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Synthesis of Half-Loaded Nitroimidazole Indocyanine Green Dyes Attached to Carbon Nanotubes

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Synthesis of Half-Loaded
Nitroimidazole Indocyanine Green Dyes
Attached to Carbon Nanotubes

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B.S., Rochester Institute of Technology, 2010

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Synthesis of Half-Loaded Nitroimidazole Indocyanine Green Dyes Attached to Carbon Nanotubes

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2012
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Dedication

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Part 1:
Synthesis of Half-Loaded Nitroimidazole Indocyanine Green Dyes Attached to Carbon Nanotubes

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University of Connecticut 2012

Part 1:
Indocyanine green dye is the only FDA approved NIR fluorescent dye that can be used in humans. 2-Nitroimidazoles are very effective radiosensitizers, have low cytotoxicity, and target hypoxic tissue. By coupling the two together, hypoxic tumors can be targeted and imaged. Previous work has been done to couple 2-nitroimidazole to both sides of the indocyanine green dye. This work focuses on expanding that concept to couple 2-nitroimidazole to only one side of the dye, thus allowing for the other end to be available to couple to another compound, generating a half loaded 2-nitroimidazole indocyanine green dye. In theory, this half-loaded dye can be coupled to various compounds resulting in unique properties for the dye. Our focus was on coupling the half-loaded 2-nitroimidazole indocyanine green dye to single-walled carbon nanotubes which also have fluorescent properties. It is hoped that more dye will be carried to the hypoxic tumor leading to an increase in the observed fluorescence, and dye retention in the tumor for an extended period of time.

Multiple half-loaded nitroimidazole ICG dyes were synthesized. The half-loaded 2-nitro ethanolamine carboxylic acid ICG was chosen to be coupled to the amine functionalized nanotubes. The biological results for this product are pending, as of the writing of this thesis.
1. Introduction

The goal of this project is to attach an unsymmetrical 2-nitroimidazole indocyanine green dye (ICG) conjugate to a single-walled carbon nanotube (SWNT). In principle, this dye-conjugate will selectively target hypoxic tumors and be retained in the tumor for a longer period of time relative to dye-conjugates not bound to SWNT. Tumor imaging will be accomplished in living mice using a near-infrared fluorescence imaging device developed by Professor Quing Zhu from the University of Connecticut Department of Electrical and Computer Engineering. Since the SWNT also have fluorescent properties, there may be an increase in the observed fluorescence of the cancerous tumor, thus creating a better dye for use in fluorescent imaging techniques.

1.1 Cancer and Hypoxia

Cancer is a term used to describe numerous diseases characterized by an unregulated growth of cells. As cells grow and divide, errors can occur in the sequencing of DNA, damaging the DNA. DNA can also be damaged from UV radiation, nuclear radiation and various chemicals. Normally, there are mechanisms in place to correct these errors, so the mutated cells do not multiply. However, at times, these mechanisms fail and the mutations are passed on to the next cell. When this happens, the cell can begin to spontaneously replicate, inhibiting apoptosis. When these mutated cells begin to form a mass, it is categorized as a tumor.¹

As the tumor grows, it forms its own network of blood vessels via angiogenesis. Since many tumors grow quickly, the result is a disorganized and leaky vascular network.
Eventually, the tumor will outgrow its vascular network, and less oxygen is available to the cells. When cells have an inadequate supply of oxygen, a tumor-specific hypoxic microenvironment can be generated and biological functions are compromised. Hypoxia is characterized by low oxygen partial pressure, low pH, and low glucose concentration.\(^2\) Tumor cells are able to adapt to this new hypoxic environment, which can lead to resistance in cancer therapies. When healthy cells are deprived of oxygen, they eventually die. As a tumor expands and outgrows its vascular network, it is able to slow its growth rate which decreases perfusion. This can inhibit the effectiveness of most chemotherapy drugs, which are essentially antiproliferation agents, because conventional drugs are generally toxic to cells at a level that is proportional to proliferation.\(^3\)

Another method in the treatment of cancer is radiation therapy. Radiation therapy uses photons to directly or indirectly ionize atoms in DNA.\(^4\) When O\(_2\) is irradiated, the ground state triplet oxygen is excited into singlet state oxygen, which generates hydroxyl radicals. These radicals kill cells in their immediate area by damaging the DNA resulting in cell death. Since singlet oxygen has a short lifetime in water, it cannot diffuse very far and, therefore, specific areas in the body can be easily targeted.\(^5\) Molecular oxygen is a potent chemical radiosensitizer, a chemical that is able to increase the effectiveness of the radiation therapy, because it is an extremely electron-affinic molecule. However, radiation has very little effect on hypoxic tumors, due to the low levels of oxygen present in the cells. The free radicals that are formed by ionizing radiation recombine without causing damage to the DNA. Hypoxic cells that lack oxygen are about three times more resistant to radiation than cells that are well oxygenated at the time of irradiation.\(^6\)
Understanding the microenvironment of a tumor is important because it dictates how effective radiation therapy will be, and how a chemotherapeutic drug can be delivered. Traditional chemotherapies do not account for this. In general, being able to identify tumor hypoxia is a key factor in determining what type of cancer therapy would be most successful for patients.

1.2 Imaging Techniques

There are several current techniques, both invasive and non-invasive, used to identify tumor hypoxia. One technique uses oxygen sensitive electrodes. Using these electrodes, the partial pressure of O\textsubscript{2} \textit{in-vivo} can be measured. However, this technique has limits. The signal generated is very small within the range of cell-threatening hypoxia since the electrode consumes oxygen by electrochemical reduction. These electrodes also require the use of computed tomography (CT), can only access tumors close to the surface of the body (such as the cervix\textsuperscript{3}, head, and neck\textsuperscript{8}), are invasive, and can damage the tissue.\textsuperscript{3}

Fiber optic probes have been used in the measurement of pO\textsubscript{2}. These probes work by using an optical fiber to carry fluorescent pulses. These pulses are inversely proportional to the partial pressure of the oxygen. Equilibrium with the pO\textsubscript{2} in the tissue allows for a continuous reading of the pO\textsubscript{2}. These probes are smaller than the oxygen sensitive electrode and are less damaging to tissues, but only a small area can be sampled at a time.\textsuperscript{9}

Non-invasive techniques exist such as positron emission tomography (PET) and magnetic resonance imaging (MRI). PET is a technique that produces a three-
dimensional image of the body. A three-dimensional image is possible because of the radiation released by a positron-emitting radioactive tracer that is introduced into the body. The most widely used PET imaging agent for hypoxia is $^{18}$F-labeled fluoromisonidazole (1), an $^{18}$F-labeled nitroimidazole. (See Figure 1) The delivery of this compound is not limited by perfusion, and its retention within tissues is dependent on nitroreductase activity the reduction status of a NO$_2$ group on the imidazole ring. Fluroromisonidazole has been shown to be retained in tumors and to cross the blood brain barrier. Drawbacks include low imaging contrast, a high retention in the liver, and a twenty-four hour resting period between repeated doses.  

![Figure 1: Structure of Fluoromisonidazole (1)](image)

An MRI uses a magnet to align nuclei in the body, and a radio frequency field is applied to systematically alter this alignment. Under these conditions, the nuclei will produce a rotating magnetic field, which is then detected and constructed into an image of the body. Using contrast agents and tracers, an estimation of tumor permeability can be determined. There are multiple types of MRIs; each type provides different characteristics of a tumor. $^{19}$F-MRI uses fluorine-containing contrast agents, which are sensitive to oxygen concentration. The MRI can then highlight the differences in oxygen concentration. Dynamic contrast-enhanced MRI (DCE-MRI) is a technique using various types of contrast agent that give information about the properties of tumors such as distribution volume, permeability, and perfusion. Blood oxygen level-dependent MRI
(BOLD-MRI) relies on the imbalance between paramagnetic deoxyhemoglobin and diamagnetic oxyhemoglobin to image tumors. However, this technique is not quantitative. Both PET and MRIs have drawbacks as well. They are both expensive techniques for routine clinical use in longitudinal imaging assessments over the course of neoadjuvant chemotherapy. Thus, a new low-cost, non-invasive technique for the analysis of tumor hypoxia is an attractive goal.

Near Infrared (NIR) Fluorescence Diffuse Optical Tomography (FDOT) is a technique that has the potential to be of great use in the detection of hypoxia. In the NIR range, human tissue has relatively low absorption and relatively high scattering, thus allowing NIR light to pass through most tissue. FDOT is a technique that has the ability to produce images of absorption and scattering. With the addition of a fluorescent dye that has the ability to target tumors, this technique can provide greater sensitivity and specificity about the tissue being analyzed due to the dye’s fluorescent signals. Fluorescent signals can be detected in tumors up to 2.0 to 3.0 cm from the skin surface, which are then reconstructed into an image, giving insight into the tumor microenvironment.

Figure 2: Image of 2-nitropiperazine ICG inside mouse tumor after 10 minutes using NIR-FDOT
From this information, the concentration of oxygen in the hemoglobin and the pH can be determined.\textsuperscript{11,12} NIR-FDOT is most successful with a fluorescent dye that has an emission in the 700 – 850 nm range, has a high extinction coefficient, and a high fluorescent yield. Other characteristics of NIR-FDOT dyes include water solubility, non-toxicity, and the ability to easily bind to biological molecules. NIR-FDOT has advantages over other techniques because it is low cost, is portable, has repeatability in longitudinal imaging, and has little background fluorescence.

