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Photoreactions of a Water Soluble Poly-isoquinolpyrrole with Plasmid DNA



Honors Thesis

Gregory Versteeg

Department: Chemistry

Advisor: Shawn Swavey, Ph.D.

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Abstract

With the goal of creating a porphyrin we reacted isoquinol pyrrole, p-fluorobenzaldehyde, and boron trifluoride etherate under appropriate conditions. Instead of a porphyrin being synthesized, we created polyisoquinol pyrrole in high yield and with great absorption properties around 600nm. We characterized this compound via UV/vis spectroscopy, proton NMR, and electrochemistry. The compound was then reacted with $[\text{Ru}(\text{bipy})_2\text{Cl}_2]$ to create a cationic compound and this molecule was tested as a possible photosensitizer for photodynamic therapy (PDT) but it was unable to photocleave plasmid DNA above 550nm. The original compound was then methylated and this new compound was also tested as a photosensitizer. This complex was successful at photocleaving DNA above 550nm and appears to be a promising photosensitizer.

Dedication

The author would like to dedicate his honors thesis to his family, especially his loving parents, Madeline and Donald Versteeg for their constant support and undying love.



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Introduction

Photodynamic therapy (PDT) has been known for many years but is currently gaining popularity due to its ability to fight many different cancers with minimal side effects. PDT is an efficient procedure for many different cancers ranging from skin and esophageal, to bladder, breast, and lung cancer.¹⁻³ Current treatments for cancer like chemotherapy and radiation have negative side effects that harm the patient. Conversely, PDT has few side effects, is minimally invasive, and targets tumor cells due to their interaction with LDL and the upregulation of LDL receptors on tumor cells.⁷ PDT requires three things; light, a photosensitizer, and molecular oxygen which separately are harmless but together can kill tumor cells.⁴ Clinically, the light can be added via a fiber optic cable to directly irradiate the tumor.¹ A photosensitizer is a molecule that absorbs light and can transfer it to other compounds.¹⁶ When the photosensitizer gets excited by the correct wavelength of light, an electron becomes excited to the triplet state.¹⁶ When this happens, four things can occur. The electron can fall down to the ground state and give off energy as fluorescence. There are three mechanisms that can occur when the electron is excited, type I is when the electron gets transferred to molecular oxygen and superoxide or other reactive oxygen species are created.⁵ Type II reaction occurs when the electron falls to the ground state, it transfers energy to triplet oxygen and creates singlet oxygen.⁵ This reaction preferably occurs because the excited photosensitizer is in the triplet state and ground oxygen is in the triplet state as well, so the energy transfer is easy.¹⁶ Singlet oxygen is both toxic in the cell and has a long half life, a little less than 0.04 μsec , and a radius of effect around 0.02 μm .⁵ This small radius ensures that PDT is a very localized reaction. Type III reaction is where the photosensitizer reacts directly with molecules in the cell by acting like an oxidizing agent.⁶ If the photosensitizer is bound to DNA, the nucleotides will be affected, most likely Guanine since it is the easiest nucleotide to oxidize. All three of these mechanisms are toxic to the cell but type II is most sought after. It is believed that singlet oxygen is what is toxic enough to kill the cell by reacting with cellular

components so this is the most sought after mechanism for the photosensitizer to undergo.¹²

The most well known, FDA approved, photosensitizer used is the hematoporphyrin derivative Photofrin (porfimer sodium). Although Photofrin is being used in the clinic, it has some disadvantages. These disadvantages include long-lasting skin photosensitivity and low singlet oxygen production at 630nm.⁸ In addition, synthesis of Photofrin suffers from purification difficulties, dark toxicity, low tissue penetration of the light, and a high concentration of compound is required in order to be effective.^{13, 14} Due to the numerous issues with Photofrin, new photosensitizers are very desirable and are currently being sought. The ideal photosensitizer should have several properties. It should have minimal dark toxicity, high quantum yield of singlet oxygen production, tumor specificity, and absorption in the 700-800nm range.¹⁷ This red region is ideal since light scattering is minimal, the depth of penetration is deeper than wavelengths that are below 600nm (the range from 600-800nm is described as the PDT window), and the photosensitizer still can be excited by the low energy of the wavelength.^{17, 18} Porphyrins are currently being researched as potential photosensitizers due to their lack of dark toxicity, their strong absorption properties, and their tumor affinity. Some porphyrins have already been proven to be more effective PDT substitutes for Photofrin however the absorption spectrum of these porphyrins remains too high in energy and thus the required light cannot penetrate far into tissue.¹¹ An additional attribute of porphyrins is their nuclear localization and specifically the ability of cationic porphyrins which have been shown to interact with DNA.¹⁰⁻¹¹ Since generic porphyrins are insoluble in water, and require higher energy light to be activated, there is a need to synthesize new novel porphyrins that overcome these obstacles.

