Developing Activatable Photosensitizers for Fluorescence-Guided Photodynamic Therapy

by

Angela Tian Hui Kwan

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Department of Chemistry
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Abstract

Photodynamic therapy (PDT) is increasingly being recognized as an attractive alternative to conventional forms of cancer therapies. Already clinically approved, this procedure is minimally invasive; by combining light, a small-molecule photosensitizer (PS), and molecular oxygen, reactive oxygen species can be produced in situ, causing damage to vital cellular components. Subsequently, cell death occurs by apoptosis or necrosis.

In practice, after a PS has been administered, an appropriate wavelength of light is irradiated at tumors and surrounding tissue. However, since PSs lack selectivity and non-specifically localize, light irradiation also destroys neighboring healthy cells.

To this regard, I developed activatable-PSs that extend beyond the conventional two-layered selectivity inherent to PDT by including biomarker-specific activation with enzymes: Glutathione S-Transferase, Carboxylesterase 2, and Azoreductase. With this heightened control, these PDT beacons will give a highly specific and localized therapeutic response by unquenching the probe to restore the PS’s native properties at the target site.
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Chapter 1
Introduction

1.1 Fluorescence Imaging

Fluorescence imaging is most commonly used to visualize fluorescent dyes, probes, or proteins as tags of biomolecules to study the structure and function of biological systems and their molecular processes. With high sensitivity and resolution, it enables a wide range of observations in a non-invasive manner – such as: the location and dynamics of gene expression, protein function and expression, and molecular interactions in tissues and cells. In parallel, the emergence of microscopic techniques allows for super-resolution live cell imaging, single-molecule sensitivity, and video-rate scans, which currently remains the diagnostic gold standard for research. Most notably, this optical modality is currently used in the field of oncology for cancer diagnosis, which involves locating the tumor tissues by employing a variety of strategies, such as designed probes that target overexpressed enzymes or monitor pH variations. Despite the increasing availability of these techniques, the overall sensitivity and detection limit of fluorescence imaging is largely dictated by the performance of the fluorescent tag.

As molecules that can be chemically or biologically attached to aid in the detection of the target biomolecule, fluorescent probes display a change in fluorescence upon non-covalent interaction with their target molecule. They can now be used in various diagnostic real-time biological applications, such as: tracing the progression of metastasis, stem cell therapy, immune cell trafficking, and molecular processes. However, their absorption and emission of light tends to fall within the ultraviolet-visible region; these types of radiation suffer from poor tissue penetration since the amount of elastic light scattering increases with decreasing wavelengths of light (190-750 nm, < 5 mm) (Figure 1.1). Thus, probes that absorb and emit strongly within the near-infrared (NIR) window are highly desirable as the scattering effect—which causes light to diffuse and spread out in many directions—is diminished, allowing for higher imaging resolution due to greater tissue penetration depth and suppression of background signal (650-1350 nm, > 5 mm).
Figure 1.1: Light-tissue interaction. Depth of light penetration into human skin; as the wavelength of light increases, the penetration depth becomes deeper. Figure retrieved from Ash, C., Dubec, M., Donne, K. & Bashford, T. Effect of Wavelength and Beam Width on Penetration in Light-Tissue Interaction using Computational Methods. *Lasers in Medical Science* **32**, 1909–1918 (2017).

Light scattering occurs inside the skin and arises from the differences in the refraction indices of different cellular components, such as the membranes and various organelles. Dermal collagen, found in the reticular layer of the dermis, is largely responsible for light scattering that occurs in our skin upon light-tissue interaction. Specifically, the cylindrical collagen fiber bundles and the tropocollagen ultrastructures—that present as banded patterns of periodic striations on these fibrils—cause Mie and Rayleigh scattering, respectively. When the diameter of the scatter becomes much larger than the wavelength of the incident light, Mie scattering occurs whereas Raleigh scattering ensues when the diameter is much smaller. Part of the light will also be reflected (4-7%) at the air–stratum corneum interface; further transmitted; and absorbed (majority) by our endogenous tissue chromophores (i.e. melanin, water, and hemoglobin). The phototherapeutic window (600-1000 nm) is where light absorption from these common endogenous chromophores and scattering of light by tissues is minimal (Figure 1.2).
Figure 1.2: Phototherapeutic window (600-1000 nm). Light absorption from common endogenous chromophores found in human skin. Figure retrieved from Niemz, M. H. Laser-Tissue Interactions. Fundamentals and Applications (Springer, 2007).

In addition to having strong absorption and emission in the NIR region, promising probes also exhibit high photostability and have a long fluorescence lifetime. Small-molecule fluorophores are usually preferred over fluorescent proteins since they can be substantially brighter, more photostable, applied to any sample, less expensive, cell permeable, and can respond rapidly to their biological target – thereby, allowing for efficient imaging, high signal-to-noise ratio, and novel synthetic designs. Examples of common NIR fluorophores used in biomedical imaging are listed in Table 1.1.
Table 1.1: Examples of common NIR fluorophores used in biomedical imaging. Figure retrieved from Hong, G., Antaris, A. L. & Dai, H. Near-Infrared Fluorophores for Biomedical Imaging. *Nature Biomedical Engineering* **1**, 0010 (2017).

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<td>807/822 nm</td>
<td>Plasma lipoprotein and atheromas, mostly non-specific</td>
<td>Negatively charged; lipophilic; QY=9.3% (serum)</td>
<td>Cardiovascular and lymphatic angiography; bile duct and GI tract imaging; tumour imaging and IGS</td>
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<td>M8</td>
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<td>Non-specific</td>
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<td>Non-specific; conjugatable with targeting ligands</td>
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<td>Conjugated copolymers</td>
<td>500-1,100 nm 700-1,400 nm</td>
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<td>Aggregation-induced emission dots</td>
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<td>Hydrophilic; QY=12.5% (TPE-TPA-DCM at 3 wt% loading in BSA)</td>
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<td>Imaging of tumour, kidney clearance kinetics and kidney dysfunction</td>
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<td>Infrared fluorescent proteins</td>
<td>690/711 nm (IFP2.0) 674/733 nm (mIRP703)</td>
<td>Liver (with adenovirus vector) or specific molecular targets</td>
<td>Expressed endogenously; QY=8.0% (IFP2.0) and 8.6% (mIRP703)</td>
<td>Imaging of liver, brain tumour, myoblast differentiation, muscle regeneration, cancer metastasis, signalling cascade, cell cycle and specific protein labelling</td>
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<td>Single-walled carbon nanotubes</td>
<td>550-1,050 nm 1,000-1,800 nm multiband</td>
<td>Non-specific; conjugatable with targeting ligands</td>
<td>Inherently hydrophobic; QY=0.4%</td>
<td>Imaging of internal organs, tumour, limb and brain vasculatures, lymphatic vessels and lymph nodes, bacterial infection and nphoxide</td>
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<td>Rare-earth nanoparticles</td>
<td>808 or 980/1,000-1,600 nm</td>
<td>Non-specific; conjugatable with targeting ligands</td>
<td>QY=90% (5F2:Nd), 50% (LaF3:Nd)</td>
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<td>IR-PEG nanoparticles</td>
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<td>Non-specific; conjugatable with targeting ligands</td>
<td>QY=1.8%</td>
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<td>EGFR+ tumour with anti-EGFR affibody</td>
<td>Water-soluble; QY=0.3%</td>
<td>Imaging of blood and lymphatic vasculatures, brain tumour and xenograft EGFR+ tumour</td>
<td>Prereclinical</td>
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1.1.1 Enzyme-Activatable Fluorescent Chemosensors

Compared to the “always on” traditional fluorogenic probes, enzyme-activatable fluorescent probes are more selective in delineating enzyme activity (Figure 1.3). In the presence of the target, the probe will be activated to the “on” state by the enzyme, which will increase the signal-to-noise ratio and permit signal amplification as there is minimal background from the non-emissive probe.8

Figure 1.3: Activatable fluorescent probe. In the signal “off” state, the fluorescent probe is non-emissive; however, after interacting with the target, it becomes switched “on” to a highly emissive state. Figure retrieved from Terai, T. & Nagano, T. Small-Molecule Fluorophores and Fluorescent Probes for Bioimaging. Pflügers Archiv - European Journal of Physiology 465, 347–359 (2013).

To generate an inactive fluorescent chemosensor, there are numerous fluorescence quenching mechanisms that can be employed such as Föster resonance energy transfer (FRET), photoinduced electron transfer (PeT), structural fluorogenicity, or changes in fluorophore conjugation.

FRET is a dynamic quenching mechanism that involves a nonradiative energy transfer between two dyes, the excited state (ES) FRET donor and the FRET acceptor (Figure 1.4).9 With light irradiation that is wavelength-specific to the donor, the fluorescence of the donor will be quenched by absorption from the acceptor, which will in turn fluoresce.10 However, upon enzymatic cleavage of the linker, the FRET pair will separate and permit fluorescence emission from the FRET donor.

Figure 1.4: FRET-based activatable fluorescent probes. Fluorescence emission is monitored at the emission wavelength of the FRET donor. In the FRET quenched state, light excitation results in fluorescence emission from the FRET acceptor. Upon separation of the FRET pairs by the target molecule, the FRET donor becomes switched “on”

PeT is a dynamic quenching mechanism where an electron is transferred from an ES donor to the acceptor fluorophore, which are in close proximity (**Figure 1.5**). In this redox reaction, a charge separation is produced within the molecule. Like FRET, PeT creates a quenched probe that emits weak fluorescence in its “off” state, which becomes strongly fluorescent only upon contact with the target molecule. However, unlike FRET, no change is observed in the shape of the excitation and emission spectra of the fluorophore in the presence of the target, which eliminates the possibility of using ratiometric measurements.

**Figure 1.5**: PeT-based activatable fluorescent probe. In the PeT quenched state, an electron is transferred from the PeT donor to the excited PeT acceptor. After interaction with the target, PeT quenching is relieved and results in a highly emissive fluorophore. Figure retrieved from Terai, T. & Nagano, T. Small-Molecule Fluorophores and Fluorescent Probes for Bioimaging. *Pflügers Archiv - European Journal of Physiology* **465**, 347–359 (2013).

Some fluorogenic probes (i.e. xanthene-containing probes) can participate in structural fluorogenicity by undergoing structural changes such as intramolecular spirocyclization after interaction with the target (**Figure 1.6**). Subsequently, the probe will be switched from a non-fluorescent colorless “closed” form to a fluorescent colored “open” form.

**Figure 1.6**: 2′-Carboxy-rhodamine-based probe. Spirolactonization between its non-fluorescent closed spirolactone form and fluorescent open zwitterionic form is governed based on the polarity of the environment. Figure retrieved from Kozma, E. & Kele, P. Fluorogenic Probes for Super-Resolution Microscopy. *Organic & Biomolecular Chemistry* **17**, 215–233 (2019).
Other fluorogenic probes contain a push-pull fluorophore with an electron donating group that is conjugated to an acceptor group via a conjugated π-system (Figure 1.7). Due to this linkage to an electron-withdrawing acceptor group, a hypsochromic shift in its absorption spectrum occurs. By participating in a photochromic modulated system, cleavage of the acceptor group will regenerate the electron donating group and undergo a bathochromic shift to its original absorption spectra.

Figure 1.7: A DCDHF class fluorogen with a push-pull system. After nitroreductase-mediated reduction of the nitro group, the electron-donating amine group is generated. Consequently, the fluorophore increases in fluorescence emission and undergoes a bathochromic shift. Figure retrieved from Kozma, E. & Kele, P. Fluorogenic Probes for Super-Resolution Microscopy. Organic & Biomolecular Chemistry 17, 215–233 (2019).

1.1.1.1 Examples of Enzyme-Activatable Fluorescent Chemosensors

An example of a Glutathione S-Transferase (GST)-activatable fluorescent chemosensor is the two-photon probe called P-GST (λ<sub>ex</sub> = 420/820 nm and λ<sub>em</sub> 550 nm) (Figure 1.8). The scaffold consists of a 2,4-dinitrobenzenesulfonate quencher group that acts as the GST substrate and serves to PeT inactivate the naphthalamide-based fluorophore. In the presence of GST and its cofactor glutathione (GSH), the 2,4-dinitrobenzene group is transferred to GSH, along with SO<sub>2</sub> release and liberation of the free fluorophore. A 40-fold increase in fluorescence was observed in MCF-7 cells.

Figure 1.8: Activatable two-photon GST probe called P-GST. After GST-mediated conjugation of GSH onto the PeT quencher group (2,4-dinitrobenzenesulfonate), the fluorophore becomes unquenched and highly emissive as it is

An example of a Carboxylesterase 2 (CES2)-activatable fluorescent chemosensor is the two-photon ratiometric probe called NCEN, which is blue-emitting in its inactive state (**Figure 1.9**). The scaffold is comprised of a naphthalamide-based fluorophore conjugated to a chloroacetyl trigger group, which will be cleaved by CES2 to release NAH—a green-emitting fluorophore via a push-pull photochromic system. NCEN was used to image human CES2 activity in HepG2 cells and mouse liver slices from depths ranging from 10 to 50 μm via two-photon microscopy (λ<sub>ex</sub> 800 nm).

![Figure 1.9: Activatable hCES2 probe called NCEN. After CES2-mediated hydrolysis of the amide linkage, the free fluorophore NAH becomes unquenched and highly emissive as it is released from the chloroacetyl trigger group. Figure retrieved from Singh, K., Rotaru, A. M. & Beharry, A. A. Fluorescent Chemosensors as Future Tools for Cancer Biology. *ACS Chemical Biology* **13**, 1785–1798 (2018).](image)

An example of an Azoreductase-activatable fluorescent chemosensor is MAR, which consists of a xanthene dye chemically attached to an azobenzene derivative (**Figure 1.10**). The azo bond in the dimethylamino-azobenzene moiety acts to quench the fluorescence of the 2-methyl-rhodamine fluorophore upon light irradiation due to energy dissipation from rapid rotation around the azo-bond. With Azoreductase-mediated reductive cleavage of the azo bond, MAR produced a 630-fold increase in fluorescence intensity, which was successful under hypoxia at 5% oxygen in A459 cells and for retinal artery occlusion in an *in vivo* mouse model.
Figure 1.10: Activatable Azoreductase probe called MAR. After azoreductase-mediated reduction of the azo bond, the free fluorophore becomes unquenched and highly emissive as it is released. This chemosensor selectively imaged hypoxic over normoxic environments in a rat model of branch retinal artery occlusion. Figure retrieved from Singh, K., Rotaru, A. M. & Beharry, A. A. Fluorescent Chemosensors as Future Tools for Cancer Biology. ACS Chemical Biology 13, 1785–1798 (2018).

1.2 Photodynamic Therapy

Photodynamic Therapy (PDT) has increasingly been recognized as an attractive alternative to conventional forms of cancer therapies (e.g. surgery, radiation therapy, and chemotherapy). This clinically approved treatment modality has many advantages over the current standard of care: it is minimally invasive, lacks ionizing radiation, has a fast healing process that leads to minimal or absent scarring, can be used in an outpatient setting, has few side effects, and can exert cytotoxicity towards malignant cells.\textsuperscript{13,14} Moreover, repeated doses of PDT can be administered without surpassing total dose limitations or introducing resistance.\textsuperscript{13} Cellular resistance to apoptosis can occur when patients undergo radiation or chemotherapy; however, PDT can overcome this limitation by preferentially inducing necrosis over apoptosis in which the PS is biased to localize in specific subcellular compartments.\textsuperscript{13}

PDT is achieved by a photochemical reaction between three components—light, a small-molecule photosensitizer drug (PS), and endogenous molecular oxygen, whereby localized reactive oxygen species (ROS) is produced to attack vital cellular components (e.g. lipids and proteins) (Figure 1.11). Through direct cellular damage, vascular shutdown, and immune response activation against the diseased site, cellular death is ultimately promoted by apoptotic or necrotic mechanisms.\textsuperscript{13} In practice, after a PS has been administered—either locally (i.e. topically) or systemically (i.e. intravenously)—an appropriate wavelength of light (usually ~600-800 nm) is irradiated only at the tumor in a temporally- and spatially-controlled manner, thus avoiding the surrounding healthy
Currently, PDT is being used in the treatment of solid tumors in the skin (i.e. basal cell carcinomas), head and neck, lungs, brain, esophagus, bladder, ocular melanoma, ovarian, prostate, renal cell, cervix, pancreas, and bone carcinomas.

**Figure 1.11:** Photochemical processes involved in PDT. During light irradiation of a wavelength specific to the PS, the PS absorbs the photons and jumps from ground state (S₀) to an excited state (e.g. S₁). Eventually, the PS will release this energy and regenerate S₀ while simultaneously inducing cellular toxicity via the conversion of tissue oxygen to singlet oxygen. Figure adapted from Lin, J. & Wan, M. T. Current Evidence and Applications of Photodynamic Therapy in Dermatology. Clinical, Cosmetic and Investigational Dermatology 145 (2014).

When light that is wavelength-specific to the PS is irradiated at that light-absorbing drug, the ground state PS becomes promoted to an excited singlet state (¹Sen*) (**Figure 1.12**). Here, the energy of ¹Sen* can be dissipated either by thermal decay or fluorescence emission. Alternatively, ¹Sen* can undergo intersystem crossing (ITC) to move to a lower-energy excited triplet state (³Sen*); the lifetime of ³Sen* is greater than ¹Sen*. At this stage, the energy of ³Sen* can be lost by phosphorescence. Otherwise, it can produce cytotoxic reactive oxygen species (ROS) via Type I or Type II processes (**Figure 1.12**). Type I PDT involves an electron transfer between the PS—acting as a photochemically activated free-radical initiator—and various receptor molecules (e.g. oxygen or biomolecules) for R• formation to eventually produce ROS (**Figure 1.12**). This mechanism involves either acquisition or donation of an electron to generate a radical cation (R⁺*) or radical anion (R⁻*). With an unpaired electron in its outer electron shell, the radical anion can react with oxygen to create a superoxide anion (O₂⁻*), which will subsequently form hydrogen peroxide (H₂O₂) through dismutation or a one-electron reduction. Another one-electron reduction will occur to yield a hydroxyl radical (HO'). The radical anion can also abstract hydrogen from other biomolecules to trigger a free-radical chain reaction. In contrast, the more relevant Type II PDT generates singlet oxygen (¹O₂) upon direct interaction between the high-energy ³Sen* and the
low-energy, ground state molecular oxygen ($^3\text{O}_2$) by a triplet-triplet annihilation mechanism (Figure 1.2). The triplet state PS will then return to ground state, where it can repeat the cycle again to generate $^1\text{O}_2$—up to a limit of $10^3$-$10^5$ before it becomes inactive. The main factor that determines whether the reaction pathway is Type I or Type II depends on the competition between molecular oxygen and biomolecules for the $^3\text{Sen}^*$ (Figure 1.2).

**Figure 1.2:** Photochemical processes involved in PDT, illustrated by a Jablonski diagram. During light irradiation of a wavelength specific to the PS, the PS absorbs the photons and jumps from ground state ($S_0$) to an excited singlet state ($S_1$). Subsequently, the PS undergoes intersystem crossing to an excited triplet state, which can then either perform type I or type II processes. For PDT, the type II process converts tissue oxygen to singlet oxygen while regenerating the PS in its ground state. Figure adapted from Hamblin, M. R. & Abrahamse, H. Inorganic Salts and Antimicrobial Photodynamic Therapy: Mechanistic Conundrums? *Molecules* **23**, 3190 (2018).

Both mechanisms will cause oxidative stress among macromolecules (e.g. lipids, proteins and amino acids, and nucleic acids) due to the high levels of ROS within the cell or when there are low levels of antioxidants (i.e. glutathione and vitamins) available in the cell, which act to quench ROS. Specifically, for protein oxidation, ROS can oxidatively cleave the polypeptide chain with distinct four mechanisms: i) cleavage of alkoxyyl peptide derivatives with the diamide pathway (see A in Figure 1.13); ii) cleavage of alkoxyyl peptide derivatives with the $\alpha$-amidation (see A in Figure 1.13); iii) oxidation of prolyl side chains (see B in Figure 1.13); and iv) oxidation of glutamyl and aspartyl side chains (see C and D in Figure 1.13).
Figure 1.13: ROS-mediated cleavage of polypeptide chains: a) alkoxy peptide derivatives cleaved by the diamide or α-amidation pathway, b) oxidative cleavage of proline side chains, c) oxidative cleavage of glutamyl side chains, and d) oxidative cleavage of aspartyl side chains. Figure retrieved from Stadtman, E. R. & Levine, R. L. Free Radical-Mediated Oxidation of Free Amino Acids and Amino Acid Residues in Proteins. *Amino Acids* 25, 207–218 (2003).

In the diamide pathway, cleavage leads to two peptides; the C-terminal residue from the N-terminal side of the protein becomes a diamide derivative and the N-terminal residue from the C-terminal side of the protein becomes an isocyanate derivative (see A in Figure 1.13). In the α-amidation pathway, the backbone Cα-N bond becomes cleaved; the C-terminal residue from the N-terminal side of the protein contains an amide group and the N-terminal residue from the C-terminal side of the protein becomes an α-ketoacyl derivative (see A in Figure 1.13). Oxidation of prolyl residues leads to formation of 2-pyrrolidone and then concomitant cleavage (see C in Figure...
Oxidation of glutamyl residues leads to formation of oxalic acid and the N-terminal residue from the C-terminal side of the protein becomes an N-pyruvyl derivative (see D in Figure 1.13). Unlike the diradical triplet state oxygen (S=1, M=3), the molecular orbital of singlet oxygen has paired electrons in its π antibonding orbital, therefore giving \(^1\text{O}_2\) a total spin quantum number of S=0 and a multiplicity state (defined by 2S+1) of M=1 (Figure 1.14 and 1.15). This violation of Hund’s rule, along with the resulting electronic repulsion between the paired electron in a single orbital, causes \(^1\text{O}_2\) to have 22.5 kcal more energy than \(^3\text{O}_2\). Singlet oxygen has a short half-life (10^-6 s) like other ROS species—such as the hydroxyl radical (10^-9 s)—in biological systems; however, this non-radical species lasts long enough to be able to react with other singlet-state, electron-rich compounds containing double bonds in close proximity. Specifically, it has a radius of approximately 20 nm as it has limited diffusion distance (10-300 nm), which helps with the spatial selectivity of PDT as damage induced by singlet oxygen is restricted to cells in which the PS has accumulated.

**Figure 1.14:** Molecular orbitals of triplet (left) and singlet oxygen (right). Triplet oxygen has the following properties: the electrons in its π antibonding orbital are unpaired, the total spin quantum number (S) is 1, the spin multiplicity (2S+1) is 3, is paramagnetic with diradical characteristics, has an energy level of 0 kcal/mole, and interacts with radical compounds. Conversely, singlet oxygen has the following properties: the electrons in its π antibonding orbital are paired, the total spin quantum number (S) is 0, the spin multiplicity (2S+1) is 1, is an electrophilic non-radical compound, has an energy level of 22.5 kcal/mole, and interacts with electron rich compounds. Figure retrieved from Min, D. B. & Boff, J. M. Chemistry and Reaction of Singlet Oxygen in Foods. *Comprehensive Reviews in Food Science and Food Safety* 1, 58–72 (2002).
Figure 1.15: Lewis-dot diagram of triplet versus singlet oxygen. Triplet oxygen is a diradical molecule due to its two unpaired electrons that have parallel spins to each other (shown in red); it also has four lone pairs of electrons and a single covalent bond. Singlet oxygen is a nonradical molecule and has a double bond (shown in red), where the electrons have antiparallel spins to each other.

Consequently, cellular components will become oxidatively damaged and eventually rendered dysfunctional—ultimately disturbing homeostasis and triggering targeted cell death via apoptosis or necrosis (Figure 1.16, 1.17, and 1.18).

Figure 1.16: Modes of cell death in PDT. The type of cellular pathway induced depends mainly on the localization of the PS. Mitochondrial PS localization will initiate a form of programmed cell death called apoptosis because the cell membrane of the mitochondria becomes damaged and leads to the release of pro-apoptotic factors. Lysosomal PS localization can either initiate apoptosis by the release of proteolytic enzymes or autophagy when the lysosomes combine with autophagosomes, which will lead to hydrolysis of damaged organelles. Necrosis becomes the dominant pathway post-PDT when apoptosis becomes defective. Figure retrieved from Mroz, P., Yaroslavsky, A., Kharkwal, G. B. & Hamblin, M. R. Cell Death Pathways in Photodynamic Therapy of Cancer. Cancers 3, 2516–2539 (2011).
The type of cellular death pathway depends on the subcellular localization of the PS and the level of PDT dosage (PS concentration x light fluence). In terms of PS localization, when PSs are localized in the plasma membrane or lysosomes, necrosis—the upregulated digestion of cell components—is triggered due to loss of plasma membrane integrity and intracellular ATP. However, PSs that localize in the mitochondria or endoplasmic reticulum will cause the release of cytochrome c, which will subsequently lead to activation of apoptosome and procaspase 3. In doing so, apoptosis will be activated. In terms of PDT dosage, a low dosage is more conducive to apoptosis due to photodamage of anti-apoptotic proteins and activation of proapoptotic proteins whereas a high dosage will inactivate enzymes and other factors that are associated with apoptosis, thus initiating necrosis.

Figure 1.17: Various modes of activation of caspases during apoptosis. The extrinsic (death receptor) pathway is initiated by the binding of ligands from the tumor necrosis-factor (TNF)-family to their respective receptors, which promotes a series of subsequent events, as shown in the diagram above. Conversely, the intrinsic (mitochondrial) pathway is induced by death signals like PDT that result in a decrease in permeability of the outer mitochondrial membrane, thereby allowing apoptotic inducers to be readily released (e.g. cytochrome c, Smac/DIABLO, and endonuclease G). Figure retrieved from Agostinis, P., Buytaert, E., BreysSENS, H. & Hendrickx, N. Regulatory Pathways in Photodynamic Therapy Induced Apoptosis. *Photochemical & Photobiological Sciences* 3, 721 (2004).
**Figure 1.18:** Major PDT-induced cellular death mechanisms. These include direct cellular damage, vascular shutdown, or immune response activation. Figure retrieved from Mroz, P., Yaroslavsky, A., Kharkwal, G. B. & Hamblin, M. R. Cell Death Pathways in Photodynamic Therapy of Cancer. *Cancers* **3**, 2516–2539 (2011).

### 1.2.1 First-Generation Photosensitizers

Photosensitizers are light sensitive drugs that are activated by light of a specific wavelength. An important factor to ensure successful PDT is the choice of a suitable PDT agent, which should meet the following requirements: it must be an effective photosensitizer (i.e. have a high singlet oxygen quantum yield, which however compromises its fluorescence quantum yield); is chemically and structurally characterized and has a single isomer; has low dark toxicity; should have preferential localization in the target area; and has a short post-irradiation clearance time to minimize photosensitivity.\(^{21}\) Lastly, the PDT agent should absorb and emit strongly in the NIR region as this region of the electromagnetic spectrum falls within the phototherapeutic window, which permits deeper tissue penetration. Two-Photon Excitation (TPE) can help circumvent situations when the required PS can only be excited at shorter wavelengths; the high-power pulsed laser used will increase the probability that the PS absorbs two photons quasi simultaneously—allowing for an excitation wavelength that is twice as long.

First generation PDT photosensitizers lack NIR wavelength absorption and induce a lengthened period of patient photosensitivity due to their poor clearance rates. The first porphyrin-type photosensitizer was prepared by reacting hematoporphyrin with 5% sulfuric acid in acetic acid, followed with alkaline hydrolysis (Figure 1.19)\(^{22}\). This reaction led to a mixture of many monomeric porphyrin derivatives and oligomers, where the higher-molecular weight oligomeric fractions were separated from the lower-molecular weight monomers as they showed *in vivo*
photodynamic effects. This mixture of porphyrin oligomers, called Photofrin, has an affinity towards tumors and strong phototoxic effects. This drug has been approved for clinical use for Photofrin (porfimer sodium)-based treatment of bladder cancer. However, Photofrin suffers from numerous drawbacks: it consists of a complex mixture of oligomers; its absorption maximum is at 630 nm, which only penetrates 4 mm deep into the skin; and it has a long clearance rate of ~4-6 weeks post-administration.

Figure 1.19: Oligomer derivatives of hematoporphyrin. This mixture called HpD represents first-generation PSs, which are obtained by subjecting hematoporphyrin to 5% sulfuric acid in acetic acid, and then base hydrolysis. Figure retrieved from Tovey, A. (1994). Third-Generation Photosensitizers: Synthesis, Characterization, and Liposome Interaction of Promising New Benzoporphyrin Derivatives. Masters. The University of British Columbia.

1.2.2 Second-Generation Photosensitizers: Improved Optical Properties

The emergence of second-generation photosensitizers led to PDT agents with improved optical properties; these mainly consisted of modified porphyrin compounds, such as: benzoporphyrin (Visudynes), chlorin (Temoporfins), and porphycene (ATMPn). These tetrapyrrolic PSs have longer absorption wavelengths than Photofrin (630 nm). Temoporfins has an absorption of 690 nm, which doubled the penetration depth as compared to 630 nm. BPDMA, a monoacid derivative of benzoporphyrin, was found to have a lower period of skin photosensitivity post-
administration unlike Photofrin (72 hours versus 4-6 weeks) (Figure 1.20).\textsuperscript{21} As well, BPDMA has a high singlet quantum yield of 0.78 in homogenous solution and 0.46 in an \textit{in vivo} environment.\textsuperscript{22}

\textbf{Figure 1.20:} Benzoporphyrin derivatives (BPD). These second-generation PSs include monoacid and diacids of BPD with ring A or ring B. Figure retrieved from Tovey, A. (1994). \textit{Third-Generation Photosensitizers: Synthesis, Characterization, and Liposome Interaction of Promising New Benzoporphyrin Derivatives.} Masters. The University of British Columbia.

Other major second-generation PSs include: mono-L-aspartyl chlorin e6 ($\lambda_{\text{max}}$ 664 nm, $\Phi\Delta$ 0.70), tin etiopurpurin ($\lambda_{\text{max}}$ 650 nm, $\Phi\Delta$ 0.65), and meso-tetra(m-hydroxyphenyl)chlorin ($\lambda_{\text{max}}$ 650 nm) (Figure 1.21).\textsuperscript{22}

\textbf{Figure 1.21:} Examples of other prominent second-generation PSs: a) Mono-L-Aspartyl Chlorin e6; b) Tin Etiopurpurin; and c) Meso-tetra(m-hydroxyphenyl)chlorin. Figure retrieved from Tovey, A. (1994). \textit{Third-Generation Photosensitizers: Synthesis, Characterization, and Liposome Interaction of Promising New Benzoporphyrin Derivatives.} Masters. The University of British Columbia.
1.2.3 Third-Generation Photosensitizers: Improved PS Localization

Recently, the development of third-generation photosensitizers resulted in an enhanced level of selectivity for cancer cells over healthy cells. One approach was to design PSs that selectively target subcellular compartments (e.g. the mitochondria) or to use targeted molecular carriers (e.g. liposomes or nanospecies). Prior to these designs, the traditionally used PSs tend to localize nonspecifically in both cancer and normal cells. As a result, they would also generate ROS in healthy tissues, causing immediate phototoxicity and side effects in patients during the treatment, thereby limiting its clinical applications. Although specific light placement confers a large amount of spatial selectivity, in practice, it is tough to apply this to microscopic tumors as there are healthy tissues present around the target site itself. Though, this problem can be circumvented if the PS has an increased affinity towards the tumor. Addition of a targeting moiety (i.e. an antibody directed against the tumor antigen or a substrate that binds to its overexpressed cell surface receptor found on the tumor) on the PS will aid in its localization, accumulation, and selective binding to the diseased site. Then even global irradiation directed at the cancer and normal cells will only cause cancer cell death. Therefore, these third-generation PSs will have double selectivity towards cancer cells from direct light placement and specific PS localization.

The structure of the PS, specifically its degree of hydrophilicity and amphiphilicity, has an impact on its pharmacokinetic profile and how it will be administrated. It has been established that a greater degree of hydrophobicity leads to a higher accumulation of the PS in tumor tissues over normal tissues at ratios of 7:1 and 8:1. Moreover, PSs that bear anionic groups, like carboxyl and sulphonate groups, have preference for the cytoplasm and will move into the nucleus when irradiated; PSs that are hydrophobic and bear cationic substituents however tend to accumulate in the mitochondria – and preferentially trigger apoptosis. Conversely, an equivalently hydrophilic PS exhibits a tumor to normal tissue ratio of merely 2:1. In terms of the route of administration, if the PS is poorly soluble in aqueous media, it would hinder their applications in physiological media and prevent their intravenous delivery into the bloodstream.

Modern PDT arises from the efforts made since the 1960s and from there, this treatment modality has made a great deal of clinical progress; there has been many approvals worldwide for new PSs for the imaging and treatment for a myriad of oncological and non-cancer diseases (i.e. actinic keratosis and age-related macular degeneration). Each generation of PSs have been developed
iteratively to improve its biological and photonic properties. In conjunction to the improvements made with the PS, light delivery technologies have also progressed from the use of old-fashion laboratory-based lamps towards the superior optic-guided delivery of laser light.

1.2.3.1 Enzyme-Activatable Photosensitizers: Improved PS Activation with Bio-Responsive Elements

PS localization and specific light placement confer two layers of selectivity to PDT. Effective PSs tend to be more hydrophobic than hydrophilic since nonpolar compounds are more likely to undergo the enhanced permeability and retention (EPR) effect. With leaky blood vessels and no lymphatic drainage, these PSs diffuse into the tumor cells and localize in intracellular compartments, such as the endoplasmic reticulum (ER) and mitochondria, more rapidly, thus giving them tumor-localizing properties. Subsequently, with directed light irradiation at these tumour cells, singlet oxygen will be generated at the target area; its short lifetime (~10-320 ns) limits its diffusion to 10-55 nm in cells. However, cell death may also occur at non-target sites since PSs can non-specifically localize to normal cells and directed light placement to microscopic tumors is difficult – especially ones with healthy tissues embedded closely around the diseased area. Therefore, PS selectivity must be enhanced in order to permit global irradiation of the tumor without the induction of side effects. This can be done by using activatable photosensitizers (aPSs).

Activatable photosensitizers are a class of PSs that have an additional layer of selectivity as a molecular activation step is incorporated to allow the aPS to be switched from an “off” to “on” state only when its target is encountered (Figure 1.22). This is achieved by integrating a bio-responsive element in their chemical design, which will ensure singlet oxygen production from aPSs inside the activation site. Otherwise, the aPS is kept in a quenched “off” state and is unable to generate singlet oxygen—even in the presence of light irradiation. These “smart drugs” therefore have enhanced selectivity towards target cells over healthy cells and are more useful probes. With the ability to isolate the photodynamic effect strictly to target cells with the distinct biomolecular signature, aPSs reduce the amount of nonspecific phototoxicity.
Figure 1.22: Activatable PS-based target cell killing. With three layers of selectivity, only cancer cells will be selectively damaged while neighboring healthy cells are unaffected (red area). The molecular activation step makes these aPSs smart drugs as their ability to generate singlet oxygen is strongly inhibited in its “off” state. Otherwise, unwanted cellular death may occur in normal cells (pink area). Figure retrieved from Lovell, J. F., Liu, T. W. B., Chen, J. & Zheng, G. Activatable Photosensitizers for Imaging and Therapy. *Chemical Reviews* **110**, 2839–2857 (2010).

One common strategy to maintaining aPS deactivation is to prevent the PS from adopting a higher excited state through contact (static) quenching (Figure 1.23). This quenching strategy works by bringing another molecule into continued contact with a PS in its ground state. Subsequently, the excitation properties of the PS changes and this often causes an absorption shift; this can also occur when the conjugation of the PS (or fluorophore) has been altered.\(^{12}\) The next deactivation point occurs after excitation, where dynamic quenching via Förster resonance energy transfer (FRET), photoinduced electron transfer (PeT), and self-quenching can be employed when the PS is in its excited state (Figure 1.23).\(^{13}\)

Figure 1.23: PDT energy diagram. The red arrows represent the photophysical pathway for PDT, which results in singlet oxygen production. The blue arrows represent potential deactivation routes. Figure retrieved from Lovell, J. F., Liu, T. W. B., Chen, J. & Zheng, G. Activatable Photosensitizers for Imaging and Therapy. *Chemical Reviews* **110**, 2839–2857 (2010).
Enzyme-activatable photosensitizers are a type of aPS that becomes activated strictly in areas that contain the active enzyme target (Figure 1.24). Otherwise, the probe remains inactive in tissues that do not express the enzyme. Since enzyme overexpression is often associated with diseases, enzymes therefore become an excellent target for aPSs as they will be switched “on” only in cancer cells in which they received sufficient activation. Furthermore, enzyme-aPS allow for high signal amplification since a single enzyme can activate numerous aPS molecules.

**Figure 1.24:** Enzyme-activatable PSs. In the “off” state, the PS is inactive and cannot generate singlet oxygen due to presence of a quencher moiety. Upon enzyme-mediated catalysis, the PS is separated from the quencher, which now becomes active and is in the “on” state, allowing for PDT applications. Figure adapted from the Beharry Lab website (http://beharrylab.com/Research).

### 1.3 Fluorescence-Guided Photodynamic Therapy

Many PSs are also fluorophores; this brings an additional advantage to PDT that is highly desirable for the detection, treatment, and resection of many types of cancer. Combined with its therapeutic ability to cause cell death via singlet oxygen production, these PSs also have diagnostic properties that permit image guided therapy (Figure 1.25).

**Figure 1.25:** Activatable PSs are inherently theranostic. These multifunctional agents can be used for fluorescence imaging and for PDT, which are undergo the following steps: administration, accumulation, light delivery via fiber optics, and finally assessment. Figure retrieved from the Hasan Program website (https://sites.dartmouth.edu/pdt/).
Such a drug can selectively distinguish and detect cancer tissues from normal tissues in real-time via the EPR effect and fluorescence, respectively. By sharing an excitation pathway for fluorescence and singlet oxygen production, this makes the PS inherently theranostic (Figure 1.26).²⁴

![Diagram of PS energy levels and pathways](image1)

**Figure 1.26:** Energy diagram for fluorescence and PDT. This Jablonski diagram highlights the pathways that are shared by fluorescence emission and singlet oxygen production. Figure retrieved from Luby, B. M., Walsh, C. D. & Zheng, G. Advanced Photosensitizer Activation Strategies for Smarter Photodynamic Therapy Beacons. Angewandte Chemie 131, 2580–2591 (2018).

