Synthesis and Photophysical Characterization of Near-Ir Probes for Bioanalytical Applications.

James Henry Flanagan Jr
Louisiana State University and Agricultural & Mechanical College

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SYNTHESIS AND PHOTOPHYSICAL CHARACTERIZATION OF NEAR-IR PROBES FOR BIOANALYTICAL APPLICATIONS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Chemistry

by

James H. Flanagan, Jr.
B.S., Spring Hill College, 1991
December, 1997

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Acknowledgments

I would like to take this opportunity to acknowledge those individuals who, over the years, have continually supported me and all of my endeavors, and have inspired me to always look forward.

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<tbody>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>A/D</td>
<td>Analog-to-Digital</td>
</tr>
<tr>
<td>b</td>
<td>pathlength</td>
</tr>
<tr>
<td>c</td>
<td>concentration</td>
</tr>
<tr>
<td>c</td>
<td>speed of light</td>
</tr>
<tr>
<td>CAPSO</td>
<td>3-cyclohexylamino-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
</tr>
<tr>
<td>CFD</td>
<td>Constant Fraction Discriminator</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
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<td>cm⁻¹</td>
<td>reciprocal centimeters</td>
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<tr>
<td>CWL</td>
<td>Continuous wavelength</td>
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<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
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<tr>
<td>ddATP</td>
<td>dideoxyadenosine triphosphate</td>
</tr>
<tr>
<td>ddGTP</td>
<td>dideoxyguanosine triphosphate</td>
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<td>ddNTP</td>
<td>dideoxynucleic acid triphosphate</td>
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<td>dimethylformamide</td>
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<td>Dimethylsulfoxide</td>
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<td>Deuterated dimethyl sulfoxide</td>
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<td>deoxynucleic acid triphosphate</td>
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<td>Φᵢ</td>
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<td>OD$_S$</td>
<td>Optical density of the singlet state</td>
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<td>OD$_T$</td>
<td>Optical density of the triplet state</td>
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<tr>
<td>PC</td>
<td>Personal computer</td>
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<td>PMT</td>
<td>Photomultiplier tube</td>
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<td>ps</td>
<td>picosecond</td>
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</table>
\begin{tabular}{ll}
$q$ & charge \\
R & Resolution \\
s & second \\
S & Spin quantum number \\
$S_0$ & Ground singlet state \\
$S_1$ & Excited singlet state \\
SNR & Signal-to-Noise ratio \\
SPAD & Single-Photon Avalanche Diode \\
t & time \\
$\tau_0$ & Intrinsic fluorescence lifetime \\
TAC & Time-to-Amplitude Converter \\
TBE & Tris-Borate-EDTA \\
TCDI & 1,1-thiocarbonyldiimidizole \\
TCSPC & Time-Correlated Single-Photon Counting \\
TEAA & triethylammonium acetate \\
TEAB & triethylammonium borate \\
TEM & Transverse electronic and magnetic \\
$\tau_f$ & Fluorescence lifetime \\
TLC & Thin Layer Chromatography \\
tm & migration time \\
Trp & tryptophan \\
UV/VIS & Ultraviolet/Visible \\
V & voltage \\
$\omega$ & frequency \\
W & watt \\
zmol & zeptomole \\
\end{tabular}
Abstract

The research presented in this dissertation involves the synthesis and photophysical characterization of a series of tricarbocyanine dyes which can be used as fluorescent labels for bioanalytical applications. All of these dyes were synthesized to contain a heavy-atom modification to enhance $k_{sec}$ through spin-orbit coupling and a functional group (isothiocyanate or succinimidyl ester) which is reactive towards primary amines.

The spectroscopic characterization of the various chromophores was examined using absorbance and fluorescence measurements, time-correlated fluorescence, and flash photolysis. The dyes exhibit absorbance and fluorescence maxima in the near-IR (750 - 810 nm) with large $\varepsilon$ (~200,000 M$^{-1}$cm$^{-1}$) and $\Phi_f$ ~ 0.05 - 0.15. Dyes which contain the heavy-atom modification possessed absorbance maxima which were similar (differences of only 1-2 nm) and similar fluorescence maxima (differences of only 1-2 nm), but have fluorescence lifetimes ($\tau_f$) which were different and which were dependent upon the heavy-atom modification ($I = 908$ ps, $F = 831$ ps). The apparent inverse heavy-atom effect was studied by examining the triplet-state photophysics of these chromophores by laser-induced flash photolysis. Various photophysical constants were determined including the intersystem crossing rate ($k_{sec}$), internal conversion rate ($k_{ic}$), and $\Phi_t$. The results from these experiments indicate that $k_{sec}$ is increasing with the heavier atom modification, but that $k_{ic}$ is decreasing possibly due to
steric interactions of the heavy-atom modification with the chromophore restricting the vibrational interconversions of the dye.

These dyes were examined as possible fluorescent labels for amino acid determination and DNA sequencing employing fluorescence detection. The experimental results with the capillary electrophoretic separation of dye-labeled amino acids indicated that in the presence of CH$_3$OH in the running buffer, the detection of the dye-labeled amino acids exhibited excellent signal-to-noise ratios corresponding to low mass detection limits for dye-labeled arginine (21 zmol). Additionally, these dyes were examined as possible dye labels for a Sanger DNA sequencing protocol employing near-IR fluorescence lifetime determination of dye-labeled dideoxynucleotides. Experimental results that these dyes efficiently react with an amine modified dideoxyguanosine triphosphate. The effect of the extension reaction conditions on the dye-labeled ddNTP was also examined.
1.1. Introduction to Fluorescence Detection

There are numerous methods of detection for analyses of biological compounds including radiography, Fourier transform infrared spectroscopy (FTIR), circular dichroism, nuclear magnetic resonance spectroscopy, mass spectrometry, electrochemical detection and laser-induced fluorescence (LIF). The low limits of detection provided by LIF makes this an attractive detection scheme for the analysis of biological compounds especially when minute quantities of analyte need to be examined. In order to understand the power of this analytical tool, it is important to explain the principles of fluorescence.

The absorption of a photon of light by a molecule and the various photophysical processes which can depopulate the excited state can be described using a Jablonski diagram (see Figure 1.1). The absorption of a photon excites the molecule from the ground singlet state (S₀) into a vibrational state of the excited singlet-state (S₁ or S_N). This transition is almost an instantaneous process (occurs on the order of 10⁻¹³ s). The Boltzmann distribution describes the number of molecules in the ν =0 and ν = 1 vibrational states and the ratio of molecules (R, where R = ν₁/ ν₀) in each state and is given by;
Figure 1.1 Jablonski diagram. Block energy diagram showing the various radiative and non-radiative processes which occur to a molecule upon the absorption of a photon of light. $h\nu_i$: incident radiation, Abs.: absorption of incident radiation by molecule; $h\nu_F$: fluorescence photon, $h\nu_P$: phosphorescence photon.
\[ R = e^{-\Delta E / kT} \]  

(1.1)

where \( \Delta E \) is the energy difference between the two levels, \( k \) is the Boltzmann constant, and \( T \) is the temperature in degrees Kelvin (K). Most molecules have typical \( \Delta E \) values on the order of 1500 cm\(^{-1}\) and at room temperature (~300 K), \( R = 0.01 \). Therefore, most molecules present are in the lowest vibrational state and the absorption of a photon of light primarily occurs from this state. Once the molecule is excited into \( S_1 \), several processes can occur which depopulate this state. From \( S_1 \), the molecule can lose energy into the surroundings as heat and relax to an excited vibrational state of \( S_0 \) which is called internal conversion (IC); the molecule can lose energy in the form of a photon of light which is fluorescence (hv\(_f\)); or the molecule can go into the triplet state (a quantum mechanically spin-forbidden transition) through the process of intersystem crossing (ISC). Once in the triplet-state, the molecule can lose energy by internal conversion or in the form of a photon in which case it is called phosphorescence. There are other processes which can occur including the loss of energy due to a conformational change in the molecule which is called photoisomerization or excited state reactions to form dimers (called eximers), excited state complexes (called exiplexes) or excited state quenching by paramagnetic species such as dissolved oxygen.

1.1.1 Characteristics of Fluorescence Spectra

Emission spectra are represented as a plot of fluorescence intensity versus the wavelength of emission. The emission spectrum is independent of the
excitation wavelength due to the rapid relaxation of the excited vibrational levels in the excited state. This relaxation is a result of the strong overlap between the vibrational states of nearly equal energy. Molecules which fluoresce in condensed media invariably show a shift of the emission spectra to longer wavelengths with respect to the absorption spectra due to a loss of energy in the excited state. Figure 1.2 illustrates this effect. G. A. Stokes first observed this in 1852, and explained this phenomenon as a result of the rapid decay of the excited vibrational levels of $S_1$. Additionally, relaxation down to vibrational levels of $S_0$ results in further losses of energy. Excited state reactions and solvent effects can also induce even larger Stokes shifts.

Most organic molecules have a total angular momentum which is used to determine the multiplicity of the various electronic states given by;

$$M = 2S + 1$$

(1.2)

where $S$ represents the spin quantum number of the molecule. For most organic molecules, $S = 0$ and $M = 1$ which is referred to as the singlet state. The selection rules for electronic transitions state that upon excitation, $\Delta S = 0$. Any transition which results in an electronic transition to a state with the same multiplicity is considered an allowed transition. A transition which results in an electronic transition to a state with a different multiplicity ($\Delta S = 1$, $M = 3$, triplet state) is considered forbidden. However, forbidden transitions can occur, but with low probabilities.
The quantum mechanical description of the intensity of an electronic transition between two states can be described by the transition dipole moment between the initial and final states as;

$$\mu_{\text{f}i} = \langle \Psi_{\text{f}}^* | Q x | \Psi_{\text{i}} \rangle$$  \hspace{1cm} (1.3)

where $\Psi_{\text{f}}^*$ and $\Psi_{\text{i}}$ are the wavefunctions of the final and initial states, respectively, and $Q$ is the particle's charge and $x$ is its position. This expression is used to derive the selection rules for electronic transitions. Radiation is only absorbed when the transition dipole is not zero. If the dipole moment is zero for a transition, the absorption band has zero intensity.

According to the Franck-Condon principle, electronic transitions occur in a time frame in which the positions of the nuclei in a molecule do not change during the transition. As a result, all electronic transitions are vertical. The quantitative form of the Franck-Condon principle is derived from the expression for the dipole moment and the wavefunctions of the initial and final states, which are the products of the respective electronic and vibrational transitions. This gives rise to the Franck-Condon factor which is the square of the transition dipole moment given by;

$$\mu_{\text{f}i}^2 = |\langle \Psi_{\text{f}}^* | R | \Psi_{\text{i}} \rangle|^2$$  \hspace{1cm} (1.4)

where $\Psi_{\text{f}}^*$ and $\Psi_{\text{i}}$ are the final and initial vibrational states, respectively, and $R$ describes the nuclear coordinates. This factor gives the probability of the transition from a vibrational level $\nu$ of the ground electronic state to a vibrational level $\nu'$ of the excited electronic state. Therefore, if a particular transition is the
most probable for the absorption of light, then the reciprocal transition is also most probable. This gives rise to the mirror image relationship between the excitation and emission spectra. The mirror image rule represented in Figure 1.2 is true in most cases with exceptions arising from different geometric arrangements of nuclei which occur in the excited state as compared to the ground state and reactions which may occur to the molecule while it is in the excited state.

1.1.2 Physical Parameters Describing Fluorescence

There are several parameters which are used to characterize the fluorescence properties of molecules. The fluorescence quantum yield ($\Phi_f$) of a molecule is the ratio of the number of molecules which fluoresce to the total number of excited molecules and can be expressed in terms of the various rate constants which depopulate the excited state;

$$\Phi_f = \frac{k_r}{k_r + k_{nr}}$$  \hspace{1cm} (1.5)

where $k_r$ is the radiative rate constant of emission (s$^{-1}$) and $k_{nr}$ is the non-radiative rate constant (s$^{-1}$). The non-radiative rate constant is composed of several non-radiative rate constants which can deactivate the excited state and is expressed as;

$$k_{nr} = k_{ic} + k_{isc} + k_p + k_{ad}$$  \hspace{1cm} (1.6)

where $k_{ic}$ is the rate constant of internal conversion, $k_{isc}$ is the intersystem crossing rate constant; $k_p$ is the rate constant of photoisomerization, and $k_{ad}$ is the
Figure 1.2 Stokes shift and the mirror image rule demonstrated for a tricarbocyanine dye in CH$_3$OH. Dye concentration = 5×10$^{-6}$ M.
solvent dependent rate constant. The fluorescence quantum yield is usually expressed as a number between 0 and 1 or as a percentage between 0 and 100%. When $k_w << k_r$ then $\Phi_f$ is close to 1, but due to Stokes energy loss resulting from vibrational relaxation, $\Phi_f$ is never unity.

The fluorescence lifetime of the excited state is given by the expression;

$$\tau_f = \frac{1}{k_r + k_{nr}} \quad (1.7)$$

However, few molecules emit all of their photons at $t = \tau_f$, and so the fluorescence lifetime is actually an average value of the time the molecule stays in the excited state. The time dependent behavior of fluorescence is given by;

$$I_f(t) = I_{f(0)}e^{-\gamma t} \quad (1.8)$$

where $I_f(t)$ is the fluorescence intensity at time $t$ following excitation, and $\gamma = \frac{1}{\tau} = \frac{1}{(\gamma + k)^{-1}}$ where $\gamma$ is the emissive rate and $k$ is the rate of non-radiative decay. The fluorescence lifetime is equated to the time required for the fluorescence intensity to decay to $1/e$ of its initial value.\(^{10}\)

The average lifetime of a molecule in the absence of any non-radiative deactivation pathways is called the intrinsic lifetime or the radiative lifetime and is defined as;

$$\tau_0 = \frac{1}{k_r} \quad (1.9)$$

Combining equation 1.5, 1.7 and 1.9 leads to the relationship between $\Phi_f$ and the lifetime;
Both $\Phi_f$ and $\tau_f$ can be modified by any factors which affect the radiative or non-radiative rate constants.

1.1.3 Variables Effecting Fluorescence

Most of the fluorescence parameters are sensitive to all processes which can occur during the excited state. An increase in the temperature of a sample can decrease $\Phi_f$ of a molecule by increasing $k_{nr}$ due to an increase in intermolecular collisions with the solvent. Large atoms (Br, I) have the ability to increase $k_{nc}$ through the process of spin-orbit coupling which can decrease $\tau_f$ and $\Phi_f$. Dissolved oxygen can decrease $\Phi_f$ and $\tau_f$ through photochemical oxidation of the molecule, but generally it quenches the fluorescence by increasing $k_{uc}$ of the molecule. This is a result of the paramagnetic properties of molecular oxygen.\textsuperscript{10}

The emission properties of many molecules, especially those which contain polar groups, are sensitive to the chemical and physical properties of the solvent. The effect of the solvent on the fluorescence of a molecule can be divided into two categories; general effects and specific effects. General effects are those which are a result of the dielectric constant ($\varepsilon$) and the refractive index of the solvent ($n$). These effects are expected to always be present and can be described by the Lippert equation;

\[
\nu_s - \nu_f = \frac{2}{\hbar c} \left[ \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \right] \frac{[\mu^* - \mu]^2}{a^3} + \text{constant} \quad (1.11)
\]
where $\hbar$ is Planck's constant, $c$ is the speed of light, $\mu^*$ and $\mu$ are the dipole moments of the molecule in the excited and ground state, respectively, and $a$ is the radius of the cavity in which the molecule resides in solution. The energy difference of the absorption and emission in cm$^{-1}$ is $\bar{v}_e - \bar{v}_f$. Although this equation is only an approximation, it can correlate spectral shifts to solvent polarity differences. The term in brackets is called the orientation polarizability ($\Delta \gamma$) and accounts for spectral shifts due to the orientation of the solvent dipoles and the redistribution of the electrons in the molecule.

Generally, an increase in $n$ will decrease the Stokes shift and an increase in $\varepsilon$ will increase the Stokes shift. More explicitly, an increase in the refractive index allows both the ground and excited states to be instantaneously stabilized by movements of electrons within the solvent molecules. The redistribution results in a decrease in the energy differences between the ground and excited states. An increase in $\varepsilon$ also stabilizes $S_0$ and $S_1$, but the stabilization in $S_1$ occurs only after the reorientation of the solvent dipoles. This requires movement of the entire solvent molecule. Therefore, the stabilization of $S_1$ due to $\varepsilon$ is dependent upon the viscosity and temperature of the solvent.$^{10}$

Specific solvent effects on the fluorescence of a molecule refer to specific chemical interactions of the solvent with the molecule. In contrast to general effects which are representative of the influence of the entire set of solvent molecules which surround the analyte, specific interactions are produced by only a few neighboring solvent molecules and are determined by the specific chemical properties of both the fluorophore and the solvent. Specific effects can be due,
but not limited to, hydrogen bonding, acid-base chemistry, or charge-transfer reactions.\textsuperscript{10}

1.1.4 Proportionality of Fluorescence Intensity with Incident Radiation

The fluorescence intensity is directly proportional to the intensity of the radiant beam that is absorbed by the molecule and is expressed as;

\[ F = \Phi_f(P_o - P) \]  

where \( F \) is the fluorescence intensity, \( \Phi_f \) is the fluorescence quantum yield, \( P_o \) is the power of the incident beam of radiation and \( P \) is the power after traversing a length (b) of the solution. Relating this to the Beer-Lambert law, this can be rewritten as;

\[ F = \Phi_f P_o (1 - 10^{-ebc}) \]  

where \( e \) is the molar absorptivity of the molecule and for dilute solutions (\( ebc < 0.02 \)) gives;

\[ F = \Phi_f 2.303 P_o ebc \]  

The intensity of the emitted fluorescence is proportional to the quantum efficiency and the concentration of the analyte with a constant incident radiation (\( P_o \)). Thus, a linear plot is obtained for the fluorescence intensity versus the concentration at low analyte concentrations. At high concentrations, the fluorescence is decreased due to reabsorption of the emitted photon if the wavelength of emission overlaps an absorption wavelength. This process is known as inner filtering effects and results in non-linearity of the calibration plot.
1.1.5 Instrumentation for Fluorescence

The basic instrumentation used in a fluorescence experiment is shown in Figure 1.3. The excitation source frequently used is a Xe arc lamp which provides an intense beam of incident light that is continuous over a broad wavelength range (250 - 600 nm) with a peak intensity occurring around 450 nm. Mercury arc lamps can be used which produce high intensity incident beams of radiation at discrete wavelengths. The incident beam is then sent through a monochromator or filter which is used to select the wavelength of excitation. Most fluorescence spectrometers employ grating monochromators as a wavelength selector.

The incident beam is then directed onto the sample cell which is fabricated from glass or silica. Fluorescence from the sample is either collected at right angle to the incident beam or at 45° from the front face of the sample cell. Fluorescence collected at right angles is subject to inner filtering effects, but does minimize scattered light.

The fluorescence radiation is then directed through another monochromator which is usually scanned over a range of wavelengths to construct a fluorescence spectrum. The selected wavelengths are usually detected using a photomultiplier tube (PMT). Since the fluorescence is of low intensity, the output of the PMT is frequently amplified and then sent to a data acquisition board on a PC. PMTs exhibit a wavelength dependent photocathode
Figure 1.3 Components of a spectrofluorometer. S: excitation source, $h\nu_{ex}$: excitation wavelength, C: sample cell, $h\nu_f$: fluorescence; Det.: detector, DA: data acquisition unit.
response, so care should be taken to select the appropriate PMT for the application.

The use of lasers as excitation sources has made fluorescence a more powerful analytical tool. Argon and He:Ne lasers provide an intense beam of monochromatic light (488, 514, and 633 nm; $P_0 \approx 1-8$ W) which can be focused to a small spot (~10 $\mu$m$^2$) to afford a high power incident beam. From equation 1.13, an increase in $P_0$ leads to an increase in the fluorescence output power ($F$) which allows for the detection of low concentrations of analyte.

1.2 Visible Fluorescence Detection

Visible fluorescence detection can utilize the advantages of a laser as the excitation source to provide a highly sensitive technique with low limits of detection in the visible region of the spectrum (350-600 nm), and in some cases, single molecule detection.$^{11,12}$ Typical lasers which are used as excitation sources are Argon ion lasers and the He:Ne laser. Argon lasers produce two major lines of radiation (488 nm and 514 nm) and the He:Ne laser produces radiation at 633 nm. The advent of microcolumn separations (capillary electrophoresis) has utilized the advantages of LIF detection. The mass detection limits for arginine and double stranded DNA in capillary electrophoresis (CE) are as low as 9 zmol ($10^{-21}$ mol) and a few yoctomoles ($10^{-24}$ mol).$^{13,14}$ The photons are detected using PMTs, charge-coupled detectors, diode array detectors, or multichannel plate detectors. Each of these provides a particular advantage over the other, but most
detection systems utilize the PMT because of its simplicity of use, large quantum efficiency in the visible region (10-20%), and their low cost.

1.2.1 Visible Fluorophores

Typical fluorophores which possess fluorescence properties in the visible region of the spectrum are the BODIPY, fluorescein, and rhodamine derivatives. A structure of each type is shown in Figure 1.4. Fluorescein is the most common chromophore for labeling biomolecules. Its wide acceptance is due to its relatively high molar absorptivity ($e \sim 80,000 \text{ M}^{-1}\text{cm}^{-1}$), excellent quantum yield ($> 0.9$), and its good water solubility. Its absorbance and fluorescence maxima are 494 nm and 520 nm, respectively. The absorbance maximum closely matches the 488 nm line of the Ar-ion laser which makes this dye an attractive fluorophore for applications which employ LIF detection.

The BODIPY dyes are comparable to the fluorescein dyes in many respects. They possess high extinction coefficients ($e \sim 80,000 \text{ M}^{-1}\text{cm}^{-1}$), high fluorescence quantum yields (close to unity), spectra which are insensitive to solvent polarity or pH, and lack of ionic charge. The dye shown in Figure 1.4 has an absorbance and fluorescence maxima of 502 nm and 512 nm, respectively, which also matches the Ar-ion laser 488 nm line.

Rhodamine dyes are similar to fluorescein dyes but possess absorption and fluorescence maxima at longer wavelengths. One major advantage of these fluorophores is their enhanced photostability when compared to other fluorophores. In addition, the absorbance and fluorescence spectra are typically
not affected by changes in pH between 4 and 10 which is an important advantage over some fluorescein derivatives.¹⁵

All of these dyes possess certain qualities which makes them excellent fluorophores. Each dye is composed of a rigid aromatic structure. This gives rise to excellent overlap between the π bonding orbitals in the structure and large fluorescence quantum yields. In addition, the rigid structure reduces non-radiative deactivation from internal conversion. In fact, it is not uncommon in these dyes to see intersystem crossing as the major non-radiative manifold.

1.2.2 Advantages and Disadvantages

The advantages of visible LIF detection include low mass detection limits, large dynamic range, and the selectivity of the technique. However, there are some problems associated with visible LIF detection. The lasers which are frequently used are not amenable to miniaturization due to their large size and power requirements. Also, visible lasers have a finite lifetime (~ 4000 h) after which the source must be replaced which increases the overall cost of use.

1.3 Near-IR Fluorescence Detection

An alternative to visible fluorescence is near-IR fluorescence detection. Near-IR fluorescence offers many attractive advantages over visible LIF including lower limits of detection, lower cost, and simplicity of instrumentation. The lower limits of detection afforded by near-IR LIF are a result of two
Figure 1.4 Representative visible fluorescent dyes used for bioanalytical applications.
properties of the near-IR region of the spectrum (700 - 1000 nm); (1) the background in the near-IR is lower due to the fact that few molecules exhibit intrinsic fluorescence in the near-IR; (2) the amount of scattered light due to Rayleigh or Raman scattering is lower in the near-IR when compared to the visible region. The first advantage is due to the fact that most biological molecules that do exhibit fluorescence do so in the visible region. There are very few molecules which exhibit intrinsic fluorescence in the near-IR. Therefore, the amount of background fluorescence is lower in the near-IR because of the lower contribution from interferences in solution.

The second property which leads to lower background is due to the fact that Rayleigh and Raman scattering is reduced at longer wavelengths. The differential cross-section for Rayleigh scattering is given by;

$$\frac{d\sigma}{d\Omega} \approx \frac{\omega^4}{(4\pi\epsilon_0hc^2)^2}$$  \hspace{1cm} (1.15)

where $\frac{d\sigma}{d\Omega}$ is the cross section for Rayleigh scattered light, $\omega$ is the frequency of the incident beam, $\epsilon_0$ is the permitivity of free space, $h$ is Planck’s constant, and $c$ is the speed of light. The differential cross-section for Raman scattering is given by;

$$\frac{d\sigma}{d\Omega} \approx \frac{\omega_1\omega_2^3}{(4\pi\epsilon_0hc^2)^2}$$  \hspace{1cm} (1.16)

where $\omega_1$ is the incident wavelength and $\omega_2$ is the scattered wavelength. From these equations, the amount of scattered light is proportional to the fourth power of the frequency of the incident light which is also $\lambda^4$ since $\omega = 1/\lambda$. Therefore,
the amount of scattered light due to Rayleigh and Raman scattering is less for longer wavelengths than at shorter wavelengths which allows for a lower background in the near-IR region when compared to the visible.

1.3.1 Instrumentation

There are instrumental advantages to working in the near-IR as well. Inexpensive solid state devices such as diode lasers and avalanche diode detectors can be used in the near-IR and are attractive sources and detectors due to their low cost, low maintenance, high output in the near-IR, simplicity of use and small size.

Diode lasers are made from semiconducting materials which are doped with either a group III or V element. The semiconducting material with a group III element doping is known as a ‘p-type’ and with a group V as an ‘n-type.’ ‘P-type’ have “holes” or electron deficient sites and ‘n-type’ are electron rich semiconductors. When these two types of semiconductors are placed in contact with one another and a ‘forward bias’ (positive potential) is placed across the material, electron/hole pairs are formed in the depletion layer and upon recombination, a photon of light is emitted. A population inversion can be sustained by confining the spatial distribution of electrons by using materials with different refractive indexes. A widely used diode laser consists of Ga$_{1-x}$Al$_x$As.

