4-1-2023

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Honors Thesis

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Abstract

A new single molecule 4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) dye designed to conjugate to the peripheral amines of proteins has been synthesized. Detailed synthesis involving a new and simple two step technique is presented. Synthesis of the carboxylic acid substituted BODIPY dye is characterized by absorption and emission spectroscopy. The new dye is reacted with N-hydroxysuccinimide to give a reactive BODIPY dye toward amine groups of bovine serum albumin. The BODIPY-N-succinimide ester was evaluated by absorption and emission spectroscopy. Covalent attachment of the BODIPY-NHS-ester with bovine serum albumin was conducted in an eppendorf tube in a phosphate buffer at a pH of 7.0. Purification by column chromatography and evaluation of the solution suggests a degree of labeling of only 50% of the bovine serum albumin.

Acknowledgements

University of Dayton Honors Program
University of Dayton Chemistry Department
College of Arts and Sciences Dean's Summer Fellowship

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Introduction

Luminescence describes the process of an excited molecule releasing energy in the form of a photon to return to ground state. The excitation of the molecule can occur through the absorption of light coming in contact with the molecule. Fluorescence, a type of luminescence, specifically describes the excitation of the molecule through light absorption followed by a rapid emission of energy. Depending on the molecular structure or the chosen fluorescent dye, the energy associated with the emission of light could fall within the visible region of the spectrum, indicating it will be visible to the naked eye.

Fluorescent dyes, or fluorophores, have been integral in research to visualize target molecules. Specifically, small molecules of fluorescent dyes can assist in localizing a region or component of a cell, which enables researchers to define cellular functions.[1] This is important to understand how a disease impacts life on a cellular level and guide future research to develop therapeutic treatment.[2] To view the cellular functions through the use of fluorescent dyes, the dye itself must be non-toxic so as to allow for observations without affecting the cell.[3] Additionally, the chosen dye must account for photostability, water solubility, quantum efficiency values, molar absorptivity, and dependence on the environmental pH.[4] When using a bio-imaging dye to observe cellular organelle properties, a more complex synthesis and design of the dye may be required.

Conjugating the use of a fluorophore to an antibody enables the targeting of a molecule. This technique of immunofluorescence enables researchers to locate an antigen as well as its distribution. Two methods of immunofluorescence are possible through the use of primary or secondary antibodies (Figure 1). With primary antibodies, the

fluorophore labeled antibody binds directly to the targeted molecule. While this method can be beneficial due to a faster process and direct conjugation, it limits the amount of fluorophore molecules that can be linked to the targeted molecule, lowering the sensitivity. With secondary antibodies, a primary antibody must first bind to the targeted molecule and secondary antibodies conjugated to the fluorophore may bind to several locations on the primary antibody. Therefore, several fluorophore molecules may be linked to the antigen, providing a greater sensitivity. Additionally, the secondary antibody method enables the use of different types of secondary antibodies to recognize the chosen primary antibody.

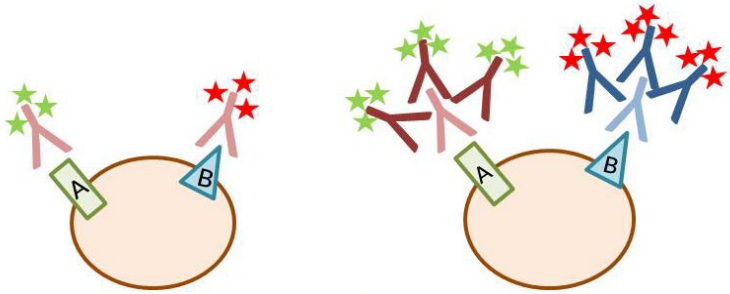


Figure 1: Primary vs. Secondary Antibody Usage

Photodynamic therapy is an emerging treatment method that relies on the use of a photosensitizer, oxygen, and light. It provides minimally invasive and highly selective imaging and treatment techniques for conditions such as cancer and bacterial infections. Additionally, it has demonstrated efficiency and effectiveness through non-specific inactivation regardless of antibiotic resistance within bacteria. However, typical photosensitizers have demonstrated issues with aggregation, shorter activation wavelengths, and poor solubility in water. To combat this, the use of a BODIPY dye was found to be effective.[5] BODIPY, or boron-dipyromethenes, dyes have been vital for research involving bio-labeling and bio-imaging. Their beneficial properties in

photodynamic therapy include longer wavelength absorption and greater water solubility.[6] Using dyes with longer wavelengths are beneficial because they reduce background absorption enabling sharper images and lessen the emission of biomolecules in a cell.[7]

To synthesize BODIPY dyes, 2,4-dimethyl pyrrole is reacted with either alkyl aldehydes or aromatic aldehydes, followed by the addition of boron trifluoroetherate (figure 2). These dyes have displayed beneficial optoelectronic properties for bio-imaging and the emission properties span the visible region of the electromagnetic spectrum.[8] The Swavey Laboratory has synthesized numerous BODIPY dyes with characteristic absorption and emission properties ranging between 588-680nm[9] and molar absorptivity values ranging from 80,000 to 190,000$M^{-1}cm^{-1}$.

