscopy are some of the technologies used in modern imaging.

Modern imaging techniques require advances in labeling compounds.[2] Focus is mainly on compounds, which can fluoresce in the near-infrared region. One of the advantages in carrying out cellular studies in the near-infrared region is that hemoglobin, water and lipid have minimum absorbance in this region. Another is that there is less autofluorescence. This feature allows for improved target illumination and less background interference. There are very few labeling compounds which fluoresce in the near-infrared region and development of such compounds are very important.[3]

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

Fluorescent probes not only allow real-time monitoring of events, but also provides spatial information about the monitored event. For such studies it is often desirable to simultaneously observe several fluorescently tagged components in a biochemical mixture, *ie* multiplexing.[4] This can be achieved by exciting all the tags using the same laser source. However excitation using a single source is difficult because dyes that absorb near the excitation source will absorb more light and dyes with absorbtion away from the excitation source will absorb less. This result in diminishing emissions from the dyes excited with a single source. Combinations of dyes arranged to maximize FRET have been used to overcome this problem.[5,6] A variety of near-infrared fluorophores based on FRET have been developed, which are largely being used in detecting early cancers or inflammatory process in mouse cells.[7,8]

1.2 Fluorescence Resonance Energy Transfer (FRET)

As early as 1920s the transfer of electronic excitation energy between well separated atoms or molecules were reported. The simplest case would be that of two atoms separated in space and the electronic excitation of one results in excitation of the other. This excitation could occur by emission of one quantum of light by the first atom and reabsorption of this quantum by the second atom. Such transfers are rarely observed and were proved to be not the case by Cairo and Franck in 1922 in their experiments on fluorescence of atoms in vapor phase. In their experiments they irradiated a mixture of mercury and thallium vapors with mercury light and obtained the emission spectra for both the atoms. Since thallium atom do not absorb mercury light, energy transfer from excited mercury atoms can excite them. Therefore a transfer by reabsorption of quantum of light is not possible in this case. The transfer of excitation energy happens over a larger distance than observed in normal collision transfer. There are a number of other examples of such excitation transfer.

Perin *et al* reported the first observation of such excitation transfer in solution in 1929. One example is described below. A solution containing both perylene (acceptor) and chloroanthracene (donor) in equal ratio was prepared. A decrease in chloroanthracene fluorescence was observed when both components were at same concentration, which means a definite transfer of excitation energy is taking place between the donor and acceptor molecule. The transfer occurs over the mean intermolecular distances between the donor and acceptor. This excitation transfer is independent of volume of solution and viscosity of the solvent and is characterized by decrease in donor fluorescence lifetime.

Mechanism of excited energy transfer can be explained with the help of Figure (1.1) where in during the absorption process, the donor is excited to a higher vibrational level of its first exited state. The donor can come down to a lower vibrational level of the same excited state by a non-radiative transition. During the deactivation process when donor returns to the ground state, it is possible that the energy of deactivation may exactly coincide with possible absorption transition in an acceptor molecule that is in the near vicinity of the deactivated donor molecule. With sufficient energetic coupling an excitation energy transfer between the donor and the acceptor molecule takes place. This type of energy transfer is referred to as “resonance transfer”. The energy transfer takes place before emission from the donor molecule takes place and requires coupling between the electronic systems of both donor and acceptor. This limits the distance over which such an energy transfer can occur. There is also literature precedent, which shows such energy transfer occurring between electronic systems of the same molecule.

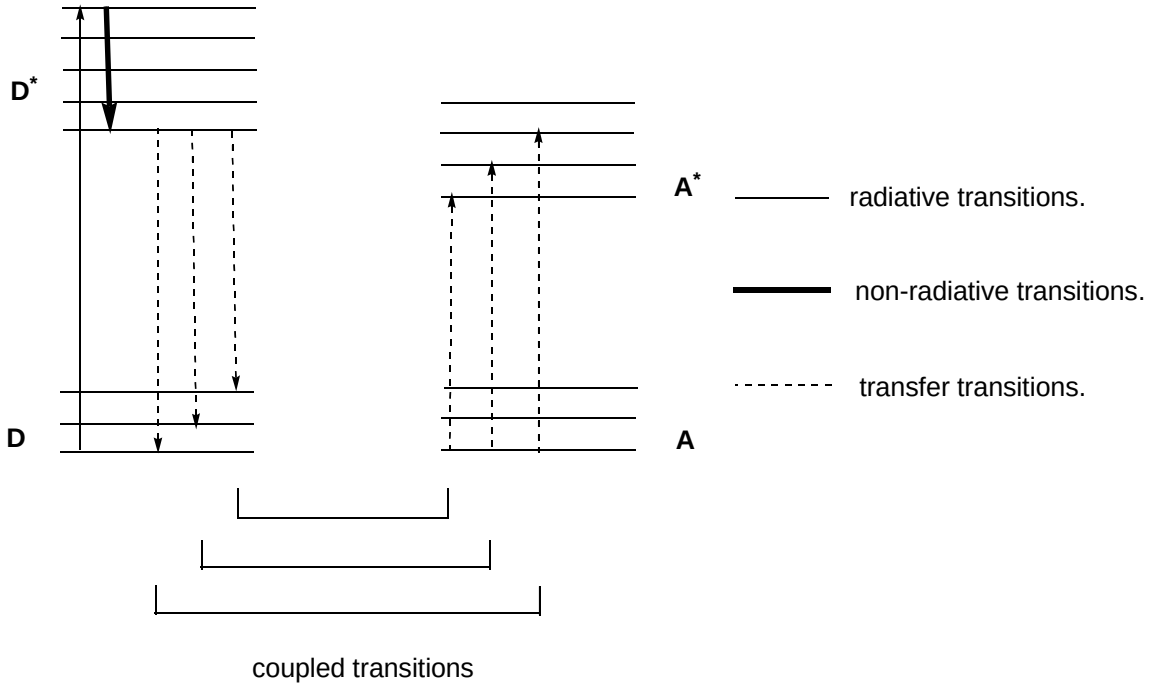


Figure 1.1 Simplified energy level diagram of energy transfer between donor (D) and acceptor (A)

This kind of excitation transfer process, when applied to fluorescent molecules can be defined as exciting a fluorescent molecule which is in proximity to a second fluorophore, results in emission occurring from the second fluorophore with minimal or almost no fluorescence seen from the excited molecule. This phenomenon is termed as Fluorescence Resonance Energy Transfer (FRET). For FRET to occur the distance between both fluorophore should be less than 100 nm. FRET was first reported by Professor Theodor Förster in 1946.[9] The excited fluorophore can be termed as a donor and the molecule emitting light can be termed as an acceptor. The energy transfer which takes place between the donor and acceptor which is separated by a distance *r*, is given by:

$$K_T(r) = Q_D\kappa^2/\tau_D\, r^6\, (9000(\ln 10)/128\pi^5 N\eta^4)_0\int^{\infty} F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda \quad \text{.....(i)}$$

Where QD = quantum yield of donor in the absence of the acceptor κ^2 = orientation factor (range from 0 to 4, usually assumed to be 0.67 for dynamic random averaging). κ^2 = 4 if the transition dipoles of the donor and acceptor are perfectly parallel and 0 when they are orthogonal. τ_D = lifetime of donor in the absence of the acceptor. N = 6.02 x 10^{23}. η = refractive index of the medium (usually assumed to be 1.4 for biomolecules in aqueous solution). The rate of energy transfer is inversely proportional to the sixth power of the distance, r, between the donor and the acceptor. The overlap integral J (λ) can be given as

$J(\lambda) = {}_0\int^{\infty} F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$.....(ii)

The extent of overlap between the emission spectrum of the donor and the absorbtion spectrum of the acceptor is given by above equation. FD(λ) is the normalized emission spectrum of the donor. $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor at wavelength λ. The Förster radius, R_o, is the distance r, at which the rate of energy transfer is equal to the rate of decay of the donor ($1/\tau_D$) in the absence of the acceptor. R_o is the distance at which FRET is 50 % efficient. At $r = R_o$, $K_T = (1/\tau_D)$. Equation 1 can be written as

$Ro = (9000(\ln 10)\, Q_D\kappa^2/128\pi^5 N\eta^4)\ {}_0\int^{\infty} F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda$.....…….(iii)

R_o is typically in the range of 20 to 60 A^o for organic fluorophores.

Knowing R_o, one can calculate the ET rate by:

$$k_T = \frac{1}{\tau_D}\left(\frac{R_o}{r}\right)^6 \quad \text{.....(iv)}$$

The efficiency of energy transfer, E, is the fractions of photons absorbed by the donor that are transformed to the acceptor. E is given by

$$E = \frac{k_T}{\tau_D^{-1} + k_T} \quad \text{.....(v)}$$

this is the ratio of the energy transfer rate to the total decay rate of the donor.

The efficiency of energy transfer can be calculated from the emission intensity of the donor in the absence and presence of the acceptor.[10]

For FRET to occur overlap of emission of donor fragment with the absorbtion of the acceptor is a must. This is a constraint, which limits the combination of dyes that can be used for multiplexing.

1.3 Through Bond Energy Transfer

Through-bond energy transfer system consists of a donor and acceptor part connected via a conjugate linker that does not allow them to be planar. This nonplanar geometry allows rapid energy transfer from the donor to the acceptor part. Through-bond energy transfer is mechanistically different from Förster basis for FRET, which requires overlap of emission of donor fragment with the absorbtion of the acceptor. There are two mechanisms proposed for the observed energy transfer. Dexter[11] and superexchange energy transfer.[12] As compared to Förster energy transfer, Dexter energy transfer is a short range phenomenon and requires the interaction between excited donor orbital with the orbital of acceptor in ground state. Superexchange energy transfer can take place over a longer distance since energy is relayed through bonds connecting the donor and the acceptor.

Thus appropriately designed through-bond energy transfer cassettes could absorb photons via a donor part and transfer the energy rapidly through the conjugate linker to the acceptor fragment that emits at a longer wavelength. There is no known constraint on the difference between the donor absorbtion and acceptor emission wavelength in this Scheme since no overlap is required for energy transfer to occur. Thus it is possible to design dyes that can absorb strongly at short wavelength and emit brightly at longer wavelength. In summary, through bond energy transfer cassettes have the potential to increase the resolution and fluorescence intensities obtained from several probes excited by laser source operating at single wavelength.

Our group has vast expertise in designing such through-bond energy transfer cassettes.[13-18] Previous members have designed four energy transfer cassettes as shown in Figure 1.2 and studied its photophysical properties as shown in Figure 1.3.[19] Excitation of these cassettes at 488 nm produces fluorescence characteristic of only the acceptor component, which proves 100 % energy transfer efficiency between the donor and the acceptor. The fluorescence intensities of **1-4** when excited at the donor component were compared to emission of molecule that resembles

only the acceptor part. Cassettes were shown to fluoresce more brightly than the acceptor proving efficient energy transfer.

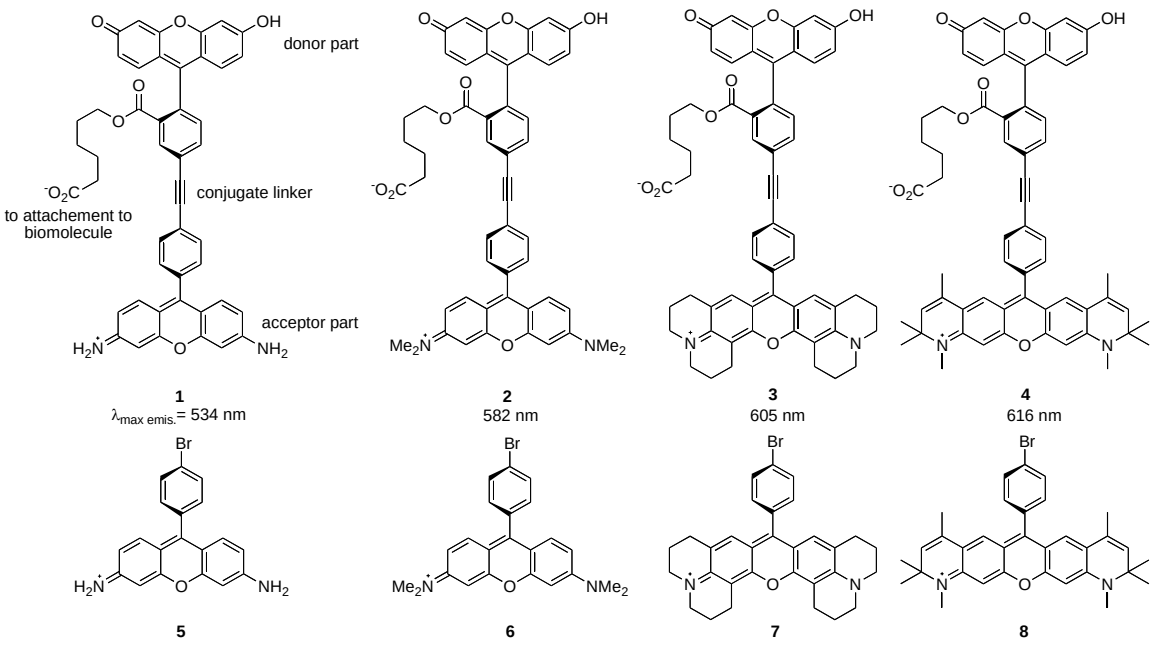


Figure 1.2 Through-bond energy transfer cassettes and acceptors synthesized by our group

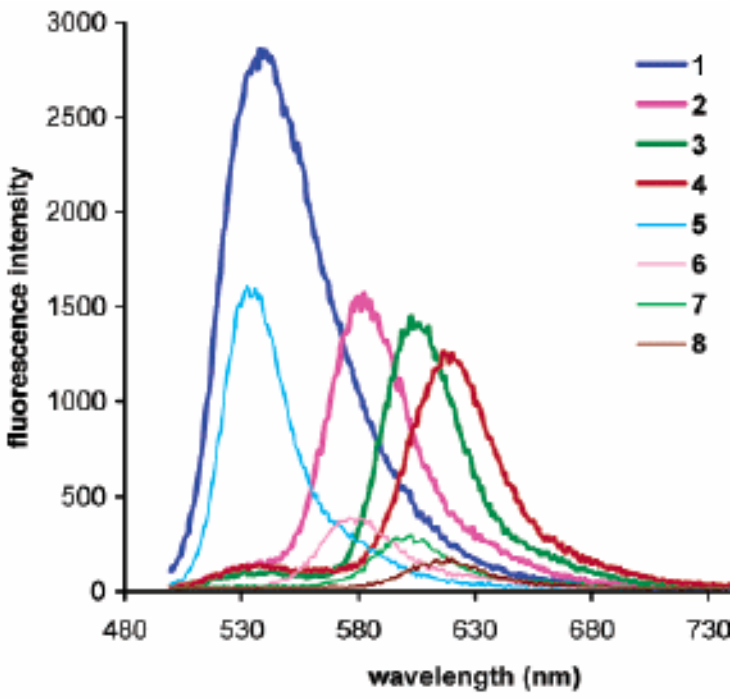
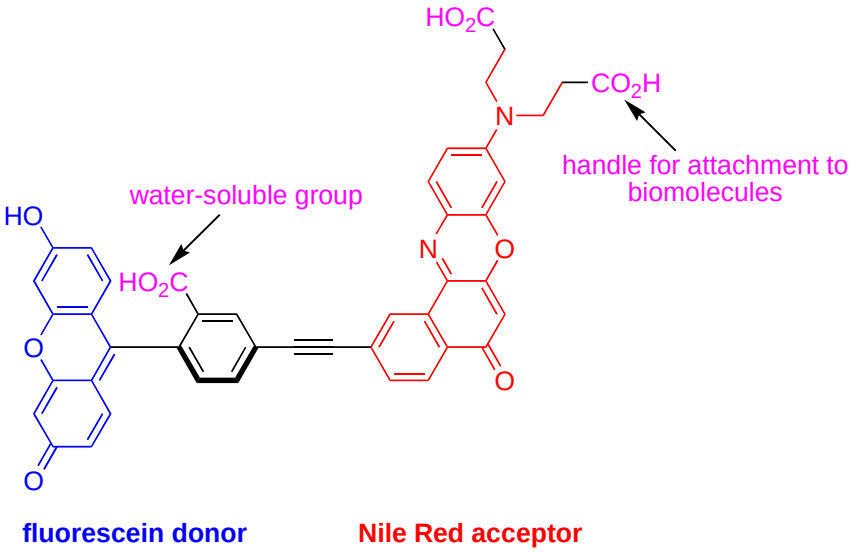


Figure 1.3 Fluorescence of equimolar EtOH solutions of 1-8 excited at 488 nm.

For the above-mentioned cassettes to be useful in cellular imaging, it should be soluble in aqueous media. To enhance its water solubility either the donor or acceptor should have water solubilising functional groups. In our quest for suitable synthons for constructing such water-soluble through bond energy transfer cassettes; we came across benzophenoxazine dyes with superior photophysical properties in organic solvents. We hypothesized that modification of these dyes by introduction of water solubilising groups will enhance its spectral properties in water, there by making it useful either as a donor or acceptor (Figure 1.4). We chose to work with Nile Red and its derivatives, a benzophenoxazine dye, which is an attractive candidate for synthetic manipulation. A detailed study of these dyes and our effort towards its modification for our specific purpose is illustrated in the chapters III and IV.

a



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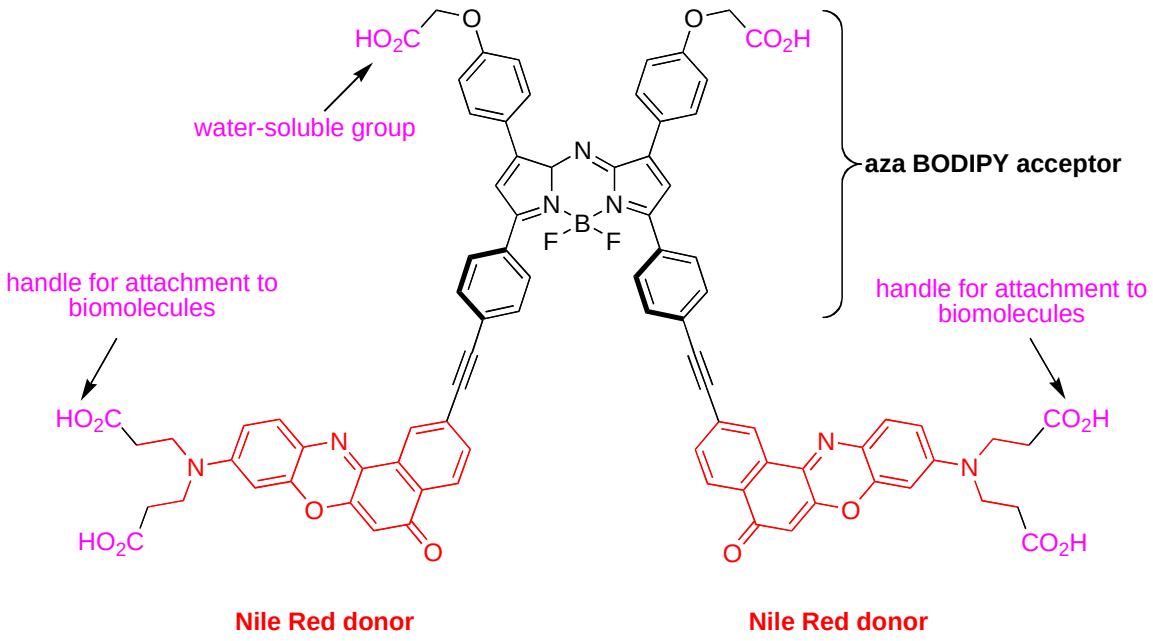


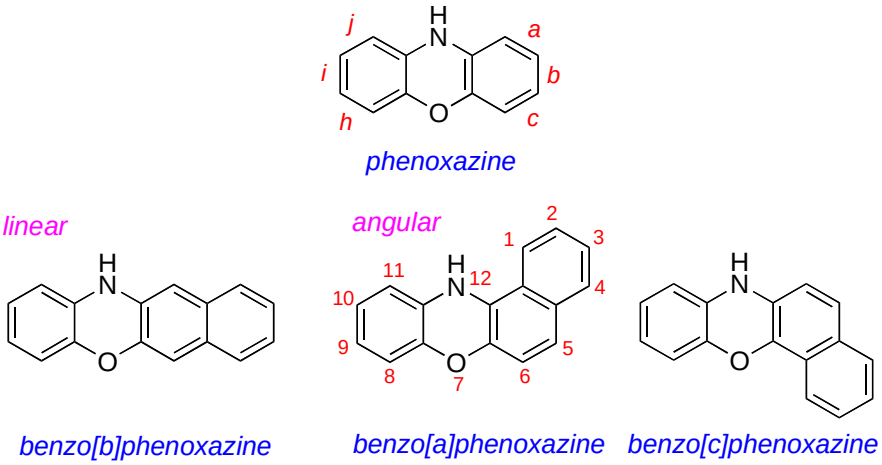
Figure 1.4 Possible water-soluble through-bond energy transfer cassettes **a** Nile Red as acceptor; **b** Nile Red as donor.

CHAPTER II
BENZOPHENOXAZINE CLASS OF DYES

2.1 Introduction

Phenoxazines to which a benzene ring is fused to the *a-c* or *h-j* faces are termed as angular or linear benzophenoxazines depending upon the orientation of the fused benzene ring.

a



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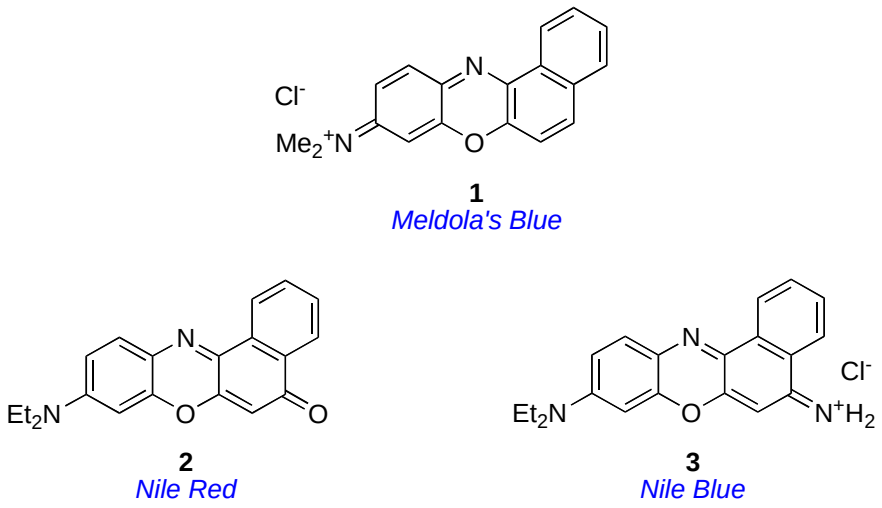


Figure 2.1. **a** Phenoxazines and benzophenoxazines. **b** Structures of the three best known fluorescent dyes in this class.

Substituents that freely donate and/or accept electron density on benzophenoxazine cores can, in some orientations, give fluorescent compounds. The first notably fluorescent compound to be discovered in this class was Meldola's Blue **1**, but Nile Red **2**[20] and Nile Blue **3**[20] (Figure 2.1) are far more frequently used in contemporary science.

Meldola's Blue, Nile Red, and Nile Blue have some desirable attributes as fluorescent probes.[21] They have reasonably high fluorescence quantum yields in apolar solvents and they fluoresce at reasonably long wavelengths. Nile Red, in particular, fluoresceces far more strongly in apolar media than in polar ones, and the fluorescent emission shows a large bathochromic (*ie* red-) shift in polar media, hence it can be used as a probe for the polarity of the environment to which it is attached.[22-24] However, these characteristics may be attributes for some applications, but limitations for others. None of these dyes is significantly soluble in aqueous media, and their quantum yields are dramatically reduced. One of the big challenges in the production of fluorescent dyes is to produce water-soluble probes that fluoresce strongly in aqueous media, particularly above 600 nm or at even longer wavelengths. Motivation for research in this area is drawn from needs for intracellular, tissue, and whole organism imaging where near-IR dyes are far more conspicuous than ones emitting at 550 nm or less.[25]

2.2 Meldola's Blue

2.2.1 Introduction

This dye is used for in the textiles, paper and paint industries, mainly as a pigment. It is not a particularly useful fluorescent dye for labeling proteins because of its poor water solubility. Further, its fluorescence is weak in all common media (*eg* EtOH) and does not give a clear indication of the surrounding polarity. However, Meldola's Blue has been used as a component in redox sensors for detection of materials such as NADH,[26] pyruvates,[27] hydrogen peroxide,[28] glucose[29] and 3-hydroxybutyrate.[28] It has also been

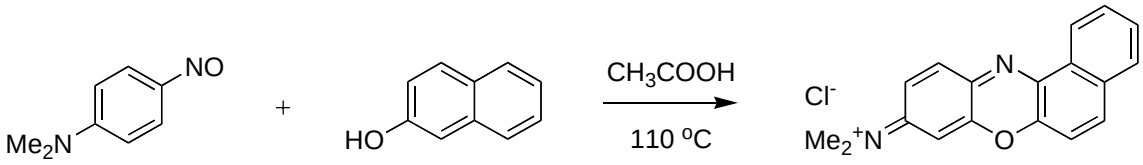
used in electrochemical experiments involving DNA wherein the dye mediates electron transport.[30]

2.2.2 Synthesis

The original, 1879, synthesis of Meldola's Blue involved condensation of a nitroso compound with 2-naphthol at elevated temperatures (Scheme 1a).[31] Details of the reaction conditions and the yield were not given. Subsequently, Meldola's Blue has been made with a variety of counter ions via reactions involving different Lewis acids (Scheme 2.1b). Counter ion modifications provide an avenue to modulate the electronic properties of solid materials for applications not directly related to labeling biomolecules.

Scheme 2.1. a Original synthesis of Meldola's Blue; and, **b** a more recent approach.

a



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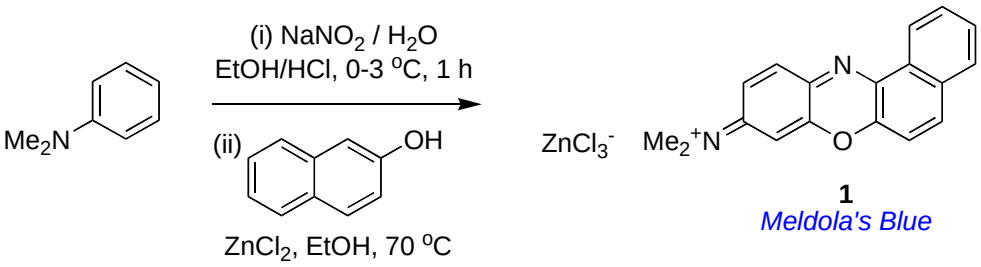


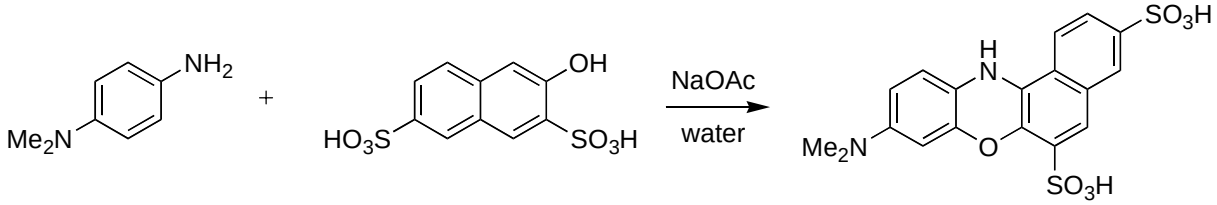
1
Meldola's Blue

The literature describes syntheses of sulfonated derivatives of Meldola's Blue.[32] However, we anticipate that some of these reactions would be hard to reproduce: the transformation shown in Scheme 2.2a, reproduced directly from the literature, seems either to have the wrong oxidation state of the starting material (perhaps it should be a

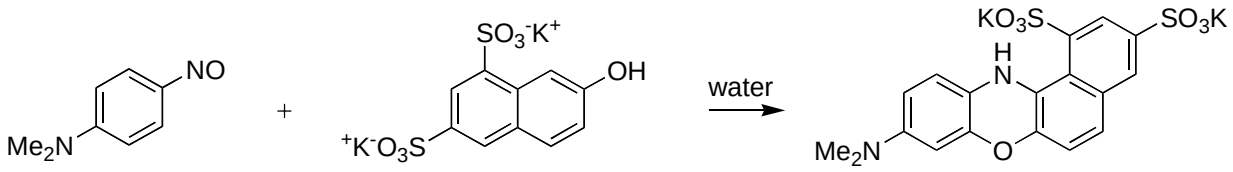
nitroso compound and not an amine), or an oxidizing step missing in the reaction conditions. Further, oxidation of the final product (which may happen in the air) is required to obtain a fluorescent material. In any event, the most difficult part of these transformations is often isolation of the product in pure form, and details of the procedures to do this are, unfortunately, often not given.

Scheme 2.2. Syntheses of sulfonated 9-(dimethylamino) benzophenoxazines: **a** 3,6-disulfonic acid; and, **b** 1,3-disulfonic acid, isomers.

a



b



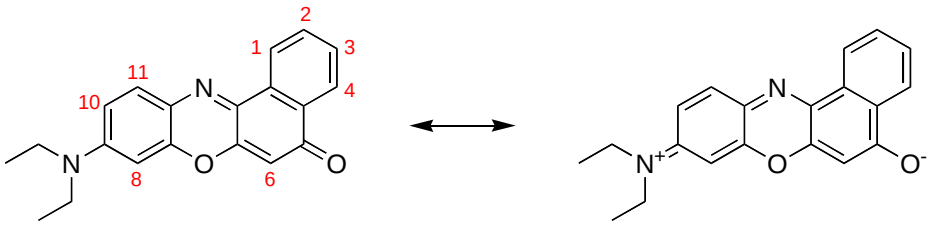
2.2.3 Photophysical Properties

Phenoxazines without strongly electron withdrawing or donating substituents have unexceptional absorbance characteristics, and they are not particularly fluorescent compounds. Fusion of a benzene ring onto the heterocycle does not alter this situation dramatically. Meldola's Blue has one dimethylamino substituent and the heterocyclic core is oxidized. These changes to the composition of the heterocycle shift its absorbtion and emission characteristics into a useful range; $\lambda_{max\ abs}$ 568 nm, $\lambda_{max\ emiss}$ 540 nm EtOH. This dyes is not known for its solvatochromic properties.[33]

2.3 Nile Red Derivatives

2.3.1 Introduction

Nile Red has a neutral oxidized phenoxazine system, *ie* it is a phenoxazinone. The 9-diethylamino substituent is able to donate electron density into the carbonyl group across the ring; this electronic arrangement probably accounts for its highly fluorescent properties (Figure 2.2).



Nile Red **2**

MeOH	*n*-heptane
$\lambda_{max\ abs}$ 554 nm	$\lambda_{max\ abs}$ 484 nm
$\lambda_{max\ emiss}$ 638 nm	$\lambda_{max\ emiss}$ 529 nm
Φ 0.38	Φ 0.48

Figure 2.2. Electron delocalization in Nile Red.

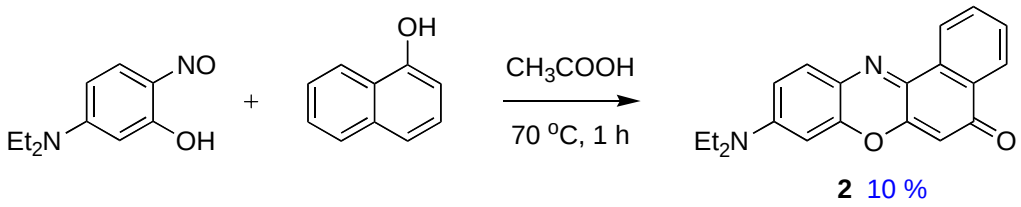
The water-solubility of Nile Red is extremely poor, but in other solvents its fluorescence maxima and intensity is a good indicator of the polarity of the dye environment. As mentioned above, this solvatochromic effect is such that polar media cause a red-shift but decreased fluorescence. This decreased fluorescence intensity is probably due to self-quenching of the dye in face-to-face aggregates. Consequently, this dye is particularly useful for studying lipids and events that involve impregnation of the dye in apolar media. Surprisingly, very few water-soluble analogs of Nile Red have been reported, and only limited fluorescence data has been given for those that have.

2.3.2 Synthesis

The first synthesis of Nile Red was essentially the same as that shown in Scheme 2.1 except that 1-napthol was used in place of 2-napthol (Scheme 2.3a). It is also possible to prepare Nile Red via hydrolysis of Nile Blue as indicated in Scheme 2.3b. The product is usually isolated via chromatography on silica.[34]

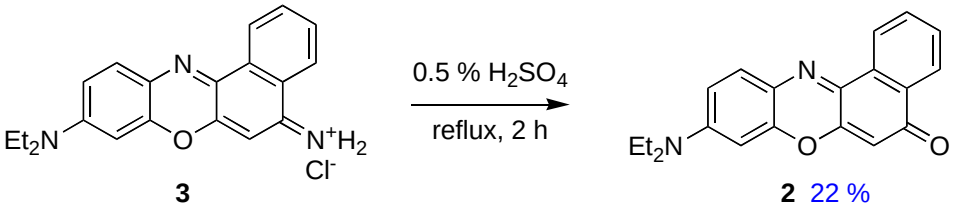
Scheme 2.3. **a** Original syntheses of Nile Red; and, **b** synthesis from Nile Blue.

a



2 10 %

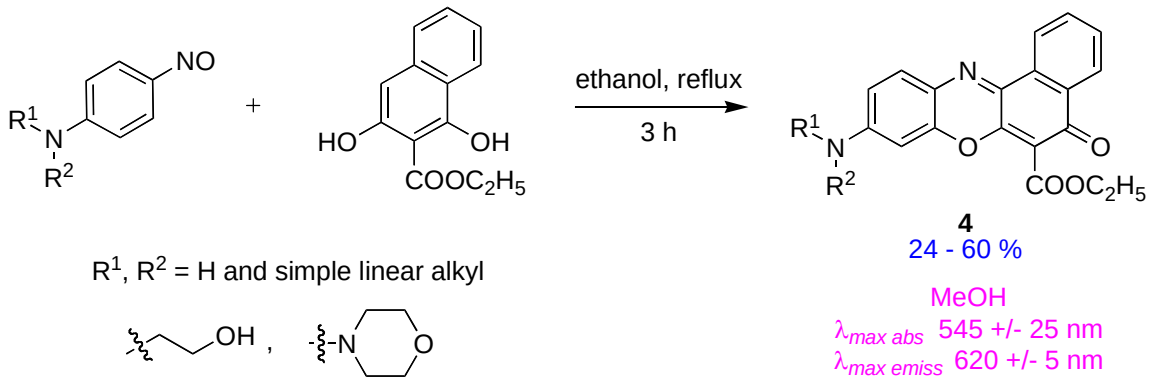
b



3 **2** 22 %

Substituted or modified Nile Red derivatives may be prepared via variations on the syntheses above, *ie de novo* methods, or by functionalizing Nile Red itself. For instance, a *de novo* approach was used to prepare the 6-carboxy ethyl derivatives **4** (Scheme 2.4). These are potentially interesting since hydrolysis of the ester would give a carboxylic acid for attachment to biomolecules; however, this does not appear to have been attempted yet.[35] The patent literature also describes a synthesis of the 2-carboxy Nile Red derivative.[36]

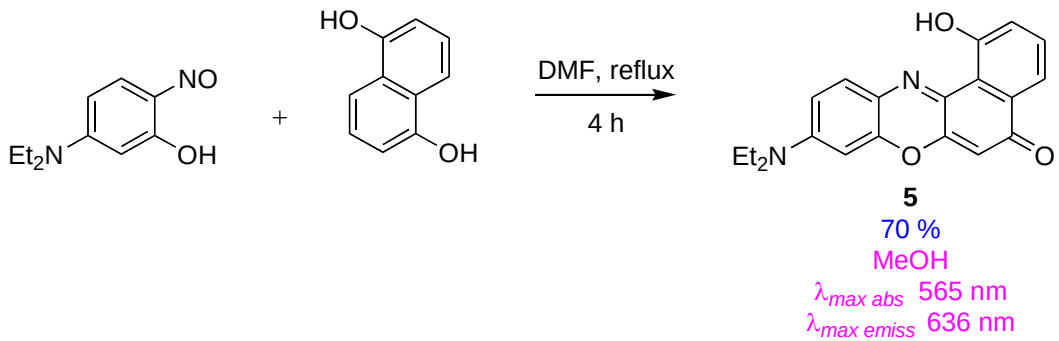
Scheme 2.4. Syntheses of 6-carboxy ethyl derivatives of Nile Red.



De novo syntheses of 1- and 2- hydroxy Nile Red, compounds **5** and **6** respectively, have also been performed, and the products are easily modified via other reactions.[24] Thus, Briggs and co-workers at Amersham prepared the parent hydroxy compounds as shown in Scheme 2.5. Some of the reactions used to derivatize these materials are also shown. In some ways the hydroxyl group of the hydroxy Nile Reds **5** and **6** is an inconvenience because the spectroscopic properties of the dye under physiological conditions become highly pH dependant. However, as a functional group to incorporate a handle for attachment to biomolecules, as in Scheme 2.5 d, this phenol is useful.

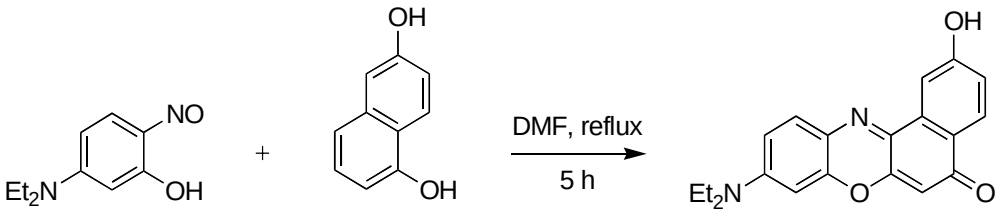
Scheme 2.5. a Synthesis of 1-hydroxy Nile Red; **b** synthesis 1-hydroxy Nile Red; and, **c** synthesis of sulfonic acid derivative of 1-hydroxy Nile Red; **d** some derivatization reactions of 1-hydroxy Nile Red.

a



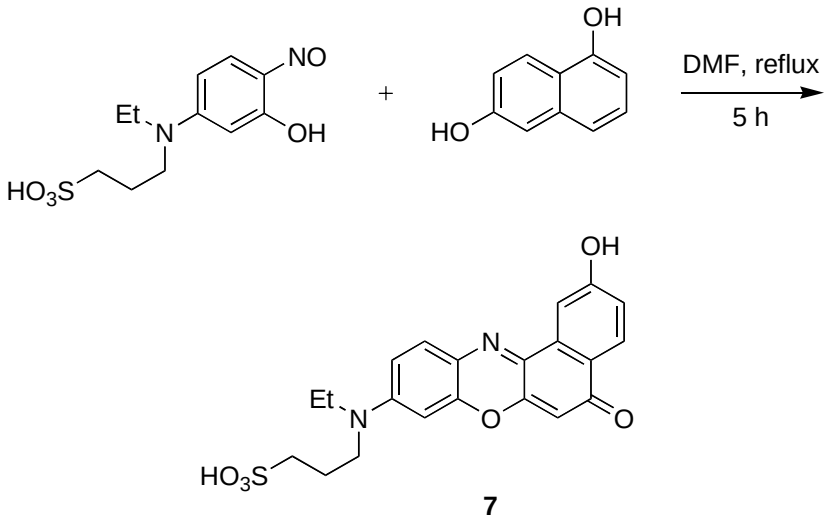
Scheme 2.5. continued.

b



6
65 %
MeOH
$\lambda_{max\ abs}$ 551 nm
$\lambda_{max\ emiss}$ 632 nm

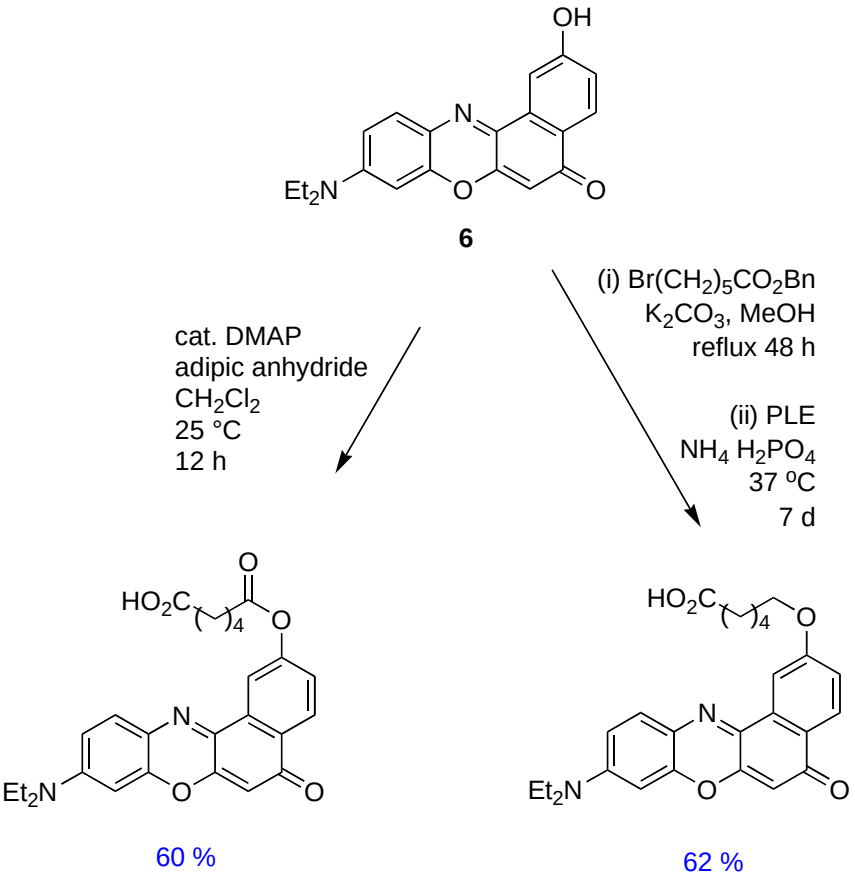
c



7
79 %
MeOH
$\lambda_{max\ abs}$ 545 nm
$\lambda_{max\ emiss}$ 618 nm

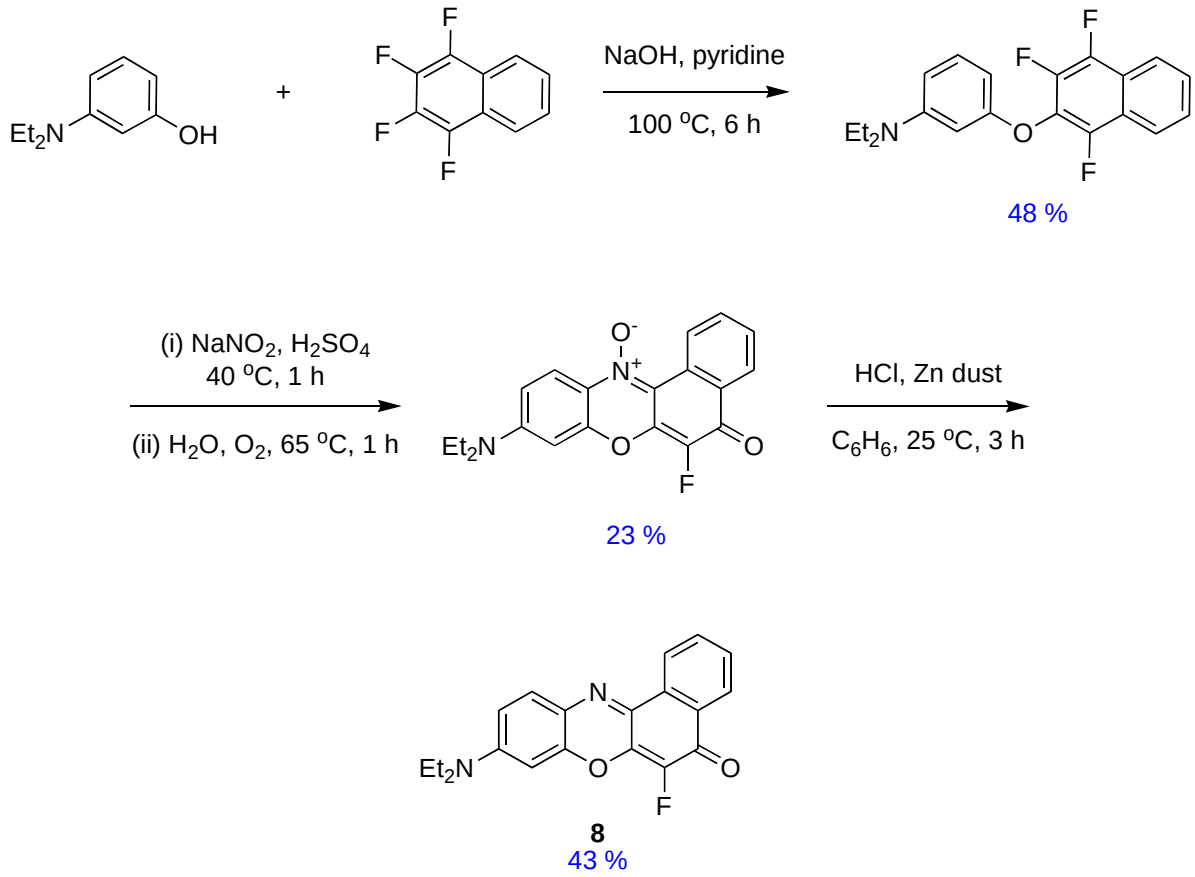
Scheme 2.5. continued.

d



A series of fluorinated phenoxazines (not shown) and benzophenoxazines (Scheme 2.6) have been prepared via sequential S_NAr substitutions on fluorinated aromatics.[37] The 6-fluoro substituent in the compound shown below slightly changes its spectroscopic properties (see below) and, presumably its pH dependence.