Diode lasers are attractive excitation sources in the near-IR because they exhibit stable, coherent beams of light with high output powers (~ 100 mW) in the near-IR and can be modified to lase at a particular wavelength. In addition,
the typical lifetime of a diode laser is on the order of 40,000 h. Compared to ion lasers, diode lasers are more cost effective over time. Diode lasers are small and can be easily used to miniaturize existing instrumentation.

Semiconductor detectors can be used in the near-IR as well as the visible. In particular, the single-photon avalanche diode (SPAD) is an attractive detector. A SPAD consists of a semiconductor material and is reversed-biased above its breakdown voltage. When a photon of light strikes the diode, it creates an electron/hole which generates a cascade of electrons. The major advantage of this device is that the detector has gain built into the system, which results from the cascade of electrons. This results in a large signal response for a single photon of light. Additionally, SPADs have large quantum efficiencies (~ 40%) in the near-IR. One drawback to the use of SPADs as detectors are their small photoactive area (~ 150 - 200 µm in diameter), but the proper use of focusing optics can alleviate this problem.

1.3.2 Near-IR Fluorophores

The advantages of near-IR fluorescence over visible fluorescence are counteracted from the fact that there are few chromophores available which can be used to label biological compounds of interest. Those dyes which are available that do posses properties in the near-IR have poor fluorescent properties and photophysics. The major type of fluorophore that exhibit near-IR fluorescence belong to the cyanine class of dyes. There are many types of cyanine dyes and their chemistry is diverse, but the most widely used and studied
are the carbocyanine dyes. The basic structure of the carbocyanine dye is shown in Figure 1.5.

These dyes possess a conjugated polymethine chain which links together two heteroaromatic fragments. The characteristic spectroscopic feature of these dyes is the bathochromic shift of the absorbance and fluorescence maxima with an increase in the length of the polymethine chain. For example, the structure, shown in Figure 1.6, demonstrates a shift in the absorbance maxima with increasing n; 422 nm (n = 0); 558 nm (n = 1); 655 nm (n = 2); 768 nm (n = 3).

According to Dyadyusha, the longest wavelength transition of symmetrical polymethines depends upon the number of vinylene groups in the chain through the linear relationship;
\[ \lambda_n = V \left( n + L + \frac{A}{n + L} \right) \]  

(1.16)

where \( A \) is specific for the vinylogous series, \( L \) is the effective length between the heteroaromatic fragments, \( V \) is the vinylene shift, and \( n \) is the number of vinylene groups in the chain. For symmetrical polymethines, the shift amounts to about 100 nm when the chain is lengthened by one double bond. In addition, the addition of different electron withdrawing or electron releasing groups onto the heteroaromatic fragments (\( X = O, \text{Se}, \text{or} \text{S} \) in Figure 1.5) or polymethine chain can either increase or decrease the absorption wavelength maxima respectively. As a consequence, these dyes exhibit absorbance and fluorescence maxima which stretches across the entire spectrum (400 - 900 nm).

The tricarbocyanine dyes (\( n = 3 \)) display absorbance and fluorescence maxima in the near-IR with large extinction coefficients (\( \sim 200,000 \text{ M}^{-1}\text{cm}^{-1} \)) but fairly poor fluorescence quantum yields. The poor quantum yields are a result of the large rate of internal conversion and in some cases, photoisomerization.

[Figure 1.6 Thiacarbocyanine Iodide. Cyanine (\( n = 0 \)), Carbocyanine (\( n = 1 \)), dicarbocyanine (\( n = 2 \)), tricarbocyanine (\( n = 3 \)).]
around the polymethine chain.\textsuperscript{18-20} Hofer \textit{et al.} have shown that most carbocyanines (n = 1), display increased steric interactions between the two heteroaromatic fragments which results in the non-radiative deactivation of the excited-state through internal conversion.\textsuperscript{20} Also, the addition of bulky groups onto the polymethine chain has been shown to increase internal conversion.\textsuperscript{21} However, increasing the chain length (n = 2) leads to larger fluorescence quantum yields due to decreased steric interactions.\textsuperscript{20} Further increases in the fluorescence quantum yields may be obtained by incorporating bridging units within the polymethine chain which stabilizes the dye to vibrational interconversions.\textsuperscript{22}

Most cationic tricarbocyanine dyes have limited solubility in H\textsubscript{2}O, and many researchers have investigated the effect of molecular aggregation on the various photophysical constants.\textsuperscript{23,24} Some researchers have shown that the addition of SO\textsubscript{3}\textsuperscript{-} groups onto the chromophore can dramatically increase the solubility which decreases the amount of molecular aggregation in aqueous solutions.\textsuperscript{25-28} In addition to aggregation effects, various researchers have studied the effects of intramolecular and intermolecular heavy-atoms on the photochemistry of polymethines.\textsuperscript{29-31} These dyes exhibited non-radiative deactivation of the excited state through intersystem crossing caused by spin-orbit coupling from the heavy-atoms either in solution or on the chromophore.

The use of these dyes as fluorescent labels has only recently been explored. Several groups have developed polymethine dyes which contain functionalities which are reactive to biomolecules. Waggoner \textit{et al.} have
developed numerous dyes which contain isothiocyanate groups and succinimidyl esters which are reactive towards primary amines.\textsuperscript{25,26,33,34} Ernst has developed dyes which contain an iodoacetamide group which is reactive towards thiols.\textsuperscript{35} Strekowski \textit{et al.} have also developed tricarbocyanine dyes which contain isothiocyanate groups for the labeling of proteins, antibodies, and DNA.\textsuperscript{27,28,36,37} The large structural diversity and the ability to tailor the absorbance and fluorescence maxima based upon changes in the dyes' structure make them excellent candidates as probes for bioanalytical applications.

1.4 Research Goals

The goals of this research are to synthesize and characterize the photophysics of new dyes for various bioanalytical applications utilizing near-IR fluorescence, especially for a single-lane, single-fluor DNA sequencing protocol which uses lifetime discrimination of labeled nucleotide bases. The dyes must possess absorbance and fluorescence properties in the near-IR region of the spectrum (700 - 1000 nm), and have a functional group to allow facile conjugation to modified DNA primers and/or dideoxynucleotides as well as other amine containing compounds. In addition, the dyes must have fluorescence lifetime values which are significantly different to be able to discriminate among the different chromophores, and must possess the same absorption maxima in order to be excited by one laser source and the same fluorescence maxima to allow for detection at one wavelength. The dyes synthesized will be characterized by absorbance, steady-state and time-resolved fluorescence
spectroscopies. The fundamental photophysical constants ($\epsilon$, $\Phi$, $\tau$, $k_a$, $k_c$) that are associated with these unique chromophores will be investigated and determined. Finally, these dyes will be applied to different bioanalytical applications including DNA sequencing and amino acid analysis.

1.5 References


Chapter 2

Synthesis of Novel Tricarbocyanine Dyes

2.1 Basal Tricarbocyanine Dyes

2.1.1 Introduction

Tricarbocyanine dyes, which have a characteristic intense light absorption in the near-IR region of the spectrum, belong to a class of dyes known as the cyanines. The term cyanine was originally named for one compound, but over time it was extended to the entire class of dyes which were renamed the polymethine dyes by W. Konig in 1922. The basic structure is shown in Figure 2.1. Basically, cyanine dyes contain two heterocyclic groups that are

![Figure 2.1 Basic structure of cyanine dyes. Y = O, S, Se, C(CH₃)₂; n = 0, cyanine; n = 1, carbocyanine, n = 2, dicarbocyanine, n = 3, tricarbocyanine.](image-url)
linked together by a polymethine chain. The nomenclature for cyanines is based upon the type of heterocycle and the length of the polymethine chain which links the two heterocycles. The simplest cyanine dye contains a methine chain linking the groups (Figure 2.1, \( n = 0 \)). The addition of another ethene unit to extend the conjugated linkage to a trimethine chain is designated as a carbocyanine dye (Figure 2.1, \( n = 1 \)). Tricarbocyanine dyes have a heptamethine chain \( (n = 3) \) which links the heterocycles. The length of the polymethine chain may extend further, but the dyes become less photochemically stable. However, the addition of bridging units within the chain helps to increase the photochemical and thermal stability of these dyes.\(^1\)

Generally, the synthesis of a polymethine dye is achieved from the condensation reaction between an activated methyl or methylene with CH acidic properties and a dianil or bisaldehyde group to form the polymethine chain. Other groups can react with enamines to form the polymethine chain including thiols and alkoxy groups. The two end groups which are linked through the polymethine chain may either be the same or different in which case the symmetric or asymmetric dye will be formed. Slominskii and Makin as well as other Soviet groups have investigated the synthesis of various cyanine dyes based upon this fundamental reaction.\(^1\)\(^2\)\(^\text{-12}\) There are extensive reviews of the synthesis of various cyanines, including derivatives, by Hamer and Tyutyulkov.\(^1\)\(^1\)\(^3\)

Early synthesis of tricarbocyanine dyes was for use in photography as sensitizers for photographic films. The dyes were able to sensitize silver chloride emulsions on photographic plates to the red and far-red region of the
Hamer has exhaustively reviewed the history and synthesis of cyanine dyes from the late 19th century into the middle of the 20th century. Interest in cyanine dyes was renewed in the 1960's for their use as lasing media, and many chromophores were developed and used for their efficient lasing properties in the red and near-IR region of the spectrum. These di- and tricarbocyanine dyes shown in Figure 2.2 such as IR-125, IR-132, diethylthiadicarbocyanine iodide (DTDCI), and many others expanded the spectroscopic range which researchers could probe with lasers. In addition, this class of dyes has gained widespread use as membrane potential probes, photochromic recording media, and as photoconductors in electronic devices.

The advent of fluorescence detection in bioanalytical application, such as protein analysis, amino acid analysis, and DNA sequencing, has prompted the synthesis of a wide variety of cyanine dyes including carbocyanines and dicarbocyanines which have absorption and fluorescence properties in the UV and visible region of the spectrum. Yet, despite the advantages provided by performing many bioanalyses in the near-IR region of the spectrum as described in Chapter 1, a small number of researchers work in this part of the spectrum because there are few dyes available which can be used as probes for bioanalytical applications. There are a couple of groups which have led the resurgence of cyanine dyes in the past decade. Waggoner and co-workers have synthesized a series of di- and tricarbocyanine dyes as probes for cellular proteins in flow cytometry and deoxynucleotide triphosphates. Patonay and co-workers have synthesized a variety of tricarbocyanine dyes as probes for proteins.
Figure 2.2 Various commercially available cyanine dyes.
amino acids, DNA sequencing, immunoassays, polymeric materials and probes for pH determinations.\textsuperscript{20-27} The basic structure of the dyes used contained two heteroaromatic fragments which were linked by a heptamethine chain. The dyes were symmetrical, which means that the heteroaromatic fragments that were linked by the polymethine chain were the same. Depending upon the application, the dyes contained sulfonate groups to make them soluble in aqueous solvents, functional groups which were reactive towards primary amines, or reactive groups (carbonyl) susceptible to changes in solvent composition.

This section will describe the synthesis of two basal tricarbocyanine dyes as well as their precursors based upon modifications of previously published synthetic procedures including the alkylation of heterocyclic fragments and the condensation reactions between the heterocyclic fragments and bisaldehydes or dianils. These dyes will serve as the foundation for a set of near-IR probes, which are described in subsequent sections, that are used for a variety of bioanalytical applications.

2.1.2 Experimental.

2.1.2.1 Chemicals.

Aniline, cyclohexanone, \textit{N,N}-dimethylformamide (DMF), 1,3-propane sultone, and iodoethane were purchased from Aldrich Chemical Co. (Milwaukee, WI). Phosphorus oxychloride (POCl\textsubscript{3}) and 2,3,3-trimethylindolenine were purchased from Kodak Co. (Rochester, NY). All other solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA).
2.1.2.2 Purification and Spectroscopic Analysis

Purification of cationic dyes was performed by flash chromatography (silica (60 - 180 mesh), CHCl₃:CH₃OH, 10:1). Purification of sulfonate dyes was performed on a Rainin model Rabbit HP preparative HPLC using a Waters C18-RP preparative column (radial compression cartridge, 5 x 10 cm); flow rate = 5 mL/min; starting at 45:55 H₂O:CH₃OH for 5 min and then a linear gradient over 20 min to 20:80 H₂O:CH₃OH followed by washing with pure CH₃OH. Analytical separations were done using a Brownlee Spheri-5 ODS reversed phase analytical column. The HPLC detector was a Shimadzu absorbance detector which was set at 770 or 790 nm. Dyes were recovered from pooled, collected HPLC fractions and the solvent removed using a rotary evaporator at 45 °C. These dyes were dried overnight in vacuo (< 0.1 torr), suspended in Et₂O and collected by filtration. The proton NMR spectra were recorded in DMSO-δ₆, CDCl₃ or CD₃OD on a Bruker FT-NMR spectrometer. Mass spectral data were acquired using a Finnigan MAT 900 Fast-atom bombardment instrument with glycerol as the sample matrix. GC-MS data was acquired on a Hewlett Packard 5971 Series II GC/MS.

2.1.2.3 General Synthesis

The basal tricarbocyanine dyes were synthesized using modifications of previously described procedures.⁵,¹⁴⁻¹⁷,²¹⁻²₃,²₆

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Scheme 2.1 Synthesis of 1-ethyl-2,3,3-triethylindolinium Iodide (1).

A mixture of 2,3,3-trimethylindolene (10 mL, 0.063 mol) and iodoethane (15 mL, 0.186 mol) were heated under reflux for 48 h. After 24 h, additional iodoethane was added (10 mL). The reaction was allowed to cool and the reddish-pink product was triturated with Et₂O (20 mL). The resulting suspension was filtered, washed with Et₂O (3 x 20 mL) and dried over night in vacuo to afford 1. Yield: 18.7 g. (95%).

Scheme 2.2 Synthesis of \(N\)-[5-Anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadiene-1-ylidene]anilinium Chloride (2).

Following a procedure outlined by Makin\(^{28}\), at \(0 \, ^{\circ}\)C, POCl₃ (11 mL, 0.12 mol) was added dropwise from a pressure equalizing addition funnel into...
anhydrous DMF (13 mL, 0.17 mol). After 30 min, cyclohexanone (5.5 mL, 0.053 mol) was added and the mixture was refluxed for 1 h. Next, with constant cooling at 20 °C, an aniline:CH$_3$CH$_2$OH (1:1 (v/v), 18 mL) mixture was added dropwise. The reaction was continued for an additional 30 min after aniline addition, and then the deep purple mixture was poured into ice cold H$_2$O:concentrated HCl (10:1, 110 mL). Crystals were allowed to form for 2 h in an ice bath, then filtered, washed with cold H$_2$O and Et$_2$O, and then dried in vacuo to give a purple powder 2. Yield 15.41 g (87%) mp: 220 °C FAB-MS calculated for C$_{20}$H$_{20}$ClN$_2$: 323.1 (M$^+$), found: 323.3. $^1$H NMR (DMSO-$d_6$, 200MHz) δ 8.5 (s, 2H), 7.6-7.2 (m, 10H), 2.74 (t, 4H, $J = 5.6$ Hz), 1.85 (m, 2H).

![Scheme](image)

**Scheme 2.3** Synthesis of 2-[4-Chloro-7-(1-ethyl-3,3-dimethylindolin-2-ylidene)-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-1-ethyl-3,3-dimethylindolinium Iodide (3).

Following a procedure outlined by Strekowski$^{21}$, a solution of anhydrous CH$_3$CH$_2$OH (60 mL), anhydrous sodium acetate (900 mg, 0.01 mol), 1 (3.51 g, 11.2 mmol) and 2 (2.0 g, 5.6 mmol) were heated under reflux for 6 h. The reaction was allowed to cool, and CH$_3$CH$_2$OH was removed on a rotary evaporator at 40 °C. The resulting residue was dissolved in CHCl$_3$ (50 mL) and
filtered from sodium acetate. The solution was concentrated and purified by flash chromatography (silica gel, CHCl₃:CH₃OH, 20:1). Pure fractions were collected, pooled, and dried in vacuo to give a green powder 3. Yield: 2.1 g (58%) FAB-MS calculated for C₃₄H₄₀ClN₂: 511.3 (M⁺), found: 511.0. ¹H NMR (CDCl₃, 400 MHz) δ 8.34 (d, 2H, J = 14.1 Hz), 7.39 (m, 4H), 7.25 (br d, 2H), 7.16 (d, 2H, J = 7.9 Hz), 6.27 (d, 2H, J = 14.1 Hz), 4.28 (q, 4H, J = 7.3 Hz), 2.78 (t, 4H, J = 6.1 Hz), 1.99 (br m, 2H), 1.72 (s, 12 H), 1.46 (t, 6H, J = 7.2).

Scheme 2.4 Synthesis of 3-[2,3,3-trimethylindolinium-1-yl]propanesulfonate (4).

Following a modified procedure outlined by Mujumdar, toluene (50 mL), 2,3,3-trimethylindolenine (10 mL, 0.06 mol), and 1,3-propane sultone (8.2 mL, 0.09 mol) were heated under reflux for 18 h. The reaction was allowed to cool to allow pink crystals to form. The resulting crystals were filtered and washed with acetone (3 x 10 mL). The filtered product was crystalized from a solution of CH₃OH and Et₂O. The crystals were collected and dried in vacuo to give a pink powder 4. Yield: 14.88 g (85%). FAB-MS calculated for C₁₄H₂₀NO₃S: 282.0 (protonated form, M⁺), found: 282.1. ¹H NMR (CD₃OD,
Scheme 2.5 Synthesis of 2-Chloro-1-formyl-3-hydroxymethylenecyclohexene (5).

Following a procedure outlined by Reynolds, at 0 °C, POCI\(_3\) (35 mL, 0.38 mol) in CH\(_2\)Cl\(_2\) (35 mL) was added dropwise from a pressure equalizing addition funnel into anhydrous DMF (40 mL, 0.52 mol) in CH\(_2\)Cl\(_2\) (40 mL). After 30 min, cyclohexanone (10.6 mL, 0.10 mol) was added and the mixture was refluxed for 3.5 h. The yellow color of the solution turned red upon refluxing. The mixture was poured into ice cold H\(_2\)O:concentrated HCl (10:1, 110 mL). Crystals were allowed to form overnight in an ice bath, then filtered, washed with cold H\(_2\)O and Et\(_2\)O, and then dried in vacuo to give a dark purple solid 5. Yield: 5.0 g (31%). FAB-MS calculated for C\(_{8}\)H\(_{16}\)O\(_2\)Cl: 173.0 (M+H\(^+\)), found: 173.1. \(^1\)H NMR (DMSO-\(d_6\), 250 MHz) \(\delta\) 8.8 (br s, 1H), 2.34 (m, 4H), 1.57 (br t, 2H).
Scheme 2.6 Synthesis of 3-[2-[4-Chloro]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3-dimethyl-3H-indolino]propanesulfonic acid (6).

Following a procedure outlined by Narayanan, a solution of 4 (1.69 g, 6 mmol), 5 (1.079 g, 3 mmol), in CH$_3$(CH$_2$)$_3$OH:Benzene (7:3, 150 mL) was fitted with a Dean-Stark trap in an N$_2$ atmosphere and heated under reflux for 12 h. The H$_2$O generated was removed as an azeotrope and collected in the trap. The remaining solvent was removed under reduced pressure and the residue was purified by preparative RP-HPLC to afford a green powder 6. Yield: 1.08 g (52%) of 6. FAB-MS calculated for C$_{36}$H$_{44}$ClN$_2$O$_6$S$_2$: 699.2 (protonated form, M$^+$), found: 699.8, 721.8 (M - H + Na)$^+$, 743.9 (M - 2H + 2Na)$^+$. $^1$H NMR (DMSO-$d_6$, 400MHz) δ 8.26 (d, 4 H, $J = 14.1$ Hz), 7.62 (d, 2H, $J = 7.4$ Hz), 7.54 (d, 2H, $J = 8.0$), 7.42 (dd, 2H, $J = 7.6$ Hz), 7.27 (dd, 2H, $J = 7.4$ Hz), 6.53(d, 2H, $J = 14.2$ Hz), 4.39 (t, 4H, $J = 7.0$ Hz), 2.75 (t, 4H, $J = 5.2$ Hz), 2.56 (br t, 4H), 2.03 (m, 4H, $J = 7.1$ Hz), 1.83 (m, 4H), 1.67 (s, 12H).

2.1.3 Results and Discussion

The synthesis of 1 was achieved through the alkylation of 2,3,3-trimethylindolenine by iodoethane. The reddish-pink cationic product was easily
recovered from the reaction in high yields as the iodo salt. The synthesis of 2 proceeded with fairly good yields, but care needed to be taken that POCl₃, was freshly opened or distilled and DMF was anhydrous. If inferior reagents were used, yields and purity of 2 were reduced. Additionally, care must be taken when adding the aniline/CH₃CH₂OH solution to the intermediate diiminium cyclohexanone complex. The formation of the anilinium product is an exothermic process and must be cooled to 0 °C before and during the addition of aniline solution.

The condensation of 1 with 2 in anhydrous CH₃CH₂OH produced 3 as the iodide salt in moderate yields. 3 has excellent solubility in CH₃CH₂OH, CH₃OH, DMF, DMSO, and CHCl₃. However, this chromophore exhibited very limited solubility in aqueous solutions. Chloro dye 3 was purified using conventional flash chromatographic techniques with mixed results. Losses from column adsorption may have been the cause of the moderate yields obtained and not the reaction conditions.

Solubility in aqueous solutions is an ideal property for dyes that are to be employed as biological probes. The lack of solubility of 3 (solubility < 1 x 10⁻⁶ M in H₂O) in aqueous solutions hinders its widespread applicability as a biological probe. In order to improve the solubility in aqueous solutions, sulfopropyl groups were added onto the nitrogen in the indolenine ring. Reaction of 2,3,3 trimethylindolenine with propane sultone gave 4 in high yields. The sulfonate indolenine 4 reacted with either 2 or 5 to furnish 6. The chloro dye 6 had excellent solubility in aqueous solutions as well as many organic solvents with the
exception of CHCl₃. Purification of chloro dye 6 was achieved using preparative reversed phase chromatography due to the increased solubility of the dye in aqueous buffers. However, complications arose because the flow rate of mobile phase was too low to effectively resolve the product from the starting materials. This lack of resolving power led to low to moderate yields of chloro dye 6.

The bisaldehyde 5 has been shown to react efficiently with the activated methine units without the need of a catalyst to form symmetrical tricarbocyanine dyes. The synthesis of 6 using this synthetic route provided the product with very little side products with moderate yields and was used as an alternative to the dianil 2 almost exclusively to synthesize 6. In addition, the reaction kinetics with 5 and other heterocyclic groups is slower which makes this bridging unit an attractive reagent for the synthesis of asymmetric cyanine dyes.

2.1.4 Summary

The synthesis of basal tricarbocyanine dyes has been described. The cationic dye 3 was not very soluble in aqueous solutions, but the addition of sulfonate groups to form 6, greatly increased the solubility in aqueous buffers. Both the cationic 3 and anionic 6 dyes contain a chlorine at the meso position on the polymethine chain through which the dyes can potentially be modified with various substituents including functional groups such as isothiocyanate or succinimidyl esters.
2.2 Modified Tricarbocyanine Dyes

2.2.1 Heavy Atom Modified Tricarbocyanine Dyes

2.2.1.1 Introduction

The heavy-atom effect is a photochemical process which can have a profound effect upon the photophysics and physical properties of a chromophore. Specifically, the heavy-atom effect occurs when an atom of large atomic size (e.g. I or Br) is brought into close proximity either through bond or through space to the conjugated system of a chromophore. The heavy-atom can induce a spin-orbit coupling which will increase $k_{uc}$ of the chromophore. Through-bond interactions can cause a profound effect upon the electronic structure of the chromophore depending upon whether electron density is donated or withdrawn from the conjugated system. Through-space interactions have less effect on the electronic structure but can still cause structural changes within the molecule to affect the absorption and fluorescence properties.

The goal of this work is to modify the basal tricarbocyanine dyes 3 and 6 with heavy-atom substituents that interact with the chromophore through-space and not through-bond to provide a set of dyes with similar absorbance and emission properties but different fluorescence lifetimes. Patonay and co-workers have shown that dyes which have a similar structure to 3 and 6 can be modified at the chlorine position with various nucleophiles to form substitution products via an $S_{RN1}$ reaction mechanism and that will be the approach that is implemented here to make the required dyes.\textsuperscript{21,26}
2.2.1.2 Experimental

- **Chemicals.** DMF, 2-iodophenol, 4-iodophenol, 2-bromophenol, 4-bromophenol, 2-chlorophenol, 2-fluorophenol, 2,6-dibromophenol, 2,6-dichlorophenol, o-cresol, and sodium hydride were purchased from Aldrich Chemical Co. (Milwaukee, WI). All other solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA) and used as received.

- **Dye Synthesis and Purification.** The addition of the heavy-atom substituted phenol onto dye 3 or 6 followed modifications of procedures outlined by Strekowski et al.\textsuperscript{21,22} The synthetic scheme for the dyes in this section is shown in Figure 2.3 The dyes synthesized are outlined in Table 2.1.

- **Synthesis of 2-[4-Phenoxy-7-\{1-ethyl-3,3-dimethylindolin-2-ylidene\}-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-1-ethyl-3,3-dimethylindolinium Iodide (7).** Sodium hydride (62 mg, 1.6 mmol) in a 60% oil dispersion and phenol (147 mg, 1.6 mmol) were added to anhydrous DMF (20 mL) to form the phenoxide over a period of 30 min. Dye 3 (50 mg, 0.7 mmol) was added and allowed to react for 1 min. The deep green solution turned a red-orange color. Dry ice was added to stop the reaction and the DMF was removed by a rotary evaporator at 40 °C. The residue was dissolved in a mixture of CHCl\textsubscript{3}/CH\textsubscript{3}OH (20:1, 2 mL) and purified by flash chromatography. Pure fractions were collected, pooled, concentrated and evaporated to dryness in vacuo overnight to give 7. Yield: 33 mg (60%). FAB-MS calculated for C\textsubscript{40}H\textsubscript{45}N\textsubscript{2}O: 569.4 (M\textsuperscript{+}), found: 569.6. \textsuperscript{1}H NMR (CDCl\textsubscript{3}, 200 MHz) \( \delta \) 7.90 (d, 2
$H, J = 14.2 \text{ Hz}$, 7.85 (br dd, 1 H), 7.62 (m, 2 H), 4.75 (q, 2 H, $J = 7.2 \text{ Hz}$), 2.85 (s, 3 H), 2.65 (t, 2 H, $J = 6.5 \text{ Hz}$), 2.17 (m, 2 H), 1.54 (s, 6 H).