\subsection*{1.3 Indocyanine Green and Nitroimidazoles}

Indocyanine green dye (ICG, 2, shown in Figure 3) meets the requirements of an NIR-FDOT dye. This NIR fluorescent dye has an emission peak \textasciitilde 807 nm, and is the only FDA approved agent that can be used in humans, due to its low toxicity and high absorbance in the NIR spectrum. Studies in animal models\textsuperscript{13,14} and in humans\textsuperscript{15,16} have demonstrated its ability to enhance tumor contrast. However, there are disadvantages to using ICG: 1) it is very non-specific due to binding to plasma proteins, which causes it to quickly wash out of the body and tumor site; 2) it is optically unstable in the body; 3) it has a low quantum yield.\textsuperscript{17,18} These issues can be mediated with a dye that can target tumors specifically, and can be retained in the body for longer durations of time.
Figure 3: Structure of Indocyanine Green (2)

The family of compounds based on nitroimidazole has a high electron affinity and shows selectivity towards hypoxic cells. This increased sensitivity is due to an enzyme-mediated four-electron reduction, which causes the nitroimidazole molecules to accumulate in hypoxic areas of a tumor. P-450 reductase is responsible for the radical reduction of nitroimidazole. The actual mechanism for this is still unknown, but a plausible mechanism has been proposed and is shown in Scheme 1. The reduced forms of nitroimidazole eventually links with macromolecules, helping to be retained in the hypoxic regions. This sensitivity to hypoxic areas differs from normal tissue because oxygen has been found to efficiently hinder the reduction of nitroimidazole by reversing it. Hypoxic areas contain low O$_2$ levels, limiting the effects of radiation therapy. This is because lower amounts of hydroxyl radicals are produced. Nitroimidazoles are bio-active radiosensitizers that make tumor cells more sensitive to radiation therapy. Radiosensitizers mimic the hydroxyl radicals, increasing the effectiveness of the radiation therapy.
Scheme 1: Plausible Mechanism for the Reduction of Nitroimidazoles

To date several members of the nitroimidazole family have been studied, the first being metronidazole, a 5-nitroimidazole, discovered in the late 1950’s. Metronidazole (3) is used primarily as an antibiotic, amebicide, and antiprotozoal. Over time, scientists have improved the effectiveness and application for nitroimidazoles. Other prominent 5-nitroimidazole compounds are tinidazole (4) and ornidazole (5). Both are antiprotozoal drugs in use today. (Figure 4)

Figure 4: Structures of 5-nitroimidazole derivatives: metronidazole (3), tinidazole (4), and ornidazole (5)

While metronidazole has been shown to be a hypoxic sensitizer, high concentrations are needed for it to be effective. Thus, other nitroimidazoles such as misonidazole (6), pimonidazol (7), and etanidazole (8) (Figure 5) are currently being analyzed. These studies have shown that 2-nitroimidazoles are very effective sensitizers, and have low cytotoxicity. Of particular interest to us is etanidazole (8); a good radiosensitizer and less toxic than misonidazole (6). It can also bind to ICG dye via an ester bond at its primary alcohol.
Figure 5: Structures of 2-nitroimidazoles derivatives misonidazole (6), pimonidazole (7), and etanidazole (8)

In order to use the nitroimidazole group to target a hypoxic tumor, a way to conjugate the nitroimidazole to the ICG dye was required. This problem was solved by using a known bis-carboxylic acid derivative of ICG (9). Figure 6

Figure 6: Structure of bis-carboxylic acid ICG (9)

Research has shown that both the bis-carboxylic acid ICG (9) and the 2-nitroimidazole ICG have an excitation peak at 755 nm and fluorescence emission peak at 778 nm. The latter has a quantum yield of 0.066, while ICG has a quantum yield of 0.012. The increase in the fluorescence quantum yield is most likely due to the formation of fluorescence-quenched aggregates that are suppressed because dye-dye interaction and aggregation is reduced by hydrophilic, non-charged but sterically demanding substituents. Several routes for the synthesis of the bis-carboxylic acid
ICG (9) have been developed by Lindsey,\textsuperscript{28} Ozinskas,\textsuperscript{31} Licha,\textsuperscript{29} and Smith.\textsuperscript{17} The Smith group used these precedents to develop a method for attaching etanidazole (8) to the bis-carboxylic ICG (9), resulting in a 2-nitroimidazole ethanolamine ICG (10).\textsuperscript{17} (Figure 7)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structure.png}
\caption{Structure of 2-nitroethanolamine ICG (10)}
\end{figure}

Based on this work, other 2-nitroimidazole derivatives could also be attached to the bis-carboxylic acid ICG (9), specifically a 2-nitroimidazole with a piperazine linker instead of an ethanolamine linker. It is believed that this derivative would be more robust \textit{in vivo} since amide bonds are less susceptible to hydrolysis. Also of note is the fact that 2-nitroimidazole ethanolamine ICG (10) is a symmetrical dye. If the dye could be altered slightly to become unsymmetrical, then the dye could have both a 2-nitroimidazole and another compound attached to it simultaneously to generate a half-loaded dye (Figure 8). This new dye would include the fluorescence ability of the ICG, the hypoxic tumor targeting ability of the nitroimidazole, and the ability of the other compound that could be attached to the half-loaded ICG dye.
1.4 Carbon Nanotubes

Several different compounds can be attached to these unsymmetrical ICG dyes (11, 12), including single-wall carbon nanotubes (SWNTs). SWNTs have unique properties due to their one-dimensional structure, including high tensile strength and resilience, plus a high thermal and current carrying capacity. The ability to functionalize these nanotubes can lead to the development of more novel or enhanced properties. Currently SWNTs are being chemically altered via oxidation of their fullerene-like caps followed by different reactions with the carboxylic groups. Another method, using free radical addition of alkyl groups ending in carboxylic acids, has allowed for the addition of functional groups to the side wall of the SWNT.
an acid functionalized nanotube exists, the addition of other functional groups such as amines is easily achieved.\textsuperscript{36} With a SWNT ending in an amine, it is possible to attach multiple unsymmetrical 2-nitroimidazole half-loaded dyes (11, 12) to the nanotube via an amide bond. This is important because it has been shown that carbonaceous materials can promote the intensification and/or prolongation of the excited states of dyes, due to charge-transfer interactions.\textsuperscript{37} Dyes that have been studied in this fashion include the phenazine dyes phenosafranine (13) and Nile Blue (14).

\begin{center}
\includegraphics[width=\textwidth]{figure9.png}
\end{center}

\textbf{Figure 9: Structures of Phenosafranine (13) and Nile Blue (14): Dyes Previously Attached to SWNTs}
2. Results and Discussion

2.1 Formation of Bis-Carboxylic Acid ICG, (9)

To the best of our knowledge, there is currently no known FDOT selective hypoxic targeting probe that fluoresces in the near infrared region (700-850 nm). Currently, the only NIR fluorescent dye approved by the FDA is indocyanine green, due to its low toxicity. Studies have shown that ICG enhances tumor contrast, but it is nonspecific and quickly eliminated from the body. The main goal of this work is the synthesis of a fluorescent ICG dye that can be used to detect and image hypoxic cancerous tumors.

The complete synthesis of the bis-carboxylic acid ICG can be seen in Scheme 2. Beginning with a Fischer-indole reaction, 4-hydrazinobenzoic acid (15) and 3-methyl 2-butanone were reacted to yield the carboxylic acid indole (16) in 68% yield. The duration of this reaction varied based on the age of the acetic acid—reaction times ranged from 12 hours to 48 hours. Next, the carboxylic acid indole (16) was heated at reflux with 1,4 butanesultone in 1,2 dichlorobenzene overnight to yield the carboxylic acid indolium salt (17) in 88% yield. The last step is a reaction in which N-[5-(Phenylamino)-2,4-pentadienyldene]aniline monohydrochloride is used as a linker to connect two of the carboxylic acid indolium salt (17) by refluxing in acetic acid and acetic anhydride with sodium acetate, forming the bis-carboxylic acid ICG (9) in 80% yield. It is easy to identify when this reaction has gone to completion because the reaction mixture changes from a brunt red to blue-green in color. The overall yield of 9 from 15 is 48%.
2.2 Formation of Methyl Ester – Acid ICG, (18)

The bis-carboxylic acid ICG (9) is a symmetrical dye, so it is difficult to perform a reaction that would allow only one of the carboxylic acid groups to react. To solve this dilemma an unsymmetrical dye must be generated to allow for the reaction of only one end of the dye at a time, resulting in a half-loaded dye. This unsymmetrical dye would require one of the carboxylic acid groups to be blocked off, so it would be unreactive towards any reaction that would add the nitroimidazole to the carboxylic acid end of the ICG. It is hoped that by protecting one end of the dye, a higher yield of mono-substituted dye can be generated. To block off one end, a methyl ester was chosen due to the ease of removal, thus forming a methyl ester–acid ICG dye (18). The formation of a methyl ester-acid dye would be useful because it would allow for two different compounds to be added to each end of the ICG dye. This ability would increase the functionality of the dye and allow it to have multiple unique properties.
Figure 10: Structure of Methyl Ester-Acid ICG Dye

The formation of the methyl ester-acid ICG (18) is similar to the formation of the bis-carboxylic acid ICG (9). In order to make the methyl ester-acid ICG (18), methodology was required that converted one of the carboxylic acid groups to a methyl ester group. One method would form a methyl ester indolium salt (19) and react it with a carboxylic acid indolium salt (17) to generate the final dye. There are three steps preceding the formation of the bis-carboxylic acid dye, and a methylated intermediate was prepared for each in an attempt to determine which was the best.

First, the 4-hydrazinobenzoic acid (15) was methylated using oxalyl chloride and methanol, but the product was methyl 4-(1,2,2-trimethylhydrazinyl)benzoate (20). Next, the carboxylic acid indoline (16) was methylated using oxalyl chloride and methanol, giving methyl ester indoline (21). This methyl ester indoline was reacted with 1,4-butanesultone to form the methyl ester indolium salt (19). However, it was very difficult to isolate the product because the solvent was 1,2 dichlorobenzene and the product would not crystallize. In addition, it was difficult to remove all of the solvent from the product. Lastly, the carboxylic acid indolium salt (17) was methylated with oxalyl chloride in
methanol. This resulted in the methyl ester indolium salt (19), which was easily isolated after stirring the crude product in ether. (Scheme 3)

**Scheme 3: Formation of Methylated Products**

The difference between the carboxylic acid indolium salt (17) and the methyl ester indolium salt (19) can be easily seen in the $^1$H NMR. At 3.98 ppm in MeOD, the methyl ester can be distinctly seen. The peak at 3.86 ppm is a side product from the reaction that could not be completely removed, but it does not carry on to the next step of the synthesis. This impurity could not be identified because it only showed a singlet in the $^1$H NMR, one peak in the $^{13}$C NMR, and a mass spec could not be obtained.
Figure 11: $^1$H NMR of Carboxylic Acid Indolium Salt (17) - Solvent: CD$_3$OD

With the methyl ester indolium salt (19), the methyl ester-acid dye (18) could be formed based on stoichiometry. The mechanism shows that the reaction between the two indolium salts is a stepwise process (Figure 13). Using one molar equivalent of methyl ester indolium salt (19), one molar equivalent of carboxylic acid indolium salt (17), and one molar equivalent of $N$-[5-(Phenylamino)-2,4-pentadienylidene] aniline
monohydrochloride, the methyl-ester acid dye (18) was generated in 83% yield, with an overall yield of 48%.

Figure 13: Proposed Mechanism for Formation of Mechanism for the Methyl Ester-Acid Dye (18)
Scheme 4: Formation of Ester-Acid ICG (18)

The synthesis of the methyl ester-acid ICG dye (18) requires specific timing. The proposed mechanism (Figure 13) shows that reaction with the indolium salts is a stepwise process. The carboxylic acid indolium salt (17) and the bis-anilide linking unit is added at the beginning of the reaction and refluxed for 30 minutes. At that time, the methyl ester indolium salt (19) is added, and the system is heated at reflux for an additional 45 minutes. If the methyl ester indolium salt (19) is added before the 30 minutes, incomplete reaction of the carboxylic acid indolium salt (17) leads to lower yields, and addition much after the 30 minutes the carboxylic acid indolium salt (17) leads to reaction on both sides. The consequence is that the product is a mixture of acid-acid dye, acid-ester dye, and ester-ester dye. Even with close monitoring of the stoichiometry and the reaction time, a very small amount of bis-carboxylic acid ICG (9) is formed.

