DESIGN AND SYNTHESIS OF FLUORESCENT DYES FOR USE IN PROTEOMIC RESEARCH

by

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Kevin James Spicka

July 2008

DEDICATION

To my parents, whose love and support throughout my life has allowed me to reach for goals beyond what I believe I am capable of.

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ABBREVIATIONS

ANS	1-anilino-8-naphthalene sulfonate
Bis-ANS	4,4'-bis(1-anilinonaphthalene 8-sulfonate)
Boc	butoxycarbonyl
BODIPY	4,4-Difluoro-4-bora-3a,4a-diaza-*s*-indacene
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DIPEA	diisopropyl ethylamine
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FTIR	Fourier transform infrared spectroscopy
HPLC	high performance liquid chromatography
HRMS-EI	high resolution mass spectrometry – electron impact
Hz	hertz
IR	infrared
LAH	lithium aluminum hydride
M	molar
MALDI-MS	matrix assisted laser desorption ionization time-of-flight mass spectrometry
mM	millimolar
mp	melting point

ABBREVIATIONS - CONTINUED

NaCl	sodium chloride
NBS	*N*-bromo succinimide
ng	nanogram
NHS	*N*-hydroxy succinimide
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance
pI	isoelectric point
pg	picogram
PTM	post-translational modification
RP	reverse phase
rt	room temperature
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TFAA	trifluoroacetic acid anhydride
Trt	trityl
µg	microgram

ABSTRACT

Proteomics is a rapidly developing field requiring powerful new technology in order to be able to detect proteins at increasingly lower concentrations. To aid in the detection of proteins at lower concentrations, DIGE dyes, a family of spectrally resolved fluorescent dyes, are currently available to proteomic researchers for 2D gel analysis. However, the demands of protein detection dictate that dyes that are even more sensitive and versatile be created. The syntheses of highly sensitive, water soluble BODIPY fluorophore dyes are described. These dyes are proposed to have the necessary sensitivity to allow for detection of proteins in much lower concentrations, providing an improvement over current protein detection limits. The BODIPY dyes that have been synthesized are available in a variety of absorbances and emissions.

While fluorescent dyes that are amine-reactive are the most popular covalently binding protein labeling markers being used in today's proteomic research, thiol-reactive fluorescent markers are gaining importance in proteomic research. Since thiol residues are less common in proteins compared to their amine counterparts, saturation labeling and quantification are more easily achieved. The syntheses of sensitive thiol- reactive fluorescent dyes are described. These syntheses allow for quick generation of thiol-reactive fluorescent markers to be used in proteomic research.

DYES IN PROTEOMICS

Background on Proteomics

In the quest to further understand how human and other biological systems function, proteomics is at the forefront of scientific discovery. The field of proteomics can be generally defined as the systematic analysis and documentation of the proteins present in biological systems under different conditions[1]. Proteomics has become of great interest to scientists since it has been realized that many more protein forms are generated than the number of genes present in the human genome.

It has been estimated that roughly 25,000 genes in the human genome ultimately encode for approximately one million different protein forms[2] in the human body. Not only can it be difficult enough to identify all the proteins that make up the proteome, the task is made even more challenging considering that a protein encoded by a specific DNA sequence can be alternatively spliced and can undergo as many as 400 chemical modifications. These changes, or post-translational modifications (PTMs), can substantially alter the makeup of a protein, giving the protein a completely different function, activity, or location in the cell or the body. The leading edges of proteomic research aim to identify the PTMs, as well as possibly identifying the binding partners, activities, quantities, etc. resulting from the modification of protein structures after the proteins have been fully translated from gene sequences[1, 3a, 3b]. The ultimate goal that comes from knowledge of the proteome is modeling of signaling and functional networks

that allows for the development of more specific and effective treatments of developmental and disease-related problems affecting mankind.

In the course of trying to identify the numerous proteins found in cell samples, it is of utmost importance to be able to separate all the different proteins in a practical and effective manner. The most common method used to separate proteins is two dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE). In 2D gels, proteins are first separated in the first dimension along a pH gradient so as to separate them by their isoelectric points (pI), also known as isoelectric focusing (IEF). This is performed by placing the proteins within the 1D strip with a built in pH gradient, followed by creating an electric field across the strip. The proteins will migrate along the pH gradient according to their charge until the proteins have gained or lost a sufficient number of protons until they become neutral overall, reaching their pI.

After isoelectric focusing, the proteins contained in the 1D strip are reduced, alkylated and treated with sodium dodecyl sulfate (SDS), which will bind to the proteins, placing a large negative charge onto the proteins, masking any charge they may possess. The treated 1D strip is placed “on top of” the polyacrylamide gel which constitutes the second dimension. An electric potential is applied to the gel, generating a positive charge on the bottom end of the gel. Due to the large negative charge of the proteins created by the surrounding SDS coat, the proteins will travel towards the positive end of the gel, separating based upon the size, or mass, of the proteins. This is due to the porosity of the gel, allowing smaller proteins to migrate farther towards the positive charge, since they will meet less resistance than larger proteins as they travel through the gel.

To detect proteins after they have been separated using 2D SDS-PAGE, the proteins are typically "stained" so they can be located on the gel and possibly removed for analysis. Proteomic researchers have several staining methods to select from for detection of proteins. Several types of dyes and stains are available, including organic colorimetric dyes, silver stains, reverse stains, metal chelates, and organic fluorophores[1].

Dyes in Proteomics

Coomassie Blue

One of the first organic dyes available to stain proteins was Coomassie Blue[4] (Figure 1.1), which still remains one of the most widely used dyes in protein detection due to its ease of use and often acceptable detection limits. After separation of the proteins by 2D SDS-PAGE, the gel is placed in a solution of Coomassie Blue in methanol. The dye molecules adhere to the proteins *via* physisorption, and free Coomassie molecules are washed from the gel with Coomassie-free methanol so as to not interfere with protein detection due to background staining.

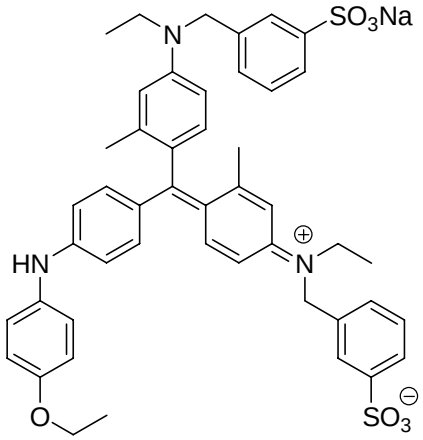


Figure 1.1. Coomassie Blue.

In an improvement to the method in 1967, Chramback and coworkers discovered that using trichloroacetic acid (TCA) in the staining protocol formed colloidal Coomassie Blue[5a, 5b]. The colloidal formation keeps fewer free dye molecules in solution, preferentially staining the proteins in the gel matrix, and less of the gel itself, reducing background staining. However, using TCA can lead to esterification of glutamic acid residues, which can hinder protein identification by mass spectrometry[6].

Coomassie Blue detection limits lie within a range of 10-100 ng protein/band[7], with a linear dynamic range (the number of orders of magnitude where the detector's response increases proportionally with protein amount) that spans only one order of magnitude. While detection limits with Coomassie Blue are sometimes sufficiently sensitive, it is not as sensitive as silver stain, which can also be used to stain proteins.

Silver Stain

Silver stain was first introduced for the staining and detection of proteins by Kerenyi and Gallyas in 1972[8]. However, their method was used for proteins separated on agarose gels, and it was not until 1979 that Switzer and coworkers developed a method for the use of silver stain for proteins separated on polyacrylamide gels[9]. The method first saturates the gels with silver ions, which binds to the proteins. Unbound silver ions are washed from the gels, followed by the reduction of the silver ions bound to the proteins to metallic silver.

Silver stain has the ability to detect between 100 pg and 1 ng of protein[7], but there are drawbacks in using silver staining for detection of proteins. While silver stain detects proteins at lower concentrations than Coomassie Blue, the linear dynamic range

of silver stain spans one order of magnitude or less. Silver stains are known to interact with cysteine residues, and the formaldehyde and glutaraldehyde used in the protocols for reductant and fixative purposes tend to alkylate α- and ε-amino groups of proteins[1, 10a-d], resulting in the prevention of Edman degradation and greatly complicating MALDI-MS mass fingerprinting[10d].

Reverse Stains

The inherent reduction of protein recovery due to the fixation step in Coomassie Blue methods, as well as in the fixation and sensitization steps in silver staining methods, reverse (negative) stains were developed with the goal of increasing protein recovery[5b]. The first method of reverse staining was reported by Wallace and coworkers in 1974[11a], in which the SDS-containing gel is cooled to 0^{o} - 4^{o}C. The free SDS in the gel will precipitate, forming an opaque background, while the SDS surrounding the proteins does not precipitate, leaving a clear protein band or spot[11b]. However, detection using this method is not very sensitive, and since the time of the publication by Wallace and coworkers, several improved reverse staining methods have become available.

One such method is reverse (negative) metal stain, which is based upon using a metal salt to form insoluble complexes with SDS. The insoluble complexes generate an opaque background, while the proteins that have bound the metal ions are seen as a clear band or spot. Common metal salts employed (with detection limits included) are copper chloride (5ng protein/band), zinc chloride and zinc sulfate (10-12 ng protein/band), potassium acetate (0.12-1.5 μg protein/band), and sodium acetate (0.1μg protein/band)[11b]. Other salts used include nickel chloride and cobalt acetate[5b]. Since there is no fixation

step involved in the reverse staining procedure, proteins can be quantitatively eluted after the metal ions are chelated with EDTA. Alternatively, a similar approach uses zinc ions in the presence of imidazole[5b,11b]. The free zinc ions form a precipitate with imidazole, forming a white background while leaving the proteins as a clear band or spot.

Metal Chelate Stains

Using metal chelate stains for detection of proteins is a relatively new method that has been used by a number of proteomic researchers. Metal chelate stains are based upon the principal that some metal ions can simultaneously bind to proteins and chelating agents in, forming highly colored complexes[12]. This method has appeal because the metal ions can be removed from the gel by a complexing agent such as EDTA, making these stains reversible. Reversible stains do not modify the proteins, and therefore do not complicate the subsequent mass spectral identification of the proteins of interest. The first paper utilizing metal complexes in staining proteins was by Graham and coworkers in 1978[13]. In that paper, it was found that use of bathophenanthroline disulfate/iron(II) generated a colored complex with proteins that could detect roughly 600 ng protein/band.

In 1982, Zapolski and coworkers introduced ^{59}Fe into the complex to lower detection limits down to 10-25 ng of protein/band[14]. However, the hazards of working with radioactive materials do not make this option of detection very attractive. Other stains used in detection of proteins using metal chelates include copper phthalocyanine tetrasulfonate[15], ferrozine/iron(II)[12] and pyrogallol red/molybdate[5b].

Due to limitations of colorimetric metal chelate stains including undesirable detection limits and use of radioactive materials to increase sensitivity, luminescent metal

chelate stains were developed to overcome such issues. The first luminescent metal chelate stain developed was SYPRO Rose, a bathophenanthroline disulfonate/europium complex[16]. SYPRO Rose does not covalently bind to proteins, so it can be easily removed prior to analysis, and is able to detect proteins in the 2-4 ng/band range. However, SYPRO Rose suffers from the fact that it also stains nucleic acids, requires a destaining step, tends to precipitate in the gel, and cannot be excited by visible light[5b].

To overcome some of the problems involved with SYPRO Rose, SYPRO Ruby (Figure 1.2) was developed, which is a ruthenium-based organic complex that binds with basic amino acid residues *via* electrostatic interaction[7,17]. SYPRO Ruby has a detection limit of 0.25-1 ng of protein/band, and does not stain nucleic acids. Additionally, SYPRO Ruby does not interfere with mass spectrometry since it can be removed and has a linear dynamic range spanning 3 orders of magnitude. Aside from being classified as metal chelate stains, the SYPRO stains represent a class of dyes that can be considered to be within the cutting edge of modern proteomic detection, which are fluorescent dyes.

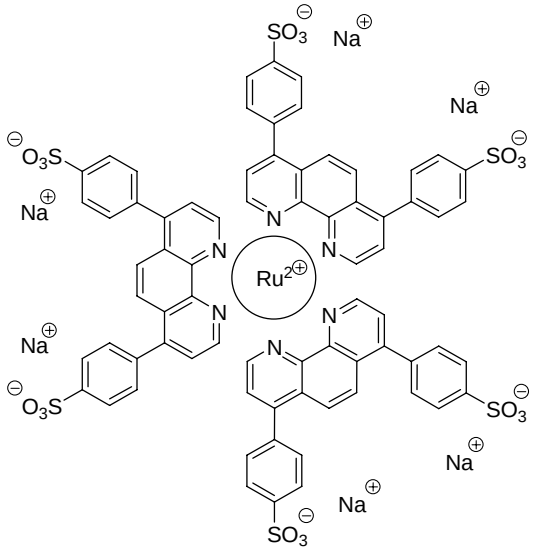


Figure 1.2. SYPRO Ruby.

Fluorescent Dyes

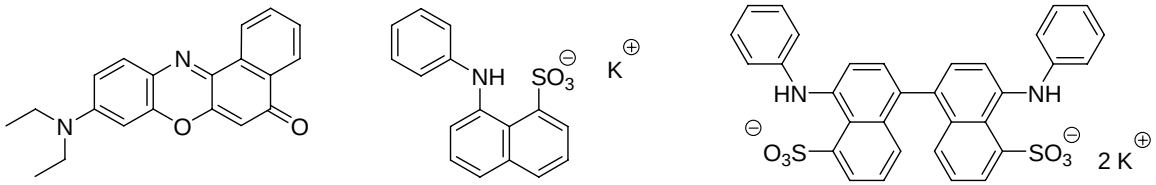
Non-Covalent Dyes: Fluorescent dyes designed for use in proteomic research are finding more application as their properties and benefits improve. Favorable fluorescent dyes possess high extinction coefficients, wide linear dynamic ranges, insensitivity to pH, good photostability and low detection limits. With several different types of fluorescent dyes available, one can choose from dyes possessing specific absorbances as well as the ability to bind to proteins covalently as an alternative to binding non-covalently.

Non-covalent fluorescent dyes do not directly interact with the proteins that they stain. Instead, they interact with the detergent coat surrounding proteins in SDS denaturing gels[5b, 7]. Even though the dyes interact with the SDS more than the protein itself, due to a fairly consistent binding of SDS to proteins in a ratio of 1.4:1, it is possible to obtain a semi-quantification measurement.

The SYPRO stains, mentioned earlier, belong to the non-covalent class of fluorescent dyes. Aside from SYPRO Rose and Ruby mentioned above, other SYPRO stains have been developed. They include SYPRO Orange, Red and Tangerine[18a, 18b, 18c]. SYPRO Orange and Red were introduced prior to SYPRO Tangerine by Molecular Probes, Inc©. The dyes are excitable at 472 nm (Orange) and 547 nm (Red), allowing for detection of proteins using visible light sources. The advantages SYPRO Orange and Red bring to protein labeling in PAGE-SDS include (1) a quick, 30-60 minute staining step with no destaining or fixation step required, (2) photostability robust enough to allow for multiple images to be recorded without reduction of the signal and (3) non-covalent binding to proteins, preventing interference with subsequent mass spectrometry analysis.

SYPRO Tangerine[18c] was introduced as a member of the SYPRO family that is friendly to the environment. Whereas most dyes require staining and/or fixation chemicals that are considered toxic, SYPRO Tangerine does not require such harmful chemicals. The staining procedure for SYPRO Tangerine is carried out in either a phosphate-buffered saline solution or 150 mM NaCl. SYPRO Tangerine possesses a detection limit of 4 to 10 ng protein, similar to that of SYPRO Orange and Red. SYPRO Tangerine is also excitable with visible light, as it possesses an absorbance maximum of approximately 490 nm.

Other available non-covalent fluorescent stains include Nile Red, 1-anilino-8-naphthalene sulfonate (ANS), 4-4'-bis(1-anilinonaphthalene-8-sulfonate) (bis-ANS) and Deep Purple[5b] (Figure 1.3).



Nile Red **ANS** **bis-ANS**

Figure 1.3. Examples of Non-Covalent Fluorescent Stains.

While Nile Red, ANS and bis-ANS bind proteins non-covalently, their performances do not compare favorably to those of the SYPRO dyes. The SYPRO dyes enjoy an advantage over Nile Red, ANS and bis-ANS due to their easy, one-step staining procedure, higher detection limits (~1 ng vs. ~100 ng), stability and better solubility (vs. Nile Red).

Covalent Dyes: Covalently binding fluorophores share the same photophysical properties (high extinction coefficients, insensitivity to pH, wide linear dynamic ranges, etc.) as non-covalently binding fluorophores. The key difference is they become physically bound to proteins, whether through primary amines or thiols. Even though many examples exist of covalently binding fluorophores, only a few will be discussed.

Deep Purple (formerly known as "Lightning Fast") contains the azophilone epicocconone derived from the fungus *Epicoccum nigrum*[10, 19]. Deep Purple binds covalently to proteins by binding through amine residues as well as binding non-covalently with the detergent coat they associate with. It is not until epicocconone reacts with amines that the dye becomes fluorescent[19a, 20] (Figure 1.4). Since more dye binds to protein than can react with amine residues, the gel is incubated in dilute ammonia to fully convert the dye to its fluorescent form. The sensitivity of Deep Purple is reported to be similar to that of SYPRO Ruby. However, the photostability of Deep Purple is limiting, with a half-life of 6 minutes, which is very problematic, as signal (and therefore detection of low concentration proteins) is lost at a fairly rapid rate.

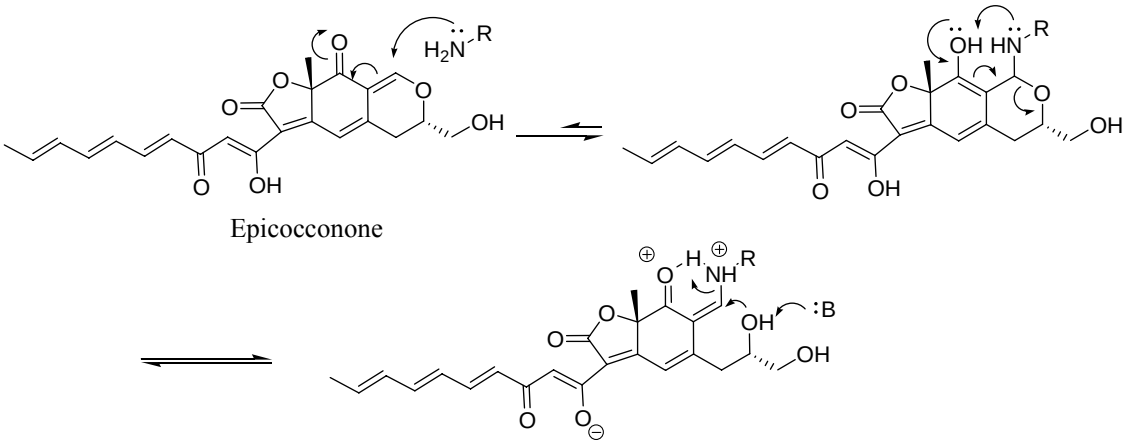


Figure 1.4. Mechanism of Epicocconone Binding.

A popular covalent fluorophore for protein labeling has been fluorescein isothiocyanate[5b] (Figure 1.5). Reaction of lysine amines with the isothiocyanate moiety results in a covalent linkage *via* a thiourea moiety. The large extinction coefficient, high quantum yield and water solubility make the fluorescein fluorophore a good choice for protein detection.

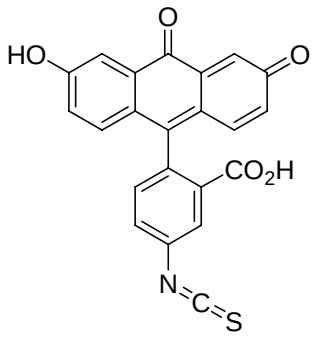


Figure 1.5. Fluorescein Isothiocyanate.

However, due to the pH sensitivity and poor photostability of fluorescein, other fluorescent dyes have been identified and synthesized. Additionally, dye "families" have been synthesized. These families are based upon a series of dyes derived from a parent fluorophore. Each dye in the family has been "tuned" by structural modification to absorb and emit at different wavelengths. This allows for simultaneous detection of different samples of experimental proteins in the same gel by utilizing the individual absorbances and emissions of the dyes. After the different protein samples have been imaged by a detector, the images can be overlaid to detect the protein forms that differ significantly between the different samples.

Currently, the GE Healthcare Typhoon imager is the most advanced fluorescence detection instrument used in 2D gel proteomic research. The Typhoon uses a blue

excitation laser at 488 nm wavelength, green excitation laser at 532 nm wavelength and red excitation laser at 633 nm wavelength to excite the fluorophores that absorb at the differing wavelengths, followed by detection of the dye emissions using different color filters. The ability to run more than one protein sample in the same gel gives researchers several advantages: (1) reduction of experimental errors from inter-gel comparisons, (2) being able to more correctly identify changes in protein abundance, as well as (3) having to run fewer gels.

The DIGE dye family of amine-reactive fluorescent dyes, sold by GE Healthcare, covalently bind to lysine residues in proteins by forming an amide linkage[7, 21]. The members of the DIGE family of dyes are based upon three chromophores: Cy2, Cy3 and Cy5, which are shown in Figure 1.6. With differing absorption maxima that allow for independent excitation of CyDyes, it is possible to label protein batches with CyDyes and analyze them on the same 2D gel using commercially available detection equipment.

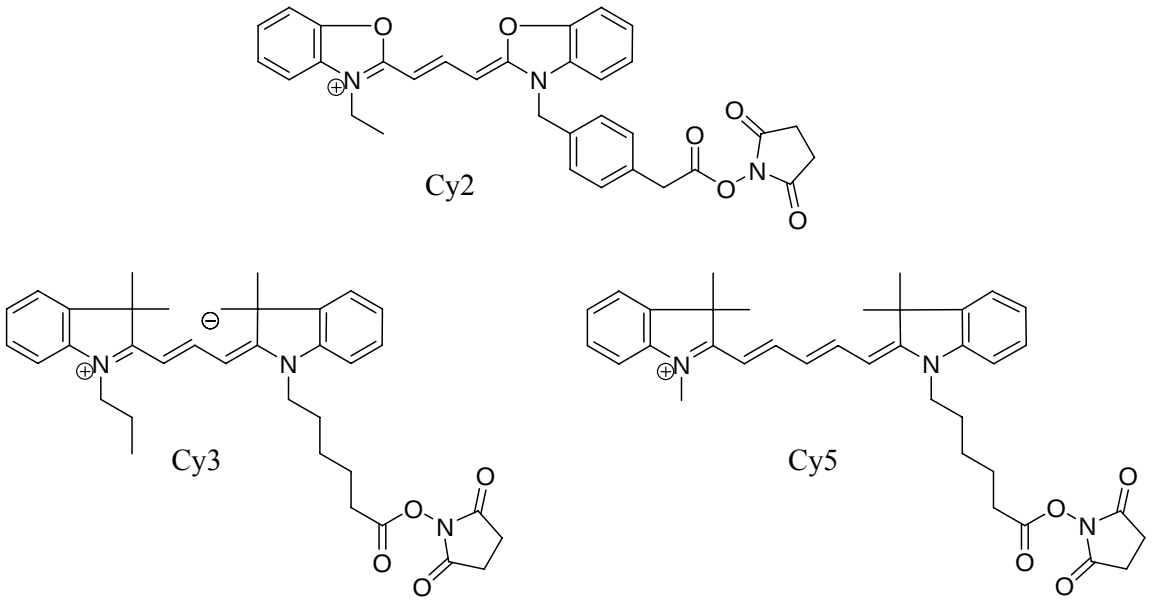


Figure 1.6. Commercially Available DIGE Dyes.

The DIGE family of dyes possesses photophysical properties that make them attractive for proteomic research. In addition to their absorption in the visible region, DIGE dyes have relatively high extinction coefficients and wide linear dynamic ranges. However, DIGE dyes have poor quantum yields and are relatively unstable, as they are susceptible to nucleophilic attack. Additionally, due to limited water solubility, protein labeling is kept at a minimum so labeled proteins will not precipitate out of solution.

A major disadvantage among the members of the DIGE family is that the dyes contain a non-titratable positive charge. This net positive charge presents a problem when performing isoelectric focusing of DIGE dye labeled proteins at high pH, as lysine residues have a pKa of approximately 10. When proteins covalently linked to CyDyes through lysine residues undergo isoelectric focusing, the net positive charge of the CyDye will persist above pH 10 and shift the pI of the proteins that have their pI at or above pH 10, complicating the analysis of certain basic proteins.

Another commercially available set of dyes is the BODIPY family of dyes, sold by Invitrogen[22] (Figure 1.7). Several members of this dye family have been synthesized that have unique absorbance and emission wavelength. The BODIPY dye family members span the visible region[22], making them versatile in diagnostic studies. However, due to limited water solubility, BODIPY dyes are not adequate for proteomic research.

The BODIPY family of dyes with amine-reactive functionalities binds to proteins by forming an amide bond with lysine residues. The photophysical properties of the BODIPY core structure make each dye in the family very effective in the detection of

labeled proteins. BODIPY dyes are known to have high extinction coefficients, high quantum yields, relatively long fluorescence lifetimes, wide linear dynamic ranges, good photostability, minimal sensitivity to solvent polarity and pH, and narrow absorbance and emission peaks that allow for detection of less than 100 pg protein[23a, 23b, 23c, 23d].

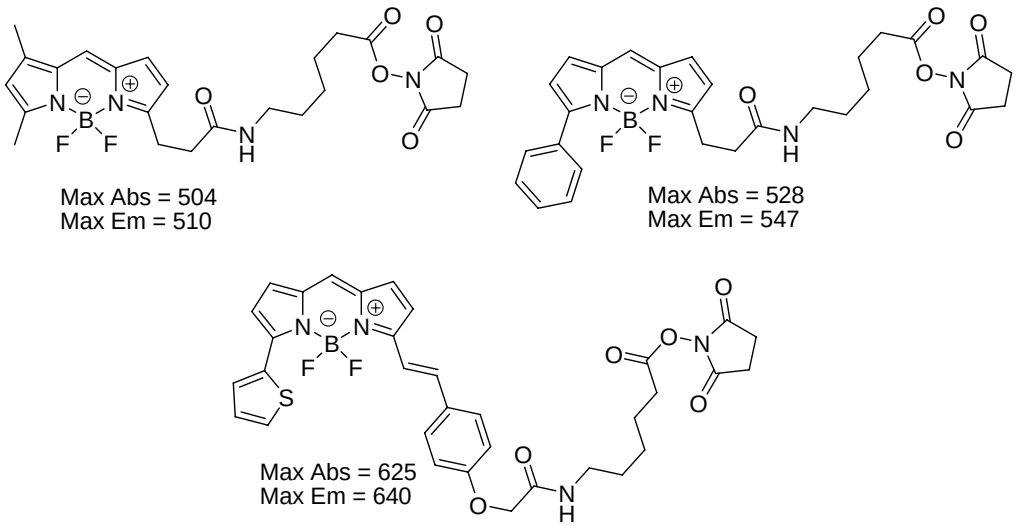
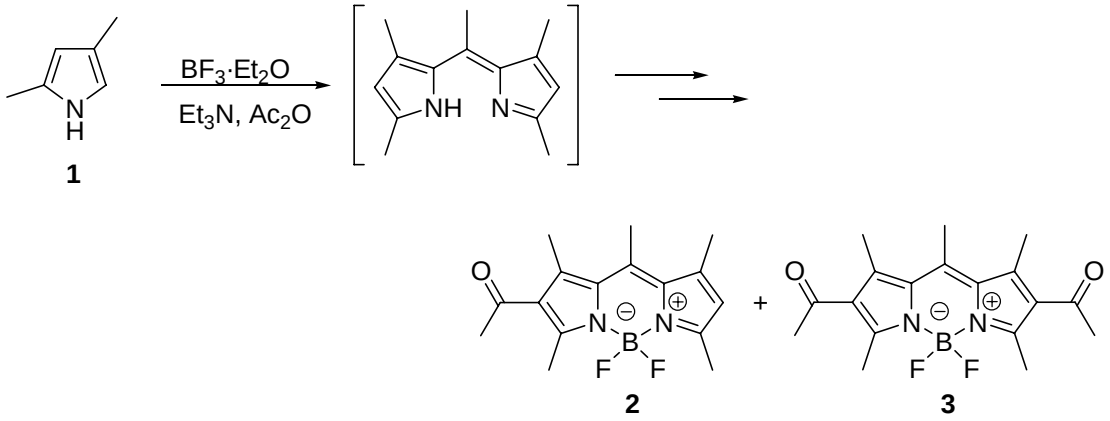


Figure 1.7. Examples of Commercially Available BODIPY Dyes

However, labeling proteins with these BODIPY dyes will change the pI of labeled proteins by converting titratable lysine amino groups into amides. Furthermore, due to limited water solubility in the commercially available BODIPY dyes, a tendency for labeled proteins to precipitate out of solution exists. If one could increase the water solubility of BODIPY dyes, one could further enhance protein detection. The first goal of this project was to synthesize a zwitterionic BODIPY dye possessing (1) increased water solubility, (2) a titratable amine to preserve the pI of labeled proteins, (3) the ability to covalently bind to proteins and (4) an absorbance maximum of approximately 633 nm, allowing excitation with a commercially available red laser.

Background on BODIPY Dyes

In 1968, Treibs and Kreuzer published the first account of the synthesis of the 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) fluorophore[24]. In their seminal paper, Treibs and Kreuzer reacted 2,4-dimethylpyrrole **1** with acetic anhydride in the presence of $BF_3{\cdot}Et_2O$ to yield the first BODIPY fluorophores **2** and **3** (Scheme 1.1).



Scheme 1.1. Formation of Initial BODIPY Dyes.

Since the report by Treibs and Kreuzer, BODIPY dyes have raised considerable interest in the synthetic community, resulting in the synthesis of a variety of BODIPY dyes, expanding not only the range of absorbance and emission wavelengths, but also the synthetic methodology and the applications of these fluorophores. The BODIPY fluorophore has been utilized for a variety of applications, including biomolecular probes[23d], pH probes[23a, 25], sensors to detect metal ions, reducing agents, or nitrogen-monoxide[23b, 26] and fluorescent switches[27]. What makes the BODIPY fluorophore so attractive in comparison to other fluorophores are the photophysical properties mentioned

earlier and the ease of tuning its absorption and emission wavelengths by simple substitution of the functional groups attached to the BODIPY core (Figure 1.8).

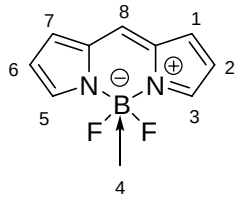


Figure 1.8. BODIPY Substitution Numbering

In 1985, Lugtenberg and coworkers prepared a BODIPY dye possessing enhanced water solubility[28] compared to the original BODIPY dyes synthesized by Treibs and Kreuzer for use in their angiographic studies. Lugtenberg *et al* reasoned that introduction of a sulfonate group at either the 2 or the 6 position (the favored sites for electrophilic substitution (Figure 1.9) on the BODIPY structure would lead to an increase in water solubility.

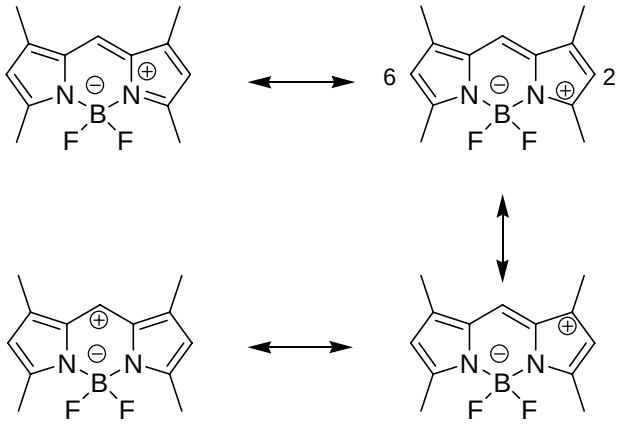
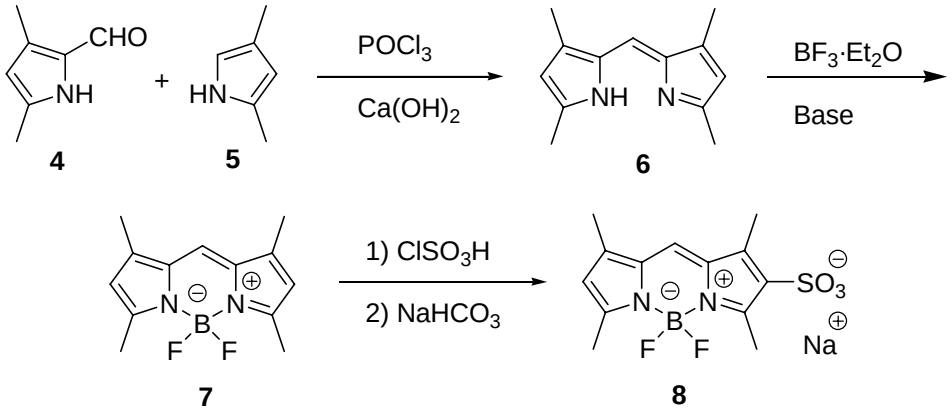


Figure 1.9. Resonance Showing Favored Sites for Electrophilic Substitution.

To generate their BODIPY dye, 3,5-dimethyl-2-formylpyrrole **4** and 2,4-dimethylpyrrole **5** were condensed in the presence of phosphorus oxychloride and calcium hydroxide to yield dipyrromethene **6** (Scheme 1.2).



Scheme 1.2. Synthesis of a Water Soluble BODIPY Dye.

Dipyrromethene **6** was treated with $BF_3{\cdot}Et_2O$ in the presence of base furnishing BODIPY fluorophore **7**. When treated with chlorosulfonic acid in dichloromethane, the monosulfonated BODIPY fell out of solution due to insolubility in dichloromethane. The dye was neutralized with sodium bicarbonate to provide BODIPY dye **8**.

Haugland and Kang contributed greatly to the synthesis of new BODIPY dyes. In the late 1980's through the 1990's, Haugland and Kang applied for a series of patents on BODIPY dyes[29a-g], covering a wide range of applications, including dyes that are able to covalently bind to proteins[29a, 29d]. The patents outline a general procedure for the syntheses of a variety of BODIPY dyes representing a wide range of absorbances and emissions. A few examples of *N*-hydroxysuccinimide activated BODIPY dyes they synthesized and are now available from Invitrogen are shown in Table 1.1 along with their absorbance and emission values[29d].

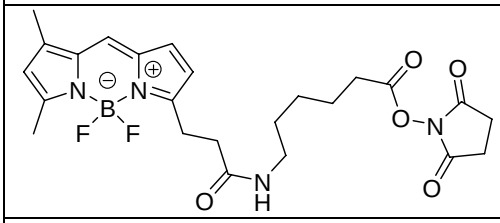
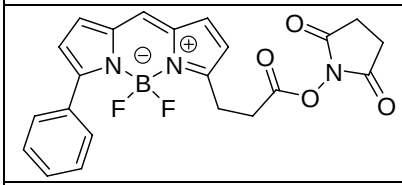
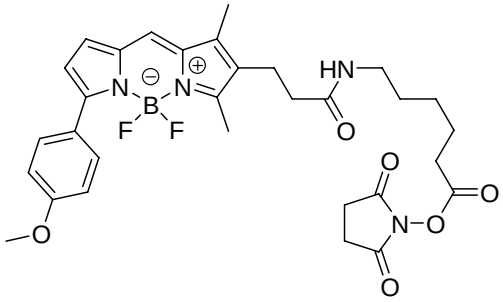
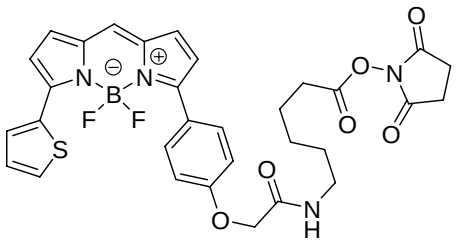
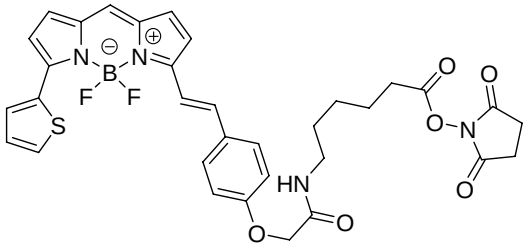
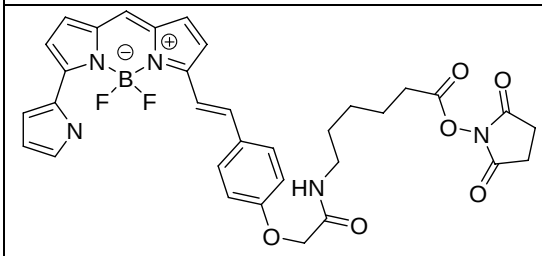
Structure	Max Absorbance (nm)	Max Emission (nm)
	504	510
	528	547
	544	570
	588	616
	625	640
	646	660

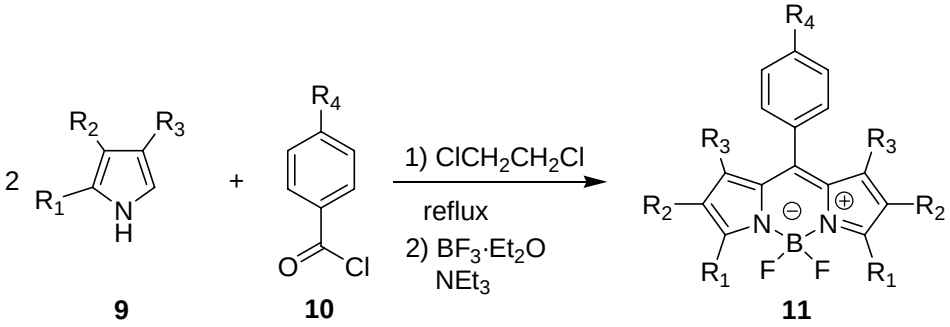
Table 1.1. Absorbance and Emission Wavelengths of Selected BODIPY Dyes.

BODIPY dyes can be tuned with the addition of substituents so their absorbances are shifted toward the red end of the visible spectrum. The absorbances can be controlled to a certain degree through the introduction of aryl groups. Review of the patents of Haugland and Kang indicates that the most common aryl substituents in BODIPY dyes are phenyl, pyrrole and thienyl groups. Although there is not a set amount as to how much red-shifting each aryl group contributes to the absorbance and emission of BODIPY dyes, by comparing the absorbances and emissions of BODIPY dyes in Table 1.1, one can see there is a general pattern as to which aryl groups will red-shift the absorbance of BODIPY fluorophores the most. Generally, phenyl groups red-shift the least, while pyrrole groups red-shift the most and thienyl groups have an intermediate effect.

Conjugation within the aryl substituents can be introduced to further the conjugation of BODIPY dyes. Two aryl groups commonly used for this purpose are the styryl and styryloxy groups, which both induce large red-shifts, as can be seen in Table 1.1. The benefit of including a styryloxy (or phenoxy) group is that the alcohol group can be alkylated with an alkyl chain containing an ester or carboxylic acid. This provides a site for activation and coupling to a desired linker.

BODIPY dyes have also been an area of interest for the Burgess group[23c, 30a, 30b, 30c]. Burgess and coworkers have focused their syntheses on symmetrical BODIPY dyes containing an aryl group substituted at the 8 position. This methodology shortens the synthesis of their BODIPY dyes, as only one substituted pyrrole is needed in the generation of the dipyrromethene core.

The common methodology utilized by Burgess involves refluxing two equivalents of a substituted pyrrole **9** in the presence of an acyl chloride **10** (Scheme 1.3). Further treatment with $BF_3 \cdot Et_2O$ in the presence of triethylamine, either *in situ* or after isolation of the dipyrromethene, results in the generation of the BODIPY dye **11**.



Scheme 1.3. Synthesis of Symmetrical BODIPY Dyes.

Several BODIPY dyes synthesized by the Burgess group contain bridged aryl systems, preventing the aryl groups from rotating freely[30b, 30c]. These restricted systems affect the fluorescent properties of the BODIPY dyes by red-shifting their absorbances and emissions. In general, the quantum yields and extinction coefficients of these constricted dyes compared to non-constricted dyes (e.g. **11**) are increased while their Stokes shifts are decreased.

The bridged BODIPY dyes are synthesized in a similar manner to the non-bridged symmetrical BODIPY dyes prepared by the Burgess group. Two equivalents of substituted pyrrole are reacted with an acyl chloride, followed by generation of the fluorophore by treatment with $BF_3 \cdot Et_2O$ in the presence of triethylamine. Examples of non-restricted and restricted dyes synthesized by Burgess and coworkers are shown in Table 1.2.

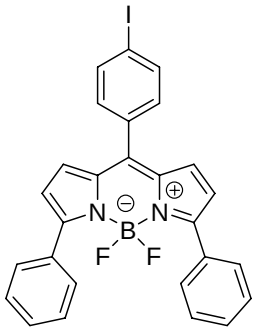
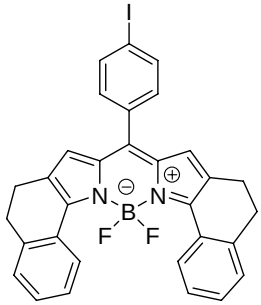
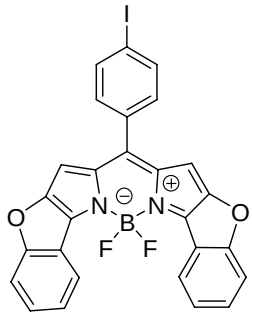
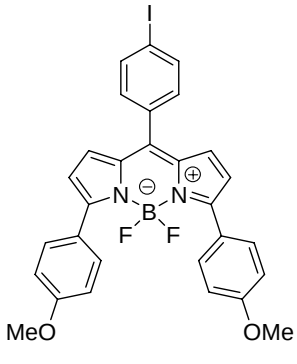
Structure	λmax (abs)	λmax (em)	Stokes shift	*E*	Φ
	558 nm	592 nm	34 nm	53,300	0.15
	634	647	13	126,250	0.38
	637	647	10	151,000	0.34
	585	629	44	54,100	0.33

Table 1.2. Comparison of Unbridged vs. Bridged BODIPY Systems.

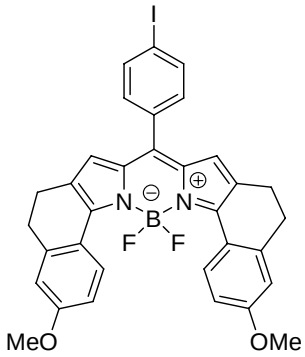
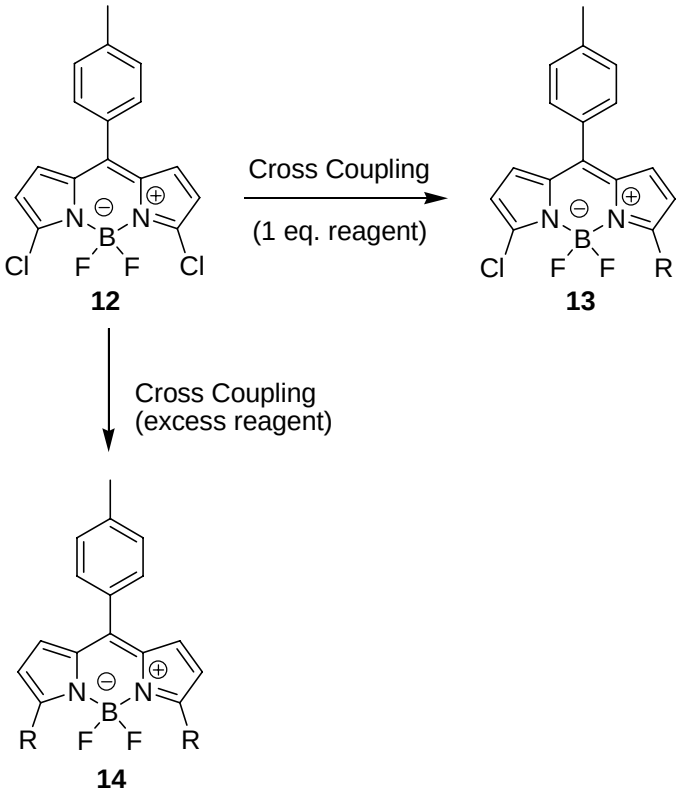
Structure	λmax (abs)	λmax (em)	Stokes shift	*E*	Φ
	658	673	15	139,500	0.13

Table 1.2. Comparison of Unbridged vs. Bridged BODIPY Systems. (Continued)

Another methodology that has recently come into use in the synthesis of BODIPY fluorophores involves palladium cross-coupling reactions with 3,5-dichloro BODIPY fluorophore **12** (Scheme 1.4)[31a, 31b]. This methodology, developed by Qin and coworkers, makes use of the well known Heck, Sonogashira, Stille, and Suzuki cross-coupling reactions. Controlling the equivalents of boronic acid added to the reaction mixture allows for formation of either a mono-substituted product **13** (1 equivalent added) or di-substituted product **14** (2 or more equivalents added). The ability to vary the aryl substituents after formation of the fluorophore is appealing as it presents a shortened synthesis compared to the number of steps that would be necessary to synthesize two appropriately substituted pyrrole sources. Yields of the mono- and di-substituted products are modest, ranging from 50% to 68%.



Scheme 1.4. Cross Coupling of Halogenated BODIPY Fluorophores.

SYNTHESIS OF A BODIPY DYE FOR USE WITH A 633 NM WAVELENGTH LASER

Design and Synthesis of the First BODIPY Dye Target

Synthetic Strategy

BODIPY-based fluorophores were targeted with the goal of synthesizing a class of fluorescent dyes that will lower detection limits of proteins, therefore enhancing proteomic research. The high extinction coefficient, quantum yield, photostability, chemical stability and spectral versatility of BODIPY fluorophores make them ideal for synthesizing a family of fluorescent dyes. In order for a new class of BODIPY fluorophores to have improved detection limits compared to current available technology, it is necessary to modify the BODIPY structure to contain structural features aimed at enhancing the performances of these dyes compared to those displayed by currently available dyes. The added structural features included in the structural design led to proposed BODIPY dye **15** (Figure 2.1).

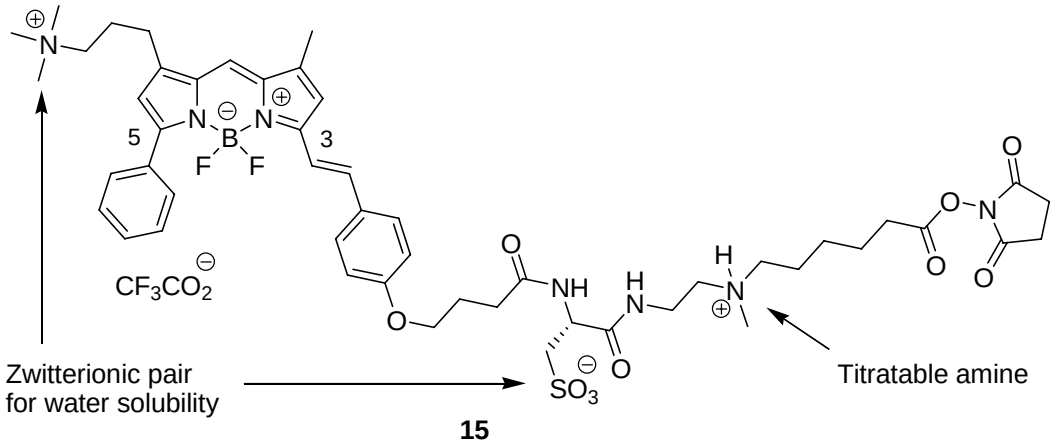


Figure 2.1. Structure and Features of Proposed BODIPY Dye **15**.

One structural feature of **15** is a zwitterionic pair, with the purpose of enhancing the water solubility of the dye. Enhanced water solubility of the dye will increase the water solubility of labeled proteins, especially at their pI where protein solubility is minimal. This enhanced solubility at the pI will allow for detection of proteins occurring in low concentrations, as higher labeling of proteins with a dye that is more water soluble will prevent the labeled proteins from precipitating out of solution.

Another structural feature is a titratable tertiary amine functionality. The purpose of this group is to mimic the pKa of the lysine residue, through which the protein binds to the dye. By mimicking the pKa of the lysine residue, this titratable functionality will prevent the pI of the labeled protein from being shifted by dye labeling and therefore dye labeling will not affect the isoelectric focusing of the protein mixture.

The final features included in the proposed structure of **15** are common to BODIPY dyes sold by Invitrogen. An *N*-hydroxysuccinimide activated ester is included for covalent linkage of the dye to protein, as well as variation of the conjugation at the 3 and 5 positions (in this case as styryloxy and phenyl groups) to allow for absorbance in the red region of the visible spectrum.

Retrosynthetic analysis of BODIPY target **15** results in fluorophore **16** and amide chain **17** (Figure 2.2). Removal of the boron difluoro group followed by cleavage of the dipyrromethene skeleton of **16** gives rise to substituted formyl pyrrole **18** and styryloxy pyrrole **19** (Figure 2.3).

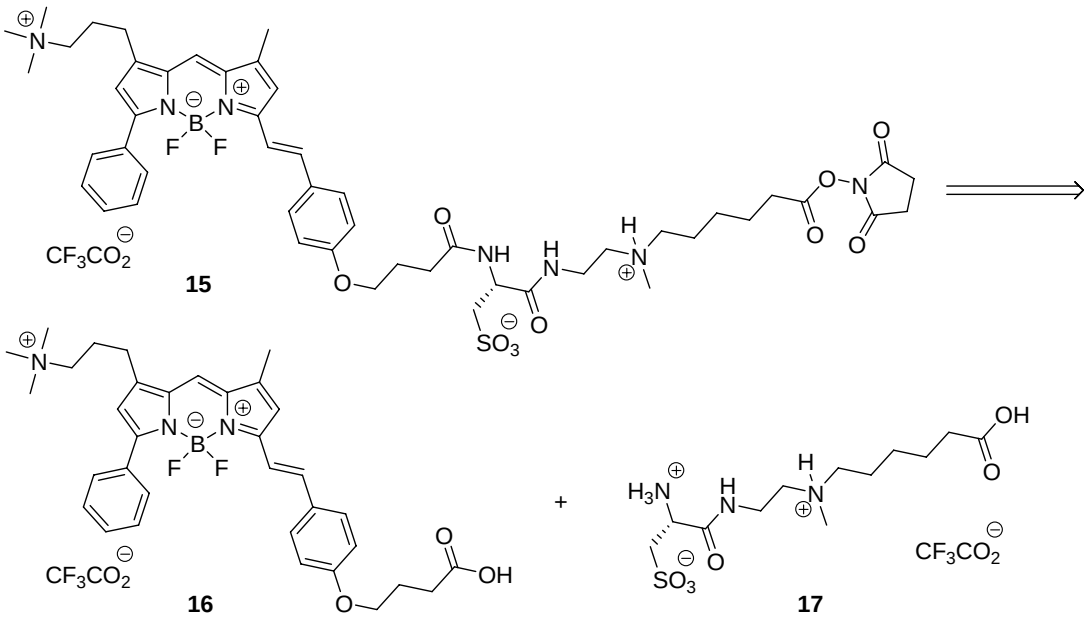


Figure 2.2. Retrosynthetic Analysis of BODIPY Dye **15**.

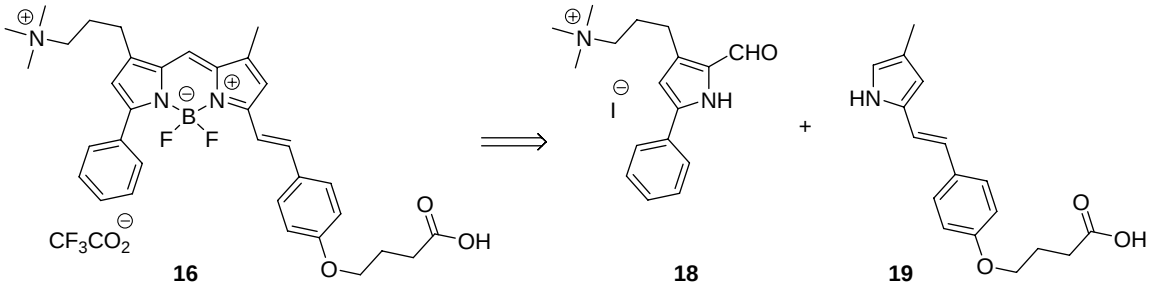
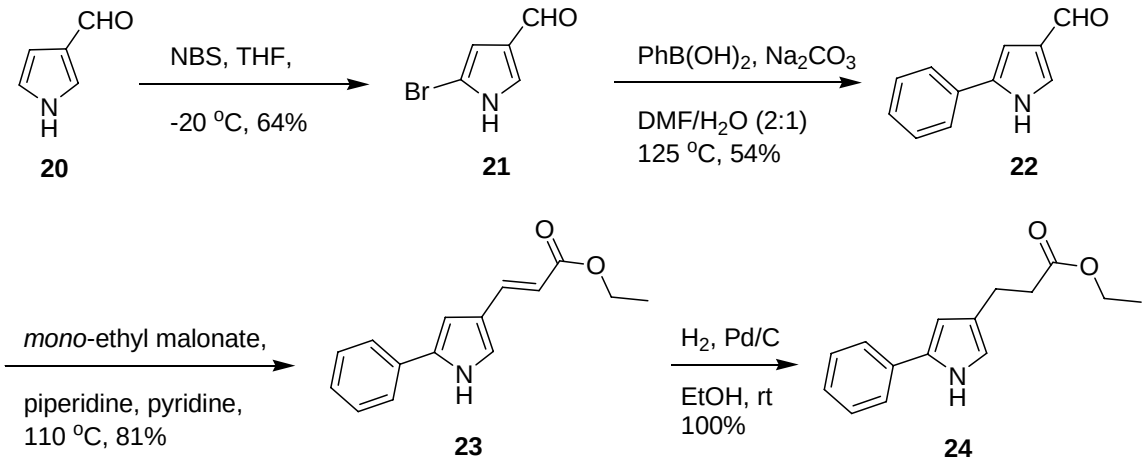


Figure 2.3. Retrosynthesis of the BODIPY Fluorophore.