- **Synthesis of 3-[2-[4-Phenoxy]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (8).** Sodium hydride (52 mg, 1.3 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) to form a slurry. After 15 min, phenol (122 mg, 1.3 mmol) was added to form the phenoxide over a period of 1 h. Dye 6 (100 mg, 0.13 mmol) was added and allowed to react for 5 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator in vacuo at 40 °C. The residue was dissolved in CH$_3$OH:H$_2$O (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried in vacuo to obtain 8. Yield: 58 mg (58%). m.p. 235 - 244 °C. FAB-MS calculated for C$_{42}$H$_{49}$N$_2$O$_7$S$_2$: 757.3 (protonated form, M$^+$), found: 757.3. $^1$H NMR (CD$_3$OD, 400 MHz) δ 7.99 (d, 2H, $J = 14.2 \text{ Hz}$), 7.36 (m, 5H), 7.19 (br m, 2H), 7.13 (d, 2H), 7.08 (t, 2H), 6.32 (d, 2H, $J = 14.2 \text{ Hz}$), 4.31 (br t, 4H), 2.94 (t, 4H, $J = 6.8 \text{ Hz}$), 2.79 (br t, 4H), 2.25 (m, 4H), 2.05 (br t, 2H), 1.34 (s, 12H).

- **Synthesis of 2-[4-(2-Iodophenyl)oxy-7-(1-ethyl-3,3-dimethylindolin-2-ylidene)-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-1-ethyl-3,3- dimethylindolinium Iodide (9).** Sodium hydride (25 mg, 0.6 mmol) in a 60% oil dispersion was added to anhydrous DMF (10 mL) to form a slurry. After 15 min, 2-iodophenol (135 mg, 0.6 mmol) was added to form the phenoxide over a
Figure 2.3 General synthesis of heavy-atom modified tricarbocyanine dyes.

Table 2.1 Heavy-atom modified tricarbocyanine dyes.

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</table>

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period of 5 min. Dye 3 (20 mg, 29 µmol) was added and allowed to react for 3 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The sample was placed on a rotary evaporator under high vacuum to evaporate the DMF. The residue was purified by flash chromatography (silica gel, CHCl₃:CH₃OH 20:1). Pure fractions were collected, pooled, concentrated and dried in vacuo overnight giving 9. Yield: 12 mg (52%). FAB-MS calculated for C₄₀H₄₄N₂O: 694.2 (M⁺), found: 695.5. ¹H NMR (CDCl₃, 200 MHz) δ 7.77 (d, 2H, J = 14.2 Hz), 7.36-7.12 (br m, 9H), 6.77 (t, 2H, J = 7.9 Hz), 6.15 (d, 2H, J = 14.1 Hz), 4.20 (q, 4H), 2.75 (br t, 4H), 2.1 (br t, 2H), 1.64 (s, 6H), 1.42 (t, 4H, J = 7.1 Hz), 1.09 (s, 6H).

- Synthesis of 3-[2-[4-(2-Iodophenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (10). Sodium hydride (52 mg, 1.3 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) to form a slurry. After 15 min, 2-iodophenol (286 mg, 1.3 mmol) was added to form the phenoxide over a period of 5 min. Dye 6 (100 mg, 0.1 mmol) was added and allowed to react for 5 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator in vacuo at 40 °C. The residue was dissolved in CH₃OH:H₂O (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried in vacuo to afford 10. Yield: 58 mg (46%). m.p. 249 - 258 °C. FAB-MS
calculated for C_{42}H_{44}IN_{2}O_{7}S_{2} calculated: 883.2 (protonated form, M'), found: 883.6. \(^1\)H NMR (CD_{3}OD, 400 MHz) \(\delta\) 8.18 (d, 2H, \(J = 14.1\) Hz), 7.48-7.23 (br m, 10H), 7.01 (t, 2H), 6.30 (d, 2H, \(J = 14.2\) Hz), 4.32 (t, 2H, \(J = 8.7\) Hz), 2.94 (br t, 4H), 2.66 (br t, 4H), 2.23 (m, 4H), 2.08 (br t, 4H), 1.98 (br t, 2H), 1.66 (s, 6H), 1.08 (s, 6H).

- Synthesis of 2-[4-(2-Bromophenyl)oxy-7-(1-ethyl-3,3-dimethylindolin-2-ylidene)-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-1-ethyl-3,3-dimethylindolinium Iodide (11). Sodium hydride (124 mg, 3.1 mmol) in a 60% oil dispersion was added to anhydrous DMF (10 mL) as a slurry. After 15 min, 2-bromophenol (363 \(\mu\)L, 3.1 mmol) was added to form the phenoxide over a period of 30 min. Dye 3 (100 mg, 0.15 mmol) was added and allowed to react for 3 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The sample was placed on a rotary evaporator under high vacuum to evaporate the DMF. The residue was purified by flash chromatography (silica gel, CHCl_{3}:CH_{3}OH 20:1). Pure fractions were collected, pooled, concentrated and dried in vacuo overnight to give 11. Yield: 48 mg (41%). FAB-MS calculated for C_{46}H_{44}BrN_{2}O: 647.3 (M'), found: 647.5. \(^1\)H NMR (CDCl_{3}, 200 MHz) \(\delta\) 8.77 (d, 2H, \(J = 13.8\) Hz), 7.98 (d, 2H, \(J = 8.6\) Hz), 7.48-7.02 (br m, 8H), 6.47 (t, 2H), 6.03 (d, 2H, \(J = 14.0\) Hz), 4.02 (t, 4H), 2.76 (br t, 4H), 2.00 (br t, 2H), 1.39 (s, 6H), 1.34 (t, 4H, \(J = 7.1\) Hz), 1.21 (s, 6H).

- Synthesis of 3-[2-[4-(2-Bromophenyl)oxy]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-
1-yl]-3,3-dimethyl-3H-indolio]propanesulfonic acid (12). Sodium hydride (52 mg, 1.3 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) to form a slurry. After 15 min, 2-bromophenol (151 µL, 1.3 mmol) was added to form the phenoxide over a period of 30 min. Dye 6 (100 mg, 0.1 mmol) was added and allowed to react for 15 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator in vacuo at 40°C. The residue was dissolved in CH₃OH:H₂O (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried in vacuo to afford 12. Yield: 30 mg (28%). m.p. 243 - 248 °C. FAB-MS calculated for C₄₂H₄₂BrN₂O₇S₂: 835.2 (M⁺), found: 836.2. ¹H NMR (CD₃OD, 400 MHz) δ 7.89 (d, 2H, J = 14.2 Hz), 7.78 (d, 1H), 7.39-7.19 (m, 10H), 6.88 (d, 1H), 6.35 (d, 2H, J = 14.2 Hz), 4.32 (t, 4H, J = 7.4 Hz), 2.94 (t, 4H, J = 6.8 Hz), 2.21 (m, 4H), 2.05 (t, 2H), 1.63 (s, 6H), 1.11 (s, 6H).

- Synthesis of 2-[4-(2-Chlorophenyl)oxy-7-(1-ethyl-3,3-dimethylindolin-2-ylidene)-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-1-ethyl-3,3-dimethylindolinium Iodide (13). Sodium hydride (124 mg, 3.1 mmol) in a 60% oil dispersion was added to anhydrous DMF (10 mL) to form a slurry. After 15 min, 2-chlorophenol (315 µL, 3.1 mmol) was added to form the phenoxide over a period of 30 min. Dye 3 (100 mg, 0.15 mmol) was added and allowed to react for 2 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The sample was placed on a rotary evaporator under high vacuum to
evaporate the DMF. The residue was purified by flash chromatography (silica
gel, CHCl₃:CH₃OH 20:1). Pure fractions were collected, pooled, concentrated
and dried in vacuo overnight giving a green powder 13. Yield: 23 mg (20%).
FAB-MS calculated for C₄₀H₄₄ClN₂O: 603.1 (M⁺), found: 603.4. ¹H NMR
(CDCl₃, 200 MHz) δ 7.80 (d, 2H, J = 14.1 Hz), 7.5-6.83 (br m, 12H), 6.15 (d,
2H, J = 14.2 Hz), 4.18 (br t, 4H), 2.81 (m, 4H), 2.08 (t, 2H, J = 5.6 Hz), 1.58
(br s, 12H), 1.32 (br t, 3H).

• Synthesis of 3-[2-[4-(2-Chlorophenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-
1-yl]-3,3dimethyl-3H-indole]propanesulfonic acid (14). Sodium hydride (54
mg, 1.3 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) to
form a slurry. After 15 min, 2-chlorophenol (151 µL, 1.3 mmol) was added to
form the phenoxide over a period of 30 min. Dye 6 (100 mg, 0.1 mmol) was
added and allowed to react for 15 min. The deep green solution turned an amber
yellow. Dry ice was added to stop the reaction and the solution returned to a
deep green color. The DMF was removed on a rotary evaporator in vacuo at 40
°C. The residue was dissolved in CH₃OH:H₂O (1:1, 3 mL) and purified by
preparative HPLC. Pure fractions were collected, pooled, concentrated and
dried in vacuo to obtain 14. Yield: 26 mg (25%). FAB-MS calculated for
C₄₂H₄₄ClN₂O₇S₂: 791.3 (Protonated form, M⁺), found: 791.8. m.p. 246-249 °C.
¹H NMR (CD₃OD, 250 MHz) δ 7.91(d, 2H, J = 14.2 Hz), 7.59 (d, 1H, J = 7.9
Hz), 7.37 (br m, 6H), 7.22 (m, 3H), 7.03 (t, 1H, J = 7.8 Hz), 6.91 (d, 1H, J = 8.0
Hz), 6.36 (d, 2H, \( J = 14.2 \) Hz), 4.33 (br t, 4H, \( J = 6.7 \) Hz), 2.94 (br t, 4H, \( J = 6.5 \) Hz), 2.81 (br t, 4H), 2.21 (br m, 4H), 2.05 (br t, 2H), 1.34 (br s, 12H).

- **Synthesis of 2-[4-(2-Fluorophenyl)oxy-7-(1-ethyl-3,3-dimethylindolin-2-ylidene)-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-1-ethyl-3,3-dimethylindolinium Iodide (15).** Sodium hydride (124 mg, 3.1 mmol) in a 60\% oil dispersion was added to anhydrous DMF (20 mL) to form a slurry. After 15 min., 2-fluorophenol (278 \( \mu L \), 3.1 mmol) was added to form the phenoxide over a period of 30 min. Dye 3 (100 mg, 0.15 mmol) was added and allowed to react for 3 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The sample was placed on a rotary evaporator under high vacuum to evaporate the DMF. The residue was purified by flash chromatography (silica gel, CHCl\(_3\):CH\(_3\)OH 20:1). Pure fractions were collected, pooled, concentrated and dried \textit{in vacuo} overnight to give 15. Yield: 16 mg (15\%). FAB-MS calculated for C\(_{40}\)H\(_{44}\)FN\(_2\)O: 587.3 (M\(^+\)), found: 587.5. \(^1\)H NMR (CDCl\(_3\), 250 MHz) \( \delta \) 7.88 (d, 2H, \( J = 14.3 \) Hz), 7.39-6.89 (br m, 12H), 6.15 (d, 2H, \( J = 14.4 \) Hz), 4.21 (br t, 4H), 2.80 (br t, 4H), 2.07 (m, 2H), 1.45 (br t, 6H), 1.38 (s, 12H).

- **Synthesis of 3-[2-[4-(2-Fluorophenyl)oxy]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3-dimethyl-3H-indolio]propanesulfonic acid (16).** Sodium hydride (52 mg, 1.3 mmol) in a 60\% oil dispersion was added to anhydrous DMF (20 mL) to form a slurry. After 15 min 2-fluorophenol (151 \( \mu L \), 1.3 mmol) was added to form the phenoxide over a period of 30 min. Dye 6 (100 mg, 0.1 mmol) was...
added and allowed to react for 15 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator in vacuo at 40 °C. The residue was dissolved in CH$_3$OH:H$_2$O (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried in vacuo to afford 16. Yield: 63 mg (59%). FAB-MS calculated for C$_{42}$H$_{48}$FN$_2$O$_7$S$_2$: 775.3 (M$^+$), found: 775.3. m.p. 240-243 °C. $^1$H NMR (CD$_3$OD, 250 MHz) δ 7.98 (d, 2H, J = 14.0 Hz), 7.41-6.93 (m, 12H), 6.36 (d, 2H, J = 14.3 Hz), 4.33 (t, 4H, J = 6.8 Hz), 2.94 (t, 4H, J = 6.7 Hz), 2.81 (br t, 4H), 2.22 (m, 4H), 2.04 (br t, 2H), 1.39 (s, 12H).

- Synthesis of 3-[2-[4-(2-Methylphenyl)oxy]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3-dimethyl-3H-indoline]propanesulfonic acid (17). Sodium hydride (52 mg, 1.3 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) to form a slurry. After 15 min, o-cresol (155 mg, 1.3 mmol) was added to form the phenoxide over a period of 30 min. Dye 6 (100 mg, 0.1 mmol) was added and allowed to react for 15 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator in vacuo at 40 °C. The residue was dissolved in CH$_3$OH:H$_2$O (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried in vacuo giving 17. Yield: 7 mg (9%). FAB-MS calculated for C$_{43}$H$_{51}$N$_2$O$_7$S$_2$: 771.3 (protonated form, M$^+$), found: 771.8. m.p. 240-243 °C. $^1$H NMR (DMSO-d$_6$, ...
200 MHz) δ 7.73 (d, 2H, J = 14.2 Hz), 7.5-7.12 (m, 8H), 6.94 (br t, 2H), 6.74 (d, 2H, J = 6.4 Hz), 6.39 (d, 2H, J = 14.1 Hz), 4.29 (br t, 4H), 2.75 (br t, 4H), 1.97 (m, 4H), 1.51 (br s, 3H), 0.97 (br s, 12H).

- Synthesis of 3-[2-[4-(2,6-Dibromophenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (18). Sodium hydride (52 mg, 1.3 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) as a slurry. After 15 min, 2,6-dibromophenol (327 mg, 1.3 mmol) was added to form the phenoxide over a period of 1 h. Dye 6 (100 mg, 0.1 mmol) was added and allowed to react for 15 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator in vacuo at 40 °C. The residue was dissolved in CH₃OH:H₂O (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried in vacuo to give 18. Yield: 63 mg (52%). FAB-MS calculated for C₄₂H₄Br₂N₂O₇S₂: 913.1 (protonated form, M⁺), found: 913.5. m.p. 239-243 °C.

- Synthesis of 3-[2-[4-(2,6-Dichlorophenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (19). Sodium hydride (52
mg, 1.3 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) as a slurry. After 15 min, 2,6-dichlorophenol (327 mg, 1.3 mmol) was added to form the phenoxide over a period of 1 h. Dye 6 (100 mg, 0.1 mmol) was added and allowed to react for 15 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator in vacuo at 40 °C. The residue was dissolved in CH₃OH:H₂O (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried in vacuo to obtain 19. Yield: 69 mg (63%). FAB-MS calculated for C₄₂H₄₂Cl₂N₂O₇S₂: 825.2 (protonated form, M⁺), found: 825.8. m.p. 238-242 °C.

¹H NMR (CD₃OD, 400 MHz) δ 7.93 (d, 2H, J = 14.0 Hz), 7.51 (d, 2H, J = 8.2 Hz), 7.38-7.35 (m, 5H), 7.21-7.13 (br m, 4H), 6.30 (d, 2H, J = 14.0 Hz), 4.31 (t, 4H, J = 7.7 Hz), 2.97 (t, 4H, J = 6.9 Hz), 2.76 (t, 4H, J = 6.0 Hz), 2.21 (m, 4H), 2.0 (br t, 2H), 1.40 (s, 12H).

- Synthesis of 3-[2-[4-(4-Iodophenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (20). Sodium hydride (55 mg, 1.4 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) to form a slurry. After 15 min, 4-iodophenol (306 mg, 1.4 mmol) was added to form the phenoxide over a period of 1 h. Dye 6 (100 mg, 0.1 mmol) was added and allowed to react for 5 min. The deep green solution turned an amber yellow. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator in vacuo at 40 °C. The
residue was dissolved in \( \text{CH}_3\text{OH}:\text{H}_2\text{O} \) (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried \textit{in vacuo} to give a green powder 20. Yield: 38 mg (29%). FAB-MS calculated for \( \text{C}_{42}\text{H}_{41}\text{IN}_2\text{O}_7\text{S}_2 \) calculated: 883.2 (protonated form, \( \text{M}^+ \)), found: 884.4. \( ^1\text{H} \) (CD\( _3\)OD, 250 MHz) \( \delta \) 7.94 (d, 2H, \( J = 14.2 \) Hz), 7.70 (d, 2H, \( J = 8.7 \) Hz), 7.38 (m, 6H), 7.21 (t, 2H), 6.97 (d, 2H, \( J = 8.7 \) Hz), 6.34 (d, 2H, \( J = 14.3 \) Hz), 4.32 (t, 4H, \( J = 6.9 \) Hz), 2.94 (t, 4H, \( J = 6.7 \) Hz), 2.78 (br t, 4H), 2.21 (m, 4H), 2.02 (br t, 2H), 1.37 (s, 12H).

- Synthesis of 3-[2-[4-(4-Bromophenyl)oxy]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3-dimethyl-3H-indolino]propanesulfonic acid (21). Sodium hydride (55 mg, 1.4 mmol) in a 60% oil dispersion was added to anhydrous DMF (20 mL) as a slurry. After 15 min, 4-bromophenol (240 mg, 1.4 mmol) was added to form the phenoxide over a period of 1 h. Dye 6 (100 mg, 0.1 mmol) was added and allowed to react for 5 min. The deep green solution turned an yellow-green color. Dry ice was added to stop the reaction and the solution returned to a deep green color. The DMF was removed on a rotary evaporator \textit{in vacuo} at 40 °C. The residue was dissolved in \( \text{CH}_3\text{OH}:\text{H}_2\text{O} \) (1:1, 3 mL) and purified by preparative HPLC. Pure fractions were collected, pooled, concentrated and dried \textit{in vacuo} to give 21. Yield: 28 mg (24%). FAB-MS calculated for \( \text{C}_{42}\text{H}_{46}\text{BrN}_2\text{O}_7\text{S}_2 \): 835.2 (protonated form, \( \text{M}^+ \)), found: 835.6. \( ^1\text{H} \) NMR (CD\( _3\)OD, 250 MHz) \( \delta \) 7.94 (d, 2H, \( J = 14.1 \) Hz), 7.53 (d, 2H, \( J = 8.9 \) Hz), 7.37 (m, 6H), 7.22 (m, 2H), 7.09 (d, 2H, \( J = 8.7 \) Hz) 6.34 (d, 2H, \( J = 14.0 \) Hz), 4.32
(t, 4H, $J = 7.0$ Hz), 2.94 (t, 4H, $J = 6.6$ Hz), 2.78 (br t, 4H), 2.21 (m, 4H), 2.03 (br t, 2H), 1.37 (s, 12H).

2.2.1.3 Results and Discussion

The nucleofugal phenoxide efficiently reacted at the meso position to form the various dyes listed in Table 2.1. The reaction proposed by Patonay involves the formation of a radical cation intermediate which is indicative of the loss of green color in the reaction solution. The quenching of the intermediate produces the newly substituted product including unidentified products.

The yields of each reaction were around 30-50%, but losses in the purification of the dyes may account for moderate yields as described in section 2.1.1.4. In addition, excessive heating above 40 °C of the reaction mixture during removal of the DMF results in the total loss of the near-infrared absorbing species and produces a product which primarily absorbs in the visible. This product was not isolated, but presumably, the excess phenol radical reacts with the chromophore to disrupt the conjugated system.

As seen before with dyes 3 and 6, the cationic heavy-atom modified chromophores were not soluble under aqueous conditions. However, the heavy-atom modified analogs of 6 were completely soluble under aqueous conditions at high concentrations (~ 1 mM) primarily due to the addition of the propylsulfonates onto the heterocyclic nitrogens.
2.2.1.4 Summary

The synthesis of a series of cationic and anionic heavy-atom modified tricarbocyanine dyes has been described and demonstrate the utility of the basal tricarbocyanine dyes 3 and 6 to undergo various modifications at the meso position by way of nucleofugal substitution with various halogenated phenols. This modification is pivotal for synthesizing dyes which contain similar electronic states, but exhibit differences in the associated radiative and non-radiative rates between the excited electronic states. The simplicity of the reaction also demonstrates the versatility of tricarbocyanine dyes to be modified to exhibit features that may be needed for various bioanalytical applications.

2.2.2 Functionalized Tricarbocyanine Dyes

2.2.2.1 Introduction

There are several factors which constitute an ideal fluorescent dye for the labeling of biomolecules. Generally, the fluorophore must be chemically and photochemically robust in solution, water soluble, have a large fluorescence quantum yield when bound to the analyte, must be specific for an analyte and must produce minimal interference on the analysis being performed (e.g. electrophoretic mobility shifts in capillary gel electrophoresis in DNA sequencing caused from differences in chromophore labels). Additionally, dyes that covalently bind to analytes of interest must possess reactive functional groups which are stable to long-term storage, but also have high labeling efficiencies with minimal side reactions and produce a stable covalent bond. Representative
labeling functionalities include isothiocyanates and succinimide esters for coupling $^1\text{a}$ and $^2\text{a}$ amines and iodoacetamides for reacting with thiols. Chromophores that non-covalently interact with analytes must possess a large fluorescence quantum yield enhancement upon interaction and a large binding constant with the analyte of interest.

The goal of this section is to describe the synthesis of several tricarbocyanine dyes which contain functional groups such as an isothiocyanate or succinimidyl ester for conjugating to biomolecules which contain primary amines. Additionally, the goal is to incorporate a heavy-atom modification which also contains an isothiocyanate functional group onto the basal tricarbocyanine dye 6 to produce a set of distinct chromophores that can label dideoxynucleotides or DNA primers to be incorporated into a Sanger DNA sequencing protocol employing near-IR fluorescence detection.

### 2.2.2.2 Experimental

\[ \begin{align*}
\text{NH}_2 & \quad \text{S} & \quad \text{N} = \text{C} = \text{S} \\
\text{OH} & \quad \text{N} \quad \text{N} & \quad \text{N} \\
\text{DMF} & \quad \rightarrow \\
\text{OH} & \quad \text{N} \quad \text{N} & \quad \text{N}
\end{align*} \]

**Scheme 2.7** Synthesis of 2-(p-Hydroxyphenyl)ethylisothiocyanate (22).
Tyramine (400 mg, 2.9 mmol) was dissolved in anhydrous DMF (10 mL) under a N₂ atmosphere. 1,1-Thiocarbonyldiimidazole (TCDI) (520 mg, 2.9 mmol) was added and the pale yellow color of the solution turned an amber red color. After 30 min, the solvent was taken off by a rotary evaporator at 40 °C to afford an orange oil. The oil was dissolved in CH₃OH:H₂O (1:3, 4 mL) to precipitate the isothiocyanate. The product was filtered and washed with cold water to afford 320 mg of 22 (62%). GC-MS calculated for C₉H₉NOS: 180.0 (M⁺), found: 180.0. ¹H NMR (DMSO-d₆, 250 MHz) δ 7.67 (s, 1H), 7.05 (d, 2H, J = 8.1 Hz), 6.70 (d, 2H, J = 8.6 Hz), 3.80 (t, 2H, J = 6.6 Hz), 2.81 (t, 2H, J = 6.6 Hz). IR: 2081 cm⁻¹ (C=S stretch).

Scheme 2.8 Synthesis of 2-(3-Iodo-4-hydroxyphenyl)ethylamine (23).

Following a procedure outlined by Evangelatos, tyramine (1.34 g, 0.01 mol) was dissolved in NH₄OH (200 mL) with stirring. A 0.96 N solution of I₂ (21 mL, 0.02 mol) was slowly added into the reaction solution from a pressure equalizing addition funnel. After 1.5 h, the solvent was concentrated to 50 mL and the solution was placed at 4 °C overnight to precipitate a white powder. The
precipitate was filtered and dried *in vacuo* to give 23. Yield: 1.65 g (62%). GC-MS calculated for C₈H₁₀INO: 263.0 (M⁺), found: 263.0.

Scheme 2.9 Synthesis of 2-(3-Iodo-4-hydroxyphenyl)ethylisothiocyanate (24).

2-(3-Iodo-4-hydroxyphenyl)ethylamine (750 mg, 2.8 mmol) was dissolved in anhydrous DMF (5 mL) under an N₂ atmosphere. 1,1-Thiocarbonyldiimidazole (606 mg, 3.4 mmol) was added and the pale yellow color of the solution turned an amber color. After 24 h, H₂O (20 mL) was added to the mixture and the product was extracted into CH₂Cl₂ (7 x 50 mL). The extract was dried with MgSO₄, filtered, and the solvent was removed *in vacuo* to afford an orange, oily mixture. The oil was purified by flash chromatography (solvent: CH₂Cl₂). Pure fractions were pooled and dried *in vacuo*. The oily residue of 24 crystalized at 4 °C overnight. Yield: 282 mg (33%). GC-MS calculated for C₉H₉NOS: 304.9 (M⁺), found: 305.0. ¹H NMR (CD₃OD, 250 MHz) δ 7.59 (s, 1H), 7.08 (d, 1H, J = 8.2 Hz), 6.78 (d, 1H, J = 8.4 Hz), 3.67 (t, 2H, J = 6.8 Hz), 2.85 (t, 2H, J = 6.6 Hz).
Scheme 2.10 Synthesis of 2-(3-Bromo-4-hydroxyphenyl)ethylamine (25).