As with the bis-carboxylic acid ICG (9), completion of the reaction can be monitored by color change. Upon addition of the first indolium salt, the reaction mixture remains red. Once the second molar equivalent of indolium salt has been added to the mixture, it begins to turn a blue green color, which is an indication that both sides of the bis-anilide linker have attached to an indolium salt. Theoretically, the methyl ester indolium salt (19) could be added first, then the carboxylic acid indolium salt (17), resulting in a small amount of ester-ester dye. The small amount of byproduct is not
separated at this point, but will be separated out after the coupling with the nitroimidazole moieties. This protocol was followed to minimize hydrolysis of the methyl ester-acid ICG dye (18) to the bis-carboxylic acid ICG (9), as tracked by TLC analysis.

Analysis of the NMR spectra shows a difference between the symmetrical and the unsymmetrical dyes. Figure 14 is the $^1$H NMR for the bis-carboxylic acid ICG (9) and Figure 15 is the $^1$H NMR for the methyl ester-acid ICG (18). Both NMRs are similar, yet a few differences can distinguish the two. The first being the CH$_3$ of the methyl ester at 3.86 ppm in the methyl ester-acid ICG (18). There is an increase in splitting showing how the molecule is no longer symmetrical. The hydrogens of the amino methylenes of the sulfonate chains, in the symmetrical dye produce a broad singlet at 4.11 ppm. These become two broad singlets at 4.11 and 4.15 ppm in the unsymmetrical dye. Increase in splitting of the aromatic protons can also be seen as well, this is thought to be due to the positioning of the carbonyl groups off the aromatic ring.
Figure 14: $^1$H NMR of Diacid ICG (9) – Solvent DMSO

Figure 15: $^1$H NMR of Methyl Ester-Acid ICG (18) – Solvent DMSO
2.3 Formation of Nitroimidazole Derivatives

Nitroimidazoles are bio-active radiosensitizers that have a high electron affinity and are selectively sensitive towards hypoxic cells. This sensitivity makes them useful for targeting hypoxic tumors. Attaching a nitroimidazole unit to the ICG dye would allow targeting of a hypoxic tumor. Carrying the fluorescent dye with it, this would allow imaging of the tumor using NIR-FDOT imaging techniques.

2-Nitroimidazole has been shown to selectively target hypoxic regions in the body, so attachment to the fluorescent dye seemed to be a reasonable goal. This required the development of synthetic methodology. However, due to the price of 2-nitroimidazole ($293.00 per gram) the methodology was tested on 4-nitroimidazole. This compound is cheaper ($1.62 per gram), but exhibits little or no hypoxic targeting capability. It is noted that 4-nitroimidazole is the normal product formed by electrophilic aromatic nitration of imidazole.

The synthetic nitroimidazole compounds are to be linked to the ICG dyes via the carboxylic acid, but there must be a linker between the nitroimidazole and the ICG dye. The first linker was an ethanolamine unit, as shown in Scheme 5. This linker could be attached to a nitroimidazole acetate derivative. Therefore, a nitroimidazole (22a, 22b) reacts with methyl α-bromoacetate to give 4-nitroimidazole methyl ester (23a) in 75% yield or 2-nitroimidazole methyl ester (23b) in 36% yield. This nitroimidazole methyl ester (23a, 23b) was then stirred with ethanolamine in methanol at room temperature to give the 4-nitroimidazole ethanolamine (24a) in 81% yield, with an overall yield of 61% and 2-nitroimidazole ethanolamine (24b) in 95% yield, with an overall yield of 34%.
Scheme 5: Synthesis of Nitroimidazole Ethanolamine Derivatives

Piperazine was analyzed as another linker in the hope it would be more robust than the ethanolamine linker due to the formation of two amide bonds. As before the nitroimidazole (22a, 22b) was converted into the methyl ester (23a, 23b), and then reacted with mono-BOC protected piperazine (25), prepared by the treatment of piperazine (26) with di-tert-butyl dicarbonate. (Scheme 6) Both mono- and di-substituted piperazine were formed, but the compounds did not require separation because the di-substituted product could not react in the next step of the synthesis. However, after several failed attempts at coupling the nitroimidazole methyl ester (23a, 23b) with the mono-BOC protected piperazine (25) using DIPEA, it was determined that the reactivity of the nitroimidazole compound had to be increased.

Scheme 6: Synthesis of Mono-BOC Protected Piperazine

To increase reactivity, the methyl ester (23a) was hydrolyzed to the carboxylic acid (27) in 97% yield by refluxing in water overnight (Scheme 7). The acid was easily
converted into the acid chloride (28) via reaction with oxalyl chloride. (Scheme 8) The acid chloride, remained in situ and was reacted with the secondary amine of the mono-protected BOC piperazine (25) to produce the 4-nitroimidazole BOC-protected piperzine (29) in 23% yield. Once the piperazine unit was attached, the compound was deprotected using TFA to give the 4-nitroimidazole piperazine TFA salt (30) in 94% yield, giving an overall yield of 16%.

Scheme 7: Synthesis of Nitroimidazole Acid

Scheme 8: Synthesis of 4-Nitroimidazole Piperazine Derivative (30)

The addition of piperazine to the 4-nitroimidazole was examined in an attempt to increase the yield, using various protecting groups as well as unprotected piperazine. We found that TMS and TES were not useful as protecting groups for piperazine. When unprotected piperazine reacted with the acid chloride (28), the products were a mixture of unreacted nitroimidazole, monosubstituted nitroimidazole piperazine, and disubstituted nitroimidazole piperazine.
Interestingly, attaching piperazine (26) to the nitroimidazole via an acid chloride works for the 4-nitroimidazole (22a), but the procedure could not be carried over to the 2-nitroimidazole (22b). After 24 hours of heating at reflux in water the 2-nitroimidazole methyl ester (23b) could not be converted into the 2-nitroimidazole acid (27b). This is thought to be because the electronics between the 4-nitroimidazole and the 2-nitroimidazole are different.

The preparation of the biologically active 2-nitroimidazole target required another procedure to attach the piperazine unit. The procedure shown in Scheme 8 was developed by Mr. Innus Mohammed. Previous experience showed that it was difficult to attach the secondary amine of the protected piperazine to the nitroimidazole with the carbonyl group already attached to it because of inactivation of the carbonyl to react with the secondary amine of the piperazine. This synthesis of 2-nitroimidazole piperazine TFA salt (31) begins with the mono-BOC protected piperzine (25), which is converted into bromoacetyl BOC-protected piperazine (32) using triethylamine and α-bromoacetyl bromide (81%). Next with sodium hydride as the base, the 2-nitroimidazole (22b) is deprotonated, then reacted with the bromoacetyl BOC-protected piperazine (32) in an $S_N2$ reaction to yield 2-nitroimidazole BOC-protected piperazine (33) with a 94% yield. The BOC protecting group is removed the same way as before using TFA, to create the 2-nitroimidazole piperazine TFA salt (31) in 86%, with an overall yield of 65%.
2.4 Formation of ICG Conjugates

With the development of the methyl ester-acid dye (18) and the nitroimidazole derivatives (24a, 24b, 30, 31), the two compounds must be coupled to give a dye-conjugate that combines the tumor targeting abilities of the nitroimidazoles with the fluorescent abilities of the dye. Initially, this coupling was attempted with DCC, with limited success, so a new procedure was developed. We found that DIPEA, PyBOP (benzotriazol-1-yl-oxytritylpyrrolidinophosphonium hexafluorophosphate), and HOBT (hydroxybenzotriazole) could be used to couple the nitroimidazoles to the dye (Schemes 10 & 11). This reaction is useful in that it can be used to couple both the ethanolamine, and piperazine linked nitroimidazoles. 24a was isolated as a dark purple solid in 20% yield and 24b was isolated as a dark purple solid in 35% yield. 35 was never completely isolated due to decomposition during workup.
Scheme 10: Coupling of the Ethanolamine-Linked Nitroimidazole (24a, 24b) to the Methyl Ester-Acid Dye (18)
Based on TLC analysis (Figure 16) multiple compounds are present after this reaction. Since this reaction goes to equilibrium, the uncoupled dye must be separated from the coupled dye, any bis-carboxylic acid ICG (9) that might be present should also be easily removed. From the reverse phase TLC, the various spots could be identified based on their R_f values. The topmost spot is the bis-carboxylic acid ICG (9) R_f = 0.76, followed by the unreacted methyl ester-acid ICG (18) R_f = 0.67, then the bis-carboxylic acid ICG that has the nitroimidazole coupled on both sides R_f = 0.36, and lastly the half loaded dye (34b) R_f = 0.28.
Figure 16: Reverse Phase TLC Analysis for Half-Loaded Dyes

Due to the polarity of these compounds, C-18 reverse phase silica gel was used to separate the product from the starting material. This greatly limits the solvent systems that can be used. Only water, methanol, and acetonitrile may be used on C-18 reverse phase silica gel. After many failed attempts to separate the compounds on a manual column, it was determined that Combi Flash, or an automated column must be used. 30% acetonitrile in water gave the best separation on TLC however, 40% methanol in water, was necessary for solubility reasons for the reverse phase column chromatography. With the hope of avoiding this difficult separation, other coupling reagents such HBTU (O-(Benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium Hexafluorophosphate) and DMTMM (4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride) will be tested to see if they improve the coupling. Both are commonly used coupling reagents used for coupling secondary amines in peptides.

NMR analysis can easily show the difference between the methyl ester-acid dye (18) (Figure 17), and a methyl ester-acid dye coupled to a nitroimidazole such as the 4-nitroimidazole ethanolamine methyl ester-acid ICG (34a) (Figure 18). Only one 4-
nitroimidazole ethanolamine has been attached to the ester acid dye based on a) the two peaks at 8.31 and 7.80 ppm which are the two hydrogens on the nitroimidazole and b) the peaks at 4.88, 4.31, and 3.52 ppm which belong to the CH$_2$ groups of the 4-nitroimidazole ethanolamine. The peak at 3.69 ppm indicating the presence of the methyl ester. It should be noted that Figure 18 has a very low concentration of the sample present.

Figure 17: $^1$H NMR of Methyl Ester-Acid ICG (18) – Solvent DMSO
Figure 18: $^1$H NMR of 4-Nitroimidazole Ethanolamine Methyl Ester-Acid ICG (34a) – Solvent DMSO

Several attempts of hydrolyzing the methyl ester were met with repeated failure. Water hydrolysis, acid-catalyzed hydrolysis using 1 M HCl, and base catalyzed hydrolysis using 2.5% NaOH all resulted in degrading the compound, based on TLC analysis. When other conditions for hydrolysis were used including barium hydroxide octahydrate (nonaqueous media) and aluminum chloride with N,N-dimethylaniline, no reaction took place. It is believed that this hydrolysis failed because these compounds are too sensitive to be hydrolyzed by an acid or base in aqueous media, and the other reagent may not have been able to overcome the activation energy to react.

