Dual Function Molecular Imaging Probes for Breast Cancer

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Dual Function Molecular Imaging Probes for Breast Cancer

Christopher DeNyse

A thesis submitted in partial fulfillment of the requirements for the degree

Master of Science in Chemistry

Supervised by

Dr. Hans Schmitthenner

School of Chemistry and Materials Science

College of Science

Rochester Institute of Technology

August 2023

Signature of the Author ______________________________

Accepted by _______________________________________

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Dr. Christina Goudreau Collison

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Dr. Michael Heagy

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Dr. Gary Skuse

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Date
Abstract

Breast cancer (BrCa) is a pressing health concern, necessitating the development of effective imaging and therapeutic agents. To address this need, we designed and synthesized molecularly targeted imaging and therapeutic agents that combine visualization and treatment of tumors in real-time. Our approach involved the development of dual-modal agents, incorporating the fluorescent near infrared (NIR) dye, IRDye78, and a chlorin-based photosensitizer (PS) dye, Mesopyropheoborbide-a (mPPa), coupled to a BrCa-targeting peptide, "18-4."

Additionally, we created a water-soluble, single-modal targeted PS probe for photodynamic therapy (PDT) of BrCa for use guided by other imaging methods.

Our methodology was based on a modular approach, wherein modules containing the dyes attached to a lysine scaffold were utilized to attach the dye payloads in two different combinations: one with the PS dye on the lysine side chain and fluorescent dye, IRDye78, on the n-terminus of lysine, and the other with reversed positions of the two dyes. Furthermore, we extended this method to synthesize palladium-metalated versions of these modules.

For the water-soluble, targeted single-modal probe, we designed a module that incorporated the PS dye on the side chain of lysine, attached to a water-solubilizing tri-sulfonated arm on the n-terminus. This module was then coupled to the BrCa peptide, resulting in a single-modal PS for PDT of BrCa.

Two probes were provided to the University of Rochester for evaluation in BrCa cells and murine models. Preliminary results confirmed the uptake of our first dual modal probe into BrCa cells through fluorescent microscopy.

This work represents the first example of receptor-targeted, dual-modal imaging and therapy agents for combined imaging and PDT of BrCa. Additionally, a new approach to water-
soluble, single-modal targeted PDT agent is introduced. Overall, this comprehensive approach offers hope for improved outcomes in lumpectomy and represents a substantial advancement in the fight against breast cancer.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AmAc</td>
<td>Ammonium Acetate</td>
</tr>
<tr>
<td>BCS</td>
<td>Breast Conserving Surgery</td>
</tr>
<tr>
<td>BrCa</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>CFM</td>
<td>Confocal Fluorescence Microscopy</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized Tomography</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode Array Detector</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEA</td>
<td>Diethylamine</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>HATU</td>
<td>1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>HOBt</td>
<td>Hydroxybenzotriazole</td>
</tr>
<tr>
<td>HPLC-MS</td>
<td>High Performance Liquid Chromatography-Mass Spectrometer</td>
</tr>
<tr>
<td>HR</td>
<td>Hormone Receptor</td>
</tr>
<tr>
<td>HRMS</td>
<td>High Resolution Mass Spectroscopy</td>
</tr>
<tr>
<td>ICS</td>
<td>Intersystem Crossing</td>
</tr>
<tr>
<td>KRT1</td>
<td>Keratin 1</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>m-THPC</td>
<td>Temoporfrin</td>
</tr>
<tr>
<td>MTPS</td>
<td>Molecularly Targeted Photosensitizer</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>NIR</td>
<td>Near Infrared</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance Spectroscopy</td>
</tr>
<tr>
<td>OBt</td>
<td>Hydroxybenzotriazole ester</td>
</tr>
<tr>
<td>OMI</td>
<td>Optical Molecular Imaging</td>
</tr>
<tr>
<td>OSu</td>
<td>N-Hydroxysuccinimide ester</td>
</tr>
<tr>
<td>PDT</td>
<td>Photodynamic Therapy</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone</td>
</tr>
<tr>
<td>Prep-HPLC</td>
<td>Preparative High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>PS</td>
<td>Photosensitizer</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid Phase Peptide Synthesis</td>
</tr>
<tr>
<td>TBTU</td>
<td>2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylammonium tetrafluoroborate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TFA</td>
<td>Tetrafluoroacetic Acid</td>
</tr>
<tr>
<td>TMIA</td>
<td>Targeted Molecular Imaging Agent</td>
</tr>
<tr>
<td>TN</td>
<td>Triple Negative</td>
</tr>
<tr>
<td>TSTU</td>
<td>N,N,N',N'-Tetramethyl-O-(N-succinimidyl)uronium tetrafluoroborate</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
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# Abbreviations of Amino Acids in Peptide 18-4

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Three Letter Abbreviation</th>
<th>One Letter Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
<td>A</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Arg</td>
<td>R</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>Glu</td>
<td>E</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
<td>Q</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
<td>L</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
<td>K</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>F</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Try</td>
<td>W</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>Y</td>
</tr>
<tr>
<td>Norleucine</td>
<td>Nle</td>
<td>X</td>
</tr>
</tbody>
</table>
Table of Contents

Abstract ............................................................................................................................................... 3

Abbreviations ................................................................................................................................... 5

Abbreviations of Amino Acids in Peptide 18-4 ............................................................................ 7

List of Figures ..................................................................................................................................... 10

1. Introduction: .................................................................................................................................. 14

  1.1 Overview ...................................................................................................................................... 14

  1.2 Breast Cancer ................................................................................................................................. 14

  1.3 The Lumpectomy reoperation epidemic ...................................................................................... 16

  1.4 Photodynamic Therapy ............................................................................................................... 17

  1.5 Peptide Based Molecularly Targeted Photosensitizers .............................................................. 20

  1.6 Peptide BrCa Targeting Probe “18-4” ....................................................................................... 21

  1.7 Solid Phase Synthesis (SPPS) of 18-4 ......................................................................................... 25

  1.8 Optical Molecular Imaging (OMI) .............................................................................................. 26

  1.9 Strategy for Modular Synthesis of TMIAs .................................................................................. 28

2. Strategy for Dual Targeted BrCa Imaging and Photodynamic Therapy .................................. 30

  2.1 Wavelength design strategy for dual OMI-PDT molecular probes ......................................... 30

  2.2 IRDye78 for Imaging of BrCa ...................................................................................................... 31

  2.3 Porphyrin Photosensitizers for PDT of BrCa ............................................................................. 33
3.0 Aim and Design of Dual targeted TMIA probes. ................................................................. 36

3.1 Design of Dual PS and NIR dye-based targeting agents .................................................... 36

3.2 Design of Dual Palladized-PS and NIR dye-based targeting agents ............................... 42

3.3 Design of Single-Dye Water-Soluble Porphyrin targeting agents .................................. 43

4. Results and Discussion ......................................................................................................... 47

4.1 Synthesis of Porphyrin & Pd-Porphyrin Puzzle Pieces .................................................... 47

4.2 Synthesis of IRDye78 Puzzle Pieces ................................................................................. 57

4.3 Synthesis of Dual-Dye: IRDye78–Lys(mPPa) modules ....................................................... 60

4.4 Synthesis of Dual-Dye: mPPa–Lys(IRDye78) modules ....................................................... 63

4.5 Synthesis of Single-Dye Water-Soluble Porphyrin modules ............................................ 64

4.6 Synthesis of Dual Dye Targeted TMIA probes ................................................................. 69

4.7 Synthesis of Targeted Single Modal MTPS for PDT of BrCa ........................................... 74

4.8 Confocal Microscopy of BrCa Cells Targeted with IRDye78-D-Lys-(mPPa)-18-4................ 74

5. Conclusion .......................................................................................................................... 78

6. Experimental Procedures .................................................................................................... 80

6.1 Materials and Methods ..................................................................................................... 80

6.2 Experimental Procedures ................................................................................................. 82

References .............................................................................................................................. 107

Appendix I. HPLC-MS, NMR, UV-Vis, and HRMS Results .................................................... 117
List of Figures

Figure 1: Modified Jablonski Energy Diagram. Sourced from Sara Shaut, MS Thesis.19...........18

Figure 2: The BrCa targeting decapeptide 18-4 developed by Dr. Kaur28 (Chapman University). Lowercase letters denote the unnatural (D) form of the amino acid utilized...23

Figure 3: The SPPS of BrCa targeting peptide 18-4 on polymeric Sieber resin29..................26

Figure 4: The CFM imaging of MDA-MB-231 TN BrCa cells. The red fluorescence is from Cy5.5-2S and the blue is the nucleus stained with Hoechst 33342. The 18-4 decapeptide was utilized to target KRT1 in MDA-MB-231 BrCa cells43.................................................................28

Figure 5: A TMIA composed of two puzzle pieces each containing a unique imaging agent conjugated to a targeting agent selective for a cancerous biomarker.................................29

Figure 6: The convergent approach to synthesize the Dual-Module Puzzle Pieces, followed by conjugation to the N-terminus of 18-4 to yield the final Dual Functional TMIA..................30

Figure 7: The structure of IRDye78 as previously described in Literature54..........................32

Figure 8: The numbering of the Porphyrin Macrocycle and different Porphyrin rings with their respective unique compositions of pi electrons.........................................................34

Figure 9: The Structure of the Chlorin-type PS dyes utilized in this project.........................36

Figure 10: The structure of a direct conjugate between a cyanine dye for OMI alongside a metalated porphyrin, as synthesized by Pandey et al. (2016)66. This dual dye conjugate was successful for the PDT of cancer cells.................................................................37

Figure 11: The synthesis of a Dual Dye TMIA utilizing the formation of a Dual Dye Puzzle Piece containing mPPa and IRDye78. The final structure elaborates the TMIA structures illustrated by their names in boxes.................................................................39
Figure 12: *The structure of the single modal TMIA with OMI dye IRDye78 (highlighted in red) directed conjugated upon the n-terminus of decapeptide 18-4, which successfully bound to MDA-MB-231 TN BrCa cells.*

Figure 13: *The obtained CFM results from Dr. Timothy Baran at the University of Rochester showcasing stained MDA-MB-231 BrCa cells with the above single modal TMIA (left stain incubation for 4 hours, right: stain incubation for 22 hours.)*

Figure 14: *The Dual Dye TMIA with the porphyrin dye upon the N-terminus of the peptide scaffold.*

Figure 15: *The structure of a dual dye TMIA with OMI dye IRDye78 and PS dye Pd-mPPa.*

Figure 16: *The design of a single dye TMIA with mPPa conjugated with 18-4 upon the sidechain of the 3’ lysine residue by MS graduate Sara Shaut\(^1\). This design has been since abandoned, in favor of attaching therapeutic agents upon the N-terminus to avoid negative binding interactions with KRT1.*

Figure 17: *The water-soluble module 25 and a water-soluble porphyrin amino acid puzzle piece 26.*

Figure 18: *The reaction scheme for the hydrogenation of Pyropheophorbide (PPa, 1) a to produce Mesopyropheophorboide a (mPPa, 2).*

Figure 19: *The UV-Vis absorbance spectrum of mPPa 2, with absorbance maxima at 407 nm and 660 nm\(^1\) in methanol.*

Figure 20: *The fluorescence spectra for mPPa 2, with a emission maxima of 661 nm\(^1\) in methanol.*

Figure 21: *The unsuccessful metalation of mPPa 3.*

Figure 22: *The conversion of mPPa 2 into mPPa-OSu 3.*
Figure 23: The mechanism of OSu Ester formation with TSTU under basic conditions. ....52
Figure 24: Quenching the reactive OSu ester by formation of the butyl amide derivative to assay the concentration of OSu ester present within the reaction by HPLC-MS analysis. ....53
Figure 25: The metalation of mPPa-OSu 3 into Pd-mPPa-Osu 13 ........................................54
Figure 26: The DAD UV-Vis spectrum of Pd-mPPa 14 .................................................................54

Figure 27: The Scheme to synthesize deprotected free base and metalated lysine-porphyrin conjugates ...........................................................................................................55

Figure 28: The mechanism of amide bond formation between primary amines and OSu Esters of mPPa under basic conditions .............................................................................56
Figure 29: The synthesis of Fmoc-Lys(IRDye78)-OH conjugates .....................................................58
Figure 30: The Fmoc deprotection of Fmoc-Lys(IRDye78) puzzle pieces ........................................59
Figure 31: The impurity resulting from the reaction between DEA and IRDye78. .........................59
Figure 32: UV-Vis DAD spectrum of H2N-D-Lys(IRDye78)-OH with absorbance maxima at 768 nm ..................................................................................................................60
Figure 33: The reactions between lysine porphyrin conjugates and IRDye78 -OSu. Yields are indicated on the right .................................................................61
Figure 34: DAD UV-Vis spectrum of the dual dye IRDye78-D-Lys(mPPa)-OH with absorbance maxima at 766nm, 650nm, and 407nm, clearly showing the presence of both IRdye78 and mPPa ..................................................................................................................62
Figure 35: DAD UV-Vis spectrum of the dual dye IRDye78-D-Lys(Pd-mPPa)-OH with absorbance maxima at 766nm, 640nm, and 393nm. clearly showing the presence of both IRDye78 and Pd-mPPa ........................................................................................................63
Figure 36: The reaction between Fmoc-D-Lys(IRDye78)-OH 21 and mPPa-Osu 3 .................64
Figure 37: The structures of the starting materials for the synthesis of the “Water-Soluble” module. ................................................................. 65

Figure 38: The synthesis of the water-soluble (WS) module 25. ................................................................. 66

Figure 39: The synthesis of a Water-Soluble lysine-porphyrin dye conjugate 26 from the TEA salt of the “Water-Soluble” module, compound 25. .......................................................................................... 67

Figure 40: The guanidinylation byproduct formed from the reaction of uronium-based coupling reagents (TSTU / TBTU) and H₂N-D-Lys(mPPa)-OH 6 under basic conditions. .... 68

Figure 41: The unsuccessful coupling between IRDye78-D-Lys(mPPa)-OH and the N-terminus of 18-4 utilizing the OSu ester. ........................................................................................................... 70

Figure 42: Uronium based coupling reagent TBTU utilized for the formation of OBt esters. .............................................................................................................................. 72

Figure 43: The reactions between dual dye lysine conjugates and the decapeptide 18-4. .... 73

Figure 44: The synthesis of a water-soluble porphyrin 18-4 MTPS. ............................................................ 74

Figure 45: The fluorescent microscopy image obtained after 1 hour from staining MDA-MB-231 TN BrCa cells with IRDye78-D-Lys(mPPa)-18-4 11 and illuminating the cells at 400 nm. ................................................................. 75

Figure 46: The fluorescent microscopy image obtained after 1 hour from staining EMT6 TN BrCa cells with IRDye78-D-Lys(mPPa)-18-4 11 and illuminating the cells at 700 nm. .... 76

Figure 47: The fluorescent microscopy image obtained after 1 hour from staining EMT6 TN BrCa cells with IRDye78- and illumination of the cells at 700nm. .............................................. 77
1. Introduction:

1.1 Overview

The goal of this thesis was the synthesis of single and dual functional targeted molecular agents designed to consists of a combination of a photosensitizer (PS) dye and near-infrared (NIR) dye for optical molecular imaging (OMI), coupled to the breast cancer (BrCa) targeting peptide 18-4 to enable the targeted imaging and photodynamic therapy (PDT) of breast cancer (BrCa) cells by the use of a single agent. Such a probe would be utilized to enable light-directed lumpectomy removal of BrCa tumors alongside PDT for removal of residual cancerous cells remaining within lesions, or occult tumors remaining in the margins.

A second goal was the synthesis of a PS dye coupled to a water soluble (WS) moiety and also to the same BrCa targeting peptide to enable solo targeted PDT of tumors using that have been mapped through other means of imaging. If successful, these would be the first known examples of a targeted single function agent for the PDT of BrCa and dual modal agents for the guided surgery and treatment of BrCa. In the synthetic design, a modular method will be utilized where the peptide is added in the final steps. Moreover, this will enable the opportunity to apply the new imaging and therapy modules created here for many different types of cancer as well.

1.2 Breast Cancer

Cancer is a leading cause of morbidity and mortality, claiming over 600,000 lives in 2022 alone\(^1\). Breast Cancer (BrCa) accounts for nearly 15% of all new cancer cases in the United States, with BrCa being diagnosed within 290,000 people in 2022\(^2\), making it the second most common cancer\(^3\). Every year in the United States 40,000 individuals will perish from BrCa and BrCa related complications\(^4\).
Treating cancerous tumor growths can be extremely difficult, as cancer cells share a large amount of similarity with normal healthy cells. Early detection increases the chances of successful treatment by identifying cancer at an early stage when the cancer may be more localized and easier to treat. The correct classification of cancer type is essential as different types of BrCa exhibit distinctive biological differences and each may necessitate a specific treatment approach.

Following a cancer diagnosis, a treatment plan for a patient is formulated by considering multiple factors surrounding their cancer type and stage, the patient’s overall health, and their treatment preferences. Therapy options for cancer patients typically encompass a combination of therapies including surgery, chemotherapy, radiation, hormonal therapy, or targeted therapy. The specific treatment plan for the patient is prescribed by their doctor depending on the specific type, location and stage of their BrCa.

Hormonal and targeted (small molecule) therapies are commonly prescribed for breast cancer patients whose cancer cells have overexpressed hormonal receptors for either estrogen (ER), progesterone (PR), or human epidermal growth factor 2 (HER2) on the cell membranes of their breast cancer cells. Patients with BrCa with either hormonal receptor (ER / PR) or HER2 receive hormonal therapy and antibody therapy, respectively, in combination with a relatively small dose of chemotherapy.

However, patients who do not overexpress any of these receptors are classified as “triple negative” (TN) BrCa patients and currently lack available targeted treatment options. Consequently, TN breast cancer patients typically receive a high dose of chemotherapy alone, as hormonal and targeted therapies do not currently exist for their specific subtype. This limitation highlights the need for further research and development of targeted treatments for these patients.
In comparison to other types of BrCa, TN BrCa accounts for only approximately 20% of cases\(^9\). However, it is the most likely to recur and metastasize. Moreover, the survival rates for metastatic TN breast cancer are significantly lower, with an average survival period of only around 1 year, as opposed to 5 years for other types of cancer\(^5\). This emphasizes the urgent need for targeted therapy options specific to TN breast cancer patients.

