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Synthesis of Near-Infrared Heptamethine Cyanine Dyes

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SYNTHESIS OF NEAR-INFRARED HEPTAMETHINE CYANINE DYES

by

JAMIE L. GRAGG

Under the Direction of Dr. Maged M. Henary

ABSTRACT

Carbocyanine dyes are organic compounds containing chains of conjugated methine groups with electron-donating and electron-withdrawing substituents at the terminal heterocycles of the general formula $[R^1-(CH)_n-R^2]^+X^-$. The synthetic methodology and optical properties of carbocyanines will be discussed.

This thesis consists of two parts: (A) synthesis and optical properties of novel carbocyanine dyes substituted with various amines *and* the synthesis of unsymmetrical carbocyanine dyes containing monofunctional groups for bioconjugation.

(B) synthesis of heptamethine carbocyanine dyes to be used for image-guided surgery.

In part A, the synthesis of carbocyanine dyes functionalized with various amines and studies of their optical properties with respect to absorbance, fluorescence, quantum yield and extinction coefficient will be presented. These property studies will aid in designing efficient dyes for future biomedical applications. Part A will also include a one pot synthesis of unsymmetrical carbocyanine dyes functionalized with mono carboxylic acid chains, useful for biomolecule (i.e. proteins, amino acids, etc.) conjugation.

Part B will describe the synthesis of novel carbocyanine dyes to be used for cancer image-guided surgery. Cancers are thus far incurable diseases, i.e. there are no drugs currently available to cure cancer; however, by designing a dye to visualize tumor cells will greatly increase the efficiency of cancer removal and hopefully increase the survival rate of cancer patients. The dyes reported in this thesis are superior to commercially available dyes used to visualize and identify various tumors invisible to the naked eye of surgeons with regards to biodistribution and clearance through kidney filtration.

INDEX WORDS: Synthesis, Near-infrared, Carbocyanine, Heptamethine, Cyanine, Dye, Polymethine, Heterocycle, Quaternary salt, Vilsmeier-Haack, Fluorophore, Imaging

SYNTHESIS OF NEAR-INFRARED HEPTAMETHINE CYANINE DYES

by

JAMIE L. GRAGG

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Art and Sciences

Georgia State University

2010

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2010

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May 2010

DEDICATION

This thesis is dedicated in memory of my late grandparents, Pauline and J.C. Green, whose strong-willed spirits have guided me, not only through this thesis but also through everyday life. You are loved and missed.

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First and foremost, I would like to thank my advisor, Dr. Maged M. Henary, whose attitude and love and for science made it possible for a once-proclaimed shy biologist to become a well-rounded, confident chemist. He has not only taught me tricks and secrets of organic synthesis, but also *how* to think. Without his paternal guidance and encouragement, this work would not have been possible.

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PART A

**SYNTHESIS AND OPTICAL PROPERTIES OF HEPTAMETHINE
CARBOCYANINE DYES SUBSTITUTED WITH VARIOUS AMINES AND
UNSYMMETRICAL CARBOCYANINE DYES CONTAINING
MONOFUNCTIONAL GROUPS**

A.1. SYNTHESIS OF CYANINE DYES, A REVIEW

A1.1. INTRODUCTION

A.1.2. Historical Background

In 1856, C. H. G. Williams synthesized the first cyanine dye [1] upon heating *N*-amyl quinolinium iodide with *N*-amyl lepidinium iodide in ammonia to produce a “magnificent blue colored” compound **1a-c** (Fig. 1). The Latin word *cyanos*, meaning blue [1], gave rise to the general cyanine dye name. In the following years, related compounds were synthesized and referred to as isocyanine **2**, pinacyanol **3**, psuedocyanine **4**, and kryptocyanine **5** (Fig. 2) [1].

Cyanine dyes are characterized as possessing two heterocyclic moieties, acting as both electron donors and acceptors, and are joined by a single or odd of number of methine groups in which $(n+1)$ bi-electrons are distributed over n atoms [1] producing a delocalized cation **1c** across the methine chain. This unique characteristic gives cyanine dyes a wider range of absorption than any other known class of dyes. Synthetic cyanines [2-5] are known to absorb between the visible and infrared regions of the electromagnetic spectrum. In addition, cyanines exhibit narrow absorption bands and high extinction coefficients. Due to these properties, cyanine dyes have been extensively employed in various applications such as photographic processes, laser printing, nonlinear optical materials, and more recently fluorescent probes for biomolecular labeling. In particular, their use in imaging technology which will be discussed in the chapters.

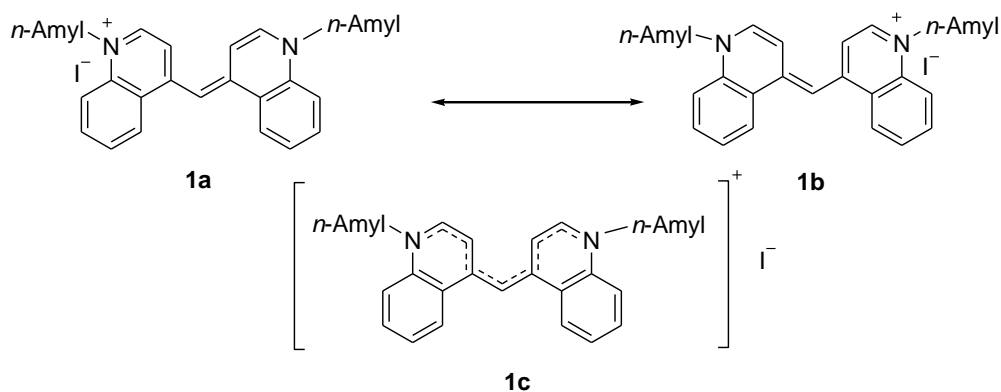


Figure 1. The first synthetic monocyano dye [1].

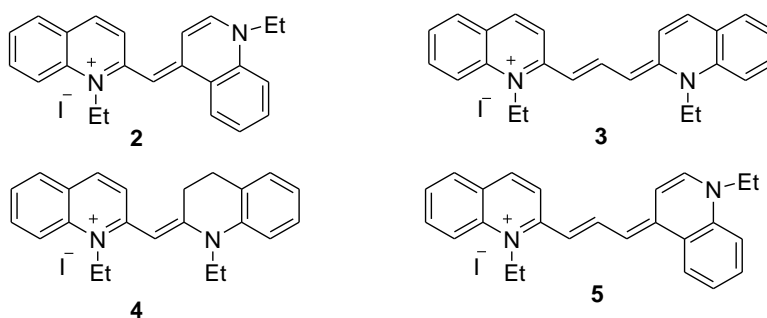


Figure 2. Various carbocyanine dye structures [1].

A.1.3. Naturally occurring cyanine dyes

Since their accidental discovery, cyanine dyes have been identified as colorants in natural products [2-5]. These natural dyes were first observed by Wyler [2,3] in the late 1960s and by Musso [4] in the late 1970s. These dyes were confirmed to contain a similar feature; a pentamethinium cyanine chromophore substituted with two chiral end groups derived from *L*- α - amino acids. Betanin **6**, which is responsible for the red-violet color of the red beet, *Beta vulgaris*, exhibits a visible absorption at 537 nm [5]. The orange-red fungus dye musca-aurin I **7**, is found in the toadstool fly agaric *Amanita muscaria*, with an absorption maximum at 475 nm (Fig. 3) [5].

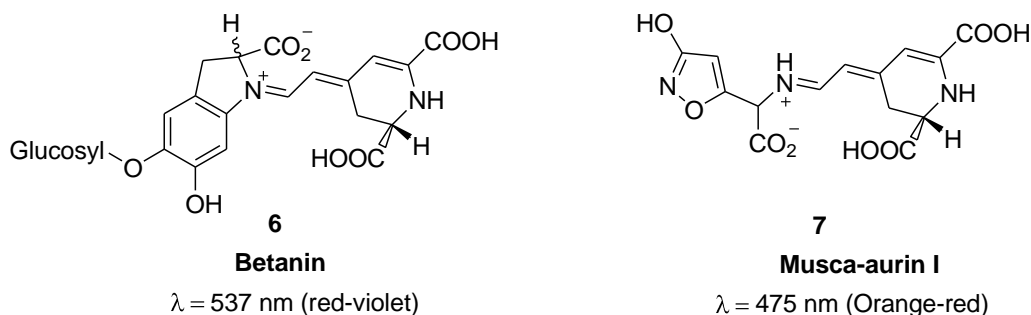


Figure 3. Natural carbocyanine dyes [5].

Cyanine dyes are cationic molecules in which two terminal nitrogen heterocyclic units are linked by a polymethine bridge as shown by the general structure **8** (Fig. 4) [6-9].

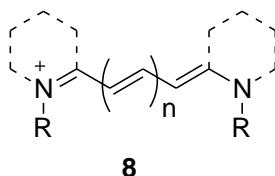


Figure 4. General structure of carbocyanine dyes [6-9].

Cyanine dyes' common names depend on the number of methine groups in the polyene chain. For example, compounds **8** with $n = 0$, $n = 1$, $n = 2$, and $n = 3$ are referred to as mono-, tri-, penta-, and heptamethine cyanines, respectively.

Many different polymethine cyanine derivatives have been synthesized during the last decade. Their syntheses are accomplished by a stepwise condensation reaction of two nucleophilic *aza*-heterocycles containing an activated methyl group with a polyene-chain precursor i.e., an unsaturated bisaldehyde or its equivalent. Structural diversity is achieved through variations in the polyene chain, nitrogen substituents, and the heterocycles. However, this general synthetic method is not compatible with a wide range of reactive groups located on the *aza*-heterocycles for a fine tuning of the solubility, reactivity, and spectroscopic properties of the corresponding cyanine dyes.

Functional groups such as carboxylic and sulfonic acids are completely inert toward the reagents and reaction conditions used for achieving the condensation reaction. An alternative synthetic approach utilized is based on the preparation of a precursor of the target functionalized cyanine dyes, or a “convertible cyanine dye”, and its subsequent post-synthetic chemical transformations to give the fluorophore bearing the desired reactive groups. This synthetic methodology was applied to the chemical derivatization of heptamethine cyanine dyes (Fig. 5).

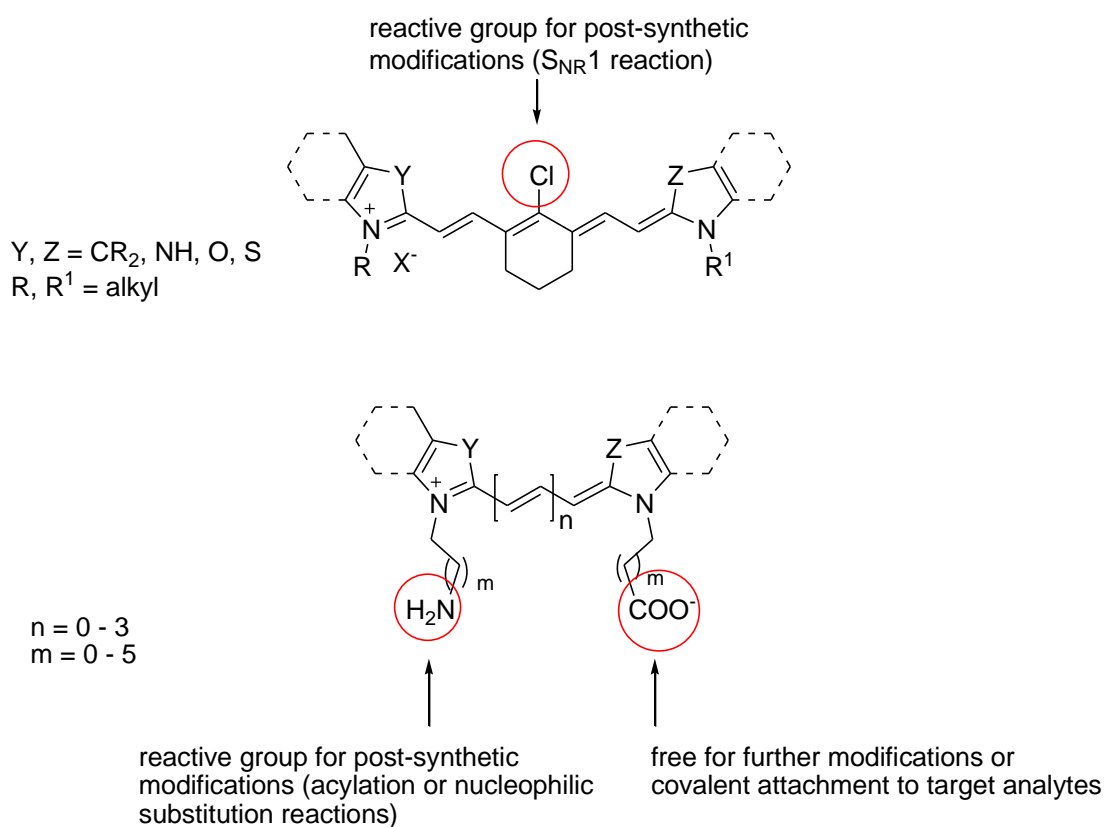


Figure 5. Reactive groups for post-synthetic modifications [7-11].

The use of a precursor having a chlorine atom at the *meso* position [7,8], can be easily replaced by various nucleophiles (alcoholates [9], amines [10,11], and thiols [12,13]) through an $S_{NR}1$ mechanism (a type of substitution reaction in which a certain

substituent on an aromatic compound is replaced by a nucleophile through an intermediary free radical species).

Some substituents such as carboxyl and amino groups attached to heptamethine dyes cyanines containing a chloro-cyclohexyl moiety in the polyene chain are important as NIR labels whose reactivity and optical properties are suitable for *in vivo* imaging [14] and DNA sequencing applications [9].

General problems with near-infrared (NIR) fluorophores compared to visible light fluorophores are: 1) significant spectral broadening as the wavelength increases, 2) low quantum yield, 3) photoinstability, 4) chemical instability with increasing red-shift, and 5) the tendency to aggregate because of hydrophobicity. The ideal NIR dyes for *in vivo* imaging should have the following characteristics: 1) a peak fluorescence close to 700-900 nm, 2) high quantum yield, 3) high chemical and photostability, 4) non-toxicity, 5) good biocompatibility, biodegradability and excretability, 6) availability of monofunctional derivatives as platform technology, and 7) commercial viability and scalable production for large quantities required for human use [15].

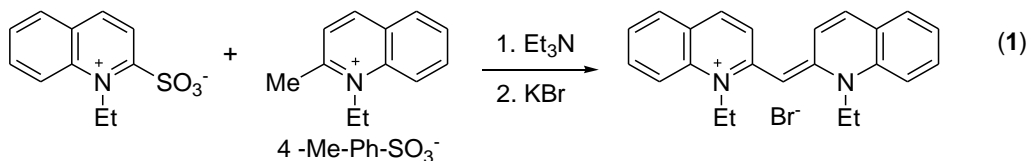
Recently, there have been extensive reports describing the synthesis and applications of polymethine dyes as non-covalent labels for nucleic acid detection [16-18]. Such dyes range between the visible and near-infrared spectral regions. There are mono-, tri- and pentamethine cyanines, used for non-covalent nucleic acid labeling. Although there are many well developed synthetic routes to monomethine and trimethine cyanines [19], the pentamethine and heptamethine dyes are generally synthesized via condensation of methyl-substituted quaternized heterocyclic compounds with an α,ω

dialdehyde or equivalent [19,20-23]. In this review chapter, various synthetic routes to produce different classes of carbocyanine dyes will be discussed.

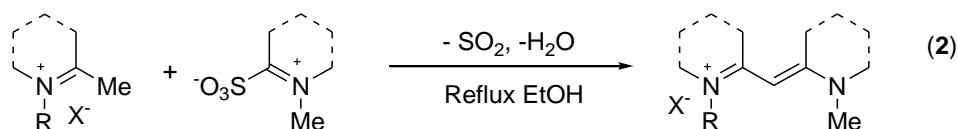
A.1.4. Monomethine cyanines

The monomethine cyanines show absorption in the visible region; the addition of one vinyl moiety to the chromophore produces a bathochromic shift of about 100 nm [23]. As the length of the polymethine chain increases, the fluorescence quantum yield decreases upon binding with nucleic acid [17].

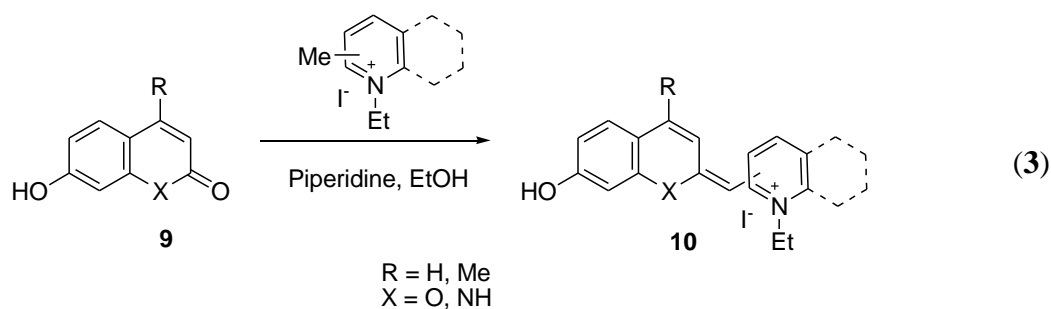
Asymmetric monomethine cyanine dyes are the best non-covalent binding nucleic acid labels due to the generation of high fluorescence signals. The synthesis of the monomethine cyanine dyes is performed upon heating sulfobetaines derived from *N*-alkylheterocyclic compounds and a quaternary salt of heterocyclic 2- or 4-methyl compounds under a basic conditions (Equation 1) [18,24].



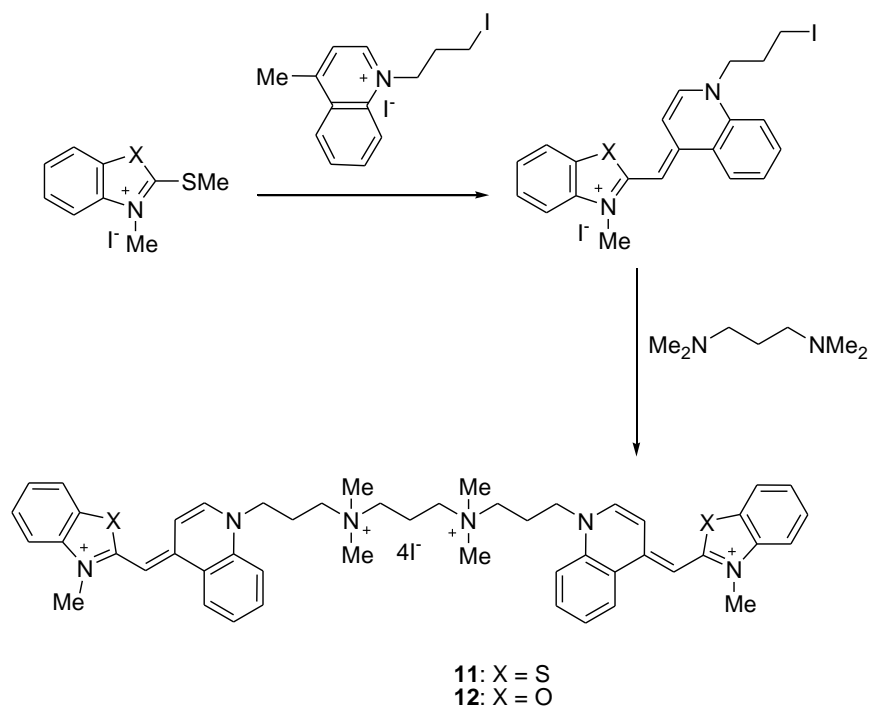
A novel method for the preparation of symmetrical and asymmetrical monomethine cyanine dyes was developed by Deligeorgiev *et al.* [25]. They found that the preparation of monomethine cyanine dyes can be carried out by melting the starting compounds e.g. a sulfobetaine derived from an *N*-alkylheterocyclic system and the quaternary salt of a 2- or 4-methyl heterocyclic salt under basic conditions. The applicability of this modification depends on the melting points of the substrates and their relative thermostability. However, for less thermo-stable intermediates, preparation in boiling polar solvent or solvent mixtures is more suitable (Equation 2) [25].



Another approach to the synthesis of monocyanyines have been suggested from the reaction of 7-hydroxy-4-methyl(*H*)coumarin **9** and 2- or 4-methyl quaternary salts e.g. ethylpyridinium and ethylquinolinium iodide [26] in the presence of piperidine as a catalyst to afford monomethine cyanine dyes **10** (Equation 3).



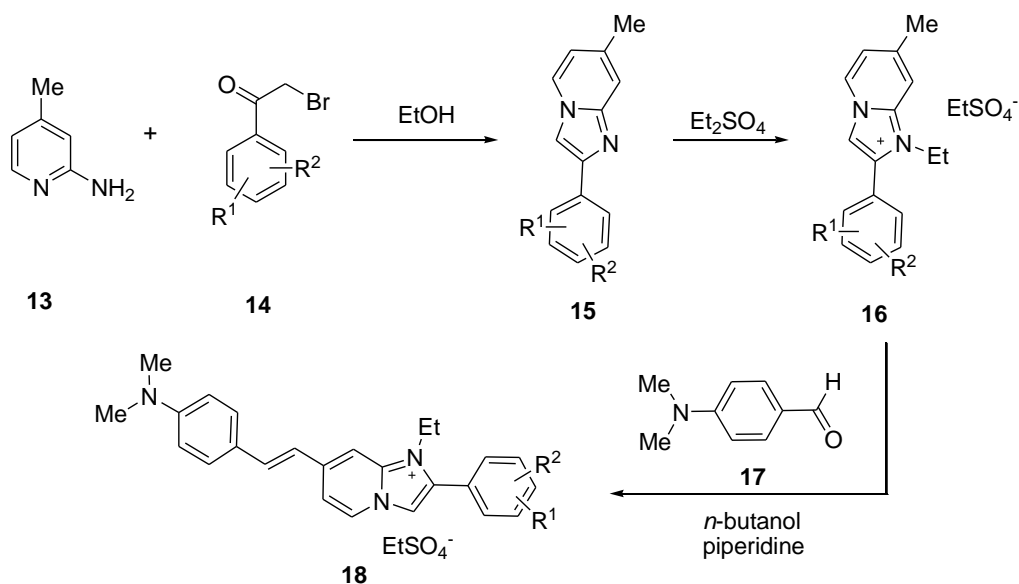
Other synthetic approaches to monomethine cyanines **11**, **12** are shown in Scheme 1 [6]. Polycationic cyanine derivatives including **11**, **12** have been synthesized as strong nucleic acid binders [27-33].



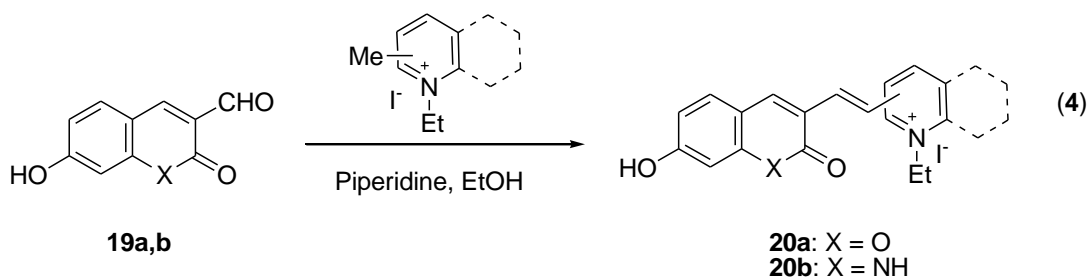
Scheme 1

A.1.5. Dimethine cyanine dyes

Kovalska *et al.* first synthesized a series of styrylcyanines containing a 2-aryl imidazo[1,2-a]pyridinium moiety and different substituents at 2-phenyl ring [34]. The general pathway for the synthesis of novel styryl imidazo[1,2-a]pyridinium dyes shown in Scheme 2. 2-Amino-4-picoline **13** was reacted with phenacyl bromide derivatives **14**, and the product **15** was treated with alkylating agent to generate quaternary salts **16**. Then the condensation reaction of **16** with benzaldehyde **17** yielded styryl dyes **18**.



Abd El-aal *et al.* reported the synthesis of dicyanines **20a,b** beginning with 3-formylcoumarin **19a** (X = O) or 3-formylquinolinone **19b** (X = NH) and 2- or 4-methyl quaternary salts e.g. ethylpyridinium and ethylquinolinium iodide under basic conditions (Equation 4) [26].



A.1.6. Trimethine cyanine dyes

A.1.6.1. Synthesis using orthoester method

The orthoester method is used as a general synthesis of trimethine cyanine dyes (Equation 5). This method was discovered by Koenig [35] and is applied only for the synthesis of symmetrical trimethines. Many classes of quaternary salts with various

substituents in the aromatic ring **21** are reacted with orthoesters **22**. Pyridine is usually used as a base [36] and in some cases mixtures of pyridine and other organic-amino bases have been reported [37].



A⁻ = counter anion

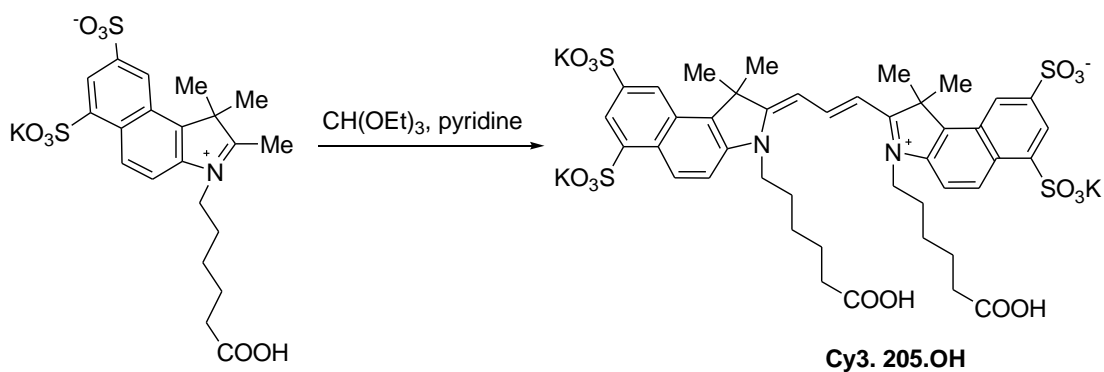
X = NR, CR₂, O, S, Se

R = alkyl

R¹ = H or alkyl

R² = alkyl or other carbon-chain functionality

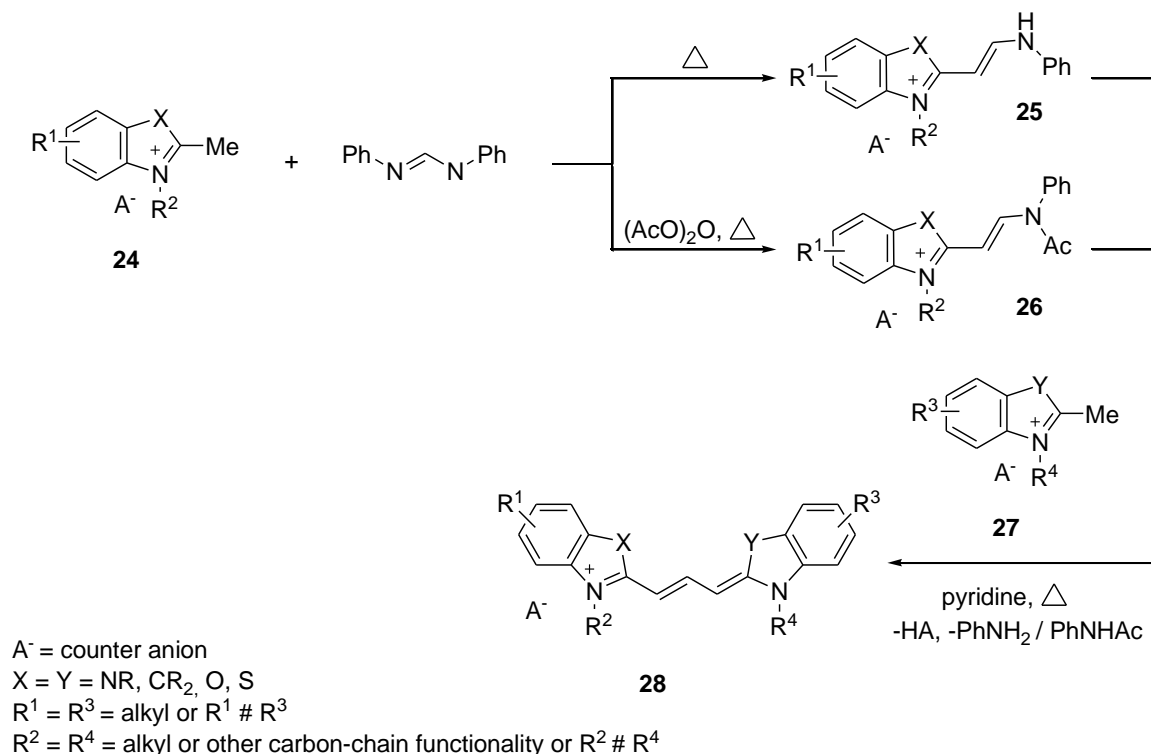
Utilizing the orthoester method for the preparation of trimethine cyanine dyes, Mujumdar *et al.* synthesized new water soluble trimethine dyes using appropriate naphthylamine derivatives as starting materials (Scheme 3) [38].



Scheme 3

A.1.6.2 Synthesis using diphenylformamidine method

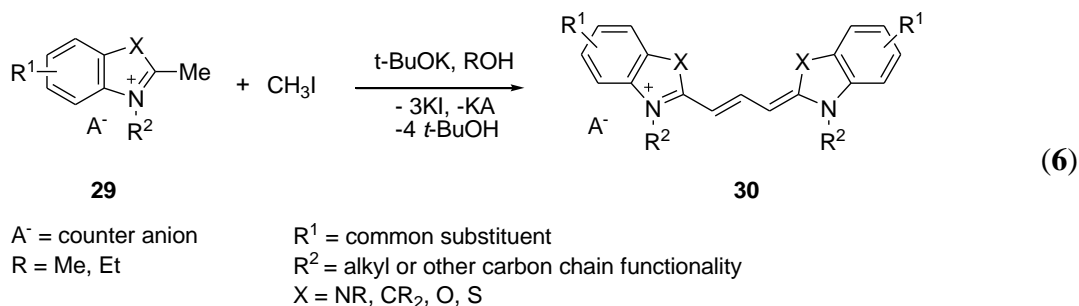
The *N,N'*-diphenylformamidine method (Scheme 4) is applied for the synthesis of symmetrical and unsymmetrical trimethines. The first step can be carried out with or without activating agents (e.g. acetic anhydride [39]) for nucleophilic attack, and yields the corresponding anilino vinyl **25** or anilido vinyl **26** compounds [37,39,40]. When the condensation is performed without acetic anhydride [41], the reactions are conducted in *n*-propanol or dimethylsulfoxide (DMSO) at high temperatures (130-180 °C) for several hours. When acetic anhydride is used, the reaction is carried out at reflux for 30-60 min. The second step is carried out similarly to the orthoester method – i.e. of pyridine the precursor **26** is coupled with another molecule of methylene base **27** under basic conditions to form the trimethine dye **28**.



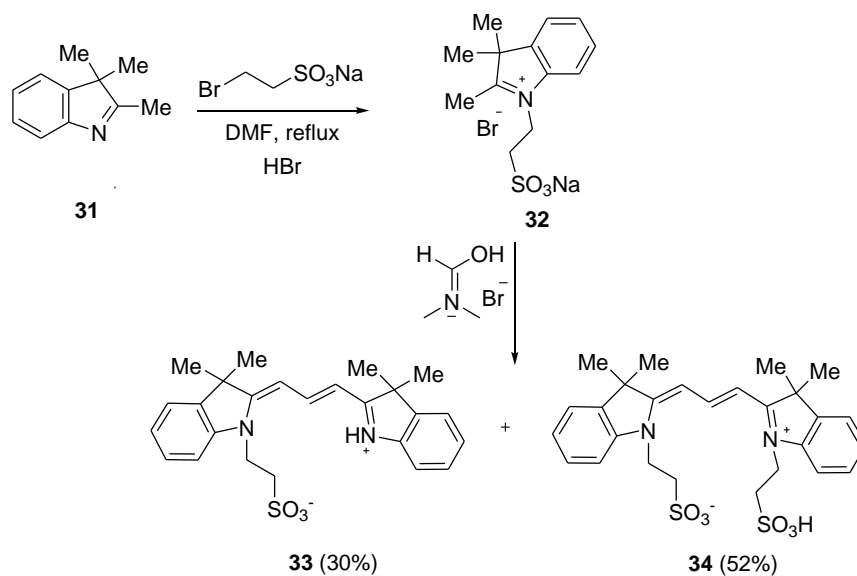
Scheme 4

A.1.6.3. Synthesis using the De Rossi Method

De Rossi *et al.* [41] reported that the preparation of trimethines requires two equivalents of indolinium salt **29** reacted with iodoform in the presence of excess of potassium or sodium *t*-butoxides to yield **30** (Equation 6).



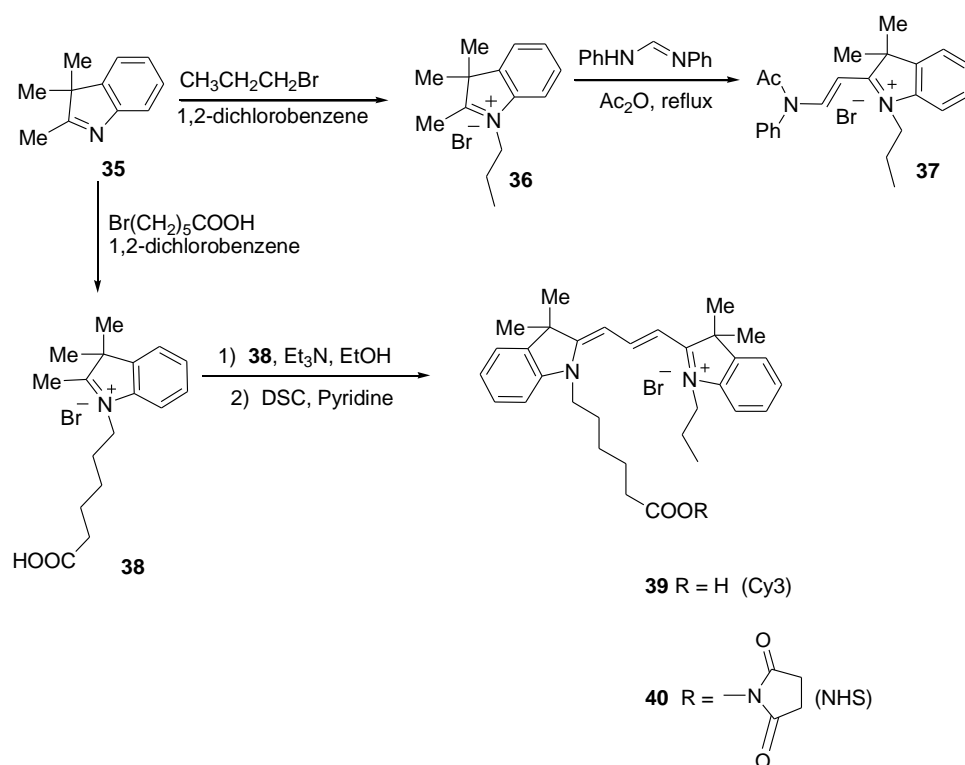
A new Vilsmeier-type reagent was generated from *N,N*-dimethylformamide (DMF) and hydro bromide (HBr), then reacted with the salt **32** to yield a mixture of indocarbocyanine pH-sensitive dye **33** and symmetric indocarbocyanine dye **34** (Scheme 5) [42].