Due to multiple failed attempts at removing the methyl ester, coupling between the bis-carboxylic acid ICG (9) and the various nitroimidazole moieties was carried out with one molar equivalence of dye, and one molar equivalence of a nitroimidazole
derivative. It was initially hoped that by using the methyl ester to block one end of the dye a higher yield could be obtained for the half load dyes, since only one end of the dye was available for coupling at a time. Using the same conditions as before, as shown in schemes 12 and 13, each of the nitroimidazoles were coupled to the bis-carboxylic acid ICG (9), and then separated on the Combi Flash, resulting in the synthesis of the half-loaded ICG dyes 36a, 36b, 37a, and 37b. The yields for the following compounds are as follows 33% for 36a, 72% for 36b, 16% for 37a, 37b was never made due to time constraints. 36b was not passed through the Combi Flash, resulting in the difference in yield.

Scheme 12: Coupling of the Ethanolamine Linked Nitroimidazole (24a, 24b) to the Bis-Carboxylic Acid Dye (9)
Scheme 13: Coupling of the Piperazine Linked Nitroimidazole (30, 31) to the Bis-Carboxylic Acid Dye (9)

Based on NMR analysis key differences can be seen the bis-carboxylic acid ICG (8) (Figure 14) and the nitroimidazole moieties. Figure 19 shows the $^1$H NMR of 4-nitroimidazole ethanolamine acid ICG (36a) key peaks to look at include a) the two peaks at 8.31 and 7.80 ppm which are the two hydrogens on the nitroimidazole and b) the peaks at 4.88, 4.31, and 3.52 ppm which belong to the CH$_2$ groups of the 4-nitroimidazole ethanolamine and c) 12.93 ppm which is the hydrogen for the acid. Differences can also be seen between the $^1$H NMR of 4-nitroimidazole piperazine acid ICG (37a) (Figure 20). Here the piperazine peaks can be seen at 3.11 and 3.60 ppm as well as the acid peak at 12.90 ppm.
Figure 19: $^1$H NMR of 4-Nitroimidazole Ethanolamine Acid ICG (36a) – Solvent DMSO

Figure 20: $^1$H NMR of 4-Nitroimidazole Piperazine Acid ICG (37a) – Solvent DMSO
2.5 Formation of ICG – Nanotubes

Single Walled Nanotubes (SWNT) have found a wide range of uses due to their unique chemical and physical properties. These compounds are incredibly strong, and can greatly vary in length. One of the most remarkable features of certain nanotubes is their ability to rapidly cross through cell membranes.\(^{38}\) This is of particular interest in the medical and biological fields as compounds that express poor diffusion into cells could be linked to SWNT to increase their delivery to cells. Nanotubes have also been shown to promote the intensification and/or prolongation of the excited states of dyes, due to charge-transfer interactions.\(^{37}\)

The SWNT used in this study were prepared by Mr. Saied Zanganeh from the University of Connecticut Department of Electrical and Computer Engineering. Commercially available SWNTs (38), were functionalized with a carboxylic acid derivative by sonicating them in sulfuric acid and nitric acid, then adding hydrochloric acid. The carboxylic acid functionalized nanotubes (39) was then converted into an acid chloride (40) via thionyl chloride which was reacted with triethylenetetramine to generate the amine functionalized single walled nanotube (41), as shown in Scheme 14.
Scheme 14: Synthesis of Amine Functionalized Nanotubes\textsuperscript{36}

In order to attach the bis-carboxylic acid ICG (9) to the SWNT, it was functionalized to give acid chloride (42) using oxalyl chloride, and then reacted with the amine functionalized SWNT (41), binding the dye to the nanotube (43). Any excess dye that did not bind to the nanotubes was washed off by immersion in water, followed by passage through a fritted crucible. Since the dye is soluble in water and the nanotubes are insoluble, the unbound dye can be easily separated. It is unknown exactly where the dye binds to the amine, or exactly how much is bound because currently there is no way to accurately quantify how many of the dye molecules have become attached to the nanotubes, other than by weight difference.
Scheme 15: Synthesis of ICG Bound Nanotubes (43)
Since the nanotubes are insoluble in any NMR solvent, other methods had to be used to analyze the compound. Analysis was attempted with two instruments, an IR spectrometer and a Raman spectrometer. IR analysis was attempted on both Nexus 670 with DTGS detector and ATR attachment and a Bruker IFS 66v/S with MCT detector using KBr pellets. Data was gathered on the bis-carboxylic acid ICG (9) and the bis-carboxylic acid bound to the nanotubes (43). However, no data could be collected on the non-coupled nanotubes (41). This is thought to be because the nanotubes are black and the laser could not penetrate the sample. Therefore, there was no baseline to compare the bis-carboxylic bound to the nanotubes (43) to. Luckily, the amine functionalized nanotubes (41) could be analyzed with a Raman spectrometer, showing two significant peaks in the Raman spectrum at 1587.48 cm\(^{-1}\) and 2666.44 cm\(^{-1}\). The bis-carboxylic bound to the nanotubes (43) shows a peak at 1594.43 cm\(^{-1}\) which is shifted from 1587.48 cm\(^{-1}\) of the amine functionalized nanotubes. (Figure 21) Therefore it can be concluded the amide peak connecting the ICG to the nanotube did form. Figure 22 shows the Raman spectrum of bis-carboxylic acid ICG (9), similarities can be easily seen between the bound and unbound ICG, specifically at 1140.39 cm\(^{-1}\) and 1373.58 cm\(^{-1}\).
Figure 21: Raman Data Showing Amide Peak for ICG-Nanotube Connection

Figure 22: Raman Data Showing ICG
2.6 Bioassay of ICG-Nanotubes

Initial biological studies in mice showed that the ICG bound nanotubes congregated on the periphery of the hypoxic tumor, instead of penetrating the tumor. Looking at the tumors under a microscope, after they have been removed from the mouse, show the difference between the bis-carboxylic acid ICG (9) and the bis-carboxylic acid ICG bound to the CNT (43). (Figure 23) This lack of penetration is thought to be due to the size of the nanotubes, they might be too large to penetrate the tumor. To see if this is truly the case, smaller nanotubes were used to attach ICG dye to it. These smaller nanotubes are a few nanometers long, as opposed to the original nanotubes which were micrometers long.

Figure 23: (a) Tumor with Bis-carboxylic acid ICG (b) Tumor with Bis-carboxylic acid ICG Bound to Long Nanotubes – Unable to Penetrate the Tumor.

With the carboxylic acid half-loaded dyes (36a, 36b, 37a), the coupling of these dyes to the amine functionalized nanotubes (41) could be achieved in the same manner (Scheme 16). Coupling was done with 36, it was functionalized to give acid chloride (44) using oxalyl chloride, and then reacted with the amine functionalized mini SWNT
Any excess dye that did not bind to the nanotubes was washed off by immersion in water, followed by passage through a fritted crucible. This resulted in the each of the half loaded dyes being bound to the mini nanotubes giving compounds 45. Using the Raman spectrometer, a peak at 1599.50 cm$^{-1}$ can be seen, indicating that the dye has bound to the nanotubes.

**Figure 24: Raman Data Showing Half Loaded 2 Nitro Ethanolamine Acid Bound to CNT**
Scheme 16: Synthesis of Half Loaded Ethanolamine ICG Dyes Coupled to Carbon Nanotubes
3. Conclusion

In conclusion the synthesis of the half-loaded nitroimidazole indocyanine green dyes (36a, 36b, 37a) were achieved in 8-10% over 6-8 steps. By coupling the nitroimidazole units to the bis-carboxylic acid ICG in 1:1 molar equivalencies, the half loaded dye conjugates with a nitroimidazole on one end and a carboxylic acid on the other were obtained. Initially, the nitroimidazole units were attached to the methyl ester-acid ICG, which had one end blocked off, in order to enhance mono-substitution. However, due to the inability to remove the methyl ester from the compound without degradation, this route was not investigated.

With the synthesis of a half-loaded ICG dye, several new variations of the ICG can be synthesized, each with unique properties depending on the substituents attached. These half-loaded dye 36 were coupled to the mini carbon nanotubes to give 45. It is hoped that a larger amount of the ICG could be brought to the hypoxic tumor at a time, result in an increase in the observed fluorescence, and be retained in the tumor for an extended period of time. As of the writing of this thesis, results for the biological testing are pending.
4. Future Work

With the synthesis of this half loaded ICG, many different derivatives can be developed with various different properties. One such derivative would be to attach a cytotoxic agent to one end of the dye, such as cisplatin (46) or carboplatin (47). Cisplatin is a platinum based chemotherapy drug that works by binding to DNA, preferably guanine, initiating cross-linking resulting in apoptosis. Carboplatin is a second-generation chemotherapy platinum agent with less side effects than cisplatin. It also binds to DNA and results in apoptosis. Both compounds are currently being used to treat various types of cancers, including ovarian, testicular, lung, and some pediatric tumors. By attaching a chemotherapeutic drug to the half-loaded dye (48), the conjugate compound would be able to target the hypoxic tumor, exhibit fluorescence to image the tumor, and kill the tumor all on one shot.

![Figure 25: Cisplatin(46) and Carboplatin(47)](image-url)
Figure 26: Structure of Theoretical Half Loaded Dye with Carboplatin

Another compound that could be attached to the half loaded dye is biotin (49). Biotin useful because it can be used as a label for protein detection and allows for easy purification. This is due to its ability to strongly bind to the proteins avidin and streptavidin. (Figure 28)

Figure 27: Structure of Biotin

Figure 28: Protein Structures for Avidin and Streptavidin
Adding a biotin unit to the half-loaded dye (49) would enable extraction and isolation of any proteins associated with the hypoxic tumor that may be bound to the nitroimidazole dye-conjugate. Once the hypoxic tumor has been excised from the mouse and after treatment with the dye-conjugate, the cells can be passed through a column containing beads of avidin. The biotinylated dye with the attached proteins should adhere to the avidin beads, immobilizing the compounds, while everything else washes through the column. If a protein is isolated, it may be possible to determine how the nitroimidazole interacts with the hypoxic tumor, providing information that may allow the synthesis of a more specific dye.

![Figure 29 Structure of Theoretical Half-Loaded Dye Biotin Conjugate](image)

**Figure 29 Structure of Theoretical Half-Loaded Dye Biotin Conjugate**

A third use for the half loaded dye would be to quantify how much of the dye can be attached to the carbon nanotubes. In order to do this, the amine would be attached to the nanotube at multiple points, leaving only one nitrogen available for coupling with the half-loaded dye. The amine would form a cage-like structure on the nanotube, with the dye attached at a single point. (Figure 30) It is hoped that the nanotube could be partially soluble so it could be analyzed by NMR. If the nanotubes still remain insoluble, it is hoped that by attaching a spacer between the amine and the half loaded dye the compound will become soluble enough to quantify the amount of dye attached to the
nanotubes via NMR. Examples of suitable amine to try to attach to the nanotube to form these amine cages are Tris[2-(methylamino)ethyl]amine (51), Bis(3-aminopropyl)amine (52), and 1,2-Bis(3-aminopropylamino)ethane (53).