Results and Discussion

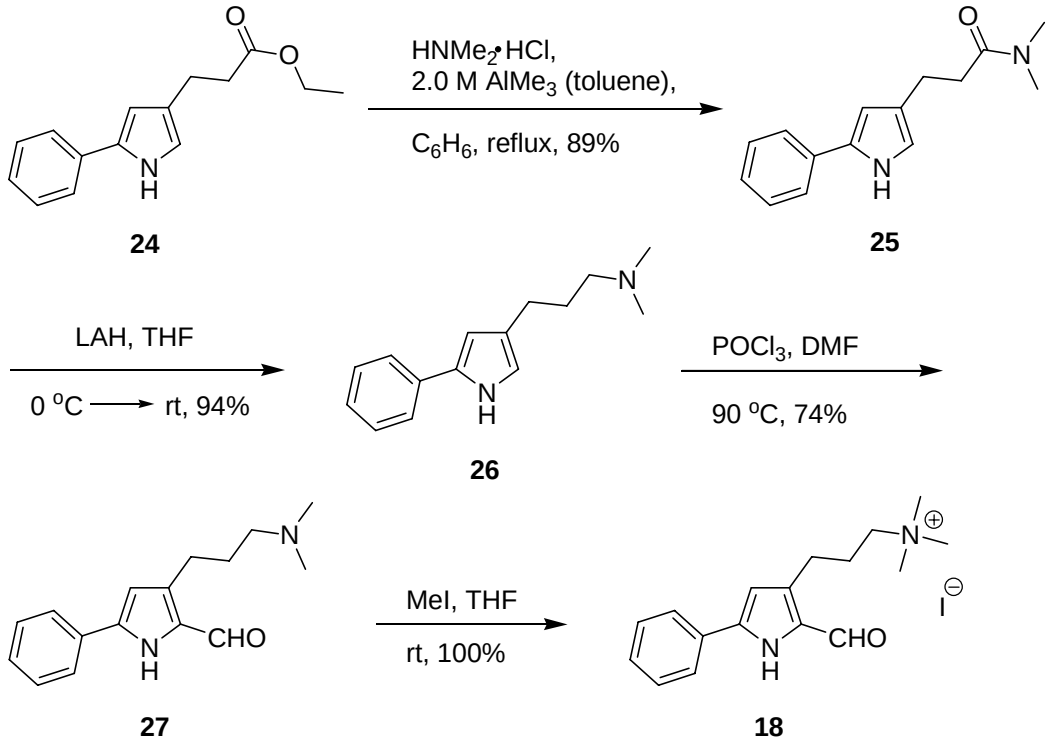
The synthesis of BODIPY dye **15** began with known pyrrole-3-carboxaldehyde **20** according to the procedure of Bray and coworkers[33] (Scheme 2.1). Utilizing the directing effect of the formyl group, treatment of **20** with NBS at -20 ^{o}C afforded the bromo formyl pyrrole **21** in 64% yield. A standard Suzuki coupling[34] carried out on **21** employing

phenylboronic acid provided phenyl pyrrole **22** in 54% yield. Conversion of **22** into acrylate **23** was achieved by Doebner modification of the Knoevenagel condensation[35], providing acrylate **23** in 81% yield. The olefin of acrylate **23** was reduced under standard hydrogenation conditions giving way to propanoate **24** quantitatively.



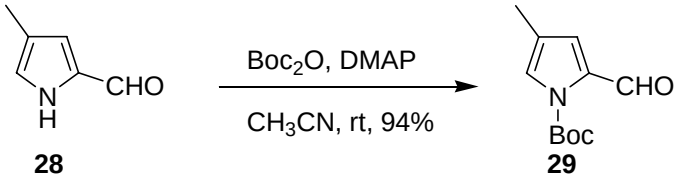
Scheme 2.1. Synthesis of Propanoate **24**.

Ester **24** was converted into amide **25** following the procedure described by Russell[36] providing amide **25** in 89% yield (Scheme 2.2). Reduction of amide **25** into amine **26** proceeded smoothly in 94% yield using lithium aluminum hydride in anhydrous tetrahydrofuran. Formylation of the α-position of pyrrole **26** was achieved by treatment with the Vilsmeier-Haack reagent, furnishing formyl pyrrole **27** in 74% yield. Subtarget **18** was reached in quantitative yield by quaternization of tertiary amine **27** using iodomethane in anhydrous tetrahydrofuran.



Scheme 2.2. Completion of Subtarget **18**.

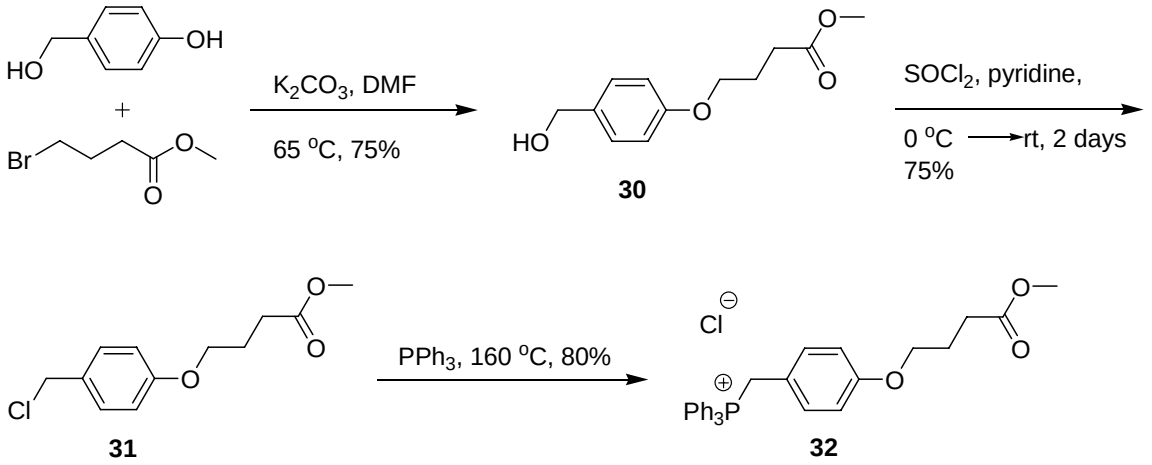
Synthesis of pyrrole **19** commenced with pyrrole **28**, previously synthesized by Muchowski and coworkers[32]. *N*-Boc protection of pyrrole **28**, employing a slight modification of the procedure of Grehn[37], led to the desired *N*-Boc pyrrole **29** in 94% yield (Scheme 2.3).



Scheme 2.3. Protection of 2-Formyl-4-Methylpyrrole.

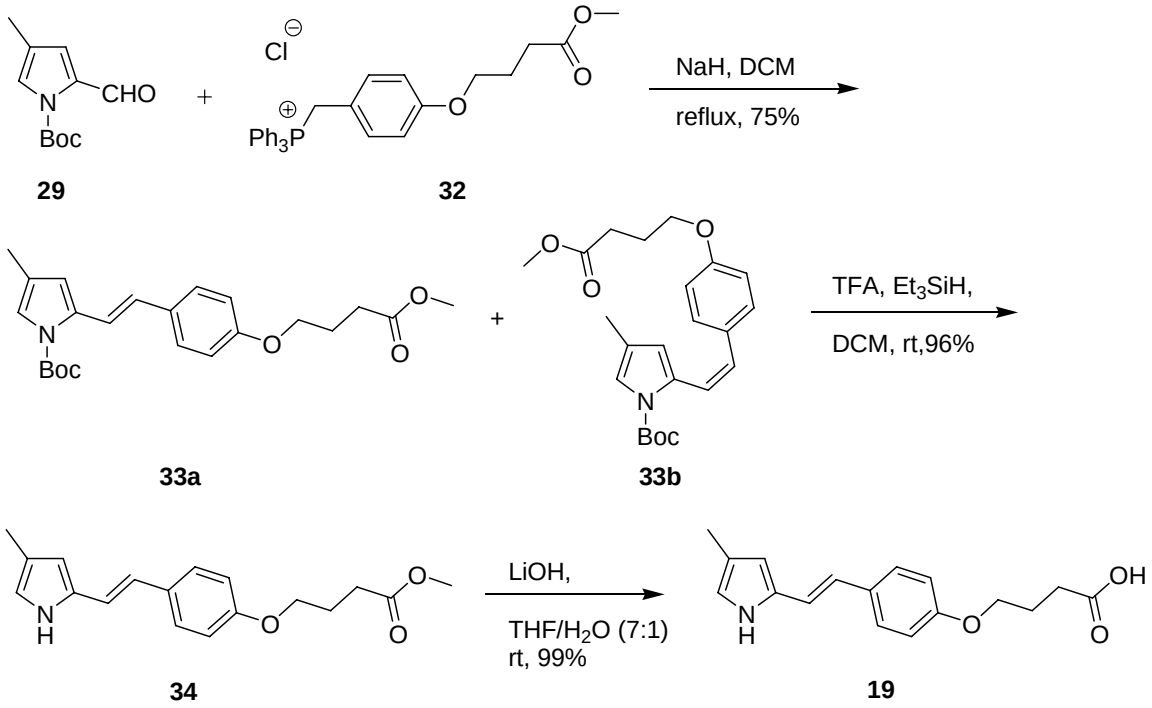
Alkylation of commercially available 4-hydroxybenzyl alcohol with methyl 4-bromobutanoate[38] provided benzyl alcohol **30** in 75% yield (Scheme 2.4). Conversion of benzyl alcohol **30** into benzyl chloride **31** was accomplished by treatment of **30** with

thionyl chloride in anhydrous toluene[39]. Stirring at ambient temperature for 1 d furnished the desired benzyl chloride **31** in 65% yield. In an attempt to improve the yield of this reaction, the reaction mixture was allowed to stir at ambient temperature for 2 d, which improved the yield of isolated **31** to 75%. With benzyl chloride **31** in hand, a Michaelis-Arbuzov reaction was performed using neat triphenylphosphine providing phosphonium salt **32** in 80% yield.



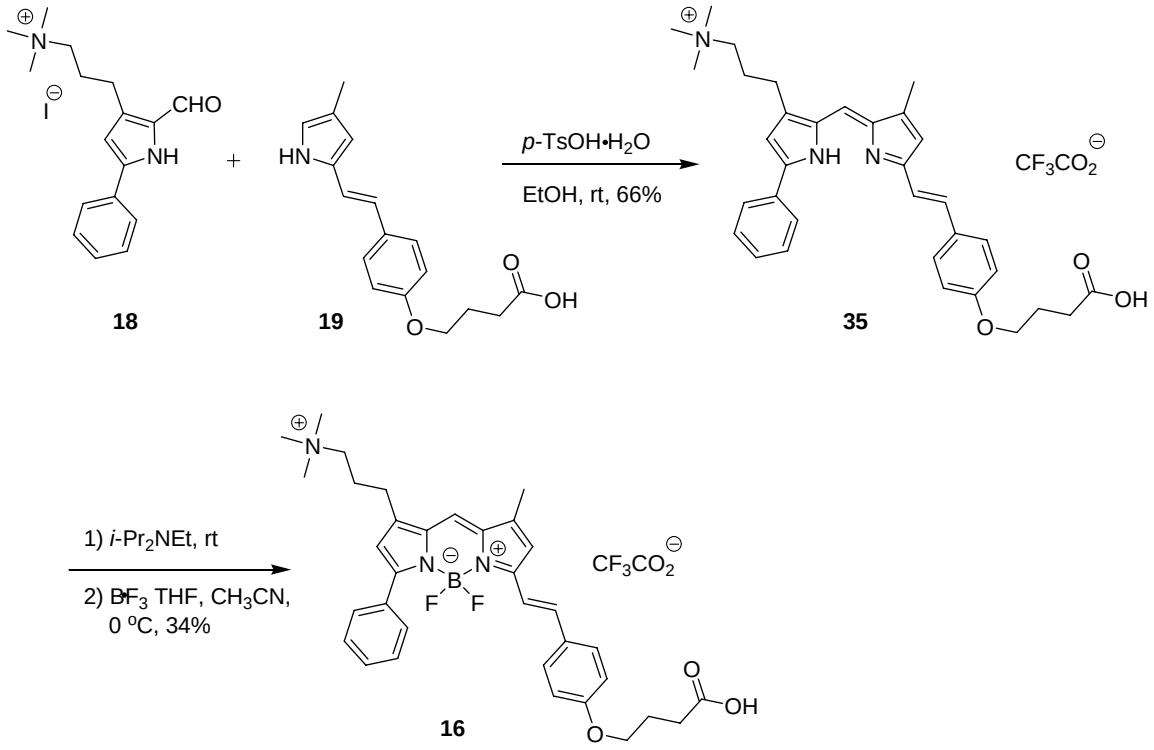
Scheme 2.4. Formation of the Phosphonium Salt.

Coupling of pyrrole **29** with phosphonium chloride **32** *via* a Wittig reaction afforded a 1:1 mixture of the (*E*)- and (*Z*)- isomers of styryloxy pyrroles **33a** and **33b** in 75% yield (Scheme 2.5). The isomers could be separated by column chromatography, but it was found that upon standing for even a few minutes neat or in solution, the pure isomers would isomerize back to a mixture. Fortunately, it was discovered that treatment of the 1:1 mixture of **33a** and **33b** with TFA and Et_3SiH[40] to effect *N*-Boc deprotection provided the desired (*E*)-styryloxy pyrrole **34** in 96% yield. Hydrolysis of the ester with LiOH in aqueous THF gave way to subtarget **19** in 99% yield.



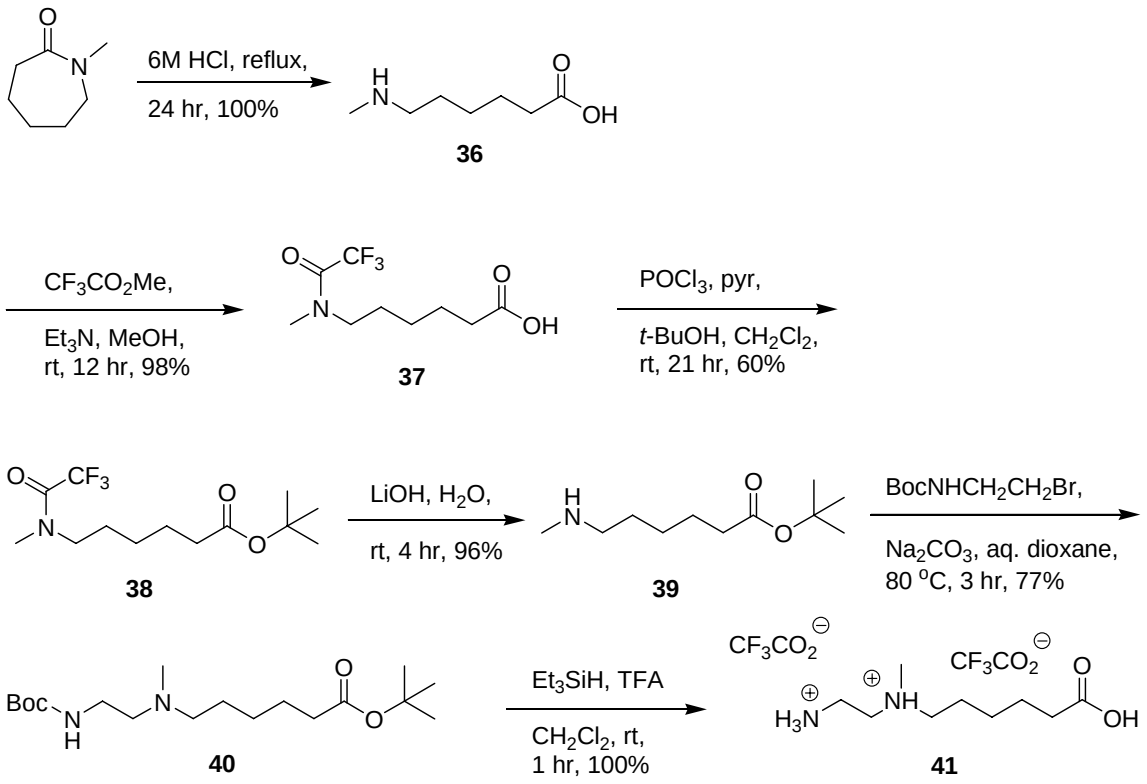
Scheme 2.5. Completion of Subtarget **19**.

With the left- and right-hand pieces of the BODIPY core structure in hand, subtargets **18** and **19** were coupled *via* an acid-catalyzed reaction providing dipyrromethene **35** in 66% yield (Scheme 2.6). Fluorophore **16** was generated by treatment of the dipyrromethene **35** with Hunig's base in anhydrous acetonitrile at ambient temperature for 5 min followed by treatment with BF_3·THF complex in an ice bath for 30 min. The solvent was removed *in vacuo* at 0 ^oC before reverse phase HPLC purification of the resulting complex mixture, giving way to fluorophore **16** in 34% yield (56% brsm). Due to the sensitivity of the BODIPY fluorophore to base, including DIPEA, it was necessary to perform the reaction and work up at 0 ^oC in order to maximize the yield of the fluoroboration step.



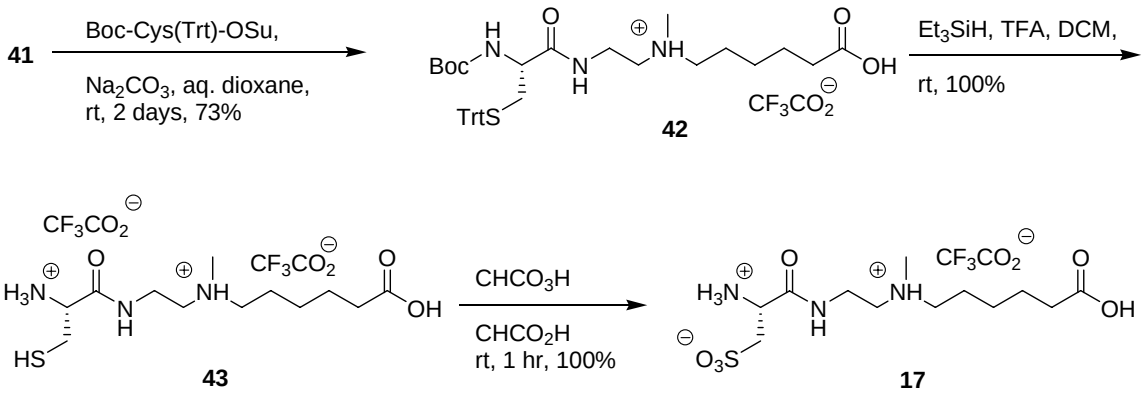
Scheme 2.6. Generation of the BODIPY Fluorophore.

To initiate the synthesis of amide chain **17**, commercially available *N*-methyl caprolactam was treated with 6M HCl, giving way to 5-(methylamino)pentanoic acid **36** in quantitative yield (Scheme 2.7). Treating the secondary amine **36** with methyl-trifluoroacetimide afforded acid **37** in 98% yield. Conversion of the acid **37** to the acyl chloride followed by trapping with *t*-butanol provided the *tert*-butyl ester **38** in 60% yield. The secondary amine was unmasked by treatment with LiOH affording amine **39** in 96% yield. The titratable amine functionality was generated by reacting the secondary amine **39** with *tert*-butyl 2-bromoethylcarbamate providing the protected amino acid **40** in 77% yield. Global deprotection of **40** was performed employing TFA and Et_3SiH[40] in CH_2Cl_2 providing amino acid **41** in quantitative yield.



Scheme 2.7. Synthesis of Amino Acid **41**.

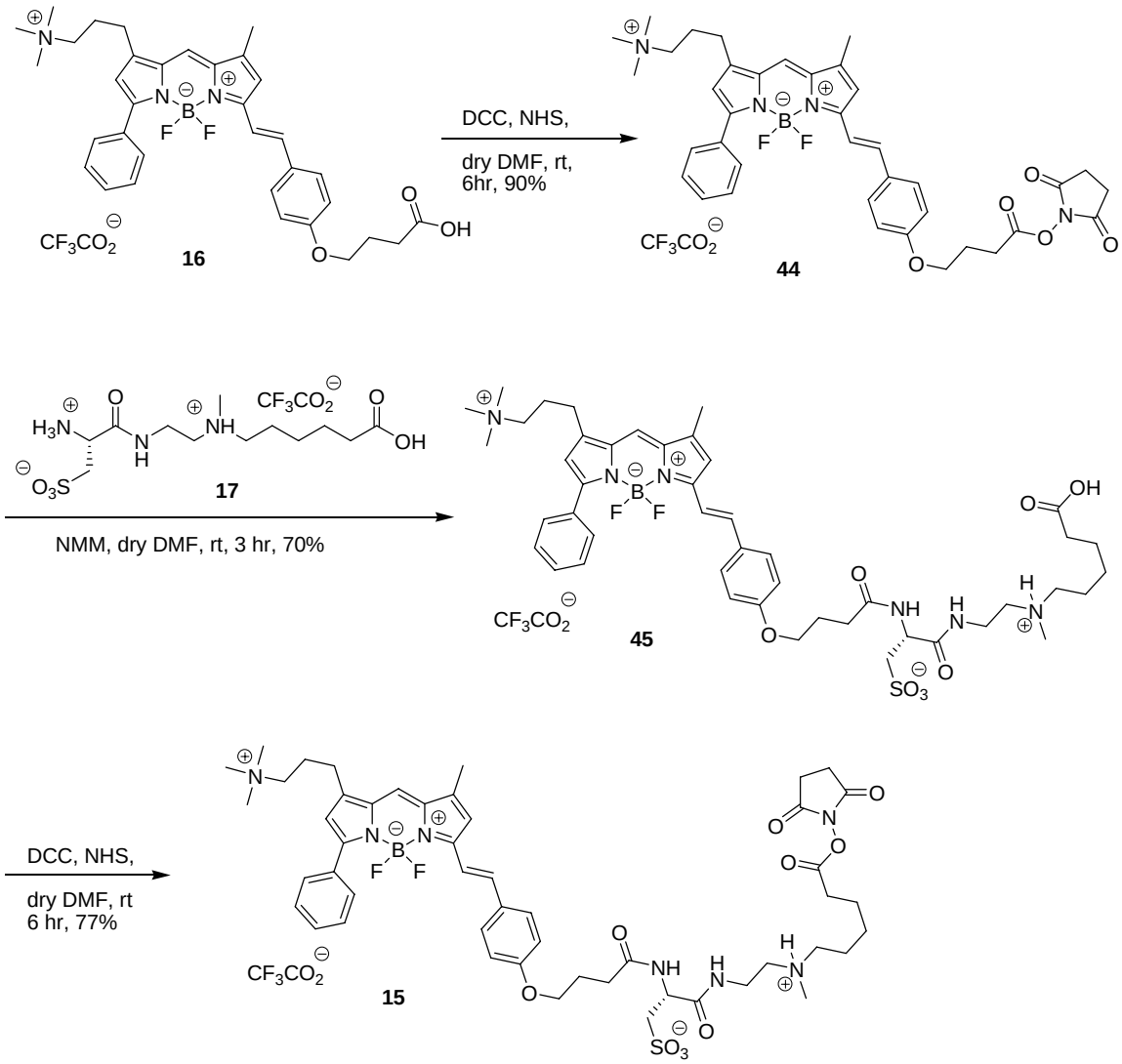
Reaction of amino acid **41** with commercially available Boc-Cys(Trt)-OSu gave way to the *N*-Boc protected, *S*-trityl protected amino acid **42** in 73% yield (Scheme 2.8). Global deprotection of **42** employing a 1:1 solution of TFA/CH_2Cl_2 in the presence of Et_3SiH[40] afforded the penultimate cysteine amide **43** in quantitative yield. Oxidation of the cysteine residue into its sulfonate by performic oxidation[41] afforded the desired amide chain **17** quantitatively.



Scheme 2.8. Completion of the Amide Chain.

With fluorophore **16** and amide chain **17** in hand, three steps remained to complete the synthesis of BODIPY dye **15**. Before coupling with the amide chain **17**, fluorophore **16** was activated as its *N*-hydroxysuccinimidyl ester by treatment with *N,N*'-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in anhydrous *N,N*-dimethylformamide affording activated fluorophore **44** in 90% yield (Scheme 2.9). Coupling fluorophore **44** with the amide chain **17** in anhydrous *N,N*-dimethylformamide was performed in the presence of *N*-methylmorpholine giving way to fluorophore **45** in 70% yield. Activation of **45** with *N,N*'-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide provided the desired BODIPY dye **15** in 77% yield.

The results shown in Table 2.1 show the maximum absorbance of BODIPY dye **15** occurs at 598 nm, with a maximum emission at 624 nm. A plot of the absorbance of BODIPY dye **15** shows minimal excitation by the red laser (Figure 2.4). Therefore, it was necessary to design and synthesize a different BODIPY dye that absorbs at the necessary wavelength.



Scheme 2.9. Completion of Targeted BODIPY Dye **15**.

Absorbance (nm)	Emission (nm)	Extinction Coefficient	Quantum Yield
598	624	77,950	80%

Table 2.1. Photophysical Properties of BODIPY Dye **15**.

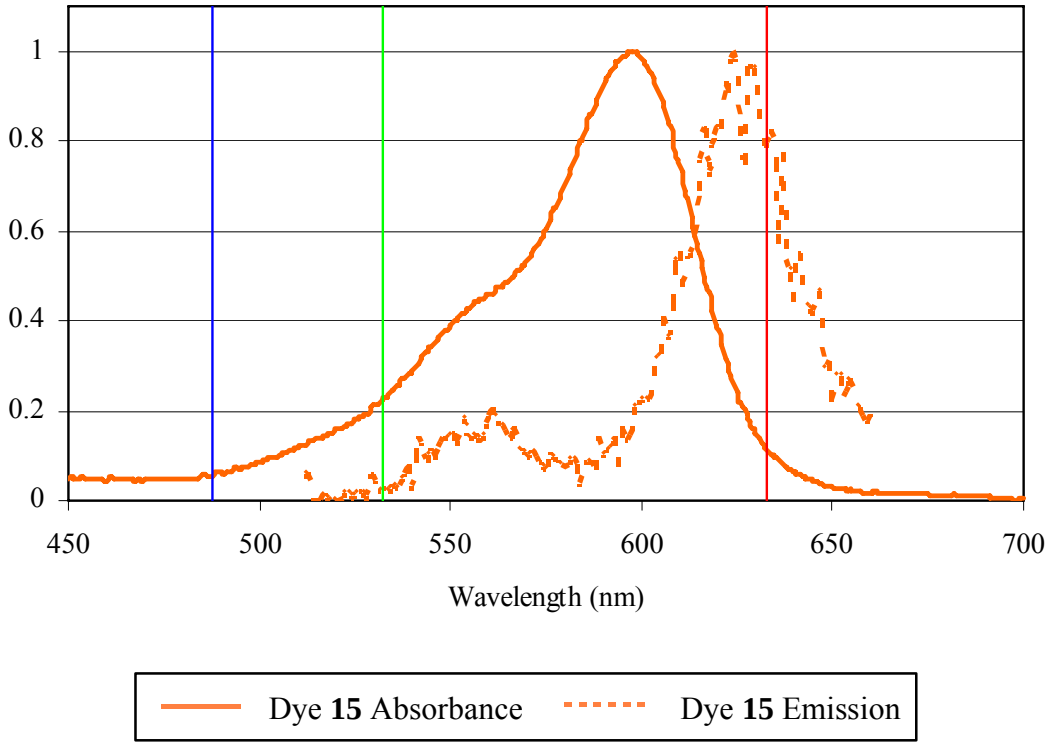


Figure 2.4. Graph of the Absorbance and Emission of BODIPY Dye **15**.

Design and Synthesis of the Second BODIPY Dye Target

Synthetic Strategy

Upon completion of the synthesis of BODIPY dye **15**, attention focused on the synthesis of a BODIPY dye more efficiently excited by the red laser. In order to obtain a dye efficiently excited by the red laser, the substituents at the 3 and 5 position of the BODIPY structure were evaluated so a suitable substitution pattern would be identified for the next target. As seen in Table 1.1, the BODIPY dye containing a thiophene aromatic ring at the 5 position and a styryloxy group at the 3 position absorbs at 625 nm.

Taking this spectral data into account, BODIPY dye **46** was proposed as the next BODIPY target (Figure 2.5).

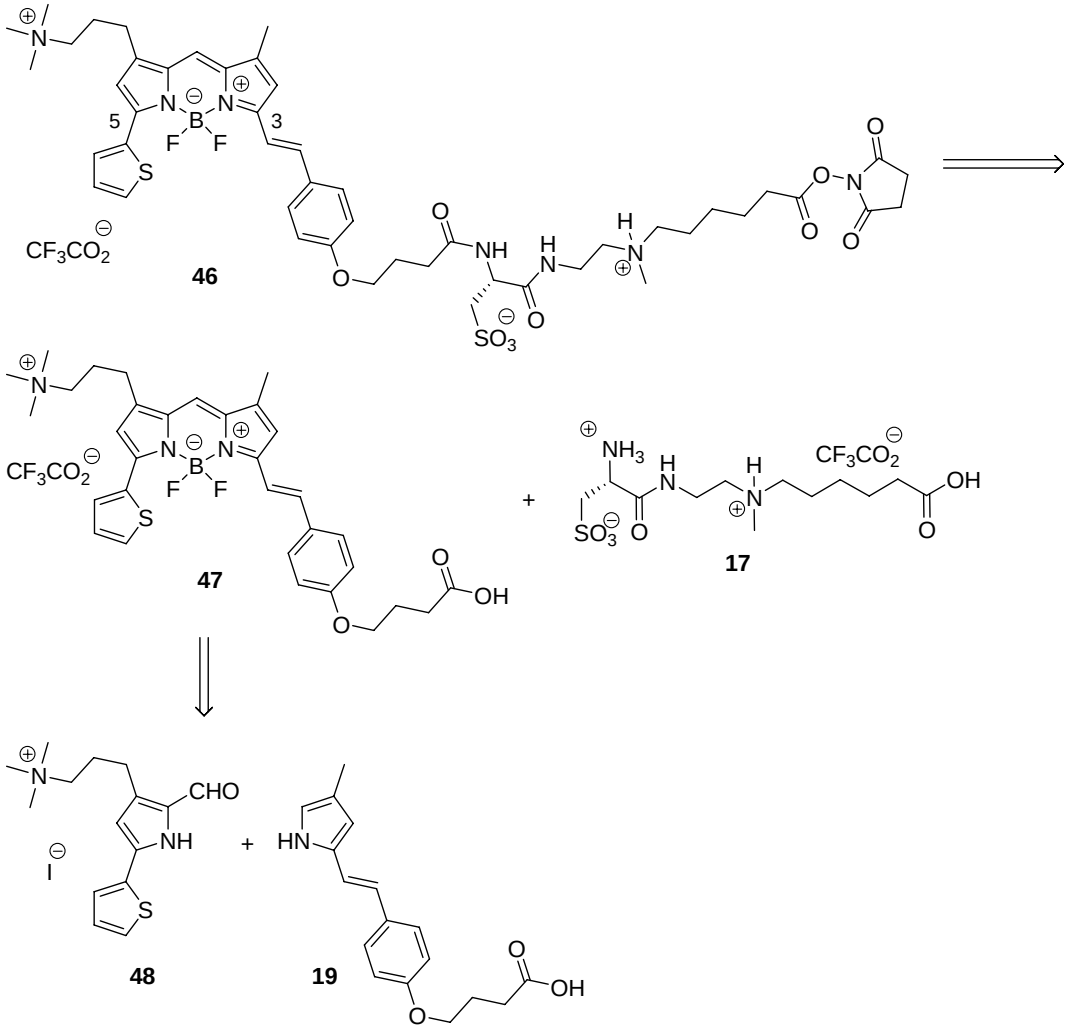


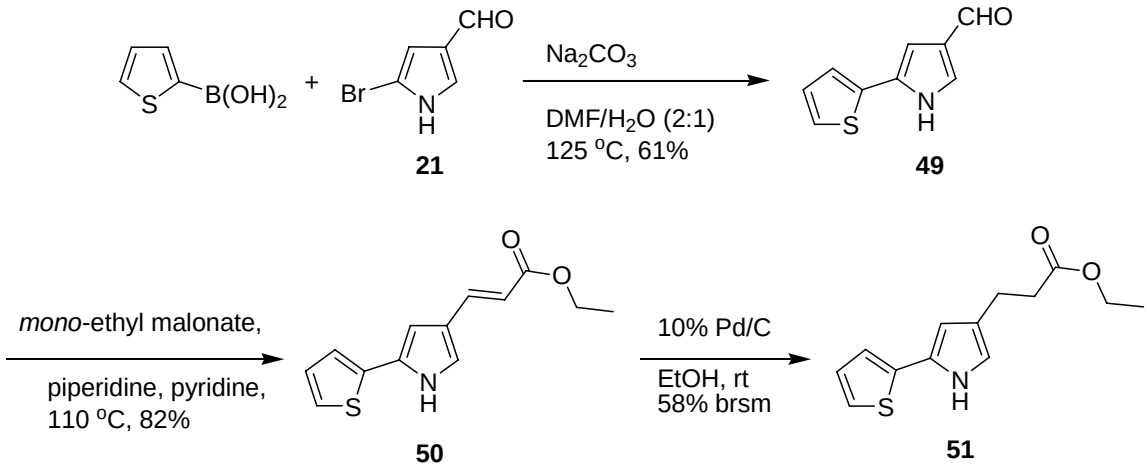
Figure 2.5. Retrosynthetic Analysis of BODIPY Dye **46**.

Due to the similarity in structures, the retrosynthetic analysis of proposed BODIPY dye **46** follows a similar route as the retrosynthetic analysis of BODIPY dye **15**. Retrosynthesis of dye **46** reveals fluorophore **47** and amide chain **17**, used in the previous synthesis (Figure 2.5). Retrosynthesis of fluorophore **47** leads to pyrrole **48** and styryloxy pyrrole **19**, also used in the previous synthesis. Pyrrole **48** is derived from a

Suzuki coupling of commercially available 2-thiopheneboronic acid and the known 2-bromo-4-formylpyrrole **21**.

Results and Discussion

Suzuki coupling of the bromo formyl pyrrole **21** with 2-thiopheneboronic acid provided the desired thiophene pyrrole **49** in 61% yield (Scheme 2.10). Conversion of **49** to acrylate **50** proceeded in a similar fashion as described in the synthesis of **15**, affording acrylate **50** in 82% yield.

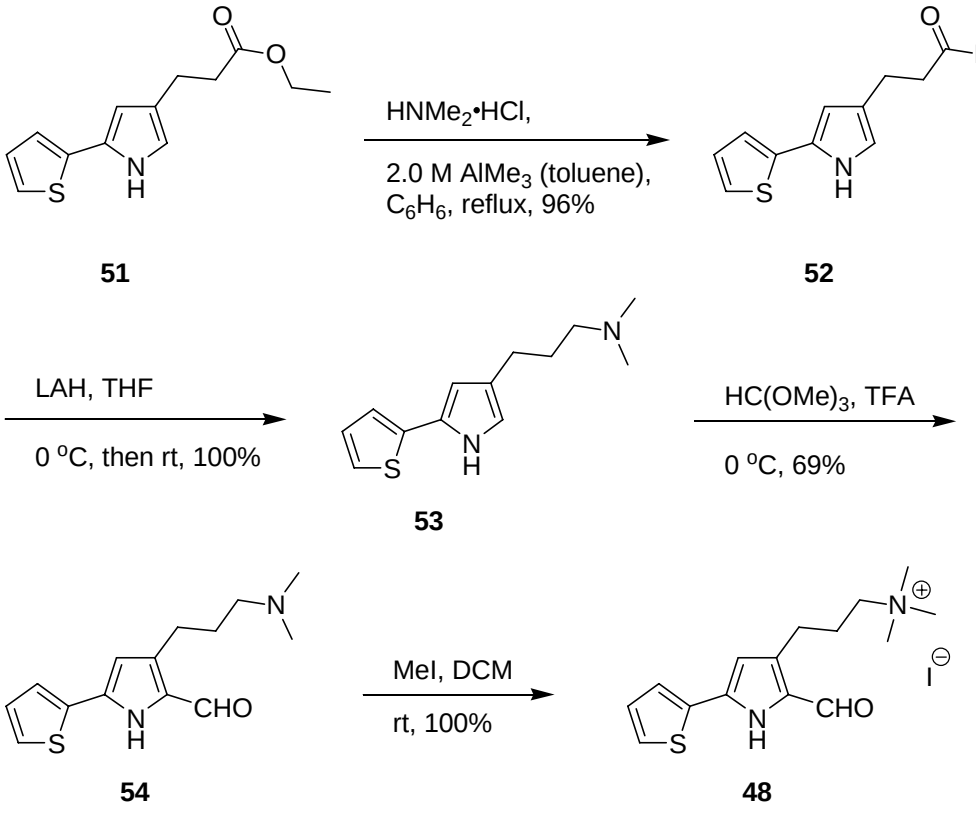


Scheme 2.10. Synthesis of Propanoate **51**.

Reduction of acrylate **50** to propanoate **51** proved to be problematic. Due to the known ability of sulfur to poison palladium catalysts, the best yield achieved in the reduction of **50** was 58% based on recovered starting material. Attempts to circumvent this problem proved fruitless. Simply adding more catalyst to the system had no effect on the consumption of starting material. Varying solvents did not improve the conversion of **50**, nor did using acid in hopes of reinvigorating the catalyst. Employing various Raney Nickel catalysts either gave no reduction of the acrylate or reduction of both the acrylate

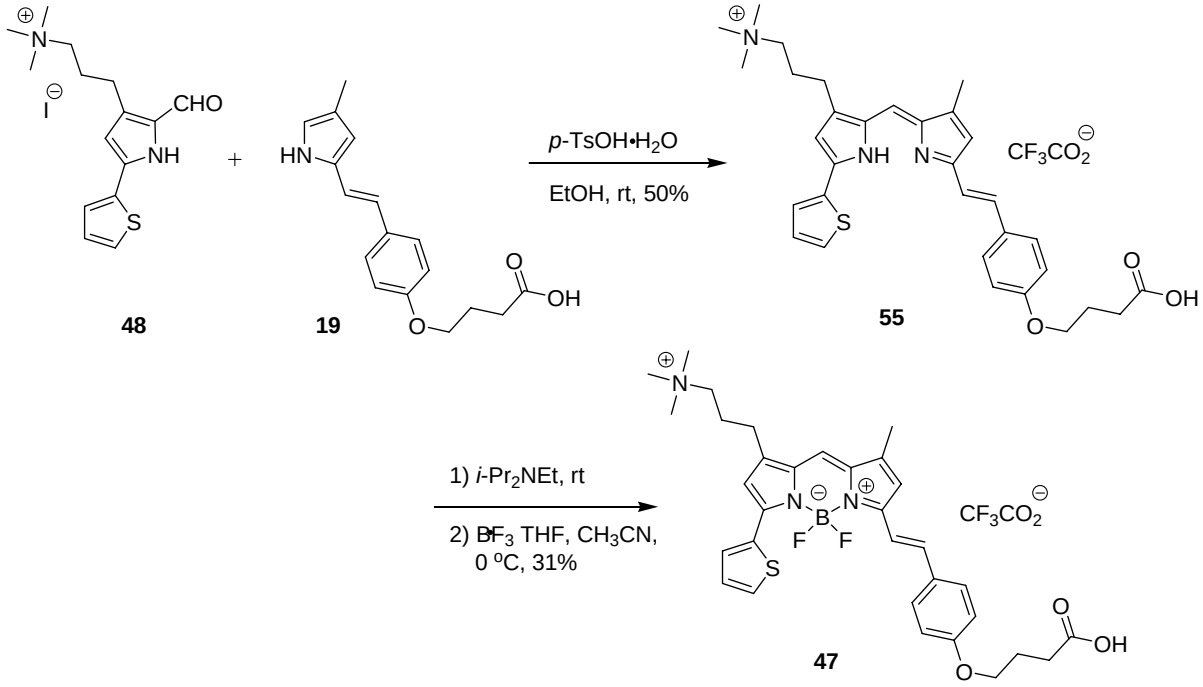
and aromatic rings. Fortunately, due to the ability to generate the starting material **50** in appreciable amounts, the modest yield of this reaction was not a major obstacle for completion of the synthesis.

Conversion of propanoate **51** using Russell's procedure[36] provided amide **52** in 96% yield (Scheme 2.11). Reduction of the amide **52** was performed using lithium aluminum hydride to furnish amine **53** in quantitative yield. Formylation of the α-position of pyrrole **53** was achieved employing trimethyl orthoformate in TFA at 0 °C[42] which provided amine **54** in 69% yield. Methylation of the amine **54** employing iodomethane afforded the desired thiophene pyrrole **48** in quantitative yield.



Scheme 2.11. Completion of Pyrrole **48**.

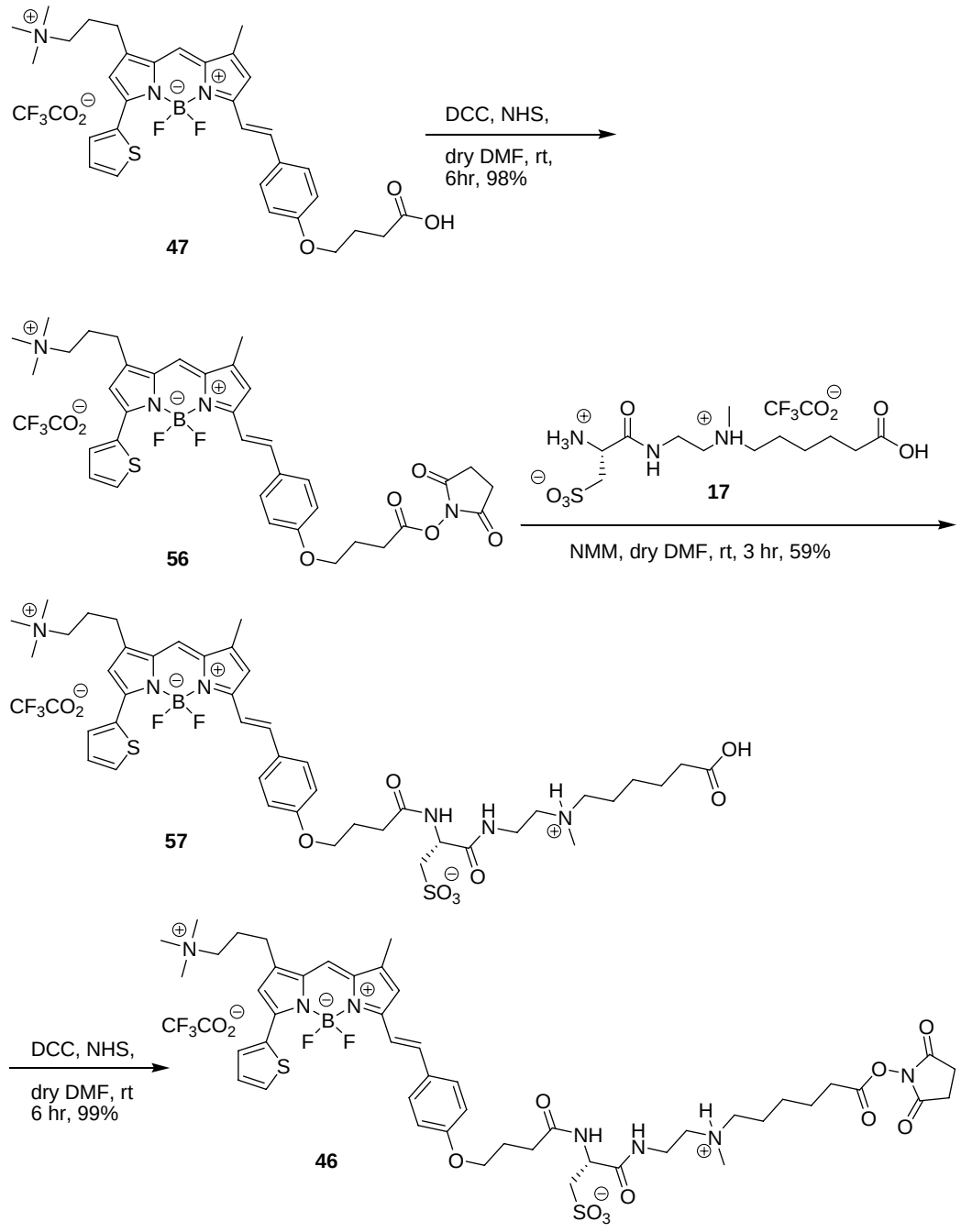
With target **48** in hand, attention was turned to formation of the fluorophore **47**. Acid catalyzed coupling of pyrrole **48** and pyrrole **19**, synthesized in conjunction with the synthesis of dye **15**, generated the dipyrromethene skeleton **55** in 50% yield (Scheme 2.12). After reverse phase HPLC purification of the resulting mixture, generation of the BODIPY fluorophore was performed in a manner similar to the formation of BODIPY fluorophore **15**, affording fluorophore **47** in 31% yield (50% brsm).



Scheme 2.12. Formation of the Second BODIPY Fluorophore.

To finish the synthesis of BODIPY dye **46**, fluorophore **47** was activated as its *N*-hydroxysuccinimidyl ester **56** in 98% yield using *N*-hydroxysuccinimide and *N,N*'-dicyclohexylcarbodiimide in anhydrous *N,N*-dimethylformamide at ambient temperature (Scheme 2.13). After dissolving the *N*-hydroxysuccinimide ester **56** in anhydrous *N,N*-dimethylformamide along with amide chain **17**, addition of *N*-methylmorpholine at

ambient temperature gave way to penultimate intermediate **57** in 59% yield. Activation of **57** with *N*-hydroxysuccinimide and *N,N*'-dicyclohexylcarbodiimide in anhydrous *N,N*-dimethylformamide furnished BODIPY target **46** in 99% yield.



Scheme 2.13. Completion of BODIPY Dye **46**.

BODIPY dye **46** exhibits a maximum absorbance at 628 nm and a maximum emission at 650 nm (Table 2.2). Using a normalized scale of the absorbance, it is seen that the excitation of BODIPY dye **46** occurs at 94% of the maximum absorbance when excited with the commercial red laser at 633 nm (Figure 2.6). With an extinction coefficient of 90,000 and a quantum yield of 55%, BODIPY dye **46** is a good candidate to be used as a red dye to label proteins in proteomic experiments.

Absorbance (nm)	Emission (nm)	Extinction Coefficient	Quantum Yield
628	650	90,000	55%

Table 2.2. Photophysical Properties of BODIPY Dye **46**.

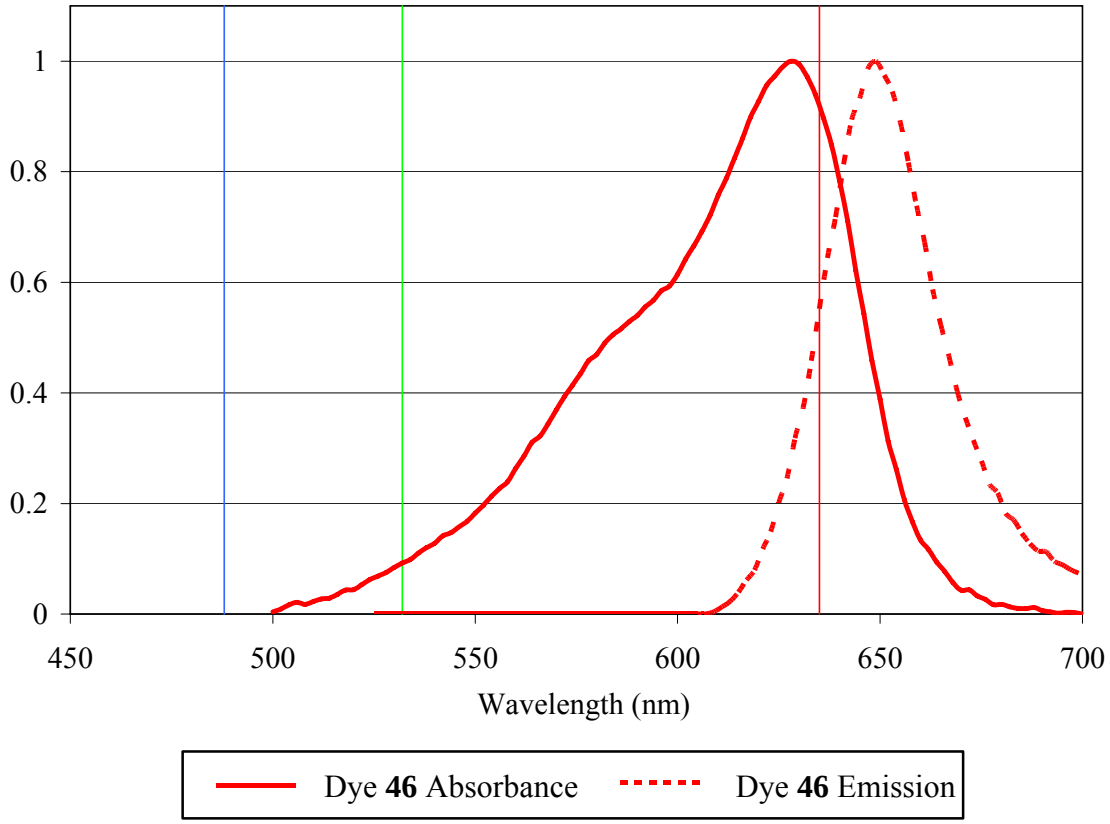


Figure 2.6. Absorbance and Emission of BODIPY Dye **46**.

Experimentation with BODIPY dyes **15** and **46** indicated a problem when the dyes were used in proteomic experiments. When exposed to the disulfide bridge reducing agents dithiothreitol or mercaptoethanol in a 50 mM solution of bicine buffered to pH 8.5, it was noticed **15** and **46** rapidly convert to dyes absorbing in the green (instead of the red) part of the visible spectrum (Figure 2.7).

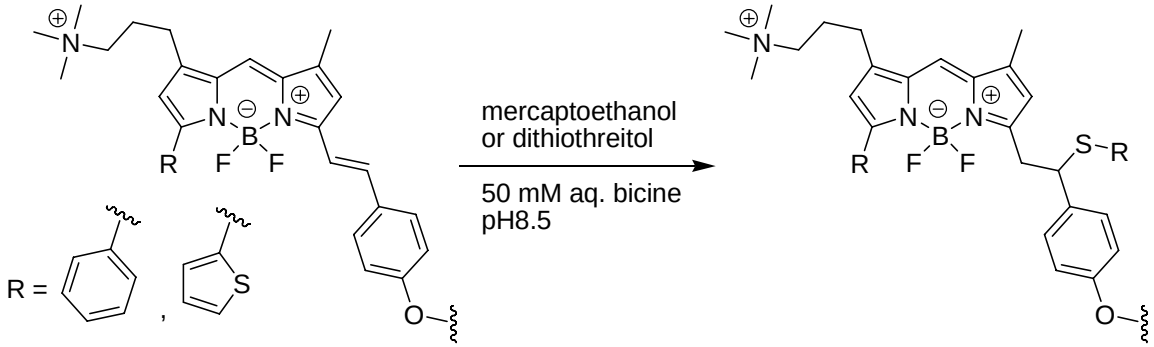


Figure 2.7. Aza-Michael Addition into the Styryloxy Double Bond.

It was speculated dyes **15** and **46** undergo 1,4-nucleophilic addition with the reducing agents. Although analysis was not performed on the product of the reaction, it is believed that 1,4-addition is occurring, as the product would interrupt the conjugation, leading to a BODIPY fluorophore that absorbs in the green visible region. In order to circumvent this problem, it was decided to design a BODIPY dye that had the possibility to absorb in the red spectrum and contained no styryl groups.

Design and Synthesis of a BODIPY Candidate to Absorb at 633 nm

Synthetic Strategy

Towards this goal, BODIPY dye **58** was designed, containing thienyl substituents at both the 3 and 5 positions (Figure 2.8). The rationale behind **58** lies in the ability of

thienyl groups to red-shift the absorbance of BODIPY fluorophores. The synthesis of BODIPY dye **58** could be readily investigated since the precursors to this fluorophore were already on hand.

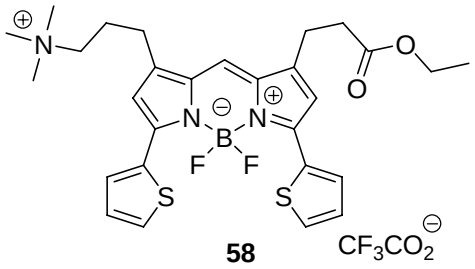
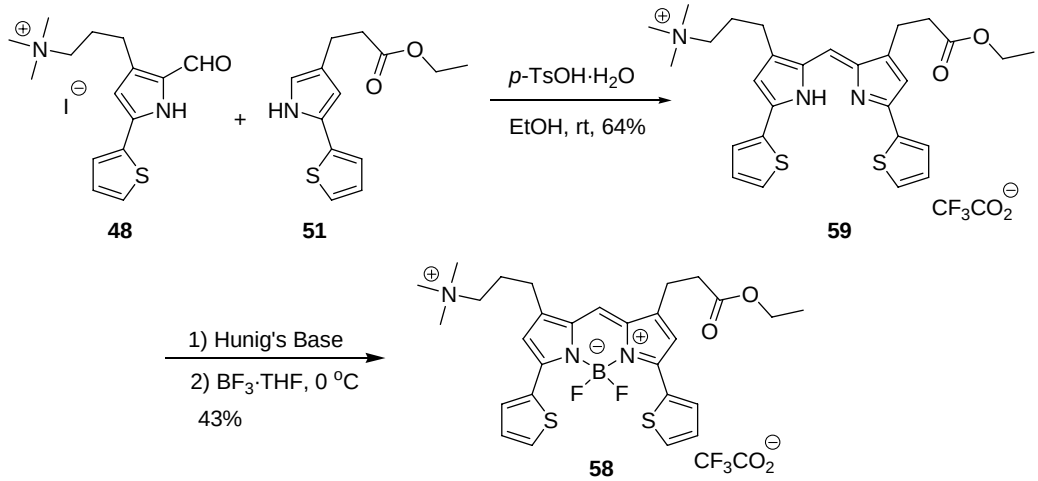


Figure 2.8. Double Thiophene BODIPY Dye.