In this report we describe our attempt to create a novel cationic porphyrin and the results of the synthesis. We describe the synthesis and characterization by ¹H NMR, UV/Vis spectroscopy, electrochemistry, and elemental analysis of a new polyisoquinol pyrrole complex with good synthetic yield and absorption properties. We investigate the ability of ruthenated and methylated derivative of

the original complex to photocleave supercoiled plasmid DNA when irradiated with low-energy. The ruthenated complex was successful at photocleaving DNA above 420nm, but not above 550nm. The methylated complex was successful at photocleaving DNA above 550nm which provides us with a photosensitizer that can be excited using low energy light.

Experimental Section

Materials. A mercury arc lamp 300 watt [Newport Model 67005], 550nm filter [Newport Model 10LWF-550-B], 420nm filter [Newport FSR-GG420] were used for the photocleavage studies. Tris(hydroxymethyl)-aminoethane (Tris Base) [Fischer], boric acid [Fischer], the plasmid pUC18 DNA [Bayou Biolabs], Hydrazine [Aldrich], Acetonitrile [Acros], Methanol [Fischer], Ethylene Glycol [Acros], Methylene chloride [Fischer], Anhydrous methylene chloride [Acros], 5-nitroisoquinoline [Acros], 4-fluorobenzaldehyde [Acros], Boron trifluoride etherate [Acros], Trifluoroacetic acid [Acros], Electrophoresis grade Agarose low EEO [Fischer}, Phosphazene base [Aldrich], Tetrabutylammonium hexafluorophosphate (Bu_4NPF_6 , used as supporting electrolyte for electrochemistry) [Aldrich], ethyl isocyanoacetate [Acros], p-chloranil [Aldrich], neutral silica gel [Selecto Scientific] were used for the synthesis and electrochemical studies. All elemental analyses were performed by Atlantic Microlab in Norcross, Ga. Mass spectrometry was performed by The Ohio State University Mass Spectrometry and Proteomics facility. *Cis*-Ru(bipy) $_2$ Cl $_2$ was synthesized as previously described.¹⁹

Isoquinone pyrrole ester. Isoquinone pyrrole ester was synthesized as previously stated.¹⁵ In a 250mL round bottom flask 2.32g (13.3 mmol) of 5-nitroisoquinoline was added with 100mL of dry THF. Massed 2.00g (17.7 mmol) of isocyanoacetate and then added it dropwise to the round bottom flask. Added 4.27mL (13.7 mmol) of phosphazene base and let the solution stir and reflux for 24 hours. The solution was black before and after refluxing. After the solution

refluxed, 100mL of methylene chloride was added to wash it. Added 150mL of deionized water to a separatory funnel and added the contents of the flask to the funnel and shook it up. The contents separated and we collected the black lower layer in a beaker. Added sodium sulfate to beaker to collect any excess water molecules and then decanted the solution into another beaker and let the solvent evaporate off. Column chromatography of the solid was performed using 1% methanol in methylene chloride. Collected orange band as it came off and used a rotator evaporator (rotovap) to remove the solvent. Collected the solid in the beaker. The solid was vacuum filtered and washed with methylene chloride. Final yield was 0.98 g.