Scheme 2.6. Preparation of 6-fluoro Nile Red.



The so-called "FLAsH dyes" feature bisarsenic(3+)-based dye precursors that are non-fluorescent, presumably due to rapid quenching of the excited state via intramolecular energy transfer. However, the disposition of the arsenic dyes is such that they are thought to react with dicysteine units engineered into modified proteins that are expressed within cells. This type of reaction has at least two effects, it: (i) alters the oxidation potential of the arsenic centers; and, (ii) constrains rotations about the C-As bonds. For whatever reason, structural changes such as this render the dye-protein complex fluorescent. Only proteins within the cell that have the special arrangement of Cys-residues are likely to become labeled in this way, so the approach allows for highly selective visualization of the engineered protein (Figure 2.3). Typically, the appropriate Cys-environment is created when two proteins interact, so the probes can be used to follow protein-protein interactions within cells.

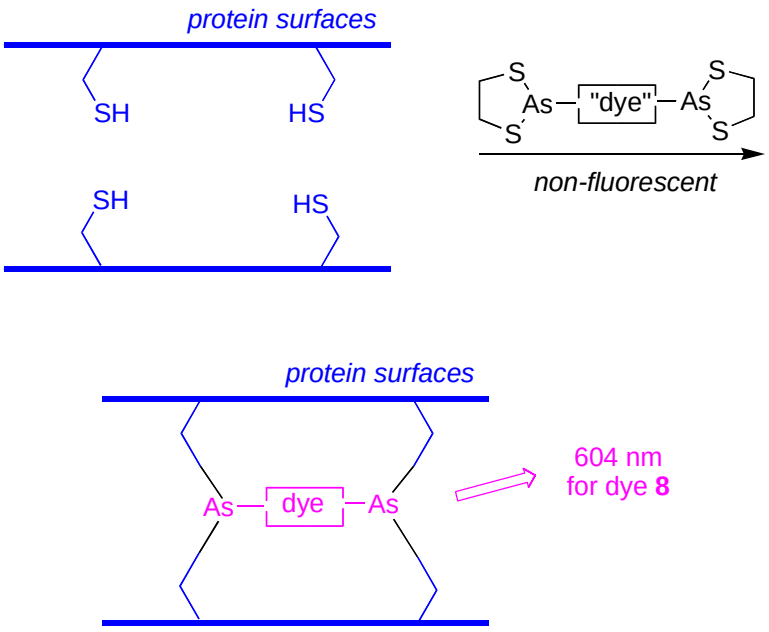


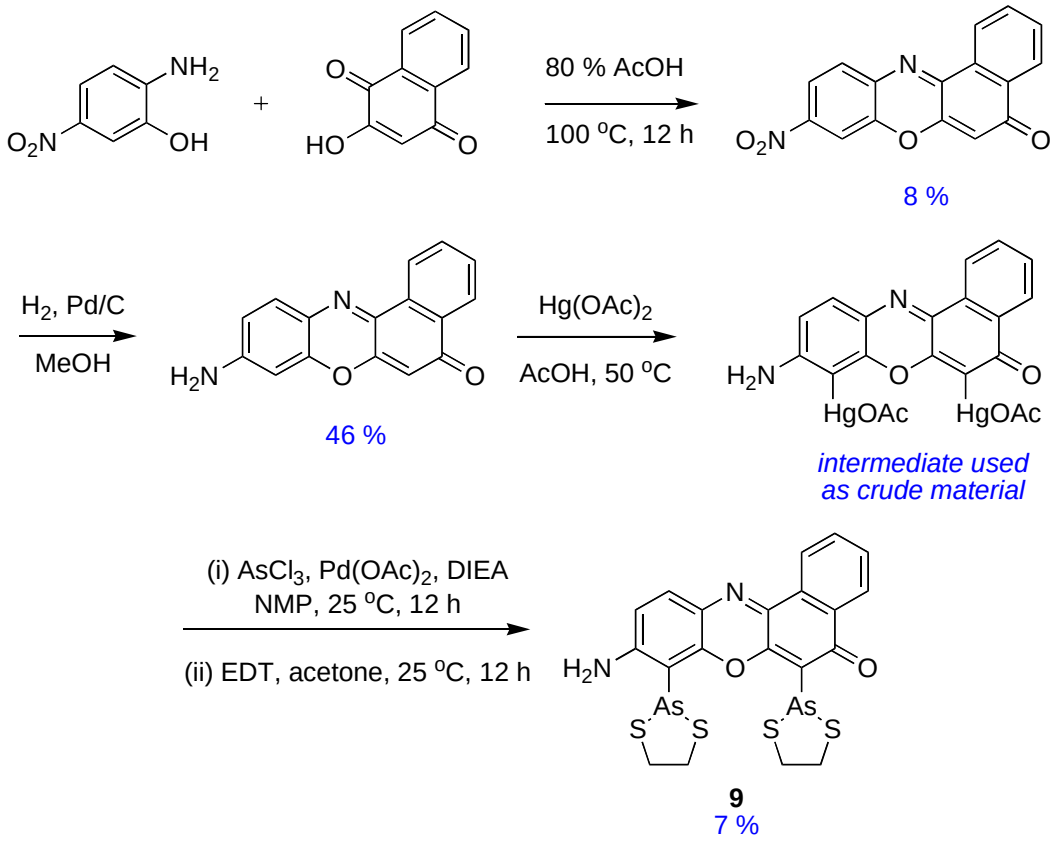
Figure 2.3. Conceptual basis of Fluorescent Arsenic dye.

The original FLAsH dyes were fluorescein derivatives.[38] A range of bisarsenic compounds on different dye skeletons have been prepared for evaluation, but only fluorescein and Nile Red derivatives have emerged as useful. This is because several parameters must be controlled tightly if this type of experiment will work. For instance, the As-to-As distance must match the disposition of the target thiols, the ethanedithiol (EDT) concentration in the cell is critical, and the dye must permeate into the cell.

The Nile Red derivative **9** was prepared as indicated in Scheme 2.7. This involves a standard condensation reaction to form the benzophenoxazine core, but without the *N*,*N*-diethyl substituents of Nile Red. These were omitted to allow more space for manipulations at the 6- and 8-positions. FLAsH dye **8** gives less fluorescent enhancement on binding than similar fluorescien dyes, but it emits at a longer wavelength (604 nm) and that, as mentioned before, is a more transparent region of the spectrum for

intracellular imaging. Calcium-induced conformational changes of appropriately modified, intracellular calmodulin have been followed using FLAsH dye **9**.[39]

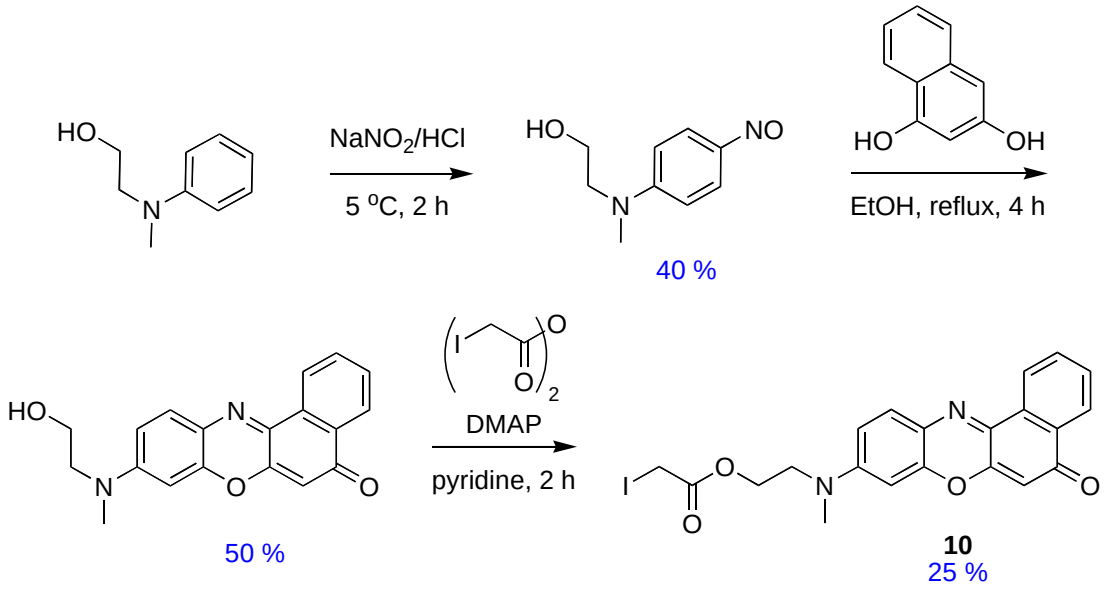
Scheme 2.7. Preparation of FLAsH dyes based on Nile Red.



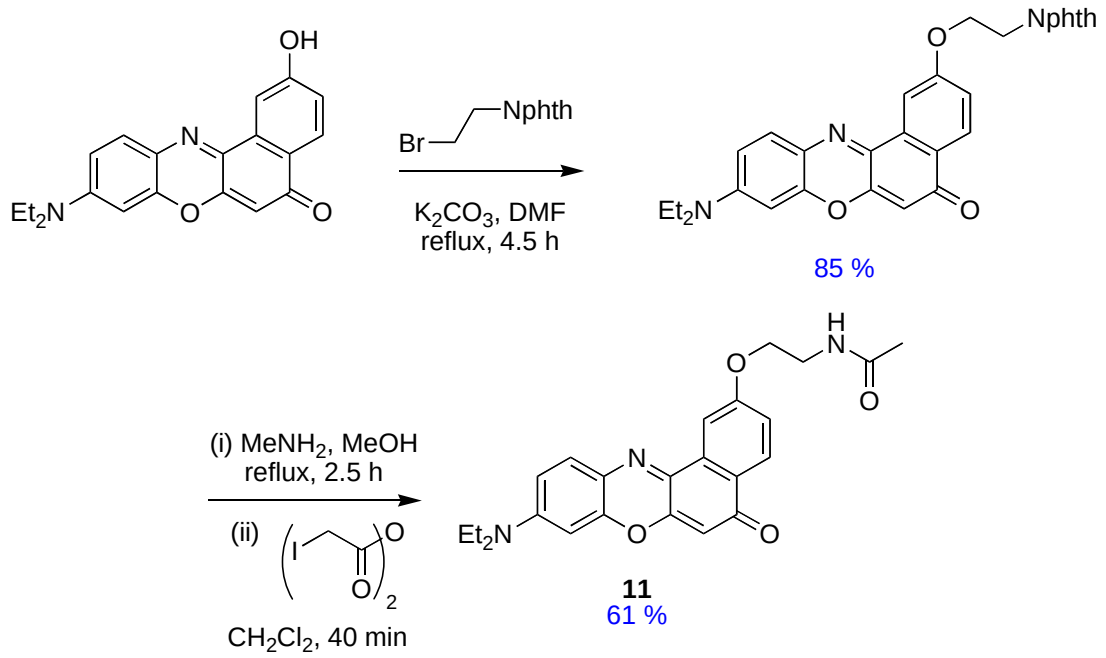
Scheme 2.8 describes syntheses of two more classical thiol-selective dyes, *ie* ones that rely on S_N2 displacement of iodide from iodoacetyl groups. Thus probes **10** and **11** were prepared from a Nile Red derivative and from a 2-hydroxy Nile Red compound, respectively. Curiously, when these were complexed to a particular Cys-residue in maltose binding protein, dye **10** showed a three-fold enhancement of fluorescence, while the emission from **11** was *reduced* by a factor of five. The authors explain this by proposing that **11** is less constrained when bound.

Scheme 2.8. Two thiol-selective dye electrophiles. **a** a Nile Red derivative; and, **b** a 2-hydroxy Nile Red derivative.

a



b



2.3.3 Spectroscopic Properties

Table 2.1 summarizes spectroscopic data for Nile Red in different solvents, all taken from the same source.[22] These data show that decreased fluorescence intensity of Nile Red correlates much more with hydrogen bonding than with solvent polarity, and the magnitude of this difference is best appreciated from the graphical presentation of only the emission wavelengths and intensities that is given in Figure 2.4. However, the bathochromic shift seems to be a function of solvent polarity, consistent with stabilization of relatively polar excited states.

Table 2.1. Solvent dependency of emission intensities and wavelengths for Nile Red **2**.

solvent	**$\lambda_{abs.}$ (nm)**	**$\lambda_{em.}$ (nm)**	**relative fluorescent intensity**
water	591	657	18
EtOH	559	629	355
acetone	536	608	687
$CHCl_3$	543	595	748
iso-amylacetate	517	584	690
xylene	523	565	685
n-dodecane	492	531	739
n-heptane	484	529	585

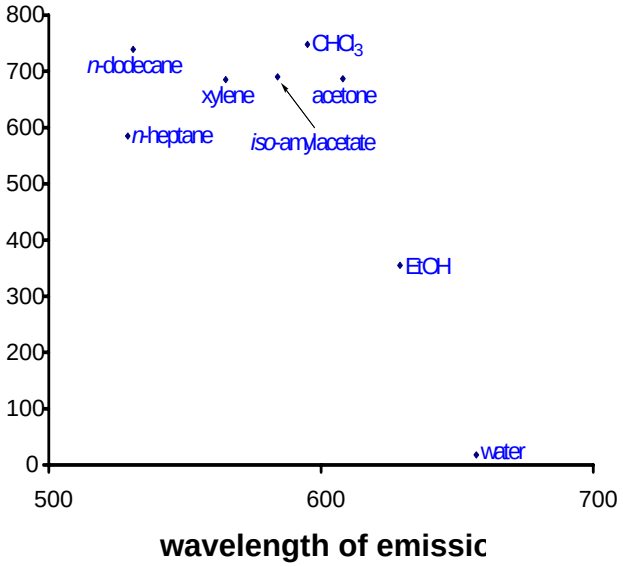


Figure 2.4. Solvent dependency of emission intensities and wavelengths for Nile Red **2**. A similar study featuring emission and absorption maxima, quantum yields, and solvent polarities have been performed for 2-hydroxy Nile Red **6**. Selected data from this study is shown in Figure 2.5. Just as with Nile Red, polar hydrogen bonding solvents correlate with reduced quantum yields and significant bathochromic shifts.

Table 2.2. Solvent dependency of emission intensities and wavelengths for 2-hydroxy Nile Red **6**.

solvent	**$\lambda_{abs.}$ (nm)**	**$\lambda_{em.}$ (nm)**	**quantum yield (Φ)**
cyclohexane	514	528	0.51
dibutyl ether	514	555	0.66
toluene	525	568	0.76
acetone	528	608	0.78
EtOH	544	634	0.58
1,2-ethanediol	584	655	0.40
CF_3CH_2OH	587	653	0.24

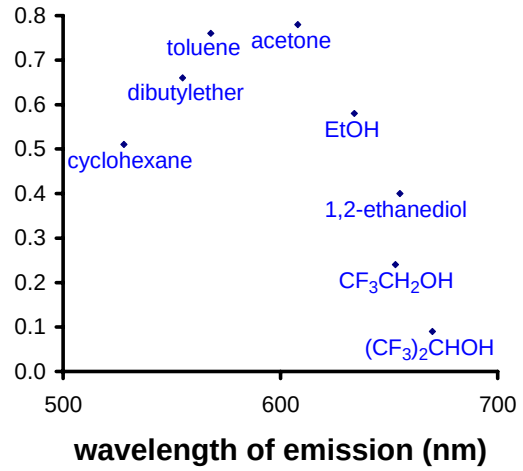


Figure 2.5. Solvent dependency of emission intensities and wavelengths for 2-hydroxy Nile Red **6**.

Fluorescence lifetimes contribute to two important physical parameters of fluorescent dyes. Dyes with long fluorescent lifetimes can emit strongly because non-radiative processes are relatively slow, and because intersystem crossing to triplet states is less competitive too.

Fluorescence lifetimes for Nile Red in different solvents have been measured. These data show that the fluorescence lifetime of Nile Red is similar to fluorescien and tetramethyl rhodamine in ethanol. The fluorescent lifetime of Nile Red does not seem to vary much with solvent polarity, but it is extremely sensitive to H-bonding. In hydrogen-bonding solvents the fluorescence lifetime of Nile Red decreases dramatically.

There are two common approaches to long wavelength dyes for imaging in tissues. The first, and most obvious, is to prepare analogs of known dyes with extended conjugated systems. Secondly, two-photon absorption can be used.[40] In this technique, two long wavelength photons absorbed by a molecule promote it to an excited state that then emits a single photon of higher energy. This is a way to excite intracellular or tissue samples at a wavelength that is more transparent to these media. Relatively few dyes are suitable for practical experiments using two-photon excitation because most do not absorb two long wavelength photons efficiently, *ie* they have poor two-photon cross sections. Unfortunately, Nile Red has a relatively poor two-photon cross-section.

One issue with two-photon excitation experiments is that the emitted light is of a relatively short wavelength, and this might not be in a convenient region to permeate out of cells of other tissues, and for detection. One strategy to circumvent this problem is to arrange a fluorescence energy transfer system (FRET) featuring a donor with an high two-photon cross section and an acceptor with a more convenient, longer wavelength, emission maxima. 2-Hydroxy Nile Red derivatives have been used in such systems. Thus a series of compounds (of which **12** and **13** are two of the simplest) have been prepared towards this end; and donor-to-acceptor energy transfer efficiencies of more than 70 % were observed (Figure 2.6).[41,42] These compounds presumably do not have the solubility and size characteristics that render them suitable for a range of applications in biotechnology, so further developments in this area would be opportune.

a

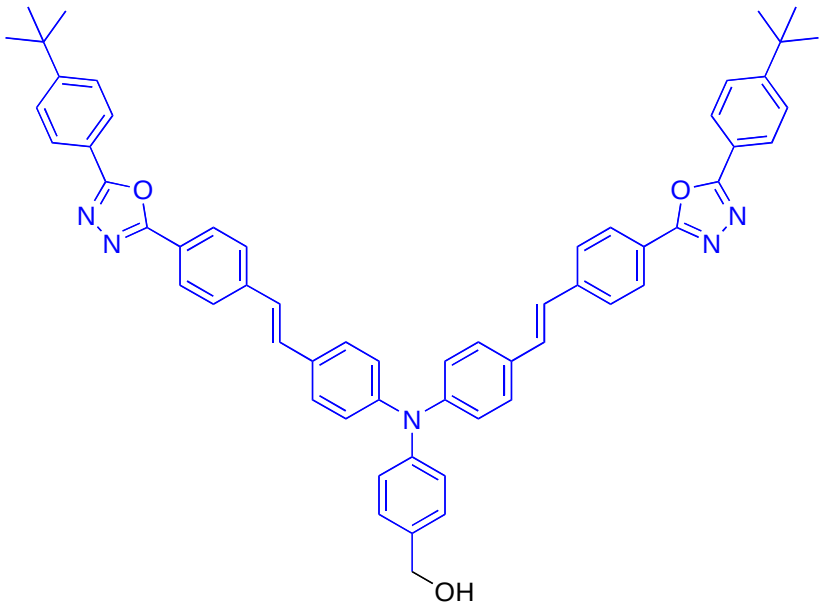
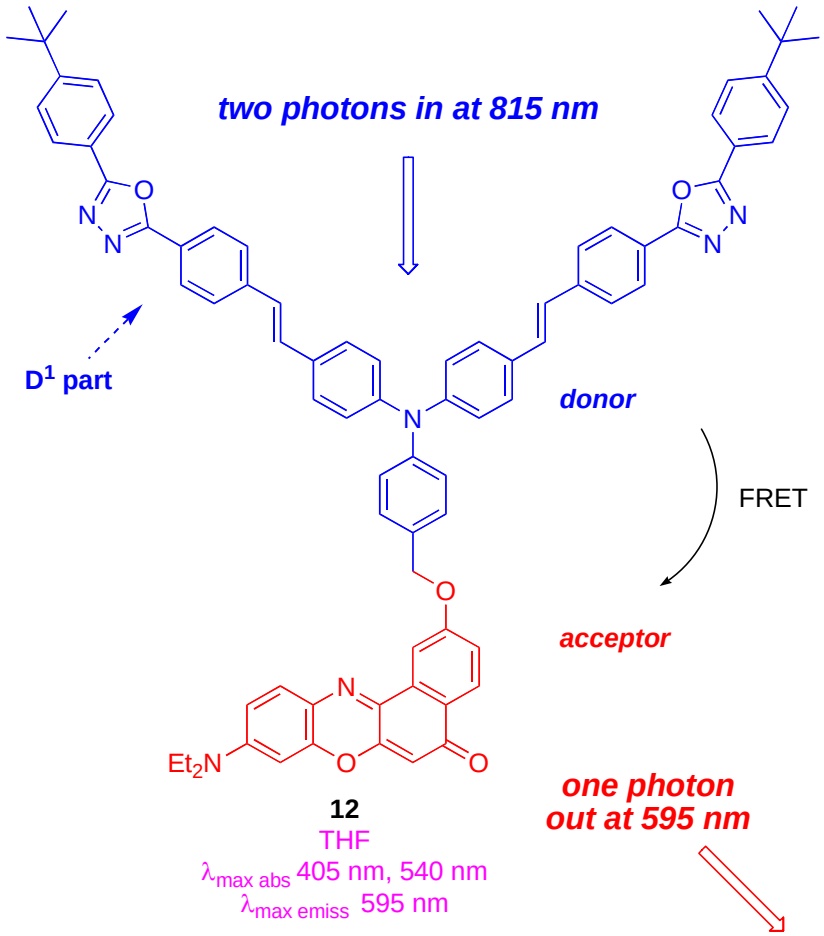


Figure 2.6. FRET system based on 2-hydroxy Nile Red as acceptor.



b

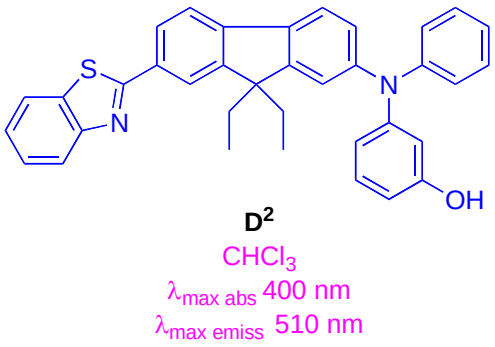


Figure 2.6. continued.

.

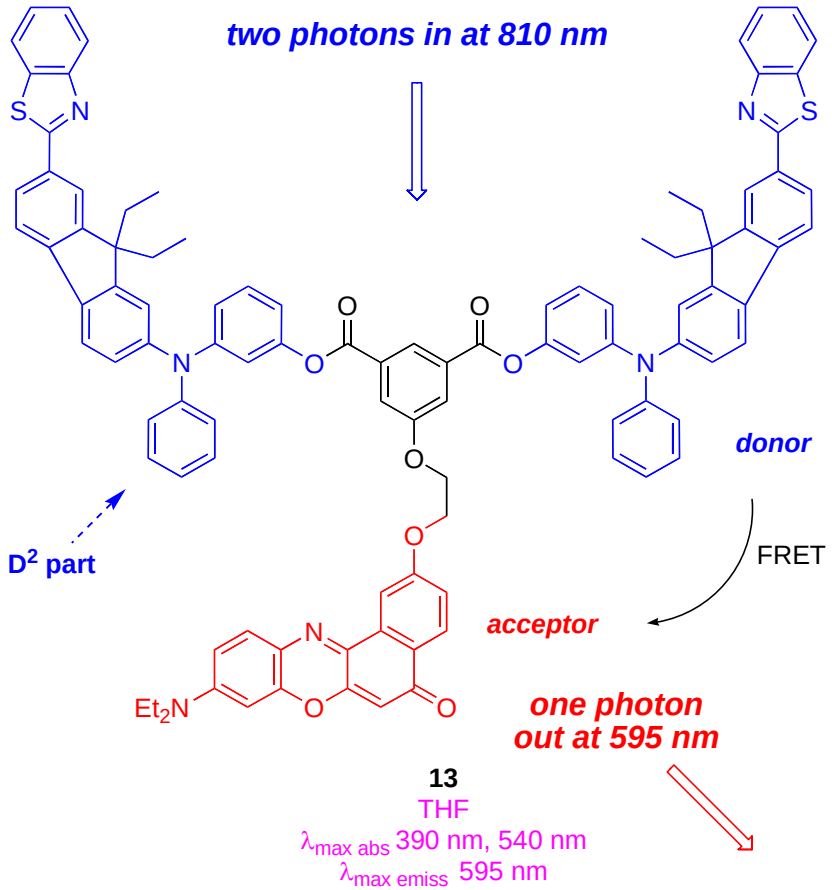
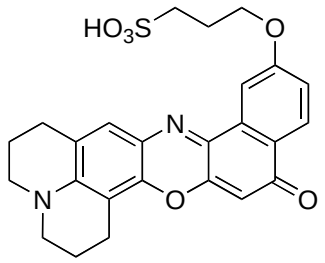


Figure 2.6. continued.

Finally, there is the possibility that the fluorescence of Nile Red is somewhat attenuated if the excited state of the benzophenoxazinone core is reduced via electron transfer from the 9-amino substituent. We mention this because the possibility has been explored via AM1 calculations.[43] These gauge the degree of charge transfer in a planar state relative to a twisted one in which the amine lone pair is not disposed to electron donation to the heterocycle. The degree of electron transfer from the 9-amino substituent will be dependant on the conformation *and* on the reduction potential of the heterocycle in its excited state. If intramolecular charge transfer were a major pathway for quenching the

fluorescence of Nile Red derivatives, it would be expected that compound **14** would be less fluorescent than Nile Red itself.



14
more or less fluorescent
than Nile Red?

2.4 Nile Blue Derivatives

2.4.1 Introduction

Nile Blue has a positively charged, oxidized, phenoxazine system, *ie* it is a phenoxazinium; the conspicuous difference between this and Nile Red **2** is that the latter is neutral. Both dyes have a 9-diethylamino substituent to donate electron density across the ring, but Nile Red **2** and Blue **3** have different electron acceptors: a carbonyl and an iminium group, respectively (Figure 2.7).

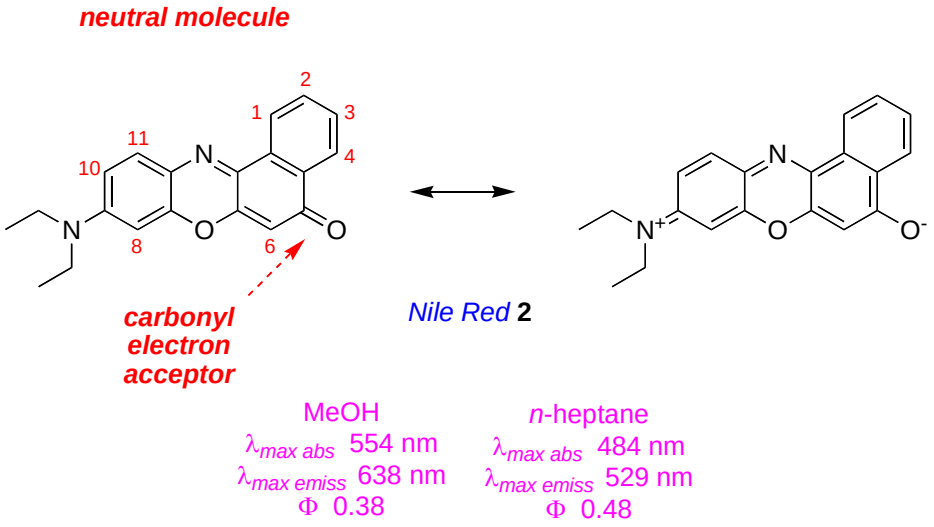


Figure 2.7. Structures of Nile-Red and Blue contrasted.

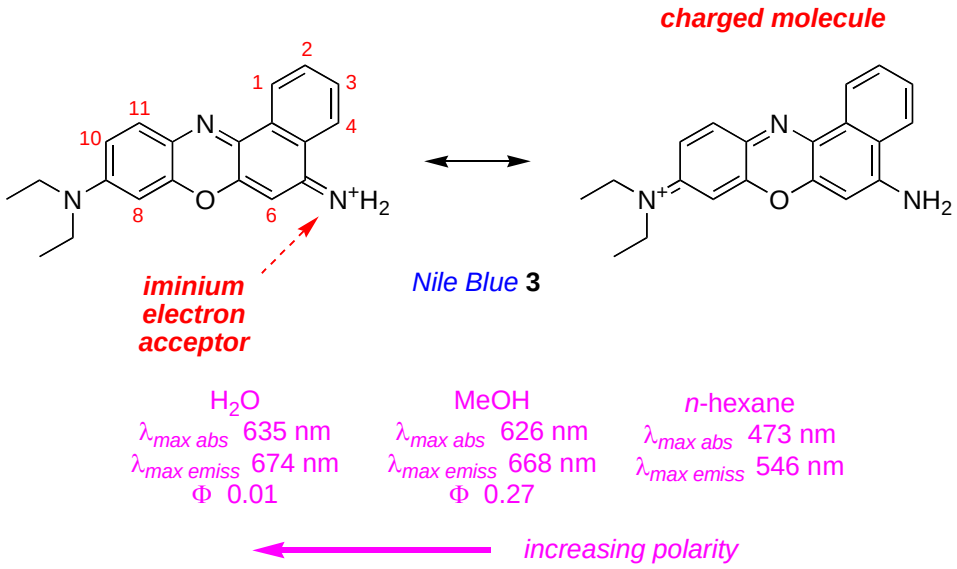


Figure 2.7. Continued.

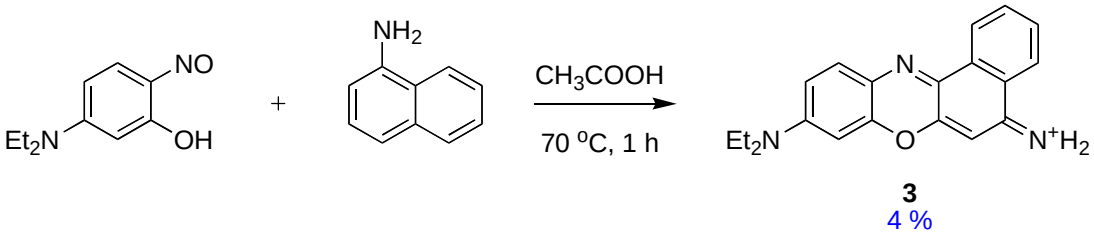
One obvious consequence of the difference in charges for Nile Red **2** and Blue **3** is that water-solubility of Nile Blue is significantly better. Like its Red cousin, the fluorescence maxima and intensity of Nile Blue are good indicators of the polarity of the dye's environment. This solvatochromic effect gives a red-shift in polar media, as would be expected for stabilization of a more charged excited state. The intensity of the fluorescence of **3** in water is about 0.01; this is a small value but, in comparison with the lack of fluorescence of Nile Red in aqueous media, it is significant.

2.4.2 Synthesis

The first reported synthesis of Nile Blue dates back to 1896 by Mohlau and Uhlmann.[20] They condensed 1-naphthylamine with 4-nitroso-*N*,*N*-diethyl-3-aminophenol to obtain a blue dye, which they later named as Nile Blue. An improvement in yield can be achieved by using perchloric acid instead of acetic acid for condensation.[44]

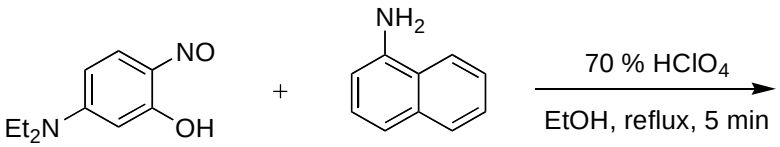
Scheme 2.9. a Original synthesis of Nile Blue; **b** modified synthesis.

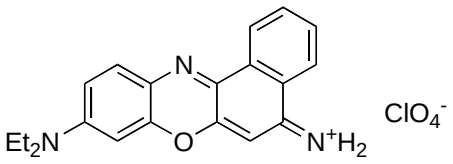
a



3
4 %

b



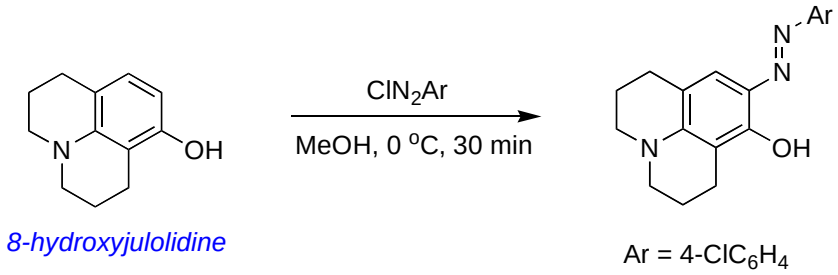


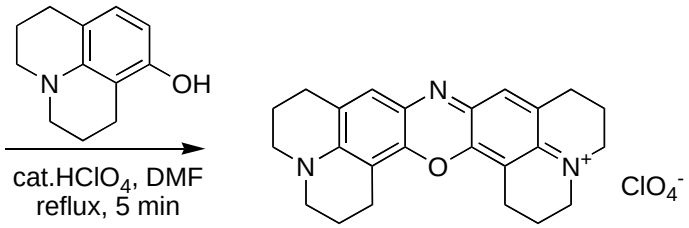
3
31 %

De novo syntheses of Nile Blue derivatives involve use of similar, but substituted starting materials. Scheme 2.10 describes syntheses of dyes **15** - **18** that incorporate 8-hydroxy julolidine.[44] That amine is a "privileged fragment" in dye syntheses because it holds the nitrogen lone pair in conjugation with the aromatic rings. This alters the reactivity of the starting material; in fact, nitrosylated 8-hydroxyjulolidine was insufficiently reactive in this synthesis so Hartmann and co-workers modified the synthon to include the reactive azo-functionality as shown. Unfortunately, the julolidine fragment is quite hydrophobic too, so the product dyes have very limited solubility in aqueous systems.

Scheme 2.10. Syntheses of Nile Blue derivatives from 8-hydroxyjulolidine.

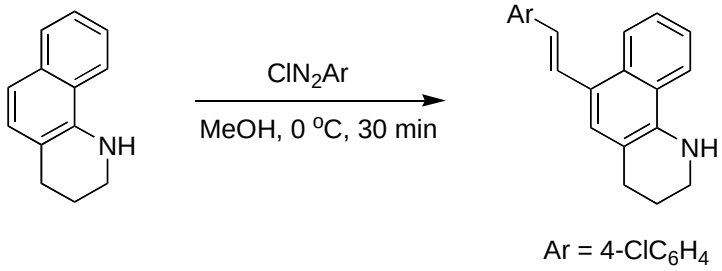
a



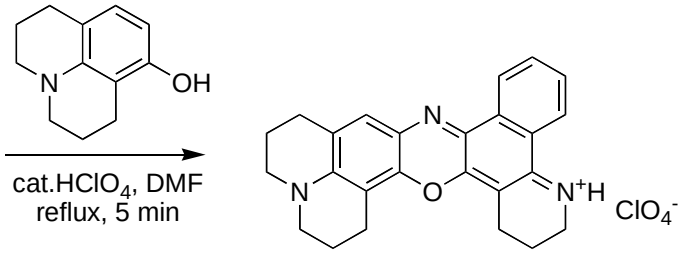


15
57 %
CH_2Cl_2; Φ 1.0
$\lambda_{max\ abs}$ 671 nm
$\lambda_{max\ emiss}$ 688 nm

b

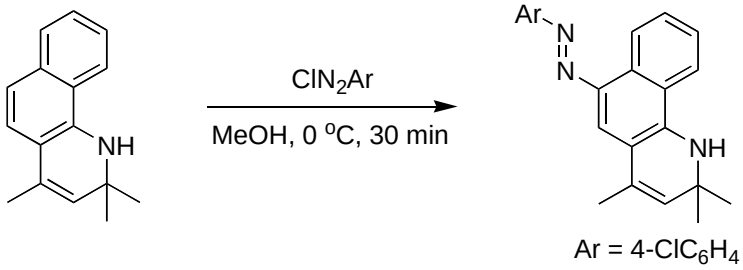


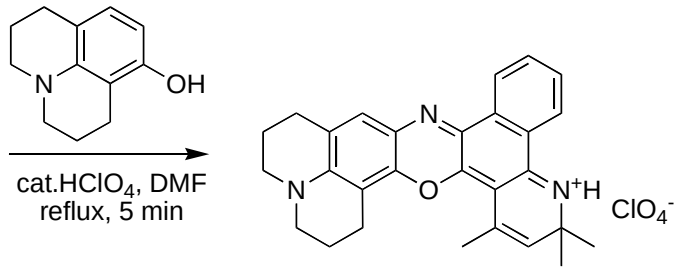
Scheme 2.10. continued.



16
10 %
CH_2Cl_2; Φ 0.35
$\lambda_{max\ abs}$ 675 nm
$\lambda_{max\ emiss}$ 712 nm

c

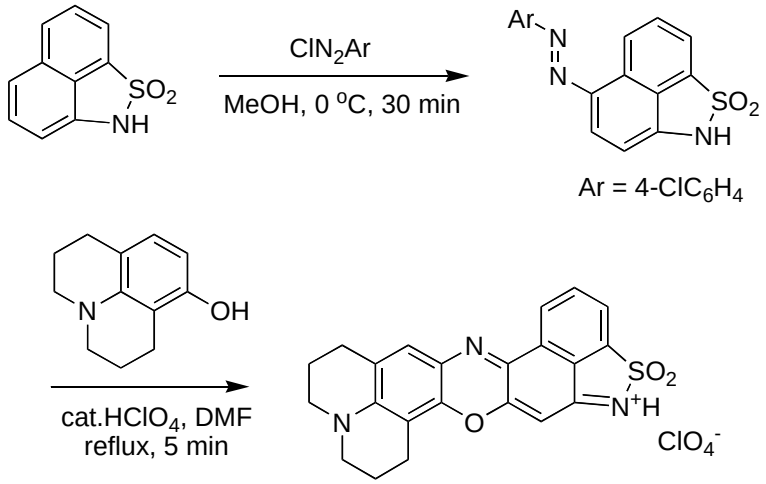




17
10 %
CH_2Cl_2; Φ 0.42
$\lambda_{max\ abs}$ 668 nm
$\lambda_{max\ emiss}$ 677 nm

Scheme 2.10. continued.

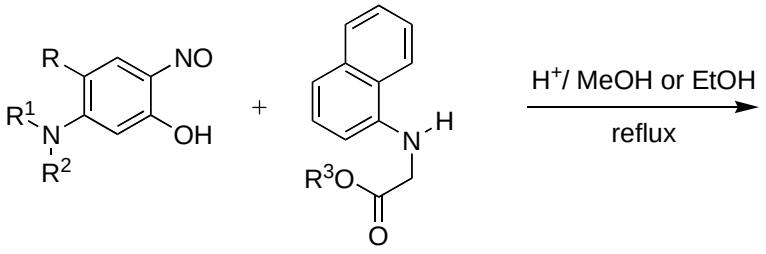
d



18
1 %
CH_2Cl_2; Φ 0.42
$\lambda_{max\ abs}$ 668 nm
$\lambda_{max\ emiss}$ 677 nm

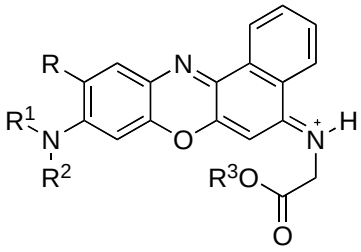
Substituted or modified Nile Blue derivatives may be prepared by *de novo* methods, or by functionalizing Nile Blue itself. A *de novo* synthesis was utilized for preparation of series of Nile Blue derivatives with carboxylated side chain as shown in Scheme 2.11.[45,46] These side chains are useful for covalent attachment of these dyes to biomolecules. High yields are reported for the acid catalyzed condensation reactions.

Scheme 2.11. Water-soluble Nile Blue derivatives with carboxylic acid side chain.



R1,R2 = H, linear alkyl
R = H, Et, R3 = H,Me, Et

Scheme 2.11. continued.



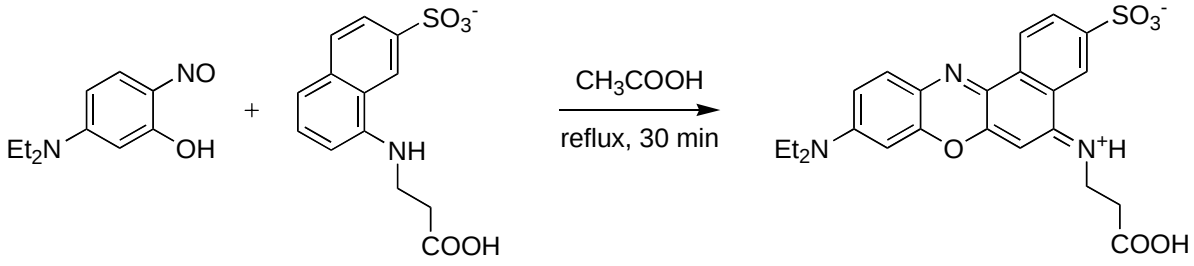
19
7-90 %

EtOH
$\lambda_{max\ abs}$ 570 +/- 60 nm
$\lambda_{max\ emiss}$ 640 +/- 30 nm

pH 7.4 buffer
$\lambda_{max\ abs}$ 570 +/- 15 nm
$\lambda_{max\ emiss}$ 638 +/- 12 nm

A *de novo* approach was utilized for the syntheses of EVOblue, a class of highly water-soluble Nile Blue derivatives.[47,48] Presence of a carboxylic acid and a sulfonic acid group in these dyes helps in preventing aggregation and also makes it useful for high-throughput screening studies. High stability of these dyes as compared to other class of dyes like BODIPY™ towards acids makes it useful in solid phase chemistry. Attachment of these dyes to peptides and subsequent cleavage of the dye-peptide conjugate from the solid support is possible with 95 % trifluoroacetic acid, without decomposition of the dye. Thus different proteins with molecular masses ranging from 10 KDa to 150 KDa have been successfully labeled with these dyes.

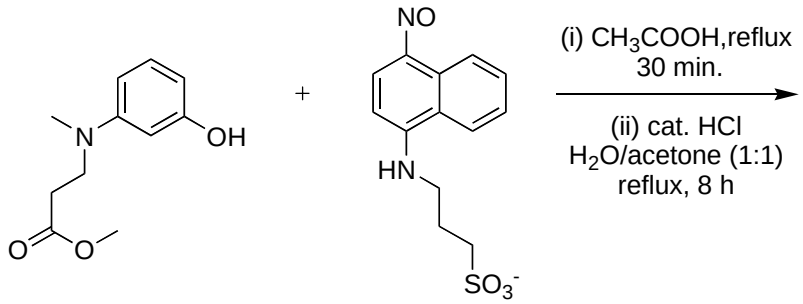
Scheme 2.12. EVOblue, a class of highly water-soluble Nile Blue derivatives.