1.3 The Lumpectomy reoperation epidemic.

A significant number of breast cancer patients undergo a surgical procedure called breast conserving surgery (BCS), commonly known as a lumpectomy\(^10\). The goal of BCS is to remove the cancerous cells whilst preserving the healthy breast tissue. However, completely removing all cancerous cells during BCS presents a significant challenge, primarily because tumors are primarily identified through visual confirmation and palpation\(^10\). This reliance on visual and palpable cues can result in the oversight of non-palpable, invisible, or occult tumors that may persist within the breast tissue even after surgery.

It is estimated that only 10-50% of BCS patients show positive cancer reduction margins after the initial surgery, necessitating multiple surgeries and often requiring chemotherapy as an additional treatment measure for many patients\(^10\). This phenomenon has given rise to what is known as a "lumpectomy reoperation epidemic," representing one of the most pressing issues in the ongoing battle against breast cancer. Addressing this problem is crucial to improving patient outcomes and reducing the need for repeated surgeries, thereby minimizing the physical and emotional burden on BrCa patients.
1.4 Photodynamic Therapy

Photodynamic Therapy (PDT) has garnered recent attention for its ability to induce cell death following targeted illumination of cancerous tumors. PDT relies on the administration of a photosensitizer (PS) dye which upon irradiation from a light source will undergo a series of photochemical reactions ultimately leading to the generation of reactive oxygen species (ROS), followed by cell death. By utilizing light activation, PDT provides a minimally invasive, non-radiative, and non-chemo form of therapy for BrCa patients. While historically PDT efficacy has been greatly hindered by poor light absorbance by PS’s\textsuperscript{11}, the advancements in PS’s, laser technology, and fiber optic technology has greatly enhanced the application of PDT, leading to notable success in treating esophageal and prostate cancers\textsuperscript{12–14}.

With respect to the mechanism of photochemistry involved, upon irradiation of a PS dye, one of its electrons is promoted into a higher-energy molecular orbital, elevating the PS from the ground state ($S_0$) into a short lived, electronically excited state ($S_n$)\textsuperscript{15}. These multiple electronic states decay quickly through internal conversion leading to the population of the first excited singlet state ($S_1$)\textsuperscript{16}. Once occupying the excited singlet state ($S_1$), the excited PS dye can undergo multiple pathways to release its energy so it can return to its ground state ($S_0$). This mostly occurs through two possible pathways, fluorescence, and intersystem crossing.

The first pathway is by emitting a fluorescent electron through fluorescence, which has a very short lifetime of $\tau_{fl}=10^{-9}–10^{-6}$ s given that transitions between the same spin states are considered “allowed” transitions according to the spin selection rule\textsuperscript{17}. Alternatively, an electron in the excited singlet state ($S_1$) may experience spin inversion and populate the lower-energy first excited triplet state ($T_1$) through a spin-forbidden process known as intersystem crossing (ISC), where the electron’s spin is no longer conserved. Following this, the excited electron can
undergo a second spin-forbidden inversion and transition back to the ground state (S0) via phosphorescence (T1 → S0), or the energy can transition to another molecule with the same spin state through a non-radiative transition\textsuperscript{18}.

Because of the spin-forbidden nature of the triplet-to-singlet transition, the lifetime of phosphorescence ($\tau_P = 10^{-3} - 1$ s) is significantly longer than that of fluorescence. These transitions, including the transition of energy to molecular oxygen, can be seen in the Jablonski energy diagram in Figure 1.

\begin{figure}[h]
    \centering
    \includegraphics[width=\textwidth]{Modified_Jablonski_Energy_Diagram.png}
    \caption{Modified Jablonski Energy Diagram. Sourced from Sara Shaut, MS Thesis.\textsuperscript{19}}
\end{figure}

Due to the long lifetime of phosphorescence, triplet state T\textsubscript{1} of PSs will undergo reactions depending on the environment surrounding the PS. These $^3$PS (triplet state PS) reactions are defined as Type-I and Type-II processes.
Type I reactions are a broad class of reactions when either \(^1\)PS (S1) or \(^3\)PS (T1) states transfers either an electron or hydrogen to the surrounding molecules. When molecular oxygen is present, it can go on to react with O\(_2\) molecules to form reactive oxygen species (ROS). ROS are a classification of oxygen molecules which contain a majority of radicals, along with superoxide anions (O\(_2^\cdot\)\)), and peroxides\(^{20}\). The presence of ROS within cells causes oxidative stress and, in high enough concentrations, results in cell death\(^{20,21}\).

While Type I generation of ROS does occur with PS dyes, the predominant mechanism which contributes more to the generation of ROS is the Type-II mechanism\(^{21}\). The Type-II mechanism differs from Type I as it is a direct energy transfer between the \(^3\)PS dye and triplet state molecular oxygen (\(^3\)O\(_2\))\). This transformation is favored and occurs readily due to the PS and O\(_2\) both existing in the same triplet spins states\(^{21}\). When \(^3\)PS dye and \(^3\)O\(_2\) interact the spin of one of molecular oxygens (\(^3\)O\(_2\)) outermost antibonding electrons inverts, generating singlet oxygen (\(^1\)O\(_2\))\(^{22}\). This process results also in the depopulation of the \(^3\)PS back into its ground state S\(_0\)\(^{22}\). This photochemical reaction is likely to occur due to the low energy barrier to turn \(^3\)O\(_2\) into \(^1\)O\(_2\)\(^{18}\), alongside the high concentration of oxygen within the cellular matrix. Ultimately, there is no singular pathway that is shown to lead to cell death after PDT treatment, however PDT destroys cells through apoptotic, necrotic, and autophagic pathways\(^{23}\).

The current market options of PS dyes used in PDT for the treatment of BrCa and other cancers have several significant drawbacks that hinder their efficacy. Widely used PS dyes like Hematoporphyrin\(^{24}\) and Photofrin\(^{15}\) suffer from limitations due to lack of selectivity for cancerous cells, leading to cell death in both cancerous and normal healthy cells. This lack of selectivity
results in potential damage to healthy tissues and can contribute to side effects associated with PDT.

Another challenge with PS dyes is their limited ability to effectively absorb light, especially when tumors are present deep within tissues. The lasers used in PDT typically have limited tissue penetration capabilities, unable to pass through more than 1 cm of tissue\textsuperscript{25}. If tumors persist beyond this depth, fiber optic diffusers are required to give the PS dye the amount of light it needs for proper activation\textsuperscript{26}. As a result of these drawbacks, PDT has been traditionally limited in application to patients with accessible tumors.

1.5 Peptide Based Molecularly Targeted Photosensitizers

It is our hypothesis that an effective molecular targeting strategy can significantly enhance the effectiveness of a PS dye. By selectively targeting unique biomarkers in cancer cell lines following systemic injection, we have the opportunity to dose cancer cells selectively. Molecular targeting in PDT enables the use of lower PS dye concentrations, reducing off-target accumulation and minimizing side effects compared to traditional untargeted PS agents.

Our initial approach in this project was to combat the “lumpectomy reoperation epidemic” through the development of an intravenous molecular targeting agent that can be used to both visualize and treat BrCa within the same operating procedure. The BrCa cells will be visualized using optical molecular imaging (OMI) using a near infrared dye (NIR) to guide the surgical removal of the tumor in a lumpectomy procedure. After the removal of cancerous tissue any remaining cancer cells in the margins will be treated with a photosensitizer (PS) dye utilized for photodynamic therapy (PDT) to induce cell apoptosis, all within the same surgical procedure.
This combination of utilizing OMI to allow for identification of the tumors in real time using fluorescence to guide surgery, in tandem with PDT for selectively inducing cell death of any residual cancer cells, will result in higher accuracy, and lower chances for recurrence and reoperation for BrCa patients. The selective uptake of the combined molecularly targeted probe (MTP) containing both the OMI and PS within BrCa will be enabled by coupling both dyes with a known molecular targeting peptide, “18-4”\(^{27,28}\), which will also serve as the scaffold for constructing our MTP.

The design for our MTP is based on a similar approach to the targeted molecular imaging agents (TMIs) previously synthesized in our lab, and published in literature\(^{29}\). This approach employs a near infrared (NIR) dye for optical molecular imaging (OMI) alongside a targeting agent for selective delivery to BrCa cells. Our approach will expand on this previously described modular method to incorporate a PS dye for PDT, to allow for the dual functionality of the MTP to impart the ability to selectively induce cell death in addition to imaging of cancer cells.

While porphyrins alone have been viewed as targeting cancer due to their significant lipophilicity which causes them to accumulate on cell surfaces, there has been no report of receptor targeted PDT using a peptide targeting agent conjugated to the PS dye. Our novel approach aims to greatly improve the efficacy of PDT through this new targeting strategy.

1.6 Peptide BrCa Targeting Probe “18-4”

The design of an effective probe has to consider bioavailability, cell penetration, clearance properties, and overall safety in order to be feasible. Current hormonal therapies available such as tamoxifen and toremifene are rife with side effects and only work on cancer cells which overexpress hormonal receptors\(^{30,31}\). Nanoparticles and antibodies have issues with nonspecific
binding to unrelated tissues, have limited biodistribution, and have cell and tissue penetration difficulties rendering them often ineffective at treating cancers. Antibodies have also been shown to occasionally produce immune responses.

A significant advantage of combining the two agents upon a singular probe is to ensure equal biodistribution of both agents. Upon uptake of the entire peptide, both dyes will be present within the cell to perform their respective functions. The concentration of both agents will always be equal within the cells, thus providing the biodistribution adequate for guiding surgery with OMI and for treatment with PDT.

In our design the molecular targeting peptide 18-4 was chosen as it has been shown as an ideal non-hormonal targeting moiety, as demonstrated by Kaur (2011). 18-4 binds to the KRT1 keratin receptor upon the cell membrane which has been found to be overexpressed in multiple BrCa cell lines, and has low expression in normal cells. Due to this high differentiation in KRT1 expression, uptake of the MTP will be significantly higher within BrCa cells versus normal healthy cells ensuring the drug is present majorly within BrCa cells. Through the exploitation of a biomarker unique for cancer cells, this allows us to selectively deliver drugs to cancerous cells minimizing off-target accumulation.
Figure 2: The BrCa targeting decapeptide 18-4 developed by Dr. Kaur (Chapman University).

Lowercase letters denote the unnatural (D) form of the amino acid utilized.

Due to the use of non-hormonal or non-antibody-based probes, 18-4 is remarkably effective at binding to most types of BrCa cells, including TN BrCa cell lines. Preliminary studies showed high affinity of 18-4 for both TN BrCa as well as cell lines which express hormonal receptors. Previous studies have validated the uptake of a 18-4-Microcin J25 (a bacterial RNA polymerase inhibitor) conjugate into BrCa lines through the use of fluorescent microscopy by testing the drug conjugates in both BrCa cell lines and in mice implanted with BrCa tumors. The mechanism of uptake has more recently been shown to be mediated through the Keratin I (KRT1) receptors, which are expressed to a high degree in active BrCa cells.

Small peptides under 4,000 molecular weight, such as 18-4, are ideal scaffolds for drug delivery as they have numerous advantages over other drug delivery scaffolds. Peptides can be proteolytically stabilized against bloodborne degradation through the incorporation of D-amino acids which makes them ideal scaffolds for attaching imaging and therapeutic moieties. Some of the benefits of peptide scaffolds include:

- High bioavailability, tissue penetration, metabolism, and clearance which allows for high efficiency of drug metabolism.
• High water and fat solubility.
• High proteolytic stability due to unnatural amino acids being utilized to optimize clearance after injection.
• Small peptides are readily synthesized with solid peptide synthesis, as well as easily purified with chromatography and identified with mass spectroscopy\(^{41}\).

These advantages make the molecular targeting peptide 18-4 an ideal candidate to serve as the targeting agent for our dually functional MTP. Peptide 18-4 was synthesized utilizing solid phase peptide synthesis (SPPS), with a procedure developed for the molecular probe M2 as described in the M.S Thesis by Xinyu Xu\(^{29}\), as well as Basant Kaur\(^{42}\) and Sara Shaut\(^{19}\) assisted by student Matthew Law. Our SPPS method has been modified from the original procedure from Kaur\(^{28}\) to allow for the use of Sieber resin. The rationale for this modification was that dyes and other imaging agents that are attached during the SPPS would survive the cleavage reaction of the peptide from the synthesis resin through the use of dilute acid cleavage that the Sieber resin enables.

In the previous research in our lab\(^{19,29,42}\), dyes were placed on the side chain of the lysine residue which is the third residue from the right on 18-4. However, when heavily sulfonate dyes were conjugated with the sidechain of the 3’ Lysine residue, the binding affinity of these dye-peptide conjugates was greatly reduced. In communications with Dr. Kaur, it was speculated that optimal binding of 18-4 to the keratin-1 (KRT1) receptor relies on positively charged residues near the C-terminus, along with the C-terminus remaining somewhat lipophilic as to allow for necessary hydrophobic interactions between to occur for binding.
Conjugating a highly charged, bulky dye payload near the C-terminus might prevent optimal binding interactions to occur, therefore it was decided to replace the 3’ lysine as utilized in our past approaches with the original arginine in 18-4, and to conjugate all new imaging and therapy groups on the N-terminus of 18-4.

The OMI and PS dyes will therefore be conjugated to the 18-4 scaffold through the use of multiple bioconjugation reactions at the N-terminus of the peptide. For the dual modal agents, the OMI and PS dyes will be conjugated together upon single a lysine amino acid and then this dual modal module will be conjugated onto the N terminus of 18-4 in order to form the TMIA.

1.7 Solid Phase Synthesis (SPPS) of 18-4

Utilizing a peptide scaffold also allows for the addition of multiple functional modules, also known as “puzzle pieces”, upon the original scaffold. As SPPS is highly efficient at linking amino acids, if lysine amino acids have their free amine coupled with a carboxylic acid of another molecule such as imaging agents via an amide bond, one could easily attach any molecule containing a carboxylic acid functional group onto the peptide. The dyes chosen for this experiment contain free carboxylic acid groups that are utilized to form modules or “puzzle pieces” constructed by attaching the dyes to the side chain of lysine amino acids. These modules can then be coupled to a targeting peptide through conventional peptide coupling methodology for the completed TMIA.

As described in detail in the MS thesis of past students Xinyu Xu and Basant Kaur, the eleven step SPPS to form the starting peptide shown in Figure 3 have been optimized and
scaled up to the gram scale for synthesis of the peptide.

\[
\text{Fmoc-Leu-NH-P}\downarrow 10 \text{ Steps in SPPS} \\
\text{Fmoc-Trp-d-Nle-Glu(}\texttt{OtBu}\text{)-Ala-Ala-Tyr(}t\texttt{Bu}\text{)-Gln-d-Lys(}\texttt{MTT}\text{)-Phe-Leu-P}\downarrow \text{deavage (also removes protecting groups)} \\
\text{Fmoc-Trp-d-Nle-Glu-Ala-Ala-Tyr-Gln-dLys-Phe-Leu-NH2}
\]

Figure 3: The SPPS of BrCa targeting peptide 18-4 on polymeric Sieber resin\textsuperscript{29}.

The SPPS of 18-4 allows for the efficient synthesis of the scaffold for the MTP. Utilizing the ‘puzzle piece’ approach developed in our lab\textsuperscript{43}, the MTP will be bioconjugated to one or two amino acid based modules, also called ‘puzzle pieces’ containing either a PS dye or OMI dye, or both dyes for dual PS & OMI. The synthesis of these puzzle pieces will be described in subsequent chapters of this thesis.

1.8 Optical Molecular Imaging (OMI)

Early detection of cancer cells is the most significant factor in favorable patient outcomes. In response to this, a plethora of imaging methods have been developed to allow doctors to visualize and treat cancerous cells. Imaging is essential for treatment as it aids in visualization and understanding of the cancerous tissue, how aggressive the cancerous growth is, if the cancer has metastasized, or how effective a treatment regime is working to reduce tumor mass.

Notably, the development of ultrasound, magnetic resonance imaging (MRI), and computed tomography (CT) have enabled doctors to screen patients and precisely localize lesions. However, these imaging methods do not generate tumor specific signals, and mostly rely on differences in anatomic structures\textsuperscript{44}. They also cannot provide doctors with real-time imaging during radiotherapy or surgery\textsuperscript{44}. Moreover, these imaging methods are expensive, and require bulky, highly specialized equipment that are not available within an operating room\textsuperscript{45}.
As a result of these limitations, there is demand for a real-time imaging method which is minimally invasive, provides tumor specific response, and can be utilized with portable and affordable equipment. In recent years, the use of optical imaging strategies to image tissues intraoperatively has garnered significant attention due to the development of targeted molecular imaging agents (TMIAs). These TMIAs are a combination of a targeting group selective for a cancerous biomarker in combination with a near-infrared fluorescent dye. By combing these two groups, the normally unspecific dye molecule will selectively undergo endocytosis in cancerous cells. This allows for real time cancer-specific visualization of tumors.

The use of such a TMIA to assist in the visualization of cancer cells during surgery is called Optical Molecular Imaging (OMI). OMI relies on the difference in expression of a cancer specific biomarker which is mutated or overexpressed within tumors to allow for selectivity. For the peptide probe 18-4, this biomarker is the KRT1 protein. Such imaging using TMIA’s is also referred to as “functional imaging” and it could be said that TMIA’s do not only describe “what is there”, but also describe “what is happening there”.

OMI will allow doctors to easily compare healthy and BrCa cells in real time through a noninvasive procedure in vivo. If the cancer cells contain a sufficient concentration of OMI dye, the doctor will be able to detect any cancerous tissue by shining a laser upon the breast tissue in order to check for any fluorescence emission. This allows for visualization of occult and non-palpable tumors in real time.

Through the use of OMI in BrCa therapy, our hope is to reduce the reoperation rate of the lumpectomy operation through the imaging and identification of cancerous cells during surgery, with a pre-administered OMI dye. The OMI dye will be selective for BrCa cells due to the attached
peptide scaffold and BrCa targeting agent ‘18-4.’ This strategy of “light directed surgery” will help combat the “lumpectomy reoperation epidemic” as described earlier through selective visualization of BrCa cells.

