

KONYA FOOD AND AGRICULTURE UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED
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DEPARTMENT OF BIOTECHNOLOGY

DEVELOPMENT OF SELF-ACTUATOR AND
ACTIVATABLE FLUOROPHORE

MASTER OF SCIENCE THESIS

N*e D*****K**

KONYA

JANUARY, 2023

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Supervisor: Assist. Prof. Dr. S*****s E***Ş Ç*****K

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JANUARY, 2023

This study titled '**Development of Self-Actuator and Activatable Fluorophore**' prepared by N****e D*****K has been accepted with consensus by the following jury on 19/01/2023 as a **MASTER THESIS** in the DEPARTMENT OF BIOTECHNOLOGY.

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ABSTRACT

DEVELOPMENT OF SELF-ACTUATOR AND ACTIVATABLE FLUOROPHORE

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Master Of Science Thesis, Department of Biotechnology

Supervisor: Assist. Prof. Dr. S*****s E****Ş Ç*****K

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In the first part of the thesis project, an auto-activatable fluorescent therapeutic agent is developed. Irradiation of photosensitizer (PS) with red light leads to production of singlet oxygen. Singlet oxygen reacts with the photosensitizer increasing the emission at 545 nm without changing the PDT efficiency, enabling self-activity reporting. Activity of the PS is shown in MCF7 cell line.

In the second part of the thesis project, dynamic covalent chemistry-controllable fluorophores that are appropriate for use in Super Resolution Fluorescence Microscopy (SCFM) are developed. Imine-amine dynamic chemistry allows a change in emission intensity and wavelength. When the pH in the MCF7 cells was lowered to 6.5 that favors imine hydrolysis, enhanced emission is observed. It was also proven that the compounds had no toxic effect on cells when applied at 10 μ M concentration.

Keywords: photodynamic therapy, auto-actuator, activatable fluorophore

ÖZET

KENDİNDEN AKTİFLEŞEBİLİR TERAPÖTİK AJANIN VE AKTİFLEŞTİRİLEBİLİR FLOROFORUN GELİŞTİRİLMESİ

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Tez projesinin ilk bölümünde, kendi floresansını aktive edebilen bir terapötik ajan geliştirilmiştir. Işığa duyarlaştırıcının kırmızı ışıkla uyarılması singlet oksijen üretimine yol açmaktadır. Singlet oksijen, fotodinamik etkiyi değiştirmeden fotoduyarlaştırıcı ile tepkime verip 545 nm'de emisyon artışına neden olmakta, bu yolla kendi aktivitesini rapor etmektedir. Fotoduyarlaştırıcının aktivitesi, MCF7 hücre hattında gösterilmiştir.

Tez projesinin ikinci bölümünde, Süper Çözünürlüklü Floresan Mikroskopide (SCFM) kullanıma uygun, dinamik kovalent kimya ile kontrol edilebilen floroforlar geliştirilmiştir. İmin-amin dinamik kimyası, emisyon yoğunluğunda ve dalga boyunda bir değişikliğe izin vermektedir. MCF7 hücrelerindeki pH imin hidrolizini teşvik eden 6.5'e düşürüldüğünde emisyon şiddetinin arttığı tespit edilmiştir. Bileşik 10 μ M konsantrasyonda uygulandığında hücreler üzerinde toksik etkisinin olmadığı da kanıtlanmıştır.

Anahtar Kelimeler: fotodinamik terapi, kendini aktifleştirebilir sistemler

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N***e D*****K

January - 2023

TEXT OF OATH

The study I presented as my Master of Science Thesis titled ‘Development of Self-Actuator and Activatable Fluorophore’ has been written without applying to any assistance inconsistent with the scientific ethics and traditions, and all sources I have benefited from were listed in bibliography and I have been used them by means of making references and I declare and confirm this with my honor.



N***e D*****K

January - 2023

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

abs	Absorbance
g	Gram
h	Hour
μ L	Mikroliter
μ M	Mikromolar
mg	Miligram
mL	Mililiter
mM	Milimolar
mmol	Minimol
min	Minute
nm	Nanometer
nM	Nanomolar

Abbreviations

AK	Actinic keratoses
ALA	Aminolaevulinic acid
AMD	Age-related macular degeneration
BCC	Basal cell carcinoma
BF ₃ OEt ₂	Borontrifluoride etherate
BODIPY	Boron-dipyrromethene
CH ₃ COOH	Acetic acid
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBF	1,3-Diphenylisobenzofuran
EDTA	Ethylenediaminetetra acetic acid
Et ₃ N	Triethylamine
EtOAc	Ethylacetate
FBS	Fetal Bovine Serum
FDA	Food and drug administration
FGS	Fluorescence guided surgery
FRET	Fluorescence Resonance Energy Transfer
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HOMO	Highest occupied molecular orbital
HPD	Hematoporphyrin derivative

HRMS	High Resolution Mass Spectroscopy
IC ₅₀	The half maximal inhibitory concentration
KOH	Potassium Hydroxide
LUMO	Lowest Unoccupied Molecular Orbital
MAL	Methyl aminolaevulinic acid
MCF-7	Michigan Cancer Foundation-7
MeOH	Methanol
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBS	N-bromo succinamide
NMR	Nuclear Magnetic Resonance
NSOM	Near-infrared Scanning Optical Microscope
OD	Optical Density
PALM	Photo-activated localization microscopy
PC	Phthalocyanine
PDT	Photodynamic Therapy
p-HBI	4-(p-hydroxybenzidiline)-5-imidazolinane
PPIX	Protoporphyrin
PS	Photosensitizer
ROS	Reactive oxygen species
RT	Room Temperature
SCC	Squamous cell carcinoma
SLM	Single-molecule Localization microscopy
SRFM	SuperResolution Fluorescence Microscopy
STED	Stimulated emission depletion
STORM	Stochastic Optical Reconstruction Microscopy
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
UV-Vis	Ultraviolet-visible
YEP	Yellow fluorescence protein

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1. INTRODUCTION

Thesis project consists of two independent works. In the first part of the thesis, an auto-actuator photosensitizer is produced with the ability of generating fluorescence output as a result of its photodynamic action. In the second part of the thesis, a blinking fluorophore based on dynamic chemistry is produced for future use in super resolution imaging.

1.1. Introduction Part I

Photodynamic Therapy (PDT) is a clinically approved therapeutic procedure with a selective cytotoxic effect against malignant cells. Clinical studies have revealed that PDT can have a healing effect, especially in early-stage diseases. It has been used successfully in dermatology, oncology, gynecology, and urology for centuries. In oncology, PDT is used to treat different kinds of cancers such as head and neck, brain, lung, pancreas, intraperitoneal space, breast, prostate, and skin cancer (Pass, 1993).

In 1999 first PDT drug Verteporfin was approved in Canada (Fong et al., 2000). Photodynamic therapy can prolong survival and significantly improve the quality of life in patients with inoperable cancer. Minimal normal tissue toxicity, negligible systemic effects, greatly reduced long-term morbidity, absence of intrinsic or acquired resistance mechanisms, and protective effects on organ function make this method a valuable therapeutic option for combination therapies (Q. Zhang & Li, 2018). Recently, use of various smart photosensitizers (e.g., stimuli-triggered, active cancer-targeting) improves the selectivity of the therapy (Chudy et al., 2018; Dougherty et al., 1998). In principle, PDT is done by applying a photosensitizer, allowing it to localize to the tumor either passively or by targeting, and then activating the agent with the light of a specific wavelength to the sensitizer. The three main components of PDT, oxygen, light and photosensitizer (PS), are not toxic alone, but together they initiate a photochemical reaction that results in the formation of a highly reactive oxygen called singlet oxygen ($^1\text{O}_2$) (Agostinis et al., 2011). The generation of highly cytotoxic singlet oxygen ($^1\text{O}_2$) is accomplished the photo-excited sensitizer in triplet state. The photosensitizer is excited when exposed to certain wavelengths of light. This excited photosensitizer emits this excess energy as fluorescence and/or heat (B. W. Henderson & Dougherty, 1992). Alternatively, an excited photosensitizer can undergo an

intersystem crossing, changes its spin state and transition to triplet state is observed especially if the molecule has heavy atom on its structure (Pass, 1993). The photosensitizer in the triplet state can either decay to the ground state without radiation or transfer its energy to molecular oxygen (O_2). Returning to the ground state, it releases energy that is transferred to oxygen to form reactive oxygen species (ROS) such as singlet oxygen and free radicals. This can cause significant oxidative toxicity that rapidly leads to cell death via apoptosis or necrosis (Kwiatkowski et al., 2018a). There are three main ways PDT mediates tumor destruction. First, ROS produced by PDT can directly kill tumor cells. Second, PDT may also damage the tumor-associated vasculature, leading to tumor infarction. Lastly, PDT can activate an immune response against tumor cells. All three mechanisms can affect each other.

Tumor localization of the photosensitizer is a strong factor determining PDT invasiveness. Since the singlet oxygen has the high reactivity and short half-life, only cells where the photosensitizer locations are directly affected by the therapy. Besides, photosensitization does not typically occur in oxygen-free areas of tissue because, the effects of almost all PDT drugs are oxygen dependent (Dougherty et al., 1998; Peng et al., 1996). According to the reports, the most effective way to target both tumor blood vessels and tumor cells is administration of photosensitizers at multiple times prior to light activation. It has been reported that multi-dose methods can induce long-term tumor growth control and result in a superior therapeutic effect compared to single-dose methods such as antivasculature PDT or antitumor cell PDT. Another way to direct the photosensitizer to a particular cell type is to use specific targeting carriers, such as conjugated antibodies to tumor-associated antigens or vascular antigens (Xiao-yan et al., 1994). Currently, PDT is being clinically tested for cancer treatments. The first photosensitizer used clinically for cancer treatment was a mixture of a water-soluble porphyrin called a hematoporphyrin derivative (HPD) and a purified form of it, porfimer sodium. This compound later became known as Photofrin (Wang et al., 2021). Photofrin is the most often used photosensitizer in the clinic today. However, this compound has several limitations. Firstly, it is difficult to reconstruct its composition, as it consists of about 60 compounds. Although the compound has an absorption value of 630 nm, high concentrations of sensitizers and light must be given to the tumor, since the molar absorption coefficient at this wavelength is low. Also, Photofrin does not have high selectivity for tumor tissue. Finally, as Photofrin is

retained by the skin, it causes long-term cutaneous photosensitivity and patients receiving Photofrin treatment should stay away from sunlight for 4-6 weeks (Josefsen & Boyle, 2008; Moan & Berg, 1991). There is a need for compounds that have higher tumor specificity and have less skin photosensitivity. To this end, many other sensitizers have recently been developed and entered clinical trials (Pogue et al., 2003; Webber et al., 1996). Within the scope of this thesis, a self-actuator which enhance emission at shorter wavelength as a result of $^1\text{O}_2$ moderated reaction is studied. Following excitation, the photosensitizer transfers its energy to molecular oxygen. Singlet oxygen dependent activity repeating was previously studied by our group with other molecular structures (Erbas-Cakmak et al., 2015; Erbas-Cakmak & Akkaya, 2014; Turkoglu et al., 2020).

1.1.1. Photochemical and Biological Mechanism of Photodynamic Therapy (PDT)

Light treatment has been practiced for more than three thousand years. Ancient Chinese, Indian, and Egyptian cultures all used light to treat various illnesses. When Oscar Raab observed the toxic effects of acridine orange in 1890, he first outlined the idea of photodynamic action. Acridine orange demonstrated activity as a photosensitizer when combined with light and oxygen by destroying *Paramecium caudatum* cells without appearing to harm the protozoa when used alone (Kim et al., 2015; Dennis et al., 2003). Niels Ryberg Finsen's discovery which was awarded with Nobel Prize in Physiology or Medicine in 1903, served as the foundation for PDT. (Chudy et al., 2018) Niels Finsen expanded on the use of "phototherapy" to heal illnesses in Denmark around the end of the nineteenth century (Dennis et al., 2003). Finsen successfully demonstrated phototherapy by treating lupus vulgaris, a skin disorder caused by tuberculosis, with heat-filtered light from a carbon-arc lamp (the "Finsen lamp") (Josefsen & Boyle, 2008). Von Tappeiner's group in Munich developed photodynamic therapy for the treatment of cancer patients with solid tumors (Sternberg et al., 1998). Another German researcher, Meyer-Betz, identified the main challenge facing photodynamic therapy in 1913. He quickly developed a general skin sensitivity after injecting himself with hematoporphyrin, an issue that persists with many photosensitizers today (Josefsen & Boyle, 2008). Thomas Dougherty and his colleagues created photodynamic treatment with hematoporphyrin derivative (HPD) in the late 1970s. They achieved some degree of HPD purification and learned that the administration of HPD followed by red light irradiation produced oxygen-dependent

tissue responses (Kim et al., 2015a). The main contributors to PDT's development to the point that it is practiced today include Dougherty.

A promising non-invasive therapeutic option, photodynamic therapy is also utilized to treat non-oncological disorders (Chudy et al., 2018). PDT is more widely used because to its effective treatment outcomes and the ability to apply it concurrently with other therapeutic protocols. PDT has been applied successfully in gynecology, urology, malignancy, and dermatology.

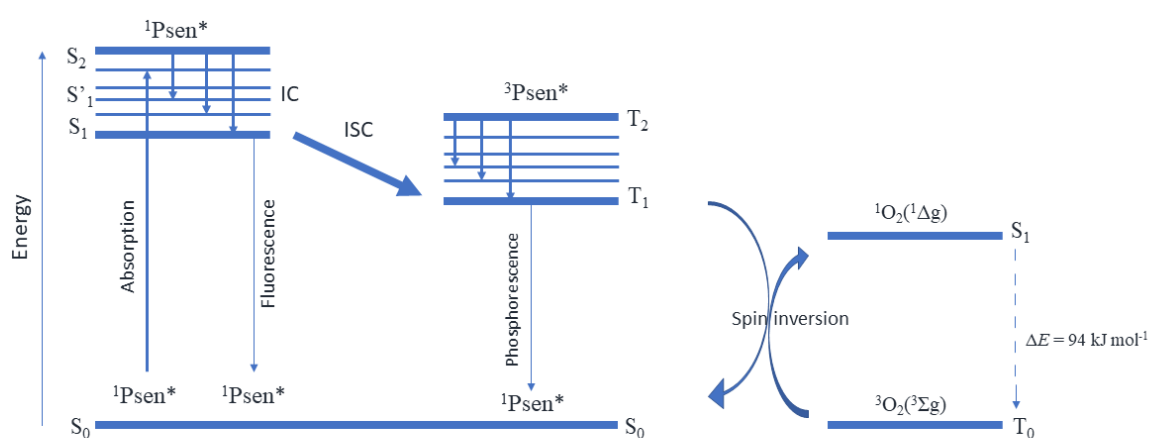


Figure 1.1. Jablonski Energy Diagram. In the case of a transition to a higher energy level, a photon is absorbed; in the case of a transition to a lower energy level, a photon is emitted. The graphic shows several distinct mechanisms—each with a unique label—through which nonradiative transitions might occur. Vibrational relaxation is the process of lowering the stimulated state's vibration to its lowest level. Since this process requires the transfer of energy from the molecule to its surroundings, it is impossible for isolated molecules to engage in it. Internal conversion (IC), a second kind of nonradiative transition, happens when the vibrational state of an electronically excited state can couple to the vibrational state of a lower electronic state.

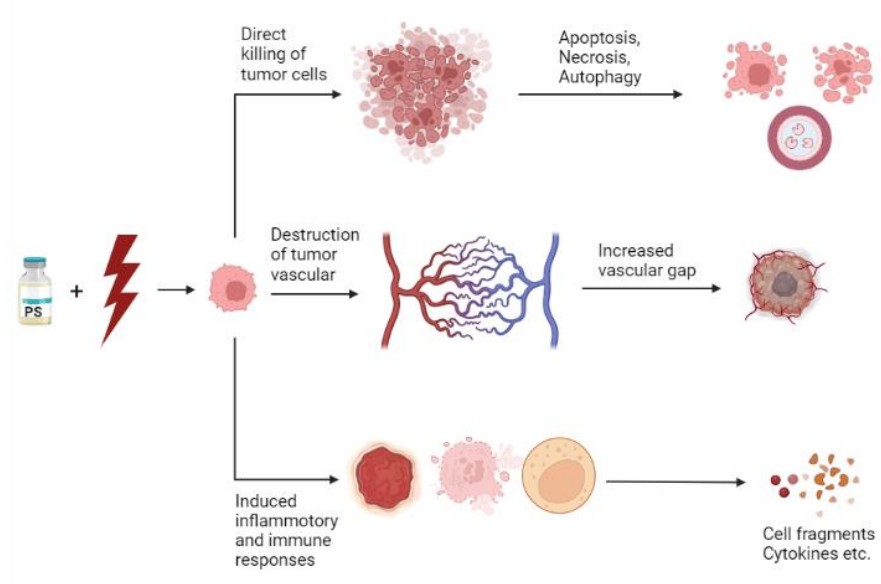


Figure 1.2. Biological responses of Photodynamic Therapy (PDT). Three mechanisms of PDT-mediated tumor injury in vivo: direct killing of tumor cells, damage to the vasculature, and inflammation and immune responses.

Reactive oxygen species (ROS), particularly singlet oxygen ($^1\text{O}_2$) and superoxides, are created when energy from the light-excited PS is transferred to oxygen molecules (O_2). These ROS damage biological molecules, triggering a series of biochemical reactions that end with the destruction and death of neoplastic cells and the damage of other biological molecules (Dougherty et al., 1998; Juzeniene et al., 2007). PDT has a wide range of physiological consequences, many of which are interrelated. PDT has been shown to have impacts on lysosomes, mitochondria, cell surfaces, and other subcellular organelles in addition to membranes. There have been direct effects on DNA and evidence of inhibition of DNA and RNA polymerase. These consequences can be significantly repaired, though. The interaction of ROS with many biological targets, which are vulnerable to oxidation, disturbance of homeostasis, alterations in lipid metabolism, and ion transportation, leads to the direct photodynamic effect against tumor cells (Dąbrowski & Arnaut, 2015a; Dobson et al., 2018; Senge & Radomski, 2013). Additionally, the cellular response to oxidative stress causes the protein kinase pathway to be activated, transcription factors and cytokines to be expressed, and the release of various mediators that are involved in the process of cell death, which can take place through apoptosis and/or necrosis. Autophagy is the third potential mechanism that may result in cell death but may also be cytoprotective

(Kwiatkowski et al., 2018a). These processes can occur simultaneously in the same cell population and are not mutually exclusive.

Radicals and activated oxygen species (ROS), that are essential to the Type II mechanism, which is expected to predominate in PDT, can only interact with molecular oxygen (O_2) when a photosensitizer is in an excited state. These species, which include singlet oxygen ($^1O_2$), hydroxyl radicals ($\cdot OH$), and superoxide (O_2^-) ions, can react with unsaturated lipids, amino acid residues, and nucleic acids, among other biological constituents. Target cell death will occur if enough oxidative damage occurs (only within the immediate area of light illumination) (Josefsen & Boyle, 2008).

Low stimulation of ROS levels may positively stimulate biological systems at low PDT levels. PDT can trigger a variety of immune cells and inflammatory cell mediators in addition to having direct phototoxic effects on target tissues. PDT with different photosensitizers has been demonstrated to modulate cytokine expression after a low light exposure and to induce immune-specific responses, which has been proven to have immunomodulatory effects in inflammatory skin disorders (Kim et al., 2015a).

1.1.2. Photosensitizers

Both natural and synthetic substances called photosensitizing agents transfer light energy to nearby molecules, especially dissolved oxygen (Allison et al., 2004a). Many photosensitizers are cyclic tetrapyrroles or structural derivatives of this chromophore, particularly porphyrin, chlorin, bacteriochlorin, expanded porphyrin, and phthalocyanine (PCs) derivatives. Several different photosensitizers, such as methylene blue, rose bengal, and acridine, are known to be effective singlet oxygen generators (Josefsen & Boyle, 2008).

There has been less research done on photosensitizers for PDT that are not based on tetrapyrrole macrocycles, although structural changes to fullerenes, cyanines, hypericins, or semiconductors have made it possible to interestingly manipulate photochemotherapeutic characteristics. However, porphyrin and phthalocyanine derivatives are still the photosensitizers being used in clinical settings for the treatment of solid tumors (Dąbrowski & Arnaut, 2015a).

1.1.2.1. Ideal Photosensitizer

A good photodynamic sensitizer should meet the following requirements: i) Lack of systemic dark toxicity is preferred. Also, no additional hazardous byproducts from the photosensitizer's metabolism should be produced. ii) The cancerous tissue should accumulate a significant amount of it. It may be advantageous to have a photosensitizer that selectively accumulates in those tissues and goes where you want it to. This presupposes that the intended target for lighting and activation is recognized. iii) It ought to be turned on at the light wavelengths required for deep tissue penetration. To avoid unintentional treatment, reliable activation by a suitable wavelength of light is required. iv) The photosensitizer shouldn't treat one illness just to cause a different one. v) To avoid potential excessive photosensitivity brought on by sunshine, there should be a minimum amount of absorption in the 400–600 nm region. vi) PS should be efficient singlet oxygen and other ROS producers. vii) It should possess the proper photophysical properties, such as a high quantum yield of triplet transition, a high singlet oxygen quantum yield, a reasonably long lifetime for the triplet state. viii) A perfect PS would enable for direct intravenous administration and transport to the intended target and have a stable, straightforward drug formulation that is soluble in biological media. If this doesn't work, a delivery mechanism should be looked for to ensure that the photosensitizer is efficiently and effectively transported to the target region via the bloodstream. Even the most effective theoretical photosensitizer must reach the desired location and activate at the appropriate moment each time.

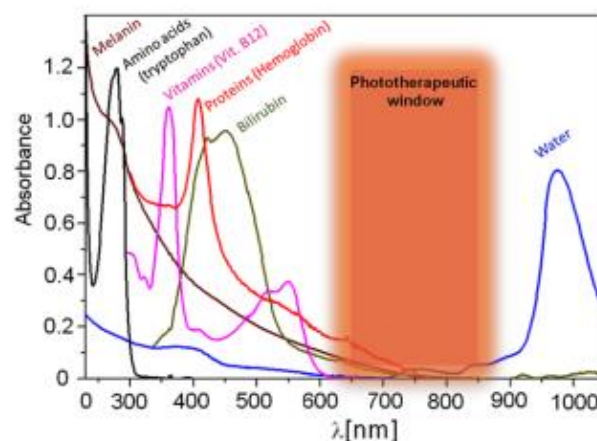


Figure 1.3. Phototherapeutic window. In the spectral band between 650 and 850 nm, where human tissues are the most transparent, electromagnetic radiation has a significant penetrating power. (Dąbrowski & Arnaut, 2015b)

ix) PS should have a quick and high yielding synthesis pathway, be single, affordable, easy to access and well-characterized compounds with a known and constant composition. x) A photosensitizer that causes discomfort during and after therapy will not enable successful outpatient PDT because PDT is performed without anesthesia and is typically performed in an outpatient setting. Patient-friendly outpatient administration and therapy. Additionally, it is economical. It's crucial to make PDT more affordable than other modalities because therapy costs are becoming a bigger factor in insurance considerations. Additionally, patients favor outpatient treatment over hospitalization. xi) The best photosensitizer is the one compatible with other treatments like surgery, radiation therapy, and chemotherapy (Allison et al., 2004a; Kwiatkowski et al., 2018b; Quirk et al., 2015).

1.1.2.2. First Generation Photosensitizers

Many photosensitizers used in PDT in the middle of the 20th century were derived from hematoporphyrin, an endogenous porphyrin produced in the initial phase of the biosynthesis of heme. Porfimer sodium, a commercial substance sold under the brand name Photofrin, has been used experimentally in both a canine glioma model and healthy dogs (Panjehpour et al., 1993; Tochner et al., 1991; Whelan et al., 1993).

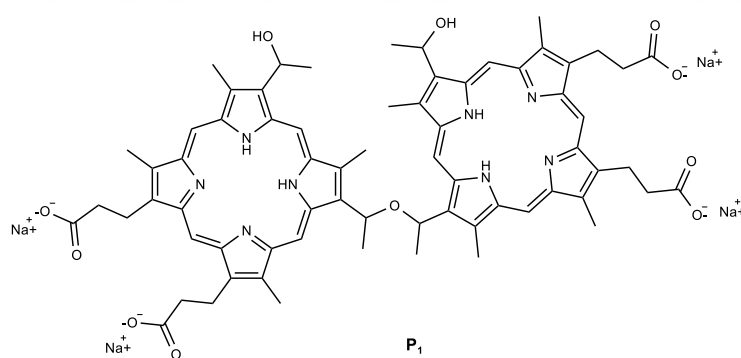


Figure 1.4. Chemical structure of P₁ (Porfimer Sodium (Photofrin))

Despite having many uses in PDT, the preparation has some restrictions on its clinical applications due to the following characteristics: low chemical purity (it is a mixture of over 60 molecules) or poor tissue penetration because the preparation absorbs at a relatively short wavelength, 630 nm, at its maximum (Kwiatkowski et al., 2018a). In addition, because PS has a long half-life and accumulates highly in the skin, there is skin hypersensitivity to light for several weeks following PDT. The drawbacks of the first-generation photosensitizers' synthesis motivate researchers to investigate

1.1.2.3. Second Generation Photosensitizers

a) 

P_2

b) 

P_3

Hematoporphyrin derivatives and synthetic photosensitizers like 5-aminolevulinic acid, benzoporphyrin derivatives, texaphyrins, thiopurine derivatives, chlorin, as well as bacteriochlorin analogues and phthalocyanines are currently included in the group of second-generation photosensitizers. ALA, marketed under the trade name Levulan by DUSA Pharmaceuticals Incorporated in Toronto, Canada, has demonstrated promise in the treatment of both malignant and non-malignant dermatological disorders via topical and intravenous injection of photodynamic therapy. ALA can only be used as a PS once it has been converted into a protoporphyrin. Because of this, ALA or its esters can be applied topically or taken internally in a variety of clinical

uses (Kwiatkowski et al., 2018a). An "occlusion time" is allowed after topical application of the photosensitizer precursor so that the medication can be digested, and porphyrins can build up before being activated by visible light. Esterified ALA derivatives with better pharmacological characteristics have been investigated to address the low bioavailability of ALA when given topically (Brown et al., 2004; Wesley M. Sharman et al., 1999). Photocure ASA (Oslo, Norway) is now marketing a methyl ALA ester under the brand name Metvix as a potential photosensitizer for basal cell carcinoma and other skin diseases (Brown et al., 2004; Detty et al., 2004). Metvix is currently FDA-approved for the treatment of immunocompetent patients' nonhyperkeratotic actinic keratosis of the face and scalp in the US. Photocure ASA has also registered benzyl (Benvix) and hexyl ester (Hexvix) derivatives for the treatment of gastrointestinal malignancies and the detection of bladder cancer (Josefsen & Boyle, 2008; Kim et al., 2015b).

