Synthesis and Characterization of Nature Inspired Fluorophores

by

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

Doctor of Philosophy

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I, Boyuan Deng, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed ...................................................

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Abstract

Oxyluciferin is the product of bioluminescence reaction of firefly luciferin. It has a highly conjugated structure and is non-toxic to animal cells. The thesis concerns the investigation of transforming the features of oxyluciferin into a bio-friendly fluorophore. The background and introduction chapters include how the fluorescence phenomenon has been discovered and a mechanistic explanation. It also covers several typical fluorophores and their applications. The second chapter starts from the design of keto and iminium derivatives of oxyluciferin with different electron-donating substituents. Then moves to their chemical synthesis includes direct condensation reaction between the corresponding ketone and dialkylamine. The third chapter covers the design of pyridine and pyridinium derivatives of oxyluciferin, the synthesis of which includes cyclisation methods of constructing thiazole and thiazolopyridine rings. Around 20 novel analogues have been successfully synthesized. The fourth part gives a brief description of optical properties of all the fluorophores prepared, includes some aggregation induced emission (AIE) fluorophores. The fluorescence of different analogues have nearly covered the entire visible light region. The bioconjugation reaction of the most promising fluorophore with an antibody illustrates the potential of its biological application. The final chapter contains detailed experimental procedures and characterisation data. The thesis is fully referenced to the primary literature.
Impact Statement

Fluorescence compounds are powerful tools to visualize and trace biological activities in cells and animal bodies. The synthesis and development of new small molecule fluorophores has been ongoing since the discovery of the first synthetic small molecule fluorophore in late 19th century. With the progress of discovery into new small molecule fluorophores, more and more micro activities can be visualized without using expensive and complicated microscopic methods. However, there are still some limitations of the fluorophore tagging method. Heavier fluorophores often have longer emission wavelength which leads to better penetration since the fluorescence with longer wavelength will not be absorbed by biomolecules, while the bioactivity of the tagged macromolecule may have changed or been reduced even completely retarded by possible conformational change which caused by the big fluorophore.

A novel series of small molecule fluorophores with emissions ranging from purple to near infrared have been developed in this project. It is rare for small molecules (M.W. < 500) to have NIR region fluorescence (>710 nm). Long wavelength emission fluorophores are very beneficial to in vivo imaging as they have excellent penetration through biological membranes and tissues. Two of the synthesized fluorophores have been connected to trastuzumab (a monoclonal antibody used to cure HER-2 positive human breast cancer) and the function of the antibody is remains unaffected which is encouraging. This developed antibody-fluorophore complex integrates selective chemotherapy and in vivo imaging together.
Acknowledgement

Firstly, I would like to thank my parents and grandmother for their support, both mentally and financially. I won't be able to finish my degree without them. Thanks to my grandfather as well, R.I.P.

It is really a great honor and a good fortune to do research under the supervision of Prof. Jim Anderson, thanks him for generous help and guidance. I would like to appreciate all past and present members of Anderson’s group, includes Dr. Helen Allan, Dr. Aisha Syed, Dr. Steven Packman, Dr. Xiangyu Zhang, Chia-Hao Chang, Anand Patel, Mikhail Pumpianskii and other people. Also thanks to friends from Bob Schroeder’s and Michael Porter’s groups. They offered me not only academic help in research, but also happiness in life.

I would like to thank Dr Kersti Karu for the mass spectrometry support and Dr Abil Aliev for the NMR spectroscopy help. Special thanks to Dr. Calise Bahou for the bioconjugation and other biochemical tests

Thanks to my friends from WCU, for making my life full of laughter. Thanks to Mr. Wu. for strengthening my mind when I was in need.

All is concealed in no words. Cheers, everyone.
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1. Introduction

1.1 Discovery and history of fluorescence

Fluorescence is the emission of light by a substance that has been excited by light or other form of electromagnetic radiation. Fluorescence was first described by Bernardino de Sahagún in 1560.[1] In his description, the *lignum nephriticum* (Latin for “kidney wood”) cup is capable of making the water inside luminescent. This kind of cup was made from wood from either, *Pterocarpus indicus* or *Eysenhardtia polystachya*. The fluorescent chemical compound glowing in the liquid was found to be matlaline, a product of the oxidation of flavonoids in the wood.[2][3] Matlaline has a conjugated π system which is shown in red.

![Chemical structure of matlaline and the *lignum nephriticum* cup with the glowing water inside. The picture was painted by William Edwin in 1916, Safford.](image.png)
Fluorescence was not only observed in organic materials, but also observed in inorganic minerals. The fluorescence phenomenon of fluorite (which is mainly calcium difluoride) was first reported by Edward D. Clarke in 1819. Later in 1852, George Gabriel Stokes noticed that uranium glass can convert invisible ultraviolet light into visible blue light. He believed this observation was the same as the glowing ability of fluorite. He called this phenomenon *fluorescence*: “I am almost inclined to coin a word, and call the appearance *fluorescence*, from fluor-spar [i.e., fluorite].” The word fluorescence was from the mineral fluorite.
1.2 Mechanistic and structural explanation of fluorescence

1.2.1 Physical principles, Jablonski diagram and quantum yield

The mechanism of fluorescence can be explained by quantum chemistry. The light excitation (by either UV light or visible light) can bring a fluorescent molecule to its excited state $S_1$ by promoting an electron from a filled to an unfilled orbital of the correct symmetry. The excited state $S_1$ can then relax back to the ground state $S_0$, along with energy loss (emission) during the relaxation. The emission process can be either radiative (light) or non-radiative (heat or other forms of energy). The whole process can be described as following:[6]

Excitation (absorption): $S_0 + h\nu_{ex} \rightarrow S_1$

Emission (fluorescence): $S_1 \rightarrow S_0 + h\nu_{em}$

Here $h$ is Planck’s constant and $\nu$ is the frequency of light. In general the frequency of the emitting light is lower than the excitation light due to internal energy loss (the Stoke’s shift). For example uranium glass absorbs higher energy, shorter wavelength UV light and emits lower energy, longer wavelength blue light. Most of the $S_0 \leftrightarrow S_1$ process in fluorescence can be rationalized through a Jablonski diagram (Figure 2).[7] As the diagram shows, a molecule can have multiple excited states $S_1 (2,3,4,…etc.)$. Those excited states can be inter-converted to each other by non-radiative transitions which ultimately reduces the energy released upon relaxation to the ground state.
Figure 2 The Jablonski diagram of excitation – emission process of fluorescence. A fluorescent molecule absorbs energy and then rises to an excited state. Normally a molecule has several excited states with different energy levels and spin multiplicities. Degradation from a higher excited stage to a lower excited state results in a non-radiative transition, which means no energy is dissipated from the molecule to the surroundings. The molecule releases energy in the form of light during the relaxation from its excited states to ground states.

The fluorescence properties of a molecule are highly related to its molecular structure. For example, the transition energy $\Delta E(S_0 \rightarrow S_n)$ is strongly dependent on the extent of conjugation. Generally, the transition energy decreases as conjugation increases making the molecule more easily excited and it fluorescing in the lower energy visible light region. Electron donating groups (e.g. $-\text{NH}_2$, $-\text{OH}$, $-\text{NR}_2$) connected to the conjugated structure benefits the fluorescence of the molecule. Non-bonding electrons of those groups can participate in the conjugation and raise the energy of the HOMO. As $\Delta E(\text{HOMO}-\text{LUMO})$ decreases, excitation of the molecule is easier. Similarly an electron withdrawing group may have the effect of lowering the energy of the LUMO. Molecular rigidity can also play a role in fluorescence. Relaxation from the excited state to ground state can be radiative (fluorescent, phosphorescence) or non-radiative (heat, vibrational, conformational change). Rigid molecules are less likely to release excess energy by vibrating or conformational change and may increase the chance of fluorescence, which means an increase in fluorescence efficacy.
The efficiency of fluorescence is described by quantum yield.[10] The quantum yield can be calculated in either ways,

\[ \varphi = \frac{\text{numbers of photons emitted}}{\text{numbers of photons absorbed}} \]

or

\[ \varphi = \frac{k_f}{\sum_i k_i} \]

where \( k_f \) is the spontaneous emission rate and \( \sum_i k_i \) is the sum of the rates of excited states decay (includes both radiative and non-radiative decay). In practice, the quantum yield of a fluorescence molecule is measured and calculated by comparing to a standard. The standard is a fluorescent molecule for which the quantum yield is already known e.g. quinine sulfate.

1.2.2 Kasha’s rule

The Kasha’s Rule is a principle in fluorescence which states that light (photon) emission can only occur from the lowest excited state of an excited molecule. It was proposed in 1950 and named after an American physicist Michael Kasha.[11]

Once a molecule absorbs a photon, depending on the wavelength of the photon, it can be excited from its electronic ground state \( S_0 \) to any of its higher electronic states \( S_n \) (\( n>0 \)). However the emission of photon is only expected from the decay of the lowest excited state \( S_1 \). An alternative statement of Kasha’s rule is that the wavelength of emission light is independent of the wavelength of the excitation photon.

Kasha’s rule can be explained by the energy difference between each electronic state of a molecule. In most cases, there is no significant energy difference between all excited states of a molecule. This fact allows internal conversion of the
molecule between several excited states. The internal conversion is so fast that the fluorescence process is not kinetically competitive. However, the energy gap between the ground state $S_0$ and the lowest excited state $S_1$ is greater, in which case once the molecule has relaxed to excited state the fluorescence occurs since it is now kinetically competitive with internal conversion. In a few cases some molecules can have large energy gaps between their excited states, which makes exception to Kasha’s rule.

1.2.3 Mirror-Image rule

In most cases, the absorption spectrum is a mirror image of the emission spectrum.$^{[12]}$ The cause of this phenomenon is that the nuclear geometry does not change significantly during electronic excitation. Hence, the energy gaps between each vibrational levels of the excited state would be similar to that of the ground state (Figure 3).

![Figure 3](image.png) The absorption and emission spectra of perylene in benzene. The mirror image phenomenon is clear. The picture is from ref [12].

However, there are several exceptions of the mirror-image rule. One exception is 1-hydroxypyrene-3,6,8-trisulfonate (HTPS) (Figure 4) which is a pH-sensitive fluorophore. Since the hydroxyl group can be either protonated or deprotonated, HTPS has different absorption spectra under different pHs. For example, it has an absorption spectrum which is similar to that of an aromatic hydrocarbon molecule under low pH (1.0). Under higher pHs (7.6 and 13.0), since the hydroxyl group is deprotonated, the
absorption spectrum changes greatly. However, the dissociation constant \((K_a)\) of hydroxyl group changes when HTPS is in its excited state, hence it can be deprotonated under neutral pH (7.0). As a result, the emission spectrum under neutral pH (7.0) is not the mirror image of the absorption spectrum under the same pH, but the mirror image of the absorption spectrum under basic pH (13.0).

Another exception of the mirror image rule is the formation of complexes. The best known example is pyrene (Figure 5). At low concentrations, the UV emission of pyrene is highly structured, while the UV emission shifts to the visible light region at higher concentration. That is due to the formation of excited state dimer, commonly abbreviated to excimer, at high concentrations.
1.2.4 Stokes shift

The emission wavelength of a molecule is always longer than wavelength of the electronic excitation. The difference in these two wavelengths is called the Stokes shift, which is named after the Irish physicist George Gabriel Stokes. The redshift of the emission wavelength is caused by the energy lost during the excitation-emission process.

The energy loss is the result of many actions. The major two are the reorganization of solvent molecules and interconversions of the molecule itself. In solution, fluorescent molecules are surrounded by many solvent molecules. When the molecule enters its excited state, the dipole moment changes. However the solvent molecules around can not adjust to this change quickly. The vibrational relaxation of the molecule will realign the dipole moment of solvent molecules, which causes the energy loss of the excited fluorescent molecule. Another interconversion that may occur during the excitation process is interconversion between several excited states. This activity dissipates energy and results in the red-shift of the emission wavelength.

Figure 5  The emission spectrum of pyrene at different concentrations. The fluorescence intensity decreases as the concentration decreases from $6.0 \times 10^{-3}$ M (top) to $9.0 \times 10^{-5}$ M (bottom). The picture is from ref [12].
If the emitted light has more energy, then the difference is called an anti-Stokes shift. This phenomenon can be found in some inorganic crystals. One example is yttrium oxysulfide which is doped with gadolinium oxysulfide.\textsuperscript{[14]} It can absorb near infrared light and emit visible light. The extra energy of the emitted light comes from the crystal cooling process when it emits thermal phonons from the crystal lattice.

1.2.5 Fluorescence quenching

The term fluorescence quenching refers to the decrease of the emission intensity of a fluorophore. This phenomenon can be caused by various actions.\textsuperscript{[15]} \textsuperscript{[16]}

One major factor is Forster resonance energy transfer (FRET), named after German physicist Theodor Forster, which is also known as fluorescence resonance energy transfer.\textsuperscript{[17]} A donor fluorophore which in its excited state, can transfer its energy to another acceptor fluorophore and bring it to its excited state, while the donor itself decays to the ground state without any fluorescence. This interaction is not an excitation-emission-re-excitation process, but a non-radiative dipole-dipole coupling, which means there no photon exchange between the donor and the acceptor.

The efficiency of FRET process is highly sensitive to the distance between the donor molecule and the acceptor molecule. The efficiency $E$ can be calculated using the following equation:

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

where $r$ is the distance between the donor molecule and the acceptor molecule and $R_0$ is the Forster critical distance of the donor-acceptor pair, which is the separation distance when the energy transfer efficiency is 50%. The $R_0$ can be calculated using:

$$R_0 = \frac{2.07 \kappa^2 Q_D}{128\pi^5 N_A \eta^4}$$
where $N_A$ is the Avogadro’s number, $\kappa$ is the dipole factor describing space orientation between donor and acceptor, $Q_D$ is the quantum yield of the donor itself (without the acceptor in the same system) and the $\eta$ is the refractive index of the medium (solvent). So $R_0$ would be:

$$R_0 = 2.11 \times 10^{-5} \sqrt[6]{\frac{\kappa^2 Q_D}{\eta^4}}$$

In practice, the FRET efficiency $E$ can be measured by:

$$E = 1 - \frac{I_{em}'}{I_{em}}$$

where $I_{em}$ is the fluorescence intensity of the donor with acceptor and $I_{em}'$ is the fluorescence intensity of the donor itself without the acceptor.

Through the above equations, the distance between the donor and the acceptor can be calculated. This phenomenon is important in biological studies because the distance can provide information related to protein-protein interactions and protein conformation.\[18][19]

After energy has been transferred to the acceptor, it can be released in either radiative or non-radiative ways. There are a series of acceptor molecule, which dissipate all transferred energy in the form of molecular vibrations (heat) and are called dark quenchers. They are dye molecules without any native fluorescence. Some of them absorb visible light with certain wavelengths while some of them suppress the entire visible spectrum (Black Hole Quenchers). Those molecules have been used in many FRET assays.\[20][23] Some typical dark quenchers and their absorption wavelengths are shown below (Figure 6):
Most dark quenchers have one or more azo bonds. The vibration of those azo bonds can suppress the radiative decay of the molecule and leads to the absence of fluorescence. This fact enables those azo bonds containing molecules to be fluorescence quenchers.

Another cause of fluorescence quenching is Dexter electron transfer, which also known as collisional energy transfer. Similar to FRET, it is also a non-radiative
energy transfer, but through excited electron transfer rather than dipole interaction. This energy transfer only occurs when there is overlap between the wavefunctions of donor and acceptor, which means it only occur over a short distance. For a fluorophore, Dexter electron transfer can happen between itself and solvent molecules, especially when there is hydrogen bonding between them. That is why water is considered a good fluorescence quencher.

As mentioned in section 1.2.3, formation of an excimer can lengthen the emission wavelength. In other terms this means that formation of an excimer would cause extra energy loss. In some cases, excimers can be non-fluorescent. Hence, formation of excimer can be a reason for fluorescence quenching. The formation of excimer can be affected by several factors, for example concentration. Generally, the fluorescence intensity increases while the concentration of fluorophore increases. However, if the concentration is extremely high that fluorophores can aggregate in the solution, the fluorescence intensity will drop significantly. In the following case of $N,N'$-bis(4-diethylamino-2-hydroxybenzylidene)-1,2-dicyano-1,2-ethenediamine 13 (Figure 7), the compound showed typical aggregation induced quenching phenomenon. Compound 13 has good solubility in organic solvents, but is poorly soluble in water. By adding water to a solution of 13 in MeCN, aggregates can be generated. In dilute solution, intramolecular hydrogen bonding forces the molecule to stay planar which enables the fluorescence. When the concentration increases, the strong intermolecular $\pi-\pi$ interaction can facilitate aggregates 15 (excimer) which leads to the disappearance of fluorescence.
Figure 7 Solutions and solid of 13 under room light and UV light, with the structure and proposed conformations in both solution and aggregates. The proportion of water increases from 0% to 98%. The picture is from ref [25].

1.2.6 Aggregation-Induced emission

Aggregation can not only quench the fluorescence, but in some situations, enhance the fluorescence. For some particular fluorescent molecules, they have weak or even no fluorescence in the solution state, but intense fluorescence in the solid state. Also, their fluorescence intensity varies greatly with the proportion of water in the solvent. This phenomena is called Aggregation-Induced Emission (AIE) and was discovered by Benzhong Tang’s team in 2011.[26] Most AIE molecules
have free-rotating groups. When those molecules are brought to their excited states, they decay to the ground states by rotating/vibrating those free-rotating groups, rather than emitting energy in the form of a photon. However, when those molecules aggregate or crystallize, the bond rotation/vibration is prevented, the radiative decay (fluorescence) then becomes competitive and the molecules glow.

For example, polyaryl compound 16 has 5 free rotatable phenyl groups (Figure 8). Due to a strong steric effect, the preferred conformation of this compound should be 17. All the phenyl groups avoid destabilizing steric interactions by rotating out of the plane of the molecule. Once the molecule is excited, the rotation of the 5 phenyl groups will consume the energy and suppress fluorescence. In aggregates or in its solid state, the rotation of those aryl groups are blocked, which allows fluorescence.

![Figure 8](image)

Figure 8  The fluorescence and structures of compound 16 in THF/water, the proportion of water changes from 0% to 90%. The fluorescence quantum yield (ΦF, %) was measured using quinine sulfate as standard. The picture is from ref [26].

A free rotatable structure is surprisingly not a necessity of AIE. For some fluorescent molecules without any free rotatable groups, AIE is still observable. An example is tetrahydro-tetrabenzoheptafulvalene (THBA) 18, which has a rigid structure.[27] Interestingly, this compound has no fluorescence in solution state but intense fluorescence in aggregates (Figure 9). Further determination has shown that the vibration of those tethered aromatic rings is indeed energy consuming.[28]
Figure 9  The UV absorption and emission spectra of THBA. The water proportion is increase from 0% to 90%. The picture is from ref [28].
1.3 Small molecule fluorophores

Fluorophores are fluorescent compounds which are capable of emitting light upon light excitation (by UV or visible light). Typical fluorophores are highly conjugated molecules which contain several aromatic groups and commonly possess an electron donor and an electron acceptor at each end of the conjugated system. Electrons in conjugated \( \pi \) bonds can be more easily excited to a higher energy level by photons and then decay to the ground state, accompanied by light emission. Usually, the molecular weights of synthetic fluorophores fall in the range of 200 – 1000 Dalton, sometimes higher depending on further modifications. Several fluorophores and their structures are shown below (Figure 10).
1.3.1 Acridine family

Acridine 19 is a nitrogen containing heterocyclic compound. It has a pyridine ring fused with two phenyl rings, the structure is planar, highly conjugated and aromatic. This particular structure renders acridine fluorescent and allows it to be used in some special applications. Acridine can insert into the DNA double helix, due to its strong π-π interaction with DNA base pairs, and alter its optical properties. Based
on this fact, some DNA fluorescent stains have been synthesized. Furthermore, many acridine based chemotherapeutic compounds have been developed due to the genotoxicity (Figure 11).[29]

Figure 11 Several acridine derivatives which been developed for different uses.

By changing the substituents on the acridine ring, the optical properties can be altered. Acridine orange 20 was the earliest member of the acridine family of fluorophores.[30] It has two dimethylamine groups in conjugation with the pyridine nitrogen. Acridine 19 has $\lambda_{ab} = 355$ nm and $\lambda_{em} = 440$ nm. The addition of two dimethylamine groups in 20 red-shifts the absorption and emission wavelength to $\lambda_{ab} = 500$ nm and $\lambda_{em} = 526$ nm. Acridine orange has good solubility only in polar solvents. To expand its application to more hydrophobic systems, nonyl acridine orange bromide (NAO) 23 has been synthesized (Figure 12).[31] A nine-carbon alkyl chain is appended to the pyridine nitrogen to increase the hydrophobicity of the molecule. The pyridine nitrogen is then positively charged, which enables it to be a mitochondria probe.[32] There are various other acridine based fluorophores that have been synthesized and developed (Figure 13).[33] One of them is acridine yellow 24, which has a similar structure and fluorescent properties to acridine orange 20.
The syntheses of members of the acridine family of fluorophores are similar. The acridine core structure can be synthesized by condensation of appropriate 1,3-phenyldiamine and carbonyl compounds.

Since acridine derivatives can insert into the double helix of DNA molecules, some of them are carcinogenic and have the potential to cause gene mutation, and so need careful handling.
1.3.2 Anthraquinone family

Anthraquinone 29 is a poly phenyl compound with a benzoquinone core structure. Compared to core structures of other synthetic fluorophores, the anthraquinone structure is much more common in naturally occurring compounds. The anthraquinone type compounds (Figure 14) have been found in plants like senna (emodin 30), aloe (aloe emodin 31), rhubarb (parietin 32) and turkey rhubarb (rhein 33). Most of them are slightly poisonous laxatives which can loosen stools and increase bowel movements.

![Figure 14](image)

**Figure 14** The structure of anthraquinone, some naturally occurring anthraquinone type molecules and their sources.

A series of drugs also have the anthraquinone core structure and are called anthracyclines (Figure 15). Most of them are antineoplastic agents which are used in chemotherapy since they can intercalate the DNA molecule to prevent cell division. The anthracycline family is considered to be the most effective antitumor agents that have ever been discovered. They are also effective against more types of cancers than any other class of chemotherapeutic agents. Though
they have serious side effects like cardiotoxicity and immune suppression, they are still widely used.

![Diagram of anthracyclines](image)

Figure 15 Some representative anthracyclines among the whole family. Mitoxantrone 39 has anthraquinone structure but its mode of action is slightly different from typical anthracyclines.

Besides naturally occurring anthraquinones and anthracyclines, anthraquinone fluorophores have been developed and synthesized over many years. Among all anthraquinone fluorophores, DRAQ5 40 dye maybe the most useful (Figure 16). DRAQ5 has been used in DNA staining and confocal microscopy.[37] It only stains the DNA of living or fixed cells. Derived from DRAQ5, DRAQ7 and CyTRAK Orange have also been developed.[38] The structures of those two anthraquinone fluorophores have not been published. Compared to DRAQ5, DRAQ7 only stains dead cells and leaves living cells unaffected, CyTRAK can demark the boundaries while staining.
Other than the three derivatives mentioned above, some new synthetic anthraquinone fluorophores have been reported recently. One example is the ion-sensing fluorophore 41.\textsuperscript{[39]} It has an aza-crown ether group which has the ability of coordinate to metal ions (Figure 17). Once coordination occurs, it alters the electronic properties of the nitrogen atom, which leads to the change of the optical properties of the whole molecule. Some other fluorophores used for enzyme detection have been reported as well.\textsuperscript{[40]}

![Figure 16](image1.png) The structure of DRAQS and the living BPAE cell in which nucleus has been stained.

![Figure 17](image2.png) The mechanism of coordination of metal ions with the fluorophore.
1.3.3 Arylmethine family

The arylmethine fluorophores have the core structure of a diarylmethane or triarylmethane. In general, the fluorophore itself is usually positively charged. The electron-donating groups on the aryl rings help to stabilize the positive charge by delocalization. Compounds in the arylmethine family are always used as dyes while not commonly as fluorophores.

One representative case is the methyl violet family 42a-c (methylated from pararosaniline 43) (Figure 18), which was first synthesized by Charles Lauth in 1861. From 1866, a mixture of the 3 isomers were manufactured by Saint-Denis-based firm of Poirrier et Chappat and marketed, under the name “Violet de Paris”. Nowadays, the most researched and developed isomer is crystal violet 42c. It has a distinct red-orange fluorescence in water. It has been used as an antibacterial agent, a reagent for Gram staining and tissue staining in light microscopy for many years. Also, as a fluorophore, it has been used in DNA gel electrophoresis as an indicator. It can be a less toxic replacement of ethidium bromide. Another advantage of using crystal violet is visible light excitation (620 nm) instead of UV activation. It is helpful in protection of sensitive biological molecules since no UV activation is required.

![Chemical structures of methyl violet family and pararosaniline](image-url)

42a $R_1=R_2=H$, Methyl violet 2B
42b $R_1=H$, $R_2=Me$, Methyl violet 6B
42c $R_1=R_2=Me$, Crystal violet
43 Pararosaniline
Another example is auramine 44 (Figure 19). It has a diarylamine core structure and a stabilized iminium moiety. Auramine has been widely used in microbiology as a stain and a screening indicator. Auramine is commonly used in cooperation with other fluorophores. Auramine-Rhodamine stain (AR stain)\(^{[46]}\) is a specific stain method for acid-fast bacilli (a series of bacteria which has resistance to common microbiological staining methods) in fluorescence microscopy. In this stain, auramine O and rhodamine B are used as the dye. During the decolorizing step, the acid-fast bacteria will maintain the dye molecules in the cytoplasm while other bacteria would be decolorized. So only acid-fast bacteria will be stained using this method. Other than rhodamine B, auramine can also be used with phenol to give the Auramine-Phenol (AP) stain.\(^{[47]}\) This stain is used to identify tuberculosis mycobacteria which belongs to mycolata group (microorganisms which can cause various tuberculosis). The cell wall of tuberculosis mycobacteria contains mycolic acid (long chain fatty acid) which can retain the AP dye molecules during the decolorizing step.
Figure 19  The structure of auramines and: a) auramine-phenol stained mycobacterium tuberculosis; b) auramine-rhodamine stained acid-fast bacteria in sputum sample. The pictures are from ref [48].

There is a limitation to using arylmethine dyes. Compounds that adopt cationic arylmethine structure are proven to be acutely toxic and carcinogenic since they have a super electrophilic center which can be attacked by the amine groups in human DNA base pairs. Crystal violet was used as a detergent in the past, however it has been replaced nowadays due to its toxicity.