1.3.1 Next-Generation Photosensitizers: Cancer Imaging and Treatment

Like aPSs, these PSs called “PDT beacons” also extend beyond the two-layered selectivity by incorporating an additional biomarker-specific activation step—with such heightened control, this results in a highly specific and localized therapeutic response (Figure 1.27).²⁴ As opposed to non-fluorescent aPSs, the binary on/off nature of fluorescence and photosensitization in PDT beacons can be modulated by activation of the rationally designed beacon with biomolecules characteristic to the target disease (Figure 1.28).

![Activatable PSs as smart PDT beacons](image2)

**Figure 1.27:** Activatable PSs as smart PDT beacons. With an additional molecular activation step, in addition to PS accumulation and light placement, highly efficient and selective eradication will be achieved. Figure retrieved from...

**Figure 1.28:** Major activation fluorescence-guided PDT strategies: a) FRET-based quenching relieved by cleavage of the covalent linker between the PS and quencher; and b) PeT quenching inhibited by binding of the biomarker to the PeT quencher, allowing the PS to become active in the “on” state. Figure retrieved from Luby, B. M., Walsh, C. D. & Zheng, G. Advanced Photosensitizer Activation Strategies for Smarter Photodynamic Therapy Beacons. *Angewandte Chemie* **131**, 2580–2591 (2018).

The fluorescence imaging aspect of these PDT beacons are advantageous during dosimetry and in real-time surgical settings for accurate diagnosis and fluorescence-guided surgery.\(^{24}\) With a high fluorescence signal-to-background ratio, such an enhancement permits the visualization of PS localization and its uptake in tumour tissues. In turn, this allows accurate imaging and identification of malignant regions, the fine-tuning and outlining of parameters during PDT treatment, and resection of tumors that are smaller in size by precisely defining tumor margins during surgical abscessions—which reduces the risk of cancer recurrence post-surgery.\(^{24}\) Hence, fluorescence-guided PDT is a powerful theranostic modality that is highly desirable for the treatment of cancer.

### 1.3.1.1 Enzyme-Activatable Fluorescent-PDT Beacons

As opposed to enzyme-aPSs, enzyme-activatable fluorescent-PDT beacons are capable of more effective PDT since its ability to fluoresce permits real-time diagnosis of tumor tissues and image-guided surgery (**Figure 1.29**).\(^{24}\) For these PDT beacons to achieve efficient deactivation and activation, they must be rationally designed to exploit enzymes or other biomarkers that are sufficiently overexpressed in cancer tissues over healthy tissues, exhibit a detectable fold change
when activated by the cellular activity being targeted, and adequately mimic the structure of the native substrate to ensure that it can still be recognized by the target enzyme.

Figure 1.29: Enzyme-activatable PDT beacons. In the “off” state, the PS is inactive and cannot fluoresce or generate singlet oxygen due to presence of a quencher moiety. Upon enzyme-mediated catalysis, the PS is separated from the quencher, which now becomes active and is in the “on” state, allowing for fluorescence-guided PDT applications. Figure adapted from the Beharry Lab website (http://beharrylab.com/Research).

An example of an Azoreductase-aPS is azoSeR, which consists of an azo bond conjugated to a seleno-rosamine dye (Figure 1.30). In the “off” state, the intact azo moiety serves to block the intersystem crossing process for $^1$O$_2$ production despite light irradiation. However, once the azo group is reductively cleaved under mild hypoxic conditions—due to the overexpression of azoreductases—$^1$O$_2$ generation is permitted, allowing for selective and efficient tumor-targeted PDT.

Figure 1.30: Activatable Azoreductase PS called azoSeR. After azoreductase-mediated reduction of the azo bond, the free PS becomes unquenched and can generate singlet oxygen as it is released. Although this seleno-rosamine dye has fluorescence properties, it has a low fluorescence quantum yield. Consequently, in cellulo fluorescence imaging relied on LIVE/DEAD staining using Calcein AM (live, green) and EthD (dead, red). Figure retrieved from Piao, W. et al. Development of an Azo-Based Photosensitizer Activated under Mild Hypoxia for Photodynamic Therapy. *Journal of the American Chemical Society* **139**, 13713–13719 (2017).
1.4 Developing New Generation Photosensitizers

1.4.1 Strategies

There are many challenges associated with the rational design of novel PSs that have desirable properties for *in vivo* applications such as fluorescence-guided surgery. For successful PDT, the PS must have a high \( ^1 \text{O}_2 \) quantum yield, high stability, selective distribution in tumor cells over healthy cells, and deep penetration depth of the absorbed light. Since singlet oxygen is the major cytotoxic ROS for the type II photoreaction in PDT, its generation can be enhanced by inhibiting the interaction between \( 3 \text{PS}\_\text{S}^* \) and free radicals or introducing heavy atoms (e.g. bromine and iodine).\(^{21}\) Current PSs tend to aggregate in solution, and this phenomenon decreases their stability since their lifetime in the triplet excited state becomes shortened—ultimately reducing the amount of singlet oxygen production.\(^{21}\) This problem can be circumvented by increasing the number of charges or introducing a central metal ion in the molecule.\(^{21}\) Moreover, the type and placement of functional groups on the PS has an impact on its lipophilicity, which will determine its tissue location. The higher the lipophilicity of the molecule, the greater the distribution towards tumor tissues.\(^{21}\) Regarding target localization of the PS, in order to achieve high efficacy and reduced off-target effects, a ligand can be introduced in the chemical design for the selective delivery of the PS to the tumor site. Lastly, a greater penetration depth of light into human tissue can be obtained by improving the PS’s absorption efficiency for red light; the incorporation of electron-rich donors will fulfill this task by expanding the molecular conjugate system, which will cause a bathochromic shift in the absorption spectra of the PS.\(^{21}\) Moreover, the PS should have a rapid clearance rate from normal tissues and no dark toxicity to diminish potential phototoxicity effects.

1.4.1.1 Heavy-Atom Effect

The heavy-atom effect is the influence of the introduced heavy atom on the intersystem crossing rate of the molecule. By synthetically substituting a heavy atom into the chemical scaffold of the PS—via minor peripheral modifications—the level of singlet oxygen generation will increase, leading to a higher singlet oxygen quantum yield.\(^{21}\) More specifically, the PS must transition from its singlet excited state to a triplet excited state upon light irradiation in order to participate in the type II photoreaction process for \(^1\text{O}_2 \) production. However, this electronic transition within a molecule is spin-forbidden since it involves two states with different spin multiplicities.\(^{21}\) To obtain such a phenomenon, a spin-orbit perturbation is required—this can be enhanced via an
external or internal heavy-atom effect. The former occurs when the molecule is placed in an environment that consists of heavy atoms whereas the latter, which is of interest in designing novel PSs, occurs by adding heavy atoms directly onto the molecule. Instead of relying on the inherent spin-orbit coupling of the PS, the addition of the heavy atom will allow full exploitation of the internal-heavy atom effect and produce a more efficient singlet-oxygen generator.

1.4.2 Conversion of Far-Red Fluorophore DDAO into a NIR PS

Many fluorogenic probes use either a coumarin or xanthene-based scaffold, which are net neutral blue or green-fluorescing molecules with a high fluorescent quantum yield, respectively. However, these two dye families have limited in vivo applications since they emit fluorescence at wavelengths outside the phototherapeutic window, specifically below 550nm. Phenoxazines, on the other hand, have a more red-shifted emission; but they tend to become chemically modified in cells by oxidation and reduction, which can drastically alter their fluorescent properties. Cyanines are a class of far-red fluorophores (e.g. Cy5, Cy7, and Alexa Fluor 647) that are advantageous in terms of their resistance to photobleaching, but they suffer from poor cell permeability due to the presence of charges on their scaffold. Carbazines are another class of far-red fluorophores; an example is DDAO, called 1,3-dichloro-7-hydroxy-9,9-dimethylacridin-2(9H)-one—a small-molecule bright NIR fluorophore that absorbs at λ_{max} of 602 nm and emits at λ_{em} of 659 nm (ε = 36,000 M^{-1}cm^{-1}, Φ = 0.39) (Figure 1.32). DDAO’s fluorescent emission in the far-red region is highly desirable for cellular imaging as a smaller degree of cellular absorption, light scattering, and autofluorescence occurs.

![DDAO chemical structure](image)

*Figure 1.32:* DDAO chemical structure. DDAO is a bright red NIR fluorophore with strong NIR fluorescence emission. Figure adapted from Jin, Q. et al. A Highly Selective Near-Infrared Fluorescent Probe for Carboxylesterase 2 and its Bioimaging Applications in Living Systems. *Drug Metabolism and Pharmacokinetics* **32**, (2017).

As mentioned above in the heavy-atom effect section, this bright NIR fluorophore can be converted into a novel NIR PS by simply adding heavy atoms, preferably bromines or iodines, on the periphery of its scaffold.
1.5 Objective and Specific Aims

Fluorescence-guided PDT is a clinical treatment in which a PS is used to simultaneously image and ablate cancer cells upon light irradiation. Despite the double layer of selectivity given by direct light placement and PS localization, there is still a major limitation with the selectivity of the PS for cancer cells over healthy cells. To overcome this, advanced activation strategies can be implemented into the design of the beacon, such as an analyte-responsive element, which provides an additional layer of selectivity as the fluorescence and photosensitization of these PDT beacons can be switched “on” or maintained “off” in the presence or absence of the biological target, respectively. Subsequently, this allows for real-time feedback on the activation state of the PS.

Another PDT limitation is the need for PSs to absorb or fluorescence strongly in the NIR region, which will allow for deeper tissue penetration for more effective PDT.

The objectives of my research are to: a) develop enzyme-activatable fluorescent-PDT beacons that can selectively image and ablate cancer cells and b) develop a novel bright NIR photosensitizer that permits deep tissue penetration for more effective fluorescence-guided PDT. To this regard, the overexpression of the following enzymes: Glutathione S-Transferases (GST), Carboxylesterases (CES), and Azoreductases in numerous human tumor tissues will be exploited to provide the third layer of selectivity for targeted cellular death. Thus far, apart from fluorescent chemosensors, there have been no reported PDT beacons developed for GST and CES. For Azoreductases, although an enzyme-activatable PS has been published, one that can also successfully fluoresce in addition to photosensitize in cellulo is needed.\textsuperscript{12}
Chapter 2
Design, Synthesis, and Characterization of GST-Activatable Fluorescent-PDT Beacons: Results and Discussion

The goal of chapter 2 is to develop a GST-activatable fluorescent-PDT beacon that can selectively kill cancer cells over healthy cells by taking advantage of the overexpression of GSTs; hence, an in-depth understanding of the GST enzyme and its different isoforms is needed in order to rationally design a GST isoform-specific probe that is clinically relevant.

2 Glutathione S-Transferases (GST)

Glutathione S-Transferases comprise a superfamily of prokaryotic and eukaryotic dimeric proteins with multiple important biological roles. These phase II metabolic isozymes participate in the cellular detoxification of xenobiotics and noxious compounds (e.g. drugs, environmental toxins, and carcinogens) by catalyzing the conjugation of GSH to these electrophiles, which neutralizes and increases their water solubility for excretion (Figure 2.1 and 2.2).27

![Figure 2.1: GST-mediated detoxification of xenobiotics. Figured retrieved from Crisóstomo, L. (2013). Pilot-Model for Oxidative Post-Competition Recovery in Swimmers. Masters. University of Porto.](image)

In cells, the GST cofactor GSH (γ-L-glutamyl-L-cysteinylglycine) exists at high levels (0.1-10mM) and is comprised of over 90% of the overall nonprotein sulfur.27

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1 Note that the MCF-7 whole cell lysates were prepared by my colleague, Nima Gharibi.
Figure 2.2: GST-mediated biotransformation of xenobiotics. After diffusing through the cell membrane, xenobiotics are subjected to phase I metabolism by the cytochrome P450 family, followed by phase II metabolism with GSTs, which conjugate GSH to these phase I-modified substances. Subsequently, they undergo phase III excretion where these compounds are actively transported out of the cell via transmembrane efflux pumps. Figure retrieved from Allocati, N., Masulli, M., Ilio, C. D. & Federici, L. Glutathione Transferases: Substrates, Inhibitors and Pro-drugs in Cancer and Neurodegenerative Diseases. Oncogenesis 7, (2018).

In eukaryotes, GSTs are categorized into different classes based on their localization in the cell. Although arising from a common ancestor, their diversity and substrate specificity have changed through mutations, gene recombination, and gene duplication. These include the microsomal GSTs (also called MAPEG: membrane-associated proteins involved in eicosanoid and glutathione metabolism), mitochondrial GSTs (also called the kappa class), and cytosolic GSTs. For microsomal GSTs, these integral membrane proteins are unrelated to the other two families on an evolutionary basis. For mitochondrial GSTs, these soluble enzymes are structurally similar to cytosolic GSTs. For cytosolic GSTs, although they share a common fold, further categorization is made based on their physical, chemical, and structural properties and sequence similarities. Within each class, members tend to share a sequence similarity of about 40% whereas for interclass sequence identities, they tend to share only \( \leq 25\% \) similarity.

In humans, the greatest level of cytosolic GST activity occurs in the liver, as compared to the kidneys (78% of liver), lungs (34% of liver), and intestines (37% of liver). The different cytosolic GST classes include the alpha (\( \alpha \))/GST A, kappa (\( \kappa \))/GST K, zeta (\( \zeta \))/GST Z, theta (\( \theta \))/GST T, mu (\( \mu \))/GST M, pi (\( \pi \))/GST p, sigma (\( \sigma \))/GST S, and omega (\( \Omega \))/GST O class (Figure 2.3). These classes are further subclassified as follows: \( \alpha \)-1,2,3,4,5; \( \mu \)-1,2,3,4,5; \( \pi \)-1; \( \Omega \)-1,2; \( \theta \)-1,2; \( \zeta \) and \( \sigma \).
In their active forms, they exist as either homodimers (e.g. denoted GST A1-1) or heterodimers (e.g. denoted GST A1-2).\(^29\) Each monomer consists of two distinct domains, which include the N-terminal thioredoxin-like (\(\beta\alpha\beta\alpha\beta\alpha\)) domain and the C-terminal alpha (\(\alpha\))-helical domain.\(^28\) Specifically, the N-terminal domain is relatively conserved among the different classes and contains a GSH binding G-site (Figure 2.4).\(^29\) Within the G-site, one specific residue—either a tyrosine or serine—is responsible for activating the thiol group of cysteine in enzyme-bound GSH through hydrogen bonding (Figure 2.4).\(^29\) It has been proposed that this residue acts as a base and abstracts the cysteinyl proton, which increases the rate of GSH conjugation by forming GS\(^-\) from GSH.\(^28\) The alpha, mu, pi (Tyr7), and sigma isoenzymes have a tyrosine residue whereas the other classes contain a serine (e.g. Ser11 in theta) or cysteine (e.g. omega).\(^29\) Unlike tyrosine or serine, the cysteine residue will participate in redox reactions by forming a mixed disulfide bridge with GSH, where these enzymes exhibit less conjugative activity towards normal GST substrates.\(^29\) The more divergent C-terminal domain, together with the N-terminal domain, contributes to the formation of the H-site (Figure 2.4).\(^29\)
Figure 2.4: Structure of GST. This diagram depicts the hGSTP1-1 isoform complex with GSH in the G site and the inhibitor, NBDHEX, in the H site. The pink region represents the N-terminal thioredoxin-like domain whereas the cyan region represents the helical C-terminal domain. Figure retrieved from Allocati, N., Masulli, M., Ilio, C. D. & Federici, L. Glutathione Transferases: Substrates, Inhibitors and Pro-drugs in Cancer and Neurodegenerative Diseases. Oncogenesis 7, (2018).

This H-site, adjacent to the G-site, is responsible for cosubstrate binding and any variation in the hydrophobic amino acids within the site is responsible for substrate selectivity as it affects the physiochemical features of the binding site (Figure 2.5). The H sites of GST A and M are mostly hydrophobic and is partially hydrophobic in GST P (Figure 2.6). More specifically, for GST A: the H-sites in GST A1 and A3 are tunnel-like and in GST A2, it is pocket-like. The residues of GST A1 (Gly14, Phe10, Leu107, Leu108, Val111, Met208, and Phe222) make the H-site inner wall very hydrophobic, especially due to the amino acids: Phe111, Met211, Leu213 and Phe220. As well, the catalytic promiscuity of GST A arises from the protein flexibility of the C-terminal α helix and the protruding ends of its a4–a5 helices (Figure 2.6). For GST M: the H-site is bigger and wider and involves the Leu12, His107, Met108, and Tyr115 residues. It has been proposed that His107 acts as a proton acceptor during the first step of catalysis; residues Leu12 and Leu203 are responsible for majority of the hydrophobic nature (Figure 2.6). Lastly, for GST P: the H-site is moderately open and partly hydrophobic. It consists of the following residues: Tyr7, Phe8, Val10, Arg13, Val35, Ile104 or Val104, Tyr106, Asn204, and Gly205, where Val10 and Gly205 contribute to the H-site floor (Figure 2.5).
Figure 2.5: Active sites of GST. The H sites are shown for the GST A1-1, M1-1, and P1-1 isoforms. Figure retrieved from Mohana, K. & Achary, A. Human Cytosolic Glutathione-S-Transferases: Quantitative Analysis of Expression, Comparative Analysis of Structures and Inhibition Strategies of Isozymes Involved in Drug Resistance. Drug Metabolism Reviews 49, 318–337 (2017).

Underexpressed and elevated levels of certain GST isoforms can serve as a biomarker of cancer (↑ GST A1-1, ↑ GST M1-1, ↑ GST P1-1), Parkinson’s disease (↑ GST P1-1), Alzheimer’s disease (↓ GST M1-1, ↓ GST T1-1, ↓ GST M3-3, ↓ GST P1-1, ↓ GST O1-1), atherosclerosis liver cirrhosis (↓ GST M1-1), and cataract development (↑GST M1-1 and ↓ GST T1-1) (Figure 2.6).28

Figure 2.6: Pathology associated with overexpression of the clinically relevant GST isoforms: GST A1-1, M1-1, P1-1, and A4-4. The x-axis refers to the types of cancer and the y-axis refers to the percentage of patients (total of 12
patients) with medium and high protein expression GST levels. The different GST isoforms are colour coded. Tissue specimens were collected with consent from patients and had been annotated to determine protein expression. Figure adapted from the Human Protein Atlas website (https://www.proteinatlas.org/ENSG00000084207-GSTP1/tissue).

Given the relevance of the A, M, and P isoforms of GST in cancer, each of these isoforms will be explored further in terms of their clinical significance.

GST A serves as a reliable biomarker of colorectal cancer, ovarian cancer, and clear cell renal cell carcinoma (RCC) – as well as in hepatotoxicity and early cisplatin induced kidney injury. The GST A1-1 class has been shown to be overexpressed in lung cancer tissues by 1.83-fold and in benign adrenocortical adenomas by 1.7- to 3.45-fold in activity – both resulting in enhanced rates of tumor growth and progression. As well, GST A1 has been found to be underexpressed in kidney cancers, which results in a pro-oxidant environment that aids in the growth of renal cell carcinomas. Overexpression of the GST A4-4 class by 2.54-fold is linked to poor prognosis of hepatocellular carcinomas.

For GST M, overexpressed and underexpressed levels are found in certain drug-resistant cell lines. The GST M2-2 class serves as a tumor suppressor for non-small cell lung cancer (NSCLC) since its overexpression in A459 cells by 45-fold results in elevated levels of Calponin 2 (CNN2) (by 6-fold), causing inhibition of cancer cell metastasis.

For GST P, the GST P1-1 class is found to be overexpressed in neoplastic tissues, lung cancer tissues, breast cancer tissues, malignant melanoma cells, malignant transitional cell carcinomas of the bladder, renal cell carcinomas, and carcinomas of the colon, ovary, pancreas, esophagus, and stomach. Moreover, GST P1-1 is highly expressed in endothelial and glial cells, which results in drug resistance against epileptic treatments; its genetic polymorphisms are associated with patient survival during treatment of metastatic colorectal cancer and a predisposition to brain tumors like high-grade pediatric astrocytoma; and its overexpression in tumors cause elevated detoxification rates of anticancer drugs, which lead to the development of atherosclerosis, liver cirrhosis, or multi-drug resistance in cancer therapies via formation of survival pathways. In a study conducted by Harrison et al. and Angus et al., 76% and 100% of their RCC patient tissue samples showed overexpressed levels of GSTP1-1 via the intense staining visualized with immunohistochemistry. In human cutaneous squamous cell carcinomas, GSTP1-1 expression was shown to be 3.45-fold greater than in normal skin cells. Furthermore, the high levels of GST
P1-1 found in ovarian cancer (54% of patients) serve as a reliable indicator of chemotherapy resistance and poor prognosis.\textsuperscript{38}

Subsequently, this led researchers to focus on two areas: 1) clarifying the role of GSTs in drug resistance and developing GST inhibitors to counteract this activity and 2) exploiting GSTs for prodrug-specific activation in cancer cells. The latter can be done by developing prodrugs—pharmacologically latent compounds which, upon enzymatic cleavage or chemical modification, becomes the active cytotoxic parent drug—that contains either a GSH/GSH-like moiety or a GSH-specific scaffold (Figure 2.7). Examples include: Canfosfamide to treat drug-resistant ovarian cancer, Metformin analogues to treat type II diabetes, nitric oxide prodrugs, and Doxorubicin analogues to treat solid tumors and malignant hematologic diseases (Figure 2.7).\textsuperscript{28}

\textbf{Figure 2.7:} GST prodrug approach: a) GSTP1-1 mediated activation of Canfosfamide into active alkylating agent; b) GST-mediated GSH conjugation on a metformin sulfonamide prodrug; c) GST-mediated GSH conjugation on a nitric oxide releasing prodrug; and d) GST-mediated sulfomidase activity on a nitrobenzenesulfonyl analogue that will
release the active agent, Doxorubicin. Figure retrieved from Allocati, N., Masulli, M., Ilio, C. D. & Federici, L. Glutathione Transferases: Substrates, Inhibitors and Pro-drugs in Cancer and Neurodegenerative Diseases. *Oncogenesis* 7, (2018).

### 2.1 Research Objective

The main objective of this project is to develop an enzyme-activatable fluorescent-PDT beacon that selectively targets the clinically relevant GST isoforms. Specifically, three different approaches were taken. First, the cis-3-(9H-purin-6-ythio) acrylic acid (PTA) prodrug was used as the GST trigger group, followed by exploration of the keto versions of PTA, trans-6-(2-acetylvinylthio)guanine (tAVTG) and cis-6-(2-Acetylvinylthio)purine (cAVTP), yielding the Class A GST-PDT beacons (*Figure 2.8*). Second, GST’s sulfonamidase activity was exploited with the use of dinitrobenzene sulfonic acid (DNBS), yielding the Class B GST-PDT beacons. Third, GSH or a GSH-derivative was utilized as the probe scaffold, yielding the Class C GST-PDT beacons.

*Figure 2.8*: Chemical structures of the PTA prodrug and the PTA keto versions: cAVTP and tAVTG.

The specific aims of this project are to: design, synthesize, and characterize enzyme-activatable fluorescent-PDT beacons that selectively target GSTA1-1, M1-1, and P1-1; determine absorption and fluorescence changes *in vitro* by conducting human GST enzyme assays and testing in MCF-7 whole-cell lysates; and measure its selectivity for the targeted GST isoform.
2.2 Class A GST-Activatable Fluorescent-PDT Beacons: Prodrug Approach

2.2.1 PTA Prodrug

PTA is a GSH-dependent prodrug that releases the cytotoxic anticancer agent, 6-mercaptopurine (6-MP), upon interaction with GSH (Figure 2.9). This reaction occurs both in vitro and in vivo and can proceed in two different ways, depending on the site where GSH attacks.

Figure 2.9: PTA activation. Pathway one involves an indirect pathway where GSH is conjugated at the C-6 carbon of the purine ring of PTA, which will release 6-MP with the aid of renal γGT dipeptidase and cysteine S-conjugate β lyase. Pathway two involves a direct pathway where PTA acts as a Michael acceptor and is attacked by GSH on the β-carbon of the acrylic acid moiety, subsequently releasing 6-MP. Figure retrieved from Ruzza, P. & Calderan, A. Glutathione Transferase (GST)-Activated Prodrugs. Pharmaceutics 5, 220–231 (2013) (left). Figure retrieved from Gunnarsdottir, S. & Elfarra, A. Glutathione-Dependent Metabolism of Cis-3-(9H-Purin-6-ylthio)acrylic Acid to Yield the Chemotherapeutic Drug 6-Mercaptopurine: Evidence for Two Distinct Mechanisms in Rats. The Journal of Pharmacology and Experimental Therapeutics 3, 950-957 (1999) (right).

If GSH attacks the purine ring at its C-6 carbon, the GSH-conjugate formed—namely, S-9H-purin-6-yl-glutathione (PG)—will be further metabolized by renal γGT dipeptidase and cysteine S-conjugate β lyase to liberate 6-MP, in an indirect way (Figure 2.9). This route occurs non-enzymatically since the introduction of human recombinant GSTs, rat liver homogenate, or rat liver GSTs did not enhance the reaction rate. However, if GSH attacks the β-carbon of the
purine’s acrylic acid moiety, which serves as a Michael acceptor, an addition-elimination reaction occurs to generate 6-MP in a direct way (Figure 2.9). This route occurs both enzymatically and non-enzymatically; the introduction of human recombinant GSTs (GST A1-1, M1-1, P1-1) and rat GSTs enhanced the reaction rate. Despite enzymatic activation, the in vitro and in vivo reaction rate is slow for these GST isoforms. The GST A1-1, M1-1, P1-1 isoforms catalyze the formation of 6-MP by only 1.7, 1.3, and 1.3-fold, respectively.

Despite this, PTA has been shown to be a successful prodrug that can target tumors that display overexpression of GSTs and GSH. Furthermore, 6-MP has been shown to treat acute lymphoblastic leukemia, but its clinical application is limited by its toxicity towards the liver and bone marrow (Figure 2.10). Hence, 6-MP prodrugs such as PTA serve to reduce general toxicity as 6-MP will be activated only in the target location.

**Figure 2.10:** Cellular death caused by 6-MP. This anticancer agent participates in the following pathways to induce cell death: a) 6-MP competes with hypoxanthine for HGPRT and in doing so, it becomes converted to TIMP and
eventually thioguanylic acid, which will be misincorporated into nucleic acids; b) TIMP can also inhibit PRPP amidotransferase, which will prevent the essential de novo pathway for purine ribonucleotide synthesis; and c) 6-MP can inhibit any IMP-related reactions by inactivating HGPRT. Figure retrieved from Avendaño Carmen & Menéndez J. Carlos. Medicinal Chemistry of Anticancer Drugs. (Elsevier Science Ltd, 2015).

2.2.1.1 Design and Synthesis of the PTA-Quencher-Photosensitizer Scaffold

The objective of the proposed scaffold is to obtain selectivity for GST by using the PTA prodrug, which specifically targets the GST A1-1, M1-1, P1-1 isozymes through a nucleophilic addition-elimination reaction (see B in Figure 2.11). 27 The PDT beacon consists of PTA in an amide linkage with a PS through the acrylic amide functional group and in another amide linkage with the corresponding FRET quencher at the N-9 nitrogen on its purine ring. An amide linkage has been shown by Qiu et al. to not affect the conjugation of GSH on this PTA derivative.

First, a Michael addition of GSH to the electrophilic β-carbon of PTA’s acrylic amide moiety occurs, producing a PTA-Q-PS-GSH conjugate. 39 Second, this enolate intermediate collapses to reform the acrylic amide and releases 6-MP-Q from GSH-PS. 39 Hence, this GST-activatable fluorescent PDT beacon switches from the “off” to the “on” state upon GST/GSH mediated activation – resulting in an increase in fluorescence and PDT capability of the PS and the release of a cytotoxic anticancer agent. Light irradiation of the PS would then permit singlet oxygen production and cellular death of cancer cells that overexpress GST.

![Figure 2.11](image)

**Figure 2.11:** Mechanism of GST-catalyzed GSH conjugation on a generic PTA-Quencher-PS scaffold.

After attempting many different synthetic pathways, the seven-step route depicted below showed to be successful in producing the PTA-Q-PS scaffold (Scheme 2.1).
Scheme 2.1: Synthetic scheme to synthesize the generic PTA-Quencher-PS scaffold.

To start, ethylenediamine was mono-protected with a tert-butoxycarbonyl (BOC) protecting group by using boc anhydride. The resulting monoBOC-protected ethylenediamine was then subjected to amide coupling using DCC as the activator with propiolic acid (PA). PA was chosen since it has a triple bond handle that is needed to form the acrylic amide functional group. Next, 6-MP was stirred with the newly synthesized monoBOC-protected PA-conjugated ethylenediamine compound. With the aid of a base such as N,N-diisopropylethylamine (DIPEA) to deprotonate the thiol group of 6-MP, a thiol-yne reaction occurs between the thiol and alkyne; purification by crystallization generated only the cis product. After this addition reaction, a PTA derivative that bears an acrylic amide moiety is produced. Lithium hydride is then added to deprotonate the N-9 position of PTA to facilitate an improved nucleophilic attack on the electrophilic carbon α to the bromine in 3-bromopropylamine hydrobromide. From this, the free amine on this N-9 linker is free to participate in an amide coupling step with any quencher in its N-hydroxysuccinimide (NHS) form. The second last step involves acid-induced deprotection of the BOC group. Trifluoroacetic acid (TFA) was used to achieve this, along with triisopropylsilane (TIS) – which acts as a scavenger to prevent side reactions with reactive t-butyl cations as it is generated in situ. Post-TFA subjection, the BOC-deprotected amine can then be coupled with a PS that bears a suitable functional group to undergo an addition reaction, resulting in the formation of the PTA-Q-PS scaffold.
2.2.1.1.1 Design and Synthesis of Compound 2.1: PTA-BHQ2-Dansyl (AK-01-067)

Figure 2.12: Mechanism of GST-catalyzed GSH conjugation on the PTA-BHQ2-Dansyl scaffold.

To demonstrate proof of concept (POC), a fluorophore was used in place of a PS to see whether this proposed generic scaffold can be successfully activated by GST (Figure 2.12). Given its clinical relevance and well-established role as a therapeutic target—especially in lung, colon, and stomach cancers—the goal was to selectively target the GST P1-1 isoform. Since the H site of the P1-1 class is only moderately open, Dansyl was selected because the small-molecular nature of this fluorophore is hypothesized to minimize any potential steric hindrance to binding at the active site. Moreover, Dansyl should be suitable for the partly hydrophobic environment in the H site as its low molecular weight helps to prevent a significant increase in the overall lipophilicity of the probe – as compared to other bulky, highly-conjugated fluorophores like tetraphenylethene (TPE). Black Hole Quencher 2 (BHQ2) was selected as the FRET quencher.

BHQ2 was synthesized and converted into its NHS form for conjugation at the N9 linker of PTA. For the last step, commercially available Dansyl chloride was stirred with the BOC-deprotected amine scaffold to yield the PTA-BHQ2-Dansyl probe (Scheme 2.2).
Scheme 2.2: Synthetic scheme to synthesize the PTA-BHQ2-Dansyl scaffold.

2.2.1.1.2 Design and Synthesis of Compound 2.2: PTA-BHQ3-Pyropheophorbide A (AK-01-170)

Figure 2.13: Mechanism of GST-catalyzed GSH conjugation on the PTA-BHQ3-Pyropheophorbide A scaffold.

After showing in vitro activation of the proposed generic scaffold (refer to Section 2.2.1.2.1 PTA-BHQ2-Dansyl Assays), a PS was incorporated in the second probe derivative for PDT applications. Pyropheophorbide A was chosen as the FRET acceptor and Black Hole Quencher 3 (BHQ3) as the FRET quencher (Figure 2.13). Given that this PS is bulkier and has greater hydrophobicity than Dansyl, this probe was designed to target GST M1-1 over GST P1-1, which has a H-site that is more hydrophobic and is larger in magnitude.
For the last step, commercially available pyropheophorbide A was pre-stirred with DIPEA and HATU to form a pre-activated ester, which was then reacted with the BOC-deprotected amine scaffold to yield the PTA-BHQ3-Pyro A probe (Scheme 2.3).43

Scheme 2.3 Synthetic scheme to synthesize the PTA-BHQ3-Pyro A scaffold.

2.2.1.1.3 Design and Synthesis of Compound 2.3: PTA-DABCYL-5FAM (AK-01-161)

Since the second probe derivative showed no activation with GST M1-1 (refer to Section 2.2.1.2.2 PTA-BHQ3-Pyro A Assays), a fluorophore that resembles Dansyl rather than pyropheophorbide A should be selected next. But, the PTA-BHQ2-Dansyl probe showed only a 2-fold increase with GST P1-1, which may be due to Dansyl’s low molar extinction coefficient and low fluorescence quantum yield (FQY). To give perspective, Dansyl glycine has a molar extinction coefficient of
4,300 cm\(^{-1}\)/M (measured at 338.5 nm in dioxane) and an FQY of 0.07 (in water).\(^{44}\) Thus, the goal of the third probe derivative is to achieve a greater fold-increase in fluorescence after GST activation. 5-Carboxyfluorescein (5FAM) was chosen as the FRET fluorophore since its molar extinction coefficient of 92,300 cm\(^{-1}\)/M (measured at 482.5nm in ethanol) and FQY of 0.79 (in ethanol) are higher in magnitude compared to Dansyl.\(^{45}\) This means that 5FAM will absorb light more strongly, will be a brighter fluorophore, and will have a stronger fluorescence signal intensity upon separation from the quencher.

DABCYL was incorporated as the FRET quencher of 5FAM (Figure 2.14). For the last step, commercially available 5FAM was pre-stirred with DIPEA and HATU to form a pre-activated ester, which was then reacted with the BOC-deprotected amine scaffold to yield the PTA-DABCYL-5FAM probe (Scheme 2.4).

**Scheme 2.4:** Synthetic scheme to synthesize the PTA-DABCYL-5FAM scaffold.
2.2.1.1.4  Design and Synthesis of Compound 2.4: PTA-BHQ2-5FAM (AK-01-221)

Like the PTA-BHQ2-Dansyl probe, the PTA-DABCYL-5FAM probe also exhibited a small increase in fluorescence post-GST-mediated catalysis; specifically, a 1.13- and 1.89-fold change was observed with GST M1-1 and GST A1-1, respectively (refer to Section 2.2.1.2.3 PTA-DABCYL-5FAM Assays). To enhance the fluorescence fold-change, a more compatible FRET quencher was selected for 5FAM since this will increase the efficiency in percent quenching between the FRET pairs and will diminish the background signal of the probe in its quenched state.

Black Hole Quencher 2 (BHQ2) was selected as the FRET quencher for the fourth probe derivative (Figure 2.15). For the last step, commercially available 5FAM was pre-stirred with DIPEA and HATU to form a pre-activated ester, which was then reacted with the BOC-deprotected amine scaffold to yield the PTA-BHQ2-5FAM probe (Scheme 2.5).
Scheme 2.5: Synthetic scheme to synthesize the PTA-BHQ2-5FAM scaffold.

2.2.1.1.5 Design and Synthesis of Compound 2.5: PTA-DABCYL-7-Coumarin-3 (AK-01-179)

Figure 2.16: Mechanism of GST-catalyzed GSH conjugation on the PTA-DABCYL-7-Coumarin-3 scaffold.

With BHQ2 as the FRET quencher over DABCYL, a 5-fold increase in fluorescence was observed with GST A1-1 due to the enhanced percent quenching efficiency between BHQ2 and 5FAM (refer to Section 2.2.1.2.4 PTA-BHQ2-5FAM Assays). Given that GST A1-1 has a H-site that is hydrophobic and more tunnel-like than the other isoforms, a fluorophore that is smaller in size than 5FAM was incorporated next to assess whether fluorophore size has an impact on substrate recognition and activation by GST A1-1.

This probe uses 7-(Diethylamino)coumarin-3-carboxylic acid (7-Coumarin-3) as the FRET acceptor and DABCYL as the FRET quencher (Figure 2.16). For the last step, commercially
available 7-Coumarin-3 was pre-stirred with DIPEA and HATU to form a pre-activated ester, which was then reacted with the BOC-deprotected amine scaffold to yield the PTA-DABCYL-7-Coumarin-3 probe (Scheme 2.6).

Scheme 2.6: Synthetic scheme to synthesize the PTA-DABCYL-7-Coumarin-3 scaffold.

2.2.1.1.6 Design and Synthesis of Compound 2.6: PTA-DABCYL-7-Coumarin-3 (Extended Linker) (AK-01-217)

Figure 2.17: Mechanism of GST-catalyzed GSH conjugation on the PTA-DABCYL-7-Coumarin-3 (extended linker) scaffold.

A 1.44-fold increase in fluorescence was observed after GST A1-1-mediated catalysis (refer to Section 2.2.1.2.5 PTA-DABCYL-7-Coumarin-3 Assays), which is suboptimal compared to the
PTA-BHQ2-5FAM probe. This may be due to GST A1-1’s poor recognition of the 7-Coumarin-3 fluorophore or 7-Coumarin-3’s low molar extinction coefficient of 24,510 cm⁻¹/M (measured at 408 nm in water) and FQY of 0.03 in water (measured at 410 nm). Next, the distance between the FRET pairs was lengthened by incorporating glycine to extend the ethylenediamine linker. Perhaps the FRET acceptor on the original chemical scaffold was too close in proximity with PTA, which may have increased the difficulty for conjugation of GSH on the acrylic acid moiety at the active site.

The sixth probe derivative consists of the same FRET pair: 7-(Diethylamino)coumarin-3-carboxylic acid (7-Coumarin-3) as the FRET acceptor and DABCYL as the FRET quencher (Figure 2.17). For the last step, a pre-made 7-Coumarin-3 glycine derivative was pre-stirred with DIPEA and HATU to form a pre-activated ester, which was then reacted with the BOC-deprotected amine scaffold to yield the PTA-DABCYL-7-Coumarin-3 (extended linker) probe (Scheme 2.7).

**Scheme 2.7**: Synthetic scheme to synthesize the PTA-DABCYL-7-Coumarin-3 (extended linker) scaffold.
2.2.1.1.7 Design and Synthesis of Compound 2.7: PTA-DABCYL-Coumarin343 (AK-01-132)

**Figure 2.18:** Mechanism of GST-catalyzed GSH conjugation on the PTA-DABCYL-Coumarin 343 scaffold.

Given that the extended linker version of the PTA-DABCYL-7-Coumarin-3 probe showed no significant fluorescence enhancement (refer to Section 2.2.1.2.6 PTA-DABCYL-7-Coumarin-3 (Extended Linker) Assays), linker length was no longer explored.