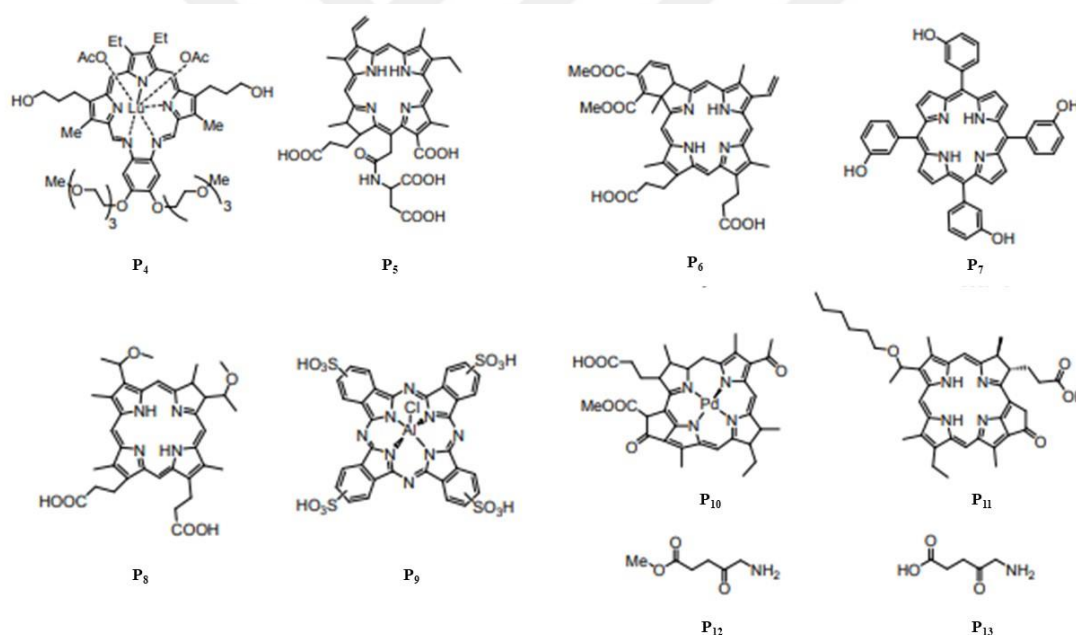


Figure 1.6. Clinically approved Second Generation Photosensitizers; P₄ (Lutrin), P₅ (Laserphryin), P₆ (Visudyne), P₇ (Foscan), P₈ (Photofrin), P₉ (Photosens), P₁₀ (Tookad), P₁₁ (HPPH (Photochlor)), P₁₂ (Metvix), P₁₃ (Levulan)

Under the brand name Foscan, Scotia Pharmaceuticals (Guildford, Surrey, UK) and BioLitec Pharma Limited developed tetra(m-hydroxyphenyl) chlorin (mTHPC) and started clinical trials in the USA and Europe (Dublin, Ireland). Trials evaluating Foscan, also known as temoporfin, as a phototherapeutic agent against head and neck tumors have been conducted. Second-generation synthetic photosensitizers, including

temoporfin or silicon phthalocyanine 4 (Pc4), have strong absorption bands at longer wavelengths, allowing treatments to reach greater depths and reducing the time the skin is photosensitive. Successful second-generation semi-synthetic photosensitizers like verteporfin and talaporfin are based on the chlorin macrocycle and have a strong red absorption band. Under the brand name Visudyne, verteporfin proved particularly effective in treating age-related macular degeneration (AMD) (Dąbrowski & Arnaut, 2015a). Verteporfin has not yet received marketing authorization for use in the treatment of cancer despite its alleged advantages over temoporfin and hematoporphyrin derivatives. Longer drug-to-light intervals lessen side effects because synthetic porphyrin derivatives remove from the tumor more slowly than they do from normal tissues (Berenbaum et al., 1993). Another second generation PS is Lutetium texaphyrin, marketed name Lutex (Pharmacyclics, Calif, USA). These enlarged porphyrins known as texaphyrins, which were synthesized for the first time in 1987 by Sessler and his team, feature a penta-aza core. This macrocyclic alteration causes a significant electromagnetic spectrum absorption in the 730–770 nm range. Since tissue transparency is greatest in this range, this area is very significant (Josefsen & Boyle, 2008).

Second-generation photosensitizers are distinguished by a higher chemical purity, a higher yield of singlet oxygen production, and improved penetration to deeply positioned tissues due to their maximal absorption in the wavelength range of 650–800 nm. They also exhibit less side effects due to a stronger selectivity for malignant tissues and a quicker removal of the photosensitizer from the body. The fundamental drawback of the second-generation PS is that they are poorly soluble in water, which severely restricts their ability to be administered intravenously and demands the development of new drug delivery strategies (Kwiatkowski et al., 2018a).

1.1.2.3. Third Generation Photosensitizers

The development of compounds with a stronger affinity for tumor tissue, with reduced effect of toxicity, forms the basis for the third generation of photosensitizers. The challenge of developing a pharmacological process that would allow the parenteral injection of photosensitizers is another obstacle to the widespread clinical application of photodynamic therapy in oncology. The bioavailability of the photodynamic technique is being effectively increased by new drug delivery technologies (Josefsen & Boyle, 2008). The following photodynamic treatment adjustments are utilized to

improve the drug's selectivity: combining compounds directed at the target receptor with second-generation photosensitizers, combinations of photosensitizers with LDL lipoprotein since proliferating tumor cells require more cholesterol for the synthesis of cell walls, the conjugation of a photosensitizer with a monoclonal antibody targeted against a particular cancer cell antigen, and the use of tumor surface markers like growth factor receptors, transferrin receptors, or hormones are among the possible treatments (e.g., insulin) (Kwiatkowski et al., 2018a). By increasing selectivity and allowing the photosensitizer to accumulate more in the affected areas, these methods provide the potential of lowering drug dosages while maintaining therapeutic efficacy.

With varying photodynamic outcomes, a wide variety of metals have been employed to build complexes with photosensitizer. Regarding the possible success of metalated photosensitizers, there doesn't seem to be a pattern of observation. Although a variety of photosensitizers are metalated, the metal ion does not necessarily give the photosensitizer any specific photoactivity. Contrary to metal-free porphyrins, the Hp complexes with copper, cobalt, iron, and zinc are all photoinactive (Ali & van Lier, 1999). For texaphyrin and PC photosensitizers, however, the opposite has been shown; only the metallo-complexes have shown effective photosensitization (Josefsen & Boyle, 2008). Only a small number of PSs with metal ions have made it to clinical trials. While Purlytin, Motexafin lutetium (Lutrin/Antrin), and TLD-1433 are currently the subject of clinical research, Photosens and TOOKAD Soluble have already received approval (McFarland et al., 2020). Clinical success has been achieved with Photosens, a sulfonated aluminum phthalocyanine, in a range of cutaneous and endobronchial conditions. It has been used to treat infections and cancer. The tongue, lip, pharynx, and larynx are just a few of the head and neck tumors that Photosens has successfully treated (Allison et al., 2004b).

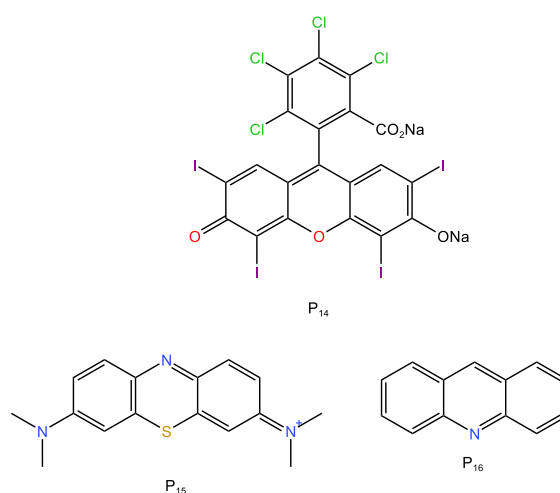


Figure 1.7. Selected examples of dye photosensitizers; P₁₄ (Rose Bengal), P₁₅ (Methylene Blue), P₁₆ (Acridine).

PDT has been used to evaluate several dye sensitizers, including porphyrin, phthalocyanine, and cyanine, for the treatment of cancer (Sun et al., 2019). In the dye family, phthalocyanines and their relatives, the naphthocyanines, are responsible for most of the activity for clinical photosensitizers. These structures are excitable by light of wavelength between 650 and 850 nm (Allison et al., 2004b; Ben-Hurt & Rosenthal, 1985). Since most dyes are hydrophobic, clinical application calls for delivery agents like a liposomal formulation. The efficacy appears to be increased when dyes are linked to various metals. The best PDT activity appears to be provided by aluminum, zinc, and silicon (Allison et al., 2004b). It's noteworthy to notice that all the clinically effective dyes to date contain porphyrin-like structures.

For PDT, a wide range of molecular structures have been investigated, including both synthetic dyes and some natural compounds including hyperacin, curcumin, hypocrellin, and riboflavin. The phenothiazinium salts, such as Methylene Blue and Toluidine Blue, have been extensively explored for both antibacterial applications and, less frequently, for anticancer activities when it comes to synthetic dyes. Another illustration is Rose Bengal (Figure 1.7), which has a long history as a photoactive dye and has been investigated for antibacterial, tissue-bonding, and anticancer applications. With delocalized molecular orbital systems, Squaraine and BODIPY dyes offer good visual absorption (Boens et al., 2012).

P₁₉

14

However, 5-ALA is converted into PPIX after being absorbed by specific cell and tissue types, such as activated T lymphocytes. PPIX can then aggregate specifically in tumor cells and kill them through PDT (Liu & Li, 2020). Rhodamine, D- π -A dyes, AIEgens (aggregation-induced-emission-based fluorescent materials), BODIPY, and other unique PSs have been the focus of recent studies that have concentrated on chemical modifications of conventional fluorophores (Hong et al., 2009; L. Li et al., 2019; Lovell et al., 2010). These resulting PSs were subsequently utilized as scaffolds to create activatable PSs by utilizing a variety of well-established turn-on mechanisms.

1.1.3. Applications of PDT

PDT is one of the most intriguing and excitingly promising methods for treating various viral, non-infectious, and oncologic disorders. Clinical effects can be shown with PDT in neoplastic illnesses such actinic keratoses, squamous cell carcinoma (SCC), basal cell carcinoma (BCC), etc. Actinic keratoses (AK) are rough, scaly lesions in places that have consistently been exposed to ultraviolet light and can develop into SCC. Actinic keratoses are the primary cause of most SCCs, and different rates of advancement have been documented. The condition known as "field cancerization" is characterized by the frequent occurrence of these lesions in multiples and is typically linked to modification of the surrounding skin (field). Only the treatment of nonhyperkeratotic AKs on the face and scalp in combination with a blue light source is FDA-approved for PDT in the United States as of 1999. After one to two treatment sessions, the clearance rate of nonhyperkeratotic actinic keratosis on the face and scalp in the initial FDA Phase II and III investigations using ALA-PDT (Levulan Kerastick; DUSA) was 85% to 90% (Ozog et al., 2016; Wan & Lin, 2014). Actinic keratosis studies are notoriously challenging to carry out. Because actinic keratoses can develop and go away on their own, it is difficult to quantify them before and after therapy. The body of research on PDT for actinic keratoses is intimidating, but ALA-PDT is convincingly effective in treating the condition and has outstanding cosmetic results, especially when compared to 5-fluorouracil and cryotherapy.

Management of SCC is categorized according to metastatic potential as low-risk cutaneous SCC or high-risk (aggressive) cutaneous SCC, where surgical excision is the standard method for the second one. Surgical excision (Mohs surgery), cryotherapy, electrosurgery, topical medications (5-fluorouracil and imiquimod), radiation therapy, and PDT are currently used as treatments for SCC. PDT should not

be used to treat invasive SCC due to the metastatic potential and reduced efficacy rates; it should only be used to treat SCC in situ. Use of red light is recommended for the best possible treatment (Wan & Lin, 2014).

The most prevalent kind of skin cancer in the US is basal cell carcinoma, which develops in the basal cell layer of the epidermis. PDT is frequently used in clinical practice to treat tiny, straightforward superficial BCCs. It has been successfully used as a solitary agent or in a neoadjuvant situation to treat basal cell carcinoma (BCC) in human patients (Berroeta et al., 2007; Rhodes et al., 2007). Topical PDT (with ALA or MAL) consistently demonstrated a higher recurrence rate for both superficial and nodular BCC when compared to surgery for BCC. The main benefit of PDT over electrodesiccation/curettage is a noticeably better appearance of the treated regions, including less clinical scarring and hypopigmentation. The main drawbacks of this procedure include the time commitment (3 hours for incubation), pain, and the requirement for two treatment sessions as opposed to one for excision or electrodesiccation (Dobson et al., 2018).

For inflammatory diseases, some have shown improvement with PDT in clinical trials. Acne vulgaris, the most prevalent dermatologic condition, is believed to be mostly brought on by sebaceous gland blockage, which encourages the growth of bacteria, primarily *Propionibacterium acnes*. Protoporphyrin IX and coproporphyrin III are naturally produced by *P. acnes*, hence using only light sources (blue light > red light) can have a direct therapeutic photodynamic impact (Johnsson et al., 1987). It is believed that ALA and MAL are preferentially absorbed by the pilosebaceous unit after being applied to the skin, enhancing the response to light therapy. Off-label therapies for acne frequently use light-emitting diode-enhanced ALA-PDT and MAL-PDT. PDT generally has a positive effect on inflammatory lesions while having little effect on comedonal/noninflammatory lesions (Wan & Lin, 2014). The outcomes of PDT in rosacea (also known as adult acne), in contrast to acne, are inconclusive at best (Bryld & Jemec, 2007).

About proliferative diseases, increased skin elastosis and collagen degradation are signs of photoaging. Clinically, rhytids, ephelides, lentigines, aberrant elastic fibers, telangiectasias, and irreversible skin darkening can all be seen (Luderschmidt C & Plewig G, 1978). According to histopathology, the papillary dermis has dilated blood

vessels and more pigment than normal. Retinoids, laser resurfacing, chemical peels, and other light therapies are current photorejuvenation procedures. Patients receiving PDT for different disease types frequently experience photorejuvenation, which has led to more research in this field. In addition to increasing skin elasticity, ALA-PDT with blue light has been shown to improve skin texture, pigmentation, fine wrinkles, and complexion (Clementoni et al., 2010; Touma et al., 2004). Following treatment, there have been no reports of changes in deep wrinkles or spotted hyperpigmentation. In one study, mottled hyperpigmentation, fine lines, skin roughness, and sallowness showed statistically significant improvement following MAL-PDT and red-light administration, while deep wrinkles, superficial angiogenesis, face erythema, and sebaceous gland hyperplasia did not. An examination utilizing high-resolution echography revealed increased skin thickness, which the scientists hypothesized to be brought on by an increase in collagen synthesis, to measure epidermal and dermal alterations during PDT (Zane et al., 2007). PDT has been demonstrated to decrease the production of type I collagen and fibroblast activity in vitro, suggesting that it may be useful in the management of scars (Karrer et al., 2003). Using MAL-PDT, several organizations have demonstrated improvements in the skin's flexibility in hypertrophic scars (Bruscino et al., 2011; Campbell et al., 2010).

If we look at other types of cancer that can be applied to PDT, prostate cancer is a desirable target. Due to the proximity of nerves, the urinary bladder, and the rectum, definitive management of early-stage prostate cancer in men with surgery or ionizing radiation therapy is linked with considerable concomitant morbidities. As a result of the ability to provide light to the entire gland utilizing interstitial cylindrically diffusing optical fibers, PDT has the potential to selectively treat the prostate gland while preserving the nearby normal tissues (Agostinis et al., 2011).

According to Mowatt et al., (2011) photo diagnosis is utilized to treat human urinary bladder malignancies, and PDT may be used to treat urinary bladder cancer as well. Human urinary bladder malignancies can be evaluated and debulked endoscopically since they are frequently superficial and multifocal. Additionally, the design of the urine bladder enables the supply of homogenous light via diffusing fibers (Dobson et al., 2018).

PDT can be used in conjunction with current standard therapies (surgery, radiation therapy, and chemotherapy) to treat brain tumors in people, according to experimental and clinical investigations (Muller & Wilson, 1995, 1996). PDT can non-invasively target tumor cells invading the nearby brain, making it a particularly valuable adjuvant to surgery. Initial tests employing different formulations of hematoporphyrin derivatives (HPD, Photofrin), ALA, and m-THPC with light sources like lamps, dye lasers, and diode lasers have shown positive results (Agostinis et al., 2011). Fluorescence guided surgery (FGS) is one of the principal applications for ALA in the treatment of human gliomas. In patients with suspected malignant gliomas, ALA-based FGS has been demonstrated to offer longer survival periods than traditional surgery (Stummer et al., 2006).

The therapeutic agent designed in the thesis project acts as a self-reporter that increases its emission depending on its activity.

1.2. Introduction Part 2

1.2.1 Microscopes

The word microscope comes from the Ancient Greek words *mikrós*, meaning "little," and *skopé*, meaning "to look (at); examine, inspect". The study of small objects and structures under a microscope is known as microscopy. From Van Leeuwenhoek's very early understanding of living individual construct with a simple microscope to the specifics of cellular processes with a range of modern advanced imaging techniques, microscopy has played a significant part in detecting the activity of cells (Hell, 2009; Sanderson et al., 2014). Watching live events with ever-increasing spatial and temporal resolution is the current driving force.

There are numerous varieties of microscopes, and they can be categorized in various ways. One technique is to explain how an instrument interacts with a sample and generates images by either delivering an electron or light beam through the sample in its optical path, detecting photon emissions from the sample, or using a probe to scan over and a short distance from the sample's surface. The optical microscope, which utilizes lenses to refract visible light that passed through a thinly sectioned sample to create an observable image, is the most popular (and first invented) type of microscope. The electron microscope, and numerous varieties of scanning probe microscopes are other important types of microscopes.

It's critical to visualize living things in vivo or in their native habitat without interference to comprehend living things at the molecular level, to clarify the mechanism of cellular bioprocesses, or to find biochemical pathways that lead to disease. Since they entail procedures that damage tissue or are only useful for monitoring surfaces, electron microscopy techniques or atomic power microscopes, which enable imaging with great resolution at 1 nm level, are not appropriate for application in live systems or for imaging dynamic processes (Yang et al., 2016). Light microscopy techniques enable the observation of dynamic biochemical processes in living animals, tissues, and cells without causing tissue damage (Stephens & Allan, 2003). The information gained from the sample in a fluorescent microscope is represented by the light gathered by the objective lens, and this information is constrained by a resolution threshold brought on by light refraction. Resolution, the standard for separating two distinct points on an object under observation, is dependent on the wavelength of the light employed (Chozinski et al., 2014).

Although the confocal method has the best resolution among fluorescent microscopy methods, the obtained resolution is above 100 nanometers due to the toxicity of high-energy UV light and its low tissue permeability. The average resolution obtained when using the highest-energy light in the visible region remains at 200 nm (Chozinski et al., 2014; Mustafa & Jaafar, 2013; Yang et al., 2016). Any optical system's resolution has a theoretical upper limit imposed by the physics of diffraction. Diffraction-limited optical systems exhibit resolution performance at the theoretical instrument limit. Limits for periodic structures with units that are obliquely lit by one of the two diffracted rays (extremely oblique illumination) and are separated by a distance (d). Abbe used the law of diffraction: $d = 0.5 \lambda / NA$, where λ is the monochromatic light's wavelength or the shortest of its mixture of wavelengths, and NA is the limiting numerical aperture of the objective or condenser (Born & Wolf, 1999). Scientists have created new microscopic techniques as a result of the fact that intracellular processes (vesicle transport, protein synthesis and monitoring, DNA replication, etc.) need for a resolution of between 10 and 100 nm. The creation of a method called Super-Resolution Fluorescent Microscopy, which significantly boosts fluorescent microscopy's resolution, was part of the work that earned Robert E. Betzig, William E. Moerner, and Stefan Hell the 2014 Nobel Prize (Möckl et al., 2014).

1.2.1.1. Super-Resolution Fluorescent Microscopy

Super-resolution fluorescence microscopy, in general, is a kind of far-field optical technology that depends on many concepts to achieve super-resolution. Super-resolution fluorescence microscopy, despite having a little history, has had a significant impact on practically all areas of life science. By regulating the photophysical transitions of the fluorophore and taking serial measurements, the Super Resolution Fluorescent Microscopes (SRFM) approach theoretically produces images with a resolution that is ten times higher than that of normal light microscopes (Hell, 2007; Huang et al., 2010; Schermelleh et al., 2010). By examining the photophysical change of the fluorophore being utilized, it is feasible to discuss more than one technique, and it is crucial for the development of novel fluorophores to comprehend how these systems work. Figure 1.9 provides an overview of the STED and PALM/STORM fluorescence technologies' application details. In the STED sub-method, a secondary laser excitation (with light of a longer wavelength) stimulates the fluorophore's emission, causing the excited electron to quickly return to ground level without producing any visible emission (Hell & Wichmann, 1994). The emission is tamed because this procedure occurs before the typical emission. Due to the torus structure, this secondary laser excitation only dampens the fluorophores nearby the focus, allowing emission from the fluorophores still inside the focus to continue. As a result, the resolution is decreased to an average of 25 nm and the signal reception region is condensed (Chozinski et al., 2014; Meyer et al., 2008; Müller et al., 2012). There are instances where the application of redox buffers causes this impact to be lowered to 1.8 nm (Kasper et al., 2010). With the use of this technique, significant advances in science have been made, including the first imaging of centrioles and the investigation of the Golgi network and lipid diffusion (D'Este et al., 2015; Erdmann et al., 2014; Honigmann et al., 2014; Lukinavičius et al., 2014; Schill et al., 2013). For STED, the most fundamental characteristics of fluorophores include photostability, high quantum efficiency, radiation in the red and near-infrared region to lessen intracellular signal noise, and high Stokes shifts (Yang et al., 2016). To decrease emission back absorption and signal noise, the Stokes shift—the difference between emission and absorbance wavelengths—must be increased (Stokes, 1852). Since the excited fluorophore receives secondary laser excitation, significant variations in absorbance and emission wavelengths are anticipated. Energy transfer is one way to

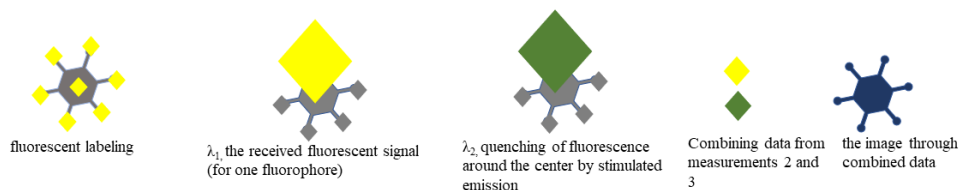
make fluorescent molecules exhibit a high Stokes shift. This application calls for fluorophores with adequate photostability due to the high laser power.

By taking numerous measurements, the Single-Molecule Localization Microscopy (SLM) technique enables the exact and high-resolution determination of each fluorophore's position (Schermelleh et al., 2010). This method marks the structure that needs to be seen with more than one fluorophore (Figure 1.9c). Each fluorophore's emission graph can be represented by a graph with Gaussian symmetry known as the point distribution function; the center of this graph, where the intensity is at its highest, indicates where the individual fluorophore is located (Fernández-Suárez & Ting, 2008). However, the position information of the fluorophore is lost due to interference from emission from several fluorophores. A specific set of fluorescent molecules is identified, its position is established, and it is then quenched once more with random fluorescent activations. A high-resolution image of the target is produced by repeating this set of operations on random fluorophores and using the information obtained to determine where the fluorophores are located on the target item. Depending on whether the fluorescent molecule is photoactivatable, or its fluorescence fluctuates randomly, the technique is referred to as either PALM or STORM (Stochastic Optical Reconstruction Microscopy) (Schermelleh et al., 2010; Yang et al., 2016).

a) Conventional Fluorescent Microscope



b) STED



c) PALM/STORM

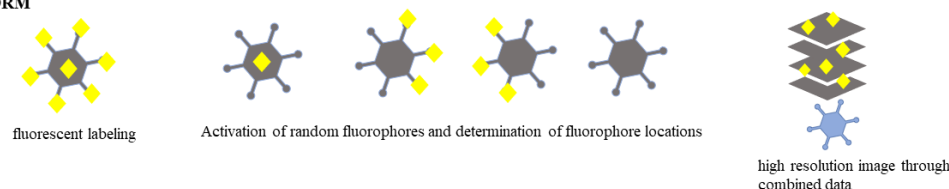


Figure 1.9. Several techniques for super-resolution fluorescence microscopy. Diffraction in the light microscope causes point distribution functions to be imperceptible, resulting in (a) decreased image resolution; (b) the STED method's operation. The center of the fluorophore can be determined, and the resolution is increased by locating the fluorophore when the fluorescence information of the fluorophore

is combined with the information obtained because of the quenching of out-of-focus fluorophores by stimulated emission; (c) In the PALM/STORM method, it is possible to locate fluorophores due to random activation of fluorophores. Fluorophores that quench, photoconvert, or photoactivate light can all be used in this technique.

Another technique used to boost resolution involves using a near-field scanning optical microscope, or NSOM for short (Fischer et al., 1988), to see the sample surface with high resolution by bringing the detector closer to the sample at a shorter distance than the light wavelength. With this technique, the numerical difference takes the place of wavelength as the primary determinant of resolution, and the resolution can drop as low as 10 nm. The major drawback of this approach is the risk of microscope components breaking as a result of measurements made too close to the sample or inaccurate surface aberrations.

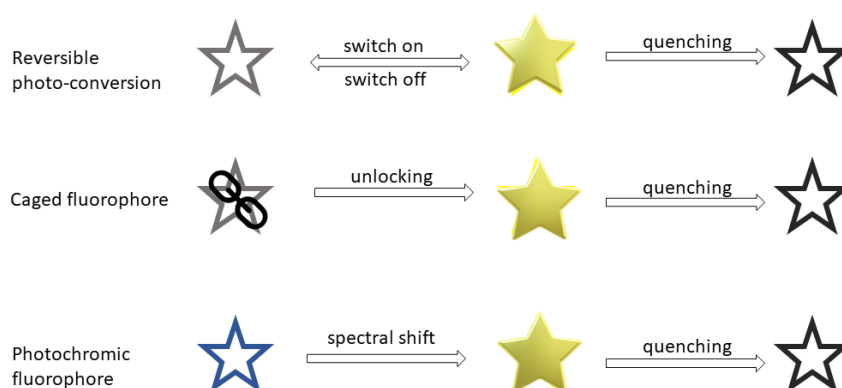


Figure 1.10. Characteristics of the several types of fluorophores utilized in sequential transitions in super-resolution fluorescent microscopy. Fluorophores that are non-emitting, emitting, emitting at various wavelengths, and quenching fluorophores, respectively, are depicted in gray, black, yellow, and blue. Fluorophores that emit with a structural change when there is no emission at first (caged fluorophores), whose emission wavelength changes depending on the external signal, and those whose emission is recorded and lost reversibly can all be employed in this microscope approach.

Figure 1.10 illustrates the activatable photophysical characteristics needed in fluorophores utilized in the Super-Resolution Fluorescence tracking approach. The most popular kind of fluorophores employed in fluorescence procedures (with STED and PALM/STORM methods) are substances with reversible photo transition and

emission that happen and vanish. Because of the significant difference between the two states, a single fluorophore is less affected by the spoofing of the signal from the surrounding fluorophores, leading to fewer false locations in these applications, where the brightness is defined as the ratio between two different emission intensities (Nienhaus & Ulrich Nienhaus, 2014). The number of repeats of the photo transitions, the interval between photo transitions, and the intensity of the light to be employed are significant factors in reversible photo transitions. For providing enough temporal resolution, an optimum photo transition time is necessary. If possible, the molecule's half-life in the emitting state should be sufficient to allow for measurements to be made between 10 and 100 ms (Uno et al., 2014). A single photo transition suffices for the PALM/STORM applications, and the presence of a caged fluorophore along with the typical characteristics of fluorophores (such as photostability, high quantum yield, and high molar absorptivity) may enable high-quality resolution (Chozinski et al., 2014; Vogelsang et al., 2010). The ratio of emission intensity between the two states of the fluorophore (emitting and non-emitting) is crucial for the quality of the information to be collected from the sample in procedures that make use of such variances in emission intensity. Depending on the application, the ratio is anticipated to be at least 70 based on the comparison between these two scenarios. Finally, depending on whether the transition is reversible or not, fluorophores whose emission wavelength varies as a result of the structural change caused by stimulation or analyte can be used in various microscopic techniques. The emission rate between the two states in reversible photo transitions should not go below 17 considering the literature examples (Nienhaus & Ulrich Nienhaus, 2014; Stiel et al., 2007). In the thesis project, spontaneously blinking fluorophore is designed for use in PALM/STORM or single molecule imaging.