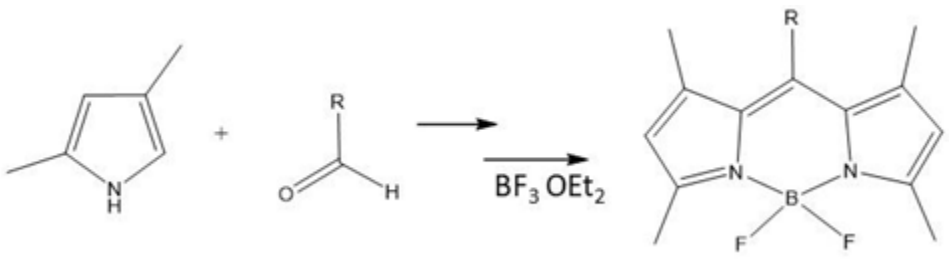


Figure 2: General Mechanism of BODIPY Dye Synthesis

This project aimed to synthesize two BODIPY dyes, one with a carboxylic acid group and the other an NHS ester, with absorption and emission properties of the visible region of the electromagnetic spectrum. These dyes were bound to bovine serum albumin.

Experimental

Materials

All chemicals were reagent grade and used without further purification. Naphtha[1,2-c]pyrrole was synthesized as previously described.[10] Chromatography was performed on a Teledyne CombiflashRf+ equipped with UV detection. Electronic absorption spectra were recorded at room temperature using an HP8453 photodiode array spectrophotometer with 2 nm resolution. All spectra were recorded at 298 K. Room temperature luminescence spectra in a 1 cm quartz spectrophotometer fluorescence cell (Starna) in DCM were run on a Cary Eclipse fluorescence spectrophotometer. Quantum yields were determined at room temperature in HPLC grade DCM relative to Rhodamine 6G as the reference (Φ= 0.95, in ethanol).[11] The quantum yields were obtained using the following equation:

$$\phi_s = \phi_r[A_r\eta_s^2 D_s/A_s\eta_r^2 D_r]$$

where s and r refer to the sample and reference, respectively, A is the absorbance at the excitation wavelength, η is the average refractive index of the solution, and D is the integrated area under the emission spectrum.

Synthesis

Naphthyl-meso-(p-benzoic acid) boron dipyrromethene (BODIPY 1)

To a 25 mL round bottom flask 50.0 mg (0.30 mmol) of naphtha[1,2-c]pyrrole or (0.21 mmol) and a molar equivalent of 4-formylbenzoic acid were combined. Approximately 1 mL of dichloromethane (other solvents i.e. methanol, acetone, chloroform also work) was added to give a homogenous solution. The solvent was then removed under reduced pressure and the flask was placed in a warm water bath preset at 70 °C for 15 min., temperatures were increased until the reactant mixture turned to a purple paste. The resulting purple paste was chromatographed on silica gel using DCM/Methanol (to

remove any polymers formed) collecting the first deep red band. The solvent was removed under reduced pressure and the flask was degassed with nitrogen. Approximately 4 mL of dry DCM was added followed by 100 µL of triethylamine and then 200 µL boron trifluoride etherate. The solution was stirred at room temperature under nitrogen overnight. The reaction mixture was washed with distilled water, dried over magnesium sulfate and chromatographed on silica gel using 95/5 chloroform/methanol as eluent, the first band was collected and the solvent removed, yield = 26 mg.

Naphthyl-meso-(p-N-succinimide ester benyl) boron dipyrromethene (BODIPY 2)

BODIPY 1 (20 mg) was placed in a 10 mL round bottom flask and dissolved with 2 mL dry acetonitrile. To this solution was added 3 equivalents of N,N'-Dicyclohexylcarbodiimide (DCC), a catalytic amount of 4-Dimethylaminopyridine, and 2 equivalents of N--hydroxy succinamide. The solution was stirred at room temperature overnight, filtered to remove the dicyclohexylurea side product. The BODIPY-NHS ester was chromatographed on silica gel to give a yield of approximately 10 mg.

Buffer and dye preparation

A sodium phosphate buffer was prepared by weighing 3.56 g (0.2 M) of $Na_2HPO_4 \cdot 2\ H_2O$ into a 100 mL volumetric flask. 2.76 g (0.2 M) $NaH_2PO_4 \cdot\ H_2O$ was weighed and delivered to a 100 mL volumetric flask. 48 mL Na_2HPO_4 was added to a new 100 mL flask followed by 2.6 mL of NaH_2PO_4 solution. Diluted to mark with MQ water to create a 0.1 M buffer solution. In an eppendorf tube was placed 5 mg of BSA in 0.5 mL of buffer. The solution was prepared in correspondence with 2 mg of BODIPY 2 and

250 µL DMSO. 100 µL of dye solution was added to the protein solution and incubated for 1 hour at room temperature.