Results and Discussion

Pyrroles **48** and **51** were dissolved in ethanol, followed by addition of *p*-TsOH·H_2O to effect the acid-catalyzed reaction giving way to dipyrromethene **59** in 64% yield (Scheme 2.14). The fluorophore was synthesized in the same manner as described in the previous syntheses by treatment with Hunig's base followed by addition of BF_3·THF complex providing the desired BODIPY dye **58** in 43% yield.



Scheme 2.14. Synthesis of the Double Thiophene BODIPY Fluorophore.

Analysis of this new BODIPY dye found the maximum absorbance to be 614 nm, with a maximum emission at 634 nm. As can be seen in Figure 2.9, dye **58** is excited with the red laser at approximately 40% of maximum absorbance. Due to the relatively low absorbance of the red laser and emission that overlays with the excitation laser, it was desired to synthesize and evaluate more candidates to be used to absorb the red laser at 633 nm.

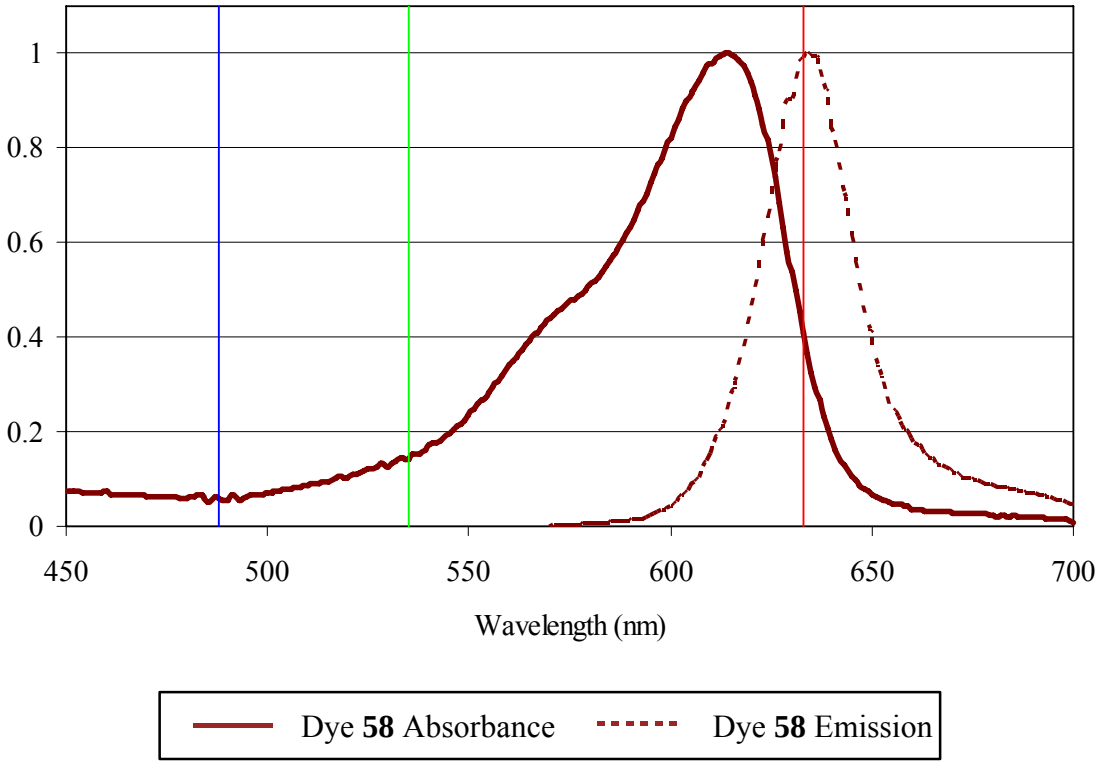
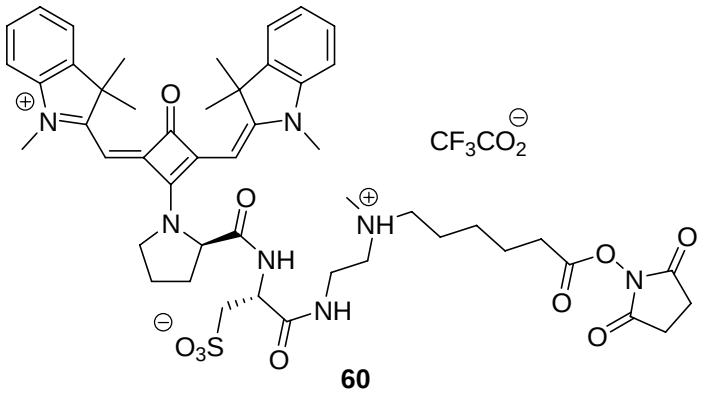


Figure 2.9. Absorbance and Emission of BODIPY Dye **58**.

While attempting to synthesize a BODIPY dye that absorbs the commercially available red laser, the Grieco group successfully synthesized squaraine dye **60**[43] for use with the red laser (Figure 2.10). This dye contains physical and spectral characteristics encompassed in the BODIPY series. The squaraine dye has increased water solubility in

the form of its zwitterionic pair. A titratable amine is included in the amide chain to maintain the pI of labeled proteins, as well as activating the dye as an *N*-hydroxysuccinimidyl ester so the dye can bind to proteins thru lysine residues.



60

Figure 2.10. Squaraine Dye to be Used with a Red Laser.

Evaluation of the photophysical properties of dye **60** indicate it possesses photophysical properties allowing **60** to be used as a dye for use with a commercially available red laser. Dye **60** has a high extinction coefficient but suffers from a low quantum yield of 3.6% (Table 2.3). Despite possessing a low quantum yield when unbound to protein, protein labeling experiments indicate the extinction coefficient of squaraine dyes increase 5-20 fold when bound to protein[44a,b]. This allows dye **60** to possess detection limits comparable to BODIPY dyes, as sensitivity experiments show dye **60** can detect a 128 pg protein/band (Table 2.4). It is theorized squaraine dyes experience fluorescent quenching in polar solvents[44a]. When bound to protein, the dyes are able to find hydrophobic pockets in the protein, lowering fluorescence quenching. This allows dye **60** to have a detection limit that is much lower than is expected for a dye possessing such a low quantum yield as free dye.

Absorbance (nm)	Emission (nm)	Extinction Coefficient	Quantum Yield
658	673	104,755	3.6%

Table 2.3. Photophysical Properties of BODIPY Dye **60**.

Protein	Dye **60**	Cy5
Myosin	128 pg/band	8 pg/band
Beta Galactosidase	128 pg/band	16 pg/band
Phosphorylase B	128 pg/band	16 pg/band
Bovine Serum Albumin	128 pg/band	16 pg/band
Ovalbumin	128 pg/band	8 pg/band
Carbonic Anhydrase	128 pg/band	8 pg/band

Table 2.4. Detection Limits of Dye **60** and Cy5 at 1x CyDye Labeling Concentration (8 pmol Dye/μg Protein).

Although the detection limit of dye **60** is not as low as Cy5 when labeling at 1x Cy5 concentration (8 pmol dye/μg protein), the ability to use dye **60** at higher concentrations than Cy5 due to increased water solubility of **60** allows for lower detection limits. Results from a proteomic experiment where proteins were labeled with dye **60** at 50x the normal concentration for labeling with Cy5 are shown in Table 2.5.

Protein	Dye **60**
Myosin	6 pg/band
Beta Galactosidase	6 pg/band
Phosphorylase B	24 pg/band
Bovine Serum Albumin	24 pg/band
Ovalbumin	6 pg/band
Carbonic Anhydrase	6 pg/band

Table 2.5. Detection Limits of Dye **60** at 50x Normal CyDye Labeling Concentration (400 pmol Dye/μg Protein).

With greater water solubility, dye **60** can be used in higher concentration to reach somewhat lower detection limits than Cy5. Considering these results, squaraine dye **60** was chosen as the fluorescent dye to be used with the red laser, and investigations into a BODIPY dye to be used with the red laser were halted.

Herein is described the syntheses of zwitterionic BODIPY based fluorescent dyes. It was desired to synthesize a BODIPY dye that absorbs the commercial red laser so it can be used as an alternative to Cy5, which can be considered the standard for proteomic dyes that absorb in the red visible region. To make these new BODIPY dyes more appealing than Cy5, they were designed to include a zwitterionic pair to increase their water solubility compared to Cy5 to lead to lower detection limits. Additionally, a titratable amine functionality was included to mimic labeled lysine residues to preserve the pI of labeled proteins.

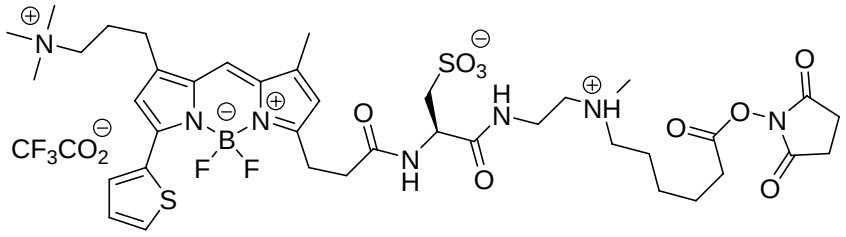
While conducting studies towards synthesizing a BODIPY dye that absorbs the red laser, BODIPY dye **46** was synthesized. It was found that **46** possesses a maximum absorbance that would make it an ideal candidate for protein labeling. However, it was found that thiol reducing agents used in proteomic experiments converted dye **46** into a dye that absorbs in the green visible region. Other BODIPY candidates that were synthesized did not possess absorbances that sufficiently absorb the red laser and had an emission spectra that overlapped with the excitation laser. Fortunately, it was discovered that indolenine-based symmetrical squaraine dyes are acceptable probes in the red visible region. This discovery led to the synthesis of squaraine dye **60**, which has the ability to detect proteins at somewhat lower concentrations than Cy5.

SYNTHESIS OF A BODIPY DYE FOR USE WITH A 532 NM WAVELENGTH LASER

Synthesis of a BODIPY Dye that Absorbs a Green Laser

Synthetic Strategy

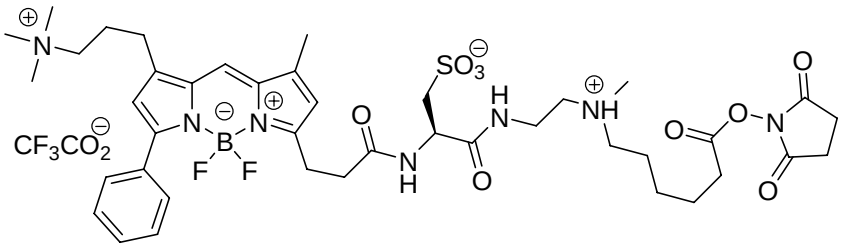
During the course of the synthesis of the BODIPY dyes **15** and **46**, the Grieco group was also investigating synthesis of a variety of BODIPY dyes that absorb in the green visible region. It was theorized BODIPY dye **61** (Figure 3.1) would appreciably absorb the 532 nm green laser due to the results observed with dye **46**. When exposed to the reducing agents mercaptoethanol and dithiothreitol, **46** undergoes nucleophilic addition converting **46** into a dye that absorbs in the green visible region (Figure 2.7).



61

Figure 3.1. Proposed BODIPY Dye **61**.

It seemed certain **61** could be readily synthesized since the major building blocks were already in hand from the synthesis of **58** and the synthesis of BODIPY dye **62**, which was previously synthesized in the Grieco lab[45] (Figure 3.2). Dye **62** was already being used in proteomic experiments and was showing promise as a substitute for Cy3.

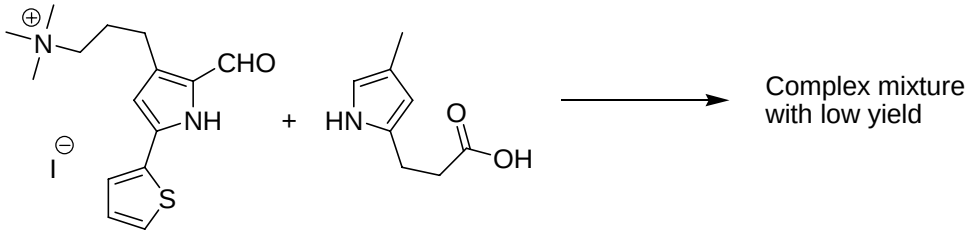


62

Figure 3.2. Previously Synthesized BODIPY Dye that Absorbs the Green Laser.

Results and Discussion

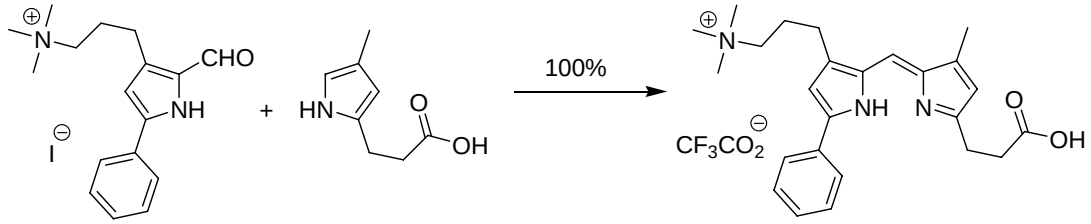
Surprisingly, coupling of pyrroles **48** and **63** gave a complex mixture of products in low yield (Figure 3.3).



48 **63**

Figure 3.3. Complication in Initial Synthetic Strategy.

This result was surprising, as pyrrole **63** was successfully coupled with substituted pyrrole **18** during the synthesis of BODIPY dye **62**, providing dipyrromethene **64** in quantitative yield (Figure 3.4).



18 **63** **64**

Figure 3.4. Previous Dipyrromethene Formation.

Since the coupling of **48** and **63** afforded the product **67** in low yield, it was decided to couple pyrroles **65** and **66** to provide the dipyrromethene **67** (Figure 3.5).

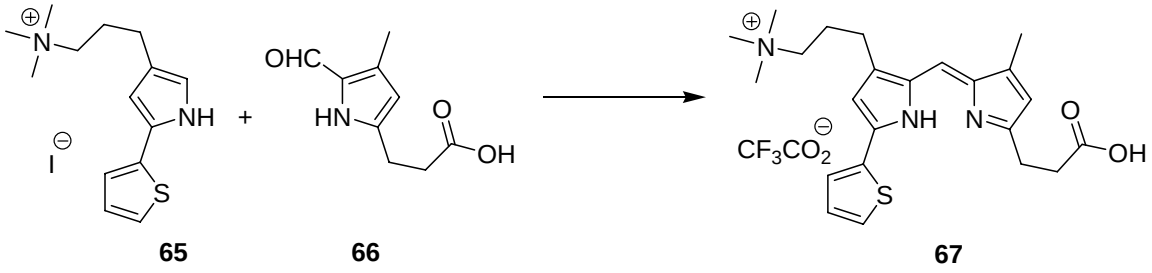
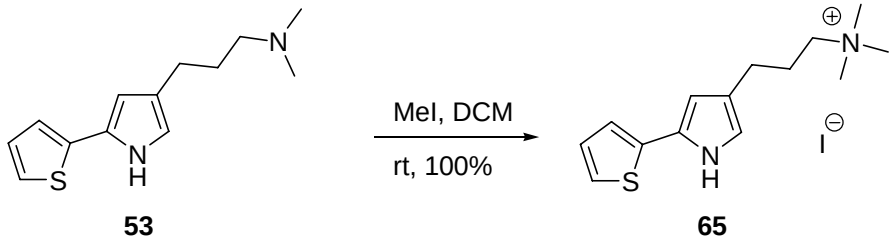


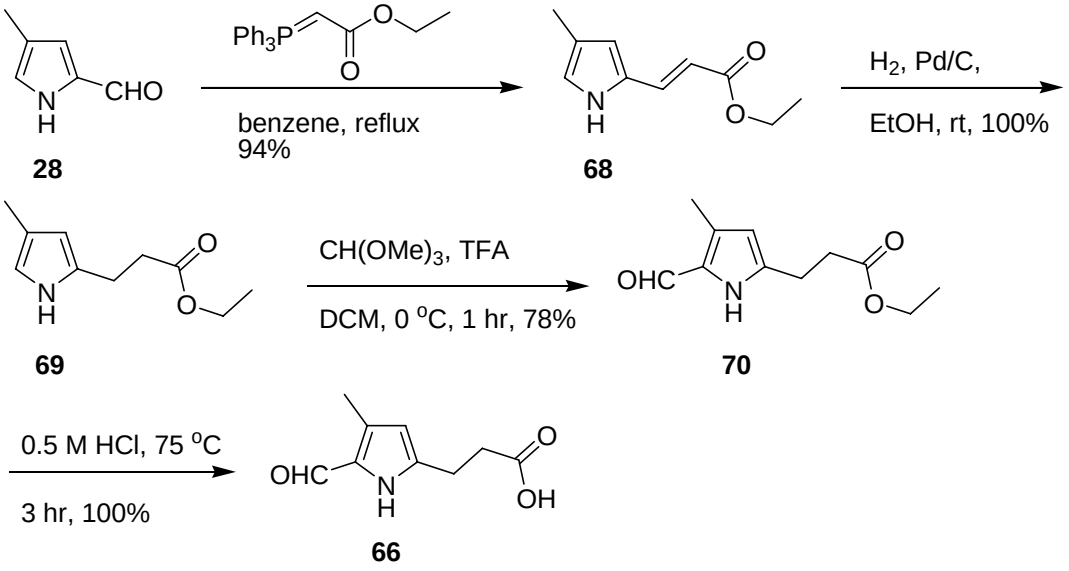
Figure 3.5. Revised Synthetic Plan for Synthesis of the Dipyrromethene.

Synthesis of the left-hand pyrrole **65** was achieved by stirring pyrrole **53** with iodomethane in anhydrous dichloromethane which afforded the desired pyrrole **65** in quantitative yield (Scheme 3.1).



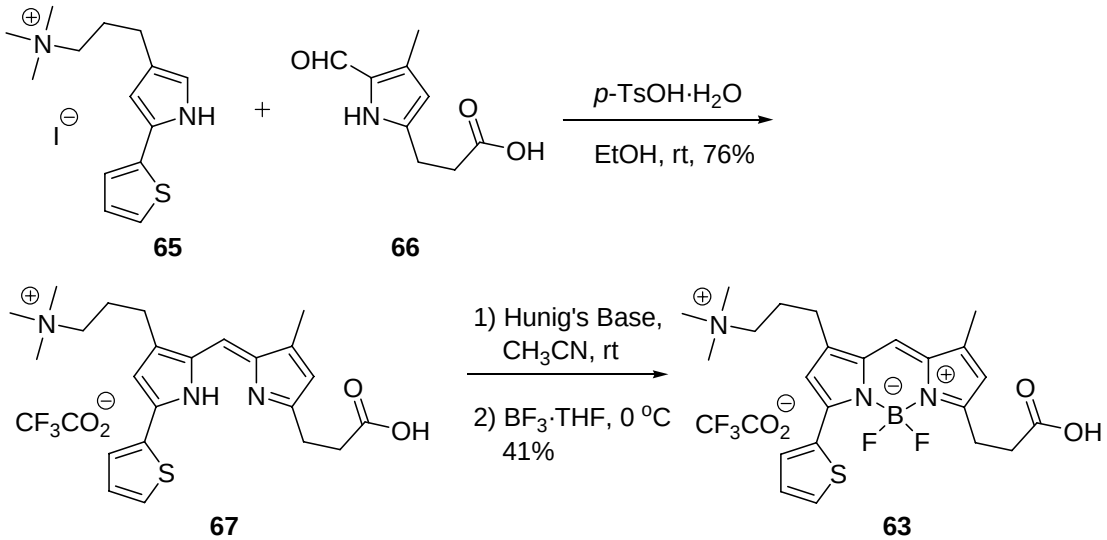
Scheme 3.1. Quaternization of Available Tertiary Amine **53**.

Synthesis of the right-hand pyrrole began with 4-methyl-2-formyl pyrrole **28**, as described by Muchowski and coworkers[32] (Scheme 3.2). A Wittig reaction using (carbethoxymethylene)triphenylphosphorane provided acrylate **68** in 94% yield. Reduction of the olefin gave way to propanoate **69** under typical hydrogenation conditions. Subsequent formylation employing trimethylorthoformate and TFA at 0 ^{o}C[42] furnished intermediate **70** in 78% yield (2 steps). Saponification afforded pyrrole **66** in quantitative yield.



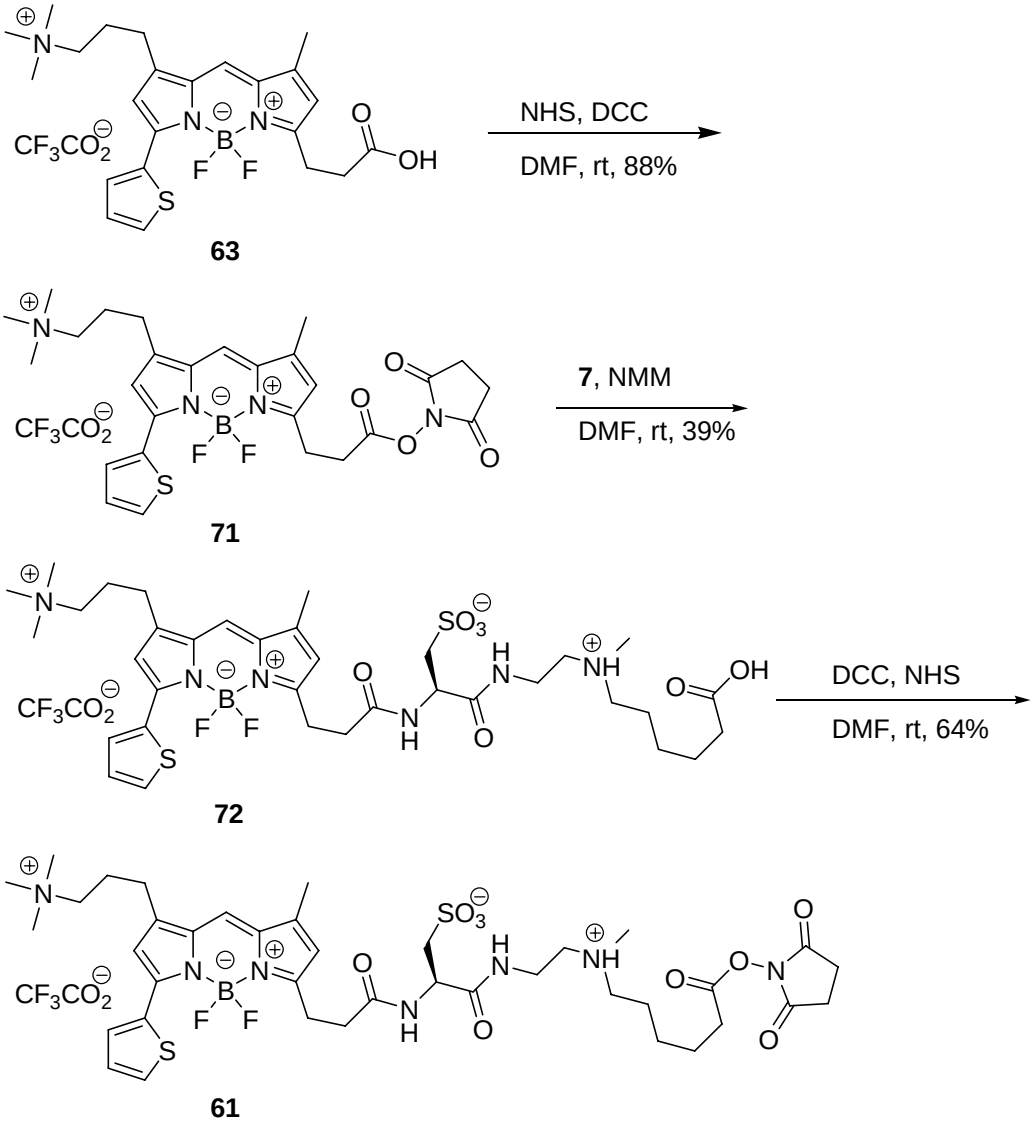
Scheme 3.2. Synthesis of Targeted Pyrrole **66**.

Coupling of pyrroles **65** and **66** afforded the dipyrromethene **67** in 76% yield (Scheme 3.3). Treatment of **67** with BF_3·THF complex in the presence of Hunig's base generated fluorophore **63** in 41% yield (49% brsm). While not an outstanding yield, this was an improvement compared to the yields encountered in the fluoroboration step of BODIPY dyes **15** and **46**.



Scheme 3.3. Synthesis of the BODIPY Fluorophore.

Fluorophore **63** was activated as its *N*-hydroxysuccinimidyl ester **71** under the usual conditions in 88% yield, followed by installation of amide chain **17** in an unoptimized 39% yield furnishing the penultimate intermediate **72** (Scheme 3.4). Activation of acid **72** was performed by stirring **72** with *N*-hydroxysuccinimide and *N,N*'-dicyclohexylcarbodiimide in anhydrous *N,N*-dimethylformamide at ambient temperature, providing BODIPY dye **61** in 64% yield.



Scheme 3.4. Completion of BODIPY Dye **61**.

The physical properties of BODIPY dye **61** are shown in Table 3.1 and Figure 3.6. With an extinction coefficient of over 52,000, a quantum yield of 96%, and absorbance of the green laser at approximately 76% of maximum absorbance, **61** is a good candidate to be used with a green laser.

Absorbance (nm)	Emission (nm)	Extinction Coefficient	Quantum Yield
560	570	52,151	96%

Table 3.1. Photophysical Properties of BODIPY Dye **61**.

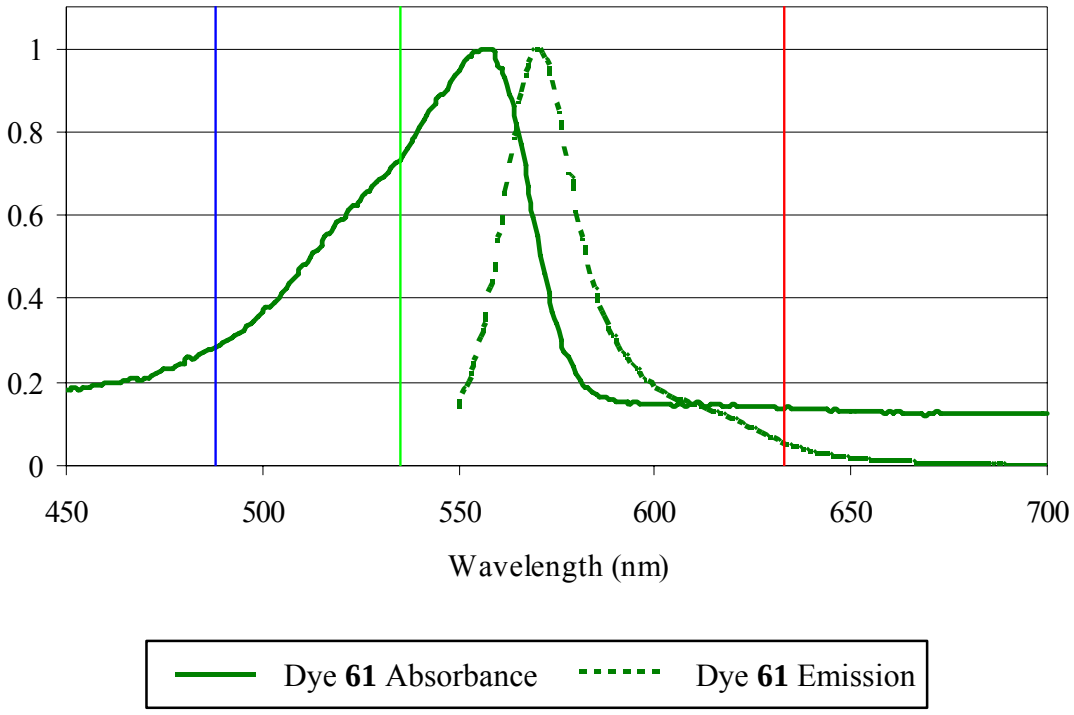


Figure 3.6. Absorbance and Emission of BODIPY Dye **61**.

Sensitivity experiments were carried out comparing BODIPY dyes **61** and **62**, as well as Cy3. The results shown in Table 3.2 provide evidence BODIPY dyes **61** and **62** synthesized in the Grieco lab have detection limits somewhat better than Cy3.

Protein	Detection Limits for Dye **61**	Detection Limits for Dye **62**	Detection Limits for Cy3
Myosin	64 pg/band	16 pg/band	32 pg/band
Beta Galactosidase	128 pg/band	8 pg/band	32 pg/band
Phosphorylase B	128 pg/band	16 pg/band	64 pg/band
Bovine Serum Albumin	N/A	16 pg/band	64 pg/band
Ovalbumin	N/A	32 pg/band	64 pg/band
Carbonic Anhydrase	N/A	32 pg/band	128 pg/band

Table 3.2. Detection Limits at 1x CyDye Labeling Concentration (8 pmol Dye/µg Protein).

Proteomic experiments were carried out with BODIPY **62** labeling at 20x the normal CyDye labeling concentration. Due to the greater water solubility of **62** compared to Cy3, dye **62** was able to achieve lower detection limits when used at 20x normal CyDye labeling concentration (Table 3.3).

Protein	Detection Limits for Dye **62**
Myosin	7.6 pg/band
Beta Galactosidase	7.6 pg/band
Phosphorylase B	7.6 pg/band
Bovine Serum Albumin	30.5 pg/band
Ovalbumin	7.6 pg/band
Carbonic Anhydrase	7.6 pg/band

Table 3.3. Detection Limits for Dye **62** at 20x CyDye Labeling Concentration (160 pmol Dye/µg Protein).

BODIPY dyes **61** and **62** exhibit excellent performances as dyes in proteomic experiments. The high quantum yields and extinction coefficients they possess allow them to detect proteins at lower limits than the widely used Cy3 cyanine dye. While Cy3 has been a standard for amine-reactive fluorescent dyes for the past several years, BODIPY dyes **61** and **62** are viable alternatives due to their low detection limits. Dyes **61** and **62** have an advantage over Cy3 due to the incorporation of polar groups to

increase water solubility, as well as a titratable amine to mimic the charge of labeled lysine residues.

The polarity of dye **62** allows the dye to be used in greater concentration, leading to lower detection limits, as was the theory behind generating fluorescent dyes with enhanced water solubility. The results in Table 3.2 and 3.3 demonstrate BODIPY dye **62** has the ability to detect proteins in lower concentration than Cy3. With BODIPY dyes **61** and **62** identified as candidates to be used with a commercially available green laser, it was deemed unnecessary to synthesize more BODIPY dyes that absorb in the green visible region.

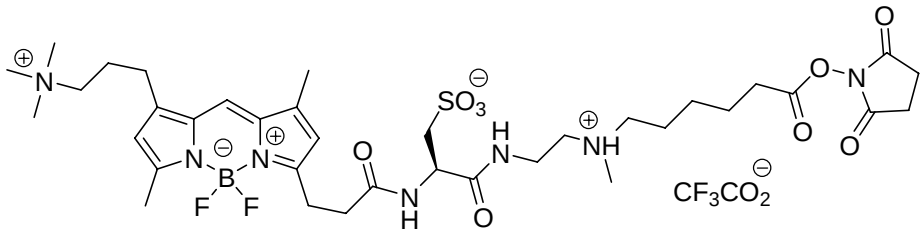
Herein is described the synthesis of a zwitterionic BODIPY based fluorophore for use with a commercial green laser in proteomic experiments. Currently, Cy3 represents the leading edge in commercially available protein labels that absorb in the green visible region. BODIPY dyes **61** and **62** were designed to possess greater quantum yields and water solubility than Cy3, allowing them to detect proteins in lower concentration that Cy3. Additionally, a titratable amine functionality was included to mimic labeled lysine residues to preserve the pI of labeled proteins. Results from proteomic experiments comparing dyes **61** and **62** to Cy3 show they have the ability to detect proteins at lower concentrations that Cy3, making them excellent candidates to be used with a commercial green laser.

SYNTHESIS OF A BODIPY DYE FOR USE WITH A 488 NM WAVELENGTH LASER

Synthesis of a BODIPY Dye that Absorbs a Blue Laser

Synthetic Strategy

During the course of synthesizing BODIPY dyes that absorb the red and green lasers, the Grieco group synthesized BODIPY dye **73** (Figure 4.1)[45], which absorbs the 488 nm (blue) laser.



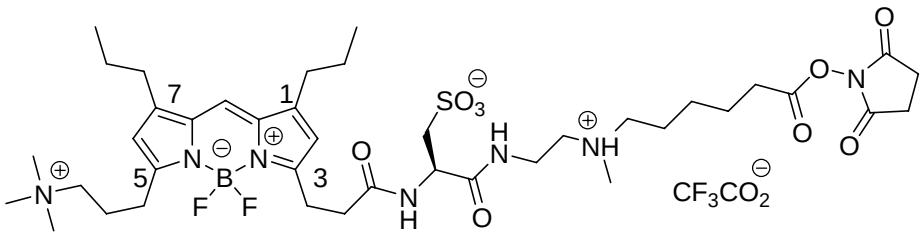
73

Figure 4.1. Previously Synthesized BODIPY Dye for Absorbance at 488 nm.

During the course of proteomic experimentation with **73**, it was noticed areas of the 2D gel would become contaminated with free dye, resulting in the inability to interpret the labeled protein spots due to interfering signals from free dye molecules. It was determined that this concentration of free dye contaminating the 2D gels resulted from dye molecules that had undergone hydrolysis during protein labeling. This is not surprising due to the basic conditions (pH 8.5) employed during protein labeling. It is theorized the free dye becomes encapsulated in SDS micelles. During the course of

electrophoresis, free dye **73** escapes from the micelles due to relatively low hydrophobicity, leading to contamination of the gel with free dye.

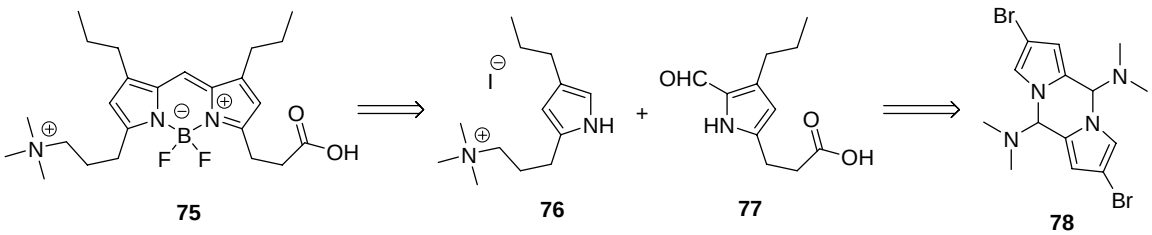
To overcome this problem, it was proposed that increasing the hydrophobicity of the dye would keep the free dye contained in the SDS micelles, preventing free dye from escaping and contaminating the 2D gel. To this end, BODIPY dye **74** was designed (Figure 4.2). Not only does dye **74** possess increased hydrophobicity, the fluorophore contains a measure of symmetry allowing for a more efficient synthesis.



74

Figure 4.2. Proposed BODIPY Dye with Increased Hydrophobicity.

Comparing dye **74** to dye **73**, the alkyl substituent at position 5 has been switched with the propyl ammonium chain at position 7. To increase the hydrophobicity of **74**, *n*-propyl chains have replaced the methyl substituents. The symmetry of the newly designed dye **74** is highlighted in the retrosynthesis of fluorophore **75** (Figure 4.3). Fluorophore **75** is derived from pyrroles **76** and **77**, which are both derived from Muchowski's dimer **78**[32].

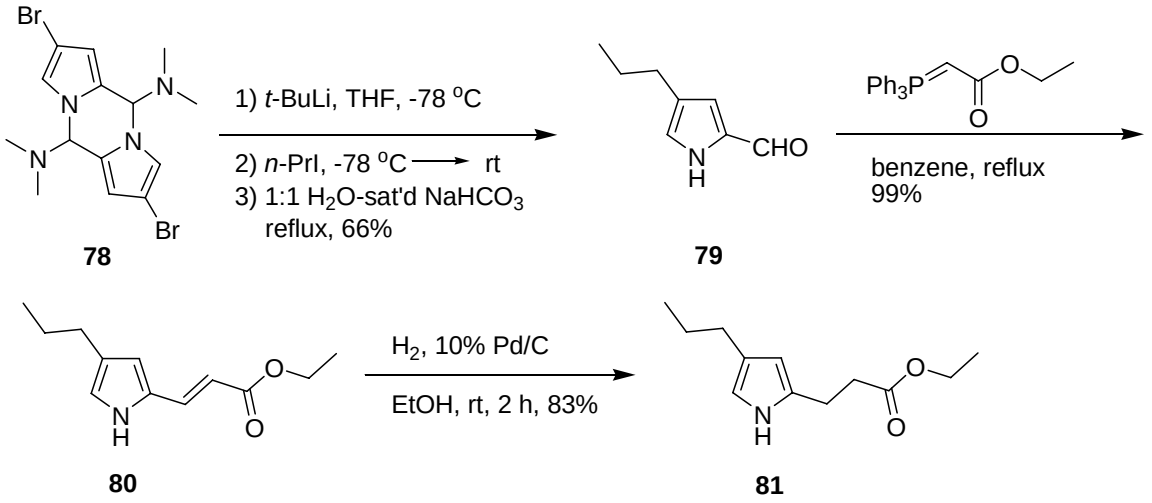


75 **76** **77** **78**

Figure 4.3. Retrosynthesis of the Proposed BODIPY Fluorophore.

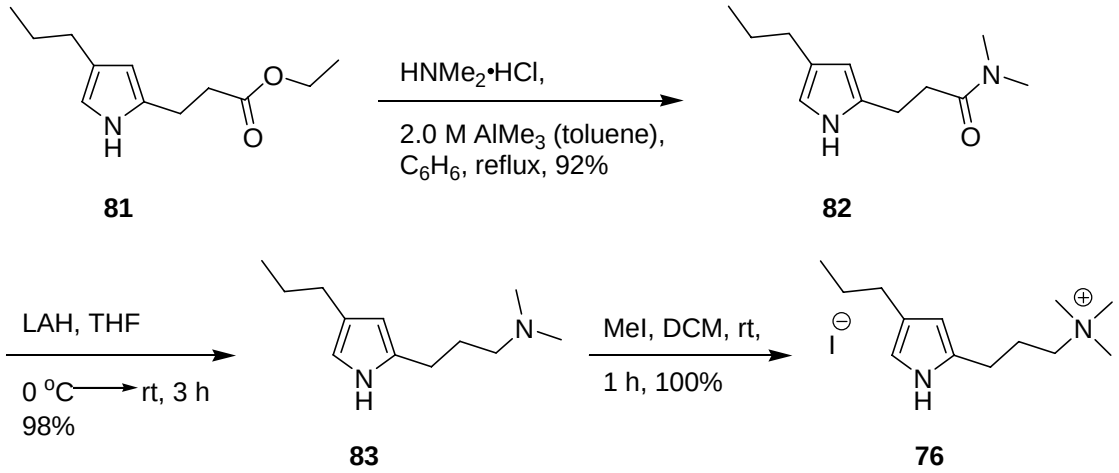
Results and Discussion

Synthesis of **76** and **77** began with treatment of Muchowski's dimer **78** with *t*-BuLi at -78 °C, followed by addition of *n*-iodopropane at -78 °C (Scheme 4.1). After allowing the reaction mixture to warm to room temperature, hydrolysis of the dimer afforded propyl pyrrole **79** in 66% yield. A Wittig reaction using (carbethoxy-methylene)triphenylphosphorane furnished acrylate **80** in 99% yield. Hydrogenation of the olefin afforded **81** in 83% yield.



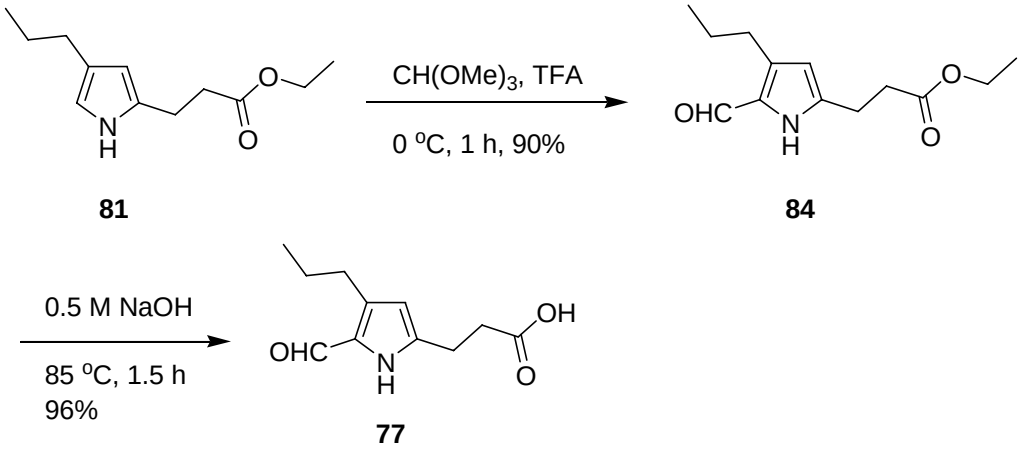
Scheme 4.1. Synthesis of Common Intermediate **81**.

The synthesis of **76** was completed by converting ester **81** into amide **82** in 92% yield employing Weinreb's conditions[36] (Scheme 4.2). Reduction of amide **82** into amine **83** was achieved using lithium aluminum hydride, followed by quaternization of tertiary amine **83**, providing ammonium salt **76** in 98% yield over the two steps.



Scheme 4.2. Synthesis of Ammonium Salt **76**.

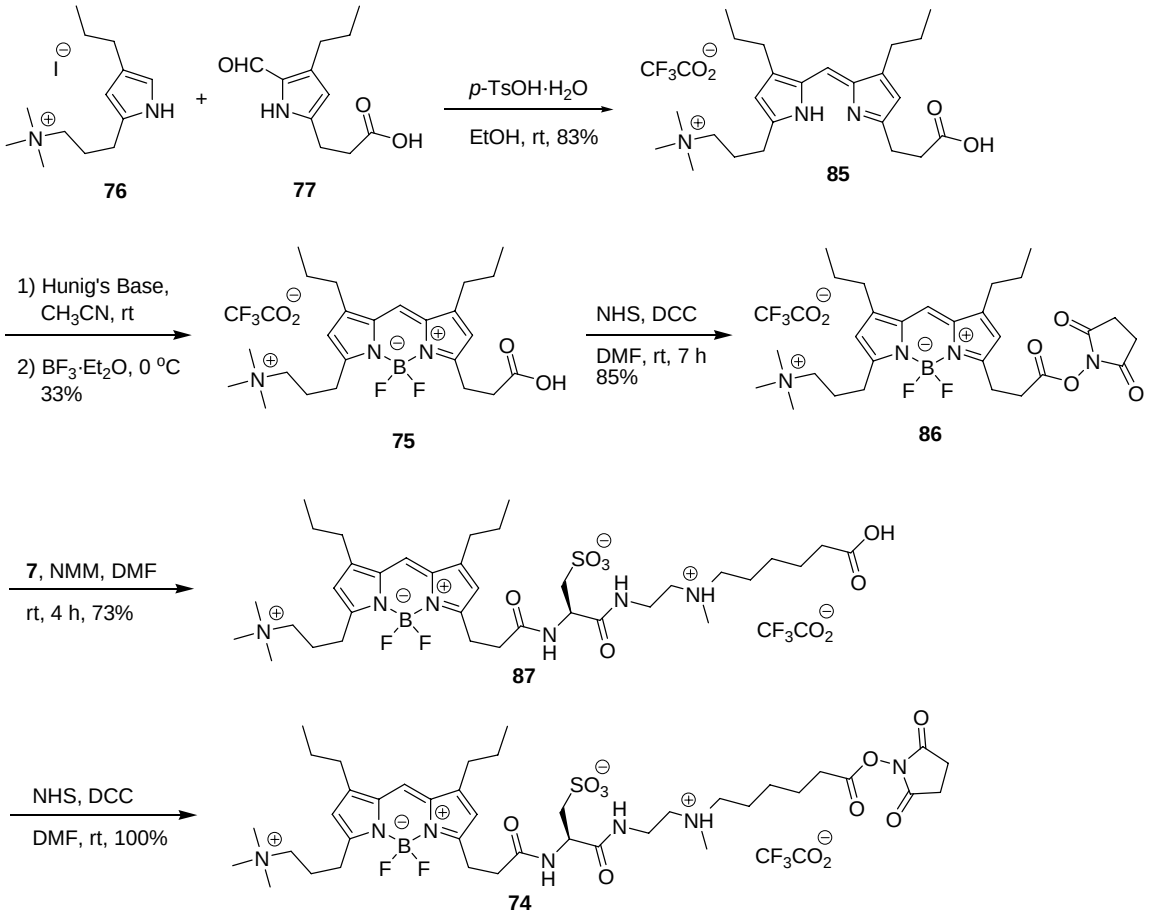
Formylation of pyrrole **81** was achieved by employing trimethylorthoformate in trifluoroacetic acid at 0 °C[42], affording formyl pyrrole **84** in 90% yield (Scheme 4.3). Saponification (0.5 M NaOH, 85 °C, 1.5 h) of ester **84** furnished the desired pyrrole **77** in 96% yield.



Scheme 4.3. Synthesis of Target **77**.

Coupling of **76** and **77** gave way to dipyrromethene **85** in 83% yield (Scheme 4.4). Installation of the boron group was initially achieved using $BF_3 \cdot THF$, generating fluorophore **75** in 19% yield. It was discovered using the more reactive $BF_3 \cdot Et_2O$

provided **75** in 33% yield (45% yield brsm). Activation of fluorophore **75** as its *N*-hydroxysuccinimidyl ester proceeded smoothly, giving way to activated fluorophore **86** in 85% yield. Installation of the amide chain **17** provided BODIPY acid **87** in 73% yield. Subsequent activation of acid **87** as it succinimidyl ester afforded BODIPY dye **74** in quantitative yield.



76 **77** **85** **75** **86** **87** **74**

Scheme 4.4. Completion of Proposed BODIPY Dye **74**.

The graph of the maximum absorbance and emission of **74** shows the maximum absorbance of **74** occurs at 507 nm, and the maximum emission occurs at 518 nm (Figure 4.4). The dye absorbs the blue laser at approximately 40% maximum absorbance. While

not optimum, it is acceptable due to **74** having good photophysical properties. Dye **74** possesses an extinction coefficient of 39,340 and a quantum yield measured at 91%.

Absorbance (nm)	Emission (nm)	Extinction Coefficient	Quantum Yield
507	518	39,340	91%

Table 4.1. Photophysical Properties of BODIPY Dye **74**.

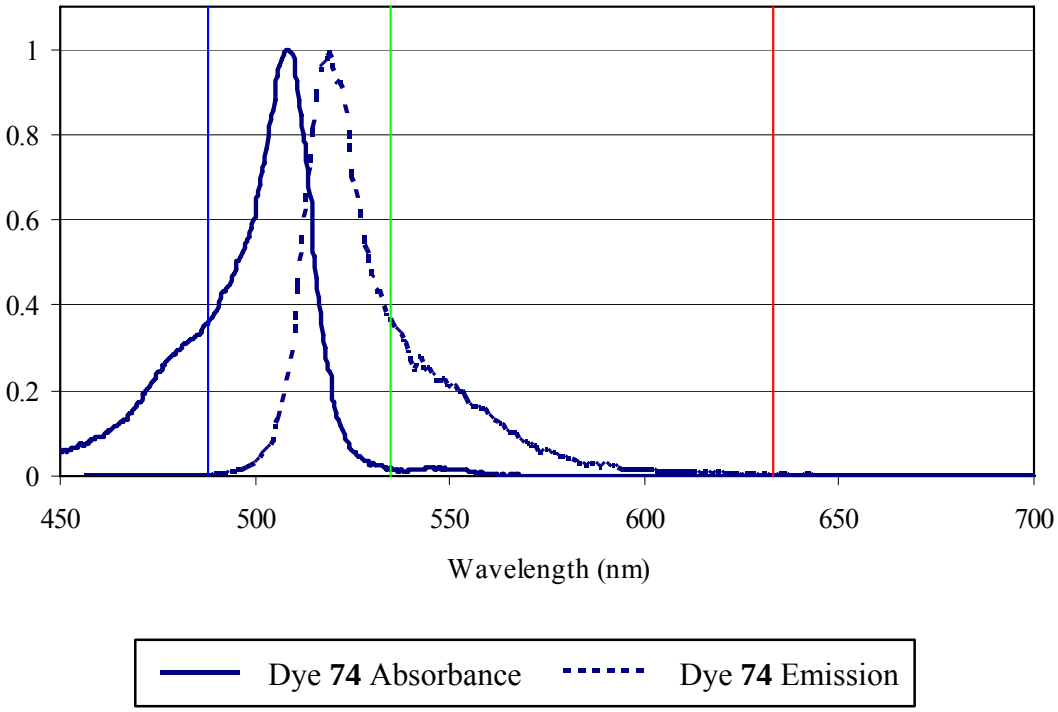


Figure 4.4. Graph of Maximum Absorbance and Emission of BODIPY Dye **74**.

Proteomic experiments carried out with BODIPY dye **74** show no evidence of free dye contaminating the 2D gel. This result, coupled with the good photophysical properties exhibited by **74**, indicates BODIPY dye **74** is a viable candidate to replace the previously synthesized dye **73**. Sensitivity experiments carried out using BODIPY **74** reveal proteins can be detected at low levels using dye **74** (Table 4.2).

Protein	Detection Limits for Dye **74**
Myosin	32 pg/band
Beta Galactosidase	64 pg/band
Phosphorylase B	16 pg/band
Bovine Serum Albumin	32 pg/band
Ovalbumin	64 pg/band
Carbonic Anhydrase	32 pg/band

Table 4.2. Detection Limits using Dye **74** at 1x CyDye Labeling Concentration.

This result is comparable to the detection limits normally found using CyDyes, as earlier results show detection limits from 16-64 pg/band. With the ability to use **74** in higher concentration than Cy2 due to **74** having enhanced water solubility, lower detection limits can be achieved using BODIPY dye **74**. It is expected **74** will possess lower detection limits similar to those seen with BODIPY dye **62**.

Herein is described the synthesis of a zwitterionic BODIPY based dye for use with a commercial blue laser. It was initially believed BODIPY dye **73** would be a suitable replacement for the widely used Cy2 cyanine dye. However, the hydrophilicity of **73** led to contamination of 2D gels during proteomic experiments. The design of BODIPY dye **74** not only provided a dye to replace **73**, it presented a shortened synthetic route. The photophysical properties possessed by BODIPY dye **74** coupled with its water solubility will allow for detection of proteins at low concentrations. The results shown in Tables 4.1 and 4.2 indicate **74** is a viable candidate to be used with a commercially available blue laser.

DESIGN AND SYNTHESIS OF THIOL-REACTIVE DYES

Introduction

Background on Thiol-Reactive Groups

While the use of amine-reactive fluorescent dyes to label proteins is common practice in proteomic research, proteins can be labeled with dyes that are thiol-reactive. Using dyes containing thiol-reactive groups allows for selective labeling of cysteine residues found in proteins. Labeling cysteine residues in proteins offers several factors that are appealing compared to labeling of lysine residues.

For example, proteins contain much fewer cysteine residues than lysine residues, making it potentially easier to perform saturation labeling of protein samples[46a,b]. More importantly, since cysteine residues do not bear a charge, labeling with a neutral fluorescent dye will have little to no effect on the pI of most of the proteins. This does not make it necessary to include a titratable charge to mimic the lysine residue of labeled proteins, as is necessary when synthesizing amine-reactive fluorescent dyes.

A major benefit of cysteine labeling is that lysine residues are not modified. When lysine residues are labeled with amine-reactive dyes, the labeled proteins cannot be fully digested by lysyl peptidase and trypsin, thus potentially affecting mass spectrometer analysis[46a]. Cysteine labeling allows full digestion of proteins by lysyl peptidase and trypsin, allowing for analysis of proteins by mass spectrometry.

Review of the literature provided several examples of thiol-reactive groups that could be incorporated into the design of fluorescent dyes. In particular, three reactive

groups are extensively employed: iodoacetimide[29d,46a,47a,b], maleimide[29d,46a, 48a-c] and dithiopyridyl[48b,49a,b] (Figure 5.1).

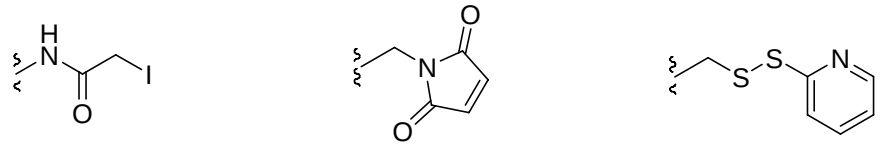
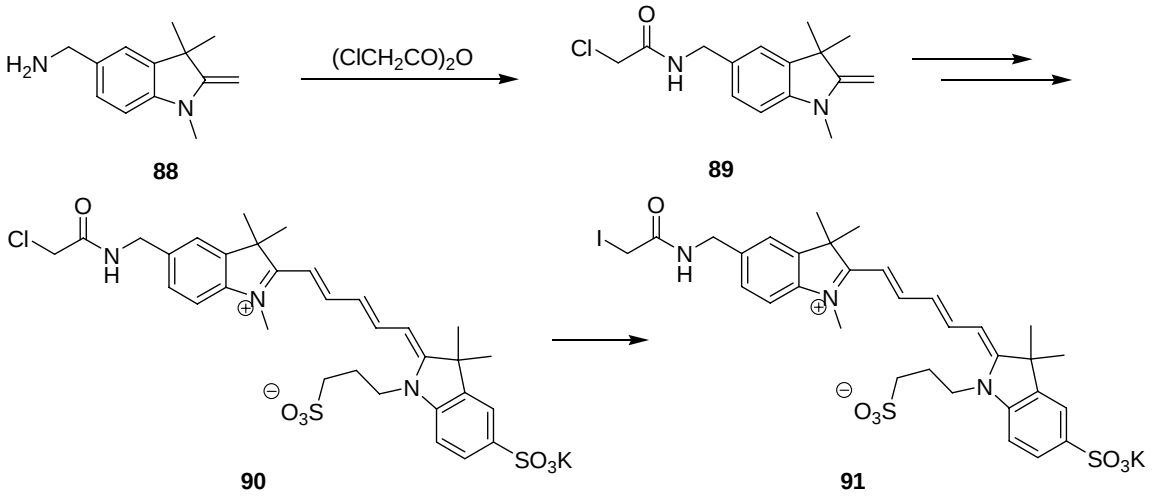


Figure 5.1. Thiol-Reactive Groups.