1.3.4 Coumarin family

Coumarin 45 is an aromatic molecule with a benzopyranone structure. It can be described as a benzene ring with a fused unsaturated δ-lactone. Coumarin type molecules are highly abundant in nature (Figure 20). They have been found in various plants e.g. strawberry and vanilla and have been proven to be responsible for the special smell of the plants.
There is a series of synthetic coumarin molecules (Figure 21), which have been used as anticoagulant (blood thinner) in medicine.\textsuperscript{[51]} The most representative member in this class is warfarin 51.\textsuperscript{[52]}

It was first marketed as a rat poison in 1948. Afterwards it was discovered to have an effect on decreasing blood clotting. This observation then led to the medical use of warfarin, which was approved by the FDA in 1954. The mechanism of warfarin analogues has been elucidated. Warfarin can block vitamin K epoxide reductase since it has similar structure to vitamin K\textsubscript{1} (57). Upon inhibiting the enzyme, it can not reproduce active vitamin K\textsubscript{1} by reducing the oxidized vitamin K\textsubscript{1} epoxide. Vitamin K\textsubscript{1} is an important cofactor for the clotting factor which helps blood clotting. The clotting factor will have decreased activity since there is insufficient vitamin K\textsubscript{1}. However, warfarin derivatives can sometimes cause internal hemorrhaging.
Coumarin type fluorophores have been researched for many years as well. AlexaFluor® is a series of fluorophores developed by Thermo Fischer® that are used for biological staining and tracking. They consist of different dyes with fluorescence wavelengths between 442 nm to 814 nm.\(^{[53]}\) AlexaFluor® 350 \(^{58}\) and AlexaFluor® 430 \(^{59}\) are two members that belong to AlexaFluor® family (Figure 22). They have coumarin core structures, with different substituents on the molecule to alter their optical properties. There are options for protein tagging since they have pendant carboxylic acid groups (succinimidyl ester version available as well) which are readily available for amine coupling.\(^{[54],[55]}\)
The dye laser is a dye molecule mediated laser. The medium inside the laser is often a liquid solution of specific fluorophores. Due to the availability of a wide-range of fluorophores, the dye laser can generate light with much wider wavelength range than other types of lasers. Coumarin fluorophores have been used as laser dyes\cite{56} since 1975 (Figure 23). This series of laser dyes provide lasers with wavelengths which fall in the range between 442 nm to 540 nm.
1.3.5 Cyanine family

Cyanine is a non-systematic name for polymethine fluorophores. Unlike other fluorophore families, there are no naturally occurring cyanine derivatives. It is a
pure synthetic fluorophore family. A typical cyanine molecule has two quaternary nitrogen atoms that are joined by a polymethine linear chain or closed cyclic polymethine moiety. The length of the chain is commonly 3, 5 or 7 carbon atoms. The molecule is positively charged where the positive charge is stabilized by delocalization.

Cyanine (Cy) dyes are the earliest members of cyanine fluorophore family (Figure 24). Their applications in chemistry and biology were first described by Alan S. Waggoner in 1979.\textsuperscript{[57]} Cy dyes can generate fluorescence wavelengths from 506 nm (cyan-green) to 808 nm (near IR) depending on their structures. The ‘0.5’ versions of each integer molecules have around 10 to 20 nm longer fluorescence, caused by extending the conjugated system. The carboxylic acid can be modified to other groups e.g. N-hydroxysuccinimide ester in order to couple with an amine in a mild way. Other modifications like alkyl, alkoxyl, acetyl and sulfonyle groups on the benzene/naphthalene ring can alter the hydrophilicity of the molecule.

![Figure 24](image_url) Structures of 6 earliest members of cyanine family fluorophores.
The original Cy dyes have two indoline (benzopyrrolidine) rings. Replacement of the pyrrolidine part by thiazolidine\(^{[58]}\), oxazolidine\(^{[59]}\), and pyridine\(^{[60]}\) has been done. Below are some examples of modified Cy3 molecules (Figure 25).

These replacements change the optical properties of the Cy dye (Table 1). The pyrrolidine and thiazolidine (85 Cy T-248) compounds have shorter absorption and emission wavelengths than the parent compound (80 Cy3), while the pyridine compound (86 Cryptocyanine) has longer wavelengths and a significantly lower quantum yield. The longer conjugation chain (same length as 7-carbon cyanine molecule) of cryptocyanine 86 could be responsible for the red-shifted emission.

**Table 1  Comparison of the optical properties of Cy3 and three modifiers.**

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>(\lambda_{\text{max}}) (absorption)</th>
<th>(\lambda_{\text{max}}) (emission)</th>
<th>(\varepsilon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 Cy3</td>
<td>(512), 550</td>
<td>570, (615)</td>
<td>0.15</td>
</tr>
<tr>
<td>84 Cy2</td>
<td>489</td>
<td>506</td>
<td>0.12</td>
</tr>
<tr>
<td>85 Cy T-248</td>
<td>527</td>
<td>578</td>
<td>n/a</td>
</tr>
<tr>
<td>86 Cryptocyanine</td>
<td>709</td>
<td>717</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Other than Cy dyes, IRDye® is another series of cyanine-type fluorophores (Figure 26). They are a set of near-IR emission fluorophores that have been used in biological studies. Compared to Cy dyes, IRDye® fluorophores have slightly longer fluorescence wavelengths. The maximum emission wavelength that has been reached is from IRDye® 800 ($\lambda_{\text{em}}$ 810 nm) cf Cy7 775 nm). The reason for the longer wavelength of emission could be the addition of a phenoxy group to the central conjugated chain.

Merocyanine-type fluorophores also have the cyanine core structure. Usually, these fluorophores are intensely coloured and have large extinction coefficients.\textsuperscript{[61]} Merocyanine 1 91 and merocyanine 540 are the earliest two members of this family (Figure 27). Merocyanine 540 was the first fluorophore that was used to determine the electric potential between both sides of a cell or other forms of biological membrane (the membrane potential).
Some merocyanine derivatives were observed to be highly sensitive to changes in their microenvironment, e.g. solvent. K. Hahn et al. have synthesized a series of merocyanine fluorophores that have been used to report the conformational change of a protein. When the conformation of a protein changes, the microenvironment of the protein package can be either more hydrophilic or hydrophobic due to the rearrangement of solvate molecules. The optical properties of reporter I-SO 93 when conjugated to a protein were shown to vary with conformational change.

1.3.6 Oxadiazole (benzofurazan) family

The benzofurazan-type fluorophore is a newer type of fluorophore compared to other fluorophore families. The first member [2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium (NBD-TMA) 94 was researched in the 1990s. This cationic fluorophore was first used in dual colour labeling in 2005 (Figure 28). Another fluorophore derived from 94 is EAM-2 95, in which a methyl group was replaced by a hydroxyethyl group, in order to modify solubility.
Figure 28  Structures of NBD-TMA, EAM-2 and the yeast cells labelled by NBD-TMA. The NBD-TMA molecule is impermeable to the membrane of yeast cell. The picture is from ref [64].

The general structure of the benzofurazan-type molecule is shown below (Figure 29). The structure contains an electron-withdrawing group and an electron-donor on each end of the conjugated chain. Different functional groups on the benzene ring have enabled different applications of the fluorophore.

One example is in metal sensing. A benzofurazan fluorophore when modified with crown ether functional group became a metal sensor. The terpyridine group plays the role of both electron-withdrawing group and metal coordinator (Figure 30). By coordinating to metal ions, the electronic state of the terpyridine group changed, and resulted in a change of fluorescent properties. The metal-fluorophore complex was robust and remained coordinated in the living cell environment.

The benzofurazan fluorophore is also used as a drug probe in the research of in vivo distribution and metabolism of cholic acid and other bile acid compounds. [67]
1.3.7 Oxazine (phenoxazine) family

Oxazine is a six-membered heterocyclic compound which contains one oxygen and one nitrogen atom. Several isomers exist, depending on the positions of nitrogen and oxygen. The 1,4-oxazine fused with two benzene rings is called phenoxazine. Phenoxazines can be found in nature. Cinnabarine 97 and cinnabaric acid 98 are two naturally occurring phenoxazine molecules (Figure 31). They can be found in Nectria cinnabarina which is a harmful fungus on broadleaf trees.[68]

![Figure 31 Isomers of oxazine and naturally occurring phenoxazines.](image)

Most of the oxazine family of fluorophores have the phenoxazine structure.[69] The most developed and researched three members of the oxazine family of fluorophores are Nile Red 99, Nile Blue A 100 and cresyl violet 101 (Figure 32). Cresyl violet is used to stain neurons in the brain spinal cord.

![Figure 32 The structures of Nile Red, Nile Blue A and cresyl violet.](image)
Nile Blue A which is an $N, N$-dialkylated derivative of cresyl violet, is used as a pH-sensitive fluorescent dye. Under strong acidic conditions, the Nile Blue A molecule is protonated again to generate red fluorescence rather than the yellow fluorescence observed under neutral or basic conditions (Figure 33a). Due to that special characteristic, it is also used to distinctively stain neutral lipids and fatty acids. Nile Red is a ketone analogue of Nile Blue A. One preparation procedure is the hydrolysis of Nile Blue A. Nile Red is a lipophilic compound and a highly solvatochromic fluorophore (colour is solvent dependent). Nile red has deep-red fluorescence in polar solvents, while the colour changes to yellow-green in non-polar solvents (Figure 33b). Based on this characteristic, Nile Red has been used in the detection of microplastic contamination of bottled water.

Figure 33 a) Nile Blue A dissolved in solvent mixture of water (bottom) and ethyl acetate (top) with different pHs. Left to right: pH 0, pH 4, pH 7, pH 10, pH 14. The picture is from ref [70]; [70]
b) Nile Red in different solvents, under natural light and UV. Left to right: water, methanol, ethanol, acetonitrile, DMF, acetone, ethyl acetate, DCM, hexane, MTBE, cyclohexane, toluene. The picture is from [71].

Oxazine type fluorophores can also be used as laser dyes (Figure 34). Compared to other laser dyes e.g. rhodamines, oxazine laser dyes have a smaller energy gap between $S_0$ and $S_1$, resulting in their quantum yield being decreased by internal conversion. Interestingly, like the three oxazine fluorophores already mentioned (Figure 32) the quantum yield of oxazine laser dyes is solvent dependent.

Further modifications have been performed on phenoxazine fluorophores. A thiazole derivative has been synthesized by K. E. Beatty et al in 2016. The replacement of the benzene ring by a thiazole moiety lead to a lower quantum yield, but a longer fluorescence wavelength (Figure 35). In addition, the fluorophore 106 was found to inhibit bacteria and is a potential novel antibacterial. Other modifications have replaced the oxygen atom for a carbon, making the phenoxazine into a carbazine (similar to acridine), which was found to lengthen the fluorescence wavelength.
A dual-function DNA probe based on the phenoxazine structure has been synthesized (Figure 36).[75] The probe consists of two parts. The tetramethylpyrrolidine oxide radical part (in blue) makes the probe detectable by electron paramagnetic resonance (EPR) (a technique used to detect unpaired electrons). The fluorophore part (in red) generates fluorescence as a normal fluorophore. The benzene ring of the phenoxazine structure was replaced by a 2-pyrimidone moiety and made the fluorophore a cytosine mimic, so that the probe could bind with guanine moieties in DNA by hydrogen bonding (structure 110). The probe has been used in the determination of dynamics of nucleic acids.[76]
1.3.8 Naphthalene family

There are lots of different fluorophores containing the naphthalene-type moiety. It is hard to draw a general structure for all of them.

Dansyl chloride 111 may be the most used fluorophore among all the naphthalene-derived fluorophores.[77] It has a reactive sulfonyl chloride group (Figure 37) toward nucleophilic substitution e.g. addition to an amine. Dansyl chloride is used in protein sequencing as a qualification probe. After reacting with dansyl chloride, the N-terminal amino acid of the protein can be cleaved to produce a dansyl-amino acid compound which has an intense green to blue fluorescence. By this operation, the type of N-terminal amino acid can be determined.

![Figure 37](image1.png) The structure of dansyl chloride and the protein sequencing method.

Another pair of well-known members of naphthalene fluorophores is prodan 112 and its derivative laurdan 113 (Figure 38), which is more lipophilic.[78] Prodan and laurdan have green fluorescence when in aqueous solution. The wavelength is shortened significantly when they are dissolved in less polar solvents e.g. cyclohexane.
Various derivatives of prodan have been synthesized. The fluorene derivative $^{114}$ is extremely solvatochromic.$^{[79]}$ It shows blue to purple fluorescence in toluene while become red in methanol. Since it is extremely sensitive to environmental change, it can be potentially useful in biological labeling. Other modifications include extending the conjugated system e.g. replacing the naphthalene ring by an anthracene moiety.$^{[80]}$ The anthradan $^{115}$ has a longer fluorescence wavelength while retaining a similar quantum yield to prodan. Interestingly, installing another fused cyclopentane moiety ($^{116}$ and $^{117}$) to prodan dramatically increased the Stokes shift of the fluorophore.$^{[81]}$

Naphthalimides are another important series of fluorophores that have a naphthalene core structure.$^{[82]}$ The general structure of naphthalimide $^{118}$ contains a rigid aromatic core which makes its quantum yields higher compared to other naphthalene containing fluorophores. It can also be modified and applied to different usages. The modified structures have been used in liquid crystal materials ($^{119}$)$^{[83]}$, soft anionic surface monitoring ($^{120}$)$^{[84]}$ and single molecule applications ($^{121a}$ and $^{121b}$)$^{[85]}$. 

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Figure 38  Structures of prodan and its analogues. Picture of different solutions of the fluorene derivative is also shown. The picture is from ref [79].

Figure 39  Structures of other prodan modifiers.
1.3.9 Phenanthridine family

Phenanthridine 122 is an aromatic compound made up of a pyridine ring fused to two benzene rings. It is fully aromatic and planar. Phenanthridine type structures are commonly natural occurring structures in alkaloids. The phenanthridine alkaloids can be partially hydrogenated to yield saturated structures (Figure 41). Most of those alkaloids have cytotoxicity i.e. suppression and inhibition of cell division. The mode of action can be explained by the ability of the alkaloid molecules to intercalate into DNA double helixes, a behavior driven by π-π interactions between the phenanthridine moiety and base pairs of DNA molecules.
Those phenanthridine alkaloids can not only insert into DNA, but also generate fluorescence under excitation. Inspired by those alkaloids, other molecules which are particularly designed for DNA detection were synthesized (Figure 42).

![Figure 42 Structures of DNA stains inspired by phenanthridine alkaloids.](image)

Ethidium bromide 127 is the most widely used DNA stain. It has an orange fluorescence under UV light, while the fluorescent intensity is magnified about 20 times when it intercalates into a DNA double helix. The significant increase of fluorescent intensity is caused by FRET from DNA to ethidium bromide. Propidium iodide 128 has a slightly different structure to ethidium bromide. The quaternary nitrogen makes the propidium iodide molecule impermeable to intact cell membrane, so only necrotic, apoptotic cells or artificially damaged cells can be stained using propidium iodide. The major limitation of ethidium bromide and propidium iodide stain are their toxicity. Both of them are intensely carcinogenic and can induce gene mutations because they can insert into DNA molecules. An ethidium bromide alternative has been synthesized. The GelRed 129 has a similar function to ethidium bromide and propidium iodide, but is less toxic.
The phenanthridine structures are also found in some dual colour fluorophores (Figure 43). The coumarin/phenanthridine fused fluorophore 130 has been synthesized and used as a lockable colorimetric fluorescence molecular switch.[91] The phenanthridine/rhodamine joint fluorophore 131 is a FRET fluorescence molecule used to label tRNA in cells since the phenanthridine moiety can interact with tRNA.[92][93]

1.3.10 Pyrene and Perylene family

Pyrene is a peri-fused polycyclic aromatic hydrocarbon compound (Figure 44). Unlike other fluorophores, pyrene produces a blue-cyan fluorescence by itself (no electron-donor and electron-withdrawing group needed). This fact enables convenient applications of pyrene, since no complicated modifications are needed.
Pyrene can form excimers when excited by electronic radiation.\cite{94} When the concentration of pyrene in solution is low, it shows nearly no fluorescence above 450 nm. While the concentration is increased to a certain amount or above, it starts to form an excimer with UV light producing an intense cyan fluorescence.

Modified pyrenes can also form excimers upon excitation. An alkyne derivative of pyrene 135 has been synthesized.\cite{95} According to the emission spectrum (Figure 45), the intensity of the first peak (~460 nm) decreases and the second peak (~490 nm) increases with increasing concentration. This is evidence for excimer formation.
Perylene has an extra benzene ring than pyrene. It forms excimers similar to pyrene under excitation. Perylene has been used in OLED application since it has good photochemical stability compared to other fluorophores. BASF has developed a series of perylene bisimide fluorophores (Figure 46). The Lumogen® series of fluorophores have excellent photochemical and thermal stabilities. They have been used in plastic products and OLED applications.
Like phenanthridine, π-π stacking interactions between perylene structure and other aromatic systems is prevalent. A perylene bisimide fluorophore has been synthesized as a supramolecular building block. The fluorophore can aggregate with itself to yield a complex upon excitation. Interestingly, its fluorescence wavelength varies depending on the concentration, even in the same solvent (Figure 47). The formation of aggregates of the fluorophore lengthens the fluorescence wavelength.

Figure 47 The structure of perylene bisimide derivative and its fluorescence with different concentrations. Left to right: 10^{-6} \text{ mol/L}, 10^{-5} \text{ mol/L}, 10^{-4} \text{ mol/L}, 10^{-3} \text{ mol/L}, 10^{-2} \text{ mol/L}. The picture is from ref [97].
1.3.11 Squaraine family

Squaraine fluorophores are derived from squaric acid 140 (Figure 48). The squaric acid core structure is rigid and electrophilic. In general, the emission of squaraine fluorophores fall in the red to near IR region which is slightly longer than other types of fluorophore. The typical structure of squaraine fluorophores is shown below (Figure 48). Two positive charges are stabilized by two electron-rich aromatic rings, while two negative charges are delocalized in two carbonyl groups. By changing the substituents on the nitrogen atoms or the phenyl rings, various derivatives with different optical properties can be generated.

![Figure 48](image)

Figure 48 The structure of squaric acid and several squaraine fluorophores. Fluorescence wavelength of some representative analogues are shown.
Some squaraine dyes suffer from non-radiative relaxation which leads to a decrease of the quantum yield, sometimes even total loss of fluorescence. That is caused by rapid rotational relaxation exacerbated by the steric of the molecule (Figure 49). Another problem is the super electrophilicity of the central carbon atoms of the squaric moiety, that can lead to unwanted nucleophilic attack. To suppress these two processes, a sterically protected squaraine dye has been synthesized and developed.\cite{100} The centered squaric structure is protected by a supramolecular cage. The specific coordination between the cage and the fluorophore is driven by several supramolecular interactions e.g. hydrogen bonding (between amides of the cage and carbonyls of the fluorophore) and π-π interaction.

![Diagram of non-radiative decay process and structure of caged squaraine fluorophore](image)

**Figure 49** The non-radiative decay process and structure of the caged squaraine fluorophore. The fluorophore is shown in blue while the cage is shown in red.
Squaraine dyes are used in catalytic processes as well since they are capable of transferring energy to other species. The encapsulation of squaraine dyes into carbon nanotubes has been reported. This encapsulation can enhance the optical properties of carbon nanotubes by transferring energy from the excited squaraine dyes to the carbon nanotubes.

Squaraine dyes can also transfer energy to oxygen species to generate reactive oxygen species. Periodic mesoporous organosilica (PMO) is a silica containing organic compound which can form a porous structure that can accommodate small organic molecules. Compound 149 is a red fluorescence emitting squaraine fluorophore which can enter PMO. When the entire system is excited by visible light, compound 149 can transfer energy to nearby oxygen species, converting them to singlet (excited and reactive) oxygen species which can oxidize phenol to benzoquinone. The azo-alkene chains on the PMO walls become trans which blocks the PMO structure and increase the catalytic efficacy.

![Structure of the reactive oxygen species generator and the catalytic process.](image)
1.3.12 Xanthene family

Xanthene 150 is a dibenzopyran (Figure 51). Xanthene fluorophores can be divided into two types, fluorescein derivatives 151 and rhodamine derivatives 152. They have similar structures except for the identity of the electron-donors on the benzene rings. In most cases the dyes are carboxylic acids (R=H). The fluorescein and rhodamine derivatives present an environmentally dependent tautomerization behavior.

In neutral and basic aqueous solutions, the molecule becomes a Zwitterion 154. Under acidic conditions, the carboxylic acid is protonated, the entire molecule becomes cationic (153). Both 153 and 154 share the same fluorophore moiety, so the absorption/emission spectra are mostly the same, except for slight differences in the quantum yields and lifetimes. In less polar solvents, the Zwitterion 154 will be converted into a non-fluorescent lactone 155 due to the interruption of the π conjugation. Due to this special characteristic, most of the xanthene fluorophores can be used as pH sensors.

Figure 51 General structures of xanthene fluorophores and its tautomerism.
Eosin Y 157 and eosin B 158 are two main derivatives of fluorescein (Figure 52). Eosin Y is a tetrabromo derivative while eosin B is a dibromo dinitro derivative. The employment of two nitro groups decreases the quantum yield significantly ($\Phi_F = 0.00045$ in water)\[^{105}\] compared to eosin Y ($\Phi_F = 0.2$ in water)\[^{106}\] since the nitro groups are electron deficient and can suppress the conjugation. Eosins are used in the haematoxylin and eosin stain (H&E stain) method, which is a widely used dual colour stain method.\[^{107}\] In this method Haematoxylin 159 can bind with basophilic substances (DNA and RNA) to produce a blue-violet colour, while eosin binds with acidophilic substances (protein and cytoplasm) and make them pink/red (Figure 52).\[^{108}\]

Other important fluorescein derivatives include erythrosine 160, rose bengal 161 and merbromin 162 (Figure 53). Erythrosine has been proven to be a potential photo sensitizer for the photodynamic therapy of oral plaque biofilms.\[^{109}\] Rose bengal is a widely used additive in photochemistry.\[^{110}\] It can convert triplet oxygen (ground state oxygen) to singlet oxygen (excited state oxygen). Singlet oxygen can undergo various unusual reactions e.g. [2+2] cycloaddition with alkenes, which is electronically forbidden under thermal conditions. Merbromin used to be an
antiseptic for many years, before its toxicity was proven and its role has been replaced by other similar but more effective antiseptics.\cite{111}

Rhodamine fluorophores have a similar structure to fluorescein, except the phenols are replaced by primary or substituted amines. Three typical rhodamine analogues are rhodamine 123 163, rhodamine 6G 164 and rhodamine B 165 (Figure 54).\cite{104} The alkylation of the amino groups leads to slight changes in optical properties of the fluorophores. In some cases, the amine groups are acetylated to yield a “caged” fluorophore, which is then used in enzyme (e.g. caspases, esterase) studies.\cite{112}

Due to the wide-ranging applications of rhodamine fluorophores, more commercially available rhodamines have been synthesized. Alexa Fluor® dyes are a set of fluorophores with different optical properties. The members of Alexa Fluor® belong to several fluorophore families, with fluorescence from blue light to the near IR region. Several Alexa Fluor® members contain the rhodamine structure (Figure 55). They are used in many biological studies.\cite{113}
Figure 55  Rhodamine family members of Alexa Fluor® dyes.
1.4 Applications of small molecule fluorophores in different fields

1.4.1 Energy, engineering and material field

1.4.1.1 Organic light-emitting diode (OLED)

An organic light-emitting diode (OLED) is a new type of light-emitting diode (LED), which was first discovered in the 1950s. Traditional LEDs are made of a semiconductor containing metalloids (e.g. gadolinium and indium), while the emissive electroluminescent layer of an OLED consists of organic molecules, which can be either small molecules or polymers.

The components of general OLEDs are shown below (Figure 56). The actual light emitting compound is settled in the emissive layer. The electrons travel from the cathode to the anode, while the positive charge carriers (“holes”) move in the opposite direction. When they are combined in the emissive layer, the light emitting compound is excited and produces light upon decay to the ground state. However, the combination of the electrons and holes may occur out of the emissive layer, which does not lead to light. To suppress this unexpected combination, an electron/hole blocking layer is needed to prevent any possible electron/hole leakage. In addition an electron/hole injection layer and an electron/hole transport layer can enhance the electron conductivity of the whole device.

![The general structure of OLED device.](image)
Small molecule OLEDs are usually metal-fluorophore complexes. The first small molecule OLED tris(8-hydroxyquinolinato)aluminum (174) (Figure 57) was synthesized in 1987 by Ching W. Tang et al.\textsuperscript{[114]} . The wavelength of electrically induced fluorescence of Alq3 falls in the range of cyan light (400-450 nm). The spectrum is also shown.

![Figure 57 The structure of Alq3 OLED and its emission spectrum. The picture is from ref [114].](image)

Similar to typical fluorescence, the wavelength of electroluminescence can be altered by modifying the molecular structure. Effective manipulations of Alq3 have given different electroluminescence signals.\textsuperscript{[115]} Several 5-substituted Alq3 compounds 175 have been synthesized (Figure 58). Both fluorescence and electroluminescence wavelengths were measured (Table 2). By comparing the fluorescence and electroluminescence wavelengths of the same molecule, it can be concluded that different excitation methods do not affect the emission wavelength significantly. The \( \text{Cd}/A_{\text{max}} \) is the ratio between the forward-directed luminance of the device and the measured current density of the device, thus the “quantum yield” of the electroluminescence.
Figure 58  The structures of derivatives of Alq3.

According to the table, it can be concluded that there is no obvious relationship between the fluorescence quantum yield and the electroluminescence quantum yield.

Table 2  Comparation of the optical properties of Cy3 and three modifiers.

<table>
<thead>
<tr>
<th>OLED</th>
<th>$\lambda_{\text{max}}$(absorption)</th>
<th>$\lambda_{\text{max}}$PL, $\Phi$</th>
<th>$\lambda_{\text{max}}$EL, Cd/A_{\text{max}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>174</td>
<td>388</td>
<td>526, 0.171</td>
<td>520, 2.57</td>
</tr>
<tr>
<td>175a</td>
<td>390</td>
<td>490, 0.533</td>
<td>479, 1.62</td>
</tr>
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<td>175b</td>
<td>410</td>
<td>501, 0.511</td>
<td>505, 0.31</td>
</tr>
<tr>
<td>175c</td>
<td>397</td>
<td>513, 0.536</td>
<td>528, 0.34</td>
</tr>
<tr>
<td>175d</td>
<td>388</td>
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<td>520, 0.69</td>
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<td>175e</td>
<td>396</td>
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<td>398</td>
<td>534, 0.298</td>
<td>541, 1.24</td>
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<td>175g</td>
<td>394</td>
<td>537, 0.234</td>
<td>531, 2.37</td>
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<td>175h</td>
<td>402</td>
<td>541, 0.201</td>
<td>531, 0.04</td>
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<td>402</td>
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<td>175j</td>
<td>389</td>
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<tr>
<td>175m</td>
<td>422</td>
<td>612, 0.008</td>
<td>616, 0.10</td>
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</table>
Other light emitters for OLEDs have been developed in recent years. Most of the OLED light emitters have highly conjugated or planar structures (Figure 59). These types of structures produce high photostability and electrostability. It has been proven that metal chelation is not essential for OLED light emitters, non-metal chelating small molecules can also be used in OLEDs.

![Chemical structures of OLED light emitters](image)

**Figure 59** Structure of some other OLED light emitters which been developed recent years.

### 1.4.1.2 Luminescent solar concentrator

A luminescent solar concentrator (LSC) is a device which can concentrate solar radiation and convert the radiation to electricity. LSC operates by collecting radiation energy over a large area and redirecting the collected energy to a smaller target.

The structure of a typical LSC can be described as follows (Figure 60). The incoming sunlight penetrates through the top surface and enters the waveguide (plastic or glass). Fluorescent molecules which are embedded in the waveguide (a layer on either the top or the bottom of the waveguide) absorb the incoming light
and re-emit it. The re-emitted light has a longer wavelength and is guided to the end of waveguide by total internal reflection. The photovoltaic material attached to the end then converts the light to electricity.

![Diagram](image.png)

**Figure 60** Structure of typical luminescent solar concentrator. The green arrow is the incoming sunlight while the red arrows are reflected re-emitted light.