Scheme 5

A simple and practical method for the synthesis of indocyanine dye **39**, useful in gel electrophoresis, was synthesized by Jung *et al.* [43]. This dye was synthesized from

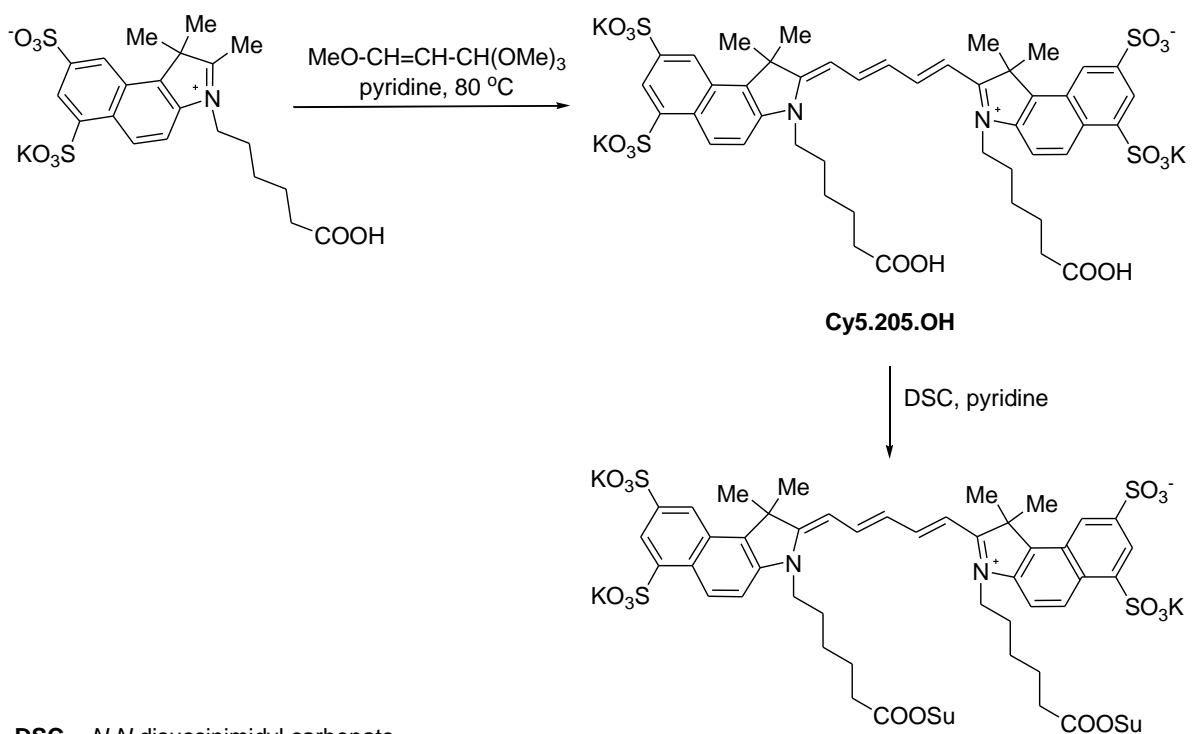
commercially available 2,3,3-trimethylindolenine **35**, which was alkylated with the appropriate alkyl halide to provide corresponding *N*-alkyl derivatives **36** and **38**. Then, the condensation of **36** with diphenylformamidinium in acetic anhydride afforded corresponding acetanilidylvinyl indolium salt **37** in excellent yield. Salt **36** was then reacted with the other salt **38** in ethanol in the presence of triethylamine to give desired dye **39** in good yield. Dye **39** was easily converted into the corresponding *N*-hydroxysuccinimide ester (NHS) **40** by treatment with *N,N'*-disuccinimidyl carbonate (DSC) under basic conditions (Scheme 6).



Scheme 6

A.1.7. Pentamethine cyanine dyes

Mujumdar *et al.* synthesized new water soluble pentamethine benz-indolenine dyes using appropriate naphthylamine derivatives as starting materials [38]. The general synthesis of the dyes is outlined in Scheme 7.

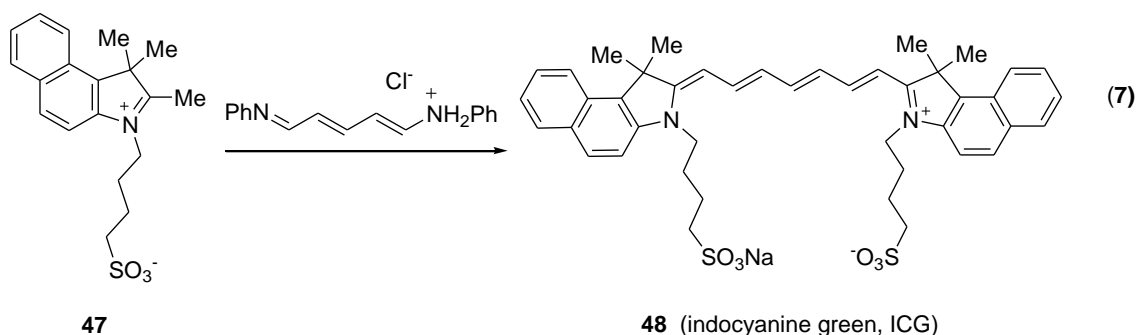


Scheme 7

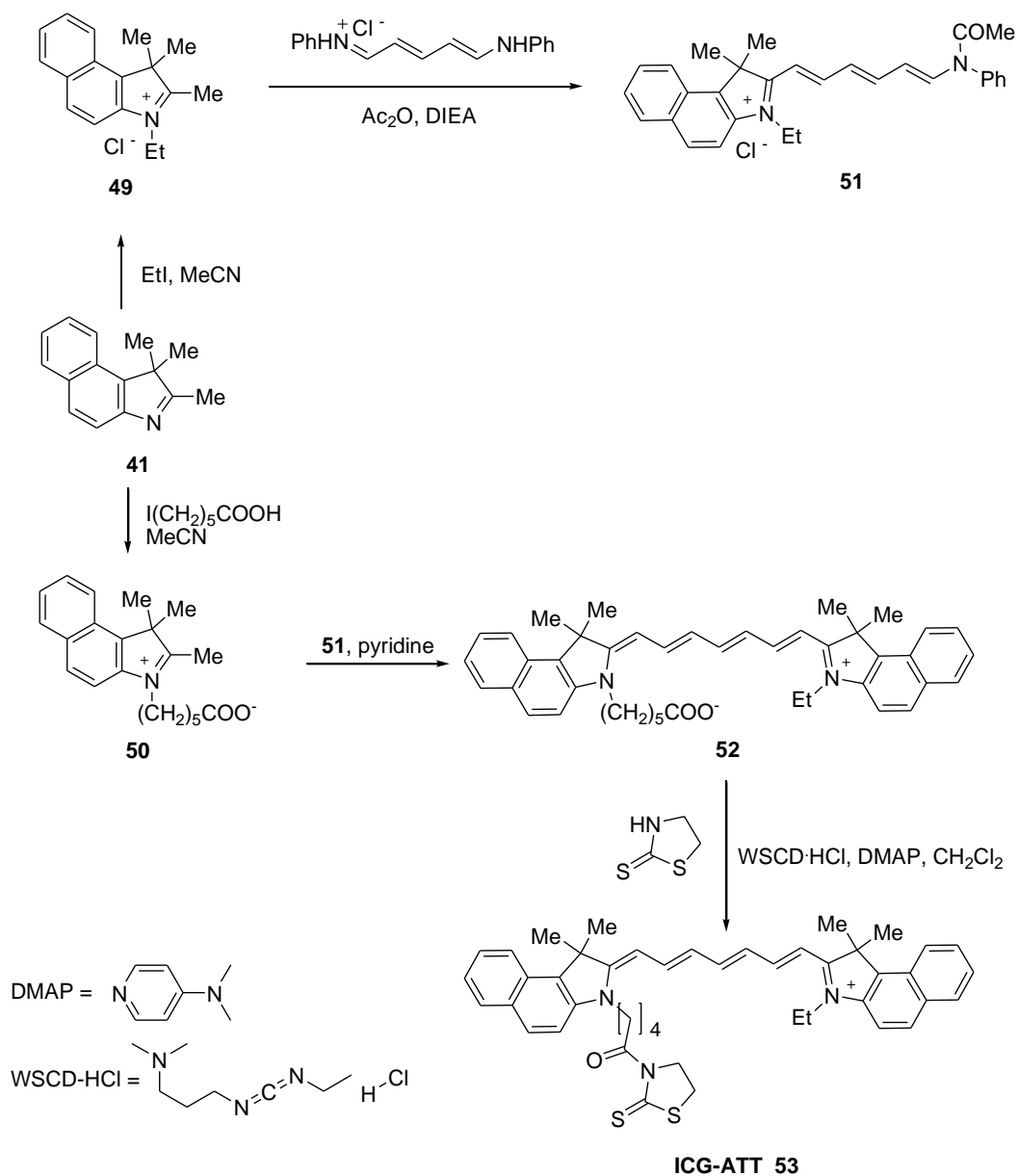
Chipon *et al.* published the first original synthetic route to new water soluble functionalized fluorescent amino acid derived from a pentamethylene cyanine dye [44]. The multi-step synthetic pathway to this dye is presented in Scheme 8. In the original report [44] 1,1,2-trimethyl-1*H*-benz[*e*]indole **41** is a common starting substrate for both iminium quaternary salt **42** and **45**. Then compound **42** was reacted with malonaldehyde dianilido hydrochloride in a mixture of acetic acid and acetic anhydride under reflux to give **43** in quantitative yield. Reaction of **43** with **45** in a mixture of acetic acid and pyridine under reflux furnished the pentamethine cyanine containing phthalimide moiety which was treated with an excess of hydrazine monohydrate to give the target cyanine-base amino acid **46**.

A.1.8. Heptamethine cyanine dyes

One of the most important heptamethine cyanine dyes, indocyanine green (ICG) dye **48** [45]. Approved in 1958 by the Food and Drug Administration (FDA), ICG is well known and has previously been clinically used to diagnose liver activity (Equation 7).

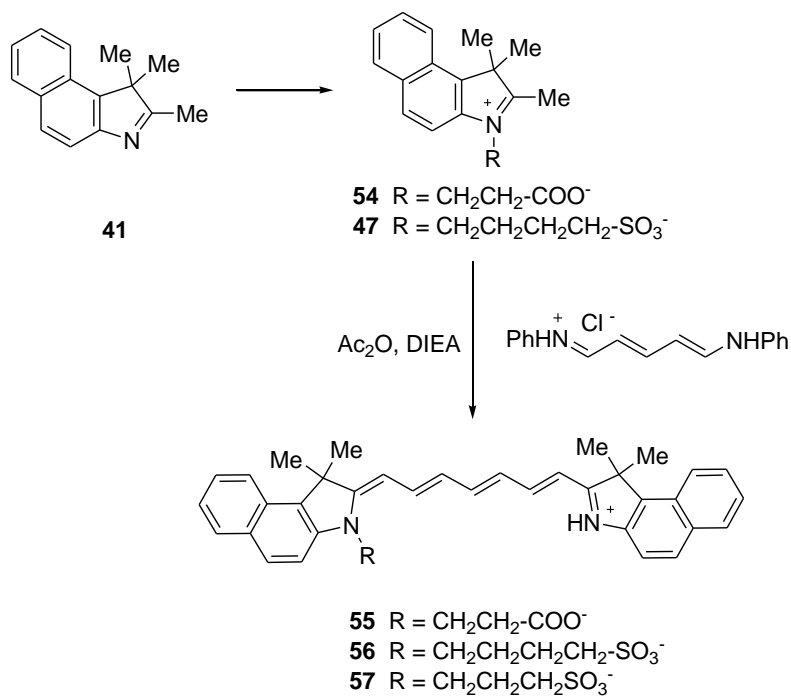


In this context, Nagao *et al.* described the synthesis of a new fluorescent labeling reagent, the indocyanine green amide derivative of 1,3-thiazolidine-2-thione (ICG-ATT) as an ICG analog [46]. The synthetic pathway for the ICG-ATT is outlined in Scheme 9. 1,1,2-Trimethylbenz[*e*]indole **41** was alkylated with ethyl iodide in acetonitrile under reflux for two days to afford compound **49** in 91% yield, which was treated with glutacanaldehyde dianilido hydrochloride in acetic anhydride at 100 °C for 1h to yield compound **51** in a quantitative yield. *N*-alkylation of **41** with 6-iodohexanoic acid in acetonitrile under reflux gave compound **50**. Reaction of **50** with **51** in pyridine at 40 °C furnished an indocyanine green derivative **52** bearing carboxylate group in 77% yield. Finally, **52** was treated with 1,3-thiazolidine-2-thione in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (WSCD·HCl) and a catalytic amount of dimethylaminopyridine (DMAP) in dichloro methane at 0 °C to give the desired ICG-ATT **53** in 87% yield.



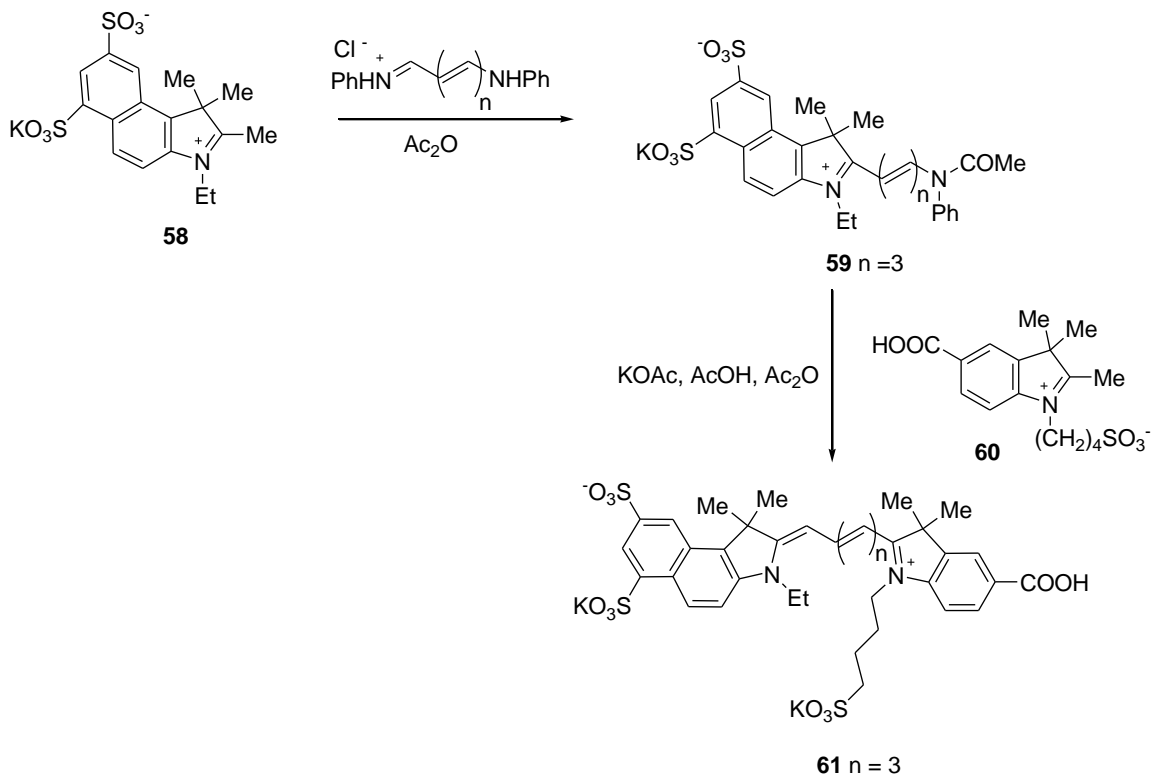
Scheme 9

In analogical way NIR fluorescent norcarboindoles (H-ICG **55** and H-cypate **56**, **57**) were synthesized as a nonspecific pH indicator or as a target-specific pH probe by conjugation the free carboxyl group with biomolecules (Scheme 10) [47].



Scheme 10

Tung *et al.* [15] published the synthesis of **61** as depicted in Scheme 11. Starting with 1,1,2-trimethylbenzindoleninium 1,3-disulfonate dipotassium salt, it was converted to **58** by treating with ethyl iodide. The reaction of **58** with malonaldehyde dianil hydrochloride or glutaconaldehyde dianil hydrochloride results in the intermediate **59** (Scheme 11). The asymmetrical dye **61** was synthesized by reacting **59** with 5-carboxy-1-(4-sulfobutyl)-2,3,3-trimethyl-3*H*-indolenine **60**.



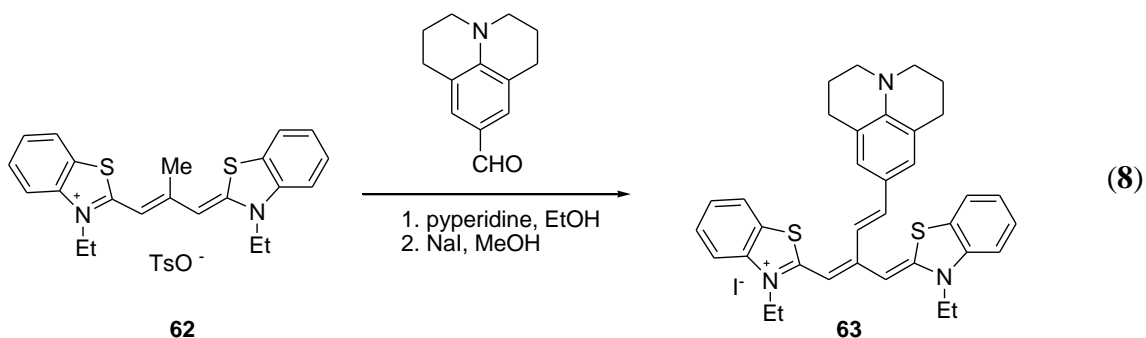
Scheme 11

A.1.9. Synthesis of *Meso*-substituted Cyanine Dyes

Some polymethine cyanine dyes substituted at the *meso*-position by alkyl or halogen groups [23] are introduced to adjust absorption wavelength and to control their aggregation property [48]. Various *meso*-substituted cyanines have been synthesized and assayed for their electronic spectral properties.

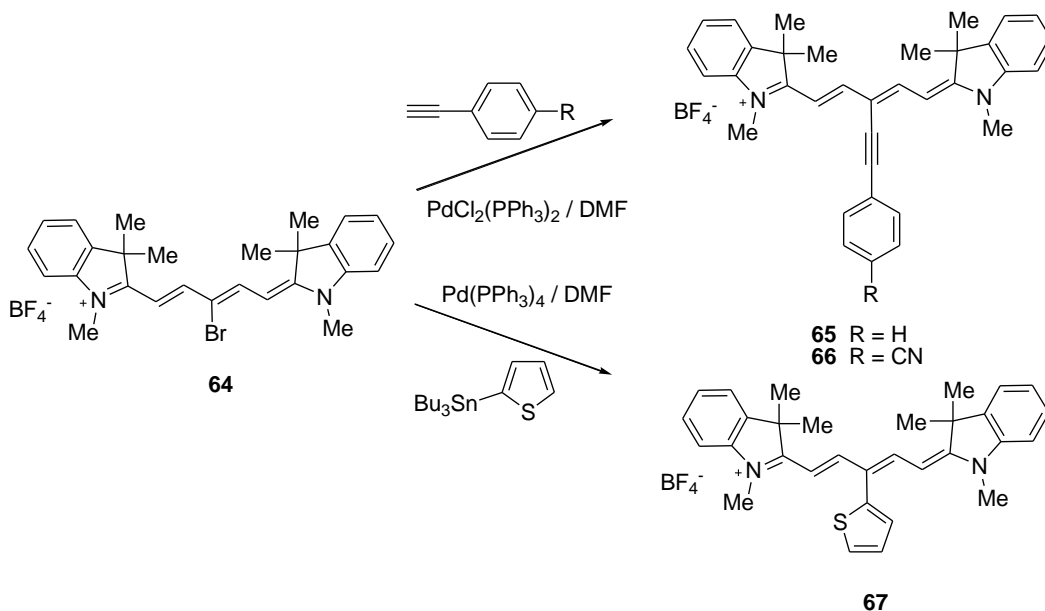
A.1.9.1. Trimethine cyanine dyes

A facile synthesis of *meso*-substituted trimethine cyanine **63** is presented in Equation 8 from the reaction of **62** with 9-formylquololidine [49].



A.1.9.2. penta- and heptamethine cyanine dyes

In the original report [50] some new indodicarbocyanines **65-67** bearing C-substituents at the *meso*-position have been synthesized by palladium-catalyzed cross-coupling reactions (Scheme 12). A much simpler and more versatile approach is available. Functionalization of the indodicarbocyanine **64** can be easily achieved by condensation of the corresponding malonaldehyde or its derivative with an indoleninium salt [51]. However, this method serves a few restrictions e.g. the availability of a suitable malonaldehyde and the survival of the desired substituents in the synthesis.



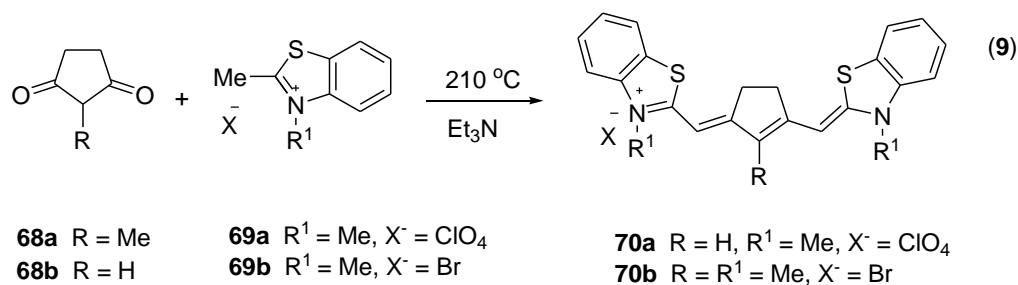
Scheme 12

A.1.9.3. Synthesis of Rigid *Meso*-substituted Cyanine Dyes

A.1.9.3.1. Pentamethine cyanine dyes

A series of pentamethine cyanine dyes with cyclohexene or cyclopentene group in the polymethine chain, assumed as DNA groove-binders, were studied as fluorescent probes for nucleic acids as well as for native and denatured proteins [52]. It was revealed that the presence of methyl or dimethyl substituents in 5 position of the cyclohexene group hinders the formation of dye-DNA fluorescent complex, while the methyl substituents in 2 position leads to the increasing of the dye-DNA complex fluorescence intensity.

Cyclopentane-1,3-diones **68a,b** were condensed with the quaternary salt of 2-methylbenzothiazolium **69a,b** at 210 °C in triethylamine to give pentamethine dyes **70a,b** (Equation 9) [53].



For the synthesis of pentamethine cyanines with the cyclohexene group in the chromophores, the condensation of the quaternary salts of various heterocycles (benzothiazole or benzoxazole) containing the active methyl group, with the 1,5-dimethoxy-1,4-cyclohexadienes or with 1,3-diethoxy-5,5-dimethyl- or 1,3-diethoxy-2,5,5-trimethyl-1,3-cyclohexanediens was carried out (Fig. 6)[54-56].

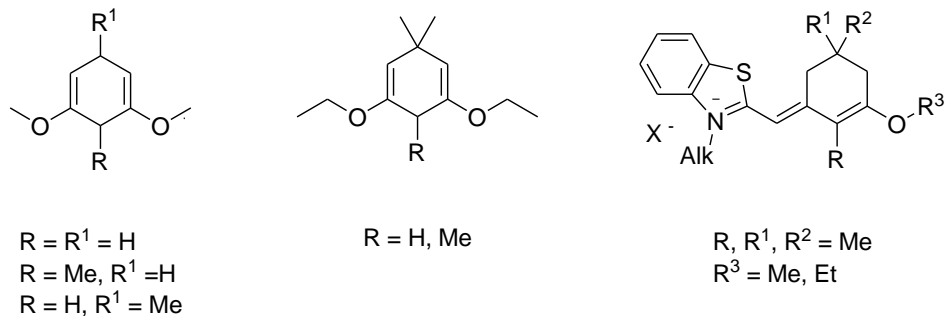
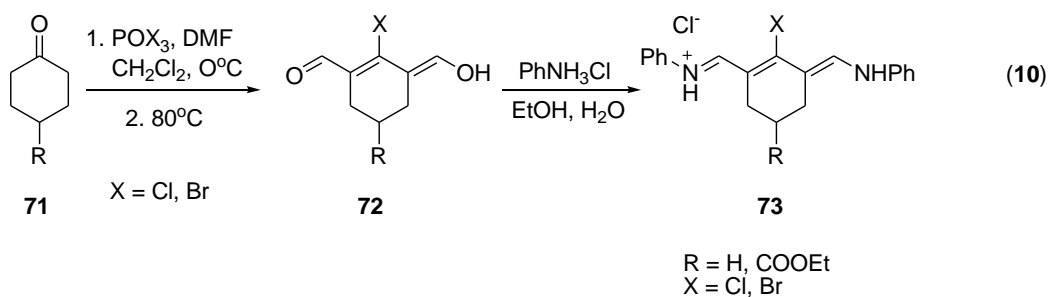


Figure 6. Various cyclohexene groups for synthesis of pentamethine cyanines [54-56].

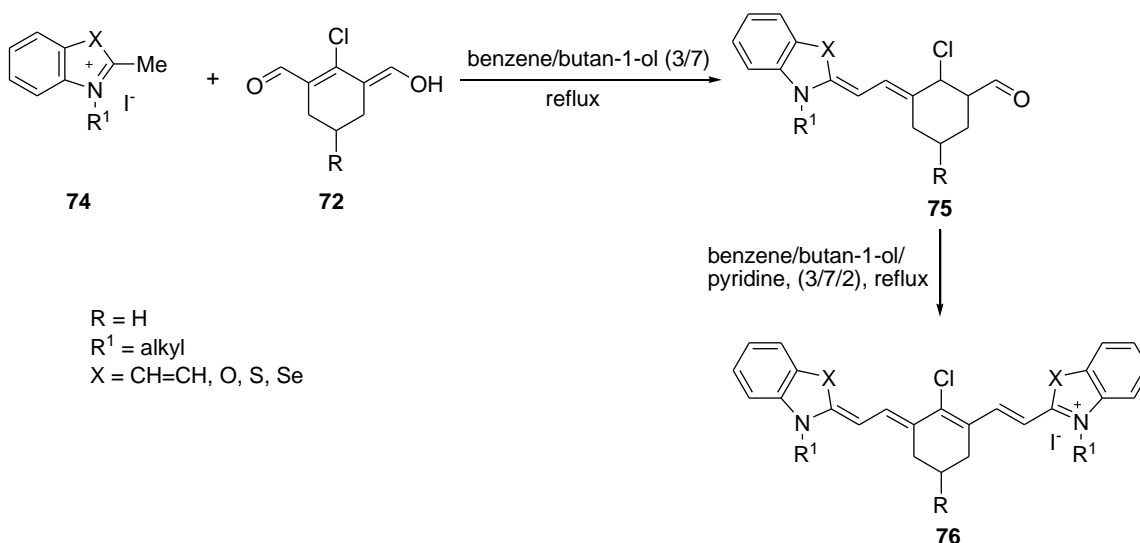
A.1.9.3.2. Heptamethine cyanine dyes

Classical synthesis of heptamethine cyanines involves condensation of a dialdehyde **72** or equivalent **73** (Equation 10) with a methyl substituted quaternized heterocyclic compound in acetic anhydride or in ethanol in the presence of base such as sodium acetate, triethylamine or pyridine.



The chloro carbocyanines **76** (Scheme 13) have traditionally been synthesized by condensation between *N*-alkyl heterocyclic bases, containing an activated methyl group in the 2- or 4-position in relation to the quaternary ammonium salt, and an unsaturated bis-aldehyde or its equivalent, usually as Schiff base. In such cases, the process is usually

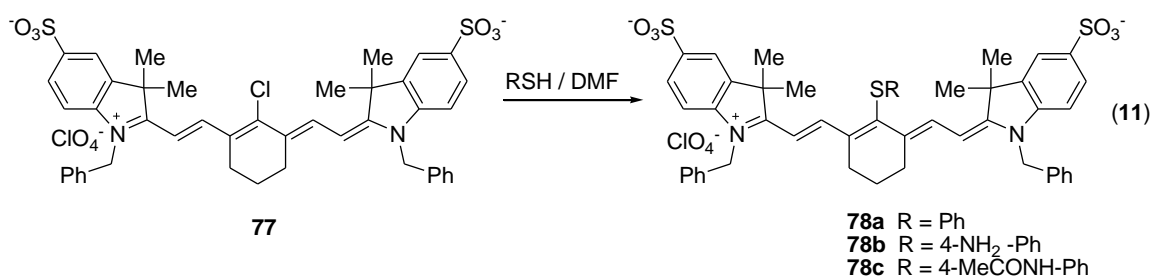
catalyzed by sodium acetate [21,57-60] or triethylamine [59-61], using a mixture of acetic acid and acetic anhydride [58,60] or ethanol [21,57,59] as solvents. More recently, an uncatalyzed synthesis of several symmetric and asymmetric chloro indocyanine dye analogous **76** ($X = \text{CMe}_2$), achieved by heating under reflux, a solution of an *N*-alkyl substituted quaternary salt derived from 2,3,3-trimethylbenzoindole and a bisaldehyde **72** in butanol/benzene (v/v, 7/3), with continuous azeotropic removal of the water formed, was described to be advantageous over the traditional method by avoiding complex mixtures [7,8]. Several chloro- and chloro-substituted indoheptamethine cyanines similar to **76** are now commercially available for use as near infrared laser dyes [62], optical recording media [63], spectrophotometric determination of trace water in organic solvents [64,65], determination of hydrophobicity of proteins [66], fluorescent labeling agents for proteins and their ultra-trace determination [7,8,57,67-72], fluorescent tags in DNA sequencing [7,8,67-68,73-74], immunoassays [71,72] and flow cytometry [75].



Scheme 13

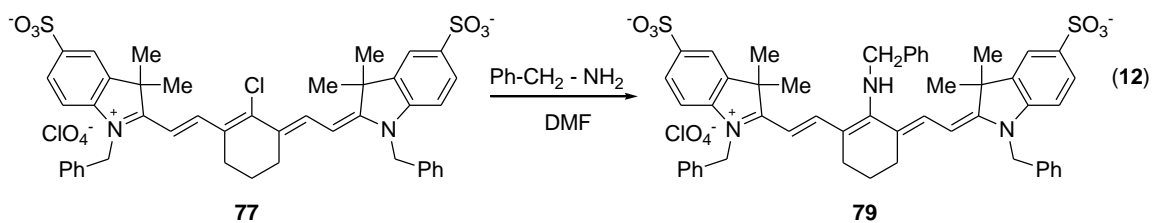
Studies show that heptamethine cyanine dyes containing a rigid chloro cyclohexenyl ring in the methine chain, can increase the photostability and fluorescence

quantum yield [65,76]. This structure also provides the dye with a reactive chloro-group for chemical substitution at the central position [69]. By substitution of the chloro atom with different nucleophiles, many heptamethine cyanine dyes were obtained and used as biosensor and fluorescent probes [76,77-79]. Some of them were employed as photo-induced electron transfer (PET) sensors. Song *et. al.* reported the synthesis of heptamethine cyanine dyes with thio-substituents in the central position such as **78** in which PET can be tuned by changing the electron-donating ability of the substituents (Equation 11) [94].

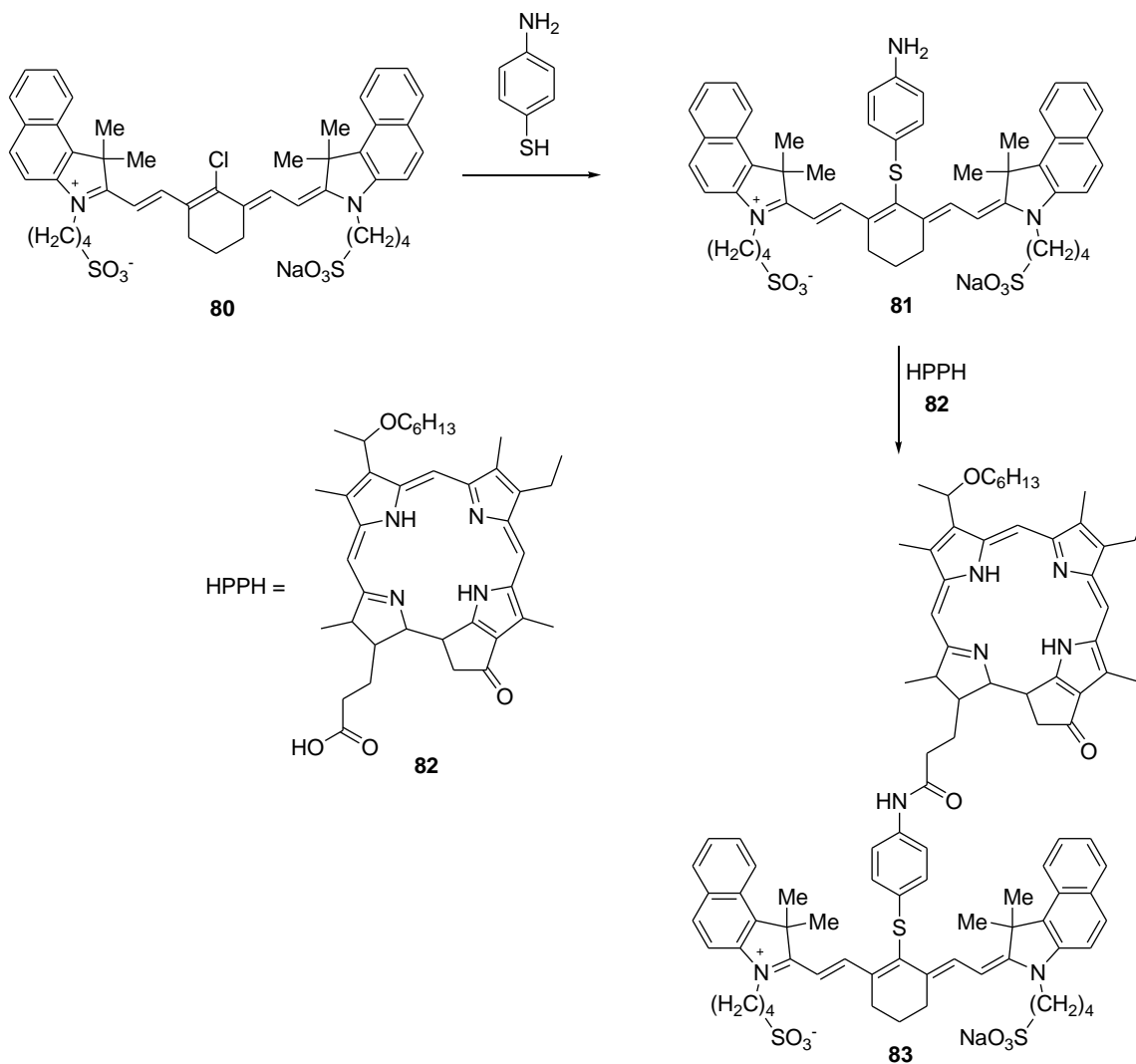


The central chlorine atom at cyclohexene ring substituted by electron-donor group can enhance the photostability of the dyes obviously [10]. Many works were done to modify the heptamethine cyanine dyes by chemical synthesis in order to obtain more advanced photochemical and photophysical properties [79,80-82].