In our prior research in this project, confocal fluorescence microscopy (CFM) has been utilized to validate the uptake of a TMIA, composed of 18-4 which had been conjugated with Cy5.5-2S (a NIR OMI dye), into the triple negative (TN) MDA-MB-231 BrCa cell line\textsuperscript{43} as shown in Figure 4 below.

![Figure 4: The CFM imaging of MDA-MB-231 TN BrCa cells. The red fluorescence is from Cy5.5-2S and the blue is the nucleus stained with Hoechst 33342. The 18-4 decapeptide was utilized to target KRT1 in MDA-MB-231 BrCa cells\textsuperscript{43}.]

1.9 Strategy for Modular Synthesis of TMIA\textsuperscript{s}

In recent years, our research lab has developed a modular synthesis of peptide-based TMIA\textsuperscript{s} utilizing modules or “puzzle piece” building blocks. These puzzle pieces consist of an amino acid with an imaging molecule conjugated upon the sidechain\textsuperscript{46,47}. These puzzle pieces are then conjugated further with a targeting group to prepare a TMIA as shown in Figure 5 below.
Figure 5: A TMIA composed of two puzzle pieces each containing a unique imaging agent conjugated to a targeting agent selective for a cancerous biomarker.

The approach we will use follows a similar process. The first dye was conjugated to the sidechain of Fmoc-Lysine-OH through an amide bond formation reaction. This will link the free carboxylic acid upon the imaging module and the amine group upon the Lysine to form an amide bond, linking the two molecules together. The resulting Fmoc-lysine module conjugate was subsequently deprotected, and then coupled with another dye on the newly liberated N-terminus of the lysine. This resulted in a dual module lysine conjugate with a free carboxylic acid group for subsequent activation and coupling upon the N-terminus of peptide 18-4 1 for the targeting of breast cancer.

An overview of this synthetic approach to creating these multifunctional imaging modules is shown in Figure 6. The targeting peptide will be synthesized separately through the use of SPPS.
Figure 6: The convergent approach to synthesize the Dual-Module Puzzle Pieces, followed by conjugation to the N-terminus of 18-4 to yield the final Dual Functional TMIA.

This approach is convergent and thus minimizes the overall amount of synthetic transformations needed to synthesize the dual TMIA-MTPS (targeted molecular imaging agent-molecular targeted photosensitizer) allowing for two different possible dye positions upon the lysine. With two arrangements available, both dual probes can be synthesized and tested to see if one is optimal over the other for the binding of the novel peptide-based conjugates to the KRT1 receptor.

2. Strategy for Dual Targeted BrCa Imaging and Photodynamic Therapy

2.1 Wavelength design strategy for dual OMI-PDT molecular probes

Both PDT and OMI, which rely on light activation, each offer distinct and captivating possibilities. By using an OMI dye and a PS dye with different absorption properties, it becomes feasible to employ one light wavelength for fluorescent imaging and another separate wavelength
for activating the photosensitizer dye (PS dye) for PDT.

When considering dyes for use in biological systems, one has to consider the “Near-infrared window” in biological tissue. Existing within biological tissue exists a diverse assortment of biological chromophores, which turn excitation into heat dissipation. One highly prevalent in vertebrate tissue is the chromophore heme, which is responsible for capturing and transporting oxygen within hemoglobin. Both the oxygenated and deoxygenated heme molecules strongly absorb light within the <600nm range, giving blood its distinctive red color. Other biomolecules such as aromatic amino acids, melanins, heterocyclic flavins, and adenine dinucleotide (NADH) all additionally contribute their own respective absorbances, preventing the use of NIR wavelengths in biological imaging. Water also has multiple vibrational overtones with absorption maxima at 970nm, 1200nm, 1450nm, and beyond 1800nm.

Considering the absorption maxima of the chromophores present within biological tissue, the spectral region of 600-850nm is most favored for fluorescence imaging, and this spectral region is denoted the “biological NIR window”. Dyes which excite within this region will not compete with chromophores naturally present within biological tissue, allowing for minimal off-target absorption robbing the PS dye from absorbing light. Additionally, it is advantageous to use dyes which have high absorption wavelength maxima, as higher wavelength lasers have increased tissue penetration. For this reason, red lasers are commonly employed in photodynamic therapy.

2.2 IRDye78 for Imaging of BrCa

The NIR dye utilized for this TMIA is IRDye78, as seen in Figure 7 below. This dye has been developed in our lab at RIT to function as an easier-to-synthesize, symmetrical analog of the clinically tested IRDye800CW®, which has seen notable attention for presenting the highest contrast in prostate cancer cells compared to other related cyanine dyes. However, the actual
synthesis of IRDye800CW® is low-yielding due to the formation of symmetrical impurities resulting in undesirable byproducts\textsuperscript{54}.

Our lab has promoted the use of this analog with the identical chromophore but symmetrical in structure through the use in our past collaborations\textsuperscript{54}. The symmetric IRDye78 as seen in Figure 7 below is readily synthesized in four steps from commercial starting materials and has high water solubility due to four sulfonate groups. IRDye78 has a $\lambda_{\text{max}}$ at 783 nm and $\lambda_{\text{emission}}$ at 805 nm in Methanol\textsuperscript{54}.

![Figure 7: The structure of IRDye78 as previously described in Literature\textsuperscript{54}.](image)

Upon cellular uptake, the peptide targeted IRDye78 will allow for the imaging of the BrCa cells through visualization of the emission photons upon excitation of the dye ($\lambda_{\text{max}}$=783nm). The fluorescence can be detected and visually observed with appropriate NIR goggles after excitation with a laser by a doctor. The dye could also be utilized \textit{in vitro} experiments by the use of confocal fluorescence microscopy (CFM), or by the use of the plate reader, molecular image station, or related instruments such as an IVIS or Odyssey instrument\textsuperscript{TM}.
2.3 Porphyrin Photosensitizers for PDT of BrCa

Porphyrin rings are a class of heterocyclic macrocycles which consist of four substituted pyrrole rings joined at their α carbons by methine carbons\textsuperscript{19}. The porphyrin ring remains one of the most widely studied macrocyclic ring systems, largely due to their unique light-absorbing energy-conversion properties, as well as the ability to act as a strong metal-complexing ligand\textsuperscript{55}. In biological applications, porphyrin rings are involved with the processes of oxygen transport and photosynthesis.

The porphyrin macrocycle is commonly substituted at the β and meso positions as seen in Figure 8. Porphyrin rings which are fully substituted with hydrogens at the β positions are known as Porphine rings. Porphyrin rings are also able to form strong metal chelates, denoted as Metalloporphyrins\textsuperscript{56}. When unchelated, the porphyrin ring is denoted as the ‘free base’ porphyrin\textsuperscript{56}.

Porphyrins are known for their extensive conjugation, which results in a highly planar and stable macrocycle. Porphyrin rings in particular have been investigated for their ability as highly potent photosensitizers due to their high degree of intersystem crossing resulting in a prolonged \( T_1 \) lifetime\textsuperscript{57}. A large population of \( T_1 \) state PS is required for the generation of \( ^3\text{O}_2 \), which is necessary for PDT to occur.
Figure 8: The numbering of the Porphyrin Macrocycle and different Porphyrin rings with their respective unique compositions of pi electrons.

Depending on the degree of saturation of the macrocycle, porphyrin rings are grouped into different classes each with their own unique degree of saturation. Porphyrin rings contain a fully unsaturated ring with 22 π electrons. Commercial porphyrin-based photosensitizers suffer from poor tissue penetration due to its poorly absorbing UV-vis absorbance maxima (630nm)\textsuperscript{58} (see table \textsuperscript{1}). Chlorin and Bacteriochlorin rings contain either one or two reduced exocyclic double bonds respectively.

Compared to porphyrin rings, chlorin rings have been seeing considerable attention for their intense absorptions in the near-infrared region (>650nm)\textsuperscript{58}. As such, these dyes are more viable for biological applications as this is well outside of the range of the absorbance of most chromophores within biological tissue.
Due to this, many chlorin-type PSs have seen success in clinical applications for PDT treatment, with talaporfin\textsuperscript{59} (mono-L-aspartyl chlorin e6) being approved in Japan for PDT treatment of lung cancer, and temoporfin\textsuperscript{60} (m-THPC) being approved in the EU for the PDT of squamous cell carcinoma of the head and neck. However, like most untargeted PSs, patients suffered from off-target accumulation and photosensitivity complications\textsuperscript{61}.

Bacteriochlorin rings are a class of tetrapyrrole macrocycle which contain two reduced exocyclic double bonds in comparison to porphyrin rings, which results in an intense red-shifted UV-Vis absorbance $\lambda_{\text{max}}$, with bacteriochlorins absorbing around 740-780nm\textsuperscript{58}. As a result of this, bacteriochlorins have received significant clinical attention, as the high absorbance maxima allows for deep tissue penetration of light.

Examples of successful bacteriochlorin PSs include Redaporphyrin which has seen success in Phase 2 clinical trials for head and neck cancer\textsuperscript{62}, and Pd-Bacteriopheophorbide (Padeliporfin) obtaining approval in the EU for the PDT of prostate cancer\textsuperscript{12}. However, use of Bacteriochlorin scaffolds has been hindered by their lack of commercial availability, as naturally derived Bacteriochlorins are extremely difficult and low yielding to extract from bacterial cultures\textsuperscript{63}.

As a result of the commercial inaccessibility of Bacteriochlorin scaffolds, PSs of the Chlorin ring type will be employed as the PS dye for the TMIA. Specifically, Mesopyropheophorbide (mPPa) \textsuperscript{2} will be utilized, as it can be synthesized in a one-step hydrogenation from commercially available Pyropheophorbide a\textsuperscript{19} (PPa) \textsuperscript{1}.

To improve the PDT ability of freebase PS dyes, the addition of metals to form metalloporphyrin PSs have demonstrated great clinical potential. Upon coordination of metals, the structure of the PS changes to become more planar, which results in a more stable porphyrin ring.
The presence of the heavy metal within the ring reduces the energy difference between the $^1S$ and $^1T$ of the PS, due to a phenomenon known as the heavy-atom effect\textsuperscript{64}. This will allow for greater ICS to occur, which results in greater production of $^1O_2$, which allows for a more effective PDT treatment.

In particular, palladized-PS dyes have seen clinical success, with notably Padeliporfin and TOOKAD\textsuperscript{®} Water-Soluble\textsuperscript{65} both proving to be highly efficient $^1O_2$ generators for PDT of cancers. As a result of this increased PDT efficacy observed in literature, the metalated chlorin Pd-Pyropheophorbide will also be investigated as an alternative viable PS dye for the TMIA. The structures of the two PS dyes which will be utilized in the final TMIA\textsuperscript{s} can be seen in Figure 9.

![Figure 9: The Structure of the Chlorin-type PS dyes utilized in this project.](image)

3.0 Aim and Design of Dual targeted TMIA probes.

3.1 Design of Dual PS and NIR dye-based targeting agents

The primary goal for the project is to synthesize novel targeted molecular probe (MTP) for the dual PDT and OMI of BrCa cells as proposed in a recently funded NIH-R15 grant proposal.
The modular method previously developed for the synthesis of TMIAs in our lab will be utilized as shown in Figure 11 and as described in Section 1.9.

There is one single example of a dual agent containing a dye for OMI and a dye for PDT, reported by the Pandey lab (at Roswell Park), described in a paper as a “bifunctional agent for tumor imaging and photodynamic therapy”. In this paper the dual-dye compound, shown in Figure 10 below was synthesized, followed by insertion of three metals, In, Ga and Pd. These were tested and importantly, were found effective agents for the PDT of basal cell carcinoma (a type of skin cancer) cells in mice models.66

![Figure 10: The structure of a direct conjugate between a cyanine dye for OMI alongside a metalated porphyrin, as synthesized by Pandey et al. (2016)66. This dual dye conjugate was successful for the PDT of cancer cells.](image)

It is noteworthy that although these agents are not targeted, lipophilic photosensitizers have been shown to accumulate within cancerous cells at a higher concentration then healthy, somatic
cells. As a result, the direct conjugates synthesized by Pandey were observed to accumulate in skin cancer cells to a greater extent than normal healthy cells. Although this can be considered as one type of targeting, the selectivity for cancer is not mediated by a receptor mediated interaction and the applicability of this approach to other cancers cannot be assured. The lack of a receptor-based targeting scheme also causes significant off-target accumulation of PS, which can potentially cause side effects in patients.

For this reason, all non-targeted PS dyes are applied to tumors through physically painting the dye upon the diseased tissue or applied with injection to the tumor mass prior to illumination with a light source. In contrast, our goal is to provide an injectable, molecularly targeted agent and one that combines imaging to direct the therapy, and the PS dye itself in the same compound. It was reassuring to find that the combination of similar imaging and PDT dyes was shown to be efficacious in the case of cancer, giving credence to our expansion to a receptor targeted scheme.

In the modular synthetic approach, the porphyrin ring will first be coupled to the side chain of Fmoc-lysine, followed by the subsequent Fmoc deprotection, and coupling of the IRDye78 to form the dual dye conjugate. This important dual-dye module or puzzle piece will then be activated for coupling to the N-terminus of peptide 18-4 as described above.
Figure 11: The synthesis of a Dual Dye TMIA utilizing the formation of a Dual Dye Puzzle Piece containing mPPa and IRDye78. The final structure elaborates the TMIA structures illustrated by their names in boxes.

In a preliminary study, the following direct conjugate as seen in Figure 12 below was synthesized by group member Micah Hrubec in our lab. This unpublished preliminary result of
this conjugate of IRDye78 to the n-terminus of 18-4 was shown in confocal fluorescence studies to successfully bind to the TN BrCa cell line MDA-MB-231 as seen in Figure 13.

Figure 12: The structure of the single modal TMIA with OMI dye IRDye78 (highlighted in red) directed conjugated upon the n-terminus of decapeptide 18-4, which successfully bound to MDA-MB-231 TN BrCa cells.

Figure 13: The obtained CFM results from Dr. Timothy Baran at the University of Rochester showcasing stained MDA-MB-231 BrCa cells with the above single modal TMIA (left stain incubation for 4 hours, right: stain incubation for 22 hours.)
Due to the success of the binding of this direct conjugate, the KRT1 receptor is thought to be somewhat tolerant of highly planar & sulfonated dyes conjugated near the n-terminus of 18-4. Therefore, it is of interest to design the next generation of dual dye conjugates to be produced with IRDye78 closer to the n-terminus of the peptide scaffold, as this result will be similar in design to probes which have been validated to bind to KRT1.

In contrast to this preliminary single modal agent, our ultimate design is a lysine module first containing two dye molecules. As lysine contains chiral carbon, the possibility for different design of chiral payloads is introduced. Part of this first aim to therefore produce both dual-dye puzzle pieces conjugated upon the side chain of both the D and L enantiomers of lysine.

It was further envisioned that the two dyes could be placed also in “reverse order”, by first placing the IRDye78 upon the sidechain of the lysine followed by coupling the porphyrin PS dye upon the new N-terminus of the lysine. An example of this final dual dye conjugate can be seen in Figure 14. While this approach results in a probe less similar to previously validated probe designs, it still serves as potential interest for understanding the receptor binding ability of the KRT1 receptor.
3.2 Design of Dual Palladized-PS and NIR dye-based targeting agents

A second overall goal for this project would be to investigate the use of palladized porphyrin dyes in use alongside OMI dye IRDye78, as Pd-porphyrin dyes have seen significant attention due to their increased PDT ability. Such a TMIA would be synthesized utilizing similar conditions as the previously proposed dual dye lysine conjugate, provided the metalated porphyrin dye is resistant to demetallation under amide coupling conditions.
3.3 Design of Single-Dye Water-Soluble Porphyrin targeting agents

A previous MS student Sara Shaut synthesized a single modal targeted PS with mPPa conjugated upon 18-4 on the third amino acid residue, D-lysine. Unfortunately, this compound proved to be completely water insoluble, hindering its ability as a therapeutic agent. Because water solubility is essential to ensure optical bioavailability for an injectable agent, a stretch goal for this project is to synthesize a more water-soluble version of the peptide-porphyrin conjugate.
Figure 16: The design of a single dye TMIA with mPPa conjugated with 18-4 upon the sidechain of the 3’ lysine residue by MS graduate Sara Shaut. This design has been since abandoned, in favor of attaching therapeutic agents upon the N-terminus to avoid negative binding interactions with KRT1.

In the case of a single porphyrin agent, the dye alone has the capability to serve as a multifunctional imaging and PDT dye alone, but the imaging is hindered by its low wavelengths for activation (600 nm) and emission (660 nm). Moreover, the molar absorptivity coefficient at this wavelength is one-tenth of a typical cyanine dye.

When searching for a design of a water-soluble module or puzzle piece, we sought to make use of sulfonates to impart additional water solubility upon the final TMIA. Dyes have been previously engineered in our lab to contain additional sulfonate groups, and as a result the water solubility of the planar dyes significantly increased. However, preliminary attempts to sulfonate
the starting material Pyropheophorbide a \( \text{I} \) proved unsuccessful, and alternative methods to add water solubility to porphyrin rings would have to proceed through the generation of another molecule containing a high amount of sulfonates for later conjugation to the porphyrin macrocycle.

This aim then developed into creating a new module or puzzle piece that was similarly based on the modular strategy using lysine, but instead of adding a second imaging group on the n-terminus of lysine, to instead add a water solubilizing group.

It should be noted that the porphyrin dye alone has the capability to serve as a multifunctional imaging and PDT dye alone, but the dye is hindered by its low absorptivity and limited emission signal. While porphyrins are rarely used as imaging dyes compared to other higher absorbing and emitting dyes, we predict there would be considerable interest in a novel approach to prepare single modal or solo water-soluble PS dyes that could be attached to targeting agents like 18-4 which also have limited water solubility.

