NITROOLEFIN FUNCTIONALIZED BODIPY DYES FOR PROTEIN LABELING

A THESIS
SUBMITTED TO THE MATERIALS SCIENCE AND NANOTECHNOLOGY PROGRAM OF THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By

HATİCE TURGUT

January, 2013

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master of Science.

…….

Prof. Dr. Engin U. Akkaya (Principal Advisor)

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master of Science.

…….

Asst. Prof. Dr. Ayşe Begüm Tekinay

I certify that I have read this thesis and that in my opinion it is fully adequate, in scope and in quality, as a thesis of the degree of Master of Science.

…….

Asst. Prof. Dr. Serdar Atılgan

Approved for the Institute of Engineering and Science:

…….

Prof. Dr. Levent Onural

Director of the Institute of Engineering and Science

ABSTRACT

NITROOLEFIN FUNCTIONALIZED BODIPY DYES
FOR PROTEIN LABELING

Hatice Turgut

M.S. in Material Science and Nanotechnology
Supervisor: Prof. Dr. Engin U. Akkaya
January, 2013

Protein labeling has significant importance in terms of visualizing dynamics of proteins, cell-cell interactions, mechanisms of life cycles of proteins, etc. Proteins are labeled by either synthetic or natural molecules with purposes such as analysis of 3D structures, determination of turnover number, covalent modifications and tracking protein-protein interactions. In addition to this, sensing and signalling thiol groups have gained popularity recently. Nitroolefin groups on dyes are good Micheal acceptors which undergo fast and selective reaction with thiol moieties. With this knowledge, in this study, we aimed to obtain derivatives of BODIPY dyes having nitroolefin substituents on its different positions. Nitroolefin functionalization of BODIPY dyes was targeted to result in conjugation of nitroolefins with thiol groups such as those belonging to cysteine residues on proteins. Three different nitroolefin functionalized BODIPY dyes have been designed, synthesized and characterized successfully. Incorporating triethylene glycol (TEG) units onto BODIPYs increased water-solubility of the molecules. To prove bioconjugation of the dyes with proteins, absorbance and emission changes were recorded after reaction with both L-cysteine and Bovine Serum Albumin (BSA) and large spectral changes were obtained. The result suggests that nitroolefin functionalization of BODIPY dyes is a promising way to sense biological thiols and hence labeling proteins having thiol groups.

Keywords: BODIPY, protein labeling, nitroolefin, thiol, cysteine, Bovine Serum Albumine (BSA), dialysis

ÖZET

PROTEİN ETİKETLEME AMAÇLI
NITROOLEFIN FONKSIYONLANDIRILMIŞ BODIPY BOYALARI

Hatice Turgut

Yükek Lisans, Malzeme Bilimi ve Nanoteknoloji Programı
Tez Yöntecisi: Prof.Dr. Engin U. Akkaya
Ocak, 2013

Protein etiketleme, protein dinamiklerini gözlemleme, hücrelerin birbirleriyle etkileşimleri, proteinlerin yaşam döngülerinin mekanizmaları ve benzeri konular açısından oldukça önem taşımaktadır. Proteinler, yapay veya doğal moleküllerle, 3 boyutlu yapı analizleri, kovalent modifikasyonlar, protein-protein etkileşimlerini belirleme, devir sayısı tayini gibi amaçlarla etiketlenirler. Buna ek olarak, tiyol gruplarının algılanması ve sinyalizasyonu da son zamanlarda popülerlik kazanmıştır. Boyaların üzerindeki nitroolefin grupları, tiyol gruplarıyla hızlı ve seçici reaksiyon veren iyi Micheal akseptörleridir. Bu bilgiye dayanarak, bu çalışmada, değişik pozisyonlarında nitroolefin bulunan BODIPY türevleri elde etmeyi hedefledik. BODIPY boyalarının nitroolefin fonksiyonlandırılmasında, nitroolefin gruplarının proteinlerin üzerindeki sistein gibi tiyol içeren gruplarla konjugasyon yapmasını amaçladık. Üç farklı nitroolefin fonksiyonlandırılmış BODIPY boyaları başarıyla dizayn edildi, sentezlendi ve karakterize edildi. BODIPY üzerine trietilen glikol ünitelerinin eklenmesi de bu moleküllerin suda çözünülürlüğünü artırdı. Boyaların proteinle biyokonjugasyon yaptığını kanıtlamak için, hem L-sistein hem de Bovin Serum Albumin (BSA) ile boyaların reaksiyonu sonrası absorbans ve ışıma değişimleri kaydedildi ve spektral karakteristiklerde geniş değişimler elde edildi. Sonuçlar, BODIPY boyalarının nitroolefin fonksiyonlandırılmasının, biyolojik tiyollerin algılanması ve dolayısıyla üzerinde tiyol grupları bulunduran proteinlerin etiketlenmesi açısından gelecek vaad eden bir yol olduğunu göstermektedir.

Anahtar Kelimeler: BODIPY, protein etiketleme, nitroolefin, tiyol, sistein, Bovin Serum Albumin(BSA), dializ

Dedicated to myself...

ACKNOWLEDGEMENTS

Firstly, my greatest thanks are for Prof.Dr. Engin Umut Akkaya for accepting me to his group and giving me chance to conduct my studies in his laboratory. I am grateful to him for listening to me all the time and trying to understand me about everything. I learnt how a person could be a really good scientist from him and I will never forget his personality and energy for life and success. I also would like to thank Prof.Dr. Mahinur Akkaya for supporting me to join this research group.

I am grateful to Dr. Murat Işık for his guidance and support in my studies. I would like to thank him deeply for being both a good teacher and a friend to me. His intelligence, knowledge and experinence influenced me so much and I will value it all throughout my life.

I would like to thank İlke Şimşek for her partnership in my studies and for her friendship.

I herein present my thanks to Sündüs Çakmak for her endless help, sincerity, generosity and smile that she kept all the time without any exception.

I would like to express further thanks for Safacan Kölemen, Ruslan Guliyev, Gizem Çeltek, Yusuf Çakmak, Elif Ertem, Ahmet Bekdemir, Ahmet Atılgan, Fatma Pir and Özge Yılmaz for their help and support. I also thank the rest of SCL (Supramolecular Chemistry Laboratory) members for everything they shared with me. I learnt a lot form them.

I thank Yusuf Keleştemur and Gözde Uzunallı for their generous help and support during my experiments.

I would like to thank UNAM (National Nanotechnology Research Center) for their financial support.

I thank Zeynep Erdoğan and Neslihan Arslanbaba for their sincerity. I also thank all the students and teachers at UNAM for the great ambiance. It was wonderful to work with them.

Lastly, my deepest thanks are for my parents and my sister for their love, support and patience all throughout my studies.. I thank my niece Melis for giving me energy, courage, love and pleasure during my time of research. I owe them a lot…

LIST OF ABBREVIATIONS

BODIPY: Boradiazaindacene

DCM: Dichloromethane

TLC: Thin layer chromotography

DCE: 1,2-Dichloroethane

GFP: Green Fluorescent Protein

NMR: Nuclear Magnetic Resonance

THF: Tetrahydrofuran

DMF: Dimethylformamide

DDQ: 2,3-Dichloro-5,6-Dicyano-1,4-benzoquinone

TEG: Triethylene glycol

BSA: Bovine Serum Albumin

HOMO: Highest occupied molecular orbital

LUMO: Lowest unoccupied molecular orbital

PeT: Photoinduced electron transfer

ICT: Internal Charge Transfer

rxn: reaction

Cys: cysteine

PBS: Phosphate buffered saline

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

DMSO: Dimethylsulfoxide

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Protein Labeling

Proteins belong to a class of major biomolecules existing in all living species. Their functions and interactions affect the whole organisation of the life. The term "protein labeling'' stands for marking proteins to follow their mechanisms. Protein labeling has great biological importance in terms of visualizing cell-cell interactions, dynamics of proteins, mechanisms of life cycles of proteins, etc. The characterization of proteins in complex mixtures, as well as analysis of their biosynthesis, processing, intracellular mechanisms and degradation, generally requires the proteins to be labeled either *in vivo* or *in vitro*. Labeled protein is then isolated and analyzed by electrophoretic techniques[1].

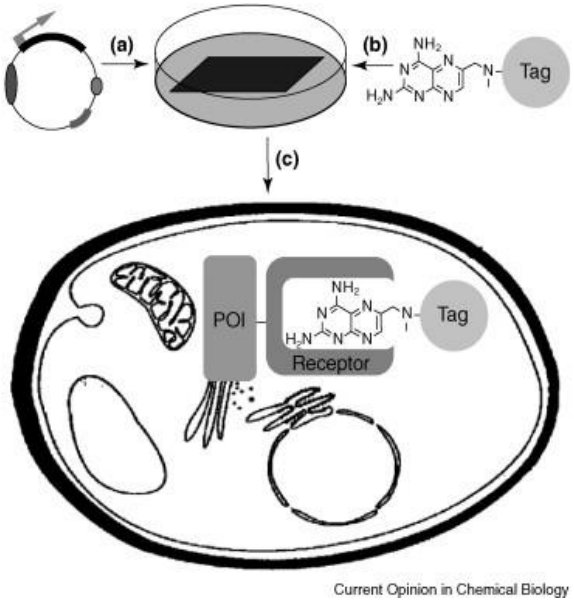
Labeling strategies include covalent or noncovalent attachment of the label to the target and since it is specific to the process, the strategy should be considered carefully in order not to disrupt the structure of the molecule. There are some factors affecting the choice of labeling strategy: Some proteins are affected negatively from fusion to another protein or peptide, some techniques are only suitable to surface proteins and others are for intracellular ones, different applications require different probes, techniques using disulfide bridges are influenced by the redox condition of the cell, etc.[2].

The purposes of protein labeling includes lowering detection limits, obtaining turnover number, covalent modifications, analysis of 3D structures (Structure determination by NMR via radioactive labelling) and protein-protein interactions, immunological tests, affinity tests by nonradioactive labeling[2].

1.1.1 *In vivo* labeling

Metabolic labeling is a strategy to label nucleic acids or proteins in a cell by culturing them with labeled nucleotides or amino acids. Cell culture in media containing labeled nucleic acids or amino acids results in all DNA, RNA or proteins becoming labeled via DNA replication, translation and protein turnover. The nucleic acid or protein of interest can later be purified for further experiments. The advantage of performing metabolic labeling is the consistent labeling of all nucleic acid or protein species. On the other hand, metabolic labeling can be toxic, depending on the type of label used, and the number of metabolic labeling reagents is not as broad as those for *in vitro* methods[3].

Protein labeling with fluorescent probes or affinity reagents has facilitated *in vitro* studies of protein structure, dynamics and interactions of them with each other[4]. Since these traditional methods require purification of protein, chemical labeling, repurification and some invasive methods such as microinjection to reintroduce into cells, they are not adequate for *in vivo* studies. The general strategy of *in vivo,* site-specific protein labeling is summarized in figure 1.



a) Living cells are transfected with DNA encoding a protein of interest which is fused to a receptor domain **b)** a cell-permeable small molecule probe consisting of a ligand coupled to a detectable tag is added to cell growth medium **c)**Analysis of protein function in living cells via fluorescent microscopy or other detection methods.

Figure 1: General strategy of in vivo, site-specific protein labeling. Picture adapted from ref 5 with permission. Copyright © 2012 Copyright Clearance Center, Inc.

There are both direct and indirect approaches to chemically modify proteins *in vivo*[5].

Most of the methods of *in vivo* labeling depends on ligand-receptor interactions. General ligand-receptor pairs include biotin-avidin system, hapten-antibody, various enzyme-antibody combinations, nitrilotriacetate(NTA)-oligohistidine sequence, biarsenical fluorophores that bind to cysteine-rich peptide sequences[5]. The biarsencial ligand/tetracysteine motif interaction studied by Roger Tsien's laboratory is the prototypical system for the specific chemical labeling of proteins *in vivo* (Figure 2a)[6-10]. This strategy depends on the subnanomolar affinity between a short tetracysteine peptide (CCXXCC, where X is any amino acid except cysteine) and a biarsenical compound such as 4′,5′-bis(1,3,2-dithioarsolan-2-yl)fluorescein (FlAsH)[5]. In this method, the FlAsH dye is administered to the cells in the presence of an excess of 1,2-ethanedithiol (EDT) which outcompetes

endogenous proteins with closely spaced cysteine pairs. Therefore it minimizes non-specific binding and toxicity. The target protein of interest is expressed with the tetracysteine motif[5]. Besides the green FlAsH biarsenical, red (ReAsH)[8], blue (ChoXAsH), and a biarsenical derivative of nile red have been synthesized[11]. In addition to direct chemical labeling, indirect approaches for modification of proteins also exist. Specific incorporation of unnatural amino acids based on suppressor tRNA technology[12-14] is one of these. Protein splicing[15-17] technique has also been adapted to protein labeling.

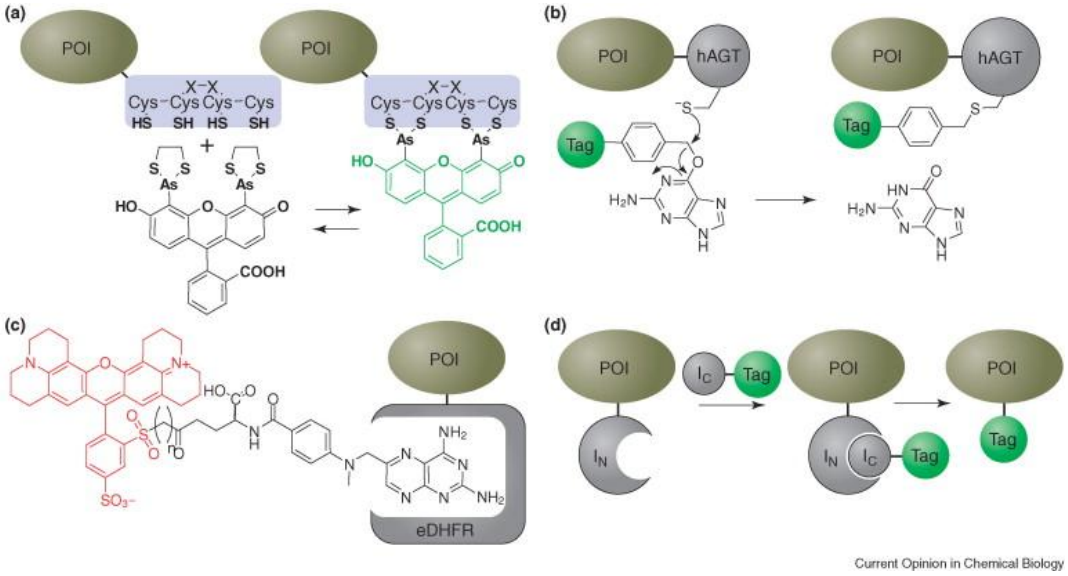


Figure 2: Some methods of labeling fusion proteins chemically in vivo

(a) FlAsH. Binding of a biarsenical fluorescein derivative to a short tetracysteine peptide fused to the protein of interest (POI) **(b)** Human *O6*-alkylguanine DNA alkyl transferase (hAGT) is labeled covalently with benzyl guanine derivatives. **(c)** Schematic representation of methotrexate-Texas Red™ dye bound to an *E. coli* dihydrofolate reductase (eDHFR) protein. **(d)** Split intein labeling[5]. Picture adapted from ref 5 with permission. Copyright © 2012 Copyright Clearance Center, Inc.

1.1.2 *In vitro* labeling

Chemical methods of protein labeling include the covalent attachment of labels to amino acids in laboratory environment. These groups, react with specific moieties on distinct amino acids. A few are also available that nonspecifically react with any amino acid at C-H and N-H bonds. Enzymatic methods are also used to label proteins and nucleic acids. These methods require the related polymerases, ATP and labeled amino acids or nucleotides. The expression of labeled proteins by *in vitro* translation can be difficult due to the requirement for proper protein length, folding and post-translational modifications that some commercial kits are unable to provide[3].

1.1.2.1 Protein Labeling Procedure

Firstly, dye & protein solution is prepared. For lysine-sensitive labeling, pH should be greater than 8; for cysteine-directed labeling, pH should be around 7 and concentration greater than 50 μm is acceptable. Then conjugation reaction takes place by stirring, under protection from light and careful monitoring considering time and temperature. To remove excess dye; gel filtration, HPLC, dialysis or precipitation is done. For characterization of the conjugate, absorbance, fluorescence spectra could be obtained or mass spectroscopy could be useful in order to determine concentration, labeling ratio, label attachment site(s). Lastly, for biological testing, SDS, native gel denaturation experiments, etc. could be performed[2].

1.1.3 Bioconjugation

Bioconjugation is the process of coupling two biomolecules via a covalent bond. Amine coupling of lysine amino acid residues, sulfhydyrl coupling of cysteine residues and photochemically initiated free radical reactions are common types of bioconjugation reactions. The product of bioconjugation is named as

“bioconjugate’’. Coupling of a small molecule such as biotin or a fluorescent dye to a protein or protein-protein conjugation such as antibody-enzyme conjugation are very common reactions of this type. Other less common molecules used in bioconjugation are oligosaccharides, nucleic acids, synthetic polymers such as polyethylene glycol[18].

1.2 Protein Labeling Techniques and Probes

Various probes and techniques exist in the literature that are useful in a number of areas such as chemical, biochemical analysis, biotechnology and immunodiagnostics in terms of protein labeling[19].

1.2.1 Chemical Labeling Techniques

There exists both covalent and noncovalent techniques to label proteins. In covalent techniques, chemical bonds are formed between the label and the protein whereas in noncovalent methods, there occurs a noncovalent interaction between the label and the target.

1.2.1.1 Covalent Labeling

One method to label cell-surface proteins is the ACP-PPTase system[20]. In this system, acyl carrier protein (ACP) undergoes post-translational modification by phosphopantetheine transferase (PPTase) as depicted in figure 3 which provides the transfer of 4′-phosphopantetheine from coenzyme A(CoA) to a serine residue of ACP[21]. This system is not commonly used to make labeling in living cells due to the activity of endogenous enzymes, limitation to cell-surface proteins and difficulty of cell permeability of the probes[20] .

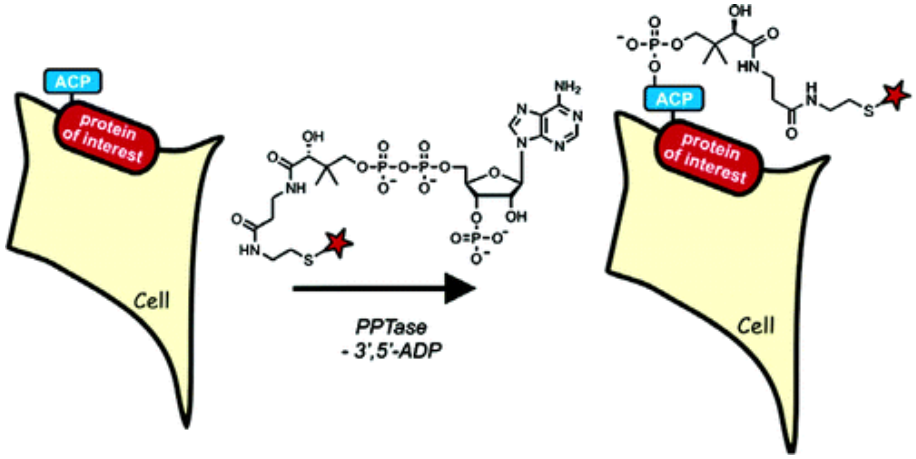


Figure 3: Labeling of ACP proteins on cell surface Picture adapted with permission from reference 22. Copyright © 2004, American Chemical Society

Secondly, biotin ligase has been used to covalently label a short, acceptor peptide which can react with hydrazide or hydroxylamine fluorescent dyes[23] and this technique could be used to biotinylate cell-surface proteins for labeling with streptavidin conjugates[24]

Another covalent labeling system depends on self alkylation reaction of human O^6-alkylguanine transferase (hAGT) to label hAGT fusion proteins with a type of fluorescent O^6-benzylguanine substrates[25,26] as depicted in figure 4.

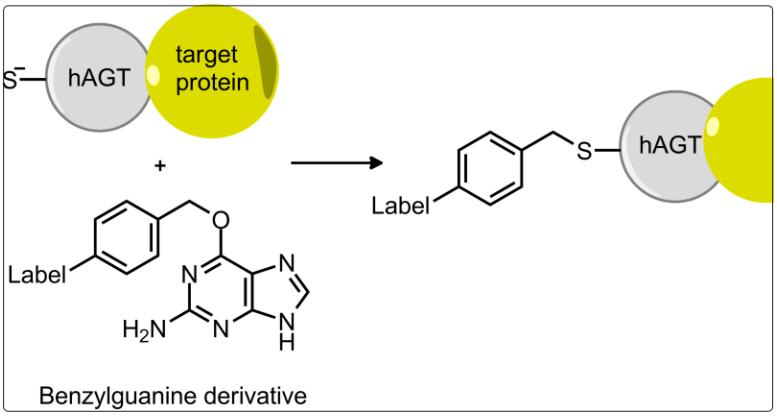


Figure 4: Covalent labeling techniques. hAGT system uses the enzyme activity to conjugate molecules containing both the label and the enzyme's substrate[20]

1.2.1.2 Noncovalent Labeling

There exists several noncovalent techniques which do not include endogenous enzymes or substrate problems faced in covalent ones. Tetracysteine-bioarsenical system that uses a nonendogenous, high affinity system is one of them. There is interaction between the enzyme dihydrofolate reductase (DHFR) and methotrexate to label DHFR fusion proteins by using a Texas-Red conjugate of methotrexate[27]. An improved system including bacterial DHFR and trimethoprim derivatives has also been published as shown in the figure 5.

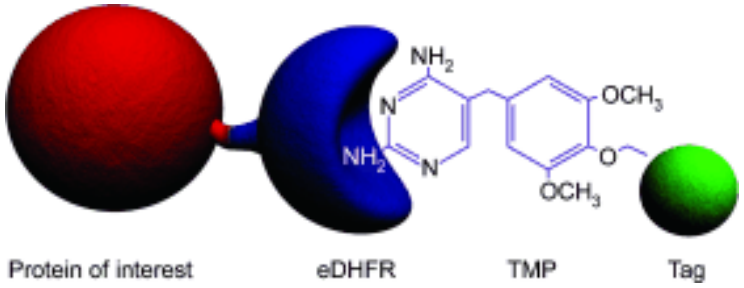


Figure 5: Schematic representation of TMP-eDHFR labeling system

TMP is covalently attached to a fluorescent tag and bound by a chimeric fusion to eDHFR. Fluorescent TMP rapidly diffuses into mammalian cells when added into the culture medium. The orthogonal interaction of TMP–eDHFR creates specific fluorescent labeling of the protein. Picture adapted from reference 28 with permission. Copyright © 2007 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

A single-chain antibody (scFV) that binds to a fluorescein conjugated hapten constitutes a specific receptor-ligand pair[29] . In this process, the protein is labeled on the secretory pathway and provides the estimation of pH in the endoplasmic reticulum (ER) but as a disadvantage, the single-chain antibodies fold poorly in

the cytosol[29]. Nitriloacetate ligands that bind reversibly to short endogenous oligohistidine sequences (NTA-His system) have been applied more recently. These ligands, however, are very weakly fluorescent; they have very low quantum yield though they absorb photons efficiently. Consequently, they could be accepted as good FRET acceptors which is a property used to map the binding site of a fluorescent serotonin receptor antagonist[30]. Furthermore, other peptide-ligand pairs created by phage display selection of peptides that have the ability to bind to Texas red derivatives and can be used for labeling of fusion proteins have also been developed[31,32]. Since Texas-red based dyes can accumulate in mitochondria, it may cause nonspecific binding. Another approach useful to overcome nonspecificity has been proposed. In this approach, a mutant of FK-binding protein 12 (FKBP12 (F36V)) binds to a fluorescent ligand which has a very high affinity towards it and this prevents binding of endogenous proteins endangering many chemical labeling techniques. The method has been used in many different cells by using different FKBP12 proteins and proved as efficient[33].

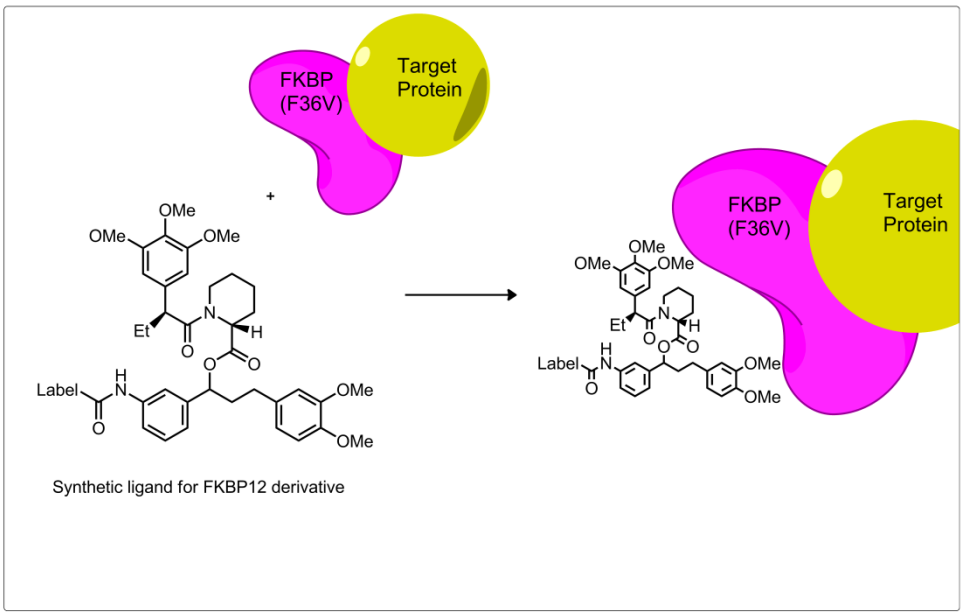


Figure 6: Non-covalent labeling techniques. A ligand binds specifically to a moiety which is conjugated to the target protein[20]

1.2.2 Biological fluorophores

Phycobiliproteins are in this category. They are complexes purified from cyanobacteria and algae. They are highly soluble in water, have large Stokes shifts, very intense emission of light, broad and high absorption of light. R-Phyco-Erythrin and C-Phyco-Cyanine are some examples[34].

Green Fluorescent Protein(GFP) is the major natural protein that is useful in protein labeling. It was first isolated from *Aequorea Victoria* jellyfish (Shimomura,1962)[35] and Tsien's research group has published many developments and they contributed to many other discoveries about GFP[36-42]. Shimomura, Tsien and Chalfie were Nobel prized for their study about GFP[43]. Though GFP has a highly efficiently emitting fluorophore with high-resolution crystals and it is useful as a marker of gene expression and protein targeting[44] it is also claimed that since GFP and GFP-like molecules (FPs) are bulky, they are restricted due to their oligomeric structure and they may be harmful to the 3D structure of the protein to which they are linked[45].

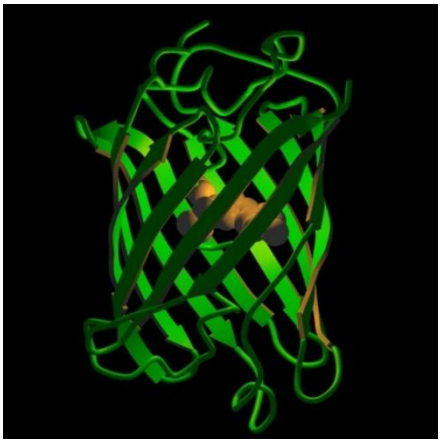


Figure 7: The Green Fluorescent Protein[46]

1.2.3 Luminescence probes for proteins

Luminescent probes have been widely used for protein labeling applications. Noncovalent fluorescent probes, near-IR fluorescent probes, Fluorescent derivatizing reagents reacting with protein at the N-terminus, rare earth ions together with their chealetes, chemiluminescence probes , resonance light scattering probes, nanoparticles and molecular beacons (MBs) are in this category[19].

Fluorescent probes are very useful for labeling due to having the following features: High molar absorbance, high photostability, large Stokes shifts, excellent solubility and stability in water and/or organic solvents, choice to cover visible and far red spectrum, available as free acid form, amine- and thio-reactive derivatives. Free carboxy group could be conjugated to amine groups on proteins, maleimide groups are able to be conjugated to thiol groups and succinimidyl esters are available for amino groups on proteins, DNA, RNA[34].

There are both covalent and noncovalent probes for protein detection. Dyes used as noncovalent probes are generally anionic dyes. These can bind to the residues of proteins carrying positive charges and pH is thus important[19]. Nile red[47] and Sypro dyes[48] are typical dyes in this category. Fluorescence quenching of dyes can also be used for protein detection[19]. For instance, Eosine Y gives strong fluorescence at pH 3.1 which is quenched after binding to proteins which could be used to detect proteins at a level of 1-100 μg[49,50].

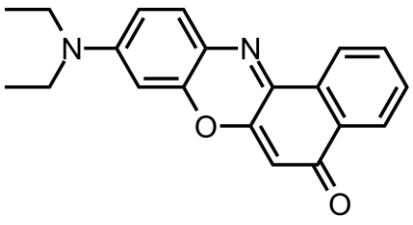


Figure 8: Structure of Nile Red

Fluorescence in near infrared region (600-1000 nm) is advantageous over ultraviolet and visible regions[19]. Low levels of background interference, reduced scatter, reduction in sample photodecomposition when longer wavelengths are used, excitation by cheap, stable, and compact diode lasers, ability to penetrate through the skin and tissues are some properties that could be observed in NIR[51,52]. NIR dyes have good photopysical properties such as high quantum yield, large Stokes shift, high molar absorptivity. There are three types of dyes in this category recorded in the literature[19] which are namely, Cyanine dyes[53-56], Squaraine dyes[57,58], Thiazine and Oxazine derivatives[59,60].