20
pH = 7.4 buffer
$\lambda_{max\ abs}$ 649 nm
$\lambda_{max\ emiss}$ 673 nm

Scheme 2.12. continued.

b

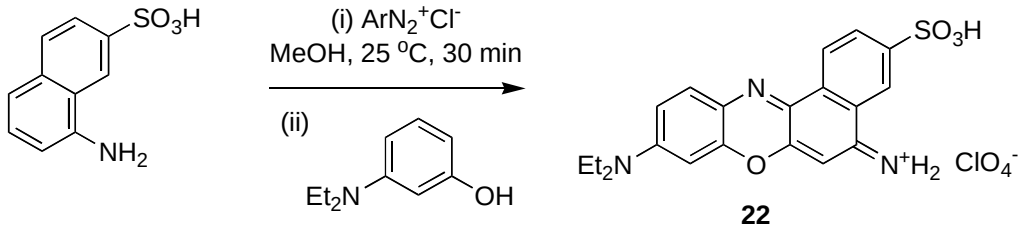


21
10 %
pH = 7.4 buffer
$\lambda_{max\ abs}$ 634 nm
$\lambda_{max\ emiss}$ 674 nm

Scheme 2.13 describes syntheses of water-soluble Nile Blue derivatives with enhanced fluorescence in aqueous media. Presence of sulfonic acid groups is reported to prevent face-to-face aggregation of dyes in water. A10-fold increase in fluorescence output in water, in comparison to Nile Blue is reported for the synthesized dyes. Short reaction time and high yields are the notable features of these dyes.[49]

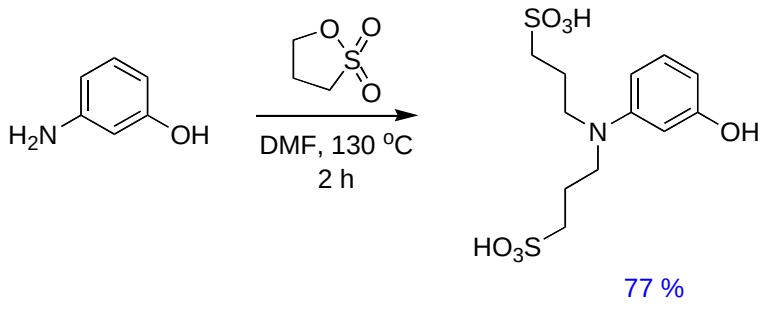
Scheme 2.13. Nile Blue derivatives with sulfonic acid groups for preventing aggregation in water.

a

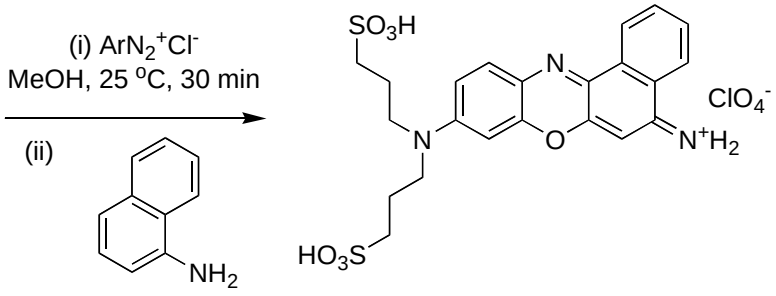


22
55 %
pH 7.4 buffer; Φ 0.03
$\lambda_{max\ abs}$ 637 nm
$\lambda_{max\ emiss}$ 677 nm

b



77 %



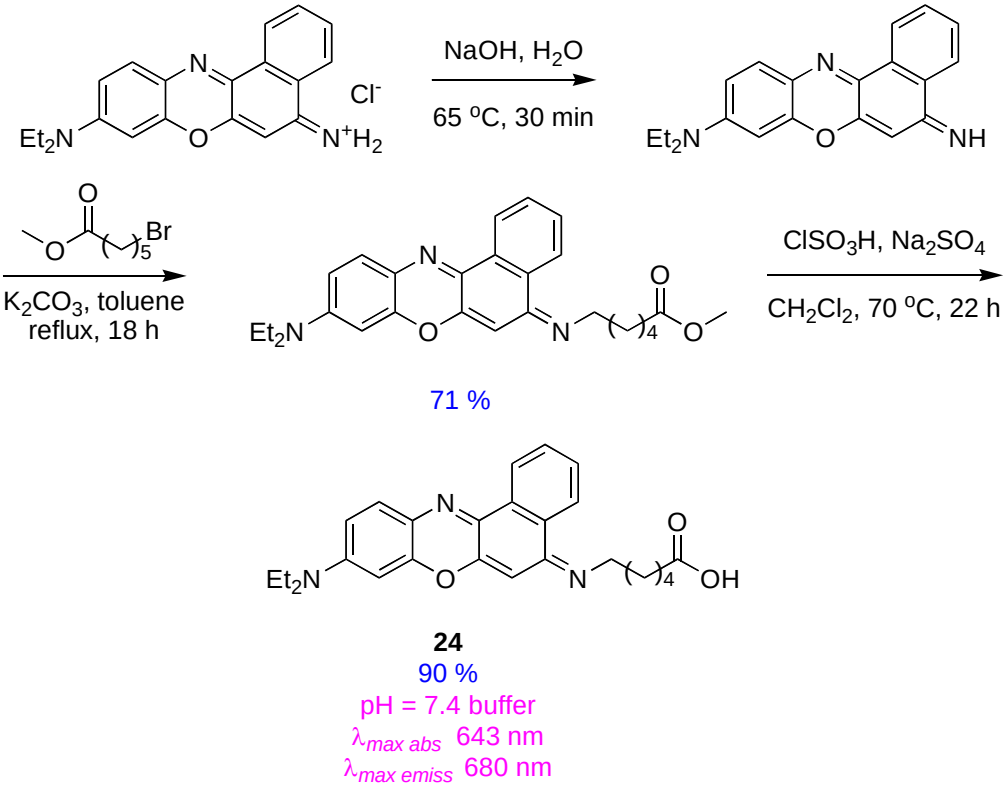
23
64 %
pH 7.4 buffer; Φ 0.10
$\lambda_{max\ abs}$ 633 nm
$\lambda_{max\ emiss}$ 675 nm

Other approaches to water-soluble Nile Blue derivatives have involved modification of the parent dye after the heterocyclic framework was assembled. For instance, Scheme 2.14a shows a patent procedure for alkylation of the 5-amino/iminium substituent; we

note that chlorosulfonic acid is an unusual choice for the ester hydrolysis reaction. In the second example, Scheme 2.14 b, an amino anthracene was used to prepare a derivative of Nile Blue that has an extended aromatic system. This modification gave a probe with absorbance and fluorescence emission shifted to the red. Unfortunately, the material **25** was probably not a pure compound since the degree of sulfonation, the regiochemistry, and the chemical/quantum yields were not given.[50]

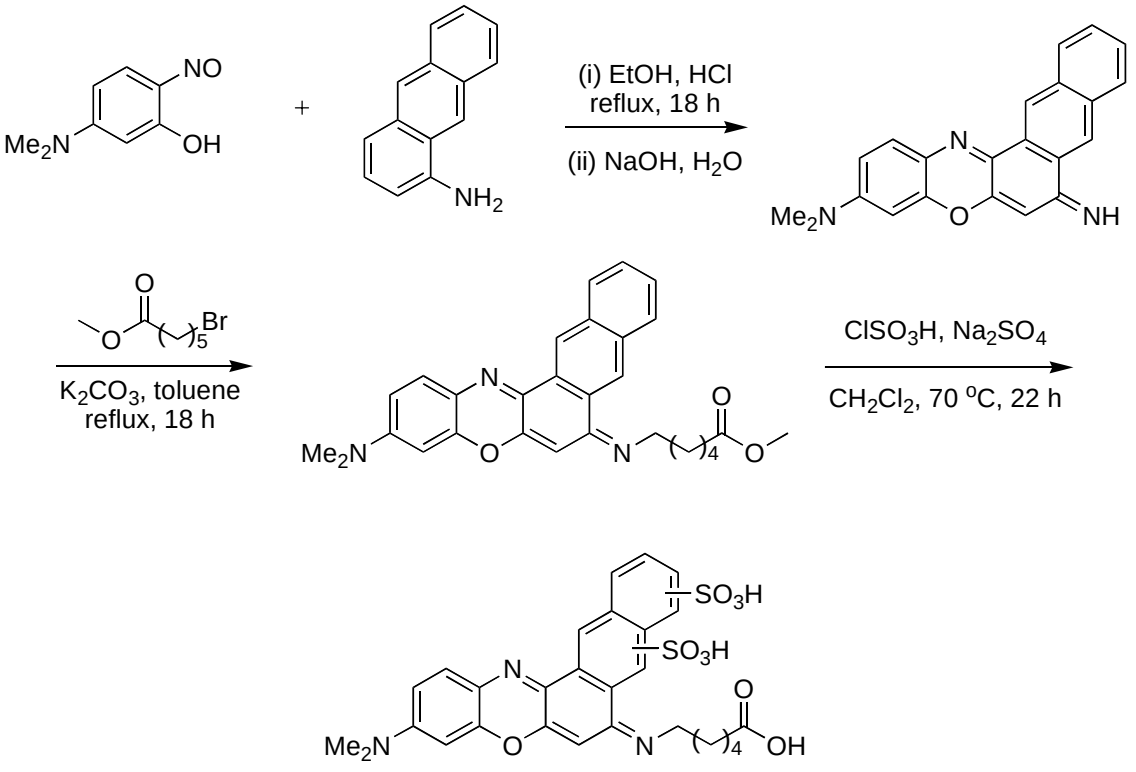
Scheme 2.14. Preparation of water-soluble Nile Red derivatives **a** with only carboxylic side chain; **b** with carboxylic acid side chain and two additional sulfonic acid groups.

a



Scheme 2.14. continued.

b



25

pH = 7.4 buffer

$\lambda_{max\ abs}$ 650 nm

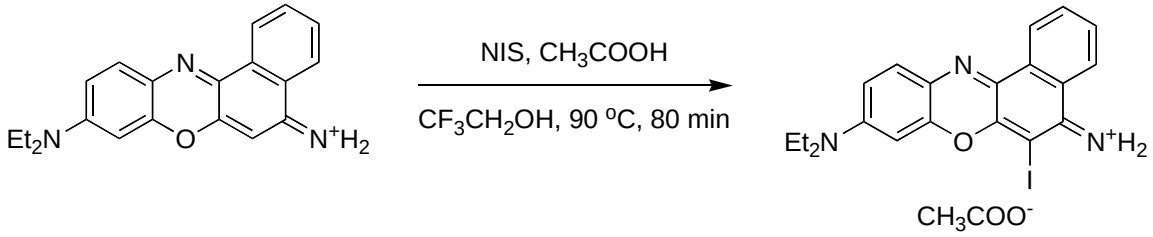
$\lambda_{max\ emiss}$ 698 nm

Direct iodination of Nile Blue is possible, and the 6-iodo derivative **26** can be prepared from this (Scheme 2.15). However, chromatography was required, the yield of the iodinated product was low, and slight variation of the conditions can lead to formation of other regioisomers. Similar considerations apply to the corresponding bromination reaction, except the product yield was better. Conversely, the *de novo* synthesis of the 2-iodo derivative **28** is unambiguous with respect to the regioisomer formed, but column chromatography is still required and the yield is also low. The diiodide **29** can be formed by a second iodination of the 2-iodide. These halogenated derivatives are potentially useful for forming more sophisticated derivatives, but they were originally

prepared as possible photosensitizers to initiate processes that are destructive to carcinogenic cells.[51,52] These photosensitizers promoted to the excited singlet state decays to the triplet state and generates singlet oxygen, which is considered to be responsible for its potent activity towards cancer cells.

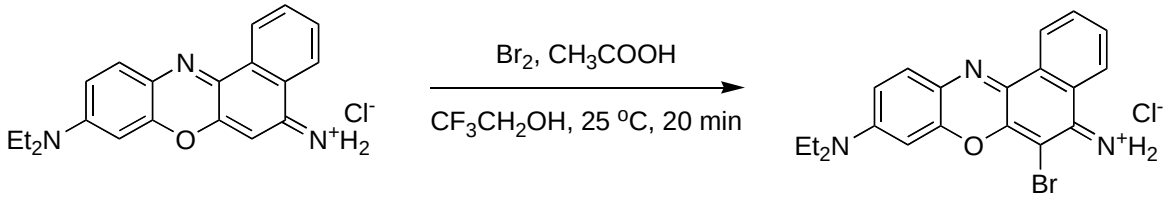
Scheme 2.15. Preparation of halogenated Nile Blue derivatives **a** 6-iodo; **b** 6-bromo; **c** 2-iodo; **d** 2,6-diiodo.

a



26
22 %
MeOH
$\lambda_{max\ abs}$ 642 nm

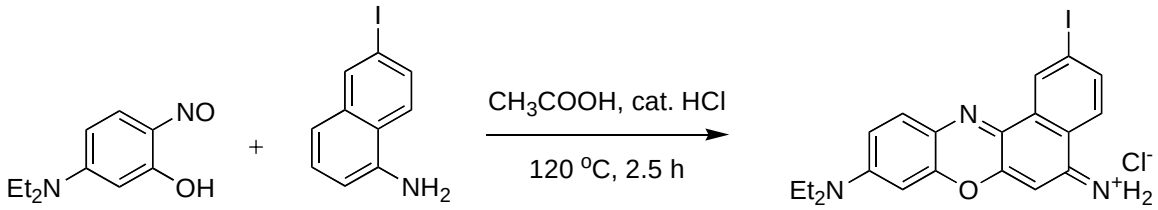
b



27
48 %
MeOH
$\lambda_{max\ abs}$ 643 nm

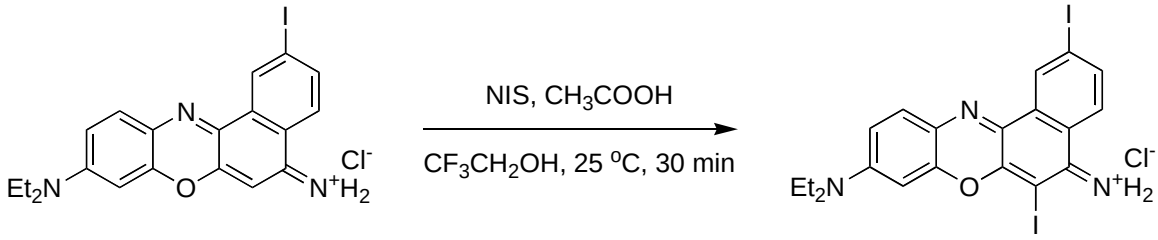
Scheme 2.15. continued.

c



28
11 %
MeOH
$\lambda_{max\ abs}$ 633 nm

d



29
38 %
MeOH
$\lambda_{max\ abs}$ 650 nm

2.4.3 Spectroscopic Properties

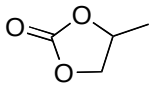
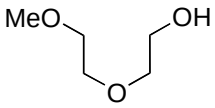
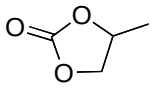
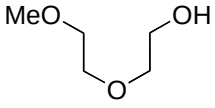
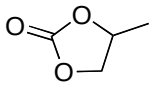
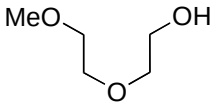
Like Nile Red **2**, Nile Blue shows progressively longer absorption and emission maxima as the solvent polarity is increased. However, the Stokes' shifts observed for the red probe **2** in different solvents is far greater; this parameter for the Blue compound can still be exceptionally high (almost 100 nm), but in polar solvents it is reduced to around 40 nm (Table 2.3).[53]

Table 2.3. UV absorption and fluorescence emission maxima of Nile Blue **3** in different solvents.

solvent	$\lambda_{max\ abs}$ **(nm)**	$\lambda_{max\ emiss}$ **(nm)**
toluene	493	574
4-chlorobenzene	503	576
acetone	499	596
DMF	504	598
$CHCl_3$	624	647
1-butanol	627	664
2-propanol	627	665
EtOH	628	667
MeOH	626	668
water	635	674
1.0 N HCl, pH 1.0	457	556
0.1 N NaOH, pH 11.0	522	668
NH_4OH, pH 13.0	524	668

Table 2.3 also reveals that the spectroscopic characteristics of Nile Blue are pH dependent. This is because under basic conditions the iminium group will be deprotonated, whereas under strongly acidic conditions the 5-amino might even become protonated.[54] Curiously, the spectroscopic properties of Nile Blue are somewhat dependent on the counter ion used Table 2.4.[55] We speculate that this could even be a reflection on intimate ion pairing influencing the solvent sphere of the dye.

Table 2.4. Study of effect of different anions on spectral properties of Nile Blue in different solvents.

anion	**solvent**	$\lambda_{max\ abs}$ **(nm)**	$\lambda_{max\ emiss}$ **(nm)**
chloride	EtOH	628	689
		632	-
		634	694
acetate	EtOH	628	689
		632	687
		634	700
benzoate	EtOH	628	691
		632	690
		634	702
iso-butanoate	EtOH	628	691
hydroxide	EtOH	515	661

The fluorescence lifetime of Nile Blue **3** in ethanol has been measured at 1.42 ns. This is shorter than the corresponding value of Nile Red (see above; 3.65 ns). The lifetime of Nile Blue is relatively invariant at dilute concentrations (10^{-3} – 10^{-8} mol dm^{-3}) but changes as the concentration is increased, and in different solvents. This is probably an indication of the interdependence of the spectroscopic properties and the degree of aggregation in solution. In support of this assertion, we note the lifetimes do not seem to be significantly impacted by the viscosity of the medium, but they are temperature dependent.[56] As far as we are aware, the two-photon cross-section of Nile Blue has not been reported.

2.5 Other Benzophenoxazine Dyes

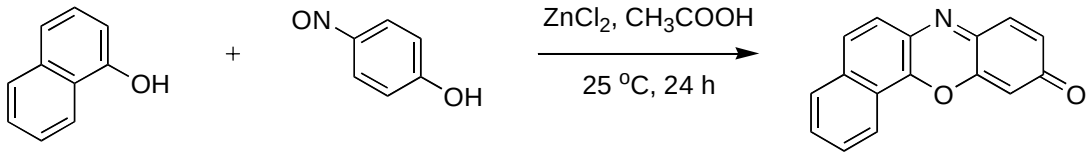
2.5.1 Introduction

There is no obvious reason why benzo[a]phenoxazines should be more fluorescent than similar compounds with different ring fusion patterns. Our interpretation of the literature is that other phenoxazines with appropriate substituents have certainly been less well studied as fluorescence probes, and are probably less synthetically accessible.

2.5.2 Synthesis

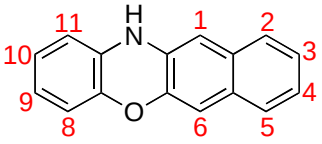
The benzo[c]phenoxazine **30** has been prepared via a route that is similar to those used for the Nile compounds **2** and **3**. 1-Naphthol is used in this synthesis (reaction 2.16)[55] rather than 2-naphthol, the isomer used for **2** and **3**. This change is necessary to obtain the different ring fusion, but it also may account for the very poor yield. This is because of the well-known reduced reactivity for 1-napthol at the 2-position in electrophilic substitution reactions, relative to the greater tendency for 2-napthol to react at the 1-position. To the best of our knowledge, the fluorescent properties of **30** have not been reported in any depth; indeed, the molecule lacks an electron donor in conjugation with the carbonyl to give it the type of extended oscillating dipole that seems to be common for fluorescent molecules.

Scheme 2.16. Synthesis of benzo[c]phenoxazinone.



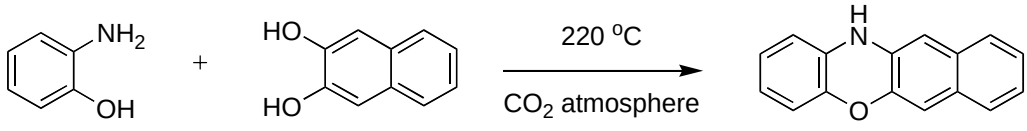
30
2-3 %
EtOH
$\lambda_{max\ abs}$ 505 nm

Benzo[b]phenoxazines are linear. Substituted derivatives of these are numbered according to the system shown below.



The linear system **31** has been prepared via the high temperature condensation process shown in Scheme 2.17.[57] This has absorption and fluorescence emission properties that are characteristic of an extended aromatic heterocycle. Like **30**, this compound does not have substituents that would allow it to be reduced to a phenoxazinone or phenoxazinium form.

Scheme 2.17. Synthesis of benzo[b]phenoxazine.



31
55 %

EtOH; Φ 0.24
$\lambda_{max\ abs}$ 365 nm
$\lambda_{max\ emiss}$ 430 nm

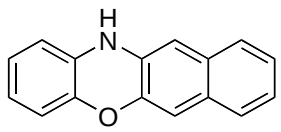
toluene; Φ 0.28
$\lambda_{max\ abs}$ 361 nm
$\lambda_{max\ emiss}$ 420 nm

Some nitro and amino derivatives of benzo[b]phenoxazines have been reported in literature, and Scheme 2.18 shows them.[58] Parts a and b show nitrosylation/oxidation

reactions that can be used to prepare nitro-substituted derivatives **33** and **34**. Predictably, these are not particularly fluorescent compounds. However, the 1,9-diaminobenzo[b]phenoxazinium **35** has the potential to be strongly fluorescent. Unfortunately, it was neutralized to **36** *then* the fluorescence properties were recorded.

Scheme 2.18. Syntheses of benzo[b]phenoxazine derivatives **a** 9-nitro; **b** 1,9-dinitro and 9-nitro-1,12-bis(benzo[b]phenoxazine); **c** 1-amino-9-iminobenzo[b]phenoxazine.

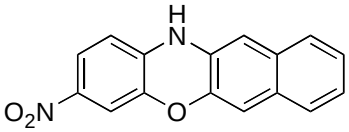
a



31

$NaNO_2$, CH_3COOH

DMF, 70 ^oC, 10 min.

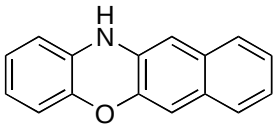


32
34 %

EtOH
$\lambda_{max\ abs}$ 436 nm
$\lambda_{max\ emiss}$ absent

toluene; Φ 0.05
$\lambda_{max\ abs}$ 438 nm
$\lambda_{max\ emiss}$ 500-530 nm

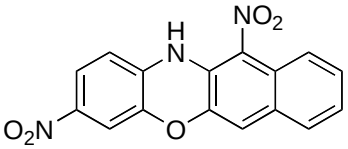
b



31

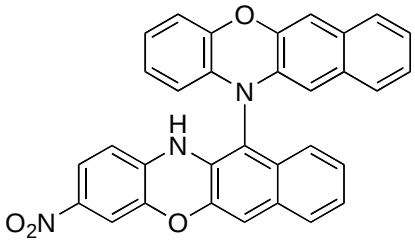
$NaNO_2$, CH_3COOH

25 ^oC, 3 h



33
30 %
toluene; Φ 0.05
$\lambda_{max\ abs}$ 470 nm
$\lambda_{max\ emiss}$ 500-610 nm

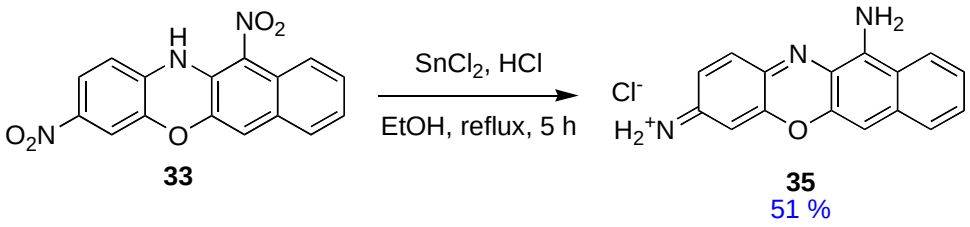
+



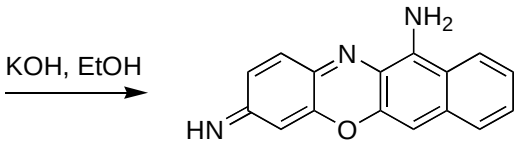
34
9 %
toluene; Φ 0.30
$\lambda_{max\ abs}$ 412 nm
$\lambda_{max\ emiss}$ 515 nm

Scheme 2.18. continued.

c



33 **35** 51 %



36
83 %
EtOH; Φ 0.05
$\lambda_{max\ abs}$ 570 nm
$\lambda_{max\ emiss}$ 760-790 nm

2.6. Aggregation

Higher the concentration of the dye, higher is the tendency to aggregate.[59-62] This is one of the major reasons for carrying out spectral studies of dyes with very dilute concentration. Aggregation can be broadly classified into two types: H and J (Figure 2.8).[63] Some dyes also form mixed aggregates, where in both type of aggregates are predominant due to which the spectral properties are greatly altered from the true value.[64] Dimers of dyes formed due to aggregation have totally different excited states. As compared to the dye monomer, dimers have two excited state energy level, one lower and other higher than the corresponding excited energy level of the monomer. Spectral properties of dimer formed are largely influenced by transition of dye molecule to these excited states. A very low fluorescence or complete quenching of fluorescence in solutions where aggregation is predominant is due to the fact that excited energy in these systems is dissipated in nonradiative processes.

2.6.1 H Type Aggregates

These are formed by dyes, which have tendency for parallel stacking also known as Sandwich type stacking. In dimers formed in such manner transition is possible only to the highest excited energy level, which results in a blue shifted absorption spectrum with respect to the dye monomer. Since transition between lowest energy excited level and ground state is not allowed these types of dimers are virtually non-fluorescent. Most benzophenoxazine dyes form H type dimers in aqueous media.

2.6.2 J Type Aggregates

Head to tail stacking of dye molecules in solution is termed as J type aggregation. In these dimers transition is possible only to the lowest energy excited state and absorption spectrum is red shifted with respect to the dye monomer due to low energy requirement for the observed transition. Such type of aggregates does not quench fluorescence completely.

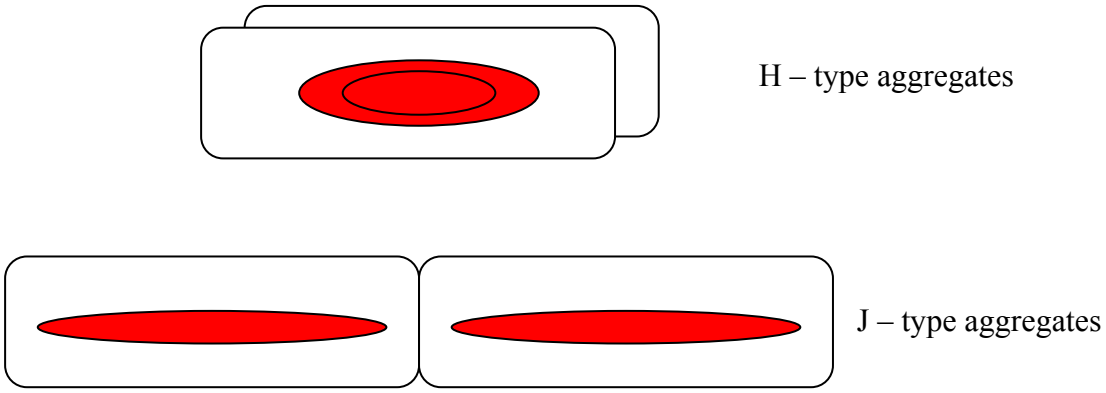


Figure 2.8. Types of dye aggregates.

2.6.3 Excimer Formation in Dyes

At high dye concentration, the fluorescence intensity is quenched by the formation of dye excimers. Excimers are dye molecules stacked together in the form of aggregates and predominant in solutions of higher concentration.[65,66] Emission and absorption of excimers is shifted with respect to monomer dye. Fluorescence emission is quenched due

to excimer formation and in some cases broadening of emission band is observed. Absorption spectra are red shifted in most cases. To avoid discrepancies due to excimer formation measurement is always done in very dilute solutions of the order of 10^{-5} M.

2.7 Conclusions

Current knowledge of fluorescent benzophenoxazine-derived probes is based almost on the benzo[a]phenoxazine ring fusion series. Almost all the syntheses feature high temperature condensation methods, and none feature contemporary synthetic methods like, for example, Buchwald-Hartwig couplings to introduce amine substituents. In fact, a lot of the synthetic methods reported are based on the procedures that are now over a century old. This, and the prevalence of patent literature in this area, means that many of the experimental procedures presented are difficult to follow, and complete spectroscopic data is rarely recorded.

Nile Red and its derivatives have some interesting spectroscopic properties (long wavelength emissions, large Stokes' shifts) but most compounds in this series have limited water solubilities. There are, however, some recent efforts to make modified compounds to redress this. Nile Blue and its derivatives tend to be more water-soluble.
Relatively little work has been done to modify benzophenoxazine dyes so that they emit even further to the red: the longest wavelength emission in the existing probes is about 700 nm. To this end, it would be useful to have access to more functionalized compounds that can be prepared easily on a gram scale, like 2-hydroxy Nile Red **6**. Other modifications might be used to give derivatives with enhanced extinction coefficients (these tend to be 10,000 or less), or improved two photon-cross-sections. These types of developments would be facilitated by more detailed studies of fundamental reactions that can be used to modify these compounds, *eg* halogenation and nitration.

As far as we can see, there is no good reason why virtually all fluorescent dyes in this series are benzo[a]phenoxazine derivatives, and not any other ring fusion. This appears to be merely a question of synthetic availability. Overall, benzophenoxazine-based probes are an intriguing subset of the fluorescent dye toolbox. They have some obvious drawbacks for applications in biotechnology, but the developments that need to be made to make more useful labels of this type are reasonably well defined.

CHAPTER III
WATER-SOLUBLE NILE RED DERIVATIVES

3.1 Introduction

Two interesting questions arise from the work on Nile Red derivatives that are described above. First, would water-soluble derivatives of Nile Red have high quantum yields in aqueous media? As already stated, most Nile Red derivatives do not fluoresce strongly in polar media, but this could be due to aggregations effects that might be avoided if the dye has some intrinsic water solubility. Second, do water-soluble Nile Red derivatives also show the pronounced bathochromic shift in polar media that is observed for compounds in the series with little or no significant aqueous solubility?

No significantly water-soluble Nile Red derivatives had been prepared to answer the questions posed above. The primary focus of this research is development of Nile Red derivatives that have:

(i) water solubility
(ii) significant quantum yields in aqueous media
(iii) fluoresce over 600 nm (the more useful region for imaging in cells and tissues)
(iv) functional groups that facilitate attachment to biomolecules.

Water solubility was identified as the critical factor to reach most of these goals, for the following reason. It is generally accepted that the reduced quantum yields of Nile Red in polar media are due to aggregation of the hydrophobic, flat, benzo[a]phenoxazinone core structures.[67] Logically, this type of aggregation could be suppressed or prevented by introducing water-solubilising groups into the dye structure. In the context of this research, that might impart water-solubility, increase the quantum yields, and facilitate the attachment of these dyes to water-soluble biomolecules. Effects on fluorescence emission maxima that occur by making the Nile Red scaffold water-soluble were unknowns that had to be tested, but we speculated that the desirable red-shift in the

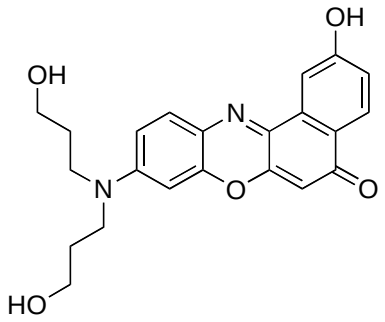
fluorescence maxima that is observed for Nile Red in polar media was less likely to be due to an aggregation effect than to stabilization of a relatively polarized excited state.

3.2 Results and Discussion (Syntheses and Spectral Studies)

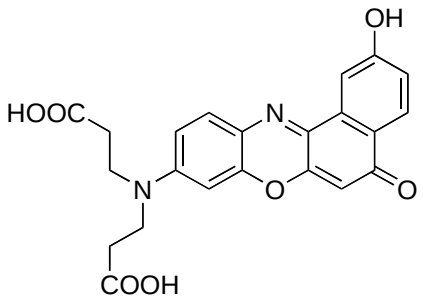
The considerations discussed above led us to attempt syntheses of the Nile Red derivatives **41**,**44**,**48** and investigate their fluorescence properties in aqueous media (if possible). As stated above, the immediate objectives of this study were to identify water-soluble Nile Red derivatives that exhibit fluorescence emission maxima above 600 nm and significant quantum yields in aqueous media; dyes of this type could have general applications in biotechnology. In the more focused context of our research on fluorescent dyes, *ie* development of the "through-bond energy transfer cassettes",[68-70] water-soluble Nile Red derivatives could be useful acceptors for syntheses of donor-acceptor cassettes. Preparation of water-soluble Nile Red derivatives, which can function either as donor or acceptor part in through bond energy transfer cassette for cellular imaging. At the onset of this work we envisioned that it would be convenient to introduce water solubilising group at the initial stage of synthesis, so that the dyes can be easily purified. Classical condensation routes were used to prepare the three Nile Red derivatives **41**,**44**,**48**. These were designed to be water-soluble, but, surprisingly, the diol/phenol **41** was not, even in aqueous base. The other two compounds do have very good water solubilites. Their spectroscopic properties are discussed in the next sub-section; but the data shown in Table 3.1 shows that the answers to both these questions were affirmative for compounds **44** and **48**.

The second part of this research involves syntheses of alkyne and iodo derivatives of Nile Red, which can serve as useful synthons for group members.

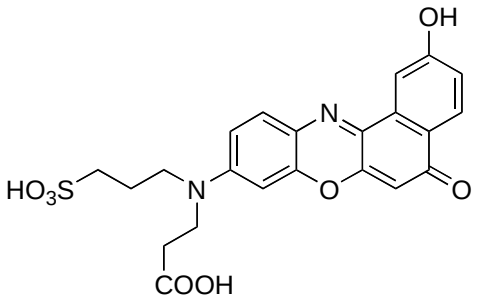
3.2.1 Syntheses



41



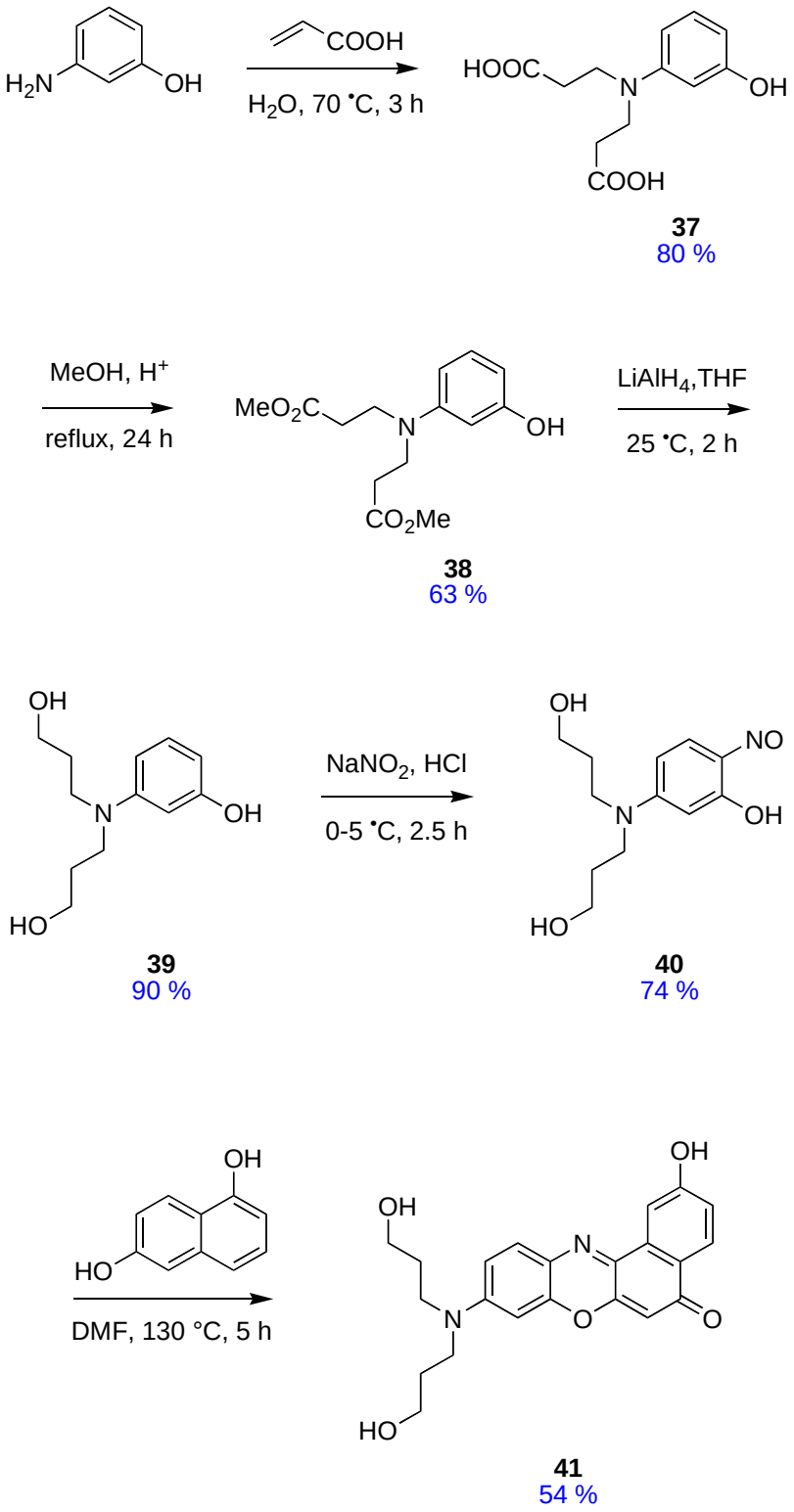
44



48

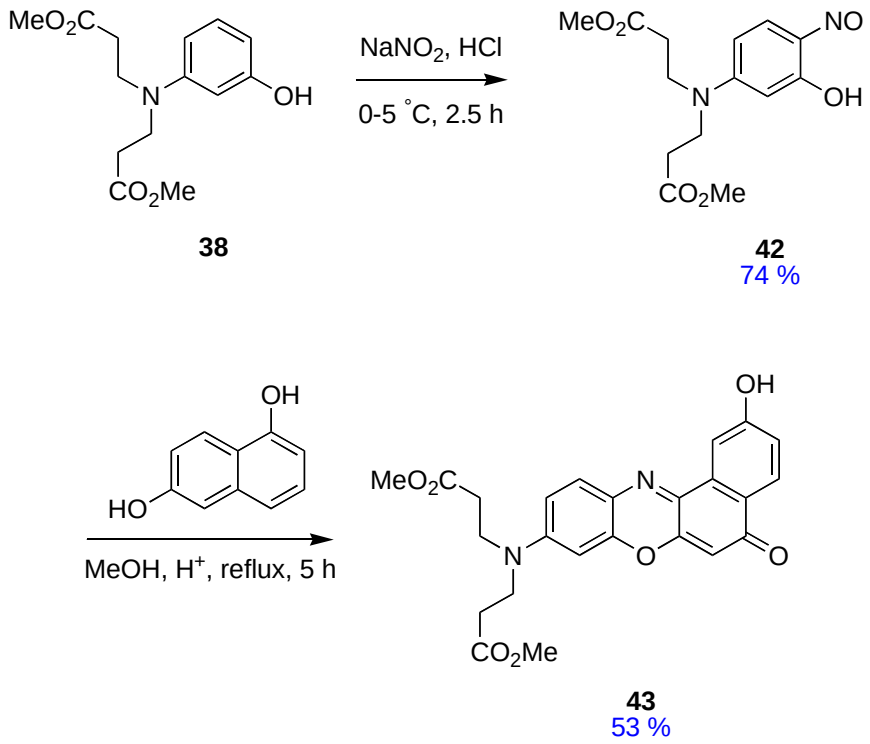
The synthesis developed for target dye **41** is shown in Scheme 3.1. 3-Aminophenol is known to undergo a facile Michael addition with acrylic acid to give a product that crystallizes out of the reaction mixture. This reaction gives good quality material on a multigram scale without necessitating chromatography. Esterification of the diacid formed in this way gives the diester **38**, which was isolated in good yields by an extraction procedure. Lithium aluminum hydride reduction of **38** gave the corresponding diol **39** that was then nitrosylated under aqueous conditions. The nitroso product **40** proved to be somewhat unstable so it was used without further purification. Assembly of the benzo[a]phenoxazinone skeleton was achieved via condensation of the nitroso compound **40** with 1,6-dihydroxynaphthalene. The product **41** was isolated via flash chromatography. This was the only chromatographic step used in the synthesis so it proved convenient to make this product on a several gram scale.

Scheme 3.1. Synthesis of Nile Red derivative **41**.

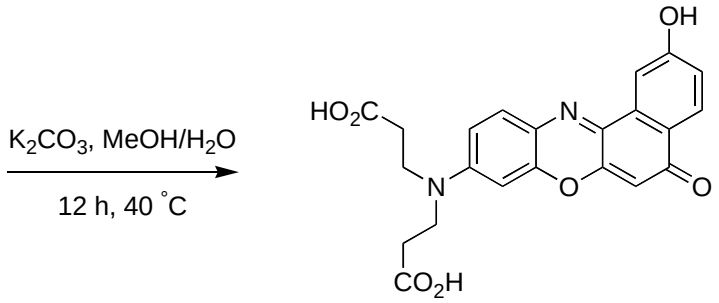


Scheme 3.2 shows the synthesis that was developed for the target dye **44**. Nitrosylation of the diester **38** (see below) afforded **42**, which was used without purification due to stability and hygroscopicity issues. Condensation of this nitroso compound with 1,6-dihydroxynaphthalene gave the benzo[a]phenoxazinone **43**. This compound was isolated via flash chromatography (the first and only one in the sequence). Aqueous hydrolysis of the diester functionalities of **43** gave the corresponding diacid **44** that could be isolated via a simple acid base extraction procedure.

Scheme 3.2. Synthesis of water-soluble Nile Red derivative **44**.



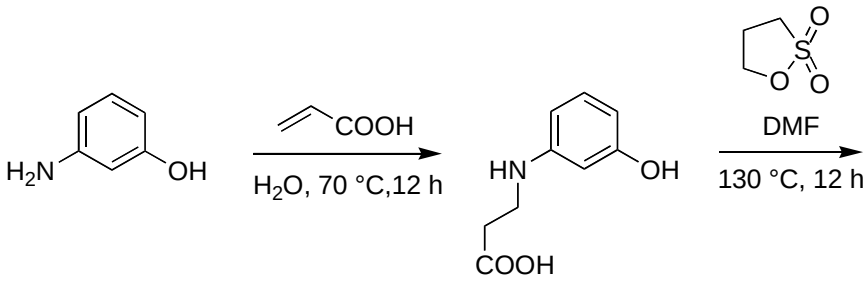
Scheme 3.2. continued.



44
60 %

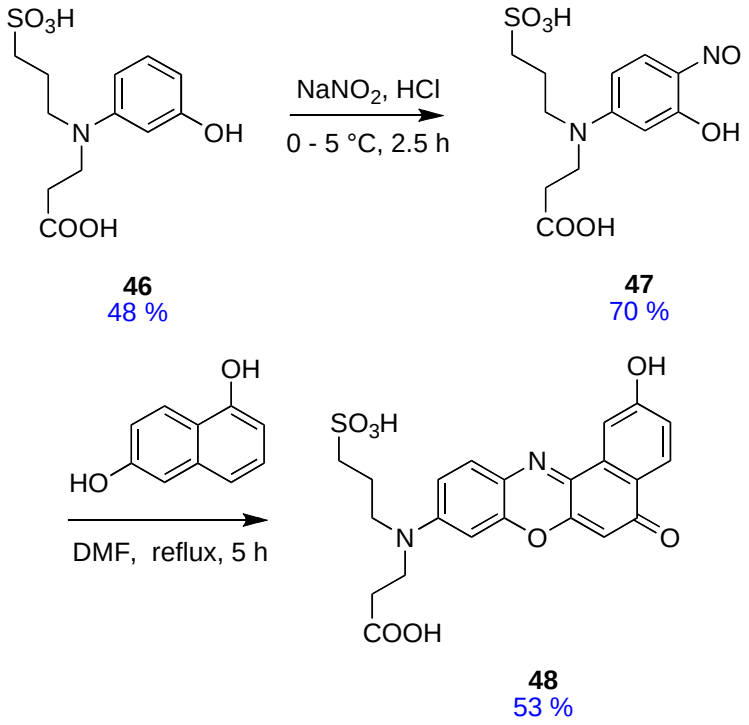
Preparation of the final target, the sulfonic acid **48**, is outlined in the Scheme 3.3. Michael addition of acrylic acid with 3-aminophenol in near stochiometric amounts gave the monoadduct **45**. Reaction of this amine with 1,3-propane sultone gave the ring-opened product **46**. This was nitrosylated to give the very unstable nitroso compound **47**, which was then condensed as above without delay. Unfortunately, flash chromatography was used in each step of this procedure but, even so, the target material **48** was isolated in about a 250 mg scale. Repetition of the sequence on a larger scale was not attempted here, but we suspect that 1 – 3 g of material could easily be made via this approach.