The availability of a water-soluble (injectable) targeted PS photosensitizer (WS-PS) dye could be used in tandem with imaging methods such as ultrasound, CT, or MRI in mapping the site of photodynamic therapy. While depth of penetration requirements of 1-2 cm would often be a problem, there are many instances in which tumors persist within that range. An example would be when tumors have metastasized to lymph nodes, or for tumors residing close to the surface of the breast. There is also a technique in which cells are irradiated utilizing interstitial fiber optics, to overcome the limited tissue penetration of lower wavelength absorbing dyes. Therefore, the development of a single-modal PS for the targeted PDT of BrCa was developed through the synthesis of the “water-soluble” module \( \text{25} \), from commercially available starting materials, as discussed in Section 4.5.
Figure 17: The water-soluble module 25 and a water-soluble porphyrin amino acid puzzle piece 26.

This water-soluble module 25 was then conjugated to the water-insoluble porphyrin-amino acid puzzle piece 6 to yield a highly water-soluble porphyrin-amino acid conjugate 26.

In the larger scope, this method of conjugating puzzle pieces to the water-soluble module could be expanded to increase the water-solubility of any lipophilic drug or peptide. This method of imparting water solubility to insoluble drug payloads or peptide conjugates would allow for drug candidates which have been previously discarded for being insoluble to be re-engineered for increased solubility to ensure optimal bioavailability.
4. Results and Discussion

4.1 Synthesis of Porphyrin & Pd-Porphyrin Puzzle Pieces

Our approach first focused on the synthesis of a stable chlorin-type PS scaffold which could be obtained in minimal steps from commercially available starting materials. The starting material Pyropheophorbide a was selected due to its overall structural simplicity of the Chlorin ring, as the ring possessed little functionality other than the required carboxylic acid needed for bioconjugation.

However, the commercial macrocycle did contain a peripheral double bond which proved reactive under acidic conditions\textsuperscript{19}. In order to prevent unwanted reactivity during further synthetic steps, the commercially available Pyropheophorbide a (PPa, 1) was hydrogenated into the reduced Meso Pyropheophorbide a (mPPa, 2) as shown in Figure 18. The naming for this compound comes from the naming of a similar reduction performed by Smith et al\textsuperscript{66}, and as described by our previous MS student Sara Shaut\textsuperscript{19}.

![Figure 18: The reaction scheme for the hydrogenation of Pyropheophorbide (PPa, 1) a to produce Mesopyropheophorboide a (mPPa, 2) ](image-url)

\textit{Figure 18: The reaction scheme for the hydrogenation of Pyropheophorbide (PPa, 1) a to produce Mesopyropheophorboide a (mPPa, 2)}
The hydrogenation reaction proved to be highly efficient and yielded a highly stable chlorin-type PS for subsequent conjugation reactions. The UV-Vis spectrum of mPPa contains multiple highly characteristic absorption bands which proved useful for identification of porphyrinoid products. Throughout our synthesis we relied on HPLC-MS and UV-Vis-NIR analysis by diode array detection (DAD) for product characterization.

The spectrum consists of an intense Soret band at 407nm ($\varepsilon = 1.1*10^5$ mol$^{-1}$ cm$^{-1}$), followed by four less intense Q bands at 503nm ($\varepsilon = 1.04*10^4$ mol$^{-1}$ cm$^{-1}$), 535nm ($\varepsilon = 1.06*10^4$ mol$^{-1}$ cm$^{-1}$), 599nm ($\varepsilon = 1.04*10^4$ mol$^{-1}$ cm$^{-1}$), 651 nm ($\varepsilon = 4.74*10^4$ mol$^{-1}$ cm$^{-1}$). Molecular absorptivity (extinction coefficient) values were obtained by diluting a sample with 1.0 AU at 407 nm in methanol by half its volume five times, thus enabling observations and calculation using the Beer-Lambert law.

![Figure 19: The UV-Vis absorbance spectrum of mPPa 2, with absorbance maxima at 407 nm and 660 nm in methanol.](image-url)
The fluorescence spectrum of 2, when irradiated at 400 nm results in a remarkable Stoke’s shift of 243 nm, distinctive of chlorin-type PS dyes\textsuperscript{19}.

\textbf{Figure 20:} The fluorescence spectra for mPPa 2, with a emission maxima of 661 nm\textsuperscript{19} in methanol.

Preliminary attempts to metalate 2, which contains a carboxylic acid proved to yield no metalation under described conditions in literature\textsuperscript{66}. Upon further investigation, it was found that literature reports few reactions of metalations upon porphyrin rings containing carboxylic acids, but many more on porphyrins that contained methyl esters or amide bonds. The few papers which showcased such transformations on acids, utilized exotic microwave conditions, but in our attempts using a conventional microwave was not successful\textsuperscript{70}. This lack of reactivity is presumed due to a competing coordination between the pyrrole nitrogens and the carboxylic acid versus the Palladium (II), hindering metalation under mild conditions.
It was hypothesized that if the carboxylic acid could be protected with an ester compatible with bioconjugation reactions, the porphyrin could be metalated under mild conditions similar to reported in literature.

In order to solve this dilemma, we turned to a popular form of active ester for coupling dye molecules, the N-hydroxysuccinimide (NHS) ester, also known as the oxy-succinimidyl ester (OSu ester). We would need to activate the acid for coupling to the side or N-terminus of Lysine in either approach, so it was logical to attempt the palladation on the active form of the ester, which we would ultimately need.

The NHS active ester is widely used due to its significant stability in neutral and acidic media. We speculated that, provided we isolate this intermediate in mildly acidic media, we could utilize it as a protecting group for the carboxylic acid. This newly protected porphyrin could then be utilized for conjugation of the porphyrin to our imaging/targeting system. The NHS ester then performs a double task as a protecting group along while serving as the reactive intermediate needed for amide bond formation in subsequent bioconjugation reactions.
In order to synthesize this NHS ester (also known as OSu ester), mPPa 2 was converted into mPPa-OSu, through the use of the uronium based coupling reagent N,N,N′,N′-Tetramethyl-O-(N-succinimidy)uronium tetrafluoroborate (TSTU).

Figure 22: The conversion of mPPa 2 into mPPa-OSu 3.

The reaction proceeded in near-quantitative conversion provided the reaction was kept sufficiently anhydrous while basic. Upon conversion of the reaction, the reaction was quenched by the addition of glacial acetic acid to protect the active OSu ester 3 from hydrolysis. Upon acidification, the resulting OSu Ester can be purified with C18 chromatography in MeOH:0.01M TFA buffer. Provided the pH remains acidic, the OSu ester 3 is resistant to hydrolysis. N,N-Diisopropylethylamine (DIPEA) is employed as the base of choice for all OSu ester formation and coupling reactions for its ability as a strong non-nucleophilic base which is unreactive towards OSu esters. It is imperative to keep the reaction conditions anhydrous (under argon in oven-dried glassware) as advantageous water reacts into OH- ions under basic conditions, and this will cause the OSu ester 3 to hydrolyze back to the starting material, the carboxylic acid mPPa 2.
Figure 23: The mechanism of OSu Ester formation with TSTU under basic conditions.

Due to the high reactivity of OSu esters, HPLC-MS analysis of OSu esters is often inconsistent, as the OSu ester is prone to hydrolysis as it passes through the C18 column. Therefore, it is difficult to differentiate between the starting carboxylic acid and how much OSu ester is present within the crude reaction mix, as the OSu ester parent mass cannot be reliably observed with ESI ionization. To monitor the formation of OSu esters, a small aliquot of the reaction is taken and treated with a solution of 0.1% butylamine in acetonitrile. This results in the reactive OSu ester reacting to form the corresponding butyl amide, which is stable and consistently observed on ESI ionization in both positive and negative ionization modes.
Figure 24: Quenching the reactive OSu ester by formation of the butyl amide derivative to assay the concentration of OSu ester present within the reaction by HPLC-MS analysis.

Throughout all amide coupling reactions described that follow, the presence of OSu or OBt esters was always assayed through generation and subsequent HPLC-MS analysis of the butyl amide derivative. The derivatization process provides an accurate, consistent way to assay the concentration of ester intermediate within in the crude reaction mixture.

The active ester form of the chlorin, mPPa-OSu 3 can be purified by C18 reverse phase preparative HPLC. Upon isolation of this material, the metalation reaction was then attempted utilizing the same mild conditions previously attempted upon the porphyrin mPPa 2, as reported in literature\textsuperscript{66}. This NHS protected macrocycle proved reactive towards metalation, and the reaction proceeded with no major byproducts or impurities.

The resulting product, Pd-mPPa-OSu 13 can be purified with C18 chromatography in MeOH:0.005M AcOH. The Pd-mPPa-OSu 13 proved resistant to demetallation & hydrolysis when dissolved in neat glacial acetic acid over 72hrs and proved to be stable under open air as a lyophilized powder. This method would serve as the source of all Pd-mPPa 13 utilized in future reactions.
Figure 25: The metalation of mPPa-OSu 3 into Pd-mPPa-Osu 13.

Upon isolation of the OSu Ester, the resulting macrocycle can be further reacted with the sidechain primary amine of Fmoc-Lysine-OH under basic conditions, resulting in amide bond formation. The reaction of both free base mPPa-OSu 3 and Pd-mPPa-OSu 13 with Fmoc-Lysine-OH yielded protected amino acid dye conjugates in good yield as seen in Figure 27.

Figure 26: The DAD UV-Vis spectrum of Pd-mPPa 14.

Puzzle pieces for PDT (PDT modules) comprised of both enantiomers of lysine conjugated to mPPa and just D-Lys for Pd-mPPa were then constructed to leverage the strategy
of utilizing peptide coupling reactions to attach the dye conjugate directly upon the N-terminus of 18-4.

Initially, we aimed to produce conjugates of varying stereochemistry as one of the enantiomers of lysine might interfere with the binding between KRT1 and 18-4 more than the other. However, for expediency we chose to focus only D-Lysine to produce metalated porphyrin conjugates in order to decrease the overall number of total compounds in synthesis and biological testing. We chose to focus on the unnatural D enantiomer of Lysine, which is preferred as unnatural amino acids are more proteolytically stable\(^{36}\). The products shown in Figure 27 will ultimately be taken on to synthesize three novel dual functional TMIAs 11, 12, 18.

![Figure 27: The Scheme to synthesize deprotected free base and metalated lysine-porphyrin conjugates.](image)

\(^{11}\) M = 2H, mPPa-O\(\text{OSu}\)  
\(^{13}\) M = Pd, Pd-mPPa-O\(\text{OSu}\)  
\(^{4}\) Fmoc-D-Lysine(mPPa)-OH - 80\%  
\(^{5}\) Fmoc-L-Lysine(mPPa)-OH - 75\%  
\(^{6}\) H2N-D-Lys(mPPa) - 71\%  
\(^{15}\) Fmoc-D-Lysine(Pd-mPPa)-OH - 64\%  
\(^{7}\) H2N-L-Lys(mPPa) - 74\%  
\(^{16}\) H2N-D-Lys(Pd-mPPa) - 79\%
Figure 28: The mechanism of amide bond formation between primary amines and OSp Esters of mPPa under basic conditions.

In order to conjugate the second dye molecule to the lysine N-terminus, the resulting Fmoc-protected amino acid dye conjugate needs to be deprotected to liberate the amine needed for conjugation.

The Fmoc-protecting group is a base-labile protecting group utilized to protect primary amines. Secondary amines are commonly used to remove Fmoc due to their notable basicity and, as secondary amines, they quickly react with the newly generated Fmoc-by product, preventing the potential formation of potential byproducts with the newly deprotected amine on the amino acid.

It was found that DMF was needed for the Fmoc deprotection as attempts to use other solvents such as DCM or ACN (which are more easily removed under vacuum) showed little to no deprotection of Fmoc over 24hrs (by HPLC-MS analysis). In contrast, when DMF was utilized as the reaction solvent the Fmoc protecting group was cleaved in only a few hours. The DMF was then removed under high vacuum, followed by purification of the product by reverse phase preparative HPLC using a C18 column.

This short synthesis results in a lysine-dye conjugate ready for conjugation upon its N-terminus with the OMI dye IRDye78.
4.2 Synthesis of IRDye78 Puzzle Pieces

In order to determine the favored structure-activity relationship in terms of the order of dye conjugation to the peptide, a method was next developed for the construction of the puzzle piece with IRDye78 on the side chain of lysine. While time prevented carrying these onto the final products, the results outlined below provided the groundwork for following students to prepare these important reverse versions of the dual modal targeted molecular probes.

In order to reverse the order of assembly of the two dyes onto 18-4, similar to the formation of Fmoc-Lys(mPPa)-OH, the imaging dye, IRDye78, was also formulated as a puzzle piece (or “imaging module”). First, IRDye78 was isolated as the OSu ester according to methods previously described by our lab. Following similar conditions utilized to couple mPPa-OSu with Fmoc-Lys-OH, IRDye78-OSu was coupled to the sidechain amine to form the lysine-dye conjugate in good yield as seen in Figure 29.

It is noteworthy that unlike lipophilic porphyrin rings, the highly sulfonated dyes allowed for the trituration of product by dilution of the reaction mixture with diethyl ether, followed by centrifugation and decantation of the supernatant to yield a dye pellet. This method allowed for removal of starting materials and DIPEA and facilitates isolation of pure product much more easily than having to utilize only C18 chromatography to isolate the analogous porphyrin-lysine conjugates.
The Fmoc deprotection of the IRDye78 lysine conjugates proved to be problematic and low-yielding due to the inherent instability of the IRDye78 under basic conditions. A significant reason for this is due to the formation of the impurity seen in Figure 30, formed by the displacement of the methoxy-propionic acid group at the center of the conjugated system by DEA. This impurity was detected by HPLC-MS analysis (UV = 620nm). Due to the instability of IRDye78 minimal DEA was utilized, and the reaction was stopped as soon as the presence of the impurity was detected on HPLC-MS. This often resulted in incomplete removal of the Fmoc protecting group, but through subsequent C18 chromatography, the impurity was separated along with starting material from the product.

Future students will need to work to optimize a mild Fmoc deprotection strategy for this puzzle piece to minimize dye degradation by products. It should also be noted that attempts to purify IRDye78 conjugates in AmAc buffer with prep HPLC yielded impurities in a significant number of column fractions, which resulted in low-yielding isolations with no significant byproducts observed on HPLC-MS before purification. Therefore, it is recommended to minimize the time in which IRDye78, and to keep all IRDye78 intermediates in mild acid during purification step to prolong the lifetime of the dye.

Figure 29: The synthesis of Fmoc-Lys(IRDye78)-OH conjugates.
Figure 30: The Fmoc deprotection of Fmoc-Lys(IRDye78) puzzle pieces.

Figure 31: The impurity resulting from the reaction between DEA and IRDye78.

Despite the instability of IRDye78, the resulting deprotected lysine-dye conjugates were ultimately synthesized in moderate yield. These were further coupled with mPPa-OSu upon the N-terminus of the lysine puzzle piece. The resulting UV-Vis spectrum of the isolated lysine IRDye78 conjugates has the same UV-Vis spectrum as previously published in literature\textsuperscript{54}. 

\[ 21 \text{H2N-D-Lys(IRDye78)-OH} \quad - 44 \% \]
\[ 22 \text{H2N-L-Lys(IRDye78)-OH} \quad - 59 \% \]
Figure 32: UV-Vis DAD spectrum of H2N-D-Lys(IRDye78)-OH with absorbance maxima at 768 nm.

4.3 Synthesis of Dual-Dye: IRDye78–Lys(mPPa) modules

Upon isolating the deprotected lysine conjugates of both enantiomers of lysine with PS dye mPPa on the lysine sidechain, the module is next coupled to IRDye78 to form the final “dual-dye puzzle pieces” for conjugation to the N-terminus of BrCa targeting peptide 18-4 as shown in Figure 33 below.
The reactions between lysine porphyrin conjugates and IRDye78-OSu. Yields are indicated on the right.

The reaction between the “active ester” IRDye78-OSu and the various lysine-porphyrin conjugates proceeded readily at room temperature, but reactions proved low yielding due to significant yield losses due to aliquots taken for HPLC-MS analysis, and additional decomposition of IRDye78 during purification with preparative HPLC. Despite near-complete conversion with no major byproducts observed through HPLC-MS, subsequent purifications of triturated crude reaction mixtures routinely yielded moderate to low recovery. This is not uncommon in cyanine dye chemistry as cyanine dyes are vulnerable to both acidic and basic disproportionation reactions back to their hydrolyzed “half dyes” which are noticeable as they are colored yellow, orange and red which can be seen in impurity fractions during preparative HPLC.

Despite the low yield, the synthesis provided sufficient quantities of dual-dye conjugates for further conjugation reactions with peptide 18-4. This was accomplished through activation of
the C-terminus carboxylic acid into a reactive ester, followed by nucleophilic attack by the N-terminus of 18-4 to yield the final Dual-Dye TMIA.

Figure 34: DAD UV-Vis spectrum of the dual dye IRDye78-D-Lys(mPPa)-OH with absorbance maxima at 766nm, 650nm, and 407nm, clearly showing the presence of both IRdye78 and mPPa.
Figure 35: DAD UV-Vis spectrum of the dual dye IRDye78-D-Lys(Pd-mPPa)-OH with absorbance maxima at 766nm, 640nm, and 393nm, clearly showing the presence of both IRDye78 and Pd-mPPa

Comparing the UV-Vis absorbance spectra of the free base and metalated porphyrin conjugates with IRDye78, it appears that the small hypochromic shift of the $\lambda_{\text{max}}$ last Q band of the Pd-metalated porphyrin versus the free base porphyrin decreases the spectral overlap between the PS and OMI dye absorbances. This hypochromic shift, as well as the decreased absorbance of the Soret band (~400nm) are consistent with literature and is a direct result of the Pd-metalation within the chlorin macrocycle.

As seen in Figure 31, IRDye78 does absorb light at 635nm, which is the wavelength utilized for PS dye activation. Therefore, in addition to being a more effective $^1$O$_2$ generator for PDT, Pd-mPPa is likely to be preferred as it would have less competition with IRDye78 for light activation which should result in a more effective PDT drug. This is due to the hypochromic shift of the metalated Pd-mPPa shifting the $\lambda_{\text{max}}$ of towards part of the spectral region where IRDye78 absorbs less. Minimizing spectral overlap allows for the specific activation of either imaging or PDT, depending on which therapy the doctor wants to utilize.