Following a modified procedure by Kajigaeshi, tyramine (1.03 g, 7.5 mmol) and CaCO₃ (830 mg, 8.3 mmol) were dissolved in CH₂Cl₂/CH₃OH (7:3, 60 mL). After the tyramine had completely dissolved, tetrabutylammonium tribromide (4.0 g, 8.3 mmol) was added with stirring. Immediately, the orange color of the solution turned brown. The reaction was followed by TLC (silica, CH₂Cl₂:CH₃OH 7:3; plates were stained with a ninhydrin solution (1% in butanol)) and was complete after 5 min. After 5 min, the reaction was filtered and the filtrate washed with Et₂O (5 x 20 mL). The ether was removed and the residue was used without further purification. Yield: 40% (by GC-MS). GC-MS calculated for C₁₀H₁₉BrNO: 215 (M⁺), found: 215.

Scheme 2.11 Synthesis of 2-(3-Bromo-4-hydroxyphenyl)ethylisothiocyanate (26).
The residue from the 2-(3-bromo-4-hydroxyphenyl)ethylamine reaction was dissolved in anhydrous DMF (5 mL) under an N\textsubscript{2} atmosphere. 1,1-Thiocarbonyldiimidazole (1.44 g, 7.5 mmol) was added and the orange color of the solution turned an amber color. The reaction was followed by TLC (silica, CH\textsubscript{2}Cl\textsubscript{2}:CH\textsubscript{3}CN, 4:1). After 30 min, the solvent was removed on a rotary evaporator at 40°C. The residue was repeatedly washed with Et\textsubscript{2}O (7 x 10 mL). The Et\textsubscript{2}O was removed to afford an orange oil which was purified by flash chromatography (silica, CH\textsubscript{2}Cl\textsubscript{2}:Hexane, 3:1). The pure fractions were collected, pooled and dried \textit{in vacuo} to afford 26. Yield: 400 mg (21%). GC-MS calculated for C\textsubscript{9}H\textsubscript{2}BrNOS: 257.0 (M\textsuperscript{+}), found: 257.0. \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 250 MHz) \(\delta\) 7.38 (s, 1H), 7.05 (d, 1H, \(J=8.5\) Hz), 6.85 (d, 1H, \(J=8.6\) Hz), 3.69 (t, 2H, \(J=6.7\) Hz), 2.86 (t, 2H, \(J=6.6\) Hz).

\[ \text{NH}_3^+ \text{Cl}^- \quad \text{m-Chloroperbenzoic Acid} \quad \text{1M HCl in DMF} \]

\[ \text{NH}_3^+ \text{Cl}^- \]

\(27\)

\textbf{Scheme 2.12} Synthesis of 2-(3-Chloro-4-hydroxyphenyl)ethylammonium Chloride (27).

Following a modification of a procedure outlined by Chung,\textsuperscript{33} tyramine hydrochloride (1.0 g, 5.8 mmol) was dissolved in a solution 1M hydrochloric acid.
in anhydrous DMF (9 mL) with stirring. m-Chloroperbenzoic acid (1.09 g, 6.3 mmol) was added slowly with stirring and the solution turned a pale yellow color. After 30 min, the reaction was poured into CH$_2$Cl$_2$ (50 mL) with stirring. The resulting white slurry was filtered and the precipitate was washed with CH$_2$Cl$_2$ (3 x 10 mL). The resulting white product 27 was dried at 70°C. Yield: 620 mg (51%). GC-MS calculated for C$_9$H$_8$ClNOS: 171.0 (free base, M$^+$), found: 171.0.

$^1$H NMR (CD$_3$OD, 250 MHz) $\delta$ 7.24 (s, 1H), 7.04 (d, 1H, $J = 8.6$ Hz), 6.89 (d, 1H, $J = 8.4$ Hz), 3.12 (t, 2H, $J = 7.2$ Hz), 2.85 (t, 2H, $J = 8.0$ Hz).

Scheme 2.13 Synthesis of 2-(3-Chloro-4-hydroxyphenyl)ethylisothiocyanate (28).

2-(3-Chloro-4-hydroxyphenyl)ethylammonium chloride (500 mg, 2.4 mmol) was dissolved in anhydrous DMF (3 mL) under N$_2$. 1,1-Thiocarbonyldiimidazole (644 mg, 3.6 mmol) was added with stirring and the solution turned a pale yellow color. After 30 min, the DMF was removed in vacuo and the residue was dissolved in CH$_2$Cl$_2$ (20 mL). This solution was washed with H$_2$O (3 x 50 mL) in a 250 mL separatory funnel. The CH$_2$Cl$_2$ layer was collected, dried with MgSO$_4$ and filtered. The CH$_2$Cl$_2$ was removed in
vacuo to afford an oil. The oil was purified by flash chromatography (silica, CH$_2$Cl$_2$:CH$_3$CN, 10:1). Pure fractions from the column were collected, pooled and concentrated in vacuo. Pure product 28 crystalized at 4 °C. Yield: 167 mg (33%). GC-MS calculated for C$_9$H$_7$ClINOS: 213.0 (M$^+$), found: 213.0. $^1$H NMR (CD$_3$OD, 250 MHz) $\delta$ 7.20 (s, 1H), 7.00 (d, 1H, $J = 7.9$ Hz), 6.87 (d, 1H, $J = 8.5$ Hz), 3.67 (t, 2H, $J = 6.8$ Hz), 2.84 (t, 2H, $J = 6.7$ Hz).

Scheme 2.14 Synthesis of 2-(3-Fluoro-4-hydroxyphenyl)ethylammonium Chloride (29).

Following a modification of a procedure outlined by Banks,$^{34}$ tyramine hydrochloride (1.0 g, 5.8 mmol) was dissolved in H$_2$O/CH$_3$OH (26 mL, 1:1) with stirring. 1-(Chloromethyl)-4-fluoro-1,4-diazaoniabicyclo[2.2.2]octane bis(tetrafluoroborate) (2.06 g, 5.8 mmol) was added and the solution turned a dark brown color. After 18 h, the solvent was taken off in vacuo and the residue was dissolved in CH$_3$OH (20 mL) and filtered. The solvent was removed from the filtrate and the crude product was precipitated from CH$_3$CN (10 mL). The crude product was used without further purification. Yield: 300 mg.

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Scheme 2.15 Synthesis of 2-(3-Fluoro-4-hydroxyphenyl)ethylisothiocyanate (30).

2-(3-Fluoro-4-hydroxyphenyl)ethylammonium chloride (300 mg, crude) was dissolved in anhydrous DMF (3 mL) under N₂ with stirring. 1,1-Thiocarbonyldiimidazole (286 mg, 1.6 mmol) was added and the reaction was complete after 30 min. The reaction was taken off stirring and H₂O (20 mL) was added. The product was extracted from the solution with CH₂Cl₂ (3 x 50 mL). The CH₂Cl₂ layer was collected, dried with MgSO₄ and filtered. The CH₂Cl₂ was removed from the filtrate to give an orange oil. The oil was purified by flash chromatography (silica, CH₂Cl₂:CH₃OH, 10:1). The pure fractions were collected, pooled, and dried in vacuo to afford 30 as an oil. Yield: 127 mg (40% of crude). GC-MS calculated for C₉H₆FNOS: 197.0 (M⁺), found: 197.0. ¹H NMR (CD₃OD, 250 MHz) δ 6.98 (d, 1H, J = 11.9 Hz), 6.88 (m, 2H), 3.69 (t, 2H, J = 6.6 Hz), 2.87 (t, 2H, J = 6.7 Hz).
Scheme 2.16 Synthesis of 3-[2-[4-(4-aminophenyl)thio]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-yldene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (31).

Following a procedure outlined by Strekowski,21 4-aminothiophenol (348 mg, 2.8 mmol) was dissolved in anhydrous DMF (30 mL) under a N₂ atmosphere. Chlorodye 6 (100 mg, 0.14 mmol) was added and the mixture was stirred for 5 min. The DMF was removed under reduced pressure, and the residue was dissolved in DMF (2 mL) and precipitated with Et₂O (10 ml). The solvents were removed and the residue was washed with Et₂O (2 x 10 mL) and purified by preparative reversed-phase HPLC. The fractions collected were concentrated and dried overnight in vacuo to give 31. Yield: 55 mg (50%). This compound was not stable to storage and was used immediately for the preparation of the isothiocyanate.
Scheme 2.17 Synthesis of 3-[2-[4-(4-isothiocyanatophenyl)thio]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (32).

Method A. The aminothioether 31 (55 mg, 0.07 mmol) and anhydrous sodium carbonate (12 mg) were dissolved in anhydrous DMF (10 mL) under a N₂ atmosphere. At 0 °C, thiophosgene (16 mg, 11 µL, 0.14 mmol) was added with stirring. After 5 min, the mixture was taken off of the ice bath and allowed to react at room temperature for 1 h. The solution was filtered and the filtrate was washed with DMF which was removed in vacuo at 40 °C. The residue was dried in vacuo overnight to afford 32. Yield: 23 mg (39 %). FAB-MS calculated for C₄₅H₄₆N₅O₆S₄: 830.2 (protonated form, M⁺), found: 830.3. ¹H NMR (CD₃OD, 250 MHz) δ 8.73 (d, 2H, J = 14.1 Hz), 7.1-7.4 (m, 12H), 6.48 (d, 2H, J = 14.0
Hz), 4.36 (br t, 4H), 2.95 (t, 4H, J = 6.5 Hz), 2.84 (br t, 4H), 2.23 (m, 4H), 2.02 (m, 4H), 1.50 (s, 12H). IR: 2108 cm⁻¹ (C=S stretch).

Scheme 2.18 Synthesis of 3-(2-[4-(4-isothiocyanatophenyl)thio]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3-dimethyl-3H-indolio)propanesulfonic acid (32).

Method B (preferred) 4-Aminothiophenol (54 mg, 0.4 mmol) was dissolved in anhydrous DMF (10 mL) under a N₂ atmosphere. Chlorodye 6 (100 mg, 0.14 mmol) was added and stirred for 5 min. The reaction was quenched with dry ice. After allowing to warm to room temperature (5 min), 1,1-thiocarbonyldiimidazole (210 mg, 1.0 mmol) was added with stirring. After 30 min, Et₂O (30 mL) was added to precipitate the dye. The resulting suspension was placed into a centrifuge for 30 s and the supernatant was discarded. The precipitate was dissolved in H₂O:CH₃OH (1:1, 1.5 mL) and purified using 5 C₁₈ Sep-Pak® Plus cartridges in series with a CH₃OH/H₂O gradient (40:60, 3 mL;
60:40 2 mL, 80:20, 4 mL). The fractions collected were concentrated and dried overnight *in vacuo* to give 32. Yield: 34 mg (29%).

Scheme 2.19 Synthesis of 3-[2-[4-(4-ethylisothiocyanato-phenyl)oxy]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diy)-1,3,5-heptatriene-1-yl]-3,3-dimethyl-3H-indolio]propanesulfonic acid (33).

Following a procedure outline by Narayanan, a 60% oil dispersion of NaH (14 mg, 0.3 mmol) was added to anhydrous DMF (4 mL) under a N₂ atmosphere at 0 °C. In a separate flask, 22 (58 mg, 0.3 mmol) was dissolved in anhydrous DMF (1 mL) under a N₂ atmosphere at 0 °C and the solution was added to the slurry of NaH. After 30 min, the phenoxide isothiocyanate solution was added to chlorodye 6 (100 mg, 0.14 mmol) dissolved in anhydrous DMF (4 mL) under N₂ atmosphere. The reaction was followed by HPLC. After 18 h, the reaction was quenched with dry ice and the solvent was removed on a rotary
evaporator at 40 °C. The residue was dissolved in CH$_3$OH:H$_2$O (1:1, 3 mL), filtered, purified by preparatory HPLC and dried in vacuo overnight to give 33. 
Yield: 37 mg (32%). FAB-MS calculated for C$_{45}$H$_{52}$N$_3$O$_7$S$_3$: 842.3 (protonated form, M$^+$), found 843.3. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 7.99 (d, 2H, $J = 14.2$ Hz), 7.04-7.38 (m, 12H), 6.31 (d, 2H, $J = 14.1$ Hz), 4.30 (t, 4H, $J = 7.6$ Hz), 3.76 (m, 4H), 2.79 (t, 4H, $J = 5.8$ Hz), 2.20 (m, 4H, $J = 5.7$ Hz), 1.31 (s, 12H).

Scheme 2.20 Synthesis of 3-[2-[4-(4-propylcarboxy-phenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyli)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (34).

3-(p-Hydroxyphenyl)-propionic acid (224 mg, 1.4 mmol) was dissolved in anhydrous DMF (10 mL) under a N$_2$ atmosphere. Sodium hydride (65 mg of a 60% oil dispersion, 2.7 mmol) was added to form the phenolate for 30 min under
constant stirring. Chlorodye 6 (100 mg, 0.14 mmol) was added to react under N\textsubscript{2} with stirring for 30 min. The reaction was quenched with dry ice and the DMF was taken off by a rotary evaporator. The residue was dissolved in H\textsubscript{2}O:CH\textsubscript{3}OH (3:2, 3 mL), filtered, and purified via HPLC. The collected fractions were concentrated on a rotary evaporator and dried \textit{in vacuo} to afford 34. Yield: 35 mg (31%) FAB-MS calculated for C\textsubscript{45}H\textsubscript{33}N\textsubscript{2}O\textsubscript{3}S\textsubscript{2}: 829.3 (protonated form, M\textsuperscript{+}), found: 829.6. \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 250 MHz) δ 8.00 (d, 2H, J = 14.2 Hz), 7.34 - 7.40 (m, 8H), 7.24 (m, 4H), 7.00 (d, 4H, J = 8.6 Hz), 6.30 (d, 2H, J = 14.2 Hz), 4.31 (br t, 4H), 2.95 (t, 2H, J = 6.6 Hz), 2.80 (br t, 4H), 2.35 (m, 2H), 2.21 (m, 4H), 2.09 (m, 2H), 1.34 (s, 12H).

Scheme 2.21 Synthesis of Cy.7.OPhEt.CO\textsubscript{2}Su (35).
N-Hydroxysuccinimide (7 mg, 60 μmol) and 34 (1 mg, 1.2 μmol) were added sequentially to anhydrous DMF (100 μL). After the free acid dye had dissolved, dicyclohexylcarbodiimide (DCC, 12 mg, 60 μmol) was added and stirred overnight in the dark. The reaction was followed by analytical HPLC, and used without further purification. The synthesis afforded 85% conversion to 35 as determined by HPLC (remaining 15% of near-IR absorbing materials was the starting free acid dye 34). The succinate ester 35 could not be isolated.

Scheme 2.22 Synthesis of 3-[2-[4-(2-Iodo-4-ethylisothiocyanato-phenyl)oxy]-7-[3,3-dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diy)-1,3,5-heptatriene-1-yl]-3,3-dimethyl-3H-indolio]propanesulfonic acid (36).

A 60% oil dispersion of NaH (14 mg, 0.3 mmol) was added to anhydrous DMF (2 mL) under a N₂ atmosphere at 0 °C. In a separate flask, 24 (128 mg,
0.4 mmol) was dissolved in anhydrous DMF (1 mL) under a N\textsubscript{2} atmosphere at 0 °C and the solution was added to the slurry of NaH. After 30 min, the iodophenoxide isothiocyanate solution was added to chlorodye 6 (100 mg, 0.14 mmol) dissolved in anhydrous DMF (3 mL) under N\textsubscript{2} atmosphere. After 30 min, the reaction was quenched with dry ice and the solvent was removed on a rotary evaporator at 40 °C. Et\textsubscript{2}O (3 x 20 mL) was added to precipitate the dye and the supernatant discarded. The crude material was dissolved in CH\textsubscript{3}OH:H\textsubscript{2}O (1:1, 2 mL) and purified using 5 C\textsubscript{18} Sep-Pak\textsuperscript{®} Plus cartridges in series with a CH\textsubscript{3}OH/H\textsubscript{2}O gradient (H\textsubscript{2}O, 10 mL; CH\textsubscript{3}OH:H\textsubscript{2}O, 6:4, 10 mL; CH\textsubscript{3}OH:H\textsubscript{2}O, 8:2, 10 mL; CH\textsubscript{3}OH, 10 mL). Pure fractions were collected, pooled, and dried \textit{in vacuo} to afford 36. Yield: 28 mg (20%). FABMS calculated for C\textsubscript{45}H\textsubscript{51}IN\textsubscript{3}O\textsubscript{7}S\textsubscript{3}: 966.2 (protonated form, M\textsuperscript{+}), found 965.8. \textsuperscript{1}H NMR (CD\textsubscript{3}OD, 250 MHz) \textdelta 7.92 (s, 1H), 7.86 (d, 2H, \textit{J} = 14.1 Hz), 7.37 (m, 5H), 7.19 (m, 4H), 6.73 (d, 1H, \textit{J} = 8.7 Hz), 6.35 (d, 2H, \textit{J} = 14.3 Hz), 4.32 (t, 4H, \textit{J} = 7.3 Hz), 3.71 (t, 2H, \textit{J} = 5.9 Hz), 2.95 (m, 6H), 2.77 (br t, 2H), 2.21 (m, 4H), 2.04 (br t, 2H), 1.66 (s, 6H), 1.12 (s, 6H).

A 60% oil dispersion of NaH (14 mg, 0.3 mmol) was added to anhydrous DMF (2 mL) under a N\textsubscript{2} atmosphere at 0 °C. In a separate flask, 26 (109 mg, 0.4 mmol) was dissolved in anhydrous DMF (1 mL) under a N\textsubscript{2} atmosphere at 0 °C and the solution was added to the slurry of NaH. After 30 min, the bromophenoxide isothiocyanate solution was added to chlorodye 6 (100 mg, 0.14 mmol) dissolved in anhydrous DMF (3 mL) under N\textsubscript{2} atmosphere. After 30 min, the reaction was quenched with dry ice and the solvent was removed on a
Scheme 2.23 Synthesis of 3-[2-[4-(2-bromo-4-ethylisothiocyanato-phenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-yldiene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (37).

rotary evaporator at 40 °C. Et₂O (3 x 20 mL) was added to precipitate the dye and the supernatant discarded. The crude material was dissolved in CH₃OH:H₂O (1:1, 2 mL) and purified using 5 Sep-Pak® Plus C₁₈ cartridges in series with a CH₃OH:H₂O gradient (H₂O, 10 mL; CH₃OH:H₂O, 6:4, 10 mL; CH₃OH:H₂O, 8:2, 10 mL; CH₃OH, 10 mL). Pure fractions were collected, pooled, and dried in vacuo to give 37. Yield: 37 mg (27%). FAB-MS calculated for C₄₅H₅₁BrN₃O₇S₃: 920.2 (protonated form, M⁺), found: 920.8. ¹H NMR (CD₃OD, 250 MHz) δ 7.89 (d, 2H, J = 14.1 Hz), 7.71 (s, 1H), 7.36 (m, 5H), 7.20 (m, 4H), 6.83 (d, 1H, J = 8.0 Hz), 6.36 (d, 2H, J = 14.0 Hz), 4.32 (t, 4H, J = 7.5 Hz), 3.74 (t, 2H, J = 5.9 Hz), 2.95 (m, 6H), 2.80 (br t, 4H), 2.21 (m, 4H), 2.05 (br t, 2H), 1.63 (s, 6H), 1.13 (s, 6H).
**Scheme 2.24** Synthesis of 3-[2-[4-(2-chloro-4-ethylisothiocyanato-phenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (38).

A 60% oil dispersion of NaH (14 mg, 0.4 mmol) was added to anhydrous DMF (2 mL) under a N₂ atmosphere at 0 °C. In a separate flask, 28 (167 mg, 0.8 mmol) was dissolved in CH₂Cl₂ (1 mL). From this solution, 539 µL (0.4 mmol) was taken out and placed into a separate flask and the CH₂Cl₂ was taken off by a rotary evaporator. The residue was dissolved in anhydrous DMF (1 mL) under a N₂ atmosphere at 0 °C and the solution was added to the slurry of NaH. After 30 min, the chlorophenoxide isothiocyanate solution was added to chlorodye 6 (100 mg, 0.14 mmol) dissolved in anhydrous DMF (3 mL) under N₂ atmosphere. After 30 min, the reaction was quenched with dry ice and the solvent was removed on a rotary evaporator at 40 °C. Et₂O (3 x 20 mL) was added to precipitate the dye and the supernatant discarded. The crude material was dissolved in CH₃OH:H₂O (1:1, 2 mL) and purified using 5 C₁₈ Sep-Pak® Plus cartridges in series with a CH₃OH:H₂O gradient (H₂O, 10 mL; CH₃OH:H₂O,
6:4, 10 mL; CH$_3$OH:H$_2$O, 8:2, 10 mL; CH$_3$OH, 10 mL). Pure fractions were collected, pooled, and dried in vacuo to give 38. Yield: 38 mg (31%). FAB-MS calculated for C$_{45}$H$_{51}$ClN$_3$O$_7$S$_3$: 876.3 (protonated form, M$^+$), found: 876.7. $^1$H NMR (CD$_3$OD, 250 MHz) $\delta$ 7.89 (d, 2H, $J = 14.2$ Hz), 7.56 (s, 1H), 7.37 (m, 5H), 7.20 (m, 4H), 6.86 (d, 1H, $J = 8.1$ Hz), 6.36 (d, 2H, $J = 14.3$ Hz), 4.33 (t, 4H, $J = 7.5$ Hz), 3.75 (t, 2H, $J = 5.8$ Hz), 2.95 (m, 6H), 2.81 (br t, 4H), 2.21 (m, 4H), 2.04 (br t, 2H), 1.29 (br s, 12H).

![Diagram](image.png)

**Scheme 2.25** Synthesis of 3-[2-[4-(2-fluoro-4-ethylisothiocyanato-phenyl)oxy]-7-[3,3dimethyl-1-[3-(sulfanopropyl)indoline-2-ylidene]-3,5(propane-1,3-diyl)-1,3,5-heptatriene-1-yl]-3,3dimethyl-3H-indolio]propanesulfonic acid (39).

A 60% oil dispersion of NaH (14 mg, 0.4 mmol) was added to anhydrous DMF (2 mL) under a N$_2$ atmosphere at 0 °C. In a separate flask, 30 (127 mg, 0.6 mmol) was dissolved in CH$_2$Cl$_2$ (1 mL). From this solution, 654 μL (0.4

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mmol) was taken out and placed into a separate flask and the CH$_2$Cl$_2$ was taken off by a rotary evaporator. The residue was dissolved in anhydrous DMF (1 mL) under a N$_2$ atmosphere at 0 °C and the solution was added to the slurry of NaH. After 30 min, the fluorophenoxide isothiocyanate solution was added to chlorodye 6 (100 mg, 0.14 mmol) dissolved in anhydrous DMF (3 mL) under N$_2$ atmosphere. After 30 min, the reaction was quenched with dry ice and the solvent was removed on a rotary evaporator at 40 °C. Et$_2$O (3 x 20 mL) was added to precipitate the dye and the supernatant discarded. The crude material was dissolved in CH$_3$OH:H$_2$O (1:1, 2 mL) and purified using 5 C$_{18}$ Sep-Pak$^\text{®}$ Plus cartridges in series with a CH$_3$OH:H$_2$O gradient (H$_2$O, 10 mL; CH$_3$OH:H$_2$O, 6:4, 10 mL; CH$_3$OH:H$_2$O, 8:2, 10 mL; CH$_3$OH, 10 mL). Pure fractions were collected, pooled, and dried in vacuo to afford 39. Yield: 28 mg (24%). FAB-MS calculated for C$_{45}$H$_{51}$FN$_3$O$_7$S$_3$: 860.3 (protonated form, M$^+$), found: 860.4.

$^1$H NMR (CD$_3$OD, 250 MHz) δ 7.96 (d, 2H, $J$ = 14.0 Hz), 7.37 (m, 5H), 7.23 (m, 4H), 7.00 (d, 1H, $J$ = 8.7 Hz), 6.90 (d, 1H, $J$ = 8.1 Hz), 6.36 (d, 2H, $J$ = 14.0 Hz), 4.33 (t, 4H, $J$ = 7.5 Hz), 3.76 (t, 2H, $J$ = 6.5 Hz), 2.94 (m, 6H), 2.80 (br t, 4H), 2.21 (m, 4H), 2.04 (br t, 2H), 1.35 (s, 12H).

2.2.2.3 Results and Discussion

Labeling dyes 32, 33, 35-39 were prepared from the base chlorocyanine dye 6 by nucleophilic substitution (via an $S_{RN1}$ mechanism)$^{21}$ of the chloride by phenolates or thiols. The inclusion of the propyl sulfonate groups on the nitrogens of the indolinen ring of 6 gives all of the dyes excellent water
solubility. The addition of \( p \)-aminothiophenol to chlorodye 6 to generate the aminothioether dye 31 occurred with moderate yields. However, pure 31 was not stable at room temperature over long periods of time, so it was generally reacted with thiophosgene (or preferably thiocarbonyldiimidazole) immediately to provide the isothiocyanate which was purified by reversed-phase chromatography. The preferred one-pot procedure for preparation of isothiocyanate dye 32 (Method B) gave the best overall results in terms of time required and the resulting purity of 32. This dye remains stable in DMSO while in the dark over a period of months, but undergoes a slow hydrolysis of the isothiocyanate in DMF, \( \text{CH}_3\text{OH} \), and aqueous solutions. The hydrolysis in DMF may be caused from the presence of free amines in the solvent. This should not be a problem if the DMF is carefully distilled prior to dissolving the dye. Dye 32 was stored as a solid in the dark at 4 °C.