Various fluorescent molecules are being used in LSCs e.g. small organic molecule fluorescent dyes, inorganic phosphors and quantum dots.[119] For a LSC, an effective luminophore must meet the following requirements:

- Broad spectral absorption (to cover the sun light spectrum as much as possible)
- High absorption efficiency
- Large Stokes shift (to avoid re-absorption)
- High quantum yield
- Good solubility in matrix

Since the first paper on LSC, organic dyes have been considered as a good choice for luminescent materials in LSCs. Compared to inorganic phosphors and quantum dots, organic dyes have excellent solubility in an organic environment, good fluorescence yields and large absorption coefficients. Several organic dyes have been widely used in LSCs (Figure 61).
However there are some limitations in using organic dyes as the LSC luminophore. One is photostability. Seybold et al. have conducted research on the photodecomposition of polymethylmethacrylate (PMMA) dyes under different conditions. It was observed that the photodecomposition of PMMA is greater in oxygen than in an inert atmosphere.

Another disadvantage of organic dyes is the limited breadth of their absorption spectrum. It can lead to insufficient absorption of solar energy. To avoid this disadvantage, combinations of different organic dyes are often used, in order to cover more breadth of sun light spectrum.

Figure 61 Some organic dyes used in LSC.

Figure 62 Absorption and emission spectra of different fluorescent dyes that can be combined in one LSC. Using different fluorescent dyes leads to broader absorption of the sunlight spectrum (375 – 750 nm).
1.4.1.3 Fluorescent penetrant inspection

An important use of these dyes is for the study of defects on aircraft metal. Defects on the surface may compromise the quality of the material or disrupt its integrity, which could lead to catastrophic stress failure. Fluorescent penetrant inspection (FPI) is a typical method of detecting defects (Figure 63). Although there are several ways which are more accurate and precise, due to the low cost and simple process, FPI is still widely used.

![Figure 63](image-url) Photograph of a steel component under UV light during FPI process. Rhodamine 123 in water is used as the penetrant. The crack can be easily observed

To conduct a FPI, a fluorescent dye is applied to the surface of the material to be inspected. The penetrant infiltrates into the defects or cracks on the surface. After the excess penetrant has been removed, defects or cracks will be readily observed using specific excitation methods (e.g. UV or laser).

After the FPI, the penetrant is thoroughly removed to prevent further contamination. Water soluble fluorophores, such as Rhodamine dyes and cyanines, are often used as the penetrant since they can be removed in a mild way (washed by water).

1.4.1.4 Fluorescence Flow tracing
A flow tracer is a substance that is used to measure the physical properties of a flowing substance (e.g. direction, velocity). There are various substances that can be used such as radioactive elements or perfluoro compounds. In some cases, small molecules fluorophores are also used.

One common use of fluorescence flow tracers is leak detection. Small leakages of water pipes can be hard to observe, especially when the pipes are in a dark environment. A fluorescent dye can make the leakage conspicuous when applied into the water pipe (Figure 64). Not only water, but pipes for long-chain hydrocarbons[^124] and polyhalogenated hydrocarbon refrigerants[^125] can also be detected in the same way.

![Figure 64](image.jpg)  Water pipe leaking detection using rhodamine dyes as the tracer.

The flow velocity can also be measured using a fluorescent flow tracer.[^126] The structure of velocimetry is shown below (Figure 65). In this technique, a “caged” fluorophore (fluorescence is suppressed) is used. The “caged” fluorophore is first dissolved uniformly in the matrix (flow). It becomes a free fluorophore when exited by a UV laser beam. Once exited the newly formed free fluorophore forms a curved surface, which can be then detected and imaged by the detector/recorder. By calculating different parameters of the curved surface, the velocity of the target flow can be calculated.
1.4.2 Life sciences

1.4.2.1 Macro molecule tracing

Visualization of macro molecules like nucleic acids, enzymes and antibodies is an essential part of biological research. Fluorescent tagging is an important method for visualizing those molecules. In most cases, a small molecule is attached to the target macro molecule, then the whole complex should be visible under light excitation.

A common method of combining target macro molecule and fluorophore is by covalent bond attachment. Most peptides contain nucleophilic side chains such as the thiol groups of cysteines. This side chains can attack fluorophores with pendant modified groups (Figure 66). However due to steric hinderance, which is generated by protein structure, not all proteins can undergo this type of labelling.
Another method of covalent attachment is by using click chemistry. The click reaction of alkynes and azides is highly specific. The protein and fluorophore are modified with an alkyne and an azide respectively in advance. If a terminal alkyne is used in this procedure, a copper catalyst is required for the reaction (Figure 67).\textsuperscript{[128]} However the copper catalyst can disrupt the protein target as it may change the structure of the protein. For this concern, another particular strained alkyne group has been developed.\textsuperscript{[129]}

Metal coordination can also combine the target macro molecule and the fluorophore. A nickel induced coordination tag has been reported.\textsuperscript{[130]} The hexahistidine tag is installed to the protein in advance (Figure 68).
Nucleic acid can also be traced by using fluorescence tagging. It has been reported that both RNA\textsuperscript{131} and DNA\textsuperscript{132} can be tagged with fluorophores, which have been mentioned in previous chapters.

1.4.2.2 Enzymatic activities monitoring

Enzymatic activities in cells can be monitored by fluorescent tagging. Most fluorophores with this function have a “switch” mechanism. The fluorophore is initially fluorogenic/non-fluorogenic, once there are specific enzymatic activities in the cell, the fluorophore is then switched to the opposite state.

Cathepsins are proteases which are found in all animals. There are around 12 members in the cathepsin family. They are distinguished by their structure and they have different catalytic mechanisms for the cleavage of proteins. Cathepsins have been implicated in cancer, ebola disease and Alzheimer’s disease\textsuperscript{133}. 
The cathepsins reporters are fluorophore-quencher molecules (Figure 69), which are non-fluorogenic molecules. Normally the fluorophore and the quencher are jointed together by specific peptide bonds. Once cathepsins are produced in the cell, they will cleave the peptide bond between the fluorophore and the quencher. The fluorophore then become “free” and fluorogenic.

![Figure 69](image)

**Figure 69** An example of a cathepsin detector, showing the peptide bond which is supposed to be cleaved highlighted by arrow.

By changing the peptide sequence, the fluorophore-quencher molecules can be used to monitor different cathepsin family members.

1.4.2.3 Redox probes

Like enzymatic activities, oxidant/reductant species in cells can also be detected by using appropriate fluorophores. The mechanism is similar using “masked” fluorophores that are “unmasked” at a specific stage.

Reactive oxygen species (ROS) are cell signaling molecules which are associated with macrophages. ROS are generated by macrophages during bacterial killing, inflammation and tissue injury.\(^{[133]}\) Hydrogen peroxide (H\(_2\)O\(_2\)) is one of the most important ROS that is related to diseases like cancer. A typical method of detecting H\(_2\)O\(_2\) is using boronic ester modified rhodamine derivatives.\(^{[134]}\) The boronic ester
is oxidized by $\text{H}_2\text{O}_2$ to a boric ester and then hydrolyzed to yield the free rhodamine (Figure 70).

![Mechanism of hydrogen peroxide detection using boronic ester modified rhodamine.](image1)

Figure 70  Mechanism of hydrogen peroxide detection using boronic ester modified rhodamine.

Hydrogen sulfide ($\text{H}_2\text{S}$) plays an important role in cellular signal transduction and intracellular redox status regulation. Due to this fact an increasing number of $\text{H}_2\text{S}$ probes have been synthesized and researched. A highly sensitive $\text{H}_2\text{S}$ probe has been reported (Figure 71).\textsuperscript{135} In this example an azide group that has been preinstalled to a fluorophore can be reduced by $\text{H}_2\text{S}$ to form an amine. This amine can then attack a pendant ester group to unmask the coumarin fluorophore.

![Mechanism of hydrogen sulfide detection using azide modified coumarin.](image2)

Figure 71  Mechanism of hydrogen sulfide detection using azide modified coumarin.
1.5 Oxyluciferin

1.5.1 Bioluminescence reaction of D-luciferin

Oxyluciferin (oxyLH$_2$) is a naturally occurring substance in firefly bioluminescence.$^{[13]}$ It is a highly conjugated molecule which is generated from the oxidation of D-luciferin 196 (LH$_2$) during the bioluminescence reaction. The process can be described as:

\[
\text{LH}_2 + \text{ATP} + \text{O}_2 \xrightarrow{\text{Mg}^{2+}} \text{oxyLH}_2 + \text{AMP} + \text{PPI} + \text{CO}_2 + \text{hv}
\]

First, the substrate D-luciferin interacts with luciferase (Luc) and ATP to form a luciferyl-adenosine-monophosphate complex (Luc-LH$_2$-AMP). Magnesium ions assist this step. The complex is then oxidized by molecular oxygen to form a peroxide intermediate. With the help of AMP (which acts as a leaving group), the peroxide intermediate cyclizes to give a highly strained four-membered ring. Finally, the four-membered ring collapses to yield the excited state of oxyluciferin 197 (oxyLH$_2^*$) and releases carbon dioxide.$^{[13]}$ Detailed mechanisms are shown below (Figure 72). This bioluminescence reaction generates oxyluciferin in its excited state, as the product. The oxyluciferin then relaxes to its ground state, and the excess energy is released in the form of a photon (light).
Figure 72  The detailed mechanism of formation of oxyluciferin (molecules which bind with luciferase enzyme is are shown in red).

The mechanism was confirmed by an $^{18}$O$_2$ feed experiment.\textsuperscript{[137]} The bioluminescent reaction was mimicked \textit{in vitro} and fed with $^{18}$O$_2$ and H$_2^{18}$O separately. C$^{18}$O$^{16}$O was observed in those tubes fed with $^{18}$O$_2$, but absent in those with H$_2^{18}$O. This experiment clarified the source of the oxygen atoms of CO$_2$, which confirmed the reaction mechanism indirectly.

D-luciferin and its synthetic analogues are widely used in bioluminescence imaging (BMI) techniques.\textsuperscript{[138]} The result of several toxicity studies in luciferin-type molecules indicates that D-luciferin and its metabolites (including oxyluciferin) are non-toxic and non-carcinogenic to living cells.\textsuperscript{[139]} Therefore, the oxyluciferin
structure may have the potential to form the basis of novel and non-toxic fluorescent dyes.

1.5.2 Synthesis of oxyluciferin-type molecules

Oxyluciferin 198 has been studied for quite a long time. In 1967, E. White et al. confirmed that the excited state of oxyluciferin was the luminescent species in the bioluminescence reaction of luciferin. After that different analogues of oxyluciferin were synthesized to elucidate the mechanism and character of the bioluminescence.

Due to stability issues (easily enolized), the synthesis of pure oxyluciferin 198 was only reported recently in its enol form 199. The ratio of keto-enol tautomers is solvent dependent, with the enol form always predominating. This fact prevents us from synthesizing pure oxyluciferin for further study.

![Synthetic route of oxyluciferin and its keto-enol tautomerism.](image)

Figure 73  Synthetic route of oxyluciferin and its keto-enol tautomerism.

Compared to oxyluciferin, the dimethyl analogue 203 was synthesized far earlier, in 1972, by N. Suzuki et al. (Figure 74). Since the 5’ carbon was blocked by two methyl groups, the whole molecule could maintain the thiazolone structure which enabled further studies on oxyluciferin.
Studies on the dimethyl oxyluciferin 203 showed that, the fluorescence properties of 203 were related to the ionization of the molecule. Once the phenol group was deprotonated, due to the stronger electron donating effect of the phenoxide ion, the quantum yield was substantially increased.

Several amine analogues of dimethyl oxyluciferin 203 have also been synthesized by an analogous method.\[^{145}\] Compared to dimethyl oxyluciferin 203, these amine analogues 204a-c have red shifted fluorescence (Figure 75). This can be explained by the stronger electron donating effect of the amino substituents reducing the energy of the HOMO.
Figure 75  Synthesis of amino oxyluciferin and their UV absorption and fluorescence spectra in different solvents. 

a. xylene  
b. benzene  
c. CHCl₃  
d. DMSO  
e. acetonitrile  
f. 2-propanol  
g. methanol

By comparing these fluorescent spectra, it could be concluded that the fluorescence is red shifted when a strong electron donating group was attached to carbon 6.

In addition to synthetic studies, some computational work has also been conducted. These studies indicated that oxyluciferin and its analogues have good potential in photochemical applications.[144],[145]
1.6 Project aims and starting point

This project aimed to synthesize oxyluciferin analogues and investigate their properties, especially the fluorescent properties. Further objectives included developing them into fluorescent dyes and applying them to practical imaging cases. The oxyluciferin structure possesses many structural attributes that may make it a versatile and useful fluorophore. For example, both nitrogen and sulfur atoms of the benzothiazole and thiazolone can coordinate to cations such as metals ions and make the molecule a chemosensor. Also, the hydroxyl group can be a linkage between the oxyluciferin molecule and other biological macromolecules such as a protein or enzyme to make the molecule a reporter. The carbonyl of the thiazolone is a hydrogen-bonding acceptor, while the benzothiazole moiety is a planar π system (that maybe suitable for π-π stacking), and these non-covalent forces are excellent for supramolecular research. These facts make the oxyluciferin a versatile target for investigation.

![versatility of the oxyluciferin molecule](image)

Figure 76  Versatility of the oxyluciferin molecule

However, oxyluciferin itself is quite labile. The oxyluciferin (enol) 199 is the predominant species because it is the relatively more stable form (aromatic) compared to oxyluciferin (keto) 198. This tautomerism shortens the π conjugation length of the whole molecule and leads to a decrease of fluorescence emission efficiency.[146]
Therefore, this project set out to made use of 5',5'-disubstituted oxyluciferin analogues. Conversion of the ketone to the iminium group should improve performance, as the iminium ion is a common feature of many fluorescent dyes. The iminium functional group should be more electron withdrawing and may enhance the conjugation of the whole molecule and increase the fluorescence intensity. As the tautomerism of iminium 205 to aromatic enamine 206 can break the conjugated \( \pi \) system and decrease the quantum yield, carbon 5' will be blocked by two alkyl groups to disable the tautomerism.

![Figure 77](image_url) The structure of proposed iminium 205 and its enol tautomer 206.
2. Results and discussion

2.1 Towards iminium oxyluciferin

2.1.1 Synthesis of methoxy ketone 208

The project started with the synthesis of iminium 207 (Scheme 1). Instead of a free hydroxyl group, iminium 207 has a methoxy group, which may make the synthesis easier due to its relative inertness. The two alkyl groups desired to stop enolisation could be methyl groups, but we decided to use two phenyl rings due to easier availability of starting material. The two phenyl groups may also increase the solubility of the molecule in organic solvents. The conversion of ketone 208 to iminium 207 was thought to be straight forward by condensing the ketone with corresponding amine. The cyclisation between thioamide 209 and halogenated carboxylic acid 210 was previously reported by E. Koltai et al.\[147\] Then thioamide 209 could be synthesized by a one-step thioamidation of nitrile 211, of which the preparation has been reported.\[148\]

![Scheme 1](image)

Scheme 1  The retrosynthetic analysis of iminium 207.

The reaction between Appel’s salt 213 and para-anisidine has been described by A. Cuadro et al. in 1994.\[149\] In our hands the reaction gave the imine precursor 214 as the product (Scheme 2). An electrocyclisation and fragmentation process of imine 214 gave nitrile 211.
The 4,5-dichloro-1,2,3-dithiazolium chloride $\text{213}$ (also known as Appel's salt) is a useful reagent in heterocycle chemistry, which was first prepared by R. Appel in 1993.\textsuperscript{[150]} Appel's salt was prepared by chlorination of chloroacetonitrile and cyclisation. Though the detailed mechanism is not clear, a plausible one was proposed by Appel (Scheme 3).

The reaction is initiated by the nucleophilic attack of chloroacetonitrile $\text{212}$ on sulfur monochloride. Once intermediate $\text{215}$ is formed, the chloride ion generated in the previous step removes a proton, which leads to the formation of dichloroacetonitrile $\text{216}$. Concerted chlorine migration and cyclisation give the intermediate $\text{218}$. The aromatization of heterocycle $\text{218}$ gives the Appel's salt $\text{213}$. The mechanism can be proved by formation of large quantity of elemental sulfur and gaseous hydrochloride in practice.
However, the mechanism above involves carbocations which may not be formed under the reaction conditions (room temperature, in CH$_2$Cl$_2$). An alternative imine-enamine tautomerism mechanism has been proposed.$^{151}$ This mechanism (Scheme 4) suggested an oxidation mechanism for the transformation of 221 to Appel’s salt 213.

![Scheme 4 Alternative mechanism of Appel’s salt formation.](image)

Determination of the purity of Appel’s salt was prevented by its air sensitivity and poor solubility in common solvents. The crude Appel’s salt was isolated by filtration. It was reacted with $p$-anisidine to give imine the precursor 214.

The imine precursor 214 was then converted to the corresponding benzothiazole nitrile by an electrocyclization reaction (Scheme 5) under thermal conditions. Collapse of the [6,5,5] tricyclic intermediate 225 is driven by the formation of the aromatic nitrile 211.
The proposed mechanism is inspired by another reaction between anthrone (Scheme 6).

The nitrile 211 was then converted to thioamide 209 in preparation for the final cyclization.\textsuperscript{152} The reagent used in this step was phosphorus pentasulfide. Compared to typical thionation reagents e.g. hydrogen sulfide and Lawesson’s reagent, phosphorus pentasulfide is easier to handle as it is a solid and also relatively odorless. It is believed that phosphorus pentasulfide reacts with ethanol first to form an active sulfur species which then performs the thioamidation
(Scheme 7). The key intermediate ketone 208 was then formed by cyclization with chlorodiphenylacetic acid in 72% yield.\[147\]

Scheme 7  Mechanism of thioamidation, which is followed by cyclization.

2.1.2 Synthesis of dimethylamine ketone 235

Amino derivatives of D-luciferin have been synthesized by S. Miller et al. and their bioluminescence was shown to be red-shifted.\[153\] It indicated that the electron rich substituent (e.g. amine or dimethylamine) on the benzothiazole ring may increase the bioluminescence wavelength. In consideration of increasing the fluorescent wavelength (better penetration through living cells), ketone 235 with dimethylamine group on the benzothiazole ring was proposed and synthesized by an analogous method to ketone 208 (Scheme 8).
The synthesis started with the reaction between commercially available aniline 230 and Appel’s salt. The electrocyclization reaction of imine 231 did not give the expected product 233. Only degraded starting material was observed. The reason could be the increased reactivity of compound 231 caused by the dimethylamine group. Under the harsh conditions (180°C for one hour), decomposition may occur instead of the desired electrocyclization. An alternative two step route, involving a palladium catalyzed C–H functionalisation reaction was adopted. and intermediate 232 was prepared for it, by reducing imine 231 using sodium thiosulphate. This coupling reaction was used to synthesize other 2-substituted benzothiazoles.

The detailed mechanism of the C–H functionalisation reaction in this transformation (232 → 233) is not clear. A possible mechanism could be concerted metalation deprotonation of 236 followed by reductive elimination. This is a general palladium (II) C–H insertion mechanism and requires a stoichiometric oxidant to convert the formed Pd(0) to the Pd(II) required to enter the catalytic cycle again. In this reaction procedure 10 mol% of palladium and only 50 mol% of copper(I) was used. The co-catalyst (copper (I) species) required to regenerate the palladium catalyst (Scheme 9) can only provide another 50 mol% of the Pd(II) catalyst required. This reaction was conducted under an inert atmosphere without any oxygen present so the oxidant can only be copper (I).
The reaction cosolvent is DMSO and it has been reported that DMSO can oxidize Cu(I) species via single electron transfer (SET).\(^{1155}\) The SET mechanism has been confirmed by EPR spectrometry. Based on that observation we propose an alternative mechanism (Scheme 10). The concerted metalation deprotonation is again directed by the thioamide nitrogen. The thiophilic copper (I) species coordinates to the sulfur and is then susceptible to SET oxidation by DMSO to form a copper (III) species. Then transmetallation and reductive elimination steps give the product. This mechanism would then only require a catalytic quantity of Cu(I) and would explain why only 50 mol\% of Cul can lead to complete reaction.
The following thioamidation to 234 and cyclisation to give the target compound 235 proceeded as expected (Scheme 8), while the yields are slightly lower than the corresponding steps in the methoxy ketone routine (Scheme 7).

An encouraging fact is that all the compounds with the dimethylamine group are visibly brighter than their corresponding methoxy derivatives. Based on this fact, it is expected that the stronger the electron-donor attached on the benzothiazole ring, the higher quantum yield of fluorescence.

2.1.3 Synthesis of hydroxyl ketone 243

In order to make an iminium oxyluciferin with a free hydroxyl group, the synthesis of ketone 244 was proposed.

The most direct route involved attempted demethylation of ketone 208. Attempted demethylation of ketone 208 using BBr₃ did not give the hydroxyketone, only decomposition of starting material was observed in the reaction. As there are several heteroatoms in the oxyluciferin structure, unexpected coordination between those atoms and BBr₃ may occur and lead to complex reactions.
Therefore, the methyl group needed to be removed at an early stage. Treatment of 211 with pyridinium chloride gave hydroxynitrile 242 in 82% yield. The hydroxynitrile was used in an analogous synthesis to that of methoxyketone 208. Formation of the thioamide 243 proceeded as before in 87% yield, but the cyclisation reaction did not proceed as expected. Only decomposed starting material was observed. It is possibly caused by the hydroxyl group which can react with acetic anhydride under the reaction condition.

2.1.4 Iminium oxyluciferin synthesis from ketone 208

The iminium synthesis started with direct condensation of an amine and oxyluciferin ketone 208 under thermal conditions, which is a general method for converting ketone to iminium. However the direct condensation did not give the desired product. The starting material was recovered after the reaction.
We were concerned about the steric effect of the diphenyl substituents during the iminium formation. Attack of $\text{Me}_2\text{NH}$ on the carbonyl would necessarily convert the planar $\text{sp}^2$ carbonyl to a tetrahedral intermediate, before elimination of water to give the iminium 207 (Scheme 13). The intermediate 245 should collapse quite rapidly and gave the product once it was formed due to its high steric energy, while the tetrahedral intermediate can be very hard to generate due to steric compression. This fact may be responsible of the failure of the reaction.

After consideration, a Vilsmeier-Haack type reaction was attempted. The Vilsmeier-Haack reaction is used to convert electron-rich aromatic compounds and alkenes to aldehydes, through the Vilsmeier reagent 252. The preparation of the Vilsmeier reagent is shown below (Scheme 14). Other than formylation, the Vilsmeier reagent can also be used in formamidinium synthesis 253.

This strategy would involve converting the ketone 208 to intermediate 255 which is analogous to the Vilsmeier reagent. The electron donating methoxy group could assist in activation of the carbonyl by oxalyl chloride to give intermediate 255. This should be more reactive than ketone 208 and may enable the attack of
dialkylamine to give intermediate 256 which has high steric compression. Two adjacent sp³ centers would make the structure highly unstable and should swiftly collapse to the desired iminium 207, but also makes intermediate 256 itself harder to generate due to the high steric energy.

In practice, the reaction did not work. Most of the starting material was recovered, with a small amount of degradation product. The high steric energy of intermediate 256 probably prohibits its formation.

Scheme 15  The proposed mechanism of Vilsmeier-type reaction.
Since the Vilsmeier-type reaction did not work, another route was proposed. An iminium ion can be also achieved by alkylation of its corresponding imine. Previous attempts indicate that condensation between the ketone 208 with an amine will not work, thus an aza-Wittig reaction was adopted instead (Scheme 16). A Wittig reaction is the reaction between a carbonyl compound and a phosphoylide, an alkene is obtained as product. In an Aza-Wittig reaction the phosphoylide is replaced by phosphoazene ($R_3P=NR'$ where $R$ and $R'$ are both alkyl or aryl) and the product is an imine.[156]

![Scheme 16 Proposed aza-Wittig reaction.](image)

The phosphoazene 258 was prepared by reacting triphenylphosphine with methyl azide (Staudinger reaction)(Scheme 17).[157] The methyl azide was prepared by an ion-exchange reaction, using methyl iodide and azide-modified resin. Due to the moisture sensitivity of phosphoazene 258, it was used directly in the next step without further purification. The presence of phosphoazene was confirmed by using $^{31}$P NMR spectrum (Figure 18).

![Scheme 17 Preparation of phosphoazene 258.](image)
According to the spectrum the major compound in the sample was triphenylphosphine oxide while the phosphaoazine 258 was the minor compound. In the Staudinger reaction, an excess of methyl azide was employed compared to the amount of triphenylphosphine. The triphenylphosphine was recrystallized prior to the reaction. The large quantity of triphenylphosphine oxide probably came from the hydrolysis of phosphaoazine 258.

Theoretically, phosphaozone 258 should attack carbonyl of ketone 208 to give the four-membered ring intermediate 259 which could then rapidly collapse to yield imine 257, which could then be alkylated to give desired 207 (Scheme 18).

Figure 78  $^{31}$P spectrum of phosphaozone 258.
However the aza-Wittig reaction did not proceed as expected, a byproduct 260 was isolated instead. The formation of byproduct 260 could be explained by the attack of phosphoazene 258 to the thiazolone ring as a nucleophile in a conjugation addition reaction. It would seem that the major steric effect of the two phenyl groups could be responsible for the inertness of the ketone to 1,2-addition. The amide anion 261 could then be methylated by excess methyl azide or remaining methyl iodide to give 262 which upon reaction with water would account for the formation of 260 (Scheme 19).

The structure of byproduct 260 was confirmed by NMR spectroscopy. According to the HSQC spectrum, the 13-H is attached to a heteroatom rather than a carbon.
The COSY spectrum indicated a strong correlation between 13-H (quartet) and 18-H (doublet).

![COSY spectrum image]

Figure 79 Partial $^1$H spectrum of byproduct 260.

2.1.5 Investigation of reactivity of ketone 208

Several attempts have been made towards iminium 207, however none of them worked. The carbonyl seems inert. The reactivity of ketone 208 was investigated in order to aid the planning of further modifications.

As mentioned in the previous chapter, oxyluciferin with two adjacent methyl groups 203 has been synthesized by H. Würfel et al.,$^{[158]}$ Their initial purpose was olefination of the carbonyl group. Treatment with isopropylmagnesium bromide gave the conjugation addition product (Scheme 20). This report indicates that the carbonyl is relatively inert compared to the 2'-carbon.
Scheme 20  The conjugate addition between dimethyl oxyluciferin 203 and Grignard reagent.

To further investigate the reactivity of ketone 208, it was treated with several different nucleophiles (Table 3).

Table 3  All nucleophilic modifications been done on ketone 208.

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>Conditions</th>
<th>Yields of 264</th>
<th>Yields of 265</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeN=PPh₃</td>
<td>MeCN, 85°C</td>
<td>0%</td>
<td>72%ᵃ</td>
</tr>
<tr>
<td>Me₂N</td>
<td>THF, 50°C</td>
<td>0%</td>
<td>0%ᵇ</td>
</tr>
<tr>
<td>MeOH</td>
<td>NaOMe (cat.), 0°C</td>
<td>0%</td>
<td>(95%)ᶜ</td>
</tr>
<tr>
<td>MeLi</td>
<td>THF, -78°C</td>
<td>0%</td>
<td>Quant.</td>
</tr>
</tbody>
</table>

ᵃ no starting material was recovered
ᵇ no reaction, with starting material recovered
ᶜ the product is unstable under room atmosphere, it degraded spontaneously

As the table shows, most nucleophiles gave the conjugate addition product. Organolithium species normally favour 1,2-addition to ketones, but in this case exclusive 1,4-addition to ketone 208 takes place. It can be concluded that when ketone 208 undergoes nucleophilic attack, the 5'-carbon is more reactive than the carbonyl.
Another contributory factor that may enhance conjugate addition in this case is the strong electron-withdrawing effect of the 6-methoxybenzothiazol-2-yl group. Evidence has been found in similar compounds that proves its strong electron withdrawing ability. Compound 266 undergoes decarboxylation at room temperature (Scheme 21) due to the strength of the electron-withdrawing group attached to it.

