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Novel Near-Infrared Cyanine Dyes for Fluorescence Imaging in Biological Systems

Nilmi T. Fernando

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NOVEL NEAR-INFRARED CYANINE DYES FOR FLUORESCENCE IMAGING IN BIOLOGICAL SYSTEMS

by

NILMI T. FERNANDO

Under the Direction of Professor Lucjan Strekowski

ABSTRACT

Heptamethine cyanine dyes are attractive compounds for imaging purposes in biomedical applications because of their chemical and photophysical properties exhibited in the near-infrared region. A series of meso amino-substituted heptamethine cyanine dyes with indolenine, benz[e]indolenine and benz[c,d]indolenine heterocyclic moieties were synthesized and their spectral properties including fluorescence quntum yield were investigated in ethanol and ethanol/water mixture. Upon substitution with amines, the absorption maxima of the dyes shifted to the lower wavelength region (~600 nm),
showed larger Stokes shifts and stronger fluorescence which can be attributed to an excited state intramolecular charge transfer (ICT). High quantum yields were observed for primary amine derivatives and lower quantum yields were observed for secondary amine derivatives. Fluorescence quantum yields are greater for dyes with $3H$-indolenine terminal moieties than for dyes with benz[e]indolenine end groups. Benz[c,d]indolenine based heptamethine cyanine dyes exhibited the lowest quantum yield due to aggregation in solution. In general, the benz[e]indolenine heptamethine cyanines showed high Stokes shifts compared to indolenine dyes. For the meso-chloro dyes, the absorption maxima for the dyes shifted bathochromically in the order of indolenine, benz[e]indolenine and benz[c,d]indolenine.

INDEX WORDS: Near-infrared, Heptamethine, Heterocycle, Indolenine, Benz[e]indolenine, Benz[c,d]indolenine, Fluorescence, Spectroscopy, Fluorophores, Photosensitizer, Benzothiazole, Quantum yield, Nonlinear, Photophysical
NOVEL NEAR-INFRARED CYANINE DYES FOR FLUORESCENCE IMAGING IN BIOLOGICAL SYSTEMS

by

NILMI T. FERNANDO

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in the College of Arts and Sciences
Georgia State University
2011
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Nilmi T. Fernando

2011
NOVEL NEAR-INFRARED CYANINE DYES FOR FLUORESCENCE IMAGING IN BIOLOGICAL SYSTEMS

by

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Committee: Professor Gangli Wang

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Office of Graduate Studies

College of Arts and Sciences

Georgia State University

December 2011
DEDICATION

This dissertation is dedicated to my loving parents, my wonderful husband, my darling son and my dearest brother.
Acknowledgement

I am deeply indebted to my supervisor Professor Lucjan Strekowski, for his sage advice, insightful criticism and patient encouragement which aided this dissertation in innumerable ways. I am grateful to Dr. Gangli Wang and Zhenghua Tang for their generosity in allowing me to use the fluorometer. I will forever be thankful to Professor Laurence Hurley and Dr. Gary Flynn for their valuable help and support to achieve my goals. A special word of thanks to Dr. Maged Henary for helping me in the beginning of my research.

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I also thank my friends Jeff Klenc, Beth Raux, Shirish paranjpe, Adam Ehalt, Reid Daniel and Jamie Gragg for their support and friendship. Thanks to the wonderful staff in the Chemistry Department for always being so helpful and friendly. People here are genuinely nice and want to help you out and I’m glad to have interacted with many.
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1. INTRODUCTION

1.1 Polymethine dyes

Polymethine dyes contain the $\pi$-electron conjugated system that includes an electron acceptor end group ($\text{EG}_1$), an electron donor end group ($\text{EG}_2$) and the polymethine chain (PC) between them.\(^1\)

![Figure 1. Polymethine dye](image)

An electron acceptor group contains an atom of high electronegativity adjacent to a double bond as, eg, $\text{N}^+ =$, $\text{O}^+ =$, $\text{S}^+ =$, $\text{O}= \text{and the carbon atom } =\text{C}$ incorporated into a heterocyclic, normally monovalent residue. The electron donor group usually contains an atom bearing a lone pair of electrons such as N, O, S and bound to the carbon atom in a heterocyclic divalent residue. The polymethine chain is constituted of $sp^2$-hybridized carbon atoms forming a chain of conjugated bonds. Conjugation between the electron-donor and the electron-acceptor groups results in the displacement of $\pi$ electrons hence bond order equalization of the chromophore. Depending on the number of carbon atoms included in the end groups the number of methine units between the end groups can be
odd or even. The total number of $\pi$ electrons is normally even and exceeds the number of atoms by one. If the end groups have the same chemical constitution the polymethine dye is called *symmetrical* and the molecule belongs to $C_{2v}$ end groups.\(^1\) Shown below (I) is a simple symmetrical polymethine called streptocyanine.

![Diagram of symmetrical polymethine dye](image1)

The electron density distribution in the chromophore of such dyes is also symmetrical; in particular, the charges on the end heteroatoms and on the symmetrical carbon atoms are equal. If the end groups differ in chemical constitution, the polymethine dye is called *unsymmetrical*. Merocyanine dyes such as (II) are examples of unsymmetric polymethine dyes.

![Diagram of unsymmetrical polymethine dye](image2)
In the above mentioned polymethine dyes, the number ofvinylene groups in the
cromophore is \( n = 0, \ 1, \ 2, \text{ etc.} \) As the value of \( n \) increases by one dye absorption
maxima normally shift to longer wavelengths by \( \sim 100 \text{ nm} \), and is termed the vinylene
shift.

Cyanine dyes are a large class of synthetic polymethine dyes with a wide variety
of colors that can show absorptions from the ultraviolet to the infrared region. Cyanine
dyes are among the oldest known classes of synthetic dyes. The first dyes were discovered
in 1856 by Williams\(^2\) who was then working at Glasgow University who noted the
tendency of quinolinium salts to give intense colors on heating with silver oxide. The
name cyanine (from the Greek word kyanos) was attributed to its beautiful blue color. The
dye was extremely fugitive to sunlight and was of no use for ordinary fabric dyeing
processes. This large class of dyes with a wide variety of colors shows absorptions that
cover the ultraviolet to the infrared region and, as a group, cover a wide span of the
spectrum than those of any other dye class. The great usefulness of cyanines was
discovered later in silver halide photography and they included the most powerful
sensitizing dyes known. Cyanines have high light absorption per molecule coupled in
many cases with a single absorption band in the visible or infrared spectral region, which
gives very color selective absorption of light. These dyes also have a tendency to
aggregate which gives rise to even narrower, more color-selective absorption of light.
Cyanine dyes may have high chemical and photochemical reactivity.

A dinuclear cyanine consists of two nitrogen centers, one of which is positively
charged and is linked by a \( \pi \)-conjugated polymethine chain of an odd number of carbon
atoms to the other nitrogen (III, IV, V). Each compound is regarded as a resonance hybrid of two canonical structures and no single formula is a complete representation. One nitrogen atom is tertiary and the other one is quaternary. Early attempts were made to determine which nitrogen was quaternary and in 1920 the concept of the virtual tautomerism of the isocyanines was introduced. This theory was then applied to thiacyanines, 2'-cyanines, indocyanines and carbocyanines until it was outdated by the resonance theory in 1939. X in the formulae (III, IV, V represents a conjugate base (anion, acid radical). R was at first and is generally an alkyl group. In 1920 it was regarded as obvious that the nitrogen atoms must carry alkyl groups in order to permit the formation of dyes capable of existence in alkaline solution.³

Both nitrogens are each independently part of heteroaromatic moieties, such as pyrrole, imidazole, thiazole, pyridine, quinoline, indole, benzothiazole, etc. There are three types of cyanine dyes, namely, streptocyanines (III), hemicyanines (IV) and closed-chain cyanines (V).⁴ Tailoring the many characteristics of a dye is a well-practised art in the cyanine class. Combinations of heterocycles, substituents and chromophore lengths can yield a series of dye structures can modulate the spectroscopic properties of the dyes. Steric features of the substituents either enhance or decrease aggregation. Solubility in either aqueous or hydrocarbon solvents can be tailored by changing the substituents. Controlling the number of conformations of the methine chain is important to enhanced infrared absorption strength.
Cyanine dyes generally have all-trans geometry in their stable form. These conjugated molecules show absorption and fluorescence that are a function of the structure of the molecules.\(^4\)

Well-developed synthetic methods allow cost-effective manufacture of cyanines for commercial applications as well as a high degree of dye-structure design for new and innovative studies such as solar cells, electrophotograph and Langmuir-Blodgett non-linear optical layers and photoreceptors for processes activated by infrared solid-state lasers. Tailoring the many characteristics of a dye is a well practiced art in the class of cyanine dyes. Combinations of heterocycles, substituents and chromophore lengths can yield a series of dye structures having parallel shifts in oxidation-reduction potentials almost independent of absorption wavelength. Steric features of substituents either
enhance or decrease aggregation. Solubility in either aqueous or hydrocarbon solvents can be provided by other substituents almost independently from those that change redox potentials or steric properties. Controlling the number of conformations of the polymethine chain, achieved by several synthetic routes is important to enhanced infrared absorption strength.

1.2 The color and constitution of cyanine dyes

Compounds of the cyanine series include sensitizers that are characterized by a single narrow absorption band. Their range is so great that not only the whole visible spectrum is covered but there are also cyanines that absorb in the ultraviolet (UV) and near-infrared (NIR). As the sensitizing and absorption bands are closely related a study of the connection between color and constitution is of prime importance for their applications. Besides the series is of unique interest because it presents a wealth of variable factors which influence the colors of the products. Such are the nature of the nuclei, the length of the polymethine chain, positions of linking, effect of substitution in the nuclei or on the chain and inclusion of part of the ring in a chain. It was pointed out in 1935 that the tautomeric formulae which had been proposed for cyanines were identical in principle with resonance formulae and it was suggested that the quantum mechanical resonance conception could be applied to explain the colors of cyanines. Resonance within conjugated systems results in a tendency towards planarity of the molecule. Since resonance can only take place in between co-planar parts of the molecule, applications of the theory have led interferences as to the steric structure of certain cyanine molecules. In 1940 it was suggested that steric inhibition of the planarity of the molecule as the cause
of modified spectral absorption. Later, replacement of the two \( NH \) groups by two \( NMe \) groups to give symmetrical cyanines necessitated a departure of the molecule from planarity and this required energy. The non-planar cyanines always absorbed less intensely than the planar ones. The color and constitution of cyanine dyes can be understood by detailed consideration of the structural components, i.e. chromophoric systems, terminal groups and solvent sensitivity of the dyes. Resonance theories have been used successfully to describe the significant trends. These trends are useful for dye chemists to design dyes with specific colors, band shape or solvent sensitivity. It is useful to review the position attained as a result of the scientific work on the constitution of the cyanine dyes and then to note briefly some developments.

1. 3 Chromophoric systems

Well known since the early 1920s, cyanine dyes cover a large wavelength region due to the fact that, for every vinylene unit an increment of \(~100\) nm in absorption towards the red region takes place. Many symmetric near-infrared cyanine dyes with heterocyclic end groups have been described during the last decade.\(^5\) There are two extreme resonance structures in which the formal charges are located at the end of the chromophore. When drawing resonance structures, intermediate resonance structures with the charges closer to the center of the chromophore or with additional dipoles are less important. Structural changes that favor intermediate forms have significant effects on the color of symmetrical dyes containing.
The parent streptopolymethine VI absorbs at more than 700 nm with \( n = 3 \). Annelation at the heterocyclic end groups results, in general, in a bathochromic shift. Generally the intensity of absorption of cyanine dyes increases.

\[
\text{VI}
\]

The longest chain cyanine dyes known are less stable than dyes of shorter chain lengths. Cyanine dyes are weakly fluorescent. The fluorescence quantum yield of cyanine dyes reaches a maximum value and then decreases on going from short to long chain lengths.

There are numerous non-symmetric near-infrared (NIR) cyanine dyes with two different heterocyclic end groups or one heterocyclic and one non-cyclic end group that likewise absorb in the NIR region. Their absorption maxima are shifted to shorter wavelengths compared to those of corresponding symmetric dyes.
1.4 Effect of the nature of the terminal heterocyclic groups

Dyes from novel terminal groups are quite numerous. The heterocycles are of two principle types: basic or electron donating and acidic or electron accepting.

1.4.1 Basic heterocycles

In addition to the early benzothiazole dyes other heterocyclic thiazoles as well as related oxazoles, pyroles and imidazoles were subsequently used for cyanines. When two different terminal groups were incorporated certain unsymmetrical dyes absorbed at unexpectedly short wavelengths whereas the symmetrical dyes absorbed at longer wavelengths. These observations resulted in the concept of deviation, which related the characteristics of absorption to the basicities of various heterocycles. For the symmetrical dye VII, Michler’s hydrol blue, the two resonance structures are equivalent. In the unsymmetrical carbocyanine ‘styryl’ dye VIII the structure with the charged heterocycle is favoured particularly for highly basic heterocycles.

\[ \text{VII} \]
The highly basic heterocycles accommodates the positive charge readily and maintain the aromatic nature of the dimethylamino benzene group the resulting bond alteration induces a polyene character to the dye chromophore and the absorption is shifted to the shorter wavelength region.\(^1\)

1.4.2 Acidic heterocycles

A similar classification is made for the acidic electron-accepting terminal groups used in dipolar (merocyanine) chromophores, **IX**.

Cyanine dyes derived from these fundamental basic and acidic terminal groups are in current use today as photographic spectral sensitizers, chemotherapeutic dyes, laser dyes and biological stains.
1.5 Effect of the length of the chain

When heptamethine cyanines were introduced in 1933, the absorption maxima of ten of them were recorded as lying at a longer wavelength by 1900-2050 Å than those of the corresponding trimethinecyanines. The bathochromic shift on passing from methine- to trimethine- cyanines varied from 1060 Å to 1400 Å, while those on passing from tri- to penta-methinecyanines varied from 940 Å to 990 Å. Besides a bathochromic shift on lengthening the chain there was an increase in intensity of absorption. When the heterocycles differed in basicity, $\lambda_{\text{cal.}} - \lambda_{\text{obs.}}$ increased with increasing chain length.

1.6 Influence of substituents

1.6.1 Effects of substituents in terminal heterocyclic end groups

Substituents in the heterocyclic units of symmetrical dyes usually give rise to the increased conjugation of the end groups thus shifting absorption maxima to longer wavelengths (bathochromic effect) as illustrated by the benzothiazole dye $X$ and table 1 below.$^1$ However in the case where the substituents in the two end groups are of opposite electronic nature, the end group electron-donor abilities are changed in opposite directions, which results in derivation and a weakened bathochromic effect of such substituents.
Table 1. Absorption maxima in relation to substituents in benzothiazole (X) end group

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>$\lambda_{\text{max}}$, nm (Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>H</td>
<td>558</td>
</tr>
<tr>
<td>N(CH$_3$)$_2$</td>
<td>N(CH$_3$)$_2$</td>
<td>612</td>
</tr>
<tr>
<td>NO$_2$</td>
<td>NO$_2$</td>
<td>585</td>
</tr>
<tr>
<td>N(CH$_3$)$_2$</td>
<td>NO$_2$</td>
<td>588</td>
</tr>
</tbody>
</table>

1.6.2 Effects of substituents in the polymethine chain

Dye absorption spectra influenced by the substituents on the methine chain obey the Förster-Dewar-Knott rule (FDK rule). These substituents cause two types of changes in the properties of dyes. First, the absorption maxima shift depends on the inductive (electronic) effect of the substituent and its position on the methine chain. Second, the substituents that change the steric properties of the dye can affect both the color and aggregation. Substituents that affect the color of dyes through steric hindrance include: (i) small alkyl groups on the methine chain that alter the equilibrium between cis and trans
isomers of the chromophore, (ii) bulky substituents on the methine chain or the heterocyclic nitrogen which cause the chromophore to become non-planar and (iii) rigidizing substituents such as the six-membered carbocyclic rings in the polymethine part of dyes. Electron-donor substituents at electron rich positions (positions 1 and 3) of the chain give rise to bathochromic shifts whereas at electron deficient positions (position 2) of the chain they lead to hypsochromic shifts. Opposite trends of spectroscopic properties are observed with electron acceptor substituents at the corresponding positions. These regularities are most vividly demonstrated using benzothiazole derivatives like XI as an example.

![Image of benzothiazole derivative](attachment:image.png)
Table 2. Effect of substituents on the polymethine chain on $\lambda_{\text{max}}$ of benzothiazole dye, \textbf{XI}

<table>
<thead>
<tr>
<th></th>
<th>2</th>
<th>3</th>
<th>$\lambda_{\text{max}}$(EtOH)</th>
<th>$\Delta\lambda$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>H</td>
<td>H</td>
<td>558</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>NH$_2$</td>
<td>H</td>
<td>471</td>
<td>-87</td>
</tr>
<tr>
<td>H</td>
<td>F</td>
<td>H</td>
<td>522</td>
<td>-36</td>
</tr>
<tr>
<td>F</td>
<td>H</td>
<td>F</td>
<td>592</td>
<td>+44</td>
</tr>
<tr>
<td>H</td>
<td>CN</td>
<td>H</td>
<td>613</td>
<td>+55</td>
</tr>
<tr>
<td>CN</td>
<td>H</td>
<td>CN</td>
<td>490</td>
<td>-68</td>
</tr>
<tr>
<td>H</td>
<td>Ph</td>
<td>H</td>
<td>560</td>
<td>+2</td>
</tr>
</tbody>
</table>

When alkyl groups and cyclic fragments are substituted on the chain, they affect the absorption spectra the same as electron donor substituents according to the FDK rule. A vinylene group substituent on the other hand, displays a profound hypsochromic effect.

Table 3. Effect of alkyl and cyclic substituents on the polymethine chain, on $\lambda_{\text{max}}$ of \textbf{XI}

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>3</th>
<th>$\lambda_{\text{max}}$</th>
<th>$\Delta\lambda$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>H</td>
<td>558</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-(CH$_2$)$_2$-</td>
<td>594</td>
<td>+36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-(CH$_2$)$_3$-</td>
<td>565</td>
<td>+7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-CH=CH-</td>
<td>470</td>
<td>-88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1,2-C$_6$H$_4$-</td>
<td>518</td>
<td>-40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.7 Solvent sensitivity of cyanine dyes

The large effect of the environment, i.e. solvent, around a dye, on the absorption spectra of dyes led to the synthesis of hundreds of dyes to investigate the relationship between structure and spectral properties. Cyanine dyes exhibit solvent sensitivity. For dyes with long chromophoric chains of –CH= (methine) groups the conformation and absorption of charged dyes may change as a function of solvent. The design of infrared dyes with increased absorption at longer wavelengths incorporates conformation restricting groups in the polymethine chain. The solvent sensitive cyanine dyes exist in two distinct conformations; one with complete charge separation and the other with at least one nonionized form. The shifts in the absorption spectra that occur in polar and nonpolar solvents are assigned to these two isomers.

1.8 Synthesis of cyanine dyes

1.8.1 Monomethine cyanine dyes

A novel method for the preparation of symmetrical and asymmetrical monomethine cyanine dyes such as XII was developed by Deligeorgiev, et al. The chemistry is illustrated in Eq. 1 and it involves melting of a quaternary heterocyclic salt containing a 2- or 4- methyl group and a 2-sulfobetaine derived from a cationic heterocyclic system under basic conditions.
1.8.2 Trimethine cyanines

The classical orthoester approach to trimethine cyanines involves condensation under basic conditions of orthoesters with quaternary heterocyclic salts substituted with an activated methyl group.\(^8\) The methodology is illustrated in Eq. 2.

In similar transformations, a central one-cardon component of the trimethine bridge is derived from \(N, N'\)-diphenylformamidine\(^{10,11}\) or iodoform.\(^{11}\) The diphenyl formamidine method allows for the synthesis of unsymmetrical dyes containing two
different end-heterocyclic subunits or two different $N$-subunits at the identical heterocyclic systems.$^{12}$ The central methine moiety of the trimethylene bridge in dyes XIV and xv can also be supplied by a novel Vilsmeier type reagent derived from $N, N$-Dimethylformamide and hydrogen bromide (scheme I).$^{13}$

Scheme 1

![Scheme 1 Diagram](image-url)
A post-synthetic modification at a meso position of a meso-methyl substituted trimethine dye is illustrated in Eq. (3) by condensation with an aldehyde to give dye XVI. An ethyl analogue undergoes a similar condensation at the α position of the ethyl group.\textsuperscript{14}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image}
\caption{Eq. (3)}
\end{figure}

1.8.3 Pentamethine cyanines

A major synthetic route to this class of dyes involves condensation of cationic heterocyclic compounds containing an activated methyl group with derivatives of malondialdehyde. Synthetic examples used recently are shown in Scheme 2 by the preparation of a water soluble dye XVII\textsuperscript{9} and in scheme 2 by the preparation of dye XVIII containing two different N-substituents.\textsuperscript{15} A modification of the latter synthetic route for the synthesis of a different pentamethine cyanine dye has also been published.\textsuperscript{12}
Scheme 2

\[
\text{Scheme 2}
\]

\[
\begin{align*}
\text{HOOC} & \quad + \quad \text{MeO-CH=CH-CH(O\text{Me})}_3 \\
\text{Pyridine} & \quad 80^\circ \text{C}
\end{align*}
\]

\[
\text{XVII}
\]
Scheme 3

\[
\text{Scheme 3}
\]

\[
\text{HOOC-} \quad \text{Br} \quad \text{Br}
\]

\[
\text{Kl, MeCN}
\text{reflux}
\]

\[
\text{H}_2\text{NNH}_2, \text{MeOH, CH}_2\text{Cl}_2
\]

\[
\text{AcOH, pyridine}
\]

\[
\text{XVIII}
\]

\[
\text{HOOC} \quad \text{NH}_2
\]

\[
\text{CF}_3\text{COO}^{-}
\]
Highly polar dyes such as **XVII** and **XVIII** were purified by reverse-phase flash chromatography, using a commercial adsorbent, C18. This and similar adsorbents are low-cost chromatographic material that can be used in low-pressure reverse-phase chromatography.