The addition of phenolate of tyramine to chlorodye 6 was envisioned as a possible strategy to obtain an isothiocyanate dye with a two carbon linker arm. Attempts to use tyramine directly in this reaction led to complicated mixtures. Apparently the free amine causes side reactions or decomposition of the dye (as in the case of free amine thioether dye 31 above). Therefore, protection of the nitrogen is required which would have to be removed and the isothiocyanate formed once the dye is assembled. Tertiary butoxy carbonyls (t-BOC) are extensively used as protecting agents for primary amines which forms a stable amide bond. Afterwards, the free amine is regenerated by cleavage of the BOC group with acid (trifluoroacetic acid). Attempts were made to synthesize the
BOC-tyramine, but this compound was an oil at room temperature. Narayanan has shown that the isothiocyanate group is stable to the moderately basic and nucleophilic reaction conditions required for the replacement of the Cl on dye 6. This strategy is much more convergent and minimizes handling of dye-containing intermediates as it avoids the need for deblocking of the amine and subsequent formation of the isothiocyanate dye. Thus, the phenolate derivative of tyramine isothiocyanate (prepared from 1,1-thiocarbonyldiimazole and tyramine) was directly added to the chlorodye 6 to provide the alkyl isothiocyanate dye 33 in moderate yield. This dye suffers from the same hydrolysis problem as 32, but is stable to hydrolysis in DMSO. This dye is usually stored as a solid in the dark at 4 °C.

The succinimide ester (35) was prepared for purposes of comparing labeling efficiency of different functional groups. Again the chlorodye reacts efficiently with the salt of 3-\((p\)-hydroxyphenyl)propanoic acid to give the carboxylic acid dye 34. The succinimide ester 35 was formed from 34, \(N\)-hydroxysuccinimide, and dicyclohexylcarbodiimide; however, this compound was difficult to isolate because the ester was highly sensitive to hydrolysis under aqueous conditions. Dye 35 was not stable to long term storage in solution and reverted back to the carboxylic dye 34. Therefore, 35 was made \textit{in situ} just prior to any experiments and used immediately.

The syntheses of heavy-atom isothiocyanate functionalized dyes was carried out under the same conditions as 33, but the halogenated phenols had to be synthesized prior to the substitution reactions on chloro dye 6. Iodination of
tyramine to form 23 was performed using a literature procedure by Evangelatos.\textsuperscript{31} This reaction produces the o-substituted product exclusively. Some 2,6 disubstitution product was observed in the GC-MS (\(\sim 1\%\)) and this product could be synthesized as the major product by adding two equivalents of I\(_2\). The functionalization of the amine group to the isothiocyanate using 1,1-thiocarbonyldiimidazole gave 24. 1,1-thiocarbonyldiimidazole is a reagent which can derivatize primary amines to isothiocyanates in high yields.\textsuperscript{35,36} This reagent is a powder and thus less volatile and safer to handle than thiophosgene. This reaction was allowed to proceed overnight, but it was complete within 1 h of the addition of the TCDI.

Bromination of tyramine proved to be more difficult due to the more reactive nature of Br\(_2\) compared to I\(_2\). Several synthetic routes were investigated including the bromination by \(N\)-bromosuccinimide\textsuperscript{37} or by using dioxane dibromide.\textsuperscript{38,39} Kajigaeshi has shown that tetralkylammonium tribromide derivatives have shown selective bromination under mild conditions on various unsubstituted and substituted phenols.\textsuperscript{32,40} The reaction mechanism is not well understood, but they suggest that the brominating agent is a methyl hypobromite intermediate generated \textit{in situ}.\textsuperscript{40} This reagent was selected due to its mild reaction conditions, minimal reaction steps, and availability. The reaction proceeded quickly and is complete within 5 min. The product, 25 is difficult to purify and is used to synthesize 26 with TCDI in low yields (\(\sim 20\%\)).

Chlorination of tyramine is more problematic because the free amine is subject to oxidation under typical conditions for this reaction. In addition, a
reagent had to be chosen which substitutes primarily at the ortho position. An extensive literature search produced the m-chloroperbenzoic acid/DMF/HCl reagent conditions as a potential chlorination procedure. The free amine was protected as the ammonium hydrochloride salt, and the reaction proceeded with fair yields (51%). The chlorinated tyraminium salt was easily purified by extraction of the reaction side-products into CH₂Cl₂ and the recovery of 27 as a white powder. The isothiocyanate, 28 was synthesized easily from 27 and TCDI in low yields (~33%).

The fluorination of tyramine was carried out under the same restrictions as the chlorination reaction. Selectfluor® (1-(Chloromethyl)-4-fluoro-1,4-diaziobiocyclo[2.2.2]octane bis(tetrafluoroborate)) is a reagent which has been shown to fluorinate various phenols under mild conditions, but it is sensitive to aqueous bases or nucleophiles in solution. In order to protect the reagent from the free amine on tyramine, the ammonium hydrochloride salt of tyramine was used. The fluorination of tyramine was carried out over 30 min, which afforded a mixture, including the unreacted starting tyramine, and the mono- and di-fluorinated products. The amount of mono-substitution product 29 was about 40% by GC-MS. This was difficult to purify from the reaction products and was used as a crude mixture for the next reaction. The crude product 29 was reacted with TCDI to synthesize 30 in fair yields (~40%).

Synthesis of heavy-atom modified, isothiocyanate functionalized tricarbocyanine dyes (36-39) was carried out by the procedure outlined by Narayanan. Each of these reactions were complete within 30 min to furnish the
final product, and the dyes were stable in the dark at 4°C. The inclusion of the isothiocyanate makes these dyes reactive towards primary amines and presents a series of tagging agents with similar structure but distinct modifications.

2.2.2.4 Summary

Various functionalized tricarbocyanine dyes have been synthesized including dyes which contain a heavy-atom modification that is spatially removed from the chromophore. In addition, halogenation of tyramine was achieved using a variety of different halogenation procedures to furnish starting materials for the heavy-atom modified, isothiocyanate functionalized chromophores. These dyes can be used as potential tagging agents or probes for a range of bioanalytical applications such as amino acid and peptide analysis, protein analysis, and DNA sequencing.

2.3 References


Chapter 3

Photophysics of Tricarbocyanine Dyes

3.1 Basal Tricarbocyanine Dyes

3.1.1 Introduction

The photophysical properties of polymethine dyes have been investigated by a number of researchers who have employed a variety of methods of analyses. Absorbance, fluorescence, time-correlated fluorescence spectroscopy, and triplet absorption spectroscopy have been used to study the electronic properties and to determine the various singlet and triplet state photophysical constants of polymethines.

Absorbance spectrophotometry is an experimental technique which measures the amount of light a molecule in solution absorbs at a particular wavelength. The Beer-Lambert law states that the absorbance of a molecule is directly proportional to the concentration \( c \) of the absorbing species in solution and pathlength \( b \) the light travels through the solution and is given by:

\[
A = \varepsilon b c
\]

(3.1)

where \( A \) is the absorbance of the solution and \( \varepsilon \) is the molar absorptivity. The molar absorptivity, \( \varepsilon \) expressed as \( \text{M}^{-1}\text{cm}^{-1} \), is a constant for a particular solvent system and is related to the amount of light that is absorbed by a molecule at a particular wavelength. Polymethine dyes have characteristic absorption maxima in the visible to near-Infrared region of the spectrum (500 nm - 900 nm) and
possess a large $\varepsilon$ ($\sim 200,000$ M$^{-1}$cm$^{-1}$) in many organic solvents, such as CH$_3$OH.$^{1,5,6,9}$ In aqueous solvent systems, polymethine dyes form aggregates in solution which results in a hypsochromic shift of the absorption wavelength and a decrease in $\varepsilon$. $^{5,10}$ However, polymethine dyes which contain polar substituents, such as sulfonates or carboxylic acids, show decreased levels of aggregation.$^{5,11,12}$

Fluorescence is the emission of light as the molecule relaxes from $S_1$ to $S_0$ and occurs approximately $10^{-9} - 10^{-8}$ s after excitation.$^{13}$ The fluorescence quantum yield ($\Phi_f$) is the ratio of the amount of molecules which fluoresce to the total number of excited molecules and is a constant for a given solvent system and temperature. The $\Phi_f$ can be defined in terms of the various rate constants which depopulate the excited state by,$^{13}$

$$\Phi_f = \frac{k_r}{k_r + k_{nr}}$$

(3.2)

where $k_r$ is the radiative rate constant and $k_{nr}$ is the non-radiative rate constant. In this case, $k_r$ is the emissive rate constant for the chromophore, and $k_{nr}$ is composed of various non-radiative rates constants which depopulate $S_1$ and is given by the expression,$^{5,13}$

$$k_{nr} = k_{ic} + k_{ic} + k_{sd} + k_p$$

(3.3)

where $k_{ic}$ is the internal conversion rate constant, $k_{ic}$ is the intersystem crossing rate constant, $k_{sd}$ is the solvent dependent rate constant and $k_p$ is the photoisomerization rate constant. The major deactivation pathway in many polymethine dyes is internal conversion, but some exhibit extensive
photoisomerization as well. 8,14-17 Also, Soper et al. have shown that polymethines display a solvent-dependent non-radiative deactivation of \( S_1 \) which can be correlated to the \( E_{30} \) value of the solvent system or H-bond donating ability of the solvent. Their results show that the solvent dependent non-radiative rate increases with an increase in H-bond donating ability of the solvent. 5 The fluorescence quantum yield of the tricarbocyanines can range from 0.3 to as small as 0.01, depending upon the solvent. 1

The fluorescence lifetime \( (\tau_f) \) of a molecule is a measure of the average time a molecule resides in the excited state \( (S_1) \) and is defined as, 13

\[
\tau_f = \frac{1}{k_r + k_{nr}} \tag{3.4}
\]

Fluorescence lifetimes range from \( 10^{-9} \) - \( 10^{-8} \) s in solution at room temperature and can be determined experimentally using a variety of techniques.

The determination of fluorescence lifetimes can be accomplished two ways; (1) the time-resolved method or; (2) frequency domain method. In the time-resolved method, the sample is excited with a brief pulse of light. The time-dependent decay of the fluorescence intensity is measured and the lifetime is determined from a least square fit of the decay profile. In the frequency domain method the sample is excited with intensity-modulated light. The phase shift and demodulation of the emission, relative to the exciting light, are used to determine the decay parameters. The time resolved method is advantageous because of its excellent timing resolution (< 0.2 ns) and its sensitivity, especially at low light levels. 13

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3.1.1.1 Time-Correlated Single-Photon Counting.

Time-correlated single-photon counting (TCSPC) is a time-resolved technique which uses a pulsed light source for excitation and monitors the time of evolution of the emission of a single fluorescent photon. After multiple excitation events, a decay profile is constructed from which the decay constants can be abstracted. Figure 3.1 diagrams the experiment as it is conducted. A trigger pulse (generated from the excitation source) which is coincident with the excitation pulse is sent to the input of a time-to-amplitude converter (TAC). This input pulse to the TAC starts the charging of a capacitor. At the same time, the sample is excited and fluoresces. The collection optics are set up to detect only one photon per excitation event. Once this photon is detected, the TAC stops charging the capacitor and an output pulse is sent from the TAC, with the amplitude of this pulse proportional to the charge on the capacitor and the time difference between the start and stop pulse. The TAC output pulse is given a numerical value after passing through an analog to digital (A/D) converter. This number is then stored at some address in a storage device. This process is repeated until a decay profile is constructed. The fluorescence lifetime is determined from the slope of a least squares fit to the decay profile.

This study will investigate the spectroscopic properties and the various photophysical constants of the basal tricarbocyanine dyes 3 and 6 using absorbance, fluorescence and time-correlated spectroscopic techniques.
Figure 3.1 Block diagram of time-correlated single-photon counting instrument. L: excitation source, T: trigger, S: sample holder, F₁F₂: filters or monochromator, DET.: detector, D₁D₂: delay lines, LED: leading edge timing discriminator, CF/D: constant fraction discriminator, TAC: time-to-amplitude converter, ADC: analog-to-digital converter, PHA: pulse height analyzer.
3.1.2 Experimental

The absorbance spectra were acquired on a Perkin-Elmer Lambda 3 spectrophotometer (Perkin-Elmer, Norwalk, CT) at dye concentrations of $5 \times 10^{-6}$ M in CH$_3$OH. The uncorrected fluorescence spectra were collected on a Spex 3000 fluorimeter (Spex, Edison, NJ) using dye concentrations of $1 \times 10^{-6}$ M in CH$_3$OH. The fluorimeter used grating monochromators for the emission and excitation wavelength discrimination. In order to increase the sensitivity of the instrument to the near-IR region, the emission monochromators were blazed for 750 nm. In addition, the photomultiplier had a high quantum efficiency (~ 10% ) and a flat response in the near-IR (Hamamatsu, R636, Hamamatsu Corp., Bridgewater, NJ). The fluorescence quantum yields were calculated relative to IR-125 in DMSO ($\Phi_r = 0.13$) following the procedure outlined by Demas and Crosby and using the formula;\textsuperscript{1,18}

\[
\Phi_f = \Phi_{f, st} \left( \frac{F}{F_{st}} \right) \left( \frac{\varepsilon}{\varepsilon_{st}} \right) \left( \frac{E_r}{E_{st}} \right) \left( \frac{n_r^2}{n_{st}^2} \right)
\]

(3.5)

where $F$ is the integrated area under the fluorescence emission profile, $\varepsilon$ is the molar absorptivity of the dye at the excitation wavelength, $E$ is the intensity of the excitation light, $n$ is the refractive index of the solvent, and the subscript $st$ refers to the parameters associated with the secondary standard.

Time-resolved fluorescence measurements were performed using a near-IR time-correlated single-photon counting instrument built in-house (Figure 3.2). The instrument consisted of a self mode-locked Ti:Sapphire laser pumped by the output of a multiline continuous wave Ar ion laser (Coherent Lasers, Palo Alto,
The Ti:Sapphire laser produced laser pulses at a repetition rate of 76 MHz and could be continuously tuned from approximately 760 to 870 nm. The laser beam was focused onto a square bore sample cell with a laser diode singlet lens (Melles Griot, Irvine, CA). The fluorescence was collected at right angles using a Nikon (Natick, MA) 40× epifluorescence microscope objective with a numerical aperture of 0.85. The fluorescence was then imaged onto a slit serving as a spatial filter to reduce the amount of scattered photons generated at the air/glass interface of the sample cell from reaching the detector. The fluorescence was further isolated from the scattering photons by an eight-cavity Fabry-Perot interference filter (Omega Optical, Brattleboro, VT) with a center wavelength of 850 nm (fwhm = 30 nm).

The fluorescence was finally focused onto the photoactive area of the detector with a 6.3× microscope objective. The photodetector was a single photon avalanche diode (EG&G Optoelectronics Canada, Vaudreuil, Canada) with a 150-μm-diameter photoactive area, which was mounted on a thermoelectric cooler and possessed dark count rates of approximately 80 counts/s. The pulses from the SPAD were amplified by a 2 GHz amplifier (Phillips Scientific, Mahwah, NJ) and conditioned with a constant fraction discriminator (CFD, Tennelec TC754, Oak Ridge, TN). The CFD pulses were directed into the gate and stop inputs of the time-to-amplitude converter (TAC, Tennelec TC863). The start input for the TAC was supplied by an intracavity diode monitoring the laser pulses and conditioned using the CFD. The fluorescence decay profiles were acquired in 4096 channels with a time
resolution of 2.88 ps/channel and constructed by digitizing the output of the TAC using PCA-II hardware and software (Tennelec Nucleus, Oak Ridge, TN).

The dye concentration used for lifetime determinations was $1 \times 10^{-8}$ M in CH$_3$OH. The fluorescence lifetime was determined by constructing a decay profile from known kinetic parameters, convolving with an instrumental prompt function, and using a reiterative, non-linear least squares fit of the experimental data to the calculated function. The quality of the fit was judged by inspection of the weighted residuals or the value of $\chi^2$, which is calculated from:

$$\chi^2 = \sum_{i=1}^{n} \omega_i \left[ R(t) - R_c(t) \right]^2$$  \hspace{1cm} (3.6)

where

$$\omega_i = \frac{1}{R(t)}$$  \hspace{1cm} (3.7)

In these expressions, $R(t)$ is the observed decay of fluorescence, $R_c(t)$ is the calculated value for the decay of fluorescence, and $\omega_i$ is a statistical weighting factor which accounts for the error in the value of $R(t)$. A value of $\chi^2 \sim 1.0 - 1.5$ indicates that the chosen decay parameters reasonably describe the experimental data. The random distribution of the residuals ($[R(t) - R_c(t)]$) around 0 indicates that fluorescence decay is adequately described by the assumed decay law.

Decay profiles were accumulated until approximately 10,000 photocounts were present in the channel with the maximum number of counts. The radiative ($k_r$) and non-radiative ($k_{nr}$) rates were calculated from;
Figure 3.2 Near-Infrared time-correlated single-photon counting instrument. Components: L, focusing lens; C, sample cell; BL, beam block; SF, spatial filter; BF, bandpass filter; SPAD, single photon avalanche diode detector; Amplifier, signal amplifier; Delay, time delay electronics; Disc., discriminator; PD, photodiode detector; TAC, time-to-amplitude converter; Start, start pulse; Stop, stop pulse; Gate, gate pulse.
3.1.3 Results and Discussion

The dyes being investigated are shown in Figure 3.3. Dye 3 is a cationic tricarbocyanine dye and 6 has two sulfonate groups giving this dye a net negative charge. As seen from Table 3.1, both dyes possess absorbance and fluorescence maxima in the near-IR region of the spectrum in CH₃OH solutions. In aqueous conditions, dye 3 shows a decrease in the absorbance as shown in Figure 3.4 A.

\[
k_r = \frac{\Phi_r}{\tau_r} \quad (3.8)
\]

\[
k_{nr} = \frac{(1 - \Phi_r)}{\tau_r} \quad (3.9)
\]

![Figure 3.3 Basal tricarbocyanine dyes](image.png)

<table>
<thead>
<tr>
<th>Dye</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>CH₂CH₃</td>
</tr>
<tr>
<td>6</td>
<td>(CH₂)₃SO₃⁻</td>
</tr>
</tbody>
</table>

Figure 3.3 Basal tricarbocyanine dyes
Figure 3.4 Absorbance spectra of dyes 3 (A) and 6 (B) in CH$_3$OH and H$_2$O. Dye concentration = $5 \times 10^{-6}$ M.
Table 3.1 Absorbance properties of basal dyes in CH\textsubscript{3}OH.

<table>
<thead>
<tr>
<th>Dye</th>
<th>λ\textsubscript{max} (nm) (in CH\textsubscript{3}OH)</th>
<th>ε (cm\textsuperscript{-1}M\textsuperscript{-1})</th>
<th>λ\textsubscript{max} (nm) (in H\textsubscript{2}O)</th>
<th>ε (cm\textsuperscript{-1}M\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>773</td>
<td>195,000</td>
<td>766</td>
<td>69,000</td>
</tr>
<tr>
<td>6</td>
<td>778</td>
<td>181,000</td>
<td>772</td>
<td>143,000</td>
</tr>
</tbody>
</table>

Previous research on polymethine dyes have shown that the charge on the nitrogen heteroatom is delocalized throughout the polymethine chain, but is more localized in the excited state, which makes it more susceptible to nucleophilic solvation.\textsuperscript{5,19} Since CH\textsubscript{3}OH is a better nucleophile than H\textsubscript{2}O, solutions of the dyes in H\textsubscript{2}O should result in a blue shift of the absorption maxima due to destabilization of the excited state, consistent with the data in Table 3.1.\textsuperscript{5} In addition, many polymethine dyes form aggregates in aqueous solutions. Aggregation is a reversible process which depends upon the dye concentration and the temperature. It can be influenced by salt effects and the solvent’s polarity as well. When these aggregates are formed, they are bonded through non-valence bonds.\textsuperscript{6} There are different types of aggregates and they are characterized as; dimers which usually absorb at shorter wavelengths than the monomer; “J-aggregates” which have red-shifted absorption maxima; “H-aggregates” which have blue shifted absorption bands.\textsuperscript{6} Based upon this definition of molecular aggregation, the observed absorption maxima change can be indicative of molecular aggregation or possibly dimer formation in solution. However, the addition of SO\textsubscript{3}\textsuperscript{-} groups onto the dye to form basal dye 6, increases the solubility in aqueous solvents reducing the amount of molecular aggregation when compared to 3 as seen in Figure 3.4.
The fluorescence spectra for the basal dyes in CH₃OH and H₂O are shown in Figure 3.5. Both dyes showed fluorescence maxima in the near-IR region. Dye 3 also showed a dramatic decrease in the fluorescence intensity in aqueous solvents. H₂O is a greater H-bonding donating solvent than CH₃OH and therefore kₘ increases which lowers Φₙ for some polymethine dyes in aqueous solvents. In addition, the decrease in ε due to molecular aggregation allows for a decreased intensity compared to the dye in CH₃OH. The decrease in the fluorescence intensity is not as dramatic for 6 presumably due to the increased solubility provided by the SO₃⁻ groups. The fluorescence quantum yield for 6 in CH₃OH is shown in Table 3.2.

**Table 3.2 Fluorescence data for basal tricarbocyanine dyes in CH₃OH.**

<table>
<thead>
<tr>
<th>Dye</th>
<th>3</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>λₘₐₓ (nm)</td>
<td>799</td>
<td>804</td>
</tr>
<tr>
<td>Φₙ</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>τᵣ(ns)</td>
<td>0.480</td>
<td>0.529</td>
</tr>
<tr>
<td>κ²</td>
<td>1.1</td>
<td>1.4</td>
</tr>
</tbody>
</table>

The average fluorescence lifetimes (τᵣ) of basal dyes 3 and 6 in CH₃OH determined by TCSPC are given in Table 3.2. These dyes possess τᵣ which are fairly short (< 1 ns) when compared to conventional visible dyes (e.g. Rhodamine 6G ~ 3.2 ns). The short lifetimes are probably a result of a large kₑₑ that is associated with polymethine dye.⁶,¹⁶,²¹
Figure 3.5 Normalized fluorescence spectra of dyes 3 (A) and 6 (B) in CH$_3$OH and H$_2$O. Dye concentration = 5x10$^{-6}$ M. Excitation and emission slit widths: 2 mm. Excitation wavelength = 710 nm.

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3.1.4 Summary

The photophysics of the basal tricarbocyanine dyes 3 and 6 have been examined using absorbance, fluorescence, and time-correlated single-photon counting. These dyes have characteristic absorption and fluorescence maxima in the near-IR with large ε and small Φ_f and τ_f in CH_3OH. The aggregation of 3 in H_2O prohibits the use of this dye as a building block for a bioanalytical probe, but 6, which contains sulfonate groups, did not exhibit aggregation making this dye an attractive starting point for the synthesis of a probe which can be used for tagging biomolecules. The increased solubility of 6 vs. 3 in aqueous solvent systems makes this basal chromophore a more attractive dye for bioanalytical applications which many times require that tagging reagent be highly soluble in H_2O.

3.2 Photophysical Characterization of Heavy-Atom Modified Tricarbocyanine Dyes

3.2.1 Introduction

The heavy-atom effect describes the influence of a heavy-atom (e.g. I, Br) on the intersystem crossing rate of a chromophore. As stated in section 3.1.1, intersystem crossing from S_1 to T_1 is a quantum mechanically forbidden transition because it violates the selection rule which states that any transition must take place between states with the same spin quantum number, S, so that ΔS = 0.\(^{22,23}\) However, intersystem crossing can still occur through the process of spin-orbit
coupling. Spin orbit coupling produces an admixture of the triplet and singlet-state to produce a wavefunction which has both singlet and triplet characteristics. An expression which approximates the wavefunction for spin-orbit coupling ($\Psi_{so}$) is given by,

$$ \Psi_{so} \propto \frac{\rho \int \Psi_s^* (\mathbf{L} \cdot \mathbf{S}) \Psi_T^*}{(E_T - E_s)} \Psi_s^* $$

(3.10)

where $\Psi_s^*$ and $\Psi_T^*$ are the singlet and triplet wavefunctions, respectively, $(E_T - E_s)$ is the energy difference between $S_1$ and $T_1$, $\mathbf{S}$ is the spin angular momentum operator, $\mathbf{L}$ is the orbital angular momentum operator, and $\rho$ is a term which is related to the potential field of the nucleus. The degree of mixing of $S_1$ and $T_1$ is dependent upon $\rho$ and $| (E_T - E_s) |$. Spin orbit coupling is more pronounced when the electron is in an orbit which has a high probability of being close to the nucleus, especially for high atomic numbers which gives rise to the heavy-atom effect. This is due to the dependency of $\Psi_{so}$ on $\rho$. As atoms become heavier, $\rho$ becomes larger which increases the amount of spin-orbit coupling. The addition of heavy-atom substituents directly onto the conjugated systems of chromophores to increase $k_{ac}$ is termed the internal heavy-atom effect. The influence of heavy-atoms that are not directly attached to the conjugated system of a molecule or are in the immediate vicinity of the chromophore (e.g. dissolved in solvents which contain heavy-atoms) is termed the external heavy-atom effect.
Researchers have studied the triplet state to determine the intersystem crossing rate and internal conversion rate of polymethine dyes. Upon the absorption of light, the triplet-state quantum yield ($\Phi_T$) of the lowest triplet state is given by:

$$\Phi_T = \frac{k_{inc}}{k_{ac} + k_r} \quad (3.11)$$

and from equations 3.4 and 3.11:

$$\Phi_T = k_{inc} \tau_f \quad (3.12)$$

The technique most commonly used to probe the photophysics of the triplet-state is termed flash photolysis. Briefly, a sample is excited by a short burst of high intensity light to populate $S_1$ (usually by a laser), and after a short period of time ($\sim 10 \text{ ns}$), the sample is excited again by another pulse of tunable light which is tuned to an electronic transition in the triplet state. The absorption of this transient is analyzed (usually by a monochromator with a photomultiplier tube) to yield information about the triplet-state population of the sample.  