The novel water-soluble near-infrared heptamethine cyanine dye **79** with C-N bond group substituted at cyclohexenyl bridge in heptamethine chain was synthesized by Peng's group (Equation 12) [83,84].



Pandey *et. al.* developed the synthesis and biological studies of target-specific bifunctional agents which could produce the photophysical properties suitable for tumor detection by optical imaging as well as photodynamic therapy (Scheme 14) [86]. Chloro heptamethine cyanine **80** was reacted with 4-aminophenylthiol to produce **81** in 80% yield, then reaction with 3-(4-hydroxyphenyl) propionic acid hydrazide (HPPH) **82** in the presence of *N,N'*-Dicyclohexylcarbodiimide (DCC) afforded HPPH-cyanine dye conjugate **83** in good yield.

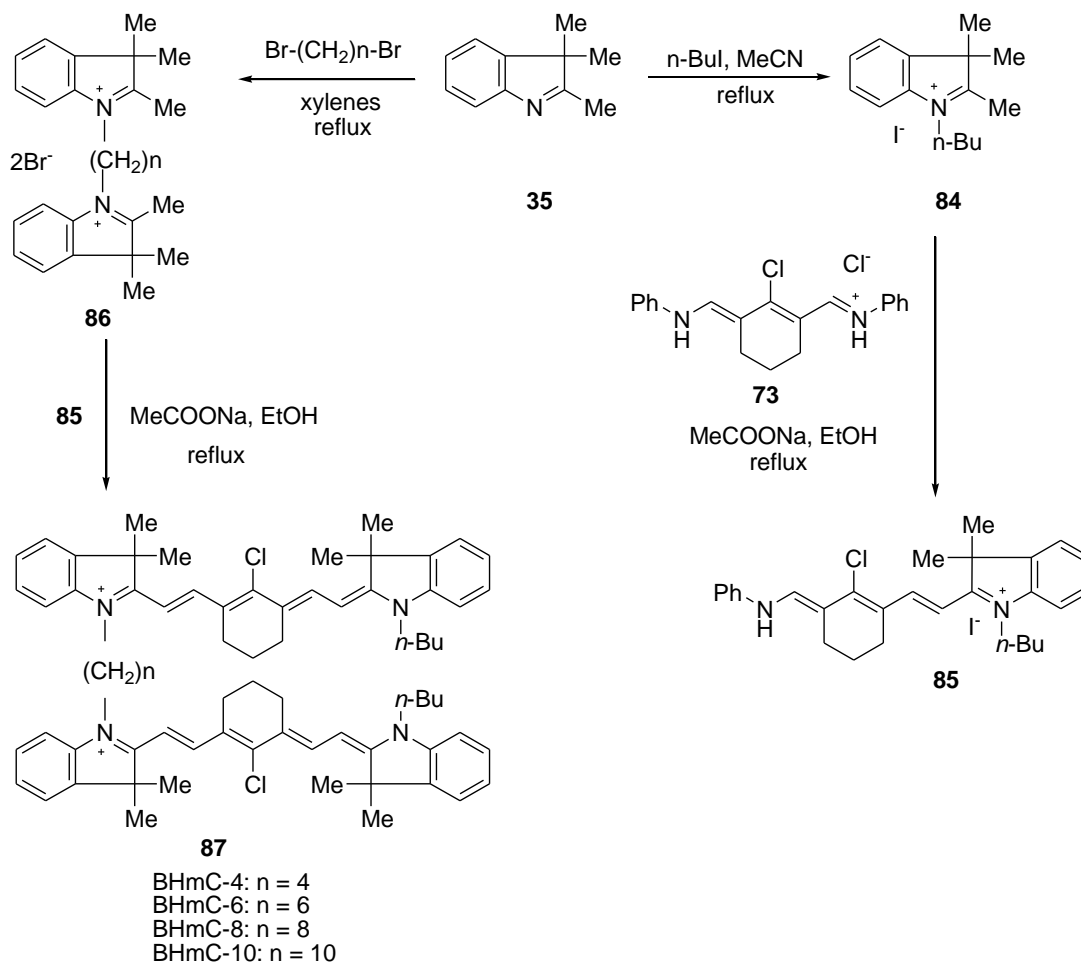


Scheme 14

A.1.9.3.3. Synthesis of Bis(Heptamethine Cyanine) Dyes

Strekowski and coworkers reported for the first time the synthesis of a novel class of near-infrared (NIR) bis(heptamethine cyanine) (BHmC) dyes containing a flexible polymethylene linker between the two cyanine subunits with versatile spectroscopic properties [88]. These bis-cyanines may be of significant bioanalytical utility due to their negligible fluorescence in aqueous solution and a strong increase in fluorescence (~1000 fold) upon binding with a protein. The synthesis of these dyes (BHmCs) such as **87** are

presented in Scheme 15. Indolenine **35** was quaternized with appropriate dibromoalkane to yield the resultant bis-indolium salt **86**, then condensed with the half dye **85**. Compound **85** was prepared by the reaction of **35** with butyl iodide to yield **84**, followed by the treatment of **84** with Vilsmeier-Haack reagent **73** [89].



Scheme 15

A.2. SYNTHESIS OF CARBOCYANINE DYES SUBSTITUTED WITH VARIOUS AMINES

Cyanine dyes are NIR chromophores possessing large molar extinction coefficients and a broad range of wavelengths. Each vinyl addition to the polymethine chain between the terminal heterocyclic groups shifts the wavelength of absorption approximately 100 nm. The polymethine chain is electron deficient due to the delocalization of the cation on the terminal heterocyclic moieties and causes the dyes to absorb in longer wavelengths. Chlorine at the *meso* carbon of the cyclohexene ring, an electron withdrawing group, pulls electrons from the polymethine chain, causing absorbance to shift to even longer wavelengths. Substitution of the chlorine atom with various nucleophiles shift the absorption wavelengths into the red or blue regions of the electromagnetic spectrum depending on the electron withdrawing or electron donating character of the substituent. By the substitution with electron donating groups, such as amines, the absorbance signal shifts into the shorter wavelengths, or blue region of the electromagnetic spectrum.

It is noted that blue shifts of wavelengths increase the photostability of the dyes. NIR cyanine dyes ($\lambda_{\text{max}} > 700$ nm) have a tendency to undergo photodegradation [90]. This is important for all practical applications of cyanine dyes involving fluorescence spectroscopy, where either high sensitivity or high signal-to-noise ratio is crucial. Recent research has placed focus on the effects of changes with regards to the substituted polymethine chains [91], substituted terminal aromatic rings [92] and the scaffold of the dyes [93]. Strekowski *et. al.* suggested the incorporation of a cyclohexene ring in the center of the polymethine chain will aid in developing a fixed conformation to the

molecule in order to enhance the photophysical properties of the NIR dyes [94,95]. Song *et. al.* demonstrated the substitution of the central chlorine atom of the cyclohexene ring with electron donor groups enhance photostability of the dyes [96]. Bertolino *et. al.* described novel heptamethine cyanine dyes with large Stokes' shifts for biological applications in the near-infrared [97].

Bertolini *et. al.* synthesized dyes containing O, S and N at the γ position of the chloro-cyclohexene ring, which induced acknowledgeable differences in the absorption and emission spectra of the dyes. It is known that ether substitution in the *meso* position shifts absorbance around 10 nm however substitution with amines shifts the absorption much more.

In spectroscopy, absorbance is $A_{\lambda} = -\log_{10}(I/I_0)$. I is the intensity of light at a specific wavelength that has passed through a sample, while I_0 is the intensity of light before it enters the sample [98]. Absorbance of a sample is proportional to the concentration of a sample. It is also important to know the molar extinction coefficient of compounds because these are parameters that define how strongly the compound absorbs light at a certain wavelength [98].

Fluorescence is a highly sensitive method; therefore, some signals observed may not be the compound of interest because of the high amplification [99]. One may be observing background fluorescence from solvents, stray light passing through the optics, or turbid solutions, just to name a few interferences. Generally, one wants to record excitation and emission spectra to study the optical properties of their compounds [99].

The emission spectra is a wavelength distribution of the emission measured at a constant excitation wavelength while an excitation spectra is the dependence of emission intensity measured at a single emission wavelength upon excitation [100]. For most fluorophores, quantum yield and emission are independent of excitation wavelengths due to rapid relaxation. Emission is a plot of fluorescence emission intensity versus the wavelength of emitted light when the fluorophore is excited with a monochromatic beam [99].

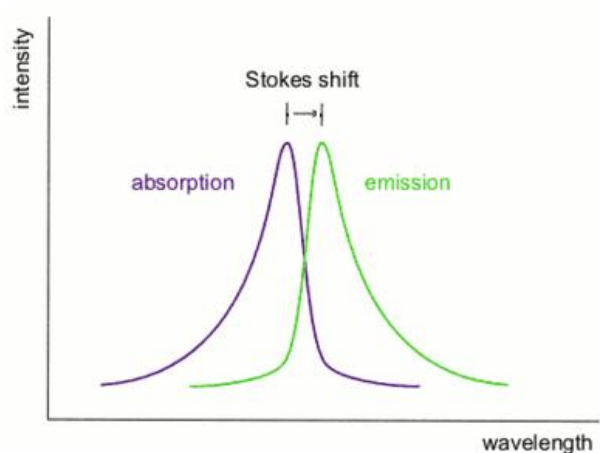


Figure 7. Stokes shift [100].

Energy losses between excitation and emission are observed for fluorescent molecules in solution [100]. Stokes shift is caused by the rapid decay to the lowest vibration level (S_1). Fluorophores generally decay to higher vibrational levels (S_0) which results in loss of excitation energy by thermalization of excess vibrational energy [100]. Fluorophores can also present Stokes shifts due to solvent effects and energy transfer.

Quantum yield is possibly one of the most important characteristics of a fluorophore [99]. The best way to estimate quantum yield of fluorophores is using a standard for comparison that has a known quantum yield. Rhodamine was used as a

standard in this research study [99]. A spectrophotometer [Fig. 8, 100] is depicted below in Figure 7. A light source, usually xenon, is used for excitation purposes and once the wavelength is emitted, a detector is used to identify the peak and create a spectrum.

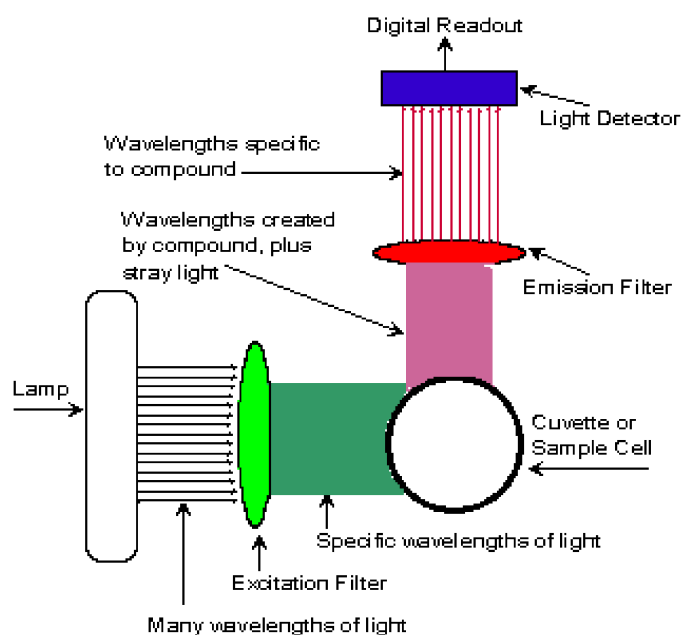


Figure 8. Spectrophotometer [100].

Fluorescence quantum yield is defined as the efficiency with which absorbed light produces some effect and the quantum yield can be defined by the equation [101]:

$$\Phi = \frac{\# \text{ photons emitted}}{\# \text{ photons absorbed}} \quad (13)$$

Experimentally, relative fluorescence quantum yields can be determined by measuring fluorescence of known quantum yields with the same experimental parameters (excitation wavelength, slit widths, etc.) [101] as the dye being studied.

The quantum yield is then calculated by:

$$\Phi = \Phi_R \times \frac{Int A_R n^2}{Int_R A n_R^2} \quad (14)$$

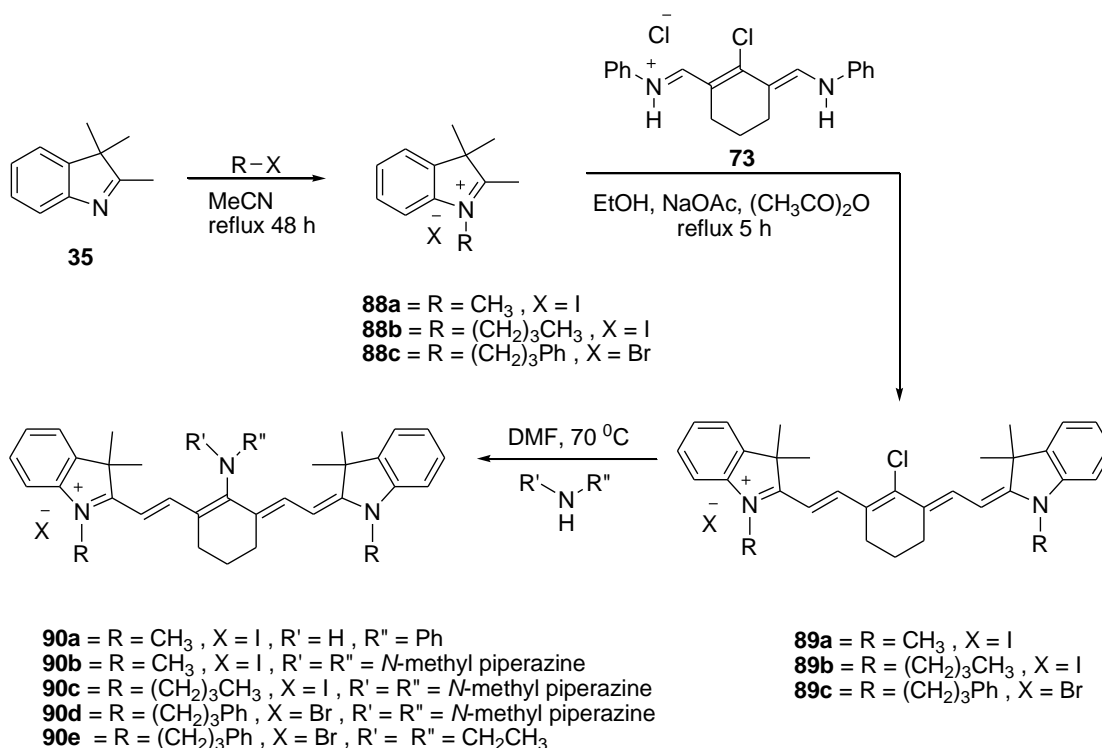
where Φ is the quantum yield, Int is the area under the emission peak, A is absorbance at the excitation wavelength, and n is the refractive index of the sample [101]. The subscript R represents reference substance. In our research, we focused on determining how amines substituted in the *meso* position of the heptamethine dyes affect the Stokes shift and fluorescence quantum yield for future use in bioanalytical applications.

Aim of the study

The aim of this study was to synthesize and characterize novel near-infrared heptamethine cyanine dyes substituted with various amines including *N*-methyl piperazine, diethyl amine, and aniline, as fluorophores possessing spectral and photophysical properties, with respect to high fluorescence quantum yield, absorption, emission, and extinction coefficients. Currently there is not much literature discussing the synthesis and photophysical properties of heptamethine cyanine dyes containing amine moieties. Chloro dyes containing various alkyl chains on the terminal heterocycles will be synthesized for amine substitution to observe differences in spectral properties. It is important to synthesize and investigate different dye analogues for comparison with the literature dyes to better understand how the optical properties change with various amine-substituted dyes for utilization in various bio-analytical applications.

A.2.1. RESULTS AND DISCUSSION

As shown in Scheme 16, the heptamethine carbocyanines possessing a chlorine atom at the *meso* carbon and alkylated with various groups such as methyl, butyl, and phenylpropyl chains on the nitrogen atom of the indole rings were synthesized.

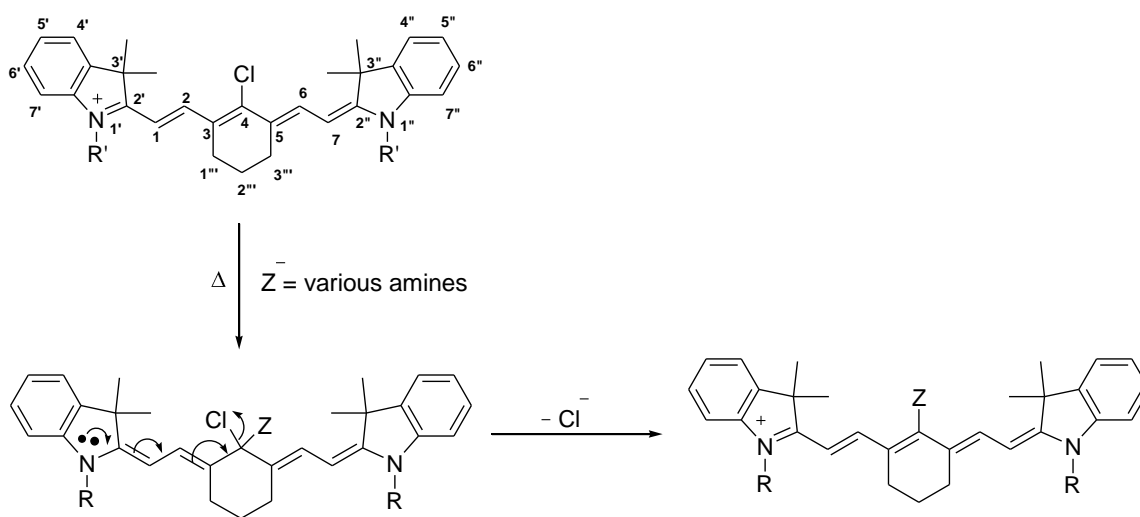


Scheme 16

The synthesis of chloro dye derivatives involved a Fischer base alkylation with various alkyl halides in boiling acetonitrile to afford quaternary ammonium salts **88a-c**. Salts **88a-c** were then condensed with Vilsmeier-Haack reagent **73** [89] under basic conditions in absolute ethanol to yield the *meso*-chloro derivatives of heptamethine carbocyanine dyes **89a-c** which then underwent nucleophilic substitution (S_{NR}1) in *N,N*-dimethylformamide to give the amine substituted dyes **90a-e**.

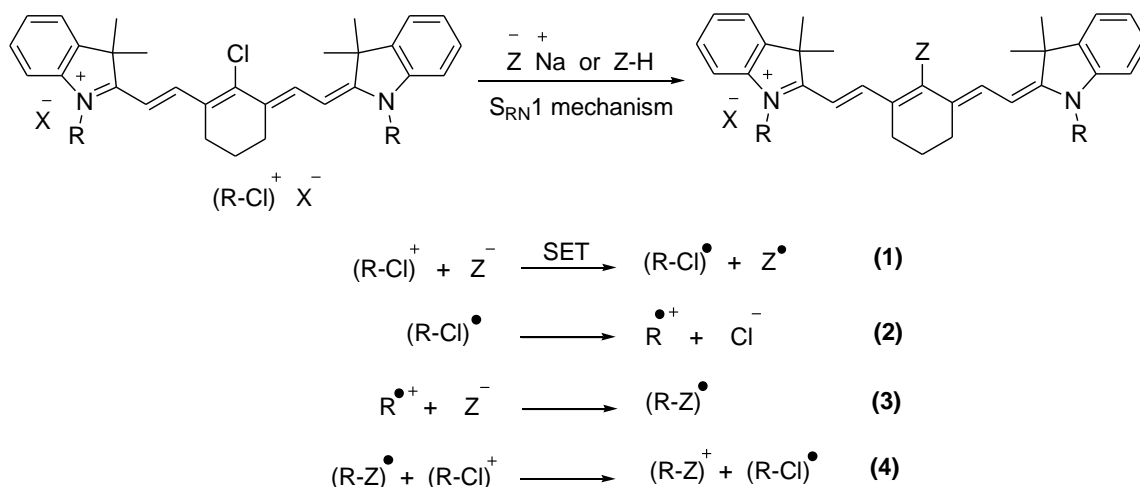
The chloro derivatives of carboyanines are susceptible to reactions with nucleophiles and redox active species due to their electron deficient π -systems. The *meso*-chlorine atom in cyanines shown in Scheme 16, undergo displacement upon treatment with various nucleophiles; however, nucleofugal group displacement may involve two mechanistic pathways [102].

The first of these pathways includes the direct addition of the nucleophile to the cationic π -system followed by elimination of the chlorine ion. Nonetheless, kinetic control of the nucleophiles addition to the polymethine chain was found to occur at the most nucleophilic site of the chromophore, the 2(2') position [103]. This is consistent with immediate decolorization that results upon reaction with hard nucleophiles such as hydroxide, alkoxides, and alkylamines [104]. This addition, although instantaneous, is reversible and the thermodynamic pathway leads to substitution at the *meso* position *via* a subsequent addition-elimination mechanism that proceeds upon heating or prolonged reaction times, as shown in Scheme 17 [102].



Scheme 17

A second suggested mechanism involves the $S_{NR}1$ pathway [102]. This process is initiated by single-electron transfer (SET) from the nucleophile species Z^- (Eqn. 1, Fig. 9) to the cationic Π -system of the chromophore to form two radical species. After dissociation of $R-Cl$ to the radical cation $R^{\bullet+}$ (Eqn. 2, Fig. 9), reaction with a nucleophile



SET = Single Electron Transfer

Figure 9. Suggested single electron transfer mechanism [102].

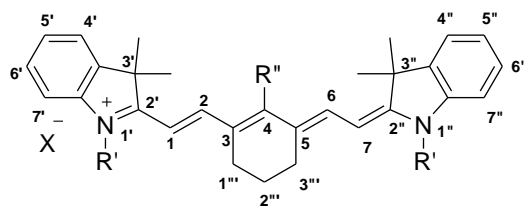
Z^- (Eqn. 3, Fig. 9) results in intermediate radical nucleophile adduct $(R-Z)^{\bullet}$ that serves as the one-electron donor in the radical propagation process (Eqn. 4, Fig. 9). This process is consistent with the cationic chromophores affinity for electrons. Cyanine dye radicals were detected in the absence of molecular traps, via bleaching studies of the respective borate salts. Further studies by Schuster [105], utilized a radical system generated *in situ* as an initiator for the free radical polymerization.

The addition followed by elimination mechanism is not favored in these reactions shown in Scheme 16 because single electron transfer is supported by the fact that the synthesis of carbocyanine dyes substituted with various amines can only be performed in polar aprotic solvents, such as *N,N*-dimethylformamide (DMF) and dimethyl sulfoxide

(DMSO). These solvents support the single electron transfer $S_{NR}1$ mechanism as shown in Figure 7 [102].

A series of carbocyanine dyes substituted with various amines were synthesized as presented in Scheme 16. In order to understand the optical properties of these dyes, spectroscopic studies were performed as outlined in Table 1, which contains numerical data of 5 carbocyanine dyes with regard to their optical properties as well as yields. It is important to study these properties to develop an ideal NIR dye for future bio-analytical applications.

The data collected in Table 1, outlines absorption, emission, Stokes' shift, extinction coefficient, and quantum yield for the amino derivatives of cyanine dyes. These dyes are substituted with different alkyl groups such as methyl, butyl, and phenylpropyl substituents on the indolenine rings.



Dye	90a	90b	90c	90d	90e
R'					
R''					
% yield	56%	70%	67%	49%	20%
Abs λ_{\max} (nm) (MeOH) \pm 1%	734	697	703	700	707
Emission λ_{\max} (nm) (MeOH)	776	782	787	791	778
Stokes' Shift (nm)	42	85	84	91	71
$\epsilon \times 10^5$ L mol ⁻¹ cm ⁻¹ (MeOH)	1.02	0.55	0.79	0.78	0.82
Φ_F (MeOH)	0.017	0.083	0.048	0.047	0.057

Table 1. Photophysical properties of novel heptamethine cyanine dyes.

The chloro dyes shown in Scheme 16 possess an absorption band at 780 nm and a fluorescence band around 800 nm; by substitution reactions of the chlorine atom with various amines, a blue shift in the electromagnetic spectrum is observed. This also leads to large Stokes shifts. As can be seen in Table 1, absorptions of *meso* substituted amine heptamethine cyanine dyes range from 697 nm to 734 nm with molar extinction coefficients ranging from 0.55 to $1.02 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

The molar extinction coefficients (ϵ) follow the decreasing order of **90a** > **90e** > **90c** > **90d** > **90b**. Dyes with lower absorption wavelengths have lower molar extinction coefficients. The enhanced molar extinction coefficient of dye **90b** ($0.55 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) is less in comparison with **90c** ($0.79 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) and **90d** ($0.78 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$).

According to the data shown above in Table 1, **90b** had a yield of 70%, an absorbance at 697 nm and an emission at 782 nm, giving this compound a Stokes shift of 85 nm. Extinction coefficient was calculated to be $0.55 \text{ M}^{-1} \text{ cm}^{-1}$ and the quantum yield was calculated to be 0.083. This dye exhibited the largest quantum yield of the 5 compounds. Dye **90a** had a yield of 56% and an absorbance at 734 nm with an emission of 776 nm. The Stokes shift (42 nm) was lowest of all compounds in Table 1; however, the extinction coefficient was found to be the highest of the 5 compounds at $1.02 \text{ M}^{-1} \text{ cm}^{-1}$. The quantum yield was the lowest at a value of 0.017 possibly due to the primary amine.

Dye **90c** had a yield of 67% and the absorbance was found to be 703 nm and the emission at 787 nm. Stokes shift was high at 84 nm and an extinction coefficient of $0.79 \text{ M}^{-1} \text{ cm}^{-1}$ was found. The quantum yield for this compound was 0.048, almost reduced by

half in comparison to **90b**. Propylphenyl dyes substituted with *N*-methylpiperazine and diethyl amine were synthesized with yields of 49% and 20% respectively. The absorbance of **90d** was found to be 700 nm and have an emission of 791 nm, giving this dye the greatest Stokes shift of 91 nm. The absorbance of **90e** was found to be 707 nm and have an emission of 778 nm, giving this dye a Stokes shift of 71 nm. Extinction coefficients for **90d** and **90e** were 0.78 and 0.82 with quantum yields of 0.047 and 0.057 respectively.

Table 1 shows Stokes' shift is significantly larger in secondary and cyclic amines than it is in primary and alkyl amines. The extinction coefficient appears highest in primary and aromatic amines and followed by secondary amines, meaning that the primary amines are absorbing more light at a given wavelength than are the secondary amines. Fluorescence quantum yield (Φ_F) exhibits a decrease from cyclic amines, to secondary amines to primary amines. This is due to the rigidity of the amine and its ability to conjugate with the polymethine chain. Secondary amines, especially cyclic ones, are more rigid and the lone pair of electrons can be conjugated with the polymethine chain, while the primary amine of aniline is conformationally flexible, allowing free rotation around the carbon bond; therefore, the lone pair of electrons are conjugated with the benzene ring rather than the polymethine chain of the dye. It is clear that the Stokes shift is primarily determined by the R'' position and not R' because of the electron donating to the polymethine chain.

All compounds were characterized by ^1H NMR, ^{13}C NMR, High Resolution Mass Spectrometry (HRMS), and melting point. Compounds **88c**, **89c**, and **90d** were chosen for complete characterization and analysis.

The initial alkylation of **35** with 3-bromophenyl propane provides **88c** which undergoes a condensation reaction with **73** to form chloro dye **89c**. Chloro dye **89c** undergoes S_{NR}1 reaction to form the amine-substituted heptamethine cyanine dye **90d**.

The ¹H NMR spectrum of **88c** is relatively simple and characteristic of indolenines with two resonances in the aliphatic region for the C-2 and C-3 methyl protons and the remaining signals resonate in the aromatic region. The aromatic region is more complex than a simple indolenine due to the phenyl rings of the alkyl chains. The remaining aliphatic carbons appear as two triplets and a multiplet to identify the propyl chain. The C-2 carbon of the 3*H*-indolium cation resonates at 196.6 ppm. There are 6 carbons in the aliphatic region and 11 carbons in the aromatic region. The melting point of **88c** was found to range between 2 degrees, 156-158 °C and HRMS was calculated for C₂₀H₂₄N [M⁺] m/z 278.1909, found 278.1915.

The ¹H NMR spectrum of **89c** revealed characteristic resonances in the aliphatic region for the C-3 methyl protons. The 1''', 2''', and 3''' hydrogens resonated in the aliphatic region as a multiplet and a triplet. The propyl chain hydrogens resonated in the aliphatic region as a multiplet and two triplets. Characteristic resonances for the polymethine chain of dyes are shown as doublets in the aromatic region around 6.01 and 8.29 ppm. The C-2 carbon of the 3*H*-indolium cation resonates at 172.2 ppm. There are 7 carbon signals in the aliphatic region while there are 15 carbon signals in the aromatic region of the spectrum. The melting point of **89c** was found to range between 2 degrees, 151-153 °C °C and HRMS was calculated for C₄₈H₅₂ N₂Cl [M⁺] 691.3819; found 691.3811.

The ^1H NMR spectrum of **90d** revealed characteristic resonances in the aliphatic region for the C-3 methyl protons. The 1''', 2''', and 3''' hydrogens resonated in the aliphatic region as a multiplet and a triplet. The propyl chain hydrogens resonated in the aliphatic region as a multiplet and two triplets while the *N*-methylpiperazine resonated at 2.48, 3.75, and 3.96 as a singlet, and two broad triplets. Characteristic resonances for the polymethine chain of dyes are shown as doublets in the aromatic region around 5.65 and 7.60. The C-2 carbon of the 3*H*-indolium cation resonates at 173.3 ppm. There are 10 carbon signals in the aliphatic region while there are 15 in the aromatic region. The melting point of **90d** was found to range between 2 degrees, 148-150 °C and HRMS was calculated for $\text{C}_{53}\text{H}_{63}\text{N}_4 [\text{M}^+]$ 755.5053, found 755.5047.

A.2.2. CONCLUSION

A series of *meso*-amine-substituted heptamethine dyes were synthesized for photophysical studies. Results show Stokes shift to be higher in the secondary amines than primary amines while the extinction coefficient was found to be higher in primary amines than secondary amines. Fluorescence quantum yield was higher in secondary amines compared to primary amines due to the conjugation of the amino group's lone pair of electrons with the polymethine chain of the dye.

A.3. SYNTHESIS OF UNSYMMETRICAL CARBOCYANINE DYES CONTAINING MONOFUNCTIONAL GROUPS

Aim of the study

The aim of this study was to synthesize mono-functional, unsymmetrical carbocyanine dyes alkylated with various carboxylic acid chain lengths attached to the terminal heterocycles. Due to the importance of unsymmetrical dyes used to conjugate bio-molecules such as proteins and amino acids, it is necessary to develop an efficient synthetic methodology for mono-functionalized unsymmetrical dyes.

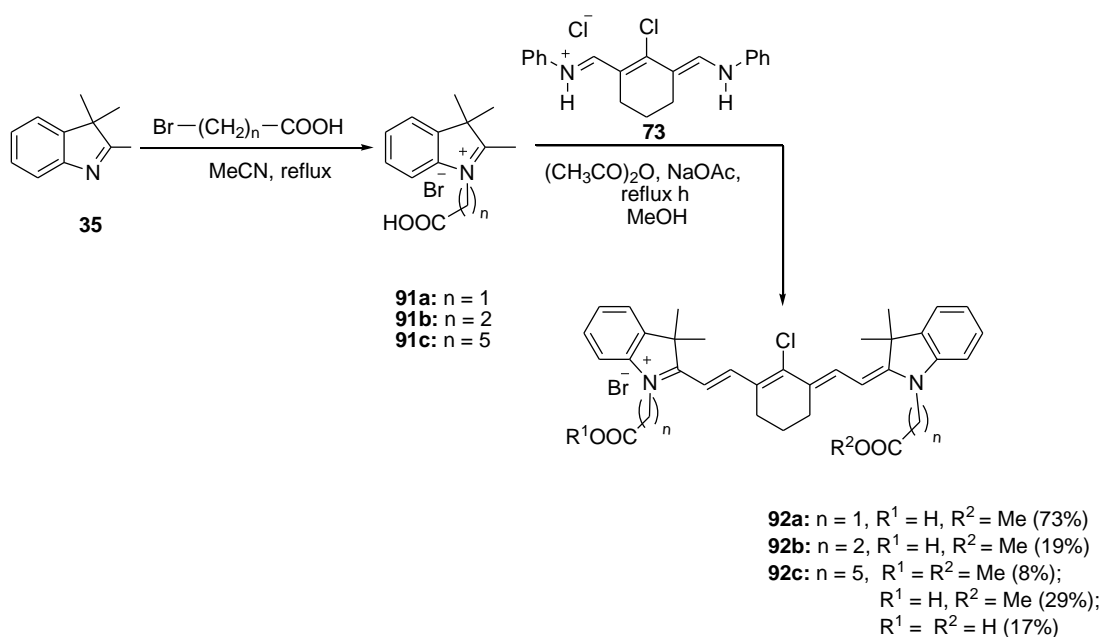
Near-infrared fluorescence-based imaging is currently of interest to scientists as it is a useful tool in early disease diagnosis, therapeutic applications, and biochemical analysis [106,107]. Ideally, there is a need for improved, brighter near-infrared water soluble dyes containing various functional groups that can be conjugated to biomolecules [108,109].

Generally, unsymmetrical dyes are synthesized *via* formation of a quaternary ammonium salt and reacted in a 1:1 ratio with Vilsmeier-Haack reagent [89] to yield a half dye which is then reacted with another equivalent of a different salt to give the final unsymmetrical dye with poor yield and tedious chromatographic separation.

As part of this research project, we developed a facile, one-pot synthesis of unsymmetrical carbocyanine dyes with mono-functional carboxylic acid groups, useful for bio-conjugation as shown in Scheme 18.

A.3.1. RESULTS AND DISCUSSION

In order to achieve a one pot synthesis of mono-functional carbocyanine dyes, 2,3,3-trimethyl indolenine **35** reacted with various brominated carboxylic acid chains ($n = 1, 2, 5$) in boiling acetonitrile under a nitrogen atmosphere to afford quaternary salts **91a-c**, which were then reacted with Vilsmeier-Haack reagent **73** [89] in acetic anhydride under basic conditions. The mixture was then quenched with methanol and products were isolated by column chromatography **92a-c**.