Purification

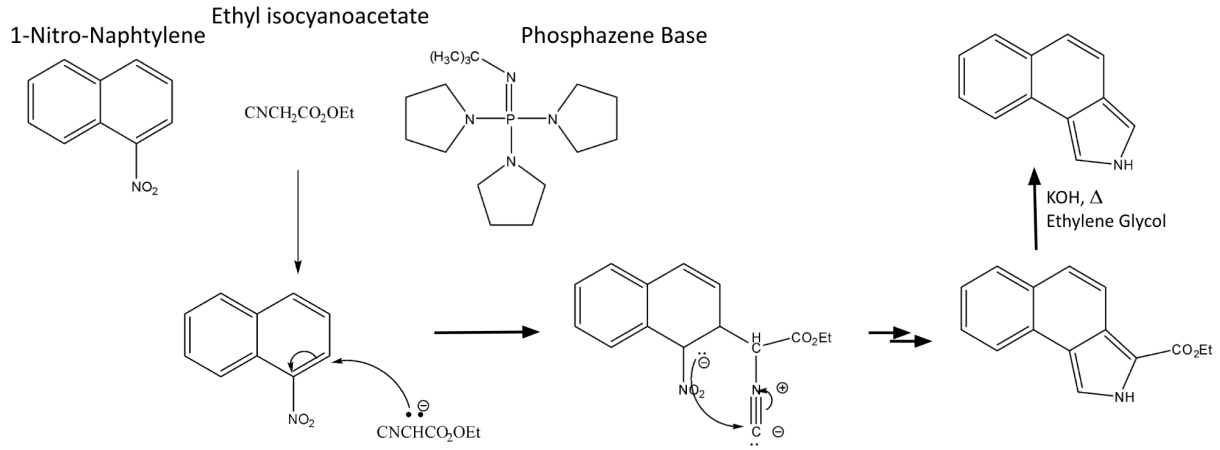
The BODIPY-BSA compound was purified using Thermo Scientific ™ Zeba™ Spin 0.5 mL Desalting Columns. These are made of low protein binding polypropylene, and are compatible with a wide range of standard laboratory instruments and consumables. The bottom plug of the column was twisted for removal and the cap loosened. The column was placed in a 2 mL collection tube and centrifuged at 1000 x g for 2 minutes to remove the storage buffer which was discarded. Approximately 0.5 mL of sample was applied to the center of settled resin. Column-tube assemblies were centrifuged at 1000 x g for 2 minutes to collect samples. The sample was washed with 0.5 mL buffer solution to maximize recovered sample.

Results and Discussion

The naphtha[1,2-c]pyrrole compound was obtained by reacting 1-nitro-naphlene, ethyl isocyanoacetate, and phosphazene base. As shown in scheme 1, the electrons on the isocyanoacetate compound, resulting from the base environment, attack the carbon on 1-nitro-naphlene causing a displacement of electron pairs. This shift of electron pairs induces a ring formation. Once reacted with potassium hydroxide and heat in an ethylene glycol solution the -CO_2Et group is removed, leaving behind naphtha[1,2-c]pyrrole. This compound was chosen for experimentation because the naphthyl group enables a pi-bond extension, which will shift the absorption and emission to longer wavelengths. This is beneficial so that the dye molecule may be selectively viewed within a cell due to the

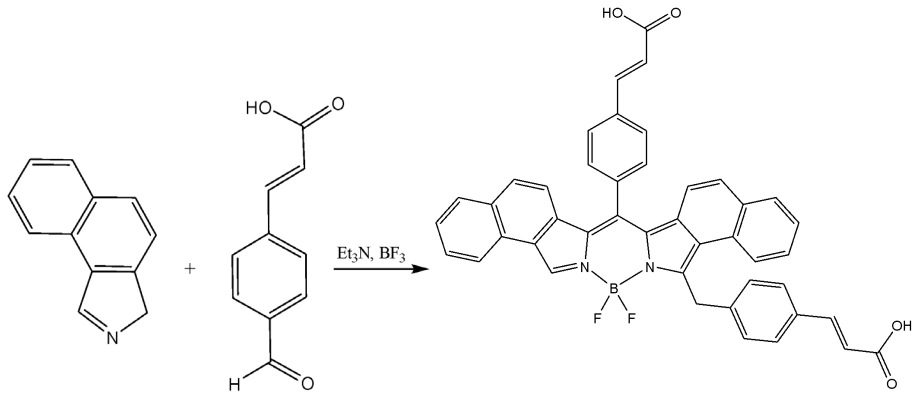
inability of cell biomolecules to absorb light efficiently at longer wavelengths. Therefore, the use of the naphthyl group allows for an increased light emission of the dye in a cell.

Scheme 1: Synthesis of Naphtha[1,2-c]pyrrole



The first target BODIPY dye of my project involved the synthesis of a cinnamic acid BODIPY. This compound was synthesized by reacting naphthylpyrrole and trans-4-formylcinnamic acid as shown in scheme 2. This compound did not produce a clear