Fluorophores that have been employed up to this point and meet some of the requirements for the SRFM monitoring method can be divided into two primary categories: low molecular weight chemical compounds and macromolecules with a protein structure. Green fluorescent protein (GFP) and yellow fluorescent protein (YFP), respectively, are included in the first group, as are additional derivatives created by genetically altering these proteins (Fernández-Suárez & Ting, 2008; Lippincott-Schwartz & Patterson, 2009; Nienhaus & Ulrich Nienhaus, 2014; Zhou & Lin, 2013). The Tsien and Moerner research teams originally discovered photo transmissible versions of these proteins, which can be excited at several distinct

wavelengths, before the advent of super-resolution microscopes (Dickson et al., 2008). The luminous feature of the protein is due to the 4-(p-hydroxybenzilidene)-5-imidazolinone (p-HBI) motif, which is typically made inside the cell from the triple peptides serine, tyrosine, and glycine (Nienhaus & Ulrich Nienhaus, 2014). In most of these proteins, reversible photo transition caused by cis-trans photoisomerization (as in Dronpa) or an irreversible conversion caused by peptide bond breaking around the chromophore or decarboxylation of amino acids (as in EosFP) are observed (Andresen et al., 2007; J. N. Henderson et al., 2009; Rg Wiedenmann et al., 2004). Proteins like IrisFP, for example, enable fluorescence monitoring by utilizing both photophysical phenomena (Adam et al., 2008). Additionally, there are fluorescent proteins with a significant Stokes shift that transfer protons in the excited state, including mNeptune, mCherry, mStrawberry, mOrange, and Mko (Nienhaus & Ulrich Nienhaus, 2014).

One of the best resolutions for measurements conducted with proteins was achieved under 60 nm imaging, and it was possible to carry it out using the citrine protein produced as a result of the alteration of the yellow fluorescent protein (Griesbeck et al., 2001; Hein et al., 2008). While the debbed Dreiklang and esEGFP proteins' photoconversion repetition was enhanced to 160 and 1000 levels, respectively, and resolution values below 40 nm were attained, the modified Dronpa protein only permits 65 times of photoconversion repetition with 50 nm resolution (Brakemann et al., 2011; Grotjohann et al., 2011; Rego et al., 2012). One of the key elements that creates contrast in microscopic monitoring is the ratio of emission intensities in fluorescent and non-fluorescent situations, and the fluorescent protein Pamcherry1 is the most successful at doing so (Subach et al., 2009). When activated by ultraviolet or violet light, these macromolecules, whose excitation wavelength spans between 490-610 nm, are typically quenched at the same wavelength and pass back to the emitting structure (Chozinski et al., 2014).

Although fluorescent proteins' polypeptide composition permits biocompatible measurements, their high molecular weight raises the likelihood of interference with the enzyme or proteins, or biological processes being observed. In addition to this drawback, developing tiny representatives is motivated by the highly time-consuming and expensive labeling sample preparation process using recombinant DNA technology. Another drawback is that because fluorescent protein concentrations in cells rely on protein expression, they may produce false results. It can be inferred from

their molar absorptivity that they are often less valuable than low molecular weight fluorophores (Chozinski et al., 2014; Nienhaus & Ulrich Nienhaus, 2014). Since the proposed fluorophores are low molecular weight substances, it is anticipated that they won't encounter the same problems as protein fluorophores in actual use.

Light-excited fluorophores between 500 and 750 nm are primarily made of cyanine, rhodamine, and oxazine derivatives in the second category of fluorophore samples, which are composed of low molecular weight organic compounds. When compared to proteins, the substances used in this approach have a high molar absorptivity, enabling spatial resolution below 20 nm (Bates et al., 2007; Chozinski et al., 2014; Fernández-Suárez & Ting, 2008). For the STED approach, where fluorophores with low energy photons and high Stokes shift are preferred, a variety of rhodamine, silicon rhodamine (SiR), and coumarin derivatives were synthesized. A Stokes shift of more than 150 nm and spatial resolution below 25 nm were attained (Schill et al., 2013; Wurm et al., 2012). It was possible to see various organelles and their underlying structures (Schill et al., 2013). Using the SiR fluorophore, the ninth symmetry of centrioles was seen for the first time (Lukinavičius et al., 2014). For the first time, a SiR derivative that binds to the cytoskeleton was found in dendrites with a sub-cortical actin lattice (D'Este et al., 2015). A BODIPY-based fluorophore created by utilizing the photo transition of spirofuran was used in a recently published study to enable the localization of organelles with a resolution of 15 nm (Y. Zhang et al., 2018).

Fluorophores with strong emission were prepared where the emission changed by the reduction of azide or nitro units in the structures of dicyanomethylene difuran-derived fluorophores to amine by light or intracellular enzymes. These activatable fluorophores were successfully used in the PALM method (H. L. D. Lee et al., 2010; M. K. Lee et al., 2013; Lord et al., 2008). These fluorophores have been used to track the activity of enzymes like nitroreductase or cytoskeletal proteins during cell division. With a resolution of under 20 nm, rhodamine derivatives can image intracellular microtubules, keratin networks, embryonic fibroblasts, and bacterial surfaces. These compounds photoconvert between the deflated spiro-lactone state and the emitting diionic state (Bossi et al., 2008; Grimm et al., 2013; M. K. Lee et al., 2014). Spirofuran or diethylene photo transitions were used to create fluorescent compounds with reversible transitions between bright and dimmed states, and the PALM technique was used to capture images with a resolution of 20 and 40 nm of

structures like tubulin and lysosome organelles, respectively (C. Li et al., 2014; H. Zhang et al., 2015). The STORM approach, which makes use of the reversible interactions of cyanine, rhodamine, and oxazine dyes with thiol derivatives and phosphines, has been used to achieve a variety of intracellular high-resolution imaging (Umezawa et al., 2017; Yang et al., 2016). In the STORM approach, a potent laser is also used, and a high thiol concentration is necessary for stochastic photo transition. Cell nuclear pore architectures might be studied by using the SiR derivative, without the requirement of external analytes, without the usage of high-power lasers, and by utilizing reversible spontaneous intramolecular Spiro cyclization (Uno et al., 2014).

The major challenge in using the low molecular weight fluorophores discussed above is to successfully mark the structure in the cell that must be monitored with the fluorophores and introduce them into the cell. For labeling, guiding units lacking substantial structures, like antibodies, and chemical alterations that permit access into the cell are needed. Covalent binding of SNAPTag and HaloTag enzymes to the desired protein using these enzymes in the structure by creating the fluorophores that contain the substrates of these enzymes using recombinant DNA techniques is the most popular strategy for accurately labeling the intended protein (Crivat & Taraska, 2012; England et al., 2015; Stephanopoulos & Francis, 2011). In the thesis project HaloTag is used for protein specific labeling purpose.

2. MATERIALS AND METHODS

Within the scope of the project, the light responsive self-actuator and the activatable fluorophore were designed, synthesized and their activity was analyzed. The design, method used for synthesis and analysis, and cell culture procedures are all described in detail below.

2.1. Molecular Design

2.1.1. Molecular Design of Self-reporting Photosensitizer

The thesis project includes chemical synthesis and characterization of the self-actuator shown in Figure 2.1. Extended conjugation of BODIPY core cinnamaldehyde was previously shown to be prone to oxidation leading to either oxidative cleavage, epoxide formation or hydroxyl substitution (Drummen et. al. 2002). These reactions are reported to cause a significant shift in emission wavelength of the compound. In the design of the thesis project, heavy atoms are introduced to a structurally similar compound F1-a to enable intersystem crossing, necessary for photodynamic action. The hypothesis is, upon irradiation, photosensitizer is expected to produce cytotoxic singlet oxygen. Oxidation of diene moieties with singlet oxygen leads to a change in emission wavelength through diene oxidation enabling self-reporting of the photosensitizer activity.

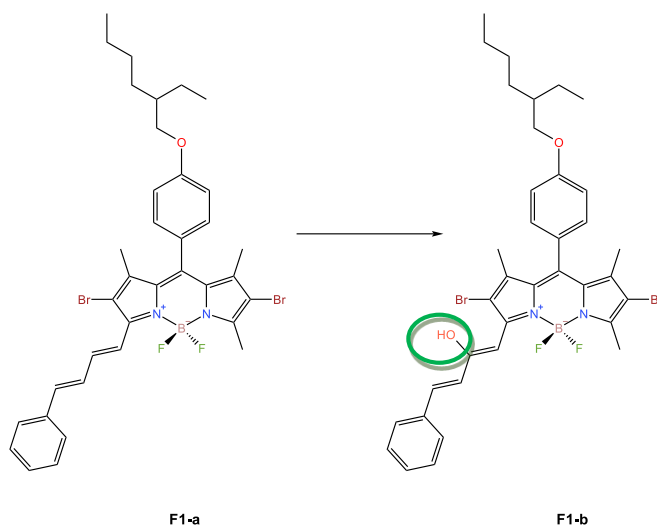


Figure 2.1. Activation mechanism of the self-actuator. F1-a consists of a phenyl moiety linked to the core by a diene linker, and a boron dipyrromethene difluoride core. Diene linker oxidation results in the formation of various oxidation products including a single hydroxyl substituted product F1-b which is expected to change emission wavelength of the compound.

This chemical structure functions using the mechanism shown in Figure 2.1 above. A core made of boron dipyrromethene difluoride, and a phenyl moiety connected to the core by a diene linker comprise the hypothesized agent in the thesis, F1-a. When exposed to oxidizing substances, the diene connectivity is the most probable candidate to experience oxidation. Diene linker oxidation results in the formation of a single hydroxyl oxidation product. It emits electromagnetic radiation in the visible region, with good spectral separation between the nonoxidized (624 nm) and oxidized (545 nm) forms. Fluorescence microscopy or fluorescence-activated cell sorting offer an elegant technique to visualize oxidizing conditions thanks to the fluorescence shift from red to green on oxidation.

2.1.2. Molecular Design of the Activatable Fluorophore (F2-a)

Within the scope of the project, a fluorophore oscillating between two steady states (Figure 2.2) with stochastically bright and dim emission suitable for use in SRFM is proposed. This molecular design works with the mechanism described below and is suitable for use in microscopic techniques such as STED and PALM/STORM. BODIPY derivative was used as fluorophore in the design. This fluorophore, which is chemically possible to adjust the absorbance and emission wavelength allowing easy functionalization, is high quantum efficient and photostable, has been designed to have various photophysical properties (Loudet & Burgess, 2007).

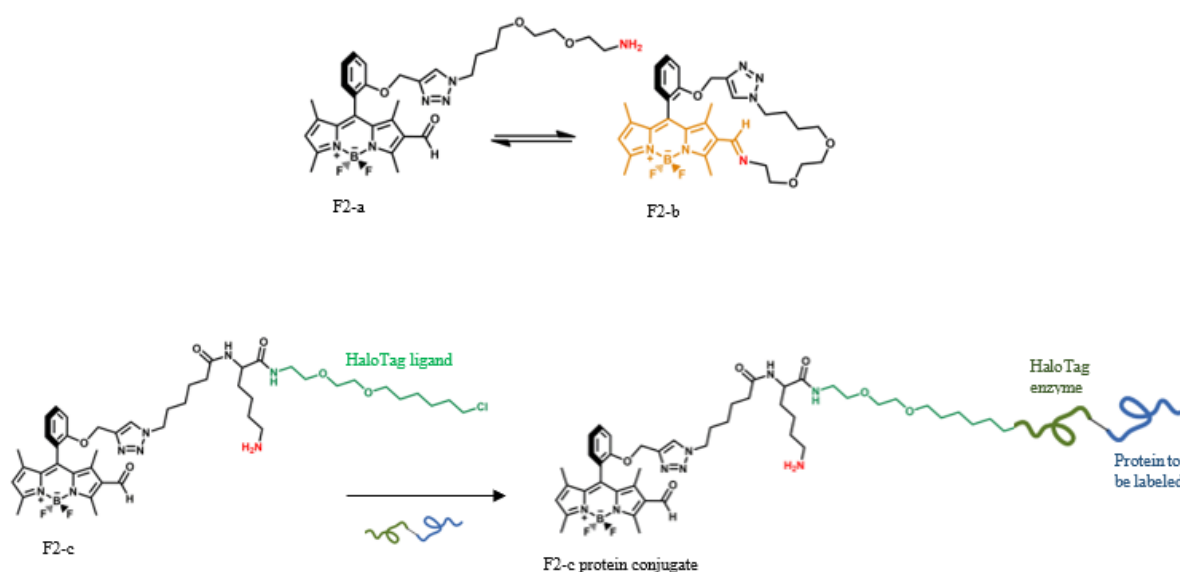


Figure 2.2. Activation mechanism of the dynamic fluorophore

The project's goal is to use dynamic covalent chemistry to manage the intensity of spontaneous emissions. Dynamic covalent chemistry is the study of processes that can be reversed under specific circumstances, such as amine-imine conversion and thiol-disulfide exchange (Ao et al., 2017; Rowan et al., 2002). There has not yet been research on a high-resolution microscopic application that, under the right circumstances, uses these spontaneous reactions to control the fluorophore emission intensity. The goal of the research is to create a new fluorophore for the STORM method, which will enable Single Molecule Monitoring due to the amine-imine dynamic chemistry and is based on the idea that instantaneous fluorescence variations can be used to pinpoint a fluorophore's location. A BODIPY derivative with high quantum yields that alters emission intensity by erratically switching between two chemical states was created for this purpose. Figure 2.2 depict the structure and mechanism of the compounds F2-a and F2-c. Photo inducing electron transfer (FeT) from the amine unit in these compounds' structure dampens their emission (Doose et al., 2009). This circumstance, which has been studied in many BODIPY derivatives and some other fluorophores, causes an electron to transfer from the nearby amine's HOMO (highest energy filled molecular orbital) to the fluorophore's LUMO (lowest energy vacant molecular orbital), which results in the excited electron. It is founded on the idea that something cannot emit emissions by coming back (de Silva et al., 2009). The feasibility of electron transfer is affected as well as the intensity of the fluorescence by altering the HOMO level of the amine unit or the LUMO level of its fluorophore through physical interactions or chemical conversion. In this work, the creation of the intramolecular imine structure will cause the amine unit's HOMO level to be decreased to a lower energy level and impede electron transport. This will make it possible to recover the emission. With imine-amine dynamic covalent chemistry, the exchange reaction that will occur within the molecule will vary the HOMO level rather than an external trigger (Belowich & Stoddart, 2012).

2.2. Synthesis and Characterization

The synthesis is carried out by following the steps shown in Figure 2.3 and Figure 2.4. ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra (Varian Inova (400 MHz), Selcuk University (Konya) and Necmettin Erbakan University (Konya)), High Resolution Mass Spectrometry (HRMS) (Agilent 6530 Accurate-Mass Q-TOF LC/MS of the Eastern Anatolia Advanced Technology Research and Application Centre

(DAYTAM, Erzurum, Turkey)) to confirm the purity of compounds. Purification was done by silica column chromatography.

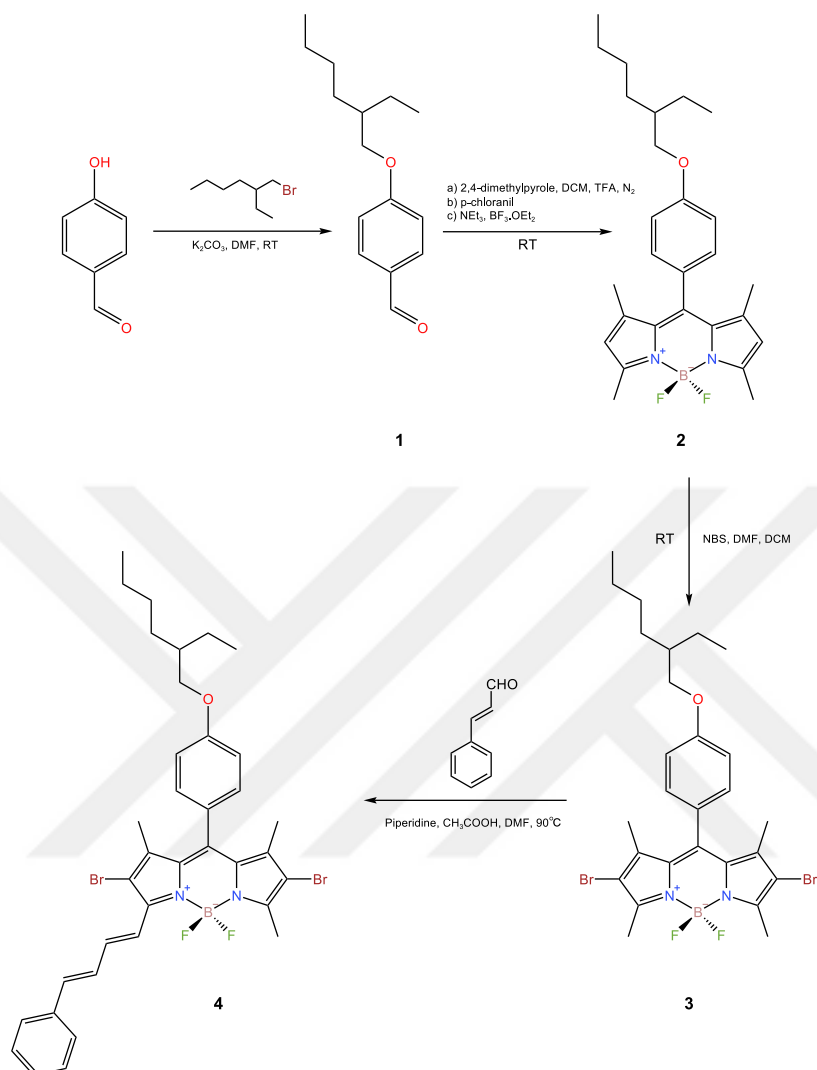


Figure 2.3. Synthesis Scheme of the Self-Actuator. (1) 4-hydroxy benzaldehyde, K_2CO_3 , 2-ethylhexyl bromide, DMF, 16 h, RT. (2) a. 2,4-dimethylpyrrole, TFA, DCM, N_2 , 16 h, RT; b. p-chloranil, 3h, RT; c. NEt_3 , $BF_3 \cdot OEt_2$, 3h, RT; (3) DMF, DCM, NBS, RT; (4) cinnamaldehyde, CH_3COOH , piperidine, benzene, Dean-Stark apparatus, $90^\circ C$

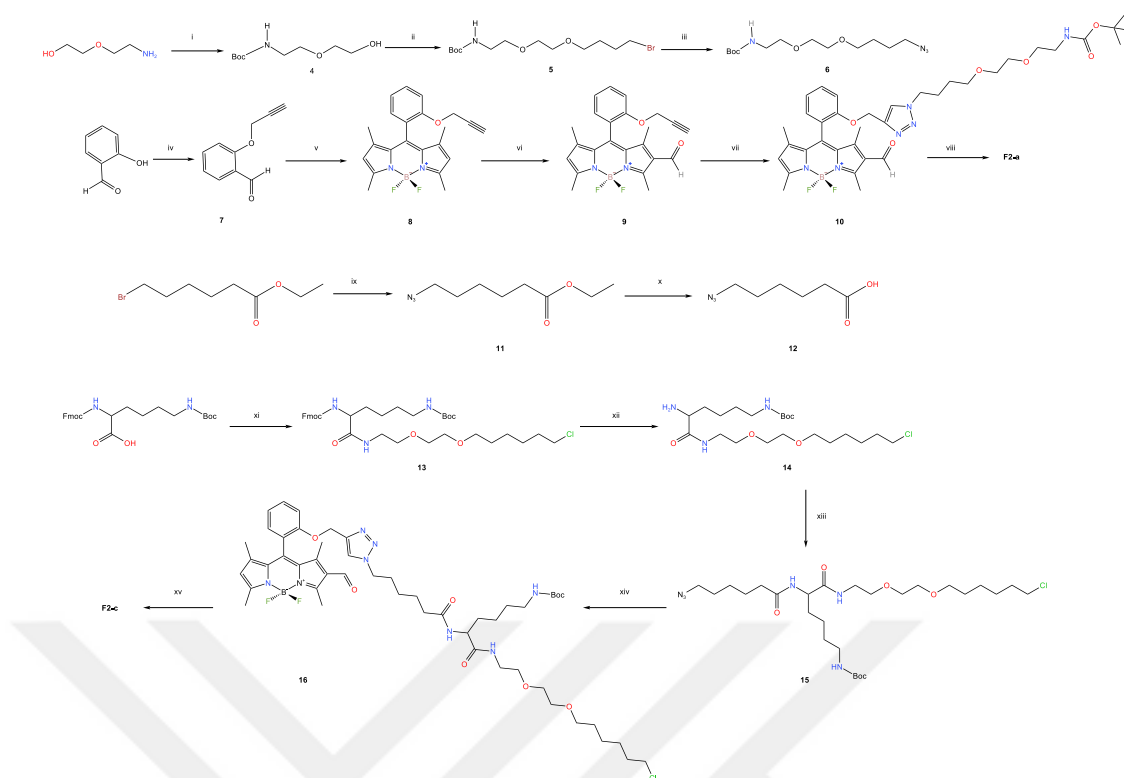


Figure 2.4. Synthesis Scheme of the activatable fluorophore. i) NaH, DMF-THF, 0°C-RT; ii) NaN₃, DMSO; iii) propargyl bromide, K₂CO₃, DMF, 0°C; iv) a. 2,4-dimethyl pyrrole, N₂, TFA, DCM; b. p-chloranil; c. Et₃N, BF₃.OEt₂; v) POCl₃, DMF, DCE, 0°C-60°C; vii) **6**, Cu(I)MeCN₄PF₆, DCM; viii) EDCI, HBTU, THF; ix) piperidine, DCM; x) **5**, DIEA, PyBOP, THF; xi) **9**, Cu(I)MeCN₄PF₆, DCM; xii) TFA, DCM.

2.2.1. Synthesis of Compound 1

2 g (16.5 mmol) 4-hydroxybenzaldehyde was dissolved in 15 mL DMF. 6.8 g (49.5 mmol) K₂CO₃ and 3.3 mL (17.5 mmol) 2-ethylhexylbromide were added. Reaction was stirred at room temperature overnight. It was filtered with filter paper. The filtrate was evaporated under reduced vacuo. The crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was purified with DCM mobile phase by silica column chromatography. The Compound 1 was obtained as colorless liquid with a yield of 2 g (55%).

¹H NMR (400 MHz, Chloroform-*d*) δ 9.74 (s, 1H), 7.69 (d, *J* = 8.8 Hz, 2H), 6.86 (d, *J* = 8.8 Hz, 2H), 3.79 (dd, *J* = 5.7, 1.1 Hz, 2H), 1.68 (m, 1H), 1.50 – 1.25 (m, 4H), 1.30 – 1.14 (m, 4H), 0.88 – 0.52 (m, 6H) (Turkoglu et al., 2020)

2.2.2. Synthesis of Compound 2

1.2 g (5.5 mmol) Compound 1 was dissolved in 150 mL DCM. The solution was purged with N₂ for 15 minutes. 2.35 mL (22.83 mmol) 2,4-dimethylpyrrole was added to the reaction. A catalytic amount (5 drops) of trifluoroacetic acid was added, and the color of the solution turned into brick-red. The reaction was left to stir 16 h at room temperature under N₂ atmosphere. 1.2 g (5 mmol) p-chloranil was added. The reaction was left to stir for 3 hours at room temperature. 4 mL triethylamine and 4 mL boron trifluoride-diethyl etherate were added. The reaction was left to stir for 3 hours at room temperature. The crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was purified with Hexane - EtoAc (4:1; v:v) by silica column chromatography. The Compound 2 was obtained as orange-red solid with a yield of 227 mg (18%).

¹H NMR (CDCl₃, 400 MHz, δ ppm) 7.16 (d, ArH; 2H), 7.01 (d, ArH; 2H), 5.97 (s, ArH, 2H), 3.89 (d, OCH₂; 2H), 2.55 (s, ArCH₃; 6H), 1.82 (m, CH; 1H), 1.45-1.35 (m, ArCH₃ + CH₂; 14H), 0.97 (t, CH₂CH₃; 6H) (Turkoglu et al., 2020)

2.2.3. Synthesis of Compound 3

226 mg (1 mmol) Compound 2 was dissolved in 7 mL DCM and 1 mL DMF. 1 equivalent 178 mg NBS was added. Reaction is checked with Thin Layer Chromatography (TLC) in every 5 min until all the starting material is consumed. The crude reaction was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was purified with Hexane - EtoAc (4:1; v:v) solvent system by silica column chromatography. The Compound 3 was obtained as pink solid with a yield of 164 mg (26%).

¹H NMR (CDCl₃, 400 MHz, δ ppm) 7.08 (d, ArH; 2H), 7.02 (d, ArH; 2H), 3.90 (d, OCH₂; 2H), 2.86 (s, ArCH₃; 6H), 1.76 (m, CH; 1H), 1.50-1.30 (m, ArCH₃ + CH₂; 14H), 0.91 (t, CH₂CH₃; 6H).

¹³C NMR (CDCl₃, 101 MHz, δ ppm) 160.40, 153.64, 142.55, 140.69, 130.86, 128.99, 125.99, 115.43, 111.66, 70.91, 39.41, 30.55, 29.17, 23.88, 23.08, 14.16, 13.95, 11.21.

2.2.4. Synthesis of F1-a

100 mg (0.16 mmol) Compound 3 was dissolved in 15 mL toluene. 1 mol equivalent 24 μ l cinnamaldehyde, 300 μ l piperidine, 1-2 crystal p-toluene sulfonic acid were added to the reaction by turns. The reaction was heated to 110°C. A color change was observed. Formation of purple product was followed by TLC. Reaction is cooled to room temperature. The crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was purified with Hexane - DCM (4:1; v:v) solvent system by alumina column chromatography. F1-a was obtained as blue-purple solid with a yield of 10 mg (8.8%).

¹H NMR (400 MHz, Chloroform-*d*) δ 8.06 – 7.82 (m, 1H), 7.68 – 7.44 (m, 1H), 7.41 (d, *J* = 7.3 Hz, 1H), 7.31 (dt, *J* = 15.8, 7.4 Hz, 2H), 7.21 (d, *J* = 7.3 Hz, 1H), 7.12 – 7.02 (m, 3H), 6.96 (d, *J* = 8.7 Hz, 2H), 6.80 (d, *J* = 15.5 Hz, 1H), 3.85 (d, *J* = 5.7 Hz, 2H), 2.58 (s, 3H), 1.70 (h, *J* = 6.1 Hz, 1H), 1.48 (s, 6H), 1.45 – 1.36 (m, 4H), 1.30 – 1.26 (m, 4H), 0.99 – 0.83 (m, 6H).