This section will examine the photophysics of a series of tricarbocyanine dyes whose structures are shown in Figure 3.6 which possess an intramolecular heavy-atom modification that is spatially removed from the conjugated system of the chromophore. The photophysical constants of the singlet and triplet state will be investigated including, $k_{inc}$ and $k_{in}$, to characterize the influence of the heavy-atom modification.
Figure 3.6 Heavy-atom modified tricarbocyanine dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>R</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>CH₂CH₃</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>8</td>
<td>(CH₂)₃SO₃⁻</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>9</td>
<td>CH₂CH₃</td>
<td>I</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>10</td>
<td>(CH₂)₃SO₃⁻</td>
<td>I</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>11</td>
<td>CH₂CH₃</td>
<td>Br</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>12</td>
<td>(CH₂)₃SO₃⁻</td>
<td>Br</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>13</td>
<td>CH₂CH₃</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
</tr>
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<td>(CH₂)₃SO₃⁻</td>
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<td>H</td>
<td>H</td>
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<td>H</td>
<td>H</td>
</tr>
<tr>
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<td>(CH₂)₃SO₃⁻</td>
<td>F</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>17</td>
<td>(CH₂)₃SO₃⁻</td>
<td>CH₃</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>18</td>
<td>(CH₂)₃SO₃⁻</td>
<td>Br</td>
<td>Br</td>
<td>H</td>
</tr>
<tr>
<td>19</td>
<td>(CH₂)₃SO₃⁻</td>
<td>Cl</td>
<td>Cl</td>
<td>H</td>
</tr>
<tr>
<td>20</td>
<td>(CH₂)₃SO₃⁻</td>
<td>H</td>
<td>H</td>
<td>I</td>
</tr>
<tr>
<td>21</td>
<td>(CH₂)₃SO₃⁻</td>
<td>H</td>
<td>H</td>
<td>Br</td>
</tr>
</tbody>
</table>
3.2.2 Experimental

The absorbance spectra were acquired on a Perkin-Elmer Lambda 3 spectrophotometer (Perkin-Elmer, Norwalk, CT) at dye concentrations of $5 \times 10^{-6}$ M in CH$_3$OH. The uncorrected fluorescence spectra were collected on the red sensitive a Spex 3000 fluorimeter (Spex, Edison, NJ) using dye concentrations of $1 \times 10^{-6}$ M in CH$_3$OH. The fluorescence quantum yields were calculated relative to IR-125 in DMSO ($\Phi_f = 0.13$) following the procedure outlined by Demas and Crosby.$^{1,18}$

Time-resolved fluorescence measurements were performed using a near-IR time-correlated single-photon counting instrument built in-house (see Figure 3.2). The flash photolysis instrument, shown in Figure 3.7, consisted of the 532 nm (second harmonic) output of a Nd:YAG laser (GCR-3, Spectra Physics, Mountain View, CA) pumping a PDL-3 pulsed dye-laser (Spectra Physics). The pulsed-dye laser used LDS-751 (Exciton, OH) as the lasing medium and was tuned to 760 nm for all triplet measurements. The output of the dye laser (135 mW, pump beam) was focused to a 0.2 cm ($1/e^2$) circular spot inside the sample cuvette. A 75 W Xe arc lamp (Oriel, Stratford, CT) was used as the probe beam. The probe beam was sent through a long-pass filter (RG-715, Edmund Scientific, Barrington, NJ) and analyzed using a single grating monochromator (Spex 220M, Spex, Edison, NJ) with a red-sensitive photomultiplier tube (Hamamatsu, R636-10, Bridgewater, NJ). The output from the PMT was sent to a digital oscilloscope (Tektronix TDS 520, Beaverton, OR), with the data transferred to a PC utilizing LabView (National Instruments, Austin, TX).
Figure 3.7 Laser flash photolysis instrument. S: shutter; C: sample cell; L₁: focusing lens; L₂: collection lens; F: longpass filter; PD: photodiode; PMT: photomultiplier tube; BL: beam block; CL: cylindrical lens.
Triplet lifetimes were determined using dye concentrations of $1 \times 10^{-5}$ M in 1 M methyl iodide dissolved in CH$_3$OH which had been purged of oxygen by bubbling dry Ar for 15 min into the sample cuvette. Methyl iodide was added to the dye solution in order to enhance the intersystem crossing rate. Triplet decay profiles were collected with a pump-probe technique using 760 nm excitation and 880 nm detection with a 4 ns delay after the excitation pulse.

Quantitative determination of $k_{\text{isc}}$ was calculated from an analysis of the triplet-triplet absorption and the time-resolved data using the triplet molar extinction coefficient ($\varepsilon_T$), the triplet quantum yield ($\Phi_T$), and $\tau_6$ using the expression:

$$k_{\text{isc}} = \frac{\Phi_T}{\tau_6}$$

(3.13)

The triplet quantum yield was calculated from:

$$\Phi_T = \frac{(\varepsilon_S/\varepsilon_T)(\text{OD}_T/\text{OD}_S)}{3.14}$$

where $\text{OD}_S$ and $\text{OD}_T$ are the optical densities of the singlet and triplet states at 880 nm, respectively, and $\varepsilon_S$ is the molar absorptivity of the singlet state at 880 nm. In order to calculate $\Phi_T$, $\varepsilon_T$ must be determined. There are various techniques to experimentally determine $\varepsilon_T$ and they have been discussed by Carmichael and Hug, and Bensasson and Land. The singlet depletion method of calculating $\varepsilon_T$ outlined by Carmichael and Hug assumes that the ground state absorption does not overlap the triplet state absorption spectra. However, attempts to measure $\varepsilon_T$ using this method provided impractical values for $\varepsilon_T$, possibly be due to singlet and triplet absorption overlap.
Michel and Mialocq have measured the $\varepsilon_T$ of some polymethines using the energy transfer method with pinacyanol chloride.$^{21,24}$ This method is based upon the principle that upon excitation from a pulsed light source, a donor molecule with a known $\varepsilon_T$ is excited and some of this population crosses into the triplet state. The acceptor molecule is excited into the triplet state via energy transfer from the donor molecule. Two major assumptions must be made for this action to be valid; all acceptor triplet states must be formed from energy transfer from the triplet donors and; one triplet acceptor molecule is formed after collision with one donor molecule. If these conditions are satisfied, then $\varepsilon_T$ is given by,$^{33}$

$$
\varepsilon_{TA} = \frac{(\Delta \text{OD}_A)(\varepsilon_{Td})}{\Delta \text{OD}_d}
$$

(3.15)

where $\varepsilon_{TA}$ is the triplet extinction coefficient of the acceptor molecule, $\Delta \text{OD}_A$ is the optical density of the acceptor molecule at the triplet absorption wavelength, $\varepsilon_{Td}$ is the triplet extinction coefficient of the donor molecule at its emission wavelength (for pinacyanol chloride $\varepsilon_{Td} = 53,000 \text{ M}^{-1}\text{cm}^{-1}$ at 635 nm), and $\Delta \text{OD}_d$ is the optical density of the donor molecule at its triplet absorption wavelength.

Using the triplet instrument shown in Figure 3.7, the frequency doubled output of the Nd:YAG laser excited the dye laser with a 10 Hz repetition rate with Rhodamine 6G as the lasing medium. The dye laser was tuned to an excitation wavelength of 560 nm with an output power of 240 mW. The output of the dye laser was passed though the sample cell. Part of the dye laser beam was sent to a photodiode detector which served as the trigger pulse for the oscilloscope. At right angles to the incident laser beam, the optical density of the
sample solution was collected using a Xe arc lamp as the excitation source. The absorbed light was collected by a single grating monochromator (Spex 220) and detected by a photomultiplier tube (PMT, Hamamatsu, R636, -1000 V). The inlet slit was set at 1.5 mm and the output slit was set at 0.8 mm. The output of the PMT was sent to the input channel of the digital oscilloscope and collected 4 ns after the laser pulse. The optical density of the solution was measured before and after the laser pulse. The sample comprised of pinacyanol chloride \((5\times10^{-7} \text{ M})\) and \(8\) \((5\times10^{-5} \text{ M})\) in methanol was purged with Ar for 10 min.\(^{35,34}\) The measured \(\varepsilon_T\) for dye 8 using this method was found to be \(6.0\times10^5 \text{ M}^{-1} \text{ cm}^{-1}\).

### 3.2.3 Results and Discussion

#### 3.2.3.1 Singlet State Photophysics.

The dyes being investigated are shown in Figure 3.6. Table 3.3 lists the absorbance and emission maxima as well as the molar absorptivities and fluorescence quantum yields (\(\Phi_f\)) for the heavy-atom modified dyes. As can been seen from the data in this Table, the absorbance and emission spectra for the heavy atom modified dye series showed that introduction of the heavy atom(s) onto the phenol substituent of the chromophore produced only minor changes in the absorption and emission maxima of the cationic and sulfonate dyes. The addition of the intramolecular heavy atom(s) produced only minor differences in the extinction coefficients (\(\varepsilon_S\)) and had no appreciable effect on \(\Phi_f\). The absorbance spectra of the dyes are shown in Figures 3.8 and 3.9 for the cationic and anionic dyes, respectively. Both the cationic and the anionic heavy-atom
modified dyes exhibit absorbance maxima in the near-IR with very large $\varepsilon_a (~180,000 \text{ M}^{-1}\text{cm}^{-1})$ in methanol.

The lack of notable differences in the electronic structure indicates that the heavy-atom substituent does not significantly perturb the energy difference between $S_0$ and $S_1$. There are slight differences in the absorbance maxima between the cationic dyes and the anionic dyes. The cationic dyes possess absorbance maxima which are blue shifted ($\sim 4\text{nm}$) when compared to the anionic dyes. This is indicative of polymethine dyes which are more susceptible to nucleophilic solvation in the excited state. The addition of the $\text{SO}_3^-$ groups on the anionic dye increased the solvation in polar solvents which lead to the bathochromic shift.\textsuperscript{19}

Table 3.3 Absorbance and fluorescence data of heavy-atom modified near-IR dyes in CH$_3$OH.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Absorbance Maximum (nm)</th>
<th>$\varepsilon$ (cm$^{-1}$ M$^{-1}$)</th>
<th>Fluorescence Maximum (nm)</th>
<th>$\Phi_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>760</td>
<td>210,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>765</td>
<td>200,000</td>
<td>790</td>
<td>0.13</td>
</tr>
<tr>
<td>9</td>
<td>764</td>
<td>182,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>768</td>
<td>181,000</td>
<td>792</td>
<td>0.13</td>
</tr>
<tr>
<td>11</td>
<td>764</td>
<td>180,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>768</td>
<td>189,000</td>
<td>793</td>
<td>0.13</td>
</tr>
<tr>
<td>13</td>
<td>764</td>
<td>175,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>768</td>
<td>179,000</td>
<td>793</td>
<td>0.11</td>
</tr>
<tr>
<td>15</td>
<td>764</td>
<td>181,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>768</td>
<td>189,000</td>
<td>793</td>
<td>0.12</td>
</tr>
<tr>
<td>18</td>
<td>764</td>
<td>146,000</td>
<td>791</td>
<td>0.11</td>
</tr>
<tr>
<td>19</td>
<td>764</td>
<td>192,000</td>
<td>791</td>
<td>0.09</td>
</tr>
<tr>
<td>20</td>
<td>764</td>
<td>184,000</td>
<td>791</td>
<td>0.14</td>
</tr>
<tr>
<td>21</td>
<td>764</td>
<td>182,000</td>
<td>791</td>
<td>0.14</td>
</tr>
</tbody>
</table>

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Figure 3.8 Absorbance spectra of cationic heavy-atom substituted dyes 7, 9, 11, 13, 15 in CH₃OH. Dye concentration: 3×10⁻⁶ M.
Figure 3.9 Absorbance spectra of anionic heavy-atom substituted dyes 8, 10, 12, 14, 16, 18-21 in CH$_3$OH. Dye concentration: 5×10$^{-6}$ M.
The fluorescence spectra for the anionic heavy-atom modified dyes are shown in Figure 3.10. All of these dyes exhibit a small Stokes shift (≈20 nm) and show a fluorescence maxima of ≈ 790 nm. An interesting feature of the fluorescence spectra is the similarities of the intensities of the monosubstituted chromophores. The dyes all display similar wavelength maxima and intensity with only minor differences. The addition of the two heavy-atoms (18,19) provided a slight decrease in the fluorescence intensity.

The fluorescence lifetimes for the cationic dyes are listed in Table 3.4 and for the anionic dyes along with the radiative ($k_r$) and non-radiative ($k_{nr}$) rates in Table 3.5. The fluorescence decay profile for 10 is shown in Figure 3.11. Relative to dye 8, the insertion of the heavy atom directly onto the chromophore (6) perturbed the photophysics of the singlet state to a much larger degree than those where the heavy atom was spatially removed from the chromophore (10, 12, 14, 16). In the latter cases, spin orbit coupling would be expected to occur predominantly through-space and not through-bond (as in 6), and thus exert a smaller influence on the singlet state photophysics, consistent with our data. Interestingly, we observed an apparent inverse intramolecular heavy-atom effect in the mono-substituted series, with $\tau_f$ increasing with increasing molecular weight of the heavy-atom, contrary to what is typically observed. Typically, the heavier atom will induce greater spin-orbit coupling which will give rise to an increase in $k_{nc}$ and a decrease in $\tau_f$. The decrease in $\tau_f$ is not what is observed for the monosubstituted series. In the case where a second heavy atom
Figure 3.10 Fluorescence spectra of anionic heavy-atom modified dyes in CH₃OH. Dye concentration: 5×10⁻⁶ M. Excitation and emission slit widths: 2 mm.
was added, the expected decrease in $\tau_f$ was seen (e.g. 18, 19 vs 8; 18 vs 12; 19 vs 14).

Table 3.4 Average fluorescence lifetimes of cationic dyes.

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\tau_f$ in CH$_3$OH (ns)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.835</td>
<td>1.3</td>
</tr>
<tr>
<td>9</td>
<td>0.852</td>
<td>1.2</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>0.798</td>
<td>1.4</td>
</tr>
<tr>
<td>15</td>
<td>0.769</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 3.5 Fluorescence lifetimes, $k_w$ and $k_r$ of anionic heavy-atom modified dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\tau_f$ in CH$_3$OH (ns)</th>
<th>$\chi^2$</th>
<th>$k_r$ (ns$^{-1}$)</th>
<th>$k_w$ (ns$^{-1}$)</th>
<th>$\tau_f$ in unpolymerized gel solution (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.906</td>
<td>1.1</td>
<td>0.12</td>
<td>0.98</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.906</td>
<td>1.3</td>
<td>0.14</td>
<td>0.96</td>
<td>0.883</td>
</tr>
<tr>
<td>12</td>
<td>0.882</td>
<td>1.2</td>
<td>0.15</td>
<td>0.99</td>
<td>0.840</td>
</tr>
<tr>
<td>14</td>
<td>0.858</td>
<td>1.1</td>
<td>0.13</td>
<td>1.04</td>
<td>0.728</td>
</tr>
<tr>
<td>16</td>
<td>0.831</td>
<td>1.2</td>
<td>0.14</td>
<td>1.06</td>
<td>0.688</td>
</tr>
<tr>
<td>18</td>
<td>0.702</td>
<td>1.2</td>
<td>0.16</td>
<td>1.27</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>0.708</td>
<td>1.3</td>
<td>0.13</td>
<td>1.29</td>
<td>-</td>
</tr>
</tbody>
</table>

The fluorescence lifetimes of the mono-substituted dyes were also measured in unpolymerized acrylamide gel solutions with 1× TBE and 30 % (v/v) formamide, a typical matrix used for DNA sequencing and these values are shown in Table 3.5. The lifetime values were smaller compared to CH$_3$OH, but the trends were similar to that seen in CH$_3$OH with the heavier atom possessing the longest lifetime. As can be seen from this data, the lifetime spread for the dye series was greater in the acrylamide solution compared to that observed in CH$_3$OH. The differences in lifetimes between the two solvent matrices are
consistent with previous work, which has shown that the tricarbocyanines display solvent dependent photophysics.\textsuperscript{5,41}

A critical question is whether the observed lifetime variation is sufficient to adequately differentiate between these dyes. The lifetimes for these dyes ranged from 688 ps (16) to 883 ps (10) when measured in an unpolymerized gel solution, with an average variation of 65 ps. Using maximum likelihood estimators, the standard deviation ($\sigma$) in the measurement can be determined from:\textsuperscript{42}

$$\sigma = \tau_f\frac{1 - e^{-T/\tau_f}}{\left[\left(1 - e^{-T/\tau_f}\right)^2 - \left(T / \tau_f\right)^2 e^{-T/\tau_f}\right]^{1/2}}$$

(3.15)

where $N$ is the total number of photocounts comprising the decay profile and $T$ is the time width of each bin (2.88 ps). If each decay profile consists of 10,000 counts and the lifetime of the base chromophore is 1.00 ns, then a lifetime difference of approximately 30 ps is required for discrimination at $3\sigma$. If the number of counts in each decay profile is doubled, the variation needed is only 21 ps ($3\sigma$). Therefore, the average variation observed for the present dye set is well within the criteria needed for facile discrimination. However, it should be noted that interferences, such as scattering and/or fluorescent impurities, can degrade the relative precision in the measurement. Recent results have indicated that with the use of near-IR fluorescence monitoring, these effects are minimal and the precision in the measurement is determined primarily by the number of counts in the decay profile.\textsuperscript{42}
Figure 3.11 Fluorescence decay profile for 10 in CH$_3$OH, fitted function and CH$_3$OH blank. Dye concentration ~ $1\times10^{-9}$ M. The weighted residuals are shown in the inset.
3.2.3.2 Non-Radiative Processes in the Heavy-Atom Dyes

Evaluation of the various components which comprise $k_{nr}$ can lead to an understanding of this apparent inverse heavy-atom effect. The values for $k_{nr}$ in Table 3.5 indicate an increase in $k_{nr}$ as the size of the heavy-atom becomes smaller. The non-radiative rate represents the sum of rates associated with non-radiative depopulation of the singlet state, and in tricarbocyanines can be represented by:

$$k_{nr} = k_{isc} + k_{ic} + k_{ad}$$

(3.16)

where $k_{isc}$ is the intersystem crossing rate constant, $k_{ic}$ is the internal conversion rate constant, and $k_{ad}$ is a solvent-dependent rate constant. The excellent fit of the fluorescence decays to a single exponential function precludes the formation of a photoisomer to any appreciable level. In addition, it has been shown previously that tricarbocyanine dyes, especially those containing bridging units in the polymethine chain, exhibit very little photoisomerization due to torsional strain imposed by the bridging units. Effectively, the major changes in $k_{nr}$ resulting from the addition of the intramolecular heavy-atom can arise from changes in the intersystem crossing and/or internal conversion rates.

3.2.3.3 Triplet State Characterization.

In order to explore the effects of the intramolecular heavy-atom on $k_{isc}$ and $k_{ic}$ for the mono-substituted series, we measured the triplet photophysics for these heavy-atom modified dyes using laser flash photolysis. A triplet-triplet...
absorption spectrum and triplet decay profile of 10 are shown in Figure 3.12. The triplet spectrum showed a negative peak at 780 nm due to ground state depletion, followed by a broad absorption profile containing a peak at 830 nm due to triplet-triplet absorption. The triplet photophysics are shown in Table 3.6, along with the calculated $k_{\text{isc}}$ and $k_{\text{ic}}$ rates. Triplet lifetimes were determined from a non-linear least squares fit of the decay profiles. The $k_{\text{isc}}$ was calculated from expression 3.16 using $\tau_f$ and $\Phi_f$. $k_{\text{ie}}$ was calculated from $k_{\text{ie}} = k_{\text{ic}} - k_{\text{isc}}$. Since all photophysics were measured in CH$_3$OH, the solvent-dependent non-radiative rate is expected to contribute equally to the non-radiative deactivation in the dye series.$^5$ As can be seen from the data in this table, the triplet lifetimes increased with decreasing size of the heavy-atom in the dyes, which is qualitatively consistent with an increase in $k_{\text{isc}}$ (i.e., normal heavy-atom effect).

Table 3.6 Triplet state photophysics of heavy-atom modified dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\tau_i$ ($\mu$s)</th>
<th>$\Phi_i$ ($\times 10^3$)</th>
<th>$k_{\text{isc}}$ (ns$^{-1}$) ($\times 10^3$)</th>
<th>$k_{\text{ic}}$ (ns$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>188</td>
<td>1.7</td>
<td>1.9</td>
<td>0.98</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>45</td>
<td>50</td>
<td>0.91</td>
</tr>
<tr>
<td>12</td>
<td>117</td>
<td>3.0</td>
<td>4.0</td>
<td>0.99</td>
</tr>
<tr>
<td>14</td>
<td>186</td>
<td>0.80</td>
<td>1.0</td>
<td>1.04</td>
</tr>
<tr>
<td>16</td>
<td>177</td>
<td>1.3</td>
<td>1.6</td>
<td>1.06</td>
</tr>
</tbody>
</table>

Inspection of Table 3.6 indicated that $k_{\text{ic}}$ decreased with the heavier atom among the monosubstituted halogenated series. Since the heavier atom results in only small increases in $k_{\text{isc}}$, and large decreases in $k_{\text{ic}}$, the net result was an apparent inverse heavy-atom effect producing longer fluorescence lifetimes with heavier atoms. Although our measurements did not allow us to evaluate $k_{\text{ad}}$.
Figure 3.12 Transient difference spectrum (A) and triplet decay profile (B) of 10. Laser wavelength: 760 nm (Lasing dye: LDS 751), 135 mW. Measurement made 4 ns after laser pulse.
independent of $k_{ic}$, it was expected that $k_{ad} \ll k_{ic}$, since previous work has shown that internal conversion was the major deactivation pathway in polymethine dyes in organic solvents.\textsuperscript{5,21,43} The observed decrease in $k_{ad}$ and longer lifetimes with heavier atoms results from the reduction of $k_{ic}$ induced by the heavier atom restricting conformational interconversions through steric interactions. Distortion of the conformation of the molecule from planarity in both the ground and excited state can enhance the rate of internal conversion, and in the present case, the heavier atom impedes such distortions through steric interactions. Since the increase in $k_{ic}$ was overcompensated by the decrease in $k_{ic}$, an apparent inverse heavy-atom effect was observed (i.e., longer $\tau_f$ with the heavier atom). This data is in contrast to what has been observed for the carbo- and dicarbocyanine dyes, where the introduction of bulky groups onto the heteroaromatics results in molecular distortion through steric interactions which increases the rate of internal conversion.\textsuperscript{44}

3.2.4 Summary

The photophysics of the heavy-atom modified dyes have been characterized by their absorbance and fluorescence data as well as time-resolved fluorescence data. The intramolecular heavy-atom substituents do not significantly affect the absorbance and fluorescence maxima of the chromophores, but do perturb the fluorescence lifetime to produce a series of chromophores with similar excitation and emission properties but different lifetimes. Interestingly, the dye set exhibited an apparent inverse heavy-atom
effect of the fluorescence lifetime values. Investigation of the triplet-state using laser flash photolysis yielded information on $k_{\text{isc}}$ and the cause of the inverse effect which was a result of the decrease in $k_{\text{isc}}$ with the addition of heavier atoms. The use of these dyes as probes, especially in DNA sequencing applications requiring multichannel operation may help to minimize instrumental complexity due to their efficient excitation with a single laser and the ability to process the fluorescence on a single channel.

3.3 Photophysical Characterization of Functionalized Heavy-Atom Modified Tricarbocyanine Dyes

3.3.1 Introduction

The dyes characterized in section 3.2 demonstrate the utility of heavy-atom modification onto the basal tricarbocyanine dyes 3 and 6 which perturbs the fluorescence lifetime without significantly altering the absorbance and fluorescence maxima. However, the use of these dyes as bioprobes is limited since they do not contain reactive functional groups such as isothiocyanates or succinimidyl esters which are typically used to label primary amines on proteins and amino acids. Dyes which contain an intramolecular heavy-atom substituent with an isothiocyanate functional group have been synthesized. In this section, the photophysics of heavy-atom modified dyes with an isothiocyanate functional group will be examined including, $\Phi_f$ and $\tau_f$. 

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3.3.2 Experimental

The absorbance spectra were acquired on a Perkin-Elmer Lambda 3 spectrophotometer (Perkin-Elmer, Norwalk, CT) at dye concentrations of $5 \times 10^{-6}$ M in CH$_3$OH. The uncorrected fluorescence spectra were collected on the red sensitive a Spex 3000 fluorimeter (Spex, Edison, NJ) using dye concentrations of $1 \times 10^{-6}$ M in CH$_3$OH. The fluorescence quantum yields were calculated relative to IR-125 in DMSO ($\Phi_f = 0.13$) following the procedure outlined by Demas and Crosby.$^{1,18}$

Time-resolved fluorescence measurements were performed using a near-IR time-correlated single-photon counting instrument shown in Figure 3.2. The dye concentration used for lifetime determinations was $1 \times 10^{-8}$ M in CH$_3$OH. The fluorescence lifetimes were calculated using a reiterative non-linear least squares algorithm written in our laboratory. Decay profiles were accumulated until approximately 10,000 photocounts were present in the channel with the maximum number of counts.

3.3.3 Results and Discussion

The dyes being investigated are shown in Figure 3.13. These dyes contain an ethyl isothiocyanate group for purposes of labeling biomolecules with primary amines. Inspection of the absorbance and emission spectra for the heavy atom modified dye series showed that introduction of the heavy atom(s) on the functionalized phenol substituent produced only minor changes in the absorption and emission maxima of the dyes. Table 3.7 lists the absorbance and emission.
maxima as well as the molar absorptivities and fluorescence quantum yields ($\Phi_f$) for the heavy-atom modified dyes. The addition of the intramolecular heavy atom(s) produced only minor differences in the extinction coefficients ($\varepsilon$) and had no appreciable effect on $\Phi_f$. The absorbance and fluorescence spectra of the dyes are shown in Figures 3.14 and 3.15.

Compared to the heavy-atom modified dyes in the previous sections (8,10,12,14,16), these dyes have slightly larger extinction coefficients in CH$_3$OH with a slight red shift in their absorbance and fluorescence maxima.