For the seventh probe derivative, Coumarin 343 was chosen to evaluate the effect of using different Coumarin derivatives as the FRET acceptor. This probe consists of Coumarin 343 as the FRET acceptor and DABCYL as the FRET quencher (Figure 2.18). For the last step, commercially available Coumarin343 was pre-stirred with DIPEA and HATU to form a pre-activated ester, which was then reacted with the BOC-deprotected amine scaffold to yield the PTA-DABCYL-Coumarin343 probe (Scheme 2.8). A 2.3-fold increase in fluorescence was observed with GST M1-1 (refer to Section 2.2.1.2.7 PTA-DABCYL-Coumarin343 Assays); however, the GST A1-1 isoform still needs to be tested in order to have a direct comparison with the 7-Coumarin-3 probe. Nevertheless, the PTA-BHQ2-5FAM probe seems to have the highest fluorescence fold-change thus far.
2.2.1.1.8 Design and Synthesis of Compound 2.8: PTA-BHQ2-RB (In-Progress)

Given the 5-fold fluorescence change seen with PTA-BHQ2-5FAM, the goal for the last probe derivative was to incorporate a PS in place of 5FAM to see whether the fold-change can still be maintained. Rose Bengal was chosen as the FRET acceptor and BHQ2 as the FRET quencher; note that RB is less bulky and less hydrophobic than pyropheophorbide A (Figure 2.19).

For the last step, commercially available Rose Bengal was pre-stirred with DIPEA and HATU to form a pre-activated ester, which was then reacted with the BOC-deprotected amine scaffold to yield the PTA-BHQ2-RB probe (Scheme 2.9).
2.2.1.2 In Vitro Assays

2.2.1.2.1 PTA-BHQ2-Dansyl Assays

The UV-Vis absorption spectrum shows that Dansyl has an $\lambda_{\text{max}}$ at 315 nm and BHQ2 has an $\lambda_{\text{max}}$ at 571 nm (Figure 2.20). Next, in vitro GST enzyme assays were used to characterize the PTA-BHQ2-Dansyl probe. Fluorescence spectra were acquired pre- and post-GST P1-1 mediated conjugation of GSH to the probe; with an excitation wavelength of 320 nm, an increase in emission at 500 nm was observed with 77 nM GST P1-1/GSH at 2-fold (Figure 2.21). This corresponds to the separation of Dansyl from its FRET quencher, BHQ2, after its interaction with GST/GSH, allowing Dansyl’s fluorescence to switch from “off” to “on.” Moreover, the addition of 213 nM GST M1-1/GSH resulted in a 1.8-fold increase in fluorescence emission over a period of 116 minutes; fluorescence spectra were also acquired pre- and post-GST M1-1-mediated conjugation of GSH to the probe (Figure 2.22).
Figure 2.20: Absorption spectrum of the PTA-BHQ2-Dansyl probe. Dansyl has an $\lambda_{\text{max}}$ at 315 nm and BHQ2 has an $\lambda_{\text{max}}$ at 571 nm. Conditions: 15 µM probe. The total DMSO concentration was 1% and the buffer used was PBS.

Figure 2.21: GST P1-1 mediated GSH conjugation on the PTA-BHQ2-Dansyl probe. Conditions: a) before spectrum (light orange): 15 µM probe, 1 mM GSH, 77 nM GST P1-1, immediately at room temperature; and b) after spectrum (maroon): 15 µM probe, 1 mM GSH, 77 nM GST P1-1, 24 hours at 37°C (excitation 320 nm, emission 500 nm). The total DMSO concentration was 1% and the buffer used was PBS.

Figure 2.22: GST M1-1 mediated GSH conjugation on the PTA-BHQ2-Dansyl probe. Conditions: a) before spectrum (light orange): 96 nM probe, at 37°C; and b) after spectrum (maroon): 96 nM probe, 2 mM GSH, 213 nM GST M1-
1, after time course at 37°C (excitation 320 nm, emission 500 nm) (left). Time course conditions: 96 nM probe, 2 mM GSH, 213 nM GST- M1-1, at 37°C (excitation 320 nm, emission 500 nm). The total DMSO concentration was 1% and the buffer used was PBS (right).

In Figure 2.21, there seems to be an extra emission band visible at 390 nm, which is not present in Figure 2.22; this may have resulted from an impurity present in the cuvette since the same probe stock was used to conduct both experiments.

Nonetheless, there was a minimal fold-change in fluorescence emission observed with GST/GSH activation. Therefore, the PTA-BHQ2-Dansyl probe was not pursued further. However, the following can be done in the future to further our understanding of this probe derivative: a negative control fluorescence assay with GSH and no enzyme; a fluorescence assay with the A1-1 isoform to see whether it has a greater effect on fluorescence fold-change or activation kinetics; a MS or HPLC experiment to confirm probe cleavage into the Dansyl-GSH product; and synthesis of a PTA-BHQ0-Dansyl probe since BHQ-0 (λ_{max} 495 nm) acts as a better FRET quencher to Dansyl (λ_{max} 315 nm) than BHQ2 (λ_{max} 571 nm).

2.2.1.2.2 PTA-BHQ3-Pyropheophorbide A Assays

The UV-Vis absorption spectrum shows that pyropheophorbide A has an λ_{max} at 428 nm and 685 nm and BHQ3 has an λ_{max} at 680 nm (Figure 2.23). Fluorescence spectra were acquired pre- and post-GST M1-1-mediated conjugation of GSH to the probe; with an excitation wavelength of 415 nm, an increase in emission at 678 nm was expected with GST M1-1. This would have corresponded to the separation of pyropheophorbide A from its FRET quencher, BHQ3, after its interaction with GST/GSH, allowing pyropheophorbide A’s fluorescence to switch from “off” to “on.” However, a decrease in fluorescence was observed with 300 nM GST M1-1; the time course was also included (Figure 2.24). Either the product, Pyropheophorbide A-GSH, was not generated or an unexpected product had formed.
Figure 2.23: Absorption spectrum of the PTA-BHQ3-Pyropheophorbide A probe. Pyropheophorbide A has an $\lambda_{\text{max}}$ at 428 nm and BHQ3 has an $\lambda_{\text{max}}$ at 680 nm. Conditions: 1.94 $\mu$M probe. The total DMSO concentration was 1% and the buffer used was PBS.

Figure 2.24: GST M1-1 mediated GSH conjugation on the PTA-BHQ3-Pyropheophorbide A probe. Conditions: a) before spectrum (maroon): 1.94 $\mu$M probe, at 37°C; and b) after spectrum (light orange): 1.94 $\mu$M probe, 2 mM GSH, 300 nM GST M1-1, after time course at 37°C (left). Time course conditions: 1.94 $\mu$M probe, 2 mM GSH, 300 nM GST M1-1, at 37°C (right). Excitation at 415 nm and emission at 678 nm. The total DMSO concentration was 1% and the buffer used was PBS.

Repetition at a lower probe concentration (from 1.94 $\mu$M to 0.19 $\mu$M) was conducted to see whether the hydrophobicity and large planar structure of the PS was causing aggregation-induced quenching (AIQ) from potential $\pi-\pi$ stacking; a decrease in fluorescence was observed with 300 nM GST M1-1 (Figure 2.25). Moreover, this phenomenon was also seen for the following stability test experiments on the probe: reduction of the temperature from 37°C to 25°C and/or addition of BSA (Figure 2.26); incubation in the water bath overnight at 37°C and/or addition of GSH (Figure 2.27); and free PS subjected to overnight water bath incubation at 37°C (Figure 2.28). From these results, my hypothesis is that the hydrophobicity of the probe causes its aggregation in phosphate
buffer, which occurs even at reduced concentrations, due to the hydrophobic nature of the pyropheophorbide A PS, as supported by Delanaye et al.\textsuperscript{44}

**Figure 2.25:** GST M1-1 mediated GSH conjugation on the PTA-BHQ3-Pyropheophorbide A probe (lower probe concentration). Conditions: a) before spectrum (light orange): 0.19 μM probe, at 37°C; and b) after spectrum (maroon): 0.19 μM probe, 2 mM GSH, 300 nM GST M1-1, after time course at 37°C (left). Time course conditions: 0.19 μM probe, 2 mM GSH, 300 nM GST M1-1, at 37°C (right). Excitation at 415 nm and emission at 678 nm. The total DMSO concentration was 1% and the buffer used was PBS.

**Figure 2.26:** Stability test on the PTA-BHQ3-Pyropheophorbide A probe (RT, lower probe concentration). Time course conditions: 0.19 μM probe, at room temperature (left). (BSA addition) Time course conditions: 0.19 μM probe, 0.5 μM BSA, at room temperature (right). Excitation at 415 nm and emission at 678 nm. The total DMSO concentration was 1% and the buffer used was PBS.
Figure 2.27: Stability test on the PTA-BHQ3-Pyropheophorbide A probe (37°C, lower probe concentration). Conditions: a) before spectrum (light orange): 0.19 μM probe, at 37°C; and b) after spectrum (maroon): 0.19 μM probe, overnight in water bath at 37°C (left). (GSH addition) Conditions: a) before spectrum (maroon): 0.19 μM probe, 2 mM GSH, at 37°C; and b) after spectrum (grey): 0.19 μM probe, 2 mM GSH, overnight in water bath at 37°C (right). Excitation at 415 nm and emission at 678 nm. The total DMSO concentration was 1% and the buffer used was PBS.

Figure 2.28: Stability test on the free pyropheophorbide A dye (37°C, lower probe concentration). Conditions: a) before spectrum (maroon): 0.19 μM free dye, at 37°C; and b) after spectrum (grey): 0.19 μM free dye, overnight in water bath at 37°C (excitation 415 nm, emission 678 nm). The total DMSO concentration was 1% and the buffer used was PBS.

Given the lack of change in fluorescence emission with GST M1-1/GSH, the PTA-BHQ2-Pyropheophorbide A probe was not pursued further. Compared to the PTA-BHQ2-Dansyl probe, it appears that the bulky nature of this PS may be causing recognition or binding problems with GST. The PSs Protoporphyrin IX ($\lambda_{\text{max}}$ 633 nm) or Tetraphenylporphyrin (TPP) ($\lambda_{\text{max}}$ 653 nm) can be tested as well – with BHQ3 ($\lambda_{\text{max}}$ 680 nm) as the FRET quencher to determine whether it is a recognition problem with pyropheophorbide A. Other less bulky PSs can also be tested to further assess the effect of PS size on substrate binding at the GST active site. Furthermore, a scaffold
with greater hydrophilicity should be considered as it would help to combat potential AIQ problems that may be contributing to the lack of activation of this probe.

### 2.2.1.2.3 PTA-DABCYL-5FAM Assays

The UV-Vis absorption spectrum shows that 5FAM has an $\lambda_{\text{max}}$ at 492 nm and DABCYL has an $\lambda_{\text{max}}$ at 454 nm (Figure 2.29). A fluorescence time course was acquired throughout the GST-mediated catalysis; with an excitation wavelength of 496 nm, an increase in emission at 520 nm was observed with GST A1-1 and M1-1. This corresponds to the separation of 5FAM from its FRET quencher, DABCYL, allowing 5FAM’s fluorescence to switch from “off” to “on.” A 1.13-fold increase in fluorescence was observed over 60 minutes with GST M1-1/GSH at 1 μM probe; this was repeated at 0.178 μM and 0.089 μM probe concentrations (Figure 2.30). Moreover, a 1.86-fold increase in fluorescence was observed with GST A1-1/GSH over 250 minutes at 10 μM probe (Figure 2.31).

**Figure 2.29:** Absorption spectrum of the PTA-DABCYL-5FAM probe. 5FAM has an $\lambda_{\text{max}}$ at 492 nm and DABCYL has an $\lambda_{\text{max}}$ at 454 nm. Conditions: 1 μM probe. The total DMSO concentration was 1% and the buffer used was PBS.

**Figure 2.30:** GST M1-1 mediated GSH conjugation on the PTA-DABCYL-5FAM probe at various concentrations. Time course conditions: 1 μM probe, 2 mM GSH, 300 nM GST M1-1, at 37°C (left). Time course conditions: 0.178
μM probe, 2 mM GSH, 300 nM GST M1-1, at 37°C (middle). Time course conditions: 0.089 μM probe, 2 mM GSH, 300 nM GST M1-1, at 37°C; the probe photobleaches (right). Excitation at 496 nm and emission at 520 nm. The total DMSO concentration was 1% and the buffer used was PBS.

**Figure 2.31:** GST A1-1 mediated GSH conjugation on the PTA-DABCYL-5FAM probe at a higher probe concentration. Time course conditions: 10 μM probe, 2 mM GSH, 231 nM GST A1-1, at 37°C (excitation 496 nm, emission 520 nm). The total DMSO concentration was 1% and the buffer used was PBS.

With a minimal fold-change in fluorescence observed with GST/GSH activation, the PTA-DABCYL-5FAM probe was not pursued further. Given that this probe derivative was activated by GST, it seems that 5FAM may be suitable to use; the effect of FRET efficiency on fold-changes in fluorescence can be explored using a better FRET quencher. This can be done by synthesizing a PTA-BHQ1-5FAM probe on the same chemical scaffold since BHQ-1 (λ_max 534 nm) has a higher percent quenching efficiency to 5FAM (λ_max 492 nm) than DABCYL (λ_max 454 nm), at the same distance apart (**Figure 2.32**).  

**Figure 2.32:** Percent quenching for 5FAM with various quenchers. Black bars represent contact quenching whereas the stripped bars represent FRET quenching. Figure retrieved from Johansson, M. K. Choosing Reporter-Quencher Pairs for Efficient Quenching Through Formation of Intramolecular Dimers. *Fluorescent Energy Transfer Nucleic Acid Probes* 17–30 doi:10.1385/1-59745-069-3:17.
In the future, a negative control fluorescence assay with GSH alone and a fluorescence assay with the A1-1 and P1-1 isoforms can be done.

2.2.1.2.4 PTA-BHQ2-5FAM Assays

The UV-Vis absorption spectrum shows that 5FAM has an $\lambda_{\text{max}}$ at 492 nm and BHQ2 has an $\lambda_{\text{max}}$ at 571 nm (Figure 2.33). A fluorescence time course was acquired throughout the GST A1-1-mediated conjugation of GSH to the probe; with an excitation wavelength of 492 nm, an increase in emission at 520 nm was observed with GST A1-1. This corresponds to the separation of 5FAM from its FRET quencher, BHQ2, allowing 5FAM’s fluorescence to switch from “off” to “on.” A 5-fold increase in fluorescence was observed over 400 minutes with 231 nM GST A1-1 at 10 μM probe (Figure 2.34).

![Absorption Spectrum](image)

**Figure 2.33**: Absorption spectrum of the PTA-BHQ2-5FAM probe. 5FAM has an $\lambda_{\text{max}}$ at 492 nm and BHQ2 has an $\lambda_{\text{max}}$ at 571 nm. Conditions: 1 μM probe. The total DMSO concentration was 1% and the buffer used was PBS.
Given the less than 10-fold-change in fluorescence emission observed with GST/GSH activation, the PTA-BHQ2-5FAM probe was not pursued further *in cellulo*; however, it showed the highest fold-increase in fluorescence among all the probe derivatives. The following can be done in the future: a negative control fluorescence assay with GSH alone and a fluorescence assay with the A1-1 and P1-1 isoforms to see whether it has a greater effect on fluorescence fold-change or activation kinetics. Replacement of DABCYL with BHQ2 exhibited an enhancement in the fluorescence fold-change. This resulted from the higher percent quenching efficiency between the new FRET pair, which helped to minimize the background signal seen in the quenched probe state; thus, the PTA-BHQ1-5FAM probe should be synthesized for *in vitro* characterization as the next iteration for the 5FAM series.

### 2.2.1.2.5 PTA-DABCYL-7-Coumarin-3 Assays

The UV-Vis absorption spectrum shows that 7-Coumarin-3 has an $\lambda_{\text{max}}$ at 409 nm and DABCYL has an $\lambda_{\text{max}}$ at 454 nm (**Figure 2.35**). Fluorescence spectra pre- and post-GST M1-1-mediated conjugation of GSH to the probe was acquired; with an excitation wavelength of 409 nm, an increase in emission at 473 nm was observed with GST M1-1. This corresponds to the separation of 7-Coumarin-3 from its FRET quencher, DABCYL, after its interaction with GST/GSH, allowing 7-Coumarin-3’s fluorescence to switch from “off” to “on.” A 2.75-fold increase in fluorescence was observed over 300 minutes with 77 nM GST M1-1/GSH at 10 μM probe (**Figure 2.36**). Next, a GST M1-1 fluorescence time course was conducted at 1 μM probe with and without 77 nM GST M1-1/GSH to assess the effect of absence of enzyme; a plateau of 250 minutes versus 500 minutes was observed, respectively (**Figure 2.37**). Lastly, a fluorescence time course with 231 nM GST A1-1/GSH at 10 μM probe showed a 1.44-fold increase in fluorescence (**Figure 2.38**).
Figure 2.35: Absorption spectrum of the PTA-DABCYL-7-Coumarin-3 probe. 7-Coumarin-3 has an $\lambda_{\text{max}}$ at 409 nm and DABCYL has an $\lambda_{\text{max}}$ at 454 nm. Conditions: 1 $\mu$M probe. The total DMSO concentration was 1% and the buffer used was PBS.

Figure 2.36: GST M1-1 mediated GSH conjugation on the PTA-DABCYL-7-Coumarin-3. Conditions: a) before spectrum (light orange): 10 $\mu$M probe, at 37°C; and b) after spectrum (light purple): 10 $\mu$M probe, 2 mM GSH, 77 nM GST M1-1, after time course at 37°C (left). Time course conditions: 10 $\mu$M probe, 2 mM GSH, 77 nM GST M1-1, at 37°C (right). Excitation at 409 nm and emission at 473 nm. The total DMSO concentration was 1% and the buffer used was PBS.
Figure 2.37: GST M1-1 mediated GSH conjugation on the PTA-DABCYL-7-Coumarin-3 probe (lower probe concentration). Time course conditions: 1 μM probe, 2 mM GSH, 77 nM GST M1-1, at 37°C (left). Time course conditions: 1 μM probe, 2 mM GSH, at 37°C (right). Excitation at 409 nm and emission at 473 nm. The total DMSO concentration was 1% and the buffer used was PBS.

Without addition of GST M1-1, the fluorescence time course plateaued at 500 minutes as compared to 250 minutes, showing that this signal is real as enzyme catalysis increased the kinetics of probe activation.

Figure 2.38: GST A1-1 mediated GSH conjugation on the PTA-DABCYL-7-Coumarin-3 probe; the probe photobleaches over time. Time course conditions: 10 μM probe, 2 mM GSH, 231 nM GST A1-1, at 37°C (excitation 409 nm, emission 473 nm). The total DMSO concentration was 1% and the buffer used was PBS.

A fluorescence assay was also attempted in MCF-7 whole-cell lysates, which resulted in a 1.73-fold increase in fluorescence (Figure 2.39). This assay should be repeated to verify that the drastic increase in fluorescence at around 100 minutes is a real signal.
Figure 2.39: GST mediated GSH conjugation on the PTA-DABCYL-7-Coumarin-3 probe in MCF7 whole-cell lysates. Time course conditions: 10 μM probe, 48 μg total protein content, lysis buffer (10 mM HEPES at pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol with 5% glycerol and 1x protease inhibitor), at 37°C (excitation 409 nm, emission 473 nm). The total DMSO concentration was 1%.

Given the minimal fold-change in fluorescence emission observed with GST/GSH activation, the PTA-DABCYL-7-Coumarin-3 probe was not pursued further. However, in the future, fluorescence assays with the A1-1 and P1-1 isoforms can be done to see whether it has a greater effect on fluorescence fold-change or activation kinetics. Linker length will be explored next to see whether an extended distance between the FRET pairs has an impact on fluorescence fold-change or binding at the GST active site.

2.2.1.2.6 PTA-DABCYL-7-Coumarin-3 (Extended Linker) Assays

The UV-Vis absorption spectrum shows that 7-Coumarin-3 has an $\lambda_{\text{max}}$ at 409 nm and DABCYL has an $\lambda_{\text{max}}$ at 454 nm (Figure 2.40). A GST A1-1 fluorescence time course was acquired; with an excitation wavelength at 409 nm, an increase in emission at 473 nm was observed. This corresponds to the separation of 7-Coumarin-3 from its FRET quencher, DABCYL, after its interaction with GST/GSH, allowing 7-Coumarin-3’s fluorescence to switch from “off” to “on.” A 2.11-fold increase in fluorescence emission was seen over 400 minutes with 231 nM GST M1-1/GSH at 2.7 μM probe (Figure 2.41).
Figure 2.40: Absorption spectrum of the PTA-DABCYL-7-Coumarin-3 probe (extended linker). 7-Coumarin-3 has an $\lambda_{\text{max}}$ at 409 nm and DABCYL has an $\lambda_{\text{max}}$ at 454 nm. Conditions: 2.7 $\mu$M probe. The total DMSO concentration was 1% and the buffer used was PBS.

Given the minimal fold-change in fluorescence emission observed with GST/GSH activation, the PTA-DABCYL-7-Coumarin-3 (extended linker) probe was not pursued further. The purpose of extending the linker length was to see whether it plays a role in enhancing GST’s recognition of the scaffold by minimizing potential steric hindrance at the active site. Based on the results above, an extension of three atoms using glycine did not significantly increase the overall fluorescence fold-change or kinetics of GST activation. However, the following can be done in the future: a control assay with GSH alone; fluorescence assays with the P1-1 isoform to see whether it has a greater effect on fluorescence fold-change or activation kinetics; and synthesis of a derivative with at least a six-nine atom extension to further explore the role of linker length. A scaffold with a decreased FRET distance between the FRET pairs can be also investigated for an effect on binding and fluorescence fold-change.

Figure 2.41: GST A1-1 mediated GSH conjugation on the PTA-DABCYL-7-Coumarin-3 probe (extended linker). Time course conditions: 2.7 $\mu$M probe, 2 mM GSH, 231 nM GST A1-1, at 37°C (excitation 409 nm, emission 473 nm). The total DMSO concentration was 1% and the buffer used was PBS.
2.2.1.2.7 PTA-DABCYL-Coumarin343 Assays

The UV-Vis absorption spectrum shows that Coumarin343 has an $\lambda_{\text{max}}$ at 438 nm and DABCYL has an $\lambda_{\text{max}}$ at 454 nm (Figure 2.42). Fluorescence spectra pre- and post-GST M1-1-mediated conjugation of GSH to the probe was acquired; with an excitation wavelength of 438 nm, an increase in emission at 493 nm was observed. This corresponds to the separation of Coumarin343 from its FRET quencher, DABCYL, after its interaction with GST/GSH, allowing Coumarin343’s fluorescence to switch from “off” to “on.” A 2.3-fold increase in fluorescence emission was observed with 300 nM GST M1-1/GSH at 89 nM probe over 167 minutes (Figure 2.43). Furthermore, a fluorescence time course was conducted at 77 nM and 154 nM GST M1-1 (Figure 2.44); no enzyme control, no enzyme/GSH control, and no GSH control (Figure 2.45); 44.5 nM probe (1.89-fold fluorescence increase with 300 nM GST M1-1 over 250 minutes) (Figure 2.46); a shutter test to minimize photobleaching of the probe (3.14-fold fluorescence increase with 300 nM GST M1-1 over 385 minutes) (Figure 2.47); and at 300 nM GST P1-1 (Figure 2.48).

Figure 2.42: Absorption spectrum of the PTA-DABCYL-Coumarin343 probe. Coumarin343 has an $\lambda_{\text{max}}$ at 438 nm and DABCYL has an $\lambda_{\text{max}}$ at 454 nm. Conditions: 89 nM probe. The total DMSO concentration was 1% and the buffer used was PBS.

Figure 2.43: GST M1-1 mediated GSH conjugation on the PTA-DABCYL-Coumarin343 probe. Conditions: a) before spectrum (light orange): 89 nM probe, at 37°C; and b) after spectrum (maroon): 89 nM probe, 2 mM GSH, 300 nM
GST M1-1, after time course at 37°C (left). Time course conditions: 89 nM probe, 2 mM GSH, 300 nM GST M1-1, at 37°C (excitation 438 nm, emission 493 nm) (right). The total DMSO concentration was 1% and the buffer used was PBS.

**Figure 2.44:** GST M1-1 mediated GSH conjugation on the PTA-DABCYL-Coumarin343 probe (lower enzyme concentrations). Time course conditions: 89 nM probe, 2 mM GSH, 77 nM GST M1-1, at 37°C (left). Time course conditions: 89 nM probe, 2 mM GSH, 154 nM GST M1-1, at 37°C (right). Excitation at 438 nm and emission at 493 nm. The total DMSO concentration was 1% and the buffer used was PBS.

**Figure 2.45:** Controls for the PTA-DABCYL-Coumarin343 probe. Time course conditions: 89 nM probe, 2 mM GSH, at 37°C (left). Time course conditions: 89 nM probe, at 37°C (middle). Time course conditions: 89 nM probe, 300 nM GST M1-1 at 37°C (right). Excitation at 438 nm and emission at 493 nm. The total DMSO concentration was 1% and the buffer used was PBS.
Figure 2.46: Analysis of kinetic rates of GST on the PTA-DABCYL-Coumarin343 probe.

No significant rate enhancement was observed when GST M1-1 was increased from 77 nM to 154 nM; for quantitative comparison, their corresponding slopes were calculated to be 0.0027 and 0.0028 (Figure 2.46). The slope increased to 0.0055 with addition of 300 nM GST M1-1 (Figure 2.46). When GST M1-1 was absent, the GSH control exhibited the slowest kinetics; the slope was calculated to be 0.0014 (Figure 2.46).

Figure 2.47: GST M1-1 mediated GSH conjugation on the PTA-DABCYL-Coumarin343 probe (lower probe concentration). Time course conditions: 44.5 nM probe, 2 mM GSH, 300 nM GST M1-1, at 37°C (excitation 438 nm, emission 493 nm). The total DMSO concentration was 1% and the buffer used was PBS.

When the probe concentration was reduced from 89 nM to 44.5 nM, the fold-increase in fluorescence also reduced from 2.3-fold to 1.89-fold.
Figure 2.48: GST M1-1 mediated GSH conjugation on the PTA-DABCYL-Coumarin343 probe (shutter test). Conditions: a) before spectrum (light orange): 89 nM probe, at 37°C; b) spectrum after 385 minutes (grey): 89 nM probe, 2 mM GSH, 300 nM GST M1-1, after time course at 37°C; and c) spectrum after 800 minutes (maroon): 89 nM probe, 2 mM GSH, 300 nM GST M1-1, after time course at 37°C (excitation 438 nm, emission 493 nm). The total DMSO concentration was 1% and the buffer used was PBS.

A shutter assay was conducted to assess whether reduction in the exposure of light would prevent or minimize the rate in which the probe was photobleaching over time. In Figure 2.48, a 3.14-fold fluorescence increase was observed with 300 nM GST M1-1 over 385 minutes as compared to the 2.3-fold seen without the use of a shutter.

Figure 2.49: GST P1-1 mediated GSH conjugation on the PTA-DABCYL-Coumarin343 probe. Time course conditions: 89 nM probe, 2 mM GSH, 300 nM GST P1-1, at 37°C (excitation 438 nm, emission 493 nm). The total DMSO concentration was 1% and the buffer used was PBS.

Figure 2.49 shows that the probe is not selective for the GST P1-1 isoform.

Since replacement of 7-Coumarin-3 to Coumarin343 did not significantly enhance the fluorescence fold-change, this probe derivative was not investigated further. Although
photobleaching was observed for the PTA-DABCYL-5FAM, PTA-DABCYL-7-Coumarin3, and PTA-DABCYL-Coumarin343 probe derivatives; this may, however, be advantageous during PDT in preventing over-treatment as any remaining PS can be eliminated during light illumination.

2.2.2 Keto-Versions of PTA Prodrug: tAVTG and cAVTP

Trans-6-(2-acetylvinylthio)guanine (tAVTG) and cis-6-(2-acetylvinylthio)purine (cAVTP) are ketone versions of the PTA prodrug that have an α, β-unsaturated ketone (Figure 2.50).

![Figure 2.50: Chemical structures of the tAVTG and cAVTP prodrugs. Figure retrieved from Ruzza, P. & Calderan, A. Glutathione Transferase (GST)-Activated Prodrugs. Pharmaceutics 5, 220–231 (2013).](image)

Upon GST-catalyzed GSH conjugation at their butenone moiety, tAVTG and cAVTP will release their cytotoxic anticancer agents, 6-thioguanine (6-TG) and 6-MP, which are widely used to treat acute lymphoblastic leukemia (ALL), respectively (Figure 2.51). Subsequently, hypoxanthine guanine phosphoribosyltransferase (HPGRT) will convert 6-TG and 6-MP into 6-thioguanosine monophosphate (6-TGMP) and ribonucleotides 6-thioinosine monophosphate (6-TIMP), which are antimetabolites that causes cancer cell death by inhibiting the de novo purine synthesis pathway (Figure 2.52).
Figure 2.51: Mechanism of GST-catalyzed GSH conjugation on the cAVTP prodrug. After addition of GSH on the butanone moiety, mono- and bis-glutathionyl derivatives are formed. Figure retrieved from Eklund, B. I., Gunnarsdottir, S., Elfarra, A. A. & Mannervik, B. Human Glutathione Transferases Catalyzing the Bioactivation of Anticancer Thiopurine Prodrugs. *Biochemical Pharmacology* 73, 1829–1841 (2007).

Figure 2.52: Metabolism of the cAVTP and tAVTG prodrugs by GST. Figure retrieved from Eklund, B. I., Gunnarsdottir, S., Elfarra, A. A. & Mannervik, B. Human Glutathione Transferases Catalyzing the Bioactivation of Anticancer Thiopurine Prodrugs. *Biochemical Pharmacology* 73, 1829–1841 (2007).

The GST isoforms that exhibit the highest activity towards tAVTG are GST M1-1 and GST A4-4 whereas GST M1-1 and GST M2-2 have the highest activity towards cAVTP (Figure 2.53). GST
M1-1 is found to be overexpressed in tumor cells that have acquired resistance to nitrogen mustards.\textsuperscript{27} Although GST M1-1 interacts well with both tAVTG and cAVTP, this enzyme is polymorphic and present in only about half of the world’s population (Figure 2.53).\textsuperscript{27} Conversely, GST P1-1, a well-established cancer biomarker, only minimally interacts with tAVTG and cAVTP (Figure 2.53).\textsuperscript{27}

<table>
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<tr>
<th>GST</th>
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<th>tAVTG</th>
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<td>A1-1</td>
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<td>1.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>A2-2</td>
<td>0.2 ± 0.04</td>
<td>0.5 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>A3-3</td>
<td>0.4 ± 0.03</td>
<td>0.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>A4-4</td>
<td>1.7 ± 0.10</td>
<td>55.3 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>M1-1</td>
<td>30.8 ± 1.0</td>
<td>23.6 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>M2-2</td>
<td>10.2 ± 0.3</td>
<td>0.5 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>M4-4</td>
<td>1.2 ± 0.03</td>
<td>8.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>M5-5</td>
<td>3.4 ± 0.1</td>
<td>9.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>O1-1</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>K1-1</td>
<td>nd</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>P1-1</td>
<td>nd</td>
<td>0.3 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>T1-1</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Z1-1</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data represents means (S.D.) of assays conducted in triplicate; nd designates non detectable.

\textbf{Figure 2.53}: Specific activity of different GST isoforms on the cAVTP and tAVTG prodrugs. Figure retrieved from Eklund, B. I., Gunnarsdottir, S., Elfarra, A. A. & Mannervik, B. Human Glutathione Transferases Catalyzing the Bioactivation of Anticancer Thiopurine Prodrugs. *Biochemical Pharmacology* 73, 1829–1841 (2007).

These ketone prodrug analogs are advantageous over PTA since PTA is too stable and only releases 10\% of 6-MP after 18 hours with 15 equivalents (15 mM) of GSH (Figure 2.54).\textsuperscript{50} At the same concentration as PTA, the cAVTP version releases 86\% of 6-MP after 135 minutes with 1 equivalent (1 mM) of GSH and 100\% of 6-MP after 30 minutes with 5 equivalents (5 mM) of GSH (Figure 2.54).\textsuperscript{50} Overall, the rate of anticancer drug release from the cAVTP prodrug is higher than the PTA prodrug.
Figure 2.54: Stability of PTA versus cAVTP. The PTA prodrug is too stable as it releases only 10% of 6-MP after 18 hours whereas cAVTP releases 86% of 6-MP after 135 minutes (with 1 mM of GSH, triangle) and 100% of 6-MP after 30 minutes (with 5 mM of GSH) (triangle). Figure retrieved from Falciani, C. et al. Design and In Vitro Evaluation of Branched Peptide Conjugates: Turning Nonspecific Cytotoxic Drugs into Tumor-Selective Agents. *ChemMedChem* **5**, 567–574 (2010).

### 2.2.2.1 Design and Synthesis of tAVTG-Quencher-Photosensitizer Scaffold

Alongside the attempt in increasing the degree of FRET quenching, the objective of the proposed scaffold is to obtain better kinetic activity for GST by using the tAVTG prodrug, which specifically targets the GST M1-1 and GST A4-4 isozymes through a nucleophilic addition-elimination reaction (Figure 2.51).\(^{50}\) The activation of tAVTG is the same as that of the cAVTP prodrug. The PDT beacon consists of tAVTG in a ketone linkage with a PS through the butenone functional group and in an amide linkage with the corresponding FRET quencher at the N-9 nitrogen on its purine ring. A ketone linkage has been shown by Eklund *et al.* to be more efficiently catalyzed by GST than the ester linkage found in GST-PTA prodrugs.\(^{50}\)

First, a Michael addition of GSH to the electrophilic β-carbon of tAVTG’s butenone moiety occurs, producing a tAVTG-Q-PS-GSH conjugate (Figure 2.55).\(^{50}\) Second, this enolate intermediate collapses to reform butenone and releases 6-TG-Q from GSH-PS (Figure 2.55).\(^{50}\) Hence, this GST-activatable fluorescent PDT beacon switches from the “off” to the “on” state upon GST/GSH mediated activation – resulting in an increase in fluorescence and PDT capability of the PS and the release of a cytotoxic anticancer agent. Light irradiation of the PS would then permit singlet oxygen production and cellular death of cancer cells that overexpress GST.
Figure 2.55: Mechanism of GST-catalyzed GSH conjugation on a generic tAVTG-Quencher-PS scaffold.

The following eight-step route depicted below was successful in producing the tAVTG-Q-PS scaffold (Scheme 2.10). The scheme is similar to that of PTA-Q-PS, but 6-TG will be used instead of 6-MP and the synthesis of the linker differs (Scheme 2.10).

Scheme 2.10: Synthetic scheme to synthesize the generic tAVTG-Quencher-PS scaffold.

2.2.2.1.1 Design and Synthesis of Compound 2.9: tAVTG-BHQ2-5FAM (AK-01-241)

This probe consists of 5FAM as the FRET acceptor and BHQ2 as the FRET quencher since this FRET pair exhibited the highest fold-increase in fluorescence in the PTA-BHQ2-5FAM probe, as compared to the other PTA-based probe derivatives (Figure 2.56 and refer to section 2.2.1.2.4. PTA-BHQ2-5FAM Assays). The goal of the tAVTG-BHQ-5FAM probe is to maintain the 5-fold fluorescence change while enhancing the kinetics probe activation by GST.
**Figure 2.56:** Mechanism of GST-catalyzed GSH conjugation on the tAVTG-BHQ2-5FAM scaffold.

**Scheme 2.11:** Synthetic scheme to synthesize the tAVTG-BHQ2-5FAM scaffold.

The linker was synthesized and conjugated to 6-TG via a thiol-yne reaction. Note that the reaction condition gives a mixture of the cis and trans product. Previously, the cis product for the PTA prodrug was isolated by purification via product crystallization; however, for the tAVTG prodrug, the trans product was not successfully isolated from the cis isoform. Next, BHQ2-NHS was conjugated to the N-9 linker of tAVTG; then the BOC group was deprotected (**Scheme 2.11**). For the last step, glycine-conjugated 5FAM was pre-activated with HATU and conjugated to tAVTG-BHQ2 to yield the tAVTG-BHQ2-5FAM probe (**Scheme 2.11**).
2.2.2.1.2  *In Vitro* Assays

The UV-Vis absorption spectrum shows that 5FAM has an $\lambda_{\text{max}}$ at 492 nm and BHQ2 has an $\lambda_{\text{max}}$ at 571 nm (Figure 2.57). Next, *in vitro* GST enzyme assays were used to characterize the tAVTG-BHQ2-5FAM probe. A fluorescence time course was acquired throughout the GST M2-2-mediated conjugation of GSH to the probe; with an excitation wavelength at 492 nm, an increase in emission was observed at 520 nm. This corresponds to the separation of 5FAM from its FRET quencher, BHQ2, after its interaction with GST/GSH, allowing 5FAM’s fluorescence to switch from “off” to “on.” A 1.4-fold increase in fluorescence emission was observed over 150 minutes with 462 nM GST M2-2/GSH at 10 μM probe (Figure 2.58). Moreover, a GSH control was performed (Figure 2.59).

![Absorption spectrum of the tAVTG-BHQ2-5FAM probe](image1)

**Figure 2.58:** Absorption spectrum of the tAVTG-BHQ2-5FAM probe. 5FAM has an $\lambda_{\text{max}}$ at 492 nm and BHQ2 has an $\lambda_{\text{max}}$ at 571 nm. Conditions: 1 μM probe. The total DMSO concentration was 1% and the buffer used was PBS.

![Fluorescence time courses](image2)

**Figure 2.59:** GST M2-2 catalysis and GSH control on the tAVTG-BHQ2-5FAM probe. Time course conditions: 10 μM probe, 2 mM GSH, 462 nM GST M2-2, at 37°C (left). Time course conditions: 10 μM probe, 2 mM GSH, at 37°C (middle). Summary plot of GST experiment and GSH control (right). Excitation at 492 nm and emission at 523 nm. The total DMSO concentration was 1% and the buffer used was PBS.

The rate of probe activation with GST M2-2 was calculated as 0.1796, which was less than that of the GSH control (0.4747). Addition of enzyme should have increased the rate of catalysis;
repetition of this assay must be done to see whether these results are replicable. Perhaps, the GST M1-1 isoform can be tested as this probe may not be recognized by GST M2-2.