1.8.4 Heptamethine cyanine dyes

The growing interest in heptamethine cyanine dyes as fluorescent biomarkers has caused active engagement in research finding various ways of synthesizing different kinds of this class of dyes. A vast majority of heptamethine cyanine dyes contain a six-membered carbocyclic system as part of the heptamethine chain such as in compound **XXI** (Eq.4). This important structural feature helps increase the rigidity of the system thus an increase fluorescence quantum yield. Sterically, this feature decreases aggregation of the dyes in solution.

![Chemical structures](image)

**XIX**: $R^1 = H, X = Cl, Br$

**XX**: $R^1 = COOEt, X = Cl$

The first heptamethine cyanine dyes were synthesized$^{16}$ by condensation of Vilsmeier-Haack reagent **XIX** derived from cyclohexanone and a heterocyclic salt.
containing an activated methyl group. An analogue of XIX derived from cyclopentanone is sometimes used for the preparation of the corresponding analogues of XXI.\textsuperscript{17} Strekowski, et al. introduced and ethoxycarbonyl substituted reagent XX for fascile functionalization of the corresponding dyes XXI (R\textsuperscript{1} = COOEt).\textsuperscript{18}

Strekowski and co-workers reported for the first time synthesis of a novel class of near-infrared (NIR) bis (heptamethine cyanine) (BHmC) dyes containing a flexible polymethylene linker between the two cyanine subunits.\textsuperscript{19, 20} Such bis cyanines are significant as bioanalytical tools due to their negligible fluorescence in aqueous solution and a strong increase in fluorescence (~1000 fold) upon protein binding. The bis dyes form an intramolecular stacking complex in solution, which quenches fluorescence, and the complex opens up upon binding of the dye proteins, which greatly increases fluorescence intensity.
Scheme 4

XXII

XXIII

MeCO₂Na, ethanol reflux

XXIV

BHmC-4: n = 4
BHmC-6: n = 6
BHmC-8: n = 8
BHmC-10: n = 10
1.9 Applications of cyanine dyes

The cyanine dyes came to the limelight in 1856\textsuperscript{2} for their application to impart light sensitivity to silver halide emulsions in a region of the spectrum to which silver halide is normally not sensitive.\textsuperscript{21} Besides their application in photography these dyes find wide applications in inorganic large band-gap semiconductors\textsuperscript{21-25}, in optical disks as recording media\textsuperscript{26-28}, in industrial paints, trapping of solar energy\textsuperscript{29}, as laser materials\textsuperscript{30-33}, in light harvesting systems of photosynthesis\textsuperscript{34-36}, as photorefractive material\textsuperscript{37}, as antitumor agents\textsuperscript{38} and as probes for biological activity.

1.9.1 Photography

Silver halide crystals have intrinsic sensitivity to blue light. In 1873, Vogel observed that certain silver halide plates had sensitivity to green light.\textsuperscript{39} This discovery led to a burst of activity to discover useful sensitizers for the new field of photography. Work advanced significantly in the 1920s when the chemical structures were elucidated and correlated with photographic activity. Dyes have been developed which can sensitize silver halide emulsions throughout the visible spectrum from 400 to 700 nm and to ca. 1300 nm in the infrared.

The sensitizing dye extends the sensitivity of silver halide crystals by adsorbing to the surface of the silver halide crystal and forming dye aggregates which absorb light at the requisite wavelength.\textsuperscript{40} The excited state of the dye then injects an electron into the conduction band of the silver halide crystal. This photoelectron then reduces an interstitial silver atom at specially designed traps composed of silver sulfide and gold.
Further reduction of silver atoms at this site produces a stable latent image. The most efficient photographic silver halide crystals can produce a stable latent image by absorbing as few as four photons.\textsuperscript{41, 42} This latent image acts as a catalytic site which then allows the selective reduction of the silver grain by a variety of specially selected reducing agents in an alkaline medium. By means of this selective reduction of silver atoms the latent image can be amplified by as much as $10^7$. This is the basis of the high sensitivity of silver halide photographic systems.

Adsorption to the silver surface is one of the most important requirements for a sensitizing dye. Intimate contact with the silver surface, facilitates electron transfer. Simple monomethine cyanines XXVI-XXVIII containing a variety of heterocyclic bases are effective blue sensitizers.
1.9.2 Imaging in biological applications

Fluorescence microscopy and fluorescence imaging are two of the most rapidly expanding areas of research in both medical and biological sciences. The detection of biological molecules in response to environmental change relies increasingly on fluorescent methods. Demand for more sensitive, more specific and more versatile reagents is increasing. Regents are required with high environmental stability, increased quantum yields for fluorescence, longer emission wavelengths, larger Stokes shifts and with good photostability. One continued area of research lies with the cyanine dyes. The cyanine dyes have been employed as fluorescent probes for several years. The generic structure of these dyes is shown below.

![Generic structure of cyanine dyes used as fluorescent probes](image)

**Figure 2.** Generic structure of cyanine dyes used as fluorescent probes

It can be seen that the cyanine dye chromophore is, in fact, a sulfoindocyanine. These sulphonated cyanine dyes have good solubility in biological media at physiological pH. Another important feature of these compounds is the site for biological attachment. N-Hydroxy-succinimidylesters are most commonly employed for labelling amino groups
present in antibodies, lipids, drugs, cell membranes and oligonucleotides, and in most cases the dyes exhibit low non-specific binding. By labelling a biological moiety with a fluorescent dye, one can track the progress of the biomolecule within a particular environment and also observe interactions with other entities using an array of fluorescent technologies. Upon excitation, sulfoindocyanines fluoresce in the visible and near infrared region of the spectrum depending upon the degree of conjugation within the chromophore employed. In general, excitation and emission wavelengths of the dye are well separated from the shorter wavelength background autofluorescence. This intrinsic characteristic of many biological specimens or indeed, scattering from cell debris, absorption of pharmaceuticals present in the analysis of medical samples or absorption from plastics present in microtitre plates can greatly interfere with emission signals and reduce sensitivity.

One technique employed to provide fluorescent probes with large Stokes' shifts utilises Energy Transfer Cassettes.\textsuperscript{44} In this method, a conjugate of a fluorescent donor or sensitiser is covalently linked to a fluorescent acceptor dye, providing an efficient mechanism for energy transfer from one dye to another. The overall Stokes' shift is dependent upon the wavelength of excitation for the donor fluor and the wavelength of emission for the acceptor fluor. Naturally, the efficiency of the energy transfer process will be dependent upon the distance between the donor and the acceptor, assuming that a Förster mechanism of energy transfer is occurring.\textsuperscript{45} Using cyanine dyes as both the donor and acceptors fluors, a range of energy transfer cassettes has been synthesised. Important criteria in the design of such constructs include an inert, covalent linker between the donor and the acceptor. This acts to hold the two complementary dyes in
close proximity. It is known that energy transfer between cyanine dyes is more efficient when contiguous dyes are oriented parallel to each other as opposed to any other configuration. Furthermore, when used in biological applications as a molecular reporter these dyes often need to be bioconjugated and therefore the presence of a biolabel is important. In many cases the label employed is the activated N-Hydroxysuccinimidyl ester. Alternatively, maleimide or other more specific labelling groups are used.

1.9.3 Cyanine dyes for information recording

The practical use of NIR dyes for optical data storage was commercialized from Taiyo-yuden as CD-R (Compact Disk Recordable) in 1988. At present two types of NIR dyes indolenine type cyanine dyes and phthalocyanine derivatives are practically used. In reversible optical storage systems, photochromic spiropyran dyes (XXIX) are used. Unlike an ordinary CD, the CD-R has an organic dye recording layer between the polycarbonate substrate and the light reflective layer. In addition, the polycarbonate substrate is etched with a spiral pre-groove. This pre-groove is used for guiding the laser beam, time measurement and various controls during recording. The laser beam, modulated by the recording signal, is focused on the groove. The beam heats and melts the recording layer of organic dye on the polycarbonate substrate, forming a series of pits. These pits are physically extremely stable, and are ideal for long-term data storage with the highest degree of reliability.
The color of the CD-R disc is related to the color of the specific dye that was used in the recording layer. The green CD-R, the cheapest of the three, uses the cyanine pigment. By itself, the pigment is blue in color, but together with the gold reflective layer, the bottom appears green. However, cyanine's ability to maintain reflectivity is poor giving it a life span of about 10 years. It also delivers the weakest reflection contrasts and thus can cause read errors when run on old CD-ROM drives.47

1.9.4 Cyanine dyes as photosensitizers in photodynamic therapy

Phototherapy is the use of visible or near-visible light in the treatment of disease. Phototherapy falls into two broad categories; direct and indirect. In the indirect method, an additional substance, a sensitizer, is administered before irradiation. The therapeutic process is initiated by light being absorbed by the sensitizer. One of the most interesting applications of NIR cyanine dyes is photodynamic therapy (PDT) of malignant tumors. This method is based on generation of singlet oxygen (\(^{1}\text{O}_2\)) at interaction of photoexcited molecule of photosensitizer (cyanine dye) with common triplet oxygen. The selective localizing of dye in tumor is achieved by administering by intravenous injection of its solution, 1-48 h prior to light treatment. The singlet oxygen is a powerful oxidant leading to necrosis of tumor tissue. The wavelength of the laser source and photosensitizer
absorption maximum is important parameters that determine the depth of photodynamic action on tissue. Owing to a red shifted wavelength absorption maximum their contribution in absorbance of sensitized tissue begins to predominate over its own absorption.46

The characteristics of the ideal photosensitizer have been discussed in recent reviews.48 They should have low levels of dark toxicity to humans and low incidence of administrative toxicity. They should absorb light in the red or far-red wavelengths in order to penetrate tissue. The PS should be a pure compound with a constant composition and a stable shelf life, and be ideally water soluble or soluble in a harmless aqueous solvent mixture. It should not aggregate unduly in biological environments as this reduces its photochemical efficiency. Cyanines have been studied as potential PDT tools during the last decade.49 Most of these compounds are cationic, in contrast to the more frequently used anionic photosensitizers such as hematoporphyrin derivatives, chlorins and sulfonated phthalocyanines. Since typically an electrical potential gradient of abouty180 mV exists across the mitochondrial membrane, cationic cyanines strongly concentrate into mitochondria, up to 1000-fold with respect to the extracellular concentration. Indoc 2 (XXX), a tumor-cell specific cyanine dye photosensitizer which reduces skin phototoxicity and damage to normal tissue, that is used in photodynamic therapy (PDT) is shown below.
1.9.5 Photovoltaic solar cells.

Traditionally new materials research for solar cell applications has been dominated by the inorganic semiconductor materials, silicon (Si) and gallium arsenide (GaAs), and influenced to a lesser extent by cadmium sulfide (CdS). These materials yield highly efficient photovoltaic devices whereby sunlight is converted directly into electricity. In the case of Si and GaAs cells, the stringent purity requirements and the complicated device fabrication techniques cause such high device costs that the practical large-scale utilization of these photovoltaic cells is economically infeasible at this time. A less popular approach to solar cell materials research has been the study of organic photoconductive compounds in the photovoltaic mode. The light harvesting dye is clearly a crucial component of the cell design and needs to fulfill several criteria; adsorption onto metal surface, overlap effectively with solar spectrum, inject electrons efficiently into metal oxide and be stable for many million cycles. Organic dyes have also been used successfully as attested by the very many articles and school projects on using fruit
berries as the dye in these cells. A range of dyes i.e. hemicaynine (XXXI), squarine (XXXII) and phthalocyanine (XXXIII) based dyes that are used as sensitizers in solar cells are shown below.

![Chemical structures with labels: XXXI, XXXII, XXXIII]

The photosensitivity of semiconductor electrodes used in photovoltaic cells, is extended to longer wavelengths when typical cyanine dyes are used to spectrally sensitize the photoinduced separation of electrons and holes. The dye-sensitized solar cell depends
on a layer of mesoporous nanoparticulate titanium dioxide to greatly amplify the surface area (200–300 m$^2$/g TiO$_2$, as compared to approximately 10 m$^2$/g of flat single crystal). The photogenerated electrons from the light absorbing dye are passed on to the n-type TiO$_2$, and the holes are absorbed by an electrolyte on the other side of the dye. The circuit is completed by a redox couple in the electrolyte, which can be liquid or solid. This type of cell allows a more flexible use of materials, and is typically manufactured by screen printing and/or use of Ultrasonic Nozzles, with the potential for lower processing costs than those used for bulk solar cells. However, the dyes in these cells also suffer from degradation under heat and UV light, and the cell casing is difficult to seal due to the solvents used in assembly. In spite of the above, this is a popular emerging technology with some commercial impact forecast within this decade.$^{51}$

1.9.6 Nonlinear optics

Non-linear optics studies the nonlinear interaction of electromagnetic radiation with a medium. The nonlinear interaction, which means the matter responds in a nonlinear manner to the incident radiation fields, endows the media a characterization to change the wavelength, or the frequency of the incident electromagnetic waves. Typically, this nonlinear interaction only observed at very high intensity (electric field) of incoming light. Figure 3 shows the schematics of linear and nonlinear interactions of waves and the media.$^{52}$
π-Electron conjugated organic systems are potentially important for various optical devices because of their fairly large third-order optical nonlinearity and their very fast response time compared with inorganic nonlinear optical materials. Several cyanine compounds have been reported\textsuperscript{53} as possible third-order optical nonlinear materials and the effect of π-conjugation length upon the nonlinear optical properties has been calculated. The compounds were symmetrical cyanines with quinoline rings (from Nippon Kankoh Shikiso Kenkyusho Co.) as shown below (XXXIV).
1.9.7 Ion recognition

The molecular design and synthesis of optical receptors with a capacity for photoswitching is a very active area of current research. A large number of molecules have been designed that consist of chromophores covalently linked to an ionophore, such as crown ethers, cryptands, and calixarenes; selective complexation of the ionophore with metal ions changes the spectral properties of the chromophore and shows photocontrollable cation binding. These dyes can be used as ion sensors and have potential application in trace metal detection in biological systems as well as for molecular data processing. Thomas et al.\textsuperscript{53} studied the photophysical properties of cyanine dyes containing an aza crown ether moiety (XXXV) and their complexation behavior with alkali-metal cations.
The chromoionophore is a useful ion sensor for the detection of alkali-metal cations in solution.

1.9.8 Application in medicine

Cyanine dyes are used for labeling of amino acids and in investigation of their transport in renal tissue, to visualize vasculature and localize endoplasmic reticulum in living cells by fluorescence probing. Indocyanine green (ICG) (XXXVI) is used in medicine for diagnosis of cardiovascular illnesses and research on kidney and liver functions.
It is used for determining cardiac output, hepatic function, and liver blood flow, and for ophthalmic angiography. It has a peak spectral absorption at about 800 nm. These infrared frequencies penetrate retinal layers, allowing ICG angiography to image deeper patterns of circulation than Fluorescein angiography. ICG binds tightly to plasma proteins and becomes confined to the vascular system. ICG has a half-life of 150 to 180 seconds and is removed from circulation exclusively by the liver to bile juice. Indocyanine green or ICG has the ability to bind 98% to plasma proteins – 80% to globulins and 20% to alpha-lipoprotein and albumin and thus, in comparison with fluorescein as a marker, has a lower leakage (slower emergence of dye from the vessels, extravasally). Because of the plasma protein binding, ICG stays for up to 20–30 minutes in the vessels (intravasally). When the eye is examined, it thus stays for a long time in tissues with a higher blood flow, such as the choroid and the blood vessels of the retina.54

1.10 Near-infrared absorbing heptamethine cyanine dyes

Recently NIR fluorescence ($\lambda_{\text{max}}: 700$-900 nm) have been used to image various biological events in-vivo. Cyanine dyes are an excellent kind of NIR fluorophore with large molar extinction coefficients and broad wavelength tenability. The spectra of cyanine dyes depend primarily on the length of the polymethine chain. The wavelengths of excitation and emission maxima shift bathochromically by ca. 100 nm with every vinylene unit. However along with the red shift of the wavelength the photostability of the dyes decreases. The tendency to undergo photodegradation becomes the common problem encountered by NIR cyanine dyes. Early analyses of structure-property
relationship of substituted cyanine dyes demonstrated a sensitive dependence of the photophysical properties on the substituents on the end heterocyclic groups. Continuous research in this area focus on effects of changes of substituted polymethine chains, substituted end groups, and the scaffold of the dyes. Strekowski, et al.\textsuperscript{55,56} found that the fixed conformation the photochemical and photophysical stabilities of NIR heptamethine cyanine dyes and introduced a chloro-cyclohexenyl group in the middle of the polymethine chain. Recently Chen, et al.\textsuperscript{57} reported that the central chlorine atom of cyclohexenyl-chain substituted by electron-donor groups can enhance the photostability of the dyes. Chen, et al also reported the effect of \textit{N}-substitution of 3H-indolenine on the photostabilities of the dyes. Electron donating groups on the \textit{N} atom of 3H-indolenine rings are favourable against photobleaching.\textsuperscript{57}

1.11 Near-infrared fluorescence spectroscopy

Fluorescence is a spectrochemical method of analysis where the molecules of the analyte are excited by irradiation at a certain wavelength and emit radiation of a different wavelength. The emission spectrum provides information for both qualitative and quantitative analysis. As shown in Figure 4 light of an appropriate wavelength is absorbed by a molecule (i.e., excitation), the electronic state of the molecule changes from the ground state to one of many vibrational levels in one of the excited electronic states. The excited electronic state is usually the first excited singlet state, \textit{S}1 (Figure 4). Once the molecule is in this excited state, relaxation can occur via several processes. Fluorescence is one of these processes and results in the emission of light.
Following absorption, a number of vibrational levels of the excited state are populated. Molecules in these higher vibrational levels then relax to the lowest vibrational level of the excited state (vibrational relaxation). From the lowest vibrational level, several processes can cause the molecule to relax to its ground state. Fluorescence corresponds to the relaxation of the molecule from the singlet excited state to the singlet ground state with emission of light. Fluorescence has short lifetime (~10^{-8} sec) so that in many molecules it can compete favorably with collisional deactivation, intersystem crossing and phosphorescence. The wavelength (and thus the energy) of the light emitted is dependent on the energy gap between the ground state and the singlet excited state. An overall energy balance for the fluorescence process could be written as:
Fluorescence intensity may also be reduced or eliminated if the luminescent molecule forms ground or excited state complexes (quenching). The quantum yield or quantum efficiency for fluorescence is therefore the ratio of the number of molecules that luminesce to the total number of excited molecules.\textsuperscript{58} According to the previous discussion, the quantum yield (\(\Phi\)) for a compound is determined by

\[
\Phi_{\text{fl}} = \frac{k_{\text{fl}}}{k_{\text{fl}} + k_{\text{i}} + k_{\text{ec}} + k_{\text{ic}} + k_{\text{pd}} + k_{\text{d}}}
\]  

\text{(6)}

relative rate constants \((k_x)\) for the processes which deactivate the lowest excited singlet states, namely, fluorescence \((k_{\text{fl}})\), intersystem crossing \((k_{\text{i}})\), external conversion \((k_{\text{ec}})\), internal conversion \((k_{\text{ic}})\), predissociation \((k_{\text{pd}})\), and dissociation \((k_{\text{d}})\).\textsuperscript{59} Owing to its sensitivity and selectivity fluorescence is used as a major analytical tool in the identification of target molecules of interest. Typically this involves using fluorophores as reporter molecules or labels. Background fluorescence from components other than the fluorophore of interest decreases the sensitivity of detection in solution. In biological systems this background fluorescence is typically from the autofluorescence of certain biological components (Figure 5).
This background fluorescence occurs at all wavelengths in the visible region and at various intensities depending on the concentration of interfering molecules present in the sample. Elimination of background fluorescence is essential where fluorescence must be detected in the presence of intervening molecules.

In fluorescence analysis excitation light can be scattered from interaction with various types of molecules at the surfaces of containers. This scattered light effect is present in all types of fluorescence detection. Scattered light contributes to significant portion of background noise, especially in biological samples, and it can be due to Rayleigh, Raman or Tyndall effects. These interferences can be minimized by using fluorophores with relatively high Stokes’ shifts (typically >40-50 nm).  

Fluorescence is known as a more sensitive and selective spectroscopic tool than absorbance because it is measured against a zero background and the magnitude of the signal $F$, at low concentration is given by the following equation.
\[ F = 2.303\Phi_I I_0 \varepsilon bC \] (7)

\( I_0 = \) excitation power, \( \Phi_I = \) fluorescence quantum yield, \( \varepsilon = \) molar absorptivity, 
\( b = \) path length, \( C = \) dye concentration

The limit of detection can be increased by a stronger excitation. However the limit of detection increases only as the \( 1/I_0^2 \) and a strong excitation source can cause photobleaching of the fluorophore. Near-infrared region (600-1100 nm) offers several advantages in fluorescence detection since noise resulting from scatter is related to wavelength of detection by the factor of \( 1/\lambda^4 \). Therefore detection in NIR region compared to UV/Vis region results in more than a six fold reduction in scatter noise. The low background interference in the NIR spectral region allows NIR fluorophores to be used as ideal probes in both biological and environmental applications.\(^6\)

1.11.1 NIR fluorophores and light absorption properties\(^6\)

In principle, the absorption property of a chromophore is a characteristic of the energy that is absorbed to cause the electronic transition. NIR-absorbing chromophores require relatively low energy for this transition and this corresponds to the longer wavelength of the electromagnetic spectrum in comparison with UV/Vis absorption. Since the transition energy is the energy required for a single electron to be excited from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LOMO), the gap between HOMO and LUMO is primarily responsible for the amount of energy required to induce this electronic transition. The shift of the absorption
maxima ($\lambda_{\text{max}}$) into the NIR region can be induced if the gap between the HOMO and the LUMO orbitals is brought close enough to give the transition energy in the range 48-26 kcal/mol. Effective structural modification of the chromophore can cause the desired bathochromic shift. Griffiths, et al.\textsuperscript{63,64} summarized in detail the general strategies to develop new NIR absorbing dyes based on this approach: (a) extending the conjugation of a chromophore (b) increasing the interaction between electron donor and electron acceptor groups within a chromophore (c) altering the electronegativity of atoms (d) introducing specific branching or bridging within a chromophore (e) metal complexation with a chromophore (f) intermolecular charge-transfer complex formation (g) formation of a free radical that is part of the chromophore.