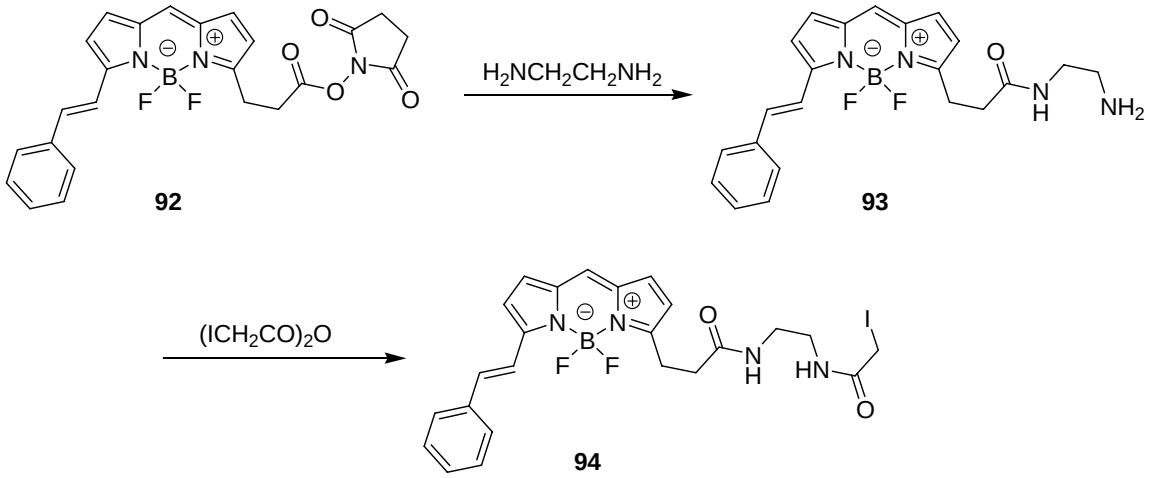
The iodoacetimide group has been installed by two different methods that are similar in approach. The method used by Toutchkine and coworkers[47a] involves treatment of a primary amine (*cf*. **88**) with chloroacetic anhydride to form the corresponding chloroacetimide (cf. **89**) (Scheme 5.1). After formation of the fluorophore (*cf*. **90**), a Finkelstein reaction is utilized converting the chloroacetimide into the iodoacetimide (*cf*. **91**).



Scheme 5.1. Example of Introduction of an Iodoacetimide Group.

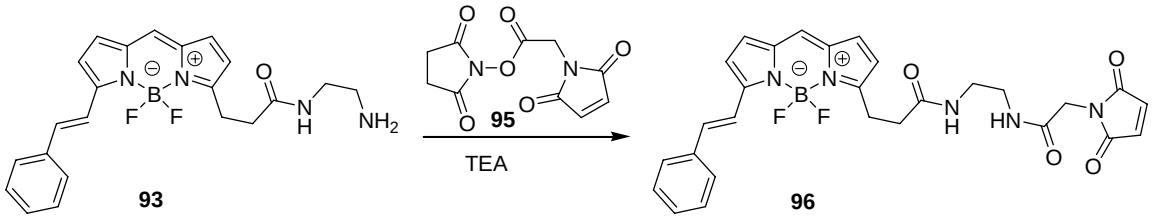
A second method for incorporating the iodoacetimide group into a fluorophore was independently developed by Sherman and coworkers[47b] and Haugland and Kang[29d].

In the synthesis of Haugland and Kang, conversion of succinimidyl ester **92** to the primary amine **93** was achieved by treatment with ethylenediamine (Scheme 5.2). Amine **93** was converted to the thiol-reactive group by treatment with iodoacetic anhydride affording the desired iodoacetimide BODIPY fluorophore **94**.



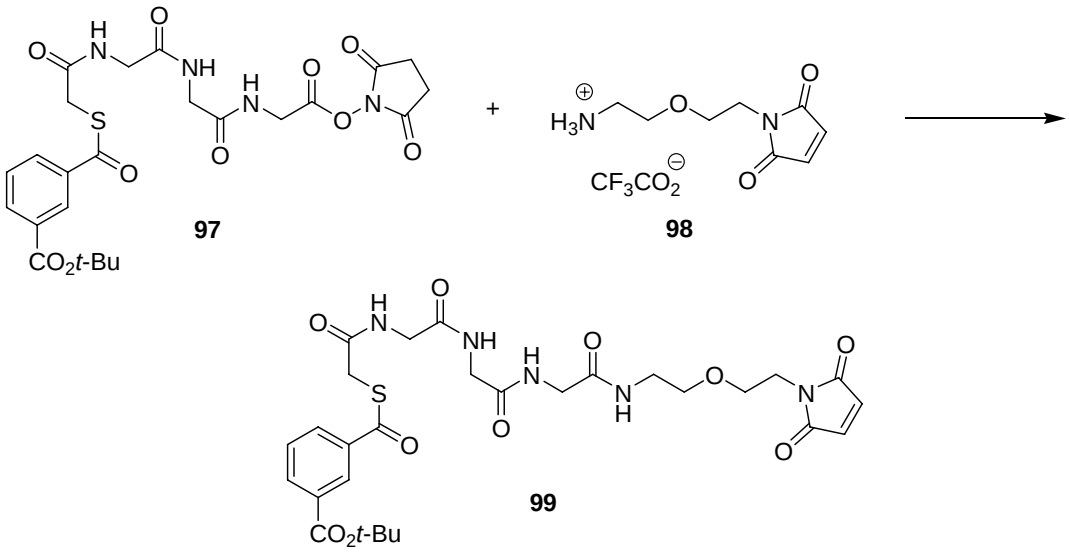
Scheme 5.2. Use of Iodoacetic Anhydride to Introduce an Iodoacetimide Group.

A protocol for the synthesis of a fluorescent dye containing a maleimide moiety is described in the patents of Haugland and Kang[29a,d]. The procedure uses a primary amine (*cf*. **93**) which was formed in conjunction with the synthesis of iodoacetimide **94**. Intermediate **93** is reacted with succinimidyl maleimidylacetate **95** in the presence of base furnishing the maleimide BODIPY dye **96** (Scheme 5.3).



Scheme 5.3. Introduction of a Maleimide Group.

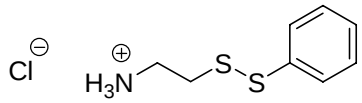
Instead of converting an *N*-hydroxysuccinimidyl activated fluorophore to a primary amine before converting to a maleimide, it was postulated that a primary amine chain containing a maleimide group could be reacted with an activated fluorophore. After searching the literature, a paper by Weber and coworkers[48a] described the synthesis and introduction of *N*-(5-amino-3-oxapentyl)maleimide trifluoroacetate **98** as the thiol-reactive group into radiolabeled markers for use in antibody studies (Scheme 5.4).



Scheme 5.4. Example of Introduction of a Maleimide Group *via* an NHS Ester.

It was proposed that maleimide **98** could be introduced into a squaraine dye using the approach described above for introduction of the amide chain **17** into a BODIPY dye (cf. **86** + **17** → **87**). In the BODIPY syntheses, the *N*-hydroxysuccinimidyl activated BODIPY fluorophores were coupled with the amide chain **7** in the presence of *N*-methylmorpholine. If maleimide **98** could be reacted with an activated *N*-hydroxy-succinimidyl ester, it would provide access to fluorescent dyes for labeling thiol residues on proteins.

Articles by Ebright and coworkers[49a], as well as Zugates and coworkers[49b], describe the synthesis and use of 2-(pyridylthio)-ethylamine hydrochloride **100** (Figure 5.2) as a thiol-reactive residue for incorporation of an EDTA-metal complex into proteins as well as in DNA delivering studies. Even though the dithio pyridyl residue was not introduced into synthetic targets by reaction with an *N*-hydroxysuccinimidyl ester, it was speculated dithio pyridyl residue **100** could be incorporated into an amine-reactive dye, providing thiol-reactive dyes.



100

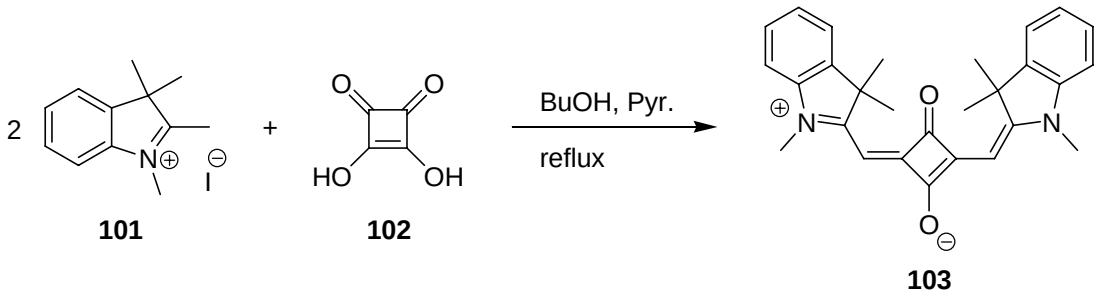
Figure 5.2. 2-(Pyridylthio)-Ethylamine Hydrochloride.

Background on Squaraine Dyes

Squaraine dyes have gained interest from the synthetic community as fluorescent probes since they absorb in the visible and near infrared regions[50]. While other fluorescent probes, such as CyDyes, are more commonly used, it is known that squaraine dyes possess greater extinction coefficients than CyDyes, and possess quantum yields, fluorescent lifetimes and photostabilities similar to conventional CyDyes[44a].

Squaraine dyes are a class of 1,3-disubstituted squaric acid derivatives that can be substituted symmetrically or unsymmetrically. A search of the literature provided two synthetic procedures to prepare squaraine dyes, depending upon whether the desired target is symmetrical[44b,46,51a-g] or unsymmetrical[44b,51c-g].

An example of a synthesis of a symmetrical squaraine dye that absorbs in the red visible region involves condensation of two equivalents of 1,2,3,3-tetramethyl-indolenium **101** with squaric acid **102** in a refluxing mixture of butanol and pyridine, leading to symmetrical squaraine **103**[52] (Scheme 5.5). Using a similar route, syntheses of other symmetrical squaraine dyes have been published[44b,51a-g]. By varying the substituents on the indolenium precursors used in the syntheses, a wide variety of symmetrical squaraine dyes have been obtained that possess different characteristics.

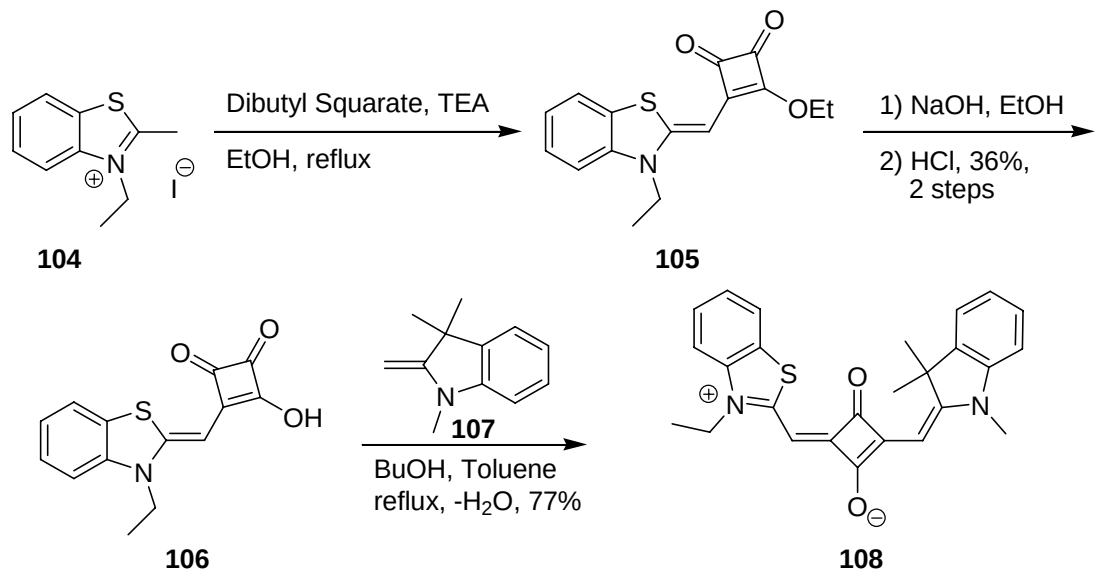


Scheme 5.5. Synthetic Strategy to Synthesize Symmetrical Squaraine Dyes.

Unsymmetrical squaraine dyes constitute a large portion of squaric dye chemistry due to the large amount of variety in physical properties one can achieve by varying the substituents on the fluorophore. The chemistry for generating unsymmetrical squaraine dyes is well established in the literature[44b,51c-g].

The synthesis of unsymmetrical squaraine **108**[51a] is illustrated. Condensation of *N*-ethyl-2-methylbenzothiazolium iodide **104** with diethyl squarate in ethanol in the presence of triethylamine generates intermediate semi-squaraine **105** (Scheme 5.6). Hydrolysis of semi-squaraine **105** provides acid **106** in 36% yield overall. Refluxing acid **106** with 1,3,3-trimethyl-2-methyleneindoline **107** in a butanol/toluene mixture gives way to unsymmetrical squaraine **108** in 77% yield. This method has been utilized to

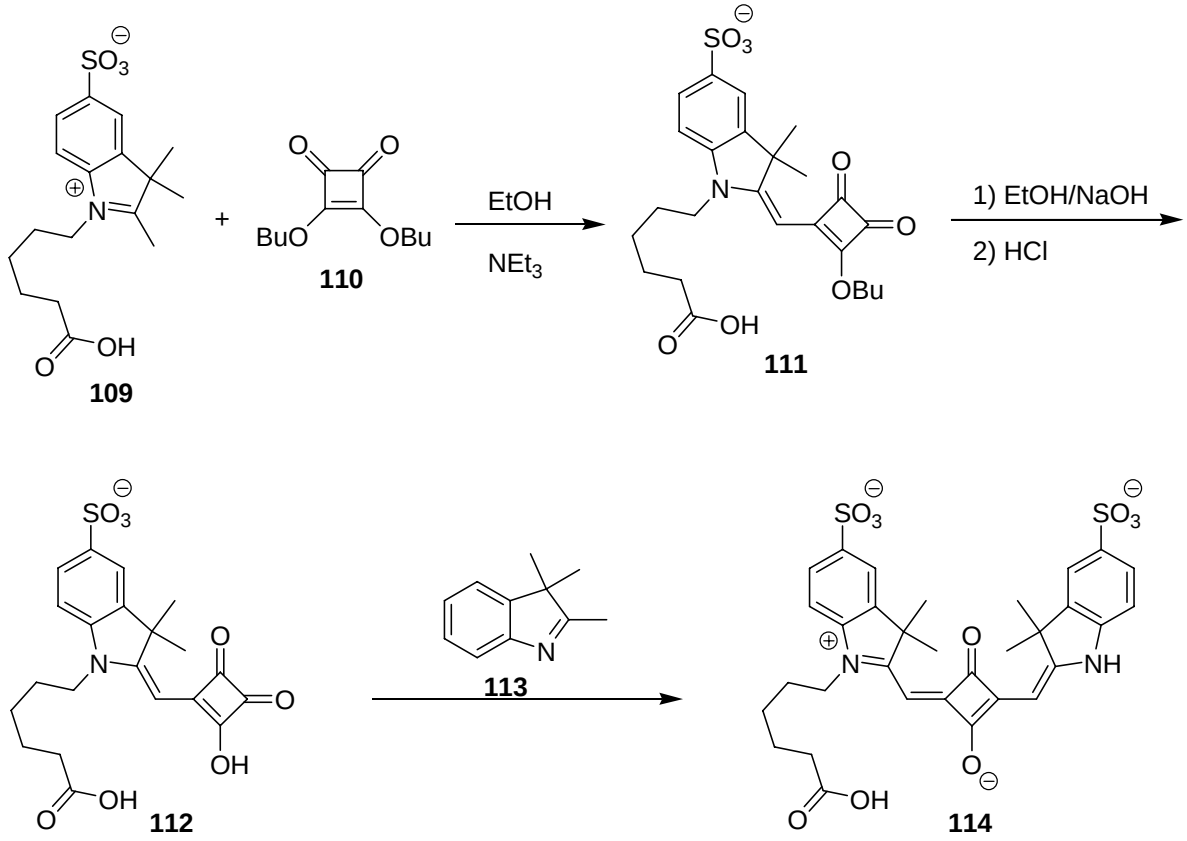
synthesize unsymmetrical squaraines absorbing in the visible region and in the near IR region[44b,51c-g].



Scheme 5.6. Synthetic Strategy to Synthesize Unsymmetrical Squaraine Dyes.

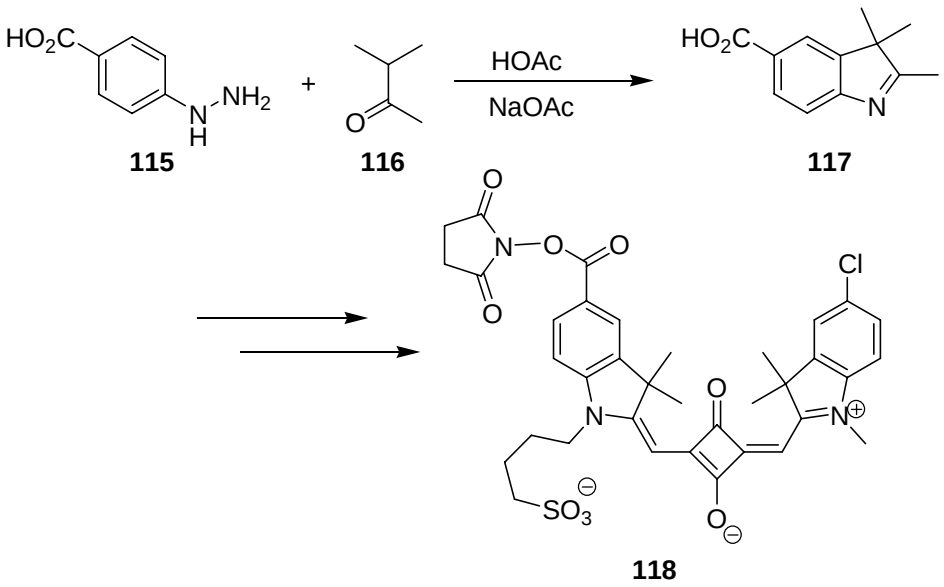
To incorporate residues **98** and **100** into squaraine dyes, it is necessary to design fluorescent dyes that contain a carboxylic acid substituent. Review of various articles describing syntheses of squaraine dyes identified three possible strategies that can be employed to incorporate carboxylic acid chains into squaraine structures. The first two strategies focus on isolation of indolenine intermediates containing a carboxylic acid substituent[44a, 51c,g, 53].

The first method incorporating a carboxylic acid uses an indoleninium salt that has been *N*-alkylated with a chain bearing a carboxylic acid residue[51c,g]. Thus condensation of indoleninium salt **109** with dibutyl squarate provided squarate **111** (Scheme 5.7). Conversion of **111** into squaric acid **112** followed by subsequent treatment with indolenine **113** furnished squaraine **114**.



Scheme 5.7. Use of an *N*-Alkylated Indoleninium Salt.

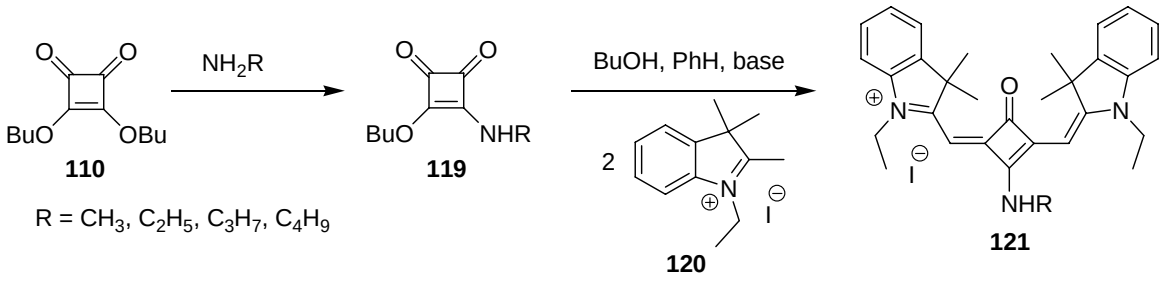
The second approach employs indoleninium salts that contain a carboxylic acid chain on the heterocycle. This approach was utilized in 1994 by Terpetschnig and coworkers[54], as they synthesized indoleninium salt **117** from *p*-hydrazinobenzoic acid and isopropylmethyl ketone as an intermediate in the synthesis of squaraine dye **118** (Scheme 5.8).



Scheme 5.8. Synthesis of Indolenines Containing a Carboxylic Acid.

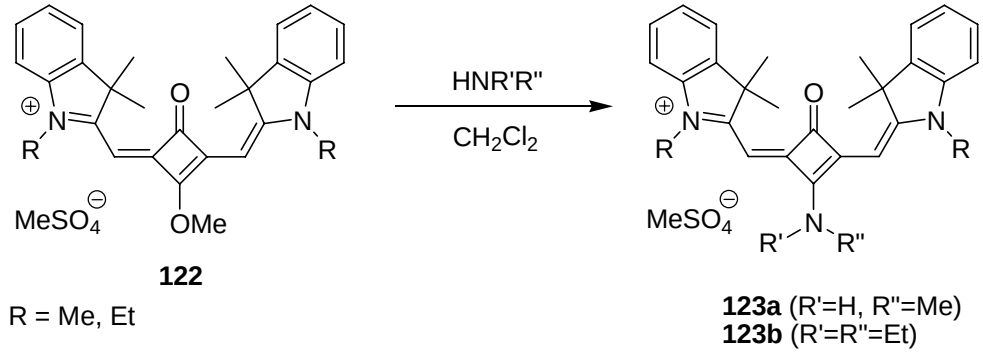
The third approach for introduction of a carboxylic acid chain into squaraine dyes, as shown in Figure 5.9, is introduction of an amino substituent into the squaric ring, leading to aminosquarylium dyes. This approach was targeted for synthesis of thiol-reactive squaraine dyes.

Two different approaches have been employed in the syntheses of aminosquarylium dyes. An account published by Kim and coworkers[51a] proceeds by treating dibutyl squarate with alkylamines in dichloromethane, giving way to alkylaminosquarates **119** (Scheme 5.9). Further treatment with indoleninium salt **120** led to the isolation of the aminosquarylium dyes **121**.



Scheme 5.9. Reaction of an Indolenium Derivative with an Alkylaminosquarate.

The second method, described by Reis and coworkers[51e] as well as Volkova and coworkers[51g], involves generating methoxy squaraine dye **122**, followed by subsequent treatment with either methylamine or diethylamine in dichloromethane providing aminosquarylium dyes **123a** and **123b**, respectively (Scheme 5.10).



Scheme 5.10. Introduction of an Amine *via* Substitution into the Squaric Ring.

It was theorized that if an amino acid chain was used, instead of a monoalkyl- or dialkylamine, for substitution on the squaric ring, one would be able to activate the resulting terminal carboxylic acid and introduce an amine residue containing a thiol-reactive group.

Design and Synthesis of Thiol-Reactive Symmetrical Squaraine Dyes

Synthetic Strategy

The first proposed structure of a thiol-reactive squaraine dye was dye **124** (Figure 5.3). A cysteic acid residue was included to provide charge balance, as well as increased water solubility. In a retrosynthetic analysis of proposed dye **124**, disconnection of the amide chain from the squaric acid moiety gives the known methylated squaraine dye **122**[49g] and amide chain **125**.

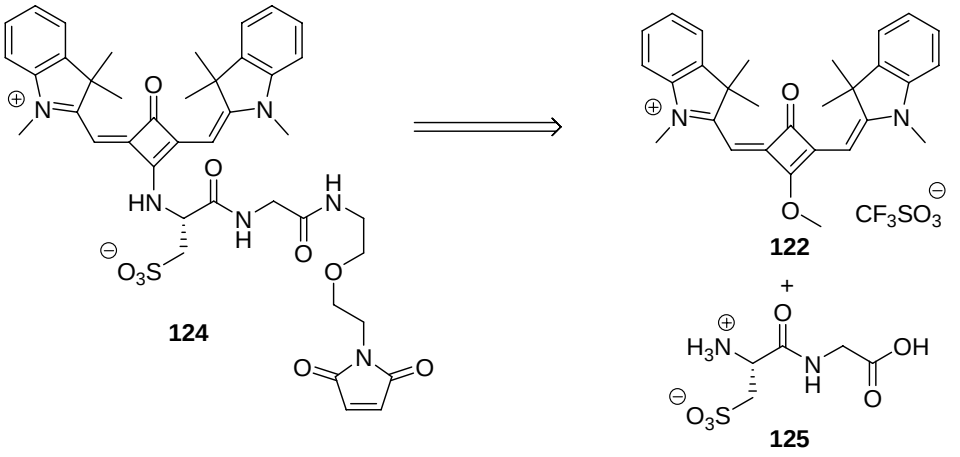
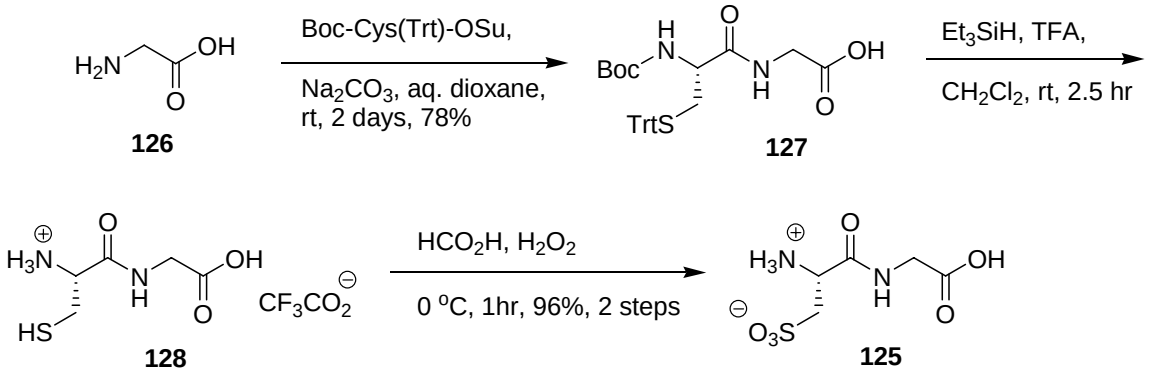


Figure 5.3. Retrosynthetic Disconnection of the Proposed Thiol-Reactive Dye.

Results and Discussion

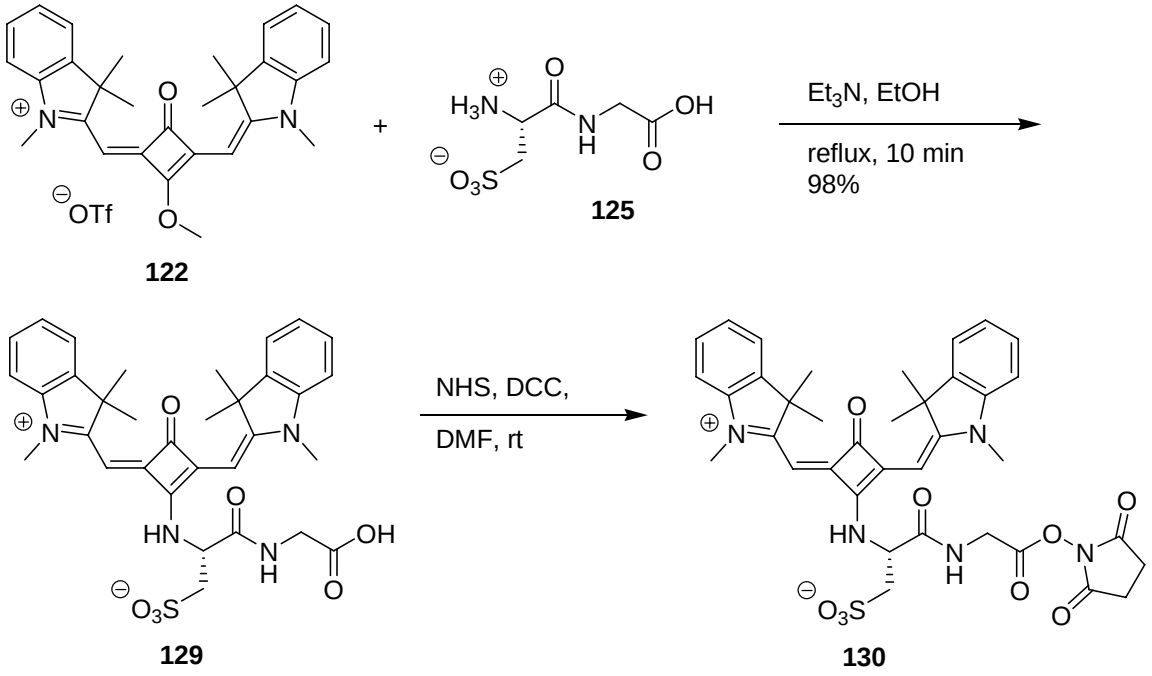
Synthesis of amide chain **125** commenced by coupling commercially available Boc-Cys(Trt)-OSu with glycine in aqueous dioxane in the presence of sodium carbonate affording protected amide **127** in 78% yield (Scheme 5.11). Deprotection of **127** was achieved employing TFA and triethylsilane in methylene chloride[40] giving way to

cysteine **128**. Subsequent performic oxidation[41] of **128** furnished amide chain **125** in 96% overall yield.



Scheme 5.11. Synthesis of the Glycine Derived Amide Chain.

Squaraine **122** and amide chain **125** were coupled by refluxing in ethanol in the presence of triethylamine, providing squaraine **129** in 85% yield (Scheme 5.12). Activation of **129** was attempted using *N*-hydroxysuccinimide and *N,N*'-dicyclohexyl-carbodiimide in anhydrous *N,N*-dimethylformamide generating ester **130**. Even though thin layer chromatography analysis indicated consumption of **129**, attempts to isolate the product by reverse phase HPLC resulted in recovered starting material, indicating that ester **130** is unstable under acidic conditions.



Scheme 5.12. Attempted Synthesis of Target **130**.

It is known that peptides can undergo cyclization to afford oxazolones[55] (Figure 5.4). After isolation of **130**, the acidic nature of the HPLC solvents allowed **130** to convert back into acid **129** while in solution. This theory is supported by ^{1}H NMR analysis of the product collected from the HPLC. ^{1}H NMR analysis of the product peak collected from the HPLC showed a mixture of **129** and **130**.

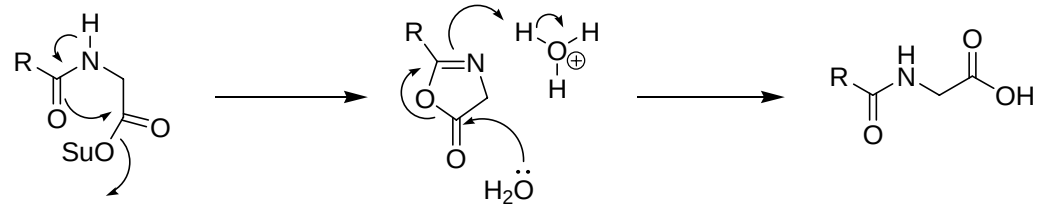
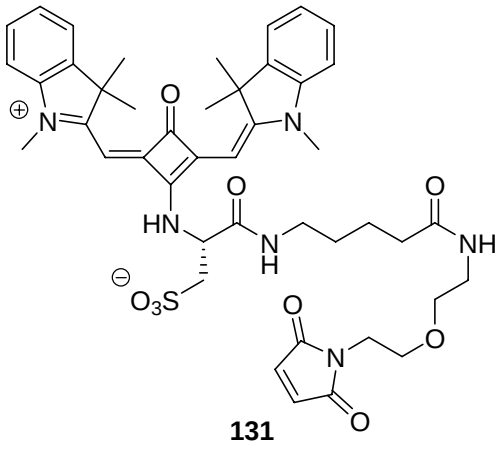


Figure 5.4. Peptide Cyclization to Form an Oxazolone.

With the presence of the succinimidyl leaving group, it is not surprising that oxazolone formation and subsequent regeneration of acid **129** would occur during reverse

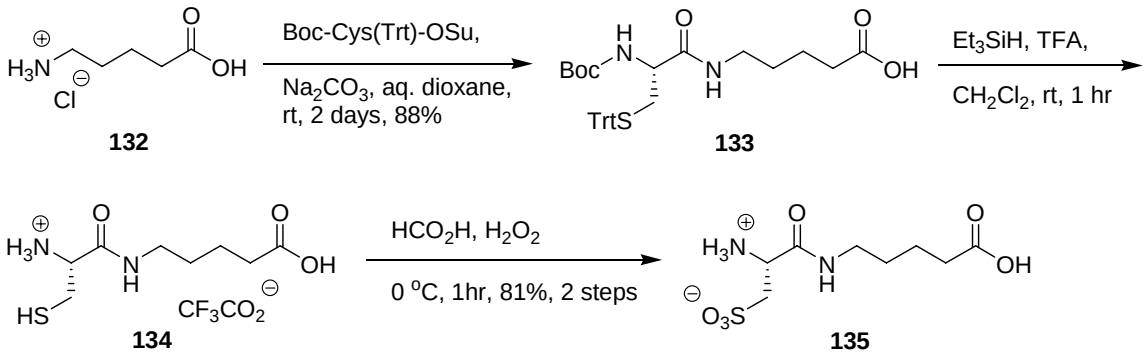
phase HPLC purification since the purification employed an acidic solvent system. Therefore, it was necessary to synthesize a squaraine dye containing a longer carboxylic acid chain, preventing oxazolone formation. Replacing the glycine residue of **124** with a valeric acid residue resulted in squaraine **131** (Figure 5.5).



131

Figure 5.5. Second Proposed Thiol-Reactive Squaraine Dye.

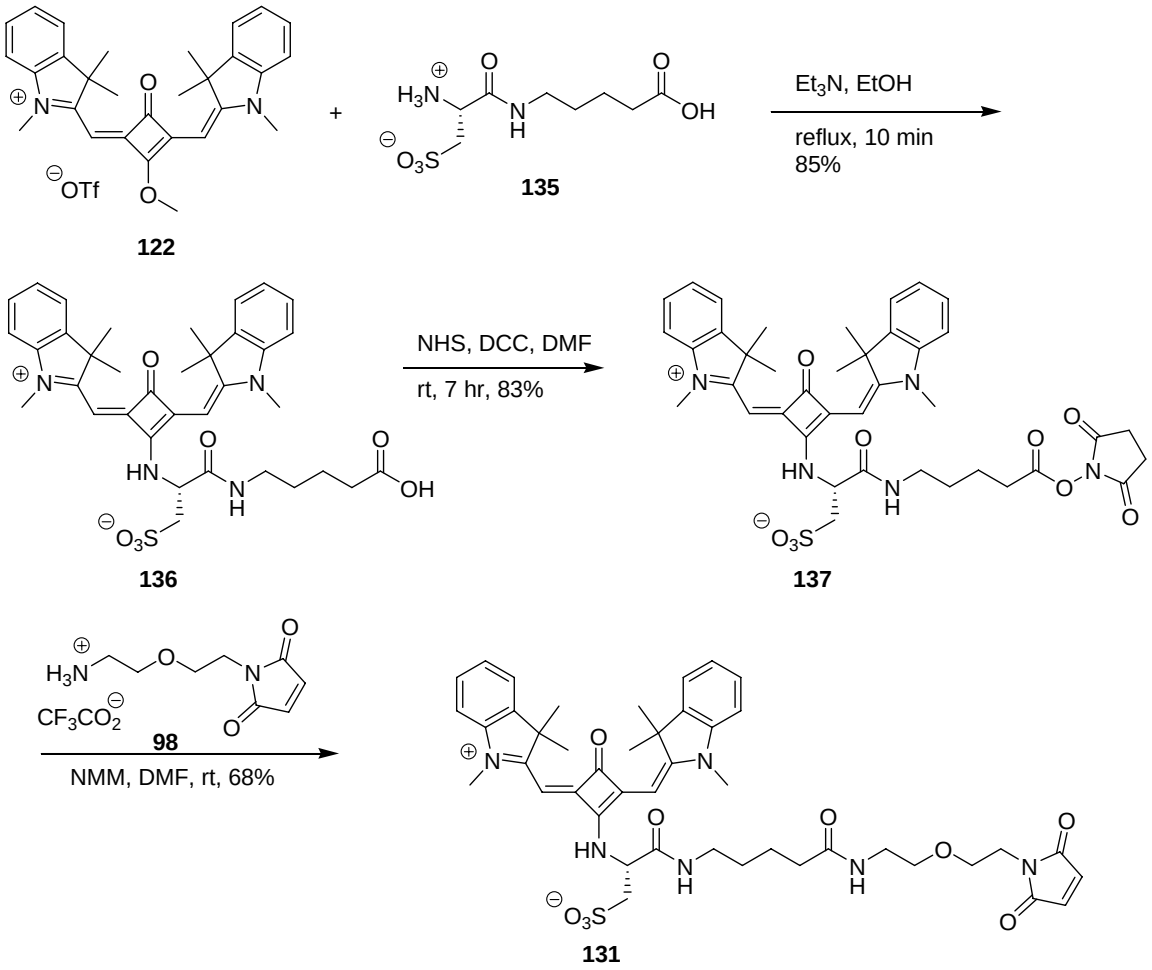
Synthesis of amide chain **135** commenced by coupling of commercially available Boc-Cys(Trt)-OSu with commercially available 5-aminovaleric acid hydrochloride providing protected amide **133** in 88% yield (Scheme 5.13). Deprotection of **133** was effected using TFA and triethylsilane in methylene chloride[40] affording cysteine **134**. Subsequent oxidation of **134** employing performic acid oxidation[41] generated amide chain **135** in 81% yield over the two steps.



Scheme 5.13. Synthesis of Amide Chain **135**.

Squaraine **122** and amide chain **135** were coupled to afford the corresponding squaraine **136** in 85% yield (Scheme 5.14). Activation of **136** as its succinimidyl ester was achieved by stirring with *N*-hydroxysuccinimide and *N,N*'-dicyclohexylcarbodiimide in anhydrous *N,N*-dimethylformamide at ambient temperature, providing activated squaraine dye **137** in 83% yield.

The final step in the synthesis of symmetrical squaraine dye **131** was incorporation of the maleimide residue **98**. Following the procedure of Weber and coworkers[48a], maleimide **98** was obtained as a white crystalline material after reverse phase HPLC purification. Reaction of activated dye **137** with maleimide **98** in the presence of *N*-methylmorpholine in anhydrous *N,N*-dimethylformamide provided the desired thiol-reactive squaraine dye **131** in 68% yield.



Scheme 5.14. Completion of the Thiol-Reactive Dye **131**.

The physical properties of squaraine **131** are shown in Table 5.1 and Figure 5.6. The results show that the symmetrical squaraine dye **131** is a fair candidate to be used with the commercially available red laser. Dye **131** possesses a high extinction coefficient and absorbs the red laser at approximately 80% of maximum absorbance. While the quantum yield is only 6.0%, it is known the quantum yields of squaraine dyes when bound to protein can increase 5-20 fold[44a,b]. This increase in quantum yield would permit **131** to be used in proteomic experiments.

Absorbance (nm)	Emission (nm)	Extinction Coefficient	Quantum Yield
645	661	140,163	6.0%

Table 5.1. Photophysical Properties of Squaraine Dye **131**.

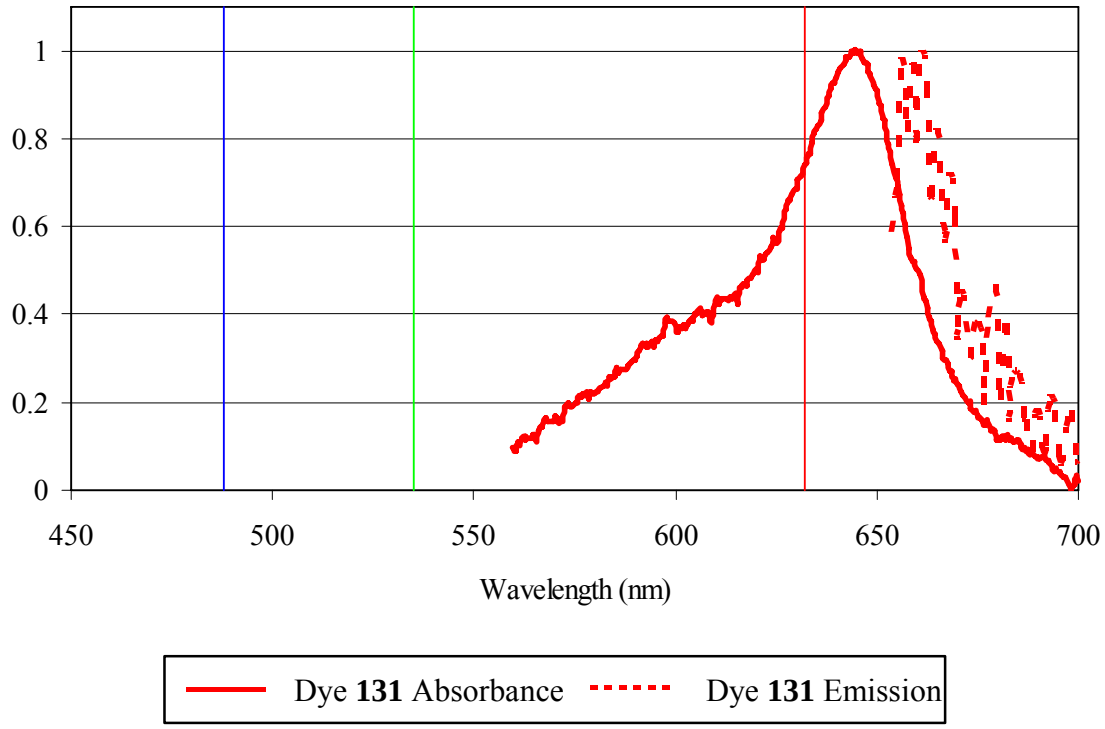


Figure 5.6. Absorbance and Emission of Squaraine Dye **131**.

Proteomic experiments carried out with squaraine dye **131** indicated that the proton in the amide bond linking the squaric moiety and the amide chain, as indicated in Figure 5.7, was acidic enough to act as a titratable proton. Since a titratable group is not desired in dyes used in thiol labeling, this result was not welcome, as it would shift the pI of labeled proteins at certain pH.

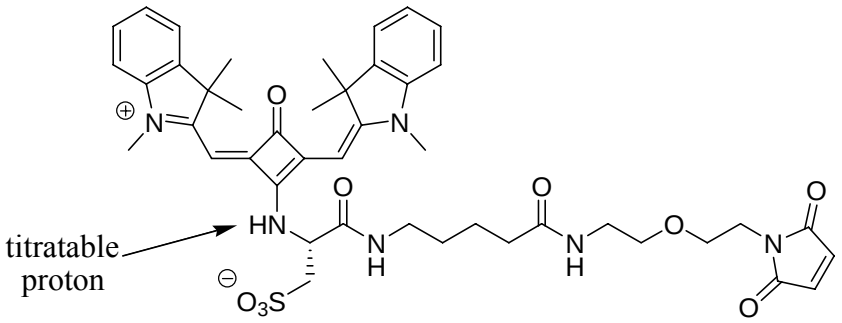


Figure 5.7. A Titratable Proton in Squaraine Dye **131**.

To circumvent this issue, it was decided the amine chain should be connected to the squaric moiety through a secondary amine. With no titratable proton present in the linkage, the pI of the labeled proteins would remain unaffected. It was determined a proline residue would provide the necessary amine source. Developing an amide chain that could insert into the squaraine moiety led to squaraine dyes **138** and **139** as the next synthetic targets (Figure 5.8).

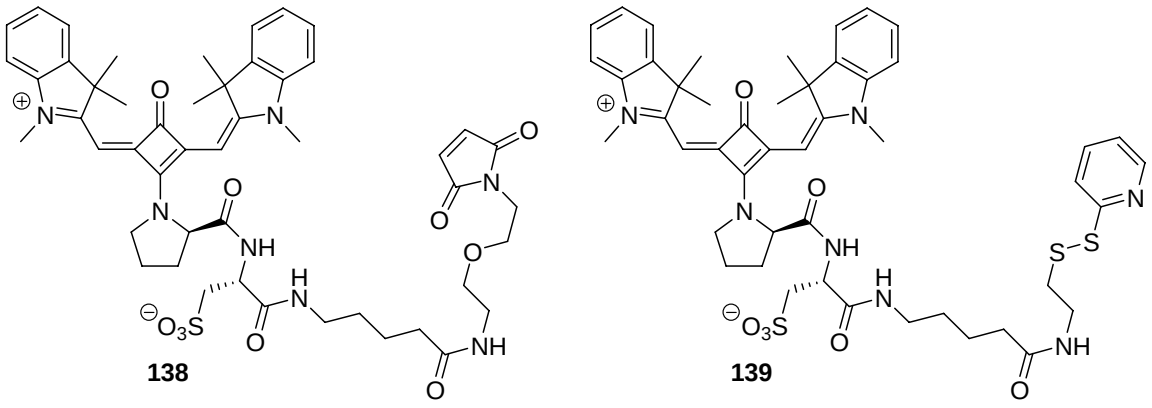
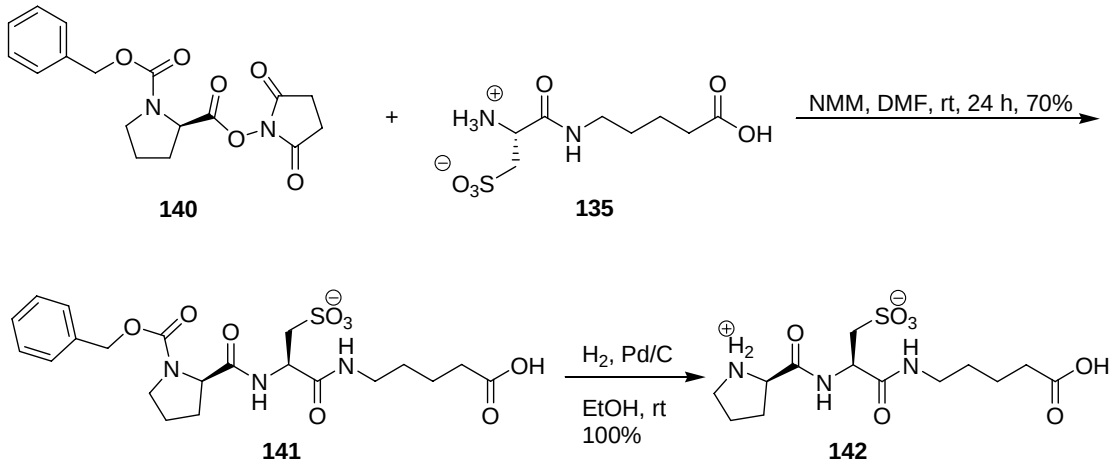


Figure 5.8. Proposed Structures of the Targeted Thiol-Reactive Dyes.

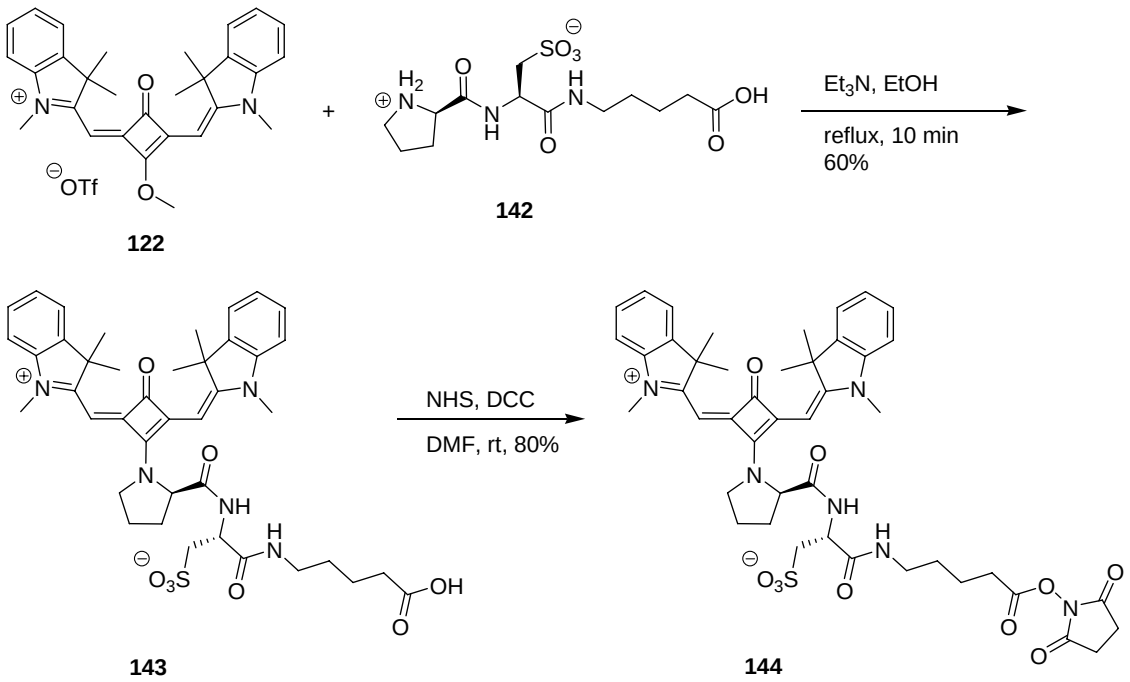
Synthesis of the amide chain **142** was readily accomplished by reaction of amide chain **135** with commercially available Cbz-(D)-Pro-OSu while stirring in the presence of *N*-methylmorpholine in anhydrous *N,N*-dimethylformamide at ambient temperature for

24 h (Scheme 5.15). Purification of the crude product by reverse phase HPLC provided the protected amino acid **141** in 70% yield. Deprotection of **141** by hydrogenation afforded amide chain **142** in quantitative yield.



Scheme 5.15. Synthesis of the Proline Amide Chain.

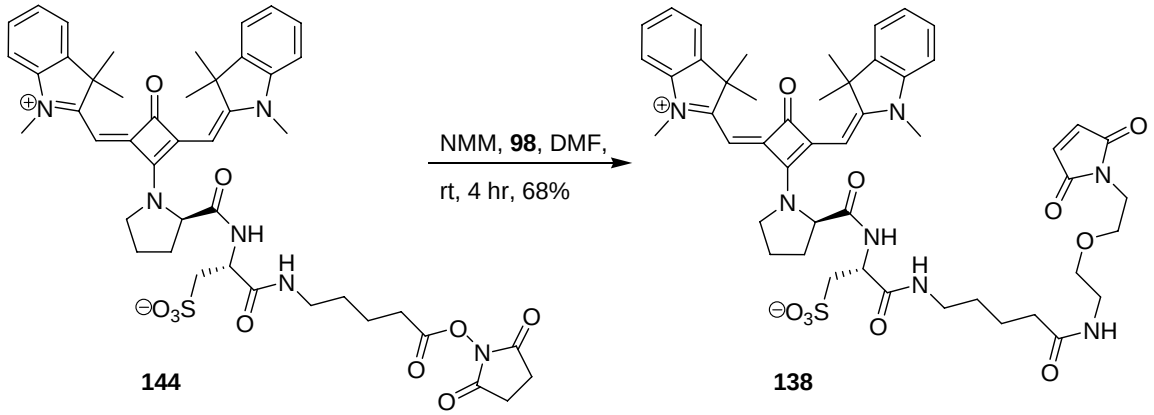
With amide chain **142** in hand, reaction with squaraine **122** in the presence of triethylamine in refluxing ethanol afforded squaraine **143** in 60% yield (Scheme 5.16). Activation of **143** as its *N*-hydroxysuccinimidyl ester was performed employing *N*-hydroxysuccinimide and *N,N*'-dicyclohexylcarbodiimide in anhydrous *N,N*-dimethylformamide at ambient temperature providing the desired activated dye **144** in 80% yield.



122 **142** **143** **144**

Scheme 5.16. Synthesis of Activated Intermediate **144**.

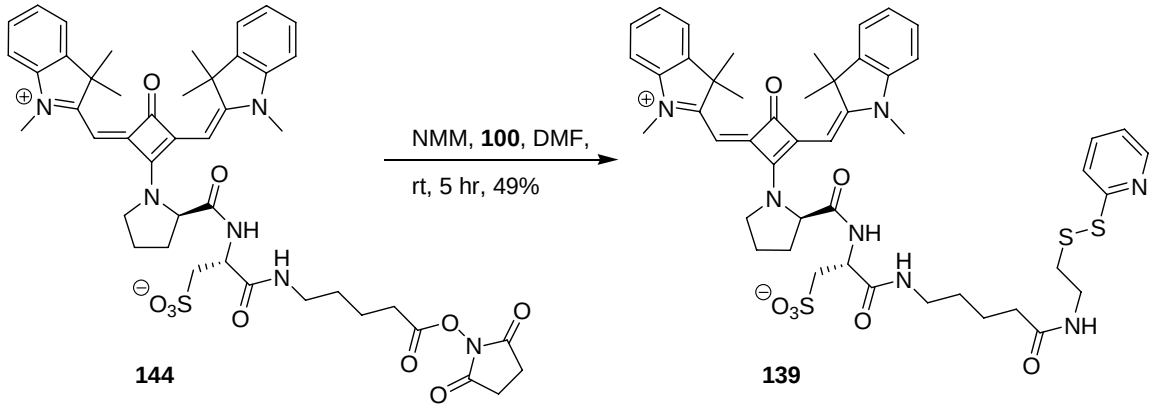
With activated dye **144** in hand, completion of the synthesis of squaraine dye **138** was achieved by stirring **144** with maleimide residue **98** in the presence of *N*-methylmorpholine in anhydrous *N,N*-dimethylformamide at ambient temperature for 4 h, furnishing the desired squaraine dye **138** in 68% yield (Scheme 5.17).



144 **138**

Scheme 5.17. Completion of the Symmetrical Squaraine Maleimide Dye.