Scheme 3.3. Synthesis of water-soluble Nile Red derivative **48.**



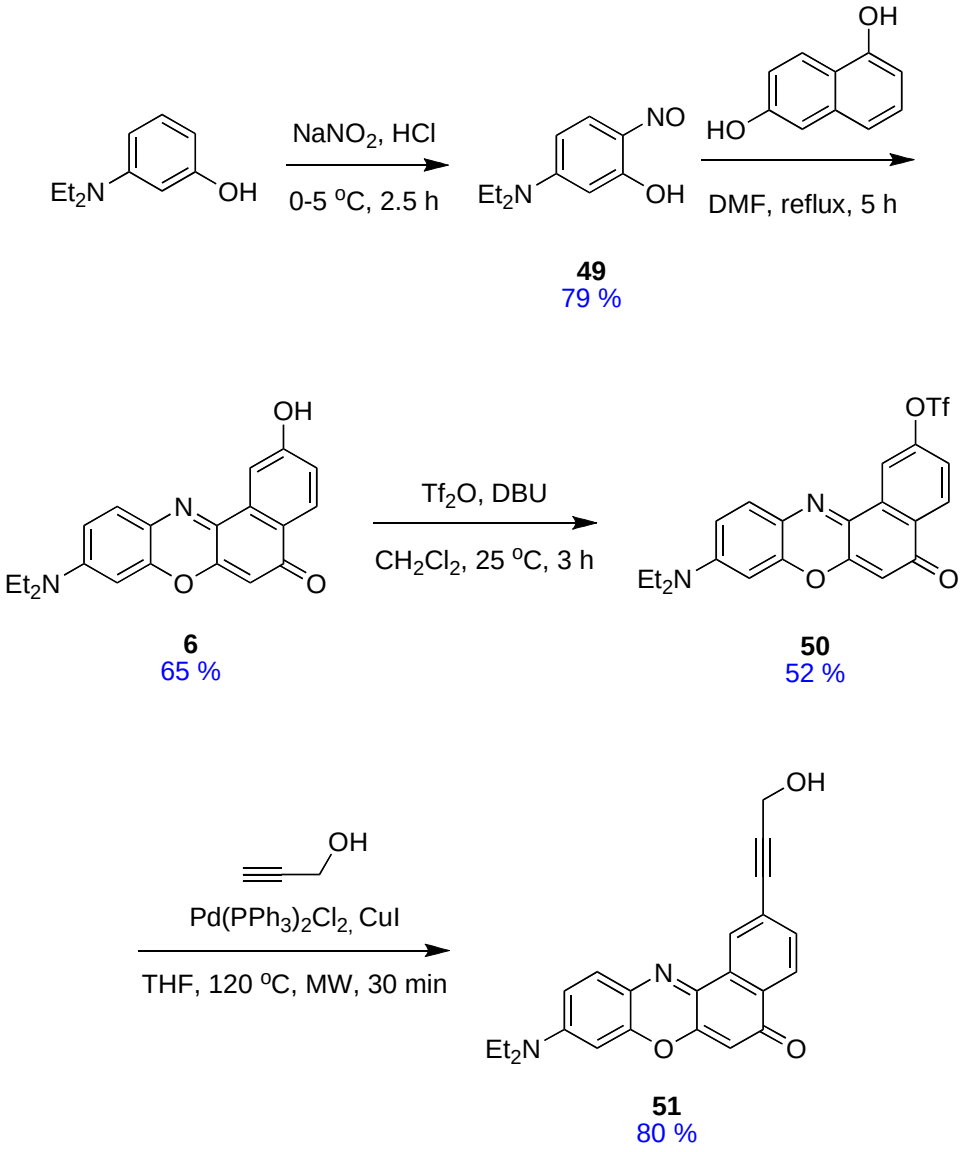
45
40 %

Scheme 3.3. continued

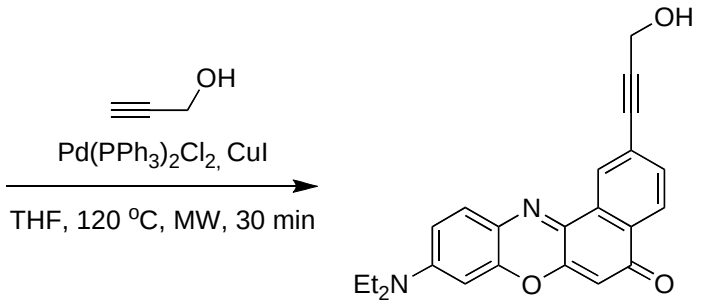


The alkyne **52** was prepared from 2-hydroxy Nile Red that was prepared following a published procedure. Triflation with triflic anhydride yielded a moderate yield of the triflate. This is because triflic anhydride is extremely moisture sensitive. Even with use of distilled anhydrous solvent the yield didn't improve much. The alkyne was obtained by Sonogashira coupling in presence of Pd (II) and copper iodide catalyst, of Propargyl alcohol with the corresponding triflate.[71] Deprotection via oxidation with manganese dioxide in presence of potassium hydroxide resulted in very low yield of the product. In spite of increasing the equivalence of manganese dioxide, yield did not show any improvement. This problem was overcome by coupling the triflate with TMS alkyne instead of propargyl alcohol as shown in the following synthetic Scheme.

Scheme 3.4. Synthesis of 9-(diethyl amino)-2-ethynyl-5H-benzo[a]phenoxazin-5-one via propargyl alcohol.



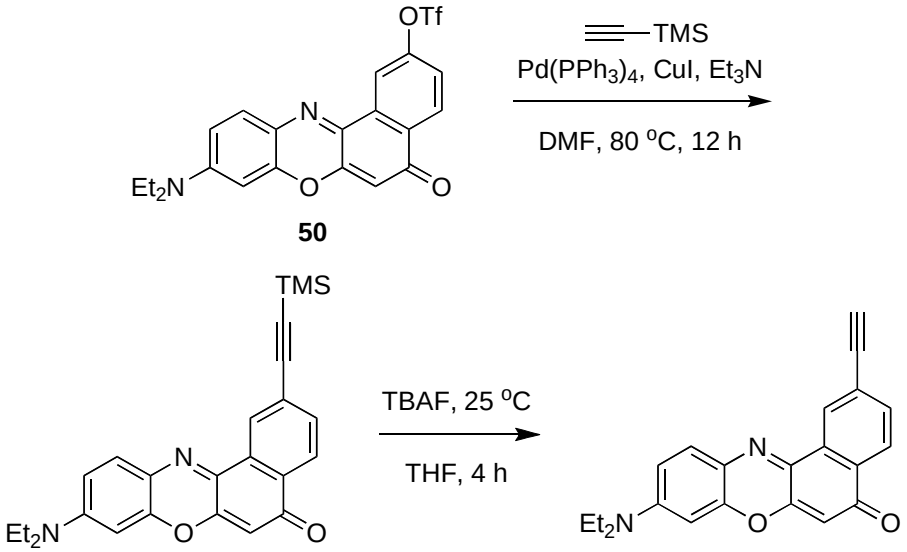
Scheme 3.4. continued.



51
80 %

In order to improve the yield of the alkyne, Sonogashira coupling was carried out with TMS alkyne instead of propargyl alcohol. Use of Pd (0) catalyst instead of Pd (II) did not show any increase in yield. Deprotection of TMS alkyne with TBAF proved very efficient yielding the alkyne in very good yield.

Scheme 3.5. Synthesis of 9-(diethylamino)-2-ethynyl-5H-benzo[a]phenoxazin-5-one via TMS alkyne.



50

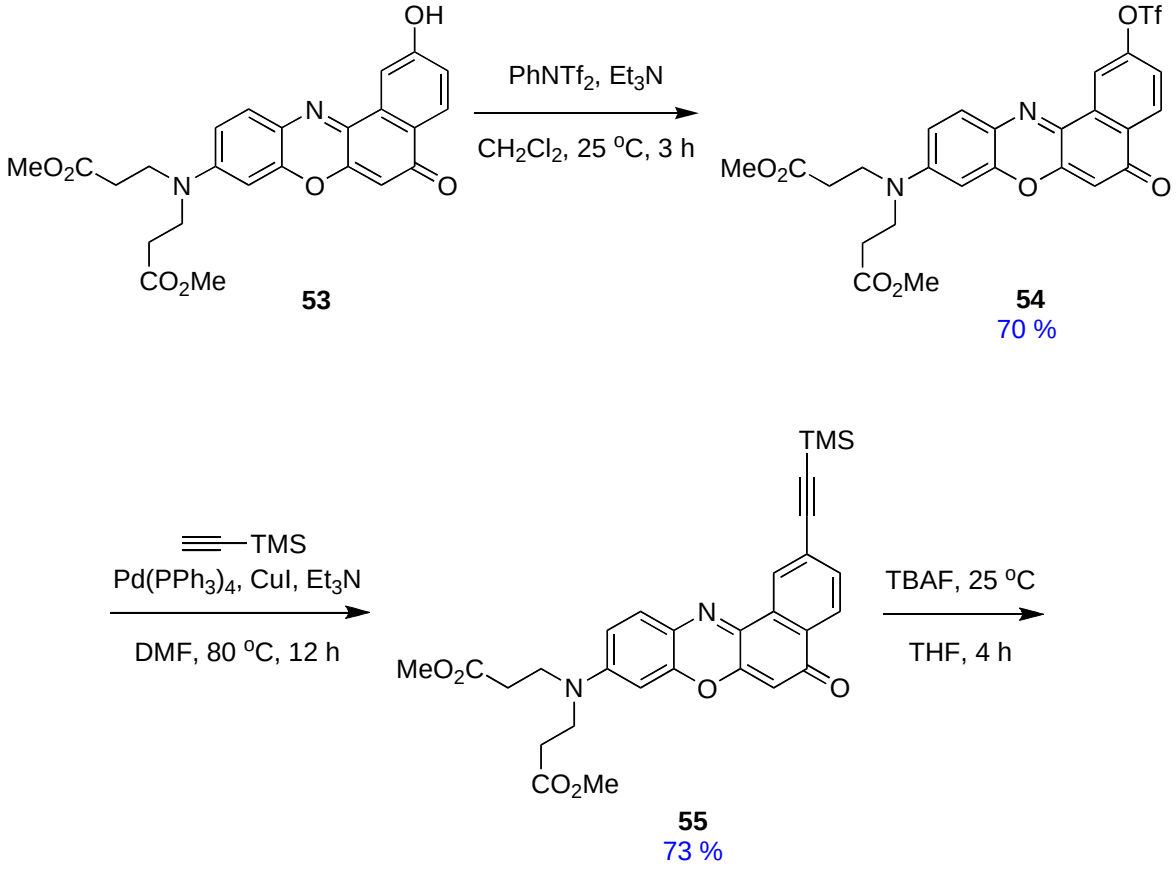
53
71 %

52
80 %

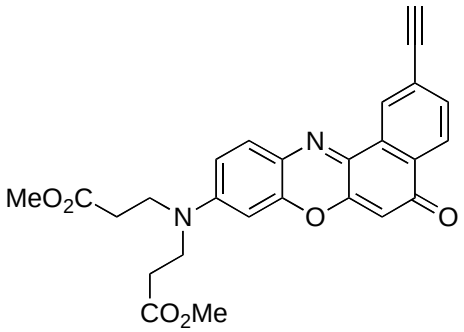
The alkyne **56** was synthesized by the same protocol using Sonogashira coupling. There are some key changes in this Scheme, which makes it more efficient than the previous

Scheme. Triflation was carried using phenyltriflamide instead of triflic anhydride, which is less moisture sensitive than triflic anhydride that resulted in improved yield of the triflate. Sonogashira coupling with Pd (II) catalyst resulted in very poor yield. Use of Pd (0) catalyst and employing freeze thaw method for removing oxygen, the yield was considerably improved. Deprotection to corresponding alkyne was accomplished with TBAF. The alkyne is not stable at room temperature and therefore has to be stored at 0 ^{o}C in a freezer.

Scheme 3.6. Synthesis of 9-[*N*,*N*-bis(2-(methoxycarbonyl)ethyl)amino]-2-ethynyl-5H-benzo[a]phenoxazin-5-one.



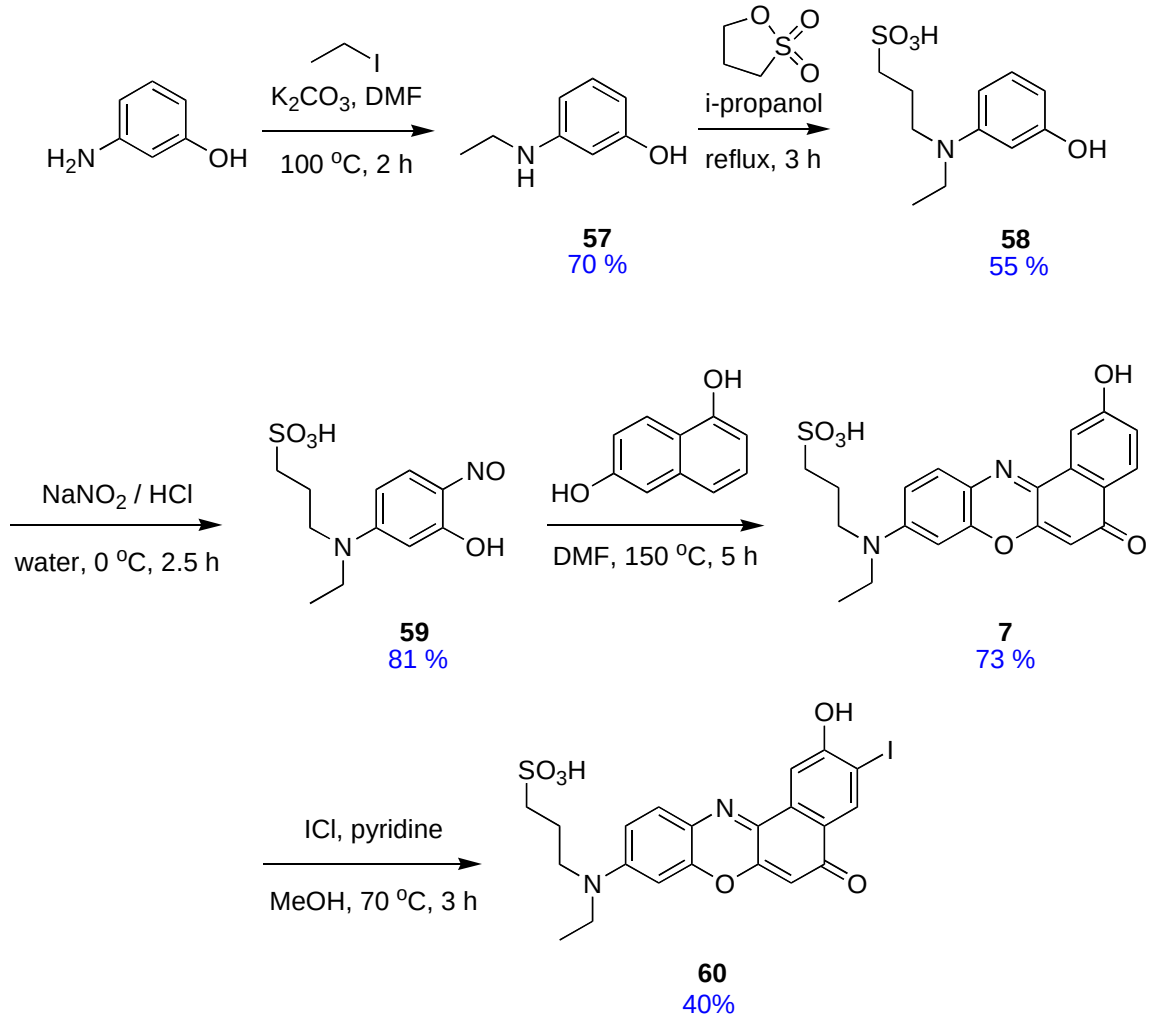
Scheme 3.6. continued.



56
80 %

The sulphonic acid derivative of Nile Red, **60** was prepared by following a published procedure. To make it a useful synthon for preparation of through bond energy transfer cassette further derivatization was necessary. Attempted synthesis of alkyne from the above dye failed due to difficulty in getting the triflated dye pure. Iodination was the most viable option since coupling of the iodo derivative with an alkyne via Sonogashira coupling to obtain through bond energy transfer cassette can be carried out at room temperature. Iodination with iodine and potassium carbonate failed to yield any desired product. Even though a high yield was not obtained using iodine monochloride and pyridine, the yield obtained was sufficient to obtain the iodinated dye in sufficient quantity.[72]

Scheme 3.7. Synthesis of 3-(*N*-ethyl-*N*-(2-hydroxy-3-iodo-5-oxo-5H-benzo[a]phenoxazin-9-yl)amino)propane-1-sulfonic acid.



3.2.2 Spectroscopic Studies

Absorbtion and emission spectra for the three dyes **41**, **44**, **48** were recorded in ethanol. The spectra for water-soluble dyes were also obtained in aqueous buffer (1.0 M phosphate buffer at pH = 7.4, or 1.0 M borate buffer at pH = 9.0). Unless otherwise noted, absorbtion spectra were recorded at a concentration of 10^{-3} M and the fluorescence spectra at 10^{-5} M to avoid aggregation and inner filter effect.

Absorbtion/Emission Spectra of Dyes in Different Solvents

In water, the Nile Red derivative **41** showed hardly any solubility at all. Surprisingly, despite having a phenolic-OH, this compound was still insoluble in pH 9 borate buffer (1.0 M) or in sodium carbonate solution. Consequently, spectroscopic data for this compound were only recorded in EtOH (Table 3.1). Compound **41** was shown to have a similar absorption and emission wavelength maxima, and a slightly better quantum yield than Nile Red **2** or the hydroxy Nile Red derivative **6** in EtOH.

Compound **44** was more interesting for the purposes of this study. This dye has good solubility (for spectroscopic purposes) in EtOH, water, and 1M phosphate buffer at pH 7.4, and in 1M borate buffer at pH 9.0. In EtOH, its spectral properties are similar to compounds **2**, **6**, and **41** except that the molar absorptivity was measured to be approximately one fifth of **41**, and the Stokes shift was slightly greater (**44**, 110 nm; **41**, 90 nm). In aqueous buffers, however, the Stokes shifts for **41** and **44** were very similar. The quantum yield of **44** at pH 7.4 was quite good (0.33), but at pH 9 it reduced to 0.07. This appears to correlate with deprotonation of the phenolic hydroxyl, possibly corresponding to quenching of the fluorescence via charge transfer.[73] The full width at half maximum height (fwhm) of the emission for **44** is 70 nm or less. Sharp fluorescence emissions facilitate resolution in experiments involving more than one probe. The fwhm for **44** compares favorably with fwhm reported for the water-soluble Nile Blue derivatives mentioned earlier (they have fwhm in the range of 86 – 93 nm).[49]

The sulfonic acid dye **48** also had good solubility in the three media studied (Table 3.1). In EtOH its spectroscopic properties were unexceptional, and the quantum yield was quite good (0.42). In both the neutral and basic buffers the absorption and emission maxima were red-shifted but by only 18 nm or less relative to ethanolic solutions of the dye. Interestingly, the quantum yield of **48** was comparable in EtOH or pH 7.4 buffer, and still quite good (0.18) at pH 9, and almost double that of **44** in this medium.

Moreover, the fwhm of the fluorescence emission peaks for the aqueous buffers were slightly lower than compound **44**.

a

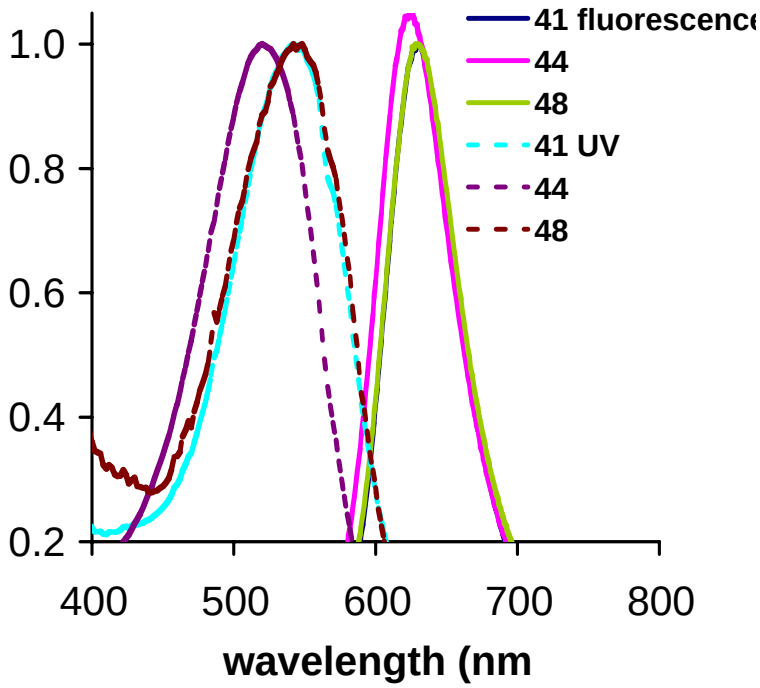
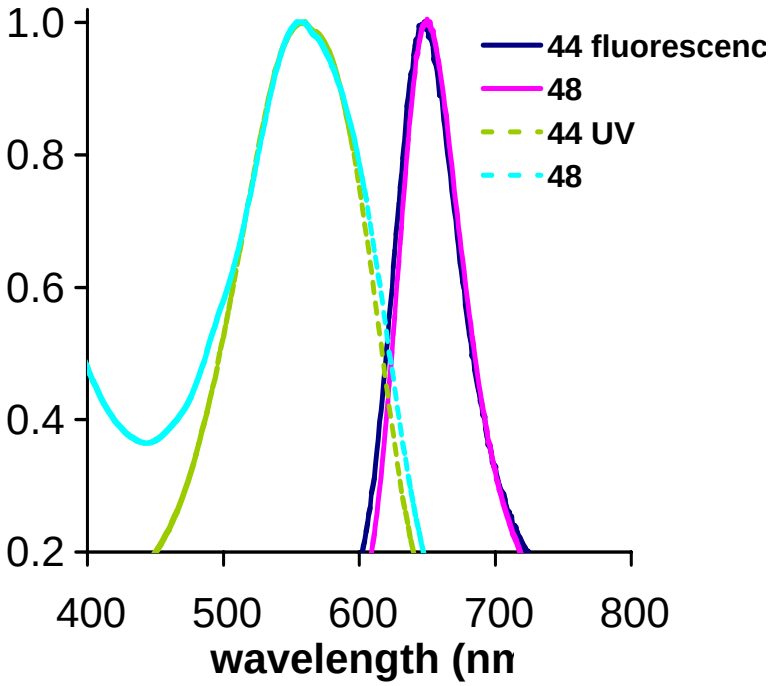


Figure 3.1. Absorption at 10^{-3} M (dashed lines) and fluorescence at 10^{-5} M (solid lines), of: **a** dyes **41**, **44**, **48** in ethanol; **b** dyes **44**, **48** in phosphate buffer (pH = 7.4); and **c** in borate buffer (pH = 9.0).

b



c

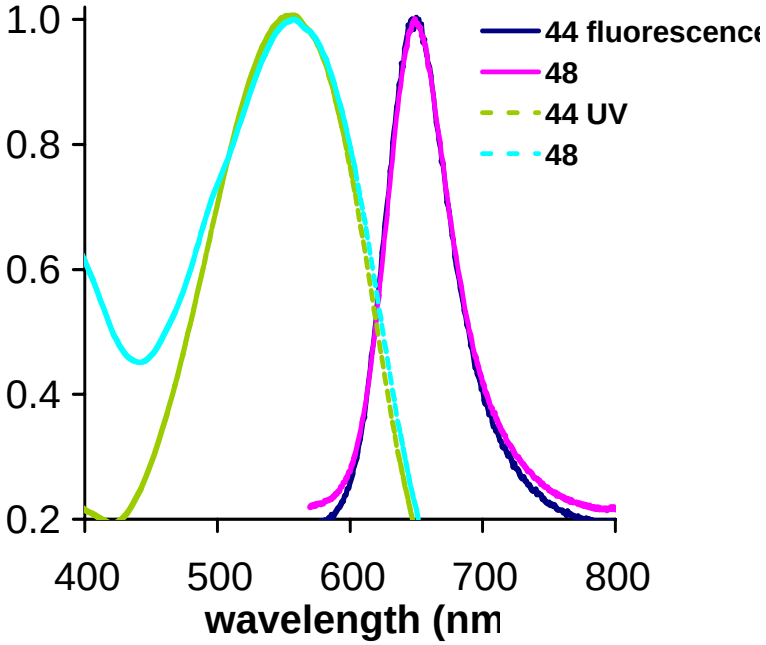


Figure 3.1. Continued

3.2.2.1 Determination of Relative Quantum Yield

One of the major problems with benzophenoxazine dyes is its low quantum yield in aqueous media. This work is directed towards improving its water solubility and also quantum yield. Measurement of quantum yield therefore is an important aspect of this work. Quantum yield measurement was done in both organic and aqueous media.

Relative quantum yield was measured rather than absolute quantum yield which is extremely difficult.[74] Rhodamine B (Φ: 0.97 in ethanol) and Rhodamine 101 (Φ: 1.0 in ethanol) was used as standard.[75]

Relative quantum yield was measured on a Photon Counting Spectrofluorometer PCI SSI instrument equipped with a R928P photomultiplier tube, which is sensitive up to 850-900 nm. The slit width was 0.5 nm for both excitation and emission. Measurements were made in both organic as well as aqueous solvent. Following equation was used to calculate quantum yield.

$$\Phi_x = \Phi_{st} (I_x/I_{st}) (A_{st}/A_x) (\eta_x^2/\eta_{st}^2)$$

where Φ_{st} is the reported quantum yield of the standard, **I** is the integrated emission spectra, **A** is the absorbance at the excitation wavelength and η is the refractive index of the solvent used (η =1 if same solvent). X subscript denotes unknown, and st denotes standard. Rhodamine B (Φ = 0.97 in ethanol) and Rhodamine 101 (Φ = 1.0 in ethanol) were used as standards.

Table 3.1. Spectral characteristics of dyes in different solvents.

dye	λ_{abs} (nm)	ε ($M^{-1}cm^{-1}$)	$\lambda_{em.}$ (nm)	fwhm (nm)	Φ[a]	solvent
41	542	24854	631	60	0.56	EtOH
44	520	4984	632	59	0.43	EtOH
44	560	7529	648	63	0.33	phos[b] 7.4
44	556	3400	647	70	0.07	bor[c] 9.0
48	548	5283	632	56	0.42	EtOH
48	558	7876	652	60	0.37	Phos[b] 7.4
48	556	6209	650	66	0.18	bor[c] 9.0

[a] Measured as specified previously.[76] [b] pH 7.4 phosphate buffer. [c] pH 9.0 borate buffer.

Calculated relative quantum yield for the three dyes are superior to those reported in the literature. Quantum yield is lower in aqueous media as compared to that in organic solvent. Also with increase in pH a drastic reduction in quantum yield is observed. We believe this is due to the anionic form of the dye, which is predominant at higher pH. The anionic form of the dye fluoresces less as reported[73] and therefore the quantum yield is low. The full width at half maximum height is much smaller than Nile Red proving lesser tendency of synthesized dyes to aggregate in aqueous media.

3.3 Conclusion

Two new water-soluble Nile Red derivatives and a water insoluble Nile Red derivative were synthesized. All dyes were obtained in moderate yield. These dyes can be used as either donor or acceptor part for the synthesis of through bond energy transfer cassettes. These dyes exhibit moderate to good quantum yield in aqueous media, which makes it useful for cellular imaging and other applications in which solubility of dyes in water is very important. Ironically the fluorescence out put of these dyes are very low at higher pH rendering them less useful for studies which involve use of higher pH. Nevertheless an improved fluorescence output around pH 7-8 is very much desirable since most biological studies are done at this pH. Presence of a sulphonic acid group is reported to reduce aggregation in aqueous media. Synthesized Nile Red derivative with sulphonic acid group shows better fluorescence properties in aqueous media as compared to the other two dyes, which proves that presence of water solubilising sulphonic acid group does prevent aggregation.

Alkyne derivatives of Nile Red constructed via Sonogashira coupling can serve as useful synthons for construction of water-soluble energy transfer cassettes. These alkynes can serve as long wavelength acceptors or donors in the syntheses of cassette. Iodo derivative of Nile Red is a potential synthon for further synthetic manipulation.

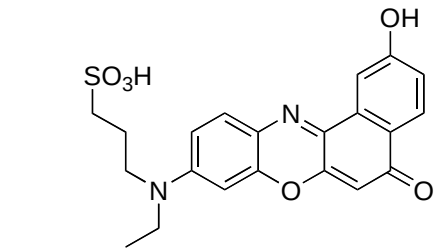
CHAPTER IV
OUTLOOK AND CONCLUSION

4.1 Outlook

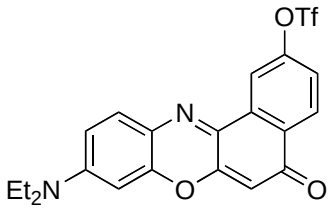
There exists further potentially interesting synthetic options with the synthesized water-soluble Nile Red derivatives. Most of these synthetic modifications will be useful in incorporating these fluorescent probes for biological applications. A further improvement in spectral properties especially in aqueous media should be the focus of research in this area. At the same time a possible means to shift the fluorescence output towards 700nm will be highly desirable. Discussed below are some of these viable synthetic routes to enhance the spectral properties of Nile Red derivatives and a means to incorporate these derivatives into water-soluble through-bond energy transfer cassettes.

4.1.1 Useful Nile Red Derivatives with Good Yield and Reproducibility

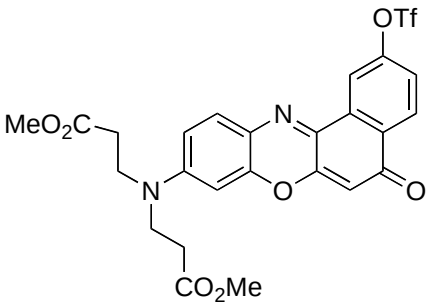
Given below is a list of compounds, which are relatively easy to synthesize in good yield (above 70%) and could be easily made in multigram scales.



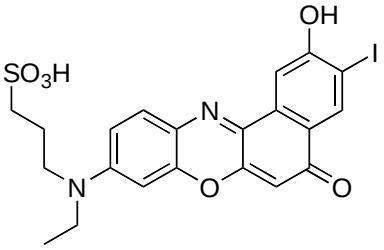
7



50



54

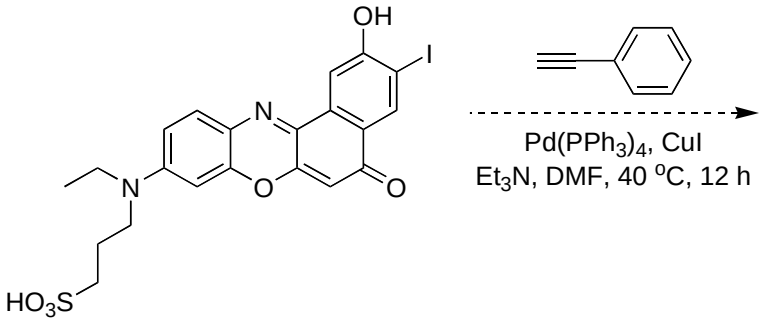


60

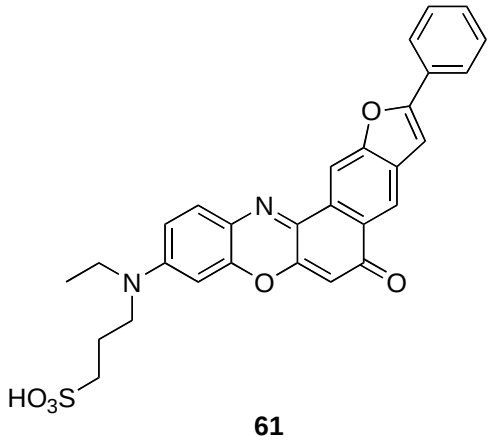
4.1.2 Dyes with Extended Conjugation with Emission between 650-700 nm

The absorption and emission maxima of the synthesized Nile Red derivatives can be further shifted towards the far visible near IR region by extending their conjugation. One such modification can be shown in the below Scheme. We hypothesize that classic Sonogashira coupling with alkynyl benzene, followed by intramolecular ring closure[77,78] will result in compound **61**, which should absorb near 560-570 nm and emit around 660-670 nm.

Scheme 4.1. Proposed synthesis of water-soluble Nile Red derivative with extended conjugation.



60

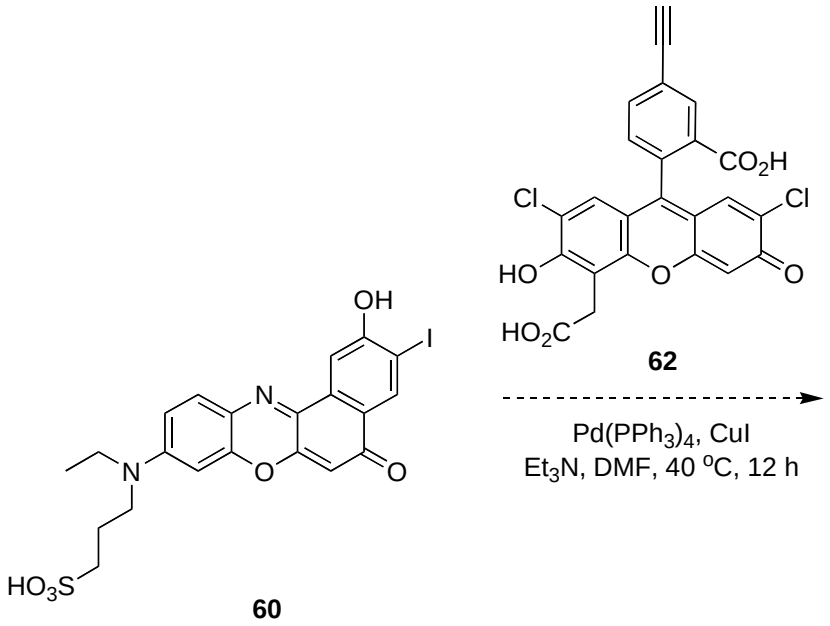


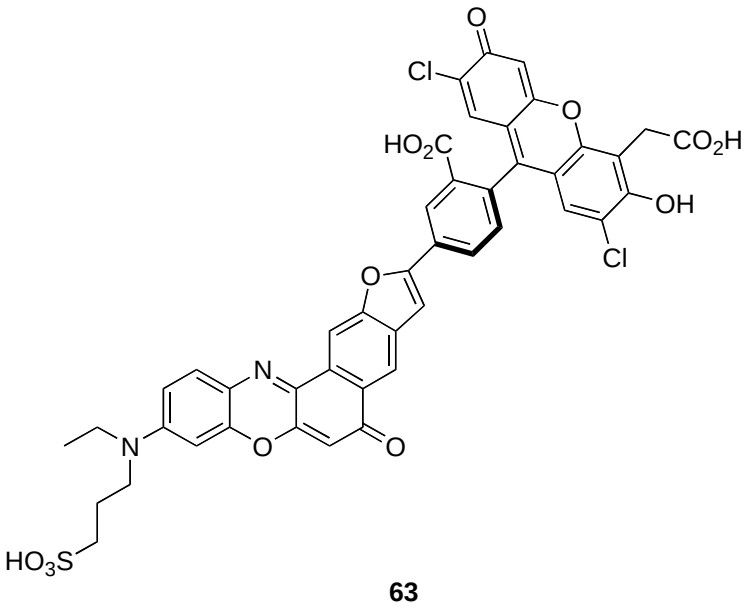
61

4.1.3. Proposed Through-bond Energy Transfer Cassette from Nile Red Derivative

The above iodo Nile Red derivative **60** can also be used for synthesis of water-soluble energy transfer cassette. Sonogashira coupling[79] with fluorescien alkyne such as **62** could be carried out to obtain the corresponding cassette as shown below in Scheme 4.2. The presence of a hydroxy group ortho to the iodo in the Nile Red derivative could result in an electrophilic cyclisation of cassette formed.

Scheme 4.2. Proposed synthesis of water-soluble cassette from iodo Nile Red derivative.





4.1.4. Bridged Nile Red Derivatives with Water-solubilising Groups

Loss in fluorescence output can be further reduced by imparting rigidity to the donor amine part in benzophenoxazines without losing water solubility. Certain proposed structural modifications of these types are shown in Figure 4.1 below. Ready availability of the respective starting materials makes them attractive synthetic targets.

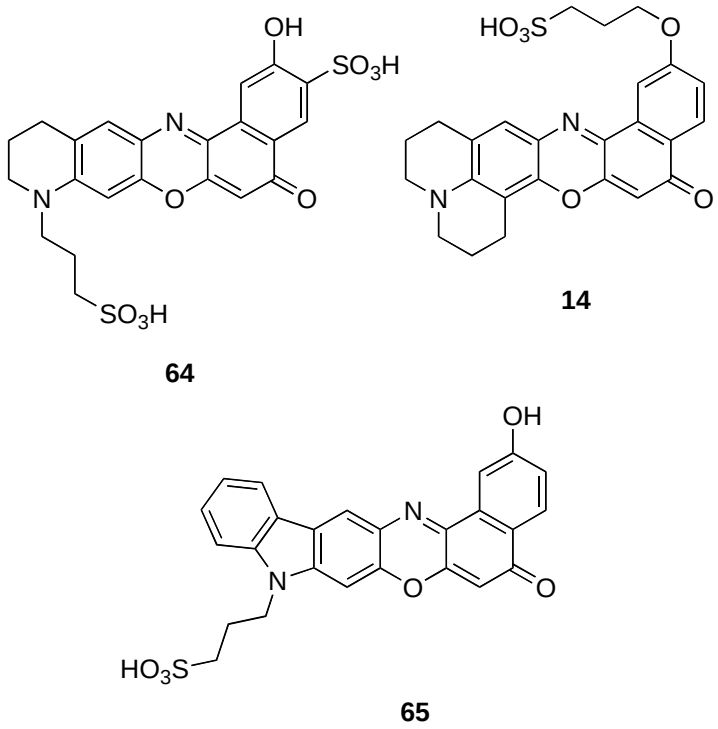
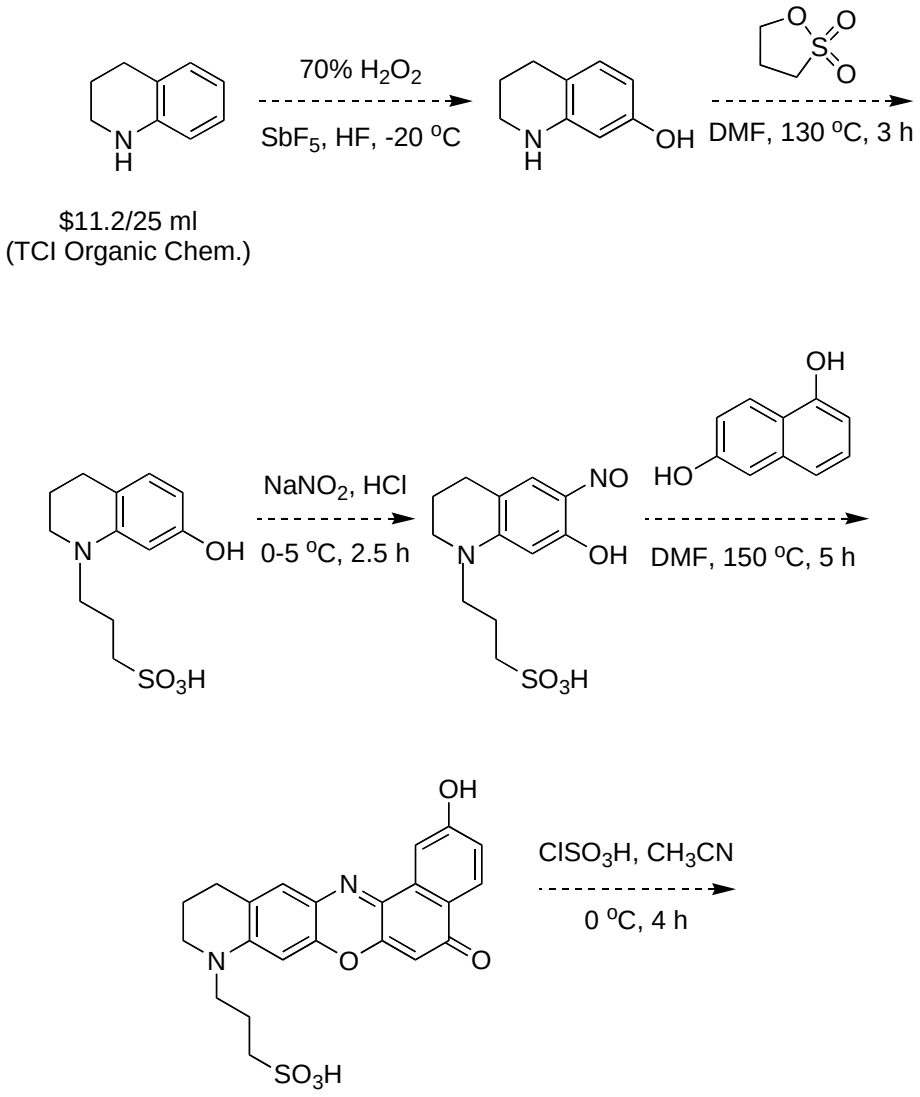


Figure 4.1. Proposed structural modifications of water-soluble Nile Red derivatives.

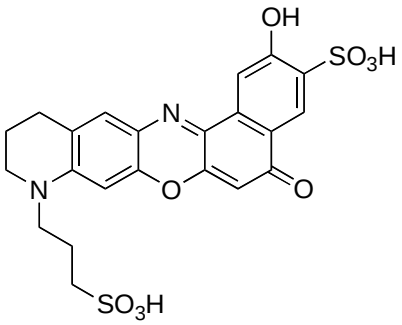
Proposed syntheses of above water-soluble Nile Red derivatives are shown in Scheme 3.3. In case of dye **65,** protection of phenolic hydroxyl group of 2-hydroxy carbazole is necessary since selective substitution at the –NH position was not possible. Deprotection, followed by nitrosylation and condensation of the nitroso compound with 1,6-dihydroxynaphthol should yield dye **65** in acceptable yields.

Scheme 4.3. Proposed synthesis of water-soluble 2-hydroxy Nile Red derivatives **a** from tetrahydroqunoline; **b** from 8-hydroxyjulolidine; **c** from 2-hydroxycarbazole.

a

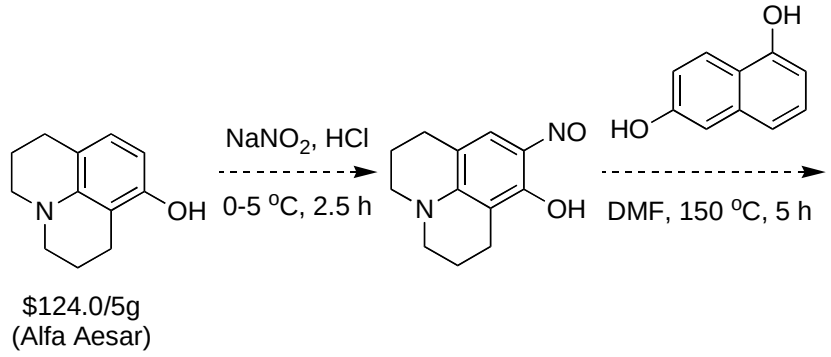


Scheme 4.3. Continued.

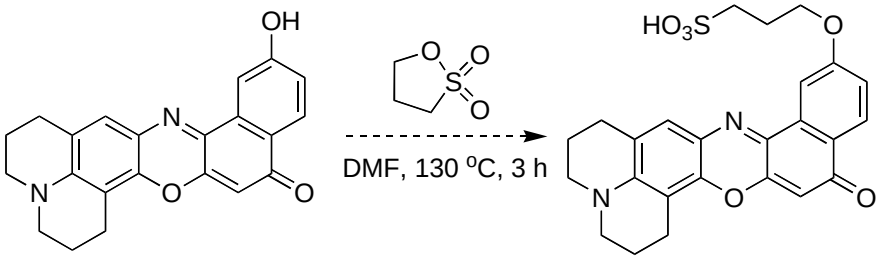


64

b



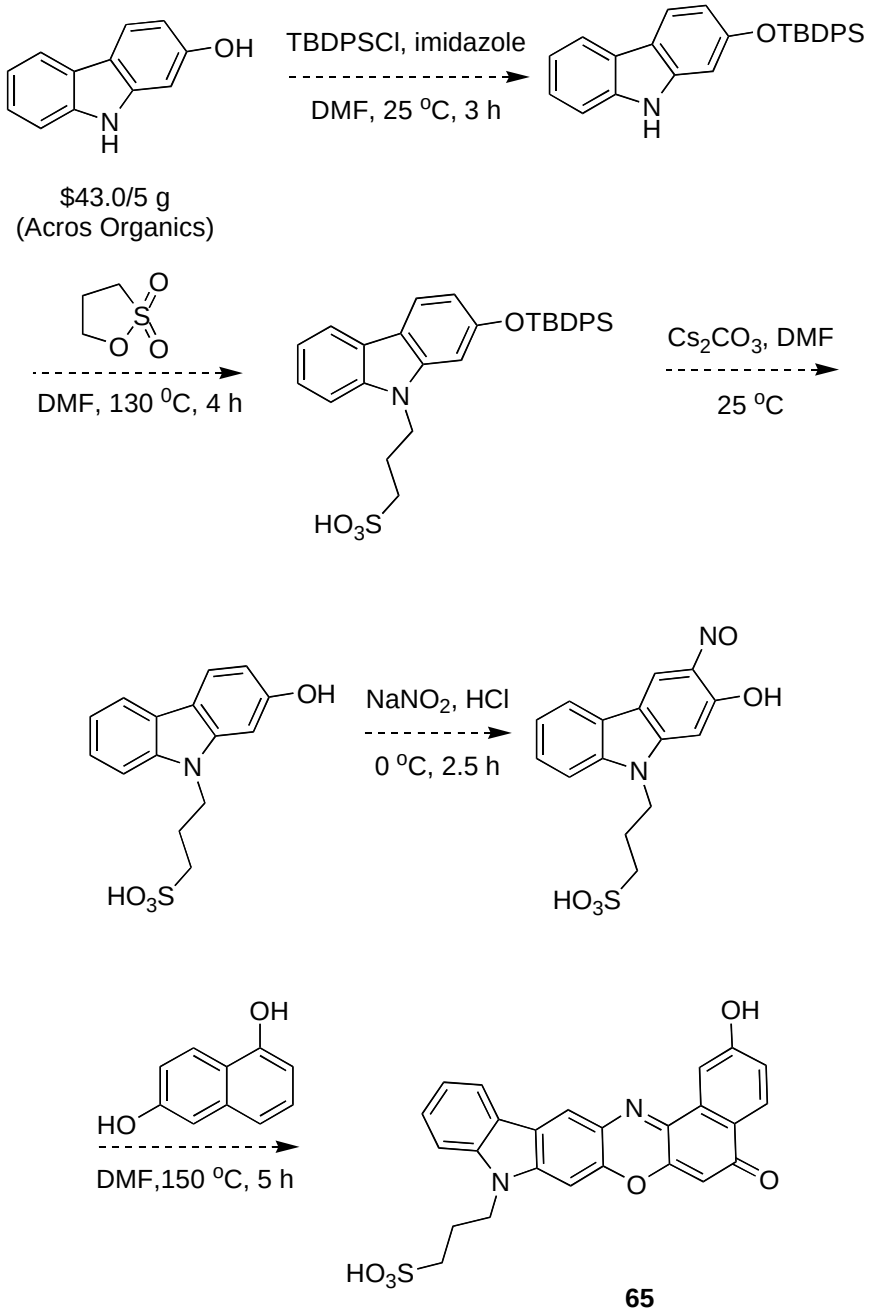
$124.0/5g
(Alfa Aesar)



14

Scheme 4.3. Continued.

c

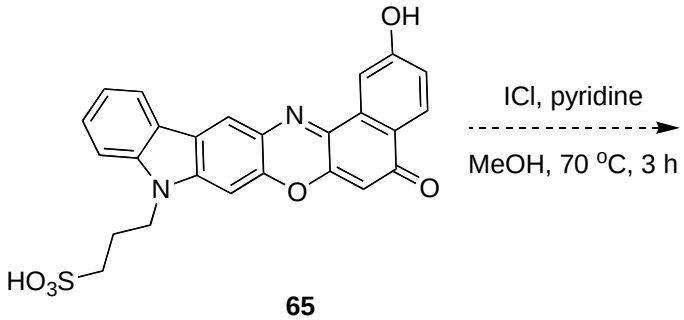


65

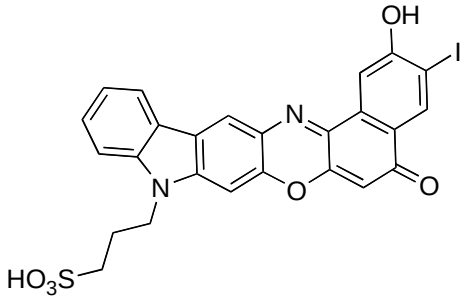
4.1.5. Possible Through-bond Energy Transfer Cassette Based on Bridged Nile Red Derivatives

Any of these above dyes **14**, **64**, **65** could be further derivatized and used as a potential donor or acceptor for synthesis of water-soluble through bond energy transfer cassette. An illustrative example of one such modification and the synthesis of cassette by coupling with a suitable donor or acceptor is shown in Scheme 4.4. Dye **65** can be iodinated and the iodinated dye **66** can then be coupled to azabodipy **67** under Sonogashira condition to obtain cassette **68**. There is a possibility of the resulting cassette being in the cyclised form **69** rather than in the open form **68**.