1.11.2 Measuring fluorescence quantum yield, $\Phi_{\text{fl}}$.

The most reliable method for recording $\Phi_{\text{fl}}$ is the comparative method reported by Williams, et al.\textsuperscript{65} This method involves the use of well-characterized standard samples with known $\Phi_{\text{fl}}$ values. Essentially, solutions of the standard and test samples with identical absorbance at the same excitation wavelength can be assumed to be absorbing the same number of photons. Hence, a simple ratio of the integrated fluorescence intensities of the two solutions (recorded under identical conditions) will yield the ratio of the quantum yield values. Since $\Phi_F$ for the standard sample is known, it is trivial to calculate the $\Phi_F$ for the test sample. In practice, the measurement is slightly more complicated than this because it must take into account a number of considerations. For example:

- The presence of concentration effects, \textit{e.g.} self-quenching
• The use of different solvents for standard and test samples

• The validity in using the standard sample and its Φ_τ value.

These considerations are answered by working within a carefully chosen concentration range and acquiring data at a number of different absorbances (i.e. concentrations) and ensuring linearity across the concentration range, including the solvent refractive indices within the ratio calculation, cross-calibrating the standard sample with another, to ensure both are behaving as expected and allowing their Φ_F values to be used with confidence.66

Cyanine dyes are considered an enormous area of chemical research because of its wide applications in analytical, biological, biomedical and various other research fields. Near-infrared absorbing cyanine dyes, in particular, have drawn much attention due to their optimal spectral, chemical and biological properties as well as their excellent safety profile.67 The advantages of NIR fluorescence are the excellent penetrating ability through biological tissue as little NIR absorption and emission exist in natural biosystems and the great decrease in autofluorescence which is always encountered in visible light excitation.68 The longer wavelength region (>800 nm) is characterized by greatly reduced background fluorescence of any complex matrix. In addition since Raman scattering shows 1/λ^4 dependence, the background is further decreased in the NIR region. The electronic spectrum of a cyanine that absorbs strongly at 700-800 nm is characterized by a strong S^0 → S^1 band (ε of up to 300, 000 M⁻¹cm⁻¹) with a relatively strong shoulder at 600-700 nm (Sorret band, ε of up to 100, 000 M⁻¹cm⁻¹) and a weak absorption in the visible region at 400-600 nm (ε < 1000 M⁻¹cm⁻¹).69
Cyanine dyes can be considered as the main class of NIR fluorescent probes for biological applications at present because of their characteristic large molar extinction coefficients, moderate-to-high fluorescence quantum yields and a broad wavelength tunability. Fluorochromes with absorption and emission maxima between 650-900 nm are ideally suited for imaging in tissue due to the minimal absorption coefficients from hemoglobin, water and lipids over this range. Compounds based on the indocyanine scaffold are widely used as fluorophores for labeling biomolecules. The spectral characteristics of these compounds depend primarily on the length of the polymethine chain linking the two aromatic heterocyclic groups. As a rule of thumb, the wavelength of excitation and emission maxima shifts to the red by ca. 100 nm with every single vinylene unit. Most polymethine cyanines have the disadvantage that their Stokes’ shifts are less than 25 nm. A small Stokes’ can cause self-quenching and measurement error by excitation light and scattered light. Both of these features can decrease the detection sensitivity to a great extent. A major factor reducing the fluorescence quantum yield of the polymethine dyes is the photo-induced cis-trans isomerization of the polymethine chain. This process which occurs via a twisted excited singlet state leads to a non-radiative dissipation of absorbed light energy. Therefore NIR dyes with a larger Stokes’ shift are needed for NIR fluorescence bioassays. The scarcity of effective and commercially available NIR labels have reduced the bio-applications to a few known dyes as Indocyanine Green (Figure 1) which has drawbacks as low photostability, metal containing fluorophores or dyes with shorter emission wavelength.

Strekowski, et al. developed heptamethine cyanine dyes with a rigid chlorocyclohexenyl ring in the methine chain which can increase the photostability and
enhance the fluorescence quantum yield. This structure also provides with a reactive site for chemical substitution at the central ring. The chloro substituent in indolium heptamethine cyanine was easily replaced via an $S_{RN1}$ mechanism by a number of nucleophiles (Nu) (Eq. 8-11). This process is initiated by a single electron transfer (SET) from the nucleophilic species $Y^-$ (Eq. 8) to the cationic $\pi$-system of the chromophore to form two radical species. This study illustrated the effect of the hard and soft nucleophiles on the mechanism of meso-substitution and provided functionalized chromophores with an absorption range of 615-820 nm.

\[
\begin{align*}
(R-\text{Cl})^+ + Y^- & \rightarrow (R-\text{Cl})^* + Y^* \\
(R-\text{Cl})^* & \rightarrow R^{\cdot+} + \text{Cl}^-
\end{align*}
\]

(8)  (9)

\[
\begin{align*}
R^{\cdot+} + Y^- & \rightarrow (R-Y)^* \\
(R-Y)^* + (R-\text{Cl})^+ & \rightarrow (R-Y)^+ + (R-\text{Cl})^*
\end{align*}
\]

(10)  (11)

After dissociation of R-Cl$^*$ to the radical cation R$^{\cdot+}$ (Eq. 9), reaction with a nucleophile Y$^-$ (Eq. 9) results in the intermediate radical nucleophile adduct (R-Y)$^*$ that serves as the one-electron donor in the radical propagation process. This process is consistent with the cationic chromophore’s affinity for electrons. This novel chemistry later became a cornerstone in the subsequent synthesis work by Strekowski and other
workers. Their approach to synthesis including SET-mediated dechlorination of NIR dyes is illustrated in Eq. 12.

The reaction of XXXVII with 4-(isothiocyanato) benzenethiol directly gives compound XXXVIIIf which is a useful reagent for labeling of proteins with a near-infrared chromophore. The mechanism of these highly efficient transformations involves a $S_{RN1}$ pathway which starts with a single-electron-transfer (SET) from a nucleophilic
species to the dye. These transformations are essentially instant for nucleophiles that are good single-electron donors, such as phenoxide or benzenethiolate ions, when conducted in a solvent that supports the SET process, such as dimethyl sulfoxide or $N,N$-dimethylformamide. Recently many heptamethine cyanines were developed as biosensors and fluorescent probes by nucleophilic substitution reactions at the central position. Phenol$^{73}$ and thiophenol$^{74}$ moieties were used to replace the chlorine in these dyes but the resulting enol and thioenol ether bond in these molecules is chemically unstable. Peng, et al$^{75}$ reported that amine-substituted tricarbocyanines have shorter wavelength of absorption, larger Stokes’ shift and stronger fluorescence intensity than non-substituted tricarbocyanines. Conversely, the synthesis of derivatized dyes is strongly inhibited in solvents, such as water and alcohols, that do not support the SET process. This analysis of the reactivity of dye XXXVII and analogs has important ramifications for future design, synthesis, and application of functionalized indolium heptamethine cyanines. Thus, dyes XXXVII and similar chloro-substituted chromophores are readily available, and their chlorine atom can be displaced by the reaction with nucleophiles under $S_{RN1}$ conditions. When used for labeling of biomolecules in aqueous media, the derivatized dyes are stable at neutral pH but may undergo a nucleophile addition reaction with the chromophore under basic aqueous conditions, as discussed above. However, the resultant adducts were quantitatively decomposed to the starting near-infrared dyes by weak acid.
In spectra (Figure 6) of the synthesized dyes three features were obvious; a large Stokes shift (~120 nm), broad and fairly structureless fluorescence spectra and no mirror image relationship between the absorption and fluorescence spectra. The authors contributed these features to an intramolecular charge transfer (ICT). A widely used criterion to identify a charge-transfer state is whether dyes have a strong solvatochromism. In contrast with dye XXXIX, a marked negative solvatochromism (a 58 nm shift from water to acetone) in the absorption spectrum of dye XLa was observed.
with increasing polarity of the solvent was observed, but no apparent solvatochromism in
the emission spectra. These properties were attributed to hydrogen-bonding interaction
between the solvents and the dye molecule. A structural change accompanying ICT from
a bridgehead amine (a locally excited state (LE) of a pyramidal geometry which is
formed after excitation) in the ground state to a flattened state (a planar configuration).\textsuperscript{76}
It was suggested that the rate of the transfer from LE to ICT is lowered in viscous polar
solvents and LE emission is dominant.

Kiyose, et al\textsuperscript{77} synthesized a series of IR-786 derivatives in order to examine the
relationship between the nature of the amine substituent and the photochemical properties
(Eq. 14). It was found that the lower the electron density of the amine the longer the
wavelength of absorption (Table 4). The results provided a rationale for the molecular
design of novel ratiometric NIR probes based on the differences in electron-donating
ability of the amine substituent before and after reaction with a biomolecule.
The importance of near-infrared cyanine dyes in fluorescence imaging in biological systems continues to increase. Also development of dyes with distinctive structural features continues to increase. The end heterocyclic bases and the nature (length and substitution) of the polymethine chain basically determine the optical properties of these dyes. Structural modification of NIR dyes while maintaining their spectral properties within the NIR region is important and requires detailed investigation.

Lee, et al. reported fluorescence life-time properties of near-infrared cyanine dyes in relation to their structure. The fluorescence life-time (FLT) measurements of heptamethine dyes with indolium, benz[e]indolium and benz[c,d]indolium heterocyclic systems (Figure 3.4) were measured. The absorption and emission maxima of indolium dyes decreased about 35 nm and 30 nm respectively compared to those of benz[e]indolium analogue. A remarkable FLT increase from 0.98 ns for XLII to 1.48 ns for XLIII was observed when the benz[e]indolium was replaced by indolium end group. The FLT of the newly developed dye XLII was 0.3 ns. The dramatic decrease in FLT was attributed to the influence of both the heterocyclic system and/or the short
polymethine chain. The steric hindrance between the heteroaromatic fragments with a shorter methine chain was reported to partly contribute to the non radiative decay of the molecule thus results in the observed FLT decrease.

The overall results showed that indolium-based dye (XLIII) generally exhibit approximately 30% longer FLT times relative to the structurally similar benz[e]indolium dye (XLII). This observation was in contrast to the suggestion made by Murphy and
Schuster\textsuperscript{79} that increase in the size of the heterocyclic system results in FLT increase by possibly retarding $k_{rot}$.

Wolinska, et al\textsuperscript{80} reported NIR bis(indolium heptamethine cyanine) dyes XLV and XLVI(a-f) with a spacer derived from oligo(ethylene glycol). The molecules were designed as improved non-covalent labels for nucleic acids and proteins. The presence of oxygen atoms in the bridge linking the two dye moieties results in an increased solubility of the bifunctional molecules in water and aqueous buffers in comparison to the more hydrophobic analogs containing a polymethylene linker. These dyes were bifunctional heptamethine cyanines that absorb and fluoresce in the near-infrared region (>700 nm).
The final yields of these highly polar products were quite low, even for the optimized procedures described in the experimental section. Nevertheless, the described preparations were inexpensive and highly reproducible, and the final dyes were analytically pure, as judged by the results of the elemental, thin-layer-chromatographic, and spectral analyses. It was of interest to compare spectral properties of the ether-linked NIR dyes with those of their oligomethylenelinked analogs, a limited number of which have been published by them previously.\textsuperscript{82-84} A striking difference is the lack of correlation between the maximum absorption wavelength and the length of the polymethylene chain.

Mader, et al.\textsuperscript{85} reported photophysical characteristics of pentamethine indocyanine dyes (Figure 7) shown below. Substituents at the aromatic system and substituents in the polymethine chain were investigated with respect to fluorescence life-times and fluorescence quantum yields. Substitutions in the polymethine chain increased the nonradiative energy dissipation of the excited singlet state and decreased the fluorescence quantum yield, relative to the unsubstituted compound. The dyes with extended aromatic systems (\textbf{S0301} and \textbf{S0430}) had the lowest fluorescence quantum yields ($\Phi_F = 0.08$) while \textbf{S0387} and Cy 5 had the highest ones ($\Phi_F = 0.18$ and $\Phi_F = 0.27$). The latter two also had the longest fluorescence life-times $\tau_F = 0.70$ and 1.0 ns. These results once again prove that the indolenine based dyes show higher fluorescence quantum yields and longer fluorescence life-times compared to dyes based on benz[\textit{c,d}]indolenine.
Figure 7. Structures of the pentamethine cyanine dyes
2. RESULTS AND DISCUSSION

The near-infrared luminescence techniques reported recently in literature were overwhelmingly bioanalytical or biologically related analyses that take advantage of the low interference of the NIR spectral region. Fluorescence \textit{in vivo} imaging has become one of the major foci of interest. Heptamethine cyanine dyes employed as fluorescence labels and sensors \textit{in vivo} have attracted immense interest because their spectra reach the NIR region where biological matrix exhibits the least absorption and auto-fluorescence background. The rationale for the research work described in this dissertation pertains to the synthesis and characterization of novel, stable cyanine chromophores that absorb and fluoresce in the NIR region of the electromagnetic spectrum. A major bottleneck in complete utilization of NIR fluorescence for many applications is the limited number of fluorophores with high fluorescence efficiency and good stability. Heptamethine cyanine dyes are a class of NIR fluorophores that have been used for many applications because of their photophysical properties in the NIR region but they have poor photostabilities compared to pentamethine and trimethine cyanine dyes.

Literature\textsuperscript{86} reports have demonstrated that incorporating a cyclohexene ring in the polymethine chain, increases photostability and minimizes non-radiative decay via cis-trans isomerization thereby increasing the quantum yield and life-time. This structure also provides the dyes with a reactive chloro-group for chemical substitution at the central position. Accordingly, a six-membered chlorocyclohexenyl group was incorporated in the polymethine chain in anticipation of increasing the fluorescence quantum yield of the dyes. The \textit{meso}-chlorine was substituted with different amines in
order to increase the Stokes’ shift of the dyes thereby reducing fluorescence quenching which in turn help improve the fluorescence quantum yield.

A condensation reaction between two equivalents of a quaternary salt of a heterocyclic base containing an activated methyl group with an unsaturated pentamethinium salt (Vilmeier-Haack reagent), furnished the heptamethine cyanine dyes described in this work. All dyes contain a trimethylene bridge at the center of the chromophore because the resultant increase in rigidity of the molecule increases the efficiency of fluorescence. A series of meso-amino substituted heptamethine cyanine dyes containing indolium, benz[e]indolium and benz[c,d]indolium heterocyclic moieties were synthesized. The large heteroaromatic subunits and the trimethylene bridge render the polymethine chain more rigid and give rise to low rates of internal conversion and cis/trans photoisomerization due to a reduced number of vibrational degrees of freedom. This, in turn, results in an increased quantum yield of fluorescence and a longer lifetime in comparison to photolabile dyes such as indocyanine green (ICG or IR-125). The indolium and benzindolium derivatives offer the additional advantage of an extrememly narrow absorption band. As a general rule, the dyes containing benz[c,d]indolium systems are more stable and show a bathochromic shift in their electronic spectra in comparison with indolium and benz[e]indolium counterparts.

The central chloro substituent in the heptamethine cyanine dyes was replaced via an $S_{RN1}$ pathway by amines that are good single electron donors. $S_{RN1}$ replacement was carried out in DMF, a solvent that supports single electron transfer, and is completely suppressed in water. Their fluorescence quantum yields were measured in ethanolic and
50% (v/v) ethanol/H$_2$O media. The relationship between the nature of the amino substituent and the photochemical properties, as well as the change of fluorescence quantum yield among heptamethine cyanine dyes with different heterocyclic bases was studied. Accordingly, the suitability of these dyes as probes in biological systems for fluorescence imaging is investigated.

The approach to synthesis and functionalization of NIR cyanine dyes is illustrated in the following schemes.

2.1 Synthesis of indolium based heptamethine cyanine dyes

2.1.1 Vilmeier-Haack-Arnold (VHA) formylation of cycloheanones\textsuperscript{87} via the system POX$_3$/CH$_2$Cl$_2$/DMF/PhNH$_3$Cl

The preparation of meso-chloro pentamethinium salt was accomplished using the system POCl$_3$/CH$_2$Cl$_2$/DMF/PhNH$_3$Cl (Scheme 5) to provide the central ring structure of indolium, benz[e]indolium, benz[c,d]indolium and benzothiazole heptamethine cyanine dyes. These intermediate dyes provide a mobile halogen atom for further derivatization of the NIR chromophore (\textit{vida infra}), as already discussed in section 1.11.2.
The bisimine 3 obtained from VHA formylation of cyclohexanone was obtained in a moderate yield of 76%. The broad peaks in the $^1$H NMR and $^{13}$C NMR spectra of the product are a result of the dynamic nature of the imine/vinyl amine moieties. Formation of this product involves the Vilsmeier-Haack reagent $N$-chloromethylene-$N,N$-dimethylammonium dichlorophosphonate formed from the reaction of DMF and POCl$_3$. Hydrolysis of iminium functionalities in the intermediate provides a bisaldehyde which is further transformed into the pentamethinium salt by treatment with anilinium hydrochloride. The mechanistic pathway to the pentamethinium salt 3 is postulated in the following reaction Scheme 6.
2.1.2 Conversion of 2, 3, 3-trimethylindolenine to N-alkylindolium iodide

To accomplish this reaction, 2,3,3-trimethylindolenine (4) was heated under reflux in acetonitrile for 48 hours in the presence of the alkyl halide. The resulting salt either precipitated out of solution or formed a solid upon concentration. The crude product was purified by treatment of the solution with diethylether which caused crystallization to obtain pink color crystals (5a-d) typically in 50-60% yield.

2.1.3 Synthesis of meso-chloro indolenine heptamethine cyanine dyes

The heptamethine chain of chloro dyes was assembled from the aldol-like condensation of the cationic heterocyclic compound containing an activated methyl group, with the iminium salt, carried out under reflux conditions in ethanol in the presence of a base, i.e. sodium acetate. The crude mixture, after concentration was purified by crystallization which furnished the pure product in reasonably good yield and
purity. The product was stable at room temperature over long periods of time. The $^1$H NMR spectra of compounds 5a-d indicated that the compounds were analytically pure to be used in the subsequent synthesis without further purification.

Scheme 7

The mechanism for the above condensation reaction between the indolenine base (5a-d) and the Vilsmeier reagent (3) is shown in the following scheme. A satisfactory yield of the product 6a-d (85%) was obtained when 3 eq. of the heterocyclic base was used. Sodium acetate was used in the reaction as a base to scavenge the acidic proton of the methyl group of the heterocyclic salt.
Scheme 8
The polymethine chain of the cyanine dyes has been shown to exist in an all-trans form. The conjugated \( \pi \)-electron system is most stable if the polymethine chain is planar. However, considerable steric hindrance can be induced by the terminal heteroaromatic nuclei. In order to alleviate this steric pressure, the chromophore can either enlarge the bond angles of the polymethine chain or the chromophore can adopt a propeller-like twist of the heteroaromatic nuclei to produce dihedral angles of up to 55°. Conformational analysis of indolium heptamethine cyanine dyes by \( ^1 \)H, NOE and decoupling experiments
and $^{13}$C spectroscopy have been conducted previously in our laboratories.\textsuperscript{88} The corresponding numbering system for the heptamethine cyanine dyes is shown below.

![Figure 9. A numbering system for the heptamethine cyanine dyes\textsuperscript{88}](image)

The molecular symmetry is exemplified by the $^1$H NMR spectrum shown in Figure 5. The overall simplicity of the spectrum is consistent with the presence of a symmetrical chromophore. The protons in the cationic polymethine chain produce two distinct doublets at 6.55 ppm (1’ (7’), $J = 14$ Hz) and at 8.38 ppm (2’(6′), $J = 14$ Hz). The magnitude of the vicinal coupling constant suggests that the all-trans character of the polymethine chain and the slight propeller-like twisting of the chromophore (Figure 5). The vast difference in the chemical shifts of the methine protons is due to the alteration of charge along the cationic $\pi$-electron system. The 2’ (6’) carbons are electron deficient and the two equivalent protons give rise to the doublet downfield (8.38 ppm). A diagnostic triplet at 4.35 ppm for the N-CH$_2$ protons also illustrates the symmetry of the chromophore.
2.1.4 **Synthesis of meso-amino derivatized indolenine heptamethine cyanine dyes**

Strekowski, et al. \(^8\) showed for the first time that the central chloro substituent in indolium and benzindolium heptamethine cyanine dyes are easily replaced via an \(S_{RN}1\) pathway by nucleophiles that are good single electron donors. Accordingly the chloro group in the following indolium heptamethine dyes was replaced by dimethylamino, hexylamino, \(N\)-methylpiperazino, \(N\)-phenylpiperazino and aniline groups. The nitrogen in the terminal groups were substituted with alkyl groups. Substitution reactions were carried out in 100 mg scale in DMF. The reaction conditions varied with the amine as well as with the chloro heptamethine dye. In some instances the reaction was accomplished with less number of equivalents of the amine and stirring at room temperature. In other instances, the reaction mixture had to be heated and the reaction time was longer for the substitution to be complete. Extraction into dichloromethane, concentration and purification by flash chromatography furnished the amines in ~20\% yield. The dyes were considerably stable at room temperature but somewhat hygroscopic as indicated by elemental analysis.