Scheme 2: Synthesis of Naphthalene Cinnamic Acid BODIPY

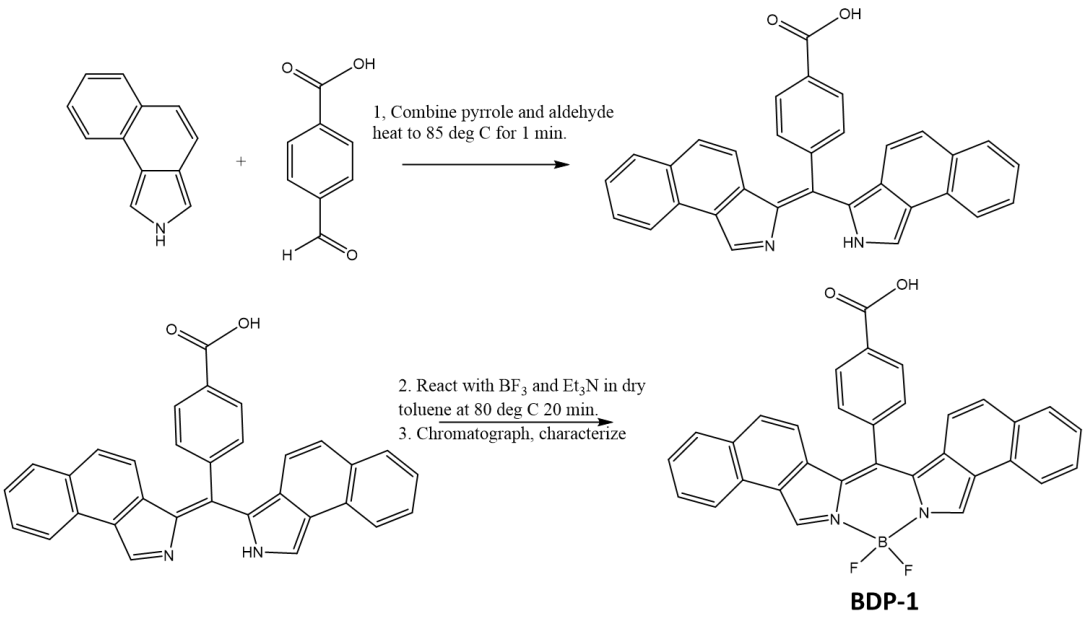


proton-NMR spectrum and mass spectrum. Regardless I continued the synthetic route to the NHS-ester; it appeared that the target dye was synthesized and when reacted with

bovine serum albumin (BSA) the solution appeared to show that the dye was bound to the protein. After sitting in the freezer, prior to further studies, it was observed that the protein had precipitated which is very unusual. Therefore, it was unclear whether the compound was successfully produced. For this reason, a second approach was pursued.

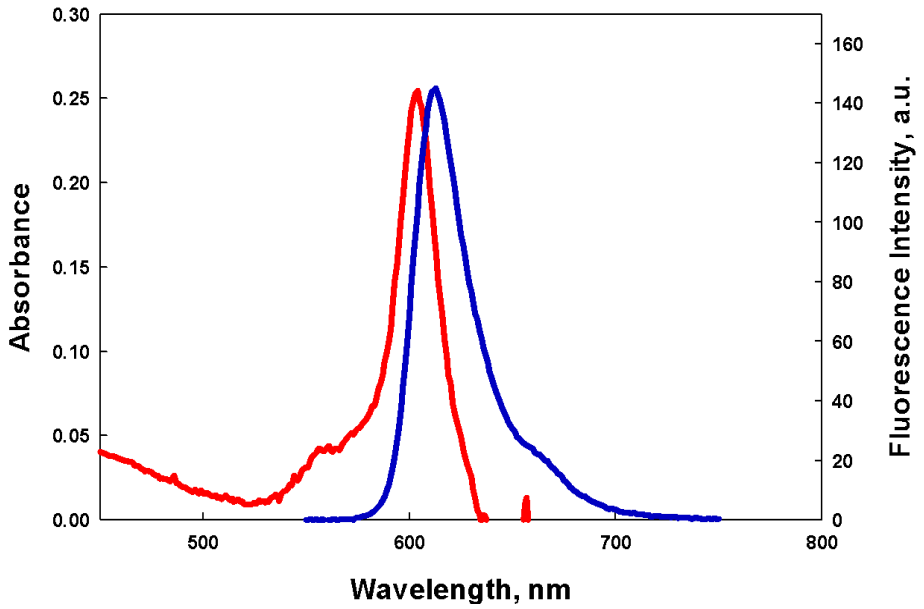
The new approach involved the synthesis of a more effective BODIPY molecule. As shown in scheme 3, the naphtha[1,2-c]pyrrole was reacted with 4-formylbenzoic acid, which is a cheaper and simpler compound compared to the previous scheme, in dichloromethane solution and heated. The resulting compound was reacted with boron trifluoride etherate and triethylamine in toluene to produce the desired compound. The product yield was found to be low at less than 9%.

Scheme 3: Synthesis of meso-benzoic acid BODIPY [BDP 1]



UV-Visible and Emission Spectroscopy were performed on BDP 1 in 1 cm quartz cuvette at room temperature in dichloromethane. Figure 1 illustrates the absorption and emission of BDP 1.

Figure 3: UV-Visible and Emission Spectrum of BDP 1 in Dichloromethane



Based on the figure, the maximum wavelength for absorption is 606 nm as shown by the sharp and intense peak of the red line. This is approximately 100 nm red-shifted compared to typical BODIPY molecules due to the extended □-system of the naphtha[1,2-c]pyrrole. The blue line represents the emission upon excitation at 606 nm. This peak occurs roughly at 615 nm. This intense peak demonstrated a quantum yield approaching 1.0. To determine the molar absorption of BDP 1 at 606 nm, the absorption spectrum was found at different concentrations and analyzed using Beer's Law in figures 4 and 5 shown below. Based on figure 5, the molar extinction coefficient was found to be 113,000 $M^{-1}cm^{-1}$.