4.4 Synthesis of Dual-Dye: mPPa– Lys(IRDye78) modules

Similarly, for the inverse order of construction, the goal was reduced to choosing one enantiomer, the D-lysine-IRDye78 module which was next coupled to mPPa to form the final inversed “dual-dye modules” for conjugation to the N-terminus of BrCa targeting peptide 18-4.

The coupling reaction between the lysine IRDye78 conjugate and mPPa-OSu was therefore also investigated to produce the dual-dye lysine with the opposite positioning of dye molecules. The reaction between H$_2$N-D-Lys(IRDye78)-OH (21) and mPPa-OSu (3) proceeded cleanly and
formed no significant impurities but, like the other purifications with IRDye78, the recovery from C18 chromatography proved to be low-yielding.

A small amount of dual-dye conjugate was produced to serve as a proof of concept from which a future student can carry on with and use these methods to scale up and synthesize dual modal probes containing IRDye78 on the sidechain of the attached lysine. The dual modal problems in the remainder of this thesis all focus on the version with the porphyrin ring on the sidechain of the attached lysine.

![Chemical structures](image)

*Figure 36: The reaction between Fmoc-D-Lys(IRDye78)-OH 21 and mPPa-Osu 3.*

### 4.5 Synthesis of Single-Dye Water-Soluble Porphyrin modules

Similar to imparting water solubility by attaching the tetra-sulfonated IRDye78 upon H2N-D-Lys(mPPa)-OH, a normally water insoluble conjugate, the existence of a “Water-Soluble” (WS) module, likewise containing multiple sulfonate groups alongside a carboxylic acid for bioconjugation, was envisioned to impart the same increased water solubility to an otherwise bulky, planar dye with no water solubility.

Molecular imaging lab member Julia Crandall introduced me to the alkylating reagent 1,3-Propanesultone, which is utilized in the synthesis of water soluble cyanine dyes (OMI dyes) to add sulfonate groups upon indole rings\textsuperscript{43} which serve as precursors for the dye molecules. This
alkylating agent was described in literature to be highly efficient for the installation of sulfonate groups upon nucleophilic heteroatoms\textsuperscript{71}.

Utilizing a similar approach as described in a patent by Bentacrot et al\textsuperscript{72} to produce water soluble phthalocyanine photosensitizers, Tert-butyl 4-aminobutrate HCl was selected to serve as the starting material to synthesize the water soluble module. This starting material was selected as it contained an unprotected nitrogen for alkylation with 1,3 propanesultone, contained a protected carboxylic acid for subsequent deprotection and amide coupling, and contained a long enough alkyl chain to distance the sulfonate groups from the N-terminus of the 18-4 peptide, as to minimize potential negative binding interactions between the charged sulfonated groups and KRT1.

The previous literature approach utilized a benzyl protecting group to protect the carboxylic acid from alkylation, but we sought to avoid this and to circumvent the requirement of catalytic hydrogenation on a large scale. We chose to utilize a t-butyl protected acid instead, which was commercially available and could be more easily cleaved with TFA.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figures.png}
\caption{The structures of the starting materials for the synthesis of the “Water-Soluble” module.}
\end{figure}
Figure 38: The synthesis of the water-soluble (WS) module 25.

The alkylation of the starting Tert-butyl 4-aminobutrate HCl proceeded quickly upon heating. The reaction rate was aided by the fact that the starting material was completely solubilized in basic alkylating agent, as 1,3 Propane sultone is liquid above 31°C. The reaction was ceased upon no more observation of the di-alkylated product by HPLC-MS analysis, as the starting material would decompose into an unknown red byproduct (UV-Vis $\lambda_{\text{max}} = 312\text{nm}$) if the reaction was left to run longer. Future process chemistry is needed to further optimize the yield of the alkylation step to minimize byproducts, but the current conditions yielded enough for further synthetic steps.

24 was deprotected with 40% TFA in DCM at room temperature. The reaction flask was left to open air so the water in air could scavenge the resulting generated carbocation from the tert butyl ester deprotection. Compound 25 has extraordinary water solubility for an organic compound, due to the high number of polar functional groups which can engage in hydrogen bonding with water. Due to this, if compound 25 is isolated as the crystalline acid (lyophilized in
0.01M TFA buffer), the resulting white crystal is insoluble in organic solvents, with no observed solubility in DMSO even with heating and extensive sonication. Therefore, as the subsequent coupling reactions would need to be run in organic solvents, the compound was lyophilized in water with a small amount of TEA, to isolate the compound as the TEA salt. This salt exhibited solubility in DMSO for use in peptide coupling reactions.

The water-soluble (WS) module was then able to be activated with TSTU into the OSu ester and coupled with H$_2$N-D-Lys(mPPa)-OH 6 in a one-pot coupling reaction, as seen in Figure 38.

![Chemical Reaction Diagram](attachment:image.png)

**Figure 39**: The synthesis of a Water-Soluble lysine-porphyrin dye conjugate 26 from the TEA salt of the “Water-Soluble” module, compound 25.

The conversion to the active OSu ester was monitored by quenching an aliquot to generate the butyl amide derivative, using a similar procedure as previously described for monitoring the formation of mPPa-OSu. However, this time a solution of 0.1% butylamine in H$_2$O was employed. Upon complete conversion of starting material to the butyl amide derivative on HPLC-MS analysis, the lysine-porphyrin conjugate H$_2$N-D-Lys(mPPa)-OH 6 was then added to yield the final water-soluble lysine porphyrin dye conjugate.
In a one-pot coupling reaction such as this, the amine should be added to the active OSu ester only upon complete conversion of the carboxylic acid to OSu ester has been assured by assaying a quenched aliquot with butyl amine to avoid formation of the byproduct arising from the reaction between free amines and the coupling reagent TSTU.

Uronium based coupling reagents like TSTU, TBTU, and HATU are highly electrophilic, readily reacting with most nucleophiles under basic conditions. If primary amines are added to the reaction before all of the TSTU has been depleted by the carboxylate, the N-terminus amine of the amino acid will quickly react with TSTU to form a guanidine group\(^7\) upon the N-terminus. Therefore, it is important to give carboxylic acids ample time to deplete the coupling reagent in the reaction mix, to avoid this byproduct. This guanidinylation byproduct also prevents the use of excess equivalents of uronium-based coupling reagents, as unreacted coupling reagent might react with your amine. An example of the observed byproduct can be seen in Figure 40.

![Figure 40: The guanidinylation byproduct formed from the reaction of uronium-based coupling reagents (TSTU / TBTU) and H\(_2\)N-D-Lys(mPPa)-OH 6 under basic conditions.](image)

The resulting conjugate between the water-soluble module 25 and the lysine porphyrin conjugate 6 exhibited exceptional water solubility, with the conjugate much more soluble in water
(>5mg/ml) compared to the water insoluble H$_2$N-D-Lys(mPPa)-OH 6. This increased water solubility prevents cumbersome aggregation in aqueous solutions, which prevented the use of the previous design of porphyrin TMIA by MS graduate Sara Shaut in biological testing.

Future students will be able to apply the methods described above towards the creation of the analogous water-soluble metalated porphyrin-lysine conjugates. This is likely of value for future studies as the metalated porphyrins have been shown to be more effective PS dye for PDT$^{66}$.

**4.6 Synthesis of Dual Dye Targeted TMIA probes**

Of the dual dye lysine conjugates that were synthesized and described earlier in this thesis, three were selected to serve as the probes for conjugation to 18-4 for preliminary biological testing. The dual dye conjugates chosen for biological testing are:

1. IRDye78-D-Lys(mPPa)-OH 9
2. IRDye78-L-Lys(mPPa)-OH 10
3. IRDye78-D-Lys(Pd-mPPa)-OH 17

These were chosen as they allowed for the comparison of effectiveness of one enantiomer of the lysine over the other, and to discern the effect of metalation of the porphyrin on binding.

Unfortunately, our preliminary attempts to couple IRDye78-D-Lys(mPPa)-OH 9 to the peptide 18-4 utilizing the OSu ester yielded no desired product. The generation of the OSu ester intermediate proceeded readily, and conversion appeared quantitative by analysis of the butyl amide derivative through HPLC-MS. However, the butyl amide derivative mass was present throughout HPLC-MS analysis of the crude reaction mixture indicating presence of active OSu within the reaction, but no coupled product was observed in the crude reaction mixture. Over time, both the peptide and dye products degraded before any product could be observed.
**Figure 41:** The unsuccessful coupling between IRDye78-D-Lys(mPPa)-OH \textbf{10} and the N-terminus of 18-4 utilizing the OSu ester.

This outcome is explained due to the low reactivity of the OSu ester. Compared to other electrophilic carbonyls such as acid chlorides or hydroxybenzotriazole (OBt) esters, OSu esters are less reactive, as they are somewhat resistant to hydrolysis in neutral water, stable under open air, and in pH 8.5 PBS Buffer\textsuperscript{74}. This stability allows for easy isolation and storage of the reactive
intermediate, but the OSu ester lacks the reactivity necessary to couple the N-terminus of 18-4 with a very bulky dye-lysine conjugate.

Therefore, it was time to abandon the traditional strategy of utilizing the OSu ester, which is informally known as the “gold standard” coupling reagent for coupling dye payloads, and instead utilize a one-pot activation and subsequent coupling utilizing a more reactive active ester. As per recommendation of MS student Andrew O’Brien, the hydroxybenzotriazole ester (OBt) was investigated next as this form of active ester is a much more reactive to N-hydroxysuccinimide and was therefore hypothesized to allow for the transformation to occur.

However, due to the increased reactivity of the OBt ester, extra care had to be taken to ensure both the starting dual dye lysine conjugate as well as the peptide 18-4 were anhydrous, as the OBt ester readily hydrolyzes upon contact with water. To obtain successful reactions with OBt esters, all dye and peptide starting materials had to be fastidiously vacuum dried over P₂O₅ to remove trace H₂O. Additionally, the use of anhydrous solvents (distilled DMF & anhydrous DMSO) and the reactions being conducted under inert atmosphere were found to be necessary to prevent hydrolysis of the OBt ester.

For the formation of the OBt ester, uronium-based coupling reagent 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) was employed similarly to how TSTU was employed for the formation of OSu esters. The generation of the OBt ester can be monitored through analysis of the butyl amide derivative followed by HPLC-MS analysis, similar to the process for monitoring the formation of OSu esters.
By utilizing the OBt ester, the resulting single and dual dye lysine conjugates were successfully coupled upon the N-terminus of 18-4 as seen in Figure 42. These products were isolated by precipitation in ether-hexane, followed by centrifugation and purified by reverse-phase C18 preparative HPLC chromatography. Pure fractions were then isolated by freeze drying.

For the pure final TMIA products, an additional step was added where the product was dissolved in water, and passed through Na ion exchange resin then re-freeze dried to yield the final dual dye TMIA as the sodium salt was desired to ensure optimal solubility and stability for biological studies.

Thus, the first reported example of a dual modal molecularly targeted probe (MTP) for the NIR imaging and treatment of breast cancer by PDT was created, and a sample was sent to the lab of Dr. Timothy Baran at the University of Rochester for testing in cells and in mice (murine model) to test the effectiveness of both imaging and therapy strategies that will ultimately be useful in lumpectomies and other treatments for breast cancer.

It is important to also note that this dual modal agent could be placed upon any targeting peptide for imaging and therapy of any cancer. One of the advantages of the modular system is
that the imaging/therapy payload is added in the final synthetic steps, making it amenable to almost any type of cancer, provided there is a targeting scheme for that cancer.

**Figure 43:** The reactions between dual dye lysine conjugates and the decapeptide 18-4.
4.7 Synthesis of Targeted Single Modal MTPS for PDT of BrCa

The same coupling strategy for the dual dye products was also utilized in the synthesis of the single-dye MTPS (molecular targeted photosensitizer) as seen in Figure 4.4. This reaction yielded a small, but adequate amount of product for preliminary biological studies.

Figure 4.4: The synthesis of a water-soluble porphyrin 18-4 MTPS.

MTPS 27 was synthesized through the 1 pot activation of the WS-D-Lys(mPPa)-OH conjugate 26. This was performed in two steps like all other 1 pot couplings, with the formation of the OBt ester monitored through assaying the butyl amide derivative on HPLC-MS. Upon activation, peptide 18-4 was then added to form product 27 in good yield. After purification through preparative HPLC, 27 was passed through Na ion exchange resin to yield the final TMIA as the sodium salt for biological testing. 27 serves as the first example in literature of a water-soluble targeted chlorin PS for BrCa. Future students should utilize this developed strategy towards the synthesis of a water-soluble metalated PS-peptide conjugate to increase the PDT efficacy of the overall probe.

4.8 Confocal Microscopy of BrCa Cells Targeted with IRDye78-D-Lys-(mPPa)-18-4

During the final weeks of this project, our collaborators in biology at the University of Rochester (Dr. Timothy Baran, assisted by RIT undergraduate JT Lapham) were able to provide
preliminary results from their work staining cultured EMT6 TN breast cancer cells with the TMIA probes described within this thesis. For preliminary testing, the probe IRDye78-D-Lys-(mPPa)-18-4 11 was utilized to stain the TN BrCa cells, and after 1 hour of staining, the fluorescence microscopy results are shown below in Figures 45 and 46 were obtained.

Figure 45: The fluorescent microscopy image obtained after 1 hour from staining MDA-MB-231 TN BrCa cells with IRDye78-D-Lys(mPPa)-18-4 11 and illuminating the cells at 400 nm.
Figure 46: The fluorescent microscopy image obtained after 1 hour from staining EMT6 TN BrCa cells with IRDye78-D-Lys(mPPa)-18-4 and illuminating the cells at 700 nm.

Figure 45 shows the TN BrCa cells excited at 400 nm, corresponding to the absorbance of PS dye mPPa. The resulting fluorescence confirms the successful uptake of the mPPa PS dye into TN BrCa cells but is weak due to the overall poor absorptivity and fluorescence of the mPPa dye.

Figure 46 shows the same TN BrCa cells excited at 700 nm, corresponding to the absorbance of IRDye78. The resulting bright fluorescence confirms the successful uptake of the IRDye OMI dye into TN BrCa cells.

Furthermore, the untargeted IRDye78 was used to stain EMT6 TN BrCa cells to test for nonspecific binding of the dye molecule. IRDye78 was observed to undergo minimal nonspecific binding as typical of porphyrin conjugates, as the uptake of the dyes is significantly lower
compared to the peptide conjugate, as seen in Figure 47 showcasing the IRDye78 fluorescence after excitation at 700nm.

![Fluorescent Microscopy Image](image)

*Figure 47: The fluorescent microscopy image obtained after 1 hour from staining EMT6 TN BrCa cells with IRDye78- and illumination of the cells at 700nm.*

The increased uptake of the peptide targeted probe versus the untargeted IRDye78 highlights the effectiveness of receptor targeted dyes versus untargeted dye molecules. The success of this preliminary work will be followed up with fluorescence microscopy of stained of BrCa cells utilizing the other TMIAs described, as well as testing in murine models with our collaborator Dr. Timothy Baran at the University of Rochester.
5. Conclusion

A modular method has been developed for the synthesis of targeted dual and single modal agents for use in the treatment of breast cancer (BrCa). The dual modal agents combine dyes for imaging and therapy by the use of lysine conjugates called modules or puzzle pieces which may be coupled to targeting agents via standard peptide coupling procedures. Using this method, lysine-based modules containing a fluorescent NIR dye along with a chlorin based photosensitizer (PS dye) were coupled to the BrCa peptide probe 18-4. In addition, metallated chlorin analogs of the dual modal agents containing palladium were also developed and conjugated to peptide 18-4.

As a stretch goal, a second invention has been the synthesis of a water-soluble lysine-based module containing the PS dye mPPa and a water solubilizing tri-sulfonated moiety. When coupled to the same BrCa targeting peptide, the resulting molecularly targeted photosensitizer (MPTS) imparted water solubility to the otherwise lipophilic peptide, which when previously combined with mPPa was shown to be completely insoluble in water. This results in the first reported water soluble, chlorin-based targeted probe for the PDT of BrCa. In the larger scope, this water-soluble module could be employed for conjugation upon a wide variety of targeting agents to engineer optimal water solubility upon lipophilic scaffolds.

The modular approach resulted in novel dual dye containing probes for the dual OMI and PDT of BrCa cells. These probes represent the first receptor-based targeting strategy for application of PDT alongside OMI for the treatment of BrCa.

The dual modal probes synthesized here are currently undergoing biological testing at the University of Rochester in the lab of Dr. Timothy Baran assisted by undergraduate JT Lapham. The successful staining of TN BrCa cells with TMIA probe 11 has paved the way for future testing.
of more dual and single functional probes. These probes will also be tested in murine (mouse) models of lumpectomy operations as described in an NIH grant.

Despite a significant number of synthetic transformations being low yielding, the synthetic methods developed here have yielded sufficient quantities of probes to be utilized in biological studies. Moreover, the synthetic methods pave the way for further process development including the optimization of reaction conditions and scaling quantities.

In the broader scope, the modular method presented here will be useful to synthesize water-soluble, injectable single and dual modal probes for use in the light directed surgery of other types of cancer. The specific aim of this proposal was to improve the outcome of lumpectomy operations for patients with breast cancer, however, the imaging modules described within can be combined with a targeting probe for any cancer type as long as the probe contains a free primary amine for conjugation, to create a multifunctional imaging and therapeutic agent for the cancer type.
6. Experimental Procedures

6.1 Materials and Methods

Chemicals were purchased from Stern Chemicals, Fisher Scientific, Alfa Aesar, Beantown Chemical, Macron, TCI, VWR, Ambeed, and Creosalus, and were used as received unless otherwise stated. All solvents were HPLC or American Chemical Society grade. Anhydrous DMF was freshly distilled prior to use. Photosensitizer starting materials were purchased from Frontier Scientific and BOC Sciences. The decapeptide “18-4” utilized was purchased from LifeTein (Somerset, NJ) and dried over P₂O₅ in vacuo before use. The HPLC-MS utilized a Waters 2695 Alliance HPLC with an Agilent XDB C18 column (3mm*100mm, 5µm particle size), a Waters 2998 Diode Array Detector, and a Waters 3100 SQ Mass Spectrometer. Mass spectra from these instruments were recorded at unit resolution with positive and negative modes at 35 V cone voltages. All aqueous mobile phases for HPLC-MS are 0.01M ammonium acetate.