The amine derivatives were identified by a characteristic absorption band at 600-700 nm, a hypsochromic shift from that of the meso-chloro dye. Emission wavelength varied from 700 to 800 nm. The amines showed a more significant Stokes shift than the parent dyes. These characteristics were useful as well as expected of the chromophoric systems that are good NIR fluorochromes suitable for fluorescence imaging. Synthesis of amines 7 a-p are shown in Eq. 12 and below. \(^1\)H NMR spectra of two of the amino derivatives of indolenine heptamethine cyanine dyes are given in Figure 11.
6a-d

\[ \text{a: } R = \text{CH}_3, X = \text{I} \]
\[ \text{b: } R = \text{CH}_3, X = \text{I} \]
\[ \text{c: } R = \text{OH}, X = \text{I} \]
\[ \text{d: } R = \text{CH}_3, X = \text{Br} \]

\[ \text{a: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{b: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{c: } A = \text{NH}_2, R = \text{CH}_3, X = \text{Br} \]
\[ \text{d: } A = \text{NH}_2, R = \text{CH}_3, X = \text{Br} \]

\[ \text{f: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{g: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]

\[ \text{h: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{i: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{j: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]

\[ \text{k: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{l: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{m: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{n: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{o: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
\[ \text{p: } A = \text{NH}_2, R = \text{CH}_3, X = \text{I} \]
Figure 10. $^1$H NMR spectra of 7a and 7j in CDCl$_3$ at 30 °C
2.2 Synthesis of benz[e]indolium heptamethine cyanine dyes

The benz[e]indolium end group with an extra phenyl group increases π-electron conjugation of the heptamethine scaffold. Increased conjugation decreases the shifts its spectra to the longer wavelength region. Also because of the increased aromatic group, planarity of the molecule is expected to increase which prevents aggregation and in turn decrease excited state fluorescence quenching.

2.2.1 Synthesis of meso-chloro benz[e]indolium heptamethine cyanine dyes

The synthesis of the chloro dyes 10a-b was accomplished by condensation of various N-substituted 2, 3, 3-Trimethyl benz[e]indolenine base with the iminium salt, 3, in the presence of triethylamine after heating for approximately 2 h at 80° C. Several attempts to synthesize the dyes using the typical conditions (sodium acetate/ethanol) gave lower yields of the product. The overall yield of the reaction significantly improved with triethylamine as base and only 2 equivalents.
\(^1\)H NMR spectrum of 10a (Figure 11) shows the additional aromatic protons that belong to the benzindolenine heterocycle.
Substitution of the benz[e]indolium end group increases the effective length of the cationic polymethine chain and shifted the absorption maximum to 820 nm.

2.2.2 Synthesis of meso-amino benz[e]indolium heptamethine cyanines

The nitrogen in the heterocyclic end group was substituted with methyl and n-butyl groups. The resulting meso-chloro dyes were treated with diethylamine, hexylamine and N-methylpiperazine to furnish the corresponding amines. The amino products obtained are listed below in Eq. 19.

\[ \text{H NMR spectra of two of the amino derivatives 11a and 11f are shown below (Figure 12).} \]
Figure 12. $^1$H NMR spectra of 11a and 11f in CDCl$_3$ at 30 °C
2.3 Synthesis of benz[c,d]indolium heptamethine cyanine dyes

As a general rule the dyes containing benz[c,d]indolium systems are more stable and show a bathochromic shift in their electronic spectra in comparison with indolium and benz[e]indolium counterparts. The large heteroaromatic subunits and the trimethylene bridge at the heptamethine chain give rise to low rates of internal conversion and cis-trans photoisomerization due to a reduced number of vibrational degrees of freedom. This in turn, results in an increased quantum yield of fluorescence and a longer lifetime of fluorescence. Not only benz[c,d]indolium derivatives show the longest $\lambda_{\text{max}}$ these are the most stable of this class of dyes (no changes in absorption were observed after the solution in methanol had been exposed to air and sunlight for 3-4 weeks). The base was synthesized starting from the commercially available benzo[c,d]indol-2(1H)-one, 12. A previously published procedure was modified to obtain the product 13 in purer form.\(^{90}\)

![Chemical Reaction](image)

The product 13 was a dark green free flowing solid in 60% yield. Compound was characterized by MS (ESI). Treatment of the sulfide 13 with methyl iodide in acetone
under reflux conditions for 1/2 h afforded 14. The reaction mixture was then cooled to room temperature and filtered. The methylthio derivative was directly used in the subsequent synthesis without further purification due to the highly unstable nature of 14.

After several attempts to obtain the product 15 in the synthetic scheme in pure form failed some modification had to be done to the original procedure. Rather than using TEA alone, a moderate amount of sodium acetate was added to the reaction mixture. The reaction was then heated to 60° C for three hours. The resulting precipitate was filtered at room temperature washed with copious amounts of ether and dried to obtain 15 as a crystalline brown solid. This compound was found to be stable over a period of time before it was substituted on the nitrogen.
The protected tricyclic base was then alkylated at the nitrogen atom by reaction with methyl iodide. The original procedure was modified to add potassium hydroxide instead of the weak amine TEA which produced 17 in low yield and difficult to purify. Reaction was carried out in DMF at 80° C for 7 h. The resulting red-brown solution was subjected to concentration and drying under vacuum and was purified on a chromatotron eluting with dichloromethane to obtain a dark red solid 17.

The protected 2, 2-dimethyl-5-(1-methylbenz[c,d]indol-2(1H)-ylidene)-1,3-dioxane-4,6-dione, 16, was then hydrolyzed under strong refluxing acidic conditions (conc. CH$_3$COOH and conc. HCl) to yield the desired heterocycle, 1,2-dimethylbenz[c,
$d$-indol-1-ium iodide, 17. Compound 16 was refluxed in glacial acetic acid for 20 min. before addition of concentrated HCl via a glass pipet until the color of the solution turned from red-orange to light green. The reaction mixture was cooled to room temperature and was treated a saturated solution of potassium iodide. The resulting brown-orange precipitate was then filtered and dried under vacuum to yield 17.

Inefficient literature preparation of 17 involved 7 steps.\textsuperscript{92} A short, albeit even more inefficient synthesis\textsuperscript{93} of 17 was based on electrophilic cyclization of 1-acetamidonaphthalene in the presence of POCl$_3$ (reflux in PhNO$_2$) to give benzindole (yield 5%) followed by alkylation.

2.3.1 Synthesis of meso-chloro benz[c,d]indolium heptamethine cyanine 18

Following a previously procedure\textsuperscript{94} procedure to synthesize this dye failed due to decomposition. The reaction afforded either negligible amount of product or unchanged starting materials after almost 2 hours of reflux. Further heating decomposed the reaction mixture. Pyridine was used as a base instead of sodium ethoxide which yielded the product very slowly or no significant amount of product. Using acetic anhydride as a
solvent worked successfully in small reaction scale but failed in moderate scale. Finally the published procedure\textsuperscript{95} was used without any added base. These reaction conditions afforded the product 18 in 2-5 h of reflux. It is noteworthy that the yield of the product under these reaction conditions was considerably high.

Figure 13. $^1$H NMR spectrum of 18 in DMSO-$d_6$ at 30° C

The $^1$H NMR (DMSO-$d_6$) of 18 shows the methine protons and the aromatic protons.
2.3.2 Synthesis of meso-amino benz[c,d]indolium heptamethine cyanine Dyes

Figure 14. $^1$H NMR spectrum of 19 in CDCl$_3$ at 30°C
The $^1$H NMR of 19 exhibits the additional $-\text{CH}_3$ protons in the amino group. The broader peaks in the spectrum indicate that the compound is aggregated in polar solvents.

Replacing the meso-chloro with amines was successful only with dimethylamine. The other amines were stable and did not show any reaction upon mixing with the starting material. The above reaction afforded the desired product 19 after stirring at room temperature for 12 h. Other amine substitution was abandoned after several attempts failed to accomplish the desired products. The reaction mixtures decomposed upon heating to 50° C for ~ 1 h. Addition of too many equivalents of the amine decomposed the material.

2.4 Synthesis of benzothiazole heptamethine cyanine dyes

Additional chromophores were obtained from the benzothiazolium salts 21a-b (scheme 10). Benzothiazolium salts, 21a-b were obtained by treating benzothiazole with methyl iodide and butyl iodide. Reaction of 21a-b with the pentamethinium salt 3 provided the expected benzothiazolium heptamethine cyanine dyes 22a-b with an absorption maximum at 804 nm. These dyes were synthesized using triethylamine as a base in acetonitrile medium. However, synthesis of the benzoazolium analog failed to provide the heterocyclic base in pure form. The salt was always mixed with the starting material. This is most likely attributed to the labile nature of the benzoazolium system that apparently undergoes hydrolysis during reaction.

The $^1$H NMR spectrum of 22b is shown below in figure 15.
Scheme 10

\[ 20 \xrightarrow{\text{R-I, MeCN, } \Delta} 21_{a-b} \xrightarrow{\text{1 eq. 3, MeCN, } 80^\circ\text{C}} 22_{a-b} \]

21a-b:
- a: R = CH₃
- b: R = (CH₃)₂CH₃

22a-b:
- a: R = CH₃
- b: R = (CH₃)₂CH₃
Several attempts to derivatize the chromophores with different amine failed due to the labile nature of the C-N bond, that results from the nucleophilic nature of the S atom in the heterocyclic end group. Therefore synthesis of amine derivatives of the benzothiazole dyes was discontinued.

Figure 15. $^1$H NMR spectrum of 22b in DMSO-$d_6$ at 30 °C
2.5 Synthesis of water soluble heptamethine cyanine dyes

2.5.1 N-oligo(ethylenoxy) heptamethine cyanine dyes

After the initial screening of the hydrophobic amino derivatives of the heptamethine cyanine dyes for fluorescence quantum yield, novel water soluble heptamethine cyanine dyes similar to the oligo (ethylenoxy) linked dimmers, were synthesized with the assumption that water soluble dyes will not aggregate and produce increased quantum yield. To make the dyes soluble in water and applicable as potential fluorescent probes in an aqueous environment, N-oligoether derivatized amine substituted dyes were synthesized and analyzed and for the above properties. Dimeric heptamethine dyes in which the two chromophoric subunits are linked by ether or an oligoether linker was described by Strekowski and co-workers. The presence of oxygen atoms in the bridge linking the two dye moieties results in an increased solubility of the bifunctional molecules in water and aqueous buffer in comparison to the more hydrophobic analogs containing methyl or polymethylene linkers. Under low concentrations in aqueous solution these dyes exist in an intramolecular clam-shell conformation. However, at increasing chromophore concentrations, these bichromophores exhibit π-π stacking interactions and tend to aggregate in solution. This aggregation would result in hypsochromic absorption and low fluorescence quantum yield, which would limit their applicability as NIR chromophores. To overcome this problem and further increase solubility under aqueous conditions, substituting two oligoether linkers at the N positions of the heterocyclic moieties was proposed. The following reaction scheme illustrates the synthesis carried out in Strekowski lab.
2.5.2 Synthesis of bridged N-oligoether derivatives of heptamethine cyanine dyes

Structurally rigid heptamethine cyanine systems are well known to improve the chemical and photostability of the dyes. To improve their spectral and photophysical properties heptamethine cyanine dyes with indolenine base were derivatized with bridged oligo (ethylenoxy) chains of various lengths (Scheme 11). This synthesis was carried out with the expectation of greatly increasing fluorescence quantum of rigidified, conformationally stable systems. The following scheme illustrates the synthesis of bridged oligo (ethylenoxy) derivatives. Similar derivatives were reported by Strekowski and co workers\textsuperscript{95} as side products of synthesis of bis (heptamethine) cyanine dyes.
Synthesis of the oligo(ethylenoxy) bridging linker was accomplished using commercially available compounds, 1-chloro-2-(2-chloroethoxy)ethane, 1,2-bis(2-chloroethoxy)ethane and 2,2′-((oxybis(ethane-2,1-diyl))bis(oxy))diethanol. 1-chloro-2-(2-chloroethoxy)ethane and 1,2-bis(2-chloroethoxy)ethane were converted to their diiodo adducts using sodium iodide under reflux conditions. The resulting precipitates were purified by filtration and washing with cold acetone. Since tetra ethylenoxy dichloride was not commercially available, the diiodo adduct had to be synthesized using tetra
ethylenglycol. Tetraethyleneglycol was first converted to its bis tosylate counterpart followed by conversion to diiodo adduct using sodium iodide. All three diiodo derivatives $23a-c$ were used in subsequent synthesis of the bis indolenine salts.

Previous attempts to synthesize the bridging groups as dibromo adducts and formation of the bis-salts failed because of the very high polarity of the products. The products were obtained as dark pink color semisolids after purification by column chromatography. Later the diiodo adducts were synthesized and the bis-salts were obtained as dark pink colored solids that were easy to purify by recrystallization in ether. The bis-salts were synthesized with analytical purity and were used for synthesis of the bridged heptamethine cyanine dyes, $25a-c$, shown in scheme 9.

The meso-chloro heptamethine cyanine dyes were synthesized using the conditions reported by Wolinska, et al. $^8$ These conditions were later found to yield impure or unsuccessful product formation. Later the conditions were modified to TEA as a base in acetonitrile/ethanol 9:1 which produced the dyes in high yields and reasonable analytical purity. The chloro dyes were not analytically pure as indicated by elemental analysis. The dyes were subsequently subjected to derivatization with amines in DMF, but failed under many different conditions. The dyes were either decomposed or did not react upon addition of amine in DMF medium. A possible reason for this unreactivity of the dyes would be their steric conformation. Because of the bridged oligo (ethylenoxy) linker the dyes may not exist in planar conformation any longer but be twisted. This twisted conformation of the dyes may hinder the reaction of the bulky amines with the meso-chloro group.
2.6 Spectral and photophysical properties of the amino derivatives of the heptamethine cyanine dyes

As the importance of NIR fluorescent cyanine dyes in biological imaging increases, dyes with distinctive structural features were developed to optimize the desired spectral properties. Structures of the heptamethine dyes under investigation are broadly categorized into three main groups based on their heterocyclic ring systems; indolium based, benz[e]indolium based and benz[c,d]indolium based heptamethine dyes. The amino derivatives of indolium heptamethine dyes were used for an initial screening of spectral properties in methanol medium. The absorption $\lambda_{\text{max}}$, emission $\lambda_{\text{max}}$ were measured and their absorption coefficients were calculated using a series of concentrations of the dyes in methanol. The respective data are presented below for the following general structure, 7.

![General Structure](image)

7
Table 5. Variation of spectral properties of different amine derivatives of indolenine heptamethine cyanine dyes in methanol

<table>
<thead>
<tr>
<th>A</th>
<th>-NH(CH₂)₃CH₃</th>
<th>-N(CH₃)₂</th>
<th>-N CH₃</th>
<th>-N Ph</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ_{abs} (nm)</td>
<td>623</td>
<td>640</td>
<td>697</td>
<td>677</td>
<td>734</td>
</tr>
<tr>
<td>λ_{fl} (nm)</td>
<td>734</td>
<td>765</td>
<td>782</td>
<td>772</td>
<td>776</td>
</tr>
<tr>
<td>ε × 10⁻⁵ M⁻¹ cm⁻¹</td>
<td>0.59</td>
<td>0.66</td>
<td>0.55</td>
<td>0.62</td>
<td>1.06</td>
</tr>
</tbody>
</table>

Absorption and emission λ_{max} increase from primary alkyl amines to secondary alkyl amines. Cyclic secondary amines show longer wavelengths of absorption and emission compared to both primary and secondary alkyl amines, i.e. hexylamine and dimethylamine. Aryl amines show the longest λ_{max} of absorption and emission. The data show that the Stokes shift of the dyes decreases in the order shown. The Stokes shift is higher for primary and secondary alkyl amine derivatives. For cyclic amines and aryl amines the Stokes shift decreases. There was no significant change in absorptivity except for the aryl amine.

The change of spectral properties with increasing N-alkyl chain length was investigated for the indolium series in methanol medium. The data are shown below in table 6.
Table 6. Variation of spectral properties with variation in length of R group in methanol

<table>
<thead>
<tr>
<th>R</th>
<th>- CH₃</th>
<th>-(CH₂)₃CH₃</th>
<th>-(CH₂)₇CH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ_{abs}(nm)</td>
<td>640</td>
<td>659</td>
<td>659</td>
</tr>
<tr>
<td>λ_{fl}(nm)</td>
<td>765</td>
<td>775</td>
<td>773</td>
</tr>
<tr>
<td>ε × 10^3 M⁻¹cm⁻¹</td>
<td>0.66</td>
<td>0.68</td>
<td>0.70</td>
</tr>
</tbody>
</table>

The absorption and emission λ_{max} and the Stokes shift show no significant change with increasing chain length of the N-alkyl group for the amino derivatives of the indolium heptamethine dyes. Therefore it is obvious that the N-substituted alkyl groups do not affect the spectral properties of this type of dyes. Electron donating substituents on the N position of the heterocyclic groups are known to protect the dyes against photobleaching. Substituting alkyl groups on the nitrogen of the heterocycle decreases the effective length of the π-conjugated system and gives photostability to the dye.

The absorption maximum λ_{max} for the dimethyl amino derivative of indolium dye 7d was measured in methanolic solutions of varying concentration of water.
Figure 16. Absorption spectra of 7d in 100% methanol (solid), 50% methanol (dashed) and 10% methanol (dotted)
With increasing concentration of water the $\lambda_{\text{max}}$ shifts to the shorter wavelength region (hypsochromic shift). This observation can be attributed to dye aggregation in polar solvents. Carbocyanines have been known for their aggregation phenomenon. These aggregates are self-assembled collections of molecules that are formed due to van der Waals interactions, hydrogen bonding or hydrophobic interactions. Two basic forms of aggregates have been described. J-aggregates (named by “E. E. Jelley” in *Nature*[^1]) are characterized by red-shifted absorption spectrum (compared to the monomer band), sharp absorption band and enhanced fluorescence. H-aggregates (hypsochromic shift) are characterized by blue-shifted absorption spectrum (compared with the monomer band), broad absorption band with negligible or low fluorescence.

The dye molecules may aggregate in a parallel way (plane-to-plane stacking) to form a sandwich-type arrangement (H-dimer) or in a head-to-tail arrangement (end-to-end stacking) to form a J-dimer. Extensive studies on J- and H- aggregates have resulted in the proposal that these aggregates exist as a one-dimensional assembly in solution that could be in (a) ladder type (b) staircase type and (c) brickwork type shown in Figure 15.

![Types of aggregates of cyanine dyes]

**Figure 17.** Different types of aggregates of cyanine dyes

J-aggregate formation depends on high aqueous solubility. Based on the blue-shift of absorption wavelength of the dye solutions upon increasing aqueous environment in can be concluded that dye 7d and possibly the other indolenine based dyes form H-type aggregates in solution. This reasoning can be further proved by the hydrophobic nature of this type of dye as exemplified by their structure.

Spectral properties including absorption (\(\lambda_{\text{max}}\)), emission (\(\lambda_{\text{em}}\)), absoprtivity and fluorescence quantum yield of the hexyl, dimethyl and N-methylpipperazine derivatives of indolenine heptamethine dyes and benz[e]indolenine heptamethine dyes with N-methyl substitution were measured and compared in ethanolic solutions. Table 7 summarizes the observed properties of these of dyes.

Spectral properties of the heptamethine cyanine dyes remained in the NIR region. As is shown in tables 7 and 8, the absorption and emission maxima (\(\lambda_{\text{abs}}\) and \(\lambda_{\text{em}}\)) of all given benzindolium dyes were higher than those of the indolium based dyes. This observation agrees with the general rule that, increase in conjugation of the scaffold increases the \(\lambda_{\text{max}}\). The extra phenyl ring in the bulky benzindolium contributes to increased conjugation in the dye molecule causing a bathochromic shift in absorption and emission. Fluorescence quantum yields are considerably greater for dyes with indolenine end groups than with benz[e]indolenine end groups and the quantum yield of the dye with benz[c,d]indolenine end group is the lowest. The lowest quantum yield was obtained for the dye with benz[c,d]indolenine end group. It is obvious from these results that although expected otherwise the fluorescence quantum yield decreases with bulky heterocycles attached to the heptamethine chain. It can therefore be concluded that bulky heterocyclic
groups destabilizes the excited singlet of these dyes. A possible and reasonable argument for this observation is that the large aromatic groups make the dyes aggregate thus causing quenching of fluorescence. These results are further proclaimed by literature reports where the indolium-based heptamethine dyes showed about 30% longer fluorescence life times compared to those of benz[e]indolium-based dyes. Their results clearly showed that the extra phenyl groups destabilized the excited state of these dyes.

The absorption coefficients of the dyes show no significant variation except for the benz[c,d]indolenine dye where it is lower. The Stokes shifts of benz[e]indolenine dyes are the highest. This observation agrees with their absorption and emission shifting to longer wavelength region. These results are in contrast though, to the observation that these dyes have lower quantum yields compared to those of indolenine based dyes. Overall, having a large Stokes shift makes these dyes suitable candidates for fluorescent probes.