Scheme 21  The decarboxylation of 266, which reaction can occur at room temperature.
2.2 Thiazolopyridine and thiazolopyridinium analogues

2.2.1 Synthesis through condensation cyclisation

Since there was no successful synthesis of an oxyluciferin iminium analogue, another similar structure 268 was proposed (Figure 80). It has a fully planar and aromatic structure which may be more stable compared to oxyluciferin iminium. The positively charged ammonium species can also be stabilized by delocalization. In addition the synthesis would not involve the formation of the iminium ion from the corresponding ketone.

![Figure 80](image)

**Figure 80** Structure of thiazolopyridinium 268 and its resonance structure.

The retrosynthesis was inspired by the synthesis of D-luciferin (Scheme 22). The thiazoline ring of D-luciferin is constructed by cyclisation of the corresponding nitrile and cysteine.\[159]\ The reaction condition is mild due to the high electrophilicity of the nitrile carbon (reason introduced in last chapter).

![Scheme 22](image)

**Scheme 22** Condensation cyclisation step in the synthesis of D-luciferin

Based upon the synthesis of D-luciferin, the retrosynthesis of methoxy thiazolopyridinium 269 was proposed (Scheme 23). The methylation step should be straightforward by stirring in methyl iodide as the reaction solvent. The isolation of the product could be convenient due to the polarity difference between 269 and
As the benzothiazole nitrile 211 is a known compound, the challenging part would be the synthesis of pyridine 271.

![Scheme 23 Retrosynthetic route to thiazolopyridinium 269.](image)

Only one synthetic route to pyridine 271 had been reported.[160] In this route, toxic (KCN) and volatile (′BuSH) reagents were used. The 6-step route, involved many functional group transformations, which contributes to its low atom economy.

![Scheme 24 Reported synthetic route of 2-amino-3-thiopyridine.](image)

To shorten the route and make the synthesis more environmentally friendly, an alternative route was devised and attempted (Scheme 25). The essential step of this route was replacing the introduction of the sulfur by nucleophilic means by introducing the sulfur through an electrophilic method with electrophilic sulfur. It was decided to use tert-butyl group as the protecting group on sulfur so that it could be removed under acidic conditions, which would be the same for the removal of...
the tert-butoxycarbonyl (Boc) group in 278. The sulfur will be introduced through deprotonation that could be directed by the NHBoc group.\[161\]

\[
\begin{array}{c}
\text{SH} & \rightarrow & \text{S}^\text{Bu} \\
271 & & \text{278} & \rightarrow & \text{S}^\text{Bu}^+ \\
& & n\text{-BuLi} & \rightarrow & 279
\end{array}
\]

Scheme 25 Alternative retrosynthesis of pyridine 271.

The deprotonation of protected aminopyridine 279 under general conditions (THF, -78°C, \(n\text{-BuLi}\)) was not successful. Several deprotonation conditions were investigated (Table 4) using quenching with elemental iodine.

\[
\begin{array}{c}
\text{N} & \text{O} & \text{O} & \rightarrow & \text{N} & \text{O} \\
279 & \text{i) n-BuLi, THF} & \rightarrow & 280 & \text{ii) I}_2
\end{array}
\]

\[\text{Table 4 Survey of deprotonation conditions of 2-Bocaminopyridine.}^a\]

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Temperature} & \text{Reaction Time} & \text{Yields of 280} & \text{Recovered 279}\text{b} \\
\hline
\text{-78°C} & 1 \text{ h} & 0\% & \text{Quant.} \\
\text{-78°C} & 2 \text{ h} & 0\% & \text{Quant.} \\
\text{-78°C} & 4 \text{ h} & 0\% & 99\% \\
\text{-40°C} & 2 \text{ h} & 0\% & 98\% \\
\text{-40°C} & 4 \text{ h} & 0\% & 90\% \\
\text{-10°C} & 2 \text{ h} & 10\% & 82\% \\
\text{0°C} & 2 \text{ h} & 51\% & 28\% \\
\text{0°C} & 4 \text{ h} & 70\% & 0\% \\
\hline
\end{array}
\]

\[^a\] 2.5 equivalents of \(n\text{-BuLi}\) was used if not specified.

\[^b\] the recovered yield was calculated without further purification, the reaction mixture was only washed with water, evaporated and dried.

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The deprotonation requires harsher conditions compared to other cases. For a naked pyridine ring, the potential deprotonation position can be proton-2 (kinetic) or proton-4 (thermodynamic). Intrinsically the 3-proton deprotonation may require higher energy to occur.

Since the deprotonation problem was solved, the synthesis moved to the sulfuration. The first sulfur electrophile that was used was di-tert-butyl disulfide (Scheme 26).[162] However, the reaction did not work as expected. The starting materials were recovered without any reaction.

The di-tert-butyl disulfide could be too hindered (tert-butyl group) to be reacted. Also the tert-butylmercaptyl group is a good but not excellent leaving group ($p$Ka [‘BuSH] = 11.22). To enhance the reactivity and possibly reduce the steric hinderance, another sulfur electrophile 282 was used.[163] The phthalate would be a better living group ($p$Ka [phthalimide] = 8.3), but the addition of the aryl anion did not work.

\[\text{Reaction between aminopyridine 279 and sulfur electrophiles.}\]
A thiol synthesis that used elemental sulfur has been reported\textsuperscript{[164]}\textsuperscript{[164]}. The reactivity of elemental sulfur as an electrophile is believed to be similar to di-tert-butyl disulfide, with less steric hinderance. Treatment of the pyridine anion 283 with elemental sulfur gave the polysulfur intermediate 284 (Scheme 27). The crude intermediate 284 was then readily reduced with sodium borohydride. After an acidic work up the desired amino pyridine thiol 271 was isolated.

\begin{center}
\includegraphics[width=\textwidth]{scheme27.png}
\end{center}

Scheme 27 Sulfuration of pyridine 279 using elemental sulfur as the electrophile.

It was observed that amino thiol pyridine 271 was oxidized rapidly to the disulfide by exposure to the atmosphere. It also decomposed during column purification, even when the amino group was protected. Therefore, amino thiol pyridine 271 was applied to the following cyclisation step without any further purification.

\begin{center}
\includegraphics[width=\textwidth]{scheme28.png}
\end{center}

Scheme 28 Attempted cyclizations on nitrile 211.
The cyclization step simply mimics the synthesis of D-luciferin. The reaction did not work under the normal conditions used for the formation of the thiazoline, with starting material being recovered (Scheme 28). The pyridine ring is electron-withdrawing and can reduce the nucleophilicity of the pendant amine group. The reaction was then repeated at 60°C. There was a compound generated however it was not the product. The new compound was elucidated to be the amide 285. It suggested that water was present in the reaction system and possibly that hydrolysis was more facile than attack by the amino thiol. The reaction was repeated after thorough removal of water. The amide did not appear, and unfortunately neither did the product.

Another attempt was made on the cyclisation reaction, by disconnecting the other benzothiazole molecule (Scheme 29). The thioaniline 286 should be relatively more nucleophilic compared to thioaminopyridine 271. The synthesis of nitrile 287 has been reported, using an analogues synthesis to the corresponding benzothiazole nitrile.

![Scheme 29 Retrosynthesis through an alternative condensation cyclisation.](image)

The synthesis started by treating 2-amino-3-bromopyridine with Appel’s salt (Scheme 30). The imine 289 was obtained in a moderate yield of 45%. However, the following cyclisation step did not proceed as planned, giving a complicated mixture of byproducts and some starting material.
Due to the difficulties of these reactions other synthetic routes were devised and investigated.

2.2.2 Convergent synthesis via $S_NAr$ substitution

Since cross coupling reactions are a general way of making poly aromatic compounds, another route towards thiazolopyridine 270 was proposed (Scheme 31). Compared to the linear synthesis in the last section, this route would be more convergent. Since benzothiazole 267 is a known compound and the cross coupling between benzothiazole and an aryl halide has been reported\textsuperscript{[166]}, the key to this proposed route was the synthesis of thiazolopyridine 290.

Generally for aromatic cross coupling reactions, aryl bromides and aryl iodides are suitable substrates. They can be prepared via a Sandmeyer reaction on aniline derivatives. A one-pot synthesis of amine 291 has been reported (Scheme 32).\textsuperscript{[167]} By reacting aminopyridine 293 with thiocyanic acid (generated in situ by reaction between isothiocyanate and strong acid), the thiourea 292 is generated. The cyclisation is induced by elemental bromine (Scheme 32)
The sulfur atom of thiourea attacks elemental bromine to give a sulfonium ion, which is highly reactive and induces an electrophilic substitution on the pyridine ring. In general cases, pyridine is not favored in electrophilic substitution. However, with the intramolecular assistance of the thiourea group which is electron rich, this reaction may occur. Driven by the force of rearomatization, the amine 291 can be achieved. Unfortunately the reaction did not work as expected. The thiourea intermediate 292 was successfully isolated, but the subsequent cyclisation could not be completed under the reported conditions. The reason for the failure is still unclear, but one possible cause is the lack of nucleophilicity of thiourea 292.

To continue the synthesis, the preparation of other thiazolopyridine compounds needed to be considered. The only reported 2-halogenated thiazolopyridine compound is 2-chlorothiazolopyridine 294.\textsuperscript{[168]} It was synthesized via chlorination of thiazolopyridine 295. The polysulfur heterocycle 295 was generated by treating pyridine 296 with potassium ethyl xanthate (Scheme 33). Since 294 is an electron-poor heterocycle (pyridine-type structure with electron-poor thiazole ring) and the leaving group (chlorine) position is in conjugation with the pyridine nitrogen (which can help in stabilizing negative charge), it should favor nucleophilic aromatic substitution reactions (\textit{S}_{\text{N}}\textit{Ar}). In line with expectations, substitution of oxygen-, sulfur- and nitrogen-based nucleophiles have been reported.\textsuperscript{[169]} However, there is no report of using carbon based nucleophiles.
Scheme 33  Synthesis of chlorinated thiazolopyridine 294 and the reported S$_n$Ar substitution reactions.

The deprotonation of benzothiazole is readily achieved as the pKa is around 29 and this could act as a suitable nucleophile for reaction with 294.$^{[170]}$ Deprotonation of 267 with n-BuLi and then treatment with 294 gave a moderate 31% yield of the desired coupled product 270 (Scheme 34). One possible reason for the low yield is that the generated nucleophile acts as a base rather than a nucleophile.

Scheme 34  Synthesis of 270 via S$_n$Ar substitution and the possible side product.

One possible reason of the low yield is the nucleophilic substitution of n-BuLi. Comparing to s-BuLi and t-BuLi, n-BuLi is slightly less basic however more nucleophilic. That’s because the n-butyl group is less steric hindered compared to other butyl groups with side chains. This hypothesis is not sufficiently evidenced
since the side product 298 has not been observed nor isolated.

Another possible reason which could be responsible for the low yield is formation of the corresponding isonitrile from the metallated benzothiazole species. A classic method of making isonitrile compounds is from the deprotonation of the corresponding benzoxazoles (Scheme 35).[171] There is a chemical equilibrium between benzoxazole anion and the isonitrile phenoxide. The phenoxide 300 can be trapped by other electrophiles and push the equilibrium to the isonitrile side.

With the benzothiazole 267, there is also the possibility of forming the thiolate isonitrile intermediate 301. Similar to phenoxide 300, thiolate 301 can be achieved via deprotonation of benzothiazole 267. However no product of the electrophile trapped thiolate was isolated, so the detailed reason of the low yield of the SnAr reaction is still unclear.

![Scheme 35](image)

**Scheme 35**  Isonitrile synthesis via deprotonation of benzoxazole and the possible side product of deprotonation of benzothiazole.

To further investigate the reactivity of chlorothiazolopyridine 294, several reactions have been conducted (Scheme 36). As suspected, the cross coupling between thiazolopyridine 294 and benzothiazole 267 was unsuccessful. Most probably the chlorine atom is too inert to undergo oxidative addition by palladium (0). The transformations of 294 to Grignard reagent and organozinc reagent are were also unsuccessful.
It can be deduced that thiazolopyridine 294 is unreactive to most of the reactions that normal halogenated arenes are susceptible to, except nucleophilic substitution.

![Scheme 36](image)

2.2.3 Dimethylamine analogues

To broaden the substrate scope, analogue 304 with a dimethylamino substituent instead of a methoxy group was proposed (Scheme 37). The synthesis route is analogous to the route to the methoxy analogue (Scheme 34).

![Scheme 37](image)

The synthesis of dimethylaminobenzothiazole 306 has been reported (Scheme 38). The selective nitration of benzothiazole proceeded in good 84% yield. There are four possible products of the nitration. It has been reported that 6- and
7-nitrobenzothiazole are two main isomers.\cite{173}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Scheme_38.png}
\caption{Reported synthesis of desired benzothiazole 306.}
\end{figure}

Generally selective nitration is achieved by the directing effect of specific substituents on the ring, as the groups can stabilize or destabilize the positive charge that generated during the nitration progress. For example, anilines favor ortho- or para-nitration, while benzonitrile favors meta-nitration as it decreases the electron density of the ortho and para positions (Scheme 39).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Scheme_39.png}
\caption{Different nitration product from different substituted benzenes.}
\end{figure}

However, for benzothiazole 307, there are no substituents on the ring. One possible origin of the selectivity is the inductive effect of the nitrogen atom. The high electronegativity of nitrogen makes it electron withdrawing and deactivates the aromatic system. The farther the distance to the nitrogen atom is, the weaker the electron withdrawing effect will be. The 6- and 7-positions are two farthest positions and they will be favored in the nitration.
The reduction of 6-nitrobenzothiazole gave 6-aminobenzothiazole in moderate 45% yield (Lit.\textsuperscript{172} yield 91%). The reaction used an iron reductant which resulted in a large quantity of iron salt. The work up procedure was difficult and complicated. Acidification and basification also caused unexpected loss of the product. The subsequent methylation was extremely low yielding as well, with less than 3% of product isolated (Lit.\textsuperscript{172} yield 22%). The lone pair of the amine group is in conjugation with the aromatic ring so its nucleophilicity is reduced. This phenomenon has been observed in the methylation of aniline. Since the benzothiazole is more electron poor than the benzene ring, the poor yield was not surprising.

An alternative synthesis of dimethylamine benzothiazole 306 has been adopted (Scheme 40).\textsuperscript{174}

![Scheme 40 Alternative synthesis of benzothiazole 306.](image)

The synthesis started with the oxidation of the electron rich aniline 310 by potassium dichromate to a reactive iminoquinone intermediate (Scheme 41). The intermediate was then attacked by thiosulfate anion to form a poly sulfur compound which readily self-aromatized (by imine-enamine tautomerism) to form thiosulfonic acid.
acid 311 in 40% yield (Scheme 41). Compound 311 then condensed with formic acid under the acidic conditions to give the final benzothiazole 305 in 73% yield.

Compared to the nitration/reduction/methylation synthesis, this route is one step shorter. It also avoided using of transition metal reductant which was hard to handle during the work up stage. The low yield and unfavorable methylation step was also bypassed.

With the desired benzothiazole 306 in hand, the next step of deprotonation/substitution was attempted. Similar to methoxy thiazolopyridine 270, the reaction proceeded as expected, but in a similar moderate 35% yield (Scheme 42).
The purification of both methoxy thiazolopyridine 270 and compound 305 was complicated by their insolubility in most solvents except DMSO. This, coupled with the low yield, would make the synthesis of large quantities of these materials challenging.

2.2.4 “Para” analogues

To broaden the substrate scope and investigate the effect of heteroatoms on fluorescence, two analogues with slightly different structures were proposed (Figure 82).

![Figure 82 Analogues with different position of pyridine nitrogen.](image_url)

In the new structures the pyridine nitrogen has been moved from the ortho-position to the para-position. These analogues could be made by following a similar synthetic route and would require the synthesis of 4-chlorothiazolopyridine 318. This has been derived from the thiazolopyridine 317. Repetition of the reported synthetic route was successful and proceeded in similar overall yield to 294 (Scheme 43).[175]

![Scheme 43 Synthesis of thiazolopyridine 294.](image_url)
The results of deprotonation/substitution reactions were also similar to previous cases (Scheme 44). Since the 2-chloro and 4-chloro thiazolopyridine have similar reactivity (both leaving group in conjugation with pyridine nitrogen), the yields were nearly identical.

![Scheme 44 Synthesis of thiazolopyridine 314 and 315.](image)

### 2.2.5 Methylation of the thiazolopyridines

In order to complete the synthesis of the four thiazolopyridine analogues the selective methylation of the pyridine nitrogen had to be achieved. The selective methylation of pyridine nitrogen has been reported and proceeds under milder conditions than that reported for the alkylation of a benzothiazole nitrogen (Scheme 45).[176]

![Scheme 45 Reported selective methylation of thiazolopyridine and benzothiazoles.](image)
For thiazolopyridine structures, the pyridine nitrogen is preferred to be methylated rather than the thiazole nitrogen. The methylation condition of benzothiazole is also harsher than the condition of thiazolopyridine. The detailed reason is not clear. Based on this fact, the methylation of thiazolopyridine analogues should have been straightforward.

Using dimethylsulfate as the methylating reagent has some disadvantages. It is highly reactive which makes it hard to handle and it is highly toxic (disrupts human DNA). An alternative methylating reagent, methyl iodide, was adopted. The methylation reactions were listed below (Table 5):
Table 5  Methylation reactions of thiazolopyridine compounds.  

\[
\begin{array}{c|c|c}
\text{Starting material} & \text{Product} & \text{Yields} \\
\hline
\text{270} & \text{269} & 76\% \\
\hline
\text{314} & \text{312} & 79\% \\
\hline
\text{305} & \text{304} & 81\% \\
\hline
\text{315} & \text{313} & 81\% \\
\end{array}
\]

\textit{a} reaction condition: MeI (as reagent and solvent) with starting material concentration of 0.01 M, room temperature, N\textsubscript{2}, 48 h

The reactions were extremely dilute (~0.02 M) since all of the thiazolopyridines have poor solubility in methyl iodide. The workup procedure was convenient and simple, requiring only evaporation. Since the thiazolopyridiniums are all ionic which made the column chromatography purification impossible, the crude products were washed with ethyl acetate, which removed any traces of starting material to leave pure products.
2.3 Pyridine, pyridinium and extended conjugation analogues

2.3.1 Pyridine analogues

D-luciferin 196 analogues have been researched for many years. Most of the analogues possess the benzothiazole-thiazoline core structure which is same to D-luciferin. However, some analogues where the benzothiazole moiety was replaced by other aryl groups have good bioluminescence (Figure 83).[177]

![Figure 83 D-luciferin and its artificial analogues, with their emission wavelengths.](image)

In a similar way, modifications of the thiazolopyridine and thiazolopyridinium compounds from the last section could lead to structures which may still possess good optical properties. Inspired by the D-luciferin analogues (Figure 83), pyridinium compounds 326-329 were proposed (Figure 84). Mimicking the compounds 323 and 325, the heteroaromatic thiazole was removed to give pyridinium analogues. It was anticipated that the syntheses may also be easier.
The retrosynthesis of the general pyridinium analogue is shown below (Scheme 46). The cross-coupling reaction on the 2-position of benzothiazole has been reported.\[178\] As the benzothiazoles and bromopyridines are known compounds, the synthesis was thought to be relatively straightforward.

The reaction mechanism for the C-2 C-H functionalization of benzothiazole is not clear, but it is believed to be similar to the Sonogashira coupling. The proposed catalytic cycle is shown below (Scheme 47):
The cross-coupling reaction of four pyridine analogues was successful (Table 6). The synthesis then only required methylation of the pyridine nucleus.
Table 6  Cross-coupling reaction between benzothiazoles and bromopyridines.\textsuperscript{a}

![Chemical structure and reaction conditions]

<table>
<thead>
<tr>
<th>Benzothiazole</th>
<th>Bromopyridine</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="267" alt="Chemical structure" /></td>
<td><img src="330" alt="Chemical structure" /></td>
<td><img src="332" alt="Chemical structure" /></td>
<td>76%</td>
</tr>
<tr>
<td><img src="267" alt="Chemical structure" /></td>
<td><img src="331" alt="Chemical structure" /></td>
<td><img src="333" alt="Chemical structure" /></td>
<td>59%</td>
</tr>
<tr>
<td><img src="306" alt="Chemical structure" /></td>
<td><img src="330" alt="Chemical structure" /></td>
<td><img src="334" alt="Chemical structure" /></td>
<td>70%</td>
</tr>
<tr>
<td><img src="306" alt="Chemical structure" /></td>
<td><img src="331" alt="Chemical structure" /></td>
<td><img src="335" alt="Chemical structure" /></td>
<td>62%</td>
</tr>
</tbody>
</table>

\textsuperscript{a} reaction conditions: PdCl\textsubscript{2} (1 mol\%), Cu(OAc)\textsubscript{2} (20 mol\%), PPh\textsubscript{3} (0.5 equiv.), K\textsubscript{2}CO\textsubscript{3} (4 equiv.), PhMe, reflux, 4-6 h.
2.3.2 Methylation of pyridine analogues

The methylation reaction of the four pyridine compounds was attempted under the same conditions as for the methylation of thiazolopyridines. The direct methylation of the para-methoxyl pyridine was successful (Scheme 48).

![Scheme 48 Methylation of pyridine analogues.](image)

The methylation of 332 did not give the desired product, only starting material was recovered. It can be caused by the steric hinderance.

However, when the methylation was attempted on compound 334, no desired product was observed. Instead only byproduct anilinium 336 was obtained in moderate yield (Scheme 49).

![Scheme 49 Methylation of dimethylamine pyridine 334 and the byproduct.](image)

According to the results of the previous methylation reactions (Scheme 48), it can be concluded that the pyridine nitrogen has good nucleophilicity. The reason for the failure to form 328 could be that steric hinderance just tips the balance to
making the methylation of the pyridine more difficult with respect to the methylation of the dimethylaniline. Also, the reaction is conducted under room temperature (kinetic condition).

An alternative synthetic route to make the desired dimethylamine pyridinium compounds by a condensation cyclisation reaction that involved the previously prepared thiosulfonic acid 311 was adopted (Scheme 50). The methylation of pyridine aldehydes has been reported.[179] The desired pyridiniums were obtained in relatively low yields by this route.

![Scheme 50 Synthesis of dimethylamine pyridiniums.](image)

2.3.3 Synthesis of pyridiniums of extended conjugation

For in vivo imaging (fluorescent tagging or bioluminescence imaging), care has to be taken when selecting the fluorophore because cells and tissues can absorb light with certain wavelengths. The absorption spectrum of a mammal’s body is shown below (Figure 85). As mentioned earlier, D-luciferin bioluminescence is a well-known and convenient in vivo imaging technique. The emission wavelength of D-luciferin 196 is 560 nm (green-yellow light), in which region mammal tissues have strong absorbance. It has become the major limitation of this imaging method. A synthetic analogue, infraluciferin 341 has been reported.[180] It has an emission wavelength of 706 nm, which falls in the near infra-red region. The near infra-red
absorbance of mammal tissues is dramatically weaker than the absorbance of green-yellow light. The reason for the red-shifted emission wavelength is believed to be due to extended molecular \( \pi \)-conjugation.

![Absorption Spectrum](image)

**Figure 85** The absorption spectrum of mammal tissues, D-luciferin and infraluciferin.

In order to achieve longer emission wavelengths of our target fluorophores, a pyridinium analogue \( 342 \) with extended \( \pi \)-conjugation structure has been proposed (Scheme 51). The proposed insertion of an alkene group between the two aromatic moieties is similar to infraluciferin. The desired pyridinium can be synthesized via a Knoevenagel condensation with a methyl pyridinium species (Scheme 51). The aldehyde \( 343 \) can be synthesized by deprotonation of a corresponding benzothiazole and addition of dimethylformamide as previously formed.

![Scheme 51](image)

**Scheme 51** Retrosynthesis of \( \pi \)-extended pyridinium species.
The synthesis proceeded as expected. The results are shown in the table (Table 6):

Table 6 Synthesis of extended conjugation pyridiniums.\(^a\)\(^b\)

\[\text{Benzothiazole \quad Aldehyde \quad Methylpyridinium \quad Final}\]

<table>
<thead>
<tr>
<th>Benzothiazole</th>
<th>Aldehyde</th>
<th>Methylpyridinium</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>[\text{267}]</td>
<td>[\text{344, quant.}]</td>
<td>[\text{346}]</td>
<td>[\text{348, 92%}]</td>
</tr>
<tr>
<td>[\text{267}]</td>
<td>[\text{344, quant.}]</td>
<td>[\text{347}]</td>
<td>[\text{349, 91%}]</td>
</tr>
<tr>
<td>[\text{306}]</td>
<td>[\text{345, 91%}]</td>
<td>[\text{346}]</td>
<td>[\text{350, 88%}]</td>
</tr>
<tr>
<td>[\text{306}]</td>
<td>[\text{345, 91%}]</td>
<td>[\text{347}]</td>
<td>[\text{351, 88%}]</td>
</tr>
</tbody>
</table>

\(\text{\(a\) formylation reaction conditions: } n{\text{-BuLi}} (1.5 \text{ equiv.}), \text{THF (dry), -78°C, 1.5 h; then DMF (5.0 equiv.), THF, overnight}\)

\(\text{\(b\) Knoevenagel condensation conditions: methylpyridinium iodide (1.0 equiv.), piperidine (cat.), EtOH, reflux, overnight}\)

In conclusion, four extended conjugation pyridinium have been made with straight forward and simple reactions.
2.4 Optical properties

2.4.1 General discussion

The optical data (absorptions, emissions, extinction-coefficients and quantum yields) of all analogues has been measured. To have a direct overview, the compounds structures with their emission wavelength are shown in a visible light spectrum (several compounds without any solvent state fluorescence are not shown in the spectrum) (Figure 86).

The emission of compounds that been synthesized in the project have nearly covered the whole visible light spectrum, from violet to red even near infrared. They exhibit different glowing colours (emission) due to their different structures.

For the same core structure, a dimethylamine group can move the emission wavelength to a redshift of about 100 - 150 nm, compared to a methoxy group. The methylation of pyridine nitrogen also lengthens the emission wavelength by 100 nm.

As expected, installation of an alkene group between the benzothiazole and the pyridinium moiety also redshifts the emission wavelength (up to 100 nm). There is around 100 nm difference in emission wavelength, between those thiazolopyridines and corresponding pyridine analogues.

With this information in hand, it is possible to propose an analogue with desired emission wavelength, by combining different moieties and installing different substituents.
Figure 86  Structure with their compound numbers and emission wavelength shown in visible light spectrum.
2.4.2 Fluorescence quenching and aggregation induced emission (AIE)

During the measurement of optical data, some compounds gave no fluorescence in their solution-state (Figure 87). For those compounds the absorption spectrum looked normal, but the fluorescence spectrum showed nearly no emission at all.

![Figure 87 Two analogues without solvent-state fluorescence.](image)

A conjugated structure is essential for a molecule to emit light under certain excitation. Disruption or removal of conjugation leads to absence of fluorescence. Conjugated structures require all the unsaturated carbons and heteroatoms to occupy the same plane. Hence, non-planar structures have less conjugation resulting in reduced or no fluorescence.

For these compounds, we propose a possible explanation for fluorescence quenching (Figure 88). The methylation of the pyridine nitrogen creates steric inhibition of resonance due to severe non-bonded between the pendant aromatic group (328 benzothiazole) in the ortho position. Upon excitation, the molecule releases the excess steric energy by orienting itself to a non-planar conformation, which can be considered as a non-radiative decay pathway.