Isoquinone pyrrole. This compound was created as previously stated.¹⁵ Started the reaction by adding 0.90 g of isoquinoline ester into a three-necked round bottom flask with 1.1 g of KOH and 20mL of ethylene glycol. Bubbled the flask under nitrogen for ten minutes. Added 10 drops of hydrazine and refluxed at 195 degrees Celsius for 30 minutes (Under nitrogen still). Poured the mixture into ice/water which causes precipitation and vacuum filtered the precipitate. Rinse the precipitate with water and vacuum filter overnight. Final yield was 0.55 g.

Poly-isoquinone pyrrole. Placed 0.36 g (2.1 mmol) of isoquinone pyrrole and 100 microliters of dry methylene chloride in a three-neck flask and stirred the solution under nitrogen. The flask was submerged in a dry ice/acetone bath. Then 230 microliters (2.14 mmol) of p-fluorobenzaldehyde and 87 microliters (0.69mmol) of boron trifluoride dietherate were added to the solution. The solution was stirred under nitrogen in the dry ice/acetone bath for two hours while being protected from light. After two hours, the flask was raised from the bath and continued to stir overnight at room temperature while still being protected from light. The next morning, we added 0.52 g of p-chloranil and continued stirring at room temperature for one hour. Poured the solution into another round bottom flask and rinsed the old flask with methylene chloride and then methanol. Rotovapped the flask to remove the solvent. Using neutral silica gel with sand on

top, we performed column chromatography on the mixture using a 90/10 methylene chloride methanol solution as eluent and collected a bright maroon purplish band. Collected the solid and vacuum filtered it. Final yield was 0.11 g of compound. Anal. Calcd For C. 73.57, H 4.84, N 15.36. Found: C 73.59, H 4.87, N 15.60. TOF MS ES+ (m/z; relative abundance): [C₃₃H₂₄N₆O₂] (537.9)

Ruthenation of poly-isoquinone pyrrole. Added 25 mg (0.050mmoles) of poly-isoquinol pyrrole, 73 mg (0.15 mmoles) and 5 mL of glacial acetic acid into a 10mL round bottom flask. Reflux and stir under nitrogen for thirty minutes. Rotovapped solution down and added 2 mL of ethanol. Refluxed and stirred under nitrogen. Added excess ammonium hexafluorophosphate to 125 mL of water. Added reduced product to solution and a red-brown precipitate. Vacuum filtered overnight. Dissolved with a little acetonitrile and then added solution to diethyl ether. Vacuum filtered again and collected 74 mg. Calculated for [C₉₃H₇₃O₂N₁₈Cl₃F₁₈P₃Ru₃·2H₂O] C, 47.05; H, 3.27; N, 10.62; F, 15.20 Found: C, 46.92; H, 3.25; N, 10.21; F, 15.22

Methylation of poly-isoquinone pyrrole. Place 1μL of DMF; 20 ml of methylene chloride, 12 mg (.0223mmoles) of poly isoquinol pyrrole, and 170 μL (1.115 mmoles) of methyl tosylate into a 50mL round bottom flask. Let stir at room temperature then reflux for 30 minutes. Gravity filtered it, a purple precipitate was left while the filtrate was bright red. Washed through with ethanol, then let evaporate to collect the solid. Deep purple solid was too hard to scrap so we used two mL of DMSO and 23mL water to dissolve and dilute product. Diluted to 100mL to a concentration of 74 μM. Anal. Calcd For C, 62.81; H, 4.96; N, 7.52. Found: C, 62.51; H, 4.85; N, 7.67. [C₅₇H₅₄N₆O₁₁S₃]

NMR Spectroscopy. ¹H NMR was performed by a Bruker 300MHz spectrometer using CDCl₃ as the solvent and TMS as the internal standard.

Electronic spectroscopy. UV/vis spectra were recorded at room temperature using a Shimadzu 1501 photodiode array spectrophotometer with 2nm resolution. Samples were run in a 1cm quartz cuvette.

Electrochemistry. Cyclic voltammograms were recorded using a one-compartment, three electrode cell, CH-Instruments, equipped with a platinum wire auxiliary electrode. The working electrode was a 2.0 mm diameter platinum disk from CH-Instruments. The working electrode was polished first using 0.30 μm alumina polish (Buehler) and then sonicated for 20s prior to use. Potentials were referenced to a Ag/Ag⁺ (0.010M, in acetonitrile) electrode. The supporting electrolyte was 0.1M tetrabutylammonium hexfluorophosphate (Bu₄NPF₆) and the measurements were made in acetonitrile.