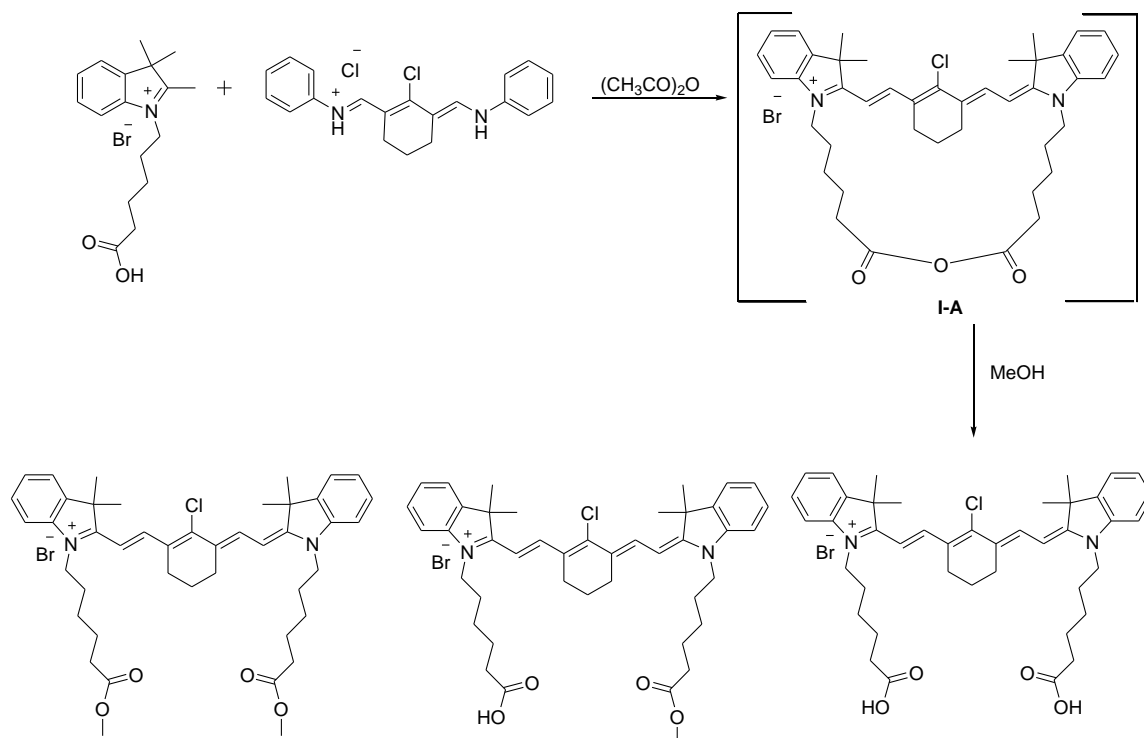


Scheme 18

As outlined in Scheme 18, and under the same conditions, bromo-ethanoic acid, bromo-propanoic acid, and bromo-hexanoic acid were used as the alkylating agents in the formation of quaternary ammonium salts **91a-c**. Both **91a** and **91b** yielded monoester heptacyanine dyes **92a** and **92b** respectively as the major compounds upon reaction with reagent **73** in acetic anhydride followed by quenching the mixtures with methanol.

However, salt **91c** yielded a mixture of three compounds, a diester, monoester, and diacid heptamethine cyanine dyes **92c** with yields of 8%, 29%, and 17% respectively.

It should be noted that the reaction of salt **91c** with Vilsmeier-Haack reagent **73** in boiling ethanol under basic condition yielded dye **92c** as diacid, as the sole product.



Scheme 19

The mechanism for the formation is thus far, unknown. As shown in Scheme 19, it is suggested that acetic acid anhydride aids in intra-molecular cyclization between the two *N*-terminal chains substituted with carboxylic acid groups to form the anhydride intermediate, **I-A**. Then by addition of methanol, cleavage of the anhydride intermediate **I-A** occurs to form monoester, diester, and diacid dyes (Fig. 8). The same conditions were then used to synthesize dyes with shorter chain lengths of carboxylic acid such as acetic and propionic acids. The acetic acid dye derivative was synthesized using the

same procedure discussed above. The results suggested there was sole formation of a monoester/monoacid product **92a**. When the same conditions were applied again to the propionic acid dye derivative, one product was isolated, monoester/monoacid **92b**.

All compounds were characterized by ^1H NMR, ^{13}C NMR, High Resolution Mass Spectrometry (HRMS), and melting point. Dye derivatives **92c** were chosen for further characterization and analysis.

The initial alkylation of **35** with 6-bromohexanoic acid provided **91c** which undergoes a condensation reaction with **73** in acetic anhydride followed by quenching with methanol to form the chloro dye derivatives **92c**.

The ^1H NMR spectrum of the diester dye **92c** shows the resonance of the 3' and 3" methyl groups as a singlet in the aliphatic region around 1.7 ppm. The 6 hydrogen from the 2 methyl groups of the ester chains are present as a singlet around 3.6 ppm. In the aliphatic region, signals are seen as the polymethine chain doublets. The carbon spectrum shows 10 signals in the aliphatic region and 12 in the aromatic. The melting point of diester dye **92c** was found to range between 2 degrees, m.p. 161-163 °C; and HRMS was calculated for $\text{C}_{44}\text{H}_{56}\text{N}_2\text{O}_4\text{Cl}$ [M^+] m/z 711.3921; found 711.3915.

The ^1H NMR spectrum of the monoester dye **92c** shows the resonance of the 3' and 3" methyl groups as a singlet in the aliphatic region. The singlet for 3 protons from the monoester group also resonates in the aliphatic region. In the aromatic region, two doublet-doublets are seen as the characteristic polymethine chain dye peaks for asymmetrical dyes. The melting point of monoester dye **92c** was found to range between 2 degrees, m.p. 165-167 °C; and HRMS was calculated for $\text{C}_{43}\text{H}_{54}\text{N}_2\text{O}_4\text{Cl}$ [M^+] m/z 697.3772; found 697.3763.

The ^1H NMR spectrum of the diacid dye **92c** shows the resonance of the 3' and 3'' methyl groups as a singlet in the aliphatic region. The alkyl hexanoic acid chains resonate in the aliphatic region as triplets and multiplets. In the aromatic region, two doublets are seen as the characteristic polymethine chain dye peaks. The carbon shows 9 peaks in the aliphatic region and 12 in the aromatic. The melting point of diacid dye **92c** was found to range between 2 degrees, m.p. 171-173 °C; and HRMS was calculated for $\text{C}_{42}\text{H}_{52}\text{N}_2\text{O}_4\text{Cl}$ [M^+] m/z 683.3616; found 683.3595.

A.3.2. CONCLUSION

A series of asymmetrical *meso*-halogen heptamethine cyanine dyes functionalized with mono-carboxylic acid groups were synthesized in a one pot reaction. The mono-functional carboxylic acid of the monoester dyes can be transformed to the active form NHS-ester as biomolecule labels for proteins, amino acids, and DNA sequencing as well as imaging applications. These compounds possess a bright fluorescence emission, water solubility, chemical stability, and a far-red/NIR absorption and emission.

GENERAL PART B

**SYTNHESIS OF CARBOCYANINE DYES UTILIZED IN IMAGE-GUIDED
SURGERY**

B.1. SYNTHESIS OF CYANINE DYES FOR CANCER GUIDED IMAGING, A REVIEW

B.1.1. INTRODUCTION

The aim of this study was to synthesize various heptamethine carbocyanine dyes to be used as agents to image cancer-guided surgery.

Cancer is a malignant mass of tumor-forming cells that typically recur and metastasize after initial excisions [110]. Thus far, many efforts to discover a cure for cancer have been unsuccessful. One of the major challenges has been differentiating tumor cells and normal cells [111]. Research has shown that cancer cells are significantly different from normal cells. These differences are the primary targets for cancer therapy [111]. Diagnostic techniques that detect cancer cells are currently being investigated including, the use of monoclonal antibodies, Photoacoustic Computed Tomography (PCT), BP-based radiotracers (bisphosphonates used to diagnose osteoblastic bone lesions), NIR fluorophores conjugated to small molecules which will be discussed in this chapter [111-114].

Optical imaging, a new imaging technique, produces high-resolution imaging of fluorophores in cancerous tissue [115]. An example of optical imaging is NIR fluorescence-based imaging. This imaging method is favorable due to its low tissue absorption and minimal auto-fluorescence of NIR light [113]. Conversely, NIR fluorescence may be able to provide a fast, inexpensive screening for breast cancer as well as other cancers [113,116-118].

As shown in Figure 10, Indocyanine green (ICG) was studied by Pauli *et. al.* ICG, approved by the United States Food and Drug Administration, is a diverse cyanine

dye that has utilization in measuring cardiac output, determining plasma volume, and studying ophthalmic angiography, hepatic function, and object localization in tissue [119]. ICG has some drawbacks including low fluorescence quantum yield of 0.01 in aqueous solution, plasma protein binding, rapid elimination through the liver, and possesses a level of cytotoxicity; therefore, it is important to synthesize a compound that possesses characteristics including high fluorescence quantum yield, rapid elimination through the kidney as opposed to the liver, and possess very little or no cytotoxicity.

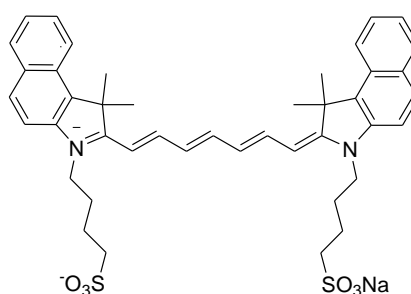


Figure 10. Indocyanine Green (ICG) [120].

Minet *et. al.* conducted studies of an amino sugar derivative of Indocyanine Green dye (SIDAG) to be used as image-guided surgery probes [120]. The absorbance and fluorescence of SIDAG are 755 nm and 790 nm respectively, shown in Figure 11 [120].

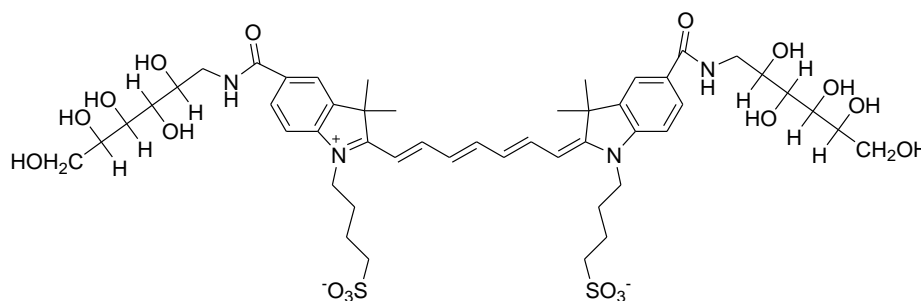


Figure 11. An amino sugar derivative of Indocyanine Green dye (SIDAG) [120].

The hydrophilic dye SIDAG has been used to demonstrate high tumor-to-normal tissue fluorescence contrast after intravenous injection [120]. It was found to bind completely to plasma proteins distributed in the intravascular space and rapidly clear from the tissue by the liver. This dye is useful in enhancing the sharpness of tumor borders and resolution of small tissue abnormalities, such as early stage tumors; however this dye is also cleared through the liver rather than the kidney.

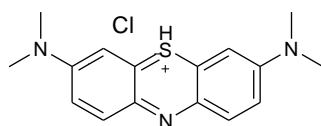


Figure 12. Methylene Blue [120].

As shown in Figure 12, a fluorescent dye considered as a potential photosensitizer in photodynamic therapy of malignant tumors is shown. Methylene blue (MB) is used primarily in cancer chemotherapy regimens as oral and intravenous doses. Peter *et. al.* studied the pharmacokinetics of the dye and discovered that in rats, higher doses of the dye traveled to the intestinal wall and liver while smaller doses traveled to the brain and whole blood. Although this dye is already on the market, these commercially available dyes are being cleared through the liver, creating a toxicity problem since it is not being cleared through the kidney.

To date, there are no efficient NIR dyes available to be used in cancer imaging technology. All the current dyes tend to clear through the liver rather than the kidney and this can cause high fluorescent signals in the gastrointestinal (G.I.) tract. The increase of fluorescent background in the G.I. tract will mislead surgeons during operations thus causing the procedure to be inefficient and unsuccessful. As a result of this, the motivation behind synthesizing novel dyes to be used as image-guided surgery

probes was to develop a dye that would not only serve the purpose to detect tumor cells but also possess little to no cytotoxicity by being eliminated through the kidney rather than the liver.

According to literature, quantum dots with zwitterionic character were synthesized to study biodistribution and excretion. Zwitterionic compounds are neutral in charge and this characteristic aids in prevention of adsorption of serum proteins, allowing the compounds to be rapidly excreted through urine. This is an important characteristic taken into account when designing dyes for biomedical applications, such as imaging cancerous tissue [121].

Based on this information, two zwitterionic heptamethine cyanine dyes were synthesized as part of this research project and the biodistribution of these dyes were evaluated by collaboration with Dr. John Frangioni, Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School.

B.2. RESULTS & DISCUSSION (HEPTACARBOCYANINE DYES FOR CANCER GUIDED IMAGING)

Aim of the study

Two unsolved, fundamental problems facing optical imaging are non-specific uptake of intravenously administered fluorophores by normal tissues and organs, and incomplete elimination of unbound targeted fluorophores from the body. As part of this research project, the goal was to synthesize a series of heptamethine indocyanine near-infrared (NIR) fluorophores that varied systematically in net charge. Interestingly, zwitterionic molecules exhibited unusual in vivo properties including no serum binding, ultra-low non-specific tissue background, and rapid elimination from the body via renal

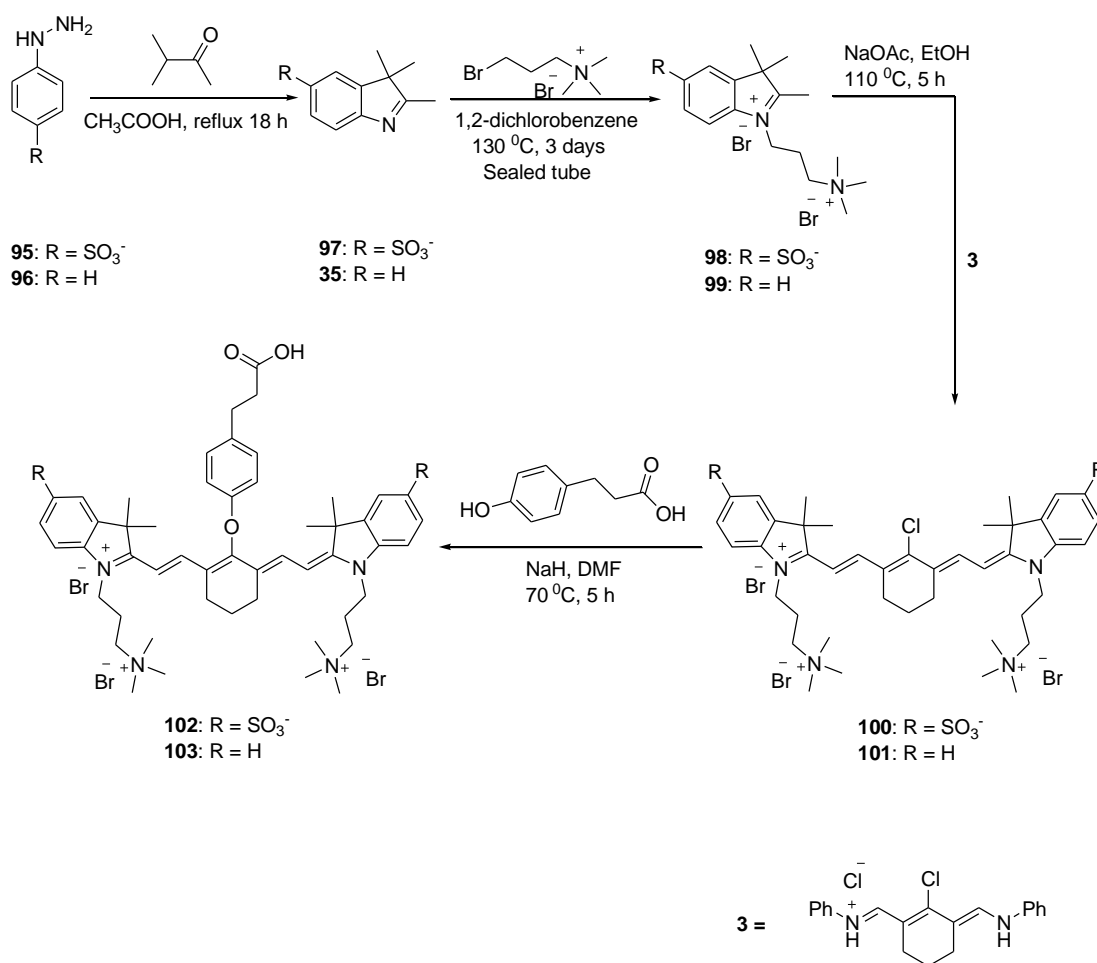
filtration. Moreover, zwitterionic molecules had outstanding optical properties including ≈ 800 nm emission, high extinction coefficients, and high quantum yields in serum. This study solves two fundamental problems associated with NIR fluorescence-guided cancer surgery and lays the foundation for targeted agents with optimal optical and *in vivo* performance.

Currently, there are few dyes that are FDA approved for use in humans. The main problems with the dyes that are commercially available are cytotoxicity due to clearance through the liver, as well as low quantum yield and low signal-to-background ratio. It was our interest to develop a dye(s) that could be used to visualize cancer and possess superior properties *in vivo* compared to the commercially available dyes. The FDA approved dyes for cancer imaging technology lacks monofunctionality; therefore, they cannot be conjugated to different target ligands in order to target different tumors. Our dyes possess carboxylic acid groups making them monofunctional for conjugation.

B.2. RESULTS & DISCUSSION (DYES FOR CANCER GUIDED IMAGING)

Heptacyanine dyes **102** and **103** were synthesized as shown in Scheme 19. Compound **97** was synthesized in 74% yield after crystallization from acetone by heating hydrazine derivative **95** under reflux in acetic acid however, the reaction of **97** and **35** were progressed in 1,2-dichlorobenzene to yield compounds **98, 99** respectively. Dyes **100, 101** were synthesized *via* condensation of Vilsmeier-Haack reagent **73** and salts **98, 99** in boiling ethanol in the presence of sodium acetate. Phenoxypropionic acid was reacted with dyes **100, 101** in DMF and sodium hydride for 5 hours to give the final dye derivatives **101,103**. ^1H NMR, ^{13}C NMR, and ESI-MS were consistent with the proposed

structures. Compounds **98**, **100**, and **102** were chosen for complete analysis and characterization



Scheme 19

The ¹H NMR spectrum of **98** revealed characteristic singlet resonances in the aliphatic region for the C-2 and C-3 methyl protons. The three methyl groups of the aliphatic chain resonated as a singlet at 3.12 ppm. The C-2 carbon of the 3H-indolium cation resonates at 199.1. ppm. The melting point of **98** was found to range between 2 degrees, mp 232-235 °C and ESI-MS was calculated for C₁₇H₂₇N₂O₃S [M]⁺ m/z 339.17, found m/z 339.17.

The ^1H NMR spectrum of **100** revealed characteristic singlet resonances in the aliphatic region for the C-3 methyl protons at 1.72 ppm. The methyl groups of the aliphatic chain resonate at 3.08 ppm. The characteristic doublets of the polymethine chain resonate at 6.36 and 8.31 ppm. The melting point of **100** was found to be in a 2 degree range, 274-277 °C and ESI-MS was confirmed for $\text{C}_{42}\text{H}_{58}\text{N}_4\text{O}_6\text{S}_2\text{Cl}$ $[\text{M}]^+$ m/z 813.35, found m/z 813.35.

Compound **102** was prepared by adding 3-(4-hydroxyphenyl)propionic acid into a solution of sodium hydroxide and water. Dye **100** and 2 equivalents of the previous mixture were dissolved in DMSO and the mixture was heated under microwave conditions to yield 98% **102**.

The ^1H NMR spectrum showed C-3 methyl group hydrogen resonance at 0.943 ppm and the 6 methyl groups of the alkyl chains resonated at 2.97 ppm. The characteristic peaks specific to heptamethine cyanine dyes resonate as doublets at 5.97 and 7.66 ppm. MS was calculated for $\text{C}_{51}\text{H}_{66}\text{N}_4\text{O}_9\text{S}_2$ $[\text{M}-\text{H}]^-$ m/z 941.43, found m/z 941.48.

***In Vivo* Biodistribution and Clearance of NIR Fluorophores 102 and 103**

The biodistribution of of the heptacyanine dyes **102** (ZW-1) and **103** (ZW-3a) were evaluated by collaboration with Dr. John Frangioni, Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School.

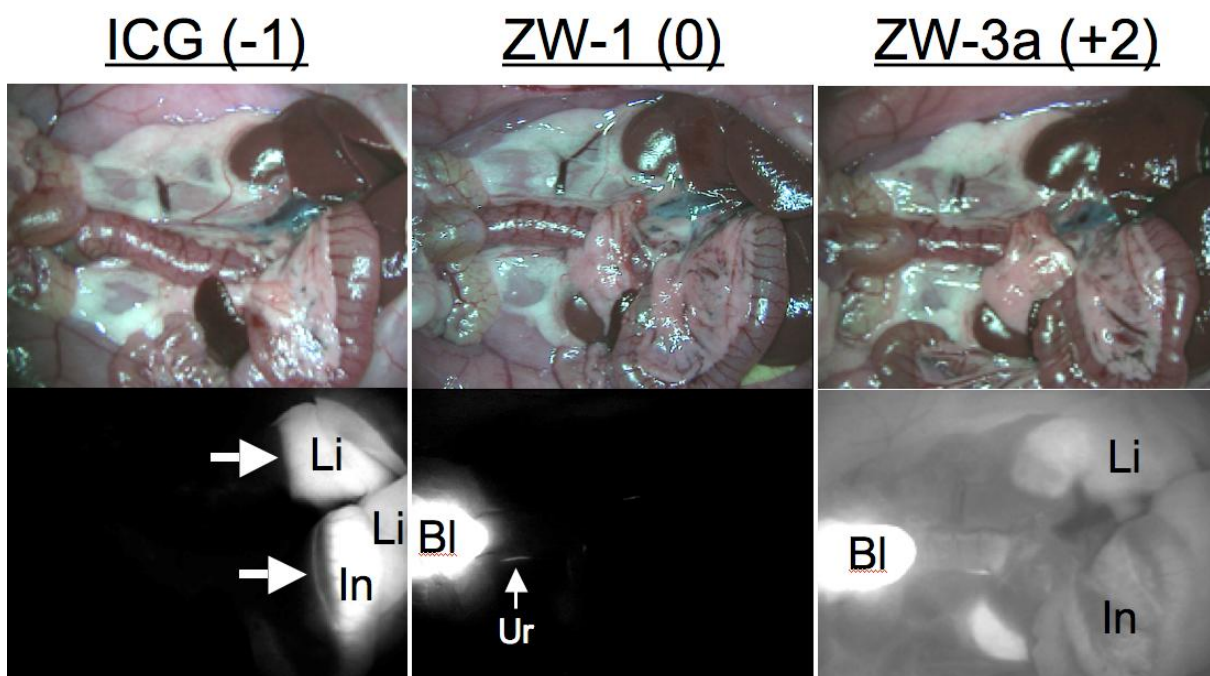


Figure 13. In Vivo Biodistribution and Clearance of NIR Fluorophores having Systematically Varying Net Charge (Parentheses) [Frangioni Lab, BIDMC, Harvard Medical School].

NIR fluorophores were injected IV into rats at 40 pmol/g (10 nmol) each, 1 h prior to imaging. Shown are color video (top row) and 800 nm NIR fluorescence (bottom row) images of surgically exposed organs and tissues. Excitation = 5 mW/cm². Camera integration time = 200 msec. NIR fluorescence images have identical normalizations. Bl = bladder; Li = Liver; In = Intestines; Ur = ureter. Note high, diffuse background for -4 and +2 dyes, and high liver and intestine background (arrows) for +2 and -1 dyes.

The *in vivo* behavior of NIR fluorophores having varying net charge is dramatically different. As shown in Figure 13, general principles of behavior emerge from studying this systematically varying family of compounds. First, heptamethine indocyanines with -1 (ICG) net charge have a high “hydrophobic moment” (i.e., one half of molecule is highly hydrophobic and the other half is hydrophilic), which results in

rapid uptake by the liver. Heptamethine indocyanines with +2 net charge **103** are cleared by kidney more than liver, however, non-specific uptake in organs and tissue is extraordinarily high. Finally, dye **102**, which has a net charge of zero, demonstrates rapid equilibration between intravascular and extravascular spaces, no measurable liver uptake, rapid renal excretion into urine, and extremely low background retention in normal tissues and organs (Figure 11).

B.3. CONCLUSION

In summary, the 800 nm zwitterionic heptamethine indocyanine NIR fluorophore **102** has remarkable *in vivo* properties, including no serum protein binding, rapid renal clearance, ultra-low non-specific tissue uptake (i.e., background), and high SBR when conjugated to tumor targeting ligands. The 700 nm NIR fluorophores with equivalent performance will be synthesized in future. This has the potential for huge impact in human cancer surgery applications; when combined with the dual-NIR wavelength capabilities of the FLARE™ image-guided surgery system, these fluorophores should make the resection of virtually any tumor and the avoidance of virtually any normal structure possible.

4 EXPERIMENTAL

All reagents were obtained from Aldrich. Melting points (open pyrex capillary) were measured on a Thomas Hoover apparatus and are uncorrected. ^1H NMR (400 MHz) and ^{13}C NMR spectra (100 MHz) were recorded on Bruker Avance spectrometer in CDCl_3 for most cyanine dyes and $\text{DMSO-}d_6$ for salts and tetramethylsilane (TMS) as an internal standard. Vis/NIR absorption spectrum was recorded on a Perkin Elmer Lambda 20 spectrophotometer in methanol for cyanine dyes and ethanol for Rhodamine references. High resolution mass spectrum (HRMS) were recorded on a VG Analytical 70-SE spectrometer.

1,2,3,3-Tetramethyl-3*H*-indolinium iodide, 88a

This compound was obtained in a 69% yield; m.p. 160-162 °C; (reported: yield 73%). mp 162 °C [122].

1-(butyl)-2,3,3-trimethyl-3*H*-indolinium iodide, 88b

This compound was obtained in a 75% yield; mp. 124-126 °C; (reported: yield 73%, mp 122-124 °C) [123].

1-(3-phenylpropane)-2,3,3-trimethyl-3*H* indolenium bromide, 88c

2, 3, 3-trimethyl indolenine (**35**, 4.00 mL, 24.9 mmol) was added to a solution of 3-bromophenylpropane (11.36 mL, 74.7 mmol) and acetonitrile (20.0 mL) under nitrogen atmosphere. The reaction mixture was heated at 110 °C for 72 hours. The reaction mixture was concentrated to dryness to give a residue which was crystallized from acetone to yield light pink crystals (**90c**, 7.06 g, 79%); mp. 156-158 °C; ^1H NMR ($\text{DMSO-}d_6$, 400 MHz) δ 0.64 (s, 6H), 1.29 (m, 2H), 1.62 (t, $J = 8.0$ Hz, 2H), 1.93 (s, 3H), 3.61 (t, $J = 8.0$ Hz, 2H), 6.33 (m, 1H), 6.40 (m, 4H), 6.74 (m, 2H), 6.95 (m, 1H), 7.07 (m,

1H); ^{13}C NMR (DMSO- d_6 , 100 MHz) ppm 14.3, 22.0, 28.8, 31.7, 47.4, 54.1, 115.4, 123.5, 126.0, 128.2, 128.3, 128.8, 129.3, 140.6, 141.0, 141.8, 196.6; HRMS (ESI) m/z calcd for $\text{C}_{20}\text{H}_{24}\text{N}$ [M^+] m/z 278.1909, found 278.1915.

1-butyl-2-((*E*)-2-((*E*)-3-((*E*)-2-(1-butyl-3,3-dimethylindolin-2-ylidene)ethylidene)-2-chlorocyclohex-1-enyl)vinyl)-3,3-dimethyl-3*H*-indolium bromide, 89b

This compound was obtained in a 69% yield; m.p. >250 °C; (reported: yield 95%, m.p. >250 °C) [123].

2-((*E*)-2-((*E*)-2-chloro-3-((*E*)-2-(3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)ethylidene)-cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-(phenylpropyl)-3*H*-indolium bromide, 89c

A solution of salt (**88c**, 3.27 g, 9.10 mmol), Vilsmeier-Haack reagent (**3**, 1.48 g, 4.12 mmol) and sodium acetate (1.22 g, 14.9 mmol) in ethanol (40.0 mL) was heated at 80 °C for 2 hours under nitrogen atmosphere. The mixture was cooled to room temperature, and the solvents were concentrated to dryness. The residue was dissolved in methylene chloride (50.0 mL) and filtered. Removal of the solvent furnished a crude product which was crystallized in ether/acetone (5:1) to provide compound (**89c**, 2.51 g, 79%); mp 151-153 °C; ^1H NMR (CDCl_3 , 400 MHz) δ 1.69 (s, 12H), 1.91 (m, 2H), 2.17 (m, 4H), 2.50 (t, $J = 6.8$ Hz, 4H), 2.88 (t, $J = 6.8$ Hz, 4H), 4.21 (t, $J = 6.8$ Hz, 4H), 6.01 (d, $J = 14.0$ Hz, 2H), 7.12 (d, $J = 14.0$ Hz, 2H), 7.27 (m, 6H), 7.35 (m, 10H), 8.29 (d, $J = 14.0$ Hz, 2H); ^{13}C NMR (CDCl_3 , 100 MHz) ppm 26.8, 28.3, 28.8, 33.0, 44.1, 49.4, 51.0, 101.7, 111.1, 122.4, 125.5, 126.6, 127.9, 128.8, 128.9, 129.0, 140.5, 141.2, 142.3, 144.3, 150.4, 172.2; HRMS (ESI) m/z calcd for $\text{C}_{48}\text{H}_{52}\text{N}_2\text{Cl}$ [M^+] 691.3819; found 691.3811.

1,3,3-trimethyl-2-((E)-2-((E)-2-(phenylamino)-3-((E)-2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-3H-indolium iodide, 90a

A solution of compound (**89b**, 0.25 g, 0.40 mmol) and aniline (0.18 mL) in *N,N*-dimethylformamide (5.00 mL) was heated at 80 °C for 18 hours under nitrogen atmosphere. The mixture was cooled to room temperature, and then, the solvents were concentrated to give an oily residue. The crude was separated by column chromatography on silica gel eluting with methanol- methylene chloride gradient from 50:1, 20:1 to provide compound (**90a**, 0.15 g, 56%); mp 131-133 °C; ¹H NMR (CDCl₃, 400 MHz) 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); ¹³C NMR (CDCl₃, 100 MHz) ppm 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, 129.8, 130.1, 140.6, 143.3, 143.5, 160.4, 170.9. HRMS (ESI) *m/z* calcd for C₃₈H₄₂N₃ [M⁺] 540.3379; found 540.3378.

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-enyl)vinyl)-3H-indolium iodide, 90b

A solution of compound (**89b**, 0.25 g, 0.40 mmol) and *N*-methyl piperazine (0.15 mL) in *N,N*-dimethylformamide (3.00 mL) was heated at 90 °C for 11 hours under nitrogen atmosphere. The mixture was cooled to room temperature, and then, the solvents were concentrated to dryness. The crude was separated by column chromatography on silica

gel eluting with methanol- methylene chloride 50:1, 20:1 to provide compound (**90b**, 0.19 g, 70%); ^1H NMR (CDCl_3 , 400 MHz) δ 1.59 (s, 12H), 1.75 (t, $J = 6.4$ Hz, 2H), 2.42 (t, $J = 6.4$ Hz, 4H), 2.46 (s, 3H), 2.79 (br. t, 4H), 3.47 (s, 6H), 3.74 (br. t, 4H), 5.69 (d, $J = 14.0$ Hz, 2H), 6.95 (d, $J = 7.6$ Hz, 2H), 7.05 (t, $J = 7.6$ Hz, 2H), 7.24 (q, $J = 7.6$ Hz, 4H), 7.57 (d, $J = 14.0$ Hz, 2H); ^{13}C NMR (CDCl_3 , 100 MHz) ppm 21.9, 25.2, 29.1, 31.6, 46.1, 48.3, 54.4, 56.4, 96.8, 109.6, 122.1, 123.9, 124.8, 128.6, 140.2, 141.6, 143.3, 170.0, 172.9. HRMS (ESI) m/z calcd for $\text{C}_{37}\text{H}_{47}\text{N}_4$ [M^+] 547.3801; found 547.3800.

1-butyl-2-((E)-2-((E)-3-((E)-2-(1-butyl-3,3-dimethylindolin-2-ylidene)ethylidene)-2-(4-methylpiperazin-1-yl)cyclohex-1-enyl)vinyl)-3,3-dimethyl-3H-indolium bromide, **90c**

A solution of compound (**89b**, 0.30 g, 0.43 mmol) and *N*-methyl piperazine (0.48 mL, 4.32 mmol) in *N,N*- dimethylformamide (5.00 mL) was heated at 65 °C for six hours under nitrogen atmosphere. The mixture was cooled to room temperature, and then, the solvents were concentrated to give an oily residue. Hexane was added to the residue to decant impurities and was then concentrated to dryness and placed on the vacuum for eight hours. The crude was separated by column chromatography on silica gel eluting with methanol- methylene chloride gradient from 0:100, 1:100, 1:50, 1:25 to provide compound (**90c**, 0.22 g, 67%); mp 182-184 °C; ^1H NMR (CDCl_3 , 400 MHz): δ 1.01 (t, $J = 7.2$ Hz, 6H), 1.48 (q, $J = 7.2$ Hz, 4H), 1.68 (s, 12H), 1.79 (t, $J = 7.2$ Hz, 4H), 1.85 (m, 2H), 2.48 (br. s, 7H), 2.73 (br. s, 4H), 3.77 (br. s, 4H), 3.96 (t, $J = 7.2$ Hz, 4H), 5.70 (d, $J = 14.0$ Hz, 2H), 7.01 (d, $J = 7.2$ Hz, 2H), 7.14 (t, $J = 7.2$ Hz, 2H), 7.31 (t, $J = 7.2$ Hz, 4H), 7.66 (d, $J = 14.0$ Hz, 2H); ^{13}C NMR (CDCl_3 , 100 MHz) ppm 14.1, 20.6, 22.0, 25.3, 29.1, 29.2, 31.1, 43.9, 48.3, 55.2, 56.8, 96.6, 109.7, 122.2, 123.8, 124.6, 128.7, 140.4,

141.7, 142.9, 169.1, 207.1; HRMS (ESI) calcd for C₄₃H₅₉N₄ [M⁺] m/z 631.4740; found 631.4712.