Scheme 4.4. Proposed synthesis of two-donor one acceptor water-soluble through-bond energy transfer cassette from **66** (donor) and azabodipy **67** (acceptor).

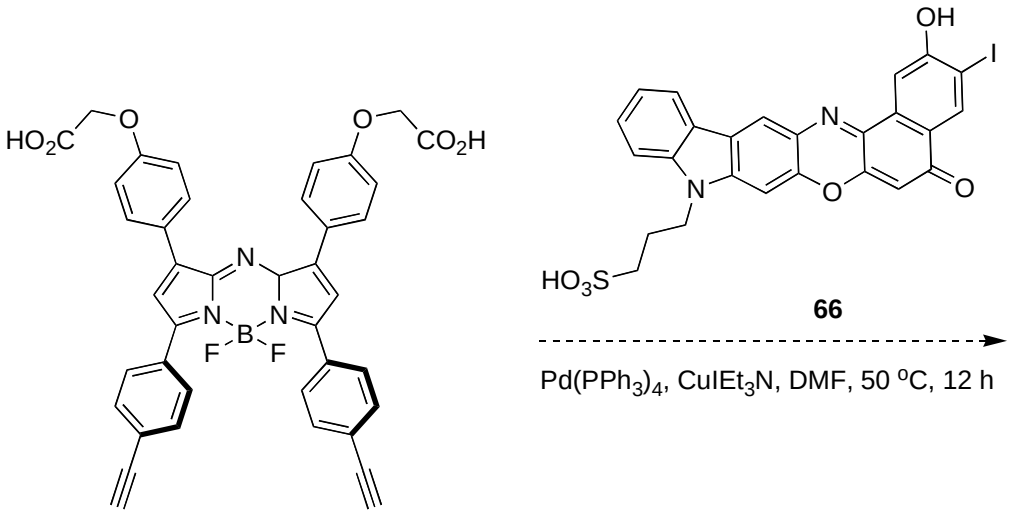


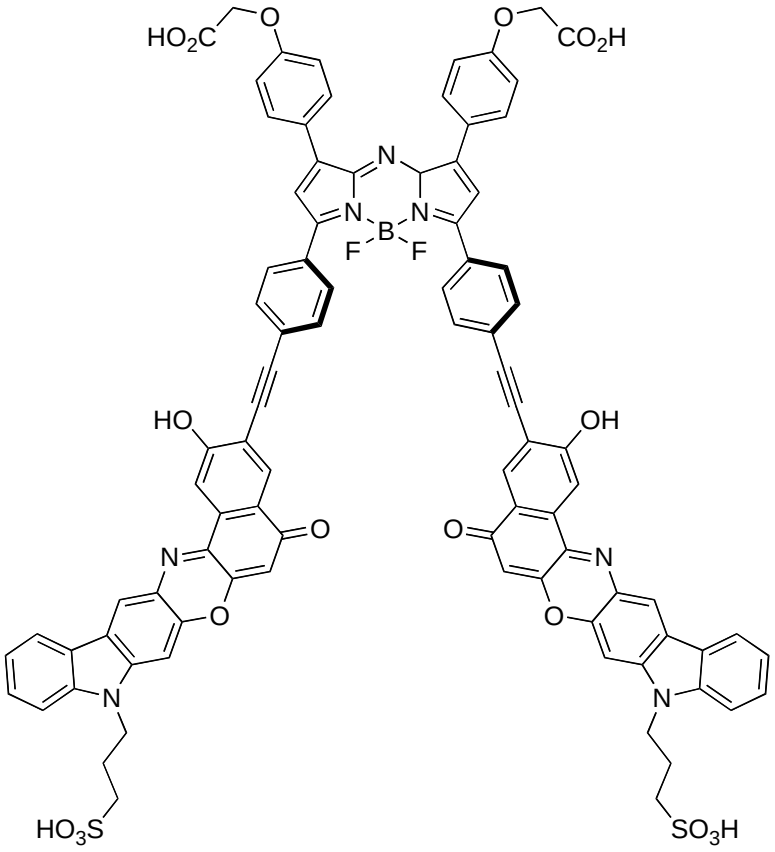
65



66

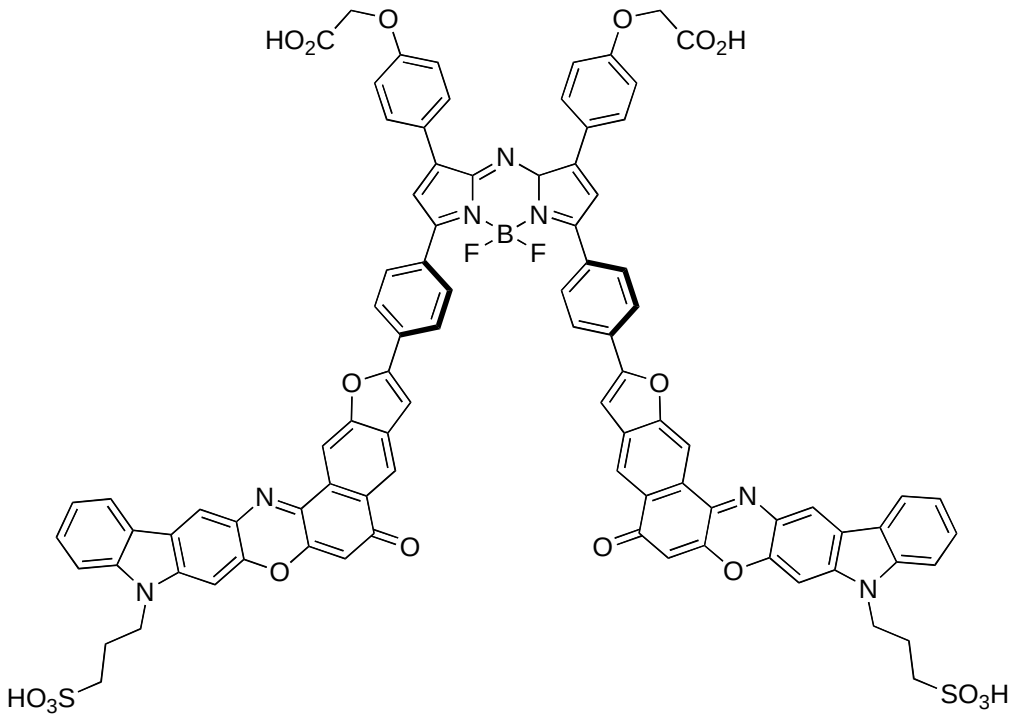
Scheme 4.4. Continued.





open form

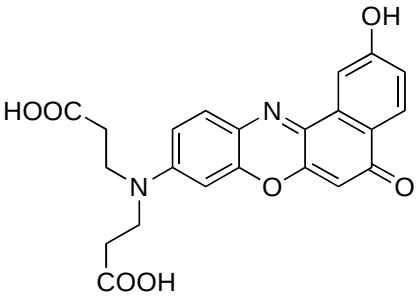
Scheme 4.4. Continued.



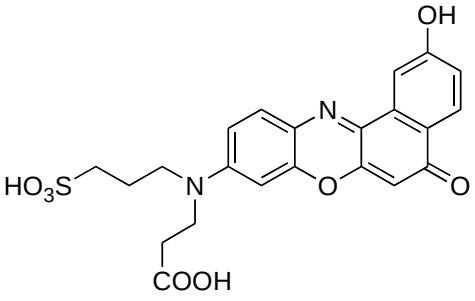
69

cyclised form

The measured quantum yields for the synthesized dyes **44** and **48** are superior to that reported for water-soluble benzophenoxazines. Improvement in quantum yield makes these dyes useful in the synthesis of through-bond energy transfer cassettes, which is the primary focus of our research group. Since these dyes fluoresce in the far visible near IR region they have the added advantage of carrying out biological studies without interference of background fluorescence from biomolecules.



44

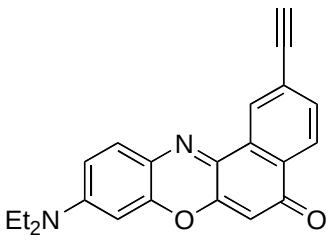


48

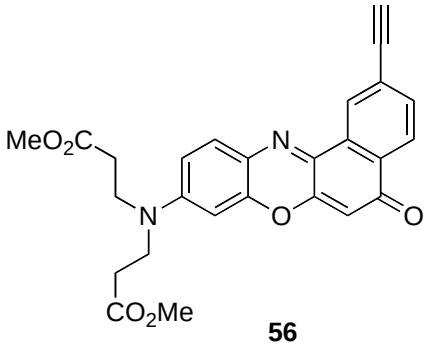
4.1.6. Potential Use of Alkynes in Syntheses of Water-soluble Through-bond Energy Transfer Cassettes

Major draw back of working with the synthesized alkynes **52** and **56** is its low stability at elevated temperature, which limits its synthetic utility. A possible solution towards this problem is to use Cadiot-Chodkiewicz coupling.[80] This reaction is used for synthesis of unsymmetrical alkynes in acceptable yields. Reaction involves coupling of one mole of terminal alkyne with another mole of haloalkyne in presence of a base and cuprous chloride in methanol. Oxygen is avoided throughout the reaction to prevent homocoupling. A drawback of this reaction is the formation of symmetrical diynes, which cannot be avoided completely under the stated reaction conditions, since cuprous chloride reacts with halo alkynes to form alkyne cuprate and dimerization of cuprate to the corresponding symmetrical diynes.

Scheme 4.5. Cadiot-Chodkiewicz reaction for synthesis of unsymmetrical diynes.

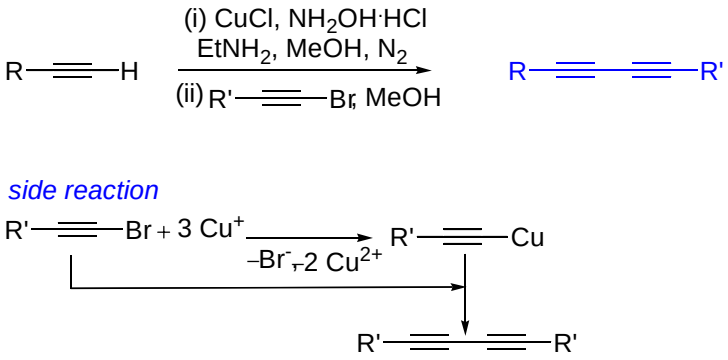


52



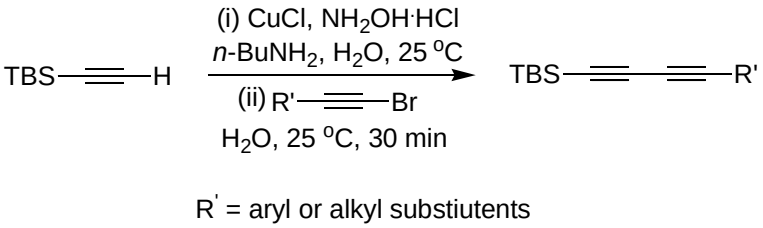
56

Scheme 4.5. Continued.



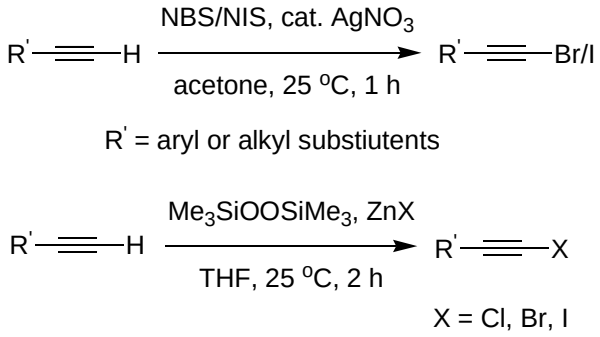
A modified procedure to overcome the problem of symmetrical diyne formation is available in literature, which involves cross-coupling reaction between bulky trialkylsilyl-protected alkynes with different bromoalkynes.[81] This reaction can be carried out at room temperature, so that the decomposition of alkynes can be avoided. The reaction conditions considerably reduces homocoupling product. Instead of ethylamine, *n*-butylamine, a less basic co solvent was used in this particular modification.

Scheme 4.6. Modified Cadiot-Chodkiewicz reaction to reduce homocoupling.



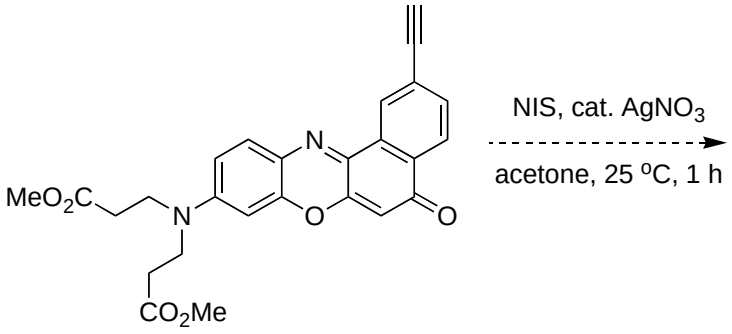
Several literature reports are available for syntheses of terminal halo alkynes.[82-84] Mild reaction conditions reported makes this an attractive means to obtain the corresponding haloalkynes.

Scheme 4.7. Syntheses of terminal halo alkynes.



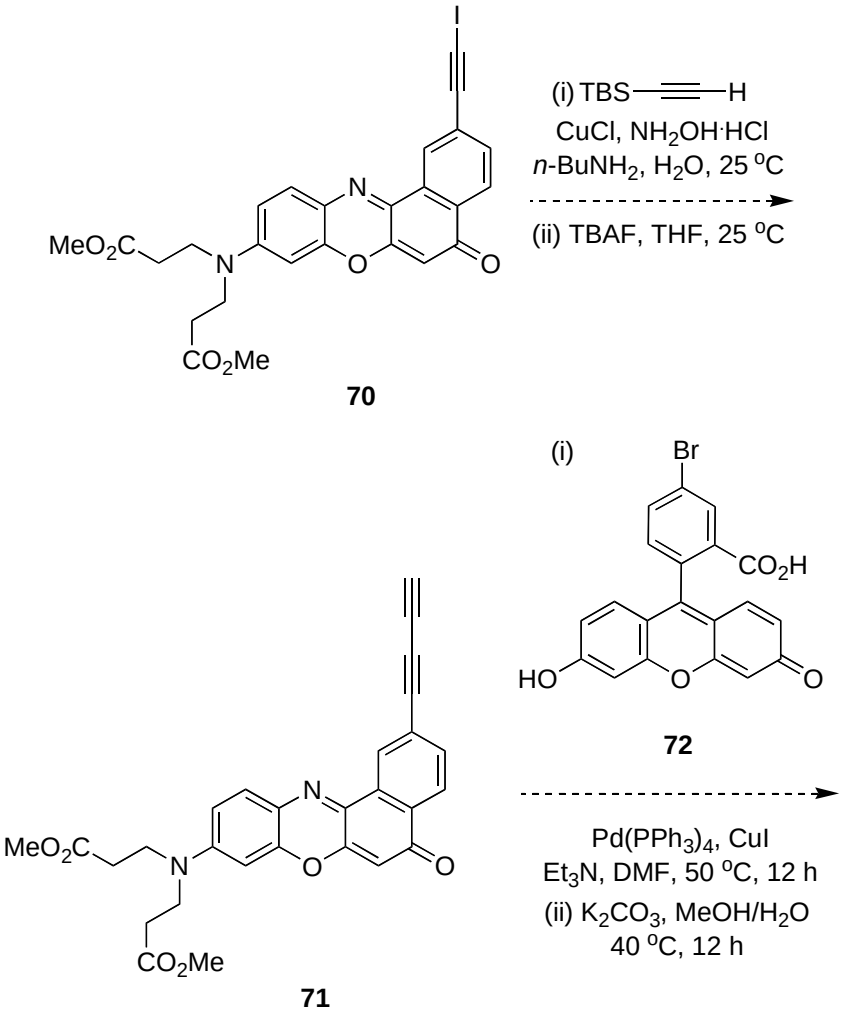
A synthetic Scheme towards a proposed cassette following the above modified Cadiot-Chodkiewicz reaction can be depicted below. Alkyne **56** can be converted to the corresponding terminal haloalkyne by following any of the two protocol from Scheme 4.7 and coupled to TBS protected alkyne to obtain a protected diyne. This diyne can be deprotected and coupled to an iodo or bromo derivative of a donor or an acceptor dye fragment to obtain the corresponding water-soluble dye via classical Sonogashira coupling.

Scheme 4.8. Synthesis of proposed cassette via modified Cadiot-Chodkiewicz and Sonogashira coupling.



56

Scheme 4.8. Continued.

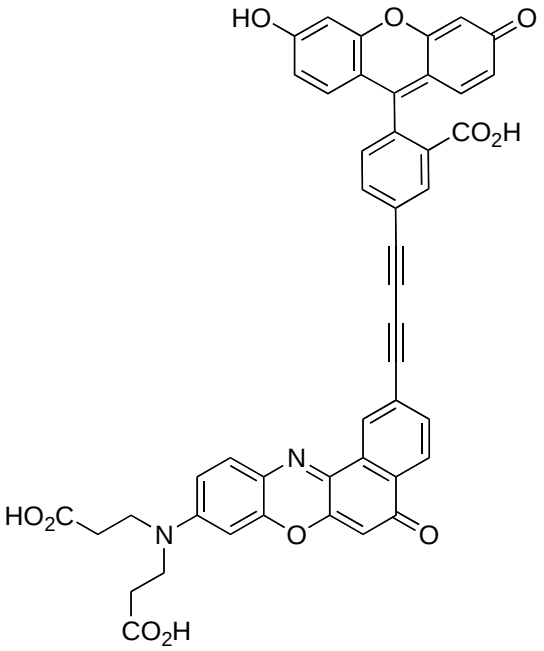


70

72

71

Scheme 4.8. Continued

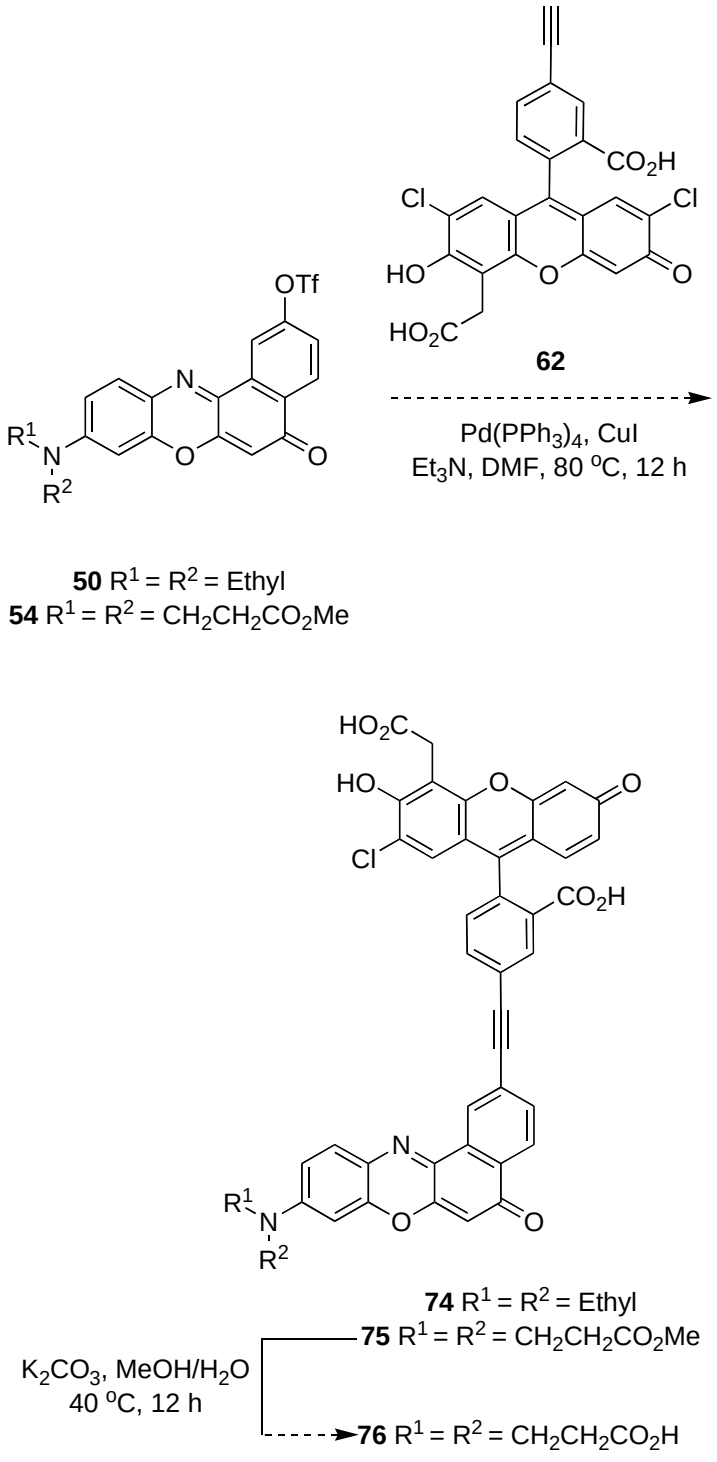


73

*4.1.7. Potential Use of Nile Red Triflates **50** and **54** in Synthesis of Cassettes.*

Nile Red triflates **50** and **54** are precursors of alkynes **52** and **56**. These are potential useful synthons for cassettes. These triflates can be prepared in gram scales and has reasonable stability at elevated temperatures (100-120 ^{o}C). Their coupling to corresponding alkynes can be affected between 80-120 ^{o}C. Water-soluble cassettes from the above mentioned triflates are shown in Scheme 4.9.

Scheme 4.9. Possible water-soluble cassettes from Nile Red triflate **50** and **54**.



4.1.8. Nile Red Derivatives with Enhanced Two-photon Absorption Cross-sections

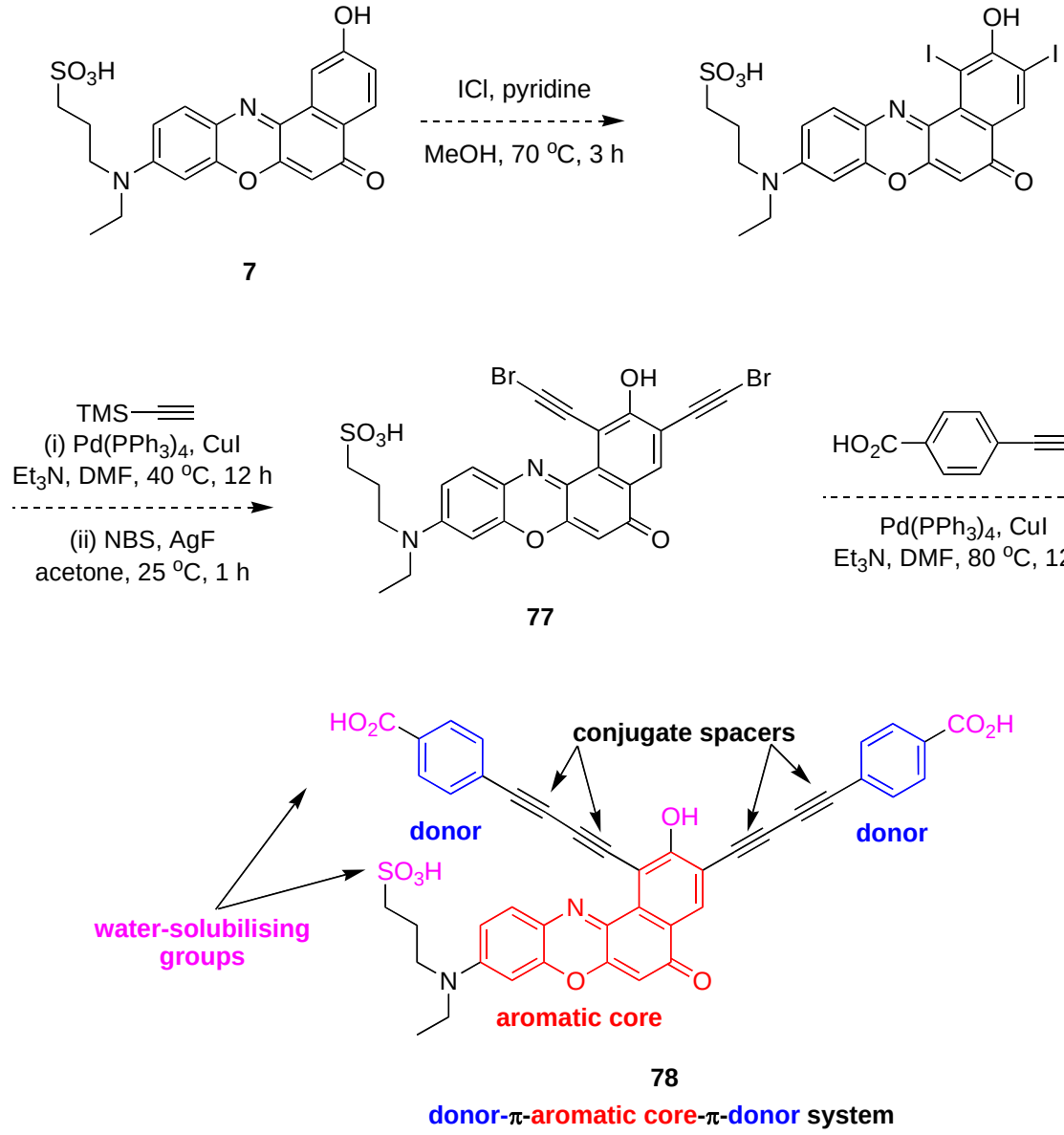
As mentioned in Chapter II, two-photon absorbtion (TPA) is an optical process in which two photons of lower energy are absorbed simultaneously by a molecule, there by promoting it to the excited state. A large TPA cross section is required for such absorbtion to take place. As such Nile Red and its derivatives does not have a large TPA cross-section, which limits it use in such studies. Therefore synthetic modification to improve their TPA cross-section is highly desired. From literature precedent it is known that a one-dimensional conjugated molecule of the general symmetrical structure D-π-aromatic core-π-D (D = donor, π = conjugated spacers) presents a high TPA cross-section. Any modification of Nile Red derivatives should be based on the above structural consideration. Imparting water solubility to such a molecule will be an added advantage since imaging studies of biomolecules are carried out in aqueous media. There are few literature precedent[85-88] on developing such water-soluble molecules with large TPA cross-section, but none based on Nile Red.

Nile Red derivative **7** can be synthetically modified to obtain a water-soluble chromophore with enhanced TPA cross-section in the region of at least a few hundred GM (Goppert-Mayer, 1GM = 10^{-50} cm^4 s $photon^{-1}$). A proposed modification is shown in Scheme 4.10.

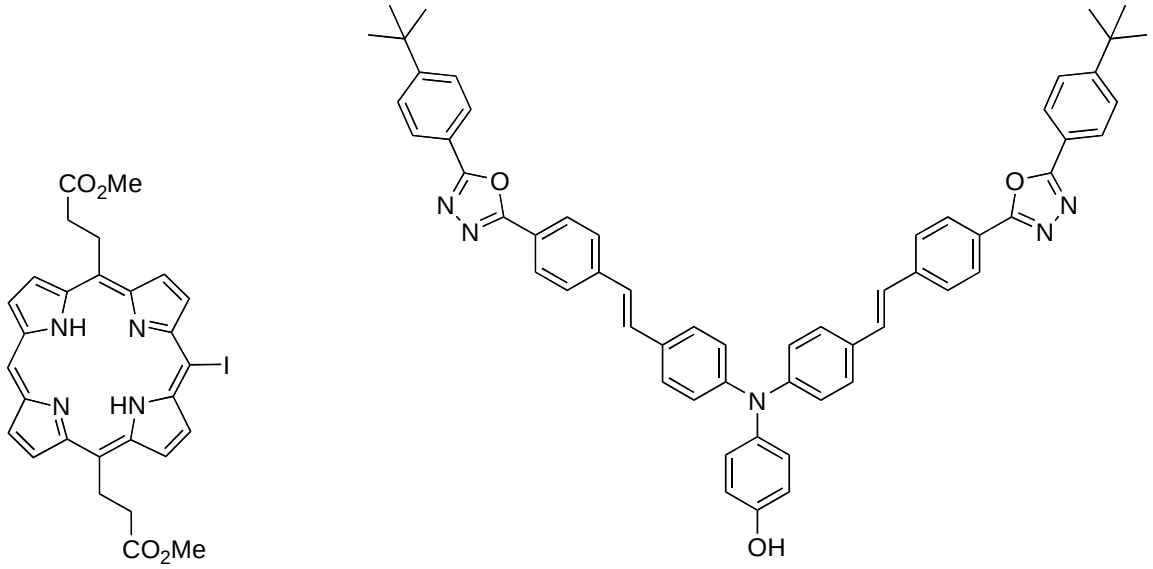
Water-soluble Nile Red derivative **7** could be diiodinated with iodine monochloride. Monoiodination of **7** was established under the same conditions using 1.1 equivalence of iodine monochloride. Therefore increasing the equivalence to 2.2 will definitely lead to diiodination. Classic Sonogashira coupling followed by bromination could afford **67** which on coupling with the 4-ethynyl benzoic acid should yield target dye **68** in acceptable yields. 4-Ethynyl benzoic acid could be easily prepared from corresponding 4-iodo benzoic acid ($67.00/25 g, TCI America) by coupling with TMS alkyne followed by deprotection. An extended conjugate spacer could further enhance the TPA cross section. Therefore 3,4 or even 5 spacers could be inserted and a relative increase in TPA

cross section can be studied. A detailed study on syntheses of such conjugated alkyne spacers is covered by Tykwinski *et al* in chapter 7 (pg 259-298) in *Acetylenic Chemistry* 2005.

Scheme 4.10. Synthesis of water-soluble red emitting chromophore **78** with improved TPA cross-section based on Nile Red derivative **7**.



Further improvement in TPA cross section could be achieved by varying the donor fragment. Some interesting donors are shown in Figure 4.2.



79 **80**

Figure 4.2. Some suitable donors for two-photon absorbing chromophore.[42,88]

4.2. Conclusion

Derivatives of Nile Red with improved water solubility were prepared and their photophysical properties studies in aqueous and organic media. With proper structural modifications it was possible, not only to improve water solubility, but also fluorescence output in aqueous media. At higher pH (around 9.0-10.0), the synthesized dyes showed very low emission, due to presence of phenolate ion, which is reported to have a very low fluorescence output. Nevertheless an improved fluorescence output around pH 7-8 is very much desirable since most biological studies are done at this pH. Presence of a sulphonic acid group is reported to reduce aggregation in aqueous media. Synthesized Nile Red derivative with sulphonic acid group shows better fluorescence properties in

aqueous media as compared to the other two dyes, which proves that presence of water solubilising sulphonic acid group does prevent aggregation.

Alkynes of 2-hydroxy Nile Red **42** and water-soluble 2-hydroxy Nile Red **46** were prepared by classic Sonogashira coupling. Further improvement in yield can be achieved by performing the coupling reaction in a copper free environment.[89] These alkynes could be incorporated into water-soluble through-bond energy transfer cassettes via Sonogashira coupling as donor or acceptor. The low stability of these alkynes at ambient temperature might be a problem in dealing with them, but they can be stored for an extended period of time in their TMS protected form. An *in situ* deprotection, followed by coupling can overcome this problem. A similar approach was reported by Brisbois R. G. *et al*.[90] The iodo Nile Red derivative **50** can also be used for synthesis of water-soluble energy transfer cassettes.

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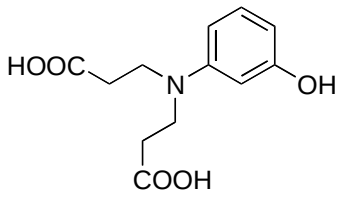
APPENDIX A
EXPERIMENTAL DATA FOR CHAPTER III

General Experimental Conditions: Unless otherwise noted, all non-aqueous reactions were carried out under an atmosphere of dry N_2 in dried glassware. When necessary, solvents and reagents were dried prior to use. THF and toluene were distilled from sodium and hexanes from calcium hydride. DMF was distilled from CaH_2 under reduced pressure.

Analytical thin layer chromatography (TLC) was performed on EM Reagents 0.25 mm silica-gel 60-F plates. Visualization was accomplished with UV light. Chromatography on silica gel was performed using a forced flow of the indicated solvent system on EM Reagents Silica Gel 60 (230-400 mesh). Nuclear Magnetic Resonance (NMR) spectra were recorded with a Mercury-300 (^{1}H at 300 MHz, ^{13}C NMR at 75 MHz) or Inova-500 (^{1}H at 500 MHz, ^{13}C NMR at 125 MHz) NMR spectrometers. Chemical shifts are reported in units of parts per million (ppm) relative to solvent (CD_3OD 3.31 ppm, CD_3COCD_3 2.05 ppm, $CD3SOCD_3$ 2.49 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants, and number of protons. Mass spectra were obtained from the Laboratory for Biological Mass spectrometry at Texas A&M University. Thin layer chromatography was performed using silica gel 60 F254 plates. Flash chromatography was performed using ultra pure silica gel (40-63 μm particle size, 230-400 mesh).THF, MeOH, CH_2Cl_2, triethylamine were distilled from appropriate drying agents.

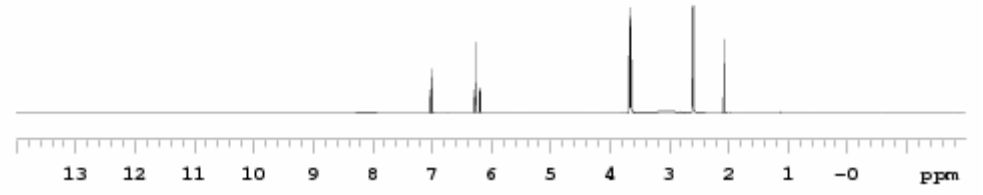


37

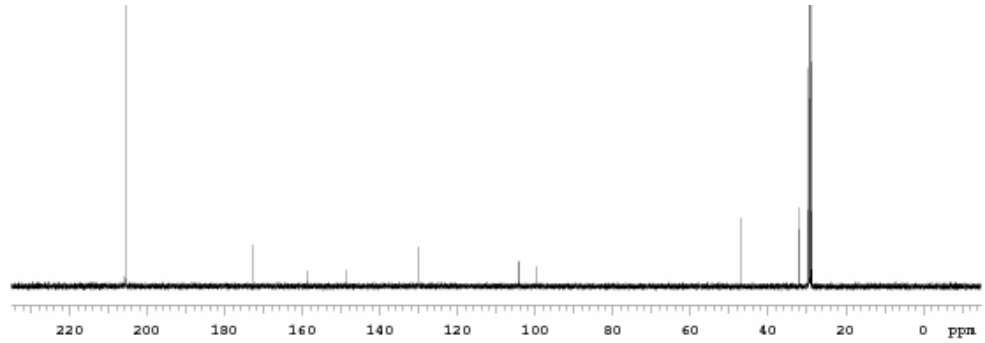
3-[(2-Carboxy-ethyl)-(3-hydroxy-phenyl)-amino]-propionic acid 37

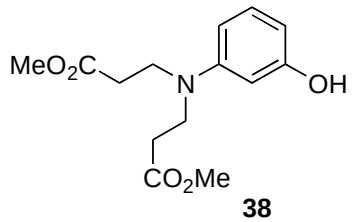
A solution of 3-aminophenol (109.0g, 1 mol) in acrylic acid (185ml, 3 mmol) and water 90 ml was heated to 70 ^{o}C for 3 h. Reaction mixture was cooled and ethanol 180 ml was added and kept at 5 ^{o}C for 12 h. Precipitate formed was filtered washed with ethanol (50 ml) and dried to obtain **37 (**200.0g, 80%). Mp = 153-154 ^{o}C (lit 149-150 ^{o}C) ^{1}H NMR (300 MHz, acetone –d_6) δ 7.02-6.98 (m, 1H), 6.28-6.24 (m, 2H), 6.19-6.17 (m, 1H), 3.64 (t, 4H, J = 4.5 Hz), 2.59 (t, 4H, J = 4.5 Hz) ^{13}C NMR (75 MHz, CD_3COCD_3) δ 172.6, 158.6, 148.6, 130.0,104.0,104.2, 99.6, 46.8, 31.7; MS (ESI) calcd for $C_{12}H_{15}NO_5$ (M^+) 253.1 found (M^+) 253.0400.

^{1}H NMR compound **37**



^{13}C NMR compound **37**



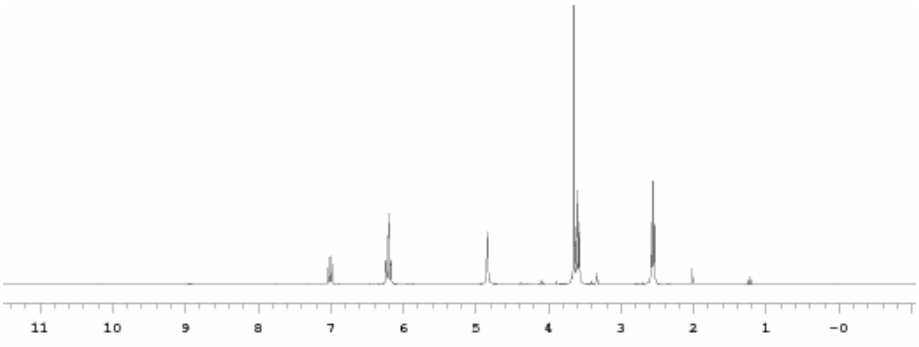


38

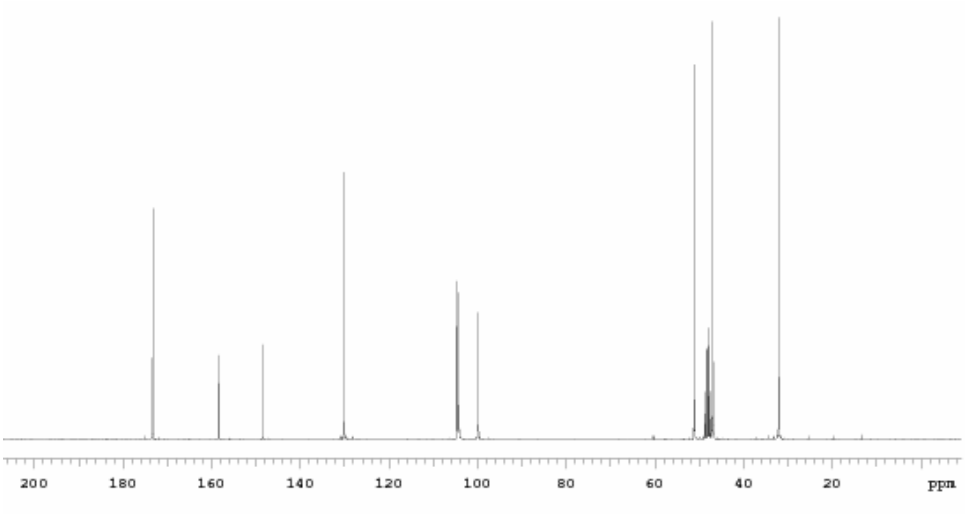
3-[(3-Hydroxy-phenyl)-(2-methoxycarbonyl-ethyl)-amino]-propionic acid methyl ester 38

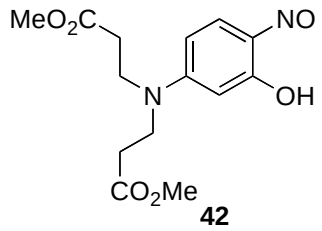
A solution of **37** (10.0g, 39.5 mmol) in methanol 500 ml along with HCl (10.0M, 1 ml) was refluxed for 12 h. Reaction mixture was cooled and MeOH evaporated under reduced pressure. Residue was dissolved in EtOAc (100 ml) and organic layer washed with water (5 x 20 ml). Organic layer was evaporated under reduced pressure to yield **38** as a yellow semisolid (7.0g, 63%). R_f = 0.7 (50% EtOAc/hexane). ^{1}H NMR (300 MHz, CD_3OD) δ 7.05-6.95 (m, 1H), 6.25-6.15 (m, 3H), 3.65 (s, 6H), 3.59 (t, 4H, J = 7.2 Hz), 2.60-2.56 (t, 4H, J = 7.2 Hz) ^{13}C NMR (75 MHz, CD_3OD) δ 173.3, 158.4, 148.5, 130.1, 104.6, 104.2, 99.9, 51.1, 46.9, 32.1; MS (ESI) calcd for $C_{14}H_{19}NO_5$ (M^+) 281.13 found (M^+)281.3614; IR(neat) 3442, 2960, 2362, 1741 cm $^{-1}$.