Similarly, completion of squaraine dye **139** was effected by stirring dithio pyridyl residue **100**[47a] and activated dye **144** in the presence of *N*-methylmorpholine in anhydrous *N,N*-dimethylformamide at ambient temperature for 5 h. The desired squaraine dye **139** was isolated in 49% yield (Scheme 5.18). Since an adequate amount of dyes **138** and **139** was synthesized for characterization and proteomic study purposes, the final steps were not revisited to improve the yields.



Scheme 5.18. Completion of the Dithio Pyridyl Symmetrical Squaraine Dye.

The physical properties of symmetrical squaraine dyes **138** and **139** are shown in Table 5.2, Figure 5.9 and Figure 5.10.

Dye	Absorbance (nm)	Emission (nm)	Extinction Coefficient	Quantum Yield
138	655	668	190,314	0.2%
139	655	673	187,471	0.6%

Table 5.2. Photophysical Properties of Symmetrical Squaraine Dyes **138** and **139**.

Dyes **138** and **139** possess photophysical properties allowing them to be used in proteomic research. They both contain high extinction coefficients and appreciably absorb the red laser. However, their respective quantum yields are quite low. As mentioned earlier, squaraine dyes experience an increase in their quantum yields when

bound to protein[44a,b]. Taking this factor into account, **138** and **139** can still be considered viable dyes for use in proteomic experiments.

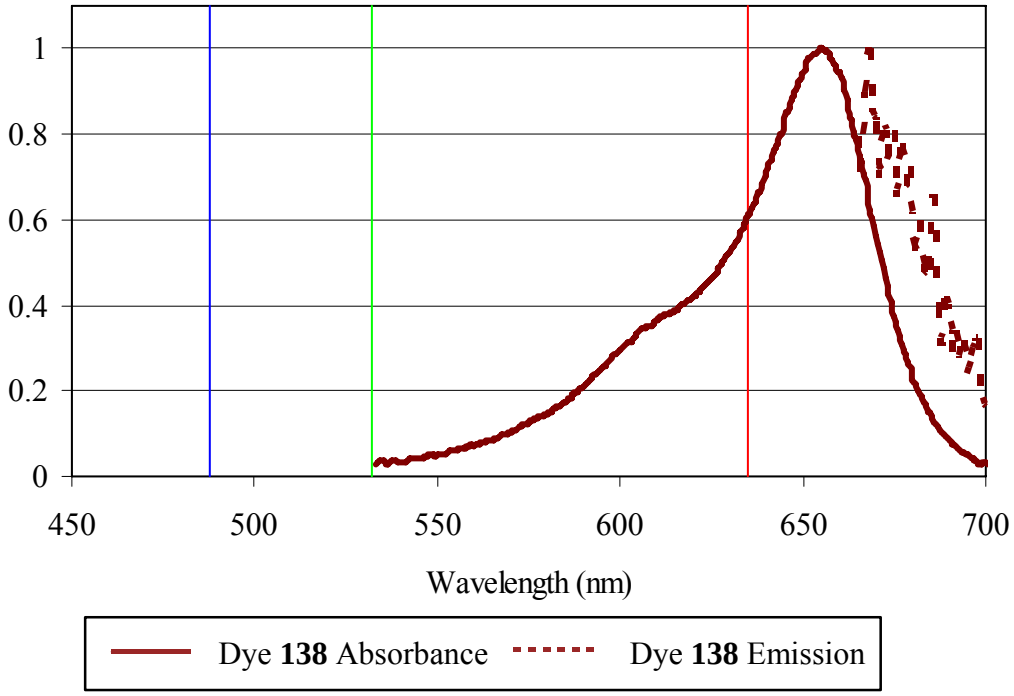


Figure 5.9. Absorbance and Emission of Dye **138**.

While the initial thiol-reactive synthetic targets met with complications, the information gleaned from the studies into targets **124** and **131** led to the development of squaraine dyes **138** and **139**. The photophysical properties expressed by **138** and **139** permit them to be used with the commercially available red laser. With dyes **138** and **139** in possession, the desire for thiol-reactive dyes absorbing the red visible region was fulfilled. It was desired to take the strategies employed for dyes **138** and **139** and apply them to synthesis of fluorescent dyes absorbing in the green visible region.

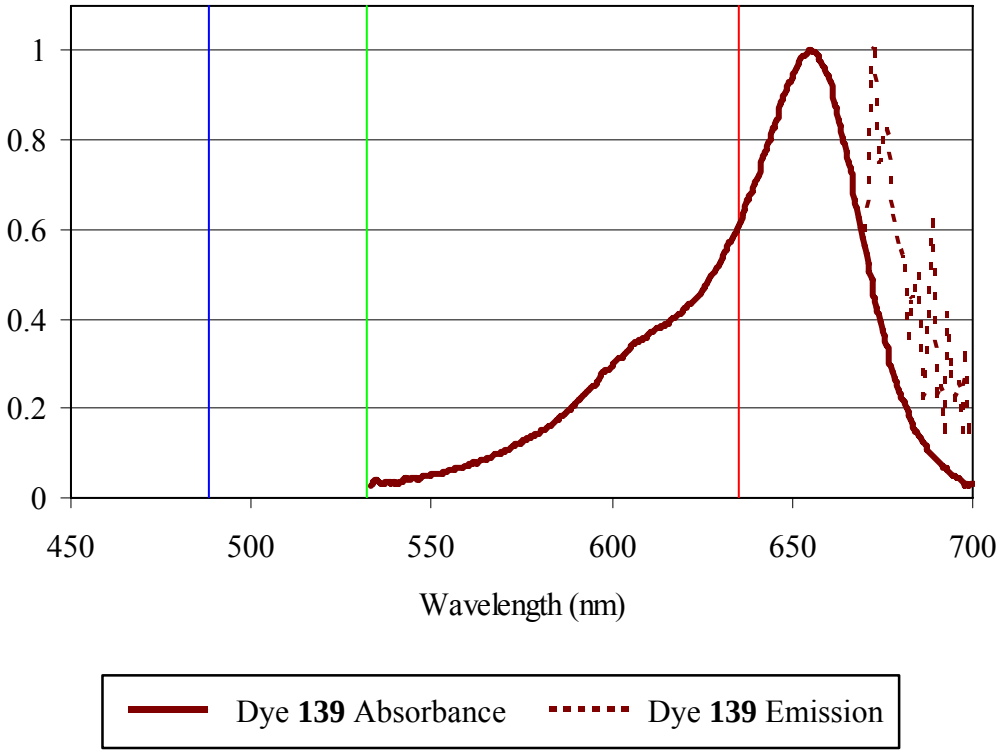
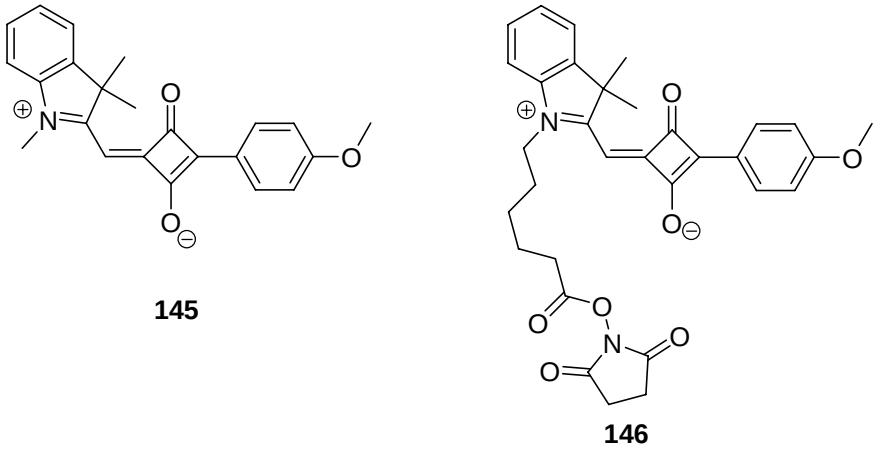


Figure 5.10. Absorbance and Emission of Dye **139**.

Design and Synthesis of Thiol-Reactive Unsymmetrical Squaraine Dyes

Synthetic Strategy

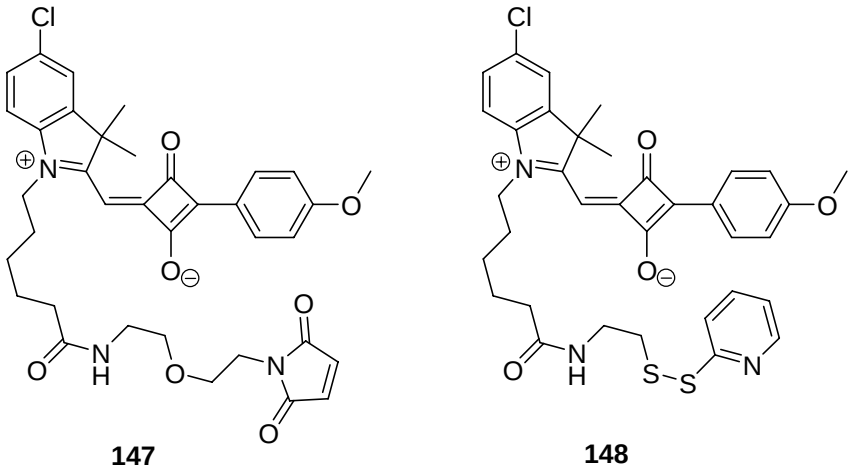
Not only can squaraine dyes absorb in the red visible region, unsymmetrical squaraine dyes have been synthesized that absorb the green visible wavelengths. Such dyes have been generated by Chen and coworkers as well as Law[56a-c]. Law synthesized a series of unsymmetrical squaraine dyes based upon indolenine derivatives, including unsymmetrical squaraine **145** shown in Figure 5.11. Based upon Law's results, unsymmetrical squaraine dye **146** was synthesized in the Grieco group[57] as a candidate to be used as an amine-reactive dye.



145 **146**

Figure 5.11. Unsymmetrical Squaraine Dye Structures Used in Design of Targets.

Based upon the structure of dye **146**, thiol-reactive unsymmetrical dyes **147** and **148** were proposed (Figure 5.12).

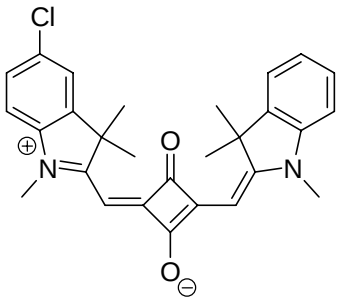


147 **148**

Figure 5.12. Proposed Structures of Thiol-Reactive Unsymmetrical Squaraine Dyes.

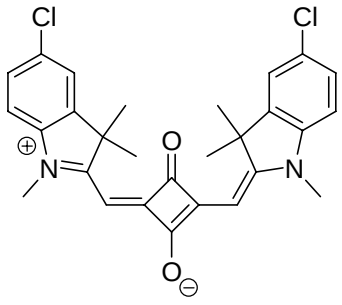
The reasoning behind the structures of **147** and **148** came from the introduction of the residues **98** and **100** into the *N*-hydroxysuccinimidyl activated ester, as had been performed previously with dyes **138** and **139**. The incorporation of a chloro substituent was inspired by the work of Terpetschnig[44b]. In their account, Terpetschnig and coworkers synthesized chloro squaraine dyes **149** and **150** (Figure 5.13).

Comparison of the quantum yields belonging to **149** and **150** showed that **150**, containing one more chloro substituent than **149**, had a quantum yield double the value of the quantum yield belonging to mono-chlorinated **149** (when bound to bovine serum albumin). It was hoped that introduction of a chloro substituent similarly affect the quantum yields of **147** and **148** relative to **146**.



149

Q.Y. = 0.34 (when bound to BSA)

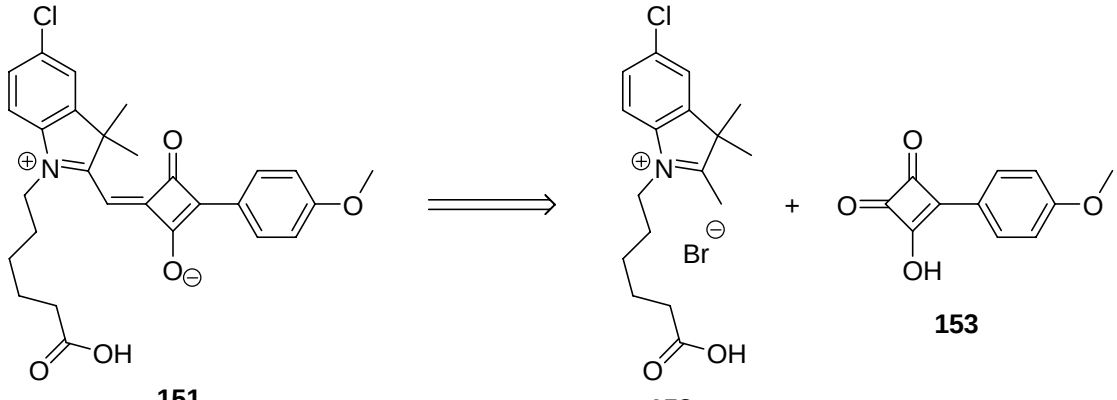


150

Q.Y. = 0.68 (when bound to BSA)

Figure 5.13. Quantum Yields of Chloro-Substituted Squaraines.

Using strategy similar to the synthesis of unsymmetrical squaraine **108**, illustrated earlier, reaction of indoleninium salt **152** with known 3-hydroxy-4-(4-methoxyphenyl) cyclobut-3-ene-1,2-dione **153** should provide the desired unsymmetrical squaraine dye **151** (Figure 5.14).

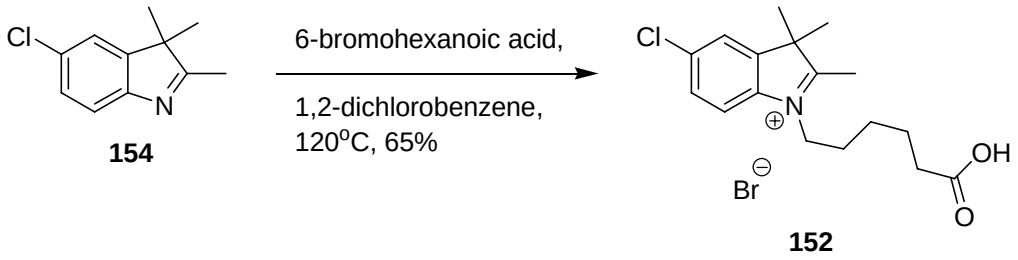


151 **152** **153**

Figure 5.14. Retrosynthetic Analysis of the Unsymmetrical Squaraine Dye Targets.

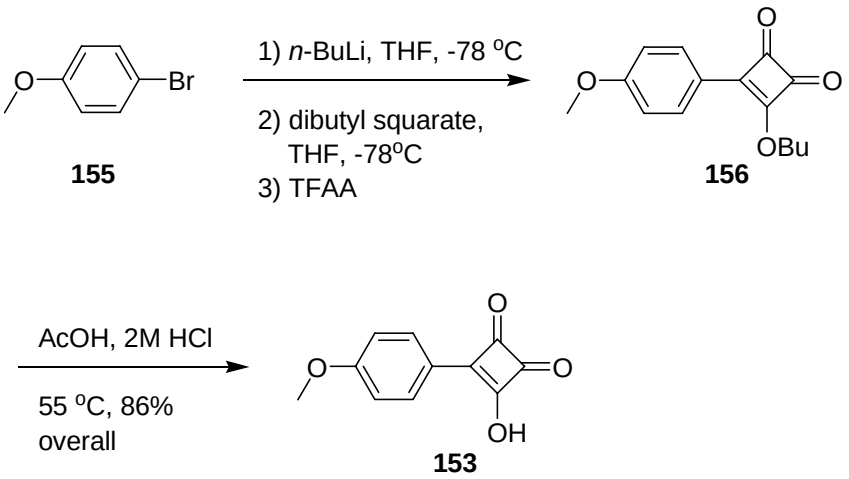
Results and Discussion

Indoleninium salt **152** was readily available *via* the alkylation of commercially available 5-chloro-2,3,3-trimethyl-3H-indole **154** with 6-bromohexanoic acid in 1,2-dichlorobenzene at 120 ºC which provided the indoleninium salt **152** in 65% yield (Scheme 5.19).



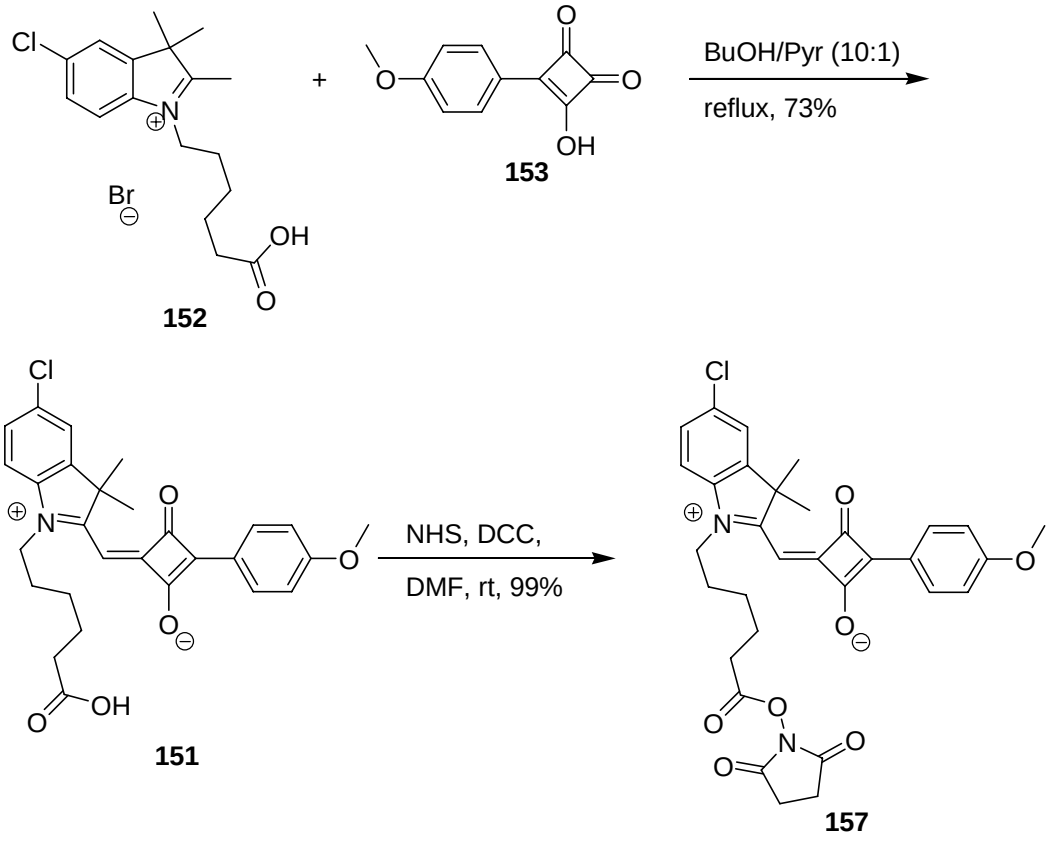
Scheme 5.19. Synthesis of the Indoleninium Salt **152**.

Following the procedure of Reed and coworkers[58], 4-bromoanisole was treated with *n*-BuLi at -78 ºC in anhydrous THF, followed by addition of the reaction mixture *via cannula* to a solution of dibutyl squarate in anhydrous THF at -78 ºC (Scheme 5.20). Subsequent treatment with TFAA and extraction provided squarate ester **156**. Hydrolysis of **156** employing 2M HCl at 55 °C provided desired acid **153** in 86% yield overall.



Scheme 5.20. Synthesis of the Substituted Squaric Acid.

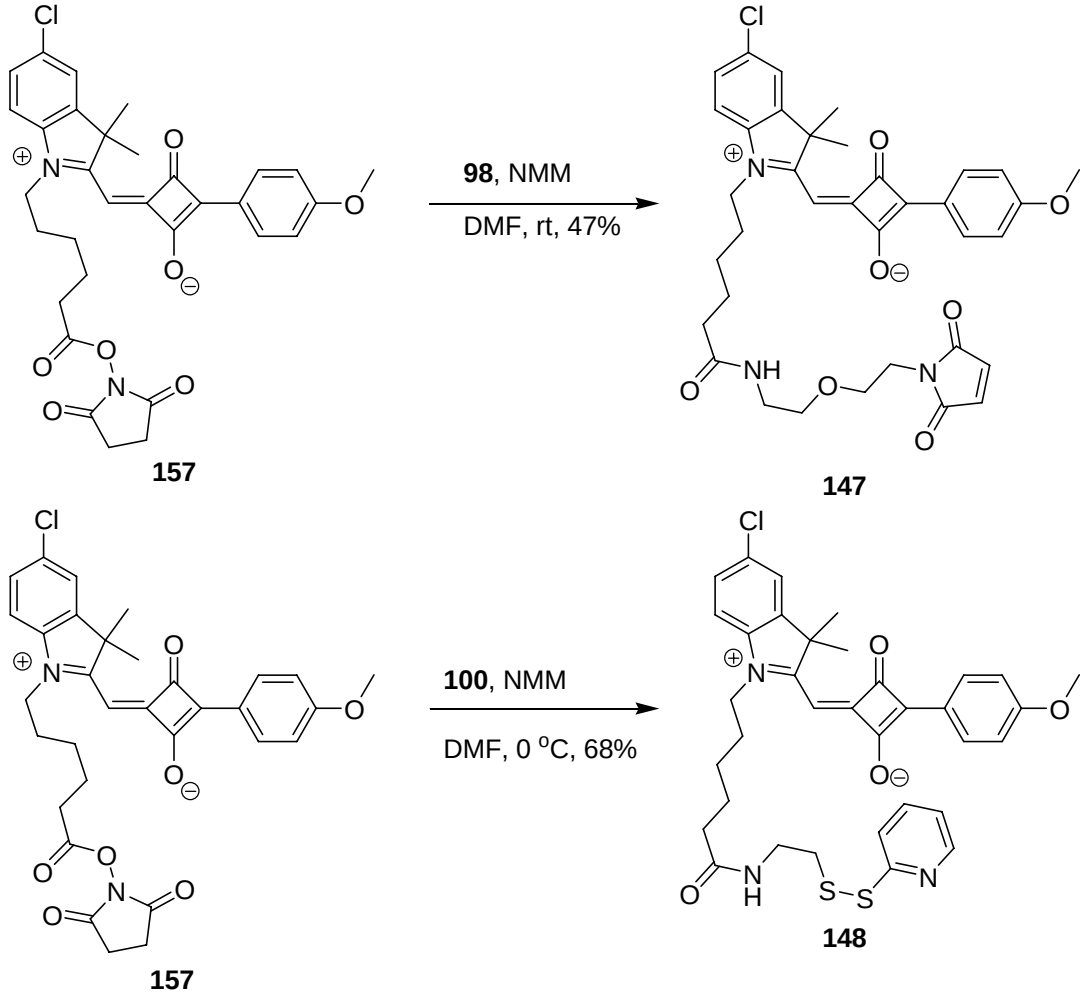
Coupling of indoleninium salt **152** and acid **153** was performed by refluxing in a 10:1 mixture of butanol/pyridine furnishing the unsymmetrical squaraine **151** in 73% yield (Scheme 5.21). Activation of **151** as its *N*-hydroxysuccinimidyl ester was achieved by treating with *N*-hydroxysuccinimide and *N,N*'-dicyclohexylcarbodiimide in anhydrous *N,N*-dimethylformamide at ambient temperature affording activated unsymmetrical squaraine **157** in 99% yield.



Scheme 5.21. Synthesis of the NHS Activated Intermediate.

To finish the syntheses of thiol-reactive unsymmetrical squaraine dyes **147** and **148**, introduction of the thiol-reactive residues **98** and **100**, respectively, was carried out in the presence of *N*-methylmorpholine in anhydrous *N,N*-dimethylformamide at ambient

temperature (Scheme 5.22). Unsymmetrical squaraine dye **147** was isolated in 47% yield, while unsymmetrical squaraine dye **148** was obtained in 40% yield.



Scheme 5.22. Completion of the Thiol-Reactive Unsymmetrical Squaraine Dyes.

When the ambient temperature was 35 °C, stirring **157** and **100** in the presence of *N*-methylmorpholine for 5 h led to a slow change in the color of the solution from red to brown. Purification by reverse phase HPLC provided no product. It was postulated that performing the reaction at lower temperature would prevent decomposition. When **157** and **100** were reacted in the presence of *N*-methylmorpholine at 0 ºC, dye **148** was isolated in 68% yield.

The physical properties of unsymmetrical squaraine dyes **147** and **148** are shown in Table 5.3 and Figures 5.15 and 5.16. It can be observed that **147** and **148** substantially absorb the green laser, but they may not be ideal candidates to be used as dyes for use in proteomic experiments due to their photophysical properties. While their extinction coefficients are desirable, their low quantum yields call into question their effectiveness as protein labels. However, if **147** and **148** experience an increase in their quantum yields when bound to protein similar to other squaraine dyes, they will be acceptable labels for proteomic studies.

Dye	Absorbance (nm)	Emission (nm)	Extinction Coefficient	Quantum Yield
147	549	579	80,688	0.9%
148	538	574	88,719	0.6%

Table 5.3. Photophysical Properties of Unsymmetrical Squaraine Dyes **147** and **148**.

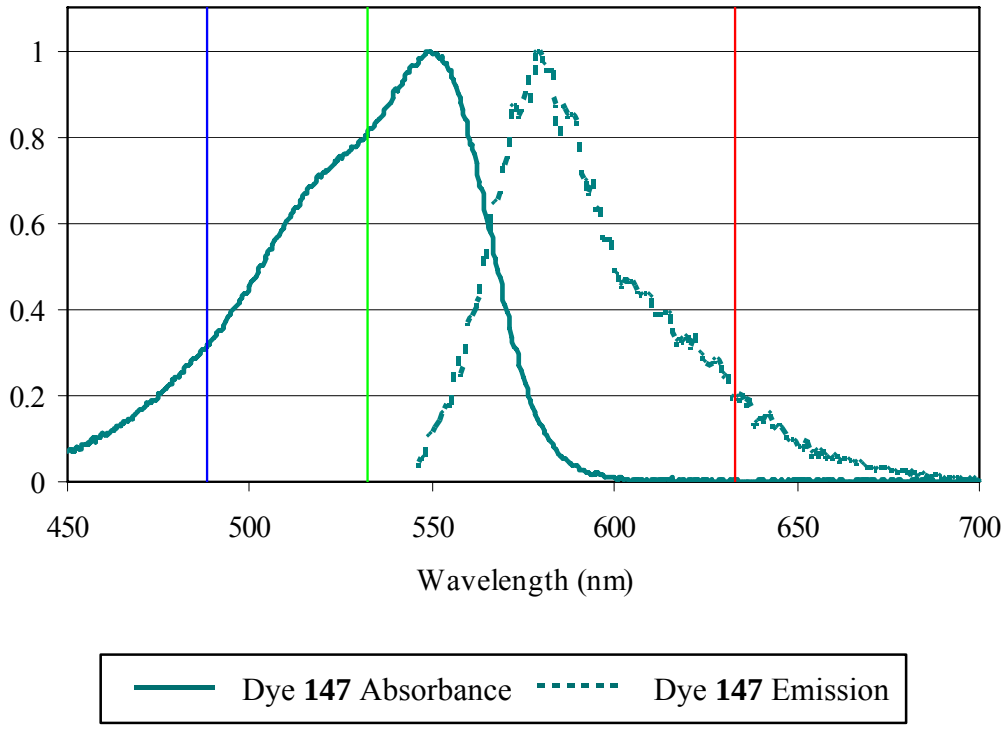


Figure 5.15. Absorbance and Emission of Unsymmetrical Squaraine Dye **147**.

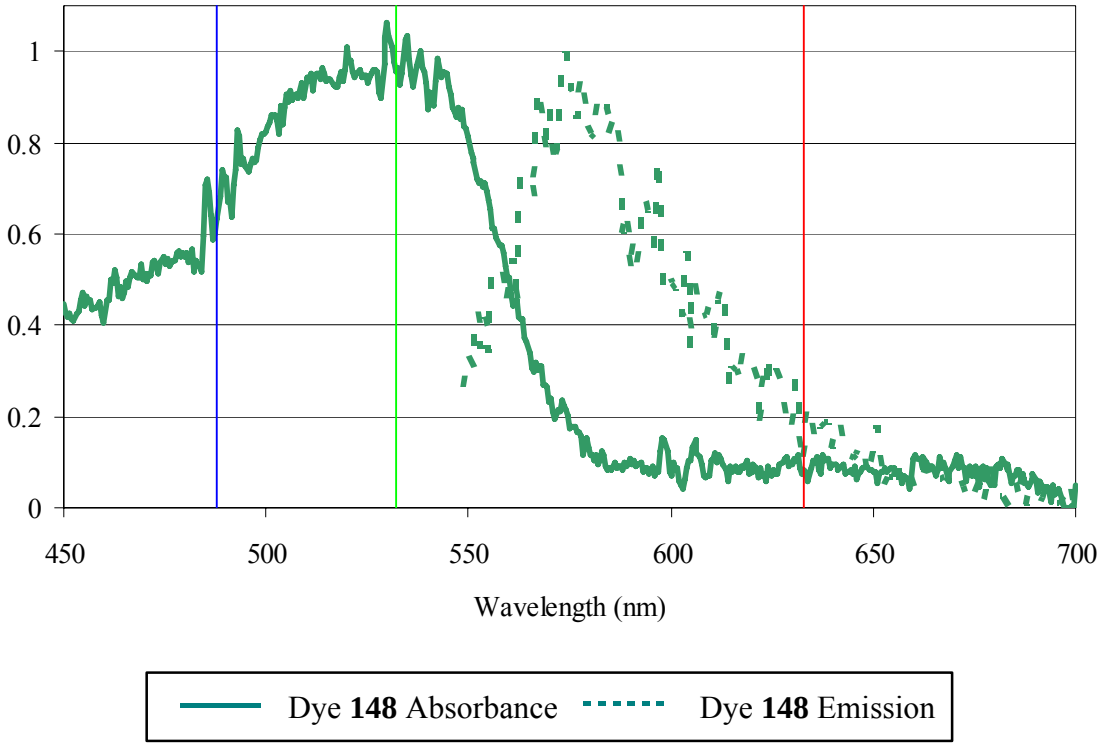


Figure 5.16. Absorbance and Emission of Unsymmetrical Squaraine Dye **148**.

Herein are described the syntheses of squaraine based fluorescent probes that can be used for cysteine labeling in proteomic research. Labeling cysteine residues is becoming a point of interest in proteomic studies since saturation labeling can be performed due to the fewer number of cysteine residues found in proteins. Additionally, labeling of cysteine residues does not prevent trypsin and lysyl peptidase digestion of proteins (as is the case with lysine labeling), allowing for more accurate identification of labeled proteins.

Investigations into the synthesis of symmetrical squaraine dyes **138** and **139**, as well as unsymmetrical squaraine dyes **147** and **148** were successful. While their quantum yields do not seem ideal while in solution, it is postulated that this is due to fluorescent

quenching of the dyes while in solution. It is hoped that when the squaraine dyes are bound to protein, the values of their quantum yields will increase, making them good candidates to be used in cysteine labeling experiments.

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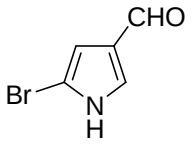
APPENDIX A

EXPERIMENTAL

General Procedure

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 300 MHz on a Bruker DPX 300 spectrometer or at 500 MHz on a Bruker DRX 500 spectrometer as indicated and are reported in parts per million (δ). ^{1}H NMR spectra were obtained in deuterated solvents and were referenced against the residual protic solvent signal as follows: acetone-d_6 ($(CD_3)_2CO$) δ 2.05, acetonitrile-d_3 (CD_3CN) δ 1.94, chloroform-d ($CDCl_3$) δ 7.26, deuterated water (D_2O) δ 4.80, dimethyl sulfoxide-d6 (DMSO-d6) δ 2.50, methanol-d_4 (CD_3OD) δ 3.31. Carbon 13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded at 75 MHz on a Bruker DPX 300 spectrometer or at 125 MHz on a Bruker DRX 500 spectrometer as indicated and are reported in parts per million (δ). ^{13}C NMR were obtained in deuterated solvents and were referenced against the residual protic solvent signal as follows: acetone-d_6 ($(CD_3)_2CO$) δ 29.84, acetonitrile-d_3 (CD_3CN) δ 118.69, chloroform-d ($CDCl_3$) δ 77.23, dimethyl sulfoxide-d6 (DMSO-d6) δ 39.51, methanol-d_4 (CD_3OD) δ 49.15. Infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrophotometer as neat samples, unless indicated otherwise. Reactions were monitored by thin layer chromatography (TLC) using E. Merck precoated silica gel 60 F-254 (0.25 mm) plates. The plates were visualized by immersion in a *para*-anisaldehyde solution and warming on a hot plate. Flash chromatography was performed as described by Still using Sorbent Technologies silica gel 60 Å (32-63 μm) mesh. Reverse phase HPLC purification was carried out using a Waters 600 Pump, a Waters 600 Controller and a Waters 2487 Dual λ Absorbance Detector utilizing a preparative reverse phase PHENOMENEX Synergi 4μ Polar RP 80Å 250 mm x 21.20 mm column.

The mobile phase was always a gradient of HPLC grade CH_3CN:H_2O containing 0.1% HPLC grade TFA as ion pairing agent. Unless otherwise stated, all experiments were run in oven-dried glassware under an argon atmosphere using anhydrous solvents. The solvents were dried and distilled as indicated below. Tetrahydrofuran, diethyl ether and toluene were purified by distillation from sodium benzophenone ketyl. Diisopropyl-ethylamine, triethylamine, dichloromethane, pyridine and *N*-methylmorpholine were distilled from calcium hydride. Acetonitrile, benzene, dimethylsulfoxide and *N,N*-dimethylformamide were purchased anhydrous as Aldrich Sure-Seal bottles. Iodo-methane was passed through a plug of potassium carbonate prior to use. Other reagents and solvents were reagent grade and were used as received.

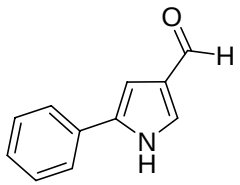


21

5-bromo-1*H*-pyrrole-3-carbaldehyde (21).

Recrystallized NBS (1.47 g, 8.26 mmol) was added to a solution of pyrrole-3-carboxaldehyde (0.783 g, 8.23 mmol) in anhydrous THF (35 mL) cooled to -78 °C. The mixture was stirred at -20 °C for 24 h. The solution was cooled to -78 °C, followed by addition of one equivalent NBS for the remaining starting material (as determined by ^{1}H NMR). The mixture was stirred at -20 °C for 24 hr. The solvent was removed *in vacuo*, and the crude product purified by flash chromatography on silica gel (19:1 hexanes-ethyl acetate) providing **21** as a white solid (0.912 g, 5.24 mmol, 64%): m.p. 119-121 °C; R_f =

0.16 (4:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2) 1495 (s), 1652 (s), 2856 (s), 2970 (s), 3075 (s), 3123(s); ^{1}H NMR (500 MHz, acetone-d_6) δ 9.71 (s, 1H), 7.67 (d, 1H, J = 2.5), 6.58 (d, 1H, J = 2.5); ^{13}C NMR (125 MHz, acetone-d_6) δ 184.75, 129.88, 129.00, 109. 10, 102.83; HRMS-EI (m/z) [M] calcd. for C_5H_4BrNO: 172.9476, found 172. 9481.

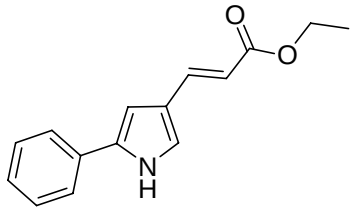


22

5-phenyl-1H-pyrrole-3-carboxaldehyde (22).

A solution of Na_2CO_3 (11.2 g, 105 mmol) in degassed water (70 mL) was added to a suspension of bromopyrrole **21** (7.50g, 43.1 mmol) and tetrakis(triphenylphosphine)-palladium(0) (1.00 g, 0.864 mmol) in degassed DMF (170 mL) under argon at ambient temperature, followed by addition of a solution of phenylboronic acid (6.00 g, 49.4 mmol) in degassed DMF (80 mL). The mixture was stirred under argon at 120 °C for 5 h. The flask was allowed to cool to ambient temperature and water (70 mL) was added. The product was isolated by extraction with CH_2Cl_2. The organic phase was washed with water (5x), brine (3x) and dried (Mg_2SO_4). The solvent was removed *in vacuo*. The resulting solid was purified by flash chromatography on silica gel (9:1 hexanes-ethyl acetate) providing **22** as a white powder (4.00g, 23.4 mmol, 61%): mp: 136-137 °C (lit 137 °C[56]); R_f = 0.06 (4:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1642 (s); ^{1}H NMR (300 MHz, acetone-d_6) δ 6.96 (s, 1H), 7.26 (t, 1H, J = 7.5 Hz), 7.40 (t, 2H, J = 7.5 Hz), 7.70 (s,

1H), 7.72 (d, 2H, J = 7.5 Hz), 9.79 (s, 1H); ^{13}C NMR (125 MHz, acetone-d_6) δ 104.17, 125.18, 127.92, 129.11, 129.81, 132.77, 135.50, 185.70; HRMS-EI (m/z): [M] calcd for $C_{11}H_9NO$ 171.0684 found 171.0680.

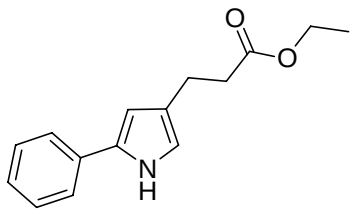


23

(E)-Ethyl 3-(5-phenyl-1H-pyrrol-3-yl)acrylate (23).

Piperidine (0.13 mL, 1.32 mmol) and mono-ethyl malonate (3.31 mL, 28.1 mmol) were added to a solution of aldehyde **22** (800mg, 4.68 mmol) in anhydrous pyridine (4.28 mL) under argon at ambient temperature. The mixture was stirred at 110 °C for 5.5 h and allowed to cool to ambient temperature. The reaction mixture was quenched with H_2O (5 mL). The mixture was acidified to pH 3 using 1M HCl, and the product was isolated by extraction with EtOAc. The organic layer was dried (K_2CO_3) and the solvent removed *in vacuo*. The crude product was purified by flash chromatography on silica gel (85:15 hexanes-ethyl acetate) providing **23** as a white solid (911 mg, 3.78 mmol, 81%): mp 67-70 °C; R_f = 0.44 (4:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1276 (s), 1625 (s), 1672 (s), 3307 (s); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.34 (t, J = 7 Hz, 3H), 4.26 (q, J = 7 Hz, 2H), 6.19 (d, J = 15.5 Hz, 1H), 6.72 (s, 1H), 7.06 (s, 1H), 7.27 (t, J = 8 Hz, 1H), 7.40 (t, J = 8 Hz, 2H), 7.51 (d, J = 8 Hz, 2H), 7.67 (d, J = 15.5 Hz, 1H), 8.96 (bs, 1H) (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.59, 60.32, 103.66, 113.89, 122.42, 122.69, 124.33, 127.22,

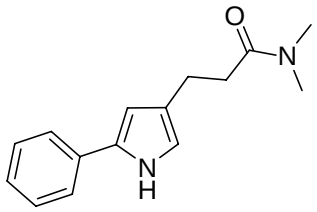
129.18, 132.04, 134.40, 138.94, 168.38; HRMS-EI (m/z): [M] calcd for $C_{15}H_{15}NO_2$ 241.1103 found 241.1102.



24

Ethyl 3-(5-phenyl-1H-pyrrol-3-yl)propanoate (24).

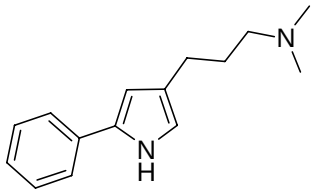
A flask containing a suspension of acrylate **23** (860 mg, 3.57 mmol) and 10% Pd/C (120mg, 0.107 mmol) in ethanol (30 mL) was charged with hydrogen. The suspension was stirred under hydrogen (1 atm) for 5 h. The catalyst was filtered and rinsed with ethanol. The solvent was removed *in vacuo* providing **24** as a white solid (866 mg, 3.56 mmol, 100%): mp 141-143 °C; R_f = 0.21 (4:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1718 (s), 3350 (s); ^{1}H NMR (500 MHz, CD_3OD) δ 1.21 (t, J = 7 Hz, 2H), 2.56 (t, J = 7.5 Hz, 2H), 2.77 (t, J = 7.5 Hz, 2H), 4.10 (q, J = 7 Hz, 2H), 6.32 (d, J = 1 Hz, 1H), 6.59 (d, J = 1 Hz, 1H), 7.10 (t, J = 7.5 Hz, 1H), 7.28 (t, J = 7.5 Hz, 2H), 7.50 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.66, 23.79, 37.24, 61.54, 106.54, 117.68, 124.59, 124.71, 126.57, 129.79, 133.24, 134.91, 175.60; HRMS-EI (m/z): [M] calcd for $C_{15}H_{17}NO_2$ 243.1259 found 243.1265.



25

***N,N*-Dimethyl-3-(5-phenyl-1H-pyrrol-3-yl)propanamide (25)**.

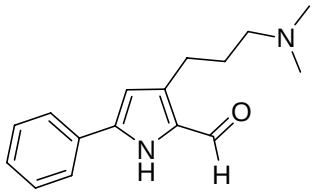
A solution of 2.0 M $AlMe_3$ in toluene (12.2 mL, 24.4 mmol) was added slowly to a flask containing a solution of dimethylamine hydrochloride (1.99g, 24.4 mmol) in anhydrous benzene (100 mL) under argon at ambient temperature. After 1 h, a solution of propanoate **24** (2.96g, 12.2 mmol) in anhydrous benzene (100 mL) was added, and the solution refluxed 15 h. The reaction was allowed to cool to ambient temperature and quenched with 1 M HCl. Water (50 mL) was added, and the product was isolated by extraction with EtOAc. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo* providing **25** as a white solid (2.60g, 10.7 mmol, 89%): mp 183-184 °C; R_f = 0.37 (ethyl acetate); FTIR (CH_2Cl_2): 1623 (s), 3250 (s); ^{1}H NMR (300 MHz, CD_3Cl) δ 2.63 (t, J = 8.5 Hz, 2H), 2.87 (t, J = 8.5 Hz, 2H), 2.97 (s, 3H), 2.99 (s, 3H), 6.41 (s, 1H), 6.69 (s, 1H), 7.19 (t, J = 7.5 Hz, 1H), 7.35 (t, J = 7.5 Hz, 2H), 7.46 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 24.27, 35.94, 36.29, 38.08, 106.64, 117.78, 124.58, 124.98, 126.58, 129.81, 133.30, 134.98 (carbonyl carbon could not be detected); HRMS-EI (m/z): [M] calcd for $C_{15}H_{18}N_2O$ 242.1419 found 242.1417.



26

***N,N*-dimethyl-3-(5-phenyl-1H-pyrrol-3-yl)propan-1-amine (26)**.

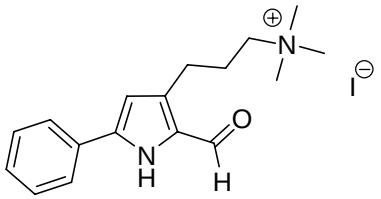
A solution of amide **25** (567 mg, 2.34 mmol) in anhydrous THF (75 mL) was added dropwise to a flask containing a suspension of LAH (454 mg, 11.9 mmol) in anhydrous THF (40 mL) under argon cooled to 0 °C. After addition was complete, the mixture was stirred at ambient temperature for 3 h. The reaction mixture was cooled to 0 °C and quenched with 1M Na_2CO_3 (200 mL). Water (50 mL) was added, and the product was isolated by extraction with EtOAc. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **26** as an off white solid (500 mg, 2.19 mmol, 94%): R_f = 0.06 (4:1 dichloromethane-methanol); FTIR (CH_2Cl_2): 1465 (s), 1513 (s), 1606 (s), 2939 (s); ^{1}H NMR (500 MHz, CD_3OD) δ 1.73 (m, 2H), 2.18 (s, 6H), 2.31 (m, 2H), 2.44 (t, J = 7.5 Hz, 2H), 6.31 (d, 1H, J = 1.5 Hz), 6.56 (d, 1H, J = 1.5 Hz), 7.07 (t, J = 7.5 Hz, 1H), 7.26 (t, J = 7.5 Hz, 2H), 7.49 (d, J = 7.5 Hz, 2H); ^{13}C NMR (75 MHz, CD_3OD) δ 26.04, 29.92, 45.53, 60.53, 106.67, 117.65, 124.54, 125.73, 126.48, 129.79, 133.13, 135.02; HRMS-EI (m/z): [M] calcd for $C_{15}H_{20}N_2$ 228.1626 found 228.1628.



27

3-(3-(dimethylamino)propyl)-5-phenyl-1H-pyrrole-2-carboxaldehyde (27).

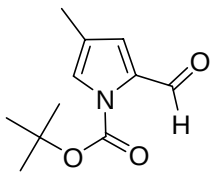
Anhydrous DMF (0.677 mL, 8.76 mmol) was added dropwise to a flask containing $POCl_3$ (0.4mL, 4.38 mmol) under argon. The mixture was stirred at ambient temperature for 1 h. 1,2-Dichloroethane (20 mL) and a solution of amine **26** (105 mg, 0.438 mmol) in $(CH_2)_2Cl_2$ (24mL) were successively added to the reaction mixture. The mixture was refluxed at 90 ^oC for 4 h. The reaction mixture was poured onto crushed ice and brought to pH 12 by addition of 10 M NaOH. The mixture was stirred at 70 ^oC for 1 h and allowed to cool to ambient temperature. The product was isolated by extraction with EtOAc. The organic phase was dried (Mg_2SO_4) and the solvent removed *in vacuo*. The crude product was purified by flash chromatography on silica gel (3:2 dichloromethane-methanol) providing **27** as a light brown solid (1.17 g, 0.325 mmol, 74%): R_f = 0.19 (1:1 dichloromethane-methanol); FTIR (CH_2Cl_2): 1632 (s) 2942 (s), 3272 (br); ^1H NMR (500 MHz, CD_3OD) δ 1.87 (m, 2H), 2.26 (s, 6H), 2.40 (m, 2H), 2.84 (t, J = 7.5 Hz, 2H), 6.60 (s, 1H), 7.34 (t, 7.5 Hz, 1H), 7.42 (t, J = 7.5 Hz, 2H), 7.74 (d, J = 7.5 Hz, 2H), 9.59 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 24.54, 30.03, 45.58, 60.26, 110.64, 126.72, 129.54, 130.15, 131.38, 132.49, 140.05, 141.58, 179.21; HRMS-EI (m/z): [M] calcd for $C_{16}H_{20}N_2O$ 256.1576 found 256.1567.



18

Trimethyl-(3-(2-formyl-5-phenyl-1H-3-pyrrolyl)-propyl)-ammonium iodide (18).

An excess of iodomethane (1 mL) was added to a flask containing a solution of aldehyde **27** (216 mg, 0.842 mmol) in anhydrous THF (5 mL) under argon at ambient temperature. The mixture was allowed to stir at ambient temperature for 30 min. The solvent was removed *in vacuo* providing **18** as a red solid (335 mg, 0.842 mmol, 100%): mp 230-232 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 2.20 (m, 2H), 2.95 (t, J = 7.5 Hz, 2H), 3.14 (s, 9H), 3.43 (m, 2H), 6.69 (s, 1H), 7.35 (t, J = 7.5 Hz, 1H), 7.44 (t, J = 7.5 Hz, 2H), 7.75 (d, J = 7.5 Hz, 2H), 9.65 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 21.98, 23.57, 52.27, 65.01, 109.35, 125.31, 127.98, 128.77, 129.83, 130.66, 138.38, 177.91; HRMS-EI (m/z): [M] calcd for $C_{17}H_{23}N_2O^+$ 271.1805 found 271.1806.

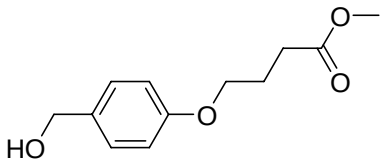


29

***tert*-Butyl 2-formyl-4-methyl-1H-pyrrole-1-carboxylate (29)**.

DMAP (204 mg, 1.66 mmol) and Boc_2O (3.26 g, 14.9 mmol) were added to a solution of 4-methyl-2-formylpyrrole (1.66 g, 15.2 mmol) in anhydrous acetonitrile (33 mL) under argon at ambient temperature. The mixture was stirred at ambient temperature

for 15 h, followed by partitioning between 0.1 M HCl (70.5 mL) and ether (150 mL). The organic layer was washed with 36 mL portions of 0.1 M HCl (5x), H_2O (1x), sat'd $NaHCO_3$ (3x), and brine (3x). The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. The crude product was purified by flash chromatography on silica gel (9:1 hexanes-ethyl acetate) providing **29** as a clear liquid (2.93 g, 14.0 mmol, 94%): R_f: 0.33 (19:1 hexanes-ethyl acetate); FTIR: 1424 (s), 1469 (s), 1669 (s), 1744 (s), 2904 (s), 2932 (s), 2980 (s); ^{1}H NMR (500 MHz, CD_3OD) δ 1.63 (s, 9H), 2.07 (s, 3H), 6.99 (s, 1H), 7.29 (s, 1H), 10.19 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 11.46, 28.25, 86.82, 123.74, 123.90, 127.08, 136.04, 149.90, 183.89; HRMS-ES (m/z): [M+Na] calcd for $C_{11}H_{15}NO_3Na^+$ 232.0950 found 232.0948.

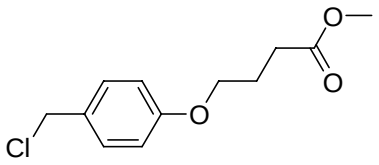


30

Methyl 4-(4-(hydroxymethyl)phenoxy)butanoate (30).

A solution of methyl 4-bromobutanoate (3.67 g, 29.6 mmol) in anhydrous DMF (72 mL) was added to a flask containing 4-hydroxy-benzyl alcohol (5.46 g, 30.2 mmol) and K_2CO_3 (30.95 g, 224 mmol) under argon at ambient temperature. The mixture was stirred at 65 °C for 16 h before being cooled to ambient temperature and filtered. The filtered solution was poured into EtOAc (300 mL) and washed with H_2O (6x150 mL). The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **30** as a clear yellow liquid (4.98 g, 22.2 mmol, 75%): FTIR: 1513 (s), 1612 (s), 1731 (s), 2875

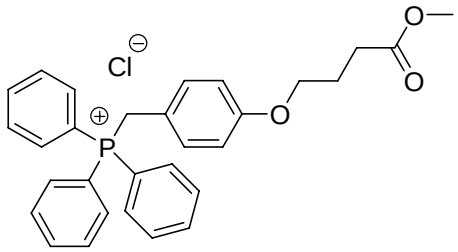
(s), 2952 (s), 3430 (br); ^{1}H NMR (500 MHz, CD_3OD) δ 2.06 (p, J = 12.5 Hz, 2H), 2.52 (t, J = 12.5 Hz, 2H), 3.67 (s, 3H), 4.00 (t, J = 12.5 Hz, 2H), 4.51 (s, 2H), 6.87 (d, J = 14 Hz, 2H), 7.25 (d, J = 14 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 25.84, 31.47, 52.22, 64.95, 67.94, 115.42, 129.69, 134.90, 159.65, 175.43; HRMS-ES (m/z): [M+Na] calcd for $C_{12}H_{16}O_4Na^+$ 247.0946 found 247.0937.



31

Methyl 4-(4-(chloromethyl)phenoxy)butanoate (31).

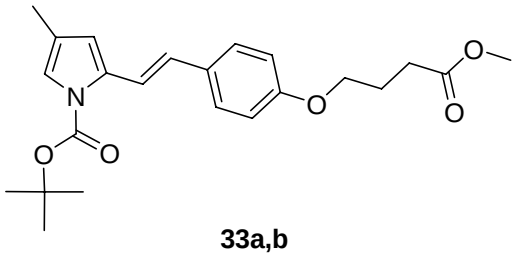
A stirred solution of butanoate **30** (7.52 g, 33.5 mmol) in anhydrous toluene (37.2 mL) under argon was cooled to 0 °C, followed by dropwise addition of $SOCl_2$ (4.9 mL, 41.0 mmol). The mixture was stirred at ambient temperature for 2 d before the solvent was removed *in vacuo*. The crude product was purified by flash chromatography on silica gel(4:1 hexanes-ethyl acetate) providing **31** as a clear liquid (6.61 g, 27.3 mmol, 81%): R_f: 0.38 (9:1 hexanes-ethyl acetate); FTIR: 1514 (s), 1611 (s), 1736 (s), 2952 (s); ^{1}H NMR (500 MHz, CD_3OD) δ 2.06 (p, J = 7 Hz, 2H), 2.51 (t, J = 7 Hz, 2H), 3.67 (s, 3H), 4.00 (t, J = 7 Hz, 2H), 4.58 (s, 2H), 6.87 (d, J = 9 Hz, 2H), 7.30 (d, J = 9 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 25.98, 31.58, 52.20, 68.14, 75.38, 115.56, 130.72, 131.71, 160.21, 175.55; HRMS-ES (m/z): [M+Na] calcd for $C_{12}H_{15}ClO_3Na^+$ 265.0607 found 265.0619.



32

(4-(Methyl-4-butanoate)benzyl)triphenylphosphonium chloride (32).

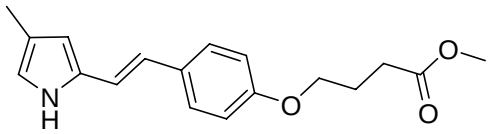
A flask containing benzyl chloride **31** (3.338 g, 13.75 mmol) and triphenyl-phosphine (3.605 g, 13.74 mmol) was placed under argon and stirred at 160 ºC for 15 h. The crude product was purified by flash chromatography on silica gel (19:1 dichloro-methane-methanol) providing **32** as a clear, viscous liquid (5.55 g, 11.0 mmol, 80%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.02 (p, J = 7 Hz, 2H), 2.48 (t, J = 7 Hz, 2H), 3.63 (s, 3H), 3.96 (t, J = 7 Hz, 2H), 5.01 (d, J = 14 Hz, 2H), 6.77 (d, J = 8.5 Hz, 2H), 7.00 (dd, J = 8.5, 2.5 Hz, 2H), 7.71 – 7.78 (m, 12H), 7.89 – 7.92 (m, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 25.68, 31.41, 52.27, 68.16, 116.12, 118.95, 119.63, 120.25, 131.46, 133.58, 135.44, 136.40, 160.53, 175.19; HRMS-ES (m/z): [M] calcd for $C_{30}H_{30}O_3P^+$ 469.1932 found 469.1935.