Plasmid Photocleavage. Buffered solution of pUC18 and pUC18/complex II or complex III at a ratio of 10:1 bp/complex were placed in a quartz cuvette and irradiated with a 300W Mercury Arc Lamp (Newport) equipped with the appropriate filter. Samples were taken at 10 minutes over an 80 minute period and run in 1% agarose gel by applying 224V for 45 minutes in approximately 300mL of Tris buffer solution. Gels were stained with ethidium bromide and photographed using UV illumination.

Results and Discussion

The original reaction of the isoquinol pyrrole was designed to create a porphyrin ring with the aldehydes incorporated. When we ran the elemental analysis, mass spectrum, NMR, and UV/vis spectrum we realized that we did not create a porphyrin. The NMR looked very similar to the starting isoquinol pyrrole. The UV/vis spectrum had a strong soret band but lacked any Q bands which should be there for a porphyrin. The elemental analysis and mass spectrum made it clear that we had created a new novel compound that was not a porphyrin. We decided

to try the reaction again and got the same results. Since the aldehyde nor the boron trifluoride dietherate were not incorporated in the compound we wondered if they were necessary for the reaction to take place. We first took out the aldehyde, reran the rest of the reaction, and the compound was not created. Next we kept the aldehyde but took out the boron trifluoride dietherate and the compound was still not created. Finally, we took out both the aldehyde and the boron trifluoride dietherate and the reaction was unsuccessful. We know that both the aldehyde and the boron trifluoride dietherate are necessary for the reaction although we are unable to explain why or how the reaction takes place. We decided to test the compound due to its great absorption properties.

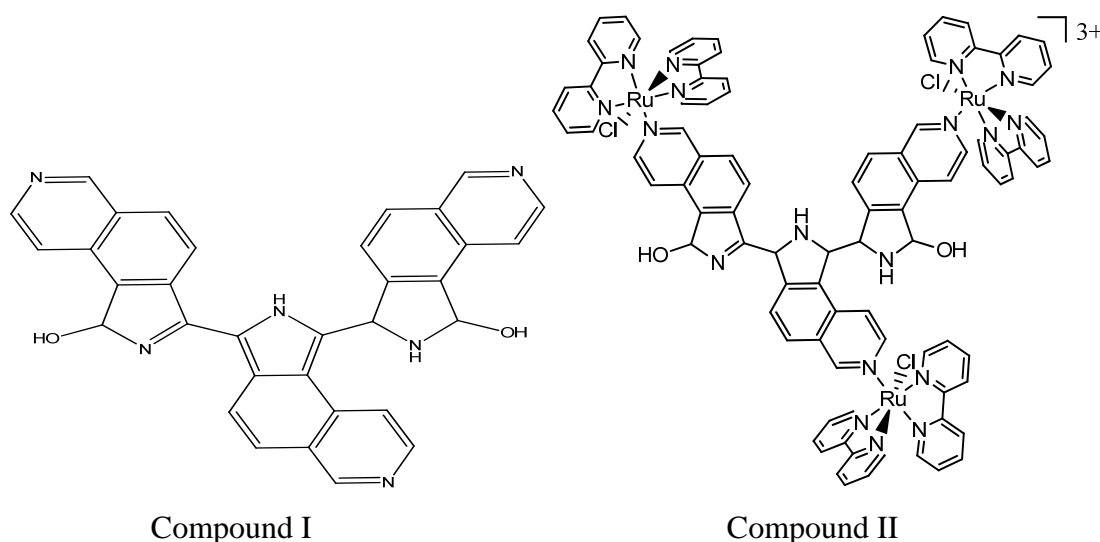


Figure 1 shows the ultraviolet visible (UV/vis spec) spectroscopy that we obtained on the compound. To get the compound in solution, we added methanol to dissolve the compound and then filled up the rest of the 25 mL flask with acetonitrile. The spectroscopy was obtained at room temperature. The red line is the compound in solution without acid and the blue line is the compound in solution with a couple drops of trifluoroacetic acid. The red line has strong absorption from 500-600 nm and is very broad. We propose that the broadness of the peak is due to stacking of the molecules in solution. Since the compound is planar and very conjugated, hydrophobic interactions are possible between molecules. The blue band is not broad and we believe that the positively charged