In the future, a fluorescence time course assay should be conducted with the same conditions as the PTA-BHQ2-5FAM probe derivative in order to assess the difference in the kinetics of probe activation by GST between the two prodrugs. Nevertheless, the same probe concentration was used, which allows for comparison in their fluorescence-fold changes; the tAVTG probe exhibited only a 1.4-fold fluorescence increase while the PTA probe showed 5-fold, with the same FRET pair (Figure 2.34 and 2.59). Therefore, given the less than 10-fold-change in fluorescence emission observed with GST/GSH activation, the tAVTG-BHQ2-5FAM probe was not pursued further. However, the following can also be done: fluorescence assay with the A1-1 and P1-1 isoforms to see whether it has a greater effect on fluorescence fold-change or activation kinetics and synthesis of a tAVTG-BHQ1-5FAM probe since BHQ-1 ($\lambda_{\text{max}}$ 534 nm) acts as a better FRET quencher to 5FAM ($\lambda_{\text{max}}$ 492 nm) than BHQ2 ($\lambda_{\text{max}}$ 571 nm).

2.2.2.2 Design and Synthesis of cAVTP-Quencher-Photosensitizer Scaffold

The objective of the proposed scaffold is to obtain better kinetic activity for GST by using the cAVTP prodrug, which specifically targets the GST M1-1 and GST M2-2 isozymes through a nucleophilic addition-elimination reaction (Figure 2.56). The PDT beacon consists of cAVTP in a ketone linkage with a PS through the butenone functional group and in an amide linkage with the corresponding FRET quencher at the N-9 nitrogen on its purine ring.

First, a Michael addition of GSH to the electrophilic $\beta$-carbon of cAVTP’s butenone moiety occurs, producing a cAVTP-Q-PS-GSH conjugate (Figure 2.60). Second, this enolate intermediate collapses to reform butenone and releases 6-MP-Q from GSH-PS (Figure 2.60). Hence, this GST-activatable fluorescent PDT beacon switches from the “off” to the “on” state upon GST/GSH mediated activation – resulting in an increase in fluorescence and PDT capability of the PS and the release of a cytotoxic anticancer agent. Light irradiation of the PS would then permit singlet oxygen production and cellular death of cancer cells that overexpress GST.
**Figure 2.60:** Mechanism of GST-catalyzed GSH conjugation on a generic cAVTP-Quencher-PS scaffold.

The following eight-step route depicted below was used in synthesizing the cAVTP-Q-PS scaffold (Scheme 2.12). The scheme is similar to that of PTA-Q-PS, but the synthesis of the linker differs (Scheme 2.12). As well, purification of the thiol-yne reaction via crystallization yielded the cis product over the trans isoform.

**Scheme 2.12:** Synthetic scheme to synthesize the generic cAVTP-Quencher-PS scaffold.

### 2.2.2.2.1 Design and Synthesis of Compound 2.10: cAVTP-BHQ2-5FAM (In-Progress)

This probe consists of 5FAM as the FRET acceptor and BHQ2 as the FRET quencher, which was converted into its NHS form for conjugation at the N9 linker of cAVTP. (Figure 2.61). The same FRET pair was used in this probe as the PTA-BHQ2-5FAM scaffold since it exhibited a 5-fold increase in fluorescence, which was the highest among the other PTA probe derivatives. As well,
the cAVTP prodrug was explored alongside the tAVTG prodrug to see which keto version of PTA yielded a probe with a more optimal GST rate of activation.

**Figure 2.61:** Mechanism of GST-catalyzed GSH conjugation on the cAVTP-BHQ2-5FAM scaffold.

**Scheme 2.13:** Synthetic scheme to synthesize the cAVTP-BHQ2-5FAM scaffold.

The linker was synthesized and conjugated to 6-TG via a thiol-yne reaction. Next, the BOC group was deprotected; then BHQ2-NHS will be conjugated to the N-9 linker of cAVTG (Scheme 2.13). For the last step, glycine-conjugated 5FAM will be pre-activated with HATU and conjugated to cAVTP-BHQ2 to yield the cAVTP-BHQ2-5FAM probe (Scheme 2.13). Note that this probe has not been fully synthesized yet; refer to the appendix.

### 2.3 Class B GST-Activatable Fluorescent-PDT Beacons: Exploiting GST’s Sulfonamidase Activity

In addition to the PTA and keto-PTA prodrug approach, the 2,4-dinitrobenzenesulfonamide (DNBS) substrate can be used to confer selectivity towards GST (Figure 2.62). By introducing an electrophilic sulfonamide bond to any PS with an amino group, the probe functions as an on/off switch during glutathionylation. The resulting by-products would be the free fluorophore and
either sulfur dioxide with GS-DNBS or GS-SO$_2$-DNBS (Figure 2.62). Below, Zhang et al. has shown the successful recognition of their fluorogenic probe, which consists of Cresyl Violet (CV) attached to DNBS, by GST A1-1 (Figure 2.63). Upon cleavage of the sulfonamide bond, the probe becomes highly fluorescent from an essentially non-emissive state as the electron-donating amine group on the fluorophore is regenerated during its release from the PeT quencher (Figure 2.63).

Figure 2.62: GST’s sulfonamidase activity on a generic sulfonamide scaffold. Upon GST-catalyzed GSH conjugation at the C-6 position on the quencher or at the sulfonamide group, the free fluorophore will be released and become turned “on” and highly emissive. Figure retrieved from Ruzza, P. & Calderan, A. Glutathione Transferase (GST)-Activated Prodrugs. Pharmaceutics 5, 220–231 (2013).

Figure 2.63: GST’s sulfonamidase activity on the CV-DNBS probe. Figure retrieved from Zhang, J. et al. Synthesis and Characterization of a Series of Highly Fluorogenic Substrates for Glutathione Transferases, a General Strategy. Journal of the American Chemical Society 133, 14109–14119 (2011).

The absorption spectra of the CV-DNBS probe shows that it is slightly blue-shifted and quenched compared to CV alone; the probe has an $\lambda_{\text{max}}$ of 594 and 607 nm (Figure 2.64). The conversion of CV-DNBS to CV by GST A1-1/GSH resulted in a 58-fold increase in fluorescence emission at 620 nm; however, this emission was significantly weakened with GSH alone and became absent without GST/GSH (Figure 2.64). Furthermore, the $k_{\text{cat}}/K_{\text{m}}$ value for GST A1-1 was high ($3.3 \times 10^8$ M$^{-1}$s$^{-1}$) and the rate enhancement was large ($2.4 \times 10^9$).
2.3.1 Summary of DNBS-Based Probes

The synthesis of the series of DNBS-based probes shown in Figure 2.65 involved a one step reaction where the F/PS was pre-stirred with sodium hydride in anhydrous DMF, followed by dropwise addition of DNBS-Cl. For RB-DNBS, DNBS-acid was converted to an acid chloride, to which a N, N’-dimethylethylenediamine handle was attached to react with Rose Bengal. Taking inspiration from the CV-DNBS probe, various commercially available fluorophores or PSs that had an available amine handle for conjugation with DNBS in a sulfonamide linkage were selected for use.

Figure 2.65: Chemical structures of the series of DNBS-based probes synthesized.

A summary of the in vitro assays for the series of DNBS-based probes (Table 2.1).
<table>
<thead>
<tr>
<th>DNBS-Based Probe</th>
<th>Absorption Spectra ($\lambda_{\text{max}}$)</th>
<th>Emission Spectra ($\lambda_{\text{emission}}$)</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Azure I-DNBS</strong></td>
<td>Azure I: $\lambda_{\text{max}}$ at 648 nm (ethanol)</td>
<td>Not tested.</td>
<td>Given that the absorption intensity of the Azure I-DNBS probe did not increase or red-shift, when compared to the free dye, with GST/GSH addition, this probe was not pursued further.</td>
</tr>
<tr>
<td>(Compound 2.11)</td>
<td>Conditions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• before spectrum: 1 µM probe, 1% DMSO, PBS (minimal absorption at 648 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• after spectrum: 1 µM probe, 2 mM GSH, 18 nM GST P1-1, 35 minutes at 37 °C, 1% DMSO, PBS (no change in absorption at 648 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Thionin-DNBS</strong></td>
<td>Not tested yet.</td>
<td>Not tested yet.</td>
<td>TBA.</td>
</tr>
<tr>
<td>(Compound 2.12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>3AIC-DNBS</strong></td>
<td>3-Amino-Iodo-Coumarin: $\lambda_{\text{max}}$ at 331 nm</td>
<td>Not tested.</td>
<td>No increase or red-shift in absorption intensity of the 3AIC-DNBS probe.</td>
</tr>
<tr>
<td>(Compound 2.13)</td>
<td>Conditions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• before spectrum: 1 µM probe, 1% DMSO, PBS (minimal absorption at 331 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• after spectrum: 1 µM probe, 2 mM GSH, 154 nM GST P1-1, overnight at 37 °C, 1% DMSO, PBS (no change in absorption at 331 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>6AP-DNBS</strong></td>
<td>6-Aminophenalenone: $\lambda_{\text{max}}$ at 605 nm</td>
<td>Not tested.</td>
<td>No increase or red-shift in absorption intensity of the 6AP-DNBS probe.</td>
</tr>
<tr>
<td>(Compound 2.14)</td>
<td>Conditions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• before spectrum: 1 µM probe, 1% DMSO, PBS (minimal absorption at 605 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• after spectrum: 1 µM probe, 2 mM GSH, 77 nM GST P1-1, overnight at 37 °C, 1% DMSO, PBS (no change in absorption at 605 nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LMB-DNBS</strong></td>
<td>Not tested yet.</td>
<td>Not tested yet.</td>
<td>TBA.</td>
</tr>
<tr>
<td>(Compound 2.15)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.1: Summary of the *in vitro* assays conducted for the series of DNBS-based probes synthesized.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Description</th>
<th>Assay Conditions</th>
<th>Results</th>
</tr>
</thead>
</table>
| **Methylene 3RAX-DNBS** (Compound 2.16) | Methylene 3RAX: $\lambda_{\text{max}}$ at 780 nm | • before spectrum: 1 μM probe, 1% DMSO, PBS (minimal absorption at 780 nm)  
• after spectrum: 1 μM probe, 2 mM GSH, 77 nM GST P1-1, overnight at 37 °C, 1% DMSO, PBS (no change in absorption at 331 nm) | Not tested.  
No increase or red-shift in absorption Methylene 3RAX-DNBS probe. |
| **RB-DNBS** (Compound 2.17) | Not tested. | • before spectrum: 1 μM probe, 1% DMSO, PBS (minimal emission at 572 nm)  
• after spectrum: 1 μM probe, 2 mM GSH, 18 nM GST P1-1, overnight at 37 °C, 1% DMSO, PBS (decrease in emission at 572 nm)  
• assay was repeated at 25 °C (decrease in emission at 572 nm) | RB-DNBS: $\lambda_{\text{emission}}$ at 572 nm  
No increase in fluorescence intensity of the RB-DNBS probe. |
| **IR780-DNBS** (Compound 2.18) | IR780: $\lambda_{\text{max}}$ at 780 nm | • before spectrum: 1 μM probe, 1% DMSO, PBS (minimal absorption at 780 nm)  
• after spectrum: 1 μM probe, 2 mM GSH, 192 nM GST P1-1, overnight at 37 °C, 1% DMSO, PBS (no change in absorption at 331 nm) | Not tested.  
No increase or red-shift in absorption intensity of the IR780-DNBS probe. |

Given the lack of activation by GST, none of these probes were pursued further. This may have resulted from purity problems with the final probes synthesized or can be a result of GST not recognizing these specific fluorophores or PSs used as substrates. Perhaps, a fluorophore similar to the structure of CV should be attempted in the next probe design; as well, CV-DNBS should be...
synthesized and tested to see whether there is a problem inherent in the conditions of my *in vitro* assays.

### 2.4 Class C GST-Activatable Fluorescent-PDT Beacons: Using a GSH or GSH-Derivative Scaffold

A GSH-independent prodrug approach can be taken, where native GSH or a GSH analog is used as the backbone of the scaffold.\(^5\) GST activation involves cleavage of the sulfone bond by a $\beta$-elimination reaction, which causes release of a vinyl sulfone by-product and the PS for applications in fluorescence-guided PDT ([Figure 2.66]).\(^5\) An example is the TLK286 probe developed by Lyttle *et al.*, which consists of a cytotoxic tetrakis(chloroethyl)phosphorodiamidate attached to the thiol group of a GSH analog ($\gamma$-Glu-Cys-Phg); oxidation of this intermediate then created a sulfone derivative.\(^5\) With addition of GST P1-1, the G site Tyr7 residue will abstract an $\alpha$-proton on the sulfone group of the GSH scaffold, which will then release a phosphoroamidate nitrogen mustard that alkylates various cellular nucleophiles like DNA ([Figure 2.67]).\(^5\) Most notably, TLK286 was shown to be successful in preclinical experiments in cell cultures and human tumor xenograft models; administration in mice at therapeutic doses also showed minimal bone marrow toxicity.\(^5\)

![Figure 2.66: Mechanism of GST P1-1 activation on a GSH or GSH-derivative scaffold by a $\beta$-elimination reaction. Figure retrieved from Ruzza, P. & Calderan, A. Glutathione Transferase (GST)-Activated Prodrugs. Pharmaceutics 5, 220–231 (2013).](image-url)
Figure 2.67: Mechanism of GST P1-1 activation on TLK286 by a β-elimination reaction. GST abstracts the α-proton off the sulfone group, which will release the alkylating agent from the GSH-Derivative scaffold and cause cell death. Figure retrieved from Lyttle, M. H. et al. Glutathione-S-Transferase Activates Novel Alkylating Agents. Journal of Medicinal Chemistry 37, 1501–1507 (1994).

2.4.1 Native GSH Scaffold

2.4.1.1 Design and Synthesis of Compound 2.21: the TPE AIEgen PS

The TPE derivative, called 2-[[4-[2,2-bis(4-hydroxyphenyl)-1-phenylethenyl]phenyl]-2-thienylmethylene]-propaedinitrile, is a propeller-shaped fluorogen/PS that undergoes AIE when in its solid or aggregate state. Consequently, this results in strong fluorescence emission due to restriction of rotation of its intramolecular bonds during π-π stacking of the AIEgen, thus preventing energy dissipation in a nonradiative manner. The light-up bioprobe developed by Yuan et al. was used for the selective imaging and ablation of cancer cells; the design includes TPECM attached to a conjugate consisting of a Cathepsin B trigger group (GFLG) and an integrin targeting moiety (cRGD) (Figure 2.68). Moreover, Yuan et al. developed a self-reporting AIE probe with a built-in singlet oxygen sensor that monitors the amount of 1O2 produced in real-time during PDT; its design consists of a red-emissive AIE PS attached to a green-emissive rhodol 1O2 reporter dye and targeting moiety by a 1O2 cleavable aminoacrylate linker (Figure 2.69).
**Figure 2.68:** Chemical structure of the light-up bioprobe called PECM-2GFLGD3-cRG. In the presence of Cathepsin B, the TPECM PS is released and switched to the “on” state, which will fluoresce and generate singlet oxygen with light irradiation. Figure retrieved from Yuan, Y. et al. Specific Light-Up Bioprobe with Aggregation-Induced Emission and Activatable Photoactivity for the Targeted and Image-Guided Photodynamic Ablation of Cancer Cells. *Angewandte Chemie* **127**, 1800–1806 (2014).

**Figure 2.69:** Chemical structure of the self-reporting AIE probe called TPEPP-AA-RhocRGD. GST activation will release the PS from the singlet oxygen rhodol dye reporter; the PS will undergo AIE in the red-region and produce singlet oxygen while the latter will emit in the green-region and provide real-time and in situ singlet oxygen quantification. Figure retrieved from Yuan, Y., Zhang, C.-J., Xu, S. & Liu, B. A Self-Reporting AIE Probe with a Built-In Singlet Oxygen Sensor for Targeted Photodynamic Ablation of Cancer Cells. *Chemical Science* **7**, 1862–1866 (2016).

Synthesis of this PS requires five steps and has not been synthesized yet (Scheme 2.14).
Scheme 2.14: Synthetic scheme for the TPE PS.

2.4.1.2 Design and Synthesis of Compound 2.19: GSH-TPE (In-Progress)

The design of the GSH-TPE probe consists of native GSH attached to a tetraphenylethene (TPE)-AIGen PS, which participates in aggregation-induced emission (AIE) after GST activation (Figure 2.70).\textsuperscript{54} Given that using GSH as part of the scaffold increases the overall molecular weight of the probe, AIE was selected as the activation mechanism since it only requires the presence of a PS—without the need of a corresponding quencher.

Figure 2.70: Mechanism of GST P1-1 activation for the GSH-TPE probe. GST P1-1 abstracts an α-proton on the sulfone group, which will release the TPE PS via a β-elimination reaction and allow for AIE.
Scheme 2.15: Synthetic scheme for the GSH-TPE scaffold.

The synthesis of the GSH-TPE probe involves the following steps: i) phosphoryl chloride was substituted with N-methylaniline at one of its chlorides by using a TEA; ii) the remaining two chlorides were replaced with two 2-bromoethanol using TEA; iii) SN2 was conducted between the thiol group of native GSH and the bromine group of the POCl3-derivative; iv) the intermediate will be oxidized using hydrogen peroxide and peracetic acid to create the sulfone group; and v) pre-synthesized TPE will be conjugated to the 2-bromoethanol handle of the POCl3-derivative (Scheme 2.15). Note that the synthesis of this probe is still in-progress; refer to appendix.

As the next probe iteration, the POCl3-derivative will be removed to test whether the GST P1-1-mediated β-elimination can still proceed successfully. The POCl3-derivative was incorporated in the design of compound 2.19 to mimic the structure of the trigger group in TLK286, as it showed to be successfully released.

2.4.2 Non-Native GSH Scaffold

2.4.2.1 Design and Synthesis of Compound 2.20: GSH Derivative-BHQ3-Pyropheophorbide A (In-Progress)

This probe consists of pyropheophorbide A as the PS, BHQ3 as the FRET quencher, and a GSH peptide derivative (Figure 2.71 and Scheme 2.16). Although the PTA-BHQ3-Pyropheophorbide A probe was not activated by GST M1-1, this FRET pair was selected for compound 2.20 because this proposed scaffold exploits a different activation approach, where GST acts as a base to promote the β-elimination reaction rather than participating in GSH conjugation. However, the
bulky nature of pyropheophorbide A could potentially cause binding problems at the GST active site. Nevertheless, pyropheophorbide A is advantageous because it serves as a PS for PDT-applications and absorbs and emits in the NIR region, allowing for deeper tissue penetration for *in vivo* applications.

![Scheme 2.16](image)

**Figure 2.71**: Mechanism of GST P1-1 activation on the GSH-Derivative-BHQ3-Pyropheophorbide A probe. GST abstracts the α-proton on the sulfone group, which will release the PS from the GSH-Quencher scaffold via a β-elimination reaction. Consequently, the PS can fluoresce and produce singlet oxygen with light irradiation.

![Scheme 2.16](image)

**Scheme 2.16**: Synthetic scheme for the GSH derivative peptide scaffold.
Scheme 2.17: Synthetic scheme for the GSH Derivative-BHQ3-Pyropheophorbide A probe.

The synthesis of the GSH Derivative-BHQ3-Pyropheophorbide A probe involves six steps, the first two steps were the same as in the GSH-TPE probe: iii) SN2 was conducted between the thiol group of the GSH derivative and the bromine group of the POCl3-derivative; iv) the intermediate will be oxidized with hydrogen peroxide and peracetic acid to make a sulfone group; v) BHQ3-NHS will be conjugated to the lysine side chain of the GSH derivative via amide coupling; and vi) pre-synthesized ethylenediamine-conjugated pyropheophorbide A will be linked to the 2-bromoethanol handle of the POCl3-derivative (Scheme 2.17). Note that the synthesis of this probe is still in-progress; refer to appendix. Perhaps a FRET pair such as BHQ2 and 5FAM can be used to test this POC scaffold before introduction of the costlier FRET pairs, BHQ3 and pyropheophorbide A.

After synthesis of the class C GST-PDT beacons, they will be tested in vitro to see whether GST activation occurs and the resulting fluorescence-fold change.
2.5 Summary and Future Directions

The goal of the GST project is to design and synthesize an enzyme-activatable PDT-beacon that is selective towards GST for fluorescence-guided PDT applications. For the class A probes, PTA-BHQ2-5FAM showed the highest increase in fluorescence emission (5-fold) upon GST/GSH activation. However, a probe derivative with a higher percent quenching efficiency should be developed before proceeding in cellulo. Perhaps a better FRET quencher such as BHQ1 can be used to improve the fold-change upon activation and to decrease the background signal in the probe’s quenched state. Linker length between the PTA prodrug and the fluorophore or quencher must be explored furthered; it can be altered to potentially enhance the kinetics of GST activation. In the future, a different prodrug can be explored like azathioprine, which is catalyzed most efficiently by GST A1-1, A2-2, and M1-1 (Figure 2.72 and 2.73).

![PTA-BHQ2-5FAM](image)

**Figure 2.72:** Future directions of the GST project. The PTA prodrug can be replaced with the azathioprine prodrug.

![Glutathione and Azathioprine](image)

**Figure 2.73:** Mechanism of bioactivation of the azathioprine prodrug. Glutathione attacks the electrophilic carbon on azathioprine, which releases the anticancer agent 6-MP along with a GS-imidazole conjugate. Figure retrieved from Modén, O. & Mannervik, B. Glutathione Transferases in the Bioactivation of Azathioprine. *Advances in Cancer Research Redox and Cancer Part A* **122**, 199–244 (2014).

For the class B probes, none of the sulfonamide probes were activated with GST/GSH. For the class C probes, the GST-TPE and the GSH Derivative-BHQ3-Pyropheophorbide A probes have not yet been fully synthesized and will be characterized in vitro as the next step. Though, other probe designs can also be explored in the future for this class. I am proposing a GSH-TPE-6MP
probe, which consists of a GSH scaffold as a GST P1-1-selective targeting moiety, a red-emissive TPE-derivative as the PS and imaging agent for PDT, a singlet oxygen cleavable linker for real-time monitoring of $^{1}O_2$ production, and 6-MP as the anticancer agent. Note that 6-MP will only be released at the target site as the singlet oxygen generated from PDT will cleave the linker that is attached to 6-MP (see Figure 2.74 and 2.75).

**Figure 2.74:** Future directions of the GST project. Design of a new GST P1-1-specific probe that can provide real-time feedback on the amount of $^{1}O_2$ produced and releases an anticancer agent alongside PDT applications with the PS.

**Figure 2.75:** Future directions of the GST project. Activation strategy for the new GST P1-1-specific probe.
Chapter 3
Design, Synthesis, and Characterization of CE-Activatable Fluorescent-PDT Beacons: Results and Discussion

The goal of chapter 3 is to develop a CES2-activatable fluorescent-PDT beacon that can selectively kill cancer cells over healthy cells by taking advantage of the overexpression of CES2; hence, an in-depth understanding of the CES2 enzyme and its different isoforms is needed in order to rationally design a CES2 isoform-specific probe.

3 Carboxylesterases (CES)

Localized in the cytoplasm and ER lumen of many tissues, Carboxylesterases (CES) are enzymes from the multi-gene serine hydrolase superfamily that participate in the xenobiotic metabolism of exogenous and endogenous compounds. They transesterify and hydrolyze compounds that contain an ester, amide, carbamate, or thioester bond. In humans, three CES isoforms have been identified: CES1, CES2, and CES3. Sharing a 47% amino acid sequence identity, CES1 and CES2 are highly expressed in the epithelial of metabolic organs but also vary in their tissue distribution; CES1 is predominately found in the liver and adipocytes while CES2 is predominately found in the small intestine and colon.

Solved by X-ray crystallography, the 3D structure of CES1 is shown to have conserved α,β-hydrolase folds; in this αβ domain, the α-helices are bordering a central parallel β-pleated sheet (Figure 3.1). CES1 also has a central catalytic domain; the catalytic residues are buried deep within the active site cleft, which creates an electrostatic gradient that guides substrates towards them (Figure 3.1). Specifically, the aromatic residues that line this 1,300 Å active site confer selectivity towards hydrophobic compounds. Furthermore, CES1 has a regulatory domain that acts as a low-affinity Z-site for surface ligand-binding. While CES1 can exist as either a monomer, trimer, or hexamer, CES2 and CES3 can only be monomers (Figure 3.1, 3.2, and 3.3). However, there are no available 3D structures for CES2 and CES3.

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2 The in cellulo work presented in chapter 2 was conducted by my colleague, Karishma Kailass.
**Figure 3.1:** 3D structure of CES1 solved by X-ray crystallography. Belonging to the α/β-hydrolase fold superfamily of proteins, CES1 has a central catalytic domain which contains the catalytic triad; a regulatory domain; and an αβ domain. Figure retrieved from Wang, D. et al. Human Carboxylesterases: a Comprehensive Review. *Acta Pharmaceutica Sinica B* 8, 699–712 (2018).

**Figure 3.2:** Human CES1 trimer, viewed in a homatropine complex. In each monomer, the catalytic domain (green, magenta, and orange), αβ domain (light green, pink, and yellow), and regulatory domain (dark green, purple, and red) are colour coded. Figure retrieved from Bencharit, S. et al. Structural Basis of Heroin and Cocaine Metabolism by a Promiscuous Human Drug-Processing Enzyme. *Nature Structural Biology* 10, 349–356 (2003).

**Figure 3.3:** Molecular properties of CES1 and CES2. Figure retrieved from Wang, D. et al. Human Carboxylesterases: a Comprehensive Review. *Acta Pharmaceutica Sinica B* 8, 699–712 (2018).
Although CES have broad substrate specificity, CES1 and CES2 exhibit preference for certain substrates; CES1 metabolizes substrates with large acyl groups and small alcohol groups (e.g. imidapril, oseltamivir, meperidine, clopidogrel, D-luciferin methyl ester, nalapril, cocaine, and heroin) (Figure 3.4).57 Also, the catalytic Ser221 residue of CES1 splits the ligand-binding site into a rigid pocket for the binding of small acyl substrates and a flexible pocket for the binding of various acyl groups.57 This indicates that CES1 is promiscuous towards a wide variety of substrates as it has the ability to hydrolyze large and small acyl groups.57 Conversely, CES2 metabolizes substrates with small acyl groups and large alcohol groups (e.g. irinotecan, capecitabine, prasugrel, fluorescein diacetate, and flutamide) (Figure 3.5).57

Figure 3.4: Representative substrates for CES1. CES1 has preference for ester-containing substrates with larger acyl groups and smaller alcohol groups. Figure retrieved from Wang, D. et al. Human Carboxylesterases: a Comprehensive Review. Acta Pharmaceutica Sinica B 8, 699–712 (2018).

Figure 3.5: Representative substrates for CES2. CES2 has preference for ester-containing substrates with smaller acyl groups and larger alcohol groups. Figure retrieved from Wang, D. et al. Human Carboxylesterases: a Comprehensive Review. Acta Pharmaceutica Sinica B 8, 699–712 (2018).
CES1 and CES2 are major players in the detoxification of drugs (e.g. Irinotecan, Oseltamivir, Capecitabine, and Clopidogrel) and environmental toxins (e.g. Pyrethroids). Researchers are focused on targeting CES2 since it is overexpressed in many human tumor tissues and is the key isoform for the intratumoral activation of major anticancer prodrugs into their metabolically active form (Figure 3.6). Specifically, CES2 is useful in prognosis and patient stratification for the following treatments: Capecitabine, Irinotecan, and neoadjuvant FOLFIRINOX therapy. For Capecitabine, CES2 selectively converts the prodrug into 5-fluorouracil in solid tumors, which inhibits thymidylate synthase and causes fluoronucleotide additions into DNA and RNA. For Irinotecan, which is popularly used to treat metastatic cancers like colorectal cancer, CES2 selectively hydrolyses the prodrug into SN-39. This active metabolite inhibits the DNA repair enzyme topoisomerase 1 and causes induction of cellular death via apoptosis. For Neoadjuvant FOLFIRINOX therapy, a combination of drugs (fluorouracil, oxaliplatin, leucovorin, and irinotecan) or a single drug (gemcitabine) selectively activated by CES2 is used to tackle metastatic pancreatic cancers like pancreatic ductal adenocarcinoma.

Figure 3.6: Pathology associated with overexpression of the clinically relevant CES2 isoform. Figure adapted from the Human Protein Atlas website (https://www.proteinatlas.org/ENSG00000172831-CES2/pathology).

Within the central catalytic domain, there is a highly conserved catalytic triad that contains the residues: Ser221, Glu243, and His468 for CES1 and Ser221, Glu345, and His457 for CES2; this triad is essential for CES-mediated catalyzation and sits at the interface between the three domains. CES hydrolysis involves a two-step base-mediated catalysis. During this process, a proton transfer occurs within the catalytic triad, which generally involves the following residues: serine, histidine, and glutamic acid (Figure 3.7). First, the charged Glu residue abstracts a proton from His under physiological conditions, which leads to a proton transfer from serine to the other available nitrogen of His (Figure 3.8). Subsequently, the alcohol group in serine becomes
deprotonated and nucleophilically attacks the carbonyl carbon of the substrate (Figure 3.8).\textsuperscript{57} An acyl-enzyme tetrahedral intermediate forms, which collapses to reform the carbonyl and releases the corresponding alcohol or amine (Figure 3.8).\textsuperscript{57} Second, water attacks the acyl-enzyme derivative to release the carboxylic acid alongside regeneration of the original state of the catalytic triad (Figure 3.8).\textsuperscript{57}

**Figure 3.7:** Proposed scheme for CES-mediated hydrolysis of ester-containing compounds. The CES1 catalytic triad consists of the following residues: Ser221, Glu354, and His468. Figure retrieved from Wang, D. et al. Human Carboxylesterases: a Comprehensive Review. *Acta Pharmaceutica Sinica B* 8, 699–712 (2018).

**Figure 3.8:** Two-step mechanism of CES-mediated ester hydrolysis. Figure retrieved from Wang, D. et al. Human Carboxylesterases: a Comprehensive Review. *Acta Pharmaceutica Sinica B* 8, 699–712 (2018).
3.1 Research Objective

The main objective of this project is to develop an enzyme-activatable fluorescent-PDT beacon for the clinically relevant CES2 isoform. Specifically, three different routes will be taken: a) using an Eosin-B based scaffold; b) Eosin-Y based scaffold; and c) 6-aminophenalenone (6AP)-based scaffold (Figure 3.9).

Figure 3.9: Chemical structure of Eosin B (left), Eosin Y (middle), and 6AP (right).

The specific aims of this project are to: design, synthesize, and characterize enzyme-activatable fluorescent-PDT beacons for CES2; determine absorption and fluorescence changes in vitro by conducting human CES2 enzyme assays; assess its selectivity for the different CES isoforms; measure fluorescence and ROS generation in cellulo using a cancer cell line; and determining its selectivity for cancer cells over healthy cells.

3.2 Eosin B-Based Probe

3.2.1 Design and Synthesis of Compound 3.1: An Eosin B-Disubstituted Probe (AK-01-260)

This probe consists of Eosin B as the PS, which will be quenched by conjugating the two hydroxyl handles with a benzoyl ester group (Figure 3.10). Consequently, Eosin B will be structurally converted to a closed lactone state that is neither absorptive or emissive. Upon CES2-mediated hydrolysis of the ester linkages, Eosin B will re-open, allowing for fluorescence emission and singlet oxygen generation (Figure 3.10).
**Figure 3.10:** Activation strategy for the Eosin B-disubstituted probe. CES2-mediated hydrolysis of the ester linkages will open the closed lactone ring to release free Eosin B.

![Activation strategy diagram](image)

**Scheme 3.1:** Synthetic scheme for the Eosin B-disubstituted probe.

Benzoyl chloride was added dropwise to a solution of Eosin B pre-stirred with TEA (Scheme 3.1).

### 3.2.1.1 *In Vitro* Assays

The UV-Vis absorption spectrum shows that the Eosin B-disubstituted probe is fully quenched by its lack of absorption at the parent dye’s characteristic $\lambda_{\text{max}}$ of 520 nm; however, upon CES2-mediated catalysis, the probe releases free Eosin B, as observed by an increase in absorption at 520 nm (Figure 3.11, Figure 3.12, and Figure 3.13). A UV-Vis time course was performed to assess the stability of the probe in DPBS buffer; over a period of 100 minutes, its absorption did not change significantly. The probe essentially remained non-absorptive, indicating that there was no probe conversion to the free parent dye, which confirms that the probe is stable in buffer. The rate of absorption change over a period of 25 minutes was calculated as 0.0014. Perhaps a singlet oxygen assay can be conducted to determine whether a significant amount of $^1\text{O}_2$ is produced by the probe after the stability test – this may provide insight on the importance of the calculated slope (Figure 3.14). Next, another UV-Vis time course was taken to monitor the absorption increase during CES catalysis over a total of 500 minutes; it shows that this probe is highly selective towards CES2 over CES1 as no probe activation was observed for CES1 (Figure 3.15). A fluorescence time course was then acquired for CES2-mediated hydrolysis of the probe; with an excitation wavelength at 520 nm, an increase in fluorescence emission was seen at 545 nm. This corresponds to opening of the closed lactone form, allowing free Eosin B to accumulate in its “on” state (Figure 3.16). A fluorescence time course was also acquired for CES1, which shows that the probe is not selective for CES1 as there is a minimal increase in fluorescence (Figure 3.17).
**Figure 3.11**: Absorption and emission spectra of Eosin B ($\lambda_{\text{max}}$ 520 nm, $\lambda_{\text{emission}}$ 545 nm).

**Figure 3.12**: Absorption spectrum of the Eosin B-disubstituted probe. Eosin B has an $\lambda_{\text{max}}$ at 520 nm ($\varepsilon = 95 000$ Lmol$^{-1}$cm$^{-1}$ at 530 nm). Conditions: 1.24 $\mu$M probe. The total DMSO concentration was 1% and the buffer used was DPBS.

**Figure 3.13**: CES2 mediated hydrolysis of the Eosin B-disubstituted probe. During the release of free Eosin B, an increase in absorbance was observed at 520 nm. Conditions: a) before spectrum (light orange): 1.24 $\mu$M probe, at 37°C; and b) after spectrum (maroon): 1.24 $\mu$M probe, 110 nM CES2, overnight incubation at 37°C. The total DMSO concentration was 1% and the buffer used was DPBS.
Figure 3.14: Stability test on the Eosin B-disubstituted probe. Time course conditions: 1.24 μM probe, at 37°C. The total DMSO concentration was 1% and the buffer used was DPBS.

![Absorbance at 520 nm over time](image)

$y = 0.0014t + 0.065$

$R^2 = 0.9534$

Figure 3.15: Selectivity test on the Eosin B-disubstituted probe. Time course conditions: a) CES2 (maroon): 1.24 μM probe, 110 nM CES2, at 37°C; and b) CES1 (light brown): 1.24 μM probe, 100 nM CES1, at 37°C scanned at five-minute intervals. The total DMSO concentration was 1% and the buffer used was DPBS.

![Absorbance at 520 nm over time](image)

Figure 3.16: CES2 mediated hydrolysis of the Eosin B-disubstituted probe. During the release of free Eosin B, an increase in fluorescence emission was observed at 545 nm. Time course conditions: 1.24 μM probe, 110 nM CES2, at 37°C (excitation 520 nm, emission 545 nm). The total DMSO concentration was 1% and the buffer used was DPBS.

![Fluorescence Intensity at 545 nm over time](image)
Figure 3.17: CES1 mediated hydrolysis of the Eosin B-disubstituted probe. During the release of free Eosin B, minimal fluorescence emission was observed at 545 nm. Time course conditions: 1.24 μM probe, 100 nM CES1, at 37°C (excitation 520 nm, emission 545 nm). The total DMSO concentration was 1% and the buffer used was DPBS.

The absorption and fluorescence CES2 time course assays should be repeated to see whether the difference in time scale of the reactions occurs again. Nevertheless, given these promising in vitro results, this probe was tested in cellulo for its ability to generate ROS.

3.2.1.2 In Cellulo ROS Imaging

The next step was to assess whether this probe can produce ROS in cancer cells that overexpress CES2. The SU86.86 CES2 cell line was selected, which contains a vector that overexpresses CES2 since SU86.86 has low levels of CES2 activity. To visualize ROS production, the fluorogenic dye 2’,7’-dichlorofluorescein diacetate (DCFHDA) was used (Figure 3.18). While non-emissive in its original state, once it successfully diffuses through the plasma membrane, cellular esterases will deacetylate DCFHDA into another non-fluorescent form called DCFH (Figure 3.18). Only in the presence of ROS will DCFH become oxidized into its green-emissive form, 2’,7’-dichlorofluorescein (DCF) (Figure 3.18).
**Figure 3.18:** Mechanism of DCFH-DA fluorescence upon interaction with ROS *in cellulo*. The non-fluorescent DCFH-DA sensor passes through the cell membrane and becomes cleaved by cellular esterases to the DCFH form, which will then become fluorescent as DCF during ROS oxidation. Figure retrieved from the Cell Biolabs Inc. website (https://www.cellbiolabs.com/reactive-oxygen-species-ros-assay).

To begin, different concentrations (0 µM, 2.5 µM, and 5 µM) of the probe was added to the SU86.86 cells, followed by three hours of incubation. Next, DCFHDA was added and incubated for 30 minutes. During this time, the overexpressed CES2 enzymes are expected to convert the probe into free Eosin B and the DCFHDA dye into DCFH. Thus, with light irradiation at 530 nm, Eosin B produced ROS, which oxidized DCFH into DCF (Figure 3.25, 3.26, and 3.27). Given that Eosin B has an extremely low fluorescent quantum yield in water (Φf 0.00045), the green emission observed comes from DCF, allowing for a quantitative measure of the amount of ROS produced. Dark experiments without light irradiation were conducted alongside the light experiments. Furthermore, Eosin B (at 0 µM, 2.5 µM, and 5 µM) was also tested; no ROS was produced as expected since Eosin B does not permeate the cell membrane (Figure 3.20, 3.24, and 3.27). As a control, light and dark experiments with the ROS sensor alone were tested; no background fluorescence was detected (Figure 3.19, 3.21, and 3.22).
Figure 3.19: Fluorescence images of SU86.86 cells after incubation with the ROS sensor, DCFHDA. Top: cells were incubated with 0 µM DCFHDA for 30 min then imaged. Bottom: cells were incubated with 0 µM DCFHDA for 30 min and then irradiated at 530 nm for 10 min (excitation 495 nm, emission 529 nm). Bright field images (left); dark field images (right).

Figure 3.20: Fluorescence images of SU86.86 cells after incubation with free Eosin B dye. Top: cells were incubated with 2.5 µM Eosin B for 3 h, followed by incubation with DCFHDA (0 µM) for 30 minutes and then imaged. Bottom: cells were incubated with 2.5 µM Eosin B for 3 h, followed by incubation with DCFHDA (0 µM) for 30 minutes and then irradiated at 530 nm for 10 min (excitation 495 nm, emission 529 nm). Bright field images (left); dark field images (right).