Figure 30: Example of Nanotube with Amine Cage Attached to Half-Loaded Dye

Figure 31: Suitable Amines to form Amine Cages on Nanotubes
5. Experimental

**General Information:** All chemicals were purchased from Sigma Aldrich or Acros. Unless indicated all chemicals and solvents were used as received. All glassware was oven dried before use, unless water was used as a reagent. $^1$H and $^{13}$C NMR were collected on Bruker Avance 300 (300.13 MHz $^1$H, 75.47 MHz $^{13}$C) or a Bruker DRX-400 (400.14 MHz $^1$H, 100.61 MHz $^{13}$C). Chemical shifts are given in ppm downfield from TMS. All RAMAN spectra were collected on a Horiba Jovin Yvon T64000 Triple Monochrometer Raman spectrometer, equipped with confocal microscope and laser-steering options, at a wavelength of 514 nm. All HRMS data was collected on a Qstar Elite (AB Sciex) using DART-AccuTOF or ESI-AccuTOF methods.

**2,3,3-Trimethyl-3H-indole-5-carboxylic acid (16):**

A 100 mL round-bottomed flask with a magnetic stirbar was charged with 4-hydrazinobenzoic acid (4.50 g, 29.6 mmol), 3-methyl 2-butanone (4.60 mL, 42.9 mmol), sodium acetate (4.86g, 59.2 mmol), and glacial acetic acid (55 mL). The mixture was placed under nitrogen and stirred for 1 hour, then heated at reflux overnight. The mixture was cooled to room temperature and the acetic acid was removed in vacuo. The resulting solid was washed with 80 mL of 10% methanol in water. The resulting precipitate was vacuum filtered off to yield a tan powder. (4.07 g, 20.0 mmol, 68%). Mp. Decompose, 205-208 °C. $^1$H NMR (CDCl$_3$): $\delta$ 1.36 (s, 6H), 2.36 (s, 3H), 7.64 (d, $J = 8.07$ Hz, 1H),
8.04 (d, \( J = 1.47 \) Hz, 1H), 8.14 (dd, \( J = 8.07 \) Hz, 1.71 Hz, 1H). \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \)

15.8, 23.1, 54.2, 119.9, 123.5, 126.9, 131.1, 145.9, 157.9, 171.4, 192.7.

**5-Carboxy-1-(\( \delta \)-sulfobutyl)-2,3,3-trimethyl-3H-indolium (17):**

\[
\text{HO} \quad \text{\includegraphics[width=0.2\textwidth]{molecule.png}} \quad \text{\text{O}} \quad \text{\text{O}} \quad \text{=} 
\]

A 100 mL round-bottomed flask with a magnetic stirbar was charged with indole acid (16) (1.00 g, 4.92 mmol) 1,4 butanesultone (2.95 mL, 28.8 mmol), and 1,2 dichlorobenzene (20 mL). The mixture was heated at reflux under nitrogen overnight. The mixture was cooled to room temperature, and then the precipitate was vacuum filtered and washed with acetone to yield a pale red powder. (1.46 g, 4.31 mmol, 88%)

Mp. Decompose, 230-232 °C. \(^1\)H NMR (MeOD): \( \delta \) 1.67 (s, 6H), 1.97 (m, 2H), 2.17 (m, 2H), 2.92 (t, \( J = 6.85 \) Hz, 2H), 4.61 (t, \( J = 7.82 \) Hz, 2H), 8.06 (d, \( J = 8.55 \) Hz, 1H), 8.32 (d, \( J = 8.55 \) Hz, 1H), 8.39 (s, 1H) \(^{13}\)C NMR (MeOD): \( \delta \) 22.6, 23.2, 27.3, 51.0, 56.2, 116.8, 125.6, 132.3, 133.7, 143.6, 145.6, 167.9, 201.1. (the single methyl group only is visible in a \(^{13}\)C DEPT NMR (MeOD): \( \delta \) 47.9)
Bis-1-1′-(4-sulfobutyl)indoletricarbocyanine-5,5′-dicarboxylic acid sodium salt (9):\textsuperscript{17}

\[
\begin{align*}
&\begin{array}{c}
\text{O} \\
\text{H} \\
\text{N} \\
\text{S} \\
\text{O} \\
\text{S} \\
\text{Na}^+ \\
\end{array}
\end{align*}
\]

A 100 mL round-bottomed flask with a magnetic stirbar was charged with the carboxylic acid indolium salt (17) (1.02 g, 3.00 mmol) and N-[5-(Phenylamino)-2,4-pentadienylidene]aniline monohydrochloride (0.344 g, 1.38 mmol). Then acetic anhydride (15 mL) and glacial acetic acid (9 mL) was added to flask. The mixture was stirred at room temperature as sodium acetate (0.40 g, 4.87 mmol) was added. The system was refluxed under nitrogen for 45 minutes. The mixture was cooled to room temperature, then poured into 150 mL of hot anhydrous ether. The solution was cooled at 5°C overnight, and then the precipitate was filtered off and washed with ether. The collected solid was recrystallized in a 1:4 mixture of water and propanol. The resulting crystals were filtered and dried for two days under vacuum to yield a green powder with a metallic yellow tinge. (0.85 g, 1.11 mmol, 80%). Mp. Decompose, 249-251°C \textsuperscript{1}H NMR (DMSO): \(\delta\) 1.64 (s, 12H), 1.73 (bd s, 8H), 4.08 (bd s, 4H), 6.5 (d, \(J = 13.5\) Hz, 2H), 6.63 (t, \(J = 12.0\) Hz, 2H), 7.48 (d, \(J = 8.56\) Hz, 2H), 7.94 (m, 5H), 8.06 (d, \(J = 1.46\) Hz, 2H), 12.87 (bs, 2H). \textsuperscript{13}C NMR (DMSO): \(\delta\) 23.1, 26.7, 27.7, 44.4, 49.1, 51.3, 105.0, 111.5, 123.9, 126.7, 131.4, 167.6.
5-Carboxylate-1-(δ-sulfobutyl)-2,3,3-trimethyl-3H-indolium (19):  

A 50 mL round-bottomed flask with a magnetic stirbar was charged with the carboxylic acid indolium salt (17) (1.01 g, 2.97 mmol), and absolute methanol (15 mL). The mixture was stirred at room temperature under nitrogen as oxalyl chloride (1.0 mL, 11.9 mmol) was added dropwise. Then the mixture was stirred for 24 hours. The mixture was reduced in vacuo to give a red paste. The crude product was stirred in ether until it became a suspension, about 24 hours. The solid was filtered and washed with ether to yield a red powder. (1.02 g, 2.89 mmol, 97%). Mp. Decompose 159 °C. $^1$H NMR (MeOD): δ 1.66 (s, 6H), 1.97 (bd s, 2H), 2.17 (bd s, 2H), 2.93 (bd s, 2H), 3.98 (s, 3H), 4.60 (bs, 2H), 8.06 (d, $J = 7.34$ Hz, 1H), 8.30 (d, $J = 6.60$ Hz, 1H), 8.39 (s, 1H). $^{13}$C NMR (MeOD): δ 22.6, 23.3, 27.3, 53.1, 56.3, 116.9, 125.5, 132.1, 133.0, 143.7, 145.8, 166.9, 201.3. HR-TOF MS: calculated for C$_{17}$H$_{24}$NO$_5$S: m/z 354.1375. Found: m/z 354.1375.
Bis-1-1’-(4-sulfobutyl)indoletetraacyanine-5-carboxy-5’-carboxylate sodium salt (18):\(^{17}\)

A 100 mL round-bottomed flask with a magnetic stirbar was charged with the carboxylic acid indolium salt (17) (0.50 g, 1.47 mmol) and \(N\)-[5-(Phenylamino)-2,4-pentadienylidene]aniline monohydrochloride (0.37 g, 1.49 mmol). Then acetic anhydride (19 mL) and glacial acetic acid (12 mL) were added to flask. The mixture was stirred as sodium acetate (0.48 g, 5.84 mmol) was added. The solution was heated at reflux under nitrogen for 30 minutes. Next the methyl ester indolium salt (19) (0.54 g, 1.52 mmol) was added to the flask, and the mixture continued to reflux for an additional 45 minutes. The mixture was cooled to room temperature, then poured into 150 mL of hot anhydrous ether. The solution was cooled at 5°C overnight, and then the precipitate was filtered and washed with ether. The collected solid was recrystallized in a 1:4 mixture of water and propanol. The crystals were filtered and dried for two days under vacuum to yield a blue green powder. (0.96 g, 1.24 mmol, 83%) Mp. Decompose 238-239 °C. \(^1\)H NMR (DMSO): \(\delta\) 1.63 (s, 12H) 1.73 (bd s, 8H), 3.86 (s, 3H), 4.15 (d, \(J = 37.2\) Hz, 4H), 6.48 (m, 4H), 7.47 (s, 1H), 7.52 (d, \(J = 9.05\) Hz, 2H), 7.84 (m, 2H), 7.97 (d, \(J = 7.34\) Hz, 2H), 8.08 (d, \(J = 7.34\) Hz, 2H), 12.96 (bs, 1H) \(^1\)\(^3\)C NMR (DMSO): \(\delta\) 23.16, 26.8, 27.7, 41.8, 49.4, 51.4, 52.8, 123.8, 124.0, 125.6, 131.3, 142.0, 147.1, 166.6, 167.7. HR-TOF MS:
Calculated for $\text{C}_{38}\text{H}_{46}\text{N}_{2}\text{O}_{10}\text{S}_{2}$: m/z 755.2594. Found: m/z 755.2736 Calculated for $\text{C}_{38}\text{H}_{45}\text{N}_{2}\text{NaO}_{10}\text{S}_{2}$: m/z 777.2413. Found: m/z 777.2540.