For dyes with both indolenine and benz[e]indolenine heterocyclic groups, the quantum yield is higher for primary amine derivatives, than for the secondary and more rigid amines. This observation can be explained in terms of an excited state intramolecular charge transfer (ICT) and an accompanying conformational change in the bridgehead amine. One important structural change accompanying intramolecular charge transfer is that the pyramidal arrangement of the bridgehead amine (Figure 19) in the ground state is considerably flattened in the ICT state.
In detail a locally excited (LE) state (pyramidal) geometry is formed after excitation\textsuperscript{76} and then it is transformed into an ICT state (planar configuration). It is suggested that the rate of the transfer from LE to ICT is lowered in secondary and cyclic amines. Therefore in such amines the pyramidal arrangement does not change at the whole process and the ICT emission disappears. Therefore the intensity and the quantum yield of fluorescence decrease in conformationally rigid amines.
Table 7. Spectral properties of indolenine dyes and benz[c,d]indolenine dye, 19 in ethanol

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\varepsilon \times 10^{-5}$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi_{\text{fl}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>655</td>
<td>773</td>
<td>0.63</td>
<td>0.47</td>
</tr>
<tr>
<td>7b</td>
<td>678</td>
<td>787</td>
<td>0.56</td>
<td>0.46</td>
</tr>
<tr>
<td>7e</td>
<td>632</td>
<td>732</td>
<td>0.74</td>
<td>0.74</td>
</tr>
<tr>
<td>7f</td>
<td>646</td>
<td>760</td>
<td>0.71</td>
<td>0.92</td>
</tr>
<tr>
<td>7i</td>
<td>695</td>
<td>789</td>
<td>0.86</td>
<td>0.33</td>
</tr>
<tr>
<td>7j</td>
<td>704</td>
<td>793</td>
<td>0.79</td>
<td>0.33</td>
</tr>
<tr>
<td>19</td>
<td>687</td>
<td>749</td>
<td>0.40</td>
<td>0.0045</td>
</tr>
</tbody>
</table>
Table 8. Spectral properties of benz[e]indolenine heptamethine cyanine dyes in ethanol

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ&lt;sub&gt;abs&lt;/sub&gt; (nm)</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt; (nm)</th>
<th>ε × 10&lt;sup&gt;-5&lt;/sup&gt; (M&lt;sup&gt;-1&lt;/sup&gt;cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Φ&lt;sub&gt;fl&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>11a</td>
<td>690</td>
<td>819</td>
<td>1.22</td>
<td>0.19</td>
</tr>
<tr>
<td>11b</td>
<td>701</td>
<td>822</td>
<td>0.77</td>
<td>0.21</td>
</tr>
<tr>
<td>11c</td>
<td>657</td>
<td>768</td>
<td>1.03</td>
<td>0.32</td>
</tr>
<tr>
<td>11d</td>
<td>667</td>
<td>791</td>
<td>0.66</td>
<td>0.51</td>
</tr>
<tr>
<td>11e</td>
<td>712</td>
<td>822</td>
<td>0.78</td>
<td>0.24</td>
</tr>
<tr>
<td>11f</td>
<td>742</td>
<td>828</td>
<td>0.94</td>
<td>0.09</td>
</tr>
</tbody>
</table>
The change of spectral properties was investigated for $N$-butyl derivatives of the heterocyclic groups of the dyes in ethanol (Tables 7 and 8). The hexylamine derivatives of $N$-butyl dye show increased quantum yield compared with the $N$-methyl dye. The dimethylamine derivatives of the $N$-butyl dyes and $N$-methyl dyes show similar quantum yield data. $N$-methylpiperazino derivative of $N$-butyl indolium dye (Table 7) and its $N$-methyl counterpart show the same quantum yield while $N$-butyl benz[e]indolium dye shows a significantly low fluorescence quantum yield compared to its $N$-methyl counterpart.

The photophysical properties of the heptamethine dyes were measured in 50% ethanol/water. The corresponding data are shown in table 9. For the $N$-methyl derivatives of indolium and benz[e]indolium heptamethine cyanine dyes, the quantum yield decreased in 50% ethanol:water compared to neat ethanol. A reasonable explanation for this observation is, because of the hydrophobic nature of these dyes, their aggregation in the more polar aqueous:ethanol medium quenches the excited state fluorescence producing a lower quantum yield. The wavelengths of absorption and emission do not show a significant change from those in ethanolic medium.
Table 9. Spectral properties of indolenine and benz[e]indolenine heptamethine cyanine dyes in 50% ethanol:water

<table>
<thead>
<tr>
<th>Dye Structure</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\varepsilon \times 10^{-5}$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi_{\text{fl}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>650</td>
<td>771</td>
<td>0.54</td>
<td>0.42</td>
</tr>
<tr>
<td>11a</td>
<td>684</td>
<td>810</td>
<td>0.64</td>
<td>0.15</td>
</tr>
<tr>
<td>7e</td>
<td>628</td>
<td>738</td>
<td>0.70</td>
<td>0.48</td>
</tr>
<tr>
<td>11c</td>
<td>657</td>
<td>773</td>
<td>0.50</td>
<td>0.27</td>
</tr>
<tr>
<td>7i</td>
<td>690</td>
<td>780</td>
<td>0.63</td>
<td>0.28</td>
</tr>
<tr>
<td>11e</td>
<td>705</td>
<td>811</td>
<td>0.66</td>
<td>0.10</td>
</tr>
</tbody>
</table>
The unsubstituted meso-chloro dye with benz[c,d]indolenine heterocyclic base, showed the highest $\lambda_{\text{abs}}$ of all the systems at 1005 nm. Its dimethylamino derivative shows a blue-shifted absorption wavelength in the same region as the other amines. This observation can be attributed to the steric hindrance caused by the dimethylamine substituent on the two bulky heterocyclic groups. The benz[c,d]indolium heterocyclic dyes are known to cause steric hindrance on each other trying to be in a planar configuration especially when the polymethine chain is shorter, i.e. trimethine. With a rigid cyclohexenyl ring in the middle of the polymethine chain the planar molecule is experiencing a lot of steric hindrance. When the chlorine atom is replaced by a bulky dimethylamine group, the hindrance caused by sterics increase, and the planarity of the molecule is skewed. This twisting of the molecule to prevent steric hindrance may disturb the $\pi$-electron conjugation of the dye. Also as indicated by $^1$H NMR (DMSO-$d_6$), this dye tends to aggregate significantly in high concentration. The resolution of NMR decreased upon increase of concentration of the sample and the peaks became broader. Aggregation of cyanine dyes changes their spectral properties from those of monomers. Therefore the change in $\lambda_{\text{max}}$ for absorption and emission changes in the aggregated state. This could be a potential reason why the $\lambda_{\text{max}}$ and $\lambda_{\text{fl}}$ of the benz[c,d]indolenine dye decreased even lower than those of some of the indolium dyes. Because the $\Phi_{\text{fl}}$ of the amine derivative of 18 significantly low, further synthesis of similar derivatives was not pursued assuming that all amine derivatives of these dyes will have insignificant fluorescence quantum yields.

The change in quantum yield for both $N$-Methyl and $N$-Butyl substituted dyes shows the same trend, the quantum yield for indolium based dyes being higher than that
of benz[e]indolium based dyes. This observation is in accordance with the results reported by Lee, et al.\textsuperscript{78} for fluorescence life-time (FLT) measurements for similar dyes, i.e. indolium based and benz[e]indolium based. The FLT of the indolium based dye showed a higher FLT than the benz[e]indolium based dye.

Dyes with $N$-Bu group show higher quantum yield for hexylamine derivative. For other amine derivatives the quantum yields are almost the same except for the methyl piperazine derivative of benz[e]indolenine dye with $N$-Bu group, whose quantum yield is considerably low.

The dyes showed increased wavelengths of absorption and emission of all derivatives of benz[e]indolenines compared to wavelengths of absorption and emission of indolenine dyes, as illustrated in below. The increased conjugation of the benzindolenine dyes lowers the energy gap between HOMO and LUMO of the molecules thus shifting their spectra to longer wavelength.

The same pattern of change of fluorescence quantum yield between indolenine based and benz[e]indolenine based dyes can be seen in 50% H$_2$O/ethanol, quantum yield of dyes with indolenine nuclei higher than those with benz[e]indolenine nuclei. In general, the quantum yields of all the dyes were decreased in 50% water/ethanol medium compared to those in ethanol which can be accounted for by the fact that these dyes tend to aggregate considerably in the aqueous medium because of their highly non-polar character. In the aggregated form dyes show completely different photophysical properties.
After a ground state \( \pi \) electron in the dye molecule has been excited, it rapidly relaxes from the higher vibrational states to the lowest vibrational state of the excited electronic state, \( S_1 \). The rate for this relaxation is on the order of picoseconds (\( 10^{12} \text{ sec.} \)) and usually occurs before measurements can be made on the system. After reaching the lowest vibrational state of the excited electronic state the excited state can decay to the ground state by a number of mechanisms. The following Jablonski diagram explains the phenomena that an excited state electron undergoes.

The system can lose the energy by internal conversion \( k_{\text{ic}} \) (heat), quenching \( k_q \) (external conversion), by emission of a photon \( k_{\text{fl}} \) (fluorescence), or by intersystem crossing \( k_{\text{isc}} \) (phosphorescence). Inter-system crossing produces a triplet state, where the spins of the excited and ground state electrons are no longer paired. Since the emission
from the triplet state is forbidden, this state is very stable and can have lifetimes (milliseconds to seconds). Most compounds decay by non-radiative processes (such as heat) and are therefore not fluorescent. Fluorescent compounds, on the other hand, decay to the ground state by the emission of light. The energy of the photon that is emitted as the electron decays to the ground state depends on the energy difference between the excited and ground state at the time of emission. The rapid decay of excited vibrational states generally implies that the state from which the system decays is independent of the excitation wavelength. However, the state to which the system decays is not necessarily the lowest vibrational state of the ground electronic state. Therefore, the emission spectra of fluorescent compounds will also show fine structure.

In theory, the energy of the transition from the lowest energy states is the same for both absorption and emission. In practice, the average energy of the emitted photon is generally less than the corresponding absorption band. This red shift is due to a change in the local environment of the excited state during its lifetime. The re-organization of solvent dipoles will lower the energy of the excited state, causing a red shift in the emission spectra. This shift in the emission spectrum is called a Stokes shift. The magnitude of the Stokes shift depends on the polarity of the solvent. Usually, solvents of higher polarity produce larger Stokes shifts.

The rate of light absorption by a compound is equal to the Einstein B co-efficient:

\[ B_{g\rightarrow e} = \frac{2\pi\mu^2}{3\hbar^2} \quad (25) \]
This is also equal to the rate of stimulated emission from the excited state to the ground state because the transition dipole operator $\mu^2$ is the same for both excitation and emission. The process of returning from the excited state to the ground state with the emission of a photon in phase with the exciting electromagnetic field is referred to as stimulated emission. If transitions in optical systems were caused by absorption and stimulated emission then we would expect the equilibrium populations of the two states to be equal since the rate constants are equal. We know that this is not the case for visible spectroscopy, thus there must be other mechanisms for the excited state to return to the ground state. One mechanism is spontaneous emission of the excited atom. Spontaneous emission from the excited state is just one of the mechanism which limits the lifetime of the excited state. This is typically quite large for excited states that emit at ultraviolet or visible wavelengths (i.e. greater than 109 sec$^{-1}$).

The rate of spontaneous emission is the rate at which the fluorescent molecule will emit light. However, the amount of fluorescence obtained from a molecule depends on the rate of spontaneous emission versus other mechanisms which return the electron to the ground state. Stimulated emission is not significant at most fluences of light used in the laboratory and need not be considered further. If other mechanisms for relaxation to the ground state besides spontaneous emission exist then the lifetime of the excited state is given by:

$$\tau_{\text{obs}} = 1 / \Sigma k_i = 1 / k_o + k_{\text{other}} + k_q[Q]$$  \hspace{1cm} (26)
Where $k_0 = 1 / A$, $k_q[Q]$ is the relaxation rate due to quenching and $k_{\text{other}}$ includes any relaxation processes besides spontaneous emission or quenching. This lifetime is the observed fluorescent life time because relaxation of the system to the ground state by any mechanism results in a loss of excited state molecules. The quantum yield is the ratio of the rate constant for fluorescent decay versus the sum of all pathways.

$$\Phi_{fl} = \frac{k_o}{k_o + k_{\text{other}} + k_q[Q]} \quad (27)$$

The quantum yield is simply the probability that an excited system will return to the ground state by the emission of a photon. For a steady state experiment the quantum yield is the number of photons emitted/photons absorbed. The quantum yield is also given by the ratio of lifetimes (this follows directly from Eq. 27).

$$\Phi_{fl} = \frac{\tau_{\text{obs}}}{\tau_o} \quad (28)$$

The definition of quenching is any process which reduces the lifetime of the excited state. A reduction in the lifetime usually implies a decrease in the quantum yield.

Detailed examination of the deactivation processes for the electronically excited cyanines indicate that the main decay routes for the singlet state are fluorescence ($k_n$) and a tortional rotation ($k_{\text{rot}}$) about one of the central carbon-carbon bonds.\(^9\) Therefore the fluorescence quantum yield ($\Phi_n$) and the fluorescence life-time ($\tau_n$) of cyanine dyes are determined in large part by the fluorescence and rotational rate constants. Typically relaxation via rotation accounts for ca. 90% of the deactivation of the excited cyanine at room temperature. Therefore this is the most important process controlling the
fluorescence quantum yield and the life-time of the dyes. Intersystem crossing ($k_{isc}$) normally contributes insignificantly to deactivation of the singlet state of cyanine dyes.

According to formula (4), fluorescence quantum yield is directly proportional to the observed fluorescence life-time. This explains the results obtained in the current work, that in general the indolenine heptamethine cyanine dyes show higher fluorescence quantum yield than the benz[e]indolenine and benz[c,d]indolenine heptamethine cyanine dyes. This same trend in observation was reported by Lee, et al.\textsuperscript{78} for fluorescence life-times of similar dyes with C-C bond at the meso-position. The extra phenyl group was reported to destabilize the singlet excited state, thus decrease the FLT. Mader, et al.\textsuperscript{85} reported similar but unsymmetric heptamethine cyanine dyes having high fluorescence quantum yields for indolenine heptamethine cyanine dyes. Therefore it is evident based on the literature data and the data obtained from the current work that the most suitable class of heptamethine cyanine dyes based on the end heterocyclic systems, i.e indolenine, benz[e]indolenine and benz[c,d]indolenine, to function as fluorescent imaging probes in biological systems are the indolenine based dyes, since these dyes have the highest fluorescence quantum yields and life-times.

In contrast to indolium dyes the benzothiazole dye 22a was inert to treatment with amines under a variety of experimental conditions and easily underwent decomposition. This observation has been previously reported by Srekowski, et al.\textsuperscript{97} Therefore making amine derivatives of benzothiazole heptamethine dyes was discontinued.
3. EXPERIMENTAL

**General.** All chemicals were purchased from commercial sources and were used without further purification. $^1$H NMR and $^{13}$C NMR data were collected on a Bruker DPX-400 spectrometer at ambient temperature in CDCl$_3$ or DMSO-$d_6$ and referenced to tetramethylsilane (TMS) as an internal standard. $^1$H NMR spectra were recorded at 400 MHz and $^{13}$C NMR spectra were recorded at 100 MHz. Low and high resolution mass spectra were obtained using electrospray ionization (ESI) mass spectrometry on a hybrid linear ion trap-Fourier transform mass spectrometer. Electronic absorption spectra were obtained from a Shimadzu UV-1700 spectrophotometer. Fluorescence data were collected on a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer. All extinction coefficient measurements were performed in spectrophotometric grade methanol or ethanol, purchased from Sigma-Aldrich. A series of dilute (10$^{-7}$ M) concentrations of the samples of which the absorbance is less than 0.1 in 10 mm cuvettes were prepared and used for Vis-NIR and fluorescence spectroscopic measurements using Rhodamine 800 (abs/emission 682/705 nm, $\Phi_f$(ethanol) = 0.21)$^{21}$ as fluorescence reference standard.

**N-((2-Chloro-3-((phenylamino)methylene)cyclohex-1-en-1-yl)methylene)benzenaminium chloride** (3) was synthesized according to a published procedure.$^{52}$

$^{1,2,3,3}$-Tetramethyl-3H-indol-1-i um iodide, (5a)$^{53}$ and 1-butyl-2,3,3-trimethyl-3H-indol-1-i um iodide (5b)$^{54}$ were synthesized according to published procedures.

$^{1,2,3,3}$-Tetramethyl-3H-indol-1-i um iodide (5a): Yield 4.37 g (81%); $^1$H NMR (DMSO-$d_6$): $\delta$ 7.92 (d, $J$ = 7 Hz, 1H), 7.84 (t, $J$ = 7 Hz, 1H), 7.63 (m, 2H), 3.99 (s, 3H), 2.79 (s, 3H), 1.54 (s, 6H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 196.4, 142.5, 142.0, 129.7, 129.2,
123.7, 115.6, 54.4, 35.3, 22.2, 14.8; HRMS: calcd for C_{12}H_{16}N⁺ m/z 174.1283, found m/z 174.1281.

**1-Butyl-2,3,3-trimethyl-3H-indol-1-iium iodide (5b):** This compound was obtained from 58 (2.5 g, 15.7 mmol) and methyl iodide (6.68 g, 47.1 mmol): Yield 4.53 g (73%); \(^1\)H NMR (DMSO-\textit{d}6): \(\delta\) 7.99 (d, \(J = 7\) Hz, 1H), 7.85 (d, \(J = 7\) Hz, 1H), 7.60 (m, 2H), 4.44 (t, \(J = 7\) Hz, 2H), 2.86 (s, 3H), 1.82 (t, \(J = 7\) Hz, 2H), 1.44 (s, 6H), 1.32 (m, 2H), 0.94 (t, \(J = 7\) Hz, 3H); \(^{13}\)C NMR (DMSO-\textit{d}6): \(\delta\) 9, 142.3, 141.5, 129.9, 129.4, 124.0, 115.9, 54.6, 47.9, 29.7, 22.5, 19.8, 14.6, 14.0; HRMS: calcd for C_{15}H_{22}N⁺ m/z 216.1752, found m/z 216.1756.

**General procedure for synthesis of 2,3,3-trimethyl-1-octyl-3H-indol-1-iium bromide (5c) and 1-dodecyl-2,3,3-trimethyl-3H-indol-1-iium bromide (5d).**

2, 3, 3-Trimethylindolenine (3g, 18 mmol) was dissolved in anhydrous acetonitrile (50 mL). The solution was stirred under an atmosphere of nitrogen and treated with 1-bromooctane (10.42 g, 54 mmol) or 1-bromododecane (13.45 g, 54 mmol). The mixture was heated under reflux for 48 h, while monitoring by thin layer chromatography, then cooled and concentrated under reduced pressure. The crude product was crystallized from ethyl ether, to give pure products 5c and 5d as light pink crystals.

**2,3,3-Trimethyl-1-octyl-3H-indol-1-iium bromide (5c):** Yield 5.05 g (84%); \(^1\)H NMR (DMSO-\textit{d}6): \(\delta\) 8.01 (d, \(J = 7\) Hz, 1H), 7.87 (d, \(J = 7\) Hz, 1H), 7.62 (m, 2H), 4.48 (t, \(J = 8\) Hz, 2H), 2.88 (s, 3H), 1.83 (m, 2H), 1.55 (s, 6H), 1.40 (s, 3H), 1.28 (m, 9H), 0.84 (t, \(J = 7\) Hz, 3H); \(^{13}\)C NMR (DMSO-\textit{d}6): \(\delta\) 196.9, 142.3, 141.5, 129.8, 129.4, 128.2, 124.0,
108

115.99, 54.6, 48.1, 31.6, 29.0, 28.9, 27.7, 26.3, 22.5, 14.6, 14.4; HRMS: calcd for C_{20}H_{32}N^{+} m/z 272.2378, found m/z 272.2365.

1-Dodecyl-2,3,3-trimethyl-3H-indol-1-ium bromide (5d): Yield 5.22g (97%); ^1H NMR (DMSO-d_{6}): δ 7.98 (d, J = 7 Hz, 1H), 7.85 (d, J = 7 Hz, 1H), 7.63 (m, 2H), 4.45 (t, J = 8 Hz, 2H), 2.84 (s, 3H), 1.85 (m, 2H), 1.54 (s, 6H), 1.25 (m, 18H), 0.85 (t, J = 8 Hz, 3H); ^13C NMR (DMSO-d_{6}): δ 196.9, 142.3, 141.5, 129.8, 129.4, 124.0, 115.9, 54.6, 48.0, 31.7, 29.4, 29.3, 29.2, 29.1, 29.0, 27.7, 26.3, 22.5, 22.5, 14.5, 14.4; HRMS: calcd for C_{23}H_{38}N^{+} m/z 328.3004, found m/z 328.3002.

2-((E)-2-((E)-2-Chloro-3-((E)-2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl) vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide (6a), 1-butyl-2-((E)-2-((E)-2-(1-butyl-3,3-dimethylindolin-2-ylidene)ethylidene)-2-chloro cyclohex-1-en-1-yl) vinyl)-3,3-dimethyl-3H-indol-1-ium iodide (6b), 2-((E)-2-((E)-2-chloro-3-((E)-2-(3,3-dimethyl-1-octylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl) vinyl)-3,3-dimethyl-1-octyl-3H-indol-1-ium bromide (6c) and 2-((E)-2-((E)-2-chloro-3-((E)-2-(1-dodecyl-3,3-dimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl) vinyl)-1-dodecyl-3,3-dimethyl-3H-indol-1-ium bromide (6d) were synthesized according to published procedures.\textsuperscript{53, 54}

2-((E)-2-((E)-2-Chloro-3-((E)-2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)ethylidene)
cyclohex-1-en-1-yl) vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide (6a): Yield 3.4g (83%); mp 220-226 °C; ^1H NMR (DMSO-d_{6}): δ 8.23 (d, J = 14 Hz, 2H), 7.61 (t, J = 7 Hz, 2H), 7.43 (d, J = 7 Hz, 4H), 7.27 (m, 2H), 6.28 (d, J = 14 Hz, 2H), 3.67 (s, 6H), 2.71 (t, J = 6 Hz, 4H), 1.84 (t, J = 6 Hz, 2H), 1.66 (s, 12H); ^13C NMR (CDCl_{3}): δ 172.9, 144.4,
109

142.7, 140.9, 129.3, 128.8, 127.5, 123.0, 122.2, 110.9, 101.5, 49.3, 32.5, 28.1, 26.7, 23.2;

1-Butyl-2-((E)-2-((E)-3-((1-butyl-3,3-dimethylindolin-2-ylidene)ethylidene)-2-chlorocyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-3H-indol-1-ium iodide (6b): Yield 1.85g (90%); mp 191-193 °C; ¹H NMR (CDCl₃): δ 8.35 (d, J = 12 Hz, 2H), 7.67 (m, 4H), 7.27 (m, 2H), 7.19 (m, 2H), 6.22 (d, J = 12 Hz, 2H), 4.20 (t, J = 8 Hz, 4H), 2.73 (br t, 4H), 2.05 (m, 2H), 1.84 (m, 4H), 1.73 (s, 12H), 1.50 (m, 4H), 1.01 (t, J = 7 Hz, 6); ¹³C NMR (CDCl₃): δ 172.3, 150.4, 144.2, 142.2, 141.0, 128.8, 127.3, 125.3, 122.2, 110.9, 101.3, 49.3, 44.9, 29.5, 28.1, 26.7, 20.7, 20.3, 13.9; HRMS: calcd for C₃₈H₄₈ClN₂⁺ m/z 567.3506, found m/z 567.3487. Anal. Calcd for C₃₈H₄₈ClN₂: C, 65.66; H, 6.96; N, 4.03. Found C, 65.58; H, 6.90; N, 4.48.