2.2.5. Synthesis of Compound 4

Compound 4 was synthesized using the procedure available in the literature (Neklesa et al., 2011). Ditet-butyl dicarbonate (Boc₂O) (4.36 g, 20 mmol) was added to a solution of 2-(2-aminoethoxy)-ethanol (2.1 g, 20 mmol) in dry ethanol (40 mL) at 0°C under nitrogen gas. The reaction mixture was brought to room temperature and stirred at this temperature for about 4 hours. The crude product was extracted with DCM and saturated brine. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. The Compound 4 was obtained as a colorless oil in quantitative yield. The substance was used in the second step without purification.

2.2.6. Synthesis of Compound 5

In a flask, 1.15 g (30 mmol, 60% in mineral oil) NaH was added to 4.13 g (20 mmol) Compound 4 dissolved in a mixture of DMF: THF (20:40). After the mixture was stirred at 0°C for half an hour, 8.63g (40 mmol) 1,4-dibromobutane was added to the reaction at the same temperature. The reaction mixture was stirred at room temperature overnight and the reaction was stopped with saturated NH₄Cl. The crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was partially

purified with Hexane - EtOAc (4:1; v:v) solvent system by column chromatography. The Compound 5 was obtained as yellow oily product in approximately 60% yield, it was passed to the next step without further purification.

^1H NMR (400 MHz, Chloroform-*d*) δ 5.08 – 4.93 (b, 1H), 3.72 – 3.35 (m, 10H), 3.30 – 3.23 (m, 2H), 2.09 – 1.83 (m, 2H), 1.76 – 1.63 (m, 2H), 1.40 (s, 9H).

2.2.7. Synthesis of Compound 6

86 mg (1.32 mmol) NaN_3 was added to 300 mg (0.88 mmol) Compound 5 dissolved in 20 mL of dimethyl sulfoxide (DMSO) at room temperature. The reaction was stirred at 60°C for 3 hours. After the reaction mixture was cooled to room temperature, the crude product was extracted with DCM and H_2O . Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. Compound 6 was obtained pure as a colorless oil in 83% yield.

^1H NMR (400 MHz, CDCl_3): δ 5.03 (b, 1H), 3.57-3.47 (m, 8H), 3.29 (m, 4H), 1.66 (m, 4H), 1.41 (s, 9H) ppm.

^{13}C NMR (100 MHz, CDCl_3) δ 156.2, 79.4, 70.9, 70.4, 70.3, 51.5, 41.2, 40.6, 28.6, 27.0, 26.0.

HRMS: Theoretical $(\text{M}+\text{Na})^+$: 325.1852, Experimental $(\text{M}+\text{Na})^+$: 325.18474, Δ = 1.41 ppm.

2.2.8. Synthesis of Compound 7

7.39 g (53.5 mmol) K_2CO_3 was added to a solution of 3.27 g (26.7 mmol) 2-hydroxybenzaldehyde dissolved in DMF. Then, 3.81 g (32 mmol) propargyl bromide was slowly added to this mixture, the reaction was heated to 70°C and stirred at the same temperature for 3 hours. After the reaction cooled down to room temperature, The crude product was extracted with DCM and H_2O . Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was partially purified with Hexane - EtOAc (4:1; v:v) solvent system by column chromatography. The Compound 7 was obtained as off-white solid with a yield of 4.12 g (96%).

2.2.9. Synthesis of Compound 8

1.5 g (9.3 mmol) Compound 7 was dissolved in 30 mL dry CH_2Cl_2 . The solution was purged with N_2 for 15 minutes. 2.41 mL (23.25 mmol) 2,4-dimethylpyrrole was added to the reaction. A catalytic amount (5 drops) of trifluoroacetic acid was added, and the color of the solution turned into brick-red. The reaction was left to stir 16 h at room temperature under N_2 atmosphere. 2.76 g (11.16 mmol) p-chloranil was added. The reaction was left to stir for 3 hours at room temperature. 9.14 mL triethylamine and 13.87 mL boron trifluoride-diethyl etherate were added. The reaction was left to stir for 3 hours at room temperature. The crude product was extracted with DCM and brine. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was purified with Hexane - EtOAc (8:1, then 7:1; v:v) solvent system by column chromatography. The Compound 8 was obtained as bright orange-green solid in 20% yield.

^1H NMR (400 MHz, CDCl_3): δ 7.45 (dt, $J = 8.6, 1.6$ Hz, 1H), 7.16 (d, $J = 8.6$ Hz, 2H), 7.12 (d, $J = 7.2$ Hz, 1H), 5.95 (s, 2H), 5.69 (s, 1H), 4.68 (d, $J = 2.4$ Hz, 2H), 2.55 (s, 6H), 1.45 (s, 6H) ppm.

^{13}C NMR (100 MHz, CDCl_3) δ 155.1, 154.1, 142.7, 138.4, 131.5, 130.3, 129.8, 124.2, 122.3, 120.9, 112.7, 78.3, 75.9, 55.3, 14.6, 13.8.

HRMS: Theoretical $(\text{M}+\text{H})^+$: 379.1793, Experimental $(\text{M}+\text{H})^+$: 379.17911, $\Delta=0.50$ ppm.

2.2.10. Synthesis of Compound 9

6 mL DMF and 6 mL POCl_3 were added to the balloon in the ice bath and the mixture was stirred at 0°C for 15 minutes, then at room temperature for about half an hour until Vilsmeier-Haack reagent was obtained. 110 mg (0.29 mmol) Compound 8 dissolved in 50 mL 1,2-dichloroethane (DCE) was added to the mixture at room temperature and heated to 60°C . The reaction was checked with TLC and completed after approximately 2 hours. The mixture was then cooled to room temperature and slowly added to saturated NaHCO_3 in an ice bath. This mixture was stirred for 2 hours. The crude product was extracted with DCM and H_2O . Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was

purified with Hexane - DCM (2:1; v:v) solvent system by column chromatography. The Compound 9 was obtained as orange solid with a yield of 100 mg (78%).

^1H NMR (400 MHz, CDCl_3): δ 10.00 (s, 1H), 7.53-7.48 (m, 1H), 7.19 (d, J = 8.8 Hz, 1H), 7.16-7.15 (m, 2H), 6.13 (s, 1H), 4.70 (d, J = 2.0 Hz, 2H), 2.82 (s, 3H), 2.62 (s, 3H), 1.74 (s, 3H), 1.49 (s, 3H) ppm.

^{13}C NMR (100 MHz, CDCl_3) δ 186.3, 161.6, 156.4, 154.1, 147.3, 142.5, 140.7, 131.2, 129.7, 126.3, 124.0, 123.5, 122.8, 113.0, 78.2, 76.4, 55.6, 15.3, 14.6, 13.2, 11.2.

HRMS: Theoretical $(\text{M}+\text{H})^+$: 407.1742, Experimental $(\text{M}+\text{H})^+$: 407.17423, Δ =0.07 ppm.

2.2.11. Synthesis of Compound 10

140 mg (0.46 mmol) Compound 6 and 86 mg (0.23 mmol) Tetrakis(acetonitrile)copper(I) hexafluorophosphate were added to a mixture of 94 mg (0.23 mmol) Compound 9 dissolved in 20 mL DCM at room temperature. The reaction was stirred at room temperature. After checking with TLC, the reaction was completed, and the crude product was extracted with DCM and H_2O . Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was purified with EtOAc - MeOH (4:1; v:v) solvent system by column chromatography. The Compound 10 was obtained as dark yellow oily substance in 88% yield.

^1H NMR (400 MHz, Chloroform- d) δ 9.99 (s, 1H), 7.49 (ddd, J = 8.4, 5.6, 3.6 Hz, 1H), 7.29 (s, 1H), 7.21 (s, 1H), 7.18 – 7.10 (m, 2H), 6.12 (s, 1H), 5.21 (s, 2H), 5.01 (b, 1H), 4.29 (t, J = 7.2 Hz, 2H), 3.60 – 3.49 (m, 6H), 3.45 (t, J = 6.2 Hz, 2H), 3.29 (q, J = 5.4 Hz, 2H), 2.82 (s, 3H), 2.61 (s, 3H), 1.97 – 1.84 (m, 2H), 1.64 (s, 3H), 1.53 (m, 2H), 1.43 (s, 3H), 1.42 (s, 9H).

^{13}C NMR (100 MHz, CDCl_3) δ 186.2, 161.6, 156.2, 156.1, 155.1, 147.4, 144.1, 142.3, 141.1, 134.6, 131.7, 129.4, 126.2, 124.0, 123.6, 122.7, 122.7, 113.9, 70.6, 70.4, 70.3, 63.6, 50.4, 40.5, 29.9, 28.6, 27.4, 26.6, 15.3, 14.7, 13.2, 11.2.

HRMS: Theoretical $(\text{M}+\text{H})^+$: 709.3696, Experimental $(\text{M}+\text{H})^+$: 706.36687, Δ =3.85 ppm.

2.2.12. Synthesis of F2-a

3 mL (1.25 M) HCl-MeOH was cooled to 0°C, and 90 mg (0.13 mmol) Compound 10 was added to this solution. The reaction was stirred at room temperature for 36h. After checking with TLC, the reaction was completed, and the crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo.

¹H NMR (400 MHz, Chloroform-*d*) δ 8.23 (s, 1H), 7.48 (ddd, *J* = 9.1, 7.3, 1.9 Hz, 1H), 7.19 (dd, *J* = 7.9, 2.5 Hz, 2H), 7.15 (d, *J* = 0.9 Hz, H), 7.11 (s, 1H), 6.04 (s, 1H), 5.49 – 4.96 (m, 2H), 4.37 (dt, *J* = 14.0, 7.1 Hz, 1H), 4.29 – 4.07 (m, 1H), 3.89 – 3.65 (m, 2H), 3.67 – 3.50 (m, 6H), 3.50 – 3.31 (m, 2H), 2.82 (s, 3H), 2.57 (s, 3H), 1.81 (m, 2H), 1.51 (s, 3H), 1.49 (s, 3H), 1.43 (m, *J* = 7.7, 2H).

¹³C NMR (100 MHz, CDCl₃) δ 158.17, 156.23, 155.40, 155.00, 145.06, 144.71, 140.50, 139.90, 131.35, 129.46, 124.65, 122.63, 122.60, 114.07, 71.15, 70.82, 70.79, 70.14, 64.08, 62.26, 50.41, 27.71, 26.91, 15.03, 14.55, 14.03, 11.57.

HRMS: Theoretical (M (imine form)+H)⁺: 591.3067, Experimental (M (imine form)+H)⁺: 591.30847, Δ = 2.99 ppm.

2.2.13. Synthesis of Compound 11

2.23 g (10 mmol) ethyl-6-bromohexanoate was dissolved in 15 mL of dimethyl sulfoxide. 1.5 equivalents 975 mg of sodium azide were added. The reaction was stirred at 60°C for 3 hours. After the reaction was cooled to room temperature, The crude product was extracted with diethyl ether and H₂O. The ether phase was extracted 3 more times with water to remove dimethyl sulfoxide. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. The next synthesis step was carried out using the product without further purification.

¹H NMR (400 MHz, Chloroform-*d*) δ 4.12 (q, *J* = 7.1 Hz, 2H), 3.27 (t, *J* = 6.9 Hz, 2H), 2.31 (t, *J* = 7.4 Hz, 2H), 1.62 (m, 4H), 1.48 – 1.34 (m, 2H), 1.25 (t, *J* = 7.1 Hz, 3H).

¹³C NMR (100 MHz, CDCl₃) δ 173.7, 60.5, 51.5, 34.3, 28.8, 26.5, 24.7, 14.5.

HRMS: Theoretical Mass (M-N₂+H)⁺: 158.1181, Experimental Mass (M-N₂+H)⁺: 158.1172; Δ = 5.69 ppm.

2.2.14. Synthesis of Compound 12

1.5 g (8 mmol) Compound 11 was dissolved in 20 mL of THF. 384 mg (16 mmol) LiOH was dissolved in 10 mL of water in a vial and slowly added to the solution. The reaction was stirred at room temperature for 12 hours. The solvent was evaporated under reduced vacuo. The pH was adjusted to 4 with 4M HCl solution. The crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. The Compound 12 was obtained as white solid with a yield of 797 mg (64%).

¹H NMR (400 MHz, Chloroform-*d*) δ 3.28 (t, *J* = 6.8 Hz, 2H), 2.37 (t, *J* = 7.4 Hz, 2H), 1.64 (m, 4H), 1.51 – 1.29 (m, 2H).

¹³C NMR (100 MHz, CDCl₃) δ 180.1, 51.4, 34.1, 28.8, 26.4, 24.4.

HRMS: Theoretical Mass (M-N₂+H)⁺: 130.0868, Experimental Mass (M-N₂+H)⁺: 130.08671; Δ =0.69 ppm.

2.2.15. Synthesis of Compound 13

500 mg (2.12 mmol) Na-Fmoc-Ne-L-Lysine and 474 mg (2.12 mmol) HaloTag ligand were dissolved in 20 mL THF. 406 mg (2.12 mmol) EDCI and 804 mg (2.52 mmol) HBTU were added to the reaction. The reaction was stirred at room temperature for 16 hours. It was filtered, the solid was washed with THF. The crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was purified with ethyl acetate by column chromatography. The Compound 13 was obtained as light-yellow solid in the yield of 694 mg (49%).

¹H NMR (400 MHz, Chloroform-*d*) δ 7.76 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.31 (td, *J* = 7.4, 1.2 Hz, 2H), 6.50 (b, 1H), 5.58 (b, 1H), 4.66 (b, 1H), 4.38 (d, *J* = 7.1 Hz, 2H), 4.20 (t, *J* = 7.0 Hz, 1H), 4.13 (q, *J* = 7.4 Hz, 1H), 3.84 – 3.34 (m, 14H), 3.10 (t, *J* = 6.6 Hz, 2H), 1.86 – 1.66 (m, 2H), 1.57 (m, 2H), 1.50-1.19 (m, 17H).

¹³C NMR (100 MHz, CDCl₃) δ 171.80, 156.37, 144.00, 141.52, 127.97, 127.32, 125.32, 120.23, 120.21, 71.49, 70.54, 70.21, 69.83, 68.87, 67.24, 55.06, 47.38, 45.29, 40.23, 39.51, 32.73, 29.91, 29.62, 28.67, 26.89, 25.60, 22.70.

HRMS: Theoretical (M (imine form)+H)⁺: 674.3572, Experimental (M+H)⁺: 674.35361, Δ =5.32 ppm.

2.2.16. Synthesis of Compound 14

621 mg (0.92 mmol) Compound 13 was dissolved in 20 mL DCM. 30 equivalents 2.73 mL piperidine was added. The reaction was stirred at room temperature and checked with TLC. When the starting compound was consumed after 2 hours, the crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. The Compound 14 was obtained as yellow solid. The next synthesis step was carried out using the product without further purification.

2.2.17. Synthesis of Compound 15

84 mg (0.52 mmol) Compound 12 was dissolved in 10 mL dry THF. 100 mg (0.22 mmol) Compound 14, 156 mg (0.26 mmol) benzotriazol-1-yloxytryrrolidinophosphonium hexafluorophosphate (PyBOP) and 115 μ L diisopropyl ethyl amine (DIPEA) were added to the reaction. It was stirred at room temperature for 16 h. The crude product was extracted with DCM and H₂O. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was partially purified with EtoAc - Hexane (1:1; v:v) solvent system by column chromatography. The next synthesis step was carried out without further purification.

HRMS: Theoretical (M+H)⁺: 591.3637 Experimental (M+H)⁺: 591.36315, Δ =0.93 ppm.

2.2.18. Synthesis of Compound 16

70 mg (118 μ mol) Compound 9 was dissolved in 10 mL DCM. 1.4 mole equivalents 142 mg (165 μ mol) Compound 15 and 1 equivalent 64 mg (118 μ mol) CuMeCN₄PF₆ was added to the reaction. The reaction was stirred at room temperature for 16 hours. The crude product was extracted with DCM and H₂O + EDTA. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. It was purified with MeOH - EtoAc (4; % v/v) solvent system by column chromatography. A second purification with the same system was performed using the preparative (prep) TLC stationary phase. The Compound 16 was obtained as orange solid substance in 50% yield.

^1H NMR (400 MHz, Chloroform-*d*) δ 9.93 (d, J = 1.3 Hz, 1H), 7.49 – 7.38 (m, 1H), 7.23 (dd, J = 8.4, 2.1 Hz, 1H), 7.13 (d, J = 2.6 Hz, 1H), 7.09 – 7.03 (m, 2H), 6.46 (b, 1H), 6.35 (dd, J = 14.1, 7.9 Hz, 1H), 6.07 (s, 1H), 5.15 (s, 2H), 4.65 (b, 1H), 4.47 – 4.24 (m, 1H), 4.18 (td, J = 7.1, 5.0 Hz, 2H), 3.58 – 3.24 (m, 8H), 3.01 (d, J = 6.6 Hz, 4H), 2.76 (s, 3H), 2.55 (s, 3H), 2.11 (dd, J = 8.4, 6.5 Hz, 2H), 1.87 – 1.65 (m, 8H), 1.64 – 1.45 (m, 8H), 1.45 – 1.06 (m, 21H).

^{13}C NMR (100 MHz, CDCl_3) δ 186.18, 186.11, 172.50, 171.62, 156.12, 154.86, 154.84, 143.97, 140.89, 131.45, 129.16, 123.91, 123.36, 122.43, 122.38, 113.66, 71.31, 70.33, 70.03, 69.60, 62.84, 62.79, 52.90, 50.17, 45.07, 40.09, 39.26, 35.95, 32.52, 32.36, 29.81, 29.66, 29.42, 28.45, 26.68, 26.03, 25.40, 24.78, 22.58, 15.14, 14.44, 12.97, 10.98.

HRMS: Theoretical ($\text{M} + \text{H}$) $^+$: 997.5301, Experimental ($\text{M} + \text{H}$) $^+$: 997.52417, Δ =5.94 ppm.

2.2.19. Synthesis of F2-c

17 mg (17 μmol) Compound 14 was dissolved in 8 mL DCM. 120 mL trifluoroacetic acid was added to the reaction. It was stirred at room temperature for 16 h. 2 mL toluene was added. The solvent was evaporated under reduced vacuo. The crude product was extracted with DCM and sodium bicarbonate saturated solution. Organic layer is collected and dried over sodium sulfate. Then, the solvent was evaporated under reduced vacuo. The solid dark orange product was obtained in quantitative yield.

^1H NMR (400 MHz, Chloroform-*d*) δ 9.90 (s, 0.28H, peak from aldehyde state), 8.13 (d, J = 3.8 Hz, 0.68H, peak from imine state), 7.71 (d, J = 13.7 Hz), 7.43 (tt, J = 7.3, 1.4 Hz), 7.19 – 6.87 (m), 6.36 (td, J = 12.7, 11.4, 7.0 Hz), 6.15 – 5.79 (m), 5.32 – 4.93 (m), 4.57 – 3.92 (m), 3.68 – 3.06 (m), 2.85 – 2.61 (m), 2.64 – 2.34 (m), 2.18 – 1.86 (m), 1.91 – 1.12 (m).

HRMS Mass Data (M (imine form) $\text{CH}_3\text{COOH} + \text{H}$) $^+$: 939.4882, Experimental Mass (M (imine form) $\text{CH}_3\text{COOH} + \text{H}$) $^+$: 939.15.

2.4. Spectroscopic Analysis

It is expected that the structure of F1-a will change as a result of light treatment. Spectroscopic analysis was used to obtain an absorbance/emission data to observe the wavelength shift of the molecule. UV-Vis absorbance and fluorescence measurements

of compound F1-a were made in the presence and absence of light. Absorbance and emission spectra were recorded using UV-Vis Absorbance (Agilent, Cary 60) and Fluorescence Spectrometry (Agilent, Cary Eclipse) respectively.

For UV-Vis absorbance – fluorescence measurements, 1 mg (1.38 mmol) F1-a compound was dissolved in dimethyl sulfoxide (DMSO) and 1 mM stock A was prepared. 300 μ l of this stock was taken and exposed to light for 24 hours in another vial, which was recorded as stock B. Stock A and B were diluted a second time in DMSO with a final concentration of 50 μ M and their emission and absorbance were measured by excitation at 500 nm.

F1-a is expected to be activated, converted to F1-b, and produce singlet oxygen upon stimulation with light. 1,3-diphenyl isobenzofuran (DPBF) molecule was used as a trap compound to prove the presence of singlet oxygen. In the presence of singlet oxygen, DPBF reacts with the molecule as given in Figure 2.5 and causes a decrease in its absorbance. (Krieg, 1993) DPBF molecule (50 μ M) was prepared in DMSO. 50 μ M DPBF and compound F1-a (50 μ M) were prepared in DMSO. While measurements were taken in the dark for the first 20 minutes, measurements were taken in the light every 10 minutes in the following minutes. To prove the presence of singlet oxygen, individual analysis of singlet oxygen production was performed with 50 μ M pre-irradiated agent (F1-b) and non-irradiated agent (F1-a). Light application to the samples was made with an LED (595 nm) light source from a distance of 15 cm for the specified time at room temperature. All spectrum measurements were performed in a quartz cuvette.

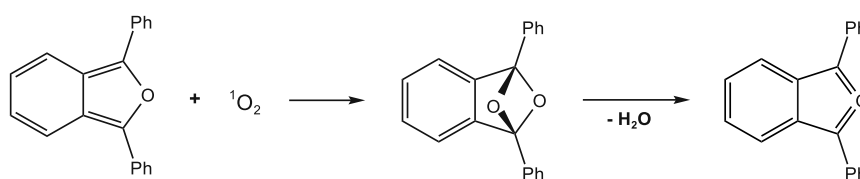


Figure 2.5. The reaction that causes a decrease in the absorbance of the molecule as a result of the reaction of the singlet oxygen and DBBF trap molecule. $^1\text{O}_2$ is produced by F1-a.

2.5. Cell Culture Experiments

Culture medium (The High Glucose DMEM for F1-a, RPMI 1640 for F2-a), which contains 10% Fetal Bovine Serum (FBS) and 1% Gentamicin, was used to cultivate MCF-7 human breast cancer cells (ATCC). Amino acids, vitamins, carbon sources,

growth factors, hormones, and inorganic salts must all be present in the cell media. In order to give the cells growth factors and hormones, FBS is added to the basal media, which already contains some nutrients including amino acids, vitamins, and carbon sources. (Segeritz & Vallier, 2017) The cells must be incubated in an environment that mimics the human body in terms of temperature, moisture, oxygen and carbon dioxide levels, and adequate hydration. The ideal conditions are 37°C, 5% CO₂, and 20% O₂. Antibiotic gentamicin is required to stop bacterial contamination in cell culture. (Elliott & Jiang, 2019)

The cells (MCF-7) stored at – 80°C were quickly transferred to the 37°C incubator, and the cryo vial was waited for a maximum of 5 minutes to dissolve. The dissolved cells were transferred from a cryo vial (2 mL) to a falcon tube (15 mL), medium was added to it at least as much as its own volume and centrifuged at 1200 rpm for 5 minutes to remove the solution. After centrifugation, without harm the cells that settled to the bottom, the liquid supernatant was poured into the waste box, the pellet was well dissolved with light hits. 3 mL of fresh medium, which we knew to be able to reproduce before, was added to it, and it was mixed without making bubbles. Cells were transferred to the T25 flask and filled with medium according to the volume of the flask. The dissolved cells were well mixed. Cells were observed under a microscope before placing them in the incubator. It was left in the incubator (37°C, 21% O₂, 5% CO₂) according to the cell's adhesion and proliferation status to the flask. Passaging and for a time medium change were performed within a few days.

Passaging cell lines was carried out because the cells must be passaged before they attain confluence. There are three distinct growth phases that cells typically go through: (a) a lag phase during which they adjust to their surroundings; (b) an exponential growth phase; and (c) a plateau, which denotes that all of the cells have confluence (Masters & Stacey, 2007).

First, the medium was carefully pipetted out of the flask using a Pasteur pipette in order to passage the cells. To separate the cells, 1 mL of trypsin-EDTA was applied to the T25 flask. The flask was placed back in the incubator for five minutes after trypsin-EDTA was introduced. Under the phase contrast microscope, the cells were examined to see if they were moving or not. The T25 flask received 2 mL of fresh media after the cells had been detached. After that, the cells were placed in a 15 mL-Falcon and

centrifuged at 1200 rpm for 5 minutes. After centrifugation, the pellet was resuspended in 1 mL of media after the supernatant was removed. The cells from the pellet were transferred to a T25 flask together with 4 mL of new medium. The cells were incubated at the normal incubator so that they were able to attach to the surface of the flasks.

2.5.1. Cytotoxicity Experiments

2.5.1.1. Cytotoxicity Experiment for F1-a

In a 96-well plate that is ideal for high-throughput screening, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction test is most frequently employed to determine cell viability. This method is based on the transformation of the tetrazolium salt MTT into formazan crystal dyes. Cellular oxidoreductases that are NAD(P)H-dependent convert the tetrazolium salt MTT into the 570 nm-absorbing formazan purple dye. (Riss et al., 2013) This conversion proves the cells are alive since it shows that the cells have mitochondrial activity. To achieve homogeneous readings, the resultant formazan crystal dyes are solubilized in DMSO or ethanol. Therefore, the optical density (OD) of an ELISA plate reader at 570 nm can be used to compute the formazan concentration, which can then be used to detect changes in the number of viable cells. The drop in viable cell counts suggests that the drug is inhibiting cell growth. The MTT assay can also be used to determine the concentration of the substance needed to achieve a 50% growth inhibition compared to the growth of the untreated control (50% inhibitory concentration, IC_{50} value).

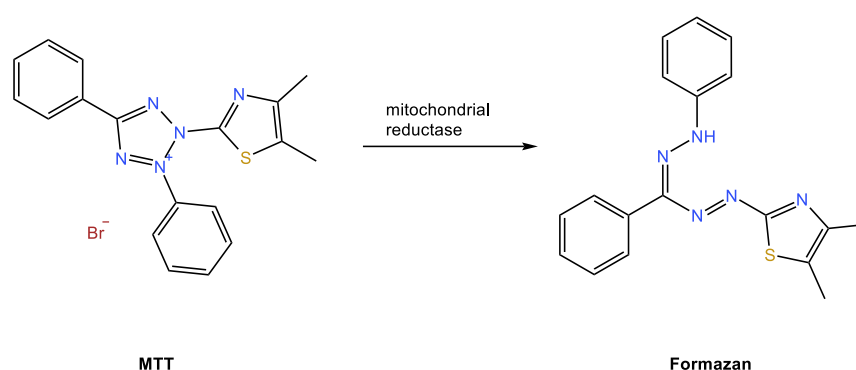


Figure 2.6. Chemical reaction happening when the MTT solution is added to the cells. The MTT is reduced by the functional mitochondria to form formazan crystals.