![Figure 88 Possible reason of the absence of solvent-state fluorescence.](image)
A similar phenomenon has been observed in tetraphenylethene (TPE) type molecules. A study focusing on the reason behind AIE was conducted by N. Tseng et al.\textsuperscript{[182]} The absence of solvent state fluorescence was believed to be caused by the following mechanism (Figure 88). Similar to previously proposed mechanisms, the rotation of the alkene bond is responsible for the lack of fluorescence resulting in an $E$-$Z$ isomerization. Hence, a direct way to confirm the mechanism is to determine the $E:Z$ ratio before and after excitation.

![Figure 88 Proposed mechanism of absence of solvent state fluorescence of TPE.](image)

To confirm the hypothesized mechanism, 1,2-diphenyl-1,2-ditolyethene (DPDTE) \textbf{353} was synthesized (Scheme 52). The $Z:E$ ratio of the synthesized DPDTE was 93:7.
The fluorescence of DPDTE was measured (Figure 89) and stilbenes were used for comparison. The DPDTE was initially dissolved in THF and exhibited no fluorescence. By the addition of water to the solution (water fraction was increased gradually), the DPDTE started to precipitate from the solution and fluorescence was observed. The quantum yield increased dramatically in 100% water. This data confirms that DPDTE does show AIE phenomenon.

![Graph showing fluorescence quantum yield versus water fraction](image)

**Figure 89** Emission spectrum of synthetic 353.

After the sample had been exposed to light excitation, $^1$H NMR data was then recorded (Figure 90). The spectra showed that the $E:Z$ ratio of the sample had not changed after exposure to the light excitation of the fluorimeter. To investigate further the origin of the change in the $E:Z$ ratio, the sample was sequentially exposed to a strong UV light source. The configurational change was then observed.
Figure 90  Proton spectra of DPDTE after [A] synthesized; [B] exposed to fluorescence spectrometer and [C] exposed to a UV light for 14 mins.

It has been confirmed that, although intramolecular rotation is highly possible when AIE occurs, the E:Z conformational change does not necessarily have to occur during the excitation.

2.4.3 Large Stokes shifts

The emission wavelength of a molecule is always longer than wavelength of the electronic excitation. The difference in two wavelengths is called the Stokes shift, named after the Irish physicist George Gabriel Stokes. The red-shift of the emission wavelength is caused by energy lost during the excitation-emission process. In this project, a few compounds have significantly large Stokes shifts (>150 nm). The data is shown below (Table 7):
Table 7  Absorption, emission and Stokes shift of selected compounds.$^a$

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$\lambda_{\text{abi}}\text{max}$ (nm)</th>
<th>$\lambda_{\text{emi}}\text{max}$ (nm)</th>
<th>Stokes shift (nm)</th>
<th>Quantum yields (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Structure 329]</td>
<td>486</td>
<td>692</td>
<td>206</td>
<td>3.4</td>
</tr>
<tr>
<td>![Structure 304]</td>
<td>528</td>
<td>726</td>
<td>198</td>
<td>6.7</td>
</tr>
<tr>
<td>![Structure 313]</td>
<td>519</td>
<td>717</td>
<td>198</td>
<td>8.1</td>
</tr>
<tr>
<td>![Structure 348]</td>
<td>402</td>
<td>570</td>
<td>168</td>
<td>24.6</td>
</tr>
<tr>
<td>![Structure 349]</td>
<td>394</td>
<td>561</td>
<td>167</td>
<td>4.6</td>
</tr>
<tr>
<td>![Structure 350]</td>
<td>487</td>
<td>747</td>
<td>260</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$ absorption data is collected in DMSO, 25°C under a concentration of 10 μM; emission data is collected in DMSO, 25°C under a concentration of 1 μM
Although all kinds of energy loss can lead to a Stokes shift, there are several mechanisms.

The energy loss caused by the vibrational shifts (due to displacement of the potential energy curve in the excited state with respect to the ground state) is relatively small (around 70 nm) in general. However, if the Stokes shift is larger than 150 nm, it is necessary to consider other mechanisms.

One possible cause of the large Stokes shift is the twisted intramolecular charge transfer (TICT). TICT is a process of electron transfer that happens upon the molecule being photoexcited. The molecule that favors TICT usually consists of a donor and acceptor pair. To enable the TICT, the donor and acceptor must be in conjugation with each other and have been connected with each other by a free rotational single bond. For example, fluorophores that been synthesized in this project are excellent models for TICT to occur.

Once the molecule is photoexcited, the electron can be transferred from the donor to the acceptor. At the meantime, the conformation of the molecule is twisted (Figure 88) which process is energy consuming. The actual photon emission is from the twisted excited molecule rather than the original excited molecule (untwisted). Comparing to the direct decay from the original excited state to the ground state, this twisted decay emits light with a more redshifted wavelength. The emissive light becomes more redshifted as the distance between the donor and acceptor grows.

The emission properties that relate to TICT are highly environmental dependent, which makes TICT-base fluorophores ideal probes for solvents or other microenvironments.
2.5 Application exploration: macro-molecule tag

To investigate the potential of the fluorophores in biological experiments we investigated their use in tagging a macro-molecule. The bioconjugation of one of the new fluorophores with trastuzumab, a monoclonal antibody used to cure HER2 positive breast cancer, was attempted.

![Compounds](image)

**Figure 91** Compounds that were chosen for the bioconjugation and their pyridinium analogues.

Two fluorophores motifs 335 and 315 were chosen as candidates since their pyridinium analogues exhibited long emission wavelengths which would lead to better penetration (Figure 91). Instead of a methyl group, a 4-butylazide group was installed to the molecule so that a bioconjugation ‘click’ reaction could be investigated (Scheme 53). The butyl azide was installed using a similar alkylation reaction to the previous methylation. Compared to the methylpyridiniums 329 and 313, 354 and 355 showed similar emission wavelengths.

![Scheme](image)

**Scheme 53** Synthesis of azide modified fluorophores.
From here on the work was performed by Dr. Calise Bahou in a collaboration with the Dr. Chudasama’s group. The trastuzumab was connected to a PD-linker in advance (Scheme 54), via cysteine residues of trastuzumab. The PD-linker was connected to the fluorophore through the reaction of the azide with a strained alkyne group. The strained alkyne encourages rapid cyclization between azides under copper-free conditions. The click reaction was successful.

![Scheme 54 Synthesis of trastuzumab-fluorophore complex.](image)

After the bioconjugation reaction, the optical data of trastuzumab-fluorophore complexes were recorded (Figure 92) in water. It has been observed that the fluorescence of 354 and 355 were not affected by the antibody ($\lambda_{max}^{Trastuzumab-354} = 682$ nm, $\lambda_{max}^{Trastuzumab-355} = 711$ nm). According to the emission spectra, the complexes still have emission in the region above 800 nm which would be very convenient for in vivo imaging.
To test the bioavailability of the trastuzumab-fluorophore complex, an enzyme-linked immunosorbent assay (ELISA) was conducted. This method tests the binding affinity between trastuzumab-fluorophores complexes and HER2 receptor (the target of trastuzumab). In this case, the aim of ELISA is to determine if the trastuzumab-fluorophore complexes can still be recognized. The result was encouraging: installation of the fluorophore to the trastuzumab did not affect its bioavailability (Figure 93).
Based on the results above, a new method of trastuzumab labelling has been developed. It can be expected that the method can be used in all monoclonal antibody labelling. For comparation, the next is to do the same bioconjugation test against those commercially available fluorophores.
3. Conclusion

In this project, several synthetic routes towards iminium ion analogues of oxyluciferin have been attempted, however, none of them proceeded as expected. The extreme steric hindrance around the thiazolone carbonyl group coupled with the strong electrophilicity of the thiazolone 2'-carbon, steered the reaction towards a more favourable conjugate addition.

In the light of this result, a second generation design of fluorophores was suggested, based upon some pyridine, pyridinium, thiazolopyridine and thiazolopyridinium analogues of oxyluciferin. Routes to these compounds were devise and many were successfully prepared. Some of the analogues have an
extended \( \pi \)-conjugated structure (348, 349, 350 and 351). The optical properties were measured. Various specific properties have been observed, includes large Stokes shift and aggregation induced emission. Two analogues with emission above 675 nm (329 and 313) were selected to investigate further modification to enable bioconjugation. The bioconjugation was successful and as expected, the fluorophore attached to trastuzumab molecule did not affect the protein’s function. The trastuzumab-fluorophore complex still possessed good emission characteristics which will enable further observation and research.
4. Future work

4.1 Oxyluciferin iminium ion

A different strategy towards the synthesis of oxyluciferin iminium ions could be attempted.

To reduce the steric hinderance from the two substituents at the tetrahedral centre adjacent to the thiazolone carbonyl a different method of blocking potential enolization could be investigated (Scheme 55). Compared to dimethyl oxyluciferin or diphenyl oxyluciferin, the proposed analogue 356 has a sp² thiazolone 5'-carbon instead of sp³. This would help in reducing steric inhibition during the formation a tetrahedral centre upon nucleophilic attack of the carbonyl group. The retrosynthetic analysis is also shown.

To avoid conjugate addition, the iminium 358 is generated in advance of cross coupling. The Ar group can be electron-rich aromatic substituents in order to stabilize iminium 358 by delocalization. The special Knoevenagel Condensation on 360 has been reported on a similar substrate.

Scheme 55  Structure of planar analogue 1 and the retrosynthesis analysis.
Fluorine atoms are small and have a strong electron-withdrawing effect. Based on that fact, an oxyluciferin iminium ion with two fluorine atoms could be considered (Scheme 56). Not only would the smaller fluorine substituents reduce steric hinderance towards nucleophilic addition to the carbonyl group, but the two electronegative atoms would make the carbonyl group more electrophilic. This could make the carbonyl more competitive towards 1,2 addition than conjugate 1,4-addition the thiazolone 2'-carbon. Some experiments towards the preparation of difluorine oxyluciferin 364 have been attempted at the very beginning of the project, but were unsuccessful.

![Structure of difluorine analogue 361 and attempted reaction towards it.](image)

**Scheme 56**  Structure of difluorine analogue 361 and attempted reaction towards it.

### 4.2 Rigid analogues with extended conjugation

Non-radiative decay of excited state molecules can reduce their fluorescence emission. The non-radiative pathway includes dissipation of energy through bond stretching, bending and rotations. Increasing molecular rigidity can increase the quantum yield by reducing non-radiative decay. This strategy has been recently confirmed in our group by the synthesis of D-luciferin type bioluminescence compounds (Figure 94). Rigid imidazole infraluciferin 366 has only one free rotatable bond while imidazole infraluciferin has two. The more rigid imidazole infraluciferin 366 was found to be 20 times brighter than imidazole infraluciferin 365 in bioluminescence studies.
Therefore the synthesis of rigid analogues of our new fluorophores may be beneficial. Compound 367 has a rigid structure with no free rotatable bonds. It would be interesting to synthesize this molecule and compare its fluorescence brightness to the non-rigid analogue 376 (Scheme 57).

The most challenging part is the oxidation of compound 369 as the product pyridine indole 368 is anti-aromatic (20 π electrons). It could be highly reactive and may decompose rapidly to an aromatic byproduct. Also, product 375 may be the more stable aromatic oxidized product.
5. Experimental Details

5.1 Compound synthesis

5.1.1 General details

All $^1$H and $^{13}$C NMR data were collected using Bruker AVANCE III NMR instruments at 300, 400 or 600 MHz for $^1$H and 75, 100 or 150 MHz for $^{13}$C respectively. All NMR samples were prepared as dilute solutions of CDCl$_3$ or DMSO-$d_6$, as stated. Spectra were recorded at 298 K unless stated. All chemical shifts (δ) were reported in parts per million (ppm), relative to solvent residual signal of CDCl$_3$ δ = 7.25, DMSO-$d_6$ δ = 2.50 for $^1$H NMR and CDCl$_3$ δ = 77.2, DMSO-$d_6$ δ = 39.5 for $^{13}$C NMR. Coupling constants (J) were reported in Hz. Multiplicities of $^1$H coupled signals were denoted as singlet (s), doublet (d), triplet (t), quartet (q) or multiplet (m). Mass spectrometry data were collected using Thermo Finnigan Mat900 xp (EI/Cl) VG-70se (FAB) or Waters LCT Premier XE (ES) mass spectrometers. Infrared spectroscopy data were collected using a Perkin-Elmer 100 FTIR spectrometer. The samples were prepared as thin films. All commercially available chemicals were used without any purification.
5.2.2 Synthesis details and compounds data

4,5-dichloro-1,2,3-dithiazolium chloride (Appel's salt, 213)[148]

\[
\text{Cl} \quad \text{Cl} \\
\text{S} \quad \text{N} \quad \text{Cl}^-. 
\]

To a solution of sulfur monochloride (101 mL, 1.26 mol) in dry CH$_2$Cl$_2$ (40 mL) was added chloroacetonitrile (20.0 mL, 0.32 mol). The yellow solution was then left for 2 h or until the gas release stopped. The darkened mixture was then left for another 48 h under nitrogen without agitation. Then the resultant dark green solid was filtered off under an argon atmosphere and washed with dry CH$_2$Cl$_2$ (3 x 50 mL). The dark green solid containing elemental sulfur was used directly without any further purification.

(Z)-4-chloro-N-(4-methoxyphenyl)-5H-1,2,3-dithiazol-5-imine (214)[149]

\[
\text{O} \\
\text{N} \quad \text{N} \\
\text{Cl} \quad \text{S} \quad \text{S} \\
\text{N} \quad \text{N} \\
\text{O} 
\]

Appel's salt (20.0 g, 0.05 mol) and para-anisidine (7.39 g, 0.06 mol) were dissolved in dry CH$_2$Cl$_2$ (150 mL) under nitrogen. The dark orange solution was stirred for 2 h. Then pyridine (9.67 mL, 0.12 mol) was slowly added to the stirred mixture and then the reaction was left for another 2 h. The reaction mixture was washed with water (3 x 50 mL) and brine (50 mL). The washed organic layer was then dried (Na$_2$SO$_4$) and evaporated to yield a dark red solid (13.50 g). The crude product
was purified by column chromatography (70% CH₂Cl₂ in hexane) to yield pure product as a yellow solid (6.70 g, 52%). m.p. 87-89°C (lit. 91-92°C); IR (film from CH₂Cl₂): ν max 2923 (C-H), 2837 (C-H), 1498 (C-H), 1441 (C-H) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.29 (2H, d, J = 9.0 Hz, ArH), 7.00 (2H, d, J = 9.0 Hz, ArH), 3.84 (3H, s, OCH₃); ¹³C NMR (100 MHz, CDCl₃) δ 158.4 (Cl-C=N), 155.8 (S-C=N), 148.6 (ArC), 143.2 (ArC), 121.9 (ArC), 114.9 (ArC), 55.7 (OCH₃); HRMS (ESI) m/z calcd for C₉H₇ClN₂O₂ ([M+H]+) 258.9761, found 258.9766. All the data was in agreement with the literature [149].

2-cyano-6-methoxybenzothiazole (211)[149]

To imine 214 (6.50 g, 25.1 mmol) was added tetramethylene sulfone (3.50 mL, 34.9 mmol). The mixture was then heated to 180°C for 20 min under nitrogen. The resultant black oil was suspended in MTBE and washed with water (3 x 30 mL) and brine (30 mL). The organic layer was dried over Na₂SO₄ and evaporated to give the crude product as a brown solid (2.94 g). The crude product was purified by column chromatography (silica, 55% CH₂Cl₂ in hexane) to yield nitrile 211 as a yellowish solid (2.01 g, 42%). m.p. 127-129°C (lit. 131-132°C); IR (film from CH₂Cl₂): ν max 2984 (C-H), 2844 (C-H), 2226 (C≡N) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.09 (1H, d, J = 9.2 Hz, ArH), 7.36 (1H, d, J = 2.4 Hz, ArH), 7.25 (1H, dd, Jₕ = 9.2 Hz, Jₖ = 2.4 Hz, ArH), 3.92 (3H, s, OCH₃); ¹³C NMR (100 MHz, CDCl₃) δ 160.5 (CN), 146.0 (ArC), 137.5 (ArC), 133.4 (ArC), 126.0 (ArC), 118.6 (ArC), 113.3 (ArC), 103.0 (ArC), 56.0 (OCH₃); HRMS (ESI) m/z calcd for C₉H₆N₂OS ([M+H]+) 191.0279, found 191.0275. All the data was in agreement with the literature [149].
6-methoxybenzo[d]thiazole-2-carbothioamide (209)[152]

![Chemical Structure](image)

A solution of phosphorus pentasulfide (350 mg, 1.58 mmol) in ethanol (20 mL) was stirred for 1 h at room temperature. To the resultant solution was added nitrile 211 (150 mg, 0.782 mmol) and the mixture was refluxed for 4 h. The reaction mixture was then cooled to room temperature, yellow needles crystallised which were collected by filtration. The crystals were then washed with ice cold ethanol (3 x 5 mL) to give thioamide 209 after dried in air (176.0 mg, 73%). m.p. 195-196°C (lit. 196-198°C); IR (film from CH₂Cl₂): v_max 3236 (N-H), 3140 (N-H), 1225 (C=S) cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ 10.14 (2H, s, br, NH₂), 7.99 (1H, d, J = 9.0 Hz, ArH), 7.72 (1H, d, J = 2.4 Hz, ArH), 7.21 (1H, dd, J₉ = 9.0 Hz, J₀ = 2.4 Hz, ArH), 3.86 (3H, s, OCH₃); ¹³C NMR (100 MHz, DMSO-d₆) δ 187.3 (ArC), 166.0 (C=S), 128.6 (ArC), 147.7 (ArC), 140.9 (ArC), 125.3 (ArC), 117.4 (ArC), 104.3 (ArC), 55.9 (OCH₃); HRMS (ESI) m/z calcd for C₉H₈N₂O₂S₂ ([M+H]^+) 225.0151, found 225.0149.

All the data was in agreement with the literature [185].

2-chloro-2,2-diphenylacetic acid (210)[147]

![Chemical Structure](image)

To a suspension of benzoilic acid (11.4 g, 50.1 mmol) in dry CH₂Cl₂ (12 mL) was added acetyl chloride (4.00 mL, 56.6 mmol). The resultant mixture was refluxed for 1 h. The mixture was cooled to room temperature. The resultant white crystal was
collected by filtration and washed with CH$_2$Cl$_2$ (3 mL x 3). Carboxylic acid 210 was obtained as white crystals (6.71 g, 54%). m.p. 117-120$^\circ$C (lit. 120-121$^\circ$C); IR (film from CH$_2$Cl$_2$): $\nu_{\text{max}}$ 3059 (O-H), 2926 (C-H), 1714 (C=O); $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.38 (10H, m, ArH); $^13$C NMR (150 MHz, CDCl$_3$) $\delta$ 170.5 (O=C=O), 140.9 (ArC), 128.3 (ArC), 77.4 (Ph$_2$C); HRMS (ESI) m/z calcd for C$_{14}$H$_{11}$ClO$_2$ ([M+H]$^+$) calcd 246.0786, found 264.0787. All the data was in agreement with the literature [147].

2-(6-methoxybenzo[d]thiazol-2-yl)-5,5-diphenylthiazol-4(5H)-one (208)

![Chemical structure of 208](image)

To a solution of thioamide 209 (99.0 mg, 0.44 mmol) and chlorodiphenylacetic acid 210 (123 mg, 0.50 mmol) in acetic acid (4 mL) was added acetic anhydride (0.50 mL, 5.29 mmol) and the mixture was refluxed for 2 h. The reaction mixture was then cooled to room temperature, a brown precipitate formed, which was filtered and washed with EtOAc (2 x 10 mL) to give the ketone 208 as a brown solid (120 mg, 66%). m.p. 187-189$^\circ$C; IR (film from CH$_2$Cl$_2$): $\nu_{\text{max}}$ 2961 (C-H), 2928 (C-H), 2837 (C-H), 1717 (C=O) cm$^{-1}$; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ 8.21 (1H, d, $J = 9.2$ Hz, ArH), 7.92 (1H, d, $J = 2.4$ Hz, ArH), 7.44 (11H, m, ArH), 3.91 (3H, s, OCH$_3$); $^13$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 187.5 (C=O), 160.2 (N=C-S-), 156.2 (CH$_3$OC), 148.0 (ArC), 139.6 (ArC), 138.9 (ArC), 129.0 (ArC), 128.6 (ArC), 128.3 (ArC), 125.9 (ArC), 118.9 (ArC), 104.7 (ArC), 74.2 (OCCPh), 56.1 (CH$_3$O); HRMS (ESI) m/z calcd for C$_{23}$H$_{16}$N$_2$O$_2$S$_2$ ([M+H]$^+$) 417.0726, found 417.0728.
(4-(dimethylamino)phenyl)carbamothioyl cyanide (232)

A solution of N,N-dimethylbenzene-1,4-diamine (1.36 g, 10.0 mmol) and Appel’s salt (3.00 g, 12.0 mmol) in acetonitrile (60 mL) and tetrahydrofuran (30 mL) was stirred under nitrogen for 1 h. Then sodium thiosulfate (4.74 g, 30.0 mmol) was added to the reaction along with water (20 mL). The reaction was stirred for another 3 h. The resultant dark solution was diluted with water (100 mL) and extracted with EtOAc (3 x 100 mL). The organics were combined, dried (Na$_2$SO$_4$) and evaporated to give the crude product as a dark oil (1.90 g). The crude product was purified by column chromatography (silica, 20% EtOAc in hexane) to give thionitrile 232 as a red solid (1.06 g, 53%). m.p. 128-131°C; IR (film from CH$_2$Cl$_2$): $\nu_{\text{max}}$ 3258 (N-H), 2808 (C-H), 2221 (C=N), 1360 (C=S) cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 13.28 (1H, s, br, NH), 7.91 (1.7H, d, $J$ = 9.0 Hz, ArH), 7.28 (0.3H, d, $J$ = 9.0 Hz, ArH), 6.76 (2H, d, $J$ = 9.0 Hz, ArH), 2.94 (6H, s, CH$_3$NCH$_3$); $^{13}$C NMR (150 MHz, DMSO-$d_6$) $\delta$ 162.8 (CN), 155.1 (CN), 150.0 (C=S), 149.4 (C=S), 127.4 (ArC), 126.0 (ArC), 124.2 (ArC), 123.3 (ArC), 114.2 (ArC), 113.0 (ArC), 112.0 (ArC), 111.4 (ArC); HRMS (ESI) m/z calcd for C$_{10}$H$_{11}$N$_3$S ([M+H$^+$]) 206.0746, found 206.0749.
6-(dimethylamino)benzo[d]thiazole-2-carbonitrile (233)

To the solution of thionitrile 232 (340 mg, 1.66 mmol) in DMSO (20 mL) and DMF (20 mL) was added PdCl$_2$ (32.0 mg, 0.19 mmol), CuI (178 mg, 0.94 mmol) and Bu$_4$NBr (1.20 g, 3.74 mmol). The resultant mixture was then heated to 120° C and stirred for 3 h under nitrogen. The resultant black solution was then cooled and diluted with EtOAc (150 mL), washed with water (4 x 50 mL) and brine (40 mL). The organic layer was dried (Na$_2$SO$_4$) and evaporated to yield the crude product as a bright yellow solid (404 mg). The crude product was purified by column chromatography (silica, 20% EtOAc in hexane) to give the dimethylaminonitrile 233 (320 mg, 95%) as a yellow solid. m.p. 132-135° C; IR (film from CH$_2$Cl$_2$) $\nu_{\text{max}}$ 2857 (C-H), 2212 (C=N), 1601 (C-H) cm$^{-1}$; $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 7.98 (1H, d, $J = 9.6$ Hz, ArH), 7.07 (1H, d, $J = 9.0$ Hz, ArH), 7.03 (1H, s, ArH), 3.10 (6H, s, CH$_3$NCH$_3$); $^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ 150.6 (CN), 144.1 (ArC), 138.8 (ArC), 129.9 (ArC), 125.3 (ArC), 115.0 (ArC), 114.0 (ArC), 101.0 (ArC), 40.9 (CH$_3$NCH$_3$); HRMS (ESI) m/z calcd for C$_{10}$H$_9$N$_3$S ([M+H]$^+$) 204.0590, found 206.0591. All the data was in agreement with the literature [186].
6-(dimethylamino)benzo[d]thiazole-2-carbothioamide (234)

\[
\begin{align*}
\text{N} & \quad \text{S} \\
\text{S} & \quad \text{N} \\
\text{NH}_2 & \\
\end{align*}
\]

234

Prepared by analogous method to nitrile 209 gave thioamide 233 as a red crystal (71.0 mg, 51%). m.p. 201-202°C; IR (film from CH\(_2\)Cl\(_2\)): \(\nu_{\text{max}}\) 3364 (N-H), 3238 (N-H) cm\(^{-1}\); ¹H NMR (600 MHz, DMSO-d\(_6\)) \(\delta\) 10.08 (1H, s, br, NH\(_2\)), 9.86 (1H, s, br, NH\(_2\)), 7.86 (1H, d, \(J = 9.0\) Hz, ArH), 7.25 (1H, d, \(J = 2.4\) Hz, ArH), 7.08 (1H, dd, \(J_a = 9.0\) Hz, \(J_b = 2.4\) Hz, ArH), 3.34 (6H, s, \(\text{CH}_3\text{NCH}_3\)); ¹³C NMR (100 MHz, DMSO-d\(_6\)) \(\delta\) 187.4 (ArC), 162.5 (C=S), 149.5 (ArC), 144.7 (ArC), 141.7 (ArC), 124.8 (ArC), 114.4 (ArC), 102.0 (ArC); HRMS (ESI) m/z calcd for C\(_{10}\)H\(_{11}\)N\(_3\)S\(_2\) (\([\text{M+H}]^+\)) 238.0467, found 238.0469.

2-(6-(dimethylamino)benzo[d]thiazol-2-yl)-5,5-diphenylthiazole-4(5H)-one (235)

\[
\begin{align*}
\text{N} & \quad \text{S} \\
\text{S} & \quad \text{N} \\
\text{O} & \quad \text{Ph} \\
\text{Ph} & \\
\end{align*}
\]

235

To a solution of dimethylaminothioamide 234 (59.0 mg, 0.25 mmol) in acetic acid (0.3 mL) was added chlorodiphenylacetic acid 210 (75.0 mg, 0.30 mmol) and acetic anhydride (30 \(\mu\)l, 0.40 mmol). The resultant mixture was refluxed for 1 h. After completion of the reaction, water (2 mL) was added and the red precipitate was collected by filtration (160 mg) thus was dissolved in acetone (50 mL), which is dried (MgSO\(_4\)) then. The acetone was evaporated to give the crude product (120 mg). The crude product was purified by column chromatography (silica, 50%
EtOAc in hexane) to yield dimethylaminoketone 235 (50.0 mg, 47%) as a dark red solid. m.p. 197-199°C; IR (film from acetone): ν<sub>max</sub> 1716 (C=O) cm<sup>-1</sup>; 1H NMR (600 MHz, DMSO-d<sub>6</sub>) δ 8.06 (1H, d, J = 9.6 Hz, ArH), 7.92 (11H, m, ArH), 7.23 (1H, dd, J<sub>a</sub> = 9.6 Hz, J<sub>b</sub> = 2.4 Hz, ArH), 3.11 (6H, s, C<sub>6</sub>H<sub>5</sub>NCH<sub>3</sub>); 13C NMR (150 MHz, DMSO-d<sub>6</sub>) δ 190.4 (C=O), 185.8 (N=C=S-), 150.9 ((CH3)2NC), 145.3 (ArC), 141.1 (ArC), 139.4 (ArC), 128.9 (ArC), 128.4 (ArC), 128.3 (ArC), 125.6 (ArC), 115.7 (ArC), 101.7 (ArC), 73.9 (OCCPh), 40.2 ((CH3)2N); MS (MALDI) m/z calcd for C<sub>24</sub>H<sub>19</sub>N<sub>3</sub>OS<sub>2</sub> ([M+H]+) 431.2 found 431.2.