![Functionalized heavy-atom modified tricarbocyanine dyes](image)

<table>
<thead>
<tr>
<th>Dye</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>H</td>
</tr>
<tr>
<td>36</td>
<td>I</td>
</tr>
<tr>
<td>37</td>
<td>Br</td>
</tr>
<tr>
<td>38</td>
<td>Cl</td>
</tr>
<tr>
<td>39</td>
<td>F</td>
</tr>
</tbody>
</table>

*Figure 3.13 Functionalized heavy-atom modified tricarbocyanine dyes.*
Table 3.7 Absorbance and fluorescence data of functionalized heavy-atom modified near-IR dyes in CH$_3$OH.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Absorbance maximum (nm)</th>
<th>$\varepsilon$ (cm$^1$M$^{-1}$)</th>
<th>Fluorescence maximum (nm)</th>
<th>$\Phi_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>33</td>
<td>765</td>
<td>230,000</td>
<td>794</td>
<td>0.07</td>
</tr>
<tr>
<td>36</td>
<td>768</td>
<td>216,000</td>
<td>796</td>
<td>0.15</td>
</tr>
<tr>
<td>37</td>
<td>768</td>
<td>254,000</td>
<td>798</td>
<td>0.14</td>
</tr>
<tr>
<td>38</td>
<td>768</td>
<td>239,000</td>
<td>797</td>
<td>0.14</td>
</tr>
<tr>
<td>39</td>
<td>768</td>
<td>221,000</td>
<td>796</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Interestingly, these dyes exhibited molecular aggregation in aqueous solutions. Figure 3.16 shows the absorbance spectra of 33 in H$_2$O and in H$_2$O with 40% CH$_3$CN. The formation of higher order aggregates is clearly demonstrated with the appearance of a second peak at 650 nm which is shifted into the blue from the monomeric peak at 764 nm. The addition of a polar organic solvent such as CH$_3$CN or CH$_3$OH results in the reappearance of the monomeric peak at 764 nm.

The fluorescence lifetimes of the functionalized heavy-atom modified dyes in CH$_3$OH are presented in Table 3.8. The functionalized heavy-atom dyes displayed the same trends as seen with the heavy-atom dyes with the heaviest modification possessing the longest $\tau_f$. Evaluation of $k_r$ and $k_{nr}$ demonstrated the same trends as seen with the previous heavy-atom dyes. The dye with the heaviest atom had the lowest $k_{nr}$ and it increased among the series as the halogen became lighter, which was consistent with the previous results for dyes 10, 12, 14, and 16. As explained before, the intramolecular heavy-atom resulted in perturbations in the singlet state photophysics (fluorescence quantum yield, $\Phi_f$).
Figure 3.14 Absorbance spectra of functionalized heavy-atom modified dyes in CH$_3$OH. Dye concentration 5x10$^{-6}$ M.
Figure 3.15 Fluorescence spectra of functionalized heavy-atom modified dyes in CH$_3$OH. Dye concentration $1\times10^{-6}$ M. Excitation and emission slit widths: 2 mm.
Figure 3.16 Dye 33 absorbance spectra in H$_2$O and H$_2$O with 40% CH$_3$CN. Dye concentration 5×10$^{-6}$ M.
A series of novel chromophores that contain an intramolecular heavy atom and a reactive functional group have been characterized by absorbance and steady-state and time resolved fluorescence spectroscopy. The dye set showed absorbance and fluorescence maxima in the near-IR and possessed fluorescence lifetime differences among the chromophores that were dependent upon the heavy-atom modification. Although they do exhibit aggregation in aqueous solutions, the addition of an organic modifier dramatically reduces the amount of aggregation to provide primarily the monomeric dye. These dyes can potentially be used in numerous bioanalyses including, amino acid analysis, protein analysis or into a DNA sequencing protocol that employs fluorescence lifetime discrimination of labeled nucleotide bases for base-calling.
3.4 References


Chapter 4

Tricarbocyanine Dyes as Fluorescent Labels for Amino Acids

4.1 Introduction

There is increasing interest in the use of fluorescent probes as a means of detection of biological and organic compounds because of the analytical sensitivity and ease of use of such probes compared to radiochemical methods. Recently, there have been several reports of near-IR fluorophores that can be used to covalently and non-covalently label biological and organic compounds, including amino acids, proteins, nucleotides, DNA primers, double-stranded DNA, thiols, and antibodies. These near-IR probes for covalent labeling are typically tricarbocyanine dyes containing an isothiocyanate or succinimidyl ester functional group for labeling of amines, or an iodoacetamide group for labeling thiols. While these chromophores do not possess large fluorescence quantum yields in H₂O, the reduced background in the near-IR results in an overall larger signal-to-noise ratio compared to the visible region. The isothiocyanate group is a versatile functionality that reacts with primary and secondary amines, and when attached to near-IR dyes, makes these dyes attractive fluorescent probes for amino acid, protein, and peptide analyses. Analyses of such biomolecules are frequently performed using capillary electrophoresis (CE) coupled to a wide variety of detection schemes including absorbance, laser-induced fluorescence, electrochemical, mass-spectral, and Raman spectroscopy. Laser
induced fluorescence is an attractive technique due to the fact that it provides high sensitivity, low detection limits, and can easily be implemented in micro-separation based techniques.

Dovichi and coworkers have pushed the detection limits in CE applications using visible fluorescent dyes to the 1-2 zmol range (600-1200 molecules) with off-column detection in a sheath flow cuvette. This method results in a reduction of the scattering photon contribution to the background. The impressive detection limits obtained by Dovichi resulted in part from the favorable photophysics associated with the dye, including large fluorescence quantum yield and extinction coefficient, and reasonable photochemical stability. The photophysics of many visible dyes, while better in organic solvents, are adequate in aqueous solvents to allow sensitive detection.

Near-IR fluorescence detection couplet to CE has been shown to be an attractive alternative to visible fluorescence detection schemes due to the intrinsically smaller backgrounds associated with this region of the spectrum. Detection at the single-molecule level has been reported in the near-IR with efficiencies superior to the visible case, even though the photophysics of the near-IR dye were found to be inferior to the visible dye used in the single molecule experiments. The improved detection efficiencies were achieved, in part, by the low background in the form of impurity fluorescence from the solvent and the lower solvent Raman cross-sections.

Difficulties associated with near-IR fluorescence in many analytical applications are the lack of sufficient labels for tagging various classes of compounds and the poor photophysical properties associated with these dyes in aqueous
solvents. In H₂O, many of these dyes show extensive ground state aggregation, fluorescence quantum yields < 0.01, and photochemical stabilities inferior to the visible dyes. However, the photophysics can be improved through the addition of organized media to the aqueous solvent or the use of nonaqueous solvents. Near-IR fluorescence detection in CE has been demonstrated for amino acids labeled with a near-IR chromophore, however, the poor labeling efficiency and photophysics associated with the dye in aqueous solvents resulted in detection limits in the picomole range, which are significantly inferior to the visible case.

This chapter of the dissertation will discuss the ultrasensitive detection of near-IR dye-labeled amino acids separated by CZE in mixed CH₃OH/aqueous running buffers with detection performed on-column. The laser-induced near-IR fluorescence detector consisted of a Ti:sapphire laser and a single photon avalanche diode (SPAD). The Ti:sapphire laser has some important attributes which make it an attractive excitation source in CE, including a TEM₀₀ mode structure allowing diffraction limited focusing, continuous wavelength tuning from 680 - 1000 nm, all solid-state components, and requires only a small frame Ar ion laser for pumping. The SPAD detector has a large single photon detection efficiency in the near-IR and can be used for visible and UV fluorescence as well. In addition, the electrophoresis was carried out in binary CH₃OH/aqueous running buffers so as to enhance the limits of detection, due to the fact that near-IR dyes show photophysics more conducive to ultrasensitive detection in organic solvents compared to aqueous solvents.
4.2 Experimental

4.2.1 Laser-Induced Fluorescence Detector

A block diagram of the near-IR LIF detector is shown in Figure 4.1. A Ti:sapphire laser (Coherent Lasers Mira 900-F, Palo Alto, CA) was used as the excitation source which was pumped by the all-lines output of a small frame Ar ion laser (Coherent Lasers, Innova 310). The laser was tuned to 790 nm with 10 mW of laser light delivered to the separation capillary. The laser light was focused to 10 μm with a diode laser singlet lens. The emission was collected in a conventional 90° format with a 40x high NA microscope objective (Nikon, Natick, MA, NA=0.85). The emission was spatially filtered (slit width = 0.4 mm) yielding a viewing distance of 10 μm along the propagation axis of the laser. The fluorescence was further isolated from scattering photons using an 8 cavity interference filter (CWL = 850 nm, HBW = 30 nm, Omega Optical, Brattleborough, VT). The fluorescence emission was then focused onto the photodetector by a 10X microscope objective producing an image of approximately 20 μm on the face of the detector. The photodetector was a single photon avalanche diode (SPAD, EG&G Electrooptics Canada, Vaudreuil, Canada) mounted on a thermoelectric cooler with a photoactive area of $1.77 \times 10^{-4}$ cm$^2$ (i.d. = 150 μm) and a dark count rate of approximately 90 counts-per-second. The pulses from the photodetector were amplified and conditioned using a discriminator (Tennelec TC 754, Oak Ridge, TN). The output from the discriminator was sent to a multichannel scalar resident in a PC486 for displaying the electropherograms (Tennelec PCA II, Oak Ridge, TN).
4.2.2 Capillary Electrophoresis System

The capillary column, 75 μm i.d., 375 μm o.d. (Polymicro, Phoenix, AZ), was cut to a length of 50 cm (40 cm to the detector) with a small portion of the polyimide coating removed to create an optical window using a low temperature flame. The column was initially rinsed with 1.0 M NaOH for 1 h followed by a 2 h wash with H₂O. Prior to the electrophoresis, the column was allowed to equilibrate with the appropriate running buffer for 30 min under high voltage conditions (300 V/cm). The high-voltage power supply was obtained from Spellman High Voltage Electronics Corp. (Plainview, NY). Buffers were composed of 10 mM borate, with the pH adjusted to 9.1. In the various CH₃OH/H₂O solutions, the ionic strength was kept constant and the apparent pH maintained at 9.1. Samples were introduced by electrokinetic injection: 5kV for 5 s. All separations were performed at ambient temperature (25 °C).

The dye solutions were made fresh daily in the appropriate running buffer from a serial dilution of stock solutions (1.0×10⁻⁴ M) which were stored in the dark at 10 °C in CH₃OH. The amino acids (arginine, glycine, glutamic acid, tryptophan and valine), boric acid, and riboflavin were purchased from Sigma Chemical Co. (St Louis, MO). DMF was obtained from Aldrich Chemical Co. (Milwaukee, WI).

4.2.3 Labeling of amino acids with isothiocyanate derivatives, 32 and 33

Capillary electrophoretic analysis of amino acids, 500 μL of a 0.1 M solution of the amino acid was dissolved in borate buffer (0.2 M, pH = 9.2) and reacted with
Figure 4.1 Block diagram of the near-IR laser-induced fluorescence detector; C = capillary column; L = focusing lens; BD = beam dump; MO = microscope objective; S = spatial filter; F = bandpass filter; SPAD = single photon avalanche diode; Disc. = discriminator; MCS = multichannel scalar.
100 μl of a 1.2×10³ M solution of dye 32 dissolved in DMF. Each amino acid was conjugated separately in a H₂O bath at 25°C for approximately 15 h. Prior to CE analysis, the amino acid reaction mixtures were pooled and serially diluted to a concentration of 1.0×10⁻⁹ M. A solution of hydrolyzed isothiocyanate dye 32 at a concentration of 1.0×10⁻⁹ M was added to the pooled amino acid solution for CE analysis. To determine conjugation efficiencies, a ten fold excess of 32 or 33 in DMSO was dissolved in a 0.1 mM solution of amino acid in borate buffer (13 mM, pH 9.3, 40% v/v CH₃CN; 100 μL ~ 10:40:50, DMSO:CH₃CN:buffer) and the solution was shaken in the dark at room temperature.

Analytical separations were performed after 12 h using a Brownlee Speri-5 ODS reversed phase analytical column, 5 μm particle size, 100 × 4.6 mm (PE Applied Biosystems, Foster City, CA). The HPLC detector was a Shimadzu absorbance detector which was set at 770 or 790 nm. H₂O-CH₃OH gradients were used in all experiments (A:H₂O, B: CH₃OH; Initial: 45% A, hold for 5 min, ramp to 20% A over 20 min, hold for 5 min, ramp to 0% A, then back down to initial conditions over 5 min.).

4.3 Results and Discussion

The conjugating dyes are shown in Figure 4.2. The major difference between these dyes is the position of the isothiocyanate (N=C=S) functionality. The isothiocyanate group is located on the aryl ring on 32. The isothiocyanate on 33 is removed from the aryl ring by an ethyl linker arm. The impetus for synthesizing 33
was to reduce steric interactions during labeling reactions with amino acids. In order to demonstrate the analytical utility of near-IR fluorescence detection in CE applications using mixed organic/aqueous running buffers, three amino acids were derivatized with a near-IR dye containing an isothiocyanate functional group (32). Scheme 4.1 illustrates the labeling reaction of 32 with glycine.

The electropherograms of the derivatized amino acids arginine, glycine, and valine in various CH$_3$OH/H$_2$O running buffers are shown in Figure 4.3. In Table 4.1, the SNR in the various running buffers are shown for dye-labeled arginine. When the amino acids were electrophoresed individually, only one peak was present, indicating the labeling efficiency was near 100%. Hydrolyzed dye 32 was also added to the amino acid mixture in order to determine the chromatographic resolution of the labeled amino acids from free dye in the mixed CH$_3$OH/H$_2$O running buffers, due to the fact that in most labeling applications, excess dye is added to the target analytes. As can be seen from this series of electropherograms and Table 4.1, the fluorescence
Scheme 4.1 Representative labeling reaction of glycine with 32.

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Figure 4.3 CE separation of the amino acids, arginine (Arg), valine (Val) and glycine (Gly) labeled with dye 32 and native dye 32 (32) in 0/100 (A), 40/60 (B), 60/40 (C) and 90/10 (D) CH₃OH/H₂O buffers. The amino acid and dye concentrations were 1 x 10⁻⁹ M in (A), (B) and (C) and 1 x 10⁻⁸ M in (D). The peaks marked with (b) are reagent blank peaks. The electrophoresis was carried out at a field strength of 450 V/cm in all cases. Electrokinetic injection: 5kV for 5 s.
Table 4.1 Signal to noise ratio (SNR), for dye-labeled arginine in various CH₃OH/aqueous borate buffers (pH = 9.1).

<table>
<thead>
<tr>
<th>% CH₃OH</th>
<th>SNR*ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>607</td>
</tr>
<tr>
<td>60</td>
<td>342</td>
</tr>
<tr>
<td>40</td>
<td>86</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

* The SNRs were determined by integrating the total number of counts in the electrophoretic peak and dividing by the square root of the average of the noise.
ᵇ The SNRs were calculated based on a dye concentration of 1 x 10⁻⁹ M.

The number of theoretical plates (N) is a term which describes the efficiency of a separation in CE and is defined as:⁴⁰

\[
N = \frac{L^2}{\sigma_T^2}
\]  

(4.1)

where L is the total distance migrated by the analyte and \( \sigma_T^2 \) is the total variance of the analyte zone. Although the concept of a theoretical plate implies that a separation is accomplished as a result of an equilibrium partitioning between two immiscible phases, this is not the case in CE. However, the idea of theoretical plates is a simple way of describing the shape of a Gaussian curve.⁴⁰ The number of theoretical plates

signal intensity is dramatically degraded when the amount of H₂O in the binary running buffer was increased. Based on an injection concentration of 1.0 x 10⁻⁹ M, the SNR for arginine was 607 in 90/10 CH₃OH/H₂O and decreased to 3 in 100% H₂O. The mass detection limit for near-IR dye-labeled arginine in 90/10 CH₃OH/H₂O was found to be 21 zmol (injection volume = 4.3 nL, SNR = 3).
per-meter for arginine, valine and glycine shown in Table 4.2 were calculated using the expression:

\[ N = 5.54 \left( \frac{t_m}{W_{1/2}} \right)^2 \]  \hspace{1cm} (4.2)

where \( t_m \) is the migration time in seconds and \( W_{1/2} \) is the width of the peak at half of its maximum height in seconds.

**Table 4.2** Calculated efficiencies of near-IR labeled amino acids separated by CE in mixed CH\(_3\)OH/H\(_2\)O running buffers.

<table>
<thead>
<tr>
<th>% CH(_3)OH</th>
<th>Arginine</th>
<th>Valine</th>
<th>Glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>85600</td>
<td>78500</td>
<td>84600</td>
</tr>
<tr>
<td>60</td>
<td>129000</td>
<td>175000</td>
<td>223000</td>
</tr>
<tr>
<td>40</td>
<td>88300</td>
<td>120000</td>
<td>179000</td>
</tr>
<tr>
<td>0</td>
<td>8480</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The resolution (R) of separated analytes in CE is given by the expression:

\[ R = \left( \frac{1}{4} \right) \left( \frac{\Delta \mu_{\text{app}}}{\mu_{\text{app,avg}}} \right) N^{1/2} \]  \hspace{1cm} (4.3)

where \( \Delta \mu_{\text{app}} \) is the difference in the apparent electrophoretic mobilities of the analytes, \( \mu_{\text{app,avg}} \) is the average apparent mobility and \( N \) is number of theoretical plates.\(^{40}\) This expression shows that resolution is made up of a selectivity term \( \left( \frac{\Delta \mu_{\text{app}}}{\mu_{\text{app,avg}}} \right) \), and an
efficiency term, N. The selectivity term is determined by properties of the analyte and the buffer, and N is dictated by the instrumentation.

From Figure 4.3 it can also be seen that the resolution is affected by the amount of H₂O in the running buffer. The resolution between glycine and valine in various mixed CH₃OH/H₂O running buffers are given in Table 4.3. In 90/10 CH₃OH/H₂O, the resolution between glycine and valine was found to be 1.9, while in 40% H₂O, the resolution decreased to 1.0. In 100% H₂O, the amino acids co-migrated and thus, a resolution value could not be calculated. The reduced resolution with higher H₂O content results from the slight loss in efficiency and the increased apparent mobilities at higher H₂O compositions.

Table 4.3 Apparent electrophoretic mobilities for valine and glycine and the resolution between the amino acids separated by CE in various CH₃OH/H₂O running buffers.

<table>
<thead>
<tr>
<th>% CH₃OH</th>
<th>Valine μₐpp (cm²/Vs)</th>
<th>Glycine μₐpp (cm²/Vs)</th>
<th>Δμₐpp (cm²/Vs)</th>
<th>μₐpp,avg (cm²/Vs)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>9.78×10⁻⁵</td>
<td>9.42×10⁻⁵</td>
<td>3.60×10⁻⁶</td>
<td>9.60×10⁻⁵</td>
<td>1.9</td>
</tr>
<tr>
<td>60</td>
<td>1.59×10⁻⁴</td>
<td>1.56×10⁻⁴</td>
<td>3.00×10⁻⁶</td>
<td>1.58×10⁻⁴</td>
<td>1.4</td>
</tr>
<tr>
<td>40</td>
<td>2.37×10⁻⁴</td>
<td>2.33×10⁻⁴</td>
<td>4.00×10⁻⁴</td>
<td>2.35×10⁻⁴</td>
<td>1.0</td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

For purposes of comparing labeling efficiency of 32 and 33 to amino acids, each dye was reacted in excess with tryptophan and glutamic acid. Initially, aryl isothiocyanate, 32 and alkyl isothiocyanate, 33 were conjugated to tryptophan and glutamic acid using aqueous borate buffers (pH = 9.3). Under these conditions, 32 exhibits moderate conjugation to tryptophan and does not conjugate to glutamic acid (see Table 4.4). Likewise, 33 shows poor
conjugation to each amino acid under these conditions. However, the addition of organic modifier (40% v/v CH$_3$CN) improves the conjugation efficiency of both dyes. The increase in percent conjugation is most likely due to the decreased aggregation of the dyes in these buffers on account of the organic modifier. Chromatograms of the conjugation of each labeling dye (32,33) to tryptophan under optimum conditions are shown in Figure 4.4. Thioether isothiocyanate dye 32 showed quantitative reaction with very little concomitant hydrolysis of the functionalized chromophore. The phenethylisothiocyanate dye 33 showed poor conjugation efficiency towards tryptophan. This may be attributed to the slower reaction kinetics of alkyl isothiocyanates when compared to aryl isothiocyanates.\textsuperscript{41}

Table 4.4 Conjugation Efficiencies of Dyes 32 and 33 to Amino Acids

<table>
<thead>
<tr>
<th>Buffer</th>
<th>tryptophan$^a$</th>
<th>glutamic acid$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>32</td>
<td>33</td>
</tr>
<tr>
<td>35% CH$_3$CN</td>
<td>10%</td>
<td>4%</td>
</tr>
<tr>
<td>40% (v/v) CH$_3$CN</td>
<td>quant.$^c$</td>
<td>20%</td>
</tr>
<tr>
<td></td>
<td>80%</td>
<td>10%</td>
</tr>
</tbody>
</table>

$^a$ The percent conjugation of reaction was determined from the following formula: ((10 x the integrated area of conjugate peak) / total integrated area of all peaks in the chromatogram) x 100%.$^b$ No reaction.$^c$ Quantitative reaction.

4.4 Summary

A series of amino acids were labeled, via a thiourea linkage, with a tricarbocyanine dye, which possessed fluorescence properties in the near-IR and separated in mixed CH$_3$OH/H$_2$O running buffers using CE. The results demonstrated enhanced efficiency, resolution, and detectability when the carrier buffer consisted of predominantly CH$_3$OH. In addition, the conjugation efficiencies of two
tricarbocyamine dyes (32,33) to amino acids (glutamic acid, tryptophan) were examined and showed modest efficiency and only minor hydrolysis. Thus, these dyes are excellent candidates as probes for biomolecules which contain primary amines.

Figure 4.4 Reversed-phase HPLC chromatograms of tryptophan labeled to dye: 32 (A) and 33 (B). Labeling reactions performed in mixed H₂O/CH₃CN buffers. Separation conditions: C₁₈ column, H₂O/CH₃OH gradient: Initial: 45% H₂O, hold for 5 min, ramp to 20% H₂O over 20 min, hold for 5 min, ramp to 0% H₂O, then back down to initial conditions over 5 min.). 20 μL injection of ~1×10⁻⁶M in H₂O. Unk (unknown), and Hyd (hydrolysis products).
4.5 References


5.1 Introduction

DNA sequencing is currently carried out following the Sanger sequencing protocol. A primer or short piece of single stranded DNA with a known sequence attaches, according to Watson-Crick base pairing rules (A to T, C to G), to a single-stranded DNA or template which has an unknown sequence. Then, a DNA polymerase enzyme is introduced with all four types (A, C, G, and T) of deoxynucleotides (dNTP) and one type (any one of the four bases) of dideoxynucleotide (ddNTP). The enzyme incorporates dNTPs until a ddNTP is inserted. At this point, extension of the unknown strand of DNA stops. If the ratio of dNTP to ddNTP is correct, a distribution of single stranded DNAs are synthesized which are complementary to the unknown strand of DNA. The generated fragments are then separated electrophoretically and the sequence is reconstructed from the complementary strands.

Conventional methods for DNA sequencing employ laser-induced fluorescence (LIF) detection of fluorescently labeled DNA strands. The use of fluorescent labels has become an integral component in DNA sequencing applications due to the fact that it can provide on-line analysis, possesses limits of detection comparable to autoradiography, is easily integrated to micro-gel separation techniques, and can potentially allow the base-calling to be performed.
in a single separation lane using spectral discrimination. The important attributes associated with the fluorescent dye set used in DNA sequencing include the following: (1) The dyes must possess unique spectral properties to allow highly efficient base calling during the fractionation step. When spectral discrimination is used for base-calling, it is advisable to have the emission bands well separated to alleviate cross-talk between detection channels. (2) The dye set must produce a uniform or constant perturbation on the electrophoretic mobility of the labeled oligonucleotide. If the mobility shift is dye-dependent, then post-run corrections must be implemented in order to avoid base shifts which result in errors during sequence reconstruction. (3) The dye series should allow facile excitation with a single laser source, and preferably, permit processing of the fluorescence on a single detection channel. The ability to use a single laser and detection channel will relieve some of the instrumental complexity associated with the sequencing device, especially in cases where high throughput sequencing is to be undertaken which requires the parallel running of many electrophoretic lanes with the associated detection channels.

The ability to perform the separation and base-calling in a single lane has become critically important, in light of the Human Genome Project, where throughput issues are a prime concern. The commonly used approach for single lane base-calling in DNA sequencing applications is spectral discrimination, where a set of four spectrally distinct chromophores, which can be attached to either the sequencing primer or the dideoxynucleotide, are identified based on unique emission maxima. The dyes typically used are the fluorescein and/or
rhodamine derivatives, which contain structural modifications to alter the absorption and emission maxima.\textsuperscript{1,3,4} A set of commercial dyes are available (FAM, JOE, TAMRA and ROX) which nearly possess the aforementioned characteristics of a dye set for sequencing applications.\textsuperscript{5} These dyes can be excited with the 488 and/or 514 nm lines from an argon ion laser with the emission spectrally isolated using a series of optical filters onto appropriate photodetectors.\textsuperscript{3,6} Some of the potential difficulties with this approach are the need for multi-line excitation, multiple detection channels, cross-talk between detection channels due to the broad emission profiles associated with the chromophores, and the non-uniform electrophoretic mobilities demonstrated by the dye-labeled oligonucleotides.