By using the MTT assay on the MCF-7 cell line after a 48-hour treatment period, the agent's cytotoxic effects were examined. MCF-7 cells (8×10^3 cells) were grown in an incubator at $37^\circ C$ and 5% CO_2 using High Glucose DMEM medium that is

supplemented with 10% FBS and 1% gentamicin (Thermo Scientific, Steri-cycle i160 CO₂ Incubator). After removing the media from the flask, PBS was used to wash the MCF-7 cells. After that, they were separated by adding 1 mL of trypsin-EDTA and incubating the mixture for 5 minutes. After adding 2 mL of fresh medium for the T25 flask, everything was moved to a 15 mL falcon. For five minutes, the falcon centrifuged at 1200 rpm. The pellet was dissolved in the remaining medium after supernatant was poured over it. 1 mL fresh medium was added to the falcon and 100 µl of that was transferred to a vial. 200 µl trypan-blue solution (0.5%; w/w) is added on it. It is mixed carefully, and 15 µl cells were loaded on both ends of the hemocytometer, then viable cells were counted and noted (2 times). After the first count, the hemocytometer was cleaned with alcohol and counted again. The total cell number was found by dividing the desired cell number. 5000 cells in each well were seeded in 2 different 96-well plates, in 200 µl medium/well, calculated to be run at least 3 times. Cells were incubated for 48 hours and adhered to the surface. MCF-7 cells were treated with different concentrations (8-64 µM) of the agent F1-a and incubated for 48 hours in 96 well-plates which contains a final volume 200 µl/well. Experiment was done with four replicates. One of the plates was kept in the dark for 48 hours, while the other plate was stimulated with 595nm light for 4 hours from a distance of 15 cm and kept in the dark for 44 hours. 10 µl MTT solution (5 mg/mL) was added to each well after 48 hours incubation and mixed gently. After 4 hours, the medium was removed and 100 µl DMSO was added to each well to solubilize the precipitate. The cells viability was estimated by measuring absorbance at 570 nm using a Quant ELISA plate reader (Bio-tek Instruments, USA).

The cell viability was also measured by using the formula;

$$\text{Relative cell viability \%} = (\text{OD}_{\text{treatment}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100 \quad \text{Formula 1}$$

2.5.1.2. Cytotoxicity Experiment for F2-a

Human Breast Cancer Cells (MCF7) were incubated in RPMI 1640 medium (Sigma, #RNB7082) in the presence of 10% Fetal Bovine Serum (FBS, Biological Industries, #2004012) and 1% Gentamycin Sulfate (Biological Industries, #1909362) at 37°C and 5% CO₂ to prevent contamination. (Thermo Scientific, Steri-cycle i160). Cells were examined with a microscope (Zeiss Axio observer), and when the flask reached 80%

fullness, it was removed from the plate and passaged using trypsin-EDTA solution (Sigma, #SLCC7772). Cell counts were performed using a hemocytometer.

Cytotoxicity analysis was performed with MCF7 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay. 5000 cells were seeded in an equal number of 96-well plates in RPMI 1640 medium, incubated at 37°C and 5% CO₂ for approximately 48 hours for cells to adhere. After the incubation, different concentrations of agents were added, and additional incubation was carried out under the same conditions for 24 hours. Stock solutions of agents were prepared in DMSO, and after adding to the cell, the DMSO concentration was diluted to be below 1%. After incubation, 0.5 mg/mL of MTT was added to each well and incubated for 4 hours in the dark. After 4 hours, the medium was carefully removed, and the formazan crystals were carefully dissolved by adding 100 μ L of DMSO. After 15 minutes of incubation in the dark, it was analyzed at 570 nm with a Quant ELISA Plate Reader (Bio-tek Instruments, USA). Each experiment was performed in at least 3 replicates.

2.5.2. Fluorescence Cell Imaging

2.5.2.1. Fluorescence Cell Imaging for F1-a

For cell fluorescence imaging, cells were seeded in High Glucose DMEM medium (10% FBS, 1% Gentamicin) at 50,000 cells per well in a 6-well plate. The agent was added after approximately 48 hours of incubation. Agent was added to the cell at a final concentration of 10 μ M. Agent stock solutions were prepared in DMSO and diluted with High Glucose DMEM medium. While the first group samples were incubated for 24 hours in the dark, the second group samples were exposed to 595 nm light from a distance of 15 cm for 4 hours and the third group samples for 8 hours. The second group was placed in the dark at the end of the 4th hour and the third group at the end of the 8th hour and incubated for a total of 24 hours. After 1 day of incubation after the treatment, the medium of the cells was removed, washed twice with Phosphate Saline Buffer solution (PBS), 2 mL of 4% formaldehyde was added, and fixated in the dark for 5 minutes. Cells were washed three times with 1 mL PBS and visualized with a Zeiss fluorescence microscope by adding 0.5 mL of PBS. Cell relative emission intensities were analyzed using ImageJ software.

2.5.2.2. Fluorescence Cell Imaging for F2-a

For cell fluorescence imaging, cells were seeded in RPMI medium (10% FBS, 1% gentamicin) at 200,000 cells per well in a 6-well plate. The agent was added after approximately 48 hours of incubation. Agent was added to the cell at a final concentration of 10 μ M. Agent stock solutions were prepared in DMSO and diluted with RPMI medium. After 1 hour of incubation after the treatment, to accelerate the imine-amine transition into one of the agent-treated wells and to understand the change in emission intensity, cell pH was reduced to 6.5 for all cells (all cells treated with F2-a at normal and low pH). And cells were incubated for additional half an hour. For this purpose, the cell medium was changed by adding dilute HCl solution, and the pH was controlled. The medium of the cells was removed, washed twice with PBS, 2 mL of 4% formaldehyde was added, and fixated in the dark for 5 minutes. Cells were washed three times with 1 mL PBS and visualized with a Zeiss fluorescence microscope by adding 0.5 mL of PBS. Cell relative emission intensities were analyzed using ImageJ software.

3. RESULTS AND DISCUSSION

3.1. Results

For two projects, agents (F1-a and F2-a) were synthesized and characterized by using ^1H NMR, ^{13}C NMR, and ESI-MS spectroscopy. According to the characterization of agents, it was verified that all the target agents were obtained. Absorbance and emission spectra of the agents were recorded by using UV-Vis Absorbance (Agilent, Carry 60) and Fluorescence Spectroscopy (Agilent, Carry Eclipse), and samples were excited at 500 nm for F1-a, 490 nm for F2-a, and F2-c to obtain data. Fluorescence peaks for F1-a were 545 nm and 624 nm, for F2-a 517 nm, and for F2-c 514 nm and 536 nm in DMSO. Absorbance peak values were 604 nm for F1-a, 499 nm for F2-a, and for F2-c 502 nm and 519 nm. MTT Assay showed that neither F2-a, nor F2-c is not cytotoxic for cells at lower ligand concentrations. Results are given below in detail.

3.1.1. Photophysical Analysis

3.1.1.1. Results of UV-Vis Absorbance and Fluorescence Spectrometry for F1-a

Compounds were dissolved in DMSO, and absorbance and emission spectra were recorded. Samples were excited at 500 nm to obtain fluorescence data. Absorbance peaks were almost the same and 604 and 603 nm for F1-a and F1-b respectively (Figure 3.2). As shown in Figure 3.1, fluorescence peak values were 624 nm for F1-a, and 545 nm for F1-b.

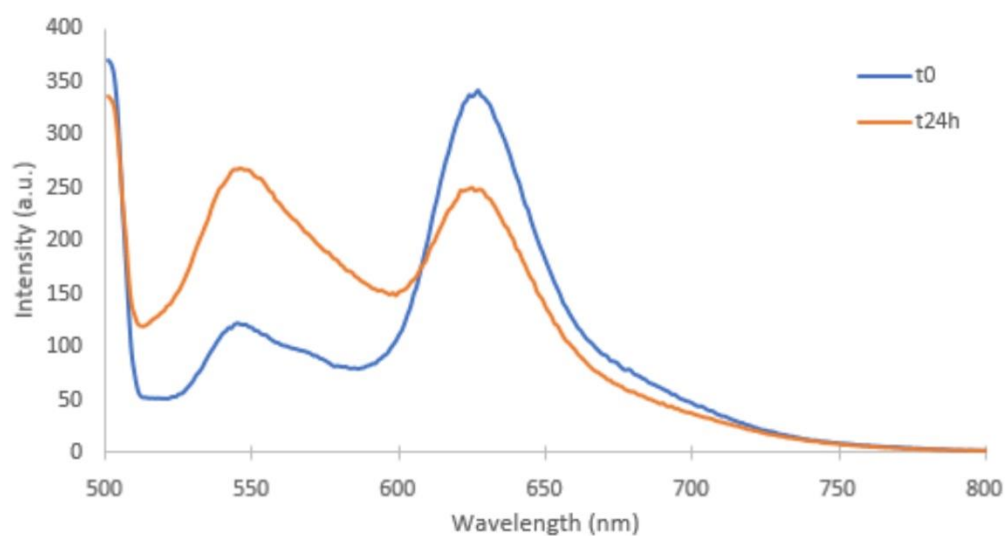


Figure 3.1. Emission spectra of F1-a (15 μ M) in DMSO before light is applied (t0), and after light is applied (t24h). (Excitation 500 nm) 595 nm light was applied to the samples at 15 cm for 24 hours.

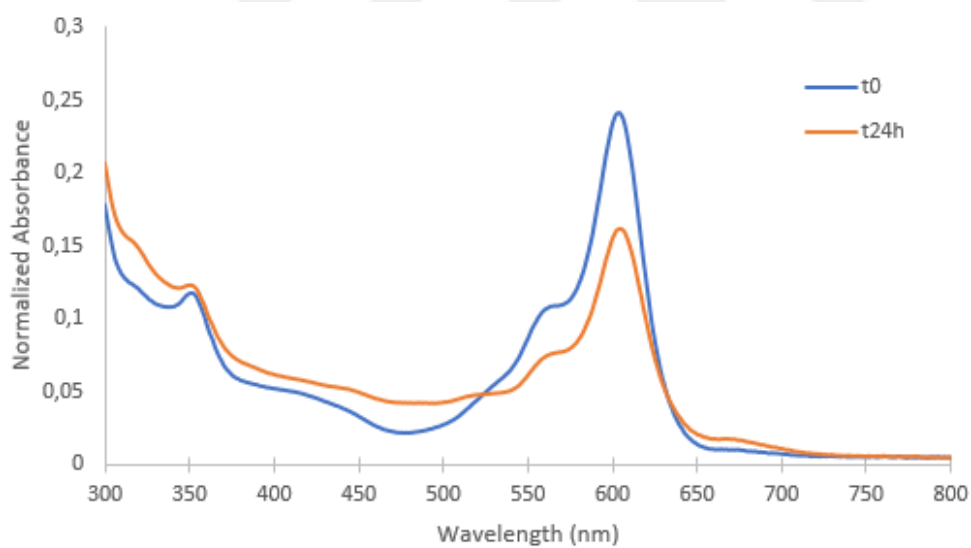


Figure 3.2. Absorbance spectra of F1-a (15 μ M) in DMSO before light is applied (t0), and after light is applied (t24h). 595 nm light was applied to the samples at 15 cm for 24h.

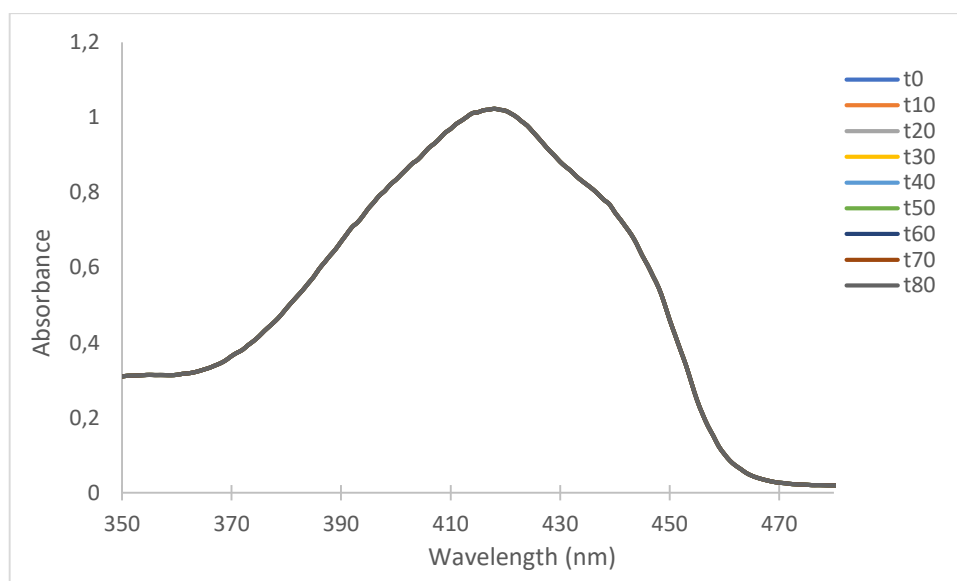


Figure 3.3. Decrease in absorbance of DPBF in DMSO. 595 nm light was applied to the samples at 15 cm.

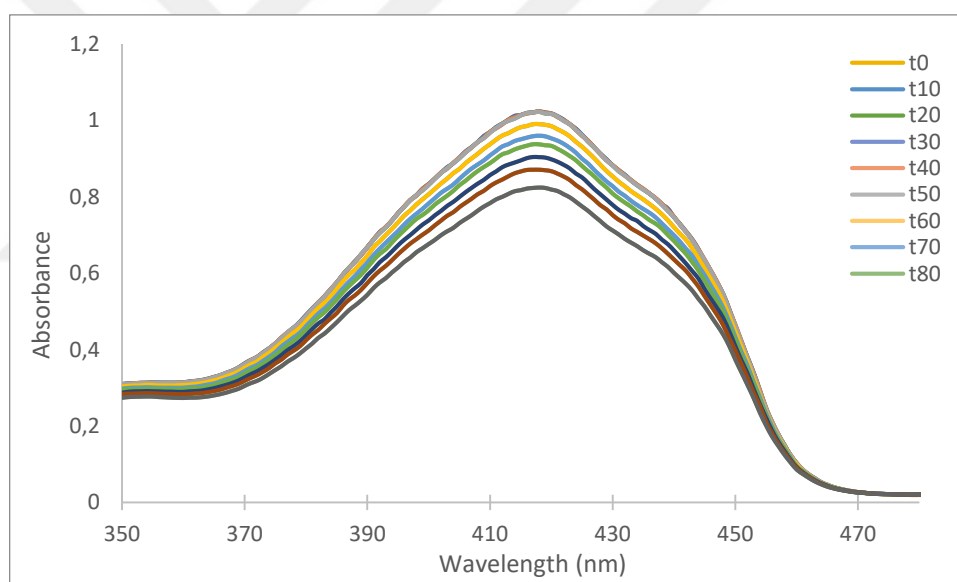


Figure 3.4. Decrease in absorbance of DPBF in DMSO in the presence of F1-a (15 μ M). 595 nm light was applied to the samples at 15 cm.

Sample labeled as F1-b is pre-light applied sample. When PDT activities of F1-a and F1-b were compared, no significant change is observed, indicating that PDT efficiency is preserved (Figure 3.6). When the sample was kept in dark for 20 min, singlet oxygen is not generated, which proves the lack of dark toxicity.

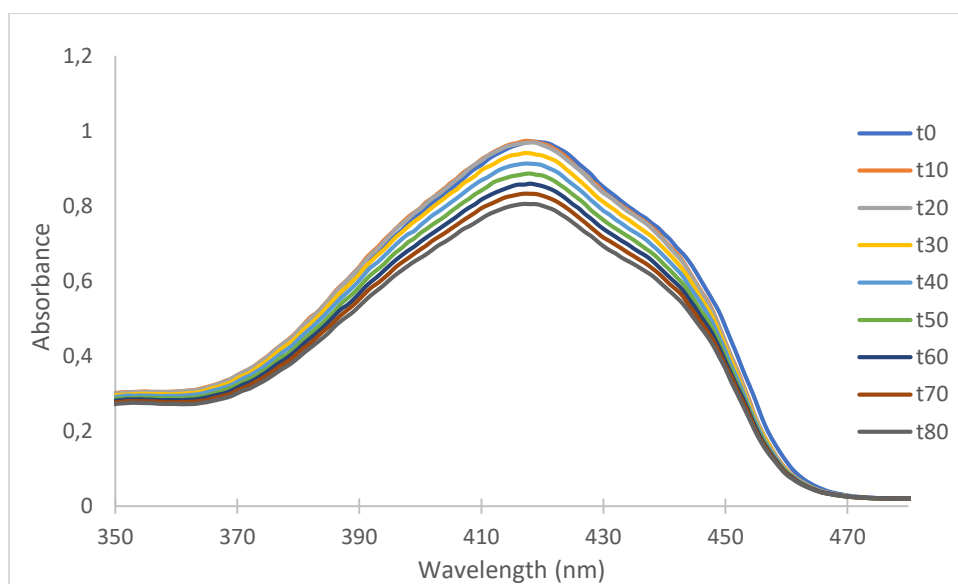


Figure 3.5. Decrease in absorbance of DPBF in DMSO in the presence of F1-b (15 μ M). 595 nm light was applied to the samples at 15 cm.

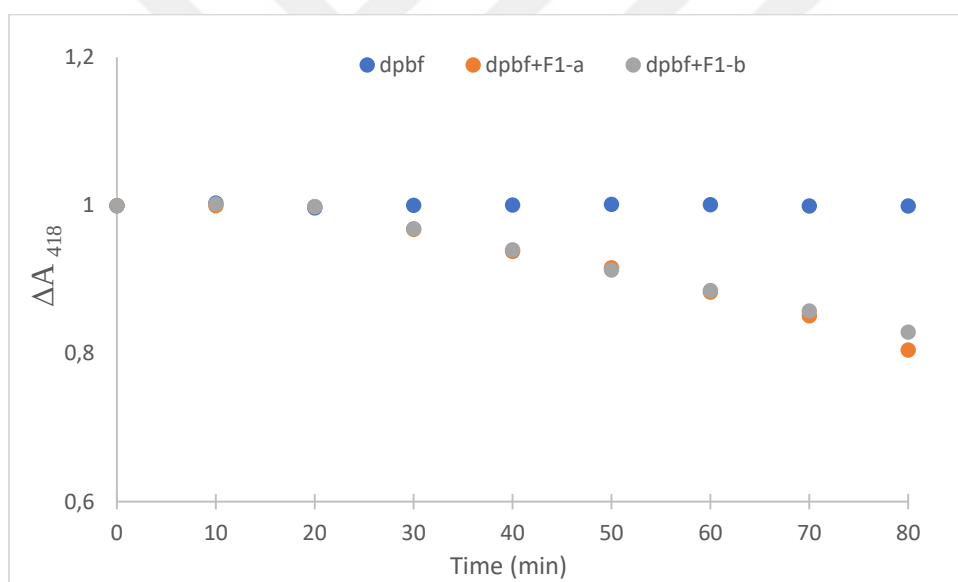


Figure 3.6. Relative $^1\text{O}_2$ generation efficiency of F1-a and F1-b in DMSO as evidenced by the decreasing absorbance of the singlet oxygen trap DPBF at 418 nm. During first 20 minutes the samples were kept in the dark

3.1.1.2. Results of UV-Vis Absorbance and Fluorescence Analysis of F2-a

Spectral analysis of F2-a, which is thought to be obtained as a mixture of imine and amine states, was performed, and the spectral change was investigated in acidic condition where the imine unit is less stable and in the presence of aldehyde, where it can form an alternative imine structure. The spectra were also compared with Compound 10 in the aldehyde structure, whose amine unit was preserved. As

predicted, a significant difference was detected in the emission intensity of F2-a relative to the control unit, in addition, spectral shift was also observed. The results are presented below.

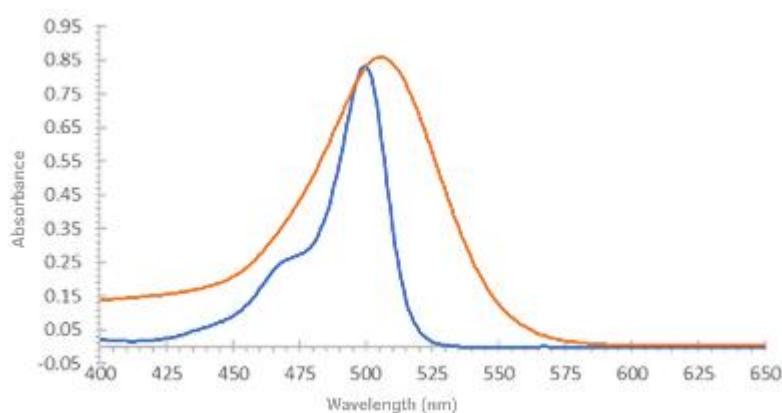


Figure 3.7. Normalized absorbance spectra of F2-a (orange) and Compound 10 (blue) in DCM.

Considering the UV-Vis absorbance values given in Figure 3.7, it was determined that F2-a showed a 6 nm bathochromic shift compared to compound 10. Compound 10, whose amine unit is protected by the Boc protecting group, has a peak absorbance at 500 nm, while F2-a has a peak at 506 nm. The broader spectrum of F2-a indicates that it may be a mixture of imine and amine units. The observation of a peak formation in the bathochromic region in F2-a in the emission spectra given in Figure 3.8 suggests that the substance is an imine-amine mixture and therefore the spectrum takes this shape as a result of the overlap of these two chemical species. When the emission intensities of 10 and F2-a with the same absorbance were compared, a dramatic decrease was observed in the emission of F2-a, which is in line with what was predicted in this project.

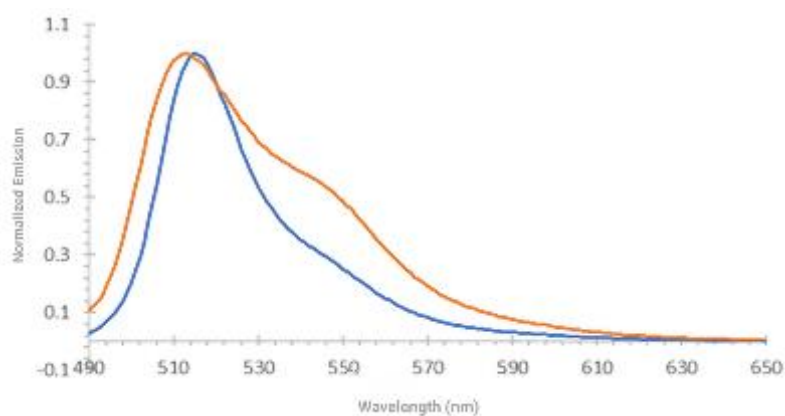


Figure 3.8. Normalized emission intensity of Compound 10 (blue, amine protected form) and F2-a (orange, amine and/or imine state). Excitation at 490 nm, in DCM.

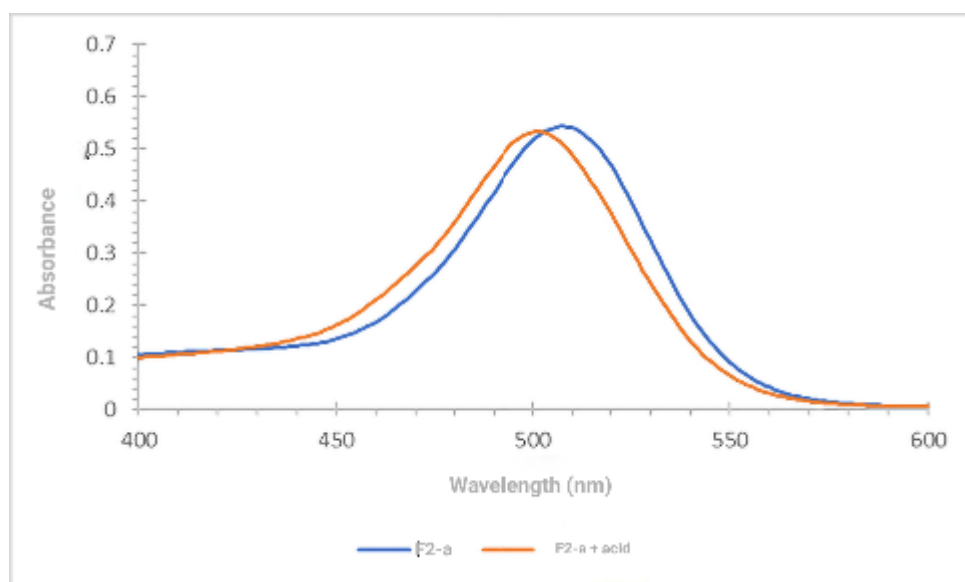


Figure 3.9. The change in the absorbance spectra of the F2-a compound (in DCM) in the presence of acetic acid (20 μ l /3mL solvent).

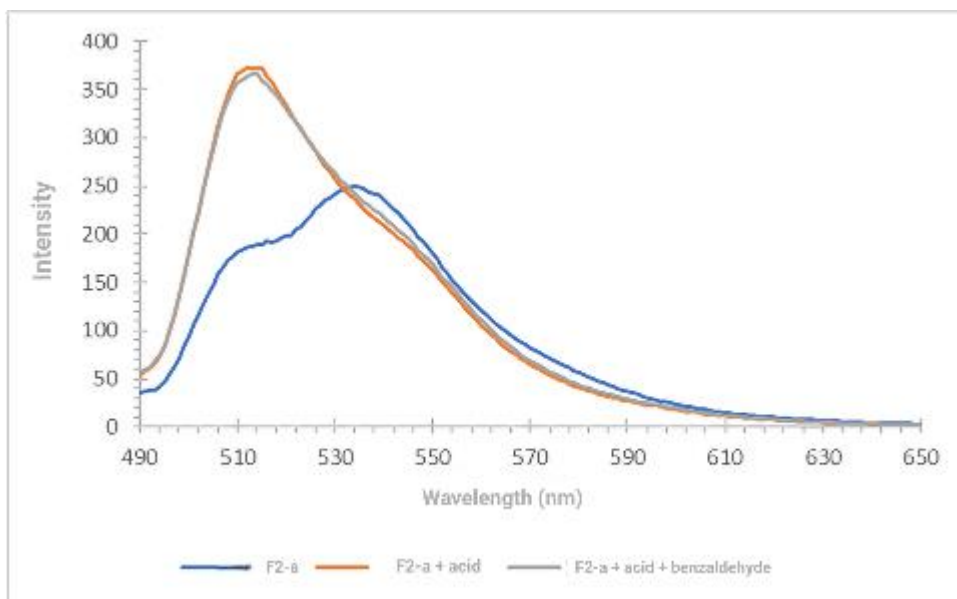


Figure 3.10. The change in the emission spectra of the F2-a compound in the presence of acetic acid (20 μ l /3mL solvent) followed by benzaldehyde (10 equivalents). Excitation at 490 nm, in DCM.

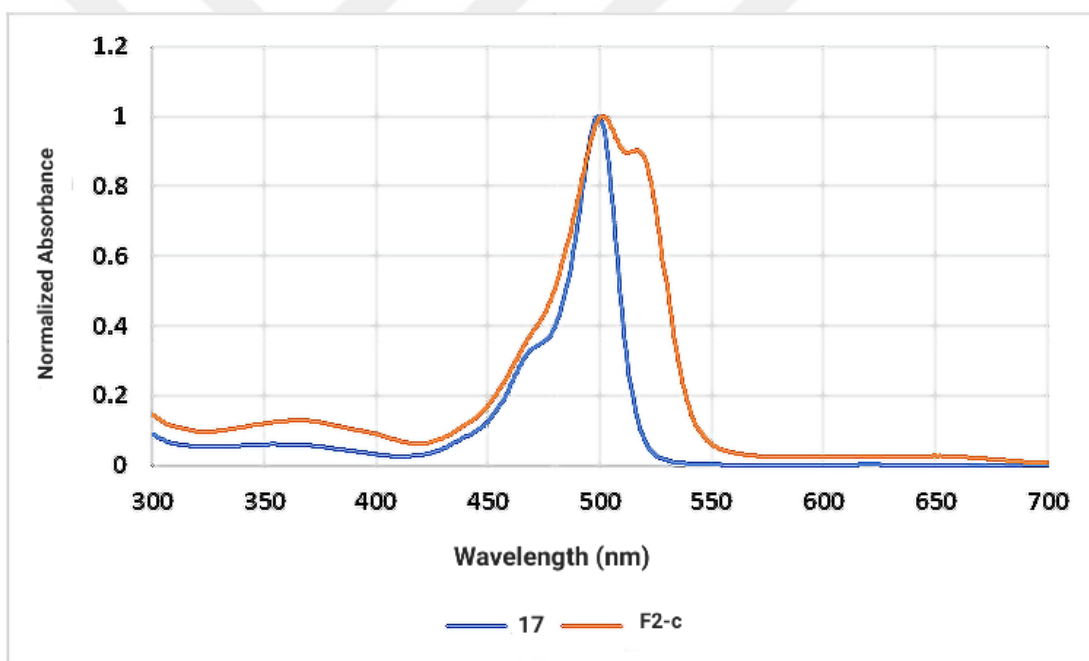


Figure 3.11. Normalized absorbance spectra of F2-c (orange) and Compound 17 (blue) in DCM.