Preparative HPLC (prep-HPLC) was carried out either with a Waters 600E system controller using a Waters 600 multi-solvent delivery system using a 30 mL/min flow rate equipped with a ThermoHypersil-Keystone HYPERSIL C18 column (250mm x 21.2mm, 5µm particle size) or a Biotage Selekt High performance automated flash system using a 50 mL/min flow rate equipped with a Biotage Sfär C18 Duo 60g column if indicated. The Biotage fraction collector UV-vis threshold was set to 10mA at the indicated wavelength and baselined with the starting eluent mixture before use.

The UV-Vis spectrophotometer was a Shimadzu UV-2600. The spectrofluorometer utilized was a Horiba Fluoromax-4 fluorescence spectrophotometer.

NMR Spectra were obtained on a Bruker Ultrashield 500 MHz NMR.
It is noteworthy that procedures on a small scale (<10mg) may incur substantial losses in yield from aliquots taken for HPLC-MS and other analyses.

High resolution mass spectra (HRMS) were obtained on a Waters Synapt G2Si (School of Chemical Sciences, University of Illinois at Urbana-Champaign) using the following parameters: Flow injection at flow rate of 0.1 ml/min, H2O/ACN/0.1% Formic Acid, positive and negative mode ESI, Cone voltage =25 V, capillary voltage = 3.0, ion source temperature = 100°C, desolvation temperature =180 °C, nebulizing gas (N2) flow = 200 L/h, cone gas (N2) flow = 5L/h.
Mesopyropheophorbide a (mPPa) Commercially available Compound 1 (50mg, 98.0 µmol, BOC Sciences) was dissolved in acetone (30mL) and added to an oven dried Parr 4590 Pressure reactor chamber, followed by the addition of 20% Pd on carbon black (5.4 mg, Strem Chemicals). The reaction chamber was sealed, and the chamber was pressurized with 20PSI with N₂ and vented thrice, followed by the reaction chamber being sealed under 35 PSI of H₂ gas. The reaction was left to stir @ 600rpm until completion. The reaction was monitored every hour by HPLC-MS (Method: Methanol 80-100%, 8min) until complete conversion of starting material for a total of 2.5 hours. The solution was then filtered through Celite Hyflo Super-cel and concentrated by rotary evaporation. The resulting black solid was purified with preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: 80-100% MeOH:0.01M TFA 20min), collecting fractions via 404nm UV absorbance. Fractions with a purple color were assayed by HPLC-MS (Method: Methanol 80-100%, 8min). Pure fractions were combined, concentrated by rotary evaporation, and dried in vacuo to yield a dark blue solid. Yield: 47.8 mg, (91%) LC-MS (LR, ESI) = Calcd. For C₃₃H₃₆N₄O₃: 536.28 m/z, found: 537.32 [M+H]+, 535.49 [M-H]-. LC-MS (HR, ESI) = Calcd. For
Mesopyropheophorbide a NHS Ester (mPPa-OSu) Compound 2 (43mg, 80.1 µmol) was dissolved in 25mL anhydrous DCM in an oven dried flask under Ar atmosphere. TSTU (26.5 mg, 88.1 µmol, Creosalus) was then added followed by DIPEA (209 µl, 72.1 µmol, Thermo Fisher). The reaction was stirred at room temperature and monitored by HPLC-MS (Method: MeOH 90-100% 8min) every 0.25 hrs for a total of 0.5hrs by treating an aliquot with 0.1% butylamine in ACN. Upon complete conversion of starting material as assayed by the butyl amide derivative, 2-3mL Glacial acetic acid was added to the flask and the reaction was concentrated by rotary evaporation until just the AcOH remained. The resulting black sludge product was diluted in 5ml 9:1 Methanol: 0.01M TFA and sonicated for 10 minutes to ensure optimal solubility, followed by purification with preparative HPLC utilizing a Biotage Sfär C18 Duo 60g column (Method:
MeOH:0.01M TFA 95-100% 20min). Fractions were collected via 404nm UV-vis absorbance. Fractions were analyzed with HPLC-MS (Method: MeOH 90-100%, 8min) for purity, by treating an aliquot with 0.1% butylamine in ACN. Pure fractions were combined, concentrated by rotary evaporation, and dried in vacuo to yield a black solid with a blue sheen. Yield 45.7 mg, (90%).

LC-MS (LR, ESI) = Calcd. For C_{37}H_{39}N_{5}O_{5}: 633.30 (m/z), found: 634.28 [M+H]^+, 632.32 [M-H]-.

UV-Vis (DAD): \( \lambda_{\text{max}}/\text{nm} = 650, 599, 534, 504, 405, 315 \)

**Fmoc-D-Lysine (Mesopyropheophorbide a)-OH** Compound 3 (32 mg, 50.5 \( \mu \text{mol} \)) was dissolved in 30ml anhydrous DCM in an oven dried flask under Ar atmosphere, followed by the addition of Fmoc-D-Lys HCl (40.9mg, 0.101 mmol, AmBeed) and DIPEA (132 \( \mu \text{l}, 0.757 \text{ mmol, Thermo Fisher} \)). The reaction was stirred and monitored by HPLC-MS (Method: MeOH 90-100%, 8min) by treating an aliquot with 0.1% butylamine in ACN every hour for a total of 5 hours. Upon complete conversion of starting material as assayed by depletion of the butyl amide derivative, the reaction was concentrated by rotary evaporation, and purified by preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: MeOH:0.01M AmAc 80-100% 20min), Fractions were collected via 404nm UV-vis absorbance. Fractions were assayed for purity with HPLC-MS (Method: MeOH 90-100%, 8min), concentrated by rotary evaporation, and dried in vacuo to yield
a black solid. Yield 35.8mg, (80%). LC-MS (LR, ESI)= Calcd. For C_{54}H_{58}N_{6}O_{6}: 886.44 (m/z), found 887.28 [M+H]+, 885.51 [M-H]. LC-MS (HR, ESI)= Calcd. For C_{54}H_{58}N_{6}O_{6}: 886.44178, found: 887.4453 [M+H]+. UV-Vis (DAD): $\lambda_{\text{max}}/\text{nm} = 650, 599, 534, 504, 405, 315, 263$. NMR (500 MHZ, CDCl3): 9.40 (s, 10-meso H), 9.19 (s, 1-meso H), 8.46 (s, 20-meso H), 7.61 (d, 30 Aryl 2H), 7.40 (t, 31 Aryl 2H), 7.30 (d, 32 Aryl 2H), 7.15 (t, Aryl 2H), 5.85 (t, 21 Fmoc NH), 5.75 (t, 27 Lysine sidechain NH), 5.24, 5.09 (d of d, exocyclic CH2), 4.48 (q, 18-H), 4.30 (m, 17-H), 4.12 (t, Lysine α CH), 3.85-3.81 (q, 3-CH2), 3.75 (q, Fmoc CH2), 3.40 (d, Fmoc CH), 3.71-3.68 (q, 8-CH2), 3.65 (s, 12-CH3), 3.29 (q, Lysine $\zeta$ CH2), (s, 7-CH3), 3.25 (s, 2-CH3), 2.75-2.70 (m, 17a CH2), 2.69-2.60 (m, 17b CH2), 2.39-2.36 (m, 17c CH2), 2.34-2.27 (m, 17d CH2), 1.82 (d, 18-CH3), 1.75, 1.70 (each t, 3-CH3 & 8-CH3), 1.31 (m, Lysine β CH2), 1.05 (s, Lysine ε,δ CH2) -1.54 (br s, NH).

Fmoc-L-Lysine(Mesopyropheophorbide a)-OH Compound 3 mPPa-OSu (29.0 mg, 64.8 µmol) was dissolved in 30ml anhydrous DCM in an oven dried flask under Ar atmosphere, followed by the addition of Fmoc-L-Lys-OH (64.5mg, 0.175 mmol) and DIPEA (141 µl, 0.810 mmol, Thermo Fisher). The reaction was monitored by HPLC-MS (Method: MeOH 90-100%, 8min) by treating an aliquot with 0.1% butylamine in ACN every hour for a total of 5 hours. Upon complete
conversion of starting material as assayed by depletion of the butyl amide derivative, the reaction was concentrated by rotary evaporation, and purified by preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: MeOH:0.01M AmAc 80-100% 20min). Fractions were collected via 404nm UV-vis absorbance. Fractions were assayed for purity with HPLC-MS (Method: MeOH 90-100%, 8min), concentrated by rotary evaporation, and dried in vacuo to yield a black solid. Yield 58.2mg, (75%). LC-MS (LR, ESI)= Calcd. For C_{54}H_{58}N_{6}O_{6}: 886.44 (m/z), found 887.35 [M+H]^+ UV-Vis (DAD): λ_{max}/nm = 651, 599, 534, 502, 405.

\[H-D-Lys(mPPa)-OH] \]

Compound 4 Fmoc-D-Lys(mPPa)-OH (60.0mg, 67.6 µmol) was dissolved in 4ml anhydrous DMF, followed by the addition of DEA (78.6 µl, 1.35 mmol, Thermo Fisher). The reaction was stirred for 3 hr and monitored via HPLC-MS (Method: MeOH 85-100%, 8min) every 1hr. Upon consumption of starting material, the reaction was concentrated under high vacuum to a sticky black film. The crude product was dissolved in MeOH with liberal sonication purified by preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: MeOH:0.01M TFA 70-100% 20min). Fractions were collected via 404nm UV-vis absorbance and analyzed for purity by HPLC-MS (Method: MeOH 85-100%, 8min). Pure fractions were combined, rotary
evaporated, and dried in vacuo to yield a dark green-black solid. Yield: 31.9mg, (71%). LC-MS (LR, ESI) = Calcd. For C_{39}H_{48}N_{6}O_{4}: 664.37 (m/z), found: 663.23 [M-H]-. LC-MS (HR, ESI) = Calcd. For C_{39}H_{48}N_{6}O_{4}: 665.38164, found: 665.3802 [M+H]^+. UV-Vis (DAD): \lambda_{\text{max}}/nm = 650, 599, 534, 504, 405, 315.

**H-L-Lys(mPPa)-OH** Compound 5 Fmoc-L-Lys(mPPa)-OH (33.0, 37.3 µmol) was dissolved in 4ml anhydrous DMF, followed by the addition of DEA (86.7 µl, 1.492 mmol, Thermo Fisher). The reaction was stirred for 3 hrs and monitored via HPLC-MS (Method: MeOH 85-100%, 8min) every 1hr. Upon consumption of starting material, the reaction was concentrated under high vacuum to a sticky black film. The crude product was dissolved in MeOH with liberal sonication and was purified by preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: MeOH:0.01M TFA 70-100% 20min). Fractions were collected via 404nm UV-vis absorbance and analyzed for purity by HPLC-MS (Method: MeOH 85-100%, 8min). Pure fractions were combined, rotary evaporated, and dried in vacuo to yield a dark green-black solid. Yield: 18.3mg,
IRDye78-D-Lys(mPPa)-OH Compound 6 H-D-Lys(mPPa)-OH (11.9mg, 17.9 µmol) was dissolved in 2ml anhydrous DMF in an oven dried reaction flask and placed under Ar atmosphere. Separately, Compound 8 IRDye78-OSu (20.0mg, 17.9 µmol) was dissolved in 4ml anhydrous DMF. The two solutions were combined under Ar, followed by the quick addition of DIPEA (46.9 µl, 0.2692 mmol, Thermo Fisher). The reaction was monitored by HPLC-MS (Method: ACN 10-100%, 8min) every 0.5hr. Upon completion of the reaction, crude product was precipitated by addition of Et₂O (30ml), vortexed thoroughly, and centrifuged. The supernatant was decanted, and the black pellet was redissolved in H₂O for purification by preparative HPLC (method MeOH:0.01M TFA 30-100%, 60min). Colored fractions were assayed with HPLC-MS(Method: ACN 10-100%, 8min), and pure fractions were combined, rotary evaporated, and lyophilized in H₂O with 50 µl TEA to yield a fluffy bright green salt. Yield: 13.4 mg, (45%). LC-MS (LR, ESI)
= Calc. for C_{86}H_{102}N_{8}O_{18}S_{4}: 1662.62 (m/z), found 1664.26 [M+H]^+, 832.97 [(M+2H/2)^2^+, 1662.63 [M-H]^-. LC-MS (HR, ESI) = Calcd. For C_{86}H_{102}N_{8}O_{18}S_{4}: 1662.61949, found: 1663.6249 [M+H]^+.

UV-Vis (DAD): λ_{max}/nm = 766, 650, 538, 504, 407, 314, 263

IRDye78-L-Lys(mPPa)-OH Compound 7 H-L-Lys(mPPa)-OH (5.7 mg, 8.6 µmol) was dissolved in 1 ml anhydrous DMF in an oven dried reaction flask and placed under Ar atmosphere. Separately, Compound 8 IRDye78-OSu (20.0 mg, 17.9 µmol) was dissolved in 4 ml anhydrous DMF. The two solutions were combined under Ar, followed by the quick addition of DIPEA (46.9 µl, .2692 mmol, Thermo Fisher). The reaction was monitored by HPLC-MS (Method: ACN 10-100%, 8 min) every 0.5 hr. Upon completion of the reaction, crude product was precipitated by addition of Et₂O (30 ml), vortexed thoroughly, and centrifuged. The supernatant was decanted, and the black pellet was redissolved in H₂O for purification by preparative HPLC (method MeOH:0.01M TFA 30-100%, 60 min). Colored fractions were assayed with HPLC-MS (Method: ACN 10-100%, 8 min), and pure fractions were combined, rotary evaporated, and lyophilized in H₂O with 50 µl TEA to yield a fluffy bright green salt. Yield: 13.4 mg, (45%). LC-MS (LR, ESI) = Calc. for C_{86}H_{102}N_{8}O_{18}S_{4}: 1662.62 (m/z), found [M+H]^+, [(M+2H/2)^2^+, [M-H]^-. UV-Vis (DAD): λ_{max}/nm = 766, 650, 538, 504, 407, 314, 263
IRDye78-D-Lys(mPPa)-18-4 The TEA salt Compound 9 (8.0mg, 3.87 µmol) was dissolved in 1 ml anhydrous DMF in an oven dried, Ar purged, reaction flask followed by the addition of TBTU (1.30 mg, 4.06 µmol, Bachem), and DIPEA (23.5 µl, 0.135 mmol, Thermo Fisher). The ester formation of compound 9 was monitored by HPLC-MS loop (direct) injection (Method: 20-100% MeOH, 2min) after treating an aliquot with 0.1% aqueous butylamine. Upon complete conversion of the starting material to the OBt ester as assayed by the butyl amide derivative, decapeptide 18-4 (5.5mg, 4.25 µmol) was added to the reaction followed by another addition of DIPEA (6.7 µl, 38.6 µmol). The reaction was monitored every hour by HPLC-MS (Method: 30-100% MeOH, 8min) by treating an aliquot with 0.1% aqueous butylamine. Upon completion of the reaction as assayed by depletion of the butyl amide derivative, the crude product was precipitated with Et₂O (15ml), vortexed thoroughly, and centrifuged. Following decanting the supernatant, the pellet was purified by preparative HPLC (Method: MeOH:0.01 AmAc 50-100%, 60min). Colored fractions were analyzed by HPLC-MS (Method: 30-100% MeOH, 8min) and pure fractions were combined, concentrated by rotary evaporation, and lyophilized into a fluffy green solid. The product was dissolved in 5ml H₂O and passed through Amberlite® IR120 Na ion-exchange resin and lyophilized to yield a fluffy, bright green salt. Yield: 2.4mg (21%). LC-MS (LR, ESI) = Calcd. for
C\textsubscript{149}H\textsubscript{189}N\textsubscript{23}O\textsubscript{32}S\textsubscript{4}: 2941.28 (m/z), found 981.46 [(M+3)/3]+, 1470.97 [(M+2)/2]+, 979.37 [(M-3)/3], 1469.27 [(M-2)/2]. LC-MS (HR, ESI)= Calcd. For C\textsubscript{149}H\textsubscript{189}N\textsubscript{23}O\textsubscript{32}S\textsubscript{4}:2940.2719, found: 1471.1431 [(M+2H)/2]+. UV-Vis (DAD): $\lambda_{\text{max}}$/nm = 766, 650, 538, 504, 407, 314, 263

**IRDye78-L-Lys(mPPa)-18-4** The TEA salt Compound 10 (2.0mg, 0.96 µmol) was dissolved in 0.5 ml anhydrous DMF in an oven dried, Ar purged, reaction flask followed by the addition of TBTU (0.32mg, 0.96 µmol, Bachem), and DIPEA (23.5 µl, .135 mmol, Thermo Fisher). The ester formation of compound 10 was monitored by HPLC-MS loop (direct) injection (Method: 20-100% MeOH, 2min) after treating an aliquot with 0.1% aqueous butylamine. Upon complete conversion of the starting material to the OBt ester as assayed by the butyl amide derivative, decapeptide 18-4 (1.35mg, 0.96 µmol) was added to the reaction followed by another addition of DIPEA (6.7 µl, 38.6 µmol). The reaction was monitored every hour by HPLC-MS (Method: 30-100% MeOH, 8min) by treating an aliquot with 0.1% aqueous butylamine. Upon completion of the reaction as assayed by depletion of the butyl amide derivative, the crude product was precipitated with Et\textsubscript{2}O (15ml), vortexed thoroughly, and centrifuged. Following decanting the supernatant, the pellet was purified by preparative HPLC (Method: MeOH:0.01 AmAc 50-100%, 60min). Colored fractions were analyzed by HPLC-MS(Method: 30-100% MeOH, 8min) and pure fractions were combined,
concentrated by rotary evaporation, and lyophilized into a fluffy green solid. The product was dissolved in 2ml H₂O and passed through Amberlite® IR120 Na ion-exchange resin and lyophilized to yield a fluffy, bright green salt. Yield: 0.98mg (51%). LC-MS (LR, ESI) = Calcd. for C₁₄₉H₁₈₉N₂₃O₃₂S₄: 2941.28 (m/z), found: 981.46 [(M+3)/3]⁺, 1470.97 [(M+2)/2]⁺, 979.37 [(M-3)/3], 1469.27 [(M-2)/2]. UV-Vis (DAD): λ_max/nm = 766, 650, 538, 504, 407, 314, 263