2-((E)-2-((E)-2-Chloro-3-((E)-2-(3,3-dimethyl-1-octylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-octyl-3H-indol-1-ium bromide (6c): Yield 2.4 g (34%); ¹H NMR (CDCl₃): δ 8.36 (d, J = 14 Hz, 2H), 7.40 (d, J = 7, 4H), 7.26 (t, J = 7 Hz, 2H), 6.22 (d, J = 14 Hz, 2H), 4.20 (br t, J = 6 Hz, 4H), 2.73 (br t, 4H), 1.99 (m, 2H), 1.86 (t, J = 4 Hz, 4H), 1.73 (s, 12H), 1.46 (m, 4H), 1.38 (m, 6H), 1.27 (m, 12H), 0.87 (t, J= 4 Hz, 6H); ¹³C NMR (CDCl₃): δ 172.3, 150.3, 144.2, 142.2, 141.0, 128.8, 127.1, 125.3, 122.2, 110.9, 101.2, 49.3, 44.9, 31.7, 29.2, 29.0, 28.1, 27.3, 26.9, 26.5, 22.5, 20.7, 14.0; HRMS: calcd for C₄₆H₆₄ClN₂⁺ m/z 679.4726, found m/z 679.4748. Anal. Calcd for C₄₆H₆₄BrClN₂: C, 72.66; H, 8.48; N, 3.68. Found C, 72.79; H, 8.73; N, 4.08.
2-((E)-2-((E)-2-Chloro-3-((E)-2-(1-dodecyl-3,3-dimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1-dodecyl-3,3-dimethyl-3H-indol-1-ium bromide (6d):

Yield 0.481 g (28%); mp 183-185 °C; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.38 (d, \(J = 14\) Hz, 2H), 7.41 (d, \(J = 7\) Hz, 4H), 7.28 (m, 2H), 7.18 (d, \(J = 7\) Hz, 2H), 6.15 (d, \(J = 14\) Hz, 2H), 4.23 (t, \(J = 7\) Hz, 4H), 2.75 (m, 4H), 2.05 (br t, 2H), 1.85 (m, 4H), 1.73 (s, 12H), 1.45 (m, 4H), 1.38 (br s, 4H), 1.27 (br d, 28H), 0.88 (t, \(J = 3\) Hz, 6H); \(^13\)C NMR (CDCl\(_3\)): 172.3, 150.2, 144.1, 142.3, 141.1, 128.8, 127.3, 125.2, 122.2, 110.9, 101.5, 49.3, 45.0, 31.8, 29.5, 29.4, 29.3, 29.3, 28.1, 27.4, 27.0, 26.6, 22.6, 20.7, 14.1; HRMS: calcd for C\(_{54}\)H\(_{80}\)ClN\(_2^+\) m/z 791.6010, found m/z 791.5995. Anal. Calcd for C\(_{54}\)H\(_{80}\)BrClN\(_2^+\): C, 74.33; H, 9.24; N, 3.21. Found C, 73.96; H, 9.06; N 3.63.

General procedure for synthesis of meso-amino derivatives of the dyes 7a-p.

To a solution of 100 mg of 6a-d in anhydrous N, N-dimethylformamide (2 mL) was added, 10 eq. of the amine (2.45 \(\times\) 10\(^{-3}\) mol) via a syringe. The mixture was heated and stirred at room temperature to 50 °C under a nitrogen atmosphere for 5 h. The reaction progress was monitored by Vis-NIR absorption changes of solutions diluted with methanol appearance of a new band 780 nm region, which corresponds to the amine. The mixture was concentrated and extracted into CH\(_2\)Cl\(_2\) and washed with water (5 \(\times\) 10 mL). The organic layers were then combined, dried under anhydrous MgSO\(_4\), filtered, and concentrated. The residue was purified by silica gel flash chromatography (CH\(_2\)Cl\(_2)/\)methanol up to 30% methanol), to obtain compounds 7a-p. Workup of the chromatography fractions included concentration followed by treatment of the residues
with CH₂Cl₂ to precipitate silica gel eluted with methanol. Product was crystallized from methanol/ether, to obtain the pure product as a dark blue solid.

2-((E)-2-((E)-2-(Dimethylamino)-3-((E)-2-(1,3,3-trimethylindolin-2-ylidene) ethylidene) cyclohex-1-en-1-yl) vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide (7a):
Yield 0.082 g (36%); ¹H NMR (CDCl₃): δ 7.45 (d, J = 13 Hz, 2H), 7.26 (t, J = 8 Hz, 4H), 7.06 (t, J = 7 Hz, 2H), 6.90 (d, J = 7 Hz, 2H), 5.58 (d, J = 13 Hz, 2H), 3.70 (s, 6H), 3.41 (s, 6H), 2.50 (t, J = 6 Hz, 4H), 1.81 (m, 2H), 1.63 (s, 12H); ¹³C NMR (CDCl₃): δ 175.7, 167.8, 143.6, 140.1, 128.2, 122.7, 122.0, 121.9, 108.4, 93.9, 76.7, 47.8, 47.6, 29.6, 29.4, 25.5, 21.5; HRMS: calcd for C₃₄H₄₂N₃⁺ m/z 492.3379, found m/z 492.3391. Anal. Calcd for C₃₄H₄₂N₃: C, 65.91; H, 6.96; N, 6.78. Found C, 65.83; H, 7.23; N, 6.48.

1-Butyl-2-((E)-2-((E)-3-((E)-2-1-butyl-3,3-dimethylindolin-2-ylidene) ethylidene)-2-(dimethylamino) cyclohex-1-en-1-yl) vinyl)-3,3-dimethyl-3H-indol-1-ium iodide (7b):
¹H NMR (CDCl₃): δ 7.46 (d, J = 13 Hz, 2H), 7.27 (m, 4H), 7.07 (t, J = 7 Hz, 2H), 6.88 (d, J = 7 Hz, 2H), 5.62 (d, J = 13 Hz, 2H), 3.83 (t, J = 7 Hz, 4H), 3.69 (s, 6H), 2.49 (t, J = 7 Hz, 2H), 1.75 (t, J = 7 Hz, 2H), 1.73(m, 4H), 1.66 (m, 12H), 1.46 (q, J = 7 Hz, 4H), 0.88 (t, J = 3 Hz, 6H); ¹³C NMR (CDCl₃): δ 140.1, 140.1, 128.1, 122.7, 122.1, 121.6, 108.4, 93.7, 47.9, 47.7, 43.1, 29.7, 29.5, 28.7, 25.5, 22.7, 21.5, 20.4, 14.1, 13.9; HRMS: calcd for C₄₀H₅₄N₃⁺ m/z 576.4318, found m/z 576.4293. Anal. Calcd for C₄₀H₅₄N₃: C, 68.26; H, 7.73; N, 5.97. Found C, 68.03; H, 7.59; N, 5.64.

2-((E)-2-((E)-3-((E)-2-(3,3-Dimethyl-1-octylindolin-2-ylidene) ethylidene)-2-(dimethylamino) cyclohex-1-en-1-yl) vinyl)-3,3-dimethyl-1-octyl-3H-indol-1-ium bromide (7c): Yield 0.039 g (79.6%); ¹H NMR (CDCl₃): δ 7.46 (d, J = 13 Hz, 2H), 7.28
(m, 4H), 7.07 (t, \(J = 8\) Hz, 2H), 6.87 (d, \(J = 8\) Hz, 2H), 5.61 (d, \(J = 13\) Hz, 2H), 3.81 (br t, 4H), 3.71 (s, 6H), 2.84 (br t, 4H), 1.83 (m, 2H), 1.76 (m, 4H), 1.66 (s, 12H), 1.40 (m, 6H), 1.28 (m, 14H), 0.87 (br t, 6H); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 170.2, 166.2, 143.0, 140.1, 128.1, 122.7, 122.1, 121.5, 108.4, 93.7, 77.2, 47.7, 47.7, 43.3, 43.1, 31.7, 29.5, 29.2, 29.1, 27.1, 26.5, 25.4, 22.6, 14.0; HRMS: calcd for C\(_{48}\)H\(_{70}\)N\(_3^+\) m/z 688.5570, found m/z 688.5555. Anal. Calcd for C\(_{48}\)H\(_{70}\)BrN\(_3\): C, 74.97; H, 9.18; N, 5.46. Found C, 74.56; H, 9.01; N, 5.28.

2-((E)-2-((Dimethylamino))-3-((E)-2-(1-dodecyl-3,3-dimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1-dodecyl-3,3-dimethyl-3H-indol-1-ium bromide (7d): Yield 0.038 g (79%); \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.46 (d, \(J = 13\)Hz, 2H), 7.29 (m, 4H), 7.08 (t, \(J = 7\) Hz, 2H), 6.89 (d, \(J = 8\) Hz, 2H), 5.64 (d, \(J = 13\)Hz, 2H), 3.83 (t, \(J = 7\) Hz, 4H), 3.67 (s, 6H), 2.48 (t, \(J = 6\) Hz, 4H), 1.84 (br t, 2H), 1.76 (m, 2H), 1.65 (s, 12H), 1.39 (m, 6H), 1.25 (m, 32H), 0.87 (t, \(J = 6\) Hz, 6H); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 175.1, 167.4, 143.0, 140.5, 140.1, 128.1, 122.8, 122.1, 121.7, 108.6, 93.9, 55.9, 47.7, 43.4, 31.8, 29.6, 29.5, 29.5, 29.4, 29.31, 27.1, 26.5, 25.2, 22.6, 21.6,14.1; HRMS: calcd for C\(_{56}\)H\(_{86}\)N\(_3^+\) m/z 800.6822, found m/z 800.6821. Anal. Calcd for C\(_{56}\)H\(_{86}\)BrN\(_3\): C, 76.33; H, 9.84; N, 4.77. Found C, 76.72; H, 9.73; N, 4.80.

2-((E)-2-((Hexylamino))-3-((E)-2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide (7e): Yield 0.060 (28%); \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.23 (br s, 1H), 7.69 (br d, 2H), 7.25 (m, 4H), 7.04 (t, \(J = 7\) Hz, 2H), 6.86 (d, \(J = 7\) Hz, 2H), 5.53 (br d, \(J = 12\) Hz, 2H), 3.86 (br d, \(J = 6\) Hz, 2H), 2.49 (t, \(J = 6\) Hz, 4H), 1.98 (m, 2H), 1.80 (m, 3H), 1.66 (m, 12H), 1.31 (m, 6H), 1.03 (m, 3H),
0.87 (br t, J = 6 Hz, 6H); $^{13}$C NMR (CDCl$_3$): δ 169.5, 167.5, 138.0, 128.1, 122.5, 121.9, 108.2, 94.1, 56.0, 50.0, 47.5, 31.9, 30.1, 29.7, 29.60, 29.05, 27.58, 26.51, 25.24, 22.61, 21.54, 14.0; HRMS: calcd for C$_{38}$H$_{50}$N$_3$ $^+$ m/z 548. 4005, found m/z 548. 3986. Anal. Calcd for C$_{38}$H$_{50}$N$_3$H$_2$O: C, 67.54; H, 7.46; N, 6.22. Found C, 67.81; H, 7.21; N, 5.98.

1-Butyl-2-((E)-2-((E)-3-((E)-2-(1-butyl-3,3-dimethylindolin-2-ylidene)ethylidene)-2-(hexylamino)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-3H-indol-1-ium iodide (7f):
Yield 0.082g (25%); $^1$H NMR (CDCl$_3$): δ 7.72 (d, J = 13 Hz, 2H), 7.26 (m, 4H), 7.04 (t, J = 7 Hz, 2H), 6.85 (d, J = 7 Hz, 2H), 5.61 (d, J = 13 Hz, 2H), 3.85 (m, 6Hz), 2.48 (t, J = 6 Hz, 4H), 1.96 (m, 2H), 1.83 (m, 2H), 1.74 (m, 4H), 1.70 (s, 12H), 1.48 (m, 4H), 1.35 (m, 4H),1.26 (s, 6H), 1.01 (t, J = 8Hz, 6H); $^{13}$C NMR (CDCl$_3$): δ 169.3, 166.8, 143.2, 137.9, 128.0, 122.4, 121.9, 120.1, 108.2, 93.9, 55.9, 50.0, 47.6, 31.4, 31.3, 29.6, 29.1, 28.6, 26.4, 25.2, 22.6, 21.6, 20.4, 14.0, 13.9; HRMS: calcd for C$_{44}$H$_{63}$N$_3$ $^+$ m/z 632. 4944, found m/z 632. 4965. Anal. Calcd for C$_{44}$H$_{63}$N$_3$: C, 69.55; H, 8.22; N, 5.53. Found C, 70.01; H, 7.86; N, 4.87.

2-((E)-2-((E)-3-((E)-2-(3,3-Dimethyl-1-octylindolin-2-ylidene)ethylidene)-2-(hexylamino)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-octyl-3H-indol-1-ium bromide (7g):
Yield 0.026g (48%); $^1$H NMR (CDCl$_3$): δ 9.72 (br s, 1H), 7.73 (br d, J = 12 Hz, 2H), 7.25 (m, 4H), 7.02 (t, J = 7 Hz, 2H), 6.79 (d, J = 7 Hz, 2H), 5.55 (br d, J = 12 Hz, 2H), 3.88 (br d, J = 6 Hz, 2H), 3.74 (m, 4H), 2.48 ( t, J = 6 Hz, 4H), 2.01 (m, 2H), 1.81 (m,3H), 1.72 (m, 18 H), 1.29 (m, 20H), (t, J = 6 Hz, 12H); $^{13}$C NMR (CDCl$_3$): δ 170.4, 140.3, 137.2, 136.8, 127.8, 122.0, 122.0, 107.8, 93.3, 49.8, 47.4, 43.0, 31.7, 31.6, 31.4, 31.3, 30.9, 29.3, 29.1, 29.0, 27.1, 27.0,26.5, 26.3, 25.5, 22.6, 22.6, 21.58, 14.0; HRMS:
calcd for C_{52}H_{78}N_{3}^{+} m/z 744. 6196, found m/z 744. 6172. Anal. Calcd for C_{52}H_{79}BrN_{3}: C, 75.69; H, 9.53; N, 5.09. Found C, 75.08; H, 9.61; N 5.26.

1-Dodecyl-2-((E)-2-((E)-3-((E)-2-(1-dodecyl-3,3-dimethylindolin-2-ylidene)-2-(hexylamino)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-3H-indol-1-ium bromide (7h): Yield 0.028 (53%); \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 9.73 (br s, 1H), 7.72 (br d, \(J = 12\) Hz, 2H), 7.25 (m, 4H), 7.02 (t, \(J = 7\) Hz, 2H), 6.79 (d, \(J = 7\) Hz, 2H), 5.55 (d, \(J = 12\) Hz, 2H), 3.89 (m, 2H), 3.75 (br m, 4H), 2.476 (t, \(J = 8\) Hz, 4H), 2.01 (m, 2H), 1.81 (m, 3H), 1.71 (br s, 18H), 1.38 (br m, 6 H), 1.26 (br m, 30H), 0.87 (t, \(J = 6\) Hz, 12H); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 170.4, 166.2, 143.2, 140.3, 137.2, 127.8, 122.0, 122.0, 120.0, 107.8, 93.3, 75.9, 49.8, 47.5, 43.0, 31.9, 31.4, 30.9, 30.1, 29.6, 29.5, 29.4, 29.5, 29.3, 29.0, 27.1, 26.5, 26.3, 25.5, 22.6, 21.6, 14.1, 14.0; HRMS: calcd for C_{60}H_{94}N_{3}^{+} m/z 856.7448, found m/z 856.7421.

1,3,3-Trimethyl-2-((E)-2-((E)-2-(4-methylpiperazin-1-yl)-3-((E)-2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3H-indol-1-ium iodide (7i): \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.64 (d, \(J = 16\) Hz, 2H), 7.32 (m, 4H), 7.13 (t, \(J = 8\) Hz, 2H), 7.05 (d, \(J = 8\) Hz, 2H), 5.82 (d, \(J = 16\) Hz, 2H), 3.80 (s, 4H), 3.55 (s, 6H), 2.74 (s, 4H), 2.51 (m, 7H), 1.84 (t, \(J = 8\) Hz, 2H), 1.67 (s, 12H); Anal. Calcd for C_{37}H_{47}IN_{4}: C, 65.87; H, 7.02; N, 8.30. Found C, 65.33; H, 6.88; N, 8.02.

1-Butyl-2-((E)-2-((E)-3-((E)-2-(1-butyl-3,3-dimethylindolin-2-ylidene)ethylidene)-2-(4-methylpiperazin-1-yl)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-3H-indol-1-ium iodide (7j): \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.66 (d, \(J = 16\) Hz, 2H), 7.33 (m, 4H), 7.15 (t, \(J = 8\) Hz, 2H), 7.02 (d, \(J = 8\)Hz, 2H), 5.85 (d, \(J = 12\) Hz, 2H), 3.96 (t, \(J = 8\) Hz, 4H), 3.78 (s, 4H),
2.74 (s, 4H), 2.50 (m, 7H), 1.82 (m, 6H), 1.68 (s, 12H), 1.48 (s, 4H), 1.02 (t, J = 8 Hz, 6H); $^{13}$C NMR (CDCl$_3$): δ (ppm) 172.1, 168.0, 141.7, 140.5, 139.2, 127.5, 123.4, 122.7, 121.0, 108.6, 95.4, 55.7, 54.1, 52.6, 47.1, 45.4, 42.7, 28.0, 24.1, 20.9, 19.4, 13.0; Anal. Calcd for C$_{43}$H$_{59}$IN$_4$. H$_2$O: C, 66.48; H, 7.91; N, 7.21. Found C, 66.32; H, 7.74; N, 6.93.

2-((E)-2-((E)-3-((E)-2-(3,3-Dimethyl-1-octylindolin-2-ylidene)ethylidene)-2-(4-methylpiperazin-1-yl)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-octyl-3H-indol-1-ium bromide (7k): Yield 0.038g (71%); $^1$H NMR (CDCl$_3$): δ 7.67 (d, J = 13 Hz, 2H), 7.34 (t, J = 8 Hz, 4H), 7.15 (t, J = 8 Hz, 2H), 7.00 (d, J = 8Hz, 2H), 5.85 (d, J = 13Hz, 2H), 3.94 (t, J = 4 Hz, 4H), 3.77 (br s, 4H), 2.78 (br s, 3H), 2.48 (t, J = 6 Hz, 6H), 1.82 (m, 6H), 1.66 (s, 12 H), 1.39 (m, 6H), 1.29 (m, 16H), 0.87 (t, J = 7 Hz, 6H); $^{13}$C NMR (CDCl$_3$): δ 169.1, 142.6, 141.5, 140.2, 128.5, 124.3, 123.7, 122.0, 109.5, 96.4, 56.6, 48.1, 47.7, 46.3, 43.8, 31.9, 29.6, 29.5, 29.0, 27.0, 26.8, 25.0, 22.6, 22.5, 21.8, 14.0; HRMS: calcd for C$_{51}$H$_{75}$BrN$_4$ $^+$ m/z 743. 5992, found m/z 743. 5967. Anal.Calcd for C$_{51}$H$_{75}$BrN$_4$. H$_2$O: C, 72.43; H, 9.22; N, 6.62. Found C, 72.64; H, 9.46; N 6.13.

1,3,3-Trimethyl-2-((E)-2-((E)-2-(4-phenylpiperazin-1-yl)-3-((E)-2-(1,3,3-trimethyl indolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3H-indol-1-ium iodide (7l): Yield 0.078 (33%); $^1$H NMR (CDCl$_3$): δ 7.75 (d, J = 13 Hz, 2H), 7.34 (m, 4H), 7.13 (m, 3H), 7.04 (br t, 6H), 5.88 (d, J =13 Hz, 2H), 3.89 (m, 4H), 3.71 (s, 2H), 3.58 (m, 2H), 3.47 (m, 4H), 2.56 (br t, 4H), 1.86 (br s, 4H), 1.65 (s, 12H); $^{13}$C NMR (CDCl$_3$): δ 169.7, 143.2, 128.5, 125.4, 123.7, 122.6, 122.0, 120.9, 116.7, 108.3, 97.1, 76.7, 54.8, 51.3, 47.8, 31.9, 29.7, 28.8, 25.6, 22.6, 21.5, 14.12; LRMS: calcd for C$_{42}$H$_{49}$N$_4$ $^+$ m/z 609.40, found
m/z 609.6. Anal. Calc. for C_{42}H_{49}IN_4: C, 65.28; H, 6.91; N, 7.05. Found C, 65.49; H, 7.03; N, 6.84.

1-Butyl-2-((E)-2-((E)-3-((E)-2-(1-butyl-3,3-dimethylindolin-2-ylidene)ethylidene)-2-(4-phenylpiperazin-1-yl)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-3H-indol-1-ium iodide (7m): Yield 0.081 (34%); \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.49 (d, \(J = 12\) Hz, 2H), 7.35 (m, 6H), 7.15 (t, \(J = 6\) Hz, 2H), 7.05 (m, 5H), 5.92 (d, \(J = 12\) Hz, 2H), 4.05 (t, \(J = 8\), 4H), 3.86 (br t, 4H), 3.49 (brt, 4H), 2.54 (m, 4H), 1.89 (m, 2H), 1.80 (m, 2H), 1.67 (s, 12H), 1.49 (m, 6H), 1.03 (t, \(J = 12\)H, 6H); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 172.1, 169.3, 142.6, 141.6, 140.3, 129.4, 125.3, 123.2, 122.0, 120.9, 116.7, 109.7, 97.0, 54.7, 51.3, 48.2, 43.8, 29.7, 29.0, 28.9, 25.5, 22.6, 21.7, 20.4, 14.1; LRMS: calcd for C_{49}H_{63}N_4\(^+\) m/z 693.49, found m/z 693.8. Anal. Calcd C_{48}H_{61}N_4\_2H_2O: C, 70.23; H, 7.59; N, 6.52. Found C, 69.81; H, 7.87; N, 6.13.