3.1.2. Results of Cell Culture Experiments

3.1.2.1. Results of Cytotoxicity Experiments

The cytotoxic effects of F1-a, F1-b, F2-a and F2-c were analyzed separately using an MTT assay in a dose-dependent manner. MCF-7 cells were treated with agents for 24 h and the cell viability was estimated by measuring absorbance at 570 nm using a Quant ELISA plate reader. It was observed that none of them is cytotoxic for the cells at lower concentrations (Figure 3.12, 3.13).

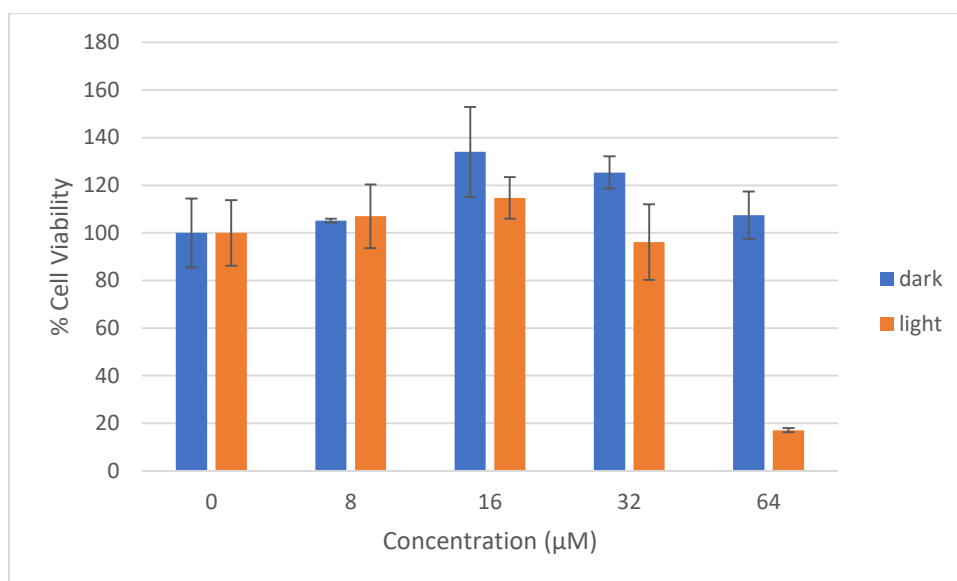


Figure 3.12. Cytotoxicity of F1-a in MCF7 cells (n=3). The light plate was treated with 595 nm light (distance of 15 cm) for 4 hours and kept in the dark for 44 hours, while the dark plate was kept in the dark for 48 hours.

MTT cell viability test was applied in order to understand whether the agent is toxic. For this purpose, cells grown in 96-well plates were exposed to varying doses of compound F1-a for 48 hours. The results of the subsequent MTT analysis are given in Figure 3.12. When the results were evaluated, it was understood that the F1-a was not toxic up to 32 μM application and the viability was preserved over 90%. Also, it is observed that PDT is incredibly effective with a mortality rate of over 80% at a concentration of 64 μM.

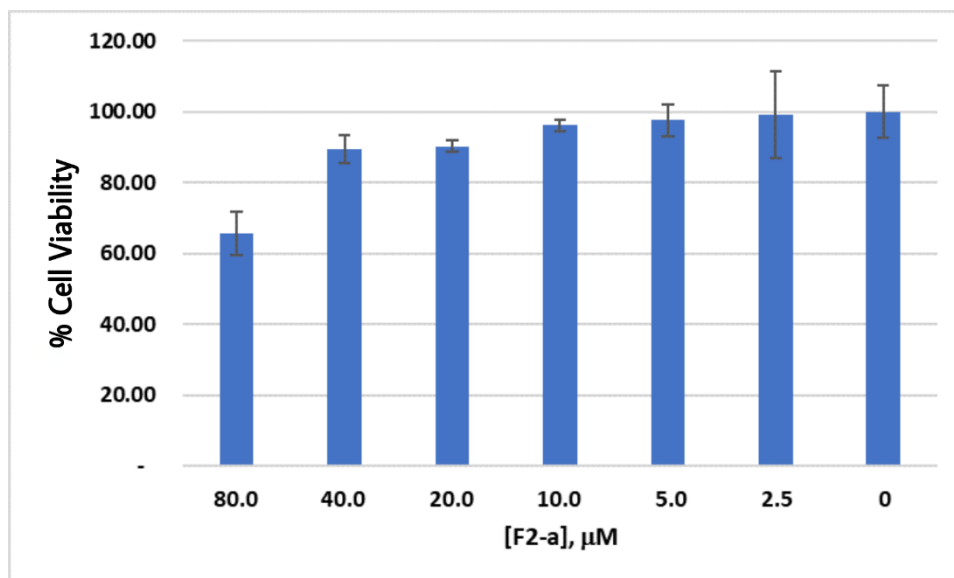


Figure 3.13. Cell viability test result of F2-a compound. It was observed that the material was not toxic at the application dose (10 μM), and the viability rate was 96% at this concentration (n=3).

The working mechanism of the F2-a compound is based on the use of the change in emission intensity and/or shift in cellular imaging as a result of the reversible spontaneous imine-amine dynamic reaction. As with many other fluorophores, the application dose is sufficient at low micromolar concentrations. The high quantum yield of BODIPY compounds allows even lower doses. MTT cell viability test was applied in order to understand whether the agent is toxic at a concentration of 10 μM , which is the application dose used in this study. For this purpose, cells grown in 96-well plates were exposed to varying doses of compound F2-a for 24 hours. The results of the subsequent MTT analysis are given in Figure 3.13. When the results were evaluated, it was understood that the F2-a fluorophore was not toxic up to 40 μM application and the viability was preserved over 90%. This value was observed as 96% at the 10 μM concentration, which is the application dose in the project.

3.1.2.2. Results of Cell Imaging Experiment

In order to analyze behavior of F1-a in cells, MCF7 cells were seeded in 24-well plates and one of them incubated at dark for 24 hours while the other ones stimulated with light for 4 and 8 hours. Then compound F1-a was added to the cells at a dose of 10 μM . In the same conditions, the control cells, which were treated with the agent, were also incubated for 24 h. When the fluorescent images of the cells were examined after fixation, a significant increase was detected in the emissions of cells exposed to light

for a longer time was observed (Figure 3.14). This result is compatible with non-cellular spectroscopic measurements.

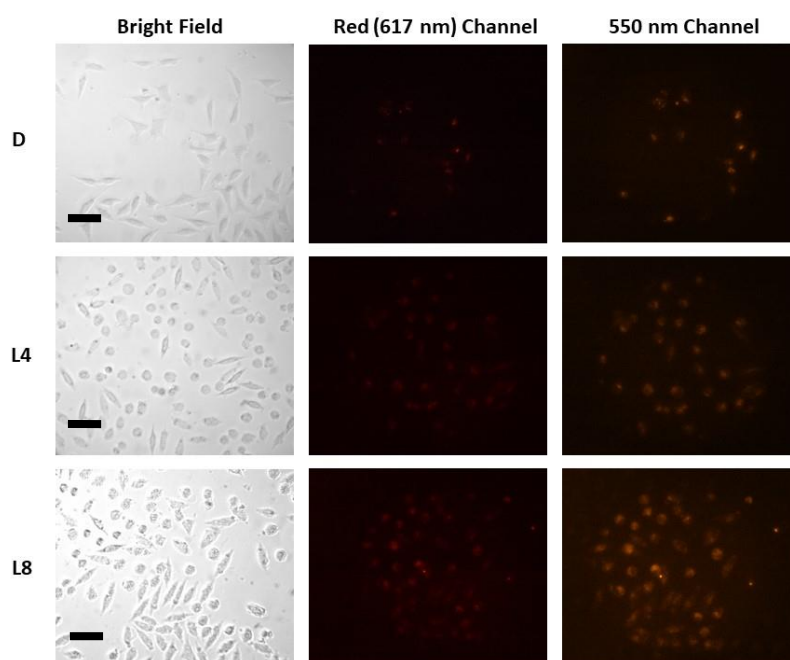


Figure 3.14. Fluorescent microscope image (40X) of compound F1-a (10 μ M) in light-stimulated (595 nm, distance of 15 cm) MCF7 cells for different durations (D: 24 h dark, L4: 4 h light + 20 h dark, L8: 8 h light + 16 h dark).

While analyzing the photophysical properties of the F2-a compound, it was determined that the emission intensity increased as a result of lowering the pH of the environment by adding acid. This can be attributed to the complete inhibition of the FeT pathway as a result of imine degradation and protonation of the entire amine. In order to analyze this behavior in cells, MCF7 cells were seeded in a 6-well plate and compound F2-a was administered at a dose of 10 μ M for 1 hour incubated. Dilute HCl solution added to the medium to adjust the pH to 6.5 was added to some of the wells and incubated for an additional 15 minutes. In the same conditions, the control cells, which were treated with the agent, were also incubated for 15 minutes at normal pH of the medium. When the fluorescent images of the cells were examined after fixation, a significant increase was detected in the emissions of the acid-treated samples which favors imine hydrolysis (Figure 3.15.). This result is compatible with non-cellular spectroscopic measurements.

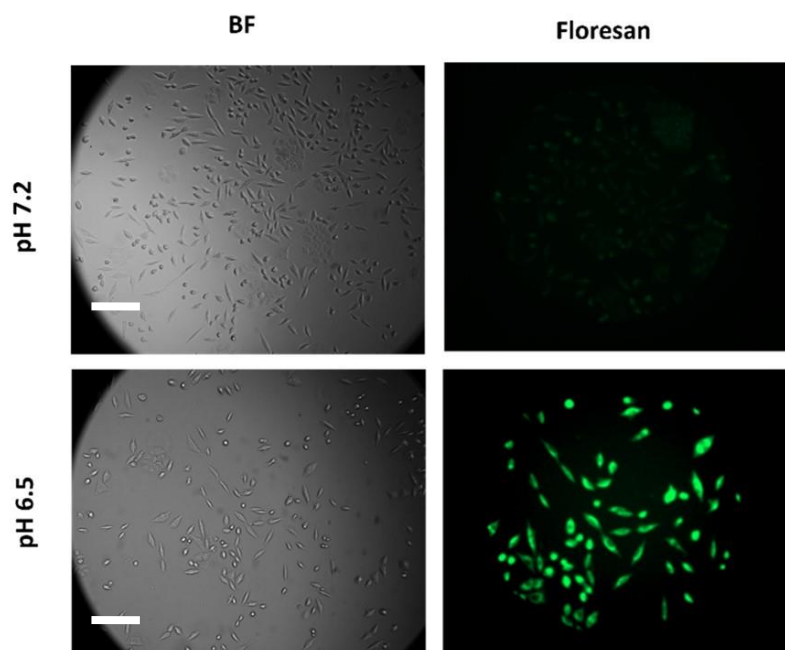


Figure 3.15. Fluorescent microscope image (20X) of compound F2-a (10 μ M) in MCF7 cell at normal pH and low pH (pH 6.5) with increased probability of imine hydrolysis.

3.2. Discussion

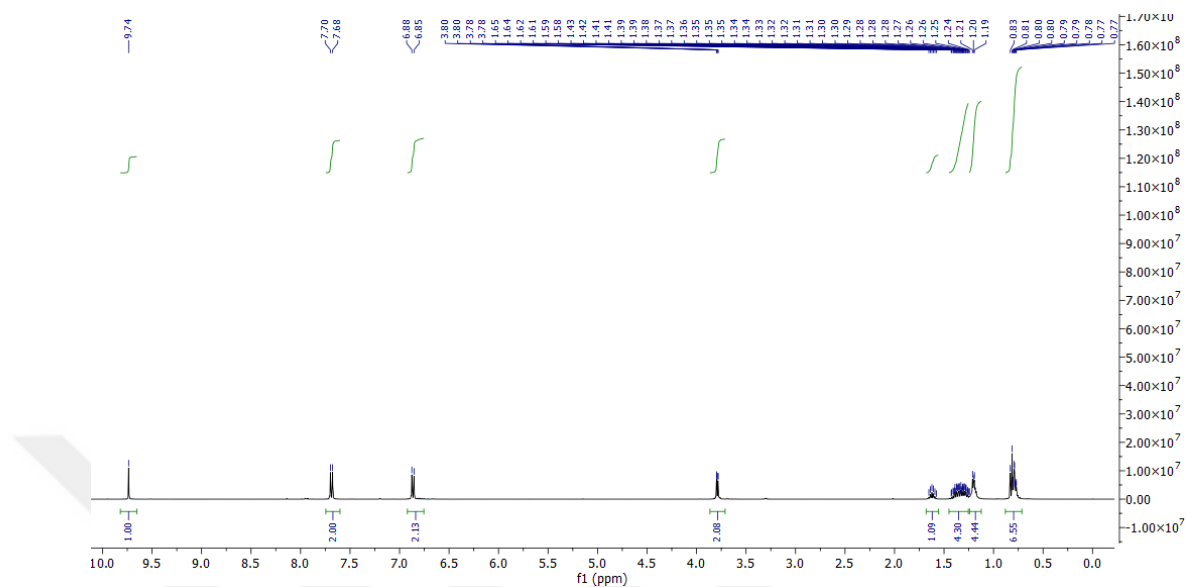
With outstanding sensitivity and selectivity, fluorescent probes are well suited to identify specific parts of intricate biomolecular interactions and processes in cellulo. Fluorescence measurements per cell are possible when used in conjunction with an appropriate method for detecting fluorescence signals, such as flow cytometry. A self-reporting photosensitizer, based on BODIPY, F1-a has the potential to be employed in the treatment of cancer while reporting its own activity. It is necessary to develop photosensitizers with lower skin photosensitivity and higher tumor selectivity. The thesis's suggested drug, F1-a, is composed of a core of boron dipyrromethene difluoride and a phenyl moiety joined to the core by a diene linker. The intact agent exhibits a strong red fluorescence with an emission peak at 595 nm because of this prolonged conjugation. The diene connectivity is the most likely candidate to undergo oxidation when confronted with oxidizing agents. As the oxidation of F1-a proceeds, the initial red fluorescence at 595 nm declines and the emission switches to much shorter wavelengths, with a peak emission at 520 nm and a shoulder at 545 nm. This behaviour enables self-actuation of fluorescence, therefore activity reporting.

Cytotoxicity analysis show a reduced viability of F1-a-treated cells under light conditions. This effect is even more obvious at higher doses of the stabilizer. By

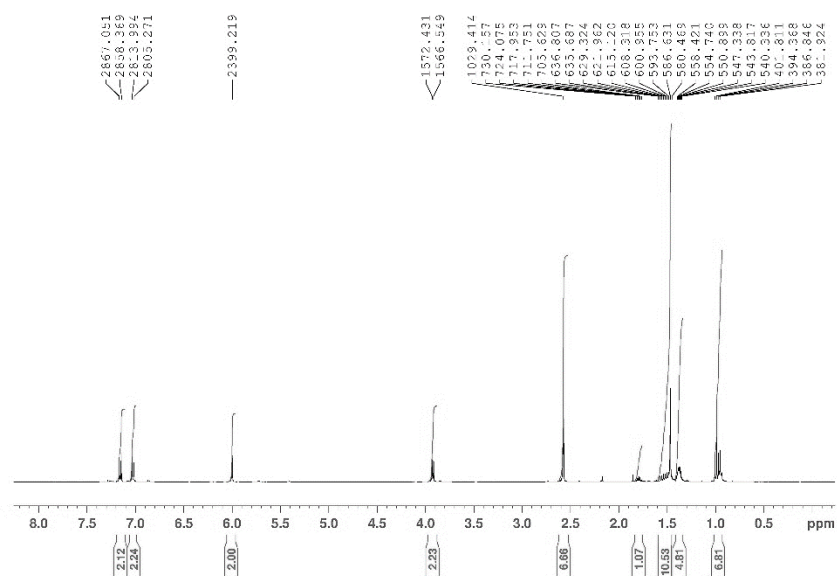
treating the agent with light, F1-a would lead cells to an apoptosis. To sum up, the self-actuator F1-a is able to activate with light, changes its structure, produces singlet oxygen, reports its activity with a strong emission.

It is expected that the photophysical properties of F2-a and F2-c compounds will change due to the dynamically formed imine structure between the aldehyde and amine units in their structures. ^1H NMR analysis, fluorescence and UV-Vis absorbance spectra indicate that these compounds are obtained in mixed imine and amine states, and show that the two structures can exist simultaneously at normal pH and are in equilibrium. When the imine structure is destroyed by the addition of acid, a photophysical difference is detected, a hypsochromic shift in the emission spectrum and an increase in the emission intensity are observed. In the MCF7 cell, this increase was clearly demonstrated when the pH of the medium was reduced to 6.5. The expected output of the project has been achieved. It was determined that the F2-a compound did not cause a significant cell death at the application dose with a HaloTag label on it, compound may be used in the future for high resolution protein imaging.

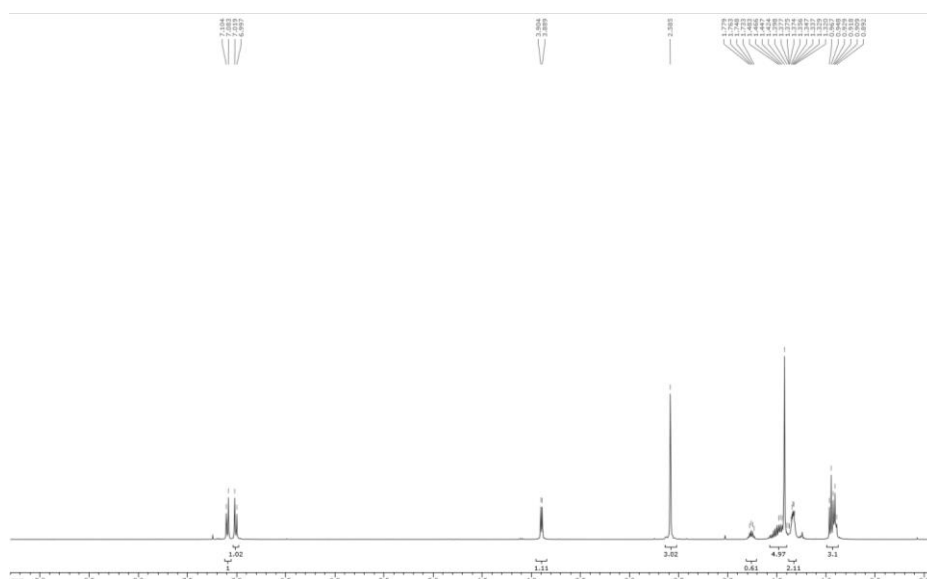
4. APPENDIX



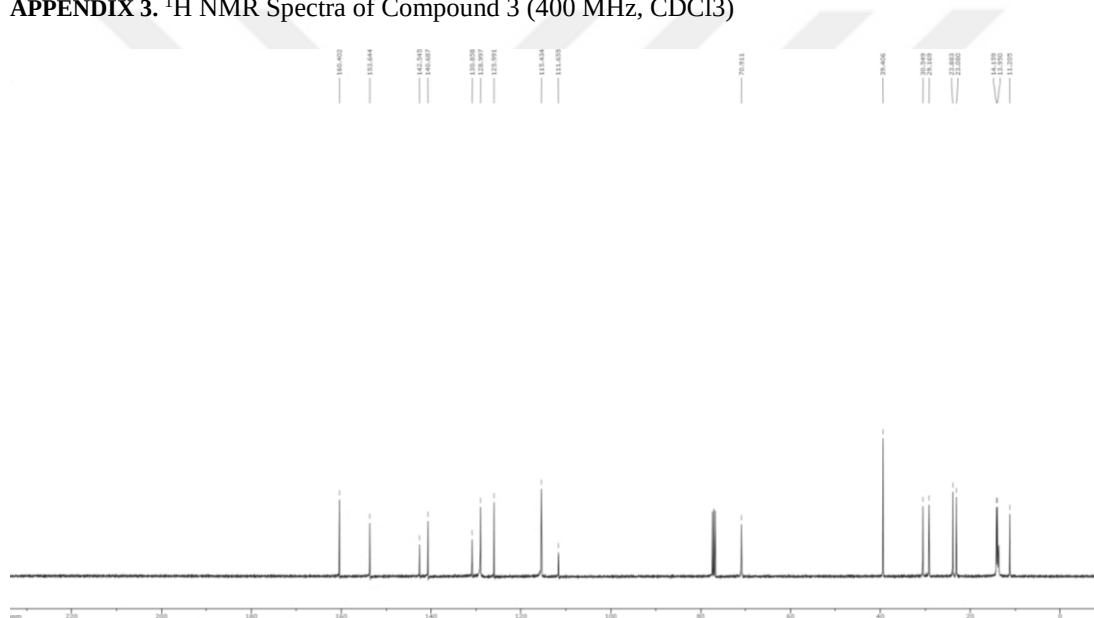
APPENDIX 1. ^1H NMR Spectra of Compound 1 (400 MHz, CDCl_3)



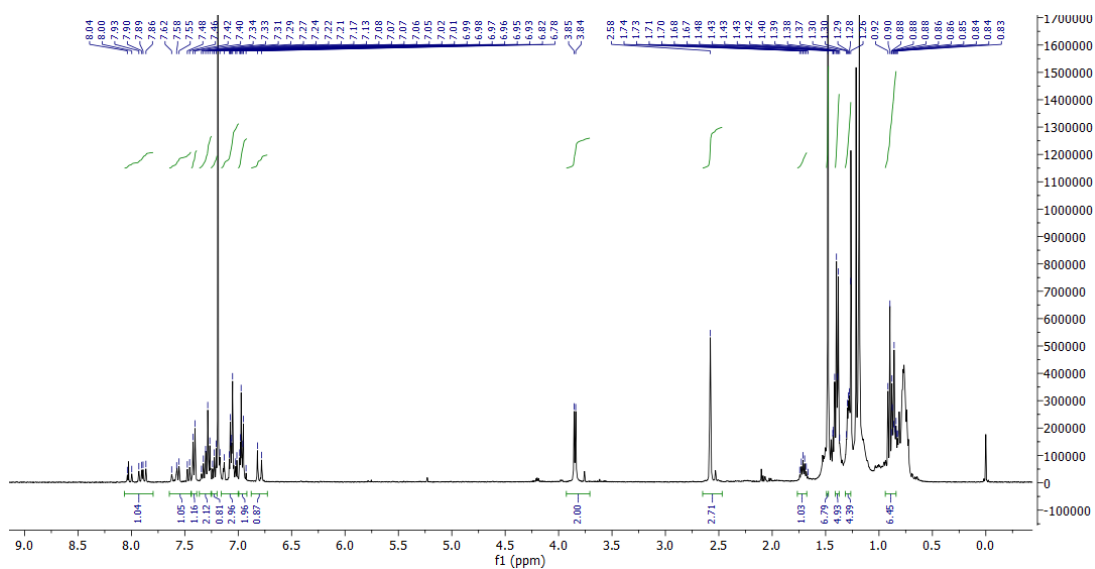
APPENDIX 2. ^1H NMR Spectra of Compound 2 (400 MHz, CDCl_3)



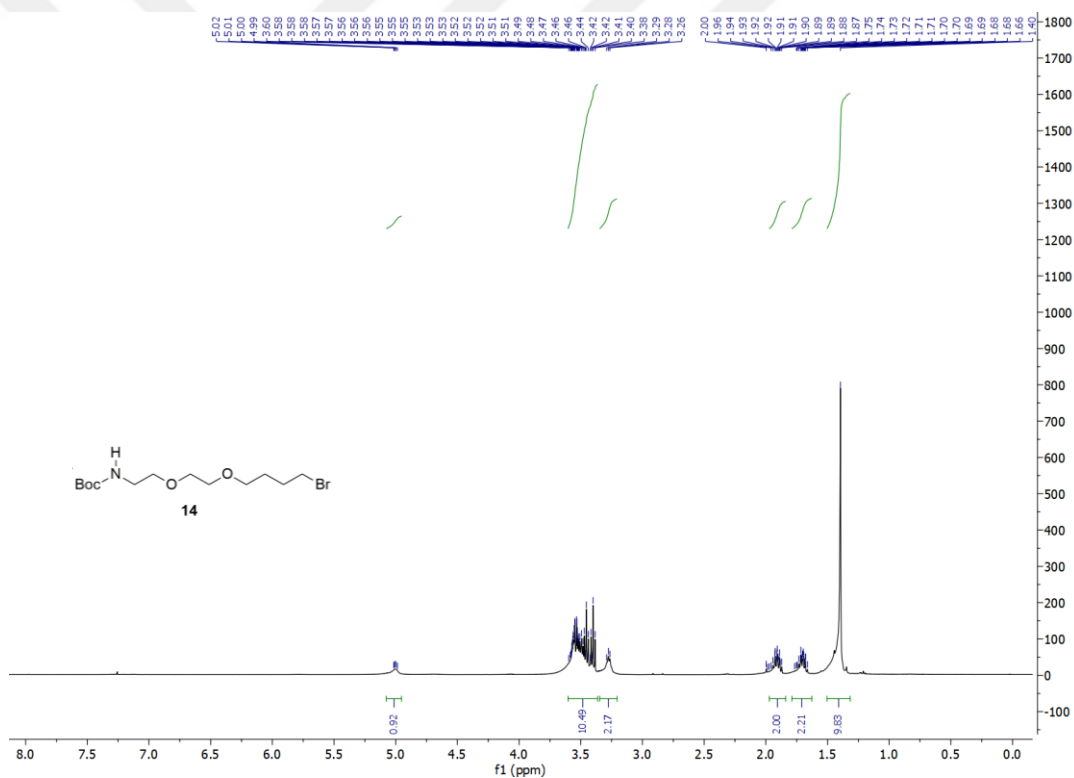
APPENDIX 3. ¹H NMR Spectra of Compound 3 (400 MHz, CDCl₃)