33a,b

***tert*-Butyl-2-(4-(3-(methoxycarbonyl)propoxy)styryl)-4-methyl-1H-pyrrole-1-carboxylate (33a,b)**.

A solution of aldehyde **29** (1.12 g, 5.35 mmol) in anhydrous DCM (7 mL) was added to a mixture of phosphonium chloride **32** (2.67g, 5.28 mmol) and sodium hydride (127 mg, 5.29 mmol) in anhydrous DCM (7 mL) under argon. The mixture was refluxed for 15 h before being cooled to ambient temperature. The crude product was purified by flash chromatography on silica gel (19:1 hexanes-ethyl acetate) providing a clear yellow oil which was a 1:1 mixture of isomers **33a,b** which were not resolved (1.58 g, 3.96 mmol, 75%): HRMS-ES (m/z): [M+Na] calcd for $C_{23}H_{29}NO_5Na^+$ 422.1943 found 422.1951.

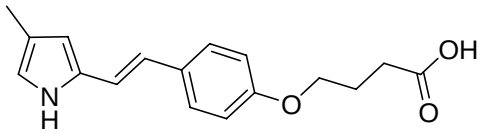


34

Methyl-4-(4-((E)-2-(4-methyl-1H-pyrrol-2-yl)vinyl)phenoxy)butanoate (34).

Triethylsilane (0.08 mL, 0.500 mmol) and TFA (2.5 mL) were added to a solution of pyrroles **33a,b** (184 mg, 0.461 mmol) in anhydrous DCM (2.5 mL) under argon at ambient temperature. The mixture was stirred at ambient temperature for 45 min

followed by quenching with H_2O (5 mL). The mixture was basified with 1 M NaOH and the product was isolated by extraction with DCM. The organic layer was washed with 1 M HCl (2x) and brine (1x). The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **34** as a purple solid (132 mg, 0.441 mmol, 96%): m.p.: 145 ^oC (decomposed); *Rf*: 0.39 (3:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1512 (s), 1722 (s); ^1H NMR (500 MHz, $CDCl_3$) δ 2.10 – 2.15 (m, 5H), 2.55 (t, J = 7 Hz, 2H), 3.71 (s, 3H), 4.02 (t, J = 7 Hz, 2H), 6.17 (s, 1H), 6.55 (s, 1H), 6.59 (d, J = 16.5 Hz, 1H), 6.78 (d, J = 16.5 Hz, 1H), 6.85 (d, J = 7.5 Hz, 2H), 7.33 (d, J = 7.5 Hz, 2H), 8.09 (bs, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.04, 24.84, 30.75, 51.83, 66.95, 109.99, 114.91, 116.86,117.44, 120.64, 122.90, 127.11, 130.76, 131.22, 158.16, 173.88; HRMS-ES (m/z): [M+Na] calcd for $C_{18}H_{21}NO_3Na^+$ 322.1419 found 322.1428.

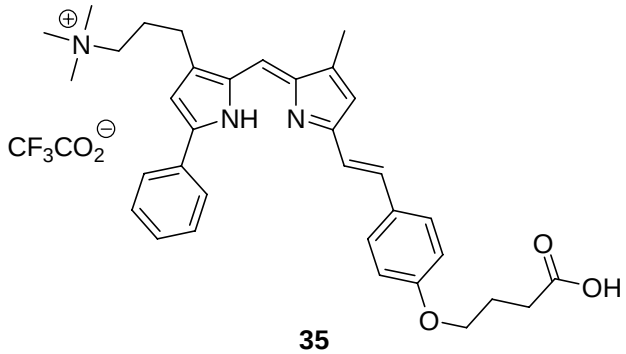


19

4-(4-((E)-2-(4-methyl-1H-pyrrol-2-yl)vinyl)phenoxy)butanoic acid (19).

A solution of aqueous LiOH (23 mg, 0.960 mmol in 1.6 mL H_2O) was added to a flask containing a solution of butanoate **34** (57 mg, 0.190 mmol) in THF under normal atmosphere at ambient temperature. The mixture was stirred at ambient temperature until the completion of the reaction was indicated by TLC (3 h). The mixture was acidified with 1 M HCl and the product was isolated by extraction with EtOAc. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **19** as a purple solid (54

mg, 0.189 mmol, 99%): mp 145 ^{o}C (decomposed); R_f: 0.33 (19:1 dichloromethane-methanol); ^{1}H NMR (500 MHz, CD_3OD) δ 1.99 – 2.06 (m, 5H), 2.46 (t, J = 7.5 Hz, 2H), 3.99 (t, J = 7.5 Hz, 2H), 6.00 (s, 1H), 6.46 (s, 1H), 6.63 (d, J = 16.5 Hz, 1H), 6.75 (d, J = 16.5 Hz, 1H), 6.83 (d, J = 9 Hz, 2H), 7.31 (d, J = 9 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 12.09, 26.08, 31.63, 68.23, 110.50, 115.90, 118.07, 119.01, 120.52, 123.19, 127.88, 132.52, 132.69, 159.40, 177.26; HRMS-ES (m/z): [M+H] calcd for $C_{17}H_{20}NO_3^+$ 286.1438 found 286.1409.

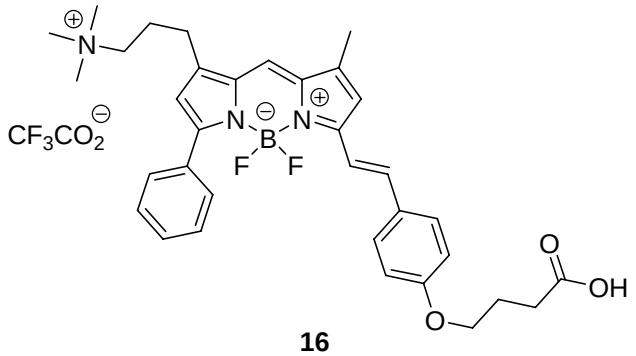


35

5-phenyl-3-(trimethylammonium trifluoroacetate)-propyl-3'-methyl-5'-(4-(4-(E)-vinylphenoxy)butanoic acid)dipyrromethene (35).

Para-toluenesulfonic acid monohydrate (67 mg, 0.352 mmol) was added to a stirred suspension of ammonium iodide **18** (140 mg, 0.352 mmol) and pyrrole **19** (100 mg, 0.350 mmol) in ethanol (10 mL) under normal atmosphere at ambient temperature. The mixture was stirred at ambient temperature for 30 min before removal of the solvent *in vacuo*. The crude product was purified *via* reverse phase HPLC using a gradient of 35:65 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 55:45 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20

mL/min, monitoring at 420 nm. The product was collected at 16 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **35** as a dark purple solid (152 mg, 0.233 mmol, 66%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.10 (p, J = 6.5 Hz, 2H), 2.29 (m, 2H), 2.50 – 2.53 (m, 5H), 2.98 (t, J = 7.5 Hz, 2H), 3.19 (s, 9H), 3.51 (m, 2H), 4.12 (t, J = 6.5 Hz, 2H), 7.04 (d, J = 9 Hz, 2H), 7.13 (s, 1H), 7.16 (s, 1H), 7.18 (d, J = 16 Hz, 1H), 7.47 (s, 1H), 7.52 – 7.60 (m, 3H), 7.69 (d, J = 9 Hz, 2H), 7.79 (d, J = 16 Hz, 1H), 8.03 (d, J = 7 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 12.39, 23.99, 24.95, 25.90, 31.46, 53.90, 67.39, 68.53, 114.38, 115.17, 116.46, 117.93, 119.79, 128.28, 129.84, 130.10, 130.54, 130.74, 131.29, 131.97, 133.29, 143.86, 147.32, 150.13, 151.98, 157.98, 163.04, 177.02; HRMS [M] calcd for $C_{34}H_{40}N_3O_3^+$ 538.3064 found 538.3071.

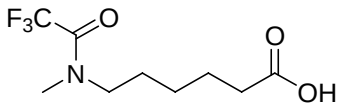


16

4-((4,4-difluoro-1-methyl-5-phenyl-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-yl)styryloxy)butanoic acid (16).

Freshly distilled *N*,*N*-diisopropylethylamine (0.87 mL, 4.99 mmol) was added to a solution of dipyrromethene **35** (82 mg, 0.126 mmol) in anhydrous acetonitrile (20.7 mL) under argon at ambient temperature, and the resulting solution was stirred at ambient

temperature for 5 min. The mixture was cooled to 0 ºC and BF_3·THF (0.11 mL, 0.99 mmol) was added. The mixture was stirred at 0 ºC for 30 min before the solvent was removed *in vacuo* at 0 ºC. The crude product was purified *via* reverse phase HPLC using a gradient of 35:65 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 55:45 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 42 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **16** as a purple solid (30 mg, 43 μmol, 34%) (56% brsm): ^{1}H NMR (500 MHz, CD_3CN) δ 2.02 (p, J = 7 Hz, 2H), 2.09 – 2.15 (m, 2H), 2.38 (s, 3H), 2.46 (t, J = 7 Hz, 2H), 3.03 (s, 9H), 3.29 – 3.33 (m, 2H), 4.05 (t, J = 7 Hz, 2H), 6.61 (s, 1H), 6.91 (s, 1H), 6.96 (d, J = 9 Hz, 2H), 7.36 (d, J = 16.5 Hz, 1H), 7.46 – 7.54 (m, 7H), 7.91 (dd, J = 9, 1.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3CN) δ 12.05, 23.35, 25.17, 25.84, 31.42, 54.44, 67.41, 68.58, 116.54, 117.20,118.57, 119.53, 121.45, 129.69, 130.24, 130.51, 130.54, 130.70, 134.61, 135.28, 137.84, 140.64, 142.72, 145.28, 155.38, 158.75, 162.00, 175.55; HRMS [M] calcd for $C_{34}H_{39}NBF_2N_3O_3^+$ 586.3053 found 586.3058.

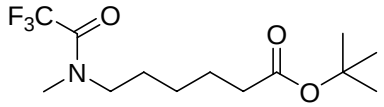


37

6-(2,2,2-Trifluoro-*N*-methylacetamido)hexanoic acid (37).

Distilled Et_3N (2.28 mL, 16.5 mmol) was added to a stirred solution of 5-(methylamino)pentanoic acid dehydrate (1.00 g, 5.49 mmol) in anhydrous methanol (5 mL) cooled to 0 ^{o}C under argon. After stirring the resulting mixture at 0 ^{o}C for 5 min,

MeO_2CCF_3 (659 µL, 6.59 mmol) was added dropwise. The resulting mixture was stirred at ambient temperature for 3 d. The solvent was removed *in vacuo*. The resulting solid was dissolved in H_2O, and the aqueous solution was acidified to pH 3 with 1 M HCl. The product was isolated by extraction with EtOAc. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **37** as a light brown oil (1.30 g, 5.39 mmol, 98%): FTIR: 1415 (s), 1691 (s), 2870 (s), 2946 (s), 3034 (s), 3105 (br); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.35 (m, 2H), 1.57-1.69 (m, 4H), 2.34-2.38 (m, 2H), 3.00 and 3.10 (2s, total of 3H), 3.38 and 3.42 (2t, J = 7.5 Hz, total of 2H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 8.65, 24.31, 24.37, 25.96, 26.08, 26.24, 28.06, 33.88, 33.91, 34.57, 34.92, 34.95, 46.32, 49.38, 49.53, 49.55, 113.24, 115.53, 115.60, 117.82, 117.88, 120.11, 120.17, 156.65, 156.93, 157.21, 179.56179.75.

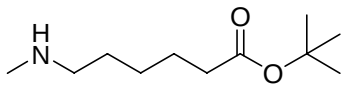


38

tert-Butyl 6-(2,2,2-trifluoro-*N*-methylacetamido)hexanoate (38).

Distilled pyridine (656 µL, 8.12 mmol) was added to a stirred solution of acid **37** (982 mg, 4.06 mmol) in anhydrous DCM (20 mL) under argon at ambient temperature followed by addition of *t*-BuOH (2.33 mL, 48.0 mmol) and $POCl_3$ (752 µL, 8.12 mmol) dropwise. The mixture was stirred at ambient temperature for 21 h. Dichloromethane (20 mL) was added, and the organic layer was washed with H_2O (3x) and brine (2x). The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo*. The crude product was purified by flash chromatography using DAVISIL (dichloromethane) providing **38** as

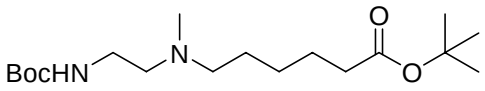
a clear oil (724 mg, 2.44 mmol, 60%): FTIR: 1421 (s), 1459 (s), 1697 (s), 1728 (s), 2939 (s), 2979 (s); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.32 (m, 2H), 1.43 (d, J = 2 Hz, 9H), 1.61 (m, 4H), 2.21 (m, 2H), 3.00 and 3.10 (2s, total of 3H), 3.37 and 3.42 (2t, J = 7.5 Hz, total of 2H); HRMS-ES (m/z): [M+H] calcd for $C_{13}H_{23}F_3NO_3^+$ 298.1625 found 298.1610.



39

tert-Butyl-6-(methylamino)hexanoate (39).

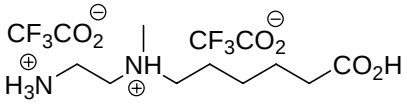
A solution of LiOH (47 mg, 1.96 mmol) in H_2O (2 mL) was slowly added to a stirred solution of hexanoate **38** (530 mg, 1.78 mmol) in MeOH (4 mL) under normal atmosphere cooled to 0 ^{o}C. The mixture was stirred at 0 ^{o}C for 5 min, followed by stirring at ambient temperature for 4 h. The MeOH was removed *in vacuo*, and water (2 mL) was added. The product was isolated by extraction with EtOAc. The organic extract was dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was purified by flash chromatography on Et_3N deactivated DAVISIL (9:1 dichloromethane-methanol) providing **39** as a yellow oil (344 mg, 1.71 mmol, 96%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.35-1.42 (m, 2H), 1.41 (s, 9H), 1.59 (p, J = 7.5 Hz, 2H) 1.85 (p, J = 7.5 Hz, 2H), 2.20 (t, J = 7.5 Hz, 2H) 2.65 (s, 3H), 2.91 (t, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.51, 25.75, 26.25, 28.27, 33.01, 35.28, 49.34, 80.39, 172.81; HRMS-ES (m/z): [M+H] calcd for $C_{11}H_{24}NO_2^+$ 202.1807 found 202.1804.



40

tert-Butyl-2-(*N*-methyl-*N*-(6-(tert-butoxycarbonyl)hexylamino))ethylcarbamate (40).

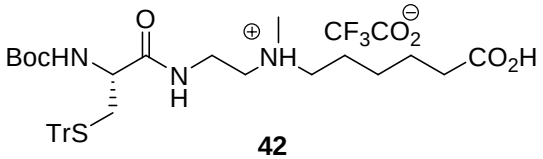
A solution of hexanoate **39** (205 mg, 1.02 mmol), *tert*-butyl 2-bromoethyl-carbamate (202 mg, 0.903 mmol) and Na_2CO_3 (316 mg, 2.97 mmol) were dissolved in a mixture of H_2O (1.8 mL) and 1,4-dioxane (1.8 mL). The mixture was stirred at 80 °C for 3 h. The mixture was allowed to cool to ambient temperature and the solvents were removed *in vacuo*. The resulting solid was partitioned between H_2O and DCM, and the aqueous phase was extracted with DCM (3x). The combined organic extracts were dried (Mg_2SO_4) and the solvent removed *in vacuo* providing the crude product **40** which was used in the next step without further purification (270 mg, 0.785 mmol, 77%).



41

6-(*N*-(2-aminoethyl)-*N*-methylamino)hexanoic acid, bishydrotrifluoroacetate (41).

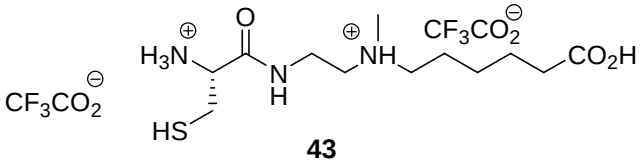
Triethylsilane (1.44 mL, 9.04 mmol) was added to a solution of carbamate **40** (1.44 g, 4.19 mmol) in TFA (10 mL) and DCM (10 mL) under argon at ambient temperature. The mixture was stirred at ambient temperature for 1 h. The solvents were removed *in vacuo* and the residue partitioned between H_2O and Et_2O. The aqueous phase was removed *in vacuo* providing the crude product **41** which was used in the next step without further purification.



42

6-(*N*-methyl-*N*-((*R*)-2-(2-tert-butoxycarbonylamino-3-tritylsulfanyl-propionamido) ethyl)amino)hexanoic acid, hydrotrifluoroacetate (42).

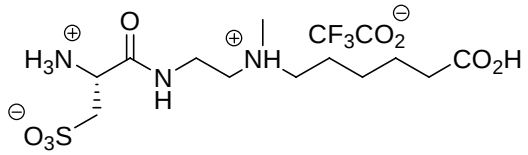
A solution hexanoic acid **41** (1.74 g, 4.19 mmol), Boc-Cys(Trt)-OSu (4.69 g, 8.37 mmol) and Na_2CO_3 (1.77 g, 16.7 mmol) in H_2O (40 mL) and 1,4-dioxane (40 mL) was stirred at ambient temperature under normal atmosphere for 48 h. The solvents were removed *in vacuo* and the resulting residue was partition between H_2O and EtOAc. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo*. The residue was purified by flash chromatography on DAVISIL (9:1 dichloromethane-methanol) providing **42** as a white solid (2g, 3.07 mmol, 73% over 2 steps): ^{1}H NMR (500 MHz, CD_3OD) δ 1.36 (p, J = 7.5 Hz, 2H), 1.44 (s, 9 H), 1.62 (p, J = 7.5 Hz, 2H), 2.21 (t, J = 7.5 Hz, 2H), 2.45-2.57 (m, 2H), 2.64 (s, 3H), 2.87 (t, J = 7.5 Hz, 2H), 2.96 (br, 2H), 3.37-3.51 (m, 2H), 3.92 (t, J = 7.5 Hz, 1H), 7.23 (t, J = 7.5 Hz, 3H), 7.29 (t, J = 7.5 Hz, 6H), 7.37 (d, J = 7.5 Hz, 6H); ^{13}C NMR (125 MHz, CD_3OD) δ 26.06, 26.45, 27.69, 28.86, 35.30, 36.77, 37.35, 41.66, 55.49, 56.87, 58.16, 68.20, 81.16, 128.11, 129.17, 130.87, 146.11, 157.58, 173.85, 180.79; HRMS-ES (m/z): [M+H] calcd for $C_{36}H_{45}N_3O_5S^+$ 634.3314 found 634.3312.



43

6-(*N*-methyl-*N*-((*R*)-2-(2-amino-3-mercapto-propionamido)ethyl)amino)hexanoic acid, bishydrotrifluoroacetate (43).

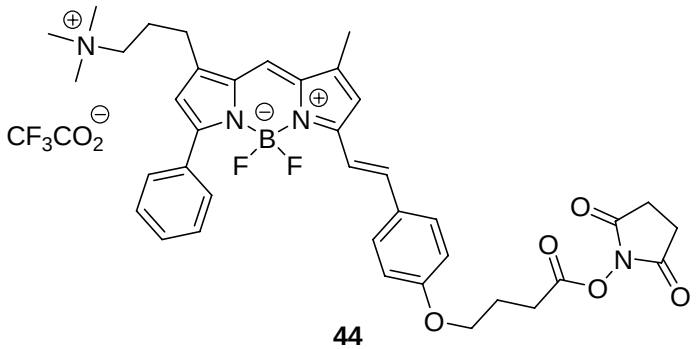
Triethylsilane (0.036 mL, 0.222 mmol) was added to a solution of hexanoic acid **42** (72 mg, 0.111 mmol) in TFA (1 mL) and DCM (1 mL) under argon at ambient temperature. The mixture was stirred at ambient temperature for 1 h. The solvents were removed *in vacuo* and the residue partitioned between H_2O and Et_2O. The aqueous phase was removed *in vacuo* providing **43** as a white solid (57 mg, 0.111 mmol, 100%): ^{1}H NMR (300 MHz, D_2O) δ 1.40 (p, J = 7.5 Hz, 2H), 1.65 (p, J = 7.5 Hz, 2H), 1.75 (m, 2H), 2.41 (t, J = 7.5 Hz, 2H), 2.92 (s, 3H), 2.98-3.50 (m, 4H), 3.08 (t, J = 6 Hz, 2H), 3.59-3.79 (m, 2H), 4.21 (t, J = 6 Hz, 1H); ^{13}C NMR (75 MHz, D_2O) δ 24.73, 25.37, 25.97, 26.92, 34.61, 35.78, 41.08, 56.12, 56.29, 57.59, 169.67, 177.37; HRMS-ES (m/z): [M+H] calcd for $C_{12}H_{25}N_3O_3S$ 292.1695, found 292.1702.



17

6-(*N*-methyl-*N*-((*R*)-2-(2-amino-3-sulfonato-propionamido)ethyl)amino)hexanoic acid, hydrotrifluoroacetate (17).

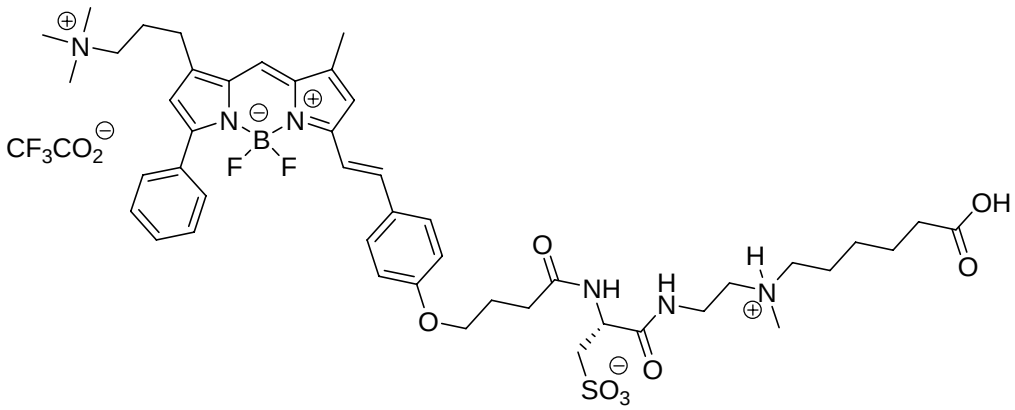
A mixture of 30% hydrogen peroxide (5 mL) and 99% formic acid (50 mL) was allowed to stand at ambient temperature for 1 h prior to use[41]. The performic acid reagent solution (55 mL) was added at 0 °C to a flask containing hexanoic acid **43** (1.5 g, 2.89 mmol). The mixture was stirred at 0 °C for 1 h. The solvent was removed *in vacuo*. The residue was dissolved in water and the solvent removed *in vacuo*. The residue was dried under vacuum providing **17** as a white crystalline solid (1.30 g, 2.89 mmol, 100%): ^{1}H NMR (500 MHz, D_2O) δ 1.41 (p, J = 7.5 Hz, 2H), 1.65 (p, J = 7.5 Hz, 2H), 1.75 (m, 2H), 2.41 (t, J = 7.5 Hz, 2H), 2.91 (d, J = 5 Hz, 3H), 3.07-3.18 (m, 1H), 3.21-3.34 (m, 2H), 3.38-3.62 (m, 2H), 3.49 (t, J = 6 Hz, 2H), 3.78-3.92 (m, 1H), 4.43 (t, J = 6 Hz, 1H); ^{13}C NMR (125 MHz, D_2O) δ 24.11, 24.72, 26.15, 34.48, 35.62, 35.68, 40.96, 41.03, 50.98, 51.14, 55.81, 57.29, 57.63, 169.37, 179.60; HRMS-ES (m/z): [M] calcd for $C_{12}H_{26}N_3O_6S^+$ 340.1542 found 340.1514.



44

4-((4,4-difluoro-1-methyl-5-phenyl-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-3-yl)styryloxy)butanoic acid, succinimidyl ester (44).

A solution of DCC (83.4 mg, 0.404 mmol) in anhydrous DMF was added to a flask containing acid **16** (30.5 mg, 43.6 μmol) and NHS (52.4 mg, 0.455 mmol) under argon at ambient temperature. The mixture was stirred at ambient temperature for 5.5 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 44 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **44** as a dark purple solid (31.4 mg, 39.4 μmol, 90%): ^{1}H NMR (500 MHz, CD_3CN) δ 2.09 (m, 2H), 2.14 (m, 2H), 2.33 (s, 3H), 2.77 (m, 5H), 2.80 (t, J = 7 Hz, 2H), 3.03 (s, 9H), 3.32 (m, 2H), 4.06 (t, J = 6 Hz, 2H), 6.59 (s, 1H), 6.85 (s, 1H), 6.94 (d, J = 8.5 Hz, 2H), 7.34 (d, J = 16 Hz, 1H), 7.44 – 7.50 (m, 7H), 7.92 (d, J = 7.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 11.73, 23.01, 24.87, 25.26, 26.51, 28.37, 54.04, 67.00, 67.44, 116.26, 116.99, 118.21, 118.41, 119.25, 121.30, 129.37, 130.10, 130.21, 130.36, 134.25, 135.00, 137.49, 140.18, 142.60, 145.00, 155.13, 158.27, 161.43, 169.97, 171.19; HRMS [M] calcd for $C_{34}H_{39}BF_2N_3O_3^+$ 683.3217 found 683.3239.

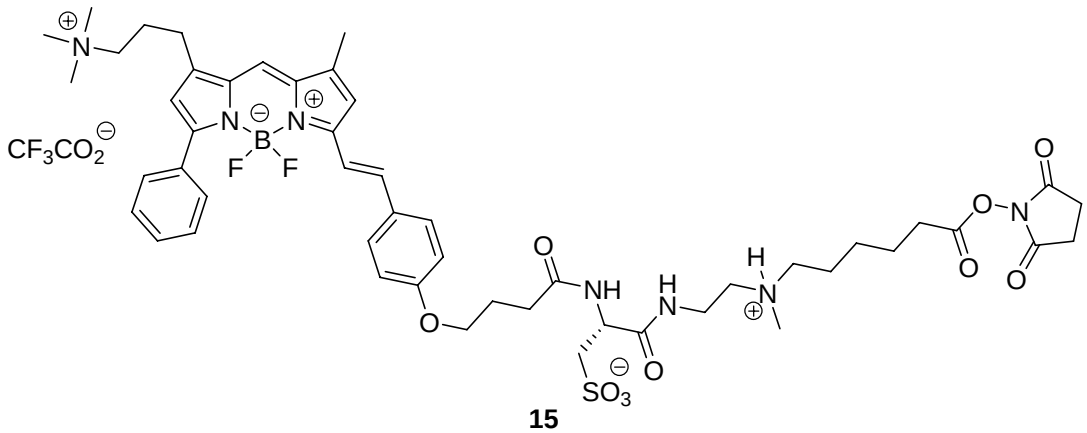


45

6-(((4,4-difluoro-1-methyl-5-phenyl-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-3-yl)styryloxy)*N*-methyl-*N*-(2-((*R*)-2-butanamido-3-sulfono-propionamido)ethyl)amino)hexanoic acid (45).

N-methylmorpholine (86 μL, 0.800 mmol) was added to a mixture of succinimidyl ester **44** (31.4 mg, 39.4 μmol) and hexanoic acid **17** (47.0 mg, 0.117 mmol) in anhydrous DMF under argon at ambient temperature. The mixture was stirred at ambient temperature for 3 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 18 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **45** as a dark purple solid (28.0 mg, 27.4 μmol, 70%): ^{1}H NMR (500 MHz, CD_3OD) δ 1.39 (m, 2H), 1.64 (p, J = 7.5 Hz, 2H), 1.74 (p, J = 7.5 Hz, 2H), 2.07 (p, J = 7 Hz, 2H), 2.18 (m, 2H), 2.32 (t, J = 7 Hz, 2H), 2.35 (s, 3H), 2.46, (t, J = 7 Hz, 2H), 2.84 (t, J = 7 Hz, 2H), 2.84 (s, 3H), 2.99 (m, 1H), 3.13 (s, 9H), 3.15-3.27 (m, 4H), 3.42 (m, 2H), 3.58-3.71 (m,

2H), 4.03 (t, J = 6 Hz, 2H), 4.69 (dd, J = 7.5, 4.5 Hz, 1H), 6.62 (s, 1H), 6.91 (d, J = 7 Hz, 2H), 6.94 (s, 1H), 7.38 (d, J = 16 Hz, 1H), 7.41-7.46 (m, 5H), 7.52 (d, J = 9 Hz, 2H), 7,92 (d, J = 7 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 11.59, 23.34, 24.71, 25.24, 25.50, 26.22, 27.08, 33.52, 34.72, 35.48, 40.93, 52.14, 53.18, 53.90, 57.24, 57.74, 67.51, 68.51, 116.35, 117.41, 118.43, 119.08, 120.47, 129.41, 130.22, 130.41, 130.50, 130.64, 134.63, 135.29, 138.02, 140.58, 142.30, 145.11, 155.69, 159.02, 162.02, 173.76, 175.42, 177.62; HRMS [M] calcd for $C_{46}H_{62}BF_2N_6O_8S^+$ 907.4413 found 907.4375.

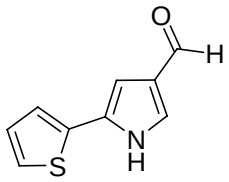


15

6-(((4,4-difluoro-1-methyl-5-phenyl-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-yl)styryloxy)*N*-methyl-*N*-(2-((*R*)-2-butan-amido-3-sulfono-propionamido)ethyl)amino)hexanoic acid, succinimidyl ester, hydrotrifluoroacetate (15).

A solution of DCC (4.7 mg, 23μmol) in anhydrous DMF (500 μL) was added to a flask containing acid **45** (2.7 mg, 2.6 μmol) and NHS (3 mg, 26 μmol) under argon at ambient temperature. The mixture was stirred at ambient temperature for 3 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via*

reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 20.5 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **15** as a dark purple solid (2.2 mg, 2.0 μmol, 77%): ^{1}H NMR (500 MHz, CD_3CN) δ 1.43 (m, 2H), 1.72 (m, 4H), 2.04 (m, 2H), 2.11 (m, 2H), 2.35 (s, 3H), 2.42 (m, 2H), 2.64 (t, J = 7.5 Hz, 2H), 2.74-2.80 (m, 9H), 2.99 (m, 4H), 3.03 (s, 9H), 3.27-3.34 (m, 4H), 3.64-3.80 (m, 2H), 4.03 (m, 2H), 4.62 (m, 1H), 6.60 (s, 1H), 6.86 (s, 1H), 6.93 (dd, J = 9, 2 Hz, 2H), 7.33 (d, J = 16.5 Hz, 2H), 7.45-7.50 (m, 7H), 7.60-7.67 (m, 1H) (NH), 7.90 (dd, J = 44.5, 7.5 Hz, 1H) (NH), 7.91 (d, J = 16.5 Hz, 2H), 8.95 (m, 1H) (NH); ^{13}C NMR (125 MHz, CD_3CN) δ 23.33, 24.21, 25.19, 26.08, 26.56, 26.84, 31.55, 33.61, 33.69, 34.99, 35.21, 41.49, 41.69, 52.07, 52.96, 53.13, 54.45, 54.48, 56.74, 57.15, 57.55, 67.38, 68.80, 116.65, 117.19, 119.57, 121.57, 126.22, 129.69, 130.23, 130.55, 130.73, 134.57, 140.71, 142.76, 145.37, 162.08, 171.57; HRMS [M] calcd for $C_{50}H_{65}BF_2N_7O_{10}S^+$ 1004.4578 found 1004.4541.

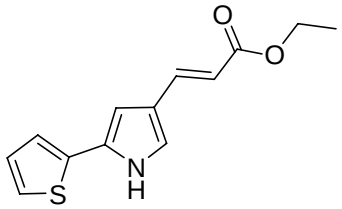


49

5-(2-thienyl)-1H-pyrrole-3-carboxaldehyde (49).

A solution of Na_2CO_3 (3.5 g, 33.0 mmol) in degassed water (22.4 mL) was added to a flask containing a suspension of 5-bromo-1H-pyrrole-3-carboxaldehyde (1.83g, 10.5

mmol) and tetrakis(triphenylphosphine)palladium(0) (570 mg, 0.493 mmol) in degassed DMF (66 mL) under argon at ambient temperature, followed by addition of a solution of 2-thiopheneboronic acid (1.96 g, 15.3 mmol) in degassed DMF (34.7 mL). The mixture was refluxed at 125 °C for 15 h. The flask was cooled down to ambient temperature and poured into DCM (155 mL). The mixture was washed with H_2O (6 x 180 mL) and the organic phase was dried (Na_2SO_4). Removal of the solvent *in vacuo* provided the crude product which was purified by flash chromatography on silica gel (7:3 hexanes-ethyl acetate) providing **49** as a yellow solid (1.13 g, 6.40 mmol, 61%): mp 198-199 ^oC; R_f: 0.20 (7:3 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1637 (s); ^1H NMR (500 MHz, CD_3OD) δ 6.70 (d, J = 1.5 Hz, 1H), 7.03 (dd, J = 5, 3.5 Hz, 1H), 7.23 (dd, J = 3.5, 0.5 Hz, 1H), 7.30 (dd, J = 5, 0.5 Hz, 1H), 7.59 (d, J = 1.5 Hz, 1H), 9.64 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 104.46, 123.79, 125.13, 128.77, 128.84, 131.24, 136.11, 187.96; HRMS-ES (m/z): [M+H] calcd for $C_9H_8NOS^+$ 178.0321 found 178.0354.

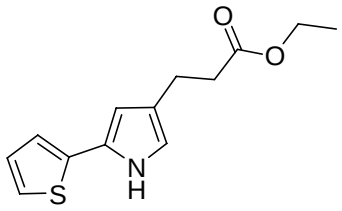


50

(E)-Ethyl 3-(5-(thiophen-2-yl)-1H-pyrrol-3-yl)acrylate (50).

Piperidine (0.13 mL, 1.32 mmol) and mono-ethyl-malonate (5.0 mL, 42.3 mmol) were added to a flask containing a solution of aldehyde **49** (1.13 g, 6.38 mmol) in anhydrous pyridine (6.6 mL) under argon at ambient temperature. The mixture was

stirred at 110 °C for 5.5 h before being cooled and quenched with H_2O (10 mL). The mixture was acidified to pH 3 using 1M HCl, followed by isolation of the product by extraction with EtOAc. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo*. The crude product was purified by flash chromatography on silica gel (85:15 hexanes-ethyl acetate) providing **50** as a white solid (1.30 g, 5.26 mmol, 82%): mp 104-105 ^oC; R_f: 0.32 (4:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1615 (s), 1684 (s), 3261 (s); ^1H NMR (500 MHz, CD_3OD) δ 1.31 (t, J = 7 Hz, 3H), 4.20 (q, J = 7 Hz, 2H), 6.10 (d, J = 16 Hz, 1H), 6.58 (d, J = 1.5 Hz, 1H), 7.02 (dd, J = 9, 5 Hz, 1H), 7.10 (d, J = 1.5 Hz, 1H), 7.18 (dd, J = 3.5, 1.5 Hz, 1H), 7.24 (dd, J = 5, 1 Hz, 1H), 7.61 (d, J = 16 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.82, 61.28, 104.46, 113.30, 122.79, 122.88, 124.21, 124.52, 128.70, 130.43, 137.05, 140.95, 170.23; HRMS-ES (m/z): [M+H] calcd for $C_{13}H_{14}NO_2S^+$ 248.0740 found 248.0781.

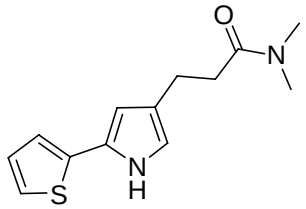


51

Ethyl 3-(5-(thiophen-2-yl)-1H-pyrrol-3-yl)propanoate (51).

A flask containing a suspension of acrylate **50** (915 mg, 3.70 mmol) and 10% Pd/C (1.00g, 0.94 mmol) in ethanol (40 mL) was charged with hydrogen. The suspension was stirred under hydrogen (1 atm) for 2 h. The catalyst was filtered and rinsed with ethanol. The solvent was removed *in vacuo*. The crude product was purified by flash

chromatography on silica gel (85:15 hexanes-ethyl acetate) providing **51** as a white solid (327 mg, 1.31 mmol, 58% brsm): mp 39-40^{o}C; R_f: 0.26 (9:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1714 (s), 3366 (s); ^{1}H NMR (500 MHz, CD_3OD) δ 1.20 (t, J = 7 Hz, 3H), 2.54 (t, J = 7.5 Hz, 2H), 2.74 (t, J = 7.5 Hz, 2H), 4.09 (q, J = 7 Hz, 2H), 6.16 (d, J = 2 Hz, 1H), 6.53 (s, 1H), 6.93 (dd, J = 5, 3.5 Hz, 1H), 7.03 (dd, J = 3.5, 1 Hz, 1H), 7.09 (dd, J = 5, 1 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.65, 23.62, 37.12, 61.54, 107.16, 117.27, 121.19, 122.80, 124.54, 127.95, 128.50, 138.44, 175.52; HRMS-ES (m/z): [M+H] calcd for $C_{13}H_{16}NO_2S^+$ 250.0896 found 250.0918.

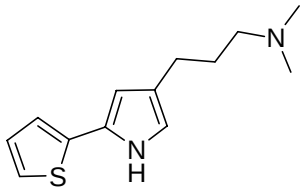


52

***N,N*-Dimethyl-3-(5-(thiophen-2-yl)-1H-pyrrol-3-yl)propanamide (52)**.

A solution of 2.0 $AlMe_3$ in toluene (1.44 mL, 2.86 mmol) was added to a flask containing a solution of dimethylamine hydrochloride (233 mg, 2.86 mmol) in anhydrous benzene (10.6 mL) under argon at ambient temperature. The mixture was stirred at ambient temperature for 1 h before addition of a solution of propanoate **51** (354 mg, 1.42 mmol) in anhydrous benzene (10.6 mL). The mixture was refluxed for 15 h. The mixture was cooled down to ambient temperature and quenched by slow addition of 1 M HCl (10 mL). Water (25 mL) was added, and the product was isolated by extraction with EtOAc. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*

providing **52** as a white solid (337 mg, 1.36 mmol, 96%): mp 107-109 ^{o}C; R_f: 0.42 (ethyl acetate); FTIR (CH_2Cl_2): 1631 (s); ^{1}H NMR (500 MHz, CD_3OD) δ 2.59 (t, J = 8 Hz, 2H), 2.73 (t, J = 8 Hz, 2H), 2.90 (s, 3H), 2.95 (s, 3H), 6.18 (s, 1H), 6.56 (s, 1H), 6.95 (dd, J = 5, 3.5 Hz, 1H), 7.04 (d, J = 3.5 Hz, 1H), 7.12 (d, J = 5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 24.08, 35.92, 36.16, 38.03, 107.29, 117.40, 121.21, 122.82, 124.88, 128.02, 128.55, 138.53, 175.77; HRMS-ES (m/z): [M+H] calcd for $C_{13}H_{17}N_2OS^+$ 249.1056 found 249.1072.

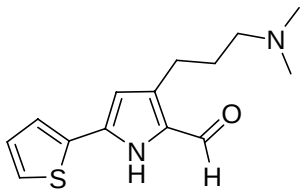


53

***N,N*-Dimethyl-3-(5-(thiophen-2-yl)-1H-pyrrol-3-yl)propan-1-amine (53)**.

A solution of amide **52** (296 mg, 1.19 mmol) in anhydrous THF (43 mL) was added dropwise to a flask containing a suspension of LAH (280 mg, 7.37 mmol) in anhydrous THF (30 mL) under argon cooled to 0 °C. The mixture was stirred at ambient temperature for 3 h before being cooled to 0 °C and quenched with 1.5 M Na_2CO_3 (5 mL). Water (30 mL) was added, and the product was isolated by extraction with EtOAc. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **53** as a light brown residue (278 mg, 1.19 mmol, 100%): R_f: 0.20 (3:2 dichloromethane-methanol); FTIR (CH_2Cl_2): 1265 (s), 2857 (s), 2931 (s), 3053 (s); ^{1}H NMR (500 MHz, CD_3OD) δ 1.74 (p, J = 7.5 Hz, 2H), 2.20 (s, 6H), 2.33 (m, 2H), 2.44 (t, J = 7.5 Hz, 2H), 6.16 (d, J = 0.5 Hz, 1H), 6.53 (s, 1H), 6.94 (dd, J = 5, 3.5 Hz, 1H), 7.04 (d, J = 3.5 Hz,

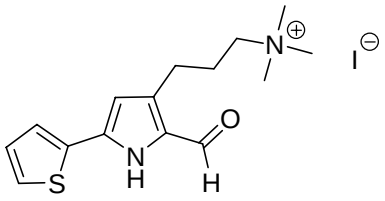
1H), 7.09 (d, J = 5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 25.91, 29.87, 45.52, 60.48, 107.28, 117.24, 121.07, 122.68, 125.59, 127.92, 128.53, 138.64; HRMS-ES (m/z): [M+H] calcd for $C_{13}H_{19}N_2S^+$ 235.1263 found 235.1280.



54

3-(3-(dimethylamino)propyl)-5-(thiophen-2-yl)-1H-pyrrole-2-carboxaldehyde (54).

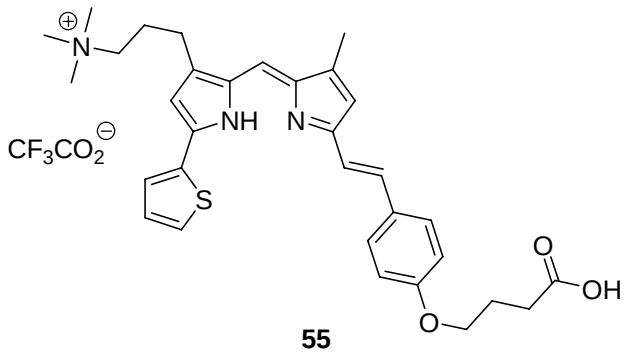
A flask containing a solution of amine **53** (280 mg, 1.19 mmol) in TFA (2.3 mL) under argon was cooled to 0 °C. Trimethyl orthoformate (0.4 mL, 3.65 mmol) was added to the solution, and the mixture was stirred at 0 °C for 20 min before being quenched with cold H_2O (10 mL). The mixture was basified using 5 M NaOH and the product isolated by extraction with EtOAc. The organic layer was dried ($MgSO_4$) and the solvent removed *in vacuo*. The crude product was purified by flash chromatography on silica gel (7:3 dichloromethane-methanol) providing **54** as a red residue (216 mg, 0.824 mmol, 69%): FTIR (CH_2Cl_2): 1468 (s), 1639 (s), 2928 (s); ^{1}H NMR (500 MHz, CD_3OD) δ 1.86 (p, J = 7.5 Hz, 2H), 2.60 (s, 6H), 2.40 (m, 2H), 2.82 (t, J = 7.5 Hz, 2H), 6.42 (s, 1H), 7.09 (dd, J = 5, 3.5 Hz, 1H), 7.43 (dd, J = 5, 1 Hz, 1H), 7.47 (dd, J = 3.5, 1 Hz, 1H), 9.56 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 24.20, 29.14, 30.87, 45.01, 59.77, 110.96, 125.88, 126.97, 129.24, 130.98, 135.35, 136.11 (carbonyl carbon could not be detected); HRMS-ES (m/z): [M+H] calcd for $C_{13}H_{19}N_2S^+$ 263.1213 found 263.1206.



48

Trimethyl-(3-(2-formyl-5-(2-thienyl)-1H-3-pyrrolyl)-propyl)-ammonium iodide (48).

An excess of iodomethane (1 mL) was added to a flask containing a solution of aldehyde **54** (216 mg, 0.823 mmol) in anhydrous DCM (5 mL) under argon at ambient temperature. The mixture was allowed to stir at ambient temperature for 30 min. The solvent was removed *in vacuo* providing **48** as a red solid (332 mg, 0.821 mmol, 100%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.15 – 2.21 (m, 2H), 2.92 (t, J = 7 Hz, 2H), 3.14 (s, 9H), 3.42 (m, 2H), 6.53 (s, 1H), 7.11 (dd, J = 5, 3.5 Hz, 1H), 7.44 (d, J = 5 Hz, 1H), 7.47 (d, J = 3.5 Hz, 1H), 9.62 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 23.57, 25.49, 53.86, 53.89, 53.92, 67.58, 111.16, 125.95, 127.05, 129.26, 131.06, 135.25, 136.06 (carbonyl carbon could not be detected); HRMS-ES (m/z): [M] calcd for $C_{15}H_{21}N_2OS^+$ 277.1369 found 277.1369.

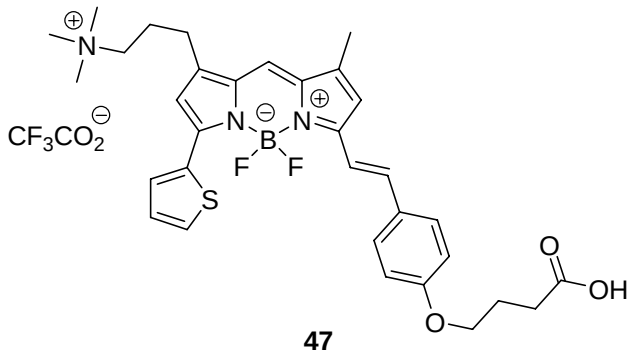


55

5-(2-thienyl)-3-(trimethylammonium trifluoroacetate)-propyl-3'-methyl-5'-(4-(4-(E)-vinylphenoxy)butanoic acid)dipyrromethene (55).

Para-toluenesulfonic acid monohydrate (17.6 mg, 92.5 μmol) was added to a stirred suspension of ammonium iodide **48** (37.5 mg, 92.8 μmol) and acid **18** (26.5 mg, 84.6 μmol) in ethanol (14 mL) under normal atmosphere at ambient temperature. The mixture was stirred at ambient temperature for 30 min before removal of the solvent *in vacuo*. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 18 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **55** as a dark blue solid (28 mg, 42.6 μmol, 50%): ^{1}H NMR (500 MHz, CD_3CN) δ 1.96 – 2.05 (m, 4H), 2.43 (s, 3H), 2.47 (t, J = 7 Hz, 2H), 2.64 (t, J = 7.5 Hz, 2H), 3.04 (s, 9H), 3.26 – 3.30 (m, 2H), 3.95 (t, J = 6.5 Hz, 2H), 6.56 (s, 1H), 6.58 (s, 1H), 6.75 (d, J = 8.5 Hz, 2H), 6.77 (s, 1H), 6.82 (d, J = 16 Hz, 1H), 7.07 (dd, J = 4, 3 Hz, 1H), 7.23 (d, J = 16.5 Hz, 1H), 7.29 (d, J = 8.5 Hz, 2H), 7.55 (d, J = 4 Hz, 1H), 7.79 (d, J = 3 Hz, 1H); ^{13}C NMR (125 MHz, CD_3CN) δ 12.76, 23.66, 24.26, 25.64, 31.35, 54.30, 67.04, 68.37, 114.43,

115.36, 116.23, 117.13, 117.88, 129.37, 129.54, 130.20, 130.36, 130.84, 131.58, 131.95, 133.31, 142.08, 145.67, 147.62, 148.97, 156.29, 162.22 (carbonyl carbon could not be detected); HRMS-ES (m/z): [M] calcd for $C_{32}H_{38}N_3O_3S^+$ 544.2628 found 544.2624.

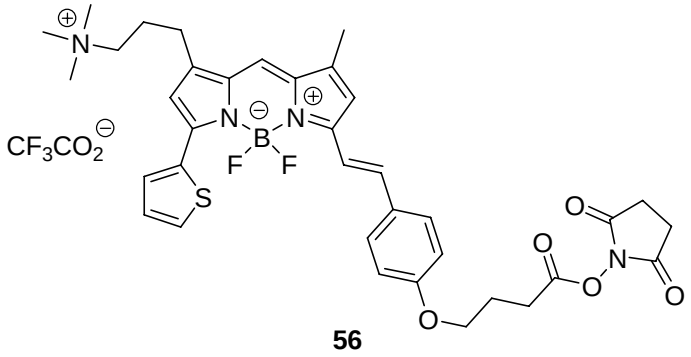


47

4-((4,4-difluoro-1-methyl-5-(2-thienyl)-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-yl)styryloxy)butanoic acid (47).

Freshly distilled *N*,*N*-diisopropylethylamine (645 μL, 3.70 mmol) was added to a stirred solution of dipyrromethene **55** (61 mg, 92.7 μmol) in anhydrous acetonitrile (15 mL) under argon at ambient temperature, and the resulting mixture was stirred at ambient temperature for 5 min. The mixture was cooled to 0 ºC and BF_3·THF (82 μL, 0.743 mmol) was added. The mixture was stirred at 0 ºC for 30 min before the solvent was removed *in vacuo* at 0 ºC. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 35 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **47** as a dark blue solid (20 mg, 28.3 μmol, 31%) (50% brsm): ^{1}H NMR (500

MHz, CD_3CN) δ 2.03 (p, J = 7 Hz, 2H), 2.07 – 2.13 (m, 2H), 2.35 (s, 3H), 2.46 (t, J = 7.5 Hz, 2H), 2.76 (t, J = 7.5 Hz, 2H), 3.02 (s, 9H), 3.27 – 3.31 (m, 2H), 4.06 (t, J = 6.5 Hz, 2H), 6.76 (s, 1H), 6.90 (s, 1H), 6.98 (d, J = 9 Hz, 2H), 7.22 (dd, J = 4.5, 3.5 Hz, 1H), 7.38 (s, 1H), 7.42 (d, J = 16.5 Hz, 1H), 7.51 (d, J = 16 Hz, 1H), 7.56 (d, J = 8.5 Hz, 2H), 7.60 (dd, J = 5, 0.5 Hz, 1H), 8.05 (d, J = 3 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 11.49, 23.26, 25.21, 25.97, 31.52, 53.82, 67.50, 68.41, 116.31, 117.61, 118.31, 118.81, 119.28, 129.36, 129.69, 130.55, 130.66, 131.16, 135.62, 135.97, 138.04, 140.15, 142.34, 144.44, 148.00, 158.58, 162.11, 177.08; HRMS-ES (m/z): [M] calcd for $C_{32}H_{37}BF_2N_3O_3S^+$ 592.2617 found 592.2609.

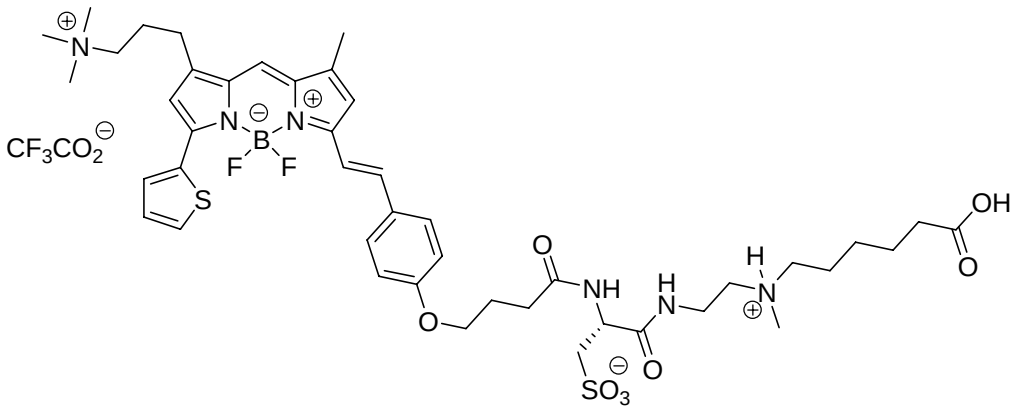


56

4-((4,4-difluoro-1-methyl-5-(2-thienyl)-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-yl)styryloxy)butanoic acid, succinimidyl ester (56).

A solution of DCC (78 mg, 0.378 mmol) in anhydrous DMF was added to a round bottom flask containing acid **47** (29.6 mg, 42.0 μmol) and NHS (48.3 mg, 0.420 mmol) under argon at ambient temperature. The mixture was stirred at ambient temperature for 5.5 h before the solvent was removed *via* the use of a lyophilizer. The crude product was

purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 41 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **56** as a dark blue solid (33 mg, 41.1 μmol, 98%): ^{1}H NMR (500 MHz, CD_3CN) δ 2.11 (m, 2H), 2.18 (p, J = 7 Hz, 2H), 2.37 (s, 3H), 2.78 (m, 6H), 2.83 (t, J = 7.5 Hz, 2H), 3.03 (s, 9H), 3.28 – 3.32 (m, 2H), 4.13 (t, J = 6.5 Hz, 2H), 6.77 (s, 1H), 6.92 (s, 1H), 7.01 (d, J = 8.5 Hz, 2H), 7.22 (dd, J = 5.5, 3.5 Hz, 1H), 7.40 (s, 1H), 7.44 (d, J = 16.5 Hz, 1H), 7.53 (d, J = 16.5 Hz, 1H), 7.58 (d, J = 8.5 Hz, 2H), 7.60 (d, J = 5.5 Hz, 1H), 8.06 (d, J = 3.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3CN) δ 11.72, 22.89, 24.75, 25.27, 26.52, 28.38, 54.06, 66.97, 67.48, 116.30, 117.08, 118.16, 119.02, 120.09, 129.82, 129.93, 130.21, 130.34, 131.00, 135.33, 135.54, 137.47, 139.86, 142.78, 144.46, 147.39, 157.71, 161.43, 170.01, 171.25; HRMS-ES (m/z): [M] calcd for $C_{36}H_{40}BF_2N_4O_5S^+$ 689.2781 found 689.2756.