In order to eliminate the need for multiple excitation sources or multi-line excitation with a single laser, the use of fluorescence energy transfer probes have been utilized in single lane DNA sequencing applications.\textsuperscript{7,8,9} In this approach, a donor (FAM) is attached to the 5' end of a sequencing primer and an acceptor (FAM, JOE, TAMRA, ROX) is covalently bound to a modified-thymidine residue eight to nine bases down the sequence. Since the primers utilize Förster resonance energy transfer, only a single excitation source is required (488 nm) with the emission sorted on appropriate detectors. Due to the structural requirements for efficient energy transfer and the need for two dyes per primer, this method will be difficult to adapt in primer walking strategies, and the use of dye-labeled dideoxynucleotides may be difficult. In addition, concerns with dye-dependent mobility shifts are present.
Figure 5.1 Electrophoretic mobility matched BODIPY dyes.
Recently, a set of electrophoretically uniform fluorescent dyes for DNA sequencing have been reported. The dyes, which are BODIPY derivatives (Figure 5.1), were attached to sequencing primers (5' end) via a unique linker structure that produced excellent sequencing data without software correction for dye-dependent mobility shifts. In addition, the dye-primer set yielded narrower spectral emission bandwidths compared to conventional dye-primer sets, resulting in smaller amounts of cross-talk between detection channels. However, as with the energy transfer dyes, this approach will not easily be amenable to primer walking strategies or dye-labeled ddNTP sequencing.

An alternative to this scheme is to use only one dye. In this case, adjustment of the molar ratio of the ddNTPs in the extension reaction allows for the identification of labeled bases based upon the peak intensities of the separated fragments. This method is more instrumentally simple requiring only one excitation source and detector. In addition, the use of one dye relieves the problems associated with electrophoretic mobility shifts. However, there are some problems with this method of analysis. Non-uniformity in the incorporation of the ddNTP's can cause errors in the base calling. Also, null signal or peaks which result from the lowest concentration can sometimes be mislabeled or lost if they are between two peaks of the largest concentration. This method also requires the use of the T7 polymerase enzyme which cannot be used for cycle sequencing and limits the size of the DNA templates which can be analyzed.

As an alternative to spectral discrimination, various groups have suggested that fluorescence lifetime discrimination can potentially serve as a
viable method for base calling in DNA sequencing applications.12-14 Using this approach, primers or dideoxynucleotides are labeled with chromophores that have unique fluorescence lifetimes. After gel electrophoresis, the sequence is reconstructed based upon the identification of the terminal base (Sanger sequencing) using lifetime discrimination and the mobility of the separated fragments. The principle advantages associated with lifetime discrimination for base-calling are; (1) the calculated lifetime is immune to concentration differences, and, as such, dye-labeled terminators can potentially be used as well as dye-primers with a wide choice in polymerase enzymes to suit the particular sequencing application; (2) lifetime values can be determined with higher precision than fluorescence intensities under appropriate conditions, potentially improving the accuracy in base-calling; (3) lifetime determinations do not suffer from broad emission profiles associated with spectral discrimination and; (4) the fluorescence can potentially be processed on a single detection channel without the need for spectral sorting to multiple detection channels.

Several problems do arise in considering lifetime discrimination for DNA sequencing, especially when utilizing the micro-separation techniques, such as capillary gel electrophoresis. The most pervasive problem is associated with the complex instrumentation required for lifetime determinations. For example, in time-domain techniques, a pulsed laser is required with a fast detector, typically a micro-channel plate photomultiplier tube, and sophisticated counting electronics. In addition, poor photon statistics (low number of photocounts) produced from low loading levels and the transient nature of the signal can produce poor
precision in the measurement. Poor precision would also be compounded by the presence of large amounts of scattering and impurity photons included into the decay profile. Finally, complex algorithms are often required for extracting the lifetime from the decay profile making on-line determinations during electrophoresis difficult.

Many of these concerns associated with lifetime-based species discrimination in DNA sequencing and other applications have been addressed using near-IR fluorescence. For example, several groups have demonstrated that semiconductor diode lasers, which can be operated in a pulsed mode and lase between 680 and 800 nm in conjunction with single photon avalanche diodes (SPADs) or photomultiplier tubes can produce a time-correlated single photon counting apparatus that is simple with performance characteristics comparable to devices using mode-locked Nd:YAG lasers and micro-channel plates.\textsuperscript{15-18}

Legendre \textit{et al.} have demonstrated that lifetime measurements can be acquired in the near-IR using solid-state components and counting electronics situated on a PC-board.\textsuperscript{19} Also, Soper \textit{et al.} have shown that simple algorithms can be used to calculate fluorescence lifetimes on-line during free solution or capillary gel electrophoresis using near-IR time-resolved fluorescence.\textsuperscript{13} The standard deviation in the lifetime measurement of C-terminated fragments labeled with a near-IR dye was found to be ±9 ps with decay profiles constructed from ~20,000 photocounts. The high precision resulted primarily from the fact that in the near-IR, the low scattering cross-sections and the minimal number of
intrinsically fluorescent components produced low numbers of interfering photocounts into the decay.

In this section, the utility of heavy-atom modified tricarbocyanine dyes as probes for DNA sequencing applications will be discussed. All of the dyes have absorbance and fluorescence maxima in the near-IR (760 - 810 nm), large molar extinction coefficients, and excellent solubility in aqueous solutions. These dyes also possess unique fluorescence lifetimes, which should permit facile identification using near-IR based time-correlated single-photon counting. The potential of these dyes for use in DNA sequencing applications has been evaluated by conjugation reactions with propargyl amine-modified dideoxynucleotides and capillary electrophoretic separations.

5.2 Experimental

5.2.1 Chemicals

Dithiothreitol and diisopropylethylamine was purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA) and used as received.

5.2.2 Spectroscopic Analysis

The absorbance spectra were acquired on a Perkin-Elmer Lambda 3 spectrophotometer (Perkin-Elmer, Norwalk, CT) with the fluorescence spectra collected using the red sensitive Spex 3000 fluorimeter (Spex, Edison, NJ) which was described in section 3.1.2.
5.2.3 Capillary Electrophoresis

Electrophoresis was performed on a Waters Quanta 4000 CE System (Millipore, Marlborough, MA) with the output signals integrated on a Perkin-Elmer LCI-100 Laboratory Computing Integrator (Norwalk, CT). Free solution separations were performed using a 75 μm i.d. capillary column (Polymicro Technologies, Phoenix, AZ). The capillary column was washed with distilled H₂O for 5 min, then 1M NaOH for 10 min, then H₂O for 5 min to insure that the silanol groups on the wall of the capillary were deprotonated. Dyes dissolved in the running buffer were electrokinetically injected (20 s with an applied voltage of 30 kV) onto the column and separated at an applied voltage of 25 kV. The analytes were detected on-column using absorbance at 254 nm. Free solution mobilities were calculated relative to the mobility of riboflavin (neutral marker) in order to correct for the electroosmotic flow.

Capillary gel electrophoresis was performed in a 5%T / 5%C polyacrylamide gel column (75 μm i.d., J&W Scientific, Folsom, CA). A mixture of the dyes (2-8) were electrokinetically injected (3 s at an applied voltage of 5 kV) onto the column and separated in reverse mode at an applied voltage of -9.13 kV. Separation in reverse mode implies that the electrode leads have been switched so that the cathode is at the injection end and the anode is at the detection end of the capillary. The addition of the polyacrylamide gel and the polymer coating on the walls of the capillary significantly diminishes the
electroosmotic flow, so that DNA fragments, which are negatively charged, preferentially migrate towards the anode.

5.2.4 Labeling of 7-(3-amino-1-propynyl)-2',3'-dideoxy-7-deazaadenosine 5'-triphosphate (40a)

7-(3-amino-1-propynyl)-2',3'-dideoxy-7-deazaadenosine 5'-triphosphate (ddATP) (0.16 μmol) and 2 (1.4 mg, 1.6 μmol) were dissolved in 67 mM sodium bicarbonate buffer (100 μL, pH = 9.3) and allowed to react in the dark at room temperature for 48 h. Diisopropylethylamine (100 μL) was added and allowed to react for 24 h to form the conjugate (see Scheme 5.1). The reaction was followed by reverse-phase HPLC.

5.2.5 Labeling of 7-(3-Amino-1-propynyl)-2',3'-dideoxy-7-deazaguanosine 5'-triphosphate (40b)

7-(3-Amino-1-propynyl)-2',3'-dideoxy-7-deazaguanosine 5'-triphosphate (ddGTP) was kindly provided by Pavel Cotofana at PE-Applied Biosystems and was prepared by methods described by Prober et al.\textsuperscript{20} The dye-ddGTP conjugate was prepared by optimization of the same method (see Scheme 5.1).\textsuperscript{20} In general, the purification required two steps. Anion exchange was used to separate free dye and the dye-labeled ddGTP. This was followed by a reverse-phase column to separate the ddGTP and the dye-labeled ddGTP. The final product was dried in vacuo and diluted with 50 mM CAPSO (750 mL), pH 9.6, to a concentration of 1.7 μM. The concentration of the dye conjugate in solution was confirmed by
Scheme 5.1 Labeling of dideoxynucleotides with near-IR dyes.
near-IR absorbance spectroscopy at 765 nm. The anionic HPLC conditions were as follows: Column: Aquapore™ AX-300, 7 μm particle size, 220 × 4.6 mm (PE Applied Biosystems); gradient: 40% CH₃CN : 60% triethylammonium bicarbonate (TEAB, 0.1M) to 40% CH₃CN : 60% TEAB (1.5 M) at 1.5 mL/min over 20 min, followed by isocratic elution. The reversed-phase separation conditions were as follows: Column: Spheri-5 RP-C18, 5 μm particle size, 220 × 4.6 mm (PE Applied Biosystems); gradient: 100% triethylammonium acetate (TEAA, 0.1 M) to 40% CH₃CN : 60% TEAA at 1.5 mL/min over 20 min followed by 40% to 100% CH₃CN at 1.5 mL/min over 5 min. with UV/VIS detection at 260 nm.

5.2.6 Experiments with DTT

Dye 8 in H₂O (1×10⁻⁶ M) was heated in a constant temperature H₂O bath for 3 min at 80 °C. Absorbance and fluorescence spectra were collected before, immediately after, and 5 h after initial heating. Dye 8 in H₂O (1×10⁻⁶ M) with 0.5 M DTT was heated in a constant temperature H₂O bath for 3 min at 80 °C. Absorbance and fluorescence spectra were collected before, immediately after, and 5 h after initial heating.

5.3 Results and Discussion

Effective use of the functionalized heavy-atom modified dyes (shown in Figure 5.2) in a DNA sequencing protocol requires the electrophoretic mobilities (μₑₑ) be uniform in order to minimize base-calling errors arising from mobility
Figure 5.2 Heavy-atom modified tricarbocyanine dyes.
shifts induced by the dye label. The $\mu_{\text{em}}$ of an analyte is defined as the steady-state velocity per unit field strength given by;

$$\mu_{\text{em}} = \frac{q}{f}$$  \hspace{1cm} (5.1)

where $q$ is the net charge of the molecule and $f$ is the translational frictional coefficient of the molecule. Therefore, differences in $\mu_{\text{em}}$ can arise from differences in the size or shape of the molecules (i.e. $f$ changes) or from differences in the net charge ($q$). The apparent mobilities ($\mu_{\text{app}}$) of the chromophores, which are a composition of the electroosmotic flow ($\mu_{\text{eo}}$) and $\mu_{\text{em}}$, were determined in free solution capillary zone electrophoresis using;

$$\mu_{\text{app}} = \frac{L_{\text{eff}}}{(t_m \times E)}$$  \hspace{1cm} (5.2)

where $L_{\text{eff}}$ is the length of the capillary column from the injection to the detection window (cm), $t_m$ is the migration time of the analyte (s), and $E$ is the field strength (V/cm). The $\mu_{\text{em}}$ were determined using the expression;

$$\mu_{\text{em}} = \mu_{\text{app}} - \mu_{\text{eo}}$$  \hspace{1cm} (5.3)

where $\mu_{\text{eo}}$ is the electroosmotic flow. The electroosmotic flow was determined from the $\mu_{\text{em}}$ of the neutral marker riboflavin which was co-injected with each dye solution. Since riboflavin is a neutral molecule, its migration is a result of only electroosmosis. In this case, $\mu_{\text{em}} = \mu_{\text{eo}}$. The calculated mobilities in Table 5.1 show that the addition of the heavy atom onto the dyes exhibits a uniform perturbation on $\mu_{\text{em}}$ (within experimental error) for the mono-substituted series (dye 8 vs. dyes 10, 12, 14, 16). The additional heavy atom on dyes 18 and 19
imposes a larger frictional drag leading to lower mobilities, since the effective charge was not altered.

The heavy-atom dyes were analyzed in a gel filled capillary column to investigate mobility differences under DNA sequencing conditions. The electropherogram is shown in Figure 5.3. As can be seen from this figure, the mono-substituted dyes co-migrated. The small late migrating peak was due to the di-substituted heavy-atom chromophores, which migrate slower due to the frictional constraints imposed on the dye by the additional heavy atom, consistent with the free solution results.

Table 5.1 Electrophoretic mobilities of heavy-atom modified dyes (8, 10, 12, 14, 16, 18, 19) in free solution.*

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\mu_{se}$ (cm$^2$/Vs) ($\times 10^4$)$^b$</th>
<th>$\mu_{app}$ (cm$^2$/Vs) ($\times 10^4$)$^b$</th>
<th>$\mu_{cm}$ (cm$^2$/Vs) ($\times 10^5$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.14</td>
<td>1.59</td>
<td>-5.5 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>2.24</td>
<td>1.73</td>
<td>-5.2 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>2.40</td>
<td>1.90</td>
<td>-5.0</td>
</tr>
<tr>
<td>14</td>
<td>2.62</td>
<td>2.14</td>
<td>-4.8 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>2.73</td>
<td>2.21</td>
<td>-5.2 ± 0.1</td>
</tr>
<tr>
<td>18</td>
<td>2.10</td>
<td>1.71</td>
<td>-3.9 ± 0.1</td>
</tr>
<tr>
<td>19</td>
<td>2.08</td>
<td>1.61</td>
<td>-4.7 ± 0.7</td>
</tr>
</tbody>
</table>

* Separation conditions: column i.d. = 75 µm, $L_{tot}$ = 58.5 cm, $L_{eff}$ = 50.0 cm, running buffer = 5 mM sodium borate (pH ~ 9.6, 50/50 CH$_3$OH/H$_2$O), $E$ = 427 V/cm, electrokinetic injection = 30 kV for 20 s, dye concentration = 5×10$^{-5}$ M, riboflavin concentration = 1×10$^{-6}$ M.

An attempt was then made to conjugate each chromophore to propynyl amino-modified ddATP 40a and ddGTP 40b analogs. Arylisothiocyanate dye 32 and succinimide ester dye 35 showed no conjugation to the ddATP. The succinimide
Figure 5.3 Capillary gel electropherogram of heavy-atom modified dyes 8, 10, 12, 14, 16, 18, 19. (1) dyes 8, 10, 12, 14, 16, (2) dyes 18, 19. Capillary gel conditions; 5% T / 5% C polyacrylamide gel; running buffer = 1× TBE; column i.d. 75 μm; $L_{tot} = 33$ cm; $L_{eff} = 26$ cm; $E = -250$ V/cm; electrokinetic injection at -5 kV for 3 s.

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ester of 35 appeared to hydrolyze before it could attach to the amino-linker arm of the dideoxynucleotide. Reversed-phase HPLC chromatograms showed the appearance of multiple unidentifiable peaks as well as a peak for the hydrolyzed 35 indicating that this dye did not attach to the dideoxynucleotide (data not shown). Legendre et al. have seen this lack of conjugation to negatively charged amino acids with a closely related arylisothiocyanate cyanine dye.21 Successful conjugation to the ddATP and ddGTP (Scheme 5.1, Figure 5.4) was achieved with alkylisothiocyanate dye 33. Increasing the length of the chain which contains the isothiocyanate functional group (33 vs. 32) improved the efficiency of conjugation to the highly negatively charged nucleotides. This may be due to the increased distance of the isothiocyanate from the chromophore in tyramine-derived 33, which decreases steric and/or electrostatic interactions that may occur between the analyte and dye. In addition, the use of an organic modifier, which inhibits aggregation of the dye, may be responsible for the more efficient conjugation of 33 with the nucleotides. The conjugates of dye 33 and ddNTP’s 41a and 41b showed no aggregation in aqueous buffer as demonstrated by absorption spectroscopy (see Figure 5.5) which exhibited only a monomeric band at 764 nm (compared to unlabeled dye at this concentration).

Strekowski has shown that aryl thiols efficiently displace phenols which are at the meso position on bridged tricarbocyanine dyes.22 Most extension reaction enzymes require the use of dithiothreitol (DTT) to efficiently incorporate dideoxynucleotides in DNA extension protocols. The action of DTT on the activity of sequencing enzymes is not completely understood, but its presence
may stabilize the tertiary structure of the enzyme allowing efficient incorporation of dideoxynucleotides. DTT is not an aryl thiol, but it does contain free alkyl thiol groups which could displace the phenol substituent at the meso position.

In order to evaluate the effect of DTT on our tricarbocyanine dyes, a series of absorbance and fluorescence experiments were performed with Dye 8 to simulate Sanger sequencing extension reaction conditions with DTT. The amount of DTT (0.5 M) was higher than that typically used in DNA sequencing applications (1 mM) in order to augment the effects for ease of observation. The absorbance and fluorescence spectra were acquired in the presence and absence of DTT before and after heating and the results are shown in Figure 5.6 and 5.7, respectively. The addition of heat does not significantly change the absorbance or fluorescence maxima of 8. However, the addition of DTT does result in a bathochromic shift of the absorbance and fluorescence maxima over time and is indicative of the formation of the thiol substitution product.2 The addition of heat with DTT results in a complete red shift in the absorbance and fluorescence maxima almost immediately. The products from the reaction without DTT and with DTT were analyzed by FABMS. Dye 9 has a molecular mass weight of 757.3 and the FAB mass spectrum of the dye sample which was heated at 80 °C without DTT confirms the presence of this sole product. However, the mass spectrum of the dye which was reacted with DTT showed the presence of a new peak at 817.6 which corresponds to the mass of a dye which contains DTT substituted at the meso position. The effect of DTT on 41a was not studied, but
should undergo the same reaction under extension conditions which would result in the cleavage of the dye from the dideoxynucleotide.

Interestingly, research by Legendre et al.\textsuperscript{19} and Steffens et al.\textsuperscript{23} has shown that a near-IR dye (IRD-40, shown in Figure 5.8) which was covalently attached to a deoxynucleotide or a DNA primer could be used for a Sanger DNA sequencing protocol reaction using DTT without noticing any detrimental effect on the conjugation. Legendre et al. incorporated IRD-40 labeled DNA primers into a sequencing protocol which utilized near-IR fluorescence lifetime discrimination of the DNA fragments.\textsuperscript{19} Steffens et al. incorporated dye-labeled deoxynucleotides into the DNA strand and detected the separated fragments by laser-induced fluorescence.\textsuperscript{23} In each case, there was no evidence which would indicate that DTT was reacting with the dye. However, Narayanan has observed the reaction of DTT with IRD-40 when the dye was attached to a deoxynucleotide.\textsuperscript{24}

5.4 Summary

The conjugation of dyes 32 and 33 to primary amines via the reactive isothiocyanate functional group allows for their use as near-IR fluorescent labels for amine modified dideoxynucleotides. Also, the mono- and un-substituted dyes (8, 10, 12, 14, 16) co-migrated under capillary gel electrophoresis conditions which will negate the need for post-run mobility correction analysis, decreasing the errors produced during sequence reconstruction. The use of these dyes may

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Figure 5.4 Reversed-phase HPLC chromatogram of ddGTP modified with a propargyl linker arm containing a primary amine covalently attached to 33. The absence of a free dye peak results from the removal of the free dye by anion exchange chromatography. Absorbance detection at 260 nm.
Figure 5.5 Absorbance spectrum of 41b in triethylammonium bicarbonate buffer (\( \sim 1.0 \text{ M, pH} = 7.0 \)). Concentration: \( 1.7 \times 10^{-3} \text{ M} \), pathlength: 1mm.
Figure 5.6 Absorbance spectra of 8 in: H₂O (-), H₂O with heating at 80°C for three minutes (■), in H₂O with 0.5 M DTT (•), with 0.5 M DTT in H₂O after 5 h (▲), with 0.5 M DTT in H₂O with heating (▼).

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Figure 5.7 Fluorescence spectra of 8 in: H₂O (●), in H₂O with heating at 80°C for three minutes (■), in H₂O with 0.5 M DTT (○), in H₂O with 0.5 M DTT after 5 h (▲), in H₂O with 0.5 M DTT with heating (▼), in H₂O with 0.5 M DTT with heating after 5 h (◆).
Figure 5.8 Structure of IRD40.
also help to minimize instrumental complexity in high throughput sequencing devices due to efficient excitation with a single laser operating at one wavelength and the ability to process the fluorescence on a single detection channel.

5.5 References


Conclusions and Future Work

The focus of this manuscript was to synthesize and characterize the photophysics of a set of tricarbocyanine chromophores which possess absorption and emission properties in the near-IR region of the spectrum (750 - 1000 nm). The primary goal of this research was to employ these dyes as fluorescent labels in different bioanalytical applications which employ laser-induced fluorescence detection, especially for DNA sequencing.

In Chapter 1, the principles of fluorescence were outlined with a focus on visible and near-IR fluorescence, and the advantages and disadvantages of performing analyses in each area were discussed. The important aspects which make near-IR fluorescence detection attractive are: (1) lower backgrounds compared to the visible case due to lower Rayleigh and Raman cross-sections and few analytes which exhibit intrinsic fluorescence in the near-IR, and (2) the use of semiconductor devices as excitation and detection components. In addition, the principle advantages and disadvantages of tricarbocyanine dyes were discussed.

In Chapter 2, the synthesis of a set of novel tricarbocyanine dyes was discussed. Generally, these were divided into two main categories: (1) heavy-atom modified tricarbocyanine dyes and (2) functionalized tricarbocyanine dyes. The heavy-atom modified dyes were synthesized according to modifications of
procedures outlined by Strekowski.¹ The synthesis involves the substitution of Cl at the meso position on the dye with various halogenated phenols. The final product of these reactions was isolated with fairly good yields (40-60%).

Functionalization of the chromophore with either an isothiocyanate or a succinimidyl ester was performed to make these dyes effective labels for covalent attachment to primary amines. The isothiocyanate was much easier to isolate than the succinimidyl ester due to the rapid hydrolysis of the ester after synthesis and, therefore could only be generated in situ. A series of isothiocyanate functionalized heavy-atom modified tricarbocyanine dyes were then synthesized according to a variety of procedures with moderate yields (20-30 %).²⁴

In Chapter 3, the various photophysical constants (ε, Φₘ, τₘ, κᵣ, κₚᵣ) for the synthesized dyes were determined using a variety of experimental techniques including absorbance, fluorescence and time-correlated single-photon counting. The general properties of these dyes include absorbance and fluorescence maxima in the near-IR with large ε (~ 190,000 M⁻¹cm⁻¹) and moderate Φₘ (0.05 - 0.15). All of these dyes exhibited molecular aggregation in H₂O, but the addition of SO₃⁻ groups onto the heterocyclic rings reduced the aggregation problem. The fluorescence lifetime was determined for each chromophore using time-correlated single-photon counting, which discovered an interesting apparent inverse heavy-atom effect with the halogenated phenol chromophores possessing the heaviest atom having the longest lifetime. In order to describe the influence of the heavy-atom on the various photophysical processes which depopulate the excited state, laser-induced flash photolysis experiments were performed. These experiments

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showed that $k_{\text{ac}}$ is increasing with increases in the weight of the heavy-atom substituent, but $k_{\text{ac}}$ decreases with a greater magnitude.

In Chapter 4, two functionalized dyes were examined as labels for amino acids separated by capillary electrophoresis in mixed CH$_3$OH/H$_2$O running buffers with laser-induced fluorescence detection. The analysis of the separated amino acids in mixed CH$_3$OH/H$_2$O buffers afforded an increase in the resolution between the valine and glycine conjugates with an increase in the number of theoretical plates with increasing CH$_3$OH content up to 60%. Additionally, the mass detection limit for dye-labeled arginine in 90/10 CH$_3$OH/H$_2$O was 21 zmol. The low limits of detection were primarily a result of the improved photophysics that were attained with running buffers with high CH$_3$OH contents.

The conjugation efficiency of an alkyl isothiocyanate and arylisothiocyanate to tryptophan and glutamic acid were determined in aqueous and mixed organic/aqueous solutions. The results demonstrated that the efficiency increases with increasing organic content which is indicative of the increased solubility of these dyes in polar organic solvents.

In Chapter 5, the functionalized dyes were evaluated as labels for DNA sequencing. Attachment of the chromophore to a ddATP and ddGTP were demonstrated. However, the DTT concentration used here was approximately 10× higher than used in DNA sequencing experiments. These results indicate that care should be taken when using DTT with these dyes.

Future work will include the attachment of the functionalized heavy-atom modified dyes to amine-modified DNA primers to be used in a Sanger DNA
sequencing experiment employing fluorescence detection of the labeled fragments. Narayanan has shown that tricarbocyanine dyes similar to those described in this manuscript which are attached to DNA primers, suffer less from the effect of DTT during extension reaction conditions. Upon successful completion of attachment, these dyes will be incorporated into a DNA sequencing detection scheme which employs fluorescence lifetime discrimination of the labeled DNA fragments that are separated by capillary gel electrophoresis.

6.1 References


Vita


In the fall of 1987, he entered Spring Hill College in Mobile, Alabama, as a Presidential Scholar and with an Athletic Scholarship. He received a Bachelor of Science degree in Chemistry in the Spring of 1991. In the fall of 1991, he began employment as a Chemist and was promoted to Process Specialist for Courtaulds Fibers, Inc. in Axis, Alabama. In the fall of 1992, he entered the graduate program in the Department of Chemistry at Louisiana State University in Baton Rouge, Louisiana. In 1997, he was awarded the James G. Traynham Award for excellence in teaching and research in the Department of Chemistry at Louisiana State University. He is currently a candidate for the Doctor of Philosophy degree in Analytical Chemistry. Upon graduation, he plans to begin his career as a Research Chemist with Transgenomics, Inc. in Omaha, Nebraska.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: James H. Flanagan Jr.

Major Field: Chemistry

Title of Dissertation: Synthesis and Photophysical Characterization of Near-IR Probes for Bioanalytical Applications

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

October 9, 1997

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