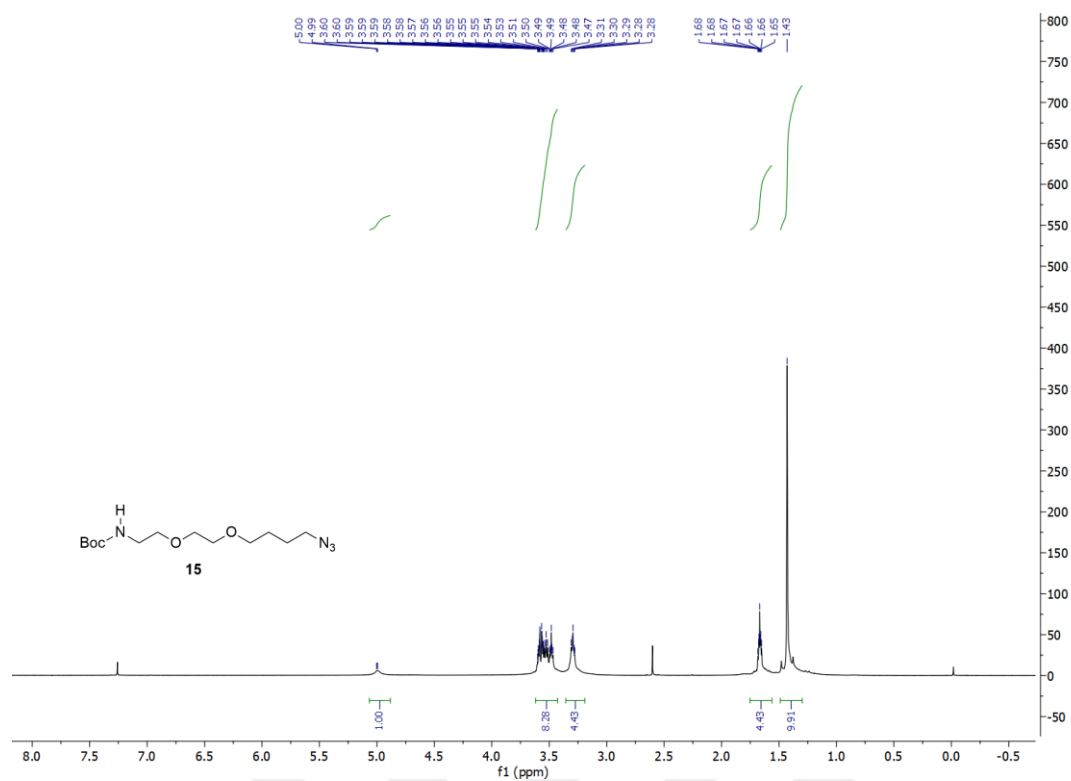
APPENDIX 4. ¹³C NMR Spectra of Compound 3 (100 MHz, CDCl₃)



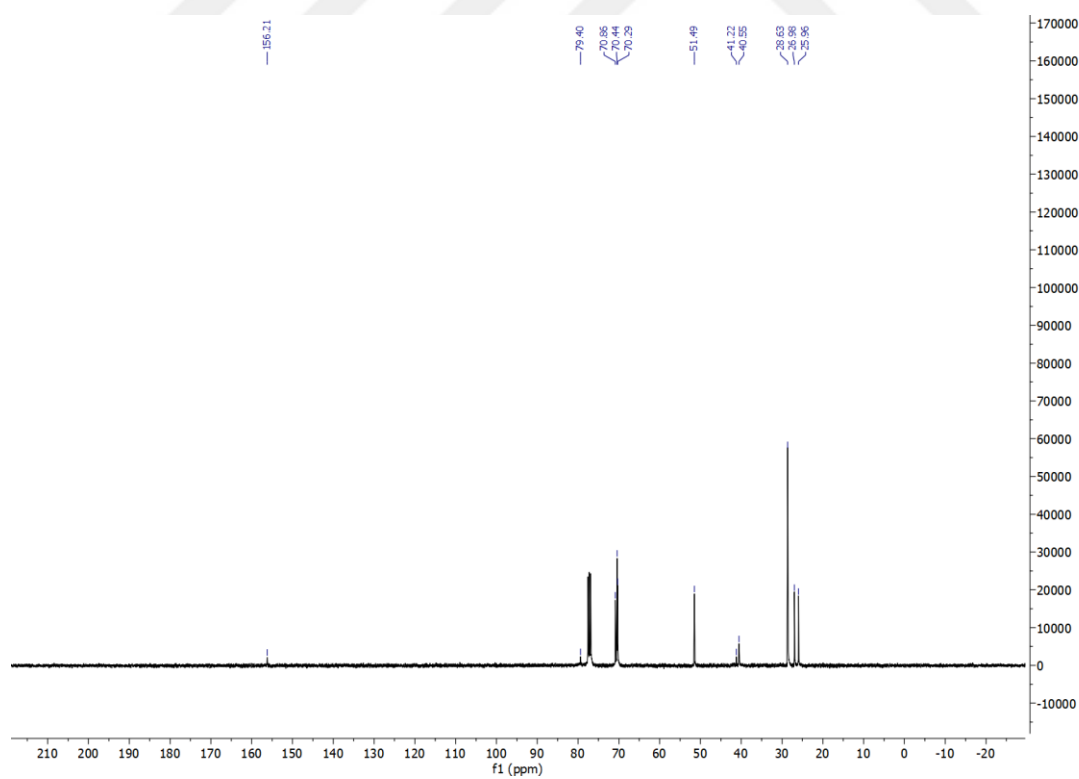
APPENDIX 5. ¹H NMR Spectra of F1-a (400 MHz, CDCl₃)



APPENDIX 6. ¹H NMR Spectra of Compound 5 (400 MHz, CDCl₃)

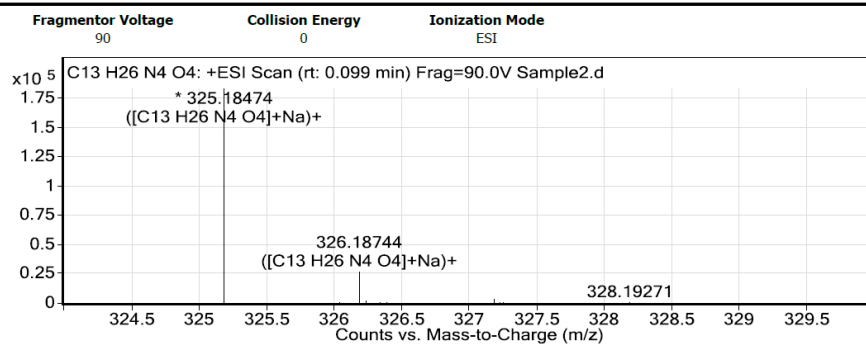


APPENDIX 7. ¹H NMR Spectra of Compound 6 (400 MHz, CDCl₃)

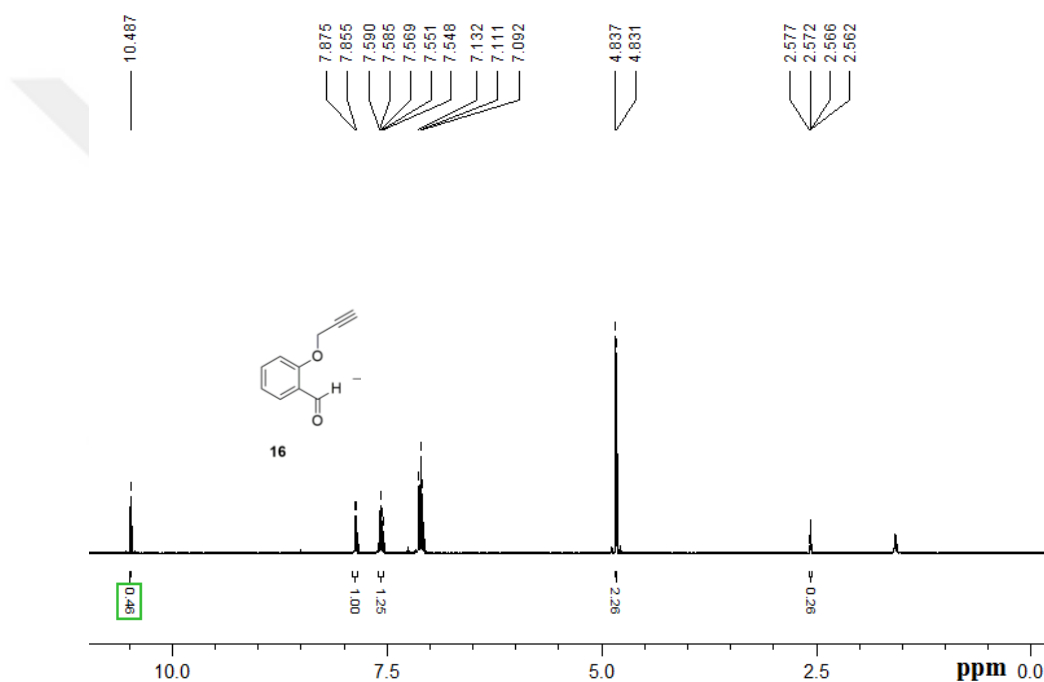


APPENDIX 8. ¹³C NMR Spectra of Compound 6 (100 MHz, CDCl₃).

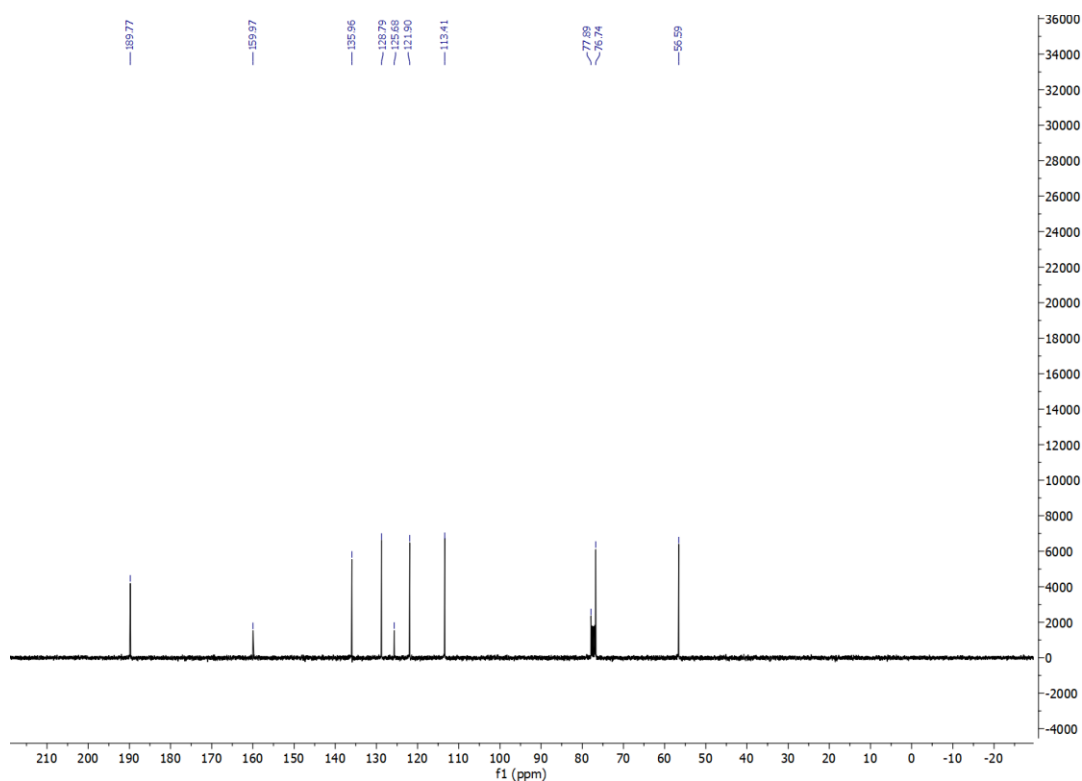
User Spectra



APPENDIX 9. HRMS Spectra of Compound 6

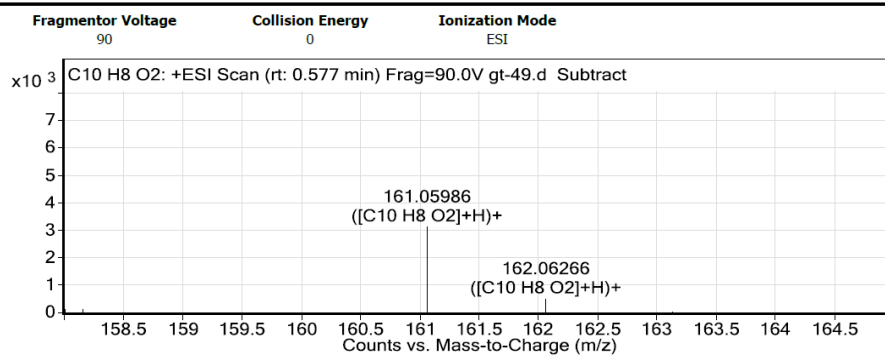


APPENDIX 10. ¹H NMR Spectra of Compound 7 (400 MHz, CDCl₃)

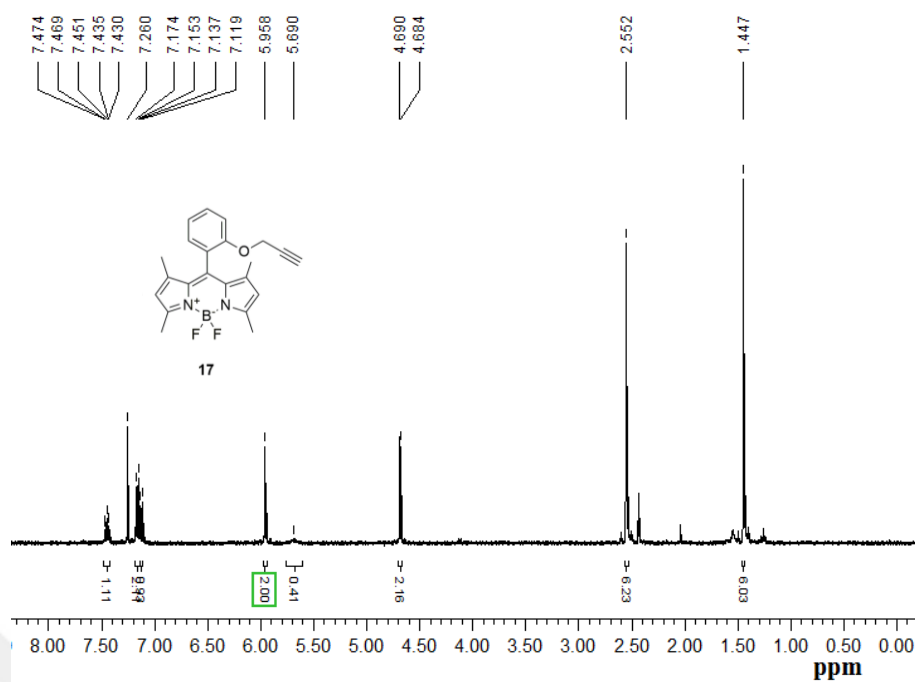


APPENDIX 11. ¹³C NMR Spectra of Compound 7 (100 MHz, CDCl₃)

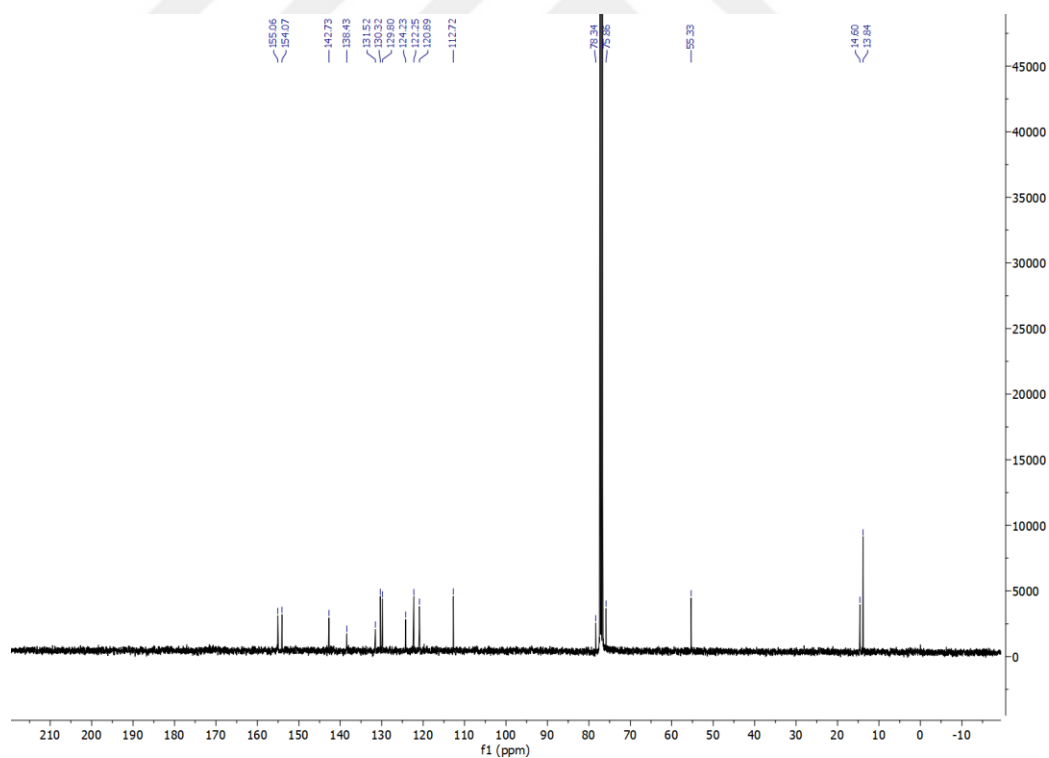
User Spectra



APPENDIX 12. HRMS Spectra of Compound 7

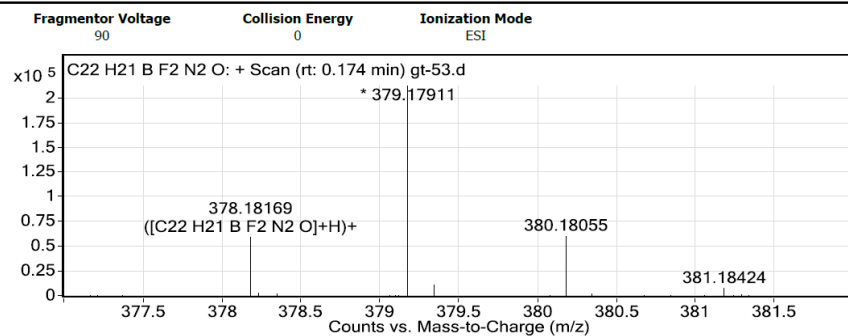


APPENDIX 13. ^1H NMR Spectra of Compound 8 (400 MHz, CDCl_3)

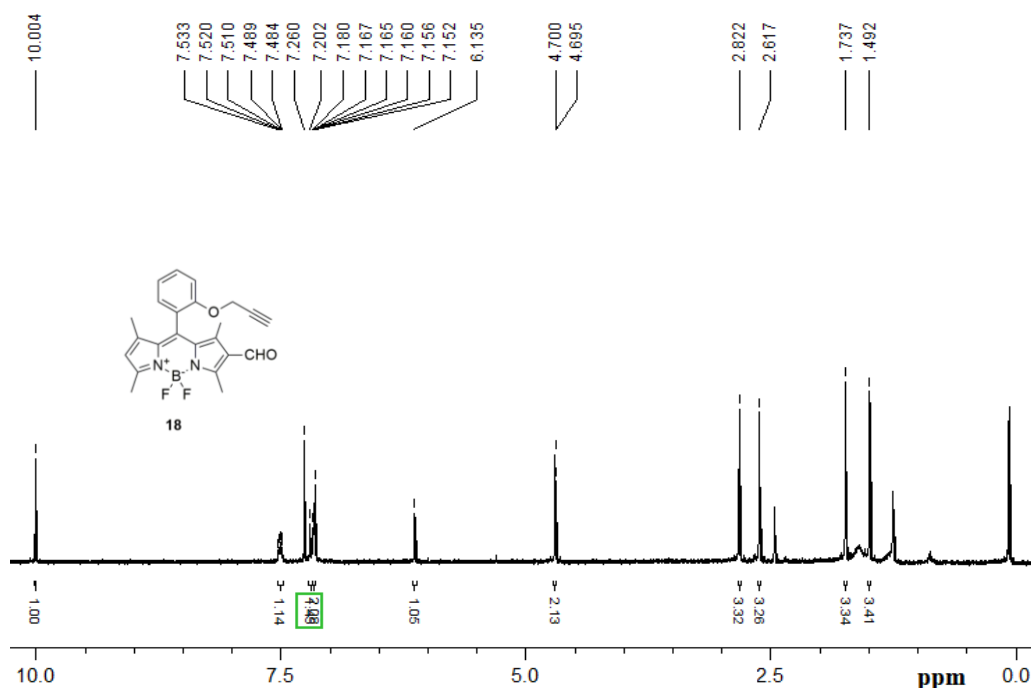


APPENDIX 14. ^{13}C NMR Spectra of Compound 8 (100 MHz, CDCl_3).

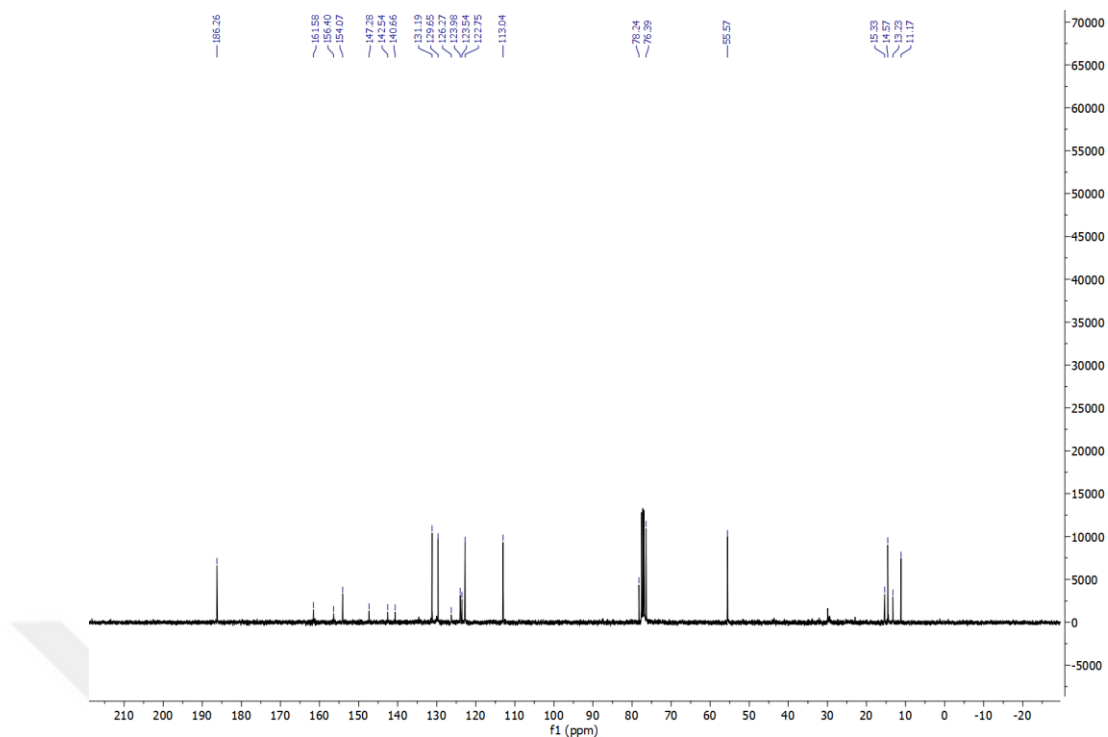
User Spectra



APPENDIX 15. HRMS Spectra of Compound 8

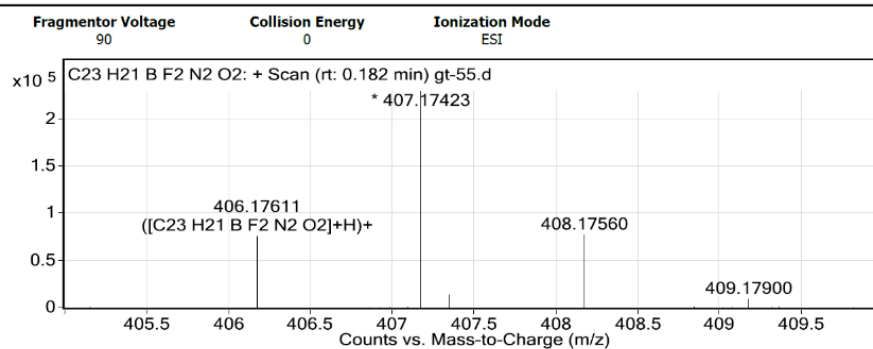


APPENDIX 16. ¹H NMR Spectra of Compound 9 (400 MHz, CDCl₃)

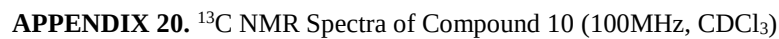
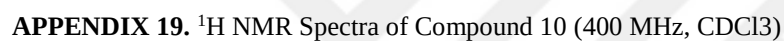


APPENDIX 17. ^{13}C NMR Spectra of Compound 9 (100 MHz, CDCl_3).