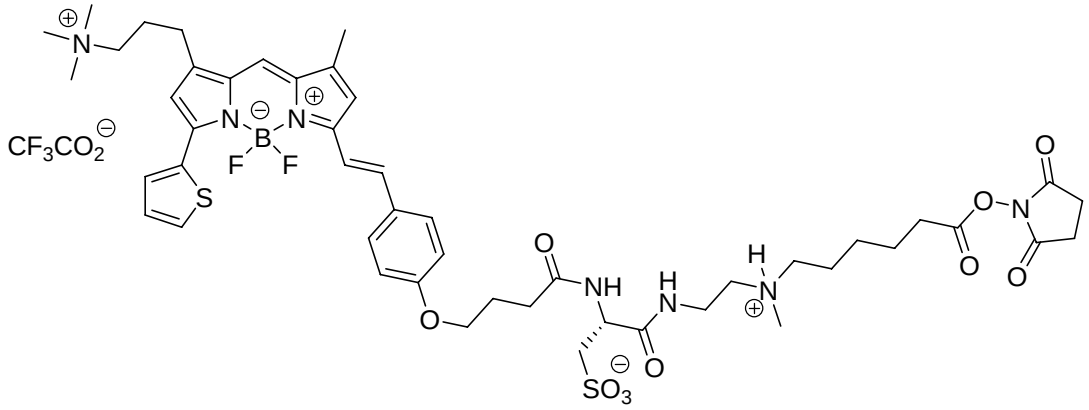


57

6-(((4,4-difluoro-1-methyl-5-(2-thienyl)-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-3-yl)styryloxy)*N*-methyl-*N*-(2-((*R*)-2-butan-amido-3-sulfono-propionamido)ethyl)amino))hexanoic acid (57).

N-methylmorpholine (91 μL, 0.847 mmol) was added to a stirred mixture of succinimidyl ester **56** (33 mg, 41.1 μmol) and hexanoic acid **17** (52 mg, 0.130 mmol) in anhydrous DMF under argon at ambient temperature. The mixture was stirred at ambient temperature for 3 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 1:4 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 33 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **57** as a dark blue solid (25 mg, 24.3 μmol 59%): ^{1}H NMR (500 MHz, CD_3OD) δ 1.38 (m, 2H), 1.64 (p, J = 7.5 Hz, 2H), 1.74 (p, J = 8 Hz, 2H), 2.03 (s, 3H), 2.06 – 2.10 (m, 2H), 2.10 – 2.16 (m, 2H), 2.29 (s, 3H), 2.31 (t, J = 7.5 Hz, 2H), 2.46 (bs, 2H), 2.76 (t, J = 7.5 Hz, 2H), 2.85 (s, 3H), 2.99 (m, 1H), 3.11 (s, 9H), 3.19 – 3.30 (m, 4H), 3.37 (m, 2H), 3.45 (m,

1H), 3.58-3.71 (bs, 2H); 4.02 (t, J = 6 Hz, 2H), 4.69 (dd, J = 7.5, 4.5 Hz, 1H), 6.74 (s, 1H), 6.86 (s, 1H), 6.93 (d, J = 9 Hz, 2H), 7.19 (dd, J = 5.5, 3.5 Hz, 1H), 7.33 (s, 1H), 7.42 (d, J = 16.5 Hz, 1H), 7.46 (d, J = 16.5 Hz, 1H), 7.52 (d, J = 8.5 Hz, 2H), 7.58 (d, J = 5.5 Hz, 1H), 8.08 (d, J = 3.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 11.57, 23.28, 24.74, 25.10, 25.54, 26.28, 27.14, 33.54, 34.68, 35.45, 40.91, 52.19, 53.25, 53.81, 57.29, 57.73, 67.47, 68.52, 89.43, 102.09, 116.38, 117.57, 118.37, 118.81, 119.31, 129.51, 129.79, 130.58, 131.20, 135.63, 136.00, 138.00, 140.12, 142.44, 144.44, 147.89, 158.40, 162.02, 173.73, 175.22, 177.28; HRMS-ES (m/z): [M] calcd for $C_{44}H_{60}BF_2N_6O_8S_2^+$ 913.3977 found 913.3991.

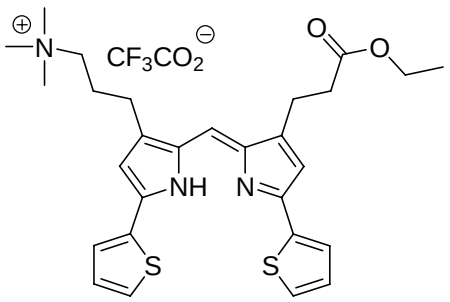


46

6-(((4,4-difluoro-1-methyl-5-(2-thienyl)-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-3-yl)styryloxy)*N*-methyl-*N*-(2-((*R*)-2-butan-amido-3-sulfono-propionamido)ethyl)amino)hexanoic acid, succinimidyl ester (46).

A solution of DCC (45 mg, 0.218 mmol) in anhydrous DMF (4.4 mL) was added to a round bottom flask, equipped with a stir bar, containing acid **57** (25 mg, 24.3 μmol) and NHS (28 mg, 0.243 mmol) under argon. The mixture was stirred at ambient

temperature for 3 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 19 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **46** as a dark blue solid (27 mg, 24 μmol, 99%): ^{1}H NMR (500 MHz, CD_3CN) δ 1.41 (m, 2H), 1.71 (m, 4H), 2.03–2.10 (m, 4H), 2.28 (s, 3H), 2.41 (m, 2H), 2.62 (t, J = 7.5 Hz, 2H), 2.71 –2.80 (m, 9H), 2.92 (m, 1H), 3.02 (s, 9H), 3.10 (m, 2H), 3.26 (m, 2H), 3.31 (m, 2H), 3.45 (m, 1H), 3.62-3.77 (m, 2H), 4.00 (m, 2H), 4.64 (bs, 1H), 6.74 (s, 1H), 6.79 (d, J = 6 Hz, 1H), 6.90 (dd, J = 8, 5 Hz, 2H), 7.20 (t, J = 4.5 Hz, 1H), 7.34 (s, 1H), 7.38 (s, 2H), 7.48 (dd, J = 8, 5 Hz, 2H), 7.58 (d, J = 5 Hz, 1H), 7.77 (d, J = 36 Hz, 1H) (NH), 7.90 (dd, J = 37, 7 Hz, 1H) (NH), 8.06 (d, J = 3.5 Hz, 1H), 8.98 (m, 1H) (NH); ^{13}C NMR (125 MHz, CD3CN) δ 11.76, 22.92, 23.88, 24.75, 25.80, 26.25, 26.52, 31.23, 33.32, 34.84, 35.01, 41.11, 41.30, 51.80, 52.97, 54.06, 56.56, 56.75, 57.19, 66.96, 68.47, 116.25, 116.92, 118.19, 118.94, 119.95, 129.85, 129.95, 130.37, 130.96, 135.27, 135.57, 137.49, 140.02, 142.62, 144.41, 147.22, 157.83, 161.62, 170.14, 171.28, 172.80, 172.97, 173.44; HRMS-ES (m/z): [M] calcd for $C_{48}H_{63}BF_2N_7O_{10}S_2^+$ 1010.4142 found 1010.4198.

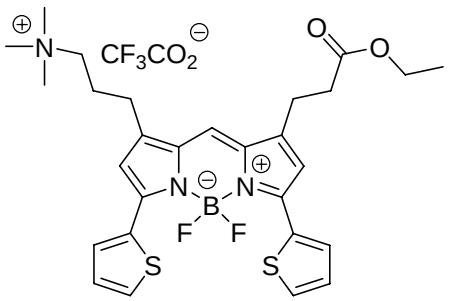


59

5-(2-thienyl)-3-(trimethylammonium trifluoroacetate)-propyl-ethyl-(3'-methyl-5'-(2-thienyl))propanoate dipyrromethene (59).

Para-toluenesulfonic acid monohydrate (27 mg, 0.142 mmol) was added to a stirred suspension of ammonium iodide **48** (56.0 mg, 0.139 mmol) and propanoate **51** (35.0 mg, 0.140 mmol) in ethanol (10 mL) under normal atmosphere at ambient temperature. The mixture was stirred at ambient temperature for 30 min before removal of the solvent *in vacuo*. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 15.5 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **59** as a blue solid (55 mg, mmol, 64%): ^{1}H NMR (500 MHz, CD_3OD) δ 1.20 (t, J = 7 Hz, 3H), 2.30 (m, 2H), 2.81 (t, J = 7 Hz, 2H), 2.99 (t, J = 7 Hz, 2H), 3.18 (t, J = 7 Hz, 2H), 3.19 (s, 9H), 3.52 (m, 2H), 4.09 (q, J = 7 Hz, 2H), 6.98 (s, 1H), 7.07 (s, 1H), 7.26 (d, J = 3.5 Hz, 1H), 7.28 (d, J = 3.5 Hz, 1H), 7.55 (s, 1H), 7.81-7.83 (m, 2H), 7.95 (t, J = 3.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.64, 22.66, 24.10, 24.99, 35.66, 53.87, 53.90, 53.93, 62.03, 67.30, 116.93, 117.00, 120.90, 130.55,

131.21 131.49, 131.62, 131.67, 132.93, 132.99, 133.20, 148.95, 149.16, 150.41, 151.64, 174.47; HRMS [M] calcd for $C_{28}H_{34}N_3O_2S_2^+$ 508.2087 found 508.2009.

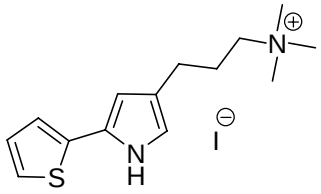


58

4,4-difluoro-3,5-di(2-thienyl)-7-(trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a-diaza-*s*-indacene-1-ethylpropanoate (58).

Freshly distilled *N*,*N*-diisopropylethylamine (600 μL, 3.44 mmol) was added to a solution of dipyrromethene **59** (51.0 mg, 0.0820 mmol) dissolved in anhydrous acetonitrile (4 mL) under argon at ambient temperature, and the solution was stirred at ambient temperature for 5 min. The solution was cooled to 0 ^{o}C and BF_3·THF (74 μL, 0.671 mL) was added. The mixture was stirred at 0 ^{o}C for 30 min before the solvent was removed *in vacuo* at 0 ^{o}C. The crude product was purified *via* reverse phase HPLC using a gradient of 35:65 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 40 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 23 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **58** as a blue solid (23.4 mg, 0.0349 mmol, 42.6%): R_f: 0.37 (90% acetonitrile in water); ^{1}H NMR (500 MHz, CD_3OD) δ 1.20 (t, J = 7 Hz, 3H), 2.07 (m,

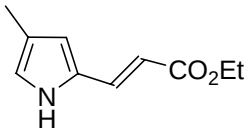
2H), 2.62-2.66 (m, 4H), 2.92 (t, J = 7 Hz, 2H), 3.07 (s, 9H), 3.27 (m, 2H), 4.07 (q, J = 7 Hz, 2H), 6.72 (s, 1H), 6.75 (s, 1H), 7.19 (d, J = 4.5 Hz, 1H), 7.20 (d, J = 4.5 Hz, 1H), 7.40 (s, 1H), 7.65 (d, J = 4.5 Hz, 2H), 8.13 (t, J = 4.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.68, 22.02, 23.30, 24.82, 35.58, 53.77, 53.80, 53.83, 61.91, 67.40, 120.14, 121.02, 129.97, 130.01, 131.01, 131.15, 132.41, 132.47, 132.52, 132.57, 132.63, 135.37, 135.42,136.37, 139.69, 144.91, 146.27, 150.52, 150.85, 174.46; HRMS [M] calcd for $C_{28}H_{33}BF_2N_3O_2S_2^+$ 556.2075 found 556.2118.



65

Trimethyl-(3-(5-(2-thienyl)-1H-3-pyrrolyl)-propyl)-ammonium iodide (65).

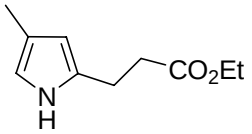
Iodomethane (1 mL) was added to a solution of amine **53** (37.6 mg, 0.160 mmol) in anhydrous DCM (3 mL). Upon addition of the iodomethane, a precipitate fell out of solution. The suspension was stirred at ambient temperature for a further 40 min before the solvent was removed *in vacuo* providing **65** as a light brown solid (54.6 mg, 0.145 mmol, 91%): m.p. 157-158 ^{o}C; ^{1}H NMR (500 MHz, CD_3OD) δ 2.07 (m, 2H), 2.59 (t, J = 7 Hz, 2H), 3.12 (s, 9H), 3.36 (m, 2H), 6.24 (s, 1H), 6.64 (s, 1H), 6.97 (dd, J = 5, 3.5 Hz, 1H), 7.08 (d, J = 3.5 Hz, 1H), 7.15 (d, J = 5 Hz, 1H); HRMS-EI (m/z): [M] calcd for $C_{14}H_{21}N_2S$ 249.1420 found 249.1421.



68

(E)-Ethyl 3-(4-methyl-1H-pyrrol-2-yl)acrylate (68).

A solution of 2-formyl-4-methyl pyrrole (2.6 g, 23.9 mmol) and (carbethoxy-methylene)-triphenylphosphorane (12.4 g, 35.7 mmol) in anhydrous benzene (250 mL) was refluxed for 6 h. The solvent was removed *in vacuo* and the crude product purified by flash chromatography on silica gel (4:1 hexanes-ethyl acetate) providing **68** as a white powder (2.17 g, 22.5 mmol, 94%): mp 65 °C; R_f = 0.44 (4:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1442 (s), 1571 (s), 1614 (s), 1682 (s), 2969 (br), 3330 (br); ^{1}H NMR (300 MHz, CD_3OD) δ 1.27 (t, J = 7.1 Hz, 3H), 2.04 (s, 3H), 4.15 (q, J = 7.1 Hz, 2H), 6.01 (d, J = 15.8 Hz, 1H), 6.31 (s, 1H), 6.68 (s, 1H), 7.43 (d, J = 15.8 Hz, 1H); ^{13}C NMR (75 MHz, CD_3OD) δ 11.87, 14.81, 61.27, 110.32, 116.95, 122.05, 122.82, 129.59, 136.54, 170.19; HRMS-EI (m/z): [M] calcd for $C_{10}H_{13}NO_2$ 179.0946 found 179.0944.

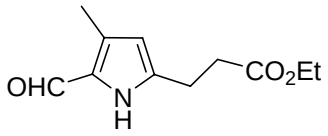


69

Ethyl 3-(4-methyl-1H-pyrrol-2-yl)propanoate (69).

A flask containing a suspension of acrylate **68** (225 mg, 1.26 mmol) and 10% Pd/C (34 mg, 0.0321 mmol) in ethanol (10 mL) was charged with hydrogen. The suspension was stirred under hydrogen (1 atm) for 3 h. The catalyst was filtered and rinsed with ethanol. The solvent was removed *in vacuo* providing **69** as a yellow oil (227

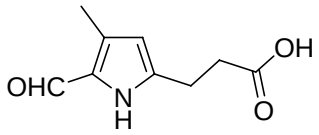
mg, 1.25 mmol, 100%): FTIR (CH_2Cl_2): 1720 (s), 2358 (s), 2926, (br), 3385 (br); R_f = 0.38 (4:1 hexanes-ethyl acetate); ^{1}H NMR (500 MHz, CD_3OD) δ 1.21 (t, J = 7.1 Hz, 3H), 2.00 (s, 3H), 2.55 (t, J = 7.5 Hz, 2H), 2.80 (t, 7.5 Hz, 2H) 4.09 (q, J = 7.1 Hz, 2H), 5.63 (s, 1H), 6.32 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 12.25, 14.62, 24.21, 35.72, 61.61, 107.37, 115.33, 118.93, 131.74, 175.10; HRMS-EI (m/z): [M] calcd for $C_{10}H_{15}NO_2$ 181.1103 found 181.1104.



70

Ethyl 3-(5-forymyl-4-methyl-1H-pyrrol-2-yl)propanoate (70).

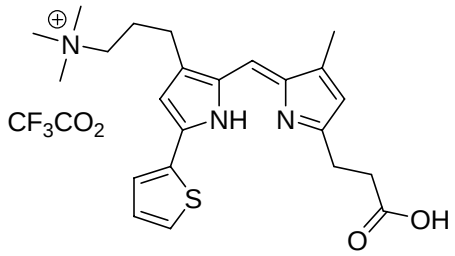
Trimethyl orthoformate (0.350 mL, 3.20 mmol) was added to a flask containing a solution of pyrrole **68** (181 mg, 1.00 mmol) in TFA (3.5 mL) under argon at 0°C. The mixture was stirred at 0°C for 1 h before being quenched with cold H_2O (5 mL). The mixture was basified using 1 M NaOH and the product was isolated by extraction with DCM. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **70** as a yellow solid (163 mg, 0.78 mmol, 78%): mp 55 ^{o}C; R_f 0.33 (7:3 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1630 (s), 1731 (s), 2848 (m), 2925 (m), 2983 (m), 3055 (m), 3255 (br); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.26 (t, J = 7 Hz, 3H), 2.33 (s, 3H), 2.65 (t, J = 7 Hz, 2H), 2.92 (t, J = 7 Hz, 2H), 4.17 (q, J = 7 Hz, 2H), 5.89 (d, J = 2 Hz, 1H), 9.52 (s, 1H), 9.55 (bs, 1H) (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.77, 14.39, 23.04, 33.70, 61.11, 111.39, 129.20, 133.64, 140.23, 173.00, 176.70.



66

3-(5-Formyl-4-methyl-1H-pyrrol-2-yl)propanoic acid (66).

A suspension of propanoate **70** (90.0 mg, 0.430 mmol) in 0.5 M NaOH (5 mL) was stirred at 85°C for 1 h. The mixture was cooled to ambient temperature and acidified to pH 3 using 1M HCl. The product was isolated by extraction with EtOAc. The organic layer was washed with brine (1x) and dried (Na_2SO_4). Removal of the solvent *in vacuo* provided **66** as a brown solid (65.7 mg, 0.429 mmol, 100%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.30 (s, 3H), 2.63 (t, J = 7.5 Hz, 2H), 2.87 (t, J = 7.5 Hz, 2H), 5.93 (s, 1H), 9.40 (s, 1H); 13C NMR (125 MHz, CD3OD) δ 20.88, 24.03, 34.32, 112.27, 130.22, 143.14, 176.30, 180.01; HRMS-EI (m/z): [M+H] calcd for $C_9H_{12}NO_3$ 182.0812 found 182.0815.

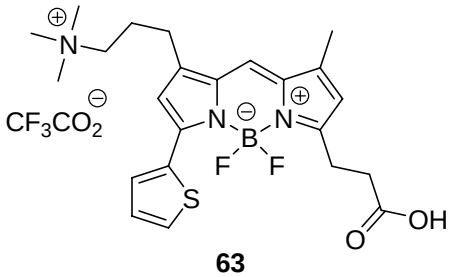


67

5-(2-Thienyl)-3-(trimethylammonium trifluoroacetate)-propyl-3'-methyl-5'-(3-propionic acid)dipyrromethene (67).

Para-toluenesulfonic acid monohydrate (20.0 mg, 0.105 mmol) was added to a stirred suspension of ammonium iodide **65** (40.0 mg, 0.106 mmol) and acid **66** (19.3 mg,

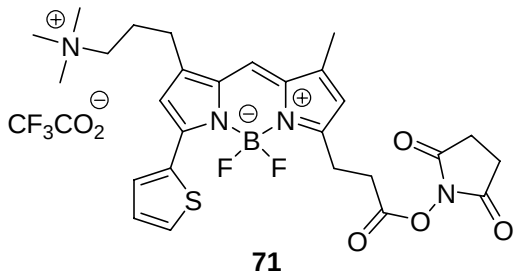
0.106 mmol) in ethanol (5 mL) under normal atmosphere at ambient temperature. The mixture was stirred at ambient temperature for 30 min before removal of the solvent *in vacuo*. The crude product was purified *via* reverse phase HPLC using a gradient of 1:4 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 1:1 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 13 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **67** as a red solid (42.2 mg, 0.0803 mmol, 76%): ^{1}H NMR (500 MHz, CD_3OD) δ 2.26 (m, 2H), 2.46 (s, 3H), 2.82 (t, J = 8 Hz, 2H), 2.95 (t, J = 7.5 Hz, 2H), 3.14 (t, J = 8 Hz, 2H), 3.18 (s, 9H), 3.50 (m, 2H), 6.51 (s, 1H), 7.06 (s, 1H), 7.27 (dd, J = 5, 4 Hz, 1H), 7.50 (s, 1H), 7.80 (d, J = 5 Hz, 1H), 7.94 (d, J = 4 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 12.39, 23.91, 24.78, 25.15, 33.04, 53.84, 53.87, 53.90, 67.26, 116.32, 118.60, 122.38, 130.06, 130.40, 130.75, 131.30, 132.58, 133.07, 148.46, 150.00, 150.42, 160.82, 175.50; HRMS [M] calcd for $C_{23}H_{30}N_3O_2S^+$ 412.2053 found 412.2037.



63

4,4-Difluoro-1-methyl-5-(2-thienyl)-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-3-propionic acid (63).

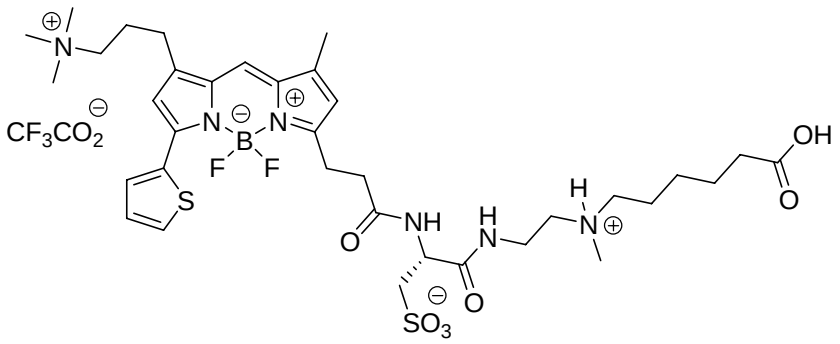
Freshly distilled *N*,*N*-diisopropylethylamine (0.500 mL, 2.87 mmol) was added to a stirred solution of dipyrromethene **67** (42.2 mg, 0.0803 mmol) in anhydrous acetonitrile (4.0 mL) under argon at ambient temperature, and the resulting mixture was stirred at ambient temperature for 5 min. The mixture was cooled to 0 ºC and BF_3·THF (70.0 µL, 0.634 mmol) was added. The mixture was stirred at 0 ºC for 30 min before the solvent was removed *in vacuo* at 0 ºC. The crude product was purified *via* reverse phase HPLC using a gradient of 1:4 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 1:1 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 26 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **63** as a red solid (18.7 mg, 0.0326 mmol, 41%) (49% brsm): ^{1}H NMR (500 MHz, CD_3OD) δ 2.16 (m, 2H), 2.32 (s, 3H), 2.73 (t, J = 7.5 Hz, 2H), 2.79 (t, J = 7.5 Hz, 2H), 3.14 (s, 9H), 3.22 (t, J = 7.5 Hz, 2H), 3.40 (m, 2H), 6.30 (s, 1H), 6.77 (s, 1H), 7.17 (dd, J = 4.5, 3.5 Hz, 1H), 7.50 (s, 1H), 7.60 (d, J = 4.5 Hz, 1H), 8.06 (d, J = 3.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 11.50, 23.18, 24.94, 25.30, 33.65, 53.80, 67.39, 119.29, 119.91, 122.25, 129.78, 130.27, 131.91, 135.48, 135.71, 135.85, 144.65, 144.82, 149.81, 162.36, 176.07; HRMS [M] calcd for $C_{23}H_{29}BF_2N_3O_2S^+$ 460.2040 found 460.2024.



71

4,4-Difluoro-1-methyl-5-(2-thienyl)-7-(3-trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-3-propionic acid, succinimidyl ester (71).

Anhydrous DMF (4 mL) was added to a flask containing acid **63** (18.7 mg, 32.6 μmol), NHS (35.0 mg, 0.304 mmol) and DCC (55.0 mg, 0.266 mmol) under argon at ambient temperature. The mixture was stirred at ambient temperature for 15 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 21 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **71** as a red solid (19.3 mg, 28.8 μmol, 88%): R_f: 0.38 (9:1 acetonitrile-water); ^{1}H NMR (500 MHz, CD_3OD) δ 2.11 (m, 2H), 2.28 (s, 3H), 2.73 (t, J = 7.5 Hz, 2H), 2.83 (s, 4H), 3.08 (t, J = 7.5 Hz, 2H), 3.11 (s, 9H), 3.33-3.37 (m, 4H), 6.34 (s, 1H), 6.76 (s, 1H), 7.17 (dd, J = 5.5, 3.5 Hz, 1H), 7.48 (s, 1H), 7.62 (d, J = 5 Hz, 1H), 8.07 (d, J = 3.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3CN) δ 11.75, 22.88, 24.46, 24.68, 26.53, 30.34, 54.05, 54.08, 54.11, 66.89, 119.72, 119.87, 123.32, 129.91, 131.02, 131.90, 134.90, 135.22, 135.69, 144.54, 145.45, 149.90, 159.71, 169.39, 171.18; HRMS [M] calcd for $C_{27}H_{32}BF_2N_4O_4S^+$ 557.2201 found 557.2202.

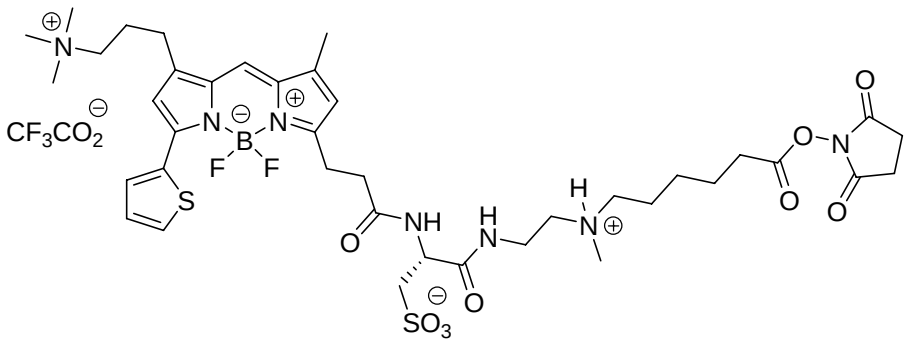


72

6-(4,4-Difluoro-1-methyl-5-(2-thienyl)-7-(trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-3-(*N*-methyl-*N*-(2-((*R*)-2-propionamido-3-sulfono-propionamido)ethyl)amino)hexanoic acid (72).

N-methylmorpholine (50 µL, 0.465 mmol) was added to a solution of succinimidyl ester **71** (17.5 mg, 26.1 µmol) and hexanoic acid **17** (31.0 mg, 77.3 µmol) in anhydrous DMF under argon at ambient temperature. The mixture was stirred at ambient temperature for 4 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 1:4 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 1:1 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]: [99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 12 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **72** as a red solid (9.1 mg, 10.2 µmol, 39%): R_f: 0.28 (1:1 acetonitrile-water, reverse phase); ^{1}H NMR (500 MHz, CD_3OD δ) 1.40 (m, 2H), 1.64 (m, 2H), 1.75 (m, 2H), 2.16 (m, 2H), 2.30 (m, 2H), 2.32 (s, 3H), 2.71 (sept, J = 7.5 Hz, 2H), 2.79 (t, J = 7.5 Hz, 2H), 2.86 (s, 3H), 3.01 (m, 1H), 3.14 (s, 9H), 3.16-3.42 (m, 9H), 3.49-3.56 (m, 1H), 3.64-3.70 (m, 1H),

4.67 (m, 1H), 6.30 (s, 1H), 6.77 (s, 1H), 7.17 (dd, J = 5, 3.5 Hz, 1H), 7.51 (s, 1H), 7.60 (d, J = 5 Hz, 1H), 8.05 (d, J = 3.5 Hz, 1H), 8.18 (m, 1H) (NH), 8.41 (m, 1H) (NH); ^{13}C NMR (125 MHz, CD_3OD) δ 11.58, 23.22, 24.75, 25.12, 25.54, 25.68, 27.15, 34.68, 35.43, 35.61, 40.96, 52.30, 53.06, 53.82, 57.24, 57.68, 67.39, 119.38, 120.20, 122.40, 129.80, 130.26, 131.96, 135.45, 135.74, 135.91, 144.67, 144.93, 149.88, 162.38, 173.69, 174.29, 177.32; HRMS [M] calcd for $C_{35}H_{52}BF_2N_6O_7S_2^+$ 781.3401 found 781.3432.

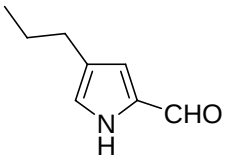


61

6-(4,4-Difluoro-1-methyl-5-(2-thienyl)-7-(trimethylammonium trifluoroacetate)-propyl-4-bora-3a,4a,diaza-s-indacene-3-(*N*-methyl-*N*-(2-((*R*)-2-propionamido-3-sulfono-propionamido)ethyl)amino)hexanoic acid, succinimidyl ester (61).

Anhydrous DMF (4 mL) was added to a flask containing acid **72** (9.1 mg, 10.2 μmol), NHS (11.5 mg, 0.100 mmol) and DCC (17.9 mg, 0.0868 mmol) under argon at ambient temperature. The mixture was stirred at ambient temperature for 15 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA])

over 30 min at a flow rate of 20 mL/min, monitoring at 420 nm. The product was collected at 13 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **61** as a red solid (6.5 mg, 6.55 μmol, 64%): R_f: 0.36 (3:1 acetonitrile-water); ^{1}H NMR (500 MHz, CD_3OD) δ 1.49 (m, 2H), 1.79 (m, 4H), 2.19 (m, 2H), 2.35 (s, 3H), 2.64-2.75 (m, 4H), 2.81-2.85 (m, 5H), 2.87 (s, 4H), 3.02 (m, 1H), 3.15 (m, 9H), 3.19-3.38 (m, 7H), 3.42 (m, 2H), 3.53-3.69 (m, 2H), 4.66 (bs, 1H), 6.31 (s, 1H), 6.80 (s, 1H), 7.17 (dd, J = 5, 4 Hz, 1H), 7.55 (s, 1H), 7.61 (d, J = 5 Hz, 1H), 8.06 (d, J = 4 Hz, 1H), 8.17 (dd, J = 21, 7 Hz, 1H) (NH), 8.42 (bs, 1H) (NH); ^{13}C NMR (125 MHz, CD_3OD) δ 11.60, 23.23, 24.49, 25.14, 25.68, 26.66, 31.40, 35.43, 35.59, 40.96, 41.02, 52.33, 53.06, 53.20, 53.84, 57.33, 57.57, 57.66, 67.39, 119.40, 120.22, 122.43, 129.80, 131.96, 135.45, 135.74, 135.92, 144.68, 144.98, 149.87, 162.43, 170.34, 172.06, 173.69, 174.31; HRMS [M] calcd for $C_{39}H_{55}BF_2N_7O_9S_2^+$ 878.3565 found 878.3566.

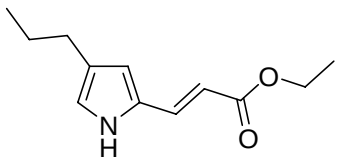


79

4-Propyl-1H-pyrrole-2-carbaldehyde (79).

A 1.7 M solution of *t*-BuLi in pentane (11.7 mL, 19.9 mmol) was added dropwise to a solution of the 3-bromo-6-dimethylamino-1-azafulvene dimer (2.00 g, 4.97 mmol) in anhydrous THF (230 mL) cooled to -78 ^{o}C under argon. The solution was maintained at -78 ^{o}C for 30 m, followed by addition of 1-iodopropane (1.95 mL, 20.0 mmol). The

solution was allowed to warm to -50 ^{o}C over 1 h, followed by further stirring at ambient temperature for 30 min. Saturated $NaHCO_3$ (88 mL) and H_2O (88 mL) were added to the solution, and the mixture was refluxed 15 h. The mixture was allowed to cool to ambient temperature, and the product was isolated by extraction with DCM. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing a brown residue. The crude product was purified by flash chromatography on silica gel (9:1 hexanes-ethyl acetate) providing **79** as a brown oil (902 mg, 6.58 mmol, 66%): R_f 0.35 (4:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 776 (s), 833 (m), 969 (m), 1131 (s), 1151 (m), 1353 (s), 1398 (s), 1446 (s), 1489 (m), 1645 (s), 2871 (m), 2930 (s), 2959 (s), 3272 (br); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.96 (t, J = 7.5 Hz, 3H), 1.61 (sext, J = 7.5 Hz, 2H), 2.47 (t, J = 7.5 Hz, 2H), 6.83 (s, 1H), 6.97 (s, 1H), 9.43 (s, 1H), 10.12 (bs, 1H) (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.92, 24.16, 28.76, 121.85, 125.89, 127.64, 132.64, 179.35; HRMS-EI (m/z): [M] calcd for $C_8H_{12}NO$ 138.0914 found 138.0909.

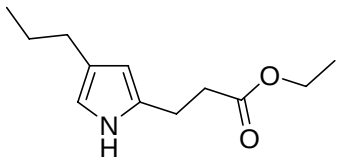


80

(E)-Ethyl 3-(4-propyl-1H-pyrrol-2-yl)acrylate (80).

A solution of aldehyde **79** (654 mg, 4.77 mmol) and (carbethoxymethylene)-triphenylphosphorane (2.49 g, 7.15 mmol) in anhydrous benzene (47 mL) was refluxed 15 h. The solution was allowed to cool to ambient temperature, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography on silica gel

(9:1 hexanes-ethyl acetate) providing **80** as a light brown oil (980 mg, 4.73 mmol, 99%): R_f 0.42 (4:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1569 (s), 1621 (s), 1677 (s), 2869 (m), 2929 (m), 2953 (m), 3302 (br); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.96 (t, J = 7.5 Hz, 3H), 1.33 (t, J = 7.5 Hz, 3H), 1.60 (sext, J = 7.5 Hz, 2H), 2.45 (t, J = 7.5 Hz, 2H), 4.26 (q, J = 7.5 Hz, 2H), 6.07 (d, J = 16 Hz, 1H), 6.43 (s, 1H), 6.72 (s, 1H), 7.56 (d, J = 16 Hz, 1H), 9.16 (bs, 1H) (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.04, 14.48, 24.24, 29.01, 60.40, 110.47, 114.46, 120.59, 127.20, 128.40, 134.92, 168.46; HRMS-EI (m/z): [M+H] calcd for $C_{12}H_{18}NO_2$ 208.1332 found 208.1335.

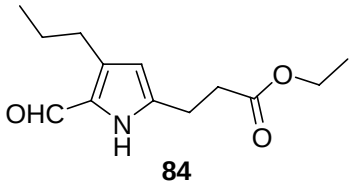


81

Ethyl 3-(4-propyl-1H-pyrrol-2-yl)propanoate (81).

A flask containing a suspension of acrylate **80** (900 mg, 4.34 mmol) and 10% Pd/C (460 mg, 0.432 mmol) in ethanol (12 mL) was charged with hydrogen. The suspension was stirred under hydrogen (1 atm) for 2 h. The catalyst was filtered and rinsed with ethanol. The solvent was removed *in vacuo* providing **81** as a clear oil (754 mg, 3.60 mmol, 83%): R_f 0.27 (9:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1445 (m), 1723 (m), 2871 (s), 2928 (s), 2957 (s), 3388 (br); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.99 (t, J = 7.5 Hz, 3H), 1.30 (t, J = 7 Hz, 3H), 1.61 (sext, J = 7.5 Hz, 2H), 2.44 (t, J = 7.5 Hz, 2H), 2.65 (t, J = 7 Hz, 2H), 2.90 (t, J = 7 Hz, 2H), 4.19 (q, J = 7 Hz, 2H), 5.82 (s, 1H), 6.46 (s, 1H), 8.30 (bs, 1H) (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.28, 14.32, 22.90, 24.40,

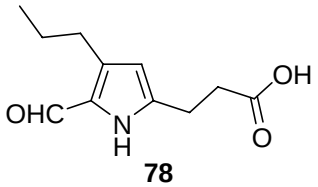
29.41, 34.70, 60.75, 106.12, 113.87, 124.49, 130.99, 174.17; HRMS-EI (m/z): [M+H] calcd for $C_{12}H_{20}NO_2$ 210.1489 found 210.1489.



84

Ethyl 3-(5-formyl-4-propyl-1H-pyrrol-2-yl)propanoate (84).

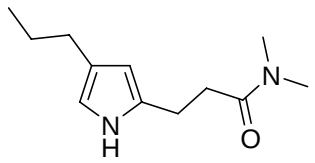
Trimethyl orthoformate (0.35 mL, 3.20 mmol) was added to a solution of pyrrole **81** (209 mg, 1.00 mmol) in TFA (3.5 mL) cooled to 0 ^{o}C under argon. The solution was stirred at 0 ^{o}C for 1 h before H_2O (2 mL) was added. The solution was basified to pH 12 using 1 M NaOH, and the product was isolated by extraction with DCM. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **84** as a yellow oil (213 mg, 0.90 mmol, 90%): R_f 0.30 (3:1 hexanes-ethyl acetate); FTIR (CH_2Cl_2): 1475 (s), 1629 (s), 1735 (s), 2871 (m), 2932 (s), 2960 (s), 3251 (br); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.94 (t, J = 7.5 Hz, 3H), 1.22 (t, J = 7.5 Hz, 3H), 1.62 (sext, J = 7.5 Hz, 2H), 2.66 (dt, J = 7.5, 1.5 Hz, 4H), 2.96 (t, J = 7.5 Hz, 2H), 4.13 (q, J = 7 Hz, 2H), 5.90 (s, 1H), 9.47 (s, 1H), 10.52 (bs, 1H), (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.96, 14.27, 23.10, 24.89, 27.48, 33.64, 60.80, 110.06, 128.02, 139.54, 141.09, 172.83, 176.59; HRMS-EI (m/z): [M+H] calcd for $C_{13}H_{20}NO_3$ 238.1438 found 238.1436.



78

3-(5-Formyl-4-propyl-1H-pyrrol-2-yl)propanoic acid (78).

A suspension of propanoate **84** (125 mg, 0.527 mmol) in 0.5 M NaOH (10 mL) was stirred at 85 ^{o}C for 1 h. The mixture was cooled down to ambient temperature and acidified to pH 3 using 1 M HCl. The product was isolated by extraction with EtOAc. The organic layer was washed with brine (1x) and dried (Na_2SO_4). Removal of the solvent *in vacuo* provided **78** as a brown solid (106 mg, 0.507 mmol, 96%): ^{1}H NMR (500 MHz, CD_3OD) δ 0.95 (t, J = 7.5 Hz, 3H), 1.63 (sext, J = 7.5 Hz, 2H), 2.64 (t, J = 7.5 Hz, 2H), 2.69 (t, J = 7.5 Hz, 2H), 2.89 (t, J = 7.5 Hz, 2H), 5.97 (s, 1H), 9.40 (s, 1H); HRMS-EI (m/z): [M+H] calcd for $C_{11}H_{16}NO_3$ 210.1124 found 210.1123.

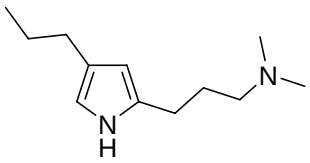


82

***N,N*-Dimethyl-3-(4-propyl-1H-pyrrol-2-yl)propanamide (82)**.

To a flask containing a solution of dimethylamine hydrochloride (163 mg, 2.00 mmol) in anhydrous benzene (7.2 mL) under argon was added a solution of 2.0 M $AlMe_3$ in toluene (1.00 mL, 2.00 mmol). The mixture was stirred at ambient temperature for 1 h before addition of a solution of propanoate **81** (210 mg, 1.00 mmol) in anhydrous benzene (7.2 mL). The mixture was refluxed 15 h. The mixture was cooled to ambient

temperature and quenched with 1 M HCl. Water (20 mL) was added, and the product was isolated by extraction with DCM. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* providing **82** as a white solid (191 mg, 0.918 mmol, 92%): mp 71-72 ^oC; HRMS-EI (m/z): ^1H NMR (500 MHz, $CDCl_3$) δ 1.00 (t, J = 7.5 Hz, 3H), 1.62 (sext, J = 7.5 Hz,2H), 2.45 (t, J = 7.5, 2H), 2.64 (t, J = 6.5 Hz, 2H), 2.93 (t, J = 6.5 Hz, 2H), 2.98 (s, 3H), 3.00 (s, 3H), 5.80 (s, 1H), 6.45 (s, 1H), 9.08 (bs, 1H) (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.12, 22.68, 24.26, 29.26, 34.06, 35.42, 36.94, 105.46, 113.49, 123.72, 131.72, 173.04; [M+H] calcd for $C_{12}H_{21}N_2O$ 209.1648 found 209.1645.

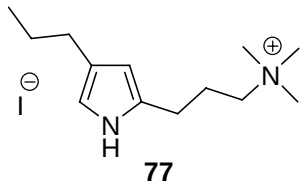


83

***N,N*-Dimethyl-3-(4-propyl-1H-pyrrol-2-yl)propan-1-amine (83)**.

A solution of amide **82** (160 mg, 0.769 mmol) in anhydrous THF (28 mL) was slowly added to a stirred suspension of LAH (150 mg, 3.95 mmol) in anhydrous THF (19.5 mL) cooled to 0 ^oC under argon. The mixture was stirred at ambient temperature for 2 h. The mixture was cooled to 0 ^oC, followed by quenching with 1.5 M Na_2CO_3. Water (30 mL) was added, and the product was isolated by extraction with EtOAc. The organic layer was washed with brine (1x) and dried (Na_2SO_4). Removal of the solvent *in vacuo* provided **83** as a brown oil (146 mg, 0.752 mmol, 98%): FTIR (CH_2Cl_2): 1464 (s), 1687 (s), 2779 (s), 2859 (s), 2928 (s), 2953 (s), 3256 (br); ^1H NMR (500 MHz, $CDCl_3$) δ 0.98 (t, J = 7.5 Hz, 3H), 1.60 (sext, J = 7.5 Hz, 2H), 1.80 (p, J = 7 Hz, 2H), 2.26 (s, 6H),

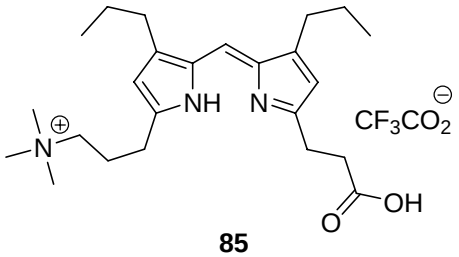
2.35 (t, J = 7 Hz, 2H), 2.44 (t, J = 7.5 Hz, 2H), 2.64 (t, J = 7 Hz, 2H), 5.79 (s, 1H), 6.44 (s, 1H), 8.78 (bs, 1H) (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.37, 24.47, 26.21, 27.39, 29.54, 45.54, 59.66, 105.56, 113.32, 124.60, 132.25; HRMS-EI (m/z): [M] calcd for $C_{12}H_{23}N_2$ 195.1856 found 195.1852.



77

Trimethyl (3-(4-propyl-1H-pyrrol-2-yl)propyl)ammonium iodide (77).

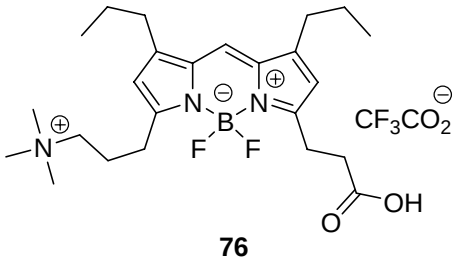
Iodomethane (1 mL) was added to a solution of amine **83** (55.5 mg, 0.286 mmol) in anhydrous DCM (1 mL) under argon. The mixture was stirred at ambient temperature for 1 h before the solvents were removed *in vacuo* providing **77** as a light brown oil (96 mg, 0.285 mmol, 100%): ^{1}H NMR (500 MHz, CD_3OD δ 0.92 (t, J = 7.5 Hz, 3H), 1.54 (sext, J = 7.5 Hz, 2H), 2.09 (m, 2H), 2.36 (t, J = 7.5 Hz, 2H), 2.67 (t, J = 7.5 Hz, 2H), 3.14 (s, 9H), 3.38 (m, 2H), 5.77 (s, 1H), 6.40 (s, 1H), 9.70 (bs, 1H) (NH); ^{13}C NMR (125 MHz, CD_3OD) δ 14.51, 24.65, 25.40, 25.62, 30.52, 54.04, 67.62, 107.16, 115.07, 125.01, 130.64; HRMS-EI (m/z): [M] calcd for $C_{13}H_{25}N_2$ 209.2012 found 209.2015.



85

5-(Trimethylammonium trifluoroacetate)-propyl-3-propyl-3'-propyl-5'-(3-propionic acid)dipyrromethene (85).

Para-toluenesulfonic acid monohydrate (8.8 mg, 0.0463 mmol) was added to a stirred suspension of ammonium iodide **77** (15.5 mg, 0.0461 mmol) and acid **78** (8.4 mg, 0.0463 mmol) in ethanol (3 mL) under normal atmosphere at ambient temperature. The mixture was stirred at ambient temperature for 30 min before removal of the solvent *in vacuo*. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 1:1 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 10 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **85** as an orange solid (19.7 mg, 0.0384 mmol, 83%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.01 (t, J = 7.5 Hz, 3H), 1.02 (t, J = 7.5 Hz, 3H), 1.71 (sext, J = 7.5 Hz, 2H), 1.72 (sext, J = 7.5 Hz, 2H), 2.28 (m, 2H), 2.81 (m, 6H), 2.95 (t, J = 7.5 Hz, 2H), 3.14 (t, J = 7.5 Hz, 2H), 3.17 (s, 9H), 3.44 (m, 2H), 6.53 (s,1H), 6.57 (s, 1H), 7.51 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.21, 14.26, 23.34, 25.10, 25.44, 25.50, 26.25, 29.49, 33.14, 53.87, 67.00, 116.89, 117.31, 123.68, 129.07, 129.42, 154.14, 154.95, 157.60, 160.56, 175.40; HRMS [M] calcd for $C_{24}H_{38}N_3O_2^+$ 400.2958 found 400.2957.

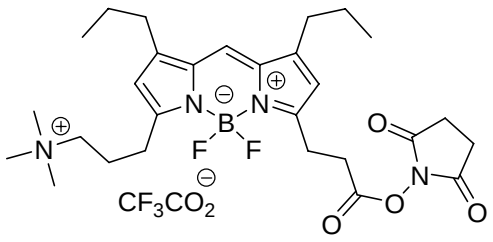


76

4,4-Difluoro-1-propyl-5-(trimethylammonium trifluoroacetate)-propyl-7-propyl-4-bora-3a,4a,diaza-s-indacene -3-propionic acid (76).

Freshly distilled *N*,*N*-diisopropylethylamine (0.100 mL, 0.574 mmol) was added to a solution of dipyrromethene **85** (15.4 mg, 0.0300 mmol) in anhydrous acetonitrile (3.0 mL) under argon at ambient temperature, and the resulting mixture was stirred at ambient temperature for 5 min. The mixture was cooled to 0 ºC and $BF_3{\cdot}Et_2O$ (25.0 μL, 0.199 mmol) was added. The mixture was stirred at 0 ºC for 30 min before the solvent was removed *in vacuo* at 0 ºC. The crude product was purified *via* reverse phase HPLC using a gradient of 35:65 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 55:45 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 18 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **76** as an orange solid (5.5 mg, 0.00979 mmol, 33%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.99 (t, J = 7.5 Hz, 3H), 1.01 (t, J = 7 Hz, 3H), 1.66 (sext, J = 7.5 Hz, 2H), 1.68 (sext, J = 7 Hz, 2H), 2.24 (m, 2H), 2.69 (m, 6H), 3.00 (t, J = 7.5 Hz, 2H), 3.14 (s, 9H), 3.18 (t, J = 7.5 Hz, 2H), 3.41 (m, 2H), 6.29 (s, 1H), 6.34 (s, 1H), 7.50 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.27, 14.33, 23.75, 25.28, 25.42, 25.49, 26.39, 28.89, 28.92,

33.85, 53.72, 67.46, 117.87, 118.27, 123.60, 134.50, 135.00, 149.05, 149.91, 158.89, 161.59, 176.06; HRMS [M] calcd for $C_{24}H_{37}BF_2N_3O_2^+$ 448.2943 found 448.2938.

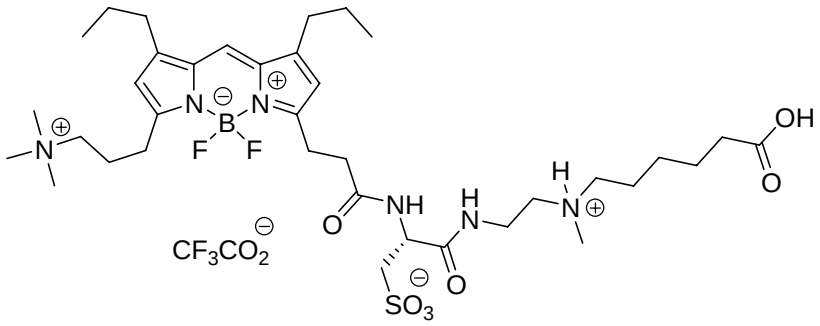


86

4,4-Difluoro-1-propyl-5-(trimethylammonium trifluoroacetate)-propyl-7-propyl-4-bora-3a,4a,diaza-s-indacene -3-propionic acid, succinimidyl ester (86).

Anhydrous DMF (4 mL) was added to a flask containing acid **76** (21.7 mg, 0.0386 mmol), NHS (35.5 mg, 0.308 mmol) and DCC (52.0 mg, 0.252 mmol) under argon at ambient temperature. The mixture was stirred at ambient temperature for 6 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 2:3 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/ 0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 18 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **86** as an orange solid (21.6 mg, 0.0328 mmol, 85%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.97 (t, J = 7.5 Hz, 3H), 0.98 (t, J = 7.5 Hz, 3H), 1.63 (sext, J = 7.5 Hz, 2H), 1.65 (sext, J = 7.5 Hz, 2H), 2.17 (m, 2H), 2.67 (dt, J = 8, 7.5 Hz, 4H), 2.78 (s, 4H), 2.95 (t, J = 7.5 Hz, 2H), 3.03 (s, 9H), 3.06 (t, J = 7.5 Hz, 2H), 3.25 (t, J = 7.5 Hz, 2H), 3.31 (m, 2H), 6.34 (s, 1H), 6.36 (s, 1H), 7.50 (s,1H);

^{13}C NMR (125 MHz, $CDCl_3$) δ 14.54, 23.08, 24.68, 25.36, 25.41, 26.25, 26.81, 28.92, 28.94, 30.69, 67.27, 118.21, 118.29, 124.50, 134.48, 134.66, 149.60, 150.12, 158.79, 160.19, 169.66, 171.42; HRMS [M] calcd for $C_{28}H_{40}BF_2N_4O_4^+$ 545.3110 found 545.3135.

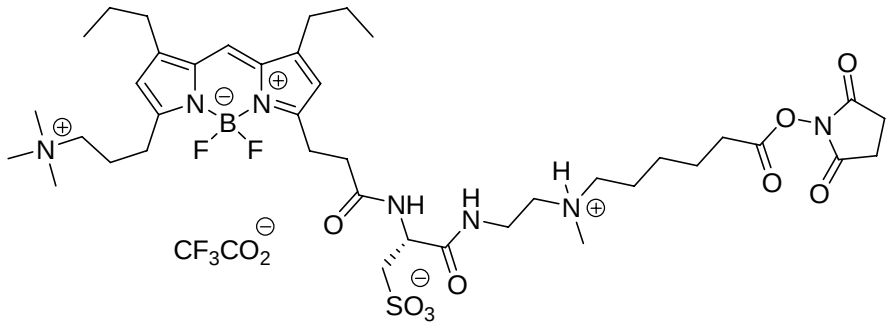


87

6-(4,4-Difluoro-1-propyl-5-(trimethylammonium trifluoroacetate)-propyl-7-propyl-4-bora-3a,4a,diaza-s-indacene-3-(*N*-methyl-*N*-(2-((*R*)-2-propionamido-3-sulfono-propionamido)ethyl)amino)hexanoic acid (87).

N-methylmorpholine (70.0 μL, 0.651 mmol) was added to a stirred mixture of succinimidyl ester **86** (21.6 mg, 0.0328 mmol) and hexanoic acid **17** (41.0 mg, 0.102 mmol) in anhydrous DMF (3 mL) under argon at ambient temperature. The mixture was stirred at ambient temperature for 4 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 14 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **87** as

an orange solid (21.1 mg, 0.0239 mmol, 73%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.99 (t, J = 7.5 Hz, 3H), 1.00 (t, J = 7.5 Hz, 3H), 1.43 (m, 2H), 1.65-1.70 (m, 6H), 1.78 (m, 2H), 2.24 (m, 2H), 2.33 (m, 2H), 2.69 (m, 6H), 2.88 (s, 3H), 3.00 (t, J = 7.5 Hz, 2H), 3.05 (m, 1H), 3.15 (s, 9H), 3.18-3.28 (m, 3H), 3.30-3.55 (m, 4H), 3.43 (m, 2H), 3.60-3.70 (m, 2H), 4.68 (m, 1H), 6.29 (s, 1H), 6.34 (s, 1H), 7.50 (s, 1H), 8.22 (m, 1H) (NH), 8.44 (m, 1H) (NH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 14.33, 23.74, 24.76, 25.42, 25.48, 25.54, 25.72, 26.42, 27.16, 28.91, 34.68, 35.44, 35.82, 40.95, 52.23, 53.16, 53.29, 53.78, 57.29, 57.80, 67.41, 117.93, 118.38, 123.62, 134.55, 134.97, 149.18, 149.90, 159.08, 161.34, 173.69, 174.34, 177.31; HRMS [M] calcd for $C_{36}H_{60}BF_2N_6O_7S^+$ 769.4301 found 769.4243.

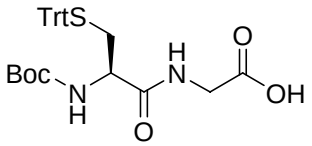


75

6-(4,4-Difluoro-1-propyl-5-(trimethylammonium trifluoroacetate)-propyl-7-propyl-4-bora-3a,4a,diaza-s-indacene-3-(*N*-methyl-*N*-(2-((*R*)-2-propionamido-3-sulfono-propionamido)ethyl)amino)hexanoic acid, succinimidyl ester (75).