^{1}H NMR compound **38**



^{13}C NMR compound **38**



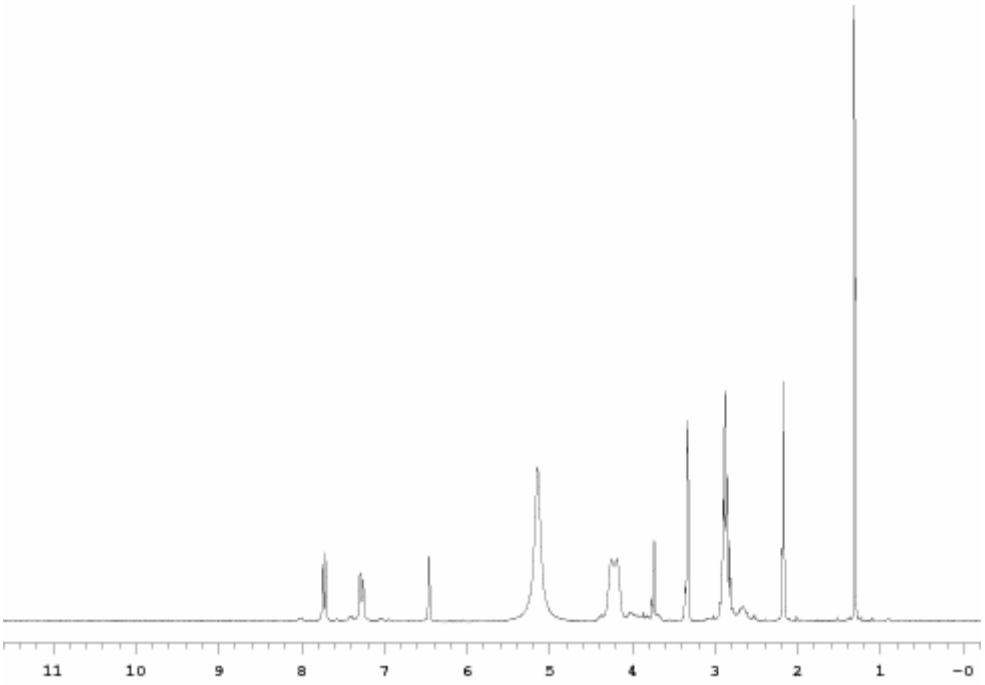


42

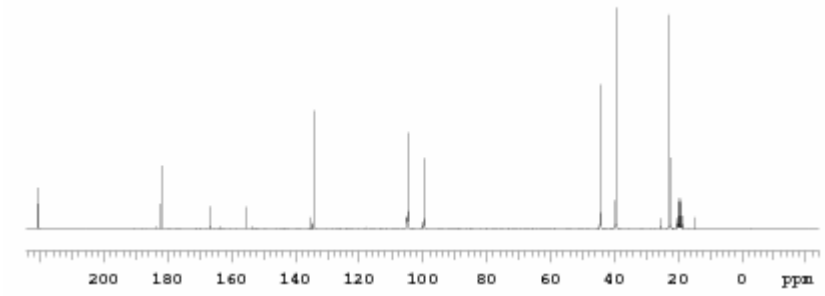
5-[*N,N*-(Dimethoxycarbonylethyl)amino]-2-nitrosophenol 42

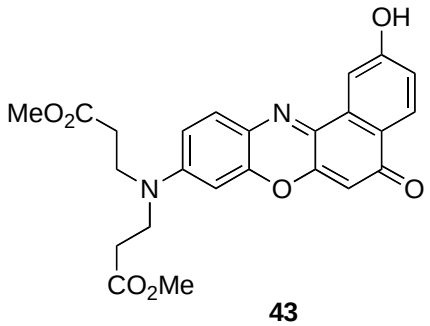
Sodium nitrite (1.32 g, 19.2 mmol) in water (10.0 ml) was added over a period of 50 min using a syringe pump at the rate of 0.2 ml per min. to a solution of **38** (5.0 g, 17.8 mmol) in HCl (6.0 ml, 10.0 M) and water (3.0 ml) at 0 ^{o}C. The mixture was stirred for 2.5 h at 0 ^{o}C and filtered to remove solid impurities. The filtrate was evaporated under reduced pressure to yield **42** (4.2 g, 74%) after drying . This somewhat unstable nitroso compound was directly used for the next step. R_f = 0.4 (90% EtOAc/MeOH). ^{1}H NMR (300 MHz, CD_3OD) δ 7.60 (d, 1H, J = 9.0 Hz), 7.15(d, 1H, J = 12.0 Hz), 6.34(s, 1H), 4.09(d, 4H, J = 27.0 Hz), 3.54(s, 6H), 2.81-2.73(m, 4H); IR (neat) 3383, 2967, 2362, 1729 cm $^{-1}$.^{13}C NMR (75 MHz, acetone-d_6) δ 182.1, 163.1, 157.5, 134.3, 104.8, 104.7, 99.7, 44.3, 39.5, 22.6; MS (ESI) calcd for $C_{14}H_{18}N_2O_6$ (M^+) 310.12 found (MH^+) 311.2341**.**

^{1}H NMR compound **42**



^{13}C NMR compound **42**



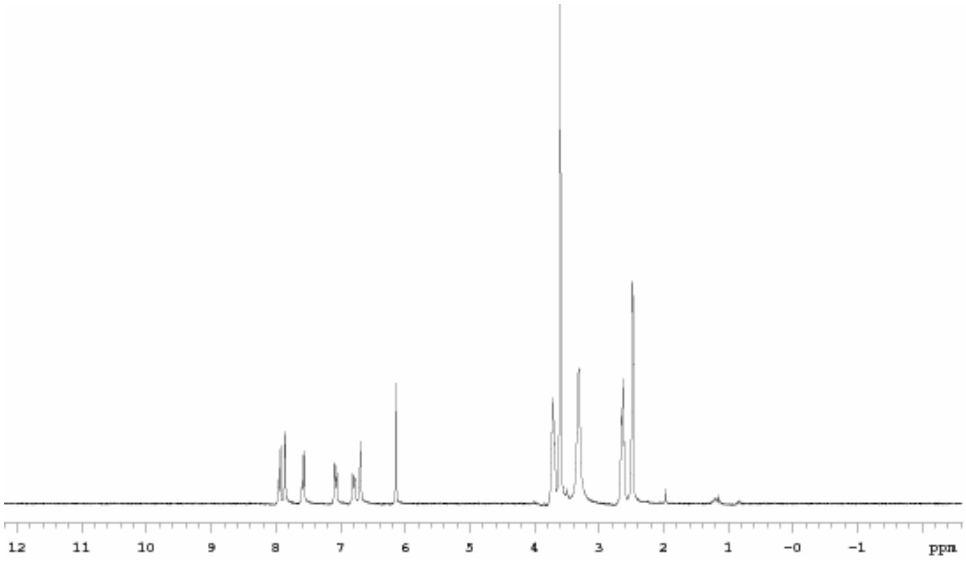


43

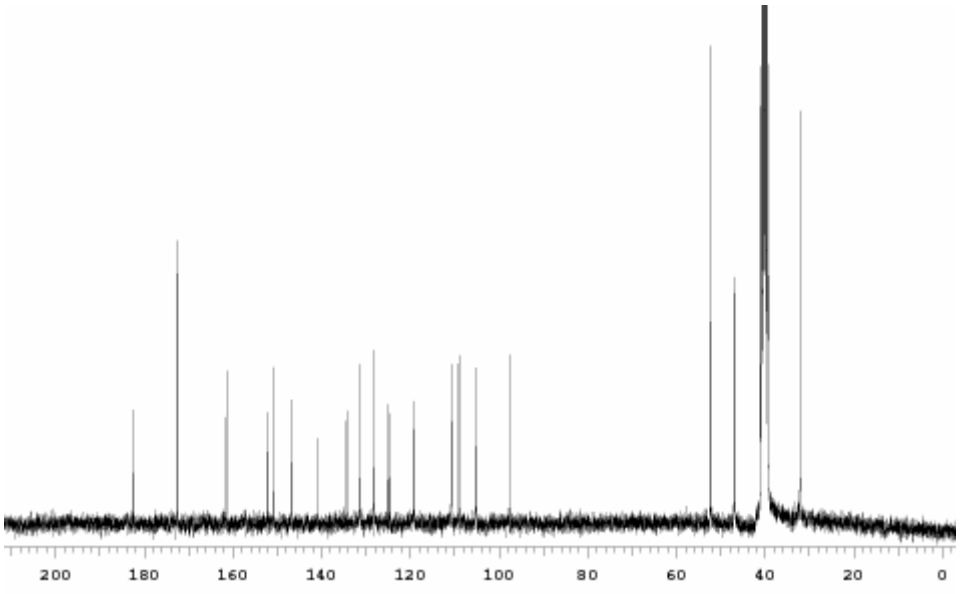
9-[*N*,*N*-Bis(2-(methoxycarbonyl)ethyl)amino]-2-hydroxy-5H-benz[a]phenoxazin-5-one 43

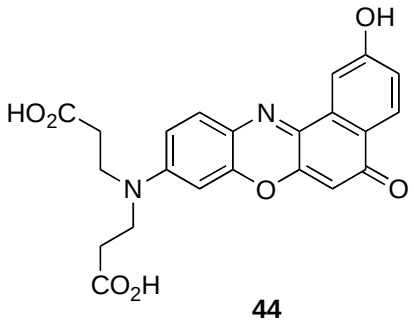
1,6-Dihydroxynaphthol (1.85 g, 11.6 mmol) with HCl (2.0 ml, 10.0 M) was added to a solution of **42** (4.0 g, 11.6 mmol) in MeOH (100.0 ml) all in one portion. The reaction mixture was refluxed for 5 h. The solvent was evaporated and the residue was purified by flash chromatography using 90% EtOAc/MeOH eluent to afford **43** as a red solid (2.8 g, 54%). R_f = 0.7 (90%EtOAc/MeOH). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.94 (d, 1H, *J* = 9.0 Hz), 7.86 (s, 1H), 7.58 (d, 1H, *J* = 9.0 Hz), 7.09-7.05 (m, 1H), 6.81-6.78 (m, 1H), 6.69 (s, 1H), 6.13 (s, 1H), 3.70 (t, 4H, *J* = 6.0 Hz), 3.60 (s, 6H), 2.65-2.60 (t, 4H, *J* = 6.0 Hz). ^{13}C NMR (75 MHz, DMSO –d_6) δ 182.4, 172.4, 161.4, 152.1, 150.9, 146.8, 140.7, 134.3, 131.4, 128.2, 124.9, 124.5, 119.3, 110.7, 108.9, 105.1, 97.7, 52.2, 46.9, 32.1; MS (ESI) calcd for $C_{24}H_{22}N_2O_7$ (M^+) 450.1427 found (M^+) 450.1459; IR (neat) 3422, 2955, 1734 cm^{-1}.

^{1}H NMR compound **43**



^{13}C NMR compound **43**



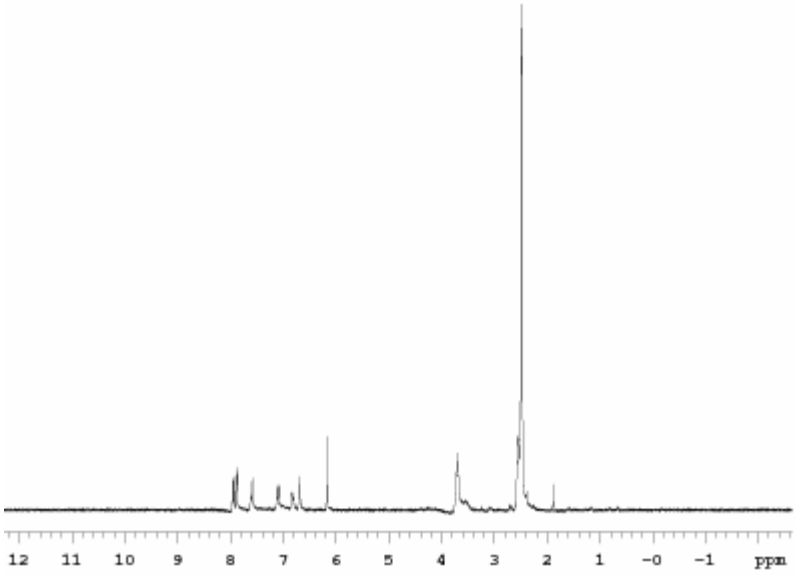


44

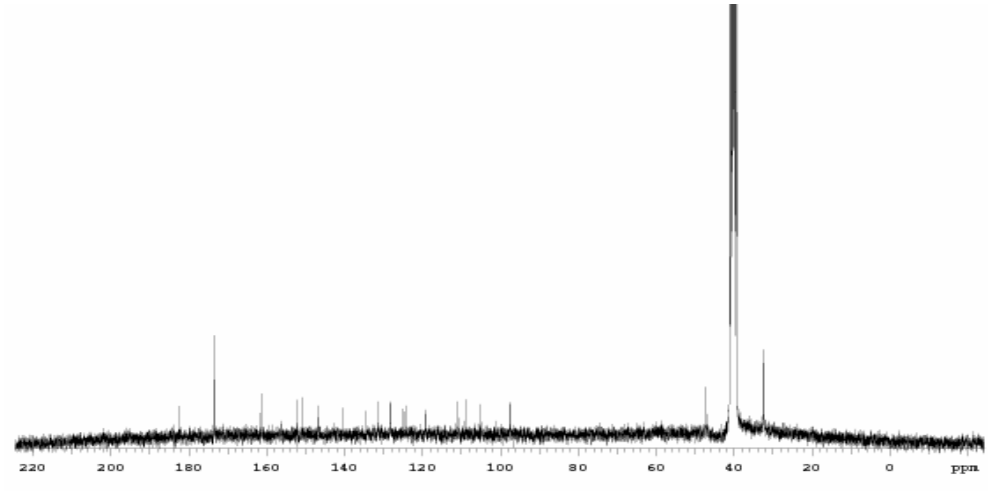
9-[*N*,*N*-Bis(2-Carboxyethyl)amino]-2-hydroxy-5H-benz[a]phenoxazin-5-one 44

A solution of K_2CO_3 (276 mg, 2 mmol) dissolved in 5.0 ml of water was added to a solution of **43** (100 mg, 0.2 mmol) in MeOH/water (1:1). The reaction mixture was heated to 40 ^oC for 36 h. The solvent was evaporated under reduced pressure, the crude mixture was then dissolved in water (10 ml) and washed with EtOAc (3 x 5 ml). The aqueous layer was acidified with HCl (4-5 drops, 10.0 M) to pH 4-5. This aqueous layer was extracted with CH_2Cl_2 /*iso*-propanol (3 x 5 ml, 1:1) and organic layer evaporated under reduced pressure to give **44** (60 mg, 60 %) as a blue solid. ^1H NMR (300 MHz, DMSO-d_6) δ 7.95 (d, 1H, *J* = 9.0 Hz), 7.88-7.87 (m, 1H), 7.62 (d, 1H, *J* = 9.0 Hz), 7.09 (dd, 1H, J = 8.7, 2.7 Hz), 6.82 (d, 1H, *J* = 9.3 Hz), 6.69 (s, 1H), 6.16 (s, 1H) 3.76-3.65 (br, 4H), 2.58-2.51 (br, 4H). ^{13}C (75 MHz, DMSO –d_6) δ 182.3, 173.5, 161.5, 152.2, 151.1, 146.9, 140.4, 134.3, 131.5, 128.2, 125.0, 124.4, 119.3, 110.9, 108.9, 105.0, 97.5, 47.2, 32.6; MS (ESI) calcd for $C_{22}H_{18}N_2O_7$ (M^+) 422.1114 found (M-H) 421.0150; IR (neat) 3425, 3059, 2928, 1637 cm^{-1}.

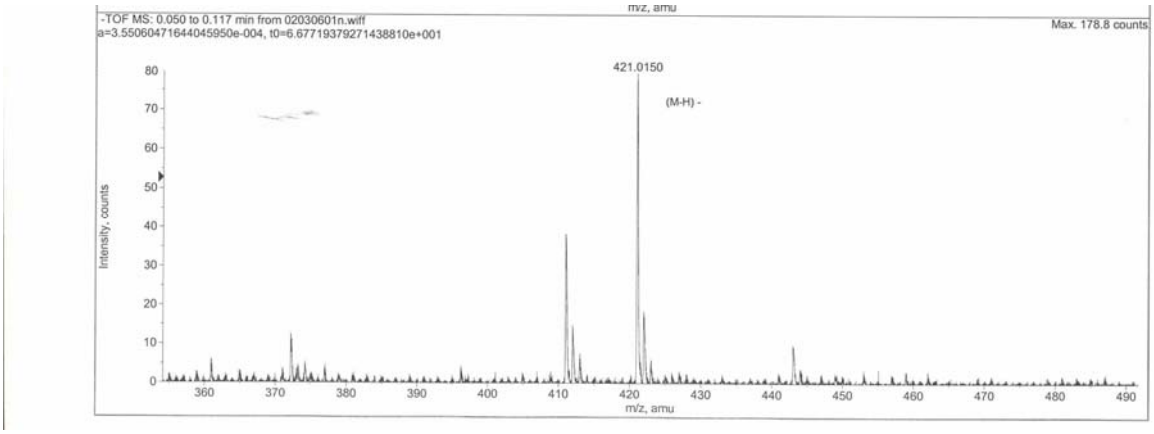
^{1}H NMR compound **44**

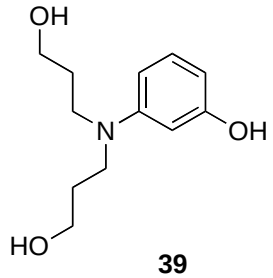


^{13}C NMR compound **44**



Mass spectra (ESI) compound **44**



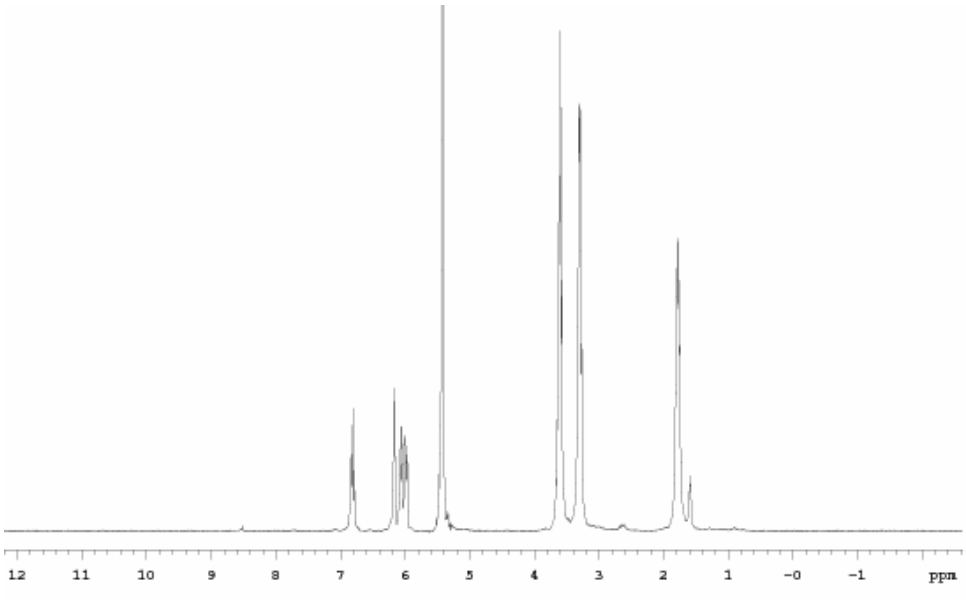


39

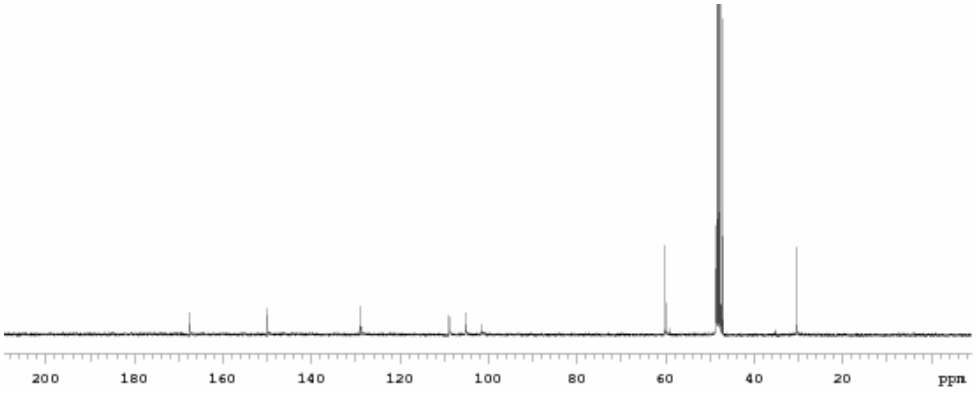
3-(Bis(3-hydroxypropyl)amino)phenol 39

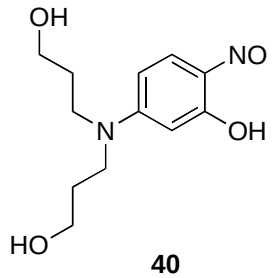
A solution of **38** (1.0 g, 3.6 mmol) in THF (15.0 ml) was added dropwise at 0 ^{o}C under vigorous stirring to a solution of $LiAlH_4$ (820 mg, 21.6 mmol) in THF (10.0 ml) in a three neck flask fitted with a reflux condenser (the reaction is exothermic); a thick white precipitate was formed almost immediately, and TLC (1:1 hexane/EtOAc) after 2 h showed complete disappearance of **38**. The reaction mixture was quenched with water and filtered to remove the solid residues. The resulting filtrate was concentrated under reduced pressure to yield **39** (730 mg, 90%) as white solid. R_f = 0.2 (1:1 hexane/EtOAc). ^{1}H NMR (300 MHz, CD_3OD) δ 6.81 (t, 1H, J = 8.1 Hz), 6.16 (s, 1H), 6.06 (d, 1H, J = 7.2 Hz), 5.98 (d, 1H, J = 8.1 Hz) 3.12 (t, 4H, J = 6.0 Hz), 2.81-2.73 (m, 4 H), 1.33 (t, 4 H, J = 6.3 Hz). ^{13}C NMR (75 MHz, CD3OD) δ 167.5, 149.9, 128.9, 108.9, 105.1, 101.5, 60.0, 48.7, 30.3; MS (ESI) calcd for $C_{12}H_{19}NO_3$ (M^+) 225.1365 found (M^+) 225.1379; IR (neat) 3350, 2921, 2854 cm^{-1}.

^{1}H NMR compound **39**



^{13}C NMR compound **39**



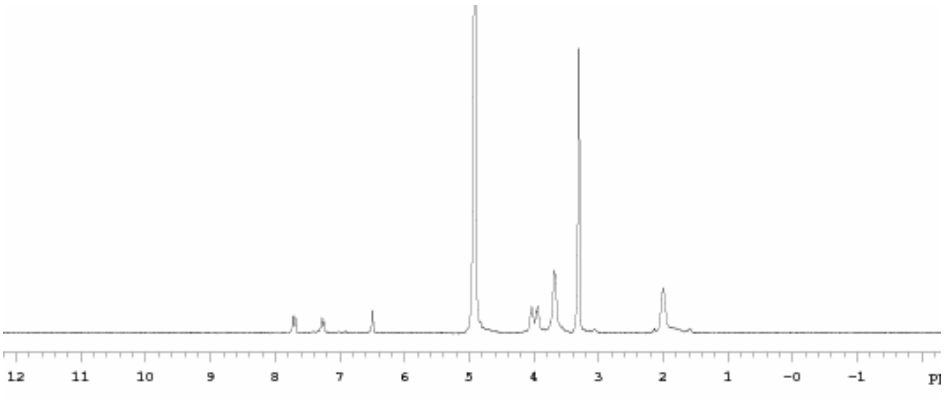


40

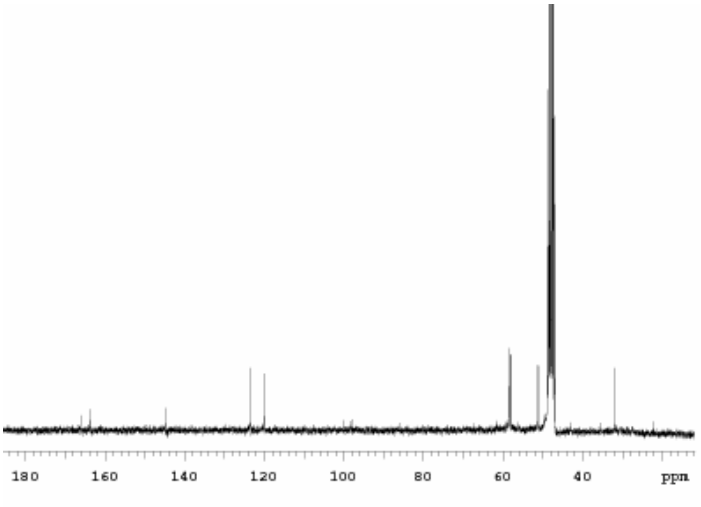
5-(Bis(3-hydroxypropyl)amino)-2-nitrosophenol 40

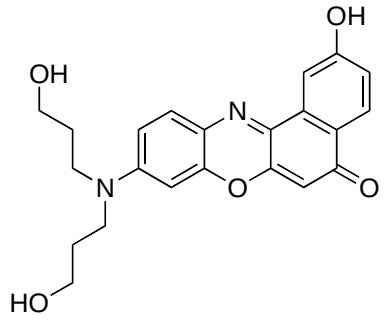
Sodium nitrite (0.92 g, 13.3 mmol) in water (11.2 ml) was added, over a period of 1 h, via a syringe pump at the rate of 0.2 ml per min, to a solution of **39** (2.0 g, 8.9 mmol) in HCl (9.0 ml, 10.0 M) and water (4.5 ml) at 0 ^{o}C. The mixture was stirred for 2.5 h at 0 ^{o}C and filtered to remove residual impurities. The filtrate was evaporated under reduced pressure and residue dissolved in methanol, dried with magnesium sulfate, filtered and methanol was evaporated to yield **40** (1.54 g, 74%). This somewhat unstable, hygroscopic nitroso compound was used in the next step without further purification. R_f = 0.4 (90% EtOAc/MeOH). ^{1}H NMR (300 MHz, CD_3OD) δ 7.70 (d, 1H, J = 10.2 Hz), 7.26 (d, 1H, J = 10.2 Hz), 6.49 (s, 1H), 3.99 (d, 4H, J = 26.7 Hz), 3.81-3.66 (br, 4H), 2.02-1.86 (br, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 166.1, 163.6, 144.8, 123.4, 120.0, 98.0, 58.3, 51.1, 31.8; MS (ESI) calcd for $C_{12}H_{18}N_2O_4$ (M^+) 254.13 found (M^+) 254.2458.

^{1}H NMR compound **40**



^{13}C NMR compound **40**





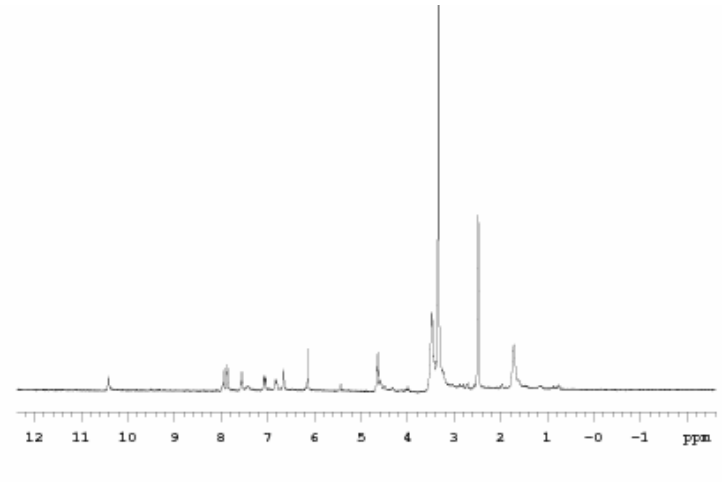
41

2-Hydroxy-9-(1,7-dihydroxyheptan-4-yl)-5H-benzo[a]phenoxazin-5-one 41

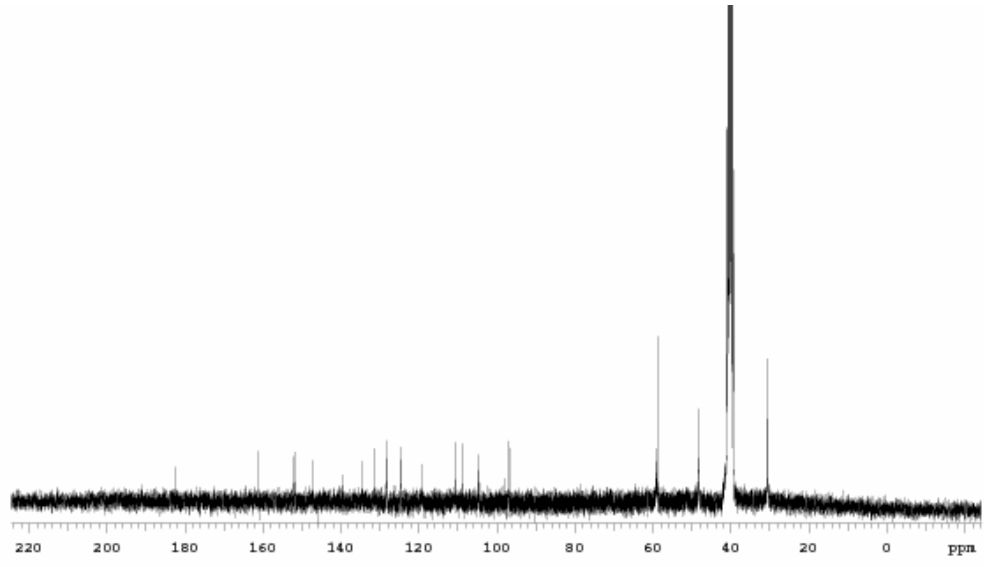
Compound **40** (1.0 g, 3.5 mmol) was dissolved in 50 ml of dry distilled DMF and solid 1,6-dihydroxynaphthol then HCl (2 ml, 10.0 M) were added in that order. The reaction mixture was heated to 130 ^{o}C for 5 h, cooled to room temperature, then the DMF was removed under reduced pressure. The residual material was purified by flash chromatography eluting with 1:10 MeOH/EtOAc to afford **41** as a red solid (740 mg, 54 %). R_f = 0.3 (90% EtOAc/MeOH). ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.41 (s, 1H), 7.94 (d, 1H, J = 8.4 Hz), 7.86 (d, 1H, J = 2.7 Hz), 7.56 (d, 1H, J = 9.0 Hz), 7.06 (dd, 1H, J = 6.0, 2.4 Hz), 6.82 (d, 1H, J = 8.4 Hz), 6.66 (s, 1H), 6.14 (s, 1H), 4.63 (d, 4H, J = 4.8 Hz),

3.47 (d, 4H, J = 1.5 Hz), 1.66-1.78 (br, 4H). ^{13}C NMR (75 MHz, DMSO –d_6) δ 182.3, 161.3, 152.3, 151.9, 147.0, 139.4, 134.4, 131.4, 128.2, 124.5, 119.1, 110.7, 108.7, 104.8, 97.2, 96.9, 58.9, 48.3, 30.6; MS (ESI) calcd for $C_{22}H_{22}N_2O_5$(M^+) 394.1529 found ($M+H^+$) 395.1616; IR (neat) 3358, 2918, 2847 cm^{-1}.

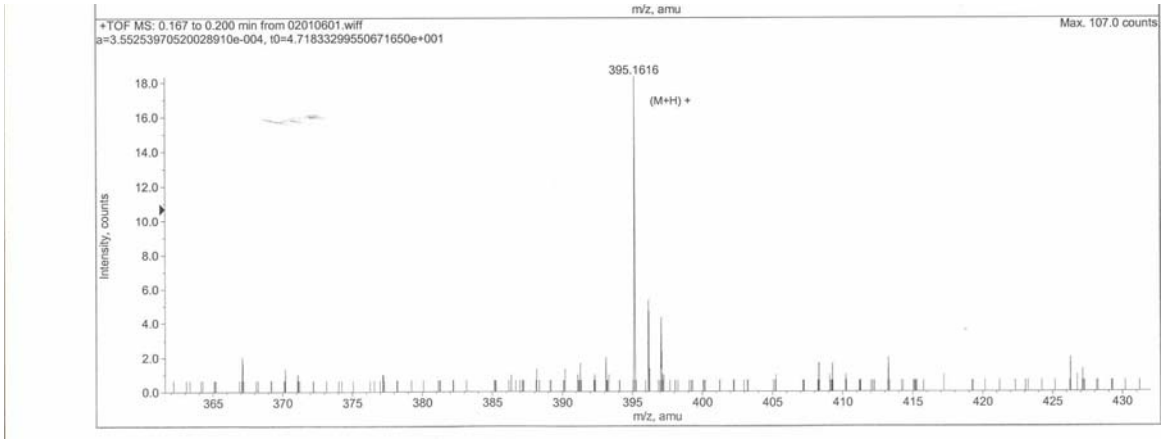
^{1}H NMR compound **41**

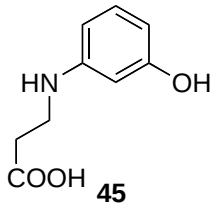


^{13}C NMR compound **41**



Mass spectra (ESI) compound **41**



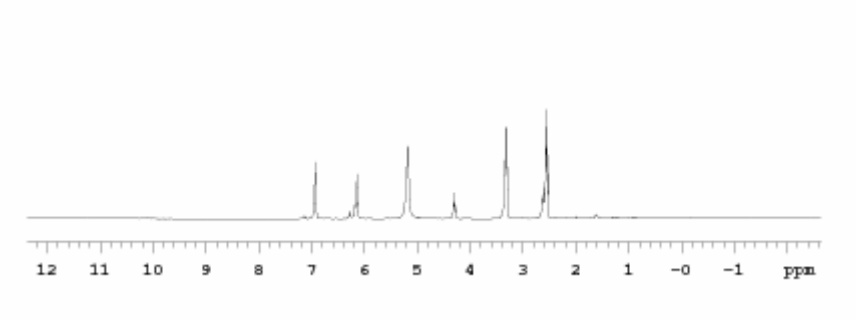


45

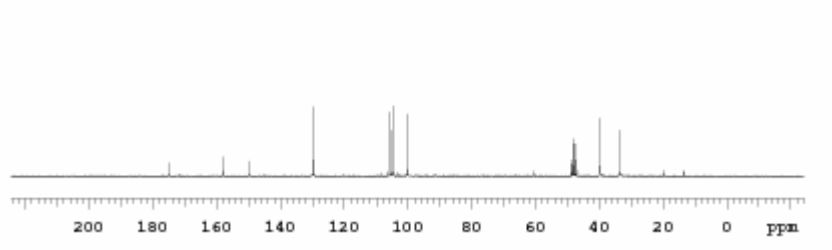
3-(3-hydroxyphenylamino)propanoic acid 45

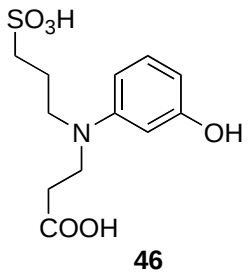
3-Aminophenol (5.0 g, 4.6 mmol) in acrylic acid (4.7 ml, 6.9 mmol) and water (3.0 ml) was heated to 70 ^{o}C for 12 h. The reaction mixture was cooled to room temperature then the solvent was evaporated under reduced pressure. The residual material was purified by flash chromatography eluting with 1:1 hexane/EtOAc to afford **45** as a white semi-solid (3.3 g, 40%) R_f = 0.3 (EtOAc). ^{1}H NMR (300 MHz, CD_3OD) δ 6.93 (s, 1H), 6.21-6.12 (m, 3H), 4.31-4.27 (m, 1H), 3.34-3.30 (m, 2H), 2.64-2.52 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 175.1, 158.1, 149.6, 129.8, 105.5, 104.8, 100.2, 39.8, 33.5; MS (ESI) calcd for $C_9H_{11}NO_3$ (M^+) 181.0739 found (M^+) 181.0829.

^{1}H NMR compound **45**



^{13}C NMR compound **45**





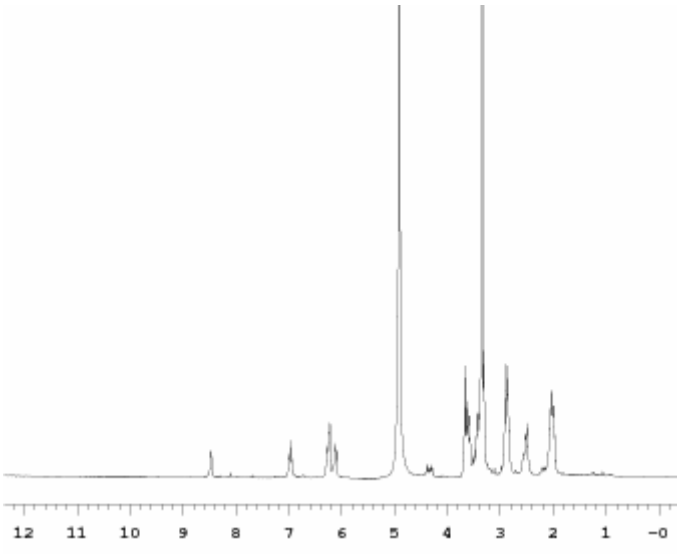
46

3-(*N*-(3-Hydroxyphenyl)-*N*-(3-sulfopropyl)amino)propanoic acid 46

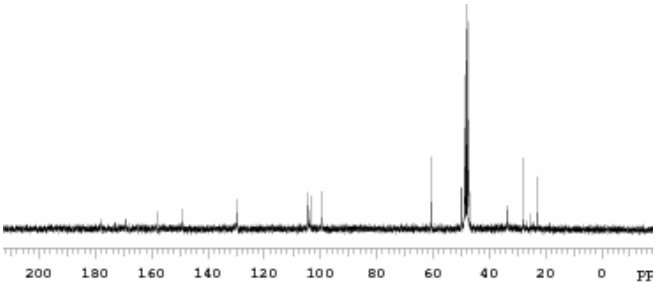
A solution of **45** (3.0 g, 16.5 mmol) and propane sultone (5.0 g, 41.2 mmol) in DMF (20 ml) was heated, with stirring, to 130 ^{o}C for 3 h. The solvent was evaporated under reduced pressure and crude residue purified by flash chromatography initially eluting with EtOAc to remove all of the unreacted sultone and then eluting with 1:1 EtOAc:MeOH to afford **46** (2.4 g, 48%) as a dark brown semi-solid. R_f = 0.2 (1:1 EtOAc:MeOH). ^{1}H NMR (300 MHz, CD_3OD) δ 8.47 (s, 1H), 7.04-6.94 (m, 1H), 6.31-6.22 (br, 2H), 6.10 (d, 1H, J = 9.0 Hz), 3.68-3.58 (m, 4H), 2.88 (t, 2H, J = 6.0 Hz), 2.5 (t, 2H, J = 6.0 Hz), 2.08-1.96 (br, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 172.8, 159.2, 149.3,

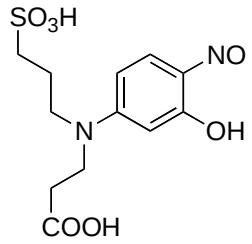
130.0, 104.6, 103.8, 99.8, 60.9, 49.7, 33.8, 28.1, 23.2 MS (ESI) calcd for $C_{12}H_{17}NO_6S$ (M^+) 303.0777 found (M-H) 302.0599; IR (neat) 3403, 2910, 1626 cm^{-1}.

^1H NMR compound **46**



^{13}C NMR compound **46**

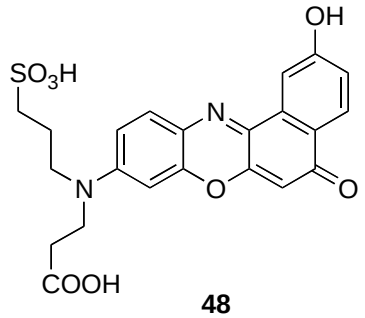




47

3-(*N*-(3-Hydroxy-4-nitrosophenyl)-N-(3-sulfopropyl)amino)propanoic acid 47

To a solution of **46** (0.5 g, 1.6 mmol) in HCl (2.0ml, 10.0M) and water (1 ml) at 0 ^{o}C was added sodium nitrite (0.13 g, 1.8 mmol) dissolved in water(2 ml) over a period of 10 minutes using a syringe pump at the rate of 0.2 ml per minute. The mixture was stirred for 3 h at 0 ^{o}C and filtered to remove residual impurities. The filtrate was evaporated under reduced pressure to yield **47** (0.4 g, 74%). The crude product was very water sensitive and therefore directly used without further purification for the next step.



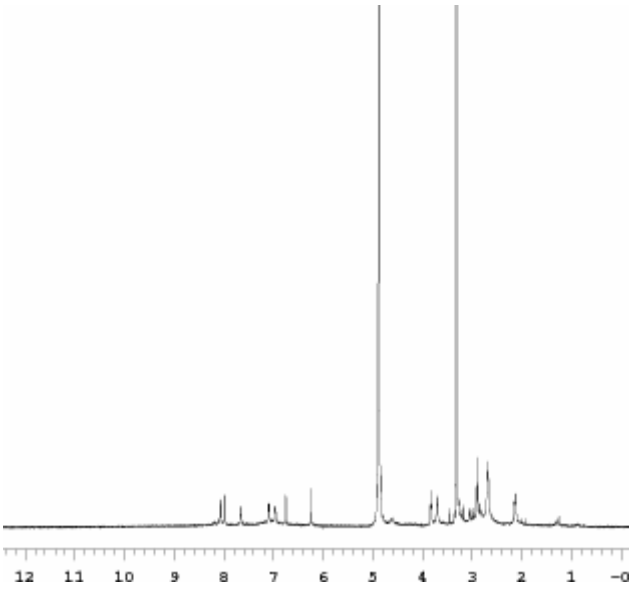
48

3-(*N*-(2-hydroxy-5-oxo-5H-benzo[a]phenoxazin-9-yl)-*N*-(3-sulfopropyl)amino) propanoic acid 48

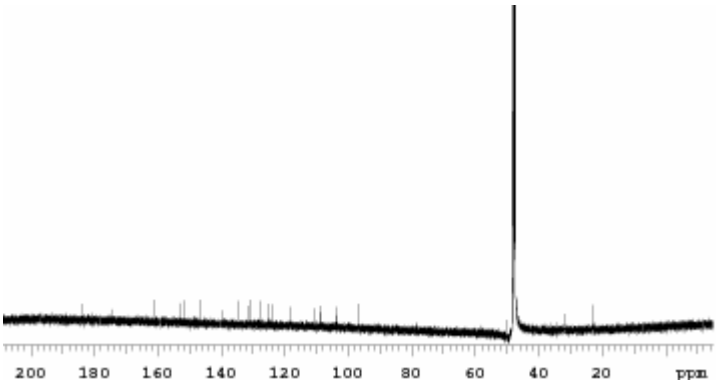
A solution of crude **37** (0.39 g, 1.1 mmol) and 1,6-dihydroxynaphthol (0.2 g, 1.2 mmol) in DMF (15 ml) was heated to reflux for 5 h. The DMF was evaporated under reduced pressure and the residual material was purified by flash chromatography; eluting with EtOAc removed any unreacted 1,6-dihydroxynaphthol and then eluted with 1:1 EtOAc:MeOH to afford **48** (0.25g, 53 %) as a red solid. R_f = 0.15 (1:1 EtOAc: MeOH). ^{1}H NMR (500 MHz, CD_3OD) δ 8.07 (d, 1H, J = 8.5 Hz), 8.00 (s, 1H), 7.65 (d, 1H, J = 9.0 Hz), 7.10 (d, 1H, J = 8.5 Hz), 6.97 (d, 1H, J = 9.0 Hz), 6.76 (s, 1H), 6.24 (s, 1H), 3.83

(t, 2H, *J* = 7.0 Hz), 3.69 (t, 2H, *J* = 8.0 Hz), 2.92-2.89 (m, 2H), 2.68 (t, 2H, *J* = 7.5 Hz), 2.14 (t, 2H, *J* = 9.0 Hz). ^{13}C NMR (75 MHz, CD_3OD) δ 184.2, 174.3, 161.3, 152.9, 151.6, 146.9, 139.6, 134.6, 131.2, 127.6, 125.4, 124.2, 118.1, 111.2, 108.6, 103.8, 96.8, 50.6, 49.6, 31.8, 22.8 MS (ESI) calcd for $C_{22}H_{22}N_2O_8S$ (M^+) 472.094 found (M-H) 471.0654.