**Methyl 2-(4-nitro-1H-imidazol-1-yl)acetate (23a):**

![Chemical Structure](image)

A 50 mL round-bottomed flask with a magnetic stirbar was charged with 4-nitroimidazole (1.00 g, 8.89 mmol), tetrabutylammonium iodide (0.075 g, 0.20 mmol), potassium carbonate (1.80 g, 13.0 mmol), and freshly distilled acetonitrile (10 mL). The mixture was stirred at room temperature under nitrogen for 30 minutes. Then methyl bromoacetate (0.84 mL, 9.14 mmol) was added dropwise and system was heated at reflux for an additional 40 minutes. The mixture was cooled to room temperature, and the solids were filtered off, and washed with acetonitrile. The filtrate and washings was removed *in vacuo* and the crude solid was recrystallized in ethyl acetate to yield a white crystalline solid. (1.24 g, 6.70 mmol, 75%). Mp, 129-130 °C. $^1$H NMR (DMSO): $\delta$ 3.74 (s, 3H), 5.11 (s, 2H), 7.85. (s, 1H), 8.37 (s, 1H). $^{13}$C NMR (DMSO): $\delta$ 48.2, 52.5, 122.6, 138.3, 146.7, 168.0. HR-TOF MS: calculated for $\text{C}_{6}\text{H}_{8}\text{N}_{3}\text{O}_{4}$: m/z 186.0515. Found: m/z 186.0532.
Methyl 2-(2-nitro-1H-imidazol-1-yl)acetate (23b):\(^{17}\)

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{NO}_2 & \\
\text{O} & \\
\text{O} & \\
\end{align*}
\]

A 50 mL round-bottomed flask with a magnetic stirbar was charged with 2-nitroimidazole (0.093 g, 0.80 mmol) tetrabutylammonium iodide (0.013 g, 0.035 mmol), potassium carbonate (0.174 g, 1.23 mmol), and freshly distilled acetonitrile (5 mL). The mixture was stirred at room temperature under nitrogen for 30 minutes. Then methyl bromoacetate (0.073 mL, 0.78 mmol) was added dropwise to the system. The solution was heated under reflux for 40 minutes. The mixture was cooled, and the solids were filtered off and washed with acetonitrile. The solvent was removed \textit{in vacuo}, and the resulting solid was placed under high vacuum to dry overnight. The crude solid was recrystallized in ethyl acetate to yield an off-white crystalline solid. (0.06 g, 0.32 mmol, 40%) Mp, 95-96 °C. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 3.79 (s, 3H), 5.10 (s, 2H), 7.04 (s, 1H), 7.18 (s, 1H). \(^{13}\)C NMR (CDCl\(_3\)): \(\delta\) 50.9, 53.4, 126.7, 128.7, 144.8, 166.7. HR-TOF MS: calculated for C\(_6\)H\(_8\)N\(_3\)O\(_4\): m/z 186.0515. Found: m/z 186.0529.
N-(2-hydroxyethyl)-2-(4-nitro-1H-imidazol-1-yl)acetamide (24a):\(^{17}\)

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{N} \\
\text{N} & \quad \text{O} \\
\text{H} & \quad \text{N} \\
\text{O} & \quad \text{OH}
\end{align*}
\]

A 50 mL round-bottomed flask with a magnetic stirbar was charged with 4-nitromethylester (23a) (0.65 g, 3.52 mmol), 6 mL of absolute methanol. Then ethanolamine (0.90 mL, 14.9 mmol) was added dropwise, and the reaction was stirred under nitrogen overnight at ambient temperature. The resulting solid was vacuum filtered off, yielding a white solid. (0.61 g, 2.85 mmol, 81%) Mp. 136-137 °C. \(^1\)H NMR (MeOD): \(\delta\) 3.35 (t, \(J = 5.62\) Hz, 2H), 3.63 (t, \(J = 5.38\) Hz, 2H), 4.88 (s, 2H), 7.73 (s, 1H) 8.14 (s, 1H) \(^{13}\)C NMR (MeOD): \(\delta\) 43.1, 50.8, 61.2, 122.76, 139.2, 168.2. HR-TOF MS: calculated for C\(_7\)H\(_{11}\)N\(_4\)O\(_4\): m/z 215.0780. Found: m/z 215.0824.

N-(2-hydroxyethyl)-2-(2-nitro-1H-imidazol-1-yl)acetamide (24b):\(^{17}\)

\[
\begin{align*}
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{O} \\
\text{H} & \quad \text{N} \\
\text{O} & \quad \text{OH}
\end{align*}
\]

A 50 mL round-bottomed flask with a magnetic stirbar was charged with 2-nitromethylester (23b) (0.06 g, 0.33 mmol), 5 mL of absolute methanol. Ethanolamine (0.08 mL, 1.32 mmol) was then added dropwise, and the reaction was stirred under nitrogen overnight at ambient temperature. The methanol and excess ethanolamine were removed \emph{in vacuo}, yielding a yellow orange solid. (0.07 g, 0.33 mmol, 95%) Mp, 163.5-
164.5 °C. $^1$H NMR (MeOD): $\delta$ 3.37 (t, $J = 5.87$ Hz, 2H), 3.62 (t, $J = 5.62$ Hz, 2H), 5.20 (s, 2H), 7.18 (s, 1H), 7.46 (s, 1H). $^{13}$C NMR (MeOD): $\delta$ 43.2, 52.8, 61.4, 128.3, 129.3, 146.3, 168.3. HR-TOF MS: calculated for C$_{7}$H$_{11}$N$_{4}$O$_{4}$: m/z 215.0780. Found: m/z 215.0800.

2-(4-nitro-1H-imidazol-1-yl)acetic acid (27):$^{42}$

![2-(4-nitro-1H-imidazol-1-yl)acetic acid](image)

A 50 mL round-bottomed flask with a magnetic stirbar was charged with 4-nitroimidazole ester (23a) (1.00 g, 5.40 mmol), and 30 mL of water. The system was heated under reflux for 24 hours. Then, the solution was cooled, and any remaining solid was filtered off. The water was removed in vacuo, and solid was placed in drying pistol with phosphorus pentoxide for 24 hours, to give a white solid (0.88 g, 5.14 mmol, 97%) Mp, 151-152 °C. $^1$H NMR (DMSO): $\delta$ 4.98 (s, 2H), 7.83 (s, 1H), 8.37 (s, 1H). $^{13}$C NMR (MeOD): $\delta$ 53.3, 122.8, 139.2, 148.4, 170.0. HR-TOF MS: calculated for C$_{5}$H$_{6}$N$_{3}$O$_{4}$: m/z 172.0358. Found: m/z 172.0369.
2-(4-nitro-1H-imidazol-1-yl)acetyl chloride (28):43

A 50 mL round-bottomed flask with a magnetic stirbar was charged with dried 4-nitroimidazole acid (27) (0.24 g, 1.37 mmol), freshly distilled tetrahydrofuran (10 mL), and dried dimethylformamide (0.20 mL, 2.58 mmol). The system was placed under nitrogen and cooled in ice bath. Then, oxalyl chloride (0.24 mL, 2.79 mmol) was added dropwise to solution. The system was warmed to room temperature and stirred for 2 hours. The solution turned a bright yellow color when the reaction was complete. This compound was used in situ. However no data could be collected on this product because while attempting to isolate this product it hydrolyzed upon removal from the solution, based on NMR and IR data.

Tert-butyl piperazine-1-carboxylate (25):

A 250 mL round bottom flask with a magnetic stirbar was charged with piperazine (3.42 g, 39.7 mmol), 47.5 mL of tert-butyl alochol, and 48.5 mL of distilled water. The flask was placed in an ice bath, and 6.3 mL of 2.5 N NaOH was added dropwise, followed by di-tert-butyl dicarbonate (3.48 g, 16.0 mmol). The contents of flask were stirred until everything had dissolved, then it was removed from the ice bath and continued to stir at
ambient temperature overnight. The mixture solvent was removed in vacuo, and the resulting solid was taken up in water. The solution was extracted with three 35 mL aliquots of DCM, then washed with three 25 mL aliquots of brine. The solution was removed in vacuo to yield a white solid. (2.11 g, 11.3 mmol, 71%) Mp, 139-141 °C. $^1$H NMR (CDCl$_3$): $\delta$ 1.48 (s, 9H), 2.83 (t, $J = 4.41$ Hz, 4H) 3.41 (t, $J = 5.37$ Hz, 4H) $^{13}$C NMR (CDCl$_3$): $\delta$ 28.7, 46.1, 79.8, 155.1.

Tert-butyl 4-(2-(4-nitro-1H-imidazol-1-yl)acetyl)piperazine-1-carboxylate (29):$^{44}$

![Chemical structure](attachment:image_url)

A solution of 4-nitroimidazole acid chloride (28) was prepared from 4-nitroimidazole acid (27) (0.45 g, 2.64 mmol). The solution was cooled in an ice bath, and monosubstituted BOC-Piperazine (25) (2.93 g, 15.7 mmol) in 10 mL of dry THF was added via canula. The mixture was stirred at room temperature for 1 hour, and then the solvents were removed in vacuo. The solid was taken up in water, and the insoluble compounds were filtered off. The remaining solid was then separated via column chromatography. The solvent system for the column consisted of DCM and MeOH. The column began with 100% DCM and in a gradient worked towards 5% MeOH in DCM, to give a fine white powder. (0.21 g, 0.622 mmol, 23%) $^1$H NMR (CDCl$_3$): $\delta$ 1.51 (s, 9H), 3.50 (bd s, 4H), 3.58 (bd s, 2H), 3.67 (bd s, 2H), 7.46 (s, 1H), 7.83 (s, 1H). $^{13}$C NMR (CDCl$_3$): $\delta$
28.2, 42.1, 44.6, 48.6, 80.6, 121.1, 137.1, 147.9, 154.2, 163.1, 180.8. HR-TOF MS: calculated for C$_{14}$H$_{22}$N$_{5}$O$_{5}$: m/z 340.1621. Found: m/z 340.1607.

**Tert-butyl 4-(2-bromoacetyl)piperazine-1-carboxylate (32):**

![Tert-butyl 4-(2-bromoacetyl)piperazine-1-carboxylate (32)](image)

A 100 ml round bottom flask with a magnetic stirbar was charged with tert-butyl piperazine-1-carboxylate (25) (0.78 g, 4.18 mmol) and dry dichloromethane (25 mL). The flask was placed into an ice bath and triethylamine (0.63 mL, 4.55 mmol) was added, then the mixture was stirred for 15 minutes. Next, α-bromoacetyl bromide (0.40 mL, 4.55 mmol) was added drop wise to the flask at 0°C and the mixture warmed to room temperature and stirred overnight. The reaction progress was monitored by TLC. After the reaction was complete, it was concentrated *in vacuo* and purified through column chromatography, (15-20% petroleum ether in ethyl acetate) to yield a white solid (0.80 g, 2.60 mmol, 81%) was isolated. $^1$H NMR (CDCl$_3$): δ 1.45 (s, 9H), 3.42 (t, $J = 12$ Hz, 2H), 3.48 (m, 4H), 3.58 (t, $J = 8$ Hz , 2H), 3.85 (s, 2H). $^{13}$C NMR (CDCl$_3$): δ 25.8, 28.6, 42.2, 46.8, 154.7, 165.7. HR-TOF MS: calculated for C$_{11}$H$_{19}$BrN$_{2}$O$_{3}$: m/z 307.0657. Found: m/z 307.0652.
Tert-butyl 4-(2-(2-nitro-1H-imidazol-1-yl)acetyl)piperazine-1-carboxylate (33):

A 50 ml round bottom flask with a magnetic stirbar was charged 2-nitroimidazole (0.10 g, 0.88 mmol) and dry DMF (1mL), and cooled to 0 °C. Then sodium hydride (0.025g, 0.11 mmol) was added and the mixture was stirred under nitrogen for 30 minutes. Next tert-butyl 4-(2-bromoacetyl) piperazine-1-carboxylate (32) (0.30g, 0.98 mmol) was added slowly and the reaction mixture was stirred at room temperature overnight. The DMF was evaporated off and water (5 mL) was added to give white precipitate, which was filtered and dried under vacuum, yielding a white powder (0.28 g, 0.825 mmol, 93%). Mp 150-174°C. ¹H NMR (CDCl₃): δ 1.46 (s, 9H), 3.46-3.45 (m, 2H), 3.50 (m, 2H), 3.60-3.57 (t, J = 12Hz, 4H), 5.21 (s, 2H), 7.05 (s, 1H), 7.19-7.18 (d, J = 4Hz, 1H). ¹³C NMR (CDCl₃): δ HR-TOF MS: calculated for C₁₄H₂₂N₅O₅: m/z 340.1621. Found: m/z 340.1608.