6-hydroxybenzothiazole-2-nitrile (242)<sup>[152]</sup>

A solution of pyridinium chloride (10.0 g, 86.50 mmol) and nitrile 211 (1.00 g, 5.26 mmol) in tetramethylene sulfone (3 mL) was heated to 180°C for 1 h. The resultant yellow oil was suspended in MTBE (150 mL) and washed with water (3 x 100 mL) and brine (50 mL). The organic layer was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to yield a yellow solid (1.80 g). The crude product was purified by column chromatography (40% EtOAc in hexane) to give hydroxynitrile 242 as a white solid (340 mg, 37%) and unreacted methoxynitrile 210 (550 mg, 55%). The yield of the reaction is 82% based on recovered starting material. m.p. 181-182°C (lit. 183-184°C); IR (film from CH<sub>2</sub>Cl<sub>2</sub>): ν<sub>max</sub> 3159 (O-H), 3017 (C-H), 2923 (C-H), 2223 (C≡N), 1458 (C-H) cm<sup>-1</sup>; 1H NMR (600 MHz, DMSO-d<sub>6</sub>) δ 10.54 (1H, s, O-H), 8.07 (1H, d, J = 9.0 Hz, ArH), 7.59 (1H, s, ArH), 7.18 (1H, d, J = 9.0 Hz, ArH); 13C NMR (150 MHz, DMSO-d<sub>6</sub>) δ 158.6 (CN), 145.4 (ArC), 137.7 (ArC), 132.4 (ArC), 125.5 (ArC), 118.7 (ArC), 113.8 (ArC), 106.7 (ArC); HRMS (ESI) m/z calcd for C<sub>8</sub>H<sub>3</sub>N<sub>2</sub>OS ([M-H]) 174.9966, found 174.9966. All the data was in agreement with the literature [152].
6-hydroxybenzo[d]thiazole-2-carbothioamide (243)

A solution of phosphorus pentasulfide (350 mg, 1.58 mmol) in ethanol (20 mL) was stirred for 1 h at room temperature. To the resultant solution was added nitrile 243 (150 mg, 0.782 mmol) and the mixture was refluxed for 4 h. The solvent was then evaporated and the resultant yellow solid was purified by column chromatography (silica, 40% - 50% EtOAc in hexane) to give hydroxythioamide 243 as a yellow solid (144 mg, 87%). m.p. 239-240°C; IR (film from CH₂Cl₂): v_{max} 3236 (N-H), 3140 (O-H), 1219 (C=S) cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ 10.21 (1H, s, br, NH₂), 10.17 (1H, s, OH), 9.98 (1H, s, br, NH₂), 7.90 (1H, d, J = 8.4 Hz, ArH), 7.40 (1H, d, J = 2.4 Hz, ArH), 7.07 (1H, dd, J₆ = 8.4 Hz, J₇ = 2.4 Hz, ArH); ¹³C NMR (100 MHz, DMSO-d₆) δ 187.4 (ArC), 164.9 (C=S), 157.0 (ArC), 146.8 (ArC), 140.9 (ArC), 125.4 (ArC), 117.4 (ArC), 106.4 (ArC); HRMS (ESI) m/z calcd for C₈H₅N₂OS₂ ([M-H]⁻) 208.9922, found 208.9921.

Preparation of azide ion exchanging resin

The Amberlite® IR 400 (Cl) (10.0 g) was washed with hot DMF (30 ml), MeOH (30 ml * 2) and deionized water (50 ml). The washed resin was then soaked in 20% NaN₃ solution (30 ml) for 10 mins, twice. The resulted resin was washed with deionized water until no chloride ion present, then washed with 30% MeOH in water, 50% in water, deionized water, 50% Et₂O in MeOH and Et₂O, three times each. After dried in air the resin was then stored in desiccator.
2-(6-methoxybenzo[cf]thiazol-2-yl)-3-methyl-2-(methylamino)-5,5-diphenylthiazolidin-4-one (260)

A flame-dried flask was filled with Amberlite® IR 400 (N₃) (5.00 g, equal to 14.8 mmol azide) and flushed with argon. To the flask was added iodomethane (0.3 ml, 4.82 mmol) and dry MeCN (6 ml). The mixture was then stirred overnight. The liquid was taken out and injected to another flame-dried flask which filled with PPh₃ (1.20 g, 4.56 mmol) and dry MeCN (6 ml). The mixture was then stirred for 2 h under room temperature before methoxy ketone 208 (40.0 mg, 0.96 mmol) was added. The resulting mixture was refluxed for 6 h or until TLC showed completion of reaction. After the mixture was cooled to room temperature, volatiles were evaporated to yield crude product as an orange solid. Further purification was achieved by column chromatography (40% EtOAc in hexane) and pure product was obtained as a yellow solid (32 mg, 72%). Rf 0.61 (30% EtOAc in hexane); m.p. 216-218°C; IR (film from acetone): νmax 2938 (C-H), 1666 (C=O) cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆) δ 7.79 (1H, d, J = 9.0 Hz, CHC≡N), 7.68 (1H, d, J = 2.4 Hz, CH₃OCC≡S), 7.20-7.38 (10H, m, ArH), 7.10 (1H, dd, Jₐ = 9.0 Hz, J₈ = 3.0 Hz, CHCHCN), 4.51 (1H, q, J = 5.4 Hz, CH₃NHC), 3.83 (3H, s, CH₃OC), 2.86 (3H, s, CH₃NCO), 2.22 (3H, d, J = 5.4 Hz, CH₃NHC); ¹³C NMR (150 MHz, DMSO-d₆) δ 171.3 (CH₃NCO), 169.9 (NCS), 157.7 (CH₃OCCH), 146.2 (CHCN), 143.4 (ArC), 142.0 (ArC), 136.6 (CHCS), 128.7 (ArC), 128.2 (ArC), 128.1 (ArC), 127.7 (ArC), 127.4 (ArC), 127.3 (ArC), 123.7 (CHCHCN), 116.0 (CHCHCN), 104.9 (CH₃OCCHCS), 87.3 (CH₃NHC), 66.8 (CH₃NCO), 55.8 (OCH₃), 28.9 (CH₃NCO).
28.0 (CH₃NHC); HRMS (ESI) m/z calcd for C₂₅H₂₃N₃O₂S₂ ([M+H]⁺) 461.1232, ([M-NHMe]⁺) 431.0888, found 431.0882.

2-(6-methoxybenzo[d]thiazol-2-yl)-2-methyl-5,5-diphenylthiazolidin-4-one (265c)

A solution of methoxy ketone (50 mg, 0.12 mmol) in dry THF (5 ml) was cooled to -78°C. To the solution was added methyllithium (1.0 M in THF/cumene) (0.15 ml, 0.15 mmol) dropwise. The reaction was stirred under the same temperature for 1 h. Saturated NH₄Cl solution (2 ml) was added to quench the reaction. The reaction mixture was then diluted with EtOAc (50 ml) and washed with water (20 ml) and brine (10 ml). The organic layer was dried over Na₂SO₄ and evaporated to yield the product as a dark yellow oil (64 mg, quant.). m.p. 186-189°C; IR (film from acetone): v max 2920 (C-H), 1678 (C=O) cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆) δ 10.16 (1H, s, NHCO), 7.84 (1H, d, J = 9.0 Hz, CHCHCN), 7.64 (1H, d, J = 2.4 Hz, CH₂OCCHCS), 7.15-7.43 (10H, m, ArH), 7.09-7.11 (1H, dd, Jₐ = 9.0 Hz, J₅ = 2.4 Hz, CHCHCN), 3.82 (3H, s, CH₃OC), 2.07 (3H, s, CH₃NHCO); ¹³C NMR (150 MHz, DMSO-d₆) δ 173.8 (CH₃NHC), 173.7 (NCS), 157.5 (CH₂OCCH), 147.1 (CHCN), 142.5 (ArC), 141.8 (ArC), 136.6 (CHCS), 128.3 (Ar), 128.1 (Ar), 127.6 (Ar), 127.5 (Ar), 123.5 (CHCHCN), 115.8 (CHCHCN), 104.9 (CH₂OCCHCS), 67.6 (HNCO), 62.7 (CH₃C), 55.8 (OCH₃), 30.8 (CH₃CNH); HRMS (ESI) m/z calcd for C₂₄H₂₀N₂O₂S₂ ([M+H]⁺) 433.1048, found 433.1045.
6-methoxybenzo[d]thiazole (267)

![Image of 6-methoxybenzo[d]thiazole (267)](image)

To a round-bottom flask was added 2-cyano-6-methoxybenzothiazole (165 mg, 1.0 mmol) followed by MeOH (10 ml). To the solution was added 4M NaOH (20 ml) slowly. The resulted mixture was stirred under nitrogen for 48 hr. The resulted solution was concentrated using rotatory evaporator. To the concentrated mixture was added conc. HCl until the pH is lower than 5. The acidified solution was then stirred under room temperature for 1 hr. The mixture was then extracted with EtOAc (2 * 20 ml) and the combined organic layers was dried over MgSO₄ and evaporated. Compound 267 has been observed as the side product (~10% by ¹H NMR). m.p. 70°C (lit.70-71°C); IR (film from acetone): v_{max} 2950, 1457 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 8.75 (s, 1H), 7.94 (d, J = 9.0 Hz, 1H), 7.32 (d, J = 2.5 Hz, 1H), 7.05 (dd, J = 9.0, 2.5 Hz, 1H), 3.82 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 158.1 (NCC), 151.5 (NCS), 148.0 (ArC), 135.2 (ArC), 124.1 (ArC), 116.0 (ArC), 104.1 (ArC), 55.9 (OCH₃); HRMS (ESI) m/z calcd for C₈H₈NOS ([M+H]⁺) 166.0322, found 166.0322. All the data was in agreement with the literature [187].

2-(N-tert-butoxycarbonyl)aminopyridine (279)[161]

![Image of 2-(N-tert-butoxycarbonyl)aminopyridine (279)](image)
To a solution of 2-aminopyridine (9.40 g, 0.10 mol) and triethylamine (12.6 g, 0.13 mol) in dry DCM (150 ml) was added trimethylacetyl chloride (12.3 g, 0.13 mol) along with dry DCM (20 ml). The reaction mixture was left stirring in an ice bath for 15 min and under room temperature for another 2 h. To the resulting mixture was added 10% NaHCO$_3$ solution (100 ml). The organic layer was separated and dried over Na$_2$SO$_4$. Crude product was obtained as a brown oil by evaporating the solvent. Further purification was achieved by recrystallization in hexane. After recrystallization, the pure product was obtained as white crystals (14.0 g, 79%).

m.p. 68-70°C (lit. 68-70°C); IR (film from acetone): $\nu_{\text{max}}$ 3304 (N-H), 2970 (C-H), 1675 (C=O) cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 9.76 (1H, s, N=CO), 8.30-8.31 (1H, m, ArH), 8.05 (1H, d, $J = 8.4$, ArH), 7.74-7.77 (1H, m, ArH), 7.07-7.09 (1H, m, ArH), 1.23 (9H, s, (C$_3$H$_7$)$_3$CCO); $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 177.9 (C=O), 175.9 (CNH), 158.15 (ArC), 153.9 (ArC), 151.8 (ArC), 147.9 (ArC), 87.2 ((CH$_3$)$_3$C), 28.3 ((CH$_3$)$_3$C); HRMS (ESI) m/z calcd for C$_{10}$H$_{14}$N$_2$O ([M+H]$^+$) 179.1179, found 179.1180. All the data was in agreement with the literature [161].

2-(N-tert-butoxycarbonyl)amino-3-iodopyridine (280)$^{[161]}$

![Image of the compound](image)

A solution of 269 (400 mg, 2.24 mmol) in dry THF (1.4 ml) was cooled to -40°C before n-BuLi (1.6 M in hexane) (4.2 ml, 6.72 mmol) was added slowly. The resulted yellow solution was warmed to -10°C by a dry ice and acetone bath and kept for 4 h. The solution was cooled to -78°C and iodine (1.20 g, 4.72 mmol) was added along with dry THF (1.6 ml). The resulted black mixture was stirred under the same temperature for 1 h before quenched with sat. Na$_2$S$_2$O$_3$ solution (5 ml). The reaction mixture was diluted with DCM (45 ml) and the organic layer was dried.
over Na$_2$SO$_4$. Evaporation of the solvent gave the crude product as a black oil (1.16 g), the crude product was then purified by column chromatography to give a pure compound as a white solid (500 mg, 71%). m.p. 147-148°C (lit. 148°C); IR (film from acetone): $\nu_{\text{max}}$ 3291 (N-H), 2970 (C-H), 1656 (C=O) cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 9.96 (1H, s, NH), 8.52 (1H, d, $J = 2.3$ Hz, ArH), 8.09 (1H, dd, $J_A = 8.6$ Hz, $J_B = 2.3$ Hz, ArH), 7.93 (1H, d, $J = 2.4$ Hz, ArH), 2.49 (9H, s, tBu); $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 177.4 (C=O), 173.7 (CNH), 153.15 (ArC), 151.6 (ArC), 145.8 (ArC), 116.24 (IC), 86.1 ((CH$_3$)$_3$C), 27.6 ((CH$_3$)$_3$C); MS (Cl) m/z calcd for C$_{10}$H$_{13}$N$_2$O$I$ ([M+H]$^+$) 305.0151, found 305.0152. All the data was in agreement with the literature [161].

**N-(tert-butythio)phthalimide (282)**

![282]

To a solution of di-tert-butyl disulfide (1.93 ml, 10.0 mmol) in pentane (20 ml) was added sulfuryl chloride (0.9 ml, 11.0 mmol) dropwise. The yellowish solution was warmed to 30°C and stirred under the same temperature for 3 hr. The yellow mixture was then added slowly to a vigorously stirring solution of phthalimide (2.65 g, 18.0 mmol), triethylamine (3.35 ml, 24.1 mmol) and DMF (3 ml). The reaction was quenched by addition of water (1 ml). The white precipitate was filtered and recrystallized in hexane to give pure product as smelly white crystals (2.71 g, 64%). m.p. 130°C (131-132°C). IR (film from acetone): $\nu_{\text{max}}$ 2979 (C-H), 2963 (C-H), 1711 (C=O) cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 7.90-7.96 (4H, m, ArH), 1.26 (9H, s, tBu); $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 168.7 (C=O), 135.1 (ArC), 131.6 (ArC), 123.7 (ArC), 50.5 ((CH$_3$)$_3$C), 29.0 ((CH$_3$)$_3$C); HRMS (ESI) m/z calcd for C$_{12}$H$_{13}$NO$_2$I
([M+H]+) 253.1005, found 253.1004. All the data was in agreement with the literature [163].

6-methoxybenzo[d]thiazole-2-carbothioamide (285)

To a round bottom flask was added 2-cyano-6-methoxybenzothiazole (19 mg, 0.1 mmol) followed by Na₂CO₃ (105.0 mg, 1.0 mmol) and MeOH/H₂O (10 ml). The mixture was heated to reflux for 6 hr. The resulted mixture was extracted with EtOAc (3 × 15 ml). The combined organic layers were evaporated under reduced pressure to yield a yellowish solid (21 mg, quant.). The solid was sent to further analysis without any further purification. m.p. 257 °C (lit. 258-260°C); IR (film from acetone): νmax 3698 (N-H), 2988, 1691 (C=O) cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆) δ 8.20 (1H, s, br, NH₂), 7.87 (1H, d, J = 9.0 Hz, ArH), 7.83 (1H, s, br, NH₂), 7.32 (1H, d, J = 2.4 Hz, ArH), 7.08 (1H, dd, Jₐ = 8.9 Hz, J₈ = 2.4 Hz, ArH), 3.81 (3H, d, OCH₃); ¹³C NMR (150 MHz, DMSO-d₆) δ 170.4, 158.6, 149.6, 144.2, 138.9, 124.1, 114.0, 102.7, 59.8; HRMS (ESI) m/z calcd for C₉H₉N₂O₂S ([M+H]+) 209.0385, found 209.0386. All the data was in agreement with the literature [144].
thiazolo[4,5-b]pyridine-2(3H)-thione (295)[168]

\[ \text{295} \]

A flame-dried flask was filled with 2-amino-3-chloropyridine (1.02 g, 7.94 mmol), potassium ethyl xanthate (1.95 g, 11.9 mmol) and DMPU (10 ml). The mixture was heated to reflux and stirred under the same temperature overnight. The resulted solution was cooled to room temperature and acetic acid (2 ml) was added, followed by water (150 ml). The precipitate was washed with EtOAc and 50% EtOH/H₂O. The washed precipitate was collected and dried in air to yield a grey solid (840 mg, 63%). The crude material was used without any further purification. m.p. 269°C (darkened) (lit. 312-315°C, decomp.); IR (film from acetone): \( \nu_{\text{max}} \) 1271 (C=S) cm\(^{-1}\); \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 14.29 (1H, br, NH), 8.35 (1H, s, ArH), 8.13 (1H, s, ArH), 7.30 (1H, s, ArH); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \( \delta \) 191.1 (CNHC=S), 153.8 (CNHC=S), 146.9 (ArC), 130.6 (CSC=S), 124.2 (ArC), 119.6 (ArC); HRMS (ESI) m/z calcd for C\(_6\)H\(_4\)N\(_2\)S\(_2\) ([M+H]\(^+\)) 168.9889, found 168.9888. All the data was in agreement with the literature [168].

2-chlorothiazolo[4,5-b]pyridine (294)[168]

\[ \text{294} \]

A flame-dried flask was filled with thione 295 (128 mg, 0.76 mmol) and flushed with argon. To the flask was added sulfuryl chloride (0.8 ml, 9.86 mmol). The reaction was then stirred overnight, before sat. NaHCO\(_3\) solution (5 ml) was used to quench
the reaction. The aqueous mixture was extracted with EtOAc, the extract was dried over Na₂SO₄ and evaporated. The crude product was obtained as a brown solid (70 mg, 54%) and used without any further purification. IR (film from acetone): v_{max} 3086, 2929, 1547 (C=N) cm⁻¹; ¹H NMR (600 MHz, DMSO-d₆) δ 8.50 (1H, s, ArH), 8.35 (1H, s, ArH), 7.29 (1H, s, ArH); ¹³C NMR (150 MHz, DMSO-d₆) δ 161.6 (NCN), 158.0 (NCCI), 148.0 (NCCH), 130.5 (SCC), 130.1 (ArC), 120.6 (ArC); HRMS (ESI) m/z cal. For C₆H₃₃ClN₂S ([M+H]+) 170.9778, found 170.9779. All the data was in agreement with the literature [168].

6-nitrobenzo[d]thiazole (308)⁶¹⁷³

![Chemical structure of 6-nitrobenzo[d]thiazole (308)](image)

To a round-bottom flask was added conc. H₂SO₄ (30 ml). The flask was then cooled by ice-bath. To the pre-cooled acid was added benzothiazole (5.8 ml, 53 mmol) dropwise. The temperature was maintained under 0°C during addition. Then to the resulted yellow solution was added conc. HNO₃ (15 ml, 0.3 mol) slowly. The temperature was maintained under 0°C. The reaction was allowed to stand for another 14 h under room temperature. The reaction mixture was then poured on to crushed ice. The resulted yellow solid was collected by filtration and purified by recrystallization (EtOH) to yield pure product as an orange solid (7.79 g, 84%). m.p. 171-174°C (lit. 176-177°C); IR (film from acetone): v_{max} 2896, 2843, 1532 (N=O) cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ 9.72 (1H, s, NCHS), 9.24 (1H, d, J = 2.0 Hz, ArH), 8.35 (1H, m, ArH), 8.26 (1H, d, J = 9.1 Hz, ArH); ¹³C NMR (100 MHz, DMSO-d₆) δ 163.6 (NCC), 157.1 (NCS), 145.2 (ArC), 135.0 (ArC), 124.2 (ArC), 121.9 (ArC),
120.2 (ArC); HRMS (ESI) m/z calcd for C₇H₅N₂O₂S ([M+H]⁺) 181.0072, found 181.0072. All the data was in agreement with the literature [173].

6-aminobenzo[d]thiazole (309)[172]

To a round-bottom flask was added 6-nitrobenzothiazole (2.50 g, 13.9 mmol) followed by 80% EtOH (63 ml) and conc. hydrochloric acid (1.93 ml, 22.7 mmol). To the solution was then added iron powder (3.70 g, 55.6 mmol). The resulted mixture was heated to reflux, allowed to stand for 1.5 h. Upon completion of the reaction (monitored by TLC), the reaction mixture was cooled to room temperature. The iron salts were filtered off and the filtrate was concentrated under reduced pressure. To the concentrated mixture was added 20% Na₂CO₃ solution (50 ml) and the aqueous layer was extracted with EtOAc (3 * 100 ml). The extracts were dried over Na₂SO₄ and evaporated to yield crude product as a brown oil. The pure product (yellowish solid) was obtained by column chromatography (939 mg, 45%). m.p. 79°C (lit. 82-84°C); IR (film from acetone): v_max 3368 (N-H), 3320 (N-H), 1667 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 8.65 (1H, s, NCH₃), 7.86 (1H, d, J = 9.0 Hz, ArH), 7.14 (1H, d, J = 2.0 Hz, ArH), 6.90 (1H, dd, Jₐ = 9.0 Hz, Jₖ = 2.0 Hz, ArH); ¹³C NMR (150 MHz, DMSO-d₆) δ 150.0 (NCS), 148.1 (NCC), 145.2 (ArC), 135.8 (ArC), 123.9 (ArC), 116.0 (ArC), 105.9 (ArC); HRMS (ESI) m/z calcd for C₇H₅N₂S ([M+H]⁺) 151.0300, found 131.0329. All the data was in agreement with the literature [172].
S-(2-amino-5-(dimethylamino)phenyl) O-hydrogen sulfurothioate (311)\textsuperscript{[174]}

![Chemical Structure](image)

To a solution of Al\textsubscript{2}(SO\textsubscript{4})\textsubscript{3}·18H\textsubscript{2}O (8.72 g, 15.0 mmol) in water (20 ml) was added \(N,N\)-dimethylphenylenediamine sulfate (6.09 g, 26.0 mmol) followed by Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} (4.40 g, 27.8 mmol) and ZnCl\textsubscript{2} (1.76 g, 12.6 mmol) along with water (20 ml). The mixture was cooled to 0°C by ice-bath. To the cooled solution was added K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} (5.00 g, 17.0 mmol) along with water (20 ml). The reaction was kept for 2 h under the same temperature. After 2 h the reaction was allowed to warm to room temperature slowly, the dark precipitate was collected by filtration. The solid was then dried in air, suspended in EtOAc (150 ml) and heated to reflux for 30 min. The grey-purple powder was then collected by filtration and dried to yield pure product as purple solid (1.67 g, 40%). m.p. 190°C (lit. 195-197°C); IR (film from acetone): \(v_{\text{max}}\) 3457 (N-H), 3556 (N-H), 1608, 1468 cm\(^{-1}\); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \(\delta\) 7.19 (1H, s, ArH), 7.02-7.07 (2H, m, ArH), 2.98 (6H, s, NCH\textsubscript{3}); \(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)) \(\delta\) 140.2 (ArC), 140.1 (ArC), 121.8 (ArC), 117.5 (ArC), 114.6 (ArC), 112.1 (ArC), 45.1 (NCH\textsubscript{3}); HRMS (ESI) m/z calcd for C\textsubscript{8}H\textsubscript{13}N\textsubscript{2}O\textsubscript{2}S\textsubscript{2} ([M+H]\textsuperscript{+}) 249.0368, found 249.0367. All the data was in agreement with the literature \[174\].

6-(\textit{N,N}-dimethylamino)benzo[\textit{d}]thiazole (306)
To a round bottom flask was added 311 (1.50 g, 6.0 mmol) and formic acid (5 ml). The mixture was heated to reflux and allowed to stand overnight. The reaction was then cooled to room temperature and neutralized by adding sat. Na$_2$CO$_3$ solution until basic. The aqueous layer was then extracted by EtOAc (3 * 50 ml). The combined organic layer was dried (MgSO$_4$) and evaporated. The crude product was then purified by column chromatography (30% EtOAc in hexane) to yield the pure product as a light yellow solid (780 mg, 73%). m.p. 73 °C (lit. 73-74°C); IR (film from acetone): $\nu_{\text{max}}$ 3001, 1548 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.67 (1H, s, N(C$_3$H$_3$)$_2$), 7.94 (1H, d, $J = 8.8$ Hz, ArC), 7.16 (1H, d, $J = 2.4$ Hz, ArH), 7.00 (1H, dd, $J_A = 9.0$ Hz, $J_B = 2.4$ Hz, ArH), 3.01 (6H, s, N(C$_3$H$_3$)$_2$); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 154.9 (NCS), 142.9 (ArC), 141.9 (ArC), 135.9 (ArC), 121.1 (ArC), 111.9 (ArC), 103.3 (ArC), 45.4 (NCH$_3$); HRMS (ESI) m/z calcd for C$_9$H$_{11}$N$_2$S ([M+H]$^+$) 179.0643, found 179.0644. All the data was in agreement with the literature [188].

**thiazolo[5,4-c]pyridine-2(1H)-thione (317)**

A flame-dried flask was filled with 2-amino-3-chloropyridine (1.02 g, 7.94 mmol), potassium ethyl xanthate (1.95 g, 11.9 mmol) and DMPU (10 ml). The mixture was heated to reflux and stirred under the same temperature overnight. The resulted solution was cooled to room temperature and acetic acid (2 ml) was added, followed by water (150 ml). The precipitate was washed with EtOAc and 50%
EtOH/H$_2$O. The washed precipitate was collected and dried in air to yield a grey solid (840 mg, 63%). The crude material was used without any further purification. m.p. 58-59°C; IR (film from acetone): $v_{\text{max}}$ 3077, 3029, 1607 cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 14.3 (1H, s, br, NH), 8.82 (1H, s, ArH), 8.39 (1H, d, $J = 6.8$ Hz, ArH), 7.43 (1H, d, $J = 6.7$ Hz, ArH); $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 190.0 (C=S), 148.9 (ArC), 144.2 (ArC), 135.8 (ArC), 116.8 (ArC); HRMS (ESI) m/z calcd for C$_6$H$_5$N$_2$S$_2$ ([M+H]$^+$) 168.9894, found 168.9895. All the data was in agreement with the literature [175].

**2-chlorothiazolo[5,4-c]pyridine (318)**

![Structure of 2-chlorothiazolo[5,4-c]pyridine](image)

To an ice-cold mixture of DMF (1.1 ml) and DCE (8 ml) was added dropwise a solution of oxalyl chloride (1.73 ml) in DCE (4 ml). A white precipitate formed, and the reaction mixture was stirred at room temperature for 5 min. Then [1,3]thiazolo[5,4-c]pyridine-2(1H)-thione (1.0 g, 6.0 mmol) was added in portions, and the reaction mixture was stirred at reflux for 3 h. After cooling to room temperature, the reaction mixture was treated with water (20 ml) and saturated aqueous NaHCO$_3$ (100 ml) and then extracted with EtOAc (2 * 100 ml). The crude product was then purified with column chromatography (50% EtOAc in hexane). The pure product was obtained as a light yellow solid (757 mg, 47%). m.p. 129-132°C; IR (film from acetone): $v_{\text{max}}$ 1756, 1688 cm$^{-1}$; $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 9.10 (1H, s, ArH), 8.67 (1H, d, $J = 5.6$ Hz, ArH), 7.85 (1H, dd, $J_A = 5.6$ Hz, $J_B = 1.0$ Hz, ArH); $^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ 156.3 (ArC), 146.5 (ArC), 143.5 (ArC), 133.4 (ArC), 117.3 (ArC); HRMS (ESI) m/z calcd for C$_6$H$_4$N$_2$S$_2$Cl.
([M+H]+) 170.9784, found 170.9784. All the data was in agreement with the literature [175].