molecules do not interact as much and do not make the peak broad. The blue band is red-shifted, or shifted to the right. We believe that this could be due to two different hypotheses. The first one is that the protonation of the hydroxyl group that is attached to the nitrogen ring without a double bond (right most nitrogen ring in the figure) creates a good leaving group. The water molecule leaves and the adjacent hydrogen gets abstracted from the nitrogen to create a double bond. The double bond increases the conjugation of the system that was previously not there and the increase in the conjugation requires lower energy to excite it (red-shift). The other hypothesis is that protonating one of the isoquinol nitrogens creates an electronegative pull on the ring. Since the rings are connected via single bonds, the conformation is continually shifting and changing. When the rings rotate they occasionally align with each other. When the quinol nitrogen gets protonated, this increases the electronegativity of that ring and when it aligns with the adjacent ring interacts with the ring and makes that conformation preferable. By making the conformation more preferable and probable, the rings can essentially increase the conjugation system by the interaction that occurs. This increase in overlapping causes the red-shift.

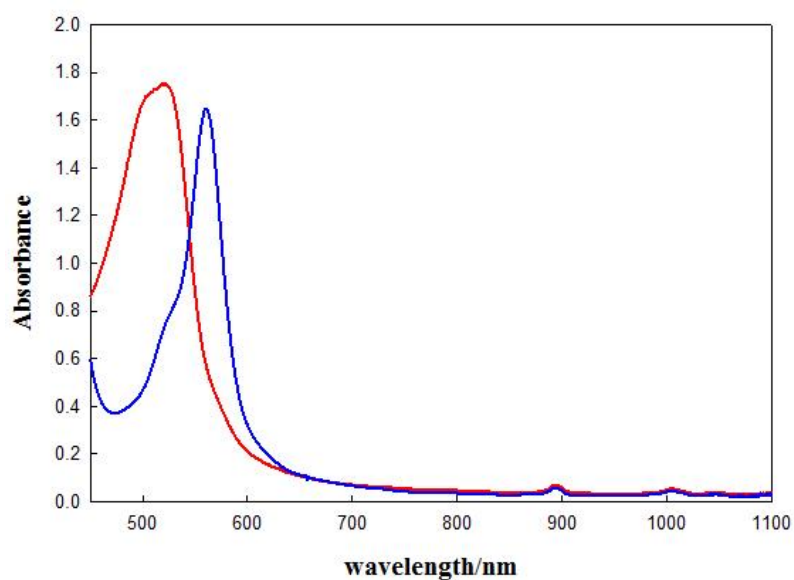


Figure 1. UV/Vis of Compound I. Red line is without acid. Blue line is after addition of TFA.

Figure 2 shows the UV/vis and fluorescence of the compound. The red line is the UV/vis and the green line is the fluorescence. The fluorescence band is the result of exciting the compound at 530 nm. The compound fluoresced well into the 600-700nm range. The fluorescence of this compound is a good sign for its availability to be used as a PDT agent.