2-((E)-2-((E)-3-((3,3-Dimethyl-1-octylindolin-2-ylidene)ethylidene)-2-(4-phenylpiperazin-1-yl)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-1-octyl-3H-indol-1-ium bromide (7n): Yield 0.020g (35%); \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 7.78 (d, \(J = 12\) Hz, 2H), 7.32 (m, 6H), 7.14 (t, \(J = 8\) Hz, 2H), 7.02 (m, 5H), 5.90 (d, \(J = 12\) Hz, 2H), 3.97 (t, \(J = 5\) Hz, 4H), 3.85 (m, 4H), 3.48 (m, 4H), 2.52 (t, \(J = 5\) Hz, 4H), 1.87 (m, 2H), 1.81 (m, 4H), 1.66 (s, 12H), 1.45 (m, 4H), 1.38 (m, 4H), 1.30 (m, 12H), 0.87 (t, \(J = 6.4\) Hz, 6H); \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 172.0, 169.4, 150.6, 142.6, 141.6, 140.3, 129.4, 128.5, 125.1, 123.9, 122.0, 120.9, 116.7, 109.7, 97.0, 54.7, 51.3, 48.2, 44.0, 31.7, 29.2, 29.1, 28.8, 27.0, 26.9, 25.1, 22.6, 21.7, 14.0; HRMS: calcd for C_{56}H_{77}N_4\(^+\) m/z 805. 6148, found m/z 805. 6136.
Anal. Calcd for C_{56}H_{77}Br_{4}. H_{2}O: C, 75.90; H, 8.76; N, 6.32. Found C, 76.21; H, 8.53; N, 5.74.

1,3,3-Trimethyl-2-((E)-2-((E)-2-(phenylamino)-3-((E)-2-(1,3,3-trimethylindolin-2-ylidene)ethyldiene)cyclohex-1-en-1-yl)vinyl)-3H-indol-1-ium iodide (7o): Yield 0.15 g (56%); {\textsuperscript{1}}H NMR (CDCl{\textsubscript{3}}): δ 1.38 (s, 12H), 1.93 (t, J = 6.4 Hz, 2H), 2.58 (t, J = 6.4 Hz, 4H), 3.52 (s, 6H), 5.79 (d, J = 14.0 Hz, 2H), 6.56 (t, J = 12.8 Hz, 1H), 6.87 (d, J = 6.4 Hz, 2H), 7.09 (t, J = 7.2 Hz, 2H), 7.24 (m, 5H), 7.38 (d, J = 7.2 Hz, 3H), 7.44 (t, J = 7.2 Hz, 2H), 8.14 (d, J = 14.0 Hz, 2H), 8.34 (s, 1H, exchangeable with D2O); {\textsuperscript{13}}C NMR (CDCl{\textsubscript{3}}): δ 21.8, 24.9, 28.5, 31.7, 48.6, 97.8, 109.4, 118.6, 121.3, 122.2, 124.0, 124.3, 128.4, 130.1, 140.6, 143.3, 143.5, 160.4, 170.9; HRMS: calcd for C_{38}H_{42}N_{3}^{+} m/z 540.3379, found m/z 540.3378. Anal. Calcd for C_{38}H_{42}N_{3}: C, 68.36; H, 6.34; N, 6.29. Found C, 67.94; H, 6.08, 5. 73.

1-Butyl-2-((E)-2-((E)-3-((1-butyl-3,3-dimethylindolin-2-ylidene)ethyldiene)-2-(phenylamino)cyclohex-1-en-1-yl)vinyl)-3,3-dimethyl-3H-indol-1-ium iodide (7p): Yield 0.032 g (59%); mp 120-123 °C; {\textsuperscript{1}}H NMR (CDCl{\textsubscript{3}}): δ 8.28 (br s, 1H), 8.12 (d, J = 14 Hz, 2H), 7.35 (d, J = 8 Hz, 2H), 7.25 (m, 6H), 7.08 (t, J = 8 Hz, 2H), 6.93 (d, J = 8 Hz, 2H), 6.77 (t, J = 8 Hz, 1H), 5.81 (d, J = 14 Hz, 2H), 3.93 (t, J = 7 Hz, 4H), 2.56 (t, J = 6 Hz, 4H), 1.95 (m, 2H), 1.75 (m, 4H), 1.43 (m, 4H), 1.37 (s, 12H), 0.96 (m, 6H); {\textsuperscript{13}}C NMR (CDCl{\textsubscript{3}}): δ 170.3, 159.9, 146.4, 143.4, 142.6, 140.8, 129.5, 128.1, 124.3, 123.7, 122.0, 120.7, 117.9, 109.3, 79.6, 48.5, 43.9, 29.0, 28.4, 24.7, 21.6, 20.3, 13.8; HRMS: calcd for C_{44}H_{54}N_{3}^{+} m/z 624.4318, found m/z 624.4330. Anal. Calcd for C_{44}H_{54}N_{3}: H, 69.96; H, 7.26; N, 5.56. Found C, 69.55; H, 6.99; N 5.22.
1,2,3-Tetramethyl-1H-benzo[e]indol-3-ium iodide, 9a and 3-Butyl-1,1,2-trimethyl-1H-benzo[e]indol-3-ium iodide (9b) were synthesized according to previously published procedures.\textsuperscript{55}

1,1,2,3-Tetramethyl-1H-benzo[e]indol-3-ium iodide (9a): $^1$H NMR (DMSO-$d_6$): $\delta$ 8.38 (d, $J = 8$ Hz, 1H), 8.29 (d, $J = 8$ Hz, 1H), 8.22 (d, $J = 8$ Hz, 1H), 8.17 (d, $J = 8$ Hz, 1H), 7.75 (m, 2H), 4.14 (s, 3H), 2.92 (s, 3H), 1.78 (s, 6H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 196.3, 139.9, 136.9, 133.4, 130.9, 130.1, 128.9, 127.5, 123.9, 123.8, 113.7, 113.6, 55.7, 21.7, 14.7; HRMS: calcd for C$_{16}$H$_{18}$N$^+$ m/z 224.1439, found m/z 224.1438.

3-Butyl-1,1,2-trimethyl-1H-benzo[e]indol-3-ium iodide (9b): $^1$H NMR (DMSO-$d_6$): $\delta$ 8.37 (d, $J = 8$ Hz, 1H), 8.29 (d, $J = 8$ Hz, 1H), 8.22 (d, $J = 8$ Hz, 1H), 8.17 (d, $J = 8$ Hz, 1H), 7.75 (m, 2H), 4.59 (t, $J = 8$ Hz, 2H), 2.97 (s, 3H), 1.88 (m, 2H), 1.76 (s, 6H), 1.47 (m, 2H), 0.94 (t, $J = 7$ Hz, 3H); $^{13}$C NMR (DMSO-$d_6$): $\delta$ 170.1, 169.4, 153.3, 147.6, 131.6, 130.7, 129.5, 129.3, 129.1, 128.1, 120.2, 117.1, 114.9, 113.3, 35.7, 33.3, 22.0, 14.1; HRMS: calcd for C$_{19}$H$_{24}$N$^+$ m/z 266.1909, found m/z 266.1921.

General procedure for synthesis of 2-((E)-2-((E)-2-Chloro-3-((E)-2-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)ethyldiene)cyclohex-1-en-1-yl)vinyl)-1,1,3-trimethyl-1H-benzo[e]indol-3-ium iodide (10a) and 3-butyl-2-((E)-2-((E)-3-((E)-2-(3-butyl-1,1-dimethyl-1H-benzo[e]indol-2(3H)-ylidene)ethyldiene)-2-chlorocyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (10b).

A mixture of 9a or 9b (1g, 1eq.) and N-((-2-chloro-3-((phenylamino) methylene)cyclohex-1-en-1-yl)methylene)benzenaminium chloride (3), (0.5 eq.) were heated to
80°C in the presence of triethylamine (1 eq.) in a solution of acetonitrile/ethanol (9:1) for 2h. The solution was subsequently removed from heat and allowed to cool down to room temperature. The solution at room temperature was filtered and the collected precipitate was washed with ether to obtain a copper-green crystalline solid, 10a and 10b.

2-((E)-2-((E)-2-Chloro-3-((E)-2-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,1,3-trimethyl-1H-benzo[e]indol-3-ium iodide (10a): Yield 28%; ¹H NMR (DMSO-d₆): δ 8.37 (d, J = 14 Hz, 2H), 8.30 (d, J = 8 Hz, 2H), 8.09 (m, 4H), 7.79 (d, J = 9 Hz, 2H), 7.67 (t, J = 8 Hz, 2H), 7.53 (t, J = 7 Hz, 2H), 6.35 (d, J = 14 Hz, 2H), 3.82 (s, 6H), 3.17 (d, J = 2 Hz, 2H), 2.77 (t, J = 6 Hz, 4H), 1.96 (s, 12H), 1.90 (m, 2H); HRMS: calcd for C₄₀H₄₀N₂Cl⁺ m/z 583.2880, found m/z 583.2870. Anal. Calcd for C₄₀H₄₀ClIN₂. H₂O: C, 65.89; H, 5.81; N 3.84. Found C, 66.00; H, 5.88; N, 4.30.

3-Butyl-2-((E)-2-((E)-3-((E)-2-(3-butyl-1,1-dimethyl-1H-benzo[e]indol-2(3H)-ylidene)ethylidene)-2-chlorocyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (10b): Yield 53%; ¹H NMR (DMSO-d₆): δ 8.38 (d, J = 14 Hz, 2H), 8.31 (d, J = 8 Hz, 2H), 8.10 (m, 4H), 7.79 (d, J = 8 Hz, 2H), 7.67 (t, J = 8 Hz, 2H), 7.54 (t, J = 8 Hz, 2H), 6.38 (d, 14 Hz, 2H), 4.36 (t, J = 6 Hz, 4H), 2.76 (m, 4H), 1.96 (s, 12H), 1.92 (m, 2H), 1.79 (m, 4H), 1.45 (m, 4H), 0.96 (t, J = 7 Hz, 6H); ¹³C NMR (DMSO-d₆): δ 173.8, 142.4, 140.2, 134.1, 131.2, 128.3, 128.0, 126.7, 112.3, 101.7, 51.2, 30.0, 27.5, 26.4, 20.0, 14.3; HRMS: calcd for C₄₆H₄₂N₂Cl⁺ m/z 667.3819, found m/z 667.3822. Anal. Calcd for C₄₆H₄₂ClIN₂. H₂O: C, 67.97; H, 6.69; N 3.44. Found C, 68.75; H, 6.60; N, 3.45.
General procedure for synthesis of meso-amino substituted benz[e]indolium heptamethine cyanine dyes, 11a-f.

Dimethylamine, hexylamine or N-methylpiperazine (5eq.) was added to a solution of 10a or 10b (100 mg, 1 eq.) in DMF (2 mL) and stirred at ambient temperature in an inert atmosphere for 12 h. The resulting solution was extracted into dichloromethane and concentrated. The crude product was purified by flash chromatography with normal phase EMD 60PF254 silica gel eluting with methylene chloride/methanol (2%) to provide compounds, 11a-f.

2-((E)-2-((E)-2-(Dimethylamino)-3-((E)-2-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,1,3-trimethyl-1H-benzo[e]indol-3-ium iodide (11a): $^1$H NMR (CDCl$_3$): $\delta$ 8.09 (d, $J = 8$ Hz), 7.85 (m, 4H), 7.55 (m, 4H), 7.32 (m, 4H), 5.68 (d, $J = 16$ Hz, 2H), 3.69 (s, 6H), 3.58 (s, 6H), 2.54 (t, $J = 4$ Hz, 4H), 1.95 (s, 12H); $^{13}$C NMR (CDCl$_3$): $\delta$ 173.8, 169.0, 139.9, 139.4, 130.4, 129.9, 129.1, 128.9, 127.4, 126.4, 122.9, 121.2, 120.9, 109.0, 93.1, 52.6, 48.6, 46.8, 28.0, 24.3, 20.7; HRMS: calcd for C$_{42}$H$_{46}$N$_3$ $^+$ m/z 592. 3692, found m/z 592. 3678. Anal. Calcd for C$_{42}$H$_{46}$N$_3$. 2H$_2$O: C, 66.75; H, 6.67; N, 5.56. Found C, 66.21; H, 6.35; N, 5.38.

3-Butyl-2-((E)-2-((E)-3-((E)-2-(3-butyl-1,1-dimethyl-1H-benzo[e]indol-2(3H)-ylidene)ethylidene)-2-(dimethylamino)cyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (11b): $^1$H NMR (CDCl$_3$): $\delta$ 8.13 (d, $J = 8$ Hz, 2H), 7.88 (t, $J = 4$ Hz, 4H), 7.63 (d, $J = 14$ Hz, 2H) 7.56 (t, $J = 8$ Hz, 2H), 7.39 (t, $J = 8$ Hz, 2H), 7.27 (t, $J = 8$ Hz, 2H), 5.72 (d, $J = 14$ Hz, 2H), 4.00 (t, $J = 4$ Hz, 4H), 3.72 (s, 6H), 2.55 (t, $J = 4$ Hz, 4H), 2.00 (s, 12H), 1.85 (m, 6H), 1.51 (m, 4H), 1.04 (t, $J = 8$ Hz, 6H); $^{13}$C NMR
(CDCl₃): δ 173.5, 168.3, 139.3, 139.2, 130.8, 129.8, 129.0, 128.8, 127.5, 126.4, 122.9, 121.0, 120.8, 108.9, 92.8, 48.6, 46.8, 42.4, 28.0, 24.4, 20.7, 19.4, 12.9; HRMS: calcd for C₄₈H₅₈N₃⁺ m/z 676.4631, found m/z 676.4651. Anal. Calcd for C₄₈H₅₈N₃.H₂O: C, 68.64; H, 7.44; N, 5.00. Found C, 68.79; H, 7.11; N, 4.98.

2-((E)-2-((Hexylamino)-3-((E)-2-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-yli dene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,1,3-trimethyl-1H-benzo[e]indol-3-ium iodide (11c): ¹H NMR (CDCl₃): δ 8.06 (d, J = 8 Hz, 2H), 7.84 (t, J = 8 Hz, 6H), 7.52 (t, J = 8 Hz, 2H), 7.35 (t, J = 8 Hz, 2H), 7.24 (m, 2H), 5.60 (d, J = 16 Hz, 2H), 3.90 (m, 2H), 3.52 (m, 4H), 2.53 (t, J = 4 Hz, 4H), 1.96 (m, 16H), 1.84 (m, 4H), 1.34 (m, 10H), 0.89 (m, 3H); ¹³C NMR (CDCl₃): δ 168.4, 139.1, 133.7, 130.8, 130.0, 129.8, 129.0, 127.6, 126.8, 126.3, 122.7, 121.0, 119.4, 113.0, 108.7, 92.8, 49.2, 48.5, 30.5, 28.7, 27.5, 26.7, 25.5, 24.3, 21.7, 20.6, 13.1; HRMS: calcd for C₄₆H₆₄N₃⁺ m/z 648.4318, found m/z 648.4315. Anal. Calcd for C₄₆H₆₄N₃.H₂O: C, 69.60; H, 7.11; N, 5.29. Found C, 69.93; H, 7.22; N, 5.81.

3-Butyl-2-((E)-2-((E)-3-((3-butyl-1,1-dimethyl-1H-benzo[e]indol-2(3H)-yli dene)ethylidene)-2-(hexylamino)cyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (11d): ¹H NMR (CDCl₃): δ 8.10 (d, J = 8 Hz, 2H), 7.84 (m, 6H), 7.54 (m, 2H), 7.36 (m, 2H), 7.19 (d, J = 8 Hz, 2H), 3.93 (m, 4H), 2.52 (t, 4H), 2.02 (m, 14H), 1.80 (m, 8H), 1.49 (m, 4H), 1.34 (m, 4H), 1.02 (m, 6H), 0.90 (t, J = 8 Hz, 4H); ¹³C NMR (CDCl₃): δ 168.8, 140.0, 136.7, 132.9, 129.8, 129.0, 127.9, 126.8, 126.3, 122.7, 121.0, 119.4, 113.0, 108.7, 92.8, 49.2, 48.5, 30.5, 28.7, 27.5, 26.7, 25.5, 24.3, 21.7, 20.6, 13.1; HRMS: calcd for C₅₂H₆₆N₃⁺ m/z 732.5257, found m/z 732.5240. Anal. Calcd for C₅₂H₆₆N₃.H₂O: C, 71.13; H, 7.81; N, 4.79. Found C, 71.15; H, 7.68; N, 4.65.
1,1,3-Trimethyl-2-((E)-2-((E)-2-(4-methylpiperazin-1-yl)-3-((E)-2-(1,1,3-trimethyl-1H-benzo[e]indol-2(3H)-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1H-benzo[e]indol-3-ium iodide (11e): $^1$H NMR (CDCl$_3$): $\delta$ 8.09 (m, 2H), 7.87 (m, 5H), 7.56 (m, 2H), 7.41 (m, 3H), 7.28 (m, 2H), 5.90 (d, $J = 12$Hz, 1H), 5.64 (d, $J = 12$Hz, 1H), 3.83 (s, 1H), 3.72 (d, $J = 8$ Hz, 5H), 3.58 (s, 2H), 2.88 (m, 1H), 2.58 (m, 5H), 1.97 (m, 12H), 1.25 (s, 1H); $^{13}$C NMR (CDCl$_3$) $\delta$ 171.6, 141.2, 140.8, 131.5, 131.0, 130.6, 130.3, 130.1, 128.7, 128.5, 127.8, 127.6, 125.3, 124.5, 124.0, 122.1, 110.5, 110.0, 97.0, 50.2, 48.1, 29.2, 25.5, 22.1; HRMS: calcd for C$_{45}$H$_{51}$N$_4^+$ m/z 647.4114, found m/z 647.4125. Anal. Calcd for C$_{45}$H$_{51}$N$_4$. H$_2$O: C, 68.17; H, 6.74; N, 7.07. Found C, 68.07; H, 6.74; N, 6.26.

3-Butyl-2-((E)-2-((E)-2-(3-butyl-1,1-dimethyl-1H-benzo[e]indol-2(3H)-ylidene)ethylidene)-2-(4-methylpiperazin-1-yl)cyclohex-1-en-1-yl)vinyl)-1,1-dimethyl-1H-benzo[e]indol-3-ium iodide (11f): $^1$H NMR (CDCl$_3$): $\delta$ 8.09 (d, $J = 8$ Hz, 2H), 7.87 (m, 6H), 7.59 (t, $J = 8$ Hz, 2H), 7.40 (m, 4H), 7.35 (d, $J = 16$ Hz, 2H), 4.11 (t, $J = 8$ Hz, 2H), 3.79 (s, 3H), 2.55 (m, 6H), 1.99 (s, 12H), 1.85 (m, 8H), 1.51 (m, 4H), 1.26 (s, 2H), 1.03 (s, $J = 8$ Hz, 6H); $^{13}$C NMR (CDCl$_3$): $\delta$ 169.8, 140.0, 139.0, 131.3, 130.3, 129.4, 129.0, 127.2, 126.6, 123.8, 123.4, 120.8, 109.4, 95.4, 55.7, 53.8, 49.0, 45.6, 42.9, 28.3, 27.6, 24.2, 20.9, 19.4, 12.9; HRMS: calcd for C$_{51}$H$_{63}$N$_4^+$ m/z 731.5053, found m/z 731.5046. Anal. Calcd for C$_{51}$H$_{63}$IN$_4$. H$_2$O: C, 69.85; H, 7.47; N, 6.39. Found C, 70.16; H, 7.29; N, 6.24.

Benzo[cd]indole-2(1H)-thione (13).

This procedure was modified from the original procedure.$^{56}$ Compound 12 (Commercial compound; Ryan Chemicals) (5.00 g, 29 mmol) and phosphorous pentasulfide (3.4 g,
7.58 mmol) were refluxed in pyridine (15 mL) for 75 min. The resulting mixture was then cooled to room temperature. Immersed the reaction flask in an ice bath and added conc. HCl (20 mL) via a pipet. To the resulting solution was added 50 mL of warm DI water. The reaction mixture was stirred 30 min. and filtered. The precipitate was washed with copious amounts of water. The resulting dark green colored precipitate was dried under reduced pressure to obtain 13 in 87% yield. LRMS: calcd for C_{11}H_{8}NS^{+} m/z 186.04, found m/z 186.1.

2-(Methylthio)-1,2-dihydrobenzo[cd]indole (14). Compound 13 (5.88 g, 31.7 mmol) was heated under reflux in acetone in the presence of iodomethane (5.98, 42 mmol) for 30 min. The resulting mixture was then removed from heat and allowed to cool to room temperature. The resulting precipitate was filtered, washed with acetone and dried in air. The compound was used the subsequent synthesis without further purification. HRMS: calcd for m/z C_{12}H_{12}NS^{+} 200.0534, found m/z 200.0533.

5-(Beno[cd]indol-2(1H)-ylidene)-2,2-dimethyl-1,3-dioxane-4,6-dione (15).