Figure 4: UV-Visible Spectrum of BDP 1 at Different Concentrations of Dichloromethane

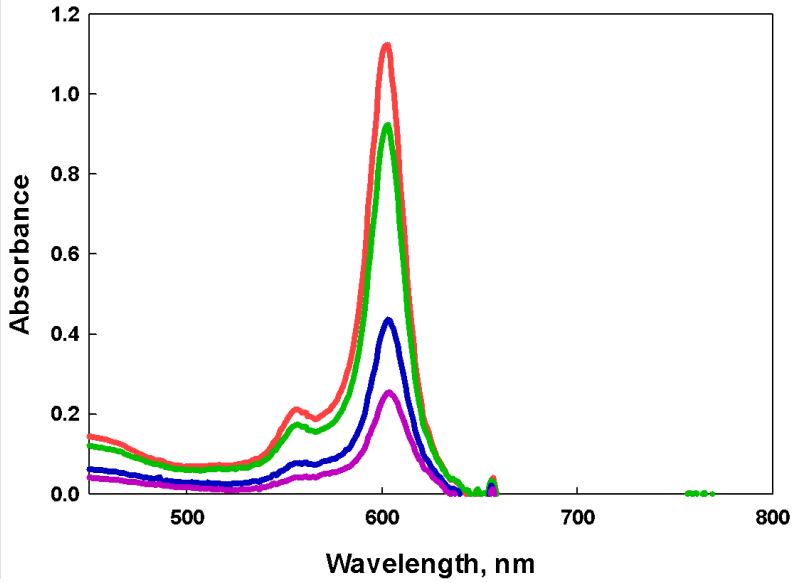
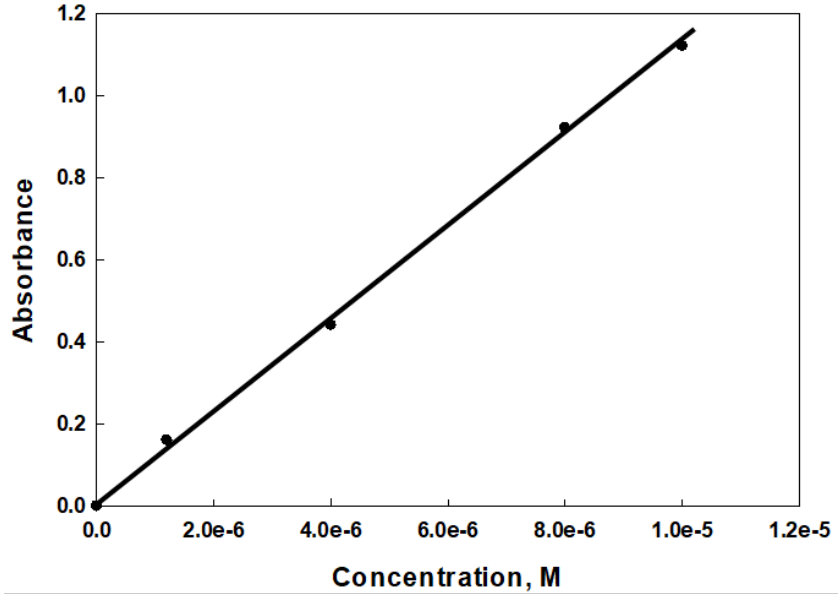
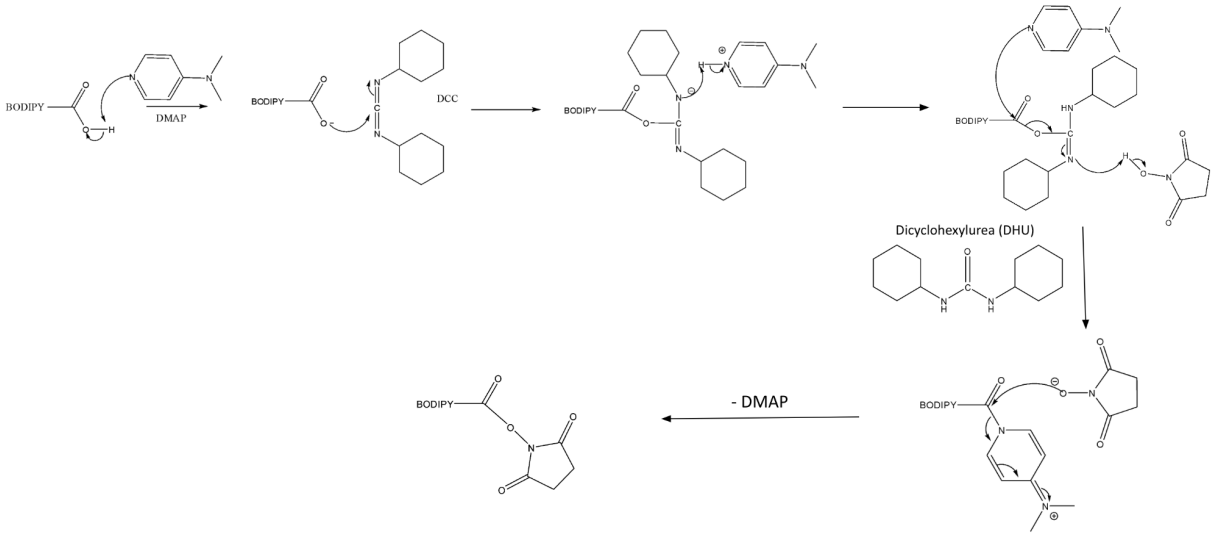