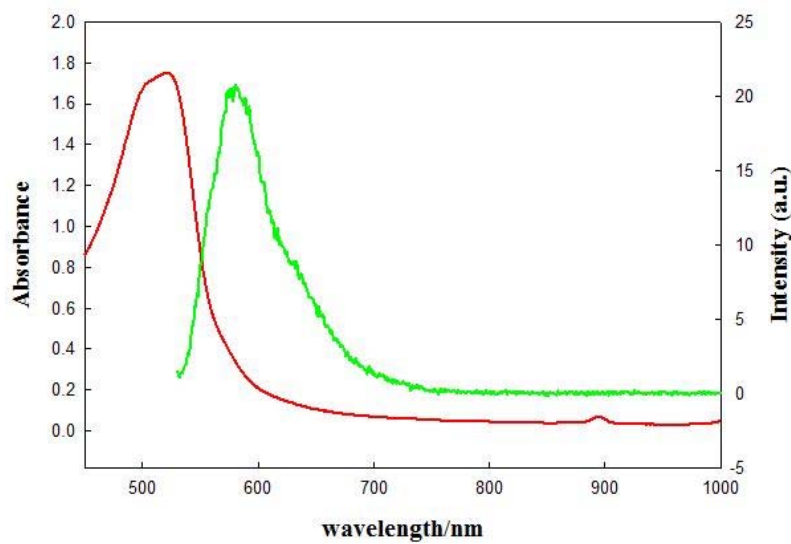


Figure 2. Red line is the UV/Vis of Compound I. Green line is the fluorescence of Compound I.

Cyclic voltammetry (CV) in the liquid phase was performed on the compound and the ruthenated compound. In Figure 3, the CV of compound I is shown. Anodically, there is a large irreversible oxidation wave of the compound around 0.8-1.0 V vs. Ag/AgCl. Cathodically there is a one-electron reduction of the compound.

Cyclic voltammetry of compound II is shown in Figure 4. Anodically there is a reversible redox couple at 0.8 V vs. Ag/AgCl that was not present in the non-ruthenated CV and is due to the Ru(III/II) couple. It does not look reversible but that is because it is overlapped by the large irreversible wave of the ligand. Cathodically there is a reduction wave that wasn't previously there that is due to the bipyridal groups that are coordinated to the rutheniums.

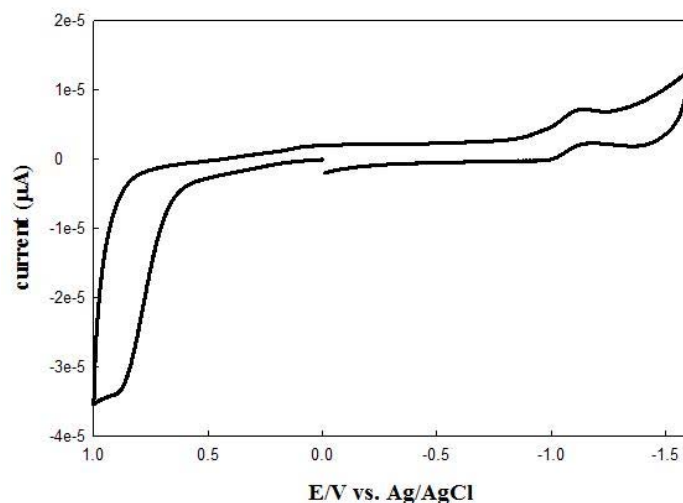


Figure 3. Cyclic voltammogram of compound I in acetonitrile with tetra-butyl ammonium hexafluorophosphate as supporting electrolyte. The working electrode is glassy carbon, scan rate = 50 mV/s.

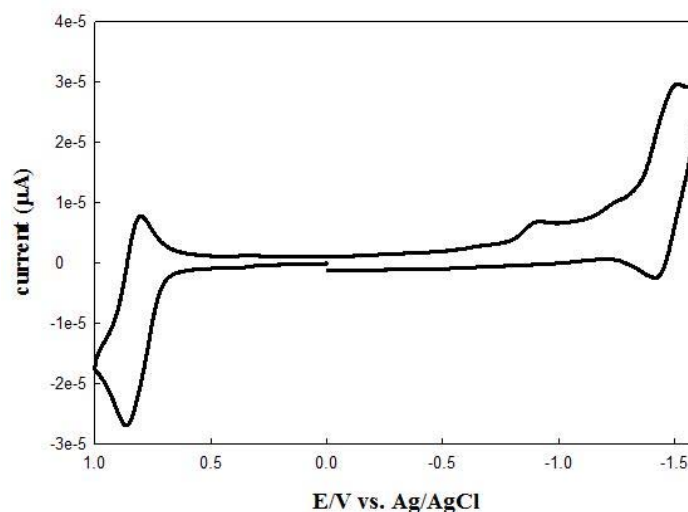


Figure 4. Cyclic voltammogram of compound I in acetonitrile with tetra-butyl ammonium hexafluorophosphate as supporting electrolyte. The working electrode is glassy carbon, scan rate = 50 mV/s.