Anhydrous DMF (3 mL) was added to a flask containing acid **87** (21.1 mg, 0.0239 mmol), NHS (8.0 mg, 0.0695 mmol) and DCC (11.0 mg, 0.0533 mmol) under argon at ambient temperature. The mixture was stirred at ambient temperature for 7 h

before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 17 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **75** as an orange solid (23.4 mg, 0.0239 mmol, 100%): ^{1}H NMR (500 MHz, CD_3OD) δ 1.00 (t, J = 7.5 Hz, 3H), 1.00 (t, J = 7 Hz, 3H), 1.52 (m, 2H), 1.68 (m, 4H), 1.81 (m, 4H), 2.24 (m, 2H), 2.69 (m, 8H), 2.82 (m, 4H), 2.89 (m, 3H), 3.00 (t, J = 7.5 Hz, 2H), 3.06 (m, 1H), 3.15 (s, 9H), 3.20 (m, 4H), 3.30-3.50 (m, 5H), 3.62-3.75 (m, 2H), 4.68 (m, 1H), 6.30 (s, 1H), 6.35 (s, 1H), 7.50 (s, 1H); ^{13}C NMR (125 MHz, CD_3CN) δ 14.27, 22.97, 23.85, 24.80, 25.07, 25.14, 25.92, 26.22, 26.50, 28.59, 31.20, 34.79, 34.95, 35.22, 41.04, 41.25, 51.77, 51.81, 52.71, 52.85, 53.99, 54.02, 56.54, 56.80, 57.18, 66.90, 117.60, 118.26, 123.68, 133.84, 134.30, 148.84, 149.61, 158.80, 161.49, 170.18, 171.36, 172.55, 172.65, 172.82; HRMS [M] calcd for $C_{40}H_{63}BF_2N_7O_9S^+$ 866.4465 found 866.4456.

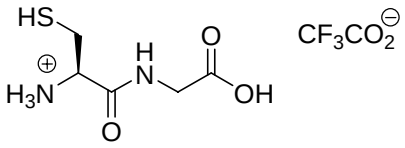


127

2-((*R*)-2-((*N*-Boc)-amino)-(3-tritylthio)-propionamido)-ethanoic acid (127).

A mixture of glycine (268 mg, 3.57 mmol), Boc-Cys(Trt)-OSu (2.0 g, 3.57 mmol) and Na_2CO_3 (757 mg, 7.14 mmol) in H_2O (21 mL) and 1,4-dioxane (21 mL) was stirred

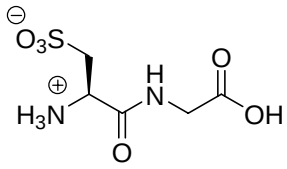
at ambient temperature for 2 days before the solvents were removed *in vacuo*. The residue was dissolved in H_2O (500 mL) and acidified to pH 2 using 1M HCl, at which point a white precipitate fell out of solution. The precipitate was filtered and washed with H_2O, followed by drying under vacuum providing **127** as a white solid (1.46 g, 2.80 mmol, 78%): ^{1}H NMR (500 MHz, *d*-acetone) δ 1.40 (s, 9H), 2.56 (dd, J = 12.5, 8.5 Hz, 1H), 2.65 (dd, J = 12.5, 5 Hz, 1H), 3.90 (m, 2H), 4.09 (m, 1H), 6.13 (d, J = 8 Hz, 1H) (NH), 7.24 (t, J = 7 Hz, 3H), 7.32 (t, J = 7.5 Hz, 6H), 7.41 (d, J = 7.5 Hz, 6H); ^{13}C NMR (125 MHz, *d*-acetone) δ 28.62, 35.06, 41.44, 41.55, 54.59, 67.42, 79.80, 127.70, 128.89, 130.47, 145.71, 145.80, 156.21, 171.07, 171.37,171.44.



128

2-((*R*)-2-amino-(3-thio)-propionamido)-ethanoic acid (128).

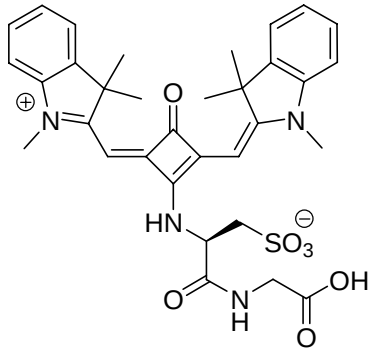
Triethylsilane (123 μL, 0.770 mmol) and TFA (1.1 mL) were added to a solution of acid **127** (200 mg, 0.384 mmol) in anhydrous DCM (1.1 mL) stirred under argon at ambient temperature. The mixture was stirred at ambient temperature for 2.5 h before the solvents were removed *in vacuo*. The white residue was partitioned between Et_2O and H_2O. The aqueous phase was removed *in vacuo* providing **128** as a clear oil which was used directly in the next step: ^{1}H NMR (500 MHz, CD_3OD) δ 3.04 (dd, J = 6, 2.5 Hz, 2H), 4.05 (dd, J = 27.5, 17.5 Hz, 2H), 4.12 (t, J = 5.5 Hz, 1H).



125

2-((*R*)-2-amino-sulfono-propionamido)-ethanoic acid (125).

A solution of 30% H_2O_2 (0.6 mL) and formic acid (5.45 mL) were allowed to stand together at ambient temperature for 1 h[41]. The solution was added to a flask containing acid **128** under normal atmosphere, and the mixture was stirred at ambient temperature for 1.5 h. The solvent was removed *in vacuo*. The resulting white solid was dissolved in water, and the water was removed *in vacuo*. The white crystalline product was dried under vacuum providing **125** as a white solid (83 mg, 0.367 mmol, 96%, 2 steps): ^{1}H NMR (500 MHz, D_2O) 3.38 (dd, J = 15, 9 Hz, 1H), 3.52 (dd, J = 15, 3.5 Hz, 1H), 4.05 (dd, J = 40, 18 Hz, 2H), 4.50 (dd, J = 9, 3.5 Hz, 1H); HRMS-ES (m/z): [M+H] calcd for $C_5H_{12}N_2O_6S^+$ 227.0332 found 227.0350.

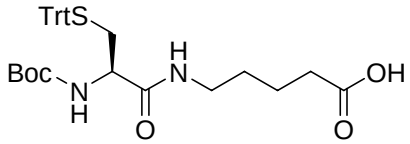


129

2-[2-(2-((*R*)-2-amino-3-sulfono-propionamido)-ethanoic acid)-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium (129).

2-[2-Methoxy-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium trifluoroacetate (92 mg, 0.156 mmol) and acid **125** (39 mg, 0.172 mmol) were placed under argon in a 25 mL rbf and dissolved in EtOH (6.0 mL). Triethylamine (130 μL, 0.934 mmol) was added to the mixture, followed by stirring at reflux for 10 min. The mixture was cooled to ambient temperature and the solvent was removed *in vacuo*. The crude product was purified *via* reverse phase HPLC using a gradient of 2:3 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 500 nm. The product was collected at 18 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **129** as a blue solid (97 mg, 0.153 mmol, 98%): ^{1}H NMR (500 MHz, *d*-DMSO) δ 1.62 (s, 3H), 1.64 (s, 3H), 1.67 (s, 3H), 1.68 (s, 3H), 3.05 (dd, J = 14, 9 Hz, 1H), 3.18 (dd, J = 13.5, 2.5 Hz, 1H), 3.67 (s, 3H), 3.71 (s, 3H), 3.76

(dd, J = 17.5, 5.5 Hz, 1H), 3.88 (dd, J = 17.5, 6 Hz, 1H), 5.04 – 5.07 (m, 1H), 6.06 (s, 3H), 6.10 (s, 3H), 7.20 – 7.30 (m, 2H), 7.41 (s, 4H), 7.55 (dd, J = 12.5, 7 Hz, 2H), 8.88 (t, J = 6, 1H), 9.29 (d, J = 9, 1H); ^{13}C NMR (125 MHz, *d*-DMSO) δ 25.75, 26.19, 31.70, 32.39, 41.08, 49.20, 49.31, 53.20, 55.50, 87.84, 89.16, 111.09, 111.20, 122.13, 124.59, 124.85, 128.10, 141.42, 141.83, 142.36, 142.75, 158.79, 159.38, 167.80, 170.15, 170.86, 172.67, 173.02, 174.00; HRMS-ES (m/z): [M+Na] calcd for $C_{33}H_{36}N_4O_7SNa^+$ 655.2197 found 655.2170.

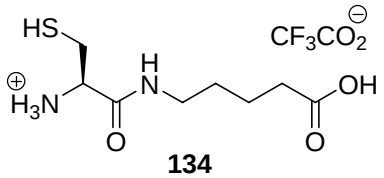


133

5-((*R*)-2-((*N*-Boc)-amino)-(3-tritylthio)-propionamido)-pentanoic acid (133).

A mixture of 5-aminovaleric acid hydrochloride (1.40 g, 9.11 mmol), Boc-Cys(Trt)-OSu (5.0 g, 8.92 mmol) and Na_2CO_3 (1.89 g, 17.8 mmol) in H_2O (52.5 mL) and 1,4-Dioxane (52.5 mL) was stirred at ambient temperature for 2 d before the solvents were removed *in vacuo*. The residue was dissolved in H_2O (1 L) and acidified to pH 2 using 1M HCl, at which point a white precipitate fell out of solution. The precipitate was filtered and washed with H_2O, followed by drying under vacuum providing **133** as a white solid (4.40 g, 7.82 mmol, 88%): FTIR (CH_2Cl_2): 1490 (s), 1527 (s), 1708 (s), 2341 (s), 2359 (s), 2931 (s), 2975 (s) 3304 (br); ^{1}H NMR (500 MHz, *d*-acetone) 1.44 (s, 9H), 1.52-1.57 (m, 2H), 1.61-1.66 (m, 2H), 2.32 (t, J = 7 Hz, 2H), 2.55-2.63 (m, 2H), 3.19-3.26 (m, 2H), 4.10-4.12 (m, 1H), 6.12 (d, J = 8 Hz, 1H), 7.27 (t, J = 8 Hz, 3H), 7.35 (t, J

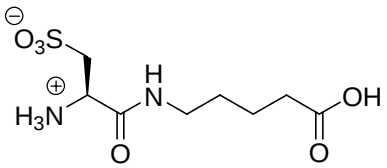
= 7 Hz, 6H), 7.45 (d, J = 7.5 Hz, 6H); ^{13}C NMR (125 MHz, *d*-acetone) 22.85, 28.63, 29.66, 33.86, 35.35, 39.56, 54.66, 67.32, 79.66, 127.63, 128.82, 130.39, 145.72, 156.03, 171.01, 174.84; HRMS-ES (m/z): [M+Na] calcd for $C_{32}H_{38}N_2O_5SNa^+$ 585.2399 found 585.2393.



134

5-((*R*)-2-amino-(3-thio)-propionamido)-pentanoic acid (134).

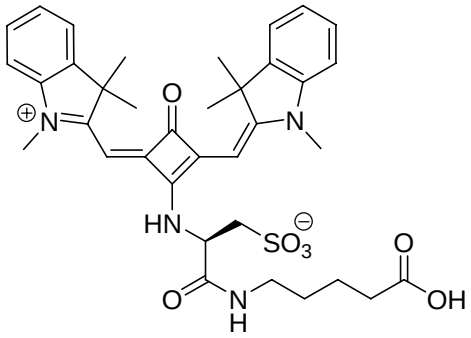
Triethylsilane (4.58 mL, 28.6 mmol) and TFA (40.4 mL) were added to a solution of acid **133** (8.075 g, 14.3 mmol) in anhydrous DCM (40.4 mL) stirred under argon at ambient temperature. The mixture was stirred at ambient temperature for 1.25 h before the solvents were removed *in vacuo*. The white residue was partitioned between Et_2O and H_2O. The aqueous phase was removed *in vacuo* providing **134** as a yellow oil which was used directly in the next step: ^{1}H NMR (500 MHz, D_2O δ) 1.60 (m, 4H), 2.41 (t, 2H, *J* = 7 Hz), 3.05 (m, 2H), 3.21-3.27 (3, 2H), 3.31-3.37 (m, 2H), 4.14 (t, 1H, 6 Hz); HRMS-EI (m/z): [M] calcd for $C_8H_{17}N_2O_3S$ 221.0960 found 221.0952.



135

5-((*R*)-2-amino-sulfono-propionamido)-pentanoic acid (135).

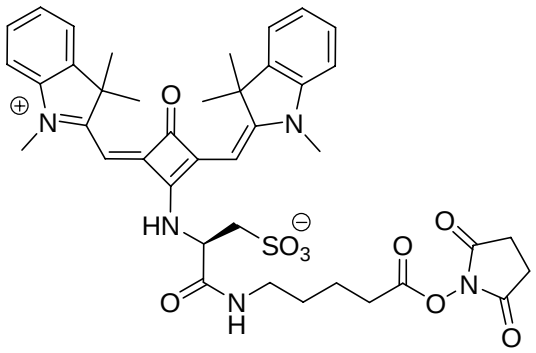
A solution of 30% H_2O_2 (22.6 mL) and formic acid (203.4 mL) were allowed to stand together at ambient temperature for 1 h[41]. The solution was used to dissolve the acid **134** from the previous step, at which point the mixture was stirred at ambient temperature for 1.5 h. The solvent was removed *in vacuo*, and the white solid was dissolved in water. The water was removed *in vacuo*. The white crystalline product was dried under vacuum providing **135** as a white solid (3.384 g, 11.6 mmol, 81%, 2 steps): ^{1}H NMR (500 MHz, *d*-DMSO) δ 1.43 (m, 2H), 1.50 (m, 2H), 2.21 (t, J = 7 Hz, 2H), 2.76 (dd, J = 13.5, 10.5 Hz, 1H), 2.96 (dd, J = 14, 2 Hz), 3.09 (m, 2H), 3.98 (d, J = 8 Hz, 1H), 8.62 (d, J = 4.5 Hz, 1H) (NH); ^{13}C NMR (125 MHz, *d*-DMSO) δ 21.77, 28.12, 33.20, 38.55, 50.24, 50.48, 166.95, 174.30; HRMS-ES (m/z): [M+Na] calcd for $C_8H_{16}N_2O_6SNa^+$ 291.0627 found 291.0609.



136

2-[2-(5-((*R*)-2-amino-3-sulfono-propionamido)-pentanoic acid)-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium (136).

2-[2-Methoxy-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium trifluoroacetate (101 mg, 0.170 mmol) and acid **135** (55 mg, 0.189 mmol) were placed under argon in a 25 mL rbf and dissolved in EtOH (6.5 mL). Triethylamine (140 μL, 1.00 mmol) was added to the mixture, and the mixture was refluxed for 10 min. The mixture was cooled to ambient temperature and the solvent was removed *in vacuo*. The crude product was purified *via* reverse phase HPLC using a gradient of 2:3 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 500 nm. The product was collected at 17 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **136** as a blue solid (97.5 mg, 0.144 mmol, 85%): ^{1}H NMR (500 MHz, *d*-DMSO) δ 1.45-1.47 (m, 4H), 1.61 (s, 6H), 1.66 (s, 3H), 1.68 (s, 3H), 2.19 (t, J = 7 Hz, 2H), 3.02-3.11 (m, 2H), 3.12-3.22 (m, 2H), 3.67 (s, 3H), 3.70 (s, 3H), 4.94-4.98 (m, 1H), 6.03 (s, 1H), 6.11 (s, 1H), 7.20-7.30 (m, 2H), 7.39-7.40 (m, 4H), 7.44 (dd, J = 13, 7.5 Hz, 2H), 8.51 (t, J = 5.5 Hz, 1H) (NH), 9.27 (d, J = 8.5 Hz, 1H) (NH); ^{13}C NMR (125 MHz, *d*-DMSO) δ 21.87, 25.66, 25.77, 28.36, 31.74, 32.48, 33.23, 38.75, 45.76, 49.22, 49.33, 53.37, 55.75, 111.09, 111.22, 122.13, 124.59, 124.90, 128.10, 141.33, 141.85, 142.36, 142.76, 158.12, 158.42, 158.75, 159.57, 167.67, 169.33, 172.76, 173.03, 173.97, 174.25; HRMS-ES (m/z): [M+Na] calcd for $C_{36}H_{42}N_4O_7SNa^+$ 697.2666 found 697.2710.

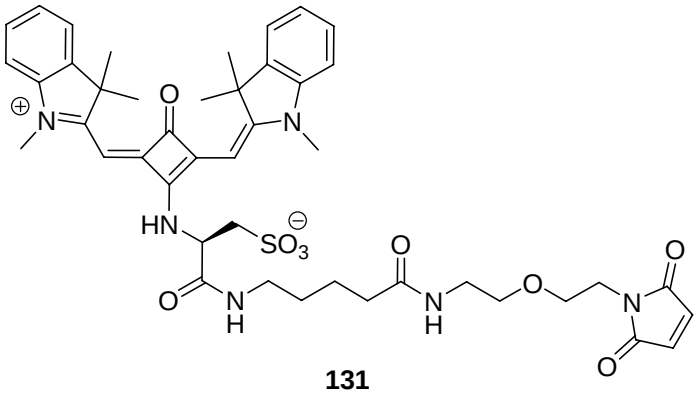


137

2-[2-(5-((*R*)-2-amino-3-sulfono-propionamido)-pentanoic acid)-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium, succinimidyl ester (137).

Acid **136** (63 mg, 93.4 μmol), NHS (54 mg, 0.469 mmol) and DCC (77 mg, 0.373 mmol) were placed under argon in a 10 mL rbf. Anhydrous DMF (1 mL) was added, and the mixture was stirred at ambient temperature for 7 h. The solvent was removed *via* the use of a lyophilizer, and the crude product was purified *via* reverse phase HPLC using a gradient of 2:3 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 1 h at a flow rate of 20 mL/min, monitoring at 500 nm. The product was collected at 23 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **137** as a blue solid (60 mg, 77.7 μmol, 83%): ^{1}H NMR (500 MHz, *d*-DMSO) δ 1.52-1.56 (m, 2H), 1.61-1.68 (m, 14H), 2.68 (t, J = 7 Hz, 2H), 2.76 (s, 4H), 3.03 (dd, J = 13.5, 8.5 Hz, 1H), 3.09 (dd, J = 13, 6 Hz, 1H), 3.16 (dd, J = 14, 3.5 Hz, 1H), 3.22 (dd, J = 13.5, 6.5 Hz, 1H), 3.66 (s, 3H), 3.70 (s, 3H), 4.95-4.99 (m, 1H), 7.25 (d, J = 6.5 Hz, 2H), 7.39-7.40 (m, 4H), 7.54 (dd, J = 12.5, 7.5 Hz, 2H), 8.55 (t, J = 5.5 Hz, 1H) (NH), 9.26 (d,

J = 8.5 Hz, 1H) (NH); ^{13}C NMR (125 MHz, *d*-DMSO) δ 21.63, 25.37, 25.66, 25.77, 26.17, 27.93, 29.76, 31.72, 32.48, 38.44, 49.22, 49.35, 53.36, 55.74, 87.90, 88.87, 111.07, 111.21, 122.12, 124.59, 124.88, 128.10, 141.36, 141.85, 142.37, 142.78, 158.13, 158.43, 158.80, 159.54, 167.67, 168.83, 169.42, 170.13, 172.74, 173.08, 173.99; HRMS-ES (m/z): [M] calcd for $C_{40}H_{46}N_5O_9S$ 772.3011 found 772.3002.

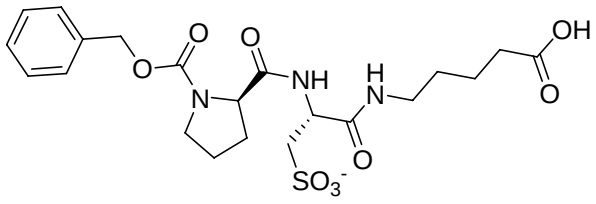


131

2-[2-(5-(*N*-maleimido-3-oxapentyl((*R*)-2-amino-3-sulfono-propionamido)-pentan-amide))-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclo-butenylidenmethyl]-1,3,3-trimethyl-3H-indolium (131).

A solution of *N*-(5-amino-3-oxapentyl)maleimide trifluoroacetate[48a] (17 mg, 0.0570 mmol) and NMM (0.15 mL, 1.4 mmol) in anhydrous DMF (2.5mL) was added to a flask containing succinimidyl ester **137** (39 mg, 0.0505 mmol) under argon at ambient temperature. The mixture was stirred at ambient temperature for 4 h. The solvent was removed *via* the use of a lyophilizer, and the crude product was purified *via* reverse phase HPLC using a gradient of 45:55 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30

min at a flow rate of 20 mL/min, monitoring at 500 nm. The product was collected at 18 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **131** as a blue solid (29 mg, 0.0343 mmol, 68%): ^{1}H NMR (500 MHz, *d*-DMSO) δ 1.37-1.49 (m, 4H), 1.62 (s, 3H), 1.64 (s, 3H), 1.67 (s, 3H), 1.68 (s, 3H), 2.03 (t, J = 7 Hz, 2H), 2.98-3.07 (m, 4H), 3.16 (dd, J = 14.5, 5 Hz, 2H), 3.32 (t, J = 5.5 Hz, 2H), 3.46 (t, J = 5.5 Hz, 2H), 3.53 (t, J = 5.5 Hz, 2H), 3.67 (s, 3H), 3.70 (s, 3H), 4.90-4.98 (m, 1H), 6.05 (s, 1H), 6.09 (s, 1H), 7.01 (s, 2H), 7.22-7.29 (m, 2H), 7.41 (s, 4H), 7.50 (dd, J = 12, 7 Hz, 2H), 7.73 (t, J = 5 Hz, 1H) (NH), 8.50 (t, J = 5.5 Hz, 1H) (NH), 9.25 (d, J = 8.5 Hz, 1H) (NH); `^{13}C NMR (125 MHz, *d*-DMSO) δ 22.60, 25.64, 25.74, 26.19, 28.45, 31.69, 34.78, 36.66, 38.22, 49.19, 49.34, 53.44, 55.65, 66.70, 68.57, 87.83, 88.92, 111.09, 111.19, 122.11, 124.58, 124.85, 128.09, 134.50, 141.33, 141.81, 142.35, 142.75, 158.81, 159.45, 167.60, 169.22, 170.85, 171.89, 172.66, 173.10, 173.98; HRMS-ES (m/z): [M+Na] calcd for $C_{44}H_{52}N_6O_9SNa^+$ 863.3409 found 863.3397.

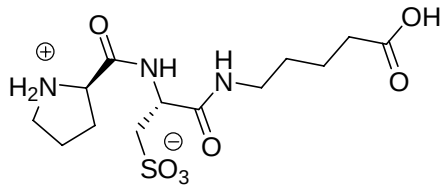


141

5-((*R*)-2-(benzyl (*R*)-2-carbamoylpyrrolidine-1-carboxyloyl)-2-carbamoylethane-sulfonato) pentanoic acid (141).

A mixture of Cbz-(D)-Pro-OSu (165 mg, 0.476 mmol), acid **135** (128 mg, 0.477 mmol) and Et_3N (2.00 mL, 14.4 mmol) in anhydrous DMF (25 mL) was stirred at

ambient temperature for 1 d. The solvent was removed *via* the use of a lyophilizer, and the crude product was purified *via* reverse phase HPLC using a gradient of 1:19 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 1:4 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 220 nm. The product was collected at 20 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **141** as white crystals (166 mg, 0.333 mmol, 70%): ^{13}C NMR (125 MHz, CD_3OD) δ 23.34, 25.80, 26.92, 27.01, 29.63, 31.01, 33.83, 34.46, 40.50, 48.31, 50.38, 52.23, 52.56, 62.50, 68.38, 128.77, 129.16, 129.67, 138.31, 157.01, 172.32, 175.27, 175.77; HRMS-ES (m/z): [M] calcd for $C_{21}H_{28}N_3O_9S^-$ 498.1552 found 498.1540.

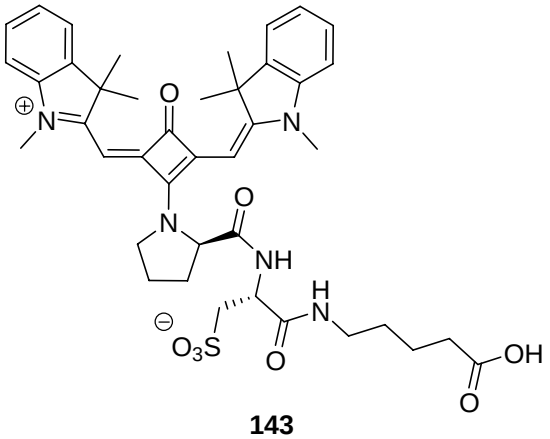


142

5-(2-carbamoyl-(*R*)-2-((*R*)-pyrrolidine-2-carboxamido)ethanesulfonyl)pentanoic acid (142).

A suspension of acid **141** (160 mg, 0.321 mmol) and 10% Pd/C (200 mg, 0.188 mmol) in EtOH (15 mL) was evacuated and charged with hydrogen gas several times before allowing the mixture to stir under hydrogen (1 atm) at ambient temperature for 3 h. The mixture was filtered, and the bluish solid which remained was rinsed with a 1:1 MeOH/H_2O mixture to dissolve the product. Removal of the solvents *in vacuo* provided **142** as a white solid (115 mg, 0.315 mmol, 100%): ^1H NMR (500 MHz, d-DMSO) δ 1.48

(m, 2H), 1.53 (m, 2H), 2.01 (p, J = 7 Hz, 2H), 2.14 (m, 1H), 2.05 (t, J = 7 Hz, 2H), 2.38 (m, 1H), 3.12-3.19 (m, 3H), 3.24-3.28 (m, 2H) 3.30-3.34 (m, 1H), 3.37-3.42 (m, 1H), 4.36 (t, J = 7 Hz, 1H), 4.71 (dd, J = 9.5, 3 Hz, 1H), 8.21 (t, J = 5.5 Hz, 1H) (NH); ^{13}C NMR (125 MHz, d-DMSO) δ 21.83, 23.37, 28.49, 28.96, 33.29, 38.40, 39.85, 51.24, 52.00, 59.22, 59.71, 167.69, 169.83, 174.41; HRMS-ES (m/z): [M+Na] calcd for $C_{13}H_{23}N_3O_7SNa^+$ 388.1149 found 388.1155.

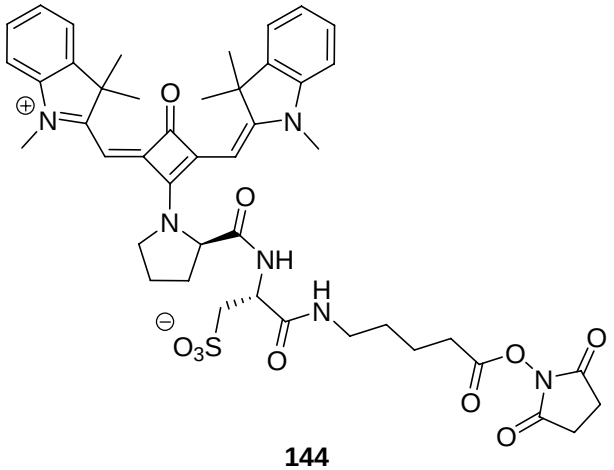


143

2-[2-(5-((*R*)-2-((*R*)-pyrrolidine-2-carboxamido)-3-sulfono-propionamido)-pentanoic acid)-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenyl-idenmethyl]-1,3,3-trimethyl-3H-indolium (143).

2-[2-Methoxy-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium trifluoroacetate (114 mg, 0.194 mmol) and acid **142** (71 mg, 0.194 mmol) were placed under argon in a 25 mL rbf and dissolved in EtOH (10 mL). Triethylamine (0.14 mL, 1.00 mmol) was added, and the mixture was stirred at reflux for 20 min before the solvent was removed *in vacuo*. The crude product was purified *via* reverse phase HPLC using a gradient of 2:3 ([95%

CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 500 nm. The product was collected at 14 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **143** as a blue solid (90 mg, 0.116 mmol, 60%): R_f: 0.30 (3:1 dichloromethane-methanol); ^{1}H NMR (500 MHz, CD_3OD) δ 1.45 (m, 2H), 1.52 (p, J = 7.5 Hz, 2H), 1.65-1.70 (m, 14H), 2.16 (m, 1H), 2.19-2.25 (m, 1H), 2.22 (t, J = 7.5 Hz, 2H), 2.42 (m, 2H), 2.98 (m, 1H), 3.05-3.16 (m, 3H), 3.70 (s, 3H), 3.74 (s, 3H), 4.21 (q, J = 8.5 Hz, 1H), 4.44 (m, 1H), 4.65 (dd, J = 8.5, 5 Hz, 1H), 5.18 (t, J = 5 Hz, 1H), 7.26 (t, J = 7.5 Hz, 2H), 7.31 (m, 2H), 7.41 (t, J = 7.5 Hz, 2H), 7.46 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 23.34, 23.43, 24.80, 26.68, 26.85, 27.07, 29.74, 29.79, 32.46, 33.82, 34.38, 34.55, 40.19, 40.26, 51.25, 51.43, 52.80, 52.86, 53.05, 65.88, 88.57, 112.14, 112.39, 112.68, 123.42, 126.37, 126.58, 129.61, 142.79, 143.32, 144.55, 144.78, 162.18, 162.38, 168.01, 171.86, 173.34, 175.93, 176.43, 177.21, 177.28; HRMS [M+Na] calcd for $C_{41}H_{49}NaN_5O_8S^+$ 794.3194 found 794.3197.

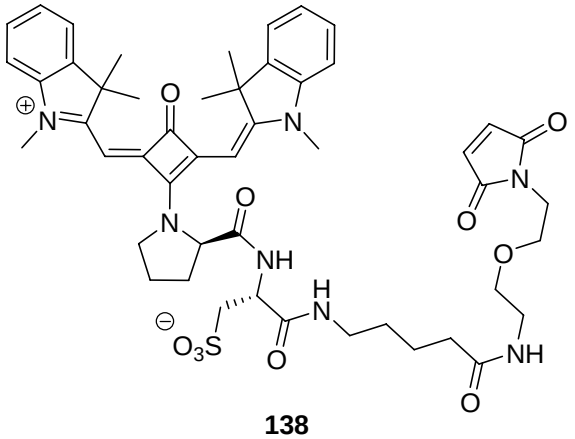


144

2-[2-(5-((*R*)-2-((*R*)-pyrrolidine-2-carboxamido)-3-sulfono-propionamido)-pentanoic acid)-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenyl-idenmethyl]-1,3,3-trimethyl-3H-indolium, succinimidyl ester (144).

Acid **143** (90 mg, 0.116 mmol), NHS (133 mg, 1.16 mmol) and DCC (200 mg, 0.969 mmol) were placed under argon in a 25 mL rbf. Anhydrous DMF (6 mL) was added, and the mixture was stirred at ambient temperature for 15 h. The DMF was removed *via* the use of a lyophilizer, and the crude product was purified *via* reverse phase HPLC using a gradient of 2:3 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 3:2 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 500 nm. The product was collected at 19 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **144** as a blue solid (81 mg, 0.0932 mmol, 80%): R_f: 0.51 (9:1 dichloromethane-methanol); ^{1}H NMR (500 MHz, DMSO) δ 1.35 (p, J = 7.5 Hz, 2H), 1.49 (p, J = 7.5 Hz, 2H), 1.61 (s, 3H), 1.63 (s, 3H), 1.64 (s, 3H), 1.65 (s, 3H), 2.03 (m, 2H), 2.27 (m, 2H), 2.54 (t, J = 7.5 Hz, 2H), 2.71 (dd, J = 13.5, 8.5 Hz, 1H), 2.76-2.84 (m,

2H), 2.79 (s, 4H), 2.95 (m, 1H), 3.67 (s, 3H), 3.68 (s, 3H), 4.19 (q, J = 8.5 Hz, 1H), 4.36 (m, 1H), 4.42 (m, 1H), 5.26 (t, J = 5 Hz, 1H), 5.69 (s, 1H), 5.99 (s, 1H), 7.21-7.27 (m, 2H), 7.40-7.41 (m, 3H), 7.46 (d, J = 8 Hz, 1H), 7.54 (d, J = 7.5 Hz, 2H), 8.02 (t, J = 4.5 Hz, 1H) (NH), 8.52 (d, J = 6 Hz, 1H) (NH); ^{13}C NMR (125 MHz, DMSO) δ 21.44, 23.10, 25.41, 25.49, 25.57, 26.00, 26.07, 27.88, 29.66, 30.77, 32.30, 32.57, 37.80, 49.10, 49.52, 51.10, 51.40, 52.19, 63.72, 87.72, 88.59; HRMS [M+Na] calcd for $C_{45}H_{52}NaN_6O_{10}S^+$ 891.3358 found 891.3346.

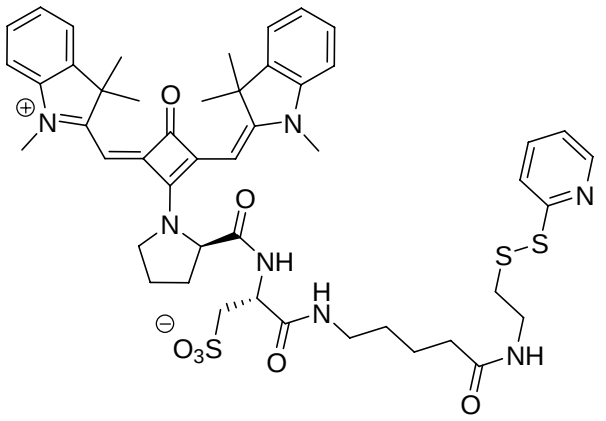


138

2-[2-(5-(*N*-maleimido-3-oxapentyl((*R*)-2-((*R*)-pyrrolidine-2-carboxamido)-3-sulfono-propionamido)-pentanamide))-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolyl-idenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium (138).

Succinimidyl ester **144** (14.0 mg, 0.0161 mmol) and *N*-(5-amino-3-oxapentyl) maleimide trifluoroacetate[48a] (7.0 mg, 0.0235 mmol) were placed under argon in a 10 mL rbf. Anhydrous DMF (2 mL) was added to the flask, followed by distilled NMM (17.0 μL, 0.158 mmol). The mixture was stirred at ambient temperature for 4 h. The solvent was removed *via* the use of a lyophilizer, and the crude product was purified *via* reverse

phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 55:45 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 500 nm. The product was collected at 26 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **138** as a blue solid (10.3 mg, 0.0110 mmol, 68%): R_f: 0.39 (9:1 dichloromethane-methanol); ^{1}H NMR (500 MHz, *d*-DMSO) δ 1.24 (p, J = 7.5 Hz, 2H), 1.35 (p, J = 7.5 Hz, 2H), 1.61 (s, 3H), 1.62 (s, 3H), 1.64 (s, 3H), 1.65 (s, 3H), 1.95 (t, J = 7.5 Hz, 2H), 2.03 (m, 2H), 2.27 (m, 2H), 2.70 (dd, J = 13.5, 8 Hz, 1H), 2.76-2.82 (m, 2H), 2.92 (dt, J = 11.5, 5.5 Hz, 2H), 3.34 (t, J = 6 Hz, 2H), 3.48 (t, J = 6 Hz, 2H), 3.54 (t, J = 5.5 Hz, 2H), 3.67 (s, 6H), 4.19 (q, J = 8 Hz, 1H), 4.36-4.43 (m, 2H), 5.24 (t, J = 5.5 Hz, 2H), 5.68 (s, 1H), 5.98 (s, 1H), 7.01 (s, 2H), 7.22-7.27 (m, 2H), 7.38-7.41 (m, 3H), 7.46 (d, J = 8 Hz, 1H), 7.54 (d, J = 7 Hz, 2H), 7.70 (t, J = 5.5 Hz, 1H) (NH), 7.96 (t, J = 5.5 Hz, 1H) (NH), 8.52 (d, J = 6 Hz, 1H) (NH); ^{13}C NMR (125 MHz, *d*-DMSO) δ 22.54, 23.06, 25.52, 25.97, 26.06, 28.45, 30.74, 32.30, 32.55, 34.82, 36.69, 38.25, 38.30, 49.10, 49.50, 51.03, 51.37, 52.23, 63.72, 66.73, 68.58, 87.70, 88.51, 110.96, 111.48, 122.15, 124.47, 125.01, 128.11, 134.50, 141.18, 141.75, 142.56, 142.79, 159.21, 159.98, 166.05, 169.80, 169.95, 170.87, 171.94, 172.27, 173.86, 174.28; HRMS [M+Na] calcd for $C_{49}H_{59}NaN_7O_{10}S^+$ 960.3936 found 960.3877.

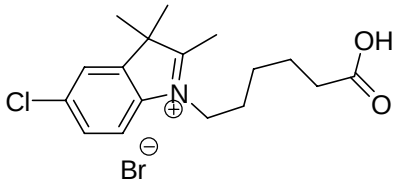


139

2-[2-(5-((2-(2-(pyridin-2-yl)disulfanyl)ethanamine)-((*R*)-2-((*R*)-pyrrolidine-2-carboxamido)-3-sulfono-propionamido)-pentanamide))-4-oxo-3-(1,3,3-trimethyl-2,3-dihydro-1H-2-indolylidenmethyl)-2-cyclobutenylidenmethyl]-1,3,3-trimethyl-3H-indolium (139).

Succinimidyl ester **144** (16.0 mg, 0.0184 mmol) and *S*-(2-pyridylthio)cysteamine hydrochloride[49a] (9.0 mg, 0.0404 mmol) were placed under argon in a 5 mL rbf. Anhydrous DMF (1mL) was added, and the mixture was stirred at ambient temperature for 5 h. The solvent was removed *via* the use of a lyophilizer, and the crude product was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]: [99.9% H_2O/0.1% TFA]) to 65:35 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/ 0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 500 nm. The product was collected at 23 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **139** as a blue solid (8.4 mg, 8.93 μmol, 48.5%): *Rf*: 0.38 (9:1 dichloromethane-methanol); ^{1}H NMR (500 MHz, *d*-DMSO) δ 1.24 (p, J = 7.5 Hz, 2H), 1.36 (p, J = 7.5 Hz, 2H), 1.61 (s, 3H), 1.62 (s, 3H), 1.64 (s, 3H), 1.64 (s, 3H), 1.96 (t, J = 7.5 Hz, 2H), 2.03 (m, 2H), 2.27 (m, 2H), 2.70 (dd, J = 13.5,

8 Hz, 1H), 2.76-2.83 (m, 2H), 2.87 (t, J = 6.5 Hz, 2H), 2.92 (p, J = 6.5 Hz, 1H), 3.29 (q, J = 6.5 Hz, 2H), 3.67 (s, 6H), 4.18 (q, J = 8 Hz, 1H), 4.36 (m, 1H), 4.42 (q, J = 6.5 Hz, 1H), 5.24 (t, J = 5 Hz, 1H), 5.67 (s, 1H), 5.98 (s, 1H), 7.22-7.26 (m, 3H), 7.38-7.40 (m, 3H), 7.45 (d, J = 8 Hz, 1H), 7.54 (d, J = 7 Hz, 2H), 7.76 (d, J = 8 Hz, 1H), 7.82 (t, J = 8 Hz, 1H), 7.97 (m, 2H), 8.44 (d, J = 4.5 Hz, 1H) (NH), 8.52 (d, J = 6 Hz, 1H) (NH); ^{13}C NMR (125 MHz, *d*-DMSO) δ 22.48, 23.07, 25.53, 25.97, 26.06, 28.40, 30.75, 32.31, 32.56, 34.89, 37.45, 37.74, 38.24, 49.11, 49.50, 51.02, 51.37, 52.25, 63.72, 87.72, 88.49, 110.98, 111.47, 119.25, 121.15, 122.15, 124.49, 125.00, 128.12, 137.80, 141.18, 141.74, 142.57, 142.79, 149.52, 159.09, 159.24, 159.97, 166.05, 169.80, 169.97, 172.06, 172.30, 173.85, 174.28; HRMS [M+H] calcd for $C_{48}H_{58}N_7O_7S_3^+$ 940.3554 found 940.3499.

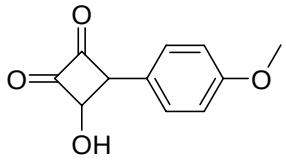


152

1-(6-Hexanoic acid)-5-chloro-2,3,3-trimethylindolium bromide (152).

A mixture of 5-chloro-2,3,3-trimethyl-3H-indole (800 mg, 4.14 mmol) and 6-bromohexanoic acid (915 mg, 4.69 mmol) in anhydrous 1.2-dichlorobenzene (7 mL) was stirred under argon at 120 ^{o}C for 24 h. The mixture was allowed to cool to ambient temperature, and the mixture was poured into ether. The resulting precipitate was collected by filtration, and the precipitate was washed with ether providing **152** as a red solid (. A portion of the collected solid was purified *via* reverse phase HPLC using a gradient of 3:7 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 65:35

([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 280 nm. The product was collected at 8 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **152** as a red solid (1.05 g, 2.70 mmol, 65%): ^{1}H NMR (500 MHz, CD_3OD) δ 1.52 (p, J = 7.5 Hz, 2H), 1.62 (s,6H), 1.70 (p, J = 7.5 Hz, 2H), 1.97 (p, J = 7.5 Hz, 2H), 2.34 (t, J = 7.5 Hz, 2H), 4.50 (t, J = 7.5 Hz, 2H), 7.66 (dd, J = 8.5, 1.5 Hz, 1H), 7.87 (s, 1H), 7.88 (d, J = 8.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 22.75, 25.49, 27.09, 27.13, 28.55, 34.38, 34.51, 56.27, 118.09, 125.41, 130.87, 137.66, 141.28, 145.44, 177.28, 198.61; HRMS-EI (m/z): [M] calcd for $C_{17}H_{23}ClNO_2$ 308.1412 found 308.1409.

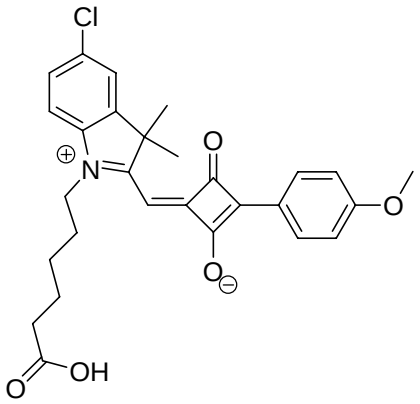


153

3-Hydroxy-4-(4-methoxyphenyl)cyclobutane-1,2-dione (153).

A flask containing *p*-bromoanisole (1.00 mL, 7.99 mmol) in anhydrous THF (40 mL) under argon was cooled to -78 ^{o}C, followed by addition of a 2.0 M solution of *n*-BuLi in pentane (4.00 mL, 8.0 mmol) dropwise. The solution was stirred at -78 ^{o}C for 20 min. The solution was added *via cannula* at -78 ^{o}C to a flask containing a solution of 3,4-dibutoxycyclobut-3-ene-1,2-dione (1.81 g, 8.00 mmol) in anhydrous THF (40 mL) cooled to -78 ^{o}C under argon. After stirring the resulting mixture at -78 ^{o}C for 20 min, TFAA (1.70 mL) and 10% NH_4Cl (8 mL) were added, and the mixture was allowed to come to ambient temperature. The product was isolated by extraction with DCM, and the organic

layer was dried (Na_2SO_4). The solvent was removed *in vacuo*. The crude was dissolved in AcOH (20 mL) and 2M HCl (4 mL) and stirred at 55 ^oC for 4 h. The solvents were removed *in vacuo* providing **153** as a light brown solid (1.41 g, 6.90 mmol, 86%), whose characteristics matched those published in literature accounts[56a].

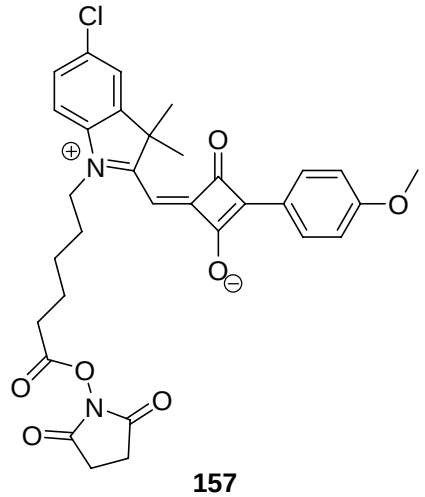


151

1-(6-hexanoic acid)-5-chloro2-[[2-hydroxy-3-(4-methoxyphenyl)-4-oxo-2-cyclobuten-1-ylidene]methyl]-3,3-dimethylindolium (151).

A mixture of indolium **152** (30.5 mg, 0.0723 mmol) and acid **153** (15.0 mg, 0.0735 mmol) in a 10:1 mixture of butanol/pyridine (5 mL:0.5 mL) was stirred at reflux for 4 h before being cooled to ambient temperature. The solvents were removed *in vacuo*, and the crude product was purified *via* reverse phase HPLC using a gradient of 1:1 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 14 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **151** as a red solid (24.3 mg, 0.0492 mmol, 68.0%): R_f: 0.33 (9:1 dichloromethane-methanol);

^{1}H NMR (500 MHz, CD_3OD) δ 1.54 (m, 2H), 1.70 (p, J = 7 Hz, 2H), 1.82 (s, 6H), 1.91 (p, J = 8 Hz, 2H), 2.33 (t, J = 7 Hz, 2H), 3.88 (s, 3H), 4.39 (t, J = 8 Hz, 2H), 6.42 (s, 1H), 7.03 (d, J = 9 Hz, 2H), 7.52 (dd, J = 8.5, 2 Hz, 1H), 7.58 (d, J = 8.5 Hz, 1H), 7.70 (d, J = 2 Hz, 1H), 8.12 (d, J = 9 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 25.72, 25.94, 27.41, 28.70, 34.73, 46.85, 53.22, 56.20, 92.63, 115.79, 115.88, 124.58, 125.31, 130.17, 130.75, 134.83, 141.35, 146.78, 163.99, 168.11, 177.38, 180.45, 182.32, 187.22, 190.00; HRMS [M+H] calcd for $C_{28}H_{29}ClNO_5^+$ 494.1729 found 494.1748.

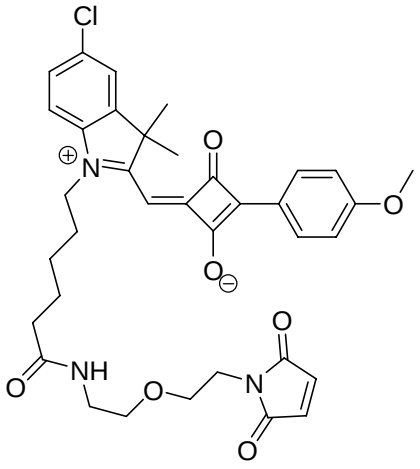


157

1-(6-hexanoic acid)-5-chloro-2-[[2-hydroxy-3-(4-methoxyphenyl)-4-oxo-2-cyclo-buten-1-ylidene]methyl]-3,3-dimethylindolium, succinimidyl ester (157).

A 25 mL rbf containing acid **151** (19.6 mg, 0.0426 mmol), NHS (50.0 mg, 0.434 mmol) and DCC (80.0 mg, 0.388 mmol) was placed under argon. Anhydrous DMF (5 mL) was added to the flask and the mixture was stirred at ambient temperature for 15 h. The solvent was removed *via* the use of a lyophilizer, and the crude product was purified *via* reverse phase HPLC using a gradient of 1:1 ([95% CH_3CN/4.9% H_2O/0.1%

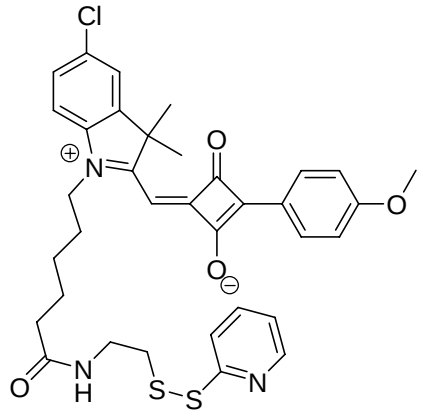
TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 20 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **157** as a red solid (19.0 mg, 0.0321 mmol, 75%): R_f: 0.50 (19:1 dichloromethane-methanol); ^{1}H NMR (500 MHz, CD_3OD) δ 1.64 (m, 2H), 1.84 (s, 6H), 1.86 (m, 2H), 1.94 (p, J = 7.5 Hz, 2H), 2.68 (t, J = 7 Hz, 2H), 2.81 (s, 4H), 3.88 (s, 3H), 4.41 (t, J = 7.5 Hz, 2H), 6.43 (s, 1H), 7.05 (d, J = 9 Hz, 2H), 7.52 (dd, J = 7.5, 2 Hz, 1H), 7.59 (d, J = 7.5 Hz, 1H), 7.71 (d, J = 2 Hz, 1H), 8.14 (d, J = 9 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 25.44, 25.95, 26.64, 26.88, 28.44, 31.44, 46.80, 53.27, 56.18, 92.66, 115.81, 115.88, 124.58, 125.38, 130.16, 130.76, 134.80, 141.43, 146.82, 164.01, 168.18, 170.26, 171.94, 180.56, 182.36, 187.18, 190.20; HRMS [M+H] calcd for $C_{32}H_{32}ClN2O_7^+$ 591.1893 found 591.1895.



147

1-(6-(*N*-maleimido-3-oxapentyl)-hexanamide)-5-chloro-2-[[2-hydroxy-3-(4-methoxyphenyl)-4-oxo-2-cyclobuten-1-ylidene]methyl]-3,3-dimethylindolium (147).

A 10 mL rbf containing succinimidyl ester **157** (16.1 mg, 0.0272 mmol) and *N*-(5-amino-3-oxapentyl)maleimide trifluoroacetate[48a] (20.0 mg, 0.0671 mmol) was placed under argon. Anhydrous DMF (1.5 mL) was added to the flask, followed by addition of freshly distilled NMM (60 μL, 0.558 mmol) at ambient temperature. The mixture was stirred at ambient temperature for 3 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 1:1 ([95% CH_3CN/4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]:[99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 14 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **147** as a red solid (8.4 mg, 0.0127 mmol, 47%): R_f: 0.32 (19:1 dichloromethane-methanol); ^{1}H NMR (500 MHz, CD_3OD) δ 1.49 (m, 2H), 1.70 (p, J = 7.5 Hz, 2H), 1.82 (s, 6H), 1.91 (p, J = 7.5 Hz, 2H), 2.22 (t, J = 7.5 Hz, 2H), 3.26 (t, J = 5.5 Hz, 2H), 3.46 (t, J = 5.5 Hz, 2H), 3.54 (t, J = 5.5 Hz, 2H), 3.62 (t, J = 5.5 Hz, 2H), 3.87 (s, 3H), 4.40 (t, J = 7.5 Hz, 2H), 6.42 (s, 1H), 6.78 (s, 2H), 7.02 (d, J = 8.5 Hz, 2H), 7.51 (dd, J = 8.5, 2 Hz, 1H), 7.58 (d, J = 8.5 Hz, 1H), 7.70 (d, J = 2 Hz, 1H), 8.11 (d, J = 8.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 25.99, 26.50, 27.29, 28.71, 36.67, 38.29, 40.42, 46.81, 53.24, 56.19, 68.95, 70.16, 92.70, 115.84, 115.90, 124.59, 125.34, 130.17, 130.74, 134.82, 135.55, 141.38, 146.78, 164.00, 168.17, 172.60, 175.93, 180.48, 182.25, 187.08, 190.11; HRMS [M+H] calcd for $C_{36}H_{39}ClN_3O_7^+$ 660.2471 found 660.2493.



148

1-(6-(2-(2-(pyridin-2-yl)disulfanyl)ethanamine)-hexanamide)-5-chloro-2-[[2-hydroxy-3-(4-methoxyphenyl)-4-oxo-2-cyclobuten-1-ylidene]methyl]-3,3-dimethylindolium (148).

A 25 mL rbf containing succinimidyl ester **157** (29.0 mg, 0.0491 mmol) and *S*-(2-pyridylthio)cysteamine hydrochloride[49a] (24.0 mg, 0.108 mmol) was placed under argon. The flask was placed in an ice bath, followed by addition of anhydrous DMF (2.50 mL). Freshly distilled NMM (54 μL, 0.502 mmol) was added, and the mixture was stirred at 0 ^{o}C for 3 h before the solvent was removed *via* the use of a lyophilizer. The crude product was purified *via* reverse phase HPLC using a gradient of 1:1 ([95% CH_3CN/4.9% H_2O/ 0.1% TFA]:[99.9% H_2O/0.1% TFA]) to 7:3 ([95% CH_3CN/ 4.9% H_2O/0.1% TFA]: [99.9% H_2O/0.1% TFA]) over 30 min at a flow rate of 20 mL/min, monitoring at 450 nm. The product was collected at 20 min. The solution containing the product was frozen, and the solvents removed *via* the use of a lyophilizer providing **148** as a red solid (24.7 mg, 0.0374 mmol, 76%): R_f: 0.35 (19:1 dichloromethane-methanol); ^{1}H NMR (500 MHz, CD_3OD) δ 1.47 (m, 2H), 1.69 (p, J = 7.5 Hz, 2H), 1.79 (s, 6H), 1.89 (p, J = 7.5 Hz, 2H),

2.21 (t, J = 7.5 Hz, 2H), 2.90 (t, J = 6.5 Hz, 2H), 3.45 (t, J = 6.5 Hz, 2H), 3.85 (s, 3H), 4.37 (t, J = 7.5 Hz, 2H), 6.38 (s, 1H), 6.98 (d, J = 9 Hz, 2H), 7.32 (t, J = 6 Hz, 1H), 7.47 (dd, J = 8.5, 1.5 Hz, 1H), 7.53 (d, J = 8.5 Hz, 1H), 7.66 (d, J = 1.5 Hz, 1H), 7.85-7.91 (m, 3H), 8.07 (d, J = 9 Hz, 2H), 8.43 (d, J = 4.5 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 26.03, 26.41, 27.28, 28.71, 36.69, 39.28, 39.40, 46.79, 53.20, 56.19, 92.69, 115.79, 115.88, 122.57, 123.15, 124.56, 125.29, 130.14, 130.73, 134.80, 140.88, 141.28, 146.70, 149.23, 160.54, 163.96, 168.21, 176.01, 180.36, 182.14, 187.00, 190.08; HRMS [M+H] calcd for $C_{35}H_{37}ClN_3O_4S^+$ 662.1909 found 662.1912.