2-(4-nitro-1H-imidazol-1-yl)-1-(piperazin-1-yl)ethanone TFA salt (30):

A 50 mL round bottom flask was charged with a magnetic stirbar 4-nitroimidazole BOC – Piperazine (29) (0.19 g, 0.551 mmol) and 13 mL of dry CHCl₃ was added.
Trifluoroacetic acid (1.0 mL, 13.0 mmol) was added dropwise, then the mixture stirred at room temperature overnight under nitrogen. The chloroform was removed \textit{in vacuo}, and the crude solid was suspended in ethyl acetate. The solution was cooled in ice bath, the flask was scratched and resulting solid was filtered, resulting in a white powder (0.123 g, 0.360 mmol, 94 \%). Mp, 213-215 °C. \textit{H} NMR (D$_2$O): \( \delta 3.34 \) (t, 2H, \( J = 5.85 \) Hz) 3.42 (t, 2H, \( J = 4.10 \) Hz), 3.88 (t, 4H, \( J = 4.97 \) Hz), 5.30 (s, 2H), 7.71 (s, 1H), 8.13 (s, 1H).

\textit{C} NMR (D$_2$O): \( \delta 39.3, 41.7, 42.9, 49.1, 123.1, 138.9, 146.7, 166.8 \). HR-TOF MS: calculated for C$_{10}$H$_{14}$F$_3$N$_5$O$_5$: m/z 240.1096. Found: m/z 240.1100.

2-(2-nitro-1H-imidazol-1-yl)-1-(piperazin-1-yl)ethanone TFA salt (31):

In 50 mL round bottom flask with a magnetic stirbar 2-nitroimidazole BOC – Piperazine (33) (0.28 g, 0.83 mmol) and 10 mL of dry CHCl$_3$ was added. Trifluoroacetic acid (1.0 mL, 23 mmol) was added dropwise, then the mixture was stirred at room temperature overnight under nitrogen. The chloroform was removed \textit{in vacuo}, and the crude solid was suspended in ethyl acetate. The solution was cooled in an ice bath, and the flask was scratched and resulting solid was vacuum filtered, resulting in a white powder (0.25 g, 0.732 mmol, 86 \%). Mp. 95-96 °C. \textit{H} NMR (D$_2$O): \( \delta 3.49 \) (t, 2H), 3.59 (t, 2H), 4.01 (t, 2H), 4.08 (t, 2H), 5.68 (s, 2H), 7.40 (s, 1H), 7.58 (s, 1H). \textit{C} NMR (D$_2$O): \( \delta 39.6, 42.2, \)
A 25 mL round-bottomed flask with a magnetic stirbar was charged acid/ester dye (18) (0.200 g, 0.257 mmol) and 2.5 mL of dry DMF, and placed under nitrogen. The flask was cooled in an ice bath, then diisopropylethylamine (0.07 mL, 0.40 mmol) was added dropwise, followed by an addition of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (0.216 g, 0.415 mmol) and hydroxybenzotriazole (HOBt) (0.053 g, 0.39 mmol). The contents were stirred at 0°C for 30 minutes, and then 4-nitroethanolamine (24a) (0.055 g, 0.26 mmol) was added. The mixture was warmed to room temperature, and continued to stir for 2 to 3 days. The DMF was slowly removed by evaporation, induced by passing a stream of air over the liquid in the flask, and then ethyl acetate was added to the resulting solid, and stirred for 20 minutes. The remaining solid was filtered off, and then stirred in CHCl₃ for 20 minutes. The solid was again filtered, and then stirred in CH₃CN for 20 minutes. The solid was filtered and then adsorbed to reverse phase silica gel. The sample was then passed through a reverse phase
automated column, using a gradient of 0% up to 40% methanol in water, resulting in dark purple solid. (0.050 g, 0.051 mmol, 20%) Mp, Decompose > 200 °C. (Because this is such a dark solid it was difficult to determine at what temperature it decomposed) $^1$H NMR (DMSO): $\delta$ 1.67 (s, 12H), 1.75 (bd s, 8H), 3.52 (bd s, 2H), 3.87 (s, 3H), 4.12 (bd s, 4H), 4.31 (t, $J$ = 5.38 Hz, 2H), 4.88 (s, 2H), 6.56 (d, $J$ = 11.49 Hz, 2H), 6.67 (t, $J$ = 11.25 Hz, 2H), 7.54 (t, $J$ = 7.34 Hz, 2H), 7.80 (s, 1H), 7.92-8.00 (m, 6H), 8.11 (d, $J$ = 5.62 Hz, 2H), 8.30 (s, 1H), 8.60 (t, $J$ = 6.84 Hz, 1H) $^{13}$C NMR: Sample has been submitted, but unable to obtain a decent spectrum. HR-TOF MS: calculated for C$_{45}$H$_{55}$N$_6$O$_{13}$S$_2$: m/z 951.3269. Found; m/z 951.3321. Calculated for C$_{45}$H$_{54}$N$_6$NaO$_{13}$S$_2$: m/z 973.3088. Found: m/z 973.3196.

Sodium 4-((2-(1E,3E,5E,7Z)-7-(3,3-dimethyl-5-(2-(2-nitro-1H-imidazol-1-yl)acetamido)ethoxy)carbonyl)-1-(4-sulfonatobutyl)indolin-2-ylidene)hepta-1,3,5-trienyl)-5-(methoxycarbonyl)-3,3-dimethyl-3H-indolium-1-yl)butane-1-sulfonate (34b):$^{46}$

![Chemical Structure](image)

A 25 mL round-bottomed flask with magnetic stirbar was charged with the acid/ester dye (18) (0.248 g, 0.319 mmol) and 2.5 mL of dry DMF, and placed under nitrogen. The flask was cooled in an ice bath, then diisopropylethylamine (0.09 mL, 0.52 mmol) was added dropwise, followed by an addition of benzotriazol-1-yl-oxytrpyrrolidinophosphonium hexafluorophosphate (PyBOP) (0.271 g, 0.520 mmol)
and hydroxybenzotriazole (HOBt) (0.072 g, 0.53 mmol). The contents were stirred at 0°C for 30 minutes, and then 2-nitroethanolamine (24b) (0.072 g, 0.33 mmol) was added. The mixture was warmed to room temperature, and continued stirring for 2 to 3 days. The DMF was slowly removed by evaporation, induced by passing a stream of air over the liquid in the flask, and then ethyl acetate was added to the resulting solid, and stirred for 20 minutes. The remaining solid was filtered off, and then stirred in CHCl₃ for 20 minutes. The solid was again filtered, and then stirred in CH₃CN for 20 minutes. The solid was filtered and then adsorbed to reverse phase silica gel. The sample was then passed through a reverse phase automated column, using a gradient of 0% up to 40% methanol in water, resulting in dark purple solid. (0.11 g, 0.11 mmol, 35%) Mp, Decompose > 200 °C. (Because this is such a dark solid it was difficult to determine at what temperature it decomposed) ¹H NMR (DMSO):  δ 1.67 (s, 12H), 1.74 (bd s, 8H), 3.53 (bd s, 2H), 3.71 (s, 3H), 4.11 (bd s, 4H), 4.29 (bd s, 2H), 5.14 (bd s, 2H), 6.54 (d, J = 13.21 Hz, 2H), 6.67 (t, J = 9.98 Hz, 3H), 7.53 (d, J = 8.31 Hz, 2H), 7.63 (s, 1H), 7.84-8.09 (m, 7H), 8.12 (s, 1H), 8.63 (t, J = 5.87 Hz 1H). ¹³C NMR: Sample has been submitted, but unable to obtain a decent spectrum. HR-TOF MS: calculated for C₄₅H₅₅N₆O₁₃S₂: m/z 951.3269. Found: m/z 951.3521. Calculated for C₄₅H₅₄N₆NaO₁₃S₂: m/z 973.3088. Found: m/z 973.3096.
A 25 mL round-bottomed flask with a magnetic stirbar was charged with the acid/ester dye (18) (0.199 g, 0.256 mmol) and 2.5 mL of dry DMF, and placed under nitrogen. The flask was cooled in an ice bath, then diisopropylethylamine (0.07 mL, 0.401 mmol) was added dropwise, followed by an addition of benzotriazol-1-yl oxytripyrrrolidinophosphonium hexafluorophosphate (PyBOP) (0.210 g, 0.403 mmol) and hydroxybenzotriazole (HOBt) (0.054 g, 0.40 mmol). The contents were stirred at 0°C for 30 minutes, then 4-nitropiperazine (30) (0.065 g, 0.27 mmol) was added. The mixture was warmed to room temperature, and continued stirring for 2 to 3 days. The DMF was slowly removed by evaporation, induced by passing a stream of air over the liquid in the flask, and then ethyl acetate was added to the resulting solid, and stirred for 20 minutes. The remaining solid was filtered off, and then stirred in CHCl₃ for 20 minutes. The solid was again filtered, and then stirred in CH₃CN for 20 minutes. The solid was filtered and then adsorbed to reverse phase silica gel. The sample was then passed through a reverse phase automated column, using a gradient of 0% up to 70% methanol in water, resulting in dark purple solid. No data was gathered on this
compound, because compound had decomposed due to excessive heating while trying to isolate. Believed to initially have compound based on TLC analysis, but was not repeated because methyl ester could not be removed.