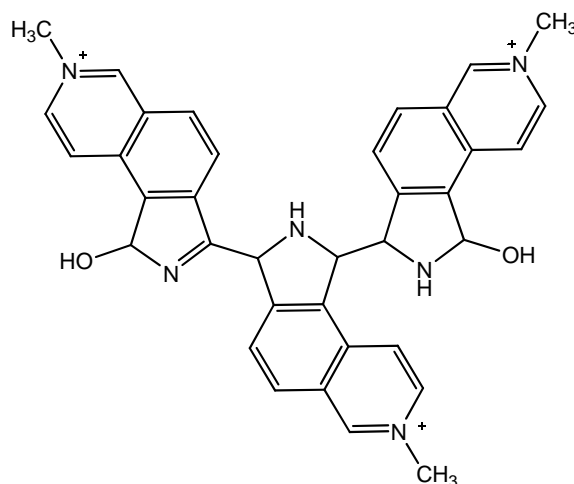
Photocleavage studies were performed with aqueous solutions of circular plasmid DNA and the ruthenated compound at a ratio 10:1 DNA base pairs to compound. Samples were removed at ten minute intervals and gel electrophoresis was performed in order to determine the ability of the ruthenated compound to photocleave DNA. There was a 420nm filter lens on the lamp. This made sure that no wavelengths lower than 420nm were allowed to penetrate the cuvette. The

lower on the gel the bands are means that the DNA are not nicked or less nicked. The first band (farthest left) is the control that contains just plasmid, water, and dye. The second band (second farthest left) is the control that contains plasmid, water, dye, and compound before being irradiated with light and since the band is so far down, this means that the compound does not affect the DNA in the dark. Every other band is after a ten minute interval. So the third band is after ten minutes, the fourth band is after twenty minutes or irradiation and so forth. The ruthenated compound was able to efficiently nick the plasmid DNA completely after the full eighty minutes due to the lack of the lower band. This is excellent data but the PDT window was not achieved. When we put the 550nm filter, the ruthenated compound was not able to photocleave the plasmid DNA. We propose that the addition of the rutheniums quenched the absorption properties of the ligand. Due to the quenching, higher energy wavelengths are required for the type II photocleavage to occur. This left shift of the absorption means that the compound is not in the PDT window and not ideal. We decided to find a different way to increase the solubility of the compound that would not quench the absorption properties.



Figure 5. Gel electrophoresis of circular plasmid DNA (pUC18) in the presence of complex I (lanes 2-10) and without complex I (lane 1) irradiated with a 300 W mercury arc lamp filtered to allow only light of 420 nm and higher to penetrate, samples taken at 10 min intervals.

To eliminate the luminescent quenching of the ruthenium polypyridyl groups but still maintain water solubility compound I was reacted with p-methyl-toluene sulfonate giving compound III.



Compound III

The methylated compound's uv/vis and fluorescence is shown below. The red band is just the methylated compound's UV/vis. It has strong absorption from 450-650nm. The blue band is the fluorescence of the compound which fluoresces well into the PDT window. This graph implies that the methyl groups do not quench the absorption properties of the ligand. Photocleavage studies are required to prove that the methylated compound can still perform photodynamic therapy in the PDT window.