Figure 5: Beer's Law Plot of BDP 1 with Different Concentrations of Dichloromethane



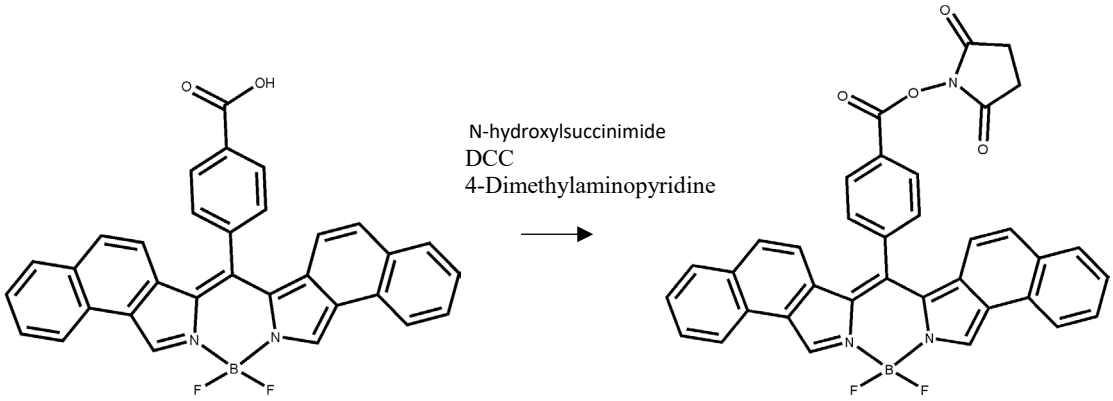
To achieve a more highly reactive BODIPY for external amine groups located on all proteins, a good leaving group is needed. Taking advantage of the meso-benzoic acid group on BDP-1, N-hydroxysuccinimide can be attached to the carboxylic acid at room temperature with additional catalysts. Steglich esterification illustrates the mechanism for attachment as shown in scheme 4 below.

Scheme 4: Steglich Esterification



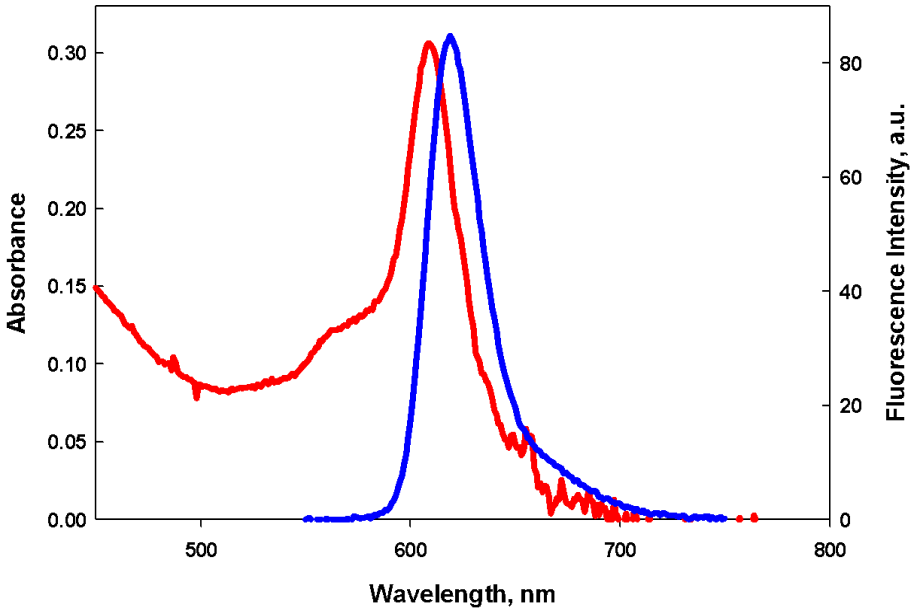
To synthesize the N-succinimide ester form of the BODIPY, BDP 1 was reacted with N-hydroxysuccinimide, N,N'-Dicyclohexylcarbodiimide (DCC), and a catalytic amount of 4-dimethylaminopyridine (DMAP), in dry acetonitrile overnight at room temperature. After purification the target BODIPY (BDP 2) was isolated in good yield. As illustrated in Scheme 4 4-dimethylaminopyridine deprotonates the carboxylic acid of the BDP 1 creating an anionic carboxylate capable of nucleophilic attack on the central carbon of DCC. The intermediate formed in this reaction then goes through a series of rearrangements leading to an insoluble urea and displacement of the DMAP catalyst by the deprotonated form of the succinimide anion. Isolation of BDP 2 occurs by first removal of the precipitated urea by filtration followed by chromatography on silica gel.

Scheme 5: Synthesis of Naphthyl-Meso-(p-benzyl N-Succinimide Ester) BODIPY[BDP 2]



UV-Visible and Emission Spectroscopy were performed on BDP 2 in 1 cm quartz cuvette at room temperature in dichloromethane. The absorption spectrum (Figure 6, red) shows an intense peak of 609 nm. The blue line (Figure 6) represents the emission, upon excitation at 609 nm, with a peak at 620 nm. Quantum yield calculations versus a rhodamine standard dye give a value approaching unity.