Compound 14 (5.13 g, 15 mmol), Meldrum’s acid (4.32 g, 30 mmol), triethylamine (3.04 g, 30 mmol) and two scoops of sodium acetate were heated at 60° C for 3 h. The reaction mixture was then removed from heat and filtered. The brown color precipitate was dried under vacuum to obtain 15 in 50% yield. ^{1}H NMR (DMSO-\textit{d}_{6}): \delta 12.77 (s, 1H), 9.40 (d, J = 8 Hz, 1H), 8.38 (d, J = 8 Hz, 1H), 7.93 (t, J = 8 Hz, 1H), 7.87 (d, J = 8 Hz, 1H), 7.77 (d, J = 8 Hz, 1H), 7.67 (t, J = 8 Hz, 1H), 1.71 (s, 6H); C NMR (DMSO-\textit{d}_{6}) \delta 159.4, 137.7, 133.6, 132.9, 128.9, 128.8, 128.5, 128.4, 123.8, 122.8, 112.7, 102.2, 84.9, 25.7; HRMS calcd for C_{17}H_{14}NO_{4}^{+} m/z 318.0742, found m/z 318.0744.
2,2-Dimethyl-5-(1-methylbenzo[cd]indol-2(1H)-ylidene)-1,3-dioxane-4,6-dione (16).

This procedure was modified from the procedure originally reported.\(^{57}\) Compound 15 (2g, 6.7 mmol) was allowed to react with iodomethane (2.88g, 20.3 mmol) in dimethylformamide (15 mL) in the presence of potassium hydroxide (1.13 g, 20.3 mmol).The reaction mixture was heated to 80 °C for 6-7 h under an inert atmosphere. The mixture was then removed from heat, filtered and concentrated. The crude compound was purified on a chromatotron eluting with 100% dichloromethane, to obtain 16 as a bright orange color solid in 85% yield. \(^{1}\)H NMR (DMSO-\(d_6\)): \(\delta\) 9.03 (d, \(J = 4\) Hz, 1H), 8.42 (d, \(J = 8\) Hz, 1H), 8.03 (d, \(J = 12\) Hz, 1H), 7.91 (m, 2H), 7.77 (t, \(J = 8\)Hz, 1H), 3.73 (s, 3H), 1.76 (s, 6H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 163.6, 161.2, 140.5, 133.8, 133.3, 130.2, 129.3, 128.3, 128.0, 125.2, 122.5, 113.0, 101.6, 81.7, 35.9, 25.8; HRMS: calcd for C\(_{18}\)H\(_{16}\)NO\(_4\)^{+} m/z 310.1079, found m/z 310.1094.

\(\mathbf{1, 2}\)-Dimethylbenzo[cd]indol-1-iium iodide (17). The above compound was synthesized according to published procedures.\(^{58}\) The product was dried under reduced pressure to obtain 17 as a light brown solid in 17% yield. \(^{1}\)H NMR (DMSO-\(d_6\)): \(\delta\) 8.98 (br d, 1H), 8.79 (br d, 1H), 8.47 (m, 2H), 8.16 (br d, 1H), 8.00 (br d, 1H), 4.21 (s, 3H), 3.20 (s, 3H); \(^{13}\)C NMR (DMSO-\(d_6\)): \(\delta\) 172.3, 138.8, 138.0, 134.5, 130.4, 129.2, 128.6, 127.7, 121.4, 120.3, 33.0, 13.7; HRMS: calcd for [M-I]^+ m/z 182.0790, found m/z 182.0964.

2-((E)-2-((E)-2-Chloro-3-((E)-2-(1-Methylbenzo[cd]indol-2(1H)-ylidene) ethylidene)cyclohex-1-en-1-yl) vinyl)-1-methylbenzo[cd]indol-1-iium iodide (18). A mixture of 17 and \(N\)-((2-chloro-3-((phenylamino) methylene)cyclohex-1-en-1-yl) methylene) benzenaminium chloride, 3, was heated under reflux in 7:3 1-
butanol/benzene for 3 h. The reaction progress was monitored by Vis-NIR spectroscopy in dilute solutions of methanol. The reaction was stopped when the only absorption shown was at 1005 nm. The reaction mixture was concentrated under vacuum and crystallized from a mixture of methylene chloride and diethylether, to obtain pure 18 as a black-green crystalline solid. $^1$H NMR (DMSO-$d_6$): $\delta$ 8.27 (d, $J = 12$ Hz, 2H), 7.97 (m, 4H), 7.78 (m, 2H), 7.33 (m, 4H), 7.23 (m, 2H), 6.43 (d, $J = 16$ Hz, 2H), 3.51 (s, 6H), 2.76 (m, 4H), 1.92 (m, 2H); HRMS: calcd for C$_{34}$H$_{28}$N$_2$Cl $^+$ m/z 499.1941, found m/z 499.1958. Anal. Calcd for C$_{34}$H$_{28}$ClN$_2$: C, 63.31; H, 4.69; N, 4.34. Found C, 63.82; H, 4.52; N, 4.43.

2-((E)-2-((E)-2-(Dimethylamino)-3-((E)-2-(1-methylbenzo[cd]indol-2(1H)-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1-methylbenzo[cd]indol-1-ium iodide (19). X g of compound 18 was dissolved in anhydrous DMF (x mL) and was added dimethylamine (10 eq.) via syringe. The above mixture was stirred at room temperature for 12 h. The resulting amine was precipitated using anhydrous ether. The precipitate was filtered and washed with ether to obtain 19 as a dark blue color solid. $^1$H NMR (CDCl$_3$): $\delta$ 8.70 (m, 2H), 8.65 (m, 2H), 7.80 (s, 4H), 7.34 (m, 6H), 6.73 (m, 2H), 5.87 (m, 2H), 4.01 (s, 6H), 3.47 (t, $J = 4$ Hz, 6H), 2.73 (m, 4H), 1.93 (m, 2H); HRMS: calcd for C$_{36}$H$_{34}$N$_3$ $^+$ m/z 508.2753, found m/z 508.2744. Anal. Calcd C, 68.03; H, 5.39; N, 6.61. Found C, 69.78; H, 5.44; N, 6.64.

Compounds 21a-b were synthesized according to previously published procedures.

2,3-Dimethylbenzo[d]thiazol-3-ium iodide (21a): $^1$H NMR (DMSO-$d_6$): $\delta$ 8.45 (d, $J = 8$ Hz, 1H), 8.30 (d, $J = 8$ Hz, 1H), 7.90 (t, $J = 8$ Hz, 1H), 7.81(t, $J = 8$ Hz, 1H), 4.21 (s, 3H),
3.18 (s, 3H); $^{13}$C NMR (DMSO-$d_6$): δ 177.1, 141.5, 129.2, 128.6, 128.0, 124.4, 116.7, 36.2, 17.1. HRMS: calcd for C$_9$H$_{10}$NS$^+$ m/z 164.0534, found m/z 164.0537.

3-Butyl-2-methylbenzo[d]thiazol-3-ium iodide (21b): $^1$H NMR (DMSO-$d_6$): δ 8.46 (d, J = 8 Hz, 1H), 8.34 (d, J = 8 Hz, 1H), 7.90 (t, J = 8 Hz, 1H), 7.81 (t, J = 8 Hz, 1H), 4.72 (t, J = 8 Hz, 2H), 3.22 (s, 3H), 1.83 (m, 2H), 1.46 (m, 2H), 0.94 (t, J = 7 Hz, 3H); $^{13}$C NMR (DMSO-$d_6$): δ 177.5, 141.3, 129.5, 128.6, 125.1, 117.3, 99.9, 49.5, 30.2, 19.7, 17.4, 17.2; HRMS: calcd for C$_{12}$H$_{16}$NS$^+$ m/z 206.1003, found m/z 206.0996.

General procedure for synthesis of 22a-b.

21a or 21b (1g, 1eq.) and N-((2-chloro-3-((phenylamino) methylene)cyclohex-1-en-1-yl)methylene)benzenaminium chloride (3), (0.5 eq.) were heated to 80°C in acetonitrile for 1 1/2 h in the presence of triethylamine (1eq.). The resulting green precipitate was then filtered, washed with diethyl ether and dried under vacuum to furnish 22a and 22b.

2-((E)-2-((E)-2-Chloro-3-((Z)-2-(3-methylbenzo[d]thiazol-2(3H)-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3-methylbenzo[d]thiazol-3-ium iodide (22a): $^1$H NMR (DMSO-$d_6$): δ 7.86 (d, J = 8 Hz, 2H) 7.62 (d, J = 13 Hz, 2H), 7.55 (d, J = 8 Hz, 2Hz), 7.37 (t, J = 8 Hz, 2H), 7.30 (t, J = 4 Hz, 2H), 6.33 (d, J = 16 Hz, 2H), 3.83 (s, 3H), 2.62 (t, J = 6 Hz, 4H), 1.85 (m, 2H); HRMS: calcd for C$_{26}$H$_{24}$N$_2$S$_2$Cl$^+$ m/z 463.1069, found 463.1058. Anal. Calcd for C$_{26}$H$_{24}$ClIN$_2$S$_2$: C, 52.84; H, 4.09; N, 4.74. Found C, 34.88; H, 3.11; N, 3.50.

3-Butyl-2-((E)-2-((E)-3-((Z)-2-(3-butylbenzo[d]thiazol-2(3H)-ylidene)ethylidene)-2-chlorocyclohex-1-en-1-yl)vinyl)benzo[d]thiazol-3-ium iodide (22b): $^1$H NMR (DMSO-
$d_6$): δ 7.97 (d, $J = 8$ Hz, 2H), 7.80 (d, $J = 16$ Hz, 2H), 7.55 (d, $J = 8$ Hz, 2H), 7.37 (t, $J = 8$ Hz, 2H), 7.38 (t, $J = 8$ Hz, 2H), 6.48 (d, $J = 16$ Hz, 2H), 4.43 (t, $J = 7$ Hz, 4H), 2.67 (t, $J = 6$ Hz, 4H), 1.85 (m, 2H), 1.71 (m, 4H), 1.41 (m, 4H), 0.94 (t, $J = 7$ Hz, 6H); $^{13}$C NMR (DMSO-$d_6$): δ 162.8, 141.1, 124.9, 124.7, 123.8, 122.6, 113.1, 99.5, 29.0, 25.9, 18.8, 13.1; HRMS: calcd for C$_{32}$H$_{36}$N$_2$S$_2$Cl m/z 547.2008, found 547.2002. Anal. Calcd for C$_{32}$H$_{36}$ClIN$_2$S$_2$: C, 56.93; H, 5.37; N, 4.15. Found C, 56.26; H, 5.33; N, 4.28.

**Bis(2-idoethyl) ether, 23a, and bis(2-idoethoxy)ethane, 23b.**

These compounds were synthesized according to a previously published procedure to obtain as yellow oil in 96% and 86% yields respectively.

**Bis(2-idoethyl) ether (23a):** $^1$H NMR (CDCl$_3$) δ (ppm) 3.77 (t, $J = 8$ Hz, 4H), 3.26 (t, $J = 8$ Hz, 4H). $^{13}$C NMR (CDCl$_3$) δ (ppm) 71.4, 2.7. HRMS (ESI) calcd for (C$_4$H$_8$IO$_2$Na)$^+$ m/z: 348.8562, found 348.8562.

**Bis(2-idoethoxy)ethane (23b):** $^1$H NMR (CDCl$_3$) δ (ppm) 3.77 (m, 4H), 3.67 (m, 4H), 3.27 (m, 4H). $^{13}$C NMR (CDCl$_3$) δ (ppm) 71.9, 70.2, 3.2. HRMS (ESI) calcd for (C$_6$H$_{12}$O$_2$I$_2$Na)$^+$ m/z : 392.8825, found 392.8824.

**Bis tosylate derivative of tetraethyleneglycol.**

Tetraethyleneglycol (15g, 77.2 mmol), p-toluenesulfonylchloride (29.4g, 154 mmol) and pyridine (24.9 mL, 308 mmol) were stirred in dichloromethane (100 mL) for 24 h. Added 25 mL of 2N HCl and extracted the crude product into ethyl acetate. The product was purified by flash chromatography using CH$_2$Cl$_2$/methanol (0-30%), to obtain the bis tosylate derivative as a colorless oil in 86% yield. $^1$H NMR (DMSO-$d_6$) δ (ppm) 7.79 (dd,
$J_1 = 8$ Hz, $J_2 = 4$ Hz, 4H), 7.46 (dd, $J_1 = 8$ Hz, $J_2 = 4$ Hz, 4H), 4.12 (m, 4H), 3.71 (m, 4H), 3.57 (m, 8H), 2.42 (s, 6H). $^{13}$C NMR (DMSO-$d_6$) $\delta$ (ppm) 145.3, 132.9, 130.5, 128.0, 72.8, 68.3, 60.7, 43.9, 21.4. HRMS (ESI) calcd for (C$_{22}$H$_{31}$O$_9$S$_2$)$^+$ m/z: = 503.1413, found 503.1410.

**Diiodo derivative of tetraethyleneglycol 23c.**

The bis-tosylate derivative (2.80g, 5.5 mmol) was dissolved in acetone. Sodium iodide (3.3g, 22 mmol) was added to the above solution and heated under reflux in an inert atmosphere for 48 h. The crude product was extracted into diethyl ether and concentrated under vacuum to obtain 23c as brown-yellow oil in 69% yield. $^1$H NMR (CDCl$_3$) $\delta$ (ppm) 3.76 (m, 4H), 3.67 (m, 8H), 3.27 (td, $J_1 = 8$ Hz, $J_2 = 2$ Hz, 4H). $^{13}$C NMR (CDCl$_3$) $\delta$ (ppm) 72.5, 71.8, 61.5, 3.1.

Compounds 24a-c were synthesized as reported previously.$^{67}$

**1,1'-(Oxybis(ethane-2,1-diyl))bis(2,3,3-trimethyl-3H-indol-1-ium) iodide (24a):** This compound was obtained in 89.7% yield. $^1$H NMR (DMSO-$d_6$) $\delta$ (ppm) 7.86 (t, $J = 8$ Hz, 4H), 7.60 (t, $J = 8$ Hz, 2H), 7.53(t, $J = 8$ Hz, 2H), 4.70 (br t, $J = 4$ Hz, 4H), 3.98 (br t, $J = 4$ Hz, 4H), 3.76 (m, 4H), 3.67 (m, 8H), 3.27 (td, $J_1 = 8$ Hz, $J_2 = 2$ Hz, 4H). $^{13}$C NMR (DMSO-$d_6$) $\delta$ (ppm) 198.4, 142.0, 141.7, 129.8, 129.3, 124.0, 115.9, 67.9, 54.7, 48.2, 22.5, 15.2. HRMS (ESI) calcd for (C$_{26}$H$_{34}$N$_2$O)$_2^+$ m/z: 390.2671, found 390.2670.

**1,1'-(Ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl))bis(2,3,3-trimethyl-3H-indol-1-ium) iodide (24b):** This compound was obtained in 82% yield. $^1$H NMR (DMSO-$d_6$) $\delta$ (ppm) 7.93 (m, 2H), 7.85 (m, 2H), 7.63 (m, 4H), 4.67 (br t, 4H), 3.57 (br t, 4H), 3.38 (br t, 4H), 2.78 (s, 6H), 1.49 (s, 12H). $^{13}$C NMR (DMSO-$d_6$) $\delta$ (ppm) 198.5, 142.0, 141.3,
129.9, 129.3, 124.0, 116.0, 70.2, 67.1, 54.7, 48.2, 22.4, 14.9. HRMS (ESI) calcd for (C\textsubscript{28}H\textsubscript{38}N\textsubscript{2}O\textsubscript{2})\textsuperscript{+} m/z : 217.1423, found 2171477.

1,1′-(((Oxybis(ethane-2,1-diy1))bis(oxo))bis(ethane-2,1-diy1))bis(2,3,3-trimethyl-3H-indol-1-ium) iodide (85c): This compound was obtained in 68% yield. \textsuperscript{1}H NMR (DMSO-\textsubscript{d}6) \textdelta (ppm) 7.99 (m, 2H), 7.86 (m, 2H), 7.63 (m, 4H), 4.78 (m, 4H), 3.87 (m, 4H), 3.47 (m, 8H), 2.79 (s, 6H), 1.55 (s, 12H). \textsuperscript{13}C NMR (DMSO-\textsubscript{d}6) \textdelta (ppm) 198.5, 142.0, 141.3, 129.9, 129.3, 124.0, 116.0, 70.2, 67.1, 54.7, 48.2, 22.5, 22.4, 14.9. HRMS (ESI) calcd for (C\textsubscript{30}H\textsubscript{42}N\textsubscript{2}O\textsubscript{3})\textsuperscript{+} m/z: 239.1597, found 239.1596.

General Procedure for Synthesis of Macrocyclic Dyes 25a-c. A mixture of Vilsmeier-Haack reagent (3) (360 mg, 1mmol), sodium acetate (82 mg, 1mmol) and the dimeric salt 24 (1mmol) in ethanol was heated under reflux for 12h in an inert atmosphere. The crude dye was purified by flash chromatography eluting with dichloromethane/methanol (98:2).

\(\text{N}, \text{N}′-(3′′-\text{Oxapentane-1}′′,5′′-\text{diyl})\{-2-[7′-(3′,3′\text{dimethylindolin-2}′-\text{ylidene})-4′-\text{chloro-3′,5′-trimethylene-1′,3′,5′-heptatrien-1}′-\text{yl}]\text{-3,3-dimethyl-3H-indol-1-ium}\}\text{iiodide (25a).}

This compound was obtained in 40% yield. \textsuperscript{1}H NMR (DMSO-\textsubscript{d}6) \textdelta (ppm) 8.18 (d, \(J = 14\) Hz, 2H), 7.56 (m, 2H), 7.35 (m, 2H), 7.23 (m, 4H), 6.31 (d, \(J = 14\) Hz, 2H), 4.45 (m, 4H), 3.91 (m, 6H), 2.65 (m, 4H), 1.85 (m, 2H), 1.57 (s, 12H).

\(\text{N,N}′-(3′′,6′′-\text{Dioxaoctane-1}′′,8′′-\text{diyl})\{-2-[7′-(3′,3′\text{dimethylindolin-2}′-\text{ylidene})-4′-\text{chloro-3′,5′-trimethylene-1′,3′,5′-heptatrien-1}′-\text{yl}]\text{-3,3-dimethyl-3H-indol-1-ium}\\text{iiodide (25b).}

This compound was obtained in 33% yield. \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \textdelta (ppm) 8.32 (d, \(J = 14\) Hz, 2H), 7.37 (m, 4H), 7.25 (m, 4H), 6.35 (d, \(J = 14\) Hz, 2H), 4.41 (m,
4H), 3.90 (m, 4H), 3.55 (m, 8H), 2.70 (m, 4H), 1.95 (m, 2H), 1.70 (s, 12H). 13C NMR (DMSO-\textit{d}_6) δ (ppm) 206.9, 143.9, 142.5, 140.8, 128.8, 125.3, 122.0, 111.4, 102.3, 70.9, 70.6, 68.3, 49.3, 45.5, 30.9, 28.2, 26.6, 20.8. λ_{\text{max}} = 783 nm (methanol). HRMS (ESI) calcd for (C_{36}H_{42}N_2O_2Cl)^+ m/z : = 569.2935, found 569.2925.

\(N,N''-(3''', 6''', 9'''-\text{Trioxaundecane-1'''}, 11'''-\text{diyl})-[2-[7'(3'', 3''-\text{dimethylindolin-2''-ylidene})-4'-\text{chboro-3',5'-trimethylene-1',3',5'-heptatrien-1'-yl}]-3,3-\text{dimethyl-3H-indol-1-ium}] \text{ iodide (25c).}\) This compound was obtained in 28% yield. 1H NMR (CDCl\textsubscript{3}) δ (ppm) 8.32 (d, \(J = 15\) Hz, 2H), 7.37-7.17 (m, 8H), 6.45 (d, \(J = 15\) Hz, 2H), 4.42 (m, 4H), 3.96 (m, 4H), 3.59 (m, 8H), 2.71 (m, 4H), 1.96 (m, 2H), 1.71 (s, 12H). λ_{\text{max}} = 783 nm (methanol).

**Measurement of fluorescence quantum yield**

**Standard samples.** The standard samples were chosen to ensure they absorb at the excitation wavelength of choice for the test sample, and, emit in a similar region to the test sample.

**Cuvettes.** Standard 10 mm path length fluorescence cuvettes were used to run the absorption and fluorescence measurements.

**Concentration range.** In order to minimise re-absorption effects, absorbances in the 10 mm fluorescence cuvette were not allowed exceed 0.1 at \textit{and above} the excitation wavelength. The concentration range was always kept at 10^{-7} M.

**Sample preparation.** All glassware were kept scrupulously clean, and solvents were of spectrophotometric grade and checked for background fluorescence.
Procedure:

1. Recorded the absorption spectrum of the reference standard solution in the 10 mm cuvette. Recorded the absorption spectrum of the sample in the 10 mm cuvette.

2. Recorded the fluorescence spectrum of the reference standard solution in the 10 mm cuvette. Recorded the fluorescence spectrum of the sample in the 10 mm cuvette.

3. Calculated the integrated fluorescence intensity (that is, the area of the fluorescence spectrum) from the fully corrected fluorescence spectrum of both the standard and the sample.

3. Repeated steps 1, 2, and 3 for five solutions with increasing concentrations of the chosen reference standard and the sample.

4. Plotted a graph of absorbance vs. concentration for each sample. Calculated the absorption coefficient using Beer’s Law.

\[ A = \varepsilon c l \]

5. Plotted a graph of integrated fluorescence intensity vs. absorbance. The result should be a straight line with gradient m, and intercept = 0.

6. Repeated steps 1-5 for the remaining samples.
Calculation of Fluorescence Quantum Yields from Acquired Data:

\[
\Phi_{\text{fl}}(x) = \Phi_{\text{fl}}(\text{std}) \left( \frac{\text{Grad}(x)}{\text{Grad}(\text{std})} \right) \left( \frac{\eta^2_x}{\eta^2_{\text{std}}} \right)
\]

Where the subscripts std and x denote standard and test sample respectively, \(\Phi_{\text{fl}}\) is the fluorescence quantum yield, Grad is the gradient from the plot of integrated fluorescence intensity vs. absorbance, and \(\eta\) the refractive index of the solvent used. If the reference standard and the sample were both prepared in the same solvent the refractive index term will be cancelled off.

For 50% ethanol/H\(_2\)O solvent system the refractive index was calculated by plotting a graph of refractive index vs. \%ethanol/H\(_2\)O.
4. REFERENCES