Figure 3.21: Fluorescence images of SU86.86 cells after incubation with the Eosin B-disubstituted probe. Top: cells were incubated with 2.5 µM probe for 3 h, followed by incubation with DCFHDA (0 µM) for 30 minutes and then
imaged. Bottom: cells were incubated with 2.5 µM probe for 3 h, followed by incubation with DCFHDA (0 µM) for 30 minutes and then irradiated at 530 nm for 10 min (excitation 495 nm, emission 529 nm). Bright field images (left); dark field images (right).

**Figure 3.22:** Fluorescence images of SU86.86 cells after incubation with the Eosin B-disubstituted probe. Top: cells were incubated with 5 µM probe for 3 h, followed by incubation with DCFHDA (0 µM) for 30 minutes and then imaged. Bottom: cells were incubated with 5 µM probe for 3 h, followed by incubation with DCFHDA (0 µM) for 30 minutes and then irradiated at 530 nm for 10 min (excitation 495 nm, emission 529 nm). Bright field images (left); dark field images (right).

**Figure 3.23:** Fluorescence images of SU86.86 cells after incubation with the ROS sensor, DCFHDA. Top: cells were incubated with 10 µM DCFHDA for 30 min then imaged. Bottom: cells were incubated with 10 µM DCFHDA for 30 min and then irradiated at 530 nm for 10 min (excitation 495 nm, emission 529 nm). Bright field images (left); dark field images (right).
Figure 3.24: Fluorescence images of SU86.86 cells after incubation with free Eosin B dye. Top: cells were incubated with 2.5 µM Eosin B for 3 h, followed by incubation with DCFHDA (10 µM) for 30 minutes and then imaged. Bottom: cells were incubated with 2.5 µM Eosin B for 3 h, followed by incubation with DCFHDA (10 µM) for 30 minutes and then irradiated at 530 nm for 10 min (excitation 495 nm, emission 529 nm). Bright field images (left); dark field images (right).

Figure 3.25: Fluorescence images of SU86.86 cells after incubation with the Eosin B-disubstituted probe. Top: cells were incubated with 2.5 µM probe for 3 h, followed by incubation with DCFHDA (10 µM) for 30 minutes and then imaged. Bottom: cells were incubated with 2.5 µM probe for 3 h, followed by incubation with DCFHDA (10 µM) for 30 minutes and then irradiated at 530 nm for 10 min (excitation 495 nm, emission 529 nm). Bright field images (left); dark field images (right).

Figure 3.26: Fluorescence images of SU86.86 cells after incubation with the Eosin B-disubstituted probe. Top: cells were incubated with 5 µM probe for 3 h, followed by incubation with DCFHDA (10 µM) for 30 minutes and then imaged. Bottom: cells were incubated with 5 µM probe for 3 h, followed by incubation with DCFHDA (10 µM) for
30 minutes and then irradiated at 530 nm for 10 min (excitation 495 nm, emission 529 nm). Bright field images (left); dark field images (right).

**Figure 3.27**: Effect of probe concentration on the fluorescence activation of DCFHDA. As the probe concentration increases, the fluorescence intensity of DCFHDA increases as more probe becomes converted to free Eosin B by CES2-mediated hydrolysis. Consequently, light irradiation of Eosin B generates ROS, which will interact with the ROS sensor and convert it to its green-emissive DCF form.

From the bar graph above, relative to the 0 µM or 10 µM DCFHDA control, a higher fluorescence intensity was seen for light experiments with probe (2.5 µM and 5 µM) and sensor at 10 µM (Figure 3.27). As expected, the light experiments had higher fluorescence intensities than their corresponding dark experiments (Figure 3.27). Therefore, this *in cellulo* ROS assay shows that the Eosin B-disubstituted probe permeates the cell membrane, becomes activated by CES2 to release free Eosin B, and generates ROS as visualized by the ROS sensor. The next steps for this probe include the following: i) quantify cellular death via an MTT assay in the SU86.86 CES2 cell line and in a cell line with low CES2 activity (SU86.86 IRES) – benzoyl acid, which is released, should be included as a control; ii) determine whether the benzoyl acids released are cytotoxic to cells; and iii) repeat these *in cellulo* assays in a healthy cell line to demonstrate the probe’s selectivity for cancer cells over normal cells.

### 3.2.2 Design and Synthesis of Compound 3.2: An Eosin B-Monosubstituted Probe (**AK-01-260**)

With the same probe design and activation strategy as the Eosin B-disubstituted probe, only one of the hydroxyl groups on Eosin B is conjugated to a benzoyl ester in compound 3.2 (Figure 3.28). This derivative was synthesized to see whether monosubstitution could effectively quench Eosin
B. As well, one less equivalent of benzoyl acid is released in this design, which will be beneficial if it contributes to cell death.

**Figure 3.28:** Activation strategy for the Eosin B-monosubstituted probe. Upon CES2-mediated hydrolysis of the ester linkages, the closed lactone state will be opened to release free Eosin B, which can fluoresce and generate singlet oxygen with light irradiation.

**Scheme 3.2:** Synthetic scheme for the Eosin B-monosubstituted probe.

Benzoyl chloride was added dropwise to a solution of Eosin B pre-stirred with TEA on ice (Scheme 3.2).

### 3.2.2.1 In Vitro Assays

The UV-Vis absorption spectrum shows that the Eosin B-monosubstituted probe is fully quenched as confirmed by its lack of absorption at a $\lambda_{\text{max}}$ of 520 nm; however, upon CES2-mediated catalysis, the probe successfully releases free Eosin B by cleavage of the mono-ester linkage (Figure 3.29). A fluorescence spectra pre- and post-CES2-mediated hydrolysis of the probe was acquired, which showed an increase in fluorescence at 545 nm (Figure 3.30). Next, a fluorescence time course was acquired for CES1-mediated hydrolysis of the probe; with an excitation wavelength of 520 nm, no increase in emission was seen at 545 nm. This shows the selectivity of the monosubstituted probe for CES2 over CES1 (Figure 3.31).
Figure 3.29: Absorption spectrum of the Eosin B-monosubstituted probe and free Eosin B dye. Eosin B has an $\lambda_{\text{max}}$ at 520 nm. CES2 mediated hydrolysis of the Eosin B-monosubstituted probe. During the release of free Eosin B, an increase in absorbance was observed at 520 nm. Conditions: a) before spectrum (dark red): 1.24 μM probe, at 37°C; and b) after spectrum (grey): 1.24 μM probe, 110 nM CES2, overnight incubation at 37°C. The total DMSO concentration was 1% and the buffer used was DPBS.

Figure 3.30: CES2 mediated hydrolysis of the Eosin B-monosubstituted probe. During the release of free Eosin B, an increase in fluorescence emission was observed at 545 nm. Conditions: a) before spectrum (light orange): 1.24 μM probe, at 37°C; and b) after spectrum (maroon): 1.24 μM probe, 110 nM CES2, after time course at 37°C (excitation 520 nm, emission 545 nm). The total DMSO concentration was 1% and the buffer used was DPBS.

Figure 3.31: CES1 mediated hydrolysis of the Eosin B-monosubstituted probe. During the release of free Eosin B, minimal fluorescence emission was observed at 545 nm. Time course conditions: 1.24 μM probe, 100 nM CES1, at 37°C (excitation 520 nm, emission 545 nm). The total DMSO concentration was 1% and the buffer used was DPBS.
Given that the monosubstituted- and disubstituted-Eosin B probes were both fully quenched, a comparison based on CES2 activation kinetics and background $^1O_2$ production should be made to determine whether the monosubstituted version should proceed to the cellular stage.

### 3.3 Eosin Y-Based Probe

#### 3.3.1 Design and Synthesis of Compound 3.3: An Eosin Y-Disubstituted Probe (AK-01-282)

With the same probe design and activation strategy as the Eosin B-based scaffolds, this probe consists of Eosin Y as the PS instead of Eosin B. The reason for using Eosin Y is because of its higher fluorescence quantum yield that will allow for enhanced *in cellulo* fluorescence imaging ($\Phi_f = 0.67$ for Eosin Y, ethanol; $\Phi_f = 4.5\times10^{-4}$ for Eosin B, water) (Figure 3.32).

![Activation strategy for the Eosin Y-disubstituted probe](image)

**Figure 3.32**: Activation strategy for the Eosin Y-disubstituted probe. Upon CES2-mediated hydrolysis of the ester linkages, the closed lactone state will be opened to release free Eosin Y, which can fluoresce and generate singlet oxygen with light irradiation.

![Synthetic scheme for the Eosin Y-disubstituted probe](image)

**Scheme 3.3**: Synthetic scheme for the Eosin Y-disubstituted probe.

Eosin Y was pre-stirred with TEA and to this solution, benzoyl chloride was added dropwise to yield the Eosin Y-disubstituted probe (Scheme 3.3).
3.3.1.1 *In Vitro* Assays

The UV-Vis absorption spectrum shows that the Eosin Y-disubstituted probe is fully quenched as confirmed by its lack of absorption at the parent dye’s characteristic $\lambda_{\text{max}}$ of 524 nm; however, upon CES2-mediated catalysis, the probe successfully releases free Eosin Y (*Figure 3.33*). A UV-Vis time course, scanned at five-minute intervals, was performed to assess the stability of the probe in DPBS buffer; over a period of 15 minutes, its absorption did not change. The probe remained non-absorptive, indicating that there was no probe conversion to Eosin Y, confirming that the probe is stable in buffer (*Figure 3.34*). Another UV-Vis time course was taken to monitor the fluorescence increase during chemical hydrolysis of the probe at pH 13; at this basic pH level, the ester linkages of the probe were hydrolyzed and released Eosin Y. This assay confirms the successful synthesis of the probe alongside NMR and MS (*Figure 3.35*). Next, a UV-Vis pre- and post-spectrum were taken with CES1-mediated catalysis; the lack of absorption increase indicates that the probe is not selective for CES1 (*Figure 3.36*). A fluorescence time course was then acquired for CES2-mediated hydrolysis of the probe; with an excitation wavelength at 525 nm, an increase in emission was seen at 545 nm during the release of Eosin Y. Lastly, a fluorescence time course was acquired for CES1; an increase in fluorescence emission was observed, but the magnitude was less than that of the CES2 run (*Figure 3.37*).

*Figure 3.33*: Absorption spectrum and CES2-mediated hydrolysis of the Eosin Y-disubstituted probe. During the release of free Eosin Y ($\varepsilon = 112,000 \text{ cm}^{-1}\text{M}^{-1}$ at 525 nm, ethanol), an increase in absorbance was observed at 525 nm. Conditions: a) before spectrum (light orange): 5.7 μM probe, at 37°C; and b) after spectrum (grey): 5.7 μM probe, 110 nM CES2, overnight incubation at 37°C. The total DMSO concentration was 0.5%; the total ACN concentration was 0.5%; and the buffer used was DPBS.
**Figure 3.34:** Stability test on the Eosin Y-disubstituted probe. Time course conditions: 5.7 μM probe, at 37°C. The total DMSO concentration was 0.5%; the total ACN concentration was 0.5%; and the buffer used was DPBS.

**Figure 3.35:** Chemical hydrolysis of the Eosin Y-disubstituted probe at pH 13.11. Time course conditions: 5.7 μM probe, five-minute intervals at 37°C (excitation 525 nm, emission 545 nm). The total DMSO concentration was 0.5%; the total ACN concentration was 0.5%; and the buffer used was DPBS.

The chemical hydrolysis test should be repeated to see whether the biphasic rate occurs again.

**Figure 3.36:** Absorption spectrum of the Eosin Y-disubstituted probe. Eosin Y has an λ_{max} at 525 nm. CES1 mediated hydrolysis of the Eosin Y-disubstituted probe. Minimal free Eosin Y was released; a negligible increase in absorbance
was observed at 525 nm. Conditions: a) before spectrum (light orange): 5.7 μM probe, at 37°C; and b) after spectrum (grey): 5.7 μM probe, 100 nM CES1, overnight incubation at 37°C. The total DMSO concentration was 0.5%; the total ACN concentration was 0.5%; and the buffer used was DPBS.

**Figure 3.37:** Selectivity test on the Eosin Y-disubstituted probe. Time course conditions: a) CES2 (light orange): 5.7 μM probe, 110 nM CES2, at 37°C; b) CES1 (red): 5.7 μM probe, 100 nM CES1, at 37°C; and c) hydrolysis test (brown): 5.7 μM probe, at 37°C (excitation 525 nm, emission 545 nm). The total DMSO concentration was 0.5%; the total ACN concentration was 0.5%; and the buffer used was DPBS.

Compared to the Eosin B-disubstituted probe, the following can be concluded for the Eosin Y-disubstituted probe: it is fully quenched in its “off” state as minimal background absorption or fluorescence was detected; its rate of activation by CES2 is the same based on the CES2 fluorescence time course assay (200 minutes); and the released Eosin Y dye will emit fluorescence at a higher intensity due to its higher FQY. The latter can be determined by repeating the CES2 fluorescence time course assays for both probe derivatives to obtain the intensity value once a plateau has been reached. However, this probe does show activation by CES1 based on **Figure 3.37**; but the activation rate and increase in fluorescence emission is less than that of CES2. Nevertheless, this probe was tested *in cellulo* for its ability to fluoresce in the SU86.86 cancer cell line – which is more advantageous than the Eosin B probe since Eosin B has an extremely low FQY in water, preventing fluorescence detection *in cellulo*.

### 3.3.1.2 *In Cellulo* Fluorescence Assay

The next step was to see whether this probe could fluoresce *in cellulo* after activation unlike the Eosin B-based disubstituted probe. The probe was added to SU86.86 CES2 cells at various concentrations and incubated for five hours, followed by fluorescence imaging (**Figure 3.38** and
3.39). As the concentration of the probe increased, the mean fluorescence intensity also increased (Figure 3.39).

**Figure 3.38:** Fluorescence images of SU86.86 cells after incubation with the Eosin Y-disubstituted probe. Cells were incubated with 0 µM (first row, left), 0.5 µM (first row, right), 1 µM (second row, left), 2.5 µM (second row, right), 5 µM (third row, left), 10 µM (third row, right), 15 µM (fourth row, left), and 20 µM (fourth row, right) probe for 5h then imaged (excitation 525 nm, emission 545 nm). Bright field image (left); dark field image (right).

**Figure 3.39:** Effect of probe concentration on mean fluorescence intensity. An increase in probe concentration led to an increase in fluorescence. The concentration in which the fluorescence emitted from Eosin Y was visible after CES2 activation and light irradiation at 530 nm was 20 µM (mean FI of 885.47).
A concentration-dependent increase in fluorescence intensity was not observed with an increasing probe concentration. This may result from CES2 having a high $K_m$ towards the probe, causing a switch-like behaviour in fluorescence at 20 µM. The next steps for this probe include the following: i) measure ROS production in the SU86.86 CES2 cell line; ii) quantify cell death via an MTT assay in the SU86.86 CES2 cell line and in a low CES2 activity cell line (SU86.86 IRES); iii) repeat the ROS imaging assay using higher probe concentrations to determine whether CES2 has a high $K_m$ towards the probe; and iv) repeat these in cellulo assays in a healthy cell line to demonstrate the probe’s selectivity for cancer cells over normal cells.

3.3.2 Design and Synthesis of Compound 3.4: An Eosin Y-Monosubstituted Probe (AK-01-282)

With the same probe design and activation strategy as the Eosin Y-disubstituted probe, only one of the hydroxyl groups on Eosin Y is conjugated to a benzoyl ester in compound 3.4 (Figure 3.40). This derivative was synthesized to see whether monosubstitution could effectively quench Eosin Y. As well, one less equivalent of benzoyl acid is released in this design, which will be beneficial if it contributes to cell death.

**Figure 3.40:** Activation strategy for the Eosin Y-monosubstituted probe. Upon CES2-mediated hydrolysis of the ester linkages, the closed lactone state will be opened to release free Eosin Y, which can fluoresce and generate singlet oxygen with light irradiation.

**Scheme 3.4:** Synthetic scheme for the Eosin Y-monosubstituted probe.
Eosin Y was pre-stirred with TEA and to this solution, benzoyl chloride was added dropwise on ice to yield the Eosin Y-monomosubstituted probe (Scheme 3.4). The next step for this probe is to conduct in vitro CES2 assays.

3.4 Summary of 6-Aminophenalenone-Based Probes

The probe design was based on the Benz-AP probe, a CES2-selective ratiometric fluorogenic probe, where 6AP is conjugated to a benzoyl group trigger group via an amide linkage (Figure 3.41).

![Figure 3.41: Activation of Benz-AP by CES2-mediated hydrolysis of the amide linkage to release 6AP as a free F/PS.](image)

The 6-aminophenalenone-based probes consist of 6-aminomethylphenalenone (6AMP) as the PS/Q and 6-aminophenalenone (6AP) as the F (Figure 3.42). Given that 6AMP is a PS, these probes can participate in PDT-applications, whereas Benz-AP can only serve as a fluorogenic probe for diagnostic purposes. In the “off” state, 6AMP loses the ability to generate singlet oxygen and 6AP becomes non-emissive. After CES2-mediated amide hydrolysis, light irradiation causes unquenched 6AP to fluoresce and 6AMP to generate singlet oxygen (Figure 3.42). Synthesis involved a two-step reaction where the Q/PS was conjugated to a trigger group and then attached to the F (Table 3.1).
Figure 3.42: Chemical structure and activation strategy for the series of proposed 6AP-based probes.

A summary of the synthesis conditions for the series of 6AP-based probes (Table 3.1).

<table>
<thead>
<tr>
<th>6AP-Based Probe</th>
<th>Synthesis Conditions</th>
<th>Conclusion</th>
</tr>
</thead>
</table>
| **Compound 3.5** | Conditions Attempted:  
  • i) 4-(chlorosulfonyl)-benzoic acid was stirred with 6AMP and TEA to generate the sulfonamylmethylamido linkage (AK-01-249)  
  • ii) the next step was attempted with the following conditions:  
  ▪ a pre-stirred solution of 6AP in sodium hydride was transferred to a pre-activated solution of AK-01-249 with HATU and DIPEA  
  ▪ sodium hydride was changed to TEA due to potential deactivation during its transfer  
  ▪ AK-01-249 was converted to an acid chloride intermediate using oxalyl chloride in THF, to which was added a pre-stirred solution of 6AP in pyridine  
  ▪ the order in which 6AMP and 6AP was attached to the trigger group was switched (in other words, 6AP was put in the sulfonamide linkage instead of 6AMP | Probe was not synthesized. |
| Compound 3.6 | Conditions Attempted:  
| | • i) 4,4’-dicarboxydiphenylether was added to a pre-stirred solution of 6AMP with HATU and TEA to generate the methyl-amide linkage (AK-01-253)  
| | • ii) the next step was attempted with the following conditions:  
| | ▪ a pre-stirred solution of 6AP with DIPEA was transferred to a pre-activated solution of AK-01-253 with HATU and DIPEA  
| | ▪ AK-01-253 was converted to an acid chloride intermediate using oxalyl chloride in THF, to which was added a pre-stirred solution of 6AP in pyridine  
| | ▪ the order in which 6AMP and 6AP was attached to the trigger group was switched | Probe was not synthesized. |
| Compound 3.7 | Conditions Attempted:  
| | • i) 4-(bromomethyl)benzoic acid was added to a pre-stirred solution of 6AMP with DIPEA for a SN2 reaction to generate AK-01-258  
| | • ii) the next step was attempted with the following conditions:  
| | ▪ a pre-stirred solution of 6AP with sodium hydride was transferred to a pre-activated solution of AK-01-258 with HATU and DIPEA  
| | ▪ AK-01-258 was converted to an DCC-intermediate with DIPEA, to which was added a pre-stirred solution of 6AP with TEA  
| | ▪ AK-01-258 was converted to an acid chloride intermediate using oxalyl chloride in THF, to which was added a pre-stirred solution of 6AP in pyridine and toluene  
| | ▪ the order in which 6AMP and 6AP was attached to the trigger group was switched | Probe was not synthesized. |

Table 3.1: Summary of the synthesis conditions conducted for the series of proposed 6AP-based probes.

Unless otherwise stated, the solvent system used was DCM for all conditions. Given the synthetic difficulties encountered with attaching 6AP, these probes were not pursued further.

3.5 Summary and Future Directions

The goal of the CES project is to design and synthesize an enzyme-activatable PDT-beacon that is selective towards CES2 for fluorescence-guided PDT applications. Two probes have been successfully synthesized and brought to the cellular stage: the Eosin B-disubstituted probe and the
Eosin Y-disubstituted probe. The former has been shown to generate ROS in the SU86.86 CES2 cell line with the use of the ROS Sensor DCFHDA. The next steps for this probe are to conduct an MTT assay to quantify cell death in cell lines that have high and low CES2 activity and to repeat these in cellulo assays in a healthy cell line to show selectivity for cancer cells over normal cells. The latter was shown to fix the low fluorescence quantum yield problem in the Eosin B-based scaffold; optimal fluorescence emission was visible at 20 μM in the SU86.86 cell line. The next step is to repeat the fluorescence imaging assay with higher probe concentrations to determine whether the switch-like increase at 20 μM can be attributed to a potential high $K_m$ of CES2 towards the probe; the $K_m$ of CES2 should be determined. The Eosin B-monosubstituted probe has been fully characterized in vitro and is ready for cell work. The remaining Eosin Y-monosubstituted probe must be characterized further in vitro to show its activation by CES2, to confirm its selectivity for CES2 over CES1, and to fluoresce upon CES2-mediated activation. The 6AP-6AMP scaffolds have not yet been synthesized due to synthetic struggles; different approaches must be taken to combat the challenge with coupling the 6AP dye. A future direction for this project is to develop an enzyme-activatable PDT-beacon that absorbs and emits in the NIR region to maximize the penetration depth of light upon irradiation; please refer to chapter 5 under “5.51 DDAO-Tetabrominated-Based CE Probe” to find the DDAO-acetyl and DDAO-benzoyl ester probes (Figure 3.43).

![Chemical structure of DDAO-acetyl and DDAO-benzoyl ester probes](image-url)

Figure 3.43: Chemical structure of the DDAO-acetyl and DDAO-benzoyl ester probes.
Chapter 4
Design, Synthesis, and Characterization of Azoreductase-Activatable Fluorescent-PDT Beacons: Results and Discussion

The goal of chapter 4 is to develop an Azoreductase-activatable fluorescent-PDT beacon that can selectively kill cancer cells over healthy cells by taking advantage of the overexpression of Azoreductases in hypoxia; hence, an in-depth understanding of the Azoreductases enzyme and PDT applications in hypoxia is needed in order to rationally design a successful Azoreductases-selective probe.

4 Reductases Associated with Hypoxia

4.1 Azoreductases

Azoreductases are a group of NAD(P)H dependent flavoenzymes that facilitate electron transport for various biochemical reactions and are made by the microbial flora in human intestines (Figure 4.1).62 They also participate in xenobiotic metabolism of pre-carcinogen azo dyes (–N=N–), nitroaromatic compounds, and azoic drugs by chemical reduction using an electron donation mechanism (Figure 4.2).62 Nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) is used as the reducing cofactor with FMN, the other flavin-containing cofactor, either present or absent.62 An example of the Azoreductase-mediated hydrolysis of the Amaranth probe, illustrated by Gawai et al., is shown below (Figure 4.3).

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Figure 4.1: Crystal structure of a dimeric flavin-dependent Azoreductase. Figure retrieved from Misal, S. A. & Gawai, K. R. Azoreductase: A Key Player of Xenobiotic Metabolism. Bioresources and Bioprocessing 5, (2018).

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3 The in cellulo work presented in chapter 4 was conducted by my colleague, Reta Bodagh.
Figure 4.2: Mechanism of activation of the Amaranth dye by azoreductases. First, a hydride is released upon oxidation of NADH, which is transferred to the FMN at the azoreductase active site. The probe binds to the active site, which becomes reduced by a hydride transfer from FMN. Second, another hydride transfer occurs from NADH through FMN to the probe. Figure retrieved from Misal, S. A. & Gawai, K. R. Azoreductase: A Key Player of Xenobiotic Metabolism. Bioresources and Bioprocessing 5, (2018).

Figure 4.3: Activation of Amaranth probe by azoreductases. The UV-Vis spectra before (blue) and after (green) azoreductase-mediated cleavage of the azo bond in 24 h, releasing the free dye (4-amino-3-hydroxynaphthalene-2,7-disulfonic acid). Figure retrieved from Misal, S. A. & Gawai, K. R. Azoreductase: A Key Player of Xenobiotic Metabolism. Bioresources and Bioprocessing 5, (2018).

4.2 PDT in Hypoxia

As a brief recap, PDT photoirradiates a PS to kill cancer cells by generation of singlet oxygen from tissue oxygen at the target area. Since type II PSs require the presence of molecular oxygen during PDT, researcher have tested whether PDT can still be successful in hypoxia – which occurs when there is a deficiency in the amount of oxygen in tissues. Mild hypoxia is typically found in solid
tumors (Oxygen levels at 5%). Urano et al. have developed an azo-based PS that becomes activated in cancer cells under mild hypoxia and undergoes PDT that selectively ablates cancer cells while leaving neighboring normoxic cells intact (Figure 4.4). The group discovered that PDT works just as efficiently at 5% oxygen when compared to healthy cells with a normal oxygen concentration at physiological conditions.

**Figure 4.4:** AzoSeR probe activation in normoxia versus hypoxia. In normoxia, the azo bond is not cleaved, which leaves the probe quenched and unable to generate singlet oxygen. Conversely, the azo bond becomes cleaved by the overexpressed levels of azoreductase in hypoxia, which releases the active photosensitizing agent, SeR. Figure retrieved from Piao, W. et al. Development of an Azo-Based Photosensitizer Activated under Mild Hypoxia for Photodynamic Therapy. *Journal of the American Chemical Society* 139, 13713–13719 (2017).

Their probe consists of an azo functional group in conjugation with a seleno-rosamine PS (Figure 4.4). The azo bond serves as a hypoxia-sensitive moiety and prevents singlet oxygen generation during light irradiation before probe activation since it undergoes ultrafast conformation changes that prevent the PS from attaining ITC; this is analogous to how azo bonds inactivate a dye’s fluorescence emission, as its $S_1$ lifetime is less than 5 picoseconds (Figure 4.5). Moreover, the dimethyl group on the azobenzene ring is needed to facilitate azo bond reduction and the xanthene-ring selenium atom serves to drive ITC after probe activation in order to increase the PS’s singlet oxygen quantum yield via the internal heavy atom effect. In a mild hypoxic environment, the azo group was reductively cleaved by the overexpressed levels of intracellular reductases and thus bioreductive reactions (e.g. azoreductases, quinones reductases, nitroreductases, and cytochrome p450s). Subsequently, PDT promote hypoxic cellular death via singlet oxygen generation from the free photosensitizing SeR dye while leaving normoxic cells intact.

**Figure 4.5:** Time constants of the azoSeR probe and free SeR dye in chloroform. Figure retrieved from Piao, W. et al. Development of an Azo-Based Photosensitizer Activated under Mild Hypoxia for Photodynamic Therapy. *Journal of the American Chemical Society* 139, 13713–13719 (2017).
4.3 Research Objective

The main objective of this project is to develop an azoreductase-activatable fluorescent-PDT beacon that has both fluorescent and photosensitizing functionalities. Currently, the azoSeR probe developed by the Urano group relies on LIVE/DEAD staining to visualize cellular death post-PDT in mildly hypoxic cells. Specifically, the two probes—which will be introduced shortly in this chapter—use a dye that is both a PS and a fluorophore, thus, allowing for fluorescence-guided PDT *in cellulo*. The specific aims of this project are to: design, synthesize, and characterize enzyme-activatable fluorescent-PDT beacons for azoreductases; determine absorption and fluorescence changes *in vitro* by conducting liver microsome assays; assess its selectivity for other reductases; measure fluorescence and ROS generation *in cellulo* using a cancer cell line; and determining its selectivity for cancer cells over healthy cells.

4.4 DABCYL-Based Probe

4.4.1 Design and Synthesis of Compound 4.1: Leucomethylene Blue-DABCYL Probe (AK-01-269)

This probe consists of methylene blue (MB) as the PS and a trigger group that contains an azo bond as the azoreductase targeting moiety (*Figure 4.6*). In the “off” state, the PS is in its reduced leucomethylene blue (LMB) form, which loses the ability to fluoresce and generate singlet oxygen (*Figure 4.7*). Cleavage of the azo linkage with Azoreductases will release LMB, which spontaneously auto-oxidizes to MB (*Figure 4.6*).^40^

*Figure 4.6:* Activation strategy for the DABCYL-based probe. Reduction of the azo bond with Azoreductases will release the trigger group from LMB. Subsequently, LMB spontaneously oxidizes to MB, which can fluoresce and generate singlet oxygen with light irradiation.
To synthesize the LMB-DABCYL probe, lithium aluminum hydride was added to DABCYL to reduce the carboxylic acid to a primary alcohol, which was converted to bromine using N-bromosuccinimide (NBS) and triphenylphosphine (TPP) (Scheme 4.1). Next, LMB was generated by heating MB at 50 °C in 50:50 toluene/water solution with sodium bicarbonate and sodium dithionite. After 30 minutes, LMB and MB separates into the toluene and water layer, respectively. LMB was then transferred to a pre-stirred solution of brominated-DABCYL in DIPEA (Scheme 4.1).

Note that the crude NMR of LMB-DABCYL shows the presence of impurities (A.164). After HPLC purification, there was not enough probe to obtain a $^1$H NMR spectrum, which may affect the in vitro photophysical assays as there is no measure of purity.

### 4.4.1.1 In Vitro Assays

The UV-Vis absorption spectrum shows that the LMB-DABCYL probe is mostly quenched as confirmed by its minimal absorption at the MB’s characteristic $\lambda_{\text{max}}$ of 609 nm and 668 nm; however, liver microsomes (75.33 ng per μL) were added to facilitate an azoreductase-mediated cleavage of the azo bond, which converted the probe to MB (Figure 4.7). A UV-Vis time course, scanned at five-minute intervals, was performed to assess the stability of the probe in 1x PBS buffer; its absorption slightly decreased at λ of 421 nm over a period of 20 minutes, which means that the probe is stable (Figure 4.8). This change in absorbance was plotted as a function of time and the slope was calculated to be -0.0087 (Figure 4.9). Next, another UV-Vis absorption spectrum was performed to assess the probe against Human NAD(P)H:quinone oxidoreductase type I (HNQO1), a cytosolic flavoprotein that reduces quinones to hydroquinone via a two-electron and becomes upregulated 50-fold in cancer cells over normal cells. HNQO1 activated the probe as seen by an absorption increase at a $\lambda_{\text{max}}$ at 609 nm and 668 nm (Figure 4.10). Fluorescence spectra were acquired pre- and post-microsomes-mediated and HNQO1-mediated catalysis of the
probe. By monitoring at the 690 nm emission channel with an using an excitation wavelength of 665 nm, an increase in emission at 690 nm was seen for microsomes and HNQO1 (Figure 4.11 and 4.12). A summary fluorescence time course was included to show the fluorescence-fold increase and the kinetics of probe activation for: probe stability in 10x PBS, a NADPH control, a liver microsomes assay, and an HNQO1 assay (Figure 4.13). Next, an in vitro singlet oxygen assay was performed with the 10-anthracenediylbis(methylene)dimalonic acid (ABDA) sensor (Figure 4.14 and 4.15). Upon interaction with singlet oxygen, the anthracene part of ABDA forms an endoperoxide via the Diels-Alder reaction of singlet oxygen. Consequently, ABDA photobleaches and can be monitored by a decrease in absorbance at λ of 380 nm. Significant amounts of singlet oxygen was produced post-HNQO1 conversion of the probe to MB; a MB control was also conducted as a reference. The quenched probe showed no singlet oxygen production, even after overnight incubation at 37 °C.

**Figure 4.7:** Absorption spectrum and rat liver microsome-mediated reduction of the DABCYL-based probe. During the release of free MB, an increase in absorbance was observed at 609 and 668 nm. Conditions: a) rat liver microsomes spectrum (yellow); b) before spectrum (light orange): 20.7 μM probe, at 37°C; and c) after spectrum (brown): 20.7 μM probe, 75.33 ng per μl rat liver microsomes, 100 μM NADPH, 48 h at 37°C. The total DMSO concentration was 0.5% and the buffer used was 10x PBS.

A MS or HPLC experiment should be done to identify the products formed after the rat liver microsome-mediated reduction assay. After 48 h of incubation, the absorption spectrum acquired has an λmax that is characteristic of MB (609 nm and 668 nm), however, the shape of the peak looks different than MB.
Figure 4.8: Stability test on the DABCYL-based probe. Time course conditions: 16.25 μM probe, five-minute intervals at 37°C (excitation 665 nm, emission 690 nm). The total DMSO concentration was 0.5% and the buffer used was 1x PBS.

Over a period of 20 minutes, there seems to be a reduction in the absorption of the DABCYL component (421 nm), at a rate of -0.0087, alongside an increase in absorption at 609 nm and 668 nm, which is characteristic of MB. Perhaps, the probe may be undergoing photoisomerization between the cis and trans form of the azobenzene.

Figure 4.9: Stability test on the DABCYL-based probe. The slope on this absorbance versus time plot was calculated to be -0.0087, showing the stability of the probe in buffer. Conditions: 16.25 μM probe. The total DMSO concentration was 0.5% and the buffer used was 1x PBS.

Figure 4.10: HNQO1-mediated reduction of the DABCYL-based probe (control). During the release of free MB, an increase in absorbance was observed at 609 and 668 nm. Conditions: a) before spectrum (maroon): 2.5 μM probe, at
37°C; and b) after spectrum (light orange): 2.5 μM probe, 1 μg per ml BSA, 1 μg per ml HNQO1, 100 μM NADPH, overnight incubation at 37°C. The total DMSO concentration was 0.5% and the buffer used was 10x PBS.

It should be noted that the appearance of the absorption peak after HNQO1 activation is characteristic of MB, unlike the spectrum acquired in rat liver microsomes.

Figure 4.11: Rat liver microsome-mediated reduction of the DABCYL-based probe. During the release of free MB, an increase in fluorescence was observed at 690 nm. Conditions: a) before spectrum (light orange): 2.5 μM probe, at 37°C; b) after 24 h spectrum (maroon): 2.5 μM probe, 75.33 ng per μl rat liver microsomes, 100 μM NADPH, 24 h at 37°C; and c) after 48 h spectrum (maroon): 2.5 μM probe, 75.33 ng per μl rat liver microsomes, 100 μM NADPH, 48 h at 37°C. The total DMSO concentration was 0.5% and the buffer used was 10x PBS.

Figure 4.12: HNQO1-mediated reduction of the DABCYL-based probe (control). During the release of free MB, an increase in fluorescence was observed at 690 nm. Conditions: a) before spectrum (light orange): 2.5 μM probe, at 37°C; and b) after spectrum (dark orange): 2.5 μM probe, 1 μg per ml BSA, 1 μg per ml HNQO1, 100 μM NADPH, overnight incubation at 37°C. The total DMSO concentration was 0.5% and the buffer used was 10x PBS.
Given these in vitro assays, it can be concluded that the LMB-DABCYL probe is successfully activated by HNQO1; however, further characterization of product formation must be done to demonstrate activation by rat liver microsomes.

Based on the singlet oxygen assay above, the slope calculated for the probe was -0.0139, which is comparable to that of MB (-0.0272). This confirms that LMB is indeed released after reduction of the azo bond of DABCYL, as it spontaneously oxidizes to MB – the active form in which singlet oxygen will be generated upon light irradiation.
**Figure 4.15:** Mechanism of ABDA detection of singlet oxygen. Interaction with singlet oxygen causes the anthracene component of the sensor to form an endoperoxide via the Diels-Alder reaction. Consequently, ABDA photobleaches and decreases in absorbance. Figure retrieved from Song, R. et al. Phytoalexin Phenalenone Derivatives Inactivate Mosquito Larvae and Root-knot Nematode as Type-II Photosensitizer. *Scientific Reports* 7, (2017).

Furthermore, it is known that azobenzenes photoisomerize during light irradiation; they undergo trans-to-cis isomerization with UV light irradiation, which promotes a \( \pi-\pi^* \) transition (at the S2 state) (Figure 4.16). The reverse, thermal cis-to-trans isomerization, can be induced in the dark, as the trans form is thermodynamically more stable, or via blue light irradiation, which promotes a n-\( \pi^* \) transition (at the S1 state) (Figure 4.16). Azobenzene photoisomerization can occur via four different pathways; the rotational route involves rupture of the azo bond to permit free rotation around the N-N bond. Hence, a photoisomerization test was conducted to see whether the probe was converted to the azo-reduced form. A UV-Vis time course, scanned at five-minute intervals, was performed while the probe was subjected to light irradiation at 415 nm; a decrease in absorption should be seen at DABCYL’s wavelength as the azo bond becomes reduced or isomerized.

**Figure 4.16:** Photoisomerization of azobenzenes. The trans-to-cis isomerization is induced by 365 nm while the cis-to-trans isomerization is induced by 440 nm. Figure retrieved from Bandara, H. M. D. & Burdette, S. C. ChemInform Abstract: Photoisomerization in Different Classes of Azobenzene. *ChemInform* 43, (2012).
Based on the UV-Vis time course, no significant decrease in DABCYL’s wavelength was observed, which suggests that the azo bond is not being cleaved nor is it photoisomerizing (Figure 4.17). However, this contrasts what was observed in the stability test; perhaps repetition of this assay is needed. A MS experiment should also be done to determine the resulting compound after performing the photoisomerization test to see whether the azo has been reduced.

In the future, an additional microsome assay can be performed using a higher concentration of microsomes (150.66 ng per μL) to assess the kinetics of probe activation. Given these promising in vitro results, the probe was tested in cellulo.

### 4.4.1.2 In Cellulo MTT Assay

An MTT assay was performed to quantify cell death with probe introduction to MCF-7 breast cancer cells; this was tested in both hypoxia and normoxia. First, various concentrations of the probe (0 μM, 1 μM, and 5 μM) were added to MCF-7 cells and incubated for two hours in hypoxic (1% oxygen) and normoxic conditions (atmospheric levels of oxygen, at 20-21%). Afterwards, PDT was performed at 625 nm for 5 minutes; the cells were then left to incubate overnight. Dark controls without light irradiation were also conducted. A colorimetric assay was performed the next day using 1 mg/ml of the yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which was used to measure cellular metabolic activity as an indicator of cell viability (Figure 4.18). Viable cells with NAD(P)H-dependent oxidoreductase enzymes will reduce MTT to formazan, a deep purple insoluble crystalline compound. Addition
of a solubilizing solution will allow for an absorbance spectrum to be acquired at 500-600 nm for quantification of cell death.65

Figure 4.18: Components of an MTT assay. In viable cells, the presence of NAD(P)H-dependent oxidoreductase enzymes will convert the MTT reagent to an insoluble crystalline compound called formazan. The absorbance of formazan is acquired and used to quantify cell death by employing the equation shown above. Figure adapted and retrieved from Sittampalam, GS, Coussens, NP, Brimacombe K, et al., editors. Bethesda. Assays User Manual. (Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2004).