**Sodium 4-(5-carboxy-2-((I,E,3E,5E,7Z)-7-(3,3-dimethyl-5-((2-(2-(4-nitro-1H-imidazol-1-yl)acetamido)ethoxy)carbonyl)-1-(4-sulfonatobutyl)indolin-2-ylidene)hepta-1,3,5-trienyl)-3,3-dimethyl-3H-indolium-1-yl)butane-1-sulfonate (36a):**

![Chemical Structure](image)

A 25 mL round-bottomed flask with a magnetic stirbar was charged with the bis-carboxylic acid ICG dye (9) (0.160 g, 0.210 mmol) and 2.5 mL of dry DMF, and placed under nitrogen. The flask was cooled in an ice bath, then diisopropylethylamine (0.050 mL, 0.35 mmol) was added dropwise, followed by an addition of benzotriazol-1-yl-oxytrypyrrolidinophosphonium hexafluorophosphate (PyBOP) (0.177 g, 0.340 mmol) and hydroxybenzotriazole (HOBt) (0.046 g, 0.34 mmol). The contents were stirred at 0°C for 30 minutes, then 1 equivalent of 4-nitroethanolamine (24a) (0.047 g, 0.22 mmol) was added. The mixture was warmed to room temperature, and continued stirring for 2 to 3 days. The DMF was slowly removed by evaporation, induced by passing a stream of air over the liquid in the flask, and then ethyl acetate was added to the resulting solid, and stirred for 20 minutes. The remaining solid was filtered off, and then stirred in CHCl₃ for 20 minutes. The solid was again filtered, and then stirred in CH₃CN for 20 minutes. The
solid was filtered and then adsorbed to reverse phase silica gel. The sample was then passed through a reverse phase automated column, using a gradient of 0% up to 70% methanol in water, resulting in dark purple solid. (0.067 g, 0.070 mmol, 33%) Mp, Decompose > 200 °C. (Because this is such a dark solid it was difficult to determine at what temperature it decomposed) $^1$H NMR (DMSO): $\delta$ 1.67 (s, 12H), 1.75 (bd s, 8H), 3.52 (bd s, 2H), 3.87 (s, 3H), 4.12 (bd s, 4H), 4.31 (t, $J = 5.38$ Hz, 2H), 4.88 (s, 2H), 6.56 (d, $J = 11.49$ Hz, 2H), 6.67 (t, $J = 11.25$ Hz, 2H), 7.54 (t, $J = 7.34$ Hz, 2H), 7.80 (s, 1H), 7.92-8.00 (m, 6H), 8.11 (d, $J = 5.62$ Hz, 2H), 8.30 (s, 1H), 8.60 (t, $J = 6.84$ Hz, 1H), 12.93 (bd s, 1H). $^{13}$C NMR: Sample has been submitted, but unable to obtain a decent spectrum. HR-TOF MS: calculated for C$_{44}$H$_{51}$N$_6$O$_{13}$S$_2$: m/z 937.3112. Found; m/z 937.3123. Calculated for C$_{44}$H$_{51}$N$_6$NaO$_{13}$S$_2$: m/z 959.2931. Found: m/z 959.2970.

Sodium 4-(5-carboxy-2-((JE,3E,5E,7Z)-7-(3,3-dimethyl-5-((2-(2-(2-nitro-1H-imidazol-1-yl)acetamido)ethoxy)carbonyl)-1-(4-sulfonatobutyl)indolin-2-ylidene)hepta-1,3,5-trienyl)-3,3-dimethyl-3H-indolium-1-yl)butane-1-sulfonate (36b):$^{46}$

![Chemical structure image]

A 25 mL round-bottomed flask with a magnetic stirbar was charged with the bis-carboxylic acid ICG dye (9) (0.192 g, 0.252 mmol) and 2.5 mL of dry DMF, and placed under nitrogen. The flask was cooled in an ice bath, then diisopropylethylamine (0.05 mL, 0.35 mmol) was added dropwise, followed by an addition of benzotriazol-1-yl-
oxytripyrrrolinophosphonium hexafluorophosphate (PyBOP) (0.176 g, 0.338 mmol) and hydroxybenzotriazole (HOBt) (0.046 g, 0.34 mmol). The contents were stirred at 0°C for 30 minutes, then 1 equivalent of 2-nitroethanolamine (24b) (0.060 g, 0.27 mmol) was added. The mixture was warmed to room temperature, and continued stirring for 2 to 3 days. The DMF was slowly removed by evaporation, induced by passing a stream of air over the liquid in the flask, and then ethyl acetate was added to the resulting solid, and stirred for 20 minutes. The remaining solid was filtered off, and then stirred in CHCl₃ for 20 minutes. The solid was again filtered, and then stirred in CH₃CN for 20 minutes. Then placed under a high vacuum to dry, resulting in a dark blue green solid, however this is a mixture of starting material and product. If ran on the Combi Flash there would not be enough material for the next step. (0.172 g, 0.179 mmol, 72%) Mp, Decompose > 200 °C. (Because this is such a dark solid it was difficult to determine at what temperature it decomposed) ¹H NMR (DMSO): δ 1.65 (s, 12H), 1.73 (bd s, 8H), 3.53 (bd s, 2H), 4.09 (bd s, 4H), 4.29 (bd s, 2H), 5.08 (bd s, 2H), 6.46 (d, J = 12.21 Hz, 2H), 6.62 (t, J = 15.23 Hz, 3H), 7.46 (t, J = 4.64 Hz, 2H), 7.75 (d, J = 8.32 Hz, 2H), 7.84 (s, 1H), 7.91-8.05 (m, 7H), 8.14 (s, 1H), 8.47 (t, J = 5.87 Hz 1H), 12.92 (bd s, 1H). ¹³C NMR: Sample has been submitted, but unable to obtain a decent spectrum. HR-TOF MS: calculated for C₄₄H₅₁N₆O₁₃S₂: m/z 937.3112. Found; m/z 937.3244. Calculated for C₄₄H₅₁N₆NaO₁₃S₂: m/z 959.2931. Found: m/z 959.2865.
Sodium 4-(5-carboxy-2-((1E,3E,5E,7Z)-7-(3,3-dimethyl-5-(4-(2-(4-nitro-1H-imidazol-1-yl)acetyl)piperazine-1-carbonyl)-1-(4-sulfonatobutyl)indolin-2-ylidene)hepta-1,3,5-trienyl)-3,3-dimethyl-3H-indolium-1-yl)butane-1-sulfonate (37a):

A 25 mL round-bottomed flask with a magnetic stirbar was charged with the bis-carboxylic acid ICG dye (9) (0.151 g, 0.202 mmol) and 2.5 mL of dry DMF, and placed under nitrogen. The flask was cooled in an ice bath, then diisopropylethylamine (0.056 mL, 0.32 mmol) was added dropwise, followed by an addition of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (0.166 g, 0.323 mmol) and hydroxybenzotriazole (HOBt) (0.0434 g, 0.32 mmol). The contents were stirred at 0°C for 30 minutes, then 1 equivalent of 4-nitropiperazine (30) (0.065 g, 0.20 mmol) was added. The mixture was warmed to room temperature, and continued stirring for 2 to 3 days. The DMF was slowly removed by evaporation, induced by passing a stream of air over the liquid in the flask, and then ethyl acetate was added to the resulting solid, and stirred for 20 minutes. The remaining solid was filtered off, and then stirred in CHCl₃ for 20 minutes. The solid was again filtered, and then stirred in CH₃CN for 20 minutes. The solid was filtered and then adsorbed to reverse phase silica gel. The sample was then passed through a reverse phase automated column, using a gradient of 0% up to 70% methanol in water, resulting in dark purple solid. (0.030 g, 0.030 mmol, 16%) Mp,
Decompose > 200 °C. (Because this is such a dark solid it was difficult to determine at
what temperature it decomposed) $^1$H NMR (DMSO): $\delta$ 1.67 (s, 12H), 1.74 (bd s, 8H),
3.13 (bd s, 4H), 3.59 (bd s, 4H), 4.11 (bd s, 4H), 4.60 (s, 2H), 5.20 (bd s, 2H), 6.49 (d, $J$
= 14.18 Hz, 2H), 6.65 (t, $J = 11.01$ Hz, 3H), 7.52 (m, 2H), 7.74 (s, 1H), 7.56-8.15 (m, 7H),
8.26 (s, 1H), 12.90 (bd s, 1H) $^{13}$C NMR: Sample has been submitted, but unable to
obtain a decent spectrum. HR-TOF MS: calculated for C$_{46}$H$_{54}$N$_7$O$_{12}$S$_2$: m/z 962.3428.
Found; m/z 962.3513. Calculated for C$_{46}$H$_{54}$N$_7$NaO$_{12}$S$_2$: m/z 984.3248. Found: m/z
984.3255.

**Amine Functionalized Single Walled Carbon Nanotubes (41):**

![Amine Functionalized Single Walled Carbon Nanotubes](image)

Prepared by Mr. Saied Zanganeh from the University of Connecticut Department of
Electrical and Computer Engineering. The SWCNTs were immersed in a mixture of
H$_2$SO$_4$ and HNO$_3$ (3:1) at room temperature. Then treated in an ultrasound bath for 2
hours and suspended for 15 hours. Next hydrochloric acid was added to the solution.
The solution was neutralized with ammonium hydroxide and filtered through a 0.22 mm
cellulose acetate membrane. The SWCNTs were washed several times with deionized
water until pH 5.5 was reached. The residue was dried in an 80 °C vacuum oven for 12
hours to obtain carboxylated carbon nanotubes SWCNT-COOH. These were then
reacted with thionyl chloride, resulting in SWCNT functionalized with acid chlorides
moieties, SWCNT-COCl. Next, the acid chloride SWCNT was reacted with triethylenetetramine generating SWCNT-NH$_2$. Addition of the functional groups is confirmed via dispersion in aqueous solutions. Non-functionalized SWCNTs do not disperse, functionalized SWCNTs do because the presence of same-sized charged particles on the surface of SWCNTs enables the SWCNTs to repel from each other, keeping them dispersed in solution.

**Bis-Carboxylic Acid ICG Coupled to Amine Functionalized Single Walled Carbon Nanotubes (43):**

Bis-carboxylic acid ICG (9) (0.192 g, 0.25 mmol) was dissolved in oxalyl chloride (1 mL, 11.7 mmol) and stirred at room temperature under nitrogen until the formation of bubbles stopped. The an additional amount of oxalyl chloride (1 mL, 22.7 mmol) was added and the solution was refluxed for 1 hour. The solution was cooled, the solvents were removed, and the resulting solid placed under high vacuum overnight. To this solid, 3 mL of dry toluene was added followed by amine functionalized SWNTs (0.064 g). The mixture was stirred at room temperature for 1 hour and then heated at reflux overnight. The mixture was cooled, and then the solid was filtered off. The solid was taken up with water and filtered. This was repeated until the filtrate is no longer colored green to give a
black and green solid (0.07g). It is unknown exactly where and how many ICG dye were bound to the SWNT. Raman Analysis: 1140.39, 1373.43, 1594.43 cm\(^{-1}\).

**Half Loaded 2-Nitroimidazole Ethanolamine ICG Coupled to Amine Functionalized Single Walled Carbon Nanotubes (45):**\(^{36}\)

A round bottom flask with a magnetic stirbar was charged with half loaded 2-nitroethanolamine ICG (36b) (0.172 g, 0.179 mmol) was dissolved in oxalyl chloride (1 mL, 11.7 mmol) and stirred at room temperature under nitrogen until the formation of bubbles stopped. The an additional amount of oxalyl chloride (1 mL, 22.7 mmol) was added and the solution was refluxed for 1 hour. The solution was cooled, the solvents were removed, and the resulting solid placed under high vacuum overnight. To this solid, 3 mL of dry toluene was added followed by amine functionalized SWNTs (0.054 g). The mixture was stirred at room temperature for 1 hour and then heated at reflux overnight. The mixture was cooled, and then the solid was filtered off. The solid was taken up with water and filtered. This was repeated until the filtrate is no longer colored green to give a black and green solid (0.099 g). It is unknown exactly where and how many half loaded
2-nitroethanolamine ICG (36b) were bound to the SWNT. Raman Analysis: 1140.52, 1373.64, 1599.50, 2693.26 cm$^{-1}$.
References


   **2003**, *30* (6), 1039-1047.


41. EMBL-EBI, European Bioinformatics Institute. (accessed 6-7-2012).