Figure 6: UV-Visible and Emission Spectrum of BDP 2



To determine the molar extinction coefficient for BDP 2 at 609 nm, absorption spectra were measured for different concentrations and analyzed using Beer's Law in figures 7 and 8 shown below. Based on figure 8, the molar extinction coefficient was found to be 80,000 $M^{-1}cm^{-1}$.

Figure 7: UV-Visible Spectrum of BDP 2 at Different Concentrations of Dichloromethane

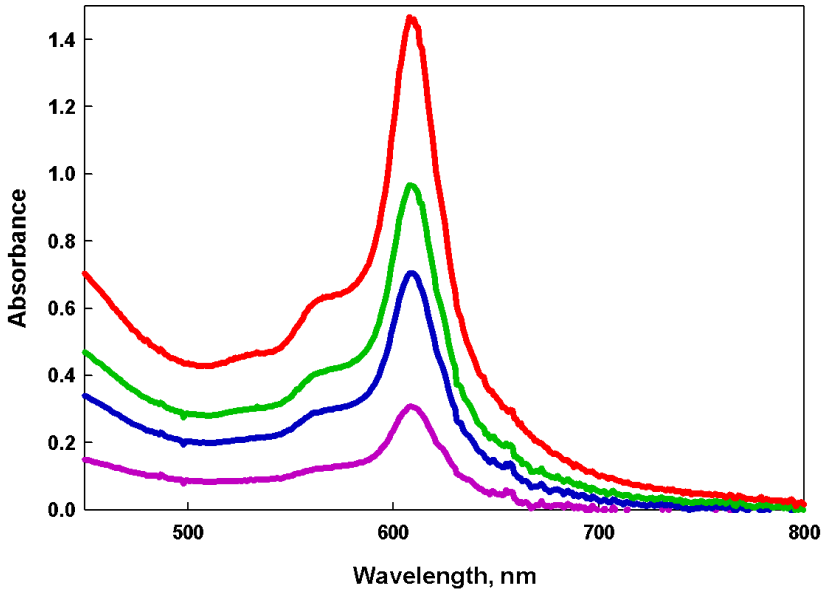
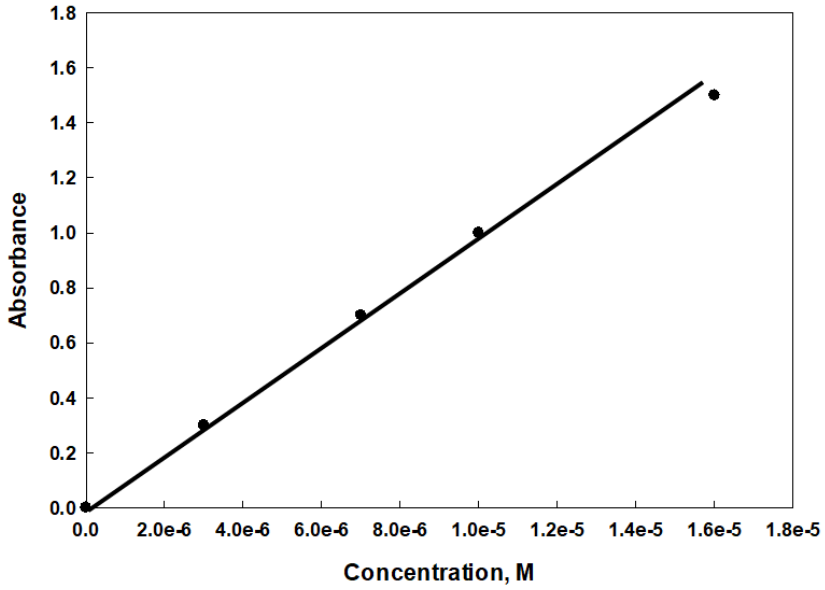


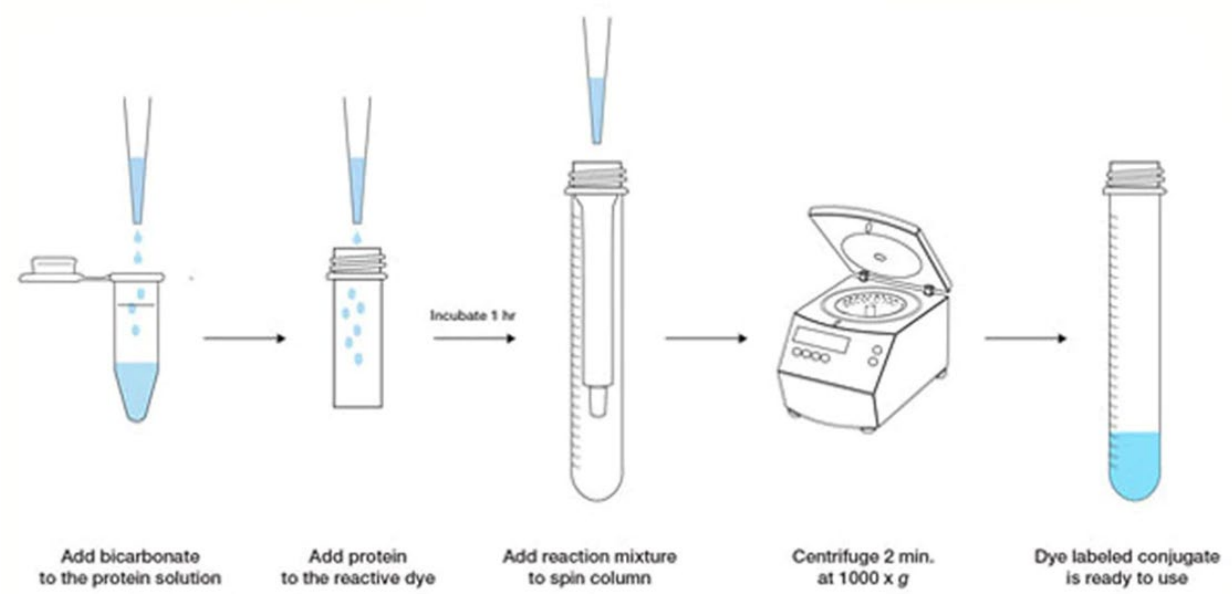
Figure 8: Beer's Law Plot of BDP 2 with Different Concentrations of Dichloromethane