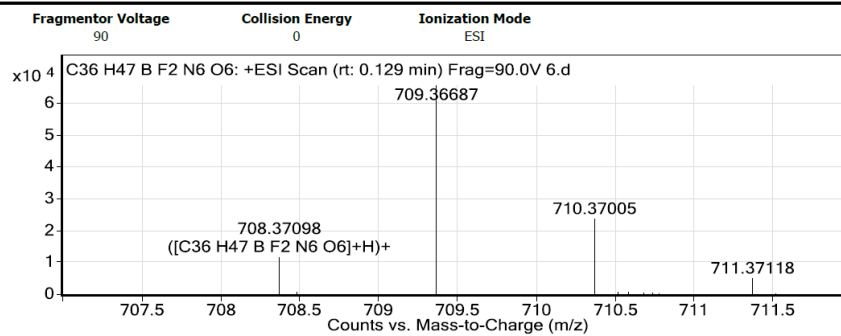
User Spectra



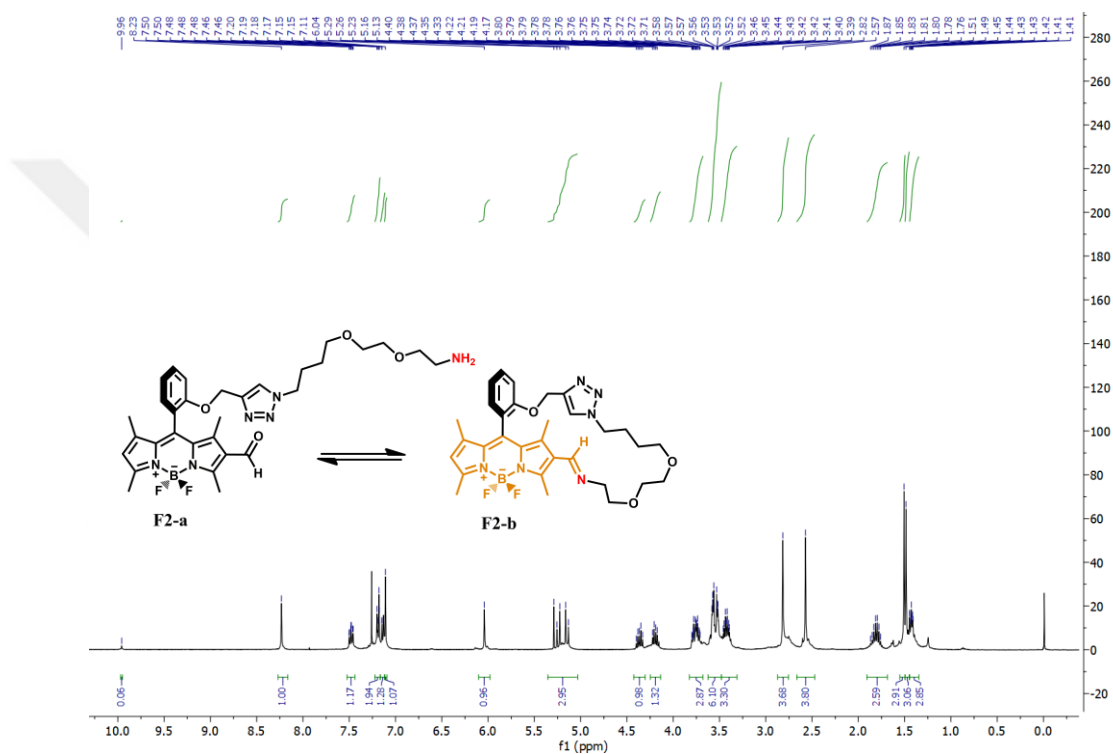
APPENDIX 18. HRMS Spectra of Compound 9



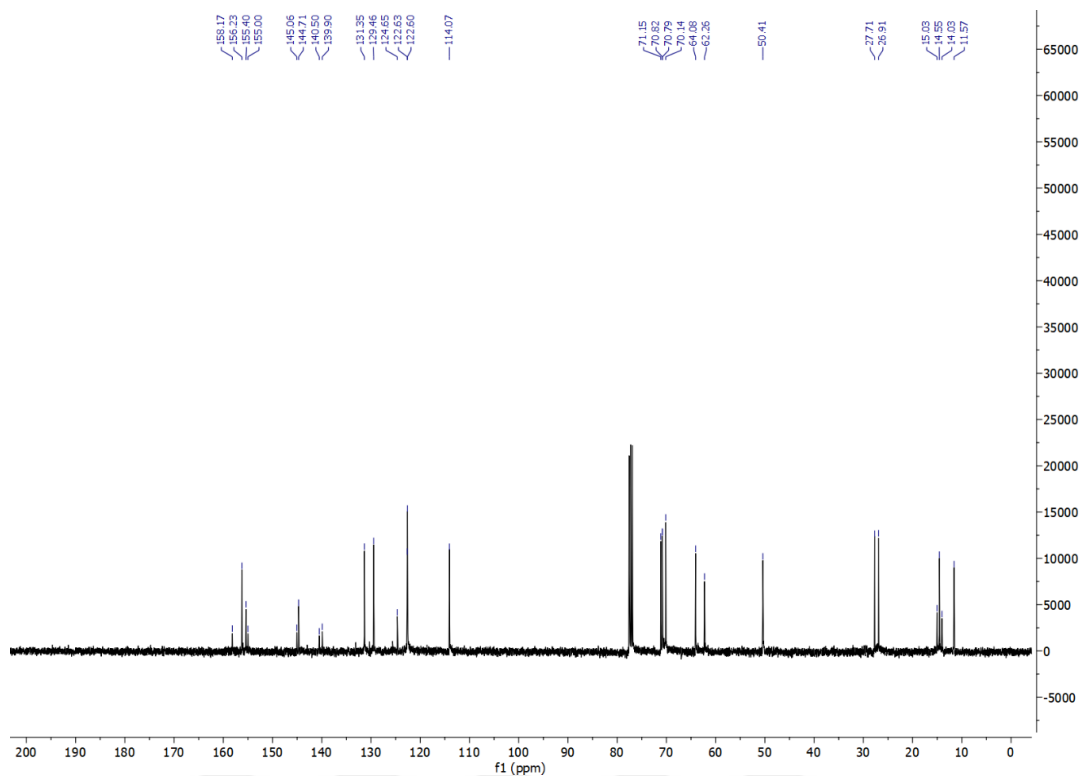
User Spectra



APPENDIX 21. HRMS Spectra of Compound 10

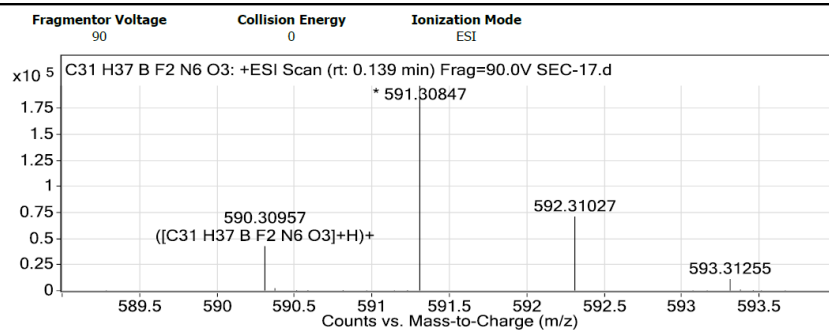


APPENDIX 22. ¹H NMR Spectra of F2-a (400 MHz, CDCl₃)

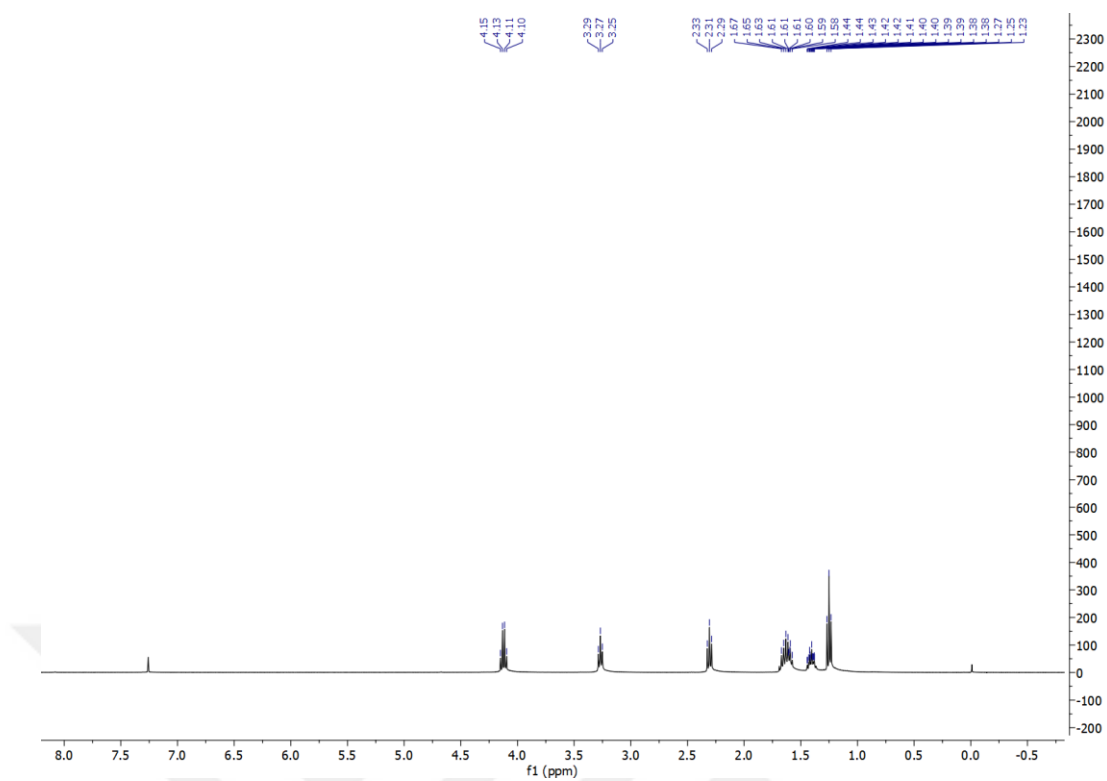


APPENDIX 23. ^{13}C NMR Spectra of F2-a (100 MHz, CDCl_3)

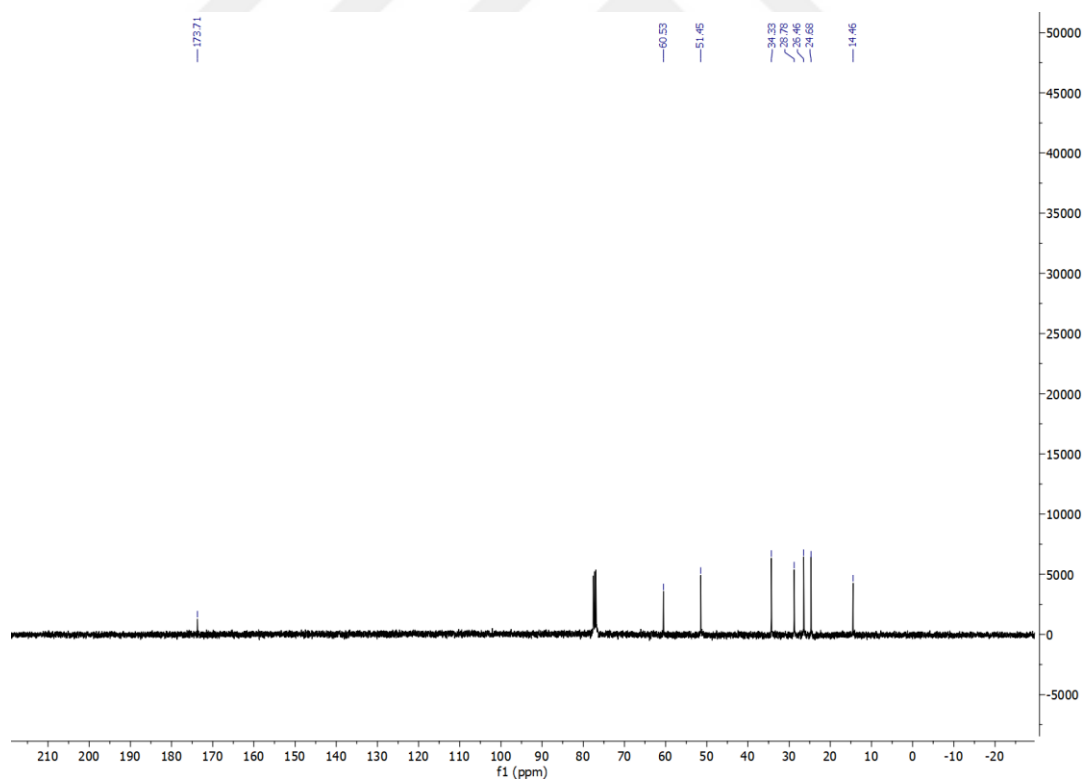
User Spectra



APPENDIX 24. HRMS Spectra of F2-a

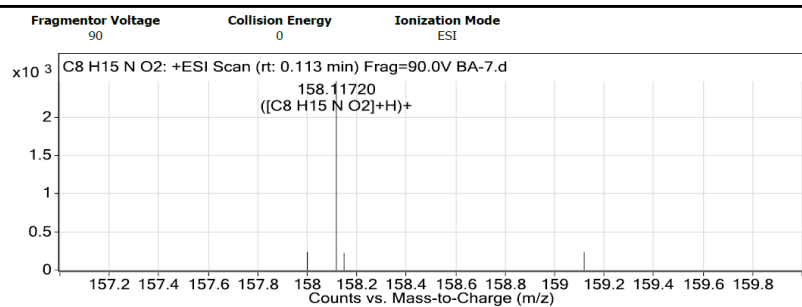


APPENDIX 25. ¹H NMR Spectra of Compound 11 (400 MHz, CDCl₃)

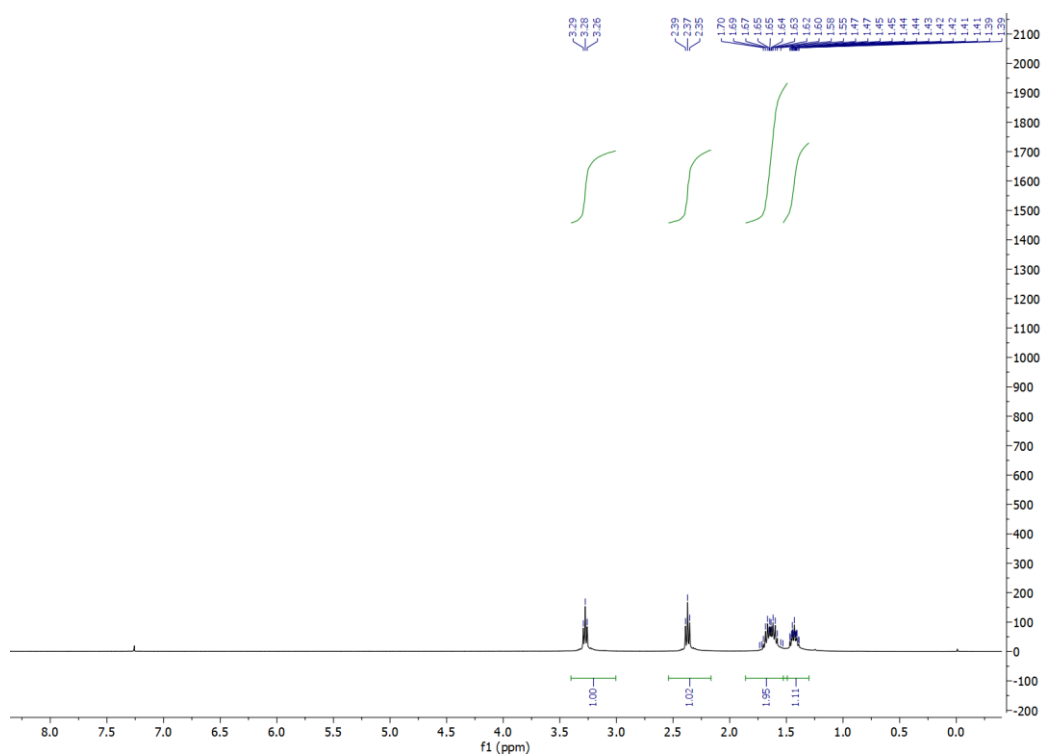


APPENDIX 26. ¹³C NMR Spectra of Compound 11 (100 MHz, CDCl₃)

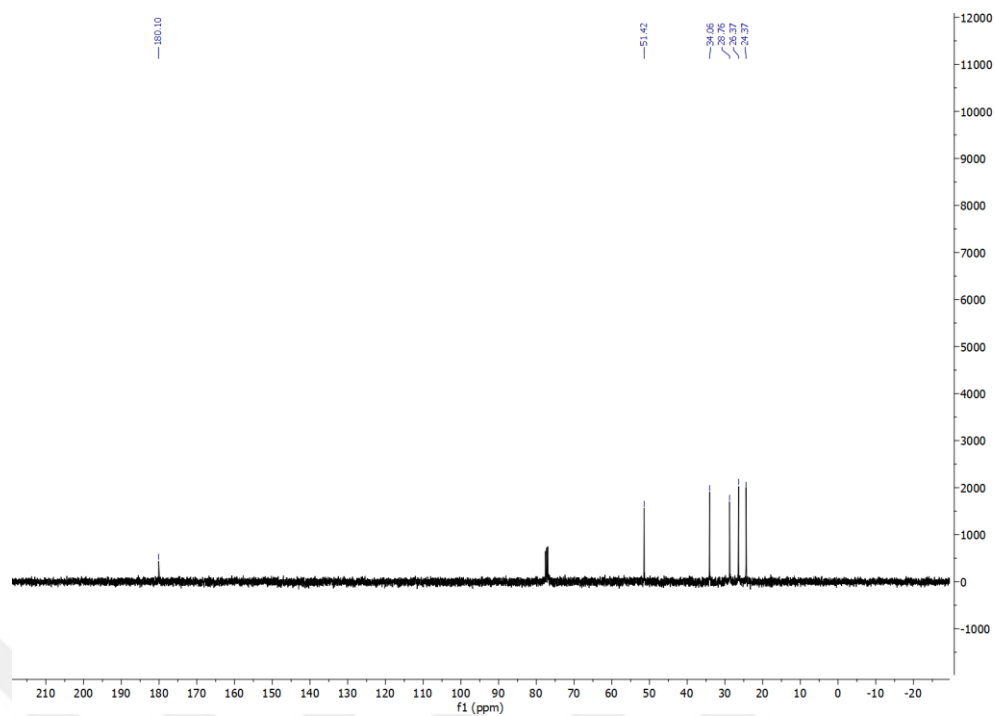
User Spectra



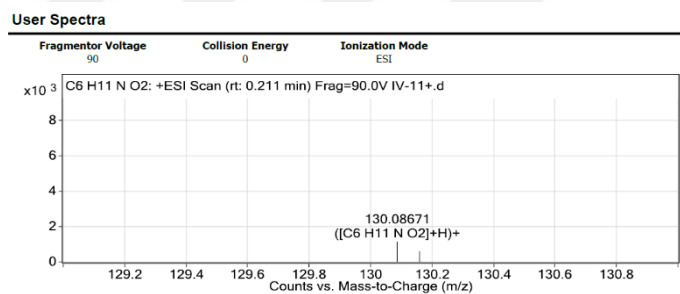
APPENDIX 27. HRMS Spectra of Compound 11



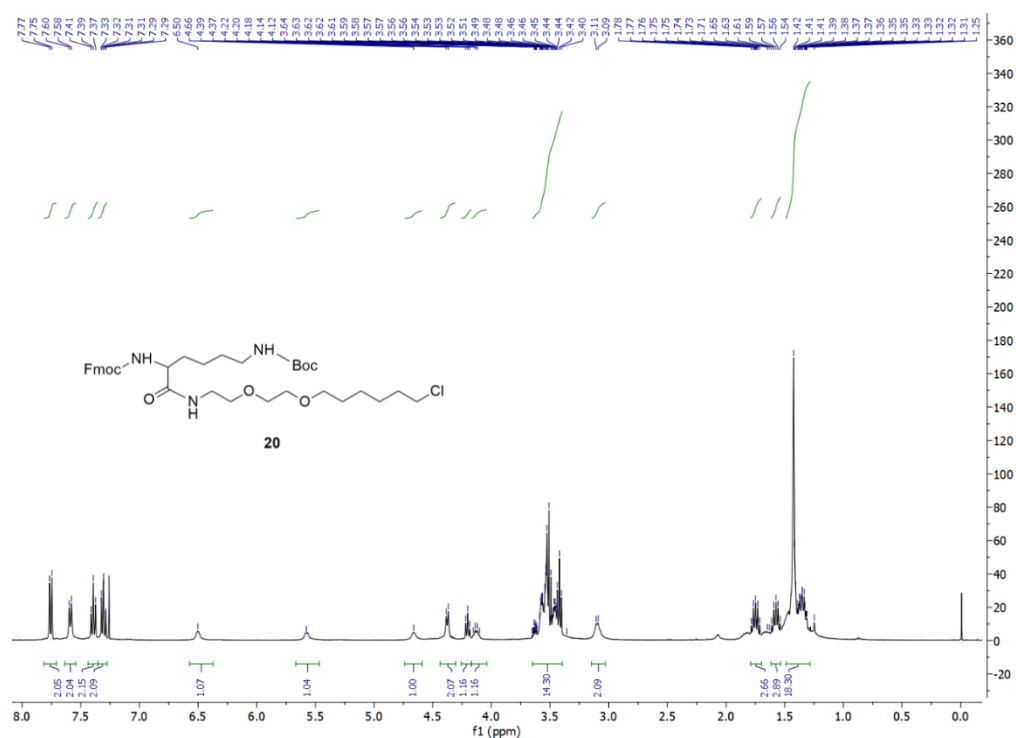
APPENDIX 28. ¹H NMR Spectra of Compound 12 (400 MHz, CDCl₃)



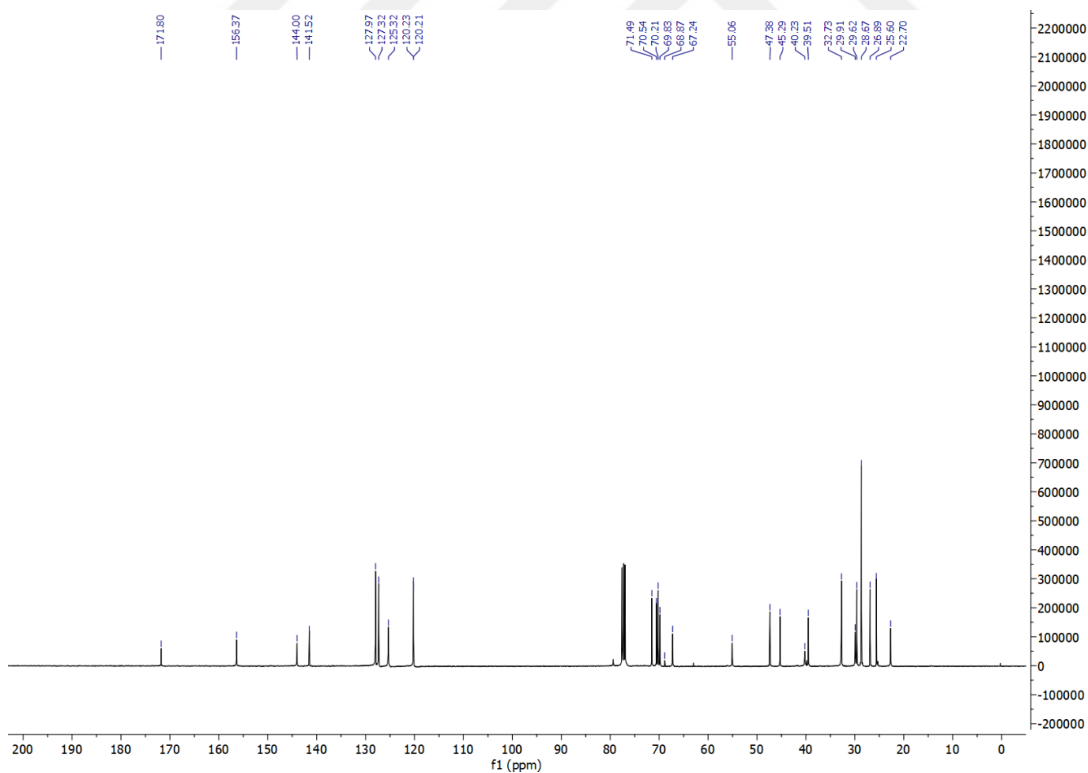
APPENDIX 29. ¹³C NMR Spectra of Compound 12 (100 MHz, CDCl₃)



APPENDIX 30. HRMS Spectra of Compound 12



APPENDIX 31. ¹H NMR Spectra of Compound 13 (400 MHz, CDCl₃)



APPENDIX 32. ¹³C NMR Spectra of Compound 13 (100 MHz, CDCl₃).

Fragmentor Voltage 90 Collision Energy 0 Ionization Mode ESI

x10⁴ C36 H52 Cl N3 O7: +ESI Scan (rt: 0.104 min) Frag=90.0V 4.d

674.35361
([C36 H52 Cl N3 O7]+H)+

676.35174
([C36 H52 Cl N3 O7]+H)+

677.35384
([C36 H52 Cl N3 O7]+H)+

Counts vs. Mass-to-Charge (m/z)

Fragmentor Voltage 90 Collision Energy 0 Ionization Mode ESI

C27 H51 Cl N6 O6: +ESI Scan (rt: 0.135 min) Frag=90.0V Sample4.d

591.36315
([C27 H51 Cl N6 O6]+H)+

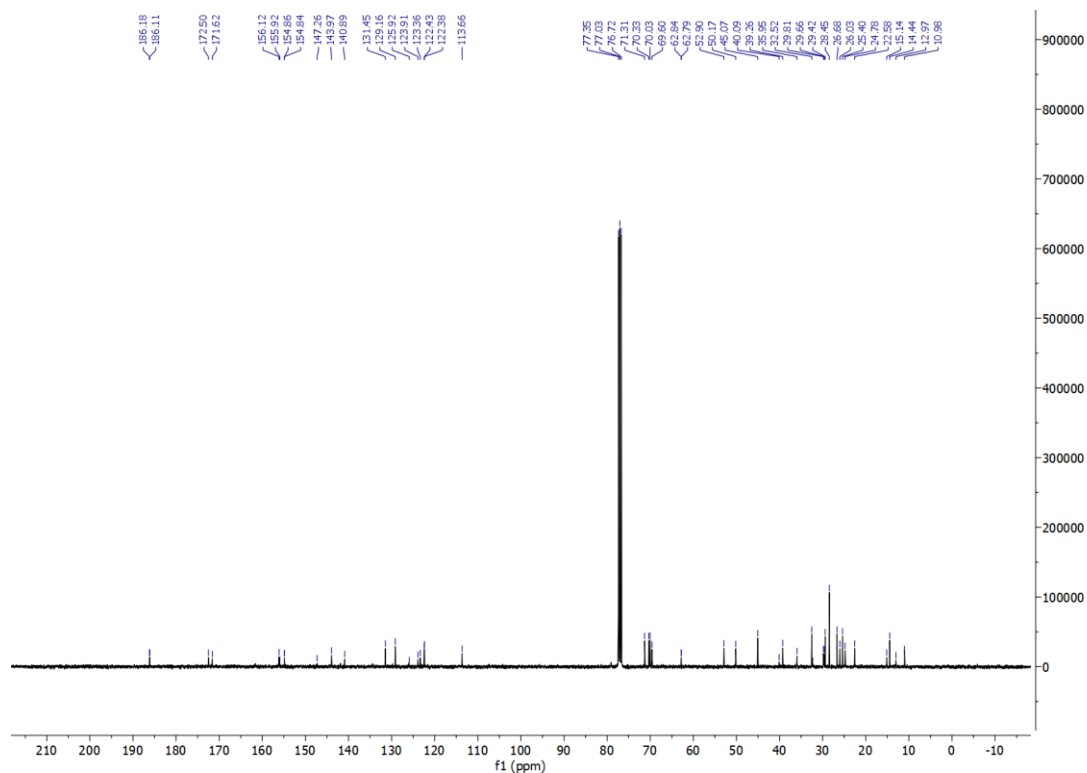
592.36750
([C27 H51 Cl N6 O6]+H)+

592.10154 592.56814

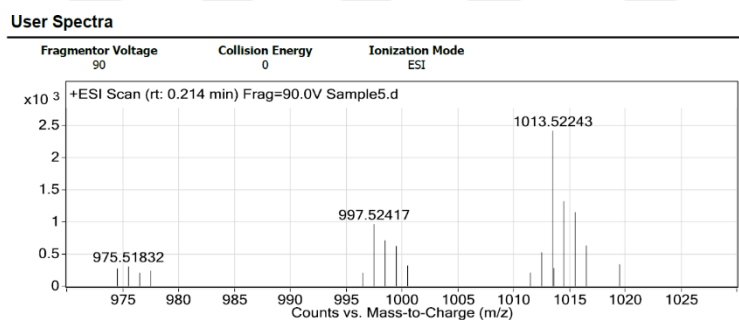
Counts vs. Mass-to-Charge (m/z)

[illegible]

75

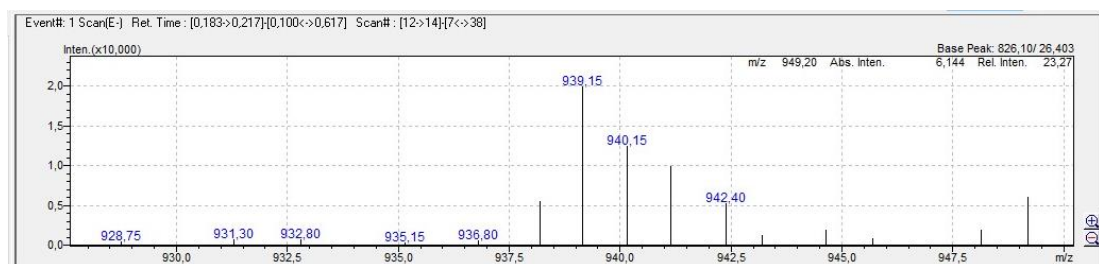


APPENDIX 36. ^{13}C NMR Spectra of Compound 16 (100 MHz, CDCl_3).



APPENDIX 37. HRMS Spectra of Compound 16





APPENDIX 40. LCMS data of F2-c

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