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\text{Pd}^{2+}[\text{mPPa-OSu}]
\]

Compound 3 (23.0mg, 36.3 µmol) was dissolved in 20mL DCM in an oven dried reaction flask and placed under Ar atmosphere. Separately, Palladium (II) Acetate (81.0mg, 0.363 mmol, Strem Chemicals) and L-ascorbic acid 6-palmitate (120mg, 0.290 mmol, TCI) were dissolved in 10mL DCM and added to the reaction flask. The reaction was monitored by HPLC-MS (Method: 98-100% MeOH, 8min) after treating an aliquot with 0.1% Butylamine in ACN. Upon complete conversion of starting material, the reaction was concentrated by rotary evaporation and purified by preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: MeOH:0.005M AcOH 95-100% 20min. Fractions were collected via 638nm UV-vis absorbance and assayed by HPLC-MS (Method: 98-100% MeOH, 8min) for purity. Pure fractions were combined, concentrated by rotary evaporation, and dried in vacuo to yield a green solid with a black tint. Yield: 22.2mg (83%) LC-MS (LR, ESI) = Calcd. For C₃₇H₃₇N₅O₅Pd: 737.18 (m/z),
found 736.14 [M+H]+, 737.73 [M-H]-. LC-MS (HR, ESI) = Calcd. For C_{37}H_{37}N_{5}O_{5}Pd: 737.18295, found: 738.1921 [M+H]+. Compound has isotope pattern indicative of the multiple stable isotopes of Pd. UV-Vis (DAD): \( \lambda_{\text{max}}/\text{nm} \) = 635, 589, 542, 498, 414, 392

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\begin{array}{c}
\text{Pd}^{2+}[\text{mPPa}] \\
\text{Compound 13 (10.0mg, 13.5 \mu\text{mol}) was dissolved in 4.5mL ACN sonicated for optimal solubility in a reaction flask for 10 minutes. Upon complete dissolution, H}_{2}\text{O (500 \mu l) and triethylamine (18.4 \mu l, 135 \mu mol, Thermo Fisher) were added to the reaction flask. The reaction flask was capped and sonicated at room temperature for 1 hour. After 1 hour the reaction was concentrated by rotary evaporation to yield a crude green powder. The resulting product was redissolved in MeOH (5mL) and purified with preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: MeOH:0.005M TFA 95-100% 20min.) Fractions were collected via UV absorbance at 638nm. Pure fractions were assayed by HPLC-MS (Method: 90-100% MeOH, 8min). Pure fractions were combined, concentrated by rotary evaporation, and dried in vacuo into a dark green film. To obtain a fluffy powder, the compound was redissolved in minimal ACN (5mL) and diluted with H_{2}O (10mL) and lyophilized to yield a fluffy green solid. Yield: 6.94 mg (80%). LC-MS (LR, ESI) = Calcd. For C_{33}H_{34}N_{4}O_{3}Pd: 640.17 (m/z), found: 641.25 [M+H]+, UV-Vis (DAD, nm): 631, 586, 414, 385.}
\end{array}
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**Pd$^{2+}$[Fmoc-D-Lys(mPPa)-OH]** Compound 13 (15.0mg, 20.3 µmol) was dissolved in DCM (20mL) in an oven dried reaction flask under Ar atmosphere. Fmoc-D-Lys-OH HCl (16.4mg, 40.6 µmol, AmBeed) was added followed by DIPEA (70 µl, 0.406 mmol, Thermo Fisher). The reaction was monitored by HPLC-MS (Method: 95-100%, 8min) after treating an aliquot with 0.1% butylamine in ACN every hour for a total of 6 hours. Upon complete conversion of the starting material as assayed by depletion of the butyl amide derivative, the reaction mixture was concentrated by rotary evaporation and purified by preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: MeOH:0.01M AmAc 80-100% 20min), collecting fractions via 638nm UV absorbance. Fractions were assayed with HPLC-MS (Method: 95-100% MeOH, 8min) for purity. Pure fractions were combined, concentrated by rotary evaporation, and dried in vacuo to yield a green solid. Yield: 12.8mg (64%). LC-MS (LR, ESI) = Calcd. For C$_{54}$H$_{56}$N$_{6}$O$_{6}$Pd: 990.33 (m/z), found 989.22 [M+H]$^+$, 987.36 [M-H]$^-$. LC-MS (HR, ESI) = Calcd. for C$_{54}$H$_{56}$N$_{6}$O$_{6}$Pd:990.32961, found: 991.3262 [M+H]$^+$. Compound has isotope pattern indicative of the multiple stable isotopes of Pd. UV-Vis (DAD): $\lambda_{\text{max}}$/nm= 638, 589, 542, 498, 414, 392, 263
**Pd\textsuperscript{2+}[H-D-Lys(mPPa)-OH]** The solid Compound 15 (7.0mg, 7.06 µmol) was dissolved in 1ml anhydrous DMF in a reaction flask, followed by addition of DEA (10.0 µl, 0.176 mmol, Thermo Fisher). The reaction was stirred and assayed every hour by HPLC-MS (Method: 98-100% MeOH) until complete conversion of starting material. Upon complete conversion of starting material by HPLC-MS (Method: 98-100% MeOH, 8min), the reaction mixture was concentrated under high vacuum to yield a black sludge with a green tint. The resulting product was dissolved in Methanol and purified by preparative HPLC (method MeOH:0.005M TFA 90-100%, 60min). Colored fractions were assayed for purity by HPLC-MS and pure fractions were combined, concentrated by rotary evaporation, and dried *in vacuo* to yield a green solid. Yield: 4.3 mg (79%). LC-MS (LR, ESI) = Calcd. For C\textsubscript{39}H\textsubscript{46}N\textsubscript{6}O\textsubscript{4}Pd: 768.26 (m/z), found 767.14 [M+H]\textsuperscript{+}. LC-MS (HR, ESI) = Calcd.
For C$_{39}$H$_{46}$N$_6$O$_4$Pd: 768.26153, found: 769.2596 [M+H]$^+$. Compound has isotope pattern indicative of the multiple stable isotopes of Pd. $\lambda_{\text{max}} = 635$ nm.

**IRDye78-D-Lys(Pd-mPPa)-OH** Compound 16 (7.0 mg, 90.9 µmol) was dissolved in 1 ml anhydrous DMF in an oven dried reaction flask under Ar atmosphere. Separately, IRDye78-OSu (11.1 mg, 100 µmol) was dissolved in 1 ml anhydrous DMF in an oven dried flask under Ar atmosphere. The two solutions were combined under Ar followed by addition of DIPEA (31.7 µl, 0.181 mmol, Thermo Fisher). The reaction was stirred and assayed by HPLC-MS (Method: 30-100% MeOH, 8 min) after treating an aliquot with 0.1% aqueous butylamine. Upon complete conversion of IRDye78-OSu as assayed by depletion of the butyl amide derivative, the crude product was precipitated by addition of Et$_2$O (15 ml), vortexed thoroughly, and centrifuged. The supernatant was decanted, and the black pellet was redissolved in H$_2$O for purification by preparative HPLC (method MeOH: 0.01 M AmAc 30-100%, 60 min). Colored fractions were assayed with HPLC-MS (Method: 30-100% MeOH, 8 min), and pure fractions were combined, rotary evaporated, and lyophilized in H$_2$O with 50 µl TEA to yield 11 as a fluffy bright green salt.

IRDye78-D-Lys(Pd-mPPa)-18-4 The TEA salt Compound 17 (0.5mg, 0.028 µmol) was dissolved in 0.5 ml anhydrous DMF in an oven dried, Ar purged, reaction flask followed by the addition of TBTU (0.95mg, 0.029 µmol, Bachem), and DIPEA (1.975 µl, 1.13µmol, Thermo Fisher). The ester formation of compound 17 was monitored by HPLC-MS loop (direct) injection (Method: 20-100% MeOH, 2min) after treating an aliquot with 0.1% aqueous butylamine. Upon complete conversion of the starting material to the OBt ester as assayed by the butyl amide derivative, decapeptide 18-4 (0.4mg, 0.031 µmol) was added to the reaction followed by another addition of DIPEA (0.5 µl, .028µmol). The reaction was monitored every hour by HPLC-MS (Method: 30-100% MeOH, 8min) by treating an aliquot with 0.1% aqueous butylamine. Upon completion of the reaction as assayed by depletion of the butyl amide derivative, the crude product was precipitated with Et₂O (10ml), vortexed thoroughly, and centrifuged. Following decanting the supernatant, the pellet was purified by preparative HPLC (Method: MeOH:0.01 AmAc 40-100%, 60min). Colored fractions were analyzed by HPLC-MS(Method: 40-100% MeOH, 8min) and pure
fractions were combined, concentrated by rotary evaporation, and lyophilized into a fluffy green solid. The product was dissolved in 2ml H$_2$O and passed through Amberlite® IR120 Na ion-exchange resin and lyophilized to yield a fluffy, bright green salt. Yield: 0.44mg (51%). LC-MS (LR, ESI) = Calcd. For C$_{149}$H$_{187}$N$_{23}$O$_{32}$PdS$_{4}$: 3045.17 (m/z), found: 1522.96 [(M+2H/2)$^+$]. LC-MS (HR, ESI) = Calcd. For C$_{149}$H$_{187}$N$_{23}$O$_{32}$PdS$_{4}$: 3044.16302, found: 1521.0684 [(M-2H/2)$^-$]. UV-Vis/nm: 775, 640, 395, 225.

IRDye78-OSu IRDye78 (20.0mg, 19.6 µmol) was used to synthesize IRDye78-OSu as previously described in literature$^{54}$. Yield: (20mg, >95%). LC-MS (LR, ESI)= Calcd. For C$_{51}$H$_{65}$N$_{3}$O$_{14}$S$_{4}$ (butyl amide derivative): 1071.33 (m/z), found: 1072.12 [M+H]$^+$, UV-Vis (DAD, nm): 768, 383.

Fmoc-D-Lys(IRdye78)-OH Compound 8 (20.0mg, 19.6 µmol) was dissolved in 2mL anhydrous DMF in an oven dried reaction flask and placed under Ar atmosphere. Then DIPEA (81.3 µl, 0.466 mmol, Thermo Fisher) and Fmoc-D-Lys-OH HCl (20.5 µmol, 8.32 mg, AmBeed) were added, and the reaction was left to stir and monitored by HPLC-MS (Method: 10-100% ACN, 8min) after
treating an aliquot with 0.1% aqueous butylamine. Upon complete conversion of starting IRDye78-OSu as assayed by depletion of the butyl amide derivative on HPLC-MS after 2.5 hr, the crude product was precipitated by addition of Et₂O (10ml) and centrifuged. The supernatant was decanted, and the black pellet was redissolved in H₂O for purification by preparative HPLC (method MeOH:0.01M TFA 10-100%, 60min). Colored fractions were assayed with HPLC-MS (Method: 10-100% ACN, 8min) and pure fractions were combined, rotary evaporated, and lyophilized to yield 11 as a fluffy bright green powder. Yield: 16.8mg (66%). LC-MS (LR, ESI) = Calcd. For C₆₈H₇₈N₄O₁₈S₄: 1366.42 (m/z), found: 1367.11 [M+H]⁺, UV-Vis (DAD, nm): 768, 383, 265.

Fmoc-L-Lys(IRdye78)-OH Compound 8 (15.3mg, 13.7 µmol was dissolved in 2mL anhydrous DMF in an oven dried reaction flask and placed under Ar atmosphere. Then DIPEA (59.9 µl, 0.344 mmol, Thermo Fisher) and Fmoc-L-Lys-OH (15.1 µmol, 5.58 mg) were added, and the reaction was left to stir and monitored by HPLC-MS (Method: 10-100% ACN, 8min) by treating an aliquot with 0.1% aqueous butylamine. Upon complete conversion of starting IRDye78-OSu as assayed by depletion of the butyl amide derivative on HPLC after 2.5 hr, the crude product was precipitated by addition of Et₂O (10ml) and centrifuged. The supernatant was decanted, and the black pellet was redissolved in H₂O for purification by preparative HPLC (method MeOH:0.01M TFA 10-
Colored fractions were assayed with HPLC-MS (Method: 10-100% ACN, 8min) and pure fractions were combined, rotary evaporated, and lyophilized to yield 11 as a fluffy bright green powder. Yield: 14.7mg (78%). LC-MS (LR, ESI) = Calcd. For C_{68}H_{78}N_{18}O_{18}S_{4}: 1366.42 (m/z), found: 1368.32 [M+H]^+, UV-Vis (DAD, nm): 768, 383, 265.

H-D-Lys(IRdye78)-OH Compound 19 (10.0mg 10.5 µmol) was dissolved in 1ml DMF followed by the addition of DEA (8.5 µl, 0.146 mmol, Thermo Fisher) and stirred. The reaction was assayed every 30 minutes by HPLC-MS (Method: 10-100% ACN, 8min). Upon complete conversion of starting material, the crude product was precipitated by addition of Et₂O (10ml) and centrifuged. The supernatant was decanted, and the black pellet was redissolved in 0.2M TFA for purification by preparative HPLC (method MeOH:0.01M TFA 10-100%, 60min). Colored fractions were assayed with HPLC-MS utilizing the previous method, and pure fractions were combined, rotary evaporated, and lyophilized to yield 11 as a fluffy bright green powder. Yield: 3.77mg (45%). LC-MS (LR, ESI) = Calcd. for C_{53}H_{68}N_{16}O_{16}S_{4}: 1144.35 (m/z), found: 1145.21 [M+H]^+, UV-Vis (DAD, nm): 768.
**H-L-Lys(IRdye78)-OH** Compound 20 (10.5mg 10.5 µmol) was dissolved in 1ml DMF followed by the addition of DEA (8.9 µl, 0.153 mmol, Thermo Fisher) and stirred. The reaction was assayed every 30 minutes by HPLC-MS (Method: 10-100% ACN, 8min). Upon complete conversion of starting material, the crude product was precipitated by addition of Et₂O (10ml) and centrifuged. The supernatant was decanted, and the black pellet was redissolved in 0.2M TFA for purification by preparative HPLC (method MeOH:0.01M TFA 10-100%, 60min). Colored fractions were assayed with HPLC-MS utilizing the previous method, and pure fractions were combined, rotary evaporated, and lyophilized to yield 11 as a fluffy bright green powder. Yield: 5.19mg (59%). LC-MS (LR, ESI) = Calcd. for C₅₃H₆₈N₄O₁₆S₄: 1144.35 (m/z), found: 1145.25 [M+H]+, UV-Vis (DAD, nm): 768.
mPPa-D-Lys(IRdye78)-OH Compound 3 mPPa-OSu 3.88 mg, 6.11 µmol) was dissolved in 1ml anhydrous DMF in an oven dried reaction flask and placed under Ar atmosphere. Then, Compound 21 H-D-Lys(IRdye78) (7.0mg, 6.11 µmol) and DIPEA (31.9 µl, 0.183 mmol, Thermo Fisher), were added to the reaction flask and the reaction was left to stir in the dark (reaction flask wrapped in tin foil). The reaction was monitored by HPLC-MS (Method: 80-100% MeOH, 8min) every hour by treating an aliquot with 0.1% butylamine in 1:1 ACN/H2O. Upon complete conversion of starting mPPa-OSu as assayed by depletion of the butyl amide derivative on HPLC-MS, the reaction was diluted with Et2O (15ml), vortexed thoroughly, and centrifuged. The supernatant was decanted, and the black pellet was purified by preparative HPLC (method MeOH:0.01M TFA 30-100%, 60min). Colored fractions were assayed with HPLC-MS(Method: 50-100% MeOH, 8min), and pure fractions were combined, rotary evaporated, and lyophilized in H2O with 50 µl TEA to yield 6 as a fluffy bright green salt. Yield: 3.05 mg, (30%). LC-MS (LR, ESI) = Calc. for C86H102N8O18S4: 1662.62 (m/z), found [M+H]+, [(M+2H/2)]2+, [M-H]+. UV-Vis (DAD, nm) λmax = 766
WS-Module Tertbutyl Tert-Butyl 4-aminobutrate hydrochloride (150.0mg, 0.76 mmol, AmBeed), 1,3 Propanesultone (465.7mg, 3.81 mmol, Alfa Aesar), and DIPEA (1.32ml, 7.63 mmol, Thermo Fisher) were combined in an oven dried reaction flask equipped with a reflux condenser under Ar atmosphere at room temperature. The reaction flask was placed in an oil bath preheated to 100C and monitored by HPLC-MS (Method: 05-30 MeOH, 8min) every 30 minutes by diluting an aliquot of the reaction with H2O. Upon no more observation of the di-alkylated product ([M+H]+ = 402.50) upon HPLC-MS, the reaction was estimated to be depleted of the starting material the reaction was left to cool to room temperature, diluted with 30mL H2O, and extracted with DCM (3x75mL). Significant resting time was needed to allow for separation of the resulting emulsion. The resulting aqueous layer was concentrated by rotary evaporation and dried in vacuo to yield a crude yellow-red oil. This oil was purified by preparative HPLC with a Biotage Sfär C18 Duo 60g column (Method: MeOH:0.01M TFA 05-30 20min). Fractions which exhibited a large deal of foaming upon agitation were assayed by HPLC-MS (Method: 05-30 MeOH, 8min) for purity. When working in large scale (>100mg), the Biotage UV-vis absorbance of a ketone (<200nm) can be observed as well to identify fractions containing product. Pure fractions were combined, concentrated by rotary evaporation, and freeze dried to yield a colorless oil. Yield: 176.7mg (44%). LC-MS (LR, ESI) Cald. For C_{17}H_{35}NO_{11}S_{3}: 525.14 (m/z), found: 526.24 [M+H]+. LC-MS (HR, ESI) = Calcd. For C_{17}H_{35}NO_{11}S_{3}: 525.13722, found: 526.1426 [M+H]+. NMR (500
MHZ, D2O), 3.44 (qu, 2-CH2), 3.31 (qu, 5-CH2), 2.92 (t, 1-CH2), 2.37 (6-CH2), 2.13 (qu, 3-CH2), 1.94 (t, 4-CH2), 1.38 (s, 7-CH3).