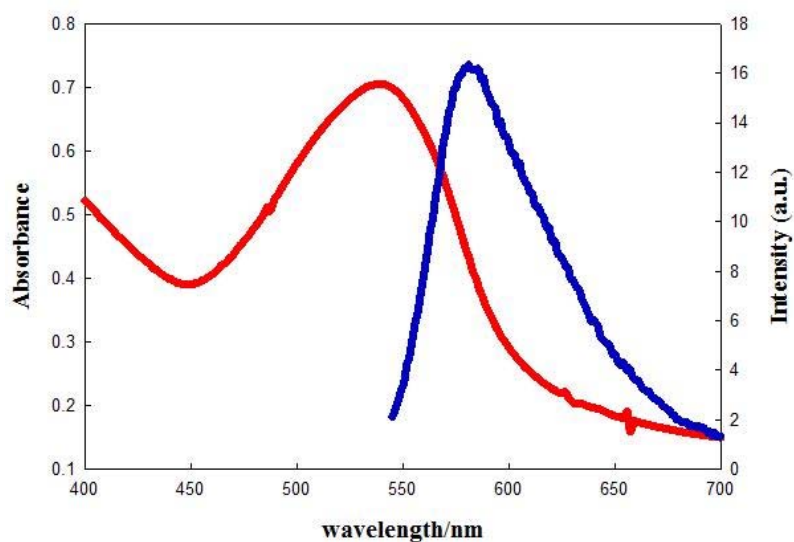


Figure 6. UV/vis (red line) and fluorescence (blue line) of the methylated polyisoquinoline.

Figure 7 displays the gel that was created during the photocleavage study when using compound III. The ratio of base pairs to compound is 5:1 and we extracted samples over ten minute intervals again, then used gel electrophoresis to determine the efficiency of the compound to photocleave DNA. We used a 550nm filter for this experiment and the compound did not quench the absorption properties of the compound as shown by the nicked bands that did not travel as far in the gel. Just like the ruthenated compound photocleavage experiment, the two controls are the same and are the farthest on the left and these lanes show that the DNA was not affected by the compound in the dark. After eighty minutes of irradiation essentially all the plasmid is nicked. Since the 550 nm filter was used, we know that no wavelength less than 550 nm was able to excite the compound so the compound was not quenched by the methyl groups and it is still able to be excited in the PDT window or very close to it. This photocleavage study proves how important this compound is, it is water soluble and cleaves in the PDT window.

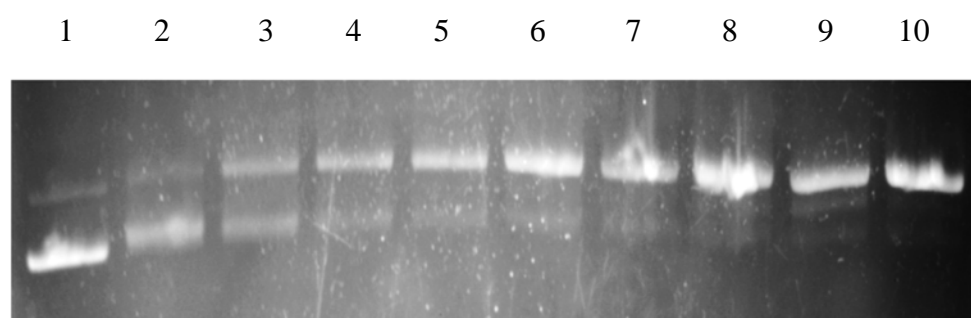


Figure 7. Gel electrophoresis of circular plasmid DNA (pUC18) in the presence of complex I (lanes 2-10) and without complex I (lane 1) irradiated with a 300 W mercury arc lamp filtered to allow only light of 550 nm and higher to penetrate, samples taken at 10 min intervals.

Conclusions

The initial desired compound was not obtained, instead a novel compound with an extended conjugated system has been synthesized in high yield and with great absorption properties. Ruthenation of the compound has been shown to photonick plasmid DNA when irradiated with wavelengths greater than 420 nm. When irradiated with wavelengths greater than 550nm the rutheniums have been shown

to quench the absorption properties and disrupt the ability of the molecule to photocleave the plasmid DNA. Methylation of the compound yielded a compound that was water soluble with great absorption and fluorescence in the PDT window. The methylated compound photolyzed plasmid DNA at wavelengths longer than 550 nm which means that the addition of the methyl groups do not quench the absorption of the ligand. Further studies are being performed to elucidate the mechanism of the reaction with the goal of understanding what is happening chemically and to synthesize more of these novel compounds to be used in PDT. Also, to determine the PDT effectiveness of the compound in normal cells and cancer cells.

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The author would like to thank his advisor, Dr. Shawn Swavey for his amazing guidance, teaching, and friendship. I would also like to thank Dr. Benin for his help with Boron NMR and always being willing to answer any questions that arose. Finally, I would like to thank the University of Dayton Chemistry and Honors departments for their generous contributions to this project.

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