2-((E)-2-((E)-3-((E)-2-(3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)ethylidene)-2-(4-methylpiperazin-1-yl)cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-(phenylpropyl)-3H-indolium bromide, 90d

A solution of compound (**89c**, 0.20 g, 0.26 mmol) and *N*-methyl piperazine (0.20 mL, 2.60 mmol) in *N,N*-dimethylformamide (3.00 mL) was heated at 65 °C for 6 hours under nitrogen atmosphere. The mixture was cooled to room temperature, and the solvents were concentrated to dryness. The crude dye was separated by column chromatography on silica gel eluting with methanol- methylene chloride gradient from 0:100, 1:100, 1:50, 1:25 to provide compound (**90d**, 0.106 g, 49 %); mp. 148-150 °C; ¹H NMR (CDCl₃, 400 MHz) δ 1.65 (s, 12H), 1.77 (m, 2H), 2.15 (m, 4H), 2.13 (t, *J* = 7.2 Hz, 4H), 2.27 (t, *J* = 7.2 Hz, 4H), 2.48 (s, 3H), 2.73 (br. s, 4H), 2.84 (t, *J* = 7.2 Hz, 4H), 3.75 (br. t, 4H), 3.96 (t, *J* = 7.2 Hz, 4H), 5.65 (d, *J* = 14.0 Hz, 2H), 6.91 (d, *J* = 7.2 Hz, 2H), 7.14 (t, *J* = 7.2 Hz, 2H), 7.31 (m, 14H), 7.60 (d, *J* = 14.0 Hz, 2H). ¹³C NMR (CDCl₃, 100 MHz) ppm 21.9, 25.1, 28.1, 29.2, 29.9, 33.2, 43.1, 48.2, 55.1, 56.8, 96.4, 109.6, 122.2, 123.9, 124.7, 126.6, 128.7, 128.7, 128.9, 140.4, 140.6, 141.5, 142.7, 168.9, 173.3; HRMS (ESI) m/z calcd for C₅₃H₆₃N₄ [M⁺] 755.5053, found 755.5047.

2-((E)-2-((E)-2-(diethylamino)-3-((E)-2-(3,3-dimethyl-1-(3-phenylpropyl)indolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-(phenylpropyl)-3H-indolium bromide, 90e

A solution of compound (**89c**, 0.25 g, 0.32 mmol) and diethyl amine (0.01 mL, 0.96 mmol) in *N,N*-dimethylformamide (3.00 mL) was stirred at room temperature for 6

hours under nitrogen atmosphere. The mixture was concentrated to dryness and the crude was washed with ether and hexanes and placed on the vacuum to dry for four hours. It was then purified by a chromatatron eluting with methanol- methylene chloride gradient from 0:100, 1:100, 1:50, 1:25, 1:10 to provide compound (**90e**, 0.051 g, 20%) m.p. 126-130 °C; ^1H NMR (CDCl_3 , 400 MHz) δ 1.29 (t, $J = 6.4$ Hz, 6H), 1.61 (s, 12H), 1.79 (t, $J = 6.4$ Hz, 2H), 2.14 (br. t, 4H), 2.27 (br. t, 4H), 2.83 (t, $J = 6.4$ Hz, 4H), 3.64 (d, $J = 6.4$ Hz, 4H), 3.96 (br. t, 4H), 5.63 (d, $J = 14.0$ Hz, 2H), 6.93 (d, $J = 6.4$ Hz, 2H), 7.04 (t, $J = 6.4$ Hz, 2H), 7.26 (d, $J = 6.4$ Hz, 6H), 7.33 (d, $J = 6.4$ Hz, 8H), 7.51 (d, $J = 14.0$ Hz, 2H); ^{13}C NMR (CDCl_3 , 100 MHz) ppm 14.9, 22.0, 24.9, 28.1, 29.2, 33.1, 43.1, 48.3, 49.6, 96.8, 109.7, 122.2, 124.0, 125.8, 126.6, 128.6, 128.7, 128.9, 140.4, 140.5, 142.6, 142.6, 169.2, 173.9; HRMS (ESI) m/z calcd for $\text{C}_{52}\text{H}_{62}\text{N}_3$ [M^+] 728.4944, found 728.4944.

1-(carboxy)-2,3,3-trimethyl-3H indolenium bromide, 91a

This compound was obtained in a 35% yield; m.p. 160-162 °C; (reported: yield 85%). mp 158-160 °C [124].

1-(3-propanoic acid)-2,3,3-trimethyl-3H indolenium bromide, 91b

2, 3, 3-Trimethyl indolenine (**35**, 1.40 mL, 8.00 mol) was added to a solution of 3- bromo propanoic acid (2.79 mL, 17.0 mmol) and acetonitrile (30.0 mL) under nitrogen atmosphere. The reaction mixture was heated at 70 °C for 72 hours. The reaction mixture was concentrated to dryness to give a reddish residue which was crystallized from acetone/ether and washed in methylene chloride to yield light beige crystals (**91b**, 1.61 g, 64%); m.p. 176-178 °C; ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 1.53 (s, 6H), 2.91 (s, 3H), 2.99 (t, $J = 6.8$ Hz, 2H), 4.66 (t, $J = 6.8$ Hz, 2H), 7.61 (m, 2H), 7.85 (br. t, 1H), 8.01 (br. t, 1H); ^{13}C ($\text{DMSO}-d_6$, 100 MHz) ppm 14.6, 21.9, 31.2, 43.6, 54.3, 115.6, 123.6, 129.0,

129.4, 140.9, 141.8, 171.6, 198.0; HRMS (ESI) m/z calcd for $C_{14}H_{18}NO_2 [M^+]$ m/z 232.1338, found 232.1334.

1-(5-Carboxypentyl)-2,3,3-trimethyl-3*H* indolium bromide, 91c

This compound was obtained in a 67% yield; m.p. 124-126 °C; (reported: yield 92%, m.p. 127-129 °C) [125].

1-(carboxymethyl)-2-((*E*)-2-((*E*)-3-((*E*)-2-(1-(carboxymethyl)-3,3-dimethylindolin-2-ylidene)ethylidene)-2-chlorocyclohex-1-enyl)vinyl)3,3-dimethyl-3*H*-indolium bromide, 92a

A solution of salt (**91a**, 2.80 g, 9.39 mmol), Vilsmeier-Haack reagent (**3**, 1.52 g, 4.23 mmol) and sodium acetate (1.45 g, 14.0 mmol) in acetic anhydride (15 ml) was heated at 80 °C for 4 hours under nitrogen atmosphere. The mixture was cooled to room temperature and then quenched with methanol. The crude dye was concentrated to dryness. The crude was dissolved in dichloromethane (20 ml) to eliminate sodium acetate. Removal of solvent furnished crude dye **92a** which was separated by column chromatography on silica gel eluting with methanol-dichloromethane gradient from 1:30, 1:20, to 1:10. The final product from the column showed the dye was converted to the monoester product (**94a**, 2.10 g, 73% yield). m.p. 150-155 °C; 1H NMR (DMSO- d_6 , 400 MHz): δ 1.18 (m, 2H), 1.58 (s, 6H), 1.65 (s, 6H), 1.67 (m, 2H), 1.90 (t, $J = 5.6$ Hz, 2H), 2.65 (br. t, 2H), 2.68 (t, $J = 5.6$ Hz, 2H), 3.70 (s, 3H), 6.08 (d, $J = 14.0$ Hz, 1H), 6.19 (d, $J = 14.0$ Hz, 1H), 7.15 (m, 3H), 7.29 (m, 5H), 7.61 (d, $J = 14.0$ Hz, 1H), 8.27 (d, $J = 14.0$ Hz, 1H).

2-((E)-2-((E)-3-((E)-2-(1-(2-carboxyethyl)-3,3-dimethylindolin-2-ylidene)ethylidene)-2-chlorocyclohex-1-enyl)vinyl)-1-(3-methoxy-3-oxopropyl)-3,3-dimethyl-3H-indolium bromide, 92b

Salt **91b** (0.331 g, 1.06 mmol), Vilsmeier-Haack reagent (**73**, 1.706 g, 0.92 mmol) and sodium acetate (0.130 g, 1.58 mmol) in acetic anhydride (15.0 mL) was heated at 85 °C for 4 hours under nitrogen atmosphere. The mixture was cooled to room temperature, and then, the reaction was quenched with methanol (5.00 ml). After 30 minutes solvents were concentrated to dryness and the solid was dissolved in dichloromethane (20.0 ml) to eliminate sodium acetate. Removal of the solvent furnished a crude dye, which was separated by column chromatography on silica gel eluting with methanol-ethyl acetate 1:9, 1:4, to 1:1. The fractions of each dye were collected and concentrated under vacuum to furnish dyes **92b**. Heptamethine cyanine dye mono-ester (120 mg, 19%) m.p. 108-111 °C; ¹H NMR (CDCl₃, 400 MHz): δ 1.71 (s, 6H), 1.75 (s, 6H), 1.95 (m, 2H), 2.68 (br. m, 2H), 2.77 (br. t, 2H), 2.87 (t, *J* = 7.2 Hz, 4H), 3.54 (s, 3H), 4.20 (m, 2H), 4.59 (br. t, 2H), 6.01 (d, *J* = 13.2 Hz, 1H), 6.68 (d, *J* = 13.2 Hz, 1H), 7.08 (d, *J* = 7.2 Hz, 2H), 7.17 (d, *J* = 7.2 Hz, 2H), 7.43 (m, 2H), **8.18** (d, *J* = 13.2 Hz, 1H), 8.36 (d, *J* = 13.2 Hz, 1H); MS (ESI) *m/z* calcd for C₃₇H₄₂N₂O₄Cl [M⁺] *m/z* 613.2833, found 613.2811.

Heptamethine cyanine dyes 92c

A solution of salt (**91c**, 2.40 g, 6.77 mmol), Vilsmeier-Haack reagent (**73**, 1.10 g, 3.05 mmol) and sodium acetate (0.833 g, 10.2 mmol) in acetic anhydride (15.0 ml) was heated at 90 °C for 3 hours under nitrogen atmosphere. The mixture was cooled to room temperature, and then, the reaction was quenched with methanol (5.00 ml). After 10 minutes the mixture began to boil and present a transparent black solution. Solvents were

concentrated to dryness and the solid was dissolved in dichloromethane (20.0 ml) to get rid of sodium acetate. Removal of solvent furnished a crude mixture of three dyes, which had λ_{max} at 781 nm. The crude was separated by column chromatography on silica gel eluting with methanol-dichloromethane gradient from 1:30, 1:20, to 1:10. The fractions of each dye were collected together and concentrated under vacuum to furnish **92c** Heptamethine cyanine dye di-ester, Heptamethine cyanine dye mono-ester, and Heptamethine cyanine dye di-acid.

2-((E)-2-((E)-2-chloro-3-((E)-2-(1-(6-methoxy-6-oxohexyl)3,3-dimethylindolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-1-(6-methoxy-6-oxohexyl)-3,3-dimethyl-3H-indolium bromide

(di-ester dye) (200 mg, 8%); m.p. 161-163 °C; ^1H NMR (CDCl_3 , 400 MHz): δ 1.54 (m, 4H), 1.73 (s, 14H), 1.86 (m, 4H), 2.00 (m, 4H), 2.36 (t, $J = 7.6$ Hz, 4H), 2.75 (t, $J = 7.6$ Hz, 4H), 3.65 (s, 6H), 4.25 (t, $J = 7.6$ Hz, 4H), 6.26 (d, $J = 14.0$ Hz, 2H), 7.18 (d, $J = 7.6$ Hz, 2H), 7.27 (m, 2H), 7.40 (m, 4H), 8.34 (d, $J = 14.0$ Hz, 2H). ^{13}C NMR (CDCl_3 , 100 MHz) ppm 20.8, 24.6, 26.4, 26.8, 27.2, 28.2, 33.7, 44.8, 49.3, 51.6, 101.7, 110.9, 122.2, 125.3, 127.8, 128.8, 141.1, 142.3, 144.2, 150.2, 172.2, 173.8; HRMS (ESI) m/z calcd for $\text{C}_{44}\text{H}_{56}\text{N}_2\text{O}_4\text{Cl} [\text{M}^+]$ m/z 711.3921; found 711.3915.

2-((E)-2-((E)-3-((E)-2-(1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)ethylidene)-2-chlorocyclohex-1-enyl)vinyl)-1-(6-methoxy-6-oxohexyl)-3,3-dimethyl-3H-idolium bromide

(mono-ester dye) (700 mg, 29%); m.p. 165-167 °C; ^1H NMR (CDCl_3 , 400 MHz): δ 1.53 (m, 4H), 1.72 (s, 14H), 1.87 (t, $J = 7.4$ Hz, 4H), 2.00 (m, 2H), 2.36 (t, $J = 7.4$ Hz, 2H), 2.42 (t, $J = 7.4$ Hz, 2H), 2.71 (m, 4H), 3.66 (s, 3H), 4.13 (m, 4H), 6.14 (d, $J = 14.0$ Hz,

2H), 7.13 (d, $J = 7.4$ Hz, 1H), 7.25 (m, 3H), 7.41 (m, 4H), 8.36 (q, $J = 14.0$ Hz, 2H). ^{13}C NMR (CDCl₃, 75 MHz) ppm 20.7, 24.5, 25.5, 26.3, 26.4, 26.5, 27.0, 28.1, 28.1, 28.5, 29.6, 33.6, 33.9, 34.5, 44.5, 44.7, 49.2, 49.5, 51.6, 100.8, 101.6, 110.7, 112.2, 122.3, 125.2, 125.6, 127.3, 125.6, 128.8, 129.0, 140.9, 141.1, 142.0, 142.2, 144.0, 144.9, 150.7, 171.9, 172.8, 173.8, 176.2; MS (ESI) m/z calcd for C₄₃H₅₄N₂O₄Cl [M⁺] m/z 697.3772, found 697.3763.

1-(5-carboxypentyl)-2-((E)-2-((E)-3-((E)-2-(1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)ethylidene)-2-chlorocyclohex-1-enyl)vinyl)-3,3-dimethyl-3H-indolium bromide

(diacid dye) (400 mg, 17%); m.p. 171-173 °C ^1H NMR (DMSO-*d*₆, 400 MHz): δ 1.56 (m, 4H), 1.71 (s, 12H), 1.76 (q, $J = 7.4$ Hz, 4H), 1.85 (q, $J = 7.4$ Hz, 4H), 2.00 (br. t, 2H), 2.46 (t, $J = 7.4$ Hz, 4H), 2.72 (br. t, 4H), 4.13 (t, $J = 7.4$ Hz, 4H), 6.20 (d, $J = 14.0$ Hz, 2H), 7.22 (m, 4H), 7.38 (m, 4H), 8.33 (d, $J = 14.0$ Hz, 2H). ^{13}C NMR (CDCl₃, 75 MHz) ppm 24.2, 25.6, 25.8, 26.7, 27.5, 33.5, 43.7, 49.0, 101.6, 111.5, 122.5, 125.1, 126.2, 128.6, 141.1, 142.0, 143.0, 148.0, 172.2, 174.3; HRMS (ESI) m/z calcd for C₄₂H₅₂N₂O₄Cl [M⁺] 683.3616; found 683.3595.

2,3,3-Trimethyl-3H-indole-5-sulfonic acid, 97

This compound was obtained in a 83% yield; mp 187-181 °C; (reported potassium 2,3,3-trimethyl-3H-indole-5-sulfonate: yield 74%, mp 292-293 °C) [126].

2,3,3-Trimethyl-1-[3-(trimethylammonio)propyl]-3H-indolium-5-sulfonate dibromide, 98

A mixture of 2,3,3-trimethyl-3H-indole-5-sulfonic acid **97** (7.17 g, 36.4 mmol) and (3-bromopropyl)trimethyl-ammonium bromide (10.5 g, 40.0 mmol) in 1,2-dichlorobenzene

(60.0 mL) was heated at 130 °C for 72 h under nitrogen atmosphere. The mixture was cooled to room temperature and the solvent was decanted. The crude product was crystallized from methanol and ether to afford pink crystals (**98**, 11.0 g, 58%); mp 232-235 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.56 (s, 6H), 2.51 (s, 3H), 3.07 (m, 2H), 3.12 (s, 9H), 3.62 (t, *J* = 7.2 Hz, 2H), 4.50 (t, *J* = 7.2 Hz, 2H), 7.71 (d, *J* = 8.0 Hz, 1H), 7.79 (d, *J* = 8.0 Hz, 1H), 8.01 (s, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 15.0, 21.6, 22.3, 45.2, 53.1, 55.0, 62.4, 115.4, 121.2, 126.8, 141.3, 142.01, 149.9, 199.1. MS (ESI) calculated for C₁₇H₂₇N₂O₃S [M]⁺ m/z 339.17, found m/z 339.17.

2,3,3-Trimethyl-1-[3-(trimethylammonio)propyl]-3*H*-indolium bromide, 99

A mixture of 2,3,3-trimethyl-3*H*-indole **35** (1.59 g, 10.0 mmol) and (3-bromopropyl)trimethylammonium bromide (2.87 g, 11.0 mmol) in acetonitrile (50.0 mL) was heated at 70 °C for 72 h under nitrogen atmosphere in a sealed tube. The mixture was cooled to room temperature, and then concentrated under reduced pressure to furnish a red residue. The crude product was crystallized from acetone/methanol (5:1) to afford a pink solid **6** (**99**, 2.52 g, 61%); mp 197-199 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.57 (s, 6H), 2.40 (m, 2H), 3.02 (s, 3H), 3.30 (s, 9H), 3.84 (t, *J* = 7.6 Hz, 2H), 4.57 (t, *J* = 7.6 Hz, 2H), 7.61 (m, 2H), 7.88 (t, *J* = 5.2 Hz, 1H), 8.25 (t, *J* = 5.2 Hz, 1H). ¹³C NMR (100 MHz, DMSO-*d*₆): δ 15.2, 22.0, 25.5, 30.6, 52.3, 54.3, 63.9, 115.7, 123.5, 128.8, 129.3, 140.9, 141.7, 197.7. MS (ESI) calculated for C₁₇H₂₈N₂ [M+H]⁺ m/z 260.22, found m/z 259.30.

Disodium-2-((*E*)-2-((*E*)-2-chloro-3-((*E*)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)propyl)-indolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)-propyl)-3*H*-indolium-5-sulfonate tribromide **100**

A mixture of bromide salt **98** (1.26 g, 2.41 mmol), Vilsmeier-Haack reagent **73** (0.433 g, 1.50 mmol), and anhydrous sodium acetate (0.37 g, 4.50 mmol) in absolute ethanol (50.0 mL) was heated under reflux for 6 h under a nitrogen atmosphere. The mixture was cooled to room temperature, and then concentrated under reduced pressure to yield a brown residue. The crude product was washed with dichloromethane to furnish a brownish-green solid **8**, which was collected, suspended in methanol (10.0 mL), filtered and dried in vacuo to yield a green solid (**100**, 1.20 g, 1.1 mmol, 73%); mp 274-277 °C. ¹H NMR (100 MHz, DMSO-*d*₆) δ 1.72 (s, 12H), 1.88 (m, 2H), 2.18 (m, 4H), 2.76 (m, 4H), 3.08 (s, 18H), 3.49 (m, 4H), 4.18 (m, 4H), 6.36 (d, *J* = 14 Hz, 2H), 7.45 (d, *J* = 8.0 Hz, 2H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.85 (s, 2H), 8.31 (d, *J* = 14 Hz, 2H). ¹³C NMR (400 MHz, DMSO-*d*₆): ¹³CNMR spectrum would not be recorded due to low solubility (if you have will be fine. Also I did dissolve MM-17 in TFAA and the C13NMR is running from today till tomorrow I will let you know). MS (ESI) calculated for C₄₂H₅₈N₄O₆S₂Cl [M]⁺ *m/z* 813.35, found *m/z* 813.35. Vis/NIR in methanol; λ_{max} = 780 nm

2-((*E*)-2-((*E*)-2-chloro-3-((*E*)-2-(3,3-dimethyl-1-(3-trimethylammonio)propyl)indolin-2-ylidene)ethyl-idene)cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)propyl)-3*H*-indolium bromide, **101**

A mixture of bromide salt **99** (840 mg, 2.0 mmol), Vilsmeier-Haack reagent **73** (359 mg, 1.0 mmol), and anhydrous sodium acetate (492 mg, 6.0 mmol) in absolute ethanol (50.0 mL) was heated under reflux at 100 °C for 5 h. The mixture was cooled to room temperature, then concentrated under reduced pressure to yield a brown residue that was washed with dichloromethane and ether (1:1) to yield dye **9** (**101**, 260 mg, 29%). δ ¹H NMR (MeOD, 400 MHz): δ 1.80 (s, 12H), 2.40 (m, 4H), 2.87 (m, 4H), 3.24 (s, 18H),

3.33 (m, 2H), 3.75 (m, 4H), 4.36 (t, $J = 7.2$ Hz, 4H), 6.44 (d, $J = 14.0$ Hz, 2H), 7.34 (t, $J = 7.2$ Hz, 2H), 7.50 (m, 4H), 7.58 (d, $J = 7.2$ Hz, 2H), 8.49 (d, $J = 14.0$ Hz, 2H). ^{13}C NMR (100 MHz, MeOD): δ 22.3, 22.5, 27.9, 28.6, 42.2, 50.9, 54.0, 64.5, 102.9, 112.6, 123.8, 126.8, 129.3, 130.2, 142.9, 143.4, 146.2, 151.7, 174.5. MS-MALDI calculated for $\text{C}_{42}\text{H}_{58}\text{ClN}_4\text{O}_6\text{S}$ $[\text{M}+\text{H}]^+$ m/z 655.40, found m/z 655.60. Vis/NIR in methanol; $\lambda_{\text{max}} = 780$ nm.

2-((*E*)-2-((*E*)-2-(4-(2-carboxyethyl)phenoxy)-3-((*E*)-2-(3,3-dimethyl-5-sulfonato-1-(3-(trimethylammonio)-propyl)indolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)-propyl)-3*H*-indolium-5-sulfonate disodium bromide, 102

Preparation of 10 (ZW-1) using sodium hydroxide (NaOH): 3-(4-oxidophenyl)propanoate (SOPP)

Hydroxyphenyl)propionic acid (33.1 mg, 0.2 mmol) and powdered sodium hydroxide (8.0 mg, 0.2 mmol) were suspended in DMSO or DMF (1.0 mL) and stirred at room temperature for 30 min under nitrogen atmosphere. Chloro dye **100** (110 mg, 0.1 mmol) was added, and the mixture was heated under microwave conditions depicted in Supplementary Table 1. The crude product was washed with methanol and ether (3x5 mL, 2:3) to yield 10 (ZW-1) as a dark green solid (82% conversion yield, 41% yield).

Preparation of 10 (ZW-1) using sodium 3-(4-oxidophenyl)propanoate (SOPP): SOPP ($\text{C}_9\text{H}_8\text{Na}_2\text{O}_3$, MW 210.14) was prepared by adding 3-(4-hydroxyphenyl)propionic acid (16.6 g, 100 mmol) into a solution of sodium hydroxide (8.0 g, 200 mmol) in water. The mixture was stirred at room temperature for 2 h, followed by lyophilization. The pale yellow solid was dried under a reduced pressure for 24 h and used for the next step

without further purification. In the following step, chloro dye **100** (110 mg, 0.1 mmol) and 2 or 10 equiv of SOPP (42 mg or 210 mg) were dissolved in DMSO or DMF (2.0 mL) under nitrogen atmosphere. The mixture was heated under microwave conditions depicted in Supplementary Table 1. The crude product was washed with methanol and ether three times each to yield 10 (ZW-1) as a dark green solid (98% conversion yield, 85% yield). ¹H NMR (150 MHz, D₂O) δ 0.943 (s, 12H), 1.71 (m, 2H), 2.06 (m, 4H), 2.35 (m, 2H), 2.45 (m, 2H), 2.63 (m, 2H), 2.97 (s, 18 H), 3.38 (m, 4H), 3.83 (m, 4H), 5.97 (d, *J* = 13.2 Hz, 2H), 6.81 (d, *J* = 7.8 Hz, 2H), 7.03 (d, *J* = 7.2 Hz, 4H), 7.45 (s, 2H), 7.56 (d, *J* = 6.6 Hz, 2H), 7.66 (d, *J* = 13.2 Hz, 2H). ¹³C NMR (150 MHz, D₂O): δ 19.8, 22.6, 23.4, 29.9, 39.9, 41.6, 42.8, 42.9, 47.5, 51.2, 51.4, 51.7, 55.5, 55.7, 60.0, 65.59, 66.2, 66.9, 103.3, 108.5, 113.4, 117.4, 118.4, 122.5, 125.4, 126.1, 127.1, 129.1, 129.6, 132.2, 132.9, 135.8, 137.7, 138.3, 139.5, 140.0, 141.5, 142.9, 143.7, 145.9, 148.4, 150.5, 152.1, 157.6, 159.1, 159.4, 160.8, 167.8, 175.0, 183.8, 184.5. MS-MALDI calculated for C₅₁H₆₆N₄O₉S₂ [M-H]⁻ m/z 941.43, found m/z 941.47. Vis/NIR in methanol; λ_{max} = 770 nm.

2-((*E*)-2-((*E*)-2-(4-(2-carboxyethyl)phenoxy)-3-((*E*)-2-(3,3-dimethyl-1-(3-(trimethylammonio)-propyl)indolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-(3-(trimethylammonio)-propyl)-3*H*-indolium bromide, **103**

Chloro dye **101** (89.6 mg, 0.1 mmol) and 10 equiv of SOPP (210 mg, 1 mmol) were dissolved in DMSO (2.0 mL) under nitrogen atmosphere. The mixture was heated at 65 °C for 30 min under microwave. The crude product was washed with ether three times to yield 11 (ZW-3a) as a dark green solid (**103**, 99% conversion yield, 92% yield); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.27 (s, 12H), 2.42 (m, 4H), 2.10 (m, 4H), 2.68 (t, *J* = 7.8 Hz, 2H), 2.74 (m, 4H), 3.07 (s, 18H), 3.58 (m, 4H), 4.16 (m, 4H), 6.21 (d, *J* = 13.8 Hz, 2H),

6.64 (d, $J = 7.8$ Hz, 2H), 6.99 (d, $J = 8.4$ Hz, 2H), 7.05 (d, $J = 8.4$ Hz, 2H), 7.23 (m, 2H), 7.40 (t, $J = 7.2$ Hz, 2H), 7.44 (d, $J = 7.8$ Hz, 2H), 7.51 (d, $J = 7.2$ Hz, 2H), 7.86 (d, $J = 13.8$ Hz, 2H). ^{13}C NMR (150 MHz, DMSO- d_6): δ 22.6, 23.5, 23.8, 25.6, 27.0, 30.4, 30.9, 33.3, 40.3, 40.6, 43.8, 47.9, 48.3, 51.4, 51.7, 55.3, 55.4, 55.6, 65.4, 66.0, 66.8, 102.4, 103.5, 114.3, 117.2, 117.6, 118.2, 124.8, 125.2, 125.6, 127.6, 128.0, 130.2, 131.6, 132.1, 132.5, 133.2, 134.6, 139.0, 140.5, 141.4, 144.0, 144.5, 144.9, 150.4, 151.1, 158.7, 160.9, 166.4, 174.8, 177.6. MS-MALDI calculated for $\text{C}_{51}\text{H}_{66}\text{N}_4\text{O}_9\text{S}_2$ $[\text{M}]^+$ m/z 784.54, found m/z 784.57. Vis/NIR in methanol; $\lambda_{\text{max}} = 765$ nm.

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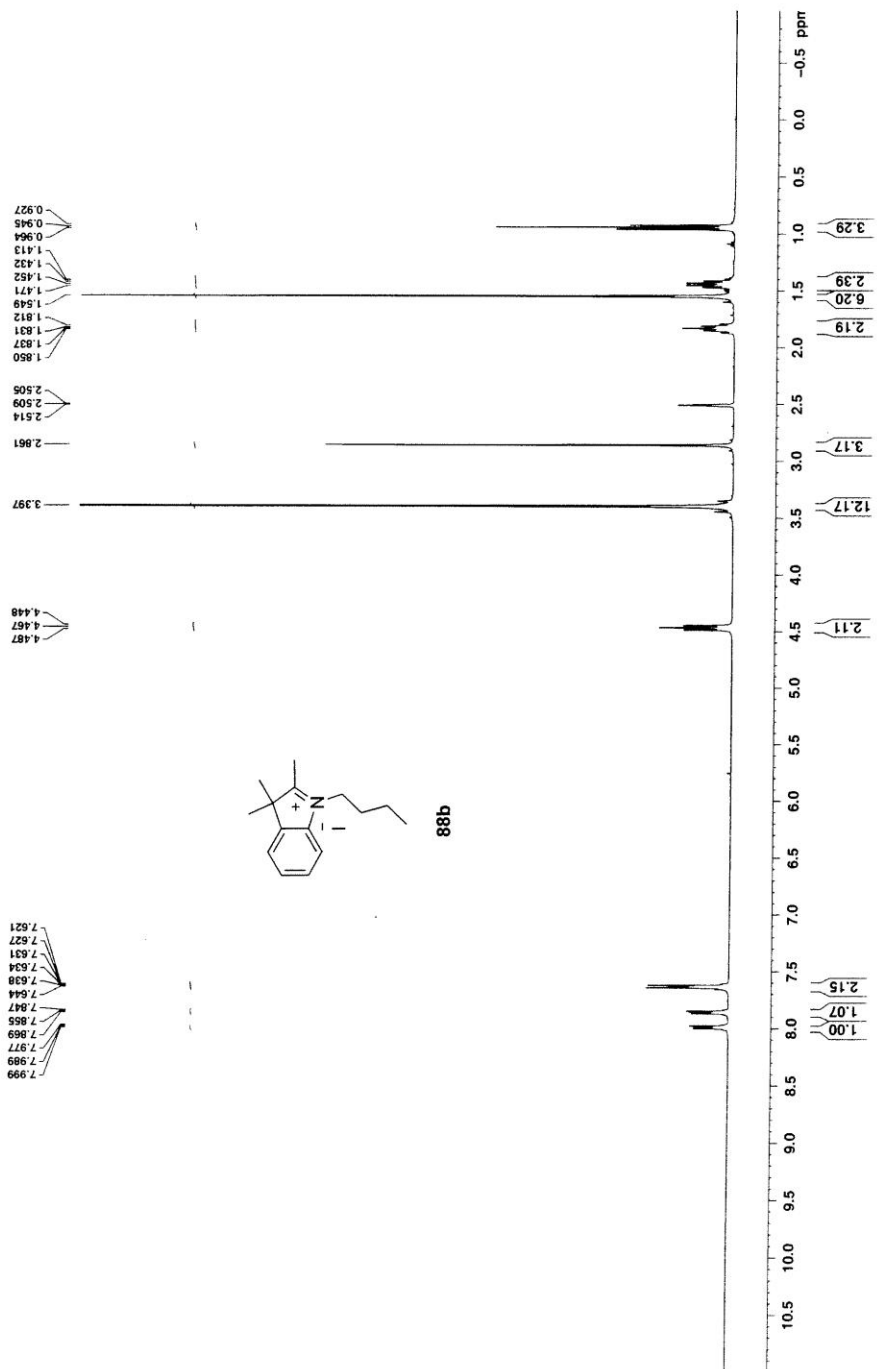
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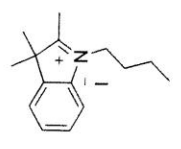
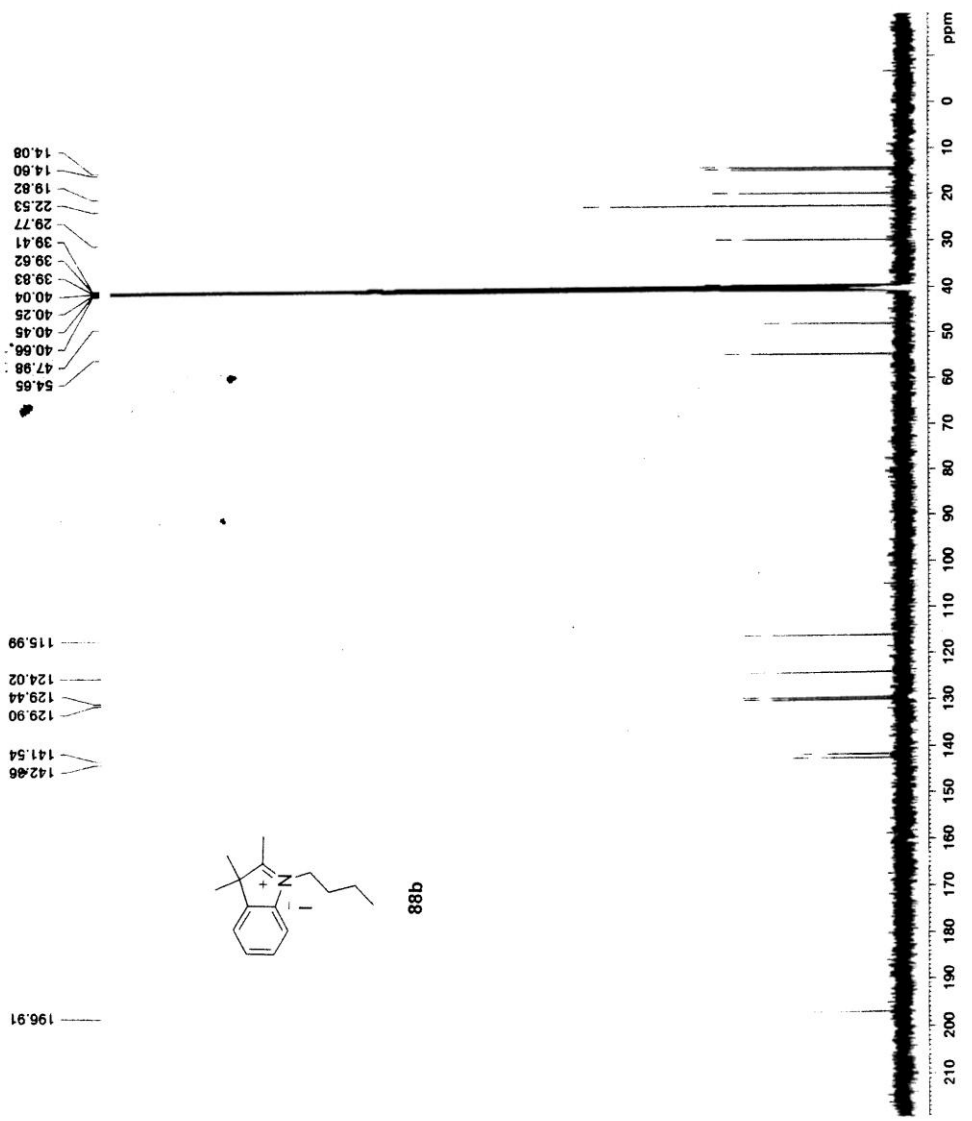
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6 APPENDICES (¹H NMR and ¹³C NMR Spectra)