According to the graph, highest cellular death seen in a hypoxic environment post-PDT as well as when probe concentration was increased; the greatest cell death was observed at 5 μM probe incubated in hypoxia with light. However, a considerable amount of cell death was also seen in hypoxia in the dark; this may have been a result of the low percentage of oxygen present (1%) induced during cell incubation. Perhaps, a mildly hypoxic environment of 5% oxygen can be attempted. In normoxia in the dark, there was minimal cellular death, as expected, since azoreductases are not overexpressed to promote probe activation. Lastly, in normoxia with light, substantial cellular death was observed, which may be due to the overexpressed levels of HNQO1 present in MCF-7 cells (Figure 4.19). An MB control was also conducted, where minimal cellular death was observed in normoxia in both light and dark experiments since MB does not penetrate the cell membrane (Figure 4.20).
Figure 4.19: MTT cell death assay on the DABCYL-based probe. The percent of viable cells were quantified at various probe concentrations (0 μM, 1μM, and 5 μM) under the following conditions: normoxia, dark (black); normoxia, light (grey); hypoxia, dark (green); and hypoxia, light (red). The greatest amount of cell death was observed at 5 μM probe post-PDT in cells under a hypoxic environment.

Figure 4.20: MTT cell death assay on MB. The percent of viable cells were quantified at various concentrations of MB (0 μM, 0.25 μM, 0.5 μM, 1μM, 2 μM, and 4 μM) under normoxia post-PDT or in the dark. Given that MB is unable to cross the cell membrane, minimal amount of cell death was observed across all tested MB concentrations.

Overall, the *in vitro* and *in cellulo* data indicates that LMB-DABCYL may serve as a HNQO1 activatable-fluorescent PDT beacon. To confirm this hypothesis, an MTT assay should be done in cells with underexpressed levels of HNQO1 to see whether the probe can be activated better by Azoreductases.
4.5  Azobenzene-Based Probe

4.5.1  Design and Synthesis of Compound 4.2: Leucomethylene Blue-Azobenzene Probe (AK-01-278)

With the same probe design, activation, and synthesis as LMB-DABCYL, an azobenzene was used instead of DABCYL to determine whether the dimethyl group is needed for probe activation and what effect it has on the rate of azo bond reduction (Figure 4.21).

![Figure 4.21: Activation strategy for the azobenzene-based probe. Upon azoreductase-mediated reduction of the azo bond, the trigger group is released and separates from LMB. Subsequently, LMB spontaneously oxidizes to MB, which can fluoresce and generate singlet oxygen with light irradiation.]

![Scheme 4.3: Synthetic scheme to synthesize the azobenzene-based probe.]

4.5.1.1  In Vitro Assays

The UV-Vis absorption spectrum shows that the LMB-azobenzene probe is mostly quenched as confirmed by its minimal absorption at MS’s characteristic $\lambda_{\text{max}}$ of 609 nm and 668 nm. Liver microsomes (75.33 ng per $\mu$L) were added to promote azo bond cleavage by Azoreductases to release MB (Figure 4.22). However, the azobenzene version was activated less by liver microsomes as compared to the DABCYL version. A UV-Vis time course, scanned at five-minute intervals, was performed to test probe stability in 1x PBS buffer. A slight absorption decrease at $\lambda$ 421 nm was observed. (Figure 4.23 and 4.24). Next, a UV-Vis absorption spectrum was conducted with Human NAD(P)H:quinone oxidoreductase type I (HNQO1), which showed probe activation as evident by an absorption increase at $\lambda_{\text{max}}$ at 609 nm and 668 nm (Figure 4.25).
Fluorescence spectra need to be acquired pre- and post-catalysis by microsomes and HNQO1. This can be done by monitoring at the 690 nm emission channel with the excitation wavelength at 665 nm; an increase in emission at 690 nm is expected to be seen for both microsomes and HNQO1. The next steps for this probe are to perform a photoisomerization test with light irradiation at 415 nm to see whether the azo bond becomes reduced or isomerizes; an in vitro singlet oxygen assay with ABDA; an additional microsome assay at a higher concentration of microsomes (150.66 ng per μL) to assess the kinetics of probe activation; and an in cellulo MTT assay to quantify cellular death post-PDT in cells with low HNQO1 activity. Furthermore, the rate of probe activation by HNQO1 and microsomes should be determined and compared between the DABCYL and azobenzene version.

**Figure 4.22:** Absorption spectrum and rat liver microsome-mediated reduction of the azobenzene-based probe. During the release of free MB, an increase in absorbance was observed at 609 and 668 nm. Conditions: a) before spectrum (light orange): 16.25 μM probe, at 37°C; and b) after spectrum (maroon): 16.25 μM probe, 75.33 ng per μl rat liver microsomes, 100 μM NADPH, overnight incubation at 37°C. The total DMSO concentration was 0.5% and the buffer used was 10x PBS.

Compared to the DABCYL version, the azobenzene version is less activated by rat liver microsomes, which suggests the need of the dimethyl group in driving the azo-bond cleavage.
Figure 4.23: Stability test on the azobenzene-based probe. Time course conditions: 16.25 μM probe, five-minute intervals at 37°C (excitation 665 nm, emission 690 nm). The total DMSO concentration was 0.5% and the buffer used was 1x PBS.

Figure 4.24: Stability test on the azobenzene-based probe. The slope on this absorbance versus time plot was calculated to be -0.0087, showing the stability of the probe in buffer. The total DMSO concentration was 0.5% and the buffer used was 1x PBS.

The slope of the absorbance versus time plot was calculated as -0.0018 versus -0.0087 for the DABCYL probe, suggesting that the azobenzene probe is more stable.

Figure 4.25: HNQO1-mediated reduction of the azobenzene-based probe (control). During the release of free MB, an increase in absorbance was observed at 609 and 668 nm. Conditions: a) before spectrum (light orange): 16.25 μM
probe, at 37°C; b) 500 min spectrum (maroon): 16.25 μM probe, 1 μg per ml BSA, 1 μg per ml HNQO1, 100 μM NADPH, 500 min at 37°C; and c) 12 h spectrum (brown): 16.25 μM probe, 1 μg per ml BSA, 1 μg per ml HNQO1, 100 μM NADPH, 12 h at 37°C. The total DMSO concentration was 0.5% and the buffer used was 10x PBS.

Alongside the rat liver microsomes, the azobenzene version is also activated less by HNQO1. Given these in vitro results, compound 4.2 was not pursued further.

### 4.6 Summary and Future Directions

The goal of the Azoreductase project is to design and synthesize an enzyme-activatable PDT-beacon that is selective towards Azoreductases for fluorescence-guided PDT applications. Two probes have been successfully synthesized: the LMB-DABCYL and LMB-azobenzene probe. The former demonstrated cell death in MCF-7 cells in hypoxia during PDT; however, this assay must be repeated under mild hypoxia as a considerable amount of cellular death was seen in the hypoxia/dark control. Moreover, a cell line with low HNQO1 activity must be selected to assess probe activity with Azoreductases. The latter requires further in vitro fluorescence characterizations.
Chapter 5
Design, Synthesis, and Characterization of a Novel Bright NIR Photosensitizer: Results and Discussion

5 Developing a NIR PS

5.1 Research Objective

The goal of chapter 5 is to develop a novel bright NIR PS that can kill cancer cells via singlet oxygen production; with this, the PS can be used to develop aPS PDT beacons to enhance light penetration in tissues for enhanced PDT. The specific aims of this project are to: design, synthesize, and characterize a library of DDAO derivatives; determine their absorption and fluorescence spectra in vitro; calculate fluorescent and singlet oxygen quantum yields in vitro; measure fluorescence and ROS generation in cellulo using in cancer cells; determine selectivity for cancer cells over healthy cells; and to design, synthesize, and characterize enzyme activatable fluorescent-PDT beacons using the DDAO PS derivative.

Many of the photosensitizers used in PDT for cancer therapy have a tetrapyrrole backbone as this structure tends to confer strong absorbance in the red to far-red spectral region (600 – 800 nm), allowing for greater penetration of light into tissues. Ideal PSs fall within this NIR range since photons of wavelengths below 600 nm suffer from poor tissue penetration while wavelengths beyond 800 nm lack the energy to convert molecular oxygen to singlet oxygen. Examples include porphyrins (red light: 630 nm), chlorins (far red light: 650 – 700 nm), bacteriochlorins (NIR light: 700 – 800 nm), and phthalocyanines (far red light: 670 nm). Despite porphyrins being most efficiently activated at the Sorbet band (400 nm), the presence of Q-bands near 630 nm permit red light administration. From porphyrins to chlorins to bacteriochlorins, double-bonds are becoming reduced, which causes a red-shift to the Q band and increases its absorption intensity. Although these tetrapyrrole compounds are NIR PSs, their bulky structure and hydrophobic scaffold may limit use in probes that require small-molecule, hydrophilic PSs.

4 The in cellulo work presented in chapter 5 was conducted by my colleague, Karishma Kailass.
Therefore, DDAO was chosen due to its small-molecule scaffold and based on inspiration from DDAB probe produced by the Yang group. Given that the DDAB probe was successful in imaging cancer cells that have overexpressed CES2 levels in cellulo and in vivo, the hypothesis was that introducing heavy atoms into the ring of the DDAO fluorophore would increase the triplet yield to produce a novel PS. Given that DDAO absorbs and emits in the far-red region, conjugation of its hydroxyl handle with the same trigger group would generate a novel NIR CES2-selective PDT beacon.

5.2 DDAO SAR

A series of DDAO derivatives were synthesized by converting the NIR DDAO fluorophore into a NIR PS (Figure 5.1). Note that the crude NMR of DDAO and the series of DDAO derivatives show the presence of impurities (A1.77, A1.84, A1.86, A1.88, A1.90, and A1.92). After HPLC purification, there was not enough probe to obtain a $^1$H NMR spectrum, which may affect the in vitro photophysical assays as there is no measure of purity.

The greatest amount of singlet oxygen produced was from tetrabrominated-DDAO (13-fold higher in SOS fluorescence than DDAO) and monoiodinated-DDAO (14-fold higher in SOSG fluorescence than DDAO). The fold-changes were calculated using the singlet oxygen sensor (SOSG), where the slope (33.28 for tetrabrominated-DDAO and 34.09 for monoiodinated-DDAO) was obtained from a fluorescence intensity versus time plot (Figure 5.2). SOSG has an anthracene and fluorescein component in its chemical structure. Upon contact with singlet oxygen, anthracene forms an endoperoxide via the Diels-Alder reaction and as a result, its fluorescence emission increases at 525 nm (Figure 5.3).$^{67}$ Fluorescent quantum yields were also obtained (Figure 5.1).

Figure 5.1: Chemical structures of the various DDAO derivatives.
Figure 5.2: Singlet oxygen assay using SOSG on the various DDAO derivatives synthesized. The slopes from the fluorescence intensity versus time graph have been calculated as: 34.09 for DDAO-monoiodinated; 33.28 for DDAO-tetrabrominated; 22.82 for DDAO-dibrominated; and 2.26 for DDAO.

Figure 5.3: Mechanism of SOSG detection of singlet oxygen. Interaction with singlet oxygen causes the anthracene component of the sensor to form an endoperoxide via the Diels-Alder reaction. Consequently, SOSG cannot PET quench the fluorescein component, causing SOSG-EP to become highly fluorescent. Figure retrieved from Kiesslich, T. et al. A Comprehensive Tutorial on In Vitro Characterization of New Photosensitizers for Photodynamic Antitumor Therapy and Photodynamic Inactivation of Microorganisms. BioMed Research International 8 (2013).

5.2.1 Synthesis of Compound 5.1: DDAO (AK-01-293)

As mentioned in the introduction, carbazines are a class of far-red fluorophores. An example is DDAO, 1,3-dichloro-7-hydroxy-9,9-dimethylacridin-2(9H)-one, a small-molecule bright NIR fluorophore that absorbs at $\lambda_{\text{max}}$ 602 nm and emits at $\lambda_{\text{em}}$ 659 nm ($\varepsilon = 36,000 \text{ M}^{-1}\text{cm}^{-1}$, $\Phi = 0.39$) (Figure 5.4). The Yang group developed a CES2-activatable fluorophore called DDAB that is sensitive and has fast kinetics. DDAB consists of the DDAO fluorophore, which is quenched by attaching a benzoyl group with its hydroxyl handle to form an ester linkage (Figure 5.5). DDAB
was successfully activated with CES2 \textit{in vitro, in cellulo,} and in an \textit{in vivo} mouses model (\textbf{Figure 5.6 and 5.7}).

\textbf{Figure 5.4:} Chemical structure of DDAO.

\textbf{Figure 5.5:} Activation strategy for the DDAB probe. Upon CES2-mediated hydrolysis of the ester bond, the free DDAO dye is released and becomes active. Figure retrieved from Jin, Q. et al. A Highly Selective Near-Infrared Fluorescent Probe for Carboxylesterase 2 and its Bioimaging Applications in Living Systems. \textit{Drug Metabolism and Pharmacokinetics} \textbf{32}, (2017).

\textbf{Figure 5.6:} Activation of the DDAB probe by CES2, monitored by its change in absorption and fluorescence. During CES2-mediated hydrolysis of the ester linkage, free DDAO becomes released, resulting in an increase in absorbance at 646 nm and fluorescence at 659 nm (alongside a decrease in absorbance at 430 nm). Figure retrieved from Jin, Q. et al. A Highly Selective Near-Infrared Fluorescent Probe for Carboxylesterase 2 and its Bioimaging Applications in Living Systems. \textit{Drug Metabolism and Pharmacokinetics} \textbf{32}, (2017).
Figure 5.7: Activation of the DDAB probe by CES2 in living cells and in vivo mouse models. Figure retrieved from Jin, Q. et al. A Highly Selective Near-Infrared Fluorescent Probe for Carboxylesterase 2 and its Bioimaging Applications in Living Systems. *Drug Metabolism and Pharmacokinetics* 32, (2017).

To synthesize DDAO, five steps were conducted: i) a tertiary alcohol was formed with the ketone in 3′-hydroxyacetophenone via a Grignard reaction with methylmagnesium bromide; ii) this intermediate was linked to the electrophilic 2,6-dichloro-N-chloro-p-benzoquinoneimine compound in 2 M of sodium hydroxide; iii) the imine formed was reduced with sodium dithionite during the workup; iv) ring closure was promoted by rapidly stirring the compound in 2M deoxygenated HCl; and v) an oxidation step was conducted by heating the resulting suspension at 100°C under argon atmosphere, resulting in a conjugated system that can be easily detected under the UV/Vis lamp by its bright red fluorescence (Scheme 5.1).

Scheme 5.1: Synthetic scheme for DDAO.

5.2.1.1 *In Vitro* Characterizations

The UV-Vis absorption spectrum shows that DDAO has a $\lambda_{\text{max}}$ of 600 nm and 650 nm (Figure 5.7). The fluorescence emission spectrum shows emission at 660 nm (Figure 5.8).
Figure 5.8: Absorption spectrum of DDAO ($\lambda_{\text{max}}$ 600 nm). Conditions: 22 $\mu$M probe. The total DMSO concentration was 1% and the buffer used was PBS.

Figure 5.9: Fluorescence spectrum of DDAO ($\lambda_{\text{em}}$ 650 nm). Conditions: 22 $\mu$M probe. The total DMSO concentration was 1% and the buffer used was PBS.

5.2.1.2 Singlet Oxygen Generation

A singlet oxygen assay was conducted to assess whether DDAO generates singlet oxygen as a type II PS. The fluorescence change of SOSG was measured after four, five-minute intervals of light irradiation at 625 nm for a total of three trials. A 1.84-fold increase in SOSG fluorescence was observed for DDAO over a period of 20 minutes and the slope was determined to be 2.51 (Figure 5.10 and 5.11). Background fluorescence from SOSG was also measured; no significant-fold increase in fluorescence was observed and the slope was determined to be -0.43 (Figure 5.10 and 5.12).
Figure 5.10: Singlet oxygen assay using SOSG on DDAO; SOSG was included as controls. The slopes from the fluorescence intensity versus time graph have been calculated as: 2.51 for DDAO and -0.43 for SOSG.

Figure 5.11: Singlet oxygen assay using SOSG on DDAO. A 1.84-fold increase in SOSG fluorescence was observed for DDAO after five-minute intervals of light irradiation at 625 nm.

Figure 5.12: Singlet oxygen assay on SOSG as a control. A minimal increase in SOSG fluorescence was observed after five-minute intervals of light irradiation at 625 nm.
Hence, DDAO can be used as a standard to compare the singlet oxygen production of the other DDAO derivatives. DDAO was found to not produce a significant amount of singlet oxygen. In the future, a total of three trials should be completed.

5.2.1.3 ROS Production Assay

In addition to the singlet oxygen assay, a ROS production assay was conducted using a general ROS sensor, 2,7-dichlorodihydrofluorescein (DCFH), to establish whether DDAO generates radical species as a type I PS. No significant background fluorescence was detected from the DCFH sensor at 523 nm; a lack of fold increase in fluorescence emission was observed after irradiation at 625 nm for five-minute intervals (Figure 5.13). A 32-fold increase in fluorescence emission was observed for DDAO and the slope was calculated as 0.74 (Figure 5.14 and 5.15).

![Figure 5.13: ROS assay on DCFDA as a control. A minimal increase in DCFDA fluorescence was observed after five-minute intervals of light irradiation at 625 nm. Conditions: 20 µM DCFDA. The total DMSO concentration was 1% and PBS buffer was used.](image)

![Figure 5.14: ROS assay using DCFDA on DDAO. A 32-fold increase in fluorescence emission was observed at 523 nm for DCFH after irradiation of DDAO at 625 nm for five-minute intervals. Conditions: 11.5 µM probe, 20 µM DCFDA. The total DMSO concentration was 1% and PBS buffer was used.](image)
Figure 5.15: ROS assay using DCFDA on DDAO. The slope from the fluorescence intensity versus time plot was calculated to be 0.74. Conditions: 11.5 µM probe, 20 µM DCFDA. The total DMSO concentration was 1% and PBS buffer was used.

Hence, DDAO can be used as a standard to compare the ROS production of the other DDAO derivatives. In the future, a total of three trials should be completed.

5.2.1.4 *In Cellulo* Fluorescence Imaging

The next step was to confirm that DDAO is cell permeable and could fluoresce *in cellulo*. The probe was added to SU86.86 CES2 cells at various concentrations (0 µM, 5 µM, 10 µM, and 20 µM) and incubated for five hours. Afterwards, fluorescence images were taken; as the concentration of the probe increased, the mean fluorescence intensity also increased. At a concentration of 20 µM, the mean fluorescence intensity emitted from DDAO was 2226.89 (Figure 5.16 and 5.17). The next step for DDAO is to conduct a ROS imaging assay *in cellulo* to show that DDAO is not a PS and does not induce cell death.

Figure 5.16: Effect of probe concentration on mean fluorescence intensity. An increase in probe concentration led to an increase in fluorescence. The concentration in which the fluorescence emitted from DDAO was visible after CES2 activation and light irradiation at 530 nm was 20 µM (mean FI of 2226.89).
Figure 5.17: Fluorescence images of SU86.86 cells after incubation with DDAO. Cells were incubated with 0 µM (row 1), 5 µM (row 2), 10 µM (row 3), and 20 µM (row 4) probe for 5h then imaged. Bright field image (left); dark field image (right).

5.2.2 Design and Synthesis of Compound 5.2: Dibrominated-DDAO (AK-01-275)

The design for this probe replaced the two chlorine groups on the periphery of DDAO’s scaffold to two bromine groups; hence, the internal heavy atom effect will be enhanced to promote ITC (Figure 5.18).
The synthesis of dibrominated-DDAO involved the same steps as DDAO, except 2,6-dibromo-N-chloro-p-benzoquinoneimine was used instead of 2,6-dichloro-N-chloro-p-benzoquinoneimine (Scheme 5.2).

Scheme 5.2: Synthetic scheme for dibrominated-DDAO.

5.2.2.1 *In Vitro* Characterizations

The UV-Vis absorption spectrum shows that dibrominated-DDAO has a $\lambda_{\text{max}}$ of 602 nm and 646 nm (Figure 5.19). The fluorescence emission spectrum shows emission at 662 nm (Figure 5.20).

Figure 5.19: Absorption spectrum of dibrominated-DDAO ($\lambda_{\text{max}}$ 602 nm). Conditions: 11 $\mu$M probe. The total DMSO concentration was 1% and the buffer used was PBS.
Figure 5.20: Fluorescence spectrum of dibrominated-DDAO ($\lambda_{em}$ 646 nm). Conditions: 11 µM probe. The total DMSO concentration was 1% and the buffer used was PBS.

5.2.2.2 Singlet Oxygen Generation

A 1.84-fold increase in SOSG fluorescence was observed for dibrominated-DDAO over a period of 20 minutes and the slope was determined to be 22.82 (Figure 5.21 and 5.22). Compared to DDAO (slope = 2.51), dibrominated-DDAO (slope = 22.82) exhibited a 9-fold higher increase in SOSG fluorescence (Figure 5.21). In the future, a total of three trials should be completed.

Figure 5.21: Singlet oxygen assay using SOSG on dibrominated-DDAO. The slope from the fluorescence intensity versus time graph has been calculated as 22.82.
Figure 5.22: Singlet oxygen assay using SOSG on dibrominated-DDAO. A 1.84-fold increase in SOSG fluorescence was observed after five-minute intervals of light irradiation at 625 nm.

5.2.2.3 ROS Production Assay

A 116-fold increase in fluorescence emission was observed for dibrominated-DDAO and the slope was calculated as 2.31 (13-fold and 0.74 for DDAO) (Figure 5.23 and 5.24). Hence, replacing the two chlorines to bromines enhanced the production of ROS.

Figure 5.23: ROS assay using DCFDA on dibrominated-DDAO. A 116-fold increase in fluorescence emission was observed at 523 nm for DCFH after irradiation at 625 nm for five-minute intervals. Conditions: 11.5 µM probe, 20 µM DCFDA. The total DMSO concentration was 1% and PBS buffer was used.
Figure 5.24: ROS assay using DCFDA on dibrominated-DDAO. The slope from the fluorescence intensity versus time plot was calculated to be 2.31. Conditions: 11.5 µM probe, 20 µM DCFDA. The total DMSO concentration was 1% and PBS buffer was used.

5.2.2.4 In Cellulo Fluorescence Imaging

The same conditions as DDAO was used. At a concentration of 20 µM, the mean fluorescence intensity for dibrominated-DDAO was 272.31, which is lower compared to DDAO (2226.89) (Figure 5.25 and 5.26). The next step is to conduct a ROS imaging assay in cellulo to assess whether ROS production can cause cell death, an MTT assay to quantify cellular death; and repetition in healthy cells to show selectivity for cancer cells over normal cells.

Figure 5.25: Effect of probe concentration on mean fluorescence intensity. An increase in probe concentration led to an increase in fluorescence. The concentration in which the fluorescence emitted from dibrominated-DDAO was visible after CES2 activation and light irradiation at 530 nm was 20 µM (mean FI of 272.31).
Figure 5.26: Fluorescence images of SU86.86 cells after incubation with dibrominated-DDAO. Cells were incubated with 0 µM (row 1), 5 µM (row 2), 10 µM (row 3), and 20 µM (row 4) probe for 5h then imaged. Bright field image (left); dark field image (right).

5.2.3 Design and Synthesis of Compound 5.3: Tribrominated-DDAO (AK-01-288)

Synthesized in the same manner as dibrominated-DDAO, tribrominated-DDAO includes an extra brominating step using liquid bromine and DABCO to introduce a third bromine at the periphery of the scaffold (Figure 5.27 and Scheme 5.3).
Figure 5.27: Chemical structure of tribrominated-DDAO.

Scheme 5.3: Synthetic scheme for tribrominated-DDAO.

5.2.3.1 *In Vitro* Characterizations

The UV-Vis absorption spectrum shows that tribrominated-DDAO has a $\lambda_{\text{max}}$ of 614 nm and 660 nm (Figure 5.28). The fluorescence emission spectrum shows emission at 670 nm (Figure 5.29).

Figure 5.28: Absorption spectrum of tribrominated-DDAO ($\lambda_{\text{max}}$ 614 nm). Conditions: 22 µM probe. The total DMSO concentration was 1% and the buffer used was PBS.
5.2.3.2 ROS Production Assay

A 33-fold increase in fluorescence emission was observed for DDAO-tribrominated and the slope was calculated as 0.99 (Figure 5.30 and 5.31). Hence, replacing the two chlorines to three bromines enhanced the production of ROS slightly; however, it decreased the production of ROS from dibrominated-DDAO (116-fold and 2.31 for dibrominated-DDAO).

Figure 5.30: ROS assay using DCFDA on tribrominated-DDAO. A 33-fold increase in fluorescence emission was observed at 523 nm for DCFH after irradiation at 625 nm for five-minute intervals.

Figure 5.31: ROS assay using DCFDA on tribrominated-DDAO. The slope from the fluorescence intensity versus time plot was calculated to be 0.99.
Given that tribrominated-DDAO produce less ROS than dibrominated-DDAO as demonstrated by their slope values of 0.99 and 2.31, tri-brominated DDAO was not pursued further.

5.2.4 Design and Synthesis of Compound 5.4: Tetrabrominated-DDAO (AK-01-288)

Synthesized in the same manner as dibrominated-DDAO, tetrabrominated-DDAO includes an extra brominating step using liquid bromine and DABCO to introduce a third and fourth bromine at the periphery of the scaffold (Figure 5.32 and Scheme 5.4).

![Figure 5.32: Chemical structure of tetrabrominated-DDAO.](image)

**Figure 5.32:** Chemical structure of tetrabrominated-DDAO.

![Scheme 5.4: Synthetic scheme for tetrabrominated-DDAO.](image)

**Scheme 5.4:** Synthetic scheme for tetrabrominated-DDAO.

5.2.4.1 *In Vitro* Characterizations

The UV-Vis absorption spectrum shows that tetrabrominated-DDAO has a $\lambda_{\text{max}}$ of 622 nm and 667 nm (Figure 5.33). The fluorescence emission spectrum shows emission at 670 nm (Figure 5.34).
**Figure 5.33:** Absorption spectrum of tetrabrominated-DDAO ($\lambda_{\text{max}}$ 622 nm). Conditions: 11 µM probe. The total DMSO concentration was 1% and the buffer used was PBS.

**Figure 5.34:** Fluorescence spectrum of tetrabrominated-DDAO ($\lambda_{\text{em}}$ 667 nm). Conditions: 11 µM probe. The total DMSO concentration was 1% and the buffer used was PBS.

### 5.2.4.2 Singlet Oxygen Generation

A 13-fold increase in SOSG fluorescence was observed for tetrabrominated-DDAO over a period of 20 minutes and the slope was determined to be 33.82 ([Figure 5.35 and 5.36](#)). Compared to DDAO (slope = 2.51), tetrabrominated-DDAO (slope = 33.28) exhibited a 13-fold higher increase in SOSG fluorescence and thus greater generation of singlet oxygen ([Figure 5.35](#)). In the future, a total of three trials should be completed.

**Figure 5.35:** Singlet oxygen assay using SOSG on tetrabrominated-DDAO. A 13-fold increase in SOSG fluorescence was observed after five-minute intervals of light irradiation at 625 nm.
Figure 5.36: Singlet oxygen assay using SOSG on tetrabrominated-DDAO. The slope from the fluorescence intensity versus time graph has been calculated as 33.28.

5.2.4.3 Fluorescent Quantum Yield (FQY)

The fluorescence quantum yield of tetrabrominated-DDAO was determined using Thionin Acetate (Φ_{ethanol} 0.5) as a standard since the absorption spectrum overlaps well with the 622 and 666 nm excitation bands of the PS. Comparing the emission spectra of the PS (0.05 A.U. at λ 600 nm) and Thionin Acetate (0.049 A.U. at λ 600 nm), it is evident that the PS is more emissive than the standard (Figure 5.37). After three trials, the FQY of tetrabrominated-DDAO was calculated as Φ_{f} 0.11 (Figure 5.37).

Figure 5.37: Fluorescent oxygen quantum yield of tetrabrominated-DDAO. Using Thionin Acetate as the standard (Φ_{ethanol} 0.5), the FQY of tetrabrominated-DDAO was calculated as Φ_{f} 0.11.
5.2.4.4 *In Cellulo* Fluorescence Imaging

The same conditions as DDAO was used. At a concentration of 20 µM, the mean fluorescence intensity emitted from tetrabrominated-DDAO was 1109.65, which is lower compared to DDAO (2226.89) (Figure 5.38 and 5.39). The next step is to conduct a ROS imaging assay *in cellulo* to assess whether ROS production can cause cell death, an MTT assay to quantify cellular death; and repetition in healthy cells to show selectivity for cancer cells over normal cells.

![Figure 5.38](image)

**Figure 5.38**: Effect of probe concentration on mean fluorescence intensity. An increase in probe concentration led to an increase in fluorescence. The concentration in which the fluorescence emitted from tetrabrominated-DDAO was visible after CES2 activation and light irradiation at 530 nm was 20 µM (mean FI of 1109.65).
Figure 5.39: Fluorescence images of SU86.86 cells after incubation with tetrabrominated-DDAO. Cells were incubated with 0 µM (row 1), 5 µM (row 2), 10 µM (row 3), and 20 µM (row 4) probe for 5h then imaged. Bright field image (left); dark field image (right).

5.2.5 Design and Synthesis of Compound 5.5: DDAO-Monoiodinated (MAMKH-01-305)

Synthesized in the same manner as dibrominated-DDAO, monoiodinated-DDAO includes an extra iodinating step using iodine (bisublimed) and a copper (II) acetate catalyst in acetic acid at reflux to introduce an iodine at the periphery of the scaffold (Figure 5.40 and Scheme 5.5).
Figure 5.40: Chemical structure of monoiiodinated-DDAO.

Scheme 5.5: Synthetic scheme for monoiiodinated-DDAO.

5.2.5.1  *In Vitro* Characterizations

The UV-Vis absorption spectrum shows that monoiiodinated-DDAO has a $\lambda_{\text{max}}$ of 601 nm and 652 nm (Figure 5.41). The fluorescence emission spectrum shows emission at 660 nm (Figure 5.42).

Figure 5.41 Absorption spectrum of monoiiodinated-DDAO ($\lambda_{\text{max}}$ 601 nm). Conditions: 11 µM probe. The total DMSO concentration was 1% and the buffer used was PBS.
Figure 5.42: Fluorescence spectrum of monoiodinated-DDAO ($\lambda_{em}$ 652 nm). Conditions: 11 µM probe. The total DMSO concentration was 1% and the buffer used was PBS.

5.2.5.2 Singlet Oxygen Generation

A 6-fold increase in SOSG fluorescence was observed monoiodinated-DDAO over a period of 20 minutes and the slope was determined to be 34.09 (Figure 5.43 and Figure 5.44). Compared to DDAO (slope = 2.51), monoiodinated-DDAO (slope = 34.09) exhibited a 14-fold higher increase in SOSG fluorescence (Figure 5.43). In the future, a total of three trials should be completed. Tetraiodinated-DDAO should be synthesized to further enhance ITC and thus increase the amount of singlet oxygen production (Figure 5.45).

Figure 5.43: Singlet oxygen assay using SOSG on monoiodinated-DDAO. A 6-fold increase in SOSG fluorescence was observed after five-minute intervals of light irradiation at 625 nm.
Figure 5.44: Singlet oxygen assay using SOSG on monoiodinated-DDAO. The slope from the fluorescence intensity versus time graph has been calculated as 34.09.

![Figure 5.44](image_url)

Figure 5.45: Chemical structure of tetraiodinated-DDAO.

5.2.5.3 Fluorescent Quantum Yield

After three trials, the FQY of monoiodinated-DDAO was calculated as $\Phi = 0.091$ (Figure 5.46). The next steps are to conduct the following: fluorescence imaging *in cellulo*; ROS imaging assay *in cellulo* to assess cellular death with ROS production; MTT assay to quantify cellular death; and repetition in healthy cells to show selectivity for cancer cells over normal cells.

![Figure 5.46](image_url)

**Figure 5.46**: Fluorescent oxygen quantum yield of monoiodinated-DDAO. Using Thionin Acetate as the standard ($\Phi_{\text{ethanol}} = 0.5$), the FQY of monoiodinated-DDAO was calculated as $\Phi = 0.091$. 

$$
\Phi_{\text{sample}} = \Phi_{\text{standard}} \frac{I_{\text{sample}}^{1/2}}{I_{\text{standard}}^{1/2}} \frac{A_{\text{standard}}}{A_{\text{sample}}} \\
\Phi_{\text{sample}} = 0.046 \cdot \frac{1}{1.3617^2} \cdot \frac{10149.33}{0.049} \\
\Phi_{\text{sample}} = 0.091
$$
5.2.6 Design and Synthesis of Compound 5.6: DDAO-Mononitrated (AK-01-307)

Synthesized in the same manner as DDAO, mononitrated-DDAO includes an extra nitrating step using urea nitrate heated using a microwave at 60 °C for 6 hours in a solution of 95:5 ACN/water; a nitro group was introduced at the periphery of the scaffold to determine its effect on SQY and FQY relative to DDAO (Figure 5.47 and Scheme 5.6).

![Diagram of mononitrated DDAO](image)

**Figure 5.47**: Chemical structure of mononitrated-DDAO.

![Scheme 5.6](image)

**Scheme 5.6**: Synthetic scheme for mononitrated-DDAO.

5.2.6.1 *In Vitro* Characterizations

The UV-Vis absorption spectrum shows that mononitrated-DDAO has a $\lambda_{\text{max}}$ of 651 nm (Figure 5.48). The fluorescence emission spectrum shows emission at 656 nm (Figure 5.49).

![Absorption spectrum](image)

**Figure 5.48**: Absorption spectrum of monoidinated-DDAO ($\lambda_{\text{max}}$ 651 nm). Conditions: 11 µM probe. The total DMSO concentration was 1% and the buffer used was PBS.
Figure 5.49: Fluorescence spectrum of monoiiodinated-DDAO (λem 656 nm). Conditions: 11 μM probe. The total DMSO concentration was 1% and the buffer used was PBS.

5.2.6.2 Singlet Oxygen Generation

A 1.4-fold increase in SOSG fluorescence was observed for mononitrated-DDAO and the slope was determined to be 2.26 (Figure 5.50 and 5.51). Compared to DDAO (slope = 2.51), mononitrated-DDAO (slope = 2.26) exhibited a 0.90-fold increase in SOSG fluorescence, suggesting that minimal singlet oxygen was produced (Figure 5.50). In the future, a total of three trials should be completed.

Figure 5.50: Singlet oxygen assay using SOSG on mononitrated-DDAO. A 1.4-fold increase in SOSG fluorescence was observed after five-minute intervals of light irradiation at 625 nm.
Given that monoiodinated-DDAO produces insignificant amounts of singlet oxygen, this derivative was not pursued further.

### 5.3 DDAO-Based-Activatable Fluorescent PDT Beacons

#### 5.3.1 Compound 5.7: Dibrominated-DDAO-Based NTR Probe (AK-01-300)

**5.3.1.1 Design and Synthesis**

This probe consists of dibrominated-DDAO as the PS, which will be quenched with a Nitroreductase (NTR) trigger group, 4-nitrobenzyl. NTR-mediated reduction of the nitro group causes release of free dibrominated-DDAO, which may fluoresce and generate singlet oxygen (Figure 5.52). NTRs are a family of flavin mononucleotide (FMN)- or flavin adenine dinucleotide (FAD)-dependent enzymes that catalyze the metabolism of nitroaromatic and nitroheterocyclic substrates by NAD(P)H reduction.⁶⁹

**Figure 5.52**: Activation strategy for the dibrominated-DDAO-based NTR probe. Upon NTR-mediated reduction of the nitro group, the trigger group is released and separates from dibrominated-DDAO. Free PS fluoresces and generates singlet oxygen with light irradiation.
Scheme 5.7: Synthetic scheme for the dibrominated-DDAO-based NTR probe.

To synthesize this probe, 4-nitrobenzyl bromide was added to a pre-stirred solution of the PS in ACN with silver (I) oxide and 4-Å molecular sieves. This silver-mediated O-alkylation condition promotes functionalization with the hydroxyl handle rather than at its ketone group; the latter occurs after adopting an alternate resonant form, as illustrated with DDAO below (Figure 5.53).^{70}

![Scheme 5.7](image)

Figure 5.53: Conversion of DDAO into fluorogenic probes via a silver-mediated O-alkylation. This reaction results in the formation of two regioisomers, DDAO-AME1 and DDAO-AME2; the site of O-alkylation can be resolved by NOESY. Figure retrieved from Tallman, K. R. & Beatty, K. E. Far-Red Fluorogenic Probes for Esterase and Lipase Detection. *ChemBioChem* **16**, 70–75 (2014).

### 5.3.1.2 In Vitro Assays

The UV-Vis absorption spectrum showed no absorption at dibrominated-DDAO’s $\lambda_{max}$ of 602 nm and 646 nm, indicating that the probe is fully quenched. After NTR-mediated reduction of the nitro group, the absorption spectrum shows the characteristic absorption peaks at 602 nm and 646 nm as the free PS becomes released, allowing for absorption (Figure 5.54). Next, a fluorescence time course was taken during NTR-mediated reduction of the probe with NADH; it showed an increase in emission at 662 nm in 200 seconds. A stability test and NADH control was conducted; both resulted in no fluorescence emission, which suggests that the probe is stable in buffer and is
activated only in the presence of NTR (Figure 5.55). Given these promising *in vitro* results, this probe will be tested in an NTR-overexpressed cancer cell line.

![Absorption spectrum of the dibrominated-DDAO-based NTR probe](image)

**Figure 5.54:** Absorption spectrum of the dibrominated-DDAO-based NTR probe. During the release of free dibrominated-DDAO, an increase in absorbance was observed at 602 and 646 nm. Conditions: a) before spectrum (blue): 1 μM probe, at 37°C; and b) after spectrum (purple): 1 μM probe, 280 nM NTR, 0.1 mM NADH, overnight incubation at 37°C. The total DMSO concentration was 1% and the buffer used was PBS.

![Fluorescence time course showing probe activation with NTR](image)

**Figure 5.55:** Fluorescence time course showing probe activation with NTR. Time course conditions: a) stability test: 1 μM probe, at 37°C; b) NADH control: 1 μM probe, 0.1 mM NADH, at 37°C; and c) NTR assay: 1 μM probe, 280 nM NTR, 0.1 mM NADH, 500 seconds at 37°C.