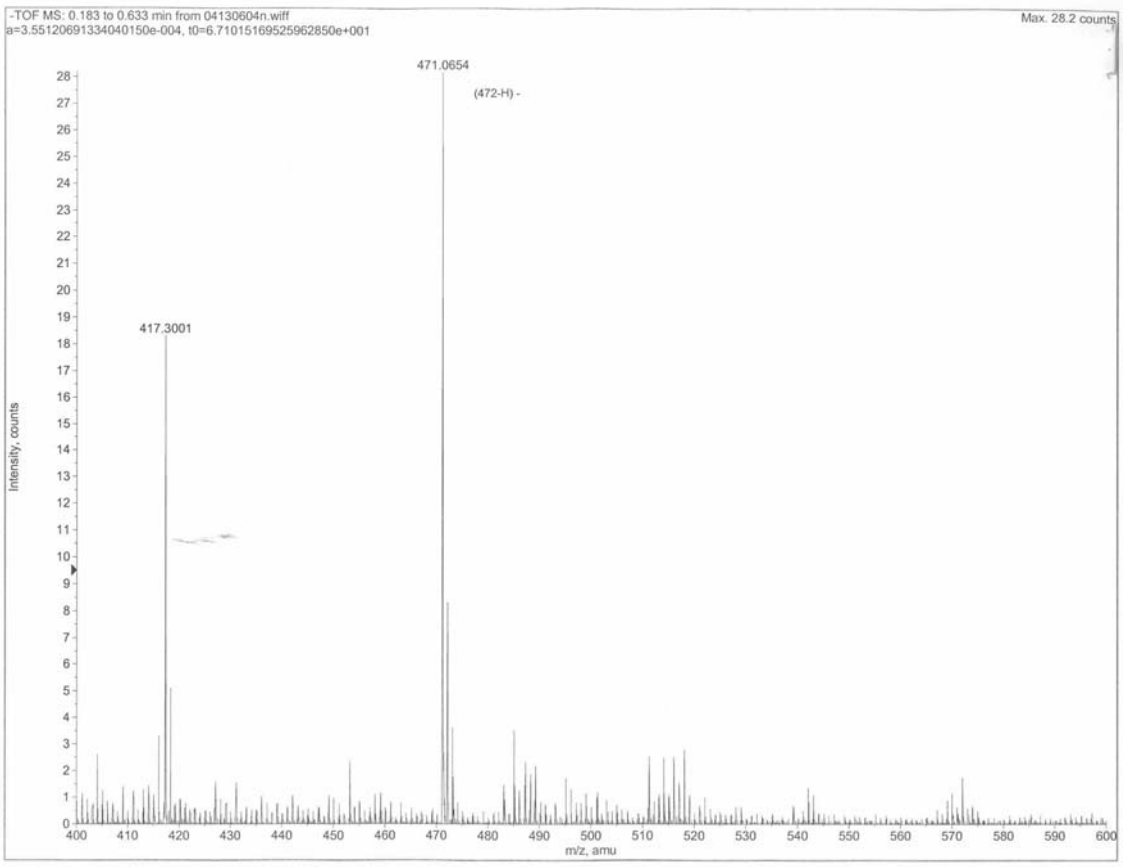
^{1}H NMR compound **48**

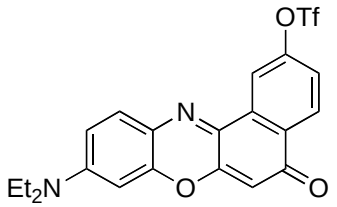


^{13}C NMR compound **48**



Mass spectra (ESI) compound **48**



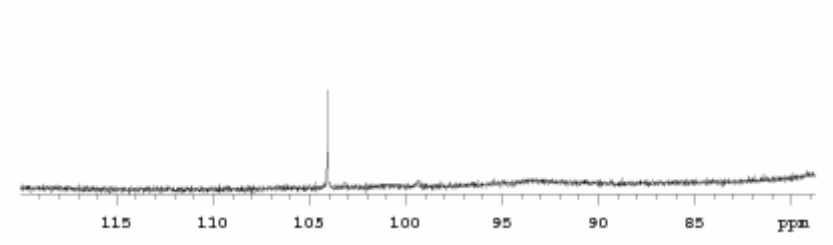


50

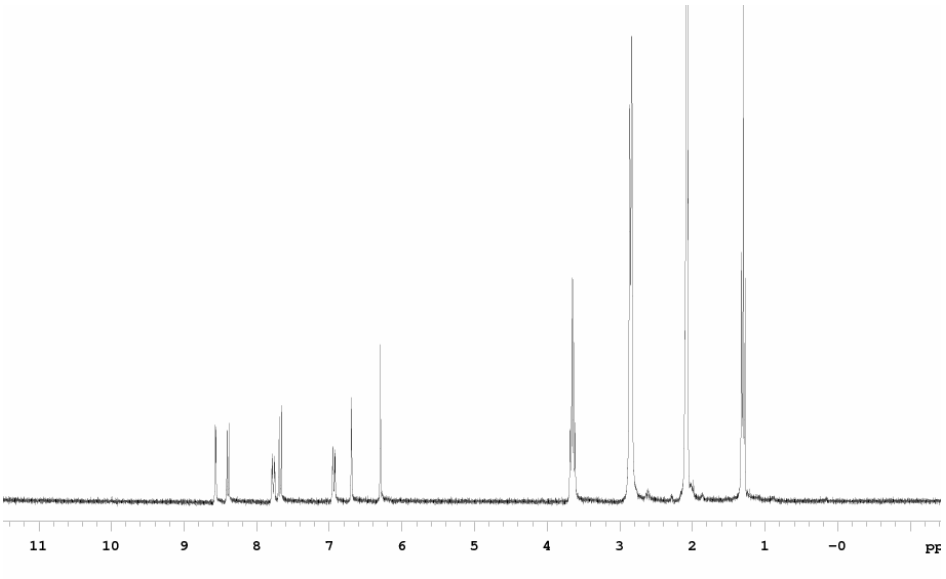
9-(Diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl trifluoromethanesulfonate 50

Triflic anhydride (0.6 ml, 3.6 mmol) was added gradually over a period of 10 minutes to the mixture of 2-hydroxyNile Red **6**(1.0g, 3.0 mmol) in 20 ml dry distilled CH_2Cl_2 and dry distilled pyridine (0.963 ml, 12.0 mmol) at 0 ^oC . The mixture was allowed to warm slowly to 25 ^oC with stirring for 12 h. Reaction mixture was washed with 0.1 M HCl (2 x 10 ml). Organic layer was evaporated under reduced pressure and purified by flash chromatography eluting with 1:1 hexane/ EtOAc to afford **50** as a red solid (720 mg, 52%). R_f = 0.7 (1:1 hexanes/EtOAc) ^1H NMR (300 MHz, acetone $-d_6$) δ 8.57 (s, 1H), 8.39 (d, J = 8.4 Hz), 7.77 (d, 1H, J = 6.0 Hz), 7.67 (d, 1H, J = 9.0 Hz), 6.94 (d, 1H, J = 6.6 Hz), 6.70 (s, 1H), 6.3 (s, 1H), 3.68-3.61 (m, 4H), 1.3 (t, 6H, J = 6.9 Hz) ^{13}C NMR (75 MHz, acetone $-d_6$) δ 181.2, 153.6, 152.4, 151.8, 147.5, 137.3, 134.9, 132.4, 131.9, 128.4, 125.3, 122.9, 116.0, 110.8, 105.2, 96.7, 45.1, 12.8 ^{19} F NMR (300 MHz, acetone $-d_6$) δ 104.2; M.S. (ESI) calcd for $C_{21}H_{17}FN_2O_5S$ (M^+) 466.08 found (M^+) 466.0777.

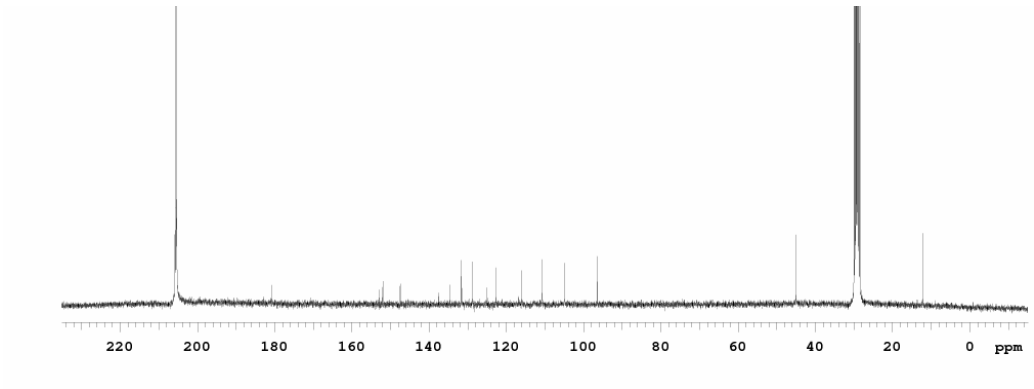
^{19}F NMR compound **50**

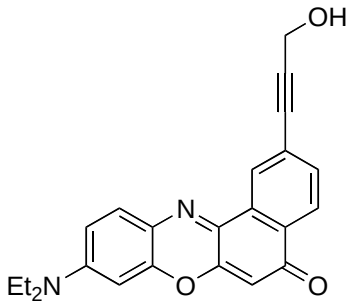


^{1}H NMR compound **50**



^{13}C NMR compound **50**



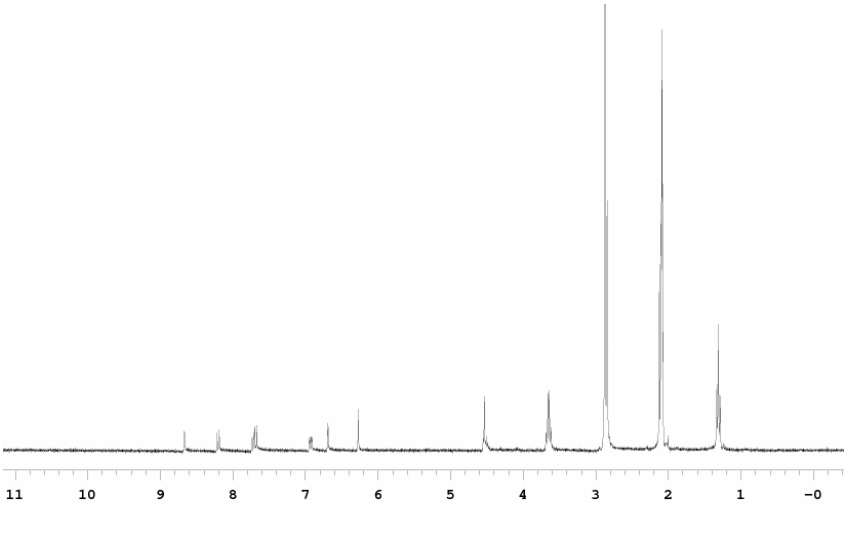


51

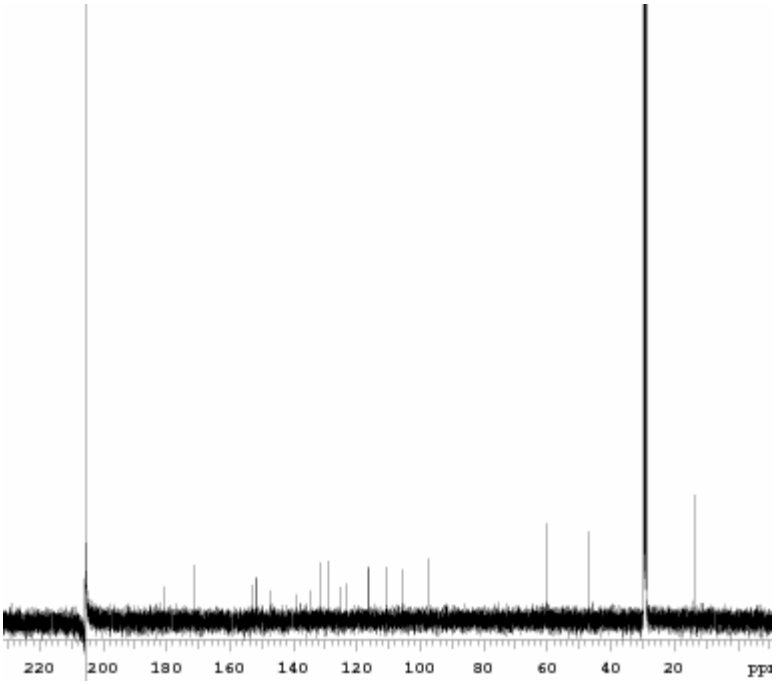
9-(Diethylamino)-2-(3-hydroxyprop-1-ynyl)-5H-benzo[a]phenoxazin-5-one 51

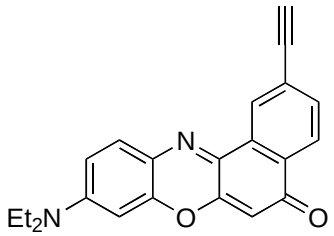
$Pd(PPh_3)_4$ (123.6 mg, 0.1mmol) and CuI (20.4 mg, 0.1 mmol) was added to a solution of compound **50** (500 mg, 1.0 mmol) in dry DMF (5.0 ml) in an oven dried Schlenk tube. To the above mixture was added an excess of propargyl alcohol (0.7 ml, 10.7 mmol) and triethyl amine (1.5 ml, 10.7 mmol). Reaction mixture was heated to 120 ^{o}C and left overnight (12h) under argon. Mixture was filtered through a sintered funnel and organic layer evaporated under reduced pressure and purified by flash chromatography 1:1 hexane/ EtOAc, yielding **51** as a red solid (253 mg, 62%). R_f = 0.50 (1:1 hexanes/EtOAc) ^{1}H NMR (300 MHz, acetone –d_6) δ 8.67 (s, 1H), 8.20 (d, 2H, J = 8.1 Hz), 7.70 (t, 2H, J = 9.0 Hz), 6.93 (d, 1H, J = 6.3 Hz) 6.69 (s, 1H), 6.27 (s, 1H), 4.53-4.50 (m, 2H), 3.68-3.61 (m, 4H), 1.31 (t, 6H, J = 6.9 Hz) ^{13}C NMR (75 MHz, acetone –d_6) δ 180.9, 171.4, 152.8, 151.8, 151.7, 151.6, 147.1, 134.2, 131.6, 131.5, 128.8, 125.3, 123.0, 116.2, 111.0, 105.4, 97.4, 60.4, 46.8, 13.8 M.S. (ESI) calcd for $C_{24}H_{22}N_2O_3$ (M^+) 386.16 found (M^+) 386.0832.

^{1}H NMR compound **51**



^{13}C NMR compound **51**

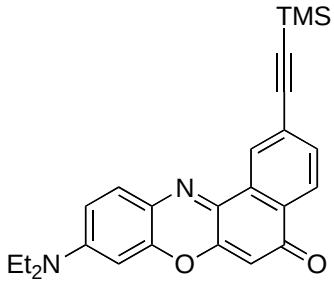




52

9-(diethylamino)-2-ethynyl-5H-benzo[a]phenoxazin-5-one 52

A solution of **51** (200.0 mg, 0.54 mmol) in 5.0 ml of dry toluene was added to18-C-6 (7.0 mg, 0.03 mmol), $KMnO_4$ (467.4 mg, 5.4 mmol) and KOH (150.4 mg, 2.68 mmol) previously dissolved in 5 ml of toluene. Reaction mixture was heated to 40 ^oC for 3h. Filtered through a sintered glass funnel to remove solid residues and organic layer evaporated under reduced pressure and purified by flash chromatography using 30% EtOAc/hexane, yielding 65 mg of **52** as a red solid (65 mg, 30%). R_f = 0.6 (70%hexanes/EtOAc)



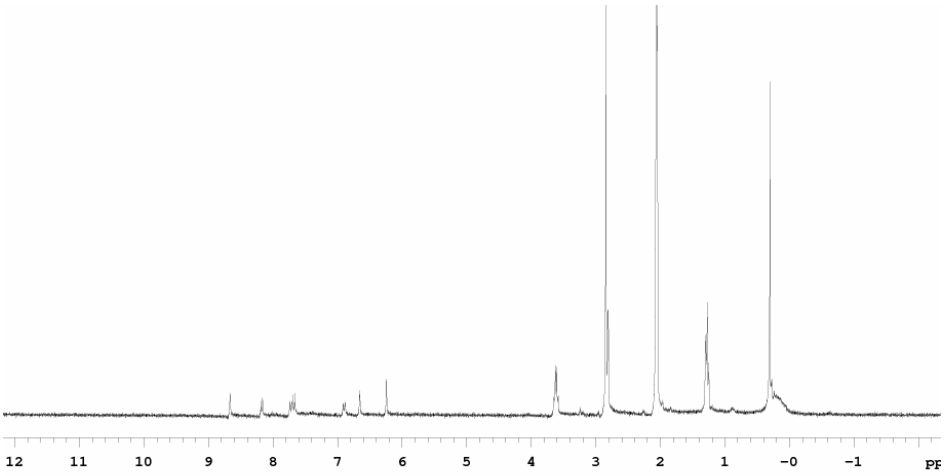
53

9-(Diethylamino)-2-(2-(trimethylsilyl)ethynyl)-5H-benzo[a]phenoxazin-5-one 53

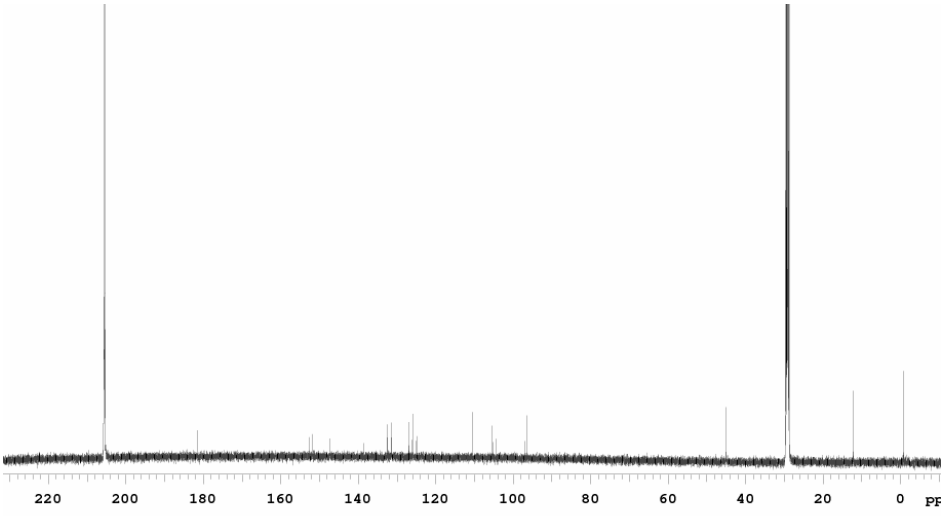
$Pd(PPh_3)_4$ (46.0 mg, 0.04mmol), CuI (7.6mg, 0.1 mmol) was added to a solution of **50** (200mg, 0.4 mmol) in 5ml of dry DMF in an oven dried Schlenk tube. To the above mixture was added an excess of TMS alkyne (0.6 ml, 4.0 mmol) and triethyl amine (0.5ml, 0.4 mmol). Reaction mixture was vacuum purged thrice after cooling to -78 ^oC in an acetone/dry ice bath. Reaction mixture heated to 80 ^oC for 24 h. Filtered through a sintered glass funnel and organic layer evaporated under reduced pressure. Purified by flash chromatography using 30% EtOAc/ hexane to obtain **53** (100 mg, 58%) as a red

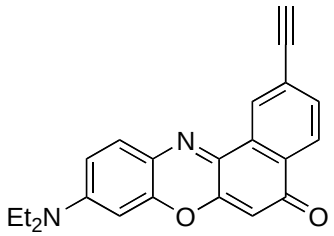
solid. R_f = 0.8 (50%hexanes/EtOAc) ^{1}H NMR (300 MHz, acetone –d_6) δ 8.66 (s, 1H), 8.17 (d, 1H, 7.8 Hz) 7.73 (d, 1H, 9.6 Hz), 7.67 (d, 1H, 9.6 Hz), 6.90 (d, 1H, 11.7 Hz), 6.66 (s, 1H), 6.24 (s, 1H), 3.63-3.60 (m, 4H), 1.27 (t, 6 H, 6.9 Hz), 0.29 (s, 9 H) ^{13}C NMR (75 MHz, acetone –d_6) δ 181.6, 152.6, 151.7, 147.2, 138.5, 132.6, 131.5, 131.4, 127.0, 126.1, 125.9, 125.1, 124.9, 110.5, 105.3, 104.4, 96.9, 96.4, 45.0, 12.2, 0.8; M.S.(ESI) calcd for $C_{25}H_{26}N_2O_2Si$ (M^+) 414.18 found (M^+) 414.2013.

^{1}H NMR compound **53**



^{13}C NMR compound **53**





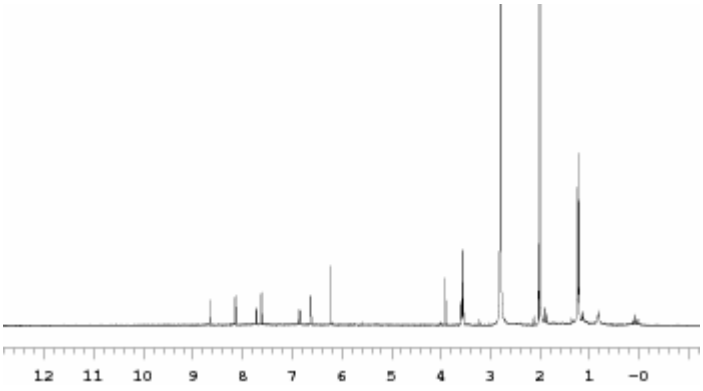
52

9-(Diethylamino)-2-ethynyl-5H-benzo[a]phenoxazin-5-one 52

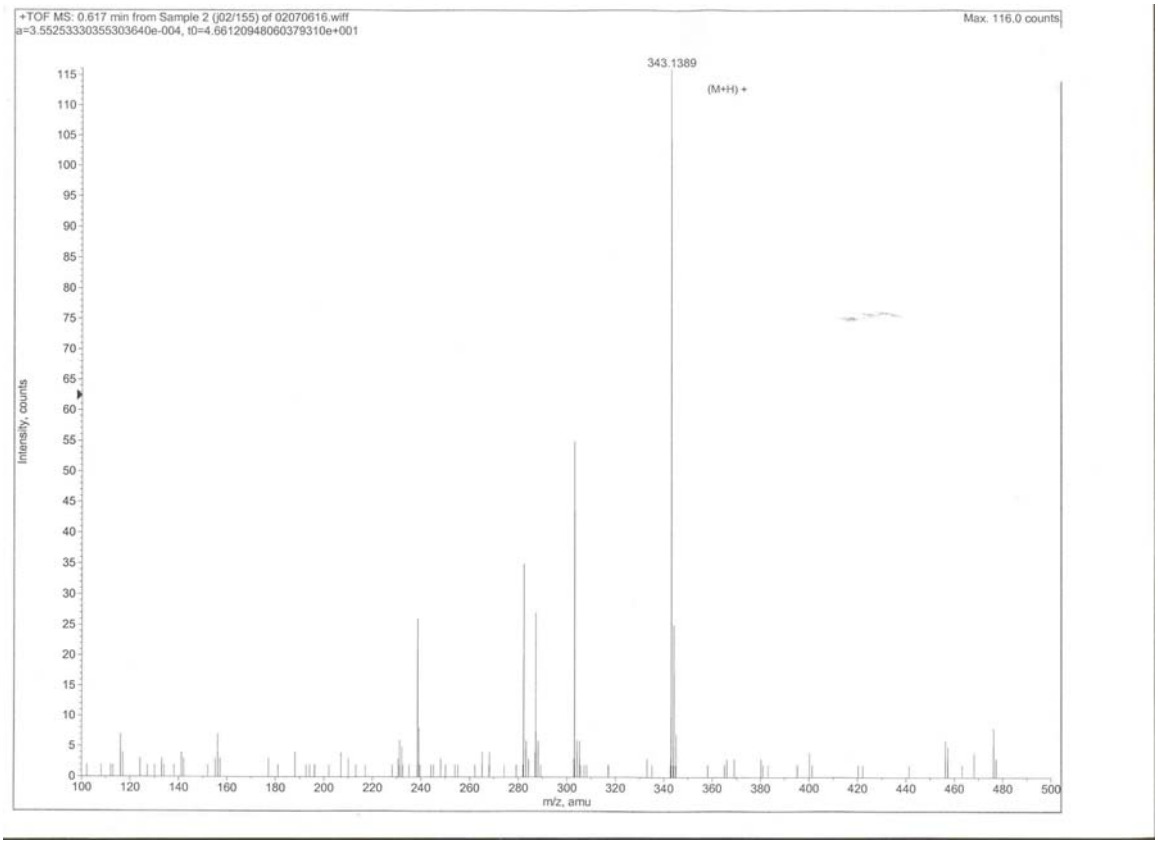
TBAF 1.0 M solution in THF (0.6 ml, 0.6 mmol) was added to a solution of **53** (100 mg, 0.2 mmol) in 10 ml of dry distilled THF at -78 ^{o}C. The reaction mixture was allowed to warm to 25 ^{o}C for 3 h. Excess TBAF was removed by washing with water

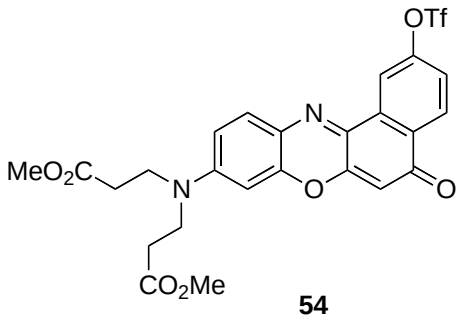
(2 x 10 ml) and organic layer evaporated under reduced pressure. Purified by flash chromatography using 30% EtOAc/ Hexane to yield **52** (59 mg, 80%) of a red solid. R_f = 0.6 (70%hexanes/EtOAc) NMR (300 MHz, acetone $-d_6$) δ 8.66 (s, 1H), 8.14 (d, 1H, J = 8.5 Hz), 7.72 (d, 1H, J = 6.0 Hz) 7.61 (d, 1H, J = 9.0 Hz), 6.86 (d, 1H, J = 6.5 Hz), 6.62 (s, 1H), 6.21 (s, 1H), 3.91 (s, 1H), 3.57-3.54 (m, 4H), 1.24-1.21(m, 6H) M.S.(ESI) calcd for $C_{22}H_{22}N_2O_2$ (M^+) 342.14 found $(M+H)^+$ 343.1389.

^{1}H NMR compound **52**



Mass spectra (ESI) compound **52**





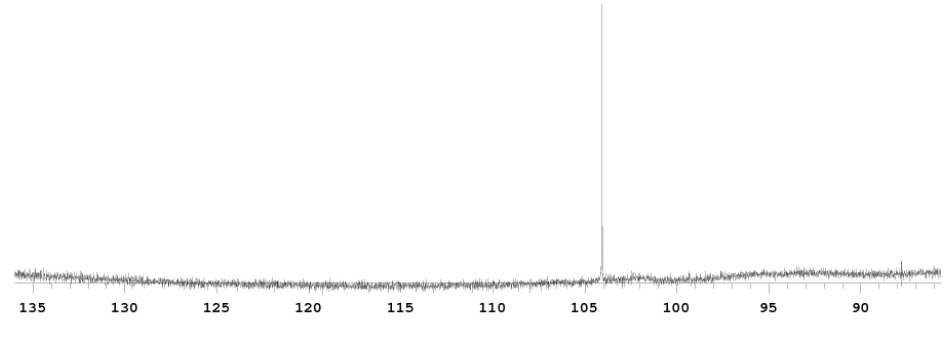
54

9-[*N*,*N*-Bis (2-(methoxycarbonyl)ethyl)amino]-5-oxo-5H-benzo[a]phenoxazin-2-yl trifluoromethanesulfonate 54

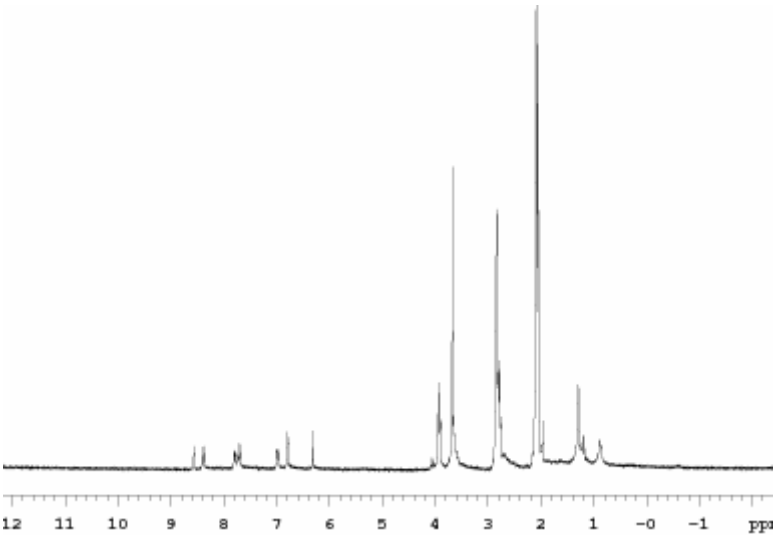
Phenyl triflamide (0.5 ml, 2.7 mmol) was added gradually over a period of 10 minutes to a solution of **43** (1.0g, 2.2 mmol) and DBU (1.3 ml, 8.8 mmol) in 20.0 ml dry distilled CH_2Cl_2 and the mixture was cooled to 0 ^oC in an ice bath. The mixture was allowed to

warm slowly to 25 ^{o}C with stirring for 3h. Reaction mixture was washed with 0.1 M HCl (2 x 10 ml). Organic layer was evaporated under reduced pressure and purified by flash chromatography eluting with 1:1 hexane/ EtOAc to afford **54** as a red solid (0.91g, 70 %). R_f = 0.4 (1:1 hexanes/EtOAc) ^{1}H NMR (300 MHz, acetone-d_6) δ 8.57 (s, 1H), 8.38 (d, 1H, J = 8.4 Hz), 7.79 (d, 1H, J = 8.1 Hz), 7.70 (d, 1H, J = 9.3 Hz), 6.98 (d, 1H, J = 9.0 Hz), 6.79 (s, 1H), 6.30 (s, 1H), 3.93 (t, 4H, 7.2 Hz), 3.67 (s, 6H), 2.78 (t, 4H, J = 7.5 Hz) ^{13}C NMR (75 MHz, acetone –d_6) δ 181.92, 173.11, 153.27, 151.97, 151.79, 146.33, 139.77, 135.72, 133.69, 129.12, 124.33, 123.43, 118.01, 111.12, 106.78, 98.23, 60.36, 49.27, 33.43. ^{19} F NMR (300 MHz, acetone –d_6) δ 104.4

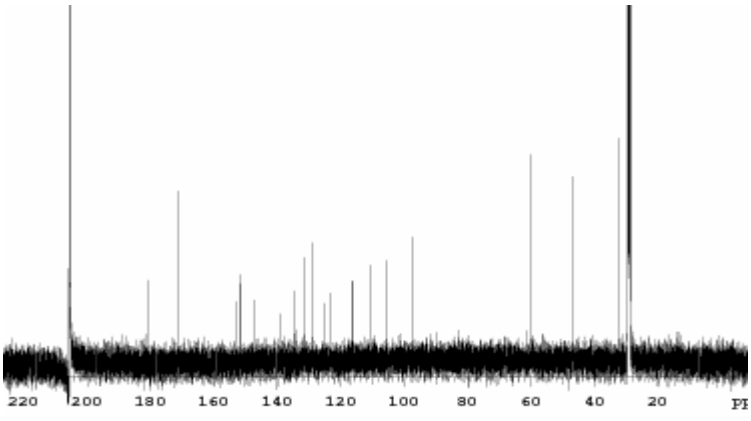
^{19}F NMR compound **54**

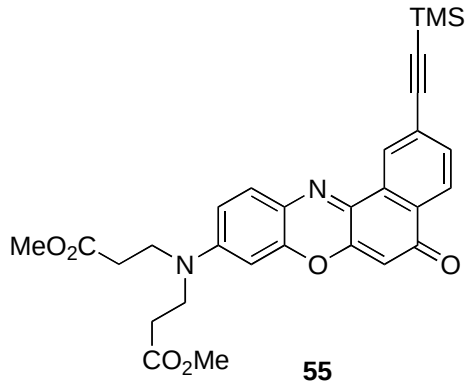


^{1}H NMR compound **54**



^{13}C NMR compound **54**





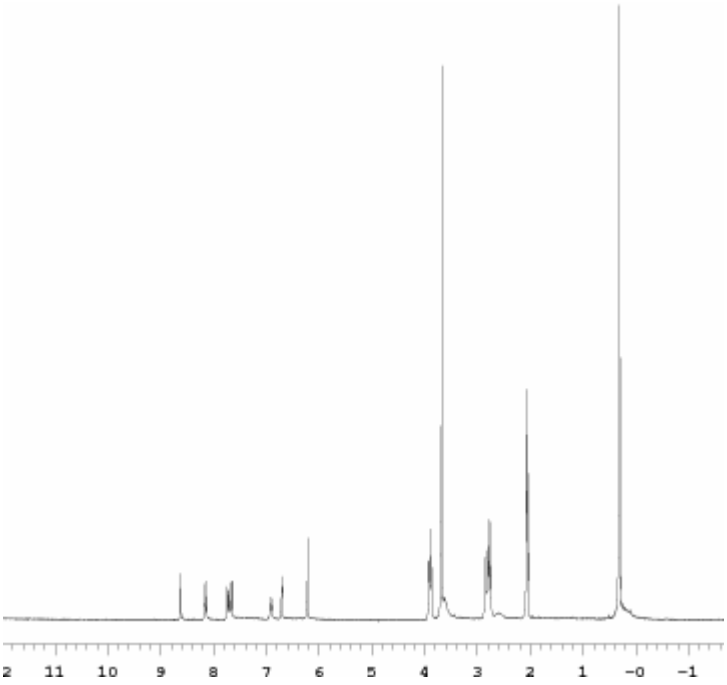
55

9-[*N,N*-Bis (2-(methoxycarbonyl)ethyl)amino]-2-(2-(trimethylsilyl) ethynyl)-5H-benzo[a]phenoxazin-5-one 55

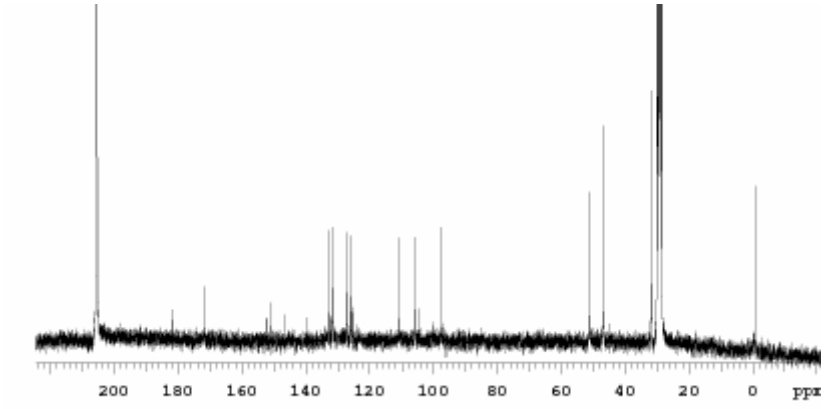
$Pd(PPh_3)_4$ (50.0 mg, 0.04mmol), CuI (8.2 mg, 0.04 mmol) was added to a solution of **54** (200mg, 0.4 mmol) in 5.0 ml of dry DMF. To the above mixture was added an excess of TMS alkyne (0.6 ml, 4.30 mmol) and triethyl amine (0.6ml, 4.3 mmol). Reaction mixture was vacuum purged thrice after cooling to -78 ^{o}C in an acetone/dry ice bath. Reaction mixture heated to 80 ^{o}C for 24 h. Filtered through a sintered glass funnel and organic layer evaporated under reduced pressure. Purified by flash chromatography using 30% EtOAc/hexane to obtain **55** (125 mg, 73%) as a red solid. R_f = 0.6 (30 % EtOAc in Hexane) ^{1}H NMR (300 MHz, acetone-d_6) δ 8.62 (s, 1H), 8.14 (d, 1H, J = 8.1 Hz), 7.72 (d, 1H, 6.6 Hz), 7.66 (s, 1H, J = 9.0 Hz), 6.90 (s, 1H, J = 6.3 Hz), 6.70 (s, 1H), 6.22 (s, 1H), 3.89 (t, 4 H, J = 7.5 Hz), 3.62 (s, 6H), 2.77 (t, 4H, J = 6.9 Hz), 0.31 (s, 9H). ^{13}C

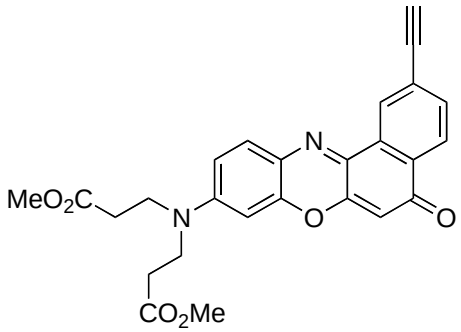
NMR (75 MHz, acetone $-d_6$) δ 181.7, 171.9, 152.4, 151.2, 146.9, 139.7, 132.9, 131.4, 127.1, 126.2, 125.9, 125.3, 110.7, 105.6, 104.3, 100.1, 97.39, 51.3, 47.0, 31.8, 0.7.

^{1}H NMR compound **55**



^{13}C NMR compound **55**



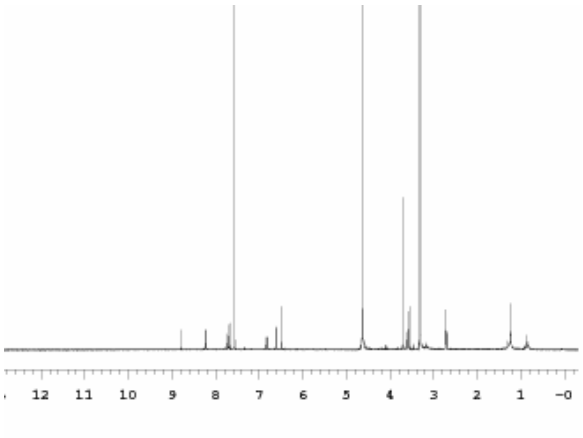


56

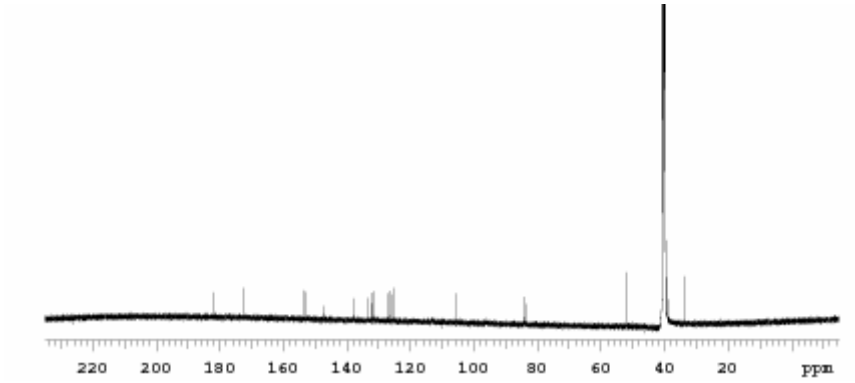
9-[*N*,*N*-Bis(2-(methoxycarbonyl)ethyl)amino]-2-ethynyl-5H-benzo[a]phenoxazin-5-one 56

TBAF 1.0 M solution in THF (0.6 ml, 0.6 mmol) was added to a solution of **55** (100 mg, 0.17 mmol) in 10 ml of dry distilled THF at -78 ^{o}C. The reaction mixture was allowed to warm to 25 ^{o}C for 3 h. Excess TBAF was removed by washing with water (2 x 10.0 ml) and organic layer evaporated under reduced pressure. Purified by flash chromatography using 50% EtOAc/ Hexane to yield **56** (63 mg, 80%) as a red solid. R_f = 0.4 (50% hexanes/EtOAc) ^{1}H NMR (300 MHz, $CD_3OD/CDCl_3$) δ 8.80 (s, 1H), 8.22 (d, 1H, 8.0 Hz), 7.72 (d, 1H, *J* = 8.0 Hz), 7.68 (d, 1H, *J* = 9.0 Hz), 6.83 (d, 1H, *J* = 6.5 Hz), 6.60 (s, 1H), 6.49 (s, 1H), 3.71 (s, 6H), 3.6 (t, 4H, *J* = 7.0 Hz), 3.53 (s, 1H), 2.718 (t, 4H, *J* = 6.5 Hz). ^{13}C NMR (75 MHz, DMSO–d_6) δ 181.77, 172.42, 153.81, 152.76, 147.48, 137.85, 133.15, 132.39, 131.83, 131.35, 127.17, 126.29, 125.60, 125.36, 105.36, 84.21, 83.43, 52.21, 39.07, 34.79; M.S.(ESI) calcd for $C_{26}H_{22}N_2O_6$ (M^+) 458.15 found $(M+H)^+$ 459.1586.

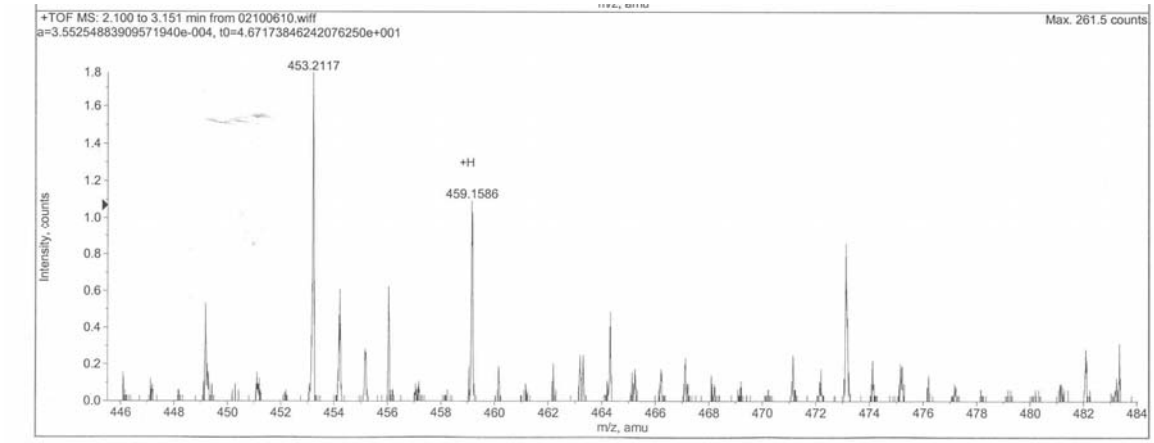
^{1}H NMR compound **56**

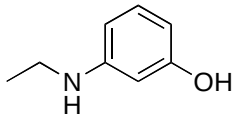


^{13}C NMR compound **56**



Mass spectra (ESI) compound **56**



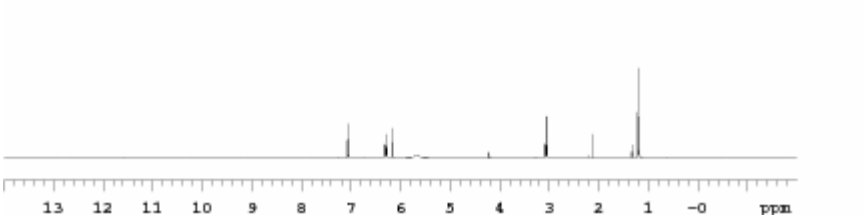


57

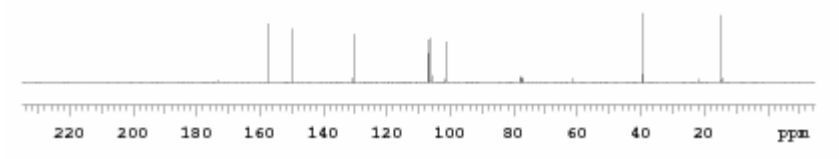
3-(Ethylamino)phenol 57[91]

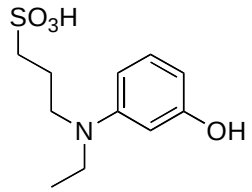
A solution of 3-aminophenol (4.0 g, 37.0 mmol) and potassium carbonate (5.0 g, 37.0 mmol) in DMF (20.0 ml) was a stirred for 15 min. Iodoethane (5.6 g, 37.0 mmol) was then added and heated to 100 ^{o}C for 2 h. Reaction mixture was then cooled to 25 ^{o}C and filtered to remove solid impurities. Water (20.0 ml) was added to the filtrate and extracted with EtOAc (20.0 ml, twice), solvent evaporated and crude reaction mixture purified using flash chromatography eluting with (20 - 50 %) EtOAc/hexane to afford **57** as brown oil (3.5g, 70%). R_f = 0.5 (1:1EtOAc/hexane) NMR (300 MHz, $CDCl_3$) δ 7.07 (t, 1H, J = 8.0 Hz), 6.32-6.28 (m, 2H), 6.16 (s, 1H), 4.23-4.21 (m, 1H), 3.09-3.05 (m, 2H), 1.21 (t, 3H, J = 7.5 Hz) ^{13}C NMR (125 MHz, CDCl3) δ 157.2, 149.9, 130.6, 106.9, 106.0, 101.5, 39.3, 14.8; MS (ESI) calcd for $C_8H_{11}NO$ (M^+) 137.08 found (M^+) 137.0843.

^{1}H NMR compound **57**



^{13}C NMR compound **57**





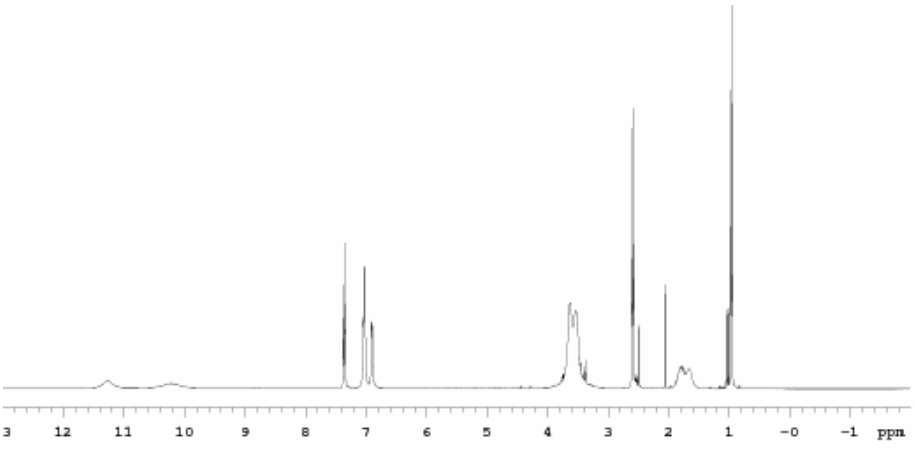
58

3-(*N*-Ethyl-*N*-(3-hydroxyphenyl)amino)propane-1-sulfonic acid 58

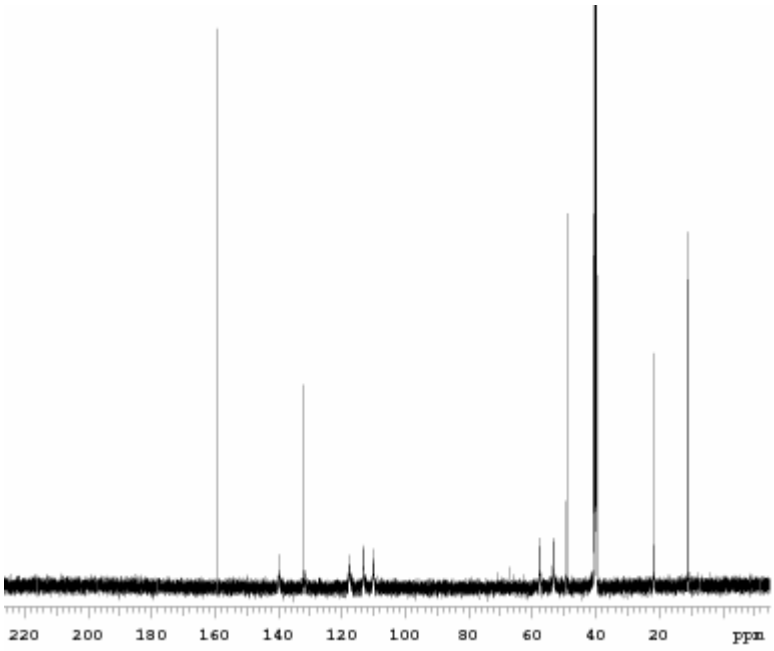
Compound **47** (3.5 g, 25.5 mmol) and 1,3-propanesultone (3.36 g, 27.5 mmol) was dissolved in 20 ml *iso*-propanol and refluxed for 3 h. The reaction mixture was cooled to 25 ^{o}C and the precipitate filtered and washed with distilled water (5.0 ml, twice) to remove any unreacted sultone to afford **58** as a whit amorphous powder (2.62 g, 60%).