General method of synthesizing 6-substituted benzo[d]thiazole-2-carbaldehyde

To a flame-dried round bottom flask was added 6-substituted benzothiazole (1.0 – 5.0 mmol) followed by dry THF which makes the reaction concentration at 0.1 M. The solution was then cooled to -78°C, before n-BuLi (1.6 M in hexane, 1.5 eq.) was added dropwise. The reaction was allowed to stand for 2 h under -55°C. The reaction was re-cooled to -78°C before DMF (5.0 eq.) was added slowly. The temperature of the reaction was maintained under -78°C during the addition. The reaction was allowed to warm to room temperature and stand overnight. The resulted mixture was quenched by adding water (10 ml). To the mixture was then added EtOAc (50 ml) and washed with brine (50 ml). The combined organic layers were dried (MgSO₄) and evaporated to yield crude product. Pure product was obtained by column chromatography (20%-30% EtOAc in hexane).

![Chemical structure](image)

yellow solid, quant. m.p. 115°C (lit.120-123°C); IR (film from acetone): \( \nu_{\text{max}} \) 2948, 1684 (C=O) cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\) \( \delta \) 10.10 (1H, s, CHO), 8.12 (1H, d, \( J = 9.0 \) Hz, ArH), 7.39 (1H, d, \( J = 2.6 \) Hz, ArH), 7.23 (1H, d, \( J_A = 9.0 \) Hz, \( J_B = 2.5 \) Hz, ArH); \(^1^3\)C NMR (100 MHz, CDCl\(_3\) \( \delta \) 185.1 (CHO), 162.9 (NCS), 160.5 (ArC), 148.3 (ArC), 138.6 (ArC), 126.5 (ArC), 118.8 (ArC), 102.9 (ArC), 56.0 (OCH\(_3\)); HRMS (ESI) m/z calcd for C\(_9\)H\(_8\)NO\(_2\)S ([M+H]+) 194.0276, found 194.0276.
bright yellow solid, 91%. m.p. 143°C (lit. 174-175°C); IR (film from acetone): $\nu_{\text{max}}$ 2998, 1680 (C=O) cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 10.05 (1H, s, CHO), 8.02-8.05 (1H, d, $J = 9.0$ Hz, ArH), 7.07 (1H, dd, $J_a = 9.0$ Hz, $J_b = 2.4$ Hz, ArH), 7.01 (1H, d, $J = 9.0$ Hz, ArH), 3.12 (6H, s, N(CH$_3$)$_2$); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 185.2 (CHO), 160.1 (NCS), 150.9 (ArC), 146.1 (ArC), 140.1 (ArC), 126.3 (ArC), 114.9 (ArC), 101.8 (ArC), 42.4 (N(CH$_3$)$_2$); HRMS (ESI) m/z calcd for C$_{10}$H$_{11}$N$_2$OS ([M+H]$^+$) 207.0592, found 207.0591.

General method of synthesizing benzothiazole-pyridine molecules

To a flame-dried flask was added 6-substituted benzothiazole (1.0 mmol), corresponding bromopyridine (0.95 mmol), palladium diacetate (0.01 mmol), copper(II) diacetate (0.2 mmol), triphenylphosphine (0.5 mmol), potassium carbonate (2.02 mmol) and toluene (5 ml). The mixture was then heated under reflux for 6 h. The resulted reaction mixture was diluted with water (10 ml) and the aqueous layer was extracted with EtOAc (5 * 10 ml). The combined organic layers were washed with brine (20 ml) and dried over MgSO$_4$. After the organics were evaporated, the crude product was then purified by column chromatography (20-50% EtOAc in hexane) to yield the pure product.
yellowish solid, 76%. m.p. 134-135°C; IR (film from acetone): $v_{\text{max}}$ 3108, 1641 (N=C) cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 8.70 (1H, dd, $J_A = 6.0$ Hz, $J_B = 1.8$ Hz, PyH), 8.26 (1H, d, $J = 7.9$ Hz, PyH), 7.94-8.02 (2H, m PyH and ArH), 7.71-7.73 (1H, m, PyH), 7.54-7.56 (1H, m, ArH), 7.12-7.15 (1H, m, ArH), 3.86 (3H, s, OC$_3$H$_3$); $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 157.8 (PyC), 157.5 (ArC), 153.4 (NCS), 149.9 (PyC), 147.6 (ArC), 137.1 (PyC), 135.1 (ArC), 125.7 (PyC), 123.5 (ArC), 120.0 (PyC), 115.7 (ArC), 104.8 (ArC), 55.8 (OCH$_3$); HRMS (ESI) m/z calcd for C$_{13}$H$_{11}$N$_2$OS ([M+H]$^+$) 243.0592, found 243.0591.

orange solid, 59%. m.p. 157-158°C; IR (film from acetone): $v_{\text{max}}$ 3211 (br), 3013, 1688 (N=C) cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 8.75-8.77 (1H, m, PyH), 7.96-8.01 (1H, m, PyH), 7.85 (1H, d, $J = 9.0$ Hz, ArH), 7.54 (1H, d, $J = 2.4$ Hz, ArH), 7.01 (1H, dd, $J_A = 9.0$ Hz, $J_B = 2.4$ Hz, ArH), 3.88 (3H, s, OCH$_3$); $^{13}$C NMR (150 MHz, DMSO-d$_6$) $\delta$ 156.7 (ArC), 150.6 (NCS), 149.8 (PyC), 145.8 (ArC), 143.7 (PyC), 136.1 (ArC), 122.6 (ArC), 121.3 (PyC), 114.6 (ArC), 104.9 (ArC), 55.8 (OCH$_3$); HRMS (ESI) m/z calcd for C$_{13}$H$_{11}$N$_2$OS ([M+H]$^+$) 243.0592, found 243.0571.

bright yellow solid, 70%. m.p. 168-169°C; IR (film from acetone): $v_{\text{max}}$ 2999, 2918, 1653 (N=C), 1532 cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 8.66 (1H, m, PyH), 8.22
orange solid, 62%. m.p. 184°C; IR (film from acetone): $\nu_{\text{max}}$ 3210 (br), 3151, 2884, 1605 (N=C) cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 8.73 (2H, m, Py$H$), 7.99 (2H, m Py$H$), 7.48 (1H, d, $J = 9.2$ Hz, Ar$H$), 6.96 (1H, d, $J = 2.6$ Hz, Ar$H$), 6.67 (1H, dd, $J_A = 9.2$ Hz, $J_B = 2.6$ Hz, Ar$H$), 3.02 (6H, s, N(CH$_3)_3$); $^{13}$C NMR (150 MHz, DMSO-$d_6$) $\delta$ 150.6 (N=C), 149.8 (Py$C$), 143.7 (Py$C$), 143.0 (Ar$C$), 140.8 (Ar$C$), 136.0 (Ar$C$), 122.5 (Ar$C$), 121.3 (Py$C$), 112.1 (Ar$C$), 103.3 (Ar$C$), 41.3 (N(CH$_3)_2$); HRMS (ESI) m/z calcd for C$_{14}$H$_{13}$N$_3$S ([M+H]$^+$) 255.0830, found 255.0834.

**General method of synthesizing benzothiazole-thiazolopyridine molecules**

To a flame-dried round-bottom flask was added 6-substituted benzothiazole (1 mmol) and dry THF which makes the reaction concentration at 0.1 M. The solution was then cooled to -78°C, before n-BuLi (1.6 M in hexane, 1.5 eq.) was added dropwise. The reaction mixture was allowed to stand for 2 h under -55°C. The reaction was re-cooled to -78°C before chlorothiazolopyridine (1.1 eq) was added along with dry THF (10 ml). The resulting mixture was kept under the same
temperature for 3 h, before water (10 ml) was added to quench the reaction. To the resulting mixture was added EtOAc (30 ml) and it was washed with brine (10 ml). The organic layer was dried (MgSO₄) and evaporated to yield crude product. Pure product was obtained by column chromatography (70%-90% EtOAc in hexane).

![Diagram of compound 270](image)

yellowish solid, 31%. m.p. 200-202°C; IR (film from acetone): \( \nu_{\text{max}} \) 3412 (br), 1701 (C=N) cm⁻¹; \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \( \delta \) 8.81 (1H, d, \( J = 4.8 \) Hz, Py\( H \)), 8.34 (1H, d, \( J = 7.8 \) Hz, Py\( H \)), 8.05 (1H, d, \( J = 9.0 \) Hz, Ar\( H \)), 7.40-7.42 (2H, m, Py\( H \) and Ar\( H \)), 7.16 (1H, dd, \( J_A = 9.0 \) Hz, \( J_B = 2.4 \) Hz, Ar\( H \)), 3.93 (3H, s, OCH₃); \(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)) \( \delta \) 165.1 (NCS), 163.9 (NCS), 159.3 (Py\( C \)), 158.3 (Ar\( C \)), 149.1 (Ar\( C \)), 148.2 (Py\( C \)), 138.4 (Py\( C \)), 131.1 (Ar\( C \)), 129.6 (Py\( C \)), 125.0 (Ar\( C \)), 120.9 (Py\( C \)), 113.4 (Ar\( C \)), 103.9 (Ar\( C \)), 56.0 (OCH₃); HRMS (ESI) m/z calcd for \( C_{14}H_{10}N_{3}O_{2} \) ([M+H]+) 300.0266, found 300.0264.

![Diagram of compound 314](image)

yellow solid, 39%. m.p. 209-210°C; IR (film from acetone): \( \nu_{\text{max}} \) 2955, 2925, 1473 cm⁻¹; \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \( \delta \) 8.60 (1H, s, Py\( H \)), 8.36 (1H, d, \( J = 6.6 \) Hz, Py\( H \)), 7.50-7.55 (2H, m, Ar\( H \) and Py\( H \)), 7.46 (1H, s, Ar\( H \)), 7.01 (1H, d, \( J = 9.0 \) Hz, Ar\( H \)); \(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)) \( \delta \) 159.9, (NCS), 159.0 (Py\( C \)), 156.1 (Ar\( C \)), 150.6 (NCS), 145.8 (Ar\( C \)), 145.6 (Py\( C \)), 143.0 (Py\( C \)), 136.1 (Ar\( C \)), 132.8 (Py\( C \)), 122.6 (Ar\( C \)), 116.8 (Py\( C \)), 114.6 (Ar\( C \)), 104.9 (Ar\( C \)), 55.8 (OCH₃); HRMS (ESI) m/z calcd for \( C_{14}H_{10}N_{3}O_{2} \) ([M+H]+) 300.0266, found 300.0264.
orange solid, 35%. m.p. 213°C; IR (film from acetone): $v_{\text{max}}$ 2413, 1644 (N=C) cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) δ 8.77 (1H, dd, $J_A = 4.6$ Hz, $J_B = 1.7$ Hz, PyH), 8.69 (1H, dd, $J_A = 8.0$ Hz, $J_B = 1.7$ Hz, PyH), 7.98 (1H, d, $J = 9.1$ Hz, ArH), 7.54 (1H, dd, $J_A = 8.0$ Hz, $J_B = 4.6$ Hz, PyH), 7.40 (1H, d, $J = 2.6$ Hz, ArH), 7.12 (1H, dd, $J_A = 9.2$ Hz, $J_B = 2.6$ Hz, ArH), 3.06 (6H, s, N(CH$_3$)$_2$); $^{13}$C NMR (150 MHz, DMSO-d$_6$) δ 164.6 (NCS), 163.4 (NCS), 154.0 (PyC), 149.9 (PyC), 149.0 (ArC), 144.5 (ArC), 138.5 (ArC), 132.4 (PyC), 128.9 (PyC), 124.2 (ArC), 121.1 (PyC), 114.3 (ArC), 102.5 (ArC), 40.3 (N(CH$_3$)$_2$); HRMS (ESI) m/z calcd for C$_{15}$H$_{13}$N$_4$S$_2$ ([M+H]$^+$) 313.0582, found 313.0582.

yellow-brown solid, 30%. m.p. 208-210°C; IR (film from acetone): $v_{\text{max}}$ 1772, 1658 (N=C), 1616 cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) δ 9.45 (1H, d, $J = 0.9$ Hz, PyH), 8.68 (1H, d, $J = 5.6$ Hz, PyH), 8.09 (1H, dd, $J_A = 5.6$ Hz, $J_B = 0.9$ Hz, PyH), 8.00 (1H, d, $J = 9.2$ Hz, ArH), 7.39 (1H, d, $J = 2.6$ Hz, ArH), 7.14 (1H, dd, $J_A = 9.2$ Hz, $J_B = 2.6$ Hz, ArH), 3.06 (6H, s, N(CH$_3$)$_2$); $^{13}$C NMR (150 MHz, DMSO-d$_6$) δ 166.7 (NCS), 157.8 (NCS), 153.5 (PyC), 150.0 (PyC), 146.2 (ArC), 145.5 (PyC), 144.5 (ArC), 138.6 (ArC), 131.5 (PyC), 124.3 (ArC), 117.2 (ArC), 114.5 (PyC), 102.4 (ArC), 40.3 (N(CH$_3$)$_2$); HRMS (ESI) m/z calcd for C$_{15}$H$_{13}$N$_4$S$_2$ ([M+H]$^+$) 313.0582, found 313.0582.
General method of synthesizing pyridiniums via direct methylation

To a round-bottom flask was added selected pyridine (0.5-1 mmol) followed by Mel to make the reaction concentration 0.02 M. The reaction was then stirred under inert atmosphere for 72 h. The precipitate was collected by filtration and suspended in EtOAc. The suspension was sonicated for 1 min and the liquid was discarded. This step was repeated several times until the unreacted starting material had been fully removed. The crude product was then dried in the air. The product was pure and did not need any further purification.

![Image 1](327)
bright orange solid, 69%. m.p. 186-189°C; IR (film from acetone): \( \nu_{\text{max}} \) 2989, 1501 cm\(^{-1}\); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \( \delta \) 8.99-9.04 (2H, m, PyH), 8.51-8.58 (2H, m PyH), 7.93 (1H, d, \( J = 9.2 \) Hz, ArH), 7.43 (1H, d, \( J = 2.5 \) Hz, ArH), 7.25 (1H, d, \( J_A = 9.3 \) Hz, \( J_B = 2.6 \) Hz, ArH), 4.35 (3H, s, N\(^+\)CH\(_3\)), 3.91 (3H, s, OCH\(_3\)); \(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)) \( \delta \) 157.9 (Ar C), 154.0 (Py C), 150.1 (NCS), 146.7 (Ar C), 146.1 (Py C), 145.1 (Ar C), 124.8 (Ar C), 124.3 (Py C), 114.9 (Ar C), 102.2 (Ar C), 57.1 (OCH\(_3\)), 47.4 (N\(^+\)CH\(_3\)); \(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)) \( \delta \); HRMS (ESI) m/z calcd for C\(_{14}\)H\(_{13}\)N\(_2\)OS ([M-I]\(^+\)) 257.0749, found 257.0750.

![Image 2](269)
orange solid, 76%. m.p. 230-231°C; IR (film from acetone): \( \nu_{\text{max}} \) 1631, 1571 cm\(^{-1}\); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \( \delta \) 9.40 (1H, d, \( J = 8.2 \) Hz, PyH), 9.24 (1H, d, \( J = 6.0 \) Hz, ArH), 7.96 (1H, d, \( J = 8.4 \) Hz, ArH), 7.44 (1H, d, \( J = 2.8 \) Hz, ArH), 4.41 (3H, s, N\(^+\)CH\(_3\)), 3.86 (3H, s, OCH\(_3\)); \(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)) \( \delta \); HRMS (ESI) m/z calcd for C\(_{14}\)H\(_{13}\)N\(_2\)OS ([M-I]\(^+\)) 257.0749, found 257.0750.
Hz, PyH), 8.22 (1H, d, J = 9.0 Hz, ArH), 8.15 (1H, dd, J_A = 8.2 Hz, J_B = 6.0 Hz, PyH), 7.93 (1H, d, J = 2.6 Hz, ArH), 7.34 (1H, d, J_A = 9.1 Hz, J_B = 2.6 Hz), 4.64 (3H, s, N^+CH_3), 3.93 (3H, s, OCH_3); 13C NMR (150 MHz, DMSO-d_6) δ 176.3 (NC), 160.0 (NCS), 155.7 (ArC), 150.8 (PyC), 147.8 (ArC), 145.2 (PyC), 141.9 (PyC), 138.7 (ArC), 133.8 (PyC), 125.4 (ArC), 122.3 (PyC), 118.6 (ArC), 105.0 (ArC), 56.1 (OCH_3), 44.2 (N^+CH_3); HRMS (ESI) m/z calcd for C_15H_12N_3S_2O ([M-I]^+) 314.0422, found 314.0423.

![Image of compound 312]

Dark orange solid, 79%. m.p. 249°C; IR (film from acetone): v_max 3001, 2949, 1629 (N=C) cm^{-1}; 1H NMR (600 MHz, DMSO-d_6) δ 9.90 (1H, s, PyH), 9.01 (1H, dd, J_A = 6.9 Hz, J_B = 1.5 Hz, PyH), 8.77 (1H, d, J = 6.8 Hz, PyH), 8.20 (1H, d, J = 9.1 Hz, ArH), 7.92 (1H, d, J = 2.6 Hz, ArH), 7.33 (1H, dd, J_A = 9.1 Hz, J_B = 2.6 Hz, ArH), 4.48 (3H, s, N^+CH_3), 3.92 (3H, s, OCH_3); 13C NMR (150 MHz, DMSO-d_6) δ 174.6 (NCS), 160.9 (NCS), 159.8 (ArC), 156.0 (PyC), 147.8 (PyC), 143.8 (ArC), 142.2 (PyC), 138.6 (ArC), 134.0 (PyC), 125.4 (ArC), 120.1 (PyC), 118.5 (ArC), 105.0 (ArC), 56.1 (OCH_3), 48.3 (N^+CH_3); HRMS (ESI) m/z calcd for C_15H_12N_3S_2O ([M-I]^+) 314.0422, found 314.0423.

![Image of compound 304]

dark solid, 81%. m.p. 251°C; IR (film from acetone): v_max 1967 (N=C) cm^{-1}; 1H NMR (600 MHz, DMSO-d_6) δ 9.32 (1H, d, J = 8.2 Hz, PyH), 9.17 (1H, d, J = 6.0 Hz, PyH), 8.06 (2H, m, PyH and ArH), 7.42 (1H, s, ArH), 7.20 (1H, d, J = 8.8 Hz, ArH), 4.58
(3H, s, NCH₃), 3.10 (6H, s, N(CH₃)₂); ¹³C NMR (150 MHz, DMSO-d₆) δ 171.2 (NCS), 155.2 (NCS), 150.9 (PyC), 150.5 (PyC), 144.9 (ArC), 144.4 (ArC), 141.1 (PyC), 140.0 (ArC), 133.6 (PyC), 125.0 (ArC), 121.6 (PyC), 115.4 (ArC), 102.0 (ArC), 43.9 (N⁺CH₃), 40.2 (N(CH₃)₂); HRMS (ESI) m/z calcd for C₁₆H₁₅N₄S₂ ([M-I]⁺) 327.0738, found 327.0734.

**General method of synthesizing pyridiniums via condensation cyclisation**

To a round bottom flask was added thiosulfonic acid 311 (2.0 mmol) and the corresponding pyridinium carboxaldehyde (2.2 mmol). To the mixture was added acetic acid (1 ml) and the reaction mixture was heated to 100°C. The reaction mixture was allowed to stand under the same temperature overnight. To the
resulted mixture was added EtOAc (20 ml). The dark precipitate was collected by filtration. The solid was then suspended in EtOAc (50 ml) and sonicated for 30 min. The crude product was then collected by filtration and dried under reduced pressure to remove acetic acid residue. The crude product is pure enough for further stage investigation.

sticky dark red solid, 26%. m.p. 218°C; IR 2986, 2871, 1554 (film from acetone): v$_{\text{max}}$ cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) δ 9.17 (1H, s, PyH), 8.55-8.67 (2H, m, PyH), 8.03-8.15 (2H, m, PyH and ArH), 7.48 (1H, s, ArH), 7.20 (1H, s, ArH), 4.59 (3H, s, N$^+$CH$_3$), 3.08 (6H, s, N(CH$_3$)$_2$); $^{13}$C NMR (150 MHz, DMSO-d$_6$) δ 158.1 (NCS), 150.6 (PyC), 149.7 (PyC), 146.5 (PyC), 145.3 (ArC), 144.9 (ArC), 138.6 (ArC), 126.3 (PyC), 126.1 (PyC), 124.1 (ArC), 115.3 (ArC), 103.1 (ArC), 45.9 (N$^+$CH$_3$), 40.9 (N(CH$_3$)$_2$); HRMS (ESI) m/z calcd for C$_{15}$H$_{16}$N$_3$S ([M-I]$^+$) 270.1065, found 270.1067.

dark green solid, 29%. m.p. 188-190°C; IR 3271, 1654 (film from acetone): v$_{\text{max}}$ cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-d$_6$) δ 8.94-9.01 (2H, m, PyH), 8.49-8.57 (2H, m PyH), 8.03 (1H, d, J = 9.2 Hz, ArH), 7.43 (1H, d, J = 2.5 Hz, ArH), 7.17 (1H, d, J$_A$ = 9.3 Hz, J$_B$ = 2.6 Hz, ArH), 4.33 (3H, s, N$^+$CH$_3$), 3.08 (6H, s, N(CH$_3$)$_2$); $^{13}$C NMR (150 MHz, DMSO-d$_6$) δ 154.0 (PyC), 150.1 (NCS), 146.4 (ArC), 146.1 (PyC), 145.3 (ArC), 139.4 (ArC), 124.8 (ArC), 123.1 (PyC), 115.0 (ArC), 102.2 (ArC), 47.4
(N\textsuperscript{+}CH\textsubscript{3}), 40.4 (N(CH\textsubscript{3})\textsubscript{2}); HRMS (ESI) m/z calcd for C\textsubscript{15}H\textsubscript{16}N\textsubscript{2}S ([M-I]\textsuperscript{+}) 270.1065, found 270.1067.

**General method for synthesizing extended conjugation pyridiniums**

To a round-bottom flask was added 6-substitutedbenzothiazole-2-carboxaldehyde (3.0 mmol) and corresponding methylpyridinium (2.8 mmol) along with EtOH (10 ml). To the mixture was added piperidine (cat.) and the mixture was heated to reflux. The reaction was stirred under the same temperature overnight. After the reaction mixture has been cooled to room temperature, the precipitate was collected by filtration. The crude product was suspended in EtOAc (50 ml) and sonicated for 5 min. After the solid has been collected by filtration and washed with excess diethyl ether, the pure product was obtained.

dark yellow-green solid, 92%. m.p. 213°C; IR 2986, 2809, 1765, 1697, 1621 (film from acetone): \( \nu_{\text{max}} \text{ cm}^{-1} \); \(^1\)H NMR (600 MHz, DMSO-d\textsubscript{6}) \( \delta \) 8.94-9.00 (2H, m, PyH), 8.38-8.44 (2H, m, PyH), 8.26 (1H, d, \( J = 16.2 \text{ Hz}, \text{CH}=\text{CH} \)), 7.98 (1H, d, \( J = 9.0 \text{ Hz, ArH} \)), 7.98 (1H, d, \( J = 16.2 \text{ Hz, CH}=\text{CH} \)), 7.79 (1H, d, \( J = 2.4 \text{ Hz, ArH} \)), 7.20 (1H, dd, \( J_A = 9.0 \text{ Hz, } J_B = 2.4 \text{ Hz, ArH} \)), 4.29 (3H, s, N\textsuperscript{+}CH\textsubscript{3}), 3.88 (3H, s, OCH\textsubscript{3}); \(^{13}\)C NMR (150 MHz, DMSO-d\textsubscript{6}) \( \delta \) 161.6 (NCS), 158.5 (ArC), 150.7 (PyC), 148.0 (ArC), 145.5 (PyC), 136.9 (ArC), 132.5 (C=C), 129.7 (ArC), 124.6 (PyC), 124.2 (C=C), 117.0 (ArC), 104.8 (ArC), 56.0 (OCH\textsubscript{3}), 43.6 (N\textsuperscript{+}CH\textsubscript{3}); HRMS (ESI) m/z calcd for C\textsubscript{16}H\textsubscript{15}N\textsubscript{2}OS ([M-I]\textsuperscript{+}) 283.0905, found 283.0906.
brown solid, 91%. m.p. 232-234°C; IR (film from acetone): $\nu_{\text{max}}$ 3010, 2871, 1543 cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 9.03-9.05 (1H, d, $J = 6.3$ Hz, PyH), 8.54-8.66 (2H, m, PyH and ArH), 7.83 (1H, d, $J = 15.8$ Hz, CH=CH), 7.81 (1H, d, $J = 2.5$ Hz, ArH), 7.72 (1H, dd, $J_A = 9.0$ Hz, $J_B = 2.6$ Hz, ArH), 4.44 (3H, s, N+CH$_3$), 3.88 (3H, s, OCH$_3$); $^{13}$C NMR (150 MHz, DMSO-$d_6$) $\delta$ 160.8 (NCS), 158.5 (ArC), 150.3 (PyC), 147.9 (PyC), 146.1 (PyC), 144.9 (ArC), 137.2 (ArC), 134.1 (C=C), 127.5 (ArC), 126.0 (PyC), 124.3 (PyC), 123.5 (C=C), 117.2 (ArC), 104.8 (ArC), 55.9 (OCH$_3$), 46.2 (N+CH$_3$); HRMS (ESI) m/z calcd for C$_{16}$H$_{15}$N$_2$OS ([M-I]$^+$) 283.0905, found 283.0906.

dark brown solid, 88%. m.p. 232°C; IR (film from acetone): $\nu_{\text{max}}$ 2801, 2779 cm$^{-1}$; $^1$H NMR (600 MHz, DMSO-$d_6$) $\delta$ 8.93 (2H, d, $J = 6.5$ Hz, PyH), 8.34-8.40 (2H, m, PyH), 8.20 (1H, d, $J = 16.2$ Hz, CH=CH), 7.85 (1H, d, $J = 9.1$ Hz, ArH), 7.63 (1H, d, $J = 16.2$ Hz, CH=CH), 7.33 (1H, d, $J = 2.5$ Hz, ArH), 7.07 (1H, dd, $J_A = 9.2$ Hz, $J_B = 2.6$ Hz, ArH), 4.28 (3H, s, N+CH$_3$), 3.04 (6H, s, N(CH$_3$)$_2$); $^{13}$C NMR (150 MHz, DMSO-$d_6$) $\delta$ 158.2 (NCS), 151.0 (PyC), 149.6 (ArC), 145.3 (PyC), 145.2 (ArC), 137.8 (ArC), 133.0 (C=C), 128.1 (ArC), 124.2 (PyC), 123.8 (C=C), 114.1 (ArC), 102.5 (ArC), 47.12 (N+CH$_3$), 40.37 (N(CH$_3$)$_2$); HRMS (ESI) m/z calcd for C$_{17}$H$_{18}$N$_3$S ([M-I]$^+$) 296.1221, found 296.1222.
dark solid, 88%. m.p. 249-250°C; IR (film from acetone): \(\nu_{\text{max}}\) 3041, 2991, 2843 cm\(^{-1}\); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \(\delta\) 9.00 (1H, d, \(J = 6.1\) Hz, Py\(H\)), 8.61 (1H, d, \(J = 8.3\) Hz, Py\(H\)), 8.53 (1H, t, \(J = 7.8\) Hz, Py\(H\)), 8.12-8.16 (1H, d, \(J = 16.0\) Hz, CH=CH), 7.97 (1H, t, \(J = 6.9\) Hz, Py\(H\)), 7.88-7.91 (1H, d, \(J = 9.0\) Hz, Ar\(H\)), 7.66-7.70 (1H, d, \(J = 15.8\) Hz, CH=CH), 7.35 (1H, d, \(J = 2.0\) Hz, Ar\(H\)), 7.07-7.10 (1H, dd, \(J_A = 9.0\) Hz, \(J_B = 2.0\) Hz, Ar\(H\)), 4.42 (3H, s, N\(^+\)C\(H_3\)), 3.35 (6H, s, N(C\(H_3\))\(_2\)); \(^{13}\)C NMR (150 MHz, DMSO-\(d_6\)) \(\delta\) 157.4 (NCS), 151.0 (Py\(C\)), 149.6 (Py\(C\)), 146.5 (Py\(C\)), 145.1 (Ar\(C\)), 144.6 (Ar\(C\)), 138.2 (Ar\(C\)), 134.6 (C=C), 126.0 (Py\(C\)), 125.6 (Py\(C\)), 123.9 (Ar\(C\)), 121.7 (C=C), 114.3 (Ar\(C\)), 102.4 (Ar\(C\)), 46.1 (N\(^+\)CH\(_3\)), 40.3 (N(CH\(_3\))\(_2\)); HRMS (ESI) m/z calcd for C\(_{17}\)H\(_{18}\)N\(_3\)S ([M-I\(^+\)]\(^\star\)) 296.1221, found 296.1222.
5.2 Optical properties

5.2.1 General details

UV spectroscopy data were collected using Varian 100 Bio UV-VIS spectrophotometer. Fluorescent spectroscopy data were collected using Varian Cary Eclipse fluorescence spectrophotometer. Extinction co-efficients of all compounds were measured in MeOH at 25°C, in quartz cuvettes of diameter of 1 cm. Quantum yields of all compounds are measured at a concentration of 1 μM in MeOH at 25°C, fluorescein sodium under the concentration of 1 μM in 0.1 M NaOH is used as the standard. The following refractive indexes of solvents are used: methanol 1.331, chloroform 1.446, water 1.333, acetonitrile 1.344, ethyl acetate 1.372, dimethylsulfoxide 1.480 and tetrahydrofuran 1.407.
### 5.2.2 Absorption and emission data

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOAc</td>
<td>THF</td>
<td>CHCl$_3$</td>
<td>MeCN</td>
</tr>
<tr>
<td>320</td>
<td>330</td>
<td>329</td>
<td>329</td>
</tr>
<tr>
<td>390</td>
<td>394</td>
<td>410</td>
<td>402</td>
</tr>
</tbody>
</table>

**Diagram:**
- Absorption spectra for different solvents (THF, Chloroform, Acetonitrile, DMSO, Methanol, Ethyl acetate).
- Normalized emission spectra for different solvents.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
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</thead>
<tbody>
<tr>
<td><img src="image" alt="structure" /></td>
<td>/ 293,367 / 413 / 386 / 380 / 386 / 379</td>
<td>10678</td>
<td>0.334</td>
</tr>
</tbody>
</table>

**Absorption**

- THF
- Chloroform
- Acetonitrile
- DMSO
- Methanol
- Water

**Normalized Emission**

- THF
- Chloroform
- Acetonitrile
- DMSO
- Methanol
- Water
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EtOAc</td>
<td>THF</td>
<td>CHCl$_3$</td>
</tr>
<tr>
<td>334</td>
<td>379</td>
<td>380</td>
<td>380</td>
</tr>
<tr>
<td></td>
<td>482</td>
<td>472</td>
<td>491</td>
</tr>
</tbody>
</table>

![Graph showing absorption spectra for different solvents](image1)

![Graph showing normalized emission spectra for different solvents](image2)
### Table

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
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</thead>
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<td>$\text{EtOAc}$</td>
<td>$\text{THF}$</td>
<td>$\text{CHCl}_3$</td>
</tr>
<tr>
<td>328</td>
<td>/</td>
<td>479</td>
<td>532</td>
</tr>
</tbody>
</table>

### Diagram

The diagram shows absorption spectra for different solvents: THF, Chloroform, Acetonitrile, DMSO, Methanol, Methanol, and Water. The x-axis represents wavelength, and the y-axis represents absorption.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
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</thead>
<tbody>
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<tr>
<td>THF</td>
<td>485 484 472 515 531 537 /</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>MeCN</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
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<td>MeOH</td>
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<tr>
<td>H$_2$O</td>
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</table>

![Absorption and Emission Spectra](image_url)
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{ab}}$ (top) / $\lambda_{\text{em}}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
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</thead>
<tbody>
<tr>
<td>EtOAc</td>
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<td>CHCl$_3$</td>
<td>MeCN</td>
</tr>
<tr>
<td>/</td>
<td>487</td>
<td>486</td>
<td>487</td>
</tr>
<tr>
<td>/</td>
<td>662</td>
<td>618</td>
<td>674</td>
</tr>
<tr>
<td>329</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$$
\begin{array}{llllllll}
\text{329} & / & 487 & 486 & 487 & 486 & 496 & 487 \\
/ & 662 & 618 & 674 & 702 & 666 & 670 & \\
\end{array}
$$

![Absorption spectrum](image1.png)

![Emission spectrum](image2.png)
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\epsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
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<tbody>
<tr>
<td></td>
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<td>![Compound Image]</td>
<td>363</td>
<td>365</td>
<td>367</td>
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<tr>
<td>270</td>
<td>428</td>
<td>430</td>
<td>430</td>
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</tbody>
</table>

![Absorption Graph](image)

![Emission Graph](image)

Boyuan Deng, University College London
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\epsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EtOAc</td>
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<td>CHCl$_3$</td>
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<tr>
<td></td>
<td>/</td>
<td>362</td>
<td>428</td>
</tr>
<tr>
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<td>/</td>
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![Absorption Spectrum](image1)

![Emission Spectrum](image2)
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<thead>
<tr>
<th>Compound</th>
<th>λ&lt;sub&gt;ab&lt;/sub&gt; (top) / λ&lt;sub&gt;em&lt;/sub&gt; (bottom) (nm)</th>
<th>ε (M&lt;sup&gt;-1&lt;/sup&gt;cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Φ</th>
</tr>
</thead>
<tbody>
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<td>442</td>
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<td>314</td>
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</table>

**Absorption Spectrum**

**Emission Spectrum**
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EtOAc</td>
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</tr>
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<td></td>
<td>/</td>
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<td>420</td>
</tr>
<tr>
<td></td>
<td>/</td>
<td>526</td>
<td>498</td>
</tr>
<tr>
<td>312</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**Diagram 1:** Absorption spectrum of compound 312 in different solvents.

**Diagram 2:** Emission spectrum of compound 312 in different solvents.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
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</thead>
<tbody>
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<td>MeCN</td>
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<td>305</td>
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<td>435</td>
<td>436</td>
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<td></td>
<td>-</td>
<td>521</td>
<td>560</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Compound</td>
<td>λ_{ab} (top) / λ_{em} (bottom) (nm)</td>
<td>ε (M^{-1}cm^{-1})</td>
<td>Φ</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------</td>
<td>------------------</td>
<td>---</td>
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<td><img src="CompoundImage.png" alt="Compound Image" /></td>
<td><img src="AbsorptionGraph.png" alt="Absorption Graph" /></td>
<td><img src="EmissionGraph.png" alt="Emission Graph" /></td>
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</tr>
<tr>
<td>Compound</td>
<td>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</td>
<td>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</td>
<td>$\Phi$</td>
</tr>
<tr>
<td>----------</td>
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<td>-------------------------------</td>
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<tr>
<td>315</td>
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<td></td>
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</tbody>
</table>

![Absorption Spectra](image1.png)

- **Absorption**
  - THF
  - Chloroform
  - Acetonitrile
  - DMSO
  - Methanol

![Emission Spectra](image2.png)

- **Normalized Emission**
  - THF
  - Chloroform
  - Acetonitrile
  - DMSO
  - Methanol
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>THF</td>
<td>CHCl$_3$</td>
</tr>
<tr>
<td>313</td>
<td>/</td>
<td>278</td>
<td>540</td>
</tr>
</tbody>
</table>

![Absorption spectra](image1.png)

![Emission spectra](image2.png)
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EtOAc  THF  CHCl$_3$  MeCN  DMSO  MeOH  H$_2$O</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>/ 397  424  402  402  397  389</td>
<td></td>
<td>21162</td>
</tr>
<tr>
<td>/ 556  532  572  570  564  564</td>
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<td></td>
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</tr>
</tbody>
</table>

The table above shows the absorption and emission data for compound 348 in different solvents. The figure illustrates the absorption and normalized emission spectra for the compound in various solvents.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
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<tr>
<td>/ 545 520 551 561 553 549</td>
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</table>

**Chart 1:** Absorption spectra in different solvents: THF, Chloroform, Acetonitrile, DMSO, Methanol, and Water.

**Chart 2:** Normalized emission spectra in different solvents: THF, Chloroform, Acetonitrile, DMSO, Methanol, Methanol, and Water.
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{abs}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\Phi$</th>
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<td>MeCN</td>
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![Absorption Spectrum](image1.png)

![Emission Spectrum](image2.png)
<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{ab}$ (top) / $\lambda_{em}$ (bottom) (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
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<td>THF</td>
<td>-    -    -    -    -    -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHCl$_3$</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MeCN</td>
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<td>-</td>
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<td>DMSO</td>
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<td>MeOH</td>
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<td>-</td>
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<tr>
<td>H$_2$O</td>
<td>-    -    -    -    -    -</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Diagram: Absorption spectrum of compound 351 in different solvents.
5.3 Bioconjugation with antibody

The following bioconjugation reaction and following biological test were conducted by Dr. Calise Bahou, department of chemistry, University College London. The experimental details are shown.

5.3.1 UV-Vis spectroscopy

UV-Vis spectroscopy was used to determine protein concentrations and pyridazinedione to antibody ratios (PDAR), using a Varian Cary 100 Bio UV-Visible spectrophotometer operating at 21 °C. Sample buffer was used as blank for baseline correction with extinction coefficients; $\varepsilon_{280} = 68,590 \text{ M}^{-1} \text{ cm}^{-1}$ for Trastuzumab Fab, $\varepsilon_{335} = 9,100 \text{ M}^{-1} \text{ cm}^{-1}$ for pyridazinedione scaffolds, $\varepsilon_{530} = 18,480 \text{ M}^{-1} \text{ cm}^{-1}$ for Fluorophore 1, $\varepsilon_{507} = 31,845 \text{ M}^{-1} \text{ cm}^{-1}$. A correction factor at 280 nm of 0.25 (at $A_{335}$) was employed for pyridazinedione scaffolds, 0.84 for Fluorophore A (at $A_{506}$) and 0.77 (at $A_{490}$) for Fluorophore B.

$$r = \frac{A_\lambda}{(A_{280} - \sum CF_\lambda \times A_\lambda)/\varepsilon_{280}}$$

With $A_\lambda$ the absorbance at the wavelength $\lambda$, and $\varepsilon_\lambda$ extinction coefficient of the relevant molecule.
Molecular masses of native and conjugated antibodies were measured using an Agilent 6510 QTOF LC-MS system (Agilent, UK). Agilent 1200 HPLC system was equipped with an Agilent PLRP-S, 1000A, 8 μM, 150 mm x 2.1 mm column. 10 μL of a protein sample (diluted to 0.2 mg/mL in d.d. H₂O) was separated on the column using mobile phase A (water-0.1% formic acid) and B (acetonitrile-0.1% formic acid) with an eluting gradient (as shown in table S1) at a flow rate of 300 μl/min. The oven temperature was maintained at 60 °C.

**Table S1 – LC-MS mobile phase gradient for A/B elution**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>15</td>
</tr>
<tr>
<td>2</td>
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<td>25</td>
<td>85</td>
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</table>

Agilent 6510 QTOF mass spectrometer was operated in a positive polarity mode, coupled with an ESI ion source. The ion source parameters were set up with a VCap of 3500V, a gas temperature at 350 °C, a dry gas flow rate at 10 L/min and a nebulizer of 30 psig. MS Tof was acquired under conditions of a fragmentor at 350 V, a skimmer at 65 V and an acquisition rate at 0.5 spectra/s in a profile mode, within a scan range between 700 and 4500 m/z. The data was then analysed by deconvoluting a spectrum to a zero-charge mass spectrum using a maximum entropy deconvolution algorithm within the MassHunter software version B.07.00.
Deconvoluted spectra was avoided where possible in the quantification of conjugates due to differing ionisation tendencies between species with significantly different masses.

5.3.3 Synthesis of PD linker

To a solution of 2,5-dioxopyrrolidin-1-yl 3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin1(2H)-yl) propanoate (50 mg, 0.11 mmol, pre-dissolved in MeCN (10 mL)), was added ((1R,8S,9S)-bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (32 mg, 0.10 mmol) and the reaction mixture was stirred at 21 °C for 16 h. After this time, the reaction was concentrated in vacuo and the crude residue dissolved in CHCl₃ (50 mL) washed with water (2 × 30 mL) and saturated aq. K₂CO₃ (30 mL). The organic layer was then dried (MgSO₄) and concentrated in vacuo. Purification of the crude residue by flash column chromatography (0% to 10% MeOH/EtOAc) afforded ((1R,8S,9S)-Bicyclo[6.1.0]non-4-yn-9-yl)methyl (2-(2-(2-(3-(4,5-dibromo-2-methyl-3,6-dioxo-3,6-dihydropyridazin1(2H)-yl)propanamido)ethoxy)ethoxy)ethyl) carbamate (46 mg, 0.07 mmol, 72%) as a yellow oil: ¹H NMR (600 MHz, CDCl₃, rotamers) δ 7.84 (s, 0.5H), 6.34 (s, 0.5H), 5.82 (s, 0.5H), 5.29 (s, 0.5H), 4.44 (t, J = 6.6 Hz, 2H), 4.14–4.12 (m, 4H), 3.73–3.71 (m, 3H), 3.60–3.38 (m, 12H), 2.62 (t, J = 6.6 Hz, 2H), 2.27 (m, 6H), 1.61–1.57 (m, 2H), 1.39–1.24 (m, 2H), 0.96–0.94 (m, 2H); ¹³C NMR (150 MHz, CDCl₃, rotamers) δ 169.1 (C), 156.9 (C), 153.1 (C), 153.0 (C), 136.4 (C), 135.5 (C), 98.9 (C), 70.4 (CH₂), 70.3 (CH₂), 69.7 (CH₂), 63.0 (CH₂), 44.6 (CH₂), 40.8 (CH₂), 39.5 (CH₂), 35.1 (CH₃), 34.1 (CH₂), 29.3 (CH₂), 29.2 (CH₂), 21.6 (CH₂), 20.2 (CH₂), 17.9 (CH), 14.3 (CH); IR (thin film) 3329, 2920, 2858, 1708, 1708, 1630, 1572, 1534 cm⁻¹; LRMS (ESI), 687 (50, [M⁵¹Br⁶¹Br+Na⁺]) 685 (100, [M⁷⁹Br⁶¹Br+Na⁺]), 683 (50, [M⁷⁹Br⁷⁹Br+Na⁺]), 663 (60, [M⁷⁹Br⁶¹Br+H⁺]); HRMS (ESI) calcd for C₂₉H₃₆Br₂N₄O₇ [M⁷⁹Br⁶¹Br+H]⁺ 663.0847; observed 663.0846.
5.3.4 Fab Digestion[84]

Immobilized pepsin (0.15 mL) was washed with acetate buffer (20 mM sodium acetate trihydrate, pH 3.1) four times and trastuzumab (0.5 mL, 6.41 mg mL⁻¹, 44.2 μM, in acetate buffer) was added. The mixture was incubated for 5 h at 37 °C whilst shaking (1100 rpm). The resin was separated from the digest using a filter column, and washed with digest buffer (50 mM phosphate, 150 mM NaCl, 1 mM EDTA, pH = 6.8) three times. The digest was combined with the washes and the combined mixture buffer swapped into digest buffer via diafiltration (4 × 15 mL, GE Healthcare, 10,000 MWCO) to remove impurities. Immobilized papain (0.5 mL, 0.25 mg mL⁻¹) was activated with 10 mM DTT in digest buffer whilst shaking (1100 rpm) for 1 h at 37 °C. The resin was washed with digest buffer (without DTT) four times and the digest was added (0.5 mL). The mixture was incubated for 16 h at 37 °C whilst shaking (1100 rpm). The resin was separated from the digest using a filter column, and washed with BBS (25 mM sodium borate, 25 mM NaCl, 0.5 mM EDTA, pH 8.0) three times. The digest was combined with the washes and the buffer exchanged completely for BBS using diafiltration columns (4 × 15 mL, GE Healthcare, 10,000 MWCO) to remove impurities. This yielded Trastuzumab Fab (0.5 mL, 42 μM, 48% yield) as confirmed by SDS-PAGE and LCMS. Concentration was determined photometrically using ε₂₈₀ = 68,590 M⁻¹ cm⁻¹ Observed mass 47637 Da.
5.3.5 Protocol for forming re-bridged species

TCEP·HCl (1.5 μL, 20 mM in d.d water, 15 eq.) was added to a solution of Trastuzumab Fab (100 μL, 20 μM, 1.0 mg/ml) and the solution was incubated at 37 °C for 2 h. After this time Methyl Strained Alkyne Dibromopyridazinedione (MepStra PD) (2.0 μL, 20 mM in DMSO, 20 eq.) was added and the solution was incubated at 37 °C for a further 4 h. Excess reagents were removed by ultrafiltration (6 × 10000 MWCO, VivaSpin®, GE Healthcare) into BBS (pH = 8.0). Expected mass: 49140; Observed mass: 49140.

UV Vis:

![Rebridging](image)

UV calculations suggest PD loading of 1.0
Mass spec of re-bridged species:
5.3.6 Protocol for forming clicked species

[Diagram showing reaction process]

**Fluorophore 1:**

Fluorophore 1 (2.0 μL, 20 mM in DMSO, 20 eq.) was added to a solution of rebridged Trastuzumab Fab (100 μL, 20 μM, 1.0 mg/ml) and the solution was incubated at 37 °C for 16 h. Excess reagents were removed using desalting columns (7000 MWCO, 0.5 mL, ZebaSpin®, Thermo Scientific) into BBS (pH = 8.0). Expected mass: 48550; Observed mass 48549.
UV:

Mass spec:
Fluorophore 2:

Fluorophore 2 (2.0 μL, 20 mM in DMSO, 20 eq.) was added to a solution of rebridged Trastuzumab Fab (100 μL, 20 μM, 1.0 mg/ml) and the solution was incubated at 37 °C for 16 h. Excess reagents were removed using desalting columns (7000 MWCO, 0.5 mL, ZebaSpin®, Thermo Scientific) into BBS (pH = 8.0). Expected mass: 48493; Observed mass 48493

UV:

Mass spec:
5.3.7 Extinction coefficient calculations for fluorophores:

Fluorophore 1:

Fluorophore 2:

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>$\varepsilon_{\text{max}}$</th>
<th>$\varepsilon_{280}$</th>
<th>Correction factor</th>
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<tbody>
<tr>
<td>1</td>
<td>6346</td>
<td>5388</td>
<td>0.84</td>
</tr>
<tr>
<td>2</td>
<td>3389</td>
<td>2490</td>
<td>0.77</td>
</tr>
</tbody>
</table>
Data used to calculate correction factor only. ε calculated was found to be concentration dependent on proteins. For protein work, a 1:1:1 ratio (protein : PD : Fluorophore) was assumed and using correction factors mentioned above and PD correction factor of 0.28, ε was estimated to provide matching concentrations of fluorophore and protein.

5.3.8 SDS-PAGE

Non-reducing glycine-SDS-PAGE at 12% acrylamide running were performed following standard lab procedures. A 4% stacking gel was used and a broad-range MW marker (10–250 kDa, Prestained PageRuler Plus Protein Standards, ThermoScientific) was co-run to estimate protein weights. Samples (10 μL at 7 μM) were mixed with loading buffer (2 μL, composition for 5 × SDS: 1 g SDS, 3 mL glycerol, 6 mL 0.5 M Tris buffer pH = 6.8, 2 mg bromophenol blue in 10 mL), heated at 75 °C for 5 minutes, and centrifuged at 16,000 RPM for 5 minutes. Samples were subsequently loaded into the wells in a volume of 5 μL. All gels were run at constant 10 mA for 15 minutes, then constant 15 mA until complete. Gels were stained using a modified Coomasie stain (25 g ammonium sulfate, 250 mg Coomassie G-250, 8.8 mL 85% ortho-phosphoric acid, 50 mL ethanol, made up to a total of 250 mL with d.d. H₂O) at room temperature for 16 h. Destained gels were imaged using a SynGene GelGenius system, with the software provided by the manufacturer. Lens aperture was set at 0.40 ms with no filter. Images were saved under default brightness, contrast, and gamma settings.
5.3.9 ELISA for Fab-Fluorophore Conjugates

Binding affinity to HER2 receptor was determined by ELISA. A 96-well plate was coated overnight at 4 °C with HER2 (Human HER2/ErbB2 Protein (His Tag) from Sino Biological) (100 μL of a 0.25 μg·mL⁻¹ solution in PBS), including coating one row of wells with PBS only for negative controls. Next, the coating solutions were removed and each well washed with PBS twice. Then, the wells were coated with a 2% BSA solution in PBS (200 μL) for 1 h at room temperature. Next, the wells were washed with PBST (0.1% Tween 20 in PBS) twice and with PBS three times. Solutions of Trastuzumab Fab and Trastuzumab Fab conjugates (in 0.2% BSA, PBST) were prepared with the following dilution series: 30 nM, 10 nM, 3.3 nM, 1.1 nM, 0.37 nM, 0.12 nM and 0.04 nM. Wells were coated with the dilution series solutions in triplicate, including a PBS only at 30 nm in the absence of HER2 as negative controls, and incubated for 2 h at room temperature. Then, the solutions were removed and the wells washed with PBST twice and with PBS three times. Detection antibody (100 μL of anti-human IgG, Fab-specific-HRP solution, prepared by taking 4 μL of a 1:5000 diluted solution and further diluting with 20mL of 0.2% BSA in PBST) was then added and incubated for 1 h at room temperature. Then, the solutions were removed and the wells washed with PBST twice and with
PBS three times. OPD solution (100 μL of 10 mg/20 mL OPD in phosphate-citrate buffer with sodium perborate, prepared by dissolving 1 capsule in 100 mL water) was added to each well. After 30 s the reaction was stopped through addition of 4 M HCl (50 μL). The absorption was measured at 490 nm and corrected by subtracting average of negative controls. The results obtained were analysed with GraphPad Prism using a regression with variable slope (four parameters).

**ELISA for Fab-Fluorophore Conjugates**

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>IC₅₀</th>
</tr>
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<tbody>
<tr>
<td>Trastuzumab Fab</td>
<td>9.12 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Trastuzumab Fab – Fluorophore 1</td>
<td>1.15 × 10⁻⁹</td>
</tr>
<tr>
<td>Trastuzumab Fab – Fluorophore 2</td>
<td>1.12 × 10⁻⁹</td>
</tr>
</tbody>
</table>
## Appendix

### Abbreviations list

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AIE</td>
<td>aggregation induced emission</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AP stain</td>
<td>auramine-phenol stain</td>
</tr>
<tr>
<td>AR stain</td>
<td>auramine-rhodamine stain</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMPO</td>
<td>5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Bu</td>
<td>butyl</td>
</tr>
<tr>
<td>COSY</td>
<td>proton-proton correlation spectroscopy</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DILSA</td>
<td>5'-O-(N-(dehydroinfraluciferyl)sulfamoyl)adenosine</td>
</tr>
<tr>
<td>DLSA</td>
<td>5'-O-(N-(dehydroluciferyl)sulfamoyl)adenosine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DMSO-&lt;sub&gt;d6&lt;/sub&gt;</td>
<td>deuterated dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPDTE</td>
<td>1,2-diphenyl-1,2-ditolethene</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FPI</td>
<td>fluorescent penetrant inspection</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FRET</td>
<td>Forster resonance energy transfer</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared</td>
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<tr>
<td>H&amp;E stain</td>
<td>haematoxylin-eosin stain</td>
</tr>
<tr>
<td>HER-2</td>
<td>human epidermal growth factor 2</td>
</tr>
<tr>
<td>HOMO</td>
<td>highest occupied molecular energy</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
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<tr>
<td>HTPS</td>
<td>1-hydroxypyrene-3,6,8-trisulfonate</td>
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<tr>
<td>'Bu</td>
<td>isobutyl</td>
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<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICT</td>
<td>intramolecular charge transfer</td>
</tr>
<tr>
<td>'Pr</td>
<td>isopropyl</td>
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<tr>
<td>iLH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>infraluciferin</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography and mass spectroscopy</td>
</tr>
<tr>
<td>LED</td>
<td>light-emitting diode</td>
</tr>
<tr>
<td>LH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>luciferin</td>
</tr>
<tr>
<td>LSC</td>
<td>luminescent solar concentrator</td>
</tr>
<tr>
<td>LUMO</td>
<td>lowest unoccupied molecular orbital</td>
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<tr>
<td>Me</td>
<td>methyl</td>
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<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
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<td>NAO</td>
<td>nonyl acridine orange</td>
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<td>NBD-TMA</td>
<td>[2-(4-nitro-2,1,3-benzoxadiazol-7-yl)aminoethyl]trimethylammonium</td>
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<tr>
<td>BuLi</td>
<td>butyllithium</td>
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<tr>
<td>NIR</td>
<td>near infrared</td>
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<tr>
<td>Nu</td>
<td>nucleophile</td>
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<tr>
<td>OLED</td>
<td>organic light-emitting diode</td>
</tr>
<tr>
<td>OPD</td>
<td>o-phenylenediamine</td>
</tr>
<tr>
<td>oxyLH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>oxyluciferin</td>
</tr>
<tr>
<td>oxyLH&lt;sub&gt;2&lt;/sub&gt; *</td>
<td>oxyluciferin (excited state)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>phosphate-buffered saline with Tween 20</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
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<tr>
<td>PMMA</td>
<td>polymethylmethacrylate</td>
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<tr>
<td>PMO</td>
<td>periodic mesoporous organosilica</td>
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<td>PP</td>
<td>pyrophosphate</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>Py HCl</td>
<td>pyridinium chloride</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SET</td>
<td>single electron transfer</td>
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<tr>
<td>S\textsubscript{Na}Ar</td>
<td>nucleophilic aromatic substitution</td>
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<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TFAA</td>
<td>trifluoroacetic anhydride</td>
</tr>
<tr>
<td>THBA</td>
<td>tetrahydro-tetrabenzooheptafulvalene</td>
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<td>THF</td>
<td>tetrahydrofuran</td>
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<tr>
<td>TPE</td>
<td>tetraphenylethene</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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References


[134] Lin, V. S.; Dickinson, B. C.; Chang, C. J. Chapter Two - Boronate-Based


2380.