5.3.2 **Compound 5.8: Dibrominated-DDAO-Based Cytochrome P450 Probe (AK-01-298)**

5.3.2.1 **Design and Synthesis**

As per the probe design for the dibrominated-DDAO-based NTR probe, the trigger group used in this probe is a methyl group for Cytochrome P450 activation (Figure 5.56). Human cytochrome P450s are membrane-bound hemeproteins that oxidize xenobiotics and drugs during phase I metabolism.
Figure 5.56: Activation strategy for the dibrominated-DDAO-based P450 probe. Upon P450-mediated demethylation free PS is released, which may fluoresce and generate singlet oxygen with light irradiation.

Scheme 5.8: Synthetic scheme for the dibrominated-DDAO-based P450 probe.

To synthesize this probe, the same procedure as the dibrominated-DDAO-based NTR probe was used; except methyl iodide was used instead of 4-nitrobenzyl bromide. The next step is to conduct in vitro absorption and fluorescence Cytochrome P450 assays to assess probe activation.

5.3.3 Tetrabrominated-DDAO-Based CES Probe

5.3.3.1 Design and Synthesis of Compound 5.9: Acetylated Probe (AK-01-303)

This probe consists of tetrabrominated-DDAO as the PS, which will be quenched by acetylating its hydroxyl handle. An acetyl group was used as a general ester group as a POC of this scaffold design; this small acyl group is also characteristic of a CES2 substrate (Figure 5.57). CES2-mediated hydrolysis of the ester linkage releases free tetrabrominated-DDAO, which may fluoresce and generate singlet oxygen (Figure 5.57).

Figure 5.57: Activation strategy for the tetrabrominated-DDAO-acetylated probe. Upon CES2-mediated hydrolysis of the acetyl group, free tetrabrominated-DDAO is released, which may fluoresce and generate singlet oxygen with light irradiation.
Scheme 5.9: Synthetic scheme for the tetrabrominated-DDAO-based CES2 probe with an acetyl trigger group.

To synthesize this probe, acetyl chloride was added to a pre-stirred solution of tetrabrominated-DDAO in DCM and TEA in a dropwise manner (Scheme 5.9).

5.3.3.2 In Vitro Assays

The UV-Vis absorption spectrum shows that the tetrabrominated-DDAO-acetylated probe is relatively quenched as confirmed by its minimal absorption at the PS’s $\lambda_{\text{max}}$ of 622 nm and 667 nm (as compared to the unquenched state) (Figure 5.58). Upon CES2-mediated ester cleavage, the probe successfully releases free tetrabrominated-DDAO as evident by its increase in absorption at 622 nm and 667 nm (Figure 5.58). A fluorescence time course was acquired for CES2 and CES1; by monitoring at the 670 nm emission channel with the excitation wavelength at 600 nm, an increase in emission at 670 nm was seen for CES2 only (Figure 5.59).

Figure 5.58: Absorption spectrum of the tetrabrominated-DDAO-based CES2 probe. During the release of free tetrabrominated-DDAO, an increase in absorbance was observed at 622 and 667 nm. Conditions: a) before spectrum (blue): 12.5 $\mu$M probe, at 37°C; b) after spectrum (purple): 12.5 $\mu$M probe, 110 $\mu$M CES2, after time course at 37°C. The total DMSO concentration was 1% and the buffer used was PBS.
Figure 5.59: Fluorescence time course showing probe activation with CES2 over CES1. Time course conditions: a) CES2 assay (light orange) 12.5 μM probe, 110 μM CES2, 15 minutes at 37°C; and b) CES1 assay (grey): 12.5 μM probe, 100 μM CES1, 15 minutes at 37°C.

Given these promising in vitro enzyme assays, this probe will be tested in the SU86.86 CES2 cancer cell line to perform in cellulo fluorescence imaging, ROS imaging, MTT cellular death assay, and repetition in a healthy cell line. Since that the released PS absorbs and emits in the NIR region, this allows for greater tissue penetration for in vivo applications of PDT – unlike the Eosin B and Eosin Y-based CES2 probes discussed in chapter 3.

5.3.3.3 Design and Synthesis of Compound 5.10: Benzoyl Ester Probe (AK-01-304)

This probe is designed similarly to the tetrabrominated-DDAO acetylated probe; however, a benzoyl ester is used as the CES2-trigger group (Figure 5.60).

Figure 5.60: Activation strategy for the tetrabrominated-DDAO-benzoyl ester probe. Upon CES2-mediated hydrolysis of the trigger group, free tetrabrominated-DDAO is released, which may fluoresce and generate singlet oxygen with light irradiation.
Scheme 5.10: Synthetic scheme for the tetrabrominated-DDAO-based CES2 probe with a benzoyl ester trigger group.

To synthesize this probe, benzoyl chloride was added to a solution of tetrabrominated-DDAO in DCM and TEA in a dropwise manner.

5.3.3.4 In Vitro Assays

The UV-Vis absorption spectrum shows that the tetrabrominated-DDAO-benzoyl ester probe is fully quenched as confirmed by its absent absorption at the PS’s $\lambda_{\text{max}}$ of 622 nm and 667 nm (Figure 5.61). Upon CES2-mediated catalysis, the probe successfully releases free tetrabrominated DDAO by cleavage of the ester linkage (Figure 5.61). A fluorescence time course was then acquired for CES2-mediated hydrolysis of the probe; with an excitation wavelength at 600nm, an increase in emission was seen at 670 nm. This corresponds to hydrolysis of the ester linkage after interaction with CES2, allowing free PS to accumulate and to be switched from “off” to “on” (Figure 5.62). A fluorescence time course was also acquired for CES1, which shows that the probe is not selective for CES1 with its minimal fluorescence increase (Figure 5.62). Compared to the acetylated probe, this version is fully quenched.

Figure 5.61: Absorption spectrum of the tetrabrominated-DDAO-based CES2 probe. During the release of free tetrabrominated-DDAO, an increase in absorbance was observed at 622 and 667 nm. Conditions: a) before spectrum (blue): 1.7 μM probe, at 37°C; b) after spectrum (purple): 1.7 μM probe, 110 μM CES2, after time course at 37°C. The total DMSO concentration was 1% and the buffer used was PBS.
Figure 5.62: Fluorescence time course showing probe activation with CES2 over CES1. Time course conditions: a) CES2 assay (light orange) 1.7 μM probe, 110 μM CES2, 15 minutes at 37°C; and b) CES1 assay (grey): 1.7 μM probe, 100 μM CES1, 15 minutes at 37°C.

Given these promising in vitro enzyme assays, this probe can be tested in the SU86.86 CES2 cancer cell line to conduct in cellulo fluorescence imaging, ROS imaging, MTT cellular death assay, and repetition in a healthy cell line. Since that the released PS absorbs and emits in the NIR region, this allows for greater tissue penetration for in vivo applications of PDT – unlike the Eosin B and Eosin Y-based CES2 probes discussed in chapter 3. Moreover, this probe is better quenched than the acetylated version, allowing for lower background signal in its quenched state.

5.4 Summary and Future Directions

The goal of this project is to design and synthesize a novel bright NIR PS and to use the best derivative—with respect to singlet and fluorescent quantum yield—to develop an enzyme-activatable PDT-beacon that is selective towards a reliable cancer biomarker for fluorescence-guided PDT applications. A library of DDAO derivatives have been synthesized which include the following: DDAO-dibrominated, DDAO-tribrominated, DDAO-tetrabrominated (ΦF 0.11), DDAO-monoiodinated (ΦF 0.091), and DDAO-mononitrated. It was found that the tetrabrominated and monoiodinated versions had the highest singlet quantum yield compared to the other derivatives. Next, an NTR-based PDT beacon was developed using the dibrominated PS; based on the in vitro NTR absorption and fluorescence assays, the probe is ready to move to the cell stage. A Cytochrome P450-based PDT beacon was developed and must still be characterized in vitro. The next two PDT beacons used tetrabrominated-DDAO and targeted CES2; an acetyl and benzoyl version were made. Both probes showed selectivity for CES2 over CES1, but the benzoyl probe was fully quenched unlike the acetyl probe. A future direction for this project is to
develop a NIR tetraiodinated-DDAO PS to increase the singlet oxygen quantum yield further as four iodine atoms are present. Subsequently, this will promote ITC better, as compared to having four bromine atoms. Moreover, this PS can then be used to make another CES2-based PDT-beacon that contains a prodrug. For CES2, Capecitabine can be used as it is used to treat various cancers like that of colon, breast, stomach, oesophageal, rectal, and pancreatic (Figure 5.63).

**Figure 5.63:** Future directions of the DDAO project. Tetraiodinated-DDAO can be synthesized to increase the SQY and Capecitabine can be incorporated as a CES2 prodrug.
Chapter 6
Experimental Section

**General Information**: All chemicals and solvents were purchased from Sigma Aldrich. Semi-preparative high-performance liquid chromatography (HPLC) was conducted on a LC-20AT Shimadzu liquid chromatograph; it contains a CBM-20A VP system controller and an SPD-M20A VD diode array detector. High-resolution mass spectrometry (HRMS) was performed by the AIMS Mass Spectrometry Laboratory at the University of Toronto. $^1$H-NMR and $^{13}$C-NMR spectra were acquired using the Bruker 400-MHz NMR spectrometer; the chemical shifts (ppm) are listed based on the tetramethylsilane (TMS) standard. The NMR characterizations are reported with the following notation: chemical shift; multiplicity: singlet (s), doublet (d), multiplet (m); coupling constant (J) in Hz; and integration. Absorbance spectra were acquired with a Shimadzu UV-1800 UV-Vis spectrometer. Fluorescence emission spectra were obtained using a Shimadzu RF-6000 spectrometer. The 415 nm, 530 nm, and 625-nm LED laser were purchased from ThorLabs; it has 18 nm bandwidth, 770 mW LED output power, and a maximum irradiance of 18.0 µW mm$^{-2}$.

**Concentration Measurements**: Probe concentration was measured using UV-Vis spectroscopy. First, DMSO was used to prepare a concentrated stock solution. Second, the probe stock was diluted 100x in PBS buffer (or 10x PBS or DPBS buffer) in a 60 µL cuvette to acquire the UV-Vis spectrum. Next, the concentration was determined; the combined extinction coefficient values for the FRET Q and FRET PS/F were used for FRET-based probes.

**Preparation of MCF-7 Whole Cell Lysates** (GST Project): As per the instructions stated by Folco et al., the following alterations were made: i) MCF-7 cells were cultured in 6 x T-75 flasks containing Dulbecco’s Modified Eagle’s medium (DMEM) with penicillin (100 U mL$^{-1}$), streptomycin (100 µg mL$^{-1}$), and 10% fetal bovine serum, using a humidified incubator at 37ºC with 5% CO$_2$ until 90% confluence (approximately 16 x 10$^6$ cells) was attained; ii) for protease inhibition, the Roche complete mini EDTA-free tablets were utilized; iii) MCF-7 cell lysis was performed by passing the cells through a 21 gauge needle (20 times) and a 16 gauge needle (15 times); iv) the cytosolic fraction was combined with the nuclear fraction; and v) the resulting extracts were divided into 60 µL aliquots and stored at -80ºC; the storage buffer contained: 10 mM HEPES (pH 7.9) with 1.5 mM MgCl$_2$, 10 mM KCl, and 0.5 mM dithiothreitol (5% glycerol and 1x protease inhibitor). A Bradford assay was used to measure the total protein concentration, with bovine serum albumin (125-2000 µg/mL) as the protein standard. This assay kit was purchased from Bio-Rad.

**Fluorescence Acquisition in Cell Extracts** (GST Project): MCF-7 whole cell lysates were diluted in 10 mM HEPES (pH 7.9) buffer with 10 mM KCl, 1.5 mM MgCl$_2$, and 0.5 mM dithiothreitol (5% glycerol and 1x protease inhibitor) to obtain a protein concentration of 46 µg/60µL.
**Fluorescence Microscopy:** Cell images were acquired using a filter cube with the 457-538 nm EGFP excitation bandpass filter (CES2 Eosin B-based probe), the 488-554 nm YFP excitation bandpass filter (CES2 Eosin Y-based probe), and the 503-557 nm AP excitation bandpass filter (DDAO probes); the >500 nm dichroic mirror; and the 502-538 nm EGFP emission bandpass filter (CES2 Eosin B-based probe), the 532-554 nm YFP emission bandpass filter (CES2 Eosin Y-based probe), or the 600-700 nm AP emission bandpass filter (DDAO probes).

**ROS Imaging** (CES2 Project: Eosin Y-Based Probe): SU86.86 CES2 cells were incubated with various probe concentrations in Opti-MEM for 3 hours and then washed once with DPBS. 10 μM of DCFHDA was added and incubated for 30 minutes, followed by three washes with DPBS. Fresh Opti-MEM was used to replace DPBS. The appropriate wells were irradiated for 10 minutes with the 530 nm LED light and then imaged using the microscope.

**Fluorescence Imaging** (CES2 Project: Eosin Y-Based Probe): SU86.86 CES2 cells were incubated with various probe concentrations in Opti-MEM for 5 hours, washed once with DPBS and then imaged using the microscope.

**Fluorescence Imaging** (DDAO Project): SU86.86 CES2 cells were incubated with various probe concentrations in Opti-MEM for 5 hours, washed once with DPBS then imaged using the microscope.

**Phototoxicity** (Azoreductase Project): MCF-7 cells were plated in 48-chambered wells at a density of 5×10^4 cells/well in EMEM supplemented with insulin, NEAA, FBS, and antibiotics. The plate was placed in a BD container with an oxygen indicator and BD anaerobic sachets to generate a <1% O_2 environment within 2 hours; this was left to incubate overnight. The next day, the cells were incubated with various concentrations of the probe (0.5 μM and 1 μM) at 37 °C for 2 hours. Then PDT was performed with light irradiation using the 625 nm LED laser (5 mW cm^-2) for 5 minutes/well. Subsequently, the cells were washed once with PBS and 250 μL of full growth medium was added. After another overnight incubation at 37 °C, 1.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 3 hours. The medium was removed and 150 μL of 10:7:3 DMSO/isopropanol/0.1 M HCl solution was added to dissolve the MTT precipitate. The solution was diluted 10x and the absorbance was recorded at 560 nm. Absorbance values for dark untreated cells at 555 nm were set to 100% cells viable.

**Fluorescence Quantum Yield** (DDAO Project): To calculate the FQY of the probe, the fluorophore Thionin Acetate (Φ_F = 0.5 in ethanol) was used as the standard as its absorption spectrum overlaps well with the excitation band of the probe. Separate solutions of Thionin Acetate in ethanol and probe in PBS were prepared to obtain an absorbance of 0.035 to 0.055 at 601 nm. Emission spectra of each sample were taken with an excitation wavelength of 601 nm (1.5 nm slit...
Fluorescence quantum yields were calculated with the formula: \( \Phi_{\text{sample}} = (\Phi_{\text{standard}}) \left( \frac{I_{\text{sample}}}{I_{\text{standard}}} \right) \times \left( \frac{A_{\text{standard}}}{A_{\text{sample}}} \right) \times \left( \frac{n_{\text{sample}}^2}{n_{\text{standard}}^2} \right) \), where \( \Phi \) is the fluorescence quantum yield, \( I \) is the integrated fluorescence intensity, \( A \) is the absorbance at the specified wavelength, and \( n \) is the refractive index of the solvent.

**GST Project:**

**PTA Prodrug:** PTA-BHQ2-Dansyl Probe (Compound 2.1: AK-01-067)

Note that the letter listed after the compound number refers to individual steps of the synthetic pathway.

**Synthesis of MonoBOC-Protected Ethylenediamine (Compound 2.1a):** Boc anhydride (2.29 mmol, 500 mg) was dissolved in anhydrous DCM (14 mL) and added dropwise to a solution of ethylene diamine (22.9 mmol, 1.53 mL) in anhydrous DCM (14 mL). The reaction was stirred overnight at room temperature, then concentrated under reduced pressure. An extraction was performed using saturated sodium bicarbonate (60 mL); the organic phase was separated. The remaining aqueous phase was extracted with DCM (3 x 560 mL), which was then combined with the previous organic phase and dried over magnesium sulphate. Next, the solvent was evaporated under reduced pressure to a colorless oil. Yield: 95% (350 mg, 2.18 mmol). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 6.74 (s, 1H), 2.91 (q, \( J = 6.2 \) Hz, 2H), 2.53 – 2.50 (m, 2H), 1.38 (s, 9H). MS (ESI positive ion): m/z = 160.91 and 183.02 for Na\(^+\) adduct (expected = 161.22) for [M]\(^+\).

**Synthesis of PTA Derivative (Compound 2.1c):** To a solution of 6-mercaptopurine (0.248 mmol, 42.2 mg) in anhydrous DMF (1.66 mL), DIPEA (0.91 mmol, 0.16 mL) was added with continuous stirring, followed by addition of monoBOC-protected PA-conjugated ethylenediamine (0.236 mmol, 50.1 mg). The solution was stirred overnight at 65 °C. Cold crystallization was performed
for the cis product. Yield: 57.3% (49.3 mg, 0.14 mmol). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.62 – 8.46 (m, 1H), 8.33 (d, $J = 31.6$ Hz, 1H), 6.86 (s, 1H), 6.33 (d, $J = 10.0$ Hz, 1H), 3.24 – 3.14 (m, 2H), 3.14 – 2.95 (m, 2H), 1.39 (d, $J = 3.5$ Hz, 9H). MS (ESI positive ion): m/z = 365.30 (expected = 365.43) for $[M]^+$ and (ESI negative ion): m/z = 363.32.

Synthesis of PTA-N9 Linker Derivative (Compound 2.1d): The PTA derivative (0.084 mmol, 30.5 mg) was added to a round bottom flask with 2 mL of anhydrous DMF under continuous stirring. Lithium hydride (0.17 mmol, 1.34 mg) was added to the solution and stirred for 5 minutes, followed by addition of 3-bromopropylamine hydrobromide (0.126 mmol, 28.1 mg). The mixture was left stirring overnight at room temperature. No work up or purification was performed. Yield: 51% (18 mg, 0.043 mmol). MS (ESI positive ion): m/z = 422.30 and 444.32 for Na$^+$ adduct (expected = 422.52) for $[M]^+$.

Synthesis of PTA-BHQ2 (Compound 2.1e): The PTA-N9 linker derivative (0.024 mmol, 10 mg) was added to a round bottom flask with anhydrous DMF (1 mL) under continuous stirring. BHQ2-NHS (0.027 mmol, 16 mg) was added to the solution was stirred for 2 minutes, followed by addition of TEA (0.12 mL). The mixture was left stirring overnight at 40 ℃. The crude was purified using silica column chromatography (gradient up to 10% methanol in DCM). Yield: 50% (10.6 mg, 0.012 mmol). $^1$H NMR (400 MHz, Chloroform-$d$) δ 8.68 (s, 0H), 8.40 (dd, $J = 9.2$, 2.3 Hz, 1H), 8.08 (d, $J = 8.9$ Hz, 1H), 7.90 (d, $J = 8.8$ Hz, 1H), 7.53 (d, $J = 32.2$ Hz, 1H), 6.77 (d, $J = 9.1$ Hz, 1H), 4.13 (d, $J = 3.3$ Hz, 3H), 3.62 – 3.31 (m, 3H), 3.11 (s, 1H), 2.42 – 2.12 (m, 1H), 2.04 (q, $J = 6.9$ Hz, 2H), 1.28 (s, 9H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities. MS (ESI positive ion): m/z = 932.70 for Na$^+$ adduct (expected = 911.03) for $[M]^+$.

Synthesis of Deprotected PTA-BHQ2 (Compound 2.1f): PTA-BHQ2 (0.012 mmol, 11 mg) was added to a round bottom flask with 0.48 mL of TFA under continuous stirring, followed by addition of triisopropylsilane (0.01 mL). The mixture was left stirring for 1 hour at room temperature. Upon completion, TFA was removed by speedvac or under reduced pressure at room temperature overnight. No purification was performed. Yield: 95% (9.5 mg, 0.012 mmol). MS (ESI positive ion): m/z = 810.46 (expected = 810.91) for $[M]^+$.

Synthesis of PTA-BHQ2-Dansyl (Compound 2.1): Deprotected PTA-BHQ2 (0.011 mmol, 9 mg) was added to a round bottom flask with 0.06 mL of DMSO under continuous stirring, followed by addition of Dansyl chloride (0.012 mmol, 3.27 mg) and then TEA (0.071 mmol, 0.01 mL). The mixture was left stirring for 2.5 hours at room temperature. The crude was purified using HPLC (ACN + 0.1% TFA and MQ + 0.1% TFA) to obtain Compound 2.1. Yield: 50% (5.8 mg, 0.0057 mmol). HRMS (ESI positive ion): m/z = 1043.17 (expected = 1044.20) for $[M]^+$.
**Compound 2.2** PTA-BHQ3-Pyropheophorbide A (AK-01-170), **Compound 2.3** PTA-DABCYL-5FAM (AK-01-161), **Compound 2.4** PTA-BHQ2-5FAM (AK-01-221), **Compound 2.5** PTA-DABCYL-7-Coumarin-3 (AK-01-179), **Compound 2.6** PTA-DABCYL-7-Coumarin-3 (Extended Linker) (AK-01-217), **Compound 2.7** PTA-DABCYL-Coumarin343 (AK-01-132), and **Compound 2.8** PTA-BHQ2-RB (In-Progress): these probes followed the same experimental procedure as the PTA-BHQ2-Dansyl probe except that the quencher-NHS is altered for step 5 and the condition for step 7 is listed below. For the PTA-DABCYL-7-Coumarin-3 (extended linker) probe, glycine was attached to the 7-Coumarin-3 dye via standard amide coupling conditions first and then conjugated to the PTA-DABCYL scaffold. For the PTA-BHQ2-RB probe, N,N'-dimethylethlenediamine was used instead of ethylenediamine.

**Synthesis of PTA-BHQ3-Pyropheophorbide A (Compound 2.2):** Pyropheophorbide A (0.0051 mmol, 2.73 mg) was added to a round bottom flask with 0.11 mL of DMF under continuous stirring, followed by addition of DIPEA (0.057 mmol, 0.01 mL) and HATU (0.0061 mmol, 2.32 mg). The mixture was left stirring for 5 minutes. Next, deprotected PTA-BHQ3 (0.0082 mmol, 7 mg) was added and left stirring overnight at 40 °C. This compound was purified using HPLC (ACN + 0.1% TFA and MQ + 0.1% TFA) to obtain **Compound 2.2**. Yield: 50% (5.7 mg, 0.0042 mmol). MS (ESI positive ion): m/z = 1368.18 (expected = 1368.71) for [M]+.

**Synthesis of PTA-DABCYL-5FAM (Compound 2.3):** 5-FAM (0.016 mmol, 6.42 mg) was added to a round bottom flask with 0.35 mL of DMF under continuous stirring, followed by addition of DIPEA (0.14 mmol, 0.025 mL) and HATU (0.020 mmol, 7.41 mg). The mixture was left stirring for 5 minutes. Next, deprotected PTA-DABCYL (0.026 mmol, 15 mg) was added and left stirring overnight at 40 °C. This compound was purified using HPLC (ACN + 0.1% TFA and MQ + 0.1% TFA) to obtain **Compound 2.3**. Yield: 50% (7.6 mg, 0.0082 mmol). MS (ESI positive ion): m/z = 931.52 (expected = 932) for [M]+. MS (ESI negative ion): m/z = 929.49.

**Synthesis of PTA-BHQ2-5FAM (Compound 2.4):** 5-FAM (0.0038 mmol, 1.51 mg) was added to a round bottom flask with 0.15 mL of DMF under continuous stirring, followed by addition of DIPEA (0.033 mmol, 0.006 mL) and HATU (0.0046 mmol, 1.75 mg). The mixture was left stirring for 5 minutes. Next, deprotected PTA-BHQ2 (0.00617 mmol, 5 mg) was added and left stirring overnight at 40 °C. This compound was purified using HPLC (ACN + 0.1% TFA and MQ + 0.1% TFA) to obtain **Compound 2.4**. Yield: 50% (2.3 mg, 0.0020 mmol). MS (ESI positive ion): m/z = 1168.62 (expected = 1169.22) for [M]+. MS (ESI negative ion): m/z = 1166.73.

**Synthesis of PTA-DABCYL-7-Coumarin-3 (Compound 2.5):** 7-Coumarin-3 (0.012 mmol, 3.14 mg) was added to a round bottom flask with 0.26 mL of DMF under continuous stirring, followed by addition of DIPEA (0.10 mmol, 0.018 mL) and HATU (0.014 mmol, 5.38 mg). The mixture was left stirring for 5 minutes. Next, deprotected PTA-DABCYL (0.0192 mmol, 11 mg) was added and left stirring overnight at 40 °C. This compound was purified using HPLC (ACN + 0.1% TFA.
and MQ + 0.1% TFA) to obtain **Compound 2.5.** Yield: 50% (4.9 mg, 0.0060 mmol). MS (ESI positive ion): m/z = 816.48 (expected = 816.96) for [M]⁺. MS (ESI negative ion): m/z = 816.42.

**Synthesis of PTA-DABCYL-7-Coumarin-3 (Extended Linker) (Compound 2.6):** 7-Coumarin-3-glycine (0.015 mmol, 4.77 mg) was added to a round bottom flask with 0.10 mL of DMF under continuous stirring, followed by addition of DIPEA (0.13 mmol, 0.023 mL) and HATU (0.018 mmol, 6.84 mg). The mixture was left stirring for 5 minutes. Next, deprotected PTA-DABCYL (0.024 mmol, 14 mg) was added and left stirring overnight at 40 °C. This compound was purified using HPLC (ACN + 0.1% TFA and MQ + 0.1% TFA) to obtain **Compound 2.6.** Yield: 40% (5.3 mg, 0.0061 mmol). MS (ESI positive ion): m/z = 873.65 and 895.70 for Na⁺ adduct (expected = 874.01) for [M]⁺.

**Synthesis of PTA-DABCYL-Coumarin 343 (Compound 2.7):** Coumarin343 (0.0065 mmol, 1.95 mg) was added to a round bottom flask with 0.14 mL of DMF under continuous stirring, followed by addition of DIPEA (0.057 mmol, 0.01 mL) and HATU (0.0078 mmol, 2.97 mg). The mixture was left stirring for 5 minutes. Next, deprotected PTA-DABCYL (0.011 mmol, 6 mg) was added and left stirring overnight at 40 °C. This compound was purified using HPLC (ACN + 0.1% TFA and MQ + 0.1% TFA) to obtain **Compound 2.7.** Yield: 40% (2.2 mg, 0.0026 mmol). MS (ESI positive ion): m/z = 840.52 and 862.44 for Na⁺ adduct (expected = 840.98) for [M]⁺.

**Synthesis of PTA-BHQ2-RB (Compound 2.8f):** PTA-BHQ2 (0.012 mmol, 11 mg) was added to a round bottom flask with 0.48 mL of TFA under continuous stirring, followed by addition of triisopropylsilane (0.01 mL). The mixture was left stirring for 1 hour at room temperature. Upon completion, TFA was removed by speedvac or under reduced pressure at room temperature overnight. No purification was performed. Yield: 95% (9.5 mg, 0.012 mmol). MS (ESI positive ion): m/z = 810.46 (expected = 810.91) for [M]⁺.

**tAVTG Prodrug:** tAVTG-BHQ2-5FAM Probe (Compound 2.9: AK-01-241)

**Synthesis of 1-(1,1-Dimethylethyl) Ester Butanedioic Acid (Compound 2.9a):** To a solution of succinic anhydride (50 mmol, 5.054 g) in anhydrous toluene (30 mL), the following was added in order: NHS (15 mmol, 1.762 g), DMAP (5 mmol, 0.617 g), dry tert-butyl alcohol (14 mL), and TEA (15 mmol, 2.11 mL). The reaction was maintained under argon atmosphere and stirred for 30 minutes at room temperature, followed by refluxing for 24 hours. Upon completion, the reaction was cooled. Ethyl acetate (80 mL) was added and this organic phase was washed with 10% citric acid (3 x 80 mL) and brine (2 x 60 mL), which was then dried with magnesium sulfate and concentrated under reduced pressure. The crude was purified using silica column chromatography (gradient up to 20% ethyl acetate in DCM). Yield: 90% (7.9 g, 45.4 mmol). ¹H NMR (400 MHz, Chloroform-d) δ 11.26 (s, 1H), 2.59 (ddd, J = 7.3, 6.0, 1.3 Hz, 2H), 2.51 (ddd, J = 7.4, 5.9, 1.3 Hz, 2H), 1.46 – 1.36 (m, 9H). MS (ESI negative ion): m/z =172.94 (expected = 174.2) for [M]⁺.
Synthesis of 4-(Methoxymethylamino)-4-Oxo-, 1,1-Dimethylethyl Ester Butanoic Acid (Compound 2.9b): To a solution of 1-(1,1-dimethylethyl) ester butanedioic acid (1.15 mmol, 200 mg) in anhydrous DMF (5 mL), HBTU (1.15 mmol, 445 mg) was added, followed by DIPEA (0.73 mmol, 0.128 mL). After stirring for 1 minute, a solution of Wienreb’s amine (1.15 mmol, 70.2 mg) in anhydrous DMF (1 mL) was added. The mixture was left to stir at room temperature for 3 hours, followed by dilution with water (30 mL) upon completion. Chloroform (3 x 30 mL) was added for an extraction; the organic phase was combined and dried on magnesium sulfate, followed by concentration under reduced pressure.

Yield: 90% (225 mg, 1.04 mmol). \(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 3.66 (s, 3H), 3.12 (s, 3H), 2.64 (t, \(J = 6.9\) Hz, 2H), 2.49 (t, \(J = 6.8\) Hz, 2H), 1.48 – 1.31 (m, 9H).

Synthesis of 4-Oxo-, 1,1-Dimethylethyl Ester 5-Hexynoic Acid (Compound 2.9c): To a solution of 4-(methoxymethylamino)-4-oxo-, 1,1-dimethylethyl ester (0.05 mL) in anhydrous THF (8 mL), 0.5 M ethynylmagnesium bromide in THF (2.4 mL) was added dropwise and stirred at 0 °C for 3 hours. Chloroform (3 x 30 mL) was added to for an extraction; the organic phase was combined and dried on magnesium sulfate, followed by concentration under reduced pressure. The crude was purified using silica column chromatography (DCM). Yield: 80% (33.7 mg, 0.185 mmol). \(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 3.26 (s, 1H), 2.90 (t, \(J = 6.6\) Hz, 2H), 2.61 (t, \(J = 6.6\) Hz, 2H), 1.47 (s, 9H).

Synthesis of tAVTG Derivative (Compound 2.9d):
To a solution of 6-thioguanine (0.10 mmol, 17.4 mg) in anhydrous DMF (0.7 mL), DIPEA (0.39 mmol, 0.068 mL) was added with continuous stirring, followed by addition of 4-oxo-, 1,1-dimethylethyl ester 5-hexynoic acid (0.099 mmol, 18.1 mg). The solution was stirred overnight at 65 °C. Yield: 60% (21 mg, 0.06 mmol). \(^1\)H NMR (400 MHz, Chloroform-d) \(\delta\) 10.09 (s, 0H), 5.33 (s, 1H), 5.07 (d, \(J = 7.4\) Hz, 0H), 4.37 – 4.30 (m, 1H), 4.13 (s, 1H), 3.45 – 3.35 (m, 3H), 2.74 – 2.48 (m, 2H), 2.36 (dt, \(J = 10.8, 7.7\) Hz, 2H), 2.02 (tt, \(J = 9.9, 5.7\) Hz, 2H), 1.68 (d, \(J = 22.3\) Hz, 4H), 0.90 (t, \(J = 6.7\) Hz, 3H), 0.12 – 0.07 (m, 6H). MS (ESI positive ion): m/z = 350.21 and 372.20 for Na\(^+\) adduct (expected = 349.41) for [M]\(^+\). MS (ESI negative ion): m/z = 348.13.

Synthesis of tAVTG-N9 Linker Derivative (Compound 2.9e): The tAVTG derivative (0.02 mmol, 7 mg) was added to a round bottom flask with 0.7 mL of anhydrous DMF under continuous stirring. Sodium hydride (0.031 mmol, 1.24 mg) was added to the solution and stirred for 5 minutes, followed by addition of 3-bromopropylamine hydrobromide (0.024 mmol, 5.36 mg). The mixture was left stirring overnight at room temperature. No work up or purification was performed. Yield: 70% (5.7 mg, 0.014 mmol). MS (ESI positive ion): m/z = 407.26 and 429.28 for Na\(^+\) adduct (expected = 407.51) for [M]\(^+\). MS (ESI negative ion): m/z = 405.22.
Synthesis of tAVTG-BHQ2 (Compound 2.9f): The tAVTG-N9 linker derivative (0.020 mmol, 8.1 mg) was added to a round bottom flask with anhydrous DMF (0.751 mL) under continuous stirring. BHQ2-NHS (0.022 mmol, 13.3 mg) was added to the solution was stirred for 2 minutes, followed by addition of DIPEA (0.57 mmol, 0.10 mL). The mixture was left stirring overnight at 40 °C. The crude was purified using silica column chromatography (gradient up to 10% methanol in DCM). Yield: 60% (10.7 mg, 0.012 mmol). MS (ESI positive ion): m/z = 895.36 (expected = 896.01) for [M]+.

Synthesis of Deprotected tAVTG-BHQ2 (Compound 2.9g): The tAVTG-BHQ2 derivative (0.0067 mmol, 6 mg) was added to a round bottom flask with 0.98 mL of TFA under continuous stirring, followed by addition of triisopropylsilane (0.02 mL). The mixture was left stirring for 1 hour at room temperature. Upon completion, TFA was removed by speedvac or under reduced pressure at room temperature overnight. No purification was performed. Yield: 90% (5.1 mg, 0.0061 mmol). MS (ESI positive ion): m/z = 838.93 (expected = 839.91) for [M]+.

Synthesis of tAVTG-BHQ2-5FAM (Compound 2.9): 5FAM-glycine (0.0053 mmol, 4.38 mg) was added to a round bottom flask with 0.11 mL of DMF under continuous stirring, followed by addition of DIPEA (0.047 mmol, 0.0082 mL) and HATU (0.0064 mmol, 2.46 mg). The mixture was left stirring for 5 minutes. Next, deprotected tAVTG-BHQ2 (0.0053 mmol, 4.38 mg) was added and left stirring overnight at 40 °C. This compound was purified using HPLC (ACN + 0.1% TFA and MQ + 0.1% TFA) to obtain Compound 2.9. Yield: 40% (2.6 mg, 0.0021 mmol).

cAVTP Prodrug: cAVTP-BHQ2-5FAM Probe (In-Progress)

Steps 1-3 followed the same procedure as the tAVTG-BHQ2-5FAM probe.

Peptide Scaffold: GSH-TPE Probe (In-Progress)

Synthesis of POCl3-N-Benzyl Derivative (Compound 2.20a): To a cooled solution (at 0 °C) of POCl3 (8 mmol, 1227 mg, 0.748 mL) in anhydrous DCM (40 mL) and TEA (1.13 mL), N-Benzyl-N-methylaniline (8 mmol, 969 mg, 1.03 mL) was added dropwise and stirred on ice for 2 hours. The crude was purified using silica column chromatography (DCM). Yield: 80% (1540 mg, 6.47 mmol). 1H NMR (400 MHz, Chloroform-d) δ 7.48 – 7.26 (m, 5H), 4.41 (d, J = 12.3 Hz, 2H), 2.84 – 2.67 (m, 3H).

Synthesis of POCl3-N-Benzyl-Bromoethanol Derivative (Compound 2.20b): The POCl3-N-benzyl derivative (2.83 mmol, 674 mg 0.499 mL) was stirred in TEA (3.98 mL), to which was added 2-bromoethanol (28.3 mmol, 3646 mg, 2.04 mL). The solution was stirred at room temperature overnight. Yield: 60% (710 mg, 1.71 mmol). 1H NMR (400 MHz, Chloroform-d) δ 7.46 – 7.22 (m, 5H), 4.40 – 4.20 (m, 5H), 3.99 – 3.69 (m, 1H), 3.68 – 3.43 (m, 5H), 2.70 – 2.56 (m, 3H).
Synthesis of (Compound 2.20c): GSH (0.715 mmol, 220 mg) was dissolved in potassium phosphate buffer at pH 9 (4.50 mL) under continuous stirring under argon atmosphere. The POCl₃-N-benzyl-bromoethanol derivative (0.858 mmol, 356 mg) was added dropwise, followed by addition of 0.1M TCEP (0.04 mL). The solution was stirred for 4 hours at room temperature. Upon completion 1N HCl was added to neutralize the solution until the pH lowered to 7, visualised by using pH paper. Yield: 40% (186 mg, 0.29 mmol).

**AIGen PS:** TPE PS (In-Progress)

Synthesis of Bromo-TPE (Compound 2.21a): To a solution of 4-bromobenzophenone (0.784 mmol, 211 mg) and 4,4'-dimethoxybenzophenone (0.607 mmol, 150 mg) in THF (3.09 mL), zinc powder (3.52 mmol, 230 mg) was added. The mixture was then cooled to -78 °C, followed by addition of titanium tetrachloride (1.73 mmol, 0.19 mL) in a dropwise manner. The temperature was slowly increased to 25 °C, then refluxed for 8 hours. Upon completion, the solution was cooled in an ice-water bath; saturated sodium bicarbonate solution (0.5 mL) was added slowly. The solution was extracted using ethyl acetate (3 x 10 mL), followed by a wash with brine (2 x 10 mL). After drying with magnesium sulfate, the crude was concentrated under reduced pressure and purified using silica column chromatography (30% ethyl acetate in hexanes). Yield: 60% (174 mg, 0.369 mmol). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.71 – 7.22 (m, 2H), 7.25 – 7.02 (m, 3H), 7.00 – 6.81 (m, 8H), 6.77 – 6.53 (m, 4H), 3.72 – 3.61 (m, 6H).

**Peptide Derivative Scaffold:** GSH-Derivative-BHQ3-Pyropheophorbide A Probe (In-Progress)

Steps 1-2 followed the same procedure as the GSH-TPE probe.