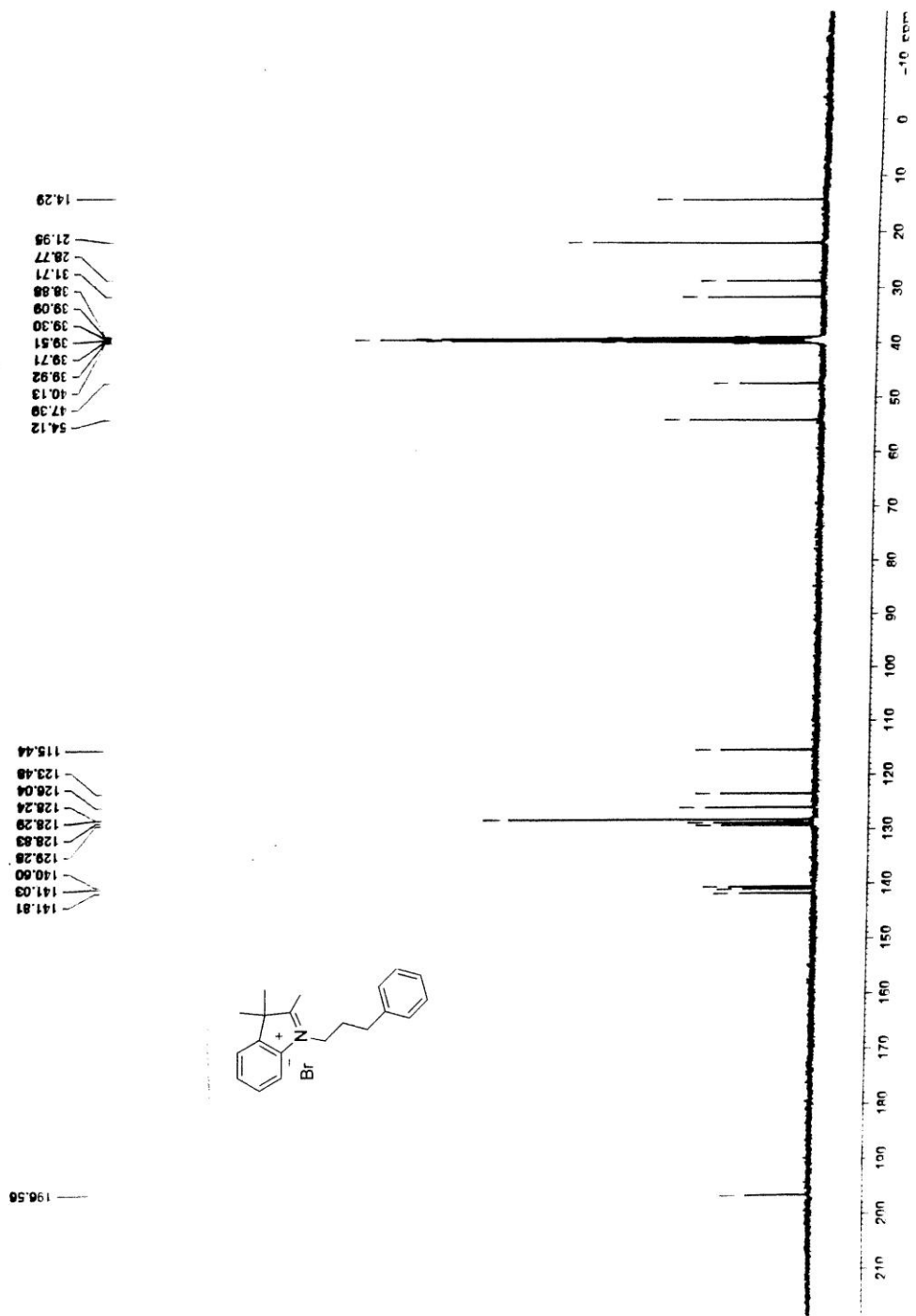
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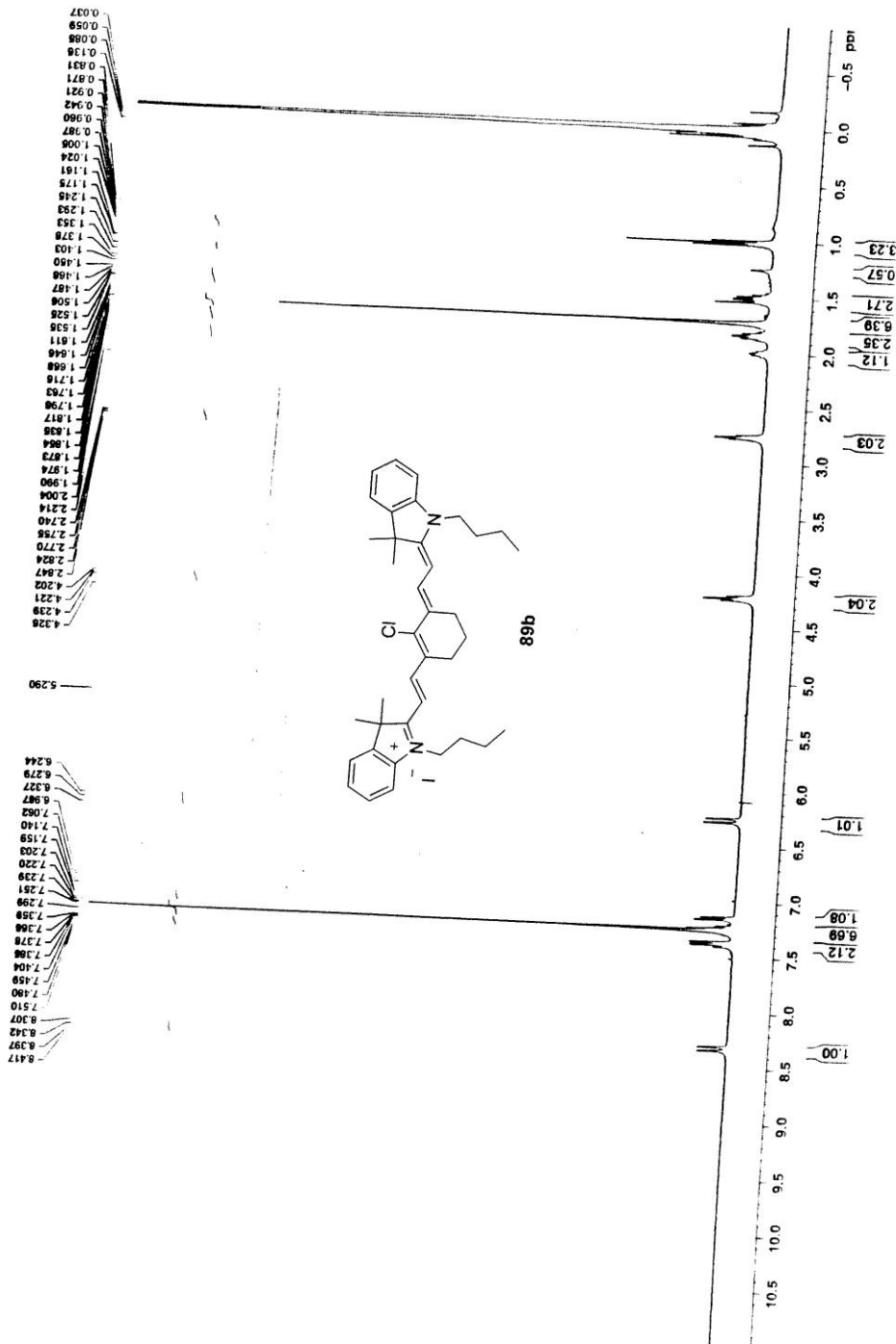
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 RG: 1819.4
 EQ: 20.850 usec
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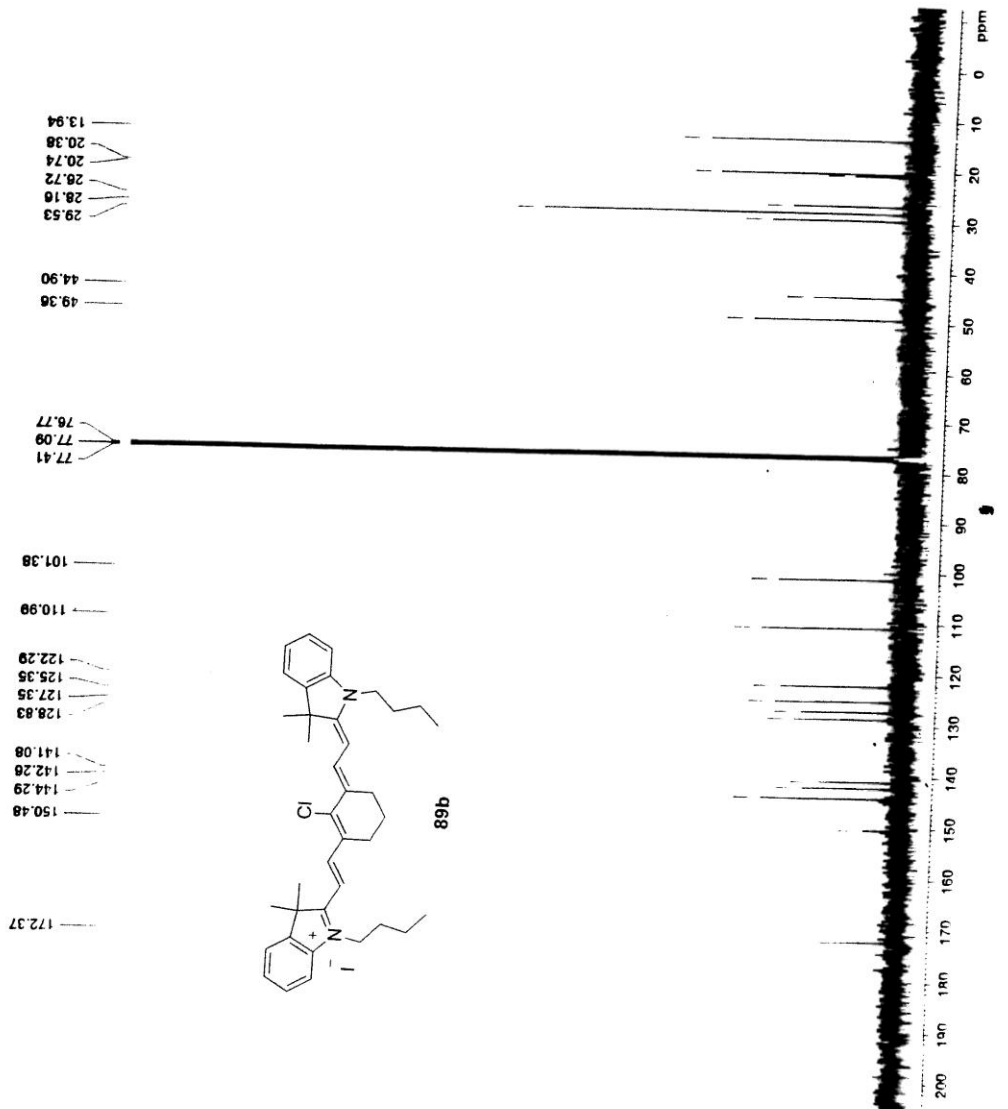
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 P2: 70.00 usec
 PL2: -1.00 dB
 PL12: 14.00 dB
 PL13: 14.00 dB
 SFO2: 400.1316005 MHz

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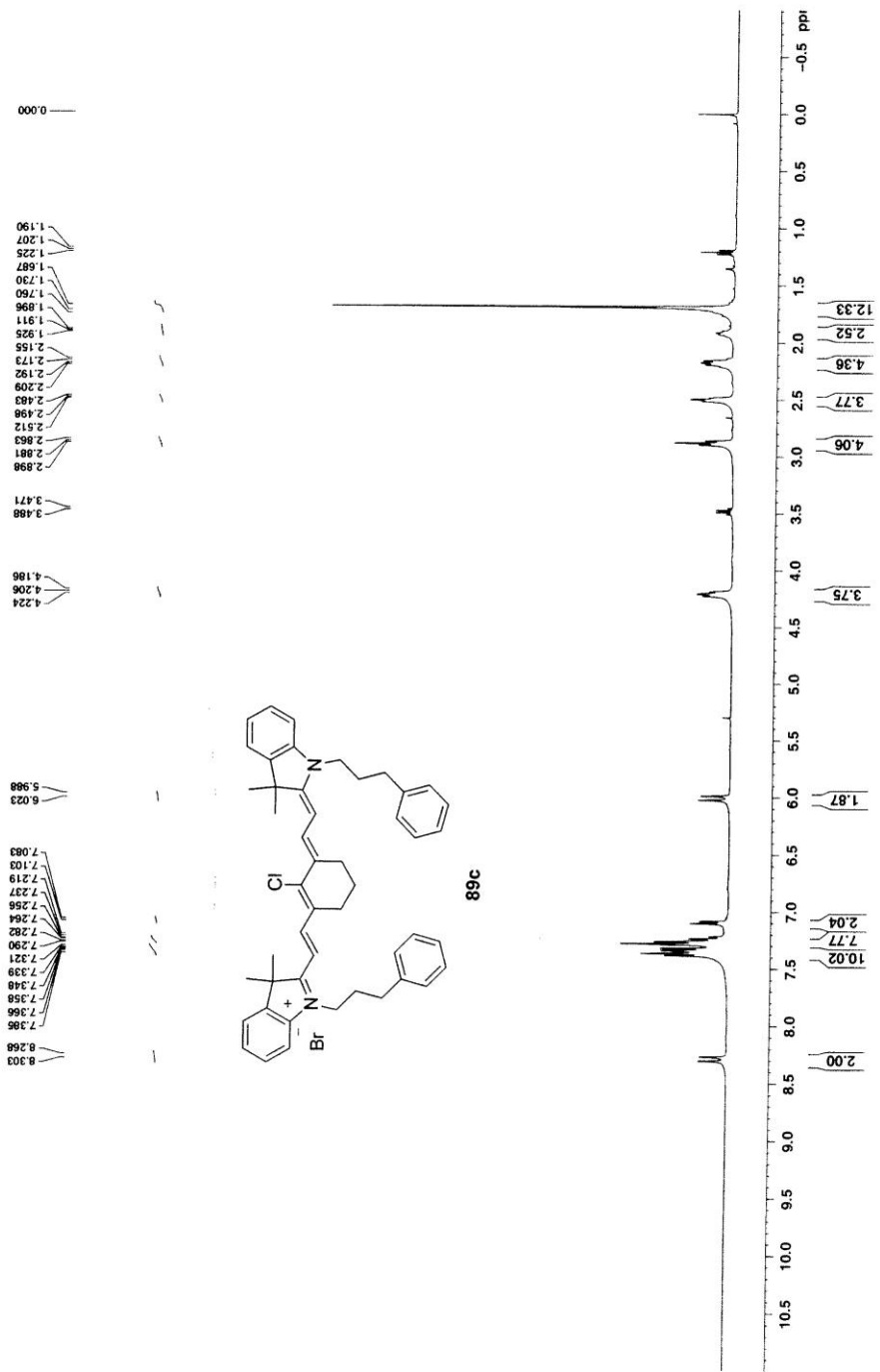


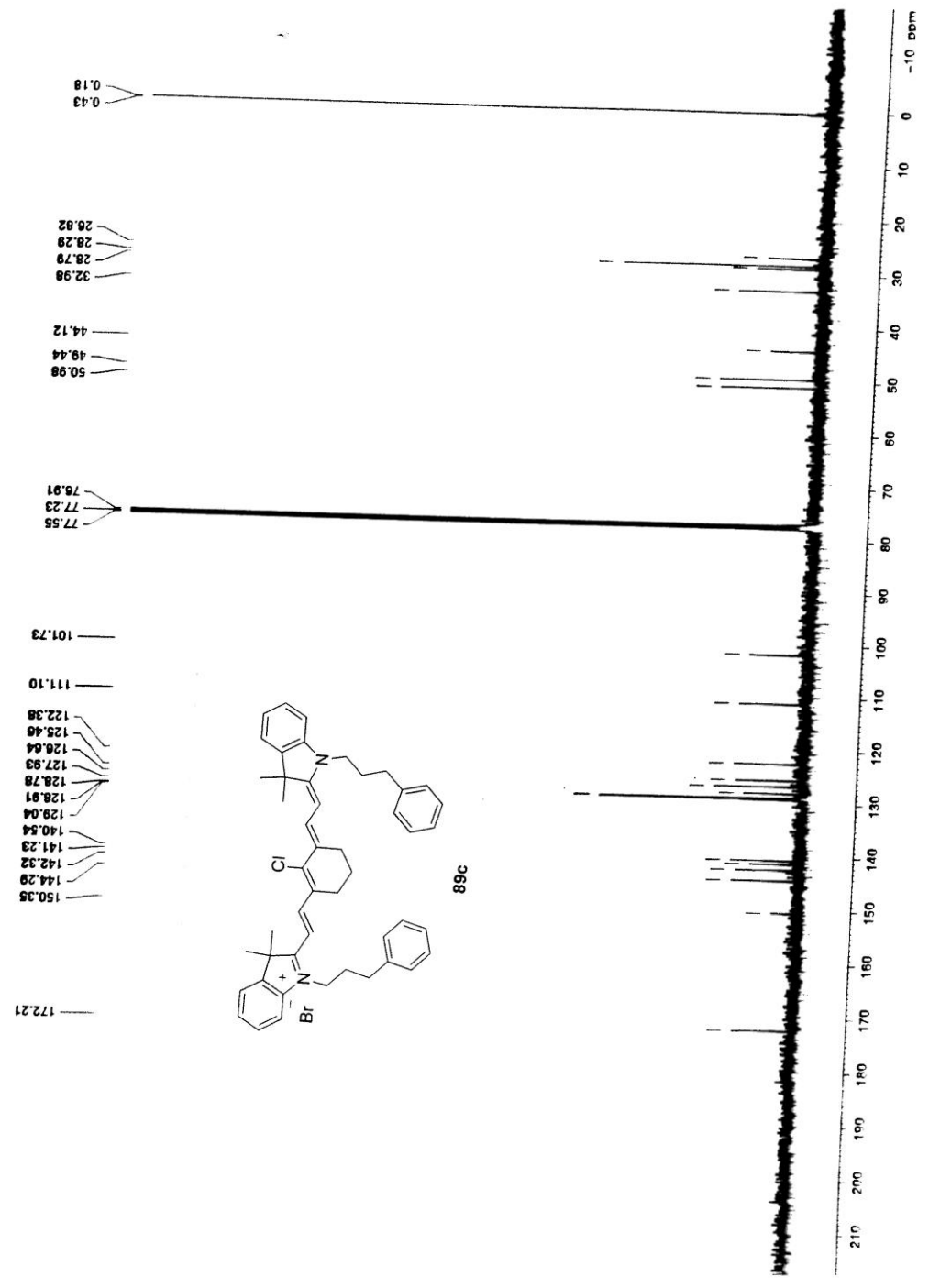
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 RG 32768
 PA 20.950 dB
 DE 2.000000 Hz
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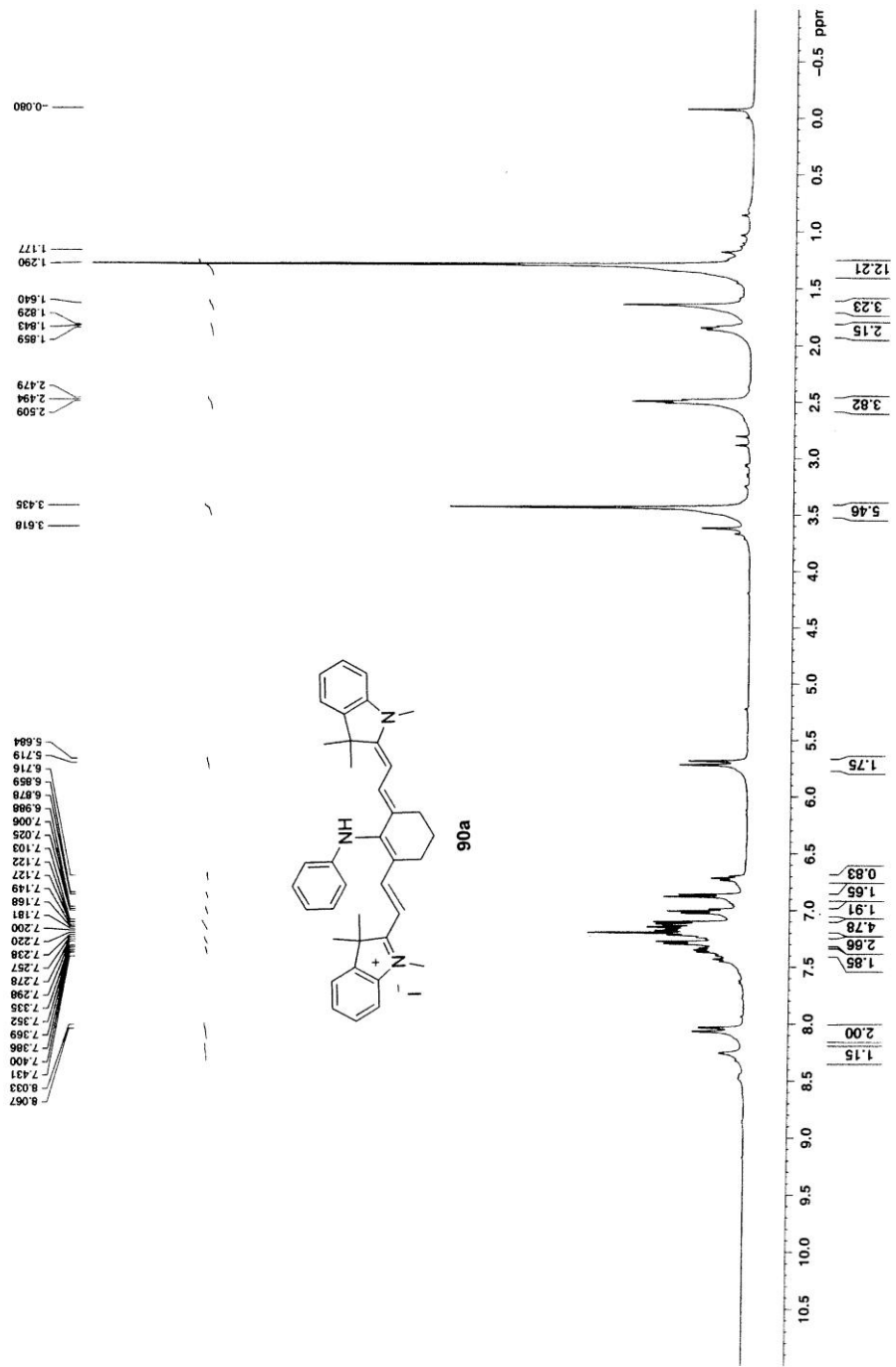
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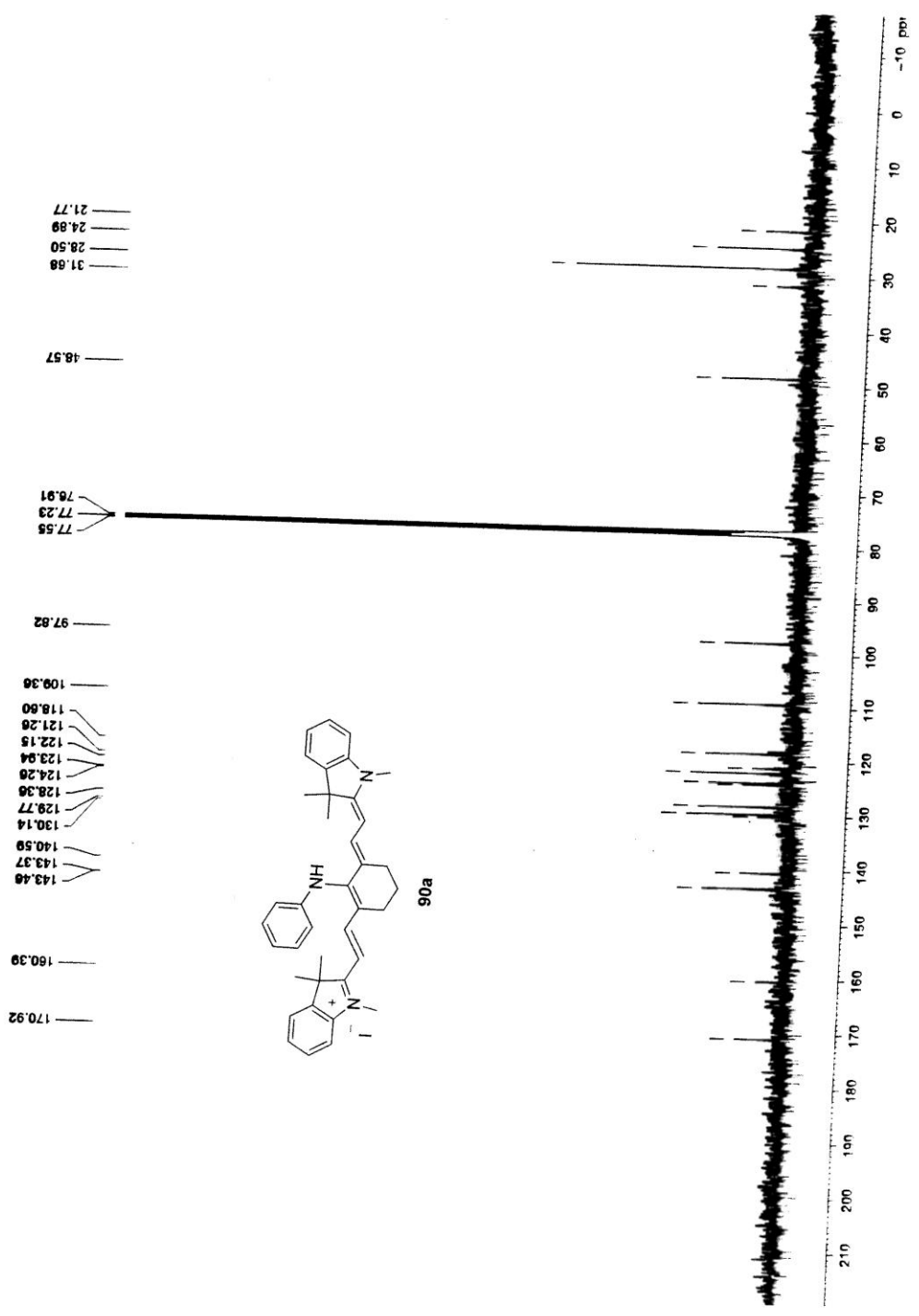
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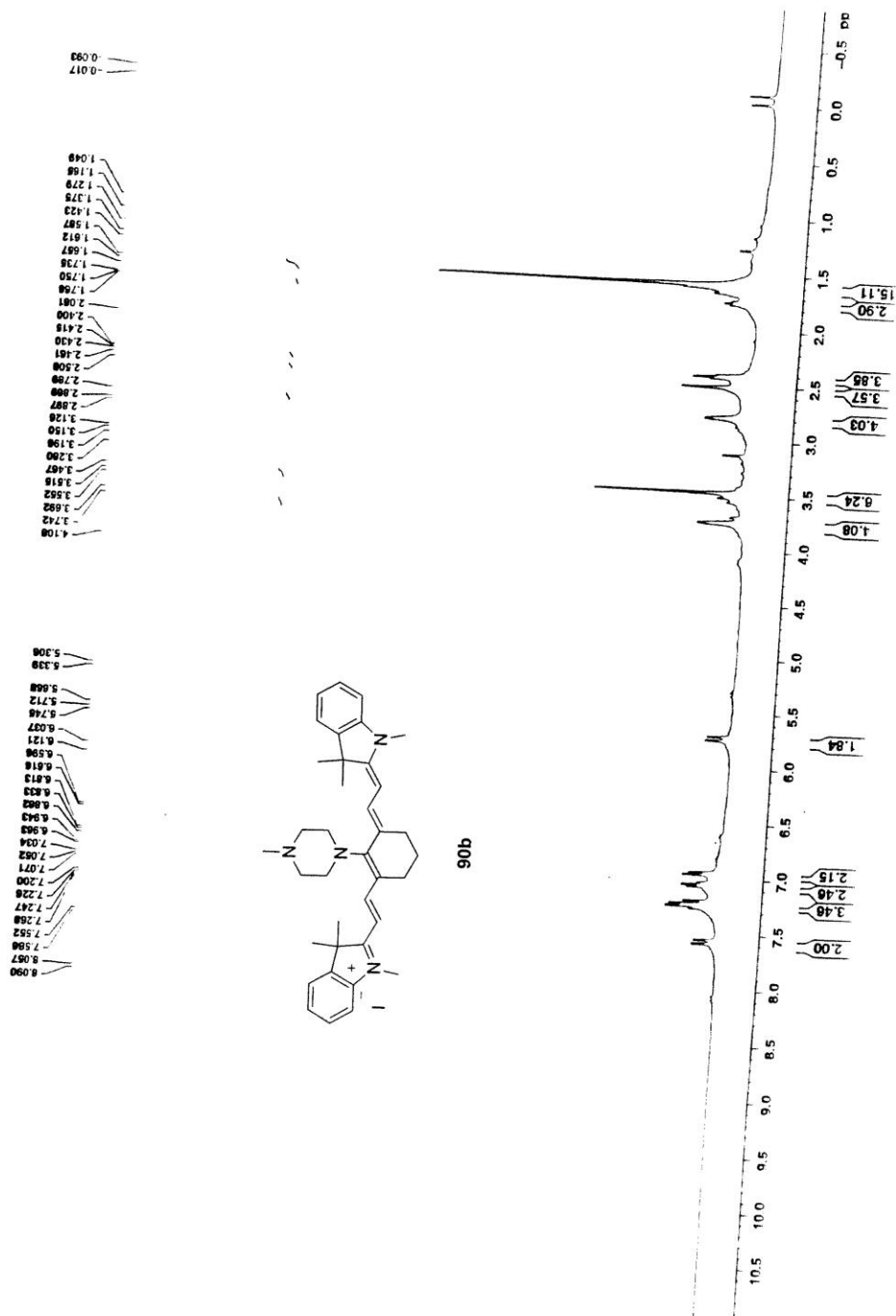
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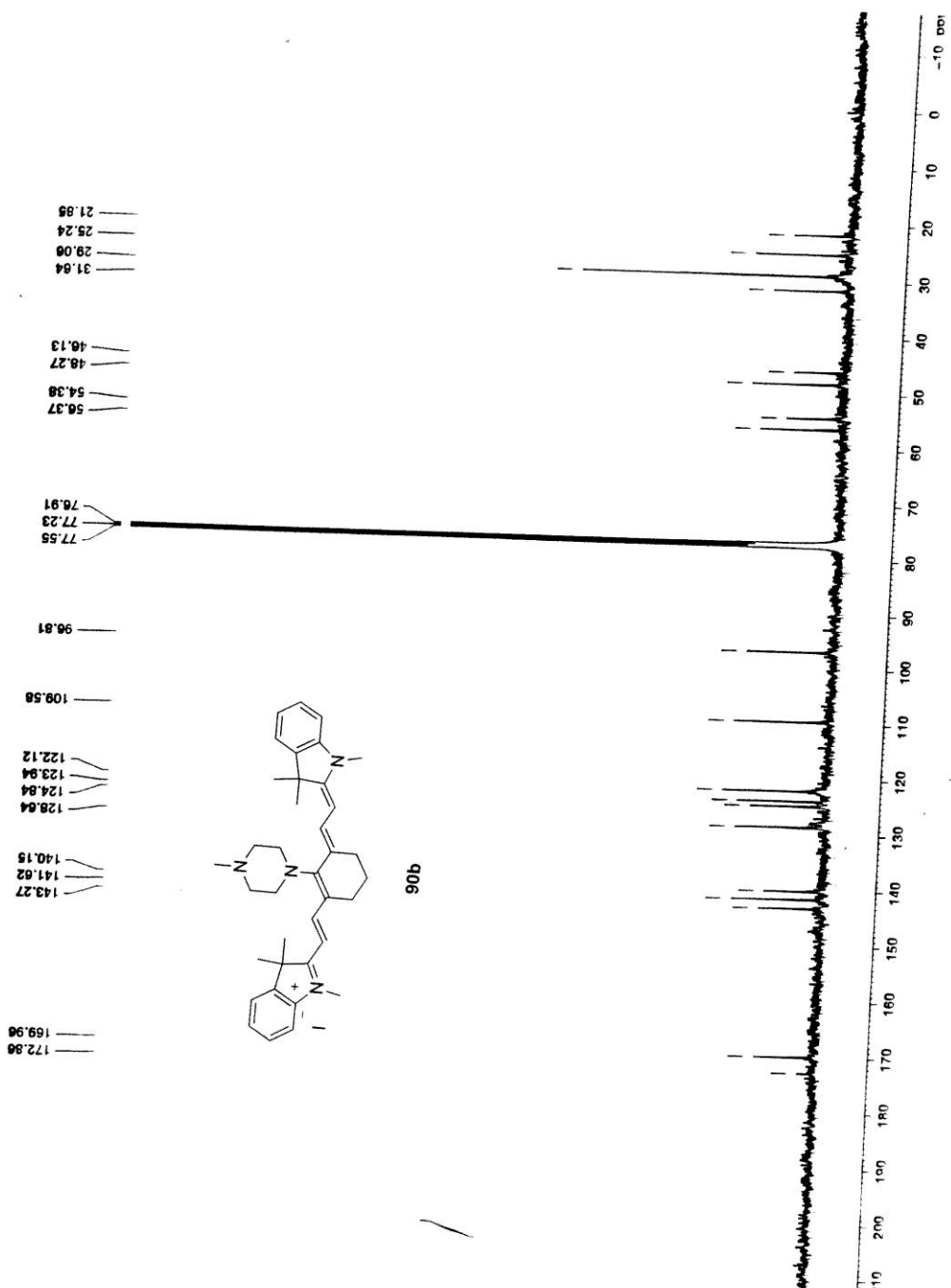


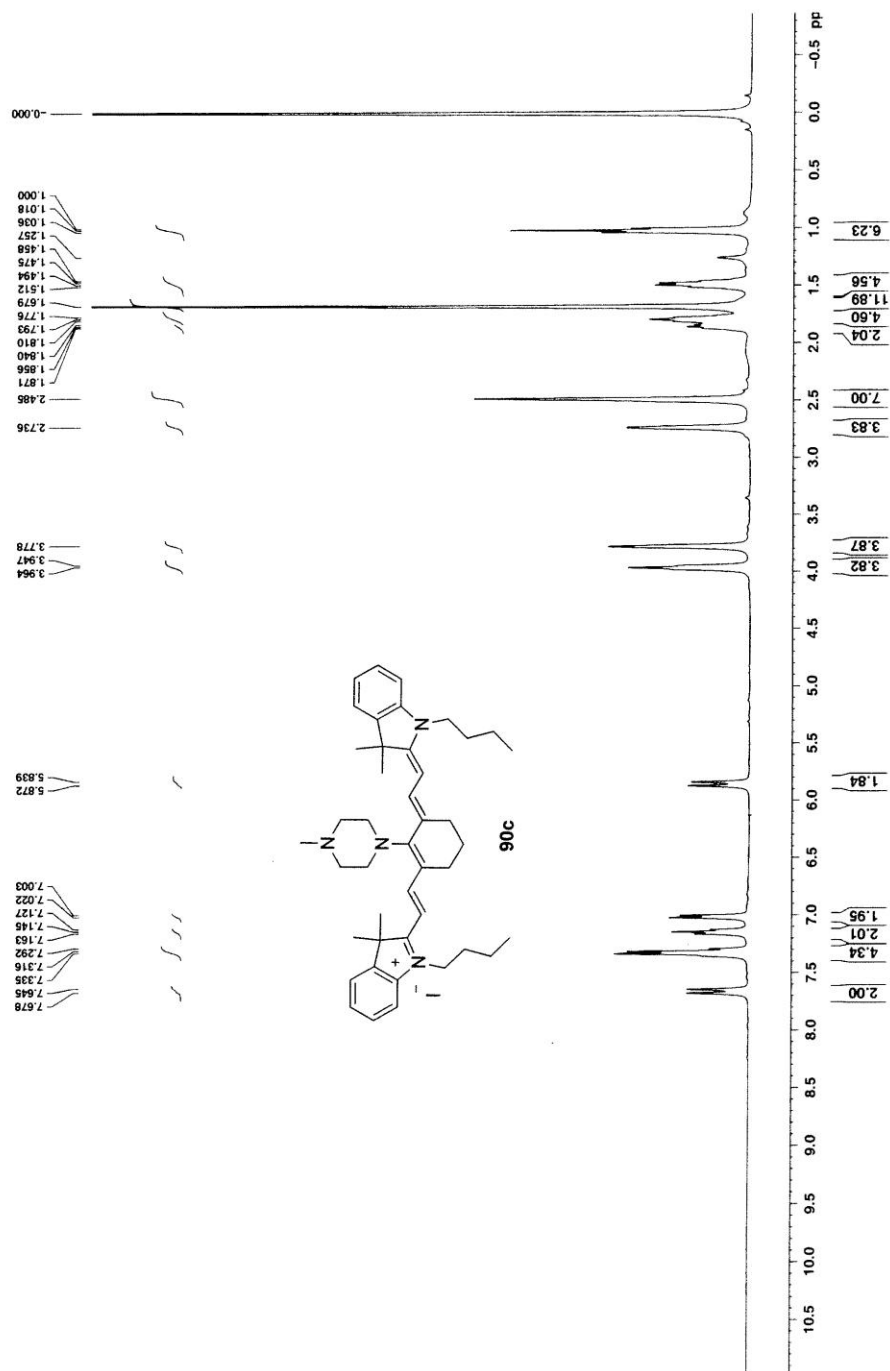


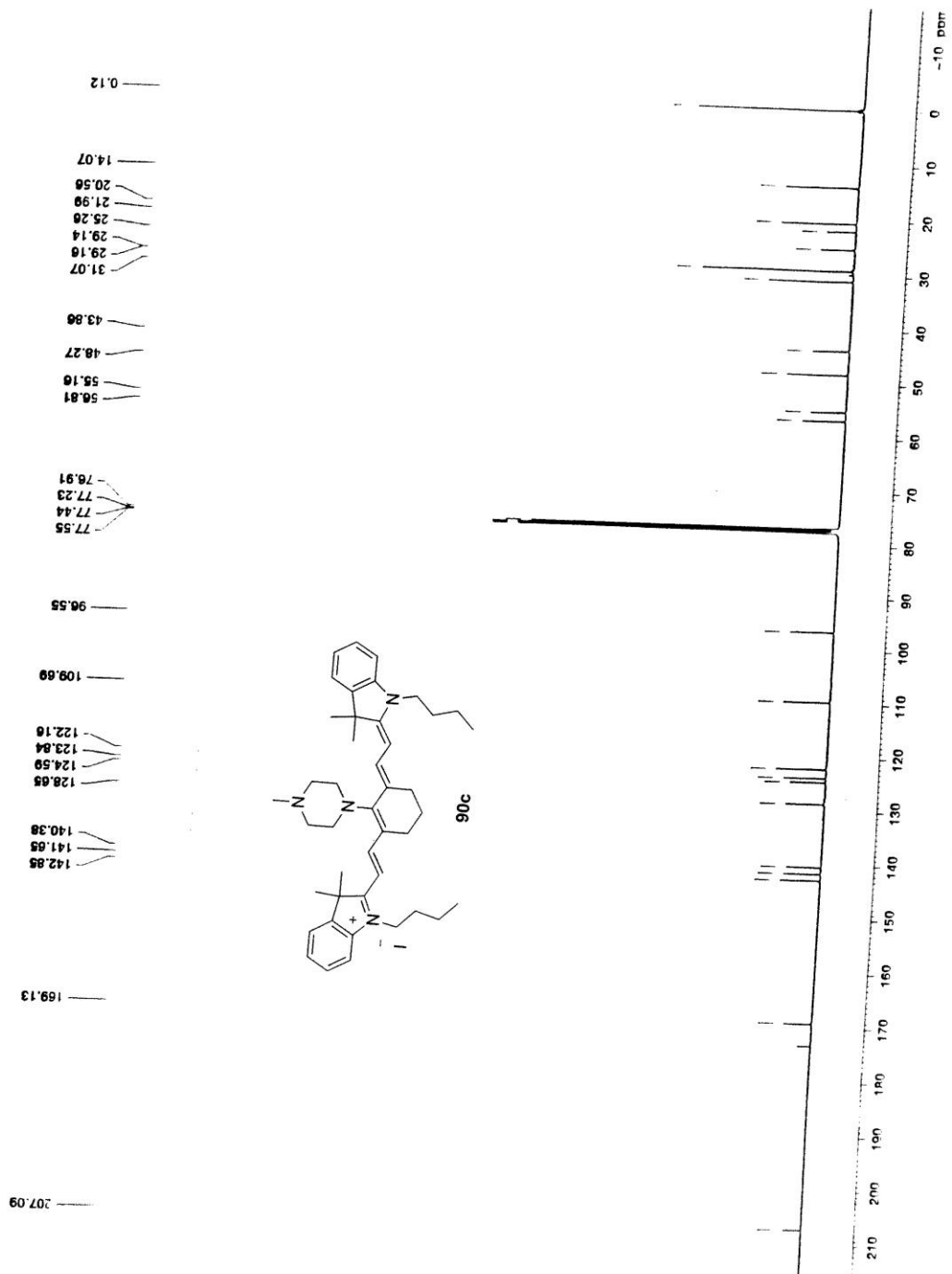


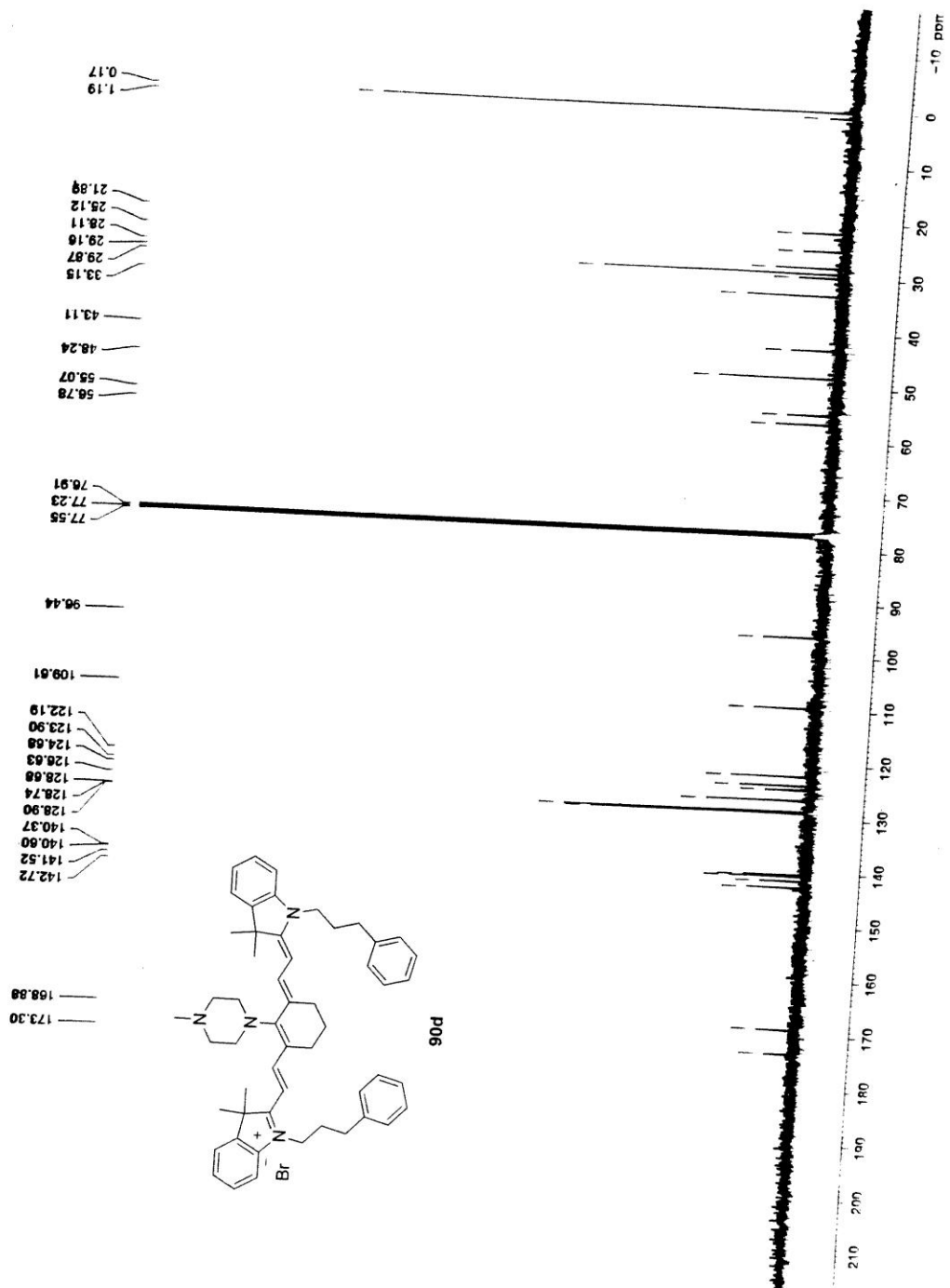


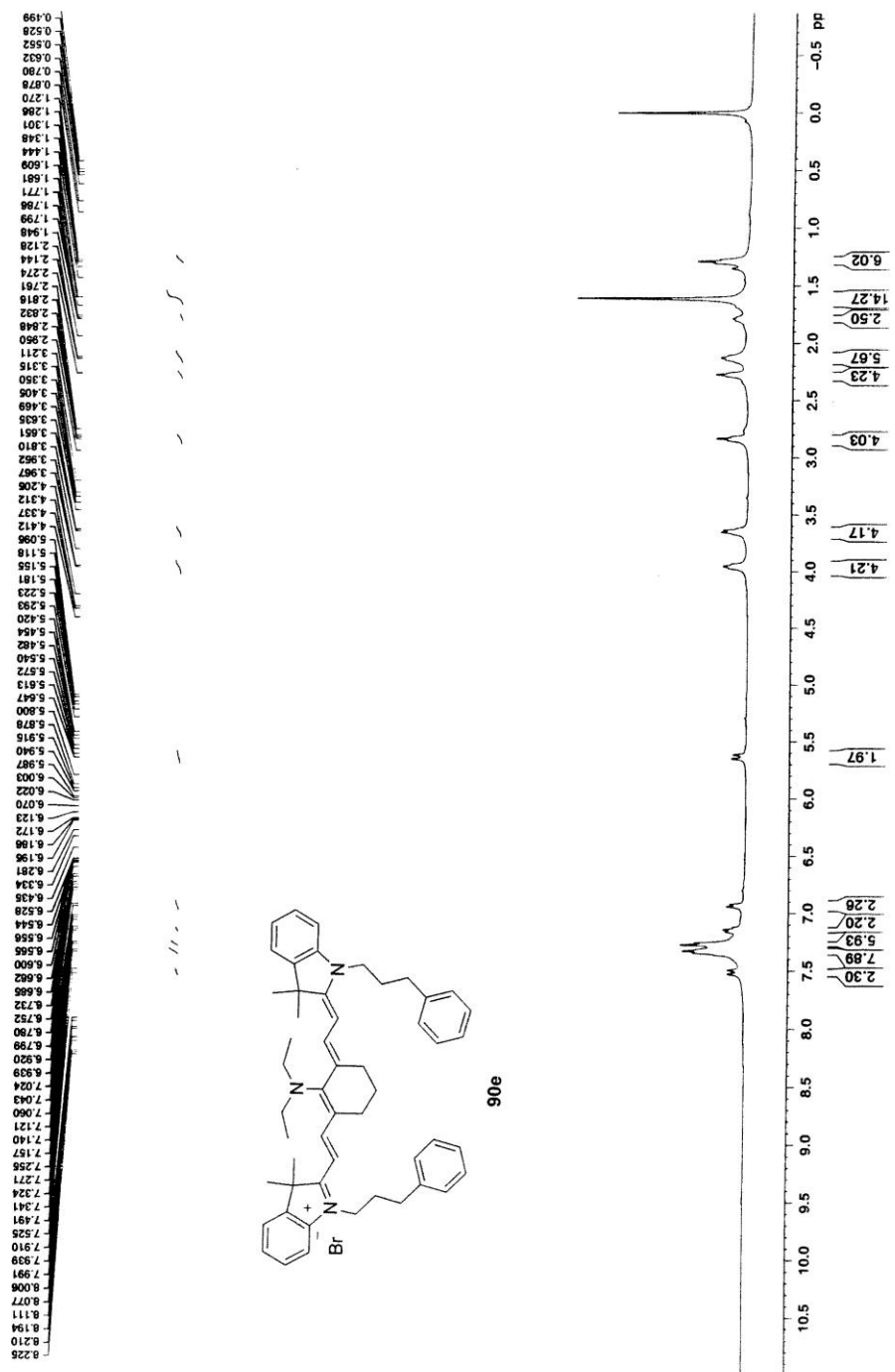


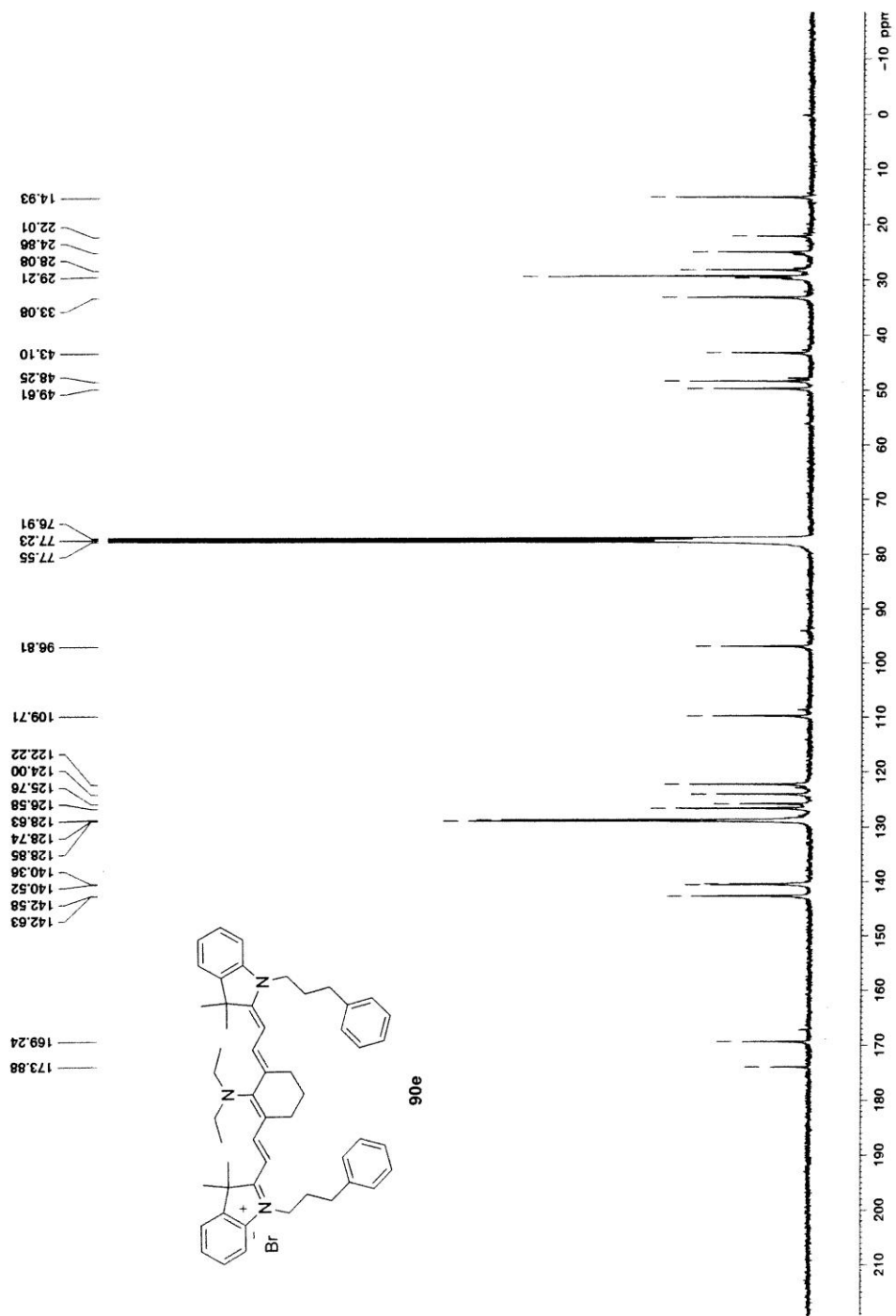


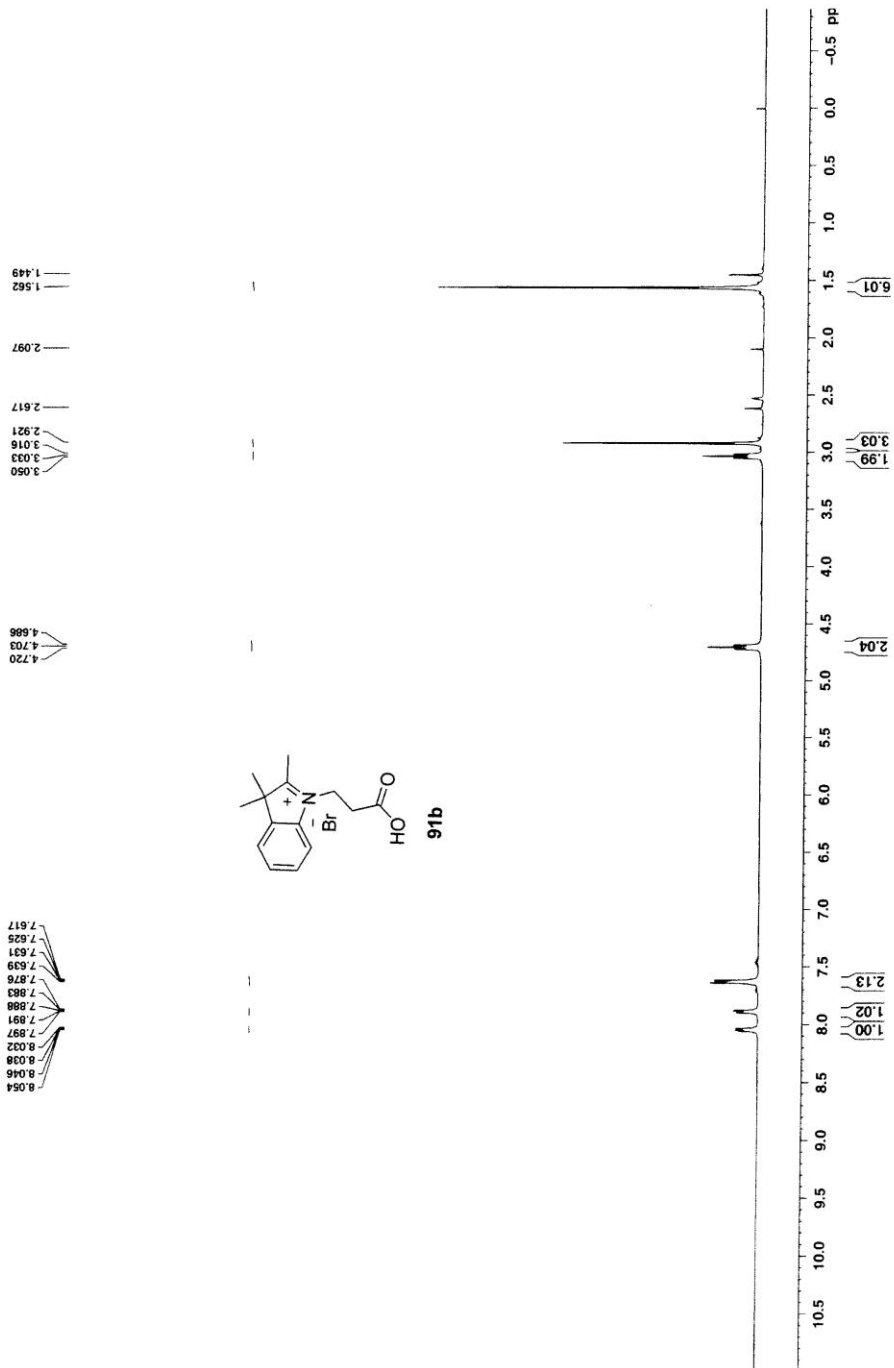




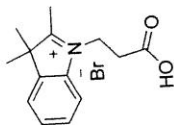
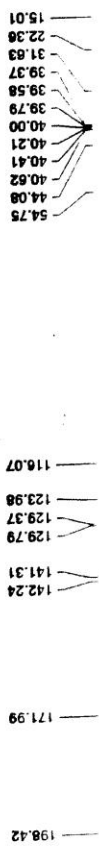








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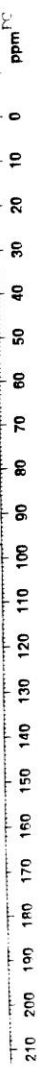


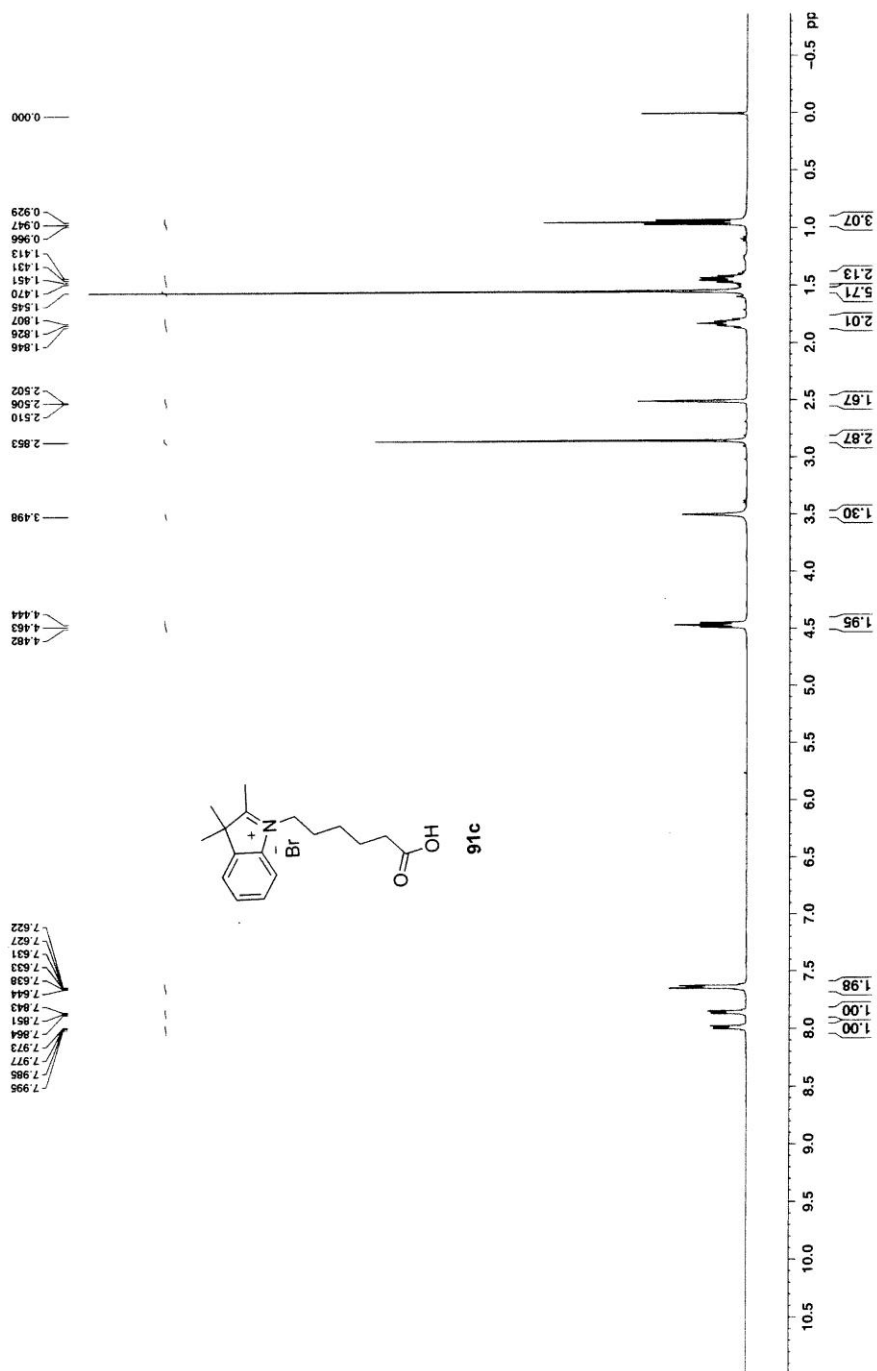
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 FIDRES 0.365918 Hz
 AQ 1.3664756 sec
 RG 32768
 DM 20.850 usec
 DE 7.00 usec
 TE 297.9 K
 d1 2.00000000 sec
 d11 0.03000000 sec
 DELTA 1.89999998 sec
 MCREST 0.00000000 sec
 MCVRK 0.01500000 sec

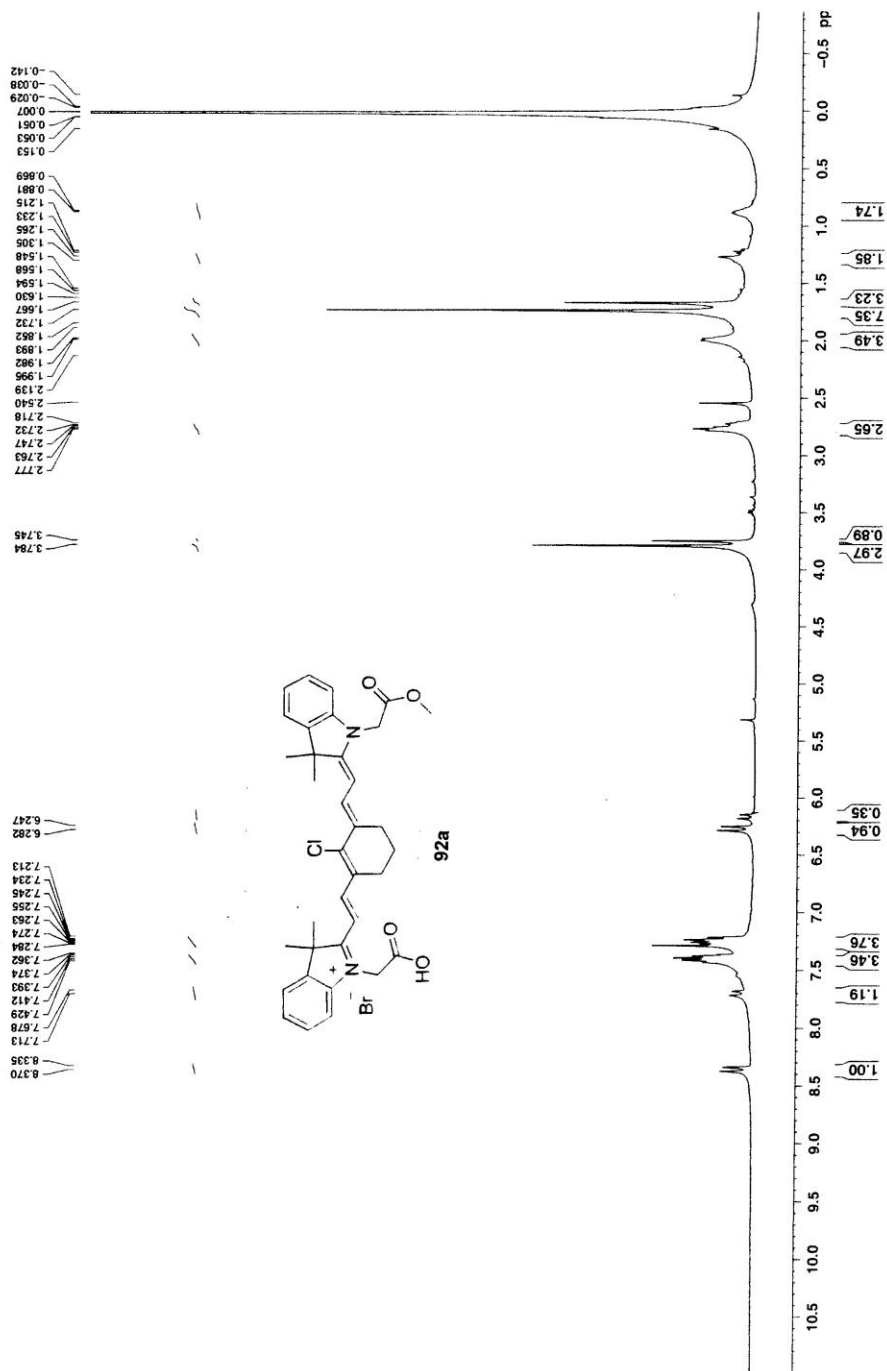
===== CHANNEL f1 =====
 NUC1 13C
 P1 8.00 usec
 PL1 -2.00 dB
 SFO1 100.6228298 MHz

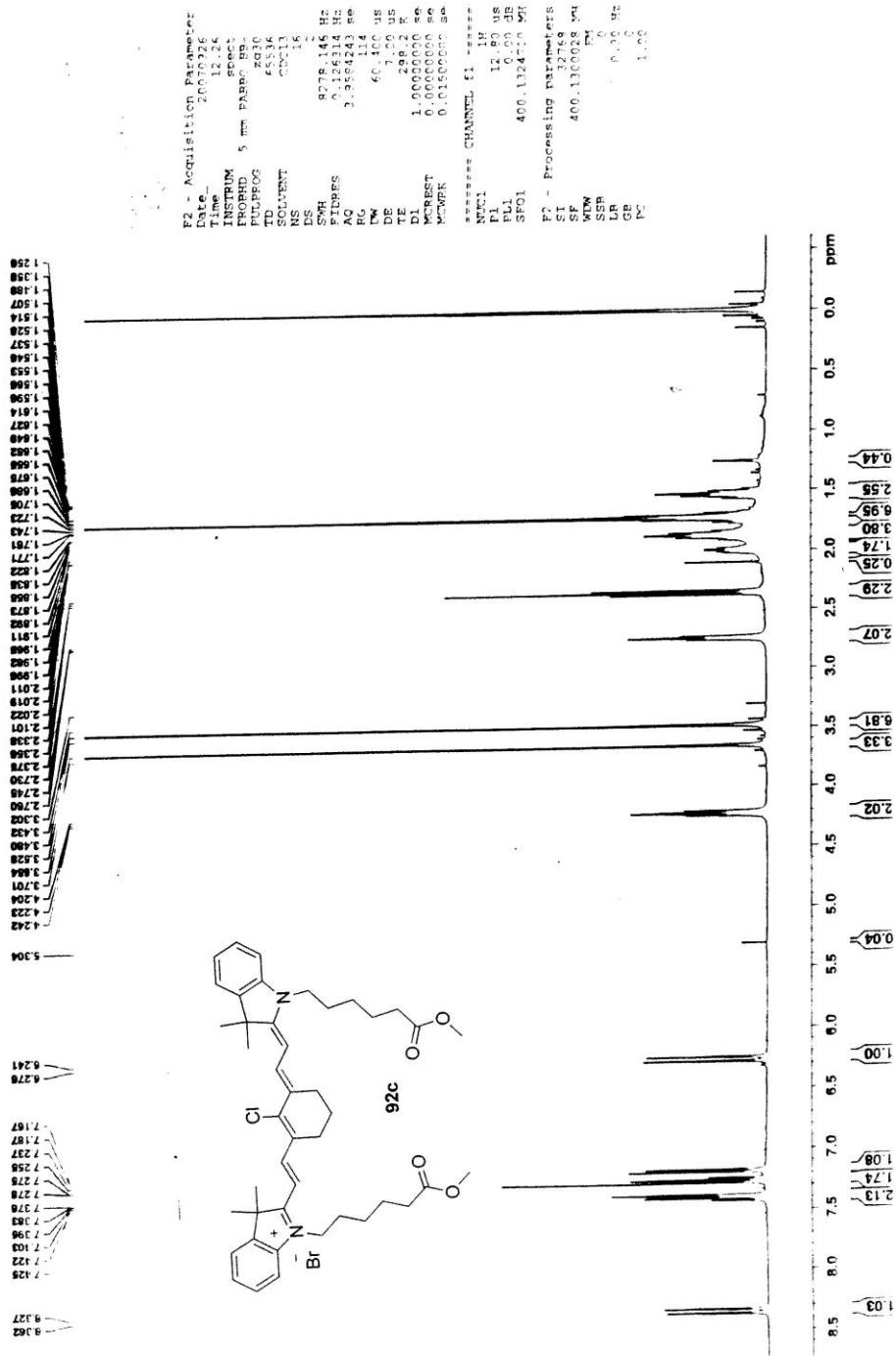
===== CHANNEL f2 =====
 CDFPRG2 waltz16
 NUC2 1H
 PCPD2 70.00 usec
 PL2 -1.00 dB
 PL12 14.00 dB
 PL13 14.00 dB
 SFO2 400.1315005 MHz

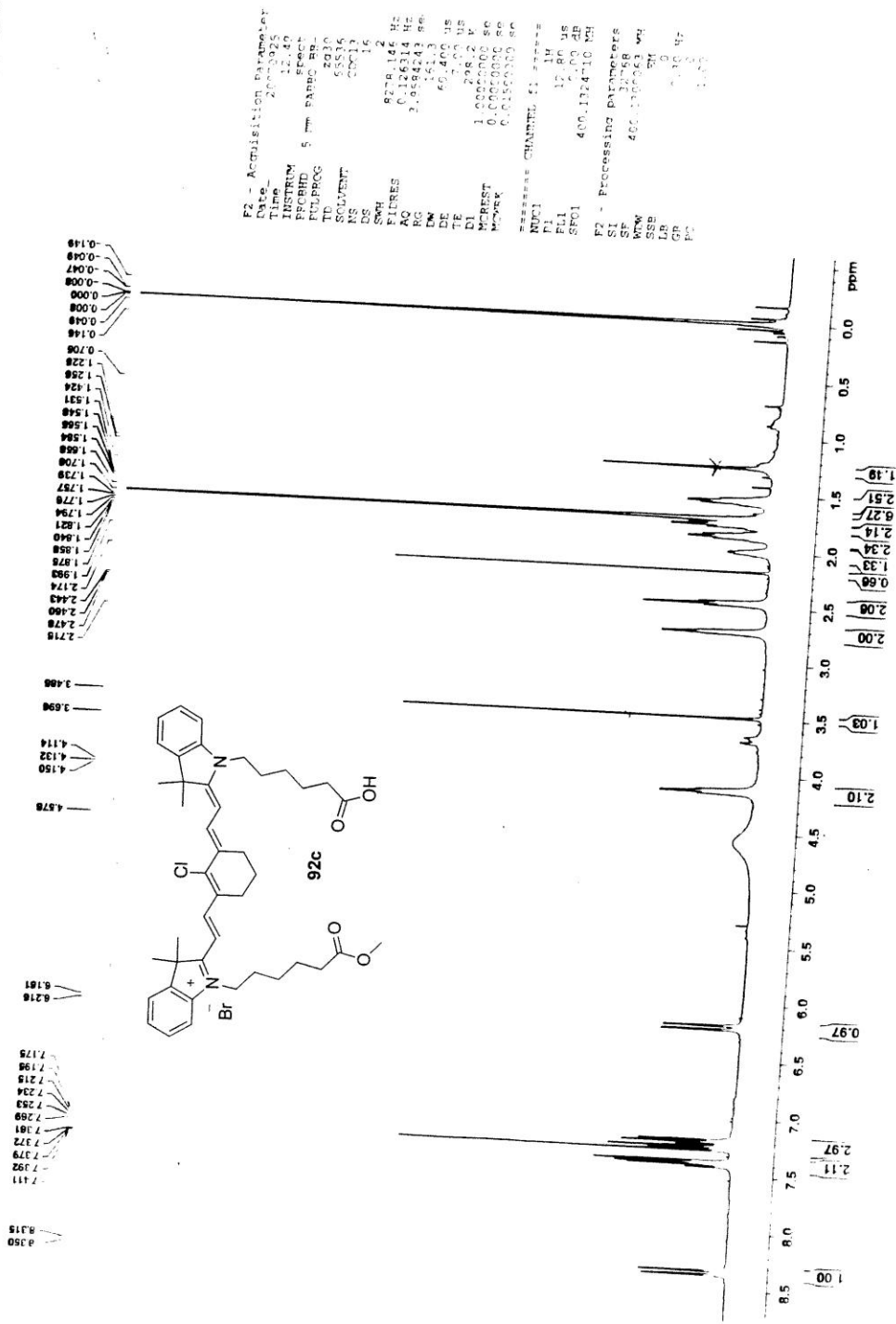
F2 - Processing parameters
 SI 32768
 SF 100.6127690 MHz
 MVM EM
 SSR 0
 LB 1.00 Hz
 GB 0
 PC 1.40



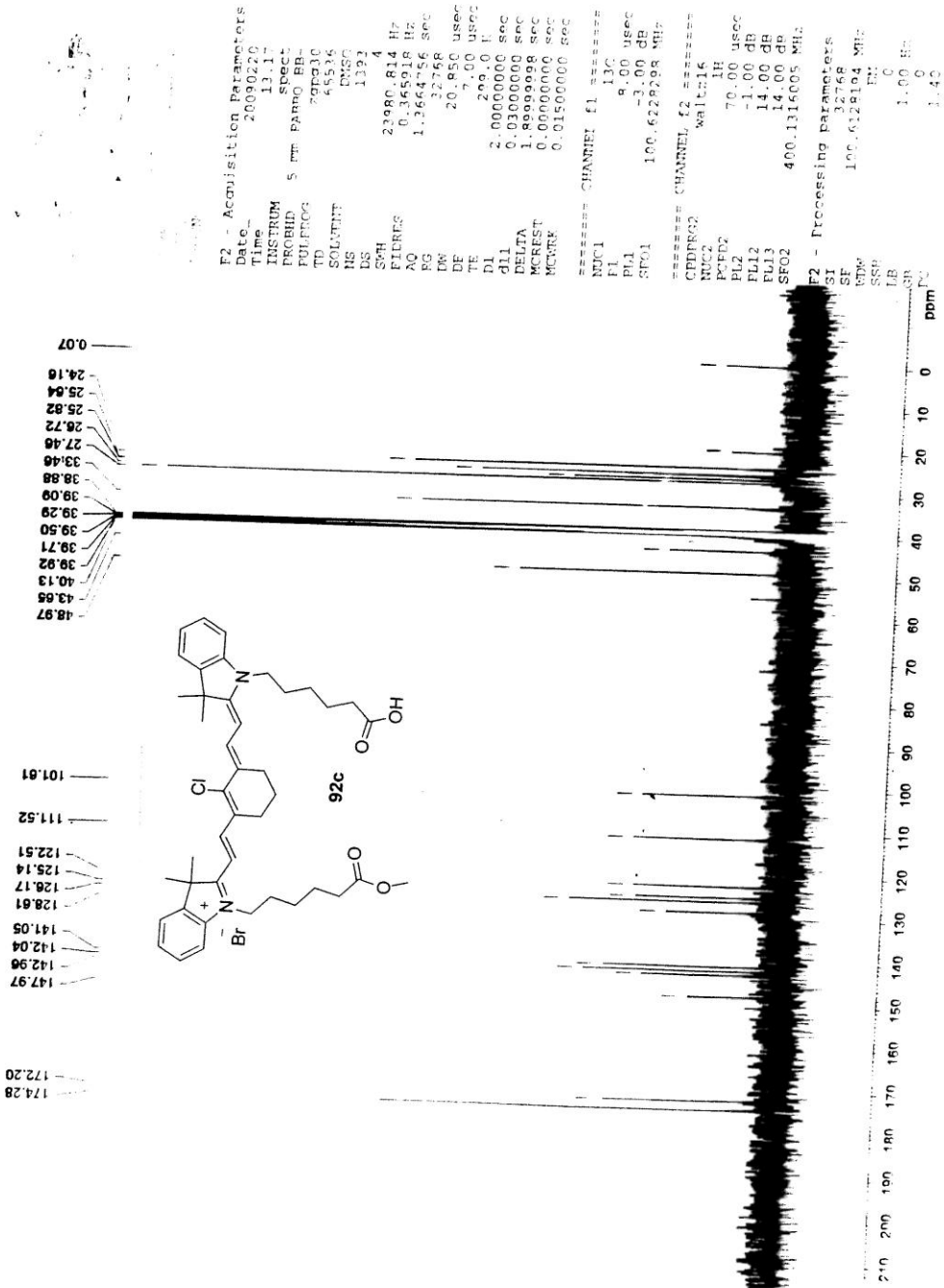


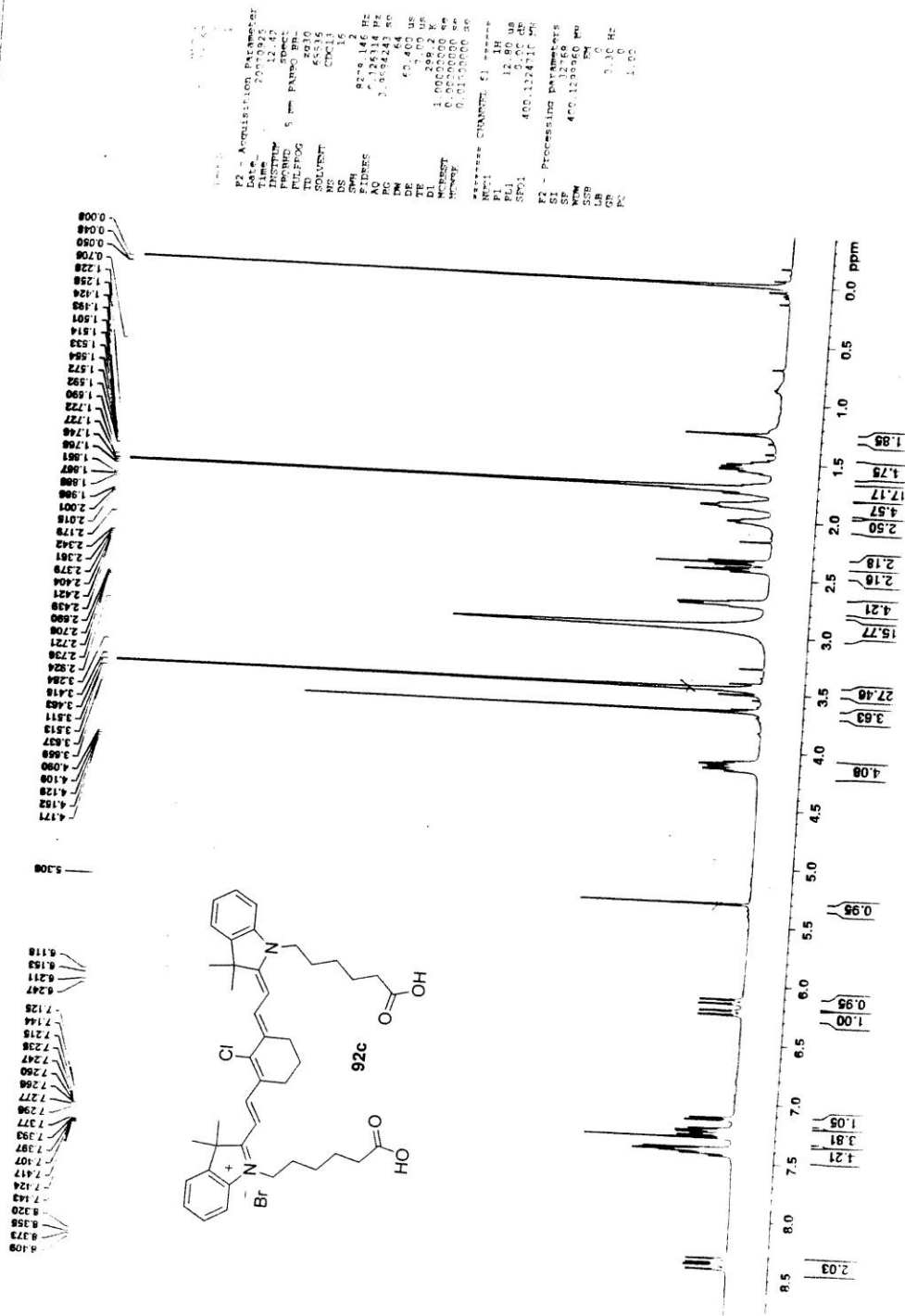


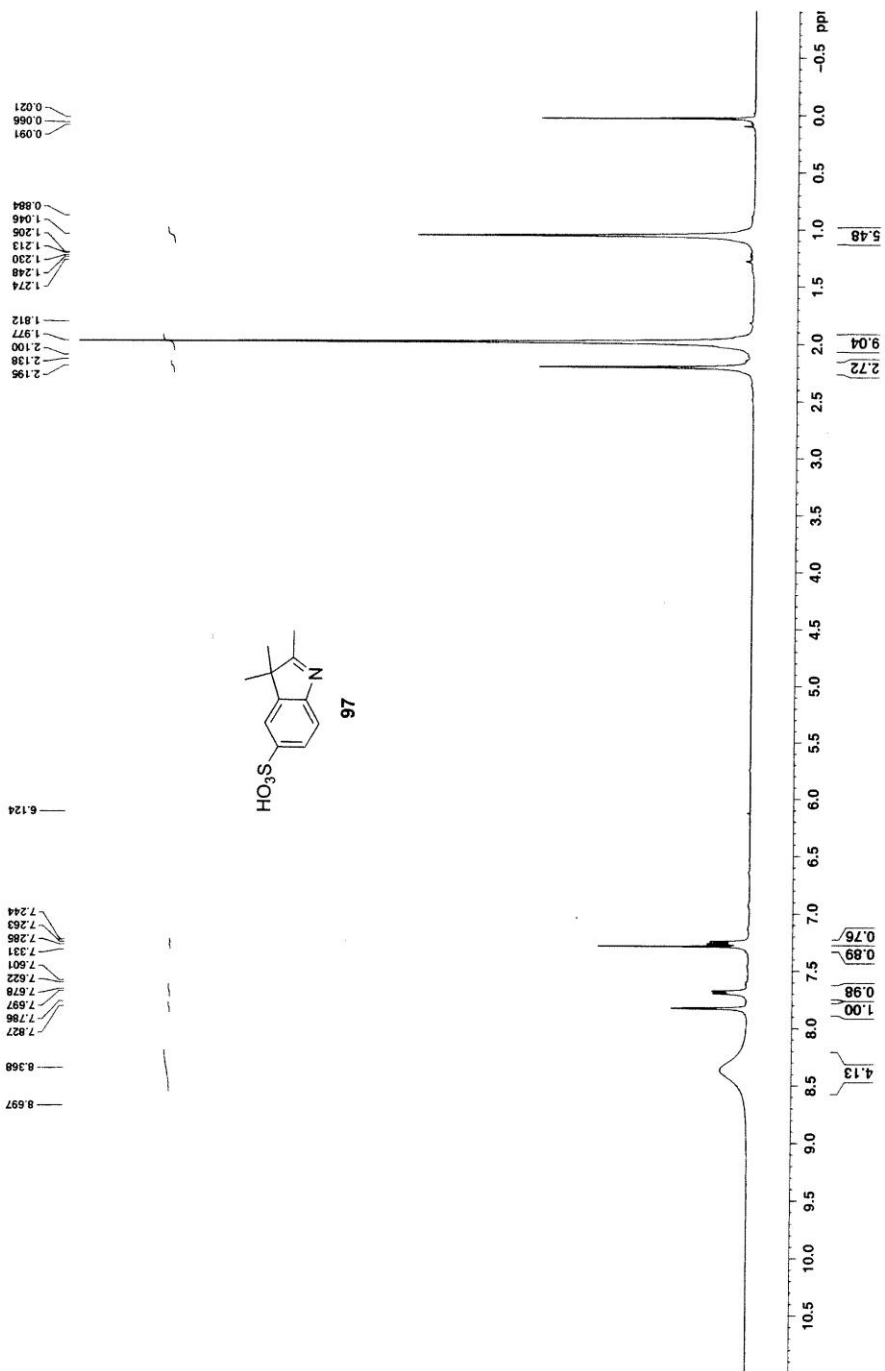


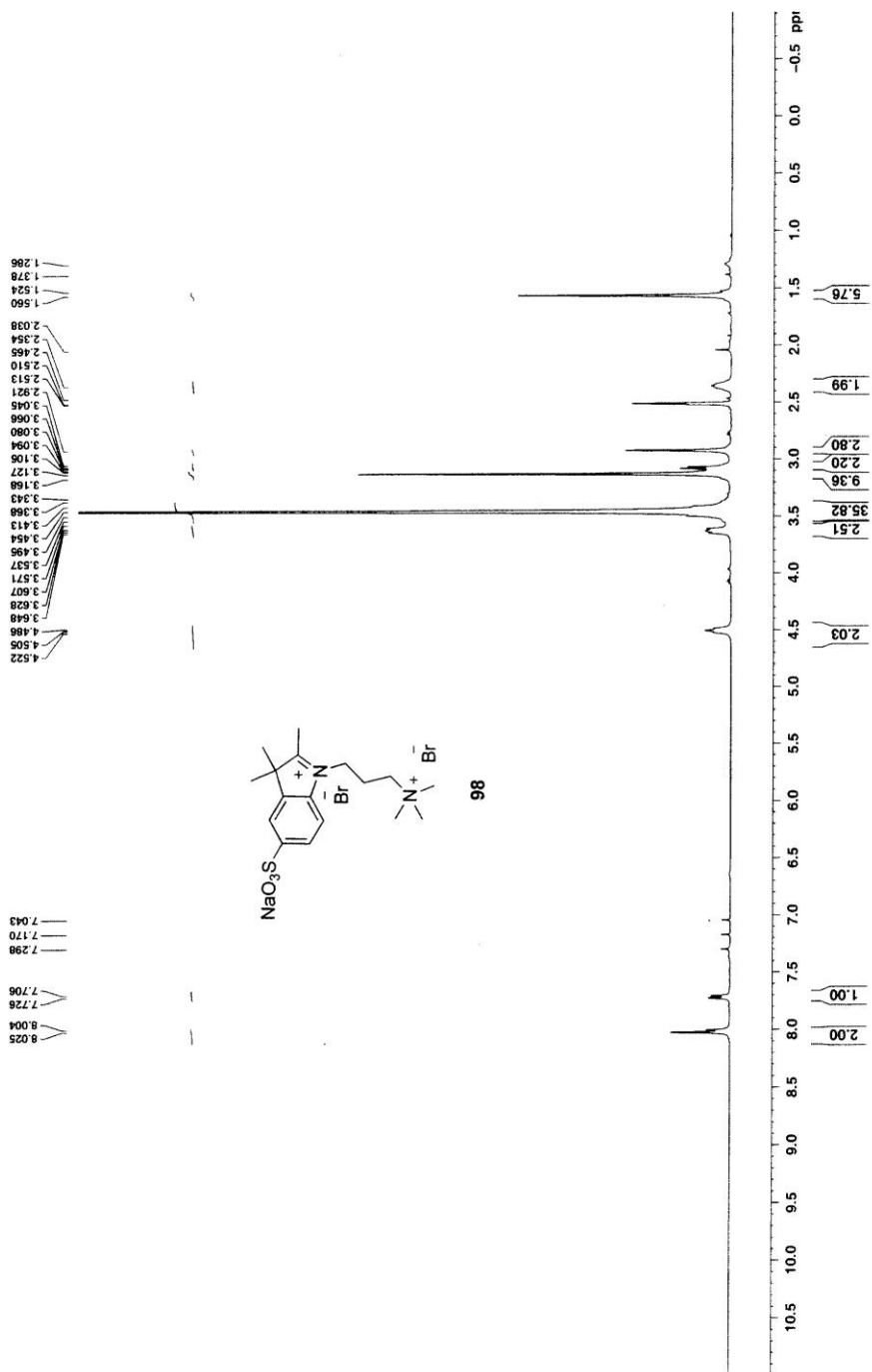


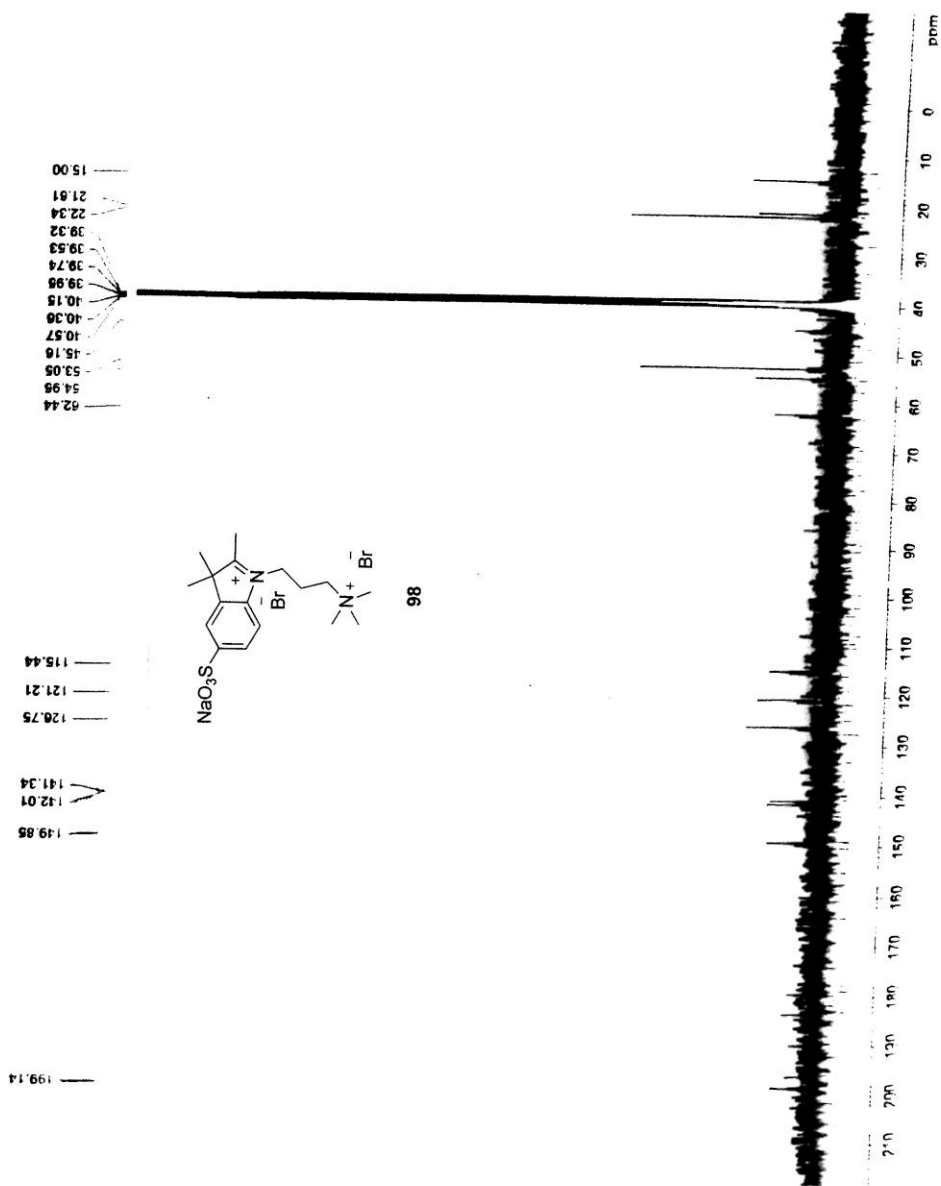
10-43-16

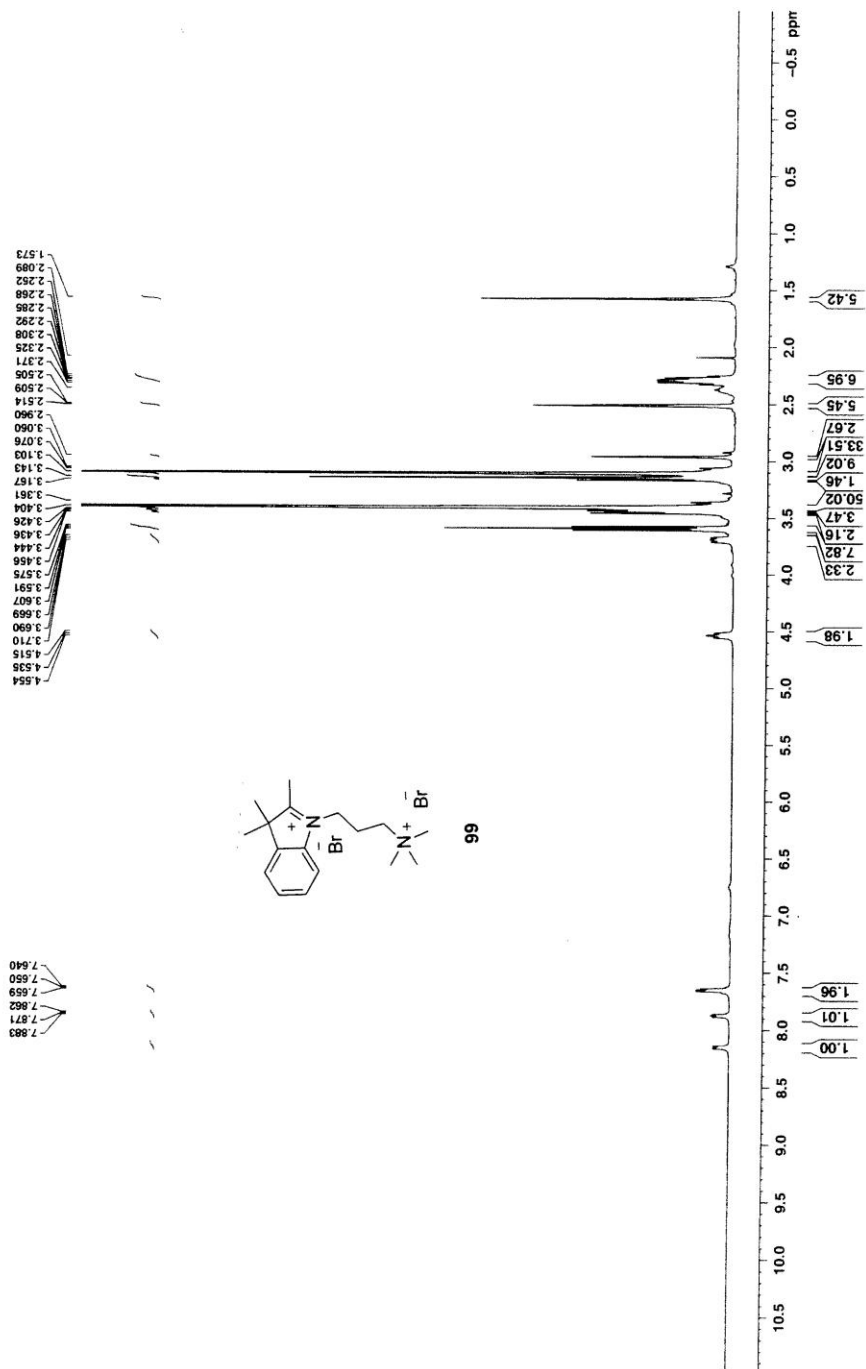


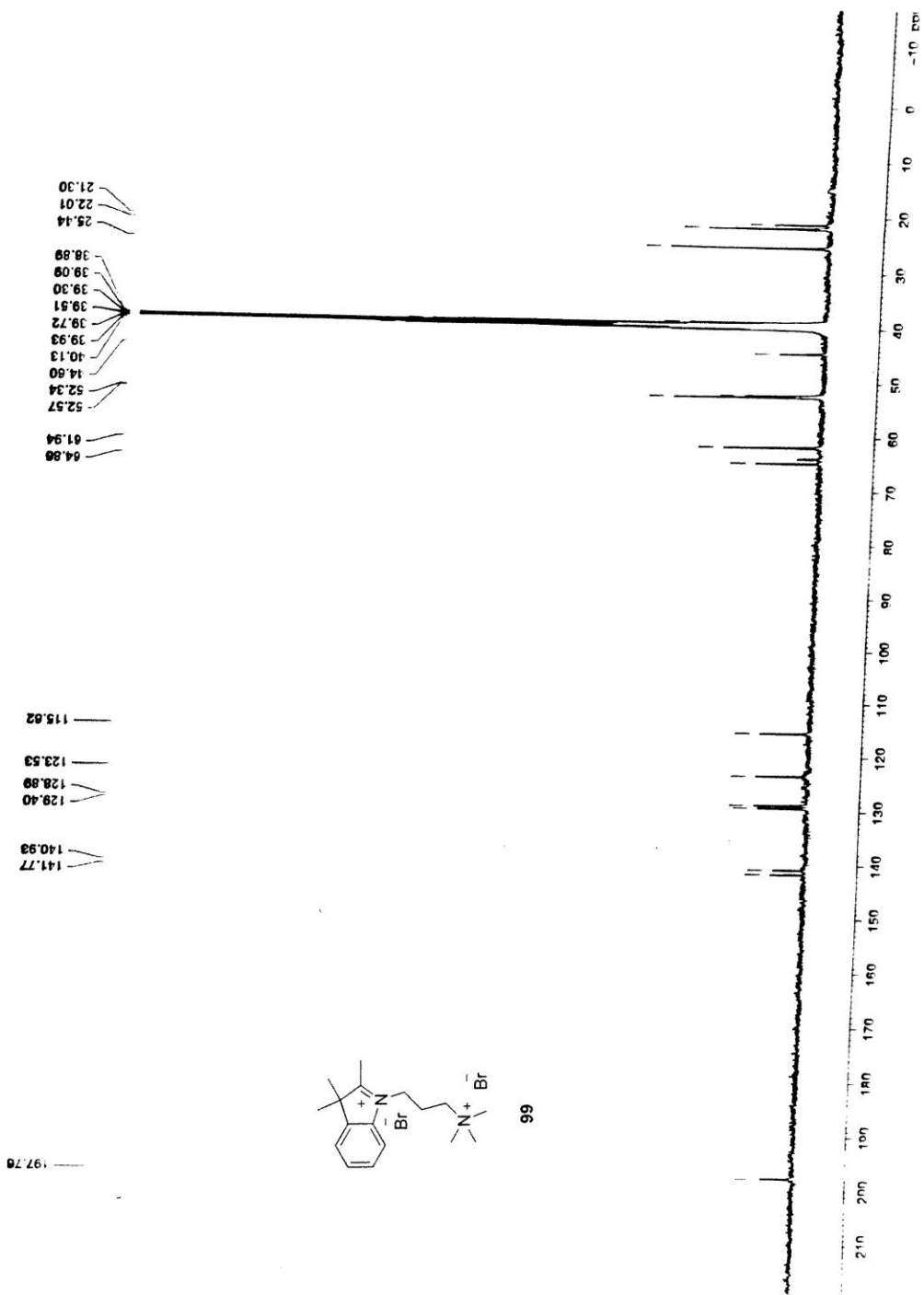


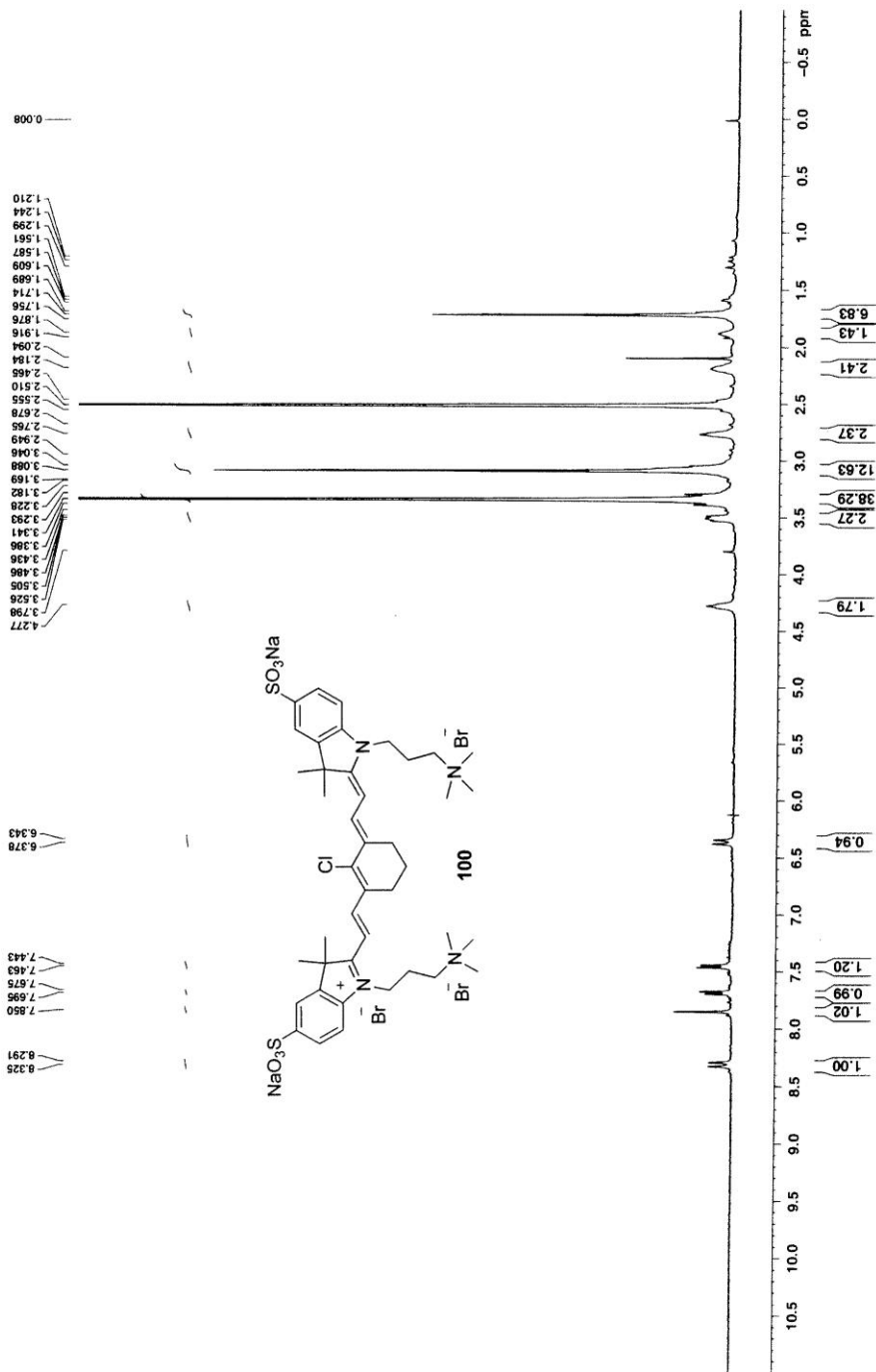


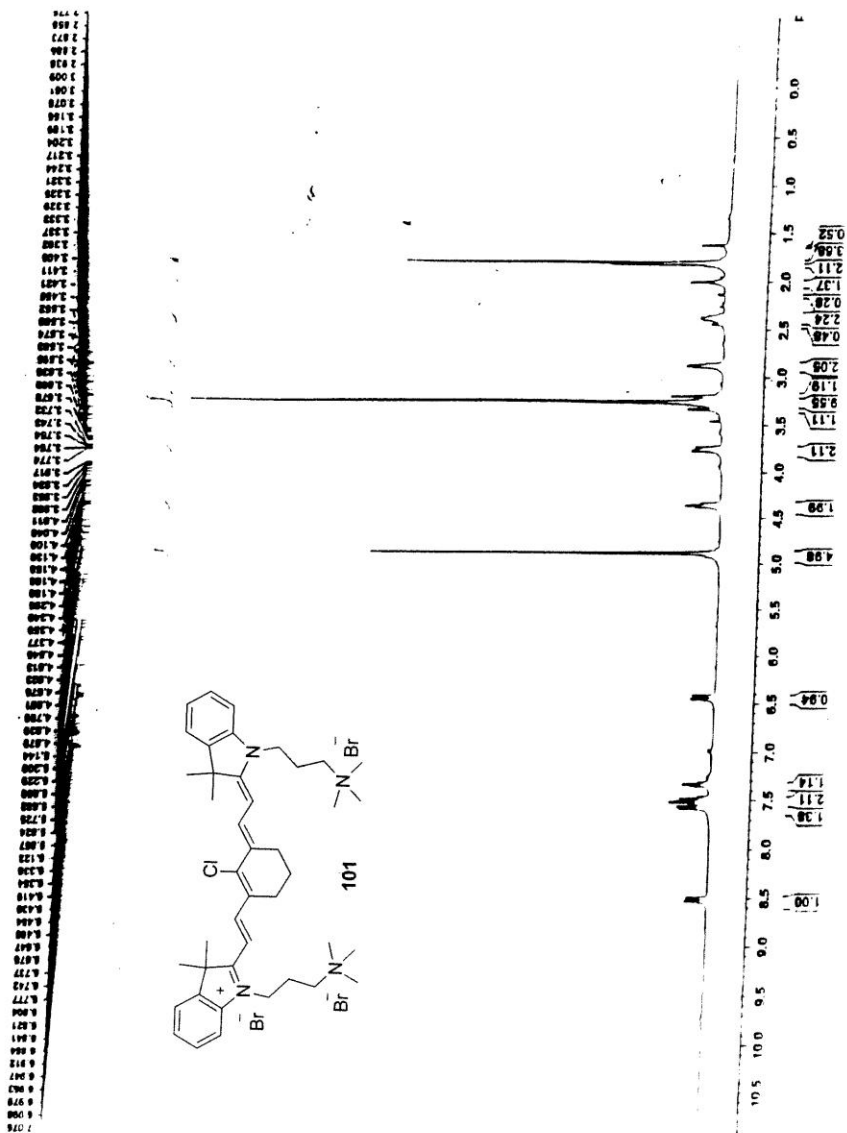












MeOD

22.27
22.51
27.93
28.62
42.20
48.51
48.72
48.83
49.16
49.30
49.67
49.79
50.07
54.01
64.53

102.83
112.56
123.77
126.82
129.32
130.21

142.68
143.38
146.23
151.74

174.45