Proteins have both N-terminus and C-terminus. Since C-terminus is not very active, it is not used in labeling applications very often. Only proteins having Phe and Trp are known to exibit natural fluorescence and the others should be derivatized or labeled by some reagents. Since N-terminus is more reactive, there exists many fluorescent derivatizing reagents reacting with proteins at the N-terminus[19]. Ninhydrin[48,49], fluorescein-5-isothiocyanate(FITC)[63,64], naphthalene-2,3-dialdehyde/cyanide (NDA)[65] are some of the reagents used in this category.

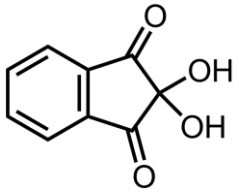


Figure 9: Structure of Ninhydrin

Rare earth ions, due to having luminescence characteristics such as narrow spectral width, large Stokes shift, long luminescence lifetime, etc. and comparable sizes to inorganic cations Ca^{2+} and Mg^{2+} are used as probes for proteins. Since they may have low sensitivity themselves, some rare earth ions (especially Eu^{3+} and Tb^{3+}) are more useful when bound to chelates such as β-diketone[19]. Phenantroline derivatives[66] and salicyclic acid derivatives[67] are in this category. A phenantroline derivative, namely, 4,7-bis(chlorosulfophenyl)-1,10-phenantroline-2,9-dicarboxylic acid (BCPDA) was a good chelator of Eu^{3+} and it was used to label BSA[68].

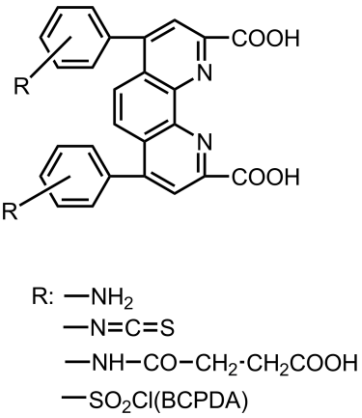


Figure 10: Structure of phenantroline derivatives

Resonance light scattering(RLS) is a phenomenon of elastic-light scattering[19]. RLS intensity of some organic compounds can be increased by biopolymers or inorganic ions due to supramolecular aggregations and some reactions and thus, the application of RLS could be used for detection of proteins[19]. Proteins are positively charged when pH is below their isoelectric point(PI) and they are more prone to aggregation on acidic dyes. Consequently, RLS intensity of dyes could be enhanced by the addition of proteins[19]. Porphyrins[69-73] and acidic dyes are under the heading of organic dyes in this phenomena. Anion surfactants, dye-nonionic surfactants and resonance double scattering method are the other tools used for detection of proteins depending on RLS method[19].

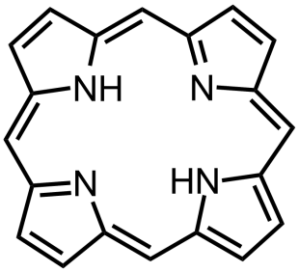


Figure 11: Structure of the simplest porphyrin

Chemiluminescence is another phenomenon which is made use of for detection of proteins. When a molecule relaxes to the ground state from an excited state, it

emits light which is called ‘’luminescence’’. If the energy to obtain the excited state is gained by a chemical reaction, it is called ‘’chemiluminescence’’[74]. Derivatization of proteins with a chemiluminogenic label could be used for labeling applications. Acylhydrazides, acridinium derivatives, dioxetanes, coelenterazines and peroxyoxalic derivatives are the five classes utilized as chemiluminescent probes[19,75].

Molecular beacons[76] and nanoparticle probes are the other types used as for protein detection. Nanoparticle probes could be subdivided as latex nanospheres[77], luminescent quantum dots (Qdots) (semiconductor nanocrsytals)[78,79] and optically active metal nanoparticles[80]. Though nanorparticles have unique physical and chemical properties especially useful for analytical chemistry, their application is still developing and will offer new oppurtuinites in the future[19].

1.2.4 Biotin and Enzymes

When one of the groups or molecules of the two having high affinty towards each other is attached to a protein, the other affinity partner makes it possible to detect the protein. The best technique with this purpose is the biotinylation of proteins[2]. Biotin is an indirect label which is popular due to is versatility for detection, purification, and amplification systems[34] and its extraordinarily strong binding to avidin, streptavidin or NeutrAvidin Protein[3]. It offers one of the strongest non-covalent interactions between a protein and a ligand and stands for one of the most compatible labels due to its relatively small size (244.3 Da). General formula of biotin derivatives and schematic representation of biotinylation are shown in the following figure.

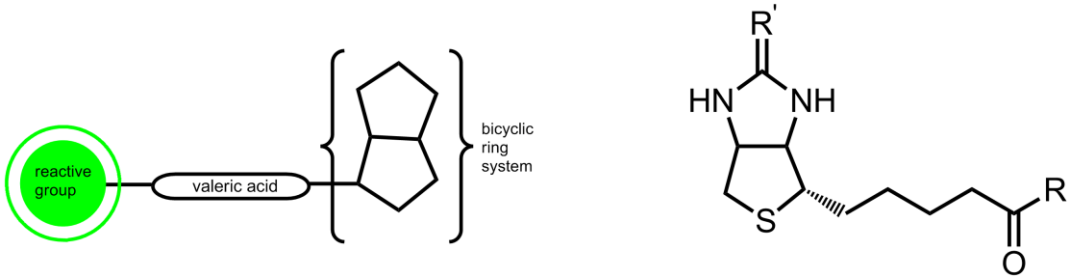


Figure 12: Schematice representation of biotinylation reactive and general formula(on the left) and general formula of biotin derivatives(on the right)[2]

Labeling proteins or nucleotides, namely biotinylation process, could be done both by chemical and enzymatic ways of which chemical ways are the most preferred. There is a biotinyl group, a spacer arm and a reactive group which is responsible for the attachment to target functional groups on proteins. Spacer arms function to link the biotin molecule to a reactive group which interacts with certain functional groups on the amino acids of the target protein[3].

There exists a wide range of biotinylation reagents with different reactive groups commercially available. Most common reactive groups with their respective targets on proteins can be listed as follows[3]:

- N-hydroxysuccinimide(NHS) and sulfo-N-hydroxysuccinimide (Sulfo-NHS)- primary amines
- Primary amines in combination with EDC-carboxyls
- Hyrdrazines and alkoxyamines- glycoproteins
- Maleimide, iodoacetyl gropus or pyridyl disulfides- sulfhydyrls

Enzyme labels are significantly larger than biotin and require the addition of a substrate to generate a chemiluminescent, chromogenic, or fluorescent signal that can be detected by different approaches. Due to multiple types of signal output, signal amplification and the wide selection of enzyme-labeled products(especially antibodies) enzymes are preferred. Horseradish peroxidase (HRP), alkaline phosphatase (AP), glucose oxidase and β-galactosidase are commonly used

enzymes for labeling. Enzyme probes can be used via conjugation to antibodies, streptavidin or other target proteins by multiple mechanisms, including glutaraldehyde, reductive amination following periodate oxidation of sugars to reactive aldehydes or by using heterobifunctional crosslinkers such as Sulfo-SMCC[3].

1.2.5 Radioactive labeling

For radioactive labeling, the protein should be purified. Biological activity is affected from radiolysis due to effects in 3D structure and radicalic reactions. ^{3}H, ^{125}I, ^{35}S, ^{32}P, ^{14}C are some important radioactive isotopes. There is only isotope difference between natural amino acids and those labeled with ^{3}H and ^{14}C. Half–life of ^{32}P is rather short. Labeling with this isotope is significant in metabolic regulation studies. Labeling with ^{35}S is important in research of newly synthesized proteins[2].

1.2.6 Novel methods

A recently developed ligand-directed tosyl (LDT) based technique allows attachment of several synthetic probes to proteins in living cells[81].This is an advantageous method in terms of its simplicity and versatility[82]. During the labeling reaction, a nucleophile from the target surface attacks the probe's electrophilic center to form a covalent adduct, with the release of the ligand that is attached to the other side of the tosyl leaving group. A series of benzenesulfonamide containing probes designed for three different detection modalities (fluorophore, biotin affinity tag or 19F NMR probe) were synthesized in order to demonstrate the efficiency of this technique. The fluorescent probes were efficient in labeling carbonic anhydrase II (CAII) in test tubes, in human red blood cells (RBCs) and in *living animals*[83].

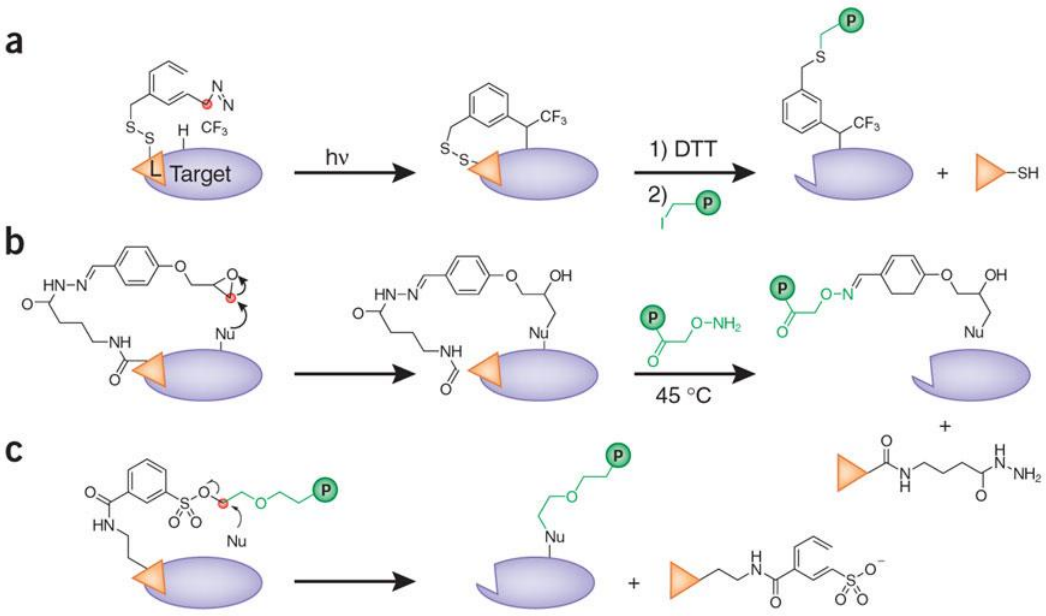


Figure 13: Some recently developed ligand-directed protein labeling strategies

a)A disulfide linker cleaved by DTT and subsequently modified by probe-containing electrophiles such as iodoacetylated dansyl group **b)**Hydrazone linker converted to oxime with the restoration of enzyme activity **c)** LDT chemistry. P: probe, L: ligand, Nu: nucleophile. Picture adapted from reference 82 with permission. Copyright © 2012 Copyright Clearance Center, Inc.

HaloTag is a new protein tagging system that is applicable to be linked onto a single genetic fusion either in solutions or in living cells or in chemically fixed cells. HaloTag is a protein which is modified haloalkane dehalogenase designed to covalently bind to synthetic ligands (Halo-Tag ligands). The synthetic ligands are based on a chloroalkane linker attached to avariety of useful molecules, such as fluorescent dyes, solid surfaces. A highly specific covalent bond formation occurs between the protein tag and the chloroalkane linker. This system is utilized for cell imaging and protein immobilization[84].

Development of affinity probes for protein labeling based on an epoxide reactive group has been achieved. Epoxide functionality possesses the special combination of stability and reactivity which makes it stable toward proteins in solution but reactive on the protein surface outside the active site (proximity-induced reactivity). Highly efficient and selective labeling of purified HCA II (human carbonic anhydrase II) was achieved by this way[85].

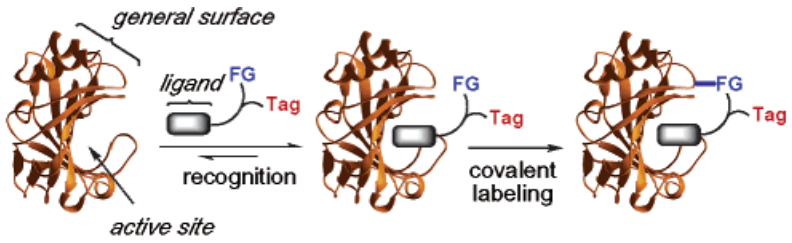


Figure 14: A concept of an affinity labeling probe. Labeling may occur either at the active site (activity site affinity label) or outside the active site (general affinity label). FG: functional group. Picture adapted with permission from reference 85. Copyright © 2003, American Chemical Society

Based on the ‘’turn-on’’ fluorescence intensity phenomena, there exists a photoactive yellow protein labeling (PYP) system in the literature. Photoactive yellow protein is isolated from purple bacteria. In this method, fluorescent probes have been develeoped which begin to give emission when attached to PYP which makes a good probe for protein detection and applicable for research to determine biological functions[86].

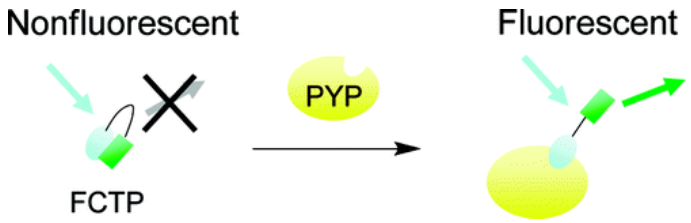


Figure 15: Principle of fluorogenic labeling system based on PYP.Picture adapted from reference 86 with permission. Copyright © 2009, American Chemical Society

There exists many other materials available to be used as probes for protein detection or startegies for protein labeling in the literature, each having its own advantages and disadvantages[87-93].

1.3 BODIPY Dyes

Boradiazaindacene (BODIPY) dyes have gained popularity among the other known fluorescent dyes within the last decade. They have been the choice of many chemists, biochemists and other scientists. Kreuzer and Treibs were first to synthesize BODIPY dyes in 1968[94]. These dyes have been developed and proposed as useful in many areas such as photodynamic therapy, light harvesting systems, energy transfer cassettes, molecular logic gates and sensitizers for solar cells.

BODIPY dyes offer many advantegous properties. Firstly, they have high molar extinction coefficients. Depending on the structure and environmental conditions, they offer strong absorption and fluorescence spectra in the visible region together with high quantum yields[95]. Secondly, they are relatively stable dyes, not sensitive to solvent polarity or pH of the solution and could be soluble in both organic and inorganic solvents depending on the functionalization of the available positions. Furthermore, BODIPY molecule has 8 positions available for functionalization which brings out new members together with different photophysical properties.

Substitution on 1th, 3th, 5th, 7th positions by condensation reactions gives out long wavelength absorbing and emitting derivatives[96.97]. Bare BODIPY core has an absorption value around 500 nm. Functionalization from *meso* does not offer significant and interesting change in photophysical properties of the dye[98].

Position numbering of BODIPY core is given in the following figure. 8th position has a special name ‘’meso’’. α and β terms could also sometimes be useful.

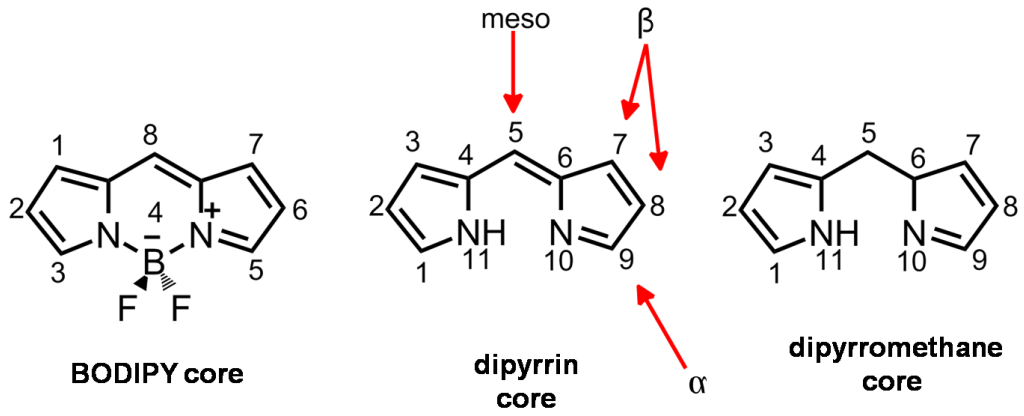


Figure 16: Position numbering of BODIPY

2th and 6th positions have relatively less positive charge than the others and therefore more prone to electrophilic attacks. Halogenation on these positions is important in Suzuki and Sonogashira coupling reactions. On the other hand, halogenations, due to heavy atom effect, decreases the quantum yield. Both mono and di-halogenations are possible and the spectrum generally shifts to red region when the dyes are halogenated[98]. 1th, 3th, 5th, 7th positions can also be functionalized by Knoavanagel reactions. Since these methlys are acidic, condensation is possible with different aldehydes. Derivatization of BODIPY dyes still continues all over the world by many research groups such as Akkaya, Ziessel and Burgess.

1.3.1 Applications of BODIPY dyes

BODIPY dyes have found many areas of application due to offering many chemical and photophysical properties. Photosensitizers for solar energy conversion[99] , through-bond (Dexter type) and through-space (Förster type) energy transfer cassettes[100,101] are some of them.

Photodynamic therapy(PDT) is a significant application area of BODIPY dyes which is a novel alternative treatment way for cancer. In PDT, e photosensitizer and a near-IR light source is used. These cause the production of singlet oxygen from molecular oxygen and since singlet oxygen is fatal to cancer tissue, the treatment is achieved. Since the skin tolerates between 650-800 nm, wavelength of light used is in this region. There is a study of Akkaya *et al.* about this subject in the literature[102]. A PDT agent were synthesized which can produce singlet oxygen when treated with 660 nm light. The molecule could also detect aciditiy and concentration of Na^+ inside the cell and is able to target the cancer tissue. The study is represented in figure 17:

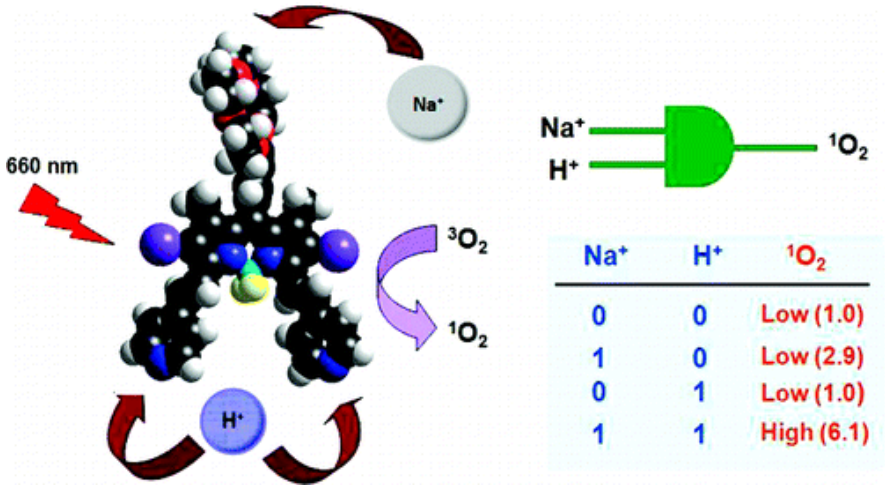


Figure 17: Photodynamic therapy agent and AND logic gate by Akkaya et al. Copyright © 2009 American Chemical Society. Adapted with permission

Light scattering is reduced at longer wavelengths; therefore fluorophores emitting beyond 650 nm are good agents for biological sensing. Two examples of red-

emitting BODIPY dyes developped by Akkaya *et al.* are given in the following figure, namely bis(2-pyridyl)-substituted boratriazaindacene (on the left)[103] and a fluorescent chemosensor for anions (on the right)[104].

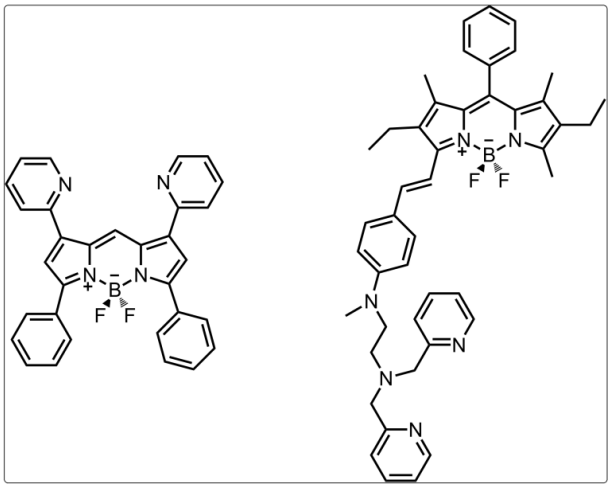


Figure 18: Examples of red-emitting BODIPY dyes

Protein labeling was one of the first applications of BODIPY dyes due to high quantum yields and photostabilities[105].

Figure 19 shows two examples from the literature. First one was published in *Org.Biomol.Chem*[106] (on the left), the other was published in *Angew.Chem.Int.Ed.*[107] (on the right) which are BODIPY dyes for protein labeling.

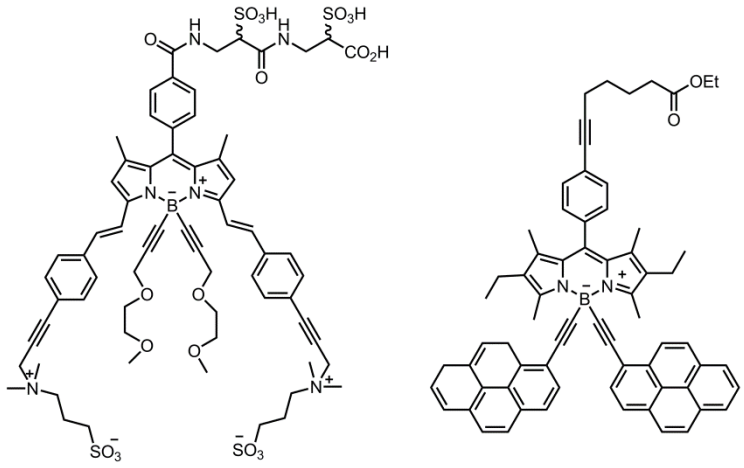


Figure 19: Literature examples of BODIPY dyes for protein labeling

1.4 Fluorescence Phenomenon

Electrons move to a higher electronic level (S_n, n>0) which is also known as ‘’excited state’’ from their ground state (S_0) when molecules are irradiated with light. The excited electron returns to its ground state via some mechanisms. The Perrin-Jablonski Diagram (Figure) is useful for visualizing possibilities of the processes that can occur after excitation. S_0 is the lowest electronic state, S_1, S_2,.. are singlet electronic states. T_1, T_2.. are triplet excited states. Lateral lines denoted as thinner than those standing for electronic states are vibrational levels. The excited electron firstly goes to S_1 (the lowest electronic state) by scattering some of its energy which is called as ‘’internal conversion’’. For movement to S_0 from S_1, there are some possibilities[108]. Turning back to ground state with emission of photon is ‘’fluorescence’’ which is the most significant of all the mechanisms. Non-radiative dissipation of energy as heat is another way. Collisional quenching is another case in which excited molecules transfer their energy to nearby moelcules. Transition from a singlet state to a triplet state T_1 is the last possibility called as ‘’internal conversion’’. The electron is then de-excited to the ground state from the excited triplet state which is namely ‘’phosphorescence’’ [108].

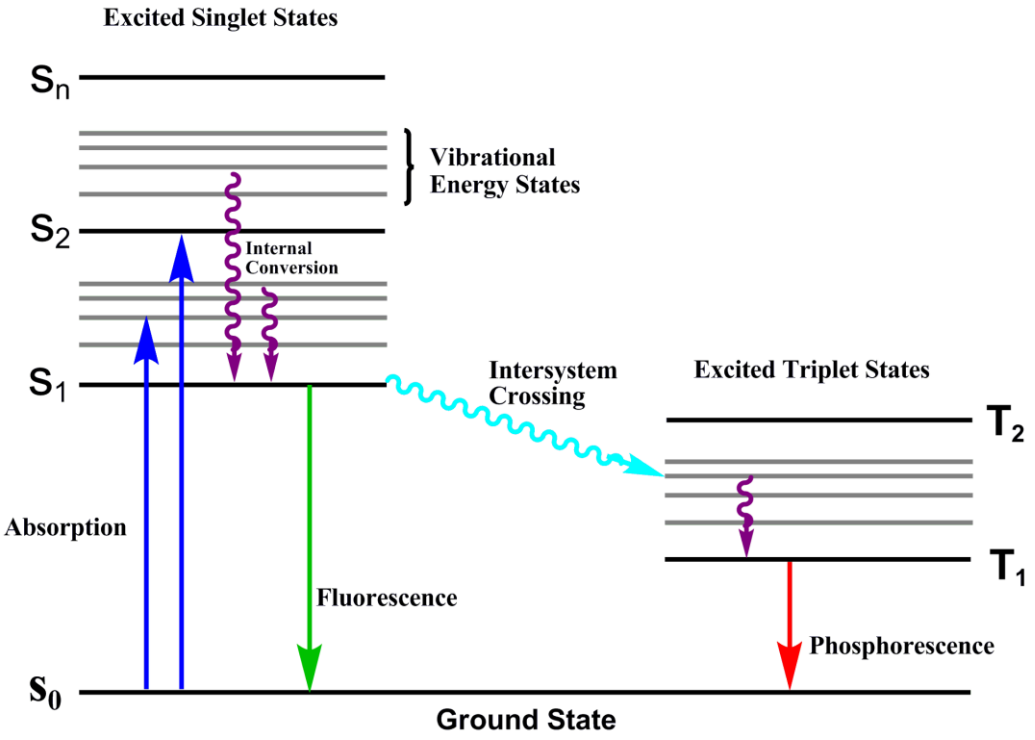


Figure 20: Jablonski diagram

As it is seen on the Jablonski Diagram, emitted light has lower energy than the absorbed light and hence it has higher wavelength than that of the absorbed light. In other words, the emission spectrum of the fluorophore is red-shifted to a higher wavelength. Fluorophore is a chromophore emitting light.

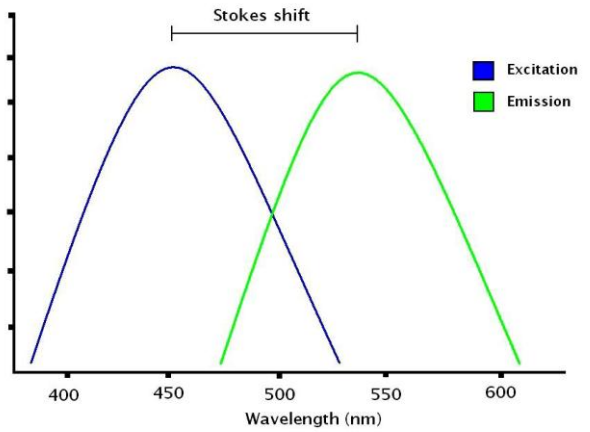


Figure 21: Simple representation of Stokes' shift

The red-shift which was firstly observed in 1852 by Sir George Stokes is known as ‘’Stokes’ shift’’ [109]. The main reason for Stokes’ shift is the rapid decay of electron to the lowest vibrational level of S_1. Solvent effects, complex formation, reactions at the excited state and energy transfer are the other causes of Stokes’ shift[110].

1.4.1 Photoinduced Electron Transfer (PeT)

Photoinduced electron transfer (PeT) is a signalling case in which emission is either quenched or enhanced, encountered in fluorophore-spacer-receptor systems[111]. In figure 22, mechanism of PeT is given. The highest occupied molecular orbital (HOMO) of the receptor is between the the lowest unoccupied molecular orbital (LUMO) and the highest occupied molecular orbital (HOMO) of the fluorophore in the figure. The receptor also contains an electron rich group. One of the electrons of the fluorophore is in the excited state due to irradiation of light. An electron from HOMO of the receptor transfers to the HOMO of the fluorophore and pairs with the single electron in HOMO of it. This situation prevents returning of the excited electron of flurophore from LUMO to HOMO and as a result, **the fluorescence is ‘quenched’.** On the other hand, if an analyte is present in the medium to stabilize the receptor, the energy level of its HOMO is lowered and it can not transfer its electron to HOMO of the fluorophore. Consequently, **strong fluorescence** is observed.

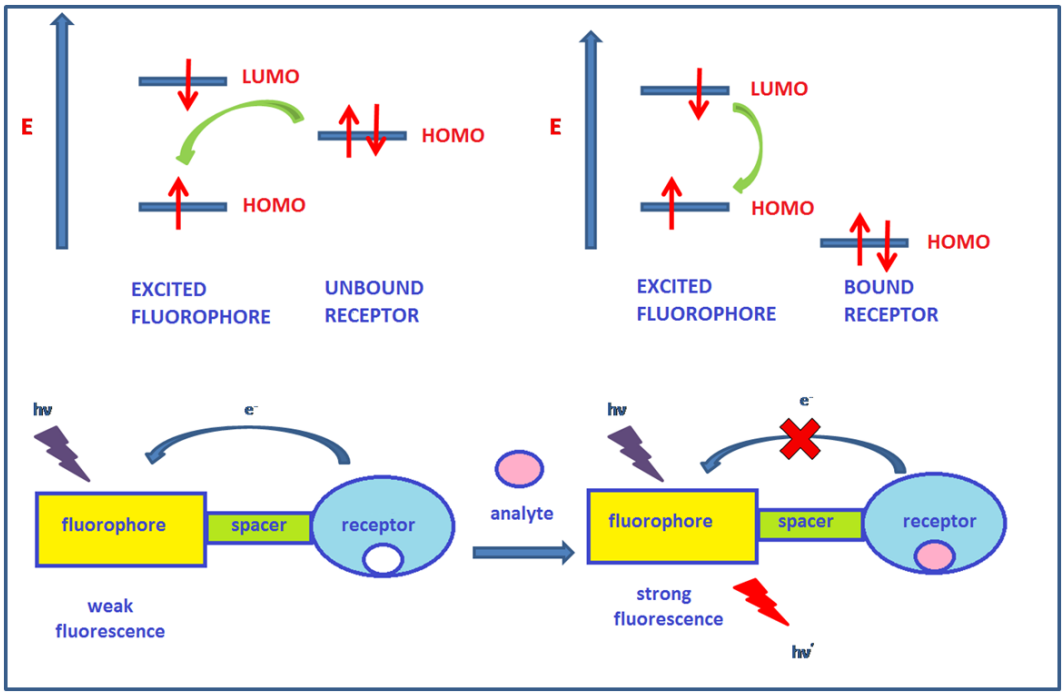


Figure 22: Reprentation of PeT working principle

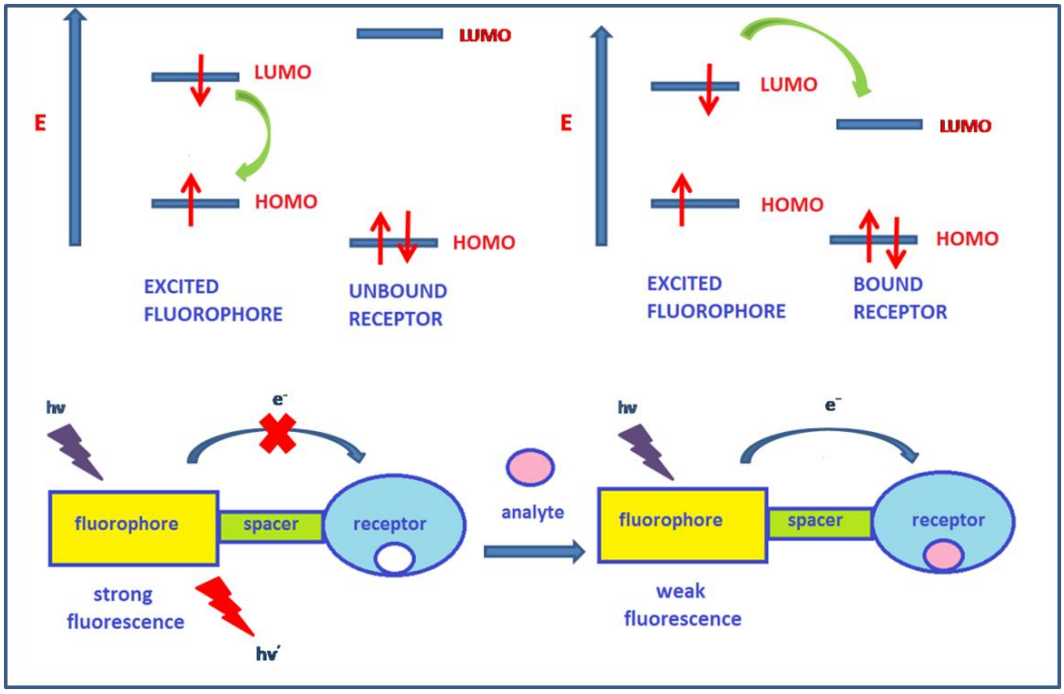


Figure 23: Representation of reverse PeT working principle

There also exists reverse PeT (oxidative PeT) mechanism. This event occurs if the receptor includes electron withdrawing group[112]. If there is no analyte in the medium, the electron in LUMO returns to HOMO and strong fluorescence is observed. However, in the presence of an analyte binding, HOMO of the receptor decreases and the electron in LUMO of the fluorophore is transferred to LUMO of the receptor and the result is weak fluorescence. In other words, fluorescence is quenched as a result of the process known as ‘’reverse PeT’’ or ‘’oxidative PeT’’ which is summarized in the figure 23.

1.4.2 Internal Charge Transfer (ICT)

In this phenomenon, there exist a fluorophore and a receptor without a spacer between them. The fluorophore has an acceptor part which is electron poor and a donor part which is electron rich. Excitation of such a fluorophore ends up with electron redistribution and creation of a dipole which result in electron donation from donor to acceptor[112].

The receptor, existing as a part of the Π-electron system of the fluorophore can be either an electron donating or electron accepting group. In the first case, consider an electron donating receptor such as an amino acid. When a cation is added to the medium, the excited state will be more destabilized as compared to the ground state and this will cause an increase in the energy gap between the excited state and the ground state. This scenerio means that the electron donating ability of the receptor decreases as a cation is present in the environment and the overall result is a ‘’blue shift’’ in the absorbance spectrum. In the second case, the receptor is an electron acceptor such as a carbonyl and the presence of a cation will enhance the electron withdrawing capacity of it. The excited state will be more stabilized as compared to the ground state and energygap will decrease causing a ‘’red shift’’ in the absorbance spectrum. The IcT phenomenon is explained in figure 24.

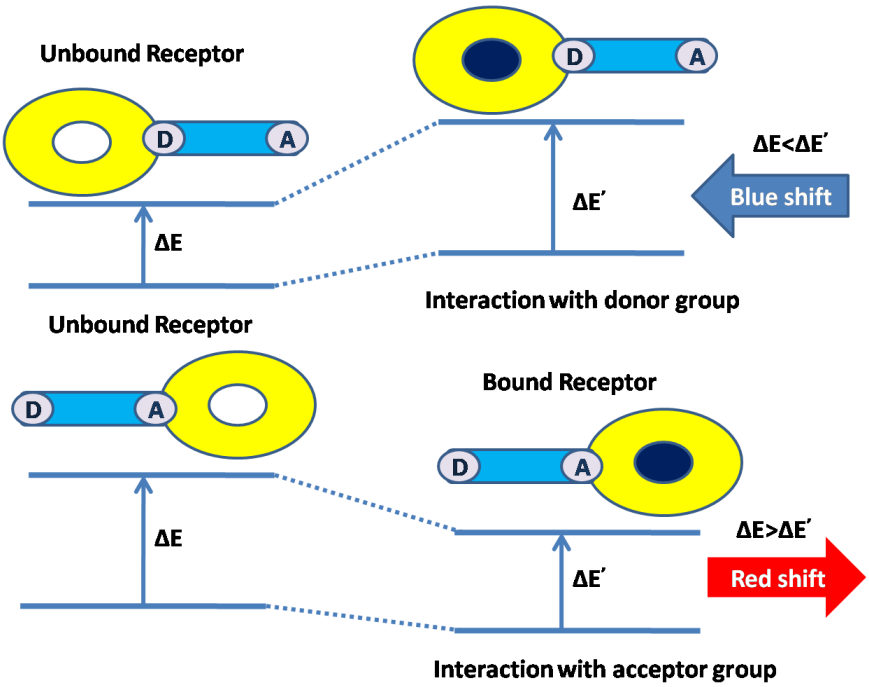


Figure 24: Schematic representation of ICT working principle

CHAPTER 2

EXPERIMENTAL

2.1 General

Chemical compounds and solvents were obtained from Sigma-Aldrich and were used without further purification. Thin layer chromatography to monitor reactions were performed by using Merck TLC Silica gel 60 F_{254}. In addition, Merck Silica gel 60 (particle size: 0.040-0.063 mm, 230-400 mesh ASTM) was used as the silica gel for column chromotography.

^{1}H NMR and ^{13}C NMR spectra were recorded on the instrument Bruker DPX-400 (operating at 400 MHz for 1H NMR and 100 MHz for 13C-NMR) at room temperature in $CDCl_3$ and DMSO-d6 with tetramethylsilane (TMS) as internal standard. Coupling constants (*J values*) are reported in Hz and chemical shifts are given in parts per million (ppm). Splitting patterns are designated as s(singlet), d (doublet), t(triplet), q(quartet), m(multiplet), and p(pentet).

Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS instrument was used to collect mass spectra data at Bilkent University, UNAM. A Varian Cary-100 spectrophotometer was used to record absorption spectra. Furthermore, to obtain emission spectra, a Varian Eclipse Spectrofluoremeter instrument was used at Bilkent University, UNAM.

2.2 Synthesis Scheme

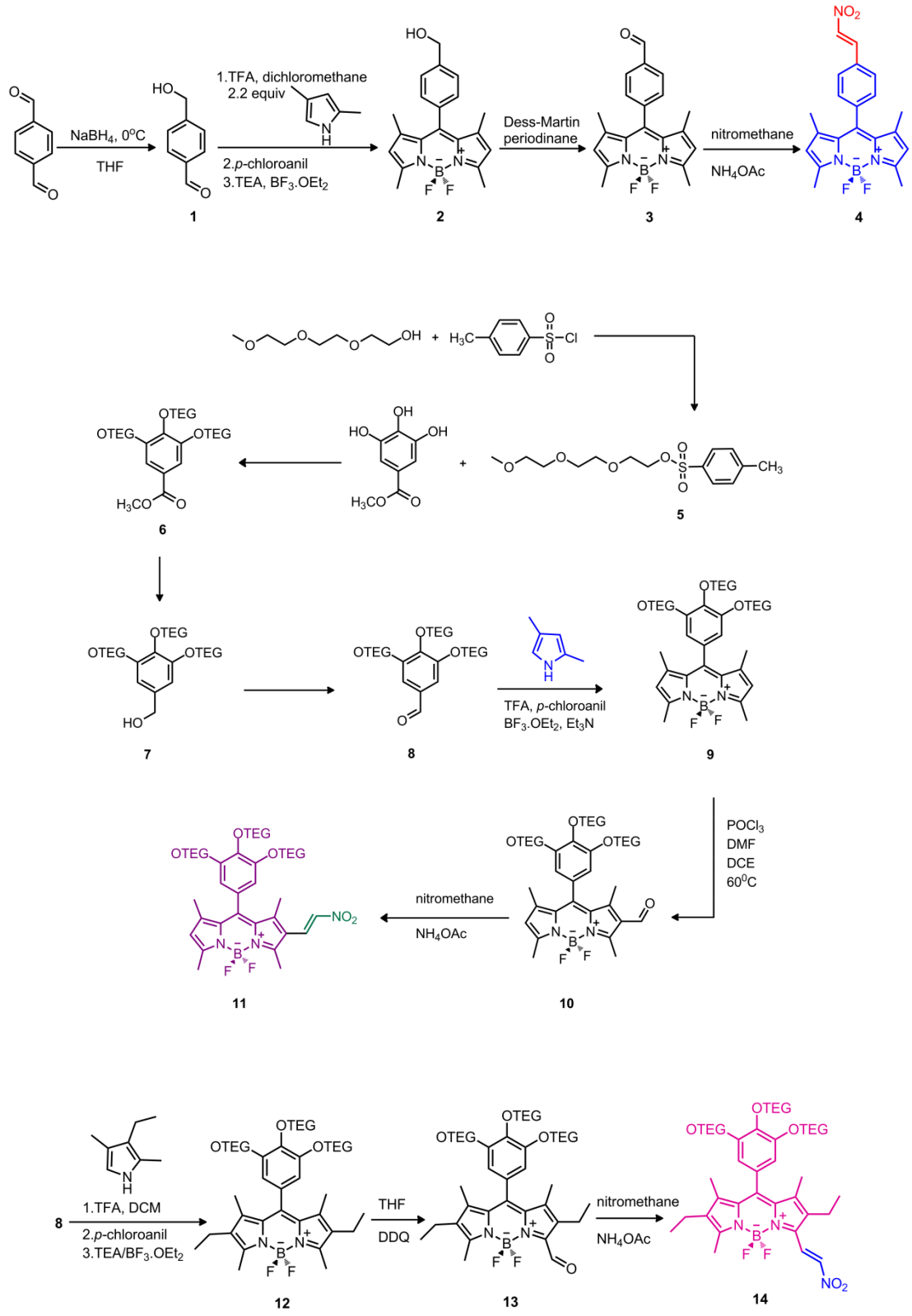


Figure 25: Synthesis Scheme

2.3 Syntheses

Three nitroolefin functionalized BODIPY dyes have been synthesized as potential protein labeling agents. Compound **1**[113] and compound **8**[114] were synthesized according to the literature.

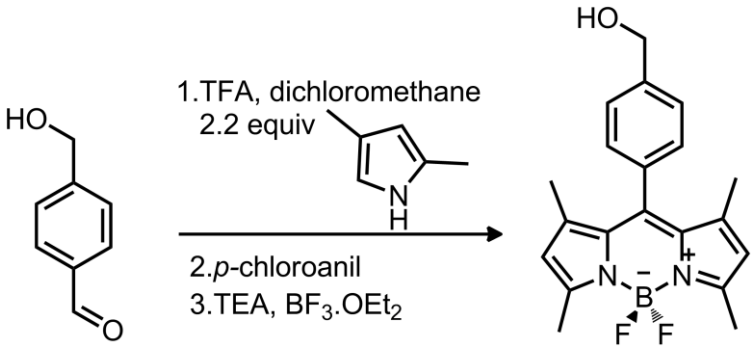
2.3.1. Synthesis of Compound 2

4-(hydroxymethyl) benzaldehyde (0.5 g, 3.68mmol, 1 equiv.) was dissolved in 400 mL Ar-degassed DCM in a 1 L round bottom flask. 2,4-dimethylpyrrole (0.83 mL, 769.5 mg, 2.2 equiv.) was added. This was followed by the addition of 1-2 drops of TFA. The mixture was stirred about 3 hr at room temperature. After TLC showed no starting material, *p*-chloroanil (903.93 mg, 1.1 equiv.) was poured into the reaction vessel. After 1 hr stirring, 6 mL TEA was added dropwise to the solution over a period of 5 min. The color turned out to be brown and it was stirred for an additional 30 min. Lastly, 6 mL $BF_3.OEt_2$ was added to the reaction in a dropwise manner over a period of 5 min, as well. The mixture was left to stir overnight at room temperature. Extraction was made with water (3x300 mL) and the organic layer was dried over anhydrous Na_2SO_4. After concentrating the organic layer *in vacuo*, it was purified by flash column chromotography with the eluant DCM. The product was obtained as red solid (380 mg, 29.2% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 (d, J = 7.8 Hz, 2H), 7.38 (d, 2H), 6.04 (s, J = 31.5 Hz, 2H), 4.83 (s, 2H), 2.60 (s, J = 17.9 Hz, 6H), 1.46 (s, J = 51.7 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 155.49, 143.08, 141.87, 141.54, 134.20, 128.18, 127.38, 121.23, 64.78, 14.47.

MS (TOF-ESI): m/z: Calcd:353.17 $[M-H]^-$, Found:353.1673 $[M-H]^-$, Δ=-7.64 ppm.



1 **2**

Figure 26: Synthesis of compound 2

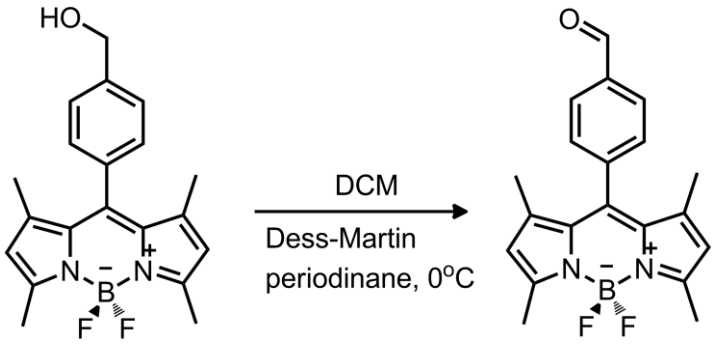
2.3.2 Synthesis of Compound 3

Compound **2** (150 mg, 0.42 mmol) was dissolved in a minimum amount of Ar-degassed DCM. Dess-Martin periodinane (359.2 mg, 0.84 mmol) was also dissolved in min. amount of Ar-degassed DCM and this was added dropwise to the previous one at 0^oC. When the addition is completed, the reaction left to stir at room temperature for about 2 hrs. When TLC showed no starting material, the mixture was quenched with 20 mL sat'd $Na_2S_2O_3$ solution. The organic layer was then washed with sat'd $NaHCO_3$ solution (2x20 mL). It was lastly washed with water (2x20 mL) and dried over anhydrous Na_2SO_4. Flash column chromotography was performed to purify the organic layer by using 95:5/ DCM:MeOH as the eluant. The product was obtained as orange solid (90 mg, 60% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 10.14 (s, J = 4.6 Hz, 1H), 8.04 (d, J = 6.2 Hz, 2H), 7.53 (d, J = 8.0 Hz, 2H), 6.019 (s, 2H), 2.58 (s, 6H), 1.37 (s, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ_C:191.43, 156.25, 142.75, 141.40, 139.68, 136.68, 130.32, 129.15, 121.63, 121.60, 14.61, 14.50.

MS (TOF-ESI): m/z: Calcd: 351.16 $[M-H]^-$, Found:351.1548 $[M-H]^-$, Δ=-14.8 ppm.



2 **3**

Figure 27: Synthesis of compound 3

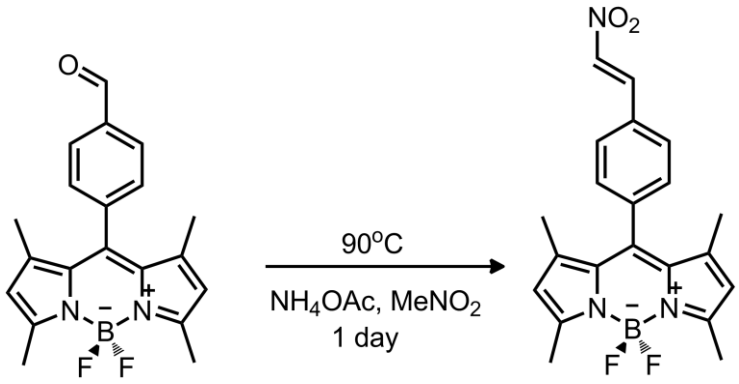
2.3.3. Synthesis of Compound 4

Compound **3** (80 mg, 0.23 mmol) was dissolved in 4 mL nitromethane. Oil bath was stabilized at 90°C. Solution was placed in oil bath and waited for 30 min. After that, a pinch of NH_4OAc was added to the reaction vessel. The reaction was completed as monitored by TLC after 24 hr stirring. Nitromethane was evaporated *in vacuo* and flash column chromotography was performed to purify the product by using 95:5/DCM:MeOH as the eluant. The nitroolefin functionalized protein labeling agent was obtained as red crystals (45 mg, 49.5% yield).

^{1}H NMR (400 MHz, d6-DMSO) δ_H: 8.35(d, *J*=18 Hz, 1H), 8.25 (d, *J*=14 Hz, 1H), 8.1(d, *J*=8 Hz, 2H), 7.6(d, *J*=8 Hz, 2H), 6.2(s, 2H), 2.45(s, 6H), 1.4(s,6H)

^{13}C NMR (100 MHz, d6-DMSO) δ_C: 156.23, 142.69, 139.66, 139.04, 138.02, 137.86, 130.95, 130.83, 129.73, 129.45, 121.60, 14.62, 14.57.

MS(TOF-ESI): m/z: Calcd: 394.16$[M-H]^-$, Found:394.1492 $[M-H]^-$,Δ=-2,74 ppm.



3 **4**

Figure 28: Synthesis of compound 4

2.3.4. Synthesis of Compound 9

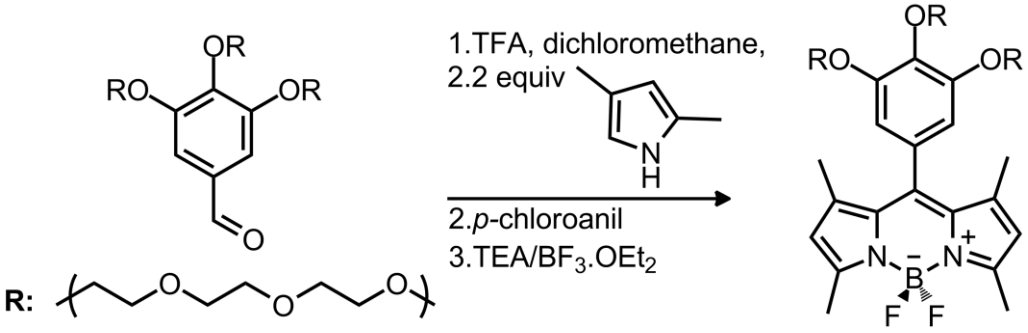
3,4,5-tris(2-(2-(2-methoxyethoxy)ethoxy)benzaldehyde (500 mg, 0.85mmol) was mixed with 2,4-dimethylpyrrole (176 mg, 1.85 mmol) in 350 ml argon degassed dichloromethane (DCM). 1-2 drops of TFA was added followingly. The mixture was stirred at room temperature for about 3 hours. When TLC showed no starting material, *p*-chloranil (229.8 mg, 0.93 mmol) was added to the reaction and it was stirred for an additional hour. Then, 7 ml Et_3N was added to the mixture dropwise over a period of 5 min and the resulting brown solution was stirred for 30 min. After that, 7 ml BF_3•OEt_2 was added dropwise over another period of 5 min to the reaction and the resulting mixture was allowed to stir overnight. The reaction mixture was washed with water (3×300 mL) and extracted into DCM. The organic layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo* and the crude product was purified by flash column chromotography packed with silica gel by using 95:5/ DCM:MeOH as the eluant. The product was obtained as a waxy dark orange solid (370 mg, 54% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 6.55 (s, 2H), 5.99 (s, 2H), 4.22 (t, *J*= 4.97 Hz, 2H), 4.12 (t, *J*= 4.72 Hz, 4H), 3.84 (t, *J*= 5.29 Hz, 6H), 3.51-3.76 (m, 25H), 3.38 (s, 2H), 3.36 (s, 6H), 2.54 (s, 6H), 1.52 (s, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 155.8, 153.7, 142.9, 141.1, 139.0, 131.1, 129.9,

121.1, 107.7, 2.7, 71.9, 71.9, 70.8, 70.7, 70.6, 70.5, 69.7, 69.2, 58.8, 29.6 14.4, 14.2.

MS (TOF-ESI): m/z: Calcd: 809.4286 $[M-H]^-$, Found: 809.4306 $[M-H]^-$, Δ= 2.47 ppm.



9

Figure 29: Synthesis of compound 9

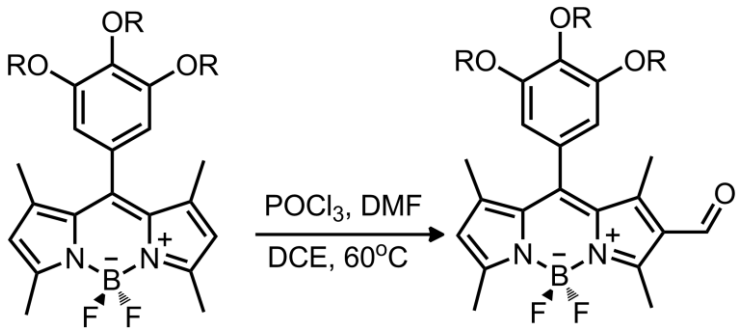
2.3.5. Synthesis of Compound 10

0.5 mL $POCl_3$ was added into anhydrous DMF (0.5 mL) dropwise in ice bath. Addition was done under N_2. This mixture (pale yellow viscous liquid) was left to stir for 30 min at room temperature. Compound **9 (**405.215mg, 0.5 mmol) was dissolved in 25 mL DCE and then slowly introduced to the previous mixture. The resulting brown solution was stirred at 60^{o}C for 3 hr. 60^{o}C is the critical temperature to get the mono-formylated product **10**, side products could be obtained otherwise at higher temperatures. Then the reaction was cooled to room temperature and poured into ice-cold saturated $NaHCO_3$ solution. Extraction was performed with DCM (2x50 mL) and the organic layer was dried over Na_2SO_4. The organic layer was concentrated *in vacuo* and purified by flash column chromotography with 98:2/DCM:MeOH as the eluant. Target compound **10** was obtained as orange crytals(185 mg, 93% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 9.98 (s,1H), 6.53 (s, 2H), 6.15 (s, 1H), 4.23 (d, *J*= 4.34 Hz, 2H), 4.12 (d, *J*= 4.29 Hz, 4H), 3.83 (d, *J*= 4.81 Hz, 6H), 3.75-3.49 (m, 25H), 3.37 (s, 2H), 3.34 (s, 6H), 2.80 (s, 3H), 2.59 (s, 3H), 1.78 (s, 3H), 1.57 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 185.81, 161.64, 156.47, 154.02, 147.17, 143.07, 142.68, 139.54, 133.98, 129.65, 128.89, 126.25, 123.92, 107.04, 72.76, 71.93, 71.88, 70.84, 70.68, 70.62, 70.55, 70.53, 69.69, 69.32, 58.96, 14.76, 11.55.

MS (TOF-ESI): m/z: Calcd: 837.4235 $[M-H]^-$, Found: 837.4094 $[M-H]^-$, Δ=-16,83ppm



9 **10**

Figure 30: Synthesis of compound 10

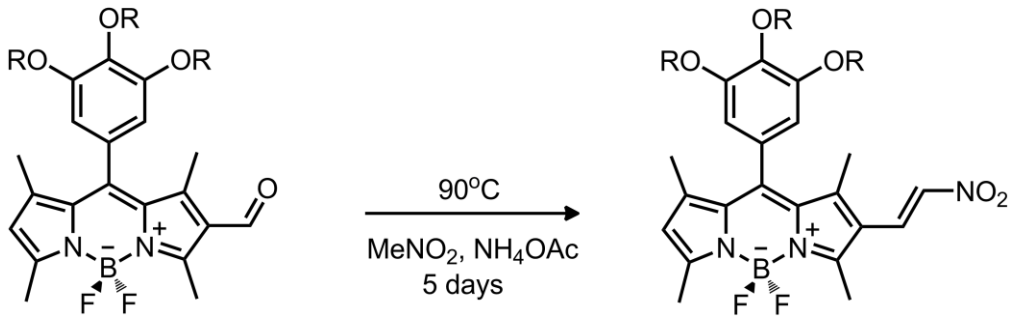
2.3.6. Synthesis of Compound 11

Compound **10** (140 mg, 0.17 mmol) was dissolved in 2 mL nitromethane. This solution was stabilized at 90 ^{o}C in oil bath in 30 min. Then NH_4OAc (1.28 mg, 0.017 mmol) was added to the mixture. The reaction was stirred for 5 days. When TLC showed no starting material, nitromethane was removed *in vacuo*. Flash column chromotography packed with silica gel was used to purify the crude product by using 95:5/ DCM:MeOH as the eluant. Compound **11** was obtained as a wine-red waxy solid (43 mg, 28.6% yield).

^{1}H NMR (400 MHz, $CDCl_3$) $\delta_{H:}$ 8.05 (d, *J*= 13.8 Hz, 1H), 7.37 (d, *J*= 13.63 Hz, 1H9, 6.56 (s, 2H), 6.18 (s, 1H), 4.26 (t, *J*= 4.64 Hz, 2H), 4.14 (t, *J*= 4.52 Hz, 4H), 3.86 (t, *J*=5.15Hz, 6H), 3.52- 3.76 (m, 25H), 3.40 (s, 2H), 3.37 (s, 6H), 2.72 (s, 3H), 2.63 (s, 3H), 1.64 (s, 3H), 1.59 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 161.54, 154.23, 154.03, 147.18, 147.22, 140.16, 139.65, 134.60, 133.71, 130.71, 130.62, 128.89, 128.84, 123.93, 120.03, 107.41, 72.81,71.96, 71.90, 70.86, 70.71, 70.68, 70.58, 70.56, 69.71, 69.36, 65.30, 59.01, 29.67, 14.72, 12.85,11.09.

MS (TOF-ESI): m/z: Calcd: 880.7612 $[M-H]^-$, Found: 880.4134 $[M-H]^-$, Δ= -2.09 ppm.



10 **11**

Figure 31: Synthesis of compound 11

2.3.7. Synthesis of Compound 12

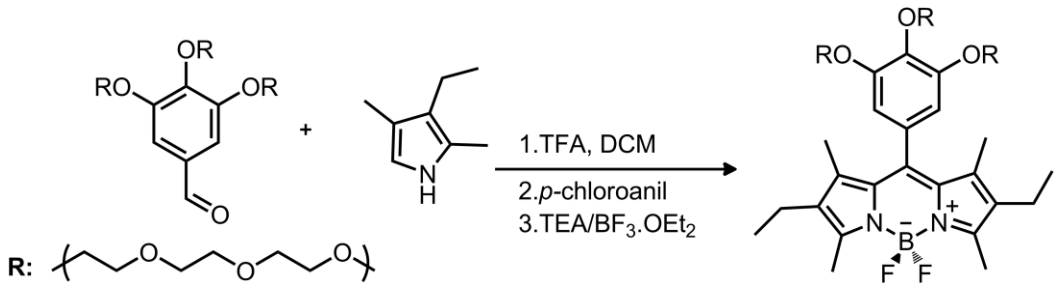
3,4,5-tris(2-(2-(2-methoxyethoxy)ethoxy)benzaldehyde (1500 mg, 2,55mmol) was dissolved in 450 ml argon degassed dichloromethane (DCM). 3-ethyl-2,4-dimethyl-pyrrole (528 mg, 5.61 mmol) was added. 1 drop of TFA was added to mixture followingly. It was stirred for about 3 hr. Reaction was monitored by TLC and when it showed no starting material, *p*-chloranil (698,4 mg, 2.79 mmol) was added in one portion. After an additional 1 hr stirring, 8 ml Et_3N was added to the mixture dropwise over a period of 5 min and the resulting brown solution was

stirred for 30 min. Thereafter, 8 ml BF_3•OEt_2 was added dropwise over another period of 5 min to the reaction and the resulting mixture was left to stir overnight at room temperature. The resultant mixture was washed with water (3×400 mL) and extracted into DCM. The organic layer was dried over anhydrous Na_2SO_4, concentrated *in vacuo* and the crude product was purified by flash column chromotography packed with silica gel by using 95:5/ DCM:MeOH as the eluant. The product was obtained as a waxy dark orange solid (1034 mg , 46.7% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 6.55 (s, *J* = 1.2 Hz, 2H), 4.24 (t, *J* = 7.4 Hz, 2H), 4.12 (t, *J* = 4.5 Hz, 4H), 3.85 (t, *J* = 4.7 Hz, 6H), 3.78 – 3.47 (m, 24H), 3.40 – 3.31 (m, 8H), 2.53 (s, 6H), 2.32 (q, *J* = 7.4 Hz, 4H), 1.42 (s, 6H), 1.00 (t, *J* = 7.35 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 153.79, 153.61, 139.66, 136.25, 132.74, 132.72, 72.70, 71.91, 70.86, 70.71, 70.64, 70.55, 69.73, 69.18, 59.01, 58.98, 17.06, 14.59, 12.47, 11.61

MS (TOF-ESI): m/z: Calcd:865.49 $[M-H]^-$, Found: 865.4904 $[M-H]^-$, Δ= -4.05 ppm.



12

Figure 32: Synthesis of compound 12

2.3.8. Synthesis of Compound 13

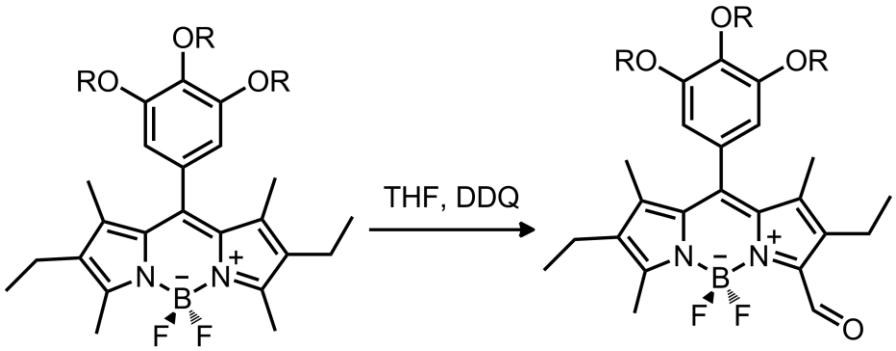
2,8-diethyl-5,5-difluoro-1,3,7,9-tetramethyl-10-(3,4,5-tris(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)-5H-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide (Compound **12**) (400 mg, 0.46 mmol) was dissolved in 18 mL, Ar-degassed THF in 100 mL round bottom flask. DDQ(420

mg, 0.54 mmol) was dissolved in 6 mL, Ar-degassed THF and 0.16 mL distilled water was added. This mixture was added dropwise to the previous one at 0°C under Ar. When the addition is completed, the reaction was left to stir for 3 days. When TLC showed no starting material, the resultant mixture was purified by flash column chromotography filled with silica gel. 95:5/ EtOAc:MeOH was used as eluant. Product was obtained as red solid(350 mg, 86% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 10.38 (s, 1H), 6.56 (s, 2H), 4.26 (s, 2H), 4.14 (s, 4H), 3.80-3.51 (m, 30H), 3.44 – 3.30 (m, 9H), 2.72 (s, 2H), 2.61(s, 3H), 2.36 (s,2H), 1.55 (s,3H), 1.41 (s, 3H), 1.05 (s, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 213.74, 185.97, 166.16, 153.90, 143.72, 141.50, 139.87, 139.23, 137.71, 137.03, 135.60, 134.33, 132.05, 129.58, 107.47, 77.37, 77.05, 76.73, 72.73, 71.90, 71.86, 70.80, 70.61, 70.59, 70.50, 70.46, 69.66, 69.23, 59.02, 59.01, 58.98, 17.59, 17.12, 14.34, 14.02, 13.68, 12.26, 10.44.

MS (TOF-ESI): m/z: Calcd: 879.47 $[M-H]^-$, Found:879.468 $[M-H]^-$, Δ= -2.52 ppm.



12 **13**

Figure 33: Synthesis of compound 13

2.3.9 Synthesis of Compound 14

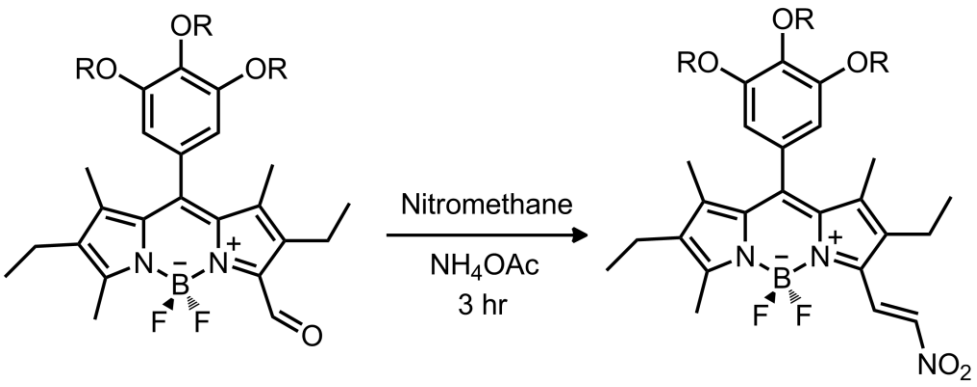
2,8-diethyl-5,5-difluoro-3-formyl-1,7,9-trimethyl-10-(3,4,5-tris(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)-5H-dipyrrolo[1,2-c:2',1'-

f][1,3,2]diazaborinin-4-ium-5-uide (Compound **13**) (340 mg, 0.39 mmol) was dissolved in 5 ml nitromethane. It was placed in an oil bath at 90^{o}C. A pinch of NH_4OAc was added. The reaction was stirred at this temperature for 3 hr. Product was obtained as violet-purple solid (90 mg, 25.2% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 8.36 (d, *J* = 13.7 Hz, 1H), 7.84 (d, *J* = 13.8 Hz, 1H), 6.55 (s, 2H), 4.29 – 4.17 (m, 2H), 4.17 – 4.01 (m, 4H), 3.90 – 3.82 (m, 6H), 3.82 – 3.33 (m, 34H), 2.72 – 2.32 (m, 7H), 2.08 – 1.68 (m, 1H), 1.50 (s, 3H), 1.46 (d, *J* = 26.5 Hz, 6H), 1.07 (m, *J* = 19.8, 7.5 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 165.16, 153.95, 143.06, 140.18, 139.36, 137.86, 137.81, 137.76, 137.51, 137.47, 136.35, 135.43, 135.09, 133.94, 129.50, 127.51, 107.56, 77.37, 77.06, 76.74, 72.76, 71.95, 71.89, 70.85, 70.70, 70.65, 70.56, 69.70, 69.27, 59.03, 59.00.

MS (TOF-ESI): m/z: Calcd: 922.48 $[M-Na]^+$, Found: 946.47 $[M-Na]^+$, Δ= -8.73 ppm.



13 **14**

Figure 34: Synthesis of compound 14

CHAPTER 3

RESULTS AND DISCUSSION

3.1.General Perspective

Starting with studies of non-covalent interactions between host and guest molecules, the field of supramolecular chemistry has enlarged to include self-assembly, molecular recognition systems, self-organization, molecular devices and many others. Proteins, comprising one of the major classes of biomolecules of life, has to be tracked in order to observe their mechanisms, determination of turnover numbers of enzymes and sometimes even for targeting a tumor tissue. As a very significant member of supramolecular chemistry field, BODIPY dyes are functionalized and derivatized to be engaged in various applications thanks to their high absorption coefficients, strong fluorescence, ease of synthesis, stability, etc. In addition to solar cells, photodynamic therapy, molecular logic gates, energy transfer cassettes and some others, protein labeling is a good area for the use of BODIPY dyes.

Akkaya *et al.* have recorded significant work in the mentioned areas with production of novel BODIPYs so far. In this study, we enriched our work and have brought three nitroolefin functionalized BODIPY dyes for protein labeling in our records.

3.2. Design and Properties of target dyes

BODIPY core has 8 positions available for functionalization as shown in figure 35.

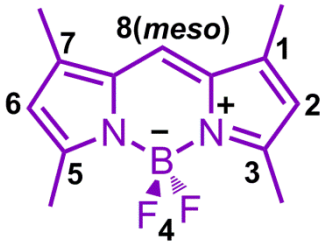


Figure 35: 8 positions of BODIPY dyes available for modification

In this study, we have designed and synthesized three different nitroolefin subsituted BODIPY dyes, namely compound 4 (dye 1), compound 11 (dye 2) and compound 14 (dye 3) as shown in the figure 36.

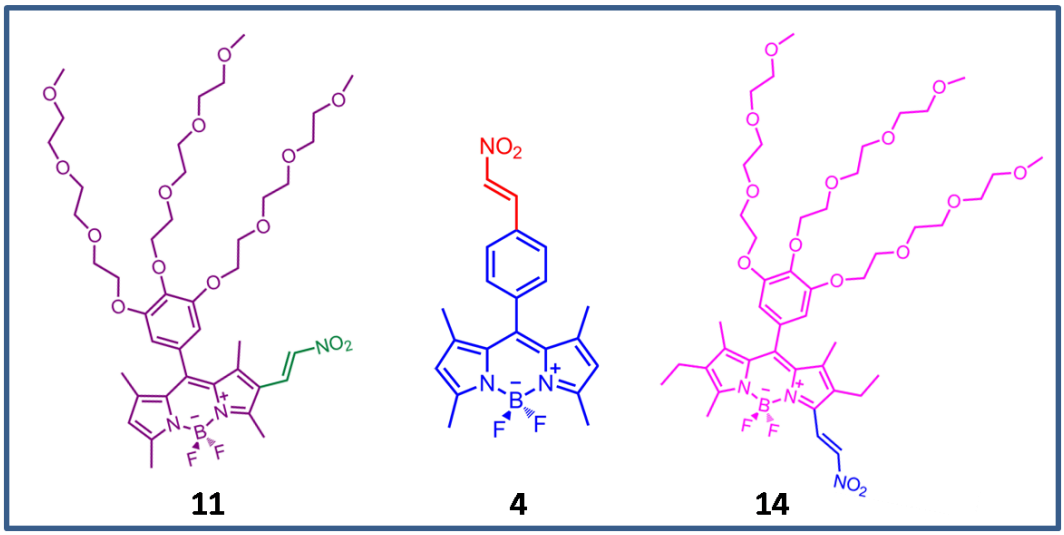


Figure 36: Target protein labeling agents

On compound **4**, nitroolefin group is on top of a benzene ring on position 8. Compound **11** has its 2nd position functionalized with the nitroolefin group and compound **14** has been incorporated with the nitroolefin from its 3rd position. These nitroolefin groups are good Micheal acceptors. Esters, nitriles, nitro groups, α,β-unsatırated ketones, etc. act as good Micheal acceptors existing in the literature[115,116]. They are good candidates for nucleophilic attack.

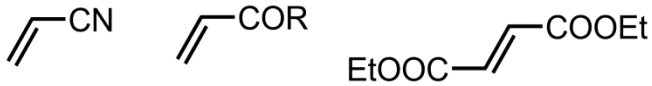


Figure 37: Examples of Micheal acceptors

Table 1 summarizes photophysical properties of the dyes. The absorption maxima of compound **4**, **11** and **14** are 503 nm, 525 nm and 570 nm respectively. Molar extinction coefficients (ε_{max}) are calculated from Beer-Lambert Law. ε_{max} were found as 105,694 $cm^{-1}M^{-1}$, 32,000 $cm^{-1}M^{-1}$ and 38,918 $cm^{-1}M^{-1}$ for compound **4**, **11** and **14** respectively.

Compound	λ_{abs} (nm)	λ_{em} (nm)	Φ_f^*	ε_{max} ($cm^{-1}M^{-1}$)
4	503	515	0.067	105,694
11	525	540	0.056	58,500
14	570	530	0.019	38,918

Table 1: Photophysical properties of dyes.* relative quantum yields. Reference dye: Rhodamine 6G in water (Φ_f:0.95)

3.2.1 Synthesis and Characterization

The synthesis of compound **4** started with reaction of 2,4-dimethylpyrrole and 4-(hydroxymethly) benzaldehyde. Then the –OH group was oxidized to the aldehyde form with Dess-Martin periodinane reagent via Dess-Martin oxidation reaction[117]. As the third step, NH_4OAc (ammonium acetate) was used as the catalyst and by using nitromethane at 90^0C, nitroolefin functionalized form was obtained declared as ‘‘dye 1’’. Our second design was compound **11** which included TEG groups to enhance its water solubility. Compound **9** was formylated on the 2nd position via Vilsmeier-Haack reaction[117]. 2-formyl BODIPY was then subsequently nitroolefinated to give the so called ‘’dye 2’’. The same procedure

was conducted for the synthesis of compound **14**, in other words, ‘’dye 3’’. This molecule also includes TEG groups but it was synthesized by using 2,4-dimethyl-3-ethyl pyrrole making it a little different from the previous BODIPY. Consequently, dye 2 & dye 3 are more soluble in water as compared to dye 1. For characterization, ^{1}H-NMR and ^{13}C-NMR spectra were recorded and results were also supported by mass spectrum as presented in appendix part. For dye 1, DMSO-d6 was used as NMR solvent. In order to prove the reaction of dyes with thiol including groups, mercaptoethanol, the simplest of all, was chosen. Figure 38 shows a comparison of aromatic region of ^{1}H-NMR of compound **4** and that of its conjugated form with mercaptoethanol. As it is clear, trans protons are removed due to the breakage of the double bonds. The same is given in figure 39 for compound **11** that shows the removal of trans protons around 8.0 ppm.

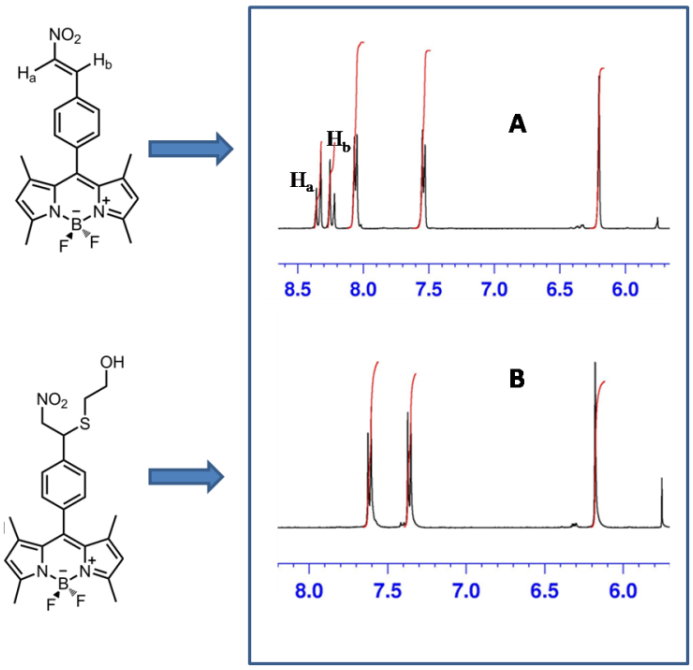


Figure 38: Comparison of aromatic regions of ^{1}H-NMR of compound 4 and that of its conjugated form with mercaptoethanol

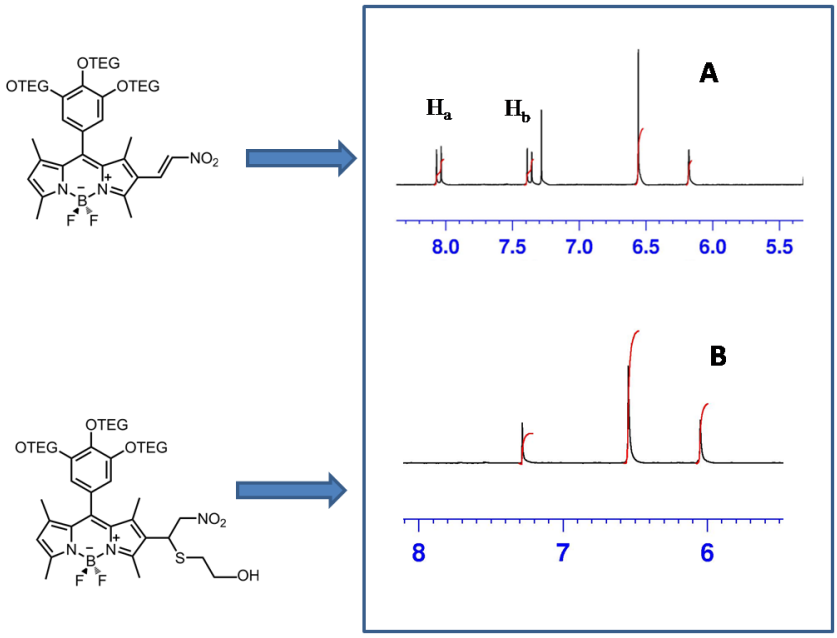


Figure 39: Comparison of aromatic regions of ^{1}H-NMR of compound 11 and that of its conjugated form with mercaptoethanol

3.3 Reactions with cysteine & spectroscopic data

First one of the three BODIPY dyes of this study, compound **4**, is the simplest but the most interesting protein labeling agent that we propose. Nitroolefin group bonded to the benzene ring on the *meso* position of our BODIPY makes it possess significant properties. Above everyhting, this is a ‘’Turn-on’’ agent showing excellent fluorometric changes upon reaction with thiol moities. It gives very fast reaction, perfectly bright color and strong signalling after the aforementioned event. Thanks to being such a small molecule and not containing any bulky groups, there is no possibility of steric hinderence to prevent desired reactions. On the other hand, since it does not contain any water-soluble units, it requires mostly organic solvents to dissolve in and therefore it may need some water-solubilising units to be a better candidate in biological applications. As a suggestion, its distyryl form could be synthesized via Knoavanagel condensation reaction with 3,4,5-(trihydroxybenzaldehyde) to increase the number of TEG units and increase

water solubility. To test spectroscopic changes after the nucleophilic attack of a thiol group, cysteine solutions were prepared and reacted with our dye 1 to collect absorbance and emission spectra. 1 µM solutions of compound **4** were used in the measurements prepared in 1x PBS:DMSO (5:95, V/V, pH:7.4 at 25^0C). Figure 40 shows UV-VIS absorbance spectra of dye 1. As it is seen, the compound itself has an absorption band at 503 nm and there is no remarkable change neither in absorbance intensity nor in the wavelength of absorbance no matter how much cysteine is added to the system. The reason is that, the benzene ring on the BODIPY core is positioned as perpendicular to the whole system as its architecture suggests and therefore it does not correspond to the Π-conjugation system of the compound as shown in the figure 41. Consequently, there is no ICT and ICT blocking phenomenon in this process and there observed no colorimetric changes.

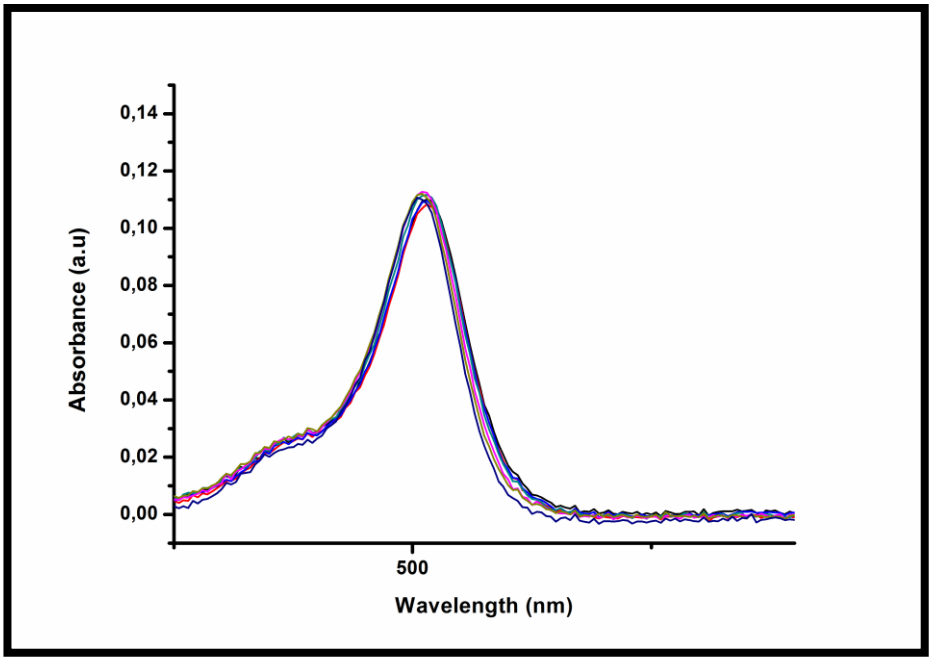


Figure 40: UV-VIS absorption spectra of compound **4** in 1x PBS:DMSO (5:95, V/V, pH:7.4 at 25^0C)

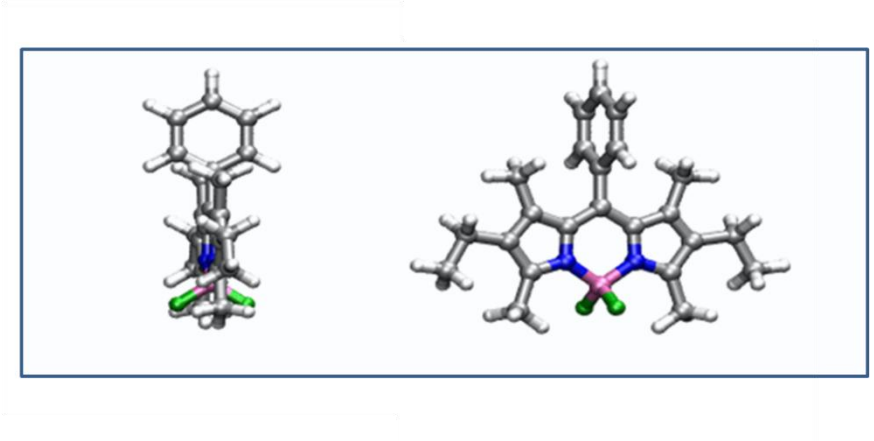


Figure 41: Representation of the perpendicular stand of benzene ring on *meso* poisiton of BODIPY

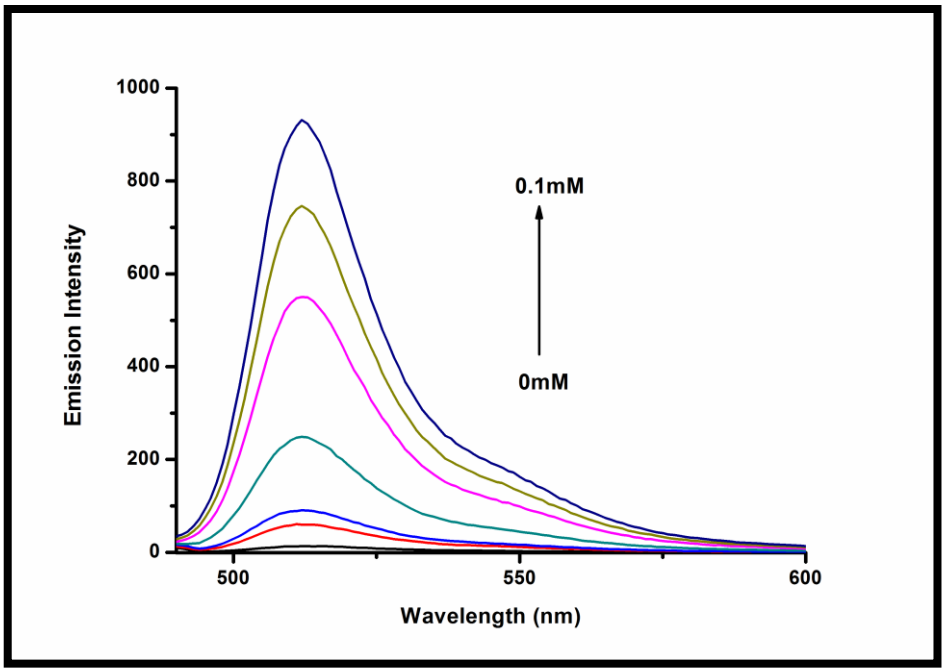


Figure 42: Fluorescence spectra of compound **4** in 1x PBS:DMSO (5:95, V/V, pH:7.4 at 25^0C) upon increased L-Cys addition

Contrary to absorbance results, fluorometric changes were expected in this study. Figure 42 shows the fluorescence spectra recorded in the same conditions as absorbance spectra were done. Excitation wavelength was chosen as 490 nm and

the slit width was 5-2.5. Because the reaction was very fast and effective, it became saturated so rapidly that it was difficult to obtain proper spectra with slit widths 5-5. Equivalents greater than 100 were not even collected in the case of 5-2.5 slit width because there was again sudden saturation due to the speed of the conjugation. As depicted in figure 42 , fluorescence intensity of dye 1 gradually increases upto 20-fold of its first value upon addition of increased L-Cys concentrations (0-100 equiv). This outcome is due to the interruption of PeT process and successful increase in fluorescence intensity is proved making it possible to declare our dye 1 as a ‘’turn-on’’ labeling agent.

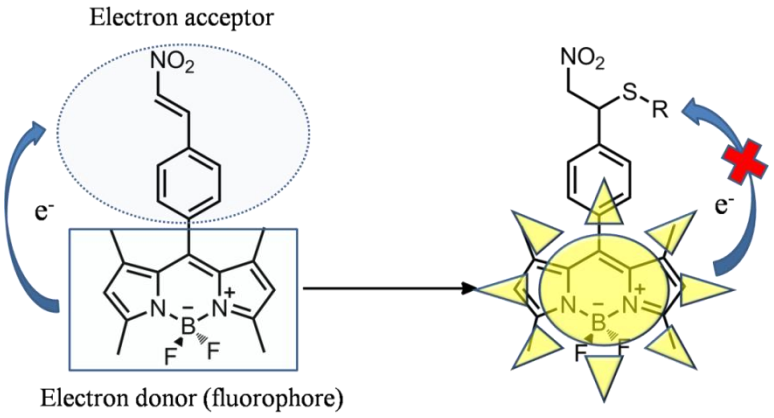


Figure 43: Representation of blocking PeT process on compound 4 upon reaction with a thiol and turn-on fluorescence

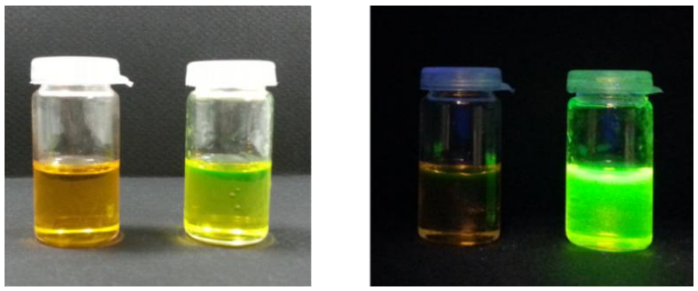


Figure 44: Compound 4 and its L-cysteine conjugated form respectively under ambient light (on the left), under UV-light (on the right)

Figure 43 shows the mechanism of emission increase during addition of a thiol group. The BODIPY core is the electron donor (fluorophore) and the *meso* substituted part is the electron acceptor. As a thiol including group is present in the medium, there appears Micheal 1,4-addition product and the double bond is broken. This is the interruption of electron donation from the fluorophore to the acceptor which is accompanied by bright signalling.
Compound 4 is normally orange, but it turns out to be yellow upon addition of L-cysteine which is shown in the figure 44. The picture taken under UV light shows that the dye has no emission initially and it turns on in the presence of L-cysteine.

Compound **11**, the second labeling agent, has a gallic acid derived unit at its *meso* position. It offers several properties. Firstly, ethylene glycolic units functionalized from the phenolic hydroxyls enhance the compound's water-solubility. Ability to readily dissolve in mostly water makes the agent attract more attention because of ease of use in bio-applications. Secondly, the gallic acid derived unit on the *meso* position of the compound can stop PeT when it is attached to BODIPY core which is electron-poor due to conjugation with a nitroolefin unit and consequently, there observed is a weak fluorescence. Reaction of this molecule with a thiol group was anticipated to disrupt the Π conjugation of the system and cause a blue shift in the absorbance spectrum. In other words, blocking the ICT process was expected. Upon nucleophilic attack of the thiol moity, disruption of PeT process and observation of strong fluorescence was another expectation.

Dye 2 had an absorption band located at 525 nm which decreased upon addition of L-Cys and a new one at 510 nm started to appear. There was a total of 15 nm blue shift in the spectra and an isosbestic point at 518 nm was present. Absorbance spectra is given in figure 45. This cysteine-triggered blue shift was the proof of disruption of ICT process. Furthermore, compound **11** (dye 2) is not fluorescent with Φ_f=0.056 as it is seen on its emission spectra in figure 46. As seen from figure 46, fluorescence intensity of the dye gradually increases upto 20-fold of its first value upon addition of increased Cys concentrations (0-400 equiv.) λ_{ex} was 500 nm at 25 °C. This is attributed to the blocking of PeT process in the presence of the reaction with the thiol group in the medium

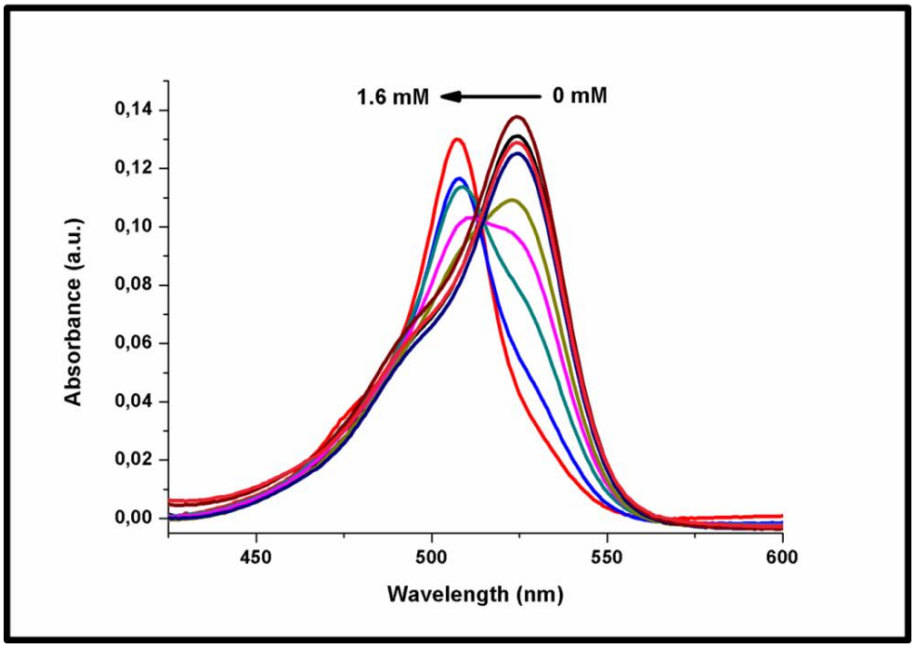


Figure 45: UV-VIS absorption spectra of compound **11** (5 µM) in 50 mM HEPES:CH_3CN (80:20, v/v, pH=7.2, at 25^0C) upon increased L-Cys concentrations

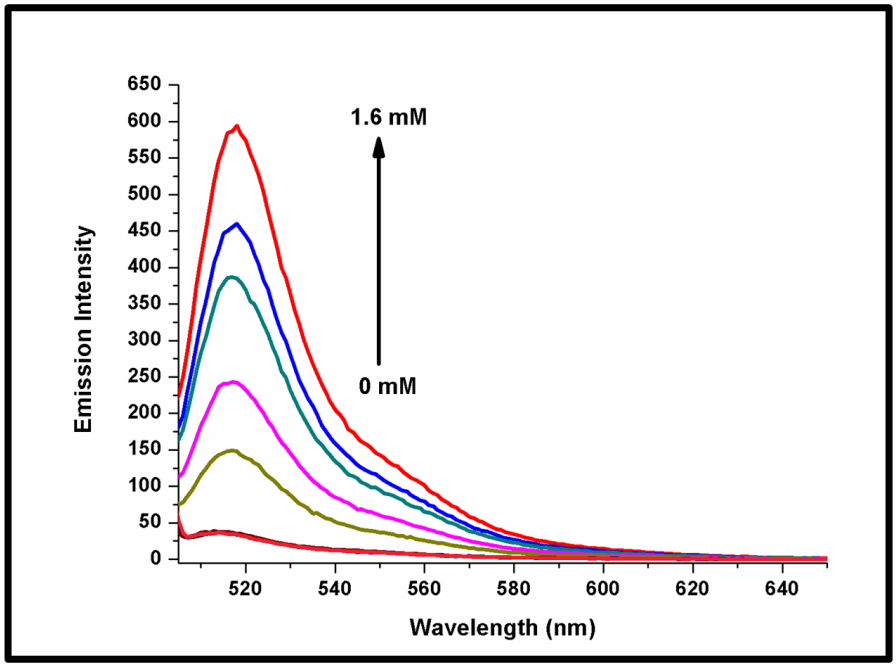


Figure 46: Emission spectra of compound **11** in 50 mM HEPES:CH_3CN (80:20, v/v, pH=7.2, at 25^0C) upon increased Cys addition

Dye 2 is red in color and when it is reacted with L-cysteine, it color turns out to be orange as shown in figure 47. The color and emission differences of the conjugated and the unconjugated dyes are also shown under UV light. The fluorescence intensity is also observed easily in the Picture taken under UV light.

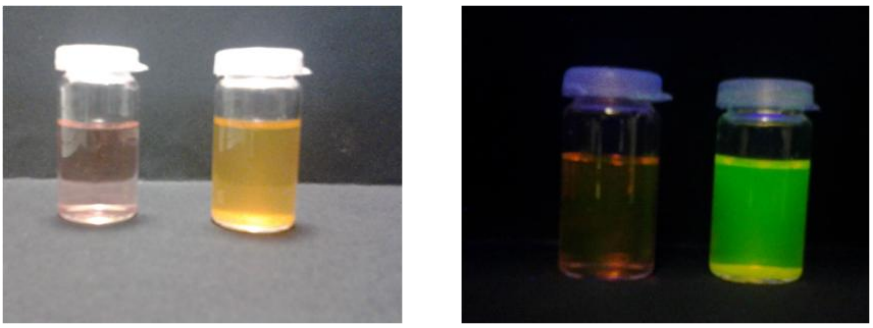


Figure 47: Compound **11** and its L-cysteine conjugated form respectively under ambient light (on the left), under UV-light (on the right)

Third target, namely compound **14** or dye 3 is another proposed labeling agent. A very similar work was published[119] this year in *ChemComm* as our work was in progress as shown in figure 48:

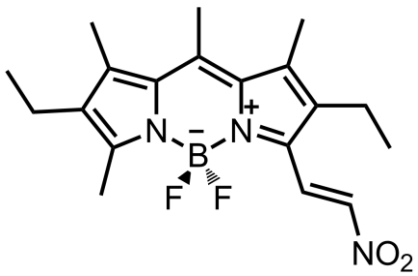


Figure 48: A nitroolefin functionalized BODIPY chemodosimeter

This BODIPY has a methyl (-CH_3) on its *meso* position whereas ours has a gallic acid derivative with TEG groups. They used 1:1 HEPES:ACN solution for their measurements, but we used 80:20 HEPES:ACN (v/v) because our molecule's water solubility is enhanced due to the TEG groups as compared to the one in this

article. Moreover, their BODIPY was for thiol sensing, ours is for protein labeling with superior solubility in inorganic environments.

Figure 49 shows absorbance spectra of compound **14** (2.5 µM) in reaction with L-L-cysteine with increasing concentrations between 0 mM-0.5 mM in 50 mM HEPES:ACN (80/20, v/v, pH:7.4 at 25 ^{0}C). Records began with the absence of cysteine and it went up to 200 equivalent L-cysteine. Dye 3 possesses an absorption band at 570 nm which declined with addition of Cys and a new one at 527 nm started to appear. There was a total of 43 nm blue shift in the spectra and an isosbestic point at 550 nm was observed. Similar to those obtained for compound **11,** this was a consequence of blocking the ICT process. Since NO_2 is fit in perfect conjugation with the Π- electron system of the BODIPY core, this change was an expected one. In other words, there was a ‘’turn-off’’ process for ICT and we recorded a hypsochromic shift in absorption spectra. PeT process is also off at this stage and the overall result is both a colorimetric and a fluorometric change.

Figure 50 shows the emission changes after L-Cys addition. Excitation is made at 520 nm. As it is clear, there is about 20-fold increase in emission intensity with the addition of 200 equivalent Cys. Figure 51 shows the color differences of the blank dye and its conjugated form to L-cysteine both in ambient light and under UV light. Dye **3** is normally pink and it turns out to be yellow with cysteine. The birght emission is depicted in the picture, as well.

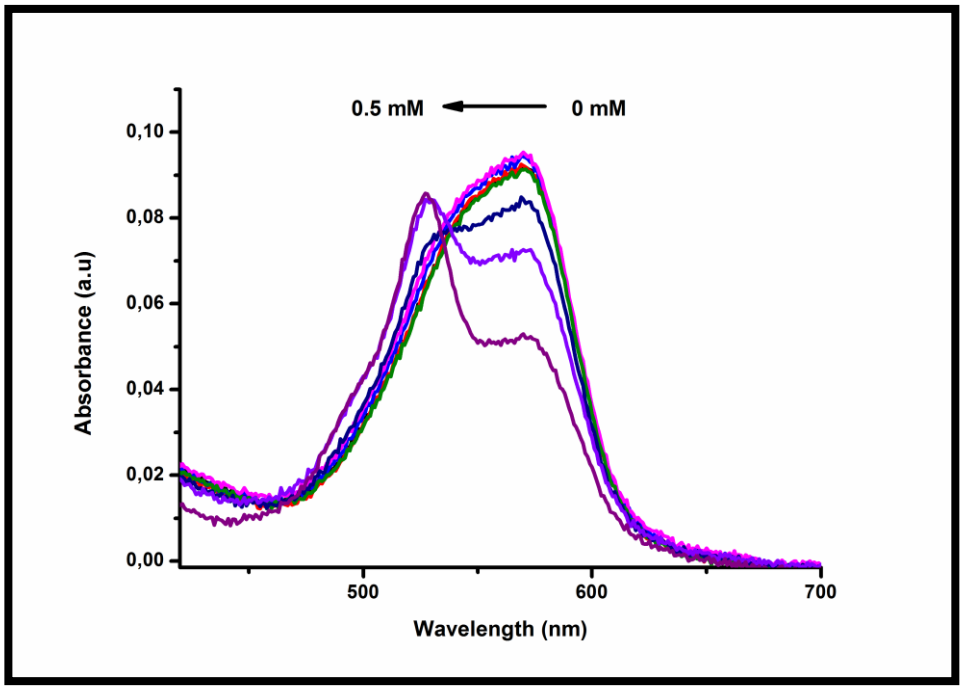


Figure 49: UV-VIS absorption spectra of compound 14 (2.5 µM) in 50 mM HEPES:ACN (80/20, v/v, pH:7.4 at 25 ^{0}C) upon increased L-Cys concentrations

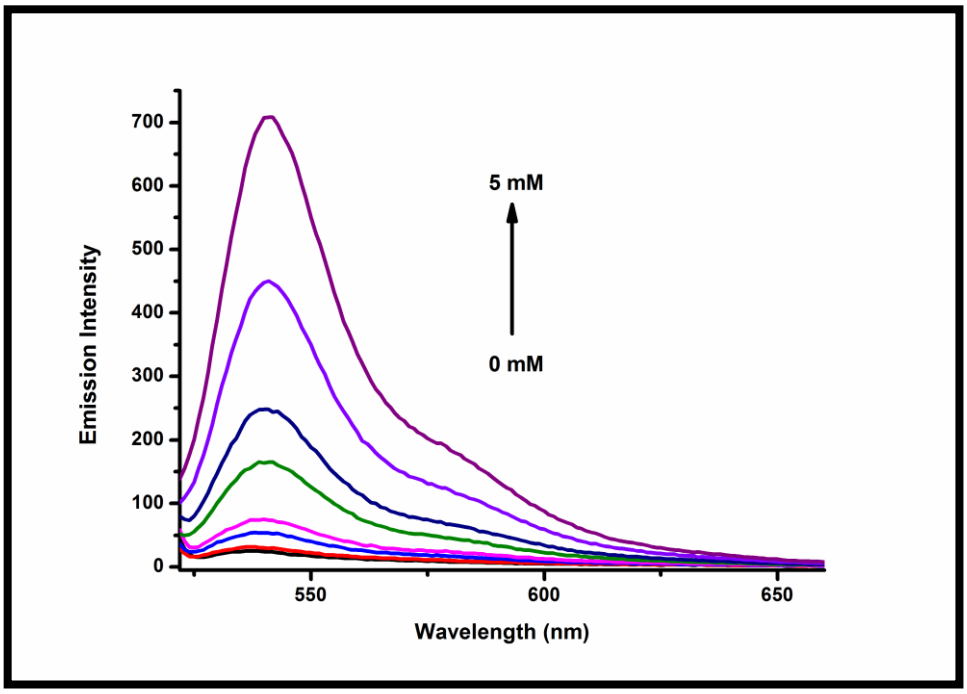


Figure 50: Fluorescence spectra of compound **14** in 50 mM HEPES:ACN (80/20, v/v, pH:7.4 at 25 ^{0}C) upon increased L-Cys concentration

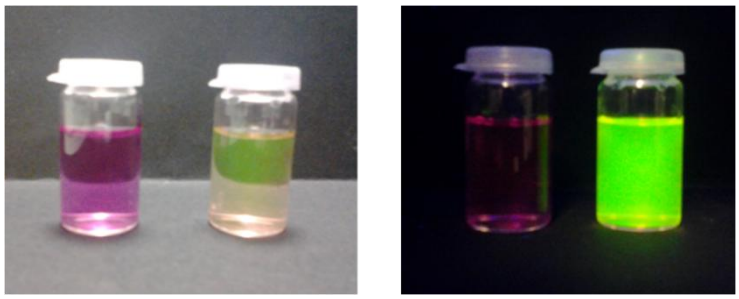


Figure 51: Compound 14 and its L-cysteine conjugated form respectively under ambient light (on the left), under UV-light (on the right)

3.4 Protein Labeling Studies & Spectroscopic Data

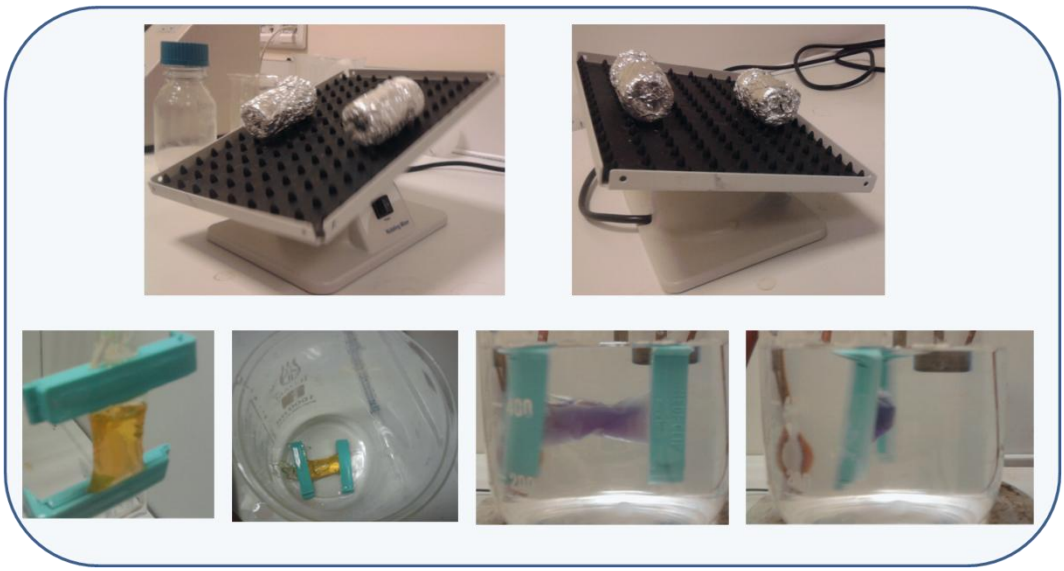


Figure 52: Incubation for protein labeling (on top) & dialysis (on the bottom)

As the last step of the study, the dyes were used to label the proteins. Bovine Serum Albumin (BSA) was chosen as the sample protein. For conjugation of the dyes with proteins, the first step was to prepare a solution of them. For studying with compound **4**, BSA was dissolved in PBS (1 mg/mL). BODIPY was added onto it as 50-fold molar excess in order to ensure the reaction. It was taken from a stock sloution prepared in DMSO. For studying with compound **11**, BSA was again dissolved in buffer, HEPES was preferred. Assuming that this could be a slower reactionas compared to the previous one, 100-fold molar excess of dye 2 was added from a stock solution in DMSO. The mixtures were protected from light, incubated overnight, and left on a nutating mixer at room temperature as depicted in the figure 52 on top. Then to remove excess BODIPY, we chose dialysis, which is a very simple method, instead of HPLC or other methods. For dialysis, a dialysis membrane with molecular weight cut off (MWCO) between 12-6000 KDa was used. Mixture was put in the membrane and dialyzed for about 12 hours. Compound **4** is 0.395 KDa, compound **11** is 0.881 KDa, compound **14** is 0.923 KDa and BSA is 66 KDa (1Da is equal to 1 g/mol). The logic behind this is very basic; particles with diameters smaller than that of the pores of the membrane escape from the membrane and those with bigger diameters stay inside and a bulk of solvent outside makes the process easier and faster. As the dialysis solvent is changed, one can remove the excess dye from the medium. The residue in the membrane is just the BODIPY-BSA conjugate which could be taken from the membrane and used in analysis.

During dialysis, the outer solvent enters inside the membrane, the one inside goes off the membrane and an equilibrium is reached. During these dyanmics, inside conjugate is diluted. An amount of it is taken from inside the membrane and used in spectroscopic analysis. In order to determine the concentration of it, we firstly measure its absorbance and by comparison with a BODIPY solution with a known concentration, one can calculate the concentration of the conjugate from Beer-Lambert Law. For compound **4** (dye 1), as figure 53 shows, absorbance at maxiumum for a 1 µM dye is 0.0672, it is 0.0551 for the conjugate and from the formula $A_1/A_2 = C_1/C_2$ we find the concentration of the conjugated dye as 0.82 µM. As it is shown on figure 54, emission at these concentrations has increased

from nearly zero to nearly 700 hunderds. (λex 490 nm at 25 °C, slit 5-2.5).This spectra is the proof of disruption of PeT as a result of conjugation of the dye to protein.

Figure 55 shows the absorbance spectra for dye 2 and for BSA-dye 2 conjugate. For compound **11** (dye 2), absorbance at maxiumum for a 2 µM dye is 0.09862, it is 0.11304 for the conjugate and from the formula $A_1/A_2 = C_1/C_2$ we find the concentration of the conjugated dye as 2.8 µM. For these concentrations, emissions were also recorded as shown in figure 56 . As it is clear, emission has increased nearly to 500 from 80 (λex 500 nm at 25 °C, slit 5-5). 8-fold increase is recorded in total.

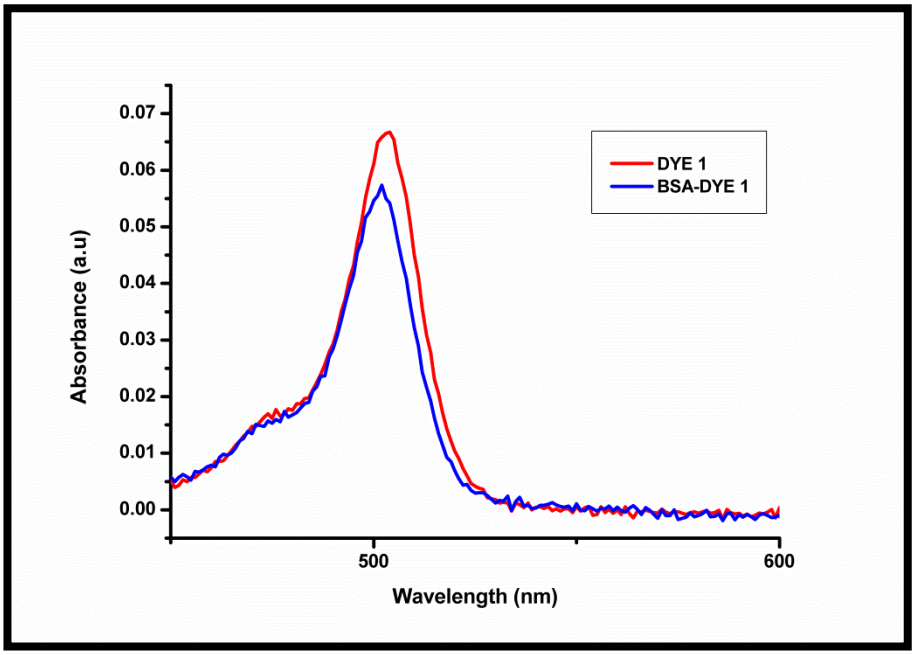


Figure 53: Absorbance spectra of dye 1 and BSA-dye 1 conjugate in 1x PBS:DMSO (5:95, V/V, pH:7.4 at 25^0C)

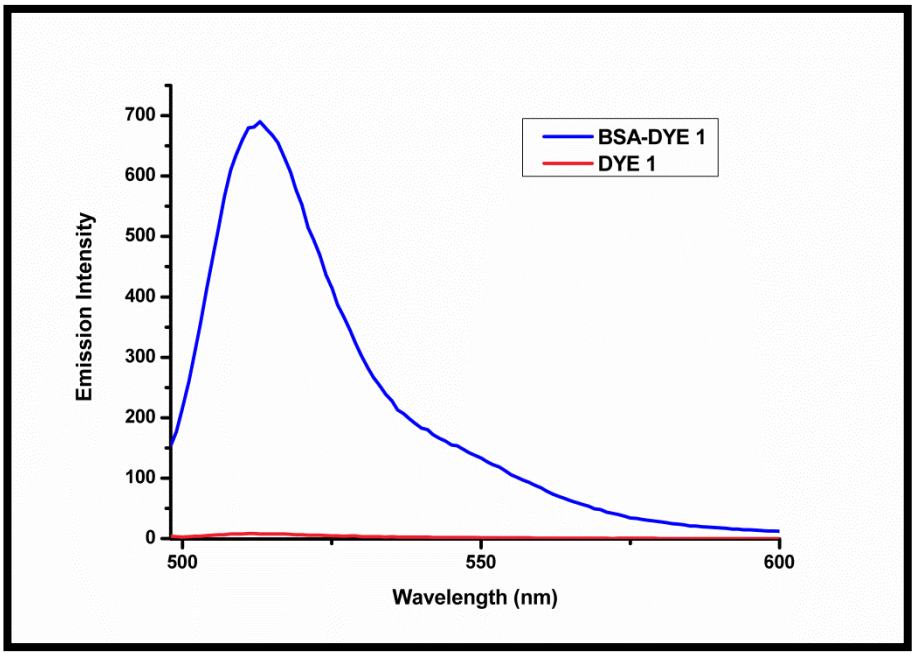


Figure 54: Emission spectra of dye 1 and BSA-Dye 1 conjugate in 1x PBS:DMSO (5:95, V/V, pH:7.4 at 25^0C)

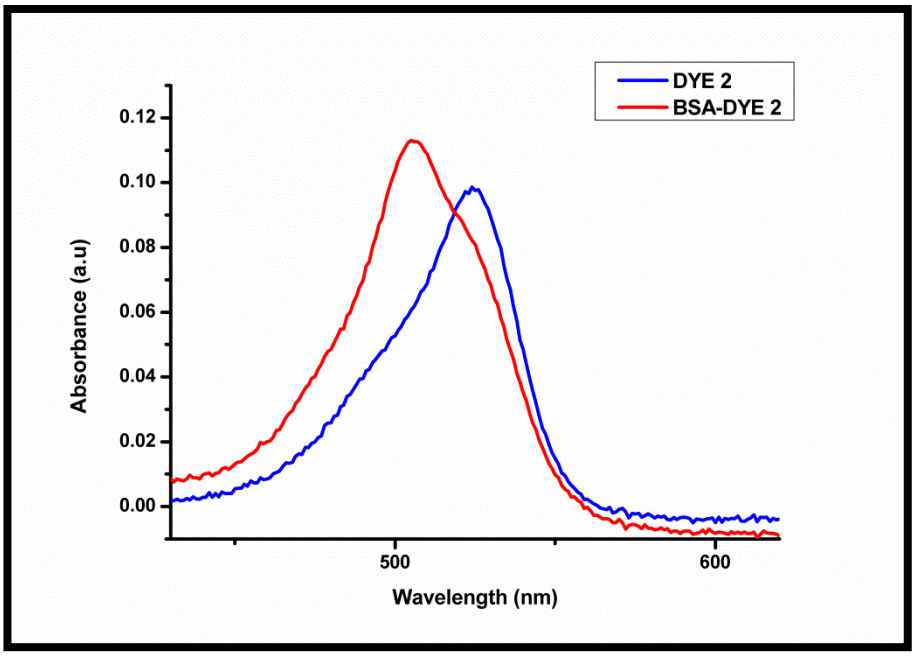


Figure 55: Absorbance spectra of dye 2 and BSA-Dye 2 conjugate in 50 mM HEPES:ACN (80/20, v/v, pH:7.4 at 25 ^{0}C

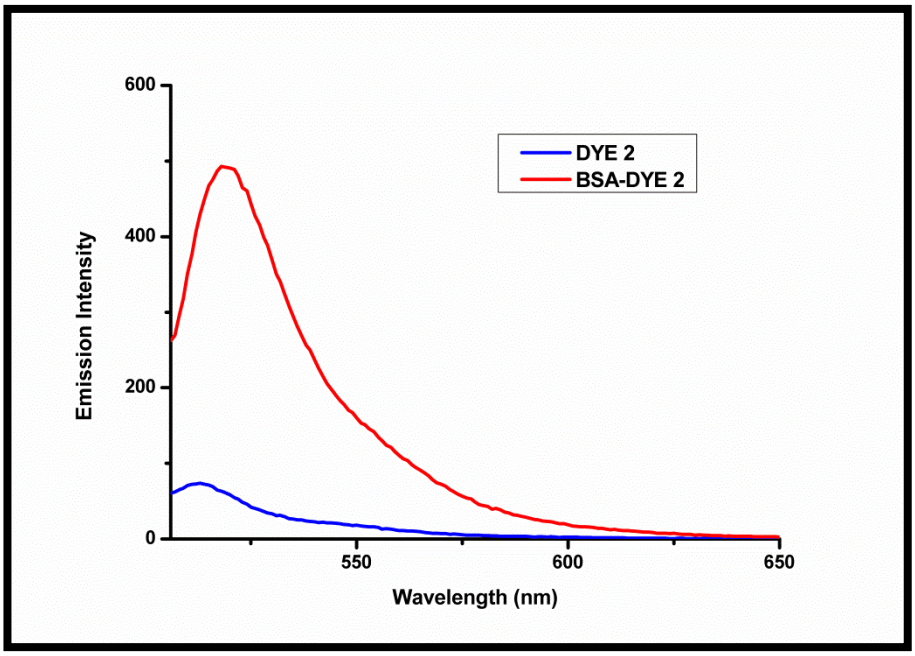


Figure 56: Fluorescence spectra of dye 2 and BSA-dye 2 conjugate in 50 mM HEPES:ACN (80/20, v/v, pH:7.4 at 25 ^{0}C

Protein labeling study for compound **14** is the future work. According to the article in reference 118, instead of nitroolefin conjugation to BODIPY dye, there occurs elimination of nitroolefin upon addition of mercaptoethanol as shown in figure 57. Disruption of ICT is stil observed and emission intensity increases but the mechanism is different. Consequently, the same result may be obtained in our case, as well.

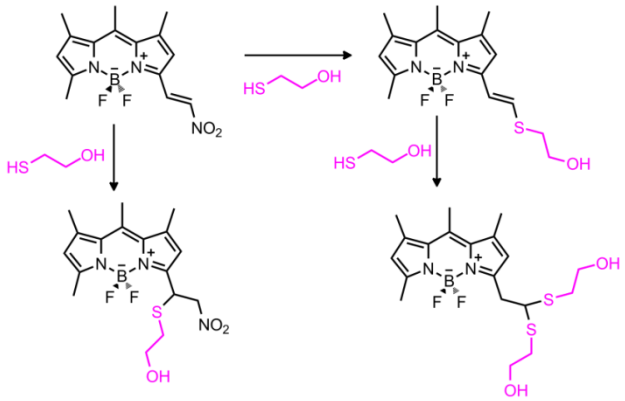


Figure 57: Elimination of nitroolefin from reference 118

CHAPTER 4

Conclusion

In this study, we have synthesized three different nitroolefin functionalized BODIPY dyes for protein labeling. Their characterization were made successfully. Since thiol groups can make nucleophilic attack easily and nitroolefin groups are good Micheal acceptors available for a nucleophilic attack, a chemical bond between them is formed and fluorescence is increased making the conjugate highly bright. This is the logic behind our study and it makes our compounds available for protein labeling. Results show that compound **4** is the most efficient in terms of fluorescence, though it is not very soluble in water. Compound **11** and compound **14** have TEG groups and therefore they are enhanced in terms of water-solubility. Protein labeling agents generally bond to proteins either from N-terminus or C-terminus, we herein reported a connection from thiol groups of proteins. We also engineered BODIPYs, incorporating nitroolefins into them and developped a path for nitroolefin functionalization reactions.

The study is a proof of principle work that basically shows the emission increase upon reaction of a nitroolefin with a thiol group based on blocking PeT and/or ICT phenomena. Our future work is expected to include protein labeling studies with compound **14** and to conduct cellular studies for all three compounds, as well. Our group is also expected to design and synthesize new protein labeling agents in the future.

REFERENCES

1) Bonifacino, J.S. (n.d.) *Protein Labeling and Immunoprecipitation.* Retrieved September 26,**2012** from http://webdoc.nyumc.org/nyumc/files/sun-lab/attachments/CPCB.ch07.Protein%20Labeling.pdf

2) Telefoncu, A. *Protein Etikleme Yöntemleri.* Retrieved September 26, **2012** from http://www.protein.ege.edu.tr/Konular/Protein%20EY.pdf

3) Thermo Scientific, Pierce Protein Research Products. Retrieved September 26, **2012** from http://www.piercenet.com/browse.cfm?fldID=4DDCADD2-5056-8A76-4E7E-2E00843BE346

4) G.T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego **(1996).**

5) Miller, L.W.& Cornish, V.W.*Current Opinion in Chemical Biology*. **2005**, 9, 56-61 (Review)

6) Griffin, B.A.; Adams, S.R.; Jones, J. and Tsien, R.Y. *Methods in Enzymology.* **2000**, 327, 565–578.

7) Griffin, B.A.; Adams, S.R. and Tsien, R.Y. *Science.***1998**, 281, 269–272

8) Adams, S.R.; Campbell, R.E.; Gross, L.A.; Martin, B.R.; Walkup, G.K.; Yao, Y.; Llopis, J. and Tsien, R.Y. *J Am Chem Soc*. **2002**, 124, 6063–6076.

9) Gaietta, G.; Deerinck, T.J.; Adams, S.R.; Bouwer, J.; Tour, O.; Laird, D.W.; Sosinsky, G.E.; Tsien, R.Y. and Ellisman, M.H. *Science*. **2002**, 296, 503–50.

10) Tour, O.; Meijer, R.M.; Zacharias, D.A.; Adams, S.R. and Tsien, R.Y. *Nat Biotechnol*. **2003**, 21, 1505–1508.

11) Nakanishi, J.; Nakajima, T.; Sato, M.; Ozawa, T.; Tohda, K. And Umezawa, Y. *Anal Chem*. **2001**, 73, 2920–2928.

12) Wang, L.; Brock, A.; Herberich, B. and Schultz, P.G.*Science*. **2001**, 292, 498–500.

13) Chin, J.W.; Cropp, T.A.; Anderson, J.C.; Mukherji, M.; Zhang, Z. And Schultz, P.G. *Science*. **2003**, 301, 964–967.

14) Zhang, Z.; Alfonta, L.; Tian, F.; Bursulaya, B.; Uryu, S.
; King, D.S. and Schultz, P.G. *Proc Natl Acad Sci USA*. **2004**, 101, 8882–8887.

15) Giriat, I.; Muir, T.W. *JACS*. **2003**, 125, 7180–7181.

16) Buskirk, A.R.; Ong, Y.C.; Gartner, Z.J. and Liu, D.R. *Proc Natl Acad Sci USA*. **2004**, 101, 10505–10510.

17) Yeo, D.S.; Srinivasan, R.; Uttamchandani, M.; Chen, G.Y.; Zhu, Q. and Yao, S.Q. *Chem Commun (Camb)*. **2003**, 2870–2871.

18) *Bioconjugation.* Retrieved September 26, 2102 from http://en.wikipedia.org/wiki/Bioconjugation.

19) Sun, C.; Yang, J.; Li,L.; Wu,X.; Liu,Y. and Liu,S. *J.jchromb.***2003,** 803, 173-190. (Review).

20) Marks, K.M.; Nolan, G.P. *Nature Methods.* **2006**, 3(8), 591-596 (Review).

21) Lambalot, R. H.; Gehring, A. M.; Flugel, R. S.; Zuber, P.; LaCelle, M.; Marahiel, M. A.; Reid, R.; Khosla, C. And Walsh, C. T. *Chem. Biol*. **1996**, 3, 923-936.

22) George, N.; Pick, H.; Vogel, H.; Johnsson, N. and Kai Johnsson. *JACS.* **2004**, 126 (29), 8896-8897.

23) Chen, I.; Howarth, M.; Lin, W. and Ting, A.Y. *Nat. Methods.* **2005**, 2, 99–104.

24) Howarth, M.; Takao, K.; Hayashi, Y. and Ting, A.Y. *Proc. Natl. Acad. Sci. USA.* **2005**, 102, 7583–7588.

25) Keppler, A. *et al. Nat. Biotechnol.* **2003**, 21, 86–89.

26) Keppler, A.; Pick, H.; Arrivoli, C.; Vogel, H. And Johnsson, K. *Proc. Natl. Acad. Sci. USA.* **2004**, 101, 9955–9959.

27) Miller, L.W.; Sable, J.; Goelet, P.; Sheetz, M.P. and Cornish, V.W. *Angew. Chem. Int. Edn Engl* .**2004**, 43,1672–1675 (2004).

28) Calloway, N.; Choob, M.; Sanz, A.; Sheetz, M.P.; Miller, L.W.; Cornish, M.W. *Chem Bio Chem.* **2007**, 8,767-774.

29) Farinas, J. & Verkman, A.S. *J. Biol. Chem.***1999**, 274, 7603–7606.

30) Guignet, E.G.; Hovius, R. & Vogel, H. *Nat. Biotechnol.* **2004**, *22*, 440–444.

31) Rozinov, M.N. & Nolan, G.P. *Chem. Biol.***1998**, 5, 713–728.

32) Marks, K.M.; Rosinov, M. and Nolan, G.P. *Chem. Biol.* **2004**, 11, 347–356.

33) Marks, K.M.; Braun, P.D. and Nolan, G.P. *Proc. Natl. Acad. Sci. USA.* **2004**, 101, 9982–9987.

34) Isolation/Modification/Labeling, protein labeling. Retrieved September 29, 2012 from http://www.interchim.fr/ft/B/BB041A.pdf

35) Shimomura, O.; Johnson, F.H. and Saiga, Y. *J. Cell. Comp. Physiol*. **1962**, 59 223-240.

36.)Tsien, R. *Annu. Rev. Biochem*. **1998**, 67, 509-544.

37) Heim, R. *et al. Proc. Natl. Acad. Sci. USA.* **1994**, 91 12501-12504.

38) Heim, R. and Tsien, R. *Curr. Biol*. **1996**, 6, 178-182.

39) Ormö, M. *et al. Science.* **1966**, 273, 1392-1395.

40) Matz, M. *et al*. *Nature Biotechnology***. 1999**, 17, 969-973.

41) Baird, G.S. *et al*. *Proc. Natl. Acad. Sci.* **2000**, ***97,*** 11984-11989.

42) Shaner, N.C. *et al. Nature Methods*. **2008**, 5, 545-551.

43) The Nobel Prize in Chemistry 2008. *Retrieved October 20, 2012, from:* http://www.nobelprize.org/nobel_prizes/nobelguide_che.pdf

44) Tsien, R.Y. *Annu.Rev.Biochem*. **1998**, 67, 509-544.

45) Fradkov,F.A.; Verkhusha, V.A.; Staroverov, D.B.; Bulina, E.M.; Yanushevich, G.Y.; Martynov, I.V.; Lukyanov, S. and Lukyanov A.K. *Biochem. J*.**2002**, 368, 17-21.

46) GFP. *Retrieved october 23, 2012, from:* http://www.tsienlab.ucsd.edu/HTML/Images/IMAGE%20-%20Rendered%20GFP%20-%201600.jpg

47) Alba, F.J.; Bermudez, A.; Bartolome, S. and Dabana, J.R. *Biotechniques.* **1996**, 21 625.

48) Steinberg, T.H.; Haugland, R.P. and Singer, V. *Anal. Biochem*. **1996**, 239, 238.

49) Hiraoka, T., Glick, D. *Anal. Biochem*. **1963**, 5, 49.

50) Maria, L.B. *Anal. Biochem*. **1973**, 53, 12.

51) Baar, M.J.; Patonay, G. *Anal. Chem*.**1999**, 71, 66.

52) Legender, B.L.; Moberg, D.L.; Williams, D.C.; Soper, S.A. *J. Chramatogr. A.* **1997**, 779, 185.

53) Liu, X.; Wang, W.S.; Wang, H.; Zhang, H.S. *J.Anal. Sci*. **2001**, 17, 346.

54) Mank, A.J.G.; Molenaar, E.J.; Lingeman, H. *Anal. Chem.* **1993**, 65, 2197.

55) Davidson, Y.Y.; Gunn, B.M.; Soper, S.A. *Appl. Spectrosc*.**1996**, 50, 211.

56) Mank, A.J.G.; Vander Laan, H.T.C.; Lingeman, H.; Gooijer, C.; Brinkman, U.A.Th.; Velthorst, N.H. *Anal.Chem.***1995**, 67, 1742.

57) Terpetsching, E.; Szmacinski, H.; Lakowicz, J.R. *Anal. Chim. Acta.***1993**, 282 633.

58) Terpetsching, E.; Szmacinski, H.; Ozinskas, A., Lakowicz, J.R. *Anal.Biochem.* **1994**, 217, 197.

59) Imasaka, T.; Fuchigami, T.; Ishibashi, N. *Anal. Sci.* **1991**, **7,** 491.

60) Brown, M.B.; Edmonds, T.E.; Miller, J.N.; Riley, D.P.; Seare, N.J. *Analyst*. **1993**,118, 407.

61) Rhodes, G.R.; Boppana, V.K. *J.Chromotogr*. **1988**, 444, 123.

62) Hiraga, Y.; Kinoshita, T. *J.Chromotogr.* **1981**, 226, 43.

63) Van den Beld, C.M.B.; Tjaden, U.R.; Reibonad, N.J.; Van der Greef, J. *J.Control. Release*. **1993**,13, 237.

64) Fierabracci, V.; Maisello, P.; Novelli, M.; Bergamini, E. *J.Chromotogr.* **1991**, 570, 285.

65) DeSilva, K.; Jiang, Q.; Kuwana, T. *Biomed. Chromotogr.* **1995**, 9, 295.

66) Evangelista, R.A.; Pallak, A.; Allore, B.; Yernplaton, E.F.; Motin, R.C. and Diamandis, E.P. *Clin. Biochem.* **1988**, 21, 173.

67) Bailey, M.P.; Rocks, B.F.; Riley, C. *Analyst.* **1984**, 109, 1449.

68) Resichstein, E.; Shami, Y.; Ramjeesingh, M.; Diamandis, E.P. *Anal. Chem*.**1988**, 60, 1069.

69) Thanabal, V. and Krishnan,V.. *JACS.* **1982**, *104* (13), 3643-3650

70) Laurence J. Boucher. *JACS.* **1970**, *92* (9), 2725-273.

71) Collman, J.P.; Zhang, X.; Wong, K.; and Brauman, J.I. JACS. **1994,** *116* (14), 6245-6251.

72) Huang, C.Z.; Li, Y.F.; Mao, J.G.; Tan, D.G. *Analyst*. **1998**, 123,1401.

73) Aratani, N.; Kim, D.; Osuka,A. *Accounts of Chemical Research.* **2009**,*42* (12), 1922-1934.

74) Dodeigne, C.; Thunus, L.; Lejeune. R. *Talanta*. **2000**, 51, 415–439.

75) Ingersoll, C.M.; Watkins, A.N.; Baker, G.A.; Bright, F.V. *Appl. Spectrosc*. **1998**,52, 933.

76) Fang, X.; Li, J.J.; Perlette, J.; Tan, W.; Wang, K. *Anal. Chem*. **2000**, 72, 747a-753a.

77) Taylor, J.R.; Fang, M.M.; Nie, S. *Anal. Chem*. **2000**, 72, 1979.

78) Murray, C.B.; Norris, D.J.; Bawendi, M.G. *J. Am. Soc.***1993,** 115, 8706.

79) Brown Jr., M.B.; Moronne, M.; Gin, P.; Weiss, S.; Alivisatos, A.P. *Science*. **1998**, 281, 2013.

80) Rangs, L.B. *Pure Appl. Chem.* **1996**, 68, 1873.

81) Tsukiji, S.; Miyagawa, M.; Takaoka, Y.; Tamura, T. And Hamachi, I. *Nat. Chem. Biol*. **2009**, 5, 341–343.

82) Lin, Q.; Lim, R.K.V. *Nature Chemical Biology.* **2009**,5,275-276.

83) Hamachi, I.; Nagase, T. and Shinkai, S. *J. Am. Chem. Soc.* **2000**, 122, 12065–12066.

84) Los,G.V.; Encell,L.P.; McDougall,M.G.; Hartzell,D.D.; Karassina,N.; Zimprich,C., *et al*. *ACS Chemical Biology.* **2008**,3,373-382.

85) Chen, G.; Heim, A.; Riether, D.; Yee, D.; Milgrom,Y.; Gawinowicz,A.M. and Sames,D. *JACS.* **2003**, 125, 8130-8133.

86) Hori, Y.; Ueno, H.; Mizukami, S. and Kikuchi,K. *Journal of the American Chemical Society*. **2009**, *131* (46), 16610-16611.

87) Hayashi,T. and Hamachi,I. *Accounts of Chemical Research.* **2012**, *45* (9), 1460-1469.

88) Hao, Z.; Hong, S.; Chen, X. and Peng, R. Chen. *Accounts of Chemical Research.* **2011**, *44* (9), 742-751.

89) Nguyen, D.P.; Elliott, T.; Holt, M.; Muir, T.W. and Jason W. Chin. *JACS.* **2011**, *133* (30), 11418-11421.

90) Truong, F.; Yoo, T.H.; Lampo, T.J. and Tirrell, D.A. *JACS*. **2012**, *134* (20), 8551-8556.

91) Liu, X.; Wang, W.S.; Wang, H.; Zhang, H.S. *J. Anal. Sci. (Chinese)*. **2001**, 17, 346.

92) Calloway, N.T.; Choob, M.; Sanz, A.; Sheetz, M.P.; Miller, L.W. and Cornish, V.W. *Chem Bio Chem.* **2007**, 8, 767-774.

93) Nonaka, H.; Tsukiji, S.; Ojida, A.; Hamachi, I. *JACS.***2007**, 129(51), 15777-15779.

94) Treibs, A.; Kreuzer, F. H. *Justus Liebigs Ann. Chem*. **1968**, *718*, 208.

95) López Arbeloa F.; Bañuelos J.; Martínez V.; Arbeloa T.; López Arbeloa I. *Int Rev Phys Chem*, **2005**, *24*, 339–374

96) Deniz, E.; Isbasar, G.C.; Bozdemir, O.A.; Yildirim, L.T.; Siemiarczuk, A. and Akkaya, E.U. *Org. Lett*. **2008,** Vol. 10, No. 16.

97) Dost, Z.; Atilgan, S.; Akkaya, E. U. *Tetrahedron.* **2006**, 62, 8484–8488.

98) Loudet, A.; Burgess, K. *Chem. Rev*. **2007**, 107, 4891–4932.

99) Ela, S.E.; Yilmaz, M.D.; Icli, B.; Dede, Y.; Icli, S.; Akkaya, E.U. *Org.Lett.* **2008**, *10* (15), 3299-3302.

100) Wan, C-W.; Burghart, A.; Chen, J.; Bergstrˆm, F.; Johansson, L.B.; Wolford, M.F.; GyumKim, T.; Topp, M.R.; Hochstrasser, R.M. and Burgess, K. *Chem. Eur. J*. **2003**, 9, 4430 ±4441.

101) Bozdemir, O.A.; Cakmak, Y.; Sozmen, F.; Ozdemir, T.; Siemiarczuk, A. and Akkaya, E.U. *Chem. Eur. J.* **2010**, 16, 6346 –6351.

102) Ozlem, S. and Akkaya, E.U. *JACS.* **2009** *131* (1), 48-49.

103) Coskun, A.; Yilmaz, M.D.; Akkaya, E. U. *Org. Lett*., **2007**, *9*, 607.

104) Coskun, A.; Deniz, E.; Akkaya, E. U. *Tetrahedron. Lett*., **2007**, *48*, 5359.

105.) Karolin, J.; Johansson, L.B.-A.; Strandberg, L.; Ny, T. J. *Am. Chem. Soc.*, **1994**, *116*, 7801

106) Niu, S.L.; Massif, C.; Ulrich, G.; Ziessel, R.; Renard, P-Y.; Romieu, A. *Org.Biomol.Chem.* **2011**, 9,66.

107) Ulrich, G.; Goze, C.; Guardigli, M.; Roda, A.; Ziessel, R. *Angew. Chem. Int. Ed.* **2005**, 44, 3694 –3698.

108) J.R. Albani, *Principles and Applications of Fluorescence Spectroscopy*, Blackwell Publishing, Oxford, UK, **2007.**

109) G. G. Stokes, *Org. Lett*., **1852**, 142, 463.

110) Lakowicz, J.R. *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, **1999.**

111) A. P. de Silva, T. S. Moody, G. D. Wright, *Analyst*, **2009**, 134, 2385.

112) B. Valeur, I. Leray, *Coord. Chem. Rev*., **2000**, 205, 3.

113) Matsumoto, T.; Urano, Y.; Takahashi, Y.; Mori, Y.; Terai, T.; Negano, T. *JOC.* **2011**, 76, 3616-3625.

114) Oar, M.A.; Serin, J.M.; Dichtel, W.R.; Frechet, J.M.J.; Ohulchanskyy, T.Y. and Prasad, P.N. *Chem. Mater*. **2005**, 17, 2267–2275.

115) Micheal Addition. *Retrieved December 16, 2012, from:* **http://www.adichemistry.com/organic/namedreactions/michael/michael-addition-1.html**

116) Micheal addition. *Retrieved December 16, 2012, from:* **http://www.organic-chemistry.org/namedreactions/michael-addition.shtm**

117) Li, J.J. *Name reactions.* Springer,4th edition, USA, 2009.

118) Zhang, M.; Wu, Y.; Zhang, S.; Zhu, S.; Wu, Q.; Jiao, L.; Hao, E. *Chem Comm.* **2012**, 48, 8925-8927.

APPENDIX A

NMR SPECTRA

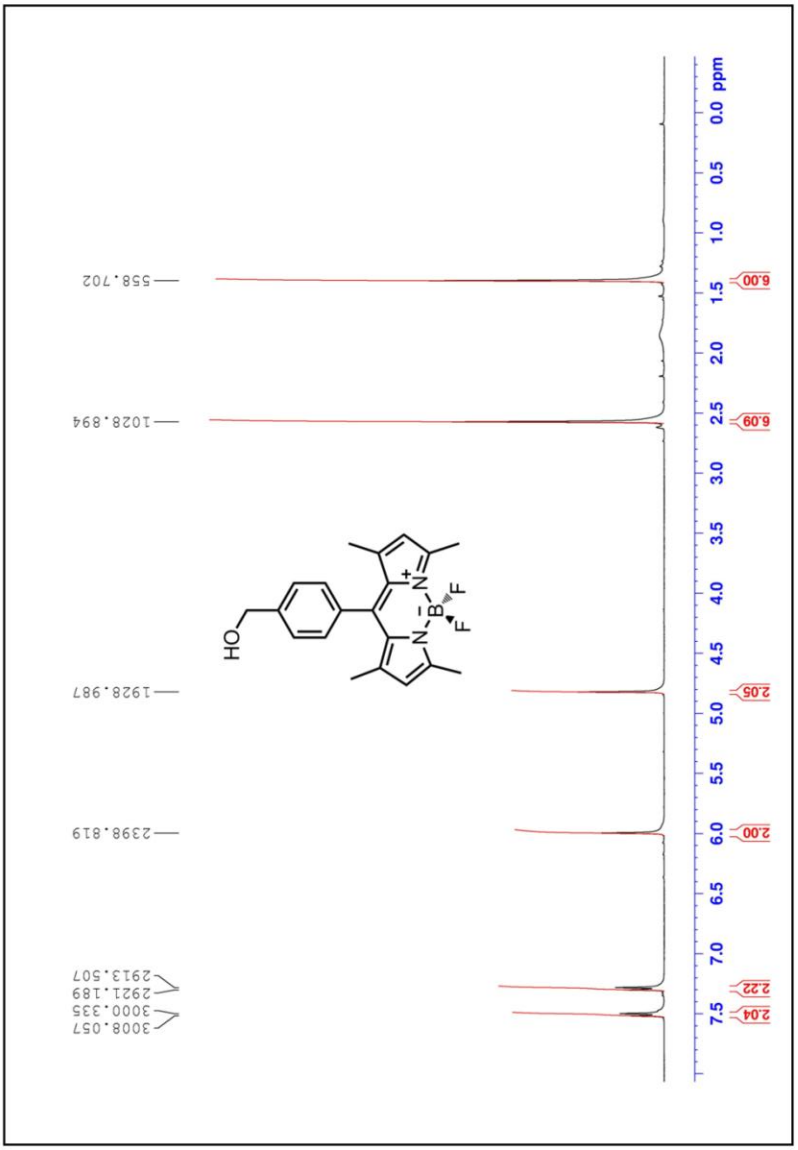


Figure 58: ^{1}H NMR spectrum of compound **2**

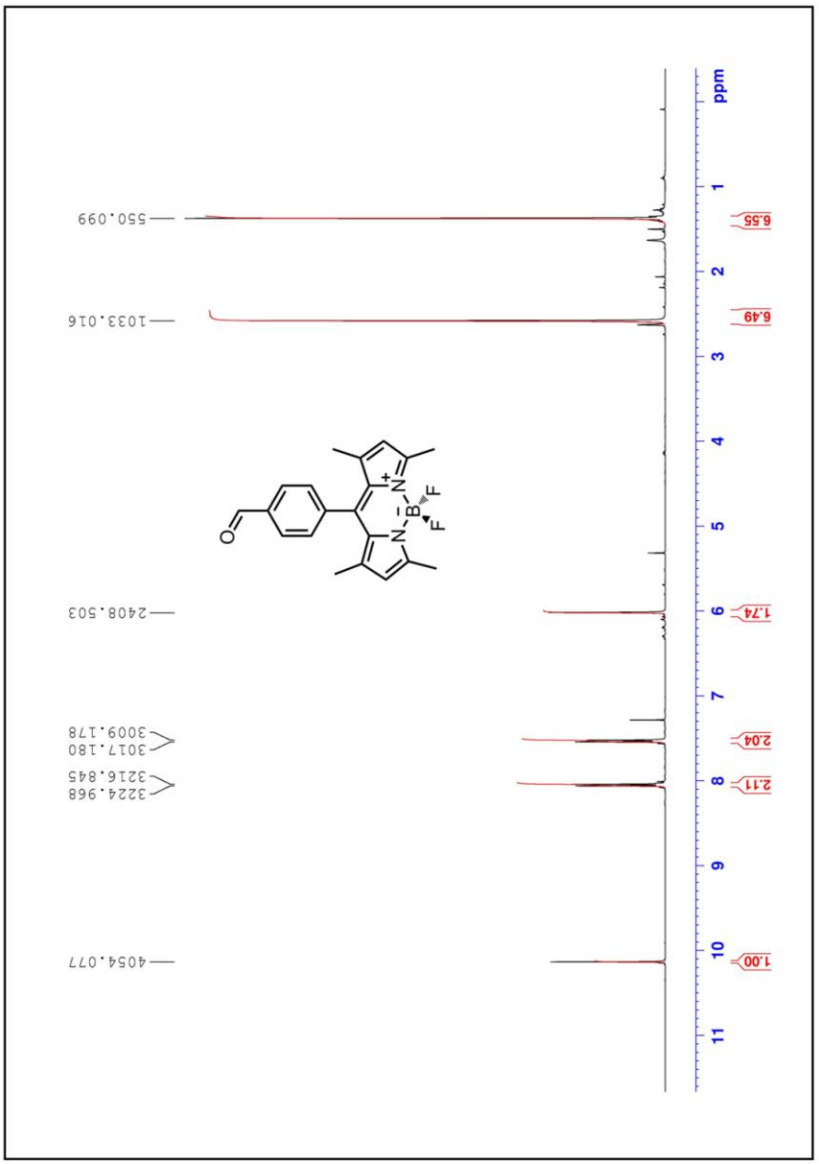


Figure 59: ^{1}H NMR spectrum of compound **3**

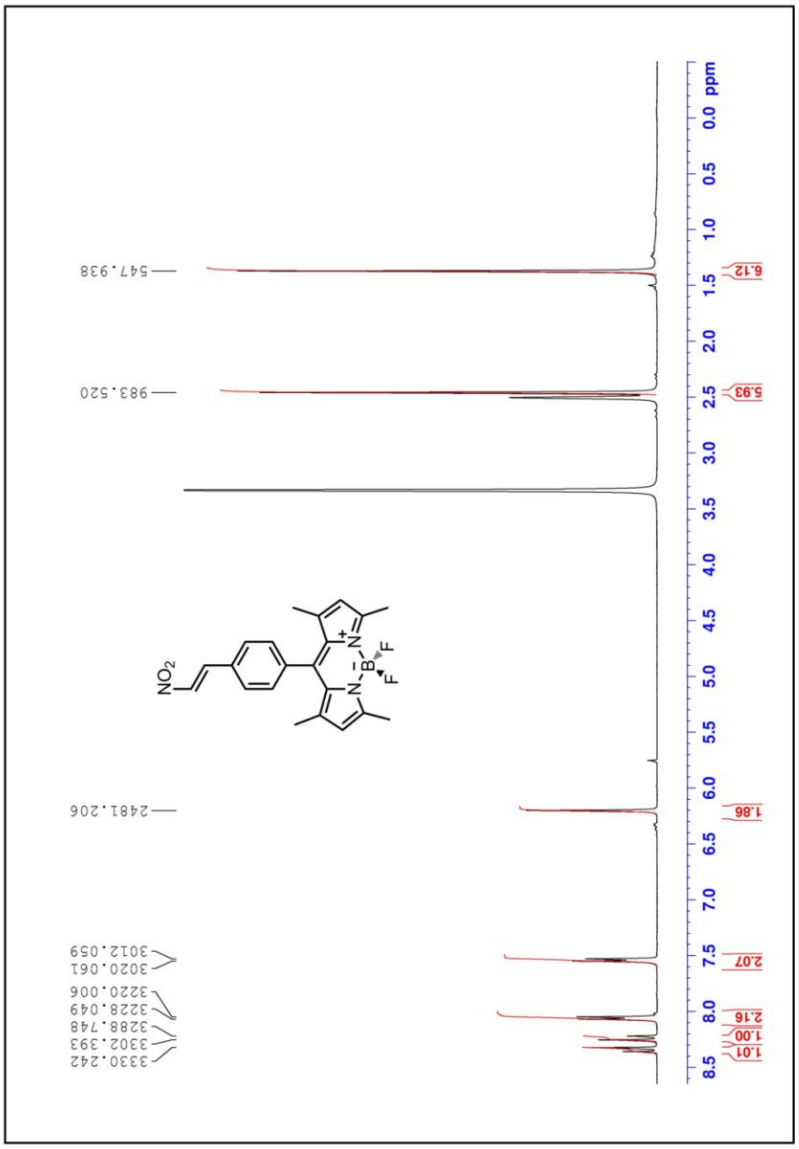


Figure 60: ^{1}H NMR spectrum of compound 4

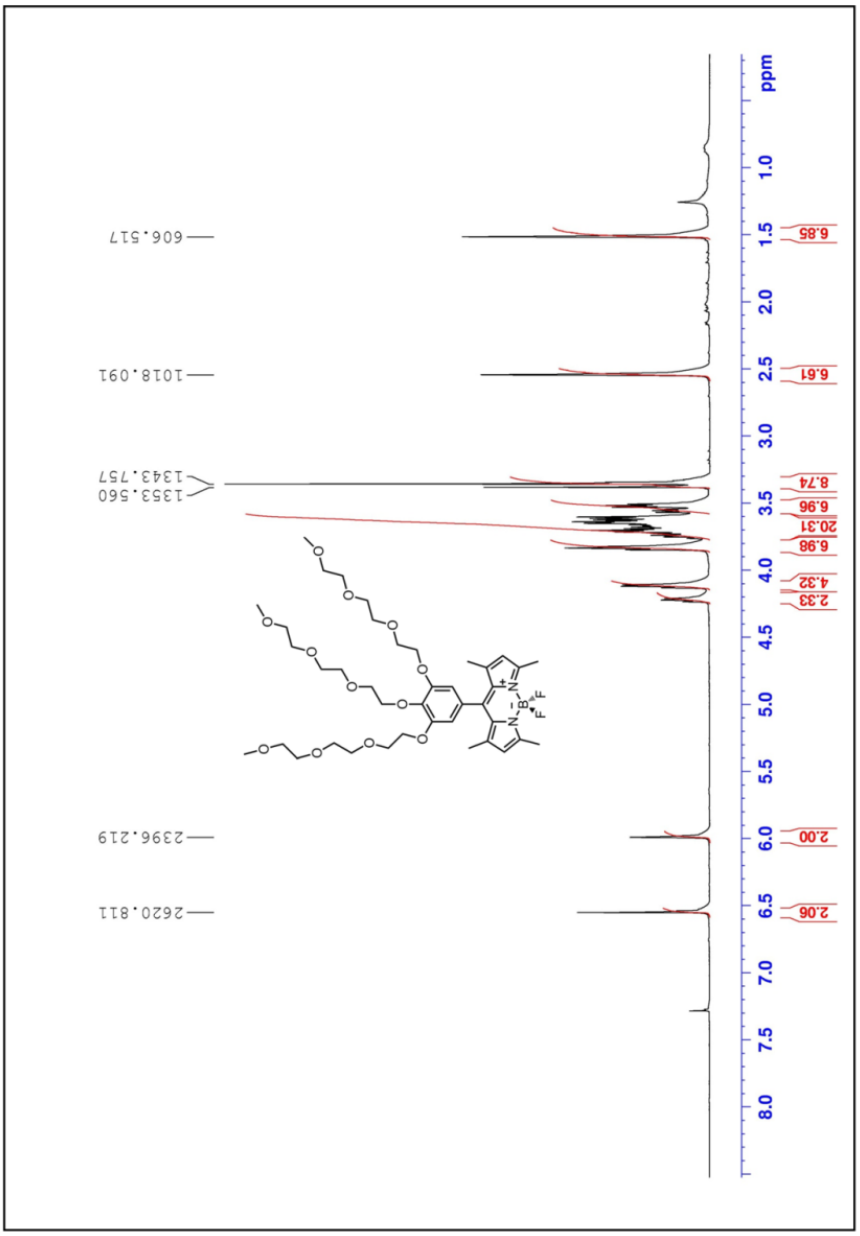


Figure 61: ^{1}H NMR spectrum of compound **9**

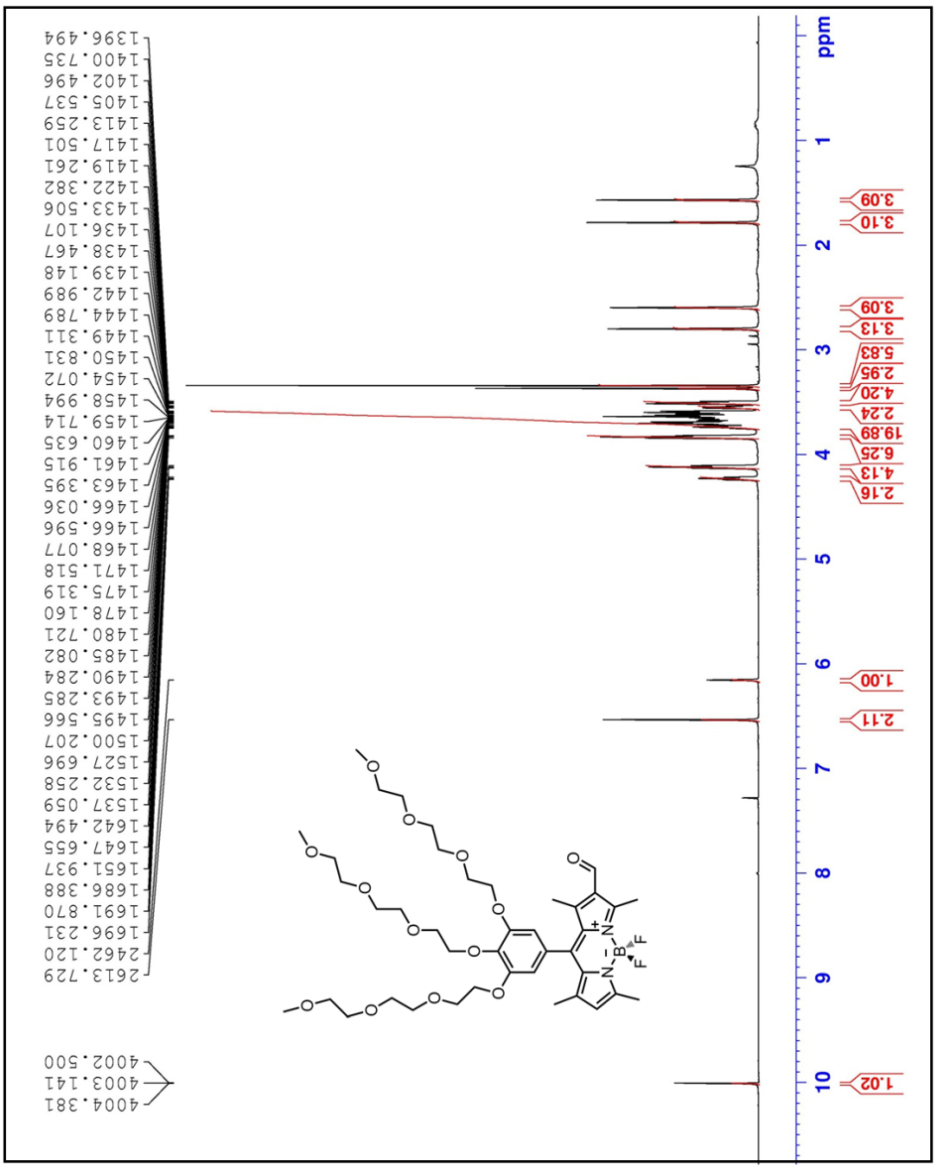


Figure 62: ^{1}H NMR spectrum of compound **10**

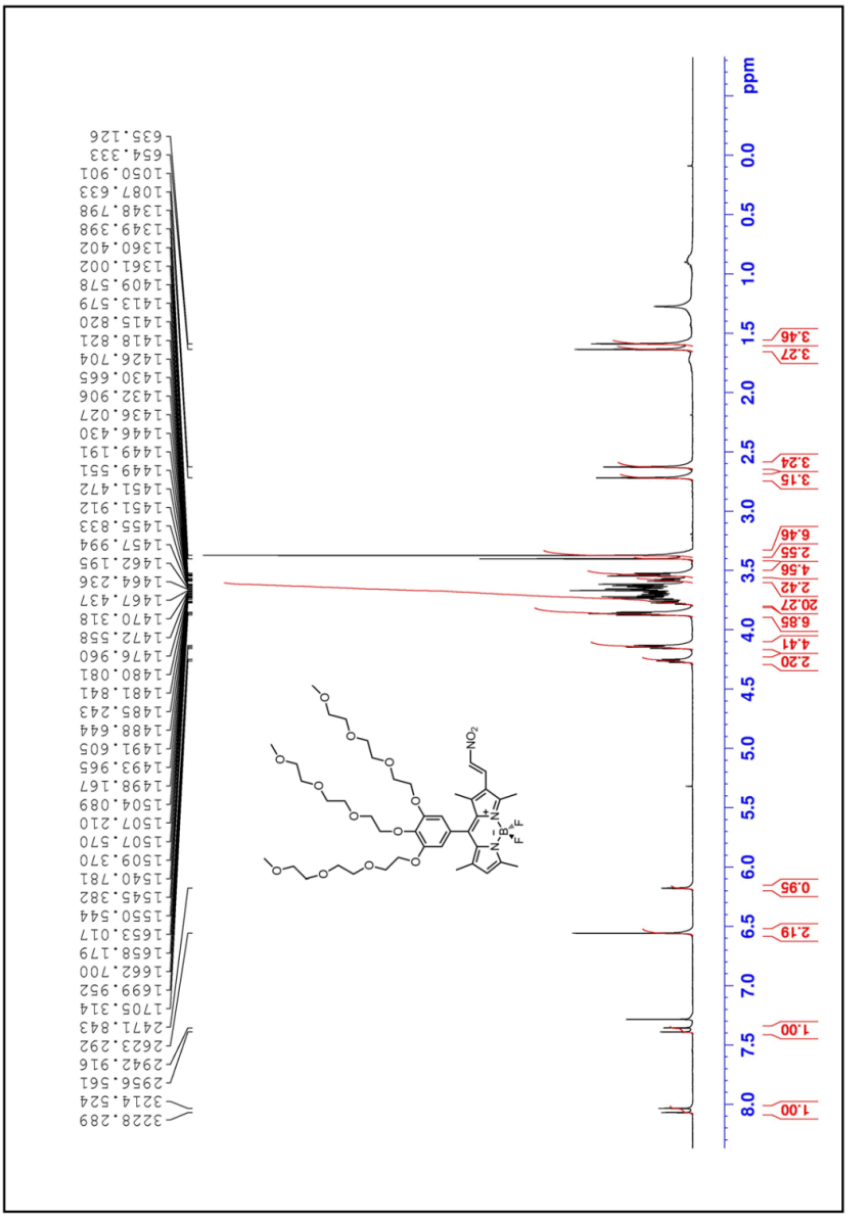


Figure 63: ^{1}H NMR spectrum of compound **11**

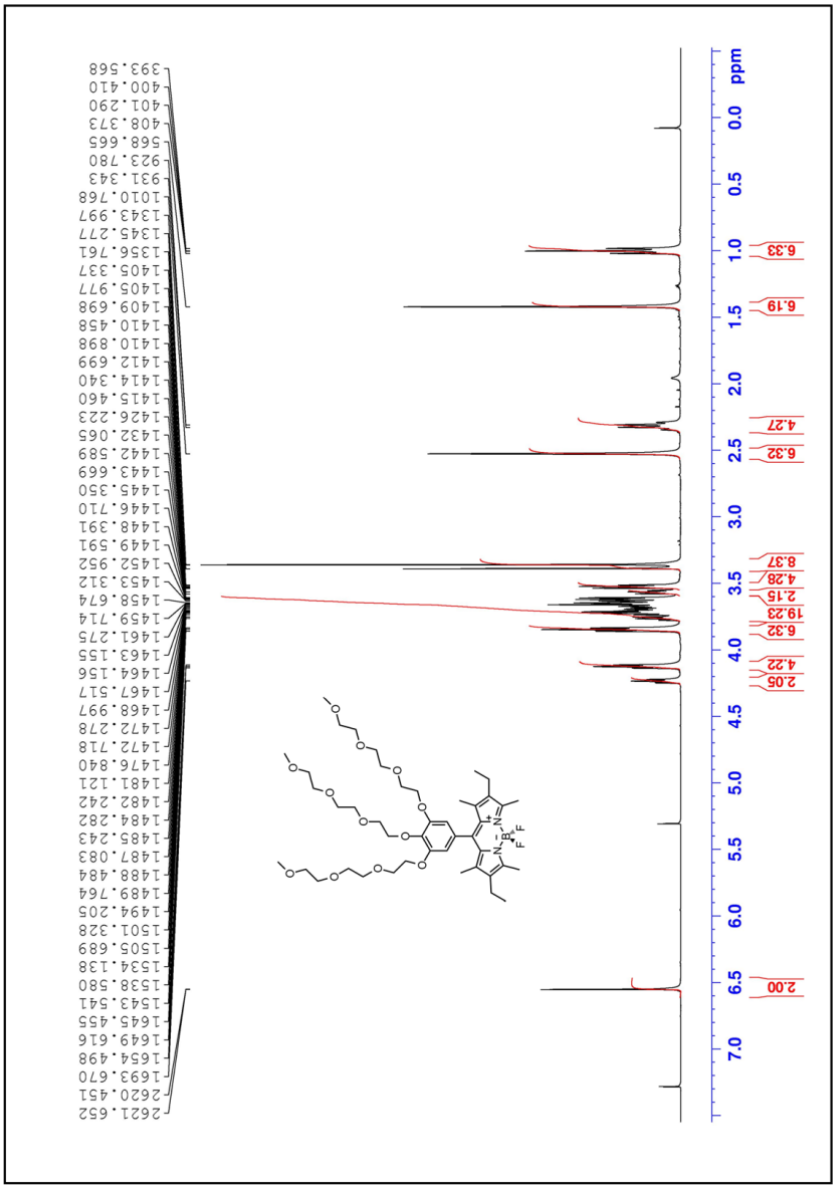


Figure 64: ^{1}H NMR spectrum of compound **12**

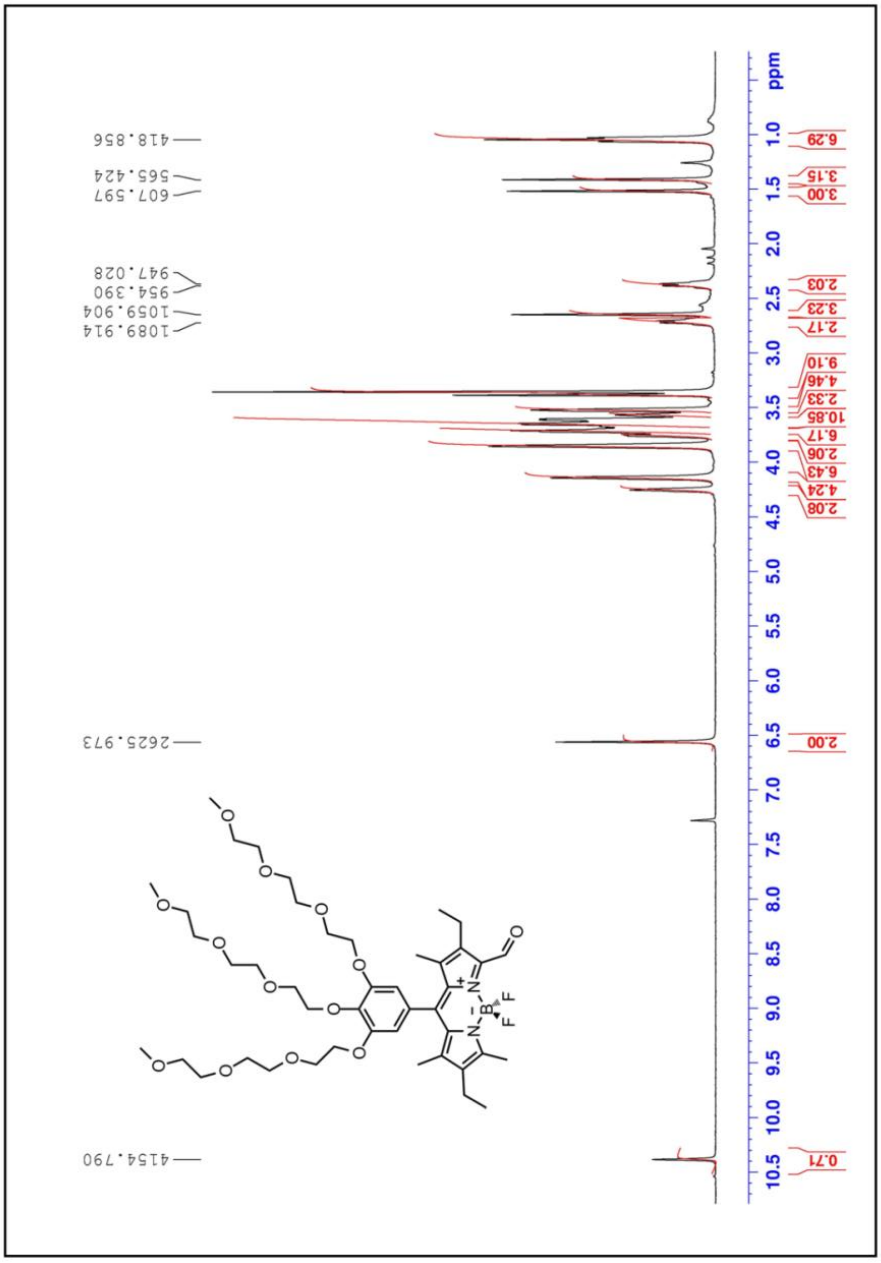


Figure 65: ^{1}H NMR spectrum of compound **13**

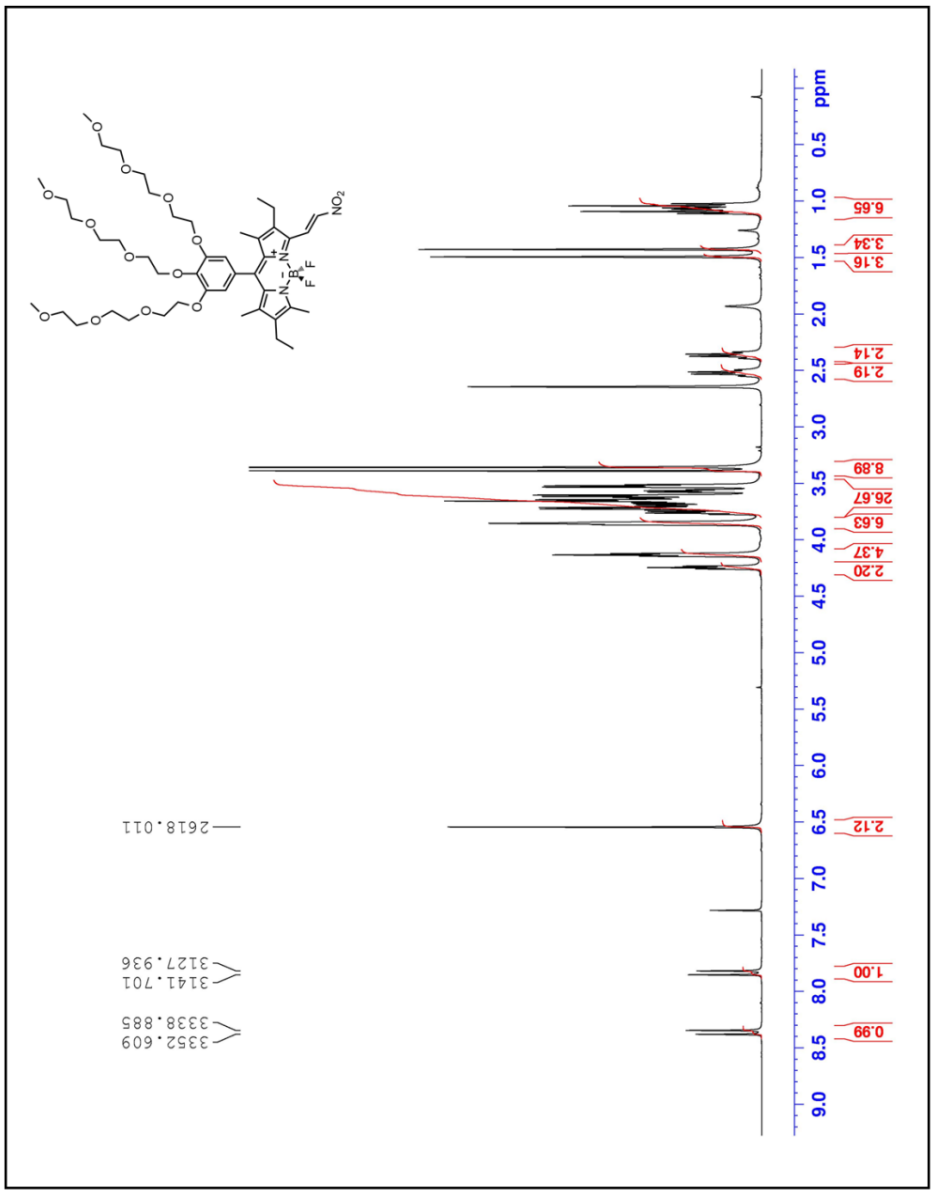


Figure 66: ^{1}H NMR spectrum of compound **14**

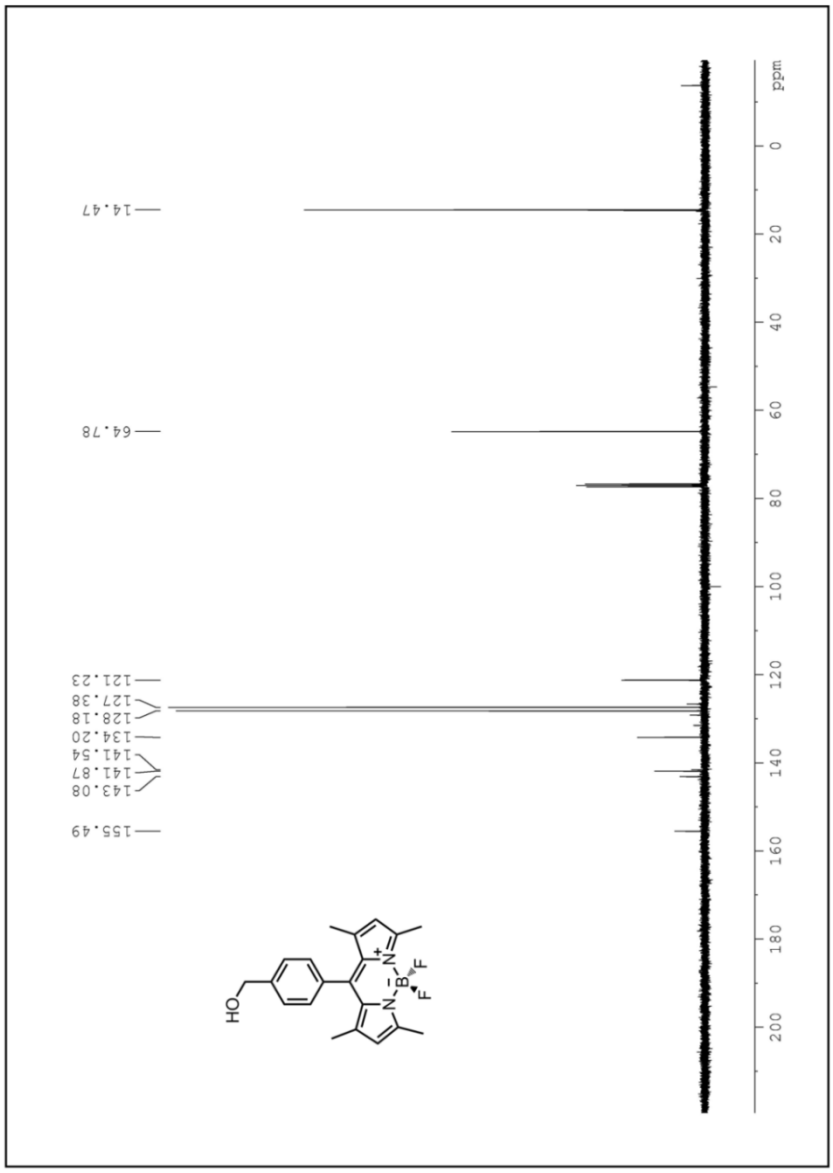


Figure 67: ^{13}C NMR Spectrum of compound **2**

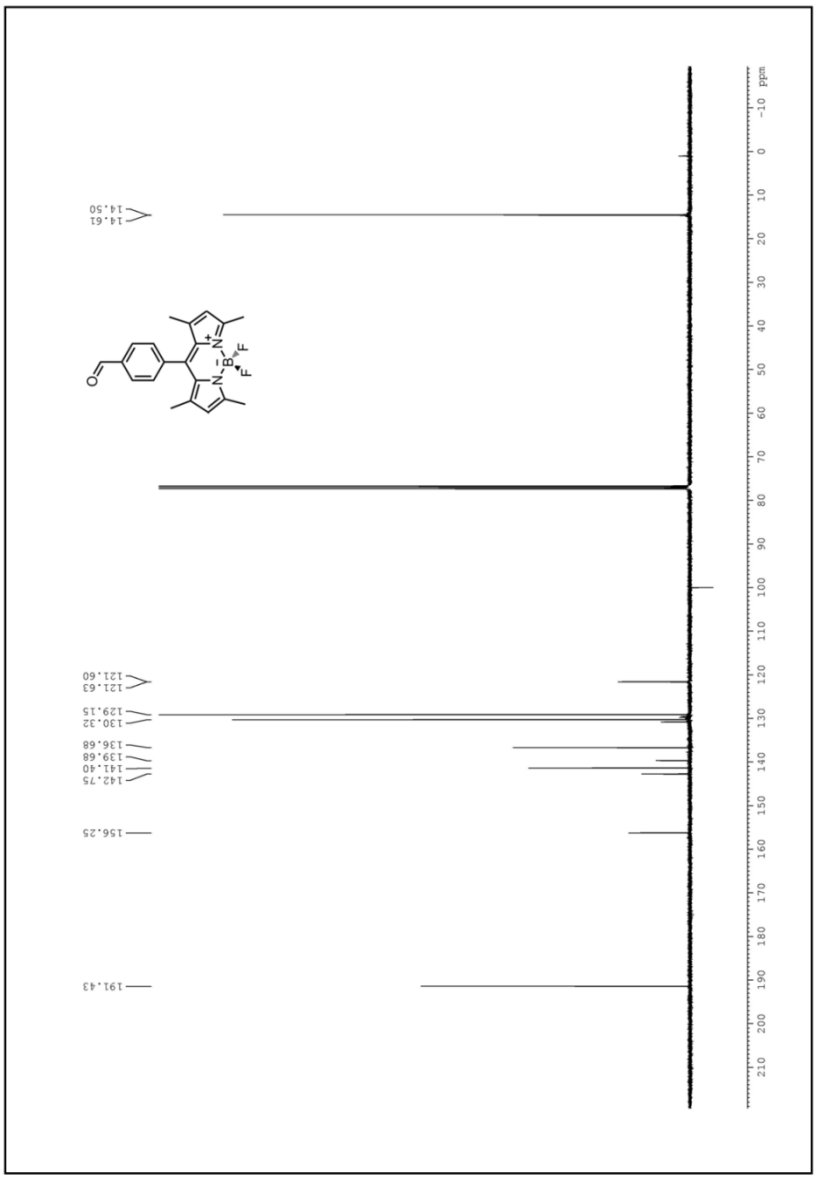


Figure 68: ^{13}C NMR Spectrum of compound **3**

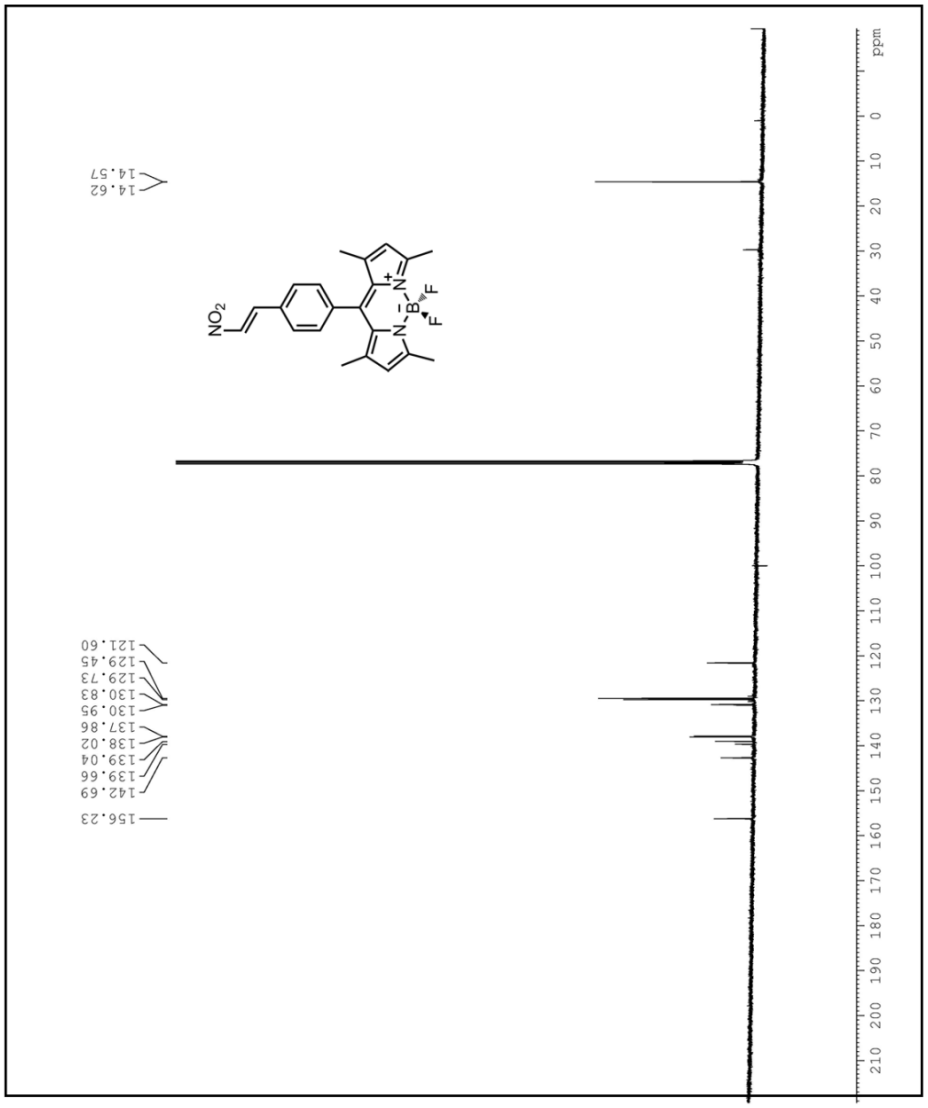


Figure 69: ^{13}C NMR Spectrum of compound **4**

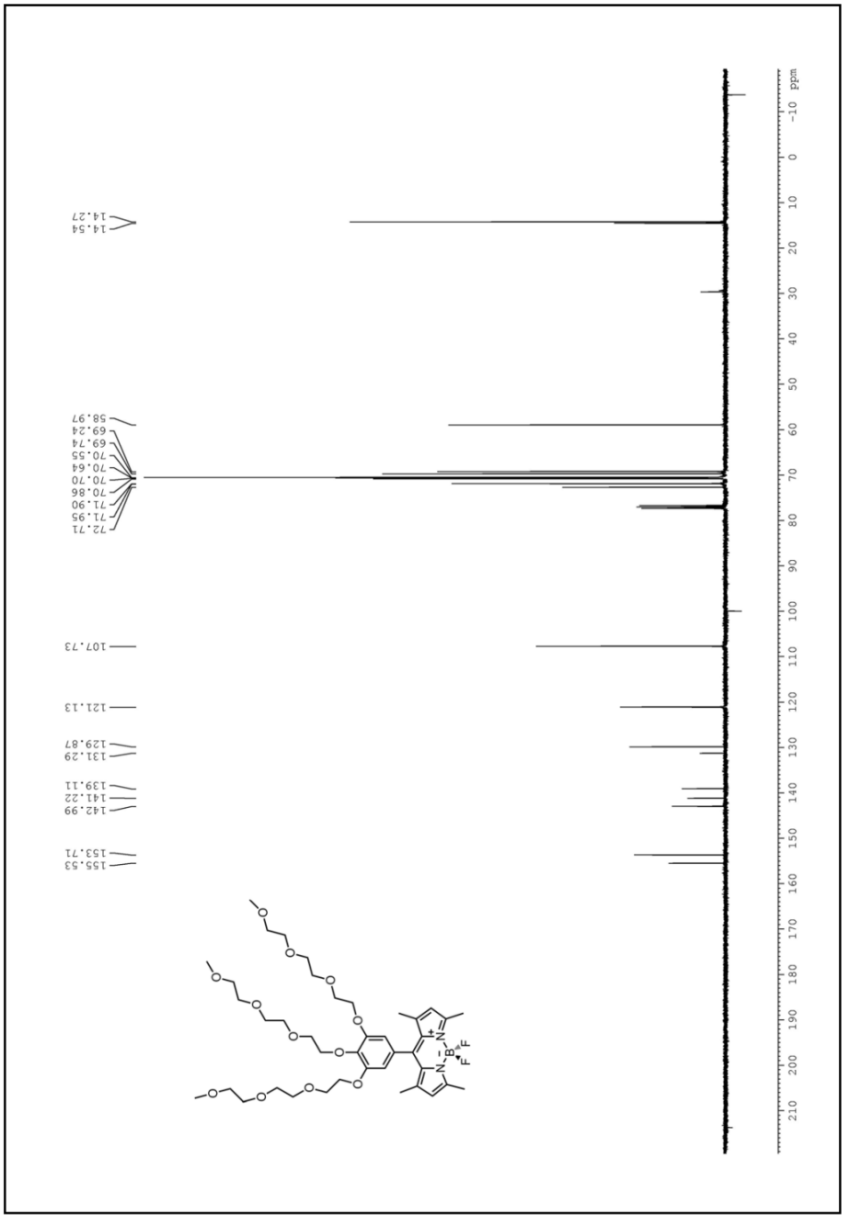


Figure 70: ^{13}C NMR Spectrum of compound **9**

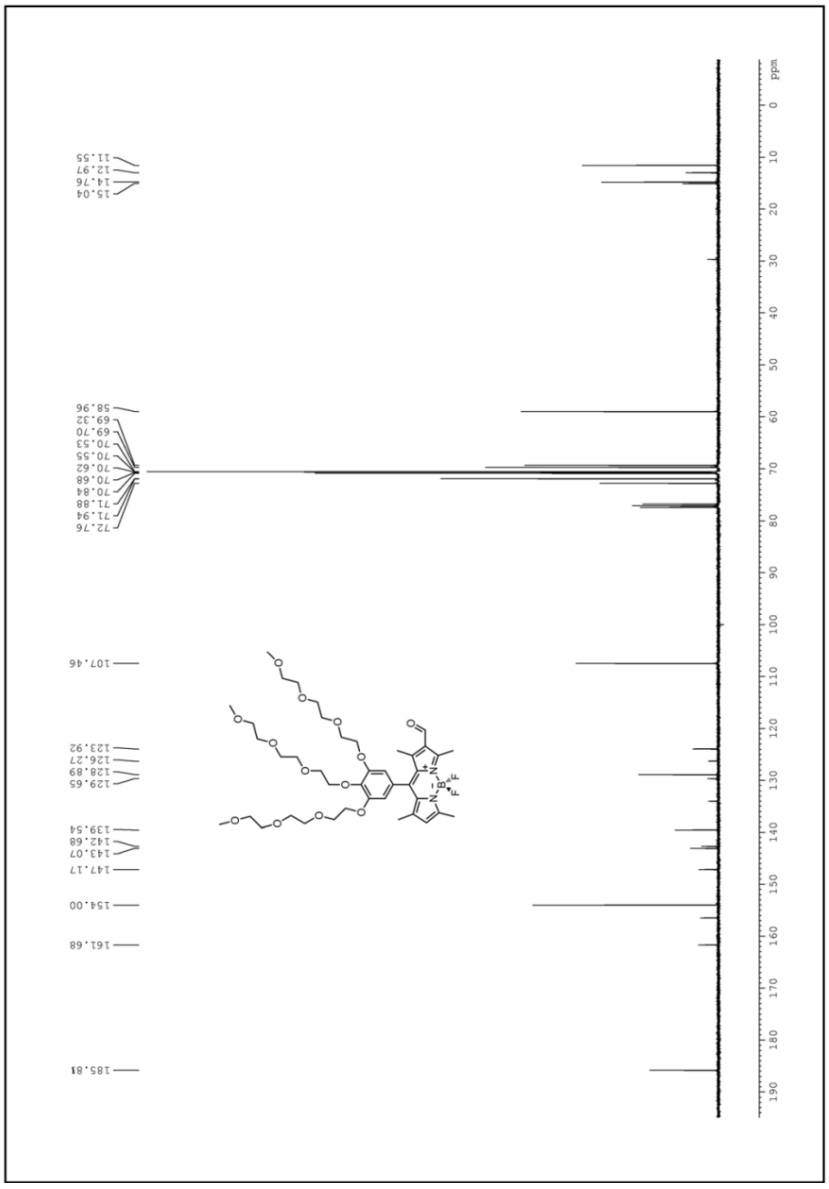


Figure 71: ^{13}C NMR Spectrum of compound **10**

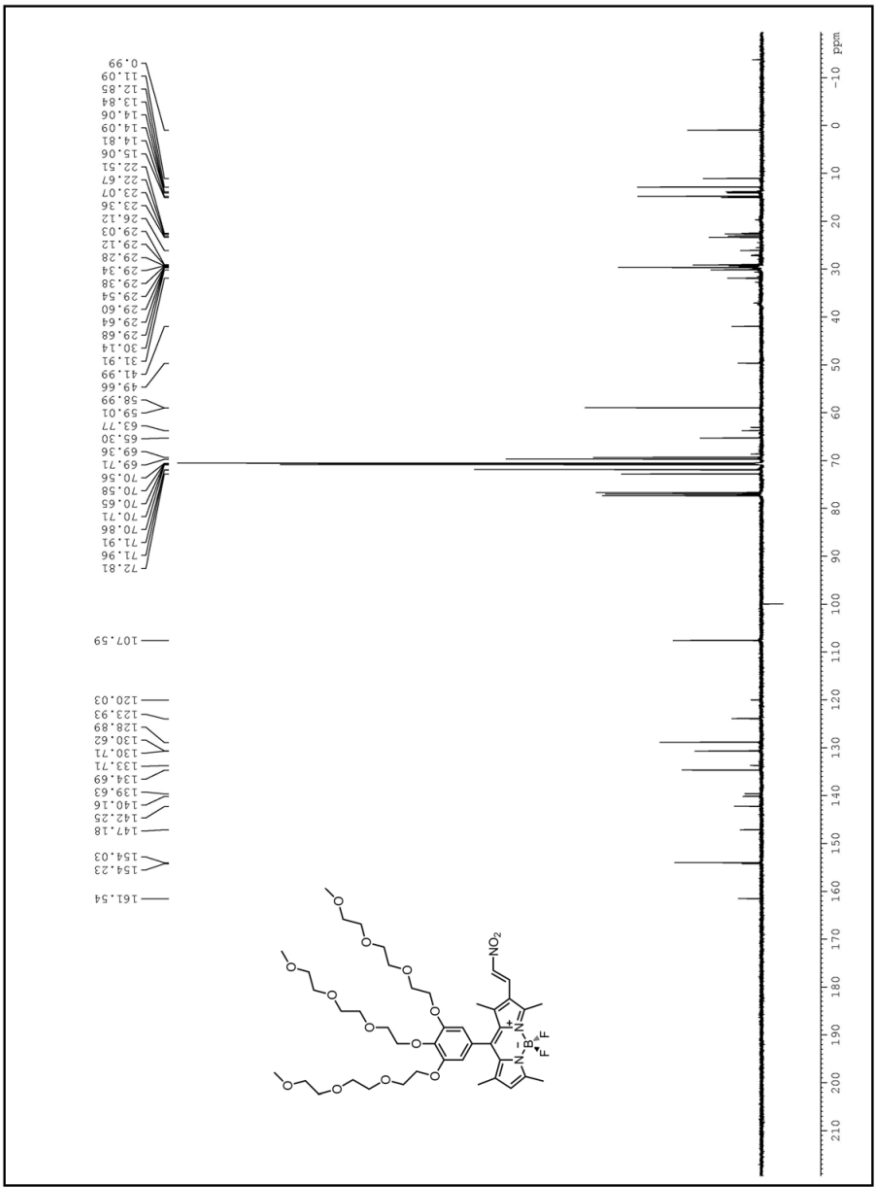


Figure 72: ^{13}C NMR Spectrum of compound 11

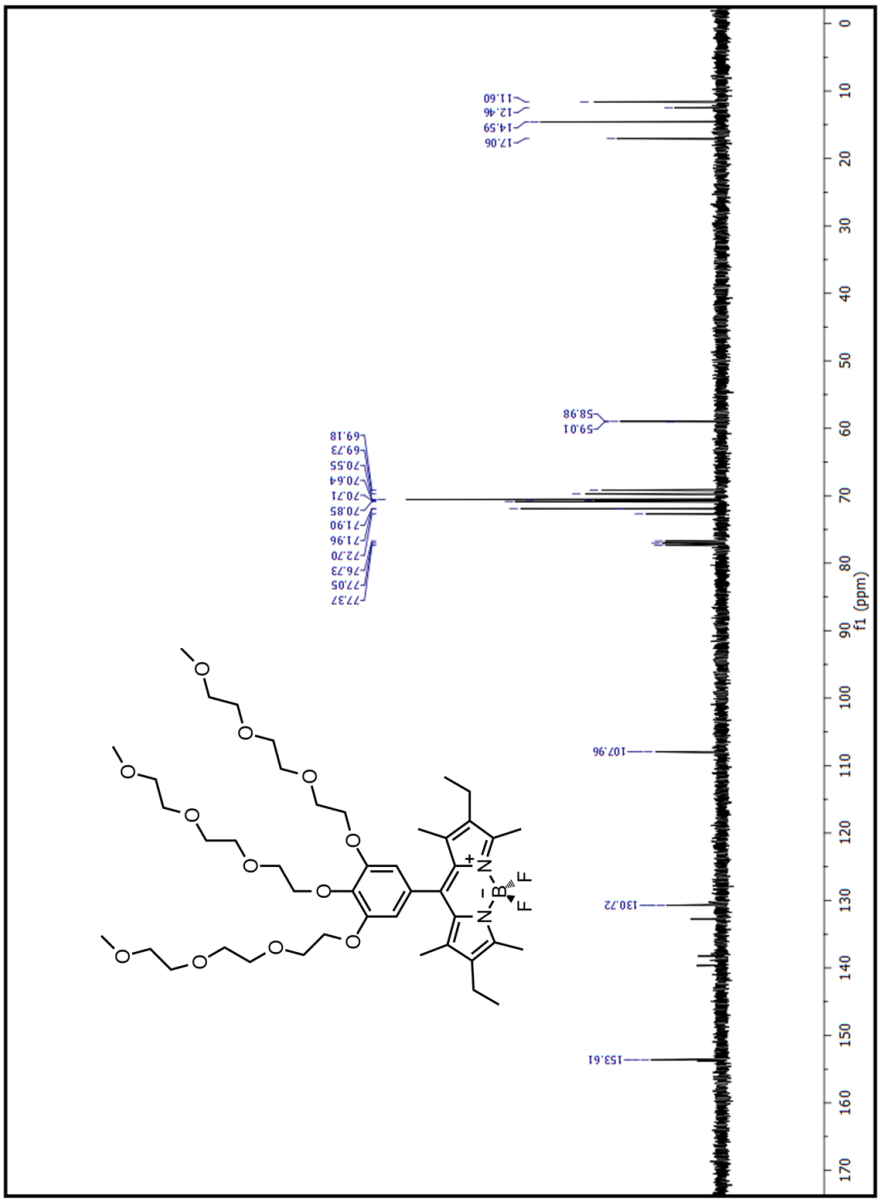


Figure 73: ^{13}C NMR Spectrum of compound **12**

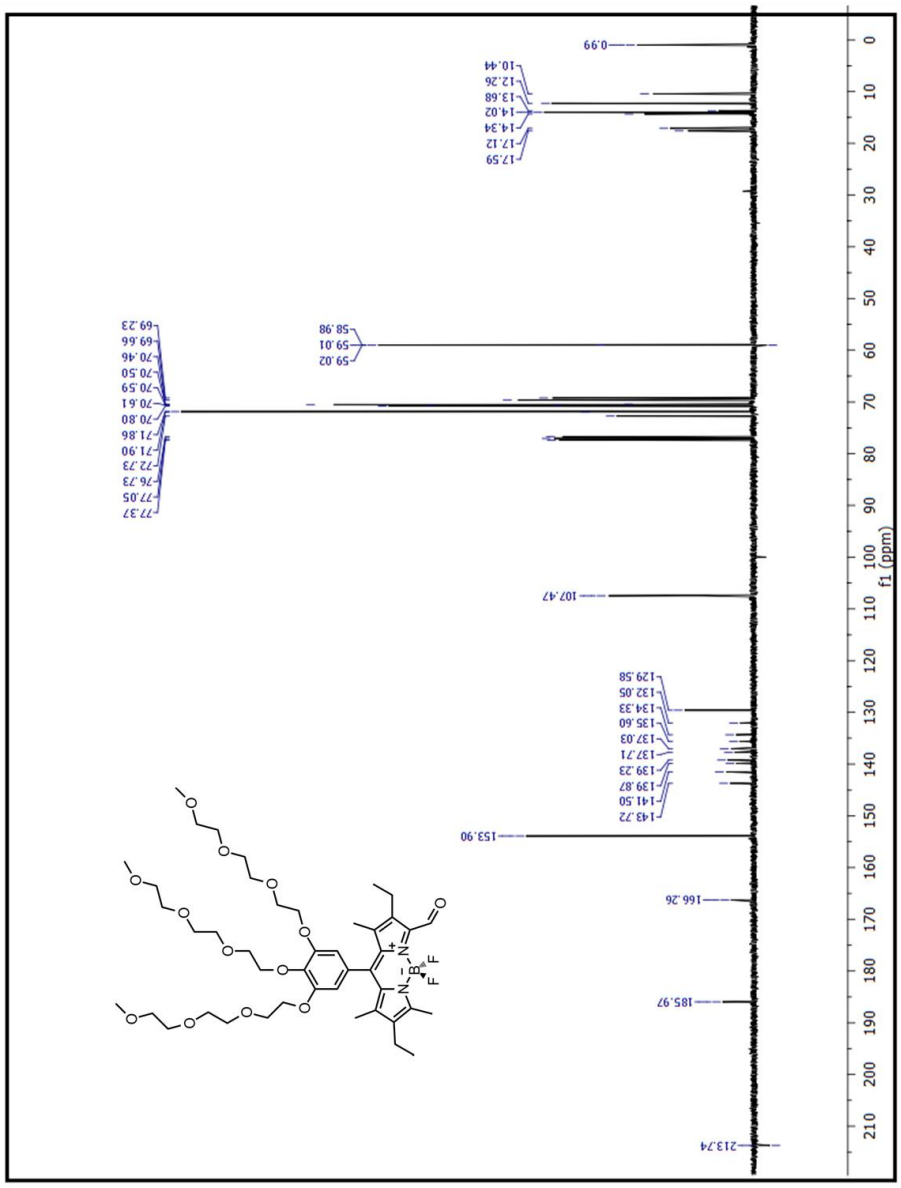


Figure 74: ^{13}C NMR Spectrum of compound **13**

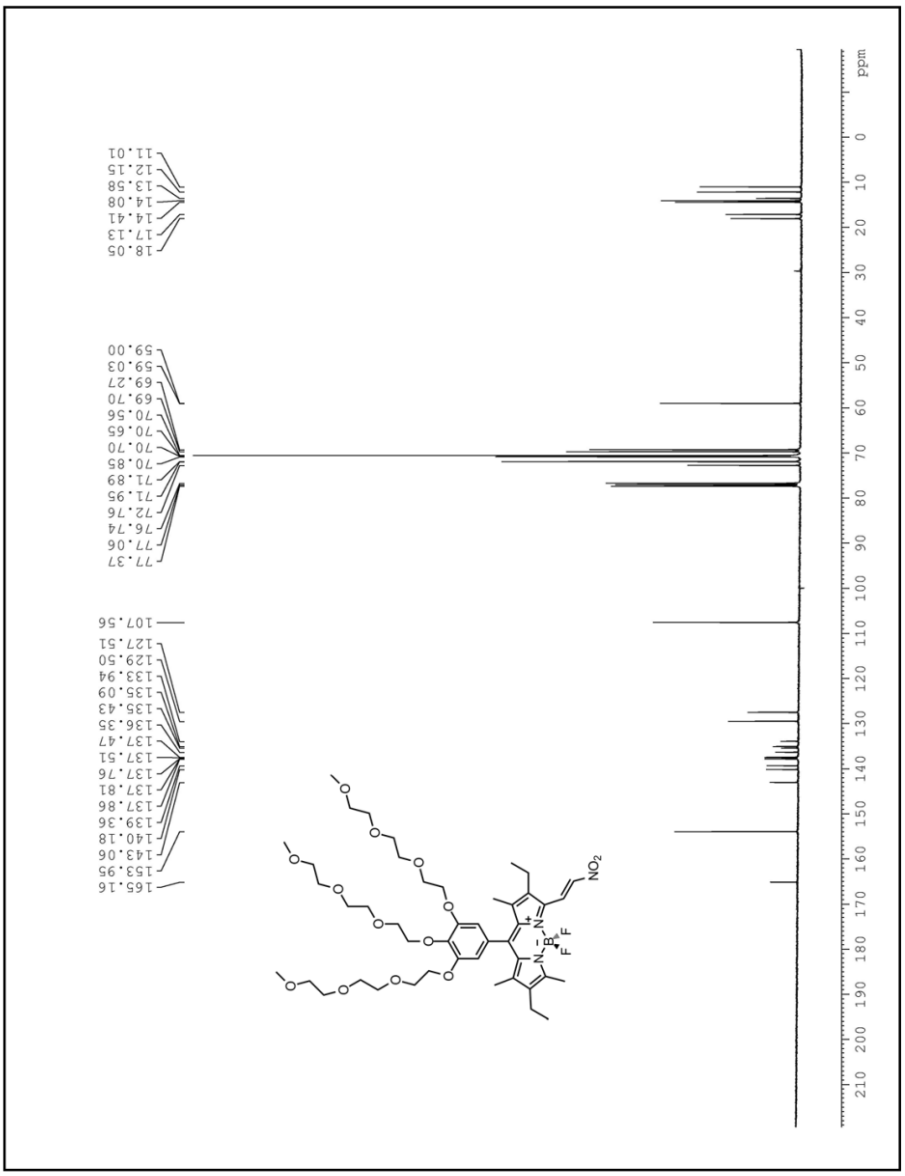


Figure 75: ^{13}C NMR Spectrum of compound **14**

APPENDIX B

MASS SPECTRA

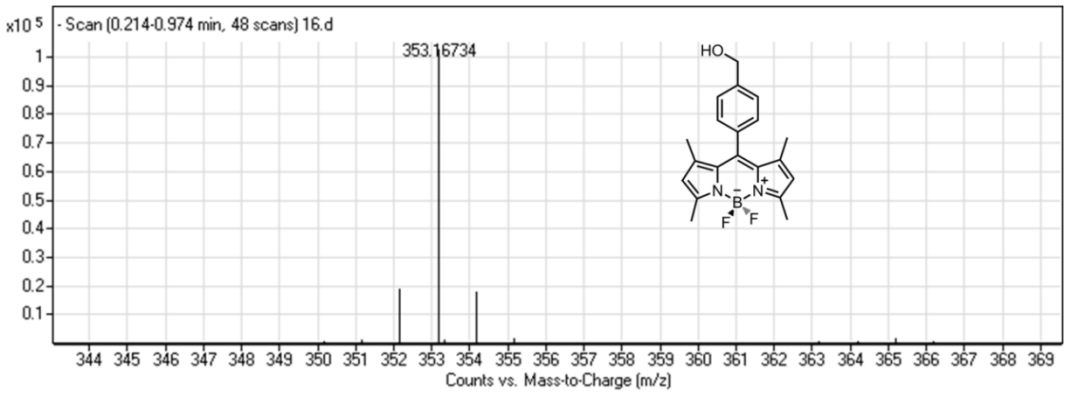


Figure 76: Mass spectrum of compound 2

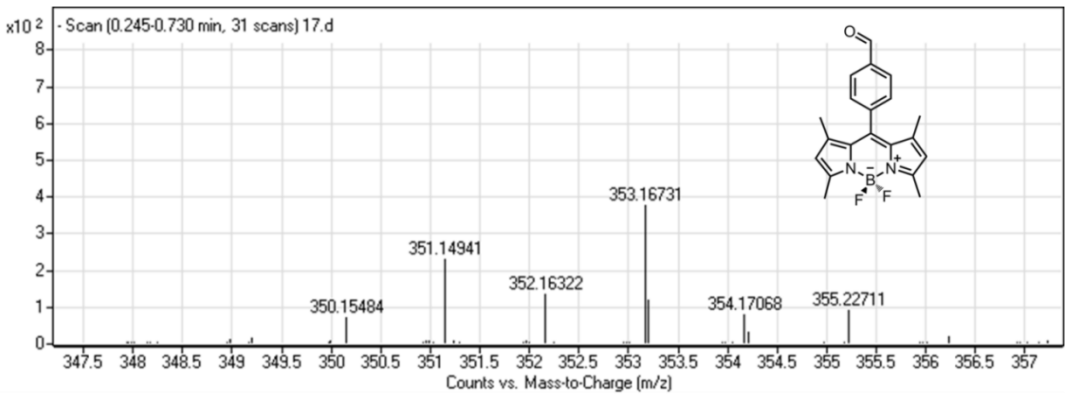


Figure 77: Mass spectrum of compound **3**

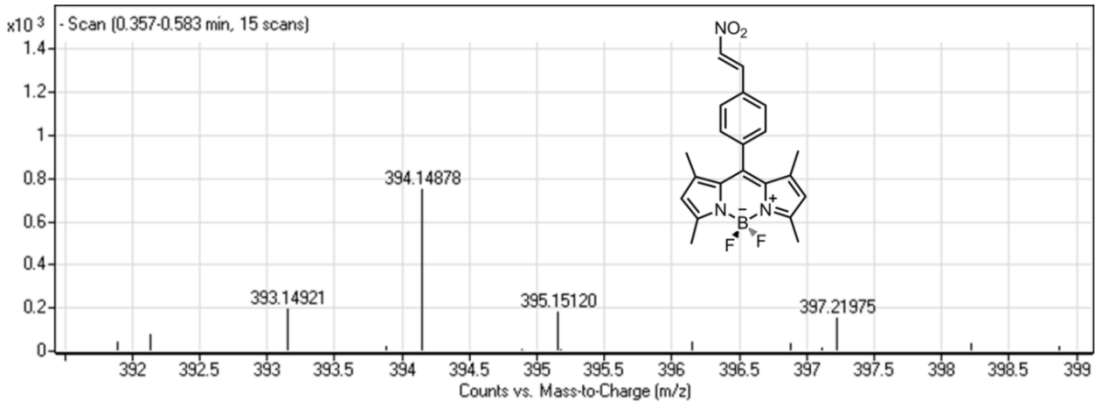


Figure 78: Mass spectrum of compound 4

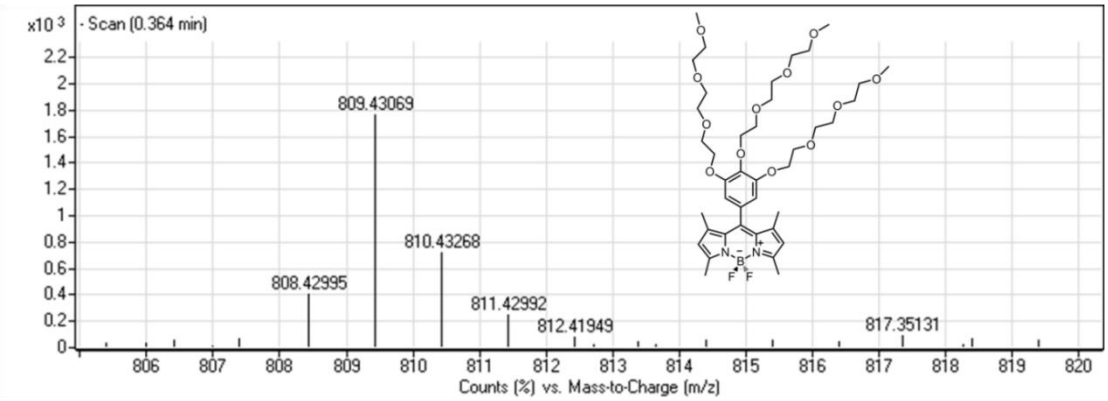


Figure 39: Mass spectrum of compound **9**

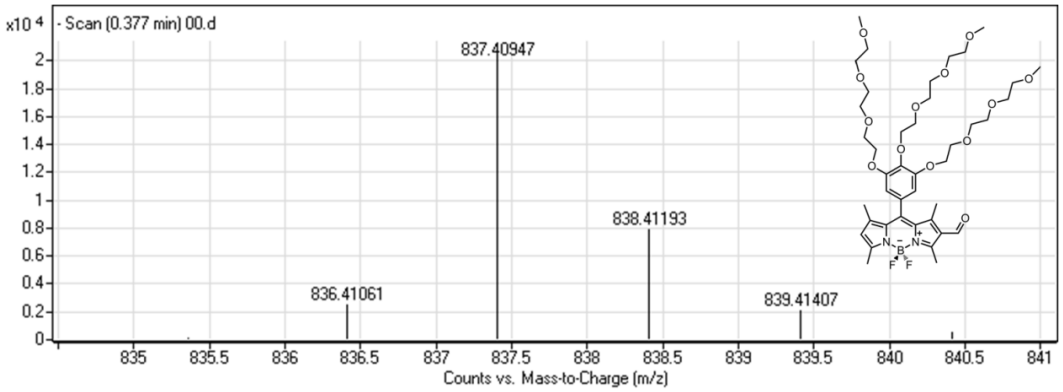


Figure 80: Mass spectrum of compound **10**

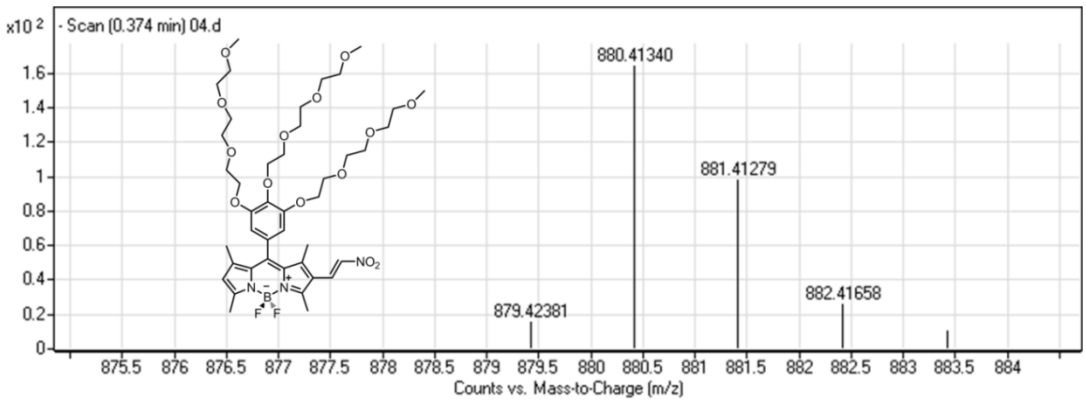


Figure 40: Mass spectrum of compound **11**

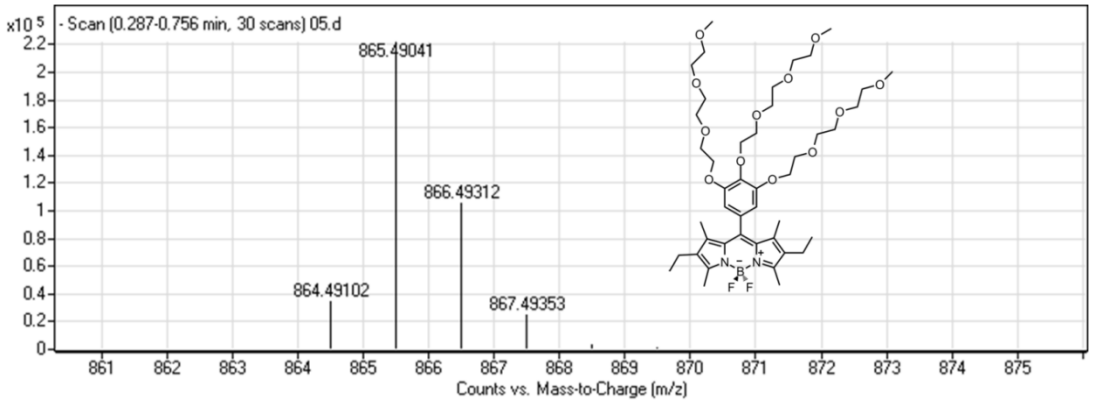


Figure 82: Mass spectrum of compound 12

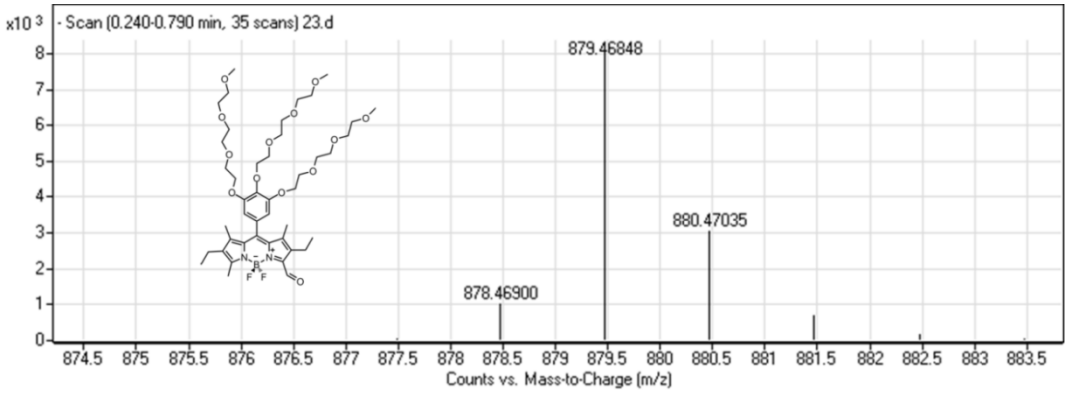


Figure 83: Mass spectrum of compound **13**

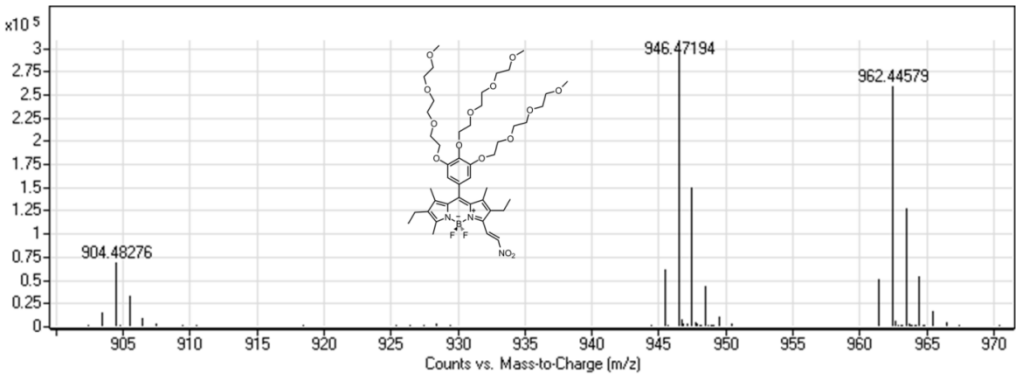


Figure 84: Mass spectrum of compound **14**