**CES2 Project:**

**Eosin B-Based Probe:** Mono- and Disubstituted-Eosin B Probes (Compound 3.1 and 3.2: AK-01-260)

Synthesis of Compound 3.1 and 3.2: To a solution of Eosin B (0.26 mmol, 100 mg) in DCM (1.5 mL), TEA (0.84 mmol, 0.0541 mL) was added and stirred for 3 minutes, followed by addition of benzoyl chloride (0.8 mmol, 0.0939 mL). The crude was purified using silica column chromatography (DCM) to obtain Compounds 3.1 (disubstituted) and 3.2 (monosubstituted). Yield (disubstituted): 40% (51 mg, 0.0657 mmol). MS (ESI positive ion): m/z = 823.10 for Na$^+$ adduct (expected = 789.32) for [M]$^+$. $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 8.32 – 8.12 (m, 6H), 7.80 – 7.65 (m, 3H), 7.56 (t, $J = 7.8$ Hz, 6H), 7.29 (s, 1H). Yield (monosubstituted): 40% (44 mg, 0.0641 mmol). MS (ESI positive ion): m/z = 687.18 (expected = 686.22) for [M]$^+$. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.27 – 8.17 (m, 3H), 8.16 – 8.05 (m, 1H), 7.98 – 7.77 (m, 4H), 7.70 – 7.60 (m, 3H), 5.77 (s, 0H).
**Eosin Y-Based Probe:** Mono- and Disubstituted-Eosin Y Probes (Compound 3.3 and 3.4: AK-01-282)

Synthesis of Compound 3.3 and 3.4: To a solution of Eosin Y (0.154 mmol, 100 mg) in DCM (1.5 mL), TEA (0.384 mmol, 0.0541 mL) was added and stirred for 3 minutes, followed by addition of benzoyl chloride (0.231 mmol, 0.0271 mL). The crude was purified using silica column chromatography (DCM) to obtain **Compounds 3.3 (disubstituted) and 3.4 (monosubstituted)**. Yield (disubstituted): 40% (53 mg, 0.0619 mmol). MS (ESI positive ion): m/z = 856.94 (expected = 857.11) for [M]+. Yield (monosubstituted): 40% (47 mg, 0.0623 mmol). MS (ESI positive ion): m/z = 754.11 (expected = 755.02) for [M]+. 1H NMR (400 MHz, Chloroform-d) δ 7.35 (d, J = 8.7 Hz, 2H), 7.28 (d, J = 3.8 Hz, 1H), 7.10 (ddd, J = 19.7, 7.9, 1.6 Hz, 8H), 5.29 (s, 1H).

**Azoreductase Project:**

**DABCYL-Based Probe:** LMB-DABCYL Probe (Compound 4.1: AK-01-269)

Synthesis of Reduced DABCYL (Compound 4.1a): DABCYL Acid (0.557 mmol, 150 mg) in anhydrous THF (0.689 mL) was added to a solution of lithium aluminum hydride (0.675 mmol, 22.9 mg) in anhydrous THF (0.2 mL) at 0 °C under argon atmosphere. The reaction mixture was warmed to room temperature and stirred overnight. Upon completion, the solution was cooled to 0 °C and quenched with water (0.6 mL), followed by addition of 10% NaOH (1.2 mL) and water (2 mL). Celite filtration was performed and then dried over magnesium sulphate. The crude mixture was concentrated under reduced pressure and was purified using silica column chromatography (20% ethyl acetate in hexanes). Yield: 70% (100 mg, 0.392 mmol). 1H NMR (400 MHz, Chloroform-d) δ 7.98 (d, J = 9.3 Hz, 2H), 7.91 (d, J = 8.3 Hz, 2H), 7.50 (d, J = 8.5 Hz, 2H), 6.85 (d, J = 8.7 Hz, 3H), 4.79 (s, 2H), 3.14 (s, 6H). MS (ESI positive ion): m/z = 256.19 and 279.00 for Na+ adduct (expected = 256.32) for [M]+.

Synthesis of Bromo-DABCYL (Compound 4.1b): Reduced DABCYL (0.345 mmol, 88 mg) in anhydrous THF (0.889 mL) was cooled to 0 °C under argon atmosphere, to which was added triphenylphosphine (0.52 mmol, 138 mg) and N-bromosuccinimide (0.52 mmol, 92.6 mg) in small alternate amounts. The solution was stirred at room temperature overnight. The crude was filtered using silica gel and concentrated under reduced pressure, then purified using silica column chromatography (20% ethyl acetate in hexanes). Yield: 70% (77 mg, 0.242 mmol). 1H NMR (400 MHz, Chloroform-d) δ 7.97 – 7.81 (m, 4H), 7.62 – 7.49 (m, 2H), 7.33 – 7.15 (m, 2H), 4.58 (s, 2H), 2.96 (s, 6H). MS (ESI positive ion): m/z = 318.14 (expected = 319.22) for [M]+.

Synthesis of LMB-DABCYL (Compound 4.1): To a solution of brominated-DABCYL (0.0339 mmol, 10.8 mg) in toluene (7 mL) and water (7 mL), sodium bicarbonate (1 mmol, 84 mg) was added, followed by addition of sodium dithionite (1 mmol, 205 mg). The mixture was heated at 50 °C for 1 hour. The upper toluene layer was transferred to a pre-stirred solution of MB (0.339 mmol, 132 mg) and DIPEA (0.0568 mmol, 0.01 mL). The crude was purified using silica column
chromatography (DCM) to obtain **Compound 4.1.** Yield: 70% (9 mg, 0.0172 mmol). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.81 (s, 8H), 7.18 – 6.98 (m, 4H), 6.73 (s, 0H), 3.99 (s, 4H), 3.06 (d, $J = 5.9$ Hz, 18H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities. MS (ESI positive ion): m/z = 522.43 (expected = 523.72) for [M]+.

**Azobenzene-Based Probe:** LMB-Azobenzene Probe (Compound 4.2: AK-01-278)

Steps 1-3 followed the same procedure for **Compound 4.2** as the LMB-DABCYL probe (refer to the appendix for MS and NMR characterizations).

**Synthesis of LMB-Azobenzene (Compound 4.2):** To a solution of brominated-azobenzene (0.091 mmol, 25 mg) in toluene (18.9 mL) and water (18.9 mL), sodium bicarbonate (2.7 mmol, 227 mg) was added, followed by addition of sodium dithionite (2.7 mmol, 553 mg). The mixture was heated at 50 °C for 1 hour. The upper toluene layer was transferred to a pre-stirred solution of MB (0.92 mmol, 357 mg) and DIPEA (0.153 mmol, 0.027 mL). The crude was purified using silica column chromatography (DCM) to obtain **Compound 4.2.** Yield: 50% (22 mg, 0.046 mmol). NMR spectrum of Compound 4.2 (step 3). $^1$H NMR (400 MHz, Chloroform-$d$) δ 7.88 – 7.67 (m, 8H), 7.51 – 7.33 (m, 10H), 2.52 – 2.43 (m, 12H).

MS (ESI positive ion): m/z = 479.37 (expected = 480.65) for [M]+.

**DDAO Project:**

**DDAO** (Compound 5.1: AK-01-293)

**Synthesis of 3-Hydroxy-$\alpha,\alpha$-Dimethyl-Benzenehemethanol (Compound 5.1a):** To a solution of 3'-hydroxyacetophenone (3.6 mmol, 500 mg) in THF (10 mL), 3M methylmagnesium bromide in ethyl ether (3.7 mL) was added dropwise at 0 °C for 3 hours. The crude was purified using silica column chromatography (gradient up to 20% ethyl acetate in hexanes). Yield: 90% (500 mg, 3.29 mmol). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.17 (s, 1H), 7.07 (t, $J = 7.8$ Hz, 1H), 6.97 – 6.81 (m, 2H), 6.57 (ddd, $J = 8.0, 2.5, 1.0$ Hz, 1H), 4.88 (s, 1H), 1.38 (s, 6H). MS (ESI positive ion): m/z = 152.10 and 175.03 for Na+ adduct (expected = 153.19) for [M]+. MS (ESI negative ion): 151.03.

**Synthesis of 2-[(3.5-Dichloro-4-Hydroxyphenyl)Amino]-5-Hydroxy-$\alpha,\alpha$-Dimethyl-Benzenehemethanol (Compound 5.1b/c):** To a solution of 2,6-dichloro-N-chloro-p-benzoquinoneimine (1.31 mmol, 404 mg) and 3-hydroxy-$\alpha,\alpha$-dimethyl-benzenehemethanol (1.31 mmol, 200 mg) in THF (0.66 mL), a solution of sodium hydroxide (74 mmol, 1.39 mL) was added and stirred at 0 °C for 1 hours. Ethyl acetate (3 x 10 mL) was added, followed by sodium dithionite (300 mg) for imine reduction. The organic phase was combined, dried on magnesium sulfate, and concentrated under reduced pressure. Yield: 98% (422 mg, 1.29 mmol). MS (ESI positive ion): m/z = 328.05 (expected = 328.19) for [M]+.
Synthesis of 1,3-Dichloro-9,10-Dihydro-9,9-Dimethyl-2,7-Acidinediol (Compound 5.1d): To a solution of hydrochloric acid (20.3 mL), 2-[(3,5-dichloro-4-hydroxyphenyl)amino]-5-hydroxy-α, α-dimethyl-benzenemethanol (0.305 mmol, 100 mg) in methanol (0.81 mL) was added and stirred under argon atmosphere for at 100 °C. Yield: 70% (67 mg, 0.216 mmol). MS (ESI positive ion): m/z = 310.10 (expected = 311.18) for [M]+.

Synthesis of DDAO (Compound 5.1e): To a solution of sodium periodate (0.1504 mmol, 32.2 mg) in water (1 mL), 1,3-dichloro-9,10-dihydro-9,9-dimethyl-2,7-acidinediol (0.0752 mmol, 30 mg) was added and stirred at room temperature for 1 hour to obtain Compound 5.1 Yield 80% (53 mg, 0.172 mmol). 1H NMR (400 MHz, Chloroform-d) δ 7.28 (s, 1H), 7.15 – 6.90 (m, 1H), 6.92 – 6.60 (m, 4H), 1.23 (d, J = 7.8 Hz, 6H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities. MS (ESI positive ion): m/z = 308.07 (expected = 308.16) for [M]+.

**DDAO Derivative:** Dibrominated-DDAO (Compound 5.2 AK-01-275)

Synthesis of (Compound 5.2): The same procedure for DDAO was followed, except 2,6-dibromo-N-chloro-p-benzoquinoneimine was used instead of 2,6-dichloro-N-chloro-p-benzoquinoneimine. Yield: 80% (24 mg, 0.0604 mmol). 1H NMR (400 MHz, Chloroform-d) δ 7.13 (d, J = 8.0 Hz, 2H), 7.03 – 6.94 (m, 1H), 6.92 – 6.74 (m, 2H), 1.68 – 1.51 (m, 6H). MS (ESI positive ion): m/z = 398.07 (expected = 398.07) for [M]+ (please refer to the appendix for MS and NMR characterizations).

**DDAO Derivative:** Tribrominated-DDAO and Tetrabrominated-DDAO (AK-01-288)

Synthesis of Tribrominated- and Tetrabrominated-DDAO (Compound 5.3 and 5.4): To a solution of dibrominated-DDAO (0.21 mmol, 84 mg) in anhydrous DCM (0.4 mL), DABCO (0.13 mmol, 14.5 mg) was added and stirred for 5 minutes at -25 °C. Next, liquid bromine was added dropwise and left to stir at -30 °C for 1 hour. The crude was purified using silica column chromatography (gradient up to 10% methanol in DCM) to obtain Compound 5.3 (tribrominated-DDAO) and Compound 5.4 (tetrabrominated-DDAO). Yield (tribrominated): 40% (24 mg, 0.0504 mmol). MS (ESI positive ion): m/z = 474.01 (expected = 476.96) for [M]+. 1H NMR (400 MHz, Chloroform-d) δ 7.89 (s, 1H), 7.84 (d, J = 6.7 Hz, 1H), 7.16 (s, 1H), 1.95 (s, 5H). Yield (tetrabrominated): 40% (28 mg, 0.0503 mmol). MS (ESI positive ion): m/z = 557.84 (expected = 557.87) for [M]+. 1H NMR (400 MHz, Chloroform-d) δ 7.29 (d, J = 2.3 Hz, 2H), 1.23 (d, J = 2.6 Hz, 7H).

**DDAO Derivative:** Monoiodinated-DDAO (MAMKH-01-305)

Synthesis of Monoiodinated-DDAO (Compound 5.59: Dibrominated-DDAO (0.0378 mmol, 15 mg) was added to acetic acid (1 mL), followed by addition of iodine (bisublimed) (0.0378 mmol, 9.59 mg) and copper acetate (0.0378 mmol, 6.87 mg) to obtain Compound 5.5. Yield: 70% (14 mg, 0.0268 mmol). 1H NMR (400 MHz, Chloroform-d) δ 7.72 (dt, J = 7.4, 3.7 Hz, 1H), 7.62 –
7.47 (m, 1H), 7.29 (s, 1H), 1.01 – 0.92 (m, 6H). MS (ESI positive ion): m/z = 524.01 (expected = 523.96) for [M]+.

**DDAO Derivative: Mononitrated-DDAO (AK-01-307)**

Synthesis of Mononitrated-DDAO (Compound 5.6): Urea (22 g) was added to water (55 mL), to which was added nitric acid (30 mL) dropwise under continuous stirring for 10 minutes. The solid was filtered with cold water and dried with open air. Urea nitrate (0.0325 mmol) was added to DDAO (0.0325 mmol, 10 mg) in acetonitrile (1 mL), which was heated in the microwave for 6 hours at 80 °C to obtain Compound 5.6. Yield: 80% (9.2 mg, 0.0261 mmol). ¹H NMR (400 MHz, DMSO-d₆) δ 7.45 – 7.32 (m, 1H), 7.32 – 7.17 (m, 1H), 6.97 – 6.77 (m, 1H), 1.70 – 1.39 (m, 6H).

**DDAO PDT Beacon: Dibrominated DDAO-based NTR Probe (AK-01-300)**

Synthesis of Dibrominated DDAO-based NTR Probe (Compound 5.7): To a solution of dibrominated-DDAO (0.07 mmol, 27.8 mg) in anhydrous THF (0.23 mL), 4-nitrobenzyl bromide (0.062 mmol, 13.5 mg) was added, followed by silver (I) oxide (0.105 mmol) and 4-Å molecular sieves (5 mg). Upon completion, the solvent was evaporated under reduced pressure and was dissolved in 1M NaOH, followed by 10% HCl and brine. The organic phase was combined and dried over magnesium sulfate, evaporated and purified using silica column chromatography (gradient up to 20% ethyl acetate in hexanes) to obtain Compound 5.7. Yield: 60% (22.4 mg, 0.0421 mmol). ¹H NMR (400 MHz, DMSO-d₆) δ 8.37 – 8.24 (m, 2H), 7.87 (dd, J = 8.7, 5.8 Hz, 2H), 7.34 (d, J = 4.1 Hz, 1H), 7.15 – 6.98 (m, 2H), 6.76 – 6.65 (m, 1H), 5.26 – 5.13 (m, 1H), 1.24 (d, J = 2.8 Hz, 6H). HRMS (ESI positive ion): m/z = 531.41 and 553.39 for Na⁺ adduct (expected = 532.19) for [M]+.

**DDAO PDT Beacon: Dibrominated DDAO-based Cytochrome P450 Probe (AK-01-298)**

Synthesis of Dibrominated DDAO-based Cytochrome P450 Probe (Compound 5.8): To a solution of dibrominated-DDAO (0.07 mmol, 27.8 mg) in anhydrous ACN (0.23 mL), iodomethane (0.105 mmol, 2.28 mg) was added, followed by silver (I) oxide (0.105 mmol) and 4-Å molecular sieves (5 mg). Upon completion, the solvent was evaporated under reduced pressure and was dissolved in 1M NaOH, followed by 10% HCl and brine. The organic phase was combined and dried over magnesium sulfate, evaporated and purified using silica column chromatography (gradient up to 20% ethyl acetate in hexanes) to obtain Compound 5.8. Yield: 40% (11.5 mg, 0.0280 mmol). ¹H NMR (400 MHz, DMSO-d₆) δ 7.28 (d, J = 3.8 Hz, 1H), 7.15 – 7.01 (m, 2H), 6.92 – 6.78 (m, 1H), 3.28 – 3.14 (m, 3H), 1.57 (d, J = 8.9 Hz, 6H). HRMS (ESI positive ion): m/z = 411.94 (expected = 412.09) for [M]+.

**DDAO PDT Beacon: Tetrabrominated DDAO-based CES2 (Acetyl) Probe (AK-01-303)**

Synthesis of Tetrabrominated DDAO-based CES2 (Acetyl) Probe (Compound 5.9): To a solution of tetrabrominated-DDAO (0.0252 mmol, 14 mg) in anhydrous DCM (1 mL), TEA (0.0313 mmol,
0.0043 mL) was added, followed by acetyl chloride (0.03 mmol, 0.00215 mL). The crude was purified using silica column chromatography (DCM) to obtain **Compound 5.9**. Yield: 80% (12.1 mg, 0.0203 mmol). $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.61 (s, 1H), 6.85 (s, 1H), 2.47 (s, 3H), 2.41 (s, 6H).

**DDAO PDT Beacon**: Tetrabrominated DDAO-Based CES2 (Benzoyl Ester) Probe (AK-01-304)

**Synthesis of Tetrabrominated DDAO-Based CES2 (Benzoyl Ester) Probe (Compound 5.10)**: To a solution of tetrabrominated-DDAO (0.0252 mmol, 14 mg) in anhydrous DCM (1 mL), TEA (0.0746 mmol, 0.0106 mL) was added, followed by benzoyl chloride (0.0756 mmol, 0.00887 mL). The crude was purified using silica column chromatography (DCM) to obtain **Compound 5.10**. Yield: 80% (13.4 mg, 0.0203 mmol). $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 12.69 (dd, $J$ = 8.3, 1.4 Hz, 2H), 12.24 – 12.16 (m, 1H), 12.07 (dd, $J$ = 8.4, 7.0 Hz, 2H), 7.30 (q, $J$ = 1.1 Hz, 6H).
Chapter 1


**Chapter 2**


45. Fluorescein Available at: https://omlc.org/spectra/PhotochemCAD/html/010.html. (Accessed: 12th April 2019)


**Chapter 3**


**Chapter 4**


**Chapter 5**


Appendices

Note that the chemical shifts labeled in the chemical structure within each NMR spectrum are predicted by ChemDraw.

**GST Project**

**PTA Prodrug:**

**Compound 2.1 PTA-BHQ2-Dansyl Probe (AK-01-067)**

**Step 1**

**A1.1:** NMR spectrum of Compound 2.1 (step 1). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 6.74 (s, 1H), 2.91 (q, $J = 6.2$ Hz, 2H), 2.53 – 2.50 (m, 2H), 1.38 (s, 9H).

**A1.2:** MS spectrum of Compound 2.1 (step 1).
Step 2

A1.3: NMR spectrum of Compound 2.1 (step 2). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 6.64 (s, 1H), 3.45 (q, $J = 5.5$ Hz, 2H), 3.33 (q, $J = 5.9$ Hz, 2H), 2.81 (s, 1H), 1.48 (s, 9H).

A1.4: MS spectrum of Compound 2.1 (step 2).
Step 3

**A1.5:** NMR spectrum of Compound 2.1 (step 3). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.62 – 8.46 (m, 1H), 8.33 (d, $J = 31.6$ Hz, 1H), 6.86 (s, 1H), 6.33 (d, $J = 10.0$ Hz, 1H), 3.24 – 3.14 (m, 2H), 3.14 – 2.95 (m, 2H), 1.39 (d, $J = 3.5$ Hz, 9H).

**A1.6:** MS spectrum of Compound 2.1 (step 3).
Step 4

A1.7: MS spectrum of Compound 2.1 (step 4).

Step 5

A1.8: NMR spectrum of Compound 2.1 (step 5). $^1$H NMR (400 MHz, Chloroform-d) δ 8.68 (s, 0H), 8.40 (dd, $J = 9.2$, 2.3 Hz, 1H), 8.08 (d, $J = 8.9$ Hz, 1H), 7.90 (d, $J = 8.8$ Hz, 1H), 7.53 (d, $J = 32.2$ Hz, 1H), 6.77 (d, $J = 9.1$ Hz, 1H), 4.13 (d, $J = 3.3$ Hz, 3H), 3.62 – 3.31 (m, 3H), 3.11 (s, 1H), 2.42 – 2.12 (m, 1H), 2.04 (q, $J = 6.9$ Hz, 2H), 1.28 (s, 9H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
A1.9: MS spectrum of Compound 2.1 (step 5).

Step 6

A1.10: MS spectrum of Compound 2.1 (step 6).
Step 7

A1.11: MS spectrum of Compound 2.1 (step 7).

**Compound 2.2 PTA-BHQ3-Pyropheophorbide A (AK-01-170)**

**Step 7**

A1.13: MS spectrum of Compound 2.2 (step 7).
**Compound 2.3** PTA-DABCYL-5FAM (AK-01-161)

**Step 5**

A1.14: MS spectrum of Compound 2.3 (step 5).

**Step 6**

A1.15: MS spectrum of Compound 2.3 (step 6).
Step 7

A1.16: MS spectrum (+ mode) of Compound 2.3 (step 7).

A1.17: MS spectrum (- mode) of Compound 2.3 (step 7).
**Compound 2.4** PTA-BHQ2-5FAM (AK-01-221)

**Step 7**

A1.18: MS spectrum (+ mode) of Compound 2.4 (step 7).

A1.19: MS spectrum (- mode) of Compound 2.4 (step 7).
Compound 2.5 PTA-DABCYL-7-Coumarin-3 (AK-01-179)

Step 7

A1.20: MS spectrum of Compound 2.5 (step 7).

Compound 2.6 PTA-DABCYL-7-Coumarin-3 (Extended Linker) (AK-01-217)

Step 7

A1.21: MS spectrum of Compound 2.6 (step 7).
Compound 2.7 PTA-DABCYL-Coumarin343 (AK-01-132)

Step 7

A1.22: MS spectrum of Compound 2.7 (step 7).
**A1.23:** HRMS spectrum of Compound 2.7 (step 7).

**Compound 2.8 PTA-BHQ2-RB (In-Progress)**

**Step 1**

**A1.24:** NMR spectrum of Compound 2.8 (step 1). $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 3.13 – 2.90 (m, 2H), 2.56 (dt, $J$ = 9.7, 2.3 Hz, 3H), 2.45 – 2.39 (m, 2H), 2.14 (t, $J$ = 4.1 Hz, 2H), 1.24 – 1.01 (m, 9H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
A1.25: MS spectrum of Compound 2.8 (step 1).

Step 2

A1.26: MS spectrum of Compound 2.8 (step 2).

Step 4

A1.27: MS spectrum of Compound 2.8 (step 4).
Step 5

**A1.28:** MS spectrum of Compound 2.8 (step 5).

Step 6

**A1.29:** MS spectrum of Compound 2.8 (step 6).

tAVTG Prodrug:

**Compound 2.9 tAVTG-BHQ2-5FAM Probe (AK-01-247)**

**Step 1**

**A1.30:** NMR spectrum of Compound 2.9 (step 1). $^1$H NMR (400 MHz, Chloroform-$d$) δ 11.26 (s, 1H), 2.59 (ddd, $J$ = 7.3, 6.0, 1.3 Hz, 2H), 2.51 (ddd, $J$ = 7.4, 5.9, 1.3 Hz, 2H), 1.46 – 1.36 (m, 9H).
A1.31: MS spectrum of Compound 2.9 (step 1).

Step 2

A1.32: NMR spectrum of Compound 2.9 (step 2). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 3.66 (s, 3H), 3.12 (s, 3H), 2.64 (t, $J = 6.9$ Hz, 2H), 2.49 (t, $J = 6.8$ Hz, 2H), 1.48 – 1.31 (m, 9H).
Step 3

A1.33: NMR spectrum of Compound 2.9 (step 3). $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 3.26 (s, 1H), 2.90 (t, $J = 6.6$ Hz, 2H), 2.61 (t, $J = 6.6$ Hz, 2H), 1.47 (s, 9H).

Step 4

A1.34: NMR spectrum of Compound 2.9 (step 4). $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 10.09 (s, 0H), 5.33 (s, 1H), 5.07 (d, $J = 7.4$ Hz, 0H), 4.37 – 4.30 (m, 1H), 4.13 (s, 1H), 3.45 – 3.35 (m, 3H), 2.74 – 2.48 (m, 2H), 2.36 (dt, $J = 10.8, 7.7$ Hz, 2H), 2.02 (tt, $J = 9.9, 5.7$ Hz, 2H), 1.68 (d, $J = 22.3$ Hz, 4H), 0.90 (t, $J = 6.7$ Hz, 3H), 0.12 – 0.07 (m, 6H).
A1.35: MS spectrum of Compound 2.9 (step 4).

Step 5

A1.36: MS spectrum (+ mode) of Compound 2.9 (step 5).
A1.37: MS spectrum (- mode) of Compound 2.9 (step 5).

Step 6

A1.38: MS spectrum of Compound 2.9 (step 6).
Step 7

A1.39: MS spectrum of Compound 2.9 (step 7).

Steps 1-3 were followed for the cAVTP-BHQ2-5FAM probe.

**Peptide Scaffold:**

**Compound 2.20** GSH-TPE Probe (In-Progress)

**Step 1**

A1.41: NMR spectrum of Compound 2.20 (step 1). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.48 – 7.26 (m, 5H), 4.41 (d, $J = 12.3$ Hz, 2H), 2.84 – 2.67 (m, 3H).
A1.42: MS spectrum of Compound 2.20 (step 1).

Step 2

A1.43: NMR spectrum of Compound 2.20 (step 2). $^1$H NMR (400 MHz, Chloroform-$d$) δ 7.46 – 7.22 (m, 5H), 4.40 – 4.20 (m, 5H), 3.99 – 3.69 (m, 1H), 3.68 – 3.43 (m, 5H), 2.70 – 2.56 (m, 3H).
Step 3

A1.44: MS spectrum of Compound 2.20 (step 3).

Steps 1-2 were followed for the GSH-Derivative-BHQ3-Pyropheophorbide A probe.

AIGen PS:

**Compound 2.21 TPE PS (In-Progress)**

**Step 1**

A1.45: NMR spectrum of Compound 2.21 (step 1). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.71 – 7.22 (m, 2H), 7.25 – 7.02 (m, 3H), 7.00 – 6.81 (m, 8H), 6.77 – 6.53 (m, 4H), 3.72 – 3.61 (m, 6H).
Peptide Synthesis:

GSH Derivative

**Step 1**

A1.46: MS spectrum of GSH Derivative (step 1).

**Step 2**

A1.47: MS spectrum of GSH Derivative (step 2).
GST Sulfonamide Approach:
Compound 2.11 Azure I-DNBS (AK-01-088)
Step 1

A1.48: MS spectrum of Compound 2.11 (step 1).

Compound 2.13 3AIC-DNBS (AK-01-213)
Step 1

A1.49: NMR spectrum of Compound 2.13 (step 1). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.96 (s, 1H), 7.08 (d, $J = 8.1$ Hz, 1H), 6.83 (d, $J = 8.0$ Hz, 1H), 5.98 (s, 1H), 5.53 (s, 1H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
A1.50: MS spectrum of Compound 2.13 (step 1).

**Compound 2.14 6AP-DNBS (AK-01-131)**

A1.51: MS spectrum of Compound 2.14 (step 1).

**Compound 2.16 Methylene 3RAX-DNBS (AK-01-105)**

Step 1
A1.52: MS spectrum of Compound 2.16 (step 1).

**Compound 2.17 RB-DNBS (AK-01-089)**

*Step 1*

A1.53: MS spectrum of Compound 2.17 (step 1).

**Compound 2.18 IR780-DNBS (AK-01-113)**

*Step 1*

A1.54: MS spectrum of Compound 2.18 (step 1).
CES2 Project

Eosin B-Based Scaffold:

**Compound 3.1 Eosin B-Disubstituted (AK-01-260)**

**Step 1**

**A1.54:** NMR spectrum of Compound 3.1 (step 1). $^1$H NMR (400 MHz, Chloroform- d) δ 8.32 – 8.12 (m, 6H), 7.80 – 7.65 (m, 3H), 7.56 (t, $J = 7.8$ Hz, 6H), 7.29 (s, 1H).

**A1.55:** MS spectrum of Compound 3.1 (step 1).
Compound 3.2 Eosin B-Monosubstituted (AK-01-260)

Step 1

**A1.56: NMR spectrum of Compound 3.2 (step 1).** $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.27 – 8.17 (m, 3H), 8.16 – 8.05 (m, 1H), 7.98 – 7.77 (m, 4H), 7.70 – 7.60 (m, 3H), 5.77 (s, 0H).

**A1.57: MS spectrum of Compound 3.2 (step 1).**
Eosin Y-Based Scaffold:

**Compound 3.3** Eosin Y-Disubstituted (AK-01-282)

Step 1

![MS spectrum of Compound 3.3 (step 1)](image)

**A1.58**: MS spectrum of Compound 3.3 (step 1).

**Compound 3.4** Eosin Y-Monosubstituted (AK-01-282)

Step 1

![NMR spectrum of Compound 3.4 (step 1)](image)

**A1.59**: NMR spectrum of Compound 3.4 (step 1). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.35 (d, $J = 8.7$ Hz, 2H), 7.28 (d, $J = 3.8$ Hz, 1H), 7.10 (ddd, $J = 19.7$, 7.9, 1.6 Hz, 8H), 5.29 (s, 1H).
A1.60: MS spectrum of Compound 3.4 (step 1).

**6AP-Based Scaffold:**

**Compound 3.5 (AK-01-249)**

**Step 1**

A1.61: NMR spectrum of Compound 3.5 (step 1). $^1$H NMR (400 MHz, Chloroform-$d$) δ 8.17 – 8.00 (m, 3H), 7.93 – 7.57 (m, 5H), 7.55 – 7.38 (m, 1H), 6.92 (d, $J = 8.1$ Hz, 1H), 6.62 (d, $J = 8.0$ Hz, 1H), 4.07 (s, 1H), 2.67 (d, $J = 3.2$ Hz, 1H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
A1.62: MS spectrum of Compound 3.5 (step 1).

**Compound 3.6 (AK-01-253)**

**Step 1**

A1.63: NMR spectrum of Compound 3.6 (step 1). $^1$H NMR (400 MHz, Chloroform-\(d\)) $\delta$ 8.38 – 8.00 (m, 5H), 8.00 – 7.76 (m, 1H), 7.76 – 7.36 (m, 4H), 7.25 – 6.97 (m, 6H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
A1.64: MS spectrum of Compound 3.6 (step 1).

Azoreductase Project

DABCYL Scaffold:

Compound 4.1 LMB-DABCYL (AK-01-269)

Step 1

A1.65: NMR spectrum of Compound 4.1 (step 1). $^1$H NMR (400 MHz, Chloroform-d) $\delta$ 7.98 (d, $J = 9.3$ Hz, 2H), 7.91 (d, $J = 8.3$ Hz, 2H), 7.50 (d, $J = 8.5$ Hz, 2H), 6.85 (d, $J = 8.7$ Hz, 3H), 4.79 (s, 2H), 3.14 (s, 6H).

Step 2

A1.67: NMR spectrum of Compound 4.1 (step 2). ¹H NMR (400 MHz, Chloroform-d) δ 7.97 – 7.81 (m, 4H), 7.62 – 7.49 (m, 2H), 7.33 – 7.15 (m, 2H), 4.58 (s, 2H), 2.96 (s, 6H).
**A1.68:** MS spectrum of Compound 4.1 (step 2).

**Step 3**

**A1.69:** NMR spectrum of Compound 4.1 (step 3). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.81 (s, 8H), 7.18 – 6.98 (m, 4H), 6.73 (s, 0H), 3.99 (s, 4H), 3.06 (d, $J = 5.9$ Hz, 18H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
A1.70: MS spectrum of Compound 4.1 (step 3).

**Azobenzene Scaffold:**

**Compound 4.2** LMB-Azobenzene (AK-01-278)

**Step 1**

A1.72: NMR spectrum of Compound 4.2 (step 1). $^1$H NMR (400 MHz, Chloroform-$d$) δ 8.04 – 7.91 (m, 4H), 7.61 – 7.43 (m, 5H), 4.82 (s, 2H).
A1.73: MS spectrum of Compound 4.2 (step 1).

Step 2

A1.74: NMR spectrum of Compound 4.2 (step 2). $^1$H NMR (400 MHz, Chloroform-$d$) δ 7.95 (ddd, $J = 11.4, 7.5, 1.8$ Hz, 4H), 7.71 – 7.40 (m, 5H), 4.58 (s, 2H).
A1.75: MS spectrum of Compound 4.2 (step 2).

Step 3

A1.76: NMR spectrum of Compound 4.2 (step 3). $^1$H NMR (400 MHz, Chloroform-d) δ 7.88 – 7.67 (m, 8H), 7.51 – 7.33 (m, 10H), 2.52 – 2.43 (m, 12H).
A1.77: MS spectrum of Compound 4.2 (step 3).

**DDAO Project**

**DDAO Fluorophore:**

**Compound 5.1 DDAO (AK-01-293)**

**Step 1**

A1.78: NMR spectrum of Compound 5.1 (step 1). $^1$H NMR (400 MHz, DMSO-d$_6$) δ 9.17 (s, 1H), 7.07 (t, $J = 7.8$ Hz, 1H), 6.97 – 6.81 (m, 2H), 6.57 (ddd, $J = 8.0$, 2.5, 1.0 Hz, 1H), 4.88 (s, 1H), 1.38 (s, 6H).
A1.79: MS spectrum of Compound 5.1 (step 1).

Step 2

A1.80: MS spectrum of Compound 5.1 (step 2).
**Step 3**

A1.81: MS spectrum of Compound 5.1 (step 3).

**Step 4**

A1.82: MS spectrum of Compound 5.1 (step 4).
Step 5

A1.83: NMR spectrum of Compound 5.1 (step 5). $^1$H NMR (400 MHz, Chloroform-d) δ 7.28 (s, 1H), 7.15 – 6.90 (m, 1H), 6.92 – 6.60 (m, 4H), 1.23 (d, J = 7.8 Hz, 6H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.

A1.84: MS spectrum of Compound 5.1 (step 5).
DDAO Derivatives:

**Compound 5.2 Dibrominated-DDAO (AK-01-275)**

**Step 2**

A1.85: MS spectrum of Compound 5.2 (step 2).

**Step 3**

A1.86: NMR spectrum of Compound 5.2 (step 3). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 6.94 – 6.86 (m, 3H), 6.83 – 6.72 (m, 2H), 1.44 (s, 6H).
A1.87: MS spectrum of Compound 5.2 (step 3).

Step 4

A1.88: NMR spectrum of Compound 5.2 (step 4). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 6.65 (d, $J = 0.8$ Hz, 3H), 6.60 – 6.45 (m, 3H), 6.11 – 5.67 (m, 6H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
A1.89: MS spectrum of Compound 5.2 (step 4).

Step 5

A1.90: NMR spectrum of Compound 5.2 (step 5). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.13 (d, $J = 8.0$ Hz, 2H), 7.03 – 6.94 (m, 1H), 6.92 – 6.74 (m, 2H), 1.68 – 1.51 (m, 6H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
**A1.91:** MS spectrum of Compound 5.2 (step 5).

**Compound 5.3 Tribrominated-DDAO (AK-01-288)**

**Step 6**

**A1.92:** NMR spectrum of Compound 5.3 (step 6). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.89 (s, 1H), 7.84 (d, $J = 6.7$ Hz, 1H), 7.16 (s, 1H), 1.95 (s, 5H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
Compound 5.4 Tetrabrominated-DDAO (AK-01-288)

Step 6

A1.94: NMR spectrum of Compound 5.4 (step 6). $^1$H NMR (400 MHz, Chloroform- $d$) $\delta$ 7.29 (d, $J = 2.3$ Hz, 2H), 1.23 (d, $J = 2.6$ Hz, 7H).
A1.95: MS spectrum of Compound 5.4 (step 6).

**Compound 5.5 Moniodinated-DDAO (MAMKH-01-305)**

**Step 6**

A1.96: NMR spectrum of Compound 5.5 (step 6). $^1$H NMR (400 MHz, Chloroform-$d$) δ 7.72 (dt, $J = 7.4, 3.7$ Hz, 1H), 7.62 – 7.47 (m, 1H), 7.29 (s, 1H), 1.01 – 0.92 (m, 6H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
A1.97: MS spectrum of Compound 5.5 (step 6).

Compound 5.6 Mononitrated-DDAO (AK-01-307)

Step 6

A1.98: NMR spectrum of Compound 5.6 (step 6). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.45 – 7.32 (m, 1H), 7.32 – 7.17 (m, 1H), 6.97 – 6.77 (m, 1H), 1.70 – 1.39 (m, 6H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.
DDAO-Based PDT Beacons:

**Compound 5.7** Dibrominated-DDAO-Based NTR Probe (AK-01-300)

**Step 6**

A1.99: NMR spectrum of Compound 5.7 (step 6). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 8.37 – 8.24 (m, 2H), 7.87 (dd, $J = 8.7, 5.8$ Hz, 2H), 7.34 (d, $J = 4.1$ Hz, 1H), 7.15 – 6.98 (m, 2H), 6.76 – 6.65 (m, 1H), 5.26 – 5.13 (m, 1H), 1.24 (d, $J = 2.8$ Hz, 6H). Note that the number of protons in this NMR spectrum do not add up according to the compound due to impurities.

A1.100: HRMS spectrum of Compound 5.7 (step 6).
Compounds 5.8 Dibrominated-DDAO-Based P450 Probe (AK-01-298)

Step 6

A1.101: NMR spectrum of Compound 5.8 (step 6). $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 7.28 (d, $J$ = 3.8 Hz, 1H), 7.15 – 7.01 (m, 2H), 6.92 – 6.78 (m, 1H), 3.28 – 3.14 (m, 3H), 1.57 (d, $J$ = 8.9 Hz, 6H).
A1.102: HRMS spectrum of Compound 5.8 (step 6).

**Compound 5.9** Tetrabrominated-DDAO-Based CES2 (Acetyl) Probe (AK-01-303)

**Step 7**

A1.103: NMR spectrum of Compound 5.9 (step 7). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 7.61 (s, 1H), 6.85 (s, 1H), 2.47 (s, 3H), 2.41 (s, 6H).
**Compound 5.10** Tetrabrominated-DDAO-Based CES2 (Benzoyl Ester) Probe (AK-01-304)

**Step 7**

A1.104: NMR spectrum of Compound 5.10 (step 7). $^1$H NMR (400 MHz, Chloroform-$d$) $\delta$ 12.69 (dd, $J = 8.3, 1.4$ Hz, 2H), 12.24 – 12.16 (m, 1H), 12.07 (dd, $J = 8.4, 7.0$ Hz, 2H), 7.30 (q, $J = 1.1$ Hz, 6H).