WS-Module-OH Compound 24 (59.0 mg, 0.112 mmol) was dissolved in 10 mL DCM in an oven dried reaction flask, quickly followed by the addition of TFA (4mL, 0.052 mol, Millipore-Sigma). The reaction was sonicated for 5 minutes to ensure optimal solubility, followed by stirring under open air. The reaction was monitored via HPLC-MS loop (direct) injection (Method: 20-100% MeOH, 2min). To make this sample, a small aliquot was concentrated by rotary evaporation, and the resulting oil dissolved in H2O. After complete conversion of starting material, the reaction was concentrated by rotary evaporation to yield a colorless oil. The oil was dissolved in 10 mL H2O with TEA (200 µl) and lyophilized to yield an off-white crystalline salt. Yield: 86mg (95%) LC-MS (LR, ESI) = Calcd. for C13H28NO11S3: 469.07 (m/z), found: 468.14 [M-H]-. LC-MS (HR, ESI) = C13H28NO11S3: 469.07462, found: 468.0666 [M-H]-.
**WS-D-Lys(mPPa)-OH** The TEA salt 25 (15.0 mg, 19.3 µmol) was dissolved in 2 ml anhydrous DMSO in an oven dried reaction flask under Ar atmosphere. Then, TSTU (5.8 mg, 19.3 µmol, Creosalus) and DIPEA (84 µl, 0.483 mmol, Thermo Fisher) were added to the reaction was left to stir for 1 hour and monitored via HPLC-MS loop (direct) injection (Method: 20-100% MeOH, 2 min) after treating an aliquot with 0.1% aqueous butylamine. Separately, compound 6 H₂N-D-Lys(mPPa)-OH (12.8 mg, 19.3 µmol) was dissolved in 1 mL anhydrous DMSO, sonicated for 5 minutes, and added to the reaction flask followed by the addition of more DIPEA (16.8 µl, 96.77 µmol). The reaction was left to stir for 3 hours, monitored every hour by HPLC-MS (Method: 70-100% MeOH, 8 min) by treating an aliquot with 0.1% aqueous butylamine. Upon complete conversion of generated OSu ester as assayed by depletion of the butyl amide derivative on HPLC-MS, the crude product was precipitated by addition of Et₂O (10 ml) and EtOAc (1 ml), thoroughly vortexed, and centrifuged. The supernatant was decanted, and the black pellet was redissolved in H₂O for purification by preparative HPLC with a Biotage Sfär C18 Duo 60 g column (Method: MeOH:0.01 M AmAc 50-100% 20 min), collecting fractions via 404 nm UV absorbance. Fractions were assayed with HPLC-MS (Method: 70-100% MeOH, 8 min) for purity. Pure fractions were combined, concentrated by rotary evaporation, lyophilized thrice with 0.005 M TFA to yield a fluffy grey solid. Yield: 12.9 mg (60%) LC-MS (LR, ESI) = Calcd. For C₅₂H₇₂N₇O₁₄S₃: 1114.43
(m/z), found: 1116.93[M+H]+, 1114.33 [M-H]. LC-MS (HR, ESI) = Calcd. For C₅₂H₇₂N₇O₁₄S₃: 1115.43776, found: 1116.4460 [M+H]+.

WS-D-Lys(mPPa)-18-4  Compound 26 (5.4mg, 3.80 µmol) was dissolved in 0.5 ml anhydrous DMF in an oven dried reaction flask followed by the addition of TBTU (1.34 mg, 4.18 µmol, Bachem), and DIPEA (23.2µl, .133 mmol, Thermo Fisher). The activation of compound 26 was monitored by HPLC-MS loop (direct) injection (20-100% MeOH, 2min) after treating an aliquot with 0.1% aqueous butylamine. Upon complete conversion of the starting material as assayed by the butyl amide derivative, decapeptide 18-4 (4.93mg, 3.80 µmol) was added to the reaction followed by another addition of DIPEA (6.7 µl, 38.6 µmol). The reaction was monitored every hour by HPLC-MS (Method: 70-100% MeOH, 8min). Upon completion of the reaction, the crude product was precipitated with Et₂O (5ml) and centrifuged. Following decanting the supernatant, the pellet was purified by preparative HPLC (method MeOH:0.01M AmAc 60-100%, 60min). Colored fractions were analyzed by HPLC-MS(Method: 70-100% MeOH, 8min) and pure fractions were combined, concentrated by rotary evaporation, and lyophilized into a fluffy grey powder. Yield: 3.73mg (41%). LC-MS (LR, ESI) Calcd. for C₁₁₅H₁₆₀N₂₃O₂₇S₃: 2392.10 (m/z), found: 798.64[(M+3)/3]+, 1197.19[(M+2)/2]+, 796.46 [(M-3)/3], 1195.87 [(M-2)/2]. LC-MS (HR, ESI) = Calcd. For C₁₁₅H₁₆₀N₂₃O₂₇S₃: 2391.10162, found: 1196.5538 [(M+2H)/2]+.
References


110


(46) Schmitthenner, H. F.; Beach, S.; Weidman, C.; Barrett, T. (54) MODULAR MAGINGAGENTS CONTAINING AMINO ACIDS AND PEPTDES.

(47) Schmitthenner, H. F.; Sweeney-Jones, A. M.; Williams, S. (54) TRANSMETALATION METHODS FOR THE SYNTHESIS OF PET AND SPECT MAGING AGENTS.


Appendix I. HPLC-MS, NMR, UV-Vis, and HRMS Results

Compound 1: Pyropheophorbide a (PPa)
The Diode Array Detector chromatogram of 1
The UV-Vis Spectrum of 1 with characteristic peaks at 395 and 667nm

The ESI positive Mass Spectra of 1 at 6.2 min, with the product at 535.34 m/z [M+H]^+

The ESI negative Mass Spectra of 1 at 6.2 min, with the product at 533.05 m/z [M-H]^-
Compound 2: MesoPyropheophorbide a (mPPa)

The proton NMR spectrum of compound 2 in CDCl3
The COSY spectrum of compound 2 in CDCl3
The Diode Array Detector chromatogram of 2

The UV-Vis Spectrum of 2 with characteristic peaks at 404 and 651 nm
The ESI positive Mass Spectra of 2 at 6.2 min, with the product at 537.32 m/z [M+H]^+

The ESI negative Mass Spectra of 2 at 6.2 min, with the product at 535.49 m/z [M-H]^−
The TOF ESI+ High resolution mass spectrum of 2 at 537.2861 amu
Compound 3: MesoPyropheophorbide N-Hydroxysuccinimide Ester (mPPa-OSu)

The Diode Array Detector chromatogram of 3

The UV-Vis Spectrum of 3 with characteristic peaks at 404 and 650 nm
The ESI positive Mass Spectra of 3 at 3.89 min, with the product at 634.28 m/z [M+H]^+

The ESI negative Mass Spectra of 3 at 3.89 min, with the product at 632.32 m/z [M-H]^−
Compound 4: Fmoc-\textit{D}-Lys(mPPa)-OH

The proton NMR spectrum of compound 4 in CDCl₃
The COSY NMR spectrum of compound 4 in CDCl3.
The Diode Array Detector chromatogram of 4

The UV-Vis Spectrum of 4 with characteristic peaks at 407 and 660 nm
The single wavelength chromatogram at 404nm of 4

The ESI positive Mass Spectra of 4 at 3.76 min, with the product at 887.28 m/z [M+H]+
The ESI negative Mass Spectra of 4 at 3.76 min, with the product at 885.51 m/z [M-H]^{-}
The TOF ESI+ High resolution mass spectrum of compound 4 with mass at 887.4453 amu
Compound 5: \( \text{H}_2\text{N-D-Lys(mPPa)-OH} \)

The Diode Array Detector chromatogram of compound 5

The UV-Vis Spectrum of 5 with characteristic peaks at 405 and 650 nm
The ESI positive Mass Spectra of 5 at 6.91 min, with the product at 666.31 m/z [M+H]^+.

The ESI negative Mass Spectra of 5 at 6.91 min, with the product at 663.23 m/z [M-H]^-. 
Compound 6: Fmoc-L-Lys(mPPa)-OH

The Diode Array Detector chromatogram of 6

The UV-Vis Spectrum of 6 with characteristic peaks at 405 and 651 nm
The ESI positive Mass Spectra of 6 at 3.13 min, with the product at 887.35 m/z [M+H]^+
The TOF ESI+ High resolution mass spectrum of 6 at 665.3802 amu
Compound 7: $\text{H}_2\text{N-}L\text{-Lys(mPPa)}\text{-OH}$

The Diode Array Detector chromatogram of 7

The UV-Vis Spectrum of 7 with characteristic peaks at 405 and 650 nm
The single wavelength chromatogram at 405 nm of 7

The ESI positive Mass Spectra of 7 at 5.55 min, with the product at 665.34 m/z [M+H]+
Compound 8: IRDye78-OSu (Assayed as the Butyl Amide derivative)

The Diode Array Detector chromatogram of 8

The UV-Vis Spectrum of 8 with characteristic peaks at 768 and 383 nm
The ESI positive Mass Spectra of 8 at 0.97 min, with the product at 1072.12 m/z [M+H]^+
Compound 9: IRDye78-\textit{D}-Lys(mPPa)-OH

The Diode Array Detector chromatogram of 9

The UV-Vis Spectrum of 9 with characteristic peaks at 766 and 407 nm
The ESI positive Mass Spectra of 9 at 6.37 min, with the product at 1664.26 m/z [M+H]^+

The ESI negative Mass Spectra of 9 at 6.37 min, with the product at 1662.63 m/z [M-H]^−
The high-resolution mass spectrum of 9, with the product at 1664.6249 amu
Compound 10: IRDye78-L-Lys(mPPa)-OH

The Diode Array Detector chromatogram of 10

The UV-Vis Spectrum of 10 with characteristic peaks at 768 and 409 nm
The ESI positive Mass Spectra of 10 at 3.92 min, with the product at 1664.29 m/z [M+H]⁺

**Compound 11: IRDye78-D-Lys(mPPa)-18-4**

The Diode Array Detector chromatogram of 11
The UV-Vis Spectrum of 11 with characteristic peaks at 768 and 409 nm

The ESI positive Mass Spectra of 11 at 6.06 min, with the product half mass at 1470.97 m/z

\[ [\text{M+2}/2]^+ \]
The ESI negative Mass Spectra of 11 at 6.06 min, with the product half mass at 1469.27 m/z

\[ [(M-2)/2]^- \]
The high resolution mass spectrum of 11, with product third mass at 981.1031 amu
Compound 12: IRDye78-L-Lys(mPPa)-18-4

The Diode Array Detector chromatogram of 12

The UV-Vis Spectrum of 12 with characteristic peaks at 768 and 409 nm
The ESI positive Mass Spectra of 12 at 4.18 min, with the product half mass at 1471.05

\[\frac{(M+2)}{2}^+\]

The ESI negative Mass Spectra of 12 at 4.18 min, with the product half mass at 1468.41

\[\frac{(M-2)}{2}^-\]
Compound 13: Pd-MesoPyropheophorbide a N-Hydroxysuccinimide Ester (Pd-mPPa-OSu)

The Diode Array Detector chromatogram of 13

The UV-Vis Spectrum of 13 with characteristic peaks at 635 and 392 nm
The ESI positive Mass Spectra of 13 at 5.33 min, with the product at 736.14 m/z [M+H]^+.

The isotope pattern of 13 caused by the multiple stable isotopes of Pd viewed by ESI.
The fluorescence spectrum comparing the freebase mPPa fluorescence (green) vs Pd-mPPa-Osu fluorescence (black). Metalation of porphyrin rings with Pd quenches fluorescence.
The TOF ESI+ High resolution mass spectrum of 13 with mass at 769.1604 amu.

The high-resolution isotope pattern of 13 showcasing the multiple isotopes of Pd.
Compound 14: Pd-Mesopyropheophorbide a (Pd-mPPa)

The Diode Array Detector chromatogram of 14

The UV-Vis Spectrum of 14 with characteristic peaks at 631 and 385 nm
The single wavelength chromatogram of 14 at 631nm

The ESI positive Mass Spectra of 14 at 6.13 min, with the product at 641.25 m/z [M+H]^+
Compound 15: Fmoc-$D$-Lys(Pd-mPPa)-OH

The Diode Array Detector chromatogram of 15

The UV-Vis Spectrum of 15 with characteristic peaks at 638 and 390 nm
The ESI positive Mass Spectra of 15 at 1.47 min, with the product at 989.22 m/z [M+H]^+

The ESI negative Mass Spectra of 15 at 1.47 min, with the product at 987.36 m/z [M-H]^−
The isotope pattern of 15 caused by the multiple stable isotopes of Pd viewed in ESI
The TOF ESI+ High resolution mass spectrum of 15 with the product at 990.3245 amu.

The high resolution isotope pattern of 15, indicative of the multiple isotopes of Pd.
Compound 16: \( \text{H}_2\text{N-}D\text{-Lys(Pd-mPPa)-OH} \)

The Diode Array Detector chromatogram of 16

The UV-Vis Spectrum of 16 with characteristic peaks at 638 and 390 nm
The single wavelength chromatogram of 16 at 638nm

The ESI positive Mass Spectra of 16 at 4.11 min, with the product at 767.14 m/z [M+H]^+
The isotope pattern of 16 caused by the multiple stable isotopes of Pd viewed in ESI
The TOF ESI+ high resolution mass spectrum of 16, with product at 767.2546 amu

The isotope pattern of 16 caused by the multiple stable isotopes of Pd
Compound 17: IRDye78-D-Lys(Pd-mPPa)-OH

The Diode Array Detector chromatogram of 17
The UV-Vis Spectrum of 17 with characteristic peaks at 768 and 638 nm

The ESI positive Mass Spectra of 17 at 2.29 min, with the product at 1766.40 m/z [M+H]^+
The ESI negative Mass Spectra of 17 at 2.29 min, with the product at 1764.62 m/z [M-H]−
The TOF ESI+ High resolution mass spectrum of 17 with the product at 1767.4995 amu
The high resolution isotope pattern of 17 with multiple stable isotopes indicative of Pd metalation
Compound 18: IRDye78-\textit{D}-Lys(Pd-mPPa)-18-4

The Diode Array Detector chromatogram of 18

![Chromatogram of Compound 18](image)

The UV-Vis Spectrum of 18 with characteristic peaks at 768 and 640 nm

![UV-Vis Spectrum of Compound 18](image)
The ESI positive Mass Spectra of 18 at 6.54 min, with the product half mass at 1522.96 m/z

$$[(M+2)/2]^+$$
The TOF ESI+ High resolution mass spectrum of 18 with the product half mass at 1520.5780 amu
The high resolution isotope pattern of 18 with multiple stable isotopes of Pd
Compound 19: Fmoc-D-Lys(IRDye78)-OH

The Diode Array Detector chromatogram of 19

The UV-Vis Spectrum of 19 with characteristic peaks 766 and 383 nm
The ESI positive Mass Spectra of 19 at 3.39 min, with the product at 1367.11 m/z [M+H]^+. 

**Compound 20: Fmoc-L-Lys(IRDye78)-OH** 

The Diode Array Detector chromatogram of 20
The UV-Vis Spectrum of 20 with characteristic peaks at 768 and 383 nm

The ESI positive Mass Spectra of 20 at 3.13 min, with the product at 1368.32 m/z [M+H]^+
Compound 21: \( \text{H}_2\text{N-D-Lys(IRDye78)-OH} \)

The Diode Array Detector chromatogram of 21

The UV-Vis Spectrum of 21 with characteristic peaks at 768 and 383 nm
The ESI positive Mass Spectra of 21 at 3.17 min, with the product at 1145.21 m/z [M+H]^+

**Compound 22: H<sub>2</sub>N-L-Lys(IRDye78)-OH**

The Diode Array Detector chromatogram of 22

Range: 2.47e+2
The UV-Vis Spectrum of 22 with peaks at 756 and 382 nm

The ESI positive Mass Spectra of 22 at 3.18 min, with the product at 1145.25 m/z [M+H]^+
Compound 23: mPPa-$D$-Lys(IRDye78)-OH

The Diode Array Detector chromatogram of 23

The UV-Vis Spectrum of 23 with characteristic peaks at 783 and 409 nm
The ESI positive Mass Spectra of 23 at 4.48 min, with the product at 1663.83 m/z [M+H]⁺
Compound 24: Water-Soluble Module Tert butyl

The proton NMR Spectrum of 24 in D2O
The COSY NMR spectrum of 24 in D2O
The Total ion chromatogram for ESI positive of 24
The Total ion chromatogram for ESI negative of 24

The ESI positive Mass Spectra of 24 at 3.43 min, with the product at 526.18 m/z [M+H]^+
The ESI negative Mass Spectra of 24 at 3.41 min, with the product at 524.44 m/z [M-H]⁻.
The TOF ESI+ High resolution mass spectrum of 24 with mass at 525.1456 amu
The Total ion chromatogram for ESI negative of 25

The ESI negative Mass Spectra of 25 at 0.83 min, with the product at 467.83 m/z [M-H]⁻
The TOF ESI+ High resolution mass spectrum of 25 with mass at 470.0813
Compound 26: Water-Soluble-D-Lys(mPPa)-OH

The Diode Array Detector chromatogram of 26

The UV-Vis Spectrum of 26 with characteristic peaks at 404 and 650 nm
The ESI positive Mass Spectra of 26 at 4.40 min, with the product at 1116.93 m/z [M+H]^+

The ESI negative Mass Spectra of 26 at 4.40 min, with the product at 1114.33 m/z [M-H]^-
The TOF ESI+ High resolution mass spectrum of 26 with mass at 1116.4460 amu
Compound 27: Water-Soluble-\textit{D}-Lys(mPPa)-18-4

The Diode Array Detector chromatogram of 27

The UV-Vis Spectrum of 27 with characteristic peaks at 407 and 651 nm
The ESI positive Mass Spectra of 27 at 4.29 min, with the product half mass at 1197.19 m/z

\[ [(M+2)/2]^+ \]

The ESI negative Mass Spectra of 27 at 4.29 min, with the product half mass at 1195.87 m/z

\[ [(M-2)/2]^− \]
The TOF ESI- High resolution mass spectrum of 27 with the half mass at 1195.5497 amu.