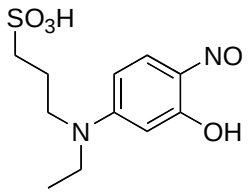
R_f = 0.3 (90%EtOAc/MeOH). NMR (300 MHz, DMSO-d_6) δ 7.36 (t, 1H, J = 48.0 Hz), 7.04 (d, 2H, J = 12.0 Hz), 6.91 (s, 1H), 3.63-3.53 (br, 4H), 2.6-2.58 (m, 2H), 1.81-1.74 (br, 2H), 1.00- 0.948 (m, 3H) ^{13}C NMR (75 MHz, DMSO-d_6) δ 159.7, 140.1, 131.8, 117.3, 113.9, 110.4, 57.9, 53.4, 49.4, 22.1, 11.2; MS (ESI) calcd for $C_{11}H_{17}NO_4$ S (M^+) 259.09 found $(M-H)^-$ 258.0167.

^{1}H NMR compound **58**



^{13}C NMR compound **58**

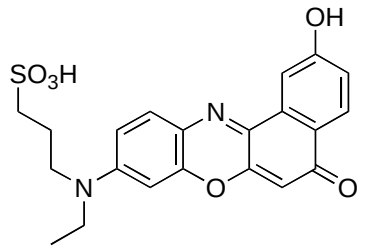




59

3-(*N*-Ethyl-*N*-(3-hydroxy-4-nitrosophenyl)amino)propane-1-sulfonic acid 59

Compound **58** (1.0 g, 3.9 mmol) in HCl (10.0ml, 10.0M) and water (5 ml), was cooled to 0 ^{o}C and sodium nitrite (0.13 g, 1.8 mmol) dissolved in water (2 ml) added over a period of 50 minutes using a syringe pump at the rate of 0.2 ml per min. The mixture was stirred for 3 h at 0 ^{o}C and filtered to remove residual impurities. The filtrate was evaporated under reduced pressure to yield **59** (1.05 g, 80 %). The crude product was very water sensitive and therefore directly used without further purification for the next step.

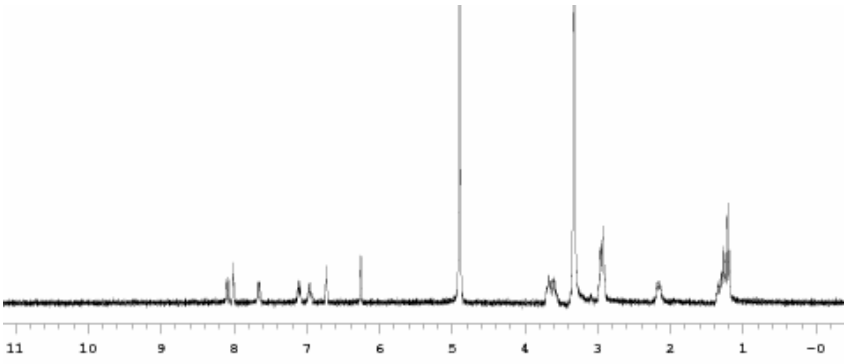


7

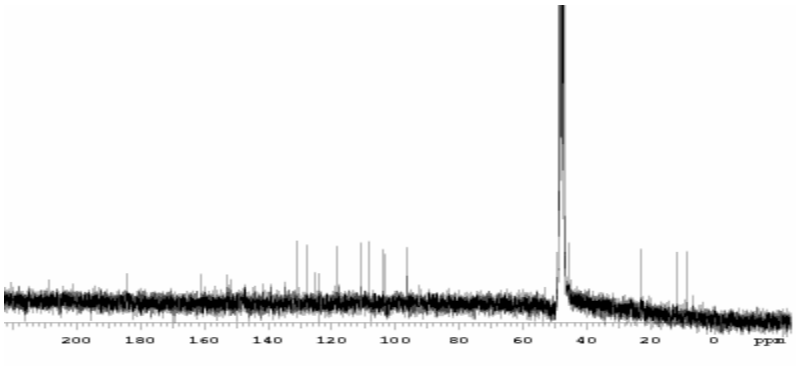
3-(*N*-Ethyl-*N*-(2-hydroxy-5-oxo-5H-benzo[a]phenoxazin-9-yl)amino)propane-1-sulfonic acid 7

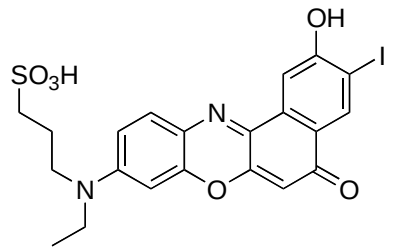
Compound **59** (1.0 g, 3.1 mmol) along with 1, 6-dihydroxyNaphthol in DMF (20.0 ml) was heated to 150 ^{o}C for 4 h. The reaction mixture was cooled to 25 ^{o}C and solvent evaporated under reduced pressure. The crude product was purified using flash chromatography eluting with 1:1 EtOAc/MeOH to yield **7** as a dark red solid (1.0 g, 73%). R_f = 0.4 (1:1EtOAc/MeOH). NMR (300 MHz, CD_3OD) δ 8.06 (d, 1H, J = 8.4Hz), 7.99 (s, 1H), 7.08 (dd, 1H, J = 6.0 Hz, J = 2.4 Hz), 6.94 (dd, 1H, J = 9.3 Hz, J = 3.2 Hz), 6.70 (s, 1H), 6.23 (s, 1H), 3.71-3.54 (m, 2H), 3.12 (q, 2H, J = 7.2 Hz), 2.92 (q, 2H, J = 6.9 Hz), 2.19-2.096 (br, 2H), 1.32-1.29 (br, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 184.2, 161.3, 151.9, 150.8, 131.2, 127.6, 125.2, 124.2, 118.1, 110.8, 108.5, 103.6, 96.3, 49.3, 45.3, 23.0, 11.4, 8.2.

^{1}H NMR compound **7**



^{13}C NMR compound **7**



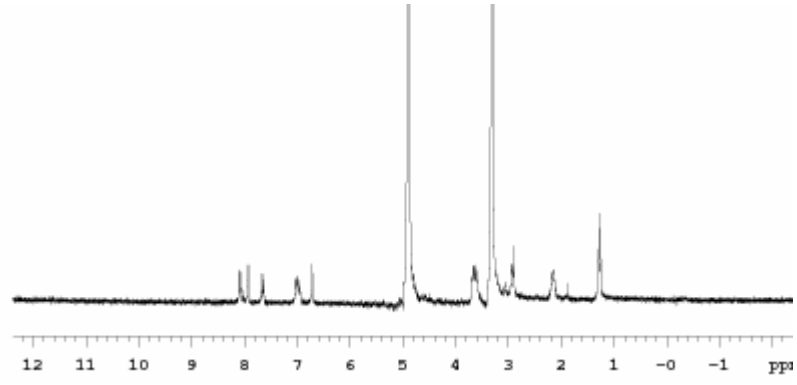


60

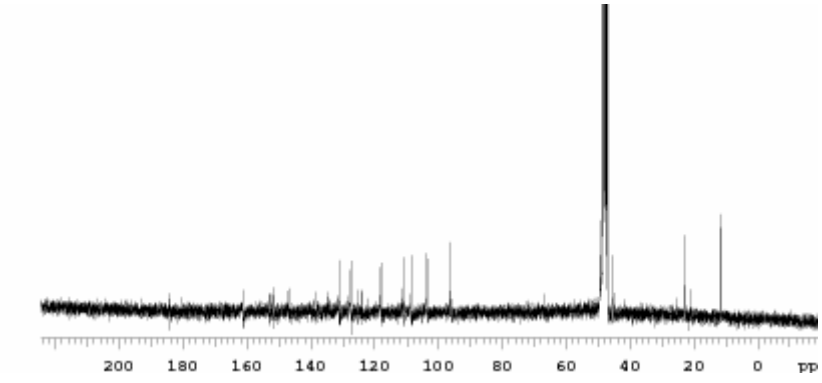
3-(*N*-Ethyl-*N*-(2-hydroxy-3-iodo-5-oxo-5H-benzo[a]phenoxazin-9-yl)amino) propane-1-sulfonic acid 60

Compound **7** (200 mg, 0.47 mmol) and pyridine (0.08 ml, 0.93 mmol) in dry distilled MeOH (16 ml) was cooled to 0 ^{o}C and ICl (0.51 ml, 0.51 mmol) added drop wise in 2 minutes and heated to 70 ^{o}C for 3 h. Reaction mixture was cooled to 25 ^{o}C and solvent evaporated under reduced pressure. Residue was dissolved in 10 ml of water, filtered to remove any solid impurities and purified using reverse phase medium pressure liquid chromatography (MPLC) with water as eluting agent to yield **60** as blue solid (0.1 g, 42 %). ^{1}H NMR (300 MHz, CD_3OD) δ 8.08 (d, 1H, J = 8.7 Hz), 7.93 (s, 1H), 7.65 (d, 1H, J = 9.6 Hz), 7.02-6.89 (m, 2H), 6.72 (s, 1H), 3.67-3.59 (m, 4H), 2.92 (t, 2H, J = 7.8 Hz), 2.18-2.12 (m, 2H), 1.27 (t, 3H, J = 7.2 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ 183.8, 161.2, 151.8, 147.0, 138.7, 134.6, 131.2, 127.5, 125.2, 124.1, 118.2, 11.3, 110.7, 108.5, 103.6, 96.2, 49.3, 45.3, 23.0, 11.46. MS (ESI) calcd for $C_{21}H_{19}N_2O_6S$ (M^+) 554.35 found $(M-H)^-$ 553.0239.

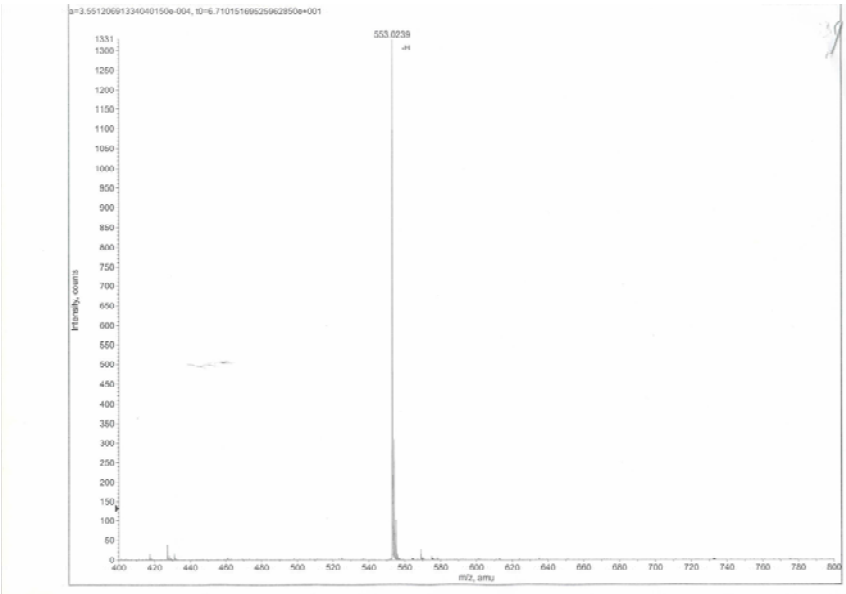
^{1}H NMR compound **60**



^{13}C NMR compound **60**



Mass spectra (ESI) compound **60**

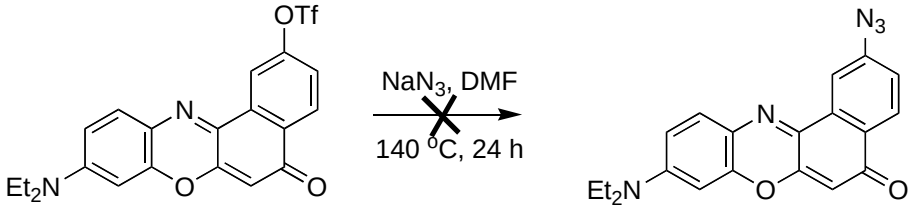


APPENDIX B
RELATED ATTEMPTED REACTIONS

This section includes those reactions, which were tried out, but was not successful under the tried reaction conditions.

A. Conversion of Nile Red triflate to azide.

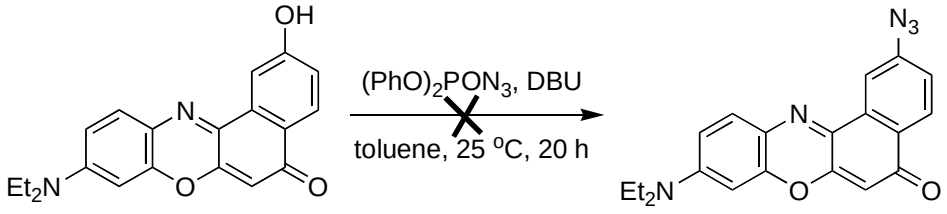
Scheme 1. Synthesis of 2-azido-9-(diethylamino)-5*H*-benzo[*a*]phenoxazin-5-one from Nile Red triflate 62.



50 **81**

Nile Red triflate **50** (20 mg, 0.04 mmol) in DMF (1ml) along with sodium azide (14.0 mg, 0.214 mm0l) was heated to 140 ^{0}C under a blast shield. The temperature was maintained at 140 ^{0}C for 24 h. TLC (1:1 hexane/EtOAc) does not show any conversion. Reaction mixture was cooled to 25 ^{o}C and solvent evaporated under reduced pressure. Mass spectra and ^{13}C NMR always showed starting material.

Scheme 2. Synthesis of 2-azido-9-(diethylamino)-5*H*-benzo[*a*]phenoxazin-5-one from 2-hydroxy Nile Red.



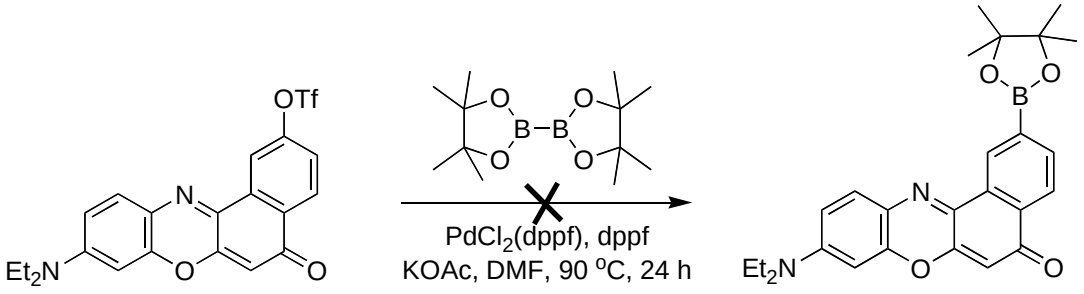
6 **81**

2-Hydroxy Nile Red **6** (50 mg, 0.15 mmol) and diphenyl phosphorazidate (48.0 μml, 0.22 mmol) in dry distilled toluene (1 ml) was cooled to 0^{o}C and DBU (33.0 μml, 0.22 mmol) was added and the reaction mixture stirred for 2 h at 0^{o}C. The temperature of the reaction

mixture was raised to 25 ^{o}C and the reaction was continued for 20 h. TLC (1:1 hexane/EtOAc) shows a faint new spot just below starting material. Toluene was evaporated under reduced pressure and residue purified by flash chromatography using 1:1 hexane/EtOAc. ^{1}H NMR was messy and mass spectrum shows no peak for the product.

B. Conversion of Nile Red triflate to Nile Red borate.

Scheme 3. Conversion of Nile Red triflate to 9-(diethylamino)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-5*H*-benzo[*a*]phenoxazin-5-one.

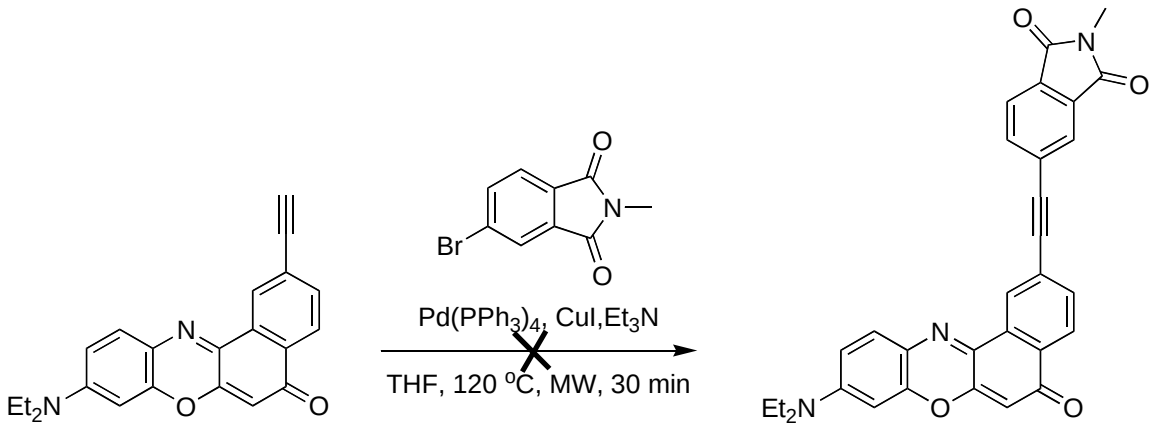


50 **82**

Nile Red triflate **40** (20 mg, 0.04 mmol) was dissolved in DMF (2ml) and Pd catalyst, 1,1'-bis(diphenylphosphino)ferrocene, potassium acetate, was added in that order. The reaction mixture was heated to 90 ^{o}C and held at 90 ^{o}C for 24 h. TLC (1:1 hexane/EtOAc) shows a faint new spot at 0.4 and Nile Red triflate spot at 0.8. Solvent was evaporated under reduced pressure and residue purified by flash chromatography using 1:1 hexane /EtOAc as eluent. The product is very unstable and ^{1}H NMR was always very messy.

B. Sonogashira coupling of Nile Red alkyne with 4-bromo-*N*-methylphthalimide.

Scheme 4. Sonogashira coupling of Nile Red alkyne with 4-bromo-*N*-methylphthalimide.



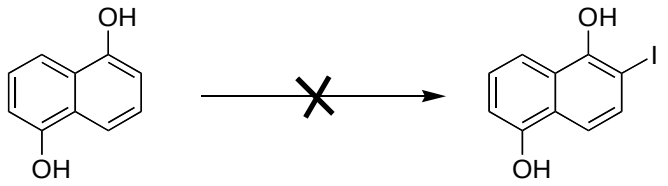
52 **83**

$Pd(PPh_3)_4$ (7.0 mg, 0.006 mmol), CuI (1.1mg, 0.0058 mmol) was added to a solution of Nile Red alkyne (20mg, 0.06 mmol) and 4-bromo-*N*-methylphthalimide in 5ml of dry DMF in an oven dried sealed tube. To the above mixture was added triethyl amine (80.8 μml, 0.6 mmol). Reaction mixture was vacuum purged thrice after cooling to -78 ^{o}C in an acetone/dry ice bath. Reaction mixture heated to 120 ^{o}C in microwave for 30 minutes. TLC (1:1 hexane/EtOAc) shows a very faint new spot Rf = 0.6. Filtered through a sintered glass funnel and organic layer evaporated under reduced pressure. Purified by flash chromatography using 1:1 hexane/EtOAc to obtain 4-5mg of a red solid. No molecular ion peak was seen in mass spectrum.

Only trace conversion was seen on TLC for the above coupling reaction. This was because Nile Red alkyne usually decomposed at elevated temperature.

D. Iodination of 1,5-dihydroxynaphthol.

Scheme 5. Iodination of 1,5-dihydroxynaphthol.



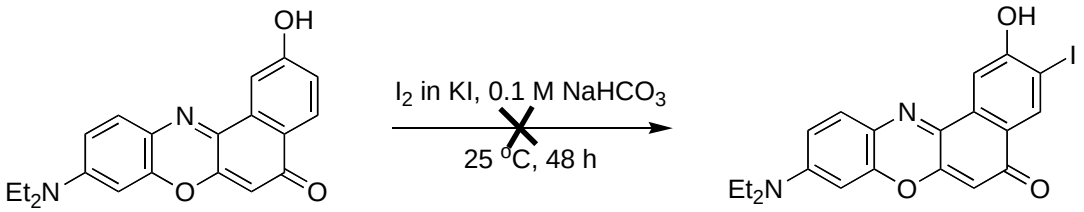
84

reagent	solvent	temp. (^{o}C)	duration
NIS	DMF	25	1 d
NIS, CF_3COOH	CH_3CN	25	1 d
NIS	chlorobenzene	25	1 d
I_2, KI	0.1 M Na_2CO_3	25	12 h
I_2, KI	0.1 M $NAHCO_3$	40	2 d
Br_2	CH_3COOH	45	2 h
Br_2, KBr	K_2CO_3	40	12 h

Iodination of 1,5-dihydroxynaphthol was attempted under the above conditions. This was a model study to optimize the best set of conditions for iodination of Nile Red derivatives. None of the above reactions worked. ^{1}H NMR of the reaction mixture always showed only starting material.

E. Halogenation of Nile Red derivatives **6** and **41**.

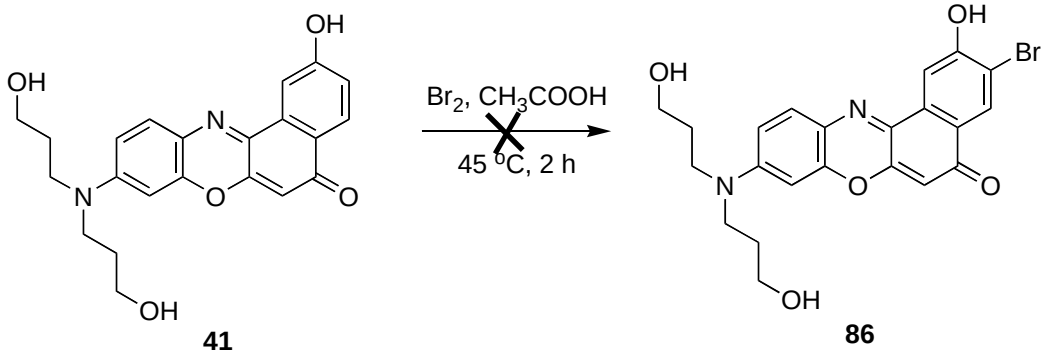
Scheme 6. Iodination of Nile Red in basic media.



6 **85**

2-Hydroxy Nile Red (50 mg, 0.15 mmol) was dissolved in 1.0M $NaHCO_3$ (5 ml) and iodine (76.1 mg, 0.3 mmol) in KI (1.0ml, 3.0M) was added. The reaction mixture was stirred for 48 h at 25 ^oC. Reaction mass was added to HCl (5.0 ml 1.0 M) in ice (100ml). The blue colored precipitate was filtered and dissolved by addition of NaOH (5.0 ml, 2.0M). To the above solution was added HCl (2 ml, 10.0 M) till solution turned acidic. The precipitate was filtered and dried to obtain 52 mg of blue solid. ^1H NMR is messy and shows too many peaks. Mass spectrum does not show molecular ion peak for mono or diiodination.

Scheme 7. Bromination of dihydroxy/phenolic Nile Red in acidic media



41 86

Dihydroxy/phenolic Nile Red (47.0 mg, 0.12 mmol) was dissolved in acetic acid (1.0 ml). Acetic acid was cooled to 0 ^oC before adding dihydroxy/phenolic Nile Red. Bromine (7.4 μml), dissolved in acetic acid (28.4 μml) was added drop wise to the above solution and temperature raised to 45 ^oC and held at that temperature for 2 h. The color of the reaction mixture was brown after 2 h indicating that the starting material decomposed.

F. Attempted synthesis of a planar benzophenoxazine dye.

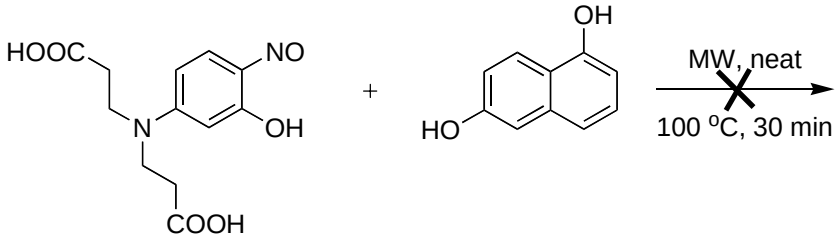
Scheme 8. Attempted synthesis of a planar benzophenoxazine dye.

49 87

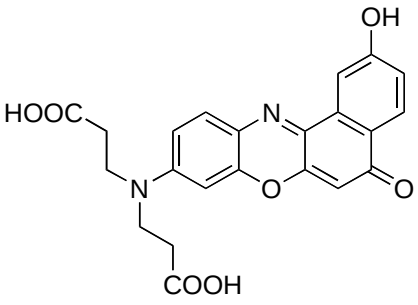
5-(Diethylamino)-2-nitrosophenol (200mg, 0.87 mmol) and 2,7-dihydroxynaphthol (140.0 mg, 0.87 mmol) was dissolved in DMF (5 ml) and heated to 150 ^{o}C for 5 h. The reaction mixture was always brown in color. No color change occurred even after 5 h. Reaction was therefore stopped and no workup was attempted. TLC in EtOAc showed no new spot. The above reactions were not successful even in neat conditions. Addition of acid should facilitate condensation.

G. Attempted synthesis of dicarboxylic acid derivative of 2-hydroxy Nile Red.

Scheme 9. Attempted synthesis of dicarboxylic acid derivative of 2-hydroxy Nile Red.



88



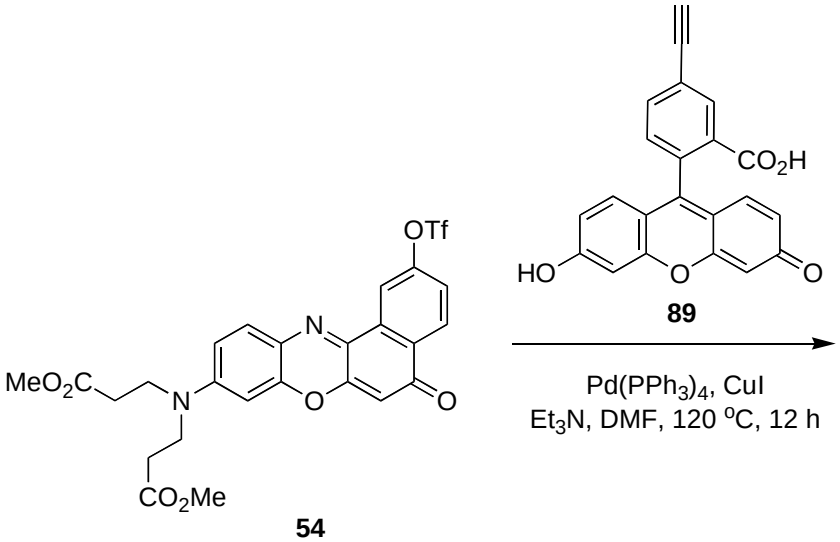
44

The above nitroso compound (50 mg, 0.16 mmol) and 1,6-dihydroxynaphthol (25.1 mg, 0.16 mmol) was heated under stirring in a sealed tube in microwave to 100 ^{o}C for 30 minutes. The UV of reaction mixture in methanol did not show any absorption between 400-600 nm. Color of the solution in methanol was brown. Decomposition of nitroso compound might have taken place at 100 ^{o}C. This reaction always needs catalytic

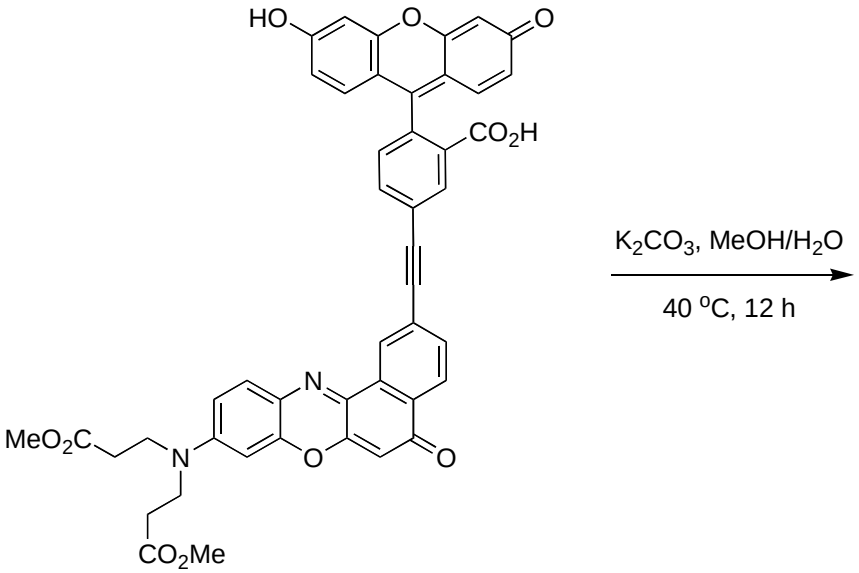
amount of acid to proceed. Also the carboxylic acid had to be protected before condensation.

H. Attempted synthesis of water-soluble through-bond energy transfer cassette from Nile Red triflate and fluorescein alkyne.

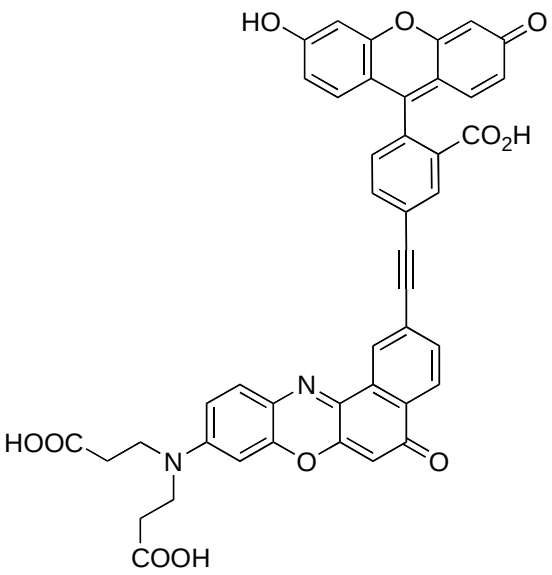
Scheme 10. Synthesis of 9-(*N*,*N*-Bis(2-carboxyethyl)amino)-2-(2-(4-(3-hydroxy-6-oxo-6H-xanthen-9-yl)phenyl)ethynyl)-5H-benzo[a]phenoxazin-5-one



Scheme 10. Continued.



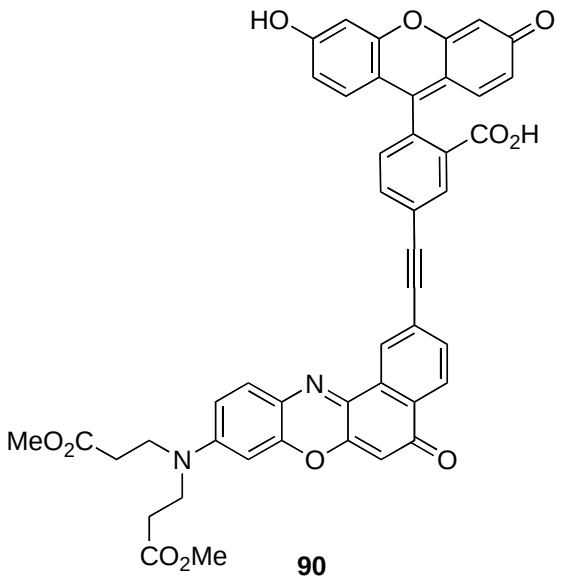
90



91

This was the only attempted Synthesis of an energy transfer cassette. Even though not part of the actual project, the model cassette was synthesized to optimize conditions for coupling of the triflate with fluorescien alkyne. After screening several conditions and temperature, the Sonogashira coupling was achieved in good yield at 120 ^{o}C in DMF. The coupling worked only in presence of Pd (0) catalyst. Use of Pd (II) catalyst resulted

in only trace amount of product (evident from TLC). Mr. Yuichiro Ueno generously and magnanimously provided the fluorescien alkyne. The purification of the final cassette proved to be difficult and was not pursued on an HPLC due to time constraints.

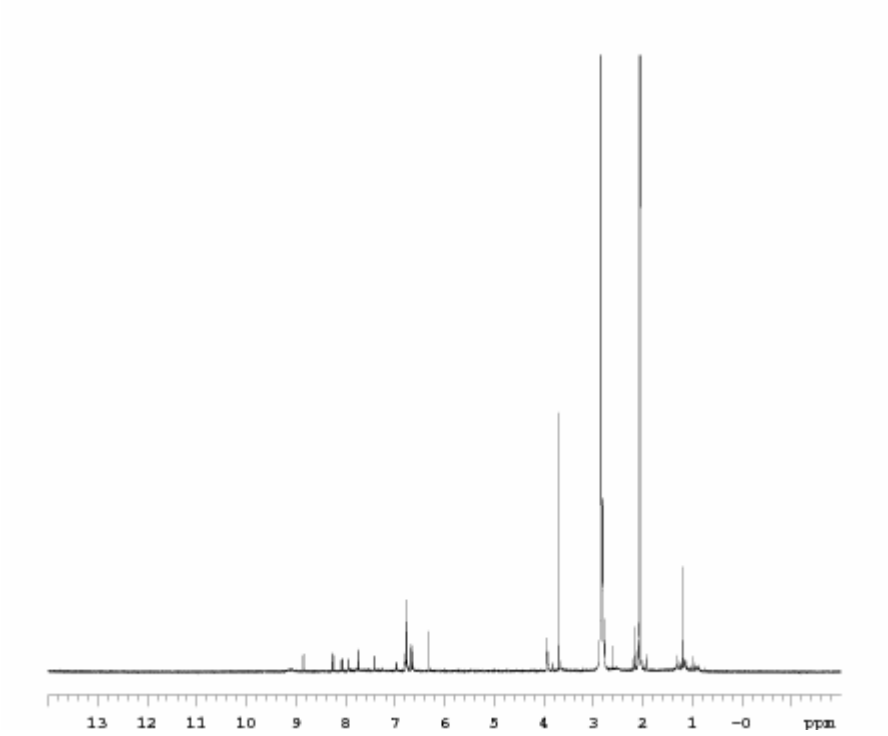


90

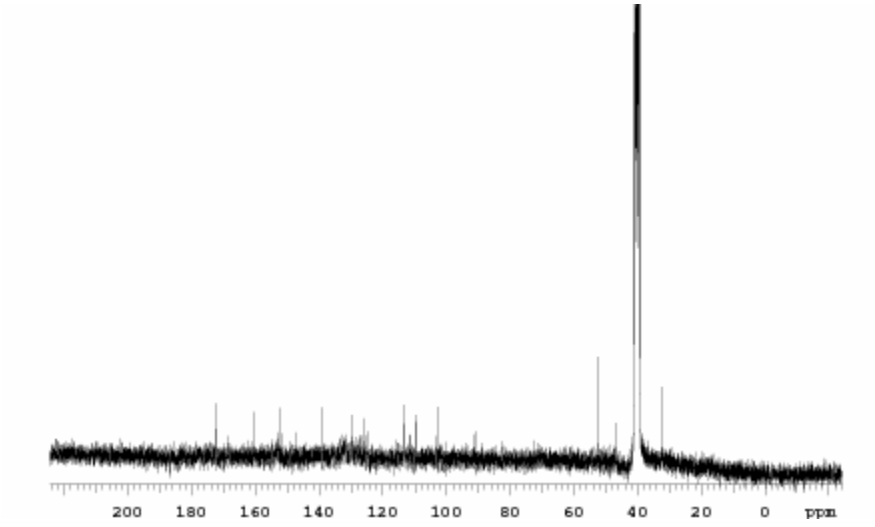
9-(*N,N*-(dimethoxycarbonylethyl)amino)-2-(2-(4-(3-hydroxy-6-oxo-6H-xanthen-9-yl) phenyl)ethynyl)-5H-benzo[a]phenoxazin-5-one 90

Compound **54** (110.0 mg, 0.2 mmol), **71**(101.0mg, 0.3 mmol), tetrakis(triphenylphosphine)Pd (22.0 mg, 0.02 mmol), copper(I)iodide (3.6 mg, 0.02 mmol) and triethylamine (265.0 mg, 1.9 mmol) were dissolved in 10 ml dry distilled DMF. The solution was freeze pump thawed three times, then heated to 120 ^{o}C for 12 h. The solution was concentrated in *vacuo* and purified by flash chromatography eluting with 30% MeOH/EtOAc to afford **90** (111.0 mg, 74%) as a red solid. R_f = 0.3(1:1 EtOAc: hexane).

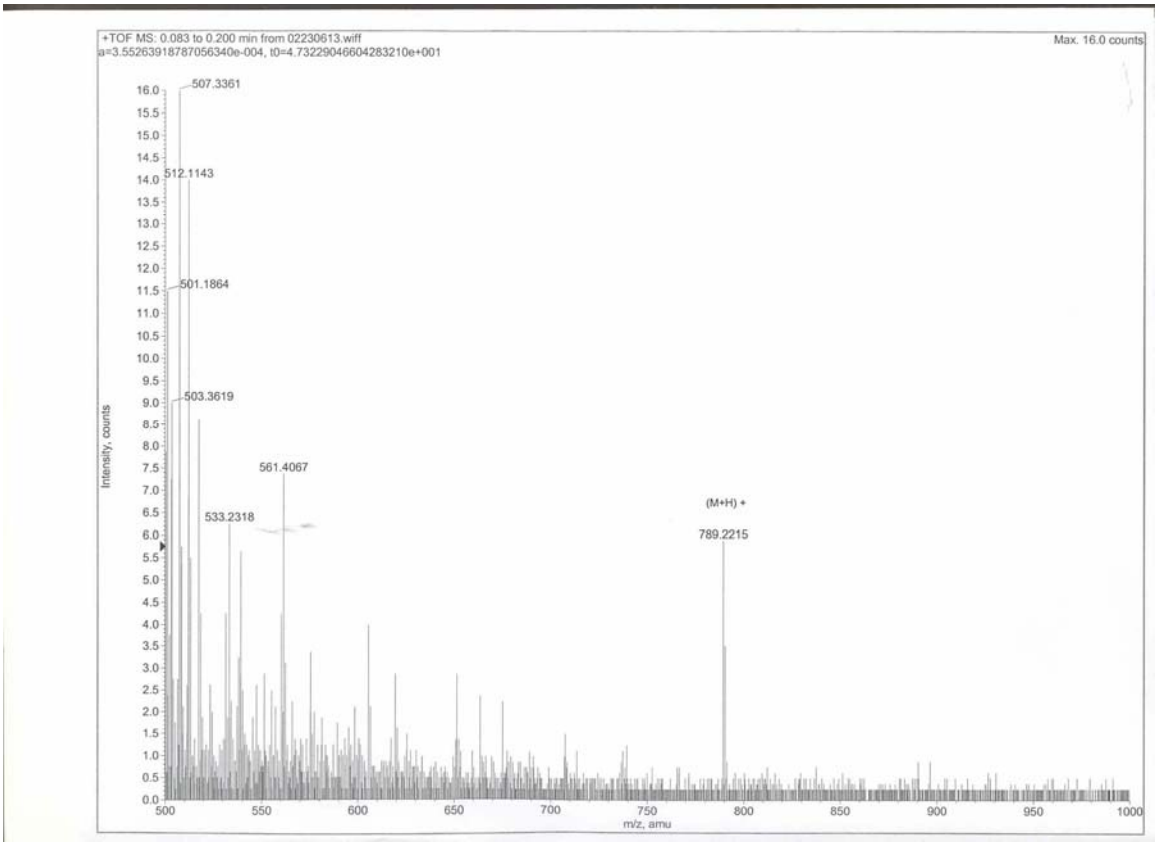
^{1}H NMR compound **90**

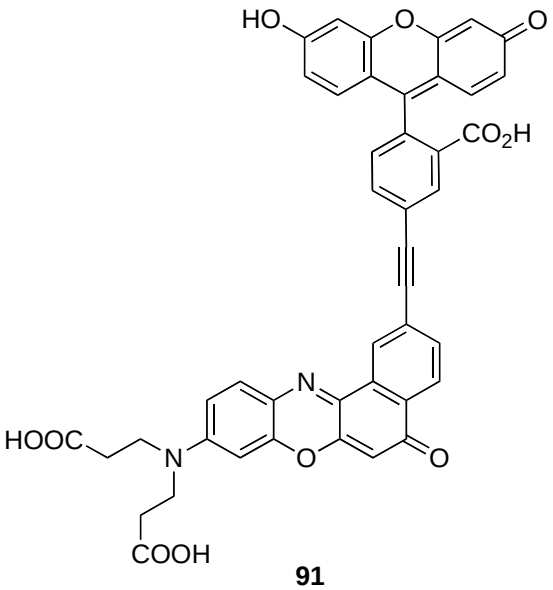


^{13}C compound **90**



Mass spectra (ESI) compound **90**



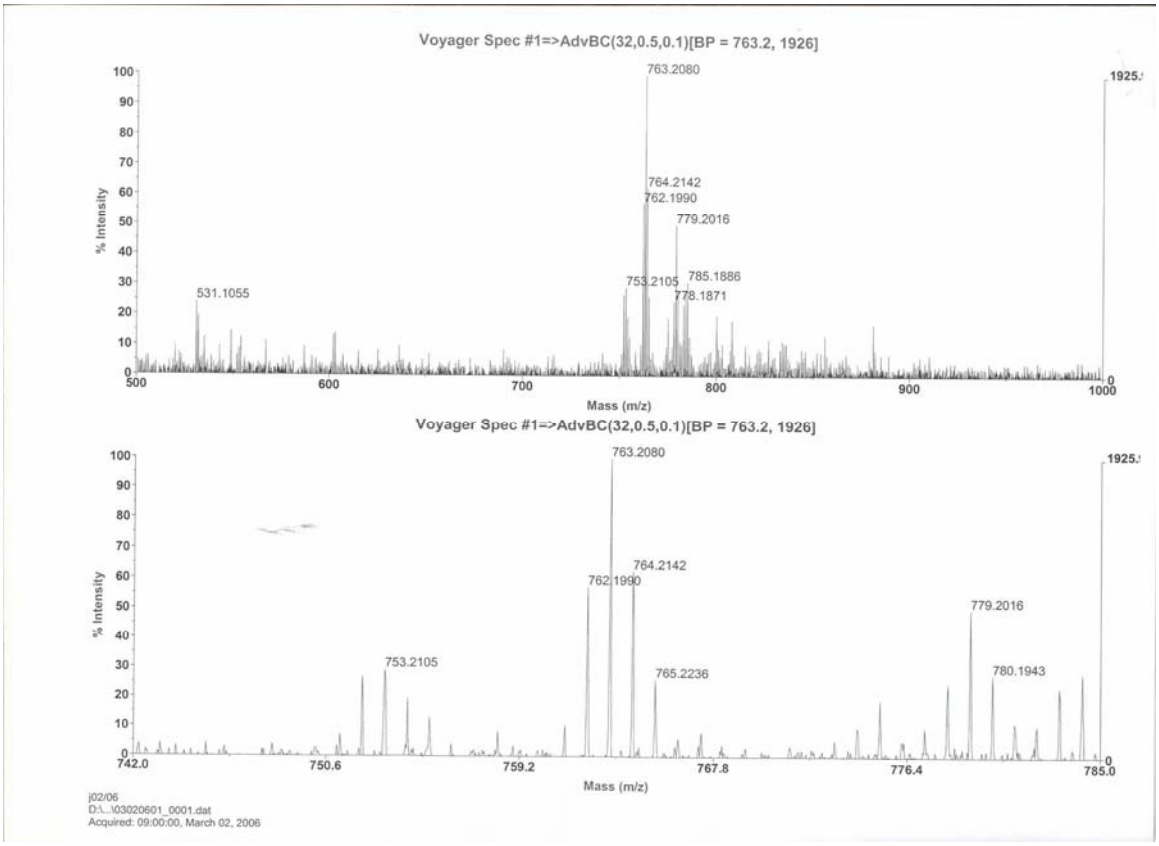


91

9-(*N,N*-Bis(2-carboxyethyl)amino)-2-(2-(4-(3-hydroxy-6-oxo-6H-xanthen-9-yl) phenyl)ethynyl)-5H-benzo[a]phenoxazin-5-one 91

Compound **90** (30 mg, 0.04 mmol) and potassium carbonate (66.7 mg, 0.48 mmol) was dissolved in MeOH/water (5 ml, 1:1) and heated to 40 ^{o}C for 12 h. The solution was Filtered to remove solid impurities and concentrated in vacuo. The crude residue was dissolved in 5 ml water and washed with ethyl acetate (5 ml, thrice) to remove any organic impurities. The pH of the aqueous layer was adjusted to 6 by careful drop wise addition of HCl (5 to 6 drops, 1.0M). The aqueous layer was then extracted with 2:1 $CHCl_3$: *iso*-propanol (5 ml, thrice). The organic extract was dried with magnesium sulfate and solvent evaporated to afford **91** (16.0 mg, 60%) as a blue solid.

Mass spectra compound **91**



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