Coordination of BDP 2 to bovine serum albumin (BSA)

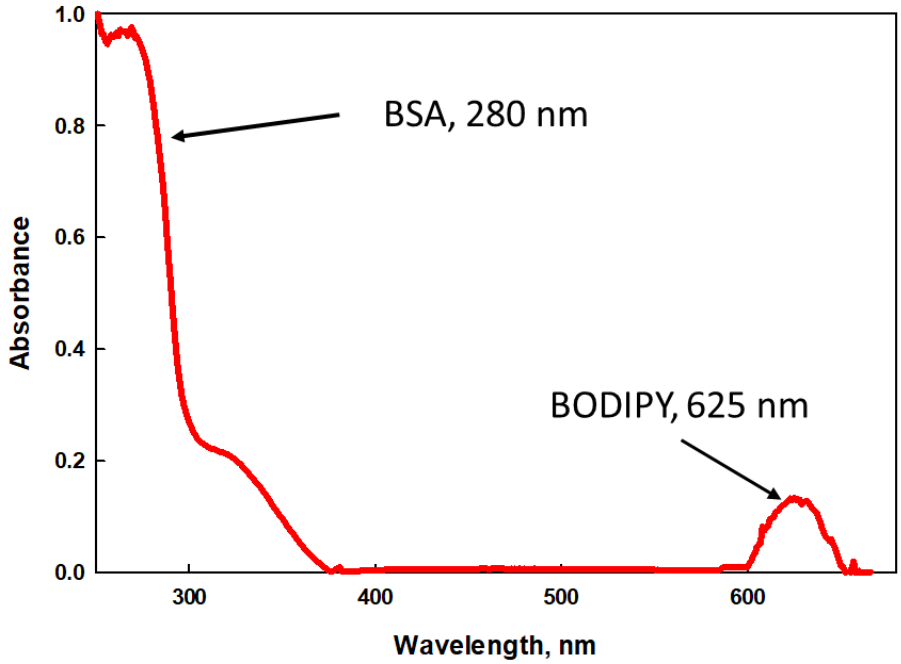
Reaction of BDP 2 and BSA was performed in an eppendorf tube. BSA was dissolved in phosphate buffer at pH of 7.0 followed by addition of an aliquot of a solution of BDP 2 in dry dimethyl sulfoxide (DMSO). The eppendorf tube containing the reactants was allowed to sit in a beaker of water at room temperature for approximately 1 hour after which time the solution was added to a Zeba desalting column specifically designed to separate unreacted BSA from the BSA-dye compound, illustrated in Scheme 6. Formation of an amide bond results from nucleophilic attack of exogenous amines on the BSA protein at the carbonyl center of the BDP 2 NHS-ester. This can be performed at room temperature in relatively short time because the N-hydroxysuccinimide of the dye is an excellent leaving group.

Scheme 6. Procedure for attaching BDP 2 to BSA in aqueous solution.



To determine the degree of labeling (DOL), which is an indication of how many dye molecules are attached to each BSA molecule, absorption spectra of the resulting purified BSA-dye solution were run, as shown in Figure 9.

Figure 9. Absorption spectra of BSA-dye aqueous solution.



BSA has an intense absorption at 280 nm with a molar extinction coefficient of 44,000 $M^{-1}cm^{-1}$. BDP 2 has an intense absorption at 625 nm with a molar extinction coefficient of 80,000 $M^{-1}cm^{-1}$. Analysis of the absorption spectra indicates that only 50% of the BSA protein molecules were tagged with BDP 2.

Conclusions

Two new BODIPY dyes were synthesized with absorption and emission properties in the near-infrared region of the electromagnetic spectrum. Attempts to attach the new NHS-ester BODIPY dye to the amine groups of bovine serum albumin (BSA) showed relatively low efficiency. To obtain the best imaging with dye-protein conjugates it is necessary to have between 2 and 5 dye molecules per protein molecule. It is unclear why so few of the BDP 2 dyes are attached to the BSA protein. Future studies are needed to explore the efficiency of this reaction with